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Hormone-Dependent Modulation of Auditory Processing and Selectivity in a Seasonally Breeding Songbird

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Abstract

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By Lisa L. Matragrano

Behavioral responses to sociosexual signals change significantly according to gonadal hormone levels. These changes may be partially explained by hormone-dependent modulation of sensory processing, which has been described in seasonally breeding songbirds. In female white-throated sparrows, sound-induced expression of immediate early genes (IEGs) in auditory areas is selective for song over behaviorally irrelevant tones only when plasma estradiol reaches breeding-typical levels. Here, I explored the neurochemical mechanisms underlying steroid-dependent plasticity in the auditory system in this species. I hypothesized that the effects of estradiol in sensory areas are mediated by monoamine systems, which are sensitive to gonadal steroids and involved in sensory processing and selective attention. Specifically, I tested whether seasonal changes in plasma levels of gonadal steroids modify monoaminergic activity in auditory areas and whether those changes are associated with changes in neural responses to behaviorally relevant stimuli. Estradiol treatment of females increased catecholaminergic and serotonergic innervation of the same areas of the auditory system in which estradiol promotes selective IEG responses, and increased levels of norepinephrine and a serotonin metabolite in the auditory forebrain. This result is consistent with the hypothesis that estradiol primes the auditory system *via* monoamines. In order to test whether monoaminergic influences on auditory responses may be due to sound-induced release, I examined whether exposure to song playback causes rapid changes in monoaminergic activity. I detected changes in monoamine synthesis and release in the auditory forebrain of estrogen-primed females after as little as 15 minutes of song, demonstrating that the monoaminergic cells innervating this region can respond rapidly to this sound and that they may play an active role in the modulation of auditory responses. Finally, I tested whether seasonal changes in gonadal steroid levels may affect auditory responses in males of the same species. Similar to estradiol in females, testosterone induced selective IEG responses in the auditory forebrain in males. Unlike estradiol, which increased monoaminergic innervation of auditory areas in females, testosterone decreased it in males. These studies show that gonadal hormones alter the selectivity of auditory IEG responses and may modulate monoaminergic activity in the auditory system in both sexes.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 THE ROLE OF HORMONES IN SENSORY PROCESSING IN VERTEBRATES

Because animals are exposed to hundreds of stimuli in the environment, the ability to extract relevant information from a barrage of stimuli is necessary for reproduction and survival. Sensory information enters the central nervous system by way of pathways that are specialized to extract and interpret that information. Every level of a sensory system receives modulatory input from other brain areas, which can alter the processing of sensory signals according to internal state. Increasing evidence has led to the hypothesis that endocrine state in particular, *via* hormonal modulation of sensory processing, influences neural and behavioral responses to a stimulus (reviewed by Maney & Pinaud, 2011 and Miranda & Liu, 2009).

Beach (1948) put forth the hypotheses that gonadal hormones such as estradiol (E2) and testosterone (T) determine whether an organism responds to a particular sensory stimulus and that these hormones can alter the frequency and intensity of the response. Since then, researchers in the field have described hormonal influences on sensory systems and behavioral responses in a number of animals including fish, frogs, birds, and mammals (Coleman et al., 1994; De Groof et al., 2010; Reeves et al., 2003; reviewed by Maney & Pinaud, 2011). It is clear that hormones have profound effects on sensory systems, including the auditory system.

In many species, plasma levels of reproductive hormones increase and decrease from season to season or from estrous to anestrous states. High levels of E2 in females and T in males are commonly associated with sexual activity and breeding. Changes in plasma levels of sex steroids may in turn affect an animal's perception of incoming stimuli and the animal's decisions regarding reproduction. In some species, for example, female mate choice is based largely on male vocalizations or other auditory cues, and females of those species display hormone-dependent preferences for conspecific male vocalizations (Puts, 2005; Sisneros, 2009; Maney & Pinaud, 2011). How a female perceives and processes the auditory signal may therefore be affected by her level of plasma E2 (reviewed by Maney, 2010).

Increases in plasma levels of gonadal steroids during periods of increased fertility may underlie plasticity of neuronal and behavioral responses to conspecific vocalizations during the breeding season. E2 and T may affect auditory processing in a number of ways, including enhancement of hearing and attention. This phenomenon may be relevant to non-seasonal breeders, such as humans. For example, hearing thresholds and efficiency were improved in women with naturally high E2 or following estrogen-replacement therapy (Davis & Ahoon, 1982; McFadden, 1998). In addition, women with low levels of E2, including women with Ullrich-Turner syndrome or who were postmenopausal, exhibited longer latencies in auditory evoked responses and a higher prevalence of hearing problems (Caruso et al., 2003; Gungor et al., 2000; Hultcrantz & Sylven, 1997; Serra et al., 2003). Similarly, female rhesus monkeys treated with exogenous estrogen showed shorter peak latencies in the auditory brainstem response (Golub et al., 2004).

Gonadal hormones might enhance higher level auditory processing and attention as well. T treatment increased sexual arousal to and enjoyment of auditory sexual stimuli in both eugonadal and hypogonadal men (Alexander et al., 1997). In women, higher levels of plasma E2 were correlated with greater attentional bias toward courtship statements (e.g., "I saw you across the room and think you're so beautiful") than neutral statements (e.g., "I was hoping you could tutor me in this class I have"; Rosen & López, 2009). Other research in humans suggests that preferences for the physical characteristics of sound itself may also change according to ovarian hormone level. During the phase of the ovarian cycle when E2 levels are highest and women are most fertile, women were more attracted to men with lower-pitched voices than to men with higher-pitched voices (Feinberg et al., 2006; Puts, 2005). Clearly, hormones enhance auditory processing but the underlying mechanisms are unclear. The techniques and procedures that can be employed in non-human primates and humans are limited and therefore an appropriate model system might be used to understand hormone-dependent plasticity and mechanisms underlying auditory processing.

1.2 SEASONALLY BREEDING SONGBIRDS AS A MODEL SYSTEM

Songbirds are a prominent model organism for studying a number of biological functions, particularly those related to vocal communication (Zeigler & Marler, 2008). The most commonly used species in songbird research is the Australian zebra finch (*Taeniopygia guttata*), an opportunistic breeder. Opportunistic breeders can begin breeding year-round

as long as the environmental conditions are favorable. To time reproductive effort, opportunistic breeders depend on short-term cues that signal optimal environmental conditions, such as rainfall, food abundance, and temperature (Davies, 1977; Zann, 1996) and seasonal breeders depend, predominantly, on day length to signal the breeding season (Bentley et al., 2000; Marshall & Serventy, 1958; Oksche et al., 1963). Seasonal breeders show marked fluctuations in gonadal hormone levels annually (Farner & Lewis, 1971; Wingfield & Farner, 1978, 1980); therefore seasonal breeders may provide valuable models for the study of hormone-induced plasticity, particularly as a model for animals that also have fixed reproductive cycles such as the annual cycle of some lizards, birds, and fish or the monthly cycle in some female mammals including rodents, primates, and humans.

The research in this dissertation will concentrate on a seasonally breeding species of songbird, the white-throated sparrow (*Zonotrichia albicollis*). Like in other seasonally breeding songbirds, in this species gonad volume and hormone levels increase during the breeding season and decrease after it ends (reviewed by Soma, 2006 and Wingfield & Farner, 1993). Plasma levels of E2 in females and T in males are highest during the spring, or breeding season, and are basal or nondetectable during the fall, or non-breeding season (reviewed by Wingfield & Farner, 1993). In the laboratory, plasma levels can be artificially elevated to those typical of the breeding season within 2 days (Moore, 1983), making this species a convenient model for studying seasonal plasticity.

Songbirds such as white-throated sparrows are good model systems for studying the auditory system because we can perform song playback experiments in the field and in the laboratory. In the wild, males sing during the breeding season when T levels are highest to attract females and defend territories; however males in the fall, or non-breeding season, do not sing in order to attract females nor to defend territories. Females' behaviors and particularly their responses to song are seasonally dependent as well. During the spring when E2 levels are highest, females respond to hearing male song with a copulation solicitation display (CSD) characterized by a wing quiver, trill, and tail lift. This display can be elicited in laboratory settings as well. Hearing a recording of male song elicits no display during the non-breeding season, but a female in non-breeding condition that is treated with E2 will produce a CSD when exposed to the same recording (Kern & King, 1972; Moore, 1983; Searcy & Marler, 1981). Many questions about how birds perceive and process sounds can be addressed with playback experiments in the laboratory because male song can elicit the behavioral and physiological responses observed in the wild.

The avian and mammalian auditory systems are anatomically and functionally similar (Butler & Hodos, 1996). Each subcortical auditory area in the mammalian brain has a homolog in the avian brain, and the pattern of connections from the cochlear nucleus to the auditory cortex/forebrain is the same in mammals and birds (reviewed by Theunissen et al., 2004). In vertebrates the auditory pathway begins in the cochlea, in which auditory information is first transduced. Auditory information then travels from the inner ear to the cochlear nucleus (CN) of the central auditory system (reviewed by Theunissen &

Shaevitz, 2006). The pathway ascends from CN to the auditory midbrain (the dorsal lateral nucleus of the mesencephalon; MLd), the avian homologue of the inferior colliculus (Karten, 1967). Similar to mammals, birds have both direct and indirect routes connecting CN and the auditory midbrain. Some afferents extend from CN to the superior olive to MLd and others extend from CN directly to the auditory midbrain. In birds MLd projects to the diencephalic nucleus Ovoidalis (n. Ov) within the thalamus, the avian homologue of the medial geniculate nucleus in mammals (Brauth et al., 1987; Karten, 1967).

The auditory pathway continues from Ov to Field L in the auditory forebrain. Field L has been divided into subdivisions that are based on their connectivity (L1, L2a, L2b, L3, and L; Fortune & Margoliash, 1992). It projects to the caudomedial nidopallium (NCM) and the caudomedial mesopallium (CMM), regions of the auditory forebrain that share reciprocal connectivity (Brauth et al., 1987; Karten, 1967; Kelley & Nottebohm, 1979). NCM and CMM are often compared to the superficial layers of the auditory cortex (Theunissen & Shaevitz, 2006) or to secondary mammalian auditory regions, such as Wernicke's area in humans (Jarvis, 2004). Much like Field L, NCM has been divided into subdivisions. It is most often divided into dorsal and ventral domains (Avey et al., 2005, 2008; Eda-Fujiwara et al., 2003; Gentner et al., 2001; Hernandez & MacDougall-Shackleton, 2004; Lynch & Ball, 2008; Maney et al., 2003; McKenzie et al., 2006; Phillmore et al., 2003; Sockman et al., 2005; Tomaszycki et al., 2006; Sockman & Salvante, 2008; Velho & Mello, 2008). Some authors have argued, however, that the connectivity, gene expression, and other neurochemical characteristics of the region

suggest that it should instead be divided into rostral and caudomedial domains (reviewed by Maney & Pinaud, 2011; for further discussion, see Chapters 2 & 3). In this research I take both conventions into consideration when possible and appropriate.

1.3 HORMONAL EFFECTS ON THE AUDITORY SYSTEM IN SONGBIRDS

Gonadal steroids induce marked changes in behavioral responses to conspecific song, (Kern & King, 1972; Moore, 1983; Searcy & Marler, 1981). This hormone-dependent behavioral plasticity suggests that gonadal hormones may alter the perceived behavioral context or salience of the auditory cue (Maney et al., 2006). Recent research has provided evidence that seasonal changes in gonadal hormone levels may alter the physiology and neurochemistry of auditory circuitry (reviewed by Maney & Pinaud, 2011). E2 induces the expression of enzymes and genes involved in synaptic plasticity (reviewed by Maney & Pinaud, 2011; Fusani et al., 2000), for example, application of E2 directly to NCM triggers rapid induction of immediate early genes (IEGs) such as *zenk* (*egr-1, NGFI-A, and krox-24*), a zinc-finger transcription factor (Tremere et al., 2009). In female white-throated sparrows, systemic E2 treatment increased the expression of ZENK, the protein product of *zenk*, in the auditory forebrain independently of sound exposure (Sanford et al., 2010). E2 therefore seems to engage processes involved in synaptic remodeling, particularly in the auditory forebrain.

The studies described above show that E2 can directly induce expression of IEGs in auditory areas; it also can modulate IEG responses to sound. IEG expression in the

auditory forebrain in response to sound stimuli has been well-described in songbirds (Bailey et al., 2002; Mello & Clayton, 1994; Stripling et al., 2001). Many independent studies have shown that, in NCM in particular, the magnitude of IEG responses is proportional to the behavioral relevance of the stimulus (e.g., Gentner et al., 2001; Sockman et al., 2002; Maney et al., 2003; Terpstra et al., 2004, 2006). Because conspecific male song is more behaviorally relevant to a female when she is in breeding condition, we have hypothesized that E2 may affect song-induced ZENK responses in NCM. In fact, we have demonstrated that in females in non-breeding condition, the ZENK response to song is not different than that to frequency-matched tones; in E2-treated birds, however, the ZENK response to song is significantly higher than that to tones. We refer to this E2-induced differential response as "selectivity" in that the ZENK response in the case of E2-treated females is selective for song over tones. Note that although the term "selective" is more commonly used in reference to a single neuron's response to stimulation, here it is used to compare responses of groups of neurons in different individuals.

To show evidence for E2-induced selectivity, females in non-breeding condition, and therefore with low plasma E2, were implanted with silastic capsules containing E2 or nothing (blank). After seven days, they heard male song or an irrelevant control sound, frequency-matched tones. The ZENK response in NCM and CMM was selective for song over tones in the E2-treated females (Maney et al., 2006; Sanford et al., 2010); however in females with blank capsules, the ZENK response to song was indistinguishable from the response to tones. Similarly, in Ov and MLd, the response to song was significantly

higher than the response to tones in the E2-treated females, but this difference was not significant in the blank-implanted females (Maney et al., 2006; Maney & Pinaud, 2011). It appears that E2 altered sound-induced responses in NCM, making the responses more selective for behaviorally-relevant sounds.

NCM is a large, heterogeneous region, and Sanford et al. (2010) reasoned that the effects of E2 may not be homogenous within it. In order to map the effects of E2 on auditory ZENK responses, they conducted a second playback study to quantify the effects of E2 on the ZENK response in five separate domains of NCM. E2 treatment alone, in the absence of a sound stimulus, increased ZENK expression in three rostral but not two caudal domains. Likewise, in the same rostral domains, the ZENK response was selective for song over tones only in the females treated with E2. In the caudal domains, E2 did not induce a ZENK response independently of sound, and the ZENK response was selective for song over tones regardless of hormone treatment. These findings provided the first map of E2-sensitive functional domains in NCM, and will help guide our explorations of the mechanisms underlying E2-dependent auditory plasticity in this region.

1.4 HORMONAL EFFECTS ON MONOAMINES AND THE ROLE OF MONOAMINES IN AUDITORY SELECTIVITY

Monoamines, specifically catecholamines and serotonin, are believed to shape responses in auditory neurons, thereby inducing selectivity and influencing auditory processing (reviewed by Castelino & Schmidt, 2010, Hurley et al., 2004, and Maney & Pinaud,

2011). The avian catecholaminergic and serotonergic systems have been extensively mapped using nonsongbirds (Bailhache & Balthazart, 1993; Challet et al., 1996; Cozzi et al., 1991; Dube & Parent, 1981; Fuxe & Ljunggren, 1965; Ikeda et al., 1971; Kaiser & Covey, 1997; Metzger et al., 2002; Reiner et al., 1994; Yamada et al., 1984). The monoaminergic cell bodies in the brainstem, including dopaminergic neurons in the substantia nigra, the ventral tegmental area, and the periaqueductal gray, noradrenergic neurons in the locus coeruleus, and serotonergic neurons in the raphe nuclei, project throughout the diencephalon and telencephalon including to the auditory midbrain, thalamus, and cortex in both the mammalian and avian brains (reviewed by Castelino & Schmidt, 2010; Appeltants et al., 2000; 2002; Balthazart & Ball, 1996; Dahlstrom & Fuxe, 1964; Durstewitz et al., 1999; Hurley & Thompson, 2001; Ikeda et al., 1971; Joel & Weiner, 2000; Klepper & Herbert, 1991; Levitt & Moore 1979; Olazàbal and Moore 1989; Reiner et al., 2004; Rouiller et al., 1989; Steinbusch, 1981; Yamada et al., 1984).

Catecholamines such as norepinephrine are involved in sensory processing and behavioral responses in both birds and mammals (reviewed by Durstewitz et al., 1999). These neuromodulators shape response properties of sensory networks involved in selective attention (reviewed by Aston-Jones & Cohen, 2005 and Hurley et al., 2004) and context-dependent modulation of forebrain plasticity (Bao et al., 2001; Cardin & Schmidt, 2004; Castelino & Ball, 2005; Cirelli & Tononi, 2004; Cirelli et al., 1996; Dave et al., 1998). In songbirds, catecholaminergic projections innervating the forebrain have been hypothesized to affect the auditory processing of sociosexual signals such as song (e.g., Appeltants et al., 2002a,b; Appeltants et al., 2005; Bharati & Goodson, 2006;

LeBlanc et al., 2007; Maney & Ball, 2003; Riters & Pawlisch, 2007). Norepinephrine, for example, enhances auditory responsiveness in the song system and may play an important role in attention and auditory processing (Cardin & Schmidt, 2003; 2004). In addition, noradrenergic denervation of the forebrain can reduce the behavioral and neural responses to and selectivity for song (Appeltants et al., 2002b; Lynch & Ball, 2008; Vyas et al., 2008).

Like norepinephrine, serotonin alters neural responses to conspecific vocalizations (Hurley & Pollack, 2005). Serotonin is released in the auditory midbrain in response to sound presentation (Hall et al., 2010) and increases auditory selectivity for conspecific vocalizations in the same brain regions (Hurley & Pollack, 2005). The role of serotonin in general has not been a focus in analogous areas in the songbird; therefore the role of the serotonergic system in the auditory pathway of songbirds is poorly understood.

Because monoamines appear to increase auditory selectivity, shape response properties, and sharpen auditory responses (reviewed by Aston-Jones & Cohen, 2005, Durstewitz et al., 1999, and Hurley et al., 2004), and because they are already thought to convey information about internal state and environmental context to the auditory system (Hurley & Hall, 2011; Hurley et al., 2004), I hypothesize that they may be involved in seasonal changes in auditory ZENK responses. Fig. 1 shows a possible model by which they may mediate the effects of gonadal steroids on auditory selectivity. Gonadal steroids may induce auditory selectivity for sociosexual signals by acting on the monoaminergic systems that innervate auditory areas.

Gonadal hormones have a variety of effects on monoamines and monoaminergic neurons, fibers, and other biomarkers. In female rhesus monkeys, for example, ovariectomy decreased the density of axons immunoreactive for a catecholaminergic enzyme, tyrosine hydroxylase (TH) in the prefrontal cortex, a region involved in selective attention (Kritzer & Kohama, 1998), and reduced the density of both TH- and serotonin-immunoreactive fibers in the basal ganglia (Kritzer et al., 2003). Replacing ovarian hormones restored immunoreactivity to normal levels in both studies. In male rats, gonadectomy decreased the density of axons immunoreactive for TH in the anterior cingulate and primary motor cortices, and T increased the immunoreactivity to normal levels (Kritzer, 2000). In the prefrontal cortex of the same species, androgens seem to have the opposite effect: gonadectomy increased TH immunoreactivity, which was reduced by T or DHT treatment (Kritzer, 2003).

Gonadal steroids may act directly on monoaminergic cell bodies (Fig. 1). Estrogen receptors (ERs) are expressed in cells in the ventral tegmental area, periaqueductal gray, locus coeruleus, and raphe nuclei (Alves et al., 1998; Maney et al., 2001; Sheng et al., 2004; Shughrue et al., 1997; Simerly et al., 1990), and androgen receptors are present in cells of all of these regions as well as in the substantia nigra (Maney et al., 2001; Sheng et al., 2004; Simerly et al., 1990). Gonadal hormones therefore may bind to receptors in the brainstem and modulate monoaminergic activity in areas such as the auditory forebrain, thalamus, and midbrain. Sex steroids can change monoaminergic synthesis, release, and

metabolism (reviewed by McEwen, 2002), and thereby have widespread actions in every region of the brain.

Because the auditory system is sensitive to monoaminergic input (Bao et al., 2001; Hurley et al., 2004) and because that input is itself sensitive to gonadal steroid level (Kritzer & Kohama 1998; 1999; Kritzer 2000; 2003), it is possible that the effects of gonadal steroids on auditory function may be mediated *via* monoamine systems (Fig. 1). In white-throated sparrows, E2 treatment increases the number of TH-immunoreactive cells in the ventral tegmental area and locus coeruleus (LeBlanc et al., 2007). Both the ventral tegmental area and locus coeruleus contain androgen receptors and ERs (Maney et al., 2001) and could be affected directly by T and E2. In songbirds, E2 increases catecholamine turnover in the auditory forebrain (Barclay & Harding, 1990) and increases the density of TH-immunoreactive fibers in NCM (LeBlanc et al., 2007). Furthermore, the effects of E2 treatment on the selectivity of the ZENK response occur in some of the same regions as E2-induced increases in the number of catecholaminergic cells (LeBlanc et al., 2007); which is consistent with a model wherein E2 mediates auditory selectivity *via* effects on catecholaminergic cells. It should be noted however that because NCM contains ERs, E2 can act directly in NCM (Fig. 1). Any effects of E2 on other auditory areas, however, are likely indirect and could result from indirect actions of catecholaminergic neurons in the brainstem. Androgen receptors are not expressed in the auditory forebrain or midbrain (Balthazart et al., 1992; Fusani et al., 2000; Zigmond et al., 1973). Thus it is unlikely that T is acting directly on the auditory forebrain in the same way that E2 might be, but instead T likely acts indirectly.

As is the case for catecholamines, the release and reuptake of serotonin appear to be regulated by gonadal hormones in mammals. E2 has a predominantly positive effect on serotonin biomarkers. E2 treatment of ovariectomized rats, for example, increased serotonin content in the dorsal raphe (Cone et al., 1981), an area rich in ERs (Alves et al., 1998; Sheng et al., 2004). E2 may elicit such effects by increasing levels of the serotonin synthetic enzyme, tryptophan hydroxylase (TPH). In ovariectomized rats, E2 treatment increased TPH mRNA expression in the dorsal raphe (Donner & Handa, 2009; Hiroi et al., 2006) and elevated TPH protein in the same region in rhesus monkeys (Bethea et al., 2000). Overall, however, the effects of E2 on serotonin markers may depend somewhat on the species and the brain region. In ovariectomized rats, E2 treatment increased serotonin turnover in the hippocampus and nucleus accumbens but many other regions were not affected (Pandaranandaka et al., 2009). Serotonergic fiber density, serotonin, and the serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA) decreased after E2 treatment in both the hypothalamus and medial preoptic area of ovariectomized rats (Lu et al., 1998). In ovariectomized rhesus macaques, E2 treatment increased serotonergic fiber density in some regions but had no effect in others (Kritzer et al., 2003; Lu et al., 2003). Ovariectomy reduced the density of serotonin-immunoreactive fibers in the basal ganglia and replacing ovarian hormones restored immunoreactivity to normal levels (Kritzer et al., 2003). In female spiny lizards, E2 treatment increased serotonin and 5-HIAA in the lateral septum but, again, not in all regions of interest (Woodley et al., 2000).

Whereas the effects of E2 on serotonin markers are generally stimulatory, the effects of T may be generally inhibitory. In male hamsters, for example, T treatment decreased the density of serotonin-immunoreactive fibers in the hypothalamus and amygdala (Grimes & Melloni, 2002; 2006). T treatment in male rats decreased serotonin, 5-HIAA, and SERT mRNA in the diencephalon (Gabriel et al., 1988; Martinez-Conde et al., 1985; McQueen et al., 1999). As is the case for E2, however, these effects were not uniform throughout the brain and there were many regions in which serotonin markers were unaffected by T treatment (e.g., see Kritzer et al., 2003). We cannot therefore predict *a priori* whether E2 or T will have stimulatory or inhibitory effects on serotonergic fiber density in sparrows. A stimulatory effect of E2 and an inhibitory effect of T would be consistent with most of the mammalian literature.

1.5 POSSIBLE HORMONAL INFLUENCES ON AUDITORY RESPONSES

I hypothesize that gonadal steroids may alter auditory responses by acting on monoamine systems. This regulation could occur by two independent mechanisms. First, hormones may increase the density of monoaminergic fibers that can release monoamines in a nonsynaptic, or paracrine, fashion. This paracrine release of monoamines might alter the response properties of neurons in auditory areas (reviewed by Beaudet & Descarries, 1978). Such changes in neurochemical makeup can be sustained over a period of time, for example during a period of high fertility, such that auditory areas may respond differently to the same sociosexual signal depending on reproductive state. In Chapter 2, I show evidence that E2 increases the density of monoaminergic innervation, and thus likely the

amount of available monoamines, in the auditory forebrain and midbrain of female white-throated sparrows.

If the role of monoamines in steroid-dependent auditory selectivity is nonsynaptic only, the monoaminergic neurons in the brainstem that project to these auditory areas need not necessarily respond to song. The slow, tonic release of monoamines may alone explain increases in selectivity. If, however, monoaminergic neurons play a more active role, for example modulating responses in the auditory pathway during sound processing, then we would expect to see evidence of this rapid engagement. Previously, the only evidence of such engagement in songbirds came from IEG studies. Maney et al. (2008) showed, for example, that hearing conspecific song induces ZENK expression in catecholaminergic areas in the brainstem. Gale and Perkel (2010) likewise showed that in male zebra finches, dopaminergic cells in the VTA fire in response to hearing song. In Chapter 3, I show evidence that hearing song has rapid effects on monoamine synthesis and release in NCM. Activity in these systems may therefore occur rapidly, in direct response to sound stimulation.

