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Differential androgen receptor expression and DNA methylation state in striatum song nucleus Area X between wild and domesticated songbird strains

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Abstract

In songbirds, a specialized neural system, the song system, is responsible for acquisition and expression of species-specific vocal patterns. We report evidence for differential gene expression between wild and domesticated strains having different learned vocal phenotypes. A domesticated strain of the wild white-rumped munia, the Bengalese finch, has a distinct song pattern with a more complicated syntax than the wild strain. We identified differential *androgen receptor* (*AR*) expression in basal ganglia nucleus Area X GABAergic neurons between the two strains, and within different domesticated populations. Differences in *AR* expression were correlated with the mean coefficient of variation of the inter-syllable duration in the two strains. Differential *AR* expression in Area X was observed before the initiation of singing, suggesting that inherited and/or early developmental mechanisms may affect expression within and between strains. However, there were no distinct differences in regions upstream of the *AR* start codon among all the birds in the study. In contrast, an epigenetic modification, DNA methylation state in regions upstream of *AR* in Area X, was observed to differ between strains and within domesticated populations. These results provide insight into the molecular basis of behavioral evolution through the regulation of hormone-related genes and demonstrate the potential association between epigenetic modifications and behavioral phenotype regulation.

Introduction

Although species-specific behavior with individual variations is always observed in animals, the precise neural mechanisms of this authenticity and variability are not known (Katz & Harris-Warrick, 1999). Consequently, characterizing its molecular basis is central to our understanding of the evolution of animal behavior (Young *et al.*, 1999; Weber *et al.*, 2013).

Approximately 3000 species of the world's oscine songbirds have species-specific song patterns that have been learned from conspecific tutors. The Bengalese finch (*Lonchura striata* var. *domestica*, BF) is derived from the wild white-rumped munia (*L. striata*, WRM) and has been domesticated for more than 250 years. The effect of domestication and selection for parenting behavior and white color morphs has produced distinct differences in the song patterns of these two strains (Fig. 1A). WRMs sing stereotypical songs that have a fixed sequence with garbled song syllables. Conversely, despite some differences among individual BFs, their songs have a complex syntax with acoustically diverse chunks of syllables interspersed with diverse transitions (Okanoya, 2004).

Regardless of the diversity in song phenotypes, these songbirds employ similar neuronal pathways for learning and expressing their songs (Fig. 1B). In particular, the song system comprises forebrain song nuclei that have similar topological, anatomical, functional and connectivity characteristics. These nuclei are subdivided into two pathways. (i) The posterior vocal pathway connects the forebrain to the brainstem vocal nuclei, which is similar to mammalian motor pathways (Jarvis *et al.*, 2005) (Fig. 1B) and consists of the robust nucleus of the arcopallium (RA) and HVC (used as a proper name) (Nottebohm *et al.*, 1976). These nuclei are integrated within a circuit with the final output from RA transmitted through the dorsal medial nucleus of the midbrain (DM) to hindbrain respiratory nuclei and the tracheosyringeal nucleus (nXIIts). This pathway controls the motor neurons that produce sounds while modulating breathing. (ii) The anterior vocal pathway forms a pallial–basal ganglia–thalamic loop, which is similar to mammalian cortical–basal ganglia–thalamocortical circuits and is necessary for song learning (Fig. 1B). The

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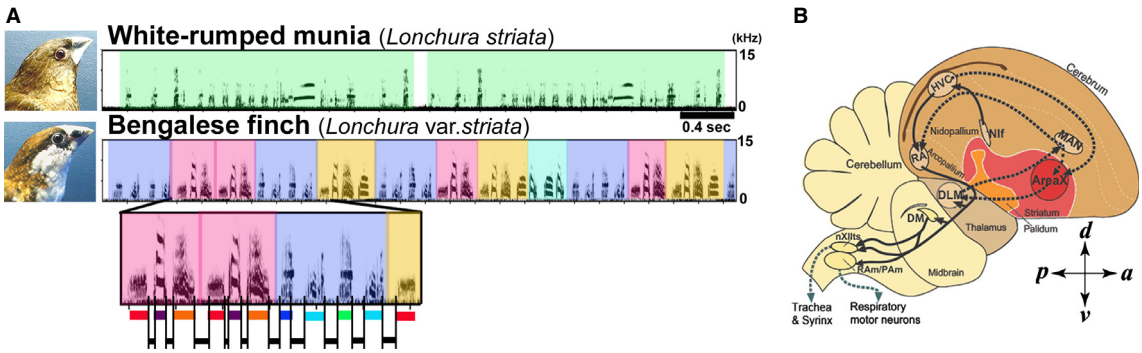

FIG. 1. Song patterns in WRM and domesticated BF. (A) A typical example of song patterns in WRM and BF. In the BF song, different colors represent different types of syllable chunks. Individual syllables are labeled with horizontal bars with different colors. Black horizontal bars represent inter-syllable durations. (B) Diagram of song pathways in the brain. Black solid lines denote connections of the posterior vocal motor pathway and black dashed lines show the anterior vocal pathway.

anterior vocal pathway consists of the following cerebral nuclei: magnocellular nucleus of the anterior nidopallium (MAN), basal ganglia nucleus Area X and dorsal lateral nucleus of the medial thalamus (DLM). Connections between these nuclei form a loop from MAN to Area X to DLM and then back to MAN. The anterior vocal pathway, which can interact with the posterior pathway through medial and lateral loops (Foster *et al.*, 1997; Jarvis *et al.*, 1998), is also involved in the real-time control of song production (Kobayashi *et al.*, 2001; Kao & Brainard, 2006), modification (Kao *et al.*, 2005) and learning (Bottjer *et al.*, 1984; Scharff & Nottebohm, 1991; Andalman & Fee, 2009).

In songbirds, androgenic hormones possess functional links that affect singing behavior and forebrain song nuclei (Arnold, 1975a,b; Nordeen *et al.*, 1987; Gahr & Konishi, 1988; Nastiuk & Clayton, 1995; Soma *et al.*, 1999). The song system is highly sexually dimorphic (Nottebohm & Arnold, 1976). Only males sing, and the song nuclei and muscles controlling song are much larger in male songbirds than in females songbirds in the temperate zone. In zebra finch (ZF), removal of the testis affects the size of the song nuclei and the amount of singing is reduced, although the birds still learn to imitate song and to sing (Arnold, 1975a, 1980). The tempo of the song is slowed by castration, and high testosterone concentrations reverse this effect (Arnold, 1975b). Furthermore, chronic excessive testosterone during development impaired song learning, resulting in a decreased number of syllables (Korsia & Bottjer, 1991). Although accumulated evidence suggests the curtailed association between androgens and song phenotypes, their causal relationships are still unknown.

