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NEURAL CONTROL OF SINGING IN THE DARK-EYED JUNCO (*JUNCO HYEMALIS*)

A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

Cynthia Corbitt Gulledge, B.S.

Fairbanks, Alaska

December 1997

NEURAL CONTROL OF SINGING IN THE

DARK-EYED JUNCO (*JUNCO HYEMALIS*)

By

Cynthia Corbitt Gulledge

RECOMMENDED:

Advisory Committee Chair

Department Head, Biology and Wildlife

APPROVED:

Dean, College of Science, Engineering and Mathematics

Dean of the Graduate School

12-1-97

Date

Abstract

This dissertation includes several discrete projects addressing various aspects of the neural control of singing in the Dark-eyed Junco (*Junco hyemalis*), a migratory songbird. I collected the birds from a local wild population during the breeding season and migration. Chapter 2 addresses the role of testosterone in controlling volumes of the brain regions that control song learning and song production (vocal control regions, VCRs), which grow and shrink seasonally and are correlated with changes in singing behavior. I found that the role testosterone plays may depend on the age of the bird and the brain region in question. Expanding on that study, I investigated the independent roles of testosterone and photoperiod in the control of VCR volumes in adolescent male juncos (Chapter 3). In seasonally breeding species, circulating androgens increase with increasing photoperiod, so increases in VCR volumes in the spring had been thought to be a result of photoperiod-induced increases in testosterone. Experimental separation of photoperiod and testosterone revealed that long photoperiod alone can have stimulatory effects on VCR growth, despite low testosterone levels. In fact, in adolescent male juncos, lengthening photoperiod may play a greater role in determining VCR volumes than testosterone does, again suggesting that the role of testosterone in the vocal control system may change with age. Other neurochemicals besides testosterone are present in the vocal control system; Chapter 4 describes the first description of opioid peptide receptor localization and density measurement in the vocal control system of adult male songbirds. I expanded that study to include nonsinging female and juvenile juncos (Chapter 5). The results of the expanded study indicate that opioids may modulate development of the vocal control system between adolescence and adulthood, as well as auditory processing throughout life.

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Acknowledgments

The completion of this dissertation would not have been possible without guidance and support from many individuals and funding agencies. The faculty of the Department of Biology and Wildlife and Institute of Arctic Biology provided a very stimulating environment in which graduate students were treated as colleagues and encouraged to develop scientifically. The office and animal quarters staffs also deserve many thanks. My thesis advisory committee (Dr. Pierre Deviche, Dr. Alison York, Dr. Kelly Drew, and Dr. Charles Geist) provided valuable instruction and encouragement throughout this whole process, and I have produced a better product for it. Specifically, I thank Dr. York for developing in me a historical perspective of my field, Dr. Drew for forcing me to learn pharmacokinetic theory, and Dr. Geist for always being a voice of reason. Dr. Pierre Deviche, my advising professor, accepted me into his laboratory as an inexperienced Masters student who had never held a bird or a Pipetman, patiently allowed me to ruin experiments and learn from my mistakes, and now is watching me leave his laboratory as a practicing neuroscientist with several published papers under my belt. I have learned a lot of science, as well as a lot about how to do science, from Pierre, for which I will always be grateful. Many students contributed to my scientific development and state of mind during the last five years. Undergradute students Martine Labelle, Cynthia Simons, and Kerry Norton, as well as high school student Daniel Eckman, provided technical assistance and listening ears. More current and former graduate students than I can name provided an extensive support network, but I specifically want to acknowledge Susan Sharbaugh, Paul Cotter, Loren Buck, Renee Crain, and Dr. Merav Ben-David for going above and beyond the call of duty. Foremost, I thank my family for their continued

support and encouragement. My husband, Jay, has been my sounding board, writing coach, advisor, confidant, and best friend.

Finally, I wish to acknowledge the funding sources for this work. Research funds were provided by two awards from the National Science Foundation and two Sigma Xi Grants-in-Aid of Research. Stipends, travel funds, and reprint fees were provided by the Department of Biology and Wildlife, the Institute of Arctic Biology, and the Graduate School.

Chapters II through V of this dissertation have been adapted from manuscripts submitted for publication or published in specialized journals. Hence, formatting varies among these chapters and references for each of these chapters are located at the end of the particular chapter. My coauthor and the journal to which each manuscript has been submitted are footnoted on the first page of each chapter.

I. Introduction

Twenty Years of Research on the Neural Control of Birdsong

Birdsong has long fascinated its listeners for its beauty, intricacy, and promise of warmer and longer days. Thorpe (1958a,b) was a pioneer in the scientific study of song development and discovered that oscines (true "songbirds") must learn their songs as they develop from juveniles to adults. Thorpe's fascinating discovery stimulated a new field of study focused on the behavioral and physiological mechanisms underlying song learning. Notable among those who pursued this question was Fernando Nottebohm, who began investigating how the oscine brain controls singing about twenty years ago. Using lesioning and tract-tracing studies, Nottebohm and his colleagues determined that singing is controlled by specific brain regions (vocal control regions, VCRs) and that these regions are interconnected to form a distinct vocal control system (Nottebohm et al., 1976; Fig. 1). Soon thereafter this system garnered notoriety when Nottebohm and Arnold (1976) reported their startling and controversial discovery that some VCRs are sexually dimorphic in species in which only males sing. VCRs in singing males are three times larger than those of non-singing females. Never before had neuroanatomical features been so strikingly shown to correspond to individual behavior patterns, and documentation of such obvious sexual dimorphism corresponding to vertebrate brain function was unprecedented. This exciting and unexpected discovery prompted a new flood of research focused on the neural control of birdsong.

Twenty years after Nottebohm and his colleagues' initial studies were published, the avian vocal control system has proven to be an excellent model for studying the neural acquisition and development of communication in higher vertebrates. This system is of

great biomedical importance as well because it shares many features with human language acquisition, such as laterality of neural control, presence of discrete developmental stages that involve learning and memorization, and presence of discrete brain regions that control the behavior (Bottjer and Arnold, 1986). Seasonal song modifications are accompanied by VCR volume changes due partly to the insertion of new neurons. Adult neurogenesis is another interesting aspect of the avian vocal control system. New neurons originate along the ventricles, migrate to a VCR, and become integrated functionally in the region. This process ceases during postnatal development in most other vertebrate taxa. Here again, the avian vocal control system is considered an important model for biomedical research. Many researchers seek to understand the neurochemical bases of avian adult neurogenesis so that medical treatments may be developed to replace neurons lost to trauma or aging in humans. As a model for studying how the brain controls behavior, researchers exploit several unique and useful characteristics of the vocal control system — VCRs control a single behavior (singing) that is easily measured, the anatomical boundaries of these regions are discrete and distinguishable using common staining procedures so that the regions' volumes are easily measured, and VCRs respond to hormones both physiologically and morphologically. Because of the scientific utility of the avian vocal control system as both a comparative and applied model, neural control of birdsong has become a lively and fruitful area of neurobiological research.

Development of singing behavior

Singing behavior develops in phases as the bird ages; the timing of these phases differs among species (reviewed by Nottebohm, 1993). In most temperate species only the males sing, yet in all photoperiodic oscines studied thus far both males and females

memorize a song template shortly after hatching in a process called the "sensory phase". Birds raised in acoustic isolation or deafened prior to song memorization do not develop a normal adult song, showing that they must learn their template from an adult tutor (Konishi, 1965). Sometime after the sensory phase begins, the birds enter a "plastic song phase", during which they attempt to match their vocalizations to the template, a process analogous to the babbling of a baby (reviewed by Nottebohm, 1993). As in the sensory phase, auditory feedback is essential because the birds must hear their "babbling" in order to compare it with the stored template (Konishi, 1965). Eventually, plastic song develops into full adult, or "crystallized" song (Nottebohm, 1993). Deafening a bird once song is crystallized does not immediately alter song production, but eventually performance degrades, indicating that maintenance of normal song production also depends on auditory feedback (Nottebohm et al., 1976; Nordeen and Nordeen, 1992). Hence, memorization, vocalization, and auditory processing are tightly integrated aspects of song acquisition and production. There are indications that the regions of the vocal control system participate in all of these processes (see below), but our knowledge of the specific roles of each region and how they function is incomplete.

Vocal control regions

The avian VCRs form two discrete yet interconnected neural pathways (Fig. 1). The motor pathway, which controls song production, includes a direct projection from the High Vocal Center (HVC) to the robust nucleus of the archistriatum (RA), which then projects to several areas in the midbrain and brainstem, and on to the avian vocal organ, the syrinx, and to respiratory motor neurons (Nottebohm et al., 1976; Vicario, 1991; Wild, 1994). These connections allow VCRs direct or indirect control over the

mechanical aspects of singing. The second pathway, called the anterior forebrain (AF) pathway, begins with projections from HVC to area X, and then continues *via* the thalamic nucleus DLM, to the lateral portion of the magnocellular nucleus of the anterior neostriatum (lMAN), and on to RA (Nottebohm et al., 1976; Bottjer et al., 1989). Lesion studies indicate that two AF nuclei (area X, lMAN) are involved in song learning, but not song production (Bottjer et al., 1984; Sorabji et al., 1990; Scharff and Nottebohm, 1991). Another lesion study found that HVC, which receives auditory input from the telencephalic Field L (Kelley and Nottebohm, 1979), is necessary for female Canaries (*Serinus canaria*) to discriminate between conspecific and heterospecific songs (Brenowitz, 1991). A recent preliminary report indicates that lesions of lMAN also disrupt song recognition abilities in female Canaries (Burt et al., 1997). Neurons in all telencephalic VCRs (area X, lMAN, HVC, RA) respond to auditory stimuli, and many are selective for the bird's own song, a characteristic that appears to develop during the plastic song phase (Margoliash, 1986; Margoliash and Fortune, 1992; Volman, 1993; Doupe, 1997). Hence, the anterior forebrain pathway is primarily responsible for song learning and recognition.

Post-hatching development of the VCRs and the two pathways they form is rapid. After hatching, the telencephalic VCRs grow rapidly by adding neurons and increasing the size of pre–existing cells (Nordeen et al., 1989; Bottjer et al., 1985; Kirn and DeVoogd, 1989; Alvarez-Buylla et al., 1994; Nixdorf-Bergweiler, 1996). In male Swamp Sparrows (*Melospiza georgiana*), neuron number in area X increases 3-fold between days 23 and 61 post-hatching (Nordeen et al., 1989). In Canaries, 99% of the neurons projecting from HVC to RA develop after hatching (Alvarez-Buylla et al., 1988). Although neurons in RA are present at hatching, RA volume increases during song

memorization as the cells enlarge and packing density decreases (Nordeen et al., 1989; Alvarez-Buylla et al., 1994). In all species studied to date in which only males sing, sexual dimorphism of VCRs is apparent shortly after hatching; these males experience accelerated cell addition and decreased cell death compared to females (Kirn and DeVoogd, 1989; Nixdorf-Bergweiler, 1996). In looking for factors that might contribute to this dimorphism, one obvious difference between the sexes is the plasma concentrations of sex steroid hormones. For example, males often have higher plasma testosterone (T) concentrations than females (Arnold et al., 1996). In mammals, this condition is commonly observed during the sexual differentiation of reproductive tissues and behaviors, leading to the organizational-activational theory of steroid action, which holds that sex steroids have organizational effects on structures during development and then have activational effects on those structures in adulthood (Phoenix et al., 1959; reviewed by Arnold, 1996). Because differences in VCR volumes are readily measured and neuroanatomical differences can often be related to specific behavioral patterns, the avian vocal control system is used frequently to examine the roles of steroid hormones in the development of sexual dimorphisms in the vertebrate brain (reviewed by Arnold, 1996). This focus has led researchers to reevaluate the strict dichotomy of the organizational-activational theory (Arnold and Breedlove, 1985).

Effects of hormones

The organizational-activational theory. Phoenix et al. (1959) administered testosterone propionate to pregnant guinea pigs and found that their daughters, as adults, did not exhibit sexual behaviors typical of females, even when given estrogen and progesterone. This study formed the basis of the organizational-activational theory of

steroid hormone action on sexual behaviors. Three essential characteristics of an effect of an *organizational* hormone are that it 1) occurs early in development, 2) occurs during a specific critical period of development, and 3) is permanent. In the case of the guinea pig, testosterone propionate permanently masculinizes the sexual behavior of females when administered during a particular period of fetal development. By contrast, *activational* hormone effects 1) are not developmental, 2) occur immediately upon or soon after hormone administration, and 3) are temporary (usually only evidenced while steroid levels are elevated). Reproductive behaviors such as lordosis and courtship, for example, can be activated by hormone administration, but typically cease once circulating hormone levels return to normal. Hence, behavioral endocrinologists have long assumed that hormone effects are either organizational or activational. Hormones stimulate behaviors in adults by acting on tissues that were organized by hormones during development.

The organizational-activational theory provided the framework for some of the major discoveries in the field of avian vocal control. Nottebohm and Arnold (1976) found that in songbird species in which only the males sing, VCRs are dramatically larger in males than in females, yet T administration can activate singing in Canaries, whether male or female (Nottebohm, 1980; Johnson and Bottjer, 1993). Arnold et al. (1976) found that many VCRs contain androgen receptors, possibly explaining this effect. Early estradiol exposure masculinizes female Zebra Finches (*Poephilia guttata*) by inducing growth of their VCRs and enabling them to respond behaviorally to T as adults (Gurney and Konishi, 1980; Gurney, 1981).

While the organizational-activational theory initially sparked development in the new field, it also hindered some hypothesis-generating because it limited the possible effects of steroids to one of two categories: either early, permanent, and organizational or late,

transient, and activational. Eventually, Nottebohm (1980, 1981) noticed that the early dramatic increases in VCR volumes are not permanent in Canaries. Typically, neuroanatomical changes were classified as organizational, and were thought to be limited to early development. In Canaries, however, structural changes could be induced in a reversible, activational fashion (Nottebohm, 1980). Subsequent experiments have not been confined by the organizational-activational theory, although it remains a paradigm for categorizing hormonal effects.

The revised organizational-activational theory. Arnold and Breedlove (1985) suggested that the classical organizational and activational effects of hormones are extremes of a continuum, rather than discrete, mutually exclusive categories, and cite some exceptions to the organizational-activational dichotomy. Some organizational effects of hormones are not limited to a tightly-defined critical period during development or are not permanent. In the adult male Canary, changes in T levels reorganize the VCRs after each breeding season. In the classical organizational sense, T and estrogen are thought to masculinize the juvenile avian song system (but see below). A perinatal steroid peak correlates with a dramatic increase in VCR volumes. Whereas these enlarged VCRs are permanent in the non-photoperiodic Zebra Finch, the "organizational" effects of sex steroids on the VCR volumes of juvenile male Canaries are not permanent.

Adult female Canaries respond to T by increasing VCR volumes and singing (Nottebohm, 1980), while adult female Zebra Finches respond to T only if their vocal control systems have been masculinized with estrogen as juveniles (Gurney and Konishi, 1980), leading researchers to propose that estrogen masculinizes the vocal control system in juvenile male Zebra Finches (Arnold et al., 1996). In reality, sexual differentiation of the Zebra Finch brain is more complicated (Arnold et al., 1996). Removal of estrogen

from developing males does not demasculinize the vocal control system (Wade and Arnold, 1994), so one hypothesis is that estrogen administered during development can masculinize the female vocal control system by triggering a cascade of events that normally occurs in males without estrogen (Arnold et al., 1996). Exactly what factors are involved in that cascade is unclear, but Arnold (1996) has proposed that sex-specific genes may regulate sexual differentiation of the vocal control system independently of sex steroid hormones.

The usual focus

As a general rule, Zebra Finches have been used to study ontogeny of the vocal control system, whereas Canaries have been used to study seasonal changes within the system (e.g., Bottjer et al., 1985; Nottebohm, 1981). This situation presents a problem for forming a coherent picture of the song system because these two species represent two very different strategies for song learning and production. Zebra Finches are not seasonal breeders but rather breed opportunistically in response to rainfall (Crews, 1993). Their early song and reproductive development is rapid compared to other species (Bottjer and Arnold, 1986), and they also fall into a category known as "critical period learners", meaning that they must learn to sing during an early, defined period, after which they are incapable of song modification. Canaries, on the other hand, breed seasonally and are "open-ended learners", meaning that they can continue to modify their songs as adults (Nottebohm et al., 1986). Hence, these models are not entirely analogous, and a complete picture of song system function and development from hatching to adulthood has yet to develop from the disparate studies on these two species. Moreover, there may be drawbacks to using domesticated species as models for singing behavior and its

neurological basis. Because these species have been bred for particular traits, especially modification or enhancement of singing, their brains may be exceptional, that is, very different from that of wild songbirds. Of course, it depends on what questions are being asked as to whether this would be a problem. On the one hand, enhancing a particular behavior through selective breeding can exaggerate the physiological bases of the behavior, thus facilitating study. If, on the other hand, one wishes to understand the typical physiological mechanisms that control a particular behavior within the context of the natural history of a taxon, then it seems undesirable to focus on systems which have been selectively bred, rather than on wild-type systems.

What's Lacking?

Because of the differences in life histories, studies on Zebra Finches tend to concern song development (e.g., Bottjer et al., 1985) and studies on Canaries often involve seasonality (e.g., Nottebohm, 1981). Comparatively few studies have included both juvenile and adult stages of a species (see Doupe, 1997; Nottebohm et al., 1986), or have used a comparative approach with non-domesticated species (see Kirn et al., 1989; Brenowitz et al., 1991; DeVoogd et al., 1993; Bernard et al., 1996; Bernard et al., 1997; Smith et al., 1997). Studying other species is important because the more we understand different patterns of song development and production, the better we will comprehend how the brain controls this behavior. Those few studies on wild birds have found that there are dramatic differences from the patterns of song learning exhibited by Zebra Finches or Canaries. For example, both of those species begin to practice singing before the end of the song memorization phase so that characteristics related to song learning and song production are difficult to separate (Nottebohm, 1993). Some other species, such as

the White-crowned Sparrow (*Zonotrichia leucophrys*; Whaling et al., 1995) and the Dark-eyed Junco (*Junco hyemalis*; Marler et al., 1962), have a long "storage phase" between song memorization as juveniles and song production shortly before the first breeding season as adults. A study on male White-crowned Sparrows during the storage phase found that birds given T five months earlier than normal produced song earlier than usual, and the songs were abnormal (Whaling et al, 1995). Clearly, T has effects on birds between song memorization and adulthood, a period referred to as adolescence (Nordeen and Nordeen, 1989), that are not understood and that may differ among species.

Further, gonadal steroids are important, but do not control all aspects of sexual differentiation, song development, and song production (reviewed by Arnold et al, 1996). Recent studies have revealed T-independent effects of long photoperiod on VCR volumes in seasonally-breeding birds (Bernard et al., 1997; Smith et al., 1997). Additionally, many other neurochemicals and/or their receptors have been found in VCRs and these substances certainly play some role in the vocal control system (Ball et al., 1988; Casto and Ball, 1994; Soha et al., 1996; Kimpo and Doupe, 1997). One neurochemical group of interest is the opioid peptides and their receptors. Opioids have many effects related to singing behavior (see below). Comparing the distribution of these chemicals in VCRs of birds in different physiological (e.g., breeding vs. non-breeding) and behavioral (e.g., singing vs. non-singing) conditions could lead to new hypotheses regarding VCR function, as well as eliminate hypotheses regarding the roles of particular neurochemicals in controlling singing behavior.

Why Juncos?

As mentioned above, during their first year juncos exhibit a distinct separation between song memorization and song production. During the intervening adolescent period, juncos must store the memorized song template without the auditory or sensorimotor reinforcement that Zebra Finches and Canaries experience as they develop their adult song. Characterizing the neurochemistry and morphology of VCRs during this time may lead to a better understanding of learning and memory in general. In addition, juncos have long been used to study photoperiodism, including the coordination of reproductive activity with changes in photoperiod (Rowan, 1925). Extending this type of investigation to the vocal control system may shed light on how changes in photoperiod affect the songbird brain, especially in light of the new research on T-independent photoperiod effects on VCR volumes in other species (Bernard et al., 1997; Smith et al., 1997). Finally, juncos are locally abundant and relatively easy to observe and capture in the wild. Information gained from studying their vocal control system can be applied toward understanding the species in its natural environment, and information gained by observing the species in the wild can be applied to interpreting results obtained in the laboratory.

Thesis Objectives

There are two broad goals of this thesis. The first is to investigate the role of testosterone in controlling VCR volumes through a male junco's first year and to determine if that role changes with age (objectives 1 – 5 below). The second goal is to investigate the presence and possible role(s) of opioid peptide receptors in the junco vocal control system (objectives 6 – 8 below). Specifically, the objectives of this thesis are to

determine:

1) if VCR volumes change seasonally through a male junco's first year
2) if those seasonal changes correlate with seasonal changes in plasma androgen levels
3) if VCR volume maintenance depends on plasma T concentrations in adult males
4) if T administration affects VCR volumes in adolescent males
5) if long photoperiod affects VCR volumes in adolescent males independently of T or photosensitivity
6) the distribution of opioid receptors in VCRs of adult male juncos
7) if opioid receptor densities vary seasonally in adult males and
8) if opioid receptors are located in VCRs of other juncos (i.e., adolescents, females).

Summary of Thesis Design and Results

Before results of experimental treatments in a biological model can be understood fully, the natural course of events must be studied. In Chapter 2 (also published as Gulledge and Deviche, 1997), the natural developmental and seasonal changes in VCR volumes and plasma androgen levels in male juncos during their first year are investigated. In addition, the effect of castration on VCR volume maintenance is studied in adult males. The main conclusion of these studies is that the effects of androgen change with development; maintenance of VCR volumes is definitely dependent on elevated androgen levels in adulthood, but not necessarily in adolescence.

The second study (Chapter 3) expands on the findings from Chapter 2 to investigate the roles of T and photoperiod in regulating VCR volume in adolescent male songbirds. Usually, males experience increases in both plasma T and photoperiod as they

enter the breeding (singing) season. This study separates elevated T and photoperiod by taking advantage of the dependence of the junco reproductive system on photoperiod. Birds were exposed to short photoperiod and implanted with capsules either filled with T (SD-T) or empty (SD-C). A third group of birds was implanted with an empty capsule and exposed to long photoperiod (LD). Because the LD birds were photorefractory (unable to respond reproductively to long photoperiod), those birds experienced long days without elevated testosterone. Results presented in Chapter 3 demonstrate that photoperiod and T can have independent effects on VCR volumes, depending on the region. Exposure to long photoperiod increased VCR volumes, even though T was low. T treatment enhanced volumes of only one VCR (RA). These results support those of Chapter 2 regarding the limited effects of T in adolescent birds and also suggest that other neurochemicals may be involved in regulating VCR volumes.

One possible alternative to T is opioid peptides and their receptors. The study described in Chapter 4 (also published as Gulledge and Deviche, 1995) is the first to measure opioid receptor densities in the vocal control system. Opioid peptides have been detected in VCRs using immunocytochemistry (Ryan et al., 1981; Ball et al., 1988; Deviche and Gunturkun, 1992; Bottjer and Alexander, 1995; Carillo and Doupe, 1995), and presence of opioid receptors would indicate that opioids play some role in the vocal control system. In other models, opioids modulate neural functions that are characteristic of the vocal control system — learning and memory (Csillag et al., 1993; Columbo et al., 1997), sensory processing (Crain and Shen, 1990), and neural differentiation and survival (Meriney et al., 1991; Hammer and Hauser, 1992). Other studies indicate that steroids and opioids can interact to control behavior (e.g., reproduction, Clark et al., 1988; feeding, Deviche, 1992). Adult male juncos were captured during and after the breeding season to

determine if VCR opioid receptor densities correlate with steroid hormone levels. No seasonal difference in opioid receptor densities was found, suggesting that steroids do not modulate opioid receptor densities and that opioids probably function in a role other than song production.

