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Inter- and intra-species differences in muscarinic acetylcholine receptor expression in the neural pathways for learned vocalization in songbirds

（鳴禽類歌神経回路において種内個体差・種間差発現を示すムスカリン性アセチルコリン受容体の研究）

A DISSERTATION

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Chinweike Norman Asogwa

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General Introduction

Species and individual differences in behaviour are unique characteristics of most animal taxa and appear to have been strongly selected for during evolution. The existence of behavioural differences even within closely related species and individuals of a species, suggests that whereas the basic organization of neural circuits is conserved, these circuits could be regulated differently in a species or individually-specific manner (Katz and Wilson, 1994; Lim et al., 2004; Goodson et al., 2005). How are evolutionarily conserved neural circuits regulated to produce different behaviours in closely related species and individuals of a species? A better understanding of this crucial question is of great interest to behavioural neuroscientists and could shed more light on potential ultimate causes of evolution of different behavioural patterns. Although it is challenging to delineate the neural mechanisms driving species and individual differences in behaviour, owing to the complexity of even the simplest nervous system, insights on how a neural circuit must be fine-tuned to produce different behaviours can be gained by comparing the central nervous system (CNS) of closely related species with different behaviours (Balaban et al., 1988; Lingle, 1992; Ryan, 1998). If the basic organization of central brain circuits generating a behaviour is similar between/among species, it suggests that variations in behaviour are produced by differential regulation of the neural circuits associated with such behaviours. If the neural circuits differ in design, which features do? Size of associated nuclei, synaptic interconnectivity between/among nuclei, cell size/number/density, or differences in the expression of regulatory genes in brain nuclei?

Several factors have been hypothesized to underlie the generation of species and individual differences in behaviour (Katz and Harris-Warrick, 1999). First, closely-related species may differ in the acquisition of sensory signals. This could involve the elaboration or loss of sensory structures such as the replacement of ancestral stretch receptors with newly evolved stretch receptors (Paul and Wilson, 1994). Secondly, the central neuronal circuits of two or more species may process sensory signals differently, which could be due to changes in the number or properties of cells and synapses in the CNS (Katz, 1991; Tierney, 1995). Lastly, effector organs may be modified adaptively in related species to respond differently to central commands (Wainwright and Turingan, 1997; Friel and Wainwright, 1999). Several lines of research suggest that the basic design of the neural circuits responsible for different

behaviours in closely related species is similar (Balaban et al., 1988; Lingle, 1993; Ryan, 1998; Lim and Young, 2004).

Although other potential contributing factors cannot be ignored, previous studies suggested that the differential expression of neurotransmitter/neuromodulator receptor genes might underlie the generation of species- and individually-specific behaviours by conserved neural circuits. For instance, species differences in the formation of social pair-bonds between males of the monogamous (*Microtus ochrogaster*) and polygamous (*M. pennsylvanicus*) species of voles (Lim et al., 2004), is regulated by differential expression of the vasopressin receptor 1A gene (V1AR) in the ventral pallidum. In addition, individual males of the monogamous species differ in the degree of pair-bonding (measured by the amount of time which their males spend with partner and stranger females) because of genetic-dependent differences in the expression level of V1AR gene (Hammock and Young, 2005). In addition, a recent study found that species and individual differences in the production of neuronal escape swim pattern in nudibranch snails is regulated by neuron-type specific differences in the expression of serotonergic neuromodulator receptor genes (Tamvacakis et al., 2018). These suggest that species and individual differences in behaviour should be regulated by differential expression of neurotransmitter/neuromodulator receptor genes in the neural circuits responsible for these behaviours.

However, the preceding examples are all innately-determined behaviours. There is much gap in knowledge about the neural mechanisms for species and individual differences in learned behaviours involving motor skills, such as human speech and birdsong. Like humans, songbirds learn their songs during a critical/sensitive period in development (Eales, 1965; Zann, 2006). There are over 4,500 species of songbirds (Marler and Slabbekoorn, 2004) that produce species-specific songs (Brenowitz and Beecher, 2005). These species-specific birdsongs are produced using a set of conserved interconnected brain nuclei called the song pathways/nuclei (Nottebohm et al., 1976). The song nuclei comprise two neural circuits: the anterior forebrain pathway (AFP) important for song learning and maintenance (Bottjer et al., 1984; Scharff and Nottebohm, 1991) and the vocal motor pathway (VMP) which is necessary for song production (Nottebohm et al., 1976). With similarly interconnected neural pathways, juveniles of the songbirds species, zebra finch (ZF) employ unique strategies to learn a song (Liu et al., 2004) and exhibit large variability in their vocalizations (Tchernichovski et al., 2001; Sato et al., 2016) These suggests that the song nuclei are regulated differently under environmental (Miller et al., 2017) or genetic (Liu et al., 2004; Sato et al., 2016) constraints

in different species and even in individuals of a species. However, the precise neural molecular mechanisms for species- and individually-specific regulation of the song pathways is not clear.

A few studies have evaluated the expression of neurotransmitter/neuromodulator receptor genes in the neural circuits for learned vocalizations in songbirds; glutamate receptors (Wada et al., 2004) and dopamine receptors (Kubikova et al., 2010). Whereas these studies found unique expression of these neurotransmitter/neuromodulator receptors in the song nuclei compared with surrounding brain areas, gene expression patterns/levels in multiple songbird species were not examined. As a result, it is not clear whether these receptor genes are expressed differently in other songbird species or not. Such analysis has the potential to illuminate the potential contribution of neurotransmitter/neuromodulator receptor genes to the evolution of species- or individual differences in learned motor skills including birdsong.

Previous studies suggested that cholinergic functions via acetylcholine receptors (AChRs), are important for species differences in behaviour (Buckley et al., 1988; Levey et al., 1991; Butt et al., 2000; Seo et al., 2009). In mammals, cholinergic projection neurons to cortical and subcortical brain areas originate in distinct basal forebrain and thalamic nuclei: the brainstem pedunculo-pontine and lateral dorsal tegmental nuclei, a subset of thalamic nuclei, the striatum, the nucleus basalis of Meynert (Mesulam et al., 1983; Woolf, 1991). In birds, cholinergic projection neurons to cortical brain areas originate in the ventral pallidum (Li and Sakaguchi, 1997; Sadananda, 2004)). Cholinergic functions are mediated by the neuromodulator acetylcholine (ACh) via ionotropic nicotinic AChRs (nAChRs) and metabotropic muscarinic AChRs (mAChRs), respectively. mAChRs belong to a family of seven transmembrane-spanning receptors (Hulme et al., 1990), which mediate neuronal signal transductions by interacting with different guanosine triphosphate (G)-binding proteins (Taylor and Brown, 1994). In mammals, mAChRs are encoded by five separate genes, named chrm1–5 (Kubo et al., 1986; Caulfield, 1993; Matsui et al., 2004). These receptor subtypes/subunits are sub-classified on the basis of different signal transduction mechanisms as mostly excitatory (acting via chrm1, 3, and 5) and inhibitory (via chrm2 and 4) subunits/subtypes (Caulfield, 1993; Caulfield and Birdsall, 1998). Whereas mAChRs have been shown to exist in insects (Schmidt-Nielsen et al., 1977; Breer and Knipper, 1984; Knipper and Breer, 1988) and molluscs (Pivovarov and Saganelidze, 1988), they show wide, but unique expression patterns in amphibian- (Butt et al., 2000), pisces- (Seo et al., 2009; Toscano-Márquez et al., 2013), and mammalian CNS (Buckley et al., 1988; Levey et al.,

1991; Caulfield, 1993). Together, these studies suggest that mAChR expression is conserved across invertebrate and vertebrate species and could perform similar functions involved in learning, memory and motor skills acquisition, including learning to sing in songbirds.

Based on above experimental evidences, I predict that mAChRs are differentially expressed in the song nuclei to modulate ACh-mediated excitability of neurons in the song pathways. In addition, these differences might be present in different songbird species and in individuals of a species. To better understand these possibilities, I examined the precise mAChRs expression in the song nuclei and their potential contributions to song learning and production through the critical period of vocal learning. In Chapter I, I found unique mAChRs expression in major brain areas and in the song nuclei. In Chapter II, I examined the developmental regulation of chrm2–5 in the song nuclei. I found that chrm4 is upregulated in HVC during development, whereas chrm2 expression level showed clear individual differences in ZFs. In addition, I showed that chrm2 expression level in HVC is neither regulated by testosterone- nor auditory-driven modulation of the timing of song crystallization in ZFs. Furthermore, I found that the parental genetic information could have potential contributions to chrm2 expression level in HVC, and that chrm2 is expressed by most types of neurons in HVC. In Chapter III, I found that there is a correlation between chrm2 expression level in HVC with the coefficient of variation (CV) of entropy variance in subsong and with song patterns in early-deafened ZFs. However, there is no clear effects of AAV9-mediated overexpression of CHRM2 protein in HVC on learned crystallized adult songs of ZFs.

Based on these findings, I will discuss the potential contributions of chrm2 and 4 to the regulation of the input of auditory signals to the song pathways. This may be important for song learning and the generation of species and individual differences in learned vocalizations.

Chapter I

The mAChRs Expression in a Songbird (zebra finch) Brain

1.1 Introduction

The biological functions of ACh in the CNS are mediated by ionotropic nAChRs and metabotropic mAChRs (Caulfield and Birdsall, 1998; Eglen, 2006). In mammals, mAChRs are encoded by five putative genes, chrm1–5 (Kubo et al., 1986; Caulfield, 1993; Matsui et al., 2004). These receptors have been shown to be present in insects (Schmidt‐Nielsen et al., 1977; Breer and Knipper, 1984; Knipper and Breer, 1988), molluscs (Pivovarov and Saganelidze, 1988), and show wide, but unique expression in the CNS of amphibians (Butt et al., 2000), fishes (Seo et al., 2009; Toscano‐Márquez et al., 2013), and mammals CNS (Buckley et al., 1988; Levey et al., 1991; Caulfield, 1993). In chicken, chrm2, 3, 4 and 5 are expressed in cardiac tissues and the retina (Gadbut et al., 1994; Tietje et al., 1989; Tietje et al., 1991; McKinnon and Nathanson, 1995; Creason et al., 2000). Autoradiographic studies using a non-specific muscarinic antagonist, N-methylscopolamine, have shown mAChRs are expressed in bird brains (Dietl et al., 1988; Ball et al., 1990). Importantly, the extensive studies of chrm1–5 expression in the brain have been performed only in mammals (Buckley et al., 1988; Levey et al., 1991; Caulfield, 1993). To the best of my knowledge, the detailed analyses of mAChR subtype/subunit-specific expression in the avian brain are lacking. In addition, a study found that chrm1 is not expressed in ocular and brain tissues of the chicken (Yin et al., 2004). As a result, it is not clear whether chrm1 is also absent in the neural and non-neural tissues of other avian species. Although cholinergic functions are important for learning and memory (Anagnostaras et al., 2003; Matsui et al., 2004; Hasselmo, 2006), motor skill acquisition and sensorimotor coordination (Conner et al., 2003; Ztaou et al., 2016), the potential contributions of specific mAChR subunits to motor skill learning and execution, such as human speech and birdsong is not clear.

Similar to human speech, birdsong is a learned motor skill acquired during a critical period in development using a set of conserved, interconnected neural circuits called the song pathways (Nottebohm et al., 1976). As a result, songbirds represent good animal models to investigate the neural molecular mechanisms for learned motor skill. In the song nuclei, a variety of neuromodulator/neurotransmitter receptor genes, such as melatonin (Fusani and Gahr, 2015), glutamate (Wada et al., 2004) and dopamine (Kubikova et al., 2010) receptor genes, have been shown to be differentially expressed in the song nuclei relative to surrounding brain areas. Such differential expression suggests that neuronal functions mediated by these receptors is different in different brain areas. However, the precise

regulation of these neuromodulator/neurotransmitter subtypes through the critical period of vocal learning is not clear.

In support of the finding that the song nuclei receive cholinergic projection neurons from the ventral pallidum (Li and Sakaguchi, 1987), acetylcholinesterase, an enzyme that degrades ACh at postsynaptic sites, is enriched in the song nuclei (Sadananda, 2004),.Therefore, AChRs may be expressed in the song nuclei to mediate the functions of ACh. However, the exact AChRs expression in the song nuclei and their potential functions remain largely unknown. Although *in situ* hybridization (ZEBrA; *www.zebrafinchatlas.org*) and DNA microarray data (Lovell et al., 2008; Lovell et al., 2018) have shown that mAChRs are expressed in the song nuclei, the detailed information on their exact expression in the whole brain and song nuclei is largely lacking. As a first step towards understanding these questions, I examined the expression of mAChRs in the songbird brain using adult male ZFs. I used ZFs for this experiment because of the ease in obtaining their brain tissues at the different stages of song development together with a complete recording of their song ontogeny.

1.2 Materials and Methods

Animals

To examine the precise mAChRs expression the song nuclei, I used adult male ZFs (> 120 post hatching day, phd) from our breeding colonies at Hokkaido University. The photoperiod was maintained at 13/11 hr light/dark cycle, while food and water were provided *ad libitum*. All animal experiments were conducted in line with the Guidelines of the Committee on Animal Experiments of Hokkaido University. The 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).

RT-PCR and cloning of mAChRs

The detailed cloning procedure has been described previously (Wada et al., 2004). Briefly, I tried to clone all five mAChRs (chrm1–5) already described in mammals (Levey et al., 1991). To avoid cross-hybridization between related receptor subtypes/subunits, I designed primer sets to amplify specific sequences of each mAChRs (**Table I–1)**. RT-PCR was performed on total RNA extracted from adult male ZF brain using primer sets. PCR products were visualized in 1.5% agarose gel, extracted when predicted sizes were observed, and cloned into pGEM-T easy vector plasmid. Chrm2, 3, 4, and 5 sequences (with assigned GenBank accession numbers: MH316766, MH316767, MH316768, and MH316769, respectively) were confirmed on BLASTN (DNA) and BLASTX (protein) and compared them with human sequences.

Collection and sectioning of brain tissues

I collected brains tissues in silent and dark, non-singing conditions before light-on hours. As a result, these birds did not sing for 10–12 hr before sacrifice. Birds were quickly sacrificed, and brains dissected into plastic moulds and compounded in OCT medium (Tissue-Tek, Sakura, USA). The mould was transferred to a dry ice box and later stored at −80^{o}C until it was sectioned. Sections (12 µm) were cut on the sagittal plane and mounted on silane-coated glass slides. These slides were stored at −80^{o}C until use for in situ hybridization.

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, Germany). Fresh frozen brain sections were fixed in 3% paraformaldehyde/phosphate-buffered saline (PBS, pH 7.0) for 5 min, washed 3 times in 1× PBS for 5 min each, acetylated for 10 min, washed 3 times in 2× SSPE for 5 min each, dehydrated in ascending ethanol concentrations (50, 70, 90, and 100%) for 5 min each, and then air-dried. Riboprobe (10^6 cpm) was mixed with 150 µl 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), and cover-slipped. Hybridization was performed in an oil bath for 14 hr at 65^{o}C. Next, the slides were washed step-wise in two different changes of chloroform, 2× SSPE/0.1% 2-mecaptoethanol for 30 min, 50% formamide/0.1% 2-mecaptoethanol for 60 min, 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%), air-dried, and exposed to BioMax MR film (Kodak, USA) for 4–5 days before development. The slides were then immersed in an NTB2 emulsion (Kodak, USA) and exposed for 3–4 weeks. This duration was optimal for avoiding the saturation of mRNA signals. The glasses were developed, counter-stained with cresyl violet, cover-slipped with Permount (Fisher Scientific, USA) in xylene, and air-dried. mRNA signals were quantified as described previously (Wada et al., 2006). Briefly, the X-ray films were digitally scanned under a microscope (Z16 Apo, Leica, USA), which was connected to a CCD camera (DFC490, Leica, USA), with a Leica Application Suite, v3.3.0 (Leica, USA). Light and camera settings were maintained for all images to ensure unbiased comparisons. The images were converted to a 256-gray scale and mRNA expression levels were quantified as mean pixel intensities using Adobe Photoshop CS2 (Adobe Systems, USA). The drawings of the areas of interest in the brain were based on Nissl-defined features, and were verified from the ZF brain atlas (Zebra Finch Song Learning Consortium; Karten et al., 2013) .

Qualitative evaluation of mRNA expression level in major brain subdivisions

To qualitatively show mAChRs expression abundance in major brain areas, I represented mRNA expression levels as very low (+/-), moderate (++), high (+++), and very high (++++).

1.3 Results

The general mAChR expression patterns in the zebra finch brain

Using brain tissues of adult ZFs and RT-PCR with oligo primers (**Table I–1**) for regions of the genome conserved among mammals, birds, and reptiles, I successfully cloned four (chrm2–5) out of the five mAChRs (**Table I–1**) known in mammals (Caulfield and Birdsall, 1998). The positions of the cloned fragments in the ZF genome were identified based on the BLAST alignment tool in the UCSC genome browser (**Figure I–3**). Even though I could not find a predicted coding region for chrm1 in the zebra finch genome (Taeniopygia_guttata taeGut3.2.4.dna.fa), I attempted to clone it using degenerate primers for chrm1 conserved regions among mammals, reptiles, and amphibians. Similar to a report in the chicken (Yin et al., 2004), I could not obtain its PCR fragment from the ZF brain, suggesting that chrm1 is absent in the ZF brain. However, I did not examine whether it is also absent in non-neural tissues of the ZF. The chrm2-5 which I cloned in this study were all identified as seven transmembrane guanine protein-coupled receptors, GPCRS **Figure I–3**), using the Blastp tool on the NCBI database

Next, I performed radioisotope *in situ* hybridization to examine chrm2–5 expression patterns in the adult ZF brains. I found that each mAChRs has unique expression pattern in the pallial brain subdivisions: hyperpallium (H), mesopallium (M), N, and A. Chrm2 expression level was similar and consistently low throughout these pallial regions (**Figures I–4 and I–5**). Chrm3 and chrm4 revealed mirrored expression patterns in the pallial regions: chrm3 expression was lower in M and A than in H and N, whereas chrm4 expression was higher 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 sub-regions. In addition, chrm3 and chrm5 expression were lower in the intercalated hyperpallium (IH) compared with H (**Figure I–5A**). Exceptions to these expression patterns were observed in the pallium for chrm2 in the dorsal nucleus of H (DNH) (Mouritsen et al., 2005), chrm3 in anterior A, and chrm4 and chrm5 in posterior A, which had higher expression levels than each pallial subdivision. In addition, expression of all subunit was low in field L2, entopallium and nucleus basorostralis (**Figure I–4 and I–5B**), which are sensory input areas analogous to layer IV of mammalian auditory, visual, and somatosensory/trigeminal cortical areas, respectively (Jarvis et al., 2013).

