SEXUAL DIFFERENTIATION OF BRAIN AND BEHAVIOR IN THE ZEBRA FINCH (*Poephila guttata*): A CELLULAR ANALYSIS

Thesis by

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Abstract

Male zebra finches sing a brief song phrase to the female during courtship. Castration of an adult male reduces the bird's frequency of singing; testosterone replacement reinstates the behavior. Testosterone treatment of female zebra finches does not activate song nor induce other elements of courtship behavior.

Correlative changes of brain and behavior in zebra finches are organized by sex hormones during development. Newly hatched zebra finch chicks were subcutaneously implanted with Silastic pellets containing either 50 μg of dihydrotestosterone or 50 μg of 17β-estradiol. Testosterone treatment activates song in adult females which were implanted with estradiol when chicks, but fails to activate song in those females which had received dihydrotestosterone. The singing females approach a sexual partner with pivoting movements, straighten to an erect posture, fluff their throat feathers, and rapidly repeat their song phrase in a behavioral sequence which closely resembles that of the male.

In zebra finches, brain nuclei of the efferent pathway for control of song show dramatic sex differences in their volume (Nottebohm and Arnold; Science 194 [1976] 211-213). 17β-estradiol treatment of genetically female chicks organizes male-like cytoarchitectonic differentiation of the telencephalic song nuclei RA, HVc, MAN, and X. Dihydrotestosterone induces masculinization of the brain stem song nuclei nXII and DM. Dendritic field spread, soma size, and the consequent volume of the brain nucleus is larger in males than females at all levels of the song system. The exposure of female chicks to either androgen or estrogen supports growth of the hormone's respective target neurons in either the brain stem or telencephalic song nuclei. These neurons reach a size identical with that of the equivalent cell type in a normal male. Anatomical comparison of normal adult male and female song systems reveals that all cell types and all identified connections are present in both sexes. Thus, the specificiation of cellular identity—i.e., position, dendritic morphology and

efferent synaptic projection—is expressed independently of the hormonal environment. Rather than selecting pathways of anatomical differentiation, androgens and estrogens exert a similar pleiotrophic effect on their respective target neurons. Although in the female song system we identify all the cell types and connections of the male, testosterone does not activate song. Thus, 17 β-estradiol may also exert a specific inductive effect on the telencephalic song neurons which renders them physiologically competent to respond to testosterone in the adult.

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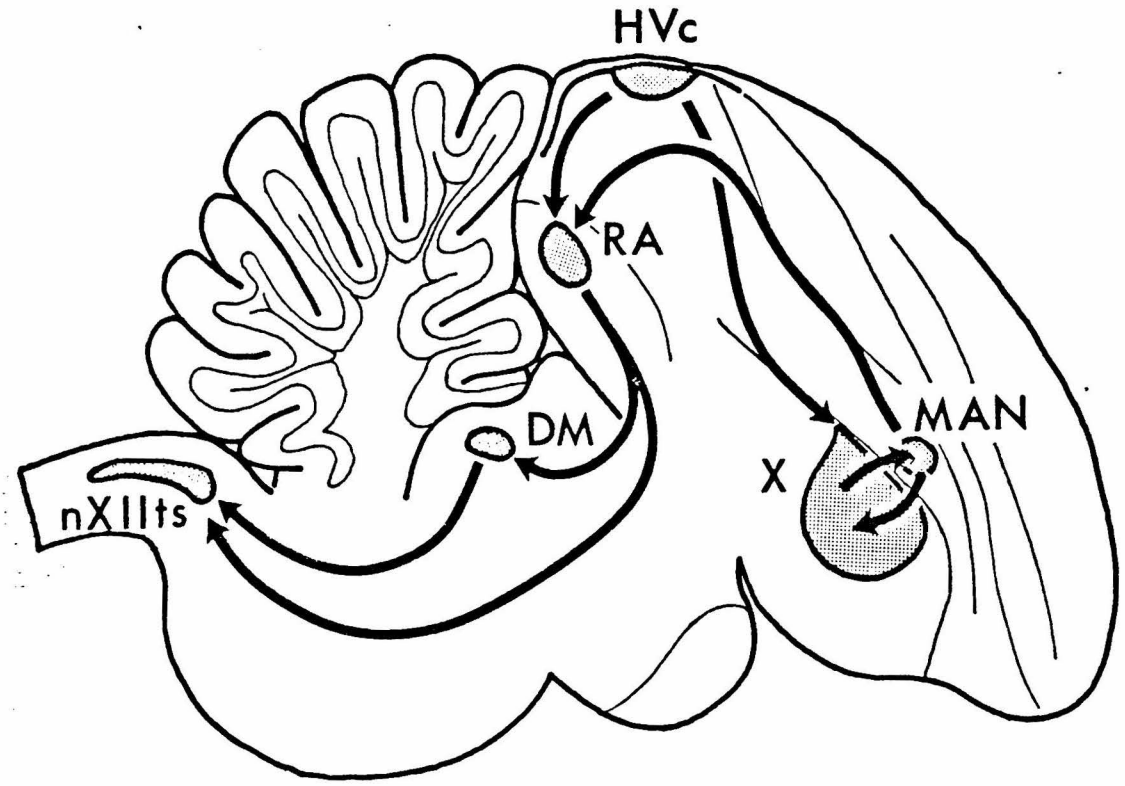


Figure 1. Schematic representation of the pattern of synaptic connectivity within the song system of the zebra finch. Lesion of HVc or RA, or section of the tracheosyringeal branch of the hypoglossal nerve (which innervates the syrinx) disrupts song (Nottebohm et al., 1976).

Biochemical Abbreviations

DHT	5α-dihydrotestosterone (5α-androstan-17 β-ol-3-one)
E_2	17 β-estradiol (1,3,5[10]-estratriene-3,17 β-diol)
T	testosterone (4-androsten-17 β-ol-3-one)

Neuroanatomical Abbreviations

AN	Anterior neostriatal nucleus
Cb	Cerebellum
CO	Chiasma opticum
CoA	Commissura anterior
DA	Tractus archistriatalis dorsalis
DLA	Nucleus dorsolateralis anterior thalami
DM	Dorsomedial intercollicular nucleus
DMP	Nucleus dorsomedialis posterior thalami
E	Ectostriatum
FRL	Formatio reticularis lateralis mesencephali
HV	Hyperstriatum ventrale
HVc	Hyperstriatum ventrale, pars caudale
ICo	Nucleus intercollicularis
LFM	Lamina frontalis suprema
LH	Lamina hyperstriatica
LMD	Lamina medullaris dorsalis
LPO	Lobus parolfactorius
MAN	Magnocellular nucleus of the anterior neostriatum
MLd	Nucleus mesencephalicus, pars dorsalis
NA	Neostriatum anterior
NC	Neostriatum caudale
NF	Neostriatum frontale
NI	Neostriatum intermedium
nXIIts	Nucleus nervi hypoglossi, pars tracheosynringealis
OI	Nucleus olivaris inferior
OM	Tractus occipitomesencephalicus
OV	Nucleus ovoidalis
PA	Paleostriatum augmentatum
PP	Paleostriatum primitivum
PTN	Nucleus pretectalis
RA	Nucleus robustus archistriatalis
Rt	Nucleus rotundus
SpM	Nucleus spiriformis medialis
TO	Tractus opticus
V	Ventricle
X	Area X

SECTION I

Introduction: The Concept of Brain Sexual Differentiation

One of the basic dichotomies of human experience finds expression in the biological division of male and female. Along with the physical attributes to each sex, we also tend to expect different patterns of behavior and personality. Human acquisition of gender identity and assumption of gender role depends in part upon chromosomal specification of gonadal sex (Wachtel, 1979), on the consequent secretion of the appropriate gonadal hormone (Imperato-McGinley et al., 1974), and on the subsequent expression of biological sex in a permissive social environment during childhood and adolescence (Money, 1978).

Concepts of human brain sexual differentiation arise from work with animal models which began with the studies of Pfeiffer in 1936. At that time, Moore and Price (1932) had developed the idea that estrous cycling in rats was of endocrine origin and depended upon a reciprocal interaction between an autonomous pituitary and ovarian hormones. Sex differences in pituitary function were implied by Goodman's (1934) finding that an ovary which had been transplanted into the eye chamber of a mature, castrated male rat did not show estrous cycling. Pfeiffer (1936) discovered that such ovarian grafts did cycle if the recipient male had been castrated immediately after his birth. Conversely, if female littermates received testicular grafts as neonates, then they became anovulatory when mature. Similar testicular grafts into adult females did not induce permanent anovulatory sterility. Based on these experiments, Pfeiffer suggested that the sexuality of the pituitary was not genetically determined, but rather bipotential and undifferentiated at birth. Through the action of the testes at birth, a masculine pattern of secretory activity could be imprinted upon the pituitary.

Testosterone was identified as a product of the testes by David et al. in 1935, and Bradbury and Gaensbauer (1939) soon showed that neonatal exposure of female rats to testosterone also induced anovulatory sterility. Harris and Jacobson (1952) later showed that the pituitary was not sexually differentiated since grafts of

pituitary tissue from male donors were as effective at reinstating ovarian function in hypophysectomized recipient females as pituitary grafts from female donors. Largely through the work of Geoffrey Harris, brain control of pituitary function was established, which thus implicated the central nervous system as a site of sexual differentiation. Androgen-dependent sexual differentiation of copulatory behavior was demonstrated soon afterwards (Phoenix et al., 1959; Harris and Levine, 1965).

Extracts of mammalian brain tissue aromatize testosterone (T) to 17β-estradiol (E_2) in vitro (Naftolin et al., 1975) and E_2 also appears as a brain metabolite of T in vivo (Lieberburg and McEwen, 1975). Sexual differentiation of gonadotropin secretion or lordosis behavior in the rat may be described as androgen-dependent (i.e., dependent on androgen secretion by the testes), but appears to be estrogen-mediated (i.e., by intracellular conversion of T to E_2, and subsequent binding of E_2 to its intracellular receptor protein [McEwen et al., 1977]). On the other hand, male copulatory behavior in rats appears to be influenced by exposure to androgen in utero, but this hormonal effect seems to be mediated by androgen (as T or DHT) rather than by E_2 (Clemens et al., 1978).

The view of brain sexual differentiation propounded by many workers in this field based on evidence from the rat (Goy and McEwen, 1980) is the following:

(1) In the absence of interaction with gonadal hormones within a limited sensitive period during their ontogeny, the target neurons of hormonal action follow an internal program which specifies a female pattern of sexual differentiation.

(2) Interaction with gonadal hormones during the sensitive period reprograms neuronal development to produce a masculine pattern of sexual differentiation.

At issue is whether gonadal hormones "organize" the anatomical differentiation of neuronal substrates for sexually dimorphic brain functions, or act as "chemical sensitizers" and program the responsiveness of these neural circuits to gonadal hormones in the adult.

Phoenix et al. (1959) originated the "organizational hypothesis" concerning "the parallel nature of the relationship, on the one hand, between androgens and the differentiation of the genital tracts, and on the other, between androgens and the organization of the neural tissues destined to mediate mating behavior in the adult (guinea pig)" (p. 369). If gonadal hormones do program the anatomical differentiation of neural circuits which underlie the pattern of brain function unique to each sex, then qualitative sex differences in behavior or physiology should result. This is the case with respect to the control of gonadotropin secretion. The hypothalamo-pituitary system of ovariectomized female rats responds to sequential injections of E_2 with a surge in the release of gonadotropin while the male's does not (Kalra et al., 1971) and perhaps this reflects an absence from the male's brain of the neural circuit which mediates the positive feedback of E_2.

The alternative view of brain sexual differentiation has been articulated by Beach (1971), "The sex hormones are best regarded not as stimuli or as organizing agents but as chemical sensitizers which alter the stimulability of critical mechanisms within the central nervous system" (p. 253). In support of this view is work describing the hormonal determinants of lordosis behavior in rats. Castrate males fail to show lordosis responding when primed with low doses of E_2 and then progesterone which would have been sufficient to induce high levels of lordosis in ovariectomized females. However, chronic exposure to higher doses of E_2 will bring lordosis responding in castrate males up to the level of estrous females (Davidson, 1969). Since males have the potential to show lordosis when primed with E_2, the neural circuits which mediate lordosis must therefore be intact. Since castrate males are quantitatively less sensitive to E_2 than ovariectomized females, this suggests that defeminization of lordosis behavior in female rats by neonatal exposure to T may raise the threshold of E_2 which is required to activate lordosis responding.

Brain sites of sexual differentiation in mammals have been implicated indirectly by brain lesion, brain stimulation, steroid antoradiography, and local hormone implants in either neonatal or adult rats (summarized in Goy and McEwen, 1980).

Such studies suggested a role for various regions of the preoptic area or hypothalamus in the control of sexually dimorphic brain functions but have not revealed precise descriptions of the neural circuits which underlie these functions. Sex differences in mammalian brain structure have also been documented (Raisman and Field, 1973; Greenough et al., 1977; Gorski et al., 1978) yet their link to sex differences in behavior or physiology is again unclear. A coherent understanding of specific hormone target sites within the developing mammalian brain, and the cellular or functional consequences of their sexual differentiation await characterization of the neural circuits which underlie sexually dimorphic brain functions.

SECTION II

Hormonal Determination of Neuronal Number and Dendritic Architecture as a Consequence of Sexual Differentiation in the Zebra Finch Song System

Neuronal differentiation may be viewed as a complex chain of causally-related events, determined in part by the expression of the neuron's internal program for development, and interdependent with a complex array of cell-cell interactions and soluble, extracellular signals. Relatively little is known concerning which aspects of the neuron's differentiated state are encoded by its internal program. Identification of such intrinsic elements depends upon demonstration of their autonomous expression in the absence of what might be considered "normal" environmental determinants. Thus, the expression of their characteristic dendritic field architecture by previously committed neurons when transferred to culture in vitro (Banker and Cowan, 1979; Messer, 1977; and Solomon, 1979) or the elaboration of dendritic spines in the absence of afferent input (Sotelo, 1973; Cullen and Kaiserman-Abramof, 1976) implies that these two aspects of a neuron's differentiated state may be determined by the neuron's internal program for development.

Sympathetic and parasympathetic neurons have been particularly amenable to culture in vitro and this has allowed considerable progress to be made in identifying soluble factors which influence aspects of their differentiation. Factors permissive for the maintenance of sympathetic (Levi-Montalcini and Angeletti, 1968) or parasympathetic neurons (Manthorpe et al., 1980) in culture have been identified, as have factors which seem to play an instructive role in neuronal differentiation. Nerve growth factor influences the elaboration of processes (Green and Tischler, 1976) and may guide their growth as well (Campenot, 1977; Letourneau, 1978; Menesini-Chen et al., 1978). Other soluble factors seem to play a role in the commitment of neurons to specific patterns of neurotransmitter metabolism (Patterson and Chun, 1977; Mudge, 1979).

Less is known about the cellular consequences of factors which act centrally in the nervous system. There exists a substantial body of literature which describes the enduring behavioral or physiological consequences of gonadal hormone action

during development (cf. Goy and McEwen, 1980) and such effects are thought to be the outward sign of a role for gonadal hormones in the patterning of neuronal differentiation within the circuits which underlie sexually dimorphic brain functions. Enduring sex differences in the functioning of the hypothalamic-pituitary axis, the expression of sexual behavior, and the pattern of aggressive, feeding, and parental behaviors are all influenced by androgens or estrogens during development. Despite the wide diversity in the behavioral expression of brain sexual differentiation, an underlying hope remains that these hormonal effects have a common cellular basis which would be grounded in the cell biology of hormone action, that is, that simple effects on neuronal development translate into the complex variety of behavioral or physiological consequences of hormone action which is observed.

In this thesis I intend to show that the rules for hormone action during neuronal differentiation in the zebra finch song system are simple ones. For the neurons within the nucleus robustus archistriatalis (RA), the pattern of their efferent projection, the architecture of their dendritic field, and their dendritic morphology are all determined independently of the hormonal environment. Estrogen (17β-estradiol [E_2]) appears to interact with the developmental program which specifies a neuron's anatomical identity in two ways: first, estrogen may determine its number of dendritic arms but not their pattern of branching and second, it may program the responsiveness of a cell to androgen in the adult. Androgen (5α-dihydrotestosterone [DHT]) influences neuronal number. This view of hormone action contrasts with the view that hormones "organize" the anatomical differentiation of neural circuits when stated in its most extreme forms, i.e., that gonadal hormones specify qualitative patterns of efferent projection or acceptance of afferents.

The zebra finch (Poephila guttata) provides a unique model in which to study the cellular consequences of sexual differentiation as they relate to behavior. In this species, only the adult male sings, the female does not. The male's song is androgen

(T or DHT)-dependent, but the female is behaviorally insensitive to the hormone. Exposure of adult females to androgen (T or DHT) is unable to induce song. The distinct advantages of this preparation are that the efferent motor pathway for song has been characterized (Nottebohm, Stokes and Leonard, 1976), and that the sex difference in behavioral capacity for song is the outward sign of profound sex differences within the brain, at all levels of this pathway (Nottebohm and Arnold, 1976).

The song system includes two telencephalic nuclei, the nucleus hyperstriatum ventrale, pars caudale (HVc) and the nucleus robustus archistriatalis (RA) (see Figure 1). Lesion of either nucleus produces a deficit in production of song (Nottebohm, Stokes and Leonard, 1976). HVc is afferent to RA, and RA has two efferent projections. One is onto the dorso-medial intercollicular nucleus (DM) and the other is onto the tracheosyringeal portion of the hypoglossal nucleus (nXIIts). DM also projects onto nXIIts. Finally, the motor neurons of nXIIts innervate the avian vocal organ, the syrinx.

The volume of HVc, RA, and nXIIts has previously been shown to be larger in males than in females (Nottebohm and Arnold, 1976). Both E_2 and DHT, when administered to female chicks at hatching, will be shown to influence the volume of song system nuclei, and the action of E_2 during development is correlated with the induction of behavioral sensitivity to androgen in females when adult.

This section of the thesis is presented in three parts. The first part compares the pattern of synaptic connectivity between song system nuclei in males and females. The second part describes an analysis of Golgi-impregnated material which questions whether or not one particular song nucleus, RA, contains the same neuronal constituents in both sexes. These studies were undertaken to provide normative data against which to evaluate the influence of gonadal hormones on sexual differentiation within the song system. The third part of this section describes the

cellular consequences of DHT- and E_2-mediated processes of sexual differentiation within RA. Exposure of females to E_2 during development induces functional differentiation of the capacity for song, and the quality of song produced by such females will be described in the final section of the thesis.

PART 1: RA Has the Same Pattern of Anatomical Connectivity in Both Sexes

Methods

Standard histological protocol

Adult zebra finches were deeply anesthetized with 0.05 cc of Equithesin, sacrificed by intracardiac perfusion with 0.13 M sodium phosphate buffered at pH 7.2, and then fixed with 4% formaldehyde in the same buffer. The brain was immedately removed from the skull and subsequently post-fixed for several days in the same solution at 4°C. The brain was then split sagitally and the right half was infiltrated with 30% sucrose (w/v) in 4% formaldehyde overnight. On the following day the brain was cut at 30 μ in the sagittal plane by frozen sectioning. Every third section was collected in 0.13 M sodium phosphate buffered at pH 7.2, mounted on Gatenby-subbed slides, and subsequently stained with 1% cresyl-violet. Shrinkage was within $\pm$1% with this protocol.

The perimeter of RA and of the other song system nuclei was defined by neuroanatomical tracing techniques as described in the **Results** section. To measure volume of a nucleus, it was located on the section, its perimeter traced with the aid of a microscopic drawing tube at a final magnification of 100X, and its cross-sectional area on each section was then determined. The volume of the nucleus was calculated from these measurements of cross-sectional area and the interval (90 μ) of sampling.

To count the number of neurons in RA, the density of neurons in the nucleus was first determined and from this measure, together with the volume of the nucleus, the number of neurons was calculated. The morphological criteria for distinguishing neurons from macroglia were established as described in the **Results** section. The count of neuron number within RA was subject to three sources of error. These were: misclassification errors in which neurons were counted as macroglia or vice-versa, counting errors in which neurons were missed or counted twice, and split-cell errors in which the unit counted may be split by the plane of section, and thus counted twice due to its appearance in two adjacent sections. In this study, nucleoli were chosen as the unit of count in 30 μ-thick frozen sections. No correction of the raw count was made for split nucleoli. Several authors have argued that nucleoli are displaced rather than cut when paraffin-embedded sections are taken (Cammermeyer, 1967) and since the quality of histological preservation is even poorer in frozen sections, split nucleoli would not seem to be a significant source of error. To calculate total neuron number, accurate measurement of RA's volume was essential. For this reason, relatively thick, frozen sections were used in preference to thin, paraffin sections as the cytoarchitectonic boundaries of the female's RA became difficult to accurately distinguish as section thickness decreased. The density of neurons in RA was estimated by repetitive measurement of neuron number within a small volume ($9.5 \times 10^3\ \mu^3$ or $57.4 \times 10^3\ \mu^3$). These volumes were large enough to insure that the measurements of neuron number were normally distributed. At least 100 measurements of neuron density were made on several sections. This sample size insured that measurement of the mean neuron density was accurate within 10% at a 95% confidence level (based on the sample variance).

To measure the somal diameter of RA neurons, the perimeter of their soma was traced with the aid of a drawing tube at a final magnification of x1000 (using a x100 oil immersion objective). As the soma of RA neurons varied from round to

ovoid, somal diameter was defined as their maximum diameter along their longest axis. Tests for kurtosis and skewness revealed that measurements of maximum somal diameter for a population of RA neurons were normally distributed. A sample of approximately 100 neurons was measured in each finch which insured that the estimate of mean somal diameter was accurate within 10% at a 95% confidence level (based on the sample variance).

^{3}H amino acid tracing of neural projections

50 μCi of [4,5,-^{3}H] leucine (48 Ci/mmol, New England Nuclear) were evaporated to dryness and then dissolved in 2 μl of 0.9% NaCl. The tip of an oil-filled, 1 μl Hamilton syringe fitted with a glass micropipette (tip diameter of 20 μ) was then lowered into this solution and 40 nanoliters drawn up (0.5-1 μCi). This volume of solution was then injected stereotoxically into the brain of a zebra finch which had been anesthetized with 0.035-0.040 cc Equithesin (i.m.). After a survival time of from 3-5 days, the zebra finch was sacrificed and perfused with 4% buffered formaldehyde and then sections were cut and mounted as described above. 30 μ-thick frozen sections were then defatted in xylene, dipped in NTB2 (diluted 1:1 with water, 45°C) and exposed in the dark at 4°C for three weeks. The slides were then developed in Kodak D-19 at 15-17°C for 2-1/2 minutes, fixed in Kodak Rapid fixer at 15-17°C for three minutes, washed, and then stained with thionin (Cowan et al., 1972).

HRP protocol

Injections of horseradish peroxidase (Boehringer-Mannheim grad I), dissolved in 40 nanoliters of 0.5% KCl were stereotoxically placed into the brain of a zebra finch using an oil-filled, 1 μl Hamilton syringe fitted with a glass micropipette. After a survival time of 12-16 hours, the zebra finch was deeply anesthetized with 0.05 cc Equithesin, and then perfused intracardially with the following solutions: 1) 20 ml of 0.13 M sodium phosphate buffered at pH 7.2; 2) 1% paraformaldehyde, 2.5%

glutaraldehyde in the same buffer for a total volume of 100 ml over 30 minutes; and then 3) 10% sucrose (w/v) in the same buffer for a total volume of 30 ml. The brain was then immediately removed from the skull, and cut at 30 microns by frozen sectioning. Every third section was collected in 0.13 M sodium phosphate buffer, pH 7.2, chilled to 4°C. The sections were then reacted with benzidine di-HCl in the following protocol, adapted from Lynch et al. (1976).

Solution A, 25 minutes at room temperature

0.2 M Na Acetate, pH 5	10 ml
100% ETOH	60 ml
$NaFe(CN_2)NO \cdot 2H_2O$	0.2 g
Benzedine di-HCl	0.1 g
Dimethyl sulfoxide	1 ml
H_2O	130 ml
30% H_2O_2	80 µl

Solution B, 20 minutes at below 0°C

0.2 M Acetate, pH 5	10 ml
$NaFe(CN_3)NO \cdot 2H_2O$	18 g
H_2O	90 ml
100% ETOH	100ml

The sections were then rinsed five times in 0.1 M sodium phosphate, mounted, and finally stained with 1% neutral red before coverslipping.

Results

The cytoarchitecture of RA

The primary goal of this study was to pursue the sexual dimorphism of RA to a cellular level of analysis, with the hope that this would provide the basis for defining the cellular consequences of androgen- or estrogen-mediated sexual differentiation. This study focused on RA in preference to the other telencephalic nuclei of the song system because its cytoarchitectonic boundary was clearly

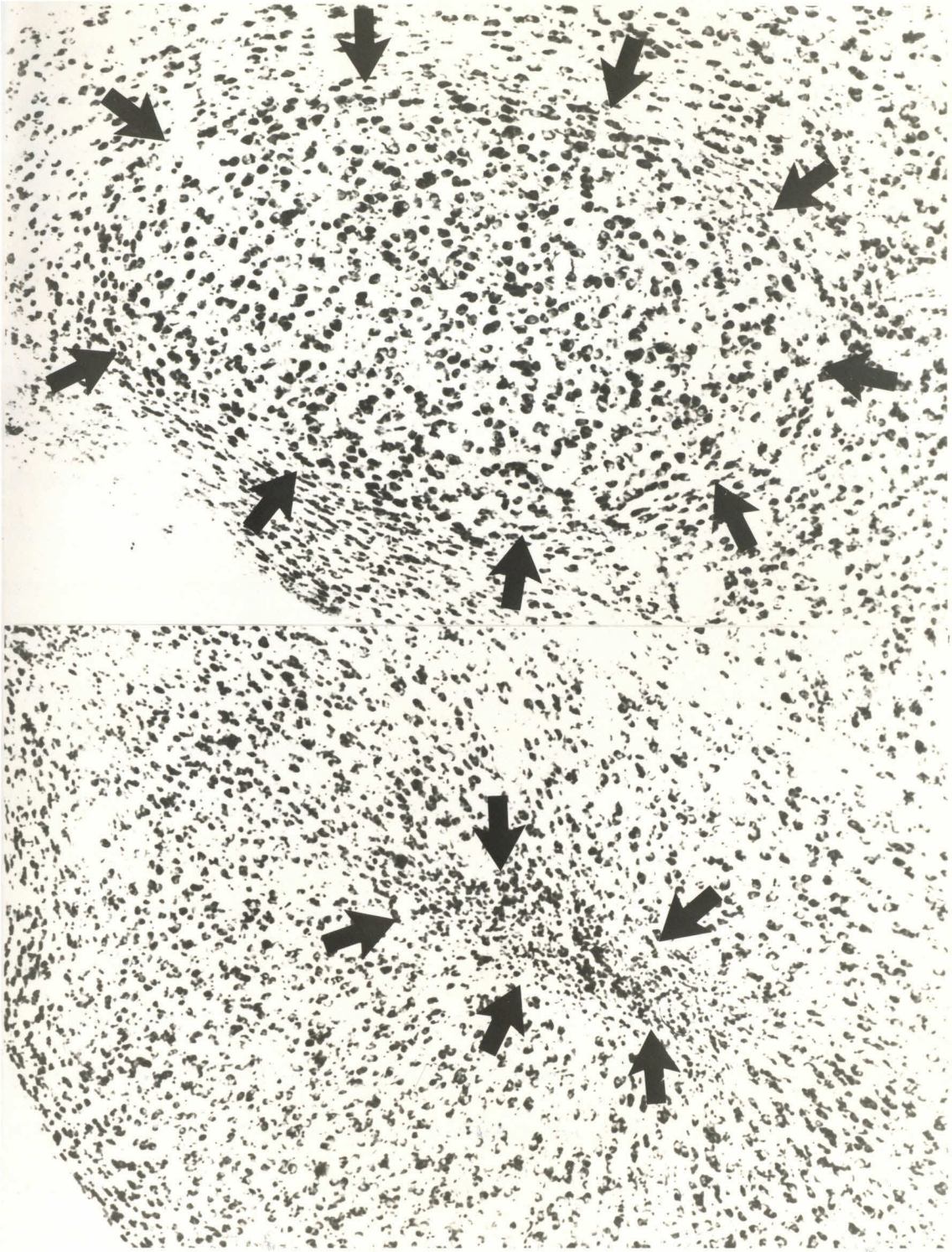
demarcated in both sexes. This was necessary for the accurate determination of neuronal number within the nucleus and to define the neuronal constituents of RA in Golgi material. The sexual dimorphism of the zebra finch song system was not at all subtle, quite the opposite, the largest difficulty in these studies has been to recognize song system nuclei in the female zebra finch brain despite their completely different cytoarchitectonic appearance.

In males, RA was a relatively large, ovoid-shaped nucleus in sagittal section (1 mm in length along its major axis, and 0.75 mm along its minor axis) which filled the dorso-medial quadrant of the archistriatum (Figure 2). Its cytoarchitecture was characterized by large cells whose Nissl substance tended to stain more intensely than that of cells in surrounding archistriatal areas. The cytoarchitecture of RA in the female was radically different. Instead of a robust, large-celled area, a thin streak (0.6 mm along its major axis and 0.15 mm along its minor axis) of densely-packed, small-bodied cells occupied an homologous position in the dorso-medial archistriatum. In view of the striking sexual dimorphism in the cytoarchitecture of RA, the question arose as to whether or not the nucleus had the same pattern of synaptic connectivity in both sexes.

RA: description of afferents

By using the Fink-Heimer technique to differentially stain degenerating fibers as a result of brain lesion, Nottebohm, Stokes and Leonard (1976) described a projection from HVc to RA. They also reported that after complete or partial lesions of area X in the frontal telencephalon, degenerating fibers could be traced dorsally into the lamina hyperstriatica where they traveled to the ventral lip of HVc, "turn ventrally and cascade down into the archistriatum entering the nucleus robustus from its dorso-lateral aspect" (p. 467). They failed to find preterminal or terminal degeneration within RA however, and so were uncertain whether that nucleus was the target of the degenerating pathway.

Figure 2. Cytoarchitecture of RA in a normal male (top) and normal female (bottom): sagittal plane, anterior to the right, 30 μ-thick frozen sections stained with cresyl-violet. Note that the cytoarchitecture of RA in the male is characterized by large, well-separated cells, while in the female RA is a thin streak of densely-packed, small-bodied cells.

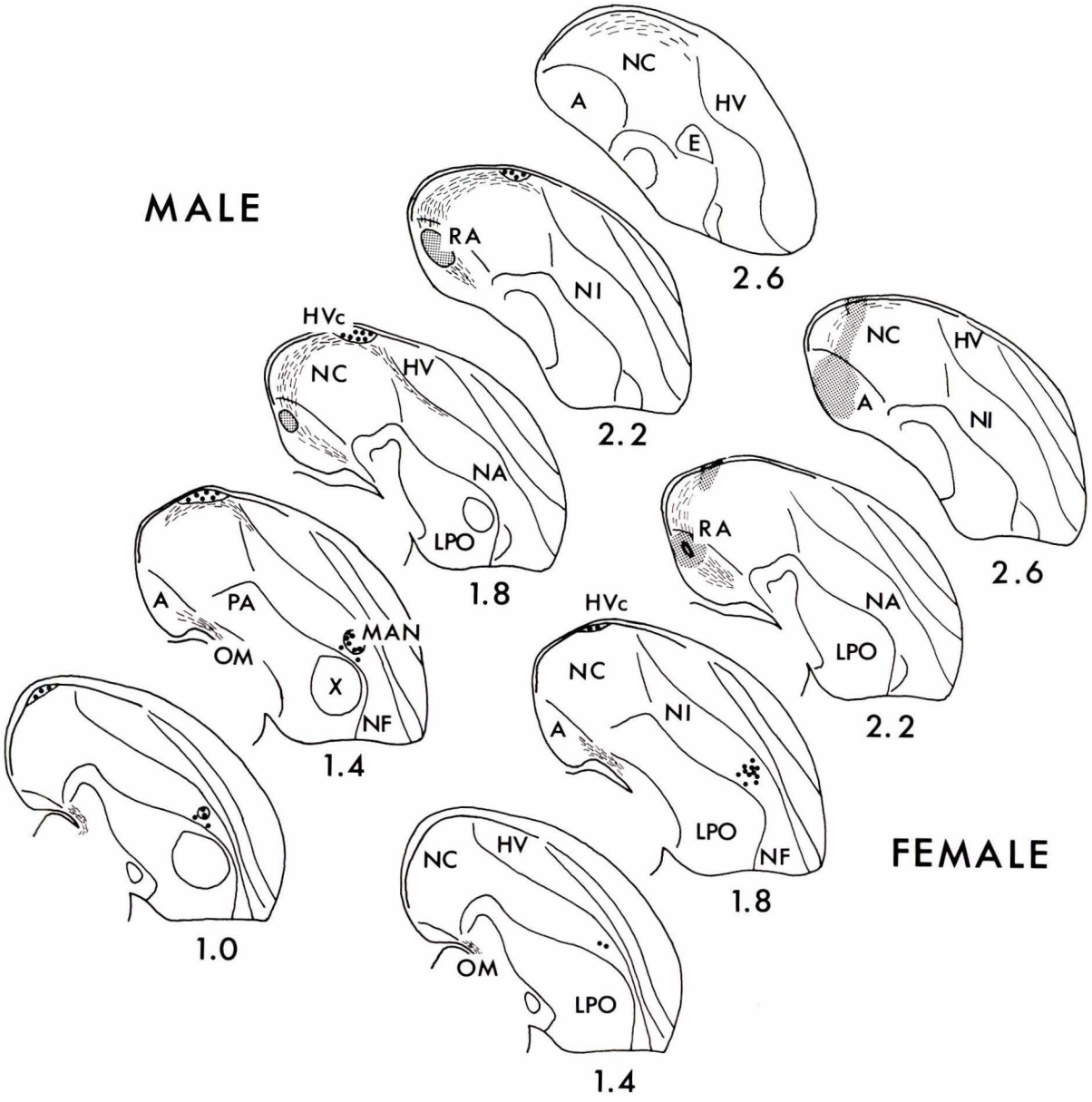


To define the brain nuclei afferent to RA in the zebra finch song system, stereotaxic injections of HRP which were centered on RA were placed in the brains of two males and three females. A lateral approach which hit RA on a slant 30° from vertical was utilized so as not to disrupt HVc, nor damage non-song system axons running in the tractus archistriatalis dorsalis (DA). Retrograde transport of HRP after injection of the enzyme into RA revealed two afferent telencephalic nuclei: HVc, as previously described, and MAN, a nucleus within the anterior neostriatum (Figure 3). This pattern of afferent projection was identical in both sexes.

