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By

Lace Ann Svec

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ABSTRACT

NEURAL MECHANISMS OF FEMALE ZEBRA FINCH MATE CHOICE: THE ROLE OF THE AUDITORY PERCEPTION SITES, THE SOCIAL BEHAVIOR NETWORK, AND THE REWARD SYSTEM

By

Lace Ann Svec

Female zebra finches are highly social. Males will court females with song, and females utilize this song stimuli to make mate choice decisions. After this decision has been made, the male and female will form a strong pair bond. The unique behaviors and characteristics associated with zebra finch affiliation and reproduction have provided an excellent opportunity to examine the intricate and complex interactions among social behavior, neural systems, and hormones. In the experiments conducted for this dissertation, I investigated the presentation and regulation of two aspects of social behaviors: song preferences and pair bonding in the female zebra finch. I used two methods to examine the neural responses of females to these two types of social stimuli: immediate early gene analysis and high performance liquid chromatography (HPLC) for dopamine neurochemistry. Additionally, I examined the effects of estrogen treatment on song responses.

Females showed preferences for high quality tutored song over untutored song, regardless of estrogen treatment. Expression of the immediate early gene, ZENK was decreased in response to estrogen treatment in the ventromedial hypothalamus. Additionally, a greater induction of ZENK following exposure to tutored versus untutored song and silence was observed in the auditory perception sites was observed, but was eliminated when females were treated with estrogen. Females performed higher

levels of associative behaviors when interacting with their mates compared to novel males. In these females, ZENK expression was correlated with two pairing behaviors, clumping and preening, in the nucleus taeniae (homologous to the mammalian medial amygdala), but not in females that were interacting with novel males. In the studies conducted for this dissertation, the activity of dopaminergic neurons was not affected by song presentation, estrogen treatment, or pairing status.

The results from this dissertation indicate that female zebra finches show different behavioral responses to certain social stimuli and these may be separately regulated by responses in two types of brain regions, auditory perception sites and the nucleus taeniae (which is part of the Social Behavior Network). In this species, dopamine activity in the nucleus accumbens or striatum did not appear to relate to responses to social stimuli in either of the behavioral paradigms. As a result, the present data indicate that these behaviors may be separately mediated by the particular neural regions examined in this dissertation, but it remains to be determined how neural systems in the avian brain integrate to fully coordinate social responses.

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CHAPTER 1: INTRODUCTION

General Introduction to Social Behavior

A variety of species live in groups in which sociality is important. Some classic examples of social behaviors involve those associated with reproduction, aggression, and parenting. When interacting with other individuals, communication and recognition are critical. Investigating reproductive behavior provides numerous opportunities to examine social communication and the interpretation of social cues. Prior to reproduction, males and females must locate one another, and often some type of courtship behavior ensues. Usually courtship involves communication from the male to the female, which may provide indication of his reproductive fitness. In the studies in this dissertation, social behavior will be investigated in the context of reproduction in the female zebra finch.

Females in a number of avian species (including zebra finches) utilize an auditory signal, male song, to determine the quality of a potential mate. For example, in song sparrows, females prefer males with a larger repertoire of songs with a species-specific structure. This preference is indicated by increased copulatory solicitation behavior in response to the preferred song types (Searcy and Marler 1981). Female songbirds also show preferences for longer songs (Neubauer 1999; Nolan and Hill 2004), songs presented at higher rates (Nolan and Hill 2004), and songs containing difficult-to-produce syllables (Ballentine et al. 2004), which all reflect increased male energy output to produce the song. Repertoire size (Buchanan and Catchpole 2000) and song rate (Dolby et al. 2005) are both correlated with high paternal effort (increased feeding of the nestlings). Also, Hasselquist et al. (1996) demonstrated a correlation

between repertoire size of a male and survival of offspring. Low song quality can be an indicator of developmental stress, and as a result, lower the fitness of a potential mate (Nowicki et al. 2002). Thus, a female songbird most likely utilizes characteristics of male song in order to examine both a male's direct (paternal effort/general health of male) and indirect (good genes) benefit to her reproductive fitness and offspring survival.

Zebra Finch Biology

Zebra finches live in colonies of 20-100, which consist of adult males, females, and their offspring (Zann 1996). Social interactions occur in many contexts, including locating food and water, evading predators, and reproducing (Zann 1996). In the colony, individuals must respond to a variety of social stimuli, which provides a number of possibilities for examining social communication in this species. There are several other important aspects of zebra finch behavior and biology that make them excellent models for these types of investigations.

First, zebra finches are opportunistic breeders; in the wild they reproduce when there is plentiful water supply. As a result, zebra finches reproduce easily in the laboratory if fresh water is provided *ad libitum*. It is unknown whether the presence of water or the grasses and grass seeds that grow as a result of rainfall in the wild stimulate the activation of breeding behavior (see Zann 1996 for review). Also, there is conflicting evidence about how quickly reproduction begins after rain, some report that courtship begins days after the first rainfall, while others state that it takes months (Zann et al. 1995; Zann 1996). It has been hypothesized that males may remain in a near-ready

state in terms of reproductive development (Farner and Serventy 1960), but this phenomenon has not been well characterized in either males or females.

Second, zebra finches, and many other songbird species, form pair bonds prior to mating. In zebra finches, these bonds can begin to form within 30 minutes of introduction, but typically take about 2-14 days to consolidate (Silcox and Evans 1982). In the wild, pairing is characterized by clumping (perching together in physical contact), allopreening (cleaning and inspecting each other for pests), and synchronized behavior (eating, drinking, perching etc. at the same time). The display of clumping and preening are often used in laboratory studies to identify individuals as paired (*i.e.* Butterfield 1970; Silcox and Evans 1982; Clayton 1990; Zann 1996; Adkins-Regan and Wade 2001; Adkins-Regan 2002). As the pair bond is consolidated, these behaviors increase in frequency and the two individuals become largely inseparable. During and after the formation of the bond, the male and female zebra finch will begin to build a nest together and eventually reproduce. Some researchers also identify pair bonds by males and females that enter a nest box together (Mansukhani et al. 1996; Adkins-Regan and Wade 2001). Tactile contact is required in order to form the pair bond, but it can be maintained with visual and auditory contact alone (Silcox and Evans 1982).

This pair bond is strong and long lasting (usually throughout life), and both members of the couple provide care for their young (Zann 1996), which is necessary at least in part due to the extremely altricial nature of zebra finches at hatching. Success of broods in another socially monogamous songbird, the reed warbler, decreases when a male parent is removed, because the female must compensate for the assistance normally

provided by the male (Duckworth 1992). It is unknown whether the same is seen in zebra finches.

Behaviors indicative of a pair bond, such as clumping, preening, or synchronized behavior are not typically displayed between unrelated single individuals (Butterfield 1970; Zann 1996). Females that have formed a pair bond react aggressively to new males, and close contact (clumping and preening) with them is inhibited (Silcox and Evans 1982). Thus, females both increase pairing behaviors displayed toward their mate, and also reduce the display of these behaviors toward other males. The pair bond in this species can be utilized to investigate a more complex social interaction and how a specific type of social bond can modify female responses to social stimuli.

In sum, the zebra finch is an excellent species in which to examine questions regarding social responses, because they are highly social, form a strong pair bond prior to copulation, and have an easily manipulated and analyzed form of auditory communication. This model provides a perfect opportunity to explore the neural mediation of female social responses. At this point, the development and characteristics of the song control system in males has been the focus of neural research in this species. The neural control of social behaviors in adult females has largely been ignored, and that is the focus of my dissertation.

Introduction to Neural Systems

Based on their likely functional associations with interpretation of social stimuli and mediation of social behaviors in mammals and possibly birds, I propose three categories of neural regions or systems that may be critical for these behaviors in the

female zebra finch: the Social Behavior Network, dopaminergic systems, and regions involved in auditory perception. These groups of brain areas are not isolated; each interacts with other systems. Regions in these three nodes may collectively integrate external and internal environmental cues to influence evaluation of and motor responses to social stimuli. The areas/systems could work together or influence each other. Alternatively, individual brain areas or groups of them might act independently to influence particular aspects of this process.

For regions in each of these categories or systems, I will highlight the existing comparative information in relation to their functional, anatomical and neurochemical characteristics. Researchers have documented numerous functional and anatomical similarities between avian and mammalian neural systems. The comparisons between birds and mammals have been extensive in some regions, but research in other regions has been much more limited. In 2004, a forum was conducted to modify the avian brain nomenclature to reflect these similarities, the results of which have been discussed in Reiner et al. (2004a, b). For each neural group, I will compare the function and anatomy of the mammalian and avian regions in relation to the social behaviors investigated in this dissertation, using the available data. Tables 1-2 will include summaries of this comparative information.

Social Behavior Network

During social interactions, female behavior is most likely mediated by integrating hormonal and sensory information to direct a specific motor response. A neural social behavior network has been proposed to serve this type of function in rodents (Newman

1999) and in other vertebrates (Goodson et al. 2005). Brain areas included in this interconnected network are responsive to gonadal steroid hormones and mediate multiple social behaviors, such as reproduction and aggression (Newman 1999). The network, as described by Newman (1999), includes the medial amygdala, bed nucleus of the stria terminalis, lateral septum, midbrain, ventromedial hypothalamus, anterior hypothalamus, and medial preoptic area. At least some of these regions are likely to be relevant to the reproductive and social behaviors examined in these studies.

In mammals, immediate early gene (IEG) induction is observed following vaginocervical stimulation or copulation with intromission in the ventromedial hypothalamus, medial amygdala, preoptic area, and bed nucleus of the stria terminalis (reviewed in Pfaus and Heeb 1997). The response within this network in rodents has also been examined following exposure to socially relevant stimuli (for rodents these stimuli are primarily olfactory, as opposed to auditory, as in songbirds). In female rats, increased IEG expression in the medial amygdala and bed nucleus of the stria terminalis is also seen when exposed to bedding with male odors (Bennett et al. 2002).

Several brain regions in this network are also implicated in the mediation of pair bonding behaviors in prairie voles. Cohabitation with a male results in increased IEG expression in the bed nucleus of the stria terminalis and preoptic area in females (Cushing et al. 2003). Following the formation of a pair bond (6 hours of mating), IEG expression is also seen in the medial amygdala, preoptic area, and bed nucleus of the stria terminalis (Curtis and Wang 2003). Thus, it appears that regions within the social behavior network are utilized in the formation/maintenance of pair bonding in another monogamous/pair bonding species.

The neuropeptides oxytocin and vasopressin, which have been strongly implicated in rodent social recognition and bonding (see Bielsky and Young 2004; Lim and Young 2006 for reviews), are present in several of the brain regions in this network. The rodent bed nucleus of the stria terminalis, septum, and amygdala contain vasopressin (DeVries et al. 1985; Caffe et al. 1987). Impaired social recognition in the Brattelboro rat, which is vasopressin deficient (Vandesande and Dierickx 1976), can be restored with vasopressin infusion directly into the lateral septum (Engelmann and Landgraf 1994). Antagonism or viral knockdown of the vasopressin 1a receptor in rats (Landgraf et al. 1995) and knockout of this receptor in mice (Bielsky et al. 2004) also causes a reduction in social recognition. Vasopressin and oxytocin are also related to sociality and monogamy in vertebrate species (see Goodson and Bass 2001 for review). Receptor levels for oxytocin are higher in monogamous than promiscuous species in the nucleus accumbens and bed nucleus of the stria terminalis, but lower in the lateral septum (Wang et al. 1996). In contrast, vasopressin receptor levels are higher in the lateral septum in monogamous species (Insel et al. 1994; Wang et al. 1996). This pattern is also observed in estrildid finches, where social species display higher levels of vasotocin (the avian homologue of vasopressin) receptor 1a binding than non-gregarious species in the lateral septum (Goodson et al. 2006).

Due to their involvement in social behaviors and specifically their role in reproductive behaviors and pair bonding in mammals, several regions in this network have been selected as the focus of these studies: ventromedial hypothalamus, preoptic area, amygdala, and bed nucleus of the stria terminalis.

Ventromedial hypothalamus

Function

The ventromedial hypothalamus has long been considered a critical region for the mediation of female receptive behavior in rodents and also a site for estrogen's action on this process (reviewed in Pfaff et al. 1994). Lesions here reduce the occurrence of lordosis (Pfaff and Sakuma 1979) and also the frequency of coital contact in female rats; possibly demonstrating reduced motivation for copulation (Emery and Moss 1984). In addition electrical stimulation of this region can facilitate lordosis in rats (Pfaff and Sakuma 1979). IEGs are induced in this region following vaginocervical stimulation in rats (see Pfaus and Heeb 1997). Additionally, the ventromedial hypothalamus plays a role in the mediation of food intake (see Bray and York 1979; Inoue and Bray 1979 for review) in mammals.

In birds, the ventromedial hypothalamus appears to mediate similar functions to mammals. Lesions to this region in ring-doves decrease the display of female courtship behaviors (Gibson and Cheng 1979). In female quail, interaction with a male induces IEG expression here, and this expression is positively correlated with the display of proceptive behaviors by the female (Meddle et al. 1999). In doves, exposure to male and female nest coos result in inhibitory activity in neurons in the ventromedial hypothalamus (Cheng et al. 1998), indicating that this region responds to auditory stimuli. In parallel, song stimuli induce IEG expression in this region in white-throated sparrows (Maney et al. 2008). Like mammals, the ventromedial hypothalamus is also implicated in mediation of food intake in bird species, as lesions here can result in hyperphagia, obesity, and an increase in insulin levels (reviewed in Kuenzel 1994).

Anatomy

Reciprocal connections exist between the ventromedial hypothalamus and the preoptic area, bed nucleus of the stria terminalis, amygdala, and lateral septum in rodents (Saper et al. 1976; Fahrbach et al. 1989; Canteras et al. 1994). Although no tract-tracing studies have been conducted from this region in birds, tracing studies from other regions have identified projections from the nucleus taeniae (Cheng et al. 1999), preoptic area (Berk and Butler 1981; Balthazart et al. 1994), auditory thalamus (Cheng and Peng 1997), and the septum (Montagnese et al. 2004) to this region. Studies concerning the neurochemical characteristics of this region are very limited in birds. However, examinations of the neural distribution of several neurotransmitters and neurochemicals have identified similarities between this region in mammals and birds. The avian ventromedial hypothalamus contains GABA (Domenici et al. 1988; Granda and Crossland 1989) and enkephalin positive neurons (Cheng and Zuo 1994); in mammals GABAergic neurons synapse with other GABAergic and enkephalin positive neurons in this nucleus (Commons et al. 1999; Commons and Pfaff 2001). In addition, glutamate (Cornil et al. 2000; Bian et al. 2008) and acetylcholine (Kow and Pfaff 1985; Watson et al. 1988) are utilized as neurotransmitters in this region in both mammals and birds. Although the functional, anatomical, and neurochemical research in this region is more limited in avian species, the available data indicates a strong similarity between the mammalian and avian ventromedial hypothalamus.

Nucleus taeniae

Function

The nucleus taeniae is an avian region with homology to the mammalian amygdala (Thompson et al. 1998; Cheng et al. 1999). In mammals, the amygdala has numerous functions involving emotional processing of sensory stimuli (reviewed in Phelps and LeDoux 2005), such as fear conditioning (reviewed in LeDoux 1998; 2003; 2007), determining stimulus-reward value (see Baxter and Murray 2002 for review), and the emotional modification of memory storage (see McGaugh et al. 1996 for review). IEGs are induced in this region following copulation or vaginocervical stimulation in rodents (Pfaus and Heeb 1997), but lesions to this region do not seem to affect sexual behavior in females (Masco and Carrer 1980). Given its importance in the processing of sensory information and emotional memory, it seems likely that this region may be critical for female neural and behavioral responses to social interactions.

Functional similarities exist between the avian nucleus taeniae and the mammalian amygdala. Cheng et al. (1999) delineated the behavioral effects of lesions to this region in female doves and starlings. Lesions increase the display of nest coos in ring doves (a female courtship behavior), possibly due to reduced fear of the aggressive behavior the male directs toward the female in response to her cooing. In starlings, lesions result in reduced social feeding, and also increased perch changes and decreased time spent in proximity to other cage-mates. These behavioral effects may be due to the cage-mate's unfavorable response to decreased social inhibition of the lesioned females. In addition, IEG expression has been detected in this region after exposure to male song stimuli in white-throated sparrows (Maney et al. 2008). It appears, through these studies, that the nucleus taeniae plays a role in the mediation of social behavior and responses to social stimuli in both mammals and birds.

Anatomy

The mammalian amygdala receives inputs from sensory systems (including thalamic and cortical components; Scalia and Winans 1975; Ottersen 1981; Canteras et al. 1994), the hypothalamus, the bed nucleus of the stria terminalis, and the ventral tegmental area/ substantia nigra (Ottersen 1980; 1981), sends outputs to several hypothalamic structures (Caffe et al. 1987; Canteras et al. 1994; Bian et al. 2008), as well as the bed nucleus of the stria terminalis (Caffe et al. 1987; Canteras et al. 1994; Dong et al. 2001), prefrontal cortex (Canteras et al. 1994), striatum (Canteras et al. 1994), nucleus accumbens (Canteras et al. 1994) and hippocampus (Caffe et al. 1987). The nucleus taeniae in birds also has reciprocal connections with the ventromedial hypothalamus, preoptic area, and bed nucleus of the stria terminalis (Cheng et al. 1999), striatum, caudolateral neostriatum (homologous to prefrontal cortex; Reiner et al. 2004a), and hippocampus (Cheng et al. 1999). The avian nucleus taeniae also appears to receive input from the caudomedial mesopallium (Cheng et al. 1999).

In rats, this region has a large amount of vasopressin innervation (DeVries et al. 1985; Sofroniew 1985), with vasopressin fibers that project to the lateral septum and hippocampus (Caffe et al. 1987). This innervation is also observed in birds (Voorhuis and de Kloet 1992; Aste et al. 1996). In addition, GABA (Wong and Moss 1992), dopamine (Young and Rees 1998), serotonin (Moore et al. 1978), and glutamate (Bian et al. 2008) act as neurotransmitters in this region in mammals. Similarly in birds, GAD-65 (a marker for GABA) is present in the nucleus (Yamamoto et al. 2005), and staining for tyrosine hydroxylase (Bottjer 1993; Appeltants et al. 2001; Roberts et al. 2002) and DARPP-32 (Roberts et al. 2002) occurs in this region, indicating dopaminergic

innervation. Serotonin innervation is also present in the nucleus taeniae (Cozzi et al. 1991; Metzger et al. 2002). Calcitonin gene-related peptide (CGRP) is also present in the amygdala in rodents (Brauth and Reiner 1991; Yasui et al. 1991) and birds (Brauth and Reiner 1991; Lanuza et al. 2000). In sum, the nucleus taeniae has similar neural connections, neurochemistry, and also appears to serve a similar function (processing the emotional context of social stimuli) as the mammalian medial amygdala.

Bed nucleus of the stria terminalis

Function

The bed nucleus of the stria terminalis is implicated in the mediation of social behaviors including reproduction in mammals. IEG expression is increased in this region during copulation or vaginocervical stimulation (reviewed in Pfaus and Heeb 1997). As IEG expression is observed here after females are exposed to conditions that promote the formation of a pair bond in female prairie voles (Curtis and Wang 2003; Cushing et al. 2003), the bed nucleus of the stria terminalis appears to play a role in pair bonding. Additionally, this region contains vasopressin innervation (see de Vries and Miller 1998; De Vries and Panzica 2006 for review).

Avian studies of the bed nucleus of the stria terminalis have been primarily anatomical, although some functional studies indicate that IEG expression is observed in this region in females following interaction with a male in quail (Meddle et al. 1999). The bed nucleus of the stria terminalis may play a role in the mediation of male reproductive and courtship behaviors in chickens (reviewed in Jurkevich and Grossmann 2003). Sexually motivated singing results in induction of IEG expression in this region

in male starlings (Heimovics and Riters 2006). IEG expression is also observed in this region following exposure to song stimuli in white-throated sparrows (Maney et al. 2008).

Anatomy

The rat bed nucleus of the stria terminalis receives inputs from vomeronasal system (Scalia and Winans 1975), hippocampus (Weller and Smith 1982), and dorsal raphe nucleus (Eiden et al. 1985). It also has reciprocal connections with the ventromedial hypothalamus and medial amygdala (Weller and Smith 1982; Gu et al. 2003; Poulin et al. 2006), and projects to the lateral septum and preoptic area in rats (Gu et al. 2003). A connection to the nucleus taeniae has been identified from the bed nucleus of the stria terminalis in birds (Cheng et al. 1999); however, other tract tracing studies have not been conducted from this region in avian species.

Vasopressin innervation is observed in this region in mammals and birds (rats, (DeVries et al. 1985; Sofroniew 1985; Veinante and Freund-Mercier 1997); quail, (Aste et al. 1998; Panzica et al. 1999; Panzica et al. 2001). In addition, this innervation is sexually dimorphic with higher density in males than females in birds (see Grossmann et al. 2002 for review, Aste et al. 1998; Jurkevich et al. 1999), as is observed in mammals (reviewed in De Vries and Panzica 2006). Serotonin and norepinephrine fibers are present in this region in rats (Phelix et al. 1992; 1992) and birds (Cozzi et al. 1991; Medina and Reiner 1994; Mello et al. 1998). Aromatase immunoreactive neurons are also common in this region in birds and mammals (birds: Balthazart et al. 1990; Foidart et al. 1994; Aste et al. 1998, rat: Sanghera et al. 1991; Jakab et al. 1993).

Preoptic area

Function

The preoptic area is considered a primary neural site for the activation of male copulatory behaviors in rodents (reviewed in Meisel and Sachs 1994), and also an important target of the hormones that mediate these behaviors (see Hull and Dominguez 2007). However, the preoptic area may also be involved in the control of aspects of female rodent reproduction, especially female proceptive behavior (reviewed in Pfaff et al. 1994; Sakuma 1995). Lesions to this region demonstrate its role in the inhibition of the lordosis reflex (see Pfaff et al. 1994; Sakuma 1995 for review) and also result in increased display of female proceptive behaviors (Whitney 1986; Hoshina et al. 1994). This region may also be important for pair bonding in prairie voles, with IEG expression observed here under conditions when pair bonds are being formed (Curtis and Wang 2003; Cushing et al. 2003).

In birds, as in mammals, the focus of functional studies in this region has been on the mediation of male copulatory behavior. In parallel with rodent research, the avian preoptic area is the site of action of testosterone (Barfield 1969; Balthazart and Surlemont 1990), and lesions to this region reduce male copulatory behavior in quail (Balthazart et al. 2001). In addition to its role in male copulatory behavior, the preoptic area may also be critical to male courtship behavior in songbirds. The display of sexually motivated song results in the induction of IEGs in male starlings in the preoptic area (Riters et al. 2004; Heimovics and Riters 2005; 2006; 2007). In starlings, lesions to this nucleus result in reduced song production in the male (Alger and Riters 2006).

The role of this structure in mediating female behaviors is less clear in birds (see discussion in Gibson and Cheng 1979). Preoptic lesions reduce egg-laying and incubation behavior in turkeys (Youngren et al. 1989), but do not affect courtship behavior. This result indicates the preoptic area is involved in reproductive behavior in birds; however, its role in proceptive and receptive behaviors is unknown at this point. In addition, neurons in this region are responsive to auditory stimuli in ring-doves and white-throated sparrows (Cheng et al. 1998; Maney et al. 2008).

Anatomy

The neuroanatomical characteristics of the preoptic area in rodents have been well described, and avian studies indicate that many of these characteristics apply to avian species as well. The rat preoptic area has reciprocal connections with the amygdala, bed nucleus of the stria terminalis, ventromedial hypothalamus, lateral septum, and ventral tegmental area (Simerly and Swanson 1986; 1988). Afferents to the POA also exist from the main olfactory bulb (Price et al. 1991). The broad inputs and outputs of the preoptic area also exist in birds, with connections between the preoptic area and the nucleus taeniae, lateral septum, ventromedial hypothalamus, and the ventral tegmental area in birds (Berk and Butler 1981; Kitt and Brauth 1986; Balthazart et al. 1994; Cheng et al. 1999).

This region is characterized by vasopressin/vasotocin innervation in rodents (DeVries et al. 1985; Caffe et al. 1987; Veinante and Freund-Mercier 1997) and birds (Viglietti-Panzica et al. 1994; Kimura et al. 1999; Absil et al. 2002), and aromatase activity/immunoreactivity in both mammals (Wagner and Morrell 1996; 1997; Roselli et al. 1998) and birds (Balthazart et al. 1990; Balthazart et al. 1996; Foidart et al. 1995;

Shen et al. 1995; Saldanha et al. 2000). The neurotransmitters norepinephrine (rats, Simerly et al. 1986; Espana and Berridge 2006, birds; Mello et al. 1998; Cornil et al. 2004), dopamine (rats, Simerly et al. 1986, reviewed in Hull et al. 1999; Dominguez and Hull 2005, quail; Cornil et al. 2004) and serotonin (rats, Simerly et al. 1986, quail, Cozzi et al. 1991) are observed in this region in both groups. A wide variety of other neurochemicals are present in neurons in this region in both groups including enkephalin (rats, Simerly et al. 1986, chickens, Blahser and Dubois 1980; de Lanerolle et al. 1981), neurotensin (Simerly et al. 1986; Absil and Balthazart 1994), and Substance P (Simerly et al. 1986; Aste et al. 1995).

