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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Abstract

Acetylcholine receptors (AChRs) abound in the central nervous system of vertebrates. Muscarinic AChRs (mAChRs), a functional subclass of AChRs, mediate neuronal responses via intracellular signal transduction. They also play roles in sensorimotor coordination and motor skill learning by enhancing cortical plasticity. Learned birdsong is a complex motor skill acquired through sensorimotor coordination during a critical period. However, the functions of AChRs in the neural circuits for vocal learning and production remain largely unexplored. Here, we report the unique expression of mAChRs subunits (chrm2–5) in the song nuclei of zebra finches. The expression of excitatory subunits (chrm3 and chrm5) was downregulated in the song nuclei compared with the surrounding brain regions. In contrast, the expression of inhibitory mAChRs (chrm2 and chrm4) was upregulated in the premotor song nucleus HVC relative to the surrounding nidopallium. Chrm4 showed developmentally different expression in HVC during the critical period. Compared with chrm4, individual differences in chrm2 expression emerged in HVC early in the critical period. These individual differences in chrm2 expression persisted despite testosterone administration or auditory deprivation, which altered the timing of song stabilization. Instead, the variability in chrm2 expression in HVC correlated with parental genetics. In addition, chrm2 expression in HVC exhibited species differences and individual variability among songbird species. These results suggest that mAChRs play an underappreciated role in the development of species and individual differences in song patterns by modulating the excitability of HVC neurons, providing a potential insight into the gating of auditory responses in HVC neurons.

Key words

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

Introduction

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

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

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

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

HVC receives cholinergic projections from the ventral pallidum of the basal forebrain that is homologous to the mammalian nucleus basalis of Meynert (Li & Sakaguchi, 1997; Reiner et al., 2004). Stimulating the cholinergic basal forebrain suppresses auditory responses to the bird's own song in HVC and RA neurons (Shea & Margoliash, 2003), suggesting a cholinergic regultation 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 chrm2 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

Materials and methods

Animals

To compare intra-specific differences and developmental patterns of mAChRs expression, we sampled male zebra finches (ZF; *Taeniopygia guttata*) at the pre-subsong (21-26 post-hatching day, phd, n = 8), subsong (30–45 phd, n = 12), plastic song (50–65 phd, n = 12), and crystalized song (> 120 phd, n = 12) stages. In addition, to understand inter-specific differences in chrm2 expression, we sampled adults of other songbird species (n = 8 each), *i.e.*, owl finch (OF; *T. bichenovii*), star finch (SF; *Neochmia ruficauda*), Bengalese finch (BF; *Lonchura striata* var. *domestica*), Java sparrow (JS; *Padda oryzivora*), and canary (CN; *Serinus canaria*) (>120 phd). Zebra and Bengalese finches were obtained from our breeding colonies at Hokkaido University. Other species were purchased from local breeders in Japan. The photoperiod was maintained at 13/11 h light/dark cycles, and food and water were provided *ad libitum*. Juvenile zebra finches were raised with their biological fathers until fledging (approximately 30 phd). All animal experiments were conducted in strict adherence to the Guidelines of the Committee on Animal Experiments of Hokkaido University, from which official permission was duly obtained. These guidelines are based on the National Regulations for Animal Welfare in Japan (Law for the Humane Treatment and Management of Animals, partial amendment number No.105, 2011).

Song recording and analysis

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

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

RT-PCR and cloning of muscarinic acetylcholine receptors

The detailed cloning procedure has been described previously (Wada, Sakaguchi, Jarvis, & Hagiwara, 2004). In brief, we tried to clone all five mAChRs (chrm1–5) already described in mammals (Levey, Kitt, Simonds, Price, & Brann, 1991). To avoid cross-hybridization among related receptor subunits, we designed primers for conserved protein coding regions of zebra finch, chicken, and humans to amplify specific sequences of each receptor subunit (**Table 1, Supplementary figure 1**). RT-PCR was performed on total RNA from an adult male zebra finch brain using the primer sets. PCR products on 1.5% agarose gel were extracted when the predicted size was obtained and were cloned into pGEM-T easy vector plasmid. Chrm2, 3, 4, and 5 sequences were confirmed on BLASTN (DNA) and BLASTX (protein) (NCBI blast, RRID: SCR_004870), and assigned GenBank accession numbers: MH316766, MH316767, MH316768, and MH316769, for chrm2, 3, 4, and 5 respectively.

