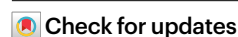


Rapid fluctuations in functional connectivity of cortical networks encode spontaneous behavior

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Experimental work across species has demonstrated that spontaneously generated behaviors are robustly coupled to variations in neural activity within the cerebral cortex. Functional magnetic resonance imaging data suggest that temporal correlations in cortical networks vary across distinct behavioral states, providing for the dynamic reorganization of patterned activity. However, these data generally lack the temporal resolution to establish links between cortical signals and the continuously varying fluctuations in spontaneous behavior observed in awake animals. Here, we used wide-field mesoscopic calcium imaging to monitor cortical dynamics in awake mice and developed an approach to quantify rapidly time-varying functional connectivity. We show that spontaneous behaviors are represented by fast changes in both the magnitude and correlational structure of cortical network activity. Combining mesoscopic imaging with simultaneous cellular-resolution two-photon microscopy demonstrated that correlations among neighboring neurons and between local and large-scale networks also encode behavior. Finally, the dynamic functional connectivity of mesoscale signals revealed subnetworks not predicted by traditional anatomical atlas-based parcellation of the cortex. These results provide new insights into how behavioral information is represented across the neocortex and demonstrate an analytical framework for investigating time-varying functional connectivity in neural networks.

Cognitive functions such as perception and attention require the dynamic activity of neuronal networks defined by synaptic connectivity over local and long-range spatial scales^{1–3}. Moreover, animals cycle through behavioral states categorized by a variety of physical markers, including pupil dilation, facial movement and locomotion, associated with changes in cognitive performance^{4–9}. Such variations in motor behaviors and arousal are themselves strongly coupled to fluctuations

in neural activity, and several recent studies have demonstrated clear modulation of spontaneous and sensory-evoked firing rates associated with transitions between behavioral states^{10–14}. Work in both human and nonhuman species has suggested that the spatiotemporal correlations between neural signals in large-scale networks spanning multiple brain regions also covary with state^{1,14–20}. These correlations are often viewed as the functional connectivity between nodes in a network, reflecting

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either true structural (synaptic) connections or common inputs²¹. From these results, behavioral state fluctuations might be viewed as shifts between distinct network configurations optimized for contextually relevant cognitive functions.

Although many analyses of state-dependent correlations in cortical networks rely on binning data within identified epochs (for example, sleep versus wakefulness or quiescence versus arousal), others have explored continuously time-varying functional connectivity^{15,22}. However, methodological challenges to monitoring large-scale brain dynamics at high spatial and temporal resolution have obscured the investigation of rapid covariations in behavioral state and network connectivity. As neurons can be exquisitely sensitive to patterned or synchronized inputs²³, it seems reasonable to hypothesize that fast changes in network correlations are closely linked to the integrative function of single cells. Furthermore, the short-term correlation between two neuronal signals is a nonlinear, second-order function of those signals expressing the dynamics of their connectivity. Thus, the temporal dynamics of activity and short-term correlations might differentially represent behaviorally relevant information. Nevertheless, the relative contributions of dynamic neural activity versus dynamic internode correlations to decoding behavior are largely unknown.

To explore this issue, we used wide-field mesoscopic and cellular-resolution two-photon calcium (Ca^{2+}) imaging, independently and simultaneously, to monitor neural activity in awake, head-fixed mice. We developed a methodological strategy that views the dynamic correlations between individual cortical regions and/or neurons as a ‘graph of graphs’, extracting their time-varying latent variables through non-Euclidean diffusion embedding²⁴. We then asked how accurately these signals could be used to decode spontaneous fluctuations in behavior (measured by pupil dilation, facial movements and locomotion) and sensory inputs. Our results showed that time-varying network correlations carry important information about behavioral metrics that can outperform analyses based on the dynamics of activity alone. In addition, the dynamic multimodal correlations between local cellular and large-scale networks are also substantially predictive of behavior. We also found that both cortical parcels and single neurons are dynamically correlated to one of two broad mesoscale networks that are distinct from traditional anatomical boundaries. Overall, these findings demonstrate that rapid fluctuations in functional connectivity across spatial scales provide a robust representation of spontaneous behavior.

Results

Monitoring spontaneous behavior and cortical dynamics

We carried out wide-field mesoscopic calcium imaging³ in awake, head-fixed mice expressing the red fluorescent indicator jRCaMP1b²⁵ (Fig. 1a). Indicator expression was mediated by neonatal injection of AAV9-Syn-jRCaMP1b into wild-type mice (Methods)^{26,27}. We simultaneously monitored cortical activity and spontaneous behavioral metrics, including pupil diameter, facial movement and locomotion (Fig. 1b–g and Methods)^{13,28}. Pupil diameter and locomotion data were extracted from video recordings of the eye and running-wheel speed, respectively. Facial movements were derived from video data by using FaceMap²⁸, and the first extracted component was used for most subsequent analyses. Although some studies have used categorical definitions of behavioral state according to thresholding of motor signals^{9,13,14,29}, our data suggest that these metrics are continuously distributed across a range of rapidly varying values (Calinski–Harabasz index values versus the number of clusters for k -means clustering using two to six clusters, $P = 0.99$, analysis of variance; Fig. 1b,c). We also found that these signals are only modestly correlated with each other (Fig. 1d and Supplementary Fig. 1). These results suggest that pupil, locomotion and facial metrics may reflect at least partially distinct, highly dynamic latent variables, such as different neuromodulatory influences¹⁴. Here, we focused on the general ability of functional connectivity to encode behavior and, therefore, separately compared

the relationships of time-varying network activity and correlations to each of these observable variables.

After normalization and hemodynamic correction of imaging data (Methods)^{14,30}, we segmented the cortex into functional parcels using a graph-theory-based approach that relies on spatiotemporal coactivity between pixels (local selective spectral clustering (LSSC); Fig. 1h and Supplementary Fig. 2)³¹. This approach yields comparable reconstruction errors for a similar number of parcels versus either the Allen Institute Common Coordinate Framework version 3 (CCFv3) anatomy-based atlas³² or a uniform grid-based segmentation (Methods). Principal component analysis (PCA) yielded similar reconstruction errors as LSSC, and localized semi-non-negative matrix factorization (LocaNMF)³³ provided near-zero errors; however, both approaches generated non-disjoint parcels that may be more difficult to interpret (Supplementary Fig. 2). We next extracted $\mathbf{x}_t$, the time-varying fluctuations in the fluorescence signal associated with each parcel (Fig. 1e and Methods). As expected, variation in activity appeared to be coupled to changes in behavioral metrics over rapid (subsecond) time-scales (Fig. 1e–h). We then calculated the time-varying, pairwise correlations $\hat{C}_t$ between LSSC parcels using a sliding 3-s window (0.1-s step size; Fig. 1f–i and Methods). On average, correlations across the cortex were high but with large fluctuations over time (average $r = 0.6 \pm 0.03$, average coefficient of variation of $r(\text{CV}_r) = 0.4 \pm 0.1$, $n = 6$ mice). Indeed, the moment-to-moment values also appeared to covary with rapid behavioral changes (Fig. 1f–i).

Dynamic functional connectivity encodes spontaneous behavior

We began with a basic assumption that behavior can be represented as some function of multidimensional, time-varying neural activity. That is:

$$b_t = f(\mathbf{x}_t). \quad (1)$$

Here, $\mathbf{x}_t$ is an N -dimensional vector corresponding to the time-varying neural activity across N cortical parcels at time t . This relationship can be approximated using the first two terms of a standard Taylor expansion (Methods):

$$b_t \approx \beta_0 + \beta_1^T \mathbf{x}_t + \sum_i \sum_j \beta_2(i,j) \hat{C}_t(i,j) + \epsilon, \quad (2)$$

where $\mathbf{x}_t$ is again the time-varying neural activity and the second-order term $\hat{C}_t(i,j)$ corresponds to the time-varying pairwise sample correlations between parcels i and j . Representing behavior as a linear combination of time-varying neural signals is a common approach^{9,34–37}, and we hypothesized that combining a linear term in activity $\mathbf{x}_t$ and correlations $\hat{C}_t$, which are a nonlinear second-order function of $\mathbf{x}_t$, would substantially improve decoding accuracy.

In an initial effort, fitting a linear ridge regression model for behavioral dynamics whose predictors are time-varying cortical activity and functional connectivity led to poor predictive power (Supplementary Fig. 2) due to overfitting caused by the high dimensionality of the pairwise correlations $\hat{C}_t$ ($\sim 10^3$ pairs per animal). Therefore, we developed a novel strategy to extract a lower-dimensional representation, ϕ_t , capturing the intrinsic dynamics of the correlational signals using Riemannian geometry and diffusion embedding²⁴. Each correlation matrix over a short temporal window (for example, 3 s) can be viewed as a graph whose N nodes are cortical parcels connected by weighted edges equal to the instantaneous pairwise correlation coefficients between parcels. Sliding the window over time ($\Delta t = 0.1$ s) produces a series of correlation matrices, which can also be viewed as a time-varying graph (Fig. 2a). We then built a graph of graphs in which each node is now a time point represented by the correlation matrix at that time (Methods). A distance measure between correlation matrices is necessary to set

