

Two cautionary points, one perhaps encouraging and another less so, emerge from our study. First, it is evident that a high mutation rate is not always beneficial. The adaptive neighbourhood of an adaptive peak can constrain evolvability, despite a high mutation rate. A high rate can lead to a high mutational load, as seen during the evolution by clone B. Thus, evolutionary changes in a population of RNA viruses, whether under clinical treatment or otherwise, should not automatically be interpreted as adaptive to the virus. Second, as had already been noted¹², if multiple peaks exist, a shift to a new adaptive peak can be triggered by a costly resistance mutation and the ensuing compensatory mutations. If some peaks are higher, drugs should be used with caution not only because resistance can evolve but also because drug treatment can lead to the evolution of viruses that are more fit rather than less. □

Methods

Strains and culture conditions

The RNA bacteriophage $\phi 6$ used in this study is a laboratory clone descended from the original isolate¹⁵. Populations A and B were generated in a previous study⁷. They are the end points of populations propagated using bottlenecks of 100 and 33 phage, respectively, to allow fitness recovery after acquisition of a common deleterious mutation. *Pseudomonas syringae* sv. *phaseolicola*, the standard host of $\phi 6$, was obtained from the American Type Culture Collection (ATCC# 21781), and *Pseudomonas pseudocaligenes* ERA, an alternate host, was obtained from L. Mindich. Details of diluting, filtering, culture and storage of phage and bacteria are published^{16,17}. All phage and bacteria were grown in LC medium at 25 °C.

Phage propagation

Details for protocol have been described⁷. Briefly, phage were plated onto a lawn of standard host *P. phaseolicola* and incubated to allow the phage to reproduce and form plaques on the lawn. After 24 h, a number of plaques were randomly chosen from the plate, phage were collected from the plaques, and then plated on a fresh lawn to start a new growth cycle. One growth cycle on the lawn corresponds to about five generations, and cycles can be repeated for as long as desired. The number of plaques chosen each cycle was 1,000 for all of the populations in this study. This number renders the effective population size of all of the populations to be equivalent by enforcing the same size of population bottleneck. A bottleneck size of 1,000 is sufficiently large to promote evolution by natural selection in $\phi 6$ (ref. 7). Viral samples from all time points were stored frozen for later fitness assays.

Because the goal of the study was to examine the evolvability of these populations, and not their co-evolvability with their bacterial host, bacterial evolution was prevented by filtering out the bacteria after each growth cycle. Thus, every growth cycle was initiated with a fresh lawn made from a frozen (non-evolving) sample of the bacteria.

Standard fitness assay

The protocol we used has been described¹⁸. A test phage and a reference phage were mixed at a ratio of roughly 1:1 and plated on a *P. phaseolicola* lawn at a density of 400 phage per plate. After a 24-h incubation, the resulting plaques were collected. Fitness was measured as $W = R_1/R_0$, where R_0 and R_1 are, respectively, the ratio of the phage (test to reference) before and after growth on the lawn. This fitness assay measures the realized growth rate of the test phage relative to the reference. A value of $W = 1.0$ or $\log_{10}(W) = 0.0$ indicates equal fitness. To differentiate the two phage, the reference phage was marked with a host range mutation that allows growth on a new host, the bacterium *P. pseudocaligenes*.

Plaque-size determination

Plaque sizes were determined by plating phage from a population on a lawn of *P. phaseolicola* and incubating for 24 h. Phage were plated at a low density (< 50 phage per plate) to ensure non-overlapping plaques. Plaques growing on the lawn, denoted test plaques, could have been used to quantify the fitness of individual viruses in the population; however, the size of a single test plaque is very sensitive to environmental factors and does not provide a precise measurement of fitness. To increase the precision, each test plaque was cut from the lawn, suspended in broth medium, diluted and plated on a scoring plate at a density of roughly 60 plaques per plate. Pictures were taken of the scoring plates and the size of plaques on each plate was determined using Scion Image Version 3b (Scion Corporation, Frederick, MD). The average plaque size on each scoring plate was then used as the measure of plaque size for the corresponding test plaque. Each variate in the distributions presented in Fig. 3 is such an average for a single test plaque.