Estrogen and the Social Behavior Network

Neurons in all of these regions are sensitive to estrogen in both mammals and birds (*e.g.* Pfaff and Keiner 1973; Vito et al. 1983; Koch and Ehret 1989; Simerly et al. 1990; DonCarlos et al. 1991; Lauber et al. 1991; Shughrue et al. 1997). A large percentage of the neurons in the preoptic area, medial amygdala, bed nucleus of the stria terminalis, and the ventromedial hypothalamus that express IEGs following vaginocervical stimulation in the rat also contain estrogen receptors (Tetel et al. 1994). Estrogen implants into the ventromedial hypothalamus result in activation of lordosis in female rats (Rubin and Barfield 1980). Specifically, estrogen treatment alone has been shown to increase IEG expression in the ventromedial hypothalamus, preoptic area, and the amygdala in rodents (Insel 1990). IEG expression can also be affected by estrogen treatment after vaginocervical stimulation. Specifically, IEG expression decreases or remains the same with estrogen treatment in the ventromedial hypothalamus (Tetel et al.

1994; Pfaus et al. 1996), decreases in the bed nucleus of the stria terminalis, or increases in the amygdala (Pfaus et al. 1996).

Similar effects of estrogen may also be observed in birds, although they have not been extensively studied. IEG responses to song in female white-throated sparrows are increased by estrogen treatment in the ventromedial hypothalamus, nucleus taeniae, bed nucleus of the stria terminalis, and preoptic area (Maney et al. 2008).

Dopaminergic Systems

Function

Dopaminergic systems in the mammalian brain include those implicated in the mediation of motor movements (Rieke 1981; Sanberg and Mark 1983; Albin et al. 1989; Hauber 1998) and reward and motivation (reviewed in Schultz et al. 1997; Akins et al. 2004; Esch and Stefano 2004; Akins and Geary 2008) in mammals. Neurons projecting from the ventral tegmental area to the nucleus accumbens (mesolimbic dopaminergic neurons) are typically considered important for determining the reward value of stimuli (Schultz et al. 1997; Akins et al. 2004; Esch and Stefano 2004). Dopaminergic neurons are also a target for addictive drugs in mammals (see Bardo 1998 for review).

These dopaminergic neurons are also activated during natural processes such as reproductive behavior. IEGs are induced in the ventral tegmental area of female rodents following reproductive behavior and/or vaginocervical stimulation (reviewed in Pfaus and Heeb 1997). Dopamine is released in the nucleus accumbens when females are allowed to pace their mating (Mermelstein and Becker 1995; Pfaus et al. 1995; Becker et

al. 2001), as measured through microdialysis. This release also occurs when females are exposed to reproductive stimuli alone (Mitchell and Gratton 1994).

The mesolimbic dopamine reward system is also critical for the display of pair bonding behavior in prairie voles. Dopamine levels increase in the nucleus accumbens in response to mating in female prairie voles (Gingrich et al. 2000). Also, dopamine antagonists presented either systemically or directly into the nucleus accumbens block the formation of a pair bond following mating (Aragona et al. 2003). Pair bonds can be formed between a male and a female without mating by treating systemically or directly into the nucleus accumbens with a dopamine agonist (Aragona et al. 2003).

The function of dopaminergic neurons appears to be conserved in birds. In avian species, motor deficits are seen with disruption of the nigrostriatal dopamine system (Rieke 1981; Sanberg and Mark 1983). In addition, drugs of addiction affect behavior in birds as they do in rodents (Levens and Akins 2004; Geary and Akins 2007; Akins and Geary 2008). In songbirds, mesolimbic dopaminergic neurons are responsive during the display of sexually motivated song (Riters et al. 2004; Heimovics and Riters 2005). Dopamine is also released in the striatum when male zebra finches are singing directed song (Sasaki et al. 2006). As a result, dopaminergic activity may play a role in sexual motivation during courtship behavior in male birds.

Dopaminergic systems are also responsive to social stimuli in birds (song stimuli, as compared to olfactory stimuli in rodents; see (Mitchell and Gratton 1994). Phosphorylated tyrosine hydroxylase increases in the ventral tegmental area when female starlings are exposed to directed song (Riters et al. 2007). Also, dopamine is

released in the NCM following exposure to song stimuli in starlings (Sockman and Salvante 2008).

Anatomy

The anatomical and physiological characteristics of dopaminergic systems are highly similar in mammalian and avian species (reviewed in Durstewitz et al. 1999; Reiner et al. 2004a).

Brainstem

The majority of dopaminergic neurons originate in the brainstem in the substantia nigra and the ventral tegmental area in mammals (Lindvall and Bjorklund 1974; Swanson 1982; Loughlin and Fallon 1983). These neurons then send projections to the basal ganglia (including the striatum and nucleus accumbens), but also to the prefrontal cortex, hippocampus, amygdala, and septum (Lindvall and Bjorklund 1974; Fallon and Moore 1978; Swanson 1982). In birds, the ventral tegmental area and the substantia nigra are also the primary origin of the dopaminergic outputs in the brain, and dopamine neurons project from these regions to the medial striatum (Kitt and Brauth 1986; Szekely et al. 1994; Metzger et al. 1996; Mezey and Csillag 2002). The avian ventral tegmental area also sends afferents to the lateral septum, preoptic area, and nucleus taeniae (Kitt and Brauth 1986), while the substantia nigra is connected to the hippocampus (Kitt and Brauth 1986). Catecholaminergic cell bodies are observed in substantia nigra and ventral tegmental area in both mammals and birds (Bailhache and Balthazart 1993; Bottjer 1993; Appeltants et al. 2001).

Striatum

The neuroanatomical and neurochemical properties of the mammalian and avian striatum have been extensively studied. Dopaminergic neurons enter the striatum from the substantia nigra and ventral tegmental area in both mammals and birds (Lindvall and Bjorklund 1974; Metzger et al. 1996). An input to the striatum also exists from the cortex in both groups (Donoghue and Kitai 1981; Veenman et al. 1995), which is primarily glutamatergic (Hassler et al. 1982; Gerfen 1992). The striatum also receives innervation from the amygdala/nucleus taeniae (Canteras et al. 1995; Cheng et al. 1999). Interneurons in this region are cholinergic (Bolam et al. 1984; Kawaguchi et al. 1995), and GABAergic (Kawaguchi et al. 1995) in rodents. These neurotransmitters are also found in the medial striatum in birds (Csillag et al. 1997; Veenman 1997; Roberts et al. 2002; Mezey and Csillag 2003). Both mammals and birds also have efferent projections from the striatum that are GABAergic (Scheel-Kruger et al. 1981; Csillag et al. 1997; Mezey and Csillag 2003), and often contain Substance P, dynorphin or enkephalin (Reiner et al. 1984; Reiner 1986; Reiner and Anderson 1990; Anderson and Reiner 1991).

Nucleus Accumbens

Anatomical and neurochemical characteristics of the nucleus accumbens have been well established in rodents (see Shirayama and Chaki 2006; Zahm and Brog 1992). This nucleus receives inputs from the ventral tegmental area and substantia nigra, the prefrontal cortex, the hippocampus, and the amygdala in the rat (Phillipson and Griffiths 1985). This nucleus also sends projections to the ventral tegmental area and substantia nigra as well as the bed nucleus of the stria terminalis, the lateral hypothalamus, ventral pallidum, and the globus pallidus (Usuda et al. 1998). Fibers in the region contain

neurotransmitters such as acetylcholine (Meredith et al. 1993), norepinephrine (Delfs et al. 1998), GABA (Christie et al. 1987; Churchill and Kalivas 1994), and glutamate (Christie et al. 1987; Fuller et al. 1987), as in the striatum. The neurochemicals enkephalin, neurotensin, and Substance P are also observed in this nucleus, although in a regional distribution (Zahm and Brog 1992).

The nucleus accumbens has not been clearly defined in avian species (see Reiner et al. 2004a), although Balint and Csillag (2007) have proposed its location and subdivision in chicks. They observed similar cholinergic innervation, DARPP-32 and calbindin immunoreactivity (Balint and Csillag 2007).

Estrogen and dopaminergic systems

In mammals, estrogen treatment increases the release of dopamine in both the nigrostriatal (Becker and Beer 1986; Becker and Cha 1989) and mesolimbic dopamine systems (Saigusa et al. 1997; Thompson and Moss 1997). Estradiol treatment also has effects on other aspects of dopaminergic neurons (reviewed in Van Hartesveldt and Joyce 1986; Kuppers et al. 2000). For example, estrogen treatment increases the density of D1 dopamine receptors (Levesque and Di Paolo 1989), D2 dopamine receptor binding (Bazzett and Becker 1994), and dopamine synthesis in the striatum (Pasqualini et al. 1995).

Estrogen receptors are present in tyrosine hydroxylase positive neurons in canary ventral tegmental area and substantia nigra (Maney et al. 2001), indicating that they are sensitive to estrogen. Estradiol does affect the activity of some dopamine neurons in songbirds, as in mammals. In white-throated sparrows, estrogen treatment increases

tyrosine hydroxylase labeling in the ventral tegmental area (LeBlanc et al. 2007). In starlings, phosphorylated tyrosine hydroxylase in the ventral tegmental area is reduced by estradiol treatment (Riters et al. 2007).

Auditory Perception

Song is an important signal in social communication in zebra finches. Male zebra finches produce directed and undirected song (described in Zann 1996). Directed song is presented during courtship and is oriented toward a nearby female. This song presentation is typically accompanied by a specific posture that can vary in its degree of intensity; the male extends his head upward, and his feathers become erect on the head, as well as the cheeks and breast. Directed song is an integral part of male zebra finch courtship behavior, and females may use this signal as an indicator in mate choice. Males may also present song that is not oriented towards another bird (undirected song), which also occurs when the male is alone. This song may function to attract females, but is not used in courtship or when males are interacting with females.

Female behavioral responses to these song presentations vary, although the courtship sequence often involves a copulation solicitation display by the female in response to directed song. Tail quivers are the primary component of the copulatory solicitation display in this and other songbird species; this behavior often directly precedes a mount attempt or successful copulation by the male (Zann 1996). Female songbirds show preferences for their father's song (*e.g.* Miller 1979; Clayton 1988; Riebel et al. 2002) and can recognize the song of their mate (Vignal et al. 2008). Females can also discriminate between songs of varying levels of quality using

characteristics such as those described in the introduction (*i.e.* bout length, song complexity).

Auditory Perception Regions

Function

As song is such an important communication tool for males of this species, the neural systems involved in the perception of song are likely vital to females in a variety of social contexts. However, this perception is especially important during courtship displays in reproduction.

Song responses in avian species have been investigated using immediate early gene (IEG; reviewed in Clayton 1997; Ball and Gentner 1998) and electrophysiology (reviewed in Theunissen and Shaevitz 2006; Pinaud and Terleph 2008). These studies have identified the importance of the caudomedial nidopallium and the caudomedial mesopallium in perception of complex auditory stimuli (Mello et al. 1992; Mello and Clayton 1994; Grace et al. 2003; Terleph et al. 2006; George et al. 2008). In adult male zebra finches, the response is selective, with higher IEG expression in these regions following exposure to conspecific compared to heterospecific song (Mello et al. 1992; Mello and Clayton 1994). These results have been confirmed with lesion studies, in which lesions to these regions eliminated the ability of individuals to recognize specific song types (MacDougall-Shackleton et al. 1998; Gobes and Bolhuis 2007).

Male zebra finches learn song from their fathers by first creating a template of his song (Nordeen and Nordeen 1992; Kroodsma and Miller 1996; Marler 1997; Brainard and Doupe 2000); research in male song perception has largely focused on the

role of auditory perception during development (when males are forming this template). Males at 30 days post-hatch, when the song template is being formed (Nordeen and Nordeen 1992; Brainard and Doupe 2000), show an increased ZENK response in the NCM and CMM to conspecific song (Bailey and Wade 2003). Female juvenile zebra finches, however, display increased c-fos expression in the NCM and CMM at day 30 to conspecific song (Bailey and Wade 2003). At day 45, after template formation, greater FOS and ZENK responses are observed in males and females following exposure to conspecific compared to heterospecific song in the NCM (Bailey and Wade 2005). Males and females at this age also present increased ZENK responses to song of high quality (from males who had tutors during development compared to those who did not; Tomaszycki et al. 2006). Thus, selective IEG responses in the auditory perception sites to song begin to develop while males are learning song from a tutor, and females appear to develop a selective response to song at this time as well.

Little is known about the neural response to song in female juveniles, and even less is known about whether or not adult female zebra finches use the auditory perception system to detect differences in song quality. Since females are required to make these types of assessments to make mate choice decisions, it is crucial to examine this process. Adult female zebra finches have an increased FOS response to conspecific compared to heterospecific song in auditory perception regions (Bailey et al. 2002), but whether or not they can detect critical differences in the quality of male zebra finch song is unknown. In other songbird species, higher IEG expression is observed in auditory perception sites in females following exposure to high compared to low quality song or

silence (Gentner et al. 2001; Maney et al. 2003; Leitner et al. 2005; Sockman et al. 2005; Terpstra et al. 2006).

In addition, the neural response to song can be affected by the context in which it is presented. For example, a new environment or added visual stimuli can eliminate the habituation that occurs when exposure to a song is repeated (Kruse et al. 2004). IEG expression can also be induced in the auditory perception regions with visual courtship stimuli alone (Avey et al. 2005). In starlings, photostimulation to bring females to an active reproductive state can increase the selectivity of the IEG response in NCM (Sockman and Ball 2009). Thus, these regions are responsive to auditory stimuli, but also take in information about the environment in which they are perceived including both internal and external cues.

Anatomy

These auditory perception regions are considered comparable to the mammalian auditory cortex (see Reiner et al. 2004a,b), which are responsive to auditory stimuli (Merzenich et al. 1975). The neural pathway for auditory processing in songbirds has largely been described through tract tracing studies. The auditory thalamus sends projections to Field L (an avian auditory region within the nidopallium) and to a lesser degree, the NCM and CMM (see Durand et al. 1992; Vates et al. 1996). The NCM and CMM are reciprocally connected (Vates et al. 1996), and along with the mammalian auditory cortex are connected to auditory thalamic regions (Huang and Winer 2000). In addition, morphological features of the neurons within the avian auditory perception regions in birds are highly comparable to the mammalian auditory cortex (Saini and

Leppelsack 1981). Neurons responsive to auditory stimuli are located within Layer IV of the auditory cortex in mammals, and the avian auditory perception regions contain microneurons that are similar to stellate calls within layer IV (Saini and Leppelsack 1981). Many neurons within the mammalian auditory cortex are also GABA-immunoreactive (Prieto et al. 1994), as are neurons in the NCM and CMM (Pinaud and Mello 2007).

Estrogen and the auditory perception system

Estrogen may act in these regions to mediate song responses. In white-throated sparrows estrogen treatment reduces IEG expression in the NCM and CMM (although only in response to tones; (Maney et al. 2006). Estrogen receptors are present along the caudal edge of the NCM and in the CMM. However, estrogen's action in these regions may occur through projections to this system from other estrogen sensitive regions. The auditory thalamus sends axons to estrogen sensitive regions such as the ventromedial hypothalamus and the nucleus taeniae (Durand et al. 1992). Sparse projections exist from the nucleus taeniae to the most lateral portion of the CMM, but apparently not the NCM (Cheng et al. 1999). It is unknown at this time whether the ventromedial hypothalamus is directly connected with these auditory regions.

Conclusions and Hypotheses

In sum, the aforementioned regions in the avian social behavior network, dopaminergic, auditory perception systems are highly comparable both in function and anatomy to those in mammals. Therefore, we can utilize the zebra finch, which is ideal

for investigating questions about social behavior, to examine the role of these three systems in mediating female behavioral responses to social stimuli. Additionally, zebra finches are opportunistic breeders, providing the opportunity to examine the hormonal mediation of behavior in a unique model system. As has been discussed, the three systems are interconnected and also estrogen sensitive, allowing us to determine how these systems work together along with estrogen when females are interpreting social stimuli.

In the following experiments, I will test two hypotheses. First, early detection of male quality is determined by a varying neural response within the auditory perception sites, the social behavior network, and the reward system to male song (the primary form of communication from males to females). Second, once a female has made a mate choice decision, her altered behavioral response to her mate is due to a change in neural responses to males after a pair bond is formed. By investigating these systems simultaneously, the role of each system and/or their interaction with one another in the mediation of female zebra finch social behavior can be elucidated. To do this, I examined behavioral and neural responses to male zebra finch song, a simple and easily controlled social stimulus, and then I utilized a more complex and natural social interaction and examined the role of pair bonding in social responses.

Table 1. Black letters indicate that the characteristic is observed in mammals and birds. Green letters indicate it is known to exist in mammals, and blue letters, indicate it is known to exist in birds. Abbreviations: Preoptic area (POA), VMH (ventromedial hypothalamus), medial amygdala (MeA), nucleus taeniae (TnA), bed nucleus of the stria terminalis (BST), lateral septum (LS), ventral tegmental area (VTA), striatum (ST), substantia nigra (SN), nucleus accumbens -rodent (NAcc), nucleus accumbens - bird (Ac), prefrontal cortex (PFC), main olfactory bulb (MOB), accessory olfactory bulb (AOB), globus pallidus (GP), ventral pallidum (VP), caudolateral nidopallium (NCL), caudomedial nidopallium (NCM), caudomedial mesopallium (CMM), hippocampus (HP), norepinephrine (NE), dopamine (DA), serotonin (5-HT), acetylcholine (Ach), glutamate (Glu), vasopressin (VP), vasotocin (VT), enkephalin (ENK), neurotensin (NT), substance P (SP), neuropeptide Y (NPY), dynorphin (DYN), Calbindin (CALB), estrogen receptor (ER), aromatase (ARO) *Images in this dissertation are presented in color.

Table 1. Summary of the functional, anatomical, and neurochemical characteristics of mammalian and analogous/homologous avian brain regions in the social behavior network examined in this dissertation.

Region	Function	Connections	Neurotransmitters	Neuropeptides	Other
VMH	Female courtship behavior, food intake, auditory perception,	POA, MEA/TnA, BST, septum, VTA, SN, auditory thalamus	GABA, Glu, ACh, NE, DA, 5-HT	ENK	ER
POA	Male copulatory behavior, female proceptive behavior, sexually motivated song production, auditory perception	VMH, MeA/TnA, BST, LS, VTA, MOB	GABA, NE, DA, 5-HT	VP/ VT, ENK, NT, SP, NPY, CGRP	ER, ARO
MEA/ TnA	Emotional processing of stimuli (stimulus-reward, fear conditioning, emotional modification of memory storage), pair bonding, Social inhibition, auditory perception	VMH, POA, BST, LS, ST, VTA, SN, Nacc, HP, PFC/NCL, AOB, CMM	GABA, Glu, DA, 5-HT	VP/ VT, ENK, CGRP	ER, ARO
BST	Female reproductive responses, pair bonding, social behavior, male reproductive behavior, sexually motivated song, auditory perception	MEA/TnA, VMH, POA, MeA, LS, AOB, Dorsal Raphe	Glu, NE, 5-HT	VP/ VT	ER, ARO

Table 2. Summary of the functional, anatomical, and neurochemical characteristics of mammalian and analogous/homologous avian brain regions (dopaminergic and auditory perception) examined in this dissertation.

Region	Function	Connections	Neurotransmitters	Neuropeptides	Other
MST	Motor control, Addiction, song production	MeA/TnA, VTA, SN, Cortex	GABA, Glu, ACh, DA	ENK, SP, DYN	
Nacc/Ac	Addiction, reward responses, reproductive behavior, response to olfactory stimuli, pair bonding	MeA, VTA, SN, GP, VP	GABA, Glu, ACh, NE, DA, 5-HT	ENK, NT, SP, CALB	
VTA	Addiction, reward responses, reproductive behavior, pair bonding, Auditory perception, song production	VMH, POA, MeA/TnA, LS, ST, Nacc, PFC	GABA, DA, NE	ENK, SP	ER
Auditory Cortex/ NCM/CMM	Auditory perception	MeA/TnA, ST, Auditory thalamus	GABA, ACh, NE, DA		

Black letters indicate that the characteristic is observed in mammals and birds. Green letters indicate it is known to exist in mammals, and blue letters, indicate it is known to exist in birds. Abbreviations: Preoptic area (POA), VMH (ventromedial hypothalamus), medial amygdala (MeA), nucleus taeniae (TnA), bed nucleus of the stria terminalis (BST), lateral septum (LS), ventral tegmental area (VTA), striatum (ST), substantia nigra (SN), nucleus accumbens -rodent (NAcc), nucleus accumbens - bird (Ac), prefrontal cortex (PFC), main olfactory bulb (MOB), accessory olfactory bulb (AOB), globus pallidus (GP), ventral pallidum (VP), caudolateral nidopallium (NCL), caudomedial nidopallium (NCM), caudomedial mesopallium (CMM), hippocampus (HP), norepinephrine (NE), dopamine (DA), serotonin (5-HT), acetylcholine (Ach), glutamate (Glu), vasopressin (VP), vasotocin (VT), enkephalin (ENK), neurotensin (NT), substance P (SP), neuropeptide Y (NPY), dynorphin (DYN), Calbindin (CALB), estrogen receptor (ER), aromatase (ARO)

CHAPTER 2: ESTRADIOL INDUCES REGION-SPECIFIC INHIBITION OF ZENK BUT DOES NOT AFFECT THE BEHAVIORAL PREFERENCE FOR TUTORED SON IN ADULT FEMALE ZEBRA FINCHES.

Abstract

Female zebra finches display a preference for songs of males raised with tutors compared to those from males without tutors. To determine how this behavioral preference may be mediated by auditory perception sites, the social behavior network, and the dopamine reward system, and whether responses of these regions are affected by estradiol, females were treated with hormone or blank implants. An auditory choice test was conducted followed by exposure to tutored or untutored song or silence to examine induction of the immediate early gene, ZENK. Birds spent significantly more time near tutored than untutored song, regardless of estrogen treatment, and estradiol significantly decreased the density of ZENK immunoreactive neurons within the ventromedial hypothalamus. These results suggest that selective neural and behavioral responses can be induced by both high quality vocalizations and estradiol, although they are not necessarily correlated.

Introduction

Song is an important signal for reproductive interactions between zebra finches (Zann 1996); females use male songs to make mate choice decisions. Females are less likely to pair with males that have been surgically altered to produce low quality song or have a lower song output (Tomaszycki and Adkins-Regan 2005). They also show preferences for particular song types, often indicated by increased time spent near a vocalization (Neubauer 1999; Lauay et al. 2004; Nolan and Hill 2004), and/or with copulatory solicitation displays (Searcy and Marler 1981; Ballentine et al. 2004). In the zebra finch, females prefer the songs of males that were raised with a male tutor and produce a normal song, compared to those from males reared without tutors (Lauay et al. 2004), which are similar in pattern and duration to tutored song but contain fewer notes and more variation in frequency, primarily due to reduced infusion of call notes with stable frequencies into the song bout (Price 1979).

This preference may be the result of a selective response to songs of higher quality in a number of neural systems. For example, immediate early genes are induced within auditory perception regions including the caudomedial nidopallium (NCM) and the caudomedial mesopallium (CMM) after exposure to song (Mello et al. 1992; Mello and Clayton 1994). In juveniles, increased immediate early gene expression is observed in these regions following exposure to conspecific versus heterospecific song (Bailey and Wade 2003; Bailey and Wade 2005) and tutored versus untutored song (Tomaszycki et al. 2006). Adult females also display an increased density of immediate early gene immunoreactive nuclei with conspecific song versus heterospecific song (Bailey et al. 2002), but responses to varying qualities of zebra finch song have not been examined in

adult females. In other songbird species, however, immediate early gene expression is induced in auditory perception regions by high quality songs that elicit a behavioral preference in females (Gentner et al. 2001; Maney et al. 2003; Leitner et al. 2005; Sockman et al. 2005).

Brain regions outside the auditory perception system, especially those important for social behavior, are likely also affected by male song. Newman (1999) describes a network of interconnected nuclei (including the medial amygdala, bed nucleus of the stria terminalis, lateral septum, midbrain, ventromedial hypothalamus, anterior hypothalamus, and medial preoptic area) that is responsive to social stimuli in a number of species (also reviewed by Goodson et al. 2005 and discussed in Maney et al. 2008). Under the influence of gonadal steroids, these areas mediate multiple social behaviors, including reproduction and aggression (Newman 1999). The response of these regions to male song stimuli has not been investigated in female zebra finches, but some areas, including the ventromedial hypothalamus and nucleus taeniae (which shares homology with a portion of the mammalian amygdale, Cheng et al. 1999), have been implicated in the control of social behaviors in ring doves (Gibson and Cheng 1979; Cheng et al. 1999) and starlings (Gibson and Cheng 1979; Cheng et al. 1999). In addition, in other avian species some regions within this network are responsive to auditory stimuli (Cheng and Peng 1997; Cheng et al. 1998), including song (Maney et al. 2008).

The mesolimbic dopamine reward system may also play a role in the response of female zebra finches to male song. It is conserved among mammals and birds (Durstewitz et al. 1999), and is activated following exposure to rewarding stimuli. For example, in female rodents, dopamine is released in this network following reproductive

interactions with males (Mermelstein and Becker 1995; Pfaus et al. 1995) and exposure to male odors (Mitchell and Gratton 1994). In birds, when male starlings are producing sexually motivated song, increased immediate early gene expression is observed within the ventral tegmental area (Riters et al. 2004; Heimovics and Riters 2006). One study in female starlings, however, indicates that the dopaminergic activity may have an inhibitory effect, which is related to breeding condition (Riters et al. 2007). Breeding females display decreased phosphorylated tyrosine hydroxylase immunoreactivity in the ventromedial hypothalamus and lateral septum when they are exposed to song compared to females exposed to no song. In contrast, non-breeding females displayed the opposite pattern, with an increased immunoreactivity in these areas in response to song.

