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Distribution of Dopamine-Beta Hydroxylase (DBH) Immunoreactivity in Male Zebra Finch Brains: Effects of Hormone and DSP-4 Treatments

by

Susanna Anita Waterman

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

2002

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This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

DISTRIBUTION OF DOPAMINE-BETA HYDROXYLASE (DBH) IMMUNOREACTIVITY IN MALE ZEBRA FINCH BRAINS: EFFECTS OF HORMONE AND DSP-4 TREATMENTS

by

SUSANNA ANITA WATERMAN

Adviser: Professor Cheryl F. Harding

The overall purpose of this research was to further examine the physiological mechanisms underlying the hormonal activation of singing behavior in male zebra finches. Previous research with zebra finches showed that hormone treatments, e.g. androstenedione (AE), which activated the highest levels of courtship behavior affected the levels and/or turnover of the catecholamines (CA), noradrenaline (NA) and dopamine (DA), in nine of the ten brain areas examined. Of the three monoamines investigated in male zebra finches, NA appears to be the most hormone sensitive.

The first experiment of this study determined the distribution of noradrenergic cell bodies and projections in the male zebra finch brain. Immunocytochemical methods were used to stain cells containing the enzyme dopamine-beta hydroxylase (DBH) which catalyzes the final step in the synthesis of NA. With the exception of the vocal control nuclei (VCN), the pattern of DBH-immunoreactive (DBH-ir) labeling was similar to that observed in other birds and in rats. DBH-ir labeled cells were restricted to hindbrain

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regions in the locus coeruleus (LoC) and ventral subcoeruleus (SCv). Within the VCN, the intensity of DBH-ir fiber staining varied.

The second experiment examined the effects of endocrine status on DBH staining. Previous research in our laboratory showed that CA levels and turnover were strongly modulated by gonadal hormones. This phase of the study examined whether gonadal hormone treatment affected NA levels by changing the amount of DBH available for NA synthesis. Castrates treated with androstenedione (AE) courted significantly more than sham-treated males. Hormone treatment did not have a significant effect upon the area of DBH-ir labeling in the VCN, but it did significantly increase the area of DBH-ir labeling in the two hypothalamic nuclei examined, the paraventricular nucleus and preoptic area.

The final experiment used DBH immunocytochemistry to investigate which brain regions were affected following treatment with the noradrenergic neurotoxin DSP-4. Brain areas affected by DSP-4 appear to differ in birds and in mammals. Extensive research in mammals found DSP-4 affected NA function only in cortical areas leaving hypothalamic areas relatively unaffected. In prior experiments, we used high performance liquid chromatography with electrochemical detection to measure NA levels in finch brains following DSP-4 treatment and found that this neurotoxin depleted NA levels in hypothalamic as well as in cortical regions. Since studies in rats have typically used DBH immunocytochemistry (ICC) to assess the effects of DSP-4 on noradrenergic neurons, we used this technique as well. Using this technique,

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the pattern of destruction following DSP-4 treatment in finches was similar to that observed in rats. The areas of DBH-ir labeling in the telencephalic VCN of the DSP-4-treated males were significantly smaller than the saline-treated males, while labeling in hypothalamic areas was not significantly affected.

These experiments demonstrated that the noradrenergic innervation of the finch brain is similar to that seen in other birds and in mammals. The one exception was that the VCN, a recently evolved neural system which controls song learning and production, appeared to receive a stronger noradrenergic innervation than surrounding brain areas.

Although prior experiments in our laboratory found that NA levels throughout the finch brain were strongly modulated by gonadal steroid levels, gonadal steroids do not appear to act by modulating levels of DBH. Only in hypothalamic nuclei did gonadal hormone treatment affect DBH staining. Finally, while prior experiments suggested that the noradrenergic neurotoxin DSP-4 had more widespread effects in finch brains than it had in the brains of other species, this appears to have been the result of the methodology used to monitor its effect on brain function. When the effects of DSP-4 treatment were monitored using DBH ICC, rather than by measuring NA levels, the effects matched those found in other species using this technique.

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There were times when I felt as though I was the only person working on these experiments, but I received some much-appreciated help from some former Harding Lab members. These include Dianne G. Coombs, Melanie Marrero, Isabelle Reinegger, Sandra-Ann Rowe, and Yelena Sorokina. I also thank Dr. Sarah Durand for her assistance with very recent photomicroscopy.

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We were very close, and I miss our frank and thought-provoking discussions about life and politics. Sadly, she passed away before I finished, and although I wish she were still around for more insight, I know she's in a better place.

Finally, where would I be without my family! Mum, Dad, Kevin Waterman, David Waterman, Heather (Dawn) Waterman, Andrew Waterman, Kimberley Waterman, Rev. Serville Waterman, Renrique Bertley, Valerie Bertley, and last but not least, my grandmother Florence Waterman. My family provided the unconditional love and support that carried me through my worst times.

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Without a doubt, my last few years as a graduate student were the most difficult times I have ever faced. So, I thank God for delivering me through those tough times and for providing the support system to see this project to its conclusion.

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DEDICATION

To St. Clair A. and Cynthia Waterman.

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CHAPTER 1:

GENERAL INTRODUCTION

In the central nervous system of most higher vertebrates, central noradrenergic innervation originates in cell groups located in the brainstem. Noradrenergic cells send highly extensive, ascending and descending axonal projections and dense terminal arborizations to major areas of the brain and spinal cord (Role and Kelly, 1991; Kitt and Brauth, 1986; Loughlin et al., 1986b). Noradrenergic neurons in the LoC send projections to the cerebellum, diencephalon and the telencephalon. The locus coeruleus (LoC) complex sends projections into the cortex via the dorsal tegmental bundle. The remaining noradrenergic cell groups form the lateral (LT) tegmental system and send their projection fibers via the central tegmental bundle which innervates areas like the hypothalamus (Role and Kelley, 1991). LT neurons also project to the spinal cord, the brainstem, cerebellum, thalamus, and, to a lesser extent the telencephalon. For the most part, projections from the LoC and the LT do not overlap. They both may innervate the same brain region, but they do not appear to innervate the same area within a given brain region (Fillenz, 1990). Projections from the LoC provide noradrenaline (NA) to most of the telencephalon (Kitt and Brauth, 1986; Loughlin et al., 1986b).

Noradrenergic cells receive input from sensory and motor pathways and modulate sleeping, dreaming, attention and arousal (Butler and Hodos, 1996). The activity of LoC neurons appear to mediate a heightened state of arousal and attention to changes in the environment by enhancing the signal to noise ratio or the gating of sensory neurons (Marshall and Finlayson, 1988). NA and the closely related catecholamine, dopamine (DA), are believed to

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modulate information processing in the brain by increasing cell responsiveness to stimuli (Servan-Schreiber et al., 1990).

NA mediates many hormone effects and has been implicated in the hormonal control or modulation of sexual behavior in both males and females (Hansen et al., 1982; Sachs and Meisel, 1988). Research in mammals, which used adrenoceptor agonists, antagonists or a noradrenergic neurotoxin strongly, suggests that NA modulates male sexual behavior. In male rats, using NA receptor agonists decreased sexual behavior while using NA receptor antagonists increased sexual behavior (Smith et al., 1987; Sachs and Meisel, 1988). In contrast, destruction of the noradrenergic terminals with the neurotoxin, DSP-4, decreased sexual behavior (Hansen et al., 1982). In females, the noradrenergic system also appears to be involved in the regulation of luteinizing hormone releasing hormone (LHRH) secretion patterns (Gore and Terasawa, 2001). LHRH stimulates gonadal hormone production, which in turn activates sexual behavior.

Noradrenergic projections from both the LoC and the LT terminate in some areas of the hypothalamus known to regulate sexual behavior and the endocrine system (Loughlin et al., 1986b; Wright and Jennes, 1993). Heritage et al. (1980) and Scott et al. (1999) showed that steroid hormones are concentrated in the nuclei of catecholaminergic (CA) neurons in the brainstem. Conversely, in the hypothalamus, CA nerve terminals surrounded neurons containing steroid receptors (Tetel and Blaustein, 1991). These findings suggest that not only are hormones signaling CA neurons, but CA neurons through their neurotransmitters, are communicating with, or regulating hormones, and/or their target tissues. We know that changes in hormone levels can lead to changes in behavior, but it appears too that changes in CA

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function can directly affect hormone levels and behavior (Barraclough and Wise, 1982; Balthazart et al., 1988; Barclay et al., 1992; Barclay et al., 1996; Carter, 1992).

Research in our laboratory focuses on the effects of changes in endogenous biochemicals (i.e. gonadal hormones and neurotransmitters) on the courtship singing of male zebra finches. Singing behavior in male zebra finches is controlled by a set of hormone-sensitive interconnected brain nuclei known as the vocal control system (VCS) (Nottebohm et al., 1976; Arnold et al., 1976; Nottebohm et al., 1982). There are two components to the VCS, a song-learning pathway or recursive loop and a motor pathway. Juvenile males use the song-learning pathway during the process of song learning, and the motor pathway controls the muscles of the syrinx, the avian vocal organ (see Figure 1 for details; Nottebohm et al., 1976). In this species, only the males can sing. Females cannot sing because they lack a song learning nucleus and their motor pathway is incomplete (Nottebohm and Arnold, 1976; Nordeen and Nordeen, 1988).

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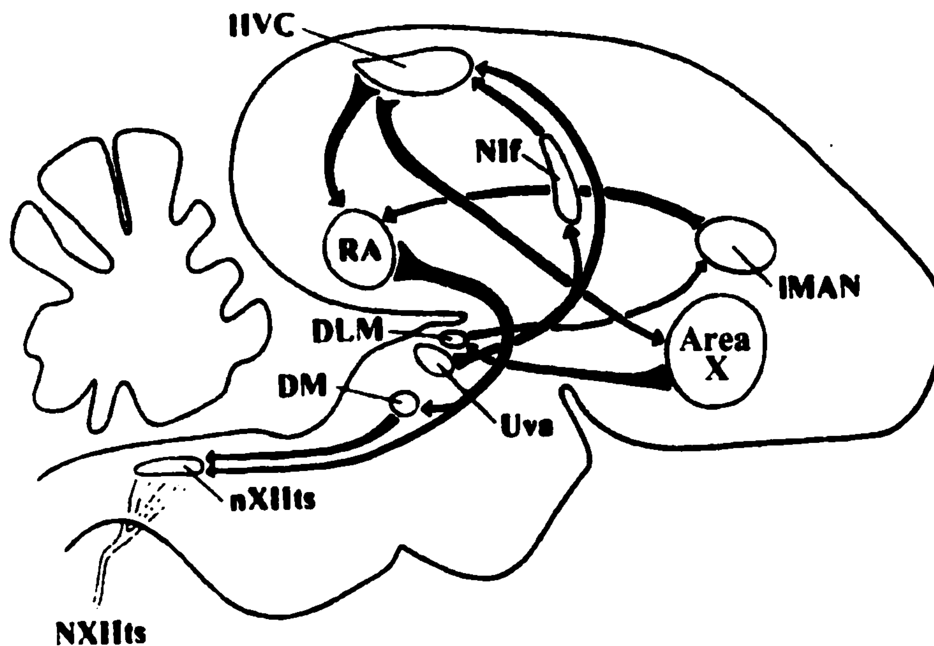

Figure 1: A schematic sagittal view of the zebra finch vocal control system (VCS). Uva-->NIf-->HVC-->RA-->nXIIts is a direct efferent path controlling the motor patterning of song. HVC-->Area X-->DLM-->IMAN is known as the song-learning pathway. MLd, which is not shown, is an auditory nucleus, which is involved in providing auditory input to the VCS. See Table 1 for abbreviations.

Our studies suggest a modulatory role of the noradrenergic system in the control of courtship singing behavior in male zebra finches. However, in order to view the whole picture, (e.g. brain regions outside of the VCS that may be involved with song production), the noradrenergic system needs to be mapped. The extent of NA innervation, particularly in the VCS and surrounding tissue can then be investigated. After mapping the noradrenergic system, the effects of hormonal manipulations and neuropharmacological techniques on the noradrenergic system can be assessed.

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Harding et al. (1983) demonstrated that treating castrates with androstenedione (AE) which provides both androgenic and estrogenic metabolites restored the highest levels of courtship behavior. AE was even better than T in restoring most courtship behaviors. Barclay and Harding (1988) found that steady-state CA function in AE-treated castrate zebra finches differed significantly from that in cholesterol-treated controls in a total of five different vocal control nuclei (VCN) and hypothalamic nuclei (HN).

A later study found the two pathways of the VCS responded differently to hormone treatment. CA turnover rates in most nuclei on the motor pathway increased with hormone treatments (estrogen & androgen) while CA turnover rates in two VCN in the song-learning pathway decreased with hormone treatment (Barclay and Harding, 1990). AE activated the highest levels of courtship singing in zebra finches and the increased CA turnover it caused in the motor pathway appears to be related to increased neuronal activity within this pathway (Harding et al., 1983; Barclay and Harding, 1988).

Administration of the noradrenergic neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) has been used to deplete telencephalic NA levels in rodents. When DSP-4 is administered systemically, it crosses the blood-brain barrier and selectively destroys telencephalic noradrenergic axon terminals which originate in the LoC but leaves noradrenergic terminals in the hypothalamus relatively unaffected (Jonsson et al., 1981; Jonsson et al., 1982; Ross, 1976; Ross and Renyi, 1976). Depending on the species, DSP-4 treatment either inhibited or enhanced male sexual behavior. In rats, DSP-4 delayed the onset of copulatory behavior (Hansen et al., 1982). Conversely, in male Japanese quail, Balthazart et al. (1988) found that copulatory behavior in T-treated castrates was enhanced by decreasing NA levels with DSP-4.

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However, in Japanese quail, NA depletion with another noradrenergic neurotoxin which was restricted to the medial preoptic area (POM) caused decreases in sexual behavior (Bailhache et al., 1993).

In zebra finches, it was hoped that DSP-4 would produce significant depletions of NA levels in the telencephalon where most of the VCN are located but leave NA function in the hypothalamus unaffected, as it does in rats (Hallman and Jonsson, 1984). These studies attempted to affect singing behavior by destroying the LoC neurons, which presumably innervate the VCS. Both systemic and central administration of DSP-4 caused significant decreases in courtship behavior in male zebra finches, but lowered both telencephalic and hypothalamic NA levels (Barclay et al., 1992; Barclay et al.,1996). Only central administration of DSP-4 significantly reduced NA levels.

The current experiments used immunocytochemistry to localize the synthetic enzyme of NA, dopamine-beta hydroxylase (DBH). The area and distribution of DBH-immunoreactive (DBH-ir) fibers and cell bodies, presumably noradrenergic neurons, was examined. The goal of the first experiment was to map the central noradrenergic system in male zebra finches. Special emphasis was given to the brain nuclei of the vocal control system and to two hypothalamic nuclei.

The second experiment examined the effects of endocrine status on DBH-ir staining intensity. In zebra finches, castration caused NA turnover rates in the motor pathway to decrease, while NA levels and/or turnover rates in the song-learning pathway increased (Barclay and Harding, 1988). The intensity of the DBH-ir innervation of the VCS and surrounding tissue in AE-treated males was compared to that in sham-treated males. The distribution

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of the DBH-ir innervation to the VCS was examined and we attempted to find the differences between presumptive noradrenergic innervation of the motor pathway and the song-learning pathway of the VCS.

Finally, the third experiment examined the extent of the damage caused by the neurotoxin DSP-4 on the noradrenergic system. Our earlier studies found deleterious effects of DSP-4 on courtship singing of male zebra finches and depletions of NA levels in VCN and HN (Barclay et al., 1992; Barclay et al., 1996). In rats, DSP-4 affected the noradrenergic innervation of the telencephalon but did not affect the noradrenergic innervation of the hypothalamus (Jonsson et al., 1981; Jonsson et al., 1982; Ross, 1976; Ross and Renyi, 1976). This experiment determined whether the differences between finches and rats in the effects of DSP-4 on noradrenergic function in the hypothalamus were caused by differences in the noradrenergic innervation of the hypothalamus or differences in the methodology used to assess NA function.

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CHAPTER 2:

A MAP OF THE NORADRENERGIC SYSTEM IN MALE ZEBRA FINCH BRAINS: DISTRIBUTION OF DOPAMINE-BETA HYDROXYLASE (DBH) IMMUNOREACTIVE CELLS AND FIBERS

ABSTRACT

Courtship singing in male zebra finches is controlled by a group of hormone-sensitive interconnected brain nuclei known collectively as the vocal control system (VCS). Previous studies from our laboratory suggested that the catecholamine noradrenaline (NA) might play a role in regulating courtship singing. The purpose of the current study was to utilize dopamine-beta hydroxylase (DBH) immunocytochemistry to study the noradrenergic innervation of the finch brain. Special attention was given to the VCS, two hypothalamic nuclei and one auditory nucleus; however DBH-immunoreactive (DBH-ir) labeling in other brain areas is also described. DBH-ir neurons were concentrated in two brainstem cell groups, the locus coeruleus (LoC) and ventral subcoeruleus (SCv). In the VCS, nucleus uvaeformis had densely-labeled DBH fibers. Heavily-labeled DBH fibers were found in the dorsomedial portion of the intercollicular nucleus, the lateral portion of nucleus magnocellularis neostriatum anterior and moderately-labeled DBH fibers in the auditory nucleus - dorsal part of the lateral mesencephalic nucleus. Finally, there were moderately-labeled fibers in the robust nucleus of the archistriatum

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and lightly-labeled fibers in the high vocal center. Area X and the surrounding parolfactory lobe were almost devoid of DBH immunoreactivity. Outside the VCS, fiber labeling was heaviest in LoC, SCv and in both hypothalamic nuclei (paraventricular nucleus and preoptic area). The granular layers of the cerebellum, ventral tegmental area and the septum were moderately labeled, while the optic tectum exhibited a layered arrangement of DBH-ir fibers ranged in staining intensity from heavily labeled to lightly labeled. A thorough description of the noradrenergic innervation is presented, and the possible involvement of this neurotransmitter in courtship behavior of male zebra finches is discussed.

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INTRODUCTION

Male zebra finches possess a discrete set of brain nuclei that control their vocalizations. These brain nuclei are hormone sensitive and are known collectively as the vocal control system (VCS) (Nottebohm et al., 1976; Nottebohm et al.,1982; see Figure 1). Several studies have established that gonadal hormones regulate both courtship singing and sexual behavior in this species (Arnold, 1975; Harding et al., 1983; Bohner et al., 1992). Precisely how gonadal hormones affect this neural-motor circuit to alter behavior remains unclear, but ultimately neurotransmitter systems have to be involved.

Research in mammals found that a class of neurotransmitters known as the catecholamines regulated the frequencies of sexual behaviors and/or displays (Carter, 1992, Sachs and Meisel, 1988; Smith et al., 1987). In ring doves, Barclay and Cheng (1992) and in Japanese quail, Balthazart et al. (1988) found that noradrenaline (NA) levels regulated vocalizations. Research in zebra finches found that catecholamine levels were strongly modulated by hormone treatments which activated high levels of singing in adult males (Barclay and Harding, 1988; Barclay and Harding, 1990). Catecholaminergic function in the VCS as measured by levels and turnover rates of NA and dopamine (DA) were extremely high during the critical period of song development (Harding et al., 1998). Pharmacological manipulations that lowered central NA levels significantly lowered the frequency of courtship singing in this species (Barclay et al., 1992; Barclay et al., 1996). These data suggested a possible role for the noradrenergic system in regulating courtship singing in male zebra finches,

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and therefore a map of this system, with special interest given to the innervation of behaviorally-relevant brain nuclei, was necessary.

The purpose of this study was to document noradrenergic innervation in male zebra finch brains. Immunocytochemical techniques were employed to localize the final enzyme in the synthesis of NA, dopamine-beta hydroxylase (DBH). The information gathered from this map should help to elucidate the role of the noradrenergic system in regulating courtship singing in male zebra finches.

Another reason for documenting noradrenergic innervation was that information about the source of the innervation of NA into the VCS was unknown but was presumed to originate from the LoC. Recently, Appeltants et al. (2000) found that LoC sends innervation to HVC. In rats, central noradrenaline has only two known sources, the locus coeruleus (LoC) and the lateral tegmental (LT) system. Recently, Mello et al. (1998) also mapped the noradrenergic innervation in zebra finches. For the most part, there is agreement between their study and the current study. However, differences are pointed out and discussed. This map provides information on central noradrenergic innervation in the VCS, the hypothalamus and surrounding tissues.

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METHODS

Subjects

Zebra finches *(Taeniopygia guttata)* were obtained from Canary Bird Farm (Englishtown, NJ). Males and females were treated with ivermectin to protect against leg-mite infections and housed in separate aviaries until needed. Bird rooms were kept on a 14:10 h light:dark cycle with the temperature controlled (24 ± 2°C) and the humidity kept over 50% to maintain optimal breeding conditions. Birds were fed a vitamin-supplemented (8 in 1, Pet Products) commercial finch seed mix, grit, water, and cuttlebone ad lib., supplemented with fresh greens and oranges. Sexually-naive adult birds were used. During the experiment, males were housed in individual cages $(56\ cm)^3$ and stimulus females were group housed until needed. All animal care, experimental procedures, and euthanasia were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee of Hunter College of the City University of New York.

Data from the saline-treated control males from the N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) experiment, which is the third study presented in this dissertation, were used for this mapping study. DSP-4 is a noradrenergic neurotoxin that was used in the third study to deplete central NA levels. All males received 20 mg/kg zimelidine dihydrochloride (20 µg/0.01 ml saline/g body weight, i.p.) to protect serotonergic neurotransmitter systems (Ross and Renyi, 1976). After thirty minutes, controls (n=8) received the saline vehicle, i.p. Drug treatments were repeated ten days later. One hour after the

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second drug administration, each male was housed with a stimulus female for the next ten days. Males were then anesthetized, perfused, and their brains processed for immunocytochemistry or cresyl-violet staining.

Perfusion

Males were mildly anesthetized with a combination of Xylazine:Ketamine (5 μg each/g body weight) in 0.5 ml saline injected into the pectoral muscle. Metofane (Pitman-Moore) was administered as necessary to keep birds deeply anesthetized during the procedure. Males were transcardially perfused with 0.2 M phosphate buffer (PB) followed by 4% paraformaldehyde in 0.1 M PB. Brains were quickly removed and post-fixed in 4% paraformaldehyde. The post-fixing solution was replaced with cryoprotectant (300 g sucrose, 10 g polyvinyl-pyrrolidone, 30 ml ethylene glycol, 2 g sodium azide, volume adjusted to 1000 ml with 0.1 M PB) and stored at 5°C. The brains stayed in the cryoprotectant until they sank (Côté et al., 1993; Beltz and Burd, 1989).

Immunocytochemistry

Brains were mounted onto frozen cryostat chucks with distilled water. Frozen brains were allowed to equilibrate in an American Optical Cryo-cut II or an IEC Minotome Plus at -15°C for an hour, then sliced into 40-micron-thick coronal sections. Three series of sections were collected into glass vials

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containing cryoprotectant. Sections were either stored at -70°C until needed or stored at 5°C for immediate immunocytochemical processing.

