

Contribution of birds to the study of sexual differentiation of brain and behavior[☆]

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ABSTRACT

Behavioral neuroendocrinology has largely relied on mammalian models to understand the relationship between hormones and behavior, even if this discipline has historically used a larger diversity of species than other fields. Recent advances revealed the potential of avian models in elucidating the neuroendocrine bases of behavior. This paper provides a review focused mainly on the contributions of our laboratory to the study of sexual differentiation in Japanese quail and songbirds. Quail studies have firmly established the role of embryonic estrogens in the sexual differentiation of male copulatory behavior. While most sexually differentiated features identified in brain structure and physiology result from the different endocrine milieu of adults, a few characteristics are organized by embryonic estrogens. Among them, a sex difference was identified in the number and morphology of microglia which is not associated with sex differences in the concentration/expression of neuroinflammatory molecules. The behavioral role of microglia and neuroinflammatory processes requires further investigations. Sexual differentiation of singing in zebra finches is not mediated by the same endocrine mechanisms as male copulatory behavior and “direct” genetic effect, i.e., not mediated by gonadal steroids have been identified. Epigenetic contributions have also been considered. Finally sex differences in specific aspects of singing behavior have been identified in canaries after treatment of adults with exogenous testosterone suggesting that these aspects of song are differentiated during ontogeny. Integration of quail and songbirds as alternative models has thus expanded understanding of the interplay between hormones and behavior in the control of sexual differentiation.

1. Introduction

Reproduction, a defining feature of living organisms, has taken multiple forms in the course of evolution. Scissiparity (reproduction by simple division of an adult) of simple primitive organisms evolved in most species in a form of reproduction based on specialized cells, the gametes. Initially these gametes were identical in all subjects of a given species (isogamy), but this mode of reproduction evolved independently in multiple lineages of plants and animals to anisogamy where one group of individuals (by definition the females) produces large gametes in relatively smaller numbers, the oocytes, while another group (by definition the males) produces a multitude of small gametes, the spermatozoa. As a consequence, the two sexes have developed different reproductive strategies (e.g., higher selectivity for partner in females than in males, development of complicated ornaments in males in order to attract females). A large amount of research has been devoted to the

analysis of the mechanisms that mediate the differentiation of these two types of subjects within a same species.

The first step in this process is usually known as sex determination. Our anthropomorphism often leads us to assume that sex determination in all animals is operated like in humans and mammals by the presence of one pair of sex chromosomes, labeled X and Y in mammals. This is, however, very far from the truth. There is across animals and plants a huge diversity of mechanisms mediating primary sex determination. This ranges from determination by a variable number of sex chromosomes that bear no relationship with the mammalian X and Y to a determination by environmental temperature or determination by the number of copies of all chromosomes (haplo-diploidy) or elimination of the paternal genome (Bachtrog et al., 2014).

Simply put, in mammals the *sry* gene (for sex-determining region of the Y) located on the male Y chromosome controls the production of a protein, called the testis determining factor (tdf), that directs the

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development of the undifferentiated gonad into a testis. In the absence of *sry*/*tdf*, this embryonic tissue will form an ovary. This is, however, a simplistic description and several other genes are also implicated in these processes (She and Yang, 2017). Once the sex of the gonads is fixed, this/these gonad(s) will secrete sex steroids that will, in most vertebrates, generate a cascade of events leading to the sexual differentiation of all tissues explaining sex differences in various aspects of the phenotype. In mammals, the newly formed testes start producing testosterone (T) early during ontogeny and this steroid or its metabolites masculinize and defeminize many features of brain and behavior. In the absence of T, a female phenotype will develop. This process was initially identified in the seminal paper of Phoenix and collaborators based on work manipulating T exposure during the early stages of life in guinea pigs, *Cavia porcellus* (Phoenix et al., 1959). In the 50 years that followed hundreds of papers confirmed the relatively general nature of this mechanism of sexual differentiation (for review: (Gerall et al., 1992; Goy and McEwen, 1980)), while identifying a few exceptions to the rule and providing a few refinements. It was namely shown that a few sexually differentiated features develop before and independently of testicular steroids (e.g., the scrotum of marsupials (Renfree et al., 1995); the dopaminergic neurons of the mesencephalon (Beyer et al., 1992; Küppers et al., 1991; Reisert et al., 1989)). In addition, the peripubertal period was demonstrated to be a second period of organization for sexually differentiated behaviors and brain features in males (Schulz et al., 2004; Sisk and Zehr, 2005) as well as in females in which estrogen secretion at the beginning of the puberty is required for the full development of female-typical behavior (Bakker et al., 2002; Brock et al., 2011).

2. Sexual differentiation in quail

Given the seemingly general nature in mammals of the mechanism initially formalized by Phoenix and colleagues, the results of the first studies analyzing this process of sexual differentiation in Japanese quail (*Coturnix japonica*) came as a surprise. Elizabeth Adkins-Regan, then a student of Norman Adler at the University of Pennsylvania, first tested the impact of sex steroids (estradiol benzoate, EB and testosterone propionate, TP) on the expression of male- and female-typical reproductive behaviors in adult subjects that had been functionally castrated by exposure to short photoperiods (Adkins and Adler, 1972). Contrary to what had been demonstrated in mammals where the expression of female receptivity is more sexually differentiated than male-typical copulatory behavior, males injected with EB displayed both male- and female-typical behavior, whereas females injected with EB or TP exhibited normal sexual receptivity but absolutely no male-typical copulatory behavior. Thus, the male quail appeared more bisexual than the female, while the opposite is observed in mammals. In addition, these experiments indicated that male-typical copulatory behavior is sexually differentiated in the organizational sense: it cannot be activated in adult females by hormonal treatments that fully activate the behavior in males. In contrast, other male-typical behaviors such as crowing and strutting, and squatting, the marker of female receptivity, can be activated by adequate treatments (with TP or EB, respectively) in both sexes. They are thus not normally expressed in both sexes, but this is only due to the lack of activation in adulthood, not the result of organization by early steroid action. The search for the organizational origin of the sex differences in copulatory behavior by investigation of the effects of very early endocrine treatments was going to reveal additional surprises.

In 1975, Adkins demonstrated that a single injection of TP or EB in quail eggs does not masculinize behaviors as observed in mammals but rather causes a demasculinization of males (Adkins, 1975). This demasculinization of males by an *in ovo* injection of EB is also restricted to a critical period of the embryonic life: adult males that had been injected with EB *in ovo* and treated with exogenous TP as adults failed to mount and copulate with females only if the EB injection was performed

on or before day 12 of incubation (Fig. 1A) (Adkins, 1979; Adkins-Regan, 1983; Schumacher et al., 1989). A detailed dose-response study also demonstrated that EB demasculinizes male behavior at much lower doses than TP: a full demasculinization of males was observed in subjects injected with 1 µg EB, but 100 µg TP or more were required to obtain the same effect (Fig. 1B) (Adkins, 1979). It could sound strange that both androgens and estrogens are having the same demasculinizing effects, but a suite of studies proved that TP demasculinizing effects are mediated via its conversion into an estrogen by aromatization. Among other things, the concurrent administration of an aromatase inhibitor or a estrogen receptor antagonist blocked these effects of TP (Adkins-Regan et al., 1982).

At that time, there was already evidence that the embryonic ovary in birds starts secreting estrogens very early during incubation (Guichard et al., 1980; Scheib et al., 1981). As a consequence, it was speculated that the absence of male-typical copulatory behavior in adult females resulted from their early exposure to endogenous estrogens produced by the ovary, a process that would be experimentally reproduced in males by the injection of exogenous estradiol, E₂ (Adkins-Regan, 1983). Some support for this model was actually found in one study demonstrating that an injection of the estrogen receptor antagonist CI-628 (nitromifene citrate) on day 9 of incubation partly masculinized the behavior of the treated females (Adkins, 1976). Six of the 9 injected females displayed head grabs and mounts and 3 of them performed cloacal contact movements, behaviors that were not seen in control females in this and in other experiments. The latency to show these behaviors was however much longer than observed in typical males. The masculinization of behavior was thus only partial and in addition, it could not be excluded that all demasculinizing effects observed so far could have resulted from non-specific toxic side effects.

Two types of evidence confirmed that in normal physiological conditions, sexual differentiation indeed proceeds by a demasculinization of females induced by their ovarian estrogens. First, radioimmunoassays of serum collected from eggs incubated for various durations indicated that E₂ circulating concentrations are significantly higher in female than in male embryos during the entire period when exogenous estrogens exert their demasculinizing effects, i.e. between day 9 (the earliest time point when sera could be collected) and day 15 of incubation (Fig. 1C; (Schumacher et al., 1988)). Secondly, availability of a newly synthesized aromatase inhibitor, R76713 (later renamed Vorozole™) allowed us to demonstrate that these endogenous estrogens are indeed responsible for the demasculinization of females. A single injection of 10 µg R76713 on day 9 of incubation resulted in females that were able, when adult and treated with exogenous T, to display the full repertoire of male-typical behavior, including mounts and copulation (Balthazart et al., 1992a). Furthermore, if this injection was combined with an injection of E₂ the effect was completely abolished (Fig. 1D).

Evidence of similar organizing effects of prenatal estrogens has to this date been collected in a few avian species, such as chicken and ring doves, and anecdotal evidence exists for quite a few other species, but available evidence is often less detailed and conclusive (see for review (Balthazart et al., 2017)).

