
Preconfigured neuronal firing sequences in human brain organoids

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Supplementary Information for

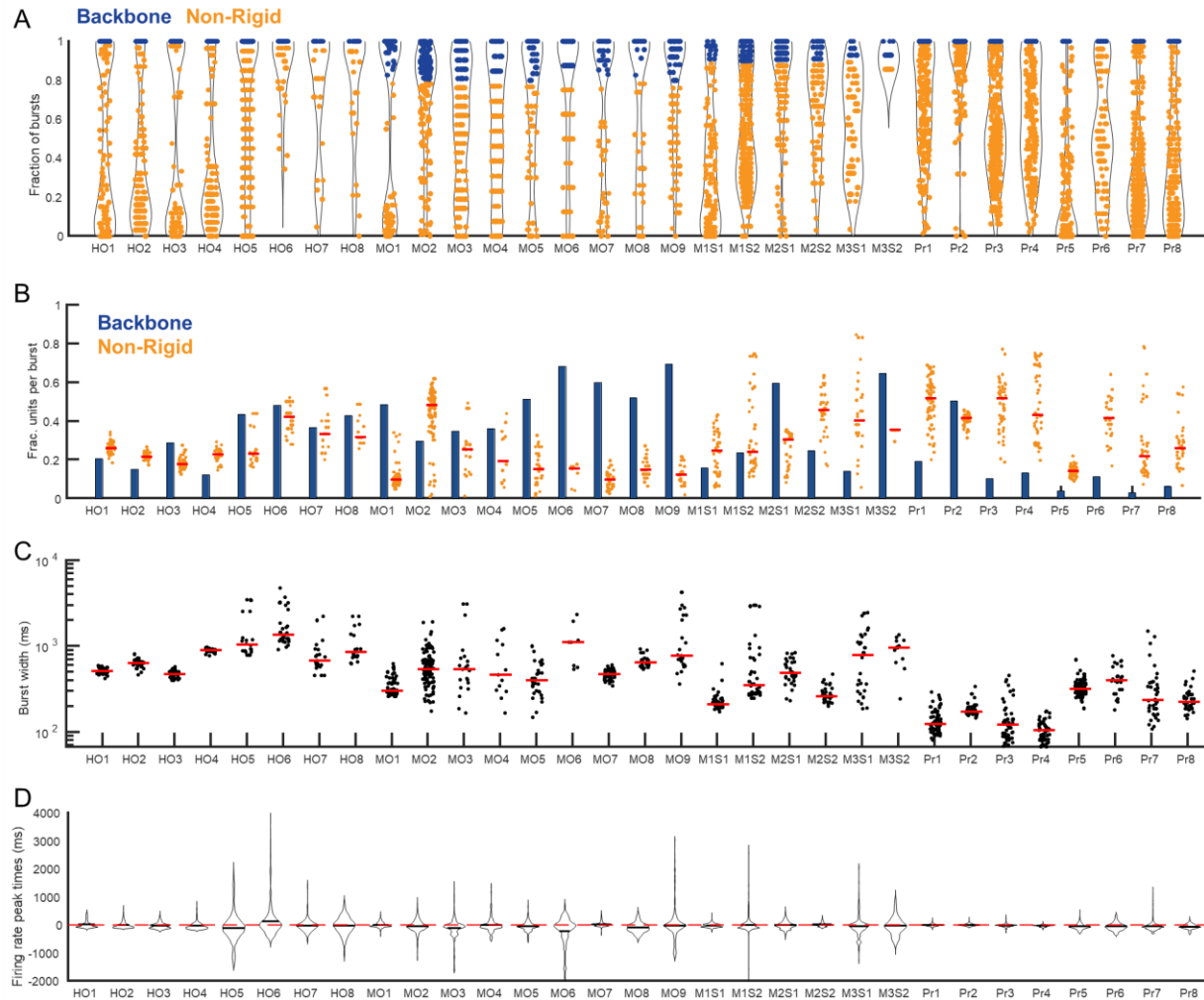
Preconfigured neuronal firing sequences in human brain organoids

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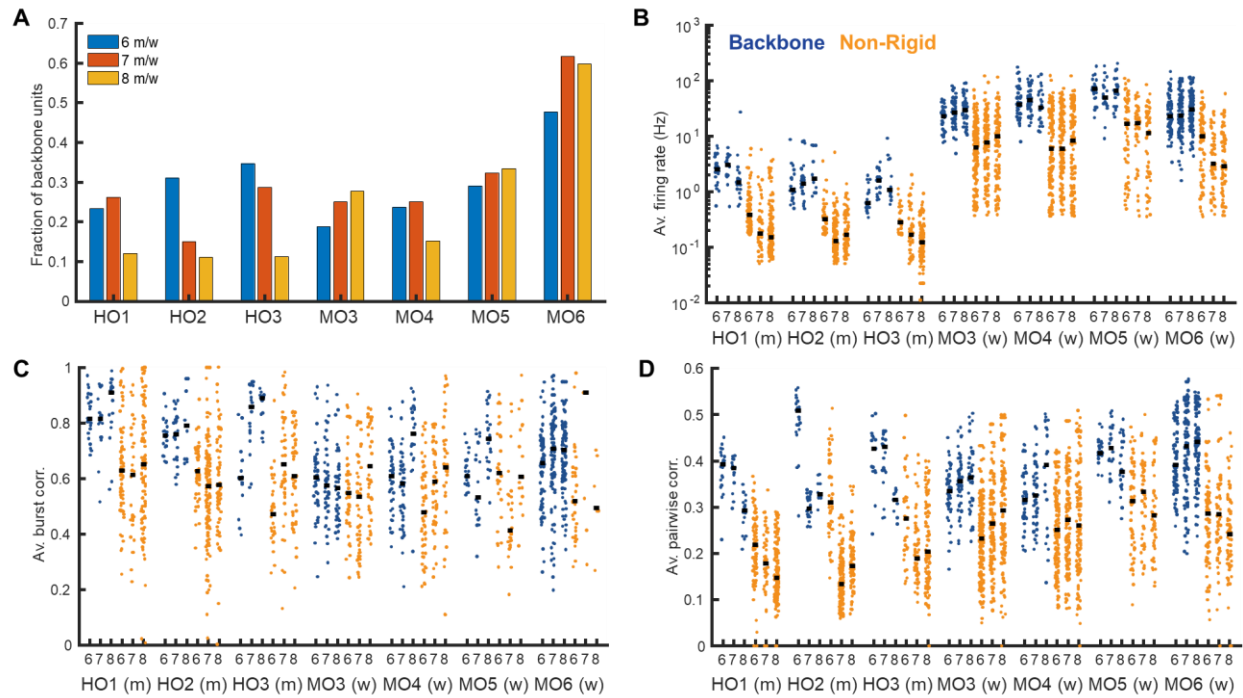
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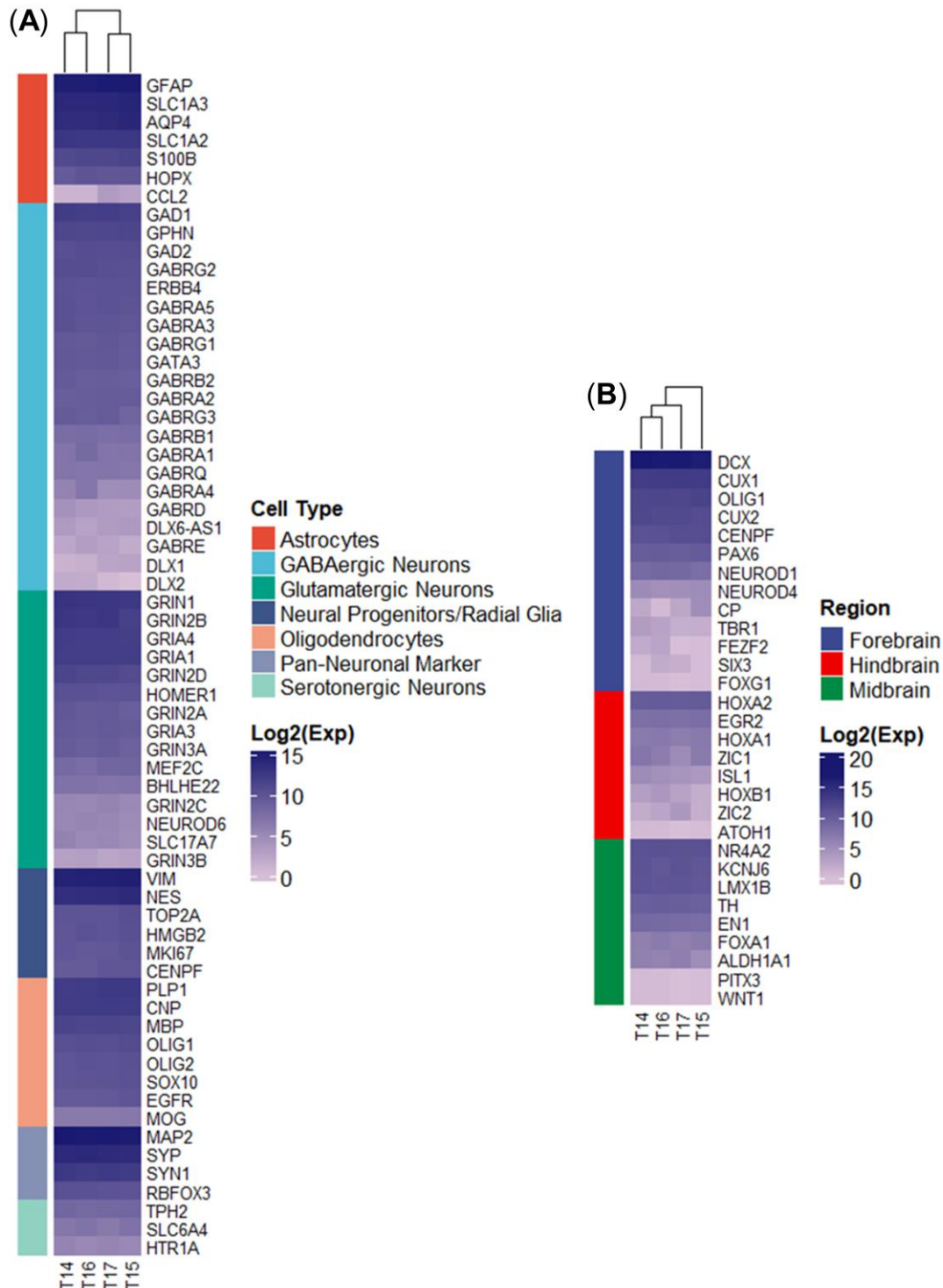
Supplementary Methods



Supplementary Figure 1. Backbone units during bursts. (A) Fraction of bursts in which a unit has at least two spikes. Every dot represents a unit. Blue dots are the backbone units and have at least two spikes in all bursts. Red dots are the non-rigid units that spike at least two spikes in less than all bursts. The backbone unit threshold for murine organoids and murine neonatal slices was lowered to at least 2 spikes in at least 80% and 90% of all bursts respectively. (B) Fraction of backbone units and non-rigid units that are active per burst. Backbone units are active in all bursts and so the fraction of backbone units is the same for each burst. The fraction of non-rigid units that fire at least two spikes in all bursts varies. Red bars indicate the distribution median. (C) Burst durations are plotted for all different recordings. Red bars indicate the distribution median (D) The distribution of single-unit firing rate peak times relative to population burst peaks for all different recordings.

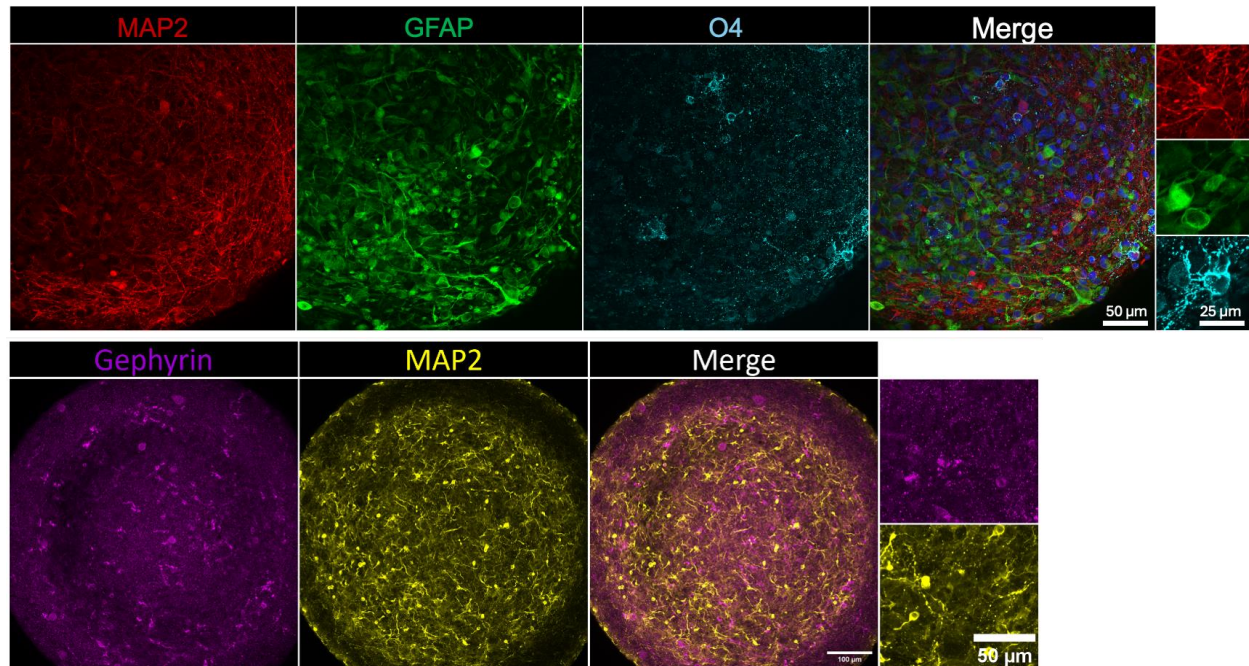


Supplementary Figure 2. Backbone units during development. (A) The fraction of backbone units decreases when the organoid matures. Human organoid (HO) ages are reflected in months whereas murine organoid (MO) ages are reflected in weeks. (B) At all recorded ages, the average firing rate per unit is on average higher for the backbone units than for non-rigid units. (C) At all recorded ages, the average burst to burst correlation per unit is on average higher for the backbone units than for non-rigid units. Furthermore, the average burst to burst correlation increases over the course of development (2-way ANOVA, impact of age on burst to burst correlation HO: $P = 4.14 \times 10^{-6}$, MO: $P = 9.45 \times 10^{-9}$). (D) At all recorded ages, the average pairwise correlation between pairs of backbone units is on average higher than for pairs of non-rigid units.

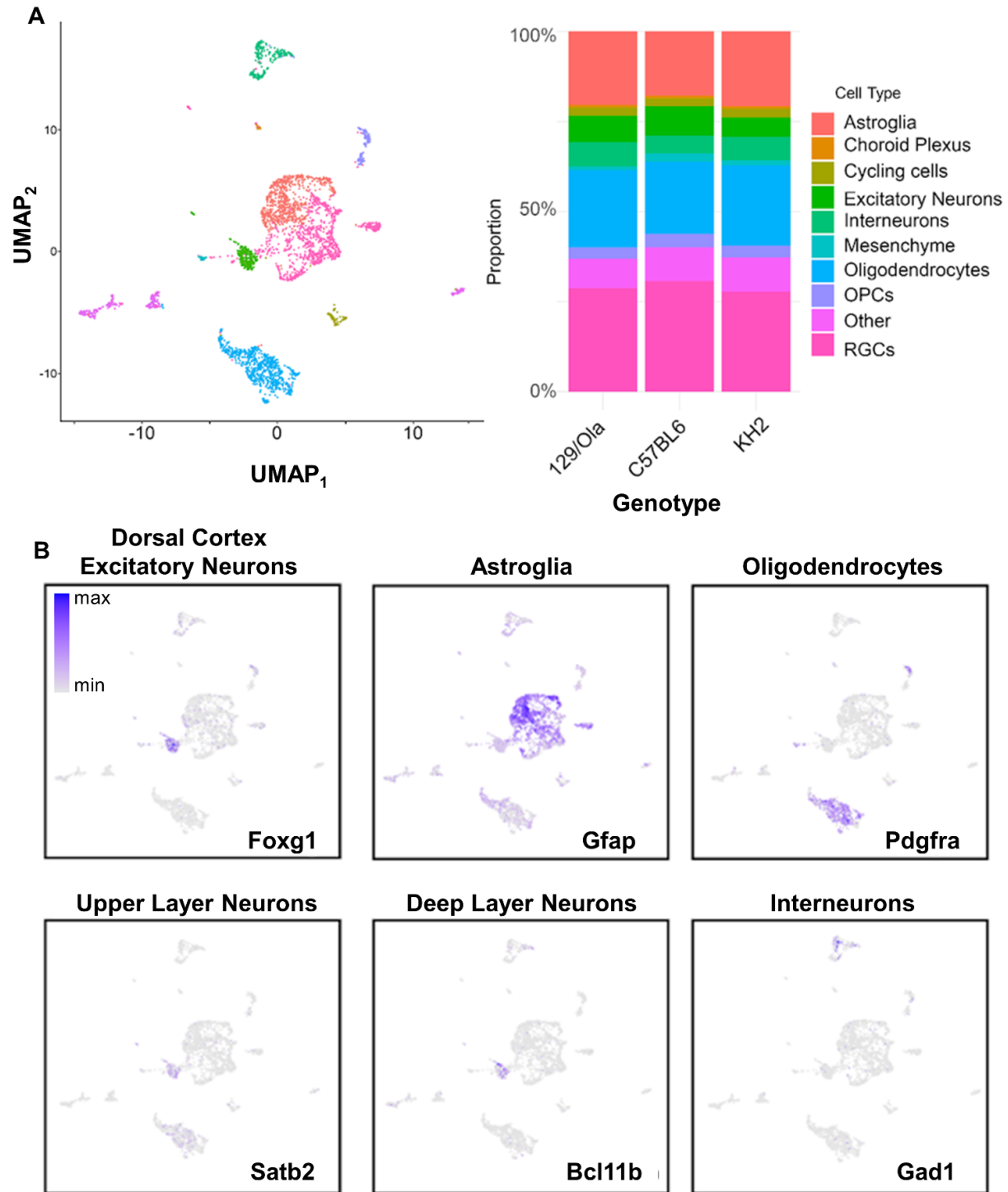


Supplementary Figure 3. Marker gene expression representing. (A) main cell lineages and (B) regions of developing brain in 8-week-old brain organoids. Heatmap represents the Log2 of relative gene expression extracted from limma-voom normalized bulk RNA-seq (data sourced from Alam El Din *et al.*). Heatmap columns represent four separate bulk RNA sequencing experiments (T14-T16) from 8-week old human brain organoids, here 50 organoids were pooled together and sequenced per replicate. The organoids show consistent expression of key genes

associated with glutamatergic (GRIN1, GRIN2A) and GABAergic neurons (GAD1, GAD2), as well as astrocytes (GFAP), oligodendrocytes (OLIG1, OLIG2) and neural progenitors (VIM, TOP2A). The region identity of neurons within these organoids contain neurons of forebrain identity (PAX6, CUX1, CUX2) as well as midbrain (TH, FOXA2) and hindbrain (HOXA2).

