A transcriptional reporter of intracellular Ca\(^{2+}\) in *Drosophila*

Xiaojing J Gao\(^1\), Olena Riabinina\(^2\), Jiefu Li\(^1\), Christopher J Potter\(^2\), Thomas R Clandinin\(^3\) & Liqun Luo\(^{1,3}\)

Intracellular Ca\(^{2+}\) is a widely used neuronal activity indicator. Here we describe a transcriptional reporter of intracellular Ca\(^{2+}\) (TRIC) in *Drosophila* that uses a binary expression system to report Ca\(^{2+}\)-dependent interactions between calmodulin and its target peptide. We found that in vitro assays predicted in vivo properties of TRIC and that TRIC signals in sensory systems depend on neuronal activity. TRIC was able to quantitatively monitor neuronal responses that changed slowly, such as those of neuropeptide F-expressing neurons to sexual deprivation and neuroendocrine pars intercerebralis cells to food and arousal. Furthermore, TRIC-induced expression of a neuronal silencer in nutrient-activated cells enhanced stress resistance, providing a proof of principle that TRIC can be used for circuit manipulation. Thus, TRIC facilitates the monitoring and manipulation of neuronal activity, especially those reflecting slow changes in physiological states that are poorly captured by existing methods. TRIC’s modular design should enable optimization and adaptation to other organisms.

Live imaging of Ca\(^{2+}\) with fluorescent indicators is a powerful technique for monitoring neuronal circuit dynamics\(^1,2\). Here we describe TRIC, which captures changes in neuronal activity over long timescales, complementing several limitations of fluorescent indicators. First, functional imaging is usually performed acutely and invasively in restrained animals. As a result, it is difficult to monitor circuits whose activities vary slowly with changes in the physiological state\(^3,4\). These circuits often use modulatory neurotransmitters or neuropeptides, and their outputs can cause the same neural network to mediate starkly different behaviors\(^5\). How the activity of modulatory neurons is regulated under natural conditions remains poorly understood given the absence of suitable tools. Second, because functional imaging and electrophysiology are time consuming, they have limited capacity to interrogate complex circuits systematically. For instance, serial electron microscopic reconstruction has revealed dozens of prominent connections in one of the flies’ visual centers, the medulla\(^6\). Systematic interrogation of these connections would involve hundreds or thousands of experiments that inactivate specific input neurons, monitor output neurons and present specific stimuli, which are extremely challenging with existing methods. Third, functional imaging is limited by the field of view accessible to a microscope. Thus, behaviorally relevant changes in neuronal activity in widely distributed brain regions can be difficult to monitor in parallel. Finally, Ca\(^{2+}\) imaging allows visualization of neuronal activity, but does not enable subsequent genetic manipulation of active cells.

An alternative approach to monitoring neural activity is based on the activation of immediate early genes (IEGs), whose expression is increased when neurons are active. Such endogenous transcriptional reporters of activity have been widely used in vertebrate models and have provided a complementary approach to live imaging of calcium signals\(^7\). In addition, other effectors have been placed under the control of IEG enhancers and promoters for genetic manipulation of active neurons\(^8-11\). However, only one IEG has recently been described in fruit flies\(^12\), and alternative methods for monitoring neuronal activity using transcriptional reporters are scarce\(^13\). Moreover, the mechanism of IEG induction by neuronal activity is still not well understood, hindering the optimization and general application of this strategy.

To address the limitations of calcium-imaging experiments, we developed TRIC to integrate changes in Ca\(^{2+}\) levels over long periods of time in freely behaving animals. TRIC takes advantage of Ca\(^{2+}\)-dependent interactions between calmodulin and its target peptides\(^14\) and reports this interaction using a binary expression system (Fig. 1a). TRIC produces a stable signal that can be monitored in fixed tissue, increasing the throughput of experiments that interrogate functional connectivity and allowing for visualization of neuronal activity in the whole brain. In addition, TRIC allows for subsequent expression of any transgenes, so that one can selectively manipulate active neurons. We tested TRIC in flies, given the well-established use of split binary systems\(^15,16\) and the potential of combining TRIC with other genetic tools. We provide proof-of-principle experiments using TRIC in cultured cells and in visual, olfactory and neuromodulatory systems *in vivo*. When applied to pars intercerebralis (PI) cells\(^17\), TRIC validated previously known regulatory factors, revealed distinct modes of PI response to different physiological conditions and enhanced stress resistance through the activity-dependent expression of a synaptic transmission blocker.

**RESULTS**

**Selecting TRIC components in cultured cells and flies**

To build a transcriptional reporter of intracellular Ca\(^{2+}\) levels, we fused calmodulin (CaM) and its target peptide\(^14\) to a transcriptional activation domain (AD) and a DNA-binding domain (DBD), respectively. When CaM binds to its target peptide in the presence of Ca\(^{2+}\),
the reconstituted transcription factor was able to express an effector (Fig. 1a).

We performed initial tests of TRIC efficiency in Drosophila S2 cells. We induced pulses of Ca\(^{2+}\) influx by heat-shocking cells transfected with a temperature-gated cation channel, dTrpA1 (ref. 18). We tested two DBDs, two ADs and three CaM-target peptides (Fig. 1b, Supplementary Table 1 and Supplementary Fig. 1a,b). We found that the TRIC version with the best signal-to-noise ratio in cultured cells consists of the codon-optimized GAL4 DBD (GAL4DBD) fused with the CaM-target peptide in CaMKII (MKII) and the p65 AD fused with CaM.

In the absence of dTrpA1, CaMKII-MKII-mediated TRIC expressed little of the co-transfected UAS-GFP reporter (Fig. 1b). In contrast, heat-induced Ca\(^{2+}\) influx through dTrpA1 resulted in robust GFP expression (Fig. 1b). The CaMKII-MKII-mediated TRIC signal was comparable to that of constitutively dimerizing split GAL4 (ref. 15) (Fig. 1b,c), which was independent of dTrpA1 (Fig. 1c).

To test TRIC in vivo, we generated transgenic flies in which MKII::GAL4DBD was expressed using a pan-neuronal promoter (nsyb) and p65AD::CaM was controlled by QUAS from the Q system (ref. 19). The p65AD::CaM was expressed by the nsyb::GAL4DBD, p65AD::CaM, nsyb::GF2, tubP::QS, UAS-mCD8::RFP, representative of ten samples). OL, optic lobe; AL, antennal lobe. In this and subsequent figures, unless specified, the maximal projections of confocal image stacks are shown and the scale bars represent 50 μm.

**Figure 1** Proof of principle of TRIC in cultured cells and transgenic flies. (a) The design of TRIC. Ca\(^{2+}\) mediates the binding of CaM and its targeting peptide, thereby bringing a transcriptional AD (fused with CaM) to the DBD of a transcription factor (here yeast GAL4, fused with CaM-target peptide) and activating transcription of an effector that is under the control of UAS. The binding of two fusion proteins depends on Ca\(^{2+}\) concentration. (b) CaMKII-MKII::GAL4DBD, ActP-p65AD::CaM. The UAS-GFP expression (right) was much weaker and sparser in the absence of ActP-dTrpA1 (left). (c) Split GAL4s that bind constitutively via leucine zippers (Zp) were used as a positive control (ActP-Zp::GAL4DBD, ActP-VP16AD::Zp). The UAS-GFP expression was independent of ActP-dTrpA1. Cells in b and c were all subjected to the same repetitive heat shocks. (d) CaMKII-MKII mediated TRIC signal in the brain of transgenic flies (nsyb-MKII::GAL4DBD, QUAS-p65AD::CaM, nsyb-GF2, tubP::QS, UAS-mCD8::RFP, representative of ten samples). OL, optic lobe; AL, antennal lobe. In this and subsequent figures, unless specified, the maximal projections of confocal image stacks are shown and the scale bars represent 50 μm.

