Single-fluorophore biosensors for sensitive and multiplexed detection of signalling activities

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Unravelling the dynamic molecular interplay behind complex physiological processes such as neuronal plasticity requires the ability to both detect minute changes in biochemical states in response to physiological signals and track multiple signalling activities simultaneously. Fluorescent protein-based biosensors have enabled the real-time monitoring of dynamic signalling processes within the native context of living cells, yet most commonly used biosensors exhibit poor sensitivity (for example, due to low dynamic range) and are limited to imaging signalling activities in isolation. Here, we address this challenge by developing a suite of excitation ratiometric kinase activity biosensors that offer the highest reported dynamic range and enable the detection of subtle changes in signalling activity that could not be reliably detected previously, as well as a suite of single-fluorophore biosensors that enable the simultaneous tracking of as many as six distinct signalling activities in single living cells.

Cell function and behaviour are shaped by the coordinated actions of multiple biochemical activities. Protein kinases in particular are implicated in regulating nearly all aspects of cellular function through their role as key nodes within intracellular signalling networks. Our understanding of these complex and intricate networks has greatly benefited from the advent of optical tools, such as genetically encoded biosensors based on fluorescence resonance energy transfer (FRET), that enable the direct visualization of numerous dynamic biochemical processes, including kinase activity, in living cells. However, fully elucidating how various signalling pathways interact to regulate complex physiological processes, such as neuronal plasticity, requires the ability to move beyond imaging these activities in isolation and has thus fuelled a growing interest in the development of strategies to simultaneously track multiple biochemical activities within living cells.

The primary obstacle to such multiplexed imaging is the limited amount of spectral space available to image multiple fluorescent biosensors1. For the most part, current approaches remain largely confined to monitoring two activities in parallel, although four-parameter imaging has been demonstrated by combining spatially separated FRET sensors with a translocating probe and a fluorescent indicator dye2. However, such hybrid strategies cannot be easily adapted to monitoring various activities throughout the cell. Alternatively, single-fluorophore biosensors based on circularly permuted fluorescent proteins (cpFPs) offer a much more straightforward path to image multiple biosensors—and hence, multiple activities—concurrently. Yet while cpFP intensity is known to be modulated by the insertion of conformationally dynamic elements for detecting Ca2+ (refs 3,4), voltage7 and other small molecules3,4, it remains unclear how easily this sensor design can be generalized for more widespread applications, such as monitoring enzymatic activities. We therefore set out to construct single-fluorophore biosensors for monitoring protein kinase activity. Here, we report a suite of single-fluorophore-based biosensors that enable more sensitive detection of dynamic kinase activities and allow us to reliably monitor multiple signalling activities in living cells, including primary neuronal cultures.

Results

Development and characterization of an excitation ratiometric kinase sensor. FRET-based kinase activity reporters typically contain a kinase-specific substrate sequence and a phosphoamino acid-binding domain (for example, FHA1) capable of binding the phosphorylated substrate and inducing a FRET change. On the basis of the hypothesis that this conformational switch could similarly modulate cpFP fluorescence (Fig. 1a), we constructed a prototype single-fluorophore enzyme activity reporter by combining the protein kinase A (PKA) substrate (LRRATLVD) and FHA1 domains of AKAR7 with cpGFP from GCaMP33 (Fig. 1b).

Following bacterial expression and purification, the resulting construct unexpectedly displayed two excitation peaks—a major peak centred around 400 nm and a second, approximately fourfold smaller peak at 509 nm with a distinct shoulder at 480 nm—both of which resulted in emission at ~515 nm (Fig. 1c). Both excitation peaks were also similarly sensitive to pH changes between 5.6 and 10 (Supplementary Fig. 1). These results are reminiscent of the neutral and anionic chromophore states found in wild-type Aequorea victoria GFP (wtGFP)11 and, given the absence of any cpGFP mutations, suggest that insertion of the PKAsub and FHA1 domains rescued wtGFP chromophore behaviour in our construct compared with GCaMP3. In addition, incubation with excess PKA catalytic subunit and ATP led to an ~80% decrease and ~30% increase in the amplitude of the first and second peak, respectively, yielding a greater than twofold excitation ratio increase (Fig. 1c). This effect
Fig. 1 | Design and characterization of ExRai-AKAR. a, Modulation of cpFP fluorescence by a phosphorylation-dependent molecular switch. b, ExRai-AKAR domain structure. c, Representative ExRai-AKAR fluorescence excitation spectra collected at 530 nm emission (left) and emission spectra collected at 380 nm (middle) or 488 nm (right) excitation without (grey) or with (green) ATP in the presence of PKA catalytic subunit. AKAR) cells pooled from 2, 2 and 11 experiments. e, ExRai-AKAR is dimmer at Ex480 (**P < 0.0001, t = 5.228, df = 136; unpaired two-tailed Student’s t-test) but brighter at Ex380 (**P < 0.0001, t = 8.826, df = 136; unpaired two-tailed Student’s t-test) versus GCaMP3. Same n as in d. a.u., arbitrary units.

f, Representative GCaMP3 or ExRai-AKAR fluorescence images. g–i, Average time courses (left) and maximum Ex480 (g) or Ex380 (ΔF/F) (h), or 480/380 ratio (ΔF/ΔF) (i) responses (right, top) in HeLa cells treated with 50 μM Fsk/100 μM IBMX (Fsk/IBMIX). n = 54 (ExRai-AKAR), 36 (ExRai-AKAR[T/A]) and 31 (ExRai-AKAR plus PKI) cells. Time courses are representative of and maximum responses are pooled from 11, 4 and 4 experiments, respectively. **P < 0.0001, F = 665.7 (d), F = 3.956 (e), F = 1.819 (f); Dfn = 2, Ddf = 119; one-way analysis of variance (ANOVA) with Dunnett’s test. Images show ExRai-AKAR Ex480 (g) or Ex380 (h) fluorescence, or 480/380 ratio (pseudocoloured) before and after stimulation (i). Warmer colours indicate higher ratios. All scale bars, 30 μm. j–l, Average time courses (left) and maximum 488-nm-excited (j) or 405-nm-excited (k) fluorescence, or 488 nm/405 nm ratio responses (right) (l) in the soma of Fsk-treated cultured rat cortical neurons. n = 57 (ExRai-AKAR) and 56 (ExRai-AKAR[T/A]) neurons pooled from 5 and 6 experiments. **P < 0.0001, unpaired two-tailed Mann–Whitney U-test. The curves are normalized to time 0 (g–i) or to the average baseline value (j–l). The solid lines indicate the mean, shaded areas, s.e.m. The bars in d, and horizontal lines in e and g–i represent mean ± s.e.m. The bar graphs in j–l show box-and-whisker plots indicating the median, interquartile range, minimum and maximum. Maximum responses are calculated as ΔF/ΔF = (Fmax – Fmin)/Fmin, or ΔR/R = (Rmax – Rmin)/Rmin (g–i) or with respect to time 0 (F/F0 or R/R0; j–k).