In the subpallium, chrm2 and chrm4 expression levels were higher in the Str than chrm3 and chrm5. Chrm2 expression was intense in P compared with other subunits (**Figure I–5B**). The differential mAChRs expression in the pallial compared with subpallial brain

subdivisions corresponds to the expression of homologous mAChRs in the pallial compared with the basal ganglia subdivisions of the mammalian brain (Levey et al., 1991). All mAChR subunits were not expressed in the dorsal thalamic nuclei (nucleus rotundus, nucleus pretectalis, and nucleus spiriformis lateralis). In the midbrain tectum opticum, chrm2 and chrm4 expression were higher than chrm3 and chrm5. In the cerebellum, chrm2 and chrm4 had higher expression than chrm3 and chrm5 from the white matter layer, granular layer, and Purkinje layer to the molecular layer (**Figure I**–**4**). These suggest that each mAChR subunit plays unique roles in different subdivisions of the telencephalon and other brain regions in the ZF.

Differential mAChRs expression in the song nuclei

Next, to examine mAChRs expression in the song nuclei of adult male ZFs, I focused on the following five major song nuclei: HVC and RA in the VMP, and LMAN, Area X and aDLM (Horita et al., 2012) in the AFP. In contrast to the unique expression of all mAChRs among pallial brain subdivisions, there was differential expression (higher or lower) of all mAChRs in at least one song nucleus compared with surrounding brain areas (**Figure I**–**6**). In the pallial song nucleus HVC, chrm2 and chrm4 expression were higher, whereas chrm3 was lower compared with the surrounding caudal N (cN) (**Figure I**–**6**). In RA, chrm3, chrm4, and chrm5 expression levels were differentially lower than in A. The expression of all mAChRs in LMAN were generally lower and non-differential than in the surrounding rostral N (rN). In Area X, only chrm4 exhibited differentially higher expression than in the surrounding Str. However, this difference in expression might be caused by a higher cell-specific mRNA expression levels, rather than cell density in Area X compared to the Str. In the thalamic song nucleus aDLM, chrm2 had lower differential expression than in the surrounding DLM. In summary, HVC had higher differential expression of chrm2 and chrm4 than the surrounding cN. In contrast, mAChR expression in RA, LMAN, and aDLM were lower than in the surrounding A, rN, and DLM, respectively. Chrm2 and chrm4 expression in Area X were higher than those of chrm3 and chrm5 (**Figure I**–**6**).

1.4 Discussion

The cholinergic system in the forebrain, mediated by AChRs is important for motor co-ordination (Ztaou et al., 2016), and motor skill learning (Conner et al., 2003). mAChRs are coupled to distinct G-protein families to modulate neuronal excitability via intracellular signal transductions (Hulme et al., 1990; Wess, 1996) In rodents, mAChRs have been shown to be expressed in brain areas important for learning and memory (Levey et al., 1995). However, the exact mAChRs expression patterns in the neural circuits for learned motor skills characterized by species and individual differences remain largely unexplored. In this Chapter, I have demonstrated a unique expression of chrm2–5 in the ZF brain, including the song pathways for song learning and production.

Availability of chrm1 in other vertebrate species

One of my key findings in this Chapter is the unique chrm2–5 expression in the ZF brain. My results are consistent with a stronger expression of chrm2 and chrm4 than chrm3 and chrm5 in the cortex and striatum compared with the thalamus and brainstem in mammals (Zhang et al., 2002). Although I cloned chrm2–5 but could not obtain chrm1 PCR fragment from the ZF brain, the existence of chrm1 in the genomes of the frog, *Xenopus tropicalis* (GenBank accession No. XM_004913660.3), and the reptile, *Alligator mississippiensis* (GenBank accession No. XM_019496993) as predicted transcripts, is evident on the National Centre for Biotechnology Information (NCBI) genome database. Furthermore, a pharmacological study (Garnier et al., 1998), suggested the presence of chrm1 in the genome of the frog, *Rana ridibunda*. These suggest that: (1) the common ancestor of birds and mammals possessed most mAChRs, and (2) chrm1 expression was greatly suppressed in neural and non-neural tissuesduring the evolution of avian species. However, it is not clear whether the suppression of chrm1 expression is conserved among other avian species.

Unique mAChRs expression in the ZF brain

Except in the subthalamic nuclei, L, E, and B, I found unique expression of chrm2–5 in the ZF brain, suggesting that each mAChR has different contributions to the regulation of cortical and subcortical brain functions. The unique mAChRs expression in the cortex and striatum compared with the subthalamic nuclei suggests that mAChRs are involved in learning, memory, and motor functions by modulating neuronal excitability in these brain areas. In contrast to the unique expression of all cloned mAChRs in major brain subdivisions,

expression was differential in the song nuclei. Chrm3 and chrm5 were very weakly expressed, whereas chrm2 and chrm4 expression were high in HVC (chrm2 and chrm4) and Area X (chrm4). HVC is a premotor song nucleus that is important for the regulation syllable sequence in a song (Yu and Margoliash, 1996), suggesting that chrm2 and chrm4 contribute to the premotor activity of HVC neurons. Chrm4 had the highest expression in Area X compared with other mAChRs in the song nuclei. Area X is part of the pallial–basal ganglia–thalamic loop that contributes to the generation of temporally unlocked neural bursts during undirected singing (Hessler and Doupe, 1999; Andalman and Fee, 2009). Therefore, chrm4 could be important for the context-dependent changes in neural activity bursts in Area X via ACh-mediated modulation. In mammals, chrm2 and chrm4 are expressed pre- and post-synaptically for modulating the release and action of ACh on postsynaptic sites (Levey et al., 1995; Quirion et al., 1995; Baghdoyan et al., 1998). In the zebra finch, cholinergic projection neurons to the HVC originate in the ventral pallidum (Li and Sakaguchi, 1997). Additionally, immunostaining for acetylcholineesterase, AChE (Sadananda, 2004) and choline acetyltransferase (Zuschratter and Scheich, 1990) have shown that the HVC is enriched with ACh-containing neurons. Therefore, the auto-modulation of ACh release by chrm2 and chrm4 could contribute to the changes in ACh concentration in HVC during song development (Sakaguchi and Saito, 1989).

1.5 Figures

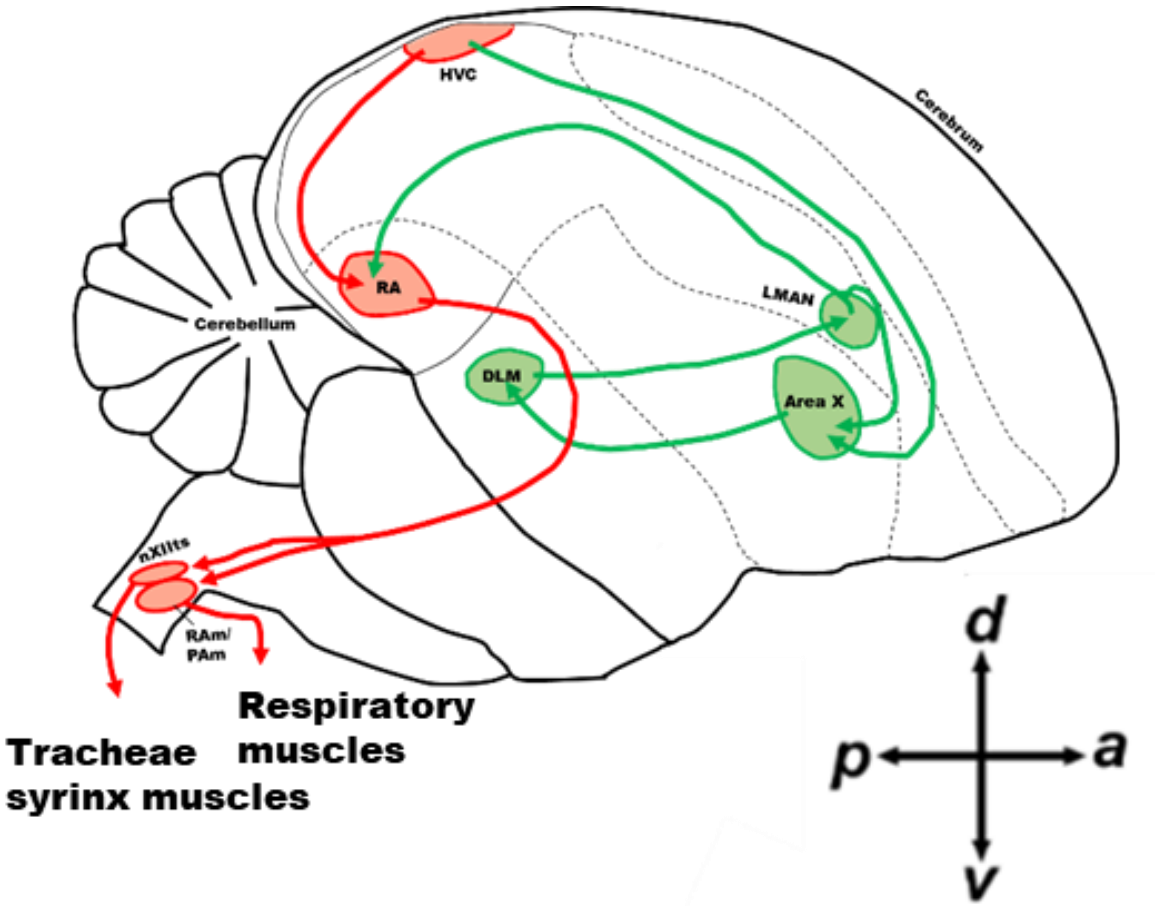

Figure I–1: The song control system of songbirds.

The vocal motor pathway (VMP): Solid red lines. The anterior forebrain pathway (AFP): Solid green lines. Axes indicate brain orientation: a = anterior, p = posterior, d = dorsal, v = ventral.

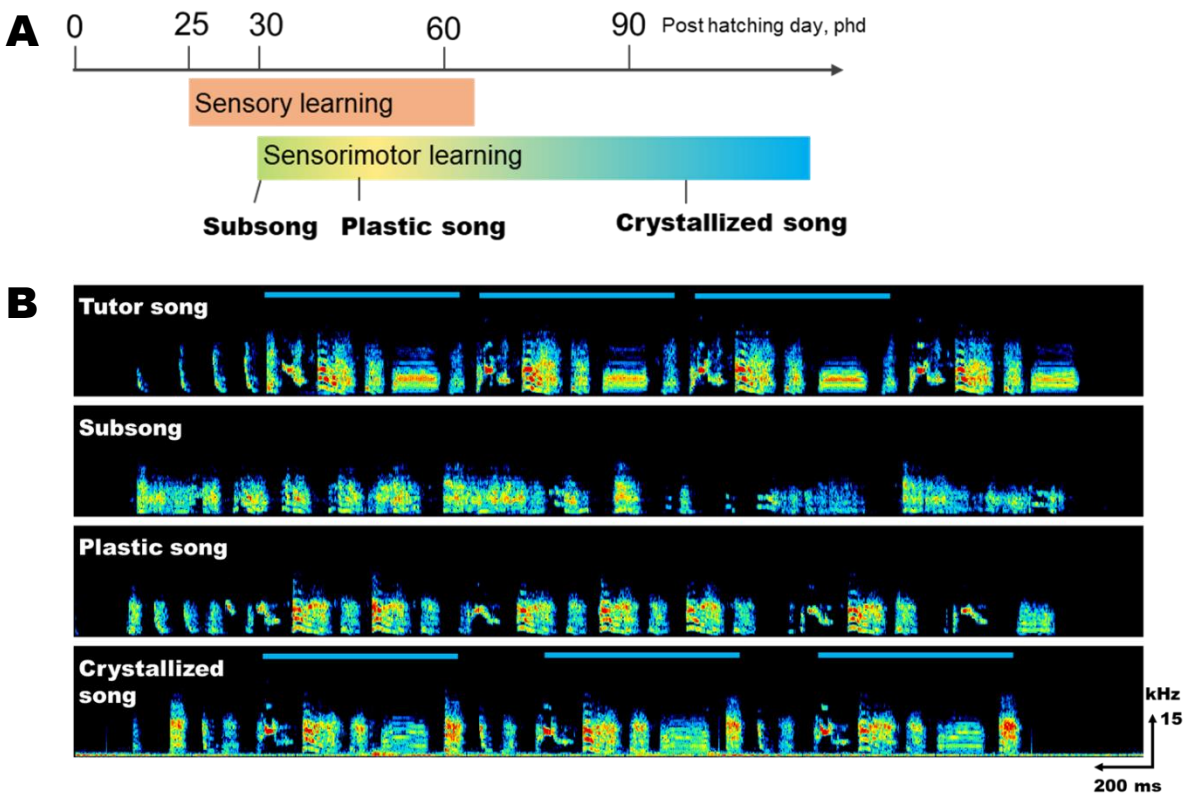

Figure I–2: Song development in ZF

A: The time-course of song development of in the zebra finch.

B: Sonograms showing the stages of song development from an adult male ZF tutor song. Blue bars indicate motif units of song.

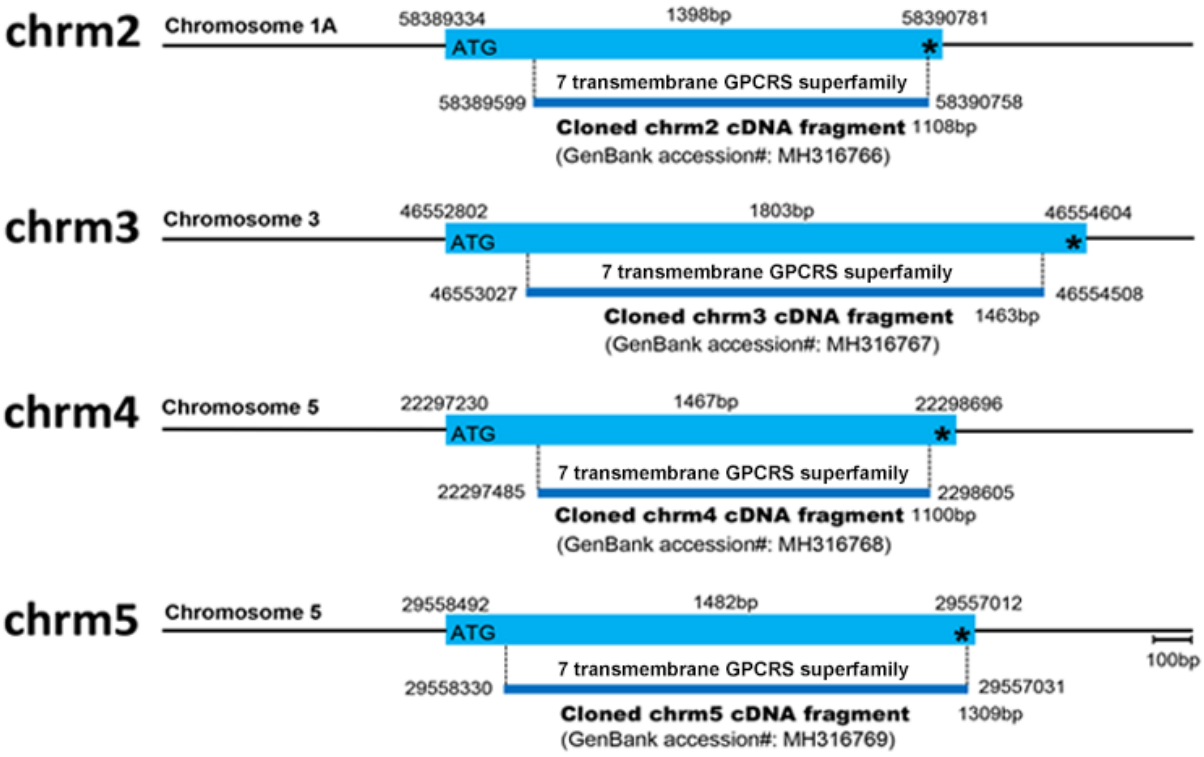

Figure I–3: Location of cloned partial cDNA fragments of chrm2–5 in the ZF genome.

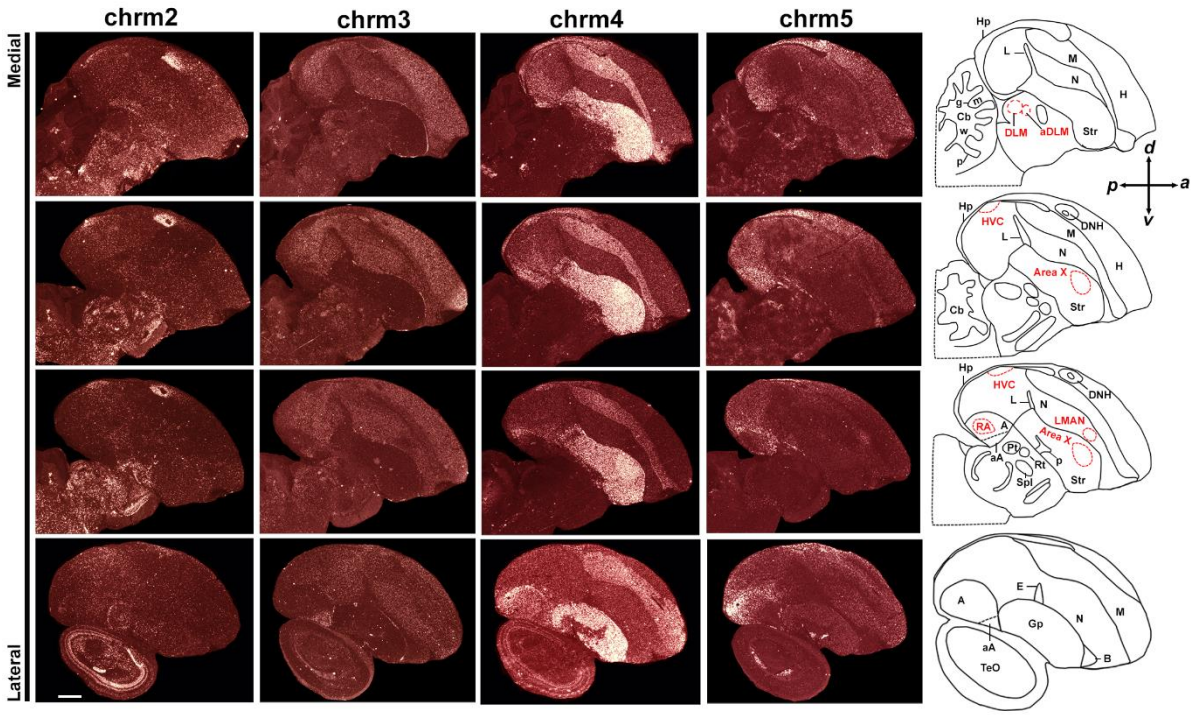

Figure I–4: Higher magnification images showing chrm2–5 mRNA expression in major brain subdivisions

Serial brain images showing the general expression of chrm2–5 (medial-lateral).