It is possible that both synaptic and nonsynaptic mechanisms of monoamine action are important. Gonadal hormones may increase nonsynaptic release of monoamines, thus priming the auditory pathway to respond to sociosexual signals in a context- and season-dependent manner. In addition, hearing song may rapidly induce monoaminergic release at synapses in the auditory forebrain, thalamus, or midbrain (Fig. 1), causing direct and rapid effects on auditory responses. Taken together, Chapters 2 and 3 may show evidence

that both mechanisms are important for seasonal regulation of auditory function in white-throated sparrows.

1.6 CONCLUSIONS AND DISSERTATION GOALS

This review argues that sex steroids alter selective responses to social signals by stimulating or inhibiting the production of neuromodulators, specifically monoamines. In this study, I begin to test the model proposed here (Fig. 1) and investigate the possible mechanisms underlying the effects of gonadal steroids on the selectivity of auditory ZENK responses. First, I test the hypothesis that seasonal changes in plasma levels of gonadal steroids modify monoaminergic activity in auditory areas in female white-throated sparrows. More specifically, in Chapter 2, I investigate whether breeding-typical levels of plasma E2 increase monoaminergic innervation of auditory areas, stimulating release in those areas. Then, in order to test whether monoaminergic influences on auditory responses may be explained in part by sound-induced release, in Chapter 3 I test whether hearing male song induces rapid monoaminergic activity in auditory areas. Finally, I explore whether the model proposed in Fig. 1 could pertain to males as well as females. In Chapter 4, I examine first whether the gonadal hormone T, which is high in males during the breeding season when they sing and listen to territorial song, alters auditory ZENK responses in male white-throated sparrows. Second, I ask whether T modulates monoaminergic innervation of the auditory system in males as E2 does in females.

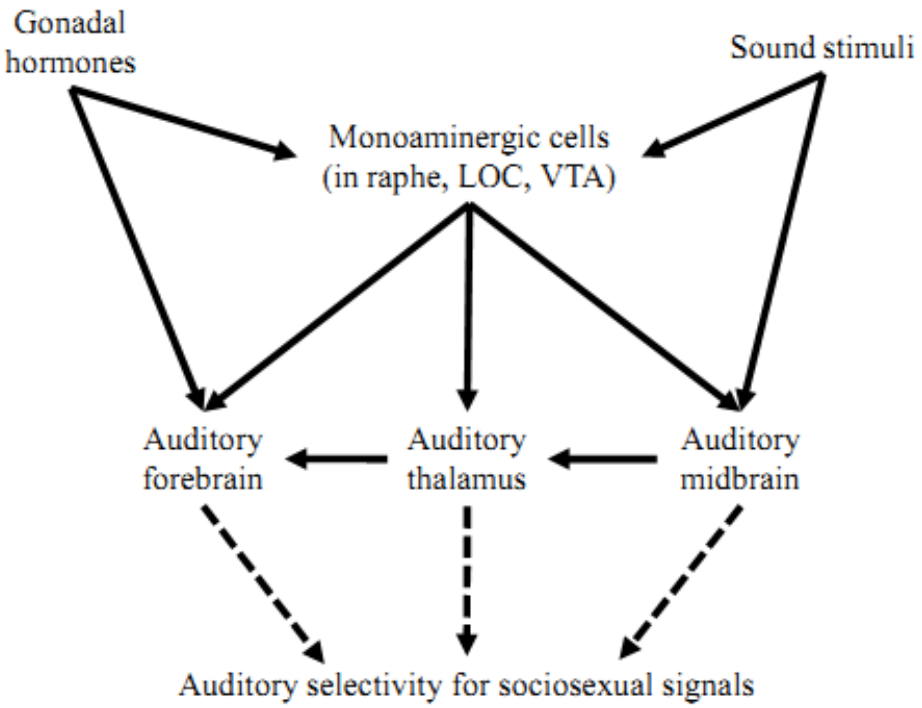

Fig. 1. Model showing how gonadal hormones may affect the processing of auditory sociosexual signals. I hypothesize that gonadal steroids associated with breeding modulate auditory ZENK responses *via* monoaminergic systems. Monoaminergic neurons (i.e. serotonin neurons in the raphe, norepinephrine neurons in the locus coeruleus (LOC), or dopamine neurons in regions such as the ventral tegmental area (VTA)) contain androgen and estrogen receptors and therefore may mediate steroid-dependent auditory selectivity in the forebrain, thalamus, and midbrain (dotted arrows). The monoaminergic cells that project to these auditory areas may fire in response to sound, rapidly inducing monoaminergic activity in auditory areas. Alternatively, steroid-dependent paracrine release of monoamines may affect response properties of the auditory forebrain independently of monoaminergic neuron firing.

CHAPTER 2

Estradiol-dependent monoaminergic innervation of auditory areas in the female white-throated sparrow

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Matragrano LL, Sanford SE, Salvante KG, Sockman KW, and Maney DL. 2011. Estradiol-dependent catecholaminergic innervation of auditory areas in a seasonally breeding songbird. European Journal of Neuroscience 34: 416-425.
and
Matragrano LL, Sanford SE, Salvante KG, Beaulieu M, Sockman KW, and Maney DL. 2012. Estradiol-dependent modulation of serotonergic markers in auditory areas of a seasonally breeding songbird. Behavioral Neuroscience 126: 110-122.

ABSTRACT

Gonadal steroids such as estradiol (E2) affect the neurochemistry not only of brain regions that are involved in reproductive behavior, but also those involved in sensory processing. Previously we found that in the auditory system of a seasonally breeding sparrow, expression of the immediate early gene ZENK was selective for conspecific song only when E2 was elevated to breeding typical levels. We hypothesize that this effect of E2 may be mediated by monoamine systems, which are sensitive to E2 in many species and involved in sensory processing and selective attention. In this study, we tested the effects of E2 on catecholaminergic and serotonergic biomarkers in the auditory areas of female songbirds. We used immunohistochemistry to estimate the density of fibers immunoreactive for tyrosine hydroxylase, dopamine beta-hydroxylase, and serotonin transporter in the auditory forebrain, thalamus, and midbrain. E2 treatment increased catecholaminergic and serotonergic innervation of the auditory forebrain and midbrain. In addition, we used high performance liquid chromatography to quantify the effects of E2 treatment on catecholamines and monoamine metabolites. Norepinephrine and the serotonin metabolite 5-hydroxyindoleacetic acid increased in the auditory forebrain, but not in the midbrain, after E2 treatment. Overall, our results suggest that increases in plasma E2 typical of the breeding season enhance monoaminergic innervation and synthesis in some regions of the auditory system, which suggests a role for catecholamines and serotonin in E2-dependent auditory plasticity in songbirds.

INTRODUCTION

Gonadal steroids are released during the breeding season and are known to regulate reproductive behaviors that occur in response to social signals. Behavioral responses to sociosexual signals may therefore be influenced according to the season. It is possible that these hormones increase the behavioral relevance or salience of these signals, therefore facilitating behavioral responses appropriate for the time of year. During the breeding season, female songbirds must attend to sensory cues, including male song, when making mate choices. During the breeding season, presentation of male song will elicit a courtship display whereas during the non-breeding season, when plasma estradiol (E2) levels are low, females do not perform the display when presented with the same song (Kern and King, 1972; Maney et al. 2008, 2009; Moore, 1983; Sanford et al., 2010). This effect of E2 on the behavioral response suggests that the hormone may alter the salience or otherwise affect the perception of male song.

The auditory system of songbirds is largely identical to that in mammals (for similarities, see Section 2 of Chapter 1). In songbirds, information is first transduced in the cochlea, ascends through the cochlear nuclei in the brainstem, and arrives at the auditory midbrain (the dorsal lateral nucleus of the mesencephalon, MLd; Fig. 1). Selectivity for behaviorally relevant sounds is already evident in the auditory midbrain, which shows greater spiking activity in response to song than to tones (Hsu et al., 2004; Woolley & Cassaday, 2004; Woolley et al., 2005). Projections from MLd neurons extend to the auditory thalamus (thalamic nucleus Ovoidalis; Ov). Similar to MLd, Ov is also tuned to behaviorally relevant sounds (Amin et al., 2010). From Ov, projections ascend to the

auditory forebrain, where the caudomedial nidopallium (NCM) receives input from the thalamorecipient Field L and is interconnected with the caudomedial mesopallium (CMM). NCM and CMM are analogous to the supragranular layers of the mammalian auditory cortex (Vates et al., 1996) or to the mammalian auditory association cortex (Pinaud & Terleph, 2008; Tremere et al., 2009). These areas have received a great deal of attention in part because they show selective responses to stimuli that are particularly salient to the listener. The response in these areas is selective for songs that are sexually stimulating (Gentner et al., 2001; Leitner et al., 2005; Maney et al., 2003; Sockman et al., 2005) and for songs that have been paired with a novel or salient stimulus (Jarvis et al., 1995; Kruse et al., 2004). Thus, activity in these areas may be related to behavioral responses and mate choices (Maney et al., 2003).

The same regions in the auditory system that show selectivity to behaviorally relevant stimuli are sensitive to E2 treatment. In female white-throated sparrows with breeding levels of plasma E2, expression of ZENK is selective for conspecific male song over frequency-matched tones. In females with low levels of plasma E2, the *zenk* responses to hearing conspecific songs and to hearing frequency-matched tones are indistinguishable (Maney et al., 2006). This E2-induced selectivity for song occurs throughout the auditory pathway including MLd, Ov, CMM, and NCM (reviewed by Maney & Pinaud, 2011). It is not the case, however, that E2 acts uniformly throughout the auditory pathway. In NCM, for example, which is a large, heterogeneous structure (Fig. 2), E2 induces selectivity for song only in the rostral domains, not in the caudomedial domain. In the caudomedial domain, a crescent-shaped area containing aromatase and estrogen receptor-

expressing cells (Gahr et al., 1993; Saldanha & Coomaralingam, 2005), the ZENK response is selective for song regardless of plasma E2 level (Sanford et al., 2010). Thus, it is possible that these domains of NCM should be considered separately (Pinaud et al., 2006; Sanford et al., 2010).

E2-dependent auditory selectivity may promote recognition of and attention to conspecific male song during the breeding season, when it is particularly relevant to the female (Maney et al., 2006). In order to identify and study the mechanisms underlying such selectivity, we must consider monoamine systems, which are understood to influence discrimination, attention, and sensory processing. Both the catecholaminergic (CA) and serotonergic systems in the avian brain have been extensively mapped (Cozzi et al., 1991; Dube & Parent, 1981; Fuxe & Ljunggren, 1965; Ikeda et al., 1971; Yamada et al., 1984) and CA and serotonergic neurons project throughout the auditory midbrain, thalamus, and forebrain (Appeltants et al., 2002a,b; Challet et al., 1996; Hurley & Thompson, 2001; Kaiser & Covey, 1997; Klepper & Herbert, 1991; LeBlanc et al., 2007; Metzger et al., 2002; Steinbusch, 1981; Zeng et al., 2007). In mammals, catecholamines and serotonin shape the response properties of neurons involved in sensory processing and selective attention (reviewed by Amin et al., 2005; Aston-Jones & Cohen, 2005; Hurley et al., 2004; McEwen & Alves, 1999; Robichaud & Debonnel, 2005). In some mammals, for example female rhesus monkeys, the density of catecholaminergic and serotonergic fibers is altered by ovarian hormones (Kritzer & Kohama, 1998, 1999). Here, we hypothesize that these systems may be sensitive to E2 in songbirds.

In songbirds, E2 regulates catecholamine levels and turnover in the brain (Barclay & Harding, 1990) and increases the density of CA innervation of NCM (LeBlanc et al., 2007). In the auditory forebrain, tyrosine hydroxylase (TH)-immunoreactive (IR) fibers surround cells containing estrogen receptor-alpha (Saldanha & Coomaralingam, 2005), suggesting that CA release may be modulated presynaptically. Less is known about the effect of gonadal steroids on serotonin in auditory areas of songbirds. In mammals, serotonin modulates auditory responses in the cochlear nucleus and auditory midbrain (Ebert & Ostwald, 1992; Hurley & Pollak, 1999, 2001, 2005). Furthermore, the serotonergic system in mammals is sensitive to E2 treatment (reviewed by McEwen & Alves, 1999). Collectively, all of this evidence suggests that modulation of the CA and serotonergic systems, specifically modulation by E2, might affect auditory processing in songbirds.

Here, we hypothesize that E2- induced selectivity in the auditory pathway is accompanied by E2-mediated changes in CA and serotonergic innervation of auditory areas. We investigated whether E2 treatment alone, without the presentation of song stimuli, alters CA and serotonergic innervation of the auditory midbrain, thalamus, and forebrain, considering each domain of NCM separately (Sanford et al., 2010). We manipulated plasma E2 and, using immunohistochemistry (IHC), measured the effects on the density of fibers immunopositive for TH-, dopamine β–hydroxylase (DBH-), and serotonin transporter (SERT-) IR. In the contralateral hemisphere of each bird, we measured the concentrations of several monoamines and their metabolites using high performance liquid chromatography (HPLC). If E2 affects sensory tuning by altering

monoaminergic activity, we might expect these monoaminergic markers to occur at higher levels in E2-treated females than in females with non-breeding levels of plasma E2. Because we hypothesize that E2-dependent selectivity is mediated by monoaminergic input, an additional goal of the present study was to test whether the neuroanatomical distribution of these effects is similar to the effects of E2 on selectivity. We previously reported that E2 treatment altered the selectivity of sound-induced ZENK responses in CMM and the rostral but not the caudal domains of NCM (Sanford et al., 2010); therefore we predicted that E2 treatment would increase the density of monoaminergic fibers in the same areas.

METHODS

Animals

All procedures in this study were approved by the Emory University Institutional Animal Care and Use Committee and adhered to NIH standards. We collected sixteen female white-throated sparrows (*Zonotrichia albicollis*) in mist nets in Atlanta, Georgia during the fall of 2007. We determined their sex by polymerase chain reaction analysis of a blood sample (Griffiths et al., 1998) and confirmed sex by necropsy at the end of the study. The birds were housed at the Emory University animal care facility in indoor walk-in flight cages and supplied with food and water *ad libitum*. We held day length constant at 10:14h light-dark, which corresponds to the shortest day the birds would experience during the winter at the capture site. We kept the birds under these conditions for two months to ensure that the birds were not photorefractory before the start of the study (Shank, 1959; Wolfson, 1958).

Hormonal Manipulation

For the study, we transferred the birds in pairs to sound-attenuated booths (Industrial Acoustics, Bronx, NY) where they were housed individually in two adjacent cages (38 x 38 x 42 cm) per room. We held the day length at 10:14 h light-dark throughout the experiment to prevent elevation of endogenous plasma E2 (Shank, 1959; Wolfson, 1958). On the day the birds were transferred to individual cages, we implanted each one with a subcutaneous silastic capsule (length 12 mm, ID 1.47 mm, OD 1.96 mm, Dow Corning, Midland, MI) sealed at both ends with A-100S Type A medical adhesive (Factor 2, Lakeside, AZ). One bird in each pair (n=8) received an empty implant and the other (n=8) received an implant containing 17β-estradiol (Steraloids, Newport, RI). This dose of E2 increases plasma levels to those typical of the breeding season within seven days in this species (Maney et al., 2006, 2008) and likely does so within two days (Moore, 1983). Seven days after implantation, we rapidly decapitated the birds and quickly harvested the brains. After removing the cerebellum in order to clearly visualize the midline, we used a clean razor blade to bisect each brain into hemispheres. We fixed one hemisphere in 5% acrolein as previously described (Maney et al., 2003, 2005) and flash-froze the other in powdered dry ice for HPLC analysis at the University of North Carolina. The hemisphere that was fixed (right or left), the room (one of six identical rooms), and the position of each bird inside the room (to the right or the left of the other bird in that room) were balanced across treatments.

Immunohistochemistry

We cut the fixed hemispheres into three series of 50 μm parasagittal sections using a freezing sliding microtome. We immunolabeled each series for TH, DBH, or SERT using standard IHC protocols (LeBlanc et al., 2007; Maney et al., 2001, 2003, 2005). Briefly, we incubated the first series of sections with an anti-TH antibody (ImmunoStar; Hudson, WI; see section on *antisera* below) diluted 1:2000 (LeBlanc et al., 2007) then labeled the TH with a biotinylated secondary antibody and the ABC method (Vector, Burlingame, CA). We visualized the immunolabeling with diaminobenzidine (LeBlanc et al., 2007). For the second series of sections, we pre-treated with avidin and biotin, incubated with anti-DBH antibody (ImmunoStar; see section on *antisera* below) diluted 1:16,000, and subsequently labeled the DBH using a biotinylated secondary antibody and the ABC method. We visualized DBH immunolabeling with nickel-enhanced diaminobenzidine (Shu et al., 1988). We processed these two series of brain sections in two separate runs of IHC in which the experimental (E2-treated) and blank (empty implants) conditions were balanced across runs.

SERT is less liable to metabolism than serotonin; therefore immunolabeling with an anti-SERT antibody is a more stable marker of serotonergic fibers and has been shown to be a better indicator of serotonin axons than an anti-serotonin antibody (Nielsen et al., 2006). Briefly, we incubated every third section with an anti-SERT antibody (ImmunoStar; Hudson, WI; see below) diluted 1:5000 (Meyer et al., 2004), labeled the SERT with a biotinylated secondary antibody and the ABC method (Vector, Burlingame, CA), and

visualized the immunolabeling with diaminobenzidine. For the series immunolabeled for SERT, we processed all of the brain sections in a single run of IHC.

Following IHC, we mounted all of the sections onto microscope slides, dehydrated them, and coverslipped in DPX (Sigma, St. Louis, MO).

Antisera

We used a mouse monoclonal antibody generated against denatured TH and purified from rat PC12 cells (ImmunoStar Cat#22941). According to the manufacturer, it recognizes a 62 kilodalton band corresponding to TH in rat, and does not cross-react with dihydropterdine reductase, DBH, phenyletholamine-N-methyltransferase, phenylalanine hydroxylase, or tryptophan hydroxylase using Western blot methods. It has wide species cross-reactivity and has been validated by preadsorption studies in a range of vertebrates (Olsson et al., 2008). This antibody labels a catecholamine-typical pattern of neurons and fibers in a wide variety of birds (e.g., Appeltants et al., 2001; Bailhache & Balthazart, 1993; Moons et al., 1994; Reiner et al., 1994; Roberts et al., 2001; Soha et al., 1996) including white-throated sparrows (Balthazart & Ball, 1996; LeBlanc et al., 2007) and was used by Reiner et al. (1994) to perform an exhaustive characterization of the distribution of TH-immunoreactivity in birds. Anti-TH antibodies from other sources and anti-dopamine (DA) antibodies produce the same neural distribution in birds (e.g., Bottjer, 1993; Metzger et al., 1996). In our sections, the antibody labels all major TH cell groups A1-A15 and fibers in a distribution typical of TH. An antibody against the phosphorylated form of TH (Genetex, Cat#16557), which we have validated in

preadsorption studies in white-throated sparrow, labels an identical distribution of cells in this species.

To label DBH we used a polyclonal antibody generated in rabbit against DBH purified from bovine adrenal medulla (ImmunoStar Cat#22806). The manufacturer notes that on Western blot the antibody detects a triplet at approximately 72-74 kilodaltons. It labels an NE-like distribution of cells and fibers in a variety of birds (e.g., Bailhache & Balthazart, 1993; Castelino & Ball, 2005; Karle et al., 1996; Sockman & Salvante, 2008), including white-throated sparrows (LeBlanc et al., 2007). Sockman & Salvante (2008) reported that preadsorption with antigen supplied by the manufacturer of the antibody eliminates all labeling in brain sections from European starling (*Sturnus vulgaris).*

To label SERT, we used a rabbit polyclonal antibody generated against a synthetic peptide sequence corresponding to amino acids (602-622) of rat SERT coupled to keyhole limpet hemocyanin (ImmunoStar Cat#24330). We validated the specificity of the SERT antibody in brain sections of a white-throated sparrow using the procedure described in Saper and Sawchenko (2003). All labeling in brain sections was eliminated at antibody concentrations of 1:25,000 by preadsorption of the antibody with 50 µg/ml of the SERT peptide (ImmunoStar Cat#24332). Omission of the primary or the secondary antibodies resulted in a complete loss of specific staining.

Regions of Interest

Our primary goals in this study were to test the effects of E2 on monoaminergic innervation of auditory areas and to determine the extent to which those effects overlap anatomically with previously described effects on the ZENK response (Sanford et al., 2010). The density of IR fibers was quantified in CMM and four domains of NCM, as well as in an auditory thalamic nucleus (n. Ovoidalis, Ov) and the auditory midbrain (MLd). Because we recently showed that E2 affects ZENK expression in rostral but not caudal NCM (Sanford et al., 2010), we wanted to map the effects of E2 on TH-, DBH-, and SERT-immunoreactivity in this region. We therefore partitioned NCM into the same domains defined by Sanford et al. (2010): rostrodorsal (rdNCM), rostroventral (rvNCM), apical (aNCM), and caudal (cNCM). As in our previous work, we also sampled from an apical domain (aNCM) located dorsal to Field L2 (Fig. 2). In the published literature, this region is usually considered part of NCM but may overlap the dorsal portion of Field L (Fortune & Margoliash, 1992). Sanford et al. (2010) further divided cNCM into dorsal and ventral domains, but because these areas are similar hodologically and neurochemically, and because E2 does not affect ZENK expression in either domain (see Introduction), we combined them into one caudal domain (Fig. 2).

We conducted all image acquisition and analyses while blind to treatment group. Regions of interest were identified with reference to Stokes et al. (1974) and Vates et al. (1996). To acquire the images, we used the 10x objective on a Zeiss Axioskop microscope attached to a Leica DFC480 camera and Macintosh G5 computer. For all regions we captured rectangular images (approximately 46 MB in size) corresponding to the field of

view of the camera (870 X 690 μm). We held the light level and exposure time constant for all photos. For each bird, we acquired images of NCM and CMM in four consecutive sections between ~350 and ~800 μm from the midline. Five separate images, each containing CMM, aNCM, rdNCM, rvNCM, or cNCM, were acquired from each of the four sections. With the exception of CMM, for which the entire photo was used, all regions of interest were selected in the photos using ImageJ (version 1.41o, National Institutes of Health, Bethesda, MD) as previously described (Matragrano et al., 2011, 2012; Sanford et al., 2010; see Fig. 3).

To acquire images of CMM and NCM, we chose the four medial-most sections, spanning 450 μm, in which CMM and the four distinct regions of NCM could be discerned (Sanford et al., 2010). The images of CMM were acquired such that the two upper corners of each photograph were positioned along the dorsal boundary of CMM and one of the lower corners was positioned adjacent to the lamina mesopallium. For NCM, an image was acquired for each domain so that there were four images per section. Using the ImageJ circle tool, we sampled the three rostral domains of NCM (Fig. 1) within circular areas (approximately 0.1 mm^2 for aNCM and 0.25 mm^2 for rdNCM and rvNCM).). For aNCM, we placed a circle approximately 350 μm in diameter dorsal to Field L and just ventral to the ventricle. For rdNCM and rvNCM, we placed two circles, each approximately 550 μm in diameter, in the dorsal and ventral portions of this region (Matragrano et al., 2011, 2012; Sanford et al, 2010) so that they did not overlap with Field L or with the region we defined as cNCM. Because we did not conduct tract tracing or another method that would enable us to discern absolutely the boundary between the

rostral domains of NCM and the adjacent subregions of Field L (Vates et al., 1996), it is possible that our samples of aNCM, rdNCM and rvNCM may have captured a very small part of L1 and L3, respectively. We are confident in any case that the rostral regions we sampled correspond exactly to those exhibiting E2-dependent selectivity as described by Sanford et al. (2010). We defined cNCM as a strip of tissue approximately 275 μm from the caudal boundary of NCM (Sanford et al., 2010) and selected it by tracing the caudal boundary with the freehand tool and using the straight line tool to define its rostral boundary. The images of cNCM captured the majority of that domain, spanning 870 μm from dorsal to ventral, but did not include the dorsal and ventral tips. The caudal domain did not overlap with the rostral domains. All five photos of CMM and NCM were viewed at the same time to ensure that the regions we selected in each section did not overlap.

In addition to the auditory forebrain, we acquired images of the auditory midbrain (MLd) and thalamus (Ov) (Figs. 3A, D). From the TH- and DBH-immunolabeled sections, we selected two consecutive sections, spanning 150 μm that contained Ov and traced the outline of the region including its shell and core (Vates et al., 1996). Because we could consistently and dependently identify three sections containing Ov in the SERT-immunolabeled sections, we photographed three consecutive sections spanning 300 μm in these sections only. Because the background staining in the core region of Ov of sections immunolabeled for SERT-IR fibers was high, and few if any fibers were present in this region (see also Belekhova et al., 2002; Kaiser & Covey, 1997); we included only the shell region in our analysis (Zeng et al., 2007). We acquired images of MLd (Figs.

