Supplementary Figure 1
Confirmation of anesthesia depth by electrocorticogram recordings. (a) ECoG recorded during awake (upper) and anesthetized (lower) periods in the same animal. (b) Power spectrum of ECoG during awake (black) and anesthetized (red) periods.
Detection of motion and comparison to ECoG recordings. (a) Infrared images of head-fixed rats during sessile (left) and awake periods (right). A semi-automated procedure tracked movement inside user-drawn whisker and forepaw ROIs (see Supplementary Methods). (b) Power in the 3-15 Hz band of the ECoG recording preceding and following motion of the animal’s paws (upper panel) or whiskers (lower panel). Negative values on the x-axis correspond to time points prior to the start of motion, and zero corresponds to all time points during motion, and positive values correspond to time points after the end of motion. Y-axis values indicate mean power in the 3-15 Hz band of the ECoG, averaged across all time points with the given latency to the start or stop of motion, binned at 1 second resolution. Error bars indicate standard error of the mean. Note the sharp drop in power 5-10 seconds before a motion, and the asymptotic return to baseline power level 10-15 seconds after; this frequency band of the ECoG is suppressed shortly before motion begins, and gradually returns to normal after motion ceases.
False positives produced by the AP detection algorithm. False positives (black line) occurred when the algorithm inferred an AP without a corresponding electrically confirmed AP (not already assigned to another inferred AP) with time difference $\leq \Delta t$. The red line shows the rate of action potentials inferred by the algorithm not within $\Delta t$ of any electrically confirmed action potential.
Supplementary Figure 4

A selection of successfully detected and assigned fluorescence transients arising from single APs, sorted by height (7 animals). All transients were confirmed by cell-attached recording, but detected based on fluorescence signals alone and classified as single APs (see Supplementary Methods). Note the success of the algorithm despite the variation in transient height and shape caused by noise in the fluorescence measurements.
Supplementary Figure 5

Measurement of the change in SR101 fluorescence from an astrocytic process as function of displacement along the z-axis. Average images (upper) were captured at 3 μm increments through an astrocytic process in an anesthetized rat. Fluorescence (lower) from a region of interest containing the astrocytic process (white rectangle in center image) increased sharply when the process was in focus, but dropped to baseline levels when the focal plane was shifted up or down by 3 μm along the z-axis. More positive values correspond to excitation focus positions closer to the pia matter.
Supplementary Figure 6

Displacement correction demonstrated on fluorescence transients. (a) Simultaneous fluorescence and electrical recording of AP firing activity under anesthesia. (b) Displacement of brain tissue relative to the objective lens occurring in an awake animal, and detected via the Lucas Kanade algorithm (see Supplementary Methods section ‘Image registration’). Displacement is shown for the x- (black) and y-directions (cyan). (c) Fluorescence transients from the period shown in ‘a’ after pixel positions have been altered according to the displacement in part ‘b,’ resulting in a distortion of optical signals. (d) Fluorescence transients from the period shown in ‘a,’ after pixel positions have been displaced as in ‘c,’ displacement has been estimated using the Lucas Kanade algorithm (see Supplementary Methods section ‘Image registration’), and pixels have been restored to their proper positions. Note the similarity with the original fluorescence signals in ‘a’ ($r = 0.96$).
Confirmation of low firing rates from distributions of line scan fluorescence values. Distributions of fluorescence increase above baseline (%ΔF/F0) of 1.5 ms line scans taken from neurons are shown for frames with putative spikes (red), frames with spikes confirmed by cell-attached recordings (blue), frames putatively without spikes (green), and frames in which silence has been confirmed by cell-attached recordings (black). Note the similarity between putative and confirmed distributions for spiking and silence, and the increase in mean and variance during spiking.
Supplementary Figure 8

Distance versus absolute firing rate difference and pairwise correlation.
(a) Distance between neurons versus absolute firing rates
difference during awake (black) and anesthetized (red) periods.
(b) Distance between neurons versus pairwise correlation
during awake (black) and anesthetized (red) periods. Error bars indicate standard error of the mean.
Supplementary figure 9

Sparseness of neurons’ AP firing as a function of firing rate, for neurons recorded in the awake (black) and anesthetized (red) states, and for a Poisson process. Sparseness decreases with firing rate in the observed neurons, but not as quickly as for a Poisson process (green line), indicating that burst firing is present.
Supplementary Movie 1

Simultaneous infrared videography (left) and two-photon imaging (right) in an awake head-fixed rat. Two-photon imaging has been motion-corrected, so that neurons are stationary while the image boundaries fluctuate. Note the successful stabilization both when the animal is moving, and when it is not.
Population imaging of ongoing neuronal activity in the visual cortex of awake rats

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Abbreviations: AP, action potential; ECoG, electrocorticogram; OGB-1, Oregon Green 488 BAPTA-1; AM, Acetoxymethyl; ROI, region of interest; AW, awake; SI, sessile; AN, anesthesia.
SUPPLEMENTARY METHODS