Sections were processed to optimize DBH staining in finch brains. Unless otherwise stated, all rinses were performed three times for five minutes each in Buffer A (0.05 M tris-buffered saline) while sections were gently agitated. In the first step, free-floating sections from each bird were rinsed then incubated for 10 minutes in 0.1% $NaBH_4$ in Buffer A. After another rinse, sections were incubated for 10 minutes in 1% H_2O_2 in Buffer A, rinsed and incubated for 10 minutes in 20% normal goat serum (NGS) in Buffer B (Buffer A with 2% NGS and 0.5% Triton X-100). Sections were incubated with the primary DBH antibody (Eugene Tech International, Inc; NJ; DBH TE103) at a dilution of 1:500 in Buffer B for 24 hrs at 5°C. The primary antibody was re-used a maximum of six times. After a rinse with Buffer B, sections were incubated with biotinylated anti-rabbit IgG at a dilution of 1:600 (Vectastain Elite ABC Kit; Vector Labs, Burlingame, CA) in Buffer B for 45 minutes at room temperature. Sections were rinsed twice for five minutes in Buffer B, rinsed twice for five minutes in Buffer A, then incubated in ABC reagent diluted in Buffer A according to the manufacturer's instructions for 40 minutes. Sections were rinsed and treated with DAB peroxidase substrate (SigmaFast 3,3'-diaminobenzidine tablet sets in Buffer A). After a final rinse, sections were sorted and mounted onto gelatin-coated slides. Slides were dehydrated in a graded alcohol series, cleared with xylene and coverslipped with Permount. Another vial containing unprocessed tissue was rinsed, sorted, mounted and

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stained with cresyl violet. This set of slides served as a cytoarchitectural reference for the DBH-immunolabeled slides. The third vial was stored as a spare.

Specificity Check

There were two checks for the specificity of DBH-antiserum in finch brains. In the positive control, the antiserum was preadsorbed with an excess of DBH (Sigma) and was used in an assay. In the negative control, incubation with the anti-DBH serum was omitted from the immunocytochemistry assay.

It is important to note that adrenergic neurons also contain the enzyme DBH. In finches, adrenergic levels in most of the VCN were below detectability, but higher levels of this neurotransmitter were found within the hypothalamic nuclei in some birds (Barclay & Harding, 1990). In rats and in another bird species, adrenergic cells were restricted to the medulla (Moore and Card, 1984; Steeves et al., 1987). Tissues from the medulla were not included in this study. Thus the great majority of immunoreactive cells and fibers seen are believed to be noradrenergic rather than adrenergic.

Figure Preparation

For Figures 2A-2J slides were observed under low power using a Nikon microscope with a reticle and were hand drawn onto graph paper. These drawings were traced using a digital pen on an 8" x 6" graphics tablet (Aiptek HyperPen 8000 USB) running the software ArtDabbler 2.1 (MetaCreations

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Corp., Carpintera, CA). Text labels were also added in this program. Images from this program were saved as bitmap files and the straight lines for all the text labels were added in Microsoft ® Paint for Windows 98 (Microsoft Corp., Seattle, WA). The photomicrographs (Figures 3-5) were the images captured by a Dage MTI CCD72 video camera interfaced via a Perceptics Pixel framegrabber to a MAC2 FX computer. Text labels and straight lines were added in NIH Image version 1.55 (written by Wayne Rasband at the U.S. National Institutes of Health), and the final layouts were arranged in Canvas Macintosh version 3.5 (Deneba Systems, Inc., Miami, FL).

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TABLE 1: List of abbreviations for zebra finch brain areas.

ABBREVIATIONS

with modifications from Stokes et al. (1974)

A	Archistriatum
Ac	Nucleus accumbens
AQ	Aqueduct
BCA	Brachium conjunctivum ascendens
BCD	Brachium conjunctivum descendens
Cb	Cerebellum
CO	Optic chiasm
CoA	Anterior commissure
CP	Posterior commissure
DBC	Decussatio brachiorum conjunctivorum
DLL	Lateral portion of the dorsolateral nucleus of the anterior thalamus
DLM	Medial portion of the dorsolateral nucleus of the anterior thalamus
DM	Dorsomedial portion of the intercollicular nucleus
DMP	Dorsomedial nucleus of the posterior thalamus
E	Ectostriatum
EW	Nucleus of Edinger-Westphal
FA	Frontal tract of the archistriatum
FDB	Diagonal band of Broca
FLM	Medial forebrain bundle
FPL	Lateral forebrain bundle
GCT	Central gray
HA	Accessory hyperstriatum
HD	Dorsal hyperstriatum
HP	Hippocampus
HV	Ventral hyperstriatum
HVC	High vocal center
ICo	Intercollicular nucleus
IM	Magnocellular portion of the isthmus nucleus
IPC	Parvocellular portion of the isthmus nucleus
LAD	Dorsal archistriatal lamina
IMAN	Lateral magnocellular nucleus of the anterior neostriatum
LMD	Dorsal medullary lamina
LoC	Locus coeruleus
LPO	Parolfactory lobe
MLd	Dorsal part of the lateral mesencephalic nucleus

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TABLE 1: List of abbreviations for zebra finch brain areas (cont'd).

ABBREVIATIONS

N	Neostriatum
NC	Caudal neostriatum
NIf	Nucleus interfacialis
nIV	Trochlear motor nucleus
NXIIts	Tracheosyringeal portion of the hypoglossal nerve which innervates the syrinx
nXIIts	Tracheosyringeal portion of the hypoglossal nucleus
OM	Occipitomesencephalic tract
OMd	Dorsal portion of the oculomotor nucleus
OMv	Ventral portion of the oculomotor nucleus
pap	Papillioform nucleus
PMH	Posterior portion of the medial hypothalamic nucleus
POA	Preoptic area
PVN	Paraventricular nucleus
RA	Robust nucleus of the archistriatum
RPgc	Gigantocellular reticular nucleus
Rt	Rotund nucleus
SCv	Ventral subcoeruleus
S	Septum
SL	Lateral septum
SLu	Semilunar nucleus
SM	Medial septum
SN	Substantia nigra
SPM	Medial spiriform nucleus
TeO	Optic tectum
TFM	Frontal thalamic and medial thalamic tract
TIO	Isthmic optical tract
Tn	Nucleus taeniae
TrO	Optic tract
TrSM	Septomesencephalic tract
Tu	Nucleus tuberis
Uva	Nucleus uvaeformis
V	Ventricle
VTA	Ventral tegmental area
Area X	Area X of the parolfactory lobe

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RESULTS

Noradrenergic map of male zebra finch brains:

Both specificity checks used in this experiment were DBH immunonegative. DBH-ir cell bodies were only found in hindbrain sections in the cell groups identified as the LoC and the SCv (Figures 2B-2D, 3B). The intensity of labeling in efferent fiber tracts varied. In the hindbrain, there were lightly-labeled DBH fibers in the brachium conjunctivum descendens (BCD) and moderately-labeled fibers in decussatio brachiorum conjunctivorum (DBC), occipitomesencephalic tract (OM) and posterior commissure (CP) (Figures 2B-2E). In the diencephalon, there were lightly-labeled fibers in OM and hyperstriatical lamina (LH) (Figure 2E, 2H). Again, CP had moderately-labeled fibers. At the level of paraventricular nucleus (PVN), fiber labeling in frontal thalamic and medial thalamic tract (TFM) was light, but at the level of preoptic area (POA), fiber labeling in lateral forebrain bundle (FPL) was stronger (Figure 2H, 2I).

In general, DBH-ir fiber staining in the hindbrain ranged in intensity from light in gigantocellular reticular nucleus to dense (LoC and SCv). In the diencephalon, fiber labeling was strong with the densest staining in periventricular hypothalamic areas (PVN and POA) (Figures 2H, 2I, 5A, 5B). Finally, in the telencephalon, fiber staining ranged from moderate (robust nucleus of the archistriatum – RA) to almost none (parolfactory lobe - LPO) (Figures 2F, 2I-2J, 4A, 5D).

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Midbrain & Hindbrain

Densely-packed DBH-ir cells and fibers were found in LoC and SCv. The cells in dorsal and ventral LoC differed in their packing arrangements and in their shapes. There were other differences between DBH-ir cells in LoC and SCv. For instance, cells in dorsal LoC were often tightly clustered together while cells in ventral LoC were diffuse, and they were even more diffuse in SCv. In terms of their morphology, LoC cells were either round or angular while those in the SCv were predominately elongated.

Sometimes one or two DBH-ir cells were found in the lower cerebellum (Cb). There was moderate DBH-ir fiber innervation to the molecular and granular layers of Cb, but the Purkinje cell layer and white matter had very few DBH-ir fibers (Figures 2A, 3A). In nucleus annularis (ANI), there were heavily-labeled DBH fibers, while in the central gray (GCT) dorsal to the nucleus of Edinger-Westphal, there were also heavily labeled fibers and heavy terminal labeling outlined the somata and processes of many DBH-immunonegative cells (Figure 2C). This pattern of staining has been referred to as "pericellular baskets" or "basket cells" by other investigators (Ball et al., 1988; Bottjer, 1993; Bailhache and Balthazart, 1993).

In optic tectum (TeO), heavily-stained layers of DBH-ir fibers were clearly visible in the outermost layers. The innermost layers of the TeO ranged in their DBH labeling intensities from moderately labeled to lightly labeled (Figures 2C-2E, 3D). The DBC and the auditory nucleus dorsal part of the lateral

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mesencephalic nucleus (MLd) were moderately stained with DBH-ir fibers. The brachium conjunctivum ascendens (BCA) and the song nuclei dorsomedial portion of the intercollicular nucleus (DM) had heavy labeling, and there were also pericellular baskets in DM. In nucleus uvaeformis (Uva) there were heavily labeled DBH fibers (Figures 2C-2E, 3C, 3D). DM also had pericellular baskets. There were moderately-stained fibers throughout the remainder of intercollicular nucleus (ICo), medial spiriform nucleus, dorsomedial nucleus of the posterior thalamus (DMP), medial portion of the dorsolateral nucleus of the anterior thalamus. There were heavily-labeled fibers in the GCT and papillioform nucleus. Both the ventral tegmental area (VTA) and the substantia nigra (SN) had moderately-labeled fibers and pericellular baskets (Figures 2E, 4C). DBH-ir fibers appeared to course through DBC and CP to innervate areas of the lower midbrain in both hemispheres (Figures 2D, 2E, 3C). There was a heavy innervation of DBH-ir fibers in dorsal OM and throughout the ventral midbrain and hindbrain (Figure 2D). However, fibers were sparse in optic tract (TrO) and optic chiasm (CO) (Figures 2H, 2I).

Hypothalamus

Some of the most intensely stained DBH-ir fibers were found in the periventricular regions of the hypothalamic nuclei. There were densely labeled DBH-ir fibers and pericellular basket labeling in the periventricular areas and the areas adjacent to posterior portion of the medial hypothalamic nucleus (PMH) and PVN (Figures 2H, 5A). Labeled pericellular baskets extended from PMH

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into the lateral hypothalamus where the fiber staining was lighter. Darkly stained pericellular baskets were also detected in the dorsal portion of nucleus tuberis and extended up along the walls of the third ventricle. There were very few DBH-ir fibers along the anterior commissure (CoA).

In POA, there were densely stained DBH fibers. From POA, moderately stained DBH fibers extended dorsally towards the septum and laterally towards the diagonal band of Broca and FPL (Figures 2I, 5B). There were a few labeled fibers in the septomesencephalic tract (TrSM) and in the CO (Figure 2I).

Mid- to Caudal Telencephalon

In accessory hyperstriatum (HA), nucleus taeniae (Tn) and in the hippocampus (HP) there were many heavily-labeled fibers that stood out from their surrounds (Figures 2F - 2H). Heavily stained DBH fibers were detected along the dorsolateral edge of the ventral hyperstriatum (HV) (Figures 2H). There were also moderately-stained fibers in the song area, robust nucleus of the archistriatum (RA), along the dorsolateral edge of the archistriatum, along the dorsal medullary lamina (LMD), and lightly-labeled fibers in the caudal neostriatum (NC) (Figures 2F - 2H, 4A). While high vocal center (HVC) and the middle of NC had some DBH-positive fibers, there were more fibers in the strip of HP dorsomedial to HVC and in NC just ventral to HVC (Figures 2G, 4B).

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Rostral Telencephalon

At the level of the song nuclei Area X and the lateral magnocellular nucleus of the anterior neostriatum (lMAN), the HA received a stronger innervation of DBH-ir fibers than the HD (Figure 2J, 5D). Moderately to heavily stained DBH fibers in some birds was observed in sections where lMAN was present. The LPO, which includes Area X was almost totally devoid of DBH-ir fibers (Figures 2J, 5D). In both the FPL and the rostral septum, there were heavily-stained DBH fibers (Figure 2I). The heavily-stained DBH fibers in the rostral septum extended along the walls of the third ventricle, and they appeared to terminate dorsally in HA and ventrally in POA. There were also heavily-labeled fibers in the TFM and lateral septum (SL), but innervation of DBH-ir fibers into medial septum (SM) was sparse (Figures 2H, 5C). DBH-ir fibers appeared to travel through the OM and TFM to innervate SL.

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TABLE 2: List of DBH-ir labeled cells and fiber density in zebra finch brain areas.

Key:	
None/Very Light	-
Light	+
Moderate	++
Intense/Heavy	+++
Dense	++++

MIDBRAIN/ HINDBRAIN	BRAIN AREA	CELL BODIES	BASKET CELLS	FIBER DENSITY
	ANI	N	N	+++
	BCA	N	N	+++
	BCD	N	N	+
	Cb - granular layer	N	N	++
	Cb - Purkinje layer	N	N	-
	CO	N	N	+
	CP	N	N	++
	DBC	N	N	++
	DLM	N	N	+++
	DM	N	Y	+++
	DMP	N	N	++
	GCT	N	Y	+++
	ICo	N	N	+++
	LoC	Y	N	++++
	MLd	N	N	++
	OM	N	N	++
	OMd	N	N	+++
	pap	N	N	+++
	RPgc	N	N	+
	SCv	Y	N	++++
	SN	N	Y	++
	SpM	N	N	+++
	TeO - in layers	N	N	+++ to +
	TrO	N	N	+
	Uva	N	N	+++
	VTA	N	Y	++
HYPOTHAL.	CO	N	N	+
	CoA	N	N	+
	DBB	N	N	++
	FPL	N	N	++
	LH	N	N	+
	LHY	N	Y	++
	OM	N	N	+
	PMH	N	Y	++++
	POA	N	Y	++++

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TABLE 2: List of DBH-ir labeled cells and fiber density in zebra finch brain areas (cont'd).

Key:	
None/Very Light	-
Light	+
Moderate	++
Intense/Heavy	+++
Dense	++++

	BRAIN AREA	CELL BODIES	BASKET CELLS	FIBER DENSITY
HYPOTHAL.	PVN	N	Y	++++
	S	N	N	++
	TFM	N	N	+
	TrSM	N	N	+
	Tu	N	Y	+++
MID-CAUDAL	A - dorsolateral	N	N	++
	HA	N	N	+++
	HP	N	N	+++
	HV	N	N	+
	HVC	N	N	+
	LMD	N	N	++
	N	N	N	++
	RA	N	N	++
	Tn	N	N	+++
ROSTRAL TELENCEPH.	Ac	N	N	+
	FPL	N	N	+++
	HA	N	N	+++
	HD	N	N	+
	HV	N	N	++
	IMAN	N	N	+++ to ++
	LPO/Area X	N	N	-
	N	N	N	++
	OM	N	N	++
	PA	N	N	-
	PP	N	N	-
	S	N	N	+++
	SL	N	N	+++
	SM	N	N	+
	TFM	N	N	+++

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Figure 2A: Distribution of DBH-ir labeling in male finch brains. DBH-ir fibers in the cerebellum. DBH-ir fibers are represented by the black dots. In all drawings, scale bar = 1mm.

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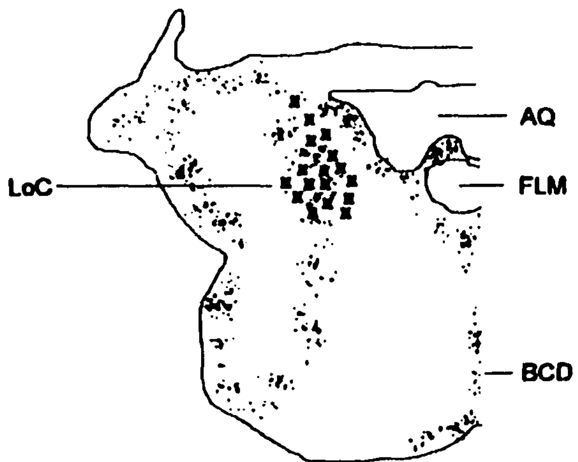

Figure 2B: Distribution of DBH-ir labeling in male zebra finch brain. DBH-ir cells (x's) and fibers (dots) in the locus coeruleus (LoC) and the surrounding brainstem.

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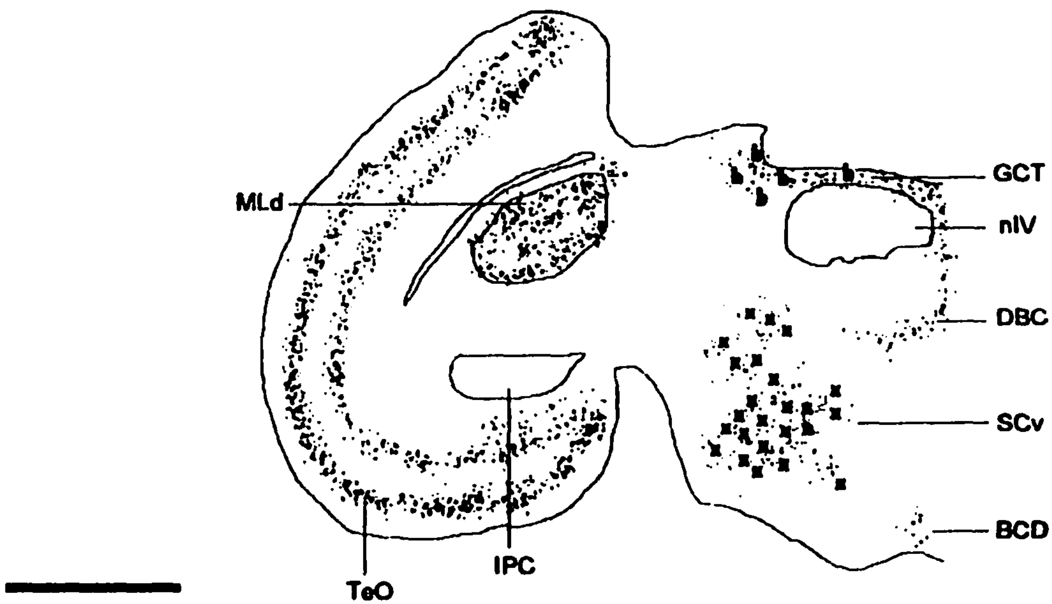

Figure 2C: Distribution of DBH-ir labeling in male zebra finch brains. DBH-ir cells (x), fibers (dots) and pericellular baskets (b) in ventral subcoeruleus (SCv), central gray (GCT), optic tectum (TeO) and an auditory nucleus (MLd).

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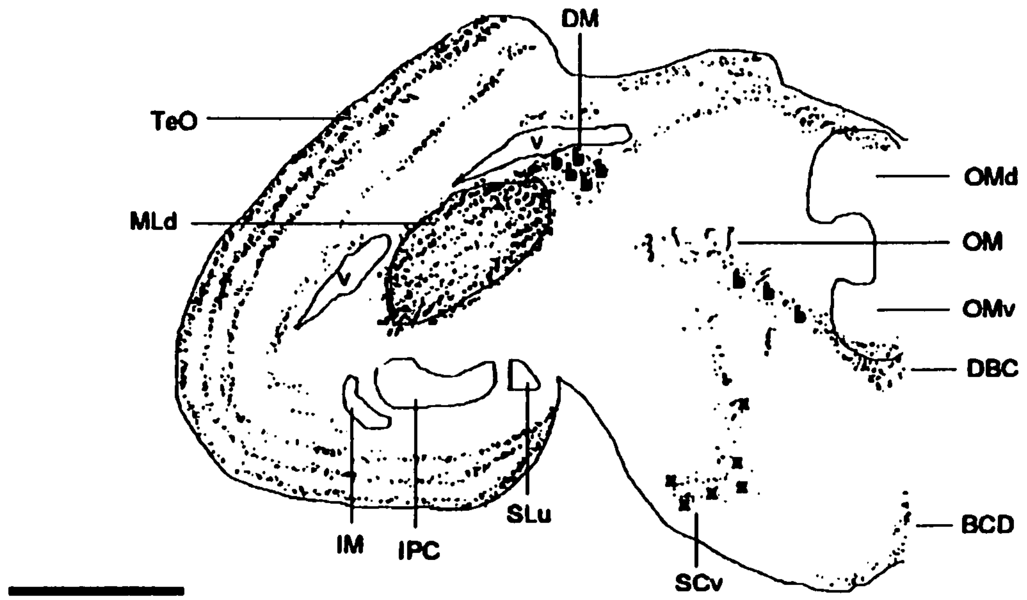

Figure 2D: Distribution of DBH-ir labeling in male finch brains. DBH-ir cells (x), fibers (dots) and pericellular baskets (b). The ventral subcoeruleus (SCv), an area involved in vocalization (DM), optic tectum (TeO) and an auditory nucleus (MLd) are shown.

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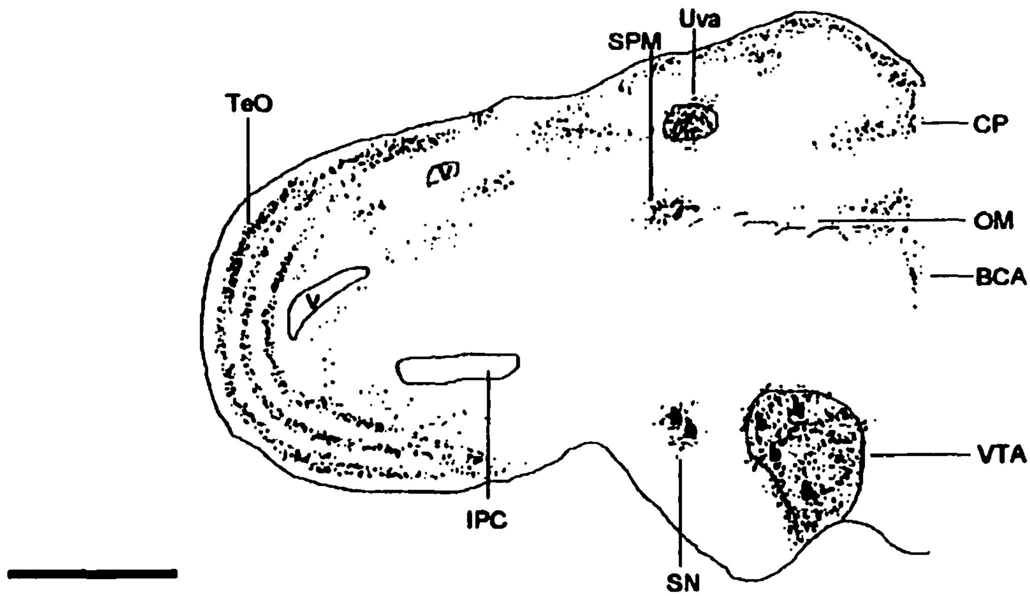

Figure 2E: Distribution of DBH-ir labeling in male finch brains. DBH-ir fibers (dots) and pericellular baskets (b) in the substantia nigra (SN) and ventral tegmental area (VTA).

