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Inter- and intra-specific differences in muscarinic acetylcholine receptor expression in the neural pathways for vocal learning in songbirds

Running title
Muscarinic AChRs in the song system

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33 **Abstract**

34 Acetylcholine receptors (AChRs) abound in the central nervous system of vertebrates.
35 Muscarinic AChRs (mAChRs), a functional subclass of AChRs, mediate neuronal
36 responses via intracellular signal transduction. They also play roles in sensorimotor co-
37 ordination and motor skill learning by enhancing cortical plasticity. Learned birdsong is
38 a complex motor skill acquired through sensorimotor coordination during a critical period.
39 However, the functions of AChRs in the neural circuits for vocal learning and production
40 remain largely unexplored. Here, we report the unique expression of mAChRs subunits
41 (chr2–5) in the song nuclei of zebra finches. The expression of excitatory subunits
42 (chr3 and chr5) was downregulated in the song nuclei compared with the surrounding
43 brain regions. In contrast, the expression of inhibitory mAChRs (chr2 and chr4) was
44 upregulated in the premotor song nucleus HVC relative to the surrounding nidopallium.
45 Chr4 showed developmentally different expression in HVC during the critical period.
46 Compared with chr4, individual differences in chr2 expression emerged in HVC early
47 in the critical period. These individual differences in chr2 expression persisted despite
48 testosterone administration or auditory deprivation, which altered the timing of song
49 stabilization. Instead, the variability in chr2 expression in HVC correlated with parental
50 genetics. In addition, chr2 expression in HVC exhibited species differences and
51 individual variability among songbird species. These results suggest that mAChRs play
52 an underappreciated role in the development of species and individual differences in song
53 patterns by modulating the excitability of HVC neurons, providing a potential insight into
54 the gating of auditory responses in HVC neurons.

55

56

57 **Key words**

58 acetylcholine, muscarinic receptors, vocal learning, sensorimotor learning, songbird,
59 individual variability, RRID:SCR_012988, RRID: SCR_014438, RRID: SCR_004870,
60 RRID: SCR_014199, RRID:SCR_006356, RRID: AB_2629439, RRID: AB_221544,
61 RRID: AB_10821150, RRID: SCR_002865, RRID: SCR_005780

62

63 **Introduction**

64 The cholinergic system in the forebrain plays pivotal roles in learning and memory
65 (Anagnostaras et al., 2003; Hasselmo, 2006; Matsui et al., 2004), motor skill acquisition
66 and sensorimotor coordination (Conner, Culberson, Packowski, Chiba, & Tuszynski,
67 2003; Ztaou et al., 2016), and selective attention (Noudoost & Moore, 2011; Sarter, Bruno,
68 & Turchi, 1999). These diverse functions are mediated by the nicotinic acetylcholine
69 receptors (nAChRs) and muscarinic AChRs (mAChRs). In mammals, mAChRs are
70 further classified into two subtypes: mostly excitatory if they stimulate phospholipase C
71 activity (via chrm 1, 3, and 5) or inhibitory if they inhibit adenylyl cyclase activity and
72 regulate K⁺ channels (via chrm 2 and 4). These receptor subtypes display a wide but
73 unique distribution in the central nervous system (CNS) (Caulfield, Robbins, Higashida,
74 & Brown, 1993). In particular, mAChRs mediate most metabotropic actions of
75 acetylcholine in the CNS (Caulfield & Birdsall, 1998; Eglen, 2006). However, the
76 contributions of AChRs to learned motor skills remain largely unexplored.

77 Birdsong is a complex vocal sequential pattern acquired during a critical/sensitive
78 period of vocal development in closed-ended learners or of seasonal vocal plasticity in
79 open-ended learners. It is characterized by the acquisition of syllable acoustics and
80 sequence under species-specific regulation. In songbirds, song learning occurs in two
81 stages: sensory and sensorimotor learning phase. During the sensory learning phase, a
82 juvenile male listens to and memorizes a tutor song model. The bird then tries to match
83 his own vocalization to that of the tutor during the sensorimotor learning phase (Doupe
84 & Kuhl, 1999; Marler, 1970). Thus, auditory input, both hearing a tutor model's song and
85 monitoring their own vocalizations, is crucial for vocal learning (Konishi, 1965). When
86 zebra finches are deafened early in development after hatching, audition-deprived birds

87 required substantially more time to crystallize their song patterns (Mori & Wada, 2015).
88 Conversely, exogenous testosterone (T) administration induces premature song
89 crystallization in juvenile zebra finches (Korsia & Bottjer, 1991; Sizemore & Perkel,
90 2011)

91 Vocal learning in songbirds is mediated by specialized neural circuits, collectively
92 called the song pathways. The song pathways comprise of two neural circuits whose
93 nuclei are interconnected: the anterior forebrain pathway important for song learning and
94 maintenance and the vocal motor pathway which is necessary for song production (Bottjer,
95 Miesner, & Arnold, 1984; Kao, Doupe, & Brainard, 2005; Nottebohm, Stokes, & Leonard,
96 1976; Scharff & Nottebohm, 1991). The anterior forebrain pathway forms a pallial–basal
97 ganglia–thalamic loop with three song nuclei: the striatal song nucleus Area X, the lateral
98 magnocellular nucleus of the anterior nidopallium (LMAN), and the medial nucleus of
99 the dorsolateral thalamus (DLM) (Luo, Ding, & Perkel, 2001). The vocal motor pathway
100 includes the premotor song nucleus HVC (proper name) and the robust nucleus of the
101 arcopallium (RA). HVC possesses two types of projection neurons: one to Area X
102 ($HVC_{(X)}$ neurons) and the other to RA ($HVC_{(RA)}$ neurons). RA is analogous to layer V
103 neurons in the human laryngeal motor cortex and projects to the tracheosyringeal part of
104 the hypoglossal nucleus (nXIIIts) that innervates syringeal muscles (Pfenning et al., 2014;
105 Vicario & Nottebohm, 1988; Wild, 1993).