3. Sexually differentiated features in brain morphology and physiology

When the principle of organizing effects of sex steroids was first formulated in the late sixties (Phoenix et al., 1959), it was broadly accepted that the brain in both sexes is very similar, if not identical, and that differences in behavior and function are only reflected at the neurophysiological level. However, small sex differences in specific aspects of neuronal morphology were progressively identified (Dörner, 1976; Dörner and Staudt, 1969; Raisman and Field, 1971; Raisman and Field, 1973) and a major shift in thinking took place when major differences in the volume of song control nuclei of canaries and zebra finches were reported (Nottebohm and Arnold, 1976). The ensuing

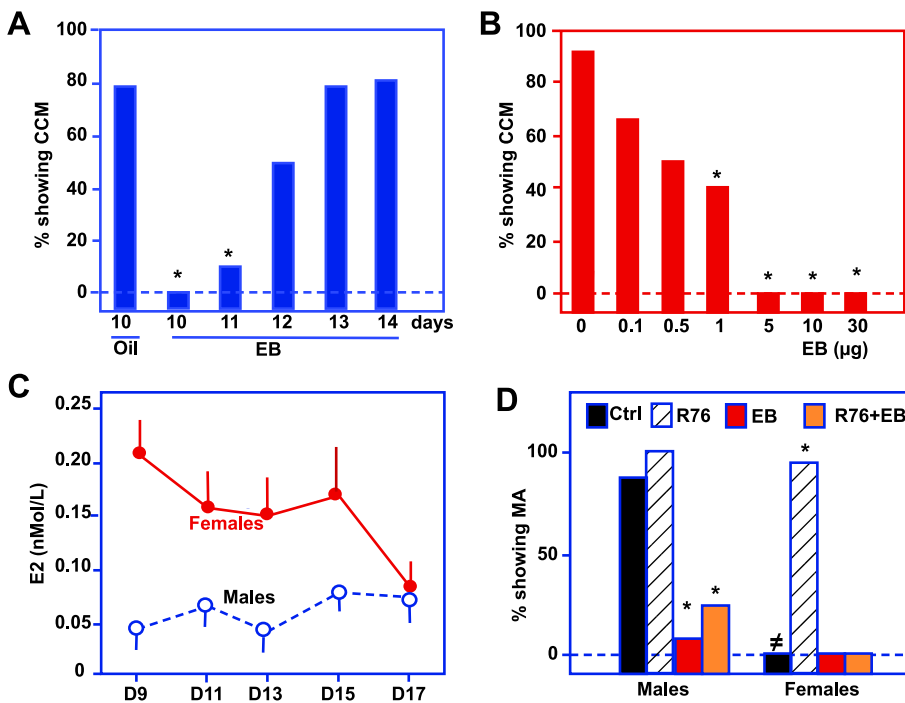


Fig. 1. Endocrine control of sexual differentiation by prenatal estrogens in quail. (A,B) Sensitive period and dose-response of the inhibition of male sexual behavior (here cloacal contact movements, CCM) by an injection in ovo of estradiol benzoate (EB). Eggs were injected with 50 μg EB or oil as a control on the different incubation days indicated (A) or with the different doses on day 10 of incubation and then treated with exogenous testosterone (T) before testing in adulthood. (C) Mean circulating concentration (±SEM) of estradiol (E₂) in male and female quail embryos between day 9 and 17 of incubation. (D). Effects on male copulatory behavior (here mount attempts of females, MA) of a single injection of male and female embryos on day 9 of incubation with either 10 μg R76713 (R76), 25 μg estradiol benzoate (EB) or both treatments combined compared with the control treatment (Ctrl). All birds were then gonadectomized at 3–4 weeks post-hatching and treated with exogenous T before behavioral tests.

*p < 0.05 compared to the control condition (same sex in panel D), ≠ p < 0.05 compared to males submitted to the same treatment (panel D only). Redrawn from data in (Adkins, 1979) (A,B), (Schumacher et al., 1988) (C) and (Balthazart et al., 1992a) (D).

experimental work quickly detected similar volumetric differences in a variety of mammalian species (e.g., (Gorski et al., 1978); for review see: (Arnold and Gorski, 1984; Tobet and Fox, 1992)). These differences often had a limited magnitude, but the comparatively much larger effects of steroids in photoperiodic species of birds and the identification of large organizing effects of steroids (see previous section) suggested that this quest might be easier and more fruitful in birds.

The demasculinization of male-typical copulatory behavior in female quail by ovarian estrogens during embryonic life has indeed proved to be one of the most reliable and reproducible mechanisms of sexual differentiation observed in vertebrates. Given the magnitude of the effect (complete and irreversible suppression of male copulatory behavior in females), it could have been anticipated that this effect would be reflected in major organized sex differences in the brain. The search for these differences in adult subjects has, however, been a bit disappointing. Indeed, although a large number of sex differences in brain structure or function have been identified in quail during the past 50 years, most of these differences disappear when birds are gonadectomized and/or placed in identical adult endocrine conditions following a same treatment of both sexes with exogenous T or E₂, suggesting they are not organized by embryonic estrogens (Balthazart et al., 1996a). It is also very difficult to relate in a causal manner the differences observed in the brain with behavior. These differences observed between sexually mature gonadally intact males and females namely concern the overall volume of a dense group of neurons corresponding to the medial preoptic nucleus (POM) (Panzica et al., 1996), the expression of sex steroid receptors (androgen receptors, estrogen receptors of the α and β subtype) as evidenced by autoradiography, immunohistochemistry or in situ hybridization of the corresponding mRNA, the expression and activity of T metabolizing enzymes, and the expression of multiple neuropeptides and neurotransmitters. These data have been reviewed and summarized on many occasions and will not be further discussed here (see for review: (Balthazart and Adkins-Regan, 2002; Balthazart et al., 2017; Balthazart et al., 1996a; Panzica et al., 1996)). These data are in fact not directly relevant to the present discussion given their activational nature.

A few brain sex differences have, however, been shown to differentiate at least in part as a result of female exposure to ovarian estrogens

during incubation. They could as a consequence explain the absence of male-typical copulatory behavior in adult females even after exposure to exogenous T. Two examples deserve a brief mention (see below) but this topic has been reviewed in detail in the past (Balthazart and Adkins-Regan, 2002).

3.1. Neuronal size in the lateral POM

As already mentioned, the volume of the POM is larger in males than in females (Viglietti-Panzica et al., 1986), but this difference disappears if both sexes are gonadectomized and treated with T (Panzica et al., 1987). In addition, injection of EB in eggs at a dose that fully demasculinizes females does not affect the adult POM volume (Panzica et al., 1987) and POM volume is not different between males and females soon after hatching or at any stage of post-natal development (Thompson and Adkins-Regan, 1992). However, detailed analyses at the cellular level indicated that neuronal size in the lateral part of the POM is markedly influenced by T in males but less so in females, raising the possibility of an organized sex difference (Panzica et al., 1991). Accordingly, treatment of male embryos with EB on day 9 of incubation, which inhibited copulatory behavior in the treated males, significantly decreased neuronal size in the lateral POM, but the same treatment on day 14 (too late to affect behavior) was ineffective. In addition, a few males, whose behavior had not been completely demasculinized by the EB injection of day 9, displayed a neuronal size in the lateral POM that had not been decreased (Aste et al., 1991). The size of these neurons could thus be a marker of the sexual differentiation of brain and behavior.

3.2. Vasotocin expression

The POM and the bed nucleus of the stria terminalis are densely innervated by vasotocin-immunoreactive (VT-ir) fibers in male quail and in males of many other avian species (Viglietti-Panzica, 1986). This dense innervation is however not observed in females (Jurkevich et al., 1996; Panzica et al., 1997) and is sensitive to T: it almost disappears in castrated birds and is restored by a treatment with exogenous T (Viglietti-Panzica et al., 1992). The sex difference is however not only the result of the lower T concentration in females because treatment of adult

females with this steroid still fails to activate a dense expression of this peptide, suggesting a participation of organizational effects (Panzica et al., 1998).

This idea was clearly confirmed in one series of experiments manipulating estrogens bioavailability during embryonic life. Injection on day 9 of incubation of EB completely suppressed the VT-ir fibers of the POM in adult males despite a treatment with T. Conversely injection on day 9 of the aromatase inhibitor R76713 generated females that displayed a full male-typical innervation of the POM after adult T treatment (Panzica et al., 1998). Similar results were obtained in chicken (Jurkevich et al., 2001). It was recently found that this organizing action of estradiol is mediated by estrogen receptor beta (Court et al., 2020).

These data thus indicate that the endocrine control of the vasotocin innervation of the POM both during ontogeny (by estrogens) and in adulthood (by T) parallels the controls of male copulatory behavior. This suggested that this sexually differentiated innervation might explain why females are unable to display male-typical copulatory behavior. However, one series of experiments failed to demonstrate a facilitatory role of vasotocin on this behavior and rather suggested an inhibitory one (Castagna et al., 1998). The reasons potentially underlying this discrepancy have been presented (Balthazart et al., 2017; Castagna et al., 1998) and more work would obviously be needed to better understand the physiological significance of the sex difference in brain vasotocin expression. Sex differences in vasotocin/vasopressin have been reported in many other vertebrate species from fish to mammals (De Vries and Panzica, 2006). Although the impact of these sex differences often remains to be unveiled, vasotocin/vasopressin play crucial roles in various aspects of social behaviors (Godwin and Thompson, 2012; Kelly and Goodson, 2014; Rigney et al., 2022).

A few other sex differences have been identified that seem to persist in adults treated with T. They concern namely the activity of aromatase and distribution of aromatase-expressing neurons (Balthazart et al., 1996b; Schumacher and Balthazart, 1986) or the concentrations and turnover of catecholamines in the medial preoptic area (Balthazart et al., 1992b). In addition, the projection of the aromatase-expressing neurons of the POM to the mesencephalic aqueductal gray is sexually differentiated in sexually mature quail (Carere et al., 2007) and it is unlikely that these projections would become similar if birds of both sexes were exposed to the same endocrine conditions because long distance projections are probably unable to grow in the adult brain. These differences are thus likely to result from organizing effects of estrogens and could mark the mechanisms that control the sexually differentiated expression of male-typical copulatory behavior but this was never formally demonstrated. This material has been reviewed in detail on several occasions (Balthazart and Adkins-Regan, 2002; Balthazart et al., 2017; Balthazart et al., 1996a).

4. The putative role of microglia and inflammation in sexual differentiation

While most attention initially focused on neurons, it turned out that the organizational actions of sex steroids can affect any cell type from glia to immune cells in the brain (Lenz et al., 2013; Lenz et al., 2018; Mong et al., 1999). Of particular interest is the role of microglia and neuroinflammation which were initially seen as the brain first line of defense against injury or pathogens until the realization about 15 years ago that they contribute to normal brain development, including sexual differentiation (Bilbo and Schwarz, 2012; Bordeleau et al., 2019; Pao-licelli and Gross, 2011; Thion et al., 2018). The discovery by Stuart Amateau and Margaret McCarthy that prostaglandins, best known for their role in inflammation and fever induction, were involved in the perinatal programming of brain and behavior by gonadal steroids provided a first hint of a role of neuroinflammation in brain sexual differentiation (Amateau and McCarthy, 2004). Indeed, female rats treated as newborns with prostaglandin E2 (PGE2) showed the full suite of male

typical behaviors after adult T treatment, while male rats in which prostaglandin synthesis had been blocked on the first day of life did not show mount or intromit as typical males do (Amateau and McCarthy, 2004). In parallel, manipulation of PGE2 in newborn pups reversed a sex difference in dendritic spine density in the POA (Amateau and McCarthy, 2004), which correlates with mounting behavior (Wright et al., 2008).

A decade later, the team led by Margaret McCarthy identified microglia as the sources of PGE2 involved in this process (Lenz et al., 2013). As it turned out, the number of microglia in the developing preoptic area differs between sexes under the influence of both PGE2 and estrogens and blocking microglial activity in newborn males prevents both preoptic dendritic spine patterning and the expression of adult male sexual behavior (Lenz et al., 2013). More recently, the same team demonstrated that gonadal steroids drive microglial phagocytosis to contribute to stable sex differences in brain and behavior (VanRyzin et al., 2019; Pickett et al., 2023).