Chapter 5 expands the project above to include an investigation of opioid receptors in adolescent male, adolescent female, and adult female juncos in addition to the adult males described in Chapter 4. The results suggest that opioids are involved in development of the vocal control system, as one or both of two adolescent groups had higher densities of opioid receptors than adults in many VCRs. Additionally, the data suggest that opioids modulate learning/memory and auditory processing.

All together, the research described in this thesis indicates that the role of androgen in the vocal control system may change with age, being very involved in VCR volume control in adult, but not in adolescent, male juncos. Certainly, other neurochemicals have roles in controlling this system. The opioidergic system (i.e., opioid peptides and their receptors) must be involved in several aspects of vocal control system function, because of its ubiquitous representation in VCRs of singing males, as well as non-singing females and adolescents.

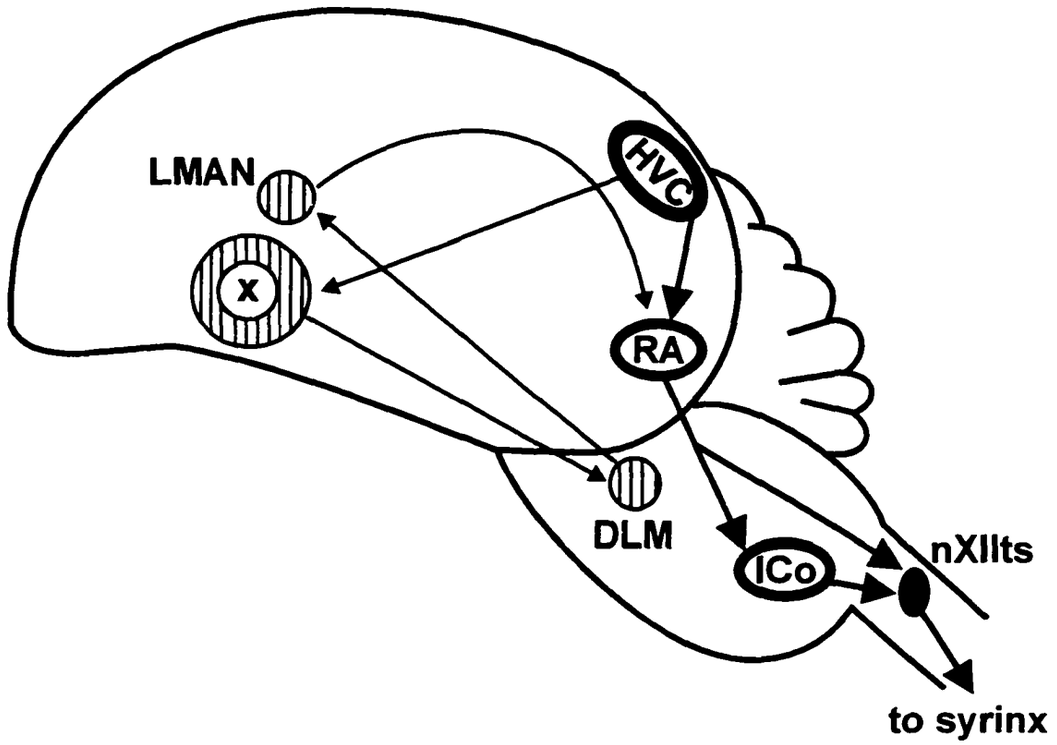

Figure 1. Diagram of the avian vocal control system. Regions outlined in black form the motor pathway and hatched regions form the anterior forebrain pathway. Abbreviations: DLM, dorsolateral n. of the medial anterior thalamus; HVC, high vocal center; ICo, n. intercollicularis; LMAN, lateral n. of the anterior neostriatum; nXIIts, tracheosyringeal portion of the hypoglossal nucleus; RA, robust n. of the archistriatum; X, area X.

II. Androgen Control of Vocal Control Region Volumes in a Wild, Migratory Songbird (*Junco Hyemalis*) Is Region and Possibly Age Dependent[†]

Introduction

In oscines, both song learning and expression are controlled by an interconnected set of brain regions called the vocal control system (Nottebohm et al., 1976; reviewed in Konishi, 1994). The best-studied vocal control regions (VCRs) are the Higher Vocal Center (HVC), robust nucleus of the archistriatum (RA), lateral magnocellular nucleus of the anterior neostriatum (lMAN), and Area X. The HVC and RA are essential for song expression (Nottebohm et al., 1976). Area X and lMAN are necessary for song development but not song production (Nottebohm et al., 1976; Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991).

Songbirds learn to sing in stages (reviewed by Marler, 1991), the timing of which varies among species. Young birds memorize the song of a tutor (sensory phase), then store that information until they begin to practice the memorized song in a form known as 'plastic song' (sensory-motor phase), which sounds similar to the song produced by adults, but is more variable and usually not as loud. Once the bird develops the plastic song into a stable, stereotyped form, the song is termed 'crystallized' (motor phase). Once song is crystallized, Area X and lMAN appear to not play any further role in song expression (Bottjer et al., 1984; Nottebohm et al., 1976). Even so, Area X occupies a large portion of the surrounding lobus parolfactorius throughout adulthood (Nottebohm et al., 1976, 1986).

[†]Published as Gulledge and Deviche (1997) *Journal of Neurobiology* 32:391-402.

During ontogeny and, in some species, between breeding seasons, VCRs grow rapidly as a result of both new neuron incorporation and increases in the sizes of existing cells (Nordeen et al., 1989; Brenowitz et al., 1991; Alvarez-Buylla et al., 1992; Smith et al., 1995). Once VCRs attain maximal size, their volumes are maintained by extending cell survival and by neuronal replacement (Alvarez-Buylla et al., 1992). Maintaining large HVC/RA and Area X may be necessary for producing crystallized song or for storing learned song, respectively. In many seasonally breeding species, large VCR volumes are maintained during the breeding (singing) season, but not afterwards, when plasma concentrations of gonadal steroids decrease (Nottebohm et al., 1986; Smith, 1996; Brenowitz et al., 1996). This observation suggests that sex steroids play a role in maintaining region sizes. One study found that testosterone (T) administration increases RA volume in female canaries (*Serinus canaria*), and that subsequent removal of exogenous T reverses that increase in RA size, suggesting that chronically elevated levels of T are necessary to maintain the enlarged volume (Brown and Bottjer, 1993). No study, however, has directly investigated the role of steroid hormones in maintenance of naturally large VCR volumes. To address this issue, the present study includes a castration and T-replacement experiment using breeding male dark-eyed juncos (*Junco hyemalis*) to determine whether breeding season concentrations of plasma T are necessary to maintain VCR volumes.

Laboratory studies on male, photoperiodic songbirds have found that, in adults, seasonal patterns in song production correlate with plasma androgen levels, as well as with changes in VCR volumes (Nottebohm, 1981; Brenowitz et al., 1991; Smith et al., 1995). Little is known, however, about the relationship between gonadal steroids and VCR volumes during the developmental period between song memorization in juveniles and their first breeding season as adults, a stage referred to as "adolescence" (Nordeen and Nordeen, 1989). A few studies have investigated seasonal and developmental aspects

of the vocal control system during adolescence (swamp sparrow, *Melospiza georgiana*: Nordeen et al., 1989; Marler et al., 1987, 1988; canary: Nottebohm et al., 1986; white-crowned sparrow, *Zonotrichia leucophrys*: Whaling et al., 1995). None of these studies, however, simultaneously measured VCR volumes and circulating androgen concentrations. Thus, whereas it is clear that VCR volumes in captive adult males correlate with plasma androgen levels (Bernard and Ball, 1995; Brenowitz et al., 1991), this relationship in adolescent birds remains unclear.

Also unclear is whether free-living birds experience the same seasonal changes in VCR volumes as those seen in captive birds exposed to breeding and non-breeding photoperiods. A previous study investigated seasonal VCR changes in free-living red-winged blackbirds *(Agelaius phoeniceus)*, and found that, in males, only the tracheosyringeal portion of the hypoglossal nucleus (which innervates the sound-producing organ, the syrinx; Konishi, 1994) was larger in the breeding season than in the following fall, although volume changes were detected in other VCRs of captive blackbirds exposed to long *vs.* short photoperiods (Kirn et al., 1989). Investigators, however, have recently reported seasonal changes in VCR volumes of wild, adult males (rufous-sided towhees, *Pipilo erythrophthalmus*: Smith, 1996; white-crowned sparrows: Brenowitz et al., 1996).

We investigated the role of androgens in regulating VCR volumes in males of a wild population of dark-eyed junco, a photoperiodic, seasonally breeding species (Deviche, 1995). For this, we determined whether VCR volumes and plasma androgen levels correlate seasonally in the wild and whether this relationship is similar in adult and adolescent males. Additionally, we determined whether VCR volume maintenance in male adults is T-dependent.

Methods

Seasonal study

Animal collection. Male dark-eyed juncos migrate to the same territory each breeding season (Deviche, unpublished data). In interior Alaska, adults arrive in early May, breed in June, molt in August, and begin emigrating in September (Deviche, unpublished data). Males were collected near Fairbanks, Alaska (64°N), using mist nets or Potter traps during three different life stages: adolescents in September (no singing, migrating, approximately 2-3 months of age, approximately 12L;12D, n=9); adults in June (singing and breeding, on breeding territories, approximately 21L;3D, n=6); and adults in October (rarely singing, post-breeding and post-molt, migrating, approximately 10L:14D, n=5). Birds collected in June were second-year males, meaning that they hatched the year before and were experiencing their first breeding season. Based on plumage, second-year males can be differentiated from older males during the breeding season but not after post-nuptial molt (Pyle et al., 1987). The age of adult males caught in October, therefore, could not be determined, but these males could be differentiated from juveniles based on plumage, eye color and amount of skull pneumatization (Pyle et al., 1987). All necessary permits were obtained prior to the collection of the birds used in this study. A blood sample (approximately 250 µl) was collected from a wing vein immediately after capture and kept on ice until processed later the same day.

Brain processing. Within hours of capture, birds received an overdose of anesthetic (ketamine/xylacine), then an intracardial injection of heparin (0.3ml; 1000 IU/ml in 0.1 M phosphate buffer (PB) solution), followed by room temperature 0.1M PB (20ml) and cold 4% paraformaldehyde solution in 0.1M PB (25ml). The brains were postfixed in 4% paraformaldehyde solution overnight, dissected from skulls, and transferred to PB containing 0.1% Na azide for 3 days. Brains were then transferred to 30% sucrose solution in PB with Na azide for 10 days, after which they were blotted dry

and weighed. Brains were coated with embedding matrix (M-1, Lipshaw) and frozen in powdered dry ice, then stored at -70°C until processed further. They were coronally sectioned (50μm) onto gelatin-coated slides in a cryostat at -15°C. The sections were dessicated overnight, then Nissl-stained with thionin.

VCR volume measurement. Vocal control regions (Area X, lateral and medial MAN, HVC, RA) and a control region not involved in vocal behavior control, the nucleus rotundus (Rt), were identified using the canary stereotaxic atlas (Stokes et al., 1974; Nottebohm et al., 1976). The "inclusive" boundaries of HVC were used to measure that region (Kirn et al., 1989). Lateral and medial MAN were measured together because the boundary between them was difficult to distinguish in most cases. Regions were measured using the M1 MCID image analysis system (Imaging Research, St. Catherines, Canada), which calculates region volume by multiplying section thickness by region area. Areas were measured by using a computer mouse to trace the borders of each region projected on a monitor. Alternate sections were measured (left and right sides separately), the volumes of all measured sections in a brain were totaled for each hemisphere and doubled. Because volumes for the left and right hemispheres do not differ (Gulledge and Deviche, unpublished data), values obtained for the left and right hemispheres were summed for each region. Telencephalon width was also measured as a control for overall brain size. For this, all brain sections with the anterior commissure (CoA) present (usually three) were measured at the widest point, and the widths were averaged for each brain. Right and left hemispheres were measured separately, then summed. All data were collected without knowledge of bird identity. Total volumes for each VCR and Rt were analyzed separately using a one way ANOVA followed by Student-Newman-Keuls multiple comparisons tests. All region data sets met ANOVA assumptions of normality and equal variance. We also determined whether differences in HVC and Area X volumes across comparable groups of birds were due to differences in the length (rostro-caudal

axis) and/or the width/height (medio-lateral or dorso-ventral axis) of these areas. For this, we counted the number of alternate brain sections containing a region (length) and we measured the largest cross-sectional area of a region on any one section (width/height). For analysis of the number of sections, a non-parametric Kruskal-Wallis one-way ANOVA was used, followed by Dunn's method tests for multiple comparisons. Measures of the largest cross-sectional area for each region (largest of right and left hemispheres averaged) were analyzed using a one-way ANOVA, followed by Student-Newman-Keuls multiple comparisons tests. Telencephalon width, brain weight, and HVC cross-sectional area data sets did not comply with equal variance assumptions, so they were ranked before analysis.

T-replacement study

Animal collection and handling. Adult male dark-eyed juncos were captured near Fairbanks, Alaska in May, 1994, when males were singing frequently and defending territories. Birds were kept in individual cages on long photoperiod (20L;4D) for 1-3 days, and they received food and water *ad libitum*. Because the gonads were initially large and heavily vascularized, birds were transferred to 8L;16D for 9 - 14 days to induce partial testicular regression, and they were then bilaterally gonadectomized under complete anesthesia. The birds remained on 8L;16D for the remainder of the experiment. One day following castration, birds received subcutaneous Silastic capsules (3 cm; 1.45mm internal diameter, 1.93mm outer diameter; Dow Corning) either filled with T (**Cx + T**, n=6) or empty (**Cx**, n=6). We refer to the 12th day after transfer to 8L;16D as day zero (D0, median day of gonadectomies). Another group of birds (**R**, n=5) was perfused (see protocol above) on D1 to provide a reference for VCR volumes of the other two groups at the time of castration. Blood samples were taken from a wing vein of all birds at capture and on day minus 2 (D-2). Additional blood samples were taken from Cx and

Cx + T groups on D22 and D47. On D47, both groups were perfused as described above.

Brain processing. Brains from the three groups were processed as in the seasonal study, except that Cx and Cx+T brains were kept in Na azide for 9 days before being weighed and transferred to 30% sucrose for 21 days, while R brains were kept in Na azide for 4 days before transfer to 30% sucrose for 10 days. They were sectioned and measured without knowledge of the individual bird identity. Volume and brain size data were analyzed using a one-way ANOVA for each region, followed by Student-Newman-Keuls multiple comparisons tests. All of these data sets met ANOVA assumptions of normality and equal variance. As for the seasonal study, the number of alternate sections per region was analyzed using a Kruskal-Wallis one-way ANOVA, followed by Dunn's method tests for multiple comparisons. Cross-sectional area measures for Area X did not pass equal variance testing, so data were ranked before analysis.

Androgen radioimmunoassay

Blood samples were centrifuged and plasma was removed and stored at -20 °C. Blood samples from both studies were assayed for androgen by direct radioimmunoassay (RIA) using a protocol modified from Barnes et al. (1988). Steroids were extracted from plasma (40–100 µl) using 5 ml of freshly distilled dichloromethane (DCMA), after ^{3}H-T (approx. 4,000 cpm; NEN, Boston) was added to each sample for calculation of steroid extraction recovery. All extraction tubes were vortexed for at least 15 seconds after adding DCMA to the plasma sample, then were allowed to sit for 2 hours before transferring the bottom (organic) phase to a dry tube. Samples were dried with N_2 in a warm bath, then resuspended in phosphate buffered saline with gelatin (PBSG) and kept at 4 °C overnight. The next day, an aliquot of each sample was counted for T recovery. Duplicate standards were made using unlabeled T (Sigma) solutions of known concentrations. All samples were divided in half. T antiserum (diluted 1:20,000; provided

by Dr. Niswender, Ft. Collins, CO) was added to all but 'total counts' and background tubes, and ^{3}H-T (approximately 17,000 cpm) was added to all tubes, which were then kept at 4°C overnight. Dextran-coated charcoal was added to all but the 'total count' tubes, and after ten minutes tubes were centrifuged and the supernatant was decanted into vials and counted for radioactivity. The T antiserum has a 69% cross-reactivity with dihydrotestosterone, so the results are reported as plasma concentrations of androgen, rather than of T. Antiserum cross-reactivity with estradiol is 0.3%. Samples from the seasonal study were assayed in a single series, which had an intra-assay coefficient of variation (CV) of 16.5%. The castration experiment samples were divided randomly between two assays that had an interassay CV of 13.3%. Average extraction recoveries for both experiments exceeded 87% and the final values for each sample were individually corrected for losses during extraction. The assay sensitivity was approximately 0.3 ng androgen/ml.

Plasma androgen concentrations in the 3 groups of birds from the seasonal study were compared using a one-way ANOVA on ranked data, followed by Student-Newman-Keuls multiple comparisons tests. Plasma androgen concentrations from the T-replacement study were analyzed using two-way repeated measures ANOVAs, followed by Student-Newman-Keuls tests for multiple comparisons. Because blood samples from the R group were only available from capture date and D-2, one ANOVA with all three treatment groups included was used for those dates and a separate ANOVA was used for Cx and Cx+T blood samples from all dates.

Results

Seasonal study

Of the regions studied, only Area X and HVC exhibited significant seasonal volume changes (Fig. 2 and 3, Table 1). The pattern of seasonal change for RA volume

was similar to that of HVC, but was not quite significant ($p=0.054$, Table 1, Fig. 4). In adults, the pattern of change followed that of plasma androgen concentrations, which were higher during than outside the breeding season (Fig. 2). Neither plasma androgen levels nor HVC volumes differed between fall adolescents and fall adults. Fall adolescents had smaller HVC volumes and lower plasma androgen concentrations than those of breeding adults. Adolescent Area X volumes, however, were similar to those of breeding adults. A comparison of the largest cross-sectional area and number of sections containing Area X and HVC indicated that differences in Area X volume among groups resulted from changes in the cross-sectional area rather than the length of this region. Changes in HVC volume between adolescence and the breeding season were due to changes in the length of this region; differences in HVC volume between the breeding season and post-breeding reflected changes in the cross-sectional area (Table 1; see Fig. 3). The volumes of neither MAN nor Rt differed significantly across seasons (Table 1). Male adolescent brains were significantly heavier than those of adult males, but brain weights in the two adult groups did not differ (Table 1). Average telencephalon width followed the same seasonal pattern, with adolescent brains being larger than those of adult males (Table 1). Brain weight and telencephalon width were positively correlated ($r^2=0.91$; $p<0.0001$), with no overlap between adolescent and adult samples (Fig. 5), confirming that overall brain size is larger in adolescent than in adult males.

T-Replacement study

Castration reduced plasma androgen levels to below detection limits (<0.3 ng/ml), and T replacement restored plasma androgen to the levels present in free-living males at the time of capture (Table 2). Keeping the birds on short photoperiod before D0 reduced plasma androgen levels (Table 2).

Removal of circulating T by castration resulted in a decrease in both Area X and

HVC volumes (Fig. 6), which in Cx birds were 31% and 36% smaller, respectively, than those of Cx+T males. T administration did not, however, increase the volume of these regions beyond the initial volumes at D1 (group R). A comparison of the largest cross-sectional area and number of sections containing Area X and HVC indicated that differences in Area X volume among groups mainly resulted from changes in the length rather than cross-sectional area, although the trend in the cross-sectional area was for Cx area to be smallest (Table 3). Changes in HVC volume, however, were in both planes, with the most striking difference in the cross-sectional area (the mean of the largest area in the Cx group was 33% smaller than that of R or Cx+T groups; Table 3; see Fig. 3). MAN volume was larger in group R than in castrated birds, but did not differ between Cx and Cx+T groups (Table 3). Rt and RA volumes did not differ significantly among groups (Table 3). Brain weights and telencephalon widths also did not differ significantly among groups (Table 3) and were similar to those of adults in the seasonal study (Table 1).

Birds used for the T-replacement study were captured in mid-May and for the seasonal study in mid-June, so birds may have been experiencing different phases of the breeding season in the two studies (*e.g.*, mate selection vs. incubation). This difference may explain why androgen levels of birds captured during the breeding season were elevated in both studies, but absolute concentrations differed (Fig. 2, Table 2; see Wingfield and Farner, 1978).

Discussion

This investigation confirms previous studies showing that VCR sizes are influenced by plasma concentrations of androgens in male birds. In addition, it reveals that the role of androgen in maintaining VCR size may be age- and region-selective. Both Area X and HVC volumes were T-dependent in adults during the breeding season, but this relationship was not apparent in Area X in adolescent males. We found that Area X,

HVC, and possibly RA, underwent seasonal volume changes in wild male juncos. These changes were VCR-specific, rather than resulting from changes in brain size as a whole. Brain size (as measured by brain weight and telencephalon width) did not change in adults between the breeding season and the following fall (Table 1), yet VCR volumes decreased during this period. Further, Area X volume was maintained and HVC volume increased from adolescence to the breeding season, even though overall brain size decreased during this time (Table 1, Fig. 5).

The seasonal change in RA volume was nearly significant ($p=0.054$) and the pattern was similar to that of HVC through the year. In other studies RA volume varied in captive and wild birds exposed to long photoperiod or exogenous T and the changes in RA volume were typically of lower magnitude than those in HVC (Nottebohm et al., 1986; Brenowitz et al., 1996; Smith, 1996), which may explain why they did not reach statistical significance in the present study. Additionally, because MAN is relatively small, we may not have been able to detect volume differences across groups for this region by measuring alternate sections.

Adults

Area X, HVC, and RA volumes in wild adult male juncos followed the seasonal pattern previously established in captive birds (canaries: Nottebohm, 1981; Nottebohm et al., 1986; red-winged blackbirds: Kirn et al., 1989; rufous-sided towhees: Brenowitz et al., 1991; white-crowned sparrows: Smith et al., 1995): they were large when circulating androgen levels were high and/or photoperiod long, and small when androgen levels were low and day length was short. This suggests that androgen and/or photoperiod plays a causal role in seasonal VCR volume regulation.

Several studies have shown that, in adults, increased plasma T levels can enlarge some VCRs. These studies involved birds with initially small VCR volumes and used T

administration (and/or exposure to long photoperiod) to induce VCR growth (Smith et al., 1995; Rasika et al., 1994; Brown and Bottjer, 1993; Brenowitz et al., 1991). Additionally, Bernard and Ball (1995) showed that elevated T is more important than long photoperiod in increasing HVC volume in the European starling (*Sturnus vulgaris*). No previous study, however, has directly explored the role of plasma T in *maintenance* of naturally large VCR volumes, although one study on female canaries found that RA volume that had increased following T administration decreased after the exogenous T was removed (Brown and Bottjer, 1993). Our T-replacement study confirms a causal relationship between breeding T levels and the maintenance of both Area X and HVC volumes in adulthood, since removing circulating T by castration decreased the size of these regions and maintaining these levels by exogenous T administration prevented the effects of castration (Fig. 6). It is not clear whether maintenance of RA volume is T-dependent in adult males, since the differences among groups in the castration study were not significant ($p=0.091$). The VCR volumes of Cx birds may not have been fully reduced, however. Johnson and Bottjer (1993) found that administering antisteroid drugs (flutamide and 1,4,6-androstratriene-3,17-dione) to castrated male canaries decreased HVC volume below that of castration alone, possibly because Cx birds still had non-gonadal sex steroids present, the actions of which were blocked by the antisteroid treatment.