**TRIC signals in the optic lobes depend on visual input**

To characterize TRIC’s ability to detect changes in neural activity, we started with the optic lobes, which process visual information. In pan-neuronal TRIC flies raised in ambient light, robust signal was detected across the optic lobes (Fig. 2a). In contrast, the signal was nearly abolished (Fig. 2a) when we introduced a norpA mutation, that eliminates phototransduction (ref. 22).

To test whether sensory experience can modulate the TRIC signal, we raised flies in darkness until eclosion, induced p65AD::CaM expression with QA, and then kept animals in ambient light or darkness for 3 d. However, the ambient light did not significantly elevate TRIC signal (P = 0.14; Supplementary Fig. 2a). We hypothesized that, unlike norpA mutants, dark-reared flies would still have spontaneous photoreceptor activity that could propagate to optic lobe neurons and be detected by TRIC. Such a low level of activity might saturate TRIC signal in darkness with an exceptionally stable mCD8::fluorescent protein (mCD8::FP) reporter. Indeed, we found significant reduction (P < 0.0001) of TRIC signal in dark rearing compared with light exposure when we used a less stable nsyb::GFP reporter (ref. 19) (Supplementary Fig. 2b,c) or when we restricted reporter availability temporally using a UAS-FRT-stop-FRT-mCD8::GFP reporter and suppressed by the ubiquitously expressed suppressor QS. Adding quinic acid (QA) relieves QS suppression, allowing us to tune the expression level of TRIC. In the test flies exposed to QA, we observed strong TRIC signal throughout the brain (Fig. 1d) and the ventral nerve cord (Supplementary Fig. 1d). Consistent with the difference in vitro (Fig. 1b and Supplementary Fig. 1a), M13, the CaM-target peptide in smooth muscle myosin light chain kinase, produced weaker signals than MKII in vivo (Fig. 1d and Supplementary Fig. 1c).

Thus, for subsequent experiments, we chose MKII::GAL4DBD and p65AD::CaM as the core TRIC components.
Figure 2 TRIC signals in the optic lobes depend on visual transduction and visual experience. (a) TRIC signal in the optic lobes was diminished in norpA mutants, in which phototransduction was blocked (genotype besides norpA: nsyb-MKII::GAL4DBDo, QUAS-p65AD::CaM, nsyb-QF2, tubP-QS, UAS-mCD8::GFP, n ≥ 5). (b) In this experiment, a core TRIC component, p65AD::CaM, was conditionally expressed following FLP-mediated recombination, which was in turn controlled by heat shock (nsyb-MKII::GAL4DBDo, QUAS-FRT-stop-FRT-p65AD::CaM, hsFLP122, nsyb-QF2, UAS-mCD8::GFP, n ≥ 5). The optic lobe exhibited strong TRIC signal in response to light exposure after heat shock (HS) induction. TRIC signal diminished in the absence of light (HS) or heat-shock (light). (c–f) Visual experiences modulated TRIC signals in the medulla (n ≥ 5, same genotype as in b). Shown are representative sections (c), the heat maps of average TRIC signals (d, color scale 0–255), total fluorescence quantification (e) and relative light-induced signal intensity in ten evenly spaced bins (f) in which circles highlight bins that showed significant differences between different stimulations. Two-tailed unpaired t test for a (P = 0.0007), b (P = 0.0061, 0.0074) and e (P = 0.0209, 0.0027, 0.0069), with Holm-Bonferroni correction for multiple comparisons; two-way ANOVA for f (interaction P < 0.0001), with Tukey post hoc comparisons. Scale bars in all panels represent 50 µm. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. The data are presented as mean ± s.e.m.; scatter plots show data points. Key changes of genetic components are colored red.

expression (Fig. 2b). Omitting either light or heat shock markedly reduced TRIC signals (Fig. 2b).

We also used FLP-regulated TRIC onset to test whether different visual stimuli trigger distinct TRIC patterns in the medulla, the second optic ganglion. We heat-shocked FLP-regulated TRIC flies and then exposed them to darkness, ambient light, moving gratings or full-field flicker; the latter two stimuli are commonly used in visual system studies45. These stimuli increased the overall fluorescent intensity in medulla to different extents (Fig. 2c–e) and different stimuli appeared to preferentially induce TRIC signal in different layers, as was evident in individual samples (Fig. 2c) or average heat maps after image registration of multiple flies (Fig. 2d,f and Online Methods). Thus, with the FLP-regulated temporal control, TRIC can report both the overall activity level of the optic lobes and layer-specific activation in the medulla in response to different visual stimuli.

Characterizing TRIC in the olfactory projection neurons

Many optic lobe neurons use graded potentials. To test whether TRIC can monitor neurons with action potentials, we turned to the spiking olfactory projection neurons (PNs) labeled by GH146-QF20. For flies raised under standard conditions, TRIC labeled PN cell bodies around, and dendrites in, the antennal lobes (Fig. 3a). These signals were markedly reduced by genetic ablation of the olfactory receptor neurons (ORNs), the primary presynaptic partners of PNs (Fig. 3a).

To abolish sensory input acutely, we removed the antennae, which contain the majority of ORNs. An nsyb::GFP reporter showed significant signal reduction 5 d after bilateral antennectomy (P < 0.0001; Fig. 3b), and this was not a result of axon degeneration (Supplementary Fig. 3a). Given that ORNs project bilaterally and that PNs only innervate the ipsilateral antennal lobe, we tested the consequence of unilateral antennectomy. This manipulation did not reduce PN signal compared with intact flies (Fig. 3b), and the signals in the ipsilateral and contralateral antennal lobes were similar (Fig. 3b and Supplementary Fig. 3b), consistent with the reported small differences between the spiking rates in PNs caused by contra- and ipsilateral inputs26. This results suggest that single-antenna input saturates PN responses in both hemispheres, reflecting a ceiling effect in PNs or TRIC. Moreover, a luciferase reporter also detected antennectomy-induced TRIC signal reduction (Fig. 3c and Supplementary Fig. 3c) and the artificial activation of PNs (Supplementary Fig. 3d).

The PNs were characterized for the only reported transcriptional indicator of neuronal activity in flies (CalExA), a NFAT-LexA chimera
whose transcriptional activity is based on the Ca\(^{2+}\)-regulated dephosphorylation and nuclear translocation of NFAT\(^{13}\). We replaced GAL4 DBD with LexA DBD to directly compare the performance of TRIC and CaLexA. We found that LexA-based TRIC labeled PNs broadly and strongly (Supplementary Fig. 4a), matching the performance of GAL4-based TRIC in cell bodies and the neuropil (Fig. 3d). In contrast, CalExA only labeled two PN classes (Supplementary Fig. 4b). Pan-neuronal CalExA signal was also much weaker and sparser than TRIC (Fig. 1d and Supplementary Fig. 4c). These results also suggest that TRIC is robust to substitution of the DBD modules.

