Cell Biology
Neuroscience

PKA regulation of neuronal function requires the dissociation of catalytic subunits from regulatory subunits

Wei-Hong Xiong, Maozhen Qin, Haining Zhong

Vollum Institute, Oregon Health & Science University, Portland, OR 97239, USA

https://en.wikipedia.org/wiki/Open_access
Copyright information

A newer version is available. **Read the latest version**.

Version of Record
November 7, 2024

Reviewed Preprint
v2 • October 21, 2024
Revised by authors

Reviewed Preprint
v1 • March 5, 2024

eLife Assessment

This **important** paper demonstrates that different PKA subtypes exhibit distinct subcellular localization at rest in CA1 neurons. The authors provide **compelling** evidence that when all tested PKA subtypes are activated by norepinephrine, catalytic subunits translocate to dendritic spines but regulatory subunits remain unmoved. Furthermore, PKA-dependent regulation of synaptic plasticity and transmission can be supported only by wildtype, dissociable PKA, but not by inseparable PKA.

https://doi.org/10.7554/eLife.93766.2.sa4

Abstract

Protein kinase A (PKA) plays essential roles in diverse cellular functions. However, the spatiotemporal dynamics of endogenous PKA upon activation remain debated. The classical model predicts that PKA catalytic subunits dissociate from regulatory subunits in the presence of cAMP, whereas a second model proposes that catalytic subunits remain associated with regulatory subunits following physiological activation. Here we report that different PKA subtypes, as defined by the regulatory subunit, exhibit distinct subcellular localization at rest in CA1 neurons of cultured hippocampal slices. Nevertheless, when all tested PKA subtypes are activated by norepinephrine, presumably via the β-adrenergic receptor, catalytic subunits translocate to dendritic spines but regulatory subunits remain unmoved. These differential spatial dynamics between the subunits indicate that at least a significant fraction of PKA dissociates. Furthermore, PKA-dependent regulation of synaptic plasticity and transmission can be supported only by wildtype, dissociable PKA, but not by inseparable PKA. These results indicate that endogenous PKA regulatory and catalytic subunits dissociate to achieve PKA function in neurons.

Introduction

Cyclic adenosine monophosphate (cAMP)-dependent kinase, or protein kinase A (PKA), regulates diverse critical functions in nearly all mammalian cells, including neurons. PKA is a tetrameric protein consisting of two regulatory subunits (PKA-Rs) and two catalytic subunits (PKA-Cs) (Francis and Corbin, 1994; Johnson et al., 2001). In the inactive state, each PKA-R binds to and inhibits a PKA-C. Binding of cAMP to PKA-R activates PKA-C. However, there are different proposals on the molecular events that follow activation.

For decades, PKA-C is thought to dissociate from PKA-R upon cAMP binding (Beavo et al., 1974; Francis and Corbin, 1994; Gold, 2019; Johnson et al., 2001; Reimann et al., 1971). Freed PKA-C molecules then move to phosphorylate their substrates. However, several studies (reviewed in Gold, 2019), including two notable recent publications (Smith et al., 2017, 2013), propose an alternative model, in which physiological concentrations of cAMP can activate PKA-C but do not result in its dissociation from PKA-R. Testing these two models will not only elucidate the biophysical mechanism of PKA activation, but also have distinct implications in how PKA may achieve its specificity, which is thought to rely on spatial compartmentalization (Wong and Scott, 2004).

We have previously found that the majority of type IIβ PKA, as defined by PKA-R, is anchored to microtubules in the dendritic shaft of hippocampal CA1 pyramidal neurons where PKA-RIIβ is bound to the abundant microtubule associated protein MAP-2 (Zhong et al., 2009). Upon activation of the β-adrenergic receptor with norepinephrine, a fraction of PKA-C dissociated from PKA-RIIβ (Tillo et al., 2017). The freed PKA-C redistributed into dendritic spines, whereas PKA-RIIβ remained anchored at the dendritic shaft (Tillo et al., 2017; Xiong et al., 2021). These results are consistent with the classical PKA activation model. However, recent studies suggest that PKA-Rs other than PKA-RIIβ may be the more abundant isoforms in CA1 neurons (Church et al., 2021; Ilouz et al., 2017; Weisenhaus et al., 2010). It remains untested whether these PKA isoforms dissociate upon physiologically relevant stimulations in neurons.

Here, we examined whether PKA-C dissociates from all major PKA-R isoforms in CA1 neurons. The rescue of function following knockdown of PKA-C was compared between wildtype dissociable PKA and an inseparable PKA variant in which PKA-C is covalently linked to PKA-R. The results support the classical model of PKA activation via dissociation.

Results

Recent studies have suggested that PKA-RIIα and PKA-RIβ may be the prevalent isoforms in hippocampal CA1 neurons. Therefore, we co-expressed C-terminally monomeric EGFP tagged PKA-C (PKA-C-mEGFP) (Tillo et al., 2017; Zhong et al., 2009) and a cytosolic marker (mCherry) with either PKA-RIIα or PKA-RIβ in CA1 neurons of organotypic hippocampal slice cultures (**Figs. 1A** and **1B**, upper left panels). At rest, PKA-C-mEGFP exhibited a distribution that was dependent on the co-expressed PKA-R: when co-expressed with PKA-RIIα, PKA-C was enriched in dendritic shafts; when co-expressed with PKA-RIβ, PKA-C was more evenly distributed (quantified using the spine enrichment indexes, or SEI, see Methods) (**Fig. 1C**). This distribution was independent of the expression level (rest conditions in **Figure 1–figure supplement 1**) and largely resembled that of the corresponding PKA-R, as visualized using expressed PKA-R-mEGFP in separate experiments (**Figs. 1A**-**1C**).

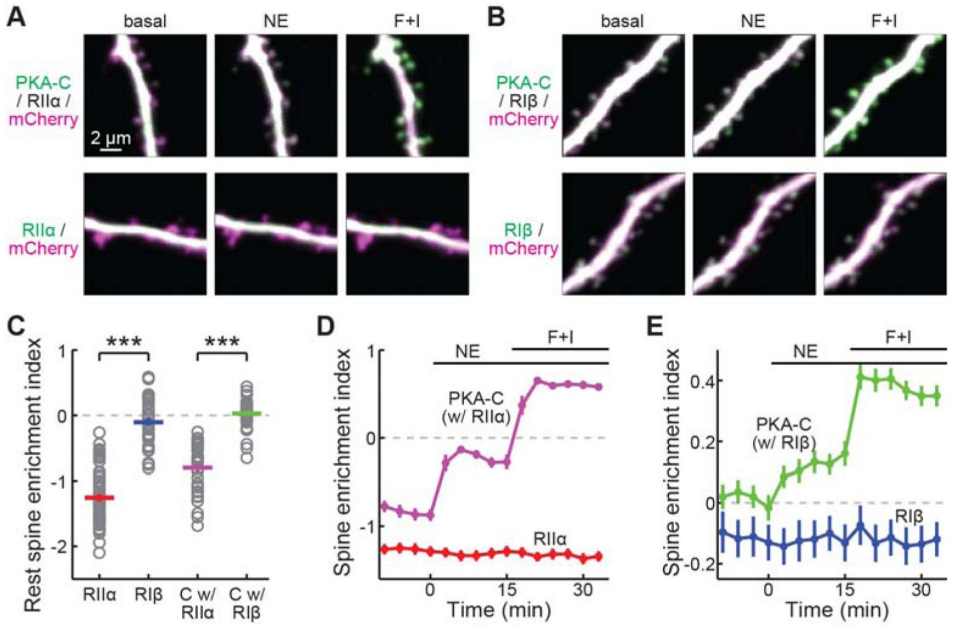


Figure 1.