Comparison of the nature of the HVC and Area X volume changes in the two experiments revealed similarities beyond a decrease in total volume. Specifically, the decrease in HVC volume after castration and, in intact males, between the breeding and non-breeding seasons, resulted from changes in cross-sectional area, without concurrent change in region length. The length of Area X was smaller in castrated males than in T-treated castrates, though there was no difference in length between adults caught in summer and fall. Cross-sectional area of Area X did not differ across groups in either

study. Additional investigations are needed to identify the specific cellular alterations (e.g., neuronal size, density, and/or number) that resulted in the observed changes in region cross-sectional area (HVC) or length (Area X). The decrease in VCR volumes measured in Cx birds used in this study was quantitatively similar to that seen in adult birds after the breeding season, when their reproductive systems were inactive and circulating T levels plummeted. Thus, while changes in photoperiod may contribute to the regulation of seasonal VCR volumes, our T-replacement study indicates that plasma androgen level manipulations (independent of photoperiod) in adult males are sufficient to produce VCR volume differences that are similar to those found in intact adult birds across seasons. The previously detected correlation between VCR volumes and T levels in captive adult males, therefore, probably reflects a causal relationship, as is the case in subjects obtained directly from a free-living population.

Adolescents

As mentioned previously, a relationship between circulating androgen levels and VCR volumes in adolescents has not been established. We found that this relationship may depend on the region being considered. Plasma androgen concentrations were much lower in adolescent juncos captured in the fall than in adults captured during the breeding season. The volume of HVC was also smaller in adolescents than in breeding adults. Further, neither circulating androgen levels nor HVC volumes differed in adolescent and adult males in the fall. Androgen levels, therefore, appear to control HVC volume in both adolescent and adult males.

In contrast, adolescent Area X volumes were as large as those of breeding adult males, even though adolescent androgen concentrations were low. This observation suggests that Area X volume is maintained in adolescent males without high plasma T concentrations. It is not clear, however, if maintenance of Area X volume is completely

T-independent in adolescent males, as plasma androgen levels were above detection limits (0.5 ± 0.2 ng/ml). Potentially, Area X may be *highly* T-sensitive, such that very low plasma T levels maintain the region volume. However, there is some evidence against this hypothesis. Androgen-concentrating cells are found in HVC, MAN and RA (reviewed in Arnold, 1992; Johnson and Bottjer, 1993), but most researchers agree that Area X does not have steroid receptors (Gahr, 1990b; but see Walters et al., 1988). Any steroid effect on Area X, therefore, is presumably indirect, possibly mediated through projections from HVC (Johnson and Bottjer, 1993; Gahr, 1990a; Gahr et al., 1993; Arnold, 1992), which in adolescent males, has not yet reached spring adult size. In addition, a previous study on castrated adolescent swamp sparrows found that song development continues normally up to the point of song crystallization, even though plasma T concentrations are below detection (Marler et al., 1988). Since song development depends on Area X (Scharff and Nottebohm, 1991; Sohrabji et al., 1990), this result indicates that Area X functions normally in adolescent swamp sparrows without detectable levels of circulating T. Thus, maintenance of Area X volume is probably T-independent at this stage. Even though Area X volume maintenance in adolescents may not be T-dependent, it may depend on estrogen acting on the HVC. Estrogen receptors are present in HVC neurons that project to Area X (Gahr, 1990a; Gahr et al., 1993), and non-gonadal estrogen is detected in the plasma of castrated adolescent swamp sparrows (Marler et al., 1988).

Functional implications

The different seasonal patterns of Area X and HVC volume changes through a bird's first year may be related to the roles of these regions. Area X is necessary for song learning, but not for production of crystallized song (Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Nottebohm et al., 1976). Lesioning this area during song learning results in abnormal song development (Sohrabji et al., 1990; Scharff and Nottebohm,

1991). In some species, Area X may also be important for modifying song repertoires between breeding seasons (Doupe, 1994; Nottebohm et al., 1990). Once a bird has crystallized its song, however, lesioning Area X does not immediately affect song quality (Nottebohm et al., 1976). Area X, however, may be important for storing memorized song until it is crystallized. This might explain why Area X would stay large from the time of song memorization through the storage phase. In addition, Area X may aid in recognition of neighboring conspecific songs (DeVoogd et al., 1995; Doupe and Konishi, 1991). This role has not been conclusively proven, but if valid, may also explain why Area X stays large through the breeding season, then shrinks after conspecifics have ceased singing.

Previous studies on VCRs in photoperiodic birds have provided conflicting results regarding the seasonal pattern of volume change, in part due to species differences, but also because of differences in the methods employed to define region boundaries. A study that defined canary HVC boundaries by backfilling with Fluoro-Gold injected into RA found that HVC volume reaches breeding size by 4 months after hatching (Alvarez-Buylla et al., 1992). Another investigation on the same species found that both HVC and RA grow until about 8 months post-hatching when measured using Nissl stain (Nottebohm et al., 1986). Recent work using VCR volume reconstruction based on cytological markers (adrenergic receptors, met-enkephalin immunoreactivity) revealed differences in these volumes that matched those using Nissl stain (Ball et al., 1995; Bernard and Ball, 1995; Smith et al., 1994), indicating that seasonal volume changes have some functional significance and represent more than a change in the amount of Nissl substance present.

Conclusions

Adolescent juncos maintained large Area X volumes until the breeding season despite low plasma T levels. There are many reasons for seasonal, migratory species to avoid high plasma androgen levels outside of the breeding season. Chronically elevated

androgen levels prevent pre-migratory fattening and molt (Deviche, 1995; reviewed by Ketterson et al., 1996) and decrease overwinter survival (Ketterson et al., 1996). Abnormally high T levels in adolescent white-crowned sparrows induce premature song crystallization, and the resulting song is abnormal (Whaling et al. 1995). Thus, in species with a prolonged storage phase between song memorization and practice, such as the junco (Marler et al., 1962), high T levels during adolescence may disrupt normal VCR development.

If T does not maintain Area X volumes in adolescent males, one or several alternative chemicals may do so. Several neurochemicals and receptors have been found in VCRs of seasonally breeding birds, including opioid peptides and receptors (Gulledge and Deviche, 1995; Ball et al., 1988), vasoactive intestinal peptide (Ball et al., 1988), adrenergic receptors (Bernard and Ball, 1995), and estrogen receptors (Gahr, 1990a, 1990b; Gahr et al., 1993). Most studies on the neuroanatomical localization of neurochemicals within the vocal control system involved adults, and the presence of these chemicals in VCRs of adolescents belonging to seasonally breeding species has not been reported.

Our results demonstrate that the role of androgen in maintaining VCR size may be age- and region-specific. Both Area X and HVC volumes are T-dependent in adults during the breeding season, but this relationship is not apparent for Area X in adolescent males. Thus, Area X volume in adolescent males may be controlled by some neurochemical that has been identified in adult VCRs, rather than by gonadal androgens.

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Table 1. Measurements from the seasonal study.

Measure	adolescent	breeding adult	fall adult	ANOVA
Area X volume (mm^3)	1.65 ± 0.28^a	1.74 ± 0.24^a	1.24 ± 0.23^b	p=0.012
HVC volume (mm^3)	0.09 ± 0.11^a	1.14 ± 0.20^b	0.71 ± 0.04^a	p<0.001
RA volume (mm^3)	0.36 ± 0.09	0.45 ± 0.085	0.33 ± 0.04	p=0.054
MAN volume (mm^3)	0.21 ± 0.07	0.18 ± 0.05	0.18 ± 0.05	p=0.42
Rt volume (mm^3)	2.24 ± 0.29	2.16 ± 0.25	2.16 ± 0.19	p=0.78
largest Area X cross-sectional area (mm^2)	1.26 ± 0.15^a	1.16 ± 0.18^{ab}	1.01 ± 0.08^b	p=0.02
number of alternate sections containing Area X	12 (10, 12)	11 (10, 13.5)	11 (9.75, 11.5)	p=0.75
largest HVC cross-sectional area (mm^2)	0.76 ± 0.07^a	0.74 ± 0.15^a	0.53 ± 0.05^b	p=0.002
number of alternate sections containing HVC	11^a (10, 11)	13^b (12, 13.5)	11^{ab} (11, 12)	p<0.001
brain weight (mg)	935 ± 58^a	785 ± 20^b	772 ± 8^b	p<0.001
telencephalon width (mm)	14.7 ± 0.4^a	13.5 ± 0.1^b	13.6 ± 0.2^b	p<0.001
n	9	6	5	

Characteristics (means ± SD, except for number of sections, which is the median and interquartile interval) of vocal control regions (Area X, HVC, RA, MAN) and one non-vocal control region (Rt), as well as total brain size, in male dark-eyed juncos obtained from a wild population at three stages of their life cycle. Differing superscripts indicate significant differences ($p<0.05$) within a given row.

Table 2. Plasma androgen concentrations (means ± SD; ng/ml) of adult male juncos that were intact (R), castrated (Cx), or castrated with T replacement (Cx+T).

Group	Capture	D-2	D22	D47	n
R	7.1 ± 2.6^a	0.5 ± 0.2^b	---	---	5
Cx+T	8.1 ± 3.1^a	1.6 ± 0.8^b	13.4 ± 4.2^a	9.1 ± 3.8^a	6
Cx	7.2 ± 4.1^a	0.7 ± 0.1^b	N.D.	N.D.	6

R: intact (killed on D1); **Cx:** castrated on D0; **Cx + T:** castrated on D0 with T replacement on D1. N.D. = non-detectable. Differing superscripts indicate significant differences ($p<0.05$) within a given row.

Table 3. Measurements from the T-replacement study.

Region	R	Cx+T	Cx	ANOVA
Area X volume (mm^3)	1.34 ± 0.38^{ab}	1.44 ± 0.31^{a}	0.99 ± 0.14^{b}	p=0.043
HVC volume (mm^3)	1.07 ± 0.10^{a}	0.94 ± 0.17^{a}	0.61 ± 0.14^{b}	p<0.001
RA volume (mm^3)	0.34 ± 0.03	0.30 ± 0.01	0.26 ± 0.02	p=0.091
MAN volume (mm^3)	0.17 ± 0.023^{a}	0.14 ± 0.024^{b}	0.12 ± 0.015^{b}	p=0.003
Rt volume (mm^3)	2.20 ± 0.10	2.26 ± 0.27	2.31 ± 0.18	p=0.63
largest Area X cross-sectional area (mm^2)	1.08 ± 0.21	1.13 ± 0.13	0.95 ± 0.10	p=0.16
number of alternate sections containing Area X	11^{ab} (9.75, 11.25)	11.5^{a} (10, 12)	9.5^{b} (9, 10)	p=0.03
largest HVC cross-sectional area (mm^2)	0.72 ± 0.07^{a}	0.70 ± 0.11^{a}	0.47 ± 0.11^{b}	p=0.001
number of alternate sections containing HVC	12^{a} (12, 12.5)	11.5^{ab} (10, 12)	10^{b} (9, 11)	p=0.007
brain weight (mg)	873 ± 42	830 ± 72	824 ± 38	p=0.3
telencephalon width (mm)	13.3 ± 0.25	13.4 ± 0.32	13.4 ± 0.33	p=0.578
n	5	6	6	

Characteristics (means ± SD, except for number of sections, which is the median and interquartile interval) of vocal control regions (Area X, HVC, RA, MAN) and one non-vocal control region (Rt), as well as total brain size, in male dark-eyed juncos in intact adult male juncos (group **R**), and 47 days after castration (**Cx**) or castration and testosterone replacement (**Cx +T**). Differing superscripts indicate significant differences ($p<0.05$) within a given row.

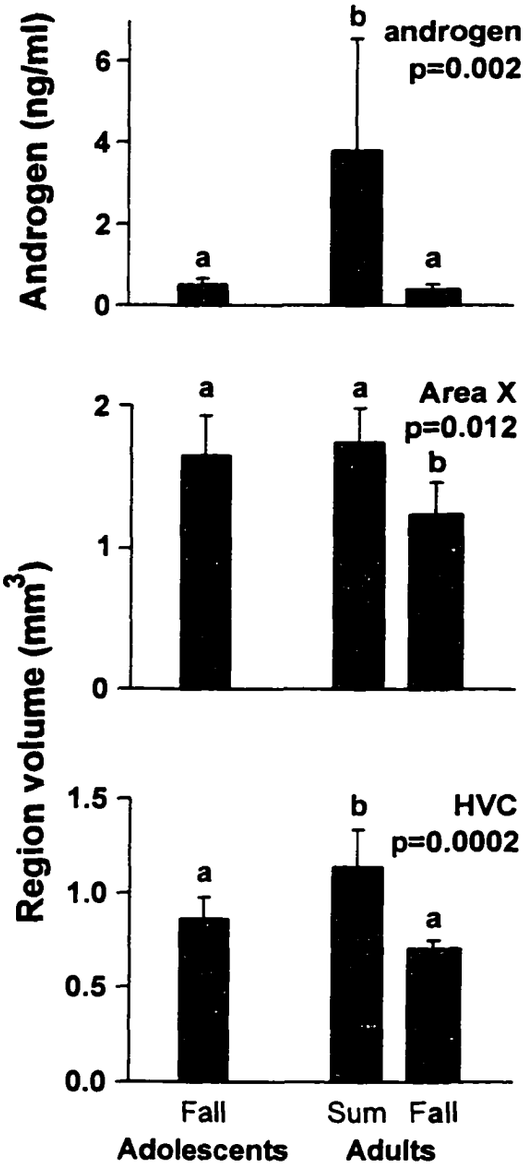

Figure 2. Plasma androgen concentrations (means ± SD; ng/ml) and volumes (means ± SD; mm^3) of Area X and HVC in male dark-eyed juncos captured from a wild population during three times of the year: September (adolescents, non-singing, n=9); June (second year adults, singing and breeding, n=6); October (fall adults, non-singing, n=5). P values refer to one-way ANOVA results. Differing superscripts indicate significant differences between groups ($p<0.05$) for each region.

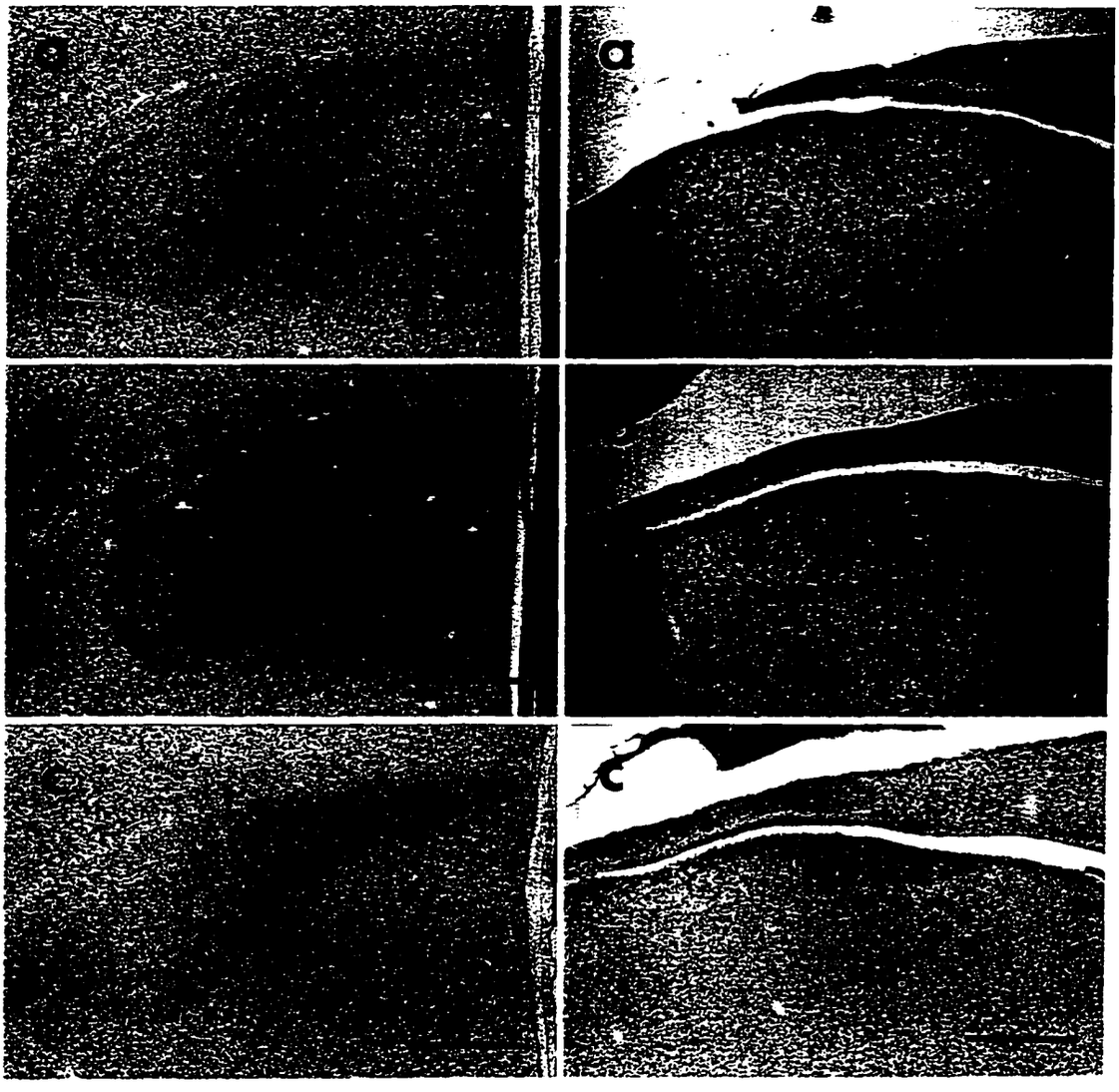

Figure 3. Nissl-stained coronal sections (50μm thick) of Area X (left) and HVC (right) in **a)** intact breeding adult male, **b)** intact post-breeding adult male, and **c)** gonadectomized adult male junco 47 days after castration. The brain with the region volume closest to the mean for the group is represented, and the section with the largest cross-sectional area for that brain is shown. Right is medial and top is dorsal. Calibration bar = 0.5 mm.

Figure 4. Nissl-stained coronal sections (50μm thick) of RA. Left is medial and top is dorsal. See legend for Fig. 3. Calibration bar = 0.5 mm.

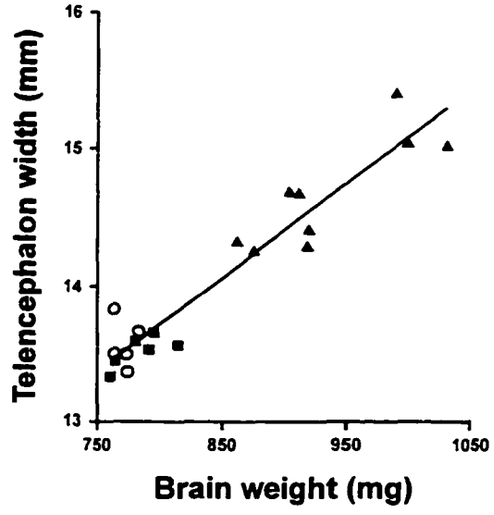

Figure 5. Correlation ($r^2 = 0.91$) between brain weight and telencephalon width in the seasonal study. There is no overlap in brain size between adolescents and adults. ▲ = adolescent males; ○ = breeding adult males; ■ = post-breeding adult males.

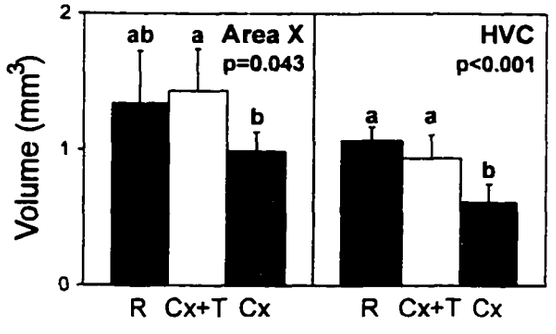

Figure 6. Volumes (means + SD: mm^3) of two vocal control regions of intact adult male juncos (group **R**, n=5), and 47 days after castration (**Cx**, n=6) or castration and testosterone replacement (**Cx +T**, n=6). P values refer to one-way ANOVA results. Differing superscripts indicate significant differences between groups ($p<0.05$; Student-Newman-Keuls multiple comparisons tests) for each region.

III. Photoperiod and Testosterone Independently Affect Vocal Control Region Volumes in Adolescent Male Songbirds[†]

Introduction

The brain regions (vocal control regions, VCRs) that control song learning and production in songbirds form an interconnected vocal control system that exhibits neuronal plasticity throughout adulthood in many species (Nottebohm et al., 1976; Nottebohm et al., 1986; Smith, 1996; Brenowitz et al., 1996a; Gulledge and Deviche, 1997; Fig. 1). Changes in VCR size result from alterations in cell number and/or cell size (Brenowitz et al., 1991; Smith, et al., 1995). In seasonally breeding adult songbirds, VCR volumes are large during the breeding (singing) season, when days are long and plasma testosterone (T) concentrations are high, and small after the breeding season, when both day length and circulating T levels decrease (Smith, 1996; Brenowitz et al., 1996a; Gulledge and Deviche, 1997). This seasonal pattern can be simulated in captive animals by exposing adult males to either long photoperiod or exogenous T, and comparing their VCR volumes to birds exposed to short photoperiod and low plasma T levels (Nottebohm, 1981; Brenowitz et al., 1991).

The effects of T are presumably mediated by intracellular androgen receptors (ARs). These receptors are located in brain nuclei that control song production (higher vocal center, HVC; robust nucleus of the archistriatum, RA; nucleus intercollicularis, ICo; tracheosyringeal portion of the hypoglossal nucleus, nXIIts), and form the "motor pathway" (Arnold et al., 1976; Smith et al., 1996; Fig. 1). They are also found in one region (lateral portion of the magnocellular nucleus of the anterior neostriatum, lMAN) that is part of a second pathway (anterior forebrain pathway) controlling song learning but not expression (Bottjer et al., 1984; Smith et al., 1996). Another anterior forebrain region, area X, is involved in song learning and changes size seasonally. Area X receives projections from

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[†]Gulledge and Deviche (1997). Submitted to *Journal of Neurobiology*.

HVC but does not contain ARs. The effects of T on this region, therefore, may be mediated indirectly via projections from HVC (Arnold, 1980; Gahr, 1990). The lMAN, which contains ARs, also sends projections to area X and may therefore also participate in effects of T on this region (Nixdorf-Bergweiler et al., 1995).

Androgen regulation of VCR volumes may be age- and region-dependent (Gulledge and Deviche, 1997). Castrating adult males during the reproductive season causes both HVC and area X to shrink compared to T-treated castrated males. In intact males, HVC volume increases from early adolescence (2 - 3 month-old birds) to adulthood in parallel with plasma T. These results suggest that HVC volume is affected by plasma T levels in adolescence, as well as in adulthood. In contrast, area X in adolescent males is the same size as in breeding adult males, even though plasma androgen levels are low in adolescence and high during the breeding season (Gulledge and Deviche, 1997). We speculated that area X volume maintenance is independent of elevated circulating T levels in adolescence, but not in adulthood. Therefore, artificially elevating T in adolescent males should not affect area X volumes, but may affect HVC volumes.