**Monitoring neuromodulatory circuits with TRIC**

TRIC relies on the relatively slow process of reporter expression. It is therefore well suited for monitoring neuromodulatory circuits, whose activities often vary with an animal’s physiological states on a time scale too long for traditional reporters. Having characterized TRIC in the sensory systems, we next turned to investigate its utility in modulatory circuits. We first tested whether TRIC signal can generally be detected in various modulatory neurons. To make TRIC compatible with existing circuits, we next focused on the PI cells, one of the most extensively studied sensory neurons, whose transcriptional activity is based on the Ca\(^{2+}\)-regulated dephosphorylation and nuclear translocation of NFAT\(^{13}\). Using the UAS-MKII::nlsLexADBDo, UAS-nsyb::GFP, and CaLexA. We found that LexA-based TRIC labeled PNs broadly and strongly (Supplementary Fig. 4a), matching the performance of GAL4-based TRIC in cell bodies and the neuropil (Fig. 3d). In contrast, CalExA only labeled two PN classes (Supplementary Fig. 4b). Pan-neuronal CalExA signal was also much weaker and sparser than TRIC (Fig. 1d and Supplementary Fig. 4c). These results also suggest that TRIC is robust to substitution of the DBD modules.

**Monitoring neuromodulatory circuits with TRIC**

TRIC relies on the relatively slow process of reporter expression. It is therefore well suited for monitoring neuromodulatory circuits, whose activities often vary with an animal’s physiological states on a time scale too long for traditional reporters. Having characterized TRIC in the sensory systems, we next turned to investigate its utility in modulatory circuits. We first tested whether TRIC signal can generally be detected in various modulatory neurons. To make TRIC compatible with existing circuits, we next focused on the PI cells, one of the most extensively studied sensory neurons, whose transcriptional activity is based on the Ca\(^{2+}\)-regulated dephosphorylation and nuclear translocation of NFAT\(^{13}\). Using the UAS-MKII::nlsLexADBDo, UAS-nsyb::GFP, and CaLexA. We found that LexA-based TRIC labeled PNs broadly and strongly (Supplementary Fig. 4a), matching the performance of GAL4-based TRIC in cell bodies and the neuropil (Fig. 3d). In contrast, CalExA only labeled two PN classes (Supplementary Fig. 4b). Pan-neuronal CalExA signal was also much weaker and sparser than TRIC (Fig. 1d and Supplementary Fig. 4c). These results also suggest that TRIC is robust to substitution of the DBD modules.

**Monitoring neuromodulatory circuits with TRIC**

TRIC relies on the relatively slow process of reporter expression. It is therefore well suited for monitoring neuromodulatory circuits, whose activities often vary with an animal’s physiological states on a time scale too long for traditional reporters. Having characterized TRIC in the sensory systems, we next turned to investigate its utility in modulatory circuits. We first tested whether TRIC signal can generally be detected in various modulatory neurons. To make TRIC compatible with existing circuits, we next focused on the PI cells, one of the most extensively studied sensory neurons, whose transcriptional activity is based on the Ca\(^{2+}\)-regulated dephosphorylation and nuclear translocation of NFAT\(^{13}\). Using the UAS-MKII::nlsLexADBDo, UAS-nsyb::GFP, and CaLexA. We found that LexA-based TRIC labeled PNs broadly and strongly (Supplementary Fig. 4a), matching the performance of GAL4-based TRIC in cell bodies and the neuropil (Fig. 3d). In contrast, CalExA only labeled two PN classes (Supplementary Fig. 4b). Pan-neuronal CalExA signal was also much weaker and sparser than TRIC (Fig. 1d and Supplementary Fig. 4c). These results also suggest that TRIC is robust to substitution of the DBD modules.

**Monitoring neuromodulatory circuits with TRIC**

TRIC relies on the relatively slow process of reporter expression. It is therefore well suited for monitoring neuromodulatory circuits, whose activities often vary with an animal’s physiological states on a time scale too long for traditional reporters. Having characterized TRIC in the sensory systems, we next turned to investigate its utility in modulatory circuits. We first tested whether TRIC signal can generally be detected in various modulatory neurons. To make TRIC compatible with existing circuits, we next focused on the PI cells, one of the most extensively studied sensory neurons, whose transcriptional activity is based on the Ca\(^{2+}\)-regulated dephosphorylation and nuclear translocation of NFAT\(^{13}\). Using the UAS-MKII::nlsLexADBDo, UAS-nsyb::GFP, and CaLexA. We found that LexA-based TRIC labeled PNs broadly and strongly (Supplementary Fig. 4a), matching the performance of GAL4-based TRIC in cell bodies and the neuropil (Fig. 3d). In contrast, CalExA only labeled two PN classes (Supplementary Fig. 4b). Pan-neuronal CalExA signal was also much weaker and sparser than TRIC (Fig. 1d and Supplementary Fig. 4c). These results also suggest that TRIC is robust to substitution of the DBD modules.
Figure 4 Stoichiometric tuning of TRIC and its application in NPF neurons. (a) Monitoring NPF neurons using LexA-based TRIC (nsyb-MKII::nlsLexADBDo, UAS-p65AD::CaM, npf-GAL4, UAS-mCD8::RFP, LexAop2-mCD8::GFP, representative of ≥6 samples). The GAL4 expression was visualized with RFP (left); the arrowheads indicate the only four cells with prominent TRIC signal (right). (b) Simulating effects of DBD/AD stoichiometry on TRIC signal. Increasing AD dose increased signal (left), whereas increasing DBD dose first increased and then decreased signal (right). (c) Validation of the scenario in b with PI cells (UAS-MKII::nlsLexADBDo, ilp2-GeneSwitch, QUAS-p65AD::CaM, nsyb-QF2, tubP-QS, UAS-mCD8::RFP, LexAop2-mCD8::GFP, n = 11). Increasing GeneSwitch-mediated DBD expression with RU486 first increased and then decreased TRIC signal. Data are presented as mean ± s.e.m. (d,e) Tuning NPF TRIC signal with more balanced expression of DBD/AD (UAS-MKII::nlsLexADBDo, UAS-p65AD::CaM [x1 in d, x2 in e], npf-GAL4, UAS-mCD8::RFP, LexAop2-mCD8::GFP, representative of ≥6 samples). The arrowheads indicate the same cells as in a, and the triangles indicate the additional signal in the fan-shaped body. (f) Ratiometric TRIC measurement revealed that sexual deprivation lowered Ca^{2+} activity in male NPF neurons in the fan-shaped body (n ≥ 12, same genotype as in e). Two-tailed unpaired t test for c (P < 0.0001, P = 0.0022) and f (P = 0.0033), with Holm-Bonferroni correction for multiple comparisons. Scale bars in all panels represent 50 μm. **P < 0.01, ****P < 0.0001. Key changes of genetic components are colored red.

on standard food, validating the identity of these cells. Furthermore, the strength of the TRIC signal negatively correlated with Ilp2 levels in individual PI cells (Fig. 5b), consistent with the expectation that higher secretory activity leads to less somatic retention of Ilps. After tuning TRIC expression level (Fig. 5c and Supplementary Fig. 6a), we found that starvation diminished TRIC signal (Fig. 5c), consistent with the reported PI response to nutrients6,35,36. As expected, PI activity was also reduced by deleting upd2 (Fig. 5c), the fly homolog of the hormone leptin that relays a satiety signal from the fat body to the brain35,36.

Larval PI cells respond to dietary amino acids, but not to sugars35, and a similar specificity has not been established in adults. To investigate this, we measured TRIC signals after feeding flies with various combinations of yeast and sucrose. We found that yeast increased the TRIC signal in PI cells, whereas sucrose had no effect either by itself or in combination with yeast (Fig. 5d), consistent with results in larvae. Given that sucrose, but not amino acid, induces upd2 expression36, we reasoned that upd2 might not be the sole satiety signal. Indeed, starvation reduced the TRIC signal in PI neurons even in upd2 mutant flies (Supplementary Fig. 6b), implying the presence of at least one additional pathway that links satiety to PI activity.

