Simultaneous cortex-wide fluorescence Ca\textsuperscript{2+} imaging and whole-brain fMRI

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Achieving a comprehensive understanding of brain function requires multiple imaging modalities with complementary strengths. We present an approach for concurrent widefield optical and functional magnetic resonance imaging. By merging these modalities, we can simultaneously acquire whole-brain blood-oxygen-level-dependent (BOLD) and whole-cortex calcium-sensitive fluorescence measures of brain activity. In a transgenic murine model, we show that calcium predicts the BOLD signal, using a model that optimizes a gamma-variant transfer function. We find consistent predictions across the cortex, which are best at low frequency (0.009–0.08 Hz). Furthermore, we show that the relationship between modality connectivity strengths varies by region. Our approach links cell-type-specific optical measurements of activity to the most widely used method for assessing human brain function.

To study the organizational principles that govern brain function, a wide array of tools has been developed. Each tool has strengths that offer insight into specific aspects of brain function, but also experimental tradeoffs. In pursuit of linking different measures of activity, complementary techniques can be combined. We present a system for performing simultaneous mesoscopic calcium (Ca\textsuperscript{2+}) and functional magnetic resonance imaging (fMRI) in mice.

With genetically encoded or virally mediated expression of Ca\textsuperscript{2+} indicators, the concerted activity of cells across a large field of view (FOV) can be examined using widefield mesoscopic Ca\textsuperscript{2+} imaging\textsuperscript{7}. The resulting signal constitutes a cell-type-specific measurement of activity because Ca\textsuperscript{2+} sensors can be genetically targeted\textsuperscript{1}. During mesoscopic imaging, neuropil is the dominant signal source\textsuperscript{2}. However, Ca\textsuperscript{2+} imaging is limited to optically accessible tissue, and the expression of Ca\textsuperscript{2+} indicators necessitates invasive manipulation of the nervous system, in practice limiting its application to animal models. Nevertheless, the ability to label specific cell types, and the high spatial and temporal resolution of the signal, make this approach informative when studying brain function.

Functional magnetic resonance imaging (fMRI) provides a non-invasive measure of activity with whole-brain coverage in both humans and animals. However, fMRI has relatively low spatial and temporal resolution, and relies on an indirect and indiscriminate index of activity through a BOLD contrast mechanism\textsuperscript{1}. Despite the indirect and non-specific nature of the BOLD signal, there is a clear relationship between neural activity and changes in BOLD signal across species\textsuperscript{4,5}. Despite the complexity of the BOLD signal, the whole-brain coverage, intrinsic contrast and ability to perform measurements in both animals and humans make this approach a prominent tool in neuroscience. Therefore, a better understanding of the cellular origins of the BOLD signal stands to have a major impact on the interpretation of fMRI studies.

Despite the success of Ca\textsuperscript{2+} imaging and fMRI individually, technical challenges have prohibited application of these techniques together. We implement these methods simultaneously and describe the innovations that enable us to collect these data longitudinally in mice. We show that trial-to-trial variations in elicited response magnitude are correlated across modalities, which adds to the growing evidence for a common underlying neural source and demonstrates the high sensitivity of our method. We test a gamma-variate fit-based convolution model\textsuperscript{6} and show that one-third of spontaneous BOLD activity can be predicted from simultaneously recorded Ca\textsuperscript{2+} signal from excitatory neurons. We find that across the cortex, the predicted parameters are consistent and that a low-frequency bandwidth (0.009–0.08 Hz) results in greater correspondence between the Ca\textsuperscript{2+} and BOLD fMRI signals.

Next, we show that functional parcellation of the cortex on the basis of connectivity derived from spontaneous activity is consistent across modalities, which provides further validation of functional connectivity, as measured with fMRI\textsuperscript{6}. Finally, we show that the relationship between connectivity strengths derived from Ca\textsuperscript{2+} activity in excitatory neurons and BOLD signal depends on the function of a given brain region. We assert that this regional functional dependence provides evidence that BOLD connectivity arises from concerted neural activity across different cell populations. Together, these results support the notion that our approach relates cell-specific activity to the signals measured via BOLD activity.

Results

Simultaneous Ca\textsuperscript{2+} imaging and MRI. Widefield Ca\textsuperscript{2+} imaging is typically performed with a conventional microscope and
camera positioned directly above the animal. To enable multi-modal imaging, we designed the optical set-up to work within the confined space and high magnetic field of the MRI scanner (Fig. 1a). This included customized optics and radio frequency (RF) MRI hardware.

We performed Ca²⁺ imaging on transgenic mice expressing GCaMP6f in cortical excitatory neurons (Slc17a7-Cre/Camk2a-tTA/Ai93). Note that our approach should be suitable for any mouse line with a sufficiently bright fluorescent signal.

We introduce two surgical preparations, one for acute and one for chronic experiments. Both are optimized to reduce fMRI signal loss due to magnetic susceptibility effects without compromising fluorescent signal transmission. For acute preparations, we built a dental cement 'well' along the circumference of the clean and intact skull surface, which we filled with an MRI-compatible transparent gel and sealed with glass (Fig. 1b). This eliminated a-dental cement and glass interface that could introduce artifacts into the magnetic field. The gel allowed the skull to be optically exposed while minimizing MR susceptibility effects, because this eliminates...
an air–tissue interface. For chronic preparations, we performed the same steps, with the addition of thinning of the skull and the replacement of gel with transparent dental cement. For both preparations, we fixed the skull to a holder that dovetailed with a radiofrequency (RF) coil and the optical-imaging apparatus (Fig. 1c). The coil was saddle-shaped to avoid obstructing the optical FOV and to optimize MR sensitivity (Extended Data Fig. 1).

We performed optical imaging with a heavily modified telecentric lens. The primary modification was the replacement of all metal parts with non-conducting plastic (Extended Data Fig. 2). A key feature of the set-up was a coherent fiber-optic bundle that relayed the fluorescent signal from within the scanner to a camera housed in an adjacent room (Fig. 1d).

Our device is different from previous implementations combining MRI with fluorescence imaging. Typically, only single fibers have been used to measure fluorescence from one or more regions of interest (ROIs)\(^4,12–16\). Here, we obtained movies (FOV, 14.5 × 14.5 mm\(^2\); resolution, 25 × 25 μm\(^2\)) of Ca\(^{2+}\) fluorescence spanning most of the mouse cortex. However, in a related approach, a fiber array was introduced into the MR environment\(^16-18\). Although there were similarities, the optical coverage with our device was greater, and we implemented the technology using mice, not rats.

We secured our set-up within an 11.7-T preclinical magnet, but this design can be scaled for larger animals and bore sizes.

To assess the optical signal-to-noise ratio (SNR), we conducted three weekly dual-imaging sessions and an additional optical-only session outside of the scanner using commercially available equipment in mice that underwent chronic window preparations. Overall, we observed no gross differences in SNR. The stimulus-response magnitude observed with the dual-imaging system and with commercially available equipment showed no difference (Supplementary Fig. 1). Furthermore, there was no difference in stimulus-response magnitude between three weekly dual-imaging sessions following a chronic preparation with skull thinning. For the cyan wavelength (Ca\(^{2+}\)-sensitive contrast), imaging 2–3 weeks after surgery (with thinning) improved in-scanner SNR relative to that at 7 d after surgery (with thinning) (\(P = 0.0003\) and \(P = 0.0447\) respectively, \(t\) test) or at 2 weeks after surgery (with thinning), compared with an acute preparation (without thinning) (\(P = 0.0214\), \(t\) test). The SNR of data collected outside of the magnet using commercially available equipment (with thinning) provided a higher SNR than did data collected after an acute preparation (without thinning) (\(P = 0.0001\), \(t\) test), or data collected in-scanner at 1 (\(P = 0.0001\), \(t\) test), 2 (\(P = 0.0019\), \(t\) test) or 3 (\(P = 0.0001\), \(t\) test) weeks after a chronic preparation (with thinning). Notably, the variances in SNR at 1, 2 and 3 weeks after surgery (with thinning) collected in-scanner were lower than in data collected subsequently outside of the magnet using commercially available equipment (\(P = 0.0015\), \(P = 0.0001\) and \(P = 0.0000\) respectively, \(f\) test) (Supplementary Fig. 2).

