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The role of sex chromosomes and sex hormones in vocal learning systems



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ABSTRACT

Vocal learning is the ability to imitate and modify sounds through auditory experience, a rare trait found in only a few lineages of mammals and birds. It is a critical component of human spoken language, allowing us to verbally transmit speech repertoires and knowledge across generations. In many vocal learning species, the vocal learning trait is sexually dimorphic, where it is either limited to males or present in both sexes to different degrees. In humans, recent findings have revealed subtle sexual dimorphism in vocal learning/spoken language brain regions and some associated disorders. For songbirds, where the neural mechanisms of vocal learning have been well studied, vocal learning appears to have been present in both sexes at the origin of the lineage and was then independently lost in females of some subsequent lineages. This loss is associated with an interplay between sex chromosomes and sex steroid hormones. Even in species with little dimorphism, like humans, sex chromosomes and hormones still have some influence on learned vocalizations. Here we present a brief synthesis of these studies, in the context of sex determination broadly, and identify areas of needed investigation to further understand how sex chromosomes and sex steroid hormones help establish sexually dimorphic neural structures for vocal learning.

1. Vocal learning behavior and circuits

Vocal learning is the rare ability to imitate and modify sounds heard and is a critical component of human spoken language. In its advanced form, vocal learning has thus far been found in five mammalian lineages (humans, cetaceans, bats, elephants, and pinnipeds) and three avian lineages (songbirds, parrots, and hummingbirds) (Jarvis, 2019). Nonhuman primates and close relatives of other vocal learners have limited or no vocal learning abilities, indicating that vocal learning evolved independently in all five mammalian lineages and all three avian lineages (Jarvis, 2019; Jarvis et al., 2014; Petkov and Jarvis, 2012). The degree of vocal learning behavior varies in different species. Some, like the zebra finch, produce one song in their entire lives, which is learned during juvenile development. Others, like the European starling, many species of parrots, and humans, have expansive vocal repertoires and continue learning new vocalizations throughout life, although still not as easily as during juvenile development (Beecher and Brenowitz, 2005; Bradbury and Balsby, 2016; Doupe and Kuhl, 1999; Jarvis, 2004). The degree of vocal learning can vary between the sexes among certain species; in some species, like humans and bay wrens, males and females learn how to imitate vocalizations relatively equally, whereas females in others range from having more limited vocal

learning abilities (Jarvis, 2004; Morton, 1996), like cardinals (Jawor and MacDougall-Shackleton, 2008; Yamaguchi, 2001), to being incapable of imitating vocalizations altogether, like zebra finches (Jarvis, 2004; Jawor and MacDougall-Shackleton, 2008; Simpson and Vicario, 1990). However, in all vocal learning species, including the zebra finch, both sexes can still produce innate calls (Simpson and Vicario, 1990; Zann, 1984; Zann, 1996). The vocal learning ability is dependent on auditory learning, which is the ability to form memories of sounds heard. However, auditory learning alone is not sufficient for vocal learning, as this ability is found in both vocal learning and vocal nonlearning species, regardless of sex (Jarvis, 2019). This is why most vertebrates can learn information about sounds heard, but cannot reproduce those sounds; for example, some pet animals can learn the meaning of auditory commands of human speech sounds but cannot reproduce them.

Mirroring observed behavior, the presence of brain pathways for vocal learning correlates with differences seen between species (Balthazart and Adkins-Regan, 2002; Jarvis, 2019), and between sexes (Arnold et al., 1986; Balthazart and Adkins-Regan, 2002; Brenowitz and Arnold, 1986; Gahr, 2000; Jawor and MacDougall-Shackleton, 2008; Nottebohm and Arnold, 1976; Wang et al., 2009). To date, only vocal learning species have been found to have distinct vocal motor learning pathways

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Received 16 February 2021; Received in revised form 22 March 2021; Accepted 23 March 2021 Available online 22 April 2021 0018-506X/© 2021 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/). in the forebrain, studied thus far in humans (Fig. 1A) and the three vocal learning bird groups (Fig. 1B). Vocal non-learning species have either rudimentary forebrain circuits or only brainstem circuits involved in the production of innate vocalizations (Fig. 1). This indicates that the vocal learning pathways of vocal learners are convergent. In species where vocal learning is more complex, there appears to be an extra duplication of this vocal learning pathway, namely the dorsal larvngeal motor cortex (dLMC) in addition to the ventral larvngeal motor cortex (vLMC) in humans (Fig. 1A) (Belyk and Brown, 2017; Conant et al., 2014; Jarvis, 2019; Pfenning et al., 2014), and the shell song system surrounding the core song system in parrots (Fig. 1B) (Chakraborty and Jarvis, 2015). The convergent vocal motor pathway is further subdivided into an anterior vocal pathway involved in learning vocalizations, and a posterior vocal pathway involved in the production of learned vocalizations (Fig. 1). In species where vocal learning is sexually dimorphic, this forebrain vocal motor learning system is well-defined in males and is atrophied or almost entirely absent in females (Fig. 1B) (Arnold et al., 1986; Balthazart and Adkins-Regan, 2002; Nealen and Perkel, 2000; Nottebohm and Arnold, 1976; Simpson and Vicario, 1990). This sex difference has been best studied in zebra finches, where the nascent vocal learning pathway forms in both male and female hatchlings, but goes on to atrophy in females during early juvenile life and into adulthood, resulting in adult females being incapable of producing learned vocalizations (Konishi and Akutagawa, 1985; Konishi and Akutagawa, 1988; Nixdorf-Bergweiler, 1996; Simpson and Vicario, 1990). Despite the atrophied nuclei, for those that can be identified in the female zebra finch, tracer injections have revealed that a rudimentary circuit is preserved (Shaughnessy et al., 2019). In contrast, both sexes of vocal learners and non-learners examined to date have been found to have a comparable auditory forebrain pathway (Fig. 1), consistent with auditory learning being more ubiquitous among species (Petkov and Jarvis, 2012; Jarvis, 2019).

