Genetically Encoded Green Fluorescent Ca\textsuperscript{2+} Indicators with Improved Detectability for Neuronal Ca\textsuperscript{2+} Signals

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Abstract

Imaging the activities of individual neurons with genetically encoded Ca\textsuperscript{2+} indicators (GECIs) is a promising method for understanding neuronal network functions. Here, we report GECIs with improved neuronal Ca\textsuperscript{2+} signal detectability, termed G-CaMP6 and G-CaMP8. Compared to a series of existing G-CaMPs, G-CaMP6 showed fairly high sensitivity and rapid kinetics, both of which are suitable properties for detecting subtle and fast neuronal activities. G-CaMP8 showed a greater signal ($F_{\text{max}}/F_{\text{min}} = 38$) than G-CaMP6 and demonstrated kinetics similar to those of G-CaMP6. Both GECIs could detect individual spikes from pyramidal neurons of cultured hippocampal slices or acute cortical slices with 100% detection rates, demonstrating their superior performance to existing GECIs. Because G-CaMP6 showed a higher sensitivity and brighter baseline fluorescence than G-CaMP8 in a cellular environment, we applied G-CaMP6 for Ca\textsuperscript{2+} imaging of dendritic spines, the putative postsynaptic sites. By expressing a G-CaMP6-actin fusion protein for the spines in hippocampal CA3 pyramidal neurons and electrically stimulating the granule cells of the dentate gyrus, which innervate CA3 pyramidal neurons, we found that sub-threshold stimulation triggered small Ca\textsuperscript{2+} responses in a limited number of spines with a low response rate in active spines, whereas supra-threshold stimulation triggered large fluorescence responses in virtually all of the spines with a 100% activity rate.

Introduction

Understanding brain function requires techniques for monitoring the spatio-temporal activity patterns of individual neurons and synapses. A promising approach for this purpose is Ca\textsuperscript{2+} imaging that can detect neuronal events as a change in Ca\textsuperscript{2+} fluorescence intensity. Recently, Ca\textsuperscript{2+} imaging using green fluorescent protein (GFP)-based genetically encoded Ca\textsuperscript{2+} indicators (GECIs) has been introduced as an alternative to using chemically synthesized fluorescent Ca\textsuperscript{2+} indicators [1–6]. GECIs offer two remarkable advantages over synthesized indicators: (i) GECIs can be targeted to specific cell types and specific subcellular compartments [7–10], and (ii) GECIs are applicable to long-term expression (over months) [4,11–13]. Although GECIs have improved, there remains a need for GECIs with greater signals and more rapid kinetics to allow the reliable detection of individual neuronal spikes.

In this study, we developed high-sensitivity and fast-respondivity GECIs, termed G-CaMP6 and G-CaMP8, by mutating existing G-CaMPs. These novel indicators allow us to reliably monitor neural spikes with larger fluorescence signals and higher temporal resolution than G-CaMP3, a recently reported variant of G-CaMP2 [4]. We also demonstrate that G-CaMP6-actin, a fusion protein of G-CaMP6 and actin, can be used to image spine-specific Ca\textsuperscript{2+} signals in response to presynaptic single spikes at the single-synapse level.