Scale bars = 1mm. Right column: Camera lucida drawing of brain areas: Hp, hippocampus; Cb, cerebellum; H, hyperpallium; M, mesopallium; N, nidopallium; DNH, dorsal nucleus of the hyperpallium; HVC (proper name),; A, arcopallium; aA, anterior arcopallium; RA, robust nucleus of the arcopallium; LMAN, lateral magnocellular nucleus of the anterior nidopallium; Area X, Area X of the striatum; Str, striatum; DLM: dorsa-lateral nucleus of the medial thalamus; aDLM, anterior dorso-lateral nucleus of the medial thalamus; L, field L; E, entopallium; B, nucleus basorostralis; Pt, nucleus pretectalis; Rt, nucleus rotundus; Spl, nucleus spiriform lateralis; Gp, globus pallidus; P, pallidum; Teo, tectum opticum. White and red colours represent mRNA and cresyl violet signals, respectively.

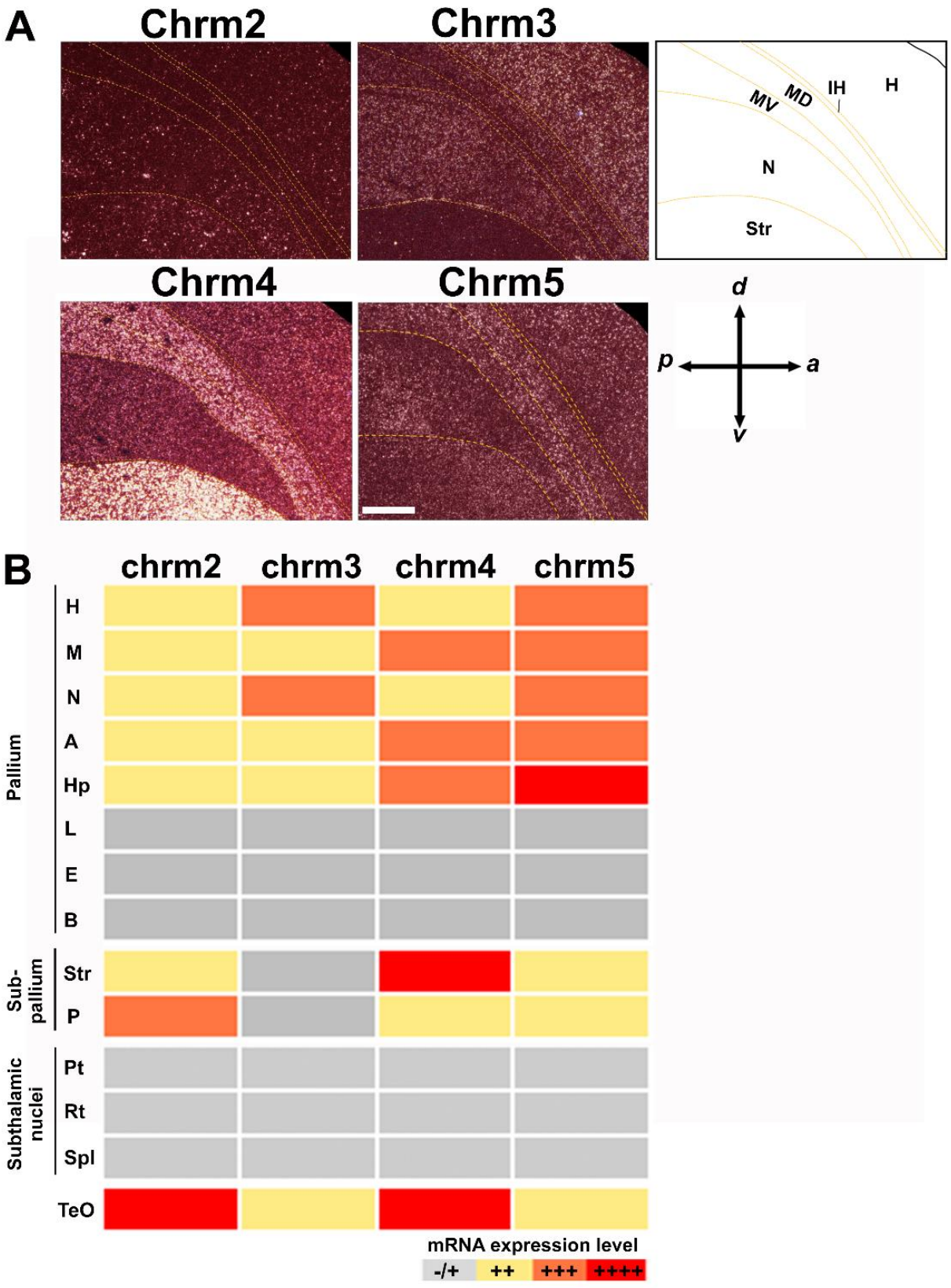
A
Chrm2
Chrm3
Chrm4
Chrm5
MV
MD
IH
H
N
Str
d
p
a
v
B
chrm2
chrm3
chrm4
chrm5
Pallium
H
M
N
A
Hp
L
E
B
Sub-pallium
Str
P
Subthalamic nuclei
Pt
Rt
Spl
TeO
mRNA expression level
-/+
++
+++
++++

Figure I–5: mAChRs expression in the pallium and striatum

A: Chrm2–5 expression in the pallium, sub-pallium and sub-thalamic nuclei. Scale bar = 3mm. Right: Camera lucida drawing of brain subdivisions. Orange dotted lines represent boundaries of the brain subdivisions: H, intercalated hyperpallium (IH), dorsal mesopallium (MD), ventral mesopallium (MV). White and red colours are mRNA and cresyl violet signals, respectively.

B: Heatmap showing chrm2–5 expression in sub-divisions of the ZF brain.

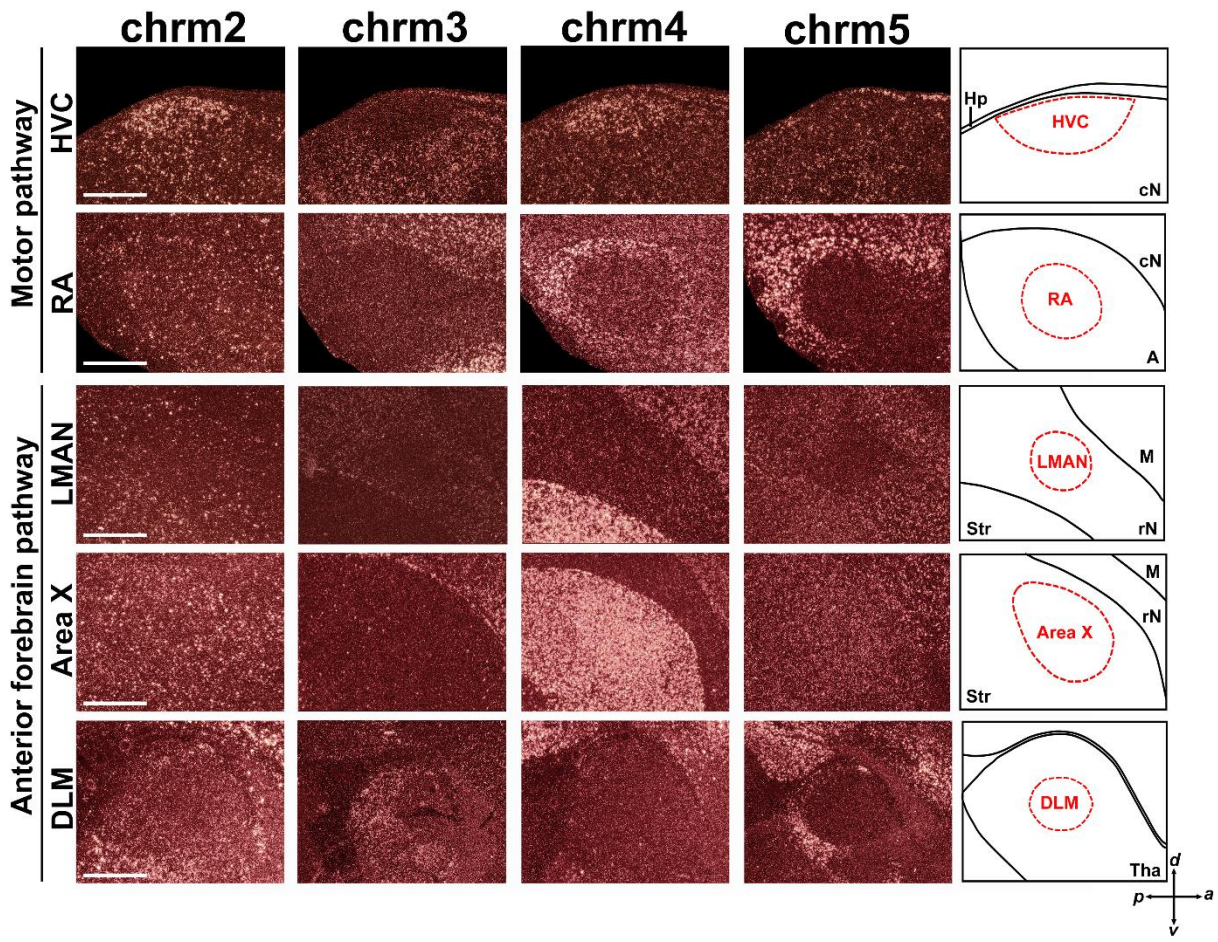

Figure I–6: Higher magnification images showing chrm2–5 mRNA expression in the song nuclei.

Scale bars: 1mm. Right column: Camera lucida drawing of brain areas: cN, caudal N; rN, rostral N. White and red colours represent mRNA and cresyl violet signals, respectively.

1.6 Tables

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

Gene	GenBank accession #	Forward primer	Reverse primer	Amplified fragment length (bp)
chrm2	MH316766	5`-ATGAACCTGTACACCCTTTAC-3`	5`-GTCATTACAAGAATATAGGAGC-3`	1108
chrm3	MH316767	5`-GGGTGGACACACTATCTGG-3`	5`-CACTTTCAAGATGCTGCT-3`	1463
chrm4	MH316768	5`-ATTTCCTCTTCAGCCTGGCC-3`	5`-TGTCAACAGCACCATCAACC-3`	1100
chrm5	MH316769	5`-CCTGTGCAGATCTTATCATTG-3`	5`-AGAGAAACTATATTGGCAGGG-3`	1309

Table I–1: Primers pairs used for cloning mAChRs with assigned GenBank accession numbers in NCBI database

Table 2: Homology (%) of cloned mAChRs fragments with existing chicken and human mAChRs sequences

Gene	Chicken		Human	
	cDNA	Protein	cDNA	Protein
Chrm2	89% NM 001030765.1	92% NP_001025936.1	78% NM 001006632.1	83% AAK68113.1
Chrm3	92% NM 205399.1	92% NP 990730.1	78% NM 001347716.1	83% NP 000731.1
Chrm4	91% NM 001031191.1	74% NP 000732.2	75% NM 000741.3	74% NP 000732.2
Chrm5	88% NM 001364658.1	89% NP 001351587.1	71% NM 001320917.1	68% NP 036257.1

Chapter II

Species and individual differences in chrm2 expression in HVC

2.1 Introduction

Birdsong is a complex motor skill characterized by syllable sequence and acoustics. It is acquired during a critical period in development using the song nuclei/pathways (Nottebohm et al., 1976). The song nuclei is composed of the AFP and the VMP (Nottebohm et al., 1976; Bottjer et al., 1984; Scharff and Nottebohm, 1991). The AFP comprises three song nuclei, Area X, LMAN, and DLM (Luo et al., 2001), whereas the VMP is made up of the premotor song nuclei HVC and RA. Song development occurs in two functional stages: sensory and sensorimotor learning phases. During the sensory learning phase (25–65 phd), juvenile male songbirds such as the ZF listen to and memorize a tutor song model. In the sensorimotor learning stage (30 to > 90 phd), they try to match their vocalizations with the memorized tutor model through repeated practice (Immelmann, 1969; Eales, 1985). This shows that auditory input (for perception of the tutor song and evaluation of their own vocal utterances) is important for song learning. Consequently, deafening impairs song learning (Konishi, 1965), delays the timing of song stabilization (Mori and Wada, 2015), and leads to the degradation of an already crystallized learned song (Lombardino and Nottebohm, 2000). In addition to auditory input, song learning is dependent on hormonal (especially testosterone) regulation. Particularly, castration slows song learning (Korsia and Bottjer, 1991) and causes song degradation (Arnold, 1975; Wang et al., 2014). In addition, application of exogenous testosterone induces faster stabilization of songs in ZFs (Korsia and Bottjer, 1991; Sizemore and Perkel, 2011).

The HVC exhibits unique responsiveness to playback of bird's own song (BOS) than to other species' songs (Margoliash, 1983; Theunissen and Doupe, 1998). Such unique response is believed to be important for auditory-mediated feedback comparison during the critical period of song learning and production (Shea and Margoliash, 2003). Furthermore, the HVC possesses various type of neurons including two glutamatergic excitatory projections neurons to Area X (HVC_X) in the AFP and to RA (HVC_{RA}) in the VMP, GABAergic local inhibitory interneurons (HVC_I) and glia, each with unique electrophysiological and pharmacological properties (Dutar et al., 1998; Kubota and Taniguchi, 1998). These neurons have unique activity patterns during singing and hearing through the critical period of song learning (McCasland and Konishi, 1981; Hahnloser et al., 2002; Kozhevnikov and Fee, 2007), suggesting that they contribute differently to HVC's functions. The neural molecular mechanisms for HVC_X-, HVC_{RA}-, and HVC_I-driven contribution to HVC's functions during song learning and production is not understood. It has been shown that stimulation of the

cholinergic basal forebrain suppresses the neural responsiveness of HVC and RA neurons to BOS (Shea and Margoliash, 2003).

There are over 4,500 species of songbirds with species-specific vocal patterns (Marler and Slabbekoorn, 2004). These species-specific songs are produced using similarly conserved song pathways (Nottebohm et al., 1976; Brenowitz and Beecher, 2005). Even within a species (such as the ZF), individuals not only exhibit differences in the strategies to learn a song (Liu et al., 2004), they also show variability in their acquired vocal patterns (Tchernichovski et al., 2001; Sato et al., 2016). Although the potential neural molecular mechanisms regulating species/individual differences in learned vocalization are unclear, it has been revealed that parental genetic information play a crucial role, suggesting that specific genes are involved. However, it is not clear which genes mediate the innate regulation of vocal learning.

The song nuclei HVC and RA receive cholinergic projection neurons from the ventral pallidum (Li and Sakaguchi, 1997). These neurons were also shown to be positive for choline acetyltransferase (Zuschratter and Scheich, 1990), the enzyme that catalyzes reuptake of choline (one of the breakdown products of ACh) back into cholinergic neurons suggesting that the HVC contains ACh-producing neurons. In the ZF, the concentration of ACh is up-regulated in the song nuclei HVC, LMAN and RA during the critical period of song learning and declined at the crystalized song stage in adulthood (Sakaguchi and Saito, 1989). However, the identities of potential candidate AChRs that could regulate the functions of ACh during the critical period of vocal learning remain elusive.

To answer these questions, I investigated mAChRs expression level in the song nuclei through the critical period of song learning in the ZF to understand their potential contributions to song development. Based on the result of mAChRs expression during song development, examined the degree of individual differences in chrm2 expression level in HVC using completely different sets of ZF brains. I further evaluated the potential contribution of the testosterone- and auditory-driven modulation of the timing of song stabilization to chrm2 expression level in HVC, and chrm2 expression among adults of other songbird species and in different types of neuron in the HVC.

2.2 Materials and methods

Animals

To understand whether the expression of mAChRs changes during song learning, I examined chrm2–5 mRNA expression levels in male ZFs at three stages of song development: subsong (30–45 phd), plastic song (50–65 phd,), and crystallized song (> 120 phd), with n = 6 birds/group. To investigate the possibility that chrm2 expression levels in the HVC vary among individual ZF, I used different sets of brain at the three song development stages and increased the animal number in each group to n = 12. Additionally, I added a fourth experimental group, the presubsong stage (21–26 phd, n = 8). The inclusion of the presubsong group enabled me to evaluate possible age-dependent regulation of the individual differences in chrm2 expression in HVC. Then, I analysed mAChRs expression levels among these groups. For these experiments, Juvenile ZFs were raised with their biological fathers until fledging (~ 30 phd). Their sexes were determined by PCR as described previously (Wada et al., 2006). To evaluate the contribution of testosterone- or auditory-driven modulation of the timing of song stabilization on chrm2 expression in HVC, I compared chrm2 mRNA expression levels in testosterone-implanted and early-deafened ZFs (collected by former students of Wada laboratory, Dr. Chihiro Mori and Shin Hayase, respectively) with those of intact, age-matched group. To understand the contribution of parental genetic information to chrm2 expression in HVC, I examined chrm2 mRNA expression levels in HVC among sibling ZFs from nine (9) different families. To further explore the relationship between genetic information and individual differences in chrm2 expression in HVC, I analysed chrm2 expression level among adults of other songbird species: star finch, SF (*Neochmia ruficauda*), Bengalese finch, BF (*Lonchura striata var. domestica*), Java sparrow, JS (*Padda oryzivora*) and the canary, CN (*Serinus canaria*) (>120 phd, n = 8/species). For this experiment, ZFs and BFs were obtained from our breeding colonies in Hokkaido University, whereas other species were purchased from local breeders in Japan. Photoperiod was maintained at 13/11 hr light/dark cycle, while food and water were provided *ad libitum*. All animal experiments were carried out in line with Guidelines of the Committee on Animal Experiments of Hokkaido University. 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 individually in sound-attenuation boxes (65cm x 27cm x 30cm) inside metallic cages (54cm x 22cm x 23cm). Songs were recorded automatically on a 24 hr basis through a microphone (SHURE SM57) connected to a computer installed with the Sound Analysis Pro 2011 program version 1.04, at 16 bits and 44 kHz sampling rate (Tchernichovski et al., 2000).

