

some similarity values were equal to zero (that is, no OTUs in common), we coded the similarity data by adding 0.01 before log transforming each value²⁹. The approach outlined in ref. 14 allowed us to use relative comparisons of bacterial community composition rather than richness to examine the taxa–area relationship; richness is very difficult to estimate accurately in hyperdiverse communities such as bacterial communities. There is no reason to believe that undersampling and/or PCR biases will co-vary with intersample distance or will result in preferential sampling of those taxa most likely to be shared among samples located close together in space; thus, these factors, although likely to be present, are unlikely to influence the z-values we observed. In addition, we can think of no model in which PCR biases and/or undersampling could generate a taxa–area relationship that was completely artefactual.

We compared the z-values for different taxon definitions by testing if the slopes of the regressions differed (see Supplementary Methods 2). We used the same distance decay approach to determine the z-value for plants, using data from the transect quadrants.

Influence of habitat heterogeneity

We used partial Mantel tests (9,999 permutations) to examine the influence of abiotic factors and aboveground plant composition on bacterial community composition, while holding geographic distance constant and vice versa.

We constructed a distance matrix for plant community composition from per cent cover estimates for the four dominant species (*Spartina alterniflora*, *Spartina patens*, *Salicornia virginica* and *Limonium nashii*). We constructed a matrix of environmental distance from the abiotic factors identified as most important to community composition (phosphate and ammonia concentrations), using BIO-ENV²⁸. The BIO-ENV procedure selects a subset of available abiotic variables to maximize rank correlation between community similarity and abiotic dissimilarity matrices. We then used these matrices to test for additional distance and plant effects.

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Correspondence and requests for materials should be addressed to M.C.H.-D. (mchd@u.washington.edu).

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Species-typical songs in white-crowned sparrows tutored with only phrase pairs

Gary J. Rose, Franz Goller, Howard J. Gritton, Stephanie L. Plamondon, Alexander T. Baugh & Brenton G. Cooper

Department of Biology, University of Utah, Salt Lake City, Utah 84112-0840, USA

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Modern theories of learned vocal behaviours, such as human speech and singing in songbirds¹, posit that acoustic communication signals are reproduced from memory, using auditory feedback². The nature of these memories, however, is unclear. Here we propose and test a model for how complex song structure can emerge from sparse sequence information acquired during tutoring. In this conceptual model, a population of combination-sensitive (phrase-pair) detectors is shaped by early exposure to song and serves as the minimal representation of the template necessary for generating complete song. As predicted by the model, birds that were tutored with only pairs of normally adjacent song phrases were able to assemble full songs in which phrases were placed in the correct order; birds that were tutored with reverse-ordered phrase pairs sang songs with reversed phrase order. Birds that were tutored with all song phrases, but presented singly, failed to produce normal, full songs. These findings provide the first evidence for a minimal requirement of sequence information in the acoustic model that can give rise to correct song structure.

Songbirds must hear conspecific song during a ‘sensitive period’ early in life to later generate a normal song; birds fail to produce normal song if raised from the egg or the nestling stage, in acoustic isolation^{3,4}. From this early acoustic experience, birds form a memory (acquired template) of the song^{5,6}. Later, during the sensorimotor phase of song development, birds use auditory feedback to compare their vocalizations with the memorized representation of the tutor song². The neural representation of this acquired template is largely unknown.

A central question with regard to the nature of the acquired template is whether a representation of the full song (Fig. 1a) is required for assembling normal song. Here we propose and test a model of how correct song structure could emerge even if the full song was never experienced. Theoretically, correct phrase order could be deduced if only information about the temporal order of adjacent phrases is represented separately in the tutor model (as phrase pairs, Fig. 1b, c), but not when birds are tutored with phrase types presented in temporal isolation. In support of the latter, Soha and Marler⁷ found that white-crowned sparrows (*Zonotrichia leucophrys*) tutored with multiple models, each consisting of repe-

titions of only a single phrase type, failed to produce normal, full songs. Birds tutored with phrase pairs, however, might be able to assemble full song; auditory experience could shape neural selectivity for phrase pairs present in tutor models. These sensory units, representing the acquired template, could then be activated by auditory feedback from self-generated vocalizations and provide reinforcement for 'correct' motor patterns (for additional details of this model, see Supplementary Information).