Statistics

All analyses were done as described¹⁹.

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Behaviourally driven gene expression reveals song nuclei in hummingbird brain

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Hummingbirds have developed a wealth of intriguing features, such as backwards flight, ultraviolet vision, extremely high metabolic rates, nocturnal hibernation, high brain-to-body size ratio and a remarkable species-specific diversity of vocalizations^{1–4}. Like humans, they have also developed the rare trait of vocal learning, this being the ability to acquire vocalizations through imitation rather than instinct^{5,6}. Here we show, using behaviourally driven gene expression in freely ranging tropical animals, that the forebrain of hummingbirds contains seven discrete structures that are active during singing, providing the first anatomical and functional demonstration of vocal nuclei in hummingbirds. These structures are strikingly similar to seven forebrain regions that are involved in vocal learning and production in songbirds and

parrots^{7–13}—the only other avian orders known to be vocal learners⁵. This similarity is surprising, as songbirds, parrots and hummingbirds are thought to have evolved vocal learning and associated brain structures independently^{5,14}, and it indicates that strong constraints may influence the evolution of forebrain vocal nuclei.

We conducted our study at a Nature Reserve of the Museu de Biologia Mello Leitão (Espírito Santo, Brazil), an enclave of the Atlantic Tropical Forest with over 30 hummingbird species¹⁵. We focused on the sombre hummingbird (*Aphantochroa cirrhochloris*) and the rufous-breasted hermit (*Glaucis hirsuta*) (Fig. 1). We identified brain areas involved in vocal communication, by monitoring expression of the transcriptional regulator ZENK (an acronym for Zif-268, Egr-1, NGFI-A and Krox-24) in freely ranging birds after hearing and vocalizing behaviours. ZENK messenger RNA synthesis in the brain is driven by neuronal depolarization, and its detection can be used to identify select regions that are activated by specific stimuli or behaviours¹⁶. This methodology has allowed us to map vocal communication areas throughout the brains of songbirds^{12,17} and a parrot¹³ (in both laboratory and natural¹⁸ settings) without disrupting the natural behaviour of the birds. This is important as hummingbird singing behaviour can be difficult to obtain under captivity, and it is currently not possible to identify relevant brain areas in freely ranging small animals using other methods such as electrophysiology.

Hummingbirds often sing while perched on a tree branch, and feed on nectar from nearby flowers between singing bouts. We located tree perches where individual birds sang most frequently, and set feeding traps consisting of a sugar-water bottle (a flower substitute) inside a small cage on nearby tree branches. The birds were caught upon entering the cage at a specific time after a certain behavioural state. We compared three groups: silent controls (birds caught early in the morning before the start of the dawn chorus); hearing only (birds caught around the same time after hearing a 25–30-min conspecific song playback, but that did not sing in response); hearing and vocalizing (birds caught early in the morning after hearing song and singing one or more song bouts

per min during the 25–30-min period) $n = 3$ males per group for *Aphantochroa* and one male per group for *Glaucis*. The birds were killed immediately upon capture and their brains processed for ZENK mRNA expression^{12,17}.

Relative to silent controls, hearing only birds showed hearing-induced ZENK expression in seven brain areas that are conserved among avian species^{13,17}: five telencephalic (NCM, CMHV, PC, Ndc, Ai); one thalamic (DIP); and one mesencephalic (MLd) (Figs 2 and 3a; for all abbreviations, see Box 1). Relative to hearing only birds, hearing and vocalizing birds showed vocalizing-induced ZENK expression in eight discrete areas. Of these, seven are telencephalic, which we named and placed into three groups: first, a ‘posterior-medial cluster’ containing two nuclei (VMN and VMH); second, an ‘anterior cluster’ containing three nuclei (VAH, VAN and VAP); and last, a ‘posterior-lateral cluster’ containing two nuclei (VLN and VA) (Figs 2 and 3a). The most marked vocalizing-induced expression occurred in VAN and VLN (Fig. 2b). The eighth structure was DM in the mesencephalon (Figs 2a and 3a), a conserved avian vocal nucleus^{13,19}. ZENK expression in these eight structures was proportional to the number of song bouts produced during the 25–30-min singing period (Fig. 3b).