Following these discoveries, it was proposed that these sex differences in microglia number and activity observed in rats may result from a maternal immune response against male fetuses (McCarthy, 2019, 2020). In this context, birds constitute a perfect model given that their sensitive window to the organizing effects of estrogens occurs in the egg, independently of maternal influence. Recent investigations of microglia in quail embryos revealed the existence of a sex difference in their number that is specific to the medial preoptic area (Delage and Cornil, 2020). Interestingly, as opposed to rats wherein microglia, in particular amoeboid microglia, is more abundant in the preoptic area of newborn males, female embryos harbor more microglia than males in quail. This sex difference was detected between embryonic days 9 to 12, that is during the sensitive period to estrogens, but not later (Fig. 2A). The number of embryonic microglia was sex-reversed by manipulations of estrogen availability during this critical period (Fig. 2B). Microglia originate from the yolk sac and enter the brain through the developing blood vessels, ventricles and meninges (Thion and Garel, 2017). The absence of sex difference in microglia lining the third ventricle suggests that estrogens do not influence microglial colonization of the brain (Delage and Cornil, 2020). Whether estrogens influence their proliferation remains to be determined.

Another question concerns the potential difference in microglia activation between male and female quail embryos and the role played by microglia in the sexual differentiation of the brain and behavior of quail. The expression of inducible nitric oxide synthase (or iNOS) in microglia was investigated as a marker of activation by inflammatory factors. Most microglia (80 %) expressed iNOS in both sexes (Delage and Cornil, 2020). Moreover, comparing the expression of a variety of microglial markers and inflammatory markers by qPCR revealed a higher expression of the proinflammatory interleukin 1 β (IL1 β) in the hypothalamus and preoptic area of female embryos at E12 (Dardenne and Cornil, Unpublished work). Although IL1 β is secreted by microglia and neurons, this observation along with the larger number of microglia in the POM of females at E12 could indicate that microglia are more active in this brain region during its organization by estrogens.

Based on the role of PGE2 in the sexual differentiation of the rat brain (Lenz et al., 2013), we investigated the potential role of prostaglandins in mediating the sexual differentiation of the brain and behavior of quail. Blocking cyclooxygenases (COX), the limiting enzymes for prostaglandin synthesis, with indomethacin during early embryonic development eliminated the sex difference in microglia and impaired the ability of males to copulate, thus partially mimicking the effects of estrogens (Fig. 3C) (Delage et al., 2021). These observations thus suggest that, as opposed to rats in which PGE2 mediates the effects of estrogens on brain sexual differentiation, PGE2 signaling in quail would mediate the masculinization process, which occurs in absence of estrogen exposure during embryogenesis. However, the absence of sex difference in the concentration of PGE2 or of any other measurable prostanoid in the HPOA of developing quail raises the question of the nature of the

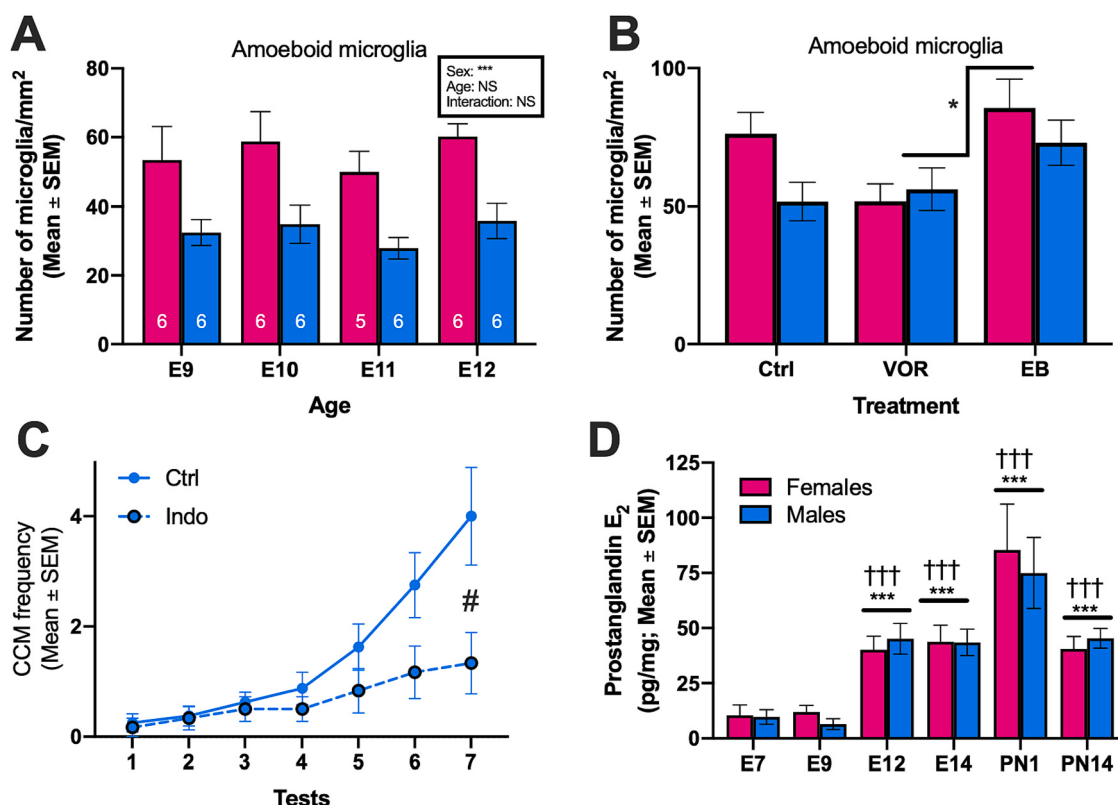


Fig. 2. Sex differences in brain microglia and effects of cyclo-oxygenase inhibition by indomethacin on the expression of male sexual behavior. **A.** Sex difference in the number of amoeboid microglia in the developing POM from embryonic day 9 (E9) to E12. The statistical insert summarizes the main results of the 2way ANOVA of these data with age and sex as factors: ** $p < 0.01$, N.S., non significant. **(B)** Effect of manipulations of in ovo estrogen bioavailability by the administration at E7 of estradiol benzoate (EB, 50 μ g) or the aromatase inhibitor, Vorozole (VOR) on the number of amoeboid microglia measured in the POM at E12. * $p < 0.05$ following significant interaction. **C.** Effect of the Cox inhibitor, indomethacin (indo, 500 μ g) administered at E7 on cloacal contact movements measured in adulthood in castrated males treated with testosterone. # $p < 0.05$ vs Indo following significant interaction. **D.** Developmental changes in the concentration of prostaglandin E₂ (PGE₂) in the POM of quail at different embryonic (E) and early post-natal (PN) developmental ages (in days). *** $p < 0.001$ vs E7, †††, $p < 0.001$ vs PN14 following significant interaction. Redrawn from (Delage and Cornil, 2020) and (Delage et al., 2021).

product of COX mediating these effects (Fig. 3D) (Delage et al., 2021). Moreover, the administration of PGE₂ to female embryos did not allow them to display male copulatory behavior following T treatment in adulthood (Delage et al., 2021). It is possible that their exposure to estrogens during development prevented the organization of circuits required to show male sexual behavior despite the action of PGE₂. Alternatively, the effect of indomethacin could be explained by its effects on endocannabinoids, which are metabolized in less active compounds by COX and are involved in neurodevelopment (Maccarrone, 2017).

Together, these observations demonstrate that microglia are sexually differentiated in the developing POM under the action of estrogens. Interestingly, the sex difference goes in the opposite direction compared to the difference reported in rats, mirroring the organizing effects of estrogens on brain and behavior. This sex difference occurring *in ovo* is difficult to reconcile with the hypothesis according to which the sexual differentiation of some aspects of the mammalian brain relates to an immune reaction of the mother towards its male embryo. Many questions remain however as a causal relationship between microglia and brain sexual organization has not been demonstrated. This endeavor is somewhat complicated by the fact that the critical period occurs *in ovo* when the brain is not easily accessible to experimental manipulations.

5. Sexual differentiation in songbirds: the zebra finch is not another quail

It was initially assumed that the mechanism of sexual differentiation based on the demasculinization by estrogens of the females that has been

best characterized in quail, would apply to all avian species. Songbirds often display an extreme sex difference in singing behavior. This sex difference is particularly prominent in zebra finches (*Taeniopygia guttata*): females never sing even if treated in adulthood with T at doses that activate a very active singing behavior in males (Balthazart and Adkins-Regan, 2002; Nottebohm and Arnold, 1976). This behavioral difference is associated with major sex differences in the brain: the volume of several nuclei of the so-called song control system including HVC (used as a proper name) and RA (the robust nucleus of the arcopallium) is significantly larger in males than in females (2 to 3 times in canaries, *Serinus canaria*, up to 5 times in zebra finches; (Nottebohm and Arnold, 1976)).

Results of the first experiments testing whether the sex difference in singing behavior develops by the same mechanism as the sex difference in quail copulatory behavior then came as a surprise. Gurney and Konishi indeed found that if zebra finch chicks are implanted soon after hatching with a Silastic capsule containing E₂, they develop as adults song control nuclei that are 2–3 times larger than in control females and almost of the same size as in males (Gurney and Konishi, 1980). Treatment of these E₂-females with androgens in adulthood then activated some singing behavior contrary to what is observed in untreated females (see Fig. 3). These results were soon confirmed in an independent study that additionally demonstrated that the E₂ treatment had to be started at or before day 4 post-hatching to be effective (Pohl-Apel and Sossinka, 1984). Treatment from day 2 until day 18 post-hatch produced females displaying songs of male-like quality whereas treatment from day 2 until day 11 only produced poor song in females. Singing and the underlying associated brain structures in zebra finches are thus masculinized by

Postnatal treatment	Song	SCN	Male copulatory behavior	Reference
None	♂ > ♀	♂ > ♀	♂ > ♀	
+E2	↗	↗	n.d.	Gurney & Konishi, 1980
+E2	↗	n.d.	n.d.	Pohl-Apel & Sossinka, 1984
+E2	↗	↗	n.d.	Simpson & Vicario, 1991a, b
+EB	↗	n.d.	↘	Adkins & Ascenzi, 1987
+ anti-ER	n.d.	↗	n.d.	Mathews et al., 1988
+ Vorozole	↘	=	=	Balthazart et al., 1994
+ Fadrozole (prenatal)	n.d.	= (?)	n.d.	Wade & Arnold, 1996

Fig. 3. Table summarizing the multiple experiments indicating that the sexual differentiation of the song control system and of copulatory behavior cannot be controlled by the same endocrine stimuli. Up and down arrows respectively indicate increases and decreases in some (but not always all) aspects of the variable under study. The equal sign indicates that no change was observed in the study. Symbols in red refer to results contradicting the idea that estrogens have masculinizing effects on all aspects of brain and behavior. Abbreviations: n.d. = non determined; SCN: song control nuclei; EB: estradiol benzoate; anti-ER: estrogen receptor antagonist. Vorozole and Fadrozole are 2 non-steroidal aromatase inhibitors.

neonatal treatment with an estrogen, in contrast with what takes place in quail and other birds in which neonatal estrogens demasculinize male-typical copulatory behavior. Another set of studies confirmed these results and additionally demonstrated that adult song in females neonatally treated with E₂ can develop in the absence of adult treatment with androgens (Simpson and Vicario, 1991a, 1991b).