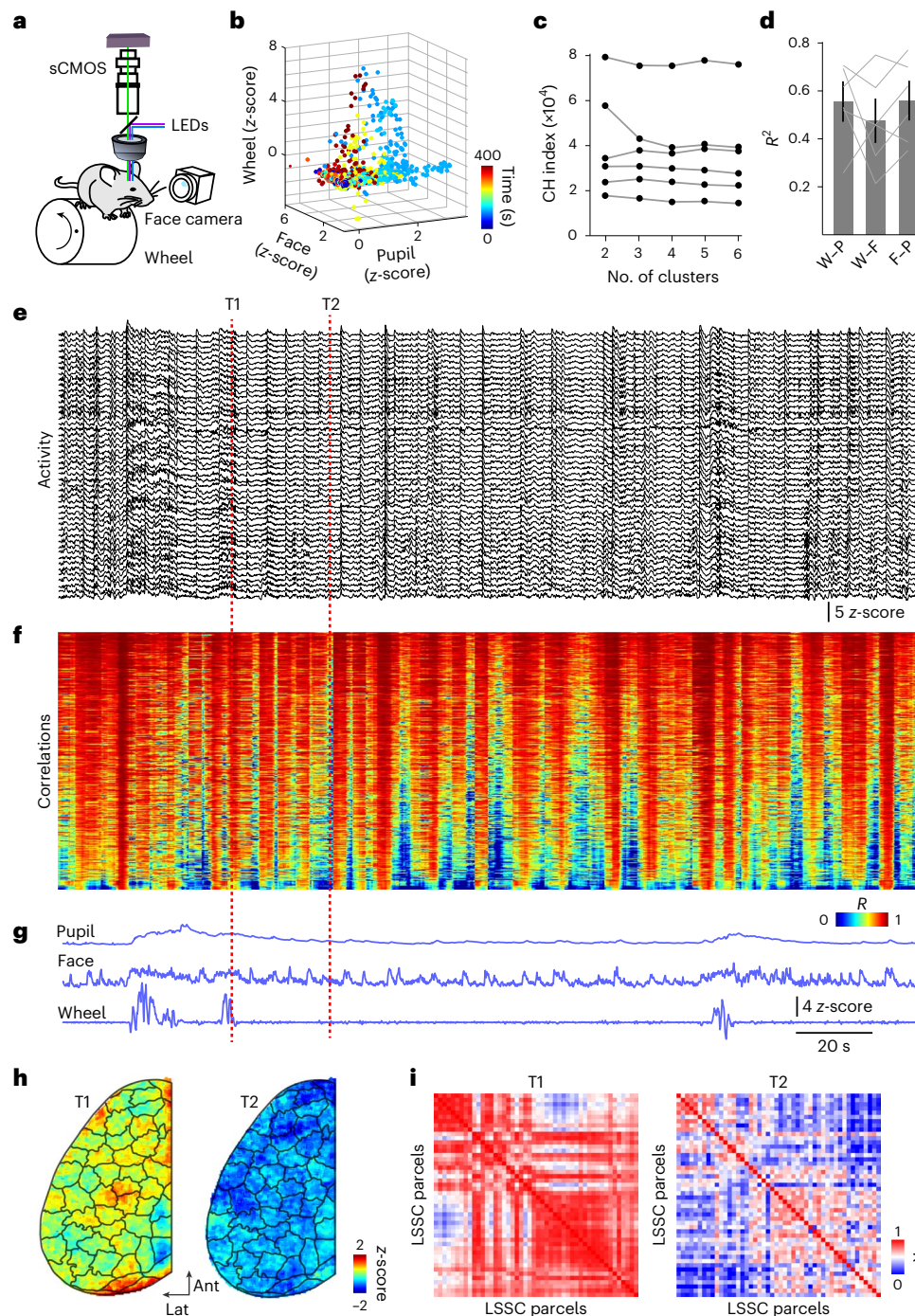


Fig. 1 | Mesoscopic imaging of cortical activity and functional connectivity.

a, Schematic showing the setup for simultaneous behavioral monitoring and mesoscopic calcium imaging. sCMOS, scientific complementary metal-oxide semiconductor; LEDs, light-emitting diodes. **b**, Scatterplot of the distribution of z-scored behavioral metric values (locomotion, facial movement and pupil size) over a 400-s window for the example mouse whose data are shown in **e–i**. **c**, Population data showing Calinski–Harabasz (CH) index values for *k*-means clustering of behavioral metrics for all subjects. **d**, Population data ($n = 6$ independent mice) showing the average ($\pm$ s.e.m.) Pearson's R^2 values for the relationships between the metrics wheel (W), pupil (P) and facial movements (F) for all subjects.

Data for individual animals shown by light gray lines. **e**, Example time series from one animal showing cortical activity across the cortex. Each trace corresponds to one LSSC-based parcel. **f**, Heat map of the time series of pairwise correlations between each parcel from **e**. Data are sorted by increasing s.d. **g**, Time series of behavioral metrics corresponding to the data shown in **e** and **f**. **h**, Example LSSC-based functional parcellation of the neocortex for the data shown above. Left and right images are for the time points (T1 and T2) indicated by vertical red lines. Lat, lateral; Ant, anterior. **i**, Example pairwise correlation matrices for the data in **e** at the time points indicated.

the edge weights of this temporal graph. As correlation matrices lie on a non-Euclidean Riemannian manifold, Euclidean distances do not properly represent similarity in this space (Fig. 2b). Therefore, we used Riemannian geometry to calculate pairwise geodesic distances between correlation matrices. We applied diffusion embedding to the

graph of graphs and extracted the low-dimensional representation ϕ_t of the temporal dynamics of functional connectivity (Fig. 2c).

We constructed a cross-validated linear regression model combining the cortical activity for all LSSC parcels ($\mathbf{x}_t$, ranging from 48 to 53 parcels per animal across six mice) and the first 20 leading

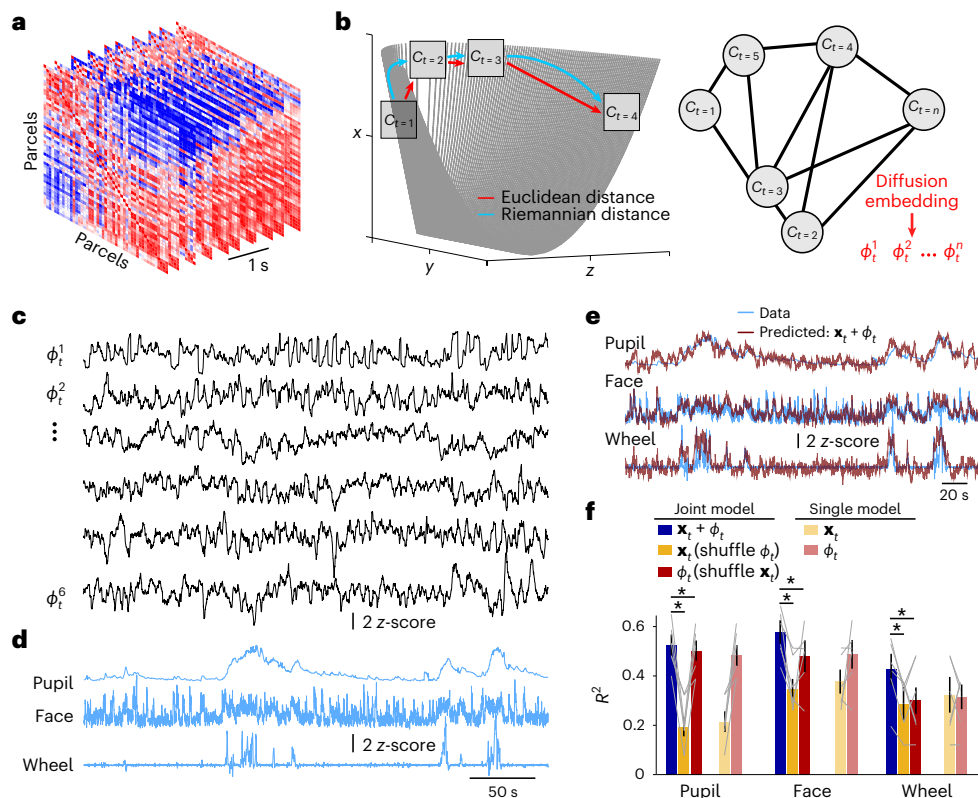


Fig. 2 | Dynamic functional connectivity encodes rapid behavioral variations.

a, Example sequential pairwise, parcel-based correlation matrices derived from a sliding window applied to neural activity across the cortex. **b**, Left, schematic of the cone-shaped Riemannian manifold used to calculate distances between correlation matrices. The Riemannian measurement reflects the geodesic distance that is ignored when using the Euclidean distance. Right, illustration of a graph of graphs, whose nodes are individual matrices and edges are weighted by the length of the geodesic arc along the Riemannian cone, used to extract diffusion-embedding (ϕ_t) components. **c**, Example diffusion-embedding (ϕ_t) components capturing the dynamics of functional connectivity. **d**, Time series for behavioral metrics corresponding to data in **c**. **e**, Example behavioral data (blue traces) from **d** showing fluctuations in pupil diameter, facial movement and

locomotion superimposed on predicted behavior (red traces) estimated using a joint model based on time-varying activity and embedded correlations. **f**, Population data ($n = 6$ independent mice) showing the average ($\pm$ s.e.m.) prediction accuracy (R^2) for modeling behavior variables using a joint model of activity and embedded correlations (blue), a joint model with shuffled ϕ_t (yellow), a joint model with shuffled activity (red), a single-predictor model using activity (pale yellow) and a single-predictor model using ϕ_t (pale red). $P < 0.05$ for a two-sided paired t test (see the main text). Full model compared to shuffling $\phi_t^{(20)}$: $P = 0.001$ for pupil, $P = 0.002$ for face and $P = 0.01$ for wheel. Full model compared to shuffling $\mathbf{x}_t$: $P = 0.02$ for pupil, $P = 0.006$ for face and $P = 0.003$ for wheel. Data for individual animals shown by light gray lines.

components of the embedded correlations, denoted by $\phi_t^{(20)}$, to predict the continuously varying behavioral signals for pupil diameter, facial movement or locomotion (Fig. 2d,e). We found that behavior can be robustly decoded by this joint model (pupil: $R^2 = 0.52 \pm 0.04$, face: $R^2 = 0.59 \pm 0.04$, wheel: $R^2 = 0.45 \pm 0.06$, $n = 6$ mice; Fig. 2e,f). Prediction accuracy decreased rapidly for higher-order components of facial movement (Supplementary Fig. 2). Additionally, accuracy was impaired when using either raw correlations, Euclidean rather than Riemannian distance for diffusion embedding, or PCA for dimensionality reduction (Supplementary Fig. 2). Smoothing or diffusion embedding of activity did not significantly affect the analysis (Supplementary Fig. 2). Moreover, predictive performance was robust to changes in model parameters, did not improve with the inclusion of >20 embedding components and did not vary appreciably for window lengths of 3–10 s (Supplementary Fig. 2). We also compared model prediction accuracy for different methods of cortical segmentation. Analyses with LSSC were similar to grid-based and anatomical (CCFv3) parcels as well as nondisjoint segmentations derived from either PCA or LocaNMF (Supplementary Fig. 2). Finally, to quantify further the potential for decoding behavior from our neural data, we considered a fully connected artificial neural network (Methods). However, this nonlinear approach showed no improvement over our joint linear model (Supplementary Fig. 2).

To investigate the relative contributions of activity versus connectivity dynamics in modeling behavior, we recreated the joint model while temporally (circularly) shuffling one of the two predictors. Shuffling either term significantly impaired prediction accuracy relative to the unshuffled full model (pupil: $R^2 = 0.2 \pm 0.04$, $P = 0.001$ for shuffling $\phi_t^{(20)}$, $R^2 = 0.48 \pm 0.04$, $P = 0.02$ for shuffling $\mathbf{x}_t$; face: $R^2 = 0.38 \pm 0.05$, $P = 0.002$ for shuffling $\phi_t^{(20)}$, $R^2 = 0.49 \pm 0.06$, $P = 0.006$ for shuffling $\mathbf{x}_t$; wheel: $R^2 = 0.31 \pm 0.07$, $P = 0.01$ for shuffling $\phi_t^{(20)}$, $R^2 = 0.32 \pm 0.05$, $P = 0.003$ for shuffling $\mathbf{x}_t$, paired t test; Fig. 2f). Surprisingly, models in which correlational data were preserved performed similarly to or better than activity-preserved models in decoding behavior, reaching significance for variations in pupil diameter (pupil: $P = 0.002$, face: $P = 0.06$, wheel: $P = 0.37$, paired t test; Fig. 2f). To examine further the ability of activity or connectivity signals to predict behavioral signals independently, we generated single-predictor models, which produced similar results (Fig. 2f).

Finally, we note that, while the time-averaged activity and pairwise correlations significantly differed for high versus low epochs of behavioral state (Methods), the temporal dynamics of averaged cortical activity (across all parcels) or correlations (across all pairs of parcels) were poorly predictive of the rapid fluctuations in behavior (Supplementary Fig. 2). Altogether, these findings indicate that inclusion of rapidly time-varying functional connectivity significantly improves the

decoding power for behavioral state modeling, suggesting that cortical network function relies not only on the absolute amount of activity but also on the dynamic coordination of activity across widespread areas.