PI cells are also activated by octopamine (OA), a neurotransmitter controlling arousal states37,38. We validated that OA feeding increased TRIC signal in PI cells and that an OA antagonist, mianserin, reversed

Figure 5 Monitoring PI cell activity with TRIC. (a) Summary of two known pathways regulating PI activity. Our data suggest an unknown pathway in parallel with Upd2 from nutrients to PI. (b) TRIC signal in the PI cells negatively correlated with the intensity of Ilp2 staining. Shown are single confocal slices. Spearman’s rank correlation for the quantification, where each dot represents one cell after normalizing it to the mean value in the corresponding animal. (c) Compared with wild-type flies on regular food (control), TRIC signal in the PI was reduced by food deprivation (starve) or hemizygous upd2 deletion (n ≥ 4). 20 mg QA per vial was used to induce TRIC expression. (d) Yeast, but not sucrose, increased TRIC signal (n ≥ 8). (e) 10 mg ml⁻¹ OA increased TRIC signal, which is antagonized by 2 mg ml⁻¹ mianserin, an OA antagonist (n ≥ 9). (f) TRIC signal was reduced by hemizygous thb mutation, which eliminated an enzyme necessary for OA synthesis (n ≥ 8). All panels are of the same genotype shown in Figure 1d, except for c and f, where mCD8::GFP was replaced with mCD8::GFP. Two-tailed unpaired t tests for c (P = 0.0041, 0.0057), e (P = 0.0032, 0.0573) and f (P = 0.0313), with Holm-Bonferroni correction for multiple comparisons. Two-way ANOVA for d. Scale bars represent 20 μm. *P < 0.05, **P < 0.01. Key changes of genetic components are colored red. Data are presented as mean ± s.e.m.
Enhancing the dynamic range of TRIC through mutagenesis

Although we validated TRIC in PI cells, the small effect sizes (Fig. 3d,e) might limit further quantitative analysis. To optimize TRIC, we screened every allele variant of MKII in S2 cells. Given that the signal of M13 in vivo was too low and the baseline of MKII too high, we reasoned that the useful variants would show signals intermediate between M13 and MKII in the presence of dTrpA1. The experiments were carried out as described in Figure 1b, except for the specific CaM-target peptides. Scale bars represent 50 μm. (b) Simultaneous quantification of TRIC and GCaMP6m signals in S2 cells in the presence (red-filled circles) or absence (open circles) of dTrpA1. TRIC normalized signal is the fluorescent intensity of tdTomato expressed by TRIC divided by the baseline fluorescent intensity of GCaMP6m. Of the three variants, MKIIK11A showed the largest fold of TRIC signal induction (n ≥ 189 cells for each condition). The fold of TRIC signal induction in experimental conditions (with dTrpA1) was labeled in percentage of control (no dTrpA1), as a direct comparison to the scale of ΔFF. (c) TRIC signal showed higher fed-to-starved ratio with MKIIK11A (n ≥ 7, compared with Fig. 5c). (d) TRIC signal varied linearly with yeast concentration (n ≥ 9), showing the Pearson correlation between yeast concentration and the mean TRIC signal at every concentration. (e) TRIC signal plateaued as OA concentration increased (n ≥ 9). (f) In the presence of 5% yeast, 10 mg ml⁻¹ OA further increased TRIC signal (n ≥ 8). (g) Signals of different TRIC variants in response to varying yeast concentrations (n ≥ 10 for each data point). All data were normalized to the signal of MKIIK11A:GAL4DBD driving mCD8::GFP, exposed to 10% yeast; MKIIK11A::GAL4DBD data were re-plotted from d. (h) Left, experimental setup. Under the experimental (red) or control (cyan) condition, flies were treated with QA 1–2 or 0–1 d before the onset of starvation (at day 0). Top right, flies with PI activity–dependent expression of shfit1 using TRIC (TRIC > shfit1) had extended longevity during starvation in the experimental condition (red) compared with control condition (cyan) (upper panel, n ≥ 94 flies). Bottom right, flies with TRIC transgenes without shfit1 did not exhibit a difference between the experimental and control conditions (lower panel, n ≥ 96 flies). Base genotype (c–f): nsyb-MKIIK11A::GAL4DBD, QUAS-p65AD::CaM, nsyb-QF2, tubP-QS, UAS-mCD8::RFP/GFP. g: colored components were used to replace corresponding transgene base genotype; h: mCD8::GFP in the base genotype was replaced with shfit1. Two-tailed unpaired t test for b (P = 0.005, 0.0005, 0.4114), c (P < 0.0001) and f (P = 0.0072) with Holm–Bonferroni correction for multiple comparisons. Log-rank test for h (P = 0.0012, 0.3411). Scale bars in c represent 20 μm. **P < 0.01, ***P < 0.001, ****P < 0.0001. Key changes of genetic components are colored red. Data are presented as mean ± s.e.m.

In summary, using TRIC, we corroborated regulations of PI activity inferred in previous studies. We also uncovered new information regarding the nutritional requirements for PI activity in adults, and our findings suggest that signaling pathways other than Upd2/leptin also mediate the PI response to satiety (Fig. 5a).

Enhancing the dynamic range of TRIC through mutagenesis

Although we validated TRIC in PI cells, the small effect sizes (Fig. 3d,e) might limit further quantitative analysis. To optimize TRIC, we screened every allele variant of MKII in S2 cells. Given that the signal of M13 in vivo was too low and the baseline of MKII too high, we reasoned that the useful variants would show signals intermediate between M13 and MKII in the presence of dTrpA1 (Fig. 6a and data not shown). To examine the relation between TRIC signal and Ca²⁺ concentration, we simultaneously measured the intensity of the fluorescent Ca²⁺ indicator GCaMP6m and a UAS-tdTomato reporter expressed by TRIC (Online Methods). dTrpA1-mediated Ca²⁺ influx induced a ΔF/F of about 100% (Fig. 6b and data not shown), comparable to in vivo physiological responses⁴⁹. Compared with the no-dTrpA1 control, TRIC signal underwent a 2.2-fold increase with the original MKII, whereas M13 resulted in a signal too weak to be significant (P = 0.41; Fig. 6b). As an example for the allele mutants, MKIIK11A reduced both the baseline and the induced TRIC signals compared with MKII, but increased the fold of induction to 3.6 (Fig. 6b).

To better understand the behavior of the MKIIK11A variant, we estimated its affinity to CaM based on in silico alanine scan (Supplementary Fig. 7d and Online Methods) and then simulated its dose response. In the simulation, MKIIK11A lowered the TRIC signal (Supplementary Fig. 7a), although the MKII and MKIIK11A dose-response curves were almost identical after normalizing to their respective maximums (Supplementary Fig. 7a). Thus, reduced affinity alone was insufficient to account for the increased induction ratio (Fig. 6b), unless some form of nonlinearity transformed the proportional decrease of signal by K11A. One possible source of nonlinearity is competition from endogenous CaM and its target peptides. Assumming the simplest case that endogenous CaM and its target peptides are expressed in equal concentrations, and that the endogenous peptides have the same affinity for CaM as MKII, simulation revealed that TRIC signal decreased as the number of endogenous competitors increased (Supplementary Fig. 7b), which again preserved the shape of the curve (Supplementary Fig. 7b). However, when the K11A variant was simulated in the presence of competition, the mutation caused a rightward shift of the response curve (Supplementary Fig. 7c),
consistent with the lower sensitivity and higher induction rate of this variant than MKII. This likely explains the performance of the alanine variant and suggests a mechanism to account for heterogeneity of TRIC efficiency in different neuronal types.