See Supplementary Table 1 for bar graph source data.
is analogous to the excitation ratiometric behaviour observed in several previously described sensors such as ratiometric pericam\(^1\), Perceval\(^6\) and GEX-GECO1\(^1\)

When expressed in HeLa cells, the resulting excitation ratiometric (ExRai)-AKAR showed moderate fluorescence at 480 nm, but again showed much stronger fluorescence under 380-nm illumination compared with either EGFP or GCaMP3 (Fig. 1d–f). Furthermore, maximally stimulating PKA in ExRai-AKAR-expressing cells using the adenyl cyclase activator forskolin (Fsk) and the pan-
phosphodiesterase inhibitor 3-isobutyl-1-methyloxanthine (IBMX) produced a modest but rapid increase in 480-nm-excited intensity (average change (ΔF/F): 24.1 ± 0.6%, \(n = 54\) (mean ± s.e.m.; \(n \) number of cells); highest response: 34.9%) (Fig. 1g and Supplementary Fig. 1), a robust decrease in 380-nm-excited intensity (average ΔF/F: −49.7 ± 0.5%, \(n = 54\); highest response: −57.9%) (Fig. 1h and Supplementary Fig. 1) and a dramatic increase in the 480 nm/380 nm excitation ratio (average ΔR/R: 143.7 ± 2.5%, \(n = 54\); highest response: 186.3%) (Fig. 1i and Supplementary Fig. 1). A robust ratiometric response was also detected in Fsk-stimulated primary cortical neurons transfected with ExRai-AKAR (Fig. 1j–l). On the other hand, cells expressing a negative-control construct in which the target threonine residue was mutated to alanine (T/A) did not respond to stimulation (Fig. 1g–l), nor did cells co-expressing the potent PKA inhibitor PKI, which acute PKA inhibition reversed the Fsk/IBMX-induced response (Supplementary Fig. 1). These data indicate that ExRai-AKAR dynamically and specifically detects PKA activity in living cells.

Robust detection of compartmentalized PKA activity in response to growth factor stimulation using ExRai-AKAR. The excitation ratiometric response of ExRai-AKAR represents a substantial improvement in performance compared with existing genetically encoded kinase activity biosensors. For example, when stimulated with Fsk/IBMX, cells expressing ExRai-AKAR exhibited a threefold higher dynamic range (143.7 ± 2.5% versus 47.0 ± 3.5%, \(P < 0.0001\)) and twofold higher signal-to-noise ratio (SNR; 316.6 ± 8.3 versus 145.8 ± 17.4, \(P < 0.0001\)) compared with AKAR\(^{11}\), as well as a 1.7-fold higher dynamic range compared with the optimized FRET sensor AKAR3e\(^{14}\) (143.7 ± 2.5% versus 83.5 ± 1.4%, \(P < 0.0001\)), with a trend towards higher SNR (316.6 ± 8.3 versus 271.5 ± 21.6, \(P = 0.0564\)) (Fig. 2a–c). Meanwhile, dose–response experiments also revealed that ExRai-AKAR responded both more sensitively and more robustly to submaximal PKA stimulation compared with AKAR4 (Fig. 2d), suggesting that ExRai-AKAR can enhance the detection of low-amplitude signalling events.

We examined this further in PC12 cells, where we previously found that nerve growth factor (NGF) and epidermal growth factor (EGF) signalling yields robust but temporally distinct PKA activity patterns at the plasma membrane, yet fails to elicit cytosolic PKA activity due to the action of cytosolic PDE3\(^{11}\). Partial inhibition of PDE3 using milrinone enables both NGF and EGF to weakly stimulate cytosolic PKA activity\(^1\), and we found that PC12 cells transfected with cytosol-targeted ExRai-AKAR (ExRai-AKAR-NESS; Supplementary Fig. 2) responded much more strongly to co-stimulation with NGF and a submaximal dose of milrinone compared with cells expressing AKAR4-NESS (18.8% versus 2.6%, \(P < 0.0001\)) (Fig. 3a–c). Thus, ExRai-AKAR greatly enhances our ability to detect subtle changes in PKA activity in living cells.

Taking advantage of this increased sensitivity, we then set out to more carefully investigate the spatial compartmentalization of growth factor-stimulated PKA activity in PC12 cells. As expected, cells expressing plasma membrane-targeted ExRai-AKAR (ExRai-AKAR-Kras; Supplementary Fig. 2) exhibited sustained and transient responses to NGF and EGF stimulation, respectively, consistent with our previous observations\(^1\) (Supplementary Fig. 2). Surprisingly, however, we also observed rapid and distinct excitation ratio responses in the nuclei of cells expressing diffusible ExRai-AKAR following treatment with either NGF (Fig. 3d) or EGF (Fig. 3e). The nuclear origin of these PKA responses was confirmed by co-localization with mCherry-tagged histone H2B\(^8\).

Overall, growth factor-induced nuclear PKA dynamics mirrored those observed at the plasma membrane, with NGF-induced responses being largely sustained while EGF induced more transient responses (Fig. 3d–e and Supplementary Fig. 2). NGF also appeared to weakly induce cytosolic PKA responses in some cells (10/17; 59%), although this was less apparent for EGF (Fig. 3d–e and Supplementary Fig. 2). Given the minimal cytosolic PKA activity, our results suggest growth factor signalling that is initiated at the plasma membrane can be directly coupled to PKA signalling in the nucleus.

