In vivo measurement of afferent activity with axon-specific calcium imaging

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In vivo calcium imaging from axons provides direct interrogation of afferent neural activity, informing the neural representations that a local circuit receives. Unlike in somata and dendrites, axonal recording of neural activity—both electrically and optically—has been difficult to achieve, thus preventing comprehensive understanding of neuronal circuit function. Here we developed an active transportation strategy to enrich GCaMP6, a genetically encoded calcium indicator, uniformly in axons with sufficient brightness, signal-to-noise ratio, and photostability to allow robust, structure-specific imaging of presynaptic activity in awake mice. Axon-targeted GCaMP6 enables frame-to-frame correlation for motion correction in axons and permits subcellular-resolution recording of axonal activity in previously inaccessible deep-brain areas. We used axon-targeted GCaMP6 to record layer-specific local afferents without contamination from somata or from intermingled dendrites in the cortex. We expect that axon-targeted GCaMP6 will facilitate new applications in investigating afferent signals relayed by genetically defined neuronal populations within and across specific brain regions.

Nerve circuits consist of large populations of cell bodies connected by axons and dendrites. Understanding circuit computations (and even the computations of single neurons) requires knowing the neural representation carried by inputs, local neurons, and outputs that lead to input–output transformations. Axons are very thin (typically less than 1 μm in diameter), branch numerous times, can vary in length, and travel the full extent of the brain, thus making activity-recording difficult. Microelectrode-based recording methods do not allow isolation of single axons and lack the genetic specificity required to identify the source of afferent inputs.

Untargeted GCaMPs (u-GCaMPs) preferentially label somatodendritic compartments and diffuse poorly to distal axons. Low expression levels of GECIs in presynaptic structures make even visualizing axons with expression difficult, and functional imaging of dimly labeled axons produces poor signal-to-noise ratios (SNRs), particularly deep in tissue where light scattering requires a high photon budget. Consequently, axonal GECI imaging is typically performed only under near-ideal conditions, such as shallow terminal fields of relatively short projections. Even so, low SNR and contamination from intermingled dendritic arbors closer to the expression source degrade imaging quality and complicate assignment of fluorescence to specific axons due to overlapping dendritic signals. Presynaptically targeted GCaMPs have been developed, but suffer substantially from photobleaching. We thus developed an approach for axonal GCaMP imaging that offers greater enrichment and photostability than previous techniques.

Results

Engineering and characterization of an axon-enriched GCaMP. To design a high-efficacy axon-enriched GCaMP, we screened a panel of targeting motifs that have been previously reported to drive axonal localization (Supplementary Fig. 1a). We chose eight motifs with diverse targeting mechanisms and fused them to either the N or C terminus of GCaMP6m. These motifs included the 20-residue tetrapalmitoylation domain found at the N terminus of growth-associated protein-43 (GAP43)21–23, the 15-residue C terminus of the amyloid precursor protein18, the ankyrin-B binding motif derived from the N and P/Q-type voltage-dependent calcium channels17, the mRNA localization zip code found within the 3′ untranslated region of the tau gene24, the 26-residue tandem palmitoylation and myristoylation domains found at the N terminus of the Src tyrosine kinase Lck22,23, the synthetic myosin VI binding domain derived from peptides found in Optineurin and in Disabled homolog21, the 20-residue palmitoylation domain found at the C terminus of Paralemmin12, and finally the full-length Synaptophysin, which was previously reported to target GCaMP effectively to axons but also rapidly photobleaches during in vivo imaging (Fig. 1a and Supplementary Fig. 1a).

We first characterized the expression pattern of each construct in dissociated rat hippocampal neuronal culture. We measured a normalized axon-to-dendrite ratio (nADR) to determine the extent to which each probe localized to axonal and somatodendritic compartments relative to a co-expressed, untargeted red fluorophore (mRuby2; Fig. 1b,c and Supplementary Fig. 1b). The 20-residue dipalmitoylation domain derived from the N terminus of GAP43 drove the highest enrichment of GCaMP6m in axons (Fig. 1b and Supplementary Fig. 1b), with ~fivefold increased nADR compared to u-GCaMP6m (nADR of GAP43-GCaMP6m, 4.83 ± 0.78; nADR of u-GCaMP6m, 0.76 ± 0.08; P = 3.09 × 10−5; Fig. 1c). The GAP43 motif also targeted GCaMP6s and GCaMP6f to axons with equally...
increased nADR compared with GCaMP6m (nADR of GAP43-GCaMP6s, 5.65 ± 1.03; nADR of GAP43-GCaMP6f, 4.58 ± 0.44; P = 8.72 × 10^{-5} and P = 2.19 × 10^{-4}, respectively). In contrast, direct tethering to a presynaptic protein (i.e., Synaptophysin-GCaMP6s or syGCaMP) as previously reported, only moderately targeted the probe to axons, producing about a twofold increase compared to u-GCaMP6m (nADR: 1.70 ± 0.08) or syGCaMP6m (1.77 ± 0.11; F_{2,51} = 26.52, P = 8.36 × 10^{-7}, n.s. = 0.23, ***P = 3.09 × 10^{-6}, respectively; one-way ANOVA with Tukey-Kramer multiple-comparisons test; n = 8-10 cells from three cultures for each construct).