According to this model, therefore, birds should be able to develop full, dialect-specific song if tutored with pairs of phrases that are normally adjacent, but not when tutored with phrases presented in isolation. We tested these predictions by tutoring (see Methods) white-crowned sparrows, 10–14 days old, with either singly presented phrases or one of two phrase-pair regimens (Fig. 1b, c). The white-crowned sparrow is an excellent system for evaluating this model because its song consists of four or five acoustically distinct segments (phrases, labelled ABCDE, Fig. 1a), a long tradition of research on song learning exists for this species and the phrase sequence varies between subspecies and dialects^{8,9}—with the exception of the whistle, which is always in the first position. Birds in the first phrase-pair tutored group were exposed to the natural phrase order (forward-order) within each phrase pair; that is, the order found in the natural song. For the other group, the phrase order was reversed (reverse-order). Phrase pairs were presented in an order such that the full forward or reversed sequence of phrases was not present across pairs. We predicted that birds in the forward-order group would develop full songs that had the natural phrase order, whereas those in the reverse-order group would construct songs with phrases in the opposite order. Birds tutored with temporally isolated phrases of all types should not, however, assemble normal songs.

Nine birds that were tutored with singly presented phrases produced crystallized songs. Across individuals, songs consisted of two to four phrase types (median, 3; Fig. 2), were of approximately normal length (median, 2.0 s; range, 1.6–2.2) and had significant forward phrase order ($P < 0.02$, binomial test). No birds, however, assembled songs of four to five phrase types placed in the correct order. The forward structure of these songs stemmed principally

from the innate predisposition^{4,7} of white-crowned sparrows to begin songs with one or more whistles (eight of nine cases), and a tendency (six of nine cases) in this study to follow an initial whistle with a B-type phrase (note complex) ($P < 0.01$, binomial test). The inclination to place the B in the second position, however, was weak; even as late as 3 weeks before crystallization, seven of the birds still repeatedly sang some songs (variants, Fig. 2) in which B did not follow the initial whistle. These results generally support the idea that birds have some inclination to assemble songs that have several different phrase types, that is, use a 'phrase diversity strategy'. Birds did not, however, include more than three representatives of the phrase types in the tutor models; most notably, C-type phrases were absent. These findings are in general agreement with those of Soha and Marler⁷, except that the crystallized songs of birds in their study consisted, on average, of two phrase types. These results support the notion that birds have an innate predisposition to produce songs of normal length that begin with whistles, and a tendency to follow the whistle(s) with a note complex, but lack an innate capacity to assemble component phrases into complete, normal songs.

According to the model, however, birds should be able to develop full, dialect-specific song if only tutored with pairs of phrases that are normally adjacent. We recorded song from five birds in the forward-order tutoring regimen. Four birds developed songs with a natural sequence of at least four phrases (ABCD) (Fig. 3). Under the experimental conditions used in this study, it is not uncommon that first-year birds do not produce the last syllable (E, in this case) in their crystallized song¹⁰. The fifth bird (bird number 45) developed a song, in which only two of the tutor syllables (AB) were combined with an improvised syllable; because only two phrases from the tutor models were copied, the song of this bird was inadequate for testing the present hypothesis. Nevertheless, as a whole, these data show that the phrase pair information in the tutor models was sufficient for birds to assemble songs with correct phrase order ($P < .001$, binomial test). Also, as a group, the songs of these birds contained significantly more forward structure than those of birds tutored with single phrases ($U = 38$, $P < .025$, Mann–Whitney U -test).

