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Estradiol Modulates Neurotransmitter Concentrations in the Developing Zebra Finch Song System

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Abstract

The neural song system in zebra finches is highly sexually dimorphic; only males sing and the brain regions controlling song are far larger in males than females. Estradiol (E2) administered during development can partially masculinize both structure and function. However, additional mechanisms, including those through which E2 may act, remain unclear. Male and female zebra finches were treated with E2 or control vehicle from post-hatching days 3 through 25, at which time norepinephrine (NE), dopamine (DA) and serotonin (5-HT) were measured in individual nuclei of the song system. Main effects of sex were not detected. However, E2 increased NE in the robust nucleus of the arcopallium (RA). In HVC (proper name), the hormone decreased 5-HT across the two sexes and increased DA in females only. These effects suggest that, while baseline levels of these neurotransmitters may not contribute to sexually dimorphic development of the song system, they could play specific roles in functions common to both sexes and/or in modification of the song system by exogenous E2.

Keywords

songbird; sexual differentiation; norepinephrine; dopamine; serotonin; steroid hormone

1. Introduction

Sex differences in brain and behavior exist across vertebrate species. In many cases, masculinization of structure and function is permanently organized by steroid hormones, estradiol (E2) in particular (*e.g.*, McCarthy, 2009). However, many downstream mechanisms remain to be elucidated.

The song control circuit in zebra finches has become a classic model for the study of sexual differentiation of brain and behavior. In this species, only males sing. This behavior is controlled by forebrain circuits. An anterior pathway that is critical for song learning includes Area X and the lateral magnocellular nucleus of the anterior nidopallium (LMAN). A motor pathway important for the song production consists of HVC (proper name; Reiner

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et al., 2004) projecting to the robust nucleus of the arcopallium (RA), which innervates the motoneurons of the vocal organ (syrinx; reviewed in Nordeen and Nordeen, 1997). The HVC and RA are substantially larger in males, who sing, compared to females who do not. Area X is not visible in females. Thus, substantial sexual dimorphisms exist in both structure and function of the zebra finch song circuit (Nottebohm and Arnold, 1976). Song is a learned behavior; juvenile males acquire song from adult tutors, commonly their fathers (Slater and Mann, 1990). Females can be partially masculinized by treatment with E2 in the first few weeks after hatching. They will develop rudimentary song and the song control nuclei will be larger than control females, although not equivalent to males (reviewed in Wade, 2001; Wade and Arnold, 2004).

Norepinephrine (NE) has long been implicated in facilitating arousal that allows adaptive change to the environment, including mediation of learning, memory, and sensory responsiveness (Castelino and Schmidt, 2010; Harley, 2004; Sara, 2009). In addition, this neuromodulator plays a role in sexual differentiation of the rodent brain. NE mediates the masculinizing effects of E2 on the sexually dimorphic nucleus of the preoptic area (SDN-POA), which is larger in males than females, and may also be involved with defeminization or feminization of behavior, depending on the receptors at which it acts (reviewed in Wilson and Davies, 2007). Thus, we considered the possibility that NE is involved in sexual differentiation of the song system in zebra finches. In further support of this idea, noradrenergic transmission facilitates the survival of new neurons in the mammalian hippocampus (Rizk et al., 2006). Masculinization of HVC and Area X both involve the addition of new neurons (reviewed in (Wade and Arnold, 2004).

Several pieces of evidence from songbirds are consistent with our hypothesis. First, a prominent source of NE in the vertebrate brain (locus coeruleus) sends projections to several portions of the song circuit, including HVC, RA and Area X (reviewed in Castelino and Schmidt, 2010). These components of the song system contain high levels of NE, as well as key receptors. Noradrenergic receptors delineate HVC, RA and Area X, with α_2 in all three regions, and $\beta_{1/2}$ in at least RA and Area X (Bernard and Ball, 1995; Riters and Ball, 2002; reviewed in Castelino and Schmidt, 2010). NE levels and turnover in RA and Area X are relatively high about 25–30 days after hatching, and gradually decline until about day 60 (NE in HVC is constant during this period; Harding et al., 1998; Sakaguchi and Saito, 1989). This developmental time-frame includes both substantial structural organization of the song circuit and the period in which males memorize their tutors' songs and use the memory to guide the sensorimotor integration that underlies vocal learning (Castelino and Schmidt, 2010).

We recently used N-(2-Chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP4) to temporarily reduce exposure of developing males to NE (Wade et al., 2013). It inhibited the development of quality songs, with some birds producing syllables that were unusually long and/or contained frequencies predominantly higher than normal, suggesting that NE is important for the acquisition of typical song. In addition to the separate pieces of evidence for roles of E2 and NE in masculinization, some data from adult males suggests they may be linked. E2 modulates NE concentration and rate of noradrenergic turnover in both Area X and RA of adult males (Barclay and Harding, 1990).

