

Self-organization of songbird neural sequences during social isolation

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Abstract

Behaviors emerge via a combination of experience and innate predispositions. As the brain matures, it undergoes major changes in cellular, network and functional properties that can be due to sensory experience as well as developmental processes. In normal birdsong learning, neural sequences emerge to control song syllables learned from a tutor. Here, we disambiguate the role of experience and development in neural sequence formation by delaying exposure to a tutor. Using functional calcium imaging, we observe neural sequences in the absence of tutoring, demonstrating that experience is not necessary for the formation of sequences. However, after exposure to a tutor, pre-existing sequences can become tightly associated with new song syllables. Since we delayed tutoring, only half our birds learned new syllables following tutor exposure. The birds that failed to learn were the birds in which pre-tutoring neural sequences were most ‘crystallized’, that is, already tightly associated with their (untutored) song.

1 Introduction

On the one hand, sensory experience is known to be essential for the normal development of brain circuits. On the other hand, genetically specified developmental processes are also essential – we learn too quickly and from too sparse data to rely on sensory experience alone [1]. Thus, it appears that the brain is able to use genetically specified predispositions to fill in gaps in its sensory experience. When typical sensory experience is absent or delayed, certain aspects of brain development proceed anyway, while other aspects are delayed. This is true both in primary sensory systems [2, 3, 4, 5], and for more cognitive behaviors such as social interaction and language [6, 7, 8]. Brain circuits acquire structure and organization even in the absence of typical training inputs.

35 Here we examine this self-organized structure, and what happens when sensory
36 experience is reintroduced, in the context of songbird vocal learning.

37 Song learning is influenced by both auditory exposure to a particular tutor
38 song, and by inherited preferences [9]. It is well known that songbirds, in the
39 absence of exposure to a tutor bird, develop ‘isolate’ songs, with highly vari-
40 able and atypical syllable rhythms [10, 11, 12]. However, when these ‘isolate’
41 songs are used as tutor songs, after two generations birds sing normally again,
42 suggesting that an ‘innate’ preference filters what aspects of a tutor song are
43 actually imitated [12]. Song imitation requires remarkably little total exposure
44 to a tutor song – approximately 75 seconds total on a single day is enough for a
45 bird to remember a song, and subsequently practice and imitate it [13]. Zebra
46 finches, like many songbird species, are able to imitate songs of birds from other
47 species, but when given a choice they prefer zebra finch song [14]. Furthermore,
48 inherited genetic predispositions have a strong effect on both the precise tempo
49 at which a zebra finch sings its song [15], as well as the particular learning styles
50 of individual birds [16]. Thus, within the songbird brain we expect to see an
51 interplay between developmentally specified and learned structure.

52 There are several possibilities for what happens in the brain during isolate
53 song, and how it compares to typical (tutored) brain development. In typical
54 birds, neurons in HVC are initially only weakly coupled to song, firing only
55 at the onsets of syllables when birds are babbling subsong [17]. Then, as the
56 song becomes more mature and repeatable, each HVC projection neuron fires at
57 its own precise moment during the song, together forming a stable sequence of
58 neural firing that tiles the song [17, 18, 19], in interplay with inhibitory neurons
59 [20, 21]. This maturation process in HVC has been modeled as an initially ran-
60 dom network of neurons that, with the right training inputs and plasticity rules,
61 assembles into a chain of sequentially connected neurons [22, 23, 17] (Figure 1A).
62 However, what happens in birds isolated from a tutor? Compared to typical
63 adult zebra finch song, isolate song has a much less stable sequence of syllables
64 and abnormally variable acoustic structure and timing [12]. In fact, aspects of
65 isolate song resemble features of early babbling (subsong). Does HVC in isolate
66 birds resemble that of subsong birds? Or does HVC mature to form sequences,
67 even without experience of a tutor, and without the behavioral stereotypy seen
68 in adult birds? We use functional calcium imaging in singing isolated birds to
69 address these questions.

70 By observing the neural activity in HVC of isolated birds, we found that the
71 HVC network activity can mature into long repeatable sequences even without
72 exposure to a tutor. However, there are some key differences between typical
73 adult HVC sequences and those found in isolated birds, suggesting which fea-
74 tures of HVC development rely on exposure to a tutor. Next, we observe HVC
75 in isolated birds immediately before and after delayed exposure to a tutor. Birds
76 isolated from a tutor are able to learn a song if exposed to a tutor before the
77 end of a critical period, typically around age 65 days post hatch (dph), but are
78 increasingly unable to learn at later ages [24, 25, 26]. Although only half of
79 our late tutored birds successfully learned from the tutor, we observed an in-
80 teresting correlation between HVC activity prior to tutoring and the degree to

81 which birds learned. Namely, birds with highly song-locked HVC activity prior
82 to tutoring typically failed to learn, while birds with less song-locked activity
83 tended to learn. In the birds that did learn, we were able to track sequences
84 throughout the course of learning. Pre-existing self-organized HVC sequences
85 persisted throughout major changes to the song, forming a substrate for newly
86 learned song elements. Together, these results point at how the brain may self-
87 organize, and at the interplay between self-organized structure and the ability
88 to incorporate new information from a tutor.

89 2 Results

90 2.1 Neural sequences are present in isolated birds, but 91 atypical

92 We first asked whether the songs of isolated birds involve the same neural path-
93 ways and neuronal sequences responsible for generating typical song. We carried
94 out functional calcium imaging of large populations of neurons in HVC of iso-
95 lated birds at a range of ages. Sequences of neuronal activity in HVC have
96 previously been analyzed by aligning neuronal activity to repeatable elements
97 of the song [27], an approach with limited utility in isolated birds due to the high
98 variability of their songs. Instead, we extract neural sequences directly from the
99 calcium signals using an unsupervised algorithm [28] to find the sequences that
100 best fit the neural data. This technique reveals the existence of significant se-
101 quential activity in HVC of isolated birds (Figure 1B,C). It also reveals long
102 continuous sequences in data acquired from typical adult HVC (Figure 1D) as
103 expected from previous work [27, 19, 18].

