Title: Heritable differences in synaptic zinc-transporter levels drive variation in learned birdsong

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Abstract:

Complex learned behaviors exhibit striking variation within populations, yet how heritable factors contribute to such inter-individual differences remains largely unknown. Here, we used behavioral-genetic analysis within a Bengalese finch population (*Lonchura striata domestica*) to investigate molecular and circuit mechanisms underlying heritable differences in the tempo of learned birdsong. We identified a genomic locus encoding the zinc transporter ZIP11 and found that *zip11*(*SLC39A11*) transcript was expressed at higher levels in song control circuitry of faster singing birds. Reducing soluble zinc increased synaptic currents in motor circuitry and accelerated song, whereas reducing ZIP11 slowed song. Our results reveal a novel zinc-dependent mechanism that modulates neural activity to drive differences in behavior and suggest that natural variation in learning may preferentially target modulatory processes rather than core neural machinery.

One sentence summary: Heritable levels of a synaptic zinc transporter drive inter-individual differences in circuit excitability and learned song

Main Text:

Natural variation in complex behaviors within a population forms a crucial substrate for selection and, more broadly, is thought to promote the overall success of a species (1). However, while recent studies have identified heritable genetic factors that contribute to inter-individual differences in innate behaviors, especially across species (2–7), understanding how such factors drive variation in complex learned behaviors within a population remains poorly understood. This reflects in part the dual challenge of needing to disambiguate which aspects of inter-individual differences in learned phenotypes reflect genetic rather than experiential factors and pinpointing the influence of those factors within the distributed neural circuitry that shapes behavior(8). Birdsong—a complex motor skill acquired through cultural transmission and subserved by well-delineated neural circuitry (9–12)—offers an attractive model(13) for studying heritable sources of variation in learned behavior. In particular, song tempo—a defining characteristic of the male’s courtship song and motor skill performance more generally (14–20)—is shaped both by experience during learning from a tutor and by heritable factors (21–28). Moreover, while the characteristic tempo at which an adult bird sings varies by only a few percent from day to day, the tempos at which different individuals sing can span more than a factor of two (14, 16, 29). Although the precision and reproducibility of song tempo at the level of individual birds has facilitated identification of the neural circuitry that controls song production and enables learning more broadly (30–39), the mechanisms that give rise to inter-individual variation, and any specific genetic constraints that govern them, remain unknown. Here, we use an unbiased behavioral-genetic approach to identify a zinc transporter linked to variation in learned song tempo, and then leverage the detailed understanding of birdsong neurobiology to uncover a novel mechanism whereby inter-individual differences in expression of this transporter drive heritable behavioral variation via zinc-dependent modulation of synaptic currents within song control circuitry.

Genetic linkage for song tempo identifies a ZIP-type zinc transporter

To investigate heritable contributions to inter-individual differences in learned song, we first quantified naturally occurring variation in tempo within a genetically heterogeneous breeding population of Bengalese finches maintained by our laboratory (29). We collected DNA and recorded the adult songs produced by each individual, as well as the father's tutor song from which they had learned (Fig. 1A). Across the study population, individuals produced songs at tempos (quantified as the median number of syllables sung per second, see Methods) that were strongly correlated with the tempos of their fathers' songs (Fig. 1B, slope = 0.43, $r = 0.39$, $P < 0.0001$, two tailed t -test). However, even within families in which brothers were tutored by the same father, there could be significant variation in tempo (Fig. 1A-C); some families had narrow, approximately Gaussian distributions of tempo, but many had broad or multi-modal distributions (Fig. 1C). Such non-Gaussian patterns within families suggest the influence of a small number of segregating genetic alleles that have a large impact on song tempo (40, 41), that might therefore be identified through genetic analysis even within modestly sized populations.

To identify genomic regions associated with heritable differences in song tempo, we examined the relationship across the population between inter-individual variation in DNA and song tempo. We first assembled a chromosome-level genome sequence for the Bengalese finch that served as a reference for the analysis (RefSeq assembly accession number GCF_005870125.1, see (42) and Methods). For each of 509 birds, from families for which we collected DNA from male offspring and one or both parents, we then sequenced ~400,000 short stretches of DNA (50-65 base pairs) at defined locations distributed throughout the genome (see Methods). We selected approximately 51,000 of these locations that exhibited DNA variation across individuals in the form of single nucleotide polymorphisms (SNPs) and could therefore be used as potentially informative markers for which specific alleles were transmitted from parents to offspring. To assess the strength of association between allelic variation at each locus and variation in tempo, we applied a Transmission Disequilibrium Test (TDT, see Methods and (43)). This method takes advantage of knowledge about parentage to restrict analysis for each family to loci that are variable within that family and is comparatively robust to issues arising from population stratification that can confound genome-wide

association studies (43, 44). However, as only a subset of families contributes to allelic variation at a given locus, TDT statistics from adjacent loci within a region are non-redundant and were therefore combined in a sliding window used to scan the genome for regions of interest. To assess statistical significance, we compared the strength of empirically observed associations between genetic variation and tempo against a null distribution of genome-wide maximum values derived from repeating the entirety of the analysis for 10,000 null data sets that preserved the allelic state and familial associations for each bird, but randomly shuffled song tempo (Fig. 1D, S1).

Significant association of allelic state with song tempo was evident for genomic regions on three chromosomes (Fig. 1D, Chromosomes 1, 18, and Z). Moreover, for each of these regions, differences in specific SNPs could account for ~10% differences in tempo across individuals (Fig. 1E, S2). Two of these regions, on Chromosomes 1 and Z, were each relatively large (on the order of megabases) and contained many genes (Fig. S2). However, the region on Chromosome 18 spanned only ~350 kilobases (Fig. 1E), suggesting that substantial variation in tempo might arise from polymorphisms within a single gene. Indeed, a closer examination of this region revealed that the specific allelic variants at five adjacent loci (Fig. 1E) accounted for substantial variation in mean song tempo; for example, at nucleotide 11605768 on Chromosome 18, birds that were homozygous for thymine ('T/T') had an average tempo that was 10.1% faster than for birds that were homozygous for cytosine ('C/C') (Fig. 1F, 7.45 ± 0.06 syl/s vs. 8.20 ± 0.13 syl/s, $P < 0.001$, two tailed t -test). These five markers all fall within introns of the avian homolog of the *SLC39A11* (*zip11*) gene, suggesting that variation within this gene or nearby regulatory regions might account for a component of inter-individual variation in learned song tempo.

The predicted avian protein encoded by the *zip11* gene displays 75% identity to the human zinc/iron-regulated transporter-related protein 11 (ZIP 11)—a zinc transporter conserved from yeast to humans and implicated in the import of extracellular zinc to the cytoplasm (45). While ZIP11 has primarily been studied in the gut of mammalian systems

118 (46), ZIP-type zinc transporters, including ZIP11, are expressed in the brain (45, 47), where
 119 the concentration and distribution of soluble zinc could contribute to neural circuit function
 120 (48). These observations thus link polymorphisms in a region that encodes the Bengalese
 121 finch homolog of the ZIP11 zinc transporter to variation in song tempo, raising the possibility
 122 that inter-individual differences in tempo stem in part from heritable differences in zinc
 123 regulation.