3B, E) from five consecutive sections, spanning 600 μm, in which MLd was clearly identifiable. We traced the perimeters of Ov and MLd in the photos using the ImageJ freehand tool. Finally, we estimated the density of fibers in a non-auditory region, the apical part of the hyperpallium (HA; see Reiner et al., 2004; Stokes et al., 1974), to test the specificity of the effects of E2 on auditory regions. Images of HA, which were acquired from the same sections used for Ov, are shown in Fig. 3C, F and as was the case for CMM, the entire photo of HA used to estimate fiber density.

Image acquisition and estimation of IR fiber density

Using ImageJ (version 1.41o, National Institutes of Health), we converted each image to 8-bit scale and selected the IR fibers using the thresholding feature (Maney et al., 2005). We set the threshold manually for each photo such that the pixels selected by ImageJ agreed with what we considered to be labeled fibers. Our method of selecting immunolabeled fibers has been fully validated and has high interrater reliability and low variability. Briefly, the thresholds selected by two independent observers were highly correlated (R = 0.98), not different from each other (P = 0.976; d = 0.0009), and were within one unit of each other in 30% of cases (Matragrano et al., 2011). In the present study, the same observer set the threshold for all images with the same lighting and computer monitor (LLM). For each region, we summed the total area covered by fibers and divided this sum by the total area measured to yield the area covered by the fibers in square microns per square mm of area measured.

Quantification of monoamines, metabolites, and total protein

The methods for quantification of catecholamines and metabolites are published elsewhere (Sockman & Salvante, 2008), and we reiterate the relevant portions here. We determined the concentration of catecholamines and metabolites by reversed-phase HPLC with electrochemical detection (Kilts et al., 1981). Because the number of brain regions we could sample *via* HPLC was limited (see below), we decided to sample regions in which we had previously shown E2-dependent selectivity of the ZENK response: NCM, CMM, and MLd (Maney et al., 2006). The chromatographic system consisted of a SM-909 isocratic HPLC pump (ANSPEC), Basic+ Marathon type 816 Autosampler (Spark, Holland), Model 400 potentiostat (EG&G Princeton Applied Research) and TurboChrom software Version 4.1 (Perkin Elmer) running on a PC. We separated the compounds using a Monochrom C18 3 μm column (100 x 4.6 mm, MetaChem) with a mobile phase consisting of sodium phosphate (7.1 g), citric acid (5.76 g), disodium EDTA (50 mg), sodium octyl sulfonate (350 mg), and methanol (130 ml) topped-up to one liter total volume with double-distilled, deionized water, with pH lowered to 3.9 with hydrochloric acid. We filtered the mobile phase through a 20 μm filter (Kontes Scientific Glassware and Instruments) before use, and flow rate was 0.8 ml/min. We maintained the electrode potential at 650 mV with respect to an Ag/AgCI reference electrode. We prepared standard solutions containing a fixed amount (30 ng) of the internal standard (isoproterenol, Sigma) and variable amounts of each of the five external standards (Sigma): DA, norepinephrine (NE), dihydroxyphenylacetic acid (DOPAC; the principal metabolite of DA), 3-methoxy-4-hydroxyphenylglycol (MHPG; the principal metabolite of NE), and 5-hydroxyindolezcetic acid (5-HIAA; the principal metabolite of serotonin). We included a five-point standard curve in each assay (5 x 50

μL injections), and used linear regression to fit a line through the standard curve points ($r^2 > 0.99$).

We sectioned the frozen, non-fixed hemispheres at 10˚C in the sagittal plane at 300 μm on a cryostat, thaw mounted the sections onto glass slides, and rapidly re-froze them on pulverized dry ice. From each of two consecutive sections starting at the midline and using chilled custom-made, thin-walled stainless steel spring-loaded punch tools, we micropunched a region of CMM (1 mm i.d.) and of NCM (1.5 mm i.d.), each for which the anatomical boundaries have been described previously (Sockman et al., 2002). Because the diameter of the punch tool was greater than the diameters of any of the individual domains within NCM, we did not further divide NCM into domains for the HPLC analysis. We took a micropunch (1 mm i.d.) from a region of MLd from two consecutive sections starting approximately 1800 μm from the midline. All of the sectioning and sampling was done in one day by the same person using the same punch tool for each region. The regions of interest were located by referring to Sockman et al. (2002) as well as two sets of Nissl-stained brain sections from white-throated sparrow. For NCM and CMM, we used Field L as a landmark. For MLd we used the tectal ventricle, which appears as a dark line in the fresh frozen tissue. We expelled the tissue punches into 1.9 ml polypropylene microcentrifuge tubes (one for each brain region), froze them on dry ice and stored them at -80˚C until assay. Immediately before assay, we added mobile phase (225 μl) containing 30 ng isoproterenol to each tube. We sonicated the samples and then centrifuged them at 16,000 *g* for 15 minutes at 4˚C. We aspirated the supernatant and injected 50 μl from each sample into the HPLC system. We

calculated catecholamine and metabolite concentrations (pg) by first correcting the peak heights for percent recovery of the internal standard (i.e., the height of the isoproterenol peak) for each sample, and then comparing the values to those obtained for the corresponding catecholamines or metabolites in the standard curve. We also measured the protein content of each sample by dissolving the remaining sample pellet in 0.2 N NaOH (100 µl) and performing the Bradford protein-dye binding assay (Quick Start Bradford Protein Assay, Bio-Rad) with bovine serum albumin as a standard (Bio-Rad) on a µQuant microplate spectrophotometer (BioTek) (Bradford, 1976). Concentrations of our compounds of interest were normalized by dividing monoamine or metabolite levels by protein content of each sample.

Statistical Analysis

Statistical analyses were conducted for the data on CA and serotonergic fibers separately. All of our dependent variables that were linked to catecholamine synthesis, and therefore related to each other, were analyzed in a single repeated-measures MANOVA that included data acquired by IHC and HPLC from all birds. Because the HPLC data set included only three regions of interest (CMM, NCM as a single region, and MLd), we could not include all of the IHC data on enzymes, which were measured in eight separate regions, in this initial analysis. The IHC data were therefore analyzed separately, with all regions of interest included, in a second analysis (see below). For the initial omnibus analysis of CMM, NCM, and MLd only, because CA innervation was measured in individual domains whereas our HPLC data were acquired from a single larger sample, we needed to collapse the NCM IHC data from individual domains into a single value for

NCM for each bird. To do so, we calculated an average percent area covered by fibers for each enzyme by summing the total area covered by immunoreactivity in rdNCM, rvNCM, and cNCM and dividing by the total area measured for all regions summed. aNCM was not included in this calculation because it lies dorsal to Field L, outside the area of the micropunch. This calculation produced an estimate of the average area covered by fibers immunoreactive for TH or DBH throughout the part of NCM that was sampled for HPLC analysis. We then conducted a repeated-measures MANOVA with region of interest (CMM, NCM, or MLd) and catecholamine measure (TH, DBH, DA, NE, DOPAC, or MHPG) as the within-subject variables, and hormone treatment (blank or E2) as the between-subject variable. In this procedure, therefore, we tested for an effect of E2 treatment on most of the related variables together as a whole before proceeding to analyze the data for each measure or individual region separately.

In order to determine the effect of E2 treatment on TH and DBH fiber innervation in our regions of interest, including Ov, HA, and the domains of NCM, we performed a separate repeated-measures MANOVA with enzyme (TH or DBH) and region of interest (CMM, rdNCM, rvNCM, aNCM, cNCM, Ov, and MLd) as the within-subjects factors, and hormone treatment and hemisphere as the between-subjects factors, followed by multivariate F-tests for each region of interest. To analyze the HPLC data in the other hemisphere, we did a repeated-measures MANOVA with region of interest (CMM, NCM, or MLd) and compound (DA, NE, DOPAC, or MHPG) as the within-subject variables, and hormone treatment (blank or E2) and hemisphere as the between-subject variables, followed by multivariate F-tests for each region of interest. Effect sizes were

calculated using Cohen's *d*. Because we intended to show that TH- and DBH-immunoreactivity in HA was unaffected by E2 treatment and thus wanted to minimize Type II rather than Type I error, we analyzed the data from that region in a separate multivariate F-test.

Data on serotonergic activity were originally presented in a manuscript separate from the CA data. Input from co-authors on the second manuscript led to a slightly different strategy for the statistical analysis, which is summarized here. For each dependent variable in the serotonergic system, we plotted histograms and performed Shapiro-Wilk tests (SPSS) to determine whether the distribution of the data was normal. In the event of significant deviation ($P < 0.05$), we performed a square-root transformation to normalize the distribution. The transformation was necessary and sufficient to normalize the distributions for 3 of the 11 variables: 5-HIAA in NCM and MLd, and SERT-immunoreactivity in MLd. The other 8 variables did not require transformation. In all cases, visual inspection of the histogram confirmed the necessity of transformation and its effectiveness.

We looked for effects of treatment on 5-HIAA concentration and the estimated density of SERT-IR fibers using a mixed-effects linear model (Stata), which uses restricted maximum likelihood to estimate parameters. For each of the 11 dependent variables (concentration of 5-HIAA in CMM, NCM, and MLd and estimated density of SERT-immunoreactivity in CMM, aNCM, rdNCM, rvNCM, cNCM, Ov, MLd, and HA), hormone treatment was a predictor and bird was nested as a random intercept and random

coefficient on treatment within pair-housed birds (Schiezelth & Forstmeier, 2009). Effect sizes were estimated using Cohen's *d* on untransformed data.

We wanted to know whether TH-, DBH-, or SERT-immunoreactivity predicted the levels of catecholamines or metabolites in each region. To find out, we ran Pearson correlation tests relating the percent area covered by TH-immunoreactivity to DA and its metabolite DOPAC, and the percent area covered by DBH-immunoreactivity to NE and its metabolite MHPG in each CMM, NCM, and MLd (total of 12 Pearson tests). We conducted an additional Pearson test relating the percent area covered by SERT-immunoreactivity to 5-HIAA in each of the three regions. For this analysis, NCM was treated as a single region and we used the averaged IHC data for individual domains (see above). We evaluated the correlations with the sequential Bonferroni correction for multiple comparisons (Rice, 1989).

RESULTS

When data on the density of TH- and DBH-immunoreactivity and the concentrations of catecholamines and their metabolites were analyzed together in a single repeated-measures MANOVA (see Methods), we found no reliable effect of treatment ($F_{1, 14}$ = 0.758; p = 0.399). There was, however, a significant interaction between hormone treatment and region (Wilks' Lambda $F_{2, 13}$ = 7.508; p = 0.007), meaning that there was a significant effect of E2 treatment that depended on the region of interest. There was also a significant interaction between hormone treatment and compound measured (Wilks' Lambda $F_{5, 10}$ = 3.713; p = 0.037), which indicated that E2 treatment may have affected

some of our measures but not others. For the data concerning the serotonergic system, we analyzed the IHC and HPLC data in an overall repeated-measures MANOVA (see Methods) and found an effect of E2 treatment ($F_{1, 12} = 5.017$; $p = 0.045$). There was no effect of hemisphere and no significant interaction between hormone treatment and hemisphere. We then proceeded to the analyses of data collected by each method (IHC or HPLC).

Catecholaminergic System

Effects of E2 treatment on catecholaminergic innervation of auditory areas

The effects of E2 treatment on TH and DBH fiber density are plotted in Fig. 4. A repeated-measures MANOVA that considered all regions of interest, including the domains of NCM, revealed a highly significant effect of E2 treatment on immunoreactivity for CA enzymes ($F_{1, 12} = 20.426$; $p = 0.001$). Post-hoc pairwise comparisons showed that E2 significantly increased TH innervation in rvNCM ($F_{1, 15} = 5.177$; $p = 0.042$; $d = 0.949$), aNCM ($F_{1, 15} = 9.615$; $p = 0.009$; $d = 1.614$), and MLd ($F_{1, 15} = 7.515$; $p = 0.018$; $d = 1.455$). DBH innervation was increased in CMM ($F_{1, 15} = 27.809$; $p < 0.001$; $d = 2.427$), rdNCM ($F_{1, 15} = 9.281$ $p = 0.010$; $d = 1.629$), rvNCM ($F_{1, 15} = 13.390$; $p = 0.003$; $d = 1.711$), aNCM ($F_{1, 15} = 15.133$; $p = 0.002$; $d = 2.006$), and MLd ($F_{1, 15} = 8.485$; $p = 0.013$; $d = 1.327$). We did not detect an effect of E2 on DBH innervation of cNCM ($F_{1, 15} = 0.288$; $p = 0.601$; $d = 0.293$).

Effects of E2 treatment on levels of catecholamines and their metabolites

A repeated-measures MANOVA with compound and region as within-subjects measures showed no main effect of hormone treatment ($F_{1, 12} = 1.472$; $p = 0.248$); however there was an interaction between treatment and region ($F_{2, 11} = 8.005$; $p = 0.007$). Post-hoc pairwise comparisons revealed that the effects of E2 treatment were limited to CMM, in which norepinephrine increased ($F_{3, 15} = 6.876$; $p = 0.022$; $d = 1.386$) (Fig. 5). We detected no other effects of hormone treatment on the concentrations of catecholamines or their metabolites in CMM, NCM, or MLd (Fig. 5).

Interhemispheric differences

Previous research has shown that some functions of the auditory system may be lateralized in songbirds (Avey et al., 2005; George et al., 2004; Phan & Vicario, 2010; Poirier et al., 2009; Remage-Healey et al., 2010). In order to control for an effect of lateralization, we included hemisphere as a factor in our analyses. The repeated-measures MANOVAs conducted on the IHC data showed an overall effect of hemisphere ($F_{1, 12} = 5.957$; $p = 0.031$), and the parallel analysis of the HPLC data showed a significant compound by hemisphere interaction ($F_{3, 10} = 4.564$; $p = 0.029$). In neither analysis was there an interaction between hemisphere and treatment or between hemisphere and other variables that included treatment. Therefore, E2 treatment affected our variables of interest equally in both hemispheres in all cases. The two interhemispheric differences we found for enzyme immunoreactivity were in the same direction; TH-immunoreactivity was higher in left than right rvNCM ($F_{1, 15} = 5.356$; $p = 0.039$; $d = 0.95$), and DBH-immunoreactivity was higher in the left than the right cNCM ($F_{1, 15} = 4.977$; $p = 0.046$; d

= 1.031). NE and its metabolite, MHPG, were both higher in the right MLd than the left (NE, $F_{1, 15} = 5.481$; $p = 0.037$; $d = 1.725$; MHPG, $F_{1, 15} = 13.298$; $p = 0.003$; $d = 1.089$). Overall, we found that E2 treatment did not affect monoaminergic markers in one hemisphere over the other.

Correlations between enzyme immunoreactivity and catecholamine levels

In order to test whether enzyme immunoreactivity predicted the levels of catecholamines and their metabolites, we looked for correlations between TH fiber density and DA or DOPAC levels and between DBH fiber density and NE or MHPG levels in CMM, MLd and NCM (rdNCM, rvNCM and cNCM combined; see Methods). Pearson correlation tests revealed that TH fiber density did not predict levels of DA or DOPAC as measured by HPLC. We detected no significant correlations between the percent area stained and DA or DOPAC levels in any of the regions of interest. Similarly, in most regions DBH fiber density did not predict levels of NE or MHPG as measured by HPLC. The only notable correlation, between DBH-immunoreactivity and the concentration of NE in CMM ($R^2 = 0.334$, $p = 0.019$) was clearly driven by the significant effect of E2 on both measures (Fig. 6). We also tried correlating the fiber density of each enzyme with the summed concentrations of all of its downstream products, in other words DBH-immunoreactivity with NE and MHPG levels summed, and TH-immunoreactivity with all four products summed, but detected no significant correlations.

Serotonergic System

Distribution of SERT-IR Fibers

The distribution of serotonergic fibers was similar to what has been described in chickens, Japanese quail, and pigeons (Challet et al., 1996; Cozzi et al., 1991; Kaiser & Covey, 1997; Metzger et al., 2002; Zeng et al., 2007). SERT-IR fibers were densely distributed throughout the entire brain and the highest densities of SERT-IR fibers were present in the striatum, diencephalon, and midbrain. The most intensely labeled structures in the brain were the pretectal nucleus, the lateral geniculate nucleus, and the nucleus taeniae of the amygdala (Fig. 7). There were additional high densities of fibers in the medial septum, lateral bed nucleus, hippocampus, ventromedial hypothalamus, habenula, and periaqueductal gray. Basket-like IR structures appeared to surround neuronal bodies in the ventral tegmental area, substantia nigra, and locus coeruleus. The song control nucleus HVC was unlabeled; in fact the density of staining in this region was lighter than in the surrounding tissue.

The auditory forebrain was densely innervated with SERT-IR fibers. In Ov, however, whereas the shell region contained clearly labeled fibers, the core region did not (Belekhova et al., 2002; Kaiser & Covey, 1997; Zeng et al., 2007; Fig. 8). The distribution of SERT-IR fibers was relatively homogenous across CMM and NCM, with clusters of fibers appearing to surround unlabeled cell bodies in some individuals. Zeng et al. (2007) reported that MLd in Bengalese finches is nearly devoid of serotonergic fibers. In contrast we found that although it was less densely labeled than the surrounding nucleus intercollicularis, it was clearly innervated by SERT-IR fibers (Fig. 8).

Effects of E2 treatment on serotonergic innervation and metabolite in auditory areas

The effects of E2 treatment on SERT fiber density are graphed in Fig. 9. E2 significantly increased the density of SERT innervation of aNCM ($z = 2.95$, $p = .003$, $d = 1.546$), rdNCM ($z = 2.14$, $p = .032$, $d = 1.186$), and MLd ($z = 4.51$, $p < .001$, $d = 1.325$). We did not detect an effect of E2 on SERT innervation in rvNCM ($z = 1.02$, $p = .306$, $d = 0.449$), cNCM ($z = -1.43$, $p = .156$, $d = 0.583$) or the shell of Ov ($z = -0.97$, $p = .333$, $d = 0.351$). Although the effect of E2 on SERT immunoreactivity in CMM was not significant, we did observe a compelling trend ($z = 1.83$, $p = .067$, $d = 0.946$). As expected, we did not detect an effect of E2 on the density of SERT-IR fibers in the non-auditory region HA ($z = 0.07$, $p = .942$, $d = 0.038$).

The effects of E2 treatment on the concentration of the serotonergic metabolite 5-HIAA was limited to CMM ($z = 2.86$, $p = .004$, $d = 1.241$) (Fig. 9). We did not detect an effect of treatment on 5-HIAA in NCM ($z = 1.07$, $p = .284$, $d = 0.383$) or MLd ($z = 0.43$, $p = .669$, $d = 0.321$; Fig. 9). Pearson correlation tests showed that 5-HIAA levels as measured with HPLC did not predict SERT-IR fiber density in CMM, NCM, or MLd (all $p > 0.183$).

DISCUSSION

Catecholamines and serotonin are involved in auditory processing in vertebrates (Appeltants et al., 2002a; Campbell et al., 1987; Hurley & Hall, 2011; Lynch & Ball, 2008). Because plasma E2 alters the CA and serotonergic system in mammals and in

birds (Kritzer & Kohama, 1999; LeBlanc et al., 2007; Pasqualini et al., 1995) as well as the behavioral response to courtship signals (Maney et al., 2006, 2008, 2009; Moore, 1983), we hypothesized that E2 would modulate monoamine markers in the auditory system of songbirds. Here, we provided evidence that in females, elevating plasma E2 from non-breeding to breeding-typical levels increased the estimated density of TH-, DBH-, and SERT-IR fibers in the auditory forebrain and midbrain. In CMM, the density of DBH-IR fibers, as well as and the concentrations of NE and 5-HIAA, increased after E2 treatment. These findings support a model in which E2 enhances the production and release of monoamines.

These effects of E2 on monoaminergic markers may be region-dependent. E2 treatment increased levels of DBH and its product, NE, in CMM but not in the other regions, suggesting that seasonal changes in forebrain noradrenergic activity may be limited to CMM. The effects of E2 on serotonergic markers also seemed to depend on region, but in a more complex manner. In NCM and MLd, E2 treatment increased the density of SERT-IR fibers, but did not increase concentrations of the serotonin metabolite 5-HIAA. In CMM, on the other hand, E2 treatment increased 5-HIAA but had no effect on SERT-IR fiber density. Our failure to detect an effect of E2 on SERT fiber density in CMM may have been related to sample size, however; note that there was a trend for an effect ($p = .067$). Further research will be necessary to determine whether the effects of E2 described here are truly region-specific.

We previously showed evidence that the effects of E2 on the auditory forebrain are restricted to certain anatomical domains (Sanford et al., 2010). NCM is a large, heterogeneous region made up of domains that differ with regard to their function, connectivity, and neurochemistry (reviewed by Maney & Pinaud, 2011). Sanford et al. (2010) found that E2 treatment induced selectivity of the ZENK response in the rostral but not the caudal domains. Here, we wanted to map the effects of E2 on monoaminergic innervation to determine whether those effects overlapped with the effects of E2 on the ZENK response. We found that in fact, E2 treatment induces selective ZENK responses and increases in monoaminergic activity in the same domains. E2 treatment increased DBH- and SERT-IR fiber density in aNCM and rNCM, but did not increase fiber density in cNCM. These findings provide evidence that the rostral and caudal domains of NCM may be functionally distinct. Furthermore, the anatomical match between the effects of E2 on the ZENK response and on monoaminergic innervation suggests that catecholamines and serotonin may mediate E2-dependent plasticity and selectivity of the ZENK response.

In the published literature, NCM is typically divided not into rostral and caudal domains, but rather dorsal and ventral domains (Avey et al., 2008; Eda-Fujiwara et al., 2003; Gentner et al., 2001; Hernandez & MacDougall-Shackleton, 2004; Lynch & Ball, 2008; Maney et al., 2003; Sockman et al., 2005; Sockman & Salvante, 2008; Velho & Mello, 2008). ZENK responses to song are higher in the dorsal than in the ventral domains (Avey et al., 2008; Phillmore et al., 2003) and the two domains show different selectivity for and sensitivity to a number of stimuli (Eda-Fujiwara et al., 2003; Gentner et al., 2001;

Maney et al., 2003; Sockman & Salvante, 2008). In this study we provide evidence that these two domains are in fact differentially sensitive to E2 treatment. Although E2 increased the density of CA fibers in both the dorsal and ventral parts of the rostral domain, the density of serotonergic fibers increased in the dorsal part only (Figs. 4 & 9). Overall, our results add to the literature suggesting that NCM is a functionally heterogeneous region that may be divided into as many as five different domains with differential responses to both sound and hormone treatment (Sanford et al., 2010).

In humans, catecholamines are known to be involved in auditory processing and attention (reviewed by Berridge & Waterhouse, 2003). Depletion of catecholamines, for example, impairs sustained attention in women (Matrenza et al., 2004). In female canaries, NE depletion decreases behavioral preferences for male song and eliminates selectivity of the ZENK response for conspecific songs (Appeltants et al., 2002b; Lynch & Ball, 2008; Vyas et al., 2008). Catecholamines may thus act as an attentional filter when released in response to relevant stimuli (Aston-Jones & Cohen, 2005). In addition to enhancing attention, catecholamines may encode information about context. In male zebra finches, for example, NE depletion eliminates social context-dependent suppression of ZENK expression in the song control pathway (Castelino & Ball, 2005). In addition, long-term exposure to high-quality male song increased DBH and catecholamine metabolite levels in NCM of female European starlings (Sockman & Salvante, 2008). These findings suggest that catecholamine activity in the auditory pathway may carry information on social context. Modulation of catecholamine activity by gonadal steroids, therefore, may dictate when a female songbird perceives song as sexual and how she chooses her mate.

In songbirds, the serotonergic system and its roles in auditory processing are less well understood than the CA system. In bats and rats, serotonin can induce changes in vocalizations as well as neuronal activity in auditory areas (Ebert & Ostwald, 1992; Hurley & Pollack, 1999, 2005; Ji & Suga, 2007). In mammals, serotonergic fiber density throughout the central nervous system is sexually dimorphic and may depend on gonadal hormones (Kojima & Sano, 1984; Patisaul et al., 2008; Simerly et al., 1984). In rhesus monkeys, for example, serotonin fiber density in the prefrontal cortex increases following ovariectomy and is reduced to normal levels with ovarian hormone replacement (Kritzer & Kohama, 1999). In ovariectomized rats, E2 treatment increases firing rate (Robichaud & Debonnel, 2005), serotonin content (Cone et al., 1981), and the expression of SERT mRNA (Donner & Handa, 2009; Hiroi et al., 2006) in the dorsal raphe. These neurons contain estrogen receptors (Gundlah et al., 2001; Lu et al., 1999, 2001; Sheng et al., 2004) suggesting that these effects may be direct. E2 may also act directly on forebrain regions to upregulate SERT *via* postranslational mechanisms (Bertrand et al., 2005; Lu et al., 2003; McQueen et al., 1997). Ongoing studies in our lab to map estrogen receptors and estradiol-dependent serotonergic activity will help elucidate sites and mechanisms of action.

Our observation that E2 increases monoaminergic fiber density could be interpreted several ways. E2 may induce axonal outgrowth in the auditory pathway as it has been shown to do in cell culture and *in vivo* (reviewed by de Lacalle, 2006). Alternatively, E2 may have altered the level of TH, DBH, or SERT protein, thus affecting our ability to

detect those axons. Regardless of whether an increase in fiber density is indicative of axonal growth, our results provide evidence that catecholamine production and serotonin reuptake activity may increase. Increases in catecholamine production in response to E2 treatment have been well-described (reviewed by Di Paolo, 1994 and Zheng, 2009). Studies using cell culture and *in vitro* preparations have demonstrated that E2 inhibits SERT activity (Chang & Chang, 1999; Koldzic-Zivanovic et al., 2004) which could lead to serotonin accumulation in the synapse and upregulation of SERT (Charoenphandhu et al., 2011). Thus, E2-induced increases in monoaminergic fiber density may be caused by a number of actions at the level of the cell bodies or the fibers. Because in this study we labeled biomarkers that can be regulated independently of structural plasticity, the increases in fiber density described here could have multiple explanations. Our findings should be confirmed using additional markers.

