SpiCee: A Genetic Tool for Subcellular and Cell-Specific Calcium Manipulation

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HAL Id: hal-02992312
https://hal.archives-ouvertes.fr/hal-02992312
Submitted on 9 Nov 2020

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SpiCee: A Genetic Tool for Subcellular and Cell-Specific Calcium Manipulation

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In Brief
Ros et al. develop SpiCee, a genetically encoded calcium chelator that enables the manipulation of this second messenger in single cells with subcellular specificity. SpiCee alters the migration of developing cortical neurons in vivo. Targeting of lipid rafts prevents axonal repulsion, whereas exclusion from this subcellular compartment does not.

Highlights
- Development of SpiCee, a genetically encoded calcium scavenger
- SpiCee inhibits calcium-dependent downstream pathways
- SpiCee enables cell-specific manipulation of calcium-dependent processes in vivo
- Subcellular targeting confers cell compartment specificity to SpiCee
SUMMARY

Calcium is a second messenger crucial to a myriad of cellular processes ranging from regulation of metabolism and cell survival to vesicle release and motility. Current strategies to directly manipulate endogenous calcium signals lack cellular and subcellular specificity. We introduce SpiCee, a versatile and genetically encoded chelator combining low- and high-affinity sites for calcium. This scavenger enables altering endogenous calcium signaling and functions in single cells in vitro and in vivo with biochemically controlled subcellular resolution. SpiCee paves the way to investigate local calcium signaling in vivo and directly manipulate this second messenger for therapeutic use.

INTRODUCTION

Alterations of calcium (Ca^{2+}) signals currently rely mostly on pharmacological strategies to prevent Ca^{2+} influx into the cytoplasm by blocking specific channels or chelate extracellular or intracellular Ca^{2+} using buffers designed decades ago (Godfraind, 2017; Tsien, 1980). Since Ca^{2+} is crucial for a wide range of signaling pathways and for the function of many components of the extracellular matrix (Averaimo and Nicol, 2014; Clapham, 2007), these strategies lack cellular specificity leading to ambiguous interpretation of their effects. The rise of optogenetics has provided a range of approaches to increase intracellular Ca^{2+} including light-sensitive channels (Nagel et al., 2003) and genetically encoded caged Ca^{2+} (Fukuda et al., 2014). This has been used to control electrical activity in excitable cells and has provided extraordinary knowledge of neuronal interactions as well as opening promising fields of therapeutics (Rajasethupathy et al., 2016; Sengupta et al., 2016; Tønnesen and Kokaia, 2017). These strategies rely on imposing additional Ca^{2+} elevations but lack the ability to manipulate endogenous Ca^{2+} levels, reducing the intracellular concentration or targeting release from internal stores. In addition, the use of optogenetics is handicapped in vivo, when light stimulation is technically challenging (e.g., during developmental stages). Reducing endogenous Ca^{2+} signals with cellular or subcellular specificity is out of the range of the available toolset (including the widely used BAPTA-AM), even though cell-specific manipulation has been successful in reducing the decay time of Ca^{2+} transients in cardiac myocytes (Wang et al., 2013). Strategies overexpressing a Ca^{2+} pump to drain this second messenger out of the cytoplasm of astrocytes (Yu et al., 2018) or altering inositol 1,4,5-triphosphate (IP_3) upstream of Ca^{2+} entry (Uchiyama et al., 2002) have successfully reduced Ca^{2+} signaling. However, these strategies lack the versatility to target a specific subcellular compartment and to be applicable to IP_3-independent Ca^{2+} modulation, respectively. Genetically encoded Ca^{2+} indicators also have secondary buffering activity (McMahon and Jackson, 2018), but their designs aim to minimalize this effect and to limit their impact on Ca^{2+} signaling. The ideal tool with which to manipulate endogenous Ca^{2+} signaling would (1) reduce free intracellular Ca^{2+} available to downstream effectors and preclude cellular processes dependent on this second messenger; (2) interact directly with Ca^{2+} rather than modifying the activity of Ca^{2+} channels; (3) be genetically encoded to enable selective subcellular localization using fusion to targeting sequences; and (4) enable cellular identification with fluorescent reporters. Here, we introduce SpiCee (sponge inhibiting Ca^{2+} signaling), a genetically encoded chelator of intracellular Ca^{2+}, which is targetable to subcellular compartments. We show that SpiCee expression is sufficient to alter Ca^{2+}-dependent signaling and cellular processes both in vitro and in vivo and demonstrate the ability of SpiCee to alter Ca^{2+}-dependent cellular behavior with subcellular specificity.
RESULTS

SpiCee Is a Bimodal Ca^{2+} Chelator

SpiCee was designed as a tetravalent chimeric Ca^{2+} buffer combining the high-affinity Ca^{2+}-binding domains of a variant of α-parvalbumin (Lee et al., 2004) and the lower affinity binding sites of calmodulin (Gifford et al., 2007) (Figure 1A). This design, based on two endogenous proteins with distinct affinities for Ca^{2+}, was chosen to ensure that SpiCee is functional in a wide variety of cellular processes. The effector domains of calmodulin were omitted to avoid gain-of-function effects. SpiCee was fused to the fluorescent mRFP protein for easy identification of SpiCee-expressing cells in fixed- or live-cell experiments (Figure 1A). The full sequence of SpiCee is provided in the STAR Methods section. To characterize the Ca^{2+}-binding properties of SpiCee, we used the tryptophan fluorescence of two variants of SpiCee (SpiCeeF134W and SpiCeeF103W). The tryptophan fluorescence of similar mutants of the native proteins (calmodulin and parvalbumin) have previously been used to determine the Ca^{2+}-binding properties of the EF-hand adjacent to the mutation, without affecting the affinity of this site (Hutnik et al., 1990; Johnson and Tikunova, 2002; Tang et al., 2003). Each of these mutants enables the evaluation of the Ca^{2+}-binding properties of either the calmodulin or the parvalbumin moiety (SpiCeeF134W and SpiCeeF103W respectively). Using Ca^{2+} titration, we found that SpiCeeF134W has a dissociation constant, K_D, of Ca^{2+} of 2.8 ± 0.9 μM, and stopped-flow experiments were performed to evaluate its dissociation rate, k_off, of 28 ± 28 s⁻¹ at 20°C (Figures 1B and 1C). The high affinity of the labeled parvalbumin site (SpiCeeF103W) prevented direct Ca^{2+} titration, and the K_D was measured by competition with EGTA. The K_D of SpiCeeF103W is 0.3 ± 0.1 nM, and k_off = 0.24 ± 0.01 s⁻¹, and k_on = 529 ± 28 s⁻¹, respectively. Representative titration fits are shown. (C) Mg^{2+} titration was used to measure the dissociation constant of Mg^{2+} of a parvalbumin- and a calmodulin-derived Ca^{2+}-binding site in the vicinity of the mutations (K_D = 2.6 ± 0.7 μM, and K_D = 0.46 ± 0.14 mM, respectively). Representative titration fits are shown. Number of replicates is provided in Table S1. a.u., arbitrary units.