124 **zip11 is expressed in song premotor circuitry at levels that correlate with song tempo**

125 Previous investigations indicate that song tempo reflects the rate of neural activity
 126 propagation within song premotor circuitry, including the telencephalic nucleus HVC (12, 30–
 127 32, 35–39). Hence, the expression of *zip11* in this circuitry would be well-positioned to
 128 influence circuit dynamics underlying song tempo. To evaluate the pattern of *zip11* expression
 129 in the avian forebrain, we performed *in situ* hybridization in sagittal brain sections containing
 130 HVC. The *zip11* probe labeled cells in both HVC and surrounding tissue (Fig. 2B; boundary of
 131 HVC indicated by dashed line). Moreover, most *zip11* labeled cells (83%) were co-labeled
 132 with a probe for the excitatory neuronal marker *vglut2* (*SLC17A6*), while most *vglut2* labeled
 133 cells (85%) were co-labeled with *zip11*, and the strength of fluorescence signals for these two
 134 probes was strongly correlated (Pearson's *r* of 0.77). Such co-extensive labeling indicates
 135 that *zip11* is widely expressed in excitatory neurons both within and around HVC, and raises
 136 the possibility that inter-individual differences in the levels of ZIP11 expression within song
 137 premotor circuitry contribute to heritable differences in learned song.

138 To determine whether levels of *zip11* expression differ across birds, and whether any
 139 differences correlate with variation in song tempo, we collected mRNA samples from the
 140 brains of 17 Bengalese finches that sang at a broad range of tempos. We separately
 141 analyzed tissue from HVC and from 'non-song' regions surrounding HVC that contained none
 142 of the specialized brain nuclei that have been implicated in song production (Fig. 2C). For
 143 each sample, we measured the levels of *zip11* transcript by quantitative polymerase chain
 144 reaction. Across individuals, *zip11* transcript levels varied by more than two-fold both in HVC

145 and in non-song regions; moreover, across individuals, transcript levels in both regions were
146 positively correlated with song tempo, with ~10% change in tempo per fold change in *zip11*
147 (Fig. 2D-E, HVC: $r = 0.56$, slope = 0.73, $P < 0.02$, two tailed *t*-test; surround: $r = 0.65$, slope =
148 0.85, $P < 0.005$, two tailed *t*-test; $n = 17$ birds from 6 nests). These correlations for non-song
149 regions raise the possibility that ZIP11 may contribute to variation in behaviors other than
150 song, while the correlations within HVC indicate that heritable differences in levels of ZIP11
151 within song control circuitry could play a causal role in determining inter-individual differences
152 in song tempo.

153 We next investigated potential mechanisms whereby ZIP11 mediated zinc transport
154 could influence neural circuitry that underlies song tempo. A role for zinc regulation has not
155 figured prominently in models of neural circuit function. However, it has long been appreciated
156 that soluble zinc is present at high levels in the brain, including avian song control regions,
157 and is co-released with glutamate at many central synapses (49–51). Moreover, zinc exerts
158 widespread modulatory effects on both voltage and ligand-gated ion channels, and several
159 recent studies indicate that zinc manipulations can alter synaptic and circuit function (52–57).
160 To investigate how ZIP11 might influence neural activity in HVC, we assessed the subcellular
161 localization of ZIP11 protein using a commercially available antibody raised against a portion
162 of mouse ZIP11 that is 99% homologous to the same region of Bengalese finch ZIP11 (see
163 Methods). Immunofluorescent labeling revealed that ZIP11 protein is enriched in discrete
164 puncta that almost completely co-localize with an antibody directed to VGLUT2, which
165 specifically marks glutamatergic synapses (Fig. 3B, MERGE, Pearson's r of 0.72 between
166 ZIP11 and VGLUT2 antibody staining; 68% of ZIP11 puncta were positive for VGLUT2 protein
167 and 70% of VGLUT2 puncta were positive for ZIP11 protein). This distribution of the ZIP11
168 transporter suggests that it could modulate activity within the HVC microcircuit by shaping the
169 level and distribution of soluble zinc at excitatory synapses.

170 We explored the potential role of zinc in modulating synaptic transmission within HVC
171 by assessing the effects of zinc chelation on evoked currents in a brain slice preparation. We
172 elicited antidromic action potentials in premotor HVC neurons that project to RA (HVC-RA
173 neurons) by stimulating the fiber tract leading to RA, outside of the boundaries of HVC (Fig.

3C). The axons of HVC-RA neurons make extensive local synapses within HVC (58–60). Hence, by antidromically activating HVC-RA neurons and measuring excitatory post-synaptic currents (EPSCs) in other downstream HVC neurons, we could assay the effects of zinc manipulation on circuit components that contribute to the propagation of neural activity within HVC and the control of song timing. We used intracellular recordings to first identify an individual HVC neuron in which EPSCs could be driven by antidromic stimulation, and then measured changes to EPSCs following bath application of a zinc chelator [TPEN [N,N,N,N-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine] or ZX1 (see Methods)]. Consistent with reports in other systems (53, 56), we found that zinc chelation in HVC increased post-synaptic drive. In some cases, there was a simple scaling in the magnitude of the EPSC elicited by antidromic stimulation (Figs. 3D and S3A). In other cases, there was also an increase in polysynaptic events after stimulation (Fig. S3B). Correspondingly, we found that zinc chelation caused an increase in both the peak amplitude of EPSCs (Fig. 3E, $18 \pm 5\%$ increase after chelation, $P < 0.005$, paired t-test, $n = 12$ experiments, $n = 6$ birds) and the total charge transfer (Fig. S3C, $26.5 \pm 10\%$ increase after chelation, $P < 0.01$, paired t-test, $n = 12$ experiments, $n = 6$ birds). These electrophysiological measurements indicate that reducing soluble zinc increases the amplitude of EPSCs within HVC and the overall excitability of the HVC microcircuit. Such marked *in vitro* effects of zinc manipulation on the excitability within the HVC circuit suggest that regulation of Zn^{2+} levels, including through ZIP11, has the potential to influence the speed of activity propagation through HVC, and prompted us to next evaluate if perturbing this system *in vivo* in adult birds would directly alter the tempo of learned song.

196 **Levels of soluble zinc and Zip11 are causally linked to song tempo**

197 We tested whether zinc levels can influence the tempo of adult song by measuring
198 changes to song following systemic injections of the zinc chelator, clioquinol (CQ), an
199 established, non-toxic means of reducing levels of soluble zinc in behaving animals (61). CQ
200 injection markedly increased song tempo across all experiments (Fig. 4A-D, top, $6.2 \pm 1.7\%$
201 average increase in median tempo for $n = 6$ birds, range 0.3 – 0.9 syl/s, $P < 0.02$ for each
202 bird, Mood's median test). In contrast, tempo was unaffected following injection of vehicle

203 alone (Fig. 4A-D, bottom; $0.59 \pm 0.25\%$ average change in median tempo for $n = 6$ birds, $P >$
 204 0.1 for each bird, Mood's median test). Notably, neither CQ (Fig. 4A, 'Experimental') nor
 205 vehicle (Fig. 4A, 'Control') caused apparent changes to the number or spectral structure of
 206 song syllables (Fig. 4B, compare 'Pre' to 'Post').