PKA-C but not PKA-R redistributes to spines upon activation. (A, B) Representative two-photon images of PKA-C-mEGFP co-expressed with PKA-RIIα or PKA-RIβ at rest, or in the presence of norepinephrine (NE) or forskolin and IBMX (F + I). mCherry (magenta) was co-expressed to reveal the neuronal morphology. (C–E) Quantification and comparison of the spine enrichment index at the resting state (C) and upon activation (D & E). As in panel C from left to right, n (spines/neurons) = 53/11, 34/7, 33/6, and 36/7.

Notably, upon application of norepinephrine (10 μM), PKA-C of both subtypes translocated to dendritic spines, but the subcellular localization of PKA-Rs remained unchanged (**Figs. 1D** & **1E**). A 5x lowered norepinephrine concentration (2 μM) also resulted in similar dynamics of PKA-C (**Figure 1–figure supplement 2**), indicating that the PKA-C translocation happens in a wide range of neuromodulator concentrations. This differential re-distribution between PKA-C and PKA-R was more prominent following activation with forskolin (25 μM) and IBMX (50 μM). The translocation of PKA-C was independent of the expression level and the effect remained when extrapolating to the zero-overexpression level using a linear fit (**Figure 1–figure supplement 1**). These results can only be explained if at least a fraction of PKA-C dissociated from both PKA-RIIα and PKA-RIβ when a physiological stimulant was used. The differential re-distribution between PKA-C and PKA-R was also observed when PKA-RIα was used (**Figure 1–figure supplement 3**), although it was less implicated in hippocampal neurons. Together with our earlier results regarding PKA-C/PKA-RIIβ, we conclude that PKA-C dissociates from PKA-R with physiologically relevant stimuli.

Next, we asked whether PKA regulation of neuronal function is dependent on the dissociation of PKA-C from PKA-R. A key experiment supporting the non-dissociating PKA activation model was that PKA regulation of cell growth could be sustained by a construct in which PKA-C was fused to PKA-RIIα in one polypeptide chain via a flexible linker (named R-C; **Fig. 2A**) (Smith et al., 2017). We therefore asked whether this R-C construct could support PKA regulation of neuronal function. When R-C-mEGFP was expressed in CA1 neurons, this construct exhibited a distribution highly similar to that of RIIα (**Figs. 2B** and **2C**). The tendency of this construct to translocate to the spine was largely diminished compared to PKA-C-mEGFP co-expressed with wildtype, unlinked PKA-RIIα (**Fig. 2D**), indicating that the catalytic subunit in R-C was indeed inseparable from the regulatory subunit.

To evaluate the function of R-C, a previously established shRNA construct was used to selectively knock down PKA-Cα in CA1 neurons in cultured hippocampal slices (Tillo et al., 2017). Given that PKA activation is required for the late phase of long-term potentiation (L-LTP) (Abel et al., 1997), we examined the structural LTP of individual dendritic spines of CA1 neurons elicited by focal two-photon glutamate uncaging (**Fig. 3**) (Matsuzaki et al., 2004). The shRNA knockdown of PKA-C resulted in attenuated LTP at 90 min after induction (**Figs. 3A**–**3C**). This attenuation was not observed when a control shRNA against LacZ was expressed (**Fig. 3C**). The attenuated structural LTP was rescued by co-expression of shRNA-resistant wild-type PKA-C-mEGFP together with PKA-RIIα (**Figs. 3A**–**3C**). However, the R-C construct in which PKA-C was also resistant to shRNA knockdown but could not leave PKA-RIIα failed to rescue the phenotype.

PKA activity has also been shown to regulate synaptic transmission. We therefore examined evoked AMPA and NMDA receptor (AMPAR and NMDAR, respectively) currents in paired, transfected and adjacent untransfected CA1 neurons in cultured hippocampal slices. As shown previously (Tillo et al., 2017; Xiong et al., 2021), neurons expressing the shRNA construct against PKA-C exhibited significantly lower AMPAR currents (**Fig. 4A**), but not NMDAR currents (**Fig. 4B**). As a result, the AMPAR/NMDAR current ratio was also reduced (**Fig. 4C**). The deficits were rescued by co-expression of shRNA-resistant, wild-type dissociable PKA-C-mEGFP and PKA-RIIα (**Fig. 4**). However, the inseparable R-C construct failed to rescue the phenotype. Taken together, the R-C construct did not support normal PKA-dependent synaptic function.

Discussion

Our results indicate that at least a fraction of PKA-C molecules dissociate from all tested PKA-R isoforms, including Iβ, IIα and previously tested IIβ, when activated by physiological stimuli. Given that PKA activity increases by two orders of magnitude when dissociated from PKA-R (Miyamoto et al., 1969), even a small fraction of PKA-C dissociation will result in a marked increase of PKA

Figure 2.

Characterization of the inseparable R-C. (A) Schematic of wildtype PKA versus R-C. In both cases PKA-C was C-terminally tagged by mEGFP. (B–C) Representative images (B), quantifications of resting distribution (C), and the distribution change upon stimulation by forskolin and IBMX (D) of R-C compared to PKA-RIIα-mEGFP and co-expressed PKA-C-mEGFP/PKA-RIIα. RIIα and RIIα+C data are from **Fig. 1C**. n (spines/neurons) = 48/10.

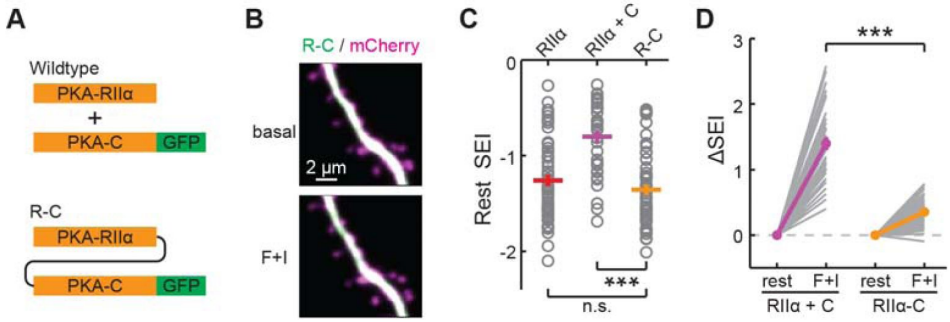
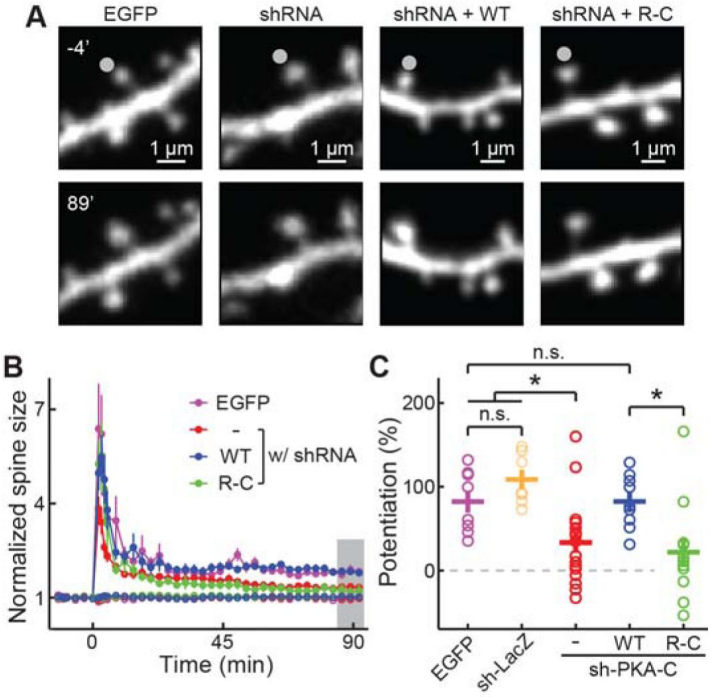


Figure 3.