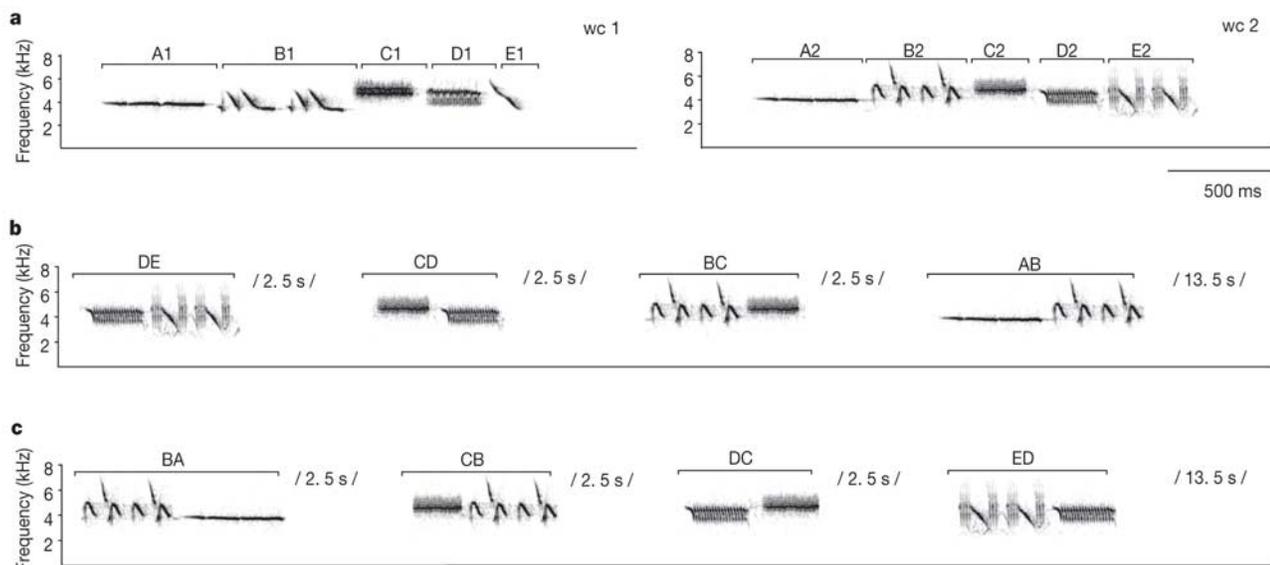


Figure 1 Sound spectrograms of songs of two white-crowned sparrows and tutor models. **a–c**, Songs consist of four to five segments, referred to as 'phrases', denoted as A, B, C, D and E. Phrases generally are composed of repeated notes or syllables. Tutoring regimens consisted of single phrases (not shown) or pairs of phrases from the songs shown in **a**.

Paired phrases were either in the normal **(b)** (for example, AB) or reversed **(c)** order; phrases in these pairs were always from the same song. Only the phrase pairs taken from the song of bird 2 are shown. wc 1 and wc 2, indicate the songs of birds 1 and 2, respectively.

Thus, white-crowned sparrows can assemble apparently normal songs despite being tutored with only pairs of phrases; exposure to the full song is not necessary. This process was undoubtedly aided, however, by the innate predisposition of these birds to begin their

songs with whistles⁷, and the tendency to follow the whistle with note complexes (Fig. 2). The second group, tutored with reverse-order pairs, further tested therefore the importance of phrase order in the tutor pairs as a determinant of song phrase-sequence development.

We recorded song from five males tutored with reverse-order phrase pairs. By the late plastic song stage, four of these birds (67, 72, 74 and 84) assembled songs with the full sequence of phrases, in reversed order (EDCBA, Fig. 4a). Of the 101 songs recorded and analysed that consisted of all five phrase types, 78 were EDCBA sequences ($\chi^2 = 7167$, $P < .001$); no ABCD or ABCDE songs were recorded.

The crystallized songs of these birds generally also consisted of phrases in reversed order, but were less complete than those shown in Fig. 4a. Bird 67 crystallized a DCBA song (Fig. 4b). Birds 72, 75 and 84 also failed to retain their EDCBA songs, but crystallized songs that lacked C-type phrases. The fifth bird (not shown) produced an improvised introductory syllable, followed by either ABA or ADB. Collectively, the songs of birds in this tutoring group showed significant reversed phrase order ($P < 0.001$, binomial test). That four of the five birds crystallized songs that ended with BA ($P < 0.001$, binomial test), is remarkable in light of their predisposition to position whistles at the beginning of the song⁷ (Fig. 2). Apparently the early experience with reverse-ordered