On the basis of our in vitro and in silico analyses, we tested five intermediate variants in PI cells (data not shown). Of these, MKIIK11A reduced PI TRIC signal in both fed and starved flies, with a larger effect on the latter. Consequently, although the TRIC signal was still robust in fed flies, it was negligible after starvation (Fig. 6c), and the ratio of TRIC signals between the fed and starved states quadrupled that of the original MKII (Figs. 5c and 6c).

We took advantage of this variant to measure the temporal characteristics of TRIC signal in PI cells using mCD8::RFP as a reporter. Shortening food induction by half reduced the TRIC signal by half (Supplementary Fig. 6c), suggesting that the signal accumulates linearly over time. To measure signal perdurance, we either examined the flies immediately after 1-d induction of PI activity by food or starved them for 1 or 2 d before dissection, and found the half-life of the TRIC signal to be 0.55 d (Supplementary Fig. 6d).

**TRIC reveals distinct modes of PI activity regulation**

Signal induction by yeast and OA was also greatly improved by MKIIK11A (Figs. 5d,e and Supplementary Fig. 6c,f), which allowed us to quantitatively compare the dose responses in PI neurons to yeast and OA exposure. PI TRIC signal increased linearly with yeast concentration (Fig. 6d). In contrast, as the OA concentration rose, the TRIC signal plateaued at a level much lower than the maximal signal induced by yeast (Fig. 6d,e). One possible explanation for this nonlinear response was that OA uptake was simply saturated. In this scenario, 10 or 20 mg ml\(^{-1}\) OA would result in the same amount of OA acting on its receptors in PI cells, and their effects should be equally inhibited by mianserin. To test this, we used an intermediate mianserin dose (Supplementary Fig. 6g) and found that 10 mg ml\(^{-1}\) OA induced a smaller TRIC signal than 20 mg ml\(^{-1}\) OA (Supplementary Fig. 6g). Thus, OA uptake was not saturated under these conditions, suggesting that the observed saturation of the TRIC signal took place at or downstream of the OA receptors.

The difference in dose-response curves suggests that yeast and OA activate PI cells through separate pathways. To test this notion, we asked whether the response to 5% yeast, which induced PI activity higher than the ceiling of OA response (Fig. 6d,e), could be further augmented by adding OA. Indeed, adding 10 mg ml\(^{-1}\) OA increased the signal induced by 5% yeast (Fig. 6f). In summary, nutrients and OA regulate PI activity through separate pathways and display distinct dose responses.

**TRIC variants cover a wide range of neural activity**

Our characterization of TRIC thus far contains several variants of TRIC. We used yeast-regulated PI activity to quantitatively compare these variants. All of the subsequent data were normalized to the TRIC signal of MKIIK11A::GAL4DBDo driving mCD8::GFP, exposed to 10% yeast.

Compared with MKIIK11A::GAL4DBDo driving mCD8::GFP, the original MKII::GAL4DBDo driving mCD8::GFP displayed a high baseline with plain agar and was saturated using 2% yeast (Fig. 6g). Replacing GAL4DBDo with nlsLexA DBDo led to a more gradual elevation of signal as yeast concentration increased (Fig. 6g); replacing the mCD8 reporter with nsyb::GFP greatly reduced the signal while also increasing the range of signal induction (Fig. 6g). Finally, combining the MKIIK11A mutation with nlsLexA DBDo, we detected no TRIC signal except at the highest yeast concentration (10%; Fig. 6g). Taken together, these variants display a wide dynamic range that can be selected by users in their neurons of interest.

**A TRIC-driven synaptic blocker enhances stress resistance**

Having achieved a high signal-to-baseline ratio of the MKIIK11A variant in PI cells, we tested whether TRIC-based expression of an effector can be used to manipulate circuit function. We used TRIC to express shi\(^{Gf1}\), a widely used mutant dynamin that disrupts synaptic transmission at restrictive temperature\(^4\). We focused on validating a well-characterized phenotype in which inactivating PI cells enhances stress resistance, as measured by survival following starvation\(^2\).

All TRIC > shi\(^{Gf1}\) flies were first kept at 25 °C on food for 2 d. The experimental group (Fig. 6h) was exposed to QA on the first day, and shi\(^{Gf1}\) was expressed on the second day in PI cells as a result of food-induced activity; the control group (Fig. 6h) was exposed to QA on the second day, and there was minimal PI activity to follow the onset of TRIC and no shi\(^{Gf1}\) expression. All flies were then starved at the restrictive temperature for shi\(^{Gf1}\). The experimental group survived significantly longer than the control group (P = 0.0012; Fig. 6h). Further controls confirmed that there was no difference in survival in the absence of the shi\(^{Gf1}\) transgene (Fig. 6h). The phenotype is like a result of expression in PI cells rather than elsewhere in the brain, given that when we visualized TRIC signal in the whole brain under these conditions, the only notable difference between the control and the experiment was in the PI cells (Supplementary Fig. 6h). TRIC can therefore mediate neural activity–dependent expression of genetic effectors that manipulate circuit function.

**DISCUSSION**

Using cultured cells and multiple in vivo assays, we found that TRIC reports changes in Ca\(^{2+}\) levels under diverse conditions in visual, olfactory and neuromodulatory systems. Our results provide quantitative assessments for choosing TRIC variants with appropriate sensitivity and stringency, and proof of principle that TRIC can be used to express a circuit manipulator. Thus, TRIC acts as a useful complement to functional Ca\(^{2+}\) imaging by integrating changes in activity over long periods of time and offering genetic access to neurons on the basis of their activity.

**Comparing TRIC with other methods**

Vertebrate IEGs, which evolved to be expressed in a high signal-to-baseline ratio in response to neuronal activation, are widely used to report neuronal activity\(^2\). However, as they rely on endogenous signaling networks, their response properties and cell-type biases are difficult to modify. TRIC can be considered a rationally designed IEG, by exogenously introducing a protein-peptide interaction to detect Ca\(^{2+}\). The modular design of TRIC renders it more amenable to optimization. TRIC reports a rise in nuclear Ca\(^{2+}\) levels, which has previously been used to monitor pan-neuronal activity in C. elegans\(^43\), and also accompanies neuronal activation in mammalian neurons likely shuttled by Ca\(^{2+}\)-binding proteins\(^44\). Our experiments indicate that nuclear Ca\(^{2+}\) correlates with activity in diverse neuronal classes in flies. It is likely that not all cell types have the same efficiency in converting cytoplasmic Ca\(^{2+}\) signal to nuclear Ca\(^{2+}\) signal. Thus, TRIC efficiency and optimization may differ for different neuronal types.

While this manuscript was in review, a Ca\(^{2+}\) integrator (CaMPARI) was reported in which the ultraviolet conversion of emission spectrum of a fluorescent protein was engineered to be contingent on
Ca\(^{2+}\) concentration\(^{45}\). CaMPARI can capture neuronal activity on a shorter time scale than TRIC or IEG. However, access of neurons to ultraviolet may limit the use of CaMPARI in deep tissues, at least in large animals, whereas TRIC and IEG report neuronal activity in the entire nervous system non-invasively. Notably, unlike CaMPARI or IEG, TRIC offers genetic access to active neurons, allowing activity-based circuit manipulation (Fig. 6h).