106 HVC receives cholinergic projections from the ventral pallidum of the basal forebrain
107 that is homologous to the mammalian nucleus basalis of Meynert (Li & Sakaguchi, 1997;
108 Reiner et al., 2004). Stimulating the cholinergic basal forebrain suppresses auditory
109 responses to the bird's own song in HVC and RA neurons (Shea & Margoliash, 2003),
110 suggesting a cholinergic regulation of auditory gating in the song nuclei. In addition,

111 acetylcholine concentration is upregulated in the song nuclei HVC, LMAN, and RA of
112 zebra finches during the critical period of song learning (Sakaguchi & Saito, 1989).
113 Acetylcholinesterase, an enzyme that breaks down acetylcholine at postsynaptic sites, is
114 highly enriched in the song nuclei HVC, RA, and LMAN during this critical period
115 (Sadananda, 2004; Sakaguchi & Saito, 1989). Therefore, these studies have shown the
116 presence of ACh in the song nuclei and suggest the presence of receptors that mediate its
117 functions during song learning and production. Although *in situ* hybridization and DNA
118 microarray data have shown the expression of mAChRs in the song nuclei (Lovell,
119 Clayton, Replogle, & Mello, 2008; Lovell, Huizinga, Friedrich, Wirthlin, & Mello, 2018)
120 (ZEBRA, www.zebrafinchatlas.org, RRID: SCR_012988), the precise distribution of
121 mAChRs in the song system during the critical period of song learning remains unclear.
122 This may reveal the song nuclei-specific contribution of mAChRs to song learning and
123 production.

124 Here, we report the unique expression pattern and developmental changes in mAChRs
125 in the song nuclei of zebra finches. In addition, we show inter- and intra-specific
126 differences in *chr2* expression in the premotor song nucleus HVC of songbirds. Our
127 results suggest a potential contribution of mAChRs to the regulation of neuronal
128 excitability in HVC during song learning and production.

129

130 **Materials and methods**

131 **Animals**

132 To compare intra-specific differences and developmental patterns of mAChRs
133 expression, we sampled male zebra finches (ZF; *Taeniopygia guttata*) at the pre-subsong
134 (21-26 post-hatching day, phd, n = 8), subsong (30–45 phd, n = 12), plastic song (50–65
135 phd, n = 12), and crystalized song (> 120 phd, n = 12) stages. In addition, to understand
136 inter-specific differences in *chr2* expression, we sampled adults of other songbird
137 species (n = 8 each), *i.e.*, owl finch (OF; *T. bichenovii*), star finch (SF; *Neochmia*
138 *ruficauda*), Bengalese finch (BF; *Lonchura striata* var. *domestica*), Java sparrow (JS;
139 *Padda oryzivora*), and canary (CN; *Serinus canaria*) (>120 phd). Zebra and Bengalese
140 finches were obtained from our breeding colonies at Hokkaido University. Other species
141 were purchased from local breeders in Japan. The photoperiod was maintained at 13/11
142 h light/dark cycles, and food and water were provided *ad libitum*. Juvenile zebra finches
143 were raised with their biological fathers until fledging (approximately 30 phd). All animal
144 experiments were conducted in strict adherence to the Guidelines of the Committee on
145 Animal Experiments of Hokkaido University, from which official permission was duly
146 obtained. These guidelines are based on the National Regulations for Animal Welfare in
147 Japan (Law for the Humane Treatment and Management of Animals, partial amendment
148 number No.105, 2011).

149

150 **Song recording and analysis**

151 Birds were housed singly in sound-attenuation boxes. Songs were automatically
152 recorded on a 24 h basis through a microphone (SHURE SM57) connected to a computer
153 installed with the sound analysis Pro 2011 program, version 1.04 (Avisoft SASLabPro,

154 RRID: SCR_014438) (Tchernichovski, Nottebohm, Ho, Pesaran, & Mitra, 2000) at 16
155 bits and 44 kHz sampling rate. Low and high frequency noises (< 0.05 and > 1.9 kHz,
156 respectively), were removed using Avisoft-SASLab Pro. Noise was further filtered using
157 Audacity Software.

158

159 **RT-PCR and cloning of muscarinic acetylcholine receptors**

160 The detailed cloning procedure has been described previously (Wada, Sakaguchi,
161 Jarvis, & Hagiwara, 2004). In brief, we tried to clone all five mAChRs (chr1–5) already
162 described in mammals (Levey, Kitt, Simonds, Price, & Brann, 1991). To avoid cross-
163 hybridization among related receptor subunits, we designed primers for conserved protein
164 coding regions of zebra finch, chicken, and humans to amplify specific sequences of each
165 receptor subunit (**Table 1, Supplementary figure 1**). RT-PCR was performed on total
166 RNA from an adult male zebra finch brain using the primer sets. PCR products on 1.5%
167 agarose gel were extracted when the predicted size was obtained and were cloned into
168 pGEM-T easy vector plasmid. Chr2, 3, 4, and 5 sequences were confirmed on BLASTN
169 (DNA) and BLASTX (protein) (NCBI blast, RRID: SCR_004870), and assigned
170 GenBank accession numbers: MH316766, MH316767, MH316768, and MH316769, for
171 chr2, 3, 4, and 5 respectively.

172

173 **Brain sampling and sectioning**

174 Brain samples were collected from individuals kept in silent, dark conditions for at
175 least 10 hours before sacrifice. Under these conditions, none of the birds were observed
176 to sing: thus, any mRNA expression observed was not due to singing/hearing song. Brains
177 were removed from the skull, placed into plastic molds, and then the mold was filled with

178 OCT medium (Tissue-Tek, Sakura, USA). The mold was transferred to a dry ice box and
179 later stored at -80°C until sectioning. Sections ($12\ \mu\text{m}$) were cut on the sagittal plane
180 and mounted on silane-coated glass slides. These slides were stored at -80°C until use.

181

182 **Radioisotope *in situ* hybridization and mRNA quantification**

183 ^{35}S -labeled riboprobes were synthesized from the T7 and Sp6 promoter sites of pGEM-
184 T easy using their respective RNA polymerases (Roche). Fresh frozen brain sections were
185 fixed in 3% paraformaldehyde/ $1\times$ phosphate-buffered saline (PBS, pH 7.0), washed 3
186 times in $1\times$ PBS, acetylated, washed 3 times in $2\times$ SSPE, dehydrated in increasing ethanol
187 concentrations (50, 70, 90, and 100%), and then air-dried. Riboprobe (10^6 cpm) was
188 mixed with $150\ \mu\text{l}$ of hybridization solution (50% formamide; 10% dextran sulphate; $1\times$
189 Denhart's solution; 12 mM EDTA, pH 8.0; 10 mM Tris-HCl, pH 8.0; 30 mM NaCl; 0.5
190 $\mu\text{g}/\mu\text{l}$ yeast tRNA; and 10 mM dithiothreitol. Hybridization was performed in an oil bath
191 for 14 h at 65°C . Thereafter, slides were washed step-wise in two changes of chloroform,
192 in $2\times$ SSPE/0.1% 2-mercaptoethanol for 30 min, in 50% formamide/0.1% 2-
193 mercaptoethanol for 60 minutes, twice in $2\times$ SSPE/0.1% 2-mercaptoethanol for 30 min
194 each, and twice in $0.1\times$ SSPE/0.1% 2-mercaptoethanol for 15 min each. The slides were
195 dehydrated in increasing ethanol concentrations (50, 70, 90, and 100%) and air-dried.
196 They were exposed to BioMax MR film (Kodak) for 4–5 days before development. Slides
197 were then immersed in an NTB2 emulsion and exposed for 3–4 weeks. These durations
198 were optimal for avoiding mRNA signal saturation. Emulsion-coated glasses were
199 developed, counter-stained with cresyl violet, cover-slipped with Permunt (Fisher
200 Scientific) in xylene, and air-dried. mRNA signals were quantified, as described before
201 (Wada et al., 2006). X-ray films were digitally scanned under a microscope (Z16 Apo,