207 To more directly link ZIP11 levels to song tempo, we evaluated the effect of reducing
 208 ZIP11 selectively in HVC by using small interfering RNAs (siRNAs, (62)). We designed
 209 several siRNAs to target *zip11* transcript and screened these in Bengalese finch primary
 210 neural cultures to identify candidates causing ~50% reduction of endogenous *zip11* transcript
 211 (Fig. S2). We then injected anti-*zip11* siRNA complexed with a transfection agent bilaterally
 212 into HVC of adult birds and measured the impact on learned song tempo (Fig. 4E-H,
 213 Experimental). For each individual injected with anti-*zip11* siRNA, a co-reared brother was
 214 injected with a control siRNA that targeted no known Bengalese finch transcript (Fig. 4E-H,
 215 Control). Neither injection caused immediate changes to song structure. However, 48 hours
 216 after the injection, the songs of all birds in the 'Experimental' group were significantly slowed
 217 relative to their pre-injection values (Fig. 4F-H, top, $-7.0 \pm 1.8\%$ average change in median
 218 tempo, $n = 4$ birds; $P < 0.02$ for each bird, Mood's median test). In contrast, the control group
 219 displayed no changes to song tempo (Fig. 4F-H, bottom, $0.90 \pm 0.5\%$ average change in
 220 median tempo), $n = 4$ brothers; $P > 0.1$ for each bird). The consistent effects of reducing *zip11*
 221 transcript on song tempo demonstrate that differences in ZIP11 levels within HVC can
 222 account causally for inter-individual differences in this complex learned phenotype.

223 Discussion:

224 It is widely appreciated that behavior is shaped by an interplay of genetics and
 225 experience (8). However, the mechanisms whereby heritable factors contribute to inter-
 226 individual differences in learned behaviors are still poorly understood. Our findings
 227 demonstrate the efficacy of an unbiased behavioral-genetic approach in proceeding from a
 228 phenotype of interest to underlying mechanisms that contribute to inter-individual variation. In
 229 the songbird, this approach enabled the identification of three genomic regions that each

230 account for ~10% variation in the tempo of learned song, and revealed a novel zinc-
231 dependent mechanism that modulates circuit function and behavior. These findings begin to
232 bridge the gap between the genetic, circuit, and behavioral levels of inter-individual variation
233 in learned behaviors and raise the possibility that one way by which evolution has achieved a
234 balance between individuality and robustness is by targeting modulatory processes rather
235 than the core machinery of circuit function.

236 Our unbiased behavioral-genetic approach to identifying specific sources of inter-
237 individual variation in song complements studies that seek a general understanding of circuit
238 function through neural recordings and circuit perturbations. For birdsong, such prior work has
239 provided a detailed account of song control circuitry and linked the rate of neural activity
240 propagation in HVC to the precise timing of song (12, 30–32, 34–38). However, despite this
241 understanding, even extensive measurements of how song circuitry differs across individuals
242 could, at best, identify only correlative evidence regarding what causes some birds to sing at
243 over twice the tempo of others (14, 28, 29, 63). Here, a behavioral-genetic approach enabled
244 us to proceed in a directed fashion from inter-individual variation in tempo towards underlying
245 causal mechanisms mediated by differences in ZIP11 levels at glutamatergic synapses in
246 HVC. While we focused in this study on *zip11*, we also identified two other regions of the
247 genome where inter-individual differences had large effects on tempo (~10%). The ability to
248 detect genomic regions that have such large effects on behavior likely derives from the
249 structure of our study, which examines how the transmission of alleles within each family
250 contributes to phenotypic variation; this approach is similar to hybridization studies or rare
251 variant studies in human pedigrees, in which a limited number of genetic variants within a
252 population may contribute to large inter-individual differences in phenotypes. In this respect,
253 we expect that examination of different Bengalese finch pedigrees, or genome-wide
254 associational studies across larger populations, would reveal additional genomic regions
255 linked to tempo. More broadly, our results support the potential of such approaches in
256 systems such as the songbird—where behavior is subserved by well-delineated neural
257 circuitry—in identifying and pinpointing novel mechanisms that drive inter-individual variation in
258 complex phenotypes.

259

260 Although zinc modulation does not play a prominent role in most contemporary models
261 of circuit dynamics, our findings support the idea that active regulation of zinc levels may
262 constitute a general mechanism for modulating behaviorally relevant neural activity. Indeed,
263 not only is zinc co-released with glutamate at many central synapses, but work in mammalian
264 systems indicates that artificial manipulation of soluble zinc by chelation can alter synaptic
265 and circuit function both *in vitro* and *in vivo* (49, 53, 55, 56, 64). While zinc interacts with a
266 multitude of biological processes that are potentially relevant to circuit function (48, 51, 52), it
267 is noteworthy in the context of our results that synaptic zinc, co-released with glutamate, can
268 act directly on glutamate receptors to attenuate excitatory post-synaptic currents (53, 56).
269 Hence, zinc chelation may increase synaptic currents in HVC and speed song by reducing
270 levels of synaptic zinc, while higher levels of ZIP11—which is a member of a class of
271 transporters that import zinc from extracellular space to the cytoplasm (45, 47, 65)—may
272 similarly contribute to faster songs by reducing levels of soluble zinc at the synapse.
273 Moreover, our finding that *zip11* levels are correlated between song and non-song regions
274 raises the possibility that heritable differences in ZIP11 may influence the excitability and
275 temporal dynamics of neural circuits underlying a variety of other behaviors in a coordinated
276 fashion. Indeed, given the widespread expression of ZIP transporters, including ZIP11, in the
277 nervous systems of species ranging from nematodes to humans, our results suggest that
278 similar zinc-dependent processes may be an evolutionarily conserved mechanism for
279 modulating multiple aspects of neural circuit function and behavior (47, 66).

280

281 The mechanisms that we have identified in adult birds may also play a role in the
282 earliest stages of song learning, as HVC is implicated not only in song production but also in
283 the sensory learning of the tutor song in juvenile birds (67, 68). It has long been hypothesized
284 that such learning is constrained by an innate “template” that establishes a bias for birds to
285 learn preferentially from songs of their own species (21, 25, 27, 69–72), and our prior work
286 has extended this idea by showing that individual birds within a species learn most effectively
287 when they are presented with tutor songs at tempos that match their individual innate
288 predispositions (28). However, while the concept of templates that constrain which stimuli are

effective in guiding learning has been highly influential, how such biases are instantiated at a circuit level remains unclear. Our results suggest that such biases could result in part from a concordance between the structure of instructive sensory stimuli and the biophysically determined properties of the circuits upon which they act(73). For example, if ZIP11 contributes to differences in the temporal dynamics of HVC circuitry(16, 36, 38, 74) even prior to tutoring, then tutor songs at the appropriately matched tempo may more effectively drive the cellular and circuit changes that instantiate sensory learning. More broadly, this suggests that innate differences in circuit biophysics may lead to a distribution, within the population, of sensitivities to specific statistics of the natural world that enable some individuals to learn better from, and perhaps seek, experiences that match their predispositions (22, 23, 26, 28, 71, 75, 76).