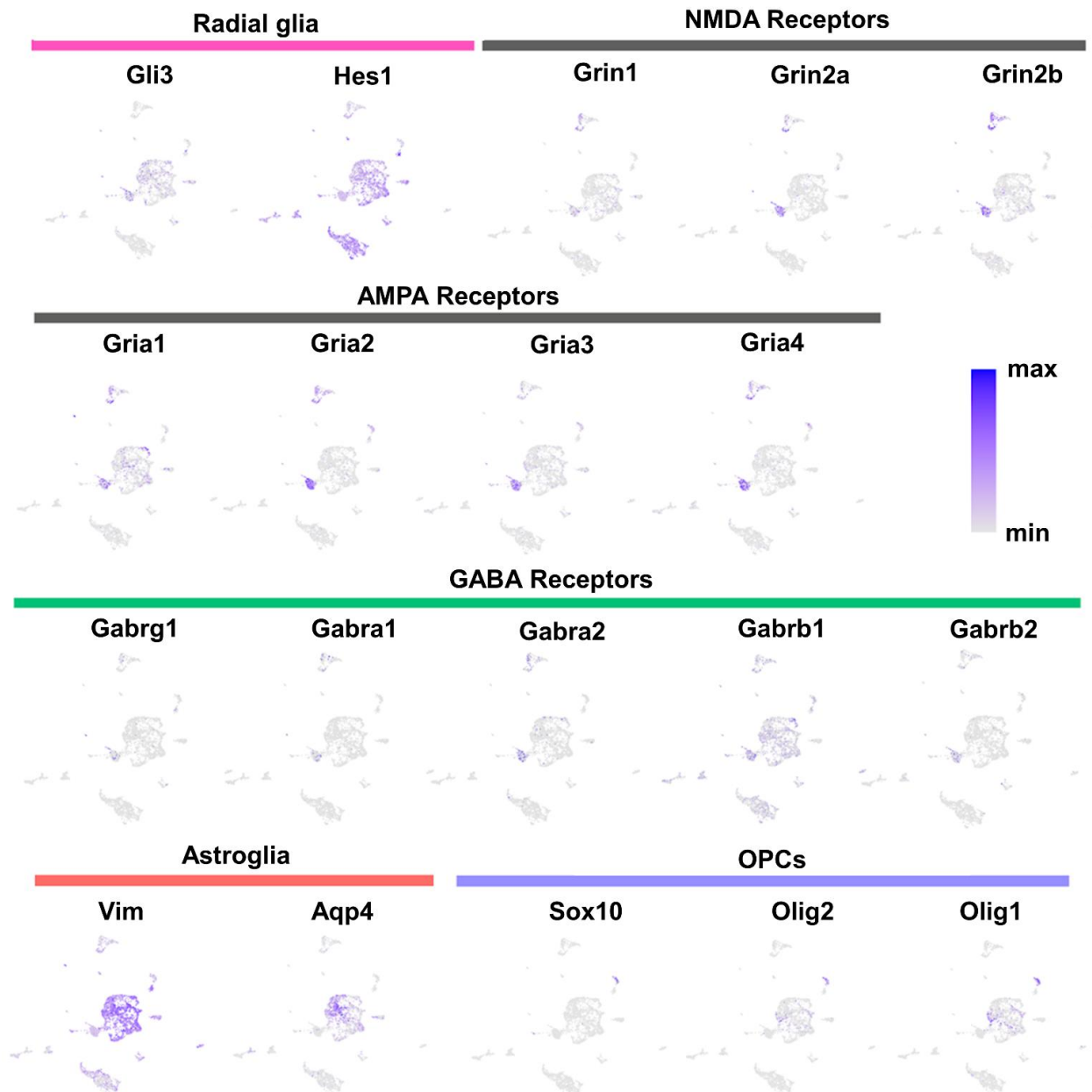


Supplementary Figure 4. Immunohistochemistry validation of human brain organoid cell types. Top row, somatodendritic marker MAP2 (red), astrocyte marker GFAP (green), oligodendrocyte marker O4 (turquoise). Nuclei are stained with Hoechst33342 (blue). Bottom row, inhibitory synaptic marker Gephyrin (purple), dendritic marker MAP2 (yellow).

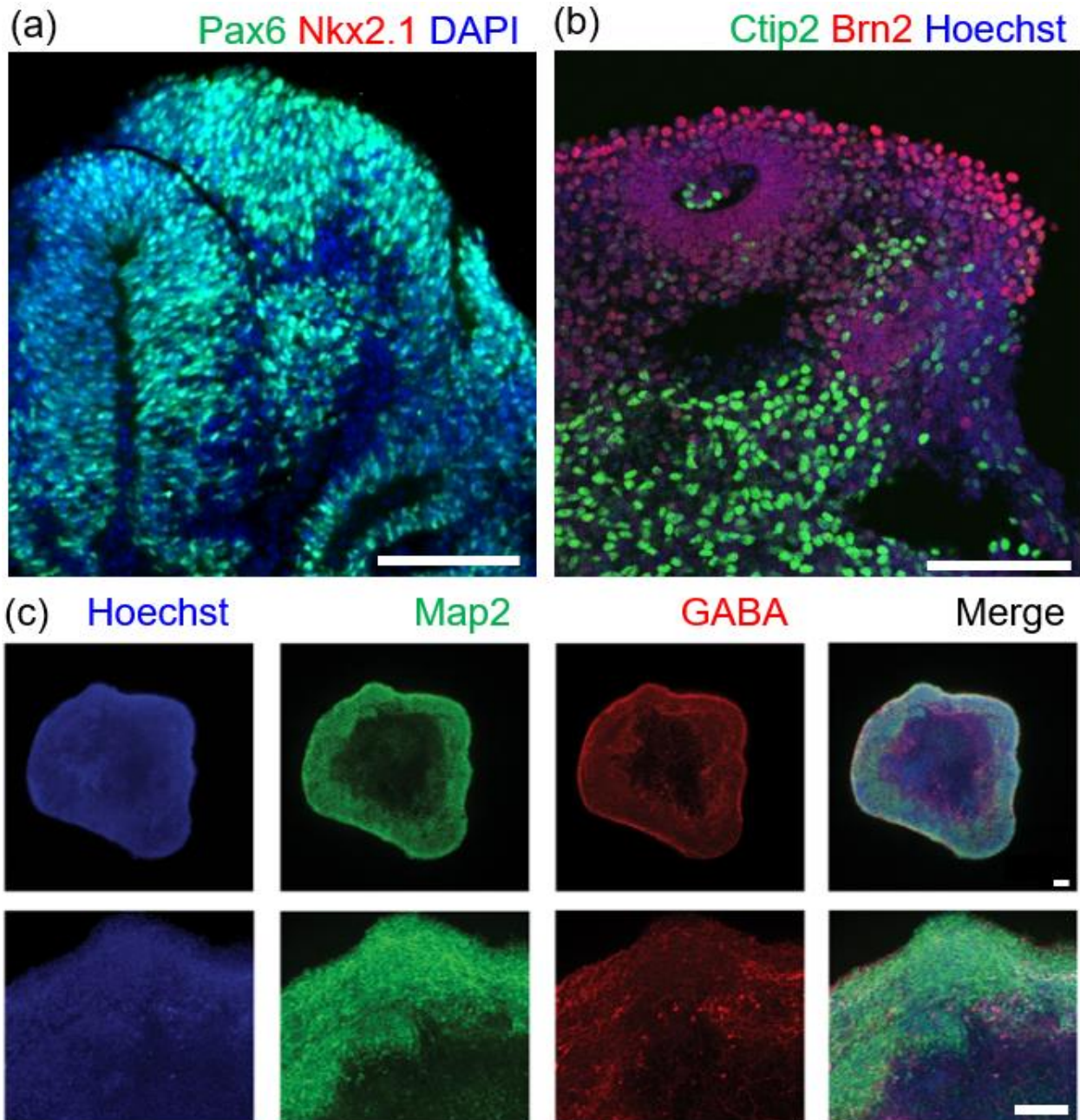


Supplementary Figure 5. Mouse ESC cortical organoid identification of cell types using single-cell sequencing. (A) Left, single-cell sequencing UMAP plot colored by cell type cluster. Right, relative proportions (fractions) of cells per cluster. Clusters are identified from 30-day-old organoids as determined by PIPseq. A total of 4031 cells were sequenced from three separate cell lines (C57/BL6, E14, and KH2 ESC, see Methods). (B) Selection of canonical markers used to identify cell types in single-cell data. UMAP plots are colored by normalized and scaled expression

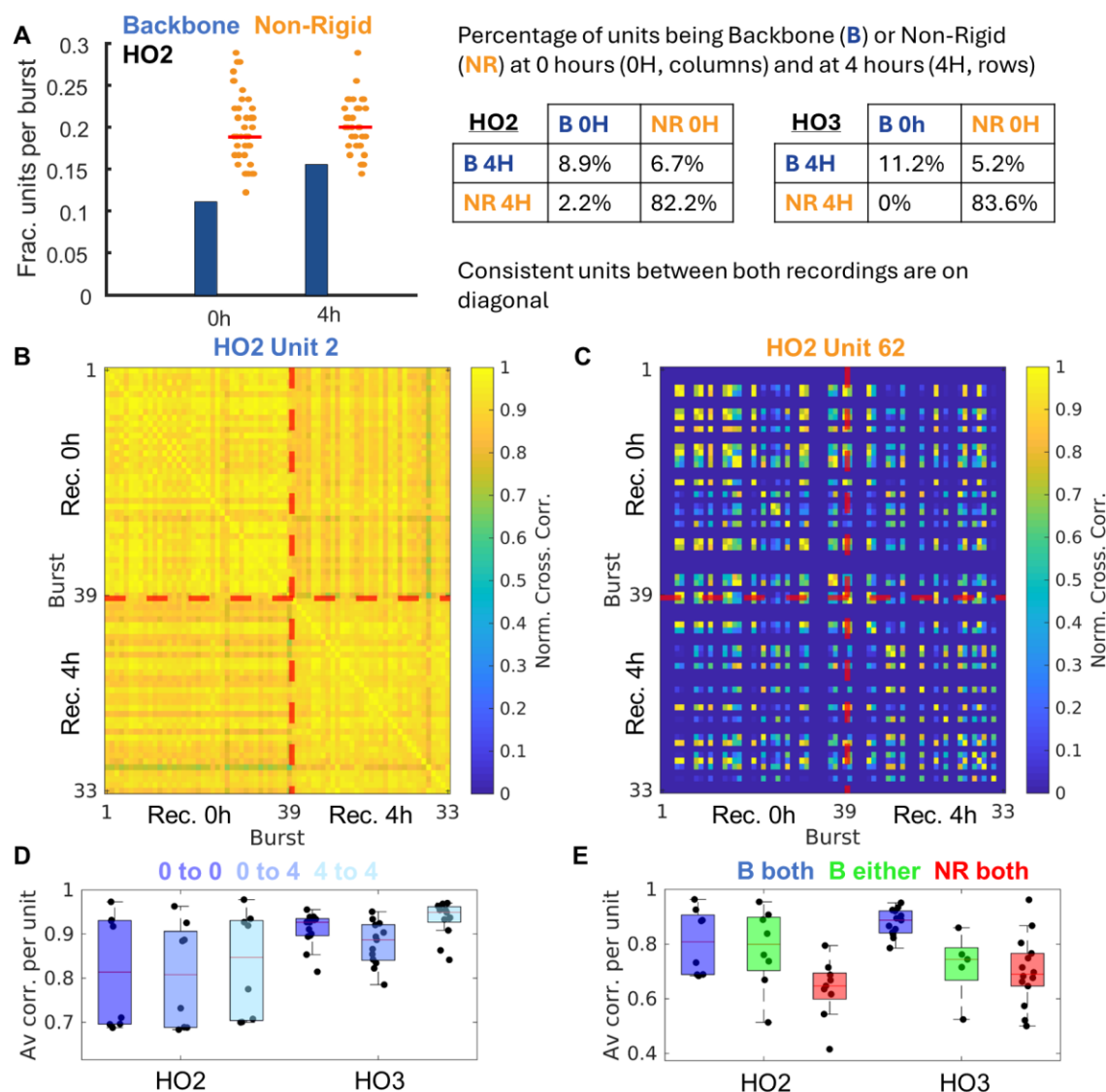
values. Expression patterns of brain-region specific marker genes reveal predominant forebrain identity of the murine brain organoids used in this study. (C) Murine organoids express multiple genes encoding subunits of AMPA- and NMDA-types of glutamate receptors as well as GABA receptor subunits and various glial cell type markers. UMAP plots are colored by expression level of known genes encoding different receptor subunits. Not detected in single-cell data were GABRA6 and GABRP.



Supplementary Figure 6. Cellular identities and neurotransmitter profiles of Mouse ESC cortical organoid determined by single-cell transcriptomics. Murine organoids express multiple genes encoding subunits of AMPA- and NMDA-types of glutamate receptors as well as GABA receptor subunits and various glial cell type markers. UMAP plots are colored by expression level of known genes encoding different receptor subunits. Not detected in single-cell data were GABRA6 and GABRP.

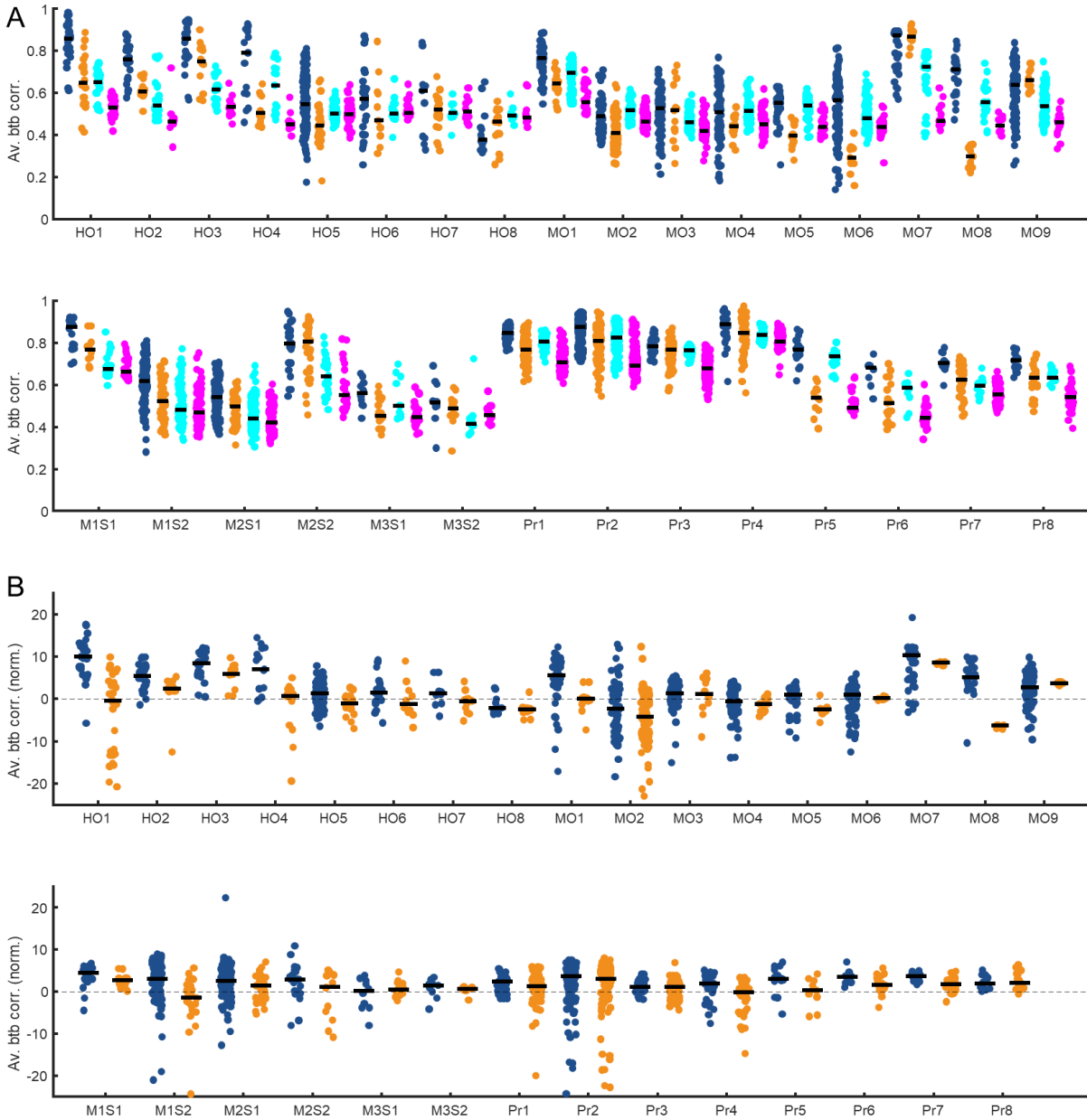


Supplementary Figure 7. Immunohistochemistry validation of mouse ESC dorsal forebrain organoid cellular identities. (A) Pax6-positive dorsal forebrain neural progenitors (green) shown in a 10-day organoid mouse cortical organoid counterstained with DAPI (cell nuclei, blue). Ventral forebrain marker Nkx2.1 is shown in red. (B) Ctip2-positive deep layer cortical neurons (green) in a 30-day cortical mouse organoid. Brn2-positive upper layer cortical neurons (red) are shown with Hoechst (cell nuclei, blue). (C) A subpopulation of GABA producing interneurons (red) are shown among MAP2-positive neurons (green) from a 30-day cortical mouse organoid. Scale bar 100 μ m.