### Multi-modal acquisition and image registration.

We recorded optical and fMR images (Fig. 2a) together with physiological data (heart rate, breath rate, arterial oxygen saturation and rectal temperature) (Supplementary Fig. 3). At the start of each fMRI volume acquisition (\(TR = 1,000\) ms, 1 Hz, 0.4 × 0.4 × 0.4 mm\(^3\)), a TTL (transistor–transistor logic) pulse from the scanner triggered the capture of 20 optical frames (20 Hz). We processed fluorescence and fMRI data using standard procedures, including motion correction, hemodynamic correction, bandpass filtering, drift correction and global signal regression (Extended Data Fig. 3).

We co-registered Ca\(^{2+}\) (two-dimensional (2D) surface) and fMR (three dimensional (3D) volume) images using the vascular anatomy on the surface of the cortex as reference landmarks (Fig. 2b). The vessels were visible in the optical images and in a time-of-flight (TOF) MR angiogram without the administration of an exogenous contrast agent. In addition, registration used an anatomical MR image with high in-plane resolution that matched the fMRI prescription (that is, they were of the same anatomy), an isotropic anatomical whole-brain MR image and a MR image of the brain tissue within the FOV of the MR angiogram (Fig. 2). We combined the latter image and the angiogram to obtain one MR projection (2D) that recapitulated the surface of the mouse brain (Fig. 2c and Extended Data Fig. 4). For each mouse, we registered all the MRI data together to create the ‘animal’s reference space’. From there, we registered the isotropic anatomical image to a common space and an atlas\(^17\).

Finally, we registered the Ca\(^{2+}\) image to the MR surface projection, thereby moving all the multi-modal data into the same space. We performed all image registration using BioImageSuite Web (www.bioimagesuite.org) using customized modules developed for this purpose.

### Evoked multi-modal Ca\(^{2+}\) and fMRI responses.

We demonstrated the sensitivity of our approach by imaging evoked responses upon unilateral hind-paw electrical stimulation (Fig. 3 and Extended Data Fig. 5)\(^14\). The stimulus ‘on’ duration (5 s) was shorter than is typical for MR experiments, to conform with optical-imaging literature\(^18-20\). With a generalized linear model, we identified responding regions for both modalities. We observed that responding regions across modalities did not completely overlap (Extended Data Fig. 6). This was likely a function of statistical threshold and physiological differences between contrast mechanisms. We would not have expected these measurements to be identical given the specificity of the Ca\(^{2+}\) signal to excitatory neurons and the non-specificity of fMRI. Furthermore, BOLD responses were maximal spatially downstream of the activation\(^14\). Finally, we collected fMRI contrast in 3D, whereas Ca\(^{2+}\) data were integrated across an uncertain depth\(^21\).

We quantified individual response amplitudes as the average signal within the responding regions and defined the time-to-peak (TTP) from 1 acquisition (10 min of data including 9 stimulii) from each of 6 mice (Ca\(^{2+}\), \(0.6 ± 0.03\) s; fMRI, \(4.7 ± 0.5\) s; mean ± s.d.; Supplementary Fig. 7). Previous work has demonstrated that changing stimulus parameters, such as strength or duration, modulates response magnitude\(^22\). Without changing the stimulus parameters, however, fluctuations still appear in both modalities. We observed that evoked response amplitudes to individual identical stimuli were correlated between modalities (Fisher’s \(z\)-transformed, Pearson’s correlation, \(z = 0.44\) and \(P < 0.003\); Fig. 3). This extends previous work by suggesting that such fluctuations are not measurement noise but are real variations in the response, and demonstrates that the quality of data from both modalities are sufficient to measure individual events.

### Transfer function between Ca\(^{2+}\) and BOLD signals.

We estimated a convolution kernel that, when convolved with Ca\(^{2+}\) signal, approximated BOLD activity, using a previously proposed approach\(^3\). This method optimized a three-parameter (amplitude, time of peak and width of peak) gamma-variate function, using minimized least-squares error, to approximate a hemodynamic response function (HRF)\(^3\). We estimated the goodness of fit using Fisher’s \(z\)-transformed Pearson’s correlation. We applied this approach using the average signal within the ROIs that respond to hind-paw stimulation. Across \(n = 6\) mice, we obtain \(z = 0.36 ± 0.20\) (mean ± s.d.) using unfiltered data (Extended Data Fig. 7). Fitting improved this value to \(z = 0.48 ± 0.19\) (Extended Data Fig. 7b) when data were filtered (0.04–0.1 Hz, MATLAB Butterworth), compared with unfiltered data (MATLAB \(t\)-test, \(P = 0.0128\))\(^3\). From this, we conclude that BOLD activity was coupled to excitatory neurons residing within functionally defined ROIs.

In the remaining sections, we present results obtained during spontaneous activity. We registered the imaging data from \(n = 6\) mice to a downsampled version of the Allen Atlas (Supplementary Fig. 8). Within each of \(n = 18\) ROIs, we applied the convolution

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Using filtered data (0.04–0.1 Hz), we found that across ROIs, scans and mice, the correlation between BOLD signals and Ca\textsuperscript{2+} signals after convolution with the optimized HRF was $z = 0.53 \pm 0.23$ (Fig. 4 and Extended Data Fig. 8). Further, we found that this relationship was consistent across the duration of each scan and across bandwidths (Extended Data Fig. 8)\textsuperscript{6,23–25}. We obtained the highest correlation using the lowest band (0.009–0.08 Hz), consistent with previous studies\textsuperscript{23,25}.

We examined the spatial distribution of the three predicted parameters (amplitude, time of peak and width of peak) (Fig. 4e). Overall, across the cortex, up to $30 \pm 9\%$ of the variance in BOLD activity was accounted for by excitatory cell activity during rest.

Functional parcellation of Ca\textsuperscript{2+} and fMRI data. We used data-driven parcellation\textsuperscript{26–29} with a multigraph $k$-way clustering algorithm to identify functional brain regions (parcels) to derive a functional atlas (parcellation) of the brain (Supplementary Fig. 9)\textsuperscript{30}. We applied this method using both Ca\textsuperscript{2+} and fMRI data in the left and right hemispheres of each mouse independently. This allowed for a within-animal estimate of reproducibility through computing cross-hemisphere similarity (Fig. 5a), quantified using the Dice coefficient. Parcellations showed strong cross-hemisphere similarity for both modalities (Ca\textsuperscript{2+}, 0.68 ± 0.05; BOLD, 0.56 ± 0.04; mean ± s.d., 10 parcels, $n = 6$ mice). Each mouse showed, relative to random parcel membership, significant cross-hemisphere similarity for both modalities ($t$ test; Ca\textsuperscript{2+} data for mouse 1–6: $P = \{0.0001, 0.0000, 0.0000, 0.0000, 0.0000, 0.0000\}$ and 0.0000; BOLD data for mouse 1–6: $P = \{0.0066, 0.0004, 0.0012, 0.0002, 0.0000$ and 0.0000; Fig. 5b).

