Appendix A - Supplementary analysis of overlapping ROIs

In single photon calcium imaging, especially with head-mounted microscopes, the treatment of regions of interest (ROIs) that overlap between days is controversial (Liberti et al 2016, Katlowitz et al 2018). In this study, canary neural signals were acquired with chronically implanted mini-microscopes and it is plausible to assume that the same sources appeared in multiple days. Due to the controversy in the literature we chose to treat ROIs independently each day. This choice is justified by two reasons. First, the numbers of identified ROIs per day often exceeded 30 (peak values exceeding 70) and these sample sizes should robustly approach the mean of the per-day distributions for the tests we carried. Second, unifying ROIs as single sources persisting across days can possibly introduce uncontrolled biases.

Never the less, we also carried an additional analysis with the following underlying assumptions:

- First, ROIs that overlapped in at least 25% of their footprint (of the smaller ROI) across consecutive days were suspected as the same source.

With this assumption, about 69% of the daily-annotated ROIs persist to the following day (Figure 1)

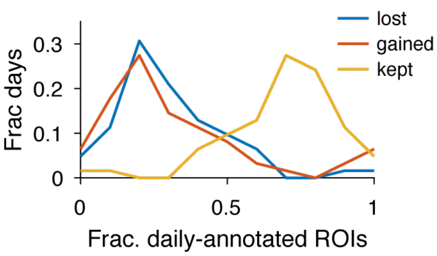


Figure 1 | Histograms of fraction of daily recorded ROIs that overlap, appear or disappear between consecutive recording days (kept, gained, lost respectively)

If we assume that the between-days persistence probability $p \in [0,1]$ is independent across ROIs and across days then the fraction of independent sources, P_{ind}, is given by:

$$P_{ind} = \sum_{n=1}^{\infty} \frac{(p^{n-1} - p^n)}{n}$$

because a ROI that appears in n consecutive days counts $\frac{1}{n}$ times in each day.

Using this equation with $p = 0.69$ we estimate $P_{ind} \approx 0.526$, or 1057 independent sources in our dataset.

- Second, sets of overlapping ROIs that showed the same phrase sequence preference in at least one context (as in the methods section of the main manuscript) were considered as the same source persisting across days and referred to as 'Unified ROIs'. This is a permissive choice since ROIs could be active in more than a single context. In doing so we only reduce the numbers of ROIs with sequence correlations and the statistical power of subsequent tests.
- In the following tests for the population of sequence correlated ROIs we repeated analyses in the main text but counted each source, the unified ROIs, only once.

There are more sequence correlates of past phrases than future phrases

We repeated the analysis in figure 2e of the main manuscript (Figure 2b, below). With unifying ROIs as one source persisting across days the numbers of representations decrease by about a third but the main observation remains the same. There are more representations of past phrases of both first and second (or higher) orders (Figure 2a, below). This bias remains highly significant.

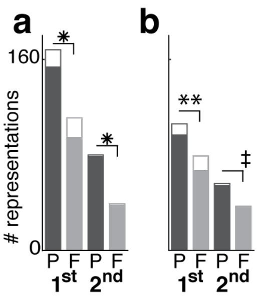


Figure 2 | **Unified ROIs represent more past contexts than future contexts. a.** Figure 2e of the main manuscript shows more individual ROI correlates to past events than to future events among N=398 sequence correlations. Numbers of significant correlations with adjacent (1st order, 2 left bars) and non-adjacent (≥2nd order, 2 right bars) phrases. The correlations are separated by phrases that precede (P) or follow (F) the phrase, during which the signal is integrated. 2-sided binomial z-test evaluate significant differences (✱:p=1.39e-6, 1.065e-7 for 1st and ≥2nd order). **b.** Unified overlapping ROIs as single sources persisting across days also have more representation of past events. This difference is significant for both 1st and ≥2nd order representations (N=255 representations). ‡: p = 0.0005, **: p = 0.0024, one-sided binomial z-tests).

Sequence correlates are predominately found in complex transitions

As in the previous section, unifying ROIs as one source persisting across days does not change the finding in the main manuscript (Figure 4c of the main manuscript, Figure 3a below). Most of the correlates are found during complex transitions (Figure 3b below).