Results

Development of Improved G-CaMPs by Site-directed and Random Mutagenesis

In an effort to create a superior GECI, we first introduced mutations from “superfast GFP” [14], which was recently reported to enhance the folding activity of GFP, into a prototype GECI, G-CaMP2 [15], because some known folding mutations improve the functionality of GECIs [16,17]. Through screening, we found that a G-CaMP2 variant with two mutations (N105Y and E124V) introduced into a prototype GECI showed a greater dynamic range ($F_{\text{max}}/F_{\text{min}} = 9.03 \pm 0.06, n = 3$) than G-CaMP2 [15] ($F_{\text{max}}/F_{\text{min}} = 4.0$) (Fig. 1B). For further improvement, mutations known to stabilize the chromophore [i.e., T203V in the calmodulin (CaM) domain] were introduced into sfG-CaMP2 [18], and this variant was termed sfG-CaMP2.02 (Fig. 1A), sfG-CaMP2.02 showed a greater signal increase ($F_{\text{max}}/F_{\text{min}} = 14.0 \pm 0.28, n = 3$)
transients did not differ among G-CaMP3, G-CaMP5.09, and G-CaMP7 
and G-CaMP8, unlike G-CaMP6 and other variants, was greater than that of G-CaMP3. The baseline fluorescence of
G-CaMP7 was similar to that of neurons expressing G-CaMP3, whereas G-
CaMP3-[4] were introduced into sfG-CaMP2.02 to examine
the expression of G-CaMPs and mCherry was driven by the CMV
promoter following transfection of the cells with the construct via
targeted single-cell electroporation [20]. Simultaneous patch-
clamp recording and confocal Ca²⁺ imaging were performed on
G-CaMP-expressing neurons 24–48 h after electroporation. The baseline
fluorescence of the neurons expressing G-CaMP6 (Fig. 2A) was similar to that of neurons expressing G-CaMP3, whereas G-
CaMP8-expressing neurons exhibited lower fluorescence intensity
than those expressing the other G-CaMPs (Fig. 2B). To monitor spike-induced Ca²⁺ responses, the neurons were current-injected
to evoke 1–6 spikes at a frequency of 50 Hz. All experiments were
conducted at room temperature (25–28°C), unless otherwise
specified. G-CaMP6 and G-CaMP8 responded to single spikes
with 100% probability. The ΔF/F amplitudes of Ca²⁺ transients
evoked by single spikes were 17.4±2.35%, 27.9±4.5%, and 37.8±5.2%,
and the signal-to-noise ratios (SNRs) were 8.0±1.5, 18.3±1.5, and 16.4±3.5 for G-CaMP3, G-CaMP6 and G-
CaMP8, respectively (Fig. 2C and D; n=7 each). The signal
amplitudes grew almost linearly as the spike number increased
(Fig. 2C and D). Over the entire stimulus range, the amplitudes of
the Ca²⁺ transients and the SNRs of G-CaMP6 and G-CaMP8 were consistently higher than those of G-CaMP3. The rise time of the spike-induced Ca²⁺ transients did not differ among G-CaMP3,
G-CaMP6 and G-CaMP8 (P<0.05, Tukey’s test). On the other
hand, the signal decay of G-CaMP6 and G-CaMP8 was significantly faster than that of G-CaMP3 (G-CaMP3, decay τ₁/, |
τ₂/ = 638±38 ms; G-CaMP6, decay τ₁/ = 457±20 ms; G-CaMP8,
decay τ₁/ = 428±11 ms; Tukey’s test; n=7 each) (Fig. 2E). The rapid
kinetics and the fairly high Ca²⁺ sensitivity (Fig. 1B and C) of G-
CaMP6 contributed to an increased temporal resolution of the
signals within spike trains up to 15–20 Hz (Fig. 2F).

The detectability of G-CaMPs was also evaluated in pyramidal
neurons in acute cortical slices. The expression of G-CaMPs in
the mouse brain was driven by in utero electroporation, as previously
described [21]. Consistent with the results presented in Figure 2C and
D, G-CaMP6 performed better than G-CaMP3 in acute
cortical slices prepared from mice at postnatal day 10–16 (Fig. 3).
This result also implies that G-CaMP6 can be stably expressed in
neurons for at least 4 weeks.

It is known that temperature significantly affects spike-de-
dependent Ca²⁺ transients: they are small and short near physiologi-
ical temperature because Ca²⁺ is removed quickly by strong Ca²⁺-
pumping activity [22]. Here, the ΔF/F value of G-CaMP6 was
~40% lower at 37°C than at 25–28°C (Fig. 4). The kinetics of G-
CaMP6 were faster at 37°C (rise τ₁/ = 51.7±2.13 ms, decay τ₁/
τ₂/ = 402±15.1 ms; n=6) than at 25–28°C (rise τ₁/
τ₂/ = 62.0±6.52 ms, decay τ₁/ = 458±23.7 ms; n=6) (Fig. 2E).

By contrast, it was confirmed that the expression of G-CaMP6
does not affect the electrophysiological properties [i.e., input
resistance, membrane capacitance, resting potential, excitatory
postsynaptic current (EPSC) amplitude, and EPSC frequency] of
hippocampal neurons (Fig. 5).

Imaging of Ca²⁺ Activity in Freely Moving Caenorhabditis elegans

We also tested G-CaMP6 in C. elegans and successfully recorded
spontaneous Ca²⁺ transients in A-type cholinergic motoneurons of
freely moving L1 worms. The peak responses (ΔAR/R) of G-CaMP6
during locomotion (ΔAR/R = 3.85±0.20, n=10 from 4 worms)
were 1.6-fold greater than those of G-CaMP3 (ΔAR/
R = 2.42±0.23, n=10 from 4 worms) (Fig. 6, Movies S1 and S2).