We registered all 6 mice so that we could compare parcel overlap across animals ($n = 20$ parcels). For all mice, Dice coefficients were correlated with parcel size (Extended Data Fig. 9), accounting...
for >89% of the variance. Hence, smaller regions showed less overlap, independent of bandwidth or mouse. This indicates that smaller regions are more difficult to localize. This fact, combined with inherent differences between hemispheres and the challenge of cross-hemisphere registration, likely drives the variance in parcellation overlap (Fig. 5b).
Fig. 4 | Gamma-variate convolution model applied within Allen Atlas ROIs. a, Average signals within example Allen Atlas regions. Ca²⁺ signal before (green) and after (blue) applying gamma-variate convolution (⊗Γ) for 50-s windows from \( n = 3 \) mice. BOLD signal (orange) for the same windows, mice and atlas regions (left and middle). Average predicted HRF across all regions and data for each mouse (right). \( W_p \) is width of peak, \( A_p \) is amplitude of peak, and \( T_p \) is time of peak. Goodness of fit is assessed by Fisher’s \( z \)-transformed Pearson’s correlation between the Ca²⁺ data convolved with the predicted HRF (blue) and the BOLD signal (orange). b, Our downsampled version of the Allen Atlas. c, Experiment timeline. “Paw” indicates which 10-min runs included unilateral hind limb stimulation. “Rest” indicates 10-min runs where no stimulation was presented. “Scan” indicates the 10-min block where data were collected. d, Correlation strength of Ca²⁺ data convolved with the predicted HRF and the BOLD signal for \( n = 6 \) mice for \( n = 18 \) Allen Atlas ROIs (\( n = \) 9 scans, \( n = 11 \) 50-s windows per scan; that is, 99 data points per mouse). Boxplot terms: central line is the median, the minimum and maximum of the box extend to the 25th and 75th percentiles, whiskers extend to all data points; and outliers (data points beyond the 25th to 75th percentiles) are denoted by ‘+’. No correction for multiple comparisons was applied. We tested for a difference between all ROIs and a null distribution (BOLD time points were shuffled) using a two-sample \( t \) test, \( P = 0.0000 \). Effects of different frequency filters are considered (Extended Data Fig. 8). e, Maps of median estimated gamma-variant parameters: amplitude (left), time of peak (middle) and width (right).
Next, we compared functional parcellations between modalities (Fig. 5a) and found a strong intermodality topological similarity (Dice: 0.54 ± 0.15, mean ± s.d., 20 parcels, n = 6 mice). This relationship was significant relative to randomly assigned parcel membership for all mice (mouse 1–6, \( P = 0.0001, 0.0000, 0.0000, 0.0000, 0.0000 \) and 0.0000). No correction for multiple comparisons was applied. We tested for a difference between Dice coefficients across ROIs using a two-sample \( t \) test. For \( \text{Ca}^{2+} \) data (mouse 1–6), \( P = 0.0066, 0.0004, 0.0012, 0.0002, 0.0000 \) and 0.0000. No correction for multiple comparisons was applied. We tested for a difference between Dice coefficients across ROIs using a two-sample \( t \) test. For BOLD data (mouse 1–6), \( P = 0.0066, 0.0004, 0.0012, 0.0002, 0.0000 \) and 0.0000. No correction for multiple comparisons was applied. We tested for a difference between Dice coefficients across ROIs using a two-sample \( t \) test. For all boxplots, the central line is the median, the minimum and maximum of the box extend to the 25th and 75th percentiles, whiskers extend to all data points and outliers (data points beyond the 25th to 75th percentiles) are denoted by ‘+’.

The relationship between \( \text{Ca}^{2+} \) and BOLD connectivity strength depends on region. Parcellations based on either modality share a topological pattern (Fig. 6), including correspondence to known brain regions. To investigate further, we projected each parcellation.
onto the data obtained using the other modality. Then, from each parcellation and the Allen Atlas, we computed a connectivity matrix for each mouse (Fig. 6a). As a control, we include analyses of data collected from \( n = 2 \) deceased mice (Supplementary Fig. 10). Data from deceased mice do not show a topological or connectivity structure, indicating that the results from live mice reflect activity, not measurement noise.

\( k \)-means clustering maximizes differences in synchrony between parcels. Thus, when \( \text{Ca}^{2+} \) data, instead of BOLD data, are used to define parcels, voxels with higher BOLD synchrony can be assigned
to different parcels, when they would have been assigned to the same parcel if BOLD data were used to define the parcellation (or vice-versa). This point is emphasized by connectivity matrices from the Allen Atlas (Fig. 6a).

To quantify the relationship between Ca\(^{2+}\) and fMRI connectivity strengths, we computed the correlation between Ca\(^{2+}\) and fMRI connectivity between regions within (intra-) and between (inter-) hemispheres using the Allen Atlas (Fig. 6b) and Ca\(^{2+}\) or BOLD parcellations (Extended Data Fig. 10). We found that the relationship between Ca\(^{2+}\) and BOLD connectivity strength varied by region and intra- versus interhemisphere. For example, Ca\(^{2+}\) and BOLD connectivity strengths were correlated (synchronous, \(P=0.0014\), Fisher’s z-transformed Pearson’s correlation) between barrel field and intrahemispheric regions (Fig. 6c) but were anticorrelated between barrel field and interhemispheric regions (asynchronous, \(P=0.0056\)). A way to interpret these data is to hypothesize that increased interhemispheric inhibitory activity could be causing a simultaneous increase in BOLD and decrease in excitatory activity.

These results demonstrate that the Ca\(^{2+}\) and fMRI signals provided partially shared information, and add to growing evidence of a neural basis for the functional connectivity metrics obtained from fMRI. In addition, these results show that the relationship between Ca\(^{2+}\) and BOLD connectivity has a regional and functional dependence which could be cell-type specific.

**DISCUSSION**

Despite widespread use, the interpretation of fMRI is hindered by our understanding of the physiological events that give rise to the BOLD signal. In animal models, we can apply an array of tools across a range of spatiotemporal scales. The challenge lies in linking measurements across scales and species. It is only through combining different techniques that we can relate many findings.

Due to the complexity of the brain and the difficulty of controlling the factors that influence activity, serial experiments, even in the same subject, are insufficient to establish definitive links between modalities. With evoked responses, averaged events can be aligned across experiments. However, the vast majority of activity in the brain is spontaneous. Elucidating the information contained within spontaneous activity is of great interest and can only be studied without temporal averaging. Subtle differences in cellular activity and hemodynamic responses are evidence of true moment-to-moment changes in brain activity that are not observable in serial experiments. In this work, we provide further evidence of this by observing correlated trial-to-trial variations in Ca\(^{2+}\) and BOLD activations and correlated spontaneous activity.

Simultaneous multi-modal methods that measure complementary features of activity, including optical and fMR imaging, have been described. However, these implementations typically employ single fibers to elicit and/or measure activity, which cannot reveal the spatial information available from imaging approaches. To do further research in systems and network-level analyses, increased area coverage with better spatial resolution is essential.

How different cell types influence hemodynamic regulation, and ultimately BOLD, has been studied for decades. BOLD arises from a complex combination of events and is indiscriminate to cell type, whereas the Ca\(^{2+}\) signal is cell-type specific. Although we labeled only excitatory neurons here, a key strength of our approach is the ability to measure the relative contributions of different cell populations to the fMRI signal.

Using the same approach, we predict BOLD activity from Ca\(^{2+}\) data to a degree similar to that of previously predicted hemodynamic activity. We account for 30±9% of the variance in BOLD, in contrast to the previously predicted ~46% (ref. 4). Notably, both the hemodynamic and neural signals previously reported were measured optically within the same spatial domain. Our results show correspondence between the temporal patterns of two independent signals arising from spontaneous activity. We anticipate that different transfer functions will be observed for each cell population in future work.

A few research groups have established protocols for awake mouse fMRI\(^{38}\). Currently, none of the awake protocols described in the literature are compatible with the dual-imaging technology described here. Even light anesthesia can have profound effects on brain activity, but in the work presented here, the anesthetized model was sufficient for demonstrating correspondence between Ca\(^{2+}\) and fMRI signals and served as a proof of principle for this dual-modality imaging approach\(^{38}\). The device and procedures have been designed to be usable for imaging awake animals. To cover a large FOV with a high-NA objective would require an objective with a much larger diameter than would fit in our 11.7-T system. For low magnification (<1 used here) the objective needs to be extremely large to get a slightly higher NA. Furthermore, for mesoscopic Ca\(^{2+}\) imaging, a typical NA is <0.1. We use an NA of 0.07. Future work could investigate optimizing the design of our optical apparatus (through increasing the NA).