In males, HVc is a large-celled nucleus which sits atop the lamina hyperstriatica and bulges up into the floor of the lateral ventricle (Figure 4). HRP-labeled cells were scattered throughout its extent, and were a distinct subset of the neuronal population within RA. They were distinguishable on the basis of their cuboid somal morphology and small diameter which averaged approximately 6-7 μ. HVc also contains at least two other types, of neurons which label with HRP after injection of the enzyme into area X. Both of these X-projecting cell types have ovoid somas and their maximum diameter averages between 10-12 μ for one and 14-17 μ for the other (data not shown). In the female, HVc is a thin crescent of small-bodied cells which is sandwiched between the floor of the lateral ventricle and the lamina hyperstriatica. After injection of a female's RA with HRP, retrogradely-labeled cells were confined within the cytoarchitectonic borders of HVc, and again, labeled cells were scattered throughout the nucleus.

MAN was first described by Arnold et al. (1976) due to the fact that it contains cells which concentrate ^{3}H-testosterone or its metabolites. Its cytoarchitecture in the male is characterized by a large round-bodied cell which averages 15 μ in diameter. These cells cluster in a compact sphere whose dorsal surface rests against the lamina hyperstriatica. Such a large-celled area was completely absent from the female's telencephalon (Figure 4). In the male, MAN occupied a medial

Figure 3. Charting of the distribution of HRP-labeled cells (indicated by black dots) after injection of the enzyme into RA. The HRP-filled micropipette was introduced at a slant 30° from vertical, so as to avoid penetrating either DA or HVc. In both sexes, scattered labeled cells were observed throughout the extent of HVc. In the male, labeled cells were also clustered within MAN. This nucleus is not recognizable in female Nissl-stained material, however, a cluster of HRP-labeled cells was observed in an homologous position within the female's anterior neostriatum (NA).


MALE
NC
A
HV
E
2.6
RA
NI
HVc
NC
HV
NA
LPO
2.2
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PA
MAN
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NF
1.4
1.0
NC
HV
A
NI
HVc
NC
HV
A
NI
2.6
RA
NA
LPO
2.2
LPO
NF
1.8
NC
HV
OM
LPO
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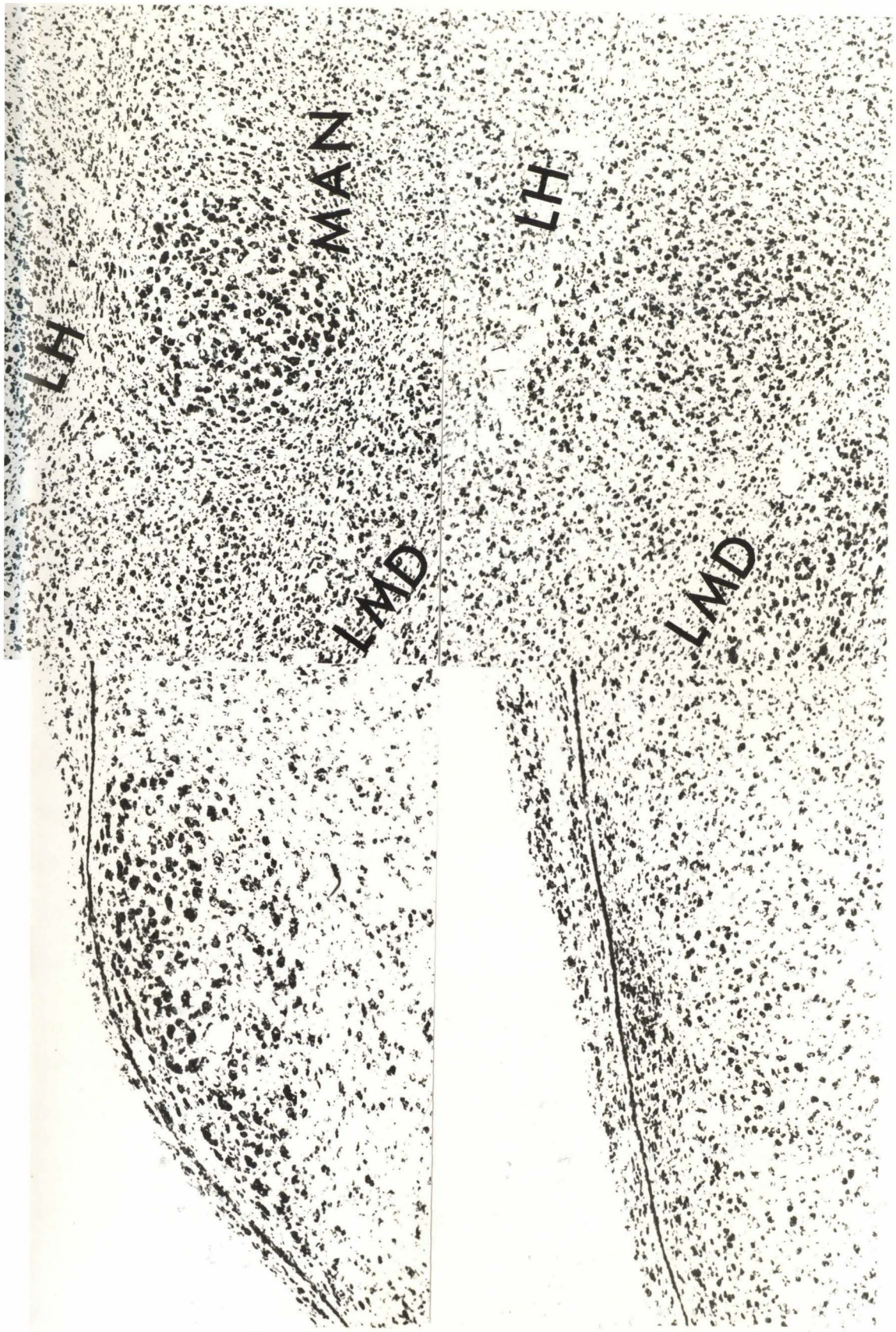
FEMALE

Figure 4. Cytoarchitecture of HVc (left) and MAN (right) in a normal male (top) and a normal female (bottom): sagittal plane, anterior to the right, cresyl-violet stain.


LH
LMD
LH
LMD

position within a dense fiber plexus which filled the anterior lip of the neostriatum. This myeloarchitectonically distinct area within the frontal telencephalon will be designated as the anterior neostriatal nucleus, abbreviated AN. AN was identifiable in both sexes of zebra finch and was sexually dimorphic in volume. In five males, the volume of AN averaged 0.643 mm^3 (range: 0.582-0.685), while in seven females its volume averaged 0.573 mm^3 (0.495-0.677). This difference was statistically significant (Mann-Whitney U-test, $p = 0.03$). For comparison, the volume of the male's MAN averaged 0.126 mm^3 (0.115-0.143). After injection of RA in males with HRP, the majority of labeled cells clustered within MAN, while a small number of labeled cells were also scattered dorsal and medial to MAN throughout the rest of the fiber plexus which defines AN (Figure 5). The distribution of labeled cells in the female after injection of RA with HRP was similar. The majority of labeled cells were again clustered in a compact ball which occupied a location within the fiber plexus of AN homologous to that of MAN in the male. In addition, scattered labeled cells were also observed throughout the rest of AN. In males, HRP labeled the large cell characteristic of MAN (this cell type also appears to be one which concentrates ^{3}H-testosterone, cf. Arnold et al., 1976, plate 1, c). In females, HRP labeled a cell whose soma was only 10 μ in diameter (vs. 15 μ in the male) which was equivalent to the somal diameter of cells in surrounding neostriatal areas. This made it difficult to define the borders of MAN in female Nissl-stained material.

Injection of MAN or HVc with ^{3}H-leucine provided a means of precisely defining the cytoarchitectonic borders of RA based on its pattern of connectivity with its afferent nuclei. Stereotaxic injections of ^{3}H-leucine were placed in either HVc or MAN in a pair of males and also in a pair of females. The terminal fields of HVc and MAN completely filled RA in either sex. In the male, the border between RA and surrounding archistriatal tissue was delimited by a thin, acellular zone at whose inner face the density of silver grains fell abruptly (Figure 6). In females,

Figure 5. HRP-labeled cells in the anterior neostriatum after retrograde transport of the enzyme from RA, male (top), female (bottom). Note that the majority of labeled cells cluster in a compact ball whose dorsal surface rests against the lamina hyperstriatica (LH). In the male, this area corresponds to MAN, while in the female this area is not cytoarchitectonically different from adjacent neostriatal tissue. Scattered labeled cells are also observed within the anterior neostriatal nucleus (AN) ventral to MAN, in both sexes.

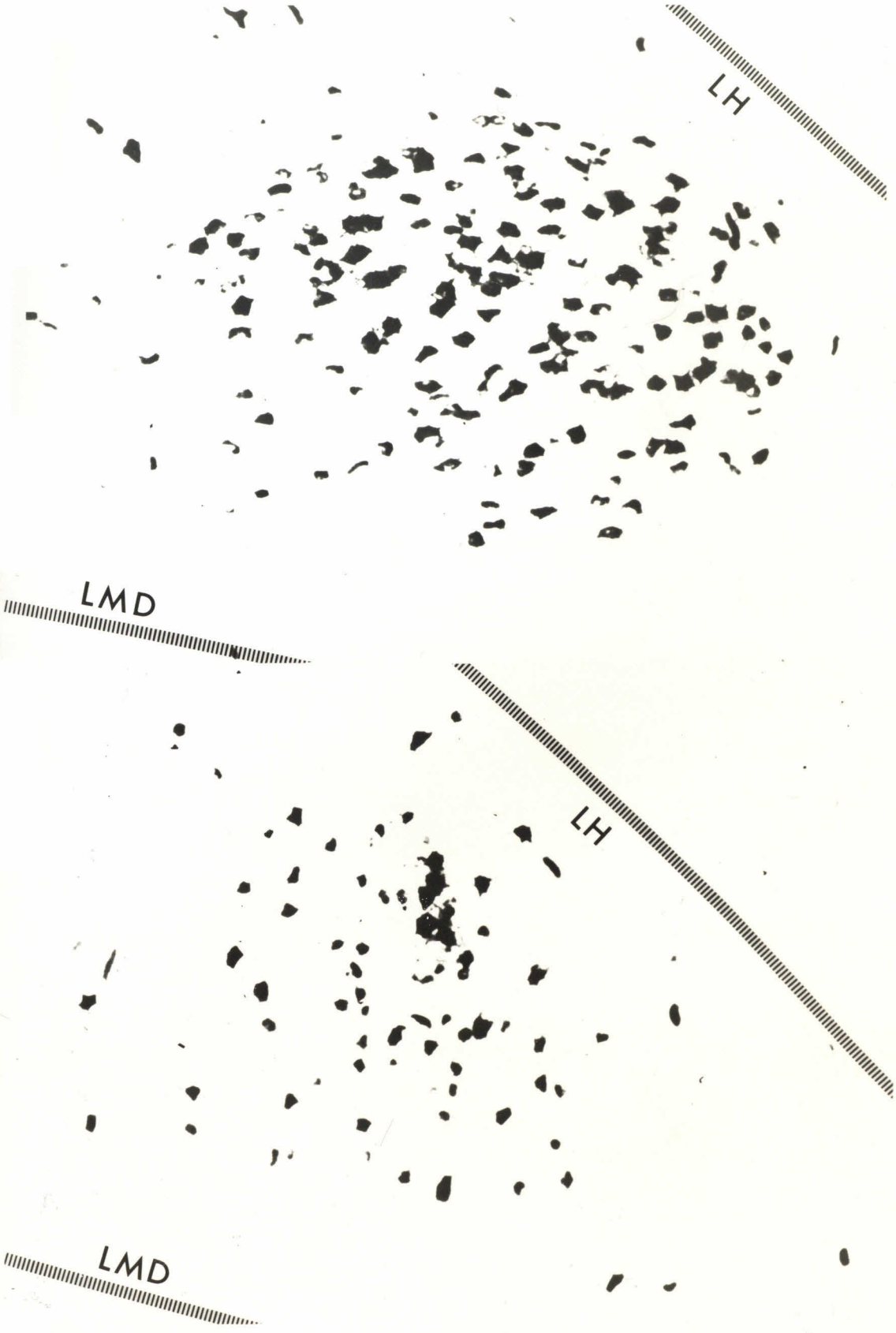
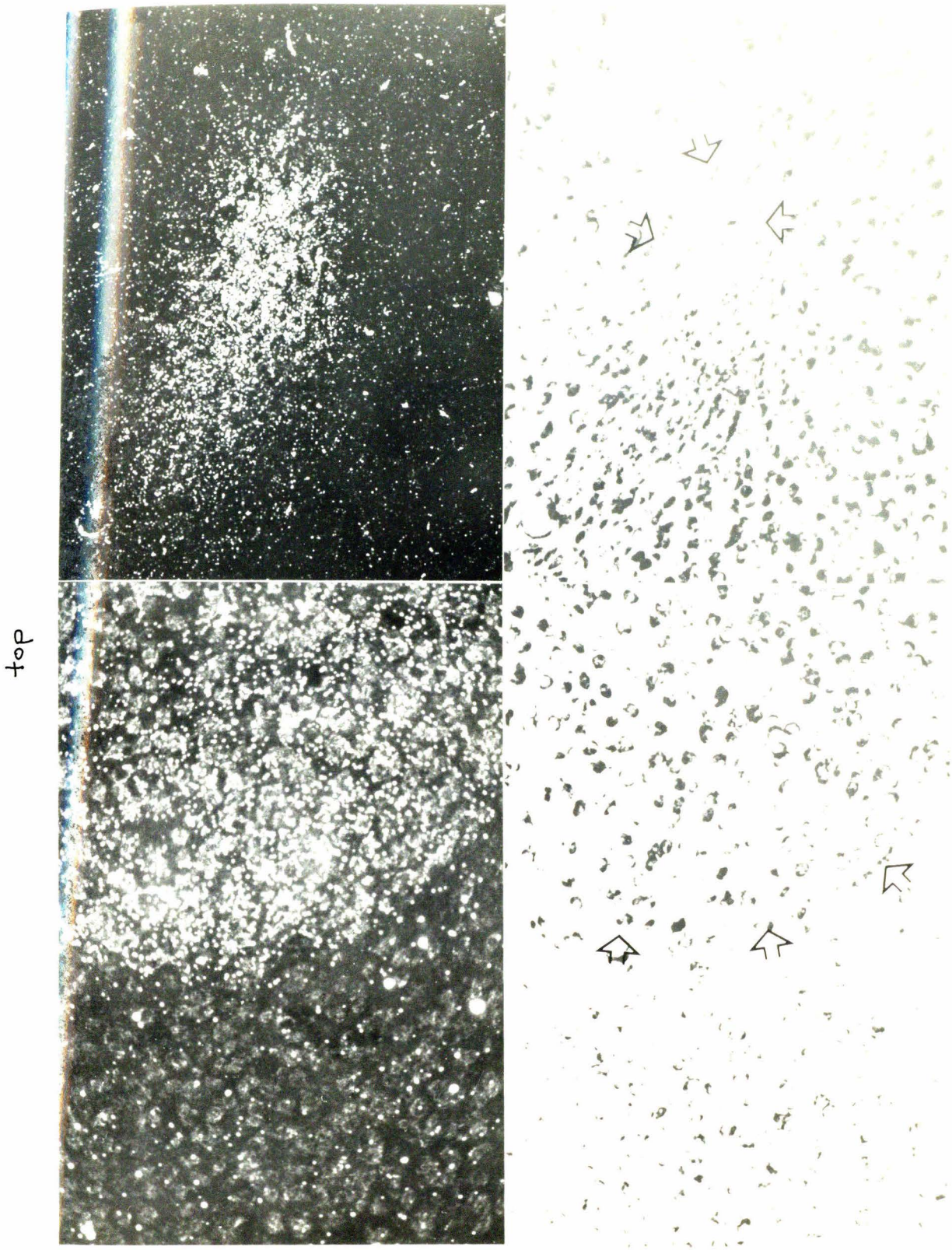

LH
LMD
LH
LMD

Figure 6. Terminal field of MAN upon RA revealed by anterograde transport of tritum-labeled material after injection of MAN with ^{3}H-leucine: (left) male, (right) female; (top) darkfield, (bottom) light field. Silver grains completely fill the interior of RA in both sexes, and their density falls abruptly at the inner face of a thin, acellular zone which rings the nucleus.


top

transported tritium-labeled material was also strictly confined to RA, and the nucleus was also surrounded by a thin, acellular zone (this is best seen in Figure 2) into which label did not penetrate. Thus, in the measurement of RA's volume, the inner face of this acellular zone was used to define the perimeter of the nucleus.

RA: description of efferents

In their study of the canary's song system, Nottebohm, Stokes and Leonard (1976) described two major efferent projections of RA. One set terminates upon the tracheosyringeal portion of the hypoglossal nucleus and degeneration of the other set of efferents results in a "field of boutons in the dorso-lateral corner of ICO just at the tip of the tectal ventricle" (p. 467). Both of these projections could be identified in the zebra finch. Though in the finch, and in sagittal section, RA is seen to terminate upon a discrete subnucleus within the nucleus intercollicularis (ICO) which lies dorsal and medial to the nucleus mesencephalicus lateralis, pars dorsalis (MLd) (Figure 7). This nucleus will be referred to as the dorso-medial intercollicular nucleus, and will be designated by the abbreviation DM. DM also projects to nXIIts (Figure 10).

To define the efferent projections of RA in the male, four adult zebra finches received bilaterally placed, stereotaxic injections of ^{3}H-leucine in RA. Figure 8 charts the distribution of label after an injection of ^{3}H-leucine which centered on the dorso-lateral portion of RA. A light distribution of silver grains was observed along the track of the micropipette through the neostriatum, though the fiber capsule which surrounds RA tended to retain the majority of label within the nucleus. From the injection site, labeled fibers collected along the rostral surface of RA and entered the occipital-mesencephalic tract (OM). Label followed OM through the diencephalon and passed immediately ventral to ICO and the nucleus spiriformis medialis (SpM). A sparse distribution of silver grains was observed over the dorso-medial thalamus. Labeled fibers then left OM in this region of the diencephalon and

Figure 7. Cytoarchitecture of DM in the male; sagittal section stained with cresyl-violet, anterior to the right. DM lies dorsal and anterior to MLd. At this level, it is shaped like a teardrop and seems to hang from the tectal ventricle. In more medial secitons, DM moves posterior and has a round cross-section. DM's cytoarchitecture is characterized by a cell which has a larger soma and more darkly-staining Nissl substance than the cells within ICO or MLd.

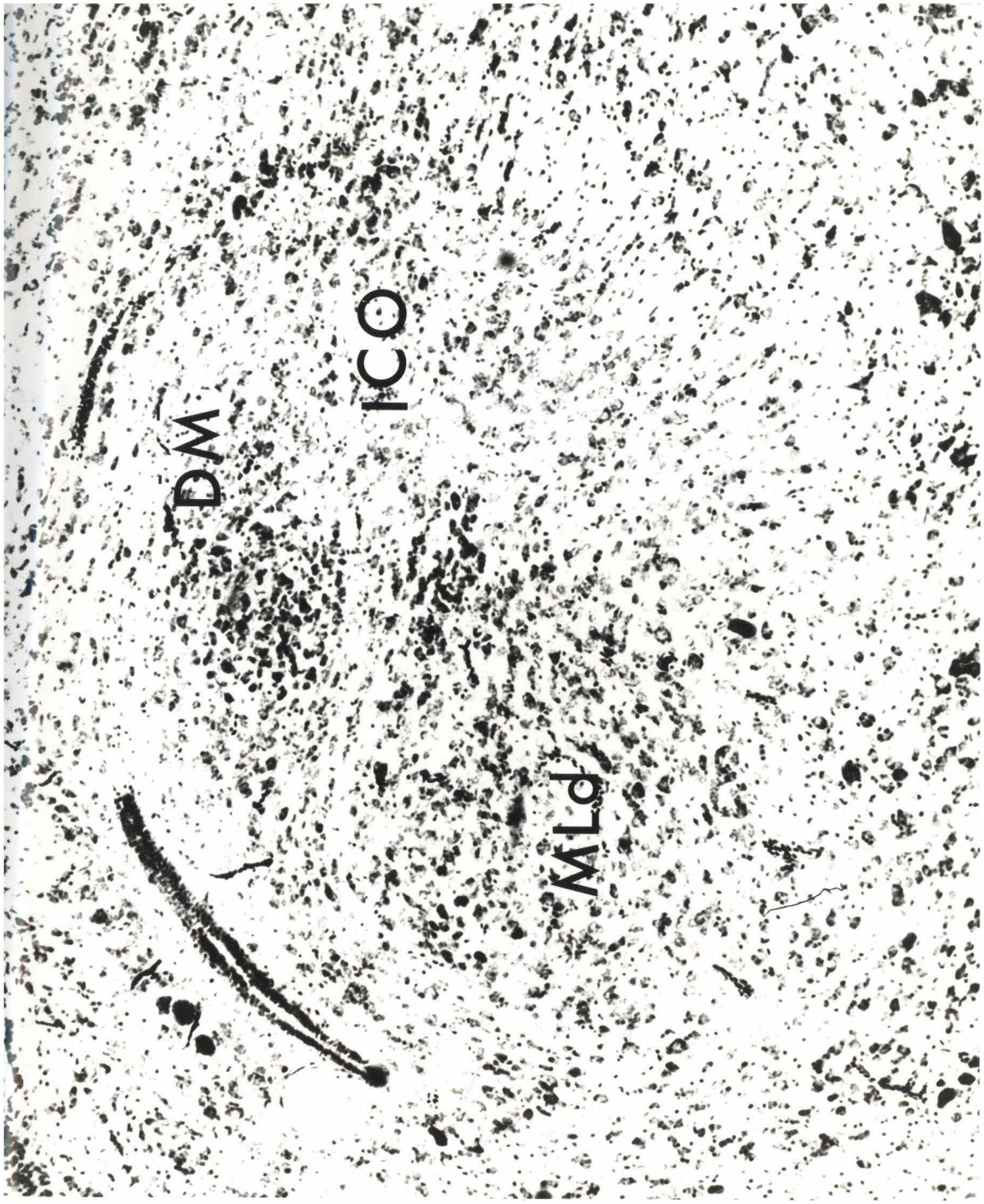
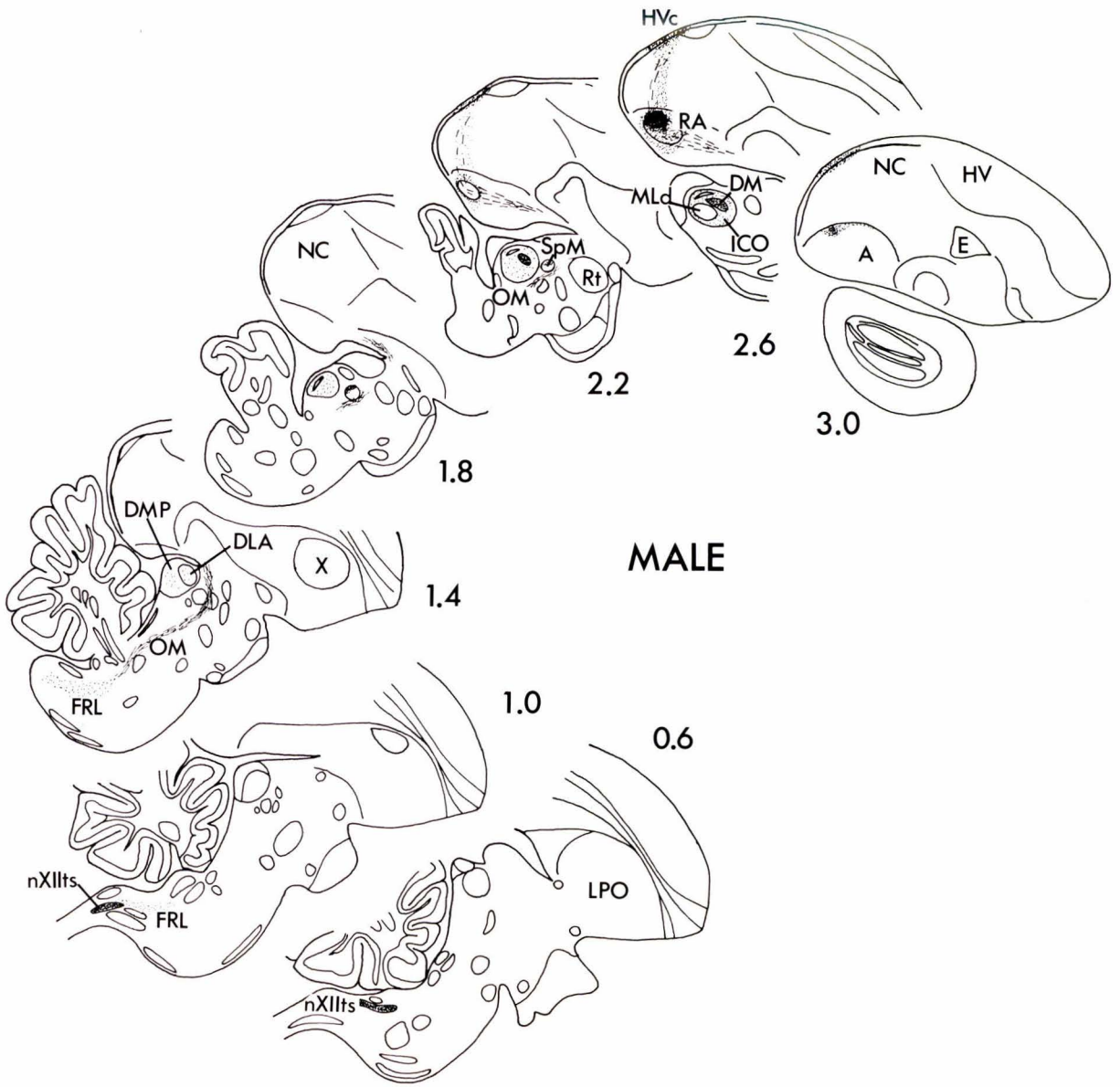

DM
ICO
MLd

Figure 8. Charting of the distribution of label after transport of tritium-labeled material from an injection of ^{3}H-leucine into RA of a male. Terminal labeling was observed over DM, ICO, SpM and nXIIts. The pattern of labeling in a female was similar.


HVc
RA
NC
HV
MLd
DM
ICO
A
E
SpM
Rt
OM
NC
2.6
2.2
3.0
1.8
DMP
DLA
X
MALE
1.4
OM
FRL
1.0
0.6
nXIIts
FRL
LPO
nXIIts

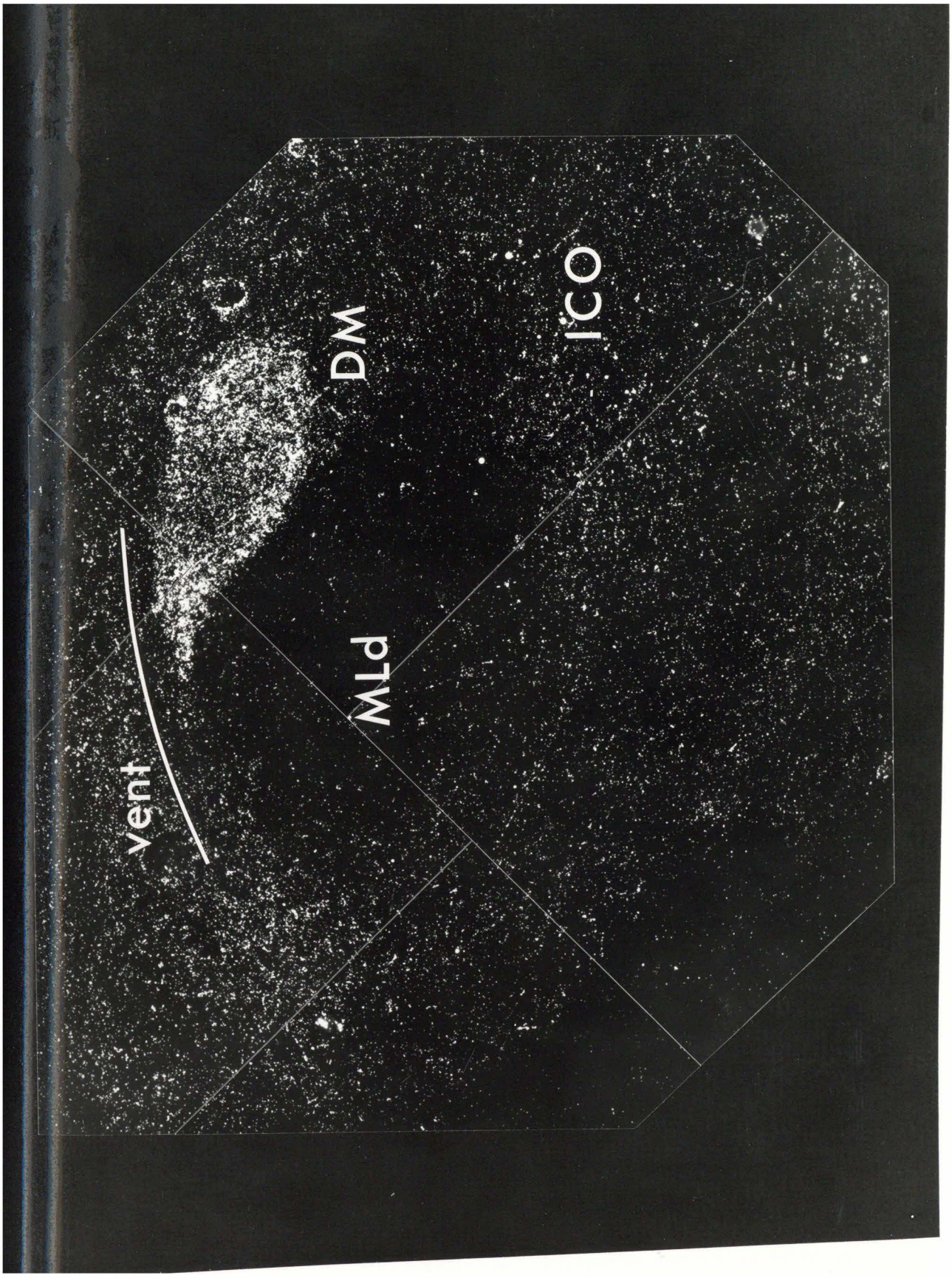
travelled dorsally to enter ICO along its medial aspect. The entire extent of ICO appeared lightly labeled while DM was covered by a dense distribution of silver grains (Figure 9). The terminal field of RA upon DM was teardrop-shaped in sagittal section and seemed to hang from the rostral tip of the tectal ventricle. MLd was completely free of terminal labeling and appeared silhouetted by the distribution of silver grains over ICO. SpM was also labeled after injection of RA. Silver grains over SpM were confined to a dorsal crescent which appeared to cap the nucleus. From the midbrain, label continued to follow OM caudally to its terminus beneath the cerebellum. At this point, fibers destined for the posterior, tracheosyringeal portion of the hypoglossal nucleus (nXIIts) left OM, traversed the reticular formation lateral to nXII, and then entered nXIIts along its lateral aspect. No labeling of the anterior, lingual portion of the hypoglossal nucleus was observed.

Neither nXIIts, SpM nor DM showed terminal labeling if the injection of ^{3}H-leucine into the archistriatum missed RA (i.e., an injection of the ventral neostriatum immediately above the lamina archistriatalis dorsalis, an injection of the archistriatum medial to RA, and an injection of OM which was just ventral to RA, all failed to label nXIIts, SpM or DM).

To determine whether or not RA completed the same pattern of efferent projections in females, the RA of three normal adult females was stereotoxically injected with ^{3}H-leucine. In the female, RA is a small ellipsoid 400 microns in length, and due to its size, the injections of ^{3}H-leucine were not confined to RA but also included adjacent regions of the dorso-medial archistriatum. After such injections, terminal labeling was observed over both DM and nXIIts. As in the male, the terminal field of RA upon either DM or nXIIts was confined to the cytoarchitectonic boundaries of these nuclei.

Due to the diffusion of ^{3}H-leucine from the injection site, it seemed desirable to place injections of HRP into the nuclei efferent to RA. This would

Figure 9. Darkfield photomicrograph of the distribution of silver grains over DM and ICO in the male after injection of RA with ^{3}H-leucine. The thin white line indicates the location of the tectal ventricle in this peri-sagittal section through the midbrain. Anterior is to the right. A heavy accumulation of label is observed over DM, ICO is sparsely labeled, while MLd is free of label and stands out in silhouette.


vent
DM
MLd
ICO

demonstrate whether or not the projection neurons whose axons terminate in DM and/or nXIIts were only located in RA or were also found in other archistriatal areas.