202 Leica, USA) and connected to a CCD camera (DFC490, Leica, USA), with Leica
203 Application Suite, v3.3.0 (Leica, USA). Light and camera settings were kept constant for
204 all images to ensure unbiased comparisons. Images were converted to a 256-gray scale,
205 and mRNA expression levels were quantified as mean pixel intensities using Adobe
206 Photoshop (CS2, Adobe Systems, RRID: SCR_014199). Boundaries of the areas of
207 interest in the brain were based on Nissl-defined features and verified from the zebra finch
208 brain atlas ([Zebra Finch Song Learning Consortium](#), RRID: SCR_006356) (Karten et al.,
209 2013).

210

211 **Fluorescence *in situ* hybridization (FISH)**

212 For double-labeling *chr2* with gene markers of HVC cell types, we used 40 ng of
213 dinitrophenol (DNP)- or 100–500 ng of digoxigenin (DIG)-labeled RNA probes for
214 *chr2*, vesicular glutamate transporter 2 (*vGlut2*; GenBank accession No. MH453476),
215 glutamate decarboxylase 2 (*Gad2*; GenBank accession No. MH453477), neurotensin
216 (NTS; GenBank accession No. MH453474), and urotensin domain binding 2 (*UTS2D*;
217 GenBank accession No. MH453475). The probes were mixed in the hybridization
218 solution (50% formamide; 10% dextran sulphate, 1× Denhart's solution; 1mM EDTA,
219 pH 8.0; 33 mM Tris-HCl, pH 8.0; 600 mM NaCl; 0.2 mg/μl yeast tRNA; 80 mM
220 dithiothreitol; and 1% N-lauroylsarcosine). Hybridization was performed in an oil bath
221 for 14 h at 65°C. Thereafter, slides were washed twice in chloroform; dipped in 2×
222 SSC/0.1% Tween 20 and 5× SSC/0.1% Tween 20 for 30 min at 65°C, in formamide I
223 solution (50% formamide/4× SSC/0.1% Tween 20) for 40 min at 65°C, in formamide II
224 (50% formamide/ 2× SSC/0.1% Tween 20) for 40 min at 65°C, and 3× in 0.1× SSC/0.1%
225 Tween 20 for 15 min each at 65°C. Then, the slides were washed in NTE buffer for 20

226 min at room temperature (RT) and three times in 1× TNT buffer for 5 min each at RT.
227 DNP probes were detected using anti-DNP horseradish peroxidase (HRP)-conjugated
228 antibody (PerkinElmer, Cat# FP1129, RRID: AB_2629439, used at 1: 300) with a TSA-
229 Alexa Fluor 488 plus system (Invitrogen, Cat# A-11094, RRID: AB_221544, used 1:100).
230 To eliminate a second fluorophore reaction, the slides were incubated in 1% H₂O₂/1×
231 TNT buffer for 30 min to inactivate the first HRP-conjugated antibody. DIG probes were
232 detected using anti-DIG HRP-conjugated antibody (Jackson, Bar Harbor, ME, USA, Cat#
233 ABIN346913, RRID: AB_10821150) with a TSA - Alexa Fluor 647 system (Invitrogen).
234 Signals were captured by fluorescence microscopy (EVOS, FL, Thermo Fisher
235 Scientific).

236

237 **Testosterone (T) administration**

238 Exogenous T was implanted as described before (Hayase & Wada, 2018). Each bird
239 was anesthetized by an intraperitoneal injection of pentobarbital (6.48 mg/mL; 60 µL/10
240 g body weight). Birds were subcutaneously implanted with a silastic tube (inner diameter,
241 1.0 mm; outer diameter, 2.0 mm; and length, 7.0 mm) (Silascon SH 100-0N, Kaneka,
242 Osaka, Japan) containing crystalline T (1.0–1.5 mg/animal) at 30 phd (T-implanted; n =
243 9). Postoperatively, the birds were placed on a heat pad in a cage until they started eating
244 and drinking. Brain sampling was performed at 9 AM after lights-on (at 8 AM) between
245 43 and 53 phd [T-implanted, 47.6 ± 2.9 (mean \pm SD)]. T-implantation caused an increase
246 in circulating T levels (10.5 ± 1.3 ng/mL at 47.64 ± 2.9 phd) compared with that in
247 normally reared birds of similar age (1–2 ng/mL) in our laboratory (Mori & Wada, 2015).

248

249 **Deafening**

250 Each bird was deafened before fledging (17–23 phd), by bilateral extirpation of
251 cochleae as described previously (Konishi, 1964; Mori & Wada, 2015). The birds were
252 anesthetized by an intraperitoneal injection of 6.48 mg/ml (0.60 μ l/g body weight)
253 pentobarbital sodium (Mori & Wada, 2015). The head was fixed on a customized
254 stereotaxic apparatus equipped with horizontal ear bars. A slight incision was made in the
255 neck muscle, at the junction of the neck and the skull bone, close to the end of the elastic
256 hyoid bone. A tiny window was made to expose the cochlear, which was then removed
257 with the aid of a hooked wire. Removal of the cochleae were confirmed based on
258 morphology under a dissection microscope. Postoperatively, the birds were returned to
259 their nests and remained with their parents until approximately 32–41 phd. The same set
260 of brain samples of deafened birds reported previously by our laboratory (Mori & Wada,
261 2015) were used for *in situ* hybridization with the chrm2 probe.