**Fig. 1** Axonal enrichment of GCaMP sensor driven by GAP43 targeting motif in dissociated cells and in vivo. **a**, Schematic representation of u-GCaMP6s, syGCaMP6s, or axon-GCaMP6s. Representative images showing enhanced axon localization of axon-GCaMP6m compared to u-GCaMP6m in dissociated neuronal culture. Results were found to be consistent across three different cultures per construct. Scale bars, 20 μm (insets); 100 μm (cell traces). **c**, Normalized nADR (see Methods for description). Average nADRs of axon-GCaMP6m (4.83 ± 0.78) represent an approximate fivefold increase compared to u-GCaMP6m (0.76 ± 0.08) or syGCaMP6m (1.77 ± 0.11; F_{2,51} = 26.52, P = 8.36 × 10^{-7}, n.s. = 0.23, ***P = 3.09 × 10^{-6}, respectively; one-way ANOVA with Tukey-Kramer multiple-comparisons test; n = 8-10 cells from three cultures for each construct).

**d-f**, Characterization of nADR of thalamocortical axons in V1. **d** Schematic representation of viral injection site of AAV encoding bicistronic constructs. **e**, Representative images showing densely labeled L4 axons projected from dLGN labeled with axon-GCaMP6s, whereas cell somata were nearly devoid of labeling. Results were consistent across four animals per construct. Scale bars, 100 μm (cortex); 500 μm (thalamus). **f**, Probability-density histogram (PDF) of nADR values derived for both constructs with medians indicated by vertical line (axon-GCaMP6s, 2.54 ± 0.28; u-GCaMP6s, 0.50 ± 0.06; P = 4.37 × 10^{-16}; Wilcoxon’s rank-sum test; n = 56 slices for axon-GCaMP6s and n = 37 slices for u-GCaMP6s, from 3 animals per construct). Data are presented as mean ± s.e.m.

We next sought to characterize the photostability and diffusibility of axon-GCaMP6m using fluorescence loss in photobleaching.
and fluorescence recovery after photobleaching assays (Fig. 2a,d and Supplementary Fig. 4a–e). Axon-GCaMP6m diffused rapidly between adjacent axonal compartments at a rate similar to that of u-GCaMP6m (mobile fraction of axon-GCaMP6m, 0.77 ± 0.02; mobile fraction for u-GCaMP6m, 0.90 ± 0.03; Fig. 2b,c and Supplementary Fig. 4d,e). High diffusibility led to fast replenishment of bleached molecules, thus greatly reducing effective photobleaching of axon-GCaMP6m (Fig. 2e). In contrast, syGCaMP6m displayed significantly reduced diffusibility, resulting in rapid photobleaching consistent with previous in vivo observations (mobile fraction for syGCaMP6m, 0.28 ± 0.01; Fig. 2c,e and Supplementary Fig. 4c). Additionally, the dipalmitoylation modification of axon-GCaMP6m provided better photostability than some of the other targeting motifs that contained either myristoylation domains (for example, Lck) or multiple binding sites for transport effectors (for example, myosin VI binding domain), which is consistent with previously reported results (Supplementary Fig. 4c).

To confirm that axon-GCaMP6m is more photostable in vivo, we labeled dLGN with rAAV encoding u-GCaMP6s, syGCaMP6s, or axon-GCaMP6s (Fig. 2f), followed by continuous two-photon
Fig. 3 | Axon-GCaMP6s improves SNR and image-wise correlations in long-distance axons. a,f. Schematic representation of viral injection in (a) dLGN and (f) LP, followed by in vivo imaging in V1 of projected axons. b,g. Representative images of L1 axons projected from (b) dLGN and (g) LP, demonstrating enhanced brightness of axon-GCaMP6s expressing axons. Experiments were performed in at least 3 animals per construct injection site pair with similar results. Scale bars, 10 μm. c–h. Left: fluorescence of a representative ROI in response to the indicated grating directions (10 trials per direction) of axon-GCaMP6s and u-GCaMP6s in (c) dLGN and (h) LP. Data presented with averages in dark colors and individual trials in light colors. Scale bars, 3 s; 100% ΔF/F. Right: polar plots of tuning properties of the selected ROIs. d,i. Trial-averaged traces of SNR time courses from all responsive ROIs to preferred stimulus direction. Point at which moving stimulus was presented indicated by vertical dotted black line. e,j. Top: cumulative distribution of normalized fluorescence intensity of axon-GCaMP6s and u-GCaMP6s in (e) dLGN and (j) LP axons projecting to V1 (dLGN: axon-GCaMP6s, 5.85; u-GCaMP6s, 0.85; LP: axon-GCaMP6s, 19.47; u-GCaMP6s, 5.08; P = 2.15 × 10^{−29} and P = 1.24 × 10^{−35}, respectively, Kolmogorov–Smirnov test). Bottom: cumulative distribution of SNRs of individual ROIs with median values indicated by vertical lines and color-coded numeric values (dLGN: axon-GCaMP6s, 2.73; u-GCaMP6s, 1.54; LP: axon-GCaMP6s, 1.07; u-GCaMP6s, 0.64; P = 9.56 × 10^{−19} and P = 1.13 × 10^{−16}, respectively, Kolmogorov–Smirnov test). In e, n = 712 ROIs from 4 animals for axon-GCaMP6s and n = 14,478 ROIs for u-GCaMP6s from 19 animals. For j, n = 1,393 ROIs from 3 animals for axon-GCaMP6s and n = 917 ROIs from 4 animals for u-GCaMP6s. k–m. Axon-GCaMP6s improves frame-to-frame correlations. (k) Representative frames from 1 imaging session per construct in L1 dLGN experiments, showing enhancements to image structure for axon-GCaMP6s. Text above and below the images indicates the procedure for calculating the frame-to-frame correlations. Scale bar, 10 μm. (l,m) Axon-GCaMP6s permits substantially improved frame-to-frame correlations for image registration. Time course of image-wise correlation values for (l) dLGN and (m) LP boutons. Data plotted as mean (solid line) ± s.e.m. (shaded regions); n = 6 imaging sessions from two animals for each construct.
excitation through a cranial window in awake, behaving animals. Upon hundreds of seconds of illumination, the fluorescence of dLGN projections in superficial V1 labeled with either u-GCaMP6s or axon-GCaMP6s remained largely unchanged (Fig. 2g and Supplementary Fig. 4f). In contrast, the fluorescence signals of syGCaMP6s-labeled projections decreased by more than 50% during in response to field-potential stimuli. We transfected axon-GCaMP6s-P2A-mRuby3 or u-GCaMP6s-P2A-mRuby3 into cultured hippocampal neurons. After 5 d of expression in dissociated neuronal culture, the ratio of green-to-red fluorescence of axons expressing axon-GCaMP6s was about fivefold higher than those labeled with u-GCaMP6s (axon-GCaMP6s, 0.100; u-GCaMP6s, 0.023; Fig. 2h and Supplementary Fig. 5a). In response to a variety of field-potential stimuli (30 Hz), fluorescence changes (ΔF/F0) of axon-GCaMP6s were similar to those of u-GCaMP6s, with higher basal fluorescence levels (Supplementary Fig. 5b) and lower noise, resulting in ~twofold enhancement in SNR (for details, see Methods) across all stimuli (Fig. 2i,j and Supplementary Fig. 5b). Kinetics (Supplementary Fig. 5c,d), size of detected regions of interest (ROIs, ~0.5–10 μm2) (Supplementary Fig. 5e,f), and fluorescence brightness from the secondary fluorophore, mRuby3 (Supplementary Fig. 5g,h), were similar between axon-GCaMP6s and u-GCaMP6s. We therefore conclude that increased probe enrichment in axons was the primary driver of the observed enhancement to SNR displayed by axon-GCaMP6s.