104 The sequences found in isolated birds are surprisingly typical in some re-
105 spects, but atypical in others, especially in their correlation to vocal output. As
106 in typical HVC sequences, neurons in isolated birds participate at characteristic
107 moments during the sequence (Figure 1E), and many neurons participate in at
108 least one sequence (Figure 1F). Neurons that participate in a sequence tend to
109 fire at a majority of sequence occurrences (Figure 1G). Neural sequences are cor-
110 related with precisely timed song features in isolated birds' song (Figure 1H, I,
111 song features calculated as in [29]). However, song locking in isolated birds was
112 only on average 0.58 times as strong as in a typically tutored adult bird (Figure
113 1J, see Methods). Finally, in isolated birds, on average only 61% of each song
114 bout is represented by a detected HVC sequence, substantially less than the
115 complete sequence coverage found in typically tutored birds [19, 18, 17] (Figure
116 1K, see Methods). HVC activity in isolated birds exhibits additional qualitative
117 differences from that in typically tutored birds. While HVC neurons generate
118 only brief bursts of spikes in tutored birds, neurons in isolated birds sometimes
119 generated extended periods of continuous activity, especially during long syllab-
120 les of variable duration (Figure 1L, 7/8 birds exhibited multiple instances of
121 persistent activity, coordinated across at least 3 neurons, and lasting at least
122 500ms). This contrasts with long syllables of typical adult song which are all

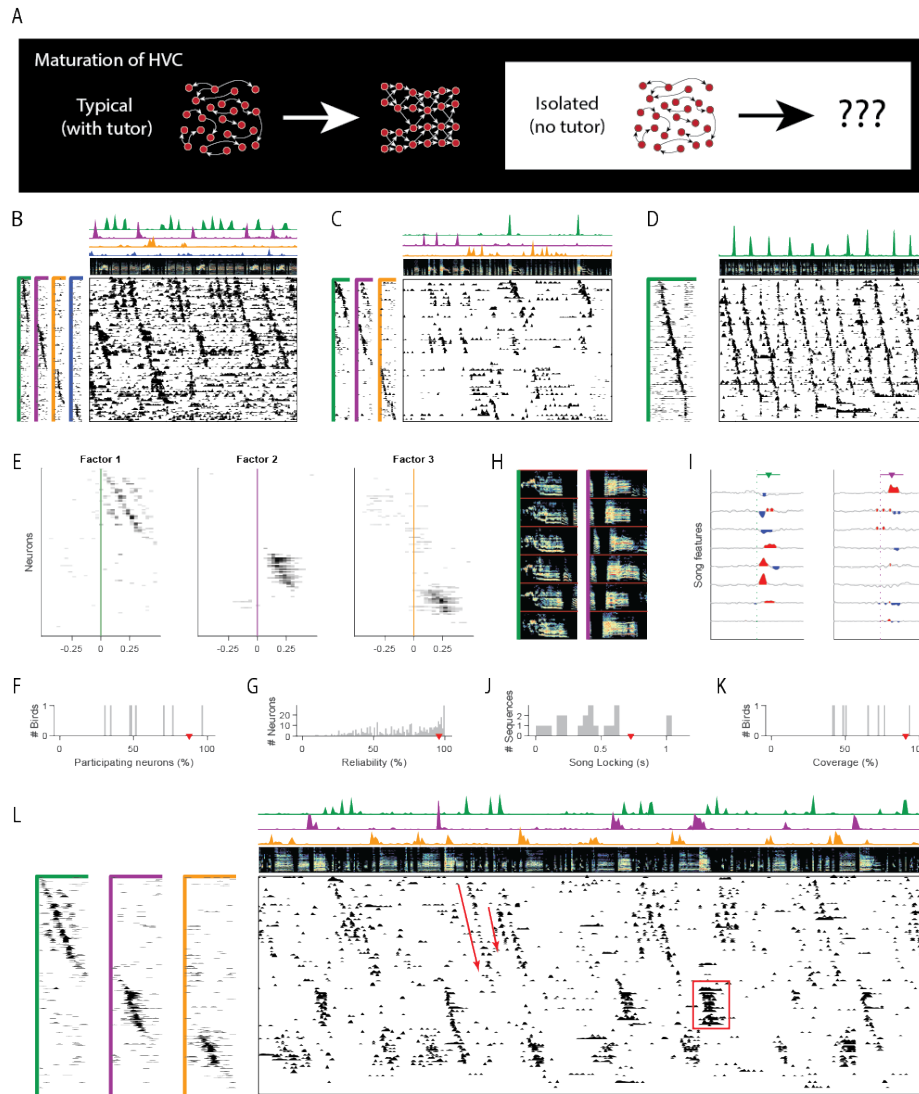


Figure 1: Sequences in isolated birds

(A) Diagram of HVC maturation. In typically tutored birds, HVC sequences appear to grow and differentiate over time. (B) Example neural sequences recorded in a singing isolated bird (older juvenile, 61 dph). Main panel (lower right), functional calcium imaging recordings from 98 neurons for a duration of 6 s. Rows (neurons) sorted according to sequences (factors) extracted by unsupervised algorithm seqNMF (see Methods). (Above) Song spectrogram (0-10 kHz). The four sequence factor exemplars and timecourses are shown to the left and above, in corresponding colors. Duration of factor exemplars: 0.5 s. (C) Same as B, for another example isolated bird (adult, 117 dph). (D) Same as in B, for a typically tutored bird (adult, 217 dph). (E) Time-lagged cross correlation between each neuron and each of the three extracted factors recorded in a singing isolated bird (older juvenile, 68 dph). Only significant bins in the cross correlation are shown ($p < 0.05$, Bonferroni corrected, compared to a circularly-shifted control). (F, G, J, K) Sequence properties in isolated birds. For reference, median for typically tutored bird in D shown in red. (F) Percent of neurons participating in at least one extracted sequence. (G) Reliability of participating neurons across sequence renditions. (H) Example song spectrograms (0.5 s) extracted at moments when neural sequences were detected in an isolated bird (older juvenile, 64 dph) (I) Correlation of these sequences with eight song features (top to bottom: amplitude, entropy, pitch goodness, aperiodicity, mean frequency, pitch, frequency modulation, amplitude modulation). Factor duration 0.5 s, indicated by colored bars above, triangle at center. (J) Strength of song locking (see Methods). (K) Percent of the song covered by some sequence. (L) Example of sequence abnormalities in an isolated bird (same as in E). Sequences of inconsistent length (8/8 isolated birds) and ensemble persistent activity (7/8 isolated birds) are annotated in red.