All seven forebrain structures with vocalizing-induced expression have distinct histological features that differentiate them from surrounding tissues (Fig. 2d). The same seven structures were found in Nissl-stained sections from males of two other hummingbird species that we caught at the Museu Mello Leitão: swallow-tailed (*Eupetomena macroura*) and white-throated (*Leucochloris albicollis*). The four species that we studied cover the two hummingbird lineages, *Glaucis* being one of the most ancient Phaethornithinae (hermits), *Aphantochroa* and *Eupetomena* two ancient Trochilinae (non-hermits), and *Leucochloris* a more recently derived Trochilinae (ref. 1; and K. L. Schuchmann and R. Bleiweiss, personal communication). Thus, forebrain vocal nuclei appear to have been

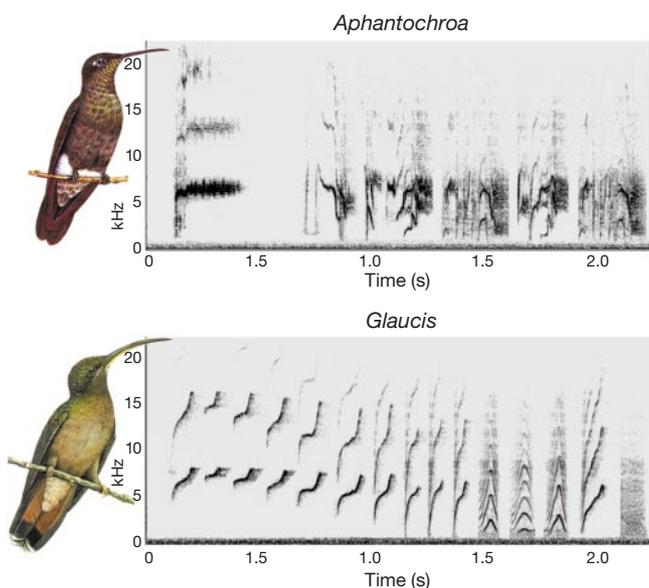


Figure 1 Song sonograms (frequency versus time) of the hummingbird species studied: *Aphantochroa cirrhochloris* and *Glaucis hirsuta*¹⁵. *Aphantochroa* has a stereotyped song, consisting of an introductory note followed by several renditions of a two-note sequence: individual notes have complex frequency modulations. *Glaucis* sings a more varied whistle-like song consisting of several introductory notes (not shown) followed by note sequences of ascending and descending frequencies that can be produced in different orders (A. Ferreira *et al.*, personal communication).