In vivo imaging of distal afferent calcium signals with axon-GCaMPs. We sought to determine whether improved properties of axon-GCaMP6 could enable in vivo applications that are difficult with u-GCaMP. Thalamocortical projections to sensory cortices, such as those projected from dLGN and lateral posterior nucleus of the thalamus (LP), are dimly labeled by u-GCaMPs. To determine the effect of axonal enrichment on SNR, we stimulated mice with drifting gratings and imaged the responses of dLGN cells (Fig. 3a–e and Supplementary Fig. 6a–c) and LP cells (Fig. 3f–j and Supplementary Fig. 6d–f) at their axon terminals in layer 1 (L1) of V1.

We successfully recorded calcium signals in thalamocortical axons in superficial layer of V1 in response to drifting grating stimuli (Fig. 3c,h). Axons labeled with axon-GCaMP6s displayed a ~sevenfold increase in normalized basal green fluorescence over u-GCaMP6s for dLGN axons (5.86 for axon-GCaMP6s, 0.85 for u-GCaMP6s; P = 2.15 × 10−19), LP axons similarly showed a ~fourfold increase (19.47 for axon-GCaMP6s, 5.08 for u-GCaMP6s; P = 1.24 × 10−13) (Fig. 3b,e,g,i) and Supplementary Video 1). While u-GCaMP6s expressing axons displayed bright fluorescent varicosities that are likely boutons6,7,29, axon-GCaMP6s fluorescence was relatively homogenous in both axonal shafts and varicosities (Fig. 3b,g). Because signal amplitude is generally lower in the axonal shaft than in varicose structures (Supplementary Fig. 7; see also ref. 22), we drew ROIs over varicosities (for details, see Methods) and measured visual responses of thalamocortical axonal populations. SNR (for details, see Methods) of axon-GCaMP6s in response to drifting gratings was significantly improved for dLGN axons (2.73 for axon-GCaMP6s, 1.54 for u-GCaMP6s; P = 9.56 × 10−18) as well as LP axons (1.57 for axon-GCaMP6s, 0.98 for u-GCaMP6s; P = 1.13 × 10−9) (Fig. 3d,e,i). Consistent with our dissociated neuronal culture data, similar ΔF/F0 values were observed across both constructs for dLGN axons (0.72 for axon-GCaMP6s, 0.40 for u-GCaMP6s; P = 5.41 × 10−7) and LP axons (0.86 for axon-GCaMP6s, 0.83 for u-GCaMP6s; P = 0.44; Supplementary Fig. 6b,c,e,f).

We next compared the sensitivity of axon-GCaMP6s with syGCaMP6s. Because syGCaMP6s bleaches more rapidly than other GCaMP6s designs (Supplementary Figs. 4f and 8a–c), we observed trial-number-dependent rundown of ΔF/F and SNR values (Supplementary Fig. 8c–e). We then compared the brightness of ROIs only in the first 25 s of imaging. At this early period of the imaging session, brightness of axon-GCaMP6s was less than two-fold higher than that of syGCaMP6s (5.57 for axon-GCaMP6s, 3.45 for syGCaMP6s; P = 3.13 × 10−3; Supplementary Fig. 8g). When averaged across all trials, syGCaMP6s exhibited comparable ΔF/F values (0.72 for axon-GCaMP6s, 0.65 for syGCaMP6s; P = 0.016) and significantly lower SNRs (2.73 for axon-GCaMP6s, 1.63 for 1276 NATURE NEUROSCIENCE | VOL 21 | SEPTEMBER 2018 | 1272-1280 | www.nature.com/natureneuroscience
**Fig. 5** Axon-GCaMP6s permits layer-specific recording of axons projected from local neurons, without contamination from somatodendritic signals. 