Sensory inputs modestly influence network connectivity

Spontaneous cortical activity likely reflects latent signals corresponding to internally generated brain processes. Thus, we asked whether extrinsic sensory information is similarly represented by large-scale networks. First, we looked at responses to visual stimulation outside any learning or task-based condition. We presented the mice with a series of drifting sinusoidal gratings (Methods) and quantified evoked activity using mesoscopic calcium imaging. Contrast-dependent responses were largest in visual areas, although they were also broadly observed across other cortical regions. However, evoked responses had minimal impact on the correlational structure of activity across the cortex (Supplementary Fig. 3). Linear modeling showed that the stimulus could be robustly decoded using activity in the visual cortex, with prediction accuracy exhibiting strong contrast dependence (Spearman's $R = 0.9 \pm 0.1$, $P = 0.0001$). However, prediction accuracy using the mesoscopic correlational structure was significantly lower ($P = 5.6 \times 10^{-5}$, paired t test) and not coupled to stimulus contrast (Spearman's $R = 0.1 \pm 0.2$, $P = 0.25$; Supplementary Fig. 3).

We then asked whether network correlational structure is more affected by sensory inputs with learned association to behavior. We adopted a visually cued eyeblink task in which the drifting grating stimulus is paired with an aversive corneal air puff (Methods). Our previous work showed that task performance is contrast dependent and requires activity in the primary visual cortex⁹. As with untrained animals, the visual stimulus evoked a large response in the primary visual cortex and surrounding areas but did not produce significant changes in the pairwise correlations between areas. However, linear modeling based on either primary visual cortex activity or mesoscopic correlations robustly predicted trial occurrence in a contrast-dependent manner (Spearman's $R = 0.91 \pm 0.05$, $P = 2.0 \times 10^{-5}$ for activity and Spearman's $R = 0.59 \pm 0.17$, $P = 0.01$ for embedded correlations), although the former again significantly outperformed the latter ($P = 5.8 \times 10^{-5}$, paired t test; Supplementary Fig. 3). These results suggest that brief sensory inputs drive large fluctuations in cortical activity, with their impact on network correlations exhibiting sensitivity to the behavioral relevance of the stimulus.

Dynamic correlations of cellular networks predict behavior

To determine the generalizability of our approach and examine encoding by neural correlations at a different spatial scale, we monitored local circuit activity using cellular-resolution two-photon calcium imaging of GCaMP6s-expressing neurons³⁸ in the primary visual cortex (Methods and Fig. 3a,b). As above, we looked at both time-varying activity, $\mathbf{x}_t$, and embedded pairwise correlations, ϕ_t , for identified neurons simultaneously with measurements of pupil diameter, facial movement and locomotion (Fig. 3c–f). Unlike large-scale network signals, correlations between neurons were broadly distributed around zero (average $r = -0.001 \pm 0.006$, average $CV_r = 6.1 \pm 1.47$, $n = 6$ mice).

We then generated a cross-validated linear model combining activity and embedded correlation dynamics across cells and attempted to predict rapid fluctuations in behavior. As with mesoscopic imaging, cellular data also robustly predicted behavior (pupil: $R^2 = 0.59 \pm 0.04$, face: $R^2 = 0.44 \pm 0.08$, wheel: $R^2 = 0.39 \pm 0.1$, $n = 6$ mice; Fig. 3). Again, modeling performance was poorer when using raw correlations and Euclidean distances for embedding but was robust to changes in model parameters (Supplementary Fig. 4).

To calculate the relative contributions of activity versus correlations in the joint model, we similarly shuffled one of the two predictors. As above, shuffling either variable significantly impaired prediction accuracy relative to the unshuffled model (pupil: $R^2 = 0.53 \pm 0.05$, $P = 0.04$ for shuffling $\phi_t^{(20)}$, $R^2 = 0.42 \pm 0.05$, $P = 0.0002$ for shuffling $\mathbf{x}_t$;

face: $R^2 = 0.39 \pm 0.09$, $P = 0.004$ for shuffling $\phi_t^{(20)}$, $R^2 = 0.32 \pm 0.05$, $P = 0.03$ for shuffling $\mathbf{x}_t$; wheel: $R^2 = 0.32 \pm 0.1$, $P = 0.04$ for shuffling $\phi_t^{(20)}$, $R^2 = 0.26 \pm 0.07$, $P = 0.02$ for shuffling $\mathbf{x}_t$, paired t test; Fig. 3g). Models preserving either activity or correlational data provided similar accuracy, with activity-based analysis showing modestly better performance for pupil fluctuations ($P = 0.025$ for pupil, $P = 0.14$ for face, $P = 0.15$ for wheel, paired t test; Fig. 3g). Single-predictor models again produced similar results (Fig. 3g). In summary, applying our approach for quantifying time-varying correlations in neural data to cellular-resolution imaging, we again found that including dynamic functional connectivity significantly enhances prediction accuracy in models linking neural signals to fluctuations in behavioral state.

Dynamic connectivity suggests distinct cortical subnetworks

The improved accuracy of behavioral prediction using embedding of mesoscopic correlation matrices suggests that these correlations may reflect underlying principles of structural organization in large-scale cortical networks. Therefore, we examined the spatial interpretation of ϕ_t by asking how the time-varying correlation for each pair of parcels is represented by the overall embedding. This approach allowed us to determine whether the embedding primarily captures subsets of pairwise correlations. We quantified the goodness of fit using $\phi_t^{(20)}$ to model the correlation time series between a target parcel and each other parcel across the cortex (Fig. 4a,b and Methods). Averaging these goodness-of-fit matrices across all animals ($n = 6$ mice) revealed substantial within-subject spatial heterogeneity that was conserved across different individuals. The overall embedding primarily represented correlations between each target parcel and one or both of a posterior and an anterolateral subdivision of the cortex (Fig. 4c and Supplementary Fig. 5). This spatial pattern was clearly evident after creating a grand average across all parcels and animals (Fig. 4d). Intuitively, this result indicates that, independent of behavior, the dynamic large-scale correlations of cortical areas are dominated by the interrelationship of each cortical parcel to one or both of these two subnetworks. Surprisingly, this functional organization is distinct from the cortical segmentation defined by traditional, anatomy-based atlases such as the CCFv3 (Fig. 4d). In particular, the anterolateral region includes rostral representations in the primary motor cortex and upper limb, mouth and nose representations in the primary somatosensory cortex. The posterior region includes visual, auditory and parietal association areas.

To examine further whether coordinated activity across this anterolateral–posterior partition preferentially encodes spontaneous behavioral variations, we systematically bisected the cortex with a line rotated about the midpoint (Fig. 4d). We modeled behavior using either (1) the activity in these two parcels or (2) the correlations between the two parcels. Then, we quantified the prediction accuracy as a function of the angle of division. Accuracy using time-varying activity was independent of angle, suggesting limited spatial heterogeneity in how behavioral metrics are encoded by fluctuations in the magnitude of cortical signals (Fig. 4e and Supplementary Fig. 5). In contrast, accuracy using the time-varying correlations between the two parcels was significantly higher for the angle matching the anterolateral–posterior division noted above (Fig. 4d). This result was relatively insensitive to the window length used for embedding (Supplementary Fig. 5). Overall, these findings suggest that encoding of spontaneous behaviors by large-scale correlations is spatially organized into distinct subnetworks in the neocortex.

Dynamic connectivity across spatial scales encodes behavior

Our analyses revealed that spontaneous behaviors can be accurately decoded from the temporal dynamics of correlations between neural signals at both the local circuit and mesoscopic spatial scales. However, single neurons are embedded in large-scale networks by virtue of long-range synaptic connections. Thus, we wondered whether the

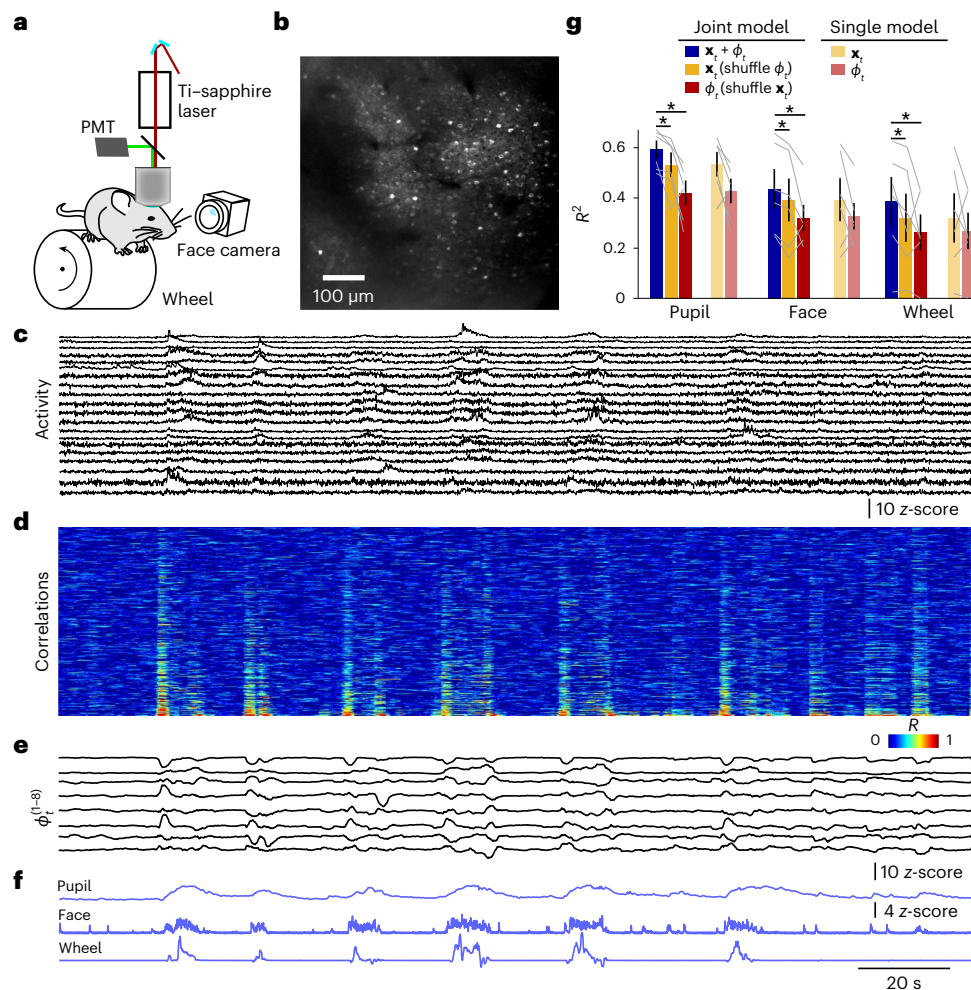


Fig. 3 | Local circuit dynamics encode spontaneous behavioral variations.