Surgical Procedures and Training. Experimental procedures were performed according to the animal welfare guidelines of the Max-Planck-Society. Six Long-Evans rats (P25-38) were anesthetized with an intraperitoneal mixture of ketamine (100 mg/kg body weight) and xylazine (5 mg/kg). Anesthesia was maintained with additional ketamine doses (25 mg/kg). The skull was exposed and cleaned and a miniature metal plate was attached, using a primer and adhesive (Optibond) applied to the exposed skull surface followed by a light-curing composite (Charisma, Heraeus Kulzer) and dental acrylic cement. Small bur holes were made in the occipital and contralateral parietal bone for ECoG wire placement. A 2-3 mm wide craniotomy was opened above binocular visual cortex 0 mm posterior of lambda and 4.5 mm lateral of midline. The craniotomy was then covered, and after full recovery (at least one day after surgery) the animal was accustomed to sitting head-immobilized in the experimental setup during multiple sessions gradually increasing in duration over three days. On the day of imaging (P29-42) the dura was cleared1 and the exposure was superfused with normal rat ringer (NRR) solution (in mM: 135 NaCl, 5.4 KCl, 1 MgCl₂, 5 HEPES, 1.8 CaCl₂ (pH 7.2 with NaOH)), filled with agarose (type III-A, Sigma; 1% in NRR), and covered with an immobilized glass coverslip. After awake imaging periods anesthesia was induced by isoflurane followed by ketamine (100 mg/kg, supplementary doses 25) applied intramuscularly and a heating blanket was inserted below the animal to maintain a body temperature of 37°C. Electrical recordings from ten additional rats were used for combined cell-attached recording and imaging experiments. Animal preparation for these experiments is described elsewhere2,3.
**Labeling Procedures.** Multi-cell bolus loading (MCBL) of neocortical cells with the calcium indicator Oregon Green 488 BAPTA-1 AM (OGB-1; Molecular Probes) and astrocyte labeling by Sulforhodamine 101 (SR101) proceeded as described previously\(^3\). MCBL was performed in superficial layer 2/3 (L2/3).

**Two-Photon Microscopy.** Two-photon imaging was performed using a custom-built two-photon laser-scanning microscope as previously described\(^3\). Excitation wavelength was 872 nm (Mira 900-F laser; Verdi-10 pump; Coherent, USA). A 20x water-immersion objective lens was used (0.95 NA; Olympus).

**Electrophysiology.** Electrocorticogram (ECoG) was recorded with the tip of a 500 μm diameter, teflon-coated silver wire through the bur hole in the contralateral bone (see above) and a reference electrode was inserted through the occipital bone bur hole. ECoG signals were acquired using a custom-built AC-coupled amplifier (input impedance 1 MΩ; bandwidth 0.1 Hz to 8 kHz). Cell-attached recordings were performed from OGB-1 loaded L2/3 neurons as previously described\(^3\).

**Arousal States.** Infrared video was used to detect movement during imaging sessions, using a Sony TVR33 digital camera at 30 frames/sec. Awake animals displayed periods of motion, including movements of the paws, whiskers, snout, and jaw. Movement of the whiskers and paws was detected by an off-line semi-automated procedure. For each type of motion the mean absolute change of pixel values in a user-drawn region of interest
(Supplementary Fig. 2a) was compared to a user defined threshold, and video frames where the mean absolute pixel change exceeded the threshold were classified as containing motion.

ECoG recordings in anesthetized animals showed high amplitude, low frequency waves with power in the frequency range of 3-15 Hz\(^4\). We quantified power in this frequency range using a 3\(^{rd}\) order elliptic filter with 2 dB of band-pass ripple and 35 dB of attenuation, applied over a sliding 4 second window advanced in 0.4 second steps. ECoG recordings in sessile\(^5\) animals showed power in this frequency range, but this activity diminished or disappeared 5-10 seconds before the onset of paw and whisker movements, and returned gradually to its baseline level 10-15 seconds after movement had ceased (Supplementary Fig. 2b). To determine periods in which the animal’s arousal state could be established with certainty, we classified periods from 5 seconds before the onset of paw and/or whisker movement to 15 seconds after movement cessation as the “awake” state, and remaining unanesthetized periods as the “sessile” state. Awake and sessile periods also showed pupil dilation and contraction, respectively.

We identified the depth of anesthesia during all anesthetized imaging sessions as stage III-3, based on lack of whisker movement, lack of tail pinch response, and a strong low-frequency component in the ECoG signal, especially in the 0.5-2 Hz range (Supplementary Fig. 1)\(^6,7\).

**Image Registration.** In all awake and some anesthetized animals, imaging sessions displayed motion of the cortical tissue relative to the objective lens. 7.1% of imaging data in awake animals exhibited constant z-shifts (movement of brain tissue toward or away
from the objective lens), and were excluded from further analysis. Z-shifts were visually detected via changes in neuron and blood vessel shape, and by the appearance and disappearance of fine neuronal and astrocytic processes in the neuropil (< 3 µm diameter). Z-shifts were not randomly interspersed among all frames, but rather occurred in contiguous periods of tens of seconds to several minutes. Z-movement was small or nonexistent during periods where Z-shifts were not visually observable, as SR101 fluorescence from fine astrocytic processes dropped off almost completely within a 3 µm step along the z-axis in both awake and anesthetized animals (Supplementary Fig. 5). This suggests that two-photon excitation provides sufficient axial resolution to detect small Z-shifts in vivo, despite the light-scattering effects of tissue.