AR (*androgen receptor*) is a member of the steroid receptor superfamily of ligand-dependent transcription factors that bind cognate DNA sequences called androgen-responsive elements (Bolton *et al.*, 2007). The transcriptional effect of *AR* prevails at hundreds of gene loci in the proximity of androgen-responsive elements (Kennedy *et al.*, 2010). *AR* expression is observed in the ZF song system from 10 days post-hatching (dph) to adulthood (Kim *et al.*, 2004). The level of *AR* expression is differentially regulated by testosterone in species- and brain region-specific manners in mammals and birds, including songbirds (Gahr & Metzdorf, 1997; Fusani *et al.*, 2000; Fraley *et al.*, 2010; Imamura, 2011). Methylation of CpG sites in the *AR* promoter has been proposed to influence the expression of *AR* in human prostate cancer cell lines (Jarrard *et al.*, 1998; Kinoshita *et al.*, 2000).

Although distinct differences in song phenotype have been reported among wild WRMs and domesticated BFs (Honda & Okanoya, 1999), no obvious differences in gene expression have been identified in their song systems. In this study, we examined the following questions. (1) Do the two strains and their individuals express *AR* differently in the brain at mRNA and protein levels, and if so, when does the difference arise during development? (2) In which cells of the song system is differential *AR* expression induced? (3) Which song features does differential *AR* expression relate to? (4) What types of molecular mechanism may have the potential to differentially regulate the level of *AR* expression in specific brain sites? To address these questions, we performed radioisotopic *in situ* hybridization and immunohistochemistry in the brains of the two strains and juvenile BFs and double fluorescence *in situ* hybridization with a GABAergic gene marker. We further examined whether differences in *AR* expression were correlated with song features across strains. We then compared the genome sequences and DNA methylation states of regions upstream of the *AR* start codon in Area X from individuals that expressed *AR* differentially.

Materials and methods

Animals

To examine *AR* expression using *in situ* hybridization, ten adult brains of each strain (> 150 dph) were collected. All BFs were laboratory-bred. After 35 dph, the birds were housed with other birds under a 14-h light/10-h dark photoperiod with temperature and humidity maintained at 20–25 °C and 40–65 %, until the experiments. Two WRMs were hatched from wild-caught parent pairs in a domestic aviary and eight were wild-caught and imported from Taiwan (n = 4) and south-eastern China (n = 4). Although accurate information about the life history of the wild-caught birds was lacking, they were kept under laboratory conditions with other laboratory-bred birds for at least 3 months until used for experiments. Juvenile male BFs (35–44 dph, n = 6) were collected throughout the year.

Before brain sampling, singing behavior was recorded at least twice at intervals of 2 weeks to 1 month. To rule out the possibility that singing behavior and/or social interaction may affect *AR* expression on a temporal scale of minutes-to-hours, the birds were housed individually in a sound-proof box overnight, and their brains were sampled before the onset of the light period under silent conditions in the morning before they began singing. After measuring the *AR* mRNA expression levels in Area X, we established three groups for CpG methylation analysis: WRM (n = 3), BF-Low *AR* (n = 4) and BF-High *AR* (n = 3). CpG methylation analysis was performed using the hemisphere of the brains opposite to that used for *in situ*

hybridization. These adults were also used for correlation analysis between *AR* expression levels and song features. Four adult male BFs and two adult male WRMs were used for immunohistochemical analysis. All bird experiments were performed according to the guidelines of the Committee on Animal Experiments of RIKEN and Hokkaido University from whom permission for the study was obtained. The guidelines are based on the national regulations for animal welfare in Japan (Law for the Humane Treatment and Management of Animals; after partial amendment No.68, 2005).

Radioisotopic in situ hybridization and quantification of mRNA expression and double fluorescence in situ hybridization (FISH)

Serial sagittal sections, 12 μm thick, were cut throughout the brain of BF and WRM males and ^{35}S-labeled riboprobe *in situ* hybridization was performed as described previously (Wada *et al.*, 2004). Riboprobes were synthesized from a ZF partial *AR* cDNA fragment (988–2019 bp, GenBank accession no. NM_001076688). Frozen sections were fixed in 3% paraformaldehyde/1 × PBS, acetylated, dehydrated in an ascending ethanol series, and then hybridized with ^{35}S-labeled riboprobes (1×10^6 cpm per slide) in hybridization solution [50% formamide, 10% dextran, 1 × Denhardt's solution, 12 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 8.0), 30 mM NaCl, 0.5 μg/μL yeast tRNA, 10 mM dithiothreitol] at 65 °C for 16–18 h. The slides were then washed and exposed to β-Max Hyperfilm (Kodak) before being immersed in NTB-2 emulsion (Kodak) for 3 weeks, developed and stained with cresyl violet. The signal intensity of *AR* mRNA in song nuclei and other brain regions was calculated by a previously described procedure (Wada *et al.*, 2004, 2006; Horita *et al.*, 2012). We used the exposed X-ray film of brain images digitally scanned from a dissecting microscope (Leica, Z16 APO) connected to a CCD camera (Leica, DFC490) with Application Suite V3 imaging software (Leica), with the same light settings across all images of the experiment. We used Photoshop (Adobe Systems) to measure the mean pixel intensities in the brain areas of interest from two or more adjacent sections after conversion to a 256 grayscale.

For statistical analysis of expression levels, an F-test was performed to examine the homoscedasticity of the expression ratio between song nuclei and their adjacent areas. We then performed Student's *t*-tests for HVC, RA and MAN, which were all homoscedastic. For Area X, which exhibited unequal variance, we applied Welch's *t*-test.

FISH was then performed with dinitrophenyl (DNP)- and digoxigenin (DIG)-labeled cRNA probes for *AR* and *GAD65*, respectively (Yamasaki *et al.*, 2010). DNP-labeled probes were detected with anti-DNP horseradish peroxidase (HRP)-conjugated antibody using a TSA DNP (AP) system (Perkin Elmer, Waltham, MA, USA) and anti-DNP-KLH Alexa 488 (Molecular Probes Inc., Eugene, OR, USA). Following treatment with 1% H_2O_2 for 30 min, which substantially inactivated the first HRP-conjugated antibody to avoid the second fluorophore reaction (data not shown), DIG-labeled probes were detected with anti-DIG HRP-conjugated antibody (Jackson Laboratory, Bar Harbor, ME, USA) and a TSA Plus Cy3 system (Perkin Elmer). Signal images were obtained by confocal laser scanning microscopy (LSM 510 Meta; Carl Zeiss).

Immunoblotting and immunohistochemistry

For immunoblotting, HVC and the caudal nidopallium (cN) of adult male BF brains were micro-dissected and homogenized in cold 1 × PBS. As a positive control, we used HEK293T cell lysates transfected using the ZF *AR* open reading frame and green fluorescent protein as a negative control. These lysates were then separated by 7% SDS–PAGE, transferred to a nitrocellulose membrane and blocked with 5% skimmed milk in 1 × PBS for 1 h. The membranes were then incubated with rabbit anti-*AR* polyclonal antibody (1:300; C-19, lot No. K0105; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. Next, the membranes were reacted with HRP-conjugated anti-rabbit secondary antibody (1:500; Life technologies) and binding was detected on X-ray films using an enhanced chemiluminescence detection system (Amersham Bioscience).