Box 1

Anatomical structures

A, archistriatum; AAC, central nucleus of the anterior archistriatum; AC, anterior commissure; ACM, caudomedial archistriatum; Ai, intermediate archistriatum; Area X, area X of the paleostriatum; Av, nucleus avalanche; Cb, cerebellum; cp, choroid plexus; CMHV, caudomedial hyperstriatum ventrale; DIP, dorsointermediate nucleus of the posterior thalamus; DLM, medial nucleus of the dorsolateral thalamus; DM, dorsomedial nucleus; DMm, magnocellular nucleus of the dorsomedial thalamus; ex, extensions of LPOm; HA, hyperstriatum accessorium; HD, hyperstriatum dorsale; Hp, hippocampus; HV, hyperstriatum ventrale; HVC, high vocal center; HVoc, complex including the oval nucleus of the hyperstriatum ventrale and surrounds; ICo, intercollicular nucleus of the mesencephalon; L2, primary telencephalic auditory area; IAHV, lateral nucleus of the anterior hyperstriatum ventrale; IAN, lateral nucleus of the anterior neostriatum; LPOm, magnocellular nucleus of the parolfactory lobe; MAN, magnocellular nucleus of the anterior neostriatum; MLd, dorsal part of the lateral mesencephalic nucleus; N, neostriatum (not the same as the mammalian neostriatum); NAoc, complex including the oval nucleus of the anterior neostriatum and surrounds; NCM, caudomedial neostriatum; Ndc, dorsocaudal neostriatum; NIDL, neostriatum intermedium pars dorsolateralis; Nlf, nucleus interfascialis; NLc, central nucleus of the lateral neostriatum; nXlts, tracheosyringeal subdivision of the hypoglossal nucleus; OC, optic chiasm; OM, occipitomesencephalic tract; OT, optic tectum; Ov, nucleus ovoidalis of the thalamus; P, paleostriatum; PC, caudal paleostriatum; PP, paleostriatum primitivum; RA, robust nucleus of the archistriatum; S, septum; T, thalamus; v, ventricle; VA, vocal nucleus of the archistriatum; VAH, vocal nucleus of the anterior hyperstriatum ventrale; VAN, vocal nucleus of the anterior neostriatum; VAP, vocal nucleus of the anterior paleostriatum; VLN, vocal nucleus of the lateral neostriatum; VMH, vocal nucleus of the medial hyperstriatum ventrale; VMN, vocal nucleus of the medial neostriatum.

present early among hummingbirds. Vocal learning has been demonstrated directly by raising birds in isolation from conspecifics for one species of the Trochilinae lineage, and indirectly through the analysis of individual variability in seven species of the Phaethornithinae lineage^{1,4-6}. Thus, it is generally assumed that vocal learning was also present early in hummingbirds.

To our knowledge, our results represent a first anatomical and functional demonstration of vocal control brain nuclei in hummingbirds. Moreover, we have identified a whole set of fore-brain vocal control structures in a vocal learning order—something that has taken years in other species using different methodologies. This now provides a map for future anatomical, physiological and behavioural investigations. Interestingly, both songbirds and budgerigars (a parrot), have also been shown to have exactly seven forebrain structures with singing-induced ZENK expression^{12,13}. Three of these nuclei are at the same relative positions in the anterior telencephalon in parrots, hummingbirds and songbirds (Fig. 4, structures in red). The other four nuclei are in different locations of the posterior and/or lateral telencephalon (Fig. 4,

structures in yellow) but within the same brain subdivisions (Table 1). These nuclei have marked morphological similarities across orders. For example, parrot NLC^{11,13}, hummingbird VLN (Fig. 2) and songbird HVC⁷ bulge into the overlying ventricle. Parrot AAC^{11,13}, hummingbird VA (Fig. 2) and songbird RA⁷ have an oval shape and constitute cytoarchitectonically very distinct nuclei within the archistriatum. The posterior-lateral nuclei in songbirds and budgerigars (Fig. 4, structures in yellow) are part of a pathway whose output is to the syrinx^{11,20}, and in songbirds controls production of learned vocalizations^{7,9,12}. The anterior nuclei (Fig. 4, structures in red) control vocal learning in songbirds⁸, and are part of a pathway comparable to cortico-basal ganglia-thalamo-cortical loops¹⁰⁻¹² in mammals, which participate in the learning and maintenance of sequential motor actions dependent on sensorimotor integration²¹.

Our findings have implications for the evolution of brain structures that control a complex behaviour. Vocal learning is a rare trait known to occur in only three groups of birds (parrots, hummingbirds and songbirds) and three groups of mammals (humans, cetaceans (whales/dolphins) and bats)^{5,6,14,22,23}. Because they are