Imaging of Spine Ca²⁺ Activity with G-CaMP6-actin

Next, we targeted G-CaMP6 to dendritic spines, the putative
synaptic sites, to reveal the dynamics of individual spine activities.
For this purpose, G-CaMP6 was fused with actin, a major
cytoskeletal protein within spines, to yield G-CaMP6-actin (Fig.
7A). G-CaMP6-actin was effectively localized to the spines in
rat hippocampal CA3 pyramidal neurons (Fig. 7B and C), as has been
reported for EGFP-actin and G-CaMP2-actin [8]. We then
electrically stimulated the granule cells of the dentate gyrus, which

Comparison of G-CaMPs in Pyramidal Neurons

We next evaluated the performance of G-CaMPs in pyramidal
neurons in cultured rat hippocampal slices. In the cultured slices,
the expression of G-CaMPs and mCherry was driven by the CMV
promoter following transfection of the cells with the construct via
targeted single-cell electroporation [20]. Simultaneous patch-
clamp recording and confocal Ca²⁺ imaging were performed on
G-CaMP-expressing neurons 24–48 h after electroporation. The baseline
fluorescence of the neurons expressing G-CaMP6 (Fig. 2A) was similar to that of neurons expressing G-CaMP3, whereas G-
innervate synapses in the striatum lucidum of CA3 region, with signals of two different strengths (Fig. 7B). Intriguingly, the sub-threshold stimulations ($D_{V_m} = 18.5 \pm 4.8$ mV) triggered small fluorescence responses ($DF/DF = 337 \pm 86\%$, $n = 256$ responses of 63 spines from 5 slices) in a limited number of spines (48.6 \pm 6.3\%) in the striatum lucidum, with a low response rate in the active spines (57.6 \pm 13.8\%) (Fig. 7D and E). In contrast, the supra-threshold stimulations triggered large fluorescence responses ($DF/DF = 443 \pm 182\%$, $n = 222$ responses of 131 spines from 5 slices) in virtually all of the spines in the imaged region including the striatum lucidum and the striatum radiatum, with a 100% activity rate (Fig. 7D and E).

One of the significant advantages of GECIs over chemically synthesized fluorescent indicators is that once indicator genes have been introduced into neurons, the stable expression of the indicator proteins allows long-term recording of the neurons [4,11–13]. To test whether G-CaMP6-actin is applicable to long-term monitoring, Ca$^{2+}$ activity was imaged in spines in slices cultured for 8 and 29 days. After 29 days in vitro (Div), the amplitudes of spine Ca$^{2+}$ transients in response to supra-threshold stimulation were not significantly different from those at 8 Div (253 \pm 30.5\% and 201 \pm 46.6\% at 8 Div and 29 Div, respectively; $n = 25$ spines, $P > 0.05$, Student’s $t$-test). These results confirmed that the expression of G-CaMP6-actin in spines remained stable after at least 4 weeks of culture (Fig. 8).

Figure 1. Characterization of G-CaMPs in vitro and in HeLa cells. A, Schematic representation. Mutations are indicated with respect to G-CaMP2. RSET and M13 are tags that encode hexahistidine and a target peptide for Ca$^{2+}$-bound CaM derived from MLCK, respectively. The amino-acid numbers of EGFP and CaM are indicated in parentheses. B, Dynamic range ($F_{max}/F_{min}$) and Ca$^{2+}$ affinity ($K_d$). Error bars, s.d. ($n$ = 3 each). C, Ca$^{2+}$ titration curve. Curves were fit according to the Hill equation. $K_d$ is shown in B. D, Normalized fluorescence and absorbance (inset) spectra of G-CaMP6 and G-CaMP8 in 1 $\mu$M Ca$^{2+}$ or 1 mM EGTA. E, Fluorescence images of HeLa cells expressing G-CaMPs. Bars, 30 $\mu$m. F, Time course of the changes ($DF/DF$) in G-CaMP fluorescence in response to 100 $\mu$M ATP. Error bars, s.d. G, Baseline fluorescence and peak responses ($DF/DF$) to ATP application in HeLa cells.

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Discussion

In this study, we developed high-sensitivity and fast-responsivity GECIs, termed G-CaMP6 and G-CaMP8, by introducing site-directed and random mutations into a prototype GECI, G-CaMP2. Both indicators showed superior performance for reliable detection of neuronal activity with larger fluorescence signals and higher temporal resolution than G-CaMP3. In addition, G-CaMP6-actin captured spine Ca\(^{2+}\) dynamics in response to the stimulation of presynaptic afferent fibers.