For immunohistochemical analysis, anesthetized birds were perfused with 1 × PBS and then 4% paraformaldehyde/1 × PBS. Sagittal sections, 24 μm thick, were cut on a freezing microtome and free-floated in 1 × PBS. Next, the brain sections were incubated in a blocking solution containing rabbit anti-*AR* polyclonal antibody (1:200) and mouse anti-*HuC/D* monoclonal antibody (1:1000; Molecular Probes) simultaneously overnight at 4 °C. After washing three times with 1 × PBS, the sections were incubated with secondary antibodies for anti-rabbit Alexa 555 and anti-mouse Alexa 488 (1:400; Invitrogen). Brain sections were then mounted in Vectashield with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, CA, USA). Signal images were obtained by confocal laser scanning microscopy (LSM 510 Meta; Carl Zeiss). To compare the levels of AR protein and mRNA expression in the same animals, we mounted adjacent brain sections on glass slides and then performed *in situ* hybridization from the acetylation step.

Quantification of song features

Before the birds were killed, we recorded individual songs using a DAT recorder (Sony ZA5ES, Japan) or a PC equipped with a microphone using Sound Analysis Pro (Tchernichovski *et al.*, 2001). Seven parameters were used to quantify song features: (i) song linearity index, (ii) song consistency index, (iii) average syllable duration, (iv) mean coefficient of variation (CV) of syllable duration, (v) average inter-syllable duration, (vi) mean CV of inter-syllable duration and (vii) number of unique syllables. We randomly selected 6–10 song bouts that were produced on different days. Each song bout ranged from 10 to 15 s in duration and contained syllables sufficient for analysing a total of 211–414 (average 273.6) syllables for each bird. Song linearity and consistency indexes were calculated as described previously (Scharff & Nottebohm, 1991). Each syllable was assigned a unique letter by three experienced observers to identify transitions of syllable connections. To calculate the mean CV of syllable duration and inter-syllable duration, the value of CV [CV = standard deviation (SD)/mean] was calculated for each song before using it for further analysis. To examine differences in song features among individuals that differed in *AR* expression in Area X, experimental BFs were separated into two groups on the basis of the *AR* expression level in Area X by *in situ* hybridization (BF-Low *AR*, $n = 3$; BF-High *AR*, $n = 3$). We then examined differences between the BF-Low *AR* and BF-High *AR* groups in the song features described above using Welch's *t*-test with the Bonferroni correction. Spearman's rank correlation analysis was performed to clarify the relationship between *AR* mRNA expression in Area X and the mean CV of inter-syllable duration for the two strains.

Cloning and sequence analysis of the region upstream of the AR start codon

To clone the region upstream of *AR* in BFs and WRMs, we first examined *AR* expression levels in Area X by *in situ* hybridization

and then extracted genomic DNA from adjacent brain slices. We used the following forward and reverse primers for conserved *AR* upstream regions in the ZF and chicken genomes: forward primer, 5′-GCAGGCTGAAGTAGACAAACC-3′; reverse primer, 5′-CCCAGCTGCACCTCCATGCT-3′. The expected size and location of the genome sequence was approximately 2100 bp, extending upstream from the ATG start codon of *AR* (see Fig. 6B). The location of the ATG start codon of *AR* in BF and WRM was obtained from ZF *AR* cDNA (GenBank accession no. NM_001076688); it was highly conserved with respect to the ATG start codon and N-terminal amino acid sequences of chicken, mouse and human (GenBank accession nos. NM_001040090, NM_013476 and M34233, respectively). The PCR conditions consisted of an initial cycle of 96 °C for 270 s, followed by 63 °C for 60 s and 72 °C for 180 s, and then 35 cycles consisting of 96 °C for 45 s, 63 °C for 30 s and 72 °C for 135 s. The PCR products were examined on 1% agarose gels before being extracted from the gels, ligated into the pGEM-T Easy plasmid (Promega), and transformed into XL1-Blue *E. coli* cells. Next, the plasmid DNA was isolated and the inserted cDNA was sequenced using vector-specific primers. The DNA sequences were BLAST-searched against the UCSC genome database and homology with ZF and chicken sequences was determined. *Cis*-teria (http://zlab.bu.edu/~mfrith/cisteria.html) and Comet (http://zlab.bu.edu/~mfrith/comet/) were used to search for putative *cis*-regulatory elements and TATA boxes.

For molecular phylogenetic analysis, the maximum-likelihood method was performed using the upstream region of *AR* in WRM ($n = 3$), BF ($n = 4$) and a ZF sequence as an outgroup, using PHYLIP software (http://evolution.genetics.washington.edu/phylip.html) after sequence alignment using ClustalW.

Sodium bisulfite sequencing with micro-dissected tissues

To examine differences in the CpG methylation status of the upstream region of the *AR* on genomic DNA in Area X cells, experimental birds were separated into three groups on the basis of the *AR* expression level in Area X by *in situ* hybridization (see *Animals* above) (WRM, $n = 3$; BF-Low *AR*, $n = 4$; BF-High *AR*, $n = 3$). Area X was micro-dissected previously (Wada *et al.*, 2006). The frozen brain hemispheres were fixed in 3% paraformaldehyde/1 × PBS at 4 °C for 14 h before being transferred to 20% sucrose/1 × PBS at 4 °C for 10 h, sectioned into 120-μm-thick sagittal slices, and dissected under a microscope using custom-made punch biopsy tools. Genomic DNA was purified by Proteinase K and RNase treatment. For the sodium bisulfite reaction, the MethyEasy Xceed Rapid DNA Bisulfite Modification Kit (Human Genetic Signatures) was used. Bisulfite-treated DNA samples were amplified using two sets of primers: (Fragment I) forward no. 1, 5′-ATAGG TTAGTAGTTATTTTATA-3′ and reverse no. 1, 5′-CAATATCCTC CATCCTACCT-3′ for Fragment I; forward no. 2, 5′-TAAGGGGA GTTTAAATTTGTT-3′ and reverse no. 2, 5′-ACCCCTACAA CTCTTAATTCG-3′ for Fragment II. The PCR products were cloned and sequenced. For each bird, 13–19 clones were sequenced for both Fragments I and II. For statistical analysis of data on the CpG methylation sites of the upstream regions of *AR* (Fragments I and II), we first performed the Kruskal–Wallis test for the percentages of CpG methylation sites between the three bird groups: WRM, BF-Low *AR* and BF-High *AR* ($P = 0.0243$ and 0.1530 for Fragment I and II regions, respectively). For total methylation percentages in Fragment I, we then applied Dunnett's test for comparisons of BF-High *AR* vs. WRM and BF-High *AR* vs. BF-Low *AR* at the 5% significance level.