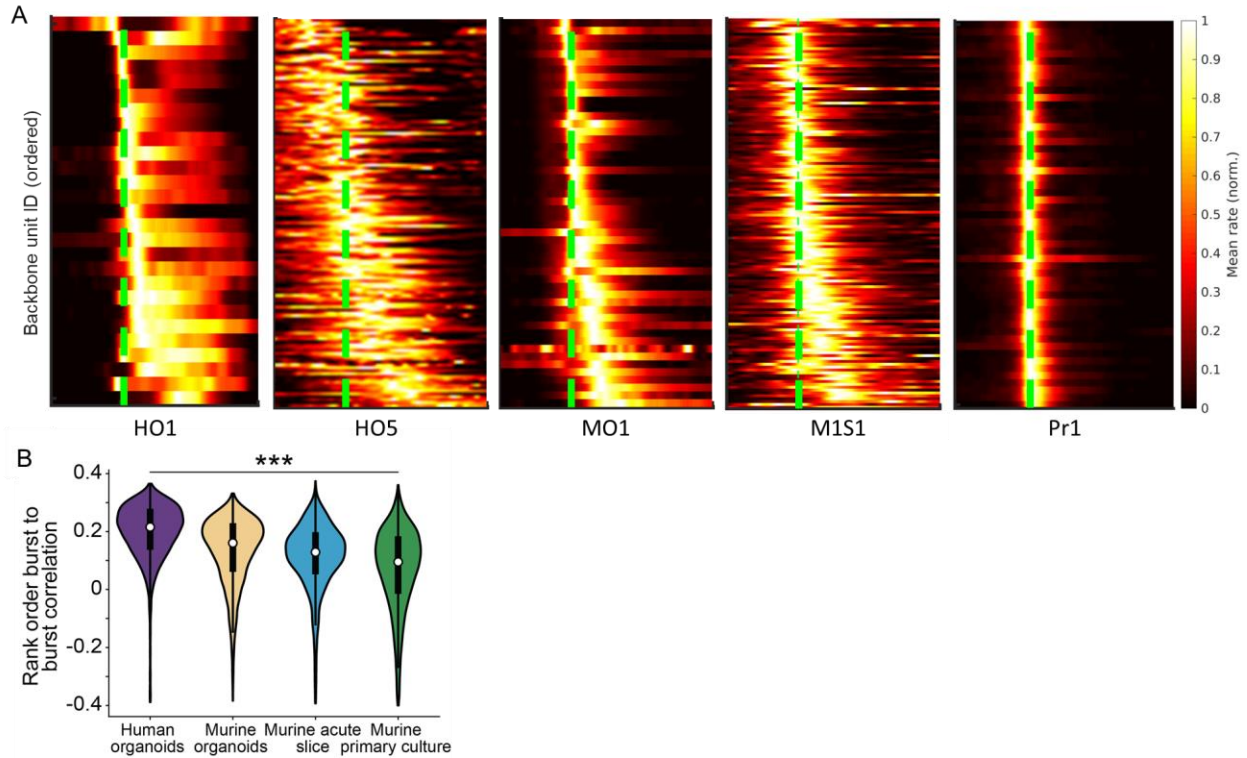
Supplementary Figure 8. Backbone unit activity is stable over multiple hours. (A) Left, fraction of backbone units and non-rigid units that fire at least 2 spikes per burst. Backbone units fire at least 2 spikes in all bursts and so the fraction of backbone units is the same for each burst. The fraction of non-rigid units that fire at least 2 spikes varies per burst. Red bars mark the distribution medians. The results are shown for two different recordings of the same organoid slice, that were made at a 4-hour interval. This data was recorded from a subset of human organoids used in this study (human organoid 2 (HO2) and organoid 3 (HO3) from Figs.1,3,5). Right, the majority of units that was detected as a backbone unit at time point 0-hour was also detected as backbone unit at time point 4-hour (top left) for both organoids. Meanwhile, the majority of units that were detected as non-rigid units at time point 0-hour were also detected as non-rigid units at time point 4-hour (bottom right) for both organoids. The percentage of backbone and non-rigid units are shown in the table on the right. (B) Pairwise burst to burst correlations for a representative example backbone unit highlight that the firing patterns of this unit are consistent between bursts in the 0-hour recording (top left), between bursts in the 4-hour recording (bottom right), and between bursts in the 0-hour and 4-hour recording (bottom left). (C) Pairwise burst-to-burst correlations for a

representative example non-rigid unit highlight that the firing patterns of this unit are inconsistent between bursts in the 0-hour recording (top left), between bursts in the 4-hour recording (bottom right) and between bursts in the 0-hour and 4-hour recording (bottom left). **(D)** The average burst to burst correlation per backbone unit, computed between bursts that occurred both in the 0-hour recording (0 to 0), between bursts that occurred in the 0-hour and 4-hour recording (0 to 4), and between bursts that occurred both in the 4-hour recording (4 to 4) reflect the consistent firing pattern of backbone units over multiple hour time spans. **(E)** Comparing the average burst-to-burst correlations computed between burst pairs of which one burst occurred in the 0-hour recording and the other in the 4-hour recording show that the backbone units (**B**) are more consistent in their firing patterns over multiple hours than the non-rigid units (NR).

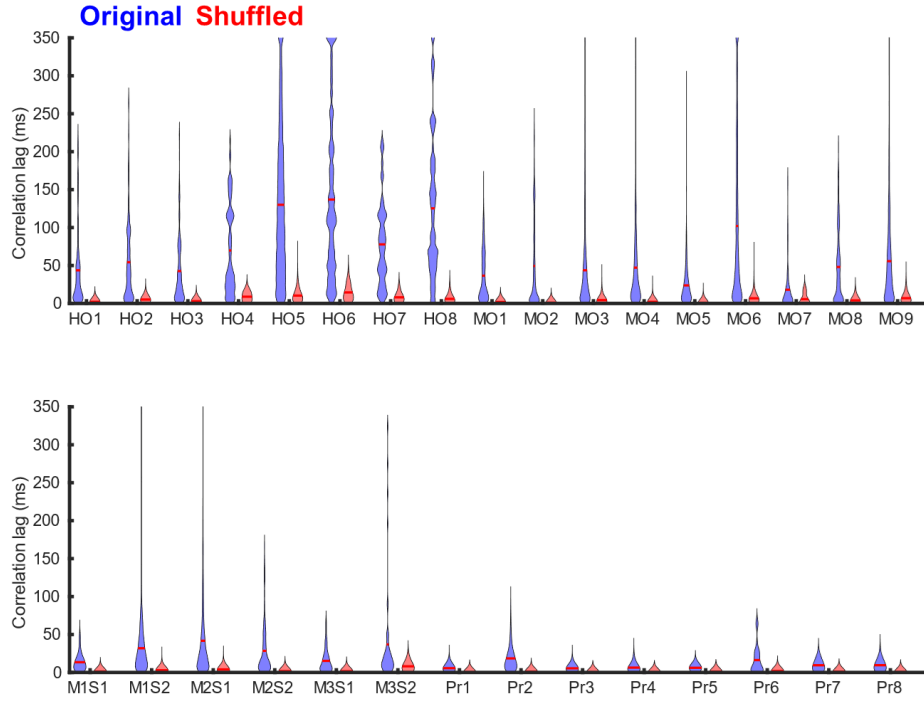


Supplementary Figure 9. Backbone units have a higher average burst-to-burst correlation after firing rate normalization for organoids and murine neonatal slices. (A) The distribution of burst-to-burst correlations per analyzed recording for backbone and non-rigid units separated. The average results for applying the same analysis on shuffled data are included in magenta and cyan. Black bars mark the distribution medians. The data is shuffled in a way that maintains the same average firing rate per unit and the same population rate per recording frame. In all cases, the backbone units have on average a higher correlation, also after shuffling. This indicates that the average firing rate differences by themselves affect the burst-to-burst correlation. HO = Human Organoid, MO = Murine Organoid, MS = Murine Slice, Pr = Murine Primary culture. (B) The distribution of burst-to-burst correlations per analyzed recording for backbone and non-rigid units separated after subtracting the average score from the shuffled recording from the original score. Black bars mark the distribution medians. In all organoid recordings and most murine neonatal

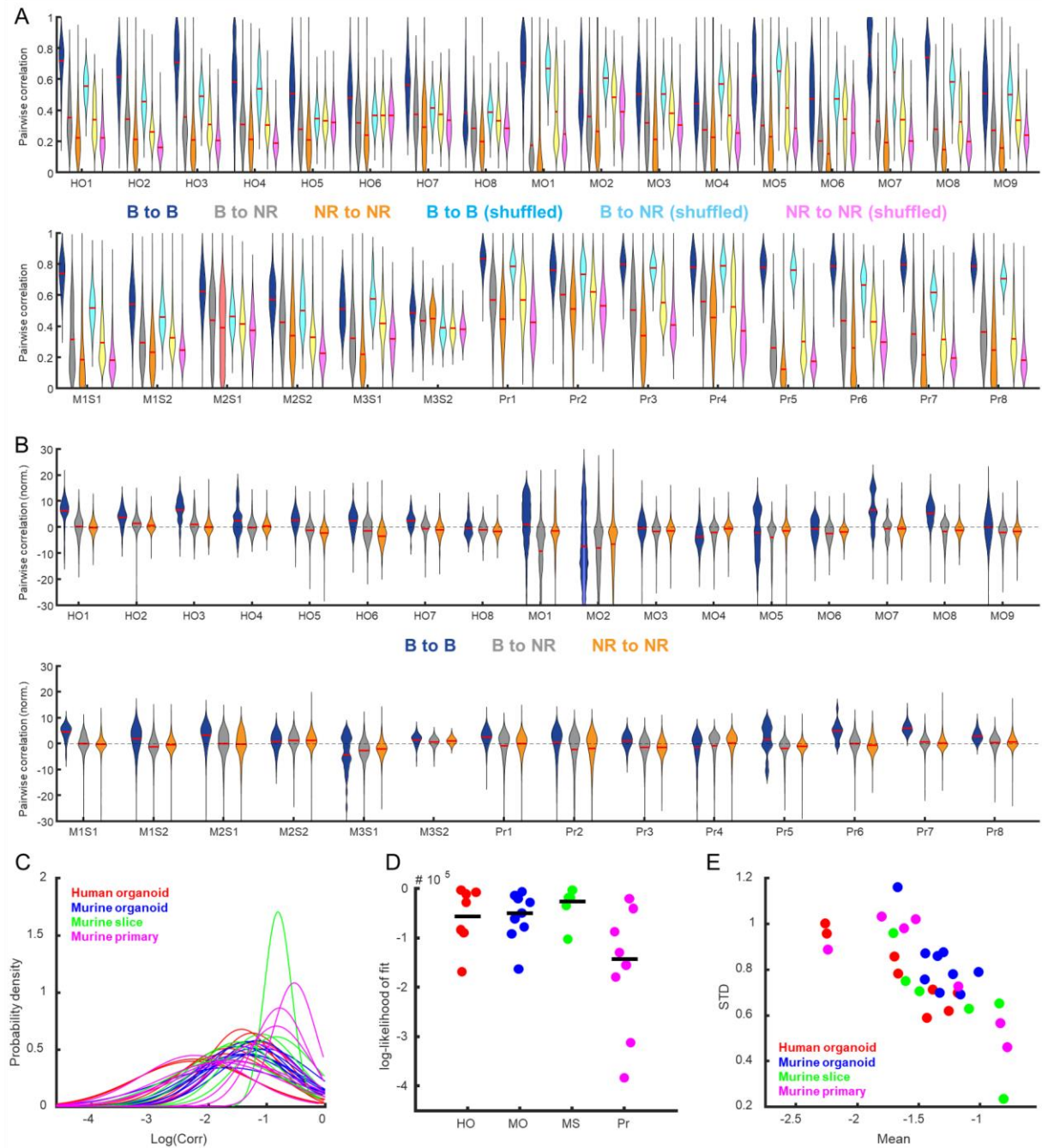
slice recordings, the average burst-to-burst correlation after average rate normalization is still higher for the backbone units than for the non-rigid units. Meanwhile, this difference is not present anymore after normalization for the murine primary cultures. See **Fig. 7B** for the results of the statistical comparison.



Supplementary Figure 10. Rank order correlations. (A) Examples of sequential firing patterns visible in the burst peak centered average firing rates of backbone units in human organoids from Sharf *et al.* (HO1), human organoids from Alam El Din *et al.* (HO5), murine organoids (MO1) and murine brain slices (M1S1) but not in murine primary cultures (Pr1). (B) Normalized Spearman rank order correlations comparing the sequential order of backbone sequences for all burst pairs over the recordings for the 4 different model types. Correlation scores are normalized relative to shuffled spike matrices such that values above 0 indicate a more consistent backbone sequence than the shuffled data (Main effect of sample type $P = 0.004$, Human organoids vs Murine Organoids $P = 0.066$, Human organoids vs Murine slices $P = 0.061$, Human organoids vs Murine primary cultures $P = 0.0004$, Murine organoids vs Murine slices $P = 0.99$, Murine organoids vs Murine primary culture $P = 0.38$, Murine slices vs Murine primary cultures $P = 0.65$, two-sided linear mixed-effect model with sample as grouping variable, $n = 31$ total samples). Box plots: boxes span the 25th–75th percentiles; the center line marks the median (50th). Whiskers extend to the most extreme values within $1.5 \times$ the interquartile range from quartile 1 and quartile 3. In violin plots, minima and maxima equal the lowest and highest observations; the shaded area shows the variable's probability density.

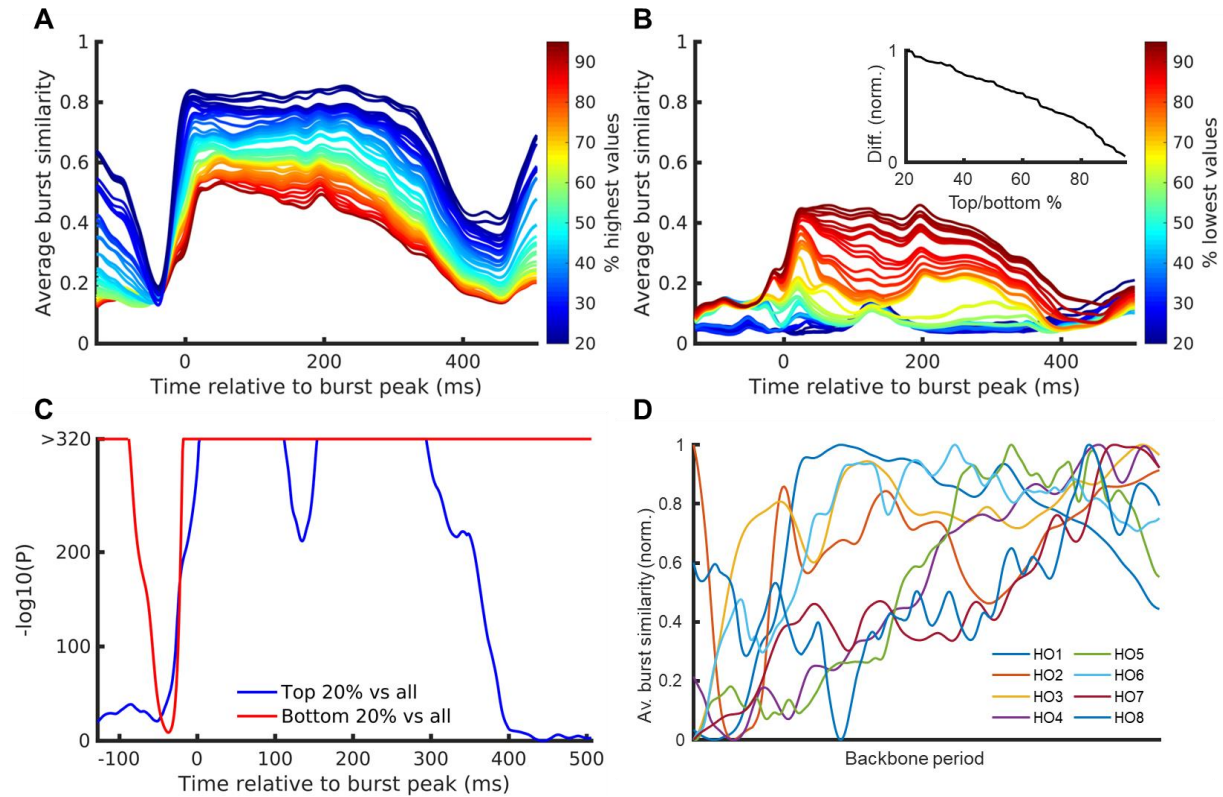


Supplementary Figure 11. Correlation lag times. Lag times corresponding to the maximum correlation when applying a cross correlation to the single unit firing rates over all pairs of backbone units. The maximum lag time was set to 350 ms, the median duration of the backbone period over all 3D models. The same computations were performed on 100 different shuffled spike matrices and the average absolute correlation lags over all 100 shuffled datasets are reported as well. Red bars mark the distribution medians. See **Fig. 7D** for the results of the statistical comparison.