Collection and sectioning of brain tissues

I collected brain tissues in silent and dark, non-singing conditions before light-on hours. Therefore, these birds did not sing for 10–12 hr before sacrifice. Brain samples were dissected into a plastic mould and compounded in OCT medium (Tissue-Tek, Sakura, USA). The mould was transferred into a dry ice box, and thereafter stored at −80^{o}C until sectioning. Sections (12 µm) were cut on the sagittal plane, mounted on silane-coated glass slides, and stored at −80°C until used for in situ hybridization.

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, USA). Fresh frozen brain sections were fixed in 3% paraformaldehyde/1× phosphate-buffered saline (PBS, pH 7.0) for 5 min, washed 3 times in 1x PBS for 5 min each, acetylated for 10 min, washed 3 times in 2× SSPE for 5 min each, dehydrated in increasing ethanol concentrations (50, 70, 90 and 100%) for 5 min each, and air-dried. Riboprobe (10^6 cpm) was mixed with 150 µl 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 hr 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 min, 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%) for 5 min each, and air-dried. The slides were exposed to BioMax MR film (Kodak, USA) for 4–5 days before development. The slides were then immersed in an NTB2 emulsion and exposed for 3–4 weeks. These durations were optimal for avoiding mRNA signal saturation. The emulsion-coated glasses were developed, counter-

stained with cresyl violet, cover-slipped with Permount (Fisher Scientific, USA) in xylene, and air-dried. mRNA signals were quantified as described previously (Wada et al., 2006).
The X-ray films were digitally scanned under a microscope (Z16 Apo, Leica, USA) which was 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 comparison. Captured images were converted to a 256-gray scale, and mRNA expression levels were quantified as mean pixel intensities using Adobe Photoshop CS2 (Adobe Systems, USA). The drawings of the areas of interest in the brain were based on Nissl-defined features and verified from the ZF brain atlas (Zebra Finch Song Learning Consortium) (Karten et al., 2013).

Testosterone (T) administration

Exogenous T was implanted as described before (Hayase and Wada, 2018). Each bird was anesthetized by an intraperitoneal injection of pentobarbital sodium (6.48 mg/mL; 60 μL/10 g body weight). The 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). After surgery, the birds were placed on a heat pad in a cage until they started eating, drinking and flying around the cage. Brain tissues were collected at 9 AM after lights-on (at 8 AM) between 43 and 53 phd [T-implanted, 47.6 ± 2.9 (mean ± SD)].

Deafening

Birds was deafened by bilateral extirpation of the cochleae as described previously (Konishi, 1964; Mori and Wada, 2015) before fledging (17–23 phd). Each bird was anesthetized by an intraperitoneal injection of 6.48 mg/ml (0.60 μl/g of body weight) pentobarbital sodium (Mori and Wada, 2015). The head was fixed on a customized stereotaxic apparatus equipped with horizontal ear bars. The skin overlying the skull bone was opened and 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 created to expose each cochlear which was removed with the aid of a hooked wire. The removed cochleae were visually confirmed under a dissecting microscope based on morphology. After the surgery, the birds were returned to their nests and remained with their parents until approximately 32–41 phd. The same sets of early-deafened ZF brains which were used for

this *in situ* hybridization with chrm2 probe were reported previously by our laboratory (Mori and Wada, 2015).

Fluorescence in situ hybridization

For double-labelling of chrm2 with gene markers of the cell-types in HVC, I used 40 ng of dinitrophenol (DNP)- or 100–500 ng of digoxigenin (Toscano‐Márquez et al.)-labelled 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) in hybridization solution (50% formamide, 10% dextran sulphate, 1× Denhart's solution, 1 mM EDTA pH 8.0, 33 mM Tris-HCL pH 8.0, 600 mM NaCl, 0.2 mg/µl yeast tRNA, 80 mM DTT, and 1% N-lauroylsarcosine). Hybridization was performed in an oil bath for 14 hr at 65ºC. Thereafter, the slides were washed twice in chloroform, dipped in 2× SSC/0.1% Tween 20, incubated in 5× SSC/0.1% Tween 20 for 30 min at 65ºC, formamide I solution (50% formamide/4× SSC/0.1% Tween 20) for 40 min at 65ºC, formamide II (50% formamide/2× SSC/0.1% Tween 20) for 40 min at 65ºC, 3x in 0.1× SSC/0.1% Tween 20 for 15 min each at 65ºC. Then, the slides were washed in NTE buffer for 20 min at room temperature (RT), three times in 1× TNT buffer for 5 min each at RT. DNP probes were detected with an anti-DNP horseradish peroxidase (HRP)-conjugated antibody with TSA plus system (PerkinElmer). To eliminate 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 with an anti-DIG HRP-conjugated antibody (Jackson, Bar Harbor, ME, USA) with a TSA system, Alexa Fluor 488 (Invitrogen). The probe signals were captured by fluorescence microscopy (EVOS, FL, Thermos Fisher Scientific, USA).

Statistical analysis

All statistical analyses were performed using the SPSS software package Ver. 16.0 (IBM Statistics). After a homoscedasticity test for the homogeneity of variances, I used a one-way analysis of variance, ANOVA, to compare the mean mAChRs mRNA expression levels in different song nuclei among the song development stages. I used a two-way ANOVA with the Games-Howell's correction (which controls for unequal samples sizes and heterogeneity of variances), to compare the mean chrm2 mRNA expression ratio (HVC/cN) among sibling

ZFs from different families. I used the unpaired Student's *t*-test to compare the mean chrm2 expression levels between intact and T-implanted ZFs (juveniles), and intact against early-deafened ZFs adults. I used one-way ANOVA with Welch test (which controls for unequal sample sizes and heterogeneity of variances) to compare the mean chrm2 mRNA expression ratio (HVC/cN) in among different songbird species. For all comparisons, statistical significance was set at $p < 0.05$.

2.3 Results

Developmental regulation of mAChRs through the critical period of song learning

To understand the potential contributions of mAChRs through the critical period of song learning, I analysed chrm2–5 mRNA expression levels in HVC, RA, LMAN, Area X, and aDLM at three song development stages (subsong, plastic song, and crystallized song) in ZFs. I found that chrm3 and chrm5 mRNA expression were consistently low through the song development stages in all song nuclei (n = 6 birds/group, one-way ANOVA, $p \geq 0.05$) (**Figure II**–**1**). In contrast, chrm4 expression was significantly changed only in HVC during song development (One-way ANOVA, $*p < 0.05$). On the other hand, chrm2 expression level in HVC showed non-significant developmental change characterized by an increase from the subsong to the plastic song stage, and a decline at the crystallized song stage. Conversely, chrm4 expression increased along with song development (gradually from the subsong stage to the crystallized song stage), suggesting that chrm2 and chrm4 could have different contributions to song learning and production. In addition, while analysing mRNA expression in these birds, I observed distinct individual differences in chrm2 expression level in HVC, which were represented as large standard errors in the bar graph (**Figure II**–**1**, standard error was used for the plots to show that high variability still existed without standard deviation). Although the developmental changes in chrm2 expression level was not significant, I further examined it in the following experiments to understand its potential contribution to species and individual differences in learned vocalizations.

Individual differences in chrm2 expression in HVC of ZFs

To evaluate potential individual difference in chrm2 expression in HVC, I increased the sample size to 12 birds at each developmental stage, added a fourth group (the presubsong stage). To minimize the impact of experimental handling on mRNA signal, I normalized mRNA expression in each song nuclei by the respective surrounding brain areas. Then, I compared chrm2 expression ratios in HVC in these groups, and chrm4 mRNA expression ratios in HVC as a control using the same sets of brains. Although there were no individual differences in chrm4 mRNA expression ratios in HVC at all four development stages (**Figure II**–**2C & D)**, I found clear individual differences in chrm2 expression levels in HVC during song development (**Figure II**–**2A, B & C**). However, when I examined chrm2 expression ratio in HVC at the pre-subsong stage, there were no clear individual differences (**Figure II**–**2B**). This age-regulated variability was reflected in a higher coefficient of variation (CV) of

chrm2 expression ratio in HVC at other song development stages compared with the presubsong stage (**Figure II-2D**, top). In contrast, the CV of chrm4 expression ratio in HVC showed no clear differences at all stages of song development (**Figure II**–**2D**, bottom).

Chrm2 expression is neither testosterone- nor audition-dependent

Individual differences in chrm2 expression in HVC clearly emerged from the subsong stage and was maintained into adulthood (**Figure II**–**2B**). Testosterone (T) has been implicated in the natural crystallization of song (Marler et al., 1988), and induces immature stabilization of song (Korsia and Bottjer, 1991; Sizemore and Perkel, 2011). To understand whether T-driven modulation of the timing of song stabilization contributes to chrm2 expression level in HVC, I used brains juvenile ZFs implanted with exogenous testosterone before the onset of first singing at 30 phd (Hayase et al., 2018). A study in our laboratory previously reported that T-implantation caused an increase in circulating T levels (10.5 ± 1.3 ng/mL at 47.64 ± 2.9 phd) compared with those of normally reared birds of similar age (1–2 ng/mL) (Mori and Wada, 2015). Then, I compared chrm2 expression ratio in the HVC between these groups of ZFs. I found no significant differences in chrm2 expression ratio in HVC between T-implanted and normally reared ZFs (Unpaired Student's *t*-test, $p \geq 0.05$). In addition, the CV of chrm2 expression ratio in HVC was similar between the two groups (**Figure II**–**3A**, right).

Auditory inputs through hearing a tutor song and self-vocalization regulate song development (Konishi, 1965; Mori and Wada, 2015) and delays the timing of song crystallization (Konishi, 1965; Mori and Wada, 2015). To evaluate if auditory-driven modulation of the timing of song stabilization contributes to chrm2 expression level in HVC, I examined chrm2 expression ratio in the HVC of early-deafened, and intact normally reared ZFs (> 120 phd). I found no significant differences in chrm2 expression level in HVC between these two groups (Unpaired Student's *t*-test, $p \geq 0.05$; **Figure II**–**3B**, left). In addition, the CVs of chrm2 expression ratio between the two groups did not show clear differences (**Figure II**–**3B,** right). However, the early-deafened ZFs similarly show individual differences in chrm2 expression levels in HVC (**Figure II**–**3B**, left). These suggest that testosterone- or auditory experience-driven modulation of the timing of song stabilization does not influence chrm2 expression level in HVC. Rather, chrm2 expression in HVC may be regulated by genetic-mediated mechanisms.

Chrm2 is expressed in most types of neurons in the HVC of ZFs

The HVC possess various types of neurons including two excitatory glutamatergic projection neurons to RA (HVC_{RA}) and Area X (HVC_X), GABAergic inhibitory neurons, each with distinct pharmacological and physiological properties (Dutar et al., 1998; Kubota and Taniguchi, 1998). To understand the potential functional contribution of chrm2 to HVC function, I examined the types of neurons in HVC that express chrm2. For this, I analysed the co-expression of chrm2 mRNA with gene markers of the types of neurons in HVC: UTS2D for HVC_{RA}, NTS for HVC_X, vglut2 and Gad2 for excitatory and inhibitory neurons, respectively (Wirthlin et al., 2015). I found that chrm2 mRNA was expressed in most HVC neurons including excitatory HVC_{RA} and HVC_X neurons and inhibitory interneurons in the zebra finch (**Figure II–4A & B**). This suggests that chrm2 have important contributions to the modulation of the excitability of HVC neurons.

Familial bias in chrm2 expression in HVC of ZF

Juvenile ZFs show individual differences in their vocal patterns as early as the subsong stage (Tchernichovski et al., 2000). Such variability has been shown to be associated with parental genetic information (Sato et al., 2016). To understand whether parental genetic information contributes to chrm2 expression level in HVC, I quantified chrm2 mRNA expression level in sibling ZFs from different breeding families. Although siblings from the same family showed individual variability in chrm2 expression, there was a significant difference in chrm2 expression level in HVC among breeding families (n = 26 birds in 9 families, two-way ANOVA with Games-Howell's test, $p = 0.035$) (**Figure II–5**). In contrast, age, or its interaction with family did not influence in chrm2 mRNA expression level among the tested families ($p = 0.216$, and $p = 0.982$ respectively). This result further supports the idea genetic-mediated mechanisms may contribute to the individual differences in chrm2 expression in HVC of ZFs.

Species-specific regulation of chrm2 expression in HVC

To further examine whether genetic mechanisms regulate chrm2 expression level in the HVC, I analysed chrm2 expression level in adults (> 120 phd) of six songbird species: ZF, OF, SF, BF, JS, and CN (**Figure II–6A**). I found significant differences in chrm2 expression among different songbird species (**Figure II–6B**, One-way ANOVA with Welch correction, $***p < 0.0001$). Although these species exhibit species-specific vocal patterns (**Figure II–6A**), I found no clear relationship between song phenotypes, especially syllable sequence and

chrm2 expression level in HVC. For example, although both CN and OF produce repetitive sequence-based songs, chrm2 expression in HVC is very high in OF but almost absent in CN. Furthermore, the species-specific chrm2 expression level in HVC was not tightly associated with evolutionary relatedness. For instance, ZF, OF, and SF belong to the same clade compared to BF, JS, and CN (**Figure II–6A**), but SF has lower chrm2 expression in HVC compared with ZF and OF (**Figure II–6B**). Additionally, I observed that only ZF and OF among the six-species examined showed large variability in chrm2 expression levels in the HVC. There were no clear individual differences in chrm2 expression in SF, BF, JS, and CN. These results indicate that the expression levels of chrm2 in the HVC are under species-specific regulation.

2.4 Discussion

One of my interesting findings in this chapter is the developmental regulation of chrm4 expression in HVC through the critical period of song learning. Chrm4 expression gradually increased from the subsong until the crystallized song stage. Even though chrm2 expression changes was not significant, it showed a trend that peaked at the plastic song stage and then declined at the crystallized song stage. However, these changes did not reach statistical significance possibly because of the apparent individual variability in chrm2 mRNA expression levels that existed at the different stages of song development. Chrm2 expression dynamics in the HVC is similar to the developmental changes in ACh concentration in the HVC of ZF during the critical period of song development (Sakaguchi and Saito, 1989). These findings suggest that the response of specific types of neurons in the HVC *in vitro* to the stimulation of mAChRs (Shea et al., 2009), could be mediated partly by chrm2 and chrm4. Chrm4 mRNA expression level was highest in Area X compared with other song nuclei and was consistently maintained through all the stages of song development. This suggest that a higher level of chrm4 mRNA is critical to mediate the potential cholinergic contributions to song development. Chrm2 and chrm4 are expressed pre- and post-synaptically, to modulate the release and action of ACh onto postsynaptic sites in mammals (Levey et al., 1995; Quirion et al., 1995; Baghdoyan et al., 1998). Therefore, auto-modulation of ACh release by chrm2 and chrm4 could contribute to the upregulation of ACh concentration in HVC during the critical period of song development (Sakaguchi and Saito, 1989). mAChRs are co-expressed with other neuromodulator/neurotransmitter receptors. Specifically, in rat striatum, chrm4 and chrm5 are co-expressed with dopaminergic terminals (Weiner et al., 1990) and glutamatergic neurons (Shin et al., 2015), respectively. Such co-expression was revealed to modulate both dopamine and glutamate release from midbrain neurons projecting to the striatum. Chrm2 was co-expressed with gene markers of excitatory glutamatergic projection neurons and inhibitory GABAergic local interneurons in HVC, suggesting that there are complementary interactions among mAChRs to regulate HVC's function. Although I did not examine the co-expression of other mAChRs with markers of neuron types in the HVC, it is possible that they are expressed in these neurons. It has been shown that the HVC receives cholinergic inputs from the ventral pallidum located in the basal forebrain (Li and Sakaguchi, 1997) and stimulation of cholinergic system in the basal forebrain shutdown the responses of HVC neurons to hearing BOS song in the anaesthetized state. (Shea and Margoliash, 2003), Such regulation could contribute to the behavioural state-dependent changes in auditory

responses in HVC (Schmidt and Konishi, 1998; Cardin and Schmidt, 2003; Shea and Margoliash, 2010). These developmental and multiple neuron-type expression patterns of chrm2 and chrm4 in the HVC suggest that these chrms play important roles in the cholinergic modulation of auditory input in HVC during development, particularly for arousal state-dependent suppression of HVC auditory responses during the sensorimotor phase of song acquisition.

Intra-species differences in chrm2 expression in HVC

Intra-species differences in behaviour are hallmarks of animal species (McIntyre et al., 2003; Zhang et al., 2004; Hammock and Young, 2005; Pantoja et al., 2016; Stern et al., 2017). A good example is the individual differences in vasopressin receptor 1a gene (V1aR) expression in the ventral pallidum, which has been shown to predict individual differences in pair-bonding behaviour (an innate behaviour) in male prairie voles (Zhang et al., 2002; McIntyre et al., 2003; Hammock and Young, 2004; Pantoja et al., 2016; Stern et al., 2017). For learned behaviours, ZFs exhibit individual differences in the song patterns they acquire from the same tutor (Tchernichovski et al., 2000). In addition, individual differences in the subsong of ZF juveniles has been attributed to parental genetic information (Sato et al., 2016). Furthermore, juvenile ZFs use individually-unique strategies to learn the same song (Liu et al., 2004). My study has revealed clear individual differences in chrm2 expression level in the HVC through the critical period of song development in ZFs. I showed that such variability was not influenced by manipulating the timing of song stabilization through testosterone administration (for acceleration) or audition deprivation (for delay). In addition, I found that chrm2 expression level is biased among siblings from different families. Currently, I could not elucidate the precise biological meaning of the individual variability in chrm2 expression in the HVC to song learning and/or production. Through the song development stages, a certain population of ZFs showed very low chrm2 expression in the HVC, suggesting that high chrm2 expression in HVC is not absolutely be necessary for song development and production, but rather have a ‘gain-of-function’ to modulate individually different excitability of HVC neurons through the process of song learning. It is certainly necessary to examine a direct causal link between chrm2 expression and song learning by viral-mediated and/or pharmacological manipulations of chrm2 activity in the HVC.