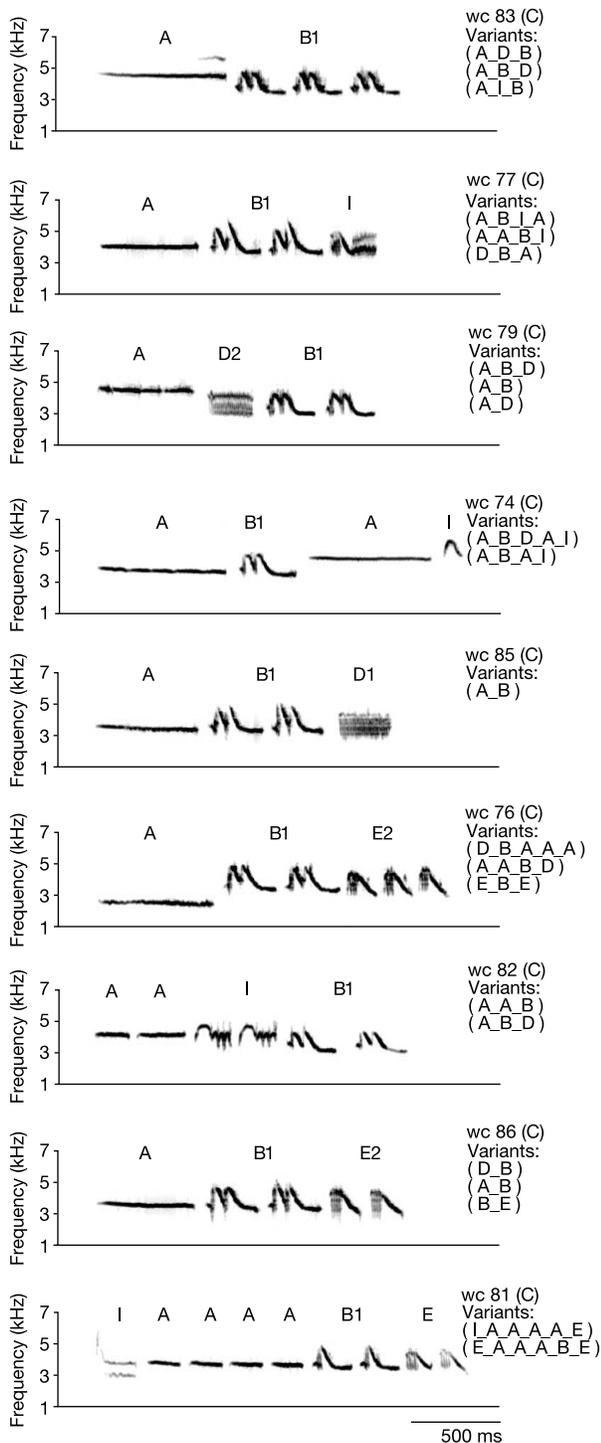


Figure 2 Sound spectrograms of representative crystallized songs. Birds were tutored with the phrase types shown in Fig. 1, but presented singly and in the order E, D, C, B, A. The songs (variants) that each bird sang before crystallization (C) are denoted by the letter sequences to the right of each spectrogram. Phrases in spectrograms are labelled with respect to the tutor phrases that they represent; improvised phrases (I) either did not appear to match a tutor phrase, or were amalgamations of two tutor phrase types.

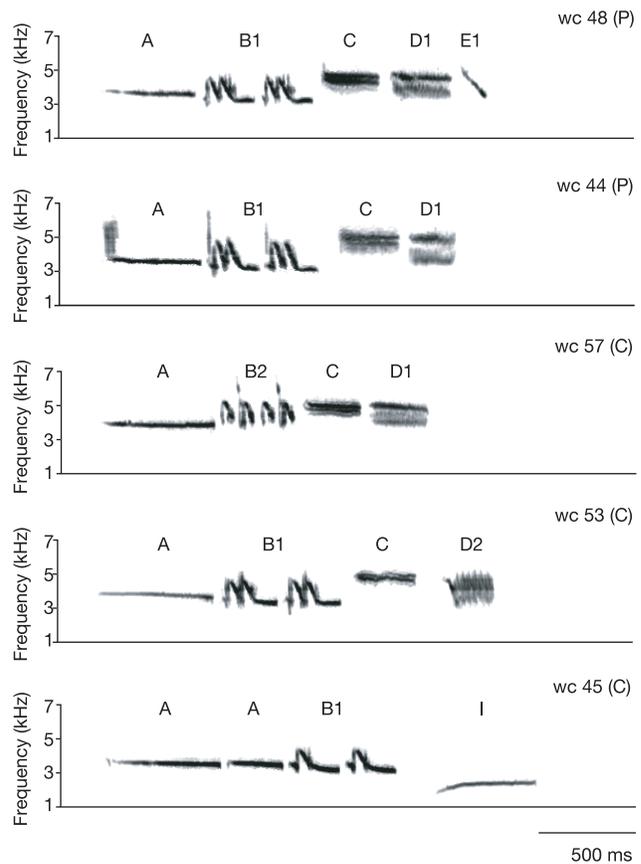


Figure 3 Sound spectrograms of representative songs of birds that were tutored with the phrase pairs DE, CD, BC and AB, presented in that order (see Fig. 1b). Late in the singing season, birds sang predominantly a single 'crystallized' (C) song. The song of bird 48, recorded during the late plastic-song stage (P), is shown to demonstrate that this bird was able to assemble a complete wild-type phrase sequence, despite ultimately crystallizing an ABCD song. Bird 44 died before crystallization, hence his late plastic-stage song is shown.