Ca^{2+}-binding properties of the EF-hand adjacent to the mutation, without affecting the affinity of this site (Hutnik et al., 1990; Johnson and Tikunova, 2002; Tang et al., 2003). Each of these mutants enables the evaluation of the Ca^{2+}-binding properties of either the calmodulin or the parvalbumin moiety (SpiCeeF134W and SpiCeeF103W respectively). Using Ca^{2+} titration, we found that SpiCeeF134W has a dissociation constant, K_D, for Ca^{2+} of 2.8 ± 0.9 μM, and stopped-flow experiments were performed to evaluate its dissociation rate, k_off, of 28 ± 28 s⁻¹ at 20°C (Figures 1B and 1C). The high affinity of the labeled parvalbumin site (SpiCeeF103W) prevented direct Ca^{2+} titration, and the K_D was measured by competition with EGTA. The K_D of SpiCeeF103W is 0.3 ± 0.1 nM, and k_off = 0.24 ± 0.01 s⁻¹ (Figures 1B and 1C). k_on was computed from K_D and k_off (k_on = (k_off / K_D)). k_on = 8 · 10^{8} M^{−1}s^{−1} ± 3 · 10^{8} M^{−1}s^{−1}, and k_off = 1.9 · 10^{8} M^{−1}s^{−1} ± 0.7 · 10^{8} M^{−1}s^{−1} for SpiCeeF103W (parvalbumin site) and SpiCeeF134W (calmodulin site), respectively. The K_Ds for Mg^{2+} of a parvalbumin-derived and a calmodulin-derived binding site were measured following the tryptophan fluorescence of SpiCeeF103W (K_D = 2.6 μM ± 0.7 μM) and SpiCeeF134W (K_D = 0.46 mM ± 0.14 mM), respectively, highlighting a high selectivity for Ca^{2+} over Mg^{2+} (Figure 1D). These features are in the range of the previously described Ca^{2+}-binding properties of the full-length parvalbumin and calmodulin proteins (parvalbumin: K_D (Ca^{2+}) = 4 nM, k_on = 2.5 · 10^{6} M^{−1}s^{−1}, k_off = 1 s^{−1}, and K_D (Mg^{2+}) = 27 μM; calmodulin: K_D = 2.4 μM, k_on = 1 · 10^{8} M^{−1}s^{−1}, k_off = 310 s^{−1}) (Johnson and
SpiCee is a bimodal Ca$^{2+}$ chelator combining low- and high-affinity sites. The reduced $K_D$ of the parvalbumin moiety of SpiCee as compared to the wild-type parvalbumin reflects the use of a high-affinity Ca$^{2+}$-binding domain of a variant of $\alpha$-parvalbumin (Lee et al., 2004).

**SpiCee Is Not Cytotoxic**

SpiCee was expressed in HEK293 cells or in retinal ganglion cells to evaluate its behavior in living cells. Expressing SpiCee did not affect cell survival in either cell type (Figure 2) or the resting Ca$^{2+}$ concentration in HEK293 cells (Figure S1), suggesting that SpiCee-expressing cells can maintain a resting Ca$^{2+}$ concentration compatible with their survival, within the previously reported range (Tong et al., 1999). SpiCee-transfected cells were also able to adapt their resting Ca$^{2+}$ concentration to the amount of Ca$^{2+}$ in the culture medium (Figure S1). These observations are similar to the reported properties of pharmacological Ca$^{2+}$ buffers (Pertusa et al., 1999; Tsien, 1980). For instance a few micromolar of BAPTA-AM is able to prevent Ca$^{2+}$ fluctuations without altering the Ca$^{2+}$ resting concentration (Collatz et al., 1997).

**SpiCee Is a Ca$^{2+}$ Scavenger in Living Cells**

We investigated SpiCee influence on the dynamic changes in Ca$^{2+}$ concentration using Twitch2B, an optimized Ca$^{2+}$ fluorescence resonance energy transfer (FRET) sensor (Thestrup et al., 2014). The FRET:CFP ratio reflects intracellular Ca$^{2+}$ concentration and was monitored in Twitch2B-expressing HEK293 cells. The release of intracellular Ca$^{2+}$ stores induced by a 20-s pulse of thapsigargin led to a 23% ± 1.8% increase in the FRET:CFP ratio, which was drastically reduced in cells co-expressing SpiCee (8.6% ± 0.8%; Figures 3A–3C; Video S1). The reduction of the thapsigargin-induced FRET:CFP ratio reveals that SpiCee competes with other Ca$^{2+}$-binding proteins, including the biosensor Twitch2B, and prevents them from binding efficiently to Ca$^{2+}$. To investigate the limits of Ca$^{2+}$ buffering by SpiCee, cells co-expressing SpiCee and Twitch2B were exposed to longer thapsigargin stimulation (1, 2, or 5 min). In all cases, SpiCee attenuated and delayed the Ca$^{2+}$ elevation detected by the biosensor (Figures S2A–S2I; Videos S2, S3, and S4). Extreme intracellular Ca$^{2+}$ elevations were induced by increasing the extracellular Ca$^{2+}$ level (from 0.2 mM to 2 mM) after a prolonged thapsigargin exposure followed by a washout of the drug. SpiCee induces a delay in the Ca$^{2+}$ elevation detected by Twitch2B (Figures S2J–S2L; Video S5). It does not, however, change the amplitude of Ca$^{2+}$ elevation of this massive and sustained elevation of Ca$^{2+}$ concentration, suggesting that SpiCee saturates in this case and confirming that the resting concentration of Ca$^{2+}$ is not affected in SpiCee-expressing cells (Figures S2J–S2L).

To verify that the SpiCee-induced reduction of the Ca$^{2+}$ elevation requires Ca$^{2+}$ binding, each SpiCee EF-hand was point-mutated twice (D52A, E63Q, D91A, E102Q, D135A, E146Q, D171A, and E182Q) to prevent their ability to bind Ca$^{2+}$ following previously described mutations of these domains (Piazza et al., 2017; Starovasnik et al., 1992). This variant was termed mutSpiCee and did not affect the Ca$^{2+}$ elevation induced by a 1-min pulse of thapsigargin (Figures S2M–S2O).

To assess whether SpiCee reduces Ca$^{2+}$ elevations induced by endogenous signaling rather than thapsigargin-induced inhibition of Ca$^{2+}$ transfer from the cytoplasm into the endoplasmic reticulum by the SERCA pump, HEK293 cells were exposed to...
ATP to increase intracellular Ca\textsuperscript{2+} concentration through the activation of purinergic receptors (He et al., 2003). SpiCee expression delayed the ATP-induced Ca\textsuperscript{2+} elevation and reduced its amplitude (Figures 3D–3F; Video S6). To evaluate whether the level of expression of SpiCee is correlated with the extent of Ca\textsuperscript{2+} scavenging, the amplitude of the ATP-induced Ca\textsuperscript{2+} elevation for individual cell was plotted against the mRFP intensity as a proxy of SpiCee expression. The FRET:CFP ratio is not correlated with SpiCee expression, suggesting that the scavenging of Ca\textsuperscript{2+} is achieved from low SpiCee expression (Figure S3A).

These results demonstrate that SpiCee is able to reduce pharmacologically induced Ca\textsuperscript{2+} elevations and physiological responses over a wide range of intracellular concentrations of this second messenger.

**SpiCee Prevents Activation of Ca\textsuperscript{2+} Downstream Signaling**

We next assessed the ability of SpiCee to preclude signal transduction downstream of Ca\textsuperscript{2+}. Thapsigargin-induced release of Ca\textsuperscript{2+} from intracellular stores activates the ERK-MAPK

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**Figure 3. SpiCee Buffers Physiological Ca\textsuperscript{2+} Transients and Downstream Signaling**

(A–C) In vitro experiments show a reduction of the Ca\textsuperscript{2+} increases induced by thapsigargin. (A and B) 20-s thapsigargin (Thaps.) exposure induces a transient elevation of the Ca\textsuperscript{2+} concentration in control HEK293 cells, monitored by the FRET:CFP ratio from the FRET biosensor Twitch2B (A, top row, FRET channel; blue trace in B). In contrast, this elevation is drastically reduced in SpiCee-expressing cells (A, bottom row, FRET channel; orange trace in B). (A) The FRET channel intensity is coded from blue (low Ca\textsuperscript{2+}) to red (high Ca\textsuperscript{2+}); red image indicates SpiCee expression (See also Video S1). (C) Both the amplitude and rate of Ca\textsuperscript{2+} elevation are reduced by SpiCee expression.

(D–F) ATP superfusion induces a transient elevation of the Ca\textsuperscript{2+} concentration in control HEK293 cells (D, top row, FRET channel; blue trace in E), monitored by the FRET:CFP ratio from the FRET biosensor Twitch2B. The ATP-induced Ca\textsuperscript{2+} increase is reduced in SpiCee-expressing cells (D, bottom row, FRET channel; orange trace in E). See also Video S6). (F) Both the amplitude and rate of Ca\textsuperscript{2+} elevation are decreased by SpiCee expression.