Results

Differential AR expression in song systems between WRM and BF

To identify an initial set of candidate genes that corresponded with learning and/or the production of strain-specific song phenotypes between WRM and BF, we first performed *in situ* hybridization experiments as part of an earlier pilot study with genes that had been cloned by our group, such as neurotransmitter/modulator receptors and transcription factors, including hormone receptors (Haesler *et al.*, 2004; Wada *et al.*, 2004, 2006; Kubikova *et al.*, 2010). Incidentally, we found a difference in *AR* expression in the song system between the two strains. As in other songbird species, *AR* mRNAs were expressed at very similar levels and patterns throughout the telencephalic vocal nuclei HVC, MAN and RA in both strains (Gahr & Metzdorf, 1999; Kim *et al.*, 2004; Liu *et al.*, 2009). However, *AR* mRNA expression in Area X differed markedly between the two strains (Fig. 2A and B), with very little *AR* expression observed in Area X of all WRMs examined (Fig. 3A) and gradual differences in *AR* expression in Area X among BFs (Fig. 3A). The individual range of *AR* mRNA expression in Area X in BFs varied from high levels, close to those in MAN and HVC, to low levels, similar to those observed in the surrounding striatum (Str) (Figs 2B, and 3A and B). These differences were Area X-specific and not observed in other song nuclei or in areas surrounding other vocal nuclei that are not directly involved in learned vocal production: rostral nidopallium (rN), arcopallium (Arch), cN and Str (Fig. 3B). *AR* mRNA was expressed consistently at low levels in Area X of all WRMs but not of BFs (Figs 2B, and 3A and B).

To quantify differences in *AR* expression, expression ratios between song nuclei and their surrounding areas were calculated. Only Area X presented a significant difference in *AR* expression between the two strains ($n = 10$ for each strain; Student's-*t* test, HVC/cN, $P = 0.793$; RA/Arch, $P = 0.346$; LMAN/rN, $P = 0.780$; Welch's *t*-test, Area X/Str, $^{**}P < 0.01$; Fig. 3C). We further compared *AR* mRNA expression in the song nuclei MAN, RA and Area X with that in HVC in WRMs and BFs to examine song nucleus-specific regulation of *AR* expression (Supporting Information Fig. S1). The results clearly demonstrated that *AR* expression in each song nucleus was differentially regulated in the two strains, and *AR* expression in Area X was also differentially regulated between the two strains ($n = 10$ for each strain; Fisher's PLSD test after ANOVA, $P < 0.0001$). *AR* mRNA expression was consistently high in HVC and MAN even in birds with low expression in Area X. Global loss of *AR* expression in song nuclei was not observed, as might occur if androgen levels are very low. Although a set of adult birds different from those used for this *in situ* hybridization study was examined, no obvious difference in the level of testosterone was detected between the two strains (WRM, $n = 11$; BF, $n = 10$; Welch's *t*-test, $P = 0.265$; Fig. S2).

Differential expression of AR in GABAergic cells in the basal ganglia nucleus Area X before song learning

To evaluate differences in the timing of the onset of *AR* expression in Area X, juvenile male BF brains were collected at 35–45 dph ($n = 6$, mean ± SD = 40.3 ± 4.6 dph) under the same conditions as described for adults. The juveniles had no subsong singing experience. *AR* mRNA expression in Area X of juveniles was similar to that of the adults, i.e. differential *AR* expression was observed among individuals in Area X, with levels ranging from higher than to the same as that in the surrounding Str (Fig. 4A).

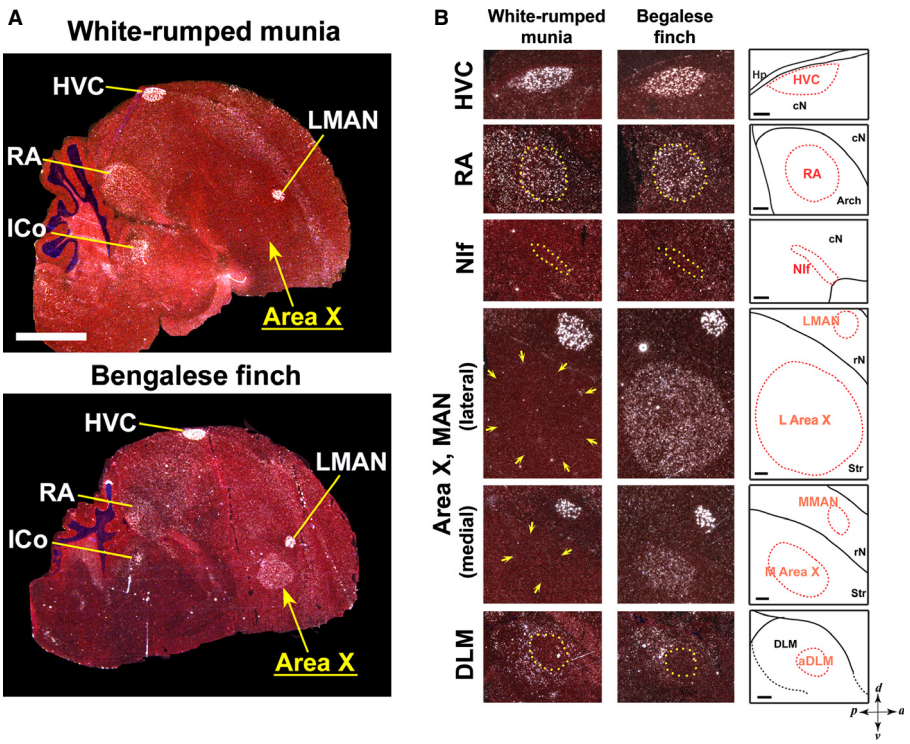

FIG. 2. *AR* mRNA expression in WRM and BF. (A) Darkfield sagittal brain images of *AR* mRNA expression in WRM and BF. Both are adult males. White color represents mRNA expression (scale bar = 2 mm). (B) *AR* mRNA expression in the song nuclei HVC, RA, NIf, Area X, MAN and DLM in WRM and BF (individuals different from those shown in A). In Area X and MAN, lateral and medial parts are shown separately. Sections are sagittal (scale bar = 200 μm).

We next examined whether individual differences in *AR* mRNA levels were reflected at the protein level in Area X. First, we used immunoblotting to test the specificity of anti-*AR* rabbit polyclonal antibody against the carboxyl-terminus of *AR* of human origin reacted to ZF *AR*. For the 94 amino acid residues in the *AR* C-terminal region, humans and ZFs have 92% homology. As with ZF recombinant *AR* protein transfected into HEK293T cells, the antibody cross-reacted with the 80-kDa BF *AR* protein (Fig. 4B, right lane and data not shown). However, a band with similar molecular size was not detected in an extract of cN, which is located adjacent to HVC (Fig. 4B, left lane). Using the *AR* antibody for immunolabeling with brain slices, we confirmed that antibody labeling matched in detail the pattern of *AR* mRNA labeling (Fig. S3). We then determined *AR* protein levels in Area X using brain slices from hemispheres opposite to those examined for *AR* mRNA expression by *in situ* hybridization (Fig. 3A and B). Most *AR* protein signals were detected in the cellular nuclei of neurons in Area X. The *AR* protein signals also exhibited individual differences matching the differential *AR* mRNA expression level observed in opposite hemispheres (Fig. 4C).