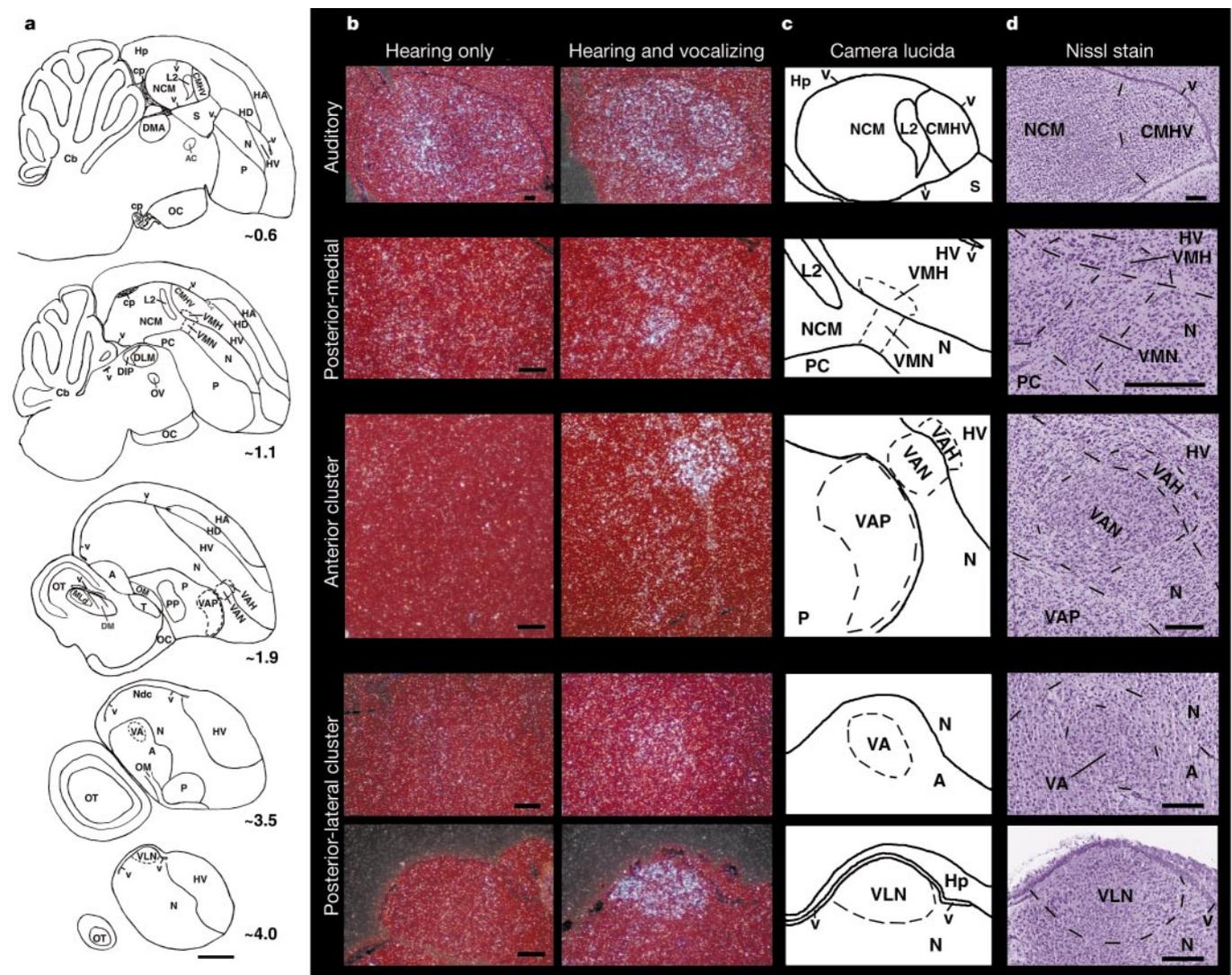


Figure 2 Identification of vocal control brain areas. **a**, Camera lucida drawings of parasagittal sections from *Aphantochroa*. Dashed lines, regions of vocalizing-induced ZENK expression. Numbers indicate mm from midline. **b**, Dark-field views of ZENK-hybridized sections. White-silver grains, ZENK expression; red background, Nissl stain. Only NCM and CMHV are shown for hearing-induced regions. **c**, Detailed drawings of brain regions from the hearing and vocalizing bird in **b**. **d**, Bright-field view of Nissl-stained reference sections containing vocalizing-induced areas, at higher magnification than in **b**

and **c** for visualization of cytoarchitectonic features: VMH contains cells larger than the surround; VMN is rectangular with cells organized into columns; VAH is small and flat; VAN is semi-round with cells larger than the surround; VAP (Nissl not shown) is crescent-shaped and contains a sub-population of large cells; VA is oval, darkly staining, and with higher cell density than the surround; VLN bulges into the overlying ventricle. Orientation: dorsal is up, anterior to the right. Scale bars, 1 mm (**a**); 0.25 mm (**b-d**). For abbreviations, see Box 1.