In the course of developing these superior G-CaMPs, we found three novel mutations for improving the GECI functionality [i.e., DH mutation in the RSET domain (in G-CaMP7 and G-CaMP8) and S205N (in G-CaMP7 and G-CaMP8) and I47F (in G-CaMP8) mutations in the circularly permutated EGFP domain]. Based on the G-CaMP2 structure, the residue Ser-205 is located in the \(\beta\)-strand of the circularly permuted EGFP domain (corresponding to the tenth \(\beta\)-strand in EGFP) and facing the inside of the chromophore [18]. This residue has been shown to interact with the chromophore in Ca\(^{2+}\)-saturated G-CaMP2 [18]. By contrast, the residue Ile-47 is located in the \(\beta\)-strand of the circularly permutated EGFP domain (corresponding to the third \(\beta\)-strand in EGFP) and facing the outside of the chromophore [18]. In addition, this residue is apart from the M13 domain and the CaM domain. Topology of the DH position in the RSET domain is unknown, because the available structural analyses of G-CaMPs based on crystallography have been performed using G-CaMP2 without the RSET domain [23] or with another tag [18]. The AR2 mutation has been known to enhance the G-CaMP fluorescence in cells by stabilizing the protein [4], but G-CaMP8

![Image](image_url)

Figure 2. Characterization of G-CaMPs in cultured hippocampal slices. A, Expression of G-CaMP6 in hippocampal CA3 pyramidal neurons. Inset: Higher-magnification views are shown in the bottom panels. B, Baseline fluorescence of hippocampal neurons expressing G-CaMP3, G-CaMP6 and G-CaMP8. No significant differences in variance were detected among the three groups (\(P > 0.05\), \(\chi^2 = 2.90\), Bartlett’s test). Error bars, s.d. (\(n = 7\) each, \(P < 0.05\), Tukey’s test). C, Representative traces of the response (\(\Delta F/F\)) to spike trains. The frequency of stimuli was 50 Hz. Right: Magnified views of single spikes. D, Mean responses (\(\Delta F/F\)) and SNRs of G-CaMP3 (black), G-CaMP6 (red) and G-CaMP8 (blue). Inset: Magnified views of 1–2 spikes. Error bars, s.e.m. (\(n = 7\) each). E, Rise and decay time constants for the responses to single spikes. Error bars, s.e.m. (\(n = 7\) each; \(^* P < 0.05\) in Tukey’s post-hoc test following one-way ANOVA). F, Trial-averaged responses of G-CaMP6 to spike trains. Gray, individual traces (\(n = 10\) trials); red, averaged traces. Bars indicate stimulus timing. Inset: Magnified views.

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Figure 3. Comparison of G-CaMP responses in acute cortical slices. A, Confocal image of G-CaMP6-expressing cortical pyramidal cells. The expression of G-CaMP6 was driven by the CAG promoter via in utero plasmid electroporation. Inset: Higher-magnification views are shown in the right panels. B, Representative \(\Delta F/F\) traces in response to 1–4 spikes evoked at 50 Hz. C, Mean responses (\(\Delta F/F\)) of G-CaMP3 and G-CaMP6 to spike trains. Error bars, s.e.m. (G-CaMP3, \(n = 4\) cells; G-CaMP6, \(n = 5\) cells).

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bearing this mutation did not show brighter fluorescence than G-CaMP7 in a cellular environment (Fig. 1E and G).