Brain sampling and sectioning

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

OCT medium (Tissue-Tek, Sakura, USA). The mold was transferred to a dry ice box and later stored at −80°°C until sectioning. Sections (12 µm) were cut on the sagittal plane and mounted on silane-coated glass slides. These slides were stored at −80°C until use.

Radioisotope *in situ* hybridization and mRNA quantification

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

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

Fluorescence *in situ* hybridization (FISH)

For double-labeling chrm2 with gene markers of HVC cell types, we used 40 ng of dinitrophenol (DNP)- or 100–500 ng of digoxigenin (DIG)-labeled RNA probes for chrm2, vesicular glutamate transporter 2 (vGlut2; GenBank accession No. MH453476), glutamate decarboxylase 2 (Gad2; GenBank accession No. MH453477), neurotensin (NTS; GenBank accession No. MH453474), and urotensin domain binding 2 (UTS2D; GenBank accession No. MH453475). The probes were mixed in the hybridization solution (50% formamide; 10% dextran sulphate, 1× Denhart's solution; 1mM EDTA, pH 8.0; 33 mM Tris-HCl, pH 8.0; 600 mM NaCl; 0.2 mg/µl yeast tRNA; 80 mM dithiothreitol; and 1% N-lauroylsarcosine). Hybridization was performed in an oil bath for 14 h at 65°C. Thereafter, slides were washed twice in chloroform; dipped in 2× SSC/0.1% Tween 20 and 5× SSC/0.1% Tween 20 for 30 min at 65°C, in formamide I solution (50% formamide/4× SSC/0.1% Tween 20) for 40 min at 65°C, in formamide II (50% formamide/ 2× SSC/0.1% Tween 20) for 40 min at 65°C, and 3× in 0.1× SSC/0.1% Tween 20 for 15 min each at 65°C. Then, the slides were washed in NTE buffer for 20

min at room temperature (RT) and three times in 1× TNT buffer for 5 min each at RT. DNP probes were detected using anti-DNP horseradish peroxidase (HRP)-conjugated antibody (PerkinElmer, Cat# FP1129, RRID: AB_2629439, used at 1: 300) with a TSA-Alexa Fluor 488 plus system (Invitrogen, Cat# A-11094, RRID: AB_221544, used 1:100). To eliminate a second fluorophore reaction, the slides were incubated in 1% H_2O_2/1× TNT buffer for 30 min to inactivate the first HRP-conjugated antibody. DIG probes were detected using anti-DIG HRP-conjugated antibody (Jackson, Bar Harbor, ME, USA, Cat# ABIN346913, RRID: AB_10821150) with a TSA - Alexa Fluor 647 system (Invitrogen). Signals were captured by fluorescence microscopy (EVOS, FL, Thermos Fisher Scientific).

Testosterone (T) administration

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

Deafening

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

Statistical analysis

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

Results

The general pattern of mAChRs expression in zebra finches

Using brain tissues of zebra finches, and RT-PCR with oligo primers for regions conserved among mammals, birds, and reptiles, we successfully cloned four out of the five mAChRs known in mammals, encompassing chrm 2–5 (Caulfield & Birdsall, 1998). The positions of these fragments in the zebra finch genome were identified based on the BLAT alignment tool in the UCSC genome browser (RRID: SCR_005780) (**Supplementary figure 1**). Although we could not find a predicted coding region of chrm1 in the zebra finch genome (Taeniopygia_guttata taeGut3.2.4.dna.fa), we attempted to clone chrm1 using degenerate primers to chrm1 conserved regions between mammals, reptiles, and amphibians. However, we could not obtain any PCR fragment from the zebra finch brain, as similarly reported in chicken (Yin, Gentle, & McBrien, 2004).