PKA regulation of synaptic plasticity cannot be sustained by an inseparable PKA variant. (A–C) Representative image (A), time course (B), and the degree of potentiation (C) at the indicated timepoints in panel B of single-spine LTP experiments as triggered by focal glutamate uncaging at the marked spines (gray dot). In panel B, both stimulated spines (solid circles) and non-stimulated control spines (open circles) are shown. As in panel C from left to right, n (spines, each from a different neuron) = 8, 7, 17, 11, 9.



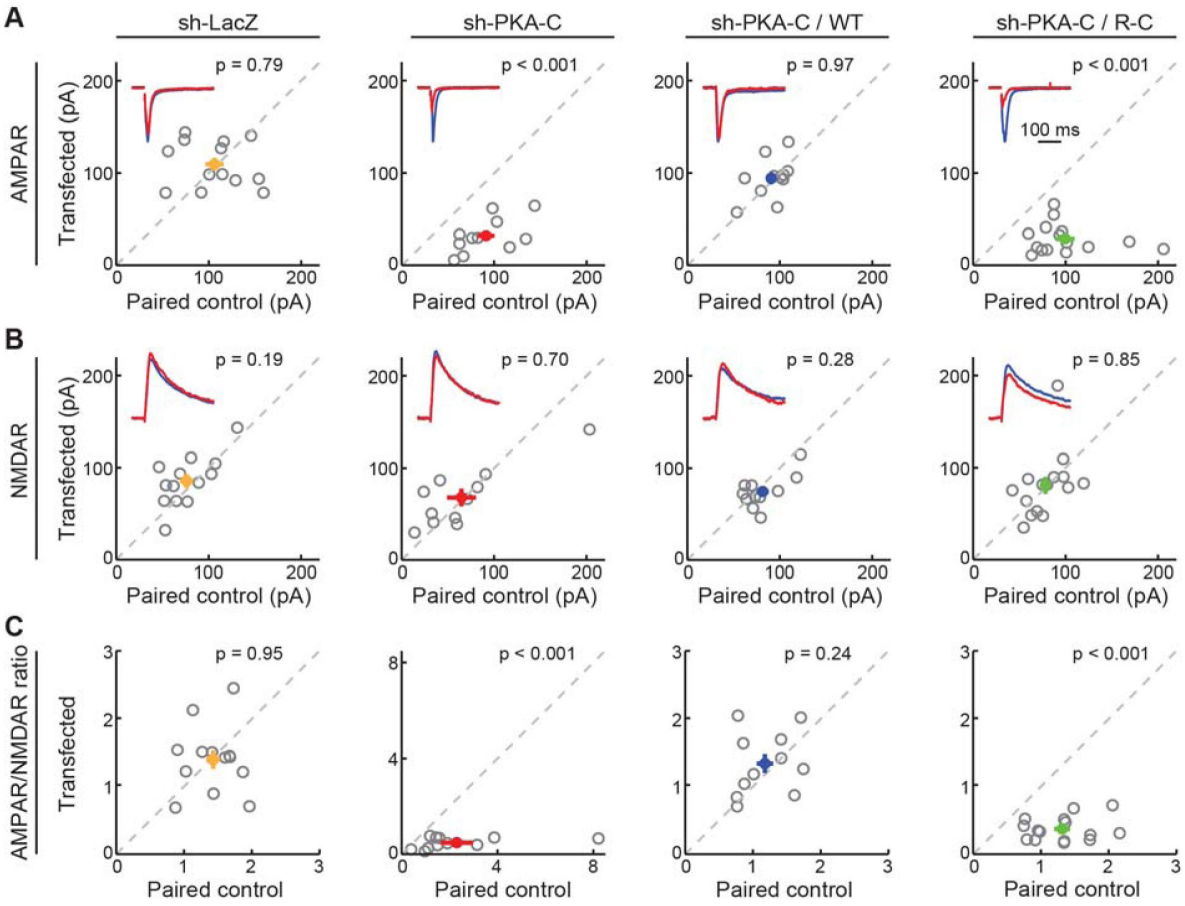


Figure 4.

AMPA receptor-mediated synaptic transmission requires wildtype dissociable PKA. (A–C) Representative traces (red) normalized to the paired control (blue) (insets) and scatter plots of paired AMPA (A) and NMDA (B) receptor currents and AMPA/NMDA receptor current ratios (C) from neighboring untransfected CA1 neurons and those transfected with shRNA against PKA-C and the indicated shRNA-resistant rescue constructs. Statistical p values were obtained using a sign test (MATLAB). From left to right, n (neuron pairs) = 13, 11, 11, and 15.

kinase activity. These results corroborate the observations in intact non-neuronal cells that used FRET imaging and biochemical measurements, respectively (Walker-Gray et al., 2017; Zaccolo et al., 2000). Furthermore, PKA-C that is covalently linked to PKA-RIIα cannot functionally replace wildtype PKA for normal neuronal transmission or plasticity. Although this R-C construct has been shown to functionally replace endogenous PKA in terms of supporting the growth of a heterologous cell line (Smith et al., 2017), it cannot support all necessary PKA functions. Overall, we conclude that PKA-C dissociation from PKA-R is essential for PKA regulation of neuronal function. Additionally, PKA specificity is mediated by spatial compartmentalization (Wong and Scott, 2004). This is likely mediated by mechanism downstream of PKA dissociation, such as membrane tethering of freed PKA-C or its buffering by extra PKA-Rs (Gaffarogullari et al., 2011; Tillo et al., 2017; Walker-Gray et al., 2017; Zhang et al., 2015).

In addition, this study shows that PKA distribution in neuronal dendrites at the resting state is subtype dependent. PKA-RIIα is enriched in dendritic shaft in a way similar to PKA-RIIβ, likely via its interaction with the abundant microtubule binding protein MAP2 (Tillo et al., 2017; Vallee et al., 1981). Note that this observation does not exclude the importance a small fraction of PKA being anchored to synaptic sites via other PKA binding proteins. In contrast, PKA-RIα and PKA-RIβ are more evenly distributed between spine and dendrites. These observations establish a spatial organizational base for understanding subtype-specific PKA function in neurons.

This study also demonstrates that PKA is essential for long-term structural LTP of individual spines. PKA has been shown to facilitate the induction of LTP (i.e., metaplasticity) (Thomas et al., 1996; Zhong et al., 2009), and is required for the maintenance of L-LTP (Abel et al., 1997), as assayed using electrophysiological recording of postsynaptic currents. However, it has been suggested that the structural and synaptic current changes may not be causally linked (Kopec et al., 2006). Our results fill the gap to show that PKA is also required for maintaining the late phase of structural LTP.