Three males and two females received bilateral injections of HRP into the medulla which included nXIIts. The distribution of labeled cells and axons observed after such an injection into a male is charted in Figure 10. RA was the only telencephalic nucleus which contained retrogradely-labeled cells after nXIIts injections in either males or females. In the midbrain, DM contained the largest number of labeled cells, but scattered labeled cells were consistently observed throughout ICO. Two males and females received large injections of HRP into the midbrain which included DM, ICO, MLd and SpM. After such an injection in either sex, the majority of retrogradely-labeled cells were observed in RA, but scattered cells in the rostromedial portion of the archistriatum were also occasionally observed (Figure 11). DM and nXII projecting neurons were distributed evenly throughout RA.

Sexually dimorphic measures of the neuronal population in RA

By retrogradly-labeling the projection neurons of RA with HRP after injection of the enzyme into DM or nXIIts, it was possible to define their somal morphology in Nissl material. In the male, HRP retrogradely labeled the large cells which provide RA its distinctive cytoarchitecture, though cells labeled from either DM or nXIIts could not be distinguished on the basis of their somal morphology. In either neutral red or cresyl-violet stained material, the projection neurons of the male's RA had an ovoid soma, possessed a large, round nucleus whose watery nucleoplasm contained one distinct nucleolus and was ringed by a distinct nuclear membrane (Figure 12a). Their somata also possessed abundant cytoplasm of pronounced basophilia. In the female, retrogradely-labeled cells were smaller and more elongate than in the male. Again, their nucleus stained palely, contained one nucleolus, and was ringed by a well-defined nuclear membrane (Figure 12b). As a consequence of their elongate soma, their cytoplasm looked as if it capped the nucleus at opposite ends.

Figure 10. Charting of HRP-labeled cells in a male after injection of the enzyme into nXII. The injection site covered nXII as well as ventral and lateral areas. Labeled fibers were observed in OM. In the midbrain, the majority of labeled cells (indicated by black dots) were confined to DM, but a few labeled cells were also scattered throughout ICO. In the telencephalon, labeled cells were only observed in RA. The distribution of labeled cells in a female after injection of nXII with HRP was identical to the male.

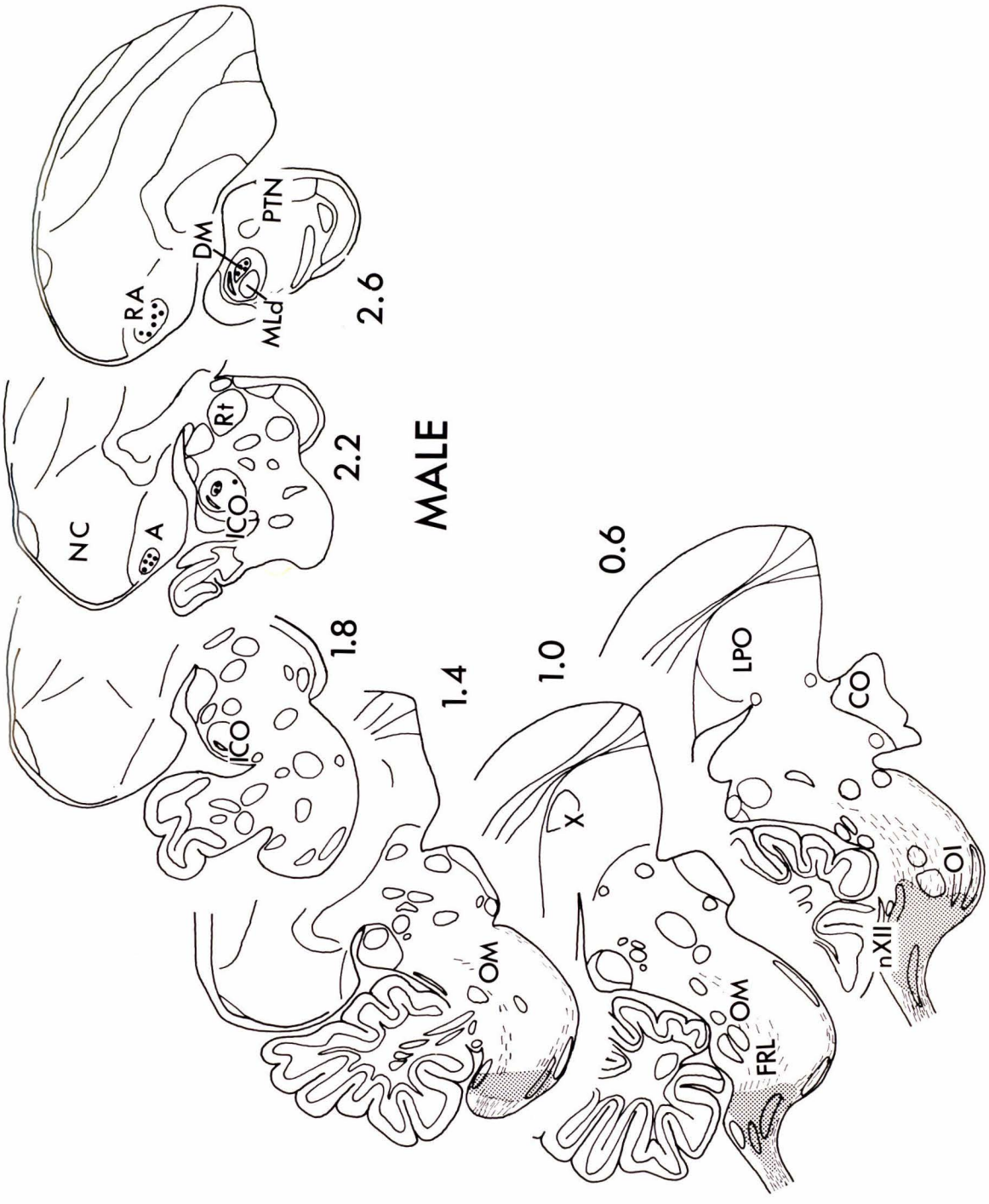

MALE
2.6
2.2
1.8
1.4
1.0
0.6
RA
DM
PTN
MLd
NC
A
Rt
ICO
ICO
X
LPO
CO
OM
OM
FRL
nXII
IO

Figure 11. Charting of HRP-labeled cells in a female after injection of the enzyme into DM. The majority of labeled cells were observed in RA, but scattered labeled cells in the rostromedial portion of the archistriatum were also observed. The distribution of labeled cells in a male after injection of DM with HRP was identical to the female.

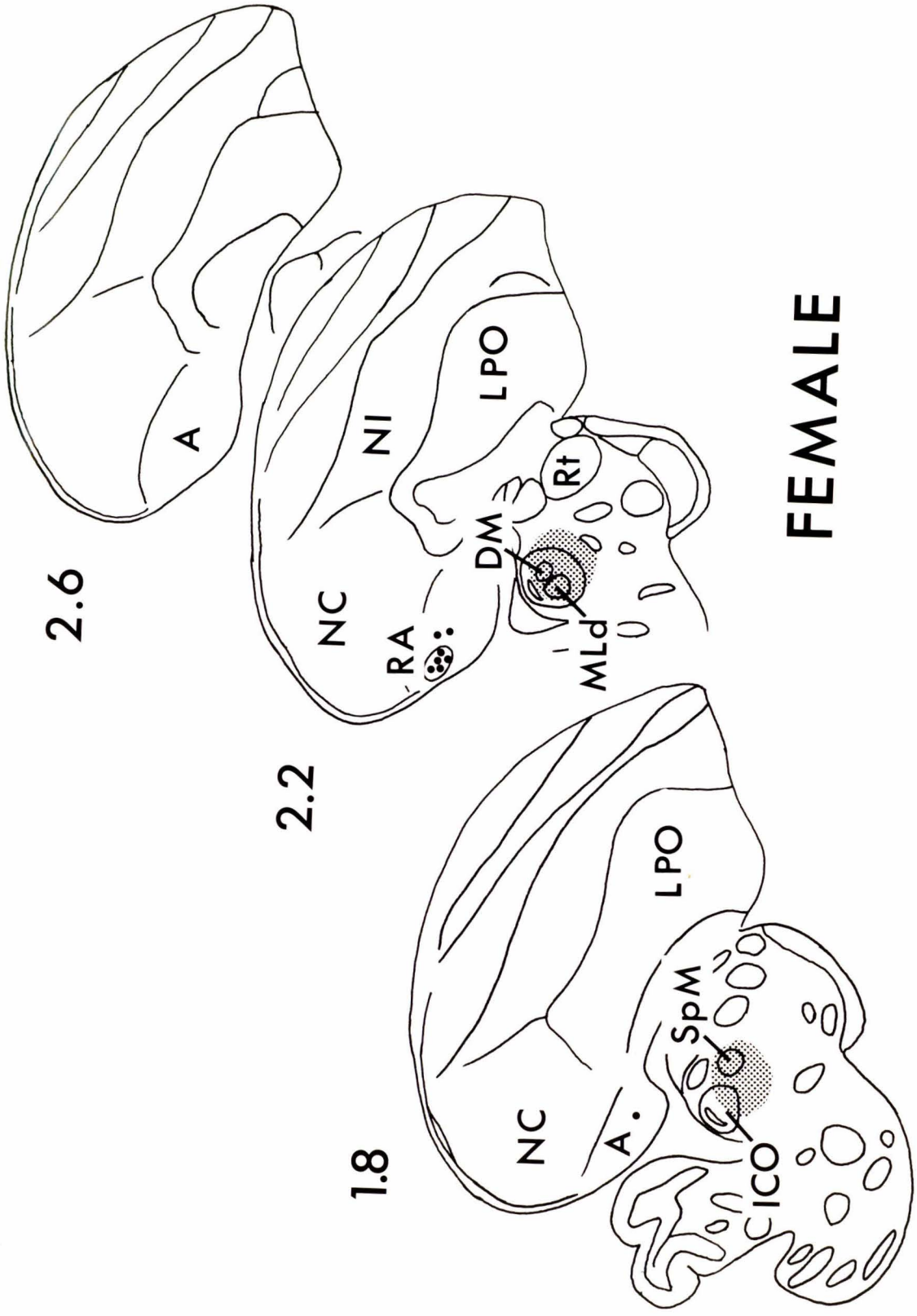
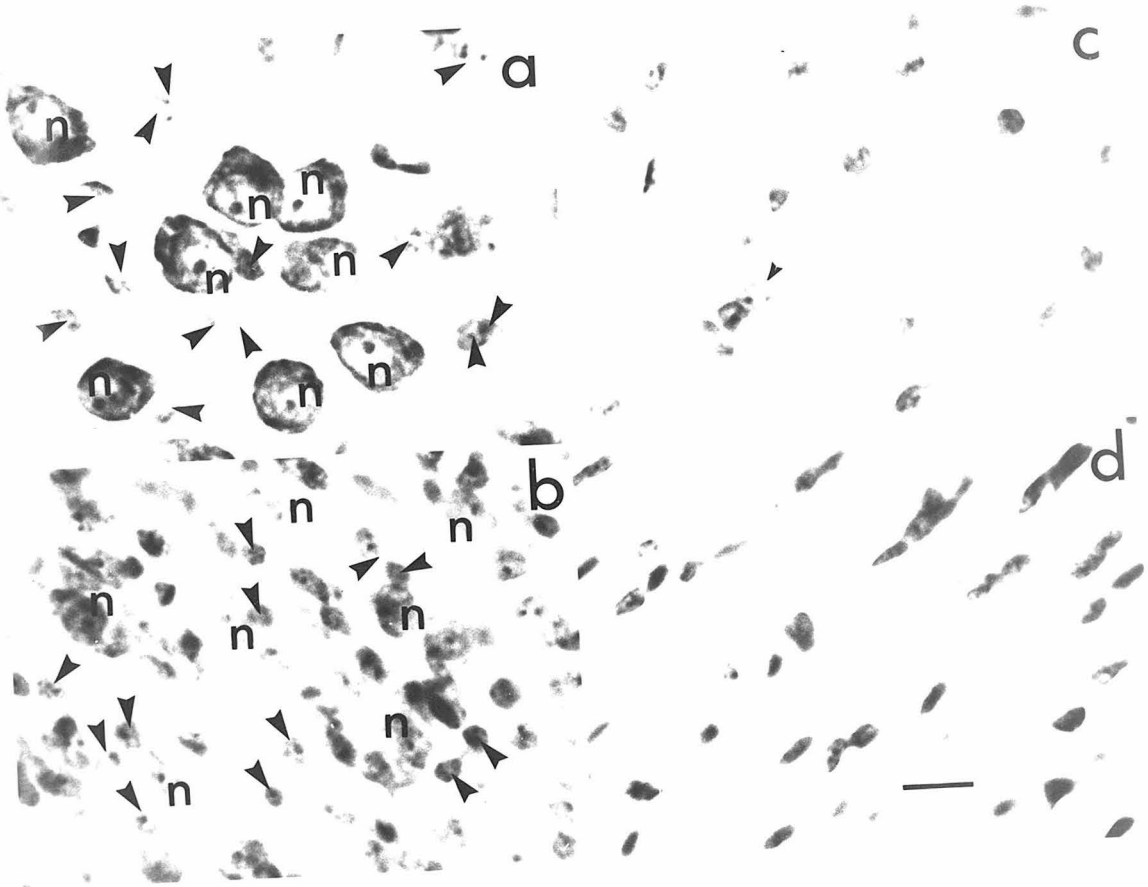

FEMALE
2.6
A
NI
LPO
NC
RA
DM
Rt
MLd
2.2
LPO
NC
A
SpM
ICO
1.8

Figure 12. Cytology of neurons and macroglia in the zebra finch in 10 μ-thick, paraffin-embedded sections which were stained with thionin; (a) male RA, (b) female RA, (c) occipito-mesencephalic tract in a male, (d) optic tract in a female. Neurons are indicated by the letter "n", macroglia are indicated by the arrowheads (for description see text). Scale bar in (d) equals 10 μ.


a
b
c
d
n
n
n
n
n
n
n
n
n
n
n
n
n
n
n

RA contained two other types of cells whose morphologies were distinct from that of the neurons described above. Both of these cell types had somata approximately 5 microns in diameter. One type had a compressed, elongate soma, lacked a distinct nucleus, and had an overall, pronounced basophilia, granular in texture. The other type of cell was ovoid and had a very lightly staining cell body with little cytoplasm. Its nuclear envelope was indistinct and its watery nucleoplasm contained from 1-5 nucleoli as well as many small, densely staining clumps of chromatin (Figure 12). Both of these cell types were also observed in the zebra finch optic tract (Figure 12d) and within the occipito-mesencephalic tract (Figure 12c) which suggested that these cells were macroglia.

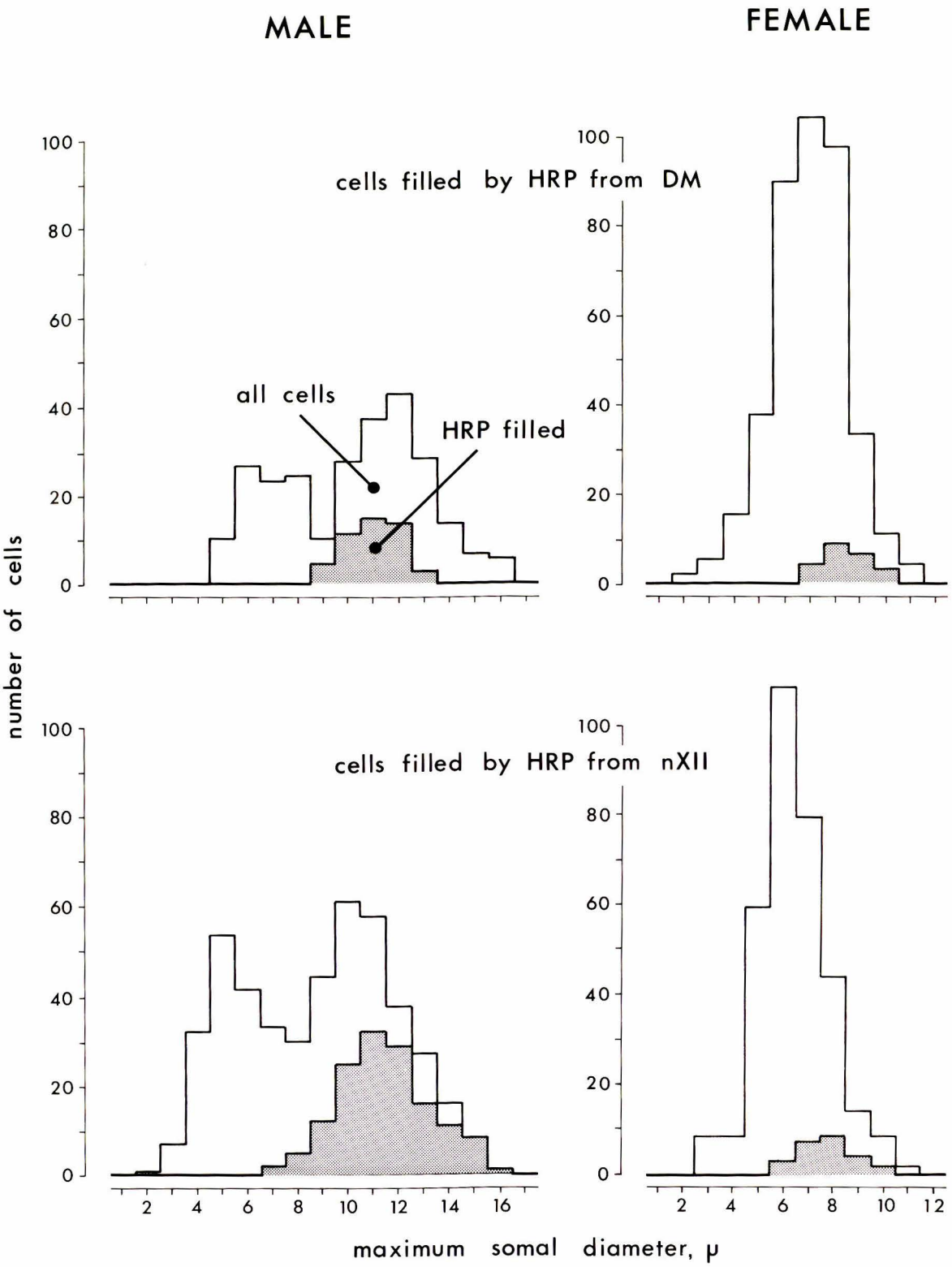
The morphology of these cell types was comparable to macroglia of mammals. Ling et al. (1973) characterized macroglia in the electron microscope and then identified their morphology in the light microscope. They described oligodendrocytes of the rat corpus callosum as elongate cells which oriented in parallel with the nerve bundles of the callosum, and which possessed a pronounced basophila. Astrocytes were described as oval cells which stained very lightly. In the zebra finch optic tract, the majority of cells were elongate (1.5 μ x 5 μ on the average) and were arranged in strands of 4-8 cells with their long axis parallel to the nerve fibers in the tract (Figure 12d). These cells lacked a distinct nucleus, and were quite basophilic. A second type of optic tract cell which was found in Nissl-stained material occurred singly, possessed an oval soma 5-6 μ in diameter, had little cytoplasm, an indistinct nuclear membrane and several nucleoli. The morphology of this cell type was similar to Ling et al.'s class of astrocytes and the first was similar to their description of basophilic oligodendrocytes. In the optic tract, oligodendrocyte-like macroglia predominated while in RA astrocytic macroglia formed the majority. Classification of these zebra finch cell types as either oligodendrocytes or astrocytes without correlative EM observations which demonstrated that the former supported myelin

sheaths or that processes of the latter were packed with filaments would be extremely tenuous. In later sections, both cell types will be denoted by the term macroglia.

RA neurons which were retrogradely-labeled from either DM or nXII were both sexually dimorphic with respect to somal diameter as can be seen in Figure 13. In the experiments illustrated, the somal diameter of RA projection neurons terminating upon either DM or nXIIts averaged roughly 8 μ in the females and 11-12 μ in the males. To compare the somal diameter of RA neurons in a large sample of individuals, the distribution of somal diameter was measured in groups of normal, gonadally intact adult males and females. The brains were processed by the standard histological protocol, and measurements were made in 30 μ-thick, cresyl-violet stained, frozen sections. In such material, the distribution of somal diameter for all RA neurons was unimodal (Figure 23). In seven males, the somal diameter of RA neurons averaged 17.8 μ (range: 16.3-18.8 μ) and in six females averaged 8.2 μ (7.2-8.6 μ) (Table 1). This difference was statistically significant (Mann-Whitney U-test, $p = 0.001$). (Both the volume of RA in males, and the mean somal diameter of its neurons tended to be larger in normal histological material than in material processed for HRP. This difference can probably be attributed to differences in the osmolarity of the fixatives used.) Thus, the sex difference in the cytoarchitecture of RA (Figure 2) is due in part to a difference in the somal size of RA neurons in males and females.

The projection neurons of RA which terminate upon DM and/or nXIIts could not be distinguished on the basis of somal diameter. However, two trends were observed in males: first, the mean size of neurons which were labeled by HRP after injection of DM tended to be smaller than the mean size of all the neurons in the microscope field (in two of three experiments which had successful transport of HRP to RA). Second, the mean size of neurons which were labeled by transport of HRP

Figure 13. Distribution of maximum somal diameter (μ) of retrogradely-labeled neurons in RA after injection of either DM or nXII with HRP (30 μ-thick, frozen sections counter-stained with neutral red). The diameter of all the cells within the microscope field was measured and the size distribution of the subset of cells labeled by HRP is shaded. Neurons and macroglia could not be distinguished with this stain.


MALE
FEMALE
cells filled by HRP from DM
all cells
HRP filled
cells filled by HRP from nXII
number of cells
maximum somal diameter, μ
0
20
40
60
80
100
2
4
6
8
10
12
14
16

from nXIIts was greater than or equal to the mean size of all the neurons in the microscope field (in two of two successful experiments). This sort of bias could be due to more efficient transport of enzyme from nXIIts by large neurons as compared to small neurons, or it could suggest that neurons of RA projecting to nXIIts have slightly larger somata than those projecting to DM. Two types of neuron were impregnated in RA by Golgi techniques and they differed with respect to the size of their soma (described in the following section). In Golgi-rapid, one cell type (RA_{II}) had a mean somal diameter of 15.4 μ ($\pm$1.8 μ SD) and the second (RA_{I}) had a mean somal diameter of 18 μ ($\pm$1.8 μ SD). The small difference between the mean somal diameters of these two cell types and the large variance within each population indicates that separation of these two populations by somal diameter alone is not possible. As a working hypothesis, it seems reasonable to assume that each of these types of neuron has a different projection. The RA_{I} neuron may terminate upon nXIIts and the RA_{II} neuron upon DM. Resolution of this question will require the simultaneous use of two retrograde tracers.

In addition to the sex difference in the somal diameter of the RA neurons described above, RA is also sexually dimorphic with respect to the number of neurons that it contains (Table 1). Males had 2.4 times as many RA neurons as females: 16,400 (range: 16,000-16,900) as compared to 6,950 (6,000-7,600). This sex difference was highly significant (Mann-Whitney U-test, $p = 0.001$). It seems unlikely that this difference in neuron number could be due to the absence of a distinct class of neurons in the female, since the female RA completes the same pattern of efferent connections as that of the male, and as shown in the next section, female Golgi-impregnated material contains the same types of RA neurons as in the male. The ratio of neurons to glia in RA was also sexually dimorphic. This ratio averaged 0.32 (0.31-0.33) in males and in females averaged 0.41 (0.37-0.46). This difference was statistically significant (Mann-Whitney U-test, $p = 0.05$). Thus, both the number of neurons and the total number of cells within RA were sexually dimorphic.

Table 1. Measures of RA's sexual differentiation: total nucleus volume, maximum somal diameter and number of RA neurons

Sex	Volume mm^3	Somal diameter μ	Number
Males (n=7)	**0.285** (0.220-0.372)[1]	**17.8** (16.3-18.8)	**16,400** (16,400-16,900)
Females (n=6)	**0.024** (0.018-0.029)	**8.2** (7.2-8.6)	**6,950** (6,000-7,600)

[1]Range: low-high.

PART 2: RA Contains the Same Cell Types in Both Sexes, Though Their Size is Sexually Dimorphic

Granted that RA completed the same efferent connections and received the same pattern of afferents in males and females, the question then arose of whether or not RA contained the same types of neurons in both sexes. The above work with Nissl-stained material showed that RA neurons were sexually dimorphic with respect to soma size, and with respect to their packing in the nucleus (Figure 2). Thus, identification of homologous cell types in males and females would need to be based on size-independent morphological criteria. If this was possible, quantitative analysis of the dendritic tree might reveal which aspects of neuronal growth were patterned by gonadal hormones.

Methods

Golgi impregnation

Whole brains were processed for Golgi-Cox by the slow procedure with optional counterstaining described by Ramon-Moliner (1975). Briefly, freshly dissected brains were impregnated for 2 weeks in a mercuric chloride/potassium dichromate solution, dehydrated, embedded and sectioned in Parlodion at a thickness of 120 microns, affixed to microscope slides, and then alkalinized on the slide. Alternate sections were counterstained with cresyl-violet. Ten female and six male brains were processed for Golgi-Cox. Of these, six of the female and four of the male brains contained well-impregnated examples of spinous RA neurons, and these were used for the quantitative comparison of dendritic field structure (Tables 2 and 3) described below.

For Golgi-rapid, birds were perfused with 4° buffered formaldehyde as described above, and the brain then post-fixed for up to a week in the same solution. The brain was then split sagittally and impregnated in a solution consisting of 80 ml

of 2.5% potassium dichromate, 16 ml of distilled water and 4 ml of 5% osmium tetroxide in darkness for one week. The tissue was then washed three times in 0.5% silver nitrate, and then transferred to 0.75% silver nitrate for a second week. The tissue was then dehydrated, embedded and sectioned in 16% Parlodion at 90 μ.

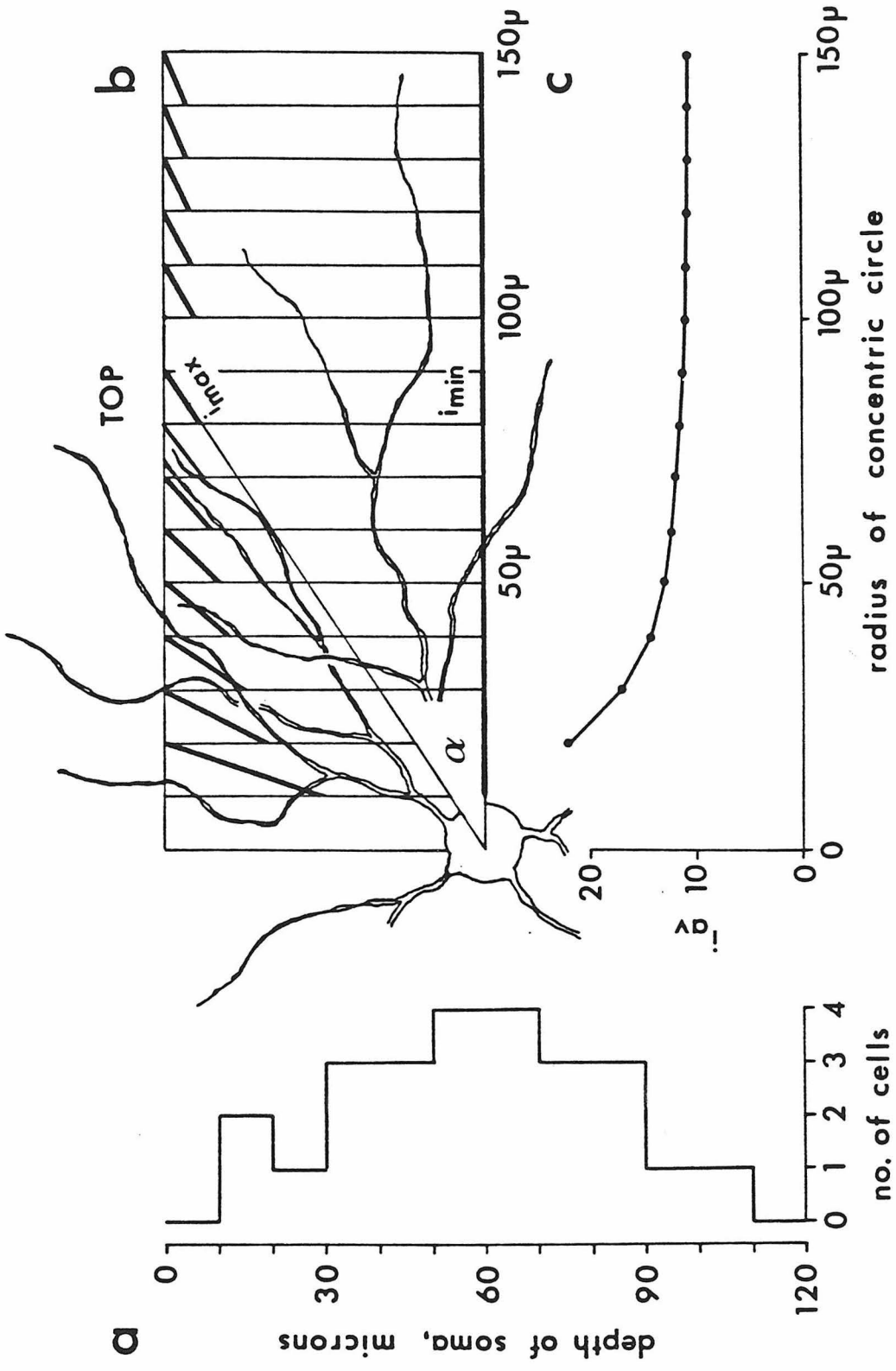
Quantitative analysis of Golgi material

The slow procedure for Golgi-Cox with alkalinization on the slide did not impregnate neurons uniformly throughout the thickness of the section. Rather, impregnation of neurons within the center of the section was favored over those located more superficially. This led to the number of impregnated neurons being normally distributed as a function of depth in the section around a maxima at the section's center (Figure 14). Perhaps because of this property of the reaction, relatively few dendritic fragments which had been severed from impregnated neurons by the knife also became impregnated.

Characteristics of a neuron's dendritic architecture which appeared to be robust to artifact were the following: i) Number of primary dendritic arms - impregnation of the proximal lengths of dendritic arms did not seem subject to vagaries in the Golgi-technique. ii) Number of branch segments - most branching occurred fairly close to the soma, thus, the bias for impregnation of neurons in the center of the section insured that if dendrites were cut by the knife, it was in their distal lengths after the majority of branching had occurred. iii) Dendritic field volume - RA neurons elaborated spherically symmetric dendritic trees. The volume of their dendritic field was defined as the minimum volume sphere, centered upon the soma, which enclosed all of a neuron's dendrites. This meant that the longest, most completely impregnated dendrite defined the radius of that sphere.

Measurement of total dendritic length was subject to severe artifact, especially in the male. The radius of a male RA_I neuron's dendritic tree approximated the thickness of the sections used in this study (130 μ radius vs. 120 μ

Figure 14. (a) Distribution of the somata of Golgi-Cox impregnated neurons as a function of depth in the section. (b) Schematic representation of a neuron's dendritic aborization viewed within the plane of the section, TOP indicates the surface of the section. The thin vertical lines represent the projection into the section of the grid of concentric circles used in the Sholl analysis. The soma of the neuron illustrated is located at a depth of 60 μ in the section. The minimum length of dendrite between a pair of consecutive lines is indicated by i_{min}, the maximum length of dendrite is indicated by i_{max}. (c) The value of the weighting coefficient, i_{av}, as a function of the radius of the second consecutive circle.


a
depth of soma, microns
0
30
60
90
120
0 1 2 3 4
no. of cells
b
TOP
i_{max}
i_{min}
α
50μ
100μ
150μ
c
$\bar{i}_{\alpha\nu}$
20
10
0
0
50μ
100μ
150μ
radius of concentric circle

section). As a consequence, a large number of dendritic segments were cut by the knife and their distal, severed fragments failed to impregnate. This source of artifact introduced a bias against the male, as female RA_I neurons tended to be less severely truncated by the knife since their dendritic field diameter (140 μ) was approximately the thickness of the section (120 μ). A final source of artifact was the quality of the impregnation. Poorly impregnated neurons characteristically possessed bloated somata and swollen dendrites whose distal lengths deteriorated into vesiculated fragments. This sort of artifact was immediately obvious and such neurons were discarded from the analysis. Less obvious was the degree to which a neuron's fine, distal dendritic segments were impregnated. The magnitude of this artifact could not be determined, although was independent of the Golgi technique used as either Golgi-Cox or Golgi-rapid seemed to impregnate RA neurons equally well.

The method of Sholl (1954) was used to represent the radial distribution of dendrites within a cell's dendritic field. Two-dimensional drawings of impregnated neurons were prepared with the aid of a drawing tube through a 40X dry objective at a final magnification of 400X. A grid of concentric circles whose spacing equalled 10 μ was then centered upon the soma and the number of dendritic intersections with each circle was counted. A simple geometric method was used to obtain an estimate of the neuron's total dendritic length from the area beneath the curve of the Sholl plot. The method assumed a raisin-and-toothpick model for the dendritic field. RA neurons had a distribution of dendrite which was radially symmetric, and dendrites generally followed straight tracks, radially outward from the soma. Thus, the minimum length of a dendrite between its intersection with two consecutive concentric rings was equal to their spacing, $i_{min} = 10$ μ (Figure 14). The maximum length would be along the line from the neuron's soma to the intersection of the second ring with the section's surface, $i_{max} = 10/\cos \alpha$, where α was calculated from

α = arc tan[t/2r] in which t = the section thickness and r = the radius of the second consecutive circle. This calculation was based on the normal distribution of impregnated neurons in the section (thus, t/2 was used to calculate α rather than t). The average length of a dendrite between two consecutive concentric circles was thus $i_{av} = [(10/\cos \alpha) + 10]/2$. The value of the weighting coefficient (i_{av}) as a function of the concentric circle radius is graphed in Figure 14c. The length of dendrite between each pair of consecutive concentric circles was then calculated from $i_{av} \times n$, where n = the number of dendritic segments crossing a pair of consecutive concentric circles.