123 generated by extended sequences of brief bursts. In addition, HVC sequences
124 in isolated birds exhibit variable durations, often truncating at different points
125 (Figure 1L, 8/8 birds), producing syllables of highly variable duration. Such
126 truncations in the middle of a syllable sequence are very unusual in typically
127 tutored birds [30]. These atypical modes of HVC activity suggest several possi-
128 ble mechanisms to understand characteristic features of isolate song, abnormally
129 long syllables and those of variable duration [12]. For example, syllables in iso-
130 lated birds may exhibit variable duration when their underlying HVC sequences
131 are truncated at different points.

132 We wondered if the existence of sequences in HVC of socially isolated birds
133 occurs only after the closure of the critical period (i.e. a product of an already
134 atypical isolate song) or whether they develop at an even earlier age when birds
135 have not yet heard a tutor song, but can still be tutored. We recorded in 5
136 birds at ages 57-64 dph, prior to tutor exposure, and found strong evidence for
137 HVC sequences (Figure S1A). There was not a significant correlation between
138 the age of the bird and any sequence features we measured (Figure S1B-F,
139 linear regression model, significance threshold $p < 0.5$, comparing to a constant
140 model). The correlations were not significant both when we restricted to birds
141 within the traditional critical period (< 65 dph), and when we included data
142 from three older isolated birds (68-117 dph). Thus, the large (several fold) bird-
143 to-bird variability in sequence properties (Figure S1B-F) is not explained by
144 age, and likely due to inter-individual variability in developmental timecourses.

145 **2.2 Prior to tutoring, birds that will learn exhibit HVC** 146 **sequences that are relatively immature and decoupled** 147 **from vocal output**

148 Next, we asked whether properties of the HVC sequences relate to the ability
149 of birds to learn a new song from a tutor. Many of our young isolated birds
150 were eventually tutored at an age around the critical period and we found that
151 half of them learned elements of their tutor song, while the others developed
152 fully isolate song. We classified birds as learners if their song had an Imitation
153 Score metric [31] greater than 0.5. The songs of non-learners remained highly
154 variable and isolate-like even after tutoring (Figure 3A). In contrast, learner
155 birds developed a new syllable within a day or two after tutoring, and ultimately
156 sang typical adult song, consisting of stereotyped motifs (Figure 3B).

157 An analysis of HVC activity revealed that sequences prior to tutoring were
158 systematically less mature/‘crystallized’ in birds that learned than in birds that
159 failed to learn. Learner birds had fewer sequences than non-learners (Figure
160 2C, average 2 sequences in learners, 3.25 sequences in non-learners, $p = 0.029$,
161 Wilcoxon rank sum test). Sequences in learner birds were more weakly corre-
162 lated to song features (Figure 2D, average 0.20 s learner, 0.55 s non-learner, p
163 $= 0.0034$, Wilcoxon rank sum test). Sequences in learners had lower autocorre-
164 lation, a measure of how repeatably/rhythmically they are produced [17], than
165 non-learners (Figure 2E, average 0.125 s learner, 0.244 s non-learner, $p = 0.018$,

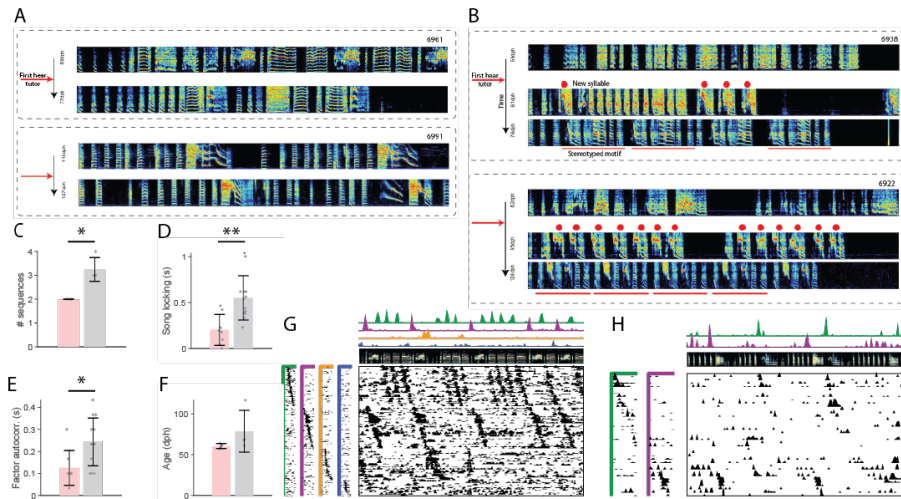


Figure 2: Relation between HVC sequence maturity and subsequent song learning
 (A) Example spectrograms for two non-learner birds, prior to tutoring and several weeks later (at least 77dph). (B) Example spectrograms for two learners, prior to tutoring, shortly after tutoring, and several weeks later. Red dots mark the new syllable. Red bars mark stereotyped motif. (C-E) Three measures of HVC sequence maturity for learners (pink) and non-learners (gray). Error bars denote standard deviation (* : $p < 0.05$, ** : $p < 0.01$). (c) Number of sequences in HVC. (D) Fraction of neurons that participate in a sequence. (E) Autocorrelation of sequence factor timecourses. (F) Age of first tutoring for learners and non-learners. (G-H) Example pre-tutoring data from two birds that were brothers. (G) A non-learner, first tutored at 61 dph. (H) A learner, first tutored at 64 dph.

166 Wilcoxon rank sum test). Three additional measures of sequence maturity, all
167 related to intrinsic sequence properties were calculated. While non-learners also
168 trended higher in these measures, the differences were not significant (Wilcoxon
169 rank sum tests, Neural participation: average 45% learner, 70% non-learner,
170 $p = 0.2$; Reliability: average 69% learner, 74% non-learner, $p = 1$; Coverage:
171 average 51% learner, 71% non-learner, $p = 0.34$).

172 The age of tutoring was not significantly correlated with whether the bird
173 was a learner or non-learner (Figure 3F, average 60.5 dph learner, 78.75 dph
174 non-learner, $p = 0.11$, Wilcoxon rank sum test). For example, one of the younger
175 birds in our dataset (61 dph) was a non-learner, and had particularly clear HVC
176 sequences before tutoring (Figure 3G). This bird's brother, tutored 3 days later,
177 was a learner, and had sequences that appear far less mature (Figure 3H). To-
178 gether, these results suggest that the presence, at the time of tutoring, of robust
179 song-locked sequences, may inhibit learning. In other words, learning may be
180 better supported by more immature sequences that are more independent from
181 vocal output.