**Generalizing the design of ExRai-AKAR.** A key feature of FRET-based sensors is their modular architecture, which can easily incorporate new domains and is readily generalized for many enzymes. In particular, swapping in various substrate sequences has facilitated the development of a multitude of kinase activity sensors\(^1\). To investigate whether the design of ExRai-AKAR could be similarly generalized, we replaced the PKA substrate in ExRai-AKAR with either a PKC\(^\text{\ast}\) or Akt/PKB\(^\text{\ast}\) substrate (Fig. 4a). The resulting probes functioned as excitation ratiometric sensors for PKC activity and Akt activity when expressed in HeLa and NIH3T3 cells, respectively (Fig. 4b,c and Supplementary Fig. 3). ExRai-CKAR produced a robust decrease in the 480 nm/380 nm excitation ratio (ΔR/R) on phorbol 12-myristate 13-acetate (PMA) stimulation in HeLa cells, with a maximum dynamic range of 156.9% (Fig. 4b). In contrast, HeLa cells expressing an ExRai-CKAR T/A mutant showed no excitation ratio response on PMA treatment, as was the case for cells pre-treated with the PKC inhibitor G66983 (Fig. 4b). Similarly, ExRai-AktAR displayed a 74.9 ± 3.0% (\(n = 13\)) increase in excitation ratio (ΔR/R), with a maximum dynamic range of 93.2%, in NIH3T3 cells treated with platelet-derived growth factor (PDGF), whereas neither cells expressing ExRai-AktAR (T/A) nor cells pretreated with the Akt inhibitor 10-DEBC responded to PDGF (Fig. 4c). Both ExRai-CKAR and ExRai-AktAR represent the highest-responding biosensor variant for their respective targets.

**Colour-shifting cpFP-based KARs for multiplexed imaging.** To maximize the spectral space available for multiparameter imaging, single-fluorophore sensors should preferably exhibit single excitation and emission maxima. In addition, for single-fluorophore sensors to succeed as a platform for multiparameter activity imaging, they must also support the incorporation of different FP colour variants. As the ExRai-AKAR excitation spectrum displayed a strong contribution from the neutral GFP chromophore (Fig. 1), we explored the possibility of generating a single-colour kinase sensor using circularly permuted T-sapphire, an enhanced long-Stokes-shift GFP variant that lacks the phenolate chromophore species\(^{26}\), by substituting cp-T-sapphire from the redox sensor Peroxid\(^{24}\) into our sensor backbone (Fig. 5a).

As expected, purified recombinant sapphireAKAR exhibited single excitation and emission maxima at ~400 nm and ~513 nm, respectively, in agreement with the published values for T-sapphire\(^{29}\) (Fig. 5b). Consistent with the behaviour of ExRai-AKAR, both peaks also decreased in amplitude when we incubated sapphireAKAR with catalytically active PKA in vitro, although the decrease was smaller (~25%). HeLa cells expressing sapphireAKAR similarly showed a ~19.5 ± 0.6% (\(n = 27\)) change in fluorescence intensity (ΔF/F) on forskolin/IBMX stimulation, with a maximum dynamic range of ~24.4%, whereas no response was observed in cells expressing sapphireAKAR (T/A) or co-transfected with PKI (Fig. 5c and Supplementary Fig. 3). Incorporating a PKC substrate in place of the PKA substrate similarly yielded sapphireCKAR (Fig. 5a), which also displayed a clear decrease in...
fluorescence intensity in HeLa cells stimulated with PMA (average $\Delta F/F$: $-21.9 \pm 1.2\%$, $n = 16$; highest response: $-33.5\%$) compared with the negative-control sensor or inhibitor pretreatment (Fig. 5d and Supplementary Fig. 3).

We then expanded our efforts by incorporating cpBFP from BCaMP, a GaCaMP variant that contains BFP chromophore mutations$^{32}$, into our sensor design (Fig. 5e). Purified blueAKAR displayed single excitation and emission peaks at ~385 nm and ~450 nm, respectively, and much like sapphireAKAR, the amplitude of both peaks decreased on phosphorylation of the sensor by PKA (Fig. 5f). Furthermore, both blueAKAR and blueCKAR showed clear fluorescence intensity decreases in HeLa cells stimulated with Fsk/IBMX and PMA, respectively (blueAKAR average $\Delta F/F$: $-16.2 \pm 0.3\%$, $n = 15$; highest response: $-21.1\%$; blueCKAR average $\Delta F/F$: $-11.8 \pm 1.1\%$, $n = 16$; highest response: $-23.1\%$ (Fig. 5g,h and Supplementary Fig. 3), and no responses were detected in the presence of inhibitor or with negative-control sensors.

An alternative design for red sensors via FP dimerization. Despite these initial successes, we were unable to generate a redshifted cpFP-based single-colour activity sensor, as incorporating cp-mRuby from the red-fluorescent Ca$^{2+}$ sensor RCAm$^{32}$ into our cpFP-AKAR
backbone yielded only a dimly fluorescent construct with no meaningful response to PKA stimulation in cells (Supplementary Fig. 4). As an alternative strategy, we utilized dimerization-dependent fluorescent proteins (ddFPs), which are a series of fluorogenic protein pairs comprising a fluorogenic FP-A and a non-fluorescent FP-B, wherein dimerization results in increased FP-A fluorescence. 

