Supplementary Notes

Characterization of the fluorescence lifetime of Green-Camuiα

We characterized the fluorescence lifetime of our CaMKIIα activity sensor Green-Camuiα in lysates of HEK293 cells transfected with the sensor using 2-photon fluorescence lifetime imaging microscopy (2pFLIM; Supplementary Fig. 1).

Application of Ca^{2+} (0.4 mM) in the presence of calmodulin (CaM) and ATP lengthened the fluorescence lifetime of Green-Camuiα (Supplementary Fig. 1a). This change in fluorescence lifetime was sustained after the removal of Ca^{2+} by adding EGTA (2 mM). Both the initial and sustained phase of the fluorescence lifetime change depended on CaM, and the sustained phase required ATP (Supplementary Fig. 1b-d). Also, mutating the autophosphorylation site of Green-Camui (T286A) removed most of the sustained fluorescence lifetime change (Supplementary Fig. 1b-d). These in vitro results indicate that the sustained fluorescence lifetime change is mostly due to autophosphorylation of CaMKIIα at the T286 site. Finally, a competitive CaMKII inhibitor (10 µM KN93), but not its inactive analog (10 µM KN92) blocked the fluorescence lifetime change in a [CaM]-dependent manner (Supplementary Fig. 1b-d).

Next, to characterize the fluorescence lifetime of Green-Camuiα in neurons, we sparsely transfected CA1 pyramidal neurons of organotypic hippocampal slices with Green-Camuiα using ballistic gene transfer, and imaged the fluorescence lifetime using 2pFLIM. We activated NMDARs with bath application of NMDA (25 µM) for 5 minutes in the absence of extracellular Mg^{2+}. The fluorescence lifetime of Green-Camuiα in dendrites increased by 0.15 ± 0.02 ns, and then decayed within 30 minutes after the inhibition of NMDAR by bath application of AP5 (50 µM) (Supplementary Fig. 11). Our method was sufficiently sensitive to visualize CaMKII activation in individual spines (Supplementary Fig. 11). Inhibition of protein phosphatases with Okadaic acid (1 µM) and FK506 (40 µM) inhibited the decay of the fluorescence lifetime change after the activation (Supplementary Fig. 11). Also, a T286A mutant of Green-Camui decayed faster (Supplementary Fig. 11). These results confirmed that the fluorescence lifetime of
Green-Camui reports CaMKII activation associated with T286 phosphorylation as well as Ca\textsuperscript{2+}/CaM binding in neurons.

To check whether Green-Camui\(\alpha\) can be polymerized into a dodecamer and localize similar to endogenous CaMKII, we performed several control experiments. First, we measured the spine-dendrite coupling of Green-Camui\(\alpha\) by replacing mEGFP with photoactivatable GFP (paGFP). After photoactivation of REACh-CaMKII\(\alpha\)-paGFP in single dendritic spines, the spine fluorescence decayed with a time course similar to that of paGFP-CaMKII\(\alpha\) (Fig. 2c) and previously reported EGFP-CaMKII\(\alpha\) \(^4\). This suggests that Green-Camui\(\alpha\) interacts with the PSD and cytoskeleton to a similar degree as EGFP-CaMKII\(\alpha\), whose activity-dependent localization has been extensively studied \(^5, 6\).