182 **2.3 Tracking HVC sequences across rapidly learned song** 183 **changes**

184 In late-tutored birds that learned, the speed with which new syllables appeared
185 was striking. These birds developed a new syllable within a day or two after
186 tutoring (Figure 2A, B), as has been previously described [32, 33, 34]. These
187 new syllables appeared to emerge *de-novo*, not by syllable differentiation as is
188 common in tutored birds.

189 We wondered if these birds, which learned a new syllable rapidly after tu-
190 toring, formed a *de-novo* HVC sequence for this new syllable, or perhaps used
191 a pre-existing sequence. We were able to track neurons in our calcium imaging
192 data throughout the course of tutoring (Figure 3A, see Methods, Gu et al., in
193 preparation), enabling us to see what happens to neural activity during rapid
194 changes in the song. We first extracted neural sequences associated with new
195 post-tutoring syllables, then followed these neurons back in time to find that the
196 sequence existed even prior to tutoring (Figure 3B,C, see Methods). However,
197 the sequence prior to tutoring was surprisingly 'latent'. That is, the sequence
198 was relatively uncoupled to vocal output, without a strong correlation to song
199 syllables. Combining data from the four birds that learned a new syllable rapidly
200 after tutoring, neural sequences extracted two days after tutoring appeared to
201 become more song locked after tutoring (Figure 3D, $p=0.0048$, Wilcoxon rank
202 sum test).

203 Next, we aimed to control for the possibility that the appearance of se-
204 quences becoming progressively more locked to vocal output after tutoring was
205 due to the fact that sequences were extracted from neural data recorded after
206 tutoring. We directly extracted HVC sequences from exclusively pre-tutoring
207 neuronal data and tracked them forward in time until a new syllable appeared.
208 Sequences that were initially relatively 'latent' persisted, becoming progressively
209 more correlated with vocal output, ultimately tightly locked to a new syllable

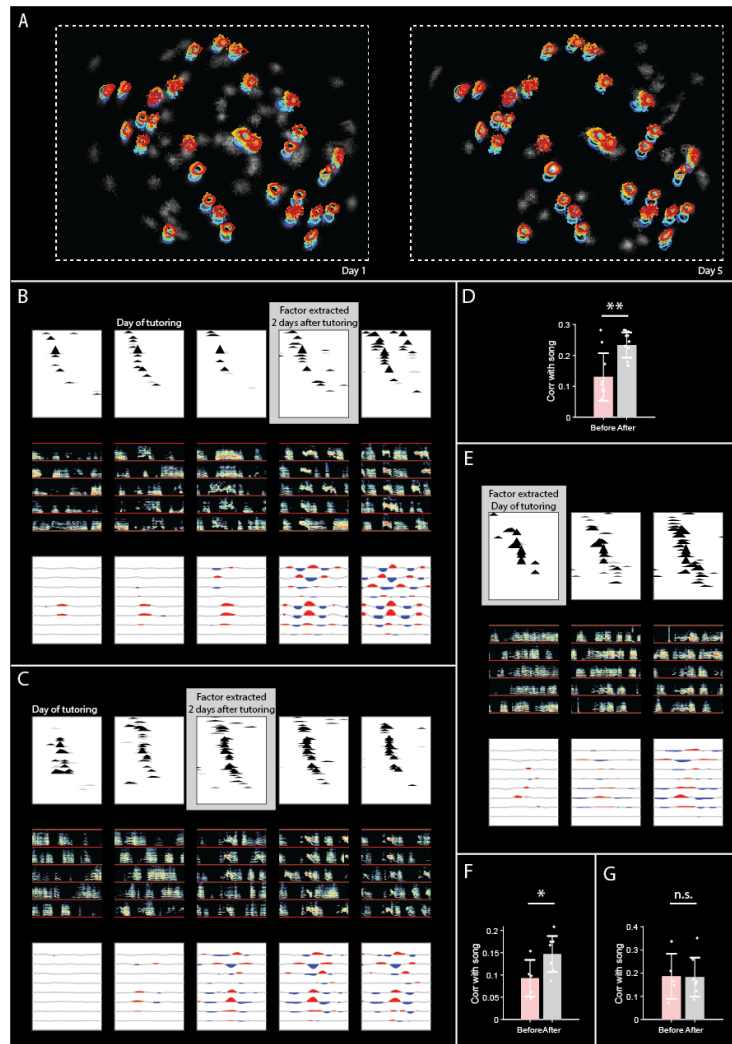


Figure 3: Tracking HVC sequences as isolated birds rapidly learn a new syllable (A) Neurons detected before (left) and after (right) tutoring shown in grayscale (CNMF_E algorithm). Colored contours indicate locations of neurons tracked across five days, from blue to red (B) Sequence in HVC, tracked before and after first tutor exposure (see Methods), through the development of a new syllable. Sequence extracted from data two days after tutoring, and neurons sorted according to participation in this sequence. (Top) On each recording day, cross correlation of neurons with the sequence that becomes associated with the new syllable. Significant bins are shown in black, non-significant bins in gray ($p=0.05$, Bonferroni corrected, compared to circularly-shifted control) (Middle) On each recording day, example spectrograms at times when the sequence occurs on each day (Bottom) On each recording day, cross-correlation of sequence with acoustic features (amplitude, entropy, pitch goodness, aperiodicity, mean frequency, pitch, frequency modulation, and amplitude modulation) (C) Same as B for a different example bird. (D) Correlation with song amplitude before (pink) and after (gray) tutoring for all sequences in learner birds extracted data when a new syllable had been learned. (E) Similar to B and C, for a different example bird. Here sequences are extracted from pre-tutoring data, then tracked forward in time. (F) Song locking (maximum cross-correlation with song amplitude) before and after tutoring for the pre-tutoring sequences that had weaker song locking. (G) Song locking before and after tutoring for the pre-tutoring sequences that started off with stronger song locking.

210 (Figure 3E). Each of the ‘learner’ birds appeared to have two HVC sequences
211 present prior to tutoring. Of these sequences, the ones that started off less
212 correlated with song amplitude exhibited a significant increase in correlation
213 with song amplitude after tutoring (Figure 3F, $p=.045$, Wilcoxon rank sum
214 test). The sequences that started off more correlated with song amplitude did
215 not significantly change their correlation with song amplitude (Figure 3G, $p=1$,
216 Wilcoxon rank sum test). Together, these results are consistent with the view
217 that the emergence of new syllables after tutoring may co-opt existing HVC
218 sequences, including relatively ‘latent’ sequences.