**a.** Schematic representation of labeling L4 neurons with Cre-dependent rAAV encoding axon-GCaMP6s or u-GCaMP6s in Scnn1a-Cre-Tg3 mice.

**b.** Expected pattern of transduction: axon-GCaMP6s strongly labeling axons, with weak labeling in the somatodendritic compartment, and the reverse pattern for u-GCaMP6s.

**c.** Representative images showing expression patterns of axon-GCaMP6s (top) or u-GCaMP6s (bottom) fused to P2A-mRuby3. Axon-GCaMP6s is enriched in axons, whereas u-GCaMP6s more strongly labels somatodendritic compartments. Inset: representative zoomed-in images in L1 and L4. This result was consistently found in 2 animals per construct. Scale bar, 100 μm (somatic image), 10 μm (inset).

**d,e.** Probability-density histograms of green-to-red ratio at (d) axons or (e) dendrites. Distribution medians indicated by vertical lines. Axon-GCaMP6s displayed significantly enhanced green-to-red ratio in axons, but dramatically decreased green-to-red ratio in dendrites, compared to u-GCaMP6s (P < 4.94 × 10⁻²⁴ and P = 1.57 × 10⁻²⁶, respectively; Wilcoxon’s rank-sum test; n = 1,200 ROIs from 2 animals for both constructs).

**f–i.** Axon-GCaMP6s enabled layer-specific tuning properties of axons across cortical layers in V1. Data were derived from 2 imaging sessions per depth in 2 animals. **f.** Representative images of axons at indicated depth with numbered circles indicating analyzed ROIs. **g.** Color-coded tuning map demonstrating pixel-wise tuning across analyzed images. **h.** Average ΔF/F traces aligned to stimulus direction of individual ROIs, with baseline indicated by dotted black line. Data plotted as mean (black line) ± s.e.m. (gray shading); n = 10 trials per direction. **i.** Tuning curves for individual ROIs.
Axon-GCaMP enables layer-specific imaging of local afferents in vivo. Lastly, we used a Cre-dependent axon-GCaMP6 to record orientation and direction tuning of axons projecting from L4 V1 neurons in each recipient cortical layer without contamination of signal from somatodendritic sources (Fig. 5). L4 excitatory neurons were labeled in Scnn1a-Tg3-Cre mice using rAAV encoding FLEX-axon-GCaMP6s-P2A-mRuby3 or FLEX-GCaMP6s-P2A-mRuby3 driven by the human Synapsin1 promoter (Fig. 5a,b). Three weeks after infection, we observed L4 axons labeled with axon-GCaMP6s ramifying across all cortical layers, whereas u-GCaMP6s primarily labeled the somatodendritic compartments of L4 neurons (Fig. 5c). L4 axons and boutons were easily identifiable from intermingled dendritic structures within different cortical layers (Fig. 5c). The average green-to-red ratio of axon-GCaMP6s in axons was about fivefold higher than that of u-GCaMP6s (axon-GCaMP6s, 2.25; u-GCaMP6s, 0.53; $P = 4.94 \times 10^{-13}$; Fig. 5d). In contrast, the green-to-red ratio of axon-GCaMP6s in dendrites was sixfold lower compared to u-GCaMP6s, indicating specific axonal enrichment of axon-GCaMP6s (axon-GCaMP6s, 0.21; u-GCaMP6s, 1.24; $P = 1.57 \times 10^{-26}$; Fig. 5e).

In agreement with our histological results, no dendritic structures were observed in the in vivo two-photon fields of view (Fig. 5f and Supplementary Fig. 14a). In contrast, animals transduced by u-GCaMP6s showed numerous intermingled dendritic structures (Supplementary Fig. 14b). Using axon-GCaMP6s, we were able to readily detect the response (Fig. 5g–i) and calculate the orientation-tuning properties of individual boutons from L4 neurons throughout their projection fields across cortical depths down to 600 μm (Fig. 5i and Supplementary Fig. 15).

In addition, axon-GCaMP6s permits layer-specific output recording of axons projected from other cortical layers as well. When rAAV-hSynapsin-FLEX-axon-GCaMP6s was introduced to V1 of Rp4-Cre or Ntsr1-Cre driver mouse lines to restrict expression to L5 or L6, respectively, single-bouton tuning properties were clearly detectable in their recipient layers (Supplementary Fig. 14). Thus, axon-GCaMP6s permits recordings of local afferents down to individual boutons even when dendritic structures of transduced cells are present within the same tissue volume.