a, Schematic showing the setup for simultaneous behavioral monitoring and two-photon calcium imaging. PMT, photomultiplier tube. **b**, Example field of view showing individual GCaMP6s-expressing neurons in the visual cortex. Similar results were obtained for each of the six mice. **c**, Example time series showing neuronal activity for all neurons in the field of view. **d**, Heat map of the time series of pairwise correlations between each neuron from **c**. Data are sorted by increasing s.d. **e**, Example of the first eight diffusion-embedding components based on data in **d**. **f**, Time series for behavioral metrics corresponding to data in

c and **d**. **g**, Population data ($n = 6$ independent mice) showing the average ($\pm$ s.e.m.) prediction accuracy (R^2) for modeling behavior variables using a joint model of activity and embedded correlations (blue), a joint model with shuffled ϕ_t (yellow), a joint model with shuffled activity (red), a single-predictor model using activity (pale yellow) and a single-predictor model using ϕ_t (pale red). * $P < 0.05$ for a two-sided paired t test (see the main text). Full model compared to shuffling $\phi_t^{(20)}$: $P = 0.04$ for pupil, $P = 0.03$ for face and $P = 0.02$ for wheel. Full model compared to shuffling $\mathbf{x}_t$: $P = 0.0002$ for pupil, $P = 0.006$ for face and $P = 0.003$ for wheel. Data for individual animals shown by light gray lines.

functional connectivity across these scales is similarly dynamic. To this end, we carried out simultaneous wide-field and cellular two-photon imaging (Fig. 5a,b and Methods)²⁶. We first analyzed these multimodal datasets separately, as described above, and quantified the accuracy with which linear models based on time-varying activity, $\mathbf{x}_t$, or embedded correlations, $\phi_t^{(20)}$, could predict behavior. Activity was predictive for mesoscopic (pupil: $R^2 = 0.13 \pm 0.03$, face: $R^2 = 0.14 \pm 0.04$, wheel: $R^2 = 0.12 \pm 0.04$, $n = 7$ mice) and cellular (pupil: $R^2 = 0.31 \pm 0.04$, face: $R^2 = 0.4 \pm 0.07$, wheel: $R^2 = 0.5 \pm 0.08$) data. Similarly, embedded correlations were also predictive for mesoscopic (pupil: $R^2 = 0.35 \pm 0.03$, face: $R^2 = 0.38 \pm 0.06$, wheel: $R^2 = 0.51 \pm 0.06$) and cellular (pupil: $R^2 = 0.14 \pm 0.03$, face: $R^2 = 0.12 \pm 0.05$, wheel: $R^2 = 0.15 \pm 0.06$) data (Fig. 5c). As above, correlation-based prediction accuracy was greater for mesoscopic data (pupil: $P = 0.002$, face: $P = 0.004$, wheel: $P = 0.0002$, paired t test, $n = 7$ mice), and activity-based prediction accuracy was greater for cellular data (pupil: $P = 0.0001$, face: $P = 0.001$, wheel: $P = 0.0004$, paired t test, $n = 7$ mice).

To explore functional connectivity across spatial scales, we developed a strategy to calculate the time-varying correlations between cells

and parcels for a sliding 3-s window followed by diffusion embedding (Fig. 5d,e and Methods). This analysis revealed considerable heterogeneity in the degree of multimodal correlation dynamics exhibited by different cells, measured as the standard deviation of correlation fluctuations averaged for a single cell across all brain parcels across the imaging session (Fig. 5e, Supplementary Fig. 6 and Methods). The variations in both the correlations and the embedding components appeared to track with behavioral metrics (Fig. 5e–g). Indeed, embedding of the correlations for the dual-imaging data predicted fluctuations in pupil diameter, facial movement and locomotion (Fig. 5h), with results robust across a range of model parameters (pupil: $R^2 = 0.29 \pm 0.06$, face: $R^2 = 0.16 \pm 0.03$, wheel: $R^2 = 0.22 \pm 0.06$, $n = 7$ mice; Supplementary Fig. 6). Shuffling any one of the correlation signals (mesoscopic, cellular or dual) did not significantly reduce model prediction accuracy, suggesting a substantial amount of overlap in the information represented across spatial scales.

We explored the spatial interpretation of the dual mesoscopic–cellular embedding by quantifying the accuracy with which the correlations between a single neuron and the mesoscopic parcels are

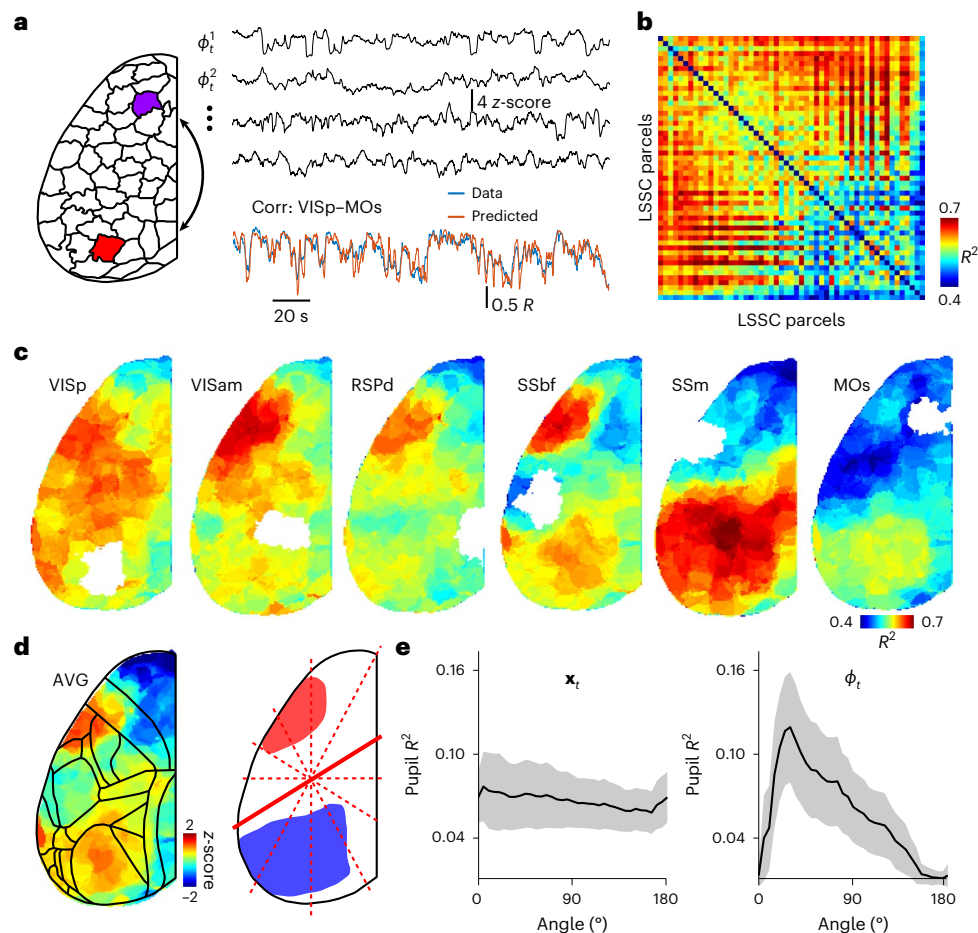


Fig. 4 | Dynamic functional connectivity reveals distinct cortical subnetworks. **a**, Left, illustration of LSSC-based parcellation, highlighting two parcels corresponding approximately to the supplementary motor cortex (MOs, purple) and primary visual cortex (VISp, red) based on the CCFv3. Right, example components of correlation embedding for one animal (black), pairwise time-varying correlation between the primary visual cortex and supplementary motor cortex (blue) and the predicted primary visual cortex–supplementary motor cortex correlation (Corr) based on embedding (red). **b**, Example matrix from one animal showing the goodness of fit (R^2) for modeling the time-varying correlations between each pair of parcels using $\phi_t^{(20)}$. **c**, Average ($n = 6$ mice) maps showing the mean R^2 values for modeling the pairwise correlations of each cortical parcel with the indicated target parcel (shown in white). VISp,

anteromedial visual area; RSPd, retrosplenial area, dorsal part; SSbf, barrel field region of the somatosensory cortex; SSm, mouth region of the somatosensory cortex–cortex. **d**, Left, grand average (AVG) map showing z-scored R^2 values as in **c**, collapsed across all animals ($n = 6$) and all cortical parcels. Right, schematic of the anterolateral (red) and posterior (blue) subnetworks derived from data in **c**. Red dashed lines indicate angles for bisecting LSSC parcels into arbitrary subnetworks, with the solid line (30°) corresponding to the anterolateral–posterior division. **e**, Left, average ($\pm$ s.e.m. (shaded area)) prediction accuracy for modeling pupil fluctuations based on the time-varying activity in two subnetworks determined by the bisecting lines in **d**. Right, average ($\pm$ s.e.m. (shaded area)) prediction accuracy for pupil fluctuations based on the time-varying correlation between two subnetworks determined by the lines in **d**.

represented by the overall embedding. In general, cells with the most dynamic correlations (largest s.d.) exhibited the strongest spatial heterogeneity in their modeling accuracy (Fig. 5i and Supplementary Fig. 6). However, the spatial pattern was generally conserved for all cells and was clearly evident after averaging across the population (Fig. 5j), again showing a division of the cortex into anterolateral and posterior subnetworks, with cells in the visual cortex being dynamically correlated most strongly with the latter. These results provide an additional independent demonstration that these subdivisions reflect a fundamental organizing principle in the cortex.

Discussion

Our results show that functional connectivity in cortical networks is highly dynamic, varying on a subsecond timescale that tracks with continuous metrics of spontaneous behaviors. Including these dynamic correlations in a linear model predicted behavioral fluctuations with high accuracy. This result was true for both large-scale networks monitored with mesoscopic calcium imaging and local

networks monitored at cellular resolution with two-photon microscopy. Moreover, combining these modalities revealed that behavior is also accurately represented by the dynamic correlations between local and cortex-wide networks. The spatial organization of dynamic correlations between either parcels or neurons and cortex-wide activity revealed two distinct subnetworks not obviously predicted from standard anatomical divisions.