262

263 **Statistical analysis**

264 All statistical analyses were performed using the SPSS software package Ver. 16.0
265 (IBM Statistics, RRID: SCR_002865). After a homoscedasticity test to confirm
266 homogeneity of variances, we used a one-way analysis of variance (ANOVA) to compare
267 mRNA expression levels of each of the mAChRs during song development. A Kruskal–
268 Wallis test was used for comparing mean mRNA expression levels, or ratio of mRNA
269 expression level in song nuclei to the surrounding areas among the different experimental
270 groups. A two-way ANOVA was used for comparing the mean chrm2 mRNA expression
271 ratio among siblings from different families. The unpaired student *t*-tests were used for
272 comparing the mean chrm2 expression levels between normal and T-implanted zebra
273 finches (juvenile) and between normal and early-deafened zebra finches (adults).

274 **Results**

275 **The general pattern of mAChRs expression in zebra finches**

276 Using brain tissues of zebra finches, and RT-PCR with oligo primers for regions
277 conserved among mammals, birds, and reptiles, we successfully cloned four out of the
278 five mAChRs known in mammals, encompassing chrM 2–5 (Caulfield & Birdsall, 1998).
279 The positions of these fragments in the zebra finch genome were identified based on the
280 BLAT alignment tool in the UCSC genome browser (RRID: SCR_005780)
281 (**Supplementary figure 1**). Although we could not find a predicted coding region of
282 chrM1 in the zebra finch genome (Taeniopygia_guttata taeGut3.2.4.dna.fa), we attempted
283 to clone chrM1 using degenerate primers to chrM1 conserved regions between mammals,
284 reptiles, and amphibians. However, we could not obtain any PCR fragment from the zebra
285 finch brain, as similarly reported in chicken (Yin, Gentle, & McBrien, 2004).

286 Next, we performed *in situ* hybridization to examine the expression patterns of chrM2–
287 5 in adult zebra finch brains (>120 phd). Each receptor had a unique expression pattern
288 in the pallial regions: hyperpallium (H), mesopallium (M), nidopallium (N), and
289 arcopallium (A). ChrM2 showed similar and consistent low expression level throughout
290 the pallial regions (**Figure 1 and 2**). ChrM3 and 4 revealed mirrored expression patterns
291 among the pallial regions: chrM3 had lower expression in M and A than in H and N,
292 whereas chrM4 had higher expression in M and A than in H and N. ChrM5 expression
293 level was gradually higher in the posterior than in the anterior parts of each pallial
294 subregion. Exceptions to these expression patterns in the pallium were observed for
295 chrM2 in the dorsal nucleus of H (DNH) (Mouritsen, Feenders, Liedvogel, Wada, & Jarvis,
296 2005), for chrM3 in anterior A (aA), and chrM4 and chrM5 in posterior A, which showed
297 higher expression levels than each pallial subdivision (**Figure 1 and 2**). In addition, all

298 subunit expressions were suppressed in field L2, entopallium, and nucleus basorostralis,
299 which are sensory input areas analogous to layer IV of mammalian auditory, visual, and
300 somatosensory/trigeminal cortical areas, respectively (Jarvis et al., 2013).

301 In the subpallium, chrm2 and chrm4 had higher expression in the striatum than chrm3
302 and chrm5. Chrm2 showed intense expression in the pallidum (P), whereas other subunits
303 did not. The differential mAChRs expression in the pallial subdivisions compared with
304 that in subpallial brain subdivisions corresponds with the expression of homologous
305 subunits in the pallial against basal ganglia subdivisions of the mammalian brain (Levey
306 et al., 1991). All subunits were absent in the dorsal thalamic nuclei [nucleus rotundus (Rt),
307 nucleus pretectalis (Pt), and nucleus spiriformis lateralis (Spl)]. In the midbrain tectum
308 opticum (TeO), chrm2 and chrm4 had higher expression than chrm3 and chrm5. In the
309 cerebellum, chrm2 and chrm4 had higher expression than chrm3 and chrm5, from the
310 white matter layer (w), granular layer (g), and Purkinje layer (p) to the molecular layer
311 (m). This suggests that each mAChR subunit plays distinct roles in subdivisions of the
312 telencephalon and other forebrain regions of the zebra finch.

313

314 **Differential mAChR expression in the song nuclei of adult zebra finches**

315 Next, to examine the expression of mAChRs in the song nuclei of adult male zebra
316 finches, we focused on the following five major song nuclei: HVC and RA in the vocal
317 motor pathway and LMAN, Area X, and anterior DLM [aDLM (Horita et al., 2012)] in
318 the pallial–basal ganglia–thalamic loop. In contrast to the unique expression of all
319 mAChRs among pallial brain subdivisions, there was differential expression (higher or
320 lower) of all mAChRs in at least one song nucleus compared with the surrounding brain
321 areas (**Figure 3**). In the pallial song nucleus HVC, chrm2 and chrm4 had higher and

322 chrm3 had lower expression than the surrounding caudal N (cN) (**Figure 3**). In RA, chrm3,
323 chrm4, and chrm5 had differentially lower expression levels than A. The expression of
324 mAChRs in LMAN was slightly lower (chrm3 and chrm5) or non-differential (chrm2 and
325 chrm4) than the surrounding rostral N (rN). In the striatal song nucleus Area X, only
326 chrm4 exhibited differential and higher expression than the surrounding striatum. In the
327 thalamic song nucleus aDLM, chrm2 had lower, differential expression than the
328 surrounding DLM. In summary, HVC had higher differential chrm2 and chrm4
329 expressions relative to the surrounding cN; RA, LMAN, and aDLM had suppressed
330 expression of one or more mAChRs; and Area X had higher chrm4 expression relative to
331 the surrounding striatum.

332

333 **Developmental regulation of mAChRs during the critical period of song learning**

334 To understand the possible contribution of mAChRs to song development, we analyzed
335 chrm2–5 mRNA expression in HVC, RA, LMAN, Area X, and aDLM at the three song
336 development stages in ZFs: subsong (35–45 phd), plastic song (50–65 phd), and
337 crystalized song (120–140 phd) (**Figure 4**). We found that chrm3 and chrm5 were
338 consistently expressed at lower levels during each developmental stage in most song
339 nuclei compared with chrm2 and chrm4. The expression level of chrm 4 was significantly
340 increased in HVC during song development (ANOVA, $*p < 0.05$). While analyzing
341 mRNA expression in these birds, we observed striking individual variability in chrm2
342 expression in HVC during song development, as evidenced by the large standard errors
343 in the bar graph (**Figure 4**). Therefore, although chrm2 expression in HVC exhibited a
344 trend to increase from the subsong to plastic song stage and decline at the crystalized song

345 stage, its expression level was not significantly different among the three song
346 developmental stages.