This simultaneous-imaging method will provide data that contribute to a firmer biological understanding of the cellular origins of the BOLD signal. The fMRI component of our approach provides a direct link from mouse to human studies, giving insights into the functional organizing principles of the brain.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at [https://doi.org/10.1038/s41592-020-00984-6](https://doi.org/10.1038/s41592-020-00984-6).

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**Methods**

**Acute surgical preparation.** All procedures were performed in accordance with the Yale Institutional Animal Care and Use Committee and are in agreement with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Animals were anesthetized with 5% isoflurane (1.5–2% during surgery). Fur was removed from the scalp and thigh (for MouseOx) using dilapidation cream (Nair). Lidocaine (0.5%, Henry Schein Animal Health V1NB-0024-6800) and marcarine and epinephrine (0.5%, Pfizer Injectables 00409175550) were used to numb the scalp prior to resection. Once the skull surface was clear of tissue, a dental cement (C&B Metabond, Parkell) ‘well’ was built around the circumference taking care not to obstruct the Ca2+ FOV. A fluorescent bead (Fluorescent green PE microspheres, UVM-S-1.00, 106–125 µm, Cospheric) was embedded within the right-anterior wall for motion correction. Dental cement was used to secure the outside edges of the well to a head plate (acrylonitrile butadiene styrene plastic, Luelbot TAZ-5 printer, with 0.35-mm nozzle) that cradled the sides of the skull and attached the head to the stereotactic burr. The head was doted with adhesive MRI coil and Ca2+-imaging hardware to minimize motion. The well was filled with an optically transparent agar substitute: 0.5% Phytagel (BioReagent, CAS.71010-52-1) with 0.5% MgSO4 in water, sealed with a glass coverslip (Carolina Biological Supply Company, no.630029) and secured with dental cement.

**Chronic surgical preparation.** Animals were anesthetized with 5% isoflurane (30% O2 and 70% medical air). Mice were head-fixed in a stereotactic frame (KOPF), and anesthesia was reduced to 2%. Paraldehyde was applied to the eyes, bupivacaine (0.1%) was injected under the scalp, a subcutaneous injection of meloxicam (2 mg per kg (body weight)) was given and fur was removed from the scalp using Nair. The head was washed 3 times using betadine and 70% ethanol. The skin and soft tissue overlying the skull were removed, and the upper portion of the neck muscle was removed from the occipital bone. Neo-Predil is applied to the skin, and isoflurane was reduced to 1.5%. The parietal and frontal plates of the skull were thinned with a 1.4-mm and 0.7-mm tip diameter hand-held drill (FST). The thinned bone was cleaned using a fine brush, and a small amount (less than one drop) of superglue was applied to the thinned surface (Loctite). When the glue dried, transparent dental cement C&B Metabond (Parkell) was applied, and the head post was attached. The head post was a double-dovetail plastic frame with a microscope slide that was hand-cut to match the size and shape of the mouse skull.

**Optical components for within-scanner imaging.** Components were composed of glass or plastic. Directly above the mouse, a prism (25 mm, uncoated, N-BK7, no. 32-336 RA-Prism, Edmund Optics) redirected the excitation and emission light by 90° so that they passed through the telecentric lens (MML-1-HR65DVI-5M, Melrotx). For MRI compatibility, we replaced the metal housing of the stock lens with plastic (Derlin Acelatin Resin and PEEK, McMaster-Carr). We further customized by replacing the beam-splitter with a dichroic filter (15 × 17 × 1 mm, 495-nm high-pass, T495lpr, lot no. 321390, Chroma). A custom port that collimated (12 mm diameter × 15 mm focal length, VIS-EXT, Inked, Plano-Convex, Edmund Optics) and redirected the excitation light by 90° into the lens was purpose-built out of Delrin and nylon (Kramer Scientific). The excitation light arrived via a 5 mm × 5 mm liquid light guide (10-10645, Lumencor) from the room neighboring the magnet where the LED light source (Lumencor SPECTRA X, Lumencor) was housed. Similarly, a custom 4.6-m-long 14.5 × 6.5 mm × 6.5 mm cross-section fiber optic bundle (NA 0.64, containing an array of multifibers (10-µm elements in a 6 × 6, 60 µm × 60 µm array, a total of ~2,000,000 fibers) (SCHOTT) transported the emission light to the same neighboring room where the Ca2+ imaging data were recorded using a sCMOS camera (512 × 512 pixels, pco.edge 4.2, PCO). An additional GFP-emission filter (ET525/50m, CHROMA) was placed between the fiber bundle and the camera converted by two blue extenders (subassemblies of a TwinCam LS Image splitter, Cairn Research).

**Optical components for out-of-scanner data acquisition.** Imaging was performed with a Zeiss Axiozoom V.16 coupled to a PlanNeofluar Z 1x, 0.25 NA objective with 56m working distance. Mice. Mice were housed on a 12-h light–dark cycle. Food and water were available ad libitum. Mice were sexed-mixed adults that were 6–8 weeks old and 25–30 g at the time of imaging. We report data from a first-generation TIGRE (genomic locus) mice (TITL2/GCaMP6f, TIGRE–Insulators–TRE2 promoter–Loc0P-Stop–Loc0P–GFP–calmodulin fusion protein 6 fast) crossbred to C57/B6J-tTA mice (calmodulin-dependent protein kinase 2 alpha 2 alpha) driven by Slc17a7 (or Vglut1)–IREs (internal ribosome entry site) 2-Cre promoter mice, all purchased from Jackson Labs (JAX stock nos. 024103, 003010 and 023517) and bred in-house. The resulting Slc17a7-Cre; Cmknk2a-tTA; A93 mice have GCaMP6f expressed in 100% of cortical excitatory neurons.

**Animal monitoring.** During data acquisition, mice were anesthetized with 0.5–1.25% isoflurane, adjusted to maintain a heart rate of 480–550 beats per minute. Mice freely breathe a mixture of O2 and medical air, adjusted to maintain an arterial O2 saturation of 94–98%. Heart and brain rate, arterial O2 saturation, and rectal temperature (Neoptix fiber) were continually monitored (MouseOx Plus Revision 1.5.25 from STARRLife Sciences) and recorded (Spick 7.07, Cambridge Electronic Design Limited) (Supplementary Fig. 3). Body temperature was maintained with a circulating water bath. During image acquisition, MRI, Ca2+-imaging and physiological-data recording were synchronized (Master-8 A.M.P.I., Spick2 Cambridge Electronic Design Limited). Hind-paw electrical stimulation was delivered at 1 mA, 5 Hz, in 5-s/5-s on/off cycles.

**Functional-imaging parameters.** Ca2+ data were recorded using CamVare V3.17 at an effective rate of 10 Hz. To enable frame-by-frame background correction, visual-evoked (EVC) and control (CTRL) time series were recorded using an echo-planar-imaging (EPI) sequence with a repetition time (TR) of 1 s, and an echo time (TE) of 9.1 ms. Data were collected at a 0.4 × 0.4 × 0.4 mm3 resolution, across 28 slices. Each run was 600 repetitions in length (10 min).

**Ray-casting algorithm for MR surface projection.** The algorithm used the 3D TOF MR angiogram to create a simulated 2D MR image (Extended Data Fig. 4). There were three steps. First, non-brain tissue was removed using signal-intensity thresholding. Second, the MR volume was rotated and translated using five degrees of freedom: three rotation angles of the axial plane of the MR volume relative to the optical imaging plane and two in-plane translations. Third, the MR data were projected along the translation perpendicular (after translation) to the optical imaging plane by shooting ‘rays’ from ‘above’. Each ray was followed until it ‘hit’ the MR brain–volume ‘surface’. For each pixel in the MR image, we computed the average signal of the first five voxels beneath the surface in the MR volume and multiplied this by the cosine between the ray and the brain surface normal to estimate the local image gradient for shading. The second and third steps were repeated iteratively. Following each iteration, the similarity of the blood vessels in the optical image and projected MR image were assessed and the five parameters estimated in the second step adjusted until convergence.