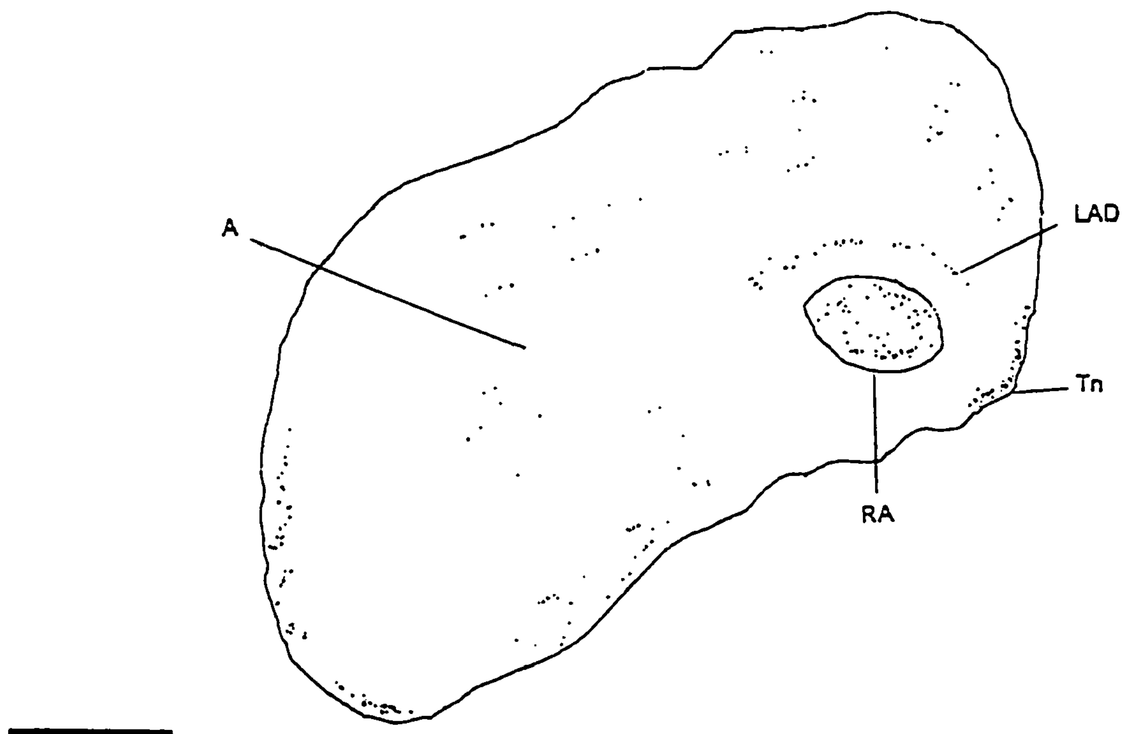

Figure 2F: Distribution of DBH-labeling in the male zebra finch brain. DBH-ir fibers (dots) in the archistriatum and the vocal control nucleus RA are shown.

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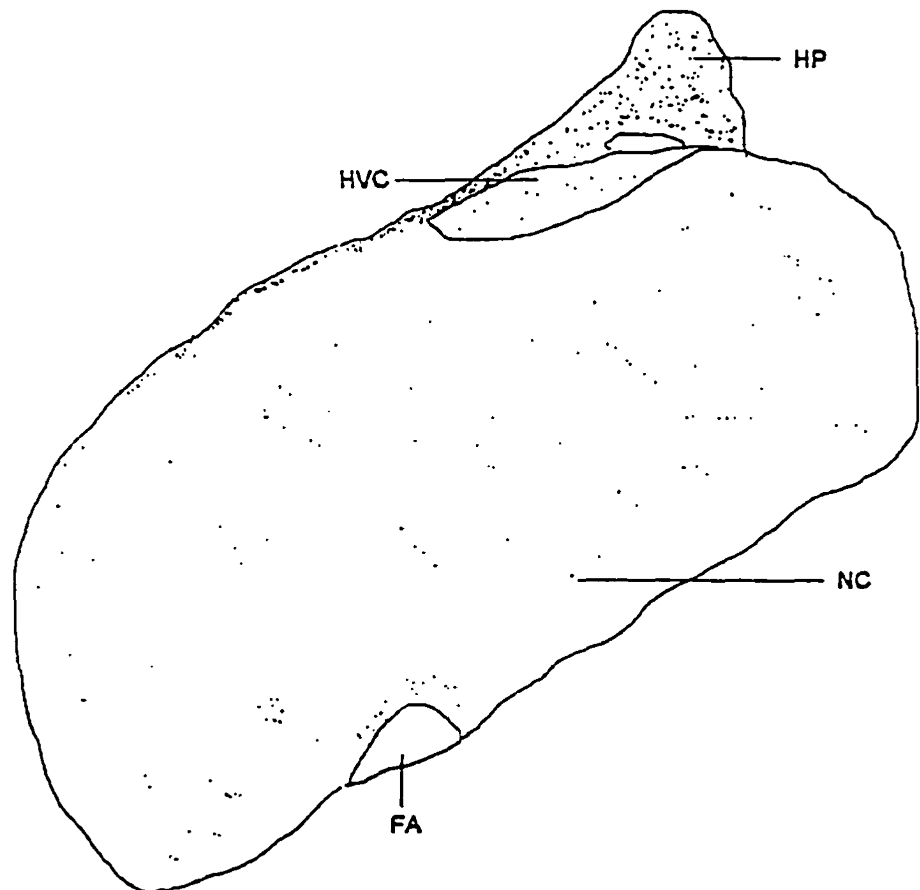

Figure 2G: Distribution of DBH-ir labeling in the male zebra finch brain. DBH-ir fibers in the hippocampus (HP), vocal control nucleus HVC, and neostriatum are represented by black dots.

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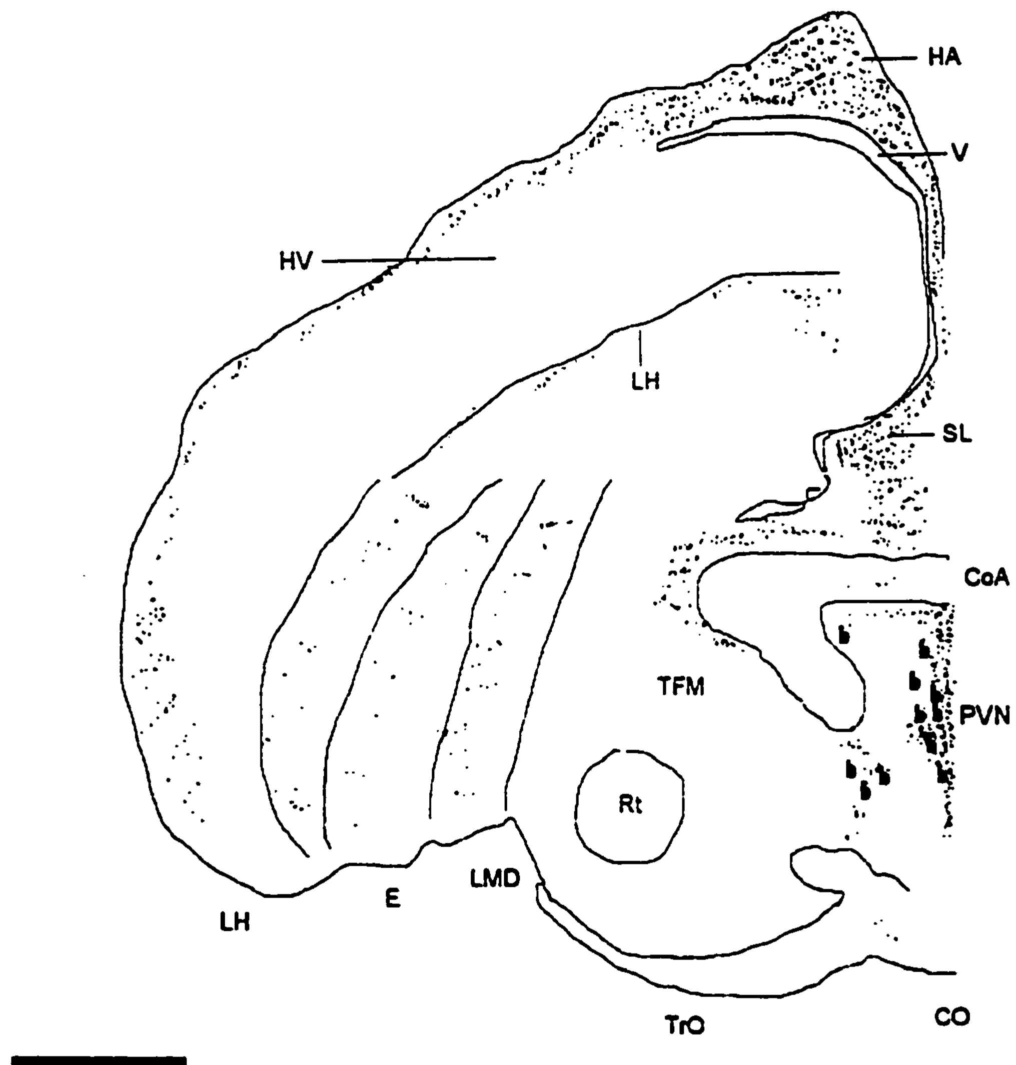

Figure 2H: Distribution of DBH-ir labeling in male finch brains. DBH-ir fibers (dots) and pericellular baskets (b) in the hypothalamic paraventricular nucleus (PVN).

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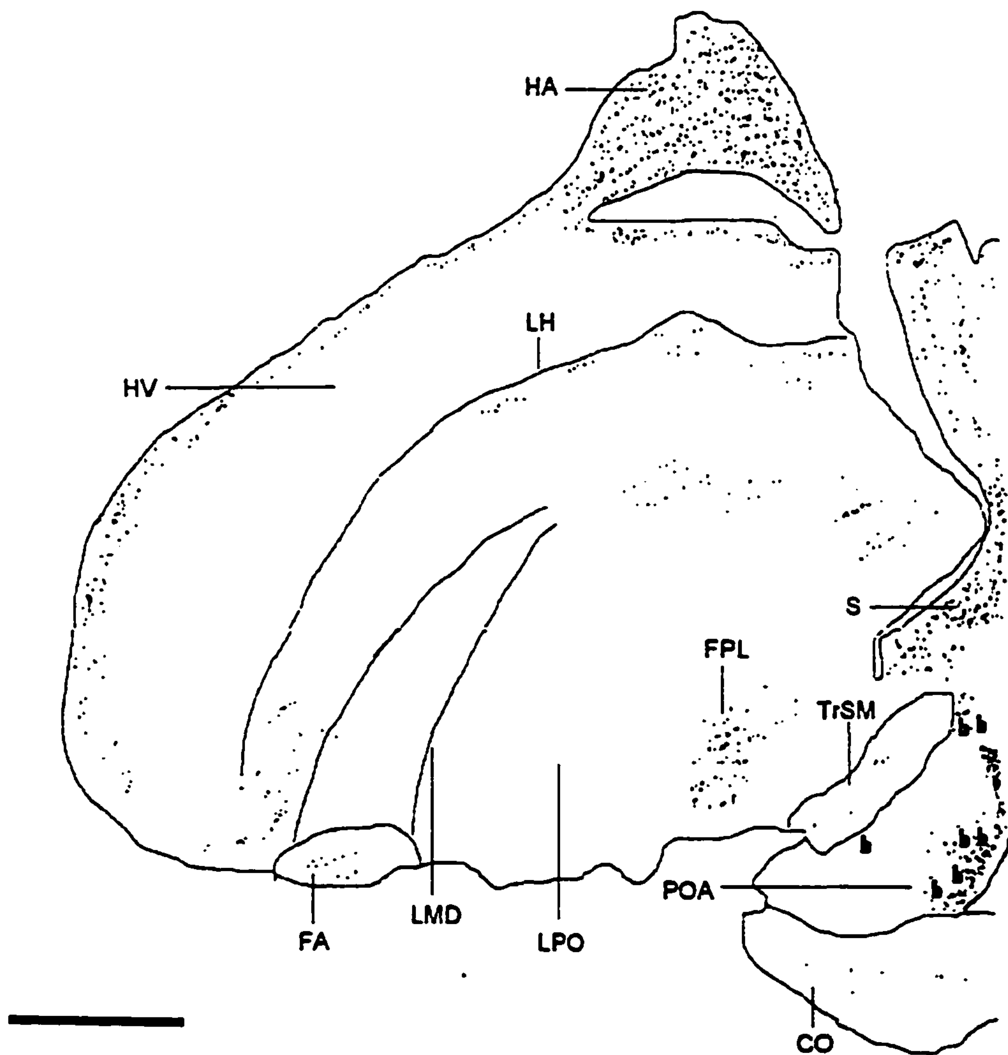

Figure 21: Distribution of DBH-ir labeling in male finch brains. DBH-ir fibers (dots) and pericellular baskets (b) in the hypothalamic nucleus preoptic area (POA).

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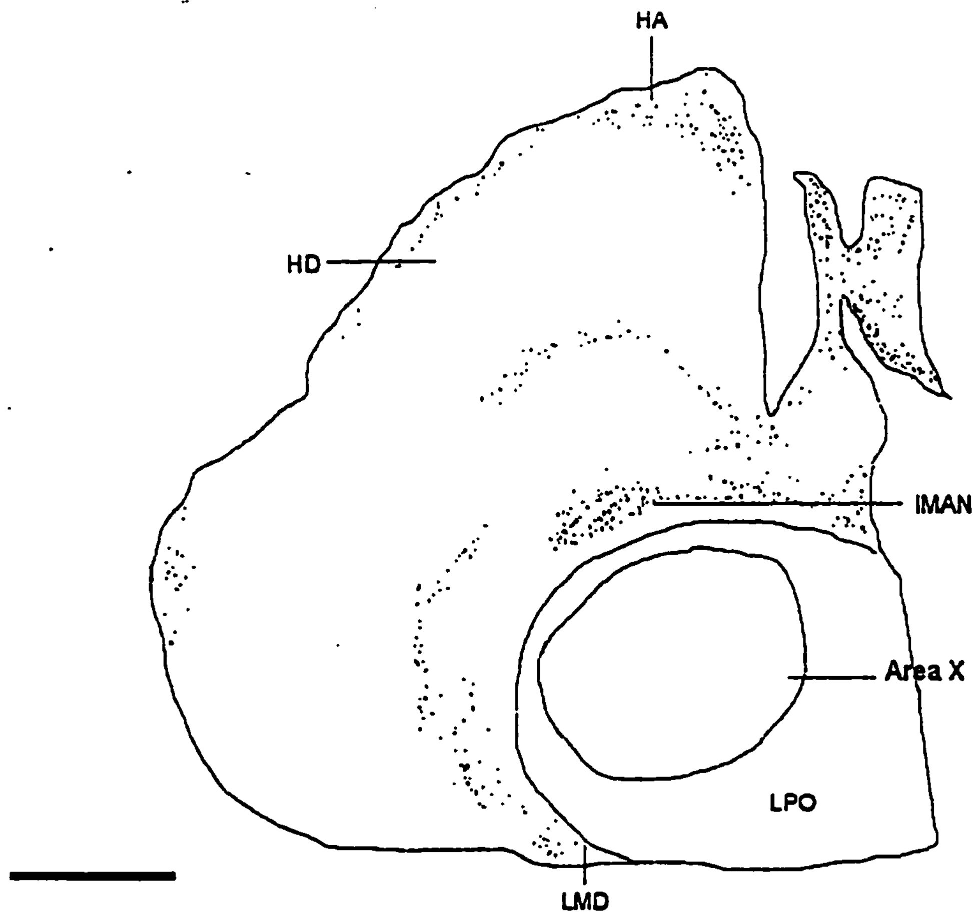

Figure 2J: Distribution of DBH-ir labeling in the male zebra finch brain. DBH-ir fibers in two vocal control nuclei, Area X and IMAN. DBH-ir fibers are represented by the black dots.

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Figure 3A

3B

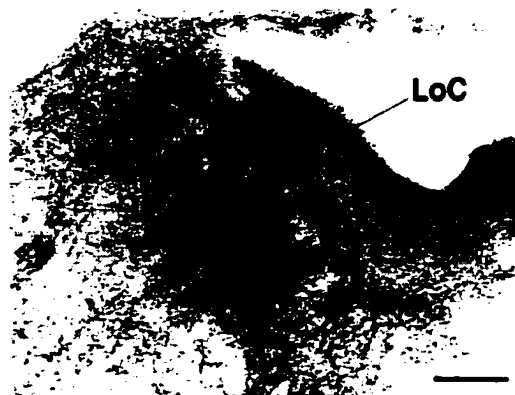

Figure 3C

3D

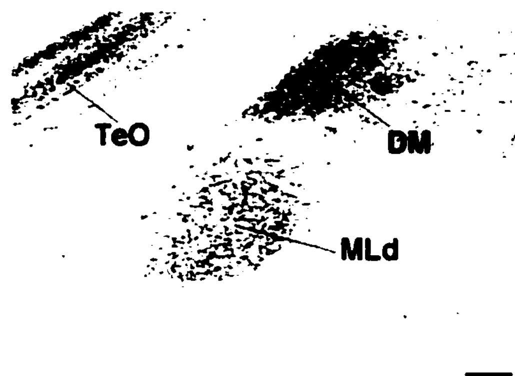

Figure 3: Photomicrographs of DBH labeling in the male zebra finch brain. **A**: DBH-ir fibers in Cb. **B**: DBH-ir cells and fibers in the LoC. **C**: DBH-ir fibers in Uva. **D**: DBH-ir fibers in DM, MLd and TeO. Scale bar = 150 μm. See Table 1 for abbreviations.

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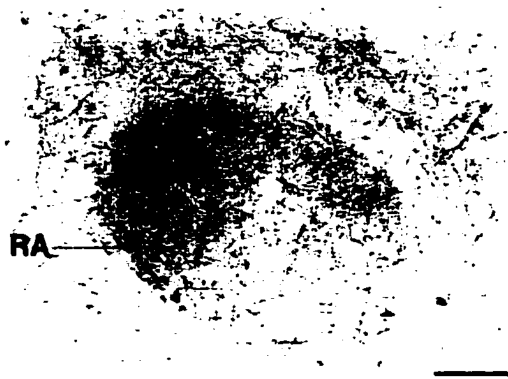
Figure 4A



4B

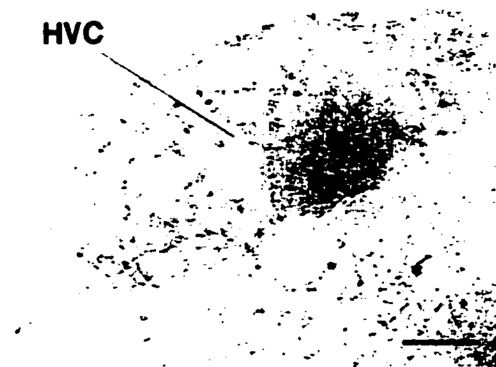


Figure 4C

Figure 4: Photomicrographs of DBH labeling in the male zebra finch brain. **A**: DBH-ir fibers in RA. **B**: DBH-ir fibers in HVC. **C**: DBH-ir fibers in VTA. Scale bar = 150 μm. See Table 1 for abbreviations.

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Figure 5A

5B

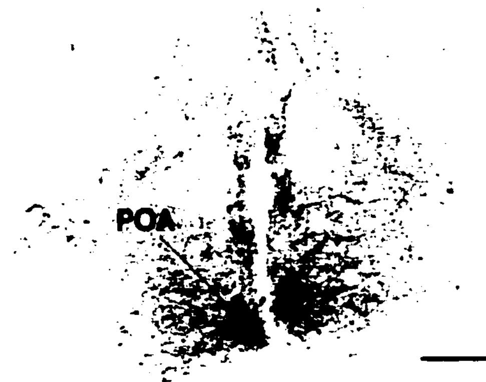
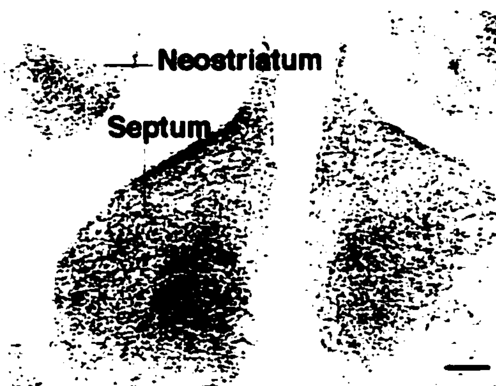


Figure 5C



5D

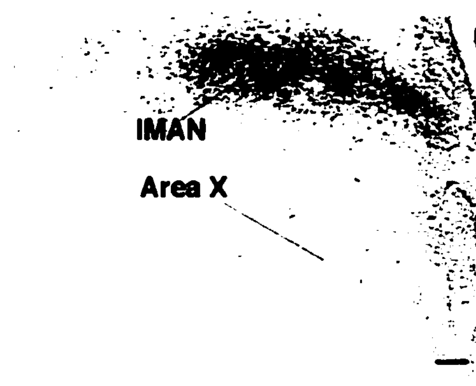


Figure 5: Photomicrographs of DBH labeling in the male zebra finch brain. **A**: DBH-ir fibers in PVN. **B**: DBH-ir fibers in POA. **C**: DBH-ir fibers in septum. **D**: DBH-ir fibers in lMAN and Area X. Scale bar = 150 μm. See Table 1 for abbreviations.

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DISCUSSION

DBH immunocytochemistry in male zebra finch brains revealed a distinct pattern of immunopositive cells and fibers. As expected, DBH-ir cells were only found in two caudal brain areas, the LoC and the SCv. These areas are known to have NA-producing cells in other species, and their fibers innervate higher brain structures via available fiber tracts (Moore and Card, 1984; Butler and Hodos, 1996; Appeltants, 2000).

DBH-ir fibers were found throughout the brain, with some notable exceptions. DBH-fiber labeling was consistently absent within paleostriatal structures, i.e. paleostriatum augmentum, paleostriatum primitivum, and LPO. There were also differences in the intensities of fiber staining. For example, there was very heavy DBH-ir staining and pericellular baskets in periventricular areas of the hypothalamus, while staining in lateral hypothalamic areas was much lighter.

In most cases, the DBH labeling observed in the current study agreed with that in Mello et al. (1998). The only differences were in the staining intensities of some brain nuclei. The lack of staining observed in the paleostriatal areas, including LPO was replicated in this study. In the current study, both Uva and MLd had more densely-labeled fibers than previously described by Mello et al. (1998). Another major difference between Mello et al. (1998) and the current study was the intensity of the DBH-ir staining of the remaining VCN and auditory nucleus (AN) relative to their surrounding areas. In the current study, Uva, MLd, DM, RA and in some cases IMAN contained

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more intense DBH-ir fibers and stood out from their immediate surround. Mello et al. (1998) reported no differences between the DBH-ir staining of the VCN and the surrounding areas.

Some of the differences between these studies could be attributable to methodological differences. In the current study, males were individually housed with females, and the working dilution of the antibody was twice as strong. However, if the antibody concentration alone accounted for these differences, then the intensity of DBH labeling should have increased uniformly in all brain areas. This was not the case; therefore the housing conditions may have contributed to the increased fiber staining in the vocal control areas mentioned.

Previous studies have shown that housing males with females resulted in significant increases in circulating androgen levels and increased singing behavior (Di Prisco et al., 1978; Harding et al., 1983). Previous studies in our laboratory have also shown that treating zebra finches with hormones which stimulated high levels of singing behavior significantly increased levels and turnover of NA in many of the VCN including NIf, HVC, RA, and DM (Barclay and Harding, 1988; Barclay and Harding, 1990). Thus, we housed males with females prior to sacrifice in hopes of increasing their gonadal hormone levels and consequently increasing NA function. We hypothesized that this social housing might increase DBH levels in the VCN and improve labeling.

In the VCN, DBH-ir fiber staining in telencephalic areas ranged in intensity from very limited, e.g. Area X, to moderate, e.g. lMAN. However, in

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Uva, which is the only diencephalic VCN, DBH-ir fiber staining was very heavy compared to its surrounding tissues. We also found that fibers passing through the CP appeared to connect Uva bilaterally. However, tract-tracing studies by Striedter and Vu (1998) found that injections into Uva produced dense terminal labeling but no labeled cells within the contralateral Uva, but there were no labeled cells. Instead, neurons from both the song nucleus DM and the rostral ventrolateral medulla, which is involved in respiration, send projections bilaterally to Uva. Therefore, the fibers that appeared to connect Uva bilaterally may have been an artifact of the plane of tissue sectioning. These axons may actually be the DBH-ir fibers that connect DM. Uva sends projections to HVC and is thought to be important in initiating singing (Nottebohm et al., 1982; Williams and Vicario, 1993). Both ICo regions also appeared to receive some contra-lateral DBH-ir projections via CP. ICo is adjacent to the primary auditory processing nucleus MLd.