(G) Phosphorylation of ERK1/2 was examined in cells treated with thapsigargin and expressing SpiCee or buffering intracellular Ca\textsuperscript{2+} with BAPTA-AM. Thapsigargin-induced phosphorylation of ERK1/2 was reduced in cells expressing SpiCee, or pre-treated with BAPTA-AM (top), while none of the treatments affected the expression of ERK1/2 (bottom).

(H) Quantification of the western blots shown in (G).

In (B), (E), and (H), data are presented as means ± SEM. Individual data points are shown in (H). For box-and-whisker plot elements in (C) and (F): center line, mean; box limits, upper and lower quartiles; whiskers, SD. In (A) and (D), scale bars, 15 μm. **p < 0.001, Mann-Whitney test in (C) and (F) and ANOVA followed by Bonferroni post hoc tests in (H). Exact p values and number of replicates are provided in Table S1. See also Figures S2 and S3.

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ATP to increase intracellular Ca\textsuperscript{2+} concentration through the activation of purinergic receptors (He et al., 2003). SpiCee expression delayed the ATP-induced Ca\textsuperscript{2+} elevation and reduced its amplitude (Figures 3D–3F; Video S6). To evaluate whether the level of expression of SpiCee is correlated with the extent of Ca\textsuperscript{2+} scavenging, the amplitude of the ATP-induced Ca\textsuperscript{2+} elevation for individual cell was plotted against the mRFP intensity as a proxy of SpiCee expression. The FRET:CFP ratio is not correlated with SpiCee expression, suggesting that the scavenging of Ca\textsuperscript{2+} is achieved from low SpiCee expression (Figure S3A).
We verified that thapsigargin induces the phosphorylation of ERK1/2, and that this signaling event is abolished by pretreatments with BAPTA-AM (Figures 3G and 3H). Like BAPTA exposure, transfection of SpiCee induced a drastic reduction in the thapsigargin-induced phosphorylation of ERK1/2, demonstrating that SpiCee buffering of Ca²⁺ prevents the activation of downstream signaling (Figures 3G and 3H). The same approach was used to evaluate whether the Ca²⁺-binding sites of Twitch2B contribute to the scavenging effect detected using FRET experiments. Co-expressing SpiCee and Twitch2B does not reduce the phosphorylation of ERK1/2 induced by thapsigargin compared to SpiCee expression alone, indicating that Twitch2B does not contribute to the Ca²⁺ scavenging effect observed using FRET approaches (Figures S3B and S3C).

Combining Low- and High-Affinity Sites Enhances Ca²⁺ Buffering Ability

To evaluate whether adding two high-affinity Ca²⁺-binding sites to the pair of lower affinity sites from calmodulin enhances SpiCee performance, a truncated form of SpiCee containing only the calmodulin-derived sites (SpiCee-C) was generated (Figure 4A). SpiCee-C induced a slight reduction in the ATP-induced Ca²⁺ elevation (Figures 4B–4D; Video S7). The reduction of the Ca²⁺ signal amplitude was enhanced, and the Ca²⁺ elevation was delayed when cells express SpiCee instead of SpiCee-C, confirming the superior performance of the 4-binding-site variant of SpiCee over calmodulin-derived Ca²⁺ buffers like SpiCee-C. SpiCee-C might be adapted for slight attenuation of Ca²⁺ elevations.
To test whether including the low-affinity binding sites from calmodulin provides an improvement of Ca\textsuperscript{2+} scavenging by SpiCee over high-affinity buffers like parvalbumin, the thapsigargin-induced Ca\textsuperscript{2+} elevation was evaluated in SpiCee- and parvalbumin-expressing cells. SpiCee and parvalbumin similarly reduce the amplitude of the Twitch2B FRET:CFP ratio detected after a 1-min thapsigargin pulse. SpiCee also delays the onset of the Ca\textsuperscript{2+} elevation (Figure 4 E–4H). This delayed Ca\textsuperscript{2+} elevation was not observed in parvalbumin-expressing cells (Figure 4 E–4H), suggesting that SpiCee provides enhanced buffering of brief Ca\textsuperscript{2+} changes.

**SpiCee Alters Neuronal Migration In Vivo**

To assess SpiCee capability to interfere with Ca\textsuperscript{2+}-dependent physiological processes in vivo, SpiCee was expressed in newly generated cortical neurons using in utero electroporation of mouse brains at embryonic day 14.5 (E14.5). The migration of early born cortical neurons relies on Ca\textsuperscript{2+} signaling (Bando et al., 2016; Uhlen et al., 2015). The positions of migrating electroporated neurons from the electroporation site were assessed at E18.5 and post-natal day 10 (P10). At both ages, no obvious morphological defects were detected in SpiCee-expressing neurons. By E18.5, the majority of control GFP and mRFP co-electroporated neurons have reached the cortical plate and formed a dense layer near the marginal zone (Figures 5 A and 5B). In contrast, many neurons co-expressing GFP and SpiCee exhibited reduced migration and were found throughout the cortical plate, including the intermediate zone (Figure 5A). Several electroporated neurons also failed to stall at the cortical plate, overshooting toward the marginal zone and causing heterotopias in 7 out of 9 animals; misplaced neurons were found in only 2 out of 10 mRFP-electroporated embryos (Figure 5B). At P10, SpiCee-expressing neurons covered a thicker lamina of the cortex than mRFP-electroporated controls (Figure 5C). Furthermore, the heterotopias detected during embryonic development were maintained at post-natal stages in nearly all SpiCee-expressing animals (10 out of 12), whereas they were found in a single mRFP-electroporated pup (5 electroporated animals; Figure 5D). Thus, Ca\textsuperscript{2+} buffering by SpiCee is sufficient to alter neuronal migration in vivo.

**SpiCee Enables Subcellular Manipulation of Ca\textsuperscript{2+}**

Ca\textsuperscript{2+} signals are often restricted to subcellular compartments (Cooper, 2015; Filadi and Pozzan, 2015). Since genetic encoding confers the ability to restrict the expression of the constructs to a specific organelle, we assessed the functionality of SpiCee in this scenario. The ability to restrict SpiCee expression to subcellular compartments was tested using the fusion with targeting sequences or full-length proteins. In developing cortical interneurons, SpiCee is found in the cytoplasm but excluded from the primary cilium. The fusion with transmembrane 5-HT6 receptor leads to the exclusive localization of SpiCee in the primary cilium (Figure S4 A). The Ca\textsuperscript{2+} pump hPMCA (human plasma membrane Ca\textsuperscript{2+} pump), recently described as a tool to prevent Ca\textsuperscript{2+} elevation in astrocytes (Yu et al., 2018), was fused to the 5-HT6 receptor to evaluate its ability to be restricted to a membrane compartment. Like SpiCee, 5-HT6-hPMCA was found in the primary cilium. The ability to control the intracellular localization of SpiCee and hPMCA was evaluated in HEK293 cells using a nuclear localization sequence (NLS) and a nuclear export sequence (NES). When not targeted, SpiCee was found in the cytoplasm and in the nucleus with variability in the nuclear level of expression. In contrast, SpiCee-NLS and SpiCee-NES were exclusively found in the nucleus and cytoplasm, respectively.
demonstrating that its intracellular localization can be controlled (Figure S4B). In the absence of targeting sequences, hPMCA was found in the plasma membrane. The attempts to restrict it or exclude it from the nucleus using NLS and NES failed, leading to the trapping of hPMCA in a cellular organelle resembling the endoplasmic reticulum (Figure S4 B).