**Custom HRF from pilot data.** The HRF passed to the generalized line model (GLM) used in the analysis of evoked responses is derived from a mouse imaging during a pilot experiment using the same imaging, anesthesia and stimulation protocol as the present work. This mouse was an Ai93 (TITL2/GCaMP6f, CaMK2a-tTA, Emx1-1RES-cre animal purchased from Jackson Labs (JAX stock nos. 024103, 003010 and 005628) and bred in-house. The Ai93/CaMK2a-tTA/Emx1-Cre mice have GCaMP6f expression in excitatory neuronal cell populations just as the Ai93/CaMK2a-tTA/Slc17a7-Cre mice (reported in ‘Results’) do. The HRF data were processed and used as a GLM (AFNI) to estimate the local image gradient for shading. The algorithm used the 3D

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**NATURE METHODS**

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Data processing. Data were motion corrected (AFNI, 3dVolReg), masked to isolate brain tissue (MATLAB, roipoly), and spatially blurred within the brain-mask (MATLAB, smooth-gaussian, full-width-half-maximum (FWHM) = 0.8 mm²). Data were filtered using 5 frequency-band windows: (1) 0.009–0.08 Hz; (2) 0.01–0.1 Hz; (3) 0.01–0.2 Hz; (4) 0.04–0.1 Hz; (5) 0.02–0.2 Hz. (MATLAB, Butterworth). The global signal was regressed (MATLAB, detrend), and the linear trend removed (MATLAB, detrend). Overall, we found our results to be unaffected by frequency filtering. Data with frame-wise motion estimates >0.4 mm are excluded (voxel size 0.4 x 0.4 x 0.4 mm³). The majority of the data we collected contained subthreshold motion (75% of evoked activity recordings and 82% spontaneous activity recordings, n=28/38) (Supplementary Fig. 4). Parameters were in agreement with previous work.[1–3]

Structural MRI parameters. High-in-plane-resolution images of MRI FOV. We used a multi-spin-multi-echo (MSEME) imaging sequence. In 10 min and 40 s using a TR/TE of 25/0/20/20 ms, we obtained 28 slices (0.4 mm thick) with an in-plane resolution of 0.1 x 0.1 mm² (2 averages). The slice prescription of these images matched those of the fMRI images (that is, were of the same anatomy).

Isotropic 3D anatomy of the whole brain. Using a MSEME imaging sequence. In 5 min and 20 s using a TR/TE of 5500/15 ms, we obtained a 0.2 x 0.2 x 0.2 mm² (single average) image of the whole brain. This sequence was repeated five times during our imaging protocol interleaved with functional acquisitions. Interleaving structural and functional acquisitions allowed recovery of the Ca²⁺ signal and more-robust responses to stimulation during functional data acquisition with evoked responses. In post-processing, the five isotropic anatomical images are concatenated (MATLAB, hconcat), motion corrected (AFNI, 3dVolReg) and averaged (MATLAB, mean) to create one image.

MR angiogram. We used a fast-low-angle-shot (FLASH) time-of-flight (TOF) imaging sequence. In 18 min, using a TR/TE of 13/0/4 ms, we obtained a 0.05 x 0.05 x 0.05 mm³ 2.0 x 1.0 x 2.5 mm³ image of the blood vessels within the cortex. High-resolution anatomy of angiogram FOV. We used a FLASH sequence. In 7 min and 30 s, using a TR/TE of 61/7.5 ms, we obtained an image with a resolution of 0.13 x 0.08 x 0.05 mm³ and FOV of 2.0 x 1.0 x 2.5 mm³ capturing the anatomy within the same FOV as the MR angiogram.

Generalized linear model for Ca²⁺ and fMRI data. For Ca²⁺ data, from each mouse, we collected 40 min of evoked responses to hind-paw stimulation during four 10-min runs, which were interspersed between spontaneous activity recordings and structural imaging. The first minute of data was excluded due to persistent effects of photobleaching in the Ca²⁺ data. Preprocessed Ca²⁺ data contained evoked responses were realigned (MATLAB, tcorr) and motion corrected (AFNI, 3dVolReg) and averaged (MATLAB, mean) to create one image.

For functional MRI data, data containing evoked responses were fit using a GLM (AFNI, 3dDeconvolve). Drift and motion parameters as well as a custom HRF were included in the model.[1] A custom HRF was used because the BOLD response was not recapitulated well by a box-car function, but the Ca²⁺ response was. In the GLM we used to identify the ROIs responding to the stimulus, we convolved the HRF with the stimulus paradigm before fitting to the fMRI data. The response map was thresholded to correct for multiple comparisons (false discovery rate, q < 0.01) and a cluster size limit (>30 contiguous voxels) was applied.

Statistics. Tests for statistical significance were computed in MATLAB (R2017b/V.38 or R2018a/C39). Errors are shown as the s.d. or the s.e., unless otherwise specified. The number of subjects and measurements are given. Data were excluded when motion artifacts (measured in fMRI) are above a predetermined threshold of 0.4 mm frame-wise displacement. This threshold was determined a priori using pilot data. In this data set, 21/28 runs (10 min of data) with stimulation, and 31/38 runs at rest were included (Supplementary Fig. 4). We test for differences between groups using a two-sample t test (MATLAB, ttest2), unless otherwise specified. For correlations, P values were from a two-sided t test (obtained using MATLAB, corr) which tests the null hypothesis that a correlation does not exist. Exact P values were given. P values < 0.0001 are reported as P = 0.0000.

Gamma-variate convolution model. The following approach was modeled after the method described by Ma et al.[2]. The original BOLD signal f(t) was upsampled x10 (MATLAB, interp) to match the sampling rate of the Ca²⁺ signal g(t).

We assumed a linear relationship between the upsampled BOLD signal f(t) and the Ca²⁺ signal g(t) by a hemodynamic response function HRF(t): $f(t) = HRF(t) \otimes g(t)$, where $\otimes$ denotes convolution and $HRF(t)$ is defined as a gamma function of the form

$$HRF(t) = A \times \frac{t^n}{\beta^{n+1} \Gamma(n+1)} e^{-t/\beta},$$

where $\alpha = \frac{t}{\beta}$ and $\beta = W^T$. $T$ represents the delay to peak, and $W$ represents the width of the HRF. The MATLAB optimization function fmincon was used to estimate the parameters $A$, $T$, and $W$, and the loss function was defined to be the L2 distance between the estimated $f_{HRF}(t)$ and the observed f(t). We consider 50-s windows of data, excluding the first 50 s of each 10-min experiment.

Ca²⁺ and fMRI parcellation with multigraph k-way clustering. The cellular elements (for example, dendrites, axons or cell bodies) that give rise to the mesoscopic Ca²⁺ signal are unknown, which makes it impossible to determine the exact depth of the signal source. For our purposes, we had to choose a reasonable depth in order to compare 3D fMRI to 2D mesoscopic Ca²⁺ data. The source of the mesoscopic Ca²⁺ signal is most likely neuropil (dendrites) and dominated by superficial cortical layers 1–5 (refs. 14, 15). On the basis of this information, we imposed a depth threshold on the fMRI data, which corresponded approximately to the fMRI data by back-projecting the Ca²⁺ FOV (Fig. 2c) using Bioimage Suite (BIS) to a fixed depth of 1.2 mm (or 3 fMRI voxels).

The parcellation of the fMRI data was computed in 3D within this mask. The parcels were then projected to the surface to compare with the Ca²⁺ data. We defined the original data set (either Ca²⁺ or fMRI) as $\{f_i\}_{i=1}^N$, where $k$ can be the index for different runs from one animal or the index for different animals. Each $P_i$ is organized as a 2D matrix $P_i = [f_{i1}, \ldots, f_{ik}]$, where each column is a time course indexed by $i$ and $N$ is the total number of pixels (2D, Ca²⁺ data) or voxels (3D, fMRI data).