In the remaining imaging data, lateral shifts were corrected (unpublished method, manuscript in preparation) as needed using the Lucas-Kanade algorithm for image registration\(^8\) to solve for x- and y-displacement every 6 milliseconds (every 4 scan lines). Lateral shifts in awake animals included slow drifts as well as rapid impulses, resulting in displacements of up to 13 µm during a single imaging frame prior to registration. On average, the total range of displacement during individual frames was 0.89 ± 0.95 µm along the rostro-caudal axis and 0.67 ± 0.74 µm along the medio-lateral axis (mean ± s.d.). Some experiments displayed considerably more displacement than others, suggesting that experimental optimizations to reduce displacement may be possible.

To ensure that errors in displacement did not introduce artifacts into fluorescence signals, we used displacement detected in awake animals to perturb the positions of pixels in simultaneous electrical and optical recordings performed in anesthetized animals. This analysis revealed that despite heavy distortions in the fluorescence induced
by displacement, our correction algorithm successfully restored the original fluorescence transients even when displacements were > 10 \mu m (Supplementary Fig. 6).

**Extraction of fluorescence signals.** OGB-1 fluorescence signals were measured using 64 x 128 pixel frames with 1.5 ms line scan duration (10.4 Hz frame rate) as previously described\(^3\). Regions of interest (ROIs) were drawn only around well-focused cell bodies (judged from the diameter compared to 3D image stacks containing the imaged neurons) because out-of-focus neurons showed a reduced signal-to-noise, lowering spike detection reliability.

**Detection of AP-evoked fluorescence transients.** AP-evoked calcium transients appeared as fluorescence increases that rose sharply and decayed exponentially\(^3\) and were detected by a novel algorithm. An initial baselining and filtering step identified frames potentially containing AP-evoked transients, a fitting step globally solved a nonconvex minimization problem to yield a spike train, and an additional filtering step eliminated spurious transients and iteratively reestimated fluorescence baseline. The initial filtering served only to make the fitting step computationally tractable and never caused electrically confirmed APs to be missed.

Denote neuron fluorescence for frame \(i\) by \(F(i)\) (note that each neuron is processed separately). Define baseline fluorescence \(F_0(i)\) as the mean of the lowest 50% of fluorescence values measured within 10 seconds before or after frame \(i\), and \(\Delta F/F_0(i)\) = \((F - F_0) / F_0\). Identify the subset of frames \(\{i_1^*, i_2^*, \ldots, i_m^*\}\) meeting the criteria:

A) \(\Delta F/F_0(i) > 6\%\)
B) $\Delta F/F_0(i) - \Delta F/F_0(i - 1) > 2\%$

C) $\Delta F/F_0(i) - \Delta F/F_0(i - 2) > 0.8\%$

D) $\Delta F/F_0(i + 1) - \Delta F/F_0(i - 1) > -3\%$

E) $\bar{F} \cdot \bar{Y} / \|\bar{Y}\| > 8\%$, with $\bar{F} = [\Delta F/F_0(i), \Delta F/F_0(i+1), \ldots, \Delta F/F_0(i+17)]$, $\bar{Y} = [e^{0\cdot dt/\tau}, \ldots, e^{-17\cdot dt/\tau}]$, $dt = 96$ ms, and $\tau = 500$ ms.

To obtain the optimal transient heights $\{h_1, h_2, \ldots, h_m\}$, we minimized the error

$$E = \sum_i [\Delta F/F_0(i) - Z(i)]^2,$$

where $Z(i) = \sum_{j=1}^m \sum_{k=0}^{17} \delta(i - k + i_j^*) h_j e^{-(k/dt)/\tau}$, where $\delta$ is the Kronecker delta, $E$ is the observed fluorescence data’s squared difference from the heights convolved by the template and $Z$ is a summing sequence of exponentially decaying fluorescence increases at frames $i_j^*$. Since single APs evoke fluorescence transients rising 9-10% above baseline $^3$ whisker, lesser heights correspond to fractions of a spike, a nonmeaningful interpretation of the fluorescence data that over-fits fluorescence noise in the absence of APs. We therefore required that $\forall j: h_j \geq 10\%$ or $h_j = 0$, to prevent fitting transients to noise fluctuations without introducing assumptions other than the expected transient height; note that $\Delta F/F_0(i_j^*) < 10\%$ does not imply $h_j = 0$. The set of allowed solutions is a disjoint union of $2^m$ half-hyperplanes of dimension 0 to $m$ corresponding to hypotheses specifying which $h_j$ are nonzero. Minimizing $E$ for each hypothesis by gradient descent (“brute force”) is impractical for large $m$.

We therefore calculated upper and lower bounds on $h_j$ that rule out most of the $2^m$ hypotheses. Consider the optimal value for some $h_j^*$ when fixing all other $h_j$. Since the template $\bar{Y} > 0$, it is easily proved that the optimal value for $h_j^*$ increases (or remains
unchanged) as the fixed values $h_j (j \neq j')$ decrease, and decreases as they increase.