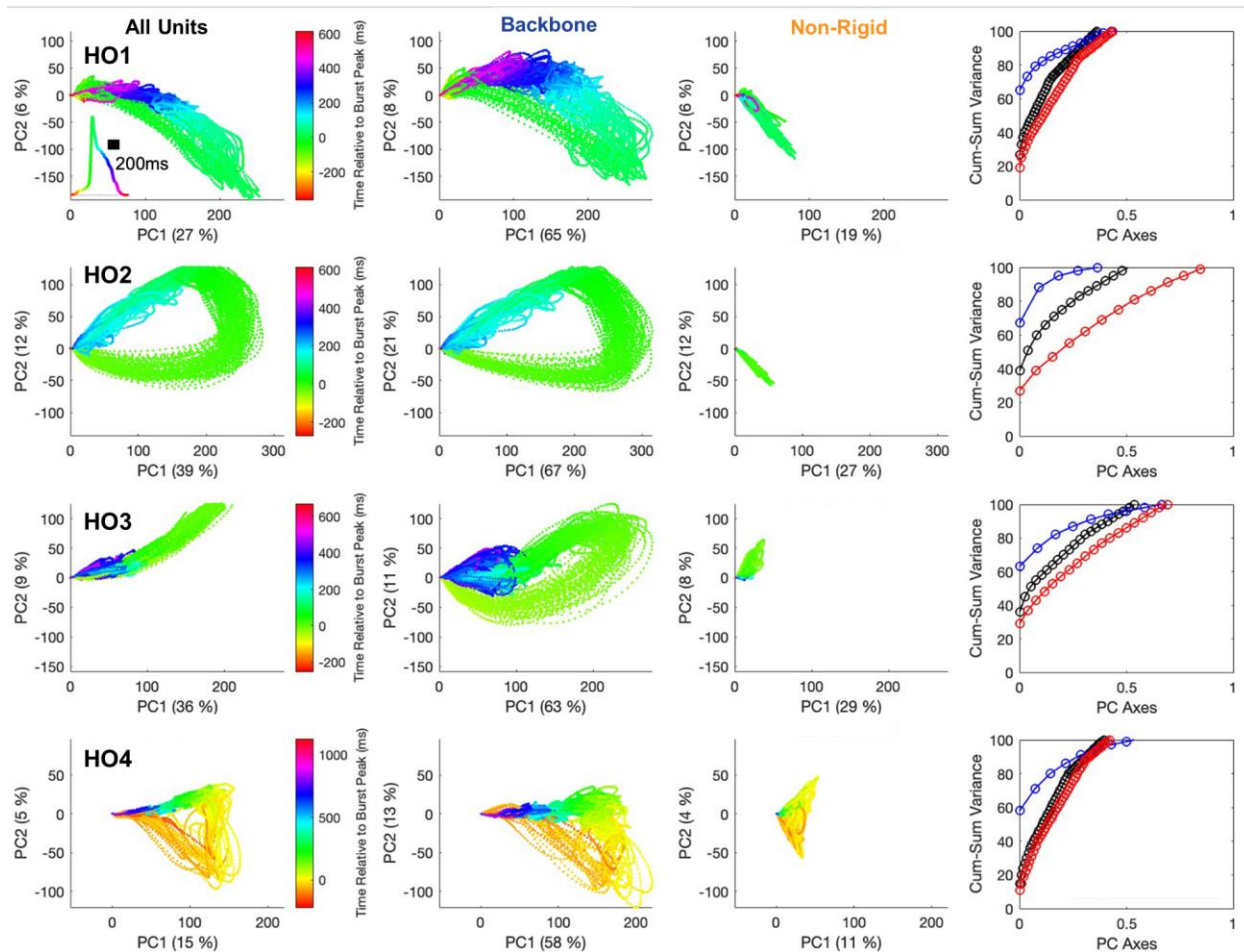
Supplementary Figure 12. Backbone unit pairs have a higher pairwise correlation after firing rate normalization for organoids and murine neonatal slices. (A) The distribution of pairwise correlations per analyzed recording for backbone unit pairs, backbone and non-rigid unit combinations and non-rigid unit pairs separated. Red bars mark the distribution medians. The average results for applying the same analysis on shuffled data are included in magenta, yellow and cyan. The data is shuffled in a way that maintains the same average firing rate per unit and the same population rate per recording frame. In all cases, the backbone unit pairs have on average a higher pairwise correlation compared to other unit type pairs, also after shuffling. This indicates that the average firing rate differences by themselves affect the pairwise correlations. B = Backbone, NR = Non-Rigid, HO = Human Organoid, MO = Murine Organoid, MS = Murine Slice,

Pr = Murine Primary culture. **(B)** The distribution of pairwise correlations per analyzed recording for backbone unit pairs, backbone and non-rigid unit combinations and non-rigid unit pairs separated after subtracting the average score from the shuffled recordings from the original score. Red bars mark the distribution medians. In all organoid recordings and most murine neonatal slice recordings, the pairwise correlations after average rate normalization are still higher for the backbone unit pairs than for the other unit pairs. Meanwhile, this difference is not present anymore after normalization for the murine primary cultures. See **Fig. 7C** for the results of the statistical comparison. **(C)** Normal distributions fitted to the distributions of the log of the pairwise correlations. **(D)** Log-likelihood for the fits in **C**. Black bars mark the distribution medians. **(E)** The mean and STD for the fits in **C**.



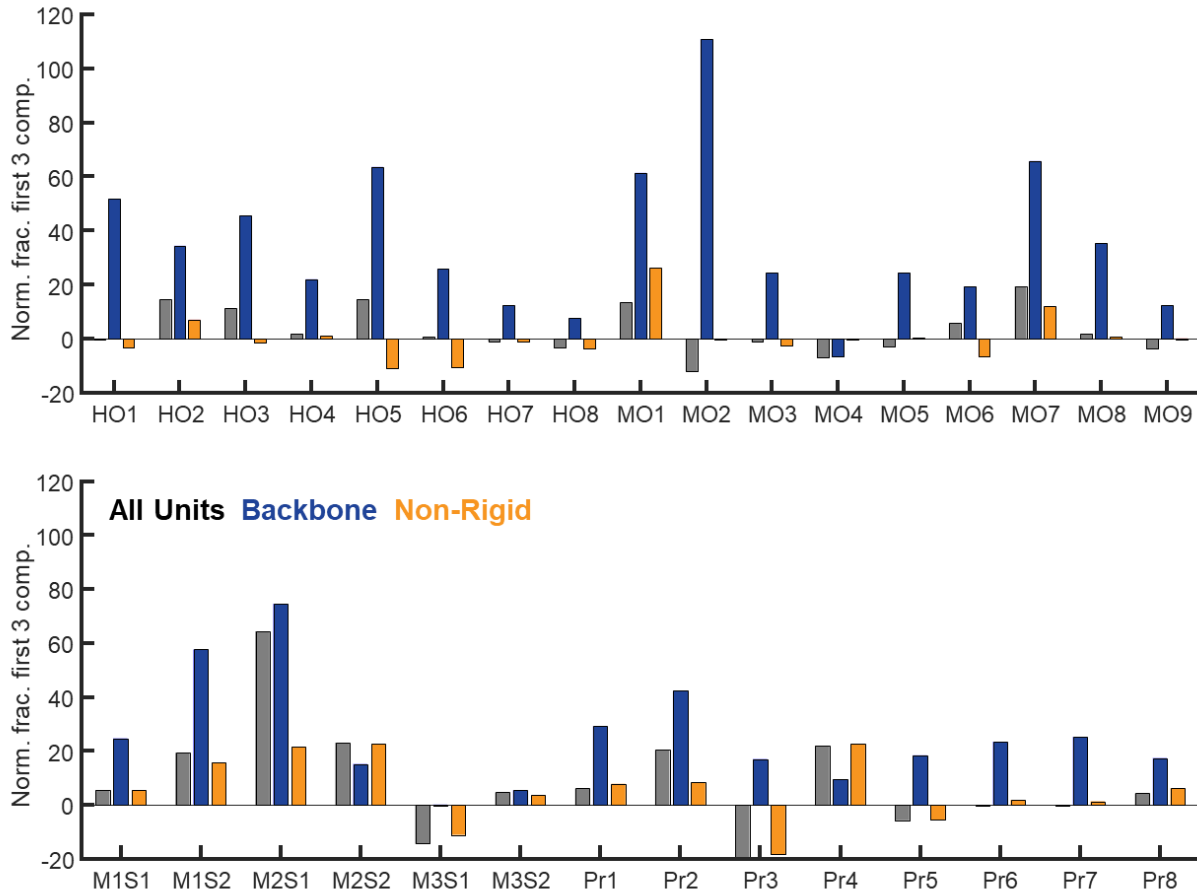
Supplementary Figure 13. Burst similarity for correlated units. **(A)** The top 20th percentile of units based on their average pairwise correlation generated higher cosine similarity scores compared to larger percentile ranges, highlighting that the burst similarity score is driven by the most correlated units. **(B)** Greater than 80% of the units with the lowest average pairwise correlation was required to get similar burst similarity score traces as all units together. The burst similarity score increase during the backbone period was not present when only the 20-40% units with the lowest average pairwise correlation was considered. The inset shows the difference in the area under the curve for the top x% of units compared to the bottom x% of units. **(C)** The $-\log_{10}(P)$ values of the paired sample t-tests comparing the burst similarity scores of the top or bottom 20% units based on their average pairwise correlation to the burst similarity scores computed over all units. A paired sample t-test is performed at every frame relative to the burst peak, comparing the distribution of burst similarity scores for all possible burst pairs between the two unit selections. **(D)** Average burst similarity computed over all units for each of the 8 organoids over their respective backbone periods. A backbone period is defined as the time of the first median firing-

rate peak time until the last median firing rate peak time relative to the burst peak over all backbone units. The burst similarity scores within the backbone period are normalized between 0 and 1 for each organoid. Note the sharp increase from the start of the backbone period for all organoids.

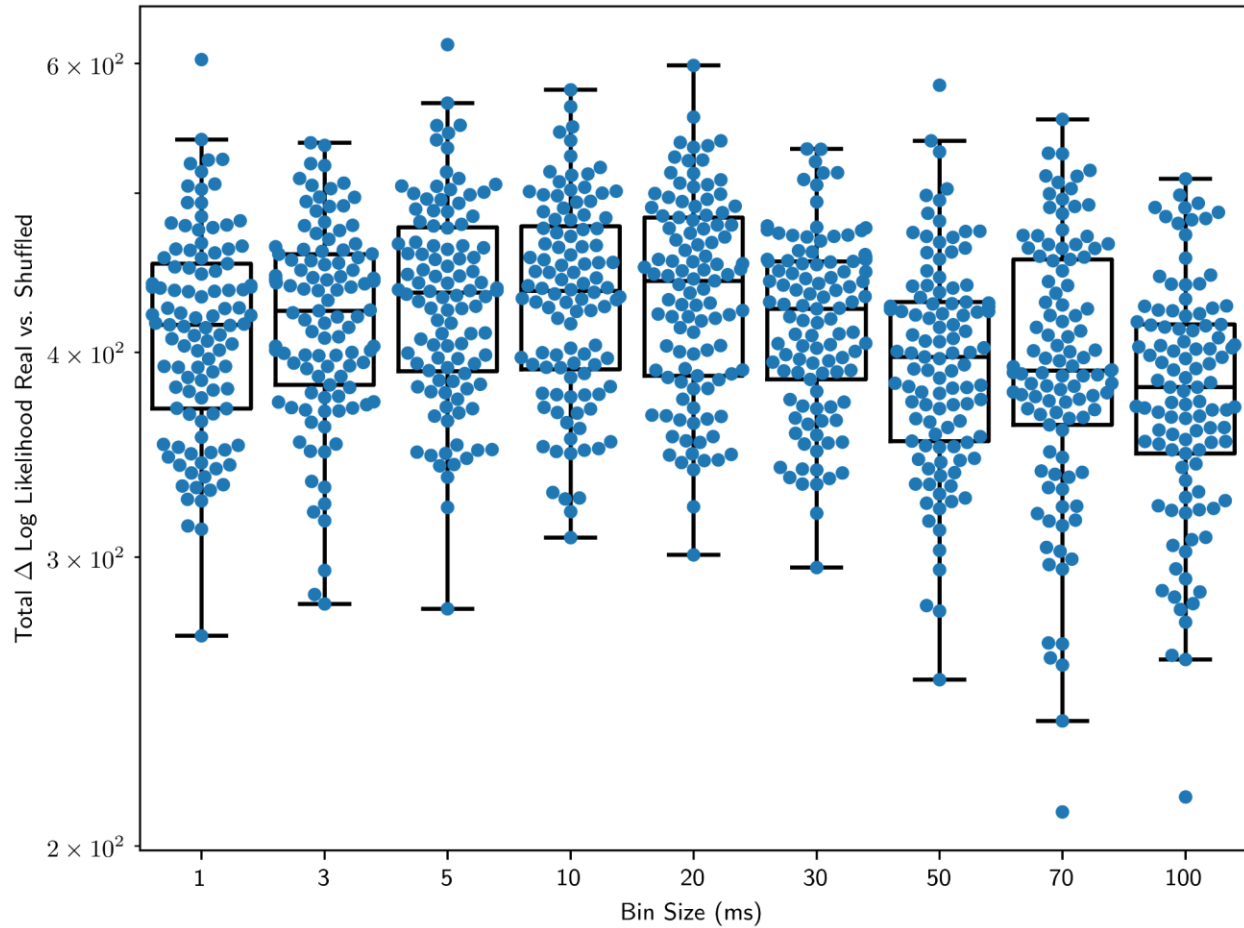


Supplementary Figure 14. PCA manifolds for different organoid recordings. Every row contains data from a different organoid. A PCA was performed on the firing rates per single unit for all units in the recording combined. Only spikes that occurred during bursts were included in the firing rate computations. Each dot represents a recording frame and is colored by the time point relative to the closest burst peak. First column, population activity projected onto its first two principal components. Note the consistent circular trajectory reflecting the burst manifold. The inset shows the burst-peak-centered average population rate colored in the same way as the PCA trajectories to indicate which parts of the burst correspond to the different coloring. Second column, same as first column but only including the backbone units. Note the similarity in the low dimensional manifold representations between the first and second column, indicating the strong contribution of the backbone units to the low dimensional activity of the whole population. Third column, same as first column but only including the non-rigid units. The low-dimensional manifold is not present anymore and the variance explained by the first to principal components is notably lower, reflecting the higher dimensional activity of the non-rigid units. Last column, the cumulative sum of the variance explained per principal component as a function of the number of first principal components included in the sum. The explained variance for backbone units only

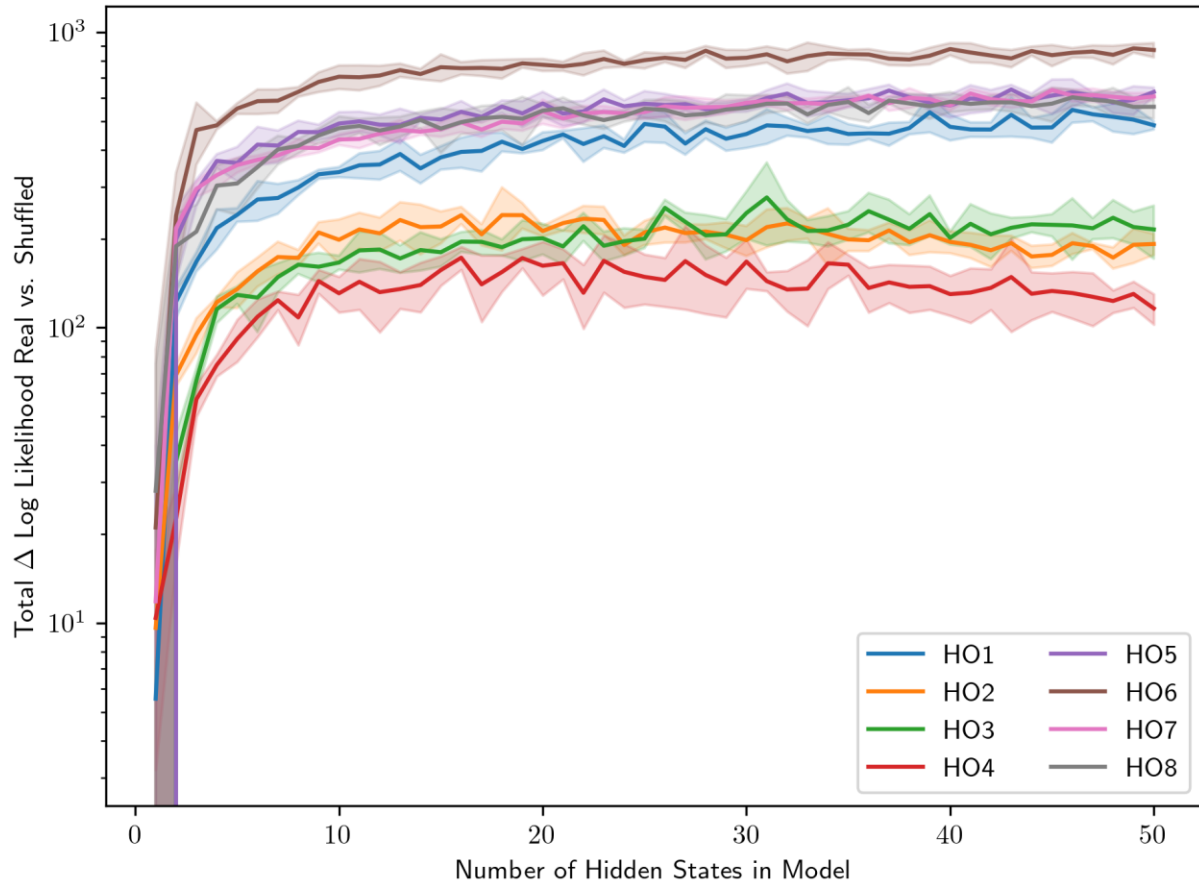
(blue) required fewer PCs compared to all units (black). Meanwhile, the opposite is true for the non-rigid units only (red).