More detailed behavioral studies performed by Elizabeth Adkins and collaborators confirmed the discrepancy between these effects of estrogens on singing and effects of estrogens on copulation in quail. Injection of zebra finches with EB during the first two weeks post-hatch produced adult males in which copulatory behavior was inhibited (demasculinized), like in quail, but at the same time singing was masculinized in adult females, as observed during sexual differentiation in mammals. As expected based on work on quail, EB-treated females did not display male-typical copulatory behaviors (Adkins-Regan and Ascenzi, 1987). These results thus raised a fundamental set of questions: are the concentrations of sex steroids, in particular estrogens, sexually differentiated during development of male and female zebra finches and more importantly how is it possible that a given hormonal milieu masculinizes singing in females and at the same time demasculinizes

copulatory behavior in males. In other words, how can the developmental trajectory produce males that sing AND copulate (Balthazart and Ball, 1995)?

It was initially speculated that singing and copulation are differentiated by estrogen action at different periods of ontogeny when circulating estrogen concentrations show opposite sex differences or that they are sensitive to different doses of the steroid but experimental exploration of these ideas failed to provide any support for these ideas (Adkins-Regan and Ascenzi, 1990; Adkins-Regan et al., 1994).

6. Towards an alternative model for the differentiation of singing

Investigations of the steroid plasma concentrations, in particular E₂ and T, during development failed to provide explanations for these conflicting results. Three separate studies investigated this question and none of them could detect a developmental period during which plasma E₂ would be reliably higher in one sex compared to the other (Adkins-Regan et al., 1990; Hutchison et al., 1984; Schlinger and Arnold, 1992b). One exception to this pattern was present in the oldest of these studies

where a peak in E₂ concentration was present on day 4 post-hatch in males (Hutchison et al., 1984). This peak was however never reproduced and might possibly just be an artifact resulting from chance fluctuations or from the fact that birds in this study contrary to others were bled from the jugular vein (Schlinger et al., 2001).

Since brain aromatase is extremely active in zebra finches and markedly influences circulating concentrations in the periphery (Schlinger and Arnold, 1991, 1992a, 1993), it was also suggested that the estrogenic stimulus that differentiates singing behavior originates in this enzymatic activity. However, no evidence of a sex difference in brain aromatase activity during ontogeny, nor actually in the activity of any other T metabolizing enzyme could ever be detected despite multiple studies (Schlinger and Arnold, 1992b; Schlinger et al., 2001; Wade et al., 1995). Functional evidence derived from brain slices in cultures was, however, presented suggesting that the synthesis of E₂ in the brain is sexually differentiated (Holloway and Clayton, 2001). This study demonstrated that the development of the neuronal projection from HVC to RA (critical for song production) in zebra finch brain slices critically depends on a local synthesis of estrogens in the male brain: a male-like projection develops in female brain slices co-cultured with male slices or exposed to exogenous estrogens and this development is blocked in both sexes by treatment with an anti-estrogen or an aromatase inhibitor. The idea that a sexually differentiated local production of estrogens can be controlling, at least in part, the sexual differentiation of brain and behavior in this species thus cannot be formally rejected but experimental evidence supporting this idea remains elusive.

Another experimental approach was also used in an attempt to explain the mechanisms underlying sexual differentiation in zebra finches (Fig. 3). Assuming that males are exposed to a larger concentration of estrogens during ontogeny (as suggested by the (Hutchison et al., 1984) study) and this explains their larger song control nuclei, Art Arnold and his collaborators investigated, in a suite of pharmacological studies, the effects on the sexual differentiation of the song control system of early treatments with estrogen receptor antagonists or aromatase inhibitors. Three different very potent antiestrogens administered soon after hatching failed to block sexual differentiation in males and actually induced a hyper-masculinization of the volume of the song control nuclei in males (Mathews and Arnold, 1990; Mathews et al., 1988). Other studies used an alternative approach based on the inhibition of estrogen production instead of blockade of their action. Administration of an aromatase inhibitor early during development similarly failed to modify the sexual differentiation of the song control system (Wade and Arnold, 1994). These studies only quantified effects on the song control system in the brain, but another similar experiment assessing the effects of early aromatase inhibition on song performance also detected very little effects: treated females still did not sing and treated males sang apparently normal songs, albeit with a slightly decreased occurrence frequency (Balthazart et al., 1994).

In a final attempt to test the idea of a masculinization of the song control system by early estrogen action, zebra finches were treated during their embryonic life (on day 5 of incubation) with the aromatase inhibitor Fadrozole. In genetic females, this treatment induced both functional ovarian and testicular tissue. In five of these seven females, the testicular tissue produced sperm in adulthood. The androgen-sensitive syrinx of these 5 females was also significantly larger than in control females indicating they had been exposed to more T during development (Wade et al., 1996). However, the presence of testicular tissue did not affect the development of the song control system and its sexual differentiation (volume of HVC and RA and neuronal soma size in these nuclei) with the exception that RA volume was slightly decreased in Fadrozole-treated males, even if it was still much larger than in females (Wade and Arnold, 1996; Wade et al., 1996).

7. The gynandromorphic zebra finch

By the end of last century, research had thus failed to find a

satisfactory explanation to the fact that early exposure to estrogens demasculinizes copulatory behavior in a variety of avian species, including the zebra finch, while the role of estrogens on the song system, if any, remained elusive. Treatments with exogenous estrogens, often at pharmacological doses, were shown to masculinize HVC and RA, as observed during the mammalian sexual differentiation, but no clear evidence for a sexually differentiated exposure to these steroids during ontogeny could be evidenced.

The exclusively hormonal theory of sexual differentiation under the control of gonadal steroids was soon to be shaken up by the discovery of a very unusual zebra finch that displayed male plumage on the right half of the body and a female plumage on the left side. The bird was identified in the avian colony of Fernando Nottebohm at Rockefeller University (New York, NY) and studied in the laboratory of Art Arnold at UCLA (Los Angeles, CA).

When housed with a female, this bird sang a male-typical song. He courted and copulated with the female who then laid infertile eggs. When housed with a male, he attacked this partner. At autopsy, the bird was found to have a normal ovary on the left side with numerous atretic follicles while the right side had a mature but disorganized testis containing both small and large seminiferous tubules, some of which contained sperm at different stages of development (Agate et al., 2003). Plasma levels of E₂ and T were undetectable.

Genetic analysis of the brain of this bird confirmed that cells on the left side were female and expressed a gene located on the W chromosome, while cells on the right side expressed at higher level than on the left side a gene located on the Z chromosome (in birds sex chromosomes in males are ZZ and are ZW in females, and sexual determination relies on a dosage difference of DMRT1 gene between sexes (Ioannidis et al., 2021)). Quite surprisingly, the volume of HVC was markedly lateralized in this bird and 1.82 times larger in the right (male) side than in the contralateral female side. This difference between the left and right side was much larger than what is normally observed in intact males (1.10 ± 0.08 times) but intermediate between the differences observed between intact males and females (4 to 7 times). It could obviously not be the result of a differential exposure of the two brain sides to gonadal steroids and had therefore to reflect direct effects of the genetic make-up of the brain. Note however that the magnitude of the difference was smaller than between control males and females, thus suggesting a potential contribution of circulating steroids.

We recently had access to another gynandromorphic zebra finch that had been raised and kept in the vivarium of the University of Bielefeld (Germany; generous gift of Barbara Caspers and Hans-Joachim Bischof). This bird presented a similar plumage asymmetry but the lateralization was reversed: it displayed a full male plumage on the left side and a female plumage on the right side (Fig. 4). The brain and genetic make-up of this bird are still under investigation but it can already be reported that a lateralization of the song control nucleus HVC was also present in the brain. It was actually more pronounced than in the initial paper. When defined by the dense cluster of parvalbumin-immunoreactive neurons and perineuronal nets, HVC was 1.9 times larger in the male side (right side) and the asymmetry also affected RA that was 0.6 times larger on the male side.

So data collected in these two gynandromorphs clearly demonstrate that neural sex differences can develop independently of sex differences in circulating sex steroids (steroids originating from the ovary on one side and the testis on the other side are obviously mixed while passing through the heart). The sex difference can only be assigned to the differential expression of male and female genes in the two brain sides. Identification of the genes that are “directly” mediating sex differences, without a mediation by gonadal sex steroids, is obviously a difficult task since gynandromorphs are rare and studies can only rely on “normal” males and females. A number of hypotheses have been presented. Some include the genes that control the local synthesis of sex steroids in the brain, in particular neuroestrogens. This approach could potentially reconcile data demonstrating direct effects of genes and effects of



Fig. 4. Gynandromorphic zebra finch hatched and raised at the Bielefeld University and studied at the University of Liege.

estrogens either systemic (Gurney and Konishi, 1980) or produced in the brain (Holloway and Clayton, 2001) on sexual differentiation. Alternative ideas are based on neurotrophins that are known to modulate brain growth (e.g., the brain-derived neurotrophic factor, BDNF and the nerve growth factor, NGF) (for review, see (Balthazart et al., 2017)).

The emergence of the idea of “direct” effects of genes on sexual differentiation has also had a huge impact on the research concerning mammalian sexual differentiation by (re)focusing it on the molecular underlying mechanisms. Art Arnold and a large network of colleagues initiated studies of the four core genotype mice that had been developed in London by Paul Burgoyne and Robin Lovell-Badge (De Vries et al., 2002). This model includes XY subjects that no longer express the testis-determining gene of the Y chromosome (XY^{sry-}) and other XX subjects in which this *sry* gene has been inserted in an autosome (XX^{sry+}). As a consequence, four types of subjects are present: XX and XY^{sry-} mice that develop ovaries and XY and XX^{sry+} mice that develop testes. Phenotyping of these mice indicated that most sex differences that relate to reproduction essentially develop by the classical mechanisms based on an early action of testes-derived T: the XY and XX^{sry+} mice are masculinized compared to the XX and XY^{sry-} mice. However, many other sex differences were discovered that develop under the control of chromosomal differences: here the subjects with XY chromosomes are different from the XX subjects independent of whether they have testes or ovaries.