347

348 **Individual differences in *chr2* expression in HVC of zebra finches**

349 To evaluate the degree of individual differences in *chr2* expression in HVC, we
350 increased the sample size up to 12 birds per developmental stage. We then compared
351 *chr2* expression with *chr4* expression, which we found to increase during song
352 development, using the same brain sets. To minimize experimental handling variability
353 during the *in situ* hybridization procedure, we normalized the mRNA expression in each
354 song nucleus by the respective surrounding brain regions, throughout which *chr2* and
355 *chr4* were similarly expressed at all developmental stages (**Supplementary figure 2**).
356 Although there were no apparent individual differences in *chr4* expression in HVC
357 compared with that in surrounding cN at all four development stages, we found clear
358 individual differences in *chr2* expression in HVC during song development (**Figure 5**).
359 However, when *chr2* expression was examined at the pre-songsong stage (21–27 phd, n
360 = 8), there were no distinct individual difference in *chr2* expression level in HVC before
361 songsong (**Figure 5C and D**). This age-regulated individual variability was reflected in
362 the coefficient of variation (CV) of *chr2* expression, but not in the CV of *chr4*
363 expression. Even when the CV of *chr2* expression in HVC and cN were analyzed
364 separately using absolute mRNA expression level, the results were similar, with high CV
365 values for *chr2* expression in HVC, but low CV values in cN from the songsong stage
366 through development (**Supplementary figure 3**).

367

368 **Chrm2 is expressed in most HVC neuron types of the zebra finch**

369 To gain further insights on the possible functional significance of individual
370 differences in *chrn2* mRNA expression levels in HVC, we examined which cell types in
371 HVC express *chrn2*. HVC possess at least two types of excitatory glutamatergic
372 projection neurons to RA [HVC_(RA) neurons] and Area X [HVC_(X) neurons], GABAergic
373 inhibitory neurons, and glial cells. These cells have distinct morphological and
374 physiological properties (Dutar, Vu, & Perkel, 1998; Kubota & Taniguchi, 1998). We
375 analyzed the co-expression of *chrn2* mRNA with gene markers of various cell types in
376 HVC: UTS2D for HVC_(RA) neurons, NTS for HVC_(X) neurons, and vGlut2 and Gad2 for
377 excitatory and inhibitory neurons, respectively (Wirthlin, Lovell, Olson, Carleton, &
378 Mello, 2015). We found that *chrn2* mRNA was expressed in most HVC neurons of the
379 zebra finch, including excitatory HVC_(RA) and HVC_(X) neurons and inhibitory
380 interneurons (**Figure 6**). This suggests that *chrn2* contributes to cholinergic modulation
381 of most HVC neurons.

382

383 **Chrn2 expression is neither testosterone- nor audition-dependent**

384 Individual differences in *chrn2* expression in HVC clearly emerged from the subsong
385 production stage and was maintained in adulthood (**Figure 5**). T has been implicated in
386 natural song crystallization (Marler, Peters, Ball, Dufty Jr, & Wingfield, 1988) and
387 induces immature song stabilization (Korsia & Bottjer, 1991; Sizemore & Perkel, 2011).
388 Therefore, we examined a possible contribution of hormonal regulation, particularly
389 androgen concentration, to the individual differences in *chrn2* expression in HVC. We
390 administrated T to juvenile zebra finches before the onset of first singing at 30 phd. We
391 observed a decrease in acoustic variability across song bouts in T-implanted juvenile
392 zebra finches 2 weeks after of T-implantation (Hayase & Wada, 2018). At this

393 developmental time point (47.64 ± 2.9 phd), we compared their *chr2* expression level
394 in HVC with that of age-matched normal juveniles. We found no significant difference
395 in the *chr2* expression level in HVC between the two groups (unpaired *t*-test, $p = 0.225$;
396 **Figure 7A** left). In addition, the CV of *chr2* expression in HVC was similar between
397 the two groups (**Figure 7A** right).

398 Song development and the timing of song crystallization is regulated by auditory input
399 from hearing both a tutor's song and the bird's own song production (Konishi, 1965; Mori
400 & Wada, 2015). To test the possible contribution of auditory experience to individual
401 variation in *chr2* expression, we deafened zebra finches before the subsong stage (17–
402 23 phd). Then, we examined *chr2* expression levels in HVC of the early-deafened birds
403 as adults. We found no significant difference in *chr2* expression levels in HVC between
404 deafened and normal adults (unpaired *t*-test, $p = 0.858$; **Figure 7B**, left). The CV
405 individual differences in *chr2* expression levels in HVC between the two groups showed
406 no clear difference (**Figure 7B**, right). These results indicate that neither T nor auditory
407 experience changed the expression level and distribution of *chr2* in HVC. As a result,
408 neither factor is likely to explain the individual differences in expression.

409

410 **Familial bias of *chr2* expression in zebra finch HVC**

411 We then examined whether familial genetics influences *chr2* expression in HVC. We
412 quantified *chr2* mRNA expression of siblings from nine breeding families. Although
413 siblings from the same families showed variability in *chr2* expression levels, there was
414 a significant difference in *chr2* expression in HVC among breeding families ($n = 26$
415 birds from nine families, two-way ANOVA, $p = 0.038$) (**Figure 8**). Conversely, age did
416 not significantly contribute to the variation in *chr2* expression in HVC between the

417 tested families ($p = 0.216$), consistent with the result on *chr2* expression not being
418 developmentally regulated in HVC (**Figure 4**). Thus, these results suggest that familial
419 genetics contribute to the individual differences in *chr2* expression in HVC of zebra
420 finches.