Inter-species differences in chrm2 expression in HVC

There are over 4,500 species of songbirds (Marler and Slabbekoorn, 2004) which learn to produce complex species-specific song patterns using similarly conserved neural circuits (Brenowitz and Beecher, 2005). My study has revealed clear species differences in chrm2 expression level in the HVC. Although there are reports of species-specific neuromodulator receptors gene expression in invertebrates (Covelli et al., 1981) and mammals (Creese et al., 1979; Insel and Shapiro, 1992; Young et al., 1997), to the best of my knowledge, my study is the first to report species specificity in the expression of a neuromodulator receptor gene in the song pathways. Differences in the expression level of V1aR predicts species-specific pair-bonding behaviour (an innate trait) differences in male voles (Young et al., 1997). The HVC is a premotor song nucleus that regulates syllable sequence of birdsong (Hahnloser et al., 2002; Fee et al., 2004). However, I could not clearly associate species differences in song patterns, particularly syllable sequence, with chrm2 expression in HVC in this study. Even though my result on the species differences in chrm2 expression in HVC was in adults, my earlier result showed that ZFs expressed chrm2 before the onset of first singing, suggesting that chrm2 expression level in the HVC could also be species-specific through the critical period of song learning. This idea needs to be further examined by future studies. In addition, the wide differences in chrm2 expression levels in the HVC between the canary (an open-ended learner) and other species is of great interest. Closed-ended learner songbirds, such as the zebra finch, have one critical period for song learning and the acquired songs remain relatively stable for the rest of their lives (Eales, 1965; Zann, 2006). In contrast, open-ended learners such as the canary, have multiple critical periods of vocal plasticity during which new song elements are incorporated into their vocal repertoire (Nottebohm et al., 1986; Brenowitz and Beecher, 2005). I found that this difference is not associated with evolutionary relatedness, suggesting that its near absence in the canary's HVC is brain area/nucleus-specific. Further examination of chrm2 expression level in other open- and closed-ended learners can reveal interesting insights into the biological contribution of chrm2 to the song learning program. It is possible that the degree of domestication under artificial selection in different species which I examined could have contributed to the species differences in chrm2 expression level in the HVC. As a result, it is important to either corroborate or contradict this finding in future studies using entirely captive songbird species.

2.5 Figures

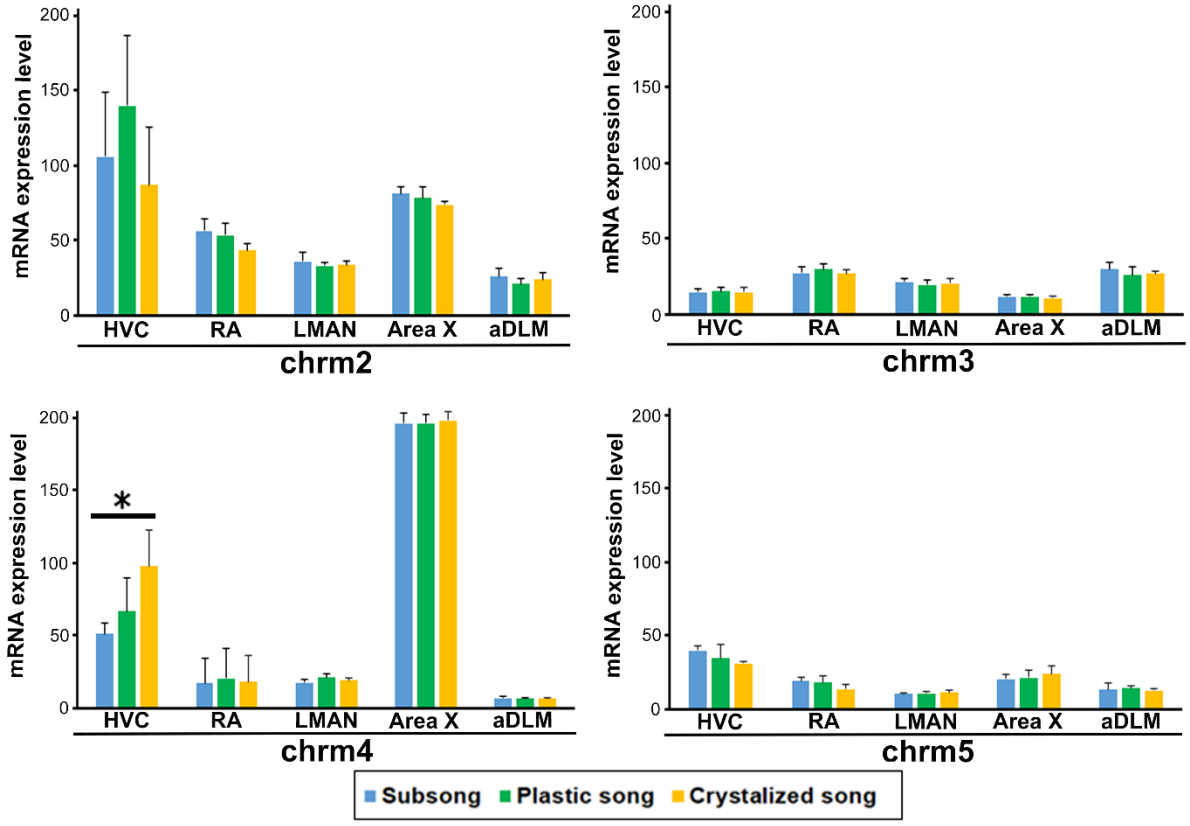

Figure II–1: mAChRs expression levels in the song nuclei during song development. Subsong (35–45 phd), plastic song (50–65 phd), and crystallized song (120–140 phd). Data: mean ± s.e.m, n = 6 birds/song development stage. One-way ANOVA, * $p \leq 0.05$.

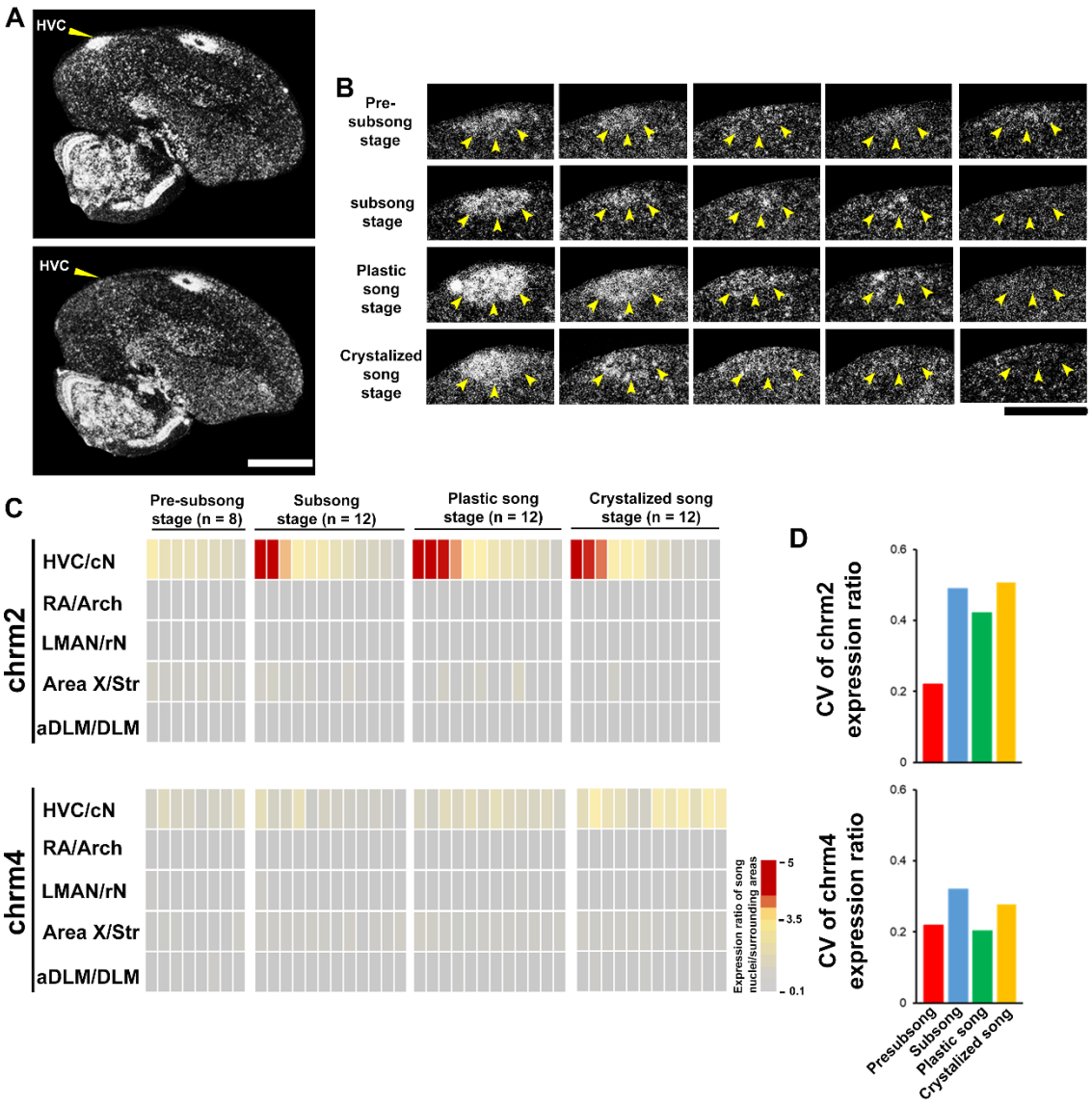

Figure II–2: Individual differences in chrm2, not chrm4 expression in HVC during song development.

A: Examples of whole brain images showing individual differences in chrm2 expression level in the HVC of two adult ZFs. Brains are sagittal, scale bar = 2 mm. White colour is mRNA signal.

B: Representative five birds showing individual differences in chrm2 expression in HVC at the four song development stages: presubsong (21–28 phd), subsong (45–46 phd), plastic song (60–65), and crystallized song (120–137 phd). Yellow arrowheads indicate HVC outlines. Scale bar = 1mm.

C: (top) Heatmap showing individual differences in chrm2 expression ratio (HVC/cN) at all

developmental stages, except at the presubsong. Bottom: Heatmap of chrm4 expression ratios in HVC. Middle: colour scale bar represents mRNA expression ratio in the HVC.

D: The coefficient of variation (CV) of chrm2 (top) and chrm4 (bottom) expression ratio in the HVC at four song development stages.

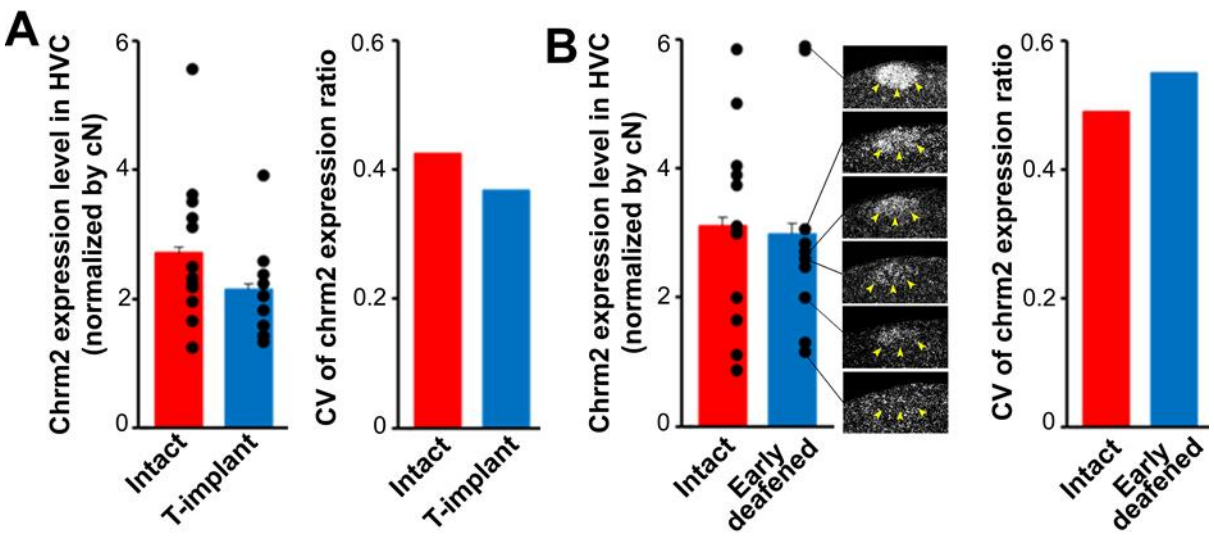

Figure II–3: Chrm2 expression level is neither T-implant nor audition-dependent.

A: (Left) normalized mRNA expression ratio of intact and T-implanted ZFs. Black dots represent individual mRNA expression ratio in the HVC. Intact (45–46 phd, n = 12); T-implant (43–49 phd, n = 9). Data: mean ± s.e.m, unpaired Student's *t*-test, $p > 0.05$. Right: CV of chrm2 expression ratio in the HVC.

B: (Left) normalized mRNA expression ratio in the HVC of intact (120–139 phd, n = 12) and early-deafened (> 1 year phd, n = 10) ZFs. Data: mean ± s.e.m, unpaired Student's *t*-test, $p > 0.05$. Black dots are individual mRNA expression ratios. Middle: higher magnifications of the HVC in six representative ZFs. The brains are sagittal. Right: CV of chrm2 expression ratio in the HVC.

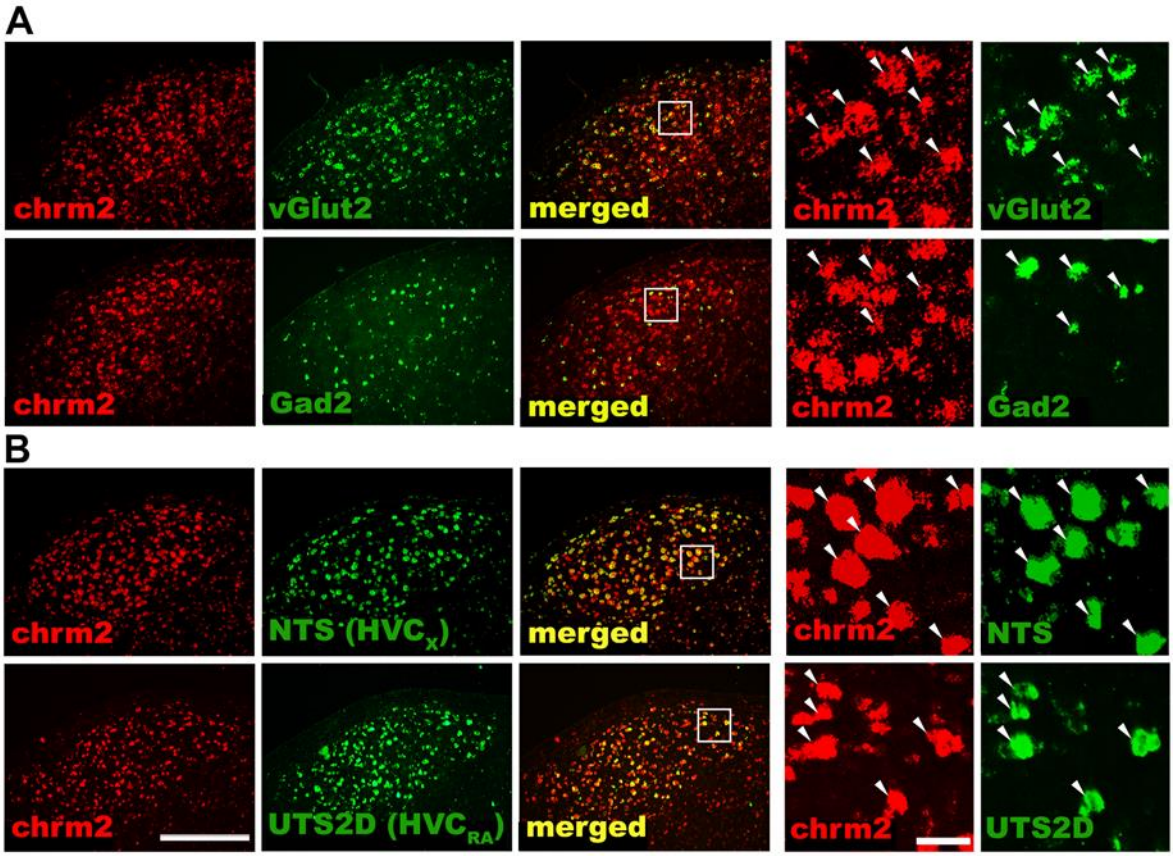

Figure II–4: Most types of neurons in HVC express chrm2.

A: Expression of chrm2 mRNA in both excitatory- (vesicular glutamate transporter 2, vGlut2 as marker) and inhibitory (glutamate decarboxylase 2, Gad2 as marker) neurons in the HVC. White squares are insets. The two extreme right columns are higher magnification of the insets. White arrowheads indicate co-expressed cells.

B: Chrm2 expression in the HVC to Area X (HVC_X; Neurotensin, NTS as marker) and HVC to RA neurons (HVC_{RA}; Urotensin-2 Domain-Containing, UTS2D as marker). Scale bars = 200 µm and 20 µm, respectively.

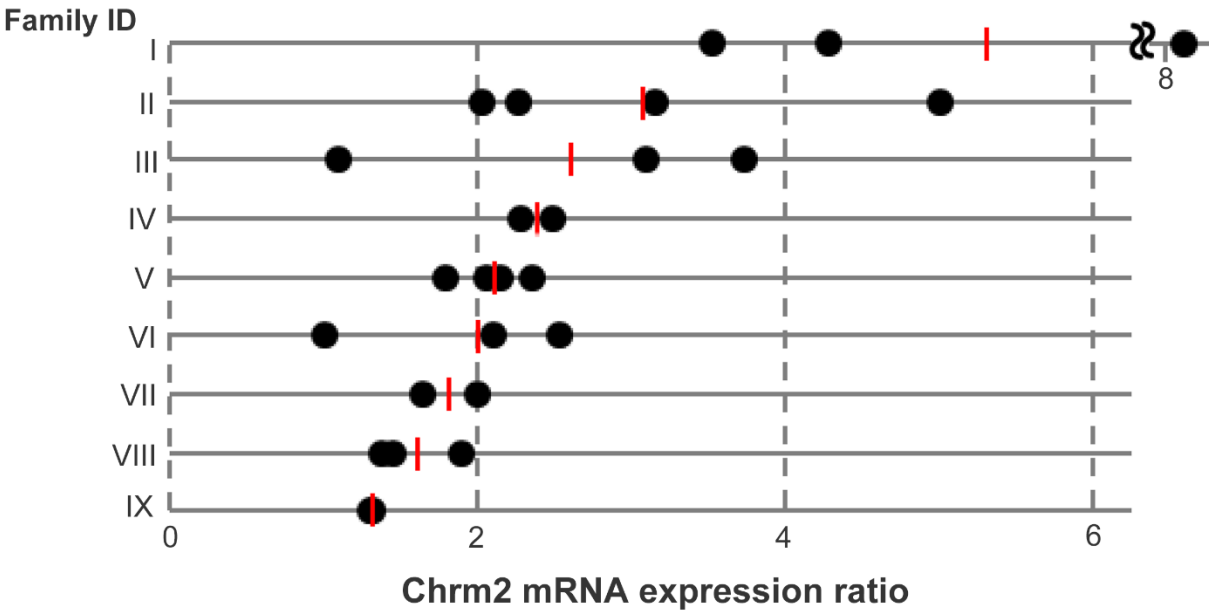

Figure II–5: Familial bias in chrm2 expression level in HVC.