Testosterone is not the sole modulator of VCR volume; photoperiod may exert effects on VCRs in adults that are independent of its temporal relationship with plasma T. For example, in castrated adult male Gambel's White-crowned Sparrows (*Zonotrichia leucophrys gambelii*), long photoperiod had small stimulatory effects on HVC volume and neural size in RA that were independent of, but also smaller in magnitude than those of, T (Smith et al., 1997). Similar T-independent effects of photoperiod on region volume were detected in HVC, RA, and area X of adult male American Tree Sparrows (*Spizella arborea*; Bernard et al., 1997). In nature, the vernal seasonal increase in circulating T concentration follows increasing photoperiod (Wingfield and Farner, 1978). Seasonal increases in VCR volumes and singing rates may result from a cumulative effect of long photoperiod and elevated T levels (Nowicki and Ball, 1989; Smith et al., 1997).

In addition to photoperiod, the photoperiodic condition of the animal may influence VCR volumes (Bernard and Ball, 1997). Songbirds hatch in a photorefractory condition.

meaning that their reproductive systems are not responsive to long photoperiods (Nicholls et al., 1988). Continuous exposure to long photoperiod maintains photorefractoriness; only exposure to short days, as in autumn and winter, allows those birds to become photosensitive, or responsive to long days. At the end of the summer breeding season, adults become photorefractory again and also must be exposed to short days to regain photosensitivity. Photorefractory adult male European Starlings (*Sturnus vulgaris*) exposed to long photoperiod did not exhibit HVC volume growth in response to T treatment, whereas photosensitive birds receiving the same T treatment did have increased HVC volumes (Bernard and Ball, 1997). In contrast, castrated, photosensitive adult birds exposed to long photoperiod had VCR volumes that increased independently of elevated T (Smith et al., 1997). Although birds in both studies were exposed to long photoperiod, they were in different photoperiodic condition (photorefractory vs. photosensitive), which may explain why VCR volumes increased in one group exposed to long days (Smith et al., 1997), but not in the other (Bernard and Ball, 1997). Together these two studies indicate that birds may need to be photosensitive in order for long photoperiod to increase VCR volumes.

Captive adolescent Dark-eyed Juncos (*Junco hyemalis*) held on short days become photosensitive by mid-November (Gulledge and Deviche, unpublished data), and in the wild respond to increasing photoperiod in midspring by producing gonadal T and producing crystallized song. Measuring the effects of long photoperiod in photorefractory adolescents with low T levels should reveal whether long photoperiod alone can influence VCR volumes independent of photosensitivity or T. Additionally, augmenting T in adolescent males exposed to short days should reveal whether T alone influences VCR volumes at this age as it does in adults. To test these predictions, we experimentally separated the effects of photoperiod/photoperiodic condition and plasma T on VCR volumes in adolescent male juncos. We compared VCR volumes of control birds (SD-C), which were short-day photosensitive and had low plasma T levels, with those of short-day photosensitive juncos with experimentally elevated plasma T levels (SD-T) and of long-day photorefractory birds with low T levels (LD).

Methods

Animal collection and handling

Adolescent male juncos that had hatched in June or July were collected near Fairbanks, Alaska (65°N, 147°W), using baited Potter traps in mid-September of 1996 (photoperiod approximately 12L;12D). These birds were not singing, and were approximately 2-3 months old. Adolescent males could be differentiated from adults based on plumage, eye color and amount of skull pneumatization (Pyle et al., 1987). All necessary permits were obtained prior to bird collection. Males were kept in individual cages and received food and water ad libitum. They were initially exposed to a photoperiod (light/dark cycle: 13L:11D) similar to that of September in Fairbanks. On September 23, they were randomly divided into 3 groups. Two groups were exposed to short photoperiod (8L:16D): SD-T birds (n=8) received T-filled subcutaneous Silastic capsules (3cm; 1.45 mm internal diameter, 1.93 mm outer diameter; Konigsberg Instruments, Inc., Pasadena CA); SD-C birds (n=14) received empty capsules. The third group (LD; n=8) also received empty Silastic capsules but was exposed to long photoperiod (16L;8D) in order to maintain photorefractoriness. Blood samples (approximately 250µl) were collected from a wing vein on September 23, 1996, and again on December 13, 1996. Samples were kept on ice until processed later the same day, at which time they were centrifuged and plasma was collected and stored at –20°C until assayed for T concentrations. On December 13, 1996, 6 SD-C birds were transferred to long photoperiod (16L;8D) to determine whether they had gained photosensitivity, and thus, whether all SD birds were photosensitive by that time. Only photosensitive birds would respond to long photoperiod exposure by singing and rapidly developing their reproductive systems (Nicholls et al., 1988), so the photostimulated SD-C birds were monitored for singing behavior daily and widths of their cloacal protuberances (CPs) were measured after six weeks to assess reproductive system activity (Schwabl and Farner, 1989; Deviche, 1992). Typically, undeveloped junco CPs are 4mm wide or smaller (Gulledge and Deviche, unpublished observations). Seven weeks after the extra SD-C birds were transferred to long photoperiod, they were euthanized and their testes were weighed.

Brain processing

On December 13, 1996, all groups except the 6 extra SD-C birds received an i.m. overdose of anesthetic (ketamine/xylacine), then an intracardial injection of heparin (0.3ml; 1000 IU/ml in 0.1 M phosphate buffer (PB) solution), followed by room temperature 0.1M PB (20ml) and cold 4% paraformaldehyde solution in 0.1M PB (25ml). After the perfusion, testes were removed and weighed. The brains were postfixed in 4% paraformaldehyde solution overnight, dissected from skulls, and transferred to PB containing 0.1% Na azide for 2 days. Brains were then transferred to 30% sucrose solution in PB with Na azide for 4 days, after which they were blotted dry and weighed. Brains were coated with embedding matrix (M-1, Lipshaw), frozen in powdered dry ice, and stored at -70°C until processed further. They were coronally sectioned (50μm) onto gelatin-coated slides in a cryostat at -15°C. The sections were dessicated overnight, then Nissl-stained with thionin.

VCR volume measurement

Vocal control regions (area X, lateral and medial MAN, HVC, RA) and a control region not involved in vocal behavior control, the nucleus rotundus (Rt), were identified on sections using the Canary stereotaxic atlas (Stokes et al., 1974; Nottebohm et al., 1976). The "inclusive" boundaries of HVC were used to measure that region (Kirn et al., 1989). Lateral and medial MAN were measured together because the boundary between them was often difficult to distinguish. Regions were measured using the M1 MCID image analysis system (Imaging Research, St. Catherines, Canada), which calculates region volume by multiplying section thickness by region area. Areas were measured by using a computer mouse to trace the borders of each region projected on a monitor. Alternate sections were measured (left and right sides separately), the volumes of all measured sections in a brain were totaled and doubled, and values obtained for the left and right hemispheres were summed for each region. Previous studies indicate that volumes from right and left sides are not different (Gulledge and Deviche, unpublished data). Telencephalon width was also measured as a control for overall brain size. For this, all brain sections with the anterior commissure (CoA)

present (usually three) were measured at the widest point, and the widths were averaged for each brain. Right and left hemispheres were measured separately, then summed. All data were collected without knowledge of bird identity. All data sets except MAN volume met analysis of variance (ANOVA) assumptions of normality and equal variance. Therefore, data for MAN were analyzed with a Kruskal-Wallis one-way ANOVA on ranks, while all other data were analyzed separately using one-way ANOVAs followed by Student-Newman-Keuls multiple comparisons tests. Data are presented as means ± standard errors, except data for MAN, which are presented as medians ± ½ interquartile intervals.

Testosterone measurement

Plasma samples were assayed for T by direct radioimmunoassay (RIA) using a Coat-A-Count Total Testosterone kit (Diagnostic Products Corporation, Los Angeles). This RIA is both sensitive (lower detection limit: 10 pg/tube) and specific (cross-reactivity: 3% with dihydrotestosterone, 0.02% with estradiol). Briefly, a standard curve was made in triplicate by adding known concentrations of T to tubes coated with antibody for T. Plasma samples (15µl to 50µl) were added in duplicate to antibody-coated tubes, and [^{125}I]T (51,000 cpm/tube) was added to all tubes. Total counts (TC) and non-specific binding (NSB) tubes (tubes containing only [^{125}I]T) were included to determine the total radioactivity added to samples and the amount of NSB to the tubes, respectively. All tubes were incubated for 3 hours at 37°C, contents of all but TC tubes were aspirated, and radioactivity was measured in a gamma counter for 2 minutes per tube. Final concentrations were corrected for amount of plasma added. The intra-assay coefficient of variation was 3.4%.

Results

In September, all birds had undetectable plasma T concentrations. In December, only birds with T implants had detectable T levels (23.6 ± 2.0 ng/ml; high end of physiological range: Gulledge and Deviche, unpublished observations). Most birds had undeveloped testes (all <5 mg, paired weight), although two SD-T birds had slightly developed testes (largest was

90 mg, paired weight). The SD-T birds began to sing about two weeks after receiving T implants. In December, the 6 photostimulated SD-C juncos responded to exposure to long photoperiod with singing and had enlarged CPs (5.5 ± .1 mm) and testes (150 ± 13 mg, paired weight) after seven weeks of photostimulation, indicating that all SD birds were photosensitive at the end of the study. The LD birds never sang, had undeveloped testes and small CPs (3.6 ± .1 mm), and undetectable plasma T concentrations at the end of the study, demonstrating that they remained photorefractory throughout the experiment.

Statistically significant differences among groups were detected in volumes of all VCRs measured except MAN (Fig. 7, Table 4). On average, LD birds had 37% larger area X volumes than both SD groups, which did not differ from each other. LD birds had 40% larger HVC volumes than SD-C birds, but HVC volumes in SD-T birds did not differ from those of either LD or SD-C birds. Both LD and SD-T groups had larger RA volumes (31% and 37% larger, respectively) than SD-C birds. Control measures (Rt volume, brain weight, and telencephalon width) did not differ among groups (Table 4), indicating that differences in VCR volumes were specific to the vocal control system and were not due to changes in overall brain size.

Discussion

The results of this study indicate that T and long photoperiod have separate effects on VCR volumes in adolescent male juncos and that, at this age, effects of T may be more limited than those of photoperiod.

Testosterone effects

Elevated T induced singing in SD-T birds. This induction was accompanied by an increase of RA volume, but volumes of other VCRs (area X, MAN, and HVC) were not affected significantly by T administration. Testosterone presumably acts on VCRs via androgen receptors (ARs). Although RA, HVC, and MAN contain ARs (Arnold et al., 1976; Smith et al., 1996), only RA responded to high T with a change in volume. MAN does not exhibit seasonal volume changes in the wild (Gulledge and Deviche, 1997), which is

consistent with its lack of response to T administration in this study. In adults, however, HVC does exhibit seasonal changes in volume and shrinks in response to castration. Additionally, HVC volume is maintained at pre-castration levels by T administration in gonadectomized adult males, indicating that maintenance of this region's volume in adult males is T-dependent (Gulledge and Deviche, 1997). In contrast, HVC volume did not respond to T administration in this study, suggesting that sensitivity of HVC volume to plasma androgen concentrations may develop after adolescence. As such, the increase in HVC volume measured between adolescence and adulthood in an earlier study (Gulledge and Deviche, 1997) may have resulted from exposure to long vernal photoperiod, rather than to increasing plasma T levels (see below). Although area X does not contain ARs, maintaining a large area X in adult male juncos requires elevated circulating T concentrations. In contrast, area X volume in adolescent male juncos is maintained although these birds have very low plasma T concentrations (Gulledge and Deviche, 1997). Apparently, then, T-dependence of area X volume develops after adolescence. The absence of ARs in area X suggests that effects of T on this region are mediated by projections from another region that contains ARs, such as HVC (Gahr et al., 1996; Smith et al., 1996). MAN and HVC are the only AR–containing regions known to project to area X (Nottebohm et al., 1976; Nixdorf-Bergweiler et al., 1995) and their volumes did not respond to T administration in the adolescent SD-T birds in this study, although HVC volume maintenance is sensitive to T concentrations in adult males (Gulledge and Deviche, 1997). These results suggest that the post-adolescent development of T–sensitivity in area X may result from age–related changes in MAN and/or HVC.

The SD-T birds were photosensitive at the end of the experiment, but we do not know *when* they became photosensitive. Based on previous studies on adolescent male juncos in our laboratory, we estimate that SD birds became photosensitive in early to mid-November (Gulledge and Deviche, unpublished data; Crain and Deviche, unpublished data). Bernard and Ball (1997) determined that T administration does not increase HVC volumes of photorefractory adult European starlings. If T has stimulatory effects on VCR

volumes only in photosensitive birds, it is possible that we could not detect an increase of HVC volumes in SD-T birds because they had been photosensitive for too short a time (2 - 4 weeks). The volume of RA, however, clearly increased in response to T administration. If photorefractoriness during the study accounts for the apparent lack of response of HVC volume to T administration, then T-induced growth of RA either was not inhibited by photorefractoriness or occurred more quickly after the onset of photosensitivity than was possible in HVC.

Photoperiod effects

Contrary to our original hypothesis, photorefractory adolescent males exposed to long photoperiod (LD) had larger VCRs than photosensitive males exposed to short photoperiod (SD-C). The effects of long photoperiod on VCR volumes often have been assumed to be indirect, resulting from a stimulation of gonadal androgen secretion (Nottebohm, 1981; Brenowitz et al., 1991). When the effects of T and photoperiod are isolated experimentally, however, VCR volumes increase in response to long photoperiod alone in photosensitive adult White-crowned Sparrows (Smith et al. 1997). Bernard and Ball (1997) suggested that photoperiodic condition, rather than photoperiod *per se*, modulates the magnitude of VCR volume changes in response to T. They found that HVC volumes increase in response to T in photosensitive birds but not in photorefractory birds. One implication of this finding is that long photoperiod, even in concert with elevated T, does not affect HVC volumes in photorefractory birds. In our study, however, neither T nor photosensitivity can explain the effects of long photoperiod on increased VCR volumes in LD birds. Rather, long photoperiod apparently stimulated morphological changes in the vocal control system through another mechanism.

This mechanism may involve melatonin, the secretion of which varies with photoperiod and coordinates various circadian and circannual processes (Binkley, 1990). Although no role for melatonin has been identified in the vocal control system, melatonin receptors are present in VCRs (including in HVC) of male but not (non–singing) female

House Sparrows (*Passer domesticus*; Whitfield-Rucker and Cassone, 1996), suggesting that melatonin modulates sexually dimorphic aspects of singing behavior in this species. A similar sexual dimorphism in melatonin receptor densities is present in HVC of adult Zebra Finches (*Poephila guttata*), which are photoperiod-independent breeders (Gahr and Kosar, 1996). There are several differences between male House Sparrows and Zebra Finches in melatonin receptor distribution in the vocal control system, however. Whereas these receptor densities are elevated in HVC, RA, and area X of male House Sparrows (Whitfield-Rucker and Cassone, 1996), in male Zebra Finches the HVC is the only VCR with elevated melatonin receptor densities and the shape of area X is noticeable for its lack of melatonin receptors compared to the surrounding parolfactory lobe (Gahr and Kosar, 1996). Differences in distribution of melatonin receptors in photoperiodic and non-photoperiodic species may reflect differences in the role of melatonin in VCRs, depending on the relative role of photoperiod in the species in mediating events in the vocal control system. Melatonin production is tightly coupled to photoperiod and its receptor densities are unaffected by castration in House Sparrows (Whitfield-Rucker and Cassone, 1996). This hormone, therefore, may account for T-independent influences of long photoperiod on VCR volumes in photoperiodic species.

Behavioral studies indicate that the adolescent vocal control system can develop to an advanced functional stage in the absence of circulating T (Kroodsma, 1986; Marler et al., 1988). Castrated adolescent male Swamp Sparrows (*Melospiza georgiana*) and Song Sparrows (*Melospiza melodia*) develop their songs through the plastic song phase independently of gonadal T, requiring this steroid only for song crystallization (Marler et al. 1988). Because VCR volumes have not been measured in castrated males throughout development to adulthood, we do not know whether morphological development of VCRs is influenced by T as adolescents mature. Hence, adolescent males may require T for song crystallization, but not for VCR growth, and in adolescent males approaching their first breeding season, lengthening spring photoperiod may increase VCR volumes via neurochemical changes that are independent of increasing plasma T concentrations. Testing

this hypothesis would require measuring VCR volumes at several time points before and after photoperiod begins to lengthen, probably in both intact and castrated adolescent males, to determine when VCR volumes naturally increase and whether T is necessary for that increase to occur. In some species, photoperiod exerts T-independent effects on VCR volumes in adults (Smith et al., 1997; Bernard and Ball, 1997; Bernard et al., 1997). Studies using adult juncos to examine the time course of the reversal of the post-breeding decrease in VCR volumes in relationship to circulating T concentrations and photoperiod, therefore, are also warranted.

Functional considerations

The present data raise the question of the functional significance of VCR volume plasticity. Typically, singing behavior and VCR volumes are positively correlated in seasonally breeding species (Nottebohm, 1980, 1981; Nottebohm et al., 1976; Brenowitz et al., 1991), and researchers tend to associate large VCRs with the ability to sing. For example, in species in which only males sing, VCRs are larger in males than in females (Arnold et al., 1986). Additionally, HVC and RA growth usually accompany T-induced singing in both male and female adults (Nottebohm, 1980; Johnson and Bottjer, 1993). In the present study, however, adolescent birds with relatively small VCRs (SD-T) sang, whereas the birds with the largest VCRs (LD) did not. Because elevated plasma T appears to be required for full song production (Arnold, 1975; Marler et al., 1988), the lack of singing by LD birds probably resulted from a lack of activation by T, despite increased VCR volumes. By contrast, the occurrence of song in the birds with smaller VCRs seems contradictory to the idea that singing is related to VCR volumes. The SD-T birds sang even though the volumes of most of their VCRs did not differ significantly from those of non-singing SD-C birds. Because area X and MAN are not required for song production once song has been learned (Scharff and Nottebohm, 1991; Sohrabji et al., 1990; Bottjer et al., 1984), the volumes of these two regions may be inconsequential as far as song expression is concerned. HVC, on the other hand, plays an essential role in song production (Nottebohm et al., 1976; Yu and

Margoliash, 1996). Changes in HVC volume may relate to song complexity rather than production. T administration induces singing in female Canaries, even though their VCRs never grow as large as those of males (Nottebohm, 1980). T-induced female songs, however, are not as complex as male songs (Nottebohm, 1980). Perhaps song production is possible with a small HVC, but more complex song or a larger song repertoire requires a large HVC (Brenowitz and Arnold, 1986, 1992; DeVoogd et al., 1993). Brenowitz et al. (1996b) suggested that female Rufous and White Wrens (*Thryothorus rufalbus*) have smaller song repertoires than males because they have smaller HVCs. Junco songs are relatively simple, generally consisting of one rapidly repeated syllable to produce a trill (Konishi, 1964; Williams and MacRoberts, 1977). In the presence of sufficiently high plasma T concentrations, this simplicity may permit normal song production with a small HVC.

Conclusions

Three VCRs (area X, HVC, RA) were larger in photorefractory birds with low T (LD birds) than in photosensitive birds with low T (SD-C birds), but T administration to photosensitive birds (SD-T) increased only RA volume. These results support the hypothesis that control of area X volumes is T-independent in adolescents, and suggest that long photoperiod may increase VCR volumes in adolescents by a mechanism that is independent of elevated plasma T and photoperiodic condition. Additionally, we were able to dissociate singing from enlarged HVC volume, raising the issue of how HVC functions in the motor production of song.

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Table 4. Measures in the photoperiod/testosterone study.

Measure	SD-T	SD-C	LD	ANOVA (p)
Area X (mm^3)	$1.02 \pm 0.08^{a,1}$	$1.04 \pm 0.08^{a,1}$	1.40 ± 0.13^{b}	0.026
MAN (mm^3)	0.07 ± 0.04^{1}	0.08 ± 0.02^{1}	0.13 ± 0.02	0.124
HVC (mm^3)	$0.48 \pm 0.02^{ab,1}$	0.42 ± 0.05^{a}	0.59 ± 0.05^{b}	0.044
RA (mm^3)	0.22 ± 0.01^{a}	0.16 ± 0.02^{b}	0.21 ± 0.02^{a}	0.022
Rt (mm^3)	1.38 ± 0.07	1.58 ± 0.07	1.47 ± 0.07	0.156
Brain weight (mg)	737 ± 19	781 ± 16	760 ± 21	0.268
Telencephalon width (mm)	12.7 ± 0.1	13 ± 0.2	13.1 ± 0.2	0.183
n	8	8	8	

Characteristics (means ± SE, except for MAN, which is the median ± ½ interquartile interval) of vocal control regions (area X, MAN, HVC, RA) and one nonvocal control region (Rt), as well as total brain size, in adolescent male dark-eyed juncos exposed to short days with (SD-T) or without (SD-C) testosterone treatment (both photosensitive) or exposed to long days without testosterone treatment (LD; photorefractory).

[1] n=7.

[a,b] Differing superscripts indicate significant differences ($p<0.05$) within a given row.

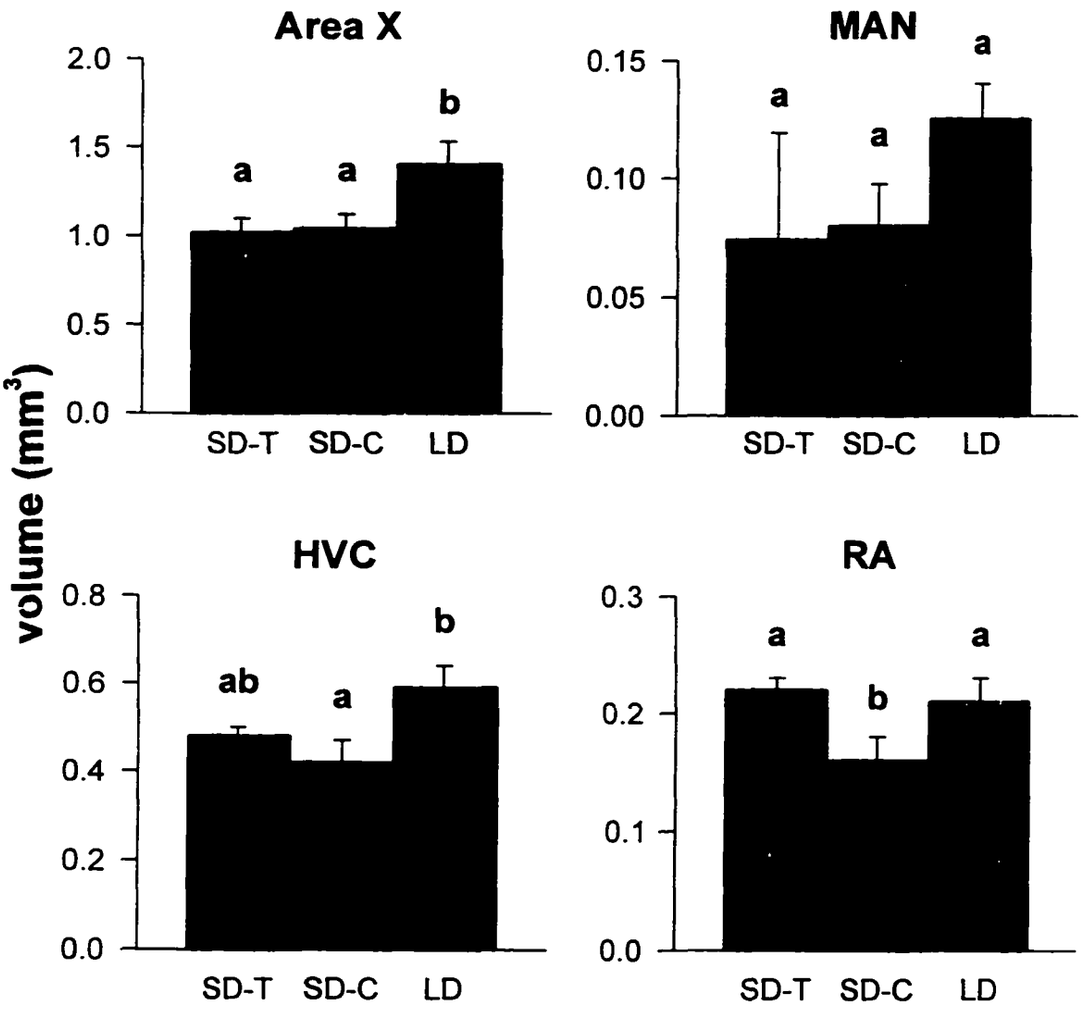

Figure 7. Characteristics (means ± SE, except for MAN, which is the median ± ½ interquartile interval) of vocal control regions (area X, MAN, HVC, RA) in adolescent male dark-eyed juncos exposed to short days with (SD-T) or without (SD-C) testosterone treatment (both photosensitive) or exposed to long days without testosterone treatment (LD: photorefractory). Differing superscripts indicate significant differences ($p<0.05$) among treatments for a particular region.