It seems likely that estradiol acts in some or all of these neural systems to facilitate a reproductive response. All brain areas in the social behavior network express estrogen receptors in zebra finches (Gahr et al. 1993; Jacobs et al. 1996; Gahr and Metzdorf 1997). They are also found in parts of the ventral tegmental area (Maney et al. 2001) and in auditory perception regions (Gahr et al. 1993; Jacobs et al. 1996; Gahr and Metzdorf 1997). In addition, high estradiol levels correlate with periods of reproductive behavior such as nest building, egg laying, and incubation in female birds (Korenbrot et al. 1974; Sockman and Schwabl 1999). In many song response studies, females are treated with estradiol to facilitate reproductive behaviors (Searcy and Marler 1981; Clayton and Prove 1989; Searcy and Capp 1997; Maney et al. 2003; Ballentine et al. 2004), but studies in canaries have indicated that this manipulation is unnecessary for the induction of copulatory solicitation displays in response to song (Nagle et al. 1993; Leboucher et al. 1994). Until now, the effect of estradiol on neural responses to song

has only been investigated in seasonal breeding birds; it increases immediate early gene expression in the auditory perception regions in white crowned sparrows (Maney et al. 2006). The influence of estradiol in neural systems in an opportunistic breeder such as the zebra finch is unknown. While hormones have not been measured across the reproductive cycle in zebra finches, presumably fluctuations in estrogen are similar to those in other avian species, which raises the question of how they alter the neural and behavioral responses of these females.

In sum, solid data from a variety of sources provide intriguing information on the neruroendocrine systems involved in perception of and responses to reproductively relevant auditory cues in female songbirds. A complete picture is still lacking, however. In an attempt to begin to synthesize and expand information about critical factors, we used a behavioral assay and immunohistochemistry for the immediate early gene ZENK in the same birds to simultaneously investigate potential roles of the auditory perception, social behavior and reward systems in the preference for tutored versus untutored song, as well as how the neural and behavioral responses may be mediated by estradiol.

Materials and Methods

Animals

Adult female zebra finches were taken from the breeding colony at Michigan State University. The birds were kept on a 12:12 light:dark cycle and provided seed and water *ad libitum*, with hard-boiled eggs mixed with bread and either spinach or orange given once a week. Females used in the study were raised in mixed sex breeding aviaries for at least 100 days, to ensure that they had reached sexual maturity and were

exposed to normal song during development. After this period, they were removed and housed in a single-sex aviary, which allowed auditory, but not visual or tactile contact with males, for at least two weeks prior to testing. All procedures were approved by the Michigan State University Animal Use and Care Committee and adhered to the guidelines of the National Institutes of Health.

Hormone treatment

Estradiol implants were produced by packing 2 mm of 17β-estradiol into 5 mm of Silastic tubing (i.d. 0.76, o.d. 1.65 mm) and sealing each end with silicone. Blank capsules were left unpacked. Females were anesthetized with isoflurane and either an estradiol or a blank capsule was inserted subcutaneously above the breast muscle. The incision was sealed with collodion adhesive, and the female was placed in an individual cage for 5 days to recover.

Song Stimuli

Recordings of tutored (males raised with other males present) and untutored (males isolated from adult males from post-hatch day 18 after hatching) zebra finch songs were received from Dr. Adkins-Regan at Cornell University (Lauay et al. 2004). Two types of sound files were produced. In one, both types of songs were used to produce ten different 20-minute sound clips for behavioral testing. Each clip was generated by combining one song of each type from the pool of six untutored and tutored males into a stereo file. Although there were large differences between the tutored and untutored songs, pairs were made of one tutored and one untutored song with similar phrase lengths and relatively high similarity scores (which indicate note type, spectral characteristics, duration, order, and time between notes; Tchernichovski et al. 1998),

determined by comparing phrases from each males song with the freeware Sound Analysis Pro (http:ofer.sci.ccny.cuny.edu/htm/body_sound_analysis.html). Each song was normalized for amplitude in Adobe Audition (Adobe Systems, Inc., San Jose, CA), and following the introductory notes, it was repeated for a total of 20 minutes. A file was then synthesized to simultaneously play a tutored song from one side of the testing chamber (see below) and an untutored one from the other side, counterbalanced across tests.

The other type of sound file was used for exposure prior to immediate early gene analysis. In this case, twelve 30-minute sound clips (six tutored and six untutored) were created in Adobe Audition. For each file, 30-second song clips from three different randomly chosen males within the same group (tutored or untutored) were played sequentially with 30 seconds of silence separating them. This compilation was repeated for a total of 30 minutes.

Choice Test

Five days after implant surgery, females were taken to a room containing a wood and plexiglass chamber of (215 cm L x 60 cm W x 60 cm H; Bailey 2006) modeled after Miller 1979). It consisted of three zones: the center (with one perch), and the left and right (with three perches each). The bird was placed into the cage through a center door and allowed to freely move between the zones. After 70 minutes for acclimation, a randomly chosen song file was broadcast from speakers, one located at each end of the chamber, for 20 minutes at approximately 60dB. Behaviors were videotaped and the time spent in each zone was quantified. In addition, two other behavioral measures (jumps between perches and calls) were taken. To ensure that the females adequately

received the song stimulus, animals that were completely unresponsive during the song presentation (*i.e.*, did not move or call) were not used (20 individuals across the groups were removed; leaving sample sizes of 20 estradiol-treated and 20 control birds).

Following song presentation, each female was captured and blood was collected by wing vein puncture, centrifuged at 10,000 rpm for 10 minutes at 4°C and plasma stored at -80°C until radioimmunoassay to quantify estradiol concentration. Females were returned to their individual cages and taken back to the colony room.

Behavioral data were compared using a mixed-model ANOVA (treatment between animals and time spent within each zone within animals). The proportion of perch jumps and flights in the tutored zone were separately analyzed between treatment groups using Mann-Whitney U tests, because some individuals did not display these behaviors and the data were not normally distributed. Statview (SAS Institute; Carey, NC) was used for all statistical analyses.

Immediate Early Gene Analysis

Two days after the choice test, the forty females were individually taken to a novel room for stimulus exposure to examine ZENK induction (Bailey et al. 2002, Bailey and Wade 2003; Bailey and Wade 2005). They remained in their individual cages during the test, which were placed within a sound-isolated box. After a 30-minute acclimation period (as in Bailey et al. 2002), a randomly chosen 30-minute file of tutored or untutored song or silence was broadcast from a speaker three inches from the cage at 60dB. Each group contained 6-8 individuals. In a few cases, a brain region could not be quantified due to histological artifact; final sample sizes are indicated in the

figures and table. To reduce habituation (Chew et al. 1995), no female was exposed to song from the same male in the choice test and stimulus exposure for ZENK induction.

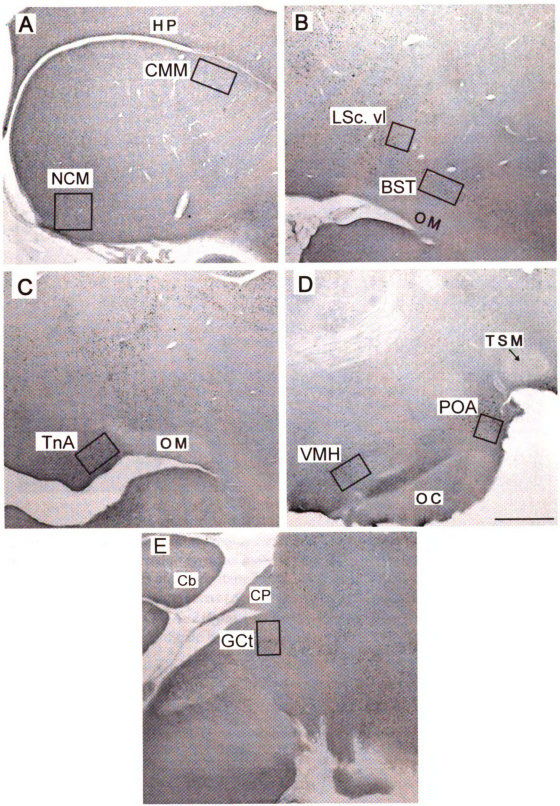
The song exposure was videotaped, and calls and receptive behaviors were later quantified. Following song presentation, the female remained in silence for 1 hour before being overdosed with 0.12 cc of equithesin and perfusion with 0.1 M phosphate buffered saline (PBS) and 4% paraformaldehyde. Brains were dissected from the skull and postfixed for 15 minutes in 4% paraformaldehyde. They were embedded in gelatin, fixed for another hour in 4% paraformaldehyde and placed in 30% sucrose in 0.1M PBS at -20°C until sectioning. The embedded tissue was sectioned frozen into four series at 30μm in the sagittal plane and stored in cryoprotectant at -20°C until immunohistochemical processing.

Immunohistochemistry was performed in a method adapted from Bailey et al. (2002) and Bailey and Wade (2003; 2005). Cryoprotected tissue was rinsed 6x5 minutes in PBS, reacted for 15 minutes in 0.5% hydrogen peroxide (H_2O_2) in PBS and blocked for 1 hour in 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS with 0.3% Triton-X-100 (PBST). The tissue was then incubated in primary antibody (Santa Cruz Biotech; catalog #sc-189, 0.1μg/ml) in PBST for two days at 4°C. It was rinsed 3x5 minutes in PBS and incubated for one hour in Biotin-SP conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories; 1:500 dilution) in PBST. After a 3x5 minute PBS rinse, tissue was exposed to an ABC reagent (Elite kit, Vector Laboratories, Burlingame, CA) for 1 hour. It was then rinsed 3x5 minutes in PBS and incubated in diaminobenzadine (Sigma, St.

Louis, MO; 0.5 mg/ml) plus 0.0075% H_2O_2 for 7 minutes. After PBS rinses, sections were mounted, dehydrated, cleared in xylene and coverslipped with DPX.

ZENK immunoreactivity in the auditory regions (NCM and CMM), social behavior areas (preoptic area [POA], ventromedial hypothalamus [VMH], bed nucleus of the stria terminalis [BST], nucleus taeniae [TnA], ventrolateral subdivision of the caudal lateral septum [LS c.vl.], and midbrain central gray [CGt]), and reward system (nucleus accumbens [Ac] and the ventral tegmental area [VTA]) was assessed using an Olympus BX60 microscope (Figures 1 and 2). The placement and size of the sampling regions, which is detailed in the figure captions, was determined after an initial scan of the tissue to locate the extent of labeling. Immunoreactive cells were hand-counted by an observer blind to treatment and auditory stimulus within the sampling regions. The NCM and CMM were analyzed within the same medial sections, which were identified by the connection of the nidopallium to the rest of the telencephalon and the presence of the Septopallio-mesencephalic tract (TSM). For the nucleus accumbens and ventral tegmental area, alternate sections in some animals were subjected to tyrosine hydroxylase immunohistochemistry to determine the correct placement of the sampling region (Figure 2). The methods were the same as those described for ZENK immunohistochemistry, but using tyrosine hydroxylase primary antibody (Immunostar; catalog # 22941, 1:10,000 dilution) and donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). As the precise border of the Ac is not completely distinguished, the box was placed in the ventromedial portion of the medial striatum in the most medial sections, as discussed in Reiner et al. (2004a).

Figure 1. Placement of sampling regions within auditory perception and social behavior areas in sagittal sections. Panel A contains boxes for the caudomedial nidopallium (NCM; 310μm x 320μm) and caudomedial mesopallium (CMM; 190μm x 345μm), panel B for the bed nucleus of the stria terminalis (BST; 190μm x 345μm) and ventrolateral subdivision of the caudal lateral septum (LSc. vl; 200μm x 200μm), panel C for the nucleus taeniae (TnA; 200μm x 320μm), panel D for the ventromedial hypothalamus (VMH; 190μm x 290μm) and preoptic area (POA; 200μm x 200μm), and panel E demonstrates for the midbrain central gray (GCt; 190μm x 290μm). The hippocampus (HP), tractus occipito-mesencephalicus (OM), tractus septopallio-mesencephalicus (TSM), optic chiasm (OC), posterior commisure (CP), and cerebellum (Cb) were used as landmarks for location of sections and placement of boxes. The more rostral portion of each photo is towards the right edge. Scale bar (in panel D) = 500μm.


A
HP
CMM
NCM
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BST
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TnA
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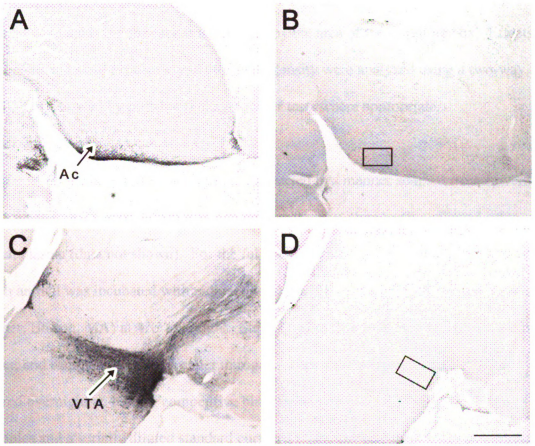

Figure 2: Placement of sampling regions within the reward system. Panels A and C contain sections subjected to tyrosine hydroxylase immunohistochemistry sliced in the sagittal plane. Panels B and D contain ZENK labeling. Panel B displays the location of the box for the nucleus accumbens (Ac; 190μm x 305μm), and panel D displays the ventral tegmental area (VTA; 210μm x 368μm). The more rostral portion of each photo is towards the right edge. Scale bar = 300μm.

The density of ZENK immunoreactive nuclei was determined by dividing the average number of immunoreactive cells for each animal (at least three sections per animal were analyzed for each brain region) by the area of the sampling box. Effects of treatment and song exposure type on ZENK density were analyzed using a two-way ANOVA followed by post-hoc Tukey-Kramer tests where appropriate.

Radioimmunoassay

A single radioimmunoassay was conducted in a manner adapted from Lovern and Wade (2003). Parallelism was first demonstrated with recently collected zebra finch plasma (data not shown). For the full assay, a mean of 31.4 μl of plasma from each animal was incubated with radioactive tracer [3H] estradiol (70Ci/mmol; Perkin Elmer, Boston, MA) at 4°C overnight. Steroids were then extracted twice with diethyl ether, and samples were dried under nitrogen. They were resuspended in PBS and stored overnight at 4°C. A competitive binding assay was completed in duplicate samples and a serially diluted standard curve (0.98 pg to 250 pg estradiol) in triplicate, by adding an estradiol antibody (NEG307H; Biogenesis, Kingston, NH) with [3H] estradiol and incubating overnight at 4°C. Water blanks were added as controls (n=4) and six aliquots of a known concentration of estradiol were used to determine intra-assay precision. The next day, dextran-coated charcoal was added and centrifuged at 2200 rpm for 10 min. at 4°C in order to remove unbound tracer. The remaining sample was combined with scintillation fluid (UltimaGold; Perkin and Elmer, Boston, MA) and analyzed with a scintillation counter (Beckman 6500, Fullerton, CA). Estradiol levels were calculated by standardizing samples for individual recovery and the volume assayed and compared to the standard curve. The intra-assay coefficient of variance

was 6%. A Mann-Whitney U test was used to compare the estradiol and blank treated groups.

Results

Behavior

Females spent significantly more time in the zone near the tutored song than that neighboring the untutored song or in the center ($F= 19.238$, $p< 0.01$;Tukey Kramer, both $p< 0.05$). However, estrogen did not affect this behavior ($F= 0.561$, $p= 0.56$), perch jumps (Mann-Whitney U, $p= 0.371$) or calls (Mann-Whitney U, $p= 0.112$; Figure 3).

During the ZENK induction, only one female displayed a tail quiver (associated with receptivity; Zann 1996), so these data were not analyzed statistically. No significant main effects of hormone, song type or interaction between them were detected in calling (data not shown).

ZENK expression

The clearest effect of estradiol was detected in the VMH. A main effect of treatment was observed, such that estradiol reduced the density of ZENK immunoreactive (IR) nuclei ($F= 8.44$, $p= 0.007$; Figure 4). No significant main effects of hormone or song type or interactions between them were observed in any of the other seven brain regions with this type of analysis (Table 3). However, the pattern observed in the auditory regions was striking. While the effect of stimulus exposure was not statistically significant (NCM: $F= 1.72$, $p= 0.194$; CMM: $F= 0.98$, $p=0.385$), the mean density of ZENK-IR nuclei in the NCM and CMM of the blank-treated birds that heard

tutored song was three times greater than those exposed to untutored song or silence (Figure 5). In fact, this increase was observed only in the one group, and a trend for estradiol treatment to reduce it existed in the CMM ($F= 3.61$, $p= 0.066$). A similar, albeit weaker, pattern was observed in the NCM ($F= 1.46$, $p= 0.236$).

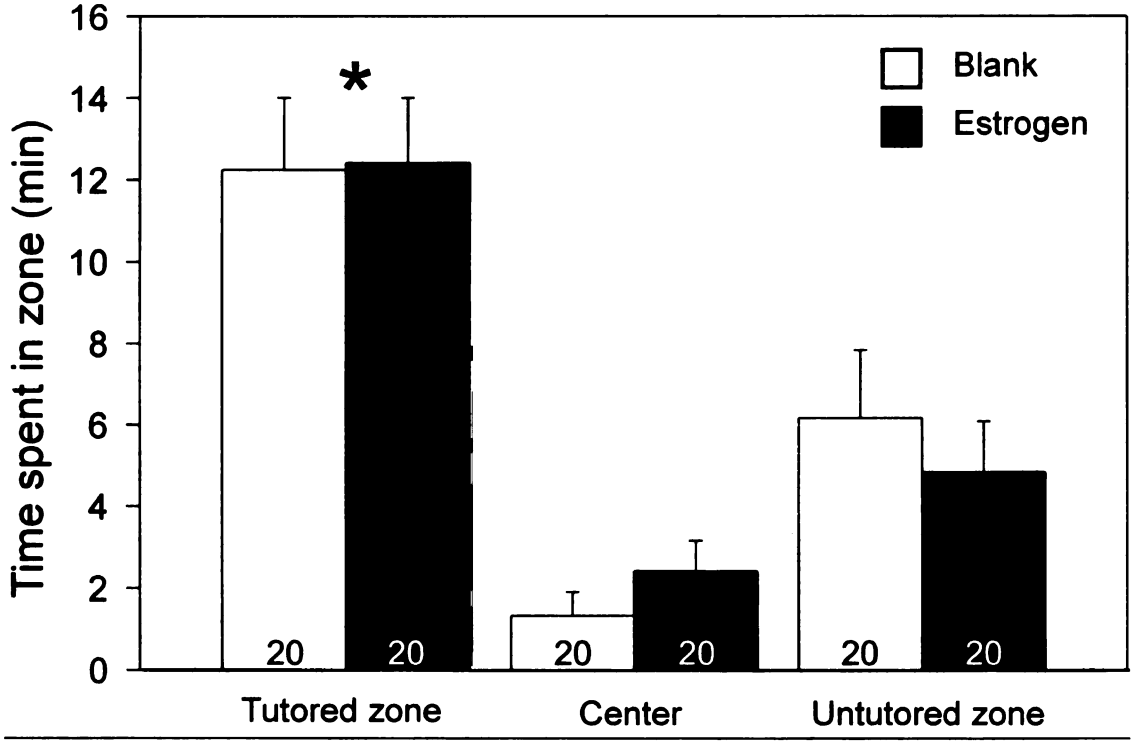

Figure 3. Total time spent within the tutored, center and untutored zones in the behavioral test. Sample sizes are indicated on the graph. * = tutored significantly greater than untutored and center zones.

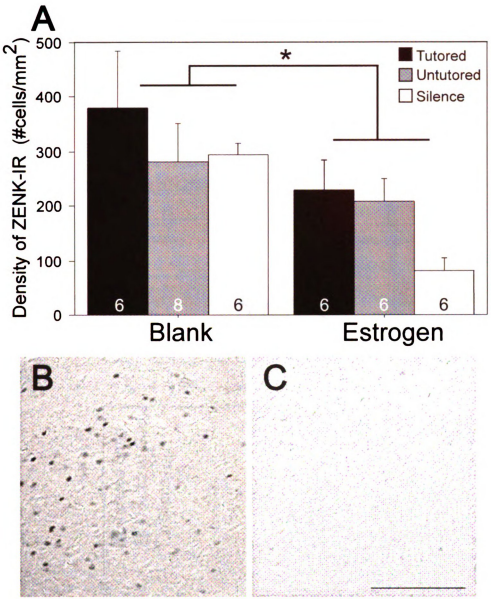

Figure 4. ZENK-IR nuclei in the ventromedial hypothalamus of female zebra finches. Panel A displays the quantification of densities of ZENK-IR for each treatment group and song exposure type. Sample sizes are indicated on the graph. A main effect of treatment was detected (*); estradiol decreased the expression of ZENK. Panels B and C depict ZENK in the ventromedial hypothalamus of birds exposed to tutored song. Panel B = blank treated female, Panel C = estrogen treated female. Scale bar = 100 μm

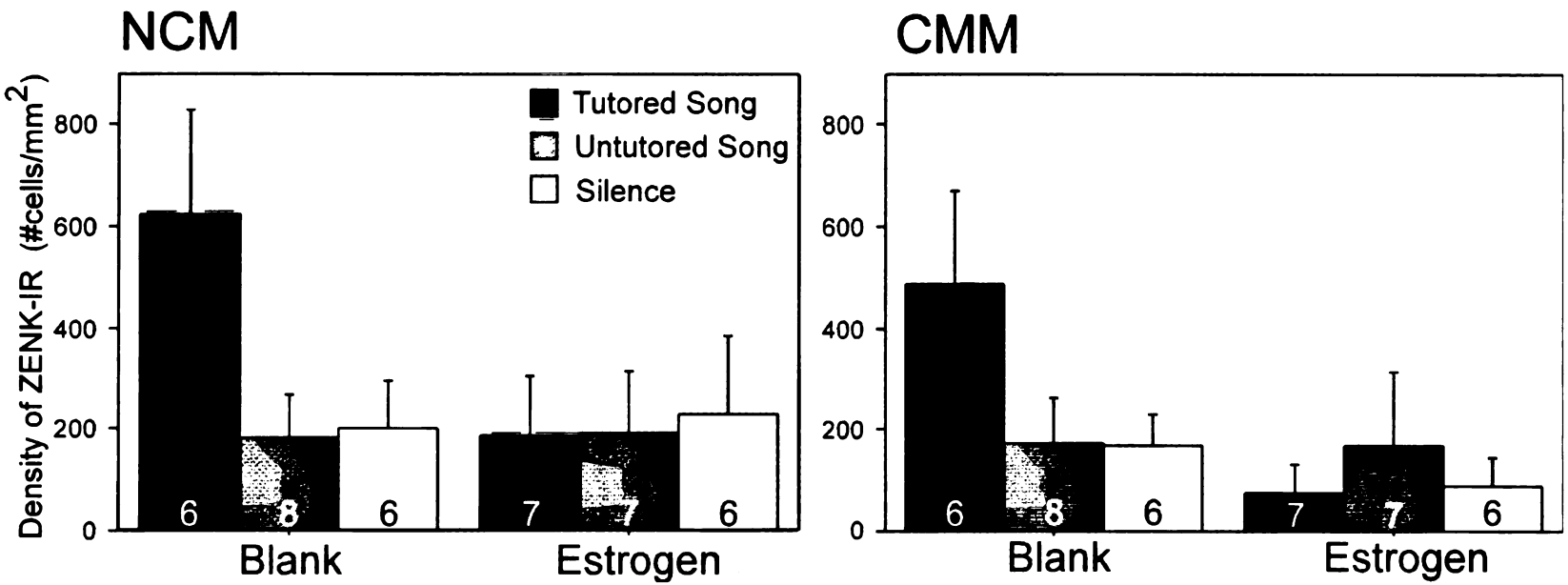

Figure 5. Densities of ZENK expression within the caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM) in female zebra finches. Sample sizes are indicated on the graphs

Table 3. Mean densities of ZENK-IR in various brain regions presented as number of IR nuclei/mm^2 (mean±standard error). Due to histological artifact, only 7 animals were analyzed in the Ac in the blank, untutored group. No main effects or interactions were detected, all $F < 3.02$, $p > 0.06$.

	Blank-Treated			**Estradiol-Treated**		
	Tutored (n=6)	**Untutored (n=7 or 8)**	**Silence (n=6)**	**Tutored (n= 6)**	**Untutored (n= 6)**	**Silence (n=6)**
POA	414.5±117	500.2±126	591.0±99	467.0±168	279.7±81	389.7±89
BST	1056.0±255	945.3±226	1106.5±254	494.8±187	935.6±338	694.4±352
TnA	125.0±73	67.3±26	30.5±11	52.8±23	91.9±46	216.4±215
LSc. vl	591.0±191	376.6±146	434.2±164	120.8±58	323.6±207	256.9±254
GCt	61.3±32	54.8±26	132.5±54	23.4±11	83.7±45	39.1±19
Ac	552.8±252	837.2±252	1286.7±224	888.4±280	1049.6±223	558.8±135
VTA	61.8±20	52.2±17	74.2±30	54.0±40	67.3±25	13.3±7

Abbreviations: preoptic area (POA), bed nucleus of the stria terminalis (BST), nucleus taeniae (TnA), ventrolateral subdivision of the caudal lateral septum (LSc. vl), GCt (midbrain central gray), nucleus accumbens (Ac), ventral tegmental area (VTA)

Plasma Estradiol

Estrogen treatment reliably increased circulating estradiol levels (Mann-Whitney U= 73, p= 0.002). Estradiol-treated birds had a mean of 5.7 ng/ml±1.3 S.E., while the value for control birds was 2.2 ng/ml±0.46 S.E. However, a number of the samples from blank-treated females fell below the range of the sensitivity of the assay, so to be conservative, the minimum detectable value (15.63 pg/sample; equivalent to an average of 1.84 ng/ml, depending on sample volume and recovery) was assigned.

Discussion

Behavioral Response

Female zebra finches spent more time near the tutored song than untutored song or silence. Lauay et al. (2004) also detected a similar propensity for increased time spent near tutored over untutored song in female zebra finches, although they did not examine the effects of estrogen or the neural response to these two types of song.