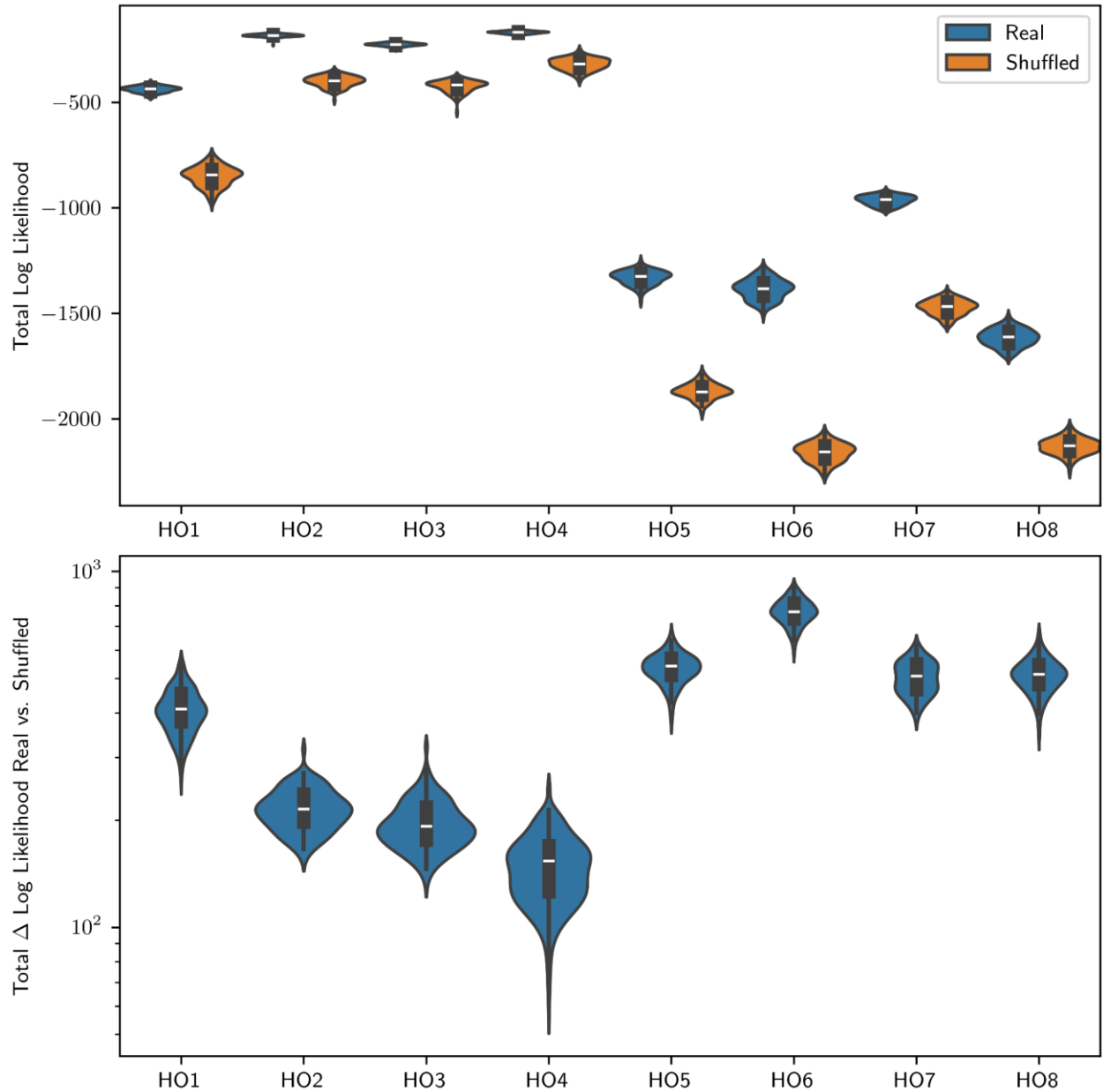
Supplementary Figure 15. Normalized sum of explained variance of first 3 components of PCA manifold. The normalized fraction of the variance explained, summed over the first three principal components for the PCA manifolds per recording. To account for differences in the total number of principal components per category, the summed explained variance for the first three principal components is divided by the summed explained variance of the first X principal components, where X is the lowest number of total principal components from the three categories: all units, backbone and non-rigid. X is defined separately per recording due to variation in the number of detected units. This summed variance is computed for the original data and the 100 different shuffled datasets after which the averages of the shuffled results are subtracted from the original results to get the final value. See **Fig. 7E** for the results of the statistical comparison.



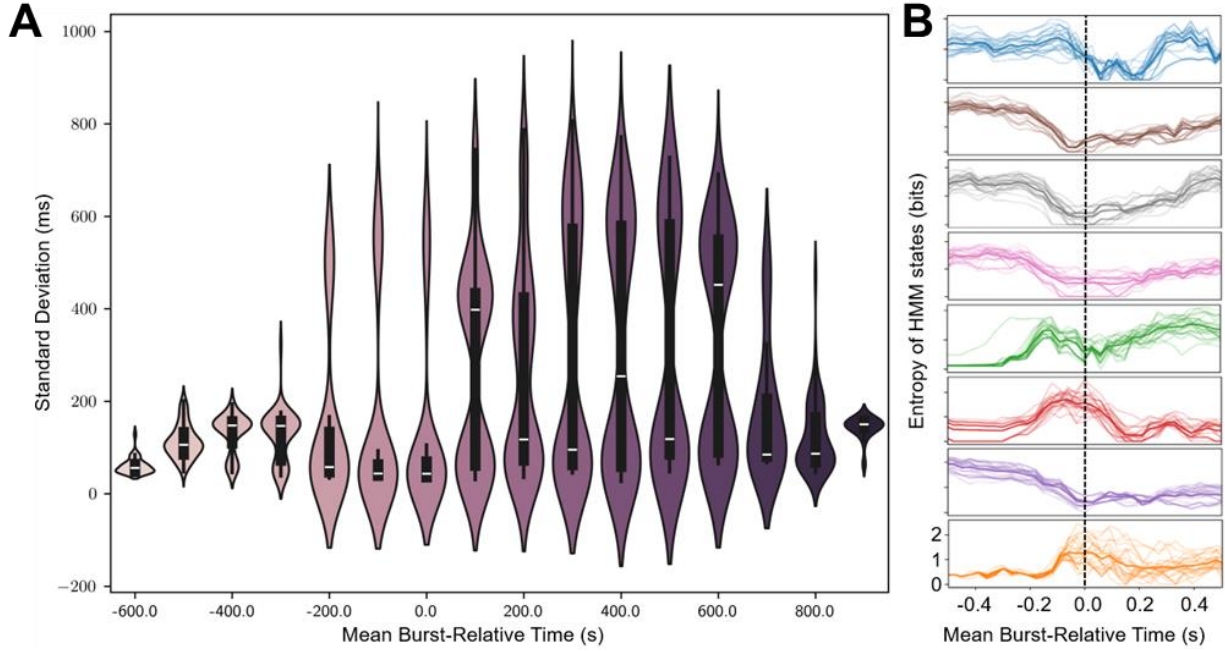
Supplementary Figure 16. Model performance with bin size. Distribution of the difference in posterior log likelihood between held-out validation data and shuffled data across 5 random cross-validation folds are shown here for Organoid 1. The number of states ranges from 10 to 30, for a total of 31 data points per bin size. Performance is relatively insensitive to bin size, just as it is insensitive to model size but depends substantially on the biological sample itself (Extended Data Figure 6 and Supplementary Figure 11). Note that in this and other supplementary figures depicting this metric, the difference in log likelihood is itself shown on a log scale. Box plots: boxes span the 25th–75th percentiles; the center line marks the median (50th). Whiskers extend to the most extreme values within $1.5\times$ the interquartile range from quartile 1 and quartile 3.



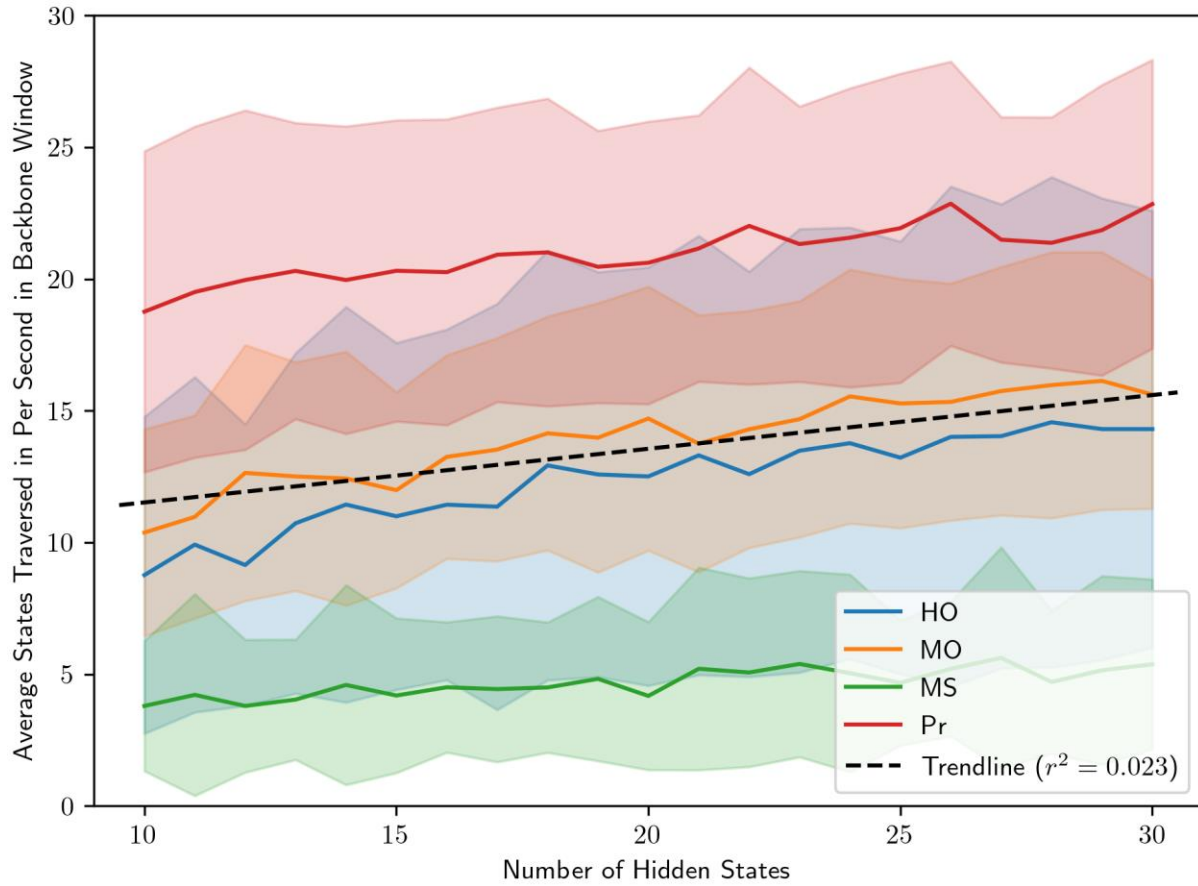
Supplementary Figure 17. Posterior log likelihood plateaus around 10 hidden states. Difference in posterior log likelihood between the held-out validation set and randomized data, for models with 30 ms time bins, as a function of the number of hidden states. Curves indicate mean values, with shaded area for ± 1 STD, as calculated across 5 random cross-validation folds as described in Methods. The posterior log likelihood of each human brain organoid recording plateaus at a different value, but this is highly consistent across multiple training runs and multiple CV folds.



Supplementary Figure 18. Log likelihood is consistently higher for real compared to randomized data. Difference in posterior log likelihood between real and randomized data as a distribution across 5 cross-validation folds for models trained on 30 ms time-binned rasters of each of the human brain organoid recordings, with the number of hidden states (K) ranging from 10 to 30. The figure compares the value of the log likelihood between the real data and the average of the shuffled data control (top), as well showing the distribution per organoid of the difference in log likelihood for each value of K (bottom). Log likelihood is always greater for real than for randomized data, with no overlap between the distributions for real and shuffled data. However, the value, as well as the magnitude of the difference, varies systematically between recordings. Box plots: boxes span the 25th–75th percentiles; the center line marks the median (50th). Whiskers extend to the most extreme values within $1.5\times$ the interquartile range from quartile 1 and quartile 3. In violin plots, minima and maxima equal the lowest and highest observations; the shaded area shows the variable's probability density

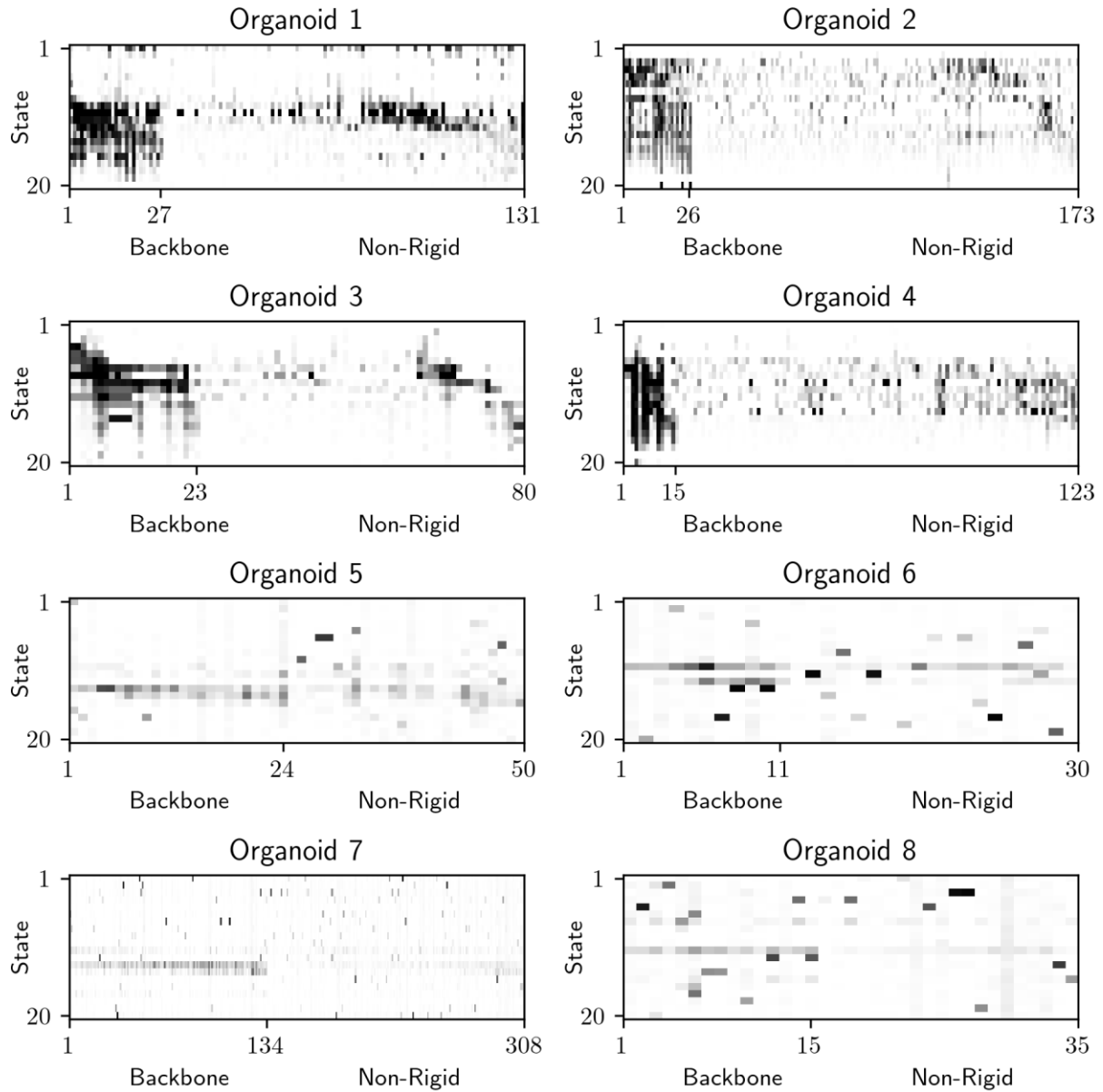


Supplementary Figure 19. Temporal spread of hidden states relative to population burst events. (A) We calculated the histogram of burst-relative times of realizations of each hidden state of each HMM fitted to each organoid recording with bin size $T = 30$ ms (see Methods), and fitted a Gaussian distribution to each histogram, parametrized by the mean and standard deviation of burst-relative times. We attempted to restrict our attention to states which are well described by the mean/STD characterization (as opposed to, e.g., having a multimodal distribution with significant weight both before and after the burst). To do this, we selected hidden states via a chi-square test, including only those which failed to reject the Gaussian fit at the 1% significance level (i.e., all states with $P > 1$). The selected states were then grouped according to their mean occurrence time with 100 ms resolution. (B) The variation in the number of hidden states is shown relative to population burst peak events across eight human brain organoid samples (Fig. 5). Hidden state diversity is quantified as a function of time relative to burst events using the Shannon entropy of the empirically observed distribution of hidden states across all burst realizations within the recording. This is calculated separately for each fitted HMM, with the average displayed in bold. Data are shown over a ± 500 ms window relative to burst peak events (gray shaded region in A). The calculated Shannon entropy in the top panel (blue) is from the organoid shown in Figure 5A. Organoids generally exhibit greater diversity of hidden states (higher entropy) outside of burst windows, although a few cases displayed trivially low entropy due to limited activity. Notably, entropy consistently approaches a local minimum near burst peaks, indicating a convergence toward specific states with higher transition probabilities during the initiation of sequential activation patterns. Box plots: boxes span the 25th–75th percentiles; the center line marks the median (50th). Whiskers extend to the most extreme values within $1.5\times$ the interquartile range from quartile 1 and quartile 3. In violin plots the shaded area shows the variable's probability density.