219 **3 Discussion**

220 We set out to determine whether the formation of sequences in HVC depends
221 on prior exposure to a tutor song. By observing the neural activity in HVC of
222 isolated birds, we found that HVC network activity can form long repeatable
223 sequences even in birds that had no prior exposure to vocal tutoring. Sequences
224 in isolate HVC exhibit some properties of typical HVC, with many neurons reli-
225 ably participating in sequences, and sequences being correlated to vocal output.
226 However, sequences in isolated birds were less reliable and less tightly corre-
227 lated with vocal output than has been described in typical birds, and exhibited
228 abnormal truncations and persistent activity.

229 We had previously hypothesized that the experience of hearing a tutor may
230 seed the formation of HVC sequences of the appropriate number and durations
231 [35], but our new data reveal that HVC sequences exist even prior to tutoring.
232 Thus, there must be a way for sequences to form without the prior storage of a
233 tutor memory. In models of Hebbian learning in HVC, sequences can form in
234 networks driven by random inputs rather than patterned inputs [22]. However,
235 in this case the distribution of sequence durations no longer matches syllable
236 durations found in typical adult birds, but is instead more consistent with the
237 highly variable and atypically long syllables that occur in birds that have never
238 heard a tutor (isolate song) [12, 10]. Thus our findings may be consistent with
239 the view that sequences can emerge in isolate birds by a combination of simple
240 Hebbian learning mechanisms together with spontaneous activity either within
241 HVC or driven by the inputs to HVC.

242 Our discovery of latent sequences suggests a separation between neural pro-
243 cesses for building a stable representation of states within a task (i.e., sequential
244 moments in time), and neural processes for associating an action with each state.
245 Thus, sequences may gradually emerge in the maturing HVC network via simple
246 Hebbian processes [23, 22, 17], but may remain relatively decoupled from
247 downstream motor neurons until a memory of the tutor song is learned and
248 reinforcement learning processes begin.

249 From a computational perspective, what do latent sequences tell us about
250 how the brain learns? By latent sequences, we mean sequences that are initially
251 only weakly correlated with vocal output, but are subsequently used to produce
252 learned song changes. In reinforcement learning models of song learning, HVC

253 sequences remain relatively stable even as the song changes [36, 37, 38, 39],
254 consistent with our observation of stable sequences. This is in contrast with
255 other models of song learning, like the ‘inverse model’ [40, 41, 42]. In the
256 inverse model, each motor neuron produces the same vocal output at different
257 times during vocal learning; song changes are caused by pre-motor neurons (e.g.
258 HVC) being activated in a different order. In contrast, we observed relatively
259 stable sequences throughout learned song changes. Our results are consistent
260 with data from primary motor cortex of macaques operating a brain-computer
261 interface—a fixed repertoire of activity patterns are associated with different
262 movements after learning [43]. Our results are also consistent with the idea
263 that the brain may use pre-existing sequential patterns to rapidly learn from
264 new experience, for example the existence of sequences in the hippocampus prior
265 to exposure to new environments [44, 45, 46, 47, 48].

266 If the brain is able to build on latent structure to learn from sparse data, es-
267 sentially implementing inductive bias, we might expect different forms of latent
268 structure for different tasks. Zebra finches are known to develop typical songs,
269 including typical syllable durations, after being tutored by atypical isolate songs,
270 relying on species-specific ‘priors’ to achieve species-typical syllable durations.
271 The latent sequences we observed tended to last on the order of a hundred mil-
272 liseconds—the same as the duration of typical zebra finch syllables. Might other
273 species that sing faster songs (e.g. grasshopper sparrow) or slower songs (e.g.
274 white-throated sparrow) exhibit latent sequences of shorter or longer durations?
275 One might imagine that the speed of latent sequences could be genetically spec-
276 ified by expression levels of ion channels with different time constants within
277 HVC. Alternatively, the duration of latent sequences could be specified by the
278 amount of time it takes for HVC to get feedback from respiratory and/or audi-
279 tory centers, which may also have their own intrinsic rhythmicity [49, 50, 51].
280 Each of these possible sources of latent HVC structure could be tested in further
281 experiments. By whatever mechanism latent sequences arise, they appear to be
282 capable of supporting song learning, at least in the case of delayed tutoring.
283 More generally, the ability of brains to generate complex learned behavior may
284 depend on the intrinsic developmental formation of appropriate latent dynamics
285 in motor and sensory circuits.

286 4 Materials and Methods

287 4.1 Table of key resources for imaging HVC sequences

288 Key resources, and references for how to access them, are listed in Table 1.

289 4.2 Animal care and use

290 For this study, Imaging data was collected in 9 male zebra finches (*Taeniopygia*
291 *guttata*) from the MIT zebra finch breeding facility (Cambridge, MA). Animal
292 care and experiments were carried out in accordance with NIH guidelines, and

Table 1: Links to key resources used for measuring HVC sequences during rapid learning

| Software/algorithm | Source | Link to code |
|--|---------------------------|---|
| seqNMF | [28] | https://github.com/FeeLab/seqNMF |
| CNMF_E (cell extraction) | [52] | https://github.com/zhoupc/CNMF_E |
| STAT (tracking neurons across days) | Gu et al., in preparation | will post preprint and submit |
| Chronux (spectrogram computation) | [53] | http://chronux.org/ |
| SAP (Sound Analysis Pro) | [29] | http://soundanalysispro.com/ |
| SI (Song Imitation) | [31] | https://doi.org/10.1371/journal.pone.0096484 |
| MATLAB | MathWorks | www.mathworks.com |
| Dataset | Source | Link to data |
| HVC, rapid learning | This paper | will post |
| Other | Source | Link |
| Zebra finches (<i>Taeniopygia guttata</i>) | MIT animal facility | |
| AAV9.CAG.GCaMP6f.WPRE.SV40 | [54] | https://pennvectorcore.med.upenn.edu |
| Miniature microscope | Inscopix nVista | https://www.inscopix.com/nvista |

293 reviewed and approved by the Massachusetts Institute of Technology Committee
 294 on Animal Care.