The correlation between *zip11* expression and song tempo even in non-song brain regions indicates that ZIP11 may contribute to variation in diverse other behaviors subserved by those regions. Most simply, this might include variation in the temporal dynamics of other movements; however, it likewise could include variation along any axis of behavior that is influenced by changes to the synaptic strength or excitability within underlying neural circuitry. This is especially interesting from the perspective that song is a courtship behavior that female birds use in part to select their mates (17, 20). The joint influences of experience and heritability on tempo render it an informative signal about the relatedness of the singer (77, 78), while the possibility that other behavioral traits covary with tempo suggests that it might additionally provide females with signals about inter-individual differences that are relevant to assessment of a male's fitness and compatibility. These considerations raise intriguing questions for future work regarding what constellation of behavioral traits covary with heritable differences in song structure and whether females are attentive to the aspects of song structure that encode such behavioral variation.

Finally, we note that the relative obscurity, from the standpoint of contemporary circuit models, of ZIP-dependent changes in circuit dynamics may reflect a more general principle regarding the generation of natural variation in behavior. In particular, while inter-individual

319 variation in behavior is an evolutionary imperative, it cannot arise at the expense of
320 fundamental circuit functionality. Thus, while circuit perturbations or mutagenesis that grossly
321 disrupt behaviors can reveal critical components of neural machinery, our findings suggest
322 that investigations of naturally occurring variation may preferentially uncover more subtle
323 mechanisms—such as the modulatory processes described here—that enable inter-individual
324 differences within a population while leaving intact the indispensable parts of brain function.

WITHDRAWN
see manuscript DOI for details

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Supplementary Materials

Materials and Methods
Figs. S1 to S4
Tables S1 to S4
Data S1

551

552 **Fig. 1. Genetic mapping identifies genomic regions influencing song tempo.**

553 (A) Example songs from a father (top) and three of his sons (bottom). The sons
554 produced copies of their father's song that were generally similar in multiple features,
555 including the repertoire of discrete syllables, their ordering, and the tempo at which they were
556 produced, but there was also variation in these features across individuals. (B) Relationship
557 between the tempo of the father's song and the tempo of songs produced by his offspring ($n =$
558 572 offspring from 54 fathers/nests, $r = 0.39$, slope = 0.43, $P < 0.0001$). The significant
559 correlation across families reflects the potential influence of both the father's tutor song, and
560 his transmitted genes. The variation within families (vertically aligned points), despite the
561 same tutor, could reflect individual differences in transmitted genes that affect tempo. (C)
562 Probability distribution of tempo for two families, one with a single mode (orange) and a
563 second with multiple modes (blue). More than one mode was present in ~40% of families and
564 is consistent with segregating genes of large effect (right, number of modes determined by
565 the minimum Bayesian information criterion from Gaussian mixture model fits). (D) Genome
566 wide scan for linkage between molecular-genetic markers and song tempo. Left Y axis
567 indicates the p-value of the TDT test statistic calculated for each sliding window of 20
568 markers. Right Y axis indicates permutation based genome-wide false positive rate.
569 Expanded views of significant peaks are shown below ($n = 509$ birds; colored lines indicate
570 permutation based genome-wide significance thresholds of $P = 0.05$ (orange), $P = 0.02$
571 (green), and $P = 0.01$ (blue)). (E) Difference in mean tempo between allelic states for single
572 markers (SNPs) on the right end of Chromosome 18 plotted against their genomic position.
573 Significance is indicated by marker color. Gene models derived from genome annotation are
574 indicated at top. Arrows indicate coding direction. Introns are shown in green, exons are
575 shown in purple. Black arrow indicates marker shown in F. (F) Distribution of song tempos for
576 birds of each of three allelic states (C/C, C/T, T/T) found at nucleotide 11605768 on
577 Chromosome 18. Birds with T/T sang significantly faster than birds with C/C (two tailed t -test;
578 ** $P < 0.02$; *** $P < 0.0001$) consistent with linkage between allelic state at this location and
579 tempo. Means indicated by cyan bars. Box shows median and inner quartiles and whiskers
580 show 5th and 95th percentiles.

Fig. 2. *zip11* mRNA transcript levels in HVC correlate with song tempo.

A) Sagittal section illustrating the brain area imaged for *zip11* transcript (red). (B) Dual *In situ* mRNA hybridization to *zip11* (*SLC39A11*) transcript (left) and *vglut2* (*SLC17A6*) transcript (middle). The merge (right) reveals co-localization indicating that *zip11* is expressed in excitatory neurons in the avian forebrain. Dashed line indicates the boundary of HVC. (C) Schematic illustrating tissue separation used to assess transcript levels. We dissected coronal sections of forebrain tissue into samples containing the song premotor nucleus HVC (blue) and surrounding non-song brain tissue anterior to the song premotor nucleus RA (black, HVC-surround). *zip11* transcript levels for each region were determined by quantitative polymerase chain reaction. (D, E) Inter-individual differences in *zip11* transcript levels in HVC (D) and HVC-surround (E) were positively correlated with the song tempo of individual birds (n = 17 birds from 6 nests; nests indicated by color). Fold expression changes are relative to the lowest expression level in the set. Parameters were fit by ordinary least squares.

Fig. 3. ZIP11 protein and soluble zinc are in a position to modulate song circuitry.

(A) Sagittal section illustrating the brain area imaged for ZIP11 protein (red). (B) Antibody staining for ZIP11 protein in HVC. Anti-ZIP11 antibody staining is shown in green (left) and anti-VGLUT2 antibody staining (a marker for excitatory synapses) is shown in red (middle). The merged image (right) reveals co-localization between ZIP11 puncta and VGLUT2 puncta, indicating that ZIP11 is present at excitatory synapses. (C-E) Chelation of soluble zinc increased the strength of synaptic currents in HVC. (C) Schematic of *in vitro* slice preparation. HVC-RA neurons were stimulated via the efferent tract to RA, and elicited excitatory post-synaptic currents (EPSCs) were recorded in downstream neurons within HVC. Chelation of zinc increased the amplitude of EPSCs as illustrated for an individual experiment (D) and summarized across experiments (E, blue indicates ZX1, black indicates TPEN, $18 \pm 5\%$ increase in peak amplitude, $P < 0.005$, paired *t*-test, $n = 12$ experiments in 6 birds).