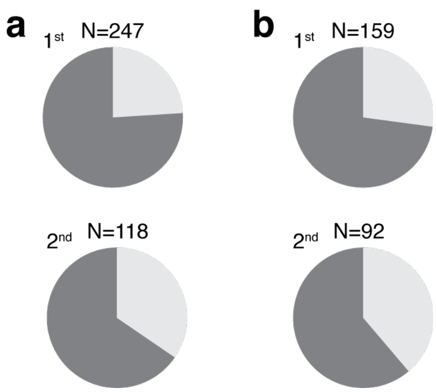


Figure 3 | **Sequence representations in unified ROIs are also predominately found during complex transitions. a.** Figure 4c of the main manuscript shows the fraction of sequence-correlated ROIs found in complex transitions. Pie charts separate 1st order and higher order (≥2nd) sequence correlations. Dark grey summarizes the total fraction for two birds. **b.** Unified ROIs also have sequence correlations mostly in complex transitions.

Appendix B – Non-parametric statistical analysis for results in main manuscript

Several analyses in the main manuscript "Hidden neural states underlie canary song syntax" are based on 1-way ANOVA tests. While statistics textbooks suggest that the requirement for normally distributed data can be relaxed without significant effects (c.f. quotes and citations in the Methods section) we repeated the manuscript's analyses with the non-parametric 1-way ANOVA (Kruskal Wallis). While fewer neurons pass the more stringent tests (~15% less), these analyses recapitulated all the findings in the manuscript. This section summarizes the statistics and results of the non-parametric tests.

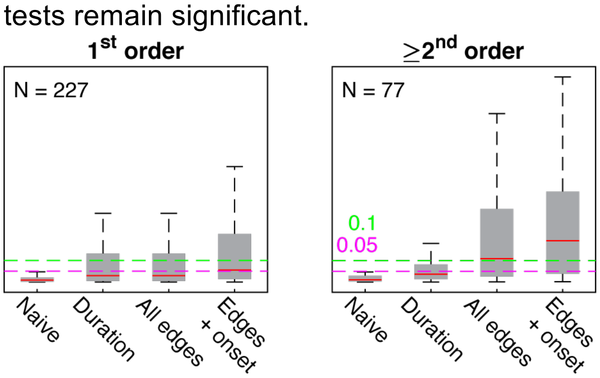
In the following, the number of categories is small and we report the 2nd number of degrees of freedom.

Statistics in Extended Data Fig. 5c showing the sequence correlations of the example neuron in Figure 2d:
Naïve: $\chi^2(35) = 27.37, p < 5e^{-5}$
Removing dependency on phrase duration: $\chi^2(35) = 13.64, p < 0.02$
Removing dependency all phrase edges in the sequence: $\chi^2(35) = 13.03, p < 0.025$
Removing dependency on all edges and the global time in song: $\chi^2(35) = 12.75, p < 0.03$

Statistics in Extended Data Fig. 5 panels:
e. $\chi^2(27) = 16.31, p < 0.0011$
f. $\chi^2(14) = 4.26, p < 0.04$
g. $\chi^2(19) = 11.11, p < 0.012$
h. $\chi^2(21) = 5.16, p < 0.024$
i. $\chi^2(14) = 6.73, p < 0.01$
j. $\chi^2(28) = 10.08, p < 0.0016$

Fractions of daily annotated ROIs with significant sequence correlations:
Total: 20.45%. 1st order: 18%, ≥2nd order: 4.65%.

Statistics in Extended Data Fig. 6h:
Accounting for all confounding variables: 51.1% of 1st order tests remain significant and 29.9% of ≥2nd order tests remain significant.



Statistic in Figure 2e showing more neurons encoding past events:
With non-parametric test the bias remains and slightly increases for high order sequence correlations. 60.6% encode the past in 1st order relations and 70.2% in ≥2nd order relations (all highly significant biases)

Statistical tests in Figure 4:
ROI #21 during first phrase: $\chi^2(13) = 10.12, p < 0.0016$
ROI #45 during first phrase: $\chi^2(13) = 4.5, p < 0.034$
ROI #50 during second phrase: $\chi^2(13) = 10.12, p < 0.0016$
ROI #36 during third phrase: $\chi^2(13) = 5.56, p < 0.019$
ROI #21 during last phrase: $\chi^2(13) = 6.72, p < 0.0016$

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ROI #45 during last phrase: $\chi^2(13) = 8.68, p < 0.0035$

In total 74% of sequence-correlated neurons are found in complex transitions.

Statistical tests in Extended Data Fig. 9:

a. $\chi^2(36) = 25.44, p < 4.1e^{-5}$
b. $\chi^2(13) = 14.24, p < 0.015$
c. $\chi^2(40) = 28.66, p < 2.65e^{-6}$
d. $\chi^2(41) = 20.37, p < 0.00011$
e. $\chi^2(23) = 20.23, p < 0.0026$