To identify the types of cells that expressed *AR* in Area X, we performed double-labeling *in situ* FISH of *AR* with probes for glutamic acid decarboxylase (*GAD65*), a GABAergic inhibitory neuronal marker. Two types of GABA-positive cells are known to be localized in Area X of songbirds, the small striatal spiny neurons and the large pallidal-like aspiny fast-firing neurons (Luo & Perkel, 1999; Doupe *et al.*, 2005; Goldberg & Fee, 2010). FISH results showed that *GAD65*(+) cells in Area X could be classified into two types on the basis of cell size and signal intensity, i.e. large cells with strong signals (Fig. 4D in the *GAD65* panel; white arrows) and small cells with weak, but distinct, signals (Fig. 4D in the *GAD65* panel; arrow heads). Most *AR*-expressing cells in Area X included both types of *GAD65*(+) neurons (Fig. 4D). The proportion of *AR*-expressing cells that were also *GAD65*(+) was 94.2 ± 0.01% (mean ± SEM), and the proportion of *GAD65*(+) cells that were also *AR*(+) was 89.2 ± 0.01% (mean ± SEM). In contrast to Area X, in the other pallial song nuclei, such as HVC, RA and LMAN, very little *GAD65* signal was observed in the *AR*-expressing cells (Fig. S4), indicating that *AR* expression was selectively regulated in GABAergic inhibitory neurons in Area X.

Correlation between AR expression in Area X and song features

To clarify whether differential *AR* expression between the two strains and within different BF populations may be related to the acquisition and/or production of different song features, we first examined differences between the BF-Low *AR* and BF-High *AR* groups in the song features described above (see Quantification of song features) (Fig. 5A). Only the mean CV of inter-syllable duration presented a significant difference between the two groups (Welch's *t*-test with the Bonferroni correction, $P < 0.05$). We further examined the possibility of generalizing the difference in the mean CV of inter-syllable duration for the two strains. We performed correlation analysis between *AR* expression in Area X and the mean CV of inter-syllable duration for the two strains (Fig. 5B). There was a significant, positive correlation between *AR* expression in Area X and the mean CV of inter-syllable duration for the two strains (Spearman's rank correlation, $r = 0.728$, $P = 0.012$, Fig. 5B).

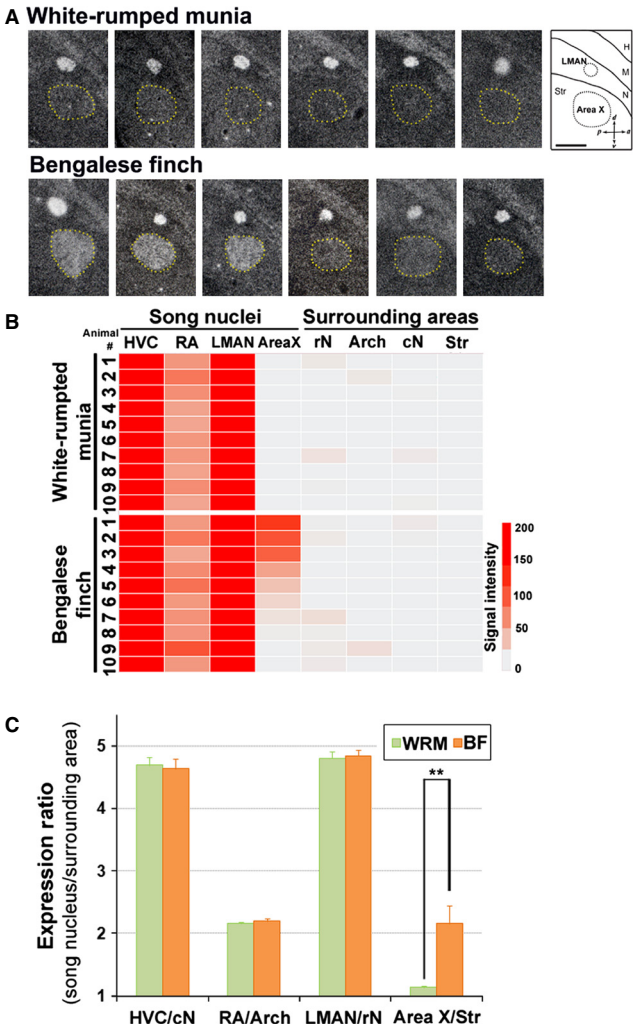

FIG. 3. Inter- and intra-strain differences in *AR* expression in Area X. (A) Magnified sagittal images of LMAN and Area X in adult males of WRMs (n = 6) and BFs (n = 6). Area X is highlighted with yellow dotted lines (scale bar = 500 μm). (B) Heat map of *AR* mRNA expression range in the song nuclei HVC, RA, LMAN and Area X and the surrounding areas (rN, Arch, cN and Str) in both strains. (C) *AR* mRNA expression ratios between four song nuclei, HVC, RA, LMAN and Area X, vs. the surrounding areas (cN, Arch, rN and Str) (mean ± SEM) (n = 10 for each strain; $^{**}P < 0.01$).

Differential DNA methylation of the region upstream of AR between the two strains

To clarify the potential molecular mechanism that regulates differential *AR* expression in Area X, we considered that genomic variations in the regulatory region upstream of *AR* probably play a role in producing the observed transcriptional variation in *AR* levels. Therefore, we cloned and compared the region 2098 bp upstream of the *AR* start codon (including the 5′ UTR sequence) in three WRMs and four BFs that had already been assayed for *AR* expression levels in Area X (Figs 3B and S5). However, phylogenetic analysis did not reveal any strain-specific or *AR* expression-related differentiation, indicating few differences in sequences between the two strains and among BFs, even among individuals that exhibited differential *AR* expression in Area X (Fig. 6A). In addition, among 71 identified single nucleotide polymorphisms (SNPs), no strain-specific SNPs were observed (Fig. S5).

Later, we recognized that the region upstream of *AR* contained a typical CpG island at the putative 5′ untranslated region, with the CpG island shore located in the putative transcription regulatory region, as reported in mammals (Jarrard *et al.*, 1998). We speculated that different DNA methylation states in the upstream region might explain the differences observed in *AR* expression in Area X. This possibility was investigated using sodium bisulfite sequencing to examine two regions, Fragments I and II, located at the shore region and at the edges of the CpG islands near the putative transcription start site of *AR*, respectively (Figs 6B and S5). Of the three *AR* expression groups, the WRM and BF-Low *AR* groups had absolute *AR* expression levels < 60 in Area X, while the BF-High *AR* group had levels > 100 in Area X (Fig. 3B). Cytosine-methylation of CpG sites in Fragment I was lower in the BF-High *AR* group than in the WRM and BF-Low *AR* groups (Dunnett's test, $P < 0.05$ for both comparisons; Fig. 6B–D). Conversely, all three of the *AR* expression groups were hypomethylated in Fragment II, located at the edges of the CpG islands (Fig. 6B–D). Although we did not clarify when the differences in DNA methylation states appeared, these results indicate an example of differences in epigenetic states corresponding to gene expression in a specific brain region associated with vocal learning.

Discussion

This study examined molecular differences in neuronal gene expression and epigenetic states between wild and domesticated songbird strains with different behavioral phenotypes, particularly learned vocal phenotypes. The song systems of the two songbird strains are very similar with respect to song nuclei size, their associated connections and cellular morphology. However, the phenotypes exhibit strain-specific features. BFs have songs with two to five song syllables organized into chunks. Several of these chunks constitute phrases formed by parsing through finite-state syntax (Fig. 1A). Consequently, song linearity is generally not high, although there are individual differences. On the other hand, WRM songs are simple and linear. These differences between song phenotypes are considered to have evolved over the last 250 years, suggesting the existence of differences in genetic predispositions for the learning and production of song patterns between strains (Okanoya, 2002). In support of this hypothesis, we identified differential *AR* expression in Area X between wild and domesticated strains and individual differences in BFs.