In the current study, another VCN, lMAN showed inconsistencies in the intensity of fiber labeling across males. In some subjects, medium-intensity DBH-ir fibers in this nucleus clearly stood out from that in the surrounding neostriatum. However, this was not true for most subjects. In the majority of cases, lMAN and the surrounding neostriatum showed the same intensity of labeling, thus making its borders harder to delineate. Unfortunately, singing behavior was not measured in these birds. However, in the second experiment presented in this dissertation, there was an indication that the intensity of DBH-ir fiber staining in lMAN was associated with prior singing

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behavior and/or courtship intensity. A male that had engaged in high-intensity courtship displays early in the pair test but prior to sacrifice showed more intense DBH-ir fiber labeling in lMAN than other males. Recently, Jarvis et al. (1998) found social context had a direct effect upon the expression of the immediate early gene transcription promoter ZENK in some VCN. When males performed directed songs, HVC had immediate early gene expression. However, when males performed undirected songs, there was labeling in Area X and in HVC. The brain areas, Area X and lMAN were once believed to only be important for juvenile males during the song learning process (Sohrabji et al., 1990; Scharff and Nottebohm, 1991), but now a possible role in singing behavior of adults has been suggested.

NA appears to play an important role in the primary processing of both visual and auditory stimuli (Rasmussen et al., 1986; Klepper and Herbert, 1991), and strong DBH fiber labeling was found in both visual (optic tectum) and auditory processing regions (MLd). This noradrenergic innervation may be important in directing males' attention toward females, since males use both visual (beak color and plumage patterns) and auditory (sexually-dimorphic vocalizations) cues in determining which birds to court. In fact, data from prior studies show that when NA levels are reduced by treating males with DSP-4, males took longer to initiate courtship and to begin singing to females (Barclay et al., 1992; Barclay et al., 1996).

Studies utilizing immunocytochemical techniques to determine which tissues contain DBH have the advantage of allowing researchers to screen

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many brain areas for DBH-ir. Image analysis software now allows quantification of these immunocytochemical data. When we compared our current data on the distribution of DBH-ir to our prior quantification of NA function in hypothalamic, vocal control and auditory nuclei, there was a major inconsistency between them for Area X. Our earlier high performance liquid chromatography with electrochemical detection (HPLC-EC) studies (Barclay and Harding, 1988; Barclay and Harding, 1990; Barclay et al., 1992; Barclay et al., 1996; Harding et al., 1998) consistently found very high NA levels in Area X, but this brain area and its immediate surround lacked DBH labeling. Given the large size of Area X and the lack of DBH-ir in the surrounding LPO, it is highly unlikely that this inconsistency can be explained in terms of inaccurate microdissection in prior studies.

Thus, the source of this inconsistency remains unknown. Smeets and Steinbusch (1990) noted that in the hypothalamus, there were instances where neurons were immunopositive for a neurotransmitter but immunonegative for its synthetic enzyme. They concluded that these neurons were accumulating the neurotransmitter but could not synthesize it. A similar hypothesis was proposed by Mello et al. (1998) to account for the lack of DBH-ir in Area X. Similar inconsistencies between levels or presence of synthetic enzymes and their resulting neurotransmitters have been documented in many other systems (Herkenham, 1991). The fact that significant levels of noradrenergic alpha-2 receptors were found in Area X (Ball, 1990; Casto and Ball, 1996) also suggests that NA is present in this area.

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Other studies have investigated central noradrenergic functioning by localizing NA receptors in songbirds. Alpha-1 receptors were found in lMAN and RA, alpha-2 receptors in Area X, lMAN, HVC, RA, and DM and both beta-1 and beta-2 receptors were found in lMAN, HVC, RA and MLd (Ball, 1990; Casto et al., 1992; Casto and Ball, 1996). Only alpha-2 receptors were found in Area X and DM; however DBH-ir fibers were found in DM. The presence of NA receptors in Area X, despite the lack of DBH labeling in that region supports our previous data that found significant levels of NA there. Unfortunately, these various methods of investigating the central noradrenergic system do not always provide data that are in total agreement. However, even when there are inconsistencies, it suggests a possible limitation of the method employed and is not necessarily indicative of a lack of NA within that brain area.

The DBH-ir found in the remaining VCN (HVC, RA, lMAN, DM) in this study was consistent with the levels of NA measured in these areas in prior studies (Barclay and Harding, 1988; Barclay and Harding, 1990). HVC had low NA levels and had the smallest area of DBH fiber staining. RA had low NA levels but had a larger area of DBH fiber staining, while lMAN had the largest area of DBH labeling and the highest NA levels of the other three VCN (NIf, RA and DM). NA levels in the MLd were also very high (Harding et al., 1998), and it had moderate DBH-ir staining. Outside the VCS, nuclei in the hypothalamus had higher levels of NA and greater areas of dense DBH-ir staining than the VCN and MLd.

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COMPARISON TO DBH-ir IN PRIOR AVIAN STUDIES

The telencephalic VCN are found only in oscine songbirds, parrots, and hummingbirds, which all learn their complex vocalizations. However, the midbrain vocal control nucleus ICo appears to be important in controlling calls in all species studied to date, including a variety of avian species (Phillips and Peek, 1975). The most obvious differences between DBH labeling in finch brains and that in chicken and quail brains were found in the telencephalon. Quail and chicken brains did not have DBH labeling corresponding to that seen in the telencephalic VCN in finches. For example, in the current study, the range in intensity of DBH labeling seen in the neostriatum in finches was not found in either quail or chicken brains. There were two DBH-labeled VCN found in the finch neostriatum; HVC which was lightly labeled and IMAN which was heavily to moderately labeled. In quail, there was no mention of any labeling within this large area. However, the lack of DBH-ir in the LPO and paleostriatal regions with the exception of the nucleus accumbens was a common characteristic of all three avian species (Mello et al., 1998; Bailhache and Balthazart, 1993; Moons et al., 1995)

Greater similarity of staining was seen in the diencephalon, where all three species showed dense labeling in the hypothalamus and some areas of the thalamus. The heaviest labeling occurred in periventricular regions with lighter labeling extending from periventricular areas into lateral hypothalamic regions. Another characteristic in staining that all three species had in common was the presence of DBH-ir pericellular baskets within the densely-

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labeled periventricular hypothalamic nuclei (Mello et al., 1998; Bailhache and Balthazart, 1993; Moons et al., 1995). In the current study, the VCN Uva had densely-stained DBH fibers. Mello et al. (1998) reported negligible staining within this nucleus. As stated previously, this difference in staining may have resulted from housing these males with females in the current study. Clearly, this type of housing arrangement would elicit the highest levels of courtship vocalizations and therefore would affect the neurochemistry within those brain areas responsible for the activation of that behavior.

DBH-ir fibers were located in POA and in some thalamic nuclei in both quail and finches. In quail, DBH-ir fibers innervated the caudal hypothalamus, the lateral edges of the CoA and the anterior hypothalamus (Bailhache and Balthazart, 1993). In finches, there was DBH-ir fiber labeling that was very dense and formed pericellular baskets (Mello et al., 1998).

DBH-ir cells and fibers were found in the brainstems of quail, chickens and finches, i.e. LoC and SCv (Bailhache and Balthazart, 1993; Moons et al., 1995; Mello et al., 1998). In all three species, DBH-ir fibers were also found in LoC, SCv, VTA, ICo and TeO. The auditory nucleus MLd is adjacent to ICo. In Japanese quail, MLd didn't show appreciable DBH-ir staining (Bailhache and Balthazart, 1993); however in the current study, MLd had moderate DBH labeling in finches.

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COMPARISON TO TH-ir IN PRIOR AVIAN STUDIES

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the synthesis of all the catecholamines, DA, NA, Adrenaline (A), and therefore is found in cells producing each of these neurotransmitters. Since DBH is only found in cells producing NA and A, comparison of the distributions of the two enzymes allows one to infer the distribution of DA-producing cells (i.e. areas which contain TH-ir but not DBH-ir). The distribution of DBH labeling in the brains of male zebra finches differed from that of TH-ir in zebra finches, Japanese quail, pigeons, and chickens in many ways. The major difference was in the location of labeled cell groups. In DBH-ir sections, cell bodies were only detected in the hindbrain in the LoC and SCv. However, TH-ir cell bodies were found throughout the brain in the cell groups that were first identified by Dahlström and Fuxe (1964) within the metencephalon, diencephalon and telencephalon (Bottjer, 1993; Bailhache and Balthazart, 1993; Reiner et al., 1994). Throughout the brain, TH-ir labeling also showed more pericellular basket cell labeling than that following DBH labeling (Bottjer, 1993; Bailhache and Balthazart, 1993; Mello et al., 1998; Reiner et al., 1994; Moons et al., 1995).

Another major difference between TH-ir and DBH-ir staining in all three avian species was that DBH immunoreactivity in the paleostriatum was limited to the nucleus accumbens. In all avian species examined, paleostriatal areas had a fine uniform covering of TH labeling. The LPO, which is part of the paleostriatum receives a strong dopaminergic innervation from the VTA (Lewis, et al., 1981; Bottjer et al., 1989). In finches, there was one region within the LPO

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that had a darker region of TH labeling. This region corresponded to the song learning nucleus, Area X. Compared to other VCN, Area X had the highest DA levels when measured by HPLC-EC techniques (Barclay and Harding, 1988; Barclay and Harding, 1990). In fact, these levels were so high that they were comparable to those seen in the hypothalamus. In addition, TH-ir cells and fibers but only DBH-ir fibers were found in the SN (Bottjer, 1993 and Bailhache and Balthazart, 1993; Mello et al., 1998).

NORADRENERGIC SYSTEM AND SINGING BEHAVIOR

As expected, DBH labeling in birds identified cells in the LoC. The activities of LoC neurons are involved with arousal and attention (Marshall and Finlayson, 1988; Aston-Jones et al., 1991; Aston-Jones et al., 1994). Studies that have manipulated NA levels suggest that the LoC is involved in species-specific learning and memory (Cornwell-Jones et al., 1988; Sullivan and Wilson; 1994). In juvenile finches, the process of song learning has two phases; sensory acquisition and sensorimotor practice (Brenowitz et al., 1997; Marler, 1997). During sensory acquisition, male juveniles attend to a male tutor in order to learn from and possibly imitate his song or some elements of his song. During development, NA levels in auditory areas and in most VCN were highest during the period of sensory acquisition (Harding et al., 1998), suggesting that NA plays a role in this process.

In finches, DBH labeling was highest in the LoC, the two hypothalamic

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nuclei, and the diencephalic VCN, Uva. DBH labeling was much lower in the remaining VCN, and almost nonexistent in Area X. Despite the low levels of DBH-ir found in most of the VCN in the current study and the relatively low NA levels found in these nuclei in previous studies (Barclay and Harding, 1988; Barclay and Harding, 1990), noradrenergic function in the VCN is correlated with singing behavior. Treatment with hormone regiments which restore high levels of singing behavior in castrated finches increases NA levels and/or turnover in three nuclei on the motor pathway, NIf, RA and DM (Barclay and Harding, 1988; Barclay and Harding, 1990).

Depletion of NA in Area X, NIf, RA, and DM following treatment with the NA neurotoxin DSP-4 was correlated with deficits in courtship singing in male zebra finches (Barclay et al., 1992). These data suggest that even minor fluctuations in NA levels are sufficient to interfere with courtship behavior. The primary effect of DSP-4 treatment was to delay the onset of courtship singing. It did not affect the motor patterning of their songs. This suggests that DSP-4-treated males take longer to attend to stimulus females and that noradrenergic innervation of sensory brain areas may play a critical role in this effect. As shown in the third experiment in this dissertation, DSP-4 treatment significantly reduced DBH-ir in both primary visual (TeO) and auditory (MLd) processing areas. Since male finches use visual cues, such as beak color and plumage patterns, and auditory cues from female calls to identify birds to court, lowering noradrenergic function in these sensory areas probably also contributes to their deficits in courtship behavior.

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CHAPTER 3:

EFFECTS OF ANDROSTENEDIONE ON THE NORADRENERGIC SYSTEM IN MALE ZEBRA FINCH BRAINS.

ABSTRACT

Data suggest that the effects of gonadal hormones on courtship singing in male zebra finches may be mediated, in part, by noradrenaline (NA). Hormone treatments that modulate singing behavior strongly modulate NA levels and turnover rates in brain areas controlling singing. To determine if gonadal hormones regulate NA function, at least in part, by regulating NA synthesis, the current study examined the effects of castration and hormone treatment on the distribution of dopamine-beta hydroxylase (DBH), the final enzyme involved in the synthesis of NA. All males in this study were castrated, then given either an empty silastic implant (sham-treated) or an androstenedione-filled implant (AE-treated). After being housed with a stimulus female for one month and being tested twice for courtship behavior, males were perfused and their brains processed for DBH immunocytochemistry. DBH-immunoreactive (DBH-ir) labeling was quantified using image analysis software in a total of nine brain regions {four vocal control nuclei, one auditory nucleus, two hypothalamic nuclei, and two additional areas, the locus coeruleus and the septum}. As expected, AE-treated males initiated courtship more rapidly and courted females more

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frequently than sham-treated castrates. AE treatment significantly increased the areas of DBH labeling only in the two hypothalamic nuclei examined, paraventricular nucleus and preoptic area compared to the sham-treated castrates. The possible involvement of NA and these hypothalamic regions in the regulation of courtship behavior in male zebra finches is discussed.

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INTRODUCTION

Gonadal hormones activate sexual behavior in many vertebrate species (e.g., Foster, 1988; Ojeda and Urbanski, 1988; Plant, 1988). It has been established by many studies that castration lowers the frequencies of sexual behaviors and that hormone-replacement therapy reinstates these behaviors to levels seen in intact animals (Leshner, 1978). Target sites for these hormones are found in the brain, and there are many hormone receptors in the hypothalamus and also in sexually-dimorphic nuclei (Arnold et al., 1976; Balthazart and Surlemont, 1990; Gorski et al., 1980). Among the many functions associated with the hypothalamus is the regulation of sexual behavior (Barfield, 1979).

Research in mammals and birds suggests that the effects of gonadal hormones are mediated, in part, by a class of neurotransmitters known as the catecholamines (CAs), which includes noradrenaline (NA). CAs have been implicated in the regulation of species-typical behaviors (Sachs and Meisel, 1988). In rats, treatments that lowered CA levels or functioning, e.g., receptor antagonists decreased sexual behavior (Smith et al., 1987; Carter, 1992). In birds, the effects of manipulating catecholaminergic function were dependent upon the species and brain area targeted. In castrated cockerels, the presence of testosterone (T) had a direct effect upon the release of endogenous CAs from hypothalamic tissues. T treatment enhanced CA release while the lack of T treatment decreased CA release (Francis et al., 1984). In Japanese quail, lowering NA levels stimulated sexual behavior, and

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in ring doves, lowering hypothalamic NA levels increased their nest-coo and bow-coo vocalizations (Balthazart et al., 1988; Barclay and Cheng, 1992).

In finches, hormone treatments which stimulate singing and courtship behavior caused striking changes in NA levels and/or turnover rates in vocal control nuclei (VCN), an auditory nucleus, and hypothalamic nuclei (Barclay and Harding, 1988; Barclay and Harding, 1990). In follow-up studies, lowering NA levels significantly impaired all measures of courtship behavior (Barclay et al., 1992; Barclay et al., 1996). This led us to propose the current study, which questioned how changes in hormone levels regulated the noradrenergic innervation.

Immunocytochemical techniques were employed to visualize the enzyme that catalyzes the synthesis of NA, dopamine-beta hydroxylase (DBH). The areas of DBH-immunoreactivity (DBH-ir) were quantified, with special interest given to the VCN and two hypothalamic nuclei. From earlier studies, it was quite clear that hormones affected NA levels, but there are many ways to achieve this. Possible mechanisms include, but are not limited to hormones increasing levels of synthetic enzymes, increasing synaptic release, or decreasing levels of degradative enzymes. The results from this study combined with the data from our high performance liquid chromatography with electrochemical detection (HPLC-EC) studies may elucidate whether the observed changes in central NA levels resulted from changes in the availability of the synthetic enzyme DBH.

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METHODS

Subjects

Zebra finches *(Taeniopygia guttata)* were obtained from Canary Bird Farm (Englishtown, NJ). Males and females were treated with ivermectin to protect against leg-mite infections and housed in separate aviaries until needed. Bird rooms were kept on a 14:10 h light:dark cycle with the temperature controlled (24 ± 2°C) and the humidity kept over 50% to maintain optimal breeding conditions. Birds were fed a vitamin-supplemented (8 in 1, Pet Products) commercial finch seed mix, grit, water and cuttlebone ad lib., supplemented with fresh greens and oranges. Sexually-naive adult birds were used. During the experiment, males were housed in individual cages $(56\ cm)^3$ and stimulus females were group housed until needed.

Males were randomly assigned to experimental and control groups. All males were castrated. Castrations were performed as described previously (Harding et al., 1984) with the following modifications. Both testes were removed through the same incision. Once the right testis was removed, the membrane between the spine and the vena cava was broken to allow removal of the left testis in similar fashion. Experimental males (n=6) received an implant of AE (5 mm of packed hormone in silastic tubing 0.76 mm ID, 1.65 mm OD) which was placed in the body cavity, after removal of the testes. Control males (n=5) received empty implants. AE was used because it was the most effective

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hormone treatment for restoring reproductive behavior in castrated finches (Harding et al, 1983).

Following implantation, each male was housed with a female for at least one month. Barclay and Harding (1988) reported that AE-treated castrates showed six times more song bouts than controls when exposed to females. Therefore, males were housed with females to facilitate pair-bonding, and hopefully maximize both the behavioral and the neurochemical differences between the two treatment groups.

Behavioral Observations

Males were observed in two post-surgery pair tests. The first was conducted three weeks after castration surgery, and the second was on the day of perfusion, which was during the fourth week. All females that were housed with the males immediately after castration surgery were removed the day before the observations to elicit male courtship behavior at the start of the observation. The same female was re-introduced into the male's home cage, and the birds were observed for fifteen minutes. After the observation, the female remained in the cage with the male. The most common behaviors recorded included courts, mounts, attempted mounts, beakwipes, headwipes, feeding and drinking.

We divided courtship singing into three intensity levels. Males performing low intensity courts sit on the perch in a relaxed posture, fluff out their sexually dimorphic feathers, and sing in the direction of females. Males performing

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medium intensity courts stand erect on the perch, fluff out their sexually dimorphic feathers, and move their heads from side to side while they sing to females. Males performing at high intensity levels start courting females from a distance, and perform a hop and turn dance toward them while singing.

A mount occurs when a male balances himself when he lands on top of a female in an attempt to make cloacal contact with her. During an attempted mount a male fails to steady himself on top of a female for any appreciable length of time (e.g., less than 2 seconds) because, he falls or she withdraws. Beakwipes are performed during feeding to remove hulls from the beak but also occur during all levels of courts. The frequency of beakwipes is typically related to the frequency of total courts. Headwipes are sometimes seen during courts but are usually performed during the act of grooming. All observations were recorded on a Marantz cassette tape recorder (model no. PMD201) for timing of latency to the first courtship song.

Perfusion

Males were mildly anaesthetized with a combination of Xylazine:Ketamine (5 mg each/kg body weight) in 0.5 ml saline injected into the pectoral muscle. Metofane (Pitman-Moore) was administered as necessary to keep birds deeply anesthetized during the procedure. Males were transcardially perfused with 0.2 M phosphate buffer (PB) followed by 4% paraformaldehyde in 0.1 M PB. Brains were quickly removed and post-fixed in 4% paraformaldehyde for two hours. The post-fixing solution was replaced with cryoprotectant (300 g

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sucrose, 10 g polyvinyl-pyrrolidone, 30 ml ethylene glycol, 2 g sodium azide, volume adjusted to 1000 ml with 0.1 M PB) and stored at 5°C. The brains stayed in the cryoprotectant until they sank (Côté et al., 1993; Beltz and Burd, 1989).

Immunocytochemistry

Brains were mounted onto frozen cryostat chucks with distilled water. Frozen brains were allowed to equilibrate in an IEC Minotome Plus at -15°C for an hour, then sliced into 40-micron-thick coronal sections. Three series of sections were collected into glass vials containing cryoprotectant. Sections were either stored at -70°C until needed or stored at 5°C for immediate immunocytochemical processing.

Sections were processed to optimize DBH staining in finch brains. Unless otherwise stated, all rinses were performed three times for five minutes each in Buffer A (0.05 M tris-buffered saline) while sections were gently agitated. To control for variations in staining intensity, tissues from experimental and control birds were always processed in the same assay. In the first step, free-floating sections from each bird were rinsed then incubated for 10 minutes in 0.1% $NaBH_4$ in Buffer A. After another rinse, sections were incubated for 10 minutes in 1% H_2O_2 in Buffer A, rinsed and incubated for 10 minutes in 20% normal goat serum (NGS) in Buffer B (Buffer A with 2% NGS and 0.5% Triton X-100). Sections were incubated with the primary DBH antibody (Eugene Tech International, Inc; NJ; DBH TE103) at a dilution of

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1:500 in Buffer B for 24 hrs at 5°C. The primary antibody was re-used a maximum of six times. After a rinse with Buffer B, sections were incubated with biotinylated anti-rabbit IgG at a dilution of 1:600 (Vectastain Elite ABC Kit; Vector Labs, Burlingame, CA) in Buffer B for 45 minutes at room temperature. Sections were rinsed twice for five minutes in Buffer B, rinsed twice for five minutes in Buffer A, then incubated in ABC reagent diluted in Buffer A according to the manufacturer's instructions for 40 minutes. Sections were rinsed and treated with DAB peroxidase substrate (SigmaFast 3,3'-diaminobenzidine tablet sets in Buffer A). After a final rinse, sections were sorted and mounted onto gelatin-coated slides. Slides were dehydrated in a graded alcohol series, cleared with xylene and coverslipped with Permount. Another vial containing unprocessed tissue was rinsed, sorted, mounted and stained with cresyl violet. This set of slides served as a cytoarchitectural reference for the DBH-immunolabeled slides. The third vial was stored as an extra.

Specificity Check

There were two checks for the specificity of DBH-antiserum in finch brains. In the positive control, the antiserum was preadsorbed with an excess of DBH (Sigma) and was used in an assay. In the negative control, incubation with the anti-DBH serum was omitted from the immunocytochemistry assay. Both specificity checks used in this experiment were DBH-immunonegative.

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It is important to note that adrenergic neurons also contain DBH. In rats and birds, adrenergic cells are restricted to the medulla (Moore and Card, 1984; Steeves et al., 1987). Tissues from the medulla were not included in this study.

Image Processing

Sections were examined using a Nikon Optiphot microscope. The image was captured by a Dage MTI CCD72 video camera interfaced via a Perceptics Pixel pipeline framegrabber to a MAC2 FX computer. Quantification of the density of DBH-ir cell bodies and/or fibers in a given brain area was performed using NIH Image version 1.55 (written by Wayne Rasband at the U.S. National Institutes of Health). Images were sharpened to focus the digitized image. Two calculations were performed. The first measured the area of DBH immunoreactivity within an outlined brain area. The density slice feature was used for thresholding and adjusted so that only immunopositive cell bodies and/or fibers were measured. The brain area of interest in each image was outlined with the freehand selection tool, using the atlas of Stokes et al. (1974) as a reference. The program then calculated the mean area of immunopositive labeling within the outlined area. The second calculation determined the area of each brain region which had been previously outlined. Spatial calibrations were performed by drawing lines of known length and the program converted the area of labeled pixels in the area of interest into square microns.