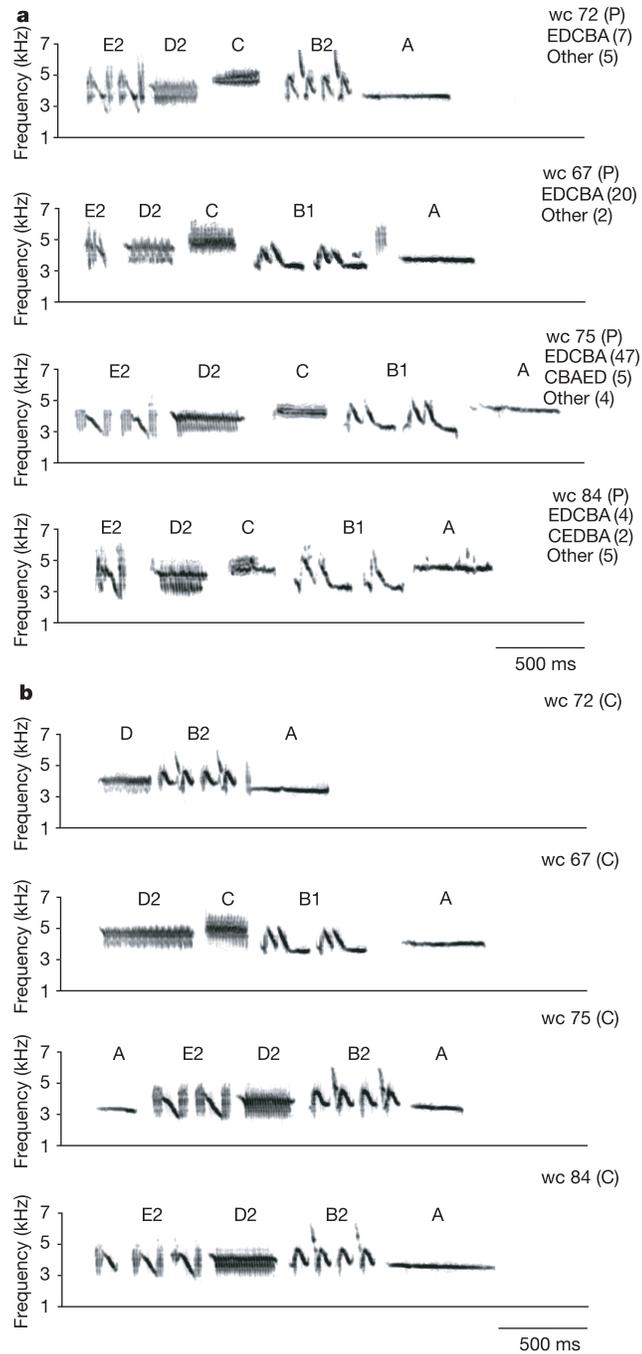


Figure 4 Sound spectrograms of representative songs of birds that were tutored with the phrase pairs BA, CB, DC, ED, presented in that order. The order of phrases in each pair, therefore, was reversed from that seen in wild-type songs. As before, (C) and (P) refer to crystallized and plastic song. **a**, Full reversed phrase-order songs produced by four of the birds during plastic song. Numbers in parentheses indicate the number of songs consisting of all five phrase types produced by each bird; single cases of particular types were grouped as 'others'. For bird number 67, 21 of the songs were recorded during the plastic stage of his second year. **b**, Crystallized songs of the same four birds.

phrases largely overrides the innate tendency to start songs with whistles.

How did birds arrive at their respective songs? Our model posits that some time early in song development, while still perfecting syllable and phrase structure, birds begin to combine phrases; first, phrases are assembled primarily into pairs, then into longer

sequences. The model also posits that, because only phrase pairs that match those in the tutor models can be reinforced maximally, over time, birds retain and refine these sequences, while eliminating other phrase pair combinations. These features of the model are generally supported by developmental data. Representative data from three birds, one tutored with forward-order phrase pairs (48) and two others tutored with reversed-order pairs (67 and 84) are shown in Fig. 5.

Early in song development, birds produced predominantly isolated phrases and phrase pairs; that is, rarely were sequences of more than two phrases produced wherein the gap between consecutive phrases was 200 ms or less (Fig. 5a–c); this conclusion also held for 'acceptance windows' of 0–100 ms and 0–300 ms. At this stage, phrases were placed in a variety of orders (Fig. 5d–f). Over time, correct phrase pairings became more common (compare solid versus dashed lines).