Since the magnitude of sex differences in vocal learning capability varies across species, a long-standing question has been what role, if any, do sex chromosomes and sex steroid hormones (i.e. estrogens and testosterone) play in the evolution, development, and function of vocal learning behavior and associated brain systems. One key discovery has shown that female zebra finches treated with extra exogenous doses of estrogen during early post-hatch development develop vocal learning systems similar to males (Gurney and Konishi, 1980); however,



Fig. 1. Vocal and auditory brain regions in (A) primates and (B) birds. Shown are drawings of brain regions necessary for vocal learning (red, yellow, and orange), auditory learning (blue), and innate vocal and auditory (grey) processing, among primates and birds. Red, anterior vocal learning pathway; yellow, posterior vocal learning pathway; orange, extra vocal learning system unique to humans and parrots; blue, auditory learning pathway; grey, innate vocal production regions. Neural connections are not shown, for simplicity. Figure is based on summaries in Jarvis (2019) & Chakraborty and Jarvis (2015). Abbreviations: aSMA: anterior supplementary motor area; aSt: anterior striatum speech area; preLMC: premotor laryngeal motor cortex; dLMC: dorsal laryngeal motor cortex; vLMC: ventral laryngeal motor cortex; A1: primary auditory cortex; A2: secondary auditory cortex; aT: anterior thalamus; PAG: peri aqueductal grey; AM: nucleus ambiguous; MO: oval nucleus of the mesopallium; MAN: magnocellular nucleus of the anterior nidopallium; CMM: caudal medial mesopallium; Av: avalanch; Nlf: interfacial nucleus of the nidopallium; L2: field L2; NCM: caudal medial nidopallium; CSt: caudal striatum; HVC: a letter based name; RA: robust nucleus of the arcopallium; aDLM: anterior medial nucleus of the dorsolateral thalamus; DM: dorsal medial nucleus of the midbrain; MLd: mesencephalic lateral dorsal nucleus; XIIts: tracheosyringeal subdivision of the hypoglossal (12th) nucleus; MOc: oval nucleus of the mesopallium complex; NAOc: oval nucleus of the anterior nidopallium complex; LAN: lateral nucleus of the anterior nidopallium; LAM: lateral nucleus of the anterior mesopallium; ACM: caudal medial arcopallium; NIDL: intermediate dorsal lateral nidopallium; MMSt: magnocellular nucleus of the anterior striatum; AAC: central nucleus of the anterior arcopallium; NLC: central nucleus of the lateral nidopallium; DMm: magnocellular nucleus of the dorsomedial thalamus; VAM: vocal nucleus of the anterior mesopallium; VAN: vocal nucleus of the anterior nidopallium; VAS: vocal nucleus of the anterior striatum; VMM: vocal nucleus of the medial mesopallium; VMN: vocal nucleus of the medial nidopallium; Ai: intermediate arcopallium; VA: vocal nucleus of the arcopallium; NDC: caudal dorsal nidopallium; VLN: vocal nucleus of the lateral nidopallium; DLM: medial nucleus of the dorsolateral thalamus.

inhibiting the production of these sex hormones in males does not prevent the development of their vocal learning systems (Choe et al., 2021; Gurney and Konishi, 1980; Holloway and Clayton, 2001; Konishi and Akutagawa, 1988; Pohl-Apel and Sossinka, 1984; Simpson and Vicario, 1991a; Simpson and Vicario, 1991b). These findings implicate sex chromosomes and estrogen for the loss of vocal learning in some species. Here we review the current state of the field regarding the roles that sex chromosomes and sex steroid hormones play in vocal learning. For context, we first briefly review the chromosomal and hormonal mechanisms of sex determination in mammals and birds.

2. Sex determination in mammals and birds

Nearly all vertebrates reproduce sexually, employing a two-sex system with each sex contributing different resources towards the production of offspring. Mammals and birds, including vocal learners among them, exclusively employ a chromosome-based system for sex determination (Gamble and Zarkower, 2012; Trukhina et al., 2013). Many non-avian reptiles utilize a temperature-based sex determining system, suggesting that mammals and birds evolved their sex chromosomes independently from different autosomes (Gamble and Zarkower, 2012; Trukhina et al., 2013) over the 300 million years separating them from their common amniote ancestor (Kumar and Hedges, 1998).

For chromosome-based sex determining systems, there are two broad categories that are characterized by whether the male or the female carries the heterogametic sex chromosome. Mammals employ the XX/XY system where males are the heterogametic sex and male sex is determined by the *SRY* (sex determining region Y) gene, also known as *TDF* (testis-determining factor), located on a non-recombining locus of the Y chromosome. Birds employ the ZZ/ZW system, where females are the heterogametic sex and female sex is determined by a non-recombining W chromosome (Fig. 2). In the mammalian XX/XY system, one of the X chromosomes in the female is mostly inactivated (Huynh and Lee, 2005; Lyon, 1961; Ohno et al., 1959) to compensate for the missing X chromosome in the male as a mechanism for gene dosage compensation (Dementyeva and Zakian, 2010); although X-inactivation escape occurs in specific instances (Souyris et al., 2018; Tukiainen et al., 2017).

In mammals, the sex determining SRY gene directs the development of male gonads by encoding a transcription factor that upregulates other transcription factors like SOX9, ultimately leading to the development of the seminiferous tubes that make sperm. A downstream target of SRY/ SOX 9 is DMRT1 (doublesex and mab-3 related transcription factor 1), a transcription factor important for Sertoli cell differentiation in the developing testis (Raymond et al., 2000; Zhao et al., 2015) and maintenance in the adult testis (Lindeman et al., 2015; Matson et al., 2011). Subsequent hormones produced in these immature testes induce the "destruction" of the proto-female reproductive structures and enhancement of the proto-male reproductive structures. In humans, DMRT1 and its paralog DMRT2 are found on chromosome 9, and haploinsufficiency (due to mutations or partial chromosome 9 loss) leads to altered or absent testes formation (Bennett et al., 1993; Muroya et al., 2000; Raymond et al., 1999). When SRY is introduced to XX embryos, they develop phenotypically male (Koopman et al., 1991). Conversely, when the SRY is removed or blocked in XY embryos, they develop phenotypically female (Fig. 2) (Jäger et al., 1990; Lovell-Badge and Robertson, 1990; McElreavey et al., 1992). X monoploidy (XO) results in female-like development, and Y monoploidy (YO) is embryonically lethal (Fig. 2). These sex chromosome-phenotype findings resulted in the "Four Core Genotype" model: 1) XX female without SRY; 2) XX male with SRY; 3) XY male with SRY; 4) XY female without SRY (Arnold, 2009, 2020; De Vries et al., 2002; Itoh et al., 2015).