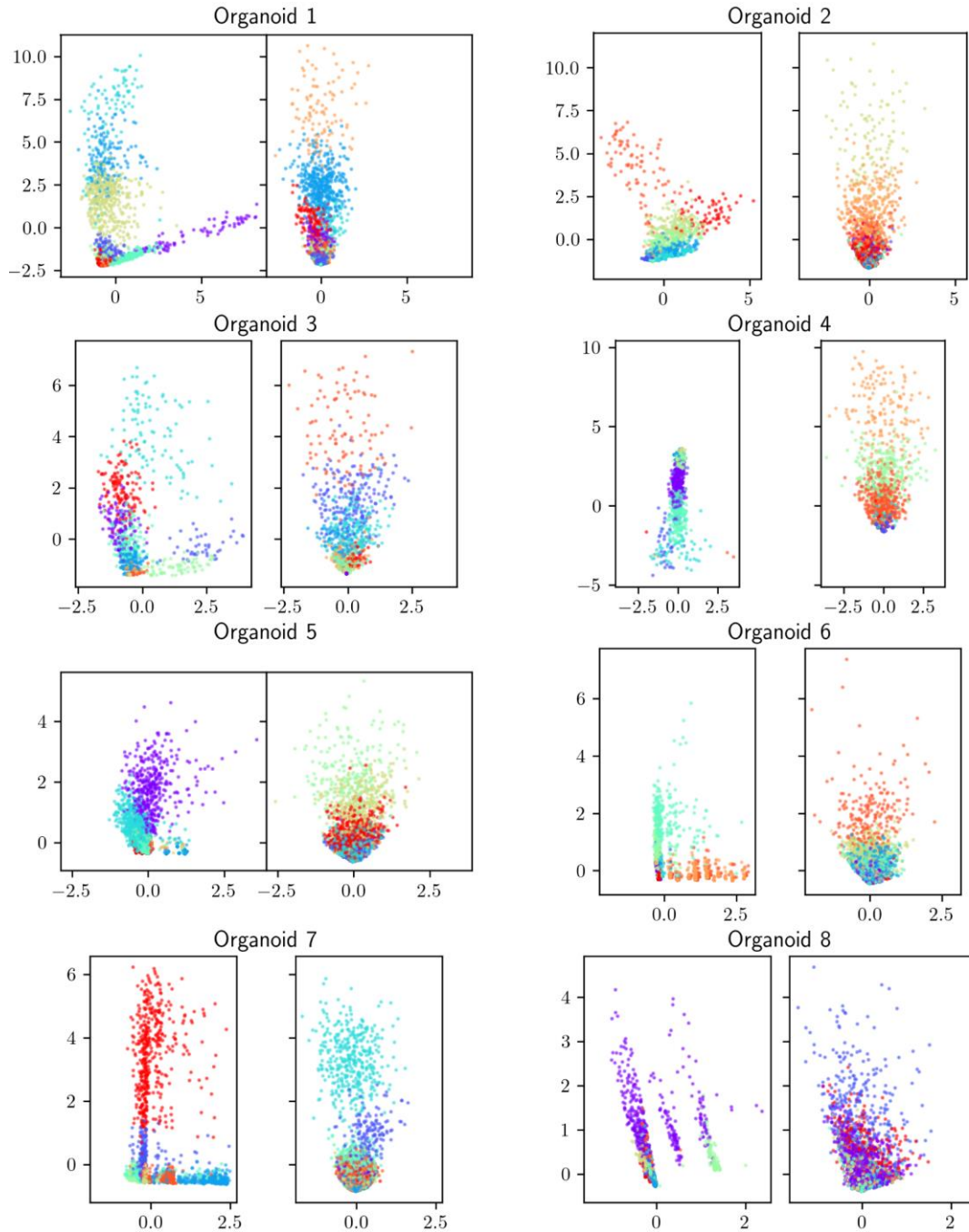


Supplementary Figure 20. State traversal rate varies little with the number of hidden states.

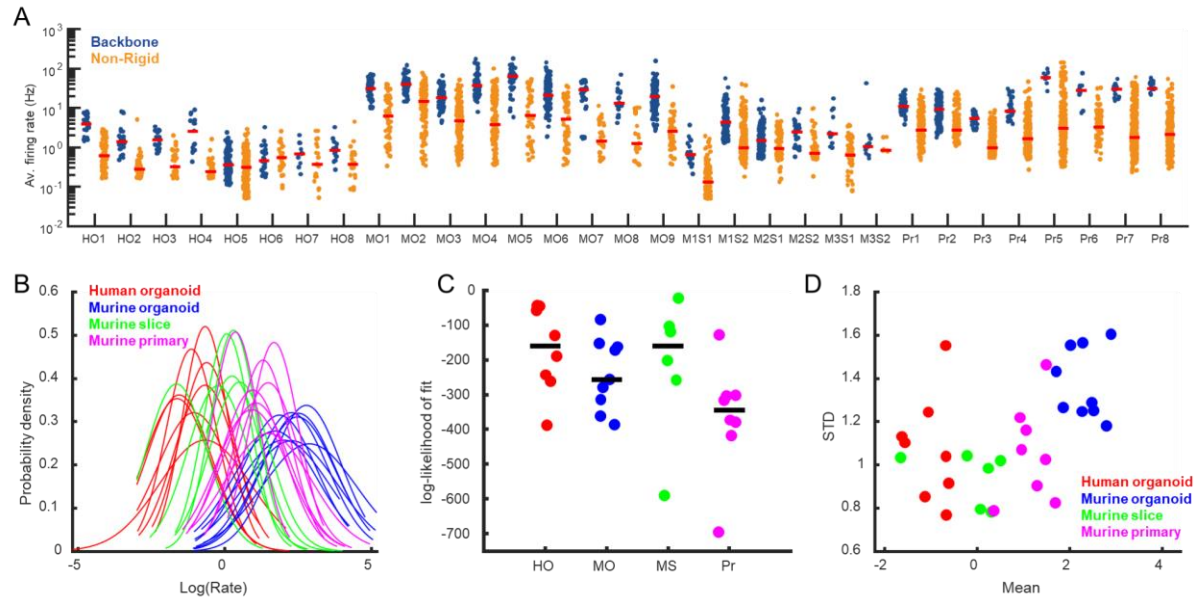
For each hidden Markov model, fitted to a given experiment, we averaged across bursts the number of *distinct* hidden states traversed during the backbone interval (see Methods) divided by its duration. An overall trendline is drawn indicating the effect of the number of hidden states on the state traversal rate. Although it is statistically significant ($P < 0.01$), the effect size is small in comparison to the variability within and between samples.



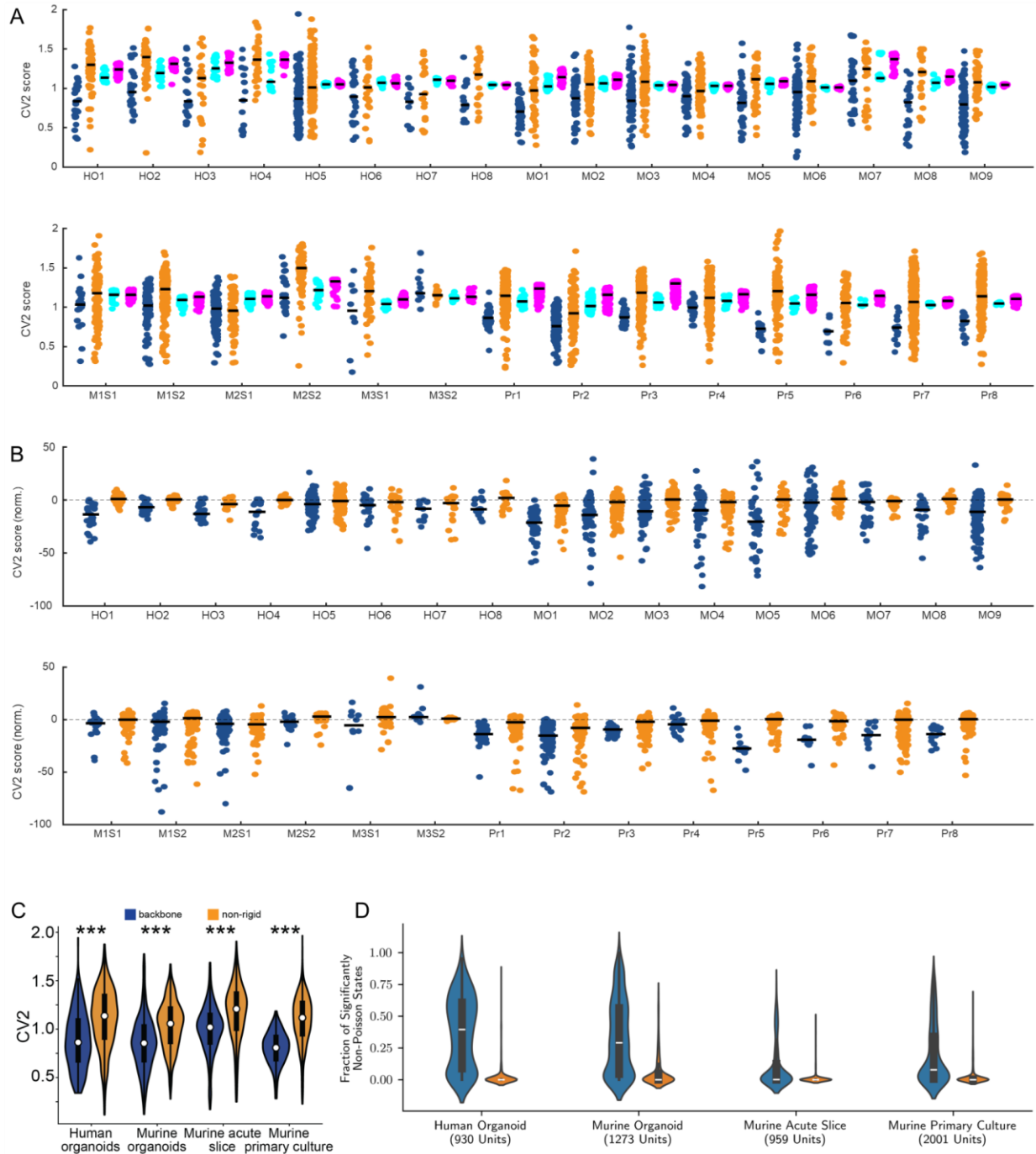
Supplementary Figure 21. Backbone and non-rigid units differ in their consistency by state. Each panel represents, for one brain organoid recording, the probability that each unit fires at least once in each of the 20 hidden states. The color scale as well as the meanings of all quantities and labels are identical to **Fig. 5D**.



Supplementary Figure 22. Randomized but not real data is explained by population rate. HMM-based reduced-dimension view of time-binned spike matrix data (see *Supplementary Methods: dimensionality of a fitted HMM*), with points colored according to the most probable hidden state according to a trained HMM with $K = 10$ hidden states. For each organoid, the left panel shows real data and the right panel shows randomized data. Note that in each case, for real data, hidden states clearly occupy different regions in the two-dimensional space, whereas they are predominantly vertically stratified in the randomized data. This vertical stratification must correspond at least approximately to the population rate, as population rate is the only temporally varying information left intact by randomization.

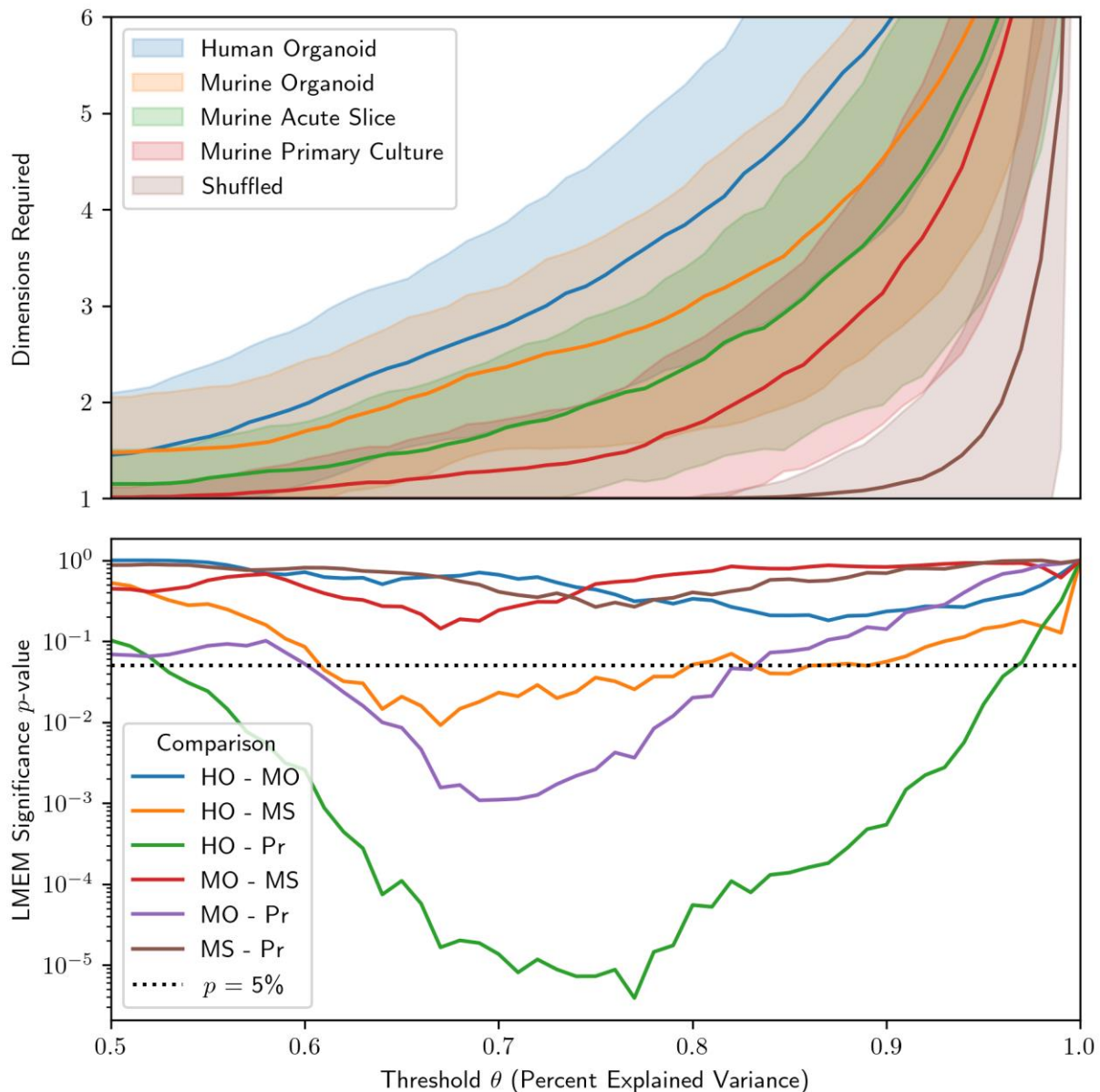


Supplementary Figure 23. Backbone units have a higher average firing rate in each individual recording. (A) The distribution of average firing rates per analyzed recording for backbone and non-rigid units separated. Red bars mark the distribution medians. In all cases, the backbone units populate the tail of the skewed average firing rate distributions. Or = organoid, MS = Murine Slice, Pr = Murine Primary culture. See **Fig. 7A** for the results of the statistical comparison. **(B)** Normal distributions fitted to the distributions of the log of the firing rate per unit for all recordings. **(C)** Log-likelihood for the fits in **B**. Black bars mark the distribution medians. **(D)** The mean and STD for the fits in **B**.