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627

629 **Fig. 4. Manipulations of zinc and *zip11* levels *in vivo* alter song tempo.**

630 (A) Schematic of *in vivo* zinc chelation. In the experimental condition (top), songs were
631 analyzed from blocks 'pre' (7-11am) and 'post' (12-8pm) injection of a solvent carrying the
632 membrane-permeant zinc chelator Clioquinol (CQ). In the control condition (bottom), the
633 same animals were injected with solvent alone. (B) Example songs produced by a bird before
634 and after chelation (top) or control (bottom) manipulation, illustrating that the gross structure
635 of song remained unchanged. (C) The median tempo for this bird increased from 8.08 syl/s
636 'pre' (blue) to 8.61 syl/s 'post' (orange) zinc chelation. Arrows indicate medians. (D) Median
637 song tempo for each bird pre and post experimental (top) and control (bottom) manipulations.
638 For all birds, the chelator caused a significant increase in song tempo ($n = 6$; * $P < 0.02$; ** P
639 < 0.002 , corrected Mood's median test). In contrast, vehicle injection did not affect tempo ($n =$
640 6 , n.s. denotes $P > 0.1$, Mood's median test). Colors of paired data correspond to individual
641 birds. (E) Schematic of experimental design for ZIP11 knockdown. For the experimental
642 group (top), all songs from day 1 were analyzed as the 'pre' condition. On day 2, HVC was
643 injected bilaterally with anti-*zip11* siRNA. All songs recorded during the first full day of singing
644 following injection (day 4 in all cases) were analyzed as the 'post' condition. For the control
645 group (bottom) treatment was identical except that the siRNA targeted no known Bengalese
646 finch mRNA. (F) Examples of songs produced by two brothers pre and post siRNA injection.
647 For both birds, there was little change to song spectral content. (G) Distribution of song
648 tempos for the same pair of birds before (blue) and after (orange) manipulation. Tempo was
649 slower following injection of anti-*zip11* siRNA (top) but unchanged following injection of
650 control siRNA (bottom). Arrows indicate medians. (H) Median song tempos pre and post
651 injection for 4 pairs of brothers. For each pair, one was injected with anti-*zip11* siRNA (top, P
652 < 0.002) while the other was injected with control siRNA (bottom). All animals injected with
653 anti-*zip11* siRNA sang significantly slower following siRNA injection (* $P < 0.02$, ** $P < 0.002$,
654 Mood's median test). Tempo was unchanged by control siRNA (bottom; n.s. denotes $P > 0.1$,
655 Mood's median test). Dashed lines indicate data from the example birds.

656 **Methods**

657

658 **Subjects**

659 Subjects were male and female Bengalese finches (*Lonchura striata domestica*) bred
 660 in our colony. They were reared and tutored by their genetic parents to the age of
 661 independence. Other than efforts to maintain separation between lineages in order to
 662 minimize inbreeding, mating pairs were randomly selected male and female birds. The initial
 663 phenotypic analysis (Fig. 1A-1C) used data from 626 male birds and the subsequent linkage
 664 analysis used data from 509 birds from families for which we were able to collect DNA
 665 markers for male offspring and one or both parents (see below). The Institutional Animal Care
 666 and Use Committee at the University of California, San Francisco, approved all protocols.

667 **Audio recording**

668 Vocalizations were digitized at 32 kHz from singly housed birds in sound isolation
 669 chambers (Acoustic Systems). Microphones were in a fixed position at the top of the cage
 670 housing the bird. Prior to analysis, songs were high pass filtered to capture sound above
 671 ~500 Hz. Filters were digitally implemented elliptical infinite impulse response with a
 672 passband edge frequency of 0.04 radians. All birds were recorded during early adulthood (90-
 673 120 days post-hatch).

674 **Calculation of song tempo**

675 Song tempo was quantified as the average number of syllables produced per second
 676 of song, a measure previously shown to be heritable (29). Within song, syllables were defined
 677 as discrete units of sound separated by silence. Amplitude traces were created by rectifying
 678 the filtered song waveform and convolving with a 2 ms square wave. Heuristic thresholds

were set automatically as three times the 10th percentile of values in the amplitude trace. We then identified 'objects' as uninterrupted sounds louder than the threshold and longer than 10ms. We merged any objects separated by silence that was less than 5ms. These final merged objects we defined as 'syllables.' This approach reliably identified syllable onsets and offsets corresponding with those identified by human evaluation. A series of syllables that had no gaps longer than 250ms was considered a song. For each song, tempo was then quantified as the number of syllables present in a given song divided by the duration of that song in ms. For each bird, all summary statistics were derived from at least 60 song renditions.

Chromosome level genome assembly

To generate contiguous DNA sequences that span the length of chromosomes, we combined data from an earlier draft genome assembly of the Bengalese finch genome (42) with newly collected data designed to capture local chromosomal architecture. Specifically, we used sequence data from Hi-C (79) libraries created by Dovetail Genomics using blood samples we provided. These libraries were sequenced to ~250x coverage using the Illumina HiSeq 4000 platform. This sequence data was then combined with the previous assembly using the Dovetail HiRise assembler. This resulted in a genome assembly with a total length of ~1.06Gb and a total of 2014 scaffolds (RefSeq assembly accession number GCF_005870125.1). The largest 31 scaffolds correspond to Chromosomes 1-15, 17-29 and Z in the Chicken reference genome and additionally to Chromosomes 1a and 4a in the zebra finch reference genome, that were previously created using Sanger sequence data. Together they account for ~97% of the total assembled DNA (National Center for Biotechnology Information BioProject PRJNA369279).

Genetic marker typing

We collected molecular-genetic marker data from each of 509 Bengalese finches used for linkage analysis. These markers were a modified form of Double Digest Restriction Associated DNA (ddRAD) (80) markers we call Single End RAD markers (seRAD). From each bird, purified genomic DNA was digested with the non-palindromic restriction enzyme BssS1 (NEB) and the 4 base restriction enzyme Mse1 (NEB) under standard double digest conditions. Digested DNA was purified using the AmpureXP system, ligated (T4 DNA ligase, NEB) to appropriate double stranded linker oligonucleotides (Table S1), then amplified in a 10 cycle PCR reaction using primers that contain an 8bp Illumina i7 compatible barcode and are specific for ligated products that reform the BssS1 and TA sites (Table S1 and S2). Amplified libraries were pooled according to DNA concentration, and size selected (200-500bp) using the BluePippin (Sage Science) system. For each locus digested with the BssS1 enzyme, one of the two ends was sequenced using a custom primer that was specific to one end of the BssS1 cut site and required correct ligation of that site and the appropriate linker (Table S1). For sequencing, 48 samples were multiplexed using the Illumina barcoding system (Table S2). All sequencing was single end 50 or 65 base pairs and performed on the Illumina HiSeq4000 platform. Sequences passing Illumina quality thresholds were then aligned to the Bengalese finch genome (RefSeq GCF_005870125.1) using the MEM algorithm (81) from the Burrows-Wheeler Aligner software package (82). Across the population of birds, there were ~400,000 distinct genomic loci associated with restriction enzyme cleavage, for each of which 50-65 base pairs were sequenced. Of these, 50,999 loci were identified as potentially informative because they both contained single nucleotide polymorphisms that could serve as markers for transmission of alleles from parents to offspring, and they were sequenced with high coverage in at least 200 individuals. For each individual, the diploid allelic state at each of these informative loci was predicted using the Bayesian framework described in McKenna et al. (83). These estimates of diploid allelic state were then used for all subsequent analyses. Across the 509 birds used for genetic mapping, each bird was unambiguously assigned a diploid allelic state at an average of $39,996 \pm 221$ and a median of 40,827 loci. This corresponds to a marker for allelic state approximately every 25 kilobases across the genome. Each locus was unambiguously assigned a diploid allelic state in an average of 399 ± 0.63 and a median of 473 birds.