Fig. 3 | ExRai-AKAR amplifies minute activity changes and reveals compartmentalized PKA signalling in growth factor-stimulated PC12 cells. a, A bar graph comparing the maximum responses ($\Delta R / R$) of AKAR4-NES ($n = 5$ cells pooled from 3 experiments) and ExRai-AKAR-NES ($n = 37$ cells pooled from 22 experiments) in PC12 cells treated with 200 ng ml$^{-1}$ NGF and a submaximal (5 μM) dose of milrinone (submil). The data are presented as box-and-whisker plots showing the median, interquartile range, minimum, maximum and mean (+). ****$P < 0.0001$; unpaired two-tailed Mann–Whitney U-test. Ratio changes are calculated as $\Delta R / R = (R_{\text{max}} - R_{\text{min}})/R_{\text{min}}$. b, Average time courses of ExRai-AKAR-NES and AKAR4-NES responses in PC12 cells treated with NGF + submil. The curves are plotted as yellow/cyan emission ratios or 480 nm/380 nm excitation ratios normalized with respect to time 0 ($R / R_0$). $n$ is the same as in a. c, Representative pseudocolour images of the AKAR4-NES (top) and ExRai-AKAR-NES (bottom) ratio responses in PC12 cells treated with NGF + submil. The arrowhead indicates drug addition. Warmer colours indicate higher ratios. The greyscale images (left) show the distribution of probe fluorescence in each channel. d, Left: average time courses of the PKA response in the nucleus (green curves) and cytosol (blue curves) in PC12 cells co-expressing diffusible ExRai-AKAR and the nuclear marker histone H2B–mCherry stimulated with 200 ng ml$^{-1}$ NGF ($n = 17$ cells pooled from 8 experiments) (d) or 100 ng ml$^{-1}$ EGF ($n = 16$ cells pooled from 6 experiments) (e). The curves are plotted as the 480 nm/380 nm excitation ratio ($R / R_0$) normalized with respect to time 0. The solid lines represent the mean; shaded areas, s.e.m. Right: representative images showing (from top to bottom) the localization of ExRai-AKAR fluorescence in the 480 nm excitation channel, the localization of mCherry-tagged histone H2B within the nucleus, merged image of ExRai-AKAR (magenta) and H2B (yellow) localization, and pseudocoloured images of the ExRai-AKAR excitation ratio before and after NGF (d) or EGF (e) stimulation. Warmer colours represent higher ratios. The arrowhead indicates the nucleus. Scale bars in c–e, 10 μm. See Supplementary Table 1 for bar graph source data.
Fig. 4 | Construction of ExRai-CKAR and ExRai-AktAR based on a generalized design. a, Domain structures of ExRai-CKAR (top) and ExRai-AktAR (bottom). b, Average time courses (left) and maximum stimulated responses (ΔR/R, right top) of HeLa cells treated with 100 ng ml⁻¹ PMA, n = 26 (ExRai-CKAR), 17 (ExRai-CKAR[T/A]) and 11 (ExRai-CKAR with PKC inhibitor pretreatment (10 μM Gö6983)) cells. The curves are representative of and maximum responses are pooled from 5, 3 and 4 experiments (** P < 0.0001, F = 351.9, DFn = 2, Dfd = 51; one-way ANOVA followed by Dunnett’s multiple-comparison test). Representative pseudocolour images show the ExRai-CKAR excitation ratio in HeLa cells before and after stimulation. Warmer colours correspond to higher excitation ratios. c, Average time courses (left) and maximum stimulated responses (ΔR/R, right top) of NIH3T3 cells treated with 50 ng ml⁻¹ PDGF, n = 15 (ExRai-AktAR), 16 (ExRai-AktAR[T/A]) and 7 (ExRai-AktAR with Akt inhibitor pretreatment (50 μM 10-DEBC)) cells. The curves and maximum responses are pooled from 12, 4 and 5 experiments (**** P < 0.0001, F = 445.3, DFn = 2, Dfd = 33; one-way ANOVA followed by Dunnett’s multiple-comparison test). Representative pseudocolour images show the ExRai-AktAR excitation ratio in NIH3T3 cells before and after stimulation. Warmer colours correspond to higher excitation ratios. Scale bars, 10 μm. Curves are plotted as 480 nm/380 nm excitation ratio normalized with respect to time 0 (R₁/R₀). The solid lines represent the mean; shaded areas, s.e.m. For the dot plots in b and c, the horizontal bars represent mean ± s.e.m. Maximum responses are calculated as ΔR/R = (Rmax − Rmin)/Rmin. See Supplementary Table 1 for bar graph source data.

As the fluorescence intensity increase following ddFP heterodimer formation broadly parallels the increased FRET between a pair of FPs brought into close proximity by a molecular switch, it should be possible to convert a range of established FRET-based biosensors into intensity-based sensors by replacing the CFP/YFP pair of EKARev14 with the RPF-A/B pair. We therefore tested the generality of this approach by replacing the FRET pair of AKAR3ev with the ddFP pair. As predicted, RAB-AKAR3ev exhibited a 17.2 ± 0.9% (n = 19) increase in fluorescence intensity (ΔF/F) in HeLa cells treated with Fsk/IBMX, with a maximum response of 26.4% (Fig. 6d and Supplementary Fig. 4). Moreover, no response was detected in cells expressing RAB-AKAR3ev (T/A) or in cells co-expressing PKI (Fig. 6d). Similarly, incorporating the RFP-A/B pair into the FRET-based cAMP sensor ICUE23 yielded RAB-ICUE (Fig. 6b), which displayed a −20.6 ± 0.8% (n = 21) change in RFP fluorescence intensity (ΔF/F) in HEK293T cells treated with Fsk, with a maximum intensity change of −24.5% (Fig. 6c and Supplementary Fig. 4).

Multiplexed activity imaging in living cells using single-fluorophore biosensors. cAMP/PKA, PKC and ERK signalling regulate numerous cellular processes and engage in frequent crosstalk, making them ideal candidates for multiplexed imaging studies, and as anticipated, the panel of single-fluorophore biosensors developed above proved apt for this purpose. For example, we were able to simultaneously image PKA, ERK and PKC activity in HeLa cells co-transfected with blueAKAR, RAB-EKARev and sapphireCKAR, which exhibited robust and discrete changes in T-sapphire, RFP and BFP fluorescence intensity on sequential stimulation using Fsk/IBMX, EGF and PMA, respectively (Fig. 7a and Supplementary Fig. 5). Simultaneous imaging of PKA, cAMP and PKC responses was also achieved by similarly co-transfecting HeLa cells with blueAKAR, RAB-ICUE and sapphireCKAR (Fig. 7b and Supplementary Fig. 5) or with the alternative combination of sapphireAKAR, RAB-ICUE and blueCKAR (Supplementary Fig. 5), highlighting the versatility and flexibility of this multiplexed imaging platform. We saw no obvious spectral contamination during co-imaging, although control experiments in cells transfected with individual sensors revealed moderate BFP bleed-through into the T-sapphire channel (Supplementary Fig. 5). Nevertheless, the
**Fig. 5 | AKAR and CKAR colour variants based on cp-T-sapphire and cpBFP.** a,e, Domain structures of sapphireAKAR and sapphireCKAR (a) and blueAKAR and blueCKAR (e). b,f, Representative fluorescence spectra of sapphireAKAR (b) and blueAKAR (f) collected without (grey) and with (teal or blue) ATP in the presence of PKA catalytic subunit. n = 3 independent experiments. c, Average time courses (left) and maximum responses (ΔF/F, right) in Fsk/IBMX-treated HeLa cells. n = 27 (sapphireAKAR), 8 (sapphireAKAR[T/A]) and 22 (sapphireAKAR plus PKI) cells. The curves are representative of and maximum responses are pooled from 6, 2 and 3 experiments. (****P < 0.0001, F = 386.4, DFn = 2, Df = 54; one-way ANOVA followed by Dunnett’s multiple-comparison test). d, Average time courses (left) and maximum responses (ΔF/F, right) in PMA-treated HeLa cells. n = 16 (sapphireCKAR), 15 (sapphireCKAR[T/A]) and 16 (sapphireCKAR with 10 μM Gö6983 pretreatment) cells. The curves are representative of and maximum responses are pooled from 5, 3 and 3 experiments (****P < 0.0001, F = 176.5, DFn = 2, Df = 44; one-way ANOVA followed by Dunnett’s multiple-comparison test). g, Average time courses (left) and maximum responses (ΔF/F, right) of blueAKAR (n = 15 cells), blueAKAR[T/A] (n = 11 cells) and blueAKAR plus PKI (n = 16 cells) (**P < 0.0001, F = 1,105, DFn = 2, Df = 39; one-way ANOVA followed by Dunnett’s test). Curves are representative of and maximum responses are pooled from 5, 2 and 4 experiments. h, Average time courses (left) and maximum responses (ΔF/F, right) of blueCKAR (n = 17 cells), blueCKAR[T/A] (n = 8 cells) and blueCKAR with Gö6983 pretreatment (n = 14 cells) (**P < 0.0001, F = 26.38, DFn = 2, Df = 36; one-way ANOVA followed by Dunnett’s multiple-comparison test). The curves are representative of and maximum responses are pooled from 6, 2 and 3 experiments. The curves are normalized with respect to time 0 (F/F0). The solid lines represent the mean; shaded areas, s.e.m. For the dot plots shown in c, d, g and h, the horizontal bars represent mean ± s.e.m. Maximum responses are calculated as ΔF/F = (Fmax − Fmin)/Fmin. See Supplementary Table 1 for bar graph source data.
potential contribution of BFP emission to the recorded T-sapphire intensity was easily removed using standard bleed-through correction (see Methods and Supplementary Fig. 5).