In order to directly assess the ability of SpiCee to alter Ca^{2+} signaling, a tandem of palmitoylation-myristoylation motifs from Lyn Kinase (Lyn-SpiCee) or the CaaX-polylysine motif derived from K-Ras (SpiCee-Kras) were fused to the N terminus or the C terminus of SpiCee, respectively, to target SpiCee to distinct plasma membrane compartments (Figure 6 A). Lyn and Kras targeting sequences have previously been used for spatial restriction of overexpressed proteins in lipid rafts and in the non-raft fraction of the plasma membrane, respectively (Averaimo et al., 2016; Depry et al., 2011; Ros et al., 2019; Zacharias et al., 2002). Both Lyn-SpiCee and SpiCee-Kras were targeted to the plasma membrane (Figure 6 B). Using sucrose gradient membrane fractionation, Lyn-SpiCee distribution peaked in fraction 3, whereas SpiCee-Kras was highly enriched in fraction 4, demonstrating that each variant of SpiCee is targeted to distinct membrane compartments. Their specific distribution matches the profile of different membrane markers, the β subunit of cholera toxin (CtB) and Caveolin respectively, although the biochemical purification used did not localize SpiCee-Kras in the non-raft fraction of the plasma membrane containing Adaptin, a protein excluded from lipid rafts (Figures 6 C and 6D; Figure S5).

Using Lyn-SpiCee and SpiCee-Kras, we investigated the potential of SpiCee to manipulate Ca^{2+}-dependent cellular processes specific to subcellular compartments. To this aim, we analyzed the response of retinal ganglion cell axons to the repellent guidance molecules slit1 and ephrinA5, known to require the modulation of Ca^{2+} concentration (Guan et al., 2007; Nicol et al., 2007) and subcellular-compartment-specific signaling in lipid rafts (Averaimo et al., 2016; Guirland et al., 2004; Nakai and...
ability of SpiCee to affect a subset of Ca$^{2+}$-dependent down-
of targeting subcellular compartments further increases the chronic manipulation of selected cell types. The possibility produced a specific and non-cytotoxic buffer that enables the Ca$^{2+}$-binding domains of endogenous proteins, we have migration in a cell-specific manner. Furthermore, by including podial actin and drastic reduction of the growth cone area (Fig-
growth cones, characterized by the depolymerization of lamelli-
ions that do not afford the opportunity for single-cell-type manipu-
spatially restricted manipulation of Ca$^{2+}$-dependent signaling cascades in subcellular compartments.

DISCUSSION

The present work introduces SpiCee, a molecular scavenger of Ca$^{2+}$. We show that SpiCee is efficient in buffering Ca$^{2+}$-transients and downstream signaling, while it does not interfere with the cellular ability to regulate the resting Ca$^{2+}$ concentration. This resembles the effect of concentrations of BAPTA that prevent the fluctuation of intracellular Ca$^{2+}$ without altering the resting concentration (Collatz et al., 1997). Since SpiCee can be expressed in a subset of cells, the use of this Ca$^{2+}$ buffer enables to investigate the role of Ca$^{2+}$ in neurodevelopmental processes such as neuronal migration in a cell-specific manner. Furthermore, by including the Ca$^{2+}$-binding domains of endogenous proteins, we have produced a specific and non-cytotoxic buffer that enables chronic manipulation of selected cell types. The possibility of targeting subcellular compartments further increases the ability of SpiCee to affect a subset of Ca$^{2+}$-dependent downstream pathways with high specificity.

Second messengers are key in the regulation of cellular responses to the environment and of their intrinsic behavior. Being able to manipulate the concentration of these signaling molecules in a precise and predictable fashion is crucial for the understanding of their downstream signaling cascades and cellular responses. Second messengers are located at the convergence of multiple signaling cascades. A simple view of these signaling molecules diffusing freely in the cytosol conflicts with the specific activation of each of their downstream effectors. The spatial confinement of second messengers emerged as a likely regulator of cellular responses (Augustine et al., 2003; Averaimo and Nicol, 2014; Averaimo et al., 2016).

Much effort has been devoted to the development of tools that allow the subcellular manipulation of cyclic nucleotides through genetically encoded tools (Averaimo et al., 2016; Lefkimmiatis et al., 2009; Ros et al., 2019) and Ca$^{2+}$-targeting agents like optical tools (Akiyama et al., 2016; Nishiyama et al., 2003; Shelly et al., 2010). In brief, SpiCee offers the possibility to study the influence of Ca$^{2+}$ signaling on the development and function of neuronal networks.

The development of SpiCee-based tools will further expand the possibilities of manipulating Ca$^{2+}$ with spatial and temporal control. Floxed transgenic rodent strains would allow precise cell-type- and developmental-stage-specific silencing of Ca$^{2+}$ signaling cascades while facilitating in vivo research by obviating surgical interventions. The temporal control of SpiCee expression could be achieved by genetically encoded switches such as the Tet-ON and Tet-OFF systems. SpiCee might be similarly imported to other animal models such as fly or zebrafish, allowing for the same refined functionality when coupled to upstream activation or silencing of Ca$^{2+}$ signaling cascades while facilitating in vivo research by obviating surgical interventions.

In conclusion, we have created a Ca$^{2+}$ scavenger that has the capability to interfere with Ca$^{2+}$-dependent physiological functions. This genetically encoded tool has the potential to alter Ca$^{2+}$ responses in a cell-specific manner and with subcellular resolution, enlarging the possibility of studying local signaling cascades in vivo. Combined with gene therapy strategies and cell-type-specific promoters, SpiCee opens promising perspectives for specific therapeutic interventions targeting Ca$^{2+}$ with limited side effects compared to pharmacological approaches that do not afford the opportunity for single-cell-type manipulation or specific cellular pathway targeting.
STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.107934.

ACKNOWLEDGMENTS

We thank Dr. O. Griesbeck for the gift of Twitch2B. We are grateful to the members of our labs and of the J. Livet lab for thoughtful discussion, to P. Gaspar and N. Spitzer for helpful critical reading of the manuscript, and to the members of the animal and imaging facilities of Institut de la Vision. This work was supported by grants from the ANR (ANR-15-CE16-0007 and ANR-18-CE16-0017), Sorbonne Université (CSC-SU IDEX SUPER SU-15-R-PERSU-17), and Retina France to X.N. This work was performed in the frame of LABEX LIFESENSES (reference ANR-10-LABX-65) and of IHU FOReSIGHT (ANR-18-131)

AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

A patent application, listing O.R. and X.N. as inventors, describing the development and applications of SpiCee as a Ca^{2+} chelator is pending.

Accepted: June 29, 2020
Published: July 21, 2020

REFERENCES


### STAR METHODS

#### KEY RESOURCES TABLE

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RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Xavier Nicol (xavier.nicol@inserm.fr).

Material Availability
Plasmid generated in this study have been deposited to Addgene under the following name and catalog number: pCX-SpiCee, #140836; pCX-Lyn-SpiCee, #140837; pCX-SpiCee-Kras, #140838; pCX-SpiCee-NLS, #140900; pCX-SpiCee-NES, #140901; pCX-mutSpiCee; #140903.

Data and Code Availability
This study did not generate any unique dataset or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
Pregnant C57BL6/J and RjOrl:SWISS mice were purchased from Janvier Labs. All animal procedures were performed in accordance with institutional guidelines and approved by the local ethics committee (C2EA-05: Comité d’éthique en expérimentation animale Charles Darwin). Animals were housed on a 12 h light/12 h dark cycle. Embryos from dated matings (developmental stage stated in each section describing individual experiments) were not sexed during the experiments and the female over male ratio is expected to be close to 1.

HEK293 cell culture
HEK293T cells (ATCC, not authenticated, free of mycoplasma contamination) were maintained in a 37°C, 5% CO2 incubator and transfected using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol and imaged the day following transfection or fixed and processed for immunocytofluorescence.

Retinal explants
Retinas of E14 mouse embryos were electroporated with mRFP, Lyn-SpiCee, SpiCee or SpiCee-Kras using two poring pulses (square wave, 175 V, 5 ms duration, with 50 ms interval) followed by four transfer pulses (40 V, 50 ms and 950 ms interpulse) with a Nepa21 Super Electroporator (NepaGene). Retinas were dissected and kept 24 hours in culture medium (DMEM-F12 supplemented with glutamine (Sigma Aldrich), penicillin/streptomycin (Sigma Aldrich), BSA (Sigma Aldrich) and glucose), in a humidified incubator at 37°C and 5% CO2. The day after, they were cut into 200 μm squares with a Tissue-Chopper (Mcllwain) and explants were plated on glass coverslips coated with poly-lysine and Laminin (Sigma Aldrich). Cells were cultured for 24 hours in culture medium supplemented with 0.4% (w/v) methyl cellulose. All animal procedures were performed in accordance with institutional guidelines and approved by the local ethics committee (C2EA-05: Comité d’éthique en expérimentation animale Charles Darwin). Embryos were not sexed during the experiments and the female over male ratio is expected to be close to 1.