Discussion

We have overcome the limitations of genetically encoded calcium indicators to allow robust imaging of afferent calcium transients deep in tissue, with structural specificity and with greater SNR. We achieved this by creating an axon-targeted GECI, axon-GCaMP6, which enriches exclusively in local or distal axons with enhanced brightness and photostability, thus expanding calcium imaging beyond the soma and dendrites. Although presynaptic-targeted GCaMPs, such as syGCaMP6, have been developed, imaging with sensors that localize tightly to presynaptic compartments suffers substantially from photobleaching (Fig. 2g). In contrast, axon-GCaMP6 is diffusible, photostable, and displays enhanced SNR. The enrichment of axon-GCaMP in axons offers enhanced frame-to-frame artifacts are frequently uncorrectable. The increased brightness facilitated the first recordings of dLGN L5/6 axons at depths down to 600 μm (Fig. 4c–e). Orientation-specific fluorescence transients ($AF/F_0$) were clearly observable, and from these transients, we calculated orientation tuning curves of these deep cortical axons (Fig. 4c–e). Similarly, axon-GCaMP6 was also brighter at all depths than u-GCaMP6s. Especially at 600 μm, axon-GCaMP6 allowed for effective recordings of orientation-specific tuning, whereas no fluorescence signal sources were observable with u-GCaMP6s (Supplementary Fig. 13). Thus, axon-GCaMP6 allows in vivo interrogation of previously inaccessible axons deep within tissue.
ers. Thalamic axons ramify in both supra- and subgranular layers. However, in vivo calcium imaging of thalamic boutons has been limited to superficial layers (<400 μm below pia) with untargeted GCaMP6s or GCaMP6f, even with the aid of adaptive optics. With axon-GCaMP6s and axon-GCaMP6f, the tuning properties of individual thalamocortical boutons in subgranular layers can be readily imaged without the aid of adaptive optics while preserving good SNR, which we expect will open the door for new studies of long-range communication in deeper tissue. As an example application, it is now established that infragranular layers, including L5,15,16 and L6,41, receive direct thalamocortical innervation from primary thalamic nuclei. Previous methods have left the axons that form these connections functionally inaccessible. Deeper imaging made possible by axon-GCaMP6 will alleviate this methodological gap.

Finally, we used axon-GCaMP6 to record orientation tuning and direction tuning of layer-specific axonal inputs projected from local neurons, while excluding signal from their extensively intermingled dendritic structures. Recording from local axonal inputs with layer specificity remained difficult, largely due to contamination from intermingled dendritic structures. Generally, it remains unclear what information is provided by local afferents on a layer-by-layer basis. Axon-GCaMP6 allows direct interrogation of these signals with requisite spatial resolution while avoiding contaminating signal from transduced dendrites.

Long-term, high-level expression of GeCIs in the mouse brain can result in prolonged response or nonfunctional indicators and can perturb circuit function. In the case of axon-GCaMP6, the enrichment in axons did not perturb neuronal physiology, as evidenced by kinetics and properties of orientation tuning remaining similar to those of untargeted GCaMP6 after long-term expression (4–6 weeks). Although we did not observe adverse effects in our experiments, physiological impact should be carefully examined in each experimental preparation. Possible interference with synaptic physiology should also be examined.

Axon-GCaMP will be useful in a number of other applications and brain regions, and the targeting tag might be transferable to other payloads. The axonal targeting sequence is only 20 amino acids, allowing easy fusion with both red-shifted13,20 and Förster resonance energy transfer-based GeCIs37–41 or other genetically encoded sensors of neural activity, such as neurotransmitter sensors42. Axonal enrichment may be particularly useful for red indicators, as this approach enhances brightness in small subcellular compartments without extensive optimization of basal fluorescence of the indicator itself. In addition, it may be possible to target actuators43 to axons to allow axon-specific activation of input signals.

Though axon-GCaMP6 is a superior tool for afferent imaging in the mammalian brain, axon-GCaMP6 is not intended as a replacement for monitoring synaptic calcium using syGCaMP. Indeed, syGCaMP indicators have proven their utility for uncovering the coupling between presynaptic calcium and vesicular release in dissociated neuronal cultures44,45, flies46, and larval zebrafish47. It has also been used to address systems-level questions in the more optically accessible larval zebrafish48,49. However, in mouse brain, we observed a high degree of photobleaching of syGCaMP (Fig. 2g), which precludes imaging for longer than a few minutes. When expressed in dissociated neurons, syGCaMP displayed significantly reduced diffusibility (P = 0.0048; Fig. 2c), limiting replenishment of photobleached molecules in boutons. The fast photobleaching in vivo is therefore presumably due to slow replenishment of bleached molecules into the imaged volume during prolonged imaging experiments. As the axon-GCaMP targeting strategy maintains fast diffusibility, it is more photostable than syGCaMP6 both in vitro and in vivo.

In vivo, axon-GCaMP6 reports axonal calcium transients in both axonal shafts and putative boutons more effectively than u-GCaMP6. We also observed that fluorescence responses of axon-GCaMP6 in shafts were smaller than those in putative boutons (Supplementary Fig. 7), consistent with previous observations that calcium signals in axons concentrate in the proximity of boutons (see also refs 29,30). When combined with post hoc structural analysis, it is possible to delineate calcium signals originating in axon shafts or putative boutons. However, when recording afferent signals from specific axons in vivo, interest is often focused on information encoded by axonal signals. Therefore, differentiating signals at synapses versus axons does not usually add additional information for answering questions at the systems level. Axon-GCaMP6 allows more effective analysis of Ca2+ signals within axonal shaft compartments, which exhibit signals that are highly correlated with those found in boutons of the same axon48,49. As axonal imaging becomes a rich area of research, requiring calcium indicators that are photostable, bright, and capable of specifically reporting axonal signals, we expect that this specialized GCaMP6 will greatly facilitate recordings from afferent signals of any length with subcellular resolution.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41593-018-0211-4.