Intracellular signalling pathways also play an important role in the brain during learning and memory, especially during synaptic plasticity, where changes in the activities of kinases such as PKA and ERK interact to induce long-lasting modulation of synaptic strength. However, the temporal and spatial dynamics of these signalling pathways are not fully understood. Using multiplexed imaging of our single-fluorophore biosensors, we can begin to explore these interactions. Here, we successfully performed three-parameter activity imaging in primary neuronal cultures (Fig. 7c,d and Supplementary Fig. 6). Specifically, we were able to simultaneously monitor changes in PKA and ERK activity, as well as Ca²⁺ influx, by co-transfecting rat cortical neurons with ExRai-AKAR, RAB-EKARev and the Ca²⁺ probe BCaMP, and found that Fsk+IBMX treatment led to a specific increase in 488-nm-excited ExRai-AKAR intensity (Fig. 7c and Supplementary Fig. 6), while application of (S)-3,5-dihydroxyphenylglycine (DHPG), which is known to activate both PKA and ERK signalling via mGluR1/5, increased the intensity of both ExRai-AKAR (488 nm excitation) and RAB-EKARev (Fig. 7d and Supplementary Fig. 6).

We were also able to easily expand into four-parameter multiplexing by combining our sensors with previously described
Fig. 7 | Multiplexed activity imaging using single-fluorophore biosensors. **a**, Three-parameter time-lapse epifluorescence imaging in HeLa cells. **a**, Time course of blueAKAR (blue), RAB-EKARev (red) and sapphireCKAR (teal) responses in a single cell treated with 50μM Fsk and 100μM IBMX (Fsk/IBMX), 100ng ml⁻¹ EGF and 100ng ml⁻¹ PMA. The data are representative of n=13 cells from 5 independent experiments. Representative images of each channel are shown below. **b**, Time course of blueAKAR (blue), RAB-ICUE (red) and sapphireCKAR (teal) responses in a single cell treated with Fsk/IBMX and PMA. The data are representative of n=17 cells from 3 independent experiments. Representative images of each channel are shown below. **c, d**, Three-parameter time-lapse confocal imaging in cultured rat cortical neurons. **c**, Time course of ExRai-AKAR (green; 488 nm excitation), RAB-EKARev (red) and BCaMP (blue) responses in the cell soma following Fsk stimulation. The data are representative of n=68 neurons from 6 independent experiments. **d**, Time course of ExRai-AKAR (green; 488 nm excitation), RAB-EKARev (red) and BCaMP (blue) responses in the cell soma of a single cultured neuron following DHPG stimulation. The data are representative of n=104 neurons from 10 independent experiments. **e, f**, Higher-order multiplexed imaging in HeLa cells. **e**, Four-parameter imaging time course of sapphireAKAR (teal), Flamindo2 (yellow), blueCKAR (blue) and RCaMP (red) responses in a single cell treated with Fsk/IBMX, PMA and 1μM ionomycin (iono). The data are representative of n=15 cells from 8 independent experiments. Representative images of each channel are shown below. **f**, Six-parameter imaging time course in a single cell co-expressing Lyn-sapphireAKAR (1, teal), sapphireAKAR-NLS (2, light blue), Flamindo2 (3, yellow), RAB-EKARev-NLS (4, red), Lyn-RAB-EKARev (5, pink) and B-GECO1 (6, blue) and treated with Fsk/IBMX, EGF and 100μM histamine. The data are representative of n=46 cells from 10 independent experiments. Representative images of each channel are shown below. All scale bars, 10μm. The curves are normalized with respect to time 0 (F/F₀; **a, b, e and f**) or with respect to the average intensity of the baseline (**c, d**). Additional curves are shown in Supplementary Figs. 5–7.
single-fluorophore probes of different colours. Specifically, we were able to simultaneously monitor changes in PKA and PKC activity, as well as cAMP and Ca\(^{2+}\) elevations, in single cells by co-transfected sapphireAKAR and blueCKAR into HeLa cells in combination with the yellow sensor Flamindo² and the red Ca\(^{2+}\) sensor RCaMP⁵. Cells expressing all four biosensors displayed robust and specific decreases in T-sapphire and YFP fluorescence intensity on stimulation with Fsk/IBMX, with subsequent PMA and ionomycin treatment yielding a clear decrease in BFP intensity and increase in RFP intensity, respectively (Fig. 7e and Supplementary Fig. 7). As above, only BFP bleed-through into the T-sapphire channel was detected in control experiments, which was easily corrected (Supplementary Fig. 7). Here, multiplexed imaging of four biochemical activities in living cells was achieved exclusively using spectrally separated biosensors.

On the basis of these results, we reasoned that even further multiplexing may be achieved by incorporating additional measures such as the physical separation of biosensors via targeting to non-overlapping subcellular structures (for example, the plasma membrane and nucleus). To test this idea, we first targeted sapphireAKAR and RAB-EKA Rev to the plasma membrane and nucleus and imaged their responses individually in HeLa cells (Supplementary Fig. 6). By applying this targeted sensor approach in conjunction with Flamindo2 and another blue Ca\(^{2+}\) probe, B-GECO1², we were then able to successfully perform sixfold multiplexing in which we simultaneously monitored membrane- and nuclear-localized PKA and ERK activity alongside cytosolic cAMP and Ca\(^{2+}\) accumulation in single living cells (Fig. 6f). All six probes were expressed well when co-transfected into HeLa cells, and each responded selectively to sequential stimulation with Fsk/IBMX, EGF and histamine (Fig. 7f and Supplementary Fig. 7). A subset of cells (12/46, 26%) also exhibited an EGF-induced Ca\(^{2+}\) transient immediately before the onset of ERK activity (Supplementary Fig. 7). The single-fluorophore biosensors developed here thus represent a powerful platform for dissecting complex signalling networks via highly multiplexed activity imaging in living cells.