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For mean labeled area and brain area measurements, all images which contained a given brain area were captured and the average was calculated. A minimum of two images of a given area per bird was used. However in most cases, more than two images were available. The areas of DBH-ir in the defined regions were also corrected for the size of the brain area measured to determine the percentage of the area that was labeled. Percent area calculations correct for the possibility that hormone treatment altered the size of the nucleus in addition to affecting the area of DBH innervation. This measurement also allowed meaningful comparisons between brain areas of different sizes. The calculation for percentage of area labeled was as follows: [(Mean area of DBH labeling)/(brain area)] * 100. DBH-ir cells were counted on a Nikon Optiphot-2 microscope (10X power) equipped with a reticle. These data were collected from all sections that contained DBH-ir cells.

Data Analyses

Most of the behavioral data and all of the differences in DBH-ir fiber staining between AE and sham treatment groups were analyzed by unpaired Student's t-tests (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). DBH-ir cell counts were corrected for double counting (Konigsmark, 1970) then analyzed by unpaired Student's t-tests. All these statistical tests were one-tailed. Courtship intensity data were analyzed non-parametrically using a one-way Friedman's ANOVA with repeated measures on courtship intensity.

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For DBH-ir cell counts, the LoC was divided into dorsal and ventral sections as follows. This division was previously done in rats by Swanson (1976) but was modified for finches. At the level of the fourth ventricle, cells located above the medial forebrain bundle (FLM) were classified as dorsal LoC, and cells located within FLM were classified as ventral LoC. Any DBH-ir cells below FLM were categorized as ventral subcoeruleus (SCv). There were other differences between DBH-ir cells in LoC and SCv. For instance, cells in dorsal LoC were often tightly clustered together while cells in ventral LoC were diffuse and cells in SCv were even more diffuse.

In terms of their morphology, LoC cells were either round or angular while those in the SCv were predominately elongated. In this study, all cells were classified based on their location. This was done simply because it was the most reliable and objective way to categorize DBH-ir cells, especially those in dorsal and ventral LoC.

Hypothalamic tissues were analyzed as half sections. This was done to increase sample size because there were not enough bilateral sections containing paraventricular nucleus (PVN) and preoptic area (POA). When bilateral sections were available, both sides were analyzed as half sections. In this study, as in our previous research, POA was defined as the area under the septomesencephalic tract (TrSM). Other authors have labeled this area in finches and an area located above PVN but directly under the anterior commissure as the medial preoptic nucleus based on aromatase labeling

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(Balthazart et al., 1996). In comparison to Mello et al. (1998), POA as defined here is similar to the anterior extent of POM, i.e. under TrSM.

RESULTS

AE-treated castrates had significantly shorter latencies to begin their first courtship song than did sham-treated castrates [$t(9)=8.644$, $p<0.0001$; see Figure 6A]. The total number of courtship displays in the AE-treated castrates was also significantly higher than that in the sham-treated castrates [$t(9)=3.129$, $p=0.0061$; see Figure 6B]. There was a main effect of hormone treatment on courtship intensities [Friedman statistic=16.30, $p=0.006$; see Figure 6C], but the post-hoc test was not significant.

Hormonal status did not have a significant effect on LoC or SCv cell counts [see Figures 7A and 7B]. Hormone treatment also did not significantly increase the mean area covered by DBH fiber labeling in any of the four VCN examined [nucleus uvaeformis (Uva), high vocal center (HVC), robust of the nucleus archistriatum (RA), lateral magnocellular nucleus of the anterior neostriatum (lMAN)]. DBH labeling was also not affected in the dorsal part of the lateral mesencephalic nucleus (MLd), an auditory nucleus, in the LoC or in the septum (S) [see Figures 8A and 8B]. However, there was a significant effect of hormone treatment in the two-hypothalamic nuclei examined, PVN and POA. AE treatment increased the area covered by DBH labeling in PVN [$t(9)=3.392$, $p=0.0040$] and in POA [$t(8)=2.925$, $p=0.0096$; see Figures 8B, 9A, 9B, 10A and 10B]. Hormone treatment had no effect on the percentage of the

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brain areas covered by DBH-ir fibers in any of the nuclei examined [see Figures 8C and 8D].

There was one hormone-treated male that displayed the high-intensity courtship display. This male had striking differences in DBH-ir fiber labeling in three VCN, RA, lMAN and Area X. These brain areas are documented in Figures 11A-C.

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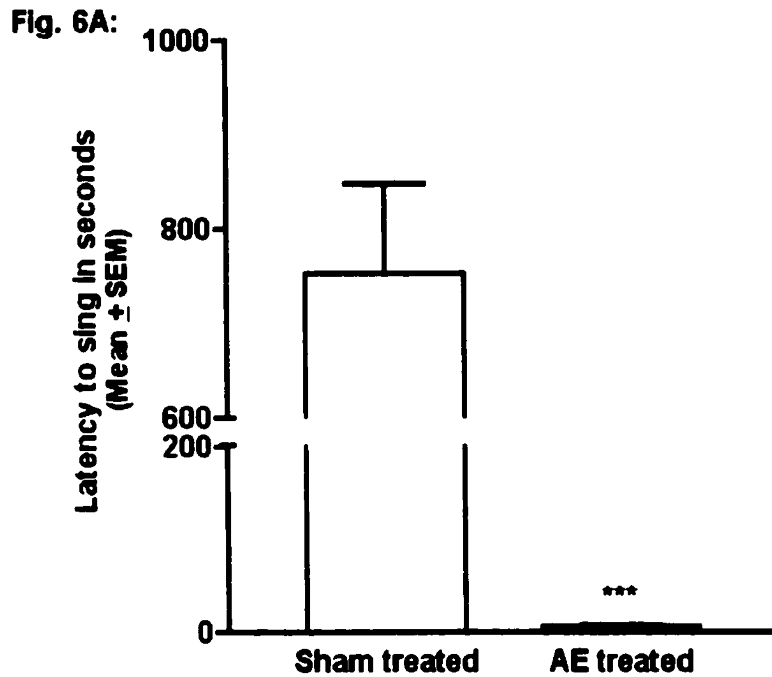

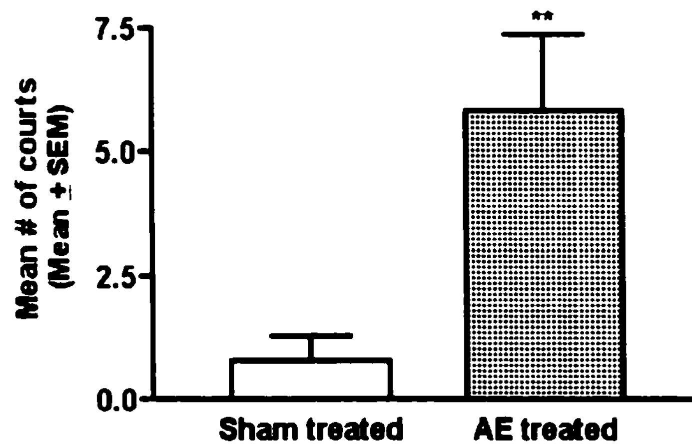

Figure 6: Courtship behavior in sham- and AE-treated castrates. Mean (± SEM) for **A**: Song latency in seconds, (note broken Y-axis), *** $p<0.001$, **B**: Total courts, ** $p=0.0061$. Significant change in number compared to sham-treated castrates.

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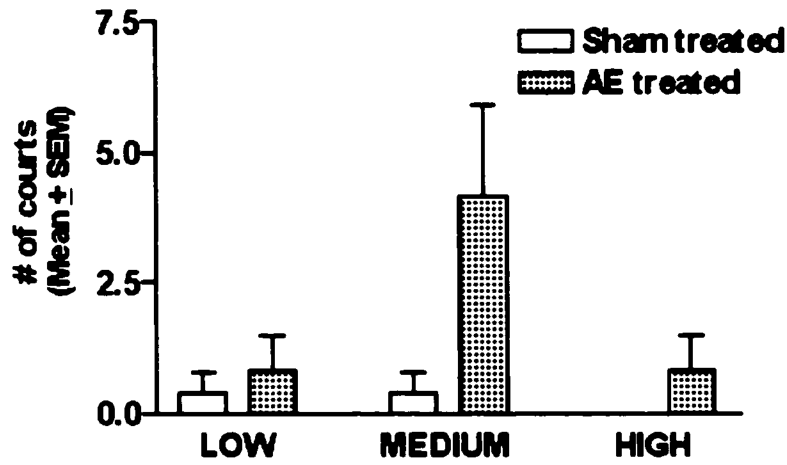

Figure 6: Courtship behavior in sham- and AE-treated castrates. Mean (± SEM) for C: Courtship intensities. Significant main effect of hormone treatment, p=0.006

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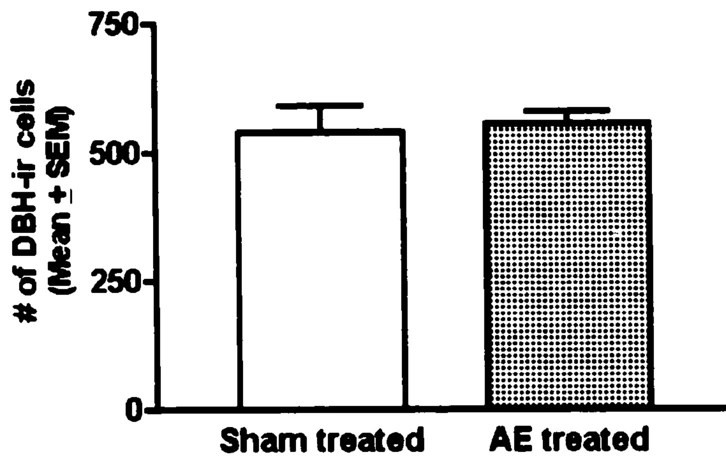

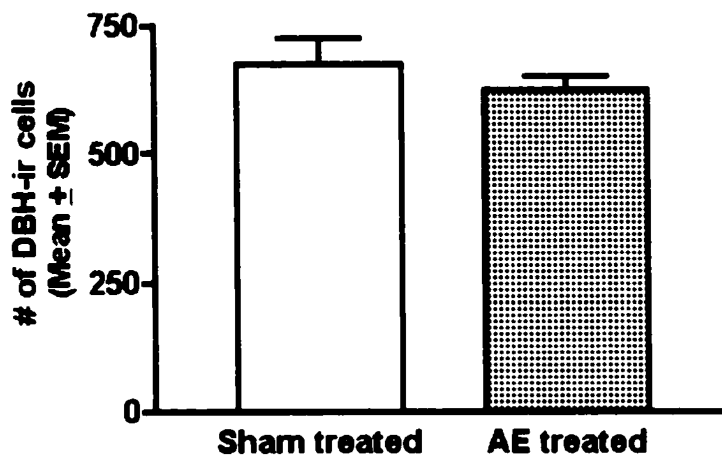

Figure 7: Mean (± SEM) corrected number of DBH-ir cells in A: LoC, B: SCv. See Table 1 for abbreviations.

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Fig. 8A:

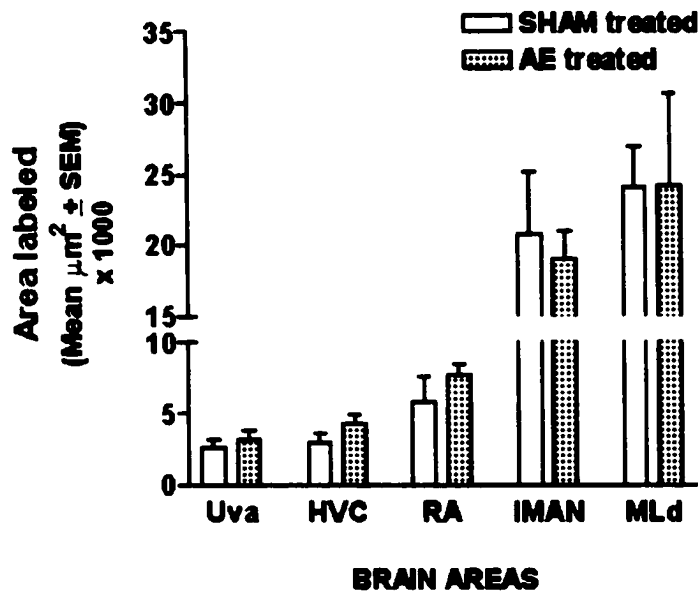

Fig. 8B:

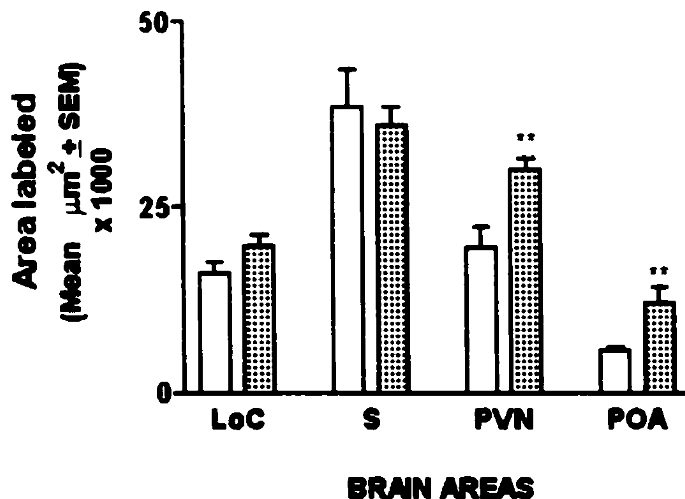

Figure 8: Mean (± SEM) area covered by DBH-ir labeling in **A**: VCN and auditory nucleus (note broken Y-axis) **B**: in hypothalamic nuclei and extra-hypothalamic nuclei in sham- and AE-treated castrate zebra finches. Significant change in area compared to sham-treated males, ** $p<0.01$. See Table 1 for abbreviations.

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Fig. 8C:

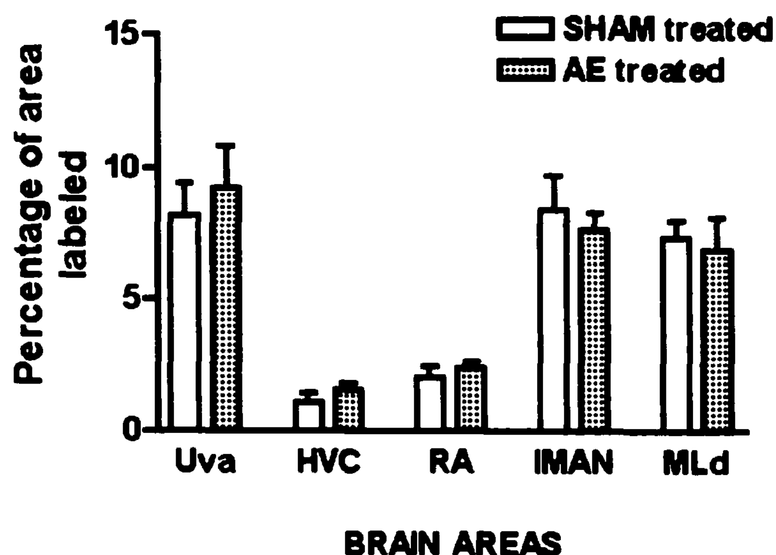

Fig. 8D:

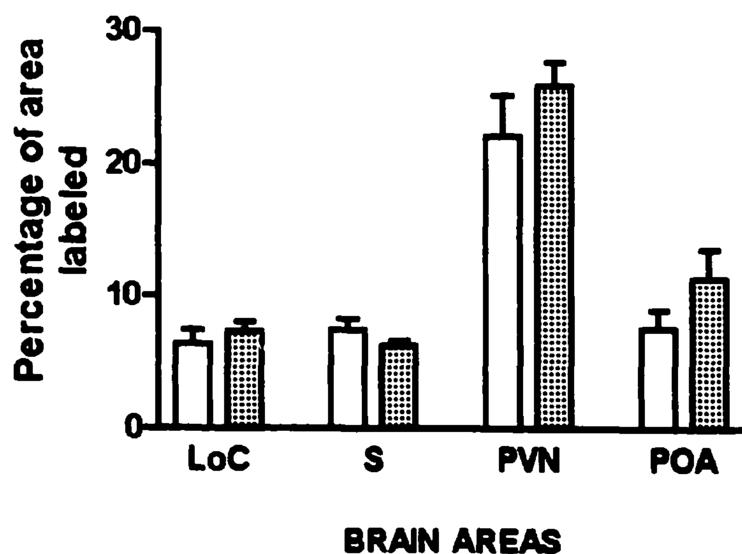

Figure 8: Mean (+ SEM) percent area covered by DBH-ir labeling C: in VCN and auditory nuclei, D: in hypothalamic nuclei and extra-hypothalamic nuclei in sham- and AE-treated castrate zebra finches. See Table 1 for abbreviations.

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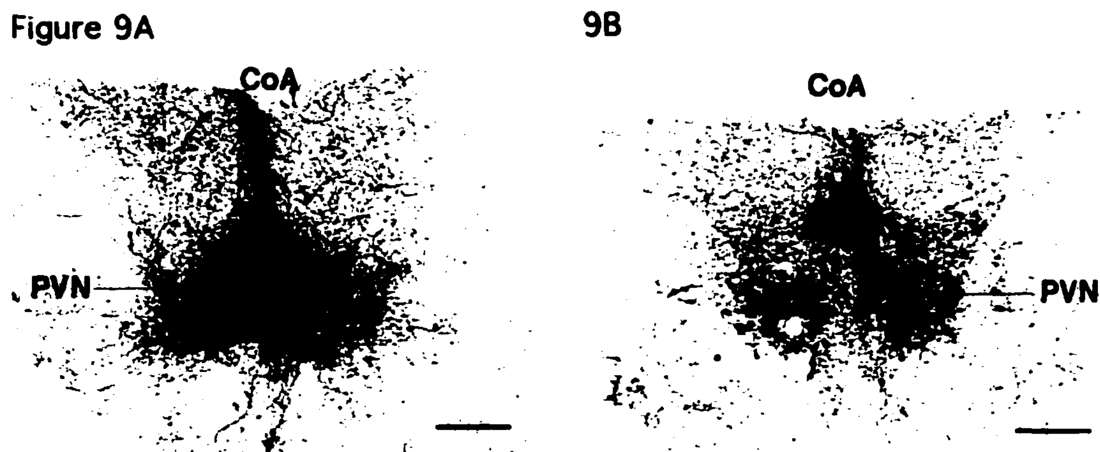

Figure 9: Photomicrographs of DBH-ir labeling in the male zebra finch brain. **A, B**: DBH-ir fibers in PVN of AE- and sham-treated castrate birds. Scale bar = 150 μm. See Table 1 for abbreviations.

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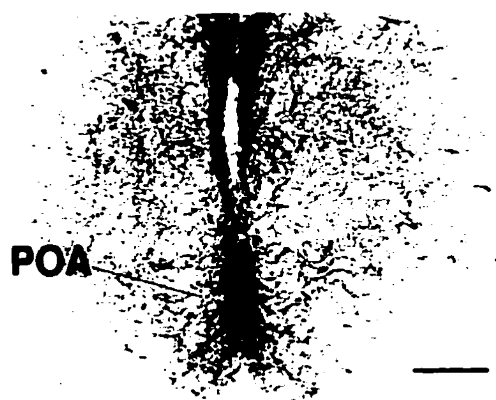
Figure 10A



10B

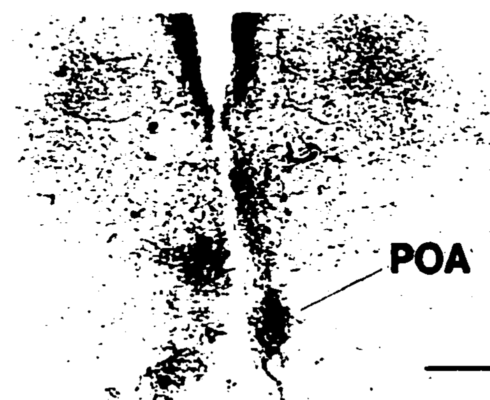


Figure 10: Photomicrographs of DBH-ir labeling in the male zebra finch brain. **A, B**: DBH-ir fibers in POA of AE- and sham-treated castrate birds. Scale bar = 150 μm. See Table 1 for abbreviations.

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Figure 11A

11B

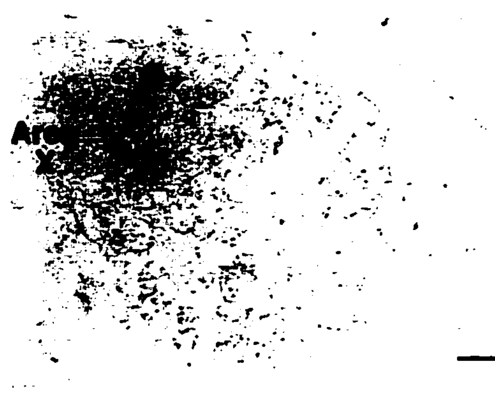

Figure 11C

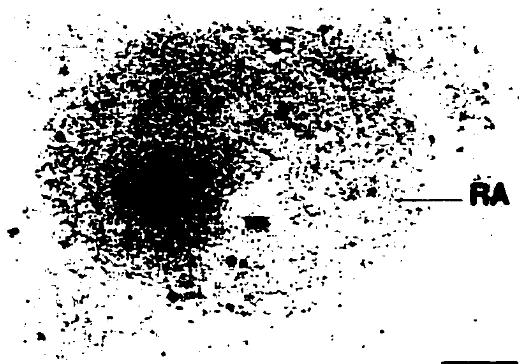

Figure 11:	Photomicrographs from AE-treated male that displayed a high-intensity court. **A:** lMAN & Area X - low magnification (4x). **B:** Area X - higher magnification (6x). **C:** RA - higher magnification (10x). Scale bar = 150 um. See Table 1 for abbreviations.

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DISCUSSION

AE is a hormone that has metabolic properties which are very similar to those of testosterone. Both hormones can be aromatized to form estrogenic metabolites, and they can also be reduced to form androgenic metabolites such as dihydrotestosterone. In zebra finches, the full repertoire of courtship behavior in castrates can only be restored by treatments like AE, which supply both classes of metabolites. AE was used in this study because it activated the highest level of courtship behavior in castrates of all treatments tested in previous experiments (Harding et al., 1983).

As expected, AE treatment had a significant effect on courtship behavior in castrates. The total number of courtship displays was significantly higher in the AE-treated group than in the sham-treated group. The data on courtship intensities showed that AE-treated castrates performed all three types of courts, i.e. high-, medium-, and low-intensities. Hormone treatment had a significant main effect on courtship intensities. As in an earlier study (Harding et al., 1983), courtship behavior was not completely eliminated in the sham-treated castrates. However, none of the courts in the sham-treated group were of high-intensity. In pair tests, AE-treated castrates began to court stimulus females much earlier than the sham-treated castrates. Hormone treatment motivated these males to perform medium- and high-intensity courts, and it helped them to attend to the stimulus females sooner.

In general, in the hormone-treated group, the diencephalic nuclei (PVN, POA and Uva) had the largest percentages of area labeled and the densest

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DBH labeling of all the areas examined. Percent area covered by DBH labeling in lMAN, MLd, LoC and S were comparable. Percent area covered by DBH labeling in the two remaining vocal control nuclei (VCN), HVC and RA were lower. In the hypothalamus, in addition to the very dense DBH terminal labeling, there was also heavy terminal labeling that outlined or surrounded the somata and processes of many DBH-immunonegative cells. This pattern of staining has been seen using other antibodies and is referred to as “basket cells” or "pericellular baskets" by other investigators (Bottjer, 1993; Bailhache and Balthazart, 1993) because the staining appears to form a basket around the unlabeled cell. The presence of DBH-ir pericellular baskets in the hypothalamus suggests that noradrenergic axons modulate neurons producing other neurotransmitters or other cell types.