In birds, no such core *SRY*-like manipulation experiments have been carried out yet, in part because transgenic technologies in birds have lagged behind mice (Han and Park, 2018; Jung et al., 2019). Nevertheless, some manipulations have been done. In birds, *DMRT1* is on the Z

		X
Q	XX XX _n XY ^{-SRY} XO XY ^{+/-DMRT1}	ZW ZZW ZZ ^{-DMRT1}
Q	XY XY _n X _n Y X _n Y _n XX ^{+SRY}	ZZ ZZZ
₽	X _n Y XY ^{+/-DMRT1}	ZW ^{+DMRT1} ZZW ZZ ^{-DMRT1}
¥	YO	ZO ZZ _n WO ZW _n

Fig. 2. Summary of genetic sex determination. Extra sex chromosomes have been denoted with "n". In mammals, male sex is determined by *SRY* on the Y chromosome and its downstream target *DMRT1*. X monoploidy results in female-like sex and Y monoploidy is lethal. X and Y polyploidy results in male sex, X polyploidy with a single Y can result in either male sex or intersex, Y polyploidy with a single X results in male sex, and homologous X polyploidy results in female sex. In birds, female sex may be determined either through a dominant W chromosome or through Z chromosome haploinsufficiency. Insufficiency of the Z chromosomal gene *DMRT1* may drive female sex or intersex development. ZZW may result in either female sex or intersex, and ZZZ results in male sex. To date, no other sex chromosome aneuploidies have been documented in living birds, suggesting their effects are lethal *in ovo*.

chromosome, as opposed to an autosome, and through incomplete gene dosage compensation, it is more abundant in males than in females (Dementyeva and Zakian, 2010). DMRT1 knockdown in developing chicken embryos causes genetically male (ZZ) animals to develop feminized gonads with loss of male gonad markers like SOX9 and gain of ovarian characteristics like elevated aromatase, the enzyme responsible for estrogen synthesis (Smith et al., 2009a). Concordantly, DMRT1 overexpression masculinizes genetically female (ZW) gonads (Lambeth et al., 2014). This suggests that DMRT1 dosage may be a primary determinant of male sex in the avian ZZ/ZW sex determining system. However, isolated cases of ZZW individuals with normal female characteristics have been documented in the wild (Küpper et al., 2012), suggesting that some mechanisms for male phenotype suppression may exist on the W chromosome. The latter finding supports a "dominant female W" hypothesis, which corresponds to the "dominant male Y" in the XX/XY system (Fig. 2). HINTW, one of the candidate genes on the avian W chromosome, failed to alter normal testes development in males when overexpressed during embryonic development (Smith et al., 2009b). It could be possible that W and Z genes exert semi-dominance, since ZZW chickens have intersex gonads (Lin et al., 1995) and ZZW songbirds have intersex plumage (de Camargo et al., 2017). Interestingly, while ZZZ chickens develop as normal (albeit sterile) males, all documented ZWW individuals terminate in ovo (Bonaminio and Fechheimer, 1993; De Boer et al., 1984; Lin et al., 1995); no birds with either Z or W monoploidy (ZO or WO) have ever been found either in the wild or in captivity, suggesting that both genotypes result in early lethality

(Graves, 2003) (Fig. 2). These limited observations support the hypothesis that the homogametic ZZ male sex is the "default" state in birds as the XX female sex is the "default" state in mammals.

3. Sex steroid hormones and the brain

Sex chromosome genes in mammals and birds help drive the development of the primary sex organs (testes or ovaries), which in turn drive secretion of sex hormones (e.g. estrogens and testosterone). These sex steroid hormones in turn, both directly and indirectly, induce specific changes in target tissues, including the brain (Lenz et al., 2012; McCarthy, 2019). In fact, it has long been known that sex steroid hormones administered during late embryonic and perinatal periods have powerful effects on adult mammalian sexual behavior (Harris and Levine, 1965; Phoenix et al., 1959; Whalen, 1964). In rodent males, the development of the testes during early development induces a surge of gonadally-derived testosterone. It is hypothesized that this transient surge of testosterone is aromatized locally within the brain to estrogen (McCarthy, 2008; McEwen et al., 1977). In addition to local conversion, both estrogen and testosterone can also be synthesized de novo within the brain (Diotel et al., 2018; Holloway and Clayton, 2001). These locally synthesized/converted sex steroid hormones are chemically identical to their gonadally derived counterparts and can interact with their cognate receptors locally; these receptors are oftentimes already differentially expressed during critical early periods (Kurian et al., 2010; McCarthy, 2008; Wu et al., 2009). These sex steroid hormones can affect target tissues through either short term mechanisms, where membrane bound receptors activate intracellular signaling cascades (Zhang et al., 2002), or long-term mechanisms, where activated receptors translocate into the nucleus to bind to DNA and initiate or suppress transcription of target genes (Mangelsdorf et al., 1995; Marino et al., 2006) or change epigenetic modifiers in the genome to also regulate gene expression (Metivier et al., 2003). Through these mechanisms, sex hormones can modify the development of sexually dimorphic neural circuits (Kurian et al., 2010; McCarthy, 2008; Nugent et al., 2011; Wu et al., 2009). Once established during development, these circuits often require hormonal activation later in adulthood, modulating appropriate initiation, execution, and maintenance of sexual and other behaviors (Balthazart et al., 1992; Juntti et al., 2010; McEwen et al., 1977).