The chrm2 mRNA expression ratios in the HVC among sibling ZFs is significantly biased across families (n = 9 families, 35–139 phd, two-way ANOVA with Games-Howell's correction, for family: **p* = 0.038; for age: *p* = 0.22; family x age interaction: *p* = 0.982. Each red bar represents the mean chrm2 expression ratio for each family.

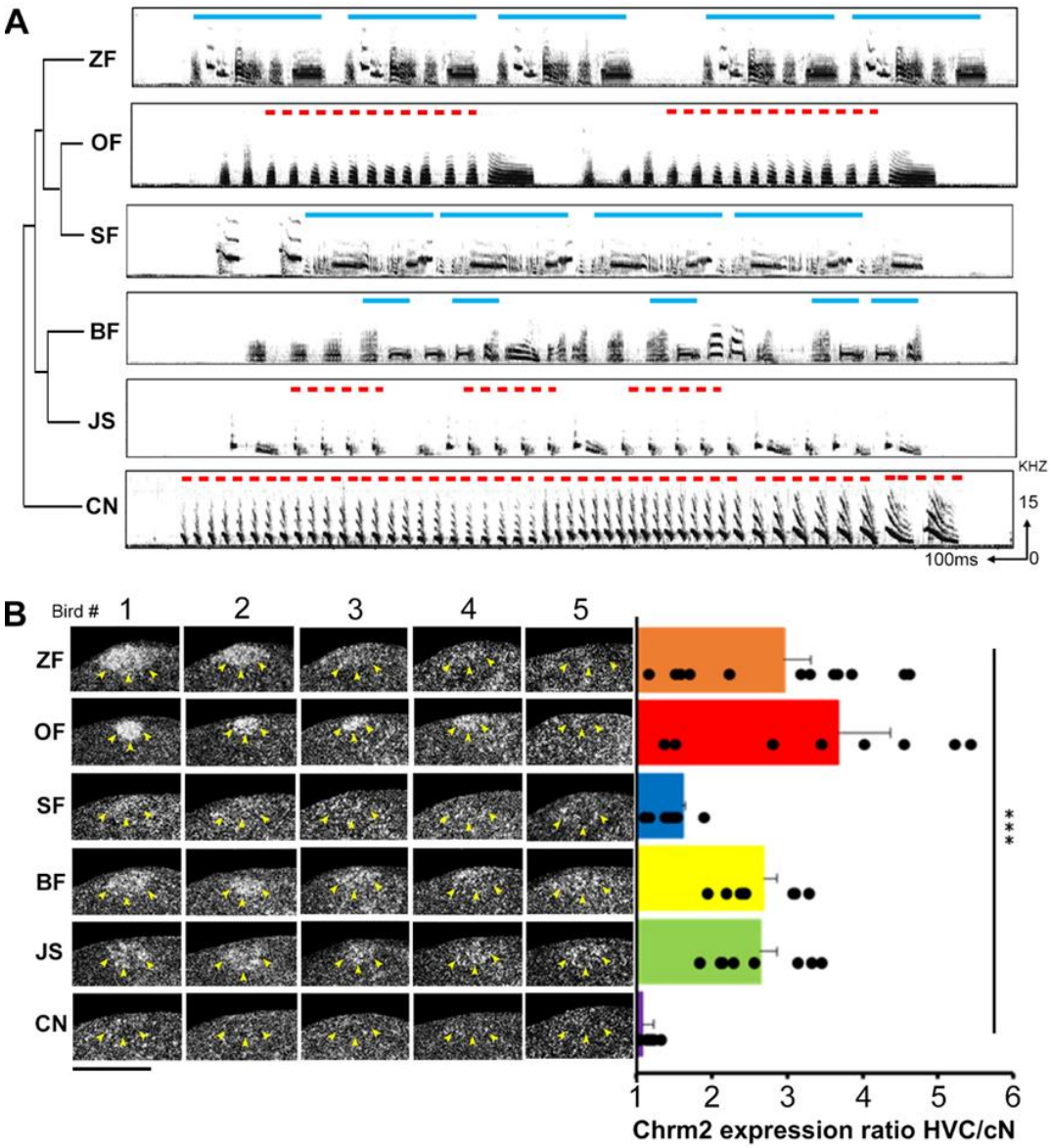

Figure II–6: Species differences in chrm2 expression ratio in HVC.

A: Phylogenetic relationships among the songbird species. ZF: zebra finch, OF: owl finch, SF: star finch, BF: Bengalese finch; JS: java sparrow; and CN: canary. Each species' sonogram is shown. Solid blue and red dotted bars indicate the motif and repetition units of songs, respectively.

B: Higher magnification images showing species differences in chrm2 expression level in HVC of six representative birds. Yellow arrowheads indicate HVC outlines. Scale bar = 1 mm. White colour is mRNA signal.

Right: Quantitative plot showing the species differences in chrm2 expression ratios (HVC/cN) among songbird species (n = 12 birds/species, data: mean ± s.e.m, One-way ANOVA, with Welch test, ***$p \leq 0.0001$). Black dots represent individual mRNA expression ratios.

Chapter III

Potential Biological Roles of chrm2 Expression in HVC

3.1 Introduction

The cholinergic system is important in selective attention (Sarter and Bruno, 1999; Noudoost and Moore, 2011), learning and memory (Anagnostaras et al., 2003; Tzavara et al., 2003; Matsui et al., 2004; Hasselmo, 2006), motor coordination (Ztaou et al., 2016) and motor skill learning (Conner et al., 2003). This suggests that the cholinergic system is important for learning motor skills, including birdsong. Birdsong is a learned motor skill acquired during a critical period in two stages: sensory and sensorimotor stages. During the sensory learning stage, male juvenile listens to, and memorizes a tutor song model, which is either his biological father's or conspecifics'. In the sensorimotor learning phase, the birds repeatedly practice singing to produce an approximate copy the tutor song (Marler, 1970; Doupe and Kuhl, 1999). The acquisition and production of birdsong is mediated by two dedicated neural circuits: AFP and the VMP. The AFP is important for song learning (Nottebohm et al., 1976; Bottjer et al., 1984; Scharff and Nottebohm, 1991). For instance, lesion of Area X results in an abnormal increase in durations and inter-syllable intervals (Nottebohm et al., 1976; Bottjer et al., 1984; Scharff and Nottebohm, 1991). On the other hand, lesion of LMAN, enhances song stabilization (Scharff and Nottebohm, 1991). These suggest that during the critical period of vocal learning, the AFP contributes to the regulation of syllables features and song stabilization. During the critical period of vocal development, song production exhibits large variability, which is typified by fluctuations in syllable acoustic entropy (Derégnaucourt et al., 2005; Hayase et al., 2018). Specifically, syllable entropy is high during the subsong and early plastic song stages, but decreases from late plastic stage to the crystallized song stage (Derégnaucourt et al., 2005). These together suggest that the degree of changes in syllable entropy predicts the timing of song stabilization. In addition, juvenile ZFs exhibit individual differences in vocal patterns as early as the subsong stage (Sato et al., 2016). This variability is reflected in variations in the distributions of syllable duration and inter-syllable intervals and was shown to be biased among breeding families (Sato et al., 2016). This suggests that parental genetic contribution influences the individual differences in learned vocal patterns of songbird species. However, the neural molecular mechanisms that regulate individual differences in learned vocalization is not clear. Interestingly, I found in Chapter II that chrm2 expression level in HVC is different among ZFs starting from subsong up to the crystallized song stage; such variability correlates with breeding families. These suggest that parental genetic information contribute to chrm2 expression level in the HVC to regulate individual differences in learned vocal patterns.

Although the concentration of ACh is upregulated in the song nuclei during song development (Sakaguchi and Saito, 1989), it remains unclear how mAChRs are involved.

Auditory information is important for song learning; when it is deprived by deafening, song learning is impaired (Konishi, 1965). HVC neurons have strong responsiveness to playback of BOS, than to conspecifics' or other species' songs (Margoliash, 1983; Theunissen and Doupe, 1998). In anaesthetized ZFs, simulation of the cholinergic system in the basal forebrain, analogous to nucleus basalis of Meynert/magnocellularis, suppresses neural responses of HVC and RA neurons to BOS (Shea and Margoliash, 2003). In addition, the response of HVC_X neurons to BOS has been shown to be mediated by G protein-coupled receptors (Rosen and Mooney, 2003), suggesting a possibility of neuron-type specific regulation of BOS in HVC by mAChRs (Shea and Margoliash, 2003). I found in Chapter II (**Figure II–4A, B**) that chrm2 is expressed in both HVC_X and HVC_{RA} neurons, suggesting that chrm2 may contribute to the regulation of auditory responses in HVC neurons. This could have crucial importance during the critical period of song learning. However, the exact contribution of chrm2 to song learning is unknown. Interestingly, I found in Chapter II (**Figure II–4A, B**), that chrm2 is expressed in both HVC's projection neurons, and local interneurons, suggesting that chrm2 modulates the excitability of HVC neurons during singing. In the ZF, cholinergic projection neurons to cortical brain areas originate from the ventral pallidum (Li and Sakaguchi, 1997). In the ZF, HVC and RA receive cholinergic projection neurons from the ventral pallidum (Li and Sakaguchi, 1997; Sadananda, 2004) and have been shown to release ACh (Zuschratter and Scheich, 1990; Sadananda, 2004). Currently, information on the extrinsic sources of cholinergic innervation to other song nuclei is not clear. Although the concentration of ACh is upregulated in the song nuclei during song development (Sakaguchi and Saito, 1989), it remains unclear whether mAChRs are involved in this regulation.

To understand the potential contributions of chrm2 to song learning, I examined the relationship between chrm2 expression level in HVC and song features at the subsong stage in ZFs. To examine the interaction between chrm2 expression level in the HVC and auditory-independent intrinsic regulation of song patterns, I examined the correlation of chrm2 expression level in the HVC with the motif and repetition indices of song in early-deafened ZFs, respectively. To evaluate chrm2 expression contribute to song production, I examined the effects of over-expressing CHRM2 by adeno-associated virus (AAV) in the HVC on the syllable distribution patterns of crystallized learned songs in adult ZFs.

3.2 Materials and methods

Animals

To examine the types of neurons in the HVC that express chrm2, I co-labelled chrm2 mRNA with marker genes for the two excitatory projection neurons, and inhibitory local interneurons in HVC using the brain of a ZF with high chrm2 expression level. To investigate a potential relationship between chrm2 expression in the HVC and song features early in development, I correlated chrm2 expression level in the HVC with the syllable duration, pitch, frequency modulation, entropy variance, and the CVs of these parameters in subsong stage of ZFs. To evaluate if chrm2 interacts with auditory inputs in shaping vocal patterns, I compared chrm2 expression levels in the HVC with the motif and repetition indices of song in early-deafened ZFs. To test if chrm2 expression contributes to song production, I over-expressed CHRM2 in adult ZFs HVC using the adeno associated virus (AAV) system. Additionally, I injected a control virus encoding the green fluorescence protein (GFP) into another group of adult ZFs. Then, I compared the syllable distribution patterns between CHRM2-virus-injectd and control virus-injected adult ZFs (> 120 phd, n = 4 birds/group).

Song recording and analysis

The birds were housed individually in a sound-attenuation box (65cm x 27cm x 30cm) inside metallic cages (54cm x 22cm x 23cm). Songs were recorded automatically on a 24 hr basis through a microphone (SHURE SM57) connected to a computer installed with Sound Analysis Pro 2011, version 1.04 (Tchernichovski et al., 2000) at 16 bits and 44 kHz sampling rate. Analysis of syllable acoustic features was performed using Sound Analysis Pro and Avisoft-SASLab Pro (Avisoft Bioacoustics). Low and high frequency noises (< 0.05 and > 1.9 kHz, respectively) were removed using Avisoft-SASLab Pro. Noise was further filtered with the Audacity Software (https://www.audacityteam.org/download/). Calls and cage noises were manually removed from selected songs. For analyses, songs were randomly selected, while syllables were automatically segmented using Avisoft-SASLab Pro. The segmented syllables were visually inspected for accuracy. A song bout was defined as a continuous production of syllables separated by at least 200 ms of silence.

To determine if chrm2 expression levels is related to the innate regulation of song patterns in early deafened ZFs, I used the syllable similarity matrix (SSM) protocol described previously (Imai et al., 2016). I selected 10 songs for each bird for SSM analysis. Syllables were saved as .WAVE file formats by automatic section labelling (threshold: 0.001V; hold

time: 0.01s, and margin: 0s) using Avisoft Saslab Pro software. The .WAVE files were then saved as separate .SON files that included information about the sequential order of the syllables in a song. Segmented syllables from two songs were exported to the Avisoft Correlator software to calculate syllable similarity scores by the round-robin comparison. Similarity score was calculated as the peak correlation coefficient between two syllables. Correlation coefficient was calculated using the formula:

$D_{KL}(Q_s||Q_k) = \sum_{m=s}^{M}\sum_{n=s}^{N} q_s(m,n) log_2 \frac{q_s(m,n)}{q_k(m,n)}$, where axy and bxy are the intensities of the spectrograms points at x and y locations, respectively. Syllable similarity scores range from 0 to 1: a value of 0 means no similarity, whereas 1 represents complete similarity between two syllables. 10 SSM were calculated for each bird. Calculated similarity scores were copied to Microsoft excel worksheet while maintaining syllable orders in the original songs. These worksheets were saved as .CSV files. To quantify the temporal structures of syllables, I calculated the occurrence rates of unique patterns of binarized 2 row x 2 columns cells in the SSM using the R software package (R Core Team, 2013). I calculated the motif- and repetition indices, respectively, by comparing two mean values: 0.86 (as similar), and 0.50 (as dissimilar). Finally, the prevalence of the binarized 2 x 2 cells for each SSM was calculated. The motif index represented paired syllable transitions which indicates the presence of two different successive syllables with the same sequential order, for instance: song 1 (AB…) vs song 2 (AB…), where A and B represent two different syllables. The repetition index indicates the presence of repetitive syllables in a song, for example: song 1 (…AA…) vs. song 2 (…AA…). Mean values were calculated for each song index for each bird and used for further statistical comparisons.

Calculation of Kullback-Leibler (K-L) distance

To evaluate the effects of over-expressing CHRM2 on song production, I calculated the acoustic features of syllables using Sound Analysis Pro 2011, version 1.04 (Tchernichovski et al., 2000), and generated scatter plots (syllable duration vs mean FM) 2 and 35 days after virus injections, for both AAV9-CHRM2 and control virus-injected ZFs (n = 500 syllables). These plots were merged to evaluate possible changes in syllable distribution patterns. Syllable duration and mean FM were previously used to evaluate the timing of song stabilization following deafening (Mori and Wada, 2015), and diurnal changes in vocal development (Ohgushi et al., 2015). To further evaluate the effects of over-expressing AAV9-CHRM2 in HVC on song production, I used the K-L distance (Wu et al., 2008) to

estimate the distance (similarity or difference) between the probability density distributions of syllable populations at two-time points (2 and 35 days after virus injections, respectively), using two acoustic song features, syllable duration (*m*) and mean FM (*n*). The probability density of each syllable distribution was estimated 2 and 35 days after (denoted as *a* and *b*, respectively) virus injection. The K-L distance was calculated as:

$D_{KL}(Q_a \| Q_b) = \sum_{m=a}^{M} \sum_{n=a}^{N} q_a(m,n) log_2 \frac{q_a(m,n)}{q_b(m,n)}$, where Q_a and Q_b are the probability density functions, $q_a(m, n)$ and $q_b(m, n)$ represent the estimated probabilities for the bins (m = 20, n = 5), 2 and 35 days after virus injection, respectively. I used 500 syllables for each time point. A larger K-L distance indicates lower similarity, whereas a K-L distance of 0 shows perfect similarity.

RT-PCR cloning of Open reading frame of CHRM2 and virus purification.

The detailed cloning procedure was as described previously (Wada et al., 2004). To over-express CHRM2 in the HVC, I cloned the open reading frame (ORF) of CHRM2 using primer sets. To avoid the amplification of related mAChRs, I designed specific primers for the predicted ORF of CHRM2 in ZF, chicken and human. RT-PCR was performed on total RNA from adult male ZF brain using primer sets. PCR products were visualized in 1.5% agarose gel and then extracted when the predicted sizes were obtained. PCR products were cloned into pGEM-T easy vector plasmid, sequenced, and the sequence was confirmed on BLASTN (DNA) and BLASTX (protein) on the NCBI genome database. The cloned ORF was ligated into Stu1 and Nhe1 restriction sites of the AAV vector (**Figure III**–**1**), purified, and sequenced.

Transfection was performed w a transfection mix containing AAV9-CHRM2 plasmid DNA (0.27 µg), pPack (0.1 µg) and pHelper (1.4 µg) plasmids, Polyethylenimine (1 mg/1mL), and made up to 20 mL with PBS. The mix was vortexed and allowed to stand for 10 min. 2 mL of transfection mix was added to HEK 293 cells in ten, 15 cm cell culture dishes containing culture medium [Dulbecco-Minimum Eagle Essential Medium (DMEM, WAKO, Japan), with 10% fetal bovine serum (FBS), 1% Glutamax, 1% penicillin and 1% Streptomycin]. After 24 hr, the cells were examined for EGFP expression, while the culture medium was changed to another medium containing 5% FBS. Cells were collected after 48 hr and stored at –80^{o}C until purification. AAV9-CHRM2 virus was purified using the OptiPrep protocol (www.axis-shield-density-gradient-media.com). Briefly, collected cells were simultaneously frozen in 100% ethanol and thawed in a water bath at 37^{o}C, for four different

times. 1 M $MgCl_2$ was added followed by Benzonase (Benzonase® Nuclease, Novagen, Germany), incubated in a water bath at 37^{o}C for 30 min, and then centrifuged at 7, 000 rpm for 1 hr at 4^{o}C. AAV9-CHRM2 virus was then purified on a density-gradient containing 54% (5 mL), 40% (5 mL), 25% (6 mL), 15% (9 mL), and 13–13.5 ml cell supernatant of virus by density-gradient ultracentrifuge at 28, 000 rpm for 6 hr at 18^{o}C. Purified virus solution was collected from the 40% fraction and stored at –80^{o}C until use.