In late plastic song (approximately 200–250 days after the last tutoring session), the decline in phrase pairs and emergence of longer sequences (Fig. 5a–c) reflected the route of song assembly by each bird. Bird 67 produced progressively more triplet sequences (DCB in particular) as the frequency of isolated and paired phrases declined. He constructed his DCBA song by then adding a whistle to the end of the DCB sequence; the appearance of quadruplet sequences in the plot reflected the tightening of the temporal coupling of the DCB and A elements. Bird 48 also produced primarily isolated phrases and phrase pairs early in development, but his assembly of an ABCD song was not preceded by a large increase in triplet sequences. Instead he primarily combined phrase pairs in various orders, eventually constructing his song by tightening the coupling between AB and CD sequences, and eliminating other arrangements; BC pairs, although quite common early in development, were not used in constructing the final ABCD song. Bird 84 assembled his final song (EDBA) by combining ED and BA phrase pairs. In mid-plastic song, the gap between ED–BA phrase pairs was approximately that seen in EDCBA songs, that is, appropriate for a C-type phrase, even though it was rarely expressed. During late plastic song, the emergence of the EDBA sequence followed the tightening of temporal coupling of these phrase pairs. DC pairings, however, were relatively rare, which may explain why this bird did not produce a DCBA song.

We have presented the first evidence that songbirds can assemble complete songs when only tutored with pairs of phrases. Furthermore, they were able to extract the correct sequence of phrases under both conditions of phrase-pair tutoring, forward and reverse. Birds crystallized the forward phrase sequence more successfully, consistent with a synergy between innate (for example, beginning songs with a whistle) and experience-based forces in the forward-order regimen. The conceptual model provides a mechanism by which motor programs for linking particular phrase types together are reinforced on a pair-by-pair basis. The reinforcement is assumed to result from activating, through auditory feedback, particular combination-sensitive neurons, although a direct influence may also be possible¹¹. Recordings in forebrain regions, particularly the HVC, of anaesthetized songbirds have shown that neural mechanisms for generating selectivity for phrase order or phrase combinations exist^{12–17}, although this selectivity emerges only during the sensorimotor phase of song development^{18–20}. Through pair-by-pair reinforcement, the correct sequence emerges as the phrases are generated, possibly by chance, in the correct order. The developmental process of comparing what is produced to the memory (template) of the tutor models may involve interaction between basal ganglia and vocal motor circuits^{1,21}. Similarly, the basal ganglia in mammals are believed to play a part in controlling sequential motor behaviour²², and, as in songbirds, are implicated in vocal learning²³. Thus the song system may be broadly relevant for understanding how the nervous system