Tuning the parameters of TRIC

Our results underscore the importance of optimizing TRIC for specific neuronal types. In this study, we have optimized TRIC for multiple cell types, and have described many variants that can help users in other cells (Fig. 6g and Supplementary Table 2). We recommend that users begin with CaM/MKII-mediated TRIC (Figs. 1, 5 and 6g) in their neurons of interest. If TRIC signal is detected, the users can attempt QA-mediated (Figs. 5 and 6) or FLP-mediated (Fig. 2) regulation of the timing of TRIC onset. The signal-to-baseline ratio can be further improved by titrating expression of TRIC using QA (Fig. 5 and Supplementary Fig. 6), choosing reporters with different stabilities (Figs. 3 and 6g, and Supplementary Fig. 2), or switching to nlsLexADDBo (Figs. 3 and 6g) or the MKIIK11A variant (Fig. 6 and Supplementary Fig. 6). Stoichiometry can also be leveraged to boost TRIC signal (Fig. 4).

With the current version of TRIC, the signal accumulates and decays over many hours (Supplementary Fig. 6c,d). To detect shorter periods of neuronal activity, an important future goal is to increase signal strength while avoiding saturation by basal Ca\(^{2+}\) concentrations. One solution to this problem would be to restrict TRIC to a narrower time window than that offered by the QA- or the FLP-mediated strategy. For example, TRIC could be split into DBD-X, Y–target peptide and CaM-AD, where X and Y are two interacting modules controlled by light\(^{46}\). One could then synchronize TRIC with a specific manipulation, or even trigger TRIC repetitively with specific behavioral features using feedback from automated tracking\(^{47}\). To preserve phasic information about neuronal activity, reporters with faster decays than CD8::GFP (Supplementary Fig. 2c) could be used or the TRIC components could be destabilized with tags for protein degradation. Given that the current TRIC was able to interact with endogenous CaM and its target peptides, another important direction is to isolate TRIC by co-engineering the CaM and MKII components to lose binding to their endogenous partners, but maintain their mutual interaction\(^{48}\). Future TRIC optimization could be achieved using high-throughput screens in cultured cells, which can predict in vivo performance (Figs. 1, 5 and 6, and Supplementary Fig. 1).

The modulation of PI activity

Previous studies\(^{17,35,36}\) used Ilp2 immunostaining, epitope-tagged Ilp2 or a secreted GFP as indirect indicators of PI activity. We validated the major conclusions of these studies using TRIC. After enhancing the dynamic range of TRIC, we gained additional insight into how PI activity is regulated. In particular, given that PI cells affect diverse processes, how do these cells determine their output according to all relevant inputs? For example, an animal may encounter conflicting metabolic needs, such as conserving energy versus defending territory in an impoverished environment. Our nutrient and OA comparison could be viewed as a minimal model of such a dilemma, as OA contributes to arousal and is necessary for ‘fight or flight’ in insects\(^{38,49}\). We found that PI cells exhibited graded, yet more readily saturated, responses to such events. In contrast, the linear PI response to nutrients extended over a wider range. These distinctions, as well as the additive interaction between yeast and OA, point to the independent operation of these two categories of inputs. To further survey the input landscape, one could genetically manipulate candidate receptors autonomously or candidate upstream neurons non-autonomously while monitoring PI activity using TRIC.

Applying TRIC to other systems

The physiological states of flies can change over hours to days and can be accompanied by changes in the activities of neurons expressing modulatory neurotransmitters\(^{3,4}\) or neuropeptides\(^{17,34}\). Although previous work has focused on the targets of modulatory neurotransmitters, inputs to these cells remain largely unknown. In addition, there are ~75 predicted neuropeptides in flies, only a small subset of which have been examined\(^{50}\). TRIC can be applied to neurons expressing specific transmitters or neuropeptides and tested in different physiological states (for example, the NPF neurons in Fig. 4). We note that the current TRIC variants might not fit the dynamic range of all neuronal types, and it might be necessary to test other AD/DBD ratios or other MKII mutants following our examples of optimization for PI cells.

Finally, TRIC can report a rise of intracellular Ca\(^{2+}\) that accompanies any cellular, developmental or physiological processes in flies and can be adapted for similar use in other model organisms. TRIC modules can be introduced as transgenes or by viral vectors, and specific stoichiometry can be achieved by specifying the number of AD and DBD sequences in multi-cistronic constructs. TRIC expression can be made contingent on recombines or other binary systems in model organisms, such as mice, where many Cre lines are available for spatiotemporal control, which can help refine activity monitoring and circuit manipulation in specific cell types.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Addgene: pActP-dtrpA1, 64713; pActP-GCaMP6m, 64714; pQUAST-p65AD::CaM, 64715; pQUAST-frt-stop-frt-p65AD::CaM, 64716; pUASTattB-p65AD::CaM, 64717; pUASTattB-MKII::nsLexADDBo, 64718; pBP-p65AD::CaM, 64719; pUASTattB-MKIIK11A::nsLexADDBo, 64720; pattB-nsyb-M13::GAL4DBDo, 64721; pattB-nsyb-MKII::GAL4DBDo, 64722; pattB-nsyb-MKIIK11A::GAL4DBDo, 64723; pQUAST-M13::GAL4DBDo, 64724; pattB-nsyb-MKII::nsLexADDBo, 64725; pattB-nsyb-MKIIK11A::nsLexADDBo, 64726.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank D. Luginbuhl (Stanford University) for generating transgenic flies, R. Alfa, J. Cao, X. Dong, Y. Fisher, D.M. Gohi, M. Lin, S. Park, C. Ran, K. Shen, M. Silies, X. Wei, Z. Yang and C. Zhou for advice and technical support, H.A. Dierick (Baylor College of Medicine), G. Dietzel (Stanford University), T. Lee (Janelia Farm), A. Rajan (Harvard University), G.M. Rubin (Janelia Farm), H.A. Dierick (Baylor College of Medicine), G. Dietzel (Stanford University), T. Lee (Janelia Farm), A. Rajan (Harvard University), G.M. Rubin (Janelia Farm), J.W. Wang (University of California, San Diego), M. Zeidler (University of Sheffield) and Bloomington Stock Center for fly strains, Addgene for plasmids, and L. DeNardo Wilke, C.J. Guenther, T.J. Mosca and X. Wang for critiques on the manuscript. X.J.G. is supported by an Enlight Foundation Interdisciplinary Fellowship. L.L. receives funding from the Howard Hughes Medical Institute. This study was also supported by US National Institutes of Health grants R01-D009982 (L.L.), R01-EY022638 (T.R.C.) and R01-DC013070 (C.J.P.), and a grant from Whitall Foundation (C.J.P.).

AUTHOR CONTRIBUTIONS

X.J.G. designed, performed and analyzed the experiments, aided by J.L. during revision. L.L. and T.R.C. supervised the project. O.R. and C.J.P provided the unpublished nsyb-QF2 line. X.J.G., L.L. and T.R.C. wrote the manuscript, with inputs from the other authors.