IV. Autoradiographic Localization of Opioid Receptors in Vocal Control Regions of a Male Passerine Bird (*Junco hyemalis*)†

Introduction

Avian vocal behavior is regulated by a well–described set of interconnected brain regions (Nottebohm et al., 1976; Vicario, 1991; Johnson and Bottjer, 1992) collectively called the vocal control system. These regions include the neostriatal higher vocal center (HVC), which projects to area X of the parolfactory lobe and to the robust nucleus of the archistriatum (RA). Area X sends efferents to the nucleus dorsolateralis anterior thalami, pars medialis (DLM), which projects to the lateral magnocellular nucleus of the anterior neostriatum (lMAN). LMAN efferents connect to the RA, which innervates the nucleus dorsomedialis (DM) of the nucleus intercollicularis (ICo). Finally, RA and DM efferents project to the hypoglossal nucleus, which innervates the trachea and syringeal muscles. These regions play specific, only partly understood, roles in song development and/or production. The HVC is essential for both song acquisition and production (Nottebohm et al., 1976). Both area X and lMAN are essential for song learning, but may not affect song production in adults, as destroying either area after song learning does not reduce song quality (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991). After song learning, lMAN volume decreases due to regional neuronal death (Bottjer et al., 1985). Area X remains large throughout adulthood, yet its role in adults is unknown.

In temperate region species, song frequency is generally highest during the breeding season, when circulating testosterone (T) levels are most elevated (Marler et al., 1987). Seasonal changes in the expression of vocal behavior correlate with modifications in vocal control region volumes (HVC, area X, RA: Nottebohm, 1981; Nottebohm et al., 1986; Kirn et al., 1989; Brenowitz et al., 1991). In many species, androgen administration stimulates

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†Published as Gulledge & Deviche (1995) *J. of Comparative Neurology* 356:408–417.

song production (Nowicki and Ball, 1989; Brenowitz and Arnold, 1990; Walters et al., 1991) and increases the volume of vocal control regions as measured from Nissl–stained sections (HVC, RA: Gurney and Konishi, 1980; Brown and Bottjer, 1993). This increase results from gonadal steroid–induced neurogenesis (Goldman and Nottebohm, 1983) and increased soma size (Brenowitz et al., 1991), and involves alterations of some morphological characteristics of neuronal populations (synaptic connections, dendritic arborization: DeVoogd and Nottebohm, 1981; DeVoogd et al., 1985; Canady et al., 1988).

Similarly, opioid peptides profoundly influence aspects of neuronal plasticity during ontogeny in mammals (Zagon and McLaughlin, 1987; Hammer et al., 1989; Hammer and Hauser, 1992) and birds (Meriney et al., 1991). Regions of the vocal control system (HVC, ICo, MAN, RA) contain opioid peptides (Ball et al., 1988, 1993; Deviche and Gunturkun, 1992; Ryan et al., 1981), but the role of these peptides in these regions is unknown, and no study has investigated whether vocal control regions possess opioid receptors. To address this question, we examined whether the HVC, area X, and ICo of a highly seasonal male passerine songbird (dark–eyed junco, *Junco hyemalis*) contain specific binding sites for the opioid receptor ligands DAMGO, pCl–DPDPE, and EKC. These compounds were selected based on the fact that in mammals, they bind selectively and with a high affinity to μ, δ, and κ opioid receptors, respectively (Mansour et al., 1986; Quirion et al., 1983; Vaughn et al., 1989). A previous study found that these ligands also bind to junco brain tissues (Deviche et al., 1993). In this species, equilibrium binding isotherm studies revealed binding sites to be specific, of high affinity (equilibrium dissociation constants $\leq$ 5 nM), and present at low concentrations (< 120 fmol/mg tissue protein). In addition, competition experiments between tritiated ligands and unlabelled competitors determined the binding of these ligands to be selective for sites that possess pharmacological characteristics similar to those of mammalian μ (^{3}H–DAMGO), δ (^{3}H–pCl–DPDPE), and κ (^{3}H–EKC in the presence of μ and δ receptor blockers) receptors.

Mammalian and avian studies have found neuroanatomical, physiological, and behavioral interactions between gonadal steroids and opioid peptides and their receptors

(Bhanot and Wilkinson, 1984; Nikolarakis et al., 1986; Clark et al., 1988; Forman and Estilow, 1988; Deviche, 1992), leading to the suggestion that some gonadal steroid–induced effects result from a modulation of opioid peptide production and/or opioid receptor densities/activity. To evaluate the possibility that gonadal androgens regulate opioid receptor densities in vocal control regions, we compared the densities of these receptors in male juncos obtained at the peak as compared to outside their annual breeding period.

Materials and Methods

Chemicals

[9–^{3}H(N)]–Ethylketocyclazocine (^{3}H–EKC; 24.5 Ci/mmol), [Tyr–3,5–^{3}H(N)]–[D–Ala^{2}, N–Me–Phe^{4}, Gly–ol^{5}]–enkephalin (^{3}H–DAMGO; 55.0 Ci/mmol), and [Tyr–3, 5–^{3}H]–[D–Pen^{2}, pCl–Phe^{4}, D–Pen^{5}]–enkephalin (^{3}H–pCl–DPDPE; 50.8 Ci/mmol) were obtained from New England Nuclear (Boston, MA). (±)Bremazocine HCl was a kind gift from Dr. Romer, Sandoz A.G., Switzerland; naloxone HCl and naltrexone HCl were kindly provided by Endo Laboratories (New York, NY). Unlabeled peptides were obtained from Peninsula Laboratories (Belmont, CA).

Specimen collection

Adult, male dark–eyed juncos were collected from a wild population in Fairbanks, Alaska at 3 stages of their reproductive cycle: spring (some singing, vernal migration: 02–10 May; N = 12), early summer (frequent singing, breeding period: 08–09 June; N = 10), and fall (rarely singing, autumnal migration: 22–23 September; N = 10). Spring and fall males were collected at seed–baited sites. Summer juncos were mist–netted on their breeding territories. Birds were sacrificed by decapitation within minutes after capture. Their brains were removed, frozen by immersion into Freon, and stored at –70°C until processed. The testes of all birds were weighed to the nearest mg, and cloacal protuberance widths (CP's, an androgen–dependent secondary sexual characteristic, Schwabl and Farner, 1989) were measured to the nearest 0.1 mm.

In vitro autoradiography

Frozen, coronal 30 µm–thick brain sections were collected on gelatin–coated microscope slides, dehydrated at 4°C under partial vacuum overnight, then stored at –70°C until further processed. For each brain, successive pairs of sections were subsequently used for incubations with each labelled ligand as described below.

All sections were first pre–incubated for 30 minutes in a large volume of buffer (pH 7.40) to dissociate and eliminate potential receptor–bound endogenous ligands (Loew et al., 1983; Kosterlitz et al., 1987, 1988). These solutions consisted of 50 mM Tris HCl buffer plus 1 mg/ml BSA and 150 mM NaCl (^{3}H–DAMGO); 50 mM Tris–HCl buffer plus 20 mg/ml bacitracin, 5 mM $MgCl_2$ and 100 mM NaCl (^{3}H–pCl–DPDPE); or 50 mM potassium phosphate buffer plus 1 mg/ml BSA and 150 mM NaCl (^{3}H–EKC).

Slides were briefly rinsed twice in plain buffer, and they were incubated in conditions such that the radioligands under study bound to µ (^{3}H–DAMGO), δ (^{3}H–pCl–DPDPE), and κ (^{3}H–EKC) receptors, respectively, with high affinity and selectivity (Deviche et al., 1993). For this, pairs of successive sections were incubated with tritiated ligand (^{3}H–DAMGO: 60 min; ^{3}H–pCl–DPDPE: overnight; ^{3}H–EKC: 60 min) in buffer (0.5 ml/slide) containing 1 mg/ml BSA and 0.5 mg/ml bacitracin (^{3}H–DAMGO); 20 mg/ml bacitracin and 5 mM $MgCl_2$ (^{3}H–pCl–DPDPE); or 1 mg/ml BSA and 100 nM each of unlabelled DAMGO and DPDPE to saturate µ and δ binding sites (^{3}H–EKC). One slide from each pair was used to measure total binding (TB), the other to measure non–specific binding (NSB). NSB was determined by including 1 mM naloxone HCl (^{3}H–DAMGO); 10 mM naltrexone HCl (^{3}H–pCl–DPDPE); or 1 mM bremazocine HCl (^{3}H–EKC) in the incubation solution. The opioid receptor ligands used to determine NSB have a high, but nonselective affinity for the three opioid receptor subtypes examined here (Corbett and Kosterlitz, 1986; Goldstein and Naidu, 1989; Takemori and Portoghese, 1984). Equilibrium binding isotherm studies using whole junco brain homogenates determined that in the present experimental conditions, the equilibrium dissociation constants (Kd's) of ^{3}H–DAMGO, ^{3}H–pCl–DPDPE, and ^{3}H–EKC are equal to 5.06 nM, 0.24 nM, and 1.27 nM,

respectively (Deviche et al., 1993). Each ligand was used at a concentration equal to twice its Kd (^{3}H–DAMGO: 11 nM; ^{3}H–pCl–DPDPE: 0.48 nM; ^{3}H–EKC: 2.48 nM). Accordingly, each labelled ligand presumably occupied a similar proportion of its respective receptors at equilibrium, and the relative densities of the 3 opioid receptor types within a specific region can be directly compared with each other.

After incubating, slides were rinsed in ice–cold buffer (^{3}H–DAMGO, 6x20 sec; ^{3}H–pCl–DPDPE, 4x4 min; ^{3}H–EKC, 8x2 min) and dipped for 2 sec in ice–cold distilled water. They were dried rapidly under a fan, and stored overnight under partial vacuum at room temperature. Slides were placed into light–proof X–ray cassettes and exposed to radioactivity–sensitive film (Ultrofilm, Cambridge Inst., Deerfield, IL). Each cassette contained a set of calibrated plastic standards (American Radiochemical Co., St. Louis, MO). After 10–12 weeks of exposure, films were developed for 4 min in Kodak D–19 developer, fixed for 5 min in Kodak Rapid Fixer, and rinsed for 30 min in running tap water.

Autoradiography data collection

Sections were Nissl–stained using Cresyl violet, and brain regions were identified using the canary (*Serinus canaria*) stereotaxic atlas (Stokes et al., 1974; Nottebohm et al., 1976). The anatomical boundaries of vocal control and non–vocal control regions were determined based on examination of the histological sections, not of the corresponding autoradiograms. Generally, the brain regions under study appeared homogenous with regard to their densities of opioid receptors, i.e. these densities did not seem to differ between the center and the outer edges. We therefore measured densities from within, rather than at, the region borders to ensure that all measures were taken from within the nuclei themselves. Vocal control regions RA and lMAN were not included in this study because, except in a few cases, their boundaries could not be precisely defined on the histological sections. For the lCo, measurements were taken and combined for the lateral (llCo) and medial (mlCo) parts of the nucleus. The mlCo included the DM, which is surrounded by the lCo proper

(De Lanerolle and Andrew, 1974; Paton et al., 1981; Wild and Arends, 1987). We also measured receptor densities in regions (n. mesencephalicus, pars dorsalis, MLd; lobus parolfactorius, LPO; posterior neostriatum, N) that are adjacent, but not part of, the vocal control system. For each individual, data were on average collected from the left and right sides of 3 (area X, LPO, HVC), 5 (ICo, MLd), and 6 (N) different sections.

Autoradiograms were analyzed by computerized microdensitometry using the M1 MCID image analysis system (Imaging Research, Canada; Deviche et al., 1993), which subtracts NSB from TB densities so that final data are expressed in μCi specifically bound (SB) ligand/g calibrated standard.

Statistical analyses

For each individual, SB data obtained for each brain region were averaged across sampled sections. The presence of seasonal differences for the morphological parameters under study and for the three opioid receptor types in each vocal control region were analyzed using one–way analyses of variance (ANOVA's) followed by multiple pair–wise comparisons (Student–Neuman–Keuls tests). Sets of data that did not comply with normality and/or variance homogeneity requirements (Kirk, 1982) were analyzed using Kruskal–Wallis ANOVA's on ranks followed by Dunn's multiple comparison tests. All data are presented as means ± standard deviations.

Results

Morphology

The three groups of birds differed from each other with regard to testis mass (Kruskal–Wallis test: $H = 34.597$, $df = 2$: $P < 0.001$) and CP size (ANOVA: $F(2,39) = 192.9$: $P < 0.001$; Fig. 8). For each parameter, values obtained for each season differed significantly from each other.

Birds caught in the spring were undergoing gonadal recrudescence, and they had partially developed CP's. Testis masses and CP's reached their maximum development in

summer, and were regressed in the fall. Thus, spring, summer, and fall juncos were in markedly different physiological condition.

Opioid receptor densities

The three vocal control regions under study contained measurable amounts of μ, δ, and κ receptors, and within each region, the three receptor types were present at different densities (Fig. 9–13). Generally, κ receptors were present at lower densities than μ and δ receptors. In area X (Fig. 11) and HVC (Fig. 12), μ receptors were more abundant than δ receptors, whereas the reverse was observed in the ICo (Fig. 13). Thus, the relative abundance of μ and δ receptors was specific to particular vocal control regions.

The ICo could be readily differentiated on μ and δ receptor autoradiograms, because the MLd contained much lower densities of these receptors (Fig. 13, Table 5: ICo–MLd receptor density differences: μ receptors: 2.37 μCi/g ; δ receptors: 3.14). Area X could be readily identified on μ receptor autoradiograms because it contained a higher receptor density than the LPO (Fig. 11, Table 5: area X–LPO μ receptor density difference: 2.85). In contrast, Area X could not be differentiated on autoradiograms for δ receptors, which were present at similar densities in area X and in the LPO (Table 5: area X–LPO δ receptor density difference: 0.26). Similarly, the lateroventral boundary of the HVC could not be precisely identified on μ and δ receptor autoradiograms because it contained these receptors at densities similar to those of the surrounding N (Fig. 12, Table 5: HVC–N receptor density differences: 0.81 and 0.05, respectively). Finally, the boundaries of the three vocal control regions under study could not easily be determined on κ receptor autoradiograms owing to the generally low densities of these receptors (see Fig. 11–13). Receptor type densities did not vary seasonally in any vocal control region studied (Table 6: $P > 0.05$ in all cases).

As mentioned earlier, the boundaries of the RA and the lMAN could often not be traced precisely, so that receptor densities in these regions were not quantified. In the few instances where these two regions could be identified with certainty, they did not appear to contain particularly high levels of any receptor type compared to the archistriatum and the

anterior N, respectively.

Discussion

This investigation provides the first demonstration that songbird brain regions (area X, HVC, and ICo) that are specifically involved in the acquisition and/or production of vocal behavior contain opioid receptors. Our results indicate that vocal control regions contain lower densities of κ than of μ and δ receptors. Further, μ receptor densities are higher than δ receptor densities in area X and HVC, whereas the reverse is true for the ICo. Thus, the distribution of μ and δ receptors is region–specific, an indication that these receptor types may control distinct physiological and behavioral functions.

The vocal behavior and the reproductive system activity of the male juncos used for this study differed markedly depending on the capture date. Birds collected during their vernal migration sang with a low frequency and had partially developed gonads and cloacal protuberances. In June, males were caught on their breeding territories; these males sang with a high frequency and had maximally developed gonads and cloacal protuberances. Finally, juncos captured in fall were not singing at the time of capture, and their reproductive systems were regressed. The present results do not reveal opioid receptor density changes as a function of reproductive system activity. Indeed, μ, δ, and κ opioid receptor densities did not vary seasonally in any area studied. These findings suggest that seasonal variations in singing frequency do not depend on seasonal changes in opioid receptor densities in these regions.

The absence of seasonal changes in vocal control region opioid receptor densities indicate that these densities are probably not controlled by circulating concentrations of gonadal steroids in adult males. This conclusion agrees with most mammalian studies showing that in males, gonadal steroids do not affect central opioid receptor concentrations in adulthood (Diez and Roberts, 1982; Cicero et al., 1983, 1987; Clark et al., 1984), although they do so during ontogeny (Hammer, 1988). It should be emphasized that even though gonadal steroids may not influence densities of opioid receptors in vocal control

regions of adults, they possibly interact with the opioid system in these regions in other fashions, such as by altering opioid production and/or release. This possibility is supported by the recent observation that T administration to female canaries enhances the density of enkephalin–like immunoreactive (enk–li ir) HVC fibers (Ball et al., 1993). In mammals, opioid–li ir neurons concentrate steroids in both sexes (Morrell et al., 1985; Akesson and Micevych, 1991; Olster and Blaustein, 1990). The rat preoptic region exhibits a steroid–sensitive sexual dimorphism in enk–li ir density (Watson et al., 1986; Simerly et al., 1988), and, in adult males, T regulates brain proopiomelanocortin gene expression (Chowen–Breed et al., 1989a,b; Adams et al., 1991) and opioid peptide content and release (Wardlaw, 1986; Almeida et al., 1987; Nakano et al., 1991). Further, gonadal steroids and opioid systems interact physiologically (Bhanot and Wilkinson, 1984; Nikolarakis et al., 1986) and behaviorally (rat: Clark et al., 1988; Forman and Estilow, 1988; male junco: Deviche, 1992). The HVC and the ICo of male songbirds contain androgen and estrogen binding sites (Arnold et al., 1976; Ball et al., 1989; Gahr et al. 1987; Brenowitz and Arnold 1989; Gahr 1990; Balthazart et al., 1992) as well as opioid peptides. Based on studies using female canaries (Ball et al., 1993) and mammals, effects of gonadal androgens on opioid innervation in these regions in male birds would, therefore, not be unexpected.

A detailed description of the autoradiographic distribution of avian brain μ, δ, and κ opioid receptors is available only for two other avian species (domestic pigeon, *Columba livia*: Reiner et al., 1989; one–day–old domestic chick, *Gallus domesticus*: Csillag et al., 1990). These studies found that similar to juncos (Deviche and Gulledge, 1993; Deviche et al., 1993), pigeons and chicks possess specific opioid receptors in a wide variety of brain regions, including the hippocampus, the neostriatum, the LPO, and, in pigeons, also the ICo and the hypothalamus. In the present study, tissue sections were exposed to μ, δ, and κ opioid receptor ligand concentrations equal to twice the respective Kd of each ligand. In contrast, Reiner et al. (1989) incubated tissue sections in the presence of equal μ, δ, and κ receptor ligand concentrations though these ligands had markedly different affinities for their respective receptors. Finally, Csillag et al. (1990) used radiolabelled ligand

concentrations that apparently were unrelated to the Kd's of these ligands as determined from studies using whole forebrain sections. As a result of these methodological differences, the relative regional abundances of the three receptor types across species cannot be directly compared. The widespread central distribution of opioid receptors nevertheless suggests that they control numerous physiological and behavioral functions.

Immunocytochemical studies have found opioid–li ir in vocal control areas including the MAN, HVC, RA, and ICo (Ryan et al., 1981; Ball et al., 1988; Deviche and Gunturkun, 1992). For example, Ryan et al. (1981) and Ball et al. (1993) reported enk–li ir fibers and diffuse terminal fields in the zebra finch (*Poephila guttata*) and the canary HVC. In addition, Ball et al. (1988) observed enk–li ir perikarya in the HVC of colchicine–treated starlings (*Sturnus vulgaris*) and song sparrows (*Melospiza melodia*), indicating that this region may possess local circuit enkephalinergic neurons. Opioid–li ir has also been observed in the ICo (enk–li ir fibers, terminal fields, and perikarya: Ryan et al., 1981; Ball et al., 1988; enk– and dynorphin–li ir fibers: Deviche and Gunturkun, 1992), suggesting that similar to the HVC, the ICo possesses intrinsic opioid innervation. With the present investigation, these results suggest that opioid peptides are released from nerve terminals in at least some vocal control regions and are able to bind to receptors located within these regions.

Both the HVC and area X receive auditory input. Specifically, the HVC receives a direct projection from the telencephalic auditory area Field L (Kelley and Nottebohm, 1979), whereas area X receives acoustic inputs more indirectly through the HVC (Doupe and Konishi, 1991; Nottebohm et al., 1976). In electrophysiological studies, HVC and area X neurons respond preferentially to the bird's own song or to conspecific song, rather than to heterospecific song (Margoliash, 1983, 1986; Volman and Konishi, 1986; Doupe & Konishi, 1991; Margoliash and Fortune, 1992; Vicario and Yohay, 1993), revealing an interconnection of the vocal control and auditory pathways (reviewed in Arnold, 1992). Lesioning the HVC causes female canaries to respond with copulation solicitation displays to both conspecific and heterospecific songs, whereas control females respond preferentially

to conspecific songs (Brenowitz, 1991). In mammals, opioids influence sensory processing (pain sensitivity, Chang et al., 1989; opioid receptor–mediated sensory neuron excitation, Crain and Shen, 1990; vision and olfaction, McGregor and Herbert, 1992), and avian auditory pathways contain opioid–li ir (Durand et al., 1993). The presence of high densities of μ opioid receptors in the HVC and area X indicates that these receptors may control auditory information processing taking place in these regions. If future studies determine this to be the case, additional investigations to examine the specific role of each receptor subtype, and whether HVC and area X receptors regulate identical aspects of the acoustical information processing, would be warranted.

The junco ICo contains opioid receptors, as does the ICo of the pigeon, a non–singing species (Reiner et al., 1989). These observations suggest that as a whole, ICo opioid receptors do not regulate song perception and/or expression. It should, however, be kept in mind that the mICo is heterogenous anatomically, neurochemically (Ball et al., 1989; Deviche and Gunturkun, 1992; Puelles et al., 1994) and functionally (Armitage and Seller, 1981; Seller, 1980). Opioid receptor densities in specific mICo subdivisions such as the DM were not determined either in Reiner et al. (1989) or in the present study. Thus, juncos and pigeons may differ with respect to these receptor densities in their DM and/or in other ICo subdivisions.