One such sexually dimorphic neural circuit is within the hypothalamus, a structure shared across vertebrates (mammals, birds, reptiles, fishes). The hypothalamus, via the hypothalamic-pituitary-adrenal/ gonadal/thyroid axis (HPA/HPG/HPT-axis), controls several reproductive functions, including male mating behavior, female ovulation, and pregnancy in viviparous species (McCarthy et al., 2015; Nishida et al., 2005; Sower et al., 2009; Tripp et al., 2020; Turano et al., 2019; Wright et al., 2010). Some of the sexually dimorphic hypothalamic nuclei in both mammals and birds include the preoptic area (POA) (Gorski et al., 1980; Viglietti-Panzica et al., 1986), the paraventricular nucleus (PVN) (Jurkevich et al., 1996; Rosinger et al., 2019), and the ventromedial nucleus (VMN) (Balthazart and Adkins-Regan, 2002; Chester-Jones et al., 2013; Matsumoto and Arai, 1986; Turano et al., 2019; Wu et al., 2009). Areas influenced by the hypothalamus include the sexually dimorphic bed nucleus of the stria terminalis (BnST) (Jurkevich et al., 1999; Wu et al., 2009), pituitary gland (Ho et al., 2020; MacManes et al., 2017; Nishida et al., 2005), and medial amygdala (MeA)/nucleus Taeniae (Voigt and Goymann, 2007; Watson and Adkins-Regan, 1989; Wu et al., 2009). Sex differences include the size and number of cells in these regions, and expression of specific genes in those cells (Bayless and Shah, 2016; Yang and Shah, 2016).

When the aforementioned estrogenic surge or downstream estrogenic signaling is prevented in male mouse pups, either pharmacologically or genetically, several regions in their brains do not properly masculinize/defeminize, like the POA, BnST and Anteroventral Periventricular nucleus of the hypothalamus (AVPV) (Juntti et al., 2010; Simerly et al., 1997), affecting subsequent adult behavior (Honda et al., 1998; McEwen et al., 1977). Conversely, when female rodent pups are supplemented with estrogen, they are rendered sterile, as their masculinized hypothalamic-pituitary system prevents the ability to enter normal estrous cycles (Wright et al., 2010). These masculinized females also go on to exhibit masculine behaviors in adulthood when given testosterone (Wu et al., 2009). Interestingly, testosterone alone is ineffective at masculinizing the brain (Juntti et al., 2010). In humans, castration in cis-gendered men alters the hypothalamic uncinate nucleus to become more female-like (Garcia-Falgueras and Swaab, 2008). These findings support the prevailing hypothesis that estrogens regulate the organization of the brains' sexual architecture during development, which is later activated by androgens.

Species specific differences have also been documented. Mice have one sex steroid hormone responsive critical period for brain sex determination, during late embryogenesis and early perinatal life (McCarthy, 2008; McCarthy et al., 2015). Humans undergo several critical periods of sex steroid hormone responsiveness in the brain; during midembryogenesis (Mathews et al., 2009; Mitsui et al., 2019), childhood (Wermke et al., 2018), and puberty (Herting and Sowell, 2017), with some plasticity retained in adulthood (Pol et al., 2006). In both species, hormone surges during puberty also lead to the maturation of the gonads and development of peripheral sex characteristics. Precocial mammals (those born with eyes open) appear to be somewhat insensitive to nonaromatizeable androgens and reliant on estrogens for development of sexually dimorphic neural structures and behavior (Wallen, 2005; Wallen and Baum, 2002). Altricial mammals (those born with eyes closed) show some sensitivity to non-aromatizeable androgens and androgen receptor inhibitors. This suggests that estrogens and androgens may work together to properly masculinize/defeminize the altricial mammalian brain (Wallen, 2005; Wallen and Baum, 2002).

Whether this precocial/altricial, estrogen/androgen mechanism is mirrored in birds is less known. In precocial birds, the critical period appears to span the time spent in ovo, whereas for altricial birds it extends to several days past hatching (Balthazart and Adkins-Regan, 2002). Additionally, in a precocial bird species like quails, when estrogen levels are exogenously increased in ovo, male embryos develop a demasculinized POA and exhibit abnormal sexual copulatory behavior that cannot be rescued with testosterone administration in adulthood. Conversely, female embryos treated with estrogen synthesis inhibitors have a masculinized POA and masculinized sexual behavior when given supplemental testosterone in adulthood. Female-typical sexual behavior can be elicited in all of the animals, regardless of sex or developmental hormone manipulation (Balthazart and Adkins-Regan, 2002; Balthazart et al., 1992). Interestingly, when juvenile male quails were castrated at 3 weeks and given replacement steroid treatments, the medial preoptic nucleus (POM) within the POA responds to both non-aromatizeable androgens and estrogens, but they show no difference in copulatory behavior compared to uncastrated controls (Aste et al., 1993). These findings support the hypothesis that the critical period for the creation of male-specific circuits precedes the time of castration in these animals. This suggests that at least in quails, the development of the male copulatory circuitry is reliant on a low estrogen milieu, contrary to what is seen in mammals. Although we have discussed the importance of estrogen in male brain development, it is important to note that estrogens are also necessary for normal female brain development (Bakker et al., 2003; Heberden, 2017; Hill et al., 2009).

Summarizing the background, the sexually dimorphic nature of the hypothalamus is an ancient trait shared among vertebrates, predating the chromosomal mammalian XY and avian ZW sex determining systems. Although divergent mechanisms may have evolved to regulate the development of this sexual dimorphism between mammals and birds, with mice and quails as exemplars, the overall outcomes for brain sex determination have remained similar. How these mechanisms evolutionarily converged upon the same endpoint presents a fascinating area of research.

4. Sex differences in vocal learning brain systems

4.1. Minor sex differences are documented in humans

In contrast to the clear hypothalamic and associated neurophysiological and behavioral differences between men and women, recent studies have revealed small but consistent sex differences in the human speech/vocal learning systems. For example, women on average have more advanced language, verbal, and music processing skills than men (Golchert et al., 2019; Miles et al., 2016; Sundermann et al., 2016) (but see Sokołowski et al. (2020) for an alternative view). Women show changes in lateralization of language brain regions and general cognition that coincides with the menstrual cycle (Hjelmervik et al., 2012; Phillips and Sherwin, 1992). During childhood, minor sex differences have been documented in the development of multisensory speech processing regions in neurotypical and atypical children (Ross et al., 2015). In a small study of eighteen 4- and 8-week-old infants, serum estradiol levels were shown to be strong predictors of acoustic complexity, suggesting that these sex differences are established early in development (Wermke et al., 2014; Wermke et al., 2018).