Therefore any set of upper bounds $h_{j'}^{\max}$ implies a lower bound $h_{j'}^{\min}$ for each $j'$, obtained by fixing $h_j = h_j^{\max}, \forall j \neq j'$, and optimizing $h_j$ over the set $0 \cup [10, \infty]$. The resulting $h_{j'}^{\min}$ can then be used to (possibly) decrease $h_j^{\max}$. To “update the bounds” we recalculated all upper (first) and lower bounds successively until no value in either bound changed by $>1\% \Delta F/F_0$, with a maximum of 15 updates for both. Proceed as follows:

0) $\forall j : h_j^{\min} = 0$. Update the bounds.

1) If $\forall j : h_j^{\max} = 0$ or $h_j^{\min} \geq 10$, minimize $E$ on the now convex set of possible solutions by gradient descent. Skip the remaining steps.

2) $\forall j' : h_j^{\max} > 0 \land h_j^{\min} = 0$, update the bounds given the alternative hypotheses $h_{j'} = 0$ and $h_{j'} \geq 10$, yielding new bounds $x_j^{\max} (j'), x_j^{\min} (j')$ and $y_j^{\max} (j'), y_j^{\min} (j')$, respectively ( $\land$ denotes logical AND).

3) Calculate $n(j') = 2^{|\{j : j_j^{\max} (j') = 0 \land x_j^{\max} (j') > 0\}|} + 2^{|\{j : j_j^{\min} (j') = 0 \land x_j^{\max} (j') > 0\}|}$, which is the number of hypotheses specifying which $h_j$ are nonzero that must be tested to minimize $E$ given the general hypotheses specifying only $h_{j'} = 0$ and $h_{j'} \geq 10$ ($|\cdot|$ denotes set cardinality).

4) $j_{\text{best}} = \text{argmin}_{j} \{n(j)\}$. Now split the problem into the two cases corresponding to the alternative bounds $x_j^{\max} (j_{\text{best}}), x_j^{\min} (j_{\text{best}})$, and $y_j^{\max} (j_{\text{best}}), y_j^{\min} (j_{\text{best}})$ and solve each starting again from step 1. The solution with lower error is the global minimum.

In datasets where updating the bounds combined with the hypotheses posed in step 2 never cause $h_j^{\min}$ (resp. $h_j^{\max}$) to reach 10% (resp. 0), all $2^m$ hypotheses must be tested in step 1 and “brute force” testing is preferable. In practice however positing a
transient (or lack thereof) at even a few points will rule out (or establish) transients at
other nearby points. In our datasets the largest number of points \( i^*_j \), passing the initial
filtering step and containing no 18-frame gap was 33, requiring error minimization over
\( 2^{33} \approx 8.6 \) billion half-hyperplanes. For this set of points our algorithm explicitly
minimized \( E \) (step 1) on roughly 700,000 half-hyperplanes (0.008\%), finding the global
minimum of \( E \) in about 2 hours (3 GHz CPU). This efficiency allowed global
minimization of \( E \) for all 56.2 neuron-hours of imaging.

Our algorithm’s final step consisted of iterative updates of fluorescence baseline
and elimination of erroneously detected transients. Having determined a final set \( i^*_j \) of
frames containing putative transients, we now re-estimate baseline by smoothing out the
fluorescence values between detected transients. Define:

\[
\begin{cases}
0, & \text{if } \exists j : 0 \leq i - i^*_j \leq 18 \\
1, & \text{otherwise}
\end{cases}
\]

Then our new baseline estimate is defined as

\[
F'_0 = \frac{(B \cdot F) \circ G_1 \circ G_2}{B \circ G_1},
\]

where \( G_1 \) and \( G_2 \) are Gaussian kernels with \( \sigma = 0.75 \) and 2 seconds
respectively and \( \circ \) denotes convolution. Proceed as follows:

I) Reestimate baseline \( F'_0 \) using \( i^*_j \) and \( F(i) \).

II) Recalculate \( \Delta F/F'_0(i) \).

III) If any frame \( i^*_j \) with a detected transient now fails the original filtering criteria A-E
above, remove the transient and return to step I.

IV) Minimize \( E \) on any remaining points \( i^*_j \) as above (steps 0-4) to yield new values \( h_j \),
possibly removing transients but not introducing new ones (requiring negligible
computation compared to steps 0-4 above). If any transients are removed (i.e. some $h_j$ are set to 0), return to step I.

V) \forall j', W_j(i) = \sum_{j\neq j'} \sum_{k=0}^{17} \delta(i - k - i_j^*) h_j e^{-(k/dt)/\tau}, that is, the full fit of transients to the data excluding the one occurring in frame $i_j^*$. Define $V_j(i) := \Delta F / F_0(i) - W_j(i)$. For all $j'$ find the least squares solution vector $c(j')$ to the system of linear equations

$$
\begin{bmatrix}
V_j(i) & W_j(1) & \ldots & W_j(i-10) \\
\vdots & \vdots & \ddots & \vdots \\
V_j(i+31) & 1 & \ldots & 1 \\
\end{bmatrix}
\begin{bmatrix}
c_1(j') \\
c_2(j') \\
c_3(j') \\
\end{bmatrix} = \begin{bmatrix}
0 & 0 & 1 \\
\vdots & \vdots & \vdots \\
0 & 0 & 1 \\
\end{bmatrix}
\begin{bmatrix}
c_1(j') \\
c_2(j') \\
c_3(j') \\
\end{bmatrix}
$$

such that $c_2(j')$, $c_3(j') \geq 0$, where $\tilde{Y} = [e^{-0\cdot dt/\tau}, e^{-1\cdot dt/\tau}, \ldots, e^{-32\cdot dt/\tau}]^T$. If any values $c_1(j') \leq 7.5\%$, remove the transient with the lowest $c_1$ value and return to step I.