Virus injections

Viruses were injected with glass capillaries (Drummond Scientific, USA) made on a Puller (Sutter Instrument Company, USA), using Nanoject II (Drummond Scientific, USA). Each ZF (phd >120) was anaesthetized by an intraperitoneal injection of pentobarbital sodium (6.48 mg/mL; 60 uL/10g body weight). Two injection points were made in each HVC, with the following coordinates starting from the mid-sagittal sinus: medial-lateral (2.3 mm), depth (0.45 mm), beak angle (0^{o}), and capillary angle (90^{o}). For the second injection point, the capillary was moved to minus 0.3 mm in the rostral-caudal direction, whereas other coordinates were maintained constant. Then, birds received bilateral injections of 1000 nl of either AAV9-CHRM2, or AVV9-CMV-GFP (control) virus/hemisphere (500 nl/injection point), at a rate of 9.2 nl/30 s. After injection, the birds recovered on a heat pad inside a cage until they started normal cage activities such as eating, drinking and flying. Virus expression was evaluated after 35 days. The effects of virus expressions were compared at 2 and 35 days post-injection. 2 days was chosen to control for possible effects of physical damage during virus injection on song.

Brain Tissue collection and sectioning

Each bird was anesthetized by an intraperitoneal injection pentobarbital sodium (6.48 mg/mL; 60 uL/10g body weight). The brain was flushed by a transcardial perfusion with 1× PBS, followed by perfusion with 4% PFA/1× PBS. Brains were dissected out and post-fixed in 4% PFA/1× PBS at 4^{o}C for 10 hr on a gentle shaker (Invitroshaker, TAITEC, Japan). The brains were transferred to 20% sucrose/4% PFA/1× PBS for 10 hr. Thereafter, the brains were sectioned on the sagittal plane at 28 µm on a freezing microtome and stored at 4^{o}C until used for fluorescence immunohistochemistry.

Fluorescence immunohistochemistry

To evaluate the rate of virus expression in the HVC, I performed fluorescence immunohistochemistry to co-label GFP positive (+) with both FLAG+ and NeuN+ cell, for the AAV9-CHRM2 and controls viruses, respectively. Fresh, free-floating brain sections (28 µm) were washed three times in 1× PBS, incubated gently on a rotary shaker (ROTARY SHAKER NR-2, TAITEC, Japan), in a blocking solution (0.3% Triton X-100/4% normal goat serum/1% BSA/1× PBS) for 30 min at RT. The brain sections were incubated overnight on a shaker (Invitroshaker, TAITEC, Japan) at 4^{o}C in 1st antibodies/blocking solutions: anti-NeuN (mouse) 1:500; anti-FLAG (Rabbit MBL) 1:250, respectively. The brain sections were washed three times in 1× PBS for 5 min each at RT and incubated for 30 min on the rotary shaker at RT in 2nd antibodies/blocking solution: anti-mouse Alexa 555 (1:500); and anti-Rabbit Alexa 555 (1:500), respectively. The brain sections were washed three times in 1× PBS at RT for 5 min each with gentle shaking. Thereafter, the brain sections were mounted on silane-coated glass slides, air-dried, and counter-stained with VECTASHIELD (with DAPI, Vector Laboratories Inc., CA, USA). The slides were cover-slipped and visualized under a fluorescence microscope (EVOS, FL, Thermos Fisher Scientific, USA). The total number of GFP+, NeuN+, and FLAG+ cells were counted in each HVC, while the proportion of AAV9-CAGS-FLAG-CHRM2-GFP co-labelled cells was calculated as GFP+/NeuN+, or FLAG+/GFP+. For the control virus, the proportion of co-labelled cells was calculated as GFP+/NeuN+.

Statistical analysis

I used the Pearson's test of significance to determine the relationship between chrm2 expression level in HVC and the features of song, and chrm2 mRNA expression levels and song indices in early-deafened ZFs. I used the Student's *t*-test to evaluate the effects of virus injections on the syllable distribution patterns of the control- and AAV9-CHRM2-injected ZFs.

3.3 Results

Chrm2 expression in HVC correlates with CV of entropy variance at the subsong stage

In Chapter II, I found that individual differences in chrm2 expression in the HVC emerged from the subsong stage. To evaluate the potential contribution of chrm2 expression level to song learning, I examined the correlation of chrm2 expression levels in the HVC with the following features of subsong: syllable duration, inter-syllable interval, pitch, frequency modulation, and entropy variance, and the CVs of these parameters. The distributions of syllable durations and inter-syllable intervals were previously shown to vary among ZFs at the subsong stage (Sato et al., 2016). In addition, syllable pitch, frequency modulation, and entropy variance were recently shown to be important predictors of song stabilization in ZFs (Korsia and Bottjer, 1991; Hayase et al., 2018). Although there were no significant correlations between chrm2 expression level in the HVC and the mean syllable duration, intersyllable interval, pitch, frequency modulation, and their CVs, I found a significant correlation with the CV of entropy variance [**Figure III–2** (Pearson's test of significance, $*p = 0.022$, n = 12 birds)].

Chrm2 expression in HVC is associated with song patterns in early-deafened ZFs

In Chapter II, I demonstrated that chrm2 expression level correlated with parental genetic information. In addition, chrm2 expression showed high degree of species-specificity among adults of other songbird species. Furthermore, early-deafened ZFs showed large individual differences in chrm2 expression in the HVC, like intact age-matched ZFs. These findings strongly suggested that chrm2 expression is regulated by innate mechanisms. To further understand whether chrm2 expression level in the HVC is regulated by genetic mechanisms, I examined mRNA expression in early-deafened ZFs. These were good models because they showed intrinsic (genetic) regulation of their song patterns. While analysing chrm2 expression in early-deafened ZFs (> 120 phd) in Chapter II, I observed a possibility that chrm2 expression level in HVC could be related to the auditory-independent intrinsic song patterns developed by early-deafened birds. To test this idea, I calculated the motif and repetition indices of song patterns and correlated them with chrm2 expression level in the HVC. I found that chrm2 expression levels in the HVC was positively and significantly correlation with the motif ($**p = 0.003$), while being negatively and significantly ($*p = 0.05$) correlated with the repetition indices of song, respectively [**Figure III– 3A & B** (n = 10 birds, Pearson's correlation test of significance)].

Over-expression of CHRM2 in HVC did not affect the syllable distribution patterns of crystallized learned song at adulthood

To understand the potential contribution of chrm2 expression to the production of crystallized learned song patterns, I over-expressed CHRM2 using AAV in the HVC of adult ZFs (> 120 phd) for 35 days. I chose to over-express, rather than to knock-down chrm2 in the HVC because ZFs show large individual differences in chrm2 expression levels at all stages of song development (in Chapter II, **Figure II**–**2A, B**). I found about 13% and 16% infection rates as average of the AAV9-CHRM2 and control viruses in HVC, respectively (n = 4 birds/group) (**Figure III**–**4A, B, C**).

Then, I examined the songs of both AAV9-CHRM2 and control virus-injected ZFs (> 120 phd, n = 4 birds/group). I found no clear changes in the syllable distribution patterns of these birds, which is shown by a plot of mean frequency modulation and syllable (**Figure III**–**5A, B**). This is highlighted in the scatter plots of the syllable distributions 2 and 35 days after virus injections (**Figure III**–**5 C**, n = 500 syllables for each time point, respectively). In addition, I found no significant difference in the mean K-L distance between the control and AAV9-CHRM2-injected ZFs [**Figure III**–**5D** (> 120 phd, n = 4 birds/group, unpaired Student's *t*-test $p > 0.05$)]. Based on the low expression rates of the viruses and the small sample size in my study, this finding may not be conclusive. However, it suggests that slight upregulation in chrm2 expression in the HVC do not have significant effects on the syllable sequence of an already crystallized learned song.

3.4 Discussion

In this Chapter, I showed that chrm2 expression level in the HVC correlates with the CV of entropy variance at the subsong stage. The acquisition and production of song is characterized by large variability among individual ZFs (Tchernichovski et al., 2001, Sato et al., 2016). Learned features of song exhibit fluctuations during the critical period of song learning in the ZF. Specifically, syllable entropy is higher in subsong and early plastic song, but declined in late plastic and crystallized songs (Derégnaucourt et al., 2005). Together, these suggest that the degree of changes in entropy variance contribute to the timing of song stabilization. In mice, differences in ACh concentration predicts individual differences in motor skill learning (McIntyre et al., 2003), suggesting that ACh activity regulates individual differences in motor skills learning. ZFs also exhibit individual differences in learning strategies for a song. My finding suggests that chrm2 expression level in the HVC contribute to song learning by modulating the timing of song stabilization via the regulation of syllable entropy variance. Although a recent study found that blockade of mAChRs in the song nucleus, RA of juvenile ZFs did not affect syllable entropy variance at the subsong stage (Puzerey et al., 2018), it showed that syllable entropy variance was greater at the crystallized song stage compared with control birds. However, the exact contribution of mAChR-specific receptor subunits to the variability in syllable entropy was not clear from this study because non-specific mAChR and nAChR antagonists were used simultaneously. Further studies using viral-mediated over-expression of CHRM2 protein expression in juvenile songbirds can reveal a causal link between CHRM2 expression in the HVC and vocal learning.

Another interesting finding in this Chapter is that chrm2 expression level in HVC is related to song patterns in early-deafened ZFs. The early-deafened ZFs retained large individual differences in chrm2 expression in the HVC similar with intact birds. In Chapter II, I found a possibility that chrm2 expression level in HVC could be regulated by intrinsic (genetic) mechanisms. My finding in this Chapter further supports the idea that chrm2 expression level in the HVC is under innate-, rather than hormonal- or auditory-driven mechanisms, suggesting that chrm2 expression in HVC is important for the genetic regulation of song patterns in songbird species. The cholinergic system in the basal forebrain regulates the input of auditory stimuli to the song nuclei through the HVC (Shea and Margoliash, 2003). In addition, auditory responses of HVC_X neurons is mediated by G protein-coupled receptors (Rosen and Mooney, 2003); mAChRs were identified as potential candidate receptors (Shea and Margoliash, 2003). Chrm2 is a G protein-coupled receptor and exhibits clear individual

differences in expression levels in the HVC at all stages of song development (Chapter II, **Figure II–2A, B**), suggesting that chrm2 plays a crucial role in regulating auditory response of HVC neurons. Although I could not find a clear relationship between chrm2 expression level in the HVC and song patterns, especially syllable sequence, in intact adult songbird species (Chapter II, **Figure II–6A**), my findings suggest that there could be more complex interactions between chrm2 expression level in the HVC and auditory input. It is possible that internal and external variables (such as hormones, breeding conditions, social interactions, etc), which I did not examine in this study have potential contributions in shaping the song patterns of ZFs with intact hearing. Further studies that will simultaneously manipulate chrm2 expression in HVC and control for these factors have the potential to reveal how chrm2 expression level in HVC interact with internal and external variables to shape acquired song patterns.

In adult ZFs, I found that over-expressing CHRM2 in the HVC for 35 days had no clear effect on the syllable distribution patterns of crystallized learned song at adulthood. Specifically, over-expressing CHRM2 in the HVC had no significant effect on crystallized learned song patterns compared with control virus-injected birds. I speculate that about 13% rate of AAV9-CHRM2 virus expression in HVC neurons and the small samples size in my study are not enough to conclude on whether CHRM2 influences song production or not. Another factor worth considering is the potential contribution of chrm4 expression to HVC function. Chrm2 and chrm4 are presynaptic auto-receptors that regulate the release and action of ACh on postsynaptic neurons (Allen and Brown, 1993; Ito and Shuman, 2008; Shin et al., 2015), suggesting that they may interact to elicit biological functions. I encourage further studies to achieve a greater over-expression of CHRM2 in the HVC with larger sample sizes, and simultaneously manipulate CHRM4 expression by gene knockdown techniques. This can illuminate the mAChRs-specific contributions to song learning and production.

3.5 Figures

Figure III–1: Overexpression of CHRM2 in ZF

Virus construct used for CHRM2 overexpression

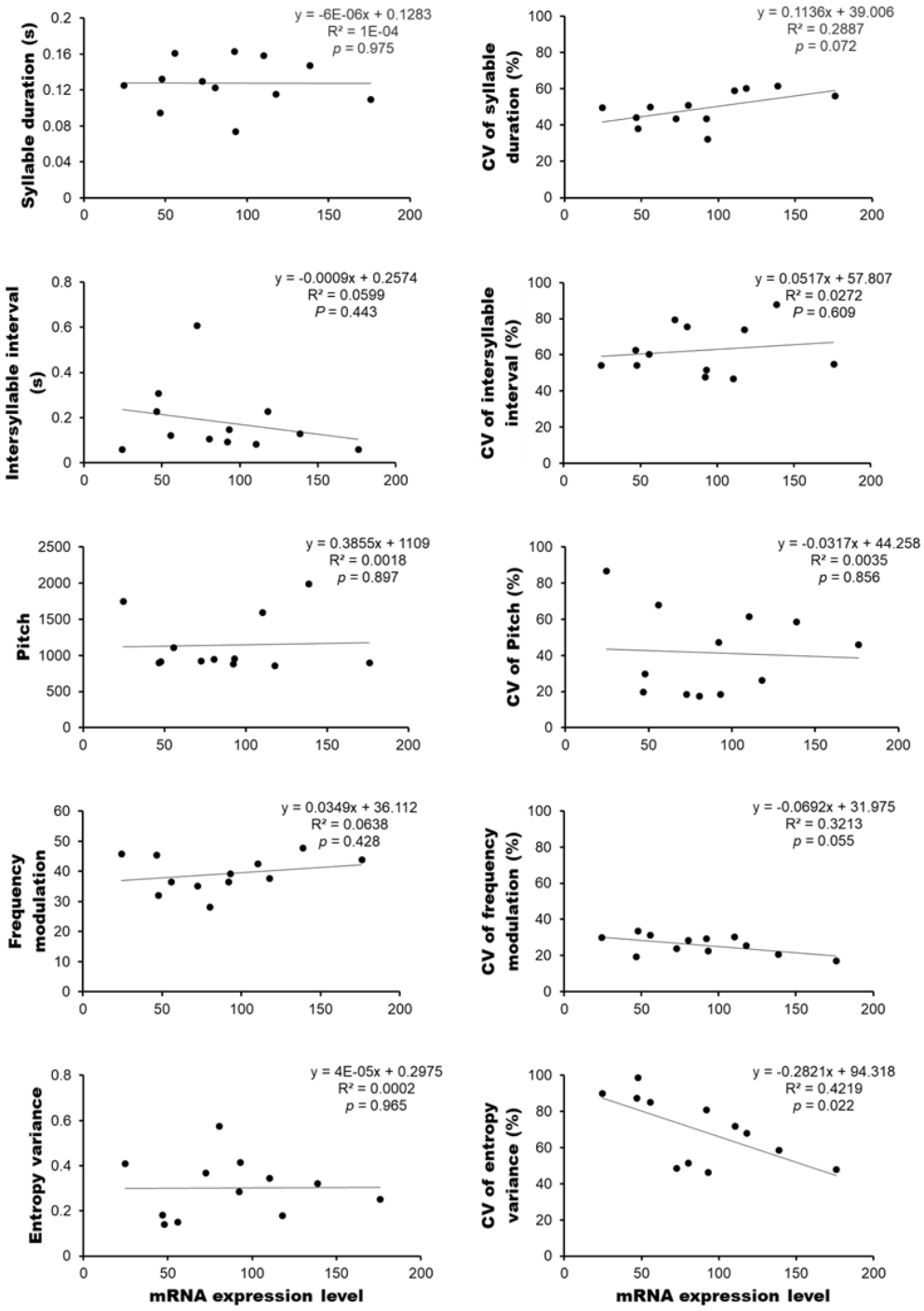

Figure III–2: Chrm2 mRNA expression level correlates with the CV of entropy variance at the subsong stage.

Left column: Correlation of absolute chrm2 mRNA expression level in the HVC with features of subsong (45–46 phd, n = 12, Pearson's correlation coefficient).

Right column: Correlation of absolute chrm2 mRNA expression level in HVC with the CV of the subsong parameters.

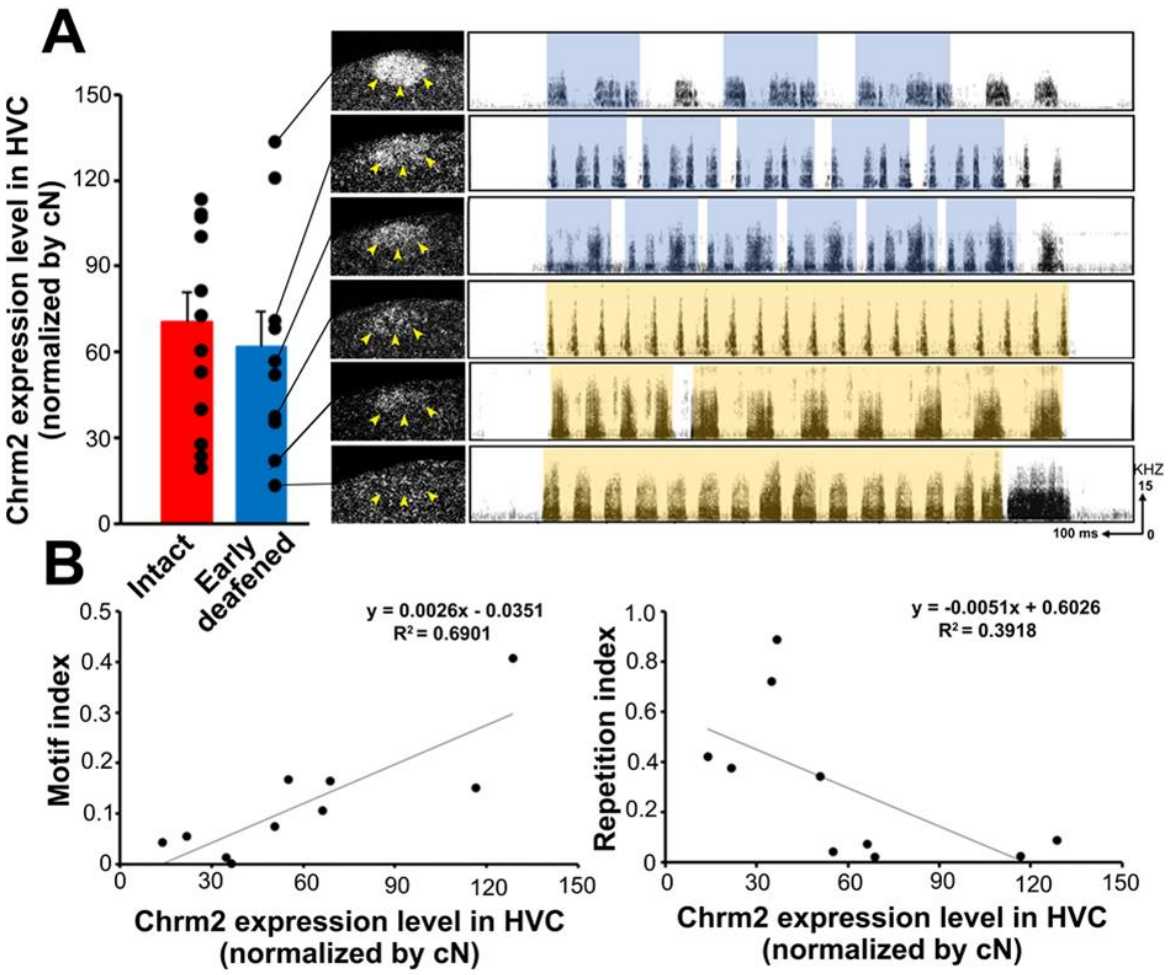

Figure III–3: Chrm2 expression correlates with song patterns in early-deafened ZFs.