Genetic mapping

To establish linkage between regions of the genome and song tempo we used an extension of the Transmission Disequilibrium Test (TDT) appropriate for quantitative phenotypes and able to utilize multiple individuals for each family (44). The TDT is a test for genetic linkage and association in outbred populations with known familial structure and uncontrolled breeding (43). The TDT statistic at any given locus is calculated only from families with segregation of allelic state at that genomic location and is thus robust to population stratification and missing marker data. In our analysis, we only included genomic locations that, across the entire population, had two (e.g. C/T and C/C) or three (e.g. C/T, C/C, T/T) allelic states comprised of two nucleotides. Where there were three allelic states, the two homozygous states (e.g. C/C and T/T) were used to calculate the TDT as these are the two states where the identities of the parents transmitting the alleles is unambiguous. TDT scores at individual polymorphic locations can be combined to assess the linkage between a larger genomic region and a phenotype (84). As each single polymorphic location uses only a subset of individuals from the population, we increased the number of individuals contributing to linkage between tempo and a larger genomic region by combining TDT statistics in sliding windows of 20 markers. The TDT is chi-square distributed and p-values for each 20 marker block were determined from a chi-square distribution with 20 degrees of freedom. This analysis was conducted genome-wide (across 50,999 single loci) on a multi-generational population of 509 (481 males and 28 females) birds where there were 105 unique parental pairs and a median of 4.88 and a maximum of 22 male offspring. Genetic data from all male offspring and (when available) their male and female parents comprised this set.

As both family overlap and genetic linkage between nearby markers may create non-independence among our linkage scores, a genome-wide significance threshold for this analysis was established by permutation test. For each of 10,000 permutations, phenotypes were shuffled relative to genotypes and the entire analysis was re-run. The maximum $-\log_{10} P$ value from each analysis was retained. This set of scores then established a distribution from

760 which significance thresholds were derived. We note that the significance values determined
761 by such permutation testing are conservative relative to those derived by a simple
762 combination of TDT statistic values.

763 **Fluorescent *in situ* hybridization**

764 Fluorescent *in situ* hybridization (FISH) was performed using the hybridization chain
765 reaction system from Molecular Instruments. Birds were euthanized with isoflurane,
766 decapitated, and debrained. Brains were flash-frozen in -70°C dry ice-chilled isopentane for
767 12 seconds within 4 minutes of decapitation, then stored at -80°C. Fresh-frozen brains were
768 cryosectioned at 16 µm onto SuperFrost Plus slides (Fisher), chilled in the cryo-chamber,
769 then melted onto the slide using a warmed metal dowel. Slides were transferred to -80°C for
770 storage. For FISH, slides were transferred from -80°C to slide mailers containing cold 4%
771 formaldehyde and incubated for 15 minutes on ice. After fixation, slides were washed three
772 times for 5 minutes using DEPC-treated PBS + 0.1% TWEEN 20 (PBST), dehydrated in 50%,
773 70%, and two rounds of 100% ethanol for 3-5 minutes each round, then dried in air. Slides
774 were then acetylated for 10 minutes (1.3% triethanolamine, 0.021 N HCl, 0.25% acetic
775 anhydride) and washed in DEPC-PBST for 10 min. Slides were dehydrated again and
776 transferred to a SlideMoat (Boekel Scientific) at 37°C. 100 µL of v3 Hybridization Buffer
777 (Molecular Instruments) was added to each slide, which was then coverslipped and incubated
778 for 10 minutes at 37°C. Meanwhile, 2 nM of each mRNA transcript specific probe was added
779 to 100 µL Hybridization Buffer and denatured at 37°C. Pre-hybridization buffer was removed,
780 100 µL of probe/buffer was added, slides were coverslipped and incubated overnight at 37°C.
781 The next day, coverslips were floated off in Probe Wash Buffer (PWB, 50% formamide, 5x
782 SSC, 9 mM citric acid pH 6.0, 0.1% TWEEN 20, 50 µg/ml heparin), then washed in 75%
783 PWB/25% SSCT (5x SSC, 0.1% TWEEN 20), 50% PWB/50% SSCT, 25% PWB/75% SSCT,
784 100% SSCT for 15 minutes each at 37°C. This was followed by 5 minutes at room
785 temperature in SSCT. Slides were incubated in 200 µL of Amplification Buffer (provided by
786 company) for 30 minutes at room temperature. Alexa fluor-conjugated DNA hairpins were

denatured for 90 seconds at 95°C then allowed to cool for at least 30 minutes in the dark at room temperature. Hairpins were added to 100 ul amplification buffer, applied to slides, and incubated overnight at room temperature. The following day, slides were washed in SSCT containing 1 ng/mL DAPI for 30 minutes at room temperature, then SSCT for 30 minutes at room temperature, followed by a final 5 minutes in SSCT at room temperature. Prolong Glass Antifade Medium (Thermofisher) was added to each slide, which was then coverslipped. Images were acquired using confocal microscopy. The degree of co-localization between objects was evaluated using Mander's co-localization coefficients (85).

Immunofluorescent staining

To prepare tissue for staining, birds were deeply anesthetized with isoflurane before being transcidentally perfused with 0.9% saline, followed by 3.7% formaldehyde in 0.025 M phosphate buffer. Brains were postfixed for 4–24 h in 3.7% formaldehyde, then cryoprotected in 30% sucrose. Forty micrometer thick sagittal sections were cut on a freezing microtome and stored in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄) at 4°C. For immunofluorescence, sections were washed 3 times for 10 min each in PBS at room temperature. Sections were then incubated on a nutator, in clearing reagent, for one hour at room temperature. Clearing agent was either PBS+0.3%triton x-100 (synaptic staining) or PBS+0.1% TWEEN 20 (*SLC39A11* staining alone). Sections were washed 3 times in PBS for 10 min each at room temperature and then transferred to PBS+2% goat serum and incubated for 2-4 hours at room temperature. Sections were transferred to fresh PBS+2% goat serum, and primary antibodies were added at a concentration of 1:500. Sections were incubated in primary antibody overnight at 4°C. The next morning, sections were washed 3 times for 10 min each in PBS at room temperature. Secondary antibodies were added at a concentration of 1:2000, and sections were incubated for 4 hours at room temperature. Following incubation, samples were washed 3 times for 10 min each in PBS at room temperature and transferred to SuperFrost Plus slides (Fisher). Excess fluid was removed, and 40l Vectashield Plus with DAPI (Vectashield) mounting media was added

before coverslips (Fisherbrand, 22mm wide, 0.13-0.17mm thick), which were sealed with clear nail polish. Slides were stored at 4°C. The polyclonal anti-ZIP11 antibody was raised in Rabbits to a fusion protein containing amino acids 93-193 of the Human ZIP11 protein (MyBioSource, MSB1497597). The monoclonal anti-VGLUT2 antibody was raised in Mice immunized to a full length recombinant protein corresponding to rat VGLUT2 (Abcam, ab79157) and has been previously shown to stain VGLUT2 in zebra finches (86). The staining patterns of these antibodies were visualized using species appropriate secondary antibodies covalent linked to Alexa fluor 488 or Alexa fluor 633. Images were acquired using confocal microscopy. The degree of co-localization was evaluated using Mander's co-localization coefficients (85).