Monoaminergic activity may affect auditory selectivity by two different mechanisms. First, CA and serotonergic neurons might fire selectively in response to sound. The immediate release of catecholamines and serotonin in the auditory system may subsequently affect local activity and facilitate selective responses. Neurons in catecholaminergic regions of the brainstem that project to the forebrain fire selectively in response to conspecific songs (Gale & Perkel, 2010; LeBlanc et al., 2007). It is not the case, however, that monoaminergic neurons must fire in response to sound in order to modulate auditory selectivity. Some cells may release monoamines in a nonsynaptic or paracrine manner, independently of sound, in turn altering sound-induced responses in the auditory forebrain (reviewed by Beaudet & Descarries, 1978). Changes in firing

activity that result from non-synaptic release of monoamines can be sustained for long periods of time (Reader et al., 1979). Because they are long-lasting, these changes may underlie long-term changes in responses to the same stimulus, for example responses to song that change according to season, without altering the selectivity of the monoaminergic neurons themselves. E2-induced changes in monoaminergic innervation may thus allow the auditory pathway of female songbirds to respond selectively to male song during the breeding season, a context in which it has high behavioral relevance.

Because TH-IR and DBH-IR fibers secrete catecholamines, we predicted that fiber density would be related to concentrations of the relevant monoamine or metabolite in the auditory midbrain and forebrain. The only significant correlation we found was between DBH-IR fiber density and NE concentration. This correlation was clearly driven by the independent effects of E2 on both variables. There were no other correlations between estimated fiber density and monoamine or metabolite concentrations. Although the level of one monoaminergic marker did not predict the other, the relationship between these markers may not be linear. Due to possible lateralization of CA activity between the hemispheres, the marker measured in one hemisphere may not relate to that measured in the contralateral hemisphere. In order to determine the relationships between monoaminergic fiber density and monoamine concentration, both IHC and HPLC should be used on tissue from the same hemisphere, for example in alternate series of sections.

In this study, we demonstrated that breeding-typical levels of plasma gonadal hormones can enhance monoaminergic fiber density as well as the concentrations of some

monoamines or monoamine metabolites in auditory areas. The E2-dependent monoaminergic activity we observed may allow for seasonal modulation of auditory tuning and appropriate behavioral responses to courtship signals (reviewed by Maney & Pinaud, 2011). In order to more fully understand the role of E2 in seasonal auditory selectivity, future studies should investigate the effects of gonadal steroids on the rapid release of catecholamines and serotonin during exposure to auditory stimuli.

ACKNOWLEDGMENTS

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FIGURES

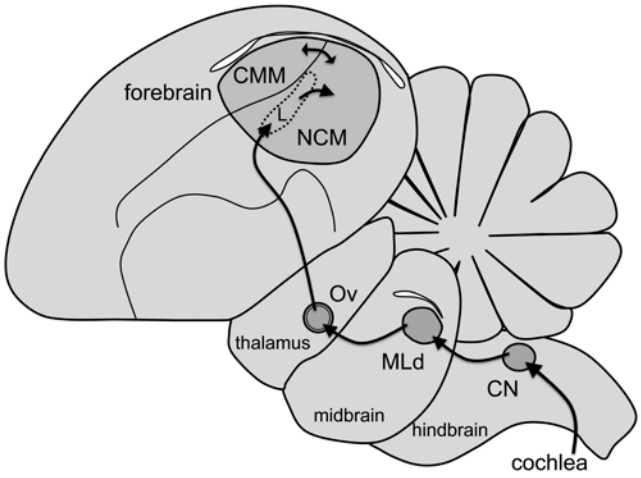

Fig. 1. Diagram showing a parasagittal view of the auditory pathway in songbirds. The auditory nerve enters the brainstem and arrives at the cochlear nucleus (CN, also called nucleus magnocellularis) which projects to the auditory midbrain (the dorsal portion of the lateral mesencephalic nucleus, or MLd). MLd projects to the core region of the auditory thalamus, also called nucleus ovoidalis (Ov). The Ov core projects to the thalamorecipient region of the auditory forebrain, Field L, which then projects to the caudomedial nidopallium (NCM). NCM is reciprocally connected to the caudomedial mesopallium (CMM). NCM and CMM are thought to be analogous to the supragranular layers of mammalian auditory cortex (Vates et al., 1996) or to mammalian auditory association cortex (Pinaud & Terleph, 2008; Tremere et al., 2009).

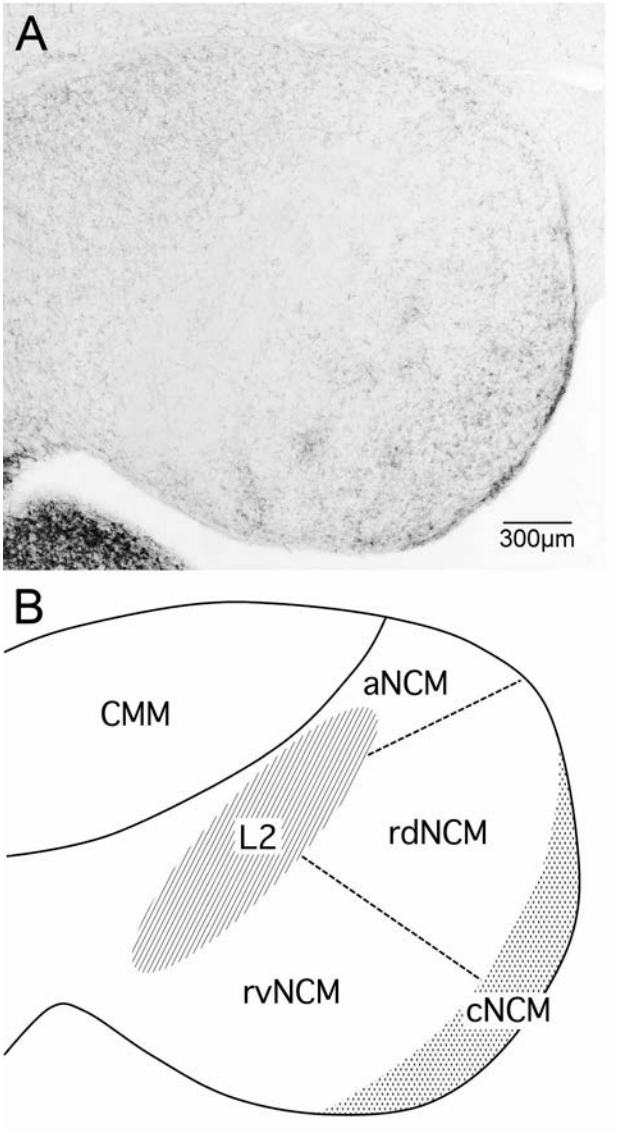

Fig. 2. Parasagittal view of tyrosine hydroxylase immunoreactivity (A) in the auditory forebrain of the white-throated sparrow. We quantified catecholamine and serotonin innervation, content, and metabolites in five regions of the auditory forebrain (B): the caudomedial mesopallium (CMM) and four domains of the caudomedial nidopallium (NCM) as defined by Sanford et al. (2010): apical NCM (aNCM), rostrodorsal NCM (rdNCM); rostroventral NCM (rvNCM) and caudal NCM (cNCM). Rostral is to the left. Scale bar = 300 µm.

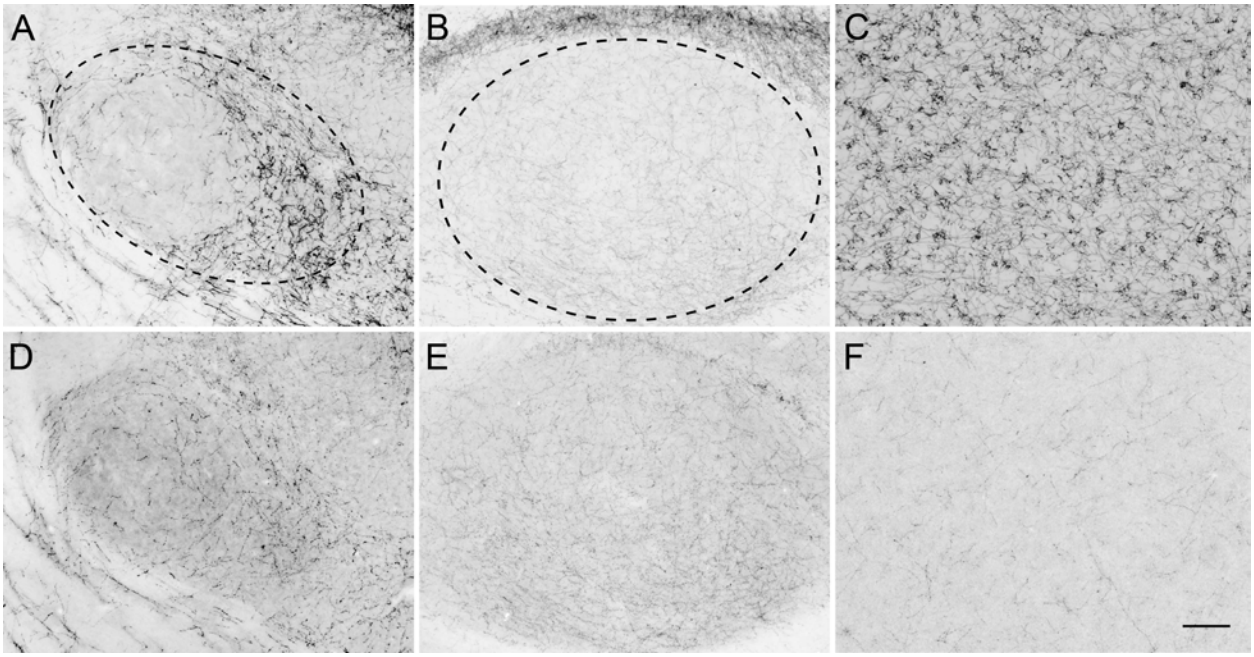

Fig. 3. Fibers immunoreactive for tyrosine hydroxylase (A, B, C) or dopamine beta-hydroxylase (D, E, F) in the auditory thalamus (Ov; A, D), the auditory midbrain (MLd; B, E) and the apical part of the hyperpallium (HA; C, F), a visual area, in an estradiol-treated female white-throated sparrow. The apparent cell bodies in HA are actually immunoreactive fibers forming basket-like structures (Metzger et al., 1996). Rostral is to the left. Scale bar = 100 μm.

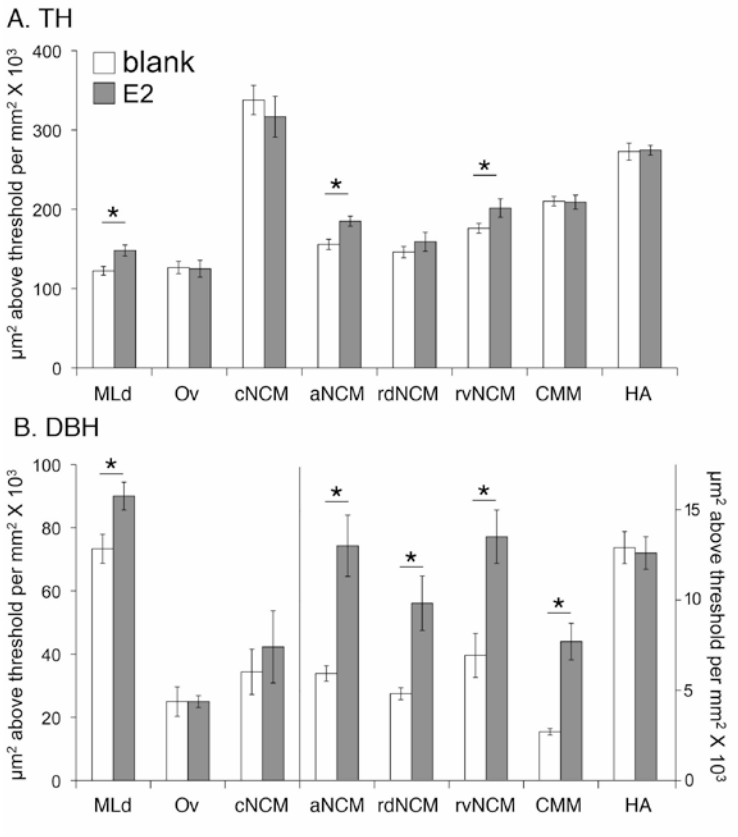

Fig. 4. Estradiol (E2) treatment increased the density of fibers immunopositive for (A) tyrosine hydroxylase (TH) in MLd, aNCM, and rvNCM; and (B) dopamine beta-hydroxylase (DBH) in MLd, aNCM, rdNCM, rvNCM, and CMM. MLd, auditory midbrain. In panel B, refer to the y-axis to the left for regions to the left of the line and refer to the y-axis to the right for regions graphed to the right of the line. HA, apical part of the hyperpallium. Ov, n. Ovoidalis. For other abbreviations see Fig. 1. *E2 condition differs from blank condition, $p < 0.02$. See text for actual p levels.

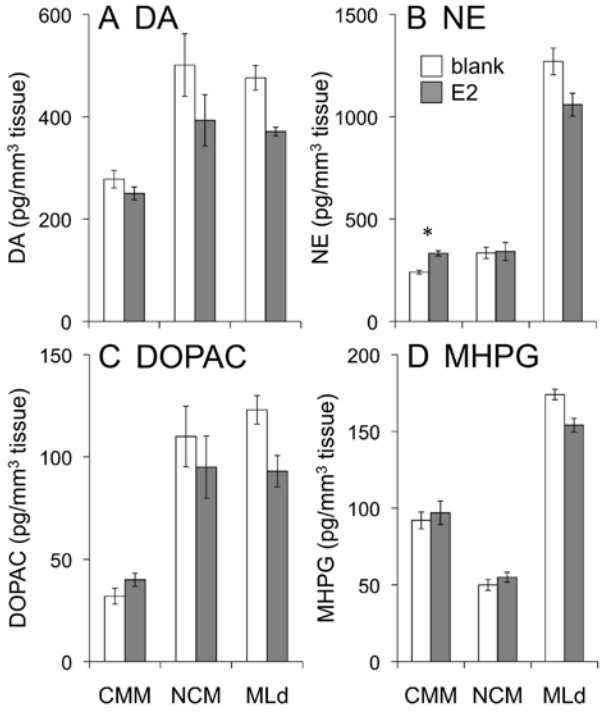

Fig. 5. The effects of estradiol (E2) treatment on (A) dopamine (DA) and (B) norepinephrine (NE) and their metabolites (C) dihydroxyphenylacetic acid (DOPAC) and (D) 3-methoxy-4-hydroxyphenylglycol (MHPG) in the caudomedial mesopallium (CMM), the caudomedial nidopallium (NCM), and the auditory midbrain (MLd) in female white-throated sparrows. Average protein content was 0.1 mg/mm^3. * $p = 0.022$.

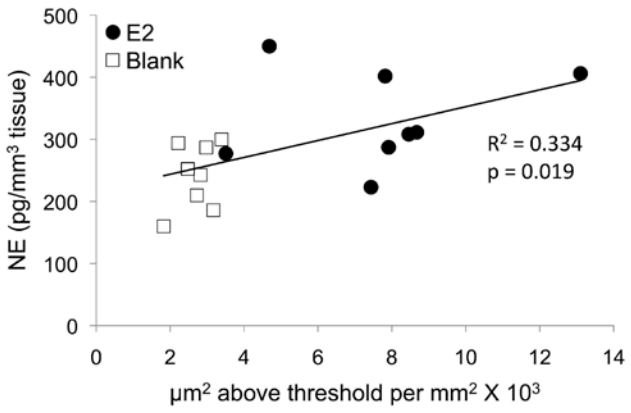

Fig. 6. Correlation between the density of dopamine beta-hydroxylase immunoreactive fibers and the level of norepinephrine (NE) in the caudomedial mesopallium (CMM). The relationship between DBH immunoreactivity and NE was likely driven by the significant effect of estradiol (E2) on both variables (see Figs. 4B, 5B).

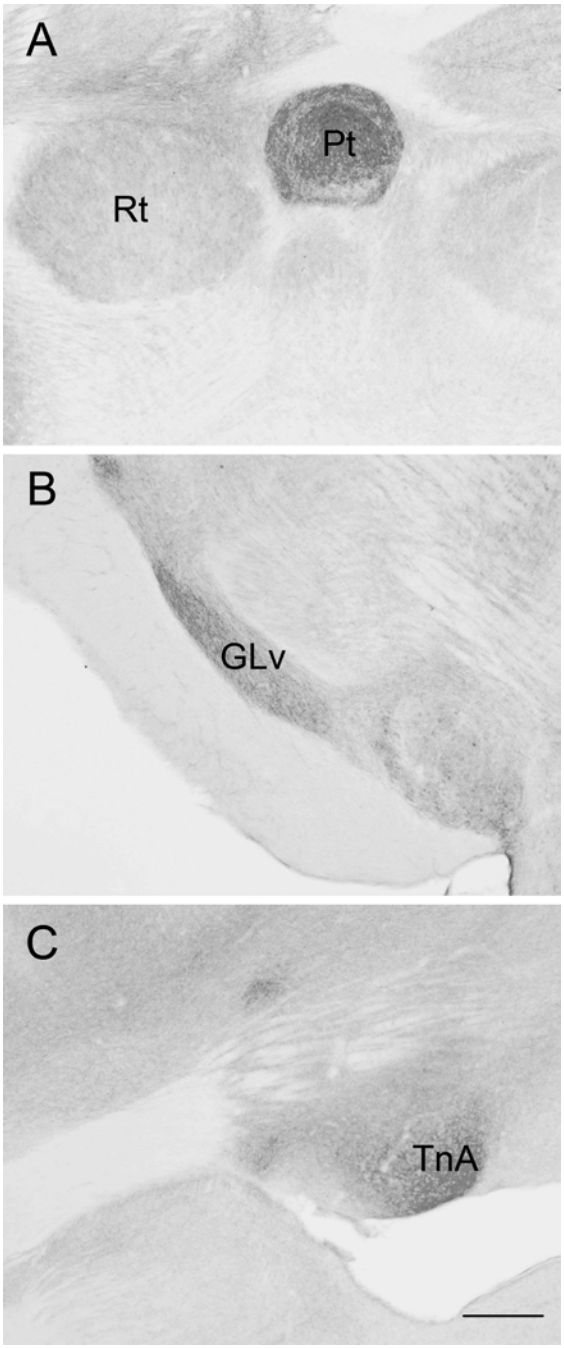

Fig. 7. Parasagittal view of serotonin transporter (SERT) immunoreactive structures in the brain – the pretectal nucleus (Pt; A), the lateral geniculate nucleus (GLv; B), and the nucleus taeniae of the amygdala (TnA; C) – from a female white-throated sparrow. Rt, nucleus rotundus. Rostral is to the left. Scale bar = 300 μm.

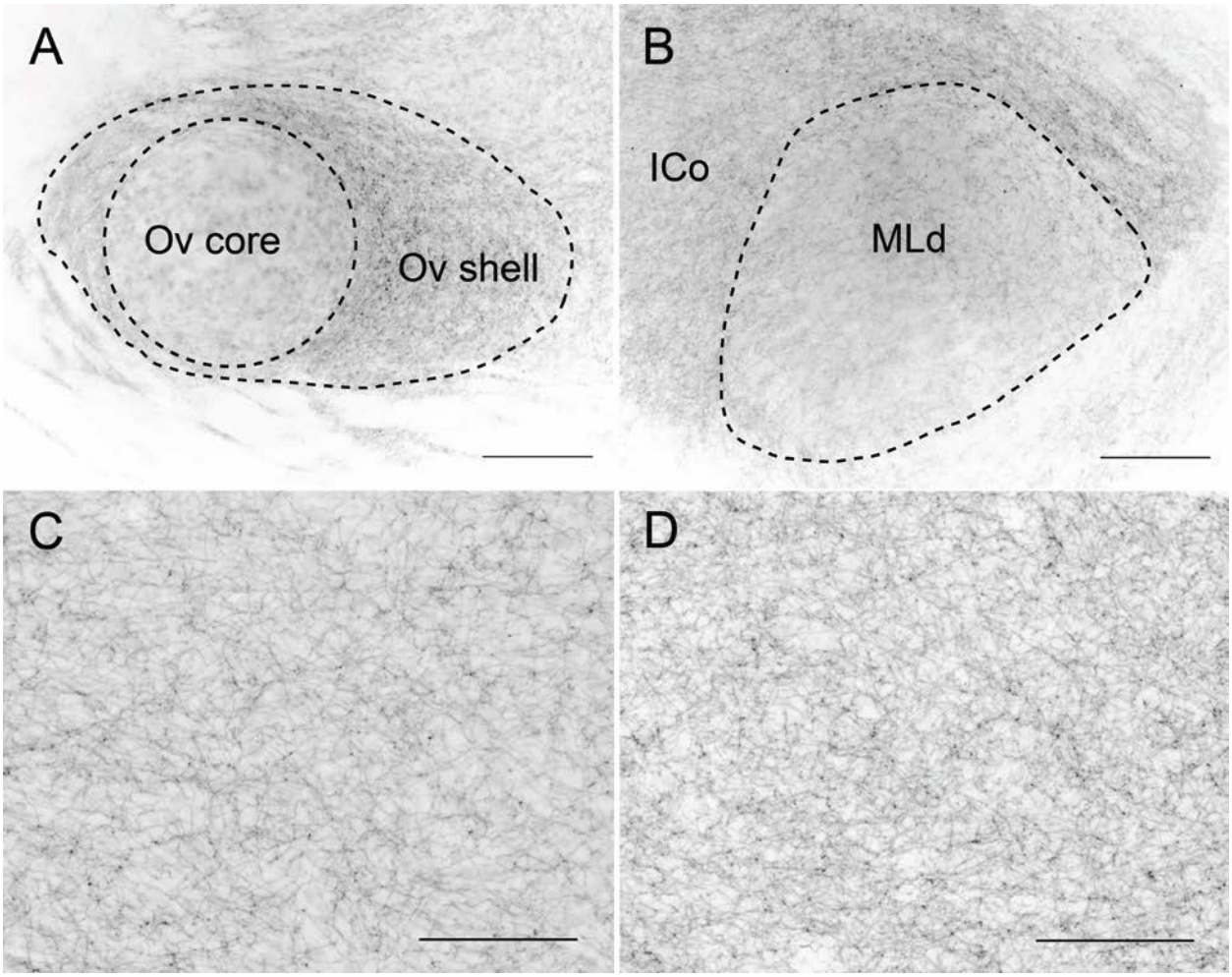

Fig. 8. Fibers immunoreactive for serotonin transporter in the auditory thalamus (Ov; A), the auditory midbrain (MLd; B), the rostrodorsal caudomedial nidopallium (rdNCM; C), and the apical part of the hyperpallium (HA; D), a visual area, in a female white-throated sparrow treated with vehicle. Note that immunolabeling in Ov is limited to the shell region; the core region is relatively devoid of fibers (see also Kaiser & Covey, 1997; Zeng et al., 2007). ICo, nucleus intercollicularis. Rostral is to the left. Scale bar = 150 µm.

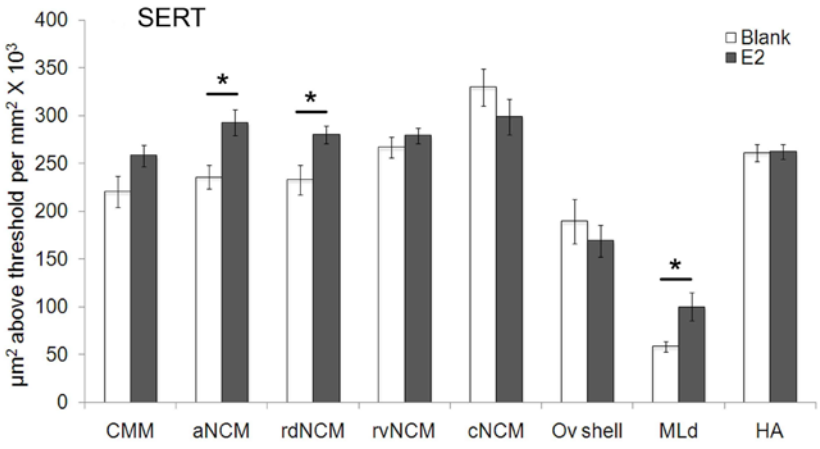

Fig. 9. The effects of estradiol (E2) on the density of fibers immunoreactive for serotonin transporter (SERT; A) and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA; B). E2 treatment increased the density of fibers immunoreactive for SERT in two domains of the caudomedial nidopallium (NCM), aNCM and rdNCM, and the auditory midbrain (MLd; A) and increased the concentration of 5-HIAA in the caudomedial mesopallium (CMM; B). aNCM, apical NCM. cNCM, caudal NCM. rdNCM, rostrodorsal NCM. rvNCM, rostroventral NCM. Ov, n. Ovoidalis. HA, apical part of the hyperpallium. * $P < 0.05$ compared to blank (placebo) condition. See text for P values.