The list of these differences controlled by chromosomes independent of gonadal steroids has grown enormously during the last decade and now includes sex differences affecting autoimmune diseases, metabolism and adiposity, cardiac and pulmonary diseases, pain sensitivity and neural tube closure defects. At the behavioral level, these chromosome effects also concern aspects of learning or social behaviors as well as the development of Alzheimer's disease (see (Schlinger, 2022) for references). Specific genes mediating these effects, mostly located on the X chromosome but escaping inactivation and thus acting via a dosage effect, have even been identified in some selected cases (see (Arnold, 2022) for review).

8. Genetic and epigenetic differences in zebra finches

For methodological reasons (difficulties in manipulating gene expression and absence of a corresponding 4 core genotypes model), the genetic bases of sex differences are not as well understood in zebra finches. However, as part of a broad collaboration between multiple laboratories in Belgium and Europe, we participated in attempts to better define the genetic bases of sexual differentiation in this species.

Changes in gene transcription (analyzed by RNA Seq) and their potential epigenomic regulation (analyzed by Reduced Representation Bisulfite Sequencing, RRBS) were quantified in the telencephalon of male and female zebra finches on day 1 (song control nuclei not yet visible), 20 (morphological differentiation of nuclei well under way, start of the sensory phase for song learning) and 65 (end of sensory and middle of the sensorimotor phase) post-hatching and in adulthood (Diddens et al., 2021). This study identified large sex- and age-related changes in transcription.

Overall, DNA methylation was however low but markedly increased over age, particularly in song control nuclei, as could be observed by separate immunohistochemical analysis with an antibody specific to methylcytosine of sections collected at 20 and 120 days post-hatch. A clear association was observed between age-dependent changes in DNA methylation and gene expression for a number of transcription factors regulating synaptic plasticity such as FOS, OTX2, AR, and EGR. Thus DNA methylation seems to regulate transcription during ontogeny via changes in transcription factors rather than via changes in the transcription of specific genes. In contrast, changes in DNA methylation only explained a very small fraction of the sex differences in transcription.

Also as indicated by previous work (Ellegren et al., 2007; Itoh et al., 2007; Itoh et al., 2010), dosage compensation appeared to be very limited in zebra finches and most of the sexually differentiated transcription was due to an incomplete dosage compensation of genes located on the Z chromosome (males in birds are ZZ and females are ZW). Very few sex differences in gene transcription concerned genes located on autosomes. A similar situation has been reported in canaries

(Ko et al., 2021). It must be noted however that many genes whose transcription was sex-biased were not dosage-compensated at 1 day post-hatch, but became dosage compensated later life. Thus, they can contribute to sex-specific patterns in development, even though they are balanced in adulthood.

These studies thus demonstrate that gene-based differentiation in the zebra finch telencephalon largely takes place by mechanisms that differ from what has been described in mammals. One set of studies indeed demonstrated that the mammalian brain feminization is supported by an active suppression of masculinization via DNA methylation (Nugent et al., 2015). In contrast, no major sex difference in global methylation levels was detected in zebra finches and no sex-specific gene methylation was observed in differentially expressed genes. Thus DNA methylation does not appear to be involved in regulating sexually differentiated gene expression. Accordingly, a recent experiment in our lab failed to identify major effects on copulatory behavior of a treatment of male and female quail embryos with the methyl transferase inhibitor, zebularine (Court Lucas and Cornil Charlotte, unpublished data). This negative result should however be considered with great caution before it is actually replicated.

9. Sexual differentiation of song in other species

The zebra finch has been a broadly used model in the study of sexual differentiation largely because this is the songbird species that most easily reproduces in captivity. This species is however peculiar in many aspects: sex differences related to singing are extreme (5 fold differences in the volume of song control nuclei with area X not or barely visible in females and adult females never sing even after treatment with T (Shaughnessy et al., 2018)). Additionally, being an Australian species living in desert areas (Zann, 1996), they do not or poorly respond to changes in photoperiod and their reproduction is largely controlled by the presence of water and green vegetation (Bentley et al., 2000; Zann, 1996). Sex differences in singing behavior are also present and have been studied in other species even if the ontogeny of these differences was rarely investigated due to the difficulty in reproducing these birds in captivity. A few field studies in cavity nesting birds were however performed (e.g., (Casto and Ball, 1996)).

The canary (*Serinus canaria*) is the most widely used species in this context. Canaries also display sex differences in singing behavior but these differences are less extreme than in zebra finches. Female canaries usually do not sing, although a few of them do occasionally (Ko et al., 2020). Additionally, female canaries display an active singing behavior when treated as adults with exogenous T (Alward et al., 2018; Boseret et al., 2006; Nottebohm, 1980). The volume of the song control nuclei HVC and RA increases in parallel in these T-treated females (Nottebohm, 1980).

An earlier study demonstrated that that even if exogenous T induces song production and increases song control nuclei volumes in females, these effects have a smaller magnitude than in males (Madison et al., 2015). It is however well known that these effects of T are partially mediated by the transformation of this androgen into an estrogen such as E₂ (Bottjer and Johnson, 1997; Fusani and Gahr, 2006). Therefore, we recently asked whether these blunted effects observed in females could be due to the fact that aromatization of T in their brain is less active than in males as observed in a variety of species even after treatment with T (Hutchison et al., 1994; Roselli and Resko, 1993; Schumacher and Balthazart, 1986).

Separate groups of castrated males and of photoregressed females (i.e., with quiescent ovaries as a consequence of exposure to short days) were subcutaneously implanted with either 2 empty 10 mm silastic capsules (Ctrl) as a control, or one empty capsule and one capsule filled with T or one capsule filled with T plus one filled with E₂. All birds were isolated in sound-attenuated boxes and the songs they produced were recorded for 3 h each week for 6 weeks before brains were collected and song control nuclei volumes were measured in Nissl-stained sections

(Dos Santos et al., 2022).

The study confirmed that song rate or duration, as well as various features of the song produced, are enhanced by T more in males than in females. Quite surprisingly these sex differences were not abolished or reduced by a concomitant treatment with E₂ and quite on the contrary, the addition of E₂ to the T treatment increased and diversified the sex differences in singing (Dos Santos et al., 2022). Overall, sex differences over the 6 weeks of treatment or significantly different changes in time in the two sexes (time by sex interactions) were indeed observed for song rate and duration, but also for additional features of song including power and bandwidth (see Fig. 5A, B). On average, E₂ actually inhibited part of the stimulating effects of T, especially in females, an effect that was in some cases statistically significant (see (Dos Santos et al., 2022) for detail of statistics).

The mechanism explaining this unexpected inhibitory effect of E₂ is unclear at this time given that E₂ alone has been shown to promote singing behavior in canaries and zebra finches (Bottjer and Johnson, 1997; Fusani and Gahr, 2006; Harding, 2008; Schlinger and Brenowitz, 2017). One study, however, showed that treatment of male canaries with the aromatase inhibitor Fadrozole increases song duration and the number of syllable repetitions within a song suggesting that estrogens inhibit these features (Fusani et al., 2003).

Additional features of song are also different in males and females and remain different in T or T + E₂ birds. They concern the rate of occurrence and various features of rapid repetitions of a same sound with a song, called trills and the diversity of syllables that males and females incorporate into their songs (Dos Santos et al., 2023).

Interestingly, the autopsy and microscopic examination of the brains further indicated that the volume of forebrain song control nuclei (HVC, RA, Area X) was increased by the two steroid treatments, but remained significantly much smaller in females than in males irrespective of the endocrine condition (see Fig. 5C for HVC). The statistical analysis of these volumes by two-way ANOVA indeed indicated the presence of significant effects of treatment and of significant sex differences but no effect of the treatment by sex interaction indicating that the sex difference was present irrespective of the endocrine condition. And almost similarly, the syrinx mass was very significantly affected by the treatments ($p < 0.001$) and by the sex of the subjects ($p < 0.001$), while the interaction between these two factors was barely significant ($p = 0.028$; Fig. 5D). Syrinx mass thus remained much smaller in females than in males independent of their endocrine condition. These morphological and behavioral differences might be causally related: T treatment might

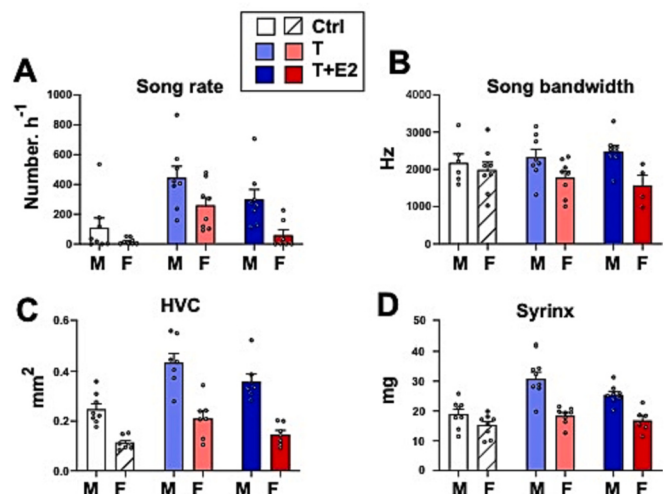


Fig. 5. Effects of treatment with T or T in association with E₂ of castrated male and sexually quiescent female canaries on representative song parameters (A-B), HVC volume (C) and syrinx mass (D). All data are means $\pm$ SEM. Redrawn from data in (Dos Santos et al., 2022).

increase song motivation in females through its action on the preoptic area (Alward et al., 2013; Alward et al., 2018; Vandries et al., 2019) so that they increase their singing behavior but in the absence of a fully developed song control system and of a fully grown syrinx, the vocal organ in songbirds, they are unable to reproduce some aspects of male song.

Taken together, these sex differences in behavior and morphology that remain present when the endocrine condition of males and females are made similar by treatment with exogenous steroids suggest the existence of organizing mechanisms that differentiate brain and behavior earlier during the ontogeny so that they respond differentially to adult steroid treatments. These sex differences are presumably organized either by early steroid action or alternatively by sex-specific gene regulation directly in the brain, as suggested by the studies of gynandromorphic zebra finches (Agate et al., 2003).

In addition, one should not forget that song is a learned behavior in songbirds. Therefore, the inability of females to produce complete male-typical songs, and in particular the lower diversity of syllables they incorporate in their song, might reflect, at least in part, the absence of song learning and practice during early ontogeny. All these ideas are easily testable but obviously require the development of a large canary-breeding colony that would reproduce these birds in relatively large numbers so that young chicks become available for experimental manipulations.