The quantitative sex differences in the dendritic field structure of spinous RA neurons reported in the **Results** section (Table 4) were extremely robust. Somal length, dendritic field volume, number of primary dendritic arms, total number of branch segments and total length of dendrite all favored the male by a factor of two. Because of the small number of impregnated neurons which were obtained from each brain, to test whether differences among the samples signified population differences or variations in samples drawn from the same population the nonparametric Kruskal-Wallis one-way analysis of variance by ranks was used (Siegel, 1956).

Results

RA: description of neuronal constituents

Impregnated neurons within the male's RA were obtained after processing of the tissue by either the Golgi-Cox or Golgi-rapid techniques. In both types of material, the boundaries of RA were identified by the following criteria. In male Golgi-rapid material, RA was distinguishable because its dense neuropil tended to absorb more silver than adjacent archistriatal tissue and the cytoplasm of large, unimpregnated neurons within RA was loaded with many small, precipitate-filled vesicles. In Golgi-Cox material, RA could be located on alternate sections which were counterstained with cresyl-violet. In both types of material, impregnated fibers

cascaded ventrally through the neostriatum, entered RA along its dorsal extent, and then interwove within the confines of the nucleus. Thus, this basketwork of fibers also served to delineate RA from adjacent archistriatal tissue. In the female, RA could be located in Golgi-Cox sections that had been counterstained with cresyl-violet. Unfortunately, the boundaries of the female's RA in Golgi-rapid material were ill-defined. This limited comparison of male and female material to tissue processed by the Golgi-Cox technique.

In the male, both the Golgi-Cox and the Golgi-rapid techniques impregnated two types of neurons within RA. One class of RA neurons was comprised of large, stellate cells whose dendrites were thick and heavily encrusted with spines. The other type of neuron was a smaller cell whose dendrites were free of spines (Figure 15). In five male finch brains processed by the Golgi-rapid technique, 22 well-impregnated spinous cells and 18 aspinous cells were obtained.

In female Golgi-Cox material, two cell types were also observed. Though the size of RA neurons was sexually dimorphic, these two cell types seemed homologous to those of the male based on their dendritic morphology. Like the male, one type of cell possessed spinous dendrites and the other aspinous dendrites (Figure 15).

Classification of neuronal elements within RA

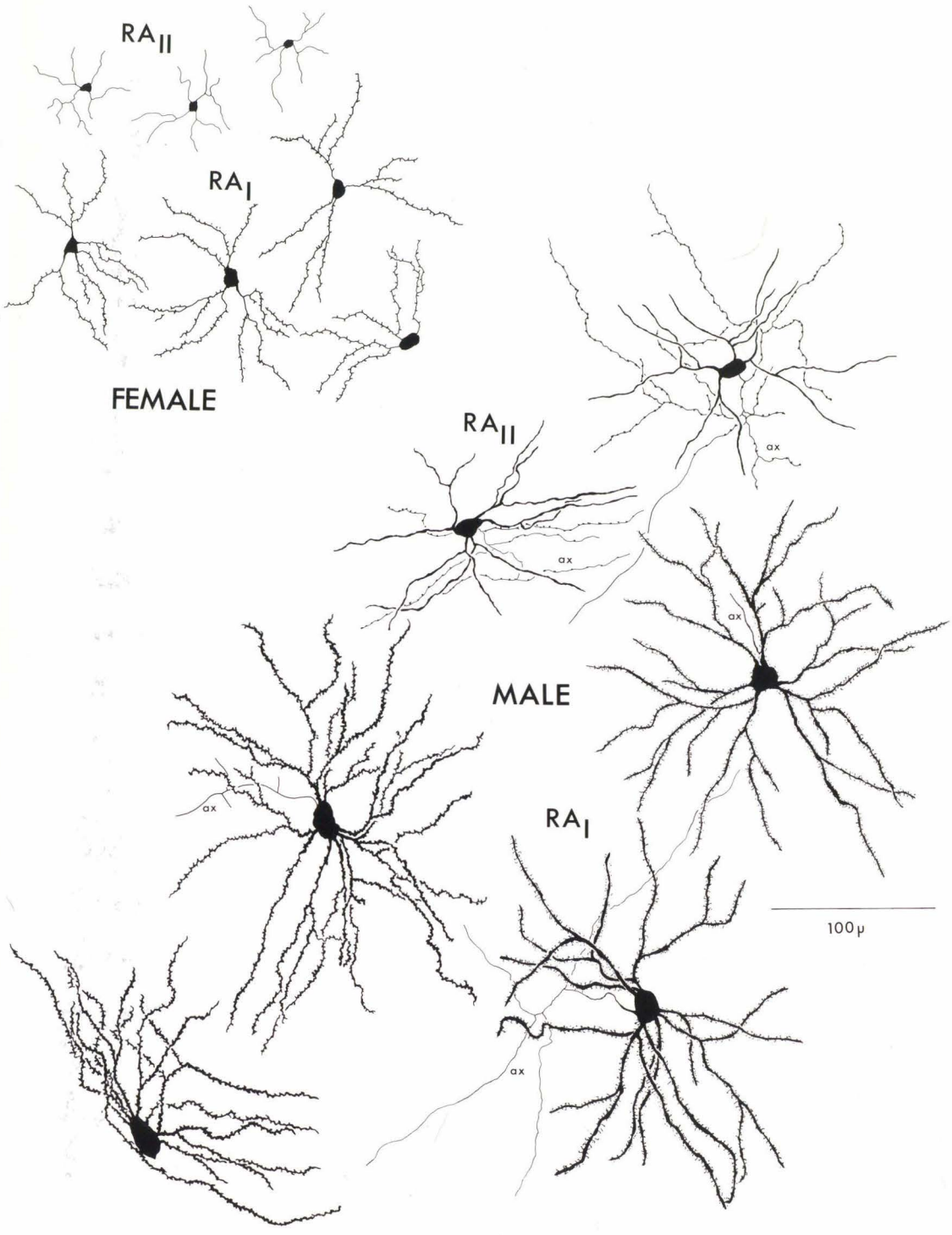
1) spinous dendrites ---------- RA_I

2) aspinous dendrites ---------- RA_{II}

Spinous RA_I neuron

The diagnostic feature of an RA_I neuron's morphology was the presence of spines along its dendrites. In male Golgi-rapid material, these spines were approximately 1 μ long and consisted of a slender stalk which was surmounted by a ball of 0.25 μ diameter (Figure 16). The spines of male and female RA_I neurons were poorly preserved in Golgi-Cox material and possessed an irregular shape. In the male,

Figure 15. Examples of Golgi-impregnated neurons in the RA of a male or female. The female RA neurons were impregnated by Golgi-Cox, as were the two male RA_I neurons in the lower left. The rest of the male RA neurons were impregnated by Golgi-rapid. The male RA_I neuron in the lower left corner was located at the periphery of the nucleus. The size of the dendritic arborization of both RA_I and RA_{II} neurons is sexually dimorphic.


RA II
RA I
FEMALE
RA II
ax
ax
MALE
ax
RA I
ax
100 μ

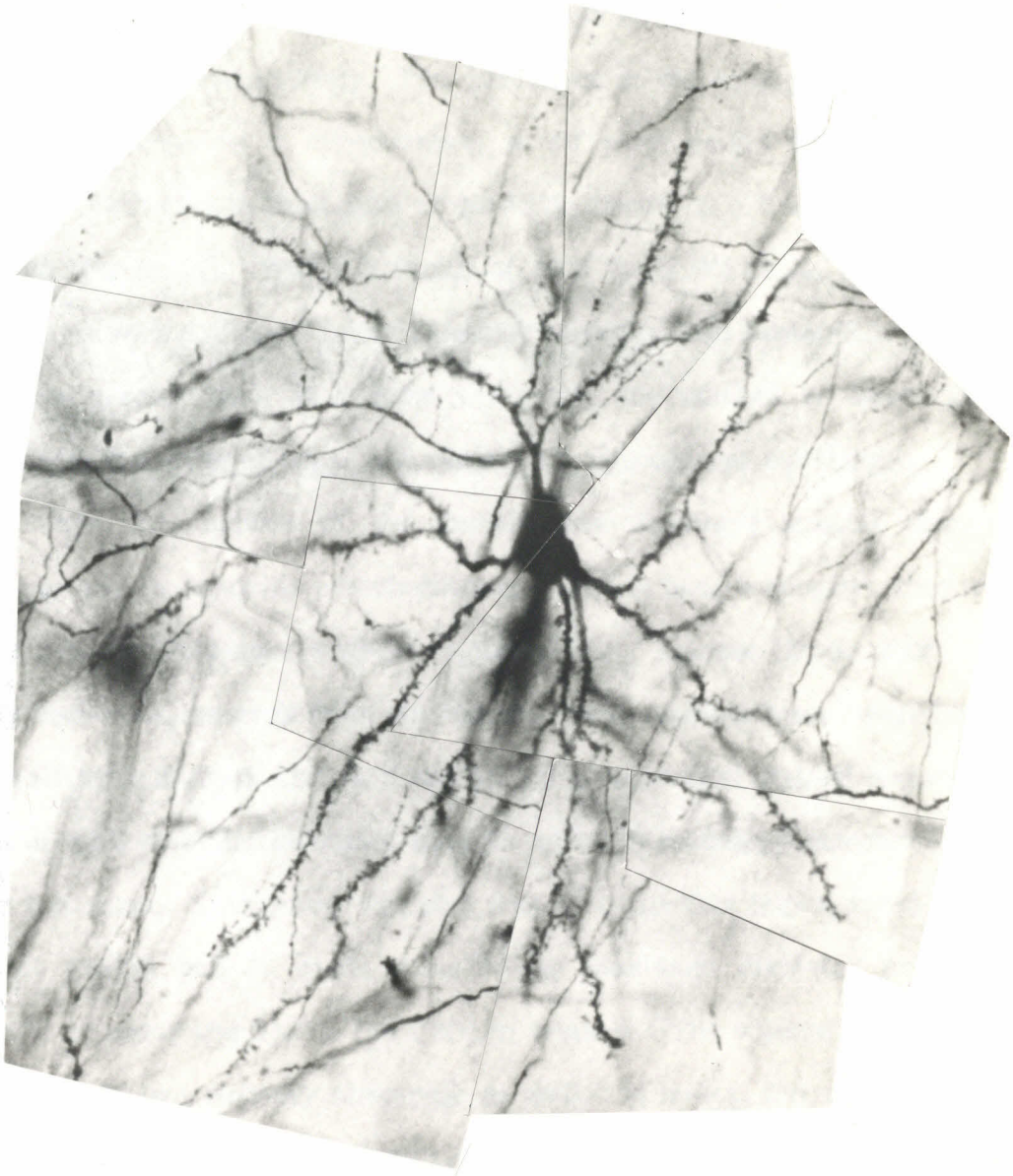
the spine density of Golgi-Cox, or Golgi-rapid impregnated RA_I neurons averaged one spine per linear micron of dendrite, while along the dendrites of female Golgi-Cox impregnated RA_I neurons, the density was much lower, an average of one spine per 3 μ. The poor preservation of spine morphology by Golgi-Cox suggests that this sex difference in the density of dendritic spines should be interpreted with caution.

In male Golgi-rapid material, the axons of several RA_I neurons could be traced until they exited the ventral border of RA. The axons of RA_I neurons gave rise to collaterals which had a smooth appearance and were of a fine caliber, 0.5 μ.

In both sexes, RA_I neurons which were located within the interior of the nucleus supported radially symmetric dendritic fields while those located along the periphery of RA possessed an asymmetrical dendritic arborization whose system of branches was strictly confined within the boundary of RA (Figure 15). The origins of a center-RA_I neuron's dendritic arms were evenly distributed over its soma, while most dendritic arms of an edge-RA_I neuron arose from the side of its soma which faced the interior of RA. These arborized inwardly, while those dendritic arms which arose from the soma near the margin of RA frequently redirected their course along the boundary of the nucleus so as to remain within it. To establish that the dendritic field of center-RA_I neurons was radially symmetric in both sexes, Sholl plots of dendritic density for Golgi-Cox impregnated neurons from sagittal or coronally sectioned material were compared. In either plane of section, the radial distribution of dendritic density was statistically equivalent (chi-square test of dendritic density at radii for which the mean number of dendritic intersections was ≥ 5).

The location of an RA_I neuron subtly affected the size of its dendritic field. Nineteen well-impregnated center-RA_I neurons and six edge-RA_I neurons were selected from male Golgi-Cox material for quantitative comparison. Edge-RA_I neurons were found to support one less dendritic arm on the average than center-RA_I neurons. The sample of edge-RA_I neurons supported a mean of 5.6 ($\pm$0.5 SE) primary

Figure 16. Dendritic morphology of a male RA_I neuron, Golgi-rapid impregnation. The spines are approximately 1 micron long and consist of a slender stalk which is capped by a ball approximately 0.25 microns in diameter. Spines have an average spacing of one spine/linear micron of dendrite.



arms while center-RA_I neurons possessed a mean of 6.6 (±0.04 SE) primary dendritic arms. This difference proved to be statistically significant (Student's t-test, $p = 0.04$). The number of secondary, tertiary, and quaternary branch segments of edge- and center-RA_I neurons did not differ significantly. Both edge- and center-RA_I neurons supported roughly the same number of branch segments, 22.3 (±1.17 SE) and 22.7 (±1.1 SE), respectively. The difference in the number of primary dendritic arms, and perhaps as a consequence, a different radial distribution of branch segments is reflected in Sholl plots of dendritic density as a function of distance from the soma (Figure 17). Within a radius of 80 μ from the soma, the arborization of edge-RA_I neurons contained fewer dendritic branches than center-RA_I neurons, but at a distance greater than 80 μ from the soma, the number of dendritic branches remaining in the arbor of edge- and center-RA_I neurons was equivalent.

The RA_I neuron of the female was strikingly smaller than its male homologue and its dendritic arborization appeared to have a simpler structure as well (Figure 15). Thirty-two well-impregnated RA_I neurons from four males and twenty-three RA_I-neurons from six females were selected from Golgi-Cox material for quantitative analysis (Tables 2 and 3). In cresyl-violet stained material, the cell bodies of RA's neuronal elements were found to be sexually dimorphic in size (Table 1) and this sexual dimorphism was also true of Golgi-Cox impregnated RA_I neurons: the RA_I neuron had an ovoid soma whose maximum diameter averaged 23.1 μ (±3.6 SD) in males and 11.8 μ (±1.2 SD) in females. The volume of the RA_I neuron's dendritic field was sexually dimorphic as well. In males, the mean dendritic field volume was 9.2×10^6 ($\pm 4.0 \times 10^6$ SD) cubic microns and in females was 1.7×10^6 ($\pm 0.8 \times 10^6$) cubic microns: both of these differences were highly significant (Table 4).

Figure 18 presents Sholl plots of dendritic density as a function of distance from the soma for male and female RA_I neurons. The two curves have a very similar shape though both their peak dendritic density and their maximum dendritic radius

Figure 17. Sholl analysis of edge-RA_I and center-RA_I neurons in the male (Golgi-Cox impregnation).

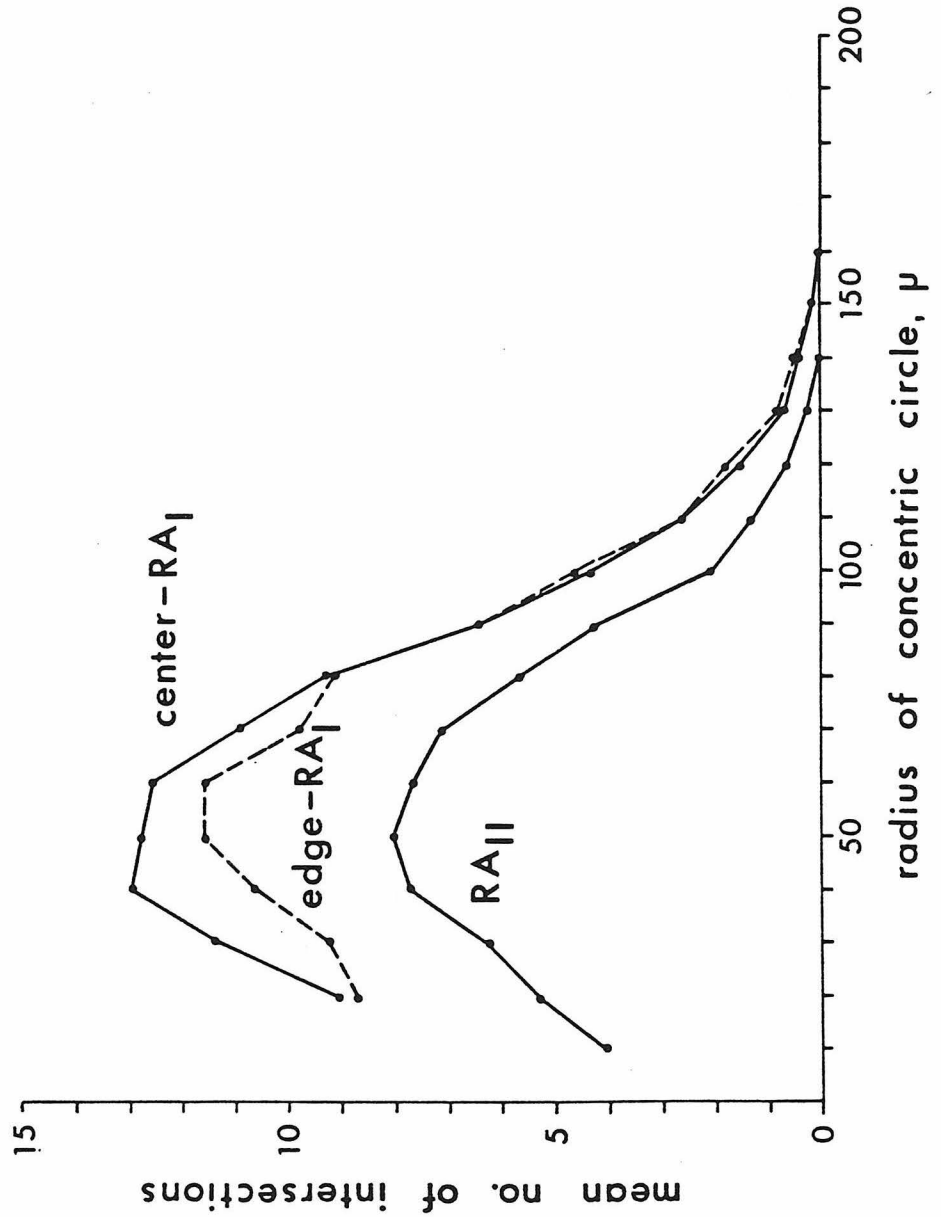

mean no. of intersections
radius of concentric circle, μ
center-RA$_I$
edge-RA$_I$
RA$_{II}$
0
5
10
15
50
100
150
200

Table 2. Male RA_I neurons: quantitative analysis of dendritic arborization (Golgi-Cox)

Male	l_s[1]	r	V_d	I	B	l_d	B/I	l_d/B
	μ	μ	$10^6 \mu^3$			μ		μ/segment
M1	25	150	14.1	7	33	1926	4.7	58
	25	120	7.2	8	28	1593	3.5	57
	23	160	17.2	7	21	1417	3.0	67
	25	140	11.5	7	21	1878	3.0	89
	18	160	17.2	8	28	1965	4.7	52
	23	120	7.2	6	24	1363	4.0	57
	23	120	7.2	6	26	1539	4.3	59
	22	110	5.6	5	25	1125	5.0	45
	22	130	9.2	8	26	1505	3.2	58
	29	130	9.2	7	19	1287	2.7	68
	23	130	9.2	6	14	1089	2.3	78
M2	20	100	4.2	6	26	1136	4.3	44
	26	110	5.6	7	21	1096	3.0	52
	25	130	9.2	6	22	1109	3.7	50
	27	110	5.6	6	22	1297	3.7	59
	23	120	7.2	5	19	843	3.8	44
	22	110	5.6	7	13	855	1.8	66
	21	110	5.6	8	22	1131	2.7	51
	21	110	5.6	6	20	1034	3.3	52
	18	110	5.6	5	23	1145	4.6	50
M3	26	130	9.2	9	41	2072	4.5	50
	21	120	7.2	7	33	1267	4.7	38
	23	150	14.1	7	53	2905	7.6	55
	18	140	11.5	5	17	921	3.4	54
	17	130	9.2	4	16	1000	4.0	62

Table 2 (continued)

M4	28	160	17.2	8	52	1988	6.5	38
	33	160	17.2	6	42	2188	7.0	52
	30	150	14.1	7	45	2325	6.4	52
	21	120	7.2	7	35	1937	5.0	55
	20	120	7.2	7	23	1188	3.3	51
	22	110	5.6	7	13	855	1.8	66
	21	110	5.6	8	22	1131	2.7	51
Mean	23.1	127	9.2	6.9	26	1442	4.0	56
±SD	±3.6	±18	±4.0	±1.6	±10	±502	±1.4	±10

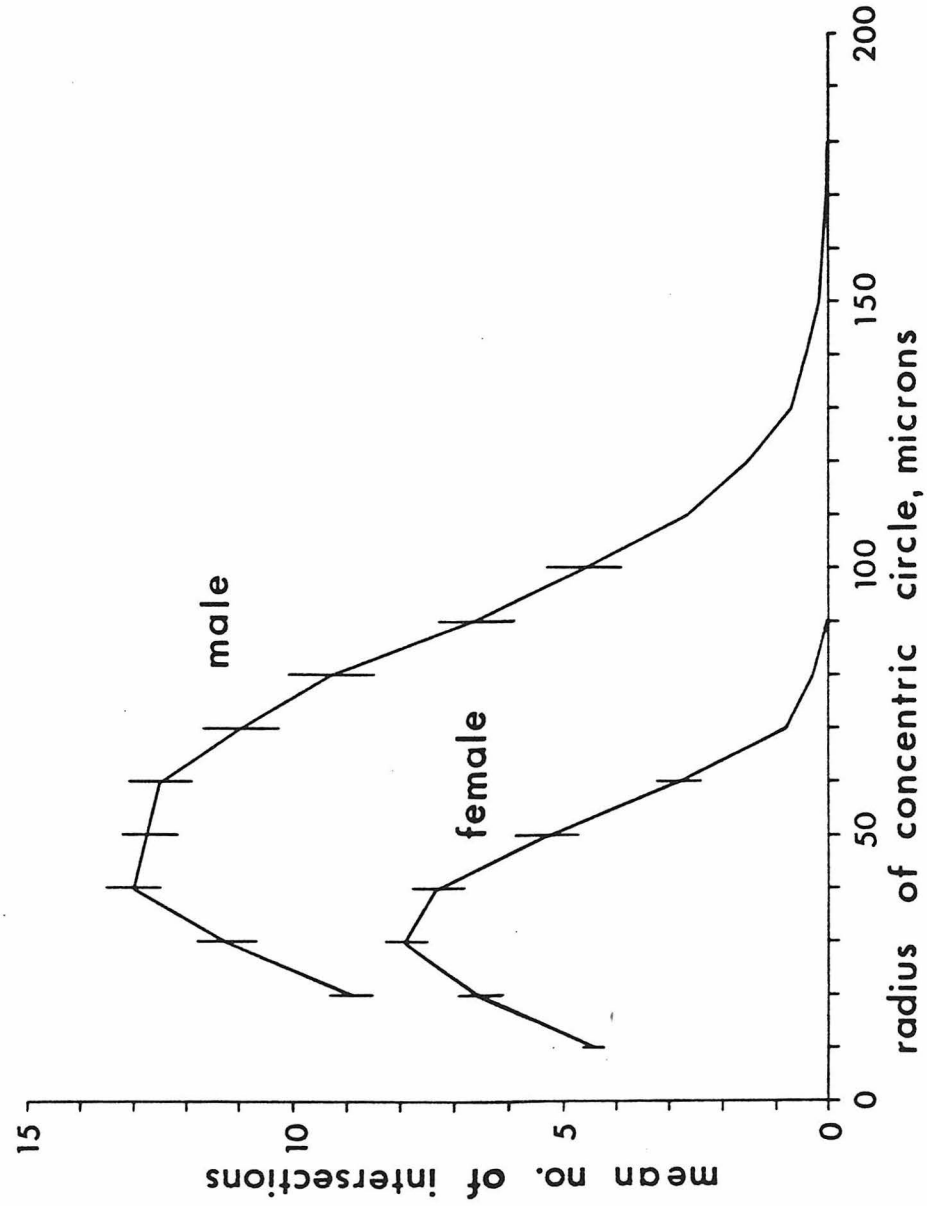
[1]Abbreviations as in Table 5.

Table 3. Female RA_I neurons: quantitative analysis of dendritic arborization (Golgi-Cox)

Female	l_s[1]	r	V_d	I	B	l_d	B/I	l_d/B
	μ	μ	$10^6 \mu^3$			μ		μ/segment
F1	10	70	1.4	3	18	505	4.5	28
	13	80	2.1	4	16	502	4.0	31
	13	90	3.1	3	14	604	3.5	43
F2	10	70	1.4	4	16	676	4.0	42
	12	80	2.1	3	14	628	3.5	45
F3	12	80	2.1	5	15	746	3.0	50
	12	70	1.4	5	13	579	2.6	45
	13	90	3.1	4	20	770	5.0	38
	12	70	1.4	4	12	587	3.0	49
	10	70	1.4	4	6	391	1.5	65
F4	12	90	3.1	4	16	906	4.0	57
	10	80	2.1	3	13	495	4.3	38
	13	70	1.4	3	11	481	3.7	44
F5	14	70	1.4	3	13	549	4.3	42
	13	90	3.1	4	12	720	3.0	60
	12	70	1.4	3	11	471	3.7	43
	12	60	0.9	4	12	472	3.0	39
	10	60	0.9	4	12	472	3.0	39
	11	60	0.9	3	15	407	5.0	27
F6	13	70	1.4	5	9	502	1.8	56
	12	60	0.9	4	12	537	3.0	45
	12	60	0.9	4	10	489	2.5	49
	12	60	0.9	4	8	375	2.0	47
Mean	11.8	73	1.7	3.8	13	559	3.4	44
±SD	±1.2	±10	±0.8	±0.7	±3	±131	±0.9	±9

[1] Abbreviations as in Table 5.

Figure 18. Sholl analysis of center-RA_I neurons in males and females. Error bars indicate $\pm$SE of the mean.


mean no. of intersections
0
5
10
15
male
female
0
50
100
150
200
radius of concentric circle, microns

were sexually dimorphic. These curves integrate the processes of dendritic branching, growth and termination. The following analysis seeks to address which of these processes determines the quantitative sex difference in the structure of the RA_I neuron's dendritic tree.

As discussed in the **Methods** section, measurement of the number of primary dendritic arms and the total number of branch segments supported by a neuron should be robust to artifact. Both of these measures of dendritic field structure were sexually dimorphic in RA_I neurons (Table 4). RA_I neurons in the female supported 3.8 ($\pm$0.7 SD) primary dendritic arms as opposed to 6.9 ($\pm$1.6 SD) primary arms supported by a male RA_I neuron. The dendritic field of female RA_I neurons also contained significantly fewer branch segments than the male: 13 ($\pm$3 SD) as compared to 26 ($\pm$10 SD).

A measure of the frequency with which the dendrites of a neuron branch is the quotient of a neuron's total number of branch segments divided by its number of primary dendritic arms (B/I). The branch segments/arm for RA_I neurons in the female was 3.4 ($\pm$0.9 SD) and for the male was 4.0 ($\pm$1.4 SD). This difference was not statistically significant (Table 4). Thus, the frequency of branching within the dendritic field of RA_I neurons was <u>not</u> sexually dimorphic, yet, since female RA_I neurons supported fewer dendritic arms, their arborization also contained fewer branch segments.

The total length of dendrite supported by RA_I neurons was also sexually dimorphic. Male RA_I neurons supported a mean of 1442 ($\pm$502 SD) linear microns of dendrite while those of the female supported only 559 ($\pm$131 SD) linear microns of dendrite (as calculated from the Sholl plot). This difference in amount of dendrite could have been simply due to the sex difference in total number of branch segments, or it could also have been due to some additional factor. These two possibilities were differentiated by comparing the average length of dendrite/branch segment (ld/B) for

Table 4. Kruskal-Wallis one-way analysis of variance by ranks of various quantitative parameters which describe the dendritic field of spinous RA_I neurons in male and female Golgi-Cox material

	Males				Females						
	M1	M2	M3	M4	F1	F2	F3	F4	F5	F6	p
l_s (μ)[1]	23.5	22.5	21.0	25	12.0	11.0	11.8	11.7	12.0	12.0	$p < 0.005$
r (μ)	124	112	134	133	80	75	76	80	68	62	$p < 0.005$
V_d ($10^6 \mu^3$)	10.4	6.0	10.2	10.6	2.2	1.8	1.9	2.2	1.4	1.0	$p < 0.001$
I	6.8	6.2	6.4	7.1	3.3	3.5	4.4	3.3	3.5	4.3	$p < 0.01$
B	24.1	20.8	32.0	33.1	16.0	15.0	13.2	13.3	12.5	9.7	$p < 0.005$
l_d (μ)	1517	1071	1633	1658	537	652	615	627	515	475	$p < 0.001$
B/I	3.7	3.4	4.8	4.7	4.0	3.7	3.0	4.0	3.7	2.3	$p > 0.1$
l_d/B (μ/segment)	62	52	52	52	34	43	49	46	42	49	$p < 0.01$

[1] Abbreviations as in Table 5. The average value of each parameter for the sample of neurons drawn from each brain is tabulated.

males and females. The mean branch segment length for RA_I neurons in males was 56 (±10 SD) linear microns and in females was 44 (±9 SD) linear microns. This difference was statistically significant (Table 4). The quantity ld/B will be a valid measure of dendritic field structure if the distribution of branch segment length for branches of different orders is fairly similar, and Lindsay (1977) has shown that this assumption holds for the basal dendrites of pyramidal neurons in rat cortex with centrifugal ordering. The sources of artifact described in the **Methods** section should have biased estimate of the mean branch segment length against the male. Yet, the sex difference in ld/B was robust enough to withstand this bias.

To summarize, the number of primary dendritic arms and the mean branch segment length of RA_I neurons are sexually dimorphic while the frequency of dendritic branching is not. This analysis is summarized in Table 5.

The aspinous RA_{II} neuron

Two characteristics distinguished RA_{II} neurons from RA_I neurons. RA_{II} neurons had aspinous dendritic arms and their axonal collaterals had a beaded appearance due to the frequent occurrence of varicosities along their length (Figure 15).

Eighteen RA_{II} neurons were obtained from male Golgi-rapid material for quantitative analysis. Golgi-rapid impregnated RA_{II} neurons supported 4.6 (±1.2 SD) primary dendritic arms which arose from an ovoid soma 15.4 μ (±1.8 SD) in diameter. Both of these measures were significantly different from Golgi-rapid impregnated male RA_I neurons (Student's *t*-test, $p < 0.05$). In Sholl plots of dendritic density as a function of distance from the soma, male RA_{II} neurons also differed from RA_I neurons in both peak dendritic density and dendritic field radius. The volume occupied by an RA_{II} neuron's dendritic field was also smaller, 5.6×10^6 cubic microns, vs. 8.5×10^6 cubic microns for RA_I neurons.

Table 5. Quantitative comparison of male and female RA_I neurons in Golgi-Cox material (mean ± SD)

	Males	Females
Length of soma (l_s), microns	23.1 ± 3.6	11.8 ± 1.2*
Radius of dendritic field (r), microns	127 ± 18	73 ± 10*
Dendritic field volume (V_d), 10^6 cubic microns	9.2 ± 4.0	1.7 ± 0.8*
Number of primary dendritic arms (I)	6.9 ± 1.6	3.8 ± 0.7*
Total number of branch segments (B)	26 ± 10	13 ± 3*
Frequency of branching (B/I)	4.0 ± 1.4	3.4 ± 0.9**
Total length of dendrite (l_d), linear microns	1442 ± 502	559 ± 131*
Mean branch segment length (l_d/B), linear microns	56 ± 10	44 ± 9*

*Significant difference, see Table 4.