Electrophysiology

We prepared brain slices from Bengalese finches (60-90 d post-hatch) raised in our breeding colony. Birds were anesthetized with isoflurane and decapitated. Brains were rapidly removed and placed in an ice-cold cutting solution consisting of (in mM): 125 $C_5H_{14}ClNO$, 3 $MgSO_4$, 0.5 $CaCl_2$, 2.5 KCl, 25 $NaHCO_3$, and 35 glucose. Sections (260-300 μm) were cut on a Leica VT1000 microtome. In preparation for electrophysiology, slices were incubated at 36°C in recording ACSF with high magnesium to reduce polysynaptic activity (consisting of (in mM): 125 NaCl, 3 $MgSO_4$, 1 $CaCl_2$, 2.5 KCl, 25 $NaHCO_3$, and 35 Glucose) for 30-60 minutes. Slices were then maintained at room temperature in well-oxygenated (95% O_2 -5% CO_2) holding chambers until transfer to the electrophysiological recording chamber. All recordings were made in oxygenated ACSF solution at room temperature (20-25°C). Glass electrodes (3-8 M Ω) were filled with intracellular solution containing (in mM) 120 KGluc, 0.1 EGTA, 40 HEPES, 5 KCl, 0.3 $MgCl_2$. Targeted recordings were made under DIC-IR visualization using an Olympus BX-51 microscope and a camera (FLIR Chameleon). HVC was identified by its visible borders under transillumination. Antidromic stimulation of the HVC neurons projecting to RA was performed with bipolar stimulating electrodes placed in the efferent tract leading to RA (100-150 μm separation, FHC, Bowdoin). Recordings were made with a Multiclamp

700B (Molecular Devices), controlled by custom acquisition software written in Matlab. Pipette capacitance and series resistance were compensated online and series resistance was monitored periodically. Recordings with monotonic changes in input resistance greater than 25% were discarded, as were recordings with monotonic changes in holding current greater than 60pA. Voltage clamp data was acquired at 10 kHz, and filtered at 3kHz. EPSCs were recorded at -70mV in the presence of the GABA-A receptor antagonist, Gabazine (SR-95531, 10-25 μ m), in the bath solution. N, N, N', N' -Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN, Sigma) was infused into the bath at 20-100 μ m. ZX1 was infused into the bath at 100 μ m. The peak amplitude of synaptic events was compared pre and post-chelation. One cell was excluded due to the appearance of an extra EPSC, induced by application of chelator, occurring before the original EPSC preventing an accurate estimate of changes in amplitude.

mRNA transcript preparation and quantification

Fresh coronal sections containing the brain region HVC were cut as described above for electrophysiology, and then transferred to microdissection dishes and kept in ice-cold cutting solution. HVC and the region surrounding HVC were separately microdissected and transferred to a 1.5ml microfuge tube and any remaining cutting solution removed. Immediately afterward, 90ul of TRIzol (ThermoFisher) was added. Samples were then stored at -80°C. When ready for analysis, samples were thawed, and tissue was macerated in the 1.5ml microfuge tube using RNase-free disposable pellet pestles (Fisherbrand). Samples were then incubated at room temperature for 5 min, 20ul chloroform was added, and samples were incubated for a further 2 min. Samples were then centrifuged at 12,000 g and 4°C for 15 min. The aqueous phase was transferred to a new tube, and the organic phase stored at -80°C. Following the addition of 10ug of RNase free glycogen, the transferred aqueous phase was incubated at room temperature for 1 min. 20ul of isopropanol was added, and the tubes were incubated at room temperature for 10 min. Samples were then centrifuged at 12,000 g and 4°C for 15 min, and the supernatant discarded. The RNA pellet was washed in 100ul

867 75% Ethanol and centrifuged at 7,500 g and 4°C for 5 min. The supernatant was removed,
868 and the RNA pellet was air-dried and stored at -80°C.

869 The ProtoScript II (NEB) reverse transcription system was used to create cDNA from
870 RNA samples. For each sample, the entire RNA pellet was solubilized in 6ul RNase free
871 water, and 2ul of the poly-T reverse transcription primer (d(T)23 VN at 50 µM) was added.
872 RNA/primer mixtures were then denatured at 65°C for 5 min and placed directly on ice. 10µl
873 ProtoScript II reaction mix and 2µl ProtoScript II enzyme mix were added to the RNA/primer
874 mixtures, and the entire reaction was incubated at 42°C for one hour. Samples were
875 incubated at 80°C for 5 minutes to heat inactivate the enzymes, and stored at -80°C.

876 For each gene, transcript levels were assessed by Quantitative PCR (qPCR).
877 Reactions were performed using the PowerUp SYBR Green Master Mix (ThermoFisher).
878 Each reaction was conducted in triplicate on a 96 well qPCR plate (ThermoFisher). For each
879 replicate reaction, 5µl of master-mix was combined with 1ml template and nuclease-free
880 water to a final volume of 10µl with 400nM final concentration of gene specific primers (Table
881 S3). Each reaction was mixed gently and stored on ice before conducting the qPCR reaction.
882 Reactions were conducted in a QuantStudio 6 Real-Time PCR machine (Applied
883 Biosystems). A standard two step PCR protocol was used. Samples were incubated at 50°C
884 for 2 minutes then heated to 95°C and incubated for 2 minutes. A cycle of two steps was
885 repeated 40 times, in which incubation at 95°C for 15 second was followed by incubation at
886 60°C for 1 minute. Data from the qPCR reactions were analyzed using the QuantStudio 6
887 software and cycle threshold (Ct) values were identified for each sample. For each sample,
888 relative Ct values for *SLC39A11* were determined by taking the difference between the
889 *SLC39A11* Ct and the Ct levels of the housekeeping gene PPIA thus controlling for variation
890 in total mRNA input.