The fact that a number of aspects of song cannot be fully masculinized in females by an adult treatment with doses of T that are fully active in males is not very surprising given that a recent study demonstrated the presence of differences in gene expression in HVC between males and females and also between spontaneously singing females and females that sing after treatment with exogenous T (Ko et al., 2021). A substantial part of the HVC transcriptome is sex-specific in canaries and many of these differentially expressed genes are also differentially expressed between singing and non-singing subjects, indicating their close relation to song production.

10. Conclusions

A few lessons can be learned from these comparative studies of sexual differentiation in avian species. The most obvious one is that the mechanisms underlying sexual differentiation are clearly not universal but rather specific to different classes of animals if not to more restricted taxonomic units. The work of Elizabeth Adkins-Regan that was confirmed and extended by many others initially pointed to the fact that male-typical copulatory behavior is demasculinized by estrogens in female birds, while it is masculinized by these hormones in male mammals. Birds are thus not feathered mammals: in both classes, sexual behavior is organized by early hormone action but the underlying mechanisms differ in birds from what is seen in mammals. It is puzzling to note that in mammals, the female is homogametic (XX) and the male is heterogametic (XY), while the opposite is true in birds (male ZZ, female ZW). Researchers initially tried to derive a rule from these observations pointing that in both classes of vertebrates it is the heterogametic sex whose phenotype develops under the organizing influence of sex steroids. A causal link has however not been identified to our knowledge and the study of song differentiation in oscines has clearly complicated the story.

The diversity in differentiating mechanisms indeed is not only related to species differences but it is also observed within a same species for different behaviors. It is indeed well established that perinatal exposure to estrogens demasculinizes copulatory behavior in zebra finches, as it does in quail, but partially masculinizes song and the song control system. This intraspecific mechanistic difference presumably relates to the fact that the organizing mechanisms concern different parts of the brain: the evolutionarily ancient preoptic area-hypothalamus for copulatory behavior and the relatively more recent hyperpallium for singing. In this latter case, steroids are however not the

full story and the analysis of the gynandromorphic zebra finch has opened a new avenue of research investigating “direct” genetic effects that are not mediated by changes in gonadal steroid production.

As noted by Elizabeth Adkins-Regan in several of her reviews and in her book (Adkins-Regan, 2005), the study of animal diversity has often generated questions that subsequently led to novel insights and deeper understanding of a given phenomenon. This has clearly been the case for studies of the gynandromorph that led to the development and study of these differentiating effects of genes in mammals via use of the four core genotypes model and others. In fact, studies of birds have been at the origin of multiple advances in various fields of biology (see (Konishi et al., 1989)). We hope that this brief overview adequately illustrates this value and will generate interest in such comparative research that unfortunately tends to be put aside by current science and funding agencies.

Data availability

No data was used for the research described in the article.

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References

- Adkins, E.K., 1975. Hormonal basis of sexual differentiation in the Japanese quail. *J. Comp. Physiol. Psychol.* 89, 61–71.
- Adkins, E.K., 1976. Embryonic exposure to an antiestrogen masculinizes behavior of female quail. *Physiol. Behav.* 17, 357–359.
- Adkins, E.K., 1979. Effect of embryonic treatment with estradiol or testosterone on sexual differentiation of the quail brain. *Neuroendocrinol.* 29, 178–185.
- Adkins, E.K., Adler, N.T., 1972. Hormonal control of behavior in the Japanese quail. *J. Comp. Physiol. Psychol.* 81, 27–36.
- Adkins-Regan, E., 1983. Sex steroids and the differentiation and activation of avian reproductive behaviour. In: Balthazart, J., Gilles, R. (Eds.), *Hormones and Behaviour in Higher Vertebrates*. Springer-Verlag, Berlin, pp. 219–228.
- Adkins-Regan, E., 2005. *Hormones and Animal Social Behavior*. Princeton University Press, Princeton, NJ.
- Adkins-Regan, E., Ascenzi, M., 1987. Social and sexual behaviour of male and female zebra finches treated with oestradiol during the nestling period. *Anim. Behav.* 35, 1100–1112.
- Adkins-Regan, E., Ascenzi, M., 1990. Sexual differentiation of behavior in the zebra finch: effect of early gonadectomy or androgen treatment. *Horm. Behav.* 24, 114–127.
- Adkins-Regan, E.K., Pickett, P., Koutnik, D., 1982. Sexual differentiation in quail: conversion of androgen to estrogen mediates testosterone-induced demasculinization of copulation but not other male characteristics. *Horm. Behav.* 16, 259–278.
- Adkins-Regan, E., Abdelnabi, M., Mobarak, M., Ottinger, M.A., 1990. Sex steroid levels in developing and adult male and female zebra finches (Poephila guttata). *Gen. Comp. Endocrinol.* 78, 93–109.
- Adkins-Regan, E., Mansukhani, V., Seiwert, C., Thompson, R., 1994. Sexual differentiation of brain and behavior in the zebra finch: critical periods for effects of early estrogen treatment. *J. Neurobiol.* 25, 865–877.
- Agate, R.J., Grisham, W., Wade, J., Mann, S., Wingfield, J., Schanen, C., Palotie, A., Arnold, A.P., 2003. Neural, not gonadal, origin of brain sex differences in a gynandromorphic finch. *PNAS* 100, 4873–4878.
- Alward, B.A., Balthazart, J., Ball, G.F., 2013. Differential effects of global versus local testosterone on singing behavior and its underlying neural substrate. *Proc. Natl. Acad. Sci. U. S. A.* 110, 19573–19578.
- Alward, B.A., Cornil, C.A., Balthazart, J., Ball, G.F., 2018. The regulation of birdsong by testosterone: multiple time-scales and multiple sites of action. *Horm. Behav.* 104, 32–40.
- Amateau, S.K., McCarthy, M.M., 2004. Induction of PGE2 by estradiol mediates developmental masculinization of sex behavior. *Nat. Neurosci.* 7, 643–650.
- Arnold, A.P., 2022. X chromosome agents of sexual differentiation. *Nat. Rev. Endocrinol.* 18, 574–583.
- Arnold, A.P., Gorski, R.A., 1984. Gonadal steroid induction of structural sex differences in the central nervous system. *Annu. Rev. Neurosci.* 7, 413–442.