Some language and learning deficits in autism are strongly correlated with sex, where being female appears to confer a protective effect (Chouinard et al., 2019; Ferri et al., 2018; Werling and Geschwind, 2013). Similarly, despite early conflicting results (Basso et al., 1982; Sarno et al., 1985; Schehter et al., 1985), it is now clear that men on average experience more severe aphasia in both speech production and comprehension after suffering damage to speech (what we consider vocal learning) and auditory brain regions than women (Sharma et al., 2019). One hypothesis for these sex differences in language deficits proposes that spoken language brain areas are more bilaterally distributed in women than in men, allowing women to recover more readily from such deficits (Wang et al., 2019). This hypothesis is consistent with differences seen in corpus callosum fibers, where women have focally larger white matter innervation superior to Broca's area, extending to the face and hand primary motor cortex regions that include the laryngeal motor cortex (Shiino et al., 2017). Differences in the functional connectivity between speech and auditory areas also exist in women relative to men (M. Xu et al., 2019), and Broca's areas in women have also been found to contain more grey matter, bilaterally, than in men (de Lima Xavier et al., 2019; Kurth et al., 2017; Weis et al., 2019). These differences could have a sex steroid hormone basis, since femaleto-male trans-men undergoing testosterone treatment have strong correlations between testosterone treatment levels and decreased grey matter volume in Broca's and Wernicke's areas (i.e. more male-like) (Hahn et al., 2016; Kurth et al., 2017). One proposed alternative mechanism for male/female differences in damage-induced communicative outcomes is that there are differences in cerebral blood flow in men versus women, rather than brain pathway differences. This may change the extent of damage possible in Broca's area, as blood flow changes can alter the metabolic profile of the brain after injury (Bai et al., 2019). However, this hypothesis cannot explain all the sex differences in language/vocal learning related functions. Thus, sex differences in the human vocal learning brain system do exist, and they appear to be, at least partially, influenced by sex steroid hormones; the influence of the sex chromosomes has yet to be determined.

4.2. Sex differences in the song system of vocal learning birds can be large depending on the species

Sex differences in the brains of vocal learning birds are better characterized than in humans, due to the former's amenability for controlled experiments. In the zebra finch, females lack or have reduced components of the song system (lack Area X, and have very small HVC and RA song nuclei) (Nottebohm and Arnold, 1976). However, females treated with exogenous doses of estrogen within the first 30 days after hatching go on to develop fully functional vocal learning systems that are similar

to those found in males. These females are capable of learning and producing courtship songs in adulthood (Simpson and Vicario, 1991a; Simpson and Vicario, 1991b). This "masculinization" is reflected in the specialized transcriptome within their vocal learning brain regions (i.e. song nuclei; Fig. 1) as shown recently by Choe et al. (2021). Estrogentreated females have many, but not all, of the same gene expression specializations in their song nuclei as males; vocal learning birds and humans have specialized differential expression for hundreds of genes specific to each vocal-learning area, relative to the surrounding nonvocal brain regions (Lovell et al., 2018; Pfenning et al., 2014). In contrast to estrogen treatment, when testosterone or dihydrotestosterone (DHT) is given during early development in place of estrogen, female zebra finches only develop very small song learning systems that are partially functional (Grisham and Arnold, 1995). Interestingly, when males are given estrogen synthesis inhibitors throughout development, the song system does not atrophy, and most of the specialized gene expression remains, although the males have poorer song learning (Choe et al., 2021). In contrast to the vocal learning pathway, blocking estrogen attenuates song-hearing induced gene expression equally in the female and male auditory pathways (Krentzel et al., 2020). Altogether, these findings suggest that unlike in the hypothalamic system, where estrogen is required for development of sex differences, in the songbird vocal learning system, estrogen regulation is linked to atrophy of the system in only one of the sexes (females).

In a less sexually dimorphic species, the canary, both sexes are capable of learning and producing songs, however males are more robust singers than females (Nottebohm, 1980; Nottebohm and Arnold, 1976). Mirroring this, the forebrain vocal learning system is present in both sexes, but is 2-3 times larger in males (Nottebohm and Arnold, 1976). Canaries are open ended learners that continue to learn new songs throughout adulthood, where syllables are added to courtship songs with each breeding cycle. Canary singing behavior also undergoes cyclical quiescence and expansion following the seasonal breeding cycle with concurrent changes in testosterone levels (Nottebohm et al., 1986). After administering testosterone or performing gonadectomy, singing frequency and complexity increases or decreases, respectively, in both sexes, confirming that the female canary song system is functional and can be activated or otherwise enhanced through elevating testosterone levels (Brown and Bottjer, 1993; Nottebohm, 1980). In the brains of species where both males and females regularly sing, higher testosterone levels in the breeding season are correlated with larger vocal learning nuclei (Jawor and MacDougall-Shackleton, 2008; Nottebohm, 1980; Schlinger, 1997; Smith et al., 1997). These findings are consistent with the activation hypothesis, where testosterone activates and enhances the vocal learning system in some species, but is not necessary to develop it (Adkins-Regan and Ascenzi, 1990).

One possible interpretation gleaned from these songbird studies is that hormonal involvement in song system development is linked with species-specific evolutionary loss in females, incorporating an estrogen dependent mechanism for sex-linked specialization similar to, but separate from, sex differences in the hypothalamus (Fig. 3). The vocal learning system evolved independently within the last \sim 30–50 million years in the three different vocal learning birds (Jarvis et al., 2014) and within the last 0.5-2.0 million years in humans (and possibly in other hominids) (Morgan et al., 2015; Uomini and Meyer, 2013). The hypothalamic-pituitary system evolved at least 400 million years ago when bony fishes diverged from other vertebrates, if not 600 million years ago with our earlier chordate ancestors (Sower et al., 2009). Sex chromosomes evolved independently in birds and mammals within the last 300 million years. The combined timelines indicate to us that the vocal learning systems evolved separately and independently from the more conserved vertebrate HPA/HPG/HPT-axis systems, but still evolved to co-opt a dependence on sex steroid hormones and likely on sex chromosomes for subsequent vocal learning sex-linked loss in females of some species, and modulation and activation in many more species.