The representation of behavioral information by time-varying cortical signals has been a focus of recent studies using diverse approaches to monitor brain activity^{1,2,10,15,22,39}. In rodent models, variations in behavioral state or arousal are coupled to changes in firing rate, pairwise correlations and neuromodulatory signaling^{11–14,40,41}. In particular, arousal- and motor-related variables (for example, pupil diameter, locomotion, whisking) are represented at the cellular and network scales^{11,12,14}. Several groups have also demonstrated that the spatial patterns of large-scale activity in the neocortex are markedly different when comparing across state^{14,16,20}. For example, spontaneous cortical activity can be decomposed, using various methods, into

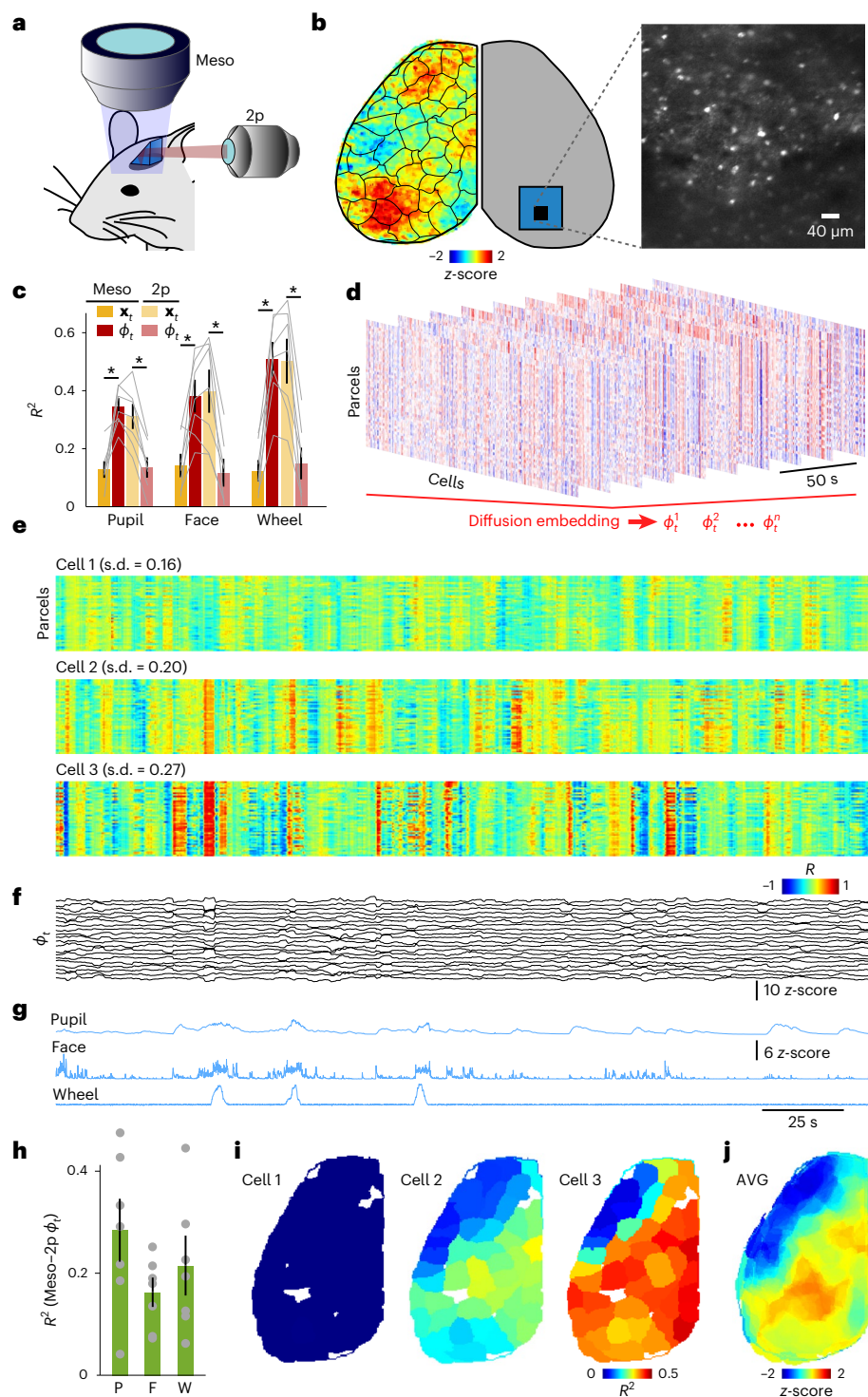


Fig. 5 | Functional connectivity across spatial scales encodes behavior.

a, Schematic showing the setup for simultaneous mesoscopic (Meso) and two-photon (2p) imaging. **b**, Left, example mesoscopic imaging frame and schematic of microprism placement in the contralateral hemisphere. Right, example two-photon imaging frame collected through the prism. **c**, Population data ($n = 7$ independent mice) showing the average ($\pm$ s.e.m.) prediction accuracy (R^2) for modeling behavior variables using either activity (yellow) or ϕ_t (red) for mesoscopic or two-photon imaging data. * $P < 0.05$ for a two-sided paired t test (see the main text). Comparing $\phi_t^{(20)}$ to $\mathbf{x}_t$ for mesoscopic data, pupil: $P = 0.002$, face: $P = 0.004$, wheel: $P = 0.0002$; for cellular data, pupil: $P = 0.0001$, face: $P = 0.001$, wheel: $P = 0.0004$. Data for individual animals shown by light gray lines. **d**, Example sequential multimodal correlation matrices, derived from a sliding window applied to neural activity from mesoscopic (parcels) and

two-photon (cells) imaging, used for diffusion embedding. **e**, Dynamic multimodal correlation time series for three example cells, where each row represents a mesoscopic parcel. The s.d. of correlation values over time, averaged across all rows, is indicated. **f**, Example of the first 20 diffusion-embedding components from the same animal ($n = 243$ cells, 47 parcels). **g**, Time series for behavioral metrics corresponding to data in **e** and **f**. **h**, Population data ($n = 7$ independent mice) showing the average ($\pm$ s.e.m.) prediction accuracy (R^2) for modeling behavior variables using ϕ_t derived from the embedding of dual mesoscopic and two-photon correlations. Data for individual animals shown by light gray dots. **i**, Example maps for the cells in **e** showing the R^2 values for modeling the correlation of the cell with each parcel using the overall diffusion embedding. **j**, Grand average map showing z-scored R^2 values as in **i**, collapsed across all animals ($n = 6$) and all cells and cortical parcels.

repeating spatiotemporal motifs that may correspond to sensory or motor signals²⁰. Similarly, in human study participants, shifts in wakefulness correspond to changes in average resting-state connectivity⁴², resting-state fluctuations predict somatosensory perception⁴, and performance in working-memory-based tasks corresponds to spatially heterogeneous variation in timescales of patterned activity^{43,44}. Time-varying functional connectivity in functional magnetic resonance imaging (fMRI) studies has recently received considerable focus, with ongoing debates on the mechanisms and behavioral relevance of coordinated signals over large-scale networks¹⁵. Indeed, a recent study also demonstrated the utility of Euclidean manifold learning in reducing the dimensionality of connectivity matrices²², suggesting the generality of this approach.

Although some prior efforts to characterize spontaneous behaviors have relied on categorical definitions of state, our data indicate that variations in pupil diameter, facial movement and locomotion do not appear to cluster into distinct regimes, a result more consistent with continuously and rapidly varying states. The exact mapping of these observed motor signals onto latent neural variables (and whether they are independent) is not well understood. For example, all three behavioral metrics are linked to variations in cholinergic and adrenergic activity^{41,45,46}. We also recently found that acetylcholine release is more robustly linked to facial movement (for example, whisking) than locomotion¹⁴. Both Stringer et al.¹² and Musall et al.¹¹ demonstrated that rapid dynamics of behavioral variables could accurately encode neural activity across the cortex. Here, we further explored the dynamics of functional connectivity expressed as the correlation between cortical parcels or neurons. Intuitively, time-varying activity and pairwise correlations can be viewed as first- and second-order terms in a Taylor expansion of a function relating behavior to neural signals. Thus, correlations cannot be linearly derived from the underlying activity and represent a potential mechanism to encode an independent component of behavioral dynamics. This conclusion is strongly supported by our results in which shuffling either activity or correlations significantly reduced modeling accuracy for both mesoscopic and two-photon imaging data.

The temporal scale of neural and behavioral dynamics is similar to synaptic integration windows for single cells, suggesting that neurons may be sensitive to convergent synaptic inputs driven by correlated large-scale activity. Our results from combining mesoscopic and two-photon imaging demonstrate that the functional connectivity across these divergent spatial scales is also dynamic and accurately predictive of fluctuations in behavior. Thus, we propose that network activity within and across spatial scales in the neocortex is coordinated as a function of spontaneous behaviors. In the near future, the ongoing development of multimodal approaches (for example, combining fluorescence imaging, fMRI and electrophysiology^{26,47–49}) will likely drive additional discoveries into the functional organization of brain networks in diverse systems.

Our method for viewing time-varying functional connectivity in cortical networks as a graph of graphs provides a conceptual and analytical framework for extracting the intrinsic dynamics of short-term correlations and uses Riemannian geometry to correctly evaluate distances between correlation matrices extracted at different time points (we note that similar analyses using Euclidean geometry yielded substantially poorer prediction accuracy). These distances are then used to set the weights of a graph of graphs, allowing us to extract a low-dimensional representation for the manifold of the correlations and capture their underlying dynamics. A similar strategy has also been applied to fMRI data²². Using this approach and including both first-order (activity) and nonlinear second-order (embedded functional connectivity) terms for modeling behavior enabled us to improve decoding power significantly. Our method was generalizable for three different datasets (mesoscopic and two-photon imaging alone and in combination) and yielded the surprising finding that higher-order

statistics (that is, correlational signals) can produce similar or better predictive accuracy than time-varying changes in activity.

Several distinct strategies have been developed to analyze the spatiotemporal organization of network activity, including singular-value decomposition and non-negative matrix factorization^{16,33}. Here, we show that parcellation of cortical regions³¹, followed by embedding of time-varying correlations based on Riemannian geometry, provides a robust means to quantify dynamic functional connectivity that accurately decodes spontaneous fluctuations in behavior. Notably, our overall findings were similar for a number of structural or functional approaches for segmenting the cortex. We found that LSSC-based parcellation provides robust results with a good balance of reconstruction errors and number of disjoint parcels. With the increasing interest in the analysis of neural manifolds, our results also highlight the necessity of considering the geometry of the manifold (that is, Riemannian versus Euclidean) on which the data lie to reveal their intrinsic representation accurately. Finally, our approach modestly outperformed a neural network-based model. This finding suggests that much of the information linking neural signals and spontaneous behavior is present in the dynamics of activity and correlations.

Surprisingly, the representation of task-independent sensory information in mesoscopic correlations was relatively weak. However, the representation of conditioned sensorimotor events was more robust, indicating that functional connectivity may be altered by external inputs with learned behavioral relevance. These findings are consistent with recent work suggesting that spontaneous behavior and external stimuli can be represented in orthogonal dimensions¹², but task-relevant behaviors are broadly present in large-scale patterns of network activity^{11,29,50}. Additionally, we and others have shown that training can modify the sensory and motor representations by single cortical neurons^{9,51–53}. Future studies must continue to explore how development, experience and learning shape the functional organization of large-scale networks.

Finally, our results show that the cortex can be spatially segmented into two broad subnetworks (anterolateral and posterior)—a functional division that emerges from the analysis of spontaneous activity and reflects variations in behavioral state metrics. We previously demonstrated such a division based on correlations between single-cell activity and mesoscopic cortical signals²⁶, a result that is also present in our dual mesoscopic and two-photon imaging data. Work from other laboratories using wide-field imaging has also provided evidence for the existence of spatially similar state-dependent subnetworks^{17,48}. Intriguingly, large-scale network activity may also be cell-type specific, as recent work found distinct patterns for different populations of layer 5 projection neurons^{29,50}. Interestingly, our functional subnetworks do not map readily onto standard anatomical segmentations of the cortex, such as the CCFv3 (ref. 32). For example, the anterolateral division distinctly omits more caudal motor and somatosensory representations, whereas the posterior division broadly encompasses visual, auditory and parietal regions while omitting retrosplenial areas. The mechanisms driving these partitions are unclear but may reflect poorly mapped intracortical connections, heterogeneous neuromodulatory signaling or indirect connections through subcortical hubs such as the thalamus^{14,54}. Indeed, recent work using BRICseq (brain-wide individual animal connectome sequencing) found anatomical evidence for inter-region connectivity that can support such disjoint subnetworks⁵⁵. Finally, we hypothesize that the dynamic modulation and plasticity of synaptic strength may support the translation between such structural and functional views of connectivity in cortical networks—a hypothesis that awaits experimental validation.