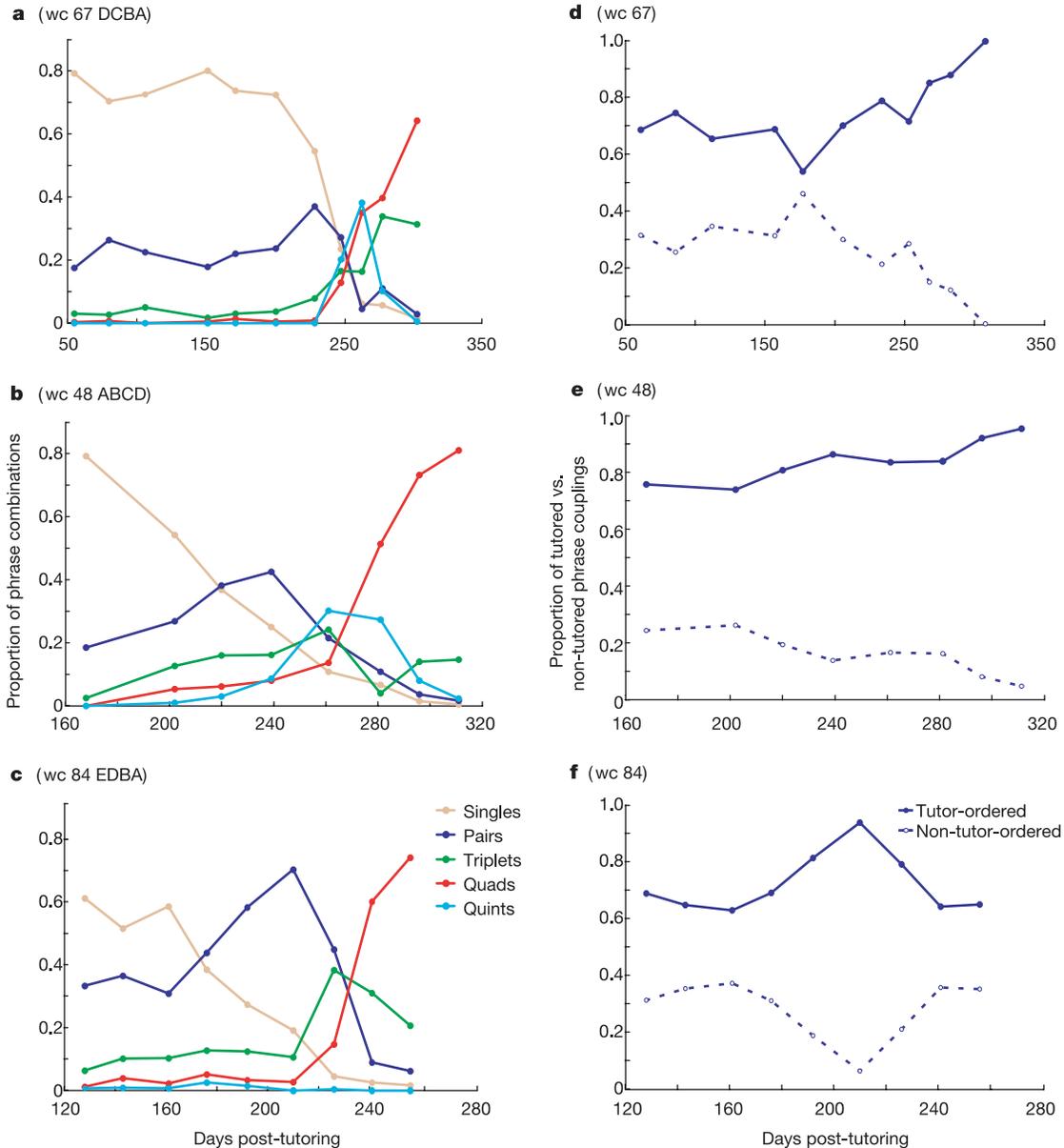


Figure 5 Song development for birds tutored with forward (bird 48) or reversed-order (birds 67 and 84) phrase pairs. **a–c**, Proportion of particular phrase groupings versus number of days elapsed from the last tutoring session; phrases were considered to be grouped if less than 200 ms transpired between the end of one phrase and the start of the subsequent phrase. **e–f**, Proportion of correct (solid lines) and incorrect (dashed lines)

phrase adjacencies over time; data were pooled over 15-day periods, with x-axis values being the first day of each of these periods. The earliest data presented varied across birds because of individual differences in the refinement of syllable and phrase structure.

encodes information about temporally related events and generates motor sequences. □

Methods

Subjects

White-crowned sparrows (*Zonotrichia leucophrys oriantha*) were collected as nestlings (5–7 days after hatching) in Wasatch County Utah during June and July of 2002 and 2003. Birds were hand fed until several days postfledging, and then given seed, puppy-chow, water and an assortment of fresh foods including crickets and mealworms. Fledglings were group-housed, 2–4 birds per cage, and tutored starting at 10–14 days of age.

White-crowned sparrows that have been exposed to song only as nestlings (less than 10 days of age), then raised in acoustic isolation, develop abnormal songs indistinguishable from those produced by 'isolate' birds that were raised from the egg⁴. Hearing song as a nestling is insufficient, therefore, for acquiring a template that can later, through auditory feedback, be used to guide song development.

Tutoring ensembles and procedures

The songs of two white-crowned sparrows (Fig. 1a), recorded at the location where hatchlings were collected, served as the starting material for constructing the tutoring ensembles. These songs were digitized (25 kHz) using Signal (Engineering Design) or SASLab Pro (Avisoft) sound analysis software. Files were then created that consisted of single phrases or pairs of normally adjacent phrases; for example, whistle-note complexes (AB), note complexes-buzz (BC) and buzz-trill (CD) (Fig. 1b). In the second ensemble, the order of phrases in these pairs was reversed from that of the natural order. A silent interval of 2.5 s separated each phrase (single-phrase tutoring experiment) or phrase pair from the preceding or following phrase or pair. The starting phrase or phrase pair for each session was selected randomly, but the order of presentation was always as shown in Fig. 1b, c; for single-phrase tutoring, phrases were presented in backwards order (E, D, C, B, A). These presentation orders were selected to ensure that birds could not use short-term memory to deduce the appropriate order that phrases should be placed. Sequences of phrases or phrase pairs were separated by 13.5 s.

In each tutoring session, the stimuli were broadcast (about 78 dB SPL) for 10 min then a minute of silence was allowed to transpire before starting the next 10-min segment. Two

tutoring sessions, each 90 min, were given each day. Birds that produced soft (subsong) vocalizations during tutoring were banded and transferred to individual custom sound attenuating chambers where tutoring continued until a total of 60 days was reached. Eight birds were tutored with the forward phrase-pair ensemble, eight others received tutoring with the reversed phrase-pair regimen. Eleven birds were tutored with singly presented phrases.