To apply the clustering algorithm, we construct a graph $G_j$ for each set of data in $P_i$. The graph consists of vertices and edges. Vertices are the N pixels or voxels and edges are the connections between each pair of vertices. Edges are characterized by their strength, which is quantified by measuring the similarity between the time courses of pairs of vertices. Accordingly, we calculate a matrix of weights $W_j$ of size $N \times N$ for a given $P_i$ and each entry $w_{ij}$ is defined by

$$w_{ij} = \exp \left(-\frac{d_{ij}^2}{2\sigma^2}\right).$$

Here, we define $d_{ij}^2 = 2 \times (1 - r_{ij})$, where $r_{ij}$ is the Pearson correlation between the time course of pixel/voxel $i$ and pixel/voxel $j$.

The optimization and computation of the clustering algorithm was performed in the spectral domain. In other words, given a $W_j$, we compute the first $m$ eigenvectors of $W_j$, denoted as $X_j = [x_{1j}, \ldots, x_{mj}]$, $X_j$ is of size $N \times m$, and each column is an eigenvector. The multigraph k-way clustering algorithm is then to solve the following optimization:

$$\min \left\{ \Phi(Y, \{R_i\}) \right\} = \sum_{k} Y_k \cdot X_k^T X_k$$

subject to $Y_j \in \{0, 1\}^N$, $Y_{1j} = 1$, and $R_k^T R_k = I_m$ where $Y_{j}$ is the m-ROI parcellation of the brain on the basis the complete sets of data from all $K$ runs, $Y_{1j}$ is a single column vector of size $N$ and $I_m$ is the m x m identity matrix. The matrix $R$ is a generalized rotation matrix in high-dimensional space. The optimization was solved iteratively. For more details, refer to Shen et al.[16].

After $Y_j$ was solved, we converted $Y_j$ to a one-dimensional (1D) label. Each row of $Y_j$ corresponds to one pixel or voxel. By definition, each row of $Y_j$ has one out of m entries equal to 1, and all other $m - 1$ entries are 0. Thus, the label for each pixel or voxel i is the column index where the ith row of $Y_j$ equals one. Finally, the 1D label is mapped to the original 2D/3D space for visualization and further analysis.

Calculation of the Dice coefficient. Given two data sets A and B, the Dice coefficient Dice(A, B) is calculated as Dice(A, B) = 2 x |A \cap B| / (|A| + |B|), where || denotes the cardinality of the set. When comparing the overlap of two parcellations, ParcA and ParcB, they are required to be in the same space. Thus, we first found the best-matched ROIi in ParcB for a given ROIi in ParcA, and then computed the weighted sum of the Dice coefficients as

$$\text{Overlap}(\text{ParcA}, \text{ParcB}) = \sum_i \frac{\text{ROI}_i(\text{ParcA}) \cap \text{ROI}_i(\text{ParcB})}{\text{ROI}_i(\text{ParcA})} \sum_j \text{ROI}_j(\text{ParcB}).$$

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

Data availability
The raw MR and optical-imaging data generated during the current study are available from the corresponding author upon reasonable request. Data are not available in a public repository at the time of this publication due to ongoing work
by the authors on these data set. The Allen Brain Atlas was downloaded from http://www.brain-map.org.

**Code availability**

Custom MATLAB code for fMRI preprocessing and MR and Ca\(^{2+}\) data post-processing (parcellation and computation of connectivity matrices) is available from the corresponding author upon reasonable request. For analyses of Ca\(^{2+}\) data, refer to: https://github.com/bioimagesuiteweb/bisweb/tree/calcium. The analysis tools for multi-modal data registration and analysis are available BioImageSuite Web at www.bioimagesuite.org.

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**Author contributions**

All authors contributed to the overall study design. E.M.R.L. and X.G. designed and constructed the imaging apparatus and collected the data. E.M.R.L. and X.G. analyzed the data. E.M.R.L., X.G., X.S., D.S. and X.P. contributed code for the analysis of the data. P.H., EMRL, and XG designed the surgical protocol for longitudinal dual-imaging experiments. PH conducted these surgeries. EMRL, X.G., F.H., J.A.C., M.J.H., M.C.C. and R.T.C. wrote the manuscript. M.C.C. and R.T.C. supervised the project.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Extended Data Fig. 1 | Assembly of MR saddle coil, mouse head-plate, and Ca\textsuperscript{2+} imaging optical apparatus. Custom saddle coil and imaging apparatus design.  

**a.** Removable saddle coil, with case that protects hardware.  

**b.** Coil in place on dual imaging sled. Coil (white) is mounted on a support system (blue) which is fixed to the sled (semi-transparent blue). A support system for the mouse (orange) is attached to the sled to which the mouse head plate (red) attaches.  

**c.** Assembled dual imaging apparatus. The telecentric lens (for Ca\textsuperscript{2+} imaging) is secured above the mouse and saddle coil. The position of the telecentric lens (and housing) can be adjusted (yellow) along the magnet B\textsubscript{0} axis to focus the Ca\textsuperscript{2+} image.
Extended Data Fig. 2 | A cross section of the optical apparatus. A diagram of the light path overlaid on a cross section of our optical apparatus. The light enters the system via a flexible liquid light guide. At the base of the telecentric lens, the light is bent by 90° degrees to enter the telecentric lens. Upon entering the telecentric lens, the excitation light reflects off of the dichroic mirror and is redirected along the length of the telecentric lens and into the prism at the end of the apparatus. The prism redirects the excitation light onto the mouse cortex. The emission light is similarly re-directed by the prism along the length of the telecentric lens (this time traveling in the opposite direction) and passes through the dichroic mirror. The fiber bundle array is mounted onto the end of the telecentric lens and transmits the light to the room neighboring the magnet where the camera is housed. To focus the Ca²⁺ image, the fiber bundle moves relative to the telecentric lens (red arrows).
Extended Data Fig. 3 | Videos of Ca$^{2+}$ data pre- and post-image processing. Representative frames from example videos (Supplementary Video 1 & 2). 

**a.** Raw (unprocessed) fluorescence signal (cyan wavelength). A fluorescent bead placed within the dental cement at the right anterior edge of the surgical preparation is indicated (white arrow). The bead is used for right and left identification and motion correction. **b.** Data from **a** after processing. **c.** Estimated motion parameters based on position of fluorescent bead.
Extended Data Fig. 4 | Ray-casting algorithm to create the TOF MR angiogram-projected surface image for multi-modal image registration. 

a. Three example views of the raw 3D MR angiogram data. Blood vessels have high MR signal intensity. 

b. Example of a maximum intensity projection (MIP) image (left) and a schematic of our ray-casting approach (right). The MIP is generated following masking which removes signal from anatomy outside of the brain. To show the curvature of the brain surface, and to isolate the blood vessels on the surface of the brain, we use the ray-casting algorithm. We project the MR data along the axis perpendicular to the optical imaging plane. Each pixel is shaded based on brain curvature. 