Materials and Methods

Key Resources Table				
Reagent type (species) or resource	**Designation**	**Source or reference**	**Identifiers**	**Additional information**
strain, strain background (Rattus norvegicus, Sprague Dawley)	Sprague Dawley rat	Charles River	Strain Code 001; RRID: RGD_734476	
recombinant DNA reagent	PKA-Cα-mEGFP (plasmid)	Addgene	# 45524	
recombinant DNA reagent	PKA-RIα-mEGFP (plasmid)	Addgene	# 45525	
recombinant DNA reagent	PKA-RIβ-mEGFP (plasmid)	Addgene	# 45526	
recombinant DNA reagent	PKA-RIIα-mEGFP (plasmid)	Addgene	# 45527	
recombinant DNA reagent	PKA-RIα (plasmid)	This paper		

recombinant DNA reagent	PKA-RIβ (plasmid)	This paper		
recombinant DNA reagent	PKA-RIIα (plasmid)	Addgene	#168492	
recombinant DNA reagent	PKA-RIIα-PKA-Cα-mEGFP (plasmid)	This paper		
recombinant DNA reagent	shPKA against PKA-Cα with DsRed co-expression (plasmid)	This paper		The shRNA was developed in Tillo et al, 2017
recombinant DNA reagent	shPKA against LacZ with DsRed co-expression (plasmid)	This paper		
recombinant DNA reagent	mCherry2 (plasmid)	Addgene	#54517	
chemical compound, drug	norepinephrine	Tocris	5169	
chemical compound, drug	forskolin	LC Labs	F-9926	

chemical compound, drug	IBMX	Sigma-Aldrich	I7018	
chemical compound, drug	MNI-glutamate	Tocris	1490	
chemical compound, drug	TTX	Tocris	1069	
chemical compound, drug	2-chloroadenosine	Sigma-Aldrich	C5134	
chemical compound, drug	GABAzine (SR 95531)	Tocris	1262	
software, algorithm	MATLAB	MathWorks	RRID: SCR_001622; https://www.mathworks.com/	
software, algorithm	SI_View	Github / Zhong Lab	https://github.com/HZhongLab/SI_View	

Plasmid constructs

Constructs were made using standard mutagenesis and subcloning methods. All previously unpublished constructs and their sequences will be submitted to Addgene. In the R-C construct, mouse PKA-RIIα and PKA-Cα were fused via a linker with residues WDPGSGSLEAGCKNFFPRSFTSCGSLEGGSAAA that were previously used (Smith et al., 2017).

Organotypic hippocampal slice cultures and transfections

Cultured rat hippocampal slices were prepared from P6 – P8 (typically P7) pups, as described previously (Stoppini et al., 1991; Zhong et al., 2009). Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Oregon Health & Science University (#IP00002274). cDNA constructs were transfected after 1.5–3

weeks *in vitro* via the biolistic gene transfer method using the Helios gene gun and 1.6 μm gold beads (**Fig. 1**) or, where long-term expression (~ 1 week) was required, with single-cell electroporation (**Fig. 2**) (Otmakhov and Lisman, 2012).

Two-photon imaging and two-photon glutamate uncaging

A custom built two-photon microscope based on an Olympus BW51WI microscope body was used. Laser beams from two different Ti:Sapphire lasers (Maitai, Newport) were aligned to allow for simultaneous two-photon excitation and photoactivation. Laser intensities were controlled by Pockels cells (Conoptics). Imaging and photoactivation were controlled by ScanImage (Vidrio Tech) (Pologruto et al., 2003). Slices were perfused during imaging in gassed artificial cerebral spinal fluid (ACSF) containing (mM) 127 NaCl, 25 $NaHCO_3$, 25 D-glucose, 2.5 KCl, 4 $MgCl_2$, 4 $CaCl_2$, and 1.25 NaH_2PO_4 with 0.5 μM tetrodotoxin (TTX). mEGFP fluorescence (green) was unmixed from that of the cytosolic marker (mCherry or DsRed Express) using a dichroic (Chroma 565DCXR) and band-pass filters (Chroma HQ510/70 for green and Semrock FF01-630/92 for red).

For single-spine structural LTP experiments, 2.25 mM MNI-caged-L-glutamate (Tocris) was added to ACSF containing 4 mM calcium, 0.05 mM magnesium, 1 μM TTX and 4 μM 2-chloroadenosine, as previously described (Harvey et al., 2008a). To trigger structural plasticity, 30 pulses of 4-ms 16-mW (at back focal plane) 720-nm laser light were delivered to the spine head at 0.5 Hz.

Image analysis was performed using custom software written in MATLAB called *SI_View* (*https://github.com/HZhongLab/SI_View*) (Ma et al., 2022). Using the software, regions of interest (ROIs) were manually drawn to isolate spines or their immediately adjacent dendritic shaft. Only the spines well isolated from the dendrite laterally throughout the entire experiments were included. Spine enrichment index was calculated as:

$$SEI = \log_2[(F_{green}/F_{red})_{spine}/(F_{green}/F_{red})_{shaft}]$$

The expression level was estimated by the maximal intensity from thick apical dendrite near the soma (typically 50-100 μm) similar to previously described (Harvey et al., 2008b; Tillo et al., 2017). To minimize the influence of noise, a line of 9 pixels (pixel size ~0.04 μm) thick was manually drawn transecting the dendrite through the visually identified, brightest region. The line profile was further smoothened by 5 pixels along the line and baseline subtracted before determining the maximal value. To combine measurements from different hardware configuration (e.g., different microscopes), the data from each configuration and experiment were internally corrected for laser stimulation intensity and then normalized to the average of all data under the same condition.

Electrophysiology

Whole-cell voltage-clamp recordings were performed using a MultiClamp 700B amplifier (Molecular Devices). Electrophysiological signals were filtered at 2 kHz and digitized and acquired at 20 kHz using custom software written in MATLAB. Slices were perfused with artificial cerebrospinal fluid containing 4 mM Ca and 4 mM Mg. The internal solution contained (in mM) 132 Cs-gluconate, 10 HEPES, 10 Na-phosphocreatine, 4 MgCl2, 4 Na2-ATP, 0.4 Na-GTP, 3 Na-ascorbate, 3 QX314, and 0.2 EGTA with an osmolarity of 295 mOsmol/kg. The junction potential was calculated to be -17 mV using a built-in function in the Clampfit software (Molecular Devices). Several less abundant anions (phosphocreatine, ATP, GTP and ascorbate) were omitted in the calculation due to lack of data in the program. The Cl reversal potential was -75 mV.

To reduce recurrent activities, cultured hippocampal slices were cut on both sides of CA1 and 4 μM 2-chloroadenosine (Sigma) was present in all recording experiments. 10 μM GABAzine (SR 95531, Tocris) was also included to suppress GABA currents. For electrical stimulation, a bipolar, θ-glass stimulating electrode (Warner Instruments) was positioned in the stratum radiatum 100–150 μm

lateral to the recorded neuron. For all recordings, a transfected neuron and an untransfected neuron located within 50 μm of each other were sequentially recorded without repositioning the stimulation electrode. Measurements were carried out on averaged traces from approximately 20 trials under each condition. For AMPAR currents, the cells were held at -60 mV (before correcting for the junction potential) and the current was measured as the baseline-subtracted peak current within a window of 2–50 ms after electric stimulation. For NMDAR currents, the average currents at 140 to 160 ms after stimulation were used when the cells were held at +55 mV (before correcting for the junction potential)

Data analysis, presentation, and statistics

Quantification and statistical tests were performed using custom software written in MATLAB. Averaged data are presented as mean ± s.e.m., unless noted otherwise. p values were obtained from one-way ANOVA tests, unless noted otherwise. In all figures, *: $p \leq 0.05$ and is considered statistically significant after Bonferroni correction for multiple tests, **: $p \leq 0.01$, and ***: $p \leq 0.001$.

Acknowledgements

We thank all members of the Mao and Zhong laboratories at the Vollum Institute for constructive discussions. We thank Drs John Williams and Michael Muniak for critical comments and edits on the manuscript. This work was supported by two NIH BRAIN Initiative awards to H.Z. (U01NS094247 and R01NS127013).