Recently, Akerboom et al. have reported new series of GECIs termed G-CaMP5s [6]. Among these indicators, they have demonstrated that G-CaMP5A, 5G and 5K outperform G-CaMP3 in a wide variety of neuronal preparations. G-CaMP5G, which shows ~3-fold greater dynamic range ($F_{\text{max}}/F_{\text{min}} = 32.7 \pm 1.5$) than G-CaMP3 ($F_{\text{max}}/F_{\text{min}} = 12.3 \pm 0.4$), is the variant which responds with the greatest signals among G-CaMP5s to maximal stimulation when expressed in cultured neurons. Indeed G-CaMP5G is reported to show ~70% greater signals ($D_F/F$) than G-CaMP3 in response to 1–5 spike trains, but its SNR is not improved with respect to that of G-CaMP3 [6]. Besides, the decay kinetics of G-CaMP5G seems to be almost the same as that of G-CaMP3, judging from the shape of trial-averaged responses of G-CaMP5G and G-CaMP3 (Fig. 2B of [6]). In contrast, G-CaMP8, of which dynamic range ($F_{\text{max}}/F_{\text{min}} = 37.5 \pm 3.6$) is similar to that of G-CaMP5G, shows ~100% greater signals than G-CaMP3 in terms of both $D_F/F$ and SNR (Fig. 2C and D) and ~2-fold more rapid decay kinetics than G-CaMP5 (Fig. 2E). On the other hand, a drawback of G-CaMP8 is its dim baseline fluorescence in neurons, which needs to be improved in the future. G-CaMP5K is the most sensitive G-CaMP5 variant ($K_d = 189 \pm 5.0$ nM) [6] and is likely to be useful for detecting small neuronal Ca$^{2+}$ signals, similar to G-CaMP6 ($K_d = 158 \pm 4.0$ nM) (Fig. 1B and C). G-CaMP5K is reported to show ~2-fold greater signals ($D_F/F$ and SNR) than G-CaMP3 in response to 1–5 spike trains [6]. G-CaMP5A is the variant with intermediate sensitivity ($K_d = 307 \pm 12$ nM) and signal amplitudes ($F_{\text{max}}/F_{\text{min}} = 17.4 \pm 1.2$) among G-CaMP5s, but is reported as the

Figure 4. Temperature dependence of G-CaMP6 signals. A, Representative traces of the fluorescence response ($\Delta F/F$) of G-CaMP6 to a single spike at 25–28°C and at 37°C. B, Mean responses ($\Delta F/F$) of G-CaMP6 to spike trains. Error bars, s.e.m. ($n = 6$ each). C, Rise and decay time constants of the responses of G-CaMP6 to single spikes. (*$P<0.05$, paired t-test).

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Figure 5. Electrophysiological properties of hippocampal neurons expressing G-CaMP6. A, Left, input resistance. Middle, membrane capacitance. Right, resting potential. Error bars, s.e.m. ($n = 6$ each). There were no significant differences between the control and G-CaMP6 groups for any of the parameters ($P>0.05$, Student’s t-test). B, Left, spontaneous current under the voltage clamp at –70 mV. Middle, amplitude of the excitatory postsynaptic current. Right, frequency of the excitatory postsynaptic current. Error bars, s.e.m. ($n = 6$ each, $P>0.05$, Student’s t-test).

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preferred variant over G-CaMP5G and G-CaMP5K for use in worm and zebrafish [6]. It is good for researchers to have the option to select the ideal GECI depending on their own applications. Because new G-CaMPs (G-CaMP6 and G-CaMP8) and G-CaMP5s have been optimized by different strategies, it may be possible to combine the mutations in the different sets of G-CaMPs to further improve them.

The detection of neuronal activity patterns with single-spike resolution is required to elucidate neural network dynamics. We demonstrated that G-CaMP6 and G-CaMP8 faithfully detected Ca\(^{2+}\) transients in response to single spikes in pyramidal neurons in hippocampal slices at 25–28°C. However, it is still unknown whether these G-CaMPs exhibit similar performance in vivo. As shown in Fig. 4, both the dynamics of intracellular Ca\(^{2+}\) and the sensitivity of Ca\(^{2+}\) indicators are temperature dependent. Indeed, it has been reported that GECI fluorescence is less intense in vivo compared to in vitro [4,24]. Another point to note is that the detectability of indicators might be affected by the expression levels of indicator proteins. Therefore, further studies are needed to determine whether similar results can be obtained in the other gene expression systems, such as transgenic mouse lines or viruses. The decay time constant of spike-induced Ca\(^{2+}\) transients of the newly-developed G-CaMPs ranged between 400 and 450 ms, which is shorter than that of G-CaMP3 [4]. We demonstrated that the rapid kinetics of Ca\(^{2+}\) indicators contribute to discrete fast individual spikes in burst-spike trains with a temporal resolution of up to 15 Hz. To our knowledge, G-CaMP6 is the most suitable