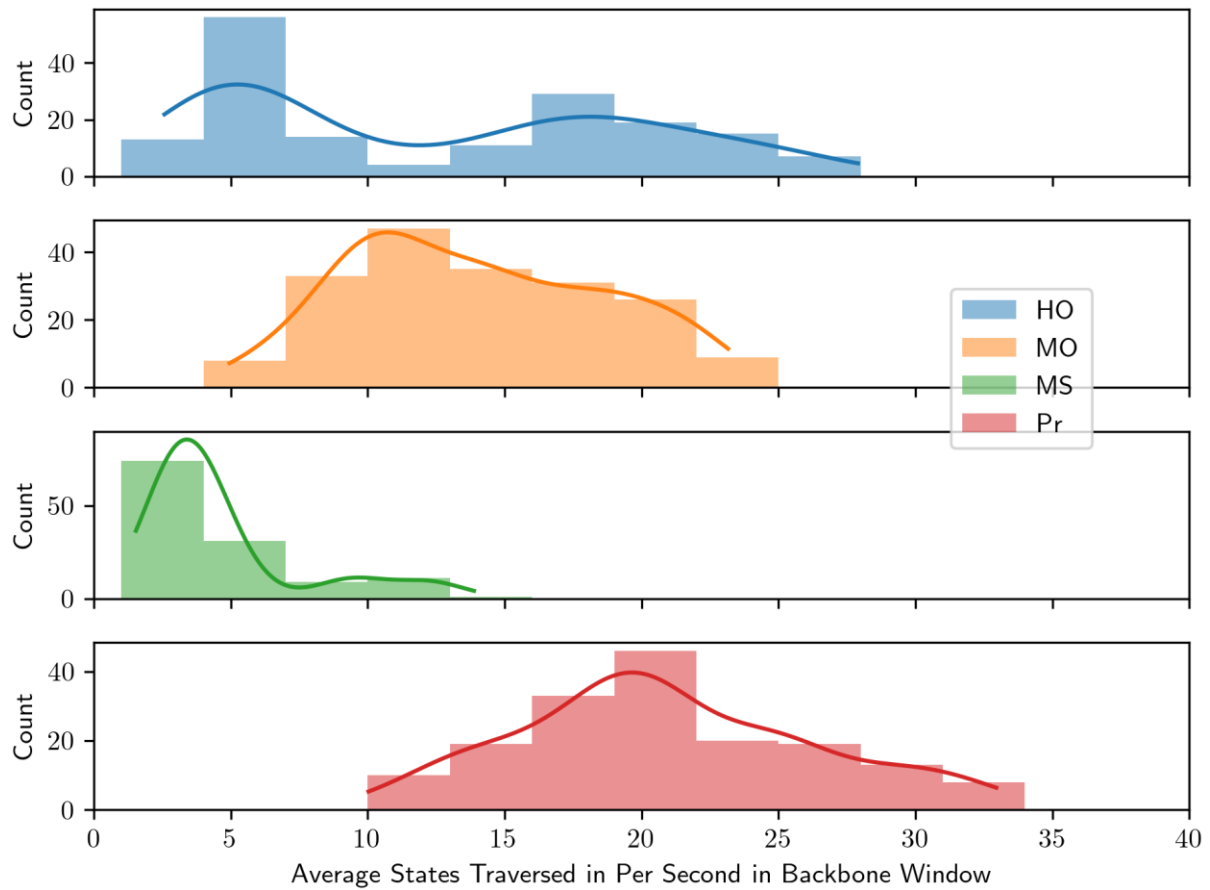


Supplementary Figure 24. Spiking variability of backbone and non-rigid units. (A) The CV2 scores of the spiking activity of each individual unit as a measure for spiking variability. The same CV2 computations were performed on 100 different shuffled spike matrices and the average over all 100 shuffled datasets are reported as well. Black bars mark the distribution medians. (B) The CV2 scores shown in A after z-score normalization using the mean and STD of the CV2 computations applied to the 100 shuffled spike matrices. Black bars mark the distribution medians. (C) The CV2 scores are significantly larger for non-rigid units compared to backbone units, reflecting a more Poisson-like firing of non-rigid units (Human organoids $P \leq 10^{-20}$, Murine organoids $P \leq 10^{-20}$, Murine slices $P = 7 \times 10^{-13}$, Murine primary cultures $P \leq 10^{-20}$, two-sided linear

mixed-effect model with sample as grouping variable, $n = 31$ total samples). **(D)** Distribution of the fraction of all hidden states across all fitted HMMs in which each unit exhibited lower firing variability than Poisson (according to a chi-square test described in *Methods: non-Poisson Units*). Whereas most backbone units have low variability in a sizable fraction of hidden states, most non-rigid units are indistinguishable from Poisson across almost all states. Box plots (C-D): boxes span the 25th–75th percentiles; the center line marks the median (50th). Whiskers extend to the most extreme values within $1.5\times$ the interquartile range from quartile 1 and quartile 3. In violin plots the shaded area shows the variable’s probability density.

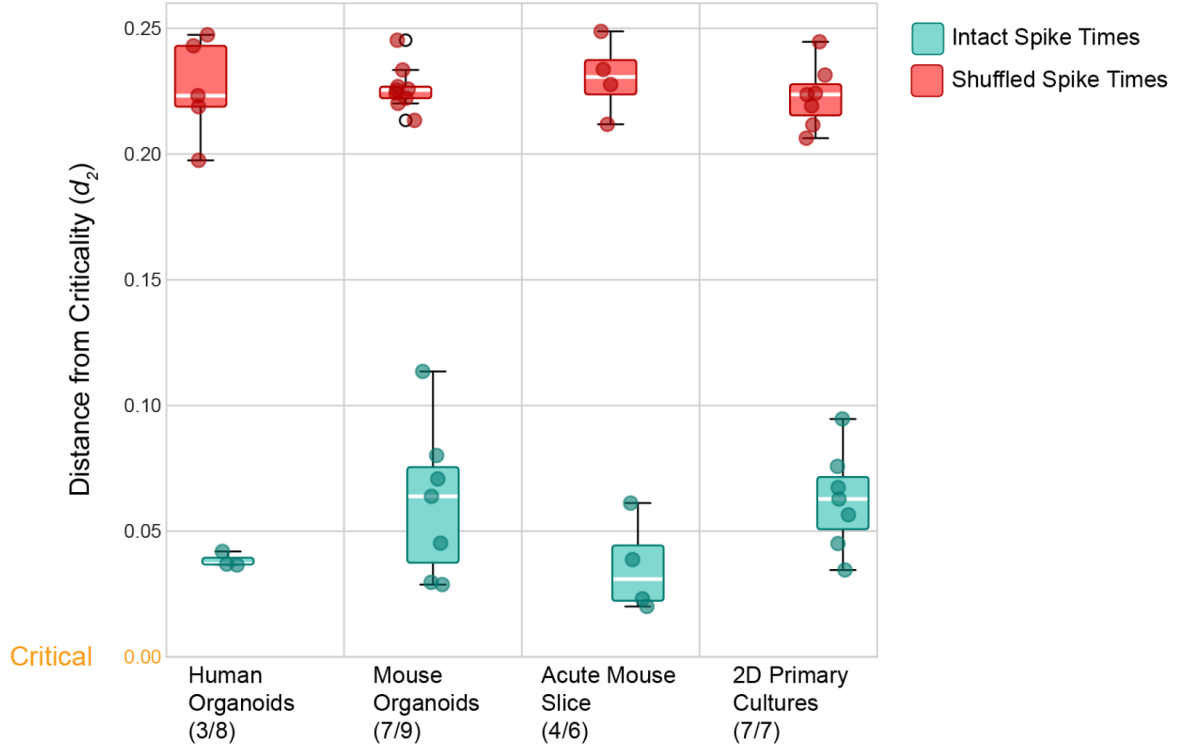


Supplementary Figure 25. Threshold Dependence of Figure 7F. Top: mean $\pm$ one standard deviation of the dimensions required to explain a given fraction θ of the variance between HMM states, as a function of θ . Bottom: statistical significance of the difference between groups in the top panel, according to a generalized linear mixed-effects model (see *Supplementary Methods: Dimensionality of a Fitted HMM*).

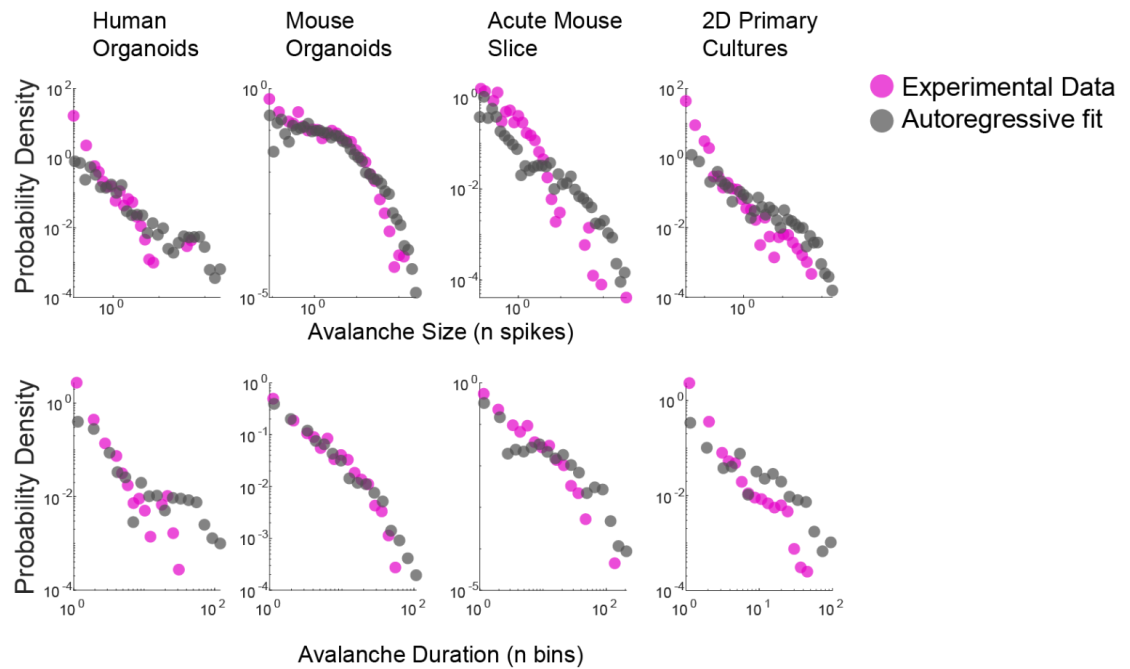


Supplementary Figure 26. State traversal rate differs significantly between models. For each hidden Markov model fitted to a given experiment, we averaged across bursts the number of *distinct* hidden states traversed during the backbone interval (see Methods) divided by its duration. The distributions appear substantially different, but not all are significant according to a linear mixed-effects model given the large inter-sample effects. In particular, human and murine organoid cannot be distinguished ($P = 0.93$), whereas the higher state traversal rate of murine primary cultures is significantly different from that of human organoids ($P = .021$) and murine acute slices ($P < 0.0001$) but not significant relative to murine organoids ($P = 0.064$).

A



B



Supplementary Figure 27. All preparations are capable of generating scale invariant dynamics consistent with a near-critical regime. (A) The distance metric d_2 quantifies how close a system is to temporal criticality. Values of d_2 between ~ 0.0 - 0.1 indicate near-critical dynamics with temporal structure spanning multiple timescales. Random (shuffled) data typically yield $d_2 \geq 0.2$. A subset of preparations from each model system generated activity well-described by the autoregressive model underlying d_2 calculation and exhibited near-critical dynamics (teal box

plots: 3/8 human organoids, 7/9 mouse organoids, 4/6 acute mouse slices, and 7/7 2D cultures). Shuffling spike times (red box plots) abolished temporal criticality (Red box plots, $P = 2.84 \times 10^{-31}$ compared to intact. 5/8 human organoids, 9/9 mouse organoids, 4/6 acute mouse slices, and 7/8 2D cultures). **(B)** Representative examples from each preparation type showing empirical data fits to the autoregressive model used for d_2 calculation. Activity patterns are analyzed as avalanches - bursts of spikes that are quantified by their duration (number of consecutive time bins with activity) and size (total number of spikes). Probability distributions of avalanche sizes (top row) and durations (bottom row) are shown for both experimental data (magenta) and the corresponding best-fit autoregressive model (gray). Mouse organoid data demonstrates particularly good agreement between empirical measurements and model predictions.

Supplementary Methods

Bulk RNA sequencing of brain organoids

Total RNA was isolated from 8-week old organoids via a Quick-RNA™ Microprep Kit (Zymo Research). Briefly, 50 organoids were pooled together per sample for bulk RNA sequencing. This organoid protocol generated organoids with $\sim 600 \mu\text{m}$ diameters and approximately 50,000 cells per organoid. The RNA integrity was assessed using the Agilent TapeStation 4200 (Agilent Technologies). A total of 500 ng of RNA was employed to generate mRNA libraries following the TruSeq Stranded mRNA Library Prep Kit (Illumina) protocol. This procedure involved isolating Poly-A mRNA, fragmenting it, synthesizing the first and second cDNA strands, adding adenylated 3' ends, and ligating TruSeq RNA Combinatorial Dual Indexes (Illumina). The ligated fragments were then amplified for 15 cycles, purified, and quantified using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific). The size and quality of the libraries were evaluated with the Agilent TapeStation 4200. Finally, the libraries were normalized, pooled, and sequenced on the NovaSeq X Plus (Illumina) using 150 bp paired-end reads, achieving approximately 100 million reads per sample.

Bulk RNA sequencing analysis

Raw FASTQ files were processed using the nf-core RNA-seq pipeline which leverages Nextflow workflows (version 23.10.1) (<https://doi.org/10.5281/zenodo.1400710>¹). Adapters and poor quality sequences were trimmed using Trim-Galore (v0.6.10)². Reads were aligned to the GRCh38 genome using ENSEMBL gene annotations (release 111) using STAR and transcripts were quantified with Salmon³⁻⁵. Post processing of reads was accomplished using SAMtools (sorting and indexing alignments)⁶, picard (duplicate identification)⁷, and BEDtools (genome coverage assessment)⁸. Read and mapping quality were assessed using RSeQC⁹, Qualimap¹⁰, dupRadar¹¹, and Preseq¹². Gene length corrected and scaled counts from Salmon were normalized using limma voom¹³. RNA-seq data from the four samples are deposited in the NCBI Gene Expression Omnibus.

Immunohistochemistry and microscopy of human brain organoids.

Organoid samples were stained following methods described in Romero *et al.*¹⁴. Briefly, after fixation in 2% PFA, organoids were permeabilized with 0.1% Triton X and blocked with 100% BlockAid™. Organoids were stained with primary antibodies (Table below) at 4°C on a shaker for 48 hours followed by staining with secondary antibodies for another 24 hours. Nuclei were stained with Hoechst 33342 trihydrochloride (Invitrogen Molecular Probes) at a concentration of

1:10,000. Organoids were imaged on a FV3000RS Olympus or a Zeiss LSM800 confocal microscope.

Antibody	Host	Clone	Company	Cat #	Dilution
Gephyrin	Mouse	Monoclonal	Synaptic Systems	147 011	1:1000
MAP2	Chicken	Polyclonal	Invitrogen	PA1-10005	1:5000
GFAP	Rabbit	Polyclonal	Dako	Z0334	1:400
O4	mouse	Monoclonal	R&D	MAB1326	1:200

Mouse ESC maintenance

Three mouse embryonic stem cell lines were used: C57BL/6, E14TG2a (129/Ola), and KH2 (129/SvJ × C57BL/6 hybrid). Mycoplasma testing using MycoAlert (Lonza # LT07-318) confirmed lack of contamination.

ESCs were maintained on recombinant human protein vitronectin (Thermo Fisher Scientific A14700) coated plates using mESC maintenance media containing Glasgow Minimum Essential Medium (Thermo Fisher Scientific 11710035), embryonic stem cell-qualified fetal bovine serum (Thermo Fisher Scientific 10439001), 0.1 mM MEM nonessential amino acids (Thermo Fisher Scientific 11140050), 1 mM sodium pyruvate (Millipore Sigma S8636), 2 mM glutamax supplement (Thermo Fisher Scientific 35050061), 0.1 mM 2-mercaptoethanol (Millipore Sigma M3148), and 0.05 mg/ml primocin (Invitrogen ant-pm-05). mESC maintenance media were supplemented with 1000 units/ml of recombinant mouse leukemia inhibitory factor (Millipore Sigma ESG1107). Media was changed every day.

Vitronectin coating was incubated for 15 min at a concentration of 0.5 µg/ml dissolved in 1× PBS pH 7.4 (Thermo Fisher Scientific 70011044). Dissociation and cell passages were done using ReLeSR passaging reagent (Stem Cell Technologies 05872) according to the manufacturer's instructions. Cell freezing was done in mFreSR cryopreservation medium (Stem Cell Technologies 05855) according to the manufacturer's instructions.

Mouse cortical organoid media composition

The cortical differentiation media used to day 5 was composed of DMEM/F12 with GlutaMAX (Thermo Fisher Scientific, #10565018), 10% Knockout Serum Replacement (Thermo Fisher Scientific, #10828028), 0.1 mM MEM Non-Essential Amino Acids (Thermo Fisher Scientific, #11140050), 1 mM Sodium Pyruvate (Millipore Sigma, #S8636), 1X N-2 Supplement (Thermo Fisher Scientific, #17502048), 2X B-27 minus Vitamin A (Thermo Fisher Scientific, #12587010), 0.1 mM 2-Mercaptoethanol (Millipore Sigma, #M3148), and 0.05 mg/mL Primocin (Invitrogen, #ant-pm-05). The medium was further supplemented with 10 µM Rho Kinase Inhibitor (Y-27632), 5 µM WNT inhibitor (XAV939, StemCell Technologies #100-1052), and 5 µM TGF-β inhibitor (SB431542, Tocris #1614).

From Day 6 to Day 12, the neuronal differentiation medium consisted of Neurobasal-A (Thermo Fisher Scientific, #10565018), BrainPhys Neuronal Medium (Stem Cell Technologies, #05790), 1X B-27 Supplement without Vitamin A (Thermo Fisher Scientific, #12587010), 1X N-2 Supplement (Thermo Fisher Scientific, #17502048), 0.1 mM MEM Non-Essential Amino Acids (Thermo Fisher Scientific, #11140050), and 0.05 mg/mL Primocin (Invitrogen, #ant-pm-05), with the fresh addition of 200 μ M Ascorbic Acid. Organoids were cultured under 5% CO₂, with medium changes performed every 2-3 days.

From Day 15 onward, the organoids were maintained in a neural differentiation medium containing BrainPhys Neuronal Medium, 1X B-27 Plus Supplement (Thermo Fisher Scientific, #A3582801), 1X N-2 Supplement, 1X Chemically Defined Lipid Concentrate (Thermo Fisher Scientific, #11905031), 5 μ g/mL Heparin (Sigma Aldrich, #H3149), and 0.05 mg/mL Primocin. Additionally, 200 μ M Ascorbic Acid was included until Day 25.