Functional links have been reported between androgens and song features. In ZF, the temporal patterning of the song is slowed by castration, and additional testosterone reverses this effect. The change in temporal patterning is attributed to an increase in the timescales of both inter-syllable intervals and syllable durations (Arnold, 1975b). This observation, however, is very similar to the finding that cooling HVC slows song speed across all timescales (Long & Fee, 2008). *AR* is expressed consistently and intensely in HVC in songbird species. In contrast, we found a correlation between *AR* expression in Area X and the mean CV of inter-syllable duration but no correlation with syllable duration and inter-syllable duration themselves. Based on this information, *AR* expression in HVC might affect the regulation of the overall tempo of song sequences. In contrast, *AR* in Area X might influence the production of variability in inter-syllable duration. This idea is consistent with a report that basal ganglia–thalamocortical circuits, including Area X, are required for the generation of the random mode producing broadly distributed durations of syllables and inter-syllables (Aronov *et al.*, 2011). Further experiments are needed to examine whether there is a direct relationship between *AR* in Area X and song features, including inter-syllable duration and its distribution.

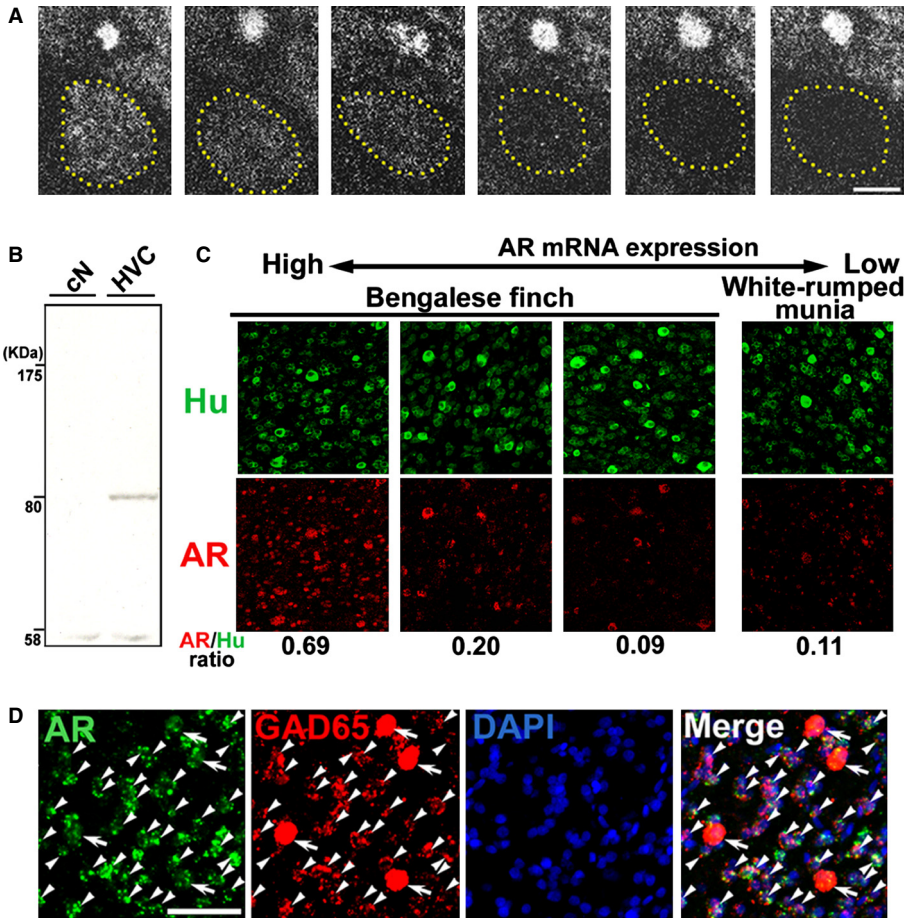

FIG. 4. Differential *AR* expression in Area X GABAergic cells before song learning. (A) Individual differences in *AR* mRNA expression in Area X in BFs at juvenile stages (35–44 dph, $n = 6$, scale bar = 500 μm). (B) Immunoblot of BF brain extracts from cN and HVC reacted with an antibody that recognizes the human *AR* carboxyl terminus. Cross-reactivity to the BF *AR* protein was found at approximately 80 kDa. (C) *AR* protein level corresponds to its mRNA expression in Area X. The *AR* protein level (red) was normalized with a neural marker, *Hu* (green), in Area X in individual birds. (D) *AR* mRNA expression in two types of GABAergic neurons in Area X. White arrows indicate GABAergic neurons having a large size and strong *GAD65* signals, whereas white arrowheads indicate GABAergic neurons having a small size and weak *GAD65* signals (scale bar = 40 μm).

Although the distribution of inter-syllable duration varies markedly among individuals in many songbird species, it is still species-specific (Catchpole & Slater, 1995; Marler & Slabbekoorn, 2004). This variability in the distribution of inter-syllable duration was also observed among different BF populations and was greater in BFs than in WRMs (Fig. 5). In ZF, developmental progression of the distribution of inter-syllable duration was observed (Aronov *et al.*, 2011; Veit *et al.*, 2011). However, it remains unknown whether the distribution of inter-syllable duration is a learned trait or is fairly constrained by inherited genetic/epigenetic mechanisms. Silent intervals in behavioral actions, such as inter-syllable duration, are considered to function as prosodic cues for segmentation and chunking when learning longer and more complex sequences (Williams & Staples, 1992; Saffran, 2002). In BF, inter-syllable duration differed significantly within chunks and at the boundaries of chunks (Takahasi *et al.*, 2010). Nevertheless, no correlations were observed between the CV of inter-syllable duration and other song features such as song linearity and consistency, which were related to sequence complexity. Therefore, it appeared that the regulation of inter-syllable duration and its distribution might have appeared as a by-product of behavioral phenotypes for learning and generating complex sequential behaviors consisting of segmented subparts.

Differential *AR* expression is selectively regulated in GABAergic inhibitory neurons in Area X. This result implies that the differential expression of transcription factor *AR* may reflect functional differences in GABAergic neurons in Area X by regulation of its downstream genes within and between the strains. At present, it is not known which *AR* target genes regulate the electrophysiological properties of neurons, but several candidates have been identified. In ZF, androgens modulate *N*-methyl-D-aspartate excitatory postsynaptic currents in the LMAN and RA nuclei, where *AR* is expressed (White *et al.*, 1999). Furthermore, the combination of *AR* activity and estrogen receptor within HVC contributed to increased firing rates of RA neurons in the white-crowned sparrow, a seasonally breeding songbird (Meitzen *et al.*, 2007). In addition, androgen and *AR*-mediated neural signaling are critically important for modulating neural action potential frequency, spontaneous inhibitory postsynaptic current amplitude, and frequency with changes in GABAergic signaling in the medial preoptic area of mice (Penatti *et al.*, 2009). These studies indicate the potential of *AR* and androgens for modulating synaptic transmission in neuronal cells at specific brain sites.