421

422 **Differential *chr2* expression in HVC among songbird species**

423 To further examine the potential genetic regulation of the individual variability in
424 *chr2* expression levels, we analyzed *chr2* expression in HVC of six songbird species
425 (> 120 phd): zebra finch (ZF), owl finch (OF), star finch (SF), Bengalese finch (BF), java
426 sparrow (JS), and canary (CN) (**Figure 9A**). We found a statistically significant
427 difference in *chr2* expression levels among the six species (**Figure 9B**, Kruskal–Wallis
428 test, $***p < 0.0001$). Although these songbird species exhibit species-unique vocal
429 patterns (**Figure 9A**) (Imai et al., 2016), we could not detect an apparent link between
430 song phenotypes, particularly the syllable sequence and *chr2* expression in HVC. For
431 example, although both CN and OF produce repetitive sequence-based song patterns,
432 *chr2* expression in HVC was high in OF but suppressed in CN. The differences in *chr2*
433 expression in HVC among species were not tightly associated with evolutionary
434 relatedness. For example, ZF, OF, and SF belong to the same clade among the species
435 tested (**Figure 9A**). However, SF showed lower *chr2* expression in HVC than ZF and
436 OF. In addition, only two of six species (ZF and OF) showed a wide range of variability
437 in *chr2* expression level in HVC between individuals. Other species, SF, BF, JS, and
438 CN, did not show clear individual variability in *chr2* expression in HVC. Taken together,
439 these results indicate that the expression level and individual variability in *chr2*

440 expression in HVC are different among songbird species and that regulation has diverged
441 rapidly.
442

443 **Discussion**

444 mAChRs belong to a distinct family of G-protein coupled receptors that modulate
445 neuronal excitability via intracellular signal transduction (Hulme, Birdsall, & Buckley,
446 1990; Wess, 1996). The forebrain ACh system has a pivotal role in motor co-ordination
447 (Ztaou et al., 2016). Lesion of the basal forebrain cholinergic system abolishes plasticity
448 in the experience-dependent cortical map that is associated with motor skill learning
449 (Conner et al., 2003), suggesting a neuromodulatory function of ACh and its receptors in
450 sensorimotor learning. However, a gap remains in understanding the potential
451 contribution of AChRs to vocal learning and production, a trait exhibited only by limited
452 animal groups, such as songbirds and humans (Jarvis, 2004).

453 Here, we describe the expression patterns of mAChRs (chr2–5) in songbird brains.
454 Chr2 expression in the premotor song nucleus HVC increased through the critical
455 period of song learning in the zebra finch, while chr2 expression level in HVC exhibited
456 striking individual variability beginning from the subsong stage. In addition, chr2 is
457 expressed in most HVC neuron types, including two types of glutamatergic excitatory
458 projection neurons and GABAergic inhibitory neurons. Chr2 expression levels in HVC
459 were not influenced by testosterone levels or auditory experience. Rather, individual
460 differences in expression seems to be associated with familial genetic background. Finally,
461 by comparing chr2 expression levels in HVC of five additional songbird species, we
462 demonstrated that expression differs greatly among species and that the intra-specific
463 differences we observed in zebra finches are also present in an additional species.

464

465 **Unique mAChRs expression in the songbird brain during song development**

466 Although we successfully cloned chrm2–5 in the zebra finch, we could not obtain
467 chrm1. Chrm1 exists in the genomes of *Xenopus tropicalis* (GenBank accession No.
468 XM_004913660.3) and *Alligator mississippiensis* (GenBank accession No.
469 XM_019496993 as a predicted transcript) on the National Centre for Biotechnology
470 Information (NCBI) genome database. A pharmacological study suggests the presence of
471 chrm1 in frogs (*Rana ridibunda*) (Garnier et al., 1998). Therefore, these information
472 suggest that (1) the common amniote ancestor of birds and mammals possessed most of
473 these receptors and (2) chrm1 was lost during avian evolution (Yin, Gentle, & McBrien,
474 2004).

475 One of the key findings of the present study is the highly unique expression patterns of
476 all cloned mAChRs (chrm2–5) in the avian brain. Our results are consistent with previous
477 reports of a greater expression of chrm2 and chrm4 compared with chrm3 and chrm5 in
478 the cortex and striatum compared with the thalamus and brainstem in mammals (Levey
479 et al., 1991; W. Zhang et al., 2002). In addition, we found that mAChRs exhibited
480 different expression patterns in the song nuclei; chrm3 and chrm5 were very weakly
481 expressed, whereas chrm2 showed high expression in HVC and chrm4 shows high
482 expression in both HVC and Area X. Chrm4 exhibited the highest expression level in
483 Area X compared with other mAChRs in the song nuclei. Chrm4 was consistently
484 expressed in Area X during the critical period of song learning. Although there have been
485 a few studies that examined the function of ACh in Area X of songbirds, it has been
486 elucidated in the mammalian striatum that ACh acts via chrm4, and its interaction with
487 dopamine signaling contributes to the modulation of neural bursts of medium spiny
488 neurons (MSNs) (Ding et al., 2006; Oldenburg & Ding, 2011). Dopaminergic modulation
489 of neurons in Area X is crucial for song learning (Gadagkar et al., 2016; Hoffmann,

490 Saravanan, Wood, He, & Sober, 2016; Leblois, Wendel, & Perkel, 2010). Therefore,
491 chrm4 may contribute to the cholinergic modulation of the changes in spiking of MSNs
492 in Area X in association with dopamine signaling.

493 Only chrm 4 showed significant differential expression changes in HVC during the
494 critical period of song learning, showing a gradual increase until the crystallized song
495 stage. On the other hand, chrm2 expression in HVC showed a trend to peak at the plastic
496 song stage and then declined. These chrm2 expression dynamics in HVC are similar to
497 the developmental changes in ACh concentration in HVC of zebra finches (Sakaguchi &
498 Saito, 1989). Chrm2 and chrm4 are known to be expressed pre- and post-synaptically to
499 modulate the release and action of ACh onto postsynaptic sites (Baghdoyan, Lydic, &
500 Fleegal, 1998; Levey, Edmunds, Koliatsos, Wiley, & Heilman, 1995; Quirion et al., 1995).
501 Therefore, such auto-modulation of ACh release by chrm2 and chrm4 could contribute to
502 the upregulation of ACh concentration in HVC during song development.

503 The cholinergic basal forebrain regulates auditory input to the song system through
504 HVC (Shea & Margoliash, 2003) and is likely to contribute to the behavioral state-
505 dependent changes in auditory responses in HVC (Cardin & Schmidt, 2003; Schmidt &
506 Konishi, 1998; Shea & Margoliash, 2010). These developmental patterns in HVC and the
507 fact that chrm2 is expressed in multiple cell types suggest that chrm2 and chrm4 play
508 developmentally critical roles in the cholinergic modulation of auditory gating in HVC,
509 particularly for state-dependent suppression of HVC auditory responses during the
510 sensorimotor learning phase of song acquisition.