In other avian species, male vocalizations enhance the female's reproductive system development (white–crowned sparrow, *Zonotrichia leucophrys*: Morton et al., 1985; budgerigar, *Melopsittacus undulatus* and canary: Hinde and Steel, 1978), but the neuroendocrine bases of this enhancement have not been elucidated. There is evidence that ICo–originating opioids exert a stimulating influence on the reproductive system. In ring doves (*Streptopelia risoria*), the female's vocal behavior (nest–coo calls) in response to the male's courtship triggers her own follicular development and ovulation (Cheng, 1987, 1992). In these females, bilateral destruction of the ICo prevents follicular development (Cohen and Cheng, 1981). The dove mICo contains enk–li ir neurons that are concentrated in, though not restricted to, the DM region (Cheng and Zuo, 1994). Some enkephalinergic

mICo neurons project to the hypothalamus, where they may stimulate the release of GnRH. The presence of opioid receptors in avian hypothalamic regions (Deviche et al., 1993; Reiner et al., 1989) is consistent with the view that opioid peptides released in these regions exert behavioral and physiological effects. Other enkephalinergic mICo neurons are adjacent to hypothalamic–projecting neurons. In doves, the mICo contains units that specifically respond to conspecific nest–coos (Cheng and Havens, 1993). Based on these observations, Cheng and Zuo (1994) proposed that intrinsic enkephalinergic mICo neurons may play a role in the dove's response to auditory stimulation. It is possible that sensory stimulation of these neurons induces the release of opioid peptides that bind to local receptors. ICo opioid receptors may, therefore, contribute to modulate endocrine responses to behavioral stimuli. Based on the information available for ring doves, it is conceivable that male vocalizations induce hypothalamic GnRH secretion and gonadal development in conspecific females through an enkephalinergic stimulation originating in the ICo.

Vocal control system opioids may additionally participate in the regulation of neuronal survival rate. Preferential neuron survival, in addition to neurogenesis, leads to apparent seasonal volume increases in some vocal control regions (reviewed in Arnold, 1992). Endogenous opioids delay normally occurring cell death in the chick ciliary ganglion (Meriney et al., 1991) and they may have a role in sparing vocal control region cells, as well.

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Table 5. Opioid receptors in vocal control and adjacent non–vocal control regions.

Region	μ	δ	κ
Area X	8.28 ± 1.53	1.13 ± 0.38	0.93 ± 0.27
LPO	5.43 ± 0.97	0.87 ± 0.30	1.30 ± 0.48
X–LPO	2.85	0.26	–0.37
HVC	4.88 ± 1.50	1.14 ± 0.51	0.52 ± 0.23
N	4.07 ± 1.33	1.09 ± 0.47	0.47 ± 0.17
HVC–N	0.81	0.05	0.05
ICo	2.98 ± 0.85	4.31 ± 1.08	0.37 ± 0.15
MLd	0.61 ± 0.29	1.17 ± 0.43	0.10 ± 0.05
ICo–MLd	2.37	3.14	0.27

Densities (means ± standard deviations; μCi/g calibrated standards) of μ, δ and κ opioid receptors in vocal control regions (Area X, HVC, ICo) and adjacent non–vocal control areas (LPO, N, MLd) in adult male dark–eyed junco (*Junco hyemalis*). Means are across seasons. This table also shows differences in receptor density means between vocal control regions and adjacent non–vocal control regions. Region pairs with receptor density difference > 2 can be distinguished on autoradiograms.

Table 6. Seasonal measures of opioid receptor densities in vocal control regions.

Receptor Type	Season	Area X	ICo	HVC
μ	Spring	7.76 ± 1.46 (12)	3.21 ± 1.12 (11)	4.12 ± 1.01 (8)
	Summer	8.45 ± 1.30 (8)	3.18 ± 0.72 (8)	5.79 ± 1.18 (7)
	Fall	8.76 ± 1.79 (8)	2.59 ± 0.55 (9)	4.73 ± 1.83 (7)
δ	Spring	1.32 ± 0.11 (9)	4.05 ± 1.10 (10)	1.23 ± 0.68 (9)
	Summer	0.95 ± 0.38 (8)	4.12 ± 0.81 (8)	0.96 ± 0.32 (8)
	Fall	1.11 ± 0.48 (8)	5.01 ± 1.21 (8)	1.23 ± 0.43 (7)
κ	Spring	0.92 ± 0.22 (9)	0.35 ± 0.17 (10)	0.62 ± 0.30(10)
	Summer	0.86 ± 0.31 (10)	0.31 ± 0.12 (9)	0.48 ± 0.22 (8)
	Fall	1.02 ± 0.25 (9)	0.44 ± 0.13 (9)	0.45 ± 0.12 (9)

Densities (means ± standard deviations; μCi/g calibrated standards) of μ, δ and κ opioid receptors in three song regions of adult male dark–eyed juncos (*Junco hyemalis*) obtained from a wild population at three stages of their annual reproductive cycle. Figures in parentheses represent the number of birds from which data were collected.

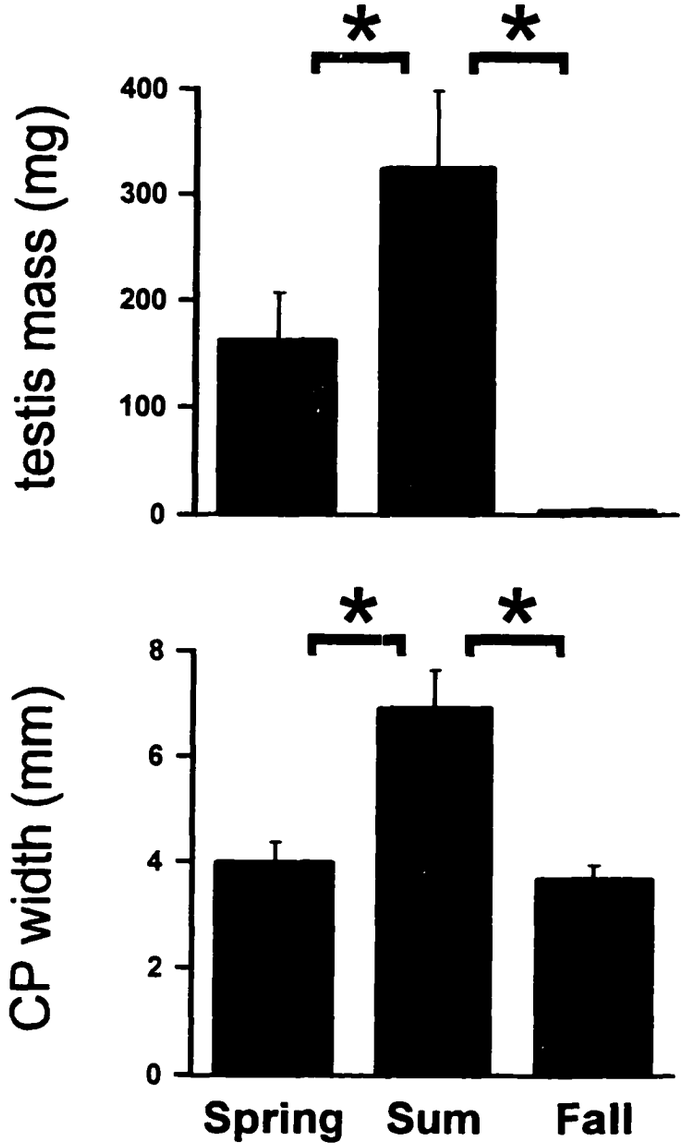

Figure 8. Adult male dark–eyed junco testis mass (mg) and cloacal protuberance (CP) width (mm). Spring: vernal migration; Sum (summer): breeding; Fall: autumnal migration. Data are shown as means ± standard deviation. *: $P < 0.001$, Dunn's (testis mass) or Student–Neuman–Keuls (cloacal protuberance) multiple pair–wise comparison tests.

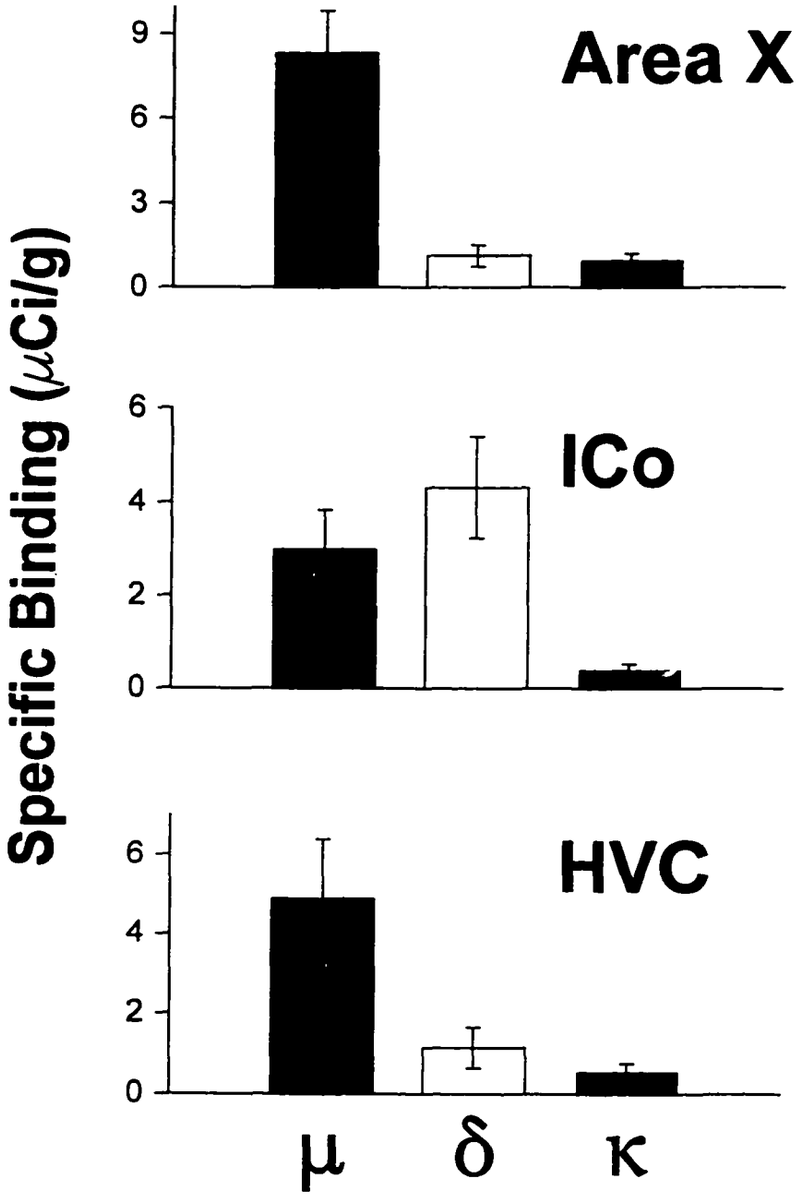

Figure 9. Specific binding across seasons (means ± standard deviation; μCi/g calibrated standard) of ^{3}H–DAMGO (μ receptor ligand), ^{3}H–pCl–DPDPE (δ receptor ligand), and ^{3}H-EKC (κ receptor ligand) to unfixed 30μm–thick brain sections in three vocal control regions (area X, HVC and ICo) of adult male dark–eyed junco (*Junco hyemalis*).

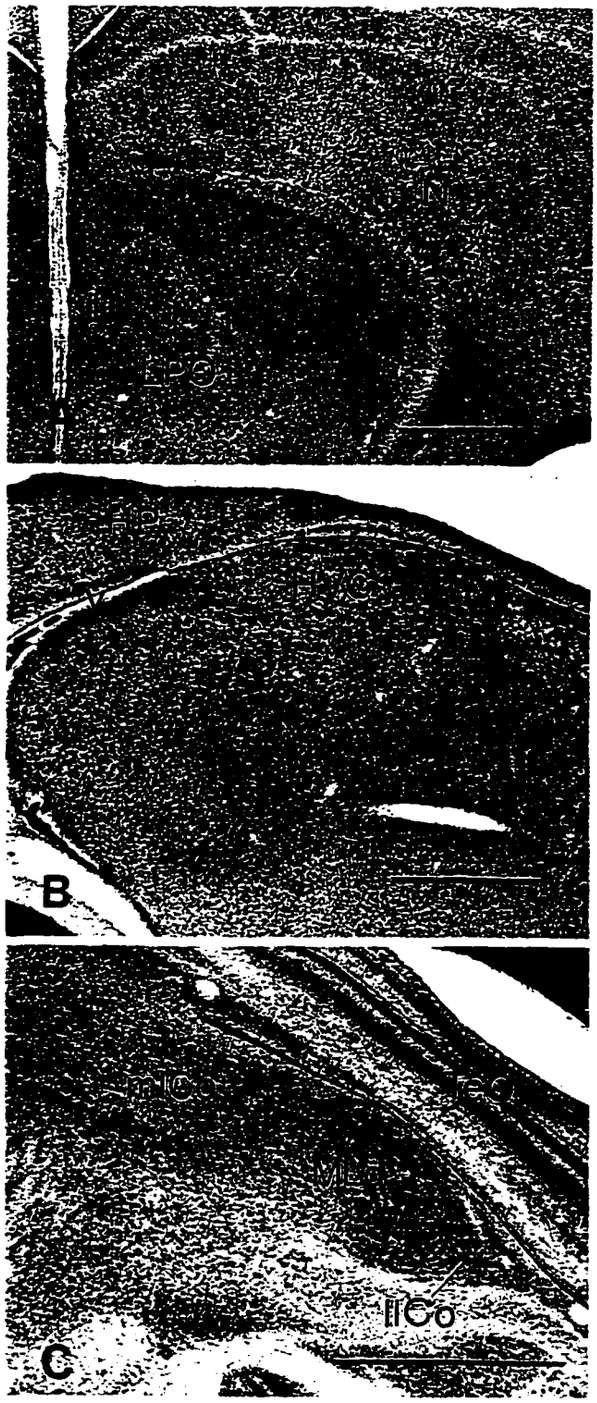

Figure 10. Photomicrographs of Nissl–stained sections illustrating three vocal control regions in adult male dark–eyed juncos. (A) area X; (B) HVC; (C) ICo. Calibration bar = 1 mm.

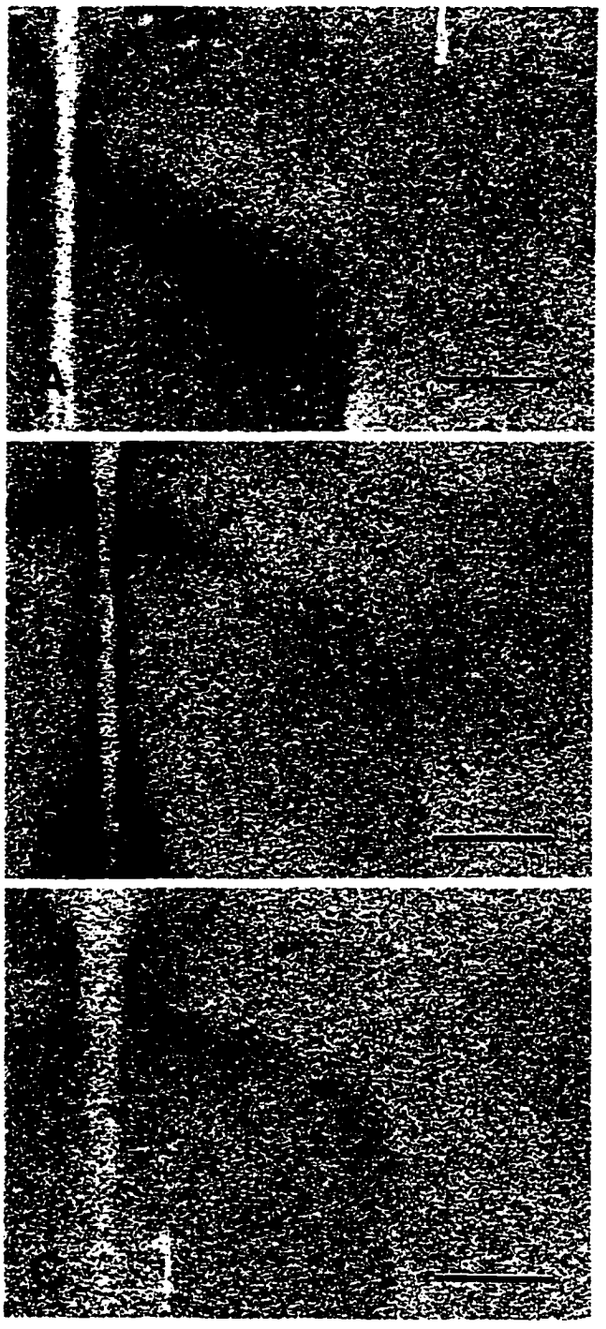

Figure 11. Autoradiograms depicting the distribution of (A) ^{3}H–DAMGO (μ receptor ligand), (B) ^{3}H–pCl–DPDPE (δ receptor ligand), and (C) ^{3}H–EKC (κ receptor ligand) binding sites in area X of the adult male dark–eyed junco. Calibration bar = 1 mm.

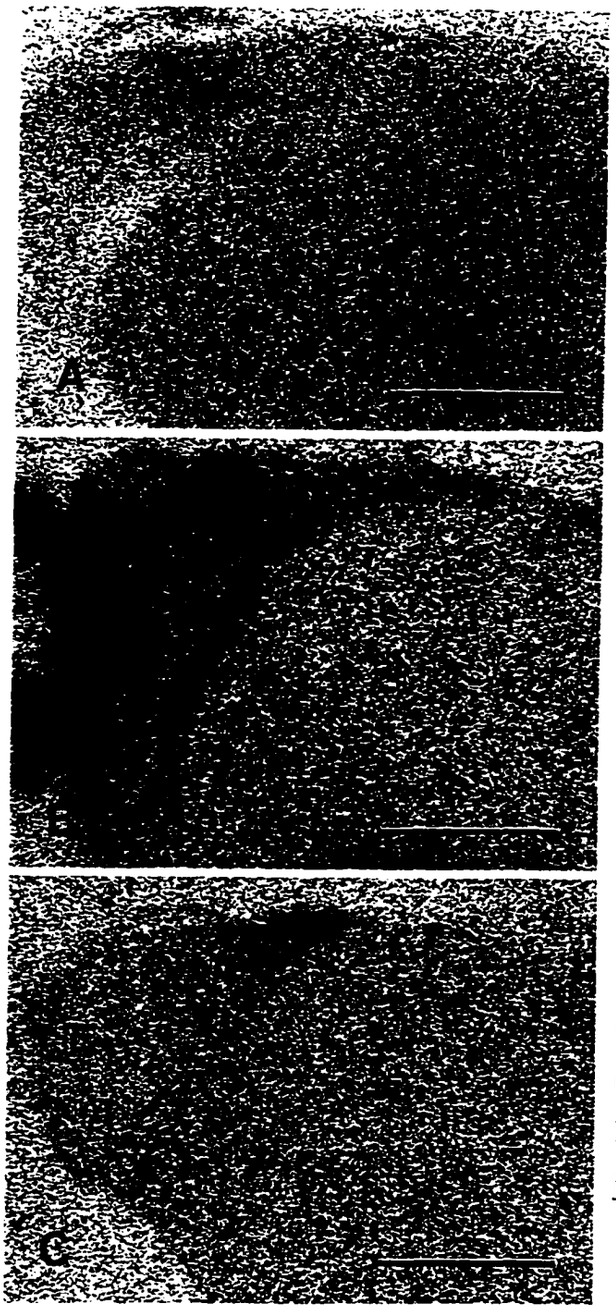

Figure 12. Autoradiograms depicting the distribution of (A) ^{3}H–DAMGO (μ receptor ligand), (B) ^{3}H–pCl–DPDPE (δ receptor ligand), and (C) ^{3}H–EKC (κ receptor ligand) binding sites in the HVC of the adult male dark–eyed junco. Calibration bar = 1 mm.

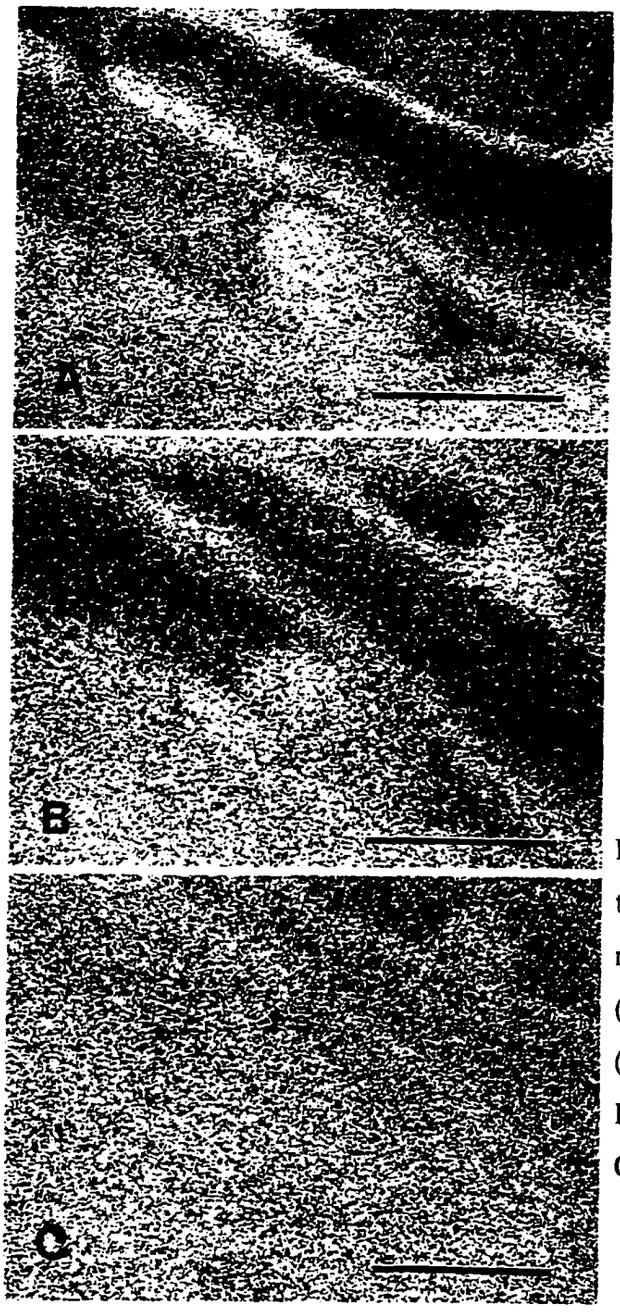

Figure 13. Autoradiograms depicting the distribution of (A) ^{3}H–DAMGO (μ receptor ligand), (B) ^{3}H–pCl–DPDPE (δ receptor ligand), and (C) ^{3}H–EKC (κ receptor ligand) binding sites in the ICo of the adult male dark–eyed junco. Calibration bar = 1 mm.

V. Age- and Sex-related Differences in Opioid Receptor Densities in the Songbird Vocal Control System[†]

Introduction

In songbirds singing is a learned behavior that is controlled by an interconnected set of discrete brain nuclei (vocal control regions, VCRs), collectively called the vocal control system (Nottebohm et al., 1976; reviewed in Konishi, 1994). Both males and females have VCRs, but VCR volumes are smaller or less well-defined in non-singing females (Nottebohm and Arnold, 1976; Bernard et al., 1993; Gulledge and Deviche, 1996). Shortly after hatching, both male and female juveniles memorize conspecific song from a tutor (usually the father), even in species in which only males sing (Slater et al., 1988; Konishi, 1965). In many species, this sensory phase overlaps with a period of song practice (sensorimotor phase; reviewed by Nottebohm, 1993). In other species, such as the Dark-eyed Junco (*Junco hyemalis*) and White-crowned Sparrow (*Zonotrichia leucophrys*), the song template is retained through a silent winter (storage phase; Whaling et al., 1995) until the males begin to practice and perfect their songs shortly before the following breeding season (Marler et al., 1962; Marler and Peters, 1982; Nottebohm, 1993). This period between song learning and adulthood is referred to as "adolescence" (Nordeen and Nordeen, 1989). During the breeding season, the males sing in order to establish and maintain territories and to attract a mate, and the females use song as one criterion for mate selection (Tomback and Baker, 1984; Grant and Grant, 1996; Nagle and Kreutzer, 1997).