Considering all of this information, it seemed reasonable to hypothesize not only that NE contributes to developmental masculinization of the structure and function of the song system, but that it is one of the mechanisms by which E2 exerts its influence. The present experiment was designed to begin to test these ideas. Hatchlings were treated with E2, and NE levels were quantified in hormone-manipulated males and females at post-hatching day 25, near the beginning of template formation and in the heart of morphological

differentiation of the song control regions. If important for masculinization, NE may be increased in the song nuclei of males compared to females, and if it is associated with E2-induced masculinization, the hormone might increase NE in critical regions. In addition, limited information is available about dopamine (DA), and particularly serotonin (5-HT), in the maturing song system. Thus, although we did not have predictions about patterns related to particular developmental mechanisms, these neurotransmitters were quantified in the same samples.

2. Results

A main effect of treatment was detected in RA, such that E2 increased the concentration of NE compared to the control manipulation ($F_{1,27} = 4.89$, $p = 0.036$; Figure 1 top). There was no main effect of sex ($F_{1,27} = 0.06$, $p = 0.813$) or sex $\times$ treatment interaction ($F_{1,27} = 1.02$, $p = 0.322$) in this brain region. NE concentration was equivalent across conditions in HVC (sex: $F_{1,28} = 1.82$, $p = 0.188$; treatment: $F_{1,28} = 0.95$, $p = 0.339$; interaction $F_{1,28} = 3.26$, $p = 0.082$; Figure 2 top) and LMAN (sex: $F_{1,27} = 1.58$, $p = 0.219$; treatment: $F_{1,27} = 2.03$, $p = 0.165$; interaction $F_{1,27} = 3.08$, $p = 0.091$; Figure 3 top).

While main effects of sex and treatment did not exist for DA in HVC ($F_{1,28} < 0.35$, $p > 0.561$), the variables interacted ($F_{1,28} = 5.88$, $p = 0.022$; Figure 2 middle). E2 induced an increase in females ($t_{13} = 2.22$, $p = 0.045$) but not males ($t_{15} = 0.22$, $p = 0.224$). Statistically significant effects for DA were not detected in RA (sex: $F_{1,27} = 0.21$, $p = 0.648$; treatment: $F_{1,27} = 0.28$, $p = 0.600$; interaction $F_{1,27} = 3.73$, $p = 0.064$; Figure 1 middle) or LMAN (sex: $F_{1,28} = 0.68$, $p = 0.442$; treatment: $F_{1,27} = 0.94$, $p = 0.341$; interaction $F_{1,27} = 1.26$, $p = 0.272$; Figure 3 middle).

The concentration of 5-HT in HVC was decreased by E2 ($F_{1,28} = 10.10$, $p = 0.004$; Figure 2 bottom), although there was no main effect of sex or interaction between sex and treatment on this measure ($F_{1,28} < 0.92$, $p > 0.347$). 5-HT did not differ across manipulations in RA (sex: $F_{1,27} = 0.56$, $p = 0.463$; treatment: $F_{1,27} = 0.36$, $p = 0.553$; interaction $F_{1,27} = 0.33$, $p = 0.572$; Figure 1 bottom) or LMAN (sex: $F_{1,28} = 0.07$, $p = 0.792$; treatment: $F_{1,27} = 1.77$, $p = 0.195$; interaction $F_{1,27} = 0.31$, $p = 0.580$; Figure 3 bottom).

3. Discussion

Main effects of sex were not detected for any of the three neurotransmitters in the brain regions investigated. Thus, it seems unlikely that fundamental differences in NE, DA or 5-HT levels at 25 days of age directly contribute to masculine development of song system structure or function. These neurotransmitters may play a role in development of functions important to both sexes, such as song perception or recognition (Castelino and Schmidt, 2010). However, it is also possible that a sex difference exists in one or more of these regions at another developmental stage, perhaps associated with sensorimotor integration of song. While females as well as males may form memories of their fathers' songs beginning around post-hatching day 25 (Miller, 1979), when tissue for the present study was collected, only male zebra finches experience this later period of learning to produce the vocalizations.

Both NE and DA have been implicated in song learning (Castelino and Schmidt, 2010; Kubikova and Kostal, 2010), but the focus has been on development of vocalizations rather than memory formation. In fact, previous research quantifying neurotransmitters during songbird development has been conducted only in males (Harding et al., 1998; Sakaguchi and Saito, 1989; Soha et al., 1996). A more detailed time course in birds of both sexes that have not been exposed to hormone manipulations would be valuable. Male-biased sex differences in tyrosine hydroxylase labeling, which reflects the capacity to synthesize

catecholamines, have been detected in the adult song systems of zebra finches and canaries (Appeltants et al., 2001; Bottjer, 1993). Determining when sex differences appear across species would allow for specific hypothesis regarding function to be generated. Rates of turnover should also be evaluated as they may provide important information on sexually dimorphic availability of monoamines. While a relationship between 5-HT and song learning is not clear, data from mammalian systems suggest that reductions in 5-HT transmission improve learning and retention of specific tasks (Di Giovanni et al., 2008). This pattern is consistent with E2 facilitating song learning via a decrease in 5-HT availability.