**Difference is not statistically significant.

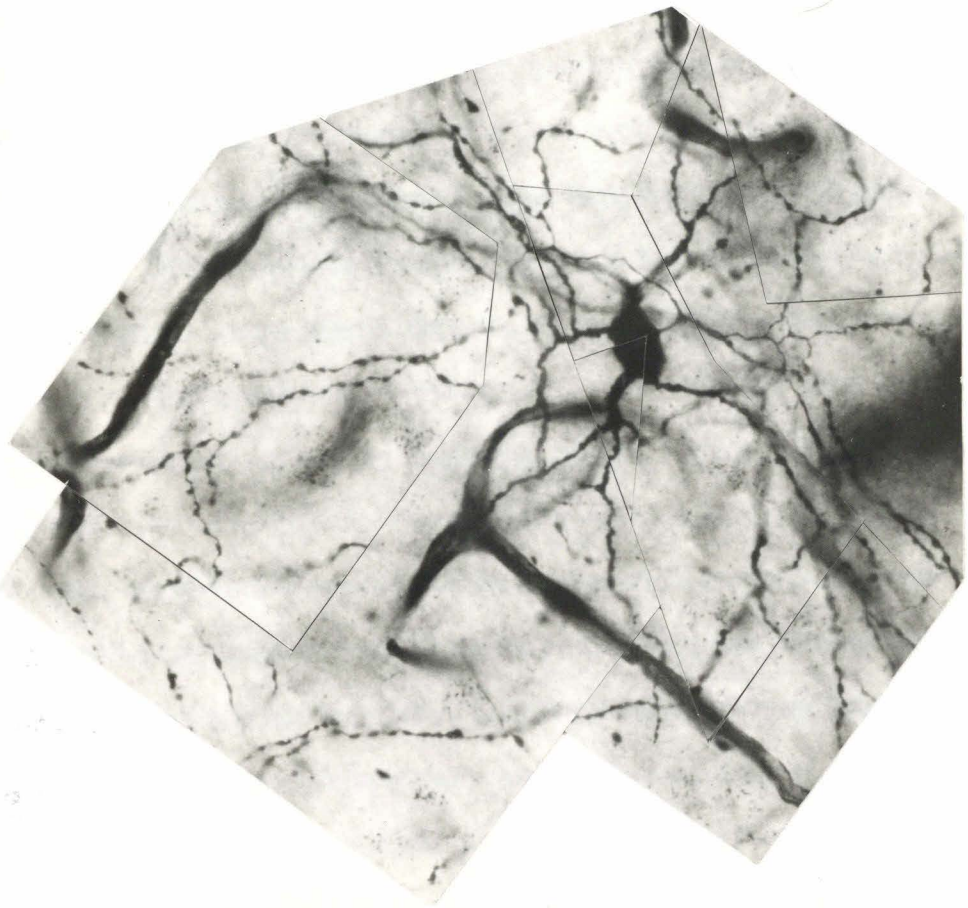
The Golgi-rapid gave an excellent impregnation of the RA_{II} neuron's axonal arborization in the male. Its axon gave off many collaterals, which branched profusely (Figure 19). The collaterals had a beaded appearance due to varicosities which were regularly spaced at 3-5 micron intervals along their length. The collaterals were of a fine caliber, approximately 0.1 microns in diameter, and swelled to a diameter of 0.5 microns at a varicosity. Axons of two RA_{II} neurons could be traced until they exited the medial border of RA.

Golgi-Cox did not impregnate RA_{II} neurons as frequently as the Golgi-rapid technique. In male Golgi-Cox material, three impregnated RA_{II} neurons were obtained and a like number were found in the female material. Qualitatively, RA_{II} neurons are also smaller in the female than in the male (Figure 15), but the small number of impregnated neurons obtained did not allow quantitative comparison.

PART 3: Hormonal Influences upon Sexual Differentiation of the Song System

Steroid effects upon somatic target tissues are of two sorts: the specific induction of a limited number of gene products, and more generalized trophic effects (Ivarie and O'Farrell, 1978; Shields, 1978). An example of the first class of effect might have been the "relabeling" of either projection or target neurons by the steroid-induced synthesis of a new spectrum of recognition molecules which would then have specified sex-specific patterns of neuronal connectivity. However, the results described above seem to discount such a possibility with respect to sexual differentiation of the zebra finch song system. An example of the second, pleiotrophic sort of effect might be the observation that a steroid hormone appears to be permissive for the maintenance of a neuronal population. In its absence, a group of neurons might die, and with them the capacity for exhibiting a sex-specific behavior.

Figure 19. Photo montage of an RA_{II} neuron from male Golgi-rapid material. Note the lack of spines along its dendrites and the frequent varicosities along its axon collaterals.



Since mammalian (Naftolin et al., 1974) and avian (Callard et al., 1978) brain tissue has the capacity to aromatize testosterone to E_2 or reduce testosterone to DHT, induction of brain sexual differentiation might be androgen-dependent (i.e., dependent upon testicular secretion of androgen during development) though it may be mediated by E_2- or DHT-dependent processes. Each of these steroids bind to distinct classes of high-affinity, low-capacity receptor proteins. The distribution of such proteins in androgen (T or DHT)- or estrogen-responsive tissues, their intracellular behavior, and pharmacological or genetic intervention with their function, suggest that such receptors mediate the genomic response to steroid hormones via specific alterations in the pattern of gene expression (Gorski and Gannon, 1976; McEwen, 1977; Wieland and Fox, 1979). DHT and E_2 are not interconverted by brain tissue (Idler, 1972) which allows aspects of sexual differentiation which might be mediated by an androgen-receptor system to be distinguished from those mediated by an estrogen-receptor system by exposure of the organism to either DHT or E_2, respectively.

The song system consists of an interconnected chain of brain nuclei which differentiate as two independent groups under the influence of either E_2 or DHT. The telencephalic song nuclei, RA, HVc, and MAN were alike in that both E_2 or DHT influenced their volume when administered to female chicks at hatching. Within RA, E_2 exerted an effect upon neuronal size, and DHT an effect upon neuronal number. HVc, RA and MAN were not influenced by E_2 or DHT in adult females. The sexual differentiation of the brainstem song nuclei, DM and nXIIts, was independent of the song nuclei in the telencephalon. Only DHT influenced their volume, not E_2, and the effect of DHT was greater when the hormone was administered to adult females rather than to female chicks. For all song nuclei, the influence of either E_2 or DHT was positive in sign, that is, administration of these hormones to females increased the volume of the various song nuclei; neither E_2 nor DHT were found to inhibit the development of any song nuclei in the male.

Methods

Zebra finch eggs were collected from the nest boxes of a zebra finch colony and transferred to an incubator. Most chicks hatched on the 14th day of incubation at 37°C and 65% relative humidity. At hatching, zebra finch chicks weighed 0.83 g (±0.15 SD) and measured approximately 15 mm in length from beak to rump. On the day of hatching, the chicks were subcutaneously implanted with a Silastic pellet which contained 50 µg of steroid (prepared as described below). This was done through a small slit in the skin over their breast muscle (this muscle was approximately 4 mm long by 2 mm wide at hatching) which was then closed with Histo-acryl Blue cement. The chicks were then transferred to the nests of Bengalese finch foster parents (Lonchura striata). The zebra finch chicks were cross-fostered because Bengalese finches were more reliable foster parents than adults of their own species. Breeding pairs of Bengalese finches were housed in individual cages, although they were in visual and acoustic contact with other zebra or Bengalese finches in the room. The young zebra finches were allowed to remain with their foster parents until sexual maturity (60-90 days of age).

Finches which were implanted with E_2 or DHT at hatching will be designated as E_2-females, or DHT-males, respectively. If a bird was implanted with hormone as an adult, the abbreviation for the hormone will be used as a suffix, e.g., female+T, male+DHT, etc.

To prepare Silastic pellets which contained steroid, a 1:5 (w/w) mixture of mortar ground steroid and RTV 738 Silastic (Dow Corning) was loaded into a 1 cc syringe, extruded through a 21-gauge needle onto a glass microscope slide to form a rope, and then cured overnight at 45°C. The final content of steroid was 50 µg/mm, and 1 mm lengths (0.6 mm dia.) were cut from the rope to be used as implants. Silastic ropes containing either testosterone, 5α-dihydrotestosterone or 17β-estradiol (Sigma) were prepared. Pellets which contained ^{3}H-labeled steroid were also

Figure 20. Release of hormone from Silastic pellets in vivo. Silastic pellets containing 50 μg of ^{3}H-labeled steroid were implanted subcutaneously over the pectoral muscle of adult female zebra finches and removed when indicated. C_t = amount of hormone in the pellet on removal, C_o = amount of hormone in the pellet before being implanted. Each data point is the average of two measurements.

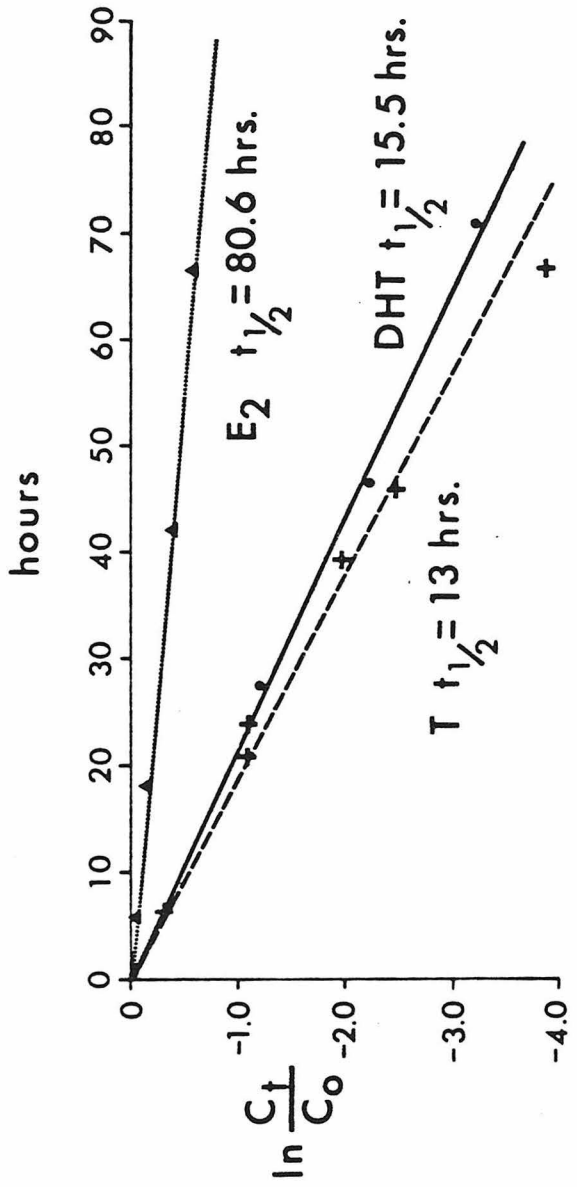

hours
0
10
20
30
40
50
60
70
80
90
$\ln \frac{C_t}{C_o}$
0
-1.0
-2.0
-3.0
-4.0
E_2 $t_{1/2}$ = 80.6 hrs.
DHT $t_{1/2}$ = 15.5 hrs.
T $t_{1/2}$ = 13 hrs.

Table 6. Half-life of hormone release

	in vivo	in vitro
E_2	80.6 hours	240 hours
DHT	15.5 hours	82 hours
T	13.0 hours	58 hours

Table 7. Survival to adulthood and sex ratio of hormone-treated and control groups

Treatment group	No. chicks	No. chicks surviving to adulthood	% Survival	Males	Females	Sex Ratio
No pellet	51	22	43	12	10	1.2
E_2	36	16	44	9	7	1.3
DHT	42	20	47	11	9	1.2

prepared. This was done by diluting 50 μCi of ^{3}H-labeled steroid in 500 μl of benzene with 50 mg of unlabeled steroid, and then Silastic pellets were prepared as described above. [1,2-^{3}H]dihydrotestosterone, [6,7-^{3}H]estradiol, and [7-^{3}H]testosterone were purchased from New England Nuclear.

To determine the rate of hormone release in vivo, a Silastic pellet containing 50 μg of steroid was subcutaneously implanted over the breast muscle of adult female zebra finches; to determine rates of release in vitro, pellets were placed in 10 ml of 1% bovine serum albumin in 0.9% NaCl. The amount of label which remained in a pellet after a given amount of time in vivo or in vitro was measured by extraction of each pellet in 15 ml of Liquifluor-toluene scintillant overnight, and then the tritium was assayed in a liquid scintillation counter.

The amount of hormone (T, DHT or E_2) remaining in the Silastic pellet fell exponentially with time in vivo or in vitro (Figure 20). The half-life of the release rate for T, DHT and E_2 in vivo or in vitro is given in Table 6. The rate of hormone diffusion from the pellet should depend on both its solubility in Silastic and on the concentration gradient which is established at the pellet/aqueous solution interface. All three steroids were released much faster in vivo than in vitro which probably reflected the efficacy of biological processes at sweeping hormone from the pellet/solution boundary layer. The difference between the release rate of E_2 and either androgen may be due to chemical differences (i.e., polarity) between these steroids which determined their rate of diffusion through the Silastic matrix.

No mortality was associated with the dose of E_2 or DHT delivered by the Silastic pellets. Unoperated controls, and groups of chicks which were implanted with Silastic pellets containing 50 μg of either DHT or E_2 had the same probability of survival to adulthood, an average of 40-50% (Table 7). Controls, and the E_2 or DHT treatment groups all had the same sex ratio as well. Before successful masculinization of RA was achieved through the use of steroid-containing Silastic pellets,

injection of E_2 in sesame oil was tried. Neither 5 μg E_2 in sesame oil injected subcutaneously into newly hatched chicks, nor sequential injection of 5 μg E_2 at hatching, 10 μg at 3 days and 15 μg at 6 days of age masculinized the cytoarchitecture of RA. When E_2-containing Silastic pellets were used, the peak amount of E_2 released on the first day after implantation was 9.6 μg and then the amount of E_2 released per day slowly declined thereafter. For a 0.8 g chick, this gave a peak dose of approximately 10 μg E_2/gm body weight. The minimum dose of TP reported to masculinize lordosis behavior in rats is 10 μg administered to 4-day-old rat pups, which when expressed in terms of body weight corresponds to about 1 μg/gm (Clemens et al., 1969).

To support song in adult E_2-females, testosterone implants in the form of Silastic capsules were used. Crystalline testosterone was tamped into Silastic tubing (Dow Corning) of 0.47 inches ID. Lengths of filled tubing containing 3 mm of crystalline steroid were then sealed at each end with RTV 738 Silastic (Dow Corning). Such implants supported E_2-females in continuous song for periods of at least 45 days.

Results

Sexual differentiation of the telencephalic song nuclei

Exposure of females to E_2 at hatching masculinized the cytoarchitecture of RA (Figure 21); it became a large-celled area within which the somal size and spacing of cells resembled that of RA in the male. In DHT-females, RA remained a small-celled area whose cytoarchitecture continued to resemble that of RA in control females. Both hormones influenced the volume of RA (Table 8). In E_2-females, RA became 5.0 times larger and in DHT-females became 1.8 times larger in volume than that of RA in normal females. Both of these effects were statistically significant (Mann-Whitney U-test, $p = 0.002$).

Exposure to E_2 at hatching also masculinized the cytoarchitecture of both MAN and HVc (Figure 22). MAN was unrecognizable in control females, and in E_2-

Figure 21. Hormonal influences upon the cytoarchitecture of RA; sagittal plane, 30 μ-thick cresyl-violet stained frozen sections, anterior is to the right. (a) male, (b) female, (c) E_2-female, (d) DHT-female.

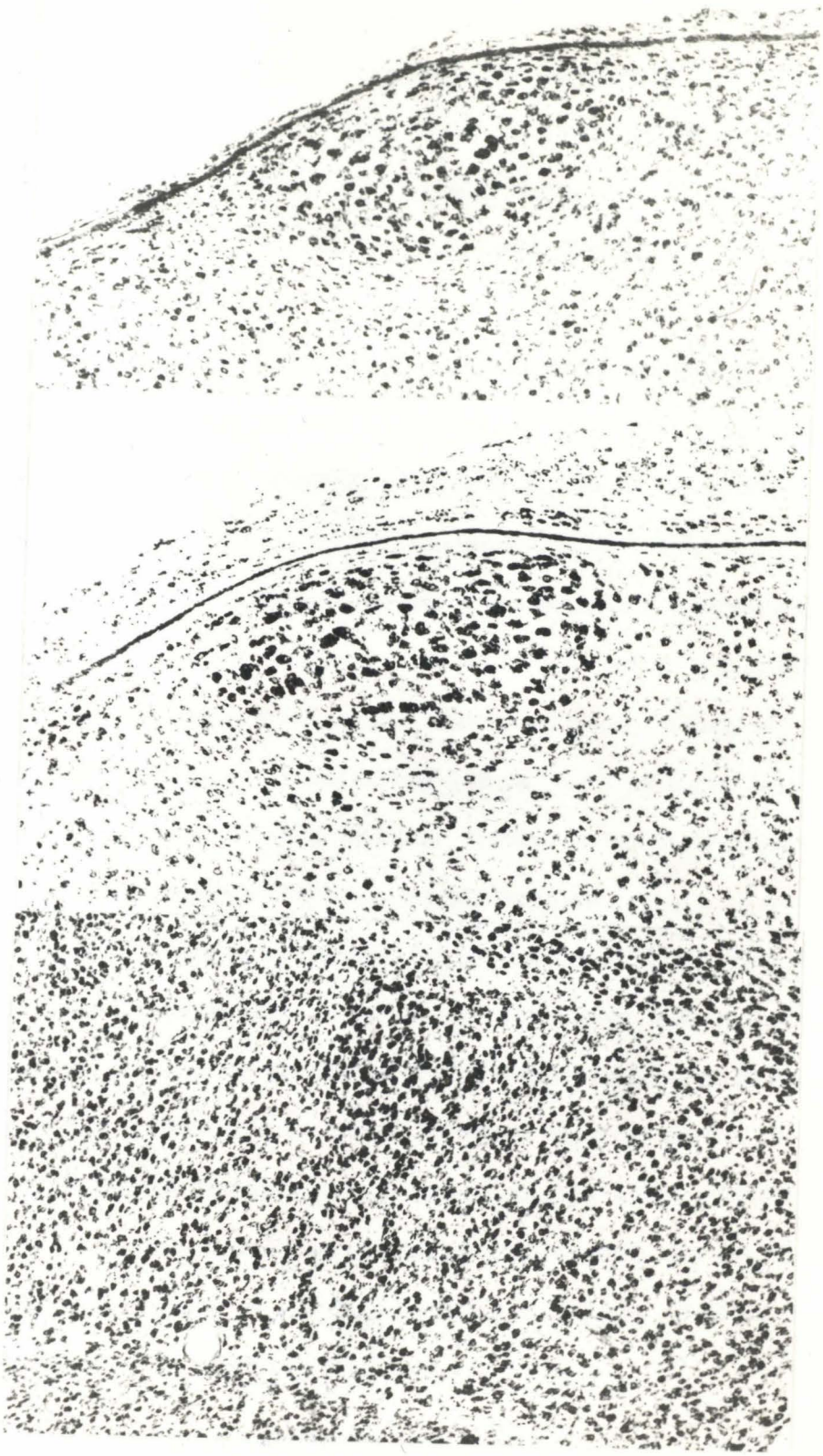
1A
1B
1C
1D

Table 8. Volumes of song system nuclei (mm^3) in experimental and control groups

	RA	HVc	MAN	DM	nXIIts
Males (n=7)	**0.285** (0.220-0.372)	**0.380** (0.264-0.466)	**0.126** (0.115-0.143)	**0.057** (0.047-0.068)	**0.123** (0.100-0.138)
Females (n=6)	**0.024** (0.018-0.029)	**0.028** (0.021-0.033)	**nm**	**0.035** (0.025-0.042)	**0.072** (0.051-0.087)
DHT-males (n=4)	**0.264** (0.102-0.357)	**0.552** (0.410-0.663)	**0.126** (0.094-0.149)	**0.058** (0.057-0.059)	**0.105** (0.099-0.109)
E_2-males (n=4)	**0.251** (0.208-0.303)	**0.444** (0.323-0.507)	**0.126** (0.090-0.161)	**0.056** (0.048-0.061)	**0.117** (0.092-0.141)
DHT-females (n=5)	**0.043** (0.035-0.047)	**0.039** (0.030-0.045)	**nm**	**0.042** (0.037-0.046)	**0.073** (0.064-0.090)
E_2-females (n=3)	**0.119** (0.101-0.133)	**0.143** (0.097-0.193)	**0.053** (0.043-0.066)	**0.035** (0.034-0.040)	**0.068** (0.061-0.081)
E_2-females+T (n=5)	**0.153** (0.126-0.190)	**0.205** (0.158-0.261)	**0.061** (0.048-0.078)	**0.053** (0.042-0.065)	**0.086** (0.076-0.100)
E_2-females+DHT (n=2)	**0.22** (0.198-0.242)	**0.308** (0.274-0.342)	**0.095** (0.075-0.116)	**0.058** (0.056-0.060)	**0.092** (0.089-0.095)
Females+DHT (n=6)	**0.028** (0.021-0.036)	**0.028** (0.024-0.035)	**nm**	**0.053** (0.042-0.067)	**0.096** (0.080-0.111)
Females+E_2 (n=4)	**0.025** (0.020-0.028)	**0.026** (0.025-0.028)	**nm**	**0.036** (0.024=0.038)	**0.069** (0.053-0.075)

nm, not measurable.

Figure 22. Hormonal influences upon the cytoarchitecture of HVc and MAN; sagittal plane, 30 μ-thick cresyl-violet stained frozen section, anterior is to the right. (Top) HVc in an E_2-female, (middle) HVc in an E_2-female+T, (bottom) MAN in an E_2-female. Compare the cytoarchitecture of these nuclei in E_2-females to that in the control males and females illustrated in Figure 4.



females it became a recognizable, compact nucleus of large-bodied cells within the anterior neostriatum. In control and DHT-females, HVc was a thin crescent of small-bodied cells, while in E_2-females, its cytoarchitecture came to resemble that of HVc in the male with respect to both the somal size and the spacing of cells within it. Like RA, the volume of HVc was similarly influenced by both E_2 and DHT at hatching: in E_2-females HVc was 5.1 times larger and in DHT-females was 1.4 times larger than in control females.

In males, neither DHT nor E_2 influenced the volume of RA or MAN when administered at hatching. However, the volume of HVc in DHT-males was significantly different from that of either control or E_2-males (Mann-Whitney U-test, $p = 0.019$). It averaged 0.552 mm^3 in DHT-males and 0.380 mm^3 in control males, while the volume of HVc in control and E_2-males was statistically equivalent.

To investigate the temporal constraints upon the ability of either E_2 or DHT to influence the volumes of the various telencephalic song nuclei, adult females were implanted with Silastic pellets containing 100 µg of either E_2 or DHT and sacrificed for histological examination 30 days later. Neither hormone treatment affected the volume of HVc, RA, or MAN (Table 8) in adult females.

The ratio of males to females for the volume of RA or HVc reported here is nearly twice as large as that reported by Nottebohm and Arnold (1976). This discrepancy can probably be attributed to differences in fixation or other histological procedures. As a control for the sex differences reported above, the volume of the nucleus ovoidalis (the thalamic relay nucleus of the avian auditory pathway) was measured in the same groups of normal males and females. No significant difference between the mean volume of ovoidalis in males, 0.077 mm^3 (0.063-0.091 mm^3) and in females, 0.078 mm^3 (0.073-0.086 mm^3) was observed.

Sexual differentiation of the brainstem song nuclei

The volume of DM or nXIIts was influenced by DHT but not by E_2 (Table 8).

When DHT was administered to females at hatching, DM attained a volume of 0.042 mm^3 which was significantly different from the volume of DM in normal females, 0.035 mm^3 (Mann-Whitney U-test, $p = 0.013$), while the volume of nXIIts was unaffected. The effect of DHT upon these nuclei was more pronounced when the hormone was administered to adult females. Silastic pellets containing 100 µg DHT were implanted over the pectoral muscle of six females and then they were sacrificed for histological examination 30 days later. In adult females+DHT, the volume of DM averaged 0.053 mm^3 which was statistically equivalent to the volume of DM in control males. The volume of nXIIts was also influenced by DHT administration to adult females. In females+DHT, nXIIts attained a volume of 0.096 mm^3 which was significantly different from that of control females (Mann-Whitney U-test, $p = 0.002$). The volume of DM and nXIIts in E_2-females or females+E_2 was equivalent to that of control females.

A second, androgen-dependent phase in the sexual differentiation of the telencephalic song nuclei

To induce song, three adult, E_2females were implanted with Silastic pellets which contained 100 µg T. The pellets were removed and replaced after a period of two weeks had elapsed, and then these females were sacrificed 30 days after initial exposure to T. Two other adult E_2-females were implanted with capsules prepared from Silastic tubing which had been filled with crystalline T. Such implants maintained these females in continuous song until their sacrifice 45 days after initial exposure to T. The volumes of HVc, RA, and MAN in adult E_2-females were all affected by exposure to T (Table 8). The differences in the volumes of these nuclei between E_2-females and adult E_2-females which received T were all statistically significant (Mann-Whitney U-test, $p < .05$). A similar increase in the volume of these nuclei was induced by exposure of adult E_2-females to DHT (two adult E_2-females were implanted with Silastic pellets which contained 100 µg DHT and then sacrificed

30 days later). The display of a response to androgen (as T or DHT) by HVc, RA, and MAN in adult E_2-females contrasts with the lack of a response by these nuclei when normal adult females are implanted with DHT. This suggests that early exposure to E_2 is a prerequisite for the development of responsiveness to androgen by these nuclei in the adult.

Cellular consequences of hormone exposure: neuronal number

DHT and E_2 influenced the sexual differentiation of RA in distinct ways. Figure 21 illustrates the cytoarchitecture of RA in adult females which had been exposed to either E_2 or DHT as chicks. The sections chosen for these photographs were ones which passed through the maximal extent of the nucleus in the sagittal plane. As can be seen, the somal size and spacing of RA neurons in E_2-females resembled the male and in DHT-females resembled the female. Since the volume of RA in E_2-females is approximately one-half that of the male, these photographs suggest that the RA of E_2-females should contain only half as many neurons as well. As for DHT-females, the volume of their RA was shown to be larger than that of normal females, which taken with the similarity in the appearance of their cyto-architecture, suggests that the RA of DHT-females may contain more neurons than that of the RA in normal females. Quantitative analysis of neuronal numbers bears this impression out.

Determination of neuronal number in RA appeared to be dominated by a DHT-mediated process. Exposure to DHT nearly doubled the number of neurons in RA from 6,950 (6,000-7,600) RA neurons in control females to 13,200 (11,900-14,200) RA neurons in DHT-females (Table 9). This difference was highly significant (Mann-Whitney U-test, $p = 0.002$). However, the number of RA neurons in DHT-females was still significantly different than the number in normal males, 16,400 (16,100-16,900) ($p = 0.001$). E_2 also influenced the number of neurons in RA, though its effect was one-tenth that of DHT. Exposure of a female to E_2 at hatching increased the number

Table 9. Measures of RA's sexual differentiation: maximum somal diameter, average volumetric spacing, and number of RA neurons

	Maximum somal diameter μm	Volumetric spacing 10^3 μ^3/neuron	Number of neurons
Males (n=7)	**17.8** (16.3-18.8)	**16.7** (13.2-20.8)	**16,400** (16,400-16,900)
E_2-females (n=3)	**15.9** (14.6-17.0)	**14.7** (13.1-16.0)	**8,120** (7,700-8,400)
E_2-females+T (n=5)	**17.3** (16.3-18.0)	**18.5** (16.2-23.8)	**8,320** (7,770-9,000)
DHT-females (n=5)	**9.3** (9.0-10.2)	**3.2** (2.7-3.6)	**13,200** (11,900-14,200)
Females (n=6)	**8.2** (7.2-8.7)	**3.4** (3.0-3.8)	**6,950** (6,000-7,600)

[1]Range: low-high

of RA neurons by 20% to 8,300 (7,700-8,250). The effect of E_2 was also statistically significant ($p = 0.001$). As will be shown in the next section, the effect of E_2 on neuron number may be related to its effect on neuronal growth, while in DHT-females, though neuronal number doubled, indices of neuronal growth such as somal size and average volumetric spacing were relatively unaffected (Table 9).

Cellular consequences of hormone exposure: neuronal size

In a previous section, the somal diameter of RA neurons was shown to be sexually dimorphic. In six males, the somal diameter of RA neurons averaged 17.8 μ (16.3-18.8) while in five females, somal diameter averaged 8.2 μ (7.2-8.7) (Table 9). The distribution of somal diameter for RA neurons in either sex was unimodal, and as can be seen in Figure 23, the distributions for males and females do not overlap. In females exposed to E_2 at hatching, the distribution of somal diameter remained unimodal, but was shifted to an average diameter of 15.9 μ (14.6-17.0, four females). The influence of E_2 on this measure of neuronal size was statistically significant (Mann-Whitney U-test, $p = 0.008$). This result indicates that all the neurons of RA responded to E_2 at hatching. If not, the distribution of somal diameter should have become bimodal with one peak at 15.9 μ (of neurons masculinized by E_2) and a second peak at 8.2 μ (corresponding to E_2-insensitive neurons which remained female-like). Exposure to DHT at hatching also influenced the somal diameter of RA neurons, though the effect was very slight. In DHT-females, somal diameter averaged 9.3 μ (9.0-10.2 μ) which was significantly different from the somal diameter of RA neurons in control females (Mann-Whitney U-test, $p = 0.008$).

To determine neuronal number, the mean density of neurons and the volume of RA was used for the calculation. The inverse of neuron density (μ^3/neuron) is a measure of their average volumetric spacing. Figure 24 presents a graph which relates the volumetric spacing of RA neurons to the volume of RA for each of the individual finches in which the number of RA neurons was measured. For each

Figure 23. Hormonal influences upon the somal size of RA neurons. Maximum somal diameter of RA neurons was measured in cresyl-violet stained, 30 μ-thick sections. (a) normal male, (b) E_2-female, (c) DHT-female, (d) normal female. Note that the distribution of somal diameter for RA neurons in each finch is unimodally distributed.