Individual differences in *AR* expression in Area X in BF were detected before juveniles started singing (Fig. 4A). The level of *AR* expression is differentially regulated by testosterone in species- and brain region-specific manners in songbirds (Gahr & Metzdorf, 1997;

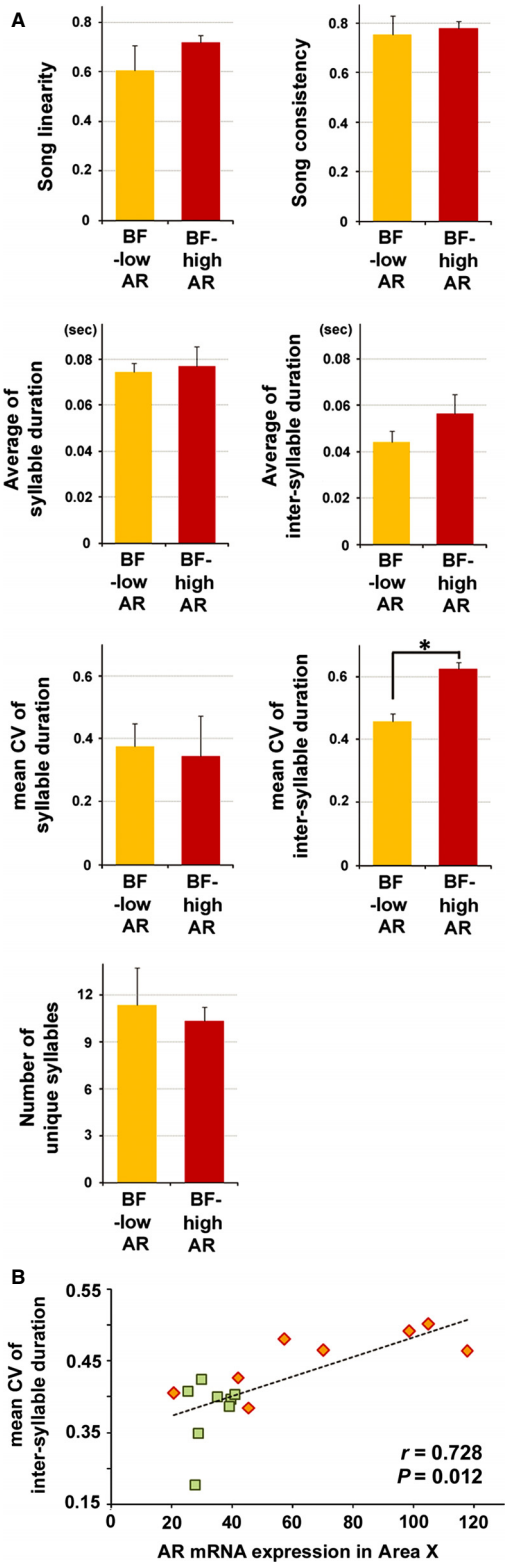

FIG. 5. Correlation between *AR* expression in Area X and mean CV of inter-syllable duration. (A) Comparisons of song features such as song linearity, song consistency, average syllable duration, average inter-syllable duration, mean CV of syllable duration, mean CV of inter-syllable duration, and number of unique syllables between the BF-Low *AR* (orange bars) and BF-High *AR* (red bars) groups (mean ± SEM) (Welch's *t*-test with the Bonferroni correction, $*P < 0.05$). (B) Correlation analysis between *AR* mRNA expression in Area X and mean CV of syllable duration. Orange diamonds and green squares represent adult BFs ($n = 8$) and WRMs ($n = 8$), respectively.

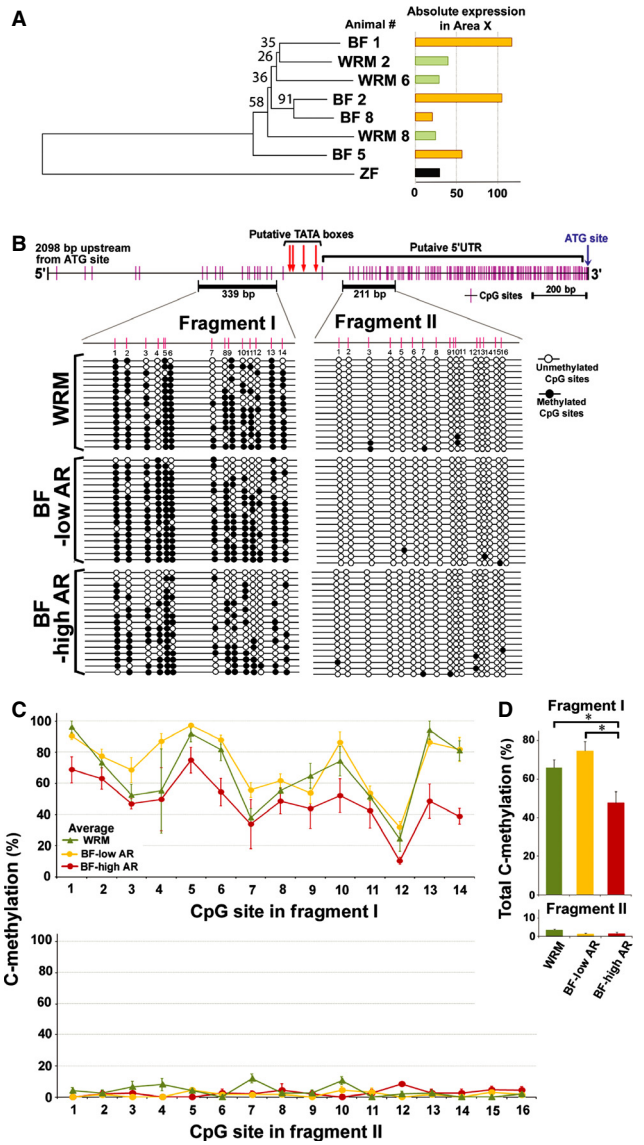

FIG. 6. Differential DNA methylation in the region upstream of *AR* between the two strains. (A) Phylogenetic analysis by genomic comparison of the regions upstream of the ATG start codon of *AR*. BFs ($n = 4$) showed different levels of *AR* mRNA expression in Area X (yellow bars). For example, BF1 had the highest *AR* expression in Area X but BF8 had low expression. In contrast, WRMs ($n = 3$) expressed similarly low levels of *AR* mRNA in Area X (green bars). Each bird ID number matches that in Fig. 3B. A ZF is used as an outgroup (black bar). The values on branches are local bootstrap probabilities from the maximum-likelihood analysis. (B) Examples of DNA methylation states in two regions upstream of *AR*. Fragment I includes 14 CpG sites located in the region upstream of the putative TATA boxes around the CpG island shore. Fragment II includes 16 CpG sites located at the putative 5′ UTR on the edge of the CpG site. White and black circles represent unmethylated and methylated CpG sites, respectively. (C) Average frequency of cytosine residues that were methylated at CpG sites in Fragments I and II of WRM ($n = 3$, green triangles), BF-Low *AR* ($n = 4$, orange circles) and BF-High *AR*($n = 3$, red circles) (mean ± SEM). (D) Frequency of methylated cytosine residues at all CpG sites in Fragments I and II of WRM ($n = 3$, green), BF-Low *AR* ($n = 4$, orange) and BF-High *AR* ($n = 3$, red) (mean ± SEM) ($*P < 0.05$, Dunnett's test).