METHOD DETAILS

Molecular Biology
SpiCee including a FLAG tag in 5’ and a 3 amino acid linker between the parvalbumin and calmodulin moieties underlined in the following sequence (5’-ATGGACTACAAAGACGATGACGACAAGTCCGGAATCGATATGTCGATGACAGACTTGCTCAGCGCTGAGGACATCAAGAAGGGCATAGGAGGAGCCTTTACTGCTCGACCTTGACCACAAAAATGTTCTTTACAGTGGGGTCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGATTGGGGTTGAAGAGTTCCACTCTGGTGGCCGAAAGCATCGATCTTAAGATGCTGACTGACCGTGGCAAGAT
two parvalbumin-derived sites were equivalent for Ca\(^{2+}\) binding.

N-terminal glutathione S-transferase (GST) tag. SpiCee F134W and SpiCeeF103W clones were prepared from SpiCee using the QuickChange II Site-directed mutagenesis kit (Agilent Technologies). Proteins were produced in BL21 (DE3) pLysS E. coli cells. The GST-tagged proteins were purified using a glutathione Sepharose 4B column. The GST tag was cleaved using recombinant tobacco etch virus N terminus domain.

Change II Site-directed mutagenesis kit (Agilent Technologies). Proteins were produced in BL21 (DE3) pLysS E. coli cells. The GST-tagged proteins were purified using a glutathione Sepharose 4B column. The GST tag was cleaved using recombinant tobacco etch virus N terminus domain.

Production of purified SpiCee\(^{134W}\) and SpiCee\(^{103W}\)

The full-length SpiCee DNA was subcloned into a pGST-Parallel2 vector (derived from pGEX-4T-1; Amersham) in frame with the N-terminal glutathione S-transferase (GST) tag. SpiCee\(^{134W}\) and SpiCee\(^{103W}\) clones were prepared from SpiCee using the QuickChange II Site-directed mutagenesis kit (Agilent Technologies). Proteins were produced in BL21 (DE3) pLysS E. coli cells. The GST-tagged proteins were purified using a glutathione Sepharose 4B column. The GST tag was cleaved using recombinant tobacco etch virus N terminus domain. The recombinant proteins were eluted in a buffer containing 30 mM MOPS (3-Morpholinopropanesulfonic acid), pH 7.2 and 100 mM KCl. Protein concentrations were deduced from A\(_{280}\) measurements using a computed extinction coefficient of 5500 M\(^{-1}\) cm\(^{-1}\) for both SpiCee\(^{134W}\) and SpiCee\(^{103W}\).

Measurement of Ca\(^{2+}\) affinity for the labeled site of SpiCee\(^{103W}\) (parvalbumin-derived)

Ca\(^{2+}\) binding to the labeled site of SpiCee\(^{103W}\) was monitored by measuring tryptophan fluorescence (285 nm excitation, 330 nm emission, 5 nm bandpass) at 20 °C in a 4 mm x 10 mm quartz cuvette (Jasco FP-8300 spectrofluorimeter). The protein was diluted to 2.25 \(\mu\)M in standard buffer (30 mM MOPS-KOH pH 7.2; 0.1 M KCl) and 254 \(\mu\)L samples were loaded in the cuvette. It was first observed that addition of CaCl\(_2\) did not increase fluorescence, whereas addition of millimolar amounts of EGTA led to large decreases (up to 27% at a final EGTA concentration of 7.5 mM). This showed that the labeled site of SpiCee\(^{103W}\) was saturated with Ca\(^{2+}\). Dissociation constant of Ca\(^{2+}\) was deduced from a competition experiment in which the protein solution was titrated with EGTA. Two \(\mu\)L samples of solutions containing increasing concentrations of EGTA (1.95 to 500 mM) were added to the cuvette. Fluorescence was measured after each addition. Fluorescence values were corrected for dilution and plotted as a function of the concentration of EGTA (Equation 1) as described (Weeks and Crothers, 1992), assuming that the two parvalbumin-derived sites were equivalent for Ca\(^{2+}\) binding.

\[
F = F_0 + (F_\infty - F_0) \left[ 1 + \frac{[\text{EGTA}]}{K_D + [\text{EGTA}]} + \frac{|\text{Ca}| + [\text{P}] - \sqrt{[\text{EGTA}] + [\text{Ca}] + [\text{P}] + [\text{Ca}] + [\text{P}] + [\text{Ca}] + [\text{P}]}}{2[P]} \right] (\text{Equation 1})
\]

where \(F_0\) is the fluorescence of the Ca\(^{2+}\)-saturated protein, \(F_\infty\) the fluorescence of Ca\(^{2+}\)-free protein, \([\text{P}]\) the concentration in high-affinity Ca\(^{2+}\)-binding sites (here 4.5 \(\mu\)M), \(K_{\text{EGTA}}\) the dissociation constant of Ca\(^{2+}\) from its complex with EGTA, \(K_D\) the dissociation constant of Ca\(^{2+}\) from its complex with the parvalbumin site, \([\text{Ca}]\) the total concentration of Ca\(^{2+}\) and \([\text{EGTA}]\) the total concentration of EGTA.

\(K_D\), \(F_0\), \(F_\infty\) and their associated standard errors were derived from non-linear least square fitting with the Origin 9.0 software. Results are the mean ± standard deviation from three independent experiments. Fitting required the input of two values. The dissociation constant of Ca\(^{2+}\) from its complex with EGTA is 0.15 \(\mu\)M in these conditions (Tsien and Pozzan, 1989). The total concentration of Ca\(^{2+}\) in the sample was calculated as the concentration of Ca\(^{2+}\) bound to the two parvalbumin sites (i.e., 4.5 \(\mu\)M) plus the concentration of free Ca\(^{2+}\) and the calmodulin-derived sites of SpiCee bound to Ca\(^{2+}\). The latter was measured using the fluorescent calcium indicator Quin-2 (50 \(\mu\)M; excitation 332 nm, emission 493 nm). At the concentrations used, the indicator was not sensitive to the parvalbumin-derived sites of SpiCee bound to Ca\(^{2+}\) because of its much lower affinity for Ca\(^{2+}\) (60 nM) (Tsien and Pozzan, 1989). A value of 2 \(\mu\)M for the concentration of free plus calmodulin-derived sites-bound to Ca\(^{2+}\) was computed from this experiment. Therefore, a total concentration of Ca\(^{2+}\) of 6.5 \(\mu\)M was used for fitting using Equation 1.

Measurement of Ca\(^{2+}\) affinity for the labeled site of SpiCee\(^{134W}\) (calmodulin-derived)

Ca\(^{2+}\) binding to the first calmodulin site of SpiCee\(^{134W}\) was monitored by measuring tryptophan fluorescence (285 nm excitation, 340 nm emission, 5 nm bandpass) at 20 °C. SpiCee\(^{134W}\) was diluted to 1.6 \(\mu\)M in standard buffer and 258 \(\mu\)L samples were loaded in the cuvette. Addition of CaCl\(_2\) increased fluorescence, whereas addition of millimolar amounts of EGTA led to fluorescence decrease, showing that the calmodulin-derived sites of SpiCee were partially filled with Ca\(^{2+}\) in the sample. The value of the fluorescence of the protein with Ca\(^{2+}\) was measured in the presence of 2 mM EGTA, a concentration found to be
saturating. Total concentration of Ca\(^{2+}\) in the sample (excluding Ca\(^{2+}\) bound to the parvalbumin-derived sites of SpiCee) was measured as described above using Quin-2 and found to be 2.5 \(\mu\)M. Dissociation constant of Ca\(^{2+}\) from the labeled site of SpiCee\(^{134W}\) was deduced from an experiment where the protein solution was titrated with increasing amounts of CaCl\(_2\) (2 \(\mu\)L additions of solutions from 0.05 to 6.25 mM). Fluorescence values were corrected for dilution, and plotted as a function of total Ca\(^{2+}\) concentration (added Ca\(^{2+}\) plus 2.5 \(\mu\)M). Data were fitted to a binding curve (equ. 2) assuming that the two calmodulin sites were equivalent for Ca\(^{2+}\) binding.

\[
F = F_0 + \frac{(F_s - F_0) [P] + [Ca] + K_D - \sqrt{([P] + [Ca] + K_D)^2 - 4[P][Ca]}}{2[P]}
\]

(Equation 2)

where \(F_0\) is the fluorescence of the Ca\(^{2+}\)-free protein, \(F_s\) the fluorescence of Ca\(^{2+}\)-saturated protein, \([P]\) the concentration in low-affinity Ca\(^{2+}\)-binding sites (here 3.2 \(\mu\)M), \(K_D\) the dissociation constant of Ca\(^{2+}\) from its complex with the labeled site of SpiCee\(^{134W}\), \([Ca]\) the total concentration of Ca\(^{2+}\).