**Discussion**

Subtle shifts in kinase activity are often critical for finely tuning the behaviour of important physiological processes, such as the modulation of synaptic plasticity²²,²³, neuronal activity oscillations²⁴, cardiac myofibril contraction²⁵ and mechano-sensitive signalling²⁶ by PKA. While these minute changes in kinase activity often approach the detection limit of genetically encoded FRET-based biosensors⁶, our results suggest that such physiologically relevant, subtle changes in kinase activity may be readily detectable using ExRai-KARs. Indeed, ExRai-AKAR not only vastly outperformed the FRET-based AKAR4 probe in reporting small cytosolic PKA activity changes in PC12 cells but also enabled the detection of endogenous, growth factor-stimulated nuclear PKA activity in these cells (Fig. 3).

The existence and regulation of nuclear PKA signalling have been a topic of extensive interest²²,²³. Our findings suggest that growth factor signalling can be directly coupled to nuclear PKA signalling, perhaps via regulation of a nuclear pool of PKA holoenzyme²⁴, and further highlight the potential for ExRai-KARs to illuminate hidden aspects of signalling biology. Notably, ExRai-AKAR also appeared to display faster response kinetics than previous AKARs (Fig. 3). Thus, ExRai-AKAR should prove similarly well suited for monitoring small, rapid changes in signalling within cellular subcompartments and nanodomains, such as local PKA activity dynamics in dendritic spines²⁶.

Complex physiological processes such as neuronal synaptic plasticity are also shaped by the convergence of multiple signalling activities²⁷, making highly multiplexed activity imaging essential for unravelling the underlying network dynamics. Yet, with few exceptions²²–²³, two-parameter multiplexing has largely remained the norm²⁸, even with single-fluorophore biosensors²⁹–³³, given their relatively narrow target profile. Similarly, although the recent development of enhanced Nano-lantern (eNL) colour variants teases the possibility of five-colour activity multiplexing³⁴, eNL-based sensors have thus far only been developed for Ca\(^{2+}\) imaging. In contrast, we successfully utilized ExRai-AKAR as a generalizable backbone to construct a suite of intensiometric single-fluorophore kinase sensors in various colours, with ddRFP serving as an alternative design strategy to obtain red sensors. Using these probes, we successfully performed threefold to fourfold multiplexed imaging of different combinations of signalling activities (for example, PKA/PKC/ERK, PKA/ERK/Ca\(^{2+}\), PKA/cAMP/PKC/Ca\(^{2+}\)) in both cultured cell lines and dissociated primary neurons (Fig. 7 and Supplementary Figs. 5–7). We were able to extend this approach even further through the supplementary use of biosensor targeting, thereby enabling us to simultaneously monitor activities in different subcellular locations and achieve sixfold activity multiplexing in single living cells (Fig. 7 and Supplementary Fig. 7).

In summary, our work presents a versatile and readily usable toolkit for obtaining a deeper understanding of kinase activity dynamics in living cells. Future efforts to expand the colour palette (for example, incorporate red and far-red cpFPs) and improve the dynamic range of these single-fluorophore KARs will further strengthen this already powerful toolkit. Together, these fluorescent biosensors will greatly enhance our ability to unravel the overlapping molecular events underlying complex processes, such as neuronal synaptic plasticity, in their physiological context.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41556-018-0200-6.

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**Author contributions**

S.M. and J.Z. conceived of the project. S.M. designed ExRai-AKAR and generated all of the biosensors used in the study, with assistance from B.T.; A.M. performed in vitro characterization of purified biosensors; S.M. and J.-Z.Z. performed live-cell imaging in HeLa, HEK293T, NIH3T3 and PC12 cells; Y.Z., R.H.R. and R.L.H. devised the neuronal experiments; Y.Z. and R.H.R. performed live-cell imaging in primary cortical neurons; R.L.H. and J.Z. supervised the project and coordinated the experiments; S.M., Y.Z., R.H.R., J.-Z.Z. and A.M. analysed the data; and S.M., Y.Z., R.H.R., R.L.H. and J.Z. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Methods