The tendency to spend increased time nearer this type of stimulus may be related to reproduction. Females prefer songs with characteristics reflecting increased male energy input (*i.e.*, long songs, increased song rate, and difficult syllables; Neubauer 1999; Ballentine et al. 2004; Nolan and Hill 2004). In addition, some of these characteristics correlate with high paternal effort (nestling feeding; Buchanan and Catchpole 2000; Dolby et al. 2005) and offspring survival (Hasselquist et al. 1996). Low song quality may also indicate developmental stress, and as a result, lower the fitness of a potential mate (Nowicki et al. 2002). It therefore seems reasonable to hypothesize that females spend more time near tutored song because it indicates a

higher quality mate than untutored song. It is possible, however, that the females simply find the untutored song aversive.

The widespread nature of song preference behavior might suggest that its function is broader than reproduction and mate choice. For example, *male* birds from a variety of species prefer song from conspecifics (Calhoun et al. 1993; Braaten and Reynolds 1999) and their own fathers (Clayton 1988; Riebel et al. 2002; Riebel and Smallegange 2003). In addition, juvenile zebra finches prefer song from their tutor (Houx and ten Cate 1999; 1999) prior to the period when they become reproductively active. The lack of an effect of estradiol on the behavioral response is also consistent with the idea that the tendency to spend time with a specific song type is dissociated from mate choice and reproduction in the female zebra finch. Alternatively, it is possible that the hormone is required to display this behavioral preference, and the concentration of circulating estradiol in control females was above the threshold (similar to untreated canaries; Nagle et al. 1993; Leboucher et al. 1994).

Neural Response

Auditory regions.

Statistically significant differences in ZENK expression due to auditory stimulus were not detected in the NCM or CMM. However, in control but not estradiol-treated animals, the average density of these cells was approximately three times greater in birds exposed to tutored song than the other two stimuli. The effect of estradiol on ZENK expression in these areas also did not reach statistical significance, and no interaction was detected between the hormone and auditory stimulus type. One

therefore needs to be very careful in suggesting conclusions about potential effects of the hormone. However, a number of factors suggest that potential biological relevance might be considered. For example, specific large mean differences in ZENK expression were seen in just the auditory areas of control birds exposed to high quality song compared to both low-quality song and silence (see Figure 5). This pattern parallels previous work in zebra finches in which immediate early gene expression in auditory regions was increased following exposure to stimuli of greater relevance (Mello et al. 1992; Bailey et al. 2002; Bailey and Wade 2003; Bailey and Wade 2005; Tomaszycki et al. 2006). If a mixed-model 3-way ANOVA is used to consider patterns across brain regions, a song x treatment x region interaction is detected. This interaction partly stems from the fact that NCM and CMM are the only regions to show this dramatic difference between tutored and untutored song and silence and that this effect is only observed in blank treated individuals. Thus, it is likely that estrogen inhibits this selective ZENK response in these regions. However, the lack of a significant main effect of song type in the auditory regions in the present study may be the result of the reduced requirement to detect variations in song in adulthood in this species. The other species in which this phenomenon has been investigated are primarily seasonal breeders, in which the females also sing (Baptista and Petrinovich 1986; Vallet et al. 1996; Pavlova et al. 2005) and/or male song is variable in adulthood (Smith et al. 1995; Riters et al. 2000; Leitner et al. 2001). Zebra finches, in contrast, are aseasonal, the females do not sing, and male zebra song is stable in adulthood (Zann 1996). A difference in ZENK response to tutored and untutored song was observed in *juvenile* female zebra finches (Tomaszycki et al. 2006). However, this plasticity may be lost

once females have formed a template and learned the basic characteristics of species-appropriate song.

From a more technical perspective, we cannot completely eliminate the possibility that the modest differences in exposure protocols during the behavior test (in which they were exposed to both song types simultaneously in a large choice cage) and ZENK induction (in which they were only exposed to one auditory stimulus in a sound isolated box) affected the animals somewhat differently, such that they showed a behavioral preference for tutored song, but not increased ZENK expression. However, in the ZENK induction test they remained within their home cage, which fit inside the sound-isolated box with the interest of reducing stress from a novel environment.

Regardless of the results in blank treated animals, it is clear that when our females were treated with estradiol, the response to all auditory stimuli was nearly identical. This result differs from the findings of Maney et al. (2006; 2008), in which estrogen treatment resulted in a greater immediate early gene response to conspecific song compared to tones. In the present study, however, we examined the difference in ZENK expression to variations in *quality of song*, whereas Maney et al. (2006; 2008) compared normal song to a tone stimulus. It is unknown how estradiol may mediate neural responses in auditory perception sites, but it is plausible that it acts on different signaling mechanisms. That is, separate pathways might (1) provide information that an auditory signal is an approximation of song, and (2) assess particular features (*i.e.*, relative quality) of the song. If so, it is also possible that estradiol facilitates a neural response in one case and inhibits it in the other. Some estrogen receptors are located along the caudal edge of the NCM, as well as within the CMM (Gahr et al. 1993; Jacobs

et al. 1996; Gahr and Metzdorf 1997), so regulation may occur directly at these sites or through connections with other brain regions. LeBlanc et al. (2007) observed an increase in density of tyrosine hydroxylase immunoreactivity in the NCM following estrogen treatment, and predicted that estradiol plays a neuromodulatory role on responses within the auditory perception regions. It will be important to further investigate all of these ideas in future experiments.

Social Behavior Network.

Although estradiol did not alter behavior in the present study, it did affect the neural response specifically in the VMH. This region contains a high concentration of estrogen receptors in a wide range of vertebrates, including rodents (*e.g.*, Pfaff and Keiner 1973; Vito et al. 1983; Koch and Ehret 1989; Simerly et al. 1990; DonCarlos et al. 1991; Lauber et al. 1991; Shughrue et al. 1997) and birds (Balthazart et al. 1989; Metzdorf et al. 1999; Halldin et al. 2006), including zebra finches (Gahr et al. 1993; Jacobs et al. 1996; Gahr and Metzdorf 1997). In addition, the VMH plays a crucial role in the control of reproduction in female rodents (Pfaff et al. 1994) and also appears to be important in birds (Gibson and Cheng 1979). However, rather than an increase in immediate early gene expression as has been observed in rodents following exposure to reproductive stimuli (Pfaus and Heeb 1997), we detected *less* ZENK-IR in the VMH with hormone administration. Treatment with estradiol alone can result in increases in immediate early gene expression (Cattaneo and Maggi 1990; Insel 1990), but similar to the present data, decreases have also been documented. For example, Tetel et al. (1994) and Pfaus et al. (1996) observed decreases in the induction of FOS-IR following small amounts of vaginocervical stimulation in the VMH when female rats were treated with

estradiol. In addition, a decrease in immediate early gene expression in the preoptic area and amygdala occurs following testosterone administration in male anole lizards (Neal and Wade 2007).

At least two potential explanations for this estradiol-induced decrease in immediate early gene expression exist. First, although these genes are often used to indicate regions that are "activated" by a certain stimulus, expression may also indicate stimulation of inhibitory neurons such as those expressing GABA. In male gerbils, a large proportion of cells expressing immediate early genes following mating are GABAergic (Simmons and Yahr 2003). Estradiol treatment can result in a decrease in bound $GABA_A$ receptor (O'Connor et al. 1988) and responsiveness of $GABA_B$ receptors (Lagrange et al. 1996) possibly resulting in disinhibition. Conversely, estrogen can cause an increase in GABA levels in the VMH of female rats (Luine et al. 1997). It is possible that estradiol reduces the activity of GABA neurons in the VMH in the present study, which is indicated by a decrease in ZENK expression. In zebra finches, song exposure results in GABA neurons expressing ZENK within the auditory perception regions (Pinaud et al. 2004; Pinaud and Mello 2007). As GABA neurons are present in female birds (Domenici et al. 1988; Granda and Crossland 1989), and can be regulated by social stimuli, as in rodents, it is possible that they may also be affected by estradiol in zebra finches. Second, it is possible that the decrease in ZENK expression observed in this study may be partially due to a decrease in activation of dopaminergic neurons in the VMH as described in Riters et al. (2007). In that study, decreased levels of phosphorylated tyrosine hydroxylase were observed following exposure to song in only breeding season female starlings. The authors hypothesize that these neurons may serve

an inhibitory function within the VMH during reproductive behavior (Riters et al. 2007).

Maney et al. (2008) conducted a study similar to ours in white-crowned sparrows; the response to song and how it is modulated by estradiol was examined in the social behavior network. In that study, increased ZENK responses were observed within the TnA, VMH, BST, and LSc. vl, and estrogen enhanced this response in all the examined social behavior network regions, except the medial VMH and the anterior medial hypothalamus. We did not observe changes in the responses of these regions to song stimuli, and we did not observe modulation of the response to song in these regions by estrogen. There are multiple potential explanations for these discrepancies. For example, housing conditions differed. Our animals were maintained in a colony room with hundreds of zebra finches, whereas the white crowned females were housed with several other females in sound-isolated booths for the extent of the study in Maney et al. (2008). Perhaps more important, however, are biological differences between zebra finches and white-crowned sparrows that may lead to differences in their responses within this network to song. Female zebra finches do not sing and adult males produce a simple and stable song in adulthood (Zann 1996). White-crowned sparrows, in contrast, have several song dialects (Marler and Tamura 1964) and females can also produce song (Kern and King 1972). It is possible that if female white-crowned sparrows produce song when hearing the song stimulus, it could affect the response of regions in the social behavior network. Some of these regions are activated in males when they sing (*i.e.* Heimovics and Riters 2006). In addition, differences in neural responses may reflect song complexity. For example, because male zebra finch

song is so simple and stable, and females do not sing, female zebra finches may not need to utilize these regions to interpret the meaning of the song stimulus, whereas birds such as white-crowned sparrows that have more variable song that can be used in more contexts might require additional regions for processing. Similarly, female zebra finches may require additional stimulation to activate these regions. Song might be interpreted by only the auditory perception regions, and other areas of the brain may only be activated when other stimuli are present, such as courtship displays (visual input) or physical bonding (tactile input). Differing effects of estrogen between the species might relate to varying levels of reliance on the hormone for reproduction in seasonal (white-crowned sparrows) and opportunistic (zebra finches) breeders. Determining which, if any, of these possibilities led to the differences in ZENK response between the present study and that of Maney et al. (2008) on white-crowned sparrows could lead to insights on the specific roles of these brain regions.

Dissociation between behavior and immediate early gene expression

Dissociations between behavior and ZENK expression were observed on two levels. First, although the females displayed a strong propensity to spend time near the tutored song, no differences in ZENK expression between auditory stimuli were observed in the social behavior network or reward system, and more work must be done on the auditory perception regions before solid conclusions can be drawn. Second, the reduction of ZENK expression by estrogen in the VMH with the lack of an effect of the hormone on behavior in the choice test suggests that an increased ZENK response within the VMH is not necessary for the display of differential behavioral responses to high quality song.

These data suggest a number of possibilities. For example, another immediate early gene may be involved in the regulation of the behavior. Consistent with that idea, some differences between the activities of FOS and ZENK have been detected in juvenile zebra finches (Bailey and Wade 2003), although similar patterns of induction of the two immediate early genes have also been observed following song presentation to adult female songbirds (Sockman et al. 2005; Velho et al. 2005). Additionally, several other immediate early genes (such as *c-jun*) exist that may also be important to the changes that occur during the display of this behavior. It is also possible that some other neural system (in addition to the three networks examined here) is vital to mediation of this behavior.

Conclusion and Future Directions

In summary, estrogen treatment does not affect the tendency to spend time near high quality, tutored song as measured in the present study, but it does suppress ZENK induction in the VMH. The present data suggest that the maintenance of this behavior does not require higher concentrations of estradiol and may not be associated with the ZENK expression detected within the examined brain regions. In addition, the fact that estrogen's inhibition of the neural response is relatively specific, and does not occur in most of the brain regions investigated, suggests that it serves some function, although the nature of it is not clear at this point. Determining the phenotype of the affected cells will help to elucidate their function(s).

Acknowledgements

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CHAPTER 3: ESTRADIOL AND SONG PRESENTATION MEDIATE BEHAVIOR OF FEMALE ZEBRA FINCHES INDEPENDENT OF DOPAMINE ACTIVITY IN THE NUCLEUS ACCUMBENS AND MEDIAL STRIATUM

Abstract

Female songbirds display preferences for certain song characteristics, but the neural and hormonal mechanisms mediating these preferences are not fully clear. The present study sought to further explore the role of estradiol, as well as assess potential roles of dopaminergic systems, on behavioral responses to song. Adult female zebra finches were treated with estradiol and exposed to tutored or untutored song or silence. Behavior was quantified and neurochemistry of the nucleus accumbens and striatum was examined with high performance liquid chromatography. As a control, the responses of these two systems to treatment with raclopride, a specific D2 receptor antagonist, were also evaluated. This manipulation did not affect dopamine (DA), but did increase DOPAC and the DOPAC/DA ratio. Estradiol reduced the display of two behaviors, long distance calls and visual scanning, but had no effect on dopaminergic responses. Auditory stimulus exposure affected other vocalizations, but song presentation did not modulate the levels of DA or its metabolite, DOPAC in the nucleus accumbens or striatum. Collectively, the results suggest that both estradiol and auditory stimuli can modify the behavioral responses of adult zebra finches, but they do so independent of changes in DA concentration and turnover. Thus, these two dopaminergic regions may not mediate the preference for high versus low quality song in this species.

Introduction

Female songbirds display preferences spending time near song with certain characteristics, and perhaps as a consequence, vary their behavioral responses to them. However, the neural mechanisms involved in these processes are not completely clear. Increased immediate early gene expression in response to male songs of higher quality are regularly seen in the auditory perception regions in a variety of songbird species (*e.g.* Gentner et al. 2001; Bailey et al. 2002; Maney et al. 2003; Leitner et al. 2005; Sockman et al. 2005; Tomaszycki et al. 2006; Svec and Wade 2009). Information on responses of other neural regions in songbirds is more limited and not as consistent. For example, results from the social behavior network (a group of interconnected nuclei that are implicated in the mediation of social behaviors and sensitive to steroid hormones (Newman 1999) differ across species. In white-throated sparrows, increased immediate early gene expression in response to song was observed in the nucleus taeniae, bed nucleus of the stria terminalis, lateral ventromedial hypothalamus, and lateral septum (Maney et al. 2008), whereas in zebra finches none of these regions were selectively responsive to high or low quality song (Svec and Wade 2009).

In the mesolimbic dopamine (DA) system, exposure to song does not induce immediate early gene expression in zebra finches (Svec and Wade 2009) or white-throated sparrows (LeBlanc et al. 2007; Maney et al. 2008), but these results do not eliminate the possibility that the regions respond to song stimuli. Quantifying the concentrations of DA and its metabolites might provide more direct assessments of their responses. This type of involvement is plausible, as song is a critical element of courtship (Zann 1996), and the mesolimbic reward system is activated in mammals

during reproduction in both sexes (reviewed in Mitchell and Gratton 1994; Melis and Argiolas 1995; Paredes and Agmo 2004), as well as in response to reproductive stimuli alone (Mitchell and Gratton 1994; Fabre-Nys et al. 1997).

The anatomical and physiological characteristics of the dopaminergic systems are highly similar in birds and mammals (reviewed in Durstewitz et al. 1999; Reiner et al. 2004a). In birds, dopaminergic neurons from the substantia nigra and ventral tegmental area project to the basal ganglia, including the medial striatum (nigrostriatal) and the nucleus accumbens (mesolimbic; Szekely et al. 1994; Metzger et al. 1996; Durstewitz et al. 1999). *In vitro*, neurons from the ventral tegmental area and substantia nigra have similar electrophysiological characteristics to mammalian dopaminergic neurons, and D2 receptor agonists block their activity (Gale and Perkel 2006). In addition, in chickens and zebra finches release of DA in striatal slices decreases with D2 receptor agonist treatment (Gale and Perkel 2005; Jackson et al. 2007). Few avian studies, however, have simultaneously examined the responses of the nigrostriatal and mesolimbic dopaminergic systems.

In addition to physiological characteristics, functional similarities exist in these systems between mammals and birds (see Reiner et al. 1998 for review). In both groups, the regions are important to the control of motor movements (see Rieke 1981; Sanberg and Mark 1983; Albin et al. 1989; Hauber 1998), as well as the mediation of reward (see Schultz et al. 1997; Akins et al. 2004; Esch and Stefano 2004; Akins and Geary 2008). In particular, these two systems are activated when males sing sexually motivated song (measured with immediate early gene expression in mesolimbic reward

regions; Riters et al. 2004; Heimovics and Riters 2005) and microdialysis in the striatum (Sasaki et al. 2006).

The response of neurons in the mesolimbic DA system to song exposure has only been examined using immediate early genes, which are either expressed to a greater degree in response to song (Maney et al. 2008) or are unchanged (LeBlanc et al. 2007; Svec and Wade 2009) in these regions. In components of the social behavior network, Riters et al. (2007) observed increased phosphorylated tyrosine hydroxylase (the rate limiting enzyme in DA synthesis) in response to male starling song. Also, DA release is increased in the auditory perception system (caudomedial nidopallium) in female starlings exposed to song (Sockman and Salvante 2008). It remains to be seen if exposure to song stimuli results in increased dopaminergic activity in the striatum and nucleus accumbens of female songbirds.

Although song is a critical component of reproduction, and estrogen is often used to prime females in song response studies (*i.e.* Searcy and Marler 1981; Clayton and Prove 1989; Searcy and Capp 1997; Maney et al. 2003; Ballentine et al. 2004), the role of steroid hormones in neural responses to song has only recently been evaluated. The effects are not completely consistent (see LeBlanc et al. 2007; Riters et al. 2007; Maney et al. 2008; Svec and Wade 2009). Zebra finch females present more distance calls in response to complex song versus other song types when they are estrogen-treated, but not control-treated (Vyas et al. 2008; 2009). In our previous study in zebra finches (Svec and Wade 2009), estradiol reduced expression of the immediate early gene ZENK in the ventromedial hypothalamus but did not affect neural responses to song, behavioral preference for tutored versus untutored male song, or general calling

behavior in response to these auditory stimuli. In contrast, a selective increase in ZENK expression in response to male song was observed following estrogen treatment in several regions in the social behavior network in white-throated sparrows (Maney et al. 2008).

Estrogen may also affect the response of dopaminergic systems. The ventral tegmental area contains estrogen receptors (LeBlanc et al. 2007). In female starlings, the activity of dopaminergic neurons in response to male song in the ventral tegmental area, as well as regions in the social behavior network, is affected by breeding condition (Riters et al. 2007). These results suggest potential for dopaminergic systems to be modified by estradiol.

The present experiments had two primary goals. First, we examined the specific behavioral responses of female zebra finches to tutored versus untutored song, and in particular how they might be affected by estradiol treatment. Second, we characterized the response of the nucleus accumbens and striatum of estrogen-treated and control females to song stimuli using high performance liquid chromatography (HPLC) to measure the levels of DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC). Prior to this main experiment, a separate group of adult females was used to verify the technique. For this, we determined that predictable changes in dopaminergic neural activity after raclopride treatment are reflected in changes in DA metabolism in the nucleus accumbens and striatum.

Methods

Animals

Adult female zebra finches were raised in mixed-sex aviaries in our breeding colony at Michigan State University. They were kept on a 12:12 light:dark cycle, and seed and water were provided *ad libitum.* Orange and spinach as well as a hard-boiled egg/bread mixture were given once per week. After reaching adulthood (at least 100 days after hatching), female birds were housed in a unisex aviary, in acoustic (but not visual) contact with adult males for at least three weeks prior to testing. All procedures were approved by the Michigan State IACUC and adhered to the guidelines of the National Institutes of Health.

Raclopride treatment

Females were removed from the all-female aviary, taken to a separate room in individual cages, and given an intramuscular injection of either 1mg/kg raclopride (1 mg/kg; Sigma-Aldrich Corp., St. Louis, MO) or a vehicle (1ml/kg). They remained in cages in the room for 70 min., after which they were rapidly decapitated and brain tissue was collected, flash-frozen, and stored at -80°C until processing.

Hormone treatment

Estradiol treatment was conducted as in Svec and Wade (2009). Briefly, females were implanted subcutaneously with 5 mm long Silastic capsules (i.d. 0.076 mm, o.d. 1.65 mm) under isoflurane anesthesia. Implants were made from Silastic tubing, which was packed with 2 mm of 17β-estradiol and sealed with silicone. Implants of this size result in hormone levels slightly above the natural range of adult female zebra finches (Adkins-Regan et al. 1990; Svec and Wade 2009). Females were not gonadectomized, as ovariectomy in this species results in an increased level of circulating estradiol (Adkins-Regan et al. 1990). Control females received empty

implants. Females were housed in individual cages in a room with males and females in group aviaries for 5 days following the surgery.

Song Stimuli

The same auditory stimuli were used as in Svec and Wade (2009), recorded by Dr. Adkins-Regan at Cornell University (also used in Lauay et al. 2004). They came from males that had been tutored (raised with adult males present) or untutored (raised in the absence of other males after 18 days of age) during development. Twelve 30-min song clips (six tutored and six untutored) were created in Adobe Audition (Adobe Systems Inc., San Jose, CA), each containing a unique combination of 30 sec of song from three randomly chosen males in each category that were separated by 30 sec of silence and repeated for 30 min.

Auditory stimulus exposure

Five days after implant surgery, females were taken to a novel room for auditory stimulus exposure. The procedure was conducted as described in Svec and Wade (2009), except that tissue was collected 10 min rather than 1 hour after the conclusion of the song presentation. Briefly, females were placed in a sound isolated box in their individual cages, allowed 30 min for acclimation, and then exposed to 30 min of one of the two types of song. A speaker with a taxidermic model of an adult male zebra finch on top broadcast the song at 60 dB three inches from the cage. As a control, some birds were not exposed to song and remained in silence during the test. The stimulus exposure was videotaped, and behaviors displayed were quantified by an observer blind to treatment condition (see Table 4 for descriptions of behaviors, largely generated from

Zann (1996). Brain tissue was collected following rapid decapitation, flash frozen, and stored at -80 °C until processing.

Table 4. Descriptions of measured behaviors

Behavior	**Description**
Distance calls	Long, loud sounds emitted by the female, often presented after song begins and shortly after song stops, often used for localizing other individuals
'Other calls'	Shorter calls, at a lower volume, including both 'tets' and 'stacks', presented frequently throughout the test in many cases
Duration of movement	The amount of time an individual bird was moving throughout the cage, excluding time in which the bird was sitting still either on the perch or on the ground for more than 10 seconds
Flights/Jumps	Movement to or from the perch or up to the sides of the cage
Beak wipes	Rhythmic movement of the beak back and forth along the perch
Allopreening	Cleaning feathers with beak
Visual Scanning	Turning head to one side, including craning the neck and/or moving one of the eyes in the direction of the speaker and bird model
Beak open	Top and bottom beak are separated slightly for more than 2 seconds
Tail quiver	Rapid movement of the tail, often associated with a bowed position on the perch

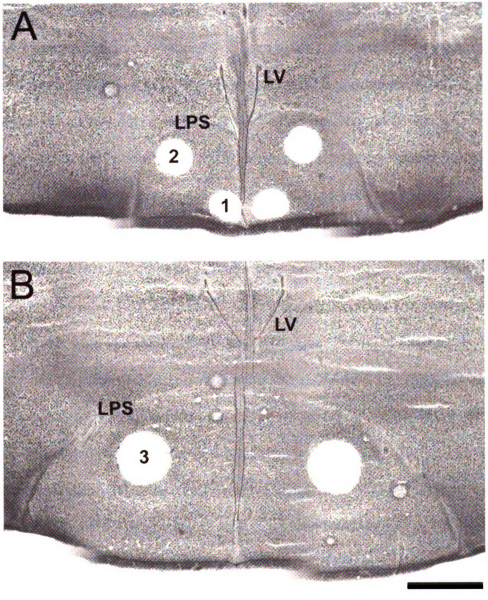

Figure 6. Examples of microdissections taken from thionin-stained coronal sections of the zebra finch brain. Panel A depicts punches of (1) the nucleus accumbens and (2) rostral medial striatum (both 21-gauge). Panel B depicts punches from the caudal medial striatum (3; 18-gauge). LV= lateral ventricle, LPS= Lamina pallio-subpallialis. Scale bar = 1 mm.

Quantification of neurochemicals

Tissue from both the preliminary study including raclopride-treated birds and those in the main experiment that were exposed to the auditory stimuli was coronally sectioned frozen (500 μm thick). Samples of the nucleus accumbens, rostral medial striatum and caudal medial striatum were microdissected from these sections using the Palkovits method (Palkovits 1973). A 21-gauge micropunch was utilized for the nucleus accumbens and rostral medial striatum, and an 18-gauge punch was used for the caudal medial striatum (Figure 6). Punches were placed in 0.1M phosphate-citric buffer in 20% methanol (pH 2.5; 50 μl for nucleus accumbens and rostral medial striatum, and 100 μl for caudal medial striatum) and stored at -20 °C.

Samples were thawed, sonicated with three 1-sec bursts (Sonicator Cell Disruptor, Heat Systems-Ultrasonic, Plainview, NY, USA) and centrifuged (12000 rpm) for 1 min. The supernatant was removed and stored at -20 °C. HPLC coupled with electrochemical detection was conducted to determine the levels of DA and DOPAC as described in Lindley et al. (1990) and Behrouz et al. (2007). Briefly, the supernatant was injected into a C-18 reverse phase analytical column and using a mobile phase of 1.0M phosphate citrate buffer with 0.1M EDTA, 0.35% sodium octylsulfate and 20% methanol. DA and DOPAC content were determined by comparing the heights of peaks generated by a Hewlett Packard Integrator (Model 3393A) with those generated the same day with standards. To determine the protein content, the pellet was dissolved in 1 N NaOH, sonicated, and assayed using the method developed by Lowry et al. (1951). Briefly, samples were compared to a standard curve serially diluted from 12.5 μg to 50 μg of protein. Samples and the standard curve were reacted with a reagent of sodium

carbonate, cupric sulfate, and KNA tartrate for 10 min and then reacted with Folin reagent (phenol in ddH_20) for 30 min. Absorbance was measured with a microplate reader (MicroQuant, Biotek Instruments, Winooski, VT).