VI) Let $u(j') = V_j(j') - V_j(j'-2)$. If any values $u(j') \leq 3\%$, remove the transient with the lowest $u$ value and return to step I.

VII) Let $A(j') = \frac{1}{2} \sum_{i= \pm 4}^{i = j} \sum_{j= \pm 4}^{j'} V_j(i)$. If any values $A(j') \leq 6.5\%$, remove the transient with the lowest $A$ value and return to step I.

VIII) Round each $h_j / 9.75\%$ to the nearest integer to yield the number of estimated APs for that transient.

**Confirmation of detected transients.** While our main evidence that observed optical signals corresponded to AP activity consisted of simultaneous cell-attached recordings in
anesthetized animals, we also performed additional controls and analyses to confirm that experimental artifacts did not compromise AP-detection or introduce false positives.

To quantify the performance of the spike-detection algorithm (Fig. 1f) we calculated AP-detection rate as the percentage of electrically confirmed APs that co-occurred with an optical transient detected by the algorithm with time difference $\leq \Delta t$ (green line), or as the percentage of electrically confirmed APs for which a corresponding AP was inferred by the algorithm (not already assigned to another electrically confirmed AP) (blue line). Note that the first measure (green line) does not require a one-to-one correspondence between electrically confirmed APs and transients. The time difference was calculated from the time when the beam focal-point passed through the center of the neuron during acquisition of the frame to which the transient was assigned and the time of the electrically confirmed AP. The spike-detection algorithm produced a low number of false positives (Supplementary Fig. 3), defined as APs inferred by the algorithm without any nearby electrically confirmed AP with time difference $\leq \Delta t$ (red line), or as APs inferred by the algorithm without a nearby electrically confirmed AP or for which all nearby electrically confirmed APs were already assigned to other inferred APs (black line). Detection and false positive rates in the text are reported for $\Delta t = 192$ ms. We considered time differences both before and after electrically confirmed APs, since noise-based fluorescence increases just before an AP could cause it to be assigned to a previous frame (detection and false positive rates were similar when requiring electrically confirmed APs to occur before APs inferred by the algorithm).

We examined the fluorescence transients to ensure that background signals did not contaminate fluorescence measurements putatively originating in neuronal somata,
since presynaptic structures in the neuropil can produce strong fluorescence signals known as the optical encephalogram (OEG) when loaded with OGB1-AM$^3$. In the present study putative AP-associated transients were not due to neuropil contamination$^2$. We ensured that the z-resolution was sufficient to detect small axial shifts (Supplementary Fig. 5, and see section ‘image registration’) and used only in-focus neurons. Despite the known correlation between presynaptic OEG signal and electrically or optically detected postsynaptic AP-firing$^3$, visual inspection confirmed that neurons did not always show putative APs during increases in OEG fluorescence, and that OEG fluorescence did not always increase during periods of putative AP-firing. As a control we quantified the component of neuron fluorescence consisting of OEG fluorescence, which we term a neuron’s “OEG contamination”, by linearly regressing neuron fluorescence against OEG fluorescence (from a large ROI in the homogenously fluorescing neuropil) and a constant term, over all time points more than 1 second before or after a given neuron’s putative APs. The standard deviation of the OEG contamination component of neuronal fluorescence kinetics was small for both awake and anesthetized periods ($1.0 \pm 0.4\% \Delta F/F_0$ for awake, $n = 20$ neurons, 3 populations; $1.7 \pm 0.7\% \Delta F/F_0$ for anesthetized, 11 neurons, 2 populations; mean ± s.d.) compared to the overall standard deviation of $\Delta F/F_0$ values ($3.85\%$ in periods of putative silence in awake animals). We also observed that OEG contamination was <1% $\Delta F/F_0$ for 77% of frames, and <2% for 93% of frames. Visual inspection showed that OEG contamination did not assume the shape of AP-related transients, as required for detection of putative APs (see above), and our AP detection algorithm did not detect any APs from the OEG contamination component (3 neurons, 4500 frames). Even in anesthetized recordings,
which showed a 70% greater standard deviation in OEG contamination, simultaneous cell-attached recordings showed that false positives in transient detection occurred only about once every 86 seconds (see results and also⁹), so whatever contamination was present was not frequently classified as AP-related.

Detection and assignment of AP activity was tested using cell-attached recordings in anesthetized animals only, though we applied our algorithm to awake animals as well. While we cannot be completely certain that calcium kinetics associated with APs are not altered by anesthesia, we did confirm that transient half-width for single APs was the same for awake and anesthetized periods (262 ± 3 ms for awake, n = 6490 transients; 257 ± 4 for anesthetized, n = 2461; P = 0.45, t-test; only transients with at least 1.75 seconds of inactivity before/after were used). Both values matched the half-width of transients from electrically confirmed single APs (261 ± 17, n = 70, P > 0.1).