Figure 6. Ca\(^{2+}\) imaging of cholinergic DA motoneurons in freely moving C. elegans. A, Confocal images of L1 larvae expressing G-CaMP6 (jqEx97) or G-CaMP3 (jqEx216) in the DA motoneurons. In both transgenic strains, DsRed-Express-1 is co-expressed in the DA motoneurons. TL, transmitted-light image. Arrows indicate the DA7 motoneuron analyzed in B. B, Representative spontaneous fluorescence responses (\(\Delta R/R\)) of G-CaMPs from DA7 cholinergic neurons in transgenic worms during locomotion. C, Mean peak responses (\(\Delta R/R\)). Error bars, s.e.m. (\(n=10\) each from 4 worms, *\(P=0.0020\), Student’s t-test). Movies of the recordings are available as supplementary information (Movies S1 and S2). doi:10.1371/journal.pone.0051286.g006
GECI currently available for detecting and isolating fast individual spikes in spike trains. Excitatory synaptic activity induces a transient \( \text{Ca}^{2+} \) increase in individual spines through the activation of voltage-sensitive \( \text{Ca}^{2+} \) channels and/or NMDA receptors. In previous studies, spine \( \text{Ca}^{2+} \) activity was imaged with synthetic indicators, such as Oregon Green BAPTA-1 [25,26]. In fly neuromuscular junctions, postsynaptically targeted G-CaMP2 (SynapG-CaMP2) has been reported to respond to excitatory postsynaptic currents [9]. In mammalian cells, Mao et al. [8] developed G-CaMP2-actin to record \( \text{Ca}^{2+} \) signals within spines but failed to detect synaptically evoked \( \text{Ca}^{2+} \) activity, presumably because of the low \( \text{Ca}^{2+} \) sensitivity of G-CaMP2. In this study, we demonstrated that G-CaMP6-actin is the first GECI that allows the visualization of \( \text{Ca}^{2+} \) signals in response to synaptic stimulation at the single-spine level. Although the exact mechanisms of spine \( \text{Ca}^{2+} \) signals remain unknown, it seems likely that sub-threshold stimulation triggers \( \text{Ca}^{2+} \) transients through postsynaptic NMDA receptors [27], while supra-threshold stimulation triggers \( \text{Ca}^{2+} \) transients at 100% of the spines by opening of voltage-gated \( \text{Ca}^{2+} \) channels through backpropagation of action potentials. In principle, we should be able to visualize spine responses to evaluate long-term plasticity, which is thought to be an elementary component of learning and memory. We expect that these novel G-CaMP technologies, together with advanced imaging systems [26,28], will facilitate our understanding of neuronal network dynamics in the brain at the single-synapse level.

Materials and Methods

Plasmid Construction

Complementary DNAs (cDNAs) encoding sfG-CaMP2, sfG-CaMP2.02, G-CaMP5.09 and G-CaMP6 were synthesized by mutagenizing the cDNA encoding the prototype GECI, G-CaMP2 [15], using the QuickChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent). cDNAs encoding G-CaMP7 and G-CaMP8 were synthesized by randomly mutagenizing the cDNAs encoding G-CaMP6 and G-CaMP7, respectively, as previously described [15]. The cDNA encoding G-CaMP3 was constructed by introducing mutations [4] into the G-CaMP2 cDNA. These cDNAs were subcloned into a pRSET<sub>B</sub> vector (Invitrogen) containing a T7 promoter [15], or into a pEGFP-N1 vector (Clontech) with a CMV promoter, as described [3] for expression in HeLa cells and cultured rat hippocampal neurons. For \textit{in utero} electroporation, cDNAs encoding G-CaMPs and mCherry (Clontech) were subcloned into a pCAGGS vector containing a T7 promoter, as described [15] for bacterial expression, or into a pEGFP-N1 vector (Clontech) with a CMV promoter, as described [3] for expression in HeLa cells and cultured rat hippocampal neurons. For \textit{in utero} electroporation, cDNAs encoding G-CaMPs and mCherry (Clontech) were subcloned into a pCAGGS vector containing a CAG promoter (CMV enhancer, \( \beta \)-actin promoter and woodchuck hepatitis virus regulatory element [WPRE]) [4]. To target G-CaMP6 to dendritic spines in neurons, a G-CaMP6-actin indicator was generated by fusing a cDNA encoding human \( \beta \)-actin (derived from pAcGFP1-actin, Clontech) to the 3′ end of a cDNA encoding G-CaMP6 via
a linker encoding the amino-acid sequence GGTTGGSRRAGTVDCRIRSLSSRSRA (in one-letter code). To generate plasmids to express G-CaMPs in the DA motoneurons in *C. elegans*, cDNAs encoding G-CaMPs were subcloned into a pFX_EGFPT vector containing the *unc-4* promoter [29]. All of the constructs were verified by sequencing.