A: Chrm2 expression ratios in intact (120–138 phd, n = 12) and early-deafened (> 120 phd, n = 10 birds) ZFs. Data: mean ± s.e.m, unpaired Student's *t*-test, $p > 0.05$. Black dots represent individual absolute mRNA expression levels. Examples of higher brain images are shown with corresponding sonograms. White colour is mRNA signal. Blue and light brown colours highlight represent motif and repetitive units of song, respectively.

B: Correlation of chrm2 mRNA expression in HVC of early deafened ZFs (> 120 phd) with the motif and repetitive indices of songs, respectively. Pearson's correlation test of significance, $p = 0.003$ and 0.053, for the motif and repetition indices of song, respectively. Black dots represent individual absolute mRNA expression levels in HVC (n = 10 birds).

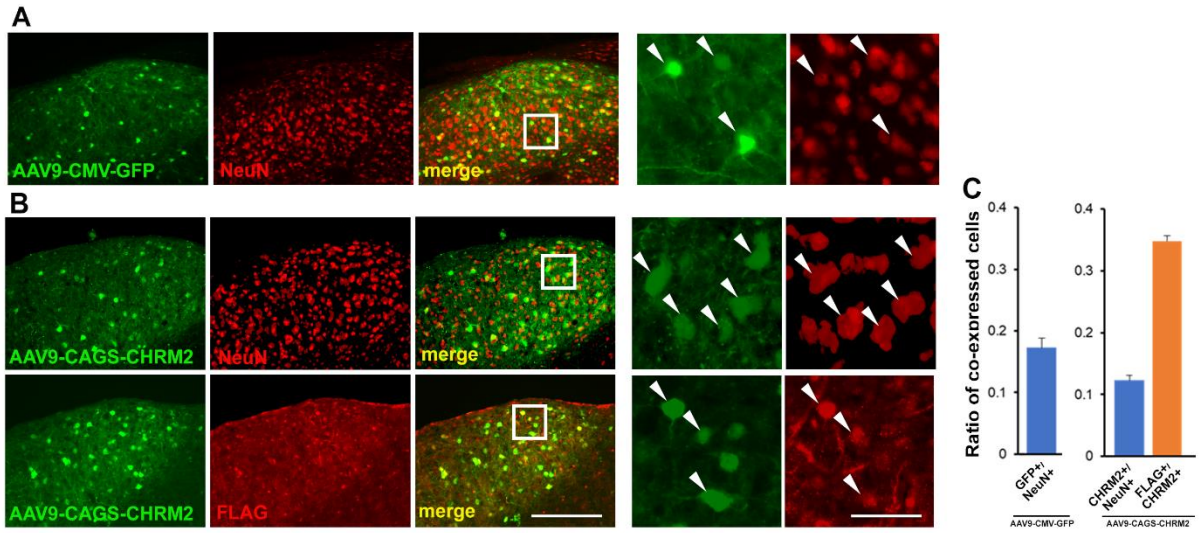

Figure III–4: Expression of AAV9-CAGS-CHRM2=GFP in adult ZF.

A and **B**: Expression of AAV9-CMV-GFP (control), and AAV9-CAGS-CHRM2-GFP respectively in the HVC. White rectangle in the merged images are insets. The two far right columns are higher magnifications of the insets.

White arrowheads show co-expressed cells (GFP+ vs NeuN+ in **A**, and CHRM2+ vs NeuN+ and FLAG+ in **B**). Scale bars = 200 µm and 10 µm, respectively.

C: Ratio of co-expressed cells in the HVC. Data: mean ± s.e.m, n = 4 birds each for control and AAV9-CAGS-CHRM2-GFP virus injected groups, respectively.

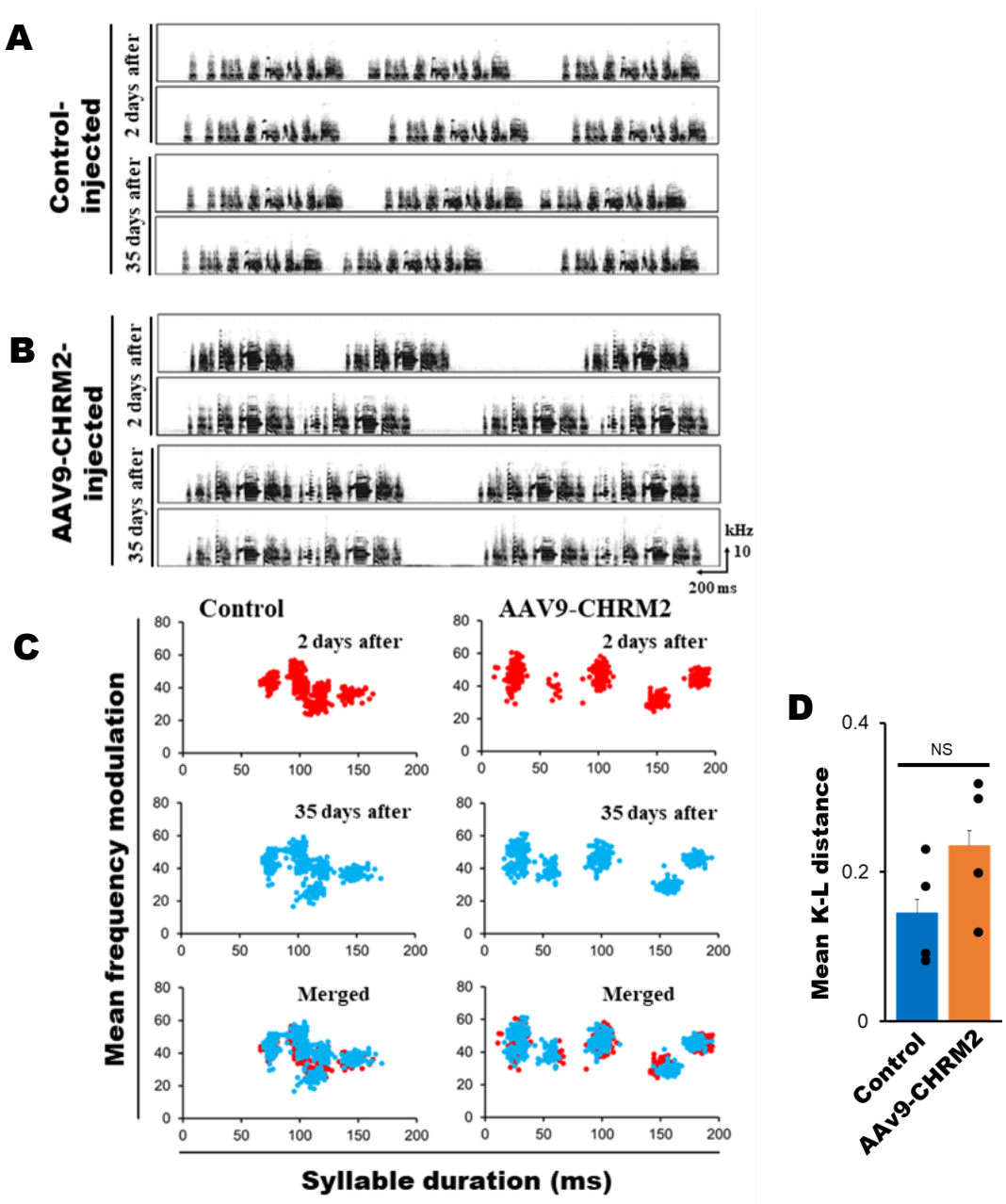

Figure III–5: Expression of AAV9-CAGS-CHRM2-GFP in the HVC did not affect the syllable distribution patterns of crystallized learned song adult ZF.

A and **B**: Examples of two sonograms each 2 and 35 days after control, and AAV9-CAGS-CHRM2-GFP virus injections in adult ZFs, respectively.

C: Syllable distribution patterns in adult ZFs 2 and 35 days after injections of the control and AAV9-CAGS-CHRM2-GFP viruses, respectively (n = 500 syllables/group). The merged plots compare syllable distributions.

D: Mean K-L distance between control and AAV9-CHRM2-injected ZFs (> 120 phd, n = 4 birds/group, NS = not significant, unpaired Student's *t*-test)

General discussion

Bird song is a learned motor skill which is unique to a few vertebrate groups and important for inter- and intra-species communications (Janik and Slater, 1997; Jarvis, 2004; Poole et al., 2005; Foote et al., 2006; Stoeger et al., 2012). Efficient vocal learning and production depends on several factors such as intact hearing, androgen signalling, and social interactions (Konishi, 1965; Korsia and Bottjer, 1991). The neural molecular mechanisms for learned motor skills remain largely unknown. Songbirds represent excellent animal models to examine the neural molecular mechanisms for the acquisition and production of motor skills because they share a conserved neural circuits and similar vocal learning process with humans (Jarvis et al., 2005). I used songbirds to investigate the precise expression patterns and potential contributions of mAChRs to vocal learning and production. In Chapter I, I found that mAChRs exhibit unique expression patterns in the songbird brain, including the neural circuits for vocal learning. In Chapter II, I revealed that chrm4 is regulated in the vocal motor song nucleus HVC during the critical period of vocal learning, whereas chrm2 expression levels in the HVC is clearly different among individual ZFs irrespective of age, and across other songbird species. In addition, I found that individual differences in chrm2 expression level in the HVC is neither regulated by serum testosterone concentration nor auditory deprivation but is rather associated with parental genetic contributions. Furthermore, I showed that chrm2 is expressed by most types of neurons in the HVC. In Chapter III, I showed that chrm2 expression level in the HVC correlated with the CV of entropy variance at the subsong stage, and with song indices in early-deafened ZFs. However, I found no clear effects of over-expressing CHRM2 in HVC on crystallized learned song of adult ZFs. My findings suggest that chrm2 and chrm4 have potential contributions to species and individual differences in learned vocalizations among songbirds.

Based on my findings I will discuss the potential contributions of chrm2 and chrm4 expression in HVC to the regulation of the input of auditory signals in the HVC, because this is an important mechanism that could be involved in species-specific vocal learning and production in songbirds.

Vocal learning and production greatly relies on intact hearing (Konishi, 1965), which enhances online comparison of self-motivated vocalizations with the memorized model of the tutor song during the critical period of song development. As a result, deafening impairs learned song (Konishi, 1964), whereas adult song degrades (Konishi, 1965) although age-dependently (Lombardino and Nottebohm, 2000). Like in humans, the acquisition and

efficient production of a spoken language is dependent on the ability of the brain to analyze auditory information from the external environment and translate it into motor output. The efficient integration of external auditory stimuli into motor output depends on the ability of the brain to filter out redundant, unnecessary stimuli from all environmental stimuli. This phenomenon referred to as auditory "gating" ability has been suggested to enable neural circuits reduce evoked response to repeated stimulus (Freedman et al., 1987; Boutros and Belger, 1999). It has also been hypothesized to be important for the song learning process and online sensorimotor comparison via auditory-mediated feedback during vocalization (Schmidt and Konishi, 1998; Rizzolatti and Craighero, 2004). This has the capability to enhance the recognition and cancellation of unimportant sensory stimuli that interfere with a preferred stimulus (Jones et al., 2016). HVC neurons exhibits remarkable sensitivity to BOS, but not to conspecific or other sounds in the anaesthetized, non-singing state (Margoliash, 1983; Theunissen and Doupe, 1998). Such response to BOS is thought to regulate the input of auditory signals to the song nuclei (Shea and Margoliash, 2003). This suggests that regulation of input of auditory signals enhances the recognition and filtering of sound stimulus that interfere with song learning and production during the critical period of vocal learning. For songbirds, it is not clear how HVC neurons in juveniles respond to the playback of a tutor song in both awake and anesthetized conditions. This can shed light on the potential contributions of chrm2 and 4 to the regulation of the input of auditory information to the song system. HVC_X neurons exhibit hyperpolarization responses to BOS in the anaesthetized state (Rosen and Mooney, 2003). Although this response was shown to be mediated by G protein-coupled receptors, the precise identities of candidate receptors remain elusive. In addition, stimulation of the cholinergic basal forebrain decreases the excitability of both HVC_X and HVC_{RA} neurons in response to the playback of BOS and was suggested to be mediated mainly by mAChRs (Shea and Margoliash, 2003). It has been revealed that HVC_X neurons exhibit auditory-vocal "mirroring" activity, by producing similar neural activities when the birds sings and when he listens to his own song played through a speaker (Prather et al., 2008; Fujimoto et al., 2011). In the awake state, this auditory-vocal "mirroring" activities of HVC_X neurons is regulated differently among species, independent of song complexity or phylogenetic relatedness (Prather, 2013). Even though auditory-vocal mirroring activity of HVC_X neurons has been examined in only a limited number of songbird species, my study has demonstrated a potential relationship between low chrm2 expression in the HVC and auditory-vocal "mirroring" activity in HVC_X neurons in some songbird species. For instance, the Canary and Bengalese finch have lower chrm2 expression in HVC than the zebra finch

that which was formally shown to be without auditory-vocal mirroring activity in HVC neurons (Kozhevnikov and Fee, 2007), (**Figure II**–**6B**). However, another study revealed that HVC_X neurons in the ZFs exhibited extremely low activity in response to playback of BOS (Prather, 2013). It will be interesting to investigate whether individual ZFs with high and low chrm2 expression level, differ in neural activity response to playback of BOS or not. Although I did not find neuron-type specific expression of chrm2 in the HVC, my study has not only illuminated the identity of potential mAChRs that potentially regulate the auditory-vocal mirroring activity of HVC neurons, it has further provided clues to the possible contribution of these receptors to the species and individual differences in learned vocalization. It is highly likely that there could be other potential internal and external factors that regulate the input of auditory signals to the song system. I strongly recommend further studies that will examine the physiological activities of the different types of neurons in the HVC, while manipulating the activities of chrm2 and chrm4 in specific types of neurons.

How similarly conserved neural circuits generate different behavioural patterns has been a crucial question in understanding the neurobiological basis of behaviour. A potential mechanism is that central neural circuits process sensory information differently (Arbas et al., 1991; Katz, 1991; Tierney, 1995). This mechanism could involve changes in the number or properties of neurons or both, and synapses in the central nervous system or in their pattern of connectivity. For innately regulated behaviours, species differences in the patterns of activity of output motor neurons underlie variability in feeding behaviour in insects (Rast and Bräunig, 2001), fishes (Konow and Sanford, 2008), and reptiles (Herrel et al., 2001). A comparative analysis of the approximate number of each type of neuron in HVC that expresses chrm2, may provide insight into its possible contribution the HVC function.

Differences in the expression levels or patterns of neurotransmitter/neuromodulator receptor types elicit profound changes in behaviour. For instance, species differences in the expression patterns of V1aR gene underlie pair-bonding behaviour in voles (Young et al., 1997). In addition, individual differences in pair-bonding behaviour in the monogamous species of vole was shown to be because of variations in the 5'-untranslated, but noncoding region of the V1aR gene. Chrm2 expression level in the HVC is strikingly different among ZFs of similar age groups, and among adults of other songbird species. In the canary, chrm2 is expressed in other brain areas like in other songbirds. However, it is nearly absent in the HVC. Although the data is not shown in this thesis, chrm4 (which is also have inhibitory functions in mammals), shows no expression in canary HVC similarly as chrm2. The canary is an open-ended learner, with seasonal-dependent vocal learning plasticity (Nottebohm et al.,

1986). In mammals, chrm2 and chrm4 are important presynaptic auto-receptors for the feedback inhibition of ACh release from cholinergic neurons. Although it is unknown whether chrm2 and chrm4 are strictly inhibitory in songbirds, a recent study found that in zebra finches, inhibitory neuronal mechanisms act to protect acquired song segments from further plasticity (Vallentin et al., 2016). In addition, it necessary to examine whether the function of chrm2 in the ZF brain is inhibitory or not. This could be by investigating which G proteins interact with chrm2 in HVC neurons. Whether the absence of an inhibitory chrm2- and chrm4-mediated mechanisms contribute to seasonal song re-learning in the canary remains unknown. It is certainly necessary to examine the effects of CHRM2 over-expression on song learning and vocal plasticity in the canary. These will further illuminate the potential contributions of inhibitory/excitatory neuronal mechanisms to how conserved song circuits can be modulated to produce species and individual differences in learned songs.

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Main Publication

Asogwa C. **N**., Mori C., Sanchez-Valpuesta M., Hayase, S and Wada K. (2018). Inter- and intra-species differences in muscarinic acetylcholine receptor expression in the neural pathways for learned vocalization in songbirds. *Journal of Comparative Neurology,* Accepted on August 24, 2018.

List of other publications

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Merullo D. P., **Asogwa C**. **N**., Sanchez-Valpuesta M., Hayase S., Pattnaik B. R., Wada K. and Riters L. V. (2018). Neurotensin and Neurotensin Receptor 1 mRNA Expression in Song-Control Regions Changes During Development in Male Zebra Finches. *Developmental Neurobiology*, 78: 671 – 686.

Oral presentation

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Poster presentations

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Merullo D. P., **Asogwa C**. **N**., Sanchez-Valpuesta M., Wada K. and Riters L. V. (2017). Neurotensin and neurotensin receptor 1 mRNA expression in song-control regions changes during development in male zebra finches. *Society for Neuroscience, Washington, DC, USA.* November 11-15, 2017.

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