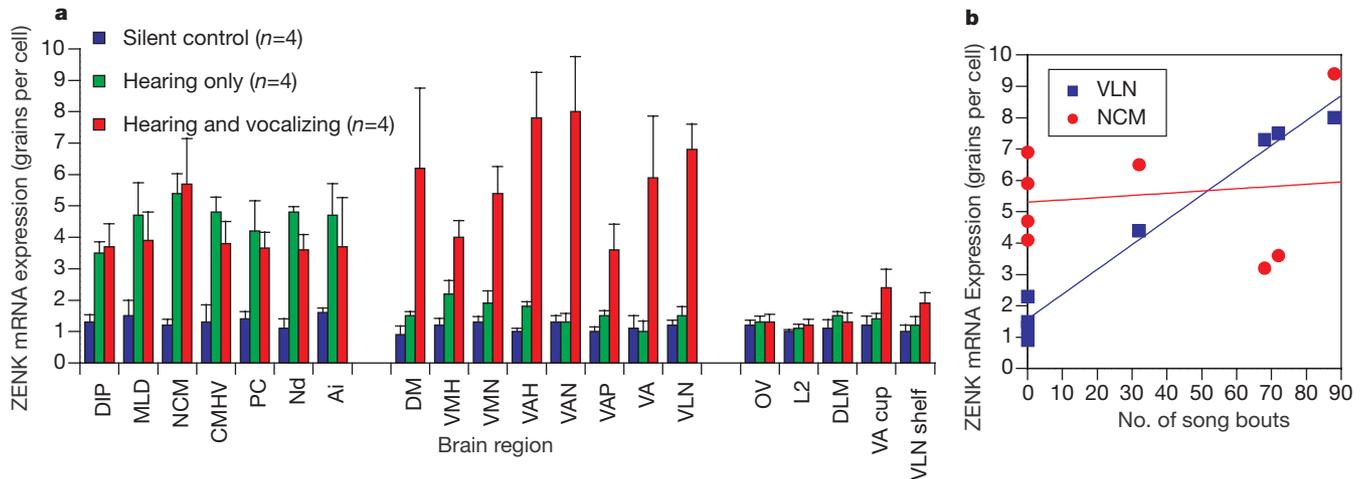


Figure 3 Quantification of ZENK expression. **a**, Regions with hearing-induced expression (left, $P, 0.03$ to <0.0001), vocalizing-induced expression (centre, $P, 0.02$ to <0.0005), or no induced expression (right, $P, 0.2$ to 0.84 ; two-tailed unpaired t -test). Of the latter, three are part of the auditory (Ov, L2) or vocal (DLM) pathways, and do not show induction in songbirds and budgerigars^{12,13,17}, two (VLN* and VA*) represent regions immediately ventral to VLN and rostroventral to VA, and correspond topographically to songbird HVC shelf and RA

cup, which show hearing-induced expression in songbirds^{12,17}. Plotted are mean \pm s.e.m. **b**, ZENK expression is proportional to amount of singing for structures with vocalizing-induced expression ($P, 0.013$ to <0.0001 ; $r, 0.819$ – 0.988) but not for those with hearing-induced expression ($P, 0.10$ to <0.93 ; $r, 0.037$ – 0.36 ; analysis of variance linear regression); VLN and NCM are representative examples. For abbreviations, see Box 1.