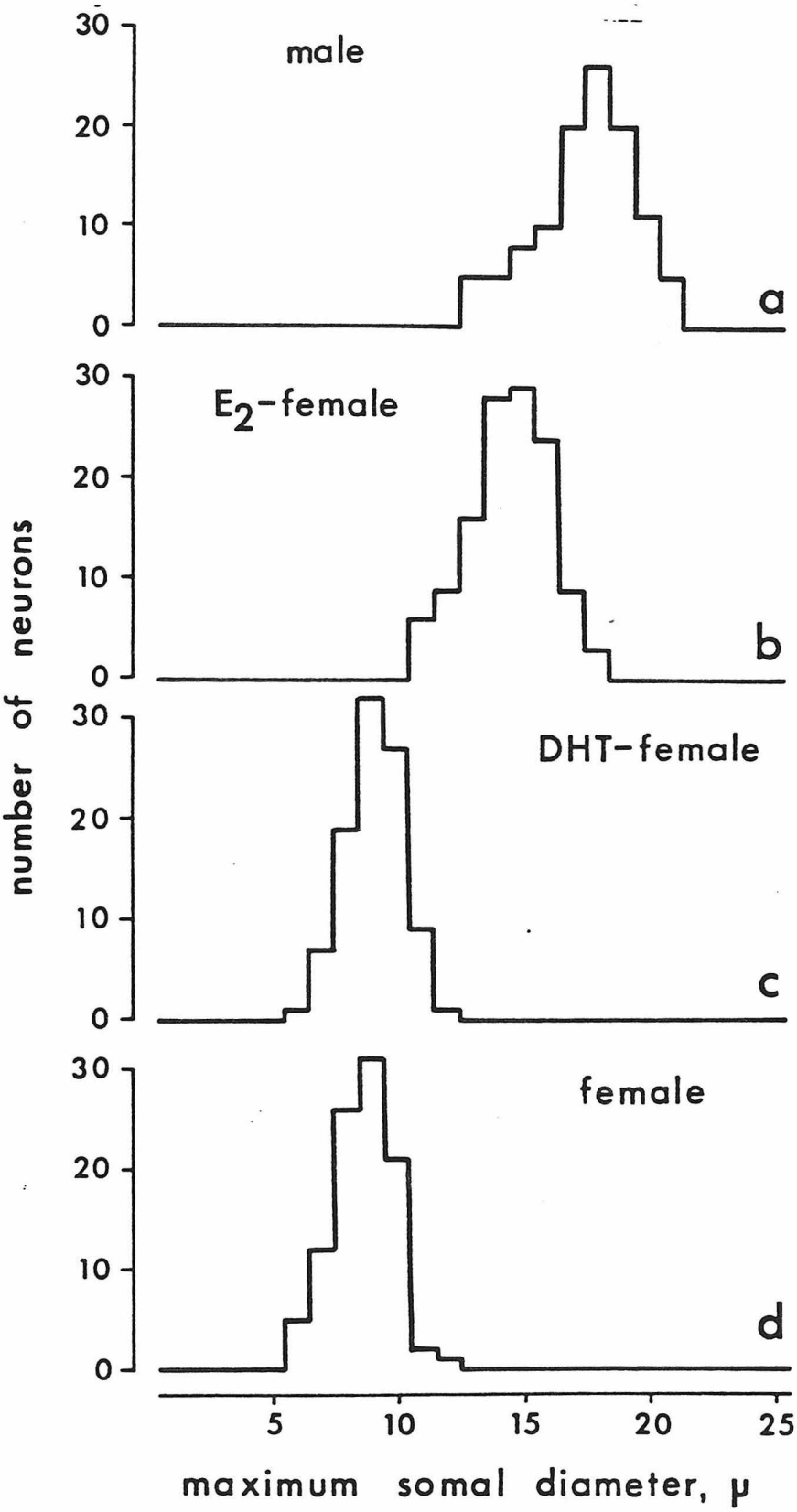
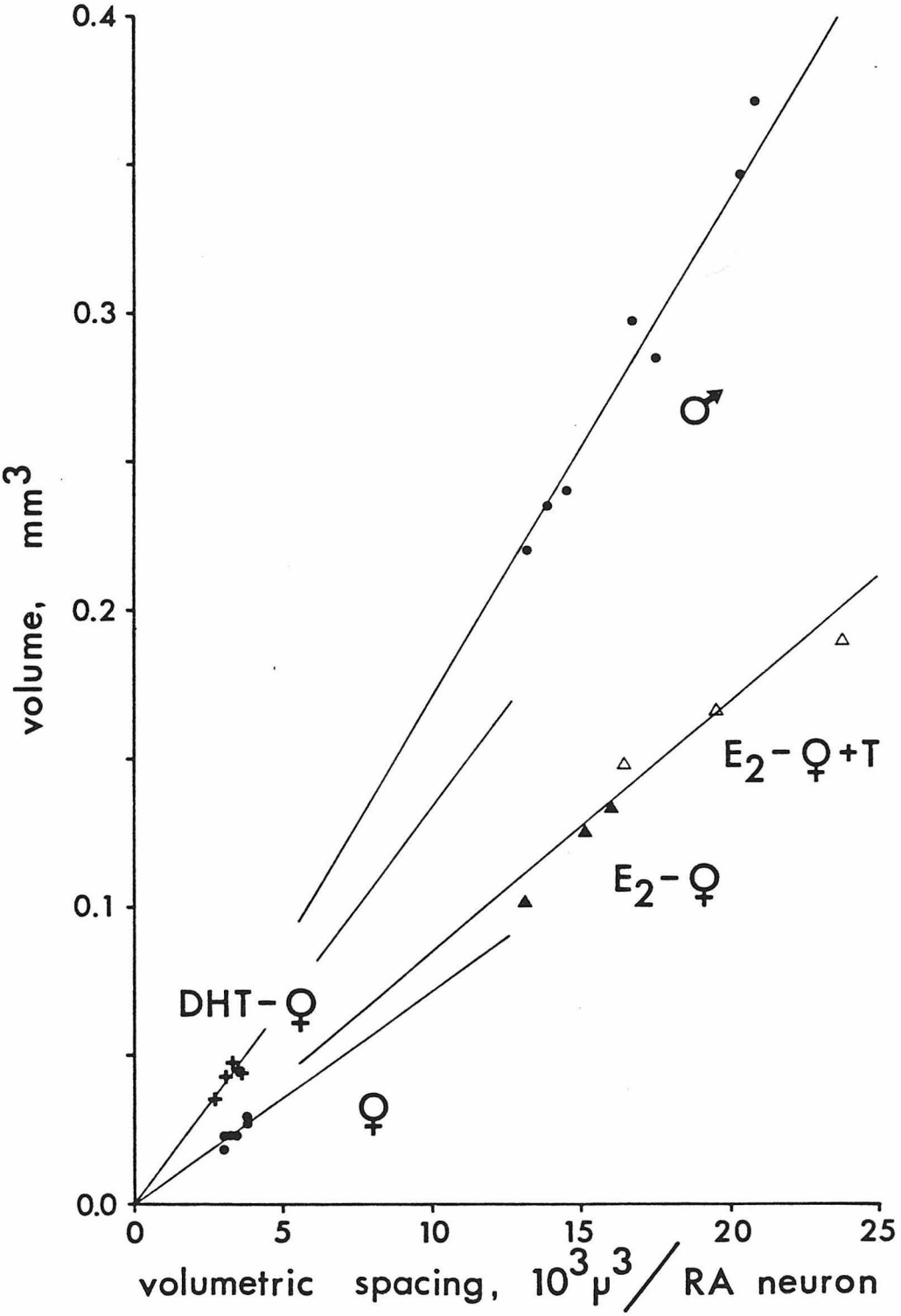

male
E_2-female
DHT-female
female
a
b
c
d
30
20
10
0
number of neurons
5
10
15
20
25
maximum somal diameter, μ

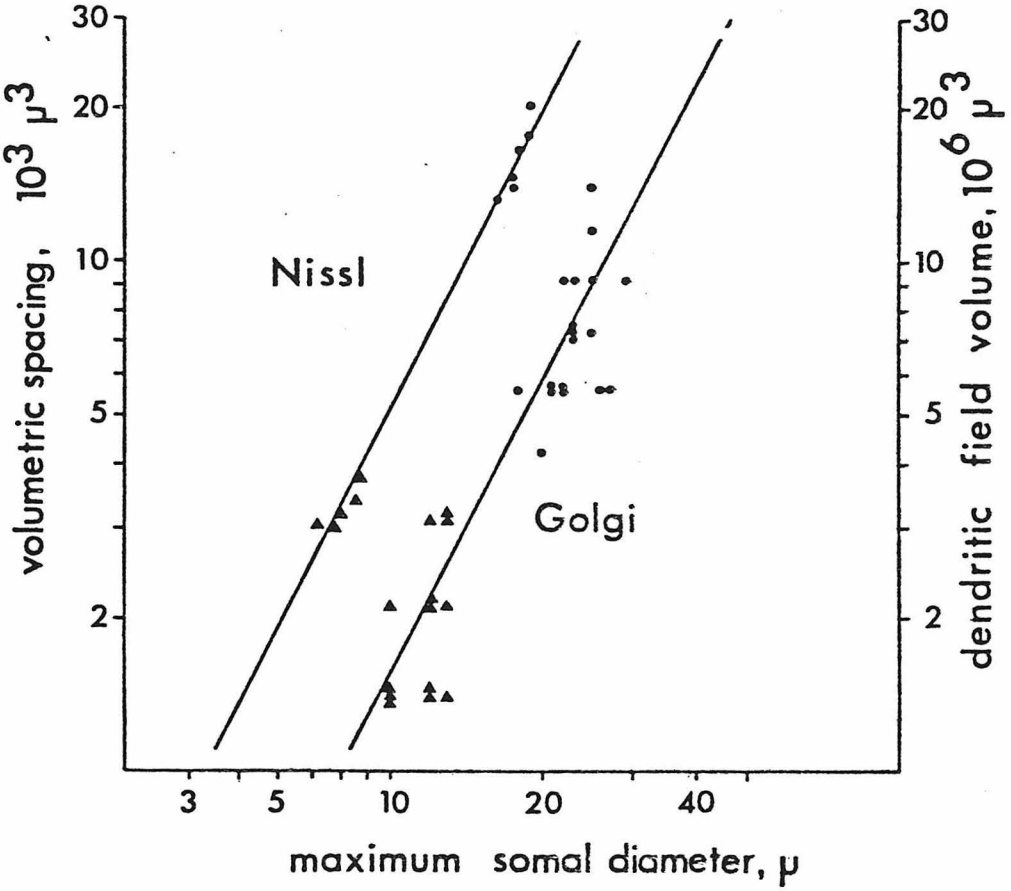
Figure 24. Graph of average volumetric spacing for RA neurons ($10^3 \mu^3$/neuron) as a function of the total volume of RA. Each point represents a single finch. The relationship of neuronal spacing to nucleus volume is linear, and the slope of the line corresponds to the number of neurons in RA.


0.4
0.3
0.2
0.1
0.0
volume, mm3
0
5
10
15
20
25
volumetric spacing, 10³μ³/ RA neuron
♂
E2-♀+T
E2-♀
DHT-♀
♀

experimental group, the relationship between the volumetric spacing of RA neurons and the volume of the nucleus was linear and intersected the origin. Its slope is equal to the number of neurons in the nucleus. DHT-females and normal females differed in their number of RA neurons, but the volumetric spacing of these neurons was not significantly different (Table 9; this can also be seen in Figure 21). The cytoarchitecture of RA in E_2-females resembled that of the male, and the volumetric spacing of neurons also fell within the range characteristic of males. Exogenous T was required to bring adult E_2-females into song, and produced a correlative expansion in the volume of RA. This increase reflected an influence of T upon the volumetric spacing of neurons in an adult E_2-female's RA and not a change in their number, as can be seen in Figure 21 (Table 9). The difference between the spacing of RA neurons in E_2-females and E_2-females which were exposed to T as adults was statistically significant (Mann-Whitney U-test, $p < 0.05$).

In Nissl-stained material from control males and females, the volumetric spacing of neurons in RA and their mean somal length were correlated (Figure 25). These two parameters were related by the power function: $Sp = 56\, l_s^{1.9}$, where Sp denotes the mean volumetric spacing of RA neurons and l_s their mean somal diameter in each individual finch. These two measures had a correlation of 0.993. In Golgi-Cox material, the dendritic field volume of an RA_I neuron and the maximum diameter of its soma were also correlated. The two were related by the power function: $V_d = 1.8 \times 10^4\, l_s^{1.95}$, where V_d denotes the dendritic field volume of an individual neuron, and l_s its somal diameter. The correlation of these two measures was 0.87. The similarity of these two relationships (Figure 25) implies that neuronal spacing in Nissl material and dendritic field volume in Golgi-Cox material are also correlated. This should not be taken to mean that "correlation implies causality." In addition to the structure of their dendritic field, the spacing of neuronal cell bodies in a brain nucleus will also be influenced by the packing of afferent axons, efferent axons and

Figure 25. The relationship of average volumetric spacing of RA neurons (in cresyl-violet stained material) and dendritic field volume (of Golgi-Cox impregnated neurons) to maximum somal diameter. Each measurement of volumetric spacing and somal diameter in Nissl material is from one finch; each measurement of dendritic field volume and somal diameter is from one Golgi-Cox impregnated neuron; triangles represent females and dots represent males.


30
20
10
5
2
volumetric spacing, 10^3 μ^3
Nissl
Golgi
3
5
10
20
40
maximum somal diameter, μ
dendritic field volume, 10^6 μ^3

their collaterals, glial cells and blood capillaries within the nucleus and by the amount of interstitial space as well. Rather, the correlation of volumetric spacing and dendritic field volume to somal diameter provides a basis for relating hormonal influences upon the cytoarchitecture of RA to the morphology of its neuronal constituents. This relationship implies that the dramatic effect of E_2 upon both the cytoarchitecture of RA and upon the somal size of RA neurons has as its correlate the restructuring of the dendritic arborization of RA neurons.

Discussion

Locus of hormone action

The efferent motor pathway for the production of song consists of several telencephalic brain nuclei whose final output to lower brain centers is channeled through RA. RA receives afferents from HVc (Nottebohm, Stokes and Leonard, 1976) and MAN, and projects in turn onto DM in the mesencephalon, and nXIIts in the medulla. DM also projects to nXIIts. The pattern of connectivity between these brain nuclei is schematized in Figure 1. This section of the thesis described the cellular consequences of androgen- and estrogen-mediated sexual differentiation within RA, as well as hormonal influences upon afferent or efferent brain centers. RA was shown to be embedded within a complex network of interacting brain nuclei, all of which are sexually dimorphic, and all of whose sexual differentiation is under the influence of androgen- or estrogen-mediated processes.

The sexual differentiation of RA is a two-step process, an early estrogen-mediated step patterns a late androgen-mediated step. Exposure of a female chick to E_2 at hatching masculinizes the cytoarchitecture of RA, i.e., with respect to the spacing of neurons in RA and the size of their somata. If later exposed to androgen (either DHT or T) when adult, a second phase in the growth of RA is initiated, which is also characterized by further expansion of the spacing between RA neurons. Androgen does not exert this effect when administered to normal, gonadally intact,

adult female zebra finches, hence early E_2 appears to be a prerequisite for responsiveness to androgen in the adult. RA is sexually dimorphic with respect to the number of neurons it contains as well as to their size and spacing. Both early E_2 and DHT influence neuronal number, though the effect of DHT is considerably larger. The effect of DHT upon neuronal number is probably not related to an effect upon the growth of RA neurons since they remain feminine with respect to their spacing and somal size in DHT-females.

The two nuclei afferent to RA, HVc and MAN, are also masculinized with respect to their cytoarchitecture by early exposure to E_2. The sexual differentiation of these nuclei is also a two-step process in which an estrogen-mediated step patterns a subsequent androgen-mediated step. HVc is also sexually dimorphic with respect to cell number, and HVc cell number is similarly enhanced in DHT-females (data not shown).

The two nuclei efferent to RA, DM and nXIIts, differentiate under the influence of androgen (T or DHT) but are not affected by E_2. These nuclei are sexually dimorphic with respect to total volume, the average volumetric spacing of neurons and their somal size, but not with respect to neuron number (data not shown). Early DHT, or DHT administered to normal, gonadally intact, adult female finches induces sexual differentiation of DM and nXIIts, and the response of these nuclei is greater in the adult than in the chick.

Biochemical consequences of sexual differentiation

In the zebra finch, Arnold et al. (1976) have shown that the motorneurons of nXIIts and perhaps cells within DM (which may correspond to the brain area labeled ICO in their Figures 8 and 9) label with [^{3}H]testosterone after injection of the hormone into adult males. Hormone uptake by the motorneurons of nXIIts is androgen specific (Arnold, 1979) and has been demonstrated in the female as well (Arnold and Saltiel, 1979). Steroid autoradiography does not quantitate levels of hormone

receptors per se, but the distribution of hormone-concentrating cells does parallel the content of hormone receptors as assayed biochemically (McEwen, 1977). Within nXIIts, androgen has been shown to influence the levels of two enzymes of neurotransmitter metabolism, acetylcholine esterase and coline acetyltransferase (Luine et al., 1980). Thus, there appears to be a conjunction between the ability of nXIIts and DM to morphologically respond to androgen, and the observation that nXIIts and perhaps DM contain neurons which possess the ability to concentrate androgen. The most compelling evidence that specific areas within the developing brain respond directly to exogenous gonadal hormones is of this sort. Toran-Allerand (1976, 1980) has shown that isolated pieces of neonatal mouse hypothalamus maintained in tissue culture can respond to exogenous sex hormones with enhanced neurite outgrowth, and that this area of outgrowth is localized to the region of the explant in which a subpopulation of hormone-concentrating cells could be identified by steroid autoradiography (Toran-Allerand et al., 1980).

Though Arnold and Saltiel (1979) found no sex difference in the number of cells within nXIIts or ICO which labeled with [^{3}H]testosterone or its metabolites, they did observe a striking sex difference in the number of HVc and MAN cells which labeled with hormone. In both HVc and MAN less than one-third as many cells labeled in the female as compared to the male. The relative paucity of steroid-concentrating cells within these nuclei in adult females correlates with the inability of exogenous androgen to stimulate their growth, and contrasts with the ability of HVc and MAN of adult E_2-females to respond to androgen. This suggests that an effect of early estrogen exposure may be to modulate the efficacy of an androgen receptor system within HVc and MAN of the adult, and that this effect is crucial for differentiation of the song system's functional capacity for androgen-mediated activation of song.

Several examples of cross-regulation of receptor levels for one hormone by a second are known in differentiated, somatic tissues (Tata, 1977). Examples of steroid

receptor cross-regulation as an event of differentiation are less common. In the hypothalamus of rats and guinea pigs, estrogen stimulation has been shown to induce appearance of progesterone receptor (Leavitt, 1977; Moguilewsky and Raynaud, 1977), and this molecular process is proposed to play a role in progesterone's facilitation of lordosis behavior in estrogen-primed female rats. In castrate, adult male rats, septal lesion followed by treatment with estrogen renders later priming with low doses of estrogen capable of inducing lordosis behavior (Nance et al., 1975). In such males, no facilitation of lordosis behavior by progesterone is observed. Also, when castrate, adult male rats are primed with high, long-term doses of estrogen, a female-like level of lordosis responding is established, yet, progesterone facilitation of the behavior is not observed (Davidson, 1969). Thus, there exists a sex difference iin the ability of progesterone to facilitate lordosis behavior which may imply a sex difference in the mechanism which mediates progesterone's action.

The temporal constraints upon the ability of E_2 to induce sexual differentiation of the telencephalic song nuclei appeared to be more stringent than those upon the ability of androgen to elicit sexual differentiation of the brainstem song nuclei. Cellular accumulation of [^{3}H]estradiol is not observed in HVc, RA or MAN of adult males (Arnold, 1979) which suggests that the sensitive period to estrogen may close due to disappearance of an estrogen-receptor system. In contrast, the lack of a limited sensitive period in DM may imply the presence of an androgen-receptor system both at hatching and in the adult.

Hormonal influences upon neuronal number

Exposure of female chicks to DHT was shown to produce a doubling in the number of RA neurons. If RA neurons are primary targets of DHT action at hatching, then DHT might function as a mitogenic stimulus and enhance neuronal proliferation, or else DHT might be a factor permissive for the maintenance of RA neurons and decrease the amount of (putative) cell death in RA.

In a preliminary series of experiments, newly hatched zebra finch chicks of both sexes were injected with ^{3}H-thymidine and then sacrificed for autoradiography as adults. Cells which are actively replicating their DNA at the time of injection incorporate labeled thymidine into their DNA (Sidman, 1970). At hatching, the zebra finch cerebellum has a pronounced external granule cell layer, and as a consequence, heavy accumulation of label was observed over granule cells in the adult brains processed for autoradiography. However, no incorporation of label by RA neurons was observed after exposure to ^{3}H-thymidine at hatching (Gurney and Konishi, 1978). Thus, the neurons of RA are post-mitotic at the time when DHT can influence their number.

Although it is possible that DHT reinitiates neuronal proliferation, it seems more likely that DHT acts to decrease the amount of cell death in RA. The locus of this effect need not be the RA neurons themselves, as both efferent targets of RA, DM and nXIIts, also differentiate under the influence of DHT with respect to their cytoarchitectonic masculinization. The target areas of neuronal populations are known to exert powerful effects on their maintenance. When the target area of a neuronal population is completely removed, death of all the neurons in the afferent population results (Cowan and Clarke, 1976; Landmesser and Pilar, 1976). Target overloading also enhances cell death (Clarke and Cowan, 1975; Detwiler, 1920) while expanding the target field (i.e., by grafting a supernumary limb onto a chick embryo) can reduce cell death (Hollyday and Hamburger, 1976). Thus, the influence of DHT on the number of RA neurons may as likely be mediated transynaptically (an increase in cell size within DM or nXIIts would have as its correlate an expansion of synaptic space, or DHT might enhance the synthesis of a trophic factor(s) permissive for the maintenance of RA neurons) as through a direct (intracellular) action upon RA neurons.

Hormonal influences upon dendritic architecture

Gonadal hormones do not influence the developmental program which specifies an RA neuron's anatomical identity. In both sexes, RA contains the same cell types and completes the same pattern of efferent projections to DM and nXIIts. E_2 and DHT exert quantitative effects upon their target populations: E_2 influences the size, and DHT the number of RA neurons.

The size of the dendritic field supported by both RA_I and RA_{II} neurons is sexually dimorphic. Quantitative measures of the dendritic field of RA_I neurons which differ between the sexes are the number of primary dendritic arms, and the mean branch segment length, while the frequency of dendritic branching is not sexually dimorphic. In neocortex of rat (Eayrs and Goodhead, 1959), rabbit (Schade et al., 1964), and human (Schade et al., 1962), the mean number of dendrites arising from the soma of pyramidal cells matures very early. Subsequent development of the basal dendritic tree is characterized by an increase in the number of branch segments, and an increase in the mean branch segment length. Based on topological analysis of the dendritic arborization of mature cortical pyramidal cells and of cerebellar Purkinje cells in the rat, Hollingworth and Berry (1975) proposed that branching is only originated from terminal branch segments. To the extent that neuritic outgrowth from sympathetic neurons in vitro applies to dendritic development in vivo, the results of Bray (1970) also suggest that new membrane is only added at the tips of neuronal processes, and that new branch points are generated by the bifuration of growth cones (Bray and Bunge, 1973).

The sequence of dendritic field maturation for these diverse types of neurons suggests that determination of the number of primary dendrites may also be an early event in the ontogeny of RA_I neurons. This feature of the RA_I neuron's dendritic field structure may be specified through the action of E_2. Since the frequency of branching/dendritic arm is the same in both sexes, each additional

dendritic arm should produce a stepwise increase in the amount of dendrite supported by an RA_I neuron and thus a consequent stepwise increase in the volume of its dendritic field. This hypothetical effect of E_2 would explain the abrupt transition in neuronal spacing from that in females to that in males or E_2-females. On the other hand, the influence of T on neuronal spacing in adult E_2-females is graded, and may indicate addition or extension of terminal branches within an RA neuron's dendritic tree.

Spatial segregation of afferents is believed to influence the orientation of the dendritic field elaborated by cerebellar Purkinje cells (Bradley and Berry, 1976; Rakic, 1974) and barrel field neurons of mouse somato-sensory cortex (Van der Loos and Woolsey, 1973; Woolsey et al., 1975). The difference in the shape of the dendritic field supported by edge- and center-RA_I neurons may reflect a similar influence of afferents from MAN or HVc on dendritic field structure. Edge-RA_I neurons also support one less dendritic arm than center-RA_I neurons. Ramon y Cajal (1929) believed that neurons initially elaborated an overabundance of dendritic processes, and subsequently resorbed many. Initial elaboration of somatic branchlets by Purkinje cells is also followed by their subsequent resorption, and may be a consequence of their deafferentation as climbing fiber synapses move onto the Purkinje cell's apical dendrites (Berry and Bradley, 1976). That edge-RA_I neurons resorb processes that fail to find afferents from HVc or MAN is an intriguing possibility.

SECTION III

Cellular Analysis of the Critical Period for Sexual Differentiation in the Zebra Finch Song System

Critical periods of sensitivity to external influences are exhibited during brain sexual differentiation (Phoenix et al., 1959; Gorski, 1968; Clemens et al., 1969; Clemens et al., 1978), the ontogeny of binocularity and selectivity for orientation in the visual cortex (Wiesel and Hubel, 1965; Blakemore and Cooper, 1970), and immunogenic tolerance to self-antigenic determinants (Lederberg, 1959; Metcalf and Klinman, 1975). The close of the critical period in each of these systems is marked by their transition from a responsive to an unresponsive state (Brockes, 1979). To determine whether this transition is timed autonomously, or whether some extracellular agent controls the termination of the critical period, two experimental strategies might be employed. One approach is to search for soluble agents of cell-cell interactions which either produce (Kasamatsu and Pettigrew, 1978), or reverse the unresponsive state (Kasamatsu et al., 1979), and then attempt to relate such effects to the close of the critical period in the unperturbed system. An alternative strategy is to examine the kinetics of a cell population's transition from responsive to unresponsive, with the hope that a mathematical description of the process will yield insight into its biological mechanism.

In the zebra finch song system, it has proved possible to follow over time the ability of individual neurons within an identified population to differentiate under the influence of 17β-estradiol (E_2). The kinetic analysis of that response, which will be presented in this section of the thesis, suggests that the closing of the critical period for E_2-mediated sexual differentiation in these neurons may be regulated autonomously.

This section also focuses upon the nucleus robustus archistriatalis (RA), which receives afferents from two other telencephalic nuclei, the hyperstriatum ventrale pars caudale (HVc) and the magnocellular nucleus of the anterior neostriatum (MAN). RA projects in turn to two brainstem nuclei, the dorso-medial intercollicular nucleus (DM) and the tracheo-syringeal portion of the hypoglossal

nucleus (nXIIts). The previous section demonstrated that exposure of female chicks to E_2 at hatching masculinizes the cytoarchitecture of RA, while when administered to normal, gonadally intact adult females, E_2 has no effect (Table 8).

Methods

To investigate the temporal constraints on responsiveness to E_2, female finches were implanted subcutaneously with a Silastic pellet containing 50 µg of E_2 either at hatching, at 3, 6, 9, 15, 30 or 40 days of age, or as adults (at least one year old). Each treatment group contained 3-4 females for a total of 28 finches. After implantation, the amount of E_2 remaining in the pellet fell exponentially with a half-life of 80.5 hours as discussed previously. Thus, a peak of 9.6 µg E_2 was released on the first day in vivo, and the amount of hormone released fell thereafter. Newly hatched chicks weighed 0.62 g (±0.05 g SD), and thus the peak dose of E_2 which they received was 15 µg E_2/g body weight/day. By 15 days, their body weight had increased 10-fold to 9.2 g (±1.0 g SD) and reached 12.1 g (±1.2 g SD) in the adult. Thus, 15-, 30-, 40-day and adult females received roughly equivalent doses of E_2 with their peak exposure averaging 1 µg E_2/g body weight/day. Zebra finches are precocial at hatching, leave the nest by 15-20 days of age, and are fledged by 30-40 days of age. The male begins to sing between 25-35 days of age and females may begin to lay eggs as early as 60 days of age. All birds were sacrificed for histological examination when sexually mature at 90 days of age with the exception of the adult females which were sacrificed 30 days after exposure to E_2. Histological protocols have been described in the previous section.

Results

A morphological assay for the ability of neurons to respond to E_2 was provided by measurement of their size. As described above, related measures of neuronal size in Nissl material for RA neurons were maximum somal diameter (the

soma of RA varied from round to ovoid) and average volumetric spacing (μ^3/neuron). Both of these parameters were sexually dimorphic. In six males, the somal diameter of RA neurons averaged 17.8 μ (range: 16.3-18.8) and their volumetric spacing averaged 16.7 x 10^3 μ^3/neuron (13.2-20.8) while in five females, somal diameter averaged 8.2 μ (7.2-8.7) and volumetric spacing averaged 3.4 x 10^3 μ^3/neuron (3.0-3.8). Both of these sex differences were statistically significant (Mann-Whitney U-test, $p = 0.002$).

The distribution of somal diameter for RA neurons in either sex was unimodal (Figure 27A). In females exposed to E_2 at hatching, the distribution of somal diameter remained unimodal, but was shifted to an average diameter of 15.9 μ (14.6-17.0, four females). The influence of E_2 on this measure of neuronal size was statistically significant (Mann-Whitney U-test, $p = 0.008$). This result indicates that all the neurons of RA respond to E_2 at hatching. If not, the distribution of somal diameter should have become bimodal with one peak at 15.9 μ (of neurons masculinized by E_2) and a second peak at 8.2 μ (corresponding to E_2-insensitive neurons which remained female-like).

In females exposed to E_2 at 40 days of age, the pattern of response to E_2 was quite different (Figure 27A). Neurons which responded to E_2 with an increase in somal size comprised only a fraction of the total neuronal population in RA, and were confined within the core of the nucleus (Figure 26). This domain of large-bodied neurons was sharply demarcated from a domain of small-bodied neurons which formed the rim of RA. The somal diameter of neurons in the rim domain averaged 8.7 μ (8.5-9.0, three females), which was equivalent in size to RA neurons in control adult females (Mann-Whitney U-test, $p = 0.35$). As a result of exposure to E_2, approximately 80% of the neurons within the core domain became masculinized with respect to somal diameter, while the rest remained female-like (Figure 27A). The distribution of somal diameter in the core domain was thus bimodal, and the mean

Figure 26. Cytoarchitecture of RA. (A) Normal male, (B) female exposed to E_2 at hatching, (C) female exposed to E_2 at 15 days of age, (D) female exposed to E_2 at 40 days of age, (E) normal adult female. Note that the cytoarchitecture of A, B, and C resemble each other. In D, the RA has a core domain of well-separated, large-bodied cells which is surrounded by a rim of small-bodied cells (indicated by the arrow). The cytoarchitecture of the core domain resembles that of RA in the male (A) or of the other E_2-treated females, (B and C), whereas the cytoarchitecture of the rim domain resembles that of the normal female (E).

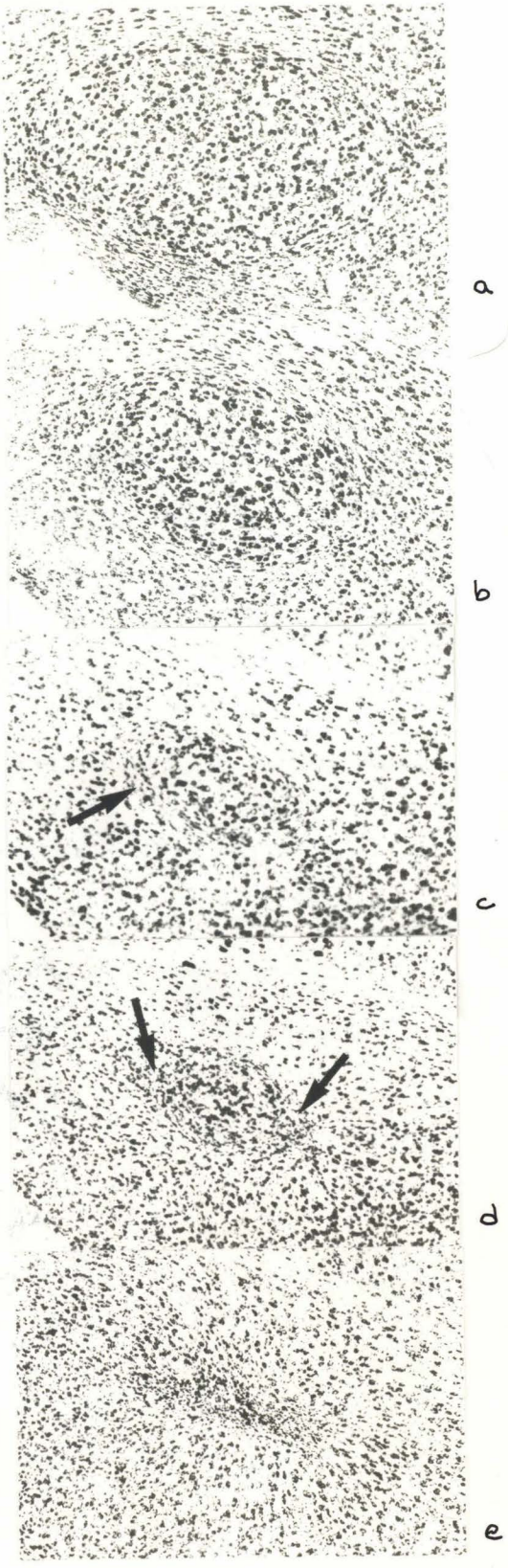
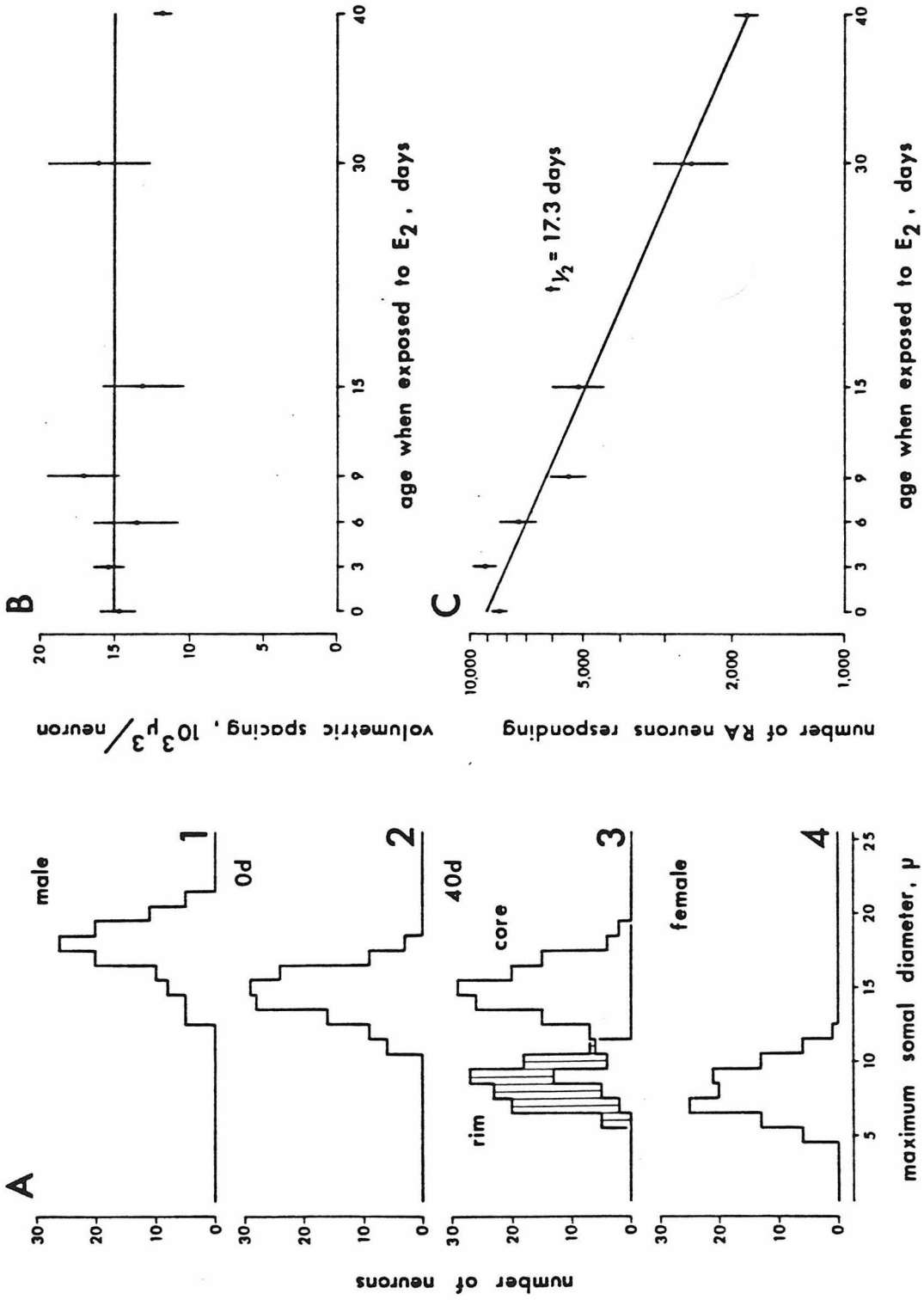

a
b
c
d
e

Figure 27. Measures of the neuronal response to E_2. (A) The distribution of maximum somal diameter for RA neurons in an adult male (1), a female exposed to E_2 at hatching (2), a female exposed to E_2 at 40 days of age (3), and a normal adult female (4). (B) The average volumetric spacing of neurons within the masculinized domain of RA as a function of the age at which a female was impalnted with E_2. (C) The number of neurons responding to E_2 as a function of age when the female was implanted with E_2.


A
male
0d
40d
rim
core
female
1
2
3
4
number of neurons
maximum somal diameter, μ
B
volumetric spacing, $10^3\mu^3$/neuron
age when exposed to E_2, days
C
number of RA neurons responding
$t_{1/2}$ = 17.3 days
age when exposed to E_2, days

somal diameter of the masculinized neurons averaged 14.6 μ (14.0-15.4, three females). The difference in mean somal diameter between masculinized core neurons and nonresponsive rim neurons in each female which had been exposed to E_2 at 40 days of age was highly significant (Student's t-test, $p < 0.001$). The distribution of somal diameter for RA neurons in untreated females which were sacrificed at 40 days of age was unimodal and equivalent in size to that of normal adult females (data not shown). It is unclear whether the separation of RA into male- and female-like domains represented reorganization of the nucleus after exposure to E_2 or a separation of responsive and nonresponsive neurons which reflected a spatial gradient in neuronal birth date, ingrowth of afferents, or some other factor.