**Measurement of Mg\(^{2+}\) affinity**

Titrations with Mg\(^{2+}\) were made in the presence of 10 mM EGTA (F103W) or 2 mM EGTA (F134W) to remove Ca\(^{2+}\) from the studied sites. The concentrations of free Mg\(^{2+}\) during titration were calculated using a dissociation constant of 19.73 mM for the Mg\(^{2+}\)-EGTA complex (Tsien and Pozzan, 1989).

\(K_D, F_0\), and \(F_s\) and their associated standard errors were derived from non-linear least square fitting with the Origin software (Origin Lab.). Results are the mean ± standard deviation from three independent experiments.

**Measurement of kinetic dissociation constants**

Values of kinetic dissociation constants (\(k_{on}\)) for the tryptophan-labeled Ca binding sites of SpiCee (SpiCee\(^{103W}\) and SpiCee\(^{134W}\)) were measured by mixing proteins with appropriate amounts of EGTA at 20°C in an Applied Photophysics stopped flow apparatus. Reacting solutions were excited at 285 nm and fluorescence was measured using a photomultiplier equipped with a 320 nm cutoff filter. For SpiCee\(^{103W}\), the protein (2.3 \(\mu\)M in standard buffer) was mixed 1:1 with 15 mM EGTA in the same buffer. For SpiCee\(^{134W}\), the protein (1.7 \(\mu\)M in standard buffer plus 50 \(\mu\)M CaCl\(_2\)) was mixed 1:1 with 4 mM EDTA in standard buffer plus 50 \(\mu\)M CaCl\(_2\). Fluorescence was recorded as a function of time and data were fitted with single exponential curves from which rate constants were derived. Reported values are the mean ± standard deviation from three measurements.

**Cell death assay**

HEK293 Cells were plated on poly-lysine-coated coverslips and transfected the following day with a pCX-mRFP or a pCX-SpiCee vector using Lipofectamine 2000 (Thermo Fisher) following the manufacturer’s instructions. Retinas of E14 mouse embryos were electroporated with mRFP or SpiCee using two poring pulses (square wave, 175 V, 5 ms duration, with 50 ms interval) followed by four transfer pulses (40 V, 50 ms and 950 ms interpulse) with a Nepa21 Super Electroporator (NepaGene). Retinas were dissected and kept 24 hours in culture medium (DMEM-F12 supplemented with glutamine (Sigma Aldrich), penicillin/streptomycin (Sigma Aldrich), BSA (Sigma Aldrich) and glucose), in a humidified incubator at 37°C and 5% CO2. The day after, they were cut into 200 \(\mu\)m squares with a Tissue-Chopper (McIlwan) and explants were plated on glass coverslips coated with poly-lysine and Laminin (Sigma Aldrich). Cells were cultured for 24 hours in culture medium supplemented with 0.4% (w/v) methyl cellulose.

Three days (HEK293 cells) or 24h (retinal explants) after plating, cells were either fixed with 4% (w/v) paraformaldehyde and processed for immunocytochemistry with the antibodies against cleaved caspase 3 (Asp175; Cell Signaling; lot # 0043) and DsRed (Ozyme), or treated with the CellEvent Caspase 3/7 Green Detection Reagent (Thermo Fisher) for 30 minutes and then fixed and labeled with an α-DsRed (Ozyme) antibody. For each experiment, the proportion of caspase3-positive over unlabeled cells in the population of mRFP- or SpiCee-positive cells was computed from 10 randomly chosen fields acquired using a 20x air objective in a DM6000 microscope (Leica Microsystems) (HEK293 cells) or from one optical section of the retinal explant acquired using a 40x dry objective in a FV1000 confocal microscope (Olympus) (retinal explants).

**Resting Ca\(^{2+}\) concentration measurement**

HEK293 Cells were plated on poly-lysine-coated 35 mm plates and transfected 24 hours later with a pCX-mRFP or a pCX-SpiCee vector using Lipofectamine 2000 (Thermo Fisher) and the recommended protocol. The following day, cells were loaded with Fura-2-AM (Thermo Fisher) for 1 hour at 37°C, washed thrice and bathed in HBS with either 0.2 or 2 mM CaCl\(_2\). Randomly chosen fields were acquired with a 40x water immersion objective (N.A. 0.8) in an Eclipse 80i microscope (Nikon). Fura-2 fluorescence from 340 and 380 nm excitation was acquired sequentially with the software Imaging Workbench 6.0 (INDEC Biosystems) controlling a Lambda DG-4 (Sutter Instruments). The emission at 594 nm was also acquired to verify the expression of mRFP and SpiCee. Calcium concentration was assessed by computing the ratio of emissions obtained from the 340nm and 380 nm excitation. Images were corrected for background fluorescence and signal induced by the mRFP bleedthrough in each of the wavelengths before ratio
calculation. Such ratio was then converted into calcium concentration using a calibration curve obtained with a Fura-2 Calcium Imaging Calibration Kit imaged in the same experimental conditions (Thermo Fisher).

**Membrane fractionation by detergent-free method**

Electroporated retinas were pelleted (195 g for 5 min at 4°C) and resuspended in 1.34 mL of 0.5 M sodium carbonate, pH 11.5, with protease inhibitor cocktail and phosphatase inhibitor cocktails 1, 2 and 3 (Sigma-Aldrich). The homogenate was sheared through a 26-gauge needle and sonicated three times for 20 s bursts. The homogenate was adjusted to 40% (w/v) sucrose by adding 2.06 mL of 60% (w/v) sucrose in MBS (25 mM MES, pH 6.4, 150 mM NaCl, and 250 mM sodium carbonate), placed under a 5%–30% (w/v) discontinuous sucrose gradient, and centrifuged at 34,000 rpm for 15–18 h at 4°C in a Beckman SW 41Ti rotor. Nine fractions (1.24 mL each) were harvested from the top of the tube mixed with 9 volumes of MBS, and centrifuged at 40,000 rpm for 1 h at 4°C (Beckman SW-41Ti rotor). Supernatants were discarded, and membrane pellets were resuspended in 100 µL of 1% (w/v) SDS.

For immunoblotting, samples were separated on a precast gel (4%–15% Mini- Protean TGX Tris-Glycine-buffer SDS-PAGE, Bio-rad) and transferred onto 0.2 µm Trans-Blot Turbo nitrocellulose membranes (Biorad). Membranes were blocked for one hour at room temperature in 1xTBS (10 mM Tris pH 8.0, 150 mM NaCl) supplemented with 5% (w/v) dried skim milk powder. Primary antibody incubation was carried out overnight at 4°C, with the following antibodies: rabbit anti-DesRed (1/200; 632476; Clontech; lot # 1306037), rabbit anti-β-Adaptin (1/200; sc-10762; Santa Cruz; lot # E1304) and rabbit anti-Caveolin (1/500; 610060; BD Transduction Laboratories; lot # GR256941-5). All primary antibodies have been previously validated for this assay (Averaimo et al., 2016). A goat anti-rabbit-HRP-coupled secondary antibody was used for detection (Jackson ImmunoResearch, West Grove, PA). After antibody incubations, membranes were extensively washed in TBS T (TBS containing 2.5% (v/v) Tween-20). Western blots were visualized using the enhanced chemiluminescence method (ECL prime Western Blotting detection reagent, Amersham).