Next, we performed *in situ* hybridization to examine the expression patterns of chrm2–5 in adult zebra finch brains (>120 phd). Each receptor had a unique expression pattern in the pallial regions: hyperpallium (H), mesopallium (M), nidopallium (N), and arcopallium (A). Chrm2 showed similar and consistent low expression level throughout the pallial regions (**Figure 1 and 2**). Chrm3 and 4 revealed mirrored expression patterns among the pallial regions: chrm3 had lower expression in M and A than in H and N, whereas chrm4 had higher expression in M and A than in H and N. Chrm5 expression level was gradually higher in the posterior than in the anterior parts of each pallial subregion. Exceptions to these expression patterns in the pallium were observed for chrm2 in the dorsal nucleus of H (DNH) (Mouritsen, Feenders, Liedvogel, Wada, & Jarvis, 2005), for chrm3 in anterior A (aA), and chrm4 and chrm5 in posterior A, which showed 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

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

Developmental regulation of mAChRs during the critical period of song learning

To understand the possible contribution of mAChRs to song development, we analyzed chrm2–5 mRNA expression in HVC, RA, LMAN, Area X, and aDLM at the three song development stages in ZFs: subsong (35–45 phd), plastic song (50–65 phd), and crystalized song (120–140 phd) (**Figure 4**). We found that chrm3 and chrm5 were consistently expressed at lower levels during each developmental stage in most song nuclei compared with chrm2 and chrm4. The expression level of chrm 4 was significantly increased in HVC during song development (ANOVA, $*p < 0.05$). While analyzing mRNA expression in these birds, we observed striking individual variability in chrm2 expression in HVC during song development, as evidenced by the large standard errors in the bar graph (**Figure 4**). Therefore, although chrm2 expression in HVC exhibited a 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 chrm2 expression in HVC of zebra finches**
349 To evaluate the degree of individual differences in chrm2 expression in HVC, we
350 increased the sample size up to 12 birds per developmental stage. We then compared
351 chrm2 expression with chrm4 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 chrm2 and
355 chrm4 were similarly expressed at all developmental stages (**Supplementary figure 2**).
356 Although there were no apparent individual differences in chrm4 expression in HVC
357 compared with that in surrounding cN at all four development stages, we found clear
358 individual differences in chrm2 expression in HVC during song development (**Figure 5**).
359 However, when chrm2 expression was examined at the pre-subsong stage (21–27 phd, n
360 = 8), there were no distinct individual difference in chrm2 expression level in HVC before
361 subsong (**Figure 5C and D**). This age-regulated individual variability was reflected in
362 the coefficient of variation (CV) of chrm2 expression, but not in the CV of chrm4
363 expression. Even when the CV of chrm2 expression in HVC and cN were analyzed
364 separately using absolute mRNA expression level, the results were similar, with high CV
365 values for chrm2 expression in HVC, but low CV values in cN from the subsong stage
366 through development (**Supplementary figure 3**).
367
368 **Chrm2 is expressed in most HVC neuron types of the zebra finch**

To gain further insights on the possible functional significance of individual differences in chrm2 mRNA expression levels in HVC, we examined which cell types in HVC express chrm2. HVC possess at least two types of excitatory glutamatergic projection neurons to RA [$HVC_{(RA)}$ neurons] and Area X [$HVC_{(X)}$ neurons], GABAergic inhibitory neurons, and glial cells. These cells have distinct morphological and physiological properties (Dutar, Vu, & Perkel, 1998; Kubota & Taniguchi, 1998). We analyzed the co-expression of chrm2 mRNA with gene markers of various cell types in HVC: UTS2D for $HVC_{(RA)}$ neurons, NTS for $HVC_{(X)}$ neurons, and vGlut2 and Gad2 for excitatory and inhibitory neurons, respectively (Wirthlin, Lovell, Olson, Carleton, & Mello, 2015). We found that chrm2 mRNA was expressed in most HVC neurons of the zebra finch, including excitatory $HVC_{(RA)}$ and $HVC_{(X)}$ neurons and inhibitory interneurons (**Figure 6**). This suggests that chrm2 contributes to cholinergic modulation of most HVC neurons.