© 2015 Nature America, Inc. All rights reserved.
ONLINE METHODS

Recombinant DNA construction. DNA construction was made using standard cloning methods. PCR amplifications were conducted with high-fidelity Phusion polymerase (NEB M6553S). The inserts were all verified by sequencing. Several intermediate constructs were not referred to in the paper.

pAC-dTrpA1. The dTrpA1 sequence was amplified from pUAST-dTrpAI (ref. 18) using primers CGATGCGCGCGCAACATGACTCTGCGCGCA and CGATGCGCGCGGCTACATGCTTATTAAGTCCG and cloned into pAC-PlacT1 using NotI/AscI.

pAC-GaCMP6m. The GaMP sequence was amplified using primers ATCCGGATCGGCGGCGGGAGTTGTTCTCATTCACTATCA and ATGGCGTTAGGGCGCGGCCCTACCTGCTCATATT and cloned between the NotI/NheI sites on CCCTTTAGCTTGCGCCGCGCATTAACATGGTGGC, and ligated between the sequence19 was generated by annealing oligos GGCCGCCACCATGGGCCGCT25

N5A mutation that increases CaM-skMLCK affinity52. The sequence was amplified from pBPnlsLexA-p65Uw by using primers CGATAGATCTCAACATGGATAAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on GaMP3 (ref. 51) using primers CGATCACTGAGCAACTGAGAAGACAGTGG and CGATGCGCGCGGCTACATGCTTATTAAGTCCG and cloned into pAC-PlacT1 using NotI/AscI and used to replace the leucine zipper in pAC-Zp: GALAD15 between NotI/NheI.

pAC-M13: GALADbo. The M13 sequence was amplified from GaMP3 (ref. 51) using primers CGATGCGCGCGGCCCAAGTTGTTCTCATTCACTATCA and ATGGCGTTAGGGCGCGGCCCTACCTGCTCATATT, and digested with NotI/NheI, and used to replace the leucine zipper in pAC-Zp: GALAD15 between NotI/NheI.

pAC-M13: GALADbo. The GALADbo (codon-optimized) sequence was amplified from pBP-p65AD2pUw by using primers CGAGGACTAAGATGAGCTGTAACAGCTG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the GALADbo in pAC-M13: GALADbo using SpeI/AscI.

pAC-MKII: GaMP. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GaMP. The MKII (codon-optimized) sequence was generated by annealing oligos GCAGAAGGCTTCATGCTGCAGGCGGACGGC and CGATGCGCGCGCGCTACATGCTTATTAAGTCCG and cloned into pAC-PlacT1 using NotI/AscI and used to replace the leucine zipper in pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GaMP. The MKII (codon-optimized) sequence was amplified from pBP-p65AD2pUw by using primers CGAGGACTAAGATGAGCTGTAACAGCTG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the GALADbo in pAC-M13: GALADbo using SpeI/AscI.

pAC-MKII: GaMP. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GaMP. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was amplified from pBP-p65AD2pUw by using primers CGAGGACTAAGATGAGCTGTAACAGCTG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the GALADbo in pAC-M13: GALADbo using SpeI/AscI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.

pAC-MKII: GALADbo. The MKII (codon-optimized) sequence was generated by annealing oligos GGCCGCCACCATGTTATACGAGAAGCGG and CGATGGCGCGCCTTACAGCCAATCTCCGTTGC, replacing the NotI/NheI sites on pAC-Zp: GALAD15 between NotI/NheI.
Brains were dissected and stained following
with a high-speed monitor designed for optomotor response59. The moving gratings
were programmed with Psychtoolbox in Matlab (Mathworks) and delivered
to three of which subject to different visual experiences. The specific visual stimula-
tions by aligning the overall mean.

They were then kept at ~25 °C for 4 d, the first of which always in dark, and the last
were exposed to three 10-min heat shocks at 37 °C, with 2-h intervals in between.
then measured fluorescent intensity in the signal channel.

doi:10.1038/nn.4016

The collected flies were used (annotated with Bloomington Stock # when available):

Fly husbandry. In addition to the flies generated with standard P element–mediated
or site-directed transgenesis for this study (Supplementary Table 2), the following
flies were used (annotated with Bloomington Stock # when available): tubP-QS
(30022), UAS-mCD8::RFP (32229), LexAop-2-mCD8::GFP (32229), UAS-mCD8::
GFP (5137), norpA (ref. 22), UAS-nshb::GFP (9263), UAS-GFP::Rpl10A (42683),
hsFLP and UAS::CD2, y>>CD8::GFP13, hsFLP122 (ref. 54), GHA46-QF (30015),
pleb1-GAL4 (ref. 55), ey-FLP (5580), UAS-w+>RTA::GFP6, UAS-luciferase (gift from
G. Dietzel), GHA46-LexA7, LexAop2-dtrapA1 (gift from G. Rubin), upd2A (ref. 58),
UAS-CaLexA and LexAop-mCD8::GFP::2A::mCD8::GFP13, TbdmH18 (ref. 39),
ple1-GAL4 (8848), trh-GAL4 (38388), tdc2-GAL4 (9313), nshb-GAL4 (25681),
dimm-GAL4 (25733), ilp2-GeneSwitch13, orco-GAL4 (26818), Ushii11 (ref. 41),
nsyb-QF2 (ref. 21).

We used the cormell, molasses and yeast medium listed on Bloomington
website. All experimental flies were raised in a dark 25 °C incubator unless speci-
fied, and collected 1–5 d after eclosion. For QA induction, 75 mg QA (or 20 mg
for all PI experiments) was dissolved in 300 µl water, every applied to the surface
of 10 ml food in a small vial, and let dry overnight. Females were used through-
out the experiments, except for those shown in Figures 2a, 4a,d,e, 5c, 6c and
Supplementary Figure 6a,b. Transgenic fly lines described in this publication
will be available at the Bloomington Stock Center.

Visual system. For the experiments that contain tubP-QS in the cross, the col-
lected flies were transferred onto QA food, and remained there for 3 d. They were either
placed on the benchtop exposed to ‘ambient light’ or enclosed in a dark
cardboard box. We quantified the fluorescence in the anterior half of the optic
lobes, where we drew identical rectangles in the counterstaining channel and
then measured fluorescent intensity in the signal channel.

For the FLP-mediated experiments, flies were raised at 18 °C. Young adult flies
were exposed to three 10-min heat shocks at 37 °C, with 2-h intervals in between.
They were then kept at ~25 °C for 4 d, the first of which always in dark, and the last
three of which subject to different visual experiences. The specific visual stimula-
tions were programmed with Psychtoolbox in Matlab (Mathworks) and delivered
with a high-speed monitor designed for optomotor response39. The moving gratings
were full-contrast sine waves with a spatial period of 2.5 cm and a frequency of 20
Hz, reversing direction every 10 s. The full-field flicker consisted of 8 blocks of 20
Hz, flipping between black and white, interpersed with 2 s of gray.

Olfactory system. For the PN experiments, antennal removal was performed
with forceps while the flies were anesthetized with CO2, and the control flies were
with forceps while the flies were anesthetized with CO2, and the control flies were

We apply to a Kimwipe.

There may be symptoms. The TRIC signal = 2' column (Supplementary
Fig. 7d). This is consistent with previous knowledge that Robetta
performs better with non-charged residues60, and suggests that future optimization
may be aided by a computational exhaustion of the parameter space.

For the simulation in the next section, we estimated the affinity of MKIIK11A,
a mutation to a charged residue, as follows. In the S2 cell assay, signal from the
MKIIK11A variant belongs to ‘Rank 2’, so we estimated the change of binding
energy caused by K11A, as the mean of the Robetta predictions for the three
non-charged residues in the same ‘TRIC signal = 2’ column (Supplementary
Fig. 7d). As a result, KCaMMKIIK11A/KCaMMKII = 1.838.

Simulating TRIC signals. The TRIC signal in Figure 4b and Supplementary
Figure 7 was calculated as follows.