Since neurons were scanned only once every 96 ms, it is conceivable that neurons firing regularly at high rates might not show any fluorescence changes. Several factors, however, show this is not the case for our data. First, cortical neurons with high firing rates display high spike count variance so that fluorescence signals should show large fluctuations around their mean values for neurons firing at high rates, whereas our recordings showed a similar size of fluorescence fluctuations in periods of putative silence and silence confirmed electrically by cell-attached recordings. Second, AP firing in ketamine-anesthetized rats is restricted to neuronal up-states, so that neurons firing at high rates under anesthesia would show periods of silence and periods of intense firing that coincided with the on-going up-state/down-state transitions. These changes would then be reflected in their fluorescence kinetics, which was not the case. Third, if neurons
fired at high baseline rates only in the awake and sessile states, we would expect to see large decreases in baseline fluorescence upon anesthesia, which were not observed. Fourth, the observed rates agree with observations made using blind cell-attached and whole-cell recordings in freely moving\textsuperscript{10}, head-fixed\textsuperscript{1}, and anesthetized rats\textsuperscript{11}.

In addition, we further looked at fluorescence changes in cell-attached neurons where the spiking was known, and compared this to the awake case, where cell-attached recordings were not obtained. While the raster scanning of imaging frames occurs at 10.4 Hz, individual line scans (5-10 per neuron per frame) last only 1.5 ms. Fluorescence signals in an individual line scan occurring just after a spike will be increased relative to the baseline defined by fluorescence values for that particular line in other frames recorded before the spike. We tested this phenomenon in imaging sessions using simultaneous cell-attached recordings, where the mean and variance of line scan fluorescence values increased significantly (P < 0.000001, t-tests and F-tests) in frames that included electrically detected APs from simultaneous cell-attached recordings. Frames including putative spikes exhibited a distribution of line scan fluorescence values similar to that observed in frames with spikes confirmed by cell-attached recording, while line scan fluorescence values from frames of putative silence in the present study matched distributions from confirmed silence in cell-attached recordings (Supplementary Fig. 7).

In neurons firing at extremely high rates for prolonged periods, the calcium dye might enter a state of constant saturation, such that even fast calcium influxes during 1.5 ms line scans might not lead to fluorescence increases. This cannot be the case in our data however, since every neuron recorded for 50 seconds in at least one arousal state showed
at least one AP-associated transient rising above a stable baseline fluorescence value (333 neurons).

**Analysis of pairwise correlation.** It was not possible to determine cross-correlations on a fine temporal timescale, since with 96 ms imaging frames each detected spike or spike burst carries a uniform temporal uncertainty across a 96 ms period. One possible way to calculate correlations would be to calculate the Pearson correlation coefficient for the number of APs detected in each frame for the two neurons:

\[
\rho = \frac{\frac{1}{n} \sum_i N_a(i)N_b(i) - \left(\frac{1}{n} \sum_i N_a(i)\right)\left(\frac{1}{n} \sum_i N_b(i)\right)}{\sigma(N_a)\sigma(N_b)}
\]

where \(N_a(i)\) and \(N_b(i)\) are the number of APs detected in frame \(i\) for neurons \(a\) and \(b\), \(n\) is the total number of frames, and \(\sigma\) denotes standard deviation. However, this would systematically underestimate correlation values for pairs of neurons separated in the \(y\)-coordinate, since APs occurring simultaneously in the two neurons could be detected in separate frames since the neurons are not raster-scanned simultaneously. Thus even if a pair of neurons separated in the \(y\)-coordinate always spike simultaneously, we will observe \(\rho < 1\), an undesirable artifact of the raster scanning. We tested pairwise correlation coefficients for the number of spikes occurring in windows of various sizes, to determine the minimum window size for which this bias was negligible. As expected, correlations across single frames did show a weak negative association with separation in the \(y\)-coordinate (\(r = -0.042, P = 0.080\) during awake periods; \(r = -0.135, P = 0.005\) under anesthesia, Spearman rank correlation tests). Over window sizes of 2-20 frames, however, there was no significant relation between correlation and \(y\)-distance for either
awake or anesthetized periods (|r| < 0.04, P > 0.10). For this reason, and also because cell-attached recordings revealed that APs were occasionally detected in the frame subsequent or prior to the one during which they actually occurred (Fig. 1f), we analyzed pairwise correlations over sliding two-frame (192 ms) windows:

$$\rho' = \frac{1}{(n-1) \sum M_a(i)M_b(i)} - \left( \frac{1}{(n-1) \sum M_a(i)} \right) \left( \frac{1}{(n-1) \sum M_b(i)} \right) M_a(i) = N_a(i) + N_a(i+1)$$
$$M_b(i) = N_b(i) + N_b(i+1)$$

We cross-validated correlation coefficients by comparing first and second halves of each recording for all three states. A highly significant association was found between correlation coefficients in the first and second halves of the time spent in each state (r > 0.4, P < 10^{-6}, over pairs recorded simultaneously for at least 400 seconds in each state, n = 1053 awake, n = 263 anesthetized, n = 23 sessile).