The hypothalamus controls many regulatory behaviors and immunocytochemical studies have found dopamine and neuropeptide cell bodies in this brain region (Voorhuis et al., 1992; Bottjer, 1993). While noradrenergic cell bodies are only found in hindbrain regions, cell bodies that are immunopositive for dopamine, vasotocin (AVT) and arginine vasopression (AVP), for example, are found in the hypothalamus of many species (Moore and Card, 1984; Voorhuis et al., 1992; Gonzalez and Smeets, 1992; Bottjer, 1993; Bailhache and Balthazart, 1993). In males, AVT which is the non-mammalian homolog of AVP, activates sexual behavior, pair-bonding and facilitates aggression (for review, see Young, 1999). With respect to the noradrenergic system, there is research that suggests that NA has a

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modulatory effect on AVT and AVP. Ji et al. (1998) found that direct administration of NA into the PVN had an inhibitory effect on AVT-secreting neurons.

In this study, hormonal status did not affect the area occupied by DBH-ir fibers except in both hypothalamic nuclei examined, PVN and POA. However, this effect was not very robust and was not seen in percent area calculations. Hormone treatments can affect the size of a nucleus (Nottebohm, 1980). These data indicate that AE treatment may have affected the size of these brain areas as well as the amount of DBH-ir inside their borders.

In contrast to TH labeling, DBH labeling did not appear to be as sensitive to changes in gonadal hormone levels in males (Kritzer, 2000). In peripheral tissues, hormone treatment restored NA levels and increased DBH activity (Bustamante et al., 1989). The available data on the effects of hormone levels on central DBH-ir staining in males is limited. In ovariectomized rhesus monkeys, Kritzer and Kohama (1999) observed increases in the density of DBH labeling in the prefrontal cortex. Hormone replacements of either estrogen alone or estrogen plus progesterone decreased DBH labeling in the prefrontal cortex and matched the density of labeling observed in the prefrontal cortex of intact females. Clarke et al. (1999) also found decreases in the intensity of DBH-ir in the POA of estrogen-treated ovariectomized ewes, at the time of the gonadotropin releasing hormone/luteinizing hormone (GnRH/LH) surge. However, these investigators

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concluded that decreased immunoreactivity indicated NA release. This conclusion was based on the research of Muscholl and Spira (1982) who found that soluble DBH is stored in vesicles along with NA and is released along with the neurotransmitter upon exocytosis.

Most of the studies used female rats as subjects, and the data suggest the noradrenergic system has a more clearly defined role in the regulation of female sexual behavior. Administration of adrenergic receptor agonists enhanced lordosis while adrenergic receptor antagonists blocked lordosis (Carter, 1992). There are many studies that suggest that the noradrenergic innervation to the hypothalamus is hormone sensitive. Heritage et al. (1977, 1980) demonstrated that noradrenergic cell groups, including those that send afferents to the hypothalamus concentrate ^{3}H-estradiol.

In addition to lowering circulating gonadal hormone levels, castration also increases plasma levels of LH (Kitahara et al., 1990). Catecholamines have been implicated in regulating LH levels and/or its releasing hormone (Jennes et al., 1982; Ramirez et al., 1984; Sharp et al., 1989; Contijoch et al., 1990). This evidence supports the idea that the effects of gonadal hormones are mediated by changes in catecholaminergic neurotransmission. In this study, castration resulted in decreased catecholaminergic function as measured by the decreased areas of DBH-ir staining in the PVN and POA.

The strongest argument for noradrenergic involvement in relaying changes in circulating gonadal hormone levels comes from the finding that NA levels affect both GnRH release (Terasawa et al., 1988) in addition to the

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expression of GnRH mRNA (Kim et al., 1994). In double-labeling studies, NA-containing fibers were found surrounding GnRH cell bodies (Jennes et al., 1982). Earlier studies suggested that GnRH cells, unlike NA neurons, did not have estrogen-receptors (Herbison et al., 1993; Lehman and Karsch, 1993). However a more recent study by Herbison and Pape (2001) indicated the presence of estrogen receptors in GnRH cells. Estrogens may regulate the activity of GnRH neurons by directly binding to these receptors. In house finches, GnRH cells were observed in both POA and septum (Cho et al., 1998). In zebra finches, DBH-ir fibers in the POA had very strong labeling which formed pericellular baskets. The septum also had strong DBH fiber staining. The distribution of DBH-ir fibers suggests there is an opportunity for interaction between GnRH cells and the noradrenergic system which may ultimately help to direct an animal's courtship behavior to a potential mate.

Incidentally, NA levels can have a direct effect on the sensitivity of cells in the hypothalamus to estrogen. In rats, treatments that lowered NA levels, decreased the concentration of cytosol estrogen receptors in the median eminence and other areas of the hypothalamus (Blaustein et al., 1986; Tetel and Blaustein, 1991). However, a later study by Brown et al. (1991) found that the administration of a DBH inhibitor had no effect on the concentration of nuclear or cytosol estrogen receptors in the brain. In zebra finches, Barclay and Harding (1990) determined that most of the hormonally-induced changes in the levels and turnover rates of NA were estrogen dependent. The concentration of androgenic receptors in the POA also appeared to be

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regulated by adrenergic neurons through alpha receptors (Handa and Resko, 1989). From these studies, there is a strong case for the effects of gonadal hormones being mediated, in part, by the activity of noradrenergic neurons.

The lack of a strong hormonal effect on the distribution of DBH-ir fibers may also be the result of sex differences. The secretion pattern of pituitary and gonadal hormones in males is tonic, while the secretion pattern of these substances in females is cyclic. During the critical periods of development, perinatal hormone exposure lays the groundwork for masculinization and defeminization of the hypothalamus. Brann and Mahesh (1991) found that the preovulatory surge could not be induced in estrogen-primed immature castrated males. Sexual differentiation of the brain could also influence catecholaminergic innervation patterns to the various sexually dimorphic nuclei (Simerly et al., 1985; Soha et al., 1996).

Hormone treatment increased the amount of DBH available to synthesize NA only in the hypothalamic nuclei PVN and POA. In rats, the PVN has been implicated in the regulation of penile erection (Chen et al., 1997). Lesioning the PVN caused decreases in temporal measures of erectile functioning, e.g. increased ejaculation latencies and decreased intromission ratios (Liu et al., 1997; Ackerman et al., 1997). POA, especially the sexually-dimorphic part of this nucleus is believed to activate male sexual behavior in different species (rats - Arendash and Gorski, 1983; Japanese quail - Balthazart and Surlemont, 1990; Bailhache et al., 1993; European starlings - Riters and Ball, 1999). Again, lesioning or lowering NA levels in this particular

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brain area had a negative effect on displays of male sexual behavior. In songbirds, the direct pathway between POA and the vocal control system (VCS) has not been established, but Foster et al. (1997) found an indirect pathway between the VCS and the lateral hypothalamus. This is the first evidence of a connection between the hypothalamus, which regulates sexual behavior, and the VCS, a hormone-sensitive neural network that controls courtship vocalizations.

There is evidence that manipulating different types of noradrenergic receptors in the PVN can directly affect sexual behavior. Pharmacological treatments that either activated alpha1-adrenoceptors or inhibited alpha2-adrenoceptors facilitated sexual responses in a variety of species – humans, monkeys and rats (Rampin, 1999). In birds, the role of NA in the regulation of male sexual behavior is dependent upon the brain area affected. For example, in quail and doves, pharmacological treatments that lowered NA levels only in the hypothalamus increased displays of male sexual behavior (Balthazart et al., 1988; Barclay and Cheng, 1992). However, in finches, decreasing levels of NA throughout the brain had a negative effect on all measures of courtship singing (Barclay et al., 1992; Barclay et al., 1996). The data from finches also indicated that even minor depletions in levels of NA were enough to significantly disrupt courtship singing behavior.

Barclay and Harding (1988, 1990) found that hormone treatments that activated high levels of courtship singing increased NA levels and/or turnover in VCN on the efferent pathway which controls the motor patterning of song

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(e.g., nucleus interfacialis - NIf, RA, dorsomedial portion of the intercollicular nucleus - DM) and in the POA. In contrast, these same hormone treatments resulted in decreased NA levels and/or turnover in VCN in the anterior forebrain pathway which are involved in song learning (e.g., Area X, IMAN), and in PVN.

Barclay and Harding (1988) found NA levels were increased in the PVN of sham-treated castrates compared to AE-treated castrate males. In POA, hormone treatment increased NA levels, NA turnover rates and DBH terminal labeling. However, in the current study, the increase of DBH labeling in PVN occurred in the AE-treated castrate group, not in the sham-treated group. Therefore, in sham-treated castrates, there was an increase in the release of NA without an increase in DBH-ir. There is some evidence that suggests that DBH levels do not necessarily correlate to NA levels. Smythe et al. (1985) found that inhibition of DBH led to an increase in hypothalamic NA activity in rats. Another study suggested that in peripheral tissues, both NA and soluble DBH are packaged in vesicles, and that both are released during exocytosis (Muscholl and Spira, 1982). Data from DBH immunocytochemistry indicate where in the brain NA is synthesized, while HPLC data measure where NA is released. Therefore, high levels of NA may not always coincide with densely-labeled DBH fibers.

Direct comparisons between Barclay and Harding (1988, 1990) and the current study are difficult. Some nuclei could not be quantified using DBH immunocytochemistry because levels of staining were so low, e.g. Area X and

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DM. This was quite puzzling given the finding that Area X had very high NA levels as measured by HPLC-EC. This was in contrast to HVC, RA and IMAN, which had low NA levels in previous studies but demonstrated greater DBH fiber staining than Area X in the current study. Although hormone treatment had a differential effect on the two hypothalamic nuclei examined, one consistency between studies was that nuclei in the hypothalamus had much higher levels and turnover rates of NA (Barclay and Harding, 1988; Barclay and Harding, 1990) and much greater DBH fiber staining than in the VCN in the current study.

Herkenham (1991) found that there were often inconsistencies between levels of neurotransmitters, their receptors and/or their synthetic enzymes. Smeets and Steinbusch (1990) noted that in the hypothalamus, there were instances where neurons were immunopositive for the neurotransmitter but immunonegative for its synthetic enzyme. They concluded that these neurons were accumulating the neurotransmitter but could not synthesize it. A similar hypothesis was proposed by Mello et al. (1998) to account for the lack of DBH-ir in Area X and its surrounding LPO region given the high levels of NA found in this area by Barclay and Harding (1988, 1990).

The percent area data allow comparison of DBH ir across brain nuclei. Hormone treatment produced an interesting shift in the concentrations of DBH-ir staining in brain nuclei. AE-treated castrates had more DBH labeling in the hypothalamus (PVN and POA) followed in descending order by Uva, IMAN, LoC, MLd, septum - S, RA and HVC. Sham-treated castrates had more DBH

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labeling in one hypothalamic nucleus (PVN) followed in descending order by lMAN, Uva, POA, S, MLd, LoC, RA and HVC. In the sham-treated castrate, the ranking of DBH labeling in POA was lowered.

In zebra finches, castration did not completely eliminate singing behavior. While untreated castrates no longer performed medium- or high-intensity courtship songs, they still performed low-intensity and undirected songs, which are song not sung directly to a female (Harding et al., 1983; Bohner et al., 1992). It appeared as though these males were not as motivated to court stimulus females. This was further evidence that gonadal hormones activated higher-intensity levels of courtship singing. Castration decreased DBH-ir in the hypothalamic nuclei PVN and POA both of which have an important role in the activation of male sexual behavior in rats (Chen et al., 1997) and birds (Balthazart and Surlemont, 1990; Bailhache et al., 1993; Riters and Ball, 1999). It appeared that hormonal status had a minor effect on the availability of DBH in these areas.

Finally, in this experiment, there was one AE-treated male that deserves special mention. This male was the only one to court at the highest-intensity level, i.e. courtship song with pivot dance. The high-intensity court, which occurred in the third minute of the fifteen-minute pair test, was the last court performed by this male. The DBH-ir staining patterns that were demonstrated were quite unique. There was light staining in nucleus RA, lMAN and most notably, in Area X. This was the first time that DBH-ir fibers were seen in Area X, even though HPLC-EC methods measured significant

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levels of NA there (Barclay and Harding, 1988; Barclay and Harding, 1990; Barclay et al., 1992; Barclay et al., 1996; Harding et al., 1998). It was also quite surprising to find DBH-ir fibers in Area X since Mello et al. (1998) did not find labeling in that brain region. The results from both the first and third experiments presented in this dissertation also agree with the data from Mello and his colleagues. This apparent "activation" of Area X also goes against the long-held belief of the function of this nucleus. Area X and IMAN were both previously thought to be associated only with the song learning processing in juvenile males (Sohrabji et al, 1990; Scharff and Nottebohm, 1991) . However, in a more recent study, Jarvis et al. (1998) found immediate early gene expression of the transcription promoter ZENK in Area X and HVC following undirected singing. In directed singing, only HVC had ZENK expression. However, in the current experiment, the staining in Area X was only seen in the one male that gave a high-intensity court which was clearly directed at the female. In support of both of these findings, Margoliash (1997) found that during the act of singing, Area X neurons may be either inhibited or excited. In the final two minutes, this male also gave a group call. However, this unique staining pattern was not observed in other males that also gave group calls.

This finding brings about two possible issues for the observed DBH staining pattern. The first issue relates to the arousal level of the animal. Males that perform at the highest courtship level are highly motivated. Did this staining pattern reflect a highly aroused state or recovery from a highly

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aroused state? The other issue relates to the time of sacrifice used in the methodology. All males were anaesthesized and perfused immediately after the final observation. This was done to have a final record of the animal's behavior prior to sacrifice. The possibility remains that this was not the optimal time for the visualization of the highest DBH labeling, or that the highly arousing sex-pair test, which probably activated noradrenergic neurons, attenuated DBH labeling by depleting NA and DBH that was stored in vesicles and subsequently released. When Jarvis et al. (1997) discovered ZENK gene expression in Area X and HVC, subjects were sacrificed 30-40 minutes after the onset of singing. Immediate early gene expression tends to be maximal 30 minutes after stimulus presentation (Kornhauser et al., 1992).

Based on the frequency of their courtship displays, these males fall into one of these four general categories. These are males that courted in 1) the beginning, middle and end, 2) the beginning and the middle, 3) the beginning and the end, or 4) just the beginning of the sex pair test. The male that had the DBH-labeled fibers in Area X fell into the last category. There were males that courted right up to the very end of the test, but their brains did not show any increased DBH-ir in the previously activated VCN. This suggests one of three possibilities. The first possibility is that the staining observed in the high-intensity courting male was the result of synthesis of DBH-ir in the activated brain nuclei. Secondly, changes in the cell membranes of this highly motivated male may have made it easier for the antibody to bind to DBH antigen in Area X. Lastly, this male was about to engage in some other

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behavior which happened to require increased DBH-ir in Area X prior to being sacrificed.

This study investigated the effect of gonadal hormones on the area of DBH-ir in male zebra finch brains. Although hormone treatment significantly increased courtship singing behavior, there was no effect on the distribution of DBH-ir within the VCN. In the hypothalamus, AE treatment increased the area of DBH-ir in PVN and POA. Thus far, only one afferent pathway has been found via the thalamus between the lateral hypothalamus and the VCS (Foster et al., 1997). It is unclear whether this increase in levels of DBH-ir in the hypothalamus affected singing behavior or is related to other processes such as the regulation of LH secretion.

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CHAPTER 4:

NEUROTOXIC EFFECTS OF *N*-(2-CHLOROETHYL)-*N*-ETHYL-2-BROMOBENZYLAMINE (DSP-4) ON THE NORADRENERGIC SYSTEM IN MALE ZEBRA FINCH BRAINS.

ABSTRACT

In our previous studies, administration of the noradrenergic neurotoxin *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4) lowered both central noradrenaline (NA) levels and the frequencies of courtship singing behavior in male zebra finches. This suggests that NA may be involved in the regulation of courtship behavior in this species. In the current study, we used dopamine-beta hydroxylase (DBH) immunocytochemistry (ICC) to quantify the effects of this neurotoxin on central noradrenergic innervation. Males were pretreated with zimelidine dihydrochloride (20mg/kg; i.p.) to protect serotonergic neurons, then injected with either DSP-4 (50mg/kg; i.p.) or saline vehicle alone, i.p. After ten days, treatments were repeated and males were housed with females for the next ten days. Males were perfused, and their brains processed for DBH ICC. DBH labeling was quantified using image analysis software in thirteen brain regions {five vocal control nuclei (VCN), one auditory nucleus (AN), two hypothalamic nuclei (HN) and five additional areas that demonstrated high DBH labeling}. DSP-4 treatment significantly decreased DBH labeling in all areas examined in the midbrain and telencephalon, while leaving the diencephalic areas examined relatively unaffected. DSP-4

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treatment significantly reduced the number of DBH-immunoreactive (DBH-ir) cells in both the locus coeruleus (LoC) and ventral subcoeruleus cell groups. It also significantly decreased the areas covered by DBH-ir fibers in four VCN - high vocal center, robust nucleus of the archistriatum, dorsomedial portion of the intercollicular nucleus, lateral magnocellular nucleus of the anterior neostriatum, an auditory nucleus - dorsal part of the lateral mesencephalic nucleus, in addition to five other brain regions - LoC, cerebellum, ventral tegmental area, optic tectum, and septum. However, as previously found in rats, the noradrenergic innervation in the diencephalon was not significantly affected by DSP-4 treatment.

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INTRODUCTION

Central noradrenaline (NA)-producing cell bodies are located in the brainstem. In rats, there are seven cell groups designated A1-A7 by Dahlström and Fuxe (1964). The largest of these noradrenergic cell groups, A6, is also known as the locus coeruleus (LoC). In rats, there are roughly 2900 NA-producing cells in the LoC (Swanson, 1976). There are three major projections from the locus coeruleus: ascending, descending and cerebellar. The majority of the highly collateralized projections ascend via the dorsal tegmental bundle. Noradrenergic innervation to the cortex/telencephalon, the cerebellum and some sensory nuclei comes exclusively from the LoC (Loughlin et al., 1986a,b; Fillenz, 1990; Role and Kelly, 1991). In both rats and pigeons, LoC projections are the primary source of NA into the cortex/telencephalon (Loughlin et al., 1986a,b; Kitt and Brauth, 1986).

The remaining central noradrenergic cell groups, which include the ventral subcoeruleus (SCv), form the lateral tegmental (LT) system (Moore and Card, 1984). In rats, LT neurons send their ascending projections via the central tegmental bundle (Role and Kelly, 1991). The diencephalon (dorsal thalamus and some areas of the hypothalamus), and septum receive their noradrenergic innervation from both LoC and LT (Fillenz, 1990). Although there is a minor contribution from LoC projection neurons to the diencephalon (Loughlin et al., 1986b), most of the noradrenergic innervation of this area is provided by the LT system (Moore and Card, 1984; Lookingland et al., 1986). In general, projections of the LT are restricted to thalamic and hypothalamic

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brain regions (Fillenz, 1990). Descending projections to the spinal cord originate from the A5-A7 cell groups (Westlund et al., 1983).

It has been suggested that NA plays a role in state-dependent behaviors and in behaviors that require heightened awareness (Marshall and Finlayson, 1988; Aston-Jones et al., 1991; Aston-Jones et al., 1994, Delini-Stula et al., 1984; Harro et al., 1995). Studies have shown the activity of LoC neurons enhanced the signal-to-noise ratio or gating of sensory neurons (Waterhouse et al., 1988; Servan-Schreiber et al., 1990; Waterhouse et al., 1993; Hasselmo et al., 1997). This appears to mediate a heightened state of arousal and attention to changes in the environment. For example, in rats, iontophoretic application of NA to the visual cortex or lateral hypothalamic neurons facilitated an excitatory response to a previously sub-threshold stimulus (Waterhouse et al., 1988).

NA belongs to a class of neurotransmitters known as the catecholamines (CAs). CAs have been implicated in modulating the actions of luteinizing hormone (LH) and/or its releasing hormone (Jennes et al., 1982; Ramirez et al., 1984). CAs also play a role in activating sexual behavior (Hansen et al., 1982; Meyerson, 1984; Smith et al., 1987; Balthazart et al., 1988; Carter, 1992; Barclay and Cheng, 1992; Barclay et al., 1992; Barclay et al., 1996). It has also been suggested that NA might be involved with some aspects of learning, e.g. discrimination (Griffin and Taylor, 1995), olfactory learning (Cornwell-Jones, 1988; Cornwell-Jones et al., 1990a and 1990b;

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Cornwell et al., 1996), olfactory memory (Sullivan and Wilson, 1994), and the acquisition of temporal learning (Ho et al., 1995; Al-Zahrani et al., 1997).

In various attempts to investigate the functions of the central noradrenergic system, N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), a noradrenergic neurotoxin, has been used successfully in different species to lower central NA levels (Balthazart et al., 1988; Barclay et al., 1992; Rodman and Karten, 1995; Barclay et al., 1996; Villani et al., 1996). When administered systemically in rats, DSP-4 crosses the blood-brain barrier and causes rapid, long-term depletion of NA levels in the cortex but leaves hypothalamic levels of NA relatively unaltered (Jonsson et al., 1981; Jonsson et al., 1982; Ross, 1976; Ross and Renyi, 1976). This differential effect on levels of NA is presumably the result of DSP-4's high selectivity for noradrenergic axon terminals that originate from the LoC (Ross, 1976; Ross and Renyi, 1976; Jaim-Etcheverry and Zieher, 1980; Jonsson et al., 1981; Hallman and Jonsson, 1984; Hallman et al., 1984). DSP-4 treatment depleted levels of NA and NA-labeled fibers within hours, but immunoreactive fibers for the enzyme that synthesizes NA, dopamine-beta hydroxylase (DBH) disappeared four days later (Fritschy et al., 1990). The neurotoxic actions of this drug start retrogradely at the axon terminals where DSP-4 competes with previously-released NA in the synapse during re-uptake, and neurons which take it up are subsequently destroyed (Hallman and Jonsson, 1984; Hallman et al., 1984; Jonsson et al., 1981). Desipramine treatment given before or concomitantly with DSP-4 prevented the loss of noradrenergic neurons presumably by preventing uptake of the

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neurotoxin at the axon terminals (Hallman and Jonsson, 1984; Villani et al., 1996). In rats, levels of NA recovered almost completely after one year (Wolfman et al., 1994), and projections that originated from the LoC were the exclusive source of re-innervation to the cortex (Fritschy and Grzanna, 1992).

Given the highly-selective actions of DSP-4 in rats, this drug was used in a songbird species, zebra finches *(Taeniopygia guttata)*, in an attempt to lower their telencephalic NA levels without significantly affecting their hypothalamic NA levels. Singing behavior in male zebra finches is controlled by a set of hormone-sensitive interconnected brain nuclei that are located primarily in the telencephalon and are known collectively as the vocal control system (VCS) (Stokes et al., 1974; Nottebohm et al., 1982; see Figure 1). Barclay and Harding (1988) found high levels of NA in the VCS. Hormone treatments, which supplied both estrogenic and androgenic metabolites and reinstated courtship singing behavior in castrates, also modulated levels and turnover of NA in the VCS (Barclay and Harding, 1990). In order to determine whether NA played a significant role in controlling singing behavior, DSP-4 was used in an attempt to lower telencephalic NA levels but leave NA levels in the hypothalamus relatively unaffected.

When DSP-4 was administered systemically, it produced significant deficits in courtship singing behavior (Barclay et al., 1992). While brain NA levels were not significantly depleted by DSP-4 treatment, levels of singing were positively correlated with the levels of NA available in three VCN (nucleus interfacialis - NIf, robust nucleus of the archistriatum - RA and

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dorsomedial portion of the intercollicular nucleus - DM). To cause significant depletions of central NA levels, DSP-4 was administered centrally via the third ventricle. Central administration of DSP-4 also caused significant deficits in courtship singing behavior and this time, NA levels were significantly depleted in three VCN (Area X of the parolfactory lobe - Area X, lateral magnocellular nucleus of the anterior neostriatum - lMAN, and NIf). However, NA levels in two hypothalamic nuclei, magnocellular portion of the paraventricular nucleus and the preoptic area (POA) were also significantly lowered (Barclay et al., 1996).