For dot blot analysis (CTB staining), 3 µL of each fraction, resuspended in MBS, were blotted on nitrocellulose membrane, dried for 1 h and blocked for 1 h with 3% (w/v) dried skim milk powder at room temperature. The membrane was incubated overnight with HRP-conjugated cholera toxin B-subunit (Sigma Aldrich C3741) and detected with ECL Prime.

**Analysis of Erk1/2 phosphorylation**

For phosphorylation studies, HEK293T cells were transfected or not with SpiCee and/or Twitch2B vectors for 24 hours after plating using Lipofectamid 2000 according to the manufacturer’s instructions (Invitrogen). For stimulation, cells were incubated for 5 minutes with 1 µM Thapsigargin or vehicle at 37°C in the presence or not of BAPTA. In this case, 30 minutes before stimulation with BAPTA-AM 50 µM or vehicle solution was applied. Thapsigargin stimulation reaction was stopped by placing cells on ice followed by extraction of proteins described before. Samples were then separated and membranes incubated with a rabbit anti-phospho-ERK1/2 (Thr202/Tyr204) (1/500; CS_9101; Cell Signaling) or a rabbit anti-ERK1/2 (1/1000; CS_4695, Cell Signaling) and visualized by extraction of proteins described before. Samples were then separated and membranes incubated with a rabbit anti-phospho-ERK1/2 (Thr202/Tyr204) (1/500; CS_9101; Cell Signaling) or a rabbit anti-ERK1/2 (1/1000; CS_4695. Cell Signaling) and visualized with goat anti-rabbit HRP as secondary antibodies and ECL Prime for the detection.

**In utero electroporation and mouse brain processing**

Timed pregnant females (Janvier Labs) were delivered to Institute’s animal facility a week prior to the surgery in order to allow a minimum of 5-day adaptation period. In utero electroporation was performed as described previously (Loulier et al., 2009). E14.5 timed pregnant females were anesthetized with Ketamine/Xylazine and a midline laparotomy was performed, exposing uterine horns and allowing visualization of embryos under oblique illumination. 1 mL of DNA containing two plasmid vectors combined 3:1 with sterile Fast Green dye (Sigma) was injected with a glass capillary pipette (75–125 µm outer diameter with beveled tip) driven by a INJECT+MATIC (INJECT+MATIC) microinjector into the lateral ventricle of each embryo. A plasmid encoding green fluorescent protein under the control of the chicken beta actin promoter (pCX-eGFP) was used to monitor the success of electroporation. The second plasmid encoded either a red fluorescent protein (mRFP) control construct or SpiCee-mRFP. The anode of 5 mm diameter tweezertrodes (Sonidel Limited) was placed above the dorsal telencephalon and four 35 V pulses of 50 ms duration were applied across the uterine sac. Following intraterine surgery, the incision site was closed with sutures (4-0, Ethicon) and the mouse was allowed to recover in a clean cage. Mice were either euthanized 4 days after surgery to harvest E18.5 embryonic brains, or allowed to give birth for analysis at P10. Embryonic brains were dissected out, immersed overnight in Antigenfix (Diapath) fixative solution and rinsed in PBS prior to sectioning. P10 mice were deeply anesthetized with sodium pentobarbital (150 mg·kg⁻¹), perfused transcardially with Antigenfix (Diapath), brains dissected out and postfixed overnight in the same solution. Embryonic and postnatal brain samples were sectioned at 200 µm thickness on a vibrating blade microtome (Leica VT 1000S) and, sections were either mounted in Vectashield+Dapi (Vector laboratories) or incubated 2 hours in 10 µg·mL⁻¹ bis-benzimide (Sigma) and mounted in Mowiol 4-88 (Sigma Aldrich). Confocal images were acquired with a 10x objective (N.A. 0.4) and a Z stack containing the whole specimen was sampled at Nyquist frequency. Images were rendered in ImageJ and Photoshop.

**Collapse assay**

Retinal explants were treated with 200 ng·mL⁻¹ rmSlit-1 or 500 ng·mL⁻¹ rmEphrinA5 (R&D Systems) for 1 hour before fixation with 4% (w/v) PFA in PB for 30 minutes.
Immunostaining
Retinal explants, or HEK cells coexpressing the targeted versions of SpiCee and GFP, were fixed with 4% (w/v) PFA in PB for 30 minutes, permeabilized and blocked with 1% (v/v) Triton and 3% (w/v) BSA in PBS, then immunized against DsRed (Clontech, lot # 1306037, previously used in a similar assay (Averaimo et al., 2016)) followed by a secondary antibody coupled to AlexaFluor 594 (Life Technologies) and GFP (Life Technologies, lot # 1789911, previously validated (Nicol et al., 2007)) or α-Tubulin (Sigma, lot # 024M4850V, validated (Nicol et al., 2007)) followed by a secondary antibody coupled to AlexaFluor 488 (Life Technologies).

FRET Imaging and analysis
Images were acquired with an inverted DMI6000B epifluorescence microscope (Leica) coupled to a 40x oil-immersion objective (N.A. 1.3) and Metamorph software (Molecular Devices). For live imaging experiments, cells transfected with Twitch2B, and co-expressing mRFP, SpiCee or its variants were perfused with HBS buffer with 0.2 or 2 mM CaCl2. Thapsigargin (Sigma-Aldrich) was used at 1 μM; ATP (Sigma-Aldrich) was used at 5 μM in HBS 1.5 mM CaCl2. Images were acquired simultaneously for the CFP (483/32 nm) and YFP (542/27) channels every 20 s, or every 5 s for ATP stimulation, while cells were continuously superfused with the medium described above. Simultaneous CFP and YFP channel acquisition was achieved using a dual chip CCD camera ORCA-D2 (Hamamatsu). The wavelength used for CFP excitation in HEK cells was 436/20 nm. Images were processed in ImageJ, corrected for background and bleedthrough from CFP into the YFP channel, and the ratio CFP/YFP was computed and normalized to the initial values for each single cell. For better evaluation of the cell morphology, the YFP channel (not the image of the ratio) is illustrated in all figures describing FRET experiments.

Imaging of SpiCee targeting
Confocal images were acquired with a 63x oil immersion objective (N.A. 1.45) and a Z stack containing the whole specimen was sampled at nyquist frequency in an Olympus confocal microscope (FV1000). Images were rendered in ImageJ and Photoshop.

Quantification and Statistical Analysis
No data were excluded from the analysis. No sample size calculation was performed. Sample size was considered sufficient after three reproducible and independent experiments, leading to n ≥ 3 since several animals, coverslips, or biochemical assays were often analyzed for the same experimental condition. Animals or cultures were equivalent and not distinguishable one from another before treatment, de facto randomizing the sample without the need of a formal randomization process. Photomicrographs were often easily traceable by eye to its experimental condition, making blind analysis of the data difficult to achieve. When careful blinding was performed, experiments reproduced the results obtained in non-blinded experiments with identical experimental conditions. Image calculation and analysis were performed using ImageJ.