These results suggested that the number of RA neurons able to respond to E_2 declined with age, yet that the magnitude of an individual neuron's response did not. To test the validity of this conclusion, four parameters of the response to E_2 were measured in females exposed to E_2 at different ages. These were: the total volume attained by the masculinized core domain within RA, the average volumetric spacing for masculinized neurons within this core, their mean somal diameter, and finally, their number. The magnitude of the increase in volumetric spacing between masculinized neurons (Figure 27B) or the increase in their somal size, induced as a result of exposure to E_2, was found to be independent of the female's age when implanted with hormone. However, both the number of neurons which became masculinized after exposure to E_2 (Figure 27C), and as a result, the volume which the masculinized core domain within RA attained, fell exponentially with increasing age of the female at implantation. RA neurons lost E_2-responsiveness with a half-life of 17.3 days. Linear regression of age at implant with ln [number of responding neurons] had a correlation of -0.964. This may be compared to regression of 1/age with the number of responding neurons which had a correlation of 0.619, and age with number which had a correlation of -0.89. Therefore, exponential decay appears to be the best mathematical model of this process.

The relationship of age at E_2-implantation to ln [number of responding RA neurons] described by the graph in Figure 27C is linear, not biphasic. This has two implications. First, it implies that the number of responding neurons was insensitive to a 10-fold difference in E_2 dose, in that females implanted at 15, 30, or 40 days of age were exposed to roughly equivalent doses of E_2, while they received only one-tenth the dose of females who were implanted with E_2 at hatching or at 3 days of age. Yet, the number of neurons responding to E_2 between 15-40 days of age or between 0-9 days of age fit the relationship described by the graph in Figure 27C equally well. Second, based on the analysis of Golgi-impregnated material described above, RA contains two classes of neurons which were both masculinized by exposure to E_2 at hatching (Figure 27A). If the time course with which each class of RA neuron lost its responsiveness to E_2 had differed greatly, then a simple relationship between age at implant and the number of responding neurons would have been unlikely. Instead, the response of both classes of RA neurons was apparently modulated in a similar way over time.

The song of adult male zebra finches is dependent upon testosterone (T) while normal adult females are insensitive to the hormone (Pröve, 1974). As will be described in Section IV of the thesis, T also activates song in females which had been exposed to E_2 at hatching. To investigate the relationship between the number of masculinized neurons in RA and the female's capacity for song, the influence of T on the vocal behavior of females which had been exposed to E_2 at hatching or at 7, 15, or 30 days of age was assessed. All groups of females produced novel, androgen-dependent vocalizations after being implanted with Silastic pellets containing 100 μg T, though those females which were exposed to E_2 earlier in development produced more male-like song. This indicated that the E_2-mediated morphological response was correlated to induction of sensitivity to androgen as assayed behaviorally.

Discussion

The data reported here suggest several testable hypotheses as to the nature of the mechanism which times the critical period transition of RA neurons from E_2-responsive to E_2-unresponsive. The segregation of RA into domains of male- and female-like cytoarchitecture when E_2 is administered to older females may be due to a reorganization in the packing of neurons within the nucleus which is related to their growth response (a geometric hypothesis) or else it may reflect spatial gradients due to ingrowth of afferents, or of neuronal birthdate, or of some other factor. If the spatial organization of responsive cells reflects processes which are also involved in timing the critical period transition, then these also need to account for the kinetics with which E_2-responsiveness is lost by the neuronal population within RA. Thus, it seems unlikely that the ingrowth of afferents into RA would be an exponential process, and therefore strongly implicated in the timing of the critical period. The spatial heterogeneity of E_2-responsive neurons within RA, and the stochastic behavior of the neuronal population, also make unlikely simple, "extracellular timing" models of the sort which would assume that all RA neurons are exposed to an homogeneous environment, and that the closing of their critical period is signalled by some systematic change in their environment which develops with age.

One tenable model assumes that a neuron's birthdate deterministically programs its transition from E_2-responsive to E_2-unresponsive. To account for the exponential loss of E_2-responsiveness from the population, this would require that RA neurons are also generated during ontogeny with exponential kinetics. That is, the bulk of RA neurons should become post-mitotic at a specific date, and then the rate of their generation should fall exponentially. The last born RA neurons should thus be the last to lose the capacity to differentiate under the influence of E_2.

An alternative "autonomous timing" model derives from the stochastic behavior of the neuronal population in RA. It assumes that the population of RA

neurons is homogeneous at hatching, and that a constant, intrinsic probability is associated with the transition of each neuron from an E_2-responsive to an E_2-unresponsive state. Intracellular estrogen-receptors are believed to mediate aspects of sexual differentiation in mammals at the genomic level (McEwen et al., 1977), and processes of this character are postulated in the zebra finch as well (as discussed above). Arnold has shown that neurons in RA of the adult male zebra finch concentrate T or DHT but not E_2 by using the technique of steroid autoradiography (Arnold, 1979). This may indicate that the transition of RA neurons from E_2-responsive to E_2-unresponsive is marked by the inactivation of their receptor mechanism for E_2 and leads to a biochemical model of the critical period. If we assume that all RA neurons are homogeneous with respect to E_2-receptor content at hatching, that E_2-receptor synthesis then ceases, and that a critical, absolute amount of occupied receptor was required to elicit sexual differentiation, then if receptor degradation proceeded with first-order kinetics, a neuron would eventually pass that threshold and lose the capacity for E_2-mediated sexual differentiation. However, for such a model to fit the data reported here, the number of occupied E_2-receptors/neuron sufficient to elicit sexual differentiation must be very low (one per cell), otherwise the stochastic loss of receptor within each cell would then cause the entire population of cells to pass the transition to E_2-unresponsive in approximate synchrony.

The data presented above suggest several testable hypotheses, and seem to indicate that the mechanism which times the critical period transition of RA neurons from E_2-responsive to E_2-unresponsive is likely to be autonomous to these cells. Such transitions, based on stochastic models, have also been postulated for the commitment to erythroid differentiation by Friend erythroleukemia cells (Gusella et al., 1976), as well as for the cell cycle transition from G_1 to S phase (Smith and Martin, 1973).

SECTION IV

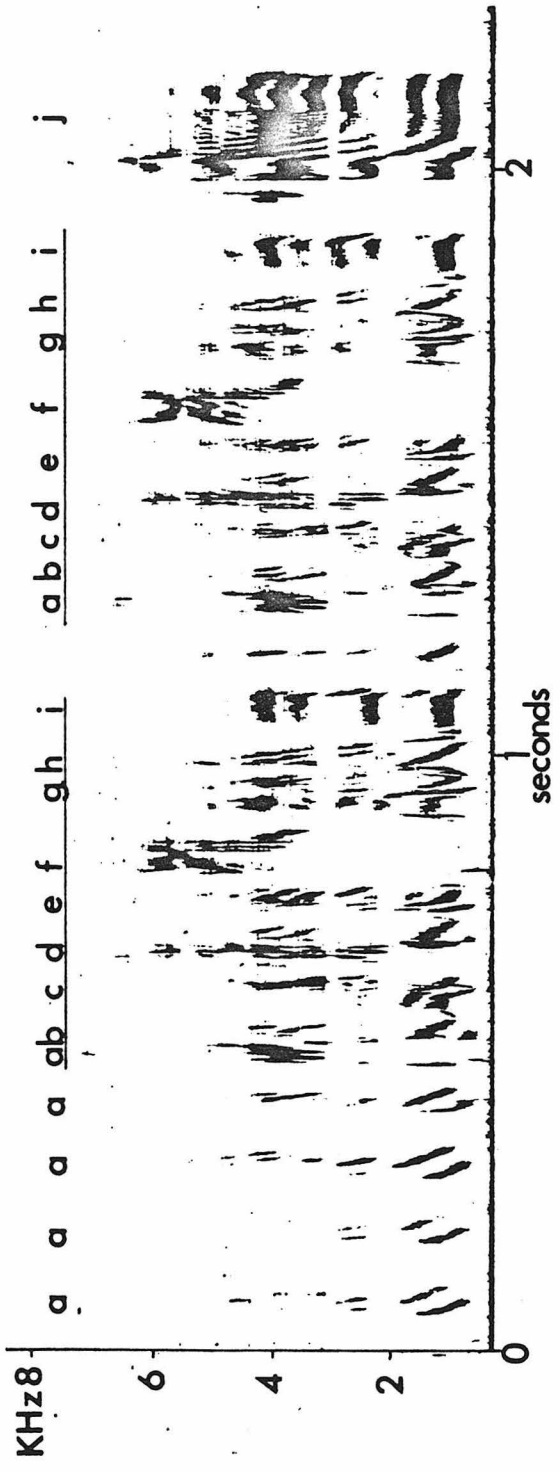
Behavioral Correlates of Brain Sexual Differentiation

The male zebra finch sings a short, stereotyped song during courtship of the female, or may render his song spontaneously. Zebra finch song is rather unmusical and usually contains many call-like notes, as well as notes of a complex harmonic structure which are ordered into a stereotyped temporal structure. Song is easily differentiated from other elements of the zebra finch vocal repertoire, both by the complexity of its multi-note structure and by its behavioral context. Both sexes have in common a short chirp which is used as an alarm call, and also a longer call note whose behavioral significance is unclear (Price, 1979). However, only the male sings. His song is androgen-dependent, while behavioral expression of the other vocalizations within the zebra finch repertoire does not seem to be influenced by the male or female's hormonal state. Castration of an adult male will abolish song behavior (Pröve, 1974), and either testosterone or 5α-dihydrotesterone (data not shown) will reinstate song. Even so, administration of either androgen to normal adult females is unable to induce song (Arnold, 1974; data below). Thus, the sex difference in functional capacity for song is not due to the trivial fact that female zebra finches lack circulating androgen. Rather, the sexual dimorphism in behavioral capacity for song is the outward sign of the sex differences within the song system which were described above.

The quality of song produced by E_2-females

A representative example of male zebra finch song (Blk 77) is illustrated in Figure 28. It consisted of several distinct components: a variable number of introductory notes (labeled by the letter "a" in Figure 28) preceded a brief stereotyped song phrase, which was then folowed by a closing note (labeled by the letter "j"). The song phrase was comprised of several elements (denoted by the letters "a-i") which had a stereotyped acoustic structure and a regular temporal order. Males constructed their songs from such simple components. Blk 77 used as many as 9 introductory notes in some renditions of his song or occasionally dispensed

Figure 28. An example of male Blk 77's song. This sonagraph was produced with a Kay Electric 6061B Sonagraph. His song consisted of several stereotyped, acoustically distinct elements which are labeled "a"-"j". The element "a" was used as an introductory note. It was rendered a variable number of times from song to song and preceded Blk 77's song phrase (elements "a"-"i", underlined). In this rendition, Blk 77 repeated his song phrase twice, and then ended his song with a closing note (element "j"). The element "j" was used in two different ways, either as punctuation to separate two song phrases or else as a closing note.


KHz8
6
4
2
0
a a a a a ab c d e f gh i
abcdefghi j
1
seconds
2

with them altogether (Table 10). He usually repeated his song phrase twice, then punctuated his song with his closing note and then might repeat his song phrase again for a third or a fourth time. The song of Blk 77 was representative of the songs produced by other zebra finch males.

An example of an E_2-female's song (G299) is illustrated in Figure 29. This E_2-female was brought into song by implantation of a Silastic capsule which contained crystalline T. This capsule maintained her in continuous song. The song illustrated in the figure was recorded 40 days after her initial exposure to androgen. Like male Blk 77, E_2-female G299 also began her song with a variable number of introductory notes, then rendered her song phrase, and also punctuated her song with an element (labeled "e" in Figure 29) whose acoustic structure resembled that used in a similar way by Blk 77. Female G299 used two different notes to introduce her song (labeled "a" or "d"), and her song phrase contained four elements which were stereotyped with respect to their acoustic structure and temporal order. This female constructed her song in a manner similar to that of a male (Table 11). She used variable numbers and combinations of introductory notes to precede her song phrase, repeated the song phrase a variable number of times, and ended her song with a complete phrase or else its first three elements.

Exposure of control or DHT-females to T when adult did not induce any novel vocalizations. The only new behavior observed as a result of exposure to T was that these females tended to carry nesting material in their beaks. This was generally a behavior that only males exhibited. The beak color of females also deepened when exposed to T or DHT. Females usually have a pale, orange-colored beak while that of the male is a deeper shade of red. Either T or DHT caused the beak of females to deepen to a shade of red like that of the male.

The quality of the song developed by E_2-females depended upon the duration of their exposure to androgen. The songs of females W201 and B315 are illustrated in

Table 10. Schematic representation of male Blk 77's songs. Each letter represents one element in the song of Blk 77 as defined in Figure 28.

4a	a b c d e f g h i	a b c d e f g h i	j			
			j			
9a						j
4a		a b c d				
5a						
3a			j			j
a			j			j
3a			j			
3a			j			j
			j			
			j			
2a			j		j	
			j		j	

Figure 29. An example of E_2-female G299's song. Like the male, her song consisted of several stereotyped, acoustically distinct elements which are labeled "a"-"e". The elements "a" and "d" were used as introductory notes, her song phrase consisted of elements "a"-"d" (underlined) and she used element "e" to separate each rendition of her song phrase. E_2-female G299 closed her song with either a complete song phrase, or else its first three elements, "a"-"c". The acoustic definition and stereotypy of elements within the song of G299 are as good as that of male Blk 77.

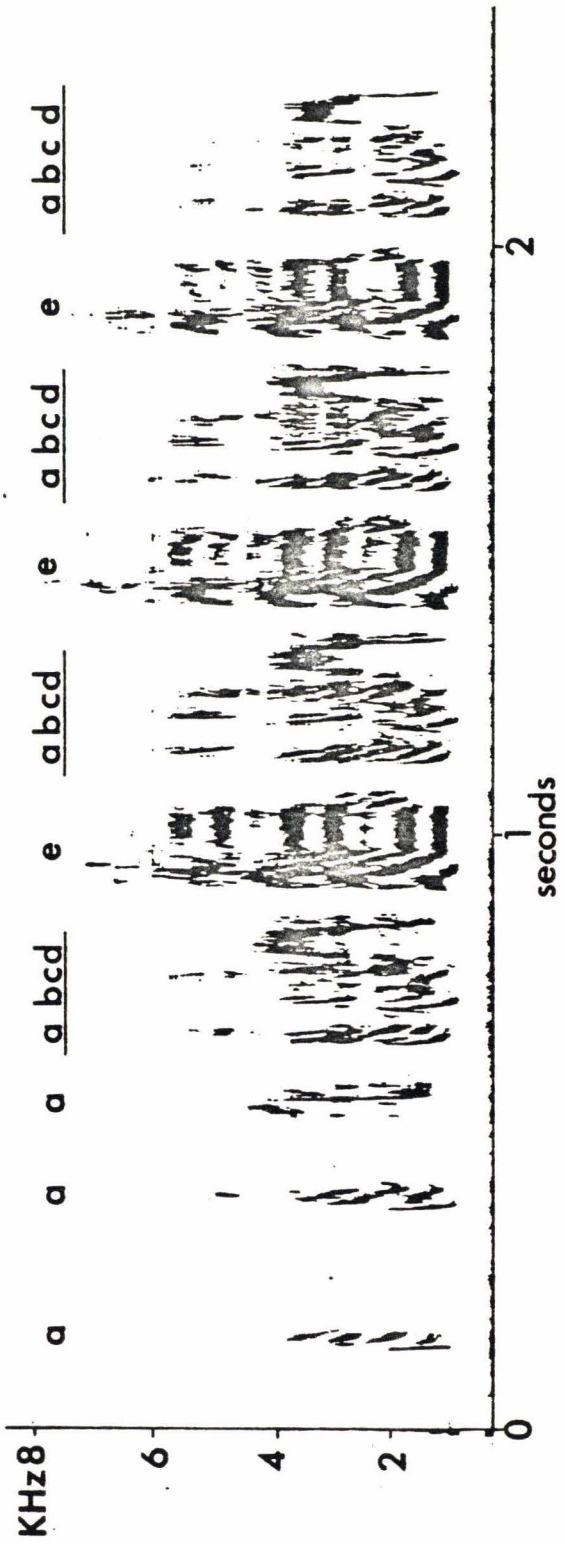

KHz8
6
4
2
0
1
seconds
a
a
a
a bcd
e
a bcd
e
a bcd
e
a bcd

Table 11. Schematic representations of female G299's songs. Each letter represents one element in the song of G299 as defined in Figure 29.

a d a d a b c d e a b c d e a b c
a _____ e _____
a a d _____ e _____ e _____
a d a d _____ e _____ e _____ e _____
a a _____ e _____ e _____ e _____
a d _____ e _____ e _____ e _____
a a d _____ e _____ e a b c
a d _____ e _____ e _____ e a b c
_____ e _____ e _____ _____ e _____
a a d _____ e _____ e _____ e _____
a a a d _____ e _____ e _____ e a b c
a d _____ e _____ e _____ e _____ e a b c
a d d _____ e _____ e _____ e a b c
a a d _____ e _____ e _____
a _____ e _____ e _____
a a d _____ e _____ e _____ e a b c

Figure 30. These two females were each implanted with a Silastic pellet containing 100 μg T which was then removed and replaced after an interval of two weeks. The songs illustrated were recorded during the fourth week after their initial exposure to T. Each female began singing on the third day after being implanted with T and continued to sing until 10 days after implantation. The songs illustrated in Figure 30 contained recognizable elements, yet their frequency structure and temporal patterning were not well stereotyped. Another female (B307) was implanted with a pellet containing 100 μg of DHT at weekly intervals. Such a schedule maintained her in continuous song. At 30 days after initial exposure to DHT, her introductory note (labeled "a" in Figure 31) and the individual elements within her song phrase (labeled "b,c,c,d") were all rendered with a stereotyped acoustic structure, although she ended her song with an element whose acoustic structure remained variable (labeled "e"). E_2-Female G299 was maintained in song by continuous exposure to T over a period of 45 days and was able to produce a song whose stereotypy was as good as that of a male as described above (Figure 29). The differences in the quality of song produced by each of these E_2-females reflects the variation in the schedules of their exposure to androgen. These differences in song quality probably reflected variations in the amount of singing practice which the different females experienced.

A male zebra finch's first song vocalizations (at about 30 days of age) had an amorphous structure. With continued practice, the acoustic definition of individual notes improved and their proper ordering and tempo gradually emerged. The young male practices his song by matching his vocal output against a model provided by an auditory memory of his father's song. Interruption of this vocal matching by deafening a young male at 30 days of age prevents him from developing a stereotyped song (M. Konishi, personal communication). The song of such a male deafened during vocal learning continues to exhibit an amorphous structure. The development of song by E_2-females resembled that of the male in that their initial, androgen-dependent

Figure 30. Examples of E_2-female song, W210 (top), B315 (bottom). These females were exposed to a schedule of exogenous T which did not maintain them in continuous song. These songs contain recognizable elements, yet their frequency structure and temporal patterning were not well-stereotyped.

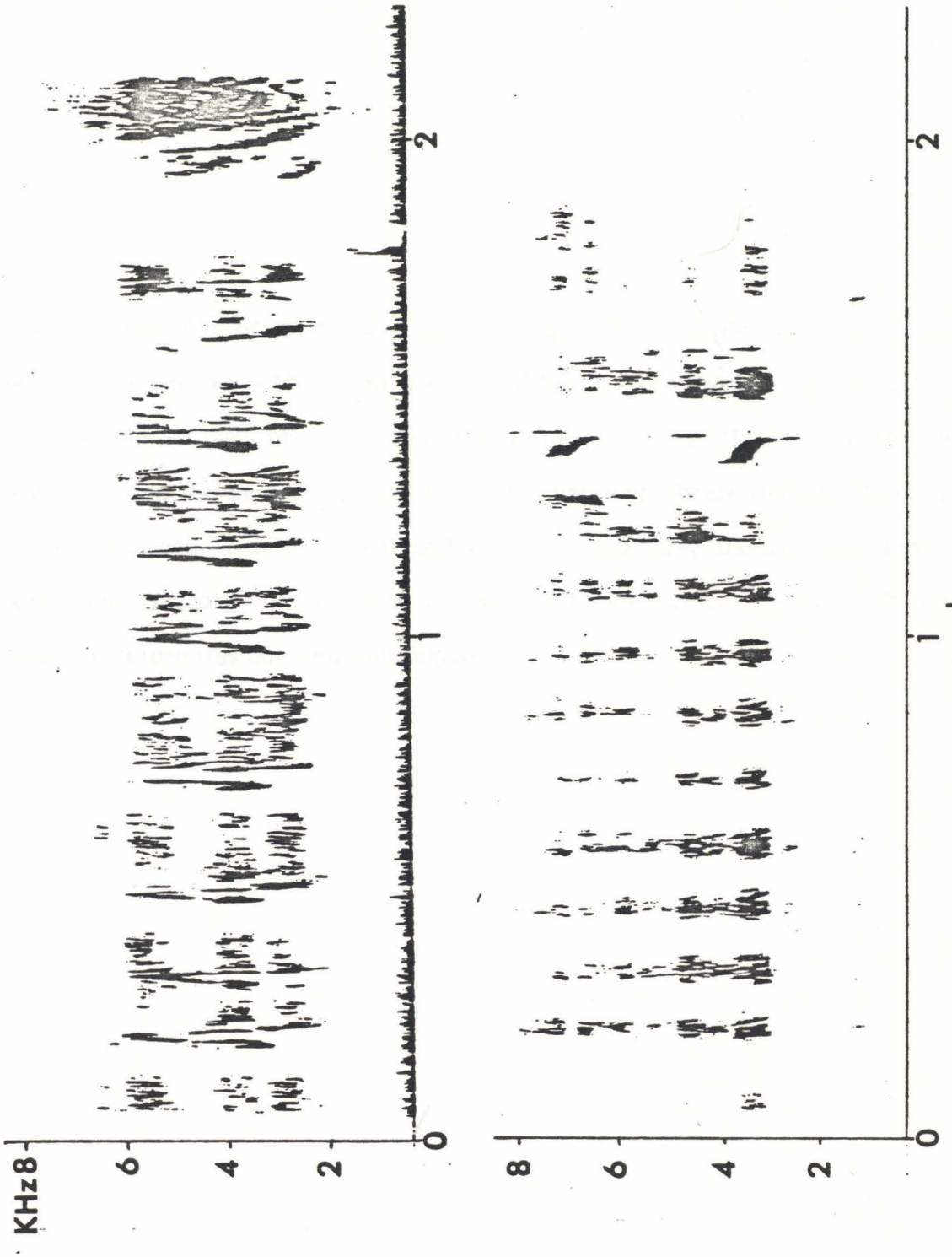
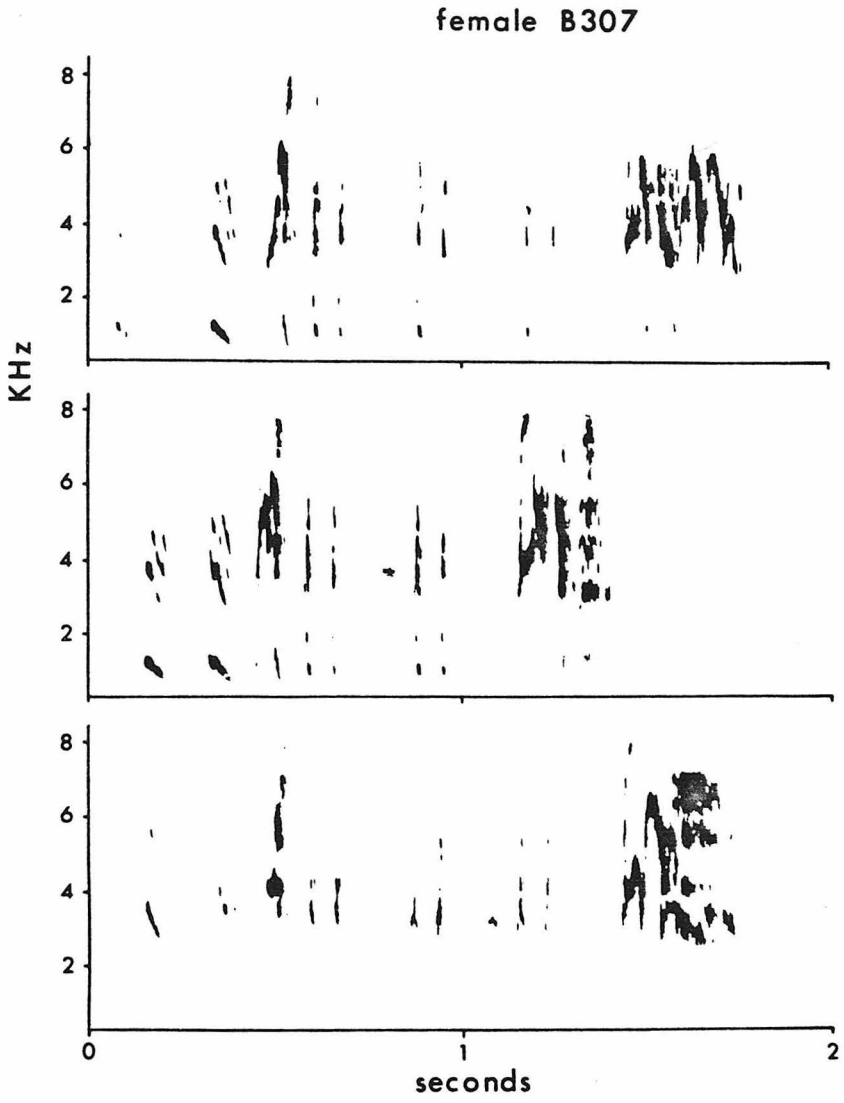

KHz
8
6
4
2
0
1
2
seconds

Figure 31. Examples of E_2-female B307's song. This female was exposed to a schedule of exogenous DHT which maintained her in continuous song. These songs were recorded 30 days after her initial exposure to DHT. Her introductory note (labeled "a") and the individual elements within her song phrase (labeled "b, c, c, d") were all rendered with a stereotyped acoustic structure, though their temporal ordering was variable. B307 closed her song with an element (labeled "e") whose acoustic structure was not well-stereotyped.


female B307
KHz
8
6
4
2
0
1
2
seconds

vocalizations had an amorphous structure from which stereotyped acoustic elements and temporal order gradually emerged. The amount of practice which the schedule of androgen exposure permitted appeared to be critical in determining the quality of an E_2-female's song.

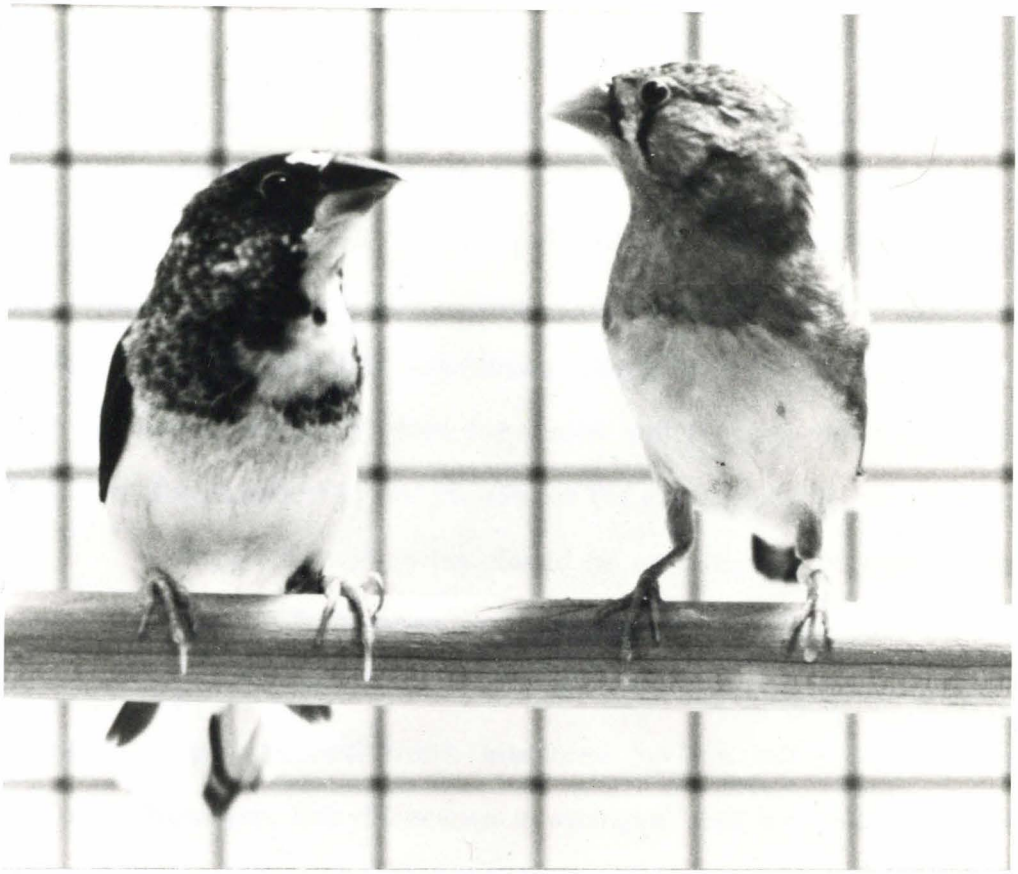
Early exposure of males to either androgen or estrogen did not affect their development, or crystallization of a stereotyped song. Both E_2-males and DHT-males were normal with respect to their vocal behavior, however, the behavioral context of their song was altered by early exposure to E_2. Male zebra finch song has two behavioral contexts which have been differentiated as "directed" and "undirected" song (Pröve, 1974). Directed songs are rendered within the context of courtship (songs directed towards the female) while undirected songs are those which are rendered either spontaneously or in the absence of other birds. No obvious differences in the acoustic structure of directed vs. undirected song were observed. Both were introduced by a number of introductory notes and contain a variable number of song phrases. When singing during courtship, the male straightened to an erect posture, sleeked the feathers upon the crown of his head, erected the feathers upon his neck and sometimes the feathers overlying his abdomen. Singing was often interspersed with the male's wiping his beak from side to side upon the perch between him and the female, as if to direct attention to his color, and he also performed a quick side-to-side hopping towards the female (Morris, 1954). This pattern of courtship behavior can be eliminated in the male by exposure to E_2 at hatching, either with a Silastic pellet which contained 50 μg of E_2, or by a single injection of 1 μg estradiol benzoate in sesame oil (Gurney and Konishi, 1978). This abolished the behavioral context for directed song, yet left undirected song intact. That is, E_2-males developed songs of normal stereotypy, but were only seen to sing spontaneously. Paradoxically, some E_2-females were only seen to sing spontaneous, undirected song when primed with T, while others both sang and courted other birds.

Such an E_2-female (B303) is illustrated in Figure 32. E_2-female B303 was cross-fostered when a chick with Bengalese finch parents. When primed with T as an adult she courted female Bengalese finches, but ignored female zebra finches. Thus, she appeared to have become sexually imprinted upon the species of her foster parents (Immelmann, 1972), which provides evidence that the behavorial context of her courtship is strongly analogous to that of a male. When courting, E_2-female B303 approached a Bengalese finch with pivoting movements, performed a characteristic beak-wiping display, straightened to an erect posture, flattened the feathers upon her crown, fluffed the feathers upon her neck and rapidly repeated her short song phrase in a behavioral sequence which closely resembled that of a normal male. Why some E_2-females exhibited courtship behavior when primed with testosterone and why E_2-males sang undirected song but did not court is unclear. One possibility might be that E_2 at hatching exerts a direct effect upon testicular maturation (Harris and Levine, 1965; Wentworth et al., 1968). That the syrinx of E_2-males is smaller in size than that of normal males (next section) supports this hypothesis as it may suggest that E_2-males have lower circulating titers of androgen. However, when E_2-males were implanted with Silastic pellets which contained 100 µg T, a regime which was sufficient to support song and courtship behavior in some E_2-females, no induction of courtship behavior was observed.

Hormonal effects upon syringeal development

The avian vocal organ, the syrinx, is located at the junction of the trachea with the two bronchia which lead to the lungs. The syrinx is bilaterally symmetrical, and on either side has two muscles which tension an internal membrane. These membranes are located at the mouth of each bronchus and sound is produced by their vibration as air is expired from the lungs. The size of the syrinx is influenced by androgen. Castration reduces the size of the syrinx in adult males, and exogenous androgen increases its size in adult females (Arnold, 1974). High-affinity, low-

Figure 32. (a) Testosterone activates song and courtship in estrogenized-females. This E_2 female (B303) when primed with a Silastic pellet containing 50 µg of T approached the Bengalese finch with pivoting movements, straightened to an erect posture, fluffed her throat feathers, and rapidly repeated her short song phrase in a behavioral sequence which closely resembles that of a normal male. This female was raised by Bengalese finches and became sexually imprinted upon that species.



capacity binding proteins for androgens have also been isolated from the zebra finch syrinx (Lieberburg and Nottebohm, 1979).

Two measures of syringeal size were found to be sexually dimorphic in the present study: its width measured at the syrinx's widest point when viewed from its ventral aspect (Arnold, 1974) and its dry weight (Table 12). To measure the syrinx's dry weight, it was severed from the trachea at the terminus of the bronchialis anticus medialis muscle, and was severed from the two bronchia at their uppermost cartilaginous ring (labeled "C" in Arnold, 1974). The syrinx was then dried at 57°C for at least one week prior to weighing. The sex differences in syringeal width or dry weight were statistically significant (Mann-Whitney U-test, $p = 0.001$). The distribution of syringeal width for males and females was non-overlapping, so this measure of syringeal size was chosen for the comparison of experimental groups.