Online content

Any methods, additional references, Nature Portfolio 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/s41593-023-01498-y>.

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Methods

All animal handling and experiments were performed according to the ethical guidelines of the Institutional Animal Care and Use Committee of the Yale University School of Medicine. Some of the mesoscopic imaging data were collected as part of a previous study¹⁴, with experimental details provided below for clarity. The analysis results presented here represent new findings and have not appeared elsewhere.

Animals

Adult (postnatal day 60 (P60)–P100) male and female C57/BL6 mice ($n = 19$ mice in total) were kept on a 12-h light–dark cycle, provided with food and water ad libitum, and housed individually following headpost implants. The animal housing facility was kept between 22 and 24 °C and 60–70% humidity. Imaging experiments were performed during the light phase of the cycle. For most mesoscopic imaging experiments, brain-wide expression of jRCaMP1b²⁵ was achieved by postnatal sinus injection, as described previously^{26,27}. Briefly, P0–P1 litters were removed from their home cage and placed on a heating pad. Pups were kept on ice for 5 min to induce anesthesia through hypothermia and then maintained on a metal plate surrounded by ice for the duration of the injection. Pups were injected bilaterally with 4 μ l of AAV9-hSyn-NES-jRCaMP1b (2.5×10^{13} genome copies per ml, Addgene). Mice were also injected with AAV9-hSyn-GRAB_{ACh3.0} to express the genetically encoded cholinergic sensor GRAB_{ACh3.0} (ref. 56), although these data were not used in the present study. Once the entire litter was injected, the pups were returned to their home cage. For two-photon imaging and eyeblink conditioning experiments, a similar procedure was used to drive cortex-wide expression of GCaMP6s³⁸. For dual mesoscopic and two-photon imaging experiments, adult (P60–P70) mice transgenically expressing GCaMP6s in cortical excitatory neurons (CaMK2a-tTA;tetO-GCaMP6s;VIP-Cre)⁵⁷ were used. These animals were also injected with adeno-associated virus driving the expression of Cre recombinase (Cre)-dependent GCaMP6s and Cre-dependent tdTomato, although all red fluorescent cells were excluded from the present analysis. For eyeblink conditioning experiments, we used adult (P60–P70) CaMK2a-tTA;tetO-GCaMP6s mice.

Surgical procedures

All surgical implant procedures were performed in adult mice (>P50). Mice were anesthetized using 1–2% isoflurane and maintained at 37 °C for the duration of the surgery. For mesoscopic imaging, the skin and fascia above the skull were removed from the nasal bone to the posterior of the intraparietal bone and laterally between the temporal muscles. The surface of the skull was thoroughly cleaned with saline, and the edges of the incision were secured to the skull with Vetbond. A custom titanium headpost was secured to the skull with transparent dental cement (Metabond, Parkell), and a thin layer of dental cement was applied to the entire dorsal skull surface. Next, a layer of cyanoacrylate (Maxi-Cure, Bob Smith Industries) was used to cover the skull and left to cure for ~30 min at room temperature to provide a smooth surface for transcranial imaging. A similar procedure was used to prepare mice for two-photon imaging, with additional implantation of a dual-layer glass window into a small (~4-mm square) craniotomy placed over the left primary visual cortex. The edges of the window were sealed to the skull with dental cement. For dual mesoscopic and two-photon imaging, a 2-mm glass micropism (Tower Optical) was placed on top of a dual-layer glass window implanted over the right primary visual cortex²⁶.

Mesoscopic imaging

Wide-field mesoscopic calcium imaging was performed using a Zeiss Axio Zoom microscope with a 1 $\times$, 0.25-numerical-aperture (NA) objective having a 56-mm working distance (Zeiss). Epifluorescent excitation was provided by an LED bank (SPECTRA X Light Engine, Lumencor) using two output wavelengths: 395/25 nm (isosbestic for GRAB_{ACh3.0})

and 575/25 nm (jRCaMP1b). Emitted light passed through a dual-camera image splitter (TwinCam, Cairn Research) and then through either a 525/50-nm (GRAB_{ACh3.0} or GCaMP6s) or 630/75-nm (jRCaMP1b) emission filter (Chroma) before it reached two sCMOS cameras (Orca-Flash V3, Hamamatsu). Images were acquired at 512 $\times$ 512 resolution after 4 $\times$ pixel binning, and each channel was acquired at 10 Hz with 20-ms exposure using HCLImage software (Hamamatsu).

Two-photon imaging

Two-photon imaging was performed using a MOM microscope (Sutter Instruments) coupled to a 16 $\times$, 0.8-NA objective (Nikon). Excitation was driven by a titanium–sapphire laser (Mai Tai eHP DeepSee, Spectra-Physics) tuned to 920 nm. Emitted light was collected through a 525/50-nm filter and a gallium arsenide phosphide photomultiplier tube (Hamamatsu). Images were acquired at 512 $\times$ 512 resolution at 30 Hz using a galvo-resonant scan system controlled by ScanImage software (Vidrio).

Dual mesoscopic and two-photon imaging

Dual imaging was carried out using a custom microscope combining Zeiss Axio Zoom (as above) and Sutter MOM (as above), as described previously²⁶. A long-working-distance objective (20 $\times$, 0.4 NA, Mitutoyo) was used to image through the implanted prism. Frame acquisitions were interleaved with an overall rate of 9.15 Hz, with each cycle alternating sequentially between a 920-nm two-photon acquisition (512 $\times$ 512 resolution), a 395/25-nm wide-field excitation acquisition and a 470/20-nm wide-field excitation acquisition. Wide-field data were collected through a 525/50-nm filter into an sCMOS camera (ORCA-Fusion, Hamamatsu) at 576 $\times$ 576 resolution after 4 $\times$ pixel binning with 20-ms exposure.

Behavioral monitoring

All imaging was performed in awake, behaving mice that were head-fixed so that they could freely run on a cylindrical wheel. A magnetic angle sensor (Digikey) attached to the wheel continuously monitored wheel motion. Mice received at least three wheel-training habituation sessions before imaging to ensure consistent running bouts. During wide-field imaging sessions, the face (including the pupil and whiskers) was illuminated with an infrared LED bank and imaged with a miniature CMOS camera (Blackfly S USB3, FLIR) with a frame rate of 10 Hz using SpinView software (FLIR).

Visual stimulation. For visual stimulation experiments, sinusoidal drifting gratings (2 Hz, 0.04 cycles per degree, 20° visual field) with varied contrast were generated using custom-written functions based on Psychtoolbox in MATLAB and presented on a liquid-crystal display monitor 20 cm from the right eye. Stimuli were presented for 2 s with a 5-s interstimulus interval.

Eyeblink conditioning. Conditioning was carried out as previously published⁹. Briefly, mice were acclimated to head fixation on the running wheel for several days. For training, each trial started with the onset of a 500-ms visual stimulus comprising a sinusoidal drifting grating (2 Hz, 0.04 cycles per degree, 20° visual field) that coterminated with a 50-ms air puff directed to the cornea. Each training day comprised 60 pairings at 100% contrast. After reaching stable performance for two to three consecutive days, mice were moved to the next stage, in which contrast was varied randomly across trials. For all sessions, trials were separated by an exponentially distributed interstimulus interval ranging from 18 to 33 s.

Data analysis

All analyses were conducted using custom-written scripts in MATLAB (MathWorks). Support vector machine (SVM) classifiers were trained using publicly available software⁵⁸. No statistical methods were used

to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications^{14,26}. Data distributions were assumed to be normal, but this was not formally tested. All animals were selected randomly from our colony for inclusion in the study, and there were no comparisons across subject groups. No blinding was used in the study, given the lack of experimental groups. No data points were excluded from the study.

Preprocessing of behavior data. Pupil diameter and facial movements were extracted from face videography using FaceMap²⁸. For subsequent analysis, facial movement was defined as the first component of FaceMap-based decomposition. Singular-value decomposition was applied to the face movie to extract the principal components explaining the distinct movements apparent on the mouse's face. Wheel position was obtained from a linear angle detector attached to the wheel axle by unwrapping the temporal phase and then computing the traveled distance (cm). Locomotion speed was computed as the differential of the smoothed distance (cm s⁻¹) using a 0.4-s window. Epochs of sustained locomotion and quiescence were extracted using change-point detection, as previously described¹⁴. High and low pupil and face epochs were extracted from within quiescence segments in which z-score-normalized values exceeded high/low thresholds of 60%/40% quantiles.

Preprocessing of mesoscopic imaging data. Imaging frames for green and red collection paths were grouped and downsampled from 512 × 512 to 256 × 256 followed by an automatic 'rigid' transformation (imregtform, MATLAB). In some cases, registration points were manually selected, and a 'similarity' geometric transformation was applied. Detrending was applied using a low-pass filter ($N = 100$, $f_{\text{cutoff}} = 0.001$ Hz). Time traces were obtained using $(\Delta F/F)_i = (F_i - F_{i,0})/F_{i,0}$, where F_i is the fluorescence of pixel i and $F_{i,0}$ is the corresponding low-pass-filtered signal.

Hemodynamic correction. Hemodynamic artifacts were removed using a linear regression method accounting for spatiotemporal dependencies between neighboring pixels¹⁴. We used the approximate isosbestic excitation of GCaMP6s or GRAB_{ACh3.0} (395 nm) as a means of measuring activity-independent fluctuations in fluorescence associated with hemodynamic signals. Briefly, given two $p \times 1$ random signals, y_1 and y_2 , corresponding to $\Delta F/F$ of p pixels for two excitation wavelengths, 'green' and 'ultraviolet', we consider the following linear models:

$$y_1 = x + z + \eta,$$

$$y_2 = Az + \xi,$$

where x and z are mutually uncorrelated $p \times 1$ random signals corresponding to p pixels of the neuronal and hemodynamic signals, respectively. η and ξ are white Gaussian $p \times 1$ noise signals and A is an unknown $p \times p$ real invertible matrix. We estimate the neuronal signal as the optimal linear estimator for x (in the sense of minimum mean-squared error):

$$\hat{x} = H \begin{pmatrix} y_1 \\ y_2 \end{pmatrix}, \quad H = \Sigma_{xy} \Sigma_y^{-1},$$

where $y = \begin{pmatrix} y_1 \\ y_2 \end{pmatrix}$ is given by stacking y_1 on top of y_2 , $\Sigma_y = \mathbb{E}[yy^T]$ is the correlation matrix of y and $\Sigma_{xy} = \mathbb{E}[xy^T]$ is the cross-correlation matrix between x and y . The matrix Σ_y is estimated directly from the observations, and the matrix Σ_{xy} is estimated by¹⁴

$$\Sigma_{xy} = \left(\Sigma_{y_1} - \sigma_\eta^2 I - \left(\Sigma_{y_1 y_2} (\Sigma_{y_2} - \sigma_\xi^2 I)^{-1} \Sigma_{y_2}^{-1} \Sigma_{y_1 y_2}^T \right)^T 0 \right),$$

where σ_η^2 and σ_ξ^2 are the noise variances of η and ξ , respectively, and I is the $p \times p$ identity matrix. The noise variances σ_η^2 and σ_ξ^2 are evaluated according to the median of the singular values of the corresponding correlation matrices Σ_{y_1} and Σ_{y_2} (ref. 59). This analysis is usually performed in patches where the size of the patch, p , is determined by the amount of time samples available and estimated parameters. In the present study, we used a patch size of $p = 9$. The final activity traces were obtained by z-scoring the corrected $\Delta F/F$ signals per pixel.