CHAPTER 3

Rapid sound-induced changes in catecholamine synthesis and release in auditory areas in the female white-throated sparrow

This chapter presents work to be published as: Matragrano LL, Beaulieu M, Phillip JO, Rae AI, Sanford SE, Sockman KW, and Maney DL. Rapid effects of hearing song on catecholaminergic activity in the songbird auditory pathway. In Review.

ABSTRACT

Catecholamines are released in the central nervous system in response to sensory stimuli and are implicated in sensory processing. We previously showed that catecholaminergic innervation of the auditory pathway of female white-throated sparrows is plastic and sensitive to plasma concentrations of the ovarian hormone estradiol. This plasticity may underlie seasonal changes in the processing of courtship signals such as song. Estradiol-sensitive catecholaminergic fibers in the auditory pathway may release their products in a nonsynaptic, paracrine fashion, independently of sound stimuli, and rapidly in response to hearing behaviorally relevant stimuli. Here, we tested whether auditory stimuli, specifically conspecific vocalizations, induce rapid effects on catecholaminergic activity in estradiol-treated females. We found that immunoreactivity for the phosphorylated form of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis, increased in the caudomedial nidopallium of the auditory forebrain after exposure to 15 and 30 min of conspecific song. In addition, 30 min of song significantly increased concentrations of the catecholamine metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid in the same region. These findings suggest that hearing male song rapidly induces catecholamine synthesis and release in the auditory pathway.

INTRODUCTION

Catecholamines such as dopamine and norepinephrine (NE) have been implicated in sensory processing, attention, and learning. In mammals, they have been shown to shape response properties of sensory networks involved in selective attention (reviewed by Aston-Jones & Cohen, 2005 and Hurley et al., 2004). Catecholaminergic (CA) cells in the midbrain themselves respond to sensory stimuli; visual, auditory, and olfactory stimuli rapidly induce firing (Dommett et al., 2005; Rasmussen et al., 1986; Steinfels et al., 1983). There is some evidence that extracellular catecholamine levels can increase rapidly in sensory areas following exposure to sensory stimuli (Rangel & Leon, 1995; reviewed by Schultz, 2007) and that the magnitude of these responses increases with the behavioral relevance of the stimulus. When a sound is paired with a foot shock, for example, the concentration of catecholamine metabolites increases in the auditory cortex (Stark & Scheich, 1997). The auditory cortex itself can undergo dramatic remodeling in part *via* dopamine-modulated plasticity (Bao et al., 2001), indicating that the CA system may be involved in the formation of learned associations.

Social animals such as some rodents and songbirds communicate with vocalizations that are vital for reproduction and survival. Because these auditory signals are behaviorally relevant, catecholamines are likely to be directly or indirectly involved in their processing. Songbirds present an interesting and convenient model in which to test this hypothesis, because song is clearly important for reproduction and the CA system in songbirds has been well-studied. The distribution of CA projections in birds is very

similar to that of mammals, and CA fibers can be found throughout most of the auditory system (Appeltants et al., 2001, 2004; Mello et al., 1998; Reiner et al., 1994). CA fibers are particularly dense in the auditory forebrain, a region analogous to the auditory cortex of mammals (Appeltants et al., 2002; LeBlanc et al., 2007). The activity of these fibers may play a role in the perception of and behavioral responses to song (Appeltants et al., 2002a,b; Cardin & Schmidt, 2004; LeBlanc et al., 2007; Riters & Pawlisch, 2007). Noradrenergic denervation of the auditory forebrain reduces behavioral and neural responses to song as well as behavioral and neural selectivity for sexually stimulating song (Appeltants et al., 2002b; Lynch & Ball, 2008; Vyas et al., 2008). Female European starlings exposed to high-quality male song have higher levels of dopamine beta-hydroxylase (DBH)-immunoreactive fibers in the auditory forebrain than those females exposed to low-quality song (Sockman & Salvante, 2008). Thus, there is evidence that catecholamines may regulate the behavioral and neural responses to song and that song itself may alter catecholamine levels in the auditory system.

Immunohistochemistry (IHC) is a valuable method for localizing and estimating the density of CA fibers. The most common strategy is to label the rate-limiting enzymes in the catecholamine synthetic pathway, TH and DBH. Although this method is an excellent way to visualize CA cells and fibers, it does not allow precise measurements of CA activity, particularly on rapid time scales. To determine whether CA cells respond rapidly to a stimulus, IHC can be used to colocalize the expression of TH or DBH and the protein product of an immediate early gene (IEG) such as ZENK (Castelino & Ball, 2005; LeBlanc et al., 2007). IEG expression does not always correlate with activity however,

and its induction can be slow. More accurate methods to measure rapid CA responses to visual, auditory, and olfactory stimuli are electrophysiology, high performance liquid chromatography (HPLC), and microdialysis (Dluzen et al., 1981; Dommett et al., 2005; Fritz et al., 2003; Steinfels et al., 1983). Although electrophysiology is a popular technique and can be used to measure responses that occur within milliseconds of stimulus presentation, a major drawback is that changes in dopaminergic neuronal activity do not predict changes in dopamine concentrations (Schultz, 2007). In fact, dopamine concentrations in the striatum can increase through local, presynaptic mechanisms without any concurrent changes in dopamine impulse activity (Schultz, 2007).

In this study, we provide evidence that IHC can be used to detect rapid changes in CA activity and to identify regions in which those changes are occurring. We took advantage of the fact that in order to synthesize dopamine, TH must be phosphorylated at a minimum of one of its four serine sites. Antibodies against the phosphorylated form (pTH) can thus be used to map and quantify active TH. The phosphorylation of TH is one mechanism by which catecholamine concentration is finely regulated; application of a stimulus *in vitro* or *in vivo* induces TH phosphorylation within minutes (Bobrovskaya et al., 2007; Dunkley et al., 2004; Haycock 1990, 1993, 1996; Haycock & Haycock, 1991; Kumer & Vrana, 1996; Liu & Arbogast, 2010; Witkovsky et al, 2004).

An increase in pTH implies an increase in catecholamine synthesis (reviewed by Dunkley et al., 2004). Dopamine synthesis and release are most highly correlated with

phosphorylation at serine site 40 (Ser40) (Harada et al., 1996; Lindgren et al., 2002; Zigmond et al., 1989) and phosphorylation of Ser 40 increases the activity of TH to a greater extent than phosphorylation of the other three serine sites (reviewed by Dunkley et al., 2004). In two previous studies, immunolabeling for TH phosphorylated at Ser40 was used to examine the effect of social stimuli on CA activity in the brain (Gammie et al., 2008; Riters et al., 2007). Here, we used the same antibody to examine the effect of auditory stimuli on CA activity in the auditory pathway.

We performed the present study to look for rapid effects of hearing a behaviorally relevant sound on CA activity in auditory areas. We hypothesized that exposure to male conspecific song would induce rapid catecholamine synthesis and release in the auditory pathway of females. To address this hypothesis, we tested whether hearing song induced a change in pTH fiber density. In order to confirm that we were able to detect changes in CA activity by using IHC, we also used HPLC to more directly quantify the concentrations of catecholamines and their metabolites.

METHODS

Animals

We collected a total of thirty female white-throated sparrows (*Zonotrichia albicollis*) in mist nets in Atlanta, Georgia during fall 2007 and 2009. We determined their sex by polymerase chain reaction (PCR) analysis of a blood sample (Griffiths et al., 1998) and confirmed sex by necropsy at the end of the study. The birds were housed at the Emory University animal care facility in indoor walk-in flight cages and supplied with food and

water *ad libitum*. We held day length constant at 10:14h light-dark, which corresponds to the shortest day the birds would experience while overwintering at the capture site. We kept the birds under these light-dark conditions for two months to ensure that birds were not photorefractory before the start of the study (Shank, 1959; Wolfson, 1958). All procedures in this study were approved by the Emory University Institute for Animal Care and Use Committee and adhered to NIH standards.

Estradiol treatment

In captivity, female *Zonotrichia* sparrows do not come into full breeding condition and do not respond to song playback with courtship displays (Lake et al., 2008; Maney et al., 2007; Moore, 1983). We therefore simulated the breeding season and stimulated courtship behavior by administration of exogenous estradiol. Seven days prior to the playback experiment, we transferred the birds in pairs to sound-attenuated booths where they were housed individually in adjacent cages (38 x 38 x 42 cm). We held day length at 10:14 h light-dark throughout the experiment to prevent elevation of endogenous plasma estradiol (Shank, 1959; Wolfson, 1958). On the day the birds were transferred to the booths, they each received a subcutaneous silastic capsule (length 12 mm, ID 1.47 mm, OD 1.96 mm, Dow Corning, Midland, MI) containing 17β-estradiol (Steraloids, Newport, RI) and sealed both ends with A-100S Type A medical adhesive (Factor 2, Lakeside, AZ). This dose of estradiol increases plasma levels to those typical of the breeding season within seven days in this species (Maney et al., 2006, 2008) and likely does so within two days (Moore, 1983).

Song Playback

We started the playback experiment seven days after estradiol treatment began. On the afternoon prior to playback, we isolated each female in a sound-attenuated booth equipped with a speaker, a video camera, and a microphone. At ~2 h after lights-on the following morning, we presented song stimuli (see below) via a speaker inside the booth. We started video and audio recordings approximately 0.5-2 h after lights-on the following morning so that at least 30 min of behaviors were recorded prior to song playback. The birds were also recorded during stimulus presentation. We later used the recordings to count the total number of vocalizations (chips, chip-up calls, trills, tseets, and songs; see Falls & Kopachena, 1994 and Maney et al., 2009 for descriptions) given by each female during the stimulus presentations. Each bird heard either 30 min of song (n = 10), 15 min of song (n = 10), or silence, i.e. no song stimuli (n = 10). For the females in the silence condition, the duration of presentation was considered to be 30 min for the purpose of recording and scoring behavior.

Song stimuli

The stimulus presentations have been previously described in detail (Maney et al., 2006, 2007, 2009; Sanford et al., 2010). Briefly, we downloaded recordings of male white-throated sparrow songs from the Borror Laboratory of Bioacoustics birdsong database and constructed presentations consisting of one song every 15 s (natural song rate). To prevent habituation to the stimulus, the identity of the singer changed to a new male every three minutes. Thus, females listening to 15 min of song heard five unique males,

and females listening to 30 min of song heard ten unique males. Each female heard the males in a unique order. All songs were presented at 70 dB, measured at the bird's cage.

Tissue collection

Immediately following the stimulus presentation, we rapidly decapitated each bird and quickly harvested the brain. We used a clean razor blade to bisect each brain into hemispheres, fixed one hemisphere in 5% acrolein as described previously (Maney et al., 2003, 2005), and flash-froze the other hemisphere to be shipped via Federal Express to the University of North Carolina for HPLC analysis (see below). The hemisphere that was flash-frozen (right or left), the booth (one of six identical booths), and the position of each bird inside the room (to the right or to the left of the other bird in that room during the seven days) were balanced across treatments.

The birds in this study were collected in two different years, 2007 (n = 18) and 2009 (n = 12). Of the animals collected in 2007, six heard 30 min of song, six heard 15 min, and six heard silence. Although the fixed hemispheres were processed successfully, the flash frozen hemispheres were misplaced by the shipping company and thawed in transit. Catecholamine content could not be ascertained in those hemispheres. We therefore repeated the study with 12 additional animals in 2009. Of these, four heard 30 min of song, four heard 15 min, and four heard silence. Our sample size was thus n = 10 in each group for the IHC component, and n = 4 in each group for the HPLC component.

Immunohistochemistry

We cut the acrolein-fixed hemispheres into three series of 50 μm parasagittal sections using a freezing sliding microtome. We immunolabeled two of the three series using standard IHC protocols (Maney et al., 2001, 2003, 2005; LeBlanc et al., 2007; Riters et al., 2007). We incubated one series of sections with an anti-pTH antibody (Genetex; Irvine, CA; see section on *antisera* below) diluted 1:1250 (Riters et al., 2007) then labeled the pTH using a biotinylated secondary antibody and the ABC method (Vector, Burlingame, CA). We visualized the immunolabeling with nickel-enhanced diaminobenzidine (Shu et al., 1988).

We incubated another series of sections with an anti-TH antibody (ImmunoStar; Hudson, WI; see section on *antisera* below) diluted 1:2000 (LeBlanc et al., 2007; Matragrano et al., 2011) then incubated the sections with a biotinylated secondary antibody and the ABC method. We visualized the immunolabeling with diaminobenzidine. We processed each series of brain sections in three separate runs of IHC in which the three playback conditions were balanced across runs. Following IHC, we mounted all of the sections onto microscope slides, dehydrated them, and coverslipped in DPX (Sigma, St. Louis, MO).

Antisera

To label pTH we used a rabbit polyclonal antibody raised against a synthetic phosphopeptide corresponding to amino acid residues surrounding the phosphorylated Ser40 of rat TH (Genetex, Cat#GTX16557). Antibodies directed against pTH label a CA-

like distribution of cells and fibers similar to the distribution of TH cells and fibers in zebra finch and white-throated sparrows (Reiner et al., 1994; LeBlanc et al., 2007; Matragrano et al., 2011). The antibodies against TH and the phosphorylated form of TH label an identical distribution of cells in white-throated sparrows. To validate the pTH antiserum, we followed the procedure of Saper and Sawchenko (2003) using tissue from two untreated females not in the study. We first determined the concentration at which labeling was barely discernable (1:20,000) and then performed the preadsorption tests at twice that concentration (Saper & Sawchenko, 2003) and at the concentration normally used to label the protein (1:1250). The diluted antibody was incubated with 50 μg/ml of TH phosphor S40 control peptide supplied by the manufacturer (Genetex, Cat#GTX30707) on an orbital shaker at least 3 hours at room temperature before use. The procedure was otherwise as described above. The specificity of the secondary antibody was validated by omitting it for some sections.

To label TH, we used a mouse monoclonal antibody generated against denatured TH purified from rat PC12 cells (ImmunoStar, Cat#22941). It immunolabels both unphosphorylated and phosphorylated TH. According to the manufacturer, the antibody recognizes a 62 kDa band corresponding to TH in rat, and does not cross-react with dihydropterdine reductase, DBH, phenyletholamine-N-methyltransferase, phenylalanine hydroxylase or tryptophan hydroxylase using Western blot methods. It has wide species cross-reactivity and has been validated by preadsorption studies in a range of vertebrates (Olsson et al., 2008). This antibody labels a catecholamine-typical pattern of neurons and fibers in a wide variety of birds (e.g., Appeltants et al., 2001; Bailhache & Balthazart,

1993; Moons et al., 1994; Reiner et al., 1994; Roberts et al., 2001; Soha et al., 1996) including white-throated sparrows (Balthazart & Ball, 1996; LeBlanc et al., 2007; Matragrano et al., 2011) and was used by Reiner et al. (1994) to perform an exhaustive characterization of the distribution of TH-immunoreactivity in birds. Anti-TH antibodies from other sources and anti-dopamine antibodies reveal the same neural distribution in birds (e.g., Bottjer, 1993; Metzger et al., 1996). In our tissue, the antibody labels all major TH cell groups A1-A15 and fibers in a distribution typical of TH.

Regions of Interest

The auditory forebrain is a large, heterogeneous region and responses to sounds and hormonal changes are not uniform throughout it (e.g., Avey et al., 2008; Eda-Fujiwara et al., 2003; Gentner et al., 2001; Maney et al., 2003; Matragrano et al., 2011, 2012; Phillmore et al., 2003; Sanford et al., 2010; Sockman & Salvante, 2008). To be consistent with our previous work, we measured the effects of song playback in the same regions and domains in which estradiol affects ZENK responses and catecholaminergic innervation (Matragrano et al., 2011; Sanford et al., 2010). Sanford et al. (2010) divided NCM both rostro-caudally and dorso-ventrally into four primary domains (Sanford et al., 2010): rostro-dorsal (rdNCM), rostro-ventral (rvNCM), caudo-dorsal (cdNCM) and caudo-ventral (cvNCM). Because the dorsal and ventral domains of NCM are similar hodologically and neurochemically, we combined them into one caudal domain. As in our previous work, we also sampled from an apical domain (aNCM) located dorsal to Field L (Fig. 1). In the published literature, this region is usually considered part of NCM but may overlap the dorsal portion of Field L (Fortune & Margoliash, 1992).

We conducted all image acquisition and analyses while blind to treatment group. To photograph our regions of interest, we used the 10x objective on a Zeiss Axioskop microscope attached to a Leica DC500 camera and Macintosh G5 computer running Leica Firecam (version 1.7.1). We captured rectangular images (approximately 46 MB in size) corresponding to the field of view of the camera (870 X 690 μm), holding the light level constant for all photos. For photographs of TH-immunolabeled sections, we used a representative field of view containing a typical number of TH fibers to set the exposure time and the black, white, and gamma (luminosity) levels (histogram clipping 0.5%; automatically set by the Firecam software). The same exposure time and luminosity levels were then used for all of the images of each region for all birds. For the pTH-immunolabeling, high background staining prevented adequate differentiation of fibers in some cases; for those images we adjusted the exposure time and luminosity levels according to those automatically determined by the software in order to better discern the area covered by fibers.

For each bird, we acquired images of NCM and CMM in four consecutive sections between ~350 and ~800 μm from the midline. Five separate images, each containing CMM, aNCM, rdNCM, rvNCM, or cNCM, were acquired from each of the four sections. For CMM, the upper corners of the field of view of the camera were positioned along the dorsal boundary of CMM, one of the lower corners was positioned adjacent to the lamina mesopallium, and the entire photo was used in the analysis (Fig. 1) For the domains of NCM, the regions of interest were selected in the photos using ImageJ (version 1.41o,

National Institutes of Health, Bethesda, MD) as previously described (Matragrano et al., 2011, 2012; Sanford et al., 2010; see Fig. 1). We defined cNCM as a strip of tissue approximately 275 µm from the caudal boundary of NCM (Matragrano et al., 2011, 2012; Sanford et al., 2010). The acquired images of cNCM captured the majority of that domain, spanning 870 µm from dorsal to ventral (Fig. 1). The remaining regions of interest in NCM were sampled relative to the location of Field L, which is obvious in TH-labeled sections as an area of low TH immunoreactivity (Reiner et al., 1994). For aNCM, we placed a circle approximately 350 µm in diameter dorsal to Field L and just ventral to the ventricle. For rdNCM and rvNCM, which were later combined into the single domain rNCM (see Matragrano et al., 2011), we placed two circles, each approximately 550 µm in diameter, into the dorsal and ventral portions of this region (Matragrano et al., 2011, 2012; Sanford et al, 2010). All five photos of each section of auditory forebrain were viewed at the same time to ensure that the regions of interest we selected in each section did not overlap.

In addition to the auditory forebrain, we estimated the density of pTH-immunoreactive (IR) and TH-IR fibers in the auditory thalamus (Ov) in three consecutive sections in which it was the largest (Matragrano et al., 2011, 2012). There was light specific labeling of pTH in the auditory midbrain; however the background was too high to select the fibers accurately (see below). We therefore did not quantify fiber density in that region.

Image acquisition and estimation of pTH- and TH-IR fiber density

We converted all the photos to 8-bit scale and selected IR fibers using the thresholding feature in ImageJ (Maney et al., 2005; Matragrano et al., 2011, 2012). The same observer set the threshold for all images with the same lighting and computer monitor (LLM). Our method of selecting immunolabeled fibers has been fully validated and has high interrater reliability and low variability (Matragrano et al., 2011). We performed this procedure on 3-5 images per region (see above). For each region, we summed the total area covered by fibers and divided this sum by the total area measured to yield the area covered by the fibers in square microns per square mm of area measured.

Quantification of Catecholamines and Metabolites

To test for effects of song presentation on the actual levels of catecholamines and their metabolites in the auditory pathway, we performed HPLC analysis on micropunched tissue from the same animals (see tissue collection, above). n. Ov was too small to sample accurately *via* micropunch, so our samples were limited to CMM, rNCM, and MLd. The methods for quantification of catecholamines and metabolites are published elsewhere (Sockman & Salvante, 2008), and we reiterate the relevant portions here. We determined the concentration of catecholamines and metabolites by HPLC with electrochemical detection (Kilts et al., 1981). We sectioned the frozen, non-fixed hemispheres at -15˚C in the sagittal plane at 300 µm on a cryostat, thaw mounted the sections onto glass slides, and rapidly re-froze them on dry ice. From each of two consecutive sections and using chilled thin-walled stainless steel spring-loaded punch tools (Fine Science Tools, Foster City, CA, USA), we micropunched a region of CMM (0.5 mm i.d.) and of rNCM (1 mm

i.d.), each for which the anatomical boundaries have been described (Sockman et al., 2002). The sample of NCM did not include aNCM and cNCM because these areas of NCM were beyond the limits of the diameter of the micropunch. We took a micropunch (0.5 mm i.d.) from a region of MLd from two consecutive sections. We expelled the tissue punches into 1.9 ml polypropylene microcentrifuge tubes (one for each brain region), froze them on dry ice and stored them at -80˚C until assay.

The mobile phase consisted of sodium acetate (3.1 g), monohydrate citric acid (8.84 g), disodium EDTA (5 mg), sodium octyl sulfonate (215 mg), HPLC grade methanol (200 ml) and double-distilled, deionized water (800 mL). Immediately before assay, we added 125 µL of mobile phase with a concentration of 1 pg/µL of isoproterenol to each tube. We sonicated the samples and then centrifuged them at 16,000 *g* for 15 minutes at 4˚C. We aspirated the supernatant and injected 100 µl from each sample into the HPLC system.

The chromatographic system consisted of a HTEC-500 HPLC machine (EICOM Corporation, Kyoto, Japan), MIDAS Autosampler (Spark Holland, Emmen, Netherlands) and EPC-500 PowerChrom software Version 2.5 (EICOM Corporation, Kyoto, Japan) running on a PC. A precolumn (PC-04, 100 x 3.0 mm i.d., EICOM Corporation, Kyoto, Japan) was applied to the system to avoid contamination of the separation column (EICOMPAK SC-30DS, EICOM Corporation, Kyoto, Japan). We separated the compounds with mobile phase and the flow rate was 350 µl/min. We maintained the electrode potential at 750 mV with respect to an Ag/AgCI reference electrode. We

prepared standard solutions containing a fixed amount (100 pg) of the internal standard (isoproterenol, Sigma) and two amounts of each of the four external standards (Sigma): dopamine, NE, epinephrine, dihydroxyphenylacetic acid (DOPAC; the principal metabolite of dopamine), homovanillic acid (HVA; a major catecholamine metabolite), and 3-methoxy-4-hydroxyphenylglycol (MHPG; the principal metabolite of NE). To calculate the amount of catecholamines and metabolites in the samples, we compared the area of each compound peak to the area of their corresponding external standards.

We measured the protein content in rNCM of each sample by dissolving the remaining sample pellet in 0.2 N NaOH (100 µl) and performing the Bradford protein-dye binding assay (Quick Start Bradford Protein Assay, Bio-Rad) with bovine serum albumin as a standard (Bio-Rad) on a µQuant microplate spectrophotometer (BioTek) (Bradford, 1976). We did not have enough tissue of CMM and MLd to obtain reliable measurements of protein content.

Statistical Analysis

Because the level of pTH presumably depends on the level of available TH, the estimates of pTH-IR fiber density were normalized using the estimates of TH-IR fiber density for each region in each bird. In other words, each pTH value was divided by the corresponding TH value prior to estimate the proportion of TH that was phosphorylated in each region of interest. These ratios were used in the analysis. To analyze the effects of song duration on this ratio and the concentrations of monoamines and their metabolites,

we used a separate general linear model for each compound and each brain region. These models use restricted maximum likelihood to estimate parameter coefficients and z tests to determine whether the value of a coefficient differs from zero. For each model, the response variable was the mean of the region's ratio of pTH-IR to TH-IR or concentration of compound (in pg per mg protein in rNCM or in pg in CMM and MLd). The predictor song duration was expanded into a dummy-variable set to model the contrasts between 0 and 15 minutes and between 0 and 30 minutes.

In order to rule out the birds' own vocalizations as a factor influencing catecholamine synthesis or release, we first calculated vocalization rates by dividing the number of vocalization behaviors during the song presentations by the duration of the presentation. We then ran Spearman correlation tests to test for relationships (1) between the rate of vocalization and the ratio of pTH- and TH-IR and (2) between the rate of vocalization and the concentrations of each compound measured *via* HPLC for each region.

RESULTS

Rapid effects of song on the ratio of pTH- to TH-immunoreactivity

The effects of song on the pTH-IR to TH-IR ratios are graphed in Fig. 3. In general the density of pTH fibers was much lower than the density of TH fibers. For no brain region were effects of song duration on TH immunoreactivity reliable ($|z| \leq 1.34$, $P \geq 0.18$). This result indicates that hearing song did not alter the availability or synthesis of TH itself. In rNCM, the ratio of pTH immunoreactivity to TH immunoreactivity increased between 0 and 15 min ($z = 4.51$, $P < 0.001$) and between 0 and 30 min ($z = 2.10$, $P = 0.035$) of song

exposure. For no other brain region were the effects of song duration on this ratio reliable ($|z| \leq 1.20$, $P \geq 0.2$).