Many studies have described neuroanatomical and functional characteristics of the vocal control system. The anterior forebrain (AF) pathway begins with projections from HVC to X, and then continues *via* the thalamic nucleus, DLM, to lMAN, and on to RA (Nottebohm et al., 1976; Bottjer et al., 1989). Lesion studies indicate that two AF nuclei (X, lMAN) are involved in song learning but not song production (Bottjer et al., 1984; Scharff

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and Nottebohm, 1991; Sohrabji et al., 1990). Another pathway ("motor pathway"), which controls song expression, includes a direct projection from HVC to RA, which then projects to several areas in the midbrain and brainstem, and on to the avian vocal organ, the syrinx, and to respiratory motoneurons (Nottebohm et al., 1976; Vicario, 1991; Wild, 1994). One RA projection travels to part of the ICo, a midbrain region that has been implicated in the control of vocalizations even in non-songbirds (Nottebohm et al., 1976; Wild, 1994). Another lesion study found that HVC, which receives auditory input from the telencephalic Field L (Kelley and Nottebohm, 1979), is necessary for female Canaries (*Serinus canaria*) to discriminate between conspecific and heterospecific songs (Brenowitz, 1991). In electrophysiological studies, X, lMAN, HVC, and RA neurons respond more selectively to the bird's own song than to conspecific song or white noise (Margoliash, 1983, 1986; Doupe and Konishi, 1991; Margoliash and Fortune, 1992; Doupe, 1997), and this selectivity develops after song template memorization (Volman, 1993; Doupe, 1997), possibly during song practice (Solis and Doupe, 1997).

Gonadal steroids play an important role in controlling neural and song development (Marler et al., 1987, 1988; Nordeen and Nordeen, 1989; Arnold et al., 1996; Gulledge and Deviche, 1997), and administering testosterone (T) to females of seasonally-breeding species results in functional and morphological effects similar to those observed during the development of the vocal control system in males (Nottebohm, 1980; DeVoogd and Nottebohm, 1981a; Brown and Bottjer, 1993; Rasika et al., 1994; Gahr and Garcia-Segura, 1996). Telencephalic VCRs are much larger in male juncos than in females (Gulledge and Deviche, 1996). In breeding males, elevated plasma androgen levels are necessary to maintain X and HVC volumes (Gulledge and Deviche, 1997). Even though overall brain size decreases during adolescence in both male and female juncos (Gulledge and Deviche, 1996, 1997), X volume is maintained and HVC volume increases during that time in males, even though plasma androgen levels are low (Gulledge and Deviche, 1997). Thus, the role of gonadal steroids in regulating VCR volume may change during development. Further, in

young birds, other neurochemicals may maintain VCR volumes when gonadal steroids are present at low circulating concentrations (Gulledge and Deviche, 1997).

Vocal control regions of adult male and female birds contain opioid peptide-like immunoreactivity (Ryan et al., 1981; Ball et al., 1988, 1993, 1995; Bottjer and Alexander, 1995; Deviche and Gunturkun, 1992; Carrillo and Doupe, 1995). VCRs of adult male juncos also contain opioid receptors (Gulledge and Deviche, 1995), indicating that opioid peptides may bind to receptors located within these regions. Opioid receptor densities in X, HVC, and ICo are similar in wild males captured either during or after the breeding (singing) season (Gulledge and Deviche, 1995), suggesting that opioids do not control song expression, however. Alternative opioid roles in the vocal control system are suggested by other studies, including those using other models. Carrillo and Doupe (1995) reported a decrease in opioid-like (leu-enkephalin) immunoreactivity in X and RA, but not in HVC or lMAN, between day 35 and adulthood in male Zebra Finches (*Taeniopygia guttata*), suggesting a role for opioids in vocal control system development. Opioids modulate learning in chicks (Csillag et al., 1993; Colombo et al., 1997) and rodents (Rigter et al., 1980; Castellano & Pavone, 1985), and they affect the structure of the nervous system during development in chicks (Meriney et al., 1991) and rats (Hammer and Hauser, 1992). In chicks, endogenous opioids can delay programmed cell death (Meriney et al., 1991) and in rats, opioid receptor activation inhibits postnatal dendrite proliferation (see review, Hammer and Hauser, 1992). Perez-Navarro et al. (1993) found that opioids may modulate the effects of nerve growth factor, which affects cell proliferation and differentiation. Opioids also have been implicated in sensory processing (Crain and Shen, 1990), and, therefore, may play a role in the auditory processing necessary for all birds to distinguish conspecific from other songs.

Adolescent juncos have memorized a song template which is stored until the breeding season; therefore, a difference in opioid receptor densities between adolescent and adult juncos could suggest a role for opioids in song learning and/or vocal control system development. Because opioid receptor densities do not differ seasonally in adult male VCRs (Gulledge and Deviche, 1995), we hypothesized that opioids do not modulate song

production. Breeding adult male juncos sing, but adult females and adolescents do not; if, contrary to our hypothesis, opioids do have a role in modulating song production, then opioid receptor densities in singing adult males should differ consistently from all groups that do not sing. To investigate the potential roles of opioid systems in avian VCRs, we used quantitative *in vitro* autoradiography to determine the anatomical distribution and to measure the densities of μ and δ opioid receptors in VCRs (X, lMAN, HVC, ICo, RA) of breeding and adolescent juncos of both sexes.

Materials and Methods

Specimen collection

Adult male (n=9) and female (n=7) Dark-eyed Juncos were collected from a wild population near Fairbanks, Alaska (65°N, 148°W) during the breeding season when males were singing on their breeding territories (May and June, 1995). Reproductive condition was assessed by measurement of cloacal protuberance size in adult males and presence of a brood patch in adult females. The cloacal protuberance is a secondary sexual characteristic in adult males (Schwabl and Farner, 1989; Deviche 1992, 1995), and presence of a developed brood patch in adult females indicates that they are producing or incubating eggs. Hatchling birds are photorefractory and show no signs of reproductive activity until they are exposed to increasing photoperiods the following spring (Nicholls et al., 1988). Adolescent males (n=11) and females (n=5) were collected during fall migration, when they were approximately 3 months old (September, 1990; October, 1995). All birds were captured in seed-baited Potter traps, then were killed by decapitation. Their brains were immediately removed, frozen by immersion into Freon, and stored at -70 °C until processed. All procedures conformed to institutional animal care and use committee guidelines, and were in accordance with federal and state animal collection permits.

Chemicals

[Tyr-3,5-^{3}H(N)]-[D-Ala^2, N-Me-Phe^4, Gly-ol^5]-enkephalin (^{3}H-DAMGO; 55.0 Ci/mmol), and [Tyr-3, 5-^{3}H]-[D-Pen^2, pCl-Phe^4, D-Pen^5]-enkephalin (^{3}H-pCl-DPDPE; 50.8 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Naloxone HCl and naltrexone HCl were provided by Endo Laboratories (New York, NY). Unlabeled peptides were obtained from Peninsula Laboratories (Belmont, CA).

In vitro autoradiography

Frozen, coronal 30 μm-thick brain sections were made in a cryostat at -15 ^{0}C and collected on gelatin-coated microscope slides, dehydrated at 4 ^{0}C under partial vacuum overnight, then stored at -70 ^{0}C until processed further. For each brain, successive pairs of sections were used for incubations with each labeled ligand as previously described (Deviche et al., 1993; Gulledge and Deviche, 1995).

Briefly, sections were pre-incubated for 30 minutes in NaCl-containing buffer (pH 7.40) to dissociate and eliminate potential receptor-bound endogenous ligands (Loew et al., 1983; Kosterlitz et al., 1987, 1988). Slides were rinsed twice in plain buffer before incubation in conditions such that the radioligands under study bound to μ (^{3}H-DAMGO) or δ (^{3}H-pCl-DPDPE) receptors with high affinity and selectivity (Deviche et al., 1993). One slide from each pair was used to measure total binding (TB), the other to measure non-specific binding (NSB). NSB was determined by including 1 mM naloxone HCl (^{3}H-DAMGO), or 10 mM naltrexone HCl (^{3}H-pCl-DPDPE) in the incubation solution. Each ligand was used at a concentration equal to twice its K_d (^{3}H-DAMGO: 11 nM; ^{3}H-pCl-DPDPE: 0.48 nM). Accordingly, each labeled ligand presumably occupied a similar proportion of its respective receptors at equilibrium, so that the relative densities of both opioid receptor types within a specific region can be directly compared with each other.

Slides were placed into light-proof X-ray cassettes with a set of calibrated plastic standards (American Radiochemical Co., St. Louis, MO) and exposed to

radioactivity-sensitive film (Hyperfilm, Amersham, Arlington Heights, IL) for 12 weeks before development.

Autoradiography data collection

Sections were Nissl-stained using thionin, and brain regions were identified using the Canary stereotaxic atlas (Stokes et al., 1974; Nottebohm et al., 1976). The anatomical boundaries of vocal control and non-vocal control regions were determined based on examination of the histological sections (Fig. 14), not of the corresponding autoradiograms. Measures were taken for all sections in which a particular region appeared. Generally, the VCRs under study appeared homogenous with regard to their densities of opioid receptors, i.e., these densities did not seem to differ between the center and the outer edges. We therefore measured densities from within, rather than at, the region borders to ensure that all measures were taken from within the nuclei themselves. The lMAN was clearly defined on histological sections that had been used for δ, but not μ, incubations, perhaps due to differences in incubation protocols. Therefore, lMAN was measured only for δ autoradiograms. For the lCo, measurements were taken and combined for the lateral (llCo) and medial (mlCo) parts of the nucleus. The mlCo included the DM which is surrounded by the lCo proper (De Lanerolle and Andrew, 1974; Paton et al., 1981; Wild and Arends, 1987). We also measured receptor densities in regions (MLd, LPO, PN, AN, Arch) that are adjacent to, but not part of, the vocal control system. The LPO, PN, AN, and Arch samples were ventral to the adjacent VCR samples, and were only measured on sections containing the corresponding VCR.

Autoradiograms were analyzed by computerized microdensitometry using the M1 MCID image analysis system (Imaging Research, Canada; Deviche et al., 1993), which subtracts NSB from TB densities so that final data are expressed in μCi specifically bound (SB) ligand/g calibrated standard.

Statistical analyses

For each individual, SB data obtained for each brain region were averaged across sampled sections. Sex and age differences in opioid receptor densities in each brain region under study were analyzed using two-way analyses of variance (ANOVAs) followed by multiple comparisons (Student-Neuman-Keuls tests). The data for δ receptors in lMAN did not comply with normality and variance homogeneity requirements (Kirk, 1982), and so were ranked before analysis using two-way ANOVAs followed by Student-Neuman-Keuls multiple comparisons tests. Data are presented as means ± standard errors, except data for lMAN, which are presented as medians ± ½ interquartile intervals.

Results

Reproductive state

Males caught in the breeding season had large cloacal protuberances (6.9 ± 0.2 mm) and developed testes (316 ± 22 mg), and adult females had brood patches as well as developed or ruptured ovarian follicles. Adolescents exhibited no signs of reproductive system activity. Thus, breeding adult and fall adolescent juncos were in markedly different physiological condition.

Opioid receptor densities

All vocal control regions studied contained measurable amounts of μ and δ receptors, and within each region, the two receptor types were present at different densities (Fig. 15 and 16; Tables 7 and 8). Opioid receptor densities differed among sex and age classes in several VCRs (Tables 7 and 8).

Densities of δ receptors. Adolescents juncos had significantly more δ receptors in X than did adult male juncos (Fig. 16, Table 7). Because δ opioid receptor densities in the surrounding LPO were similar in all groups and not much different from those measured in X in adults, X stood out visually from the surrounding LPO only on adolescent autoradiograms (Fig. 15b). Higher δ receptor densities were present in lMAN of adult females than of all

males, but not than of adolescent females (Table 7). Even the highest density of δ receptors in lMAN were barely above background, and statistically significant differences in this region will not be discussed further. Densities of δ receptors in HVC and the ICo did not differ among groups (Table 7). On most δ autoradiograms, the shape of HVC could be discerned as an area appearing lighter than the surrounding neostriatum (Fig 15, e-f), which includes the songbird auditory cortex analogue depicted in Capsius and Leppelsack (1996). On δ (as well as μ) autoradiograms, the ICo was obvious compared to the MLd that it surrounds, as well as the surrounding midbrain (Fig. 15, i-l). Adolescent males had almost 3 times more δ receptors in RA than other groups, which did not differ from each other (Fig. 16, Table 7). The shape of RA was often obvious on autoradiograms, appearing exactly as on the corresponding histological section and distinct as either lighter or darker than the surrounding Arch (Fig. 15, m-n).

Densities of μ receptors. Densities of μ receptors were higher than δ receptors in X, but did not differ significantly among groups (Fig. 16, Table 8). In contrast to δ autoradiograms, X stood out as being darker than the surrounding LPO on μ receptor autoradiograms for all groups (Fig. 15, c-d). On all μ autoradiograms, the medial neostriatum, including the HVC and auditory cortex analogue, had high receptor densities (Fig. 15, g-h), but μ receptor densities did not differ among groups in HVC (Table 8). Significant differences among groups in μ receptor densities were found in the ICo and RA (Table 8). Adolescent females had a higher density of ICo μ receptors than adults, but not than adolescent males. Additionally, adolescent females had almost twice the μ opioid receptor density in RA as other groups (Fig. 16, Table 8). This difference was specific to RA, as opposed to the surrounding area; although ANOVA results indicated that Arch μ receptor densities were significantly higher in adolescents than in adults, pairwise comparisons did not reveal specific differences between any two groups (Table 8). As on δ autoradiograms, the shape of RA often could be discerned as being lighter than the immediate surrounding area (Fig. 16p).

Discussion

Vocal control regions of all groups studied contained both μ and δ receptors, the densities of which varied regionally within the brain. The differences in μ and δ receptor patterns suggest that the two receptor subtypes play different roles in the various VCRs in which they occur. The current findings are consistent with those of our previous study of opioid receptors in adult male VCRs (Gulledge and Deviche, 1995). Comparing additional age-sex classes in this study allowed us to determine that VCR opioid receptor densities are age- and sex-dependent. We found that δ receptor densities were higher in X of adolescents than adults. Further, adolescent females had more μ receptors in the ICo than adults and more μ receptors in RA than any other age-sex class. The reciprocal condition occurred in adolescent males, which had more δ receptors in RA than any other age-sex class. Opioid receptor densities in breeding adult males, the only group that presumably had elevated T levels and produces song, never were markedly higher or lower than all other groups. These data support our previous hypotheses, based on lack of seasonal changes in opioid receptor densities in adult males, that opioid receptor densities are not modulated by T levels and also that these receptors do not control song production (Gulledge and Deviche, 1995). Furthermore, the data suggest that opioid receptors modulate other aspects of vocal behavior, such as auditory processing, development, and/or learning, that are extant in the various non-singing groups.

Auditory processing

Opioids exert a broad range of short-term effects on neural systems, often by altering the release of other neurochemicals (*e.g.*, acetylcholine, Jhamandas et al., 1970; dopamine, Loh et al., 1976; nerve growth factor, Perez-Navarro et al., 1993). Inhibitory effects of opioids include inhibition of adenylyl cyclase activity and modulation of G protein-associated ion channels, which result in decreased neuronal activity, membrane hyperpolarization and subsequent blockade of neurotransmitter release (Sarne et al., 1996; Yu, 1996). Recent reports also indicate that opioid receptors can stimulate neuronal activity

and neurotransmitter release through coupling to stimulatory G proteins and Ca^{++} channels (see review, Sarne et al., 1996).

In the present study, μ receptor densities in X and HVC were consistently high but did not differ among the sex-age classes (Fig. 16, Table 8). We did not measure opioid receptor densities in the medial telencephalic auditory centers depicted by Capsius and Leppelsack (1996) because these areas are defined functionally rather than anatomically, but inspection of autoradiograms indicate that the areas contained high densities of opioid receptors (Fig. 15, e-h). The ubiquitous presence of opioid receptors in regions that receive and respond to auditory information (X, HVC, ICo, RA, songbird auditory cortex analogue; Fig. 15) suggest that opioids exert strong effects on acoustic information processing.

One such effect of opioids may be the filtering of auditory information as it is processed within VCRs. A common effect of opioids is to inhibit neuronal activity (reviewed in Sarne et al., 1996). Neurons in HVC, X, lMAN, and RA of adult males are more responsive to the bird's own song than to other sounds (Doupe, 1997). Opioids may be involved in inhibiting non-selective neuronal responses to auditory stimuli within VCRs, or may inhibit non-preferred auditory information from being passed on to the next nucleus in the pathway, thus preventing the target nucleus from responding to the sound. Doupe (1997) found that lMAN neurons are more sound-selective than X neurons and suggested that this difference may result from inhibitory processing taking place in X, the only known source of auditory input to lMAN (via DLM). Thus, opioid receptors on X neurons may play a role in filtering auditory information through the AF pathway.

Sexual dimorphism

Opioids exert not only short-term, but also long-term influences on neuronal pathways. The latter effects may play an important role in the sexual differentiation of the vocal control system. The primary mechanisms involved in this differentiation are enhanced cell survival and lower programmed cell death in males than females (Kirn and DeVoogd, 1989). In species with sexually dimorphic singing behavior, sexual dimorphism of VCRs is

observed at both cellular and regional levels (Nottebohm and Arnold, 1976; DeVoogd and Nottebohm, 1981b; Arnold et al., 1986; Kirn et al., 1989; Ball et al., 1995; Gulledge and Deviche, 1996). For example, in adult Canary RAs, dendrites are longer in males than in females, and RA volumes are much larger in males (DeVoogd and Nottebohm, 1981b; Nottebohm and Arnold, 1976). Opioid activity inhibits postnatal dendrite proliferation in rats (Hammer and Hauser, 1992). Therefore, the elevated μ receptor densities in the RA of adolescent females (Fig. 16) may inhibit expansion of dendrites and overall volume in RA of these females. On the other hand, endogenous opioids can delay programmed cell death in chicks during development (Meriney et al., 1991). In this study, densities of δ receptors were higher in RAs of adolescent males than in any other group (Fig. 16). Opioids may maintain VCR volumes in adolescent males by prolonging cell survival, contributing to the sexual dimorphism observed in many junco VCRs (Gulledge and Deviche, 1996).

Learning and memory

Studies on chicks point to a role for opioids in learning and memory (Schulteis et al., 1990; Csillag et al., 1993; Columbo et al., 1997). Area X, a part of the LPO present only in oscines, is specialized for song learning and modification (Sohrabji et al., 1990; Scharff and Nottebohm, 1991). Several studies on chicks indicate that the LPO itself is used for memory storage: bilateral LPO lesions given 1 hour post-training inhibit task performance 24 hours later (Gilbert et al., 1991), and training alters metabolic activity (2-deoxyglucose usage and glycoprotein synthesis; Kossut and Rose, 1984; Lössner and Rose, 1983) and synaptic morphology in the LPO (Stewart et al., 1987). Csillag et al. (1993) found that training-induced changes in the LPO included an increase in δ, but not μ, receptor densities. In juncos we found that adolescents had higher δ receptor densities than adults in X, but that μ receptor densities in X did not differ among age-sex classes (Fig. 16). Although we lack behavioral data on the exact timing of junco song learning, the fact that we found age differences in δ receptor densities in X is consistent with a role for opioids in song learning, just as opioids modulate memory storage in the chick LPO.

Conclusions

We found that all vocal control regions measured contained δ and μ receptor types, and that in some of those regions receptor densities differed between adolescents and adults, as well as between males and females. Densities of δ receptors in both X and RA differed between young and adult males. This result complements a study that found a decrease in leu-enkephalin (preferential δ receptor ligand; Lord et al., 1977) immunoreactivity in X and RA, but not in lMAN or HVC, between 35 day-old and adult male Zebra Finches (Carrillo and Doupe, 1995). Most of the differences we observed in this study occurred in adolescents, which, along with the results of Carrillo and Doupe (1995), point to developmental roles for opioids in the vocal control system. Those roles could involve song learning, sexual differentiation, or possibly a more subtle aspect of vocal control system maturation. In VCRs where densities of one or both opioid receptor subtypes do not differ among age-sex classes, opioid receptors may modulate functions common to both male and female songbirds throughout life, such as processing auditory information. Our suggestion that opioid receptors in the avian vocal control system are involved in short-term effects such as auditory processing, as well as long-term effects such as learning and development (*e.g.*, inhibition of dendrite proliferation, delay of programmed cell death), is consistent with previous findings regarding the variety of opioid receptor actions at the molecular level. Further study on the roles of opioids in the vocal control system will require examination of the effectors coupled to the receptors for these peptides.

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Table 7. Densities of δ opioid receptors in vocal control regions and adjacent non-vocal control regions

Region	Adolescent Males	Adult Males	Adolescent Females	Adult Females	ANOVA Age	ANOVA Sex	ANOVA Age x Sex
X	1.32 ± 0.14^{a}	0.69 ± 0.08^{b}	1.27 ± 0.19^{ac}	0.86 ± 0.11^{bc}	p = 0.0008	p = 0.68	p = 0.43
LPO	0.80 ± 0.08	0.62 ± 0.08	0.75 ± 0.09	0.67 ± 0.09	p = 0.16	p = 0.99	p = 0.59
lMAN	0.17 ± 0.10^{a}	0.18 ± 0.07^{a}	0.22 ± 0.12^{ac}	0.46 ± 0.04^{bc}	p = 0.038	p = 0.042	p = 0.046
AN	0.27 ± 0.04	0.28 ± 0.06	0.33 ± 0.07	0.44 ± 0.09	p = 0.38	p = 0.12	p = 0.47
HVC	1.11 ± 0.16	0.73 ± 0.10	0.90 ± 0.10	0.86 ± 0.06	p = 0.12	p = 0.46	p = 0.33
PN	1.40 ± 0.16	1.32 ± 0.22	0.87 ± 0.16	1.46 ± 0.16	p = 0.19	p = 0.32	p = 0.085
lCo	4.83 ± 0.44	3.44 ± 0.34	4.15 ± 0.75	4.61 ± 0.39	p = 0.34	p = 0.61	p = 0.06
MLd	1.00 ± 0.12	0.92 ± 0.08	0.86 ± 0.18	1.06 ± 0.19	p = 0.68	p = 0.999	p = 0.32
RA	4.87 ± 0.62^{a}	1.89 ± 0.23^{b}	1.99 ± 0.44^{b}	1.57 ± 0.23^{b}	p = 0.0023	p = 0.0037	p = 0.0168
Arch	2.48 ± 0.37	1.44 ± 0.21	1.54 ± 0.28	1.66 ± 0.28	p = 0.18	p = 0.28	p = 0.09

Specific binding (means ± standard errors; μCi/g calibrated standards) of ^{3}H-pCl-DPDPE to δ opioid receptors in vocal control regions (Area X, HVC, lCo, RA) and adjacent non-vocal control areas (LPO, PN, MLd, Arch) in dark-eyed juncos (*Junco hyemalis*). Densities reported for the vocal control region lMAN are medians ± ½ interquartile intervals (see methods). Densities reported for the adjacent control area AN are means ± standard errors. Reported p values represent two-way ANOVA results. Superscripts indicate significant differences among groups within a region as determined by multiple comparisons tests.