**Biosensor construction.** To construct ExRai-AKAR, cpGFP was PCR-amplified from pGMP3 + using the forward primer 5′-GGACGCGTCACTACGCGCGCTAGCTCTATACGACG-3′ and the reverse primer 5′-GGACGCGATCCCTGCTGAGCGGATCCGATGCGGTCGCGCCG-3′ (SacI and Spht sites underlined), and FHA1 with a stop codon was PCR-amplified from plasmid DNA using the forward primer 5′-GCCGACTGATAAGTTTCTACAGGA-3′ and the reverse primer 5′-GGACGCGATCCCTGCTGAGCGGATCCGATGCGGTCGCGCCG-3′ (SacI and Spht sites underlined) and cpEFP from BCAmp + (forward primer 5′-GGACGCGATCCCTGCTGAGCGGATCCGATGCGGTCGCGCCG-3′ and reverse primer 5′-GGACGCGATCCCTGCTGAGCGGATCCGATGCGGTCGCGCCG-3′) (SacI and Spht sites underlined) or cpEFP from BCAmp + (forward primer 5′-GGACGCGATCCCTGCTGAGCGGATCCGATGCGGTCGCGCCG-3′ and reverse primer 5′-GGACGCGATCCCTGCTGAGCGGATCCGATGCGGTCGCGCCG-3′) (SacI and Spht sites underlined). The resulting PCR fragments were then ligated into SacI/EcoRI-digested backbone containing a PKA substrate sequence. Vector backbones containing PKA and Akt substrate sequences were similarly used to generate ExRai-AKAR and ExRai-AktAR, respectively. The original cpGFP sequence in ExRai-AKAR and ExRai-AKAR was then replaced by subcloning with a SacI/SphI-digested PCR fragment encoding either cpGFP or ‘sponge from Poredox’ (forward primer 5′-GGACGCGATCCCTGCTGAGCGGATCCGATGCGGTCGCGCCG-3′ and reverse primer 5′-GGACGCGATCCCTGCTGAGCGGATCCGATGCGGTCGCGCCG-3′) (SacI and Spht sites underlined) or cpEFP from BCAmp + (forward primer 5′-GGACGCGATCCCTGCTGAGCGGATCCGATGCGGTCGCGCCG-3′ and reverse primer 5′-GGACGCGATCCCTGCTGAGCGGATCCGATGCGGTCGCGCCG-3′) (SacI and Spht sites underlined) to generate sapphireAKAR and sapphirePKC, or blueAKAR and bluePKC. RAB-ARKh was generated by subcloning a BglII/Sacl-digested fragment containing the FHA1 domain, Eevee linker and PKA substrate sequence from FLINC-AKAR + and a SacI/EcoRI-digested fragment encoding the dEFP-B sequence into a RAB-EKAR vector backbone cut out with BglII and XhoI (generated by subcloning a BglII/SacI-digested fragment encoding the conformational switch from ICUE2, corresponding to amino acids 149–881 of Epac1-25, amplified using the forward primer 5′-GGATCTGAAAGTTCTGGGTAGAAGAGACATAGAGT-3′ and reverse primer 5′-GGAGGCGGATCCTCAGCCGCTTAGCCTGCGGCCCCG-3′ (SacI and Spht sites underlined), between dEFP-B and dEFP-B via BglII and SacI digestion. Biosensors were constructed in the pRSETB vector (Invitrogen) and then subcloned into pcDNA3 (Invitrogen) behind a Kozak sequence via BamHI/EcoRI digestion for mammalian expression or into pcAG for neuronal expression. Non-phosphorylatable (T/A) mutant ExRai-AKAR was generated by subcloning a cpGFP-FHA1 fragment into a SacI/EcoRI-digested backbone containing a mutated PKA substrate. ExRai-, sapphire-, and blueCKAR (T/A) (primer 5′-GGATCTGAAAGTTCTGGGTAGAAGAGACATAGAGT-3′ and reverse primer 5′-GGATCTGAAAGTTCTGGGTAGAAGAGACATAGAGT-3′) were generated by site-directed mutagenesis. Tyrosolic and plasma membrane-targeted ExRai-AKAR, as well as plasma membrane- and nuclear-targeted sapphireAKAR and RAB-EKAR, were generated by subcloning into pcDNA3 backbones containing a carboxy-terminal nuclear export signal (EFLPPLERTL), the C-terminal targeting sequence from KRs (KKKKKKRTKRVM), the amino-terminal 11 amino acids from Lyn kinase (MGCIKSKRKDK) or a C-terminal nuclear localization signal (PKKKVRKVEDA). All constructs were verified by sequencing.

**Other plasmids.** mCherry-tagged PKK1, AKAR4 +, AKAR4-NES + and RAB-EKAR were provided by S. S. Taylor, UC San Diego, La Jolla, CA) for 30 min at 30 °C in kinase assay buffer (New England Biolabs; 50 mM Tris-HCL pH 7.5, 10 mM MgCl₂, 0.1 mM EDTA, 2 mM DTT, 0.01% Brij 35) without ATP for dephosphorylated spectra or with 200 μM ATP for phosphorylated spectra. pK values were determined by measuring the excitation spectra of ExRai-AKAR diluted into buffer (100 mM citric acid/200 mM dibasic sodium phosphate; 50 mM Tris-HCL, 50 mM glycine) with different pH values.

**Cell culture and transfection.** HeLa and HEK293T cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Gibco) containing 1 g l⁻¹ glucose and supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma) and 1% (v/v) penicillin-streptomycin (Pen-Strep, Sigma-Aldrich). NIH3T3 cells were cultured in DMEM (Gibco) containing 1 g l⁻¹ glucose and supplemented with 10% (v/v) fetal calf serum and 1% (v/v) Pen-Strep (Sigma-Aldrich). All cells were cultured in DMEM (Gibco) containing 1 g l⁻¹ glucose and supplemented with 10% (v/v) FBS (Sigma), 5% donor horse serum (DHS, Gibco) and 1% (v/v) Pen-Strep (Sigma-Aldrich). All cell lines were cultured in a humidified incubator at 37 °C with 5% CO₂ atmosphere. Before transfection, cells were plated onto sterile 35-mm glass-bottomed dishes and grown to 50–70% confluence. Cells were then transfected using Lipofectamine 2000 (Invitrogen) and grown for an additional 24 h (HeLa, HEK293T) or 48 h (PC12) before imaging. NIH3T3 cells were changed to serum-free DMEM immediately before transfection and sustained for 24 h before imaging. PC12 cells were changed to serum-free DMEM medium (5% FBS, 1% DHS) 24 h before imaging. For six-parameter imaging experiments, HeLa cells were transfected using the calcium phosphate transfection method.

Cortical neurons obtained from Sprague–Dawley rats at embryonic day 18 were plated onto poly-l-lysine-coated 18-mm glass coverslips in standard 12-well tissue culture dishes at a density of 250,000 cells per well and grown in glia-conditioned neurobasal media supplemented with 2% B-27, 2 mM Glutamax, 50 mM U-133 Pen-Strep and 1% horse serum. Cultured neurons were fed twice per week. At 12–13 days in vitro, cortical neurons were transfected with kinase sensors and GFP or diRed using Lipofectamine 2000 (Invitrogen). All experimental procedures involving animals were conducted according to the National Institutes of Health guidelines for animal research and were approved by the Animal Care and Use Committee at Johns Hopkins University School of Medicine.

**Time-lapse fluorescence imaging.** Epifluorescence imaging. Cells were washed twice with Hank’s balanced salt solution (HBSS, Gibco) and subsequently imaged in HBSS in the dark at 37 °C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was added to reduced medium (5% FBS, 1% DHS) 24 h before imaging. For six-parameter imaging experiments, raw fluorescence images were corrected by subtracting the background fluorescence intensity of a cell-free region from the emission intensities of biosensor-expressing cells. GFP emission ratios (Fmax/Fmin) or yellow/orange emission ratios at 503/500 nm were then calculated at each time point. These values were normalized by dividing the ratio or intensity at each time point by the basal value at time zero (F0, or R0), which was defined as the time point immediately preceding drug addition. Maximum intensity (ΔF/F) or ratio (ΔR/R) changes were calculated as (Fmax – Fmin)/Fmin or (Rmax – Rmin)/Rmax, where Fmax and Fmin or

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$R_{\text{max}}$ and $R_{\text{min}}$ are the maximum and minimum intensity or ratio value recorded after stimulation, respectively. The SNRs in Fig. 2 were calculated from single-cell ratio time courses by dividing the maximum stimulated ratio change by the standard deviation of the baseline before drug addition. Graphs were plotted using GraphPad Prism 7 (GraphPad Software).