To check the robustness of our analyses with respect to the correlation measure employed, we also computed correlation by convolving transient detection times (which were different for neurons with y-separation) with a uniform kernel with amplitude equal to the number of detected APs and width 96 ms. Correlation coefficients were then calculated on the resulting continuous functions of time. This measure produced the same trends for the comparison of correlation values across arousal states (P < 0.01, Kruskal-Wallis nonparametric ANOVA followed by Wilcoxon rank sum tests), the firing rate-correlation association (P < 0.01, t-tests, regression slope ratio 9.6 for anesthetized compared to awake periods), and the lack of significant association between correlation and x-, y-, or overall distance (P > 0.05, Spearman rank correlation tests with Bonferroni correction, strongest association observed for the awake state with $r = -0.048, P = 0.048$).
For both correlation measures, correlation and distance did not show a stronger relationship after accounting for the relationship between correlation and firing rate.

**Analysis of population synchrony.** For each imaged population we randomly selected as many as possible non-overlapping groups of 7 neurons recorded simultaneously for at least 200 seconds. We compared the distribution of number of spikes / 192 ms observed for 7-neuron populations to three theoretical distributions: independent multinomial, independent Poisson, and correlated Poisson. Each distribution was defined on the full set of population activation states \((n_1, n_2, ..., n_7)\) where \(n_i\) is the number of spikes fired by neuron \(i\) in a given 192 ms time window.

The independent multinomial distribution was defined by setting the probability of observing a given network state to the product \(P(n_1 = k_1, n_2 = k_2, ..., n_7 = k_7) = \Pi_i P(n_i = k_i)\), with the product terms \(P(n_i = k_i)\) on the right hand side taken directly from the observed data. This distribution preserves firing rates and sparseness for individual cells (and thus burst index), but removes all correlations between neurons’ activity.

The independent Poisson distribution was defined by setting \(P(n_1 = k_1, n_2 = k_2, ..., n_7 = k_7) = \Pi_i e^{-\overline{n}_i n_i^{k_i}} / k_i!\), where \(\overline{n}_i\) is the mean number of APs per 192 ms time window observed experimentally for neuron \(i\). This distribution preserves firing rates, removes all correlations, and sets sparseness to the value \(1/((\overline{n}_i + 1))\) expected for a Poisson process with random spike times (and therefore sets burst index to zero).

For the correlated Poisson distribution, we first defined latent variables \(q_i\), with \(q_i = 0\) when \(n_i = 0\), and \(q_i = 1\) otherwise. Next, generalized iterative scaling\(^{12}\) was used to
explicitly determine the maximum entropy distribution on the variables \(q_i\) satisfying the constraints:

\[
P(q_i = 1) = 1 - e^{-\overline{n}_i}
\]

\[
P(q_i = 1, q_j = 1) = \frac{(\rho_{ij}^{\text{data}} \sqrt{n_i n_j + \overline{n}_i \overline{n}_j})(1 - e^{-\overline{n}_i})(1 - e^{-\overline{n}_j})}{n_i n_j},
\]

where \(\rho_{ij}^{\text{data}}\) denotes the correlation observed experimentally between the \(i^{\text{th}}\) and \(j^{\text{th}}\) neurons and the experimentally observed mean rates \(\overline{n}_i\). Iteration ceased when the constraints were satisfied within a 0.001 margin of error and none of the \(2^7 = 128\) activation states changed its probability by \(>0.01\) through one iteration; iteration completed successfully in every case. We then set \(P(n_i = k \mid q_i = 1) = \frac{e^{-\overline{n}_i} \overline{n}_i^k}{(1 - e^{-\overline{n}_i}) k!}\), with the variables \(n_i\) conditionally independent for fixed values of \(q_i\). Thus the marginals of \(n_i\) are

\[
P(n_i = k) = \frac{e^{-\overline{n}_i} \overline{n}_i^k}{k!}, \text{ so each } n_i \text{ is Poisson. Solving for } \rho_{ij}^{\text{data}}, \text{ we have:}
\]