To control for variation in size of each sample, concentrations of DA and DOPAC were determined by dividing the absolute quantity of each neurochemical by the protein content within the sample. The DOPAC/DA ratio was also calculated as a measure of the ratio of metabolized to stored DA. No differences in the values were observed between the two portions of the striatum, so they were pooled to form a total striatum value.

Statistics

Two-way ANOVAs were conducted to examine effects of estrogen and auditory stimulus type on behaviors. These were followed by Tukey-Kramer post- hoc tests for pairwise comparisons as appropriate. The HPLC results were analyzed with mixed-model ANOVAs to compare DA and DOPAC concentrations and the DOPAC/DA ratio across treatments and song exposure (between animals) and brain regions (within animals) for both the raclopride and song exposure studies. All statistical analyses were conducted using StatView (SAS Institute; Carey, NC). Sample sizes for each group are indicated in the legends to Figures 7-9 and Table 5.

Results

Estrogen and Song Exposure - Behavior

A main effect of treatment was observed, such that estradiol decreased the frequency of long distance calls ($F= 5.12$, $p= 0.031$) and visual scanning ($F= 6.26$, $p= 0.018$), regardless of type of stimulus exposure (Figure 7, data pooled across the groups

of auditory stimuli since they did not differ). In addition, a main effect of auditory stimulus was observed in the frequency of 'other calls' ($F= 5.93$, $p= 0.006$); females produced more of them when exposed to untutored song than silence (Tukey Kramer, $p< 0.05$; Figure 8). No main effects of song or treatment, or interactions between these variables, were observed for any of the other behaviors (all $F< 3.99$, $p> 0.055$).

Raclopride

A main effect of treatment was observed in the concentration of DOPAC ($F= 23.35$, $p= 0.001$) and the DOPAC/DA ratio ($F= 25.95$, $p= 0.001$); raclopride increased both compared to the control manipulation (Figure 9). An interaction between brain region and treatment indicated that the effect of raclopride on DOPAC concentration was greater in the striatum than in the nucleus accumbens ($F= 10.14$, $p= 0.008$). In addition, the concentrations of DOPAC and DA were higher in the striatum than in the nucleus accumbens ($F= 39.70$, $p< 0.001$; $F= 40.48$, $p< 0.001$, respectively). As expected, no effect of raclopride treatment on DA concentration was observed ($F= 2.43$, $p= 0.145$).

Estrogen and Song Exposure - Neurochemistry

A main effect of brain region was observed such that concentrations of DA ($F= 250.314$, $p< 0.001$) and DOPAC ($F= 82.24$, $p<0.001$) were greater in the striatum than in the nucleus accumbens. In addition, the DOPAC/DA ratio was higher in nucleus accumbens than in the striatum ($F= 58.69$, $p< 0.001$). A trend for an interaction among treatment and song type was detected in concentration of DA ($F= 03.26$, $p= 0.051$), but one-way ANOVAs between treatments or among song exposure groups revealed no significant differences (all $F< 4.44$, $p> 0.059$). No other effects of song or treatment on neurochemistry approached statistical significance (all $F< 1.92$, $p> 0.16$; Table 5).

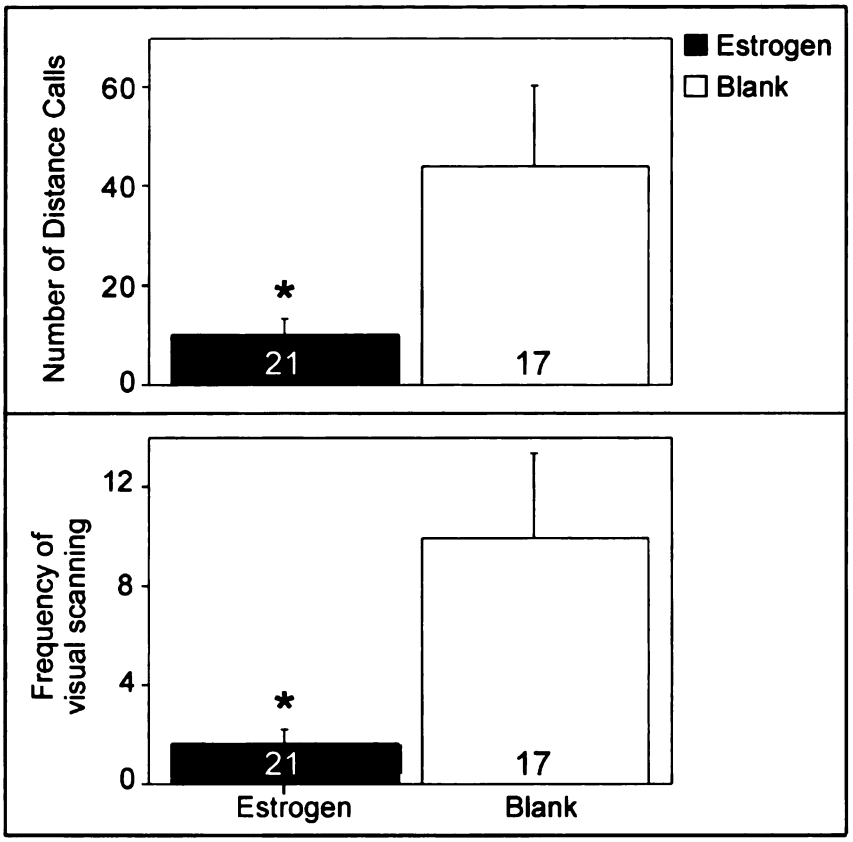

Figure 7. Effects of estradiol on long distance calls (top) and visual scanning behavior (bottom). Data (mean±S.E.) were pooled across song exposure groups, as the effects of the auditory stimuli did not differ among them. Sample sizes: Estradiol-treated tutored song = 6, estradiol-treated untutored song = 8, estradiol-treated silence = 7, blank-treated tutored song = 5, blank-treated untutored song = 6, blank-treated silence = 6. * $p < 0.031$.

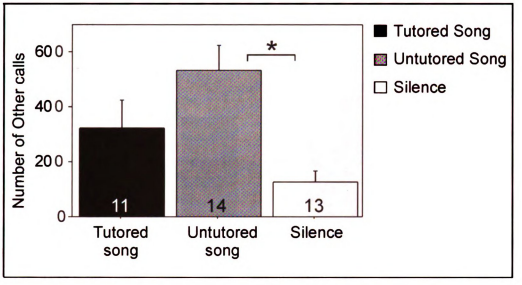

Figure 8. Effect of auditory stimulus on 'other calls' (mean±S.E.). Other calls include "tets" and "stacks", which are shorter calls presented throughout the test. Data were pooled across treatment groups, as estradiol did not affect the number of these vocalizations. Sample sizes: Estradiol-treated tutored song = 6, estradiol-treated untutored song = 8, estradiol-treated silence = 7, blank-treated tutored song = 5, blank-treated untutored song = 6, blank-treated silence = 6. * p= 0.006

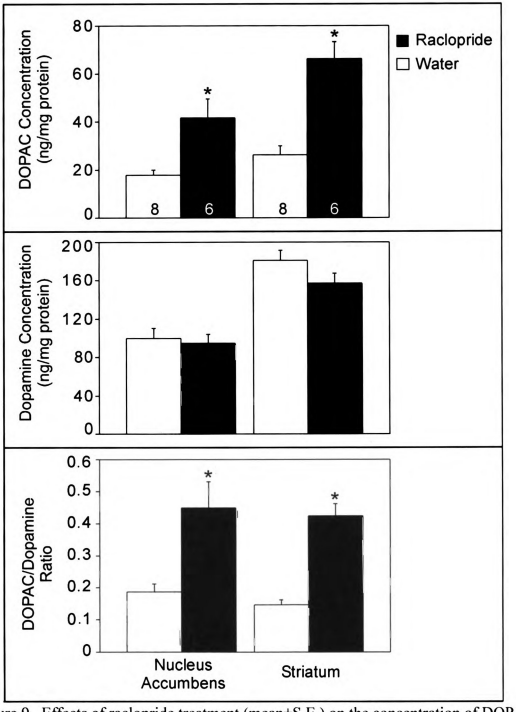

Figure 9. Effects of raclopride treatment (mean±S.E.) on the concentration of DOPAC (top) and DA (middle), and the DOPAC/DA ratio (bottom). Sample sizes are indicated at the bottom of each bar; * $p < 0.008$.

Table 5. Concentrations of DOPAC, Dopamine and the DOPAC/DA ratio in the nucleus accumbens and striatum following song presentation and estrogen treatment. No main effects or interactions were detected, all $F < 1.92$, $p > 0.16$.

Brain Region	Treatment	Song	DOPAC concentration (ng/mg)±SE	Dopamine concentration (ng/mg)±SE	DOPAC/ DA ratio±SE
Nucleus Accumbens	Estrogen	Tutored n= 7	11.95±1.85	20.63±3.15	0.6±0.10
		Untutored n= 7	12.26±2.37	25.83±6.25	0.51±0.06
		Silence n= 5	14.61±3.88	21.64±4.06	0.67±0.13
	Blank	Tutored n= 6	12.51±2.38	29.85±5.43	0.46±0.09
		Untutored n= 8	9.70±1.96	17.12±1.91	0.55±0.80
		Silence n= 7	12.6±2.86	23.04±5.93	0.74±0.16
Striatum *	Estrogen	Tutored	22.88±4.51	85.07±12.37	0.29±0.06
		Untutored	30.24±5.70	137.89±20.94	0.22±0.02
		Silence	37.40±4.85	113.43±10.63	0.22±0.02
	Blank	Tutored	29.49±4.92	125.58±18.83	0.26±0.05
		Untutored	32.98±5.34	110.15±8.88	0.29±0.04
		Silence	38.81±9.52	131.95±16.58	0.24±0.05

* Sample sizes same as in Nucleus Accumbens

Discussion

Summary

Estradiol decreased long-distance calls and visual scanning behavior, and across hormone manipulations, females presented a greater number of 'other calls' when hearing untutored song compared to silence. Through the raclopride study, we verified our measures of dopaminergic activity in the nucleus accumbens and striatum. However, neither estrogen treatment nor exposure to either tutored or untutored song affected these measures in the two measured neural centers.

Behavioral response to estrogen treatment and song exposure

The present results provide a more detailed analysis of the effect of estradiol on behavior than our previous work on female zebra finches (Svec and Wade 2009). In the earlier study, we did not observe an effect of these manipulations on a general call measure. However, vocalizations were not separated into long distance and 'other calls' in that study, and visual scanning behavior was not quantified. Distance calls and 'other calls' have two very distinct functions and by examining them separately, we were able to determine whether the song stimuli or treatment affected these two types of behaviors separately. In the current study, the estrogen-induced decrease in long distance calls and visual scanning behavior clearly indicates that behavioral effects of the hormone do occur. These behaviors may be associated with the same function, involving a reduced need or desire for locating other individuals. Visual scanning behavior would be consistent with this idea, and distance calls are typically used for localization of other birds and often uttered when an individual is isolated (Zann 1996). It is interesting to note that this reduction in behavior parallels a general reduction of ZENK expression in

the VMH following estrogen treatment in a previous study (Svec and Wade 2009).

At least three potential explanations exist for the observed reductions in behaviors. First, it is possible that estrogen treatment results in decreased social interest. Perhaps the visual cues received from the model zebra finch were sufficient for estrogen-treated females and increased attempts to contact or find an individual are not required. Second, estradiol facilitates receptive behaviors in a variety of vertebrate species (see Pfaff et al. 1994; Ball and Balthazart 2002; Godwin and Crews 2002 for reviews). Perhaps an increased focus on mating results in a decrease in the amount of time the female spends on other behaviors such as calling or scanning their environment. Third, it is possible that estrogen-treated females have decreased anxiety, as has been observed in mammals (reviewed in Walf and Frye 2006). This reduction in anxiety may then result in a diminished response to the novelty of the testing environment, which is manifested in fewer long distance calls and visual scanning. Although modulated by estradiol treatment, these behaviors were unaffected by auditory stimulus type. This result suggests that these behaviors may be displayed in response to some other aspect of the testing situation, perhaps isolation or one or more features of the novel environment.

Our results differ from those of Vyas et al. (2008; 2009) who showed that estrogen treatment resulted in increased selectivity of the behavioral response to song (*i.e.* increased number of distance calls in response to high versus lower quality song). However, several important differences exist between their studies and ours. First, Vyas et al. (2008; 2009) utilized three song stimuli: prototypical song (normal song), long bout song (which had additional motifs), and complex song (which included

acoustically different song motifs and high numbers of unique syllables). Thus, female responses to normal song were compared to those of artificially higher quality. In our study, we compared behavior following exposure to normal versus lower quality song. It is possible that females employ distinct mechanisms to distinguish between the features, and estradiol mediates these mechanisms in different ways. Second, we exposed females to only one song type during the behavioral test, whereas in the other studies, females were exposed to all three song types during each test, and proportional responses to them were compared across the groups. Third, no silence group was utilized by Vyas et al. (2008; 2009).

In contrast, other types of calls (tets and stacks collectively) were not affected by estradiol treatment in our study, but were increased in response to untutored song compared to silence. This result is consistent with the idea that females are responding to the unfamiliarity of this song type, which they would not have previously heard. In parallel, exposure to novel stimuli can result in increased locomotion. Tets are used as contact calls and are often associated with movement, and stacks are frequently presented when taking off in flight (Zann 1996). Although we did not detect an effect of song type on movement, it may have been undetected due to the small size of the cage in which the test was conducted. However, it is also possible that hearing this unusual and novel stimulus, untutored song, induces females to increase their attempts to contact other birds.

Dopaminergic response to raclopride treatment

The increased levels of DOPAC and DOPAC/DA ratio in both the striatum and nucleus accumbens of raclopride-treated birds indicates that activity of these neurons

was increased by blocking D2 dopaminergic receptors. In mammals, D2 receptors serve as autoreceptors, and raclopride treatment results in an increase in DOPAC in both of the regions (Ogren et al. 1986; Eaton et al. 1992). Based on the results of several *in vitro* studies, it appears that this also holds true in birds. For example, DA release decreases when D2 receptor agonists are administered to chicken hyperstriatal slices (Jackson et al. 2007), and the activity of striatal dopaminergic neurons is decreased with activation of the D2 receptor (Ding and Perkel 2002). The results here confirm that this effect is also observed following *in vivo* treatment with raclopride, validating the use of DOPAC concentration and the DOPAC/DA ratio as reliable indicators of dopaminergic activity in birds, as in mammals.

Dopaminergic response to estrogen treatment

The lack of effects of estrogen treatment on DA, DOPAC, and the DOPAC/DA ratio was surprising, as estradiol increases DA release in both the striatum (Becker and Beer 1986; Becker and Cha 1989) and the nucleus accumbens (Saigusa et al. 1997; Thompson and Moss 1997) in mammals. Estradiol also increases labeling for tyrosine hydroxylase, the rate-limiting enzyme for DA synthesis, in white-throated sparrows in the ventral tegmental area, the location of dopaminergic cell bodies (LeBlanc et al. 2007). However, as zebra finches are opportunistic breeders, hormones may not play the same role as in seasonal breeders like sparrows. The level of estrogen in adult female zebra finches may remain above threshold, reducing the effect of changes in circulating estradiol. It is unlikely that the treatment itself was ineffective, since we observed changes in behavior due to the estrogen implants.

Parallel to the lack of DA response, we did not detect an effect of estrogen on

ZENK expression in response to song in either the social behavior network or mesolimbic dopaminergic system (Svec and Wade 2009). Similarly, ZENK expression in catecholamine neurons (tyrosine hydroxylase positive neurons which may be dopaminergic or noradrenergic) following song exposure was unaffected by estrogen treatment in white-throated sparrows (LeBlanc et al. 2007). Perhaps in this system estradiol is involved with slower or more long-term modulation, but short-term neurochemical responses within these regions are less sensitive to the hormone.

Dopaminergic responses to song exposure

To our knowledge, the present study is the first to concurrently examine the effects of song perception on dopaminergic neurons in the striatum and nucleus accumbens in songbirds. Concentrations of DA, DOPAC and the DOPAC/DA ratio were not affected by song exposure in the nucleus accumbens or striatum. This result corresponds with the fact that presentation of the same auditory stimulus did not increase the density of ZENK immunoreactive nuclei in the nucleus accumbens or the ventral tegmental area in zebra finches (Svec and Wade 2009), and ZENK expression in other catecholiminergic brain regions was also unaffected by song presentation in white-throated sparrows (LeBlanc et al. 2007). Similarly, in female starlings, male song had no effect on immunoreactivity of phosphorylated tyrosine hydroxylase (an indicator of dopaminergic activity) in the ventral tegmental area (Riters et al. 2007) in starlings. In contrast, dopaminergic neurons in some brain regions do appear to be involved in song perception. For example, the level of DOPAC in the auditory perception regions increases following exposure to high quality song stimuli (Sockman and Salvante 2008), and phosphorylated tyrosine hydroxylase increases after exposure to male song

in the breeding season in the lateral septum and ventromedial hypothalamus of starlings (Riters et al. 2007).

Perception of zebra finch song may differ from what is observed in other songbirds in part because adult male zebra finch song is simple and highly stable in adulthood (Zann 1996). Therefore, it may not be as critical for female zebra finches to utilize additional neuronal processing (such as the activity of these dopaminergic systems) to assess the relative value of or distinguish among song types. Alternatively, females may utilize auditory perception systems to determine whether or not song is present and then process other cues such as visual courtship or tactile stimuli to determine the relative value of those signals via the dopaminergic system.

We also cannot completely rule out the possibility that the time point at which we collected the tissue may have affected our results. However, DA levels rapidly increase and remain high for 30-40 minutes in Area X (in the medial striatum) of male zebra finches after singing (Sasaki et al. 2006). As tissue for the present experiment was collected 10 min after the conclusion of the song presentation, it seems likely that the DA levels would still be elevated at this time point.

Conclusions and future directions

The results from this study, along with other recently collected data from this lab, reveal that estrogen has specific effects on both behavior and neural activity in adult female zebra finches. Estrogen decreased the display of long distance calls and visual scanning (present study) and inhibited the expression of ZENK in the ventromedial hypothalamus (Svec and Wade 2009). These effects, however, appear independent of auditory stimulus presentation, and therefore may result from some

other aspect of the behavior testing environment. As estradiol in the ventromedial hypothalamus in other model systems affects both anxiety and sexual receptivity (see above), either of these factors could have influenced the present behavioral results. Other vocalizations were increased in response to untutored song (present study), and female zebra finches spend more time near tutored than untutored song (Svec and Wade 2009). Neither the effects of estradiol nor those of song exposure appear related to dopaminergic responses in the nucleus accumbens or the medial striatum. However, DA neurons are also present in cortical regions in songbirds (*i.e.* Bottjer 1993; Appeltants et al. 2001), and it is possible that their activity plays a role in mediating responses to song.

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CHAPTER 4: PAIR BONDING IN THE FEMALE ZEBRA FINCH: A POTENTIAL ROLE FOR THE NUCLEUS TAENIAE

Abstract

Male and female zebra finches are highly social and form pair bonds typically associated with reproduction. To determine how these bonds affect a female's behavioral response to future interactions, females were paired with a male for two weeks, separated for 48 hours, and then exposed to the same or a novel male. Control females were left unpaired and introduced to a novel male. Behaviors, as well as neural ZENK expression, were quantified. Females displayed higher levels of behaviors associated with pair bonds (clumping and preening) toward their mates than novel males, and display of these behaviors was correlated with expression of the immediate early gene ZENK in the nucleus taeniae of one group of females, those interacting with their mates. Behaviors of the stimulus males were largely unaffected, but those interacting with an unpaired female attempted to mount more than those interacting with their mates. The results indicate that the nucleus taeniae may play some role in the maintenance of pair bonds in this species. Additionally, females may provide some signal to influence elements of the behavior of males.

Introduction

The formation of social bonds is important to an individual's health and success in many social species, including humans (Lucas et al. 2003). In monogamous species, the reproductive sequence commonly involves formation of a pair bond. The neural mediation of these bonds has only been examined in a few model systems, including monkeys and prairie voles (see Mason and Mendoza 1998; Wang and Aragona 2004 for reviews). Zebra finches form strong pair bonds prior to reproduction. They are formed in 2-14 days, can be maintained through auditory contact alone (Silcox and Evans, 1982), and are characterized by close contact, allopreening, and synchronized behaviors (Zann 1996). Appearance of these behaviors typically occurs shortly after the male and female are introduced, and they increase in frequency by the second day (Silcox and Evans 1982). These behaviors are not typically displayed between unrelated single individuals (Zann 1996; Butterfield 1970). Females that have formed a pair bond react aggressively to new males, and close contact (clumping and preening) with them is inhibited (Silcox and Evans 1982). Pair bonding seems to affect female behavior in two ways. It increases the incidence of pairing behaviors directed toward her mate and reduces the display of them toward other males. Studies designed to examine the responses of females to pairing manipulations are limited. Variations in female responses toward males that are observed following pair bonding are likely mediated by multiple neural systems, including those related to auditory perception, social behavior, and reward.

Responses to interactions with males have been investigated in several species using immediate early genes (IEGs; see Pfaus and Heeb 1997; Meddle et al. 1999),

although research on the neural correlates of behaviors associated with pairing in avian species is limited. Increased IEG expression is observed in female rodents following sexual interactions with males in regions in the social behavior network (defined by Newman 1999) as brain areas involved in the mediation of social behaviors which are responsive to steroid hormones), including the ventromedial hypothalamus, bed nucleus of the stria terminalis, and amygdala (Pfaus and Heeb 1997). In female Japanese quail, interaction with a male results in increased Fos expression in the ventromedial hypothalamus, intercollicular nucleus, and mesopallium (Meddle et al. 1999), at least some of which are involved in the control of reproductive and social behavior in birds (Gibson and Cheng 1979; Cheng et al. 1999). The mesolimbic dopamine reward system is also implicated in the regulation of female reproductive behaviors in rodents (see Melis and Argiolas 1995; Paredes and Agmo 2004 for reviews). Dopamine levels increase following paced copulation in female rats (*i.e.* Becker et al. 2001; Mermelstein and Becker 1995; Pfaus et al. 1995). Although work on the reward system in female birds is limited, singing behavior by male starlings results in increased IEG expression in the ventral tegmental area during the breeding season (Riters et al. 2004; Heimovics and Riters 2005; 2006).

In the monogamous rodent, the prairie vole, IEG expression is seen in the bed nucleus of the stria terminalis and preoptic area when females cohabitate with a novel male (Cushing et al. 2003), and in the medial amygdala, preoptic area, and bed nucleus of the stria terminalis after 6 hours of mating, which is typically sufficient for the formation of a pair bond in this species (Curtis and Wang 2003). The reward system is also vital to the regulation and formation of the pair bond in these animals. Dopamine

levels increase in the nucleus accumbens in response to mating in female prairie voles (Gingrich et al. 2000). Dopamine antagonists presented either systemically or directly into the nucleus accumbens block the formation of a pair bond following mating (Aragona et al. 2003). Similarly, pair bonds can be formed between a male and a female without mating by treating systemically or directly into the nucleus accumbens with a dopamine agonist (Aragona et al. 2003). Thus, regions in both the social behavior and mesolimbic reward system mediate pairing behaviors in the prairie vole.

Studies in birds have tended to focus on the role of auditory perception in female mate choice and pair formation. Females behaviorally discriminate between mate and stranger calls (Vignal et al. 2008), and auditory cues from males are critical for pair bond formation (Silcox and Evans 1982; Tomaszycki and Adkins-Regan 2005). Exposure to male song results in increased IEG expression in the auditory perception regions in numerous songbird species including zebra finches (Mello et al. 1992; Mello and Clayton 1994; Bailey et al. 2002), and these regions display differential levels of IEG expression based on variations in male song (*e.g.* Gentner et al. 2001; Maney et al. 2003; Leitner et al. 2005; Sockman et al. 2005).

The present study was designed to examine the mediation of pair bonding behavior in the *female* zebra finch. Although studies have confirmed that females change their behavior as pair bonds are formed, the only study to examine how pairing affects responses to new individuals was conducted with *male* zebra finches (Caryl 1976). To investigate whether forming a pair bond changes how a female behaves during subsequent social interactions with males, we evaluated behavioral responses in three groups of birds – females re-exposed to their partners after a brief break, and

previously paired and unpaired females exposed to novel males. More importantly, to assess potential roles of the auditory perception, social behavior, and mesolimbic reward systems in the mediation of these behavioral responses, we examined neural correlates by measuring expression of the IEG, ZENK after these social interactions.

Methods

Animals

Adult female and male zebra finches were raised in our breeding colony at Michigan State University. Animals were kept on a 12:12 light:dark cycle, and provided seed and water *ad libitum*. Their diets were enriched with orange and spinach and a hard-boiled egg/bread mixture provided once a week. Adults were raised in mixed-sex aviaries until they had reached adulthood (at least 100 days of age). They were then housed in unisex aviaries, in acoustic but not visual contact with the opposite sex, for at least three months prior to testing.