295 In order to control exposure to a tutor song, 8 birds were foster-raised by
 296 female birds, which do not sing, starting on or before post-hatch day 15 (15
 297 dph). Starting between 40 dph and 50 dph, these birds were housed singly in
 298 custom-made sound isolation chambers. An additional bird was tutored by his
 299 father, as is typical. After a couple of days of acclimation to the lab environ-
 300 ment, birds were anesthetized with isoflurane, and were given a surgery to inject
 301 virus to express the functional indicator GCaMP6f and implant a GRIN (gradi-
 302 ent index) lens (see below). Analgesic (Buprinex) was administered 30 min
 303 prior to the surgery, and for 3 days postoperatively. After at least a week for
 304 virus expression, an Inscopix miniscope baseplate was attached to the existing
 305 implant. Birds were acclimated to the miniscope for several days. Once birds
 306 started singing with the miniscope, functional calcium signals were recorded for
 307 several days. To avoid photobleaching, short files (approximately 10 seconds)
 308 were obtained, typically fewer than 50 files per day. Once some pre-tutoring
 309 singing data had been obtained, birds were tutored briefly (5-10 song bouts
 310 from a tutor bird) each day.

311 4.3 Expression of functional calcium indicator GCaMP6f

312 The calcium indicator GCaMP6f was expressed in HVC by intercranial injection
 313 of the viral vector AAV9.CAG.GCaMP6f.WPRE.SV40 [54] into HVC. In the
 314 same surgery, a cranial window was made using a relay GRIN (gradient index)
 315 lens (1mm diameter, 4mm length, Inscopix) implanted on the surface of the
 316 brain, after the dura was removed. After at least one week, in order to allow
 317 for sufficient viral expression, recordings were made using the Inscopix nVista
 318 miniature fluorescent microscope.

319 **4.4 Extraction of neuronal activity and background sub-** 320 **traction using CNMF_E**

321 Neuronal activity traces were extracted from raw fluorescence movies using a
322 constrained non-negative matrix factorization algorithm, CNMF_E, that is spe-
323 cialized for microendoscope data by including a local background model to re-
324 move activity from out-of-focus cells [52]. Custom software (Shijie Gu, Emily
325 Mackevicius, Pengcheng Zhou) was used extend the CNMF_E algorithm to com-
326 bine batches of short files (BatchVer) and track individual neurons over the
327 course of multiple days (STAT, Gu, et. al., in preparation, see below).

328 **4.5 Unsupervised discovery of neural sequences using seqNMF**

329 We addressed the challenge of needing to detect neural sequences in HVC with-
330 out relying on aligning neural activity to the song by developing an unsupervised
331 algorithm, seqNMF [28]. This was necessary because juvenile songs are highly
332 variable and difficult to parse into repeatable syllables, and because we wanted
333 to allow for the possibility that HVC activity might be more stereotyped than
334 the song. Briefly, seqNMF factorizes data into exemplar sequence factors (\mathbf{W} 's).
335 Each sequence factor has a corresponding timecourse (\mathbf{H}). Convolving each ex-
336 emplar with its respective timecourse produces an approximate reconstruction
337 of the original data ($\tilde{X} = \mathbf{W} \circledast \mathbf{H}$). SeqNMF returns a factorization that min-
338 imizes reconstruction error, subject to a penalty term that encourages simpler
339 factorizations.

340 **4.6 Preprocessing calcium traces prior to running seqNMF**

341 We performed several preprocessing steps before applying seqNMF to functional
342 calcium traces extracted by CNMF_E. First, we estimated burst times from the
343 raw traces by deconvolving the traces using an AR-2 process. The deconvolution
344 parameters (time constants and noise floor) were estimated for each neuron using
345 the CNMF_E code package [52]. Some neurons exhibited larger peaks than
346 others, likely due to different expression levels of the calcium indicator. Since
347 seqNMF would prioritize the neurons with the most power, we renormalized
348 by dividing the signal from each neuron by the sum of the maximum value of
349 that row and the 95th percentile of the signal across all neurons. In this way,
350 neurons with larger peaks were given some priority, but not much more than
351 that of neurons with weaker signals.

352 **4.7 Estimating the number of significant sequences in each** 353 **dataset**

354 The number of sequences present in real neuronal datasets can be slightly am-
355 biguous, so we used several methods to arrive at and validate an estimate for
356 the number of significant neural sequences present in each dataset. It is impor-
357 tant to note that, since our datasets are short, there may be additional neural

358 sequences in HVC that do not appear, or do not achieve significance, in our
359 datasets. In order to cross-validate sequences on held-out data, we split each
360 dataset into a training set (75%) and a test set (25%). Sequences were detected
361 in the training set, and significance was measured in the test set by assessing
362 how much the overlap of the sequences with the test data compared to null
363 (time-shifted) sequences. In order to choose a value for the seqNMF param-
364 eter λ that balances reconstruction cost with correlation (redundancy) cost, we
365 swept λ with $K = 10$ and $L = 0.5$ seconds to find λ_0 , the cross-over point that
366 balances these cost terms (Figure S2A). Based on analysis on simulated data
367 [28], where values of λ at or slightly above λ_0 yielded the correct number of
368 sequences, we looked at the distribution of significant sequences at $\lambda = \lambda_0$ and
369 $\lambda = 2\lambda_0$ (Figure S2B), and chose as our estimate a number between the peaks of
370 these two distributions. We validated these estimates in two ways. First, we ran
371 seqNMF with K equal to this estimate and $\lambda = 0$, and confirmed that the result-
372 ing sequences tended to be significant on held-out data. Next, we ran seqNMF
373 on the entire dataset at this K from 25 different random initial conditions, and
374 confirmed that the sequences were consistent across the different runs (Figure
375 S2C). Consistency measures the extent to which there is a one-to-one mapping
376 between the factors of two different factorizations [28]. When this analysis was
377 run at a K higher than the estimated K , results tended to be less consistent
378 (Figure S2D).

379 4.8 Selecting a consistent factorization

380 For each dataset, we selected the most consistent factorization on which to
381 perform all further analysis. Once we had selected an appropriate number of
382 sequences for each dataset, using the analyses described above, we ran seqNMF
383 25 times at this value of K from different random initial conditions, and picked
384 the factorization that was most consistent with the other factorizations (Figure
385 S2D). Factorizations at K chosen by the above methods tended to be more
386 consistent than factorizations at higher K (Figure S2D).