Author contributions

H.Z. conceived the project. W.X. and H.Z. designed the experiments. W.X. and H.Z. performed the experiments and data analyses. M.Q. produced critical reagents used in the experiments. H.Z. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Figures and figure legends

Figure 1–figure supplement 1.

Spine enrichment indexes and the movement of PKA-C into spines upon activation are not dependent on the protein expression level. (A–B) Averaged spine enrichment indexes within individual neurons of PKA-C co-expressed with RIIα (A) and RIβ (B), and their linear fit at rest or under the indicated stimulation.

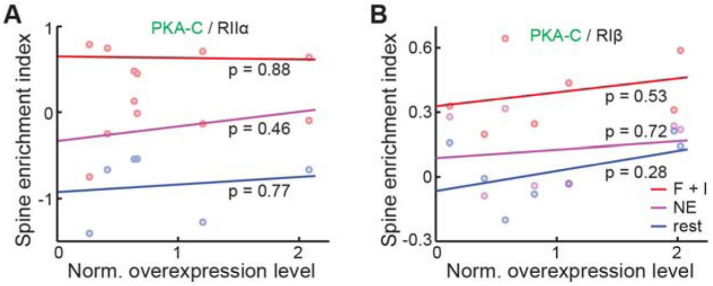


Figure 1–figure supplement 2.

PKA-C translocation can be driven by norepinephrine at low concentrations. (A, B) Representative two-photon images (A) and the collective trace of PKA-C-mEGFP co-expressed with PKA-RIIα at rest, or in the presence of 2 μM norepinephrine (NE) or forskolin and IBMX (F + I). DsRed Express (magenta) was co-expressed to reveal the neuronal morphology. n (spines/neurons) = 16/4.

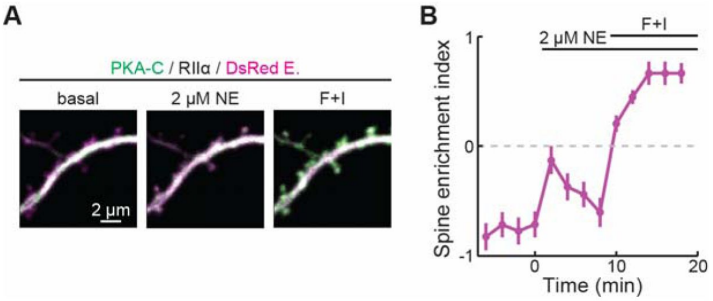
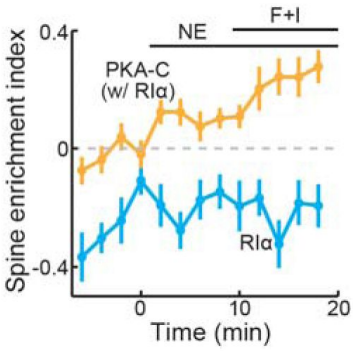


Figure 1–figure supplement 3.

PKA-C and PKA-RIα differentially re-distributed upon activation. The collective trace of PKA-C-mEGFP co-expressed with PKA-RIα (orange) and PKA-RIα-mEGFP (light blue) at rest, or in the presence of 10 μM norepinephrine (NE) or forskolin and IBMX (F + I). DsRed Express was co-expressed to reveal the neuronal morphology. n (spines/neurons) = 20/5 for PKA-C and 16/4 for RIα.



References

1. Abel T, Nguyen P V., Barad M, Deuel TAS, Kandel ER, Bourtchouladze R (1997) **Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory** *Cell* **88**:615–626 https://doi.org/10.1016/S0092-8674(00)81904-2

2. Beavo JA, Bechtel PJ, Krebs EG (1974) **Activation of protein kinase by physiological concentrations of cyclic AMP** *Proc Natl Acad Sci U S A* https://doi.org/10.1073/pnas.71.9.3580

3. Church TW, Tewatia P, Hannan S, Antunes J, Eriksson O, Smart TG, Kotaleski JH, Gold MG (2021) **Akap79 enables Calcineurin to directly suppress protein Kinase a activity** *Elife* **10** https://doi.org/10.7554/eLife.68164

4. Francis SH, Corbin JD (1994) **Structure and Function of Cyclic Nucleotide-Dependent Protein Kinases** *Annu Rev Physiol* https://doi.org/10.1146/annurev.ph.56.030194.001321

5. Gaffarogullari EC, Masterson LR, Metcalfe EE, Traaseth NJ, Balatri E, Musa MM, Mullen D, Distefano MD, Veglia G (2011) **A myristoyl/phosphoserine switch controls cAMP-dependent protein kinase association to membranes** *J Mol Biol* https://doi.org/10.1016/j.jmb.2011.06.034

6. Gold MG (2019) **Swimming regulations for protein kinase A catalytic subunit** *Biochem Soc Trans* https://doi.org/10.1042/BST20190230

7. Harvey CD, Yasuda R, Zhong H, Svoboda K (2008) **The spread of Ras activity triggered by activation of a single dendritic spine** *Science (80-)* **321**:136–140 https://doi.org/10.1126/science.1159675

8. Harvey CD, Yasuda R, Zhong H, Svoboda K (2008) **The spread of Ras activity triggered by activation of a single dendritic spine** *Science (80-)* **321**:136–140 https://doi.org/10.1126/science.1159675

9. Ilouz R, Lev-Ram V, Bushong EA, Stiles TL, Friedmann-Morvinski D, Douglas C, Goldberg G, Ellisman MH, Taylor SS (2017) **Isoform-specific subcellular localization and function of protein kinase a identified by mosaic imaging of mouse brain** *Elife* **6** https://doi.org/10.7554/eLife.17681

10. Johnson DA, Akamine P, Radzio-Andzelm E, Madhusudan Taylor SS (2001) **Dynamics of cAMP-dependent protein kinase** *Chem Rev* https://doi.org/10.1021/cr000226k

11. Kopec CD, Li B, Wei W, Boehm J, Malinow R (2006) **Glutamate receptor exocytosis and spine enlargement during chemically induced long-term potentiation** *J Neurosci* **26**:2000–2009 https://doi.org/10.1523/JNEUROSCI.3918-05.2006

12. Ma L, Day-Cooney J, Benavides OJ, Muniak MA, Qin M, Ding JB, Mao T, Zhong H (2022) **Locomotion activates PKA through dopamine and adenosine in striatal neurons** *Nature* **611**:762–768 https://doi.org/10.1038/s41586-022-05407-4

13. Matsuzaki M, Honkura N, Ellis-Davies GCR, Kasai H (2004) **Structural basis of long-term potentiation in single dendritic spines** *Nature* **429**:761–766 https://doi.org/10.1038/nature02617

14. Miyamoto E, Kuo JF, Greengard P (1969) **Cyclic nucleotide-dependent protein kinases. 3. Purification and properties of adenosine 3',5'-monophosphate-dependent protein kinase from bovine brain** *J Biol Chem* **244**:6395–6402

15. Otmakhov N, Lisman J (2012) **Measuring CaMKII concentration in dendritic spines** *J Neurosci Methods* https://doi.org/10.1016/j.jneumeth.2011.09.022

16. Pologruto TA, Sabatini BL, Svoboda K (2003) **ScanImage: Flexible software for operating laser scanning microscopes** *Biomed Eng Online* **2** https://doi.org/10.1186/1475-925X-2-13

17. Reimann EM, Brostrom CO, Corbin JD, King CA, Krebs EG (1971) **Separation of regulatory and catalytic subunits of the cyclic 311, 511-adenosine monophosphate-dependent protein kinase(s) of rabbit skeletal muscle** *Biochem Biophys Res Commun* **42** https://doi.org/10.1016/0006-291X(71)90086-6