Parcellation of mesoscopic data using LSSC. Functional parcellation of mesoscopic data was performed primarily using LSSC³¹. Briefly, this method identifies areas of coactivity by building a graph in which nodes are pixels and edge weights are determined by pairwise similarities between activity traces of pixels obtained by the following kernel:

$$K(i, j) = \exp \left\{ -\|(\Delta F/F)_i - (\Delta F/F)_j\|^2 / \sigma^2 \right\},$$

where σ is a parameter expressing a similarity radius. A row-stochastic matrix P is obtained by normalizing the rows such that $P = D^{-1}K$, where $D(i, i) = \sum_j K(i, j)$. The matrix P can be viewed as a transition matrix of a Markov chain of the graph, where $P(i, j)$ is the probability to jump from node (pixel) i to node (pixel) j . We obtain a nonlinear embedding of pixels by calculating the d right eigenvectors with the largest eigenvalues of P :

$$(\Delta F/F)_i \rightarrow \psi^{(n)}(i) = \begin{pmatrix} \psi^1(i) \\ \vdots \\ \psi^n(i) \end{pmatrix}.$$

Overall, by taking n to be significantly smaller than the number of time samples, every pixel is represented by a lower-dimensional embedding, $\psi^{(n)}$.

We evaluate the embedded representation $\psi^{(n)}$ and calculate the spectral embedding norm⁶⁰ of every pixel, $s_i = \|\psi^{(n)}(i)\|$. LSSC uses an iterative approach for parcellating the brain, in which the inputs are the embedded representation of all pixels, $\psi^{(n)}$; their corresponding norms, s_i , $i = 1, \dots, p$; and a list of all pixels sorted by decreasing order of the embedding norm, denoted by l . On each iteration, the following operations are performed until coverage of at least θ of the masked brain pixels is assigned to parcels:

1. Select the first item on list l (the pixel having the maximal norm, noted by i^*).
2. Select the axes in which i^* has the largest values; that is, the subset $L_{i^*} = \{\ell_1, \ell_2, \dots, \ell_{d_i}\}$ such that $|\psi^{\ell_1}(i^*)| \geq |\psi^{\ell_2}(i^*)| \geq |\psi^{\ell_3}(i^*)| \geq \dots$
3. Obtain the pixels whose embeddings are closer to $\psi^{(n)}(i)$ than to the origin based on the axes L_{i^*} and assign them to the cluster k ; that is:

$$C_k = \left\{ j \mid \|\psi^{L_{i^*}}(i^*) - \psi^{L_{i^*}}(j)\|^2 < \|\psi^{L_{i^*}}(j)\|^2 \right\}.$$

4. Remove the set C_k from the list l : $l \leftarrow l \setminus C_k$.
5. $k \leftarrow k + 1$.
6. If at least θ of the mask of the brain is assigned to a specific parcel, then break.

Therefore, the output is a set of clusters, $\{C_k\}$, in which each cluster contains the pixels in that cluster. To increase robustness, we divided every session into ten disjoint segments (folds), extracted the embedding on every fold and evaluated the embedding norm as the maximal value across all ten folds. We refined the brain parcellation by merging parcels whose time traces are correlated more than a given threshold. Overlapping pixels were assigned to the parcel with the closest centroid (in the embedding space). Additionally, unassigned isolated

pixels (if any) were assigned to the (spatially) closest parcel. Isolated pixels within the borders of more than one parcel were assigned to the closest cluster (in the embedding space). Data from each animal and session were parcellated to reach 95% coverage of the mask of the brain, where clusters were merged based on a threshold of 0.99, resulting in ~45 parcels per hemisphere. Time series for parcels were extracted by averaging values for all pixels within the parcel (see ‘Preprocessing of mesoscopic imaging data’ above).

As a comparison to LSSC, we used PCA to reduce the dimension of the wide-field signal and used LocaNMF³³ for the parcellation of mesoscopic data. As an additional comparison, we spatially decimated the mesoscopic data by a factor of 8, which is equivalent to using a fixed grid dividing the brain into patches of 8×8 pixels, used as ‘grid’ parcels.

Cellular region-of-interest extraction by LSSC. We also used LSSC to identify cell bodies from two-photon imaging data. The overall approach is similar to the parcellation process except for the stopping condition, in which iterations continue until a maximal number of cells is reached. In the refinement stage, identified cells smaller than 15 pixels were discarded, and overlapping regions were resolved by demixing³¹.

Taylor expansion for estimating behavior as a function of neuronal activity. We formulate the link between temporal dynamics of neuronal activity, $\mathbf{x}_t \in \mathbb{R}^N$, and an observed behavior, b_t , as

$$b_t = f(\mathbf{x}_t),$$

where f is an unknown function. Assuming that $f(\mathbf{x}_t)$ is two-times differentiable, we can write its second-order Taylor’s expansion as

$$\begin{aligned} b_t &= f(\mathbf{x}_t) \approx f(\bar{\mathbf{x}}) \\ &+ \sum_i \left. \frac{\partial f}{\partial x_i} \right|_{\bar{\mathbf{x}}} (x_t(i) - \bar{x}(i)) + \frac{1}{2} \sum_i \sum_j \left. \frac{\partial^2 f}{\partial x_i \partial x_j} \right|_{\bar{\mathbf{x}}} \\ &(x_t(i) - \bar{x}(i))(x_t(j) - \bar{x}(j)) + \epsilon, \end{aligned}$$

where $\bar{\mathbf{x}}$ is the average neuronal activity (across time) and ϵ is the error of neglecting higher orders of $\mathbf{x}_t$. Simplifying this equation leads to

$$b_t \approx \beta_0 + \sum_i \beta_1(i) x_t(i) + \sum_i \sum_j \beta_2(i, j) C_t(i, j) + \epsilon, \quad (3)$$

where $C_t(i, j) = (x_t(i) - \bar{x}(i))(x_t(j) - \bar{x}(j))$ is the time trace of the instantaneous interaction between brain regions i and j and β_n , $n = 0, 1, 2$, are the model parameters. Overall, equation (3) proposes a linear model for behavior based on two temporal signals—the activity $x_t(i)$ and the pairwise interaction $C_t(i, j)$, which is a nonlinear second-order function of elements of $\mathbf{x}_t$. As the elements $x_t(i)$ and $C_t(i, j)$ are linearly independent for all $i, j \in [1, N]$ can measure the decoding power of each of these two components of $\mathbf{x}_t$ and C_t independently.

In equation (3), the instantaneous interactions $C_t(i, j)$ are evaluated based on a single time point. In practice, estimation of all pairwise interactions at a single point is highly sensitive to noise. Thus, we evaluate the interactions over a short sliding time window to obtain the sample covariance $\hat{C}_t(i, j)$ as a smoothed and more robust estimation for the temporal evolution of $C_t(i, j)$:

$$\hat{C}_t(i, j) = \frac{\sum_{\tau=t-N_t/2}^{t+N_t/2} (x_\tau(i) - \bar{x}_t(i))(x_\tau(j) - \bar{x}_t(j))}{\sqrt{\sum_{\tau=t-N_t/2}^{t+N_t/2} (x_\tau(i) - \bar{x}_t(i))^2} \sqrt{\sum_{\tau=t-N_t/2}^{t+N_t/2} (x_\tau(j) - \bar{x}_t(j))^2}}, \quad (4)$$

where $\bar{x}_t(j)$ is the smoothed averaged activity:

$$\bar{x}_t(i) = \frac{1}{N_t} \sum_{\tau=t-N_t/2}^{t+N_t/2} x_\tau(i).$$

Inserting $\hat{C}_t(i, j)$ into equation (3) leads to equation (1). Overall, $\hat{C}$ is a three-dimensional tensor of parcels by parcels by time, where each element $\hat{C}_t(i, j)$ is a time trace of the instantaneous correlation coefficient between parcels i and j . For most analyses, N_t was 30 (corresponding to a 3-s moving window). In all cases, the time step was set to be one frame (0.1 s).

Riemannian projection of correlation matrices. Correlation matrices are symmetric and positive definite (SPD; that is, symmetric and full rank), and their underlying geometry is a manifold shaped like a cone with a Riemannian metric (Supplementary Fig. 2)^{61,62}. The distance between two correlation matrices on this cone is defined by the geodesic distance, the length of the arc connecting these matrices, whereas the Euclidean distance is not an accurate measure for this geodesic distance. To accurately capture distances between SPD matrices, Riemannian geometry is often used to project them onto a tangent Euclidean space where the geodesic length is approximated by the Euclidean distances between the corresponding projections. This evaluation becomes more accurate when the plane is tangent to the cone at a point that is relatively close to all relevant matrices, usually taken as their Riemannian mean.

Briefly, let $\{C_k\}$ be a set of K SPD matrices. Denote $\bar{C}$ as the Riemannian mean of the set and $\bar{S}$ as its equivalent in the tangent plane. $\bar{C}$ and $\bar{S}$ are calculated using the following iterative equations:

$$\bar{S}_n = \frac{1}{K} \sum_{k=1}^K \bar{C}_n^{-1/2} \log \left(\bar{C}_n^{-1/2} C_k \bar{C}_n^{-1/2} \right) \bar{C}_n^{1/2},$$

$$\bar{C}_{n+1} = \bar{C}_n^{-1/2} \exp \left(\bar{C}_n^{-1/2} \bar{S}_n \bar{C}_n^{-1/2} \right) \bar{C}_n^{1/2},$$

where $\log(\cdot)$ and $\exp(\cdot)$ are the matrix logarithm and matrix exponential, respectively, and where the Euclidean mean is used to initialize $\bar{C}_0 = \frac{1}{K} \sum_{k=1}^K C_k$. Convergence is obtained when the Frobenius norm of $\bar{S}_n$ is smaller than a preset parameter ϵ : $\|\bar{S}_n\|_F < \epsilon$.