- Aste, N., Panzica, G.C., Viglietti-Panzica, C., Balthazart, J., 1991. Effects of in ovo estradiol benzoate treatments on sexual behavior and size of neurons in the sexually dimorphic medial preoptic nucleus of Japanese quail. *Brain Res. Bull.* 27, 713–720.
- Bachtrog, D., Mank, J.E., Peichel, C.L., Kirkpatrick, M., Otto, S.P., Ashman, T.L., Hahn, M.W., Kitano, J., Mayrose, I., Ming, R., Perrin, N., Ross, L., Valenzuela, N., Vamori, J.C., Tree of Sex, C., 2014. Sex determination: why so many ways of doing it? *PLoS Biol.* 12, e1001899.
- Bakker, J., Honda, S.I., Harada, N., Balthazart, J., 2002. The aromatase knock-out mouse provides new evidence that estradiol is required during development in the female for the expression of sociosexual behaviors in adulthood. *J. Neurosci.* 22, 9104–9112.
- Balthazart, J., Adkins-Regan, E., 2002. Sexual differentiation of brain and behavior in birds. In: Pfaff, D.W., Arnold, A.P., Etgen, A.M., Fahrbach, S.E., Rubin, R.T. (Eds.), *Hormones, Brain and Behavior*. Academic Press, San Diego, CA, pp. 223–301.
- Balthazart, J., Ball, G.F., 1995. Sexual differentiation of brain and behavior in birds. *Trends Endocrinol. Metab.* 6, 21–29.
- Balthazart, J., De Clerck, A., Foidart, A., 1992a. Behavioral demasculinization of female quail is induced by estrogens: studies with the new aromatase inhibitor, R76713. *Horm. Behav.* 26, 179–203.
- Balthazart, J., Foidart, A., Sante, P., Hendrick, J.C., 1992b. Effects of α -methyl-para-tyrosine on monoamine levels in the Japanese quail: sex differences and testosterone effects. *Brain Res. Bull.* 28, 275–288.
- Balthazart, J., Absil, P., Fiasse, V., Ball, G.F., 1994. Effects of the aromatase inhibitor R76713 on sexual differentiation of brain and behavior in zebra finches. *Behaviour* 131, 225–260.
- Balthazart, J., Tlemçani, O., Ball, G.F., 1996a. Do sex differences in the brain explain sex differences in the hormonal induction of reproductive behavior? What 25 years of research on the Japanese quail tells us. *Horm. Behav.* 30, 627–661.
- Balthazart, J., Tlemçani, O., Harada, N., 1996b. Localization of testosterone-sensitive and sexually dimorphic aromatase-immunoreactive cells in the quail preoptic area. *J. Chem. Neuroanat.* 11, 147–171.
- Balthazart, J., Arnold, A.P., Adkins-Regan, E., 2017. Sexual differentiation of brain and behavior in birds. In: Pfaff, D., Joels, M. (Eds.), *Hormones, Brain and Behavior*, 3rd ed. Academic Press, New York, pp. 185–224.
- Bentley, G.E., Spar, B.D., MacDougall-Shackleton, S.A., Hahn, T.P., Ball, G.F., 2000. Photoperiodic regulation of the reproductive axis in male zebra finches, *Taeniopygia guttata*. *Gen. Comp. Endocrinol.* 117, 449–455.
- Beyer, C., Kolbinger, W., Froehlich, U., Pilgrim, C., Reisert, I., 1992. Sex differences of hypothalamic prolactin cells develop independently of the presence of sex steroids. *Brain Res.* 593, 253–256.
- Bilbo, S.D., Schwarz, J.M., 2012. The immune system and developmental programming of brain and behavior. *Front. Neuroendocrinol.* 33, 267–286.
- Bordeleau, M., Carrier, M., Luheshi, G.N., Tremblay, M.E., 2019. Microglia along sex lines: from brain colonization, maturation and function, to implication in neurodevelopmental disorders. *Semin. Cell Dev. Biol.* 94, 152–163.
- Boseret, G., Carere, C., Ball, G.F., Balthazart, J., 2006. Social context affects testosterone-induced singing and the volume of song control nuclei in male canaries (*Serinus canaria*). *J. Neurobiol.* 66, 1044–1060.
- Bottjer, S.W., Johnson, F., 1997. Circuits, hormones, and learning: vocal behavior in songbirds. *J. Neurobiol.* 33, 602–618.
- Brock, O., Baum, M.J., Bakker, J., 2011. The development of female sexual behavior requires prepubertal estradiol. *J. Neurosci.* 31, 5574–5578.
- Carere, C., Ball, G.F., Balthazart, J., 2007. Sex differences in projections from preoptic area aromatase cells to the periaqueductal gray in Japanese quail. *J. Comp. Neurol.* 500, 894–907.
- Castagna, C., Absil, P., Foidart, A., Balthazart, J., 1998. Systemic and intracerebroventricular injections of vasotocin inhibit appetitive and consummatory components of male sexual behavior in Japanese quail. *Behav. Neurosci.* 112, 233–250.
- Casto, J.M., Ball, G.F., 1996. Early administration of 17 β -estradiol partially masculinizes song control regions and α 2-adrenergic receptor distribution in European starlings (*Sturnus vulgaris*). *Horm. Behav.* 30, 387–406.
- Court, L., Vandries, L., Balthazart, J., Cornil, C.A., 2020. Key role of estrogen receptor β in the organization of brain and behavior of the Japanese quail. *Horm. Behav.* 125, 104827 <https://doi.org/10.1016/j.yhbeh.2020.104827>. Sep.
- De Vries, G.J., Panzica, G.C., 2006. Sexual differentiation of central vasopressin and vasotocin systems in vertebrates: different mechanisms, similar endpoints. *Neuroscience* 138, 947–955.
- De Vries, G.J., Rissman, E.F., Simerly, R.B., Yang, L.Y., Scordalakes, E.M., Auger, C.J., Swain, A., Lovell-Badge, R., Burgoyne, P.S., Arnold, A.P., 2002. A model system for study of sex chromosome effects on sexually dimorphic neural and behavioral traits. *J. Neurosci.* 22, 9005–9014.
- Delage, C.I., Cornil, C.A., 2020. Estrogen-dependent sex difference in microglia in the developing brain of Japanese quail (*Coturnix japonica*). *Dev. Neurobiol.* 80, 239–262.
- Delage, C.I., Nys, G., Fillet, M., Cornil, C.A., 2021. Effect of cyclo-oxygenase inhibition on embryonic microglia and the sexual differentiation of the brain and behavior of Japanese quail (*Coturnix japonica*). *Horm. Behav.* 134, 105024.
- Diddens, J., Coussement, L., Frankl-Vilches, C., Majumdar, G., Steyaert, S., Ter Haar, S. M., Galle, J., De Meester, E., De Keulenaer, S., Van Crielinge, W., Cornil, C.A., Balthazart, J., Van Der Linden, A., De Meyer, T., Vanden Berghe, W., 2021. DNA methylation regulates transcription factor-specific neurodevelopmental but not sexually dimorphic gene expression dynamics in zebra finch telencephalon. *Front. Cell. Dev. Biol.* 9, 583555.
- Dörner, G., 1976. *Hormones and Brain Differentiation*. Elsevier, Amsterdam, Oxford, New York.
- Dörner, G., Staudt, J., 1969. Structural changes in the hypothalamic ventromedial nucleus of the male rat, following neonatal castration and androgen treatment. *Neuroendocrinol.* 4, 278–281.
- Dos Santos, E.B., Ball, G.F., Cornil, C.A., Balthazart, J., 2022. Treatment with androgens plus estrogens cannot reverse sex differences in song and the song control nuclei in adult canaries. *Horm. Behav.* 143, 105197.
- Dos Santos, E.B., Logue, D.M., Ball, G.F.C., C. A., Balthazart, J., 2023. Does the Syrinx, a Peripheral Structure, Constrain Effects of Sex Steroids on Behavioral Sex Reversal in Adult Canaries? *BiorXiv*. <https://doi.org/10.1101/2023.04.19.537462>.
- Ellegren, H., Hultin-Rosenberg, L., Brunstrom, B., Dencker, L., Kultima, K., Scholz, B., 2007. Faced with inequality: chicken do not have a general dosage compensation of sex-linked genes. *BMC Biol.* 5, 40.
- Fusani, L., Gahr, M., 2006. Hormonal influence on song structure and organization: the role of estrogen. *Neuroscience* 138, 939–946.
- Fusani, L., Metzendorf, R., Hutchison, J.B., Gahr, M., 2003. Aromatase inhibition affects testosterone-induced masculinization of song and the neural song system in female canaries. *J. Neurobiol.* 54, 370–379.
- Gerall, A.A., Moltz, H., Ward, I.L., 1992. *Handbook of Behavioral Neurobiology*. In: *Sexual Differentiation*, vol. 11. Plenum Press, New York.
- Godwin, J., Thompson, R., 2012. Nonapeptides and social behavior in fishes. *Horm. Behav.* 61, 230–238.
- Gorski, R.A., Gordon, J.H., Shryne, J.E., Southam, A.M., 1978. Evidence for a morphological sex difference within the medial preoptic area of the rat brain. *Brain Res.* 148, 333–346.
- Goy, R.W., McEwen, B.S., 1980. *Sexual Differentiation of the Brain*. The MIT Press, Cambridge, MA.
- Guichard, A., Scheib, D., Haffen, K., Mignot, T.M., Cedard, L., 1980. Comparative study in steroidogenesis by quail and chick embryonic gonads in organ culture. *J. Steroid Biochem.* 12, 83–87.
- Gurney, M.E., Konishi, M., 1980. Hormone-induced sexual differentiation of brain and behavior in zebra finches. *Science* 208, 1380–1383.
- Harding, C.F., 2008. Hormonal modulation of singing behavior: methodology and principles of hormone action. In: Zeigler, H.P., Marler, P. (Eds.), *Neuroscience of Birdsong*. Cambridge University Press, Cambridge, pp. 305–319.
- Holloway, C.C., Clayton, D.E., 2001. Estrogen synthesis in the male brain triggers development of the avian song control pathway in vitro. *Nat. Neurosci.* 4, 170–175.
- Hutchison, J.B., Wingfield, J.C., Hutchison, R.E., 1984. Sex differences in plasma concentrations of steroids during the sensitive period for brain differentiation in the zebra finch. *J. Endocrinol.* 103, 363–369.
- Hutchison, J.B., Beyer, C., Green, S., Wozniak, A., 1994. Brain formation of oestrogen in the mouse: sex dimorphism in aromatase development. *J. Steroid Biochem. Mol. Biol.* 49, 407–415.
- Ioannidis, J., Taylor, G., Zhao, D., Liu, L., Idoko-Akoko, A., Gong, D., Lovell-Badge, R., Guoli, S., McGrew, M.J., Clinton, M., 2021. Primary sex determination in birds depends on DMRT1 dosage, but gonadal sex does not determine adult secondary sex characteristics. *Proc. Natl. Acad. Sci. U. S. A.* 118, 1–10.
- Itoh, Y., Melamed, E., Yang, X., Kampf, K., Wang, S., Yehya, N., Van Nas, A., Replogle, K., Band, M.R., Clayton, D.F., Schadt, E.E., Lusis, A.J., Arnold, A.P., 2007. Dosage compensation is less effective in birds than in mammals. *J. Biol.* 6, 2.
- Itoh, Y., Replogle, K., Kim, Y.H., Wade, J., Clayton, D.F., Arnold, A.P., 2010. Sex bias and dosage compensation in the zebra finch versus chicken genomes: general and specialized patterns among birds. *Genome Res.* 20, 512–518.
- Jurkevich, A., Barth, S.W., Aste, N., Panzica, G.C., Grossmann, R., 1996. Intracerebral sex differences in the vasotocin system in birds: possible implication in behavioral and autonomic functions. *Horm. Behav.* 30, 673–681.
- Jurkevich, A., Grossmann, R., Balthazart, J., Viglietti-Panzica, C., 2001. Gender-related changes in the avian vasotocin system during ontogeny. *Microsc. Res. Tech.* 55, 27–36.
- Kelly, A.M., Goodson, J.L., 2014. Social functions of individual vasopressin-oxytocin cell groups in vertebrates: what do we really know? *Front. Neuroendocrinol.* 35, 512–529.
- Ko, M.C., Van Meir, V., Vellema, M., Gahr, M., 2020. Characteristics of song, brain-anatomy and blood androgen levels in spontaneously singing female canaries. *Horm. Behav.* 117, 104614.
- Ko, M.C., Frankl-Vilches, C., Bakker, A., Gahr, M., 2021. The gene expression profile of the song control nucleus HVC shows sex specificity, hormone responsiveness, and species specificity among songbirds. *Front. Neurosci.* 15, 680530.
- Konishi, M., Emlen, S.T., Ricklefs, R.E., Wingfield, J.C., 1989. Contributions of bird studies to biology. *Science* 246, 465–472.
- Küppers, E., Pilgrim, C., Reisert, I., 1991. Sex-specific schedule in steroid response of rhombencephalic catecholaminergic neurons in vitro. *Int. J. Dev. Neurosci.* 9, 537–544.
- Lenz, K.M., Nugent, B.M., Haliyur, R., McCarthy, M.M., 2013. Microglia are essential to masculinization of brain and behavior. *J. Neurosci.* 33, 2761–2772.
- Lenz, K.M., Pickett, L.A., Wright, C.L., Davis, K.T., Joshi, A., McCarthy, M.M., 2018. Mast cells in the developing brain determine adult sexual behavior. *J. Neurosci.* 38, 8044–8059.
- Maccarrone, M., 2017. Metabolism of the endocannabinoid anandamide: open questions after 25 years. *Front. Mol. Neurosci.* 10, 166.
- Madison, F.N., Rouse Jr., M.L., Balthazart, J., Ball, G.F., 2015. Reversing song behavior phenotype: testosterone driven induction of singing and measures of song quality in adult male and female canaries (*Serinus canaria*). *Gen. Comp. Endocrinol.* 215, 61–75. <https://doi.org/10.1016/j.ygcen.2014.09.008>.
- Mathews, G.A., Arnold, A.P., 1990. Antiestrogens fail to prevent the masculine ontogeny of the zebra finch song system. *Gen. Comp. Endocrinol.* 80, 48–58.