Rapid effects of song on catecholamines and their metabolites

Hearing song influenced the concentrations of some monoamines and their metabolites, depending on the region of the brain examined (Fig. 4). In rNCM, DOPAC increased between 0 and 30 min of song playback ($z = 2.40$, $P = 0.016$). Although the effect was not yet significant at 15 min ($z = 1.84$, $P = 0.07$), by 30 min HVA was also elevated ($z = 3.83$, $P < 0.001$). In MLd, the level of epinephrine was significantly lower after 15 min ($z = -2.42$, $P = 0.015$) and after 30 min ($z = -2.63$, $P = 0.008$) of song playback. Dopamine also showed a compelling downward trend in MLd after 30 min ($z = - 1.91$, $P = 0.056$). We did not find a reliable effect of playback duration on any of the other monoamines or their metabolites in rNCM or MLd, nor did we find a reliable effect of playback duration on any of the catecholamines or their metabolites in CMM (each analysis: $|z| \leq 1.76$, $P \geq 0.08$).

Explanatory value of vocalization behavior

We did not detect any significant relationships between the ratios of pTH/TH immunoreactivity and the rate of vocalization behaviors ($P \geq 0.200$). We did not find any significant correlations between the number of vocalization behaviors and the levels of catecholamines or their metabolites in regions where song playback did affect these variables.

Validation of antiserum

Pre-adsorption of primary antisera with the pTH protein (see Methods) completely abolished labeling of somata and fibers. Immunoreactive cells and fibers were clearly discernable in sections incubated with anti-pTH at 1:10,000 and 1:1250, and this labeling was absent in sections incubated with primary plus protein. Omission of the secondary antibody resulted in abolition of staining of fibers.

DISCUSSION

The purpose of this study was to test for rapid effects of auditory stimulation on CA activity in the auditory pathway. In this study, we found that hearing 15 or 30 min of song induced rapid changes in catecholaminergic activity. Our findings demonstrate that catecholamine activity increased, in a domain-specific manner, after 15 or 30 min of male song. In NCM, specifically in the rostral domain, we found an effect of song on pTH fiber density and catecholamine metabolite concentrations. We did not find an effect of song on these catecholamine markers in the caudal domain of NCM. TH phosphorylation and concentrations of the catecholamine metabolites DOPAC and HVA increased over a period of 15 to 30 min. Increases in metabolites can indicate catecholamine release (Commissiong, 1985; Houdouin et al., 1991; Kopin, 1985). Whereas HVA is a metabolite of all catecholamines, DOPAC is a metabolite of dopamine only. An increase in both compounds thus suggests that dopamine may have been released. It is possible that NE and epinephrine were also released during playback, but we detected no increase in the NE metabolite MHPG. NE is known to be involved in the perceptual processing of song and more generally, of salient environmental stimuli in songbirds and other social species

(Berridge & Waterhouse, 2003; Castelino & Schmidt, 2010; Furlow et al., 1980). Our results point most definitively, however, to a role for dopamine. The effects of song on pTH immunoreactivity in rNCM were mirrored in the contralateral hemisphere by increases in dopamine metabolites.

Previous studies have demonstrated that auditory stimuli can induce CA activity in both songbirds and mammals. The presentation of song or other social stimuli, for example, induced IEG expression in TH neurons of the brainstem in zebra finches (Bharati & Goodson, 2006; Goodson et al., 2009; Nordeen et al., 2009). Sound-induced CA activity has also been described in the auditory system, even in peripheral structures. In guinea pigs, TH immunoreactivity was up-regulated in the cochlea after 24 hours of exposure to a 1 kHz tone (Niu & Canlon, 2002). In rats, exposure to 45 min of mildly intense white noise increased concentrations of the NE metabolite MHPG in the cochlear nuclei, but not the inferior colliculus or primary auditory cortex (Cransac et al., 1998). In European starlings, one week of exposure to high-quality conspecific male song increased concentrations of NE and dopamine metabolites as well as the density of DBH-IR fibers in NCM (Sockman & Salvante, 2008). Our current findings provide further evidence that song stimulates CA activity in the auditory system and shows that its effects can be observed after only 15 minutes.

Rapid regulation of TH activity is possible *via* TH phosphorylation (Kumer & Vrana, 1996), which occurs within minutes of stimulus application (Bobrovskaya et al., 2007; Dunkley et al., 2004; Haycock 1990, 1993, 1996; Haycock & Haycock, 1991; Kumer &

Vrana, 1996; Liu & Arbogast, 2010; Witkovsky et al, 2004). The IHC method we used here has been used in only two earlier studies, one in mice and the other in birds. Gammie et al. (2008) immunolabeled pTH fibers in the brains of mice and found that the density of pTH fibers in a dopamine-associated area, zona incerta, was elevated in neglectful mothers. Riters et al. (2007) examined TH phosphorylation in response to an auditory stimulus in European starlings. They found that the density of pTH fibers decreased in the ventromedial nucleus of the hypothalamus and ventral tegmental area – brain regions involved in social behavior – in breeding females that heard 20 min of male song (Riters et al., 2007). Here, we used the same technique to show that hearing song for a similar duration also affects TH phosphorylation in the auditory forebrain. Immunolabeling of pTH may therefore represent an accurate and convenient method for assessing rapid effects of sensory stimuli on CA activity.

Our IHC and HPLC data together provide evidence for catecholamine release in the auditory forebrain of females during song presentation. After 15 min of song playback, we observed an increase in TH phosphorylation, then after 30 min of song playback we observed an increase in the metabolites DOPAC and HVA. We did not, however, see an increase in the catecholamines themselves. Future studies making use of earlier or more frequent sampling might be done to determine more definitively if and when dopamine is released. In addition, we found that exposure to song caused a decrease in epinephrine in the auditory midbrain (Fig. 4B). In birds, adrenergic cell bodies are found primarily in the C1-C3 cell groups located in the caudal medulla (Reiner et al., 1994; Steeves et al., 1987). These cells are thought to have a primarily autonomic function and have been

shown to project to the paraventricular nucleus as well as other regions involved in homeostatic regulation (Marino-Neto & Armengol, 2000). Projections elsewhere, however, are not well-described, perhaps because adrenergic axons are so thin that there is not enough immunoreactive product to detect them (reviewed by Marino-Neto & Armengol, 2000). Blocking axonal transport with a compound like colchicine, then combining IHC with anterograde tract-tracing might remedy this problem (Marino-Neto & Armengol, 2000). If adrenergic axons do project diffusely throughout the brain, they may innervate auditory structures. In a previous study in rats, intraperitoneal administration of epinephrine potentiated evoked auditory responses in the cortex (Berntson et al., 2003); however the effect was deemed peripheral because epinephrine does not easily cross into the brain.

In this study, we described rapid effects of hearing song on CA activity in the auditory forebrain of a female songbird. Because we did not measure pTH outside the auditory pathway, we do not know whether it might have increased in other regions as well. We did not however observe an effect of hearing song in every auditory region we examined; therefore the rapid effects of hearing song are probably not occurring universally throughout the brain. Because song was the only stimulus we presented here, we do not know whether sound-evoked responses depend on the type or behavioral relevance of the stimulus. Future studies in our laboratory will be conducted to determine whether these sound-induced responses are selective for song.

ACKNOWLEDGEMENTS

We thank Sarah Green, Arundhati Murthy, David Lee, Allison Reid, Katy Shepard, and Said Saab for technical assistance in Atlanta and Dr. Richard B. Mailman and Stan B. Southerland for providing and helping with the HPLC system. This research was supported by NINDS R01 NS055125 to KWS, NSF IBN-0346984 to DLM, and the Center for Behavioral Neuroscience IBN-9876754.

FIGURES

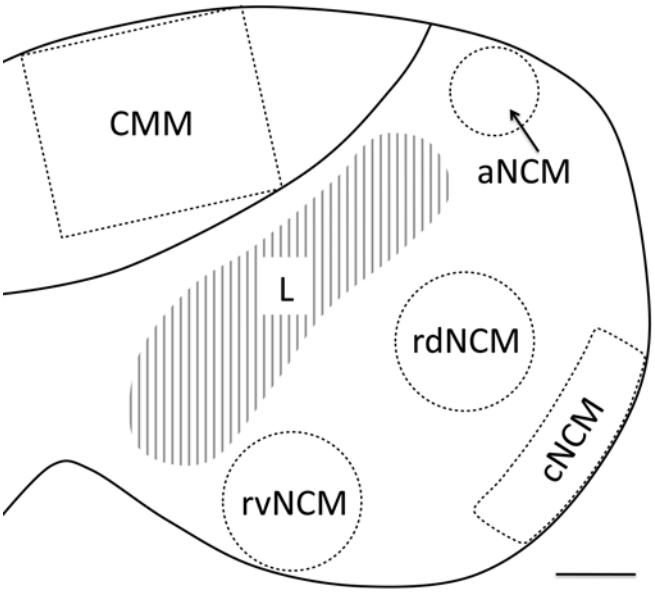

Fig. 1. Regions of interest depicted in a parasagittal view of the auditory forebrain. Dashed lines delineate the areas we sampled when measuring immunoreactivity. We sampled from four domains within the caudomedial nidopallium (NCM): apical NCM (aNCM), rostrodorsal NCM (rdNCM), rostroventral NCM (rvNCM), and caudal NCM (cNCM). rdNCM and rvNCM were combined to give an overall value for immunoreactivity in the rostral NCM (Chapter 2). Rostral is to the left. Scale bar, 300 μm.

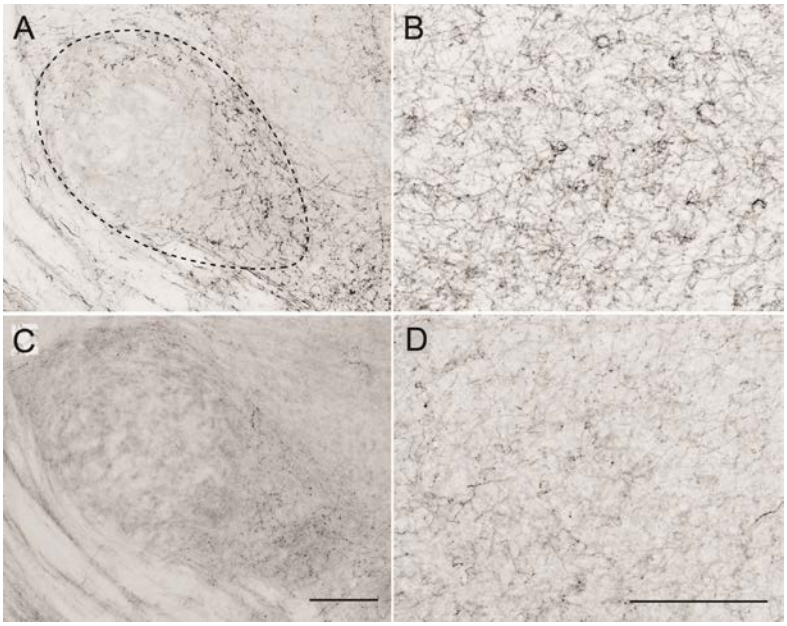

Fig. 2. Fibers immunoreactive for tyrosine hydroxylase (A, B) or phosphorylated tyrosine hydroxylase (C, D) in the auditory thalamus (n. Ovoidalis, Ov; A, C) and the auditory forebrain (rostral domain of caudomedial nidopallium, rNCM; B, D) in a female white-throated sparrow. Ov, which is made up of a core region surrounded by a shell, is encircled by the dotted line in panel A. Scale bar, 150 μm.

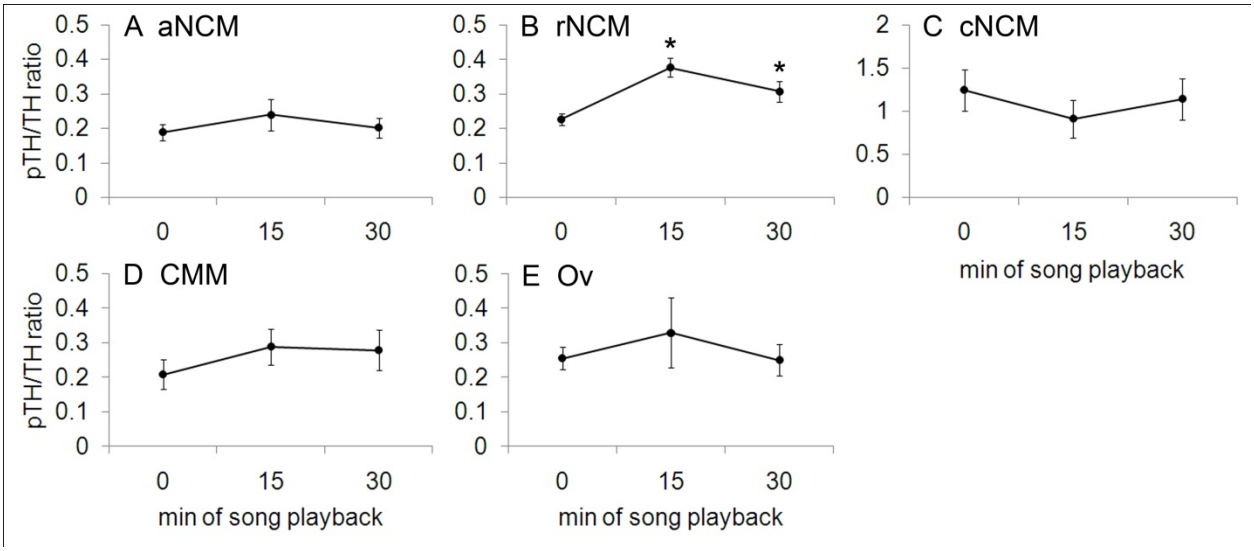

Fig. 3. The effects of song presentation on phosphorylated tyrosine hydroxylase (pTH) immunoreactivity relative to tyrosine hydroxylase (TH) immunoreactivity in the caudomedial nidopallium (NCM; A-C), the caudomedial mesopallium (CMM; D), and n. Ovoidalis (Ov; E). aNCM, apical NCM. cNCM, caudal NCM. rNCM, rostral NCM. *significantly higher than in the silence (0 min song) condition, $p < 0.05$. See text for actual p levels.

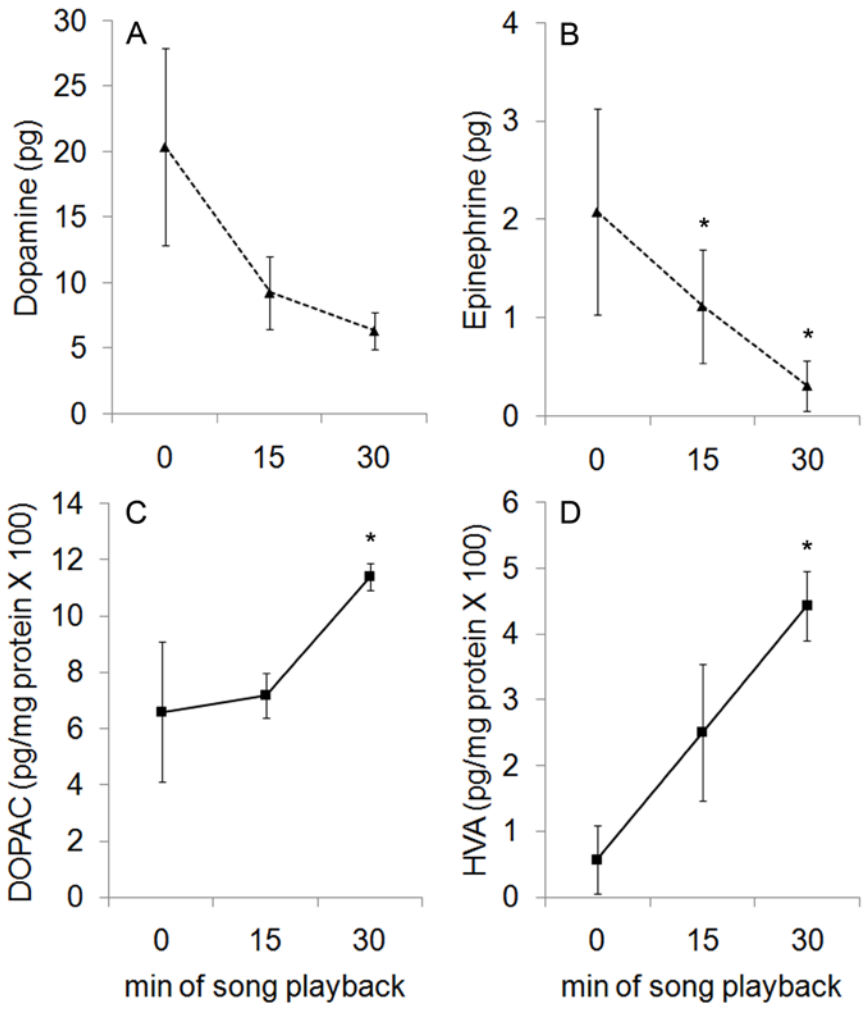

Fig. 4. The effects of song presentation on concentrations of the catecholamines dopamine (A) and epinephrine (B) in the auditory midbrain (MLd; denoted by triangles) and the catecholaminergic metabolites dihydroxyphenylacetic acid (DOPAC; C) and homovanillic acid (HVA; D) in the rostral domain of the caudomedial nidopallium (rNCM; denoted by squares) in female white-throated sparrows. Values given for rNCM were corrected for the total protein contained in the sample. The protein content of the MLd samples could not be obtained, thus the data for MLd were graphed without correcting for total protein. * Hearing song differs from hearing silence, $p < 0.02$. See text for actual p levels.

CHAPTER 4

Testosterone modulates genomic responses to song and monoaminergic innervation of auditory areas in the male white-throated sparrow

This chapter presents work from: Matragrano LL, LeBlanc MM, Chitrapu A, and Maney DL. 2011. Testosterone modulates genomic responses to song and serotonergic innervations of auditory areas in a seasonally breeding songbird. Abstract for poster presentation. Annual Society for Neuroscience Meeting, Washington DC.

ABSTRACT

Behavioral responses to social stimuli vary between seasons, possibly because the perceived context of the stimuli changes according to the hormonal condition of the animal. In songbirds, for example, behavioral responses to conspecific song depend on the plasma levels of estradiol in females and testosterone in males which change dramatically between the breeding and non-breeding seasons. Our previous work has suggested that such changes in behavior may be due to an effect of hormones on sensory processing. In the auditory forebrain of female white-throated sparrows, the expression of the immediate early gene ZENK (egr-1) is higher in response to conspecific song than a control sound only when estradiol levels are elevated to breeding-typical levels. Along with this change in selectivity, we have observed an increase in the density of monoaminergic fibers in the same areas following estradiol treatment. We hypothesize, therefore, that reproductive hormones may promote selective responses to auditory stimuli by acting on monoaminergic systems. This possibility has not been examined in males. In this study, we looked for evidence that testosterone alters (1) the selectivity of auditory ZENK responses as well as (2) monoaminergic innervation of auditory areas in male white-throated sparrows. We found that, like in females, selective ZENK responses in the caudomedial nidopallium were observed only in males with breeding-typical levels of steroids. Responses in the caudomedial mesopallium were selective regardless of hormone treatment. Whereas we previously reported that estradiol treatment increased monoaminergic fiber density in females, testosterone decreased monoaminergic fiber density in auditory areas in males. Although both sexes show selective ZENK responses to songs when gonadal hormones are at levels observed during breeding season, the

density of monoaminergic fibers decreases only in males. These findings suggest that the mechanisms underlying seasonal changes in auditory selectivity may differ between females and males.

INTRODUCTION

Gonadal hormones such as testosterone (T) and estradiol (E2) modulate sensory processing in fish, birds, and mammals (Kelliher et al., 1998; Miranda & Wilczynski, 2009; Sisneros et al., 2004; reviewed by Maney & Pinaud, 2011). In the past, research has focused on the effect of E2 in sensory systems, but relatively fewer studies provide evidence that T affects these systems (reviewed by Ball & Balthazart, 2004; Zheng, 2009). In male ferrets, T treatment significantly augmented the expression of the immediate early gene (IEG) *Fos* in the main olfactory bulb in response to females' odors (Kelliher et al., 1998). In the male European starling, in which gonadal hormones rise during the breeding season, T treatment increases the volume of the olfactory bulb. Breeding males can detect some odors that non-breeding males cannot (De Groof et al., 2010). Thus, seasonal changes in olfactory sensitivity may be mediated by circulating levels of gonadal steroids.

The auditory system may also be sensitive to sex steroids. Much of the research concerning the modulation of auditory processing *via* sex steroids has focused on the effects of E2. In seasonally breeding songbirds, behavioral responses to conspecific song change dramatically as plasma E2 rises in the spring. Females produce copulation displays in response to song only when E2 is increased to breeding levels (Kern & King,

1972; Moore, 1983). We have hypothesized that this effect of E2 on responses to auditory stimuli may reflect an effect on the physiology and function of auditory circuits (reviewed by Maney & Pinaud, 2011). E2 applied directly to the auditory forebrain can induce IEG expression (Maney & Pinaud, 2011; Tremere et al., 2009). In females of a seasonally breeding species, expression of the IEG *zenk* (protein product ZENK) is higher in the auditory system in response to song than to frequency-matched tones only when plasma E2 levels are increased to mimic breeding condition (Maney et al., 2006; Sanford et al., 2010).

The auditory forebrain in birds contains estrogen receptors (Gahr, 2001; Gahr et al., 1993), and E2 could act directly to alter auditory responses. Alternatively, or perhaps in addition, E2 may act *via* a variety of neuromodulatory systems known to affect auditory responses. For example, monoamines such as serotonin and catecholamines are important for alteration of auditory processing (Cransac et al., 1998; Ebert & Ostwald, 1992; Kahkonen et al., 2002). Catecholamines are known to shape context-dependent modulation of forebrain plasticity in zebra finches (Castelino & Ball, 2005) and serotonin similarly enhances plasticity of the auditory system in bats and mice (reviewed by Hurley & Hall, 2011). The catecholaminergic and serotonergic systems have been mapped extensively in birds (Challet et al., 1996; Cozzi et al., 1991; Dube & Parent, 1981; Fuxe & Ljunggren, 1965; Ikeda et al., 1971; Kaiser & Covey, 1997; Metzger et al., 2002; Yamada et al., 1984; reviewed by Durstewitz et al., 1999), and in songbirds, auditory areas are innervated by immunopositive fibers (LeBlanc et al., 2007; Reiner et al., 1994; Zeng et al., 2007). These monoamines are particularly good candidates for mediating the

effects of gonadal steroids because they are already known, particularly in mammals, to be sensitive to these hormones. Outside the auditory circuit, T decreases monoamine concentrations and downregulates monoaminergic receptors in mammals and birds (Riters et al., 2002; Shemisa et al., 2006). Previously, we found that E2 increases the density of catecholaminergic and serotonergic fibers innervating the auditory pathway in female white-throated sparrows (LeBlanc et al., 2007; Matragrano et al., 2011, 2012). Few studies, however, have been conducted to determine the effects of T on the monoaminergic innervation of the auditory system.

Steroid-dependent changes in the selectivity and innervation of auditory areas suggest that auditory perception may depend on season. Such plasticity may be important when similar sounds have different meanings according to the time of year. In female white-throated sparrows, for example, song is used during the breeding season as a courtship signal, but outside the breeding season it is more likely to communicate aggression (reviewed by Maney & Goodson, 2011). Plasma E2 may thus act on the auditory system to promote behavioral responses that are seasonally appropriate. In males, although song is likely to be used in an agonistic context at all times of the year, its purpose during the breeding season does differ from the non-breeding season in that it is more likely to signal defense of a physical territory. Seasonally breeding males are more likely to have a territorial response to song during the breeding season (Falls & Kopachena, 1994; Wingfield & Farner, 1978; reviewed by Maney & Goodson, 2011) and these aggressive behaviors are often accompanied by or correlated with increased plasma T (reviewed by Maney & Goodson, 2011 and Wingfield et al., 1990). Thus, like females, males alter their

behavioral responses to conspecific song according to context and season. We therefore hypothesized that selectivity and monoaminergic innervation of auditory areas may be enhanced by T treatment in males, just as it is enhanced by E2 in females.

In the current study, we investigated whether T modulates the ZENK response in the auditory system of males and whether these effects might be mediated by monoaminergic innervation of the same regions. In females, ZENK responses in the auditory forebrain, thalamus, and midbrain are selective for male song when plasma E2 reaches breeding levels. Here, we predicted that T-treatment would induce selectivity in the same regions of the auditory pathway in males. Further, if T-induced selectivity is mediated by monoamines, the density of monoaminergic fibers may be altered in the same areas. To test these predictions, we treated male white-throated sparrows with T or vehicle, and exposed them to either conspecific male song or frequency-matched tones (Maney et al., 2006). We then examined how T affected (1) the ZENK response to sound and (2) the densities of noradrenergic and serotonergic fibers in the auditory pathway.

METHODS

Animals

All procedures in this study adhered to NIH standards and were approved by the Emory University Institutional Animal Care and Use Committee. We collected twenty-three male white-throated sparrows in mist nets in Atlanta, Georgia. We determined their sex by polymerase chain reaction analysis of a blood sample (Griffiths et al., 1998) and confirmed sex by necropsy at the end of the study. The birds were housed at the Emory

University animal care facility in indoor walk-in flight cages and supplied with food and water *ad libitum*. We held day length constant at 10:14h light-dark, which corresponds to the shortest day the birds would experience during the winter at the capture site. We kept the birds under these conditions for at least four months to ensure that they were not photorefractory prior to the start of the study (Shank, 1959; Wolfson, 1958).