Chrm2 expression is neither testosterone- nor audition-dependent

Individual differences in chrm2 expression in HVC clearly emerged from the subsong production stage and was maintained in adulthood (**Figure 5**). T has been implicated in natural song crystallization (Marler, Peters, Ball, Dufty Jr, & Wingfield, 1988) and induces immature song stabilization (Korsia & Bottjer, 1991; Sizemore & Perkel, 2011). Therefore, we examined a possible contribution of hormonal regulation, particularly androgen concentration, to the individual differences in chrm2 expression in HVC. We administrated T to juvenile zebra finches before the onset of first singing at 30 phd. We observed a decrease in acoustic variability across song bouts in T-implanted juvenile 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 chrm2 expression level
394 in HVC with that of age-matched normal juveniles. We found no significant difference
395 in the chrm2 expression level in HVC between the two groups (unpaired *t*-test, $p = 0.225$;
396 **Figure 7A** left). In addition, the CV of chrm2 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 chrm2 expression, we deafened zebra finches before the subsong stage (17–
402 23 phd). Then, we examined chrm2 expression levels in HVC of the early-deafened birds
403 as adults. We found no significant difference in chrm2 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 chrm2 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 chrm2 in HVC. As a result,
408 neither factor is likely to explain the individual differences in expression.
409
410 **Familial bias of chrm2 expression in zebra finch HVC**
411 We then examined whether familial genetics influences chrm2 expression in HVC. We
412 quantified chrm2 mRNA expression of siblings from nine breeding families. Although
413 siblings from the same families showed variability in chrm2 expression levels, there was
414 a significant difference in chrm2 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 chrm2 expression in HVC between the

tested families (p = 0.216), consistent with the result on chrm2 expression not being developmentally regulated in HVC (**Figure 4**). Thus, these results suggest that familial genetics contribute to the individual differences in chrm2 expression in HVC of zebra finches.

Differential chrm2 expression in HVC among songbird species

To further examine the potential genetic regulation of the individual variability in chrm2 expression levels, we analyzed chrm2 expression in HVC of six songbird species (> 120 phd): zebra finch (ZF), owl finch (OF), star finch (SF), Bengalese finch (BF), java sparrow (JS), and canary (CN) (**Figure 9A**). We found a statistically significant difference in chrm2 expression levels among the six species **(Figure 9B**, Kruskal–Wallis test, ***p < 0.0001). Although these songbird species exhibit species-unique vocal patterns (**Figure 9A**) (Imai et al., 2016), we could not detect an apparent link between song phenotypes, particularly the syllable sequence and chrm2 expression in HVC. For example, although both CN and OF produce repetitive sequence-based song patterns, chrm2 expression in HVC was high in OF but suppressed in CN. The differences in chrm2 expression in HVC among species were not tightly associated with evolutionary relatedness. For example, ZF, OF, and SF belong to the same clade among the species tested (**Figure 9A**). However, SF showed lower chrm2 expression in HVC than ZF and OF. In addition, only two of six species (ZF and OF) showed a wide range of variability in chrm2 expression level in HVC between individuals. Other species, SF, BF, JS, and CN, did not show clear individual variability in chrm2 expression in HVC. Taken together, these results indicate that the expression level and individual variability in chrm2

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

Discussion

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

Here, we describe the expression patterns of mAChRs (chrm2–5) in songbird brains. Chrm4 expression in the premotor song nucleus HVC increased through the critical period of song learning in the zebra finch, while chrm2 expression level in HVC exhibited striking individual variability beginning from the subsong stage. In addition, chrm2 is expressed in most HVC neuron types, including two types of glutamatergic excitatory projection neurons and GABAergic inhibitory neurons. Chrm2 expression levels in HVC were not influenced by testosterone levels or auditory experience. Rather, individual differences in expression seems to be associated with familial genetic background. Finally, by comparing chrm2 expression levels in HVC of five additional songbird species, we demonstrated that expression differs greatly among species and that the intra-specific differences we observed in zebra finches are also present in an additional species.

Unique mAChRs expression in the songbird brain during song development

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

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

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

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

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

Intraspecific differences in chrm2 expression in HVC

Individual variability in behavior is a hallmark of various animal species. Some behavioral patterns are known to be regulated by differential distribution of neurotransmitter/neuromodulator and receptor expression (Hammock & Young, 2005; McIntyre, Marriott, & Gold, 2003; Pantoja et al., 2016; Stern, Kirst, & Bargmann, 2017; Zhang, Beaulieu, Sotnikova, Gainetdinov, & Caron, 2004). Zebra finches, for instance, show clear individual differences in their acquired songs patterns from the same tutor (Tchernichovski, Nottebohm, Ho, Pesaran, & Mitra, 2000). Juvenile zebra finches can also use individually unique strategies to learn the same song (Liu, Gardner, & Nottebohm, 2004). Individual differences in vocal temporal patterns were observed at the subsong stage in zebra finch juveniles, and this variability is biased among breeding families (Sato, Mori, Sawai, & Wada, 2016). Our study has uncovered a fascinating individual variability in chrm2 expression levels in HVC during song development in zebra finches. Chrm2 mRNA expression level was not affected by manipulating song stabilization timing through T administration (for acceleration) or audition deprivation (for delay). Instead, chrm2 expression level varied across individuals depending on their family background, consistent with the idea that genetic differences among individuals drive the differences in chrm2 expression levels. However, further experiments are necessary to rule out the potential contribution of other factors, such as differences in parental care (*e.g.*, nutrition and tutoring), or the degree of social interactions, which were not monitored in this study.