**Bacterial Protein Expression and in vitro Characterization**

*E. coli* KRX (Promega) transformed with pRSETB-G-CaMP was grown at 37°C, and protein expression was induced by adding 0.1% rhamnose and incubating for an additional 5 h at 20°C. The indicator proteins with N-terminal histidine tags were purified, dialyzed against KM buffer containing (in mM) 100 KCl and 20 MOPS (pH 7.5) and used for in vitro characterization [15]. Spectral analyses were performed as previously described [16,17]. The term “dynamic range” was defined as $F_{\text{max}}/F_{\text{min}}$, where $F_{\text{max}}$ is the fluorescence intensity at saturating [Ca$^{2+}$], and $F_{\text{min}}$ is the fluorescence intensity at nominally zero [Ca$^{2+}$] with 1 mM EGTA. The Ca$^{2+}$ titration experiments were performed at pH 7.2 with 10 mM solutions of K$_2$H$_2$EGTA and Ca$_2$EGTA from the Ca$^{2+}$ Calibration Kit #1 (Invitrogen), as previously reported [30].

**Ca$^{2+}$ Imaging in HeLa Cells**

HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s manual. Fluorescence images of cells expressing G-CaMPs were acquired with a fluorescence microscope (IX71, Olympus) equipped with a CCD camera (ORCA-ER, Hamamatsu), as previously described [16,17]. The cells were perfused with HEPES-buffered saline (HBS) containing (in mM) 135 NaCl, 5.4 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 glucose and 5 HEPES (pH 7.4), and after reading the baseline fluorescence, 100 uM ATP was bath-applied for 1 min. The images were analyzed using Aqua Cosmos version 2.0 software (Hamamatsu). The transient increase in fluorescence ($\Delta F/F$) was calculated after subtracting the background fluorescence.

**Ca$^{2+}$ Imaging in *C. elegans***

The expression plasmid carrying G-CaMP6 (*Punc-4::G-CaMP6*) or G-CaMP3 (*Punc-4::G-CaMP3*) was co-injected with the plasmid carrying DsRed-Express-1 (*Punc-4::DsRed-Express-1*) [29] into wild-type N2 worms using a standard protocol [31]. The *jqEx97* (G-CaMP6) strain and the *jqEx216* (G-CaMP3) strain were used in this study. Ca$^{2+}$ imaging was performed in worms on a 1.5% agar pad placed on a glass slide (76×26 mm, 1.0- to 1.2-mm thickness, Matsunami). L1 animals were placed in M9 buffer [32] and dropped onto the agar pad, and the glass slide was covered by a cover glass (24×24 mm, 0.12- to 0.17-mm thickness, Matsunami). The worms were then subjected to imaging analyses using an A1R laser confocal microscope (Nikon) and NIS-Elements AR 3.2 image acquisition software (Nikon). The images were captured with manual movement of the X and Y positions of the stage to

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**Figure 8. Long-term imaging of Ca$^{2+}$ activity in spines in a cultured hippocampal pyramidal neuron.** A, Z-projection of a representative CA3 pyramidal neuron expressing G-CaMP6-actin at 8 (upper) and 29 (lower) days in vitro (Div). After 7 days in vitro, the G-CaMP6-actin plasmid was introduced into the neuron via single-cell electroporation. Two spines of interest (S1, S2) are indicated by yellow circles. B, Changes in fluorescence at S1 and S2 upon supra-threshold electrical stimulation (Stim). The average spine $\Delta F/F$ ratios in response to supra-threshold stimulation were 253±30.5% and 201±46.6% at 8 Div and 29 Div, respectively (n = 25 spines, $P<0.05$, Student’s t-test).

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track the worms. Confocal images (512x512 pixels) of cholinerge
DA motoneurons were captured at 15 frames per second (fps) with
a water immersion objective (40x, 1.15 NA, Nikon). After the
subtraction of background noise, the fluorescence ratio changes
(DR/R0) of G-CaMPs or G-CaMP3 against DsRed-Express-1 were
calculated as (R1 - R0)/R0, where R1 is the fluorescence ratio at any
time point and R0 is the baseline fluorescence ratio.