The purpose of the current study was to document which regions of noradrenergic innervation in finch brains were affected following systemic DSP-4 treatment. From our previous studies (Barclay et al., 1992; Barclay et al., 1996), the highly-selective action which DSP-4 demonstrated in rats was in question in finch brains. Systemic administration of the standard rat dosage failed to significantly lower central NA levels. One possible explanation is that in finch brains, catecholamine levels have been shown to be about ten times higher than in comparable brain areas in rats (Barclay and Harding, 1988). Only when DSP-4 was injected directly into the brain were NA levels significantly decreased in some VCN; however hypothalamic NA levels were also lowered. This suggests three possibilities; 1) the source of NA innervation to the hypothalamus differs between zebra finches and rats, 2) DSP-4 destroys both noradrenergic LoC and LT projections in zebra finches, 3) or some combination of these hypotheses.

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To measure the effects of DSP-4 treatment in male zebra finch brains, standard immunocytochemical procedures were used to visualize DBH-containing neurons. This technique has been used successfully in rats and goldfish to document the extent of the neurotoxic effects of DSP-4 on central noradrenergic neurons (Fritschy and Grzanna, 1989; Fritschy et al., 1990; Villani et al., 1996). In rats, this technique also provided direct evidence that the noradrenergic innervation of the hypothalamus was spared following drug treatment (Grzanna et al., 1989). Based on the results of our previous studies (Barclay et al., 1992; Barclay et al., 1996), we predicted that DSP-4 would affect noradrenergic innervation in both the telencephalon and hypothalamus of male zebra finch brains.

METHODS

Subjects

Zebra finches *(Taeniopygia guttata)* were obtained from Canary Bird Farm (Englishtown, NJ). Males and females were treated with ivermectin to protect against leg-mite infections and housed in separate aviaries until needed. Bird rooms were kept on a 14:10 h light:dark cycle with the temperature controlled (24 + 2°C) and the humidity kept over 50% to maintain optimal breeding conditions. Birds were fed a vitamin-supplemented (8 in 1, Pet Products) commercial finch seed mix, grit, water and cuttlebone ad lib., supplemented with fresh greens and oranges. Sexually-naive adult birds were

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used. During the experiment, males were housed in individual cages $(56\ cm)^3$ and stimulus females were group housed until needed. All animal care, experimental procedures, and euthanasia were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee of Hunter College of the City University of New York.

DSP-4 Injections

The two drugs used in this study were zimelidine dihydrochloride, a serotonergic re-uptake blocker, and N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4; generous gifts of Trevor Archer, Astra Pharmaceuticals, Ltd.). Males were randomly assigned to either experimental or control groups. All males received 20 mg/kg zimelidine dihydrochloride (20 μg/0.01 ml saline/g body weight, i.p.) to protect serotonergic neurons (Ross and Renyi, 1976). After thirty minutes, experimentals (n=8) received 50 mg/kg DSP-4 (50 μg/0.01 ml saline/g body weight, i.p.) and controls (n=8) received the saline vehicle alone, i.p. DSP-4 was used within 10 minutes of being mixed with saline to prevent the formation of the non-toxic azridinium derivative (Lookingland, et al., 1986). To maximize noradrenergic depletion, drug treatments were repeated ten days later. One hour after the second drug administration, each male was housed with a stimulus female for the next ten days to maximize neurochemical differences between treatment groups. Males were then anesthetized, perfused, and their brains processed for immunocytochemistry or cresyl violet staining.

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Perfusion

Males were mildly anesthetized with a combination of Xylazine:Ketamine (5 mg each/kg body weight) in 0.5 ml saline injected into the pectoral muscle. Metofane (Pitman-Moore) was administered as necessary to keep birds deeply anesthetized during the procedure. Males were transcardially perfused with 0.2 M phosphate buffer (PB) followed by 4% paraformaldehyde in 0.1 M PB. Brains were quickly removed and post-fixed in 4% paraformaldehyde. The post-fixing solution was replaced with cryoprotectant (300 g sucrose, 10 g polyvinyl-pyrrolidone, 30 ml ethylene glycol, 2 g sodium azide, volume adjusted to 1000 ml with 0.1 M PB) and stored at 5°C. The brains stayed in the cryoprotectant until they sank (Côté et al., 1993; Beltz and Burd, 1989).

Immunocytochemistry

Brains were mounted onto frozen cryostat chucks with distilled water. Frozen brains were allowed to equilibrate in an American Optical Cryo-cut II microtome or an IEC Minotome Plus at -15°C for an hour, then sliced into 40-micron-thick coronal sections. Three series of sections were collected into glass vials containing cryoprotectant. Sections were either stored at -70°C until needed or stored at 5°C for immediate immunocytochemical processing.

Sections were processed to optimize DBH staining in finch brains. Unless otherwise stated, all rinses were performed three times for five minutes

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each in Buffer A (0.05 M tris-buffered saline) while sections were gently agitated. To control for variations in staining intensity, tissues from experimental and control birds were always processed in the same assay. In the first step, free-floating sections from each bird were rinsed then incubated for 10 minutes in 0.1% $NaBH_4$ in Buffer A. After another rinse, sections were incubated for 10 minutes in 1% H_2O_2 in Buffer A, rinsed and incubated for 10 minutes in 20% normal goat serum (NGS) in Buffer B (Buffer A with 2% NGS and 0.5% Triton X-100). Sections were incubated with the primary DBH antibody (Eugene Tech International, Inc; NJ; DBH TE103) at a dilution of 1:500 in Buffer B for 24 hrs at 5°C. The primary antibody was re-used a maximum of six times. After a rinse with Buffer B, sections were incubated with biotinylated anti-rabbit IgG at a dilution of 1:600 (Vectastain Elite ABC Kit; Vector Labs, Burlingame, CA) in Buffer B for 45 minutes at room temperature. Sections were rinsed twice for five minutes in Buffer B, rinsed twice for five minutes in Buffer A, then incubated in ABC reagent diluted in Buffer A according to the manufacturer's instructions for 40 minutes. Sections were rinsed and treated with DAB peroxidase substrate (SigmaFast 3,3'-diaminobenzidine tablet sets in Buffer A). After a final rinse, sections were sorted and mounted onto gelatin-coated slides. Slides were dehydrated in a graded alcohol series, cleared with xylene and coverslipped with Permount. Another vial containing unprocessed tissue was rinsed, sorted, mounted and stained with cresyl violet. This set of slides served as a cytoarchitectural

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reference for the DBH-immunolabeled slides. The third vial was stored as an extra.

Specificity Check

There were two checks for the specificity of DBH-antiserum in finch brains. In the positive control, the antiserum was preadsorbed with an excess of DBH (Sigma) and was used in an assay. In the negative control, incubation with the anti-DBH serum was omitted from the immunocytochemistry assay. Both specificity checks used in this experiment were DBH-immunonegative.

It is important to note that adrenergic neurons also contain DBH. In rats and birds, adrenergic cells are restricted to the medulla (Moore and Card, 1984; Steeves et al., 1987). Tissues from the medulla were not included in this study.

Image Processing

Sections were examined using a Nikon Optiphot microscope. The image was captured by a Dage MTI CCD72 video camera interfaced via a Perceptics Pixel pipeline framegrabber to a MAC2 FX computer. Quantification of the density of DBH-ir cell bodies and/or fibers in a given brain area was performed using NIH Image version 1.55 (written by Wayne Rasband at the U.S. National Institutes of Health). Images were sharpened to focus the digitized image. Two calculations were performed. The first measured the area of DBH-ir within an outlined brain area. The density slice

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feature was used for thresholding and adjusted so that only immunopositive cell bodies and/or fibers were measured. The brain area of interest in each image was outlined with the freehand selection tool, using the atlas of Stokes et al. (1974) as a reference. The program then calculated the mean area of immunopositive labeling within the outlined area. The second calculation determined the area of each brain area which had been previously outlined. Spatial calibrations were performed by drawing lines of known length and the program converted the area of labeled pixels in the area of interest into square microns.

For mean labeled area and brain area measurements, all images which contained a given brain area were captured and the average was calculated. A minimum of two images of a given area per bird was used. However in most cases, more than two images were available. The areas of DBH-ir in the defined regions were also corrected for the size of the brain area measured to determine the percentage of the area that was labeled. This corrects for the possibility that DSP-4 treatment altered the size of the nucleus in addition to affecting the area of DBH innervation. This measurement also allowed meaningful comparisons between brain areas of different sizes. The calculation for percentage of area labeled was as follows: [(Mean area of DBH labeling)/(brain area)] * 100. DBH-ir cells were counted on a Nikon Optiphot-2 microscope (10X power) equipped with a reticle. These data were collected from all sections that contained DBH-ir cells.

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Data Analyses

Differences between the treatment groups in mean area of DBH-ir fiber staining and percent area covered by DBH-ir were analyzed by unpaired Student's t-tests (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, California, www.graphpad.com). Cell counts were corrected for double counting (Konigsmark, 1970) then analyzed by unpaired Student's t-tests. All statistical tests were one-tailed.

The LoC was divided into dorsal and ventral sections. This division was previously done in rats by Swanson (1976) and was modified for finches as follows. At the level of the fourth ventricle, cells located above the medial forebrain bundle (FLM) were classified as dorsal LoC, and cells located within FLM were classified as ventral LoC. Any DBH-ir cells below FLM were classified as SCv. There were other differences between DBH-ir cells in LoC and SCv. For instance, cells in dorsal LoC were often tightly clustered together while cells in ventral LoC were diffuse, and cells were even more diffuse in SCv. In terms of their morphology, LoC cells were either round or angular while those in the SCv were predominately elongated. In this study, all cells were classified based on their location. This was done simply because it was the most reliable and objective way to categorize DBH-ir cells, especially between those in ventral LoC and SCv. Based on Dahlstrom and Fuxe's nomenclature, dorsal and ventral LoC would include the A4 and A6 cell

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groups. It is not clear whether the A7 cell group exists in birds (Reiner et al., 1994; Mello et al., 1998).

Hypothalamic tissues, the paraventricular nucleus (PVN) and preoptic area (POA) were analyzed as half sections. This was done to increase the sample size because there were not enough tissue sections containing the bilateral PVN or POA. When bilateral sections were available, they were also analyzed as half sections. In this study, as in our previous research, POA was defined as the area under the septomesencephalic tract (TrSM). Other authors have labeled this area in finches and an area located above PVN but directly under the anterior commissure as the medial preoptic nucleus based on aromatase labeling (Balthazart et al., 1996). In comparison to Mello et al. (1998), POA as defined here is similar to the anterior extent of POM, i.e. under TrSM.

RESULTS

In the LoC, the total number of DBH-ir cells in the DSP-4-treated group was significantly lower than that in saline controls [$t(14)=8.893$, $p<0.0001$; see Figures 12A, 14A, 14B]. Significant loss of DBH-ir cells occurred in both dorsal LoC [$t(14)=6.146$, $p<0.0001$; see Figure 12B] and in ventral LoC [$t(14)=2.522$, $p=0.0122$; see Figure 12C]. DBH-ir cells in the SCv cell groups were also significantly decreased in the drug-treated group [$t(14)=7.765$,

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$p<0.0001$; see Figure 12D]. Drug treatment also significantly reduced the areas of DBH-ir cells and fibers in the LoC [$t(14)=2.668$, $p=0.0092$], and DBH-ir fibers in cerebellum (Cb) [$t(12)=7.148$, $p<0.0001$], ventral tegmental area (VTA) [$t(12)=2.641$, $p=0.0108$], optic tectum (TeO) [$t(12)=3.720$, $p=0.0015$] and septum (S) [$t(11)=2.566$, $p=0.0131$; see Figures 13A, 14A, 14B, 16A, 16B, 17, 18A, 18B]. DSP-4 treatment significantly decreased the mean area covered by DBH-ir fibers in four of the five VCN examined, {high vocal center (HVC) [$t(12)=2.641$, $p=0.0108$], robust nucleus of the archistriatum (RA) [$t(11)=4.375$, $p=0.0006$], dorsomedial portion of the intercollicular nucleus (DM) [$t(9)=9.920$, $p<0.0001$], lateral magnocellular nucleus of the anterior neostriatum (lMAN) [$t(8)=4.206$, $p=0.0015$] and one auditory nucleus - dorsal part of the lateral mesencephalic nucleus (MLd) [$t(12)=4.313$, $p=0.0005$]}; see Figures 13B, 15, 16C, 16D, 17A, 17B]. DSP-4 treatment did not significantly affect the mean area covered by DBH-ir fibers in any of the three diencephalic nuclei examined, PVN and POA and the nucleus uvaeformis (Uva), which is a VCN (Figure 13A, 13B, 14C, 14D, 18C, 18D, 19).

When brain nuclei were corrected for size, the outcomes did not change. Percent area calculations correct for the possibility that DSP-4 treatment altered the size of the nuclei in addition to depleting DBH innervation. Outside the vocal control system, LoC [$t(14)=2.766$, $p=0.0076$], PVN and POA had the greatest percentage of their areas labeled with DBH-ir fibers (see Figure 13C). These areas were followed in descending order of area covered by VTA [$t(12)=2.467$; $p=0.0148$], Cb[$t(12)=6.714$, $p<0.0001$], S

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[$t(11)=2.512$, $p=0.0144$], and TeO [$t(12)=3.738$, $p=0.0014$; see Figure 13C]. Once again, the percent of the brain areas showing DBH-ir fibers were not significantly affected by drug treatment in the three-diencephalic nuclei examined: Uva, PVN and POA. Of the VCN and auditory nuclei, Uva had the greatest percentage of its area labeled with DBH-ir fibers followed by MLd [$t(12)=4.473$, $p=0.0004$], DM [$t(9)=7.929$, $p<0.0001$], lMAN [$t(8)=4.302$, $p=0.0013$, RA [$t(11)=4.378$, $p=0.0006$] and finally, HVC [$t(12)=2.506$, $p=0.0138$; see Figure 13D].

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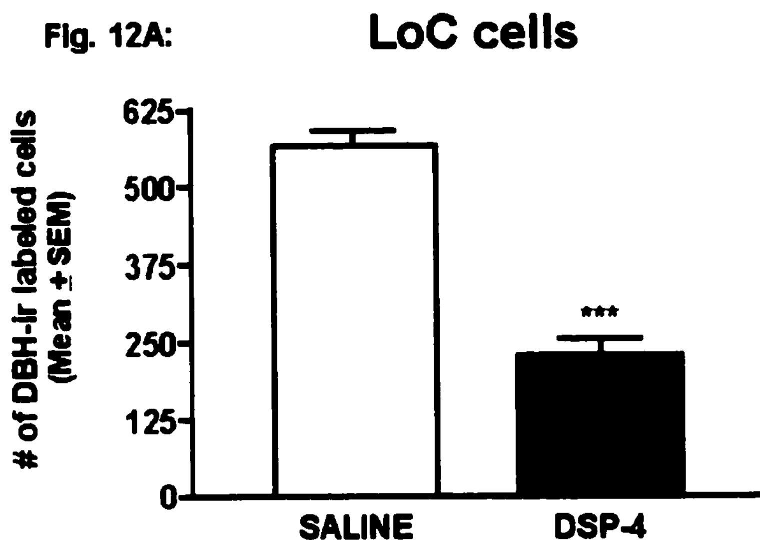

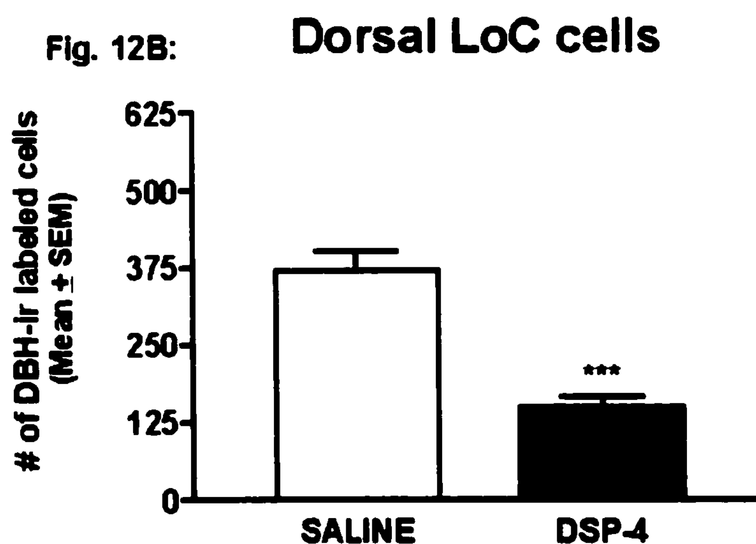

Figure 12: Mean (± SEM) corrected number of DBH-ir cells in **A**: locus coeruleus (LoC), **B**: dorsal LoC. Significant change in number compared to saline-treated males, *** $p<0.001$. See Table 1 for abbreviations.

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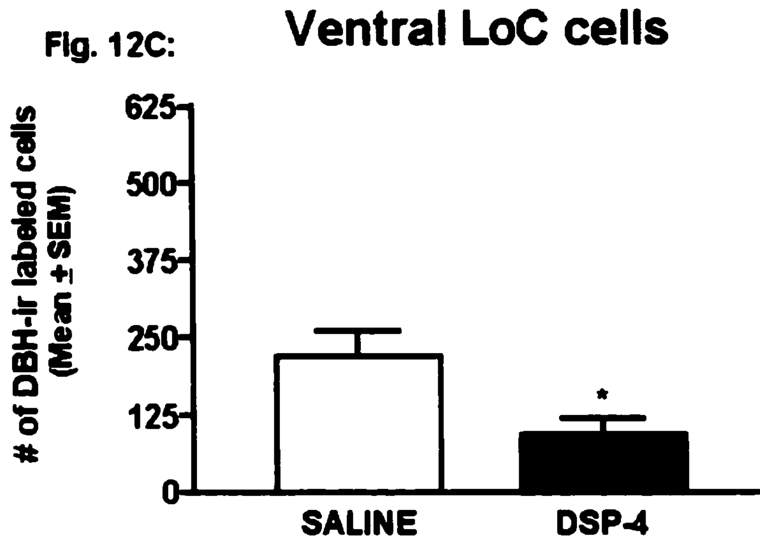

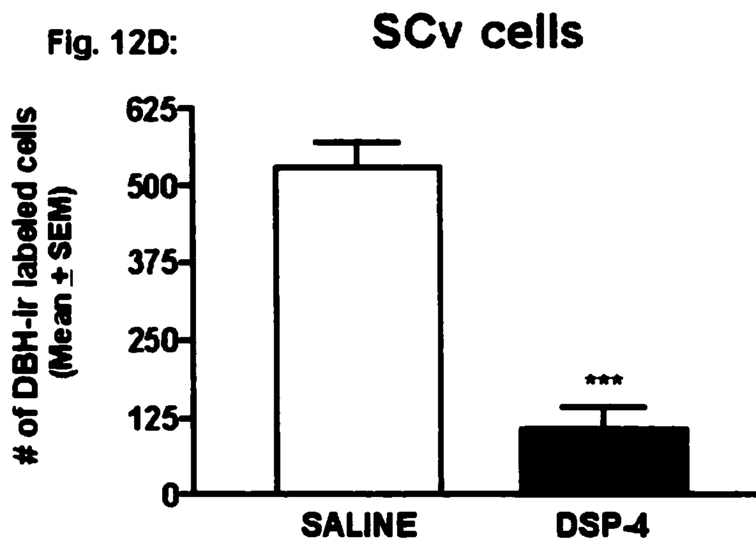

Figure 12: Mean (± SEM) corrected number of DBH-ir cells in **C**: ventral LoC and **D**: SCv. Significant change in number compared to saline-treated males, * p=0.0122, *** p<0.001. See Table 1 for abbreviations.

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Fig. 13A:

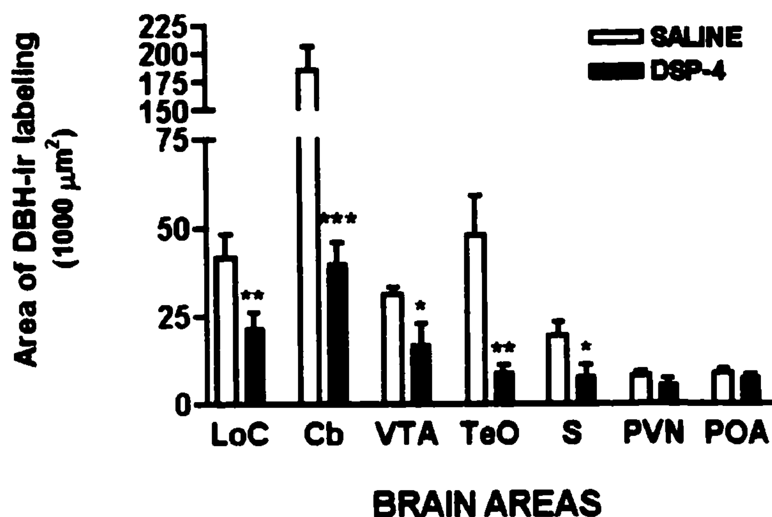

Fig. 13B:

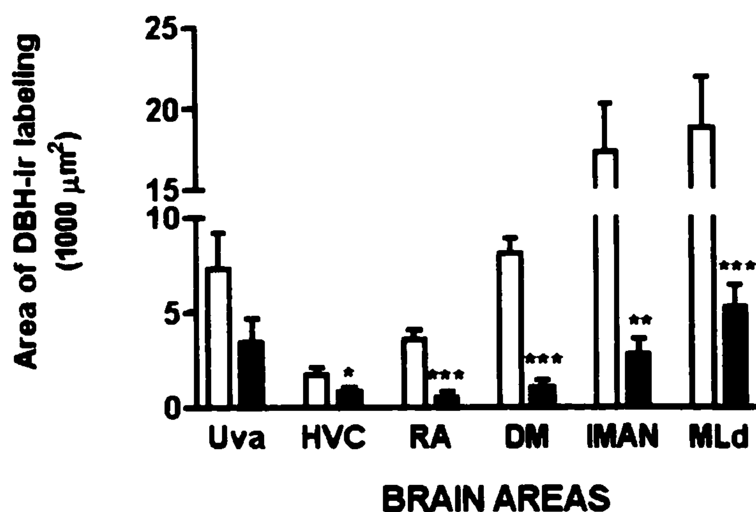

Figure 13: Mean (+ SEM) area covered by DBH-ir labeling in **A**: hypothalamic nuclei and extra-hypothalamic nuclei (note broken Y-axis), **B**: in VCN and auditory nucleus in saline- and DSP-4-treated male zebra finches (note broken Y-axis). Significant change in area compared to saline-treated males, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. See Table 1 for abbreviations.