- Mathews, G.A., Brenowitz, E.A., Arnold, A.P., 1988. Paradoxical hypermasculinization of the zebra finch song system by an antiestrogen. *Horm. Behav.* 22, 540–551.
- McCarthy, M.M., 2019. Sex differences in neuroimmunity as an inherent risk factor. *Neuropsychopharmacology* 44, 38–44.
- McCarthy, M.M., 2020. A new view of sexual differentiation of mammalian brain. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 206, 369–378.
- Mong, J.A., Glaser, E., McCarthy, M.M., 1999. Gonadal steroids promote glial differentiation and alter neuronal morphology in the developing hypothalamus in a regionally specific manner. *J. Neurosci.* 19, 1464–1472.
- Nottebohm, F., 1980. Testosterone triggers growth of brain vocal control nuclei in adult female canaries. *Brain Res.* 189, 429–436.
- Nottebohm, F., Arnold, A.P., 1976. Sexual dimorphism in vocal control areas of the songbird brain. *Science* 194, 211–213.
- Nugent, B.M., Wright, C.L., Shetty, A.C., Hodes, G.E., Lenz, K.M., Mahurkar, A., Russo, S. J., Devine, S.E., McCarthy, M.M., 2015. Brain feminization requires active repression of masculinization via DNA methylation. *Nat. Neurosci.* 18, 690–697.
- Panzica, G.C., Viglietti-Panzica, C., Calagni, M., Anselmetti, G.C., Schumacher, M., Balthazart, J., 1987. Sexual differentiation and hormonal control of the sexually dimorphic medial preoptic nucleus in the quail. *Brain Res.* 416, 59–68.
- Panzica, G., Viglietti-Panzica, C., Sanchez, F., Sante, P., Balthazart, J., 1991. Effects of testosterone on a selected neuronal population within the preoptic sexually dimorphic nucleus of the Japanese quail. *J. Comp. Neurol.* 303, 443–456.
- Panzica, G.C., Viglietti-Panzica, C., Balthazart, J., 1996. The sexually dimorphic medial preoptic nucleus of quail: a key brain area mediating steroid action on male sexual behavior. *Front. Neuroendocrinol.* 17, 51–125.
- Panzica, G.C., Aste, N., Castagna, C., Balthazart, J., Viglietti-Panzica, C., 1997. Sexual dimorphism, steroid-induced plasticity and behavioral significance of the vasotocin innervation of the avian brain. In: Korf, H.W., Usadel, K.H. (Eds.), *Neuroendocrinology: Retrospect and Prospect*. Springer, Heidelberg, Berlin, pp. 127–150.
- Panzica, G.C., Castagna, C., Viglietti-Panzica, C., Russo, C., Tlemçani, O., Balthazart, J., 1998. Organizational effects of estrogens on brain vasotocin and sexual behavior in quail. *J. Neurobiol.* 37, 684–699.
- Paolicelli, R.C., Gross, C.T., 2011. Microglia in development: linking brain wiring to brain environment. *Neuron Glia Biol.* 7, 77–83.
- Phoenix, C.H., Goy, R.W., Gerall, A.A., Young, W.C., 1959. Organizational action of prenatally administered testosterone propionate on the tissues mediating behavior in the female guinea pig. *Endocrinology* 65, 369–382.
- Pickett, L.A., VanRyzin, J.W., Marquardt, A.E., McCarthy, M.M., 2023. Microglia phagocytosis mediates the volume and function of the rat sexually dimorphic nucleus of the preoptic area. *Proc. Natl. Acad. Sci. U. S. A.* 120, e2212646120.
- Pohl-Apel, G., Sossinka, R., 1984. Hormonal determination of song capacity in females of the zebra finch. *Z. Tierpsychol.* 64, 330–336.
- Raisman, G., Field, P.M., 1971. Sexual dimorphism in the preoptic area of the rat. *Science* 173, 731–733.
- Raisman, G., Field, P.M., 1973. Sexual dimorphism in the neuropil of the preoptic area of the rat and its dependence on neonatal androgens. *Brain Res.* 54, 1–29.
- Reisert, I., Engele, J., Pilgrim, C., 1989. Early sexual differentiation of diencephalic dopaminergic neurons of the rat in vitro. *Cell Tissue Res.* 255, 411–417.
- Renfree, M.B., Harry, J.L., Shaw, G., 1995. The marsupial male: a role model for sexual development. *Philos. Trans. R. Soc. Lond. [Biol.]* 350, 243–251.
- Rigney, N., de Vries, G.J., Petrusis, A., Young, L.J., 2022. Oxytocin, vasopressin, and social behavior: from neural circuits to clinical opportunities. *Endocrinology* 163.
- Roselli, C.E., Resko, J.A., 1993. Aromatase activity in the rat brain: hormonal regulation and sex differences. *J. Steroid Biochem. Mol. Biol.* 44, 499–508.
- Scheib, D., Guichard, A., Mignot, T.M., Cedard, L., 1981. Steroidogenesis by gonads of normal and of diethylstilbestrol-treated quail embryos: radioimmunoassays on organ cultures. *Gen. Comp. Endocrinol.* 43, 519–526.
- Schlenger, B.A., 2022. Arthur Arnold. In: Nelson, R.J., Weil, Z.M. (Eds.), *Biographical History of Behavioral Neuroendocrinology*. Springer Nature, Cham, Switzerland, pp. 321–336.
- Schlenger, B.A., Arnold, A.P., 1991. Brain is the major site of estrogen synthesis in a male songbird. *Proc. Natl. Acad. Sci. U. S. A.* 88, 4191–4194.
- Schlenger, B.A., Arnold, A.P., 1992a. Circulating estrogens in a male songbird originate in the brain. *Proc. Natl. Acad. Sci. U. S. A.* 89, 7650–7653.
- Schlenger, B.A., Arnold, A.P., 1992b. Plasma sex steroids and tissue aromatization in hatchling zebra finches: implications for the sexual differentiation of singing behavior. *Endocrinology* 130, 289–299.
- Schlenger, B.A., Arnold, A.P., 1993. Estrogen synthesis in vivo in the adult zebra finch: additional evidence that circulating estrogens can originate in brain. *Endocrinology* 133, 2610–2616.
- Schlenger, B.A., Brenowitz, E.A., 2017. Neural and hormonal control of birdsong. In: Pfaff, D.W., Joels, M. (Eds.), *Hormones, Brain and Behavior*, 3rd ed. Academic Press, Oxford, pp. 255–290.
- Schlenger, B.A., Soma, K.K., London, S.E., 2001. Neurosteroids and brain sexual differentiation. *Trends Neurosci.* 24, 429–431.
- Schulz, K.M., Richardson, H.N., Zehr, J.L., Osetek, A.J., Menard, T.A., Sisk, C.L., 2004. Gonadal hormones masculinize and defeminize reproductive behaviors during puberty in the male Syrian hamster. *Horm. Behav.* 45, 242–249.
- Schumacher, M., Balthazart, J., 1986. Testosterone-induced brain aromatase is sexually dimorphic. *Brain Res.* 370, 285–293.
- Schumacher, M., Sulon, J., Balthazart, J., 1988. Changes in serum concentrations of steroids during embryonic and post-hatching development of male and female Japanese quail (*Coturnix coturnix japonica*). *J. Endocrinol.* 118, 127–134.
- Schumacher, M., Hendrick, J.C., Balthazart, J., 1989. Sexual differentiation in quail: critical period and hormonal specificity. *Horm. Behav.* 23, 130–149.
- Shaughnessy, D.W., Hyson, R.L., Bertram, R., Wu, W., Johnson, F., 2018. Female zebra finches do not sing yet share neural pathways necessary for singing in males. *J. Comp. Neurol.* 527, 843–855.
- She, Z.Y., Yang, W.X., 2017. Sry and Sox9 genes: how they participate in mammalian sex determination and gonadal development? *Semin. Cell Dev. Biol.* 63, 13–22.
- Simpson, H.B., Vicario, D.S., 1991a. Early estrogen treatment alone causes female zebra finches to produce learned, male-like vocalizations. *J. Neurobiol.* 22, 755–776.
- Simpson, H.B., Vicario, D.S., 1991b. Early estrogen treatment of female zebra finches masculinizes the brain pathway for learned vocalizations. *J. Neurobiol.* 22, 777–793.
- Sisk, C.L., Zehr, J.L., 2005. Pubertal hormones organize the adolescent brain and behavior. *Front. Neuroendocrinol.* 26, 163–174.
- Thion, M.S., Garel, S., 2017. On place and time: microglia in embryonic and perinatal brain development. *Curr. Opin. Neurobiol.* 47, 121–130.
- Thion, M.S., Ginhoux, F., Garel, S., 2018. Microglia and early brain development: an intimate journey. *Science* 362, 185–189.
- Thompson, R.R., Adkins-Regan, E., 1992. Ontogeny of a sexually dimorphic nucleus in the preoptic area of the Japanese quail (*Coturnix japonica*). *Dev. Brain Res.* 70, 231–237.
- Tobet, S.A., Fox, T.O., 1992. Sex differences in neuronal morphology influenced hormonally throughout life. *Handbook of behavioral neurobiology*. In: Gerall, A.A., Moltz, H., Ward, L.L. (Eds.), *Sexual Differentiation*, vol. 11. Plenum Press, New York, pp. 41–83.
- Vandries, L.M., Ghorbanpoor, S., Cornez, G., Shevchouk, O.T., Ball, G.F., Cornil, C.A., Balthazart, J., 2019. Testosterone or estradiol when implanted in the medial preoptic nucleus trigger short low-amplitude songs in female canaries. *eNeuro* 6.
- VanRyzin, J.W., Marquardt, A.E., Argue, K.J., Vecchiarelli, H.A., Ashton, S.E., Arambula, S.E., Hill, M.N., McCarthy, M.M., 2019. Microglial phagocytosis of newborn cells is induced by endocannabinoids and sculpt sex differences in juvenile rat social play. *Neuron* 102 (435–449), e436.
- Viglietti-Panzica, C., 1986. Immunohistochemical study of the distribution of vasotocin reacting neurons in avian diencephalon. *J. Hirnforsch.* 27, 559–566.
- Viglietti-Panzica, C., Panzica, G.C., Fiori, M.G., Calcagni, M., Anselmetti, G.C., Balthazart, J., 1986. A sexually dimorphic nucleus in the quail preoptic area. *Neurosci. Lett.* 64, 129–134.
- Viglietti-Panzica, C., Anselmetti, G.C., Balthazart, J., Aste, N., Panzica, G.C., 1992. Vasotocinergic innervation of the septal region in the Japanese quail: sexual differences and the influence of testosterone. *Cell Tissue Res.* 267, 261–265.
- Wade, J., Arnold, A.P., 1994. Post-hatching inhibition of aromatase activity does not alter sexual differentiation of the zebra finch song system. *Brain Res.* 639, 347–350.
- Wade, J., Arnold, A.P., 1996. Functional testicular tissue does not masculinize development of the zebra finch song system. *Proc. Natl. Acad. Sci. U. S. A.* 93, 5264–5268.
- Wade, J., Schlenger, B.A., Arnold, A.P., 1995. Aromatase and 5 β -reductase activity in cultures of developing zebra finch brain: an investigation of sex and regional differences. *J. Neurobiol.* 27, 240–251.
- Wade, J., Springer, M.L., Wingfield, J.C., Arnold, A.P., 1996. Neither testicular androgens nor embryonic aromatase activity alters morphology of the neural song system in zebra finches. *Biol. Reprod.* 55, 1126–1132.
- Wright, C.L., Burks, S.R., McCarthy, M.M., 2008. Identification of prostaglandin E2 receptors mediating perinatal masculinization of adult sex behavior and neuroanatomical correlates. *Dev. Neurobiol.* 68, 1406–1419.
- Zann, R.A., 1996. *The zebra Finch: A Synthesis of Field and Laboratory Studies*, 0 ed. Oxford. Univ. Press, Oxford.