Cultured Slice Preparation and Single-cell Electroporation
All experiments were performed with the approval of the animal
experiment ethics committee at the University of Tokyo (approval
number: 19–43) and according to the University of Tokyo
guidelines for the care and use of laboratory animals. Hippocam-
pal slices from postnatal day 7 Wistar/ST rats (SLC) were
prepared, as previously described [33], according to the guidelines
for laboratory animal care and safety of the University of Tokyo.
Briefly, rat pups were chilled with ice and decapitated. The brains
were removed and cut horizontally into 300-μm slices using a
D1TK-1300 vibratome (Dosaka) in acrared, ice-cold Gey’s
balanced salt solution supplemented with 25 mM glucose. The
entorhino-hippocampal stumps were excised and cultivated on
Omnipore membrane filters (JHWP25900, Millipore) that were
placed on plastic O-ring disks. The cultures were incubated in a
humidified incubator at 37°C in 5% CO2 with 1 ml of 50% minimal
essential medium, 25% Hanks’ balanced salt solution
(HBSS), 25% horse serum (Cell Culture Laboratory) and
antibiotics. The medium was changed every 3.5 days. On days
3–5 in vitro, G-CaMPs and mCherry under the control of the
CMV promoter were introduced into the neurons via targeted
single-cell electroporation [20]. Briefly, borosilicate glass pipettes
(tip resistance, 5–7 MΩ) were filled with HBSS containing
1–2 μg/μl plasmid DNA. After the tip of the pipette was placed in
close proximity to the soma, electroporation was performed with
50 rectangular pulses (5 V, 0.3-ms duration) at a frequency of
50 Hz. Single-cell electroporation was applied sequentially to up
to 10 cells using the same pipette within 5 min. Imaging was
performed 24–48 h after electroporation.

In utero Electroporation and Acute Slice Preparations
Day-14 pregnant ICR mice (CLEA Japan) were deeply
anesthetized, and their intrauterine embryos were removed
surgically, as previously described [21]. To express G-CaMPs
and mCherry, expression plasmids under the control of the CAG
promoter (2 μg/μl) were injected into the lateral ventricle of the
intrauterine embryos, and 2 days after birth. For sub-threshold stimulation, the stimula-
tion intensity was adjusted so that <50% of the spines exhibited
Ca2+ transients (40–120 μA, 50 μs). For supra-threshold stimulations, the intensity was raised to more than 200 μA so that almost all neurons generated action potentials.

Electrophysiology and Ca2+ Imaging in Cultured
Hippocampal Slices
Hippocampal slices were mounted in a recording chamber and perfused at a rate of 1.3–3 ml/min with aCSF containing in mM: 127 NaCl, 26 NaHCO3, 3.3 KCl, 1.24 KH2PO4, 1.0 MgSO4, 1.0 CaCl2 and 10 glucose, bubbled with 95% O2 and 5% CO2. All recordings were performed at room temperature (24–28°C), unless otherwise specified. Patch-clamp recordings were collected from
hippocampal CA3 pyramidal neurons using a MultiClamp 700B
amplifier and a Digidata 1440A digitizer controlled by
pCLAMP10 software (Molecular Devices). Epifluorescence mi-
croscopy was used to select cells showing stable mCherry
expression with a fluorescence intensity ranging from 103 to 125
(arbitrary units). Borosilicate glass pipettes (3–7 MΩ) were filled with a solution containing (in mM) 135 K-gluconate, 4 KCl, 10
HEPES, 10 phosphocreatine-Na2, 0.3 Na2-GTP and 4 Mg-ATP
(pH 7.2). The signals were low-pass filtered at 1–2 kHz and
digitized at 20–100 kHz. Data were discarded if the access resistance changed by more than 20% during the experiment.

Supporting Information
Movie S1 L1 larvae (jqEx97) co-expressing G-CaMP6 and
dsRed-Express-1 in the DA neurons during locomotion (corresponds to Fig. 6). Green, red and transmitted-light images were overlaid. Images were taken at 15 fps. (AVI)

Movie S2 L1 larvae (jqEx216) co-expressing G-CaMP3 and
dsRed-Express-1 in the DA neurons during locomotion (corresponds to Fig. 6). Green, red and transmitted-light images were overlaid. Images were taken at 15 fps. A worm was kept in the imaging field by manual adjustment of the x-y stage. (AVI)
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Conceived and designed the experiments: MO YI JN. Performed the experiments: MO TS JS CK KG-A YK-N YI JN. Analyzed the data: MO TS JS CK KG-A YK-N YI JN. Contributed reagents/materials/analysis tools: MO JS. Wrote the paper: MO TS JS CK KG-A YK-N YI JN.