18. Smith FD *et al.* (2017) **Local protein kinase A action proceeds through intact holoenzymes** *Science (80-)* **356** https://doi.org/10.1126/science.aaj1669

19. Smith FD, Reichow SL, Esseltine JL, Shi D, Langeberg LK, Scott JD, Gonen T (2013) **Intrinsic disorder within an AKAP-protein kinase A complex guides local substrate phosphorylation** *Elife* **2013** https://doi.org/10.7554/eLife.01319.001

20. Stoppini L, Buchs PA, Muller D (1991) **A simple method for organotypic cultures of nervous tissue** *J Neurosci Methods* https://doi.org/10.1016/0165-0270(91)90128-M

21. Thomas MJ, Moody TD, Makhinson M, O'Dell TJ (1996) **Activity-dependent β-adrenergic modulation of low frequency stimulation induced LTP in the hippocampal CA1 region** *Neuron* **17**:475–482 https://doi.org/10.1016/S0896-6273(00)80179-8

22. Tillo SE *et al.* (2017) **Liberated PKA Catalytic Subunits Associate with the Membrane via Myristoylation to Preferentially Phosphorylate Membrane Substrates** *Cell Rep* **19**:617–629 https://doi.org/10.1016/j.celrep.2017.03.070

23. Vallee RB, DiBartolomeis MJ, Theurkauf WE (1981) **A protein kinase bound to the projection portion of MAP 2 (microtubule-associated protein 2)** *J Cell Biol* **90** https://doi.org/10.1083/jcb.90.3.568

24. Walker-Gray R, Stengel F, Gold MG (2017) **Mechanisms for restraining cAMP-dependent protein kinase revealed by subunit quantitation and cross-linking approaches** *Proc Natl Acad Sci U S A* **114**:10414–10419 https://doi.org/10.1073/pnas.1701782114

25. Weisenhaus M, Allen ML, Yang L, Lu Y, Nichols CB, Su T, Hell JW, Mcknight GS (2010) **Mutations in AKAP5 disrupt dendritic signaling complexes and lead to electrophysiological and behavioral phenotypes in mice** *PLoS One* **5** https://doi.org/10.1371/journal.pone.0010325

26. Wong W, Scott JD (2004) **AKAP signalling complexes: Focal points in space and time** *Nat Rev Mol Cell Biol* https://doi.org/10.1038/nrm1527

27. Xiong WH, Qin M, Zhong H (2021) **Myristoylation alone is sufficient for PKA catalytic subunits to associate with the plasma membrane to regulate neuronal functions** *Proc Natl Acad Sci U S A* **118** https://doi.org/10.1073/pnas.2021658118

28. Zaccolo M, De Giorgi F, Cho CY, Feng L, Knapp T, Negulescu PA, Taylor SS, Tsien RY, Pozzan T. (2000) **A genetically encoded, flourescent indicator for cyclic AMP in living cells** *Nat Cell Biol* **2**:25–29 https://doi.org/10.1038/71345

29. Zhang P, Ye F, Bastidas AC, Kornev AP, Wu J, Ginsberg MH, Taylor SS (2015) **An Isoform-Specific Myristylation Switch Targets Type II PKA Holoenzymes to Membranes** *Structure* https://doi.org/10.1016/j.str.2015.07.007

30. Zhong H, Sia GM, Sato TR, Gray NW, Mao T, Khuchua Z, Huganir RL, Svoboda K (2009) **Subcellular Dynamics of Type II PKA in Neurons** *Neuron* **62**:363–374 https://doi.org/10.1016/j.neuron.2009.03.013

Editors

Reviewing Editor
Kang Shen
Stanford University, Howard Hughes Medical Institute, Stanford, United States of America

Senior Editor
Amy Andreotti
Iowa State University, Ames, United States of America

Reviewer #1 (Public review):

Summary:

This is a short self-contained study with a straightforward and interesting message. The paper focuses on settling whether PKA activation requires dissociation of the catalytic and regulatory subunits. This debate has been ongoing for ~ 30 years, with renewed interest in the question following a publication in Science, 2017 (Smith et al.). Here, Xiong et al demonstrate that fusing the R and C subunits together (in the same way as Smith et al) prevents the proper function of PKA in neurons. This provides further support for the dissociative activation model - it is imperative that researchers have clarity on this topic since it is so fundamental to building accurate models of localised cAMP signalling in all cell types. Furthermore, their experiments highlight that C subunit dissociation into spines is essential for structural LTP, which is an interesting finding in itself. They also show that preventing C subunit dissociation reduces basal AMPA receptor currents to the same extent as knocking down the C subunit. Overall, the paper will interest both cAMP researchers and scientists interested in fundamental mechanisms of synaptic regulation.

Strengths:

The experiments are technically challenging and well executed. Good use of control conditions e.g untransfected controls in Figure 4.

Weaknesses:

The novelty is lessened given the same team has shown dissociation of the C subunit into dendritic spines from RIIbeta subunits localised to dendritic shafts before (Tillo et al., 2017).

Nevertheless, the experiments with RII-C fusion proteins are novel and an important addition.

https://doi.org/10.7554/eLife.93766.2.sa3

Reviewer #2 (Public review):

Summary:

PKA is a major signaling protein which has been long studied and is vital for synaptic plasticity. Here, the authors examine the mechanism of PKA activity and specifically focus on addressing the question of PKA dissociation as a major mode of its activation in dendritic spines. This would potentially allow to determine the precise mechanisms of PKA activation and address how it maintains spatial and temporal signaling specificity.

Strengths:

The results convincingly show that PKA activity is governed by the subcellular localization in dendrites and spines and is mediated via subunit dissociation. The authors make use of organotypic hippocampal slice cultures, where they use pharmacology, glutamate uncaging, and electrophysiological recordings.

Overall, the experiments and data presented are well executed. The experiments all show that at least in the case of synaptic activity, distribution of PKA-C to dendritic spines is necessary and sufficient for PKA mediated functional and structural plasticity.
The authors were able to persuasively support their claim that PKA subunit dissociation is necessary for its function and localization in dendritic spines. This conclusion is important to better understand the mechanisms of PKA activity and its role in synaptic plasticity.

Weaknesses:

While the experiments are indeed convincing and well executed, the data presented is similar to previously published work from the Zhong lab (Tillo et al., 2017, Zhong et al 2009). This reduces the novelty of the findings in terms of re-distribution of PKA subunits, which was already established, at least to some degree.

https://doi.org/10.7554/eLife.93766.2.sa2

Reviewer #3 (Public review):

Summary:

Xiong et al. investigated the debated mechanism of PKA activation using hippocampal CA1 neurons under pharmacological and synaptic stimulations. Examining all major PKA-R isoforms in these neurons, they found that a portion of PKA-C dissociates from PKA-R and translocate into dendritic spines following norepinephrine bath application. Additionally, their use of a non-dissociable form of PKA demonstrates its essential role in structural long-term potentiation (LTP) induced by two-photon glutamate uncaging, as well as in maintaining normal synaptic transmission, as verified by electrophysiology. This study presents a valuable finding on the activation-dependent re-distribution of PKA catalytic subunits in CA1 neurons, a process vital for synaptic functionality. The robust evidence provided by the authors makes this work particularly relevant for biologists seeking to understand PKA activation mechanisms, its downstream effects, and synaptic plasticity.

Strengths:

The study is methodologically robust, particularly in the application of two-photon imaging and electrophysiology. The experiments are well-designed with effective controls and a comprehensive analysis. The credibility of the data is further enhanced by the research team's previous works in related experiments. The study provides sufficient evidence to support the classical model of PKA activation via dissociation in neurons.