Hormonal manipulation

We transferred the birds to individual cages (38 x 38 x 42 cm) in sound-attenuated booths (Industrial Acoustics, Bronx, NY) before the start of the experiment. Each booth held 4-6 males. Hormone treatment was balanced within housing groups. On the day each bird was transferred, we implanted each bird with a subcutaneous silastic capsule (length 15 mm, ID 1.47 mm, OD 1.96 mm, Dow Corning, Midland, MI) sealed at both ends with A-100S Type A medical adhesive (Factor 2, Lakeside, AZ). Males received either an empty implant (n = 11) or an implant containing T (n = 12; Steraloids, Newport, RI). T-filled implants increase plasma levels within a physiological range in castrated birds of this genus (Smith et al., 1997; Tramontin et al., 2003; Wikelski et al., 1999).

Sound stimuli

The playback protocol has been previously described (Maney et al., 2006, 2007, 2009; Sanford et al., 2010). Briefly, we used recordings of singing male white-throated sparrows from the Borror Laboratory of Bioacoustics birdsong database. We edited the recordings so that there were 15 seconds of silence between each male song and each

recording of a unique male lasted for exactly 3 min. These 3 min segments were spliced together to form the song presentations and each song presentation contained the songs of 14 males for a total of 42 min. We played a variety of male songs with the intention to help overcome habituation to the stimulus (Stripling et al., 1997). For each of the song recordings described above, the frequencies for each whistle of each song were measured using AudioXplorer (Arizona Software, San Francisco, CA) and sinusoidal tones were generated at these frequencies, each of equal duration. Tone sequences were made to be equivalent to individual songs in total duration, duty cycle, and number of onsets and offsets of sound. The tone sequences were spliced together with 15 s of silence between each tone sequence and contained 14 sequences for a total of 42 min as described above for the song sequences.

Playback experiment

We started the playback experiment seven days after hormone treatment began. On the afternoon prior to stimulus presentation, each bird was isolated in an empty sound-attenuated booth equipped with a microphone, speaker, and video camera. Sound playback began approximately 1 h after lights-on the following morning. Birds were randomly assigned to a stimulus group such that half of each treatment group heard either songs (T-treated, $n = 6$; blank, $n = 6$) or tones (T-treated, $n = 6$; blank, $n = 5$). The order of the song or tone stimuli was unique. All sound stimuli were delivered *via* the speaker inside the booth at a peak level of 70 dB measured at the front of the bird's cage. The stimulus presentation was followed by 18 min of silence. Video and audio recordings were made of each bird for 42 min prior to and 42 min during stimulus presentation. We

later used the recordings to count the total number of vocalizations (chip-ups, trills, tseets, and songs; see Falls & Kopachena, 1994 and Maney et al., 2009 for descriptions) given by each male.

Tissue collection

Sixty min after the start of sound playback, each bird was deeply anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL) and we collected a blood sample from the jugular vein into a heparinized StatSampler tube (Iris, Westwood, MA). We centrifuged the samples to isolate the plasma and stored the samples at -20°C. We used blood samples from each male in a radioimmunoassay to determine plasma T levels using a commercially prepared kit (Diagnostic Systems Laboratories, Webster, TX; #DSL-4000) to verify that the T implants effectively increased plasma T levels to the range of free-living males (Spinney et al., 2006). Brains were harvested, immersion-fixed in 5% acrolein, cryoprotected in 30% sucrose, and frozen at -20°C until sectioning (Maney et al., 2003; 2005).

Immunohistochemistry

We cut the fixed brains into three series of 50 μm coronal sections using a freezing sliding microtome. We immunolabeled the three series for ZENK, dopamine beta-hydroxylase (DBH), or serotonin transporter (SERT) using standard immunohistochemistry (IHC) protocols (LeBlanc et al., 2007; Maney et al., 2001, 2003,

2005). The procedure for ZENK immunolabeling was described previously by Maney et al. (2003). To label for ZENK, SERT, and DBH protein, we incubated sections with an antibody against ZENK (anti-egr-1; Santa Cruz Biotechnology, Santa Cruz, CA; see section on *antisera* below) diluted 1:8000, an antibody against SERT (ImmunoStar; Hudson, WI; see *antisera* section below) diluted 1:5000 (Meyer et al., 2004), or an antibody against DBH (Immunostar, Hudson, WI; see section on *antisera* below) diluted 1:16,000, respectively. All sections were subsequently incubated using a biotinylated secondary antibody against rabbit IgG (Vector, Burlingame, CA) diluted 1:250, followed by the ABC method (Vector, Burlingame, CA). We visualized ZENK and DBH immunolabeling with nickel-enhanced diaminobenzidine (Shu et al., 1988) and SERT immunolabeling with diaminobenzidine (without nickel). We processed each series of brain sections in three separate runs of IHC in which the treatment (T-treated or empty implants) and sound stimulus (tones or songs) conditions were balanced across runs. Following IHC, we mounted all of the sections onto microscope slides, dehydrated them, and coverslipped in DPX (Sigma, St. Louis, MO).

Antisera

We labeled ZENK with egr-1 antisera (Santa Cruz Biotechnology Cat#sc189; Santa Cruz, California). The specificity of this antibody has been validated previously in songbirds and specifically in white-throated sparrows (Mello & Ribeiro, 1998; Saab et al., 2010). All labeling in brain sections was eliminated at antibody concentrations of 1:25,000 by preadsorption of the antibody with 50 µg/ml of the egr-1 peptide (Santa Cruz Biotechnology Cat#sc189-P; Santa Cruz, California; Saab et al., 2010).

Immunolabeling for SERT is considered a more stable marker of serotonergic fibers and has been shown to be a better indicator of serotonin axons than immunolabeling for serotonin because SERT is less likely to be metabolized than serotonin (Nielsen et al., 2006). To label SERT we used a rabbit polyclonal antibody generated against a synthetic peptide sequence corresponding to amino acids (602-622) of rat SERT coupled to keyhole limpet hemocyanin (ImmunoStar Cat#24330). Immunolabeling in the rat brain is completely abolished by pre-absorption with synthetic rat SERT (602-622). We validated its specificity in white-throated sparrow brain sections using the procedure described in Saper and Sawchenko (2003). All labeling in brain sections is eliminated at antibody concentrations of 1:25,000 by preadsorption of the antibody with 50 µg/ml of the SERT peptide (ImmunoStar Cat#24332). The specificity of each of the primary or the secondary antibodies was validated by omitting it in some sections and observing a complete loss of specific staining.

We used a polyclonal antibody generated in rabbit against DBH purified from bovine adrenal medulla (ImmunoStar Cat#22806). According to the manufacturer, the antibody detects a triplet at approximately 72-74 kilodaltons in a Western blot. The antibody labels a norepinephrine-like distribution of cells and fibers in a variety of birds (e.g., Bailhache & Balthazart, 1993; Castelino & Ball, 2005; Karle et al., 1996; Sockman & Salvante, 2008), including white-throated sparrows (LeBlanc et al., 2007). Sockman & Salvante (2008) reported that preadsorption with antigen supplied by the manufacturer of the

antibody eliminates all labeling in brain sections from European starling (*Sturnus vulgaris).*

Regions of interest

Our regions of interest were within the NCM and CMM in the auditory forebrain, the core and shell of auditory thalamus (Ov), and the auditory midbrain (MLd). We acquired the images of all regions of interest using the 4x objective (MLd) or the 10x objective (NCM, CMM, Ov) on a Zeiss Axioskop attached to a Leica DFC480 camera and Macintosh G5 computer. All light and shutter speed settings were held constant for each region. We acquired 3-5 consecutive images of each region spanning 300-600 µm (see below). Images were each approximately 46 MB in size and were converted to 8-bit with ImageJ (version 1.41o, National Institutes of Health, Bethesda, MD).

We photographed five consecutive sections of NCM spanning 600 µm. A domain of NCM with dense labeling was sampled in all 5 photos with the circle tool in ImageJ to select a circle with a diameter of 550 µm that was approximately 350 µm from the medial edge of NCM (Maney & Pinaud, 2011; sampled within the box in Fig. 1). CMM was photographed in four consecutive sections spanning 450 µm and MLd was photographed in three or four consecutive sections (see below) spanning 300-450 µm. We photographed the region of CMM approximately 400 µm from the midline at the level where the lamina arcopallialis dorsalis meets the lateral ventricle (Maney et al., 2006;

Figs. 1 and 2). Using the freehand tool in ImageJ, the area of hippocampus in each image was subtracted before the density of immunoreactive (IR) fibers was estimated. For MLd, we photographed the three (ZENK) or four (SERT and DBH) sections that contained the largest cross-sections of MLd and traced its obvious borders with respect to the nucleus intercollicularis and surrounding tissue using the freehand tool of ImageJ (Maney et al., 2006; Fig. 2). Ov was photographed in three consecutive sections spanning 300 μm. We identified the core and shell of Ov in coronal sections with reference to Durand et al. (1992). In previous research the core of Ov did not contain specific staining of serotonin immunoreactivity (Zeng et al., 2007). We found that the core of Ov contained minimal specific staining of SERT-IR fibers and the background in the region was too high to select the fibers accurately; therefore only the shell of Ov was selected in sections immunolabeled for SERT. The density of ZENK-IR and DBH-IR fibers was estimated in both the core and shell of Ov. The brain from one bird was not fixed properly and the tissue had to be excluded from the analysis of the data from the forebrain and thalamus for all antibodies. Finally from SERT- and DBH-IR sections, we photographed a nonauditory area, the apical hyperpallium (HA; Reiner et al., 2004; Stokes et al., 1974), in two sections spanning 150 μm. These photos were collected to test the specificity of the effects of T treatment on auditory regions. The entire photo of HA used to estimate fiber density.

ZENK-IR cell nuclei and DBH- and SERT-IR fibers were selected in each image by a blind observer using the thresholding feature in ImageJ as previously described (LeBlanc

et al., 2007; Maney et al., 2003, 2006; Matragrano et al., 2011, 2012). The threshold was set manually for each image such that selected pixels agreed with what the observer considered to be labeled nuclei or fibers. Because of cell clumping the cells were not counted individually and instead the density of labeled cells was estimated (see below). As was done for the ZENK analysis, the area covered by DBH- or SERT-IR fibers with an optical density higher than threshold was measured within each area of interest. Our method of selecting immunolabeling has been validated and shown to have high interrater reliability and low variability (Matragrano et al., 2011). The threshold was set for all images by one of three observers with the same lighting and computer monitor. To estimate the density of immunoreactivity, we divided the total area covered by cells or fibers, in square microns per square mm, by the total area measured for each region.

Statistical analysis

Data were square root transformed to normalize their distribution. We first performed a repeated-measures MANOVA with region of interest (NCM, CMM, Ov shell, or MLd) and immunolabel (ZENK, SERT, or DBH) as the within-subject factors, and sound stimulus (song or tones) and hormone treatment (blank or T) as the between-subjects factors. Because we hypothesized that ZENK-, SERT-, and DBH-IR for the visual region we sampled, HA, would be unaffected by hormone treatment and sound stimuli and because we wanted to minimize Type II rather than Type I error, we analyzed the data from that region in a separate multivariate *F*-test. We did not predict an effect of sound stimulus on the monoaminergic markers thus the density of DBH- and SERT-IR fibers

was estimated and univariate *F*-tests for both markers were performed with hormone treatment as the fixed factor. Then we performed a MANOVA for each label with IHC run (i.e. 1st, 2nd, or 3rd group of sectioned brains that was immunolabeled) as the between-subject factor. The area covered by labeling was dependent on IHC run only for DBH; therefore, IHC run was included as a random factor in subsequent *F*-tests for DBH immunolabeling. For the ZENK data, we performed univariate *F*-tests with sound stimulus and hormone treatment as the fixed factors. Because we did not predict an effect of sound stimulus on the density of monoaminergic fibers, we performed univariate *F*-tests for each compound in each region of interest with hormone treatment as the only fixed factor. Effect sizes were calculated on untransformed data using Cohen's *d.* Finally, in order to determine whether the birds' own behavior influenced ZENK expression, we ran Spearman correlation tests between the percent area covered by ZENK-IR cells within each auditory region and the total number of vocalizations given during the 42 min playback as well as the 84 min period (42 min of silence followed by 42 min of song) of behavioral recording.

RESULTS

When we analyzed IHC data in a repeated-measures MANOVA (see Methods), we found an effect of treatment (Wilks' lambda, $F_{1, 18} = 8.996$; $P = 0.008$) and an effect of sound stimulus (Wilks' lambda, $F_{1, 18} = 10.622$; $P = 0.004$). There was no interaction between hormone treatment and sound stimulus.

Effects of T treatment and sound stimulus on ZENK immunoreactivity in auditory areas

Post hoc univariate *F*-tests with T treatment and sound stimulus as fixed factors showed an effect of stimulus in NCM ($F_{1, 18} = 6.438$, $p = 0.021$) and CMM ($F_{1, 18} = 19.966$, $p < 0.001$) of the auditory forebrain. In NCM, the ZENK response in T-treated males hearing song was significantly greater than in T-treated males hearing tones ($F_{1, 18} = 6.493$, $p = 0.029$, $d = 1.256$) but there was no significant difference between blank-treated males that heard song or tones ($F_{1, 18} = 1.091$, $p = 0.327$, $d = 0.636$). In CMM, ZENK-IR in males hearing song was higher than in males hearing tones in both T- ($F_{1, 18} = 9.462$, $p = 0.012$, $d = 1.702$) and blank-treated birds ($F_{1, 18} = 11.812$, $p = 0.009$, $d = 2.298$). Finally there was a trend for the stimulus to affect ZENK-IR in the auditory midbrain ($F_{1, 18} = 3.588$, $p = 0.074$) and no other notable effects in the auditory midbrain and thalamus. The effects of T treatment and sound stimuli on ZENK-IR are graphed in Fig. 3.

Effects of T treatment on monoaminergic immunoreactivity in auditory areas

T treatment significantly decreased SERT-IR fiber density in NCM ($F_{1, 20} = 4.466$, $p = 0.047$, $d = 0.907$), the shell of Ov ($F_{1, 20} = 10.417$, $p = 0.004$, $d = 1.257$), and MLd ($F_{1, 20} = 12.060$, $p = 0.002$, $d = 1.446$). The effects of T treatment on SERT fiber density are graphed in Fig. 3. T treatment significantly decreased DBH-IR fiber density in the auditory midbrain ($F_{1, 20} = 343.489$, $p < 0.001$, $d = 0.586$) but not in the auditory forebrain and thalamus (Fig. 4). There was no effect of treatment on SERT- or DBH-IR fiber density in the visual region, HA.

Correlations between vocalizations and ZENK-IR

There were no significant correlations between the number of vocalizations (the total tseets, trills, chip-up, or song and the total of all vocalizations) and ZENK-IR in each of the auditory regions of interest (NCM, CMM, Ov, and MLd), either during the playback period or the total 84 min of behavioral recording.

DISCUSSION

For any organism that relies on auditory signals for communication, plasticity within the auditory system is necessary particularly when the behavioral relevance of those signals changes from season to season. We have hypothesized that reproductive hormones alter the salience of reproductively-relevant signals, facilitating responses to these important stimuli during the breeding season. In previous studies we showed that E2, when increased to breeding-typical levels in female white-throated sparrows, causes sound-induced ZENK responses in NCM to become selective for songs over tones (Maney et al., 2006; Sanford et al., 2010). In this study we found that in males, the ZENK response in NCM was selective for song over tones only when T was increased to breeding levels. NCM thus appears to respond more selectively to conspecific song during the breeding season in both males and females. It is possible that T-induced selectivity may occur in another seasonally breeding songbird, the black-capped chickadee. In CMM and the dorsal and ventral domains of NCM of male chickadees, two domains that partially overlap with our sampled domain of NCM, the ZENK response was higher to conspecific than to heterospecific song only when males were in breeding condition (Phillmore et al.,

2011). A role for reproductive hormones in the tuning of auditory ZENK responses to behaviorally relevant stimuli may thus generalize to other species.

The effect of T on the selectivity of ZENK induction in NCM was not attributable to T-induced increases in the response to song. Hearing song resulted in similar ZENK induction in both T-treated and placebo-treated birds (Fig. 3). Rather, T may have affected selectivity by reducing the ZENK response to tones. In black-capped chickadees, the ZENK response to heterospecific song was strikingly lower throughout the auditory forebrain in breeding males than in non-breeding males (Phillmore et al., 2011). Similarly, in female white-throated sparrows, E2 treatment inhibited the ZENK response to non-relevant synthetic tones (Maney et al., 2006). These results suggest steroid-dependent modulatory input to the auditory forebrain that induces a selective response to behaviorally relevant signals by inhibiting responses to non-relevant stimuli.

Although we have evidence that the selectivity of the ZENK response may be altered by T, the mechanism of action is still unclear. There is no evidence of androgen receptors in the auditory forebrain and midbrain (Balthazart et al., 1992; Fusani et al., 2000; Zigmond et al., 1973), thus it is unlikely that T is acting directly on receptors in these regions. Instead T may be acting indirectly. T can be converted by the enzyme aromatase to E2, which may act on estrogen receptors (ERs). Most of the constituents of the auditory pathway in songbirds, for example the cochlear nuclei, MLd, Ov, Field L, and CMM, do not contain ER-positive cells (Gahr, 2001; Gahr et al., 1993) thus indirect modulation by

aromatization may not occur in these regions. NCM, however, does contain ERs. ER-alpha is expressed along the dorsal caudomedial edge of NCM, and ER-beta is expressed uniformly throughout the region (Gahr et al., 1993; Jeong et al., 2011; Saldanha & Coomaralingam, 2005). Because aromatase and ER mRNA are expressed in high levels in NCM (Appeltants et al., 2004; Balthazart & Ball, 1996; Fusani et al., 2000; Jeong et al., 2011; Schlinger, 1997) and because we previously showed that E2 increased the selectivity of the ZENK response in NCM of females (Maney et al., 2006; Sanford et al., 2010), it is possible that the effects we observed in this study were ultimately due to aromatization of T to E2.

An indirect effect of T on auditory selectivity may be mediated by serotonergic systems. T treatment decreases the density of serotonin-IR fibers in male hamsters (Grimes & Melloni, 2002; 2006). In gonadectomized female rats, T treatment decreases the density of serotonin-IR fibers to a level similar to that observed in males (Simerly et al., 1985). T decreases the level of additional serotonergic biomarkers as well. For example, T treatment of male rats decreases levels of SERT mRNA, serotonin, and the serotonin metabolite 5-hyroxyindoleacetic acid (5-HIAA) in a number of regions throughout the brain (Gabriel et al., 1988; Martinez-Conde et al., 1985; McQueen et al., 1999). We previously found that in female white-throated sparrows, E2 treatment increases the density of SERT-IR fibers (Matragrano et al., 2012; see Chapter 2), but here we found that elevated T in males decreases SERT-IR fiber density. Thus, like in mammals, T appears to suppress serotonergic projections in this seasonally breeding songbird.

In addition to decreasing serotonergic fiber density in the auditory midbrain, T also decreased noradrenergic fiber density. The noradrenergic system appears to influence the auditory system (reviewed by Castelino & Schmidt, 2010). For example, noradrenergic antagonists blocked modulation in the auditory forebrain of male songbirds (Cardin & Schmidt, 2004). We previously found that E2 treatment increased the density of DBH-IR fibers in the auditory midbrain and forebrain (Matragrano et al., 2011; see Chapter 2). Here we found that T treatment decreased DBH-IR fiber density in the auditory midbrain which could result in decreased levels of norepinephrine and reduced activity in the noradrenergic system. In rats, castration increased norepinephrine levels and T treatment significantly reduced norepinephrine levels in the hypothalamus (Gabriel et al., 1988). In regions involved in song control in male starlings, plasma T was inversely related to the density of alpha(2)-noradrenergic receptors (Riters et al., 2002) and male birds in breeding condition, that likely have increased plasma T, had a lower density of this noradrenergic receptor in the song control pathway (Heimovic et al., 2011). The adrenergic system might be modulated by testosterone as well. In male rats, testosterone treatment downregulated alpha2A-adrenergic receptor expression in the brainstem where adrenergic cell bodies that project to the cortex are located (Dygalo et al., 2002). Further experimentation in our laboratory is being conducted to characterize the distribution and steroid modulation of catecholaminergic receptors in the auditory pathway.

Whereas T and E2 have similar effects on the selectivity of the ZENK response in males and females, respectively, they seem to have opposite effects on monoaminergic innervation of the auditory pathway. If steroid-dependent selectivity is driven by steroid-induced changes in monoaminergic activity, therefore, seasonal changes in selectivity may be mediated *via* different monoaminergic mechanisms in males and females. In this study we provide evidence that whereas E2 treatment increases catecholaminergic innervation of auditory structures in females, T treatment downregulates the same innervation in males. E2 and T have previously been hypothesized to exert opposite effects on the dopaminergic system in mice (Shemisa et al., 2006) and there are a number of sex differences in dopaminergic function in mammals (reviewed by Becker, 1999). A number of neurotoxins that affect the dopaminergic system do so in a sexually dimorphic manner, suggesting that E2 has a neuroprotective effect whereas T may instead exacerbate the effects of the toxins (Brooks et al., 1989; Dluzen et al., 2001; Dluzen & McDermott, 2002; Dluzen et al., 2003; Freyaldenhoven et al., 1996; Wagner et al., 1993; Yu & Wagner, 1994). For example in mice, induction of neurotoxicity causes more degeneration in the catecholamine system in males than in females (Dluzen et al., 2003). The differential sensitivity of dopaminergic systems in males and females suggests that E2 and T may have disparate effects on these systems. Our results are consistent with this model, and show further that such effects may occur independently of neurotoxicity.

Gonadal hormones are known to have a priming effect that facilitates breeding-typical behavioral responses in reproductive contexts. This priming is likely to involve monoamines, particularly serotonin. In female rats, gonadal hormones such as E2

modulate serotonin activity in areas involved in female sexual receptivity (reviewed by Uphouse, 2000). E2 treatment reduced the density of serotonin-IR fibers as well as serotonin levels in the medial preoptic area and ventromedial nucleus of the hypothalamus (Lu et al., 1998). The decrease in extracellular serotonin may have been caused by an E2-dependent enhancement of serotonin reuptake (Maswood et al., 1999); E2 is known to increase SERT mRNA in the dorsal raphe nucleus (McQueen et al., 1997). Gonadal hormones may similarly modulate serotonergic (and noradrenergic) input to regions important for the processing of socially relevant information and thereby facilitate reproductive behaviors in the spring. E2 increases SERT-IR fiber density in regions necessary for processing song (Matragrano et al., 2012). Hearing conspecific vocalizations is associated with a rapid increase in serotonin in at least one auditory area in mice (Hall et al., 2010) and an increase in a serotonin metabolite in NCM in female white-throated sparrows (Matragrano et al., 2012; See Chapter 5). It is yet unknown whether the steroid-induced monoaminergic activity described in this study ultimately affects behavioral responses. Future studies in songbirds should focus on establishing a causal link between monoaminergic activity and the neural and behavioral responses to sociosexual signals.

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FIGURES

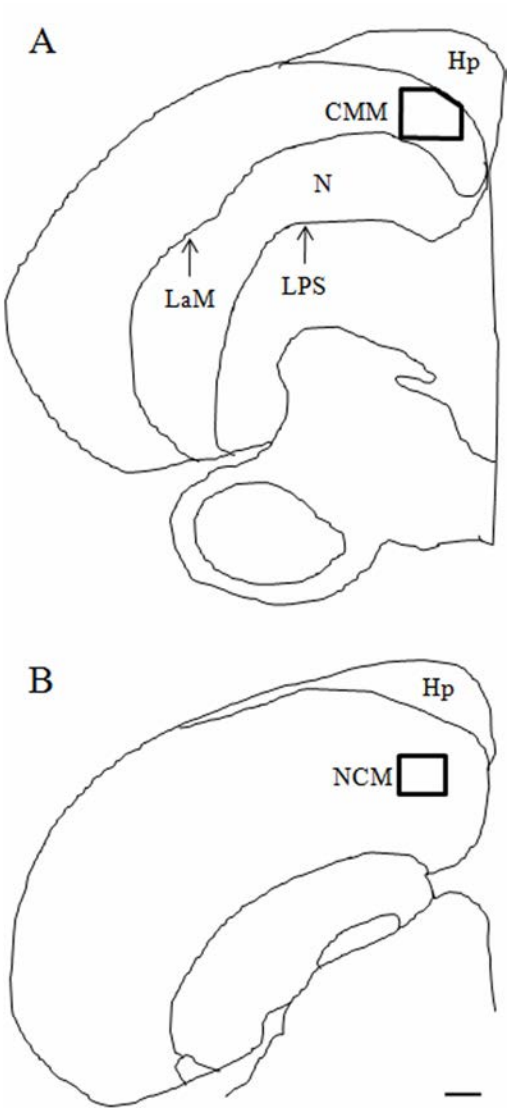

Fig. 1. We estimated the density of immunoreactive cells and fibers in coronal sections in the caudomedial mesopallium (CMM; A) and the caudomedial nidopallium (NCM; B). Hp, hippocampus. LaM, lamina mesopallialis. LPS, lamina pallio-subpallialis. N, nidopallium. Scale bar, 500 μm.