511

512 **Intraspecific differences in chrm2 expression in HVC**

513 Individual variability in behavior is a hallmark of various animal species. Some
514 behavioral patterns are known to be regulated by differential distribution of
515 neurotransmitter/neuromodulator and receptor expression (Hammock & Young, 2005;
516 McIntyre, Marriott, & Gold, 2003; Pantoja et al., 2016; Stern, Kirst, & Bargmann, 2017;
517 Zhang, Beaulieu, Sotnikova, Gainetdinov, & Caron, 2004). Zebra finches, for instance,
518 show clear individual differences in their acquired songs patterns from the same tutor
519 (Tchernichovski, Nottebohm, Ho, Pesaran, & Mitra, 2000). Juvenile zebra finches can
520 also use individually unique strategies to learn the same song (Liu, Gardner, & Nottebohm,
521 2004). Individual differences in vocal temporal patterns were observed at the subsong
522 stage in zebra finch juveniles, and this variability is biased among breeding families (Sato,
523 Mori, Sawai, & Wada, 2016). Our study has uncovered a fascinating individual variability
524 in *chrn2* expression levels in HVC during song development in zebra finches. *Chrn2*
525 mRNA expression level was not affected by manipulating song stabilization timing
526 through T administration (for acceleration) or audition deprivation (for delay). Instead,
527 *chrn2* expression level varied across individuals depending on their family background,
528 consistent with the idea that genetic differences among individuals drive the differences
529 in *chrn2* expression levels. However, further experiments are necessary to rule out the
530 potential contribution of other factors, such as differences in parental care (*e.g.*, nutrition
531 and tutoring), or the degree of social interactions, which were not monitored in this study.

532 Some zebra finches showed little *chrn2* expression in HVC during the critical period
533 of song learning. This finding may suggest that *chrn2* expression in HVC represents a
534 “gain-of-function” to modulate individual differences in the excitability of HVC neurons.
535 We do not understand the precise contribution of such variability in *chrn2* expression in
536 HVC, making it necessary to examine direct causal links between *chrn2* expression and

537 song variables. This could be done, for example, by comparing the degree/rate of song
538 crystallization in relation to mAChR subunit-specific gene and/or pharmacological
539 manipulations.

540

541 **Interspecific differences in chrm2 expression in HVC**

542 There are over 4,000 species of songbirds that produce complex species-specific song
543 patterns (Brenowitz & Beecher, 2005; Marler & Slabbekoorn, 2004). We found clear
544 species differences in the expression of chrm2 in HVC among songbird species, and these
545 differences seemed to have rapidly evolved in the songbird species we investigated. Most
546 of the songbird species used for our study are closely related (**Figure 9A**), suggesting that
547 a broader comparison of chrm2 expression among species may reveal even greater
548 differences. To the best of our knowledge, this is the first report of species differences in
549 a neuromodulator receptor gene expression in the song system. This has the potential to
550 link individual differences with species differences in a complex learned behavior. There
551 are studies that have reported species-specific expression of neuromodulator receptors in
552 invertebrates (Covelli, Memo, Spano, & Trabucchi, 1981) and mammals (Creese, Stewart,
553 & Snyder, 1979; Insel & Shapiro, 1992; Young, Winslow, Nilsen, & Insel, 1997). For
554 example, species differences in the expression of vasopressin receptor 1A gene predict
555 pair-bonding behavior (an innate trait) in voles. However, there are very few reports on
556 species-specific gene expression in neural circuits related to learned behaviors such as
557 birdsong. Even though the species differences in chrm2 expression were observed in
558 adults, zebra finch expressed this receptor gene even before they produced their first
559 songs. Therefore, there is a high possibility of the existence of species difference in chrm2
560 expression in HVC before the critical period of song learning. There are other potential

561 factors that might explain the species differences in *chrn2* expression in HVC, such as
562 differences in cell densities or in the proportion of HVC cell types. Further studies are
563 necessary for examining these possibilities.

564 HVC is a premotor song nucleus that regulates syllable sequence (Fee, Kozhevnikov, &
565 Hahnloser, 2004; Hahnloser, Kozhevnikov, & Fee, 2002). We could not clearly associate
566 species differences in song patterns, particularly syllable sequence, with *chrn2*
567 expression in HVC in this study. Since we examined mostly closely related songbird
568 species, our findings set the stage for further examination of *chrn2* expression in distantly
569 related species. However, we would like to propose a potential contribution of *chrn2* to
570 the modulation of species differences in auditory gating in the awake state, which may
571 underlie the auditory–vocal mirroring activity in $HVC_{(x)}$ neurons. Auditory-vocal
572 mirroring is a phenomenon whereby $HVC_{(x)}$ neurons exhibit similar patterns of neural
573 activity when a bird sings and listens to the playback of the same song (Prather, Peters,
574 Nowicki, & Mooney, 2008). In multiple songbird species, $HVC_{(x)}$ neurons are active
575 during singing (Fujimoto, Hasegawa, & Watanabe, 2011; Kozhevnikov & Fee, 2007;
576 Prather, Peters, Nowicki, & Mooney, 2008). However, the auditory response of $HVC_{(x)}$
577 neurons in awake birds differs across the songbird species. The species differences in
578 state-dependent auditory gating of $HVC_{(x)}$ neurons neither appears to be phylogenetically
579 dependent nor based on song complexity (Hessler & Okanoya, 2018; Prather, 2013;
580 Prather et al., 2008). Based on previous physiological studies of auditory responses to
581 song in HVC neurons and our present results on the species differences in *chrn2*
582 expression, we found an evidence for a potential relationship between *chrn2* expression
583 and auditory responses in HVC: songbird species with low *chrn2* expression in HVC
584 may exhibit auditory responses in HVC neurons when awake. For instance, the canary

585 and Bengalese finch have relatively low *chr2* expression in HVC and HVC_(x) neurons
586 of both species have auditory responses in the awake state, which represents an auditory-
587 vocal “mirroring” activity. In contrast, zebra finches have relatively high *chr2*
588 expression in HVC (shown in **Figure 9**) and do not show auditory activity in HVC_(x)
589 neurons when awake. Although this relationship is speculative based on a limited number
590 of songbird species tested for auditory-vocal mirroring in HVC(x) neurons, further
591 comparative analyses of the potential relationship between species differences in auditory
592 responses and *chr2* expression level in HVC could help to elucidate the molecular basis
593 of auditory-vocal “mirror” neuron activity. While the functional significance of the
594 species differences in *chr2* expression in HVC needs to be examined within the context
595 of song learning, the present results provide insight into the potential contribution of ACh
596 and its receptors to the evolution of acoustic communication with learned vocalization.
597