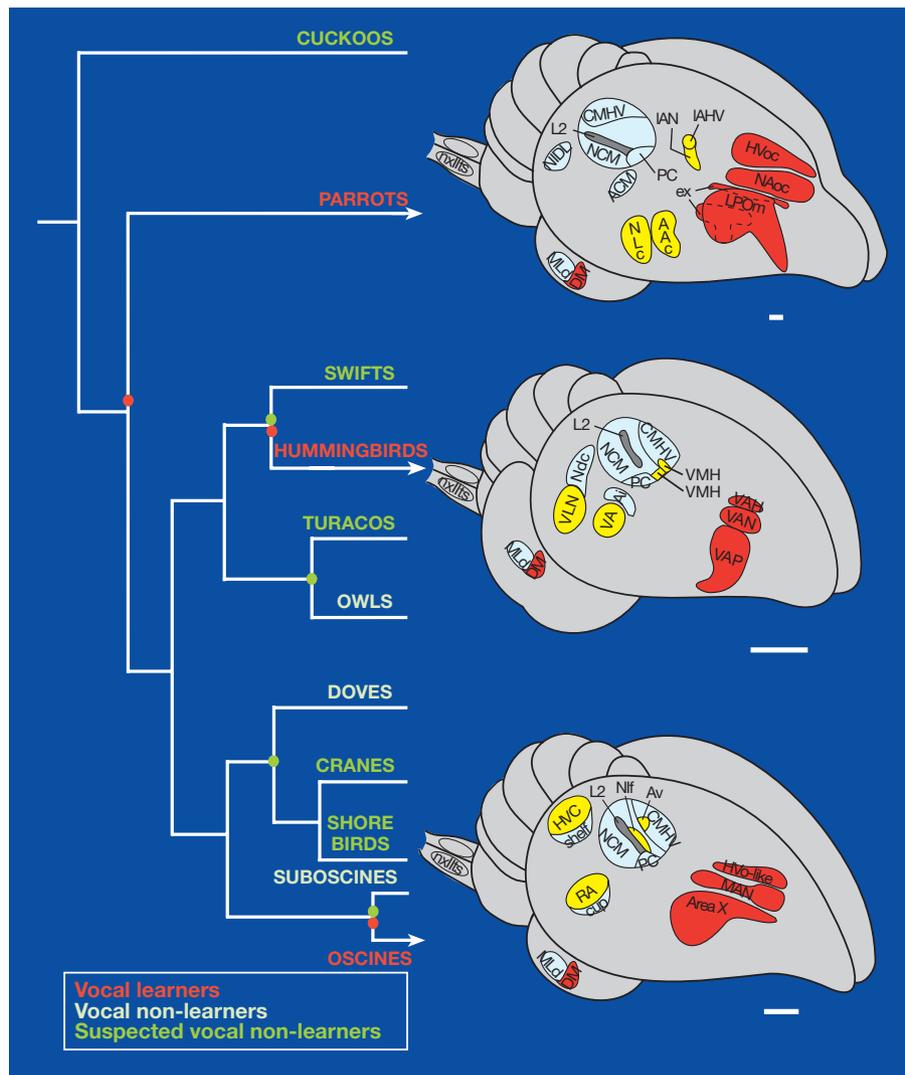


Figure 4 Comparative brain anatomy of hearing- and vocalizing-induced ZENK expression in avian vocal learners. Left, partial phylogenetic tree based on Sibley and Ahlquist²⁴, with one common species name per order; colours indicate evidence for vocal learning^{5,6,27–30}. Red dots, ancestral nodes of possible independent gain of vocal learning; green dots, ancestral nodes of possible independent loss of vocal learning. Parrots are the

oldest vocal learning order, oscine songbirds the most recent²⁴. Right, semi three-dimensional brain renditions of vocal learners. Blue, hearing-induced regions; red, similarly positioned vocalizing-induced regions; yellow, differently positioned vocalizing-induced regions^{12,13}. Scale bars, 1 mm. For abbreviations, see Box 1.