c. The resulting 2D projection of the MR image.
Extended Data Fig. 5 | Average responses to unilateral hind-paw stimulation. a, Average responses to nine stimuli across N = 6 mice. Stimulus onset is denoted by black triangles. Ca\textsuperscript{2+} data (top) and the corresponding fMRI data (bottom) are plotted. The fMRI signal is normalized to the mean. The standard deviation within the responding ROI is shown as shading. b, The average normalized (to peak amplitude), stimulus response across N = 6 mice, n = 9 stimuli each. This shows the different temporal dynamics of these two modalities. The fMRI signal is delayed, relative to stimulus presentation and the Ca\textsuperscript{2+} signal. c) A zoomed in view of the Ca\textsuperscript{2+} and BOLD signals. Since the Ca\textsuperscript{2+} signal is collected at a relatively high temporal resolution (10 Hz), it appears in to be noisy. By zooming in the fast kinetics of these data are shown. No filtering of the Ca\textsuperscript{2+} signal has been applied.
Extended Data Fig. 6 | Localization of Ca$^{2+}$ and fMRI responses to stimuli. a, A surface projection of the down-sampled Allen Atlas overlaid on the optical data of an example mouse. The ROI expected to respond to the presented unilateral hind limb stimuli is indicated with a dotted line. b, Responding ROIs from all mice from both modalities normalized to the maximum response amplitude. Calculated as follows if we had two instead of six mice: if voxel $(i,j)$ for mouse #1 on average showed a 40% response relative to the maximum responding voxel for that mouse, and voxel $(i,j)$ for mouse #2 on average showed a 60% response relative to the maximum responding voxel for mouse #2, then voxel $(i,j)$ would be color-coded to 50%. The expected responding ROI from the Allen Atlas is shown as a dotted line. c, An example responding ROI from one mouse overlaid on the optical data. d, The same example responding ROIs (from the same mouse) overlaid on the projected MRI data.
Extended Data Fig. 7 | Gamma-variant convolution model applied within responding ROIs identified by GLM. a, Three example 50-second windows from \( N = 3 \) mice (left and middle panels). \( \text{Ca}^{2+} \) signal, averaged within the responding ROI, before (green) and after (blue) applying the gamma-variant convolution. BOLD signal (orange), averaged within the responding ROI. The average predicted hemodynamic response function (HRF) from these three examples (right panel). Goodness of fit assessed by correlating (Fisher’s Z transformed Pearson’s correlation) the \( \text{Ca}^{2+} \) signal convolved with the predicted HRF (blue) and the BOLD signal (orange). b, The correlations for \( N = 6 \) mice (\( n = 4 \) session, \( n = 11 \) windows, that is 44 data points per mouse) using filtered (0.04-0.1 Hz) (left) or unfiltered (middle) data. Each 50-second window contains the presentation of one unilateral hind-paw stimulus. The \( \text{Ca}^{2+} \) and BOLD responding ROIs are not fixed across mice, as illustrated (right) [reproduced from Extended Data Fig. 6]. For the boxplots, the central line is the median, the minima and maxima of the box extends to the 25th and 75th percentiles, whiskers extend to all data points, and outliers (data points beyond the 25th to 75th percentiles) are denoted by ‘+’.
Extended Data Fig. 8 | Convolution model applied within Allen Atlas ROIs is not affected by window, frequency band, scan number or mouse.
Correlation between Ca\textsuperscript{2+} and BOLD signals. **a**, Correlation strengths compared across nine scans spanning the duration of our acquisitions. Scans where no stimulus are presented (grey), and during unilateral hind-paw stimulation (green), are different from the null (BOLD time points scrambled). **b**, Correlation strengths compared across mice. **c**, Correlation strengths compared between windows. **d**, Correlation strengths compared within different frequency filters. All show the same relationship to the null using a two-samples t-test. For boxplots, the central line is the median, the minima and maxima of the box extends to the 25\textsuperscript{th} and 75\textsuperscript{th} percentiles, whiskers extend to all data points, and outliers (data points beyond the 25\textsuperscript{th} to 75\textsuperscript{th} percentiles) are denoted by ‘+’. No correction for multiple comparisons was applied.
Extended Data Fig. 9 | Parcellation results are independent of frequency filter across mice and parcel, variance is caused by parcel size. **a**, Variance across parcels for each mouse for $n = 5$ frequency filters. **b**, Variance due to parcel for each filter for $N = 6$ mice. Neither frequency filter, nor mouse, captures the variance in the Dice coefficient observed. **c**, Variance is highly correlated with parcel size. **d**, Variance across frequency filters for $N = 6$ mice. For the boxplots, the central line is the median, the minima and maxima of the box extends to the 25th and 75th percentiles, whiskers extend to all data points, and outliers (data points beyond the 25th to 75th percentiles) are denoted by ‘+’.
Extended Data Fig. 10 | Inter- vs. intra-hemisphere connectivity strength patterns between Ca2+ and BOLD vary regionally and by brain functional area. a, and b, are reproduced for reference from Fig. 6. c, and d, are equivalent plots to b) showing the same information (inter-, purple, and intra-, grey, hemisphere regional connectivity strengths) for the three parcellations shown in a). b) shows results from the Allen atlas, c) shows results from the Ca2+ parcellation, and d) shows results from the BOLD parcellation. For the boxplots, the central line is the median, the minima and maxima of the box extends to the 25th to 75th percentiles, whiskers extend to all data points, and outliers (data points beyond the 25th to 75th percentiles) are denoted by ‘+’. 
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

| The magnetic resonance imaging (MRI) data were collected on a Bruker 11.7T magnet using ParaVision 6.0.1 software. Rectal temperature was measured using a Neoptix ReFlex fiber optic cable. Physiological measurements [heart rate, breath rate, and arterial oxygen saturation] were recorded using MouseOx Plus Revision 1.5.25. Temperature, and physiological measurements were synchronized with MRI and calcium fluorescence recordings using Spike2 7.07. Calcium fluorescence data were recorded using CamWare V3.17. Illumination was controlled by LE 7Ch Controller from lumencor. For optical data collected outside of the scanner we used a Zeiss Axiozoom V.16. |

Data analysis

| MRI data were processed using MATLAB R2018aV 39, and AFNI (Analysis of Functional Neuroimages) Version 16.0.00. Calcium data were processed using MATLAB R2017bV 38 and ImageJ FijI 1.8.0 64bit. Multi-modal image registration was performed using BioimageSuite Web [www.bioimagettesuite.org]. Final results and significance testing were computed using MATLAB R2018aV 39. |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data generated during the current study are available from the corresponding author upon request. The Allen Brain Atlas was downloaded from http://www.brain-map.org
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
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Life sciences study design

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

Sample size
- Sample size was not determined a priori. Given the nature of the present work, the demonstration of a novel simultaneous multi-modal imaging technique, no group-wise comparisons are made. The relationship of interest was between the simultaneously recorded calcium fluorescence and BOLD signals. With N=6 mice we observed significant correspondences between signals within individual animals and across the group. We assert that for the purposes of the present work the sample size is sufficient. N=6 was arbitrary.

Data exclusions
- Data were excluded if motion artifacts were above a predetermined threshold of 0.4mm frame-wise displacement. This threshold was determined a priori using pilot data. 21/26 runs (10 minutes of data) with stimulation, and 31/36 runs at rest were included. These data are summarized in Supplementary Figure 6. The first stimulus response (for all runs, which include 10 stimuli) was excluded due to photobleaching artifacts in the optical data.

Replication
- N=6 for chronic and N=6 for acute experiments. The present work examines the relationship between the simultaneously recorded calcium fluorescence and BOLD signals. Thus, each animal within our study is a replication. No attempts at the experiment failed.

Randomization
- Randomization was not relevant to this study because there was one experimental group being investigated.

Blinding
- Blinding was not relevant to this study because there was one experimental group being investigated.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

- n/a
- Involved in the study
- Antibodies
- Eukaryotic cell lines
- Palaeontology and archaeology
- Animals and other organisms
- Human research participants
- Clinical data
- Dual use research of concern

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
- Mice were housed on a 12-hour light/dark cycle. Food and water were available ad libitum. We used A93 mice [or TIt2L-GCaMP6f, TIGRE - Insulators - TRE2 promoter - LoxPStop1LoxP - GFP CalModulin fusion Protein 6 fast] crossed to CaMK2a-tTA mice (CalModulin dependent protein Kinase 2 alpha) driven by SLC17A7 (or Vglut1) - IRES (internal ribosome entry site) 2 - Cre promoter mice, all purchased from Jackson Labs (JAX stock numbers: 024103, 003010 and 023517) and bred in-house. The resulting SLC17A7-Cre; CamK2a-tTA; A93 mice have GCaMP6f expressed in cortical excitatory neurons. The N=6 mice reported in Results were mixed gender adults (6-8 weeks old at the time of imaging).

Wild animals
- This study did not involve wild animals.

Field-collected samples
- This study did not involve samples collected from the field.