891 **Primary neural culture and siRNA screening**

892 All siRNA molecules used for *in vivo* manipulations of gene expression were first
893 screened for efficacy *in vitro*; all sequences were tested for their ability to reduce the targeted
894 gene transcript in cultured Bengalese finch neuronal cells. Prior to cell preparation, tissue
895 culture treated flat bottom 96 well plates (Corning) were incubated in a thin layer of 1mg/ml
896 Laminin (Sigma) solubilized in HBSS with magnesium and calcium (Life Technologies, no
897 phenol red) for 2 hours at 37°C followed by 3 washes in HBSS alone. Chambers were
898 incubated with a thin layer of Poly-L-Ornithine (Life Technologies) at 37°C overnight and
899 washed 3 times with HBSS alone the following day. 1-14 day old birds were euthanized with
900 isofluorane and brains were removed in ice cold HBSS. Following removal of the dura, brain
901 cells were dissociated in pre-warmed papain (Worthington, 20U/ml in HBSS) at 37°C for
902 45min. After incubation, tissue was triturated with a 5ml Pasture pipette 15 times then
903 centrifuged at 1200rpm for 5 min. The supernatant was aspirated and the pellet was washed
904 with 7mg/ml Ovomucoid Trypsin inhibitor (Worthington) in HBSS and centrifuged again at
905 1200rpm for 5 min. Supernatant was aspirated and the pellet was resuspended in 5ml warm,
906 filtered Neurobasal A Complete media (NBAC; Neurobasal A, N2 supplement, B27
907 supplement, Glutamax, penicillin and streptomycin; Life Technologies). The solution was then
908 triturated 15 times with a fire polished 5ml Pasteur pipette and filtered through a 1000µm cell
909 strainer (Corning). The concentration of cells was measured using a hemocytometer (Fisher
910 Scientific) and cells were diluted to a concentration of 5×10^5 cells per ml in warm NBAC. 400µl
911 of cell suspension was placed in each well of the 96 well plate and incubated at 37°C for 5
912 hours at which point the culture media was removed and replaced with 500µl of fresh warm
913 NBAC. Cultures were then incubated at 37°C and 5% CO₂. 50% of media was exchanged every
914 3-4 days.

915 Once neural processes formed (after ~1 week) the cell cultures were used to test
916 siRNA molecules. For each siRNA tested, knockdown experiments were conducted in
917 duplicate using the RNAiMAX (Life Technologies) transformation reagent. For each siRNA
918 25µl of Opti-MEM Medium (Life Technologies) was mixed with 1.5µl of RNAiMAX reagent.
919 Separately, 25µl of Opti-MEM Medium was mixed with 5pmol paired siRNA molecules
920 (Dharmacon). These two mixtures were then combined and incubated at room temperature

921 for 5min. Following incubation, 10µl of the RNAiMAX/siRNA mixture was added to each of two
922 wells of neural cell culture. Cultures were then incubated at 37°C and 5% CO₂ for 2 days.
923 After incubation, RNA from each well was purified, converted to cDNA, and quantified using
924 the protocol described in mRNA transcript preparation and quantification (above). The mRNA
925 expression level of the 'target' gene was compared between cells exposed to experimental
926 siRNA molecules and cells exposed to a control siRNA molecule. The siRNA resulting in
927 maximal knockdown of *SLC39A11* transcript (Table S4 and Fig. S4) was then used for all *in*
928 *vivo* experiments.

929 **siRNA injections**

930 Prior to injection, siRNAs were complexed with the transfection reagent BrainFectIN
931 (Oz Biosciences). siRNA molecules were added to BrainFectIN at a ratio of 1 µg siRNA:1.5
932 µg BrainFectIN and incubated at room temperature for 20 min and then used within 20min.
933 Concurrently, birds were deprived of food and water for 1 hour, and then anesthetized with an
934 intramuscular injection of 30–40 µl of equithesin (0.85 g of chloral hydrate, 0.21g of
935 pentobarbital, 0.42g of MgSO₄, 2.2 ml of 100% ethanol, and 8.6 ml of propylene glycol to a
936 total volume of 20ml with water). Following craniotomy and removal of the dura, siRNA
937 complexes were injected into HVC bilaterally. The stereotactic coordinates used were 2.0ml
938 medial/lateral, 0 dorsal/ventral relative to the y-sinus, with the head oriented with a beak angle
939 of 50 degrees. siRNA complexes were introduced at multiple points between 700-250 µm
940 below the surface of the brain using a Nanoject-2 (Drummond Scientific), culminating in a
941 total injection volume of 10 µl per hemisphere. In each hemisphere, after the final injection,
942 the pipette was left in place for ~10 minutes before full retraction. Baseline, 'pre' manipulation
943 song tempo data was collected for a full day prior to injection, and 'post' manipulation data
944 was analyzed from the first full day of singing (the second day post-surgery in all cases). All
945 siRNAs were synthesized by Dharmacon. Control and experimental siRNA sequences are
946 presented in Supplemental Table 4.

947 **Clioquinol injections**

948 Clioquinol (CQ) was solubilized in DMSO at a concentration of 40mg/ml. For all birds
 949 and all experiments, 6 μ l of either DMSO or DMSO+CQ was injected intramuscularly at 11
 950 am. The tempo of songs produced before 11am were then compared to the tempo of songs
 951 produced after 12pm.

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Supplementary Figures

Fig. S1. Permutation based genome-wide null probability distribution for association between song tempo and allelic state.

Null probability distribution resulting from 10,000 permutations of the complete linkage analysis as shown in Figure 1D. Before each permutation, the phenotype and bird identity were randomly shuffled; thus, each analysis retained the genetic architecture of the overall population but dissociated this architecture from song tempo. The distribution comprises the TDT statistic for the single most significant sliding window (of 20 makers) from each permutation. Critical values for genome-wide significance were calculated as the $100 \times (1 - \alpha)$ percentile of this distribution where α is the false positive rate. Colored arrows indicate the most significant sliding window for each of three regions (see Figure 1) significantly linked to song tempo.

Fig. S2. Effect size associated with individual markers within genomic regions on Chromosomes 1 and Z.

Difference in mean phenotype associated with allelic state at polymorphisms within regions on Chromosome 1 (A) and Chromosome Z (B) that were significantly linked to song tempo. P values for linkage at each locus are indicated by the color of the corresponding point and the heat maps at right. Gene models within each region as determined by genome annotation (National Center For Biotechnology Information BioProject PRJNA369279) are indicated at top. Detail for gene models are adjusted due to differences in gene number and density. Coding (dark green) and intronic (light green) regions are indicated in A while the length of a gene model (both coding and non-coding) are indicated by dark green in B.

Fig. S3. Zinc chelation increases synaptic currents in HVC.

(A-B) Chelation of zinc increased the amplitude of synaptic events as illustrated for an individual experiment (A) and, in some cases, increased the number of synaptic events as shown in a different experiment (B). (C) Summary of increased synaptic charge transfer across experiments (blue indicates ZX1, black indicates TPEN, $26.5 \pm 10\%$ increase in total charge transfer, $P < 0.01$, paired t -test, $n = 12$ experiments in 6 birds).

Fig. S4. Fraction of *zip11* expression following siRNA mediated knockdown in neural culture.

Fraction of control expression level of *zip11* following knockdown with one of 3 siRNAs. Expression following knockdown is normalized to levels from neural culture exposed to a control siRNA (supplemental table 4) designed to target no known Bengalese finch transcript.