Syringeal size in males should be an indicator of their serum testosterone level. Control and DHT-males had syrinxes of equivalent size (Table 13), while the syrinx of E_2-males was significantly different (Mann-Whitney U-test, $p = 0.03$). In control males, syringeal width measured 3.4 mm while in E_2-males it measured 3.1 mm. However, this difference in syringeal size was not reflected in the males' testicular development. The combined weight of both testes in control males averaged 45.5 mg (36-52 mg), in E_2-males averaged 50.2 mg (40-50 mg), and in DHT-males averaged 47.3 mg (35-56 mg).

The syringeal widths of control, E_2- and DHT-females were all equivalent. Neither E_2-females+T nor females+DHT possessed syrinxes whose widths were significantly different from that of control females either (Table 13). These results indicate that a dose of T or DHT sufficient to exert an influence on the volume of song nuclei sensitive to androgen in the adult and to support song behavior may be below that required for a morphological response in the syrinx. This would suggest that the influence of DHT upon the volume of nXIIts or DM is not mediated through a

Table 12. Measures of syrinx size

	Males (n=7)	Females (n=6)
Width (mm)	3.4 ± 0.2	2.8 ± 0.1
Dry weight (mg)	5.1 ± 0.9	2.9 ± 0.5

Table 13. Comparison of syringeal width in experimental and control groups

Group		Syrinx width, mm (range: low-high)
Males	(n=7)	3.4 (3.2-3.8)
E_2-males	(n=4)	3.1 (2.8-3.4)[1]
DHT-males	(n=4)	3.6 (3.4-4.0)[2]
E_2-females	(n=4)	2.85 (2.8-2.9)
E_2-females+T	(n=4)	3.0 (2.9-3.2)[3]
DHT-females	(n=7)	2.8 (2.5-3.1)
Females+DHT	(n=6)	2.85 (2.7-3.0)
Females	(n=6)	2.8 (2.6-3.0)

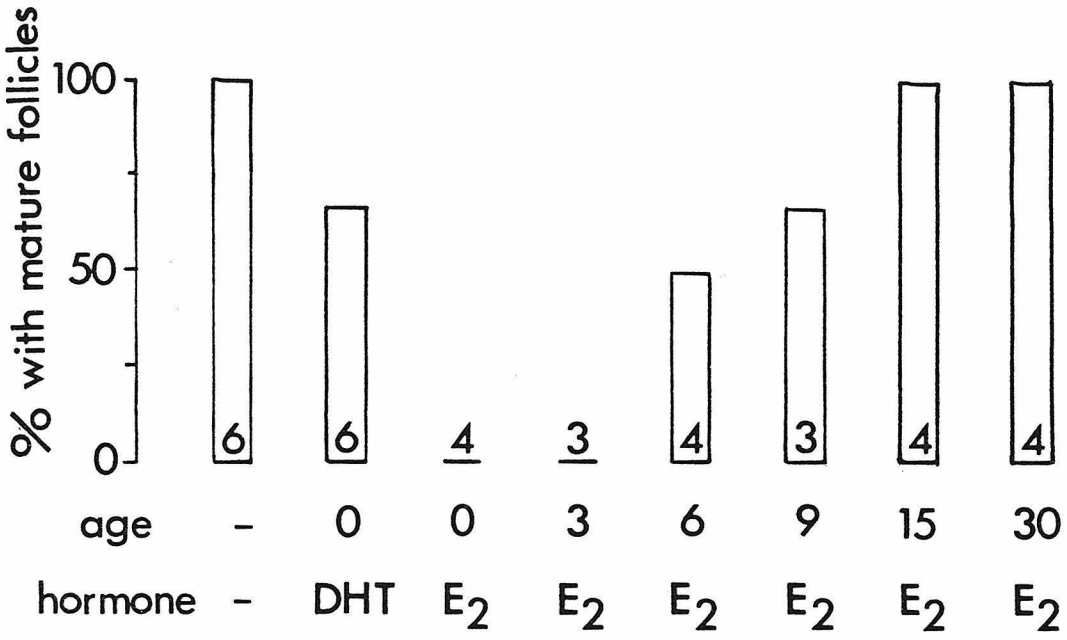
[1] H_o: E_2-males = males, P(U) = 0.03

[2] H_o: DHT-males = males, no significant difference

[3] H_o: E_2-females = E_2-females+T, no significant difference

[4] H_o: Males = female, P(U) = 0.002

Figure 33. Effect of early exposure to E_2 upon ovarian maturation. Mature follicles were those which showed deposition of yolk. The age at implant with hormone, and the type of hormone are indicated beneath the bar graph. The number of females in each group is indicated by the numeral at the base of each bar.


% with mature follicles
100
50
0
6
6
4
3
4
3
4
4
age - 0 0 3 6 9 15 30
hormone - DHT E_2 E_2 E_2 E_2 E_2 E_2

retrograde influence from the syrinx, and also that syrinx size is not a constraint upon E_2-female song behavior.

Hormonal effects upon ovarian maturation

The ovary of mature female zebra finches in breeding condition contained developing follicles which bulged conspicuously from its surface. Most follicles were small and whitish in color, while a few of the larger follicles deepened to yellow. These were the follicles in which deposition of yolk was occurring. In contrast, the ovary of either non-breeding adult females or sexually immature females weighed less, and all of its follicles were small and white.

Control, E_2- and DHT-females were similarly housed in groups of mixed sex of four to six birds in cages measuring: length, 62 cm; width, 36 cm; and height, 41 cm. At sacrifice, all six control females had ovaries which showed follicular development, four of six DHT-females showed follicular development, while no E_2-female showed deposition of yolk in any follicle. The ability of E_2 to suppress ovarian development was under temporal constraint. E_2 administered to females at hatching or at 3 days of age suppressed ovarian development in all treated females. This effect gradually diminished, and females exposed to E_2 at 15 or 30 days of age all had normal ovaries in which follicular deposition of yolk was observed (Figure 33). The limited period during which E_2 suppresses ovarian development contrasts with the extended period over which RA is sensitive to that hormone (Section IV of the thesis).

Discussion

Sexual differentiation of brain and behavior

The touchstone of these anatomical correlates of sexual differentiation, and the unique advantage of the zebra finch preparation, is the well-defined functional role of the song system. The profound sex differences at all levels of the zebra finch song system are the inward sign of outward, behavioral sex differences in the

functional capacity for song. Only the male sings; a normal, gonadally intact female is songless. The adult male's song is androgen-dependent (T or DHT) (Gurney and Leverette, 1980; Pröve, 1974), though exogenous androgen is unable to elicit song in adult female finches.

Without exception, in both males and females the influence of either E_2 or DHT upon the sexual differentiation of the telencephalic and brainstem song nuclei and consequent behavioral capacity for song was positive in sign. One implication of the results reported in this thesis is that masculine sexual differentiation of the zebra finch song system should prove to be actively dependent upon hormone. Secretion of testosterone at hatching by the male chick's gonads should be required for masculinization of his song system; metabolism of testosterone to 17 β-estradiol (Callard et al., 1978) is implicated in the masculinization of dendritic architecture and of the functional capacity for song, while either testosterone or its androgenic metabolite, 5α-dihydrotestosterone should mediate masculinization of neuronal number within RA. In the zebra finch, the female would appear to be the "hormone-independent" or "neutral" sex with respect to sexual differentiation of the song system (Toran-Allerand, 1980, and others propose that low levels of gonadal hormones are necessary to support neuronal maturation in the "neutral" sex as well.) An active dependence upon hormone for masculine sexual differentiation within the song system contrasts with the influence of steroid hormones upon the sexual differentiation of male copulatory behavior in Japanese quail, Coturnix coturnix japonica (Adkins, 1975). Early exposure to E_2 appears to suppress the emergence of copulatory behavior in male quail, and thus their behavior is said to be feminized. By induction, the lack of male copulatory behavior in female Japanese quail is thought to be due to its active suppression through ovarian secretion of estrogen during normal development, and conversely the development of copulatory behavior by the male should be hormone-independent. In mammals, the situation is the reverse of that in the quail. In the rat

for example, neonatal castration of a male leads to "hormone-independent" emergence of lordosis behavior, while testosterone replacement, or exposure of female neonates to testosterone leads to suppression of lordosis behavior in the adult. Based on work with quail (Adkins, 1975), chickens (Wilson and Glick, 1970), and various species of mammals (Goy and McEwen, 1979), it has seemed that the hormonally neutral sex in Aves and Mammals is the homogametic sex. The female possesses two similar sex chromosomes in mammals, while in Aves, the male is the homogametic sex (Beatty, 1964). Unless estrilid finches are unlike the rest of the avian class, this generalization (Adkins, 1978) will need to be modified.

The E_2-females resemble males with respect to their functional capacity for song. The quality of the song produced by an E_2-female as a result of exposure to androgen can be as good as that of a male in terms of its tempo, the acoustic definition of its individual elements and its stereotypy. Thus, E_2-mediated cyto-architectonic masculinization of RA and the other telencephalic song nuclei is correlated with the functional masculinization of the female's behavioral capacity for song. Early E_2 is also a prerequisite for the response of RA, HVc and MAN to androgen in the adult. Their response is characterized by an increase in total nucleus volume which is due to an androgen-dependent increase in the average volumetric spacing of their constituent neurons, and not due to an effect on neuronal number in the adult. This morphological response relates to the ability of androgen to activate song in E_2-females. Androgen cannot activate song in normal adult females, and neither can androgen elicit a morphological response within their HVc, RA, or MAN. Androgen is also unable to elicit song in DHT-females. Thus, the DHT-mediated doubling of neuronal number in RA does not have a behavioral correlate. In light of this, interpretation of the functional correlates of brain sexual dimorphisms reported in mammals, i.e., in the morphology and organization of preoptic area neurons (Greenough et al., 1977; Gorski et al., 1978) and in synaptic numbers (Raisman and

Field, 1971; Matsumoto and Arai, 1976), should proceed with caution as the neural pathways which these brain regions participate in are poorly characterized and thus, although exposure to gonadal hormones appears to "organize" aspects of mammalian brain differentiation, the functional implication of these changes for the sexuality of behavior is as yet unclear.

In terms of categories of behavior, i.e., male (song) or female (no song), E_2 actively induces or "organizes" maleness. At an anatomically gross level of analysis, E_2 does not "organize the neural substrate" for song behavior. RA completes the same pattern of efferent projections in both males and females, and contains the same types of neurons. Further, electrical stimulation of HVc in either sex elicits EMGs in the syrinx, which indicates that conduction through the female's song system is physiologically functional as well (Arnold, 1978). From the anatomy, it isn't obvious why female zebra finches cannot sing, nor why E_2-mediated masculinization of cytoarchitecture should have as its correlate induction of the capacity for song. The complete behavioral dichotomy between male and female zebra finches seems to have as its molecular basis the ability (in the male) or inability (in the female) of androgen (T or DHT) to activate song in the adult. After early estrogenization, the female's unresponsiveness to androgen is reversed: androgen is then able to activate song in E_2-females and also induces correlate growth in RA, HVc, or MAN.

Based on behavioral assay of sexual differentiation, Phoenix et al. (1959) drew attention to "the parallel nature of the relationship, on the one hand, between androgens and the differentiation of the genital tracts, and on the other, between androgens and the organization of the neural tissues destined to mediate mating behavior in the adult" (p. 369). Later workers in this field have taken the view that hormones "organize" the anatomical differentiation of neural pathways which mediate sexually dimorphic brain functions (cf. Goy and McEwen, 1980). Phoenix and his coworkers were ambiguous as to the meaning of the term "organize"; in another

section of their paper they argued that "androgen administered perinatally has an organizing action...in the sense of producing a responsiveness to exogenous hormones which differs from that of normal adult females" (p. 369). Beach (1971) has cogently argued this position with respect to sexual differentiation in mammals, and this report also supports that view. In terms of organizing the maleness (song) or femaleness (no song) of zebra finch behavior, estrogen does so by determining the ability of the song system to respond to androgen in the adult.

Unlike mating behavior, song is learned. The learning of song has both auditory and motor components. The young male zebra finch learns the acoustic structure of his song from his father (Immelmann, 1969). In the zebra finch, there is first an early sensitive period for the frequency structure of individual notes in the father's song which closes by 40 days of age, and this then overlaps slightly with a second sensitive period during which the young male learns the sequence, tempo, and duration of the song. The second sensitive period begins between 25-35 days of age at the same time that the young male begins to produce "song" vocalizations and lasts until approximately 80-90 days of age. The initiation of singing correlates to a rise in serum testosterone titer which also occurs around 30 days of age in the male (Pröve and Sossinka, 1978), and also to a doubling in the volume of HVc and RA which occurs between 25 and 40 days of age (data not shown). The male's first song vocalizations are of an amorphous structure. With continued practice, the young male matches his vocal output against his auditory memory of his father's song, and the acoustic definition of individual notes improves and their proper ordering and tempo emerges. The final song of the adult male zebra finch is completely stereotyped with respect to frequency patterning, sequence, and tempo, and is a close copy of his father's song (Immelmann, 1969; Price, 1979). Young males deafened at 30 days of age continue to produce amorphous "song" vocalizations of unstable structure, though deafening of adult male zebra finches after emergence of their full, stereotyped song does not

change its quality (M. Konishi, personal communication). Thus, motor development of song by the zebra finch male is a bona fide example of learning with reference to auditory feedback (Konishi, 1965). Since lesion of the song system in canaries produces profound deficits in song (Nottebohm et al., 1976) and electrical stimulation within the zebra finch song system elicits EMGs in the syrinx (Arnold, 1978), this pathway is the motor substrate for song and may participate in storage of the motor program for song as well. HVc and RA attain normal volumes in males whose song learning was disrupted by deafening at 30 days of age (Gurney and Konishi, 1978), hence, the gross development of RA and HVc is independent of learning. However, the late, androgen-mediated phase of growth in HVc and RA which is due to the expansion of neuropil within these nuclei may be the substrate for learning. Male RA_I neurons have a larger dendritic field and a higher density of spines along their dendrites, and so have a larger synaptic field than in the female. These characteristics appear to differentiate under the influence of early estrogen and then late androgen. Thus, hormonal determination of dendritic architecture may be of primary importance for organizing a song system capable of learning to sing but of secondary importance for determining its sexuality.

References

Adkins, E. K. (1975). Hormonal basis of sexual differentiation in the Japanese quail. J. Comp. Physiol. Psychol. **89**, 61-71.

Adkins, E. K. (1978). Sex steroids and the differentiation of avian reproductive behavior. Amer. Zool. **18**, 501-509.

Arnold, A. P. (1974). Behavioral effects of androgen in zebra finches (Poephila guttata) and a search for its sites of action. Ph.D. Dissertation, Rockefeller University.

Arnold, A. P. (1978). Anatomical and electrophysiological studies of sexual dimorphism in a passerine vocal control system. Proc. XVII Int. Cong. Ornithol. Berlin, Germany, in press.

Arnold, A. P. (1979). Hormone accumulation in the brain of the zebra finch after injection of various steroids and competitors. Soc. Neurosci. Abstr. **5**, No. 1474.

Arnold, A. P., F. Nottebohm, D. W. Pfaff. (1976). Hormone concentrating cells in vocal control and other areas of the brain of the zebra finch (Poephila guttata). J. Comp. Neurol. **165**, 487-512.

Arnold, A. P. and A. Saltiel. (1979). Sexual difference in pattern of hormone accumulation in the brain of a songbird. Science **205**, 702-705.

Banker, G. A. and W. M. Cowan. (1979). Further observations on hippocampal neurons in dispersed cell culture. J. Comp. Neurol. **187**, 469-493.

Beach, F. A. (1971). Hormonal factors controlling the differentiation, development, and display of copulatory behavior in the Ramstergig and related species. In The Biopsychology of Development (E. Toback, L. R. Aronson and E. Shaw, eds.) Academic Press, pp. 249-296.

Beatty, R. A. (1964). Chromosome deviations and sex in vertebrates. In Intersexuality in Vertebrates Including Man (C. N. Armstrong and A. J. Marshall, eds.) Academic Press, London, pp. 311-348.

Berry, M. and P. Bradley. (1976). The growth of the dendritic trees of Purkinje cells in the cerebellum of the rat. Brain Res. **112**, 1-35.

Blakemore, C. and G. F. Cooper. (1970). Development of the brain depends on the visual environment. Nature **228**, 477-478.

Bradbury, J. T. and T. Gaensbauer. (1939). Masculinization of the female rat by gonadotropic extracts. Proc. Soc. Exp. Biol. and Med. **41**, 128-131.

Bradley, P. and M. Berry. (1976). The effects of reduced climbing and parallel fibre input on Purkinje cell dendritic growth. Brain Res. **109**, 133-151.

Bray, D. (1970). Surface movements during the growth of single explanted neurons. Proc. Natl. Acad. Sci. USA **65**, 905-910.

Bray, D. and M. B. Bunge. (1973). The growth cone in neurite extension. In Locomotion of Tissue Cells, CIBA Foundation Symposium 14 (new series), Elsevier, Amsterdam, pp. 195-209.

Brockes, J. P. (rapporteur) (1979). Consequences of signal reception (outcome). In Report of the Dahlem Workshop on the Role of Intercellular Signals: Navigation, Encounter, Outcome (J. G. Nichols, ed.) Verlag-Chemie, Weinheim, Ger., 1979, pp. 285-300.

Callard, G. V., Z. Petro and K. J. Ryan. (1978). Conversion of androgen to estrogen and other steroids in the vertebrate brain. Amer. Zool. **18**, 511-523.

Cammermeyer, J. (1967). Artifactual displacement of neuronal nucleoli in paraffin sections. J. Hirnforschung **9**, 209-224.

Campenot, R. B. (1977). Local control of neurite development by NGF. Proc. Natl. Acad. Sci. USA **74**, 4516-4519.

Clarke, P. G. H. and W. M. Cowan. (1975). Ectopic neurons and aberrant connections during neural development. Proc. Natl. Acad. Sci. USA **72**, 4455-4458.

Clemens, L. G., M. Hiroi and R. A. Gorski. (1969). Induction and facilitation of female mating behavior in rats treated neonatally with low doses of testosterone proprionate. Endocrinology **84**, 1430-1438.

Clemens, L. G., B. A. Gladne and L. P. Coniglio. (1978). Prenatal endogenous androgenic influences on masculine sexual behavior and genital morphology in male and female rats. Horm. and Behavior **10**, 40-53.

Cowan, W. M. and P. G. H. Clarke. (1976). The development of the isthmo-optic nucleus. Brain Behav. and Evol. **13**, 345-375.

Cowan, W. M., D. I. Gottlieb, A. Hendrickson, J. L. Price and T. A. Woolsey. (1972). The autoradiographic demonstration of axonal connections in the central nervous system. Brain Res. **37**, 21-51.

Cullen, M. J. and I. R. Kaiserman-Abramof. (1976). Cytological organization of the dorsal lateral geniculate nuclei in mutant anopthalamic and postnatally enucleated mice. J. Neurocytol. **5**, 407-424.

David, K., E. Dingmanse, J. Frend and E. Laquer. (1935). Uber krystallinisches männliches Hormon aus Hoden (Testosteron) wirksamer als aus Horn oder aus cholesterin bereitetes Androsteron. Hoppe-Seylers Z. Physiol. Chem. **233**, 281-282.

Davidson, J. (1969). Effects of estrogen on the sexual behavior of male rats. Endocrinology **84**, 1365-1372.

Detwiler, S. R. (1920). On the hyperplasia of nerve centers resulting from excessive peripheral loading. Proc. Natl. Acad. Sci. USA **6**, 96-101.

Eayers, J. T. and B. Goodhead. (1959). Postnatal development of the cerebral cortex of the rat. J. Anat. **93**, 385-402.

Goodman, L. (1934). Observations on transplanted immature ovaries in the eyes of adult male and female rats. Anat. Rec. **59**, 223-251.

Gorski, R. A. (1968). Influence of age on the response to paranatal administration of a low dose of androgen. Endocrinology **82**, 1001-1004.

Gorski, J. and F. Gannon. (1976). Current models of steroid action: a critique. Ann. Rev. Physiol. **1976**, 425-450.

Gorski, R. A., J. H. Gordon, J. E. Shryne and A. M. Southam. (1978). Evidence for a morphological sex difference within the medial preoptic area of the rat brain. Brain Res. **148**, 333-346.

Goy, R. W. and B. S. McEwen. (1980). Sexual Differentiation of the Brain, MIT Press, Massachusetts.

Green, L. A. and A. S. Tischler. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA **73**, 2424-2428.

Greenough, W. T., C. S. Carter, C. Steerman and T. J. DeVoogd. (1977). Sex differences in dendritic patterns in hamster preoptic area. Brain Res. **126**, 63-72.

Gurney, M. E. and M. Konishi. (1978). Development and sexual differentiation of the song system in the zebra finch. Soc. Neurosci. Abstr. **4**, No. 332.

Gurney, M. E. and M. Konishi. (1980). Hormone induced sexual differentiation of brain and behavior in zebra finches. Science, in press.

Gurney, M. E. and J. P. Leverette. (1980). Hormonal specificity in the activation of song and courtship in the zebra finch. Manuscript in preparation.

Gusella, J., R. Geller, B. Clarke, V. Weeks and D. Housman. (1976). Commitment to erythroid differentiation by Friend erythroleukemia cells: a stochastic analysis. Cell **9**, 221-229.

Harris, G. W. and D. Jacobson. (1952). Functional grafts of the anterior pituitary gland. Proc. Roy. Soc. London, B **139**, 263-276.

Harris, G. W. and S. Levine. (1965). Sexual differentiation of the brain and its experimental control. J. Physiol. **181**, 379-400.

Hollingworth, T. and M. Berry. (1975). Network analysis of dendritic fields of pyramidal cells in the neocortex and Purkinje cells in the cerebellum of the rat. Phil. Trans. B, **270**, 227-262.

Hollyday, M. and V. Hamburger. (1976). Reduction of naturally occurring motor loss by enlargement of the periphery. J. Comp. Neurol. **170**, 311-320.

Idler, D. R. (ed.) (1972). Steroids in Nonmammalian Vertebrates, Academic Press, New York.

Immelmann, K. (1969). Song development in the zebra finch and other estrildid finches. In Bird Vocalizations (R. Hinde, ed.) Cambridge Univ. Press, Cambridge, England, pp. 61-74.

Immelmann, K. (1972). The influence of early experience upon the development of social behavior in estrilidine finches. Proc. XV Int. Ornith. Congress, Berlin, Germany, 316-337.

Imperato-McGinley, J., L. Guerraro, T. Gantier and R. E. Peterson. (1974). Steroid 5α-reductase deficiency in man: an inherited form of male pseudohermaphroditism. Science **186**, 1213-1215.

Ivarie, J. D. and P. H. O'Farrell, (1978). The glucocorticoid domain: steroid mediated changes in the rate of synthesis of rat hepatoma proteins. Cell **13**, 41-55.

Kalra, P. S., L. Krulich, M. Quijada, S. P. Kalra, C. P. Fawcell and S. M. McCann. (1971). Feedback effects of gonadal steroids on gonadotropins and prolactin in the rat. In Hormonal Steroids (E. James and R. Martini, eds.) MIT Press, Cambridge, Mass., pp. 708-715.

Kasamatsu, T. and J. D. Pettigrew. (1978). Depletion of brain catecholamines: Failure of ocular dominance shift after monocular occlusion in kittens. Science **194**, 206-209.

Kasamatsu, T., J. D. Pettigrew and M. L. Ary. (1979). Restoration of visual cortical plasticity by local microperfusion of norepinephrine. J. Comp. Neurol. **185**, 163-182.

Konishi, M. (1965). The role of auditory feedback in the control of vocalization in the white crowned sparrow. Z. Tierpsychol. **22**, 770-783.

Landmesser, L. and G. Pilar. (1976). Fate of ganglionic synapses and ganglion cell axons during normal and induced cell death. J. Cell Biol. **68**, 357-374.

Leavitt, W. W., T. J. Chen, T. C. Allen and J. O. Johnston. (1977). Regulation of progesterone receptor formation by estrogen action. Ann. N.Y. Acad. Sci. **286**, 21-225.

Lederberg, J. (1959). Genes and antibodies. Science **129**, 1649-1653.

Letourneau, P. C. (1978). Chemotactic response of nerve fiber elongation to nerve growth factor. Develop. Biol. **66**, 183-196.

Levi-Montalcini, R. and P. V. Angeletti. (1968). Nerve growth factor. Physiol. Rev. **48**, 534-569.

Lieberburg, I. and B. S. McEwen. (1975). 17 β-estradiol: a metabolite recovered in cell nuclei from limbic areas of neonatal rat brains. Brain Res. **85**, 165-170.

Lieberburg, I. and F. Nottebohm. (1979). High-affinity androgen binding proteins in syringeal tissues of songbirds. Gen. and Comp. Endo. **37**, 286-293.

Lindsay, R. D. (1977). Tree analysis of neuronal processes. In Computer Analysis of Neuronal Structures (R. D. Lindsay, ed.) Plenum Press, New York, pp. 149-164.

Ling, F. A., J. A. Patterson, A. Privat, S. Mori and C. P. LeBlond. (1973). Investigation of glial cells in semithin sections. J. Comp. Neurol. **149**, 43-72.

Luine, V., F. Nottebohm, C. Harding and B. S. McEwen. (1980). Androgen affects cholinergic enzymes in syringeal motor neurons and muscle. Brain Res., in press.

Lynch, G., C. Gall, P. Mensah and C. Cotman. (1974). Horseradish peroxidase histochemistry: A new method for tracing efferent projections in the central nervous system. Brain Res. **65**, 373-380.

Manthorpe, M., S. Skaper, R. Adler, K. Londa and S. Varon. (1980). Cholinergic neurotrophic factors: Fractionation properties of an extract from selected chick embryonic eye tissues. J. Neurochem. **34**, 69-76.

Matsumoto, A. and Y. Arai. (1976). Effect of estrogen on early postnatal development of synaptic formation in the hypothalamic arcuate nucleus of female rats. Neurosci. Letters **2**, 79-82.

McEwen, B. S. (1977). Gonadal steroid receptors in neuroendocrine tissues. In Receptors and Hormone Action, Vol. 1 (B. W. O'Malley and L. Birnbaumer, eds.) Academic Press, New York, pp. 353-400.

McEwen, B. S., I. Lieberburg, N. Maclusky and L. Plapinger. (1977). Do estrogen receptors play a role in the sexual differentiation of the rat brain? J. Steroid Biochem. **8**, 593-601.

Menesini-Chen, M. G., J. S. Chen and R. Levi-Montalcini. (1978). Sympathetic nerve fibers ingrowth in the central nervous system of the neonatal rat upon intracerebral NGF injections. Arch. Ital. Biol. **116**, 53-84.

Messer, A. (1977). The maintenance and identification of mouse cerebellar granule cells in monolayer culture. Brain Res. **130**, 1-12.

Metcalf, E. S. and N. R. Klinman. (1975). In vitro tolerance induction of neonatal murine B cells. J. Exp. Med. **143**, 1327-1340.

Moguilewsky, M. and J. P. Raynand. (1979). Estrogen-sensitive, progestin binding sites in the female rat brain and pituitary. Brain Res. **164**, 165-175.

Money, J. (1978). Phylogeny and ontogeny in gender identity differentiation. In Perspectives in Endocrine Psychobiology (F. Brambilla, P. K. Bridges, E. Endroczi and G. Henser, eds.) Wiley and Sons, New York, pp. 467-478.

Moore, C. R. and D. Price. (1932). Gonad hormone functions, and the reciprocal influence between gonads and hypophysis with its bearing on the problem of sex hormone antagonism. Am. J. Anat. **50**, 13-71.

Morris, D. (1954). The reproductive behavior of the zebra finch (Poephila guttata). Behavior **6**, 271-322.

Mudge, A. W. (1979). Somatostatin and Substance P levels in cultured sensory neurons are influenced by environmetnal factors. Soc. Neurosci. Abstr. **5**, No. 1803.

Naftolin, F., K. J. Ryan, I. J. Davies, V. V. Reddy, F. Flores, Z. Petro, R. J. White, Y. Takaoka and L. Wolin. (1975). The formation of estrogens by central neuroendocrine tissues. Rec. Prog. Hormone Res. **31**, 295-319.

Nance, D. M., J. Shryne and R. A. Gorski. (1975). Facilitation of female sexual behavior in male rats by septal lesions: an interaction with estrogen. Horm. and Behavior **6**, 289-299.

Nottebohm, F. and A. P. Arnold. (1976). Sexual dimorphism in vocal control areas of the songbird brain. Science **194**, 211-213.

Nottebohm, F., T. M. Stokes and C. M. Leonard. (1976). Central control of song in the canary serinus canarius. J. Comp. Neurol. **165**, 457-486.

Patterson, P. H. and L. L. Y. Chun. (1977). The induction of acetylcholine synthesis in primary cultures of dissociated rat sympathetic neruons. Develop. Biol. **56**, 263-280.

Pfeiffer, C. A. (1936). Sexual differences of the hypophyses and their determination by the gonads. Am. J. Anat. **58**, 195-225.

Phoenix, C. H., R. W. Goy, A. A. Gerall and W. C. Young. (1959). Organizing action of prenatally administered testosterone proprionate on the tissues mediating mating behavior in the female guinea pig. Endocrinology **65**, 396-382.

Price, P. H. (1979). Developmental determinants of structure in zebra finch song. J. Comp. Physiol. Psychol. **93**, 260-277.

Pröve, E. (1974). Der einfluss von kastration und testosteron-substitution auf das sexual verhalten mannlicher Zebrafinken. J. für Ornithol. **115**, Heft 3, 338-347.

Pröve, E. and R. Sossinka. (1978). Kurzberichte aus der lanfenden Forschung. J. für Ornithol. **119**, Heft 2, 235.

Raisman, G. and P. M. Field. (1971). Sexual dimorphism in the preoptic area of the rat. Science **173**, 731-733.

Rakic, P. (1974). Intrinsic and extrinsic factors influencing the shape of neurons and their assembly into neuronal circuits. In Frontiers in Neurology and Neuroscience Research 1974 (P. Seeman and G. M. Brown, eds.) Univ. of Toronto, pp. 112-132.

Ramon y Cajal, S. (1929). Studies on Vertebrate Neurogenesis, Thomas, Ill.

Ramon-Moliner, E. (1970). The Golgi-Cox technique. In Contemporary Research Methods in Neuroanatomy (W. J. H. Nauta and S. O. E. Ebbesson, eds.) Springer-Verlag, New York.

Schade, J. P., K. Meeter and W. B. von Groeningen. (1962). Maturational aspects of the dendrites in human cerebral cortex. Acta Morphol. Neerl. Scand. **5**, 37-48.

Schade, J. P., H. van Backer and E. Colon. (1964). Quantitative analysis of neuronal parameters in the maturing cerebral cortex. Prog. in Brain Res. **4**, 150-175.

Shields, R. (1978). Pleiotypic effects of hormones. Nature **276**, 440-441.

Sholl, D. A. (1954). Dendritic organization in the neurons of the visual and motor cortices of the cat. J. Anat. **87**, 387-407.

Sidman, R. L. (1970). Autoradiographic methods and principles for the study of the nervous system with thymidine-H^3. In Contemporary Research Methods in Neuroanatomy (W. J. H. Nauta and S. O. E. Ebbesson, eds.) Springer-Verlag, New York.

Siegel, S. (1956). Nonparametric Statistics for the Behavioral Sciences, McGraw-Hill Book Co., New York, pp. 184-189.

Smith, J. A. and L. Martin. (1973). Do cells cycle? Proc. Natl. Acad. Sci. USA **70**, 1263-1267.

Soloman, F. (1979). Detailed neurite morphologies of sister neuroblastoma cells are related. Cell **16**, 165-169.

Sotelo, C. (1973). Permanence and fate of paramembranous synaptic specializations in 'mutants' and experimental animals. Brain Res. **62**, 345-351.

Tata, J. R. (1977). Modulation of hormone receptors. Nature **269**, 757-758.

Toran-Allerand, C. D. (1976). Sex steroids and the development of the newborn mouse hypothalamus and preoptic area in vitro: Implications for sexual differentiation. Brain Res. **106**, 407-412.

Toran-Allerand, C. D. (1980). Sex steroids and the development of the newborn mouse hypothalamus and preoptic area in vitro. II. Morphological correlates and hormonal specificity. Brain Res. **189**, 413-427.

Toran-Allerand, C. D., J. L. Gerlach and B. S. McEwen. (1980). Autoradiographic localization of ^{3}H-estradiol related to steroid responsiveness in cultures of the newborn mouse hypothalamus and preoptic area. Brain Res., in press.

Van der Loos, H. and T. A. Woolsey. (1973). Somatosensory cortex; structural alternations following early injury to sense organs. Science **179**, 395-398.

Wachtel, S. S. (1979). Immunogenetic aspects of abnormal sexual differentiation. Cell **16**, 691-695.

Wentworth, B. C., B. G. Hendricks and J. Sturtevant. (1968). Sterility induced in Japanese quail by spray treatment of eggs with mestranol. J. Wildl. Man. **32**, 879-887.

Wieland, S. J. and T. O. Fox. (1979). Putative androgen receptors distinguished in wild-type and testicular feminized (tfm) mice. Cell **17**, 781-787.

Wiesel, T. N. and D. H. Hubel. (1965). Comparison of the effects of unilateral and bilateral eye closure on cortical unit responses in kittens. J. Neurophysiol. **28**, 1029-1040.

Wilson, J. A. and B. Glock. (1970). Ontogeny of mating behavior in the chicken. Amer. J. Physiol. **218**, 951-955.

Woolsey, T. A., M. L. Dierker and D. F. Wann. (1975). Mouse SmI cortex: Qualitative and quantitative classification of Golgi-impregnated barrel neurons. Proc. Natl. Acad. Sci. USA **72**, 2165-2169.