The projections of $\{C_k\}$ onto the tangent plane to the cone at the Riemannian mean are given by

$$S_k = \bar{C}^{-1/2} \log \left(\bar{C}^{-1/2} C_k \bar{C}^{-1/2} \right) \bar{C}^{1/2}, \quad k = 1, \dots, K.$$

As presented previously⁶³, the pairwise distances $d_R^2(C_k, C_l)$ on the cone between correlation matrices $\{C_k\}$ can be approximated by the Euclidean distances between their corresponding projections $\{S_k\}$:

$$d_R^2(C_k, C_l) \cong \|\bar{S}_k - \bar{S}_l\|^2,$$

where $\bar{S}_k = \log \left(\bar{C}^{-1/2} C_k \bar{C}^{-1/2} \right)$. This method requires that all matrices $\{C_k\}$ be full rank^{64,65}. In practice, this is not always the case when the number of time points for the evaluation of correlation matrices is smaller than p (that is, $N_t < p$). Therefore, we add a regularization term, λI , to each correlation matrix C_k (ref. 66), where λ is set to the median of the singular values of $\mathbf{x}_t$ (ref. 59).

Dimensionality reduction by diffusion embedding. The series of matrices C_t are symmetrical; therefore, the dimension of their projections, $\{S_t\}$, is equal to $\left(\frac{p}{2}\right)$, resulting in a high-dimensional signal. To

analyze the dynamics of this signal, we used diffusion geometry to obtain a low-dimensional representation capturing the dynamical properties of the correlation traces. Unlike LSSC, in which we reduce the dimension across time samples, we reduce the dimension of parcels in this case; we evaluated the $N_t \times N_t$ kernel matrix of $\{S_t\}$:

$$A(i, j) = \exp\{-\|S_i - S_j\|^2 / \sigma^2\}, \quad (5)$$

where σ is a scale parameter evaluated as the median of pairwise distances between each projected matrix and its k -nearest neighbors, where $k = 20$. Note that our results are highly robust to variations in this parameter (Supplementary Fig. 2).

Normalizing the kernel A to be row stochastic and taking the right eigenvectors lead to the low-dimensional representation for the correlation traces:

$$S_t \rightarrow \phi_t^{(n)} = \begin{pmatrix} \phi_t^1 \\ \vdots \\ \phi_t^n \end{pmatrix}.$$

The size of the kernel matrix A is determined by the available time points recorded in each experiment ($N_T \approx 10^4$ for all sessions). To reduce computational complexity involving eigenvalue decomposition of large matrices, we used Nyström out-of-sample extension⁶⁷, as follows: we randomly choose a smaller subset of time points, $\{t_i\}_{i=1}^N$, where $N < N_T$. We obtain the normalized kernel using these time points and extract its eigendecomposition, $\lambda_k, \phi_{t_i}^k, k = 1, \dots, n$. We then extend this low-dimensional representation to all time points:

$$\phi_t^k = \frac{1}{\lambda_k} \sum_{i=1}^N \exp\{-\|S_t - S_{t_i}\|^2 / \sigma^2\} \phi_{t_i}^k.$$

For comparison to using Riemannian geometry for calculating distances in the kernel, we carried out similar diffusion embedding based on Euclidean distances (Supplementary Fig. 2).

Dimensionality reduction by PCA. As a comparison to LSSC, we also used PCA to reduce the dimensionality of wide-field data (Supplementary Fig. 2). Principal components were derived using the ‘pca’ function in MATLAB.

Visual response analysis. Visual responses for unconditioned and conditioned experiments were evaluated, per parcel, as the difference between the peak response during stimulus presentation and the average activity during the preceding 2 s. The responses were averaged per contrast value and normalized by the response to 100% contrast. To quantify the accuracy with which visual responses are encoded by visual activity or embedded network activity/correlations, we trained a binary classifier (linear SVM, LIBSVM) to separate the visual response and the 2 s before stimulus onset. We used tenfold cross-validation to estimate the classification accuracy for every contrast value based on each predictor.

Modeling behavior. Behavioral variables (pupil diameter, facial movements, running speed) were modeled using linear ridge regression (unless otherwise mentioned) with tenfold cross-validation. Each session was divided into ten disjoint continuous segments, where, on each fold, one segment was set aside for testing and the other segments were used for training. We assessed the predictive power of neuronal activity using the following predictors: raw activity and smoothed activity (using a 3-s moving window) and diffusion embedding based on Euclidean distances. For pairwise correlations, we used raw correlation traces, PCA with the number of components selected to account for >95% variance, and diffusion embedding of correlation traces using either Euclidean or Riemannian distances. To compare the predictive power of activity versus embedded correlations directly, we combined these predictors and evaluated the goodness of fit of the joint model. We then circularly shuffled either activity or embedded correlations through time and trained the resulting model to assess $R^2_{\text{shuffled activity}}$ and $R^2_{\text{shuffled } \phi_t}$.

As a comparison to linear modeling, we trained three fully connected artificial neural networks for the prediction of pupil/FaceMap/

running speed based on neuronal activity traces as predictors. We performed a grid search for the optimal configuration for the prediction of behavior based on tenfold cross-validation in which, on each fold, 80% was used for training, 10% was used for parameter tuning and 10% was used for testing. Overall, optimal performance was achieved with two inner layers of 64 and 4 units, a learning rate of 0.05 and ReLU (rectified linear unit) activation.

Modeling correlation data by embedding. To quantify the relationship between the embedding of functional connectivity across the cortex, ϕ_c , and the time-varying correlation between specific pairs of parcels, we used linear regression (tenfold cross-validation) and obtained an R^2 value for every pairwise correlation trace. To match LSSC parcels across animals, we identified the LSSC parcels whose center of mass was closest to each CCFv3 brain parcel (23 parcels overall in a single hemisphere) and extracted a 23×23 matrix of R^2 values per session. We averaged these matrices across animals and extracted the rows corresponding to individual parcels. Each row was then represented as a separate brain map image, color-coded by the R^2 value corresponding to the correlation between the target (specific to that image) and each of the other parcels.

Evaluating predictive dynamics of cortical subnetworks. We evaluated the average activity (across parcels) of two subcortical networks using an arbitrary partition of the brain with a line bisecting the neo-cortex. We then used these two time traces to predict pupil size, facial movement and locomotion speed. By rotating the line at 30°, 60°, 90°, 120° and 150°, we measured the R^2 values for the prediction of behavioral variables based on different ways of partitioning the brain into subnetworks. We then evaluated the time trace of the correlation between these two subnetworks using a sliding window of 3 s. This time, we used the correlation trace to predict each behavior and evaluated the R^2 values as a function of the angle. For the correlations, we also evaluated R^2 as a function of the analysis window length (varying from 0.5 to 15 s) for an angle of 30°.

Instantaneous multimodal connectivity. Denote $\mathbf{x}_t^{(1)} \in \mathbb{R}^{N_1}$ as the neuronal activity of N_1 brain parcels at time t and denote $\mathbf{x}_t^{(2)} \in \mathbb{R}^{N_2}$ as the neuronal activity of N_2 cells at time t . We define $\Sigma_t(i, j)$ as a three-dimensional tensor of N_1 over N_2 over time, expressing the dynamics of multimodal connectivity between cells and parcels:

$$\Sigma_t(i, j) = \frac{\sum_{t=t-\frac{N_T}{2}}^{t+\frac{N_T}{2}} (\mathbf{x}_t^1(i) - \bar{\mathbf{x}}_t^1(i)) (\mathbf{x}_t^2(j) - \bar{\mathbf{x}}_t^2(j))}{\sqrt{\sum_{t=t-\frac{N_T}{2}}^{t+\frac{N_T}{2}} (\mathbf{x}_t^1(i) - \bar{\mathbf{x}}_t^1(i))^2} \sqrt{\sum_{t=t-\frac{N_T}{2}}^{t+\frac{N_T}{2}} (\mathbf{x}_t^2(j) - \bar{\mathbf{x}}_t^2(j))^2}},$$

$$i = 1, \dots, N_1, j = 1, \dots, N_2, t = 1, \dots, N_T,$$

where N_T is the number of time points. For a given time point t , the matrix Σ_t is nonsymmetric and, therefore, not bound to the Riemannian cone (as opposed to the sample covariance matrices $\hat{C}_t$, which are SPD). Therefore, we used Euclidean distances to evaluate the diffusion kernel between correlation matrices related to different time points:

$$\tilde{A}(t, t') = \exp\{-\|\Sigma_t - \Sigma_{t'}\|^2 / \sigma^2\}, \quad (6)$$

where σ is a scale parameter evaluated as the median of pairwise distances between each correlation matrix and its k -nearest neighbors, where $k = 200$. Note that, as for embedding of correlation matrices, our results here are also highly robust to variations in k (Supplementary Fig. 6). Here, we again reduce computational complexity using the Nyström extension and evaluate the kernel $\tilde{A}$ based on a smaller randomly selected subset of time points, $t' = 1, \dots, N$, where $N < N_T$. We normalize the kernel matrix to be row stochastic and obtained its eigendecomposition:

$$\Sigma_{t'} \rightarrow \varphi_{t'}^{(n)} = \begin{pmatrix} \Phi_{t'}^1 \\ \vdots \\ \Phi_{t'}^n \end{pmatrix},$$

$$\lambda_k, \quad k = 1, \dots, n.$$

We then extend this representation to all time points, $t = 1, \dots, N_T$:

$$\varphi_t^k = \frac{1}{\lambda_k} \sum_{t'=1}^N \exp \left\{ -\|\Sigma_t - \Sigma_{t'}\|^2 / \sigma^2 \right\} \varphi_{t'}^k.$$

Multimodal connectivity s.d. We estimate the average variability of correlations between a given cell and the mesoscopic cortical network as

$$\sigma_j = \frac{1}{N_1} \frac{1}{N_t} \sum_{i=1}^{N_1} \sum_{t=1}^{N_t} ((\Sigma_t(i, j) - \bar{\Sigma}(i, j)))^2, j = 1, \dots, N_2,$$

where $\bar{\Sigma}(i, j) = \frac{1}{N_T} \sum_{t=1}^{N_T} \Sigma_t(i, j)$.

Reporting summary

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

Data availability

The full datasets generated and analyzed in this study are available from the corresponding author on reasonable request. Data for mesoscopic imaging experiments with CCFv3-based parcellation have been deposited on the figshare archive (https://figshare.com/projects/Benisty_Higley_2023/175317).

Code availability

Custom-written MATLAB scripts used in this study are available on GitHub (https://github.com/cardin-higley-lab/Benisty_Higley_2023).

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Author contributions

H.B., R.R.C., G.M., M.C.C., J.A.C. and M.J.H. designed the study. H.B., D.B., R.R.C., G.M. and M.J.H. developed the analytical approach. H.B. carried out all analyses. D.B., A.H.M., S.L. and L.T. collected experimental data. H.B. and M.J.H. wrote the manuscript.

Competing interests

The authors declare no competing interests.

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Data analysis Data was analyzed using MATLAB (v2019b, Mathworks) and GraphPad Prism (v9.0.1 Graphpad Software). All MATLAB scripts used are deposited on Github (https://github.com/cardin-higley-lab/Benisty_Higley_2023) and also available upon request.

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Blinding Blinding not required as our study did not use separate subject groups.

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
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

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

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	Adult (P60-100) male and female c57/Bl6 mice, maintained in our colony between 22-24 degrees C and 60-70% humidity.
Wild animals	Did not involve wild animals.
Reporting on sex	Animals included both sexes, and no differences were found, thus subjects were pooled for final analyses.
Field-collected samples	Did not involve field-collected samples.
Ethics oversight	Ethical oversight provided by Yale IACUC.

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