Statistical tests were calculated using GraphPad Prism (GraphPad Software Inc.). Table S1 summarizes the tests used, P values and number of replicates for all the data shown in Figures 1, 2, 3, 4, 5, and 6 and Figures S1, S2, S3, S4, S5, and S6.
Supplemental Information

SpiCee: A Genetic Tool for Subcellular and Cell-Specific Calcium Manipulation

Oriol Ros, Sarah Baudet, Yvrick Zagar, Karine Loulier, Fiona Roche, Sandrine Couvet, Alain Aghaie, Melody Atkins, Alice Louail, Christine Petit, Christine Metin, Yves Mechulam, and Xavier Nicol
Figure S1. SpiCee-transfected cells adapt to maintain a resting concentration of Ca^{2+} similar to their controls. Related to Figure 2. SpiCee- or RFP-transfected HEK293 cells were loaded with the ratiometric calcium sensor Fura-2, enabling to evaluate their intracellular calcium concentration. (A) Fura-2 was sequentially excited at 340 nm (Ca^{2+}-bound) and 380 nm (Ca^{2+}-free), and the intracellular Ca^{2+} concentration was computed based on the fluorescence of calibrated Ca^{2+} solutions. No difference in the resting Ca^{2+} concentration was detected between SpiCee- and RFP-transfected cell in culture media containing either (A) 0.2 mM or (B) 2 mM Ca^{2+}. (C) The Ca^{2+} resting concentration is higher in both SpiCee- and RFP-transfected cells grown in 2mM extracellular Ca^{2+} as compared to cells kept in 0.2 mM Ca^{2+}, suggesting that SpiCee does not prevent the regulation of the intracellular resting concentration of this ion. (A, B) Scale bar, 20 µm. The scale bar in (B) applies to (A). (C) Box-and-whisker plot elements: center line, mean; box limits, upper and lower quartiles; whiskers, s.d.; *** P ≤ 0.001, Kruskal-Wallis test followed by Dunn’s multiple comparison test. Exact P values and number of replicates are provided in Table S1.
Figure S2. SpiCee reduces the elevation of Ca\textsuperscript{2+} concentration induced by pulses of thapsigargin and delays massive and sustained elevations of intracellular Ca\textsuperscript{2+} concentration. Related to Figure 3. HEK293 cells were bathed with a low Ca\textsuperscript{2+} medium and challenged with a 1-minute (A-C) (See also Video S2), 2-minute (D-F) (See also Video S3) and 5-minute (G-I) (See also Video S4) pulse of thapsigargin. SpiCee-expressing cells display a reduced FRET/CFP ratio elevation compared to controls, and a delayed maximum response. The decay of the response is also delayed in SpiCee-expressing cells, reducing the sharpness of the response. (J) A change of extracellular Ca\textsuperscript{2+} concentration (from 0.2 mM to 2 mM)
generates a massive elevation of fluorescence in the Twitch2B FRET channel in HEK293 cells. The numbers on top of each column reflect the time of acquisition for each image and match the position of the numbered time point in panel K. (K, L) SpiCee-expressing cells exhibit a change in the FRET/CFP ratio of similar magnitude, but delayed as compared to cells devoid of SpiCee (See also Video S5). (M) mutSpiCee, a variant in which the calcium binding sites have been mutated, does not prevent the elevation of fluorescence in the Twitch2B FRET channel induced by a 1-minute pulse of thapsigargin. (N, O) The FRET/CFP ratio elevation is not affected by the expression of mutSpiCee. Scale bars, 20 µm. (B, E, H, K, N) Data are mean ± s.e.m. (C, F, I, L, O) Box-and-whisker plot elements: center line, mean; box limits, upper and lower quartiles; whiskers, s.d.; *** P ≤ 0.001. (C, F, I, L) Mann-Whitney test, (O) Kruskal-Wallis test. Exact P values and number of replicates are provided in Table S1.
**Figure S3. Ca\textsuperscript{2+} buffering by SpiCee is not affected by the level of expression or by co-expression of Twitch2B. Related to Figure 3.**

(A) The amplitude of the ATP-induced elevation in the FRET/CFP ratio was plotted against the mRFP fluorescence as a proxy of SpiCee or mRFP expression level. The dataset shown in Figure 3D-F was used. The level of mRFP fluorescence is correlated with the amplitude of the FRET/CFP ratio elevation neither in mRFP- (blue data) nor in SpiCee-expressing (orange data) cells. Data points were fitted to a straight line. In both cases the fit leads to close to horizontal line with a minimal r\textsuperscript{2} coefficient.

(B) HEK293 cells expressing either SpiCee or SpiCee and Twitch2B were exposed to thapsigargin and the level of ERK1/2 phosphorylation was assessed. The calcium-induced elevation of ERK phosphorylation is not reduced in SpiCee and Twitch2B co-expressing cells compared to cells expressing SpiCee alone.

(C) Data are mean ± s.e.m. Individual data points are shown. ** P ≤ 0.01; ANOVA followed by Bonferroni post hoc tests. Exact P values and number of replicates are provided in Table S1.
Figure S4. SpiCee can be targeted to membrane and intracellular compartments, in contrast to hPMCA. Related to Figure 6. (A) In cortical interneurons, SpiCee is found throughout the cytoplasm and excluded from the primary cilium stained with Arl13b (higher magnification in insets). When fused to the 5HT6 receptor, it is targeted to the primary cilium (middle). Like SpiCee, hPMCA can be targeted to this plasma membrane compartment using the 5HT6 receptor (right). (B) In HEK293 cells, SpiCee is found throughout the cytoplasm and to lesser extent in the nucleus whereas hPMCA expression is restricted to the plasma membrane. SpiCee expression can be restricted to the nucleus using a nuclear localization sequence (NLS) or to the cytoplasm using a nuclear export sequence (NES). In contrast, attempts to restrict or exclude hPMCA expression from the nucleus failed and led to its trapping to a compartment resembling the endoplasmic reticulum. Scale bars, 20 µm; in insets 5µm.
Figure S5. Biochemical characterization of SpiCee targeting. Related to Figure 6. (A, B) Lyn-SpiCee peaks in fractions 3 in a membrane fractionation assay. (A) It coincides with the expression profile of the β subunit of cholera toxin (CtB, enriched in fractions 3 and 4, peaks in fraction 3) and (B) differs from the expression of Caveolin (enriched in fraction 4). SpiCee-Kras is mostly enriched in fraction 4, with a profile (C) distinct from CtB, and (D) resembling Caveolin. (E) The Kras-targeted variant of Twitch2B was co-expressed with either mRFP (top row) or SpiCee-Kras (bottom row) in HEK293 cells. SpiCee-Kras reduces the thapsigargin-induced FRET channel fluorescence elevation of Twitch2B-Kras. (F) FRET/CFP ratio and (G) amplitude and slope quantifications from cells co-expressing either Twitch2B-Kras and mRFP (blue trace and boxes) or Twitch2B-Kras and SpiCee-Kras (orange trace and boxes). (A-D, F) Data are mean ± s.e.m., (G) Box-and-whisker plot elements: center line, mean; box limits, upper and lower quartiles; whiskers, s.d.; * P ≤ 0.05, *** P ≤ 0.01, **** P ≤ 0.001, (A-D) Two-way ANOVA and Bonferroni post hoc tests, (G) Mann-Whitney test. Exact P values and number of replicates are provided in Table S1. Data shown in Figure 6D is included here for additional comparisons. Individual data points are shown in Figure 6D when n ≤ 10.
Figure S6. Cytosolic SpiCee and Lyn-SpiCee but not SpiCee-Kras prevent ephrinA5- and Slit1-induced growth cone collapse. Related to Figure 6. Non-electroporated RGC axons display a fan-shaped growth cone at their tip which is not affected by the expression of mRFP, SpiCee, Lyn-SpiCee or SpiCee-Kras. When exposed to (B) ephrinA5 or (F) slit1, mRFP-expressing growth cones exhibit the same collapse response than their non-electroporated neighbors of the same coverslips, (C, G) SpiCee- and (D, H) Lyn-SpiCee-expressing axons are resistant to the repellent activity of ephrinA5 and slit1, that is observed in the non-electroporated axons from the same coverslip. (E, I) In contrast, SpiCee-Kras-expressing axons exhibit...
similar growth cone remodeling to their controls. Box-and-whisker plot elements: center line, mean; box limits, upper and lower quartiles; whiskers, s.d. Scale bar, 10 µm. (A) No significant differences are detected; Kruskal-Wallis test. (B-I) * $P \leq 0.05$; *** $P \leq 0.001$; Wilcoxon paired test. Exact $P$ values and number of replicates are provided in Table S1. A subset of the data shown in Figure 6E and 6F is included here for additional comparisons with control growth cones from the same coverslips.