387 4.9 Significance testing for cross-correlation analyses

388 Several of our results involve analyzing the temporal relationship between differ-
389 ent timecourses (factors and neurons; factors and song acoustic features; factor
390 autocorrelations). These analyses involve testing the significance of the cross-
391 correlation between two timeseries, compared to null cross-correlation values
392 that could occur if the signals were circularly shifted relative to each other by
393 a random large timelag. Before measuring cross-correlations, we centered each
394 signal to have zero mean. If we are assessing the cross-correlation at lags in the
395 range from $-L$ to L , we want to compare values measured here to null values
396 measured at random lags longer than L . We compute the cross-correlation at
397 each lag ℓ in the range $-T < \ell < T$, where T is the length of the timeseries,
398 by circularly shifting one of the timeseries by ℓ and computing the dot prod-
399 uct with the other timeseries. We then use the cross-correlations at null lags

400 $(-(T - L) < \ell < -L \text{ or } L < \ell < (T - L))$ to determine a Bonferroni-corrected
401 significance threshold. The threshold is the $100 \times (1 - p/Num)^{th}$ percentile of
402 the absolute value of these null cross-correlations, where Num is the number
403 of comparisons ($2L$ times the number of tests being run), and p is the p -value.
404 Significance is achieved for lags at which the measured cross-correlation exceeds
405 this value.

406 **4.10 Assessing song locking, the cross-correlation between** 407 **each factor and acoustic song features**

408 Several of our results involve quantifying the temporal relationship between
409 sequence timecourses (\mathbf{H} 's) and the song. To do this, we measured the cross-
410 correlation of sequences with song acoustics using 8 acoustic features common
411 in the songbird literature [29]: amplitude, entropy, pitch goodness, aperiodicity,
412 mean frequency, pitch, frequency modulation, and amplitude modulation. Each
413 of these acoustic features is measured from the song at 1ms resolution using
414 standard software (Sound Analysis Pro, <http://soundanalysispro.com/>, [29]).
415 The seqNMF \mathbf{H} 's are upsampled to this resolution, then cross-correlation be-
416 tween each \mathbf{H} and each song feature is assessed using the above procedure, with
417 $L = 1$ second, $p = 0.05$, and Bonferroni correction (2000 timebins) \times (8 features)
418 \times (K sequences). The overall measure of song locking is computed by integrat-
419 ing the number of seconds that a given sequence has significant correlation with
420 each of the song features.

421 **4.11 Assessing which neurons participate in each sequence**

422 Several of our results involve assessing which neurons participate in each se-
423 quence. In order to do this, we measure whether there is a significant cross-
424 correlation between each neuron and each factor (with $L=0.5$ seconds, $p = 0.05$,
425 and Bonferroni correction (30 timebins) \times (N neurons) \times (K sequences)). Note
426 that, since seqNMF is run on the neural data, it is guaranteed that some neurons
427 will be correlated with the factors—the primary aim of this test is to assess
428 which neurons are in which sequences.

429 **4.12 Tracking HVC projection neurons over the course of** 430 **major song changes**

431 A core motivation for using calcium imaging methods instead of other methods
432 was the possibility to track HVC projection neurons over the course of major
433 song changes. HVC projection neurons are particularly difficult to record with
434 electrophysiological methods—current methods are unable to record an HVC
435 projection neuron for more than a few hours, and tend to record one, or at most
436 three, projection neurons at a time [55, 17]. Previous studies of song-locked HVC
437 activity throughout the learning process could only track changes in the neural
438 population that occurred at a timescale slower than a week, because population
439 statistics had to be compiled from single-neuron recordings [17]. This technique

440 misses rapid changes that can happen within a day [32], and is unable to assess
441 the stability of HVC sequences.

442 Stability of HVC sequences over time can be assessed using calcium imag-
443 ing, though some challenges remain due to the potential for errors in tracking
444 neurons across days. Single-photon calcium imaging methods have been used
445 to address the stability of HVC sequences in adult birds with stable songs, ob-
446 serving stable song-locked activity in slightly more than half of HVC projection
447 neurons, and unstable song-locked activity in slightly less than half of HVC pro-
448 jection neurons [56]. This measure is likely an underestimate of the stability of
449 HVC activity, since noise in tracking cell locations across days could lead to per-
450 ceived instability. Thus, HVC sequences appear relatively stable in birds with
451 stable song, but what about birds whose songs are changing? The potential for
452 errors in tracking neurons across days was one factor in our decision to record
453 in birds undergoing very rapid learning. It was necessary for us to expand upon
454 previous methods for tracking neurons recorded by calcium imaging over time
455 [57], likely due to the relatively short individual file sizes in our dataset from
456 singing juvenile birds (we recorded many short files each day, when the birds
457 happened to sing, instead of longer continuous files).

458 We tracked the activity of populations of HVC neurons over multiple days
459 using Spatial Tracking Across Time (STAT, Gu et al., in preparation). This
460 method builds off of previous methods [57], where individual cell pairs' shape
461 spatial correlation and distance are used to determine the correspondences be-
462 tween cells extracted from different sessions. STAT also considers local neigh-
463 borhood motion consistency in computing the optimal tracking of cells across
464 sessions, and requires less manual supervision. The local motion consistency is
465 optimized using the Hungarian Method, a combinatorial optimization algorithm
466 that solves assignment problems in polynomial time. Cells that have no good
467 match are excluded, as are cells with abnormal coefficient of variations. Finally,
468 the results of the matching algorithm are checked manually.

469 **4.13 Tracking sequences extracted on one subset of a dataset** 470 **to another subset of the dataset**

471 In order to track a sequence, \mathbf{W} , extracted in one subset of a dataset (\mathbf{X}_1 ,
472 for example before tutoring) to another subset of the dataset (\mathbf{X}_2 , for example
473 after tutoring), we first mean-subtract \mathbf{W} and \mathbf{X}_2 along the time dimension,
474 then estimate $\tilde{\mathbf{H}}_2 = \mathbf{W}^\top \circledast \mathbf{X}_2$. In order to assess whether a neuron significantly
475 participates in \mathbf{W} in dataset \mathbf{X}_2 , we bootstrap using control datasets \mathbf{X}_2^{shuff} , in
476 which data from each neuron is circularly shifted in time by a different random
477 amount. We then ask whether the neuron participates more strongly in the
478 real dataset compared to participation calculated on control datasets (p=0.05
479 significance threshold, Bonferroni corrected for the number of neurons and the
480 number of time-lags). Specifically, we compare $\tilde{\mathbf{W}}_2 = \mathbf{X}_2 \tilde{\mathbf{H}}_2^\top$ to $\tilde{\mathbf{W}}_2^{shuff} =$
481 $\mathbf{X}_2^{shuff} \tilde{\mathbf{H}}_2^{shuff^\top}$.