Ethics oversight
- All procedures were performed in accordance with the Yale Institutional Animal Care and Use Committee and are in agreement with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Note that full information on the approval of the study protocol must also be provided in the manuscript.
## Magnetic resonance imaging

### Experimental design

**Design type**
Resting-state and event related (sensory response to unilateral hind-paw electrical stimulation).

**Design specifications**
For each mouse (N=6) we conducted one imaging session. During each acquisition, we collected functional and structural data. Functional data was collected in 30 minute runs which were interleaved with the acquisition of structural data (ref. below to acquisition sequence and imaging parameters). In total, we collected 90 minutes of functional data (5 resting-state runs, and 4 hind-paw stimulation runs). Five to 18 minutes of structural data was collected between each functional run (depending on the structural sequence being interleaved). During the hind-paw stimulation functional runs, hind-paw stimulation was delivered at 1mA, 5Hz, in 5/55 seconds ON/OFF cycles (10 stimuli were delivered during each functional run with hind-paw stimulation). The total experiment time was approximately 2.5 hours.

**Behavioral performance measures**
Behavioral performance was not evaluated in the present study. Evoked responses to sensory stimulation (hind-paw electrical stimulation) were assessed.

### Acquisition

**Imaging type(s)**
Functional and structural

**Field strength**
11.7T

**Sequence & imaging parameters**
Functional magnetic resonance imaging (fMRI) data were collected using a gradient-echo, echo-planar-imaging (EPI) sequence with a repetition time (TR) of 1 second, and echo time (TE) of 9ms. EPI data were collected at 0.4x0.4x0.4mm3 resolution, across 28 slices. The 28 slices cover from the middle of the olfactory bulb to the middle of the cerebellum, providing effective whole-brain coverage. Each EPI acquisition was 600 repetitions in length (10 minutes). From each mouse (N=6 in results), we collected five resting-state acquisitions and four hind-paw stimulation acquisitions (90 minutes of fMRI data). In addition, we collected a high in-plane resolution image of the fMRI field of view (FOV) using a multi-spin-multi-echo (MSME) imaging sequence. In 10 minutes, 40 seconds, using a TR/TE of 2500/20ms, we obtained 28 slices (0.4mm thick) with an in-plane resolution of 0.1x0.1mm2 (two averages). We also collected an isotropic 3D anatomy image of the whole brain using a MSME imaging sequence. In 5 minutes, 20 seconds, using a TR/TE of 5500/15ms, we obtain a 0.2x0.2x0.2mm3 (single average) image of the whole brain (including the entire olfactory bulb and cerebellum which are needed for registration purposes). This sequence was repeated throughout our imaging protocol interleaved with functional acquisitions. Interleaving allows recovery of the calcium signal and more robust responses to stimulation. We also acquire a time-of-flight angiogram using a fast-low-angle-shot (FLASH) imaging sequence. In 18 minutes, using a TR/TE of 130/4ms, we obtain a 0.05x0.05x0.05mm3 2.0x1.0x2.5cm3 image of the blood vessels within the cortex. Finally, we acquire a high resolution anatomy image of the angiogram FOV. Also using a FLASH sequence. In 7 minutes, 30 seconds, using a TR/TE of 61/7.5ms, we obtain an image with resolution 0.1x0.08x0.08mm3 and FOV 2.0x1.0x2.5cm3 capturing the anatomy within the same FOV as the fMRI angiogram.

**Area of acquisition**
Depending on the purpose of each scan, we collected data from the whole brain or part of the brain. Please refer to the description of the sequences and imaging parameters provided above.

**Diffusion MRI**
- **Used**: Not used

### Preprocessing

**Preprocessing software**
Functional MRI data were preprocessed using MATLAB R2018a, and AFNI (Analysis of Functional Neuroimages) 16.0.00. Multi-modal image registration was performed using BioimageSuite Web (www.bioimagesuite.org). Modules within which were custom developed in-house for this purpose and are freely available on-line.

**Normalization**
To ease comparison with fluorescence imaging data and between animal comparisons, response amplitudes (to hindlimb electrical stimulation) were normalized to the peak amplitude or to the mean signal amplitude for display purposes. Only the time courses of the mean signal within the responding ROI was normalized.

**Normalization template**
No normalization template was needed. All data analysis was conducted in the subject’s native or individual space.

**Noise and artifact removal**
Data were motion corrected (AFNI, Analysis of Functional Neuroimages, 3dVolReg), masked to isolate brain tissue (MATLAB, roiopoly), and spatially blurred within the brain-mask (MATLAB, smooth-gaussian, full-width-half-maximum, FWHM, 0.8mm). Data were filtered (0.01-0.2Hz, MATLAB, butterworth), the global signal regressed (MATLAB, detrend), and the linear trend removed (MATLAB, detrend). Data with frame-wise motion estimates >0.4mm were excluded (voxel size 0.4x0.4x0.4mm3). The majority of the data we collect contains sub-threshold motion (75% of evoked activity recordings and 82% spontaneous activity recordings, N=28/38).

**Volume censoring**
No volume censoring was applied. Runs (10 minutes each) were excluded if frame-to-frame motion estimates exceeded our threshold (0.4mm). Enough runs per animal were collected to conduct our analyses using multiple runs from each animal meeting this criterion.
### Model type and settings

Data containing evoked responses are fit using a generalized linear model (GLM). (AFNI, 3dDeconvolve). Drift and motion parameters as well as a custom hemodynamic response function (HRF) were included in the model. The HRF was derived from the average evoked response recorded from a mouse imaged during a pilot experiment (not one of the N=6 reported in Results). The response map was thresholded to correct for multiple comparisons (false discovery rate q<0.01 and p<0.03) and a cluster size limit (>30 contiguous voxels) was applied. The data used to generate the customized HRF were processed following the same steps as described above for the data included in the Results (motion correction, blurring, global signal regression, and low-pass filtering), and fit using a GLM (AFNI, 3dDeconvolve) which included drift, and motion parameters. Likewise, the same threshold was applied to the resulting evoked response map to correct for multiple comparisons (false discovery rate q<0.01 and p<0.03) and a cluster size limit (>30 contiguous voxels). The single difference between the analysis of the data reported in Results and the data from this animal is that a box-car time-locked to stimulation onset used in place of an HRF. Signal from responding voxels in the contralateral cortex were averaged. We isolate the first four responses to hind-paw stimulation because we find that in a typical experiment the first four responses are the most robust. The averaged, smoothed, and normalized (MATLAB) trace is used as our HRF for the analyses of the N=6 animals presented in Results.

### Effect(s) tested

We tested the correlation between the magnitudes of simultaneously recorded calcium fluorescence changes and BOLD signal changes in response to hind-paw electrical stimulation. To test for a relationship, we used Pearson’s correlation (MATLAB corr) and report the r and p-values. Using simultaneous multi-modal recordings of spontaneous activity, we test the within modality (cross-hemisphere) and between modality correspondence of our k-mean clustering parcellation results. To test for significance, we compute the Dice coefficient (cross-hemisphere or cross-modality) and test for a difference between our results and a null distribution (random parcel membership assignment). Computation of the Dice coefficient is described within the manuscript. To test for a difference between the distributions we use ANOVA (MATLAB anova1) and report p-values. Finally, we measure the correspondence between modalities in connectivity strengths (ie. do the same regions which show high/low synchrony in calcium fluorescence data also show high/low synchrony in BOLD data). To test for correspondence we use Pearson correlation (MATLAB corr) and report the r and p-values.

#### Specify type of analysis:

- [ ] Whole brain
- [ ] ROI-based
- [x] Both

#### Anatomical location(s):

The responding ROI to the hind-paw stimulation was determined using a generalized linear model.

### Statistic type for inference

(See [Eklund et al. 2016](#)) Functional parcellation was based on a voxel-wise analysis. Regions of interest (for hind paw responses to electrical stimulation) were determined using a generalized linear model (described in detail above in model type and settings) consistent with Eklund et al. 2016.

### Correction

To correct for multiple comparisons, when identifying the responding ROI, we applied a false discovery rate threshold (q<0.01 and p<0.03) and a cluster size limit (>30 contiguous voxels).

### Models & analysis

#### n/a Involved in the study

- [x] Functional and/or effective connectivity
- [ ] Graph analysis
- [x] Multivariate modeling or predictive analysis

#### Functional and/or effective connectivity

Pearson correlation (MATLAB corr).