While prior to the present study endocrine regulation of neurotransmitter levels in birds had only been investigated in adults, it is clear that steroid hormones can influence the noradrenergic system and that they can act via this pathway to modulate behavioral responses to song (reviewed in Castelino and Schmidt, 2010). However, research on E2 effects on neurotransmitters has largely focused on regions outside of the song control system. For example, the hormone increases catecholamine innervation and NE concentration selectively in regions of the auditory cortex in female white-throated sparrows (Matragrano et al., 2011). Also in these animals, 5-HT fiber density and concentration of a 5-HT metabolite is increased in particular auditory regions by E2 (Matragrano et al., 2012). Interestingly, we found no effect of E2 on DA concentration in the nucleus accumbens and striatum of adult female zebra finches (Svec et al., 2009). Some effects of E2 in adult songbirds can be quite rapid, including modulation of neural responses to auditory stimuli, which are transmitted to HVC to alter perception and production of song. These effects may depend on catecholamines (Remage-Healey, 2012). However, they appear to be limited to the mature brain, as the ability of neuroestrogens to enhance the response of HVC to a bird's own song occurs in adults but not late juveniles (Remage-Healey and Joshi, 2012).

It is intriguing that E2 modulated all three neurotransmitters that we quantified during development and particularly that the effects were specific. That is, E2 increased NE only in RA and decreased 5-HT only in HVC. The pattern for DA was even more selective, as E2 increased the concentration of this neurotransmitter only in HVC and only in females. These data are consistent with estrogen receptor alpha expression patterns during early development. The mRNA is present at relatively high levels in HVC from post-hatching days 10–25 and in limited quantities in RA at day 25, whereas it was undetectable through this period in LMAN (Jacobs et al., 1999). Thus, while the hormone may be affecting rate of production in the cell bodies synthesizing the neurotransmitters, this pattern suggests the possibility that E2 might also act locally to influence neurotransmitter availability in the motor regions, HVC and RA.

The specificity of the effects of E2 in the present study allows us to generate hypotheses regarding potential mechanisms through which the hormone might act to masculinize structure of the song circuit. Increased addition of cells in the HVC of developing males and increased death of cells in the RA of developing females are key features of the sexual differentiation process (reviewed in Wade and Arnold, 2004). Thus, it is possible that the up-regulation of NE by E2 in RA inhibits cell death. Similarly, the increase in DA and decrease in 5-HT in HVC might facilitate cell addition/survival. These ideas would need to be tested, including assessments of the availability and activity of E2 in the brain regions both synthesizing and responding to the neurotransmitters in males and females. More detailed, downstream mechanisms should also be investigated.

The female-specificity of the DA effect in HVC will also important to consider further. One possibility is that the effects of E2 in males on this measure are at a ceiling. However, on average E2 induced a 32% decrease in DA in this region in males. While this decline was

not statistically significant, the pattern does not provide strong support for a ceiling effect. No consistent evidence exists for differences in either circulating levels or whole brain or telencephalic production of E2 during development (reviewed in Wade, 2001). One intriguing study, however, indicated that increased E2 produced in slice cultures from juvenile males is important for growth of the projection from HVC to RA (Holloway and Clayton, 2001). In addition, obvious differences in estrogen receptor expression do not exist in the brains of developing males and females (Jacobs et al., 1999), but sample sizes in that study were too small to fully evaluate this idea. Thus, whether this difference between the sexes in the response of HVC reflects variability in sensitivity or availability of E2 requires further investigation.

In conclusion, the present results indicate that E2 during juvenile development can selectively modulate monoamine levels in the motor pathway for song production (HVC and RA). In one case this effect was sex-specific. However, in general, levels of NE, DA and 5-HT were equivalent in the song system of 25-day-old males and females. This result raises questions of the ways in which these brain regions may be functionally similar between the sexes. For example, HVC may be involved in song discrimination by females birds, but the data are inconsistent across species (Brenowitz, 1991; del Negro et al., 1998; Lynch et al.; MacDougall-Shackleton et al., 1998), and even less information is available about other regions such as RA. Perhaps it is worth further consideration of why some of these relatively large structures are retained in zebra finch females which do not sing.

4. Experimental Procedures

4.1 Animals

Zebra finches were raised in large colony aviaries, each containing approximately 7 adult animals of each sex and their young. The light cycle was 12:12, and animals were provided seed and water *ad libitum*. Hard boiled chicken eggs mixed with bread, and fresh spinach and oranges, were also provided weekly. Nest boxes were checked each day. Procedures were approved by the Michigan State University IACUC.