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### Author contributions

L.P. and L.T. initiated the project. G.J.B. and L.T. designed the projects; developed axon-targeted GCaMP6; performed characterization of ADR, photostability, and SNR in vitro in dissociated neuronal culture; and performed histological characterization of ADR and expression in vivo. Y.L. G.M., and N.J. performed in vivo characterization of dLGn axons in V1, deep-brain imaging, and layer-specific cortical axonal recording in V1. M.E. and L.P. performed in vivo characterization of L.I. axons in V1 projecting from LA and area LM and examined frame-to-frame correlations. G.J.B. analyzed data from in vivo imaging with help from Y.L., M.E., and G.M. E.K.U. performed viral injections. X.X. performed pilot experiments for sensor characterization. G.J.B. and L.T. wrote the manuscript with critical input from all authors.

### Competing interests

The authors declare no competing interests.

### Additional information

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Methods

**Protein engineering.** All constructs were designed using a combination of overlap extension cloning and gBlock gene fragments (Integrated DNA Technologies). All constructs were flanked by BamHI with a downstream Kozak sequence and start codon, as well as HindIII for final subcloning onto pAAV-hSynapsin1-FLEX vectors. Alternatively, constructs were flanked by ScaI and ALLI sites for subcloning into custom pAAV-hSynapsin1-FLEX vectors.

**Concurrent two-color fluorophore expression** was achieved by the use of either two plasmids with mRuby2 as the red reference fluorophore or a bicistronic approach in which the GCaMP construct was separated by a P2A sequence from a downstream mRuby3. The two-plasmid approach was used for all in vitro characterization, while the bicistronic approach was used for in vivo characterization. Functional assays were performed with no reference fluorophore.

**Dissociated hippocampal neuronal culture.** For ADR and diffusion analyses, primary hippocampal neuronal cultures were prepared as described previously. Briefly, P0 or E18 pups were decapitated, and the brains were dissected into ice-cold neural dissection solution (NDS, 10 mM HEPES (Sigma) in Hank’s Balanced Salt Solution (HBSS, Thermo Fisher Scientific), pH 7.4). Hippocampi were removed, enzymatically digested with papain (~60 units), washed with prewarmed plating medium (PM, Minimal Essential Medium (Thermo Fisher Scientific) supplemented by 10% FBS (Thermo Fisher Scientific) and 100 U/mL penicillin–streptomycin (Thermo Fisher Scientific)) and then mechanically digested by trituration. Cells were plated on 35 mm MatTek glass bottom dishes (MatTek) coated with Matrigel matrix (BD Biosciences) or a mixture of poly-l-ornithine (Sigma-Aldrich) and laminin (Sigma-Aldrich), and kept at 37 °C, 5% CO2 in PM for ~24 h and then in Neurobasal medium (Thermo Fisher Scientific) supplemented with 10% B27 (Thermo Fisher Scientific) for the experiment duration, with half medium exchanges every 4 d. On the fifth day in vitro (DIV), cells used for ADR and FRAP/FLIP experiments were transfected by Effectene (Qiagen), following the manufacturer’s recommendations with the following modification: for 100 μL transfection complex, we included only 5 μL Effectene reagent. Such cells were subsequently used for imaging experiments between 2 and 5 d post-transfection. Cells used for SNR analysis were transduced on DIV5 by rAAV-hSynapsin-GCaMP6s-P2A-mRuby3-WPRE-SV40 or rAAV-hSynapsin-axon-GCaMP6s-P2A-mRuby3-WPRE-SV40 to drive near-complete transduction of all cultured neurons. All cultures were imaged on a laser confocal microscope (Zeiss 710) equipped with 405-, 488-, 514-, 561-, and 633-nm laser lines. Band limits for detection of GCaMP fluorescence were set within the indicated structure. Normalizing to the distribution of red fluorescence within the bleached circle. For these experiments, all fields of view included an axon that was not directly attached to the bleached segment. Fluorescence within this control axon segment was monitored to ensure that imaging did not cause greater than a 5% decrease in baseline fluorescence. Imaging sessions in which the control process fluorescence dropped by greater than this threshold were discarded.

**FRAP** was also performed to extract diffusion coefficients of axonal GCaMP variants. Imaging and bleaching parameters were identical to those used in the FLIP experiments, except that a single bleaching pulse was applied. To drive sufficient bleaching, experiments were conducted in imaging medium supplemented by 5 μM of the calcium ionophore ionomycin, effectively increasing the extinction coefficient of the GCaMP. Normalized postbleach profiles, \( f_{\text{postbleach}}(t) \), were determined by dividing the fluorescence profile directly following the bleaching epoch by that preceding the bleach event. Diffusion occurring between consecutive imaging acquisitions was then corrected for by fitting the effective bleach radius, \( r_c \), and bleach depth, \( K \), of the normalized bleach profile to the equation:

\[
\hat{f}_{\text{postbleach}}(t) = 1 - K e^{-t^2 / (4D)}
\]

FRAP curves were then determined by fitting the recovery time course, \( F(t) \), to the equation

\[
F(t) = (1-a)(F_p-F_e) e^{-t/\tau_{slow}} + a(F_p-F_e) e^{-t/\tau_{fast}} + F_e
\]

where \( a, F_p, F_e, \tau_{slow}, \) and \( \tau_{fast} \) are free parameters corresponding to the double exponential fraction, the initial postbleach fluorescence, the steady-state fluorescence recovery level, and the slow and fast exponential recovery rates, respectively. The time to half recovery, \( t_{1/2} \), was then determined as the timepoint where \( F(t_{1/2}) = \frac{F_e+4\Delta F}{5} \). Finally, the diffusion coefficient, \( D \), was recovered using the nominal user selected bleach radius, \( r_c \), with the expression

\[
D = r_c^2 + r_c^2 \frac{5}{8\Delta F / F_e}
\]