\[
\rho_{ij}^{\text{data}} = \frac{P(q_i = 1, q_j = 1) - \frac{\overline{n}_i}{(1 - e^{-\overline{n}_i})} - \frac{\overline{n}_j}{(1 - e^{-\overline{n}_j})} - \overline{n}_i \overline{n}_j}{\sqrt{n_i n_j}} = \frac{\overline{n}_i \overline{n}_j - \overline{n}_i \overline{n}_j - \overline{n}_i \overline{n}_j}{\sqrt{\text{var}(n_i) \text{var}(n_j)}} = \rho(n_i, n_j), \text{ i.e. the correlation matrix for the variables } n_i \text{ matches the correlation matrix observed experimentally (covariance and the expectation of the product } \overline{n}_i \overline{n}_j \text{ need not match experimentally observed values when experimentally observed marginals are non-Poisson). Thus, this distribution preserves firing rates and pairwise correlations between neurons while setting sparseness to the value } 1/(\overline{n}_i + 1) \text{ expected for a Poisson process (and therefore sets bursting index to zero).}
The firing rates observed in our recordings were low, and we demanded only 200 seconds (1041 2-frame samples) from a discrete distribution on $2^7 = 128$ values for the analysis of population synchrony. In contrast, previous applications of maximum entropy methods to binary population spiking data in vitro incorporated longer recordings, greater temporal resolution, and higher firing rates. Therefore, it was necessary to introduce some regularization to avoid overfitting, especially for the case of neuronal pairs which were never simultaneously active (simply setting certain activation states’ probabilities to zero and maximizing entropy over the remaining possibilities can lead to severe overfitting). To this end, for any neuronal pair which was never simultaneously active, we calculated the probability of never observing simultaneous activity for that pair assuming independence and given the rates $\bar{n}_i, \bar{n}_j$ and number of time windows $m$: $p_{\text{disjoint}} = [1 - (1 - e^{-\bar{n}_i})(1 - e^{-\bar{n}_j})]^m$. For all neuronal pairs $(i, j)$ never observed to be simultaneously active and with $p_{\text{disjoint}} > 0.1$, we removed the constraint on $P(q_i = 1, q_j = 1)$ before commencing generalized iterative scaling (which is not equivalent to setting $P(q_i = 1, q_j = 1) = P(q_i = 1)P(q_j = 1)$). This weak regularization does not modify the distribution greatly since only weak significance ($\alpha = 0.1$) is required of the observed correlations. Indeed, the resulting absolute deviation in correlation from experimental observations was < 0.001 for all pairs. Note that despite this regularization step it is still possible to observe data which cannot be fit by the class of multivariate Poisson distribution described here, as some datasets can lead to desired values of $P(q_i = 1, q_j = 1)$ that are < 0 or greater than the theoretical maximum $\min(P(q_i = 1), P(q_j = 1))$. However, the AP activity for all populations analyzed
produced valid values for $P(q_i = 1, q_j = 1)$ and was successfully fit to correlated Poisson distributions. These distributions are by no means unique among joint distributions with the given correlation matrix and Poisson marginals. Higher order correlations can also be imposed on $q_i$, and even given a fully specified distribution on $q_i$ there may be several possible resulting distributions on $n_i$ consistent with the observed correlation matrix and Poisson marginals, since in general multivariate Poisson distributions have more degrees of freedom than multivariate binary distributions.

**Quantification of Spike Train Sparseness.** We quantified the sparseness of a neuron’s AP discharges using the Treves-Rolls measure $S = 1 - \frac{\left(\sum \frac{a_i}{m}\right)^2}{\sum \frac{a_i^2}{m}} = 1 - \frac{\bar{a}^2}{\bar{a}^2}$, where $m$ is the number of two-frame (192 ms) windows and $a_i$ is the number of APs in the $i$th window. For a Poisson process $Var(a) = \bar{a}^2 - \bar{a}^2 = \bar{a}$ so that $S_{\text{poisson}} = 1/(\bar{a} + 1)$. We performed a sample size correction $^{18}$, dividing $S$ by $(1 - 1/m)$, but the effect was negligible for our data since the number of windows was large.

**Data Analysis.** Analysis of firing rates was performed on neurons recorded for at least 50 seconds in a given state (434 ± 255 seconds per neuron awake, 337 ± 231 anesthetized, 231 ± 90 sessile, mean ± s.d.), while pairwise correlations and population synchrony required 200 seconds (466 ± 191 seconds per pair awake, 452 ± 160 anesthetized, 268 ± 79 sessile). The significance of firing rate changes was the same when using parametric (Student’s t) and nonparametric (Wilcoxon rank sum) tests. 95% confidence intervals for slopes and y-intercepts of linear regressions were obtained as described in $^{19}$. 
Box-and-whisker plots indicate the median and the 5\textsuperscript{th}, 25\textsuperscript{th}, 75\textsuperscript{th}, and 95\textsuperscript{th} percentiles of a distribution. We report mean +/- standard error of the mean unless otherwise noted. In the main text, the significance of all comparisons was quantified using Wilcoxon rank sum tests, preceded by a Kruskal-Wallis nonparametric ANOVA when more than two quantities were simultaneously compared. We tested the significance of the associations between individual neurons’ firing rates and individual pairs’ correlations before and after anesthesia using a t-test. For the association between distance and absolute firing rate difference a Spearman rank correlation test was used.
SUPPLEMENTARY DISCUSSION

The findings related to spatial distance between neurons (Supplementary Fig. 8) are surprising and merit further investigation in future work. Because there does not seem to be any spatial organization in the orientation tuning of L2/3 neurons in the rat visual cortex\textsuperscript{20}, it is not surprising that there is no effect of distance on pairwise correlations in AP-firing as nearby neurons are likely to receive different constellations of synaptic inputs. We recently found that for barrel cortex L2/3 neurons located in a column, distance does have a strong effect on pairwise correlations in sensory evoked AP-responses\textsuperscript{9}. This difference is most likely due to the barrel being a well defined columnar structure whose neurons receive similar inputs. To our knowledge the present work is the first to directly compare the relationship between spatial distance between neurons and spontaneous firing rate differences. The fact that different neuronal types are closely intermixed does not preclude similar average spontaneous firing rates of nearby neurons. Although the finding remains noisy, after averaging across all neurons for each 20 \textmu m distance bin, it is highly significant ($P < 10^{-6}$) for the awake state due to the large number of neuronal pairs (> 1000) we were able to record. In addition, because two photon population imaging provides high spatial resolution we were able to accurately measure distances between neurons at small graduations, something other multi-unit recording techniques cannot achieve. Note that the findings reported here are based on spontaneous firing activity and that a different picture may emerge during the presentation of visual stimuli.
References:
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