Fig. 3. Interplay between genetic sex and estrogen (E2) on (A) HPA-axis and (B) song system sex dimorphism. Female song system develops normally in some species and requires estrogen induced rescue in others. Black, normal phenotype without experimental manipulation. Blue is E2 depleted and red is E2 supplemented, which can induce feminization or defeminization/masculinization, depending on species and neural system, during the critical period. Green, genetically manipulated feminization or defeminization. Bar-headed lines indicate incomplete phenotype, and arrow-headed lines indicate complete phenotype.

5. Specializations of hormone receptors in vocal communication circuits

All three vocal learning bird groups (songbirds, parrots, and hummingbirds) show specialized gene expression patterns within their vocal learning circuits, including the sex steroid hormone receptors, which are not seen in their closest vocal non-learning relatives or in the distantly related Galliformes (chicken and quail) (Gahr, 2000). In songbirds, estrogen receptor 1 (ERa) is upregulated in HVC and its descending fibers relative to the surrounding nidopallium neurons, whereas androgen receptor (AR) is upregulated in most forebrain song nuclei, including the HVC, RA, Nif, LMAN, and Area X. AR is also upregulated in the nXIIts of the brainstem, the nucleus that is the output to the avian vocal muscles (Frankl-Vilches and Gahr, 2018; Gahr and Metzdorf, 1999; Kim et al., 2004). The G-protein coupled estrogen receptor (GPER) is widely expressed in the brain but elevated within HVC and RA (Acharya and Veney, 2012). Within the auditory learning pathway, estrogen receptor 2 (ER β) and aromatase are enriched in NCM (caudomedial nidopallium) (Jacobs et al., 1999; Perlman and Arnold, 2002; Shen et al., 1995).

We are not aware of any study that sought to determine if there was specialized expression of hormone receptors in human speech brain regions. So we performed a qualitative analysis of the sex steroid receptors using the Allen Institute for Brain Science surface gene expression maps (Fig. 4). We did not note any overt consistent specialized expression patterns across individuals in either the dLMC or vLMV, two regions that have convergent specialized expression of other genes with the RA analog of all three avian vocal learning lineages (Nevue et al., 2020; Pfenning et al., 2014).

The causal molecular mechanisms that drive the specialization of these hormone receptors and other genes in vocal learning brain areas are not known, although ongoing work in our lab indicates that epigenetic mechanisms are involved (Cantin et al., 2018). The downstream target genes of these hormone receptors in vocal learning brain regions are also not known, but it is possible that the target genes are involved in modulating the maturation and activation of vocal learning systems, as the specialized expression for some of these genes change developmentally (Wang et al., 2015). Such functions for estrogen and testosterone mediated regulation in the song system may include the closing of the critical vocal learning period and priming of the song system for singing (Cornez et al., 2020a; Cornez et al., 2020b; Konishi and Akutagawa, 1988). Incidentally, this closing off of the critical period is similar to the window for native language formation in humans, which closes near the onset of puberty (Doupe and Kuhl, 1999; Piekarski et al., 2017; Wermke et al., 2018). In summary, the specialized expression of sex steroid hormone receptors in vocal learning systems suggests specialized modulation of vocal learning by the sex steroid hormones.

6. Mechanisms for formation and evolution of sexually dimorphic vocal learning systems

Both hypothalamic sexual dimorphism in mammals and vocal learning sexual dimorphism in songbirds utilize apoptosis and proliferation as key mechanisms of differentiation. In the mammalian hypothalamus, males and females have the same number of cells shortly after birth, but specific cell populations during early development die via apoptosis, which can be either induced (AVPV) or suppressed (POA) in response to estrogen or aromatize-able testosterone (Arai et al., 1996; Davis et al., 1996; McCarthy, 2008; Waters and Simerly, 2009). In the song system, female and male zebra finches develop similarly during early development (0-20 days), but thereafter, increased cell proliferation occurs in male vocal learning nuclei and apoptosis mediated atrophy occurs in female vocal learning nuclei (Konishi and Akutagawa, 1985). When female zebra finches are given higher than normal doses of estrogen, this prevents the apoptosis mediated atrophy of their vocal learning system (Choe et al., 2021; Konishi and Akutagawa, 1988). Unlike the mammalian hypothalamus, estrogen deprivation/inhibition does not cause a substantial change in the male vocal learning system of songbirds (Bender and Veney, 2008; Choe et al., 2021; Tehrani and Veney, 2018). These findings indicate that the mechanism for hypothalamic atrophy in female mammals and birds may be similar to the mechanism of vocal learning nuclei atrophy in female zebra finches; however, the mechanism in male vocal learning nuclei development is different from the hypothalamus.

A final piece of the puzzle linking sex chromosomes and sex steroid hormones to the formation of vocal learning systems can be gleaned from rare avian gynandromorphs. In these animals one half is male and the other is female (Morris et al., 2018; Peer and Motz, 2014), with a clear middle separating the two halves despite sharing the same hormonal milieu. One side contains testes and the other ovaries (Agate et al., 2003; Morris et al., 2018). Interestingly, this type of gynandromorphism has only been documented in species with the ZZ/ZW sex determining system, including birds, butterflies, and lobsters, even though the ZZ/ZW chromosomes in these species are not derived from a common ancestral autosome (Chace Jr. and Moore, 1959; Narita et al., 2010; Scriber et al., 2009). Agate et al. (2003) observed that beyond plumage, the vocal learning system in a gynandromorphic zebra finch was also split by the corresponding sex genotype: the song nuclei in the male side were larger than those in the female side. However, HVC was actually larger than normal on the male side, and the song nuclei on the female side were at the upper end of that seen in normal females. This suggests that male and female song nuclei characteristics are strongly influenced via cell intrinsic and extrinsic mechanisms in ZZ and ZW cells even when sharing a similar circulating hormone environment. These findings also support that vocal learning in males, as the homogametic

ESR1



Fig. 4. Expression surface map from the Allen Brain Atlas. Human microarray expression data from 24yo Black male (left), 49yo Hispanic female (center) and 55yo White male (right) (Hawrylycz et al., 2012). Each shape is a different sample. Purple is higher expression and green is lower expression relative to the whole cortex. Arrows point to dorsal (yellow) and ventral (blue) laryngeal motor cortices (dLMC, vLMC).

sex may be the "default" state, and its loss in females of some species is the derived state.