4.2 Treatment and Tissue Collection

Hatchling male and female zebra finches were administered a subcutaneous implant of 17 β -estradiol (E2) or vehicle control on post-hatching day 3 (Tang and Wade, 2009; Tang and Wade, 2012). Each implant contained approximately 100 μ g of E2 in silicone sealant (Dow Corning, Midland MI) or just the sealant. Birds were returned to their home aviaries and raised by their parents until post-hatching day 25. At this point, they were rapidly decapitated, and the brains immediately frozen in methyl-butane. The tissue was stored at -80°C until use. Group sizes were initially 9 control males, 8 E2-treated males, 8 control females, and 7 E2-treated females. The RA sample from one E2-treated male and the LMAN sample from one E2-treated female were lost during processing, resulting in n=7 and n=6, respectively.

Brains were sectioned at 300 μ m, and punches were collected from HVC, RA, and LMAN using a stainless steel cannula (0.5mm diameter, Stoelting Co., Wood Dale IL). These brain regions were identified using surrounding landmarks in the issue (as in Tang and Wade, 2012). Punches were collected on both sides of the brain in each section that brain regions were available, resulting in an average of 3.7 (0.14 S.E.) for LMAN, 5.9 (0.06) for HVC, and 3.4 (0.04) for RA. The number of tissue punches collected for each region was equivalent across sexes and treatments ($F < 2.95$, $p = 0.09$). Area X was not investigated, as it is not present in females.

4.3 Sample Processing

Samples were immediately placed in a 0.03 M citrate buffer containing 15% MeOH and 0.05 M Na phosphate (pH 2.49). They were stored at -80°C until analysis. At that point, the samples were thawed, sonicated and centrifuged at $14,000 \times g$ for 3 minutes. The supernatant was stored at -80°C until processing by high performance liquid chromatography (HPLC) with electrochemical detection (King et al., 2008). HPLC was performed with a commercial system (ESA Biosciences, Chemsford MA) with a Coulochem III detector. Three electrodes were used, with the first at 200mV, the second at 100mV, and the detection electrode at -350mV . Thus, reduction rather than oxidation was quantified.

NE, 5-HT and DA were separated on a HR-80 reversed-phase column (Thermo Scientific, Pittsburgh PA). The mobile phase was Cat-A-Phase II (Thermo), and optimum flow rate was 1.1 mL/min. The separation column was maintained at 35°C , and the limit of detection for NE and DA was 0.1ng/ml, for 5-HT it was 0.5ng/ml. Standard curve calibrations for all three neurotransmitters produced R^2 values of greater than 0.9997.

Protein concentrations were determined from the pellet following the addition of RIPA complete lysis buffer (Santa Cruz Technology, Santa Cruz CA). These samples were sonicated and spun at $10,000 \times g$ at 4°C for 10 minutes. The supernatant was evaluated with the Micro BCA protein assay kit (Thermo Scientific, Waltham MA), and compared to a standard curve to determine total protein concentration.

4.4 Analyses

Neurotransmitter concentrations were divided by protein concentrations to generate corrected values of NE, DA and 5-HT (ng/mg protein). Two-way ANOVAs were used to determine the effects of sex and treatment on this variable within each brain region (SPSS version 20).

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Highlights

- Norepinephrine, dopamine and serotonin are present in the song system of juvenile zebra finches.
- Levels of these neurotransmitters are equivalent in males and females across song nuclei.
- Estradiol increases norepinephrine in the robust nucleus of the arcopallium.
- In HVC, estradiol increases dopamine in females only and decreases serotonin across the two sexes.

Figure 1. Concentrations of NE (top), DA (middle) and 5-HT (bottom) in the RA of 25-day-old zebra finches. Birds had been treated with estradiol or vehicle control (blank) since 3 days after hatching, and the hormone significantly increased NE only, across the two sexes.

Figure 2.

NE (top), DA (middle) and 5-HT (bottom) concentrations in the HVC of juvenile zebra finches. No significant effects were detected for NE. However, compared to the control manipulation (blank implant), estradiol increased dopamine in females only (denoted by asterisks within the white bars) and decreased serotonin across the two sexes (single asterisk).

Figure 3.

Concentrations of NE (top), DA (middle), and 5-HT (bottom) in the LMAN of 25-day-old zebra finches. No statistically significant effects of sex or treatment were detected, and the variables did not interact. The mean and variability of DA in E2-treated males were greatly increased by the presence of one individual with a level of 519 ng/mg protein. While a mathematical outlier, we had no technical or biological reason for excluding the sample, so it is included in the statistics reported. The presence of this sample does not change the pattern of results. The dashed line in the right bar of this histogram indicates the mean if this value is deleted from the calculations – it is very similar to the other three groups.