Behavior

Females were placed in an individual cage (30 cm x 23 cm x 38 cm) either alone or with a sexually mature male zebra finch for two weeks. This time period is sufficient for the formation of a pair bond in this species (Silcox and Evans 1982). A test was then conducted to confirm the formation of a pair bond. The cage containing two birds was taken to a separate testing room, and after one half hour for acclimation, behaviors of both individuals were video-recorded for one hour. Male and female behaviors (Table 6) were quantified by an observer blind to experimental group based on descriptions in Zann (1996) and Adkins-Regan and Ascenzi (1987). They were

classified as bonded if they displayed either clumping or preening during the test. These criteria are routinely used to identify paired birds in this species (Butterfield 1970; Silcox and Evans 1982; Clayton 1990; Zann 1996; Adkins-Regan and Wade 2001; Adkins-Regan 2002), and are uncommon among unrelated, unpaired adult birds (Zann, 1996). Females housed alone were also taken to the testing room for the same time period, although their behavior was not videotaped. Following the test, cages were returned to the colony room, and males were removed and housed individually within visual and acoustic contact with the female for 48 hours. Novel stimulus males were also placed in individual cages at this time, but they were housed out of visual contact with the experimental females. Birds that were not classified as bonded in this first test were not included in the study.

After the 48-hour separation, a second behavior test was conducted. Paired females were taken into the testing room in their individual cages. After 30 minutes of acclimation, they were exposed to either a familiar male (n=7) or a novel male (n=10). Individually housed (unpaired) females were exposed to a novel male (n=10). Behaviors were videotaped for one hour, and those of both individuals were evaluated. A principle components analysis was conducted to determine associations among the behavioral variables. Several of the behaviors were infrequently displayed. If fewer than 35% of the individuals displayed a specific behavior *(e.g.*, males preening the female, copulation, and producing undirected song), we excluded it from the principle components analysis. This procedure allowed us to create groups of related variables that could then be examined as a whole to determine whether they were affected by our

Table 6. Descriptions of measured behaviors. Frequencies were assessed for all; durations were also measured for the behaviors with an asterisk *.

Behavior	**Description**
Pairing	
Clumping *	Male and female perch in physical contact, facing the same direction
Individual in proximity of another *	Male or female moves to perch less than a body width apart; they face the same direction
Preening *	One individual cleans another's feathers with beak
Reproductive	
Tail quiver	Rapid back and movement of the tail. In the female often associated with a bowed position on the perch, but also may be performed by males following copulation
Attempted Mount	Male perches at least briefly on the back of the female, but no cloacal contact is observed
Copulation	Male perches on the back of the female and cloacal contact is observed
Beak Wipe	Beak is moved back and forth in a rhythmic fashion along the perch
Other	
Directed Singing *	Male produces song facing the female
Undirected singing *	Male produces the song, but is not facing the female
Beak fencing	Male and female swipe beaks with one another, can be an aggressive behavior
Approach	Direct movement toward the other individual with an upright posture (often followed by clumping or courtship behavior)

experimental manipulations. To do this, we compared the principle components scores using one-way ANOVAs across exposure groups, followed by post-hoc Tukey/Kramer tests where appropriate. Further analyses focused only on behaviors with high loadings for the principle components which were affected by group. Kruskal-Wallis tests were then used to compare the frequencies and durations of these specific behaviors among the three exposure groups. Pair-wise comparisons were conducted, as appropriate, with Mann-Whitney U tests.

ZENK

The female was removed from the cage one half hour after the conclusion of the second test, overdosed in the testing room with 0.12 cc equithesin, and perfused intracardially with 0.1 M phosphate-buffered saline (PBS) and 4% paraformaldehyde. The brain was removed, fixed in 4% paraformaldehyde for 15 minutes, embedded in gelatin, fixed for one hour in 4% paraformaldehyde, and then placed in 30% sucrose overnight. It was then sagittally sectioned into four series at 30μm on a freezing microtome. Tissue was stored in cryoprotectant at -20°C until immunohistochemistry was performed.

The methods for visualizing ZENK were as described in Svec and Wade (2009), modified from Bailey et al., (2002) and Bailey and Wade (2003; 2005). Briefly, sections were rinsed in PBS, incubated in 0.5% hydrogen peroxide in PBS, rinsed, blocked in 5% normal donkey serum in PBS with 0.3% Triton-X-100 (PBST) for 1 hour, and then incubated with primary antibody (Santa Cruz biotech; catalog #sc-189, 0.1 μg/ml) in PBST overnight at 4°C. After primary incubation, sections were rinsed, incubated in Biotin-SP conjugated donkey-anti-rabbit antibody (Jackson

ImmunoResearch laboratories; 1:500 dilution) in PBST for 1 hour, exposed to ABC reagent (Elite kit, Vector Laboratories, Burlingame, CA), and reacted with diaminobenzadine with 0.0075% hydrogen peroxide for 3 minutes. Tissue was then rinsed, mounted, dehydrated, cleared with xylenes, and coverslipped with DPX.

ZENK immunoreactivity was assessed using brightfield microscopy. Sampling regions were placed in brain areas within three neural systems: the auditory perception regions (caudomedial nidopallium and caudomedial mesopallium), the social behavior network (ventromedial hypothalamus, preoptic area, nucleus taeniae, and bed nucleus of the stria terminalis), and the reward system (ventral tegmental area and nucleus accumbens) as described and pictured in (Svec and Wade 2009). A number of anatomical landmarks were utilized to identify these regions and place the boxes within the same portion of the region in each animal. Box sizes were as follows: caudomedial nidopallium (310μm x 320μm), caudomedial mesopallium (190μm x 345μm), ventromedial hypothalamus (190μm x 290μm), preoptic area (200μm x 200μm), nucleus taeniae (200μm x 320μm), bed nucleus of the stria terminalis (190μm x 345μm), nucleus accumbens (190μm x 305μm), and ventral tegmental area (210μm x 368μm). Immunoreactive cells were manually counted within each sampling region. The average number of immunoreactive cells was calculated from at least three sections per animal, and the density of ZENK immunoreactivity was calculated by dividing this average by the area of the sampling box. A mixed-model ANOVA was conducted to compare the density of ZENK immunoreactivity across brain regions (within animals) and groups (between animals), followed by Tukey post-hoc tests where appropriate. To further investigate the relationship between behavior and ZENK expression, correlation

analyses within each exposure group were used to determine associations between the densities of immunoreactive cells in each brain region with behaviors that differed among the groups.

An outlier was detected among the paired females interacting with their mates in the caudomedial mesopallium and one was also detected in the paired females interacting with novel males in the bed nucleus of the stria terminalis and ventral tegmental area using Dixon's test (Rohlf and Sokal 1981); data reported exclude them.

The principle components (PC) analysis and ANOVA of the PC scores was conducted in SPSS, while other statistical analyses were conducted with Statview (SAS Institute; Carey, NC).

Results

Behavior

Principle Components Analysis

Six PCs were revealed (Table 7). The first two explained almost 50% of the variation in behavior. The first component (female PC) had high loadings for behaviors in which females increased proximity to or initiated contact with males. The second (PC2) involved reproductive behaviors and female beak wipes. ANOVAs revealed a main effect of group on scores for the female PC ($F= 3.56$, $p= 0.044$), such that paired females interacting with their mates displayed higher principle component scores than paired females exposed to novel males (Tukey HSD, $p= 0.037$). A main effect of group on scores for the second PC was also detected ($F= 3.92$, $p= 0.034$), but post-hoc tests revealed no significant differences between pairs of groups (all $p> 0.059$). Scores for

Table 7. Principle Components Analysis. Loadings over 0.5 are indicated with bold type.

	Female PC	PC 2	PC3	PC4	PC5	PC6
Directed Singing						
Frequency	-0.401	**0.759**	-0.331	-0.187	0.187	-0.038
Duration	-0.395	**0.693**	-0.412	-0.186	0.071	0.067
Beak wipe to female	-0.172	0.395	**0.552**	0.409	0.243	0.391
Beak wipe away from female	-0.140	0.309	**0.724**	0.160	-0.027	0.464
Beak wipe toward male	0.146	**0.573**	**0.637**	-0.225	0.020	-0.348
Beak wipe away from male	0.259	**0.545**	**0.604**	-0.269	0.119	-0.367
Male approaching female	-0.192	**0.745**	-0.386	-0.030	-0.170	0.142
Female approaching male	**0.844**	0.292	-0.119	-0.013	-0.043	0.017
Male in proximity of female						
Frequency	0.067	0.427	-0.142	**0.792**	0.219	-0.159
Duration	-0.102	0.082	-0.155	**0.830**	0.200	-0.360
Female-imitated clumping						
Frequency	**0.866**	0.052	-0.151	0.062	-0.069	0.183
Duration	**0.560**	-0.066	-0.105	0.238	-0.098	0.296
Female in proximity of male						
Frequency	**0.725**	0.267	0.020	0.123	-0.375	-0.126
Duration	**0.722**	0.142	-0.068	0.053	-0.358	-0.124
Female preening male						
Frequency	**0.819**	0.120	-0.034	-0.157	0.361	0.002
Duration	**0.852**	0.185	-0.119	-0.091	0.297	0.184
Attempted Mount	-0.263	**0.780**	-0.242	-0.072	-0.277	0.128
Beak Fencing	0.178	-0.075	-0.220	-0.252	**0.790**	0.040
Eigen values	4.828	3.525	2.247	1.881	1.435	1.009

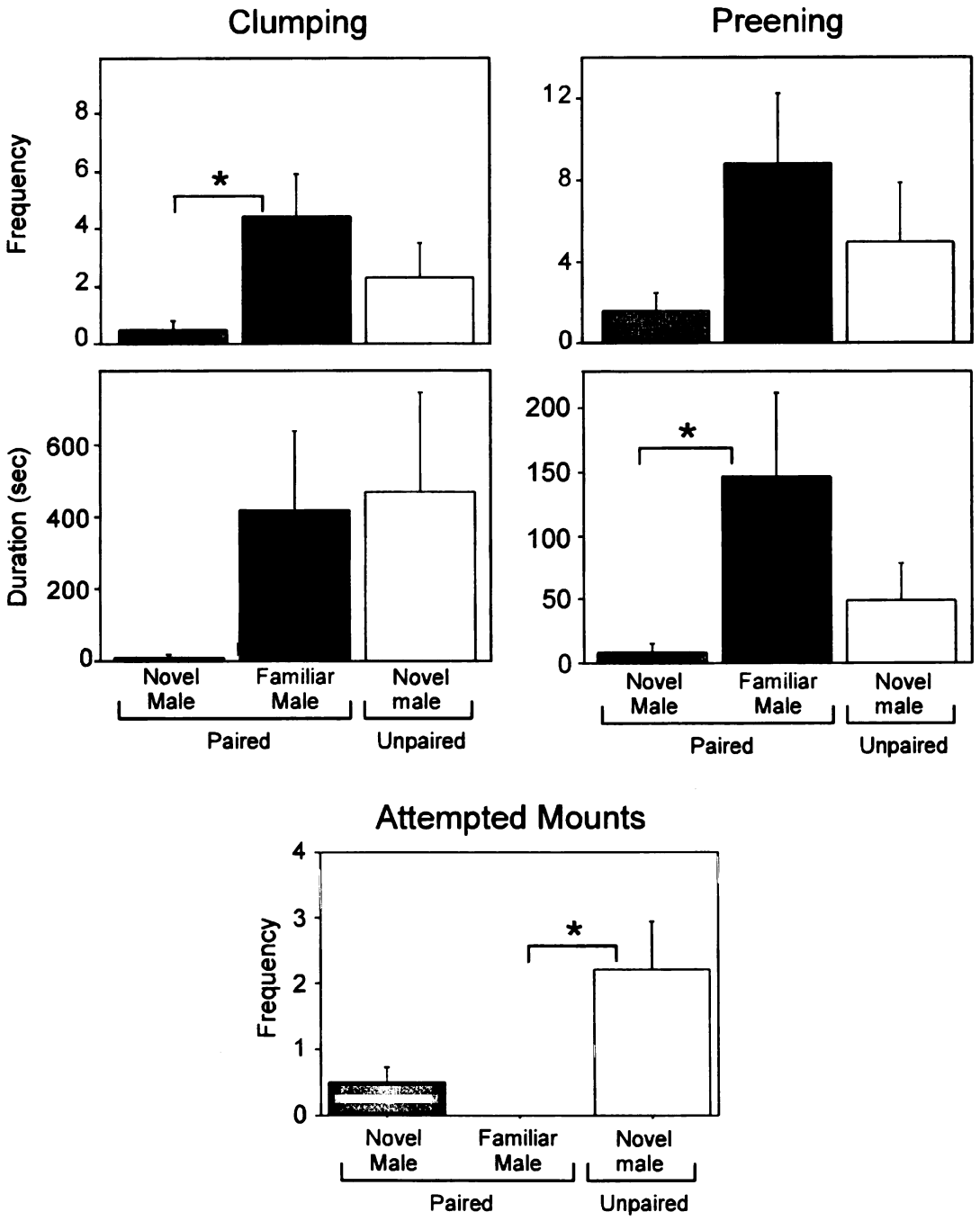

Figure 10. Female pairing behavior in the social interaction test. The two left panels depict the frequency (top) and duration (bottom) of clumping to the male. The right two panels depict the frequency (top) and duration (bottom) of preening the male. The bottom center panel depicts attempted mounts by the male. * signifies $p < 0.05$.

the other four principle components did not differ among the groups (all $F < 1.78$, $p > 0.190$).

Female Principle Component

Among the individual behaviors with high loadings for the female PC, a significant effect of group was detected in the frequency of female-initiated clumping (Kruskall-Wallis: $H = 6.49$, $p = 0.039$) and the duration of females preening males ($H = 5.43$, $p = 0.043$). Both were higher in paired females interacting with their mates compared to novel males (Mann-Whitney $U = 12$, $p = 0.012$, Mann-Whitney $U = 11$, $p = 0.015$, respectively; Figure 1). A trend also existed for paired females to spend more time preening their mates than unpaired females preened novel males (Mann-Whitney $U = 17$, $p = 0.064$). Finally, Kruskall-Wallis tests revealed trends for an effect of group on the duration of female-initiated clumping ($H = 4.74$, $p = 0.057$) and the frequency of females preening males ($H = 4.92$, $p = 0.086$; Figure 10).

Principle Component 2

From the analyses of behaviors with high loadings for the second principle component, the only statistically significant effect detected was a difference among the groups on attempted mounts ($H = 5.429$, $p = 0.027$), such that novel males attempted to mount unpaired females more frequently than familiar males did their mates (Mann-Whitney $U = 14$, $p = 0.016$).

Neural responses

A main effect of brain region existed such that density of ZENK immunoreactive cells was higher in the nucleus accumbens and the bed nucleus of the stria terminalis than all of the other areas investigated ($F = 15.22$, $p < 0.001$, Figure 11). No main effect of group

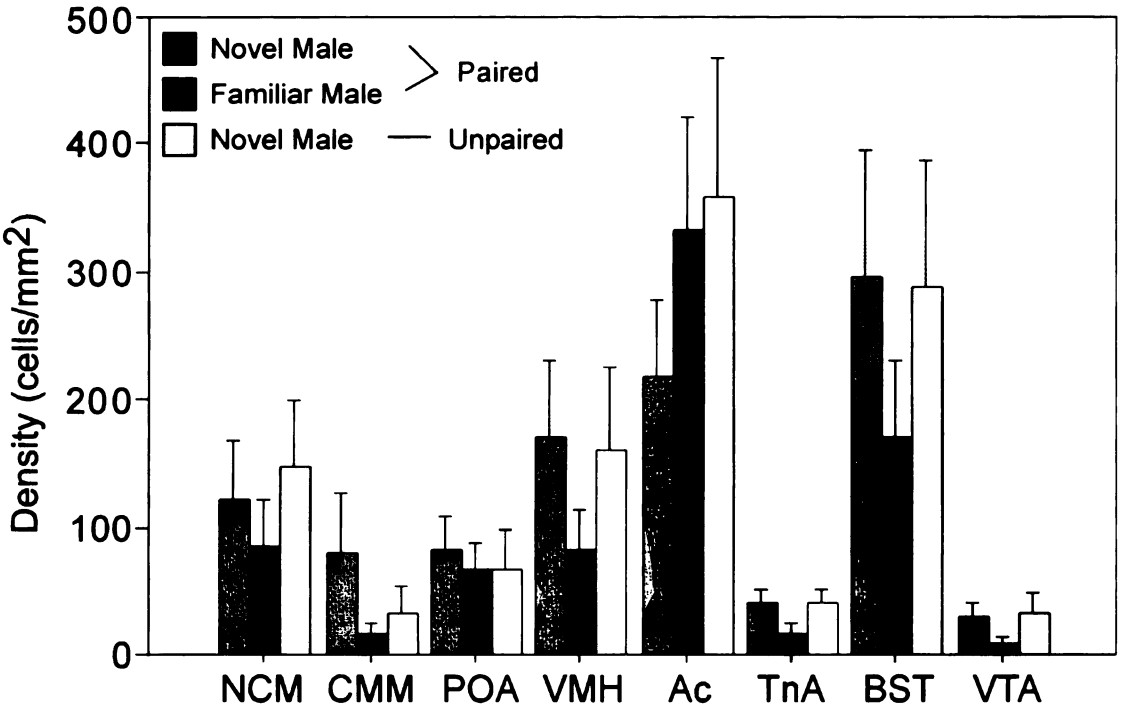

Figure 11. ZENK immunoreactivity across the brain regions investigated in the three groups of females (paired females exposed to novel males or their partner, and unpaired females exposed to novel males). A main effect of region was detected; the density of calls was highest in the nucleus accumbens and bed nucleus of the stria terminalis. A main effect of group and interaction between group and region were not detected. Abbreviations: caudomedial nidopallium (NCM), caudomedial mesopallium (CMM), preoptic area (POA), ventromedial hypothalamus (VMH), nucleus accumbens (Ac), nucleus taeniae (TnA), bed nucleus of the stria terminalis (BST), ventral tegmental area (VTA).

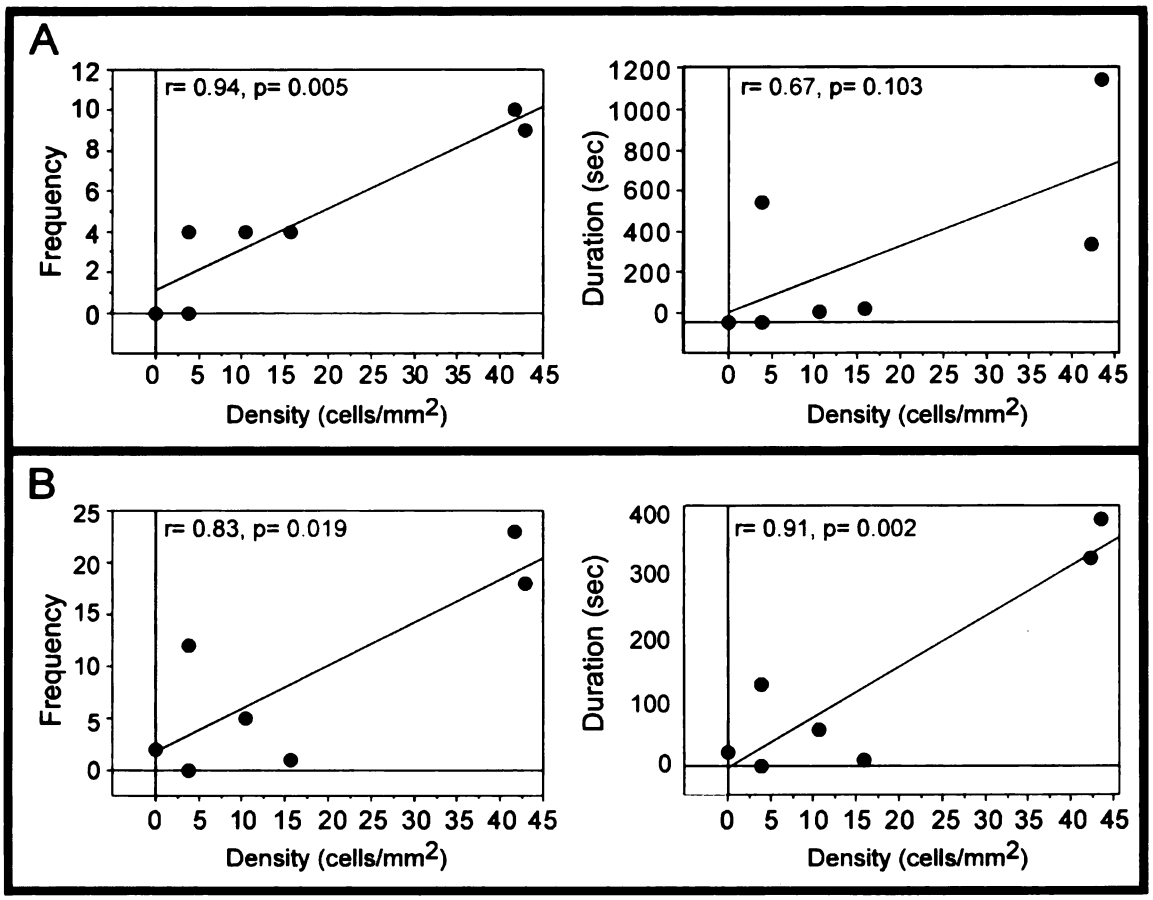

Figure 12. Correlation analyses of the density of ZENK immunoreactive cells in the nucleus taeniae with female pairing behavior in the social interaction test. The figure includes only females interacting with their mate. Panel A depicts the frequency and duration of clumping to the male. Panel B depicts the frequency and duration of preening the male. Three of the four correlations are statistically significant.

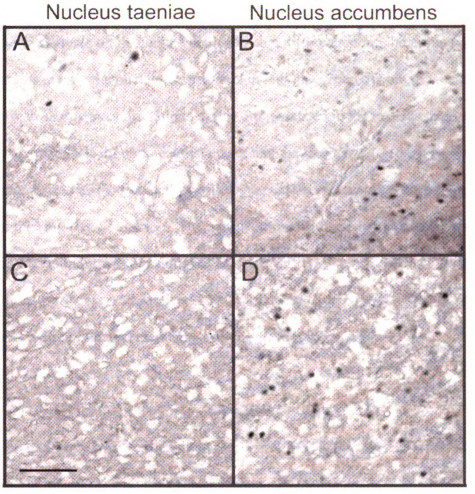

Figure 13. ZENK immunolabeling in the nucleus taeniae (Panels A and C) in the nucleus accumbens (Panels B and D) of females interacting with familiar males. The density of ZENK immunoreactivity was significantly higher in the nucleus accumbens than nucleus taeniae. Labeling within cells also appeared darker in the nucleus accumbens. ZENK immunoreactivity was more apparent in the caudal portion of nucleus taeniae (left side of photos), but was more homogenous in the nucleus accumbens. The top two panels (A and B) depict labeling from animals that displayed high levels of pairing behavior. The bottom two panels (C and D) depict labeling in animals that displayed low levels of pairing behaviors. Scale bar = 50 μm.

or interaction between group and brain region was observed ($F < 0.73$, $p > 0.700$). Correlation analyses on the female behaviors that differed among the groups revealed associations in only the nucleus taeniae within only the group of paired females exposed to their mates. Positive correlations in this group existed in the nucleus taeniae between the density of ZENK immunoreactive nuclei and the frequency ($r = 0.94$, $p = 0.005$) of clumping, as well as the frequency ($r = 0.83$, $p = 0.019$) and duration ($r = 0.91$, $p = 0.002$) of preening (Figures 12 and 13). The correlation between ZENK expression and duration of clumping was not statistically significant ($r = 0.67$, $p = 0.103$). No significant correlations were observed for these behaviors in any of the other groups (unpaired females or paired females interacting with novel males) or brain regions.

Discussion

In this study, we detected higher levels of behaviors indicative of a pair bond in females that interacted with their mates compared to novel males. While that is not particularly surprising, we were excited to observe specific correlations between those behaviors and ZENK immunoreactivity in the nucleus taeniae only in females interacting with their mates. These selective results suggest that the relationship between neural activity in nucleus taeniae and behavior differs among the groups, and that this particular brain region may play a role in maintaining pair bonds in zebra finches. Immunoreactivity was substantially higher in the bed nucleus of the stria terminalis and nucleus accumbens than nucleus taeniae, but the responses in those areas occurred with the introduction of any male stimuli, indicating that they were not

specific to pair bonding; they might be important in more general responses to social interaction, however.

Behavior

The increased levels of female preening and clumping we observed in response to familiar males parallels the results from other studies examining pairing in zebra finches (Butterfield 1970; Caryl 1976; Silcox and Evans 1982). Most of our data confirmed that females change their behaviors toward their partners after pairing, as occurs with male zebra finches (Caryl 1976). We defined birds as partners if they presented clumping and/or preening behavior in after an initial period of co-habitation. Other studies generally utilize one or both of these criteria when defining a bonded pair (Butterfield 1970; Silcox and Evans 1982; Clayton 1990; Zann 1996; Adkins-Regan and Wade 2001; Adkins-Regan 2002). However, if a more stringent method for classifying males and females as paired is used following the initial test (criteria involving a certain number of instances of proximity, clumping and preening, as in Ikebuchi and Okanoya 2006), the sample sizes in the present study are reduced limiting statistical power, but the same pattern of results is detected. This fact suggests that the criteria commonly employed are appropriate for defining paired animals, and that the behavioral results we observed in the paired groups likely reflected the effects of a pair bond.

It is intriguing that the amount of clumping overall did not differ between unpaired and paired females in the final behavioral test. When the behaviors are more closely examined, the average latency for unpaired females to clump was two times greater than for the females interacting with their mates. In addition, the pattern

throughout the course of the test differed. If the data from the hour-long test are examined in ten minute bins, it is clear that duration of clumping in females interacting with their mates is high in the first 20 minutes, but decreases over the next 40 minutes. In contrast, in unpaired females, the duration of clumping increases after 10 minutes and then remains consistent. The duration of preening was consistently higher in females interacting with their mates than that of unpaired females throughout the test period. Most pairing behaviors were initiated by females in all three groups, but males did display low levels of both clumping and preening. However, only males interacting with their mates clumped and preened for long durations.