**Fluorescence bleed-through correction.** To assess potential bleed-through of fluorescence intensity in the multi-parameter imaging experiments, cells were individually transfected with each single-fluorophore biosensor and then imaged under the same conditions used during multi-parameter imaging. For these experiments, obvious bleed-through was detected only in the T-sapphire channel in cells expressing BFP (for example, blueAKAR or blueCKAR). Bleed-through correction factors were determined by first plotting the pixel intensity of the T-sapphire channel versus that of the BFP channel and then extracting the slope from a linear fit of the resulting $XY$ scatter in MATLAB (MathWorks). Corrected T-sapphire intensities were calculated as $I_{\text{TsR}}' = I_{\text{TsR}} - (C \times I_{\text{BFP}})$, where $I_{\text{TsR}}'$ and $I_{\text{TsR}}$ are the corrected and uncorrected T-sapphire intensity, respectively, $I_{\text{BFP}}$ is the BFP intensity and $C$ is the correction factor. To avoid possible over-correction, bleed-through correction was applied only to cells with $I_{\text{TsR}} < 1,000$ and $I_{\text{TsR}}/I_{\text{BFP}} < 2$.

**Confocal imaging of cortical neurons.** Two days after transfection, neurons were imaged on a Zeiss spinning-disc confocal microscope using a 40×/1.6 NA oil objective. Neurons were mounted on a heated, custom-built perfusion chamber and continuously perfused with artificial cerebrospinal fluid (ACSF; 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 30 mM d-glucose and 25 mM HEPES, pH 7.4) warmed to 37 °C. Images were acquired every 2 min, and at least 3 baseline images were acquired before stimulation with either 10µM Fsk or 100 µM DHPG for 6 min followed by washout with ACSF for 15 min. Neurons were pre-incubated in 37 °C ACSF for 60 min before mounting on the microscope. For DHPG stimulation experiments, ACSF used during pre-incubation, baseline and washout was supplemented with 100µM (2R)-a-amino-5-phosphonovaleric acid. At the end of each experiment, neurons were stimulated with 10–50 mM KCl to test for general excitability. For image analysis, background-subtracted intensity values of the cell soma were normalized to the average intensity during baseline. Maximal intensity or ratio changes were calculated with respect to the initial value at time 0 ($F/F_0$ or $R/R_0$). Only neurons with stable baseline intensities were included in the analysis. Image analysis and quantification was performed using ImageJ.

**Statistics and reproducibility.** Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software). All data were tested using the D’Agostino–Pearson normality test. For Gaussian data, pairwise comparisons were performed using Student’s $t$-test or Welch’s unequal variance $t$-test, and comparisons between three or more groups were performed using ordinary ANOVA followed by Dunnett’s test for multiple comparisons. Non-Gaussian data were analysed using the Mann–Whitney $U$ test for pairwise comparisons or the Kruskal–Wallis test followed by Dunn’s multiple-comparison test for analyses of three or more groups. Statistical significance was set at $P < 0.05$. Unless otherwise noted, experiments were repeated at least three times with similar results. Average time courses shown in Figs. 1g,h, 2a, 4b, 5 and 6 depict individual representative experiments. Average time courses shown in Figs. 3 and 4c depict combined data sets due to the low number of cells per experiment. Average time courses from cultured rat cortical neurons (Fig. 1j–l and Supplementary Fig. 6a–d) depict combined data sets.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
Source data for bar graphs shown in Figs. 1–6 and Supplementary Fig. 6 have been provided as Supplementary Table 1. All other data supporting the findings of this study are available upon reasonable request.

**References**
40. Mo, G. C. H. et al. Genetically encoded biosensors for visualizing live-cell biochemical activity at super-resolution. *Nat. Methods* **14**, 427–434 (2017).
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| A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons | x |
| A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) | x |
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| Give $P$ values as exact values whenever suitable. | |
| For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings | |
| For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes | |
| Estimates of effect sizes (e.g. Cohen's $d$, Pearson's $r$), indicating how they were calculated | |
| Clearly defined error bars | |
| State explicitly what error bars represent (e.g. SD, SE, CI) | |

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Software and code

Policy information about availability of computer code

Data collection
- Live-cell imaging data were collected using METAFLUOR 7.7. Fluorimetry data were collected using FelixGX 4.1.2 (Horiba Scientific).

Data analysis
- Raw imaging data were analyzed using METAFLUOR 7.7 or ImageJ as described in the Methods. In vitro fluorimetry data were analyzed using Microsoft Excel. Statistical analyses were performed using GraphPad Prism 7. Bleedthrough correction was performed using standard calculations in MATLAB. No custom code was used.

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Life sciences study design

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| Sample size       | No sample-size calculations were performed. Sample sizes were similar to those generally employed in the field. |
|-------------------|----------------------------------------------------------------------------------------------------------|
| Data exclusions   | Cells that did not respond to stimulation or that did not express all of a given set of constructs (for multi-parameter imaging experiments) were excluded from analysis. Neurons that did not exhibit stable baseline intensities were excluded from analysis. All exclusion criteria were pre-determined. |
| Replication       | Unless otherwise noted, all experiments were repeated at least three times. All replication attempts were successful. |
| Randomization     | Cells were randomly seeded into experimental groups after resuspension. |
| Blinding          | Blinding was not performed as it was not necessary. Objective quantitative data was generated and analyzed. |

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a Involved in the study
- [x] Unique biological materials
- [ ] Antibodies
- [x] Eukaryotic cell lines
- [x] Palaeontology
- [ ] Animals and other organisms
- [x] Human research participants

Methods

n/a Involved in the study
- [x] ChIP-seq
- [ ] Flow cytometry
- [x] MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

All plasmids will be made freely available by direct request from the investigators, as well as through Addgene.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

HeLa, HEK293T, NIH3T3, and PC12 cells were used in this study. All cell lines were obtained from ATCC.

Authentication

Cell line identities were verified via STR analysis by the commercial source and then maintained separately and isolated from one another to avoid cross-contamination.

Mycoplasma contamination

All cell lines were determined to be free of mycoplasma contamination based on weekly DNA staining.

Commonly misidentified lines

HEK cells were used in some experiments. However, the identity of the cells was not central to the outcome of these experiments.

(See ICLAC register)
### Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

| Category              | Description |
|-----------------------|-------------|
| Laboratory animals    | Sprague-Dawley rats at embryonic day 18 were used to obtain primary cortical neurons. Tissue was pooled from both male and female embryos. |
| Wild animals          | The study did not involve wild animals. |
| Field-collected samples | The study did not involve samples collected from the field. |