598 **Abbreviations**

599 A, arcopallium

600 Area X, striatum song nucleus Area X

601 B, basorostralis

602 Cb, cerebellum

603 DLM, dorsolateral nucleus of medial thalamus

604 DNH, dorsal nucleus of the hyperpallium

605 E, entopallium

606 g, granular layer in the cerebellum

607 Gp, globus pallidus

608 H, hyperpallium

609 Hp, hippocampus

610 HVC, acronym as proper name

611 IH, Intercalated hyperpallium

612 L, field L

613 LMAN, lateral magnocellular nucleus of the anterior nidopallium

614 m, molecular layer in the cerebellum M, mesopallium

615 MD, dorsal mesopallium

616 MV, ventral mesopallium

617 N, nidopallium

618 p, Purkinje layer in the cerebellum

619 P, pallidum

620 Pt, nucleus pretectalis

621 RA, robust nucleus of the arcopallium

- 622 Rt, nucleus rotundus
- 623 Spl, nucleus spiriformis lateralis
- 624 Str, striatum
- 625 TeO, tectum opticum
- 626 Tha: thalamus
- 627 w, white matter layer in the cerebellum

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629

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877
878

879 **Table**

880 **Table 1: PCR primers used for cloning mAChRs in zebra finch**

881

882

883 **Figure legends**

884 **Figure 1: Muscarinic acetylcholine receptors (mAChRs) expression in the zebra**
885 **finch brain**

886 **A:** Serial whole brain images showing *chrn2–5* expression. Brain views are sagittal. The
887 white color represents the mRNA signal. The red lines are borders of song nuclei in a
888 camera lucida drawing of brain areas. *a*: anterior; *p*: posterior; *d*: dorsal; and *v*: ventral.
889 Scale bar = 1mm.

890 **B:** Expression heat map of *chrn2–5* in brain subregions.

891

892 **Figure 2: mAChRs mRNA expression in the pallium and striatum**

893 *Chrn2–5* expression in the pallium and striatum. Scale bar = 3mm. Right: Camera lucida
894 drawing of brain subdivisions. Orange dotted lines represent boundaries of the brain
895 subdivisions: hyperpallium (H), intercalated hyperpallium (IH), dorsal mesopallium
896 (MD), ventral mesopallium (MV), nidopallium (N), and striatum (Str).

897

898 **Figure 3: mAChRs mRNA expression in the song system of adult male zebra finch**

899 *Chrn2–5* expression in HVC, RA, LMAN, Area X, and aDLM. White color represents
900 mRNA signal. Brain views are sagittal. Dotted red lines are borders of song nuclei in
901 camera lucida drawing of brain areas. Scale bars = 1mm.

902

903 **Figure 4: mAChRs expression in the song nuclei during song development**

904 Chrm2–5 expression in HVC, RA, LMAN, Area X, and aDLM at the subsong (35–45
905 phd; blue), plastic song (50–65 phd; green), and crystalized song (120–140 phd; yellow)
906 stages. Data: mean \pm SEM. n = 6 birds/song development stage. One-way analysis of
907 variance ANOVA, * $p < 0.05$.

908

909 **Figure 5: Individual difference of chrm2 expressions in HVC during song**
910 **development**

911 **A:** Examples of whole brain images showing individual differences in chrm2 expression
912 in HVC of adult zebra finches. Scale bar = 2 mm. The white color represents the mRNA
913 signal.

914 **B:** Representative brain images of six birds showing individual differences in chrm2
915 expression in HVC at the four song development stages: pre-subsong (21–26 phd),
916 subsong (45–46 phd), plastic song (60–65), and crystalized song (120–137 phd). The
917 yellow arrowheads indicate HVC outline. Scale bar = 1 mm.

918 **C:** Expression heatmaps of chrm2 and chrm4 in the song nuclei compared with those
919 in the surrounding brain areas at the four song development stages. Each column
920 represents mRNA expression for an individual bird (pre-subsong, n = 8 birds; subsong,
921 n = 12 birds; plastic song, n = 12 birds; and crystalized song n = 12 birds).

922 **D:** Coefficient of variation (CV) of chrm2 and chrm4 in HVC at different song
923 developmental stages.

924

925 **Figure 6: HVC neurons expressing chrm2**

926 **A:** Chrm2 expression in both excitatory and inhibitory neurons in HVC. vGlut2 and Gad2
927 are gene markers for glutamatergic excitatory and GABAergic inhibitory neurons,
928 respectively. The two extreme right columns indicate higher magnification of the insets.
929 The white arrowheads indicate co-expressing cells.

930 **B:** Chrm2 expression in HVC to Area X and HVC to RA neurons. NTS and UTS2D are
931 gene markers for HVC_(X) and HVC_(RA) neurons, respectively. Scale bars = 500 μ m (left)
932 and 20 μ m (right).

933

934 **Figure 7: Chrm2 expression in HVC under testosterone administration or auditory**
935 **deprivation**

936 **A:** (Left) Chrm2 expression in HVC of intact and T-implanted birds at the plastic song
937 stage (43–49 phd). The black dots represent individual mRNA expression in HVC
938 compared with that in cN. Data: mean \pm SEM. Unpaired *t*-test, $p \geq 0.05$. (Right) CV of
939 chrm2 expression ratio in HVC.

940 **B:** (Left) Chrm2 expression in HVC of intact and early-deafened birds at adult stage
941 (>120 phd). Data: mean \pm SEM. Unpaired *t*-test, $p \geq 0.05$. Middle: Examples of chrm2
942 expression in HVC in six early-deafened birds. Brain views are sagittal. (Right) CV of
943 chrm2 expression ratio in HVC.

944

945 **Figure 8: Familial bias of chrm2 expression in HVC**

946 Chrm2 expression in HVC among siblings from different breeding families (n = 26 birds
947 from nine families, 35–139 phd). The red vertical lines represent the mean of chrm2
948 expression in siblings from the same family. Two-way ANOVA, $*p = 0.038$.

949

950 **Figure 9: Differential expression level and distribution of chrM2 in HVC among**
951 **songbird species**

952 **A:** Phylogenetic relationship and examples of song spectrograms of the songbird species
953 examined: zebra finch (ZF), owl finch (OF), star finch (SF), Bengalese finch(BF), java
954 sparrow (JS), and canary (CN).

955 **B:** Examples of species difference in chrM2 expression in HVC of six birds. The yellow
956 arrowheads show HVC outlines. Scale bar = 1 mm. Right: A quantitative plot showing
957 species difference in chrM2 expression ratio (HVC/cN) among songbird species (n = 12
958 birds/species; Mean \pm SEM; Kruskal–Wallis test, *** $p < 0.0001$). The black dots
959 represent the individual mRNA expression ratio of HVC/cN.

960