Independent variables. [Ca2+]: Ca2+ concentration (normalized to the dis-
sociation coefficient between Ca2+ and CaM). [AD]: the total number of AD:
CaM proteins (normalized to the dissociation coefficient between DBD and its
target sequence). [DBD]: the total number of peptide::DBD proteins (normalized
to the dissociation coefficient between DBD and its target sequence). [CaM]: the
total number of endogenous CaM (normalized to the dissociation coefficient
between DBD and its target sequence). [peptide]: the total number of endogenous
CaM-target peptide (normalized to the dissociation coefficient between DBD and
its target sequence).

Parameters. KCaMpeptide = KCaMMKII, KCaMMKIIK11A: dissociation coeffi-
cients between Ca2+-bound CaM and endogenous target peptides, MKII and
MKIIK11A, respectively (normalized to the dissociation coefficient between
DBD and its target sequence).

Initialization. [DBD] = 1.5 (for “1 DBD”), [AD] = 1.5 (for “1 AD”), [CaM] = 1.5
(for “1 × competition”) and [peptide] = 1.5 (for “1 × competition”). We arbitrarily
set KCaMMKII = 1, and the corresponding KCaM/MMKIIK11A = 1.838 according to
the estimation described in computational alanine scanning.

Assumptions. [Ca2+] >> [AD] + [CaM]. Complete cooperativity between the four
Ca2+–binding sites on CaM, as illustrated in Figure 1a. [DBD] >> its target
sequence. KCaMpeptide = KCaMMKII.

Calculations. [CaM+Ca2+] = ([AD] + [CaM]) × [Ca2+]^4 / (1 + [Ca2+]^4), find the
solution to [DBD+CaM+Ca2+] following five equations.

\[
\begin{align*}
[\text{DBD}]_{\text{free}} & \times [\text{CaM}+\text{Ca}_2^+] \times [\text{peptide}] \times [\text{CaM}+\text{Ca}_2^+] \\
& = K_{\text{CaMMKII}} (\text{or } K_{\text{CaMMKIIK11A}}) \\
[\text{DBD}]_{\text{free}} + [\text{DBD}+\text{CaM}+\text{Ca}_2^+] &= [\text{DBD}] \\
[\text{peptide}]_{\text{free}} \times [\text{CaM}+\text{Ca}_2^+] & = K_{\text{CaMpeptide}} \\
[\text{peptide}]_{\text{free}} + [\text{peptide}+\text{CaM}+\text{Ca}_2^+] & = [\text{peptide}] \\
[\text{CaM}+\text{Ca}_2^+]_{\text{free}} & + [\text{DBD}+\text{CaM}+\text{Ca}_2^+] + [\text{peptide}+\text{CaM}+\text{Ca}_2^+] = [\text{CaM}+\text{Ca}_2^+] \\
\end{align*}
\]

then:

\[
\text{signal} = [\text{DBD}+\text{CaM}+\text{Ca}_2^+] / (1+[\text{DBD}]) \times [\text{AD}] / ([\text{AD}]+[\text{CaM}])
\]

Immunohistochemistry. Brains were dissected and stained following standard
procedures61. Primary antibodies: Mouse nc82 (DSHB mAbnc82, 1:30), chicken anti-GFP
(Aves Labs GFP-FP10, 1:1,000), rabbit anti-dsRed (Clontech 632496, 1:500). Brains from the same condition were processed within the same
tube.
**Imaging brains.** Images were taken on LSM 510 or 780 confocal microscopes with 20× objectives (Zeiss). For experiments in the same panel, we adjusted the gain so that the sample with the strongest signal barely saturates the dynamic range of the PMT, and imaged all the brains with the same settings and as close as possible time-wise. We did not adjust the offset.

**Image analysis.** Images were analyzed with Fiji. For fluorescence intensity quantification, the Z stacks of the sum of TRIC signals were generated. We then manually selected the region of interest (see details under the subheadings for each manipulation) to measure the total intensity.

Comparisons were made between data collected either from parallel experiments, with exactly the same master mix of solutions, timeline and imaging conditions, or from different batches of experiments normalized to common controls. For example, data for Figure 6g were collected separately, but within each batch there was always one group of MKIJK11a::GAL4DBDo > mCD8::GFP exposed to 10% yeast, whose signal intensity served as the common denominator. Each experiment was repeated at least twice, and we only presented qualitatively consistent results. The data passes Jarque-Bera test whenever normality was assumed.

Custom Matlab scripts were used to assist the averaging of medulla images. The confocal stacks were loaded into Matlab. For each sample, we visually selected a slice corresponding to the same anterior-posterior position according to the nc82 counterstain pattern. Matlab then masked the image using a user-defined threshold. We manually selected the block of neuropil corresponding to the medulla, and Matlab traced its outline. We then specified the outer and inner rims of the medulla by clicking on their starting and ending points, and Matlab generated ten equally spaced control points on each rim. These twenty control points were used to register the medulla to a common fan-shaped template. After registration, the sums of all pixel-by-pixel fluorescent intensities within medulla were used for Figure 2e. For Figure 2f, we pooled data into ten evenly spaced bins along the proximal-distal axis (which do not exactly correspond to the 10 layers), and added the fluorescent intensities within each bin. The total signal intensity in each bin was then normalized to the overall sum.

**Statistics.** No statistical methods were used to determine sample sizes, but our sample sizes are similar to those generally employed in the field. Individual flies were randomly assigned to treatment groups. Data collection and analysis were not performed blind to the conditions of the experiments.

A Supplementary Methods Checklist is available.

---

51. Tian, L. et al. Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nat. Methods 6, 875–881 (2009).
52. Montigiani, S., Neri, G., Neri, P. & Neri, D. Alanine substitutions in calmodulin-binding peptides result in unexpected affinity enhancement. J. Mol. Biol. 258, 6–13 (1996).
53. Bischof, J., Maeda, R.K., Hediger, M., Karch, F. & Basler, K. An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. Proc. Natl. Acad. Sci. USA 104, 3312–3317 (2007).
54. Berdnik, D., Chihara, T., Couto, A. & Luo, L. Wiring stability of the adult Drosophila olfactory circuit after lesion. J. Neurosci. 26, 3367–3376 (2006).
55. Sweeney, L.B. et al. Temporal target restriction of olfactory receptor neurons by Semaphorin-1a/PlexinA-mediated axon-axon interactions. Neuron 53, 185–200 (2007).
56. Smith, H.K. et al. Inducible ternary control of transgene expression and cell ablation in Drosophila. Dev. Genes Evol. 206, 14–24 (1996).
57. Lai, S.L., Awasaki, T., ItO, K. & Lee, T. Clonal analysis of Drosophila antennal lobe neurons: diverse neuronal architectures in the lateral neuroblast lineage. Development 135, 2883–2893 (2008).
58. Hombria, J.C., Brown, S., Hader, S. & Zeidler, M.P. Characterization of Upd2, a Drosophila JAK/STAT pathway ligand. Dev. Biol. 288, 420–433 (2005).
59. Katsov, A.Y. & Clandinin, T.R. Motion processing streams in Drosophila are behaviorally specialized. Neuron 59, 322–335 (2008).
60. Kim, D.E., Chivian, D. & Baker, D. Protein structure prediction and analysis using the Robetta server. Nucleic Acids Res. 32, W526–W531 (2004).
61. Wu, J.S. & Luo, L. A protocol for dissecting Drosophila melanogaster brains for live imaging or immunostaining. Nat. Protoc. 1, 2110–2115 (2006).