Weaknesses:

No specific weaknesses are noted in the current study; future research could provide additional insights by exploring PKA dissociation under varied physiological conditions, particularly in vivo, to further validate and expand upon these findings.

https://doi.org/10.7554/eLife.93766.2.sa1

Author response:

The following is the authors' response to the original reviews.

New Experiments

(1) Activation-dependent dynamics of PKA with the RIα regulatory subunit, adding to the answer to Reviewers 1 and 2. To determine the dynamics of all PKA isoforms, we have added experiments that used PKA-RIα as the regulatory subunit. We found differential translocation between PKA-C (co-expressed with PKA-RIα) and PKA-RIα (Figure 1–figure supplement 3), similar to the results when PKA-RIIα or PKA-RIβ was used.

(2) PKA-C dynamics elicited by a low concentration of norepinephrine, addressing Reviewer 3's comment. We have found that PKA-C (co-expressed with RIIα) exhibited similar translocation into dendritic spines in the presence of a 5x lowered concentration (2 μM) of norepinephrine, suggesting that the translocation occurs over a wide range of stimulus strengths (Figure 1-figure supplement 2).

> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *This is a short self-contained study with a straightforward and interesting message. The paper focuses on settling whether PKA activation requires dissociation of the catalytic and regulatory subunits. This debate has been ongoing for ~ 30 years, with renewed interest in the question following a publication in Science, 2017 (Smith et al.). Here, Xiong et al demonstrate that fusing the R and C subunits together (in the same way as Smith et al) prevents the proper function of PKA in neurons. This provides further support for the dissociative activation model - it is imperative that researchers have clarity on this topic since it is so fundamental to building accurate models of localised cAMP signalling in all cell types. Furthermore, their experiments highlight that C subunit dissociation into spines is essential for structural LTP, which is an interesting finding in itself. They also show that preventing C subunit dissociation reduces basal AMPA receptor currents to the same extent as knocking down the C subunit. Overall, the paper will interest both cAMP researchers and scientists interested in fundamental mechanisms of synaptic regulation.*
>
> *Strengths:*
>
> *The experiments are technically challenging and well executed. Good use of control conditions e.g untransfected controls in Figure 4.*

We thank the reviewer for their accurate summarization of the position of the study in the field and for the positive evaluation of our study.

> *Weaknesses:*
>
> *The novelty is lessened given the same team has shown dissociation of the C subunit into dendritic spines from RIIbeta subunits localised to dendritic shafts before (Tillo et al., 2017). Nevertheless, the experiments with RII-C fusion proteins are novel and an important addition.*

We thank the reviewer for noticing our earlier work. The first part of the current work is indeed an extension of previous work, as we have articulated in the manuscript. However, this extension is important because recent studies suggested that the majority of PKA-RIIβ are axonal localized. The primary PKA subtypes in the soma and dendrite are likely PKA-RIβ or PKA-RIIα. Although it is conceivable that the results from PKA-RIIβ can be extended to the other subunits, given the current debate in the field regarding PKA dissociation (or not), it remains important to conclusively demonstrate that these other regulatory subunit types also support PKA dissociation within intact cells in response to a physiological stimulant. To complete the survey for all PKA-R isoforms, we have now added data for PKA-RIα (New Experiment #1), as they are also expressed in the brain (e.g., https://www.ncbi.nlm.nih.gov/gene/5573). Additionally, as the reviewer points out, our second part is a novel addition to the literature.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *PKA is a major signaling protein that has been long studied and is vital for synaptic plasticity. Here, the authors examine the mechanism of PKA activity and specifically focus on addressing the question of PKA dissociation as a major mode of its activation in dendritic spines. This would potentially allow us to determine the precise mechanisms of PKA activation and address how it maintains spatial and temporal signaling specificity.*
>
> *Strengths:*
>
> *The results convincingly show that PKA activity is governed by the subcellular localization in dendrites and spines and is mediated via subunit dissociation. The authors make use of organotypic hippocampal slice cultures, where they use pharmacology, glutamate uncaging, and electrophysiological recordings.*
>
> *Overall, the experiments and data presented are well executed. The experiments all show that at least in the case of synaptic activity, the distribution of PKA-C to dendritic spines is necessary and sufficient for PKA-mediated functional and structural plasticity.*
>
> *The authors were able to persuasively support their claim that PKA subunit dissociation is necessary for its function and localization in dendritic spines. This conclusion is important to better understand the mechanisms of PKA activity and its role in synaptic plasticity.*

We thank the reviewer for their positive evaluation of our study.

> *Weaknesses:*
>
> *While the experiments are indeed convincing and well executed, the data presented is similar to previously published work from the Zhong lab (Tillo et al., 2017, Zhong et al 2009). This reduces the novelty of the findings in terms of re-distribution of PKA subunits,*

> *which was already established. A few alternative approaches for addressing this question: targeting localization of endogenous PKA, addressing its synaptic distribution, or even impairing within intact neuronal circuits, would highly strengthen their findings. This would allow us to further substantiate the synaptic localization and re-distribution mechanism of PKA as a critical regulator of synaptic structure, function, and plasticity.*

We thank the reviewer for noticing our earlier work. The first part of the current work is indeed an extension of previous work, as we have articulated in the manuscript. However, this extension is important because recent studies suggested that the majority of PKA-RIIβ are axonal localized. The primary PKA subtypes in the soma and dendrite are likely PKA-RIβ or PKA-RIIα. Although it is conceivable that the results from PKA-RIIβ can be extended to the other subunits, given the current debate in the field regarding PKA dissociation (or not), it remains important to conclusively demonstrate that these other regulatory subunit types also support PKA dissociation within intact cells in response to a physiological stimulant. To complete the survey for all PKA-R isoforms, we have now added data for PKA-RIα (New Experiment #1), as they are also expressed in the brain (e.g., https://www.ncbi.nlm.nih.gov/gene/5573). Additionally, as Reviewer 1 points out, our second part is a novel addition to the literature.

We also thank the reviewer for suggesting the experiments to examine PKA's synaptic localization and dynamics as a key mechanism underlying synaptic structure and function. We agree that this is a very interesting topic. At the same time, we feel that this mechanistic direction is open ended at this time and beyond what we try to conclude within this manuscript: prevention of PKA dissociation in neurons affects synaptic function. Therefore, we will save the suggested direction for future studies. We hope the reviewer understand.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *Xiong et al. investigated the debated mechanism of PKA activation using hippocampal CA1 neurons under pharmacological and synaptic stimulations. Examining the two PKA major isoforms in these neurons, they found that a portion of PKA-C dissociates from PKA-R and translocates into dendritic spines following norepinephrine bath application. Additionally, their use of a non-dissociable form of PKC demonstrates its essential role in structural long-term potentiation (LTP) induced by two-photon glutamate uncaging, as well as in maintaining normal synaptic transmission, as verified by electrophysiology. This study presents a valuable finding on the activation-dependent re-distribution of PKA catalytic subunits in CA1 neurons, a process vital for synaptic functionality. The robust evidence provided by the authors makes this work particularly relevant for biologists seeking to understand PKA activation and its downstream effects essential for synaptic plasticity.*
>
> *Strengths:*
>
> *The study is methodologically robust, particularly in the application of two-photon imaging and electrophysiology. The experiments are well-designed with effective controls and a comprehensive analysis. The credibility of the data is further enhanced by the research team's previous works in related experiments. The conclusions of this paper are mostly well supported by data. The research fills a significant gap in our understanding of PKA activation mechanisms in synaptic functioning, presenting valuable insights backed by empirical evidence.*

We thank the reviewer for their positive evaluation of our study.