In terms of evolution, the songbird phylogenetic tree provides some clues regarding the cause of this sex-linked and hormonally modulated differentiation of the song system. In the majority of species for which there is documented singing behavior, both males and females are capable of producing apparent learned song (Fig. 5A). The tree topology suggests that female song loss occurred independently and frequently, especially in clades that experience extreme sexual or environmental competition (Jarvis, 2006; Morton, 1996; Odom et al., 2014). Coincidentally, there is evidence for rapid evolution both within the songbird W and Z chromosomes compared to other avian lineages (L. Xu et al., 2019; Zhou et al., 2014). These rapidly evolving sex chromosomes may contain genes or regulatory networks that contribute to multiple independent vocal learning losses in females. This could include gene products originating from the female W chromosome that "turn off" the development of the song system in species where females lost song learning (Fig. 5B). This "off" switch could later be overturned or rescued by estrogen signaling in these song learning regions, via specialized expression of estrogen and androgen receptors in both male and female songbirds. This rescue would presumably occur downstream of a Wdominant "off" switch. Several genomic evolution mechanisms to consider include pseudogenization, changes in promotors and other regulatory regions, and changes in synteny through duplication, deletion, inversion, and translocation.

This hypothesis is similar to a disassortative mating phenomenon seen in the white throated sparrow (*Zonotrichia albicollis*). In this species, there is a chromosomal inversion that leads to two different forms of chromosome 2, one of which carries a "supergene" locus that behaves in a dominant fashion. The "supergene" locus carries mutations in ER α and other genes that impact behavior. Furthermore, the evolutionary trajectory of these two forms of chromosome 2 is quite similar to that of sex chromosomes (Thomas et al., 2008; Tuttle et al., 2016). Those that carry the "supergene" have a dose-dependent increase in aggressive behavior; this increased aggression is lowered to the levels seen in individuals without the "supergene" when ER α is knocked-down (Merritt et al., 2020). This dominant effect is also accompanied by an allelic imbalance where the expression of the "supergene" locus is favored, caused by cisacting elements and epigenetic changes within this region. This incredible phenomenon was only discovered with the aid of in-depth molecular characterization and a haplotype-phased genetic experiment (Davis et al., 2011). In order to properly examine our proposed "W-off" hypothesis, we argue the need for high-quality heterogametic genome assemblies, with transcriptomic data from multiple individuals, paired with similar gene manipulation studies.

7. Proposals for future data and experiments

The biggest unanswered question, we believe, is: What are the mechanisms that cause sex dimorphism in vocal learning circuits for those species that have it at extreme levels, such as the zebra finch, versus those that have minor differences, such as some parrots and humans?

One understudied area that may help address this question is epigenomics. For example, epigenetic differences have been shown to be involved in sexual dimorphism for other traits (Day and Bonduriansky, 2004; Gregg et al., 2010; Patten and Haig, 2009; Rice and Chippindale, 2001), so one can ask, are there sex-linked epigenetic differences in the sex chromosomes for genes that control development of vocal learning circuits? Trans-acting elements and inter-chromosomal interactions have been documented between sex chromosomes and autosomes in mammalian genomes (Kalhor et al., 2012; Kaufmann et al., 2015; Zhao et al., 2006); do these interactions also exist in birds, and can these interactions impact vocal learning and its sex distribution? Some specific genomic features to examine could include the hormone receptor binding sites that are scattered across the genome. To our knowledge, only one study has looked at androgen and estrogen-responsive elements in a vocal learning context, within the canary (Frankl-Vilches et al., 2015). But where are the hormone receptor binding sites in the genomes of other vocal learning species and their closely related vocal nonlearning relatives? Are there differences in the binding affinity or availability of hormone-receptor binding sites between vocal learners,



Fig. 5. Possible source of sexual dimorphism in singing behavior. (A) A phylogenetic tree of songbird lineages with known song behavior from males and females. Species with singing behavior in both sexes are colored in red, and species with singing behavior exclusive to males are colored in blue. The majority of species have song capable females, and only species with extreme sexual competition (e.g. birds of paradise) or with extreme natural selection (e.g. shrikes & honey eaters) have song incapable females. This indicates strong evidence that female singing behavior is ancestral to all extant songbirds (from Odom et al. (2014)). (B) Both the Z and W chromosome are undergoing rapid evolution in songbirds. If singing behavior is the default ancestral state of the song system, then elements on the sex chromosomes may drive sexual dimorphism in species where female song capability was lost, which can be rescued through administration of exogenous estrogen (from Choe et al., 2021). Incomplete gene dosage or haploinsufficiency of Z chromosome genes in females may drive the decay of the song system. Alternatively, genes that have obtained a dominant-negative role (coding or non-coding lnRNA/miRNA), a gain of function, or haplodominance on the W chromosome may drive decay of the song system.

non-learners, and sexually dimorphic learners? Are there temporal dynamics involved throughout development? Answering these questions will benefit greatly from high throughput genomic technologies, like ChIP-sequencing for estrogen and androgen receptor binding sites in the genome, along with ATAC-sequencing for assessing all open chromatin, Hi-C for chromosome interactions, and RNA-sequencing for assessing all differentially expressed genes, the latter of which was recently done by Choe et al. (2021) for song-regions at one developmental time point in zebra finches.

Another understudied area is in comparative evolutionary genomics, and here too there are many specific questions that could direct future research endeavors. There is evidence that some novel genes and gene duplications may be unique to some vocal learning lineages (e.g. oscine songbirds) when compared to closely related vocal non-learning lineages (e.g. suboscine songbirds) (Wirthlin et al., 2014). And some genes and regions within the autosomes and avian Z-chromosomes of some vocal learning species show evidence of accelerated/selective evolution (Balakrishnan et al., 2013; Kong et al., 2010; Li et al., 2007; Yuri et al., 2008). Whether these changes have causal impacts on vocal learning circuits and behavior is still unknown.