Fusani *et al.*, 2000; Fraley *et al.*, 2010). For example, increased testosterone levels increase *AR* mRNA in HVC, but not in MAN and RA, in the white-crowned sparrow (Fraley *et al.*, 2010). In contrast, in domesticated canaries, *AR* expression in HVC does not differ

between April and November when levels of circulating testosterone are different (Fusani *et al.*, 2000). Although we could not directly determine whether differential *AR* expression in Area X was conserved among juvenile BFs until the adult stage, the result obtained in juvenile BFs suggests the possibility that differential *AR* expression in Area X could be regulated independent of age, hormonal maturation and/or breeding. Supporting this idea, circulating testosterone concentrations in the two strains were not significantly different (Fig. S2). Furthermore, *AR* mRNA expression in Area X in the presence of excessive quantities of testosterone was still low in some birds (Fig. S6). These results indicate that the level of androgen does not simply correlate with *AR* mRNA expression in Area X.

The molecular mechanisms underlying the differential regulation of specific genes in specific brain areas are still not well understood. However, in voles, inter- and intra-specific differences in social behaviors related to gene expression patterns in the brain have been reported (Young *et al.*, 1999; Hammock & Young, 2005). Pair-bonding behavior in male voles has been correlated with expression of the gene for *arginine vasopressin 1a receptor* (*avpr1a*) in specific brain regions. Variations in the expression difference of *avpr1a* are considered to be related to polymorphic microsatellites in the *cis*-regulatory regions of the gene (Hammock & Young, 2005). Furthermore, in rats, maternal care influences individual differences in *glucocorticoid receptor* (*GR*) expression in the hippocampus through epigenetic programming, DNA methylation and histone modification at promoter sites in *GR* (Weaver *et al.*, 2004). Such individual differences in *GR* expression and its epigenetic states are strongly associated with the hypothalamic–pituitary–adrenal response to stress (Szyf *et al.*, 2005).

Although we compared regions of approximately 2.1 kbp that were located upstream of the *AR* start codon in the two strains to examine any polymorphisms that might explain strain-specific and/or *AR* expression-related differentiation, there was no distinct difference in the polymorphisms, including microsatellites and SNPs. In contrast, we observed that differences in *AR* expression within and between strains corresponded to the DNA methylation states of CpG island shores near the *AR* transcription start site in adult birds. Variation in DNA methylation was reported in a similar region upstream of the *AR* transcription start site in mammalian cells (Kinoshita *et al.*, 2000; Moverare-Skrtic *et al.*, 2009). However, the causal relationship between these methylation states and the regulation of *AR* expression was not clarified. Although dissociation between DNA methylation and gene expression has recently been reported at specific loci in the human genome (Weber *et al.*, 2007; Gibbs *et al.*, 2010), DNA methylation near the transcription start sites of genes is generally known to be critical for transcriptional silencing (Suzuki & Bird, 2008; Guo *et al.*, 2011). After careful consideration of cell-type specificity in Area X, future studies need to examine the association between differential DNA methylation states and *AR* expression.

This study did not examine whether differential DNA methylation and *AR* expression were transgenerationally inherited in BF. However, the differential *AR* expression observed in Area X before the initiation of singing implies several possibilities, including social interactions between siblings and parents in the nest, hatching dates, and/or the existence of an inherited or *in ovo* mechanism. Further studies are needed to elucidate the causal factors that regulate individual differential *AR* expression. While our knowledge of the extent to which expression and epigenetic profiles could be inherited is still limited, stable inheritance of epigenetic variants has been demonstrated in rodents, humans and birds (Franklin & Mansuy, 2010; Franklin *et al.*, 2010; Natt *et al.*, 2012). Epigenetic variation has recently been considered a driving evolutionary force (Feinberg & Irizarry, 2010). Variably methylated regions (VMRs) are present across species, individuals and tissues (Irizarry *et al.*, 2009). In addition, VMRs are rich in development-related genes and have been found to be related to phenotype, at least as far as the proximate gene (within approximately 500 bases from the start codon) (Feinberg & Irizarry, 2010). The variability observed in the methylation state of the region upstream of *AR* examined in this study thus indicates sharing of some characteristics of VMRs. Furthermore, our findings suggest that VMRs may affect phenotypes not only at the tissue level but also at the specific cell type level.

Similar differences in *AR* expression in Area X have been observed in populations of ZF (Gahr, 2004) and songbirds such as the spice finch (*Lonchura punctulata*), chipping sparrow (*Spizella passerina*) and ovenbird (*Seiurus aurocapillus*) (data not shown); however, the reasons for this variability have not yet been clarified. It therefore appears that differential *AR* expression in the basal ganglia nucleus is not restricted to BFs and WRMs. Many species potentially have similar variations in *AR* expression among individuals, with differences in the distribution bias of *AR* expression varying in a species-specific manner. It may be that in specific neuron types, the distribution of key functional gene expression levels may have drifted between individuals and/or dispersed to other strains by epigenetic regulation of VMRs under specific environmental conditions, such as domestication. Epigenetic differences mediated by such important changes in gene expression could be a powerful catalyst for the evolution of behavior in phylogenetically constrained nervous systems.

In conclusion, although the functional significance of differential *AR* expression and DNA methylation in the basal ganglia nucleus Area X needs to be examined within the context of song learning and production, the present results provide insight into the neural mechanisms underlying learnability, and this in turn contributes to the understanding of the evolution of behavior at the molecular levels of gene expression and epigenetic regulation.

Supporting Information

Additional supporting information can be found in the online version of this article:

Fig. S1. *AR* mRNA expression ratios between song nuclei in WRM and BF.
Fig. S2. Circulating testosterone levels in WRM and BF.
Fig. S3. *AR* protein expression in Bengalese finch song nuclei.
Fig. S4. *AR* and *GAD65* expression in Bengalese finch song nuclei.
Fig. S5. Genome comparison of the upstream region of *AR* between WRM and BF.
Fig. S6. *AR* expression in Area X in the presence of excessive quantities of testosterone.

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Abbreviations

AR, *androgen receptor*; Arch, arcopallium; BF, Bengalese finch; cN, caudal nidopallium; CV, coefficient of variation; DIG, digoxigenin; DLM, dorsal

lateral nucleus of the medial thalamus; DM, dorsal medial nucleus of the midbrain; DNP, dinitrophenyl; dph, days post-hatching; FISH, fluorescence *in situ* hybridization; *GR*, *glucocorticoid receptor*; HRP, horseradish peroxidase; MAN, magnocellular nucleus of the anterior nidopallium; nXIIts, hindbrain respiratory nuclei and the tracheosyringeal nucleus; RA, robust nucleus of the arcopallium; rN, rostral nidopallium; SNPs, single nucleotide polymorphisms; Str, striatum; VMRs, variably methylated regions; WRM, white-rumped munia; ZF, zebra finch.

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