Second, anti-GFP immunoprecipitation of Green-Camui\(\alpha\) co-immunoprecipitated nonlabeled CaMKII\(\alpha\) in lysates of HEK-293 cells transfected with Green-Camui\(\alpha\) and non-labeled CaMKII\(\alpha\) (1:1), indicating that Green-Camui\(\alpha\) and CaMKII\(\alpha\) can be co-assembled (Supplementary Fig. 9). Third, the diffusion constant of Green-Camui\(\alpha\) was measured in lysates of HEK-293 cells transfected with this sensor using fluctuation correlation spectroscopy \(^7\). The diffusion constant of Green-Camui\(\alpha\) was 13 ± 0.3 \(\mu\text{m}^2/\text{s}\), a value similar to that obtained for mEGFP-CaMKII\(\alpha\) (15 ± 0.3 \(\mu\text{m}^2/\text{s}\)), but a little smaller (slower) than the diffusion of CaMKII\(\alpha\) dodecamer previously measured (25 \(\mu\text{m}^2/\text{s}\)) \(^8\). This reduction is presumably due to the increase of mass caused by the addition of the fluorescent proteins (Supplementary Fig. 10). When Green-Camui\(\alpha\) was coexpressed with nonlabeled CaMKII\(\alpha\) at a 1:10 ratio, we obtained the diffusion constant of 22 ± 0.3 \(\mu\text{m}^2/\text{s}\), a value close to that of CaMKII\(\alpha\) dodecamer \(^8\) (Supplementary Fig. 10), suggesting that Green-Camui\(\alpha\) can co-polymerize with non-labeled CaMKII\(\alpha\) to form a dodecamer. The T286A Green-Camui\(\alpha\) mutant acted as dominant negative for LTP induction in a slice culture (Fig. 1c, f), suggesting that a mutation in Green-Camui\(\alpha\) can affect the function of a CaMKII holoenzyme. From these results, we concluded that Green-Camui\(\alpha\) can be incorporated into a CaMKII holoenzyme, and thus behaves similarly to endogenous CaMKII.
Effects of the inclusion of Green-Camuia in a holoenzyme

To test if our measurements of the activation and the mobility of CaMKII are affected by the inclusion of Green-Camuia in a holoenzyme, we changed the number of Green-Camuia in a holoenzyme by co-transfecting Green-Camuia together with non-labeled CaMKIIα and CaMKIIβ (2:1:1). Consistent with the low expression level of Green-Camuia (see below; Supplementary Fig. 2), the kinetics and amplitude of the fluorescence lifetime change under this condition were similar to those measured in neurons expressing Green-Camuia alone (Supplementary Fig. 5a). The mobility of the mixture measured by FRAP was also similar to that of Green-Camuia (Supplementary Fig. 5b, c). Thus, the measured activity kinetics and the mobility of CaMKII in our experiments are likely to be similar to those of endogenous CaMKII holoenzymes.

Measurements of the expression level of Green-Camuia

Overexpression of Green-Camuia might change the activation kinetics and mobility of this molecule. Also, if inter-subunit or inter-molecule FRET occurs, the fluorescence lifetime may depend on the overexpression level. Thus, we measured the expression level of Green-Camuia compared to endogenous CaMKIIα using immuno-fluorescence (Supplementary Fig. 2). We identified transfected cells with green channel, and measured the immuno-fluorescence (Texas-red) in the transfected cell (\(I_{\text{Transfected}}^{\text{Texas-red}}\)) and average of 2 – 4 adjacent non-transfected cells (\(I_{\text{Untransfected}}^{\text{Texas-red}}\)) with red channel under 2-photon microscopy. Then, their ratio \(\left(I_{\text{Transfected}}^{\text{Texas-red}} / I_{\text{Untransfected}}^{\text{Texas-red}}\right)\) was calculated as the relative immuno-fluorescence in transfected cells to be 1.25 ± 0.09 for Green-Camuia (\(N = 18\)) and 1.39 ± 0.17 for mEGFP-CaMKII (\(N = 11\)) (Supplementary Fig. 2). The expression level of exogenous CaMKIIα compared to endogenous CaMKIIα was calculated as:

\[
\text{Overexpression level} = \frac{I_{\text{Transfected}}^{\text{Texas-red}}}{I_{\text{Untransfected}}^{\text{Texas-red}}} - 1.
\]

Because some portion of the immuno-fluorescence could be due to non-specific immuno-reaction, this overexpression level is the lower limit. To estimate the non-specific
immuno-staining, we also measured immuno-fluorescence in neurons expressing short hairpin- (sh-) RNA against CaMKII\(\alpha\) \((I_{\text{shRNA}})\) compared to non-transfected cells. The upper limit of the relative proportion of non-specific immuno-fluorescence was calculated as \(I_{\text{shRNA}} / I_{\text{Untransfected}} = 0.50 \pm 0.05\ (N = 22; \text{Supplementary Fig. 2})\). Thus, overexpression level was calculated as:

\[
\left(\frac{I_{\text{Transfected}}}{I_{\text{Untransfected}}} - 1\right)/\left(1 - \frac{I_{\text{shRNA}}}{I_{\text{Untransfected}}}\right) \times \text{Overexpression level} = \frac{I_{\text{Transfected}}}{I_{\text{Untransfected}}} - 1.
\]