Characterization of performance in dissociated neuronal culture. To quantify performance of GCaMP-labeled cell culture, we performed electrical field-stimulation experiments in dissociated hippocampal culture. In cultures densely transduced by virus, confocal imaging was performed on intermingled fields of axons within fields of view more than 100 μm from the nearest visible dendritic segment. Axons imaged for this study were first masked using the mRuby3 fluorescence signal. These masks were segmented using an IFC plugin to split ROI1 into ROIs with normal distribution and mean size around that of typical ROIs used in vivo imaging experiments (0.8–10 μm²). Time-lapse calcium imaging stacks were acquired from these axons based on the GCaMP fluorescence signal. After a 5 s baseline, 1, 2, 5, 10, 20, or 40, 80 field potentials were applied as 1-V, 1 ms square-wave pulses at 30 Hz under the control of Ephus software. mRuby3-based ROIs were then averaged with the baseline of average fluorescence, \( F_e \), calculated to use the fluorescent transients \( \Delta F/F_e = (F-F_e)/F_e \). SNR was then calculated as the maximum \( \Delta F/F_e \), divided by the s.d. of the baseline period.

**Animal procedures.** All animal procedures were conducted according to the Protocol and Direcção General de Veterinaria and the United States National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at the University of California, Davis, the Howard Hughes Medical Institute, or the Champalimaud Center for the Unknown. Animal Care and Use Committee at the University of California, Davis, the Howard Hughes Medical Institute, or the Champalimaud Center for the Unknown. The protocols for the Institutional Animal Care and Use Committees at the University of California, Davis, the Howard Hughes Medical Institute, or the Champalimaud Center for the Unknown.

**Viral injections.** Injection procedures were essentially identical to those described in refs 44, 49 with a few exceptions. Briefly, virus injection was performed using a glass pipette beveled at 45° with a 15- to 20-μm opening and backfilled with mineral oil. A fitted plunger controlled by a hydraulic manipulator (Narashige, MO16; or World Precision Instruments, UMP3) was inserted into the pipette and used to load the virus construct to inject the viral solution.

For confirmation of enhanced axonal expression of axon-GCaMP6s in living mice, ~20–40 nL of rAAV2/1-hSynapsin1-GCaMP6s-P2A-mRuby3-WPRE-SV40 (~2 × 10¹¹ viral genome (vg) per mL) or rAAV2/1-hSynapsin1-axon-GCaMP6s-P2A-mRuby3-WPRE-SV40 (~3.2 × 10¹³ vg per mL) were slowly injected into the right auditory cortex of mice (12.5 mm lateral from midline, 2.3 mm anterior to bregma, 2.5 mm below pia). For axon-specific labeling of local cortical connections in primary visual cortex (V1), ~20–40 nL of rAAV2/1-hSynapsin1-axon-GCaMP6s-WPRE-SV40 (~3.2 × 10¹³ vg per mL) were injected to cortical L4 (Snn1a-Tg3-Cre mice: three injection sites in left
hemi-sphere centered at 3.4 mm posterior to bregma; 2.7 mm lateral from midline; 0.3 mm below pia; injection sites are ~ 250 μm apart). Additionally, we produced these viruses under a 2/5 serotype to test differences in transduction efficiency. We did not find apparent differences across serotypes (data not shown).

For calcium imaging of thalamocortical and LM corticocortical projections, ~ 20–80 nL of rAAV2/1-hSynapsin1-ΔCaMP6s-WPRE-SV40 (~ 2–4 × 10^10 infectious units per mL) or rAAV2/1-hSynapsin1-axon-ΔCaMP6s-WPRE-SV40 solution (~ 4 × 10^10 infectious units per mL) were slowly injected into dLGN, LP (1.5 mm posterior to bregma; 1.1 mm lateral from midline; 2.7 mm below bregma), or LM (coordinates determined by intrinsic optical imaging19–21). For imaging of cortical neurons and local axon projections – 20–40 nL of rAAV1-hSynapsin1-ΔFLEx-ΔCaMP6s-WPRE-SV40 (~ 2–4 × 10^10 infectious units per mL) or rAAV1-hSynapsin1-ΔFLEx-axon-ΔCaMP6s-WPRE-SV40 (~ 3.1 × 10^10 infectious units per mL) were injected in per injection site into V1 for cortical L5 (Schnitt and Garaschuk22; mGBA and a bimodal Gaussian function56 to fit the angular tuning of each image across the drifting directions (one-sided t-test between the fluorescence values during the baseline and stimulus period resulted in significance at an alpha value of 0.001).

In vivo image analysis. Lateral motion artifact in two-photon imaging stacks was corrected using a cross-correlation-based registration algorithm23, where cross-correlation was used to detect and correct for translation shifts in stacks. In each image stack, the mean projection was used as the registration reference. Several rounds of registration and re-averaging of the stack were performed to decrease ∑(Δx_i + Δy_i), where Δx and Δy represent the horizontal and vertical shifts of the kth image, respectively. This process was repeated once for LP and LM data and for up to seven iterations for the dLGN data.

ROI selection. In average or s.d. images of registered image stacks, labeled axons appeared as bright spots in u-ΔCaMP6s and as filled axonal shafts and varicosities in axon-ΔCaMP6s. For analysis of both u-ΔCaMP6s and axon-ΔCaMP6s experiments, circular ROIs (0.8–4 μm) were manually drawn over varicosities that likely correspond to presynaptic boutons17–18.