Until recently, sex chromosomes have not been investigated in many non-model organisms. Therefore it is unknown whether genetic differences exist within the W-chromosomes of specific avian clades/species where females retained or lost the vocal learning ability. A future goal would be to produce more high-quality genomes of songbird species where females have independently lost song and have close sister species that maintained it, beginning with specific clades identified by Odom et al. (2014) and the female birdsong project (Odom and Benedict, 2018), to find the genomic origin of vocal learning loss in females, potentially both within the sex chromosomes and autosomes. This can of course be expanded to other avian vocal learning lineages (parrots and hummingbirds) where sex differences exist between closely related species. As argued by Jourjine and Hoekstra (2021), comparative evolutionary genomics can reveal new principles into neurobiology by expanding model organisms beyond just species, but to whole clades. Such experiments may also help identify candidate factors in the evolution and function of vocal learning circuits.

Investigating these questions necessitates the availability of highquality genomes and high-quality sex-specific transcriptomic and epigenomic data (Ratnu et al., 2017). This is currently being addressed by



Fig. 6. 2nd vs 3rd generation genome assemblies. (A) Previous reference genomes made from medium length (Sanger) and short length (Illumina) reads scaffolded together versus new genomes made from long reads (Pac-Bio). Newer genomes from the VGP resolve erroneous repeat sequences (green) due to differences in haplotypes as well as gaps (in black). From Korlach et al. (2017). (B) Genomes assembled using a reference genome risk missing chromosome rearrangements and syntenic shuffling by artificially assuming conformity. Here, hypothetical species A was used to create the genome for hypothetical species B. If a chromosome rearrangement point exists after the first gene in species B, this would be missed in the species A referenced genome but would be identified in a de novo assembled genome. (C) Hi-C read interaction maps showing near complete chromosomal level scaffolds in the zebra finch (bTaeGut2) and the platypus (mOrnAna1). Bands at the top identify sex chromosomes: blue is X/Z and green is Y/W. From Rhie et al. (2021).

the efforts of the Vertebrate Genomes Project (VGP), which to date has produced over 130 high-quality, chromosome level genomes that include the full sex chromosome complements (Rhie et al., 2021; https://www.ncbi.nlm.nih.gov/bioproject/489243; https://vgp.github. io/). The first high-quality genomes, including human, mouse, and fruit fly, were first generated using expensive "first generation" sequencing technologies with 400–700 bp read lengths (Rhie et al., 2021). These were followed by less expensive but lower quality "second generation" or "next-gen" sequencing technologies, with 30-200 bp short read lengths. The primary downsides of first and second-generation sequencing platforms are that they are poor at resolving whole chromosomes, which often contain highly repetitive and GC-rich regions, leading to large gaps (Rhie et al., 2021). These gaps have been better addressed through incorporating third-generation, high-throughput, long-read sequencing technologies, like the Pacific Biosciences' SMRT technology, which can sequence through long repetitive regions and GCrich regions (Korlach et al., 2017; Roberts et al., 2013). When merged with long-range scaffolding data, like Hi-C and optical maps, they have allowed for the assembly of more complete, near error-free, haplotype phased chromosomes (Fig. 6) (Rhie et al., 2021).

While the vocal learning circuit has been beautifully characterized, it does not work in isolation. The vocal learning circuit has been proposed to sit inside of a motor learning circuit from which it independently evolved in different species, explaining some of the similarities that they share in divergent species (Feenders et al., 2008). These surrounding circuits are considered the same in males and females, and have some input into the song system. Future investigations could find out when during development the vocal learning circuits diverge from the surrounding circuits, and whether that divergence point is associated with functional dependence on sex chromosomes or sex steroid hormones. In the avian brain we are still resolving the origins of the pallial subdivisions (Gedman et al., 2020), and other such experiments would further resolve development origins of the avian brain. While we have some region-specific transcriptomic data collected during specific periods of juvenile development among studies (Choe et al., 2021; Hayase et al., 2018; Louder et al., 2018; Lovell et al., 2018; Shi et al., 2020), this does not span across the entire developmental window. Lastly, better molecular tools can aid in refining our understanding of how the hypothalamus interacts with vocal learning circuits. For example, Riters and Alger (2004) and others (Alward et al., 2013; Alward et al., 2016; Nottebohm et al., 1976; Riters and Ball, 1999) have shown that the hypothalamus drives motivation, which increases practice, which in turn may drive some aspects of the sex differences seen in the song system.

Behavior is influenced by genetic variation; although no one specific gene is likely the cause of any one specific behavior, minute differences in various bodily systems will invariably modulate behavior (Niepoth and Bendesky, 2020). The identification of sex differences in regulatory and coding sequences through comparative genomics also requires large sample datasets, with greater characterization of sex differences in vocal learning behavior across many species. Candidates identified from such genome wide association studies (GWAS) across species could then be used to pave the way for targeted mechanistic studies. In humans, GWAS studies have unearthed evidence that specific genetic variations may be linked to substance abuse and addictive behaviors (Crist et al., 2019; Evans et al., 2020; Moses et al., 2020) or vulnerability to developing depression and other forms of mental health conditions with shared comorbidities like insomnia, anxiety and suicide (Lind and Gehrman, 2016; Musliner et al., 2019; Purves et al., 2020; Ruderfer et al., 2020; Stein et al., 2018). Performing mechanistic studies is difficult in humans, but variants for similar genes and their functional partners could be manipulated in other vocal learning species.

8. Conclusion

There is much to be determined about sexually dimorphic structures

and circuits within the brain. Although we have learned much from rodent models, additional work needs to be done in other species with rare and complex behaviors, like vocal learning and spoken language. Even though songbirds, parrots, and hummingbirds have >300 million years of evolution separating them from humans, and 66 million years separating them from each other, they presently represent the most tractable models for studying the underpinnings of vocal learning and spoken language. In humans, spoken language delays are sexually biased, with more boys experiencing communicative disorders. If we could understand how sex influences brain development, we could understand why being female at birth is more protective against these disorders. However, in order to understand how sex differences occur in the human brain, we need to more broadly understand how sex differences occur in the vertebrate brain, and to do that, more genomes and basic functional research are needed in both male and female subjects.

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