Some zebra finches showed little chrm2 expression in HVC during the critical period of song learning. This finding may suggest that chrm2 expression in HVC represents a "gain-of-function" to modulate individual differences in the excitability of HVC neurons. We do not understand the precise contribution of such variability in chrm2 expression in HVC, making it necessary to examine direct causal links between chrm2 expression and

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

Interspecific differences in chrm2 expression in HVC

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

561 factors that might explain the species differences in chrm2 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 chrm2
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 chrm2 expression in distantly
569 related species. However, we would like to propose a potential contribution of chrm2 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 chrm2
582 expression, we found an evidence for a potential relationship between chrm2 expression
583 and auditory responses in HVC: songbird species with low chrm2 expression in HVC
584 may exhibit auditory responses in HVC neurons when awake. For instance, the canary

585 and Bengalese finch have relatively low chrm2 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 chrm2
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 chrm2 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 chrm2 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

Abbreviations

A, arcopallium

Area X, striatum song nucleus Area X

B, basorostralis

Cb, cerebellum

DLM, dorsolateral nucleus of medial thalamus

DNH, dorsal nucleus of the hyperpallium

E, entopallium

g, granular layer in the cerebellum

Gp, globus pallidus

H, hyperpallium

Hp, hippocampus

HVC, acronym as proper name

IH, Intercalated hyperpallium

L, field L

LMAN, lateral magnocellular nucleus of the anterior nidopallium

m, molecular layer in the cerebellum M, mesopallium

MD, dorsal mesopallium

MV, ventral mesopallium

N, nidopallium

p, Purkinje layer in the cerebellum

P, pallidum

Pt, nucleus pretectalis

RA, robust nucleus of the arcopallium

Rt, nucleus rotundus

Spl, nucleus spiriformis lateralis

Str, striatum

TeO, tectum opticum

Tha: thalamus

w, white matter layer in the cerebellum

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Table

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

Figure legends

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

A: Serial whole brain images showing chrm2–5 expression. Brain views are sagittal. The white color represents the mRNA signal. The red lines are borders of song nuclei in a camera lucida drawing of brain areas. *a*: anterior; *p*: posterior; *d*: dorsal; and *v*: ventral. Scale bar = 1mm.

B: Expression heat map of chrm2–5 in brain subregions.

Figure 2: mAChRs mRNA expression in the pallium and striatum

Chrm2–5 expression in the pallium and striatum. Scale bar = 3mm. Right: Camera lucida drawing of brain subdivisions. Orange dotted lines represent boundaries of the brain subdivisions: hyperpallium (H), intercalated hyperpallium (IH), dorsal mesopallium (MD), ventral mesopallium (MV), nidopallium (N), and striatum (Str).

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

Chrm2–5 expression in HVC, RA, LMAN, Area X, and aDLM. White color represents mRNA signal. Brain views are sagittal. Dotted red lines are borders of song nuclei in camera lucida drawing of brain areas. Scale bars = 1mm.

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

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

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

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

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

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

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

Figure 6: **HVC neurons expressing chrm2**

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

B: Chrm2 expression in HVC to Area X and HVC to RA neurons. NTS and UTS2D are gene markers for $HVC_{(X)}$ and $HVC_{(RA)}$ neurons, respectively. Scale bars = 500 μm (left) and 20 μm (right).

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

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

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

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

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

Figure 9: **Differential expression level and distribution of chrm2 in HVC among songbird species**

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

B: Examples of species difference in chrm2 expression in HVC of six birds. The yellow arrowheads show HVC outlines. Scale bar = 1 mm. Right: A quantitative plot showing species difference in chrm2 expression ratio (HVC/cN) among songbird species (n = 12 birds/species; Mean ± SEM; Kruskal–Wallis test, ***$p < 0.0001$). The black dots represent the individual mRNA expression ratio of HVC/cN.