482 5 Assessing sequence coverage of song bouts

483 Sequence coverage quantifies the observation that sequences in isolated birds ap-
484 pear to pop on and off at somewhat arbitrary moments in bouts, leaving some
485 sections of some bouts with no clear sequences present. First, the moments when
486 each sequence occurs is estimated by computing when $\tilde{\mathbf{H}} = \mathbf{W}^\top \circledast \mathbf{X}$ is larger
487 than expected by chance (Bonferroni-corrected 95% percentile of $\tilde{\mathbf{H}}^{shuff} =$
488 $\mathbf{W}^\top \circledast \mathbf{X}^{shuff}$). Next, the sequence is convolved with the corresponding \mathbf{W} .
489 Finally, the total number of seconds when some sequences was present is di-
490 vided by the total number of seconds in the bout, and multiplied by 100, to get
491 the percent of the bout covered by some sequence. Note that sequence cover-
492 age is distinct from previously described measures of burst coverage within a
493 repeatable adult song motif [18].

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678 **Supplementary figures**

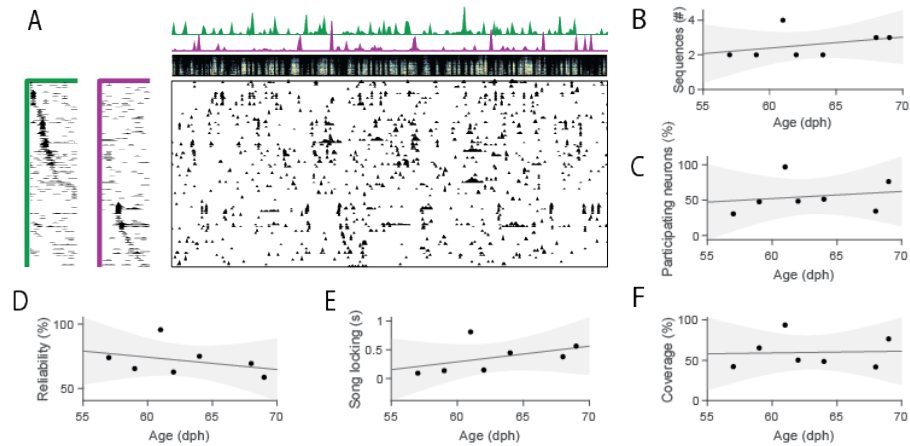


Figure S1: HVC sequences exist even in young isolated birds
(A) Example HVC sequences recorded in a young isolated bird (59 dph) (B-F) Sequence properties as a function of age in 7 juvenile isolated birds (5 birds recorded prior to the closing of the traditional critical period (<65 dph), and 2 older juvenile birds (65 dph - 90 dph)). Line denotes least squares fit, gray area 95% confidence interval. (B) Number of HVC sequences extracted. (C) Percent of neurons participating in at least one sequence. (D) Reliability of neural participation across sequence renditions. (E) Song locking. (F) Percent of the song covered by at least one sequence.

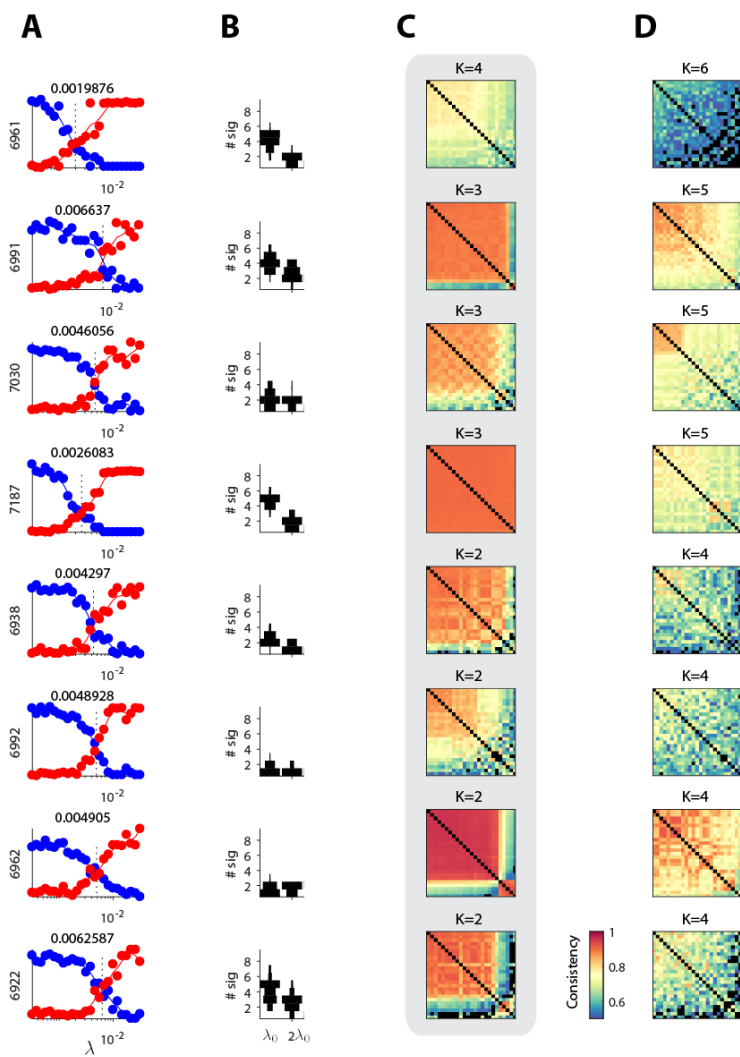


Figure S2: Supplementary Figure 1. Estimating the number of significant sequences in each dataset

(A) Reconstruction cost (red) and correlation cost (blue) as a function of λ (with $K=10$, $L=0.5$ seconds) for 8 datasets (pre-tutoring data from 8 different birds). The crossover point, λ_0 , is stated and marked by a dashed line. (B) Histogram of the number of significant sequences at λ_0 and $2\lambda_0$ for these datasets. (C) For the chosen K , and $\lambda = 0$, consistency across 25 runs of seqNMF from different random initializations. Factorizations are sorted from most to least consistent. (D) Consistency matrix for 25 runs at K above the estimated K .