Table 1 Telencephalic subdivisions and their vocalizing-activated nuclei across vocal learning avian orders*

Brain Subdivision	Parrot	Hummingbird	Songbird
Hyperstriatum	HVoc IAHV	VAH VMH	HVo-like Av
Neostriatum	NAoc NLc IAN	VAN VLN VMN	MAN HVC Nif
Archistriatum	AAC	VA	RA
Paleostriatum	LPOm	VAP	Area X

*Correspondences are based on relative position and cytoarchitectonic features for the three orders, in addition to connectivity data for songbirds and parrots (budgerigars)^{17,11–14,20}. For abbreviations, see Box 1.

phylogenetically separated by vocal non-learners²⁴ (Fig. 4), it is thought that the three avian vocal learning groups, and presumably the mammalian ones, evolved vocal learning independently^{5,14}. Similarly, the associated forebrain vocal control structures are absent in avian vocal non-learners, and it is believed that songbirds, parrots and presumably hummingbirds evolved such structures independently^{14,25}. Modern birds evolved from a common ancestor thought to have lived about 65 million years ago near the Cretaceous/Tertiary transition²⁶. Of the descendants, parrots are the oldest vocal learning order, followed by hummingbirds and then oscine songbirds²⁴ (Fig. 4). According to the dominant hypothesis^{5,14}, our results indicate that within the past 65 million years 3 out of 23 avian orders²⁴ may have independently evolved 7 similar forebrain vocal structures for a complex behaviour (Fig. 4). This would suggest that the evolution of these structures is under strong epigenetic constraints; in which case, similar structures may have also evolved in vocal learning mammals (humans, cetaceans and bats). Alternatively, vocal learning and associated brain structures may have been present in a common ancestor to avian vocal learners. In this regard, there is a shift in the posterior forebrain vocal structures from more anterior-lateral to posterior-medial positions, in accordance with the relative age of the vocal learning orders (Fig. 4). This hypothesis requires that the forebrain vocal structures were lost in the intervening vocal non-learning orders at least four times independently (Fig. 4). Such a loss could be due to the considerable expense required to maintain vocal learning and associated brain structures, with many birds possibly evolving in adaptive zones that did not require complex learned vocalizations. Another alternative hypothesis is that avian vocal non-learners have some rudimentary form of forebrain vocal areas that were previously missed by Nissl and tract tracing studies^{14,19,25}. If true, this would constitute a challenge to the hypothesis that forebrain vocal structures are unique to vocal learners¹⁴. □

Methods

Behaviour

The behaviour of all birds was monitored by video taping: only those birds that could be monitored during the entire observation period were captured. Video recordings were used to score number of song bouts produced by the hearing and vocalizing group during the observation period. For the hearing only group, playback of digitally recorded conspecific song (three song bouts per min, each 3–4 s long, from a bird of another locale) were presented from a nearby speaker (3–4 ft). To capture the birds, a feeding bottle containing 24% sucrose inside a cage was used. After 25–30 min in one of the behavioural conditions, a string attached to a stick holding the cage door open was pulled and the bird caught on a regular visit to the feeding bottle. The birds were immediately killed by decapitation, and their brains were dissected, placed in cryogenic embedding medium, and frozen in dry ice; sex was confirmed by direct inspection of the gonads.

Gene expression

Serial parasagittal (right hemisphere) and frontal (left hemisphere) frozen sections (10 µm) were cut throughout the entire brain of each bird. One section every 0.1 mm of each brain (~100 sections per brain, totalling 1,200 sections) was processed for ZENK expression by *in situ* hybridization with a ³⁵S radioactively labelled riboprobe for canary ZENK, followed by emulsion autoradiography^{21,17}. Unhybridized adjacent sections (20 µm) were stained with cresyl violet and used as reference for identification of cytoarchitectonic boundaries (Fig. 2d). Quantification (Fig. 3) was performed by counting silver grains over cells¹². Regions where ZENK expression was significantly higher in

hearing only compared with silent control animals represent areas activated by hearing conspecific song; regions where ZENK expression was higher in hearing and vocalizing compared with hearing only animals represent areas activated by singing. This strategy reveals all known telencephalic vocal control nuclei in songbirds and parrots, and identified previously undetected ones^{12,13}. No obvious differences were detected between *Aphantochroa* and *Glaucis*, and thus their values were grouped (total *n* = 4 per group).

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