All of these data lead to some interesting possibilities that need to be further explored. For example, the shorter latency compared to unpaired females and decrease in clumping over time in females interacting with their mates may indicate that initially the display of the behavior is important for re-establishment of the bond after a short absence, but is not required for continual maintenance. In contrast, previously unpaired females might be more gradually attempting to form new bonds when males are introduced. It is also plausible that the unpaired females displayed clumping behavior they might not otherwise simply due to a heightened need for social contact. This group was isolated 12 days longer prior to the test than the paired females. In addition, the fact that the pattern of clumping, but not preening, differs through the test seems to indicate that two behaviors may serve different functions. For example, clumping might be more important in the social aspects associated with re-establishing or forming a new bond, whereas preening may be vital to the maintenance of an established pair bond.

Pairing itself did not have a strong effect on a female's response to *novel* males, given that both clumping and preening were similar between paired and unpaired females interacting with novel males. This result corresponds with observations by Caryl (Caryl 1976) that males courted novel females the same amount after pairing as they did before forming a bond. Finally, although clumping and preening are consistently used as indicators of pair bonding in this species (Butterfield 1970; Silcox and Evans 1982; Clayton 1990; Zann 1996; Adkins-Regan and Wade 2001; Adkins-Regan 2002), it is unknown whether additional levels or functions of these behaviors might exist.

Although the present experiment was designed to examine female behavior, we were also able to observe how manipulations of a female's pairing status affected male behavior. Females play a vital role in mate choice decisions in this species (Butterfield 1970; Clayton 1990; Zann 1996), and in the present study they initiated 66% of the instances of clumping and 71% of the preening. This result is consistent with the idea that females control a large amount of the behavior associated with pair bonds. Male actions are likely influenced by signals from the females. This possibility is supported by the fact that unpaired males attempted more mounts with novel unpaired females than paired males did with their mates. Unpaired females may have provided different cues to the males. Of course, as the pairing status also differed between the males in these two groups, either the fact that they had formed a bond or had not been in contact with females recently could have contributed to the differential response.

Relationship between behavior and IEG expression in the nucleus taeniae

The relationship observed between clumping and preening and ZENK-IR was highly specific. Correlations were observed only in one group, females interacting with their mates, and in only one brain region, the nucleus taeniae. This region is comparable to the mammalian amygdala in several ways. It contains some sensory inputs and sends projections to the hypothalamus and hippocampus (Cheng et al. 1999). It also expresses GAD 65, LAMP, and COUP-TF II like the mammalian amygdala (Yamamoto et al. 2005). Functionally, nucleus taeniae appears to mediate similar behaviors in birds as the amygdala does in mammals, such as social (Kollack-Walker and Newman 1995; Numan and Sheehan 1997; Cheng et al. 1999; Reiner et al. 2004a) and reproductive behaviors (Pfaff et al. 1994; Thompson et al. 1998; Newman 1999; Reiner et al. 2004a). The mammalian amygdala has also been implicated in the mediation of interpretation of social cues, especially the reward and emotional significance of these cues (for review see Baxter and Murray 2002; Phelps and LeDoux 2005; Phelps 2006; Murray 2007).

In animals that form pair bonds, this region shows either increases (prairie vole; Curtis and Wang 2003; Cushing et al. 2003) or decreases in activity (*e.g.* human; Bartels and Zeki 2000, monkey; Bales et al. 2007) with regards to pairing. In the prairie vole, formation of a pair bond is correlated with an increase in fos expression in the amygdala (Curtis and Wang 2003), and lesions to the region decrease affiliative behaviors (Kirkpatrick et al. 1994). Neurochemical mediation of formation and maintenance of pair bonds differs, however. Formation involves D2 receptor binding (Wang et al. 1999; Gingrich et al. 2000), whereas bond maintenance involves altered dopamine D1 receptor density and binding in the nucleus accumbens (Aragona et al.

2006). It is possible that in the zebra finch, neurons within the nucleus taeniae are involved in the *maintenance* of a pair bond, whereas another system may be vital to the mediation of *initial formation* of a bond. It remains to be investigated whether similar relationships between neural activity in nucleus taeniae and behavior occur in other social situations.

Several potential explanations exist for the function of the relationship between bonding behaviors and ZENK induction in the nucleus taeniae of female zebra finches observed in the present study. First, this association between ZENK expression with clumping and preening may indicate that nucleus taeniae actively facilitates the production of these pairing behaviors or inhibits the presentation of other behaviors that are not related to bonding. The context of a specific behavior can affect IEG expression (as in singing behavior in starlings in breeding conditions vs. non-breeding conditions; *i.e.* Heimovics and Riters, 2005, 2006, Riters et al., 2004), so it is reasonable that this correlation was only detected when the female was interacting with her mate. However, it is also possible that the correlation might reflect sensory stimulation related to the behavior, perhaps affecting how sensory information is perceived when the female is in contact with an individual she remembers as her mate (see above references on mammals), or it might represent activity in cells that inhibit responses to less salient sensory stimuli. In either case, the increased neuronal response could serve to increase the focus of the female to the relevant cues and aid in maintaining the pair bond. These possibilities need to be explored further and could be partly elucidated by determining the neuronal subtypes of the responsive neurons within this brain region.

Neural response in other brain regions

Although no effects of our treatment groups were detected in any of the other brain regions, it is intriguing that we observed higher levels of ZENK immunoreactivity in the nucleus accumbens and bed nucleus of the stria terminalis compared to the other measured regions. There are at least two potential explanations for the higher level of immunoreactivity in these regions. First, it may have functional relevance, as females in all the groups interacted with a male during the behavior test. The relatively high neural activity in these brain regions may be associated in social interactions, whereas the nucleus taeniae might respond more specifically depending on whether or not a pair bond has been formed between the male and female that are interacting. Second, it is possible that these regions have a higher general level of activity than the other examined regions, resulting in greater baseline immediate early gene expression. These ideas and the specific roles of the nucleus accumbens and bed nucleus of the stria terminalis in social situations warrant further investigation.

Summary and conclusions

The present data provide information about both behavioral interactions and their neural mechanisms. Although pair bonding is generally identified and defined by the presence of specific behaviors (clumping and preening) in zebra finches, their functions appear to be more complex. Our results indicate that clumping and preening might serve different roles and can be displayed differentially. In addition, a region in the social behavior network (nucleus taeniae) was identified in which immediate early gene expression is strongly correlated with the behaviors only in females interacting with their mates. Thus, the neural signal associated with the behaviors depends on the contexts in which they occur. Finally, the bed nucleus of the stria terminalis, a region in

the social behavior network, and the nucleus accumbens, a region in the mesolimbic dopamine reward system, may be implicated in responses to female-male (or perhaps even other) social interactions in general. In contrast, the auditory perception regions investigated in this study appear to play little if any role in mediating the formation or maintenance of social bonds under the conditions tested here.

Acknowledgements

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CHAPTER 5: PAIR BONDING BEHAVIOR IN FEMALE ZEBRA FINCHES IS DISSOCIATED FROM DOPAMINERGIC ACTIVITY IN THE NUCLEUS ACCUMBENS AND MEDIAL STRIATUM

Abstract

Female zebra finches display higher levels of pairing behavior towards their mates than to novel males. To determine the role of dopaminergic systems in this behavior, we paired females with males for two weeks, and then they were separated from their mates for two days. These birds were then separated for two days and either their mates or a novel male was introduced to the female for 30 minutes. Novel males were introduced to additional unpaired females as controls. The content of dopamine and its metabolite, DOPAC, in the nucleus accumbens and striatum was determined using high performance liquid chromatography. Although females appeared to demonstrate higher levels of clumping and preening when interacting with their partners, dopaminergic activity was not affected by our behavioral manipulations in either of these regions. These results indicate dissociation between the activity of the mesolimbic dopaminergic reward system and the behaviors potentially involved in the maintenance of pair bonds in the zebra finch.

Introduction

Social bonds are formed in many species, although their neural mediation has been investigated in relatively few animals (reviewed in Mason and Mendoza 1998; Wang and Aragona 2004; Adkins-Regan 2009). Bonds are particularly common among songbirds (see Black 1996). Zebra finches are opportunistic breeders, and form pair bonds prior to mating (Zann 1996). Extra pair fertilizations are relatively low in this species (Birkhead et al. 1989; Birkhead and Hunter 1990), suggesting that copulations are uncommon among unpaired birds. Pair bonds in this avian species are characterized by clumping (sitting in close proximity), preening (cleaning another individuals feathers), and synchronized behavior. Laboratory investigations of pair bonding typically utilize the presence of clumping and preening to identify bonded individuals (Butterfield 1970; Silcox and Evans 1982; Clayton 1990; Zann 1996; Adkins-Regan and Wade 2001; Adkins-Regan 2002), as they are rarely displayed among unpaired individuals (Butterfield 1970; Zann 1996).

The formation of a pair bond affects the display of both male and female behaviors. Courtship display decreases with the formation of a bond, but increases upon reunion following a period of separation (Butterfield 1970). Males and females are also more likely to clump and preen as pair bonds are formed (Caryl 1976; Silcox and Evans 1982; Svec et al. 2009). An unpaired female is unlikely to preen novel individuals, but may display clumping if she interacts with a male for a longer amount of time (Svec et al. 2009). These results indicate that while clumping and preening are both associated with pair bonds, they may represent different aspects of the bond.

The mesolimbic dopaminergic reward system may be critical for mediating the display of clumping and preening in paired individuals. Dopaminergic neurons are involved in the interpretation of rewarding stimuli (see reviews in Schultz et al. 1997; Schultz 2000; Ikemoto 2007), including those associated with reproduction (reviewed in Paredes and Agmo 2004; Mitchell and Gratton 1994; Melis and Argiolas 1995). In monogamous species, including humans, this system is active when individuals are exposed to their partners, or representations of them (Bartels and Zeki 2000; Gingrich et al. 2000; Bartels and Zeki 2004; Aron et al. 2005).

In the prairie vole, one of the relatively few monogamous mammals, pair bonds can be formed after a day of cohabitation with copulation. This bond is typically characterized in laboratory tests by a preference for spending time with the partner in a choice test (see Williams et al. 1992; Young et al. 1998). When a male and female are housed together and begin to copulate, dopamine (DA) is released in the nucleus accumbens in female prairie voles (Gingrich et al. 2000). Preference for the mating partner can be eliminated in these females by infusing DA D2 receptor antagonists into the cerebrospinal fluid or directly into the nucleus accumbens prior to the mating period (Aragona et al. 2003). Conversely, treatment with D2 agonists can induce a female's preference for a male even if they did not copulate (Aragona et al. 2003).

A variety of similarities exist between the reward systems of birds and mammals, suggesting similar dopaminergic mechanisms may be involved in the pair bonding of zebra finches and mammals (prairie voles). For example, the majority of dopaminergic neurons emerge from the brainstem in the substantia nigra and the ventral tegmental areas and project to the basal ganglia including the striatum and the nucleus

accumbens in both avian and mammalian species (reviewed in Veenman 1997; Ikemoto 2007)). The activity of dopaminergic neurons in the striatum is mediated through D2 autoreceptors in birds (Gale and Perkel 2005; Jackson et al. 2007), as in mammals. In parallel, we demonstrated that the regulation of dopaminergic activity through D2 autoreceptors can be reliably measured using a micro-punch sampling technique and high-performance liquid chromatography (HPLC) in the nucleus accumbens and striatum (Svec et al. submitted). Functionally, addictive drugs known to affect dopaminergic systems in mammals have similar behavioral effects in birds (Levens and Akins 2004; Geary and Akins 2007; Akins and Geary 2008). In addition, both the striatal and mesolimbic DA systems respond during the production of sexually motivated song in male birds (Riters et al. 2004; Heimovics and Riters 2005; Sasaki et al. 2006), indicating that their importance for motivated behaviors.

While immediate early gene expression in the nucleus taeniae (homologous to the mammalian amygdala; Cheng et al. 1999) correlates with clumping and preening in female zebra finch females interacting with their partners, no relationship with pairing behaviors existed in the nucleus accumbens or ventral tegmental area 2009). Similarly, in female prairie voles the mating bouts that occur during formation of a pair bond do not result in a corresponding change in immediate early gene expression in the nucleus accumbens (Curtis and Wang 2003; Cushing et al. 2003). However, dopamine release occurs in this region following copulation in female prairie voles (Gingrich et al. 2000), indicating that immediate early gene expression may not be the best indicator of dopamine release. Based on these results and the limitations of immediate early genes

in their use as markers for neural activity (see Pfaus and Heeb 1997), a more direct examination of the response of the dopaminergic systems seemed prudent.

The current study was designed to investigate whether differences in female responses to males after the formation of a pair bond are related to changes in activity of the dopaminergic systems. Females were paired with individual males, and control individuals were unpaired. After a period of isolation, each female was exposed to either her partner (if one existed) or a novel male. Dopaminergic activity was measured by determining the concentrations of DA and DOPAC in samples from the nucleus accumbens and striatum using high performance liquid chromatography (HPLC).

Methods

Animals

Adult male and female zebra finches were raised in the breeding colony at Michigan State University. They were kept on a 12:12 light dark cycle and were fed seed and water *ad libitum* supplemented with a mixture of hard-boiled eggs and bread, as well as spinach and orange once a week. Birds were raised in mixed-sex aviaries with about seven adult males and females, and their offspring. After they reached sexual maturity, the birds were housed in single-sex aviaries for at least one month prior to testing. They were in acoustic, but not visual, contact with members of the opposite sex. All procedures were approved by the Michigan State University Animal Use and Care Committee and adhered to the guidelines of the National Institutes of Health.

Behavior

Stimulus exposure and behavior testing were conducted as in Svec et al. (in press). Briefly, females were housed alone or with a sexually mature male for two weeks in an individual cage (30 cm x 23 cm x 38 cm) in the room with the group aviaries. To confirm formation of a bond between the cohabitating birds, they were taken to a sound-isolated room, given 30 min for acclimation, and then videotaped for one hour. A pair was classified as bonded if the individuals displayed clumping, preening, or copulation during this hour. Those that had not bonded were excluded from analyses. Each cage was then returned to the aviary room, and the male was removed and housed individually in acoustic and visual contact with the female. Females housed alone were taken to the testing room for the same period of time, but not videotaped. At this time, additional stimulus males were placed in individual cages and housed in the same aviary room in acoustic, but not visual, contact of the females.

Two days later, the female was re-acclimated to the testing room for 30min, and then either the same male or a new male was introduced to their cage for 30 min. Behavior was videotaped and later analyzed by an observer blind to group (paired female with partner, paired female with novel male, or unpaired female with novel male). Measured behaviors are described in detail in Svec et al. (in press), and include those associated with pairing (clumping, preening, approach) and courtship (directed singing and beak wipes), as well as those more directly related to reproduction (tail quivers, attempted mounts, and copulation). Additional analyzed behaviors included undirected song and beak fencing. After the conclusion of the test, the female was

rapidly decapitated. The brain tissue was flash-frozen in methyl-butane, and stored at -80 °C until processing.

HPLC Analysis

As in Svec et al. (submitted), tissue was sectioned coronally at 500 μm, and portions of the nucleus accumbens (21-gauge), and rostral (21-gauge) and caudal (18-gauge) medial striatum were microdissected using the Palkovits punch method (Palkovits 1973). Tissue was placed in 50 μl of 0.1M phosphate-citric buffer in 20% methanol (pH 2.5) and stored at -20 °C. Prior to HPLC, samples were centrifuged, sonicated with three 1-sec bursts, centrifuged for 1 min, and the supernatant was removed and stored at -20 °C. HPLC coupled with electrochemical detection was conducted as in Lindley et al. (1990), Behrouz et al (2007), and Svec et al. (submitted). Supernatant was first run through a c-18 reverse phase analytical column and then electrodes with 1.0M phosphate citrate buffer with 0.1M EDTA, 0.35% sodium octylsulfate and 20% methanol. Peaks were generated for each sample with a Hewlett Packard Integrator (Model 3393A). Standards containing known amounts of DA and DOPAC were run on the same day and were used to determine the content of DA and DOPAC within each sample.

A Lowry assay (Lowry et al. 1951) was used to determine protein content. The pellet from each sample was dissolved in 0.1M NaOH and sonicated. Two reactions were then conducted on the samples and a standard curve of protein serially diluted from 12.5 μg to 50 μg. First, they were exposed to sodium carbonate, cupric sulfate, and KNA tartrate for 10 minutes, and then Folin reagent (phenol in ddH_20) was added for 30 minutes. A microplate reader (MicroQuant, Biotek Instruments, Winooski, VT)

was used to determine absorbance of the samples and standard curve, and these values were used to determine the protein content of each sample. DA and DOPAC values were divided by the protein content for each sample to determine the concentration of the neurochemicals. The DOPAC/DA ratio was also calculated using the absolute quantity of DA and DOPAC for each sample.

Statistics

A principal components (PC) analysis was conducted to determine relationships among the behaviors (see Svec et al. 2009). Behaviors which were presented by less than 25% of individuals were not included in this analysis. The scores for each PC were then compared among the three groups with one-way ANOVAs. As in Svec et al. (in press), the frequency and duration of female-initiated clumping and preening and the frequency of attempted mounts were also compared with non-parametric Kruskall-Wallis tests. HPLC data for each metabolite were analyzed with mixed-model ANOVAs to compare data across brain regions (within individuals) and across the groups (between individuals). Tukey-Kramer post-hoc tests were conducted for pair-wise comparisons, as appropriate.

Results

Behavior

Six PCs were revealed in the PC analysis (Table 8). Collectively, the first two explained 42 percent of the variance in the data. The first principal component (PC1) included female-initiated clumping (frequency and duration), preening (both male and

Table 8. Principal components analysis. Behaviors loading above 0.5 are in bold.

	PC 1	PC 2	PC3	PC4	PC5	PC6
Directed Singing						
Frequency	-0.278	**0.748**	0.353	-0.357	-0.12	-0.028
Duration	-0.335	**0.728**	0.382	-0.332	-0.06	0.036
Beak wipe to female*	-0.015	0.009	**0.670**	0.105	0.413	0.371
Beak wipe away from female	-0.180	-0.28	0.156	0.128	0.061	**0.739**
Beak wipe toward male	-0.005	0.208	0.156	**0.727**	-0.28	0.026
Beak wipe away from male	-0.062	0.070	0.243	**0.709**	-0.07	0.047
Male approaching female	**0.563**	-0.28	**0.700**	-0.04	0.148	-0.04
Female approaching male	-0.225	**0.553**	0.221	-0.305	-0.28	-0.196
Male-initiated Clumping						
Frequency	0.314	**0.769**	-0.17	-0.168	-0.28	0.288
Duration	0.222	0.037	-0.25	-0.166	-0.37	**0.707**
Male in proximity of female						
Frequency	0.279	**0.501**	-0.020	**0.521**	0.492	0.065
Duration	0.170	0.307	-0.22	0.261	**0.573**	-0.026
Female-imitated clumping						
Frequency	**0.840**	-0.25	0.389	-0.177	-0.01	-0.138
Duration	**0.922**	-0.03	-0.17	-0.191	-0.06	0.230
Female in proximity of male						
Frequency	**0.642**	0.355	0.104	0.314	-0.45	-0.129
Duration	**0.388**	0.236	-0.02	**0.553**	-0.47	-0.127
Male preening female						
Frequency	**0.590**	0.409	**-0.52**	-0.091	0.292	-0.010
Duration	**0.611**	0.329	**-0.54**	-0.042	0.306	-0.012
Female preening male						
Frequency	**0.908**	-0.19	0.300	-0.136	-0.05	0.028
Duration	**0.900**	-0.18	0.239	-0.192	-0.01	-0.127
Beak Fencing	0.012	**0.643**	0.457	0.005	0.279	0.057
Eigenvalues	5.321	3.538	2.613	2.325	1.792	1.441

*Unless otherwise indicated, values represent frequencies.

Table 9. Durations of male and female behaviors displayed following introduction of a male (mean±S.E.). Results are taken from the 30 min test in the present study and from the first 30 minutes of the behavior tests in Svec et al. in press. Values from the two experiments are summed and presented on the right.

	Present study			Svec et al. in press			Combined Data		
Behavior	Paired with same male (n=10)	Paired with new male (n=6)	Unpaired (n=12)	Paired with same male (n=7)	Paired with new male (n=10)	Unpaired (n=10)	Paired with same male (n=17)	Paired with new male (n=16)	Unpaired (n=22)
Female-initiated Clumping									
Duration (sec)	147.8±62	28.7±29	15.3±10	215.7±102	10.3±8	192.2±129	175.8±54	17.2±11	95.8±60
Female Preening Male									
Duration (sec)	83.7±40	21.2±20	22.3±21	204.1±130	8.2±7	8.4±5	133.3±58	13.1±8.3	24.6±14
Male-initiated Clumping									
Duration (sec)	83.1±73	0.0±0	3.2±2	147.8±62	8.4±5	5.2±3	129.9±88	5.3±3	4.1±2
Male Preening Female									
Duration (sec)	18.5±13	0.0±0	0.1±0	27.1±21	0.0±0	3.8±4	22.1±11	0.0±0	1.8±2

female-initiated, frequency and duration), female approaching the male (frequency), and female in proximity of male (frequency and duration). The second principal component (PC2) included primarily male behaviors: directed singing (frequency and duration), male approaching the female (frequency), male-initiated clumping (frequency and duration), male in proximity of female (frequency and duration), and beak fencing (frequency). No group effects were detected with one-way ANOVAs conducted on any of the PC scores (all $F < 2.30$, $p > 0.123$). However, a trend for an effect of group on PC1 ($F = 2.79$, $p = 0.083$) was detected, levels appeared increased in females interacting with their mates. Kruskall-Wallis tests revealed no significant effects of group on the five behaviors analyzed in Svec et al. (in press); all $H < 3.66$, all $p > 0.16$). Selected behavioral data (durations) are presented in Table 9, along with a portion of the data from our previous study (Svec et al. 2009) for comparison.

HPLC

A main effect of brain region on DOPAC concentration was detected ($F = 7.91$, $p = 0.001$; Figure 14). Across the groups, it was higher in the nucleus accumbens than the rostral striatum (Tukey-Kramer, $p < 0.05$). In contrast, the concentration of DA was higher in both the rostral and caudal striatum than the nucleus accumbens ($F = 22.43$, $p = 0.42$; Tukey Kramer $p < 0.05$). In addition, a main effect of brain region was observed in the DOPAC/DA ratio ($F = 72.09$, $p < 0.001$), such that it was higher in the nucleus accumbens than both the rostral and caudal striatum, and higher in the caudal than the rostral striatum (Tukey-Kramer, $p < 0.05$). A trend for an interaction between group and brain region was also observed within the concentration of DOPAC ($F = 2.32$, $p < 0.07$).

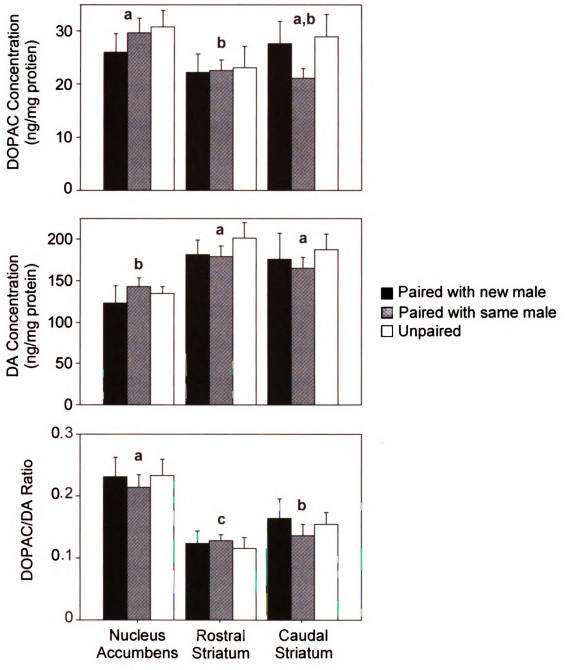

Figure 14. Concentrations of DOPAC (top), DA (middle), and the DOPAC/DA ratio (bottom) in the nucleus accumbens (left), rostral striatum (center), and caudal striatum (right). Significant effects of brain region were observed for each measure, indicated by different letters above the bars. No effects of group or interactions among group and brain region were observed. Sample sizes: paired with new male, n=6, paired with same male, n= 10, unpaired, n= 11.

While the groups responded similarly in each region, the DOPAC seemed greater in the nucleus accumbens than both portions of the striatum in females exposed to their partners ($F= 13.83$, $p= 0.0002$; both Tukey-Kramer, $p< 0.05$), and greater than the rostral striatum in unpaired females ($F= 4.64$, $p= 0.022$; Tukey-Kramer, $p< 0.05$). No other effects of group or interactions among the groups and brain regions were detected for any of the neurochemical measures (all $F< 0.994$, $p> 0.42$).

Discussion

The present study was designed to investigate the response of the dopaminergic reward system in female zebra finches associated with pairing behaviors and/or pairing status. Upon introduction, paired females behave differently toward their partners than toward novel males. However, we did not detect statistically significant changes in activity of this system with pairing and interactions with males. These results parallel those in a previous study conducted in our lab (Svec et al. submitted), documenting a lack of relationship between DA neurochemistry and female responses to high quality song, which induced a preference behavior. Together, these studies suggest that the role of this system in mediating behavioral preferences for specific social stimuli may be limited in this species.

Behavior

Unlike our previous study (Svec et al. 2009), we did not detect a significant effect of pairing on female clumping and preening with males. The only difference between the two experiments was the duration of the behavioral test (30 min after male

introduction in the present study, which was required for neurochemical analysis, compared to 60 min in the previous study). Group differences in the display of some behaviors may not have been detected in the current study because of the shorter testing period. In fact, our earlier work suggested that pairing behaviors emerged later in the test, particularly in unpaired individuals (Svec et al. 2009). Still, the general patterns of behavior seemed similar between the two studies, with higher levels of clumping and preening in paired females interacting with their mates.