Table 8. Densities of μ opioid receptors in vocal control regions and adjacent non-vocal control regions

Region	Adolescent Males	Adult Males	Adolescent Females	Adult Females	ANOVA Age	ANOVA Sex	ANOVA Age x Sex
X	9.06 ± 0.86	7.57 ± 0.73	10.59 ± 0.84	9.09 ± 1.45	p = 0.21	p = 0.20	p = 0.99
LPO	6.53 ± 0.65	5.61 ± 0.42	7.96 ± 0.59	6.19 ± 1.02	p = 0.12	p = 0.23	p = 0.61
HVC	5.36 ± 0.37	5.73 ± 0.61	7.52 ± 0.61	5.50 ± 0.88	p = 0.21	p = 0.15	p = 0.076
PN	4.97 ± 0.36	5.33 ± 0.71	6.42 ± 0.69	5.42 ± 0.65	p = 0.60	p = 0.22	p = 0.28
ICo	3.34 ± 1.15^{ab}	2.66 ± 0.90^{a}	4.80 ± 1.06^{b}	3.36 ± 0.92^{a}	p = 0.02	p = 0.016	p = 0.38
MLd	0.67 ± 0.07	0.61 ± 0.01	0.79 ± 0.11	0.62 ± 0.07	p = 0.21	p = 0.48	p = 0.56
RA	4.38 ± 0.55^{a}	3.44 ± 0.45^{a}	7.50 ± 0.54^{b}	3.79 ± 0.44^{a}	p = 0.0006	p = 0.007	p = 0.027
Arch	4.34 ± 0.66	3.80 ± 0.69	5.97 ± 0.39	3.27 ± 0.76	p = 0.0465	p = 0.48	p = 0.18

Specific binding (means ± standard errors; μCi/g calibrated standards) of ^{3}H-DAMGO to μ opioid receptors in vocal control regions (Area X, HVC, ICo, RA) and adjacent non-vocal control areas (LPO, PN, MLd, Arch) in dark-eyed juncos (*Junco hyemalis*). Reported p values represent two-way ANOVA results. Superscripts indicate significant differences among groups within a region as determined by multiple comparisons tests.

Figure 14. Histological sections representing VCRs depicted in Figure 15. (a) X/LPO, (b) HVC/PN, (c) ICo/ MLd, (d) RA/Arch. Adult and adolescent males have larger X, HVC, and RA volumes than adult and adolescent females. Left is medial and top is dorsal. Scale bars = 2.5 mm.

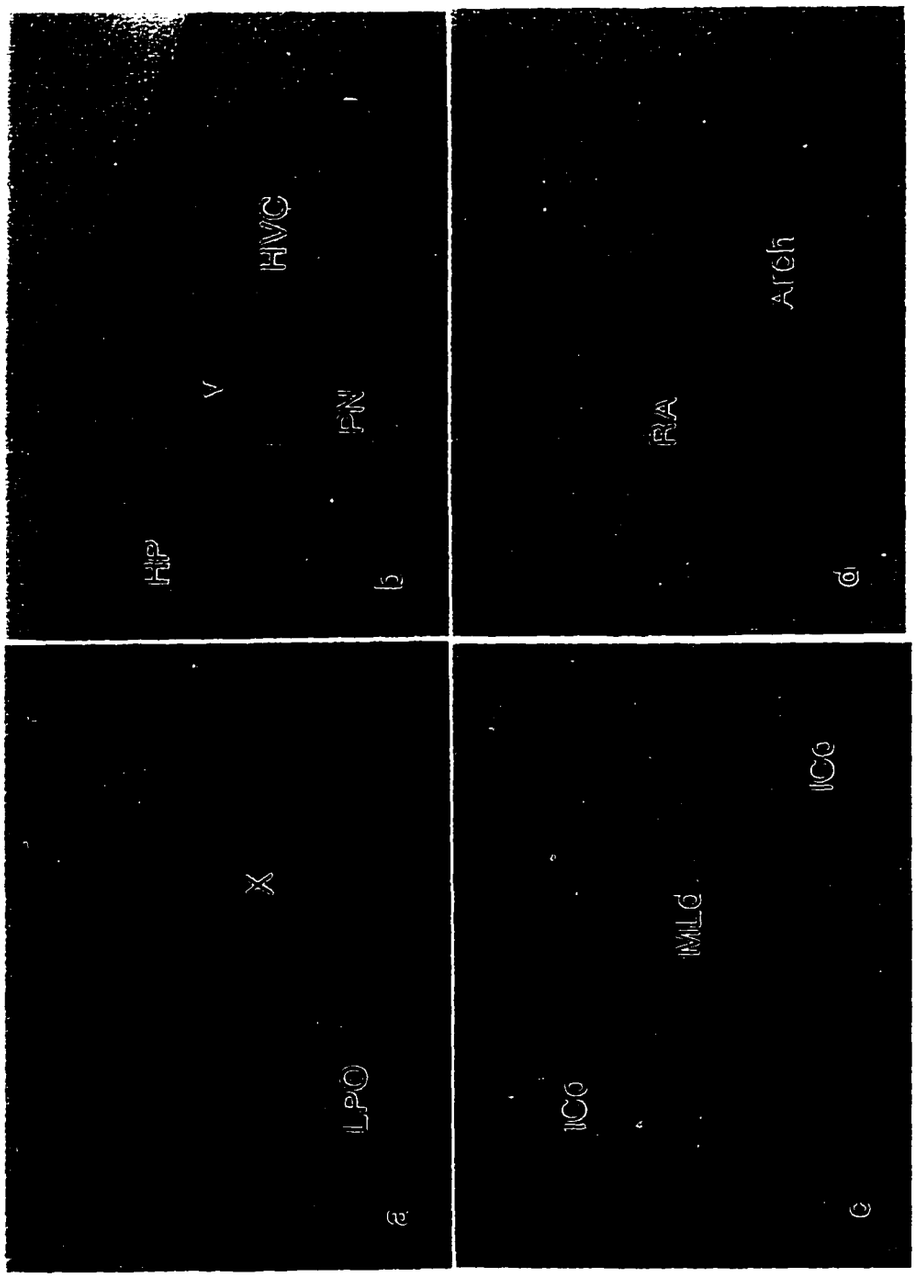
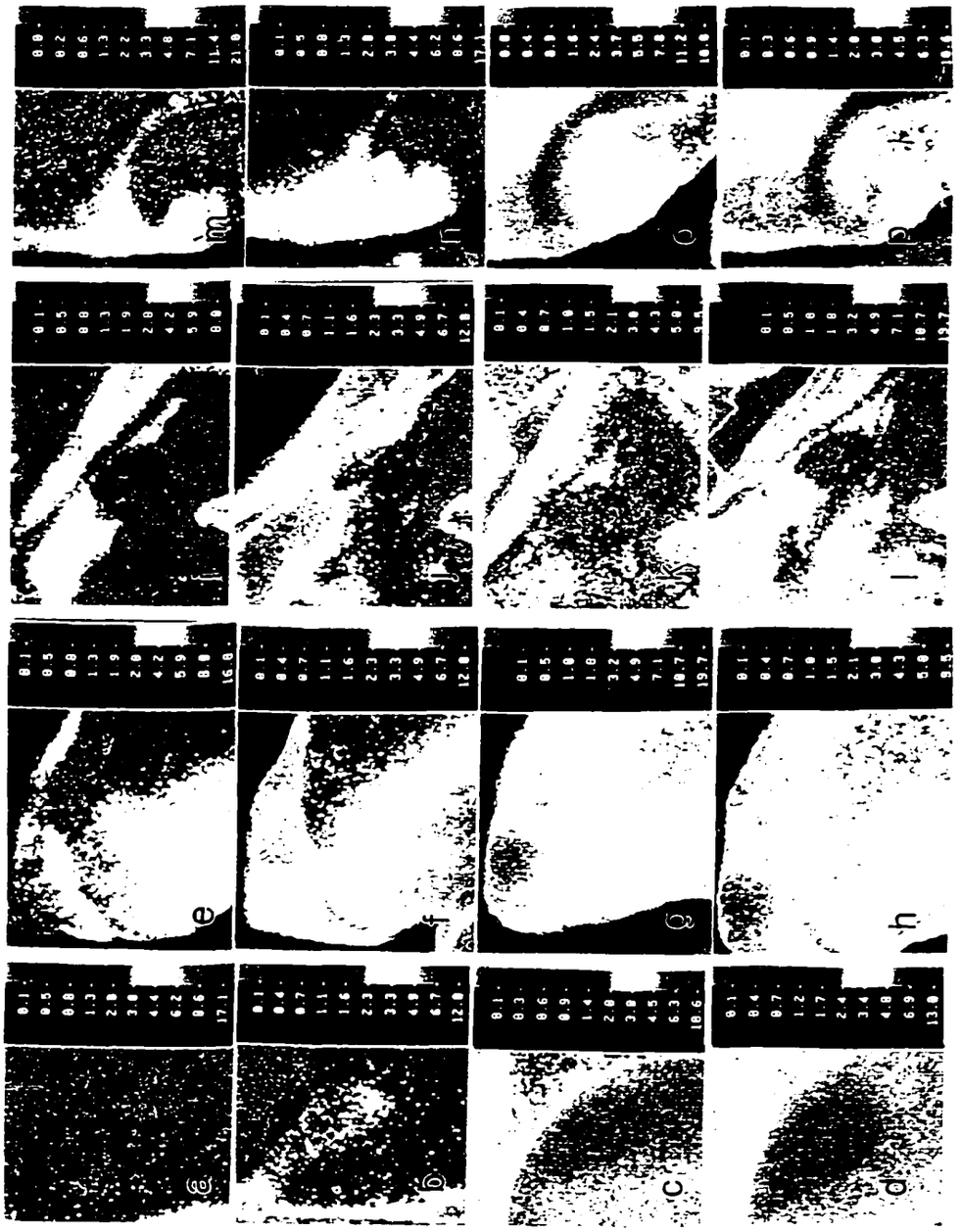


Figure 15. Autoradiograms depicting the distribution of ^{3}H-pCl-DPDPE (δ receptor ligand; top two rows) and ^{3}H-DAMGO (μ receptor ligand; bottom two rows) specific binding in VCRs and surrounding areas. For each ligand and region, a brain section from a group with significantly higher receptor densities (if any) and another representing the other groups are shown. (a-d) X/LPO (e-h) HVC/PN, (i-l) ICo/MLd, (m-p) RA/Arch. AM=adolescent male; BM=breeding adult male; AF=adolescent female; BF=breeding adult female. a) BF; b) AF; c) BM; d) AM; e) BM; f) AM; g) AF; h) BM; i) BM; j) AF; k) BF; l) AF; m) AF; n) AM; o) AF; p) AM. Calibration bars indicate relative radioactive ligand binding (μCi/g calibrated standard) with blues and greens indicating low receptor densities and yellows and reds indicating high receptor densities.



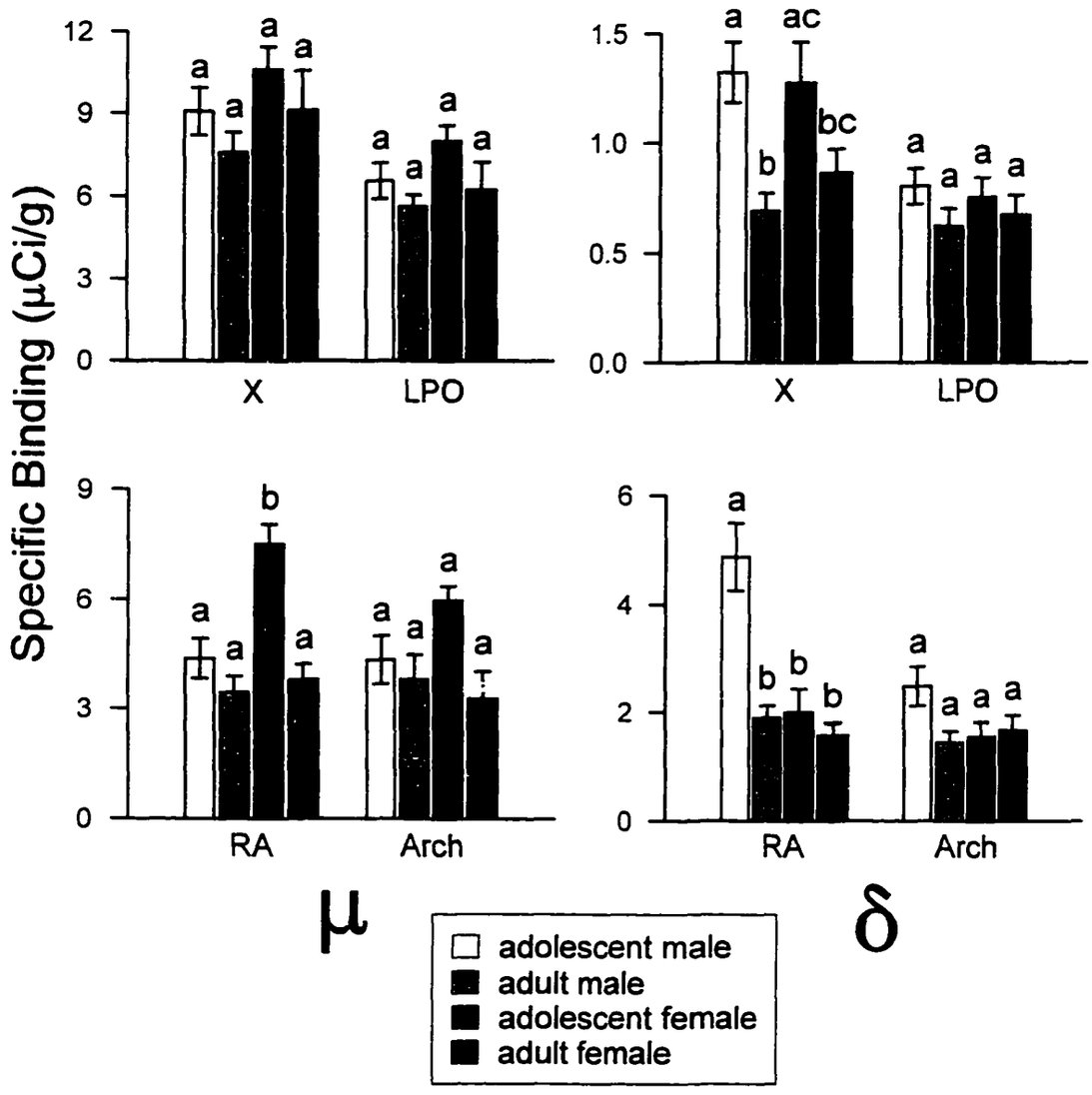


Figure 16. Specific binding (means ± standard error; μCi/g calibrated standard) of ^{3}H-DAMGO (μ receptor ligand; left column), and ^{3}H-pCl-DPDPE (δ receptor ligand; right column) to unfixed 30μm-thick brain sections in vocal control regions (area X and RA) and their respective surrounding areas (LPO and Arch) in the Dark-eyed Junco (*Junco hyemalis*). Note differences in Y axis scales.

VI. Conclusions

In the introductory chapter, I mentioned several areas in which previous research on the avian vocal control system had been lacking. Briefly, these were deficiencies in

- a comparative (i.e., non-domesticated) perspective;
- an understanding of age-related differences in testosterone effects on VCR volumes;
- investigations of non-steroidal effects on the vocal control system.

The previous four chapters help form a more complete picture of vocal control system function, particularly as songbirds age from adolescence to adulthood, a stage of life that had largely been ignored by researchers in this field. Below I discuss what I believe to be the most important conclusions from my thesis work, as well as the future directions of the field as a whole.

A Comparative Perspective

Dark-eyed Juncos (*Junco hyemalis*) are an excellent model for studying the avian vocal control system. As expected, this photoperiodic, seasonally-breeding species exhibits seasonal patterns of VCR volume modification (Chapter 2). In that respect, they resemble the well-studied Canary (*Serinus canaria*; Nottebohm, 1980). Canaries, however, have been bred to sing during most of the year, and as such, have a very short photorefractory period in the late summer/early fall (Nottebohm et al., 1986). We cannot be certain that their seasonal changes reflect what is seen in wild birds. For example, only one month after the end of its post-breeding decline, the HVC in Canaries is restored to the volumes found in breeding adults (Nottebohm et al., 1994). This may reflect their short photorefractory period, rather than represent the timing found in most wild songbirds. Juncos follow a seasonal photoperiodic pattern typical of most wild songbirds residing in temperate regions (Rowan, 1925). It is important now to expand this comparative approach to a broader spectrum of wild songbirds to determine the typical pattern of seasonal VCR changes. I suspect results from the junco will represent this pattern well.

Testosterone Through the Ages

Because T administration or exposure to long photoperiod can stimulate adolescent male juncos to sing crystallized song, we at first thought of these birds as young adults, just waiting for spring to come. Experiments described in the previous four chapters indicate that this is not the case. The adolescent period involves more than just killing time until days are long and breeding begins. For example, the effects of T on the VCRs of adolescent males differ from those detected in birds just a few months older (Chapters 2 and 3). This difference may reflect the effects on VCR neurons of exposure to elevated plasma T during the first breeding season. Before then, T administration does not cause most VCR volumes to increase (Chapter 3), and exposure to only low T concentrations does not prevent area X from being maintained at breeding adult sizes in adolescent males (Chapter 2). In contrast, giving T to adult Canaries in fall-like conditions causes VCR volumes to increase (Nottebohm, 1980; Johnson and Bottjer, 1993), and removing T during the breeding season by castration causes area X and HVC volumes to decrease to post-breeding sizes (Chapter 2). These results indicate that maintenance and modification of VCR volumes in adult males depend on T, or at least can be affected by T levels, whereas the effects of T on these processes in adolescent males are limited. It is possible that exposure to high T levels during the breeding season causes certain VCR neurons to become dependent on those elevated levels. Nottebohm et al. (1994) determined that, in Canaries, HVC neurons born during the fall when T is low have longer life spans than those born during the spring when T levels are elevated; many HVC cells born in the spring die after that same breeding season. They proposed that neurons born in the presence of high T require T to stay alive, so that a sharp decrease in T, as occurs after breeding, may prompt programmed cell death. Perhaps VCR neurons born in adolescent males with low T levels do not require T for survival. Neurons in HVC are continually replaced throughout life in Canaries (Kirn et al., 1991; Kirn and Nottebohm, 1992; Alvarez-Buylla et al., 1994). Therefore, some neurons are added during the breeding season when T is elevated. Those neurons may be the ones dying at the end of the breeding season (Nottebohm et al., 1994; Chapter 2) or after castration during the breeding season (Chapter 2).

Non-steroidal Effects on VCRs

One major theme from Chapters 2 and 3 is that the effects of T on VCR volumes are limited during adolescence. The results from Chapter 3 indicate that long photoperiod may be more important than T in determining VCR volumes throughout the vocal control system in adolescent male juncos, as 3 of 4 VCRs measured were larger in adolescent males exposed to long photoperiod than in those exposed to short photoperiod, whereas only RA volume increased in response to T administration. In adults of other species, T appears to supercede photoperiod in affecting VCR volumes (Smith et al., 1997; Bernard et al., 1997). Experiments from Chapter 3 should be repeated using adult male juncos to determine whether the relative importance of T and long photoperiod in controlling VCR volumes is opposite in adolescent and adult males of this species. The neurochemicals that are affected by photoperiod have not been identified. Melatonin is an interesting possibility, as its receptors have been measured in VCRs of adult male House Sparrows (*Passer domesticus*) and its production is regulated by light/dark cycles (Whitfield-Rucker and Cassone, 1996; Binkley, 1990).

Other neurochemicals and their receptors have been detected in the avian vocal control system (Bernard et al., 1993; Casto and Ball, 1994; Ball et al., 1994; Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997; Li et al., 1997). We chose to focus on opioids because their functions in other systems mimic characteristics found in singing behavior — learning, sensory processing, prolonged neural survival — and because opioid peptides had already been detected in some VCRs (Ryan et al., 1981; Ball et al., 1988). Results presented in Chapters 4 and 5 indicate that opioids probably do not control song production; opioid receptor densities do not differ between singing and non-singing adult males (Chapter 4) or between singing adult males and non-singing adult females (Chapter 5). This finding has shifted our focus to other potential opioid actions in VCRs. The results described in Chapter 5 indicate that opioid roles may vary with region and age. Developmental changes in opioid receptor densities occurred in area X, RA and ICo. Changes in area X, in particular, are intriguing because of that region's role in song learning and previous studies on other species that implicate an opioid role in learning (Csillag et al., 1993; Janak et al., 1994; Columbo et al., 1997). Differences in opioid receptor densities were not apparent in many regions that

contain auditory neurons. Future research on opioid roles in VCRs should focus on song learning and auditory processing. For instance, one could ask whether central application of opioid antagonists during adolescence would prevent or disrupt normal plastic song, or whether opioid antagonists would alter auditory response properties of VCR neurons, making them more or less selective to the bird's own song.

The Future of the Field

Briefly skimming through the latest book of abstracts for the 1997 Society for Neuroscience Annual Meeting (Volume 23), it is immediately apparent that this field is expanding and, twenty years after the initial studies describing the neural pathways for avian vocal control, continues to capture the interest of researchers from many backgrounds and perspectives. Fields of study include sexual differentiation, coordination of the motor pathway and respiration, induction of immediate early gene expression, neurogenesis, and similarities between vocal learning in songbirds and humans. Roles of sex steroids continue to be a focus of many researchers, particularly those interested in sexual differentiation, but more and more researchers are investigating actions of non-steroidal factors in the vocal control system. The ever-expanding use of molecular techniques makes those studies more powerful and popular. For example, recent results include findings of immediate early genes (Fos and ZENK) and mRNA for brain-derived neurotrophic factor being expressed during motor, but not sensory, activities in certain VCRs (Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997; Li et al., 1997). Surely, future studies will attempt to determine the function of these substances in the vocal control system.

The Canary and the Zebra Finch (*Poephila guttata*) remain useful models for studying the vocal control system, but more and more researchers are studying a variety of wild songbirds to enhance the comparative perspective in the field. This year's Society for Neuroscience meeting will include papers on White-crowned Sparrows (*Zonotrichia leucrophrys*; Hough and Volman, 1997; Tramontin et al., 1997; Soma et al., 1997), European Starlings (*Sturnus vulgaris*; Chaiken and Cheng, 1997; Casto and Ball, 1997), Cardinals (*Cardinalis cardinalis*; Goller and Suthers, 1997; Suthers et al., 1997), Carolina Wrens (*Thryothorus ludovicianus*; Nealen and Perkel, 1997), and of course, Dark-eyed Juncos

(Gulledge and Deviche, 1997). These represent a minority of the presentations on the vocal control system, but reflect a growing interest in understanding neural control of singing in natural populations. I believe this trend will enhance our ability to relate neurological idiosyncracies to the specific behavioral patterns and unique natural histories of different songbirds. This approach offers a degree of insight into how neurophysiology controls behavior that would be unavailable if we limited our studies to two domesticated models.

Ultimately, one goal for many researchers is to replicate controlled neurogenesis in humans suffering from brain disease, trauma, or aging. Those researchers tend to focus on avian neurogenesis and its hormonal control. Many others in the field work toward understanding how changes in neurons and groups of neurons induce changes in behavior — how the brain controls behavior. The avian vocal control system remains an excellent model for this pursuit.

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