> *Weaknesses:*

> *The physiological relevance of the findings regarding PKA dissociation is somewhat weakened by the use of norepinephrine (10 μM) in bath applications, which might not accurately reflect physiological conditions. Furthermore, the study does not address the impact of glutamate uncaging, a well-characterized physiologically relevant stimulation, on the redistribution of PKA catalytic subunits, leaving some questions unanswered.*

We agreed with the Reviewer that testing under physiological conditions is critical especially given the current debate in the literature. That is why we tested PKA dynamics induced by the physiological stimulant, norepinephrine. It has been suggested that, near the release site, local norepinephrine concentrations can be as high as tens of micromolar (*Courtney and Ford, 2014*). Based on this study, we have chosen a mid-range concentration (10 μM). At the same time, in light of the Reviewer's suggestion, we have now also tested PKA-RIIα dissociation at a 5x lower concentration of norepinephrine (2 μM; New Experiment #2). The activation and translocation of PKA-C is also readily detectible under this condition to a degree comparable to when 10 μM norepinephrine was used.

Regarding the suggested glutamate uncaging experiment, it is extremely challenging because of finite signal-to-noise ratios in our experiments. From our past studies, we know that activated PKA-C can diffuse three dimensionally, with a fraction as membrane-associated proteins and the other as cytosolic proteins. Although we have evidence that its membrane affinity allows it to become enriched in dendritic spines, it is not known (and is unlikely) that activated PKA-C is selectively targeted to a particular spine. Glutamate uncaging of a single spine presumably would locally activate a small number of PKA-C. It will be very difficult to trace the 3D diffusion of these small number of molecules in the presence of surrounding resting-state PKA-C molecules. Finally, we hope the reviewer agrees that, regardless of the result of the glutamate uncaging experiment, the above new experiment (New Experiment #2) already indicate that certain physiologically relevant stimuli can drive PKA-C dissociation from PKA-R and translocation to spines, supporting our conclusion.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *It was a pleasure reading your paper, and the results are well-executed and well-presented.*
>
> *My main and only recommendations are two ways to further expand the scope of the findings.*
>
> *First, I believe addressing the endogenous localization of PKA-C subunit before and after PKA activation would be highly important to validate these claims. Overexpression of tagged proteins often shows vastly different subcellular distribution than their endogenous counterparts. Recent technological advances with CRISPR/Cas9 gene editing (Suzuki et al Nature 2016 and Gao et al Neuron 2019 for example) which the Zhong lab recently contributed to (Zhong et al 2021 eLife) allow us to tag endogenous proteins and image them in fixed or live neurons. Any experiments targeting endogenous PKA subunits that support dissociation and synaptic localization following activation would be very informative and greatly increase the novelty and impact of their findings.*

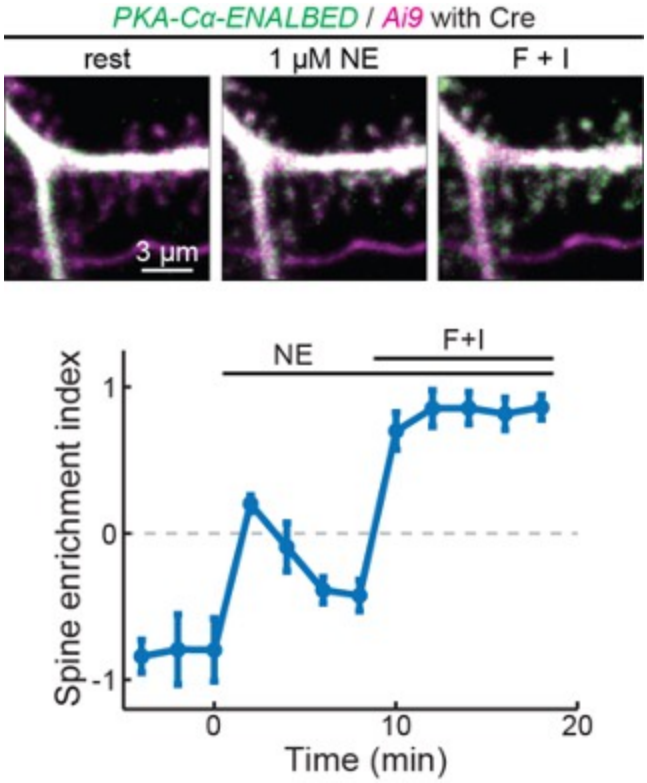
We agreed that addressing the endogenous PKA dynamics is important. However, despite recent progress, endogenous labeling using CRISPR-based methods remains challenging and requires extensive optimization. This is especially true for signaling proteins whose endogenous abundance is often low. We have tried to label PKA catalytic subunits and regulatory subunits using both the homologous recombination-based method SLENDR and our own non-homologous end joining-based method CRISPIE. We did not succeed, in part because it is very difficult to see any signal under wide-field fluorescence conditions, which makes it difficult to screen different constructs for optimizing parameters. It is also possible

that, at the endogenous abundance, the label is just not bright enough to be seen. Nevertheless, for both PKA type Iβ and type IIα that we studied in this manuscript, we have correlated the measured parameters (specifically, Spine Enrichment Index or SEI) with the overexpression level (Figure 1-figure supplement 1). We found that they are not strongly correlated with the expression level under our conditions. By extrapolating to non-overexpression conditions, our conclusion remains valid.

To overcome the inability to label endogenous PKA subunits using CRISPR-based methods, we have also attempted a conditional knock-in method call ENABLED that we previously developed to label PKA-Cα. In preliminary results, we found that endogenously label PKA were very dim. However, in a subset of cells that are bright enough to be quantified, the PKA catalytic subunit indeed translocated to dendritic spines upon stimulation (see Additional Fig. 1 in the next page), corroborating our results using overexpression. These results, however, are not ready to be published because characterization of the mouse line takes time and, at this moment, the signal-to-noise ratio remains low. We hope that the reviewer can understand.

Author response image 1.

Endogeneous PKA-Cα translocate to dendritic spines upon activation.



> *Second, experiments which would advance and validate these findings in vivo would be highly valuable. This could be achieved in a number of ways - one would be overexpression of tagged PKA versions and examining sub-cellular distribution before and after physiological activation in vivo. Another possibility is in vivo perturbation - one would speculate that disruption or tethering of PKA subunits to the dendrite would lead to cell-specific functional and structural impairments. This could be achieved in a similar manner to the in vitro experiments, with a PKA KO and replacement strategy of the tethered C-R plasmid, followed by structural or functional examination of neurons.*

> *I would like to state that these experiments are not essential in my opinion, but any improvements in one of these directions would greatly improve and extend the impact and findings of this paper.*

We thank the reviewer for the suggestion and the understanding. The suggested in vivo experiments are fascinating. However, in vivo imaging of dendritic spine morphology is already in itself challenging. The difficulty greatly increases when trying to detect partial, likely transient translocation of a signaling protein. It is also very difficult to knock down endogenous PKA while simultaneously expressing the R-C construct in a large number of cells to achieve detectable circuit or behavioral effect (and hope that compensation does not happen over weeks). We hope the reviewer agrees that these experiments would be their own project and go beyond the time and scope of the current study.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *Please elaborate on the methods used to visualize PKA-RIIα and PKA-RIβ subunits.*

As suggested, we have now included additional details for visualizing PKA-Rs in the text. Specifically, we write (pg. 5): "…, as visualized using expressed PKA-R-mEGFP in separate experiments (Figs. 1A-1C).".

https://doi.org/10.7554/eLife.93766.2.sa0