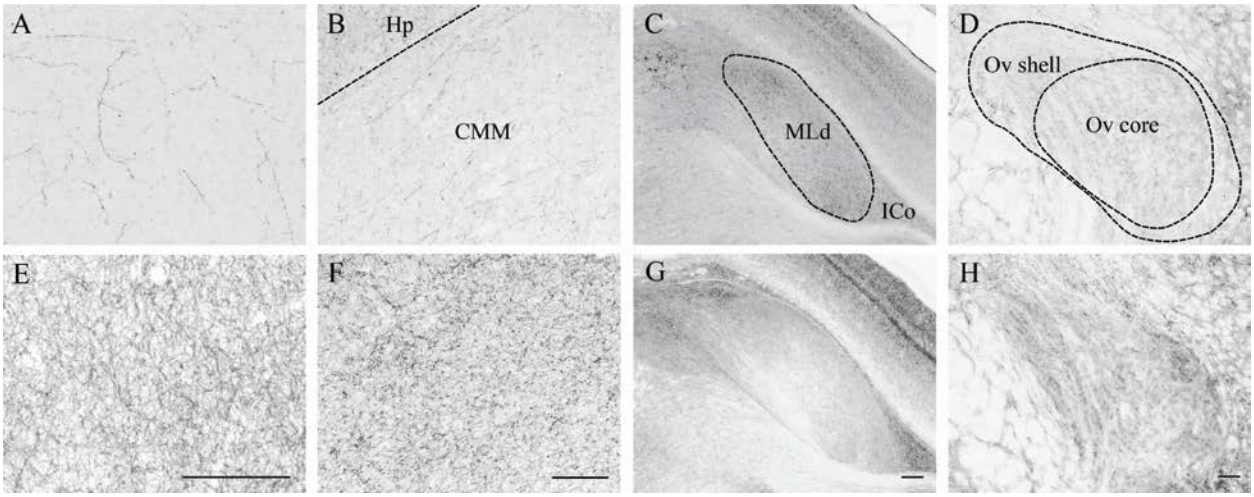

Fig. 2. Fibers immunoreactive for dopamine β-hydroxylase (DBH; A-D) and serotonin transporter (SERT; E-H) in the caudomedial nidopallium (NCM; A, E), the caudomedial mesopallium (CMM; B, F), the auditory midbrain (MLd; C, G), and the auditory thalamus (Ov shell and Ov core; D, H). All images are from a T-treated male. Hp, hippocampus; ICo, nucleus intercollicularis. Scale bar, 150 μm.

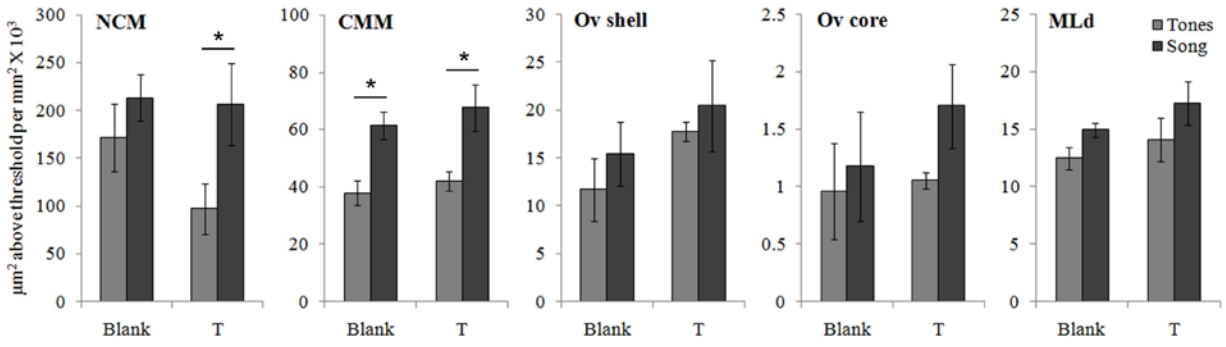

Fig. 3. The effects of testosterone (T) treatment and auditory stimulus on the protein product of the immediate early gene ZENK in the caudomedial mesopallium (CMM), the caudomedial nidopallium (NCM), shell and core of the auditory thalamus (Ov shell, Ov core), and the auditory midbrain (MLd). * $p < 0.05$ compared to blank condition. See text for actual p levels.

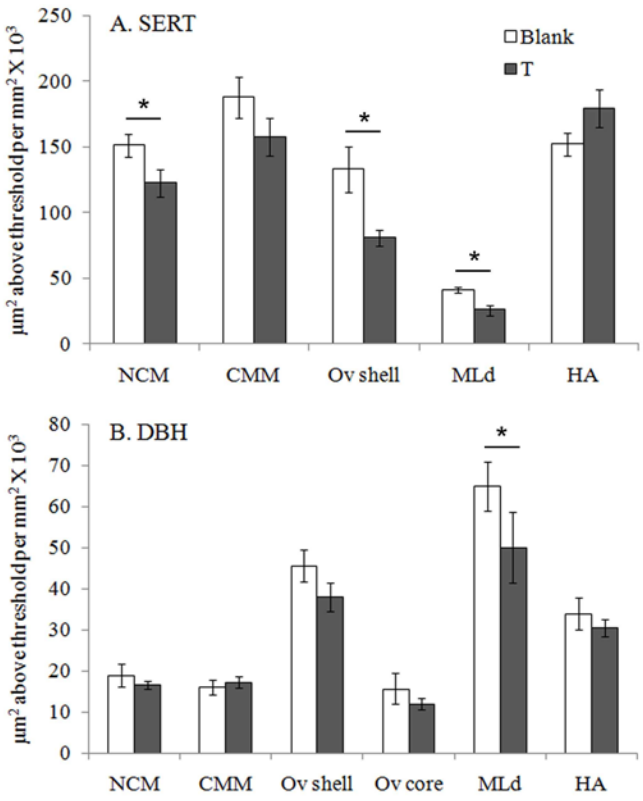

Fig. 4. The effects of testosterone (T) treatment on the density of fibers immunoreactive for serotonin transporter (SERT, A) and dopamine beta-hydroxylase (DBH, B) in the caudomedial mesopallium (CMM), the caudomedial nidopallium (NCM), shell and core of the auditory thalamus (Ov shell, Ov core), and the auditory midbrain (MLd) in males. Data from both stimuli groups were combined here. Because there was high background and little specific binding in the core of Ov in sections immunolabeled for SERT, the density of SERT fibers could not be reliably estimated. * $p < 0.05$ compared to blank condition. See text for actual p levels.

CHAPTER 5

CONCLUSIONS

5.1 SUMMARY OF KEY FINDINGS

In some species, the behavioral relevance of an auditory stimulus may change over time such that the same sound may have a different meaning according to social context. In songbirds, for example, song may be used as a communication signal year-round but typically indicates courtship only during the breeding season. Seasonally breeding songbirds are thus excellent models to study the mechanisms underlying how perceiving and responding to sociosexual stimuli can be altered by context. Gonadal hormones, because their levels in plasma change dramatically according to season, are good candidates for mediating the effect of social context on auditory responses. We previously reported that estradiol (E2) alters sound-induced expression of the immediate early gene, *zenk* (protein, ZENK) in the auditory pathway of female white-throated sparrows (Maney et al., 2006; Sanford et al., 2010). The ZENK response was selective for song over a control sound in non-breeding birds only when E2 was increased to breeding-typical levels. In addition, E2 treatment increased the expression of ZENK in the auditory pathway even in birds not exposed to sound. My dissertation replicated this finding in males treated with testosterone (T), in that the ZENK response was higher in response to song than to a control sound only when T was increased to breeding-typical levels; thus gonadal hormones appear to increase auditory selectivity in both sexes.

Because gonadal hormones are known to modulate monoaminergic activity, I hypothesized that hormone-dependent auditory selectivity may be mediated by monoaminergic systems. A major finding of my dissertation was that E2 and T treatment

altered catecholaminergic and serotonergic fiber density in the auditory pathway. In females, E2 treatment increased monoaminergic innervation of the auditory forebrain and midbrain, and in males, T treatment decreased monoaminergic innervation of the same regions. These results suggest that seasonal changes in gonadal hormones alter auditory perception and processing in both sexes, and that T and E2 may do so *via* different mechanisms.

5.2 EFFECTS OF E2 ON SOUND-INDUCED AUDITORY RESPONSES IN FEMALES: CONCLUSIONS & IMPLICATIONS

As described in Chapter 1, hormones may affect the processing of auditory sociosexual signals. I proposed a model wherein gonadal steroids act *via* monoamine systems to alter selectivity of auditory ZENK responses (Chapter 1, Fig. 1). To test this model, I first asked whether and where the gonadal hormone E2 modulates monoaminergic innervation of the auditory system. In Chapter 2, I treated females with E2 or vehicle for a week in order to determine the effect of systemic E2 treatment on monoaminergic activity in auditory regions. I found evidence that E2 increases not only the levels of at least one monoamine and metabolite, but also the density of monoaminergic fibers. E2 treatment increased the density of fibers immunoreactive (IR) for tyrosine hydroxylase (TH), dopamine beta-hydroxylase (DBH), or serotonin transporter (SERT) in the auditory forebrain and midbrain, and also increased the concentrations of norepinephrine and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) in one region of the auditory forebrain, the caudomedial mesopallium (CMM). Because both DBH-IR fiber density

and norepinephrine concentrations increased with E2 treatment in CMM, and because DBH is necessary to convert dopamine to norepinephrine, I concluded that E2 may increase the production and possibly the release of norepinephrine in that region.

E2 appears to sharpen genomic responses to behaviorally relevant signals (Maney et al., 2006; Sanford et al., 2010). Gonadal hormones may achieve this effect by acting *via* monoaminergic cell bodies in the brainstem. In vertebrates, estrogen receptors have been described in the dorsal raphe, locus coeruleus, and ventral tegmental area (Alves et al., 1998; Maney et al., 2001; Sheng et al., 2004; Shugrue et al., 1997; Simerly et al., 1990); therefore E2 can have direct effects on these neurons. The dorsal raphe, locus coeruleus, ventral tegmental area, substantia nigra, and other monoaminergic cell groups extend projections to many regions of the brain, including the auditory pathway (Durstewitz et al., 1999; Hurley & Thompson, 2001; Joel & Weiner, 2000; Klepper & Herbert, 1991; Levitt & Moore, 1979; Olazàbal & Moore 1989; Reiner et al., 2004; Rouiller et al., 1989; Steinbusch, 1981). In the ventral tegmental area, estrogen receptors are expressed on the cells that contain TH (Kritzer, 1997). E2 promotes axonal sprouting and growth *in vitro* in hypothalamic cell cultures, in the hippocampus and arcuate nucleus of ovariectomized rats, and in the medulla of ovariectomized monkeys (reviewed by Toran-Allerand et al., 1999; Vanderhorst et al., 2002). Thus E2 may increase the projections that extend from brainstem monoaminergic cell groups and innervate auditory areas in female songbirds.

In Chapter 2, I showed that breeding levels of E2 enhance monoaminergic innervation of several auditory areas and may increase release. It is possible that the release of

monoamines in a paracrine or nonsynaptic fashion may be sustained over prolonged periods, for example throughout a period of high fertility, such that the auditory system is primed to respond differently to the same input depending on social context or season. In addition, E2 priming could potentially promote selective firing of the monoaminergic cells in response to song, yielding rapid effects on the auditory pathway that contribute to selective auditory responses. In this way, monoaminergic activity could also have rapid, short-lived effects on auditory responses. In Chapter 3, I investigated this possibility by testing whether hearing song induces rapid monoaminergic activity. I found that just 15 min of song playback increased phosphorylation of TH in NCM, suggesting rapid increases in catecholamine synthesis. Hearing song also increased levels of the catecholamine metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid in NCM, suggesting catecholamine release. Interestingly, these effects were observed in the same auditory regions in which E2 increases catecholaminergic fiber density (Chapter 2). Other research has shown that hearing song induces activity in the catecholaminergic cell groups likely to project to the auditory forebrain (Gale & Perkel, 2010; Maney et al., 2008). The resulting transmitter release may rapidly alter auditory responses. In summary, the findings reported in Chapters 2 & 3 suggest that E2 increases the density of monoaminergic terminals that may be capable of releasing monoamines either in a nonsynaptic fashion, not coupled with firing, or rapidly in response to sound. It still remains to be seen whether the sound-induced rapid responses observed are dependent on E2.

In the caudomedial nidopallium (NCM) of the auditory forebrain, serotonergic fibers appeared to be denser than the catecholaminergic fibers we immunolabeled. This difference may be due either to differences in the methods used to label each, or to functional differences between the neuromodulators. In mammals, the density of serotonergic innervation of the auditory system varies according to region and can also differ from the density of catecholaminergic fibers (Campbell et al., 1987; Takeuchi & Sano, 1983). In monkeys, serotonergic innervation of the auditory cortex is denser than catecholaminergic innervation (Campbell et al., 1987) which is consistent with my observation in white-throated sparrows. It is possible that differential innervation of auditory areas may allow for differential enhancement of auditory responses by catecholaminergic versus serotonergic systems.

The auditory forebrain contains two similar but separate regions, CMM and NCM, which are heavily interconnected (Brauth et al., 1987; Karten, 1967; Kelley & Nottebohm, 1979). The two regions are considered to be analogous to the superficial layers of the mammalian auditory cortex (Theunissen & Shaevitz, 2006) or to secondary mammalian auditory regions (Jarvis, 2004). My studies suggest that they may have functionally distinct roles. E2 treatment increased both noradrenergic fiber density and the concentration of norepinephrine in CMM, but not NCM. On the other hand, song presentation rapidly increased both catecholaminergic activity and metabolism in NCM, but not CMM. These results are consistent with a model wherein E2 upregulates catecholaminergic activity in NCM on a rapid time scale, concurrent with song exposure and in CMM over longer periods of time. The catecholaminergic innervation may

function differently in these two regions in that catecholamines may be released in response to song in NCM but released in a paracrine manner determined by plasma hormone levels in CMM (Chapter 1, Fig. 1). The functional implications of this finding are unclear, however, because few researchers have attempted to assign unique functional roles to each region. There is some evidence to suggest that CMM is more sensitive to social context, whereas NCM is more sensitive to familiarity (Woolley & Doupe, 2008); note however that selective responses to familiar songs have also been reported in CMM (Maney et al., 2003). Much more research will be needed to differentiate the unique roles of CMM and NCM in auditory processing.

5.3 EFFECTS OF E2 ON SOUND-INDUCED RESPONSES IN FEMALES: FUTURE DIRECTIONS

In my studies, I identified possible sites of monoamine action by labeling axon fibers. Because monoamines can be released in a nonsynaptic, paracrine fashion, however, localizing the fibers does not necessarily pinpoint the exact sites of action. We recently completed a study mapping the distributions of two serotonin receptor mRNAs, serotonin-1B and -1E. We found that serotonin-1B and -1E are present in CMM and NCM of the auditory forebrain and absent from the auditory midbrain. Serotonin-1B receptor is present in both the core and shell of the auditory thalamus. Catecholamine receptors, such as adrenergic and dopaminergic receptors, have been described throughout the avian brain (Ball et al., 1989; Kubikova et al., 2010; reviewed by Castelino & Schmidt, 2010). Their distribution may depend on sex and plasma levels of

gonadal hormones (Riters et al., 2002). For example, the density of adrenergic receptors is sexually differentiated in zebra finches (Riters & Ball, 2002). In mammals, serotonin receptor density is sexually dimorphic (Fischette et al., 1983) and serotonergic and adrenergic receptor expression can be regulated by gonadal steroids (reviewed by Bethea et al., 2002; Davies & Lefkowitz, 1984; Gundlah et al., 1999; Kugaya et al., 2003; Rubinow et al., 1998). Two weeks of E2 treatment in female rats resulted in an increase in 5-HT_2 receptors, but there was a concomitant decrease in 5-HT_1 and beta-adrenergic receptors (Biegon et al., 1983). These findings were obtained in homogenized samples of whole brains thus the sites of these effects are unknown. 5-HT_{2A} receptor binding decreased in ovariectomized female rats, and E2 treatment either partially or completely restored 5-HT receptor binding density to control levels in the frontal cortex (Cyr et al., 1998). In future work, the effects of E2 treatment on monoamine binding in the avian brain should be quantified with the use of radioactive ligand binding assays.

In Chapters 2 and 3, I demonstrated that the rapid, song-induced increases in the catecholamine metabolites DOPAC and HVA occurred in the same auditory regions in which E2 increased catecholaminergic fiber density (Chapter 2). In that experiment, we measured not only catecholamines and their metabolites, but also serotonin and its metabolite, 5-HIAA. Because that chapter focused entirely on catecholaminergic systems, the results of the serotonin analysis were not included there. They are shown in Fig. 1 below. In addition to increasing the concentrations of two catecholamine metabolites (Chapter 3), hearing 30 min of song also increased the concentration of 5-HIAA (Matragrano et al., 2012; Fig. 1). This increase occurred in NCM, the same region in

which DOPAC and HVA increased. Thus, the overall results of the playback study suggest that, in this region, both catecholamines and serotonin are released in response to hearing song. Because all females in the playback study were treated with E2, and because none were exposed to a control sound such as frequency-matched tones, it remains unclear whether this rapid response to sound depends on E2 or is selective for biologically relevant stimuli. A future project could expand this study to include an E2 manipulation as well as control sounds. Such an experiment would benefit from a larger sample size which would increase statistical power and help verify the effects I have reported here, particularly in light of the fact that we were not able to measure monoamine and metabolite concentrations in all of the birds that heard song using HPLC.

In addition to HPLC, I used IHC to immunolabel phosphorylated TH. Immunolabeling phosphorylated TH provides an excellent method with which to examine rapid responses to any number of stimuli. I encountered a number of challenges however in this study, such as inconsistent levels of background between sections in the same tray and "sticky" sections that would fold on themselves and cause nonuniform immunolabeling. It is still unclear why these problems occurred. Additional methods may be used to measure rapid responses of the catecholaminergic system. Both voltammetry and cerebral microdialysis can be used to measure the rapid release of catecholamines *in vivo*. Microdialysis has been used to measure rapid release of dopamine in songbirds and mammals, and voltammetry has been used *in vivo* in mammals (reviewed by Kubikova & Kostal, 2010). These techniques would not only provide new methods to measure rapid release in birds,

but would also help verify the sound-induced responses of the catecholaminergic system we reported using our methods.

The best methods for studying auditory selectivity make use of within-subject designs that allow researchers to compare responses of the same cell or animal to different stimuli. Using a within-subject design, for example, I could play song, tones, and silence to the same individual and compare the effects of different sounds within an individual bird. Similarly, the same animal could be tested in different reproductive states. With IHC, however, the effects of multiple sounds on a single individual cannot be compared. Another method, such as magnetic resonance imaging (MRI), could be employed (reviewed by Van der Linden et al., 2009). Blood oxygen level-dependent (BOLD) functional MRI (fMRI) has been used to study sound-induced activity in the auditory forebrain of songbirds (Van der Linden et al., 2004). Recent fMRI studies with zebra finches have shown that regions such as CMM and NCM, as well as some of the domains within these regions, can be imaged and the responses to sound quantified (Poirier et al., 2011). A future experimental plan might include fMRI to map the effects of E2 on auditory BOLD responses to song. fMRI would allow quantification of activity in the auditory pathway of the same individual in response to several sounds and under different hormonal conditions, ultimately leading to more powerful statistical comparisons.

5.4 EFFECTS OF TESTOSTERONE ON AUDITORY ZENK RESPONSES IN MALES: CONCLUSIONS & IMPLICATIONS

In Chapter 4, I begin to test a model (Chapter 1, Fig. 1) explaining how gonadal hormones may induce auditory selectivity to sociosexual signals in males. In nonbreeding males treated with T, ZENK responses were selective for song over frequency-matched tones. This T-induced selectivity was, however, limited to NCM. In CMM, the ZENK response was selective for song over tones regardless of hormone treatment. Selective ZENK responses were not observed in the auditory thalamus or midbrain in either T-treated or untreated males. This result, together with the findings in E2-treated females (Maney et al., 2006; Sanford et al., 2010), shows that T and E2 have similar effects on the selectivity of sound induced ZENK responses in NCM. Unlike E2 in females, however, T in males decreased the density of SERT-immunoreactive innervation throughout the auditory pathway and decreased DBH-IR fiber density in the midbrain. Thus, although the two gonadal hormones both induce selectivity, the mechanisms by which this selectivity is mediated are likely very different (See Discussion in Chapter 4). In mammals, males and females differ with respect to dopaminergic and serotonergic function (reviewed by Becker, 1999; Fischette et al., 1983; Sheng et al., 2004) and these differences may be attributable to sex differences in plasma levels of gonadal hormones. In rats, for example, E2 enhances dopamine release in the striatum in females but has no effect in males (Becker, 1999). I did not test either steroid in both sexes, so it is unclear whether they would have dimorphic effects in the auditory system. The behavioral relevance of conspecific song should be similar in males

and females during the non-breeding season, but during the breeding season the sexes have quite different responses to it. Perhaps the disparate effects of gonadal steroids on song processing areas can be attributed to the sexually dimorphic contexts in which song is heard and interpreted.

5.5 EFFECTS OF TESTOSTERONE ON AUDITORY ZENK RESPONSES IN MALES: FUTURE DIRECTIONS

In Chapter 4, I describe T-dependent monoaminergic innervation and auditory selectivity separately but not how or whether they are related. I cannot yet conclude definitively that monoamines mediate steroid-induced auditory selectivity. To establish a causal role, the playback study described in this dissertation could be replicated following blockade of monoaminergic activity in the auditory forebrain *via* direct injection of monoamine receptor antagonists. The results of these studies may provide a causal link between monoaminergic activity and selective ZENK responses in the auditory forebrain of songbirds.

Because only serotonergic and noradrenergic fibers were immunolabeled in my study with males, it remains unclear whether T modulates dopaminergic fiber density. Appeltants et al. (2003) found that T treatment increases the density of fibers immunopositive for TH in regions involved in song control in female canaries and suggested that dopaminergic and/or noradrenergic projections may be playing a neuromodulatory role in these regions. Here, I found an effect of T on DBH-

immunoreactivity which provides evidence that, in the auditory pathway, noradrenergic projects may be the catecholamine that is modulated by T. In order to ask whether T affects dopaminergic activity, additional experiments could be done to examine the effects of T on the concentrations of dopamine and its metabolites as measured *via* HPLC and on the density of related biomarkers that can be visualized *via* IHC.

5.6 FINAL CONCLUSIONS

Sex steroids have widespread actions throughout the brain. The studies described in this dissertation provide evidence that E2 and T modulate monoaminergic innervation of auditory structures as well as auditory responses in those structures. The monoaminergic cells that project to auditory areas may fire in response to sound, rapidly inducing monoaminergic activity in auditory areas (Chapter 3). Although not yet addressed experimentally, this rapid response may be dependent on plasma hormone levels. In addition, steroid-dependent paracrine release of monoamines may affect response properties of the auditory forebrain independently of monoamine neuronal firing (Chapter 2).

Previous research showed that after E2 treatment, ZENK responses in the auditory pathway of females are selective for song over behaviorally irrelevant control sounds (reviewed by Maney & Pinaud, 2011). The research presented here showed that in males, T induced similar selectivity in some of the same auditory regions. Overall our work has supported the hypothesis that breeding-typical levels of gonadal steroids promote

auditory selectivity for sounds that are most relevant during the breeding season. We also showed evidence that this plasticity could be mediated by monoaminergic systems. E2 treatment increased and T treatment decreased the density of monoaminergic fibers innervating the auditory pathway; therefore steroid-dependent selectivity might be occurring *via* different mechanisms in males and females. Alternatively, selectivity, may arise independently of monoaminergic activity. In the future, studies using both songbirds and other animals in which the behavioral relevance of social signals changes over time will be necessary to understand how behavioral context alters the perception of auditory signals and the role gonadal hormones play in that plasticity.

FIGURE

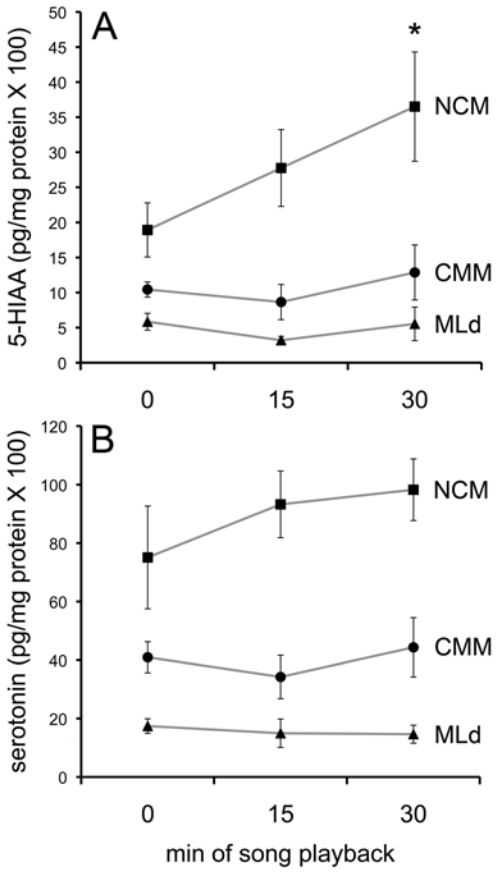

Fig. 1. Effects of song presentation on the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) and serotonin in auditory areas of female white-throated sparrows. 5-HIAA (A) and serotonin (B) concentrations in CMM, NCM, and the auditory midbrain, MLd after exposure to 15 or 30 min of conspecific song. Hearing 30 min of song significantly increased levels of 5-HIAA in NCM (A). * significantly different from levels at time 0, $P = 0.024$. Total protein content was available for all samples only for NCM (see Chapter 3, Methods); for consistency in the graphs, plotted values for CMM and MLd have been normalized using the average protein values for those regions.

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