Birds were sexed in the following summer either by microsatellite DNA analysis²⁴ or autopsy; five of the birds in the reversed phrase-order group and seven in the forward phrase-order group were confirmed to be males.

Recording and analysis of vocalizations

The vocalizations of each bird were recorded using a small condenser microphone that was located in each sound-attenuating chamber. Beginning at approximately 30 days after tutoring, recordings were made frequently, with intervals not exceeding 9 days. These signals were digitized, displayed as spectrograms in real time and stored as computer files (Avisoft Recorder). Across renditions, the crystallized songs of birds often varied in length, with the last phrase sometimes not included. Spectrograms of crystallized songs that are displayed in this paper were chosen to show all of the phrase types of typical length found in these songs. Songs were scored with regard to whether adjacent phrases were placed in correct versus incorrect relative position; these values were then evaluated with respect to what would be expected by chance.

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Correspondence and requests for materials should be addressed to G.R. (rose@bioscience.utah.edu).

Early motor activity drives spindle bursts in the developing somatosensory cortex

Rustem Khazipov^{1,2,3*}, Anton Sirota^{2*}, Xavier Leinekugel^{1,2,4*}, Gregory L. Holmes³, Yehezkel Ben-Ari¹ & György Buzsáki²

¹INMED, INSERM U29, Avenue de Luminy, B.P. 13, 13273 Marseille, France

²CMBN, Rutgers University, 197 University Avenue, Newark, New Jersey 07102, USA

³Section of Neurology, Neuroscience Center at Dartmouth, Dartmouth Medical School, One Medical Center Drive Lebanon, New Hampshire 03756, USA

⁴INSERM EMI 224, 105, Boulevard de l'Hôpital 75013 Paris, France

* These authors contributed equally to this work

Sensorimotor coordination emerges early in development. The maturation period is characterized by the establishment of somatotopic cortical maps^{1,2}, the emergence of long-range cortical connections³, heightened experience-dependent plasticity^{4–7} and spontaneous uncoordinated skeletal movement^{8,9}. How these various processes cooperate to allow the somatosensory system to form a three-dimensional representation of the body is not known. In the visual system, interactions between spontaneous network patterns and afferent activity have been suggested to be vital for normal development^{10,11}. Although several intrinsic cortical patterns of correlated neuronal activity have been described in developing somatosensory cortex *in vitro*^{12–14}, the *in vivo* patterns in the critical developmental period and the influence of physiological sensory inputs on these patterns remain unknown. We report here that in the intact somatosensory cortex of the newborn rat *in vivo*, spatially confined spindle bursts represent the first and only organized network pattern. The localized spindles are selectively triggered in a somatotopic manner by spontaneous muscle twitches^{8,9}, motor patterns analogous to human fetal movements^{15,16}. We suggest that the interaction between movement-triggered sensory feedback signals and self-organized spindle oscillations shapes the formation of cortical connections required for sensorimotor coordination.

We examined the nature of early sensory signals and self-generated activity in the primary somatosensory (S1) cortex, using extracellular mapping and patch-clamp recordings in neonatal rats *in vivo* (postnatal days 1–8). In contrast to the adult neocortex¹⁷, activity in the neonatal rat was characterized by intermittent network bursts (mean±s.d. = 0.65±0.15 s), separated by long silent periods (Fig. 1; 7.2±1.93 s; *n* = 19 non-anaesthetized pups). The multiple unit bursts were associated with a single sharp potential or spindle-shape field oscillations (10.8±1.2 Hz). Unitary discharges were rare between these field events (Fig. 1a, d). Sharp potentials occurred in isolation or preceded spindles by 100–200 ms. Simultaneous recording of field events in various S1 cortical layers with silicon probes showed a reversal of both the sharp potential and spindle pattern between superficial and deep layers (Fig. 1f; *n* = 2 pups). Spindle activity was associated with rhythmic multiple unit discharges (Fig. 1b; see Supplementary Figures).

To explore the synaptic mechanisms underlying the generation of S1 network bursts, we employed patch-clamp recordings in urethane-anaesthetized animals (Fig. 2; see Supplementary Figures). Glutamatergic excitatory postsynaptic currents (EPSCs) and GABA (γ -aminobutyric acid) receptor A (GABA_A) receptor-mediated PSCs were separated by using whole-cell recordings in voltage-clamp mode with low-chloride internal solution. The basic parameters of EPSCs and GABA_A-PSCs were similar to that described *in vitro* (Supplementary Table)¹⁸. A characteristic feature of synaptic activity in S1 neurons was the grouping of both EPSCs