A comparison of the durations of each behavior between the first 30 min of the two studies indicated that in each case they were statistically equivalent (Table 9). In a collective analysis, paired females clumped longer with their partners than with novel males (Kruskall-Wallis $H= 7.3$, $p= 0.03$, Mann-Whitney U, $p= 0.01$), but males did not differ among groups in the duration of clumping (Kruskall-Wallis $H= 1.3$, $p= 0.53$). Overall, males initiated very few episodes of clumping. However, both males and females preened longer when interacting with their mates then they did when the male was novel (with both paired and unpaired females; both Mann-Whitney U, $p< 0.05$).

The combined data, which allowed a more powerful analysis of the effect of pairing condition on the presentation of these behaviors, suggest the idea that clumping and preening may serve quite different functions. As clumping appears more frequently in unpaired individuals than preening, it is possible that this behavior indicates a more general level of social interaction. Clumping can be observed between sick individuals or juvenile siblings (Zann 1996), indicating it may represent something more social than primarily reproductive. Preening, in contrast, was not displayed between unpaired

individuals, but only after formation of a pair bond, suggesting it is more specific to pairing.

It is intriguing that no effect of group was observed in male-initiated clumping; indicating pairing status may affect males and females differently. However, males and females in the unpaired group experienced different situations prior to the test. It is possible that the longer durations of female-initiated clumping displayed by females that were unpaired prior to this interaction may have resulted from the reduced social interaction these females experienced during the two weeks prior to the test. The fact that the females differed from males in clumping but not preening, lends more support to the possibility that clumping might serve a more general social function than preening.

Neural correlates

The neurochemical results indicate that dopaminergic systems likely do not play a role in mediating the behavioral responses associated with pairing. Although females appeared to display higher levels of pairing behaviors when interacting with their mates, this effect did not correspond with a difference in dopaminergic activity in the striatum or nucleus accumbens. Several biological, as well as methodological explanations can be proposed for the lack of relationship between these behaviors and the activity of the dopaminergic systems.

First, zebra finches are highly social (Zann 1996), and separation from mates has both behavioral and physiological effects (Butterfield 1970; Remage-Healey et al. 2003). The effect of social isolation alone on stress and corticosterone levels has not

been examined in this species. However, the intense sociality of this species may modify how females respond to individuals following isolation, suggested by the behavioral differences between unpaired females and stimulus males in this study. It is possible that interaction with another individual following the short social isolation prior to the behavior test resulted in a dopaminergic response in all of the groups of females.

Second, dopamine responses may relate more to copulation than other aspects of bond formation or maintenance. In mammals, increased mesolimbic dopaminergic responses are typically observed after copulation (Mermelstein and Becker 1995; Pfaus et al. 1995; Gingrich et al. 2000). Studies in the prairie vole have examined the response of dopaminergic neurons following pair formation resulting from a period of copulation. Female prairie voles can form bonds in the absence of mating, if they cohabitate with an individual for long periods (24-48 hours; Williams et al. 1992) or have decreased levels of corticosterone (DeVries et al. 1995). However, the neural mechanisms mediating the formation of pair bonds in these cases have not been elucidated. In zebra finches, copulation does not appear to play an important role in bond formation, as it is rarely observed prior to the establishment of a pair bond (Zann 1996). As a result, the importance of the response of mesolimbic dopamine neurons may be reduced compared to what has been previously observed in prairie vole studies.

Third, the present study investigated the role of this system in mediating responses to individuals *after* formation of a pair bond, rather than during the formation of these bonds, which is commonly investigated in prairie voles. The formation of a pair bond results in increased levels of D-1 receptor binding in male prairie voles

(Aragona et al. 2006). A parallel change in receptors might occur in the zebra finch with bond formation, and if so, it would provide for selective dopamine response in the absence of a change in neurotransmitter release.

Finally, we cannot rule out the possibility that the timing of tissue collection might have affected the present results. We based the duration of behavioral exposure on a study by Sasaki et al (Sasaki et al. 2006), who observed increased DA levels in the striatum for 30 min when males produced directed song. Females interacted with the introduced male throughout the 30 min period in the present study. Thus, while we cannot be sure, it seems unlikely that any DA responses would have returned to baseline.

Conclusions and Future Directions

Female zebra finches exhibit specific changes in behavioral responses to males after they have formed a pair bond. The results indicate that preening and clumping may be separately mediated or indicate different motivational states – bonding for social purposes rather than bonding specifically for the purpose of reproduction, perhaps. Modifications of dopaminergic activity or turnover do not appear to be involved in these varied behavioral responses, however. That does not mean that DA plays no role; changes in receptor levels may be associated with pair bonds, which would increase responsiveness to DA release. This possibility should be explored in future studies. Another potential mechanism for the mediation of these behaviors involves other neurotransmitters or neuromodulators, such as arginine vasotocin, the avian homologue of vasopressin. This neuropeptide is implicated in social recognition (see Bielsky and

Young 2004) for review) and pair bonding (reviewed in Lim and Young 2006) in mammals, and may well regulate social responses in zebra finches. Vasotocin mediates aggressive behaviors (Goodson et al. 2004), and vasotocin-positive neurons in the bed nucleus of the stria terminalis respond when male zebra finches interact with females (Goodson et al. 2009). To fully elucidate mechanisms associated with formation and maintenance of pair bonds, it will be vital to examine the role of a variety of neurochemicals and associated molecules in bonding in both sexes and across diverse species.

Acknowledgements

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CHAPTER 6: DISCUSSION

The unique behaviors and characteristics of the zebra finch have provided an excellent opportunity to examine the intricate and complex interactions among social behavior, neural systems, and hormones. In the experiments conducted for this dissertation, I have investigated the presentation and regulation of two types of social behavior: song preferences and pair bonding in the female zebra finch. Females showed behavioral preferences for high value stimuli in both paradigms (exposure to tutored song or their partners). Also, I observed relationships between IEG expression and behaviors: (1) increased ZENK expression in response to tutored song with a parallel preference for that song in blank treated birds; and (2) a positive correlation between ZENK expression and clumping and preening behaviors in the nucleus taeniae. Estradiol treatment also reduced the induction of ZENK in the ventromedial hypothalamus. However, the neural correlates for song preference and pairing differed in that song preference corresponded with changes in the auditory perception regions, while pairing behaviors related to a region in the social behavior network, the nucleus taeniae. In this species, dopamine activity in the nucleus accumbens or striatum did not appear to relate to behavioral responses in either of the paradigms.

Two behavioral paradigms

I have verified that female zebra finches display behavioral preferences for certain social stimuli using two different behavioral paradigms. First, females spend more time near tutored than untutored song, and also display higher levels of clumping and preening towards their partners after a pair bond has been formed. These

preferences were strong and repeatable and fit with previous research on both song preference (Neubauer 1999; Lauay et al. 2004; Nolan and Hill 2004) and pair bonding (Silcox and Evans 1982; Clayton 1990; Zann 1996). To fully understand the regulation and mediation of these two behaviors, it is important to consider how they relate to one another.

In a zebra finch colony, single males court single females. This courtship includes directed singing, some dancing, and perhaps eventually copulation attempts by the male (Zann 1996). Early in this period, it is important for the female to acquire information from the male as to whether the courtship should continue to the formation of a pair bond. In the studies conducted for this dissertation, approximately 25% of the pairs housed together for two weeks did not form a pair bond (according to our criteria, defined in Chapters 4 and 5). One characteristic the female may utilize to make this decision is the quality of the male song, which can indicate reproductive fitness (Hasselquist et al. 1996; Buchanan and Catchpole 2000; Nowicki et al. 2002; Dolby et al. 2005). Females show preferences for high quality song (Neubauer 1999; Lauay et al. 2004; Searcy and Marler 1981; Ballentine et al. 2004; Nolan and Hill 2004), and male zebra finches producing poor quality songs are less likely to form pair bonds with females (*e.g.*, Tomaszycki and Adkins-Regan 2005).

After the male and female begin courtship, a pair bond can begin to form (Zann 1996), and the value of social stimuli may begin to change. Once a bond is formed a female will recognize her partner and present different behaviors towards him than to other males (Chapter 3 and 5, Silcox and Evans 1982). Females remain bonded with males surgically altered males that do not producing song or produce low quality song,

indicating that the importance of the quality of song is diminished after a pair bond has developed (Tomaszycki and Adkins-Regan 2006). Females can identify their mate's song (Vignal et al. 2008), but they may also utilize additional visual or tactile stimuli to recognize their mate. Thus, song preferences likely represent the first stage of courtship and pairing responses represent a later stage of reproduction, in which decisions about relative male quality have already been made.

Some important similarities between the two paradigms must be noted. The presentation of both responses to courtship song and pairing behavior is most likely mediated by the female making a determination of the value of the social stimulus. To make this determination, females in both situations may compare the new stimulus to a memory stored as a template. Female zebra finches isolated during a sensitive period do not display preferences for tutored song (Lauay et al. 2004), and juvenile zebra finches can memorize both conspecific and heterospecific song (Braaten et al. 2006; Braaten et al. 2008), indicating that templates are formed in development in these birds. The role of memory has been unexplored in terms of pair bonding in birds, but it is logical that a memory of one or more aspects (including visual, tactile and or behavioral cues) of the partner is formed and stored in the brain of the female and other males are compared to this template when females interact with them.

However, the types of stimuli differ. Song stimuli are simple auditory cues, whereas interactions with males involve a number of sensory stimuli that the female must interpret. Increased IEG responses to a variety of types of preferred song stimuli are observed in males, females, and juveniles (Bolhuis et al. 2001; Bailey and Wade 2003; Stripling et al. 2003; Bailey and Wade 2005; Tomaszycki et al. 2006), indicating

that these neural responses may be primarily sensory and may not involve high levels of integration or be uniquely associated with reproduction. A greater amount of integration is likely required to determine value of a stimulus in a social interaction with a variety of sensory inputs, such as that observed in the pair bonding paradigm. Thus, females may utilize different mechanisms to mediate the behavioral responses in these two paradigms.

Neural Correlates

Methodological Issues

Immediate early genes as indicators of neural response

IEG expression results can be somewhat difficult to interpret (see Pfaus and Heeb 1997 for discussion). At a basic level, IEGs indicate that a signal transduction event has occurred (Morgan and Curran 1989). The induction of IEGs can represent either excitation or inhibition in a specific brain region. IEG expression may indicate a role for a brain region in either the presentation of a behavior or the interpretation of sensory information. By coupling examination of IEG expression with the expression of a specific neurotransmitter within a brain region, researchers may better be able to determine the precise meaning of the IEG expression. In addition, more direct studies such as those using lesions, microdialysis or pharmacological treatments could be utilized in the implicated brain regions to verify their precise role in a behavior. Finally, lack of IEG expression does not completely rule out the possibility that a brain region may be involved in a behavior (see discussions in Hoffman et al. 1993; Pfaus and Heeb 1997; Hoffman and Lyo 2002; Kovacs 2008). IEG expression may be dissociated

from neuronal stimulation (*i.e.* Figueiredo et al. 2003). Additionally, specific IEGs (*e.g.* c-jun, c-fox, Arc) may be upregulated in different contexts (i.e. Guzowski et al. 2001; Bailey and Wade 2005).

In sum, IEG analyses provide excellent starting points for examining neural correlates of behavior, given that an investigator can efficiently examine a wide variety of regions and determine which, if any, might be involved in certain behaviors. However, due to the limitations in interpretation of IEG results, other methods are required to verify the role of certain brain regions and also to more closely investigate responses identified by immediate early gene induction.

Neurochemical analyses

In this dissertation, I followed up IEG studies with neurochemical investigations of the dopaminergic reward system. Dopamine was predicted to be important for the mediation of these behaviors, because of its role in motivation and reward. By examining neurochemistry in micropunches of specific brain regions, I was able to clearly investigate the response of dopaminergic regions in these systems and determine if dopaminergic activity in these regions played a role in song preference and pair bonding in this species. Neurochemical analyses, unlike IEG analyses, can provide direct measures of the activity of neurons in a specific region and allow predictions about neurotransmitter release. However, unlike microdialysis, these analyses only allow for the quantification at a specific time point, and if the neuronal activity is transient, then it is possible that rapid responses may be undetected.

Three Neural Systems

Social Behavior Network

Although some of the regions in this network displayed high constitutive levels of ZENK during the behavior tests, most did not exhibit selective ZENK responses during song presentation or interactions with males. Only in one region of this network, the nucleus taeniae (homologous to the mammalian medial amygdala, see Chapter 1), was a correlation with behavioral responses displayed towards female's partners. No regions in this network were associated with song preference.

There are several reasons why the social behavior network may be involved in mediating responses to interactions with male partners and not song preferences. First, song stimuli are much less complex than the multiple stimuli received during a full social interaction. Male zebra finches produce a fairly simple and stable song, and only one sensory modality is needed to interpret this stimulus. In addition, a strong connection may not exist between song preference and pair bonding. Male and juvenile songbirds also display song preferences (Clayton 1988; Calhoun et al. 1993; Braaten and Reynolds 1999; Houx and ten Cate 1999; 1999; Riebel et al. 2002; Riebel and Smallegange 2003), indicating they may be dissociated from reproduction and might instead simply reflect a general social response to the song stimulus. If song playback does not involve a clear reproductive response from the female, then the activation of the social behavior network during the presentation of this preference behavior may be more limited.

In the pairing paradigm, the situation is much more complex. Females are interpreting a much larger suite of social cues, including auditory, tactile, and visual stimuli. In addition, the behavioral response to the stimulus is varied between the two

paradigms used in this dissertation. In the song response studies, females presented a very limited repertoire of behaviors that primarily consisted of presentation of calls. In contrast, in the pairing paradigm females presented behaviors to the males including beak wipes, calls, and even copulatory solicitation displays. Perhaps the social behavior network is primarily utilized to mediate interpretation of more complex social stimuli, which require or reflect more complex behavioral responses.

The identification of the nucleus taeniae as an important node in the behavioral preference for partners in the zebra finch is novel. This idea parallels data from the mammalian homologue (medial amygdala), which is utilized in interpreting the emotional context of sensory stimuli (for review see Baxter and Murray 2002; Phelps and LeDoux 2005; Phelps 2006; Murray 2007). As the neural mediation of these types of behaviors in zebra finches has been largely unexplored, the nucleus taeniae will be an important region to further examine the neural mediation of reproduction and other social interactions in females.

Dopaminergic Systems

Responses to the social stimuli in the two paradigms investigated in this dissertation were not reflected by changes in dopaminergic responses, using either IEGs or neurochemistry. These results may indicate that dopaminergic systems are not important for determining the value of social stimuli or that they do not play a role in the behavioral responses I observed. Although dopaminergic systems have been implicated in reproductive behavior (reviewed in Mitchell and Gratton 1994; Melis and Argiolas 1995; Paredes and Agmo 2004), responses to reproductive stimuli (Mitchell

and Gratton 1994), and pair bonding (Gingrich et al. 2000) in mammals, there are some notable differences between mammalian model systems and zebra finches.

In mammal studies, increased dopaminergic activity is observed following copulatory behavior in males and female rodents (Pfaus et al. 1990; Pleim et al. 1990; Damsma et al. 1992; Pfaus et al. 1995; Kohlert and Meisel 1999). Sexually naïve individuals will demonstrate a lower level of dopamine release, which will increase with more sexual experience (Kohlert and Meisel 1999). In some cases, these responses can be observed following exposure to sensory stimuli alone without reproduction (i.e. Louilot et al. 1991; Damsma et al. 1992; Mitchell and Gratton 1994; Fabre-Nys et al. 1997). In the medial preoptic area, dopamine release in response to reproductive odors is not observed in prepubertal male hamsters (Schulz et al. 2003), indicating that a connection with copulatory behavior may be present for this release to occur. In contrast, both behavioral paradigms used in the studies in this dissertation have limited relationships with copulation. Song preferences may not relate to reproduction, but instead might reflect a general sensory preference for familiarity, which could occur at a number of levels including recognition of a member of the same species, a particular type of conspecific song previously heard (tutored vs. untutored), or one's father or mate. Similarly, pair bonding has a strong social component, perhaps indicated by the presentation of clumping behavior, and this bond may be somewhat dissociated from copulatory behavior, as well. As a result, these behaviors (song preference and pair bonding) may have reduced association with activity of the mesolimbic dopaminergic systems in female zebra finches.

Auditory Perception Regions

In the song preference paradigm, IEG results in blank-treated birds replicated a number of studies in which increased IEG expression is observed in the auditory perception regions in response to high quality or preferred song stimuli (*e.g.* Gentner et al. 2001; Bailey et al. 2002; Maney et al. 2003; Leitner et al. 2005; Sockman et al. 2005; Tomaszycki et al. 2006). Electrophysiological studies have verified that neurons within these regions display selective responses to high quality song (reviewed in Theunissen and Shaevitz 2006; Pinaud and Terleph 2008).

However, dissociation between IEG expression in these regions and behavioral preferences was also observed (Chapter 2). When females were estrogen treated, the selective neural response was eliminated without affecting the behavior preference for tutored song. In addition, although females identify and respond differently to their mate's song (Vignal et al. 2008), IEG expression in the auditory regions did not differ between exposures to song produced by different males in the pair bonding study (Chapter 4). Both of these studies (Chapter 2 and 4) demonstrate that a selective IEG response may not be required for the presentation of a behavioral preference for tutored song or a familiar mate. It may be that these preferences are so pervasive and critical that redundancy in their neural mediation exists. Thus, a preference may be displayed regardless of a differential neural response in the auditory perception regions specifically.

Neural systems summary

For this dissertation, I proposed that components in three neural systems might interact in a number of ways to mediate behavioral responses to social stimuli. The results of the four studies indicate that components of the social behavior network, the

nucleus taeniae in particular, and auditory perception sites may act independently to mediate behavioral responses in both of the behavioral paradigms. It is likely, however, that additional brain areas that were not investigated play some role. Presumably, the presentation of complex behavioral responses requires the coordination of several brain regions and systems, although that was not detected in these studies. In addition to evaluating more sites, further work using other techniques or modifications to these paradigms may help in elucidating some of these more complex relationships between neural systems.

Both the auditory perception sites and the nucleus taeniae/amygdala have been implicated in memory processes using immediate gene induction as a marker (McGaugh 2004; Richter-Levin 2004; Gobes and Bolhuis 2007). Thus, they may be utilized to determine the salience of a stimulus, perhaps through accessing some type of memory. Immediate early genes have been implicated in the formation of memories (reviewed in Tischmeyer and Grimm 1999). IEG expression is observed during learning processes (Anokhin and Rose 1991; Brennan et al. 1992), and antagonism of immediate early gene with antisense-oligonucleotides can block learning and association in rodents (Tischmeyer et al. 1994; Lamprecht and Dudai 1996; Swank et al. 1996). The connection with memory formation fits with the hypothesis that the behavioral responses I observed result from the connection of a stimulus with a memory template. To more fully investigate the neural mediation of these behaviors, it would be prudent to utilize the expression of several immediate early genes such as c-fos or c-jun, or take a more broad approach by measuring responses of additional neurochemicals within several brain regions.

Role of Estrogen in Brain and Behavior

Estrogen did not affect preference for tutored song, but treatment resulted in decreased long distance calls and visual scanning behavior. These data may be connected with the estradiol-induced decrease of ZENK in the ventromedial hypothalamus. The ventromedial hypothalamus contains estrogen receptors in zebra finches (Gahr et al. 1993; Jacobs et al. 1996), suggesting that the hormone can act there directly. This change in ZENK expression may be associated with increased receptivity, perhaps due to disinhibition in this brain region (see Commons and Pfaff 2001). This function might enable the female to focus more on reproductive behaviors or stimuli than song. Alternatively, the reduction in ZENK expression in the ventromedial hypothalamus could be due to the novel conditions in which the testing occurred. In mammals, estradiol can modulate the binding of oxytocin in the ventromedial hypothalamus (reviewed in Johnson 1992), which has been implicated in the reduction of anxiety (see McCarthy 1995; Neumann 2008 for review). However, estradiol-treated female rats infused with oxytocin into the ventromedial hypothalamus display reduced sexual behaviors, without a decrease in anxiety-related behaviors (Bale et al. 2001). At this point, the role of estradiol and the ventromedial hypothalamus in the mediation of anxiety behavior in songbirds is unknown. It will be important to test whether their interaction contributes directly to behavioral changes.

Additionally, estrogen reduced the selective ZENK response in the auditory perception regions. This result parallels the estrogen induced decrease of ZENK in the ventromedial hypothalamus (see above). Perhaps these neural responses are linked and reflect a shift from a focus on the details of song to receptive behavior once a mate has

been selected. Although NCM and CMM have very few estrogen receptors, the auditory system is connected to the ventromedial hypothalamus (Cheng and Peng 1997), and mediation by estradiol may occur through this connection.

The uniqueness of the zebra finch model

The work presented here highlights some of the unique qualities of the zebra finch model and provide a number of directions to be explored in future research in this model.

First, as zebra finches are opportunistic breeders, it is possible that the role of estrogen in mediating their behaviors may be reduced. The song preference studies indicate that this may be this case, as behavior was completely unaffected by estrogen treatment. It is unknown at this time how estrogen levels fluctuate in female zebra finches. Regardless of reproductive state, measures of GnRH activity appear to remain at a relatively high level in male zebra finches (Perfito et al. 2006). A similar phenomenon may also be observed in females.

The regulation of song preference behavior by estradiol may also be reduced by the fact that zebra finches form pair bonds prior to reproduction. If estradiol levels fluctuate in this species, they are likely to be highest just prior to egg-laying. As song preference does not appear to be critical during this time period, it is unlikely that these behaviors are mediated by changes in estradiol.

The pair bond in this species also has some unique characteristics that are important to investigate further. While bonds are typically formed for reproduction, they also may serve other purposes related more generally to sociality. The experiments

conducted in this dissertation shed light onto this distinction, indicating that clumping behavior may be a less specific social behavior, whereas preening is likely more specific to pair bonded individuals.

Conclusions and Future Directions

Using IEGs, brain regions (the auditory perception regions and the nucleus taeniae) were identified that may play a role in the presentation of song preference and pair bonding behaviors. Since IEG results can be difficult to interpret, a more direct investigation must be conducted in these brain regions to determine the meaning of the IEG response. Co-localization studies with specific neuropeptides and/or neurotransmitters in these regions should eventually be conducted in these regions to identify the specific factors regulating these neural responses and behaviors. Some brain regions that were not examined in this dissertation may also be investigated, including the lateral septum (which has been implicated in social behaviors in mammals and birds; Insel et al. 1994; Wang et al. 1996; Goodson et al. 2005) and the prefrontal cortex (which is a site of dopamine release; Bannon and Roth 1983). The caudolateral nidopallium (NCL) in birds is considered analogous to the prefrontal cortex, based on function and anatomy (Mogensen and Divac 1982; Reiner et al. 2004a). Perhaps some of these other neural systems are vital to the overall neural coordination of these types of social responses. They, and their potential interactions with the three circuits investigated, should be considered further.

Although dopamine activity in the reward system does not appear to play a role in either of these behaviors, there are some other neurochemicals on which future

research can focus. For example, vasopressin has been implicated in pairing behaviors in mammals (see Lim et al. 2004), and its avian homologue, arginine vasotocin, also plays a role in mediating social behaviors in avian species (Goodson et al. 2004; Goodson et al. 2009). It is also critical to social recognition (Bielsky and Young 2004), and this behavior may be important to both song preference and pair bonding in the zebra finch. Vasotocin receptor knockout mice have difficulty recognizing conspecifics (Bielsky et al. 2004). These receptors are found in the nucleus taeniae in birds (Voorhuis and de Kloet 1992; Aste et al. 1996), and action of vasotocin in this region may be critical for the recognition of partners by paired females. Oxytocin is also critical for social recognition (see Bielsky and Young 2004), and recognition of mate quality as oxytocin knockout mice cannot distinguish between healthy and parasitized males (Kavaliers et al. 2003). Oxytocin has also been implicated in mediating pair bonding behaviors in prairie voles (Insel and Hulihan 1995), although its role has only been examined in the context of mating-induced pair formation. Mesotocin, the avian homologue of oxytocin, may function as oxytocin does in mammals. Goodson et al. (2004) found no role for vasotocin and mesotocin in individual recognition in male and female zebra finches, but the cohabitation time used in their study was much shorter than is usually required for the formation of a bond in this species.

One other molecule, norepinephrine, might be also be critical in regulation of these behaviors. Norepinephrine is important for attention and focusing on salient information (reviewed in Berridge and Waterhouse 2003), which is critical to the presentation of preference in both of the behavioral paradigms examined in this dissertation. Mammalian studies have demonstrated a role for norepinephrine in

increasing the magnitude and latency of responses to specific stimuli and perhaps reducing spontaneous neuronal firing (reviewed in Berridge and Waterhouse 2003), which can result in the ability to distinguish salient information from noise. In addition, norepinephrine can enhance long-term potentiation (see Berridge and Waterhouse 2003) and mediates emotional memory formation in the amygdala (Cahill and McGaugh 1996). In birds, norepinephrine mediates the ability of females to respond to song when auditory distracters are present (Appeltants et al. 2002), and its release and innervation is increased after long-term exposure to high quality song (Sockman and Salvante 2008).

In conclusion, in these studies I have noted several unique behavioral characteristics of zebra finches. In addition, I have identified a brain region (the nucleus taeniae) that may be critical for the maintenance or response to pairing in female zebra finches. Finally, I observed an effect of estrogen on calling and visual scanning behavior and response of the auditory perception systems and the ventromedial hypothalamus. All of these results are novel, and warrant future investigation to more fully elucidate the mechanisms associated with mate choice and subsequent responses once a partnership has been formed.

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