From this equation, the expression level compared to endogenous CaMKII\(\alpha\) is obtained to be 25 – 50 % and 40 – 80 % for Green-Camu\(\alpha\) and mEGFP-CaMKII\(\alpha\), respectively. We also measured the concentration of Green-Camu\(\alpha\) and mEGFP-CaMKII\(\alpha\) in the same neurons using the fluorescence intensity of mEGFP at the thick dendrite compared to purified mEGFP (see Methods). The concentration of Green-Camu\(\alpha\) and mEGFP-CaMKII\(\alpha\) was measured to be 20 ± 4 µM \((N = 18)\) and 19 ± 4 µM \((N = 11)\), respectively. This suggest that endogenous CaMKII level ([fluorescent CaMKII] / Overexpression level) is ~10 – 100 µM.

We also measured the concentration of Green-Camu\(\alpha\) used for glutamate uncaging (Supplementary Fig. 4). The concentration was 14.6 ± 4.3 µM for wildtype Green-Camu\(\alpha\) \((N = 11)\), 5.8 ± 1.8 µM for T286A mutant \((N = 9)\) and 21.9 ± 5.7 µM for T305D mutant \((N = 7)\). Because the overexpression level of T286A mutant was ~40 % as high as that of wild type, the overexpression level of T286A mutant is estimated to be 10 – 20 %.

References
1. Takao, K., et al. Visualization of synaptic Ca\(^{2+}\)/calmodulin-dependent protein kinase II activity in living neurons. \textit{J. Neurosci.} \textbf{25}, 3107-3112 (2005).
2. Sumi, M., et al. The newly synthesized selective Ca\textsuperscript{2+}/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. *Biochem. Biophys. Res. Commun.* **181**, 968-975 (1991).

3. McAllister, A.K. Biolistic transfection of neurons. *Sci. STKE* **2000**, PL1 (2000).

4. Okamoto, K., Narayanan, R., Lee, S.H., Murata, K. & Hayashi, Y. The role of CaMKII as an F-actin-bundling protein crucial for maintenance of dendritic spine structure. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 6418-6423 (2007).

5. Shen, K. & Meyer, T. Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. *Science* **284**, 162-166 (1999).

6. Otmakhov, N., et al. Persistent accumulation of calcium/calmodulin-dependent protein kinase II in dendritic spines after induction of NMDA receptor-dependent chemical long-term potentiation. *J. Neurosci.* **24**, 9324-9331 (2004).

7. Thompson, N.L., Lieto, A.M. & Allen, N.W. Recent advances in fluorescence correlation spectroscopy. *Curr. Opin. Struct. Biol.* **12**, 634-641 (2002).

8. Gaertner, T.R., et al. Comparative analyses of the three-dimensional structures and enzymatic properties of alpha, beta, gamma and delta isoforms of Ca\textsuperscript{2+}-calmodulin-dependent protein kinase II. *J. Biol. Chem.* **279**, 12484-12494 (2004).

9. Matsuzaki, M., Honkura, N., Ellis-Davies, G.C. & Kasai, H. Structural basis of long-term potentiation in single dendritic spines. *Nature* **429**, 761-766 (2004).

10. Sobczyk, A. & Svoboda, K. Activity-dependent plasticity of the NMDA-receptor fractional Ca\textsuperscript{2+} current. *Neuron* **53**, 17-24 (2007).

11. Chen, Y., Muller, J.D., Ruan, Q. & Gratton, E. Molecular brightness characterization of EGFP in vivo by fluorescence fluctuation spectroscopy. *Biophys. J.* **82**, 133-144 (2002).
Supplementary Fig. 1: Characterization of fluorescence lifetime of Green-Camuiα in vitro

**a.** Fluorescence lifetime curves of Green-Camuiα before and after Ca\(^{2+}\) application in lysate of Hela cells expressing Green-Camuiα.

**b.** Fluorescence lifetime of Green-Camuiα in response to application of Ca\(^{2+}\) (0.5 mM) and EGTA (2 mM) at times indicated by arrows in the graph. Error bars are s.e.m. of 3 measurements from 3 different preparations. CaM and ATP concentrations are indicated in micromolar. Note that KN93 (10 µM) blocks the fluorescence lifetime change at [CaM] = 0.2 µM, but not at [CaM] = 1 µM, because KN93 is a competitive inhibitor against CaM binding to CaMKII\(^{2+}\). KN92, an inactive analog of KN93, did not affect the fluorescence lifetime change.