Characterizing ∆F/F and SNR in vivo. For stacked data from LP and LM axons, F0 was calculated as the average of the baseline fluorescence for each stimulus presentation. For dLGN axons, the mode of the fluorescence intensity histogram from each ROI was used as F0. Fluorescence transients were then calculated as ∆F/F = (F(t) – F0)/F0. Averaged ∆F/F traces were then derived from the mean data across ten trials of the same orientation. Resultant traces were then averaged over the period of stimulus presentation to derive a ∆F/F value for each ROI. The s.d. of the ∆F/F signal during the period before presenting the moving visual stimulus was calculated across trials, and the resultant values were averaged together. SNR was calculated by dividing the ∆F/F value by the s.d. To characterize the tuning of axonal boutons, we followed the previously described procedure24. Briefly, the response R of each ROI to a visual stimulus was defined as the average ∆F/F across the window of drifting gratings. For ROIs with significantly different responses across the drifting directions (one-way ANOVA, P < 0.05), we fit their normalized response tuning curves to grating drifting angle θ with a bimodal Gaussian function

\[
R(θ) = R_{\text{cor}} \frac{\text{ang}(0 - θ)^2}{2σ^2} + R_{\text{uni}} \frac{\text{ang}(0 - θ + 180)^2}{2σ^2}
\]

Here θ_{cor} is the preferred orientation, R_{cor} is a constant offset, and R_{uni} and R_{cor} are the responses at θ = 0 and θ = 180 degrees, respectively. ang(0) = min(0, x, 360) wraps angular values into the interval 0° to 180°. For the dLGN data, only ROIs whose tuning curves were well fit by the bimodal Gaussian function were considered orientation selective. In LP and LM data, ROIs were considered visually responsive if a two-sided t test between the fluorescence values during the baseline and stimulus period resulted in significance at an alpha value of 0.001.

For determining the frame-to-frame correlations, we calculated the 2-D correlation between frames of registered image stacks using the Matlab function corr2. The brightness of imaged varicosities from the dLGN thalamocortical projections was determined by normalizing pixel fluorescence value to the square of the excitation laser intensity. Contrast was then determined by taking profiles across axonal structures and normalizing these profiles to the peak intensity.

Statistical methods. All statistical analyses were performed in Matlab (MathWorks) or Prism (GraphPad). We used parametric and nonparametric ANOVA, Wilcoxon’s rank-sum tests, and Kolmogorov–Smirnov test. All tests were two-tailed. Error bars are s.e.m. or s.d. as indicated in figure legends and main text. No statistical methods were used to predetermine sample size. However, sample sizes are consistent with those reported in previous publications43,44. Data collection was randomized in the organization of the experimental conditions and visual stimulus presentations. Data collection and analysis were not performed blind to the progression of the experiments. Data distribution was assumed to be normal, but this was not formally tested. No animals and no data points were excluded from the analyses.

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

Data, reagent and code availability. The data that support the findings of this study are presented in the paper and the supplementary materials and all raw data are available at the following URL (https://github.com/author1/author2).
imaging data are available upon request. All routine analysis methods are included in the Methods section, and Matlab codes are deposited in GitHub (https://github.com/gerardj-broussard/BroussardEtAl2018.git). Plasmids encoding axon-GCaMP and bicistronic variants (accession numbers: MH282423, MH282424, MH282425, MH282426, MH282427, MH282429, MH282430, MH282432) have been made available in the Addgene plasmid repository (plasmid numbers 111261–112010).

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- **☐** 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)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

| Data collection | Scanimage software in a Matlab environment and Zeiss Zen software were used for data collection. |
|-----------------|--------------------------------------------------------------------------------------------------|
| Data analysis   | Matlab and PRISM software were used for all data processing and statistical analyses. All Matlab code is deposited in Github and also available upon request. |

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The data that support the findings of this study are presented in the paper and the supplementary materials and all raw imaging data are available upon request. All routine analysis methods are included in the Methods section and MATLAB codes are deposited in github (https://github.com/gerardj-broussard/)
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size       | No statistical methods were used to predetermine sample size. However, sample sizes are consistent with those reported in previous publications (12,13). |
|-------------------|-------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions   | No data were excluded from this study                                                                                              |
| Replication       | Each experiment was performed on multiple cultures or animal subjects. All attempts were successful.                             |
| Randomization     | Animals and cultures were randomly assigned for transduction with the different constructs described in the manuscript.          |
| Blinding          | Blinding was not used in this study. The extreme differences in the expression patterns of targeted and non-targeted constructs made it immediately apparent which construct was transduced in each subject/culture. |

Reporting for specific materials, systems and methods

Materials & experimental systems

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

Methods

- n/a Involved in the study
- □ ChIP-seq
- □ Flow cytometry
- □ MRI-based neuroimaging

Animals and other organisms

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

Laboratory animals

For derivation of primary neuronal cultures, P0 or E18 Sprague Dawley pups were used. For in vivo imaging and histological experiments, C57Bl6, Scnn1a-Tg3-Cre, Rbp4-Cre and Ntsr1-Cre mice between the ages of 8 and 30 weeks of age with weights ranging from 19g-27g were used. Use of male and female animals was matched between tests of the two constructs.

Wild animals

The study did not involve wild animals.

Field-collected samples

The study did not involve in field-collected samples.