Single-cell RNA sequencing of cortical organoids

PIPseq library preparation

Murine cortical organoid library prep was carried out following the methods described in Clarke *et al.*¹⁵. Ten murine cortical organoids per cell line (3 cell lines defined above) were pooled together and dissociated using Worthington Papain Dissociation System (Worthington # LK003150) according to the manufacturer's instructions. Briefly, 20 units of papain per ml, 1mM L-cysteine and 0.5mM EDTA were resuspended in Earle's Balanced Salt Solution (EBSS). The enzyme solution was activated by incubating for 30 minutes at 37° C. After activation, we included 200 units of DNase I per ml. We transferred the tissue into the papain and DNase I solution and incubated for 30 minutes at 37° C. Every 10 minutes the samples were agitated by gently shaking the tube. The tissue was mechanically dissociated using flamed glass Pasteur Pipets (Fisher Scientific # 13-678-6B). The tissue was centrifuged at 300 RCF for 3 minutes. The supernatant was removed and approximately 1mL of 1X PBS containing 0.1% Bovine Serum Albumin (Millipore Sigma # A3311) was added and the cells were resuspended, then put through a 40 μ m cell strainer (Corning # 431750) into a 6 well ultra low-adhesion plate (Millipore Sigma # CLS3471). Cells were then manually counted on a hemocytometer and 3.3K cells from each of the three dissociated organoid cell lines were pooled to make a total of 10K cells. These pooled cells were then combined into a single sequencing reaction using the PiPseq T2 kit and carried out according to the manufacturer's instructions.

PIPseq data analysis

Samples were pooled and sequenced on the AVITI PE75 Flowcell ~900M reads at the UCDavis Technologies Core. The sequencing data was then processed using the PIPseeker pipeline (PIPseeker v3.3, Fluent BioSciences), using the mouse genome GRCm39 (GENCODE vM29 2022.04, Ensembl 106)¹⁶. We used the default parameters to process the FASTQ files, perform mapping, transcript counting and cell calling.

Sensitivity 5 matrices were used for downstream analysis using R package Seurat (version 5.1.0)¹⁷. Souporecell was used for genotype demultiplexing and doublet identification¹⁸. Doublets were also identified using the R package DoubletFinder v2.0.4¹⁹.

Cells were filtered based on doublet identification, with greater than 20% mitochondrial genes, less than the 5th percentile unique genes detected, and greater than 50,000 total RNA counts. The

raw gene count matrices were normalized with SCTransform function while regressing out mitochondrial genes identified the top 3,000 highly variable genes. Principal component analysis was performed and clusters were identified by the FindNeighbors function using 40 principal components (chosen based on visual inspection of the elbow plot). The function FindClusters was run with resolutions of 2, 1, and 0.5 with leiden clustering. The clusters were then embedded and visualized with Uniform Manifold Approximation and Projection (UMAP)²⁰ and the resolution of 0.5 was chosen based on visual inspection of marker genes to represent the best approximal cell type. Annotation of the dataset was performed by marker gene expression from the FindMarkers function by the Wilcoxon Rank Sum test and reference to canonical marker genes from the Allen Brain Atlas Whole Cortex and Hippocampus²¹ visualized using the UCSC Cell Browser²².

Immunohistochemistry and microscopy

Organoids were collected and fixed in room temperature 4% Paraformaldehyde (PFA) (Thermo Fisher Scientific # 28908) and cryopreserved in 30% Sucrose (Millipore Sigma # S8501). They were then embedded in a solution containing 50% of Tissue-Tek O.C.T. Compound (Sakura # 4583) and 50% of 30% sucrose dissolved in 1X Phosphate-buffered saline (PBS) pH 7.4 (Thermo Fisher Scientific # 70011044). Organoids were then sectioned to 20 µm using a cryostat (Leica Biosystems # CM3050) directly onto glass slides. After 2 washes of 5 minutes in 1X PBS the sections and 1 wash in deionized water (Chem world #CW-DW-2G) sections were incubated in blocking solution 5% v/v donkey serum (Millipore Sigma # D9663), and 0.1% Triton X-100 (Millipore Sigma # X100) for 1 hour. The sections were then incubated in primary antibodies overnight at 4° C. They were then washed 3 times for 10 minutes in PBS and incubated in secondary antibodies diluted in blocking solution for 90 minutes at room temperature. They were then washed 3 times for 10 minutes in PBS and coverslipped with Fluoromount-G Mounting Medium (Thermo Fisher Scientific # 00-4958-02). Additional histology was performed on sections on fixed tissue that were not cryopreserved (Supplementary Fig. 7). Organoid samples were fixed in 4% paraformaldehyde (Thermo Fisher Scientific # 28908) overnight at 4 °C. After rinsing, samples were embedded in 4% low melting point agarose (invitrogen # 16520-050) in PBS and sectioned (50 µm) using a VT1000s vibratome (Leica, Lumberton, NJ). Sections were then incubated in an initial blocking solution consisting of 5% v/v donkey serum (Millipore Sigma # D9663), BSA 1% (Millipore Sigma # A7906), and 0.5% Triton X-100 (Millipore Sigma # X100) for 1 hour in 4°C. The initial blocking solution was then carefully removed, and sections were then placed in an antibody blocking solution with primary antibodies composed of 2% v/v donkey serum (Millipore Sigma # D9663), and 0.1% Triton X-100 (Millipore Sigma # X100) overnight. Sections were removed from the antibody blocking solution and were washed four times in 1X PBS for 15 minutes at room temperature. Sections were then placed with secondary antibodies diluted in antibody blocking solution, as previously described, at room temperature for 30 minutes. Another wash in 1X PBS was performed, followed by Hoechst 33342 nucleic acid counterstain for 15 minutes at room temperature. Three final washes were conducted and sections were mounted on glass slides using Fluoromount-G Mounting Medium (Fisher Scientific OB100-01).

Primary antibodies used were: rabbit anti-Map2 (Proteintech # 17490-1-AP, 1:2000); mouse anti-Pax6 (BD Biosciences # 561462, 1:100); rabbit anti-Nkx2.1 (Abcam # ab76013, 1:400); rat anti-Ctip2 (Abcam # ab18465, 1:250); rabbit anti-Brn2 (Thermofisher # PA530124, 1:400); anti-Gaba (Thermo Fisher Scientific # PA5-32241, 1:375). Secondary antibodies were of the Alexa

series (Thermo Fisher Scientific), used at a concentration of 1:750. Nuclear counterstain was performed using 1.0 µg/ml Hoechst 33342 (Thermofisher # H1399).

Imaging was done using an inverted confocal microscope (Zeiss 880) and Zen Blue software (Zeiss). Images were processed using Zen Black (Zeiss) and ImageJ software (NIH).

Synaptic Blocker experiments

Preparation of stock solutions to block components of slow and fast synaptic transmission: the AMPA receptor blocker NBQX (Abcam) was solubilized in DMSO, the NMDA receptor blocker R-CPP (Abcam) and the GABA receptor blocker Gabazine (Abcam) were prepared in ultrapure distilled water (Life Tech) at 1000x the desired working concentration. The working concentrations were 10, 20 and 10 µM for NBQX, R-CPP and Gabazine respectively. Recordings were made from murine brain organoids with gabazine 15 minutes after addition of the compound. Subsequent recordings were performed after the addition of NBQX and R-CPP. Data was spike sorted and analyzed as described in the Methods.

Firing rate peak rank order correlations

For each burst, the firing rate peak time of all backbone units was taken and a rank was assigned to each unit. Next a Spearman rank order correlation was computed between every pair of bursts to assess the similarity in the firing rate peak sequences of the backbone units (using the built in MATLAB function `corr` with “Type” = “Spearman”). The same computations were performed on 100 different shuffled the spike matrices and the results from the original spike matrices were normalized using the $(A-B)/(A+B)$ strategy, where A is the measured value and B is the averaged value computed over the 100 shuffled datasets.

Burst clustering

For all the detected bursts, the firing rate per unit was selected for a window of -250 ms until 500 ms relative to the burst peak. Similar to Segev *et al.*²³, the pairwise correlations in the firing rates were computed for all unit pairs, using the firing rates for this single burst window. Subsequently, the bursts were clustered by performing a k-means++ clustering on the pairwise correlation matrices. The optimal number of clusters was selected using the elbow method. The clustering results were assessed by projecting the pairwise correlation matrices per burst onto the first two principal components, labeled by their cluster and cluster separability was confirmed. Next, to assess variability in the firing of a unit between different burst clusters, the average firing rate per unit was computed for each burst cluster and the CV score (STD/mean) was computed to quantify the firing rate differences between burst clusters per unit. This difference was compared between backbone units and non-rigid units using the statistical analysis as described in *Methods: statistical analyses for model comparisons*.

Hidden Markov model analysis

Hidden Markov models (HMMs) have been widely used in computational biology, ranging from protein modeling²⁴ to determining evolutionarily conserved genomic elements across species²⁵. More recently, this approach has been utilized to characterize the firing patterns of neuronal ensembles of specific brain states during motor function²⁶, deciphering neural codes of sleep²⁷ and uncovering temporal structure in hippocampal outputs²⁸.

A hidden Markov model is a statistical characterization of a discrete-time random process in terms of a discrete “hidden” state, which cannot be directly observed, but which changes the probability distribution of the observations. At each time step, the value of the hidden state depends only on the previous hidden state. The observation distribution and transition probabilities together make up the parametrization of the HMM. These parameters are fitted using the Expectation-Maximization (EM) algorithm to assign the parameters of the observation distribution and transition probabilities in order to co-optimize the posterior likelihood of the observations and the sequence of hidden states.

For neuronal spiking data, HMMs are typically applied to time-binned spike matrices, where at each time step, the observation is a population activity vector consisting of the number of spikes produced by each unit within that time bin. If the bin size is significantly larger than the neuronal refractory period, the spike counts can be modelled as independent Poisson-distributed observations per neuron, with rate parameter λ . In this case, the probability that a given neuron fires n times in a time bin Δt is given by $p(n) = (\lambda \Delta t)^n \cdot e^{-\lambda \Delta t} / n!$. Our analysis is carried out in the Python programming language, version 3.11, using the implementation of an HMM with Poisson observations provided by the package SSM.

We validated that HMMs capture information from the spiking data via 5-fold cross-validation by comparing the posterior log likelihood of a held-out validation set to that of the randomized surrogate data for the held-out region²⁸. Each “fold” consists of fitting the model parameters to a random 80% subset of the data and evaluating the fitted model on the remaining 20%. Log likelihood is always greater for the real than the surrogate data (Supplementary Fig. 18), indicating that the HMM is modeling transitions using information present in the real data but not the surrogate.

Hidden Markov Model Hyperparameters

Although the parameters of the observation and state transition distributions are selected by EM, there are two hyperparameters as well: the bin size T for converting spiking data into a discrete-time observation vector, and the number of states K for the HMM itself. Both must be chosen independently of the EM fitting process, so we treat them as hyperparameters and evaluate performance for a range of values using the 5-fold cross-validation method described above. We performed this validation across all 8 human brain organoid recordings. We first chose a default bin size of 30 ms based on the characteristic time scale of bursts. Under this condition, fit performance is insensitive to the number of hidden states above 10 (Supplementary Fig. 17), so all further analysis is conducted across models with the number of hidden states ranging from 10 to 30. We next evaluated performance across bin sizes of 10, 20, 30, 50, 70, and 100 ms, for numbers of hidden states ranging from 10 to 50 (Supplementary Fig. 16). Performance is also relatively insensitive to bin size near 30 ms, but significantly larger or smaller bin sizes do exhibit somewhat worse performance. The analysis reported on in the main paper uses a fixed bin size of 30 ms, but the number of hidden states ranges from 10 to 30.

Hidden State Trajectories

The ability of a HMM to capture the stereotyped dynamics of bursts in human brain organoids is explored in Figure 5A-C. Given a fitted HMM, we estimate the most probable sequence of hidden states for a given time-binned spike matrix using the forward-backward algorithm, a standard technique for maximum likelihood estimation of hidden state. Then, this sequence of

hidden states is registered relative to the peaks of all the bursts in the recording, and trimmed into fixed-length subsequences corresponding to a fixed time window surrounding each peak. For visualization purposes, the parameters were set to 300 ms before and 600 ms after. We then computed the empirical probability distribution over hidden states as a function of time relative to the burst peak by counting how many times each state appears at each position across all subsequences, divided by the total number of subsequences. Results are shown for one representative HMM (with 20 hidden states) in the first organoid we analyzed (with 30ms time bins).

We also measured the rate of hidden state traversal during the burst backbone time period (*Methods: firing rate sequences and burst backbones*). We split the maximum-likelihood state sequence into subsequences corresponding to one backbone period around each burst peak, and calculated the total number of hidden states divided by the duration of the burst backbone period. The distribution of these state traversal rates was significantly different for murine primary cultures compared to human brain organoids and murine slices (Supplementary Fig. 26), but much more similar between different 3D models.

Consistency of units within a Hidden State

We refer to each time bin of the spike matrix in which a given hidden state is most probable as a “realization” of that state. We then computed the consistency of each unit in each hidden state as the fraction of realizations of that hidden state in which the unit fires at least once. This procedure yielded an array of consistency scores with one row for each of the model’s K hidden states, and one column for each unit (Fig. 5D, Supplementary Fig. 21).

We view the columns of this array as vectors in a K -dimensional space; Figure 5E visualizes a concrete example of this space, using PCA dimensionality reduction to demonstrate that backbone and non-rigid units are almost linearly separable. We measured the degree of this approximate linear separability by determining an optimal decision boundary within the original space using a linear support vector machine (SVM).

Linear separability was quantified as the accuracy of the linear classifier according to leave-one-out cross-validation: for each unit, the model was fitted to all other units, and its accuracy was evaluated on the single held out unit. The single-unit accuracy (always either 0 or 1) was then averaged across all units. Then, as a null hypothesis to control for the fact that backbone units’ fire more overall than non-rigid units, we calculated the linear separability of backbone and non-rigid units based solely on their overall firing rate. This does not depend on the fitted HMM, so the null hypothesis has only one score per organoid (the linear separability across all units), rather than the distribution across models produced by the SVM-based linear separability metric.

Dimensionality of a Fitted HMM

To evaluate the ability of HMMs to represent non-trivial activity manifolds beyond simple variations in population firing rate, we estimated the dimensionality of the observation model in each fitted HMM. The population observation matrix is an array of shape [number of hidden states] $\times$ [number of units], where each entry represents the parameter λ of the Poisson distribution estimated for the firing of that unit in that state. We performed a singular value decomposition (SVD) on this matrix so that a principal component analysis with any desired

number of dimensions d could be acquired by projecting only the first d components of the SVD. Furthermore, this same transformation can be applied to the time-binned spike matrix itself in order to yield a dimension-reduced version for visualization purposes (Supplementary Fig. 22).

We defined the “dimensionality” of the trained HMM for a given dataset to be the number of principal components required to meet a given threshold θ in the percent explained variance on the HMM states themselves. The statistical significance of this finding was calculated using a generalized linear mixed-effects model (*glmer* from the lme4 R package) with Poisson-family observations and a logarithmic linkage function. This differs from other results using a standard linear mixed-effects model due to the dimension being a discrete quantity, which cannot reasonably be approximated as Gaussian. For the same reason, normalization against the randomized baseline was not performed in this analysis; the original integer dimensionality values were modeled.

Non-Poisson Units

Under the null hypothesis, which was originally assumed in fitting HMMs to our data in the first place, each unit produces Poisson firing at a rate which depends on the hidden state. For a Poisson unit, for each hidden state of each fitted HMM, the number of firings of the unit in each realization of the hidden state should follow a Poisson distribution. This distribution has mean and variance both equal to its single parameter λ , so we calculated the mean number of firings of a unit across all realizations of a hidden state, and performed a one-sided chi-square test to identify whether the variance in firings for that unit was less than under the Poisson null hypothesis at the $P = 1\%$ significance level. This value was then averaged across all hidden states (weighted by the number of realizations of each state) to yield an overall measurement of the conditional deviation from Poisson statistics given the hidden state information (Supplementary Fig. 24D).

d_2 calculation

To assess temporal scale-invariance in network dynamics, we calculated the distance metric d_2 following methods developed by Sooter *et al.*²⁹. For each recording, we first created a population activity time series by counting all spikes across all neurons in 30 ms time bins. This time series was z-scored (mean subtracted and normalized by standard deviation). We then fit an order-20 autoregressive (AR) model to the z-scored time series using the Yule-Walker method. The AR model describes how current activity depends on past activity through a history kernel that spans 20 time steps, capturing temporal correlations across multiple timescales. For time-shuffled controls, we randomly shuffled the spike times of each neuron independently while maintaining their firing rates, then performed the same binning, z-scoring, and AR model fitting procedures.

The AR model coefficients were used to calculate d_2 , which quantifies the Euclidean distance from the model parameters to the $\beta=2$ critical fixed point in the temporal renormalization group framework. This fixed point represents a type of criticality characterized by power-law temporal correlations with exponent $\beta=2$. Values of d_2 near zero indicate dynamics close to criticality, characterized by scale-invariant temporal fluctuations that persist across a broad range of timescales. In contrast, larger values of d_2 indicate deviation from criticality, reflecting dynamics dominated by a characteristic timescale or lacking long-range temporal correlations. Time-shuffled controls, which destroy temporal correlations while preserving basic firing statistics, typically yield d_2 values greater than 0.2. The geometric interpretation of d_2 is

the minimum distance between the AR model coefficients and a hyperplane in coefficient space that contains all models that flow to the $\beta=2$ fixed point under the temporal renormalization group transformation.

Not all neural activity patterns are well-described by autoregressive models. For example, highly bursty dynamics with long silent periods punctuated by brief population-wide activation can violate the AR model's assumptions about temporal continuity. Therefore, before calculating d_2 , we assessed the quality of the AR model fit for each preparation. We compared the avalanche size distributions, avalanche duration distributions, and power spectra of the original data with those generated by simulating the best-fit AR model. Preparations were excluded from d_2 analysis if the AR model failed to capture these key statistical features of the empirical data, specifically if the model-generated distributions deviated from the empirical distributions by more than one order of magnitude. This approach excluded 5/8 human organoid preparations, 2/9 mouse organoid preparations, 2/6 acute slice preparations, and 0/7 2D culture preparations from d_2 analysis, ensuring that our assessment of criticality was based only on preparations whose temporal structure was accurately captured by the AR framework.

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