**c.** Change in fluorescence lifetime due to Ca\(^{2+}\) application (3.5 min after Ca\(^{2+}\) application).

**d.** Sustained change in fluorescence lifetime after EGTA application (15.5 min after Ca\(^{2+}\) application).
Supplementary Fig. 2. Immuno-fluorescence of CaMKIIα in hippocampal slices.

a, Immuno-fluorescence of CaMKIIα in cultured slices transfected with shRNA against CaMKIIα, Green-Camuα or mEGFP-CaMKIIα.

b, Quantification of immuno-fluorescence in transfected cells compared to adjacent non-transfected cells.
Supplementary Fig. 3. Green-Camuía enrichment into spines during LTP
2-photon fluorescence measured in neurons transfected with Green-Camuía (a) or mEGFP-CaMKIIα (b) together with mCherry. Green and red fluorescence were simultaneously measured in the stimulated spine during structural plasticity of spine induced by 2-photon uncaging in the absence of extracellular Mg$^{2+}$. 

Supplementary Fig. 3
Supplementary Fig. 4. Green-Camuiα activation during spine volume change.

**a**, Averaged time course of fluorescence lifetime change of Green-Camuiα (wildtype [WT], T286A and T305D) in the stimulated spine. The data were obtained from a different set of experiments from Fig. 1. The number of samples (spines / neurons) is 31 / 11 for WT, 34 / 9 for T286A, and 23 / 7 for T305D.

**b**, Averaged time course of spine volume change in stimulated spines and adjacent spines. We did not measure the full time course for these experiments.

**c**, CaMKII activation (average over 0 – 40 s). Stars denote statistical significance ($p < 0.05$) between wildtype and mutants.

**d**, Transient volume change (volume change averaged over 1.5 – 2 min subtracted by that averaged over 25 – 30 min).

**e**, Sustained volume change (volume change averaged over 25 – 30 min).

**f**, Expression level of Green-Camuiα measured using green fluorescence.
Supplementary Fig. 5. Effects of the inclusion of Green-Camuiα in a holoenzyme

a, CaMKII activation and inactivation following brief (16 s) stimulation of spines in zero extracellular Mg\(^{2+}\). Measurements were performed in neurons overexpressing Green-Camuiα only and Green-Camuiα plus CaMKIIα and CaMKIIβ (cDNA ratio = 2:1:1). Data for Green-Camuiα only is from Fig 2b. Data were fitted with double exponential functions. The time constants were 4.7 s (61 %) and 136.6 s (39 %) for Green-Camuiα only and 4.8 s (70 %) and 154 s (30 %) for mixture. The number of samples (spines / neurons) is 16 / 8 for Green-Camuiα only and 16 / 3 for mixture.

b, Time-lapse images of Green-Camuiα fluorescence during fluorescence recovery after photo-bleaching (FRAP). The spine indicated by the arrowhead was bleached at time zero.

c, FRAP of neurons overexpressing Green-Camuiα only and Green-Camuiα plus CaMKIIα and CaMKIIβ (cDNA ratio = 2:1:1).
Supplementary Fig. 6. Calcium transients during glutamate uncaging paired with depolarization.

a. Images of a cell loaded with a Ca\(^{2+}\)-insensitive red dye (0.2 mM Alexa-594; excited at 920 nm) and a Ca\(^{2+}\)-sensitive green dye (0.5 mM Fluo-4FF) before and after depolarization and subsequent glutamate uncaging (0.5 Hz). Images were acquired every 32 ms. Green and red images were averaged over 3 and 30 frames, respectively. Images were further smoothed (6 x 6 pixels). The numbers indicate the time elapsed from
depolarization. For glutamate uncaging, images were averaged over the first four trials (uncaging at 16, 18, 20 and 22 s. The time for the first trial is indicated). Extracellular [Mg^{2+}] is 1 mM.

b, [Ca^{2+}] transients in the stimulated spine and parent dendrite in response to cell depolarization and subsequent glutamate uncaging (30 times). The intensity was measured on non-filtered images. Average of 13 spines from 6 neurons.
c, Close view of [Ca^{2+}] transients in response to depolarization and 1st, 2nd, 29th and 30th stimulation. Decrease of Ca^{2+} transients over time may be due to Ca^{2+} dependent depression of NMDARs.
d, Average [Ca^{2+}] transients in response to cell depolarization in spines (left) and dendrites (right) in the absence (closed) or presence (open) of Nimodipine, L-type calcium channel blocker in spines (left) or dendritic shaft (right) (5 spines and dendrites from 5 neurons, interleave experiments).

Supplementary Fig. 7

Supplementary Fig. 7: Effect of whole-cell patch on CaMKII activation. Fluorescence lifetime change of Green-Camuia in spines in response to glutamate uncaging (6 ms, 4 mW, 45 pulses, 0.5 Hz) before and after (> 15 min) whole-cell patch clamp (same neurons). Experiments were performed using K+ internal solution in a current-clamp mode. Number of spines: 13 (before patch), 13 (after patch). Number of neurons: 4.
Supplementary Fig. 8

Supplementary Fig. 8. Dependency of uncaging evoked postsynaptic current (uEPSC) on the position of uncaging laser.

a, b, Location of laser spot to uncage glutamate (circles, a) and corresponding postsynaptic current evoked by 4 ms laser pulse (b). Current in b was averaged over 4 uncaging trials, and filtered with 2 ms window. In other experiments, we locate the laser spot at the tip of a spine (white circle in a).

b, c, Peak uEPSC (average of 6 ms around the peak) normalized to zero distance. 4 spines from 4 neurons.

Supplementary Fig. 9

Supplementary Fig. 9: Incorporation of Green-Camuia in CaMKII holoenzyme

Immunoblot analysis of anti-GFP immuno-precipitates using anti-CaMKII antibody in HEK-293 cells expressing Green-Camuia and CaMKIIa (1:1). Lanes 1-4 are the input (10%) for lanes 5-8. Lane 8 is anti-non-immune IgG immunoprecipitates as a negative control.
Supplementary Fig. 10: Fluctuation correlation spectroscopy (FCS) analysis of Green-Camuiα and mEGFP-CaMKIIα.

a, FCS curve under 2-photon FCS for mEGFP, mEGFP-CaMKIIα, and Green-Camuiα in lysate of HEK293 transfected with these proteins. Black curves are fitting with:

\[ G(\tau) = G(0) \left[ 1 + \left( \frac{\tau}{\tau_D} \right) \right]^{-1} \left[ 1 + \left( \frac{w_{xy}}{w_z} \right)^2 \left( \frac{\tau}{\tau_D} \right) \right]^{-1/2} \]

where \( G(0) \) is the correlation at time 0, \( \tau \) is the correlation time, \( w_{xy} \) (0.42 µm) and \( w_z \) (1.46 µm) is the radial and axial \( 1/e^2 \) radii of the excitation volume, and \( \tau_D \) is the diffusion correlation time constant. The diffusion coefficient \( D \) was obtained from \( \tau_D \) as

\[ D = \frac{w_{xy}^2}{8\tau_D} \]

The FCS curves were normalized to \( G(0) \). We obtained \( D = 74 \pm 2 \) µm\(^2\)/s for mEGFP, \( 13 \pm 0.3 \) µm\(^2\)/s for EGFP-CaMKIIα, \( 15 \pm 0.3 \) µm\(^2\)/s for Green-Camuiα. The value for mEGFP is consistent with previous measurements\(^{11}\).

b, Diffusion coefficient of Green-Camuiα or mEGFP-CaMKIIα co-transfected with non-labeled CaMKIIα with various cDNA ratios.
Supplementary Fig. 11. Characterization of Green-Camui in neurons
Fluorescence lifetime of Green-Camui in dendrites of pyramidal neurons in cultured hippocampal slices. Top panels: Fluorescence lifetime images of dendrites. Bottom panel: Changes in fluorescence lifetime of Green-Camui (WT; N = 10) and T286A mutant Green-Camui (T286A; N = 8). The stimulation was started by application of NMDA and stopped by 50µM AP5. Application of AP5 before NMDA abolished the signal (AP5; N = 6). Incubation of slices with phosphatase inhibitors 1 µM Okadaic acid and 40 µM FK-506 (OK + FK; N = 5) for 2 hours prior to the stimulation abolished the decay of the fluorescence lifetime change.