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Fig. 13C:

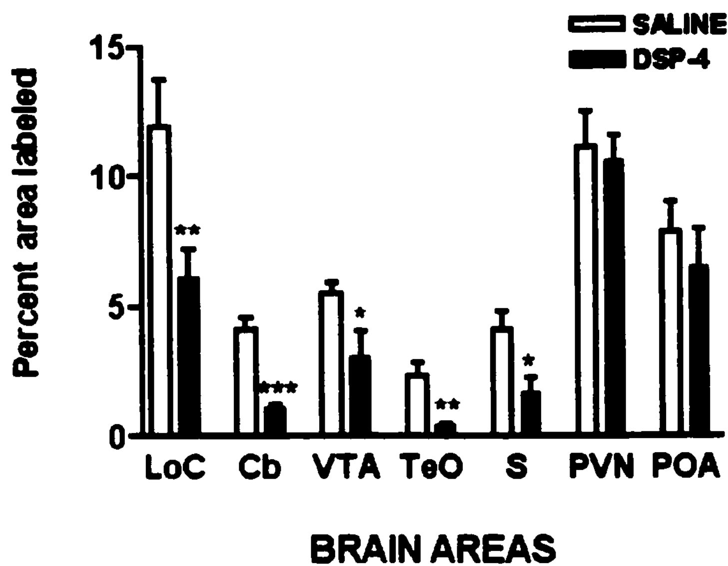

Fig. 13D:

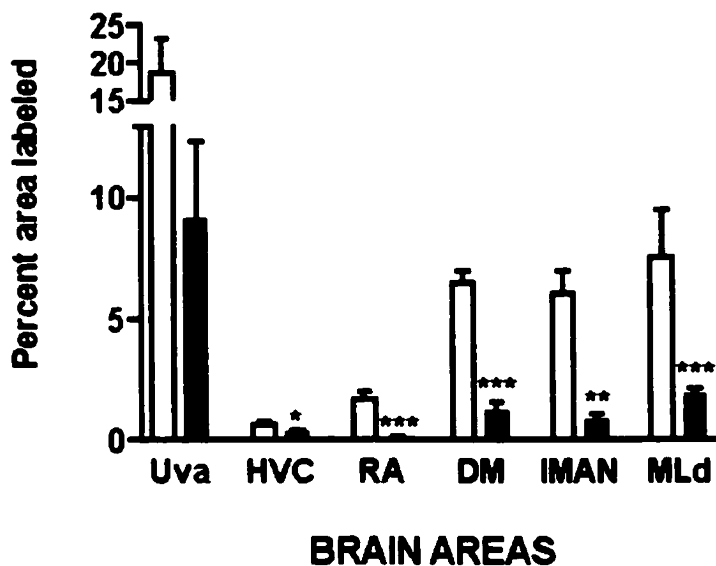

Figure 13: Mean (± SEM) percent of each brain area covered by DBH-ir labeling **C:** in hypothalamic nuclei and extra-hypothalamic nuclei, **D:** in VCN and auditory nucleus saline- and DSP-4-treated male zebra finches (note broken Y-axis). Significant change in area compared to saline-treated males, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. See Table 1 for abbreviations.

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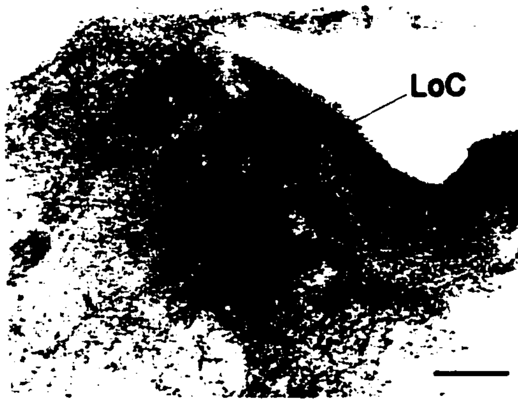
Figure 14A



14B

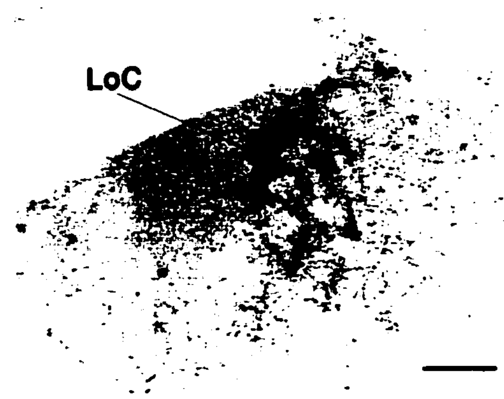


Figure 14C

14D

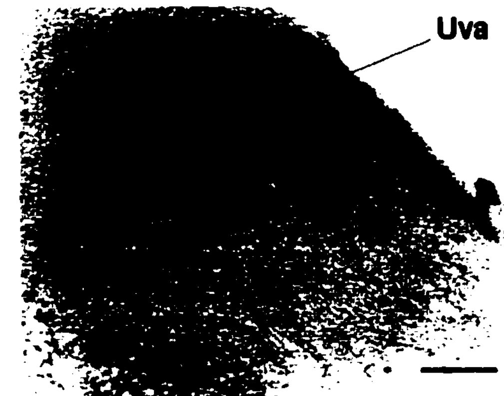


Figure 14: Photomicrographs of DBH-ir labeling in the male zebra finch brain. **A, B**: DBH-ir cells and fibers in the LoC of saline-treated and DSP-4-treated birds. **C, D**: DBH-ir fibers in Uva in saline-treated and DSP-4-treated birds. Scale bar = 150 μm. See Table 1 for abbreviations.

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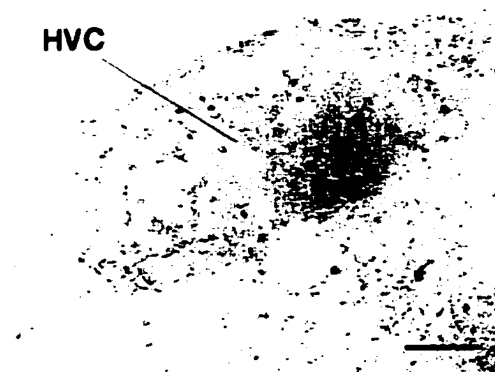
Figure 15A



15B

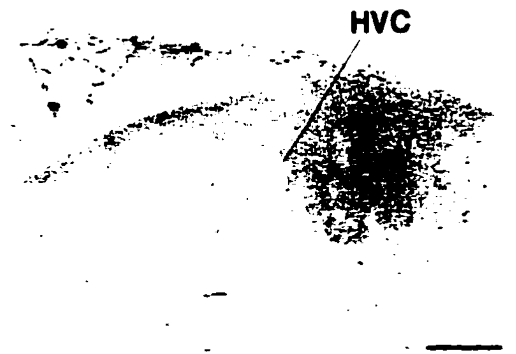
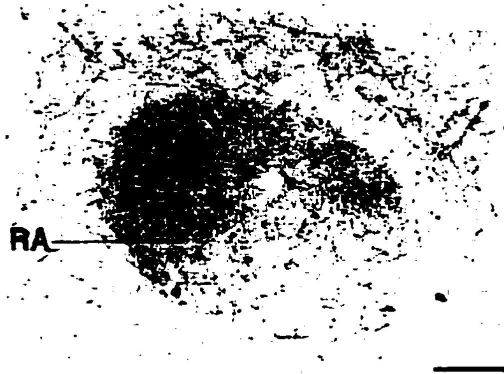


Figure 15C



15D

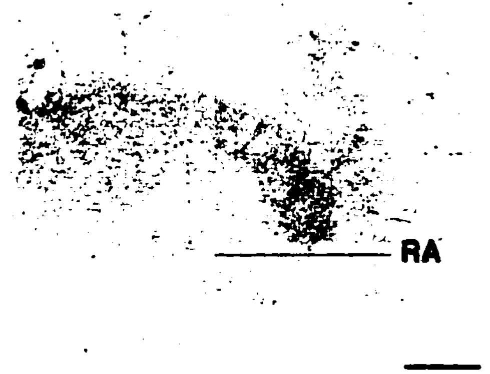


Figure 15: Photomicrographs of DBH-ir labeling in the male zebra finch brain. **A, B**: DBH-ir fibers in HVC in saline-treated and DSP-4-treated birds. **C, D**: DBH-ir fibers in RA in saline-treated and DSP-4-treated birds. Scale bar = 150 μm. See Table 1 for abbreviations.

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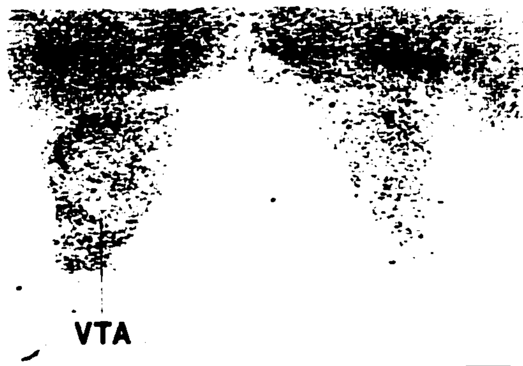
Figure 16A



16B

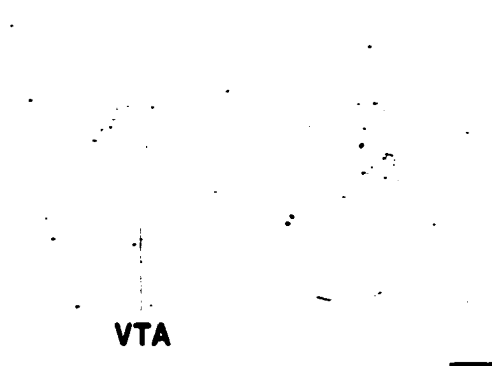
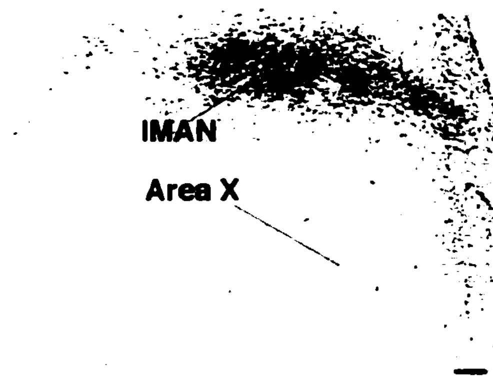


Figure 16C



16D

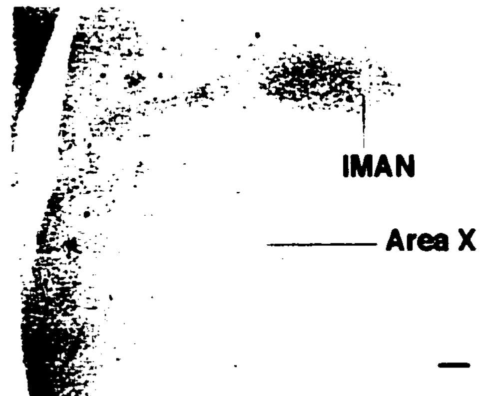


Figure 16: Photomicrographs of DBH-ir labeling in the male zebra finch brain. **A, B**: DBH-ir fibers in VTA in saline-treated and DSP-4-treated birds. **C, D**: DBH-ir fibers in IMAN in saline-treated and DSP-4-treated birds. Scale bar = 150 μm. See Table 1 for Abbreviations.

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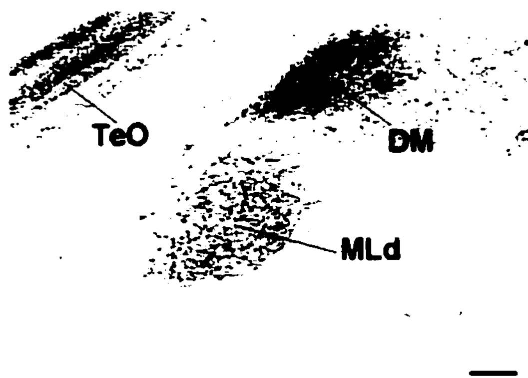
Figure 17A



17B

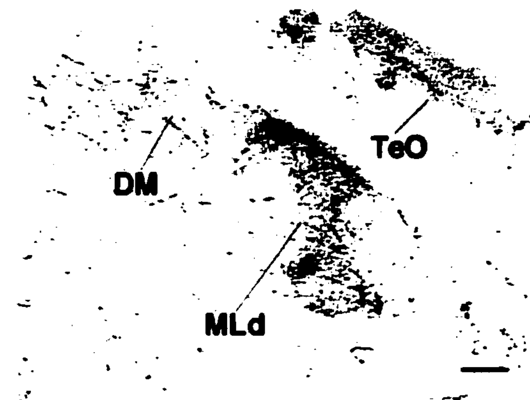
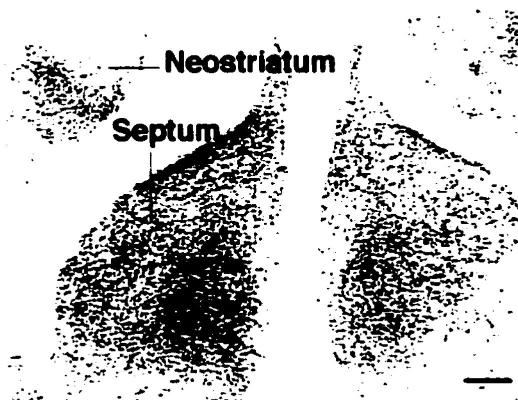


Figure 17C



17D

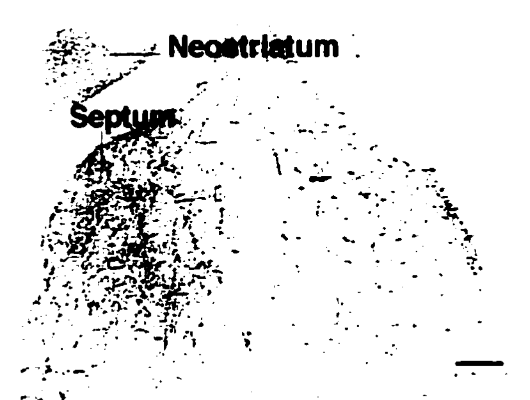


Figure 17: Photomicrographs of DBH-ir labeling in the male zebra finch brain. **A, B**: DBH-ir fibers in DM, MLd and TeO in saline-treated and DSP-4-treated birds. **C, D**: DBH-ir fibers in septum in saline-treated and DSP-4-treated birds. Scale bar = 150 μm. See Table 1 for abbreviations.

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Figure 18A

18B

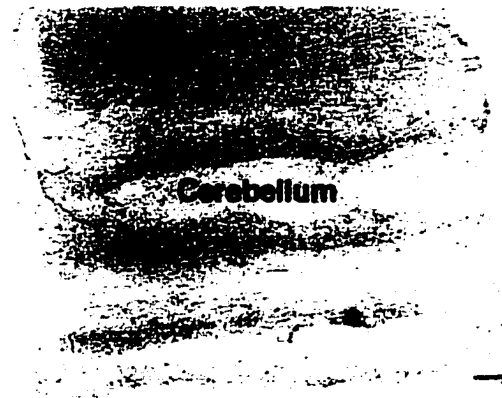
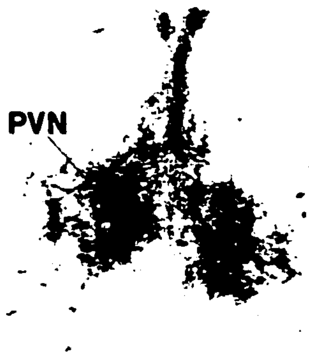


Figure 18C



18D

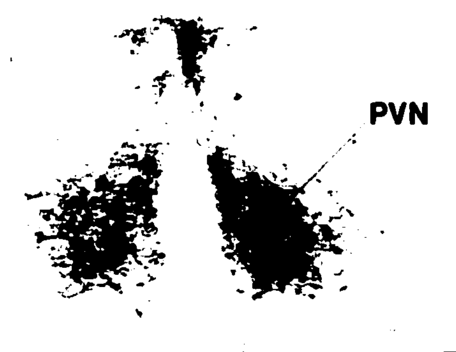


Figure 18: Photomicrographs of DBH-ir labeling in the male zebra finch brain. **A, B**: DBH-ir fibers in Cb in saline-treated and DSP-4-treated birds. **C, D**: DBH-ir fibers in PVN in saline-treated and DSP-4-treated birds. Scale bar = 150 μm. See Table 1 for abbreviations.

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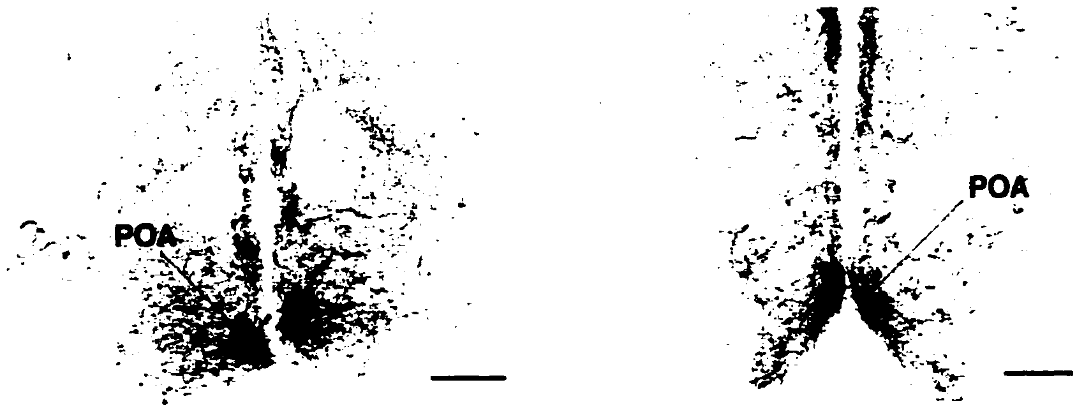

Figure 19: Photomicrographs of DBH-ir labeling in the male zebra finch brain. **A, B**: DBH-ir fibers in POA in saline-treated and DSP-4-treated birds. Scale bar = 150 μm. See Table 1 for abbreviations.

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DISCUSSION

This study used DBH immunocytochemistry to document the extent of DSP-4's neurotoxic effects on the noradrenergic innervation in male zebra finch brains. DSP-4 treatment significantly reduced the number of DBH-ir cell bodies in both the LoC and SCv cell groups. The decrease in the number of labeled cells following DSP-4 treatment is considered indicative of the destruction of NA neurons (Fritschy and Grzanna, 1991b). In finches, this dramatic loss of cells occurred within twenty days of the first drug treatment. This was incredibly fast compared to the effects of DSP-4 on DBH-ir cell bodies in the LoC in rats where it took up to six months after drug treatment to achieve a comparable magnitude of cell loss (Fritschy and Grzanna, 1992).

One possible explanation for the rapid loss of DBH-ir cells in this study compared to that seen in rats was that these finches were treated twice with the standard dose typically used in rat studies. This was done because in an earlier study by Barclay et al. (1992), a single systemic dose of the neurotoxin failed to significantly deplete central NA levels in finches as it does in rats. In finch brains, catecholamine levels were found to be approximately ten times higher than those found in comparable brain areas in rats (Barclay and Harding, 1988). This may account for the ineffectiveness of the administration of a single standard rat dosage. Another possible explanation for the rapid effect of DSP-4 in finches is their brains are smaller and less elongated than rat brains. Therefore, retrograde transport of the neurotoxin from the axon terminals to the cell body probably occurred more rapidly in finches.

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In birds and mammals, the telencephalon receives NA primarily from LoC projection neurons (Kitt and Brauth, 1986; Loughlin et al., 1986a,b). Because DSP-4's neurotoxic effects occurred first at the axon terminals, then worked their way back to the soma, the decrease in the number of labeled-cell bodies suggested there was complete retrograde destruction of noradrenergic neurons (Fritschy and Grzanna, 1991b). In saline-treated finches, estimates of total LoC DBH-ir cell numbers were less than half of the 1300 reported in Japanese quail and in chickens (Bailhache and Balthazart, 1993; Moons et al., 1995) and much lower than the 2900 reported in albino rats (Swanson, 1976). By comparison, finch brains are much smaller than quail, chicken and rat brains, so it is possible that fewer LoC cells are needed to provide noradrenergic projections throughout the brain.

In finches, there were also differences in the intensity of DBH labeling of cell bodies and fibers within the LoC and SCv of the saline-treated and drug-treated groups. Some cells were intensely stained while others were very lightly stained. The intensity of DBH staining may indicate the levels of available enzyme. In the drug-treated group, DBH-ir cells in the LoC were not so clustered, probably because the neurotoxin destroyed many cells. By comparing the DBH-ir cells in the LoC in the two groups, it was observed that it was the lightly-stained cells that were not detected in the DSP-4-treated males. This suggested that this subgroup of neurons which may have lower levels of DBH enzyme and/or NA levels were less able to survive the neurotoxic effects of DSP-4 treatment.

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In rats, morphologically distinct cell populations have been identified within the LoC (Swanson, 1976; Grzanna and Molliver, 1980) that appeared to have different efferent targets (Loughlin et al., 1986b). Loughlin et al. (1986b) found that dorsal LoC cells sent their projections to the cortex, hippocampus and hypothalamus, while ventral LoC cells sent their projections to the cerebellum. To determine if there were regional differences in DSP-4's effect on LoC cells in finches, it was divided into dorsal and ventral regions, as was previously done by Swanson (1976) in rats. There were fewer DBH-ir cells in the ventral than in the dorsal LoC. However, in terms of percent reduction, DBH-ir cells in both areas were decreased by approximately 50% in DSP-4-treated males. In finches, DSP-4 treatment destroyed DBH labeling in the cerebellum and telencephalon, areas which are reported to be LoC targets in rats (Loughlin et al., 1986b).

As might be expected from the fact that DSP-4-treated males had fewer DBH-ir cell bodies, they also had fewer DBH-ir fibers in several brain areas compared to the saline-treated controls. The differences in DBH fiber staining between the treatment groups were obvious and in some brain regions could be seen without the aid of a microscope. In general, the distribution of DBH-ir fibers in the saline-treated finches formed a network of lightly- to densely-stained fibers. DSP-4-treated males did not have this fibrous network, and their remaining DBH-ir fibers appeared turgid and fragmented, as described by Fritschy et al. (1990) in DSP-4-treated rats. These authors hypothesized that these swollen fragments resulted when the highly-branched axon collaterals,

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which are characteristic of disintegrated central noradrenergic neurons, thus halting axonal transport. These fragments, in addition to the presence of axonal sprouts on some swollen axons, were offered as further evidence of DSP-4's neurotoxic effects on DBH-ir fibers.

In finches, DSP-4 treatment had greater effects on the telencephalic and mesencephalic nuclei leaving the diencephalic nuclei examined relatively unaffected. Drug treatment was effective in reducing the area covered by DBH-ir fibers in four VCN (IMAN, DM, HVC and RA) and in an auditory nucleus (MLd). Other areas that DSP-4 affected included the LoC, SCv, TeO, VTA, Cb and S. However, the areas of fiber labeling in the two-hypothalamic nuclei (PVN and POA) and the diencephalic VCN Uva, were not significantly affected.

DBH labeling in the auditory nucleus and all but one VCN, HVC, stood out from their respective backgrounds. For instance, there were moderately-stained DBH-ir fibers in MLd, RA and densely-stained fibers in Uva. Therefore, in most birds, DBH-ir staining was a reliable marker of these nuclei. These striking differences in staining intensities were not observed in most instances by Mello et al. (1998). Three possible reasons for this phenomenon lie in the differences in methodology. Firstly, the concentration of the antibody used in this study was twice as strong as that used by Mello et al. (1998). However, if the staining differences were attributable only to the stronger concentration of antibody, then one would expect just an overall increase in staining intensity across brain areas, which wasn't the case. Secondly, Mello

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et al. (1998) performed their assay on slide-mounted tissue rather than free-floating sections. Free-floating sections have two surfaces and increase the opportunity for the antibody to bind to available antigens. Finally, all males in this study were housed with females to maximize the neurochemical differences between the treatment groups. These males were given the opportunity to court and mate with females. Since our prior studies suggested that high levels of singing behavior were correlated with high levels of NA in the VCN, we expected that housing with females would increase levels of DBH in the saline-treated males, maximizing differences between treatment groups. Mello and his co-workers found the highest density of DBH labeling in the hippocampus (HP). While there was heavy staining in the HP in this study that tended to be much stronger than that in HVC, there were additional areas that stood out in comparison to the other brain regions examined. It should also be noted that Mello et al. (1998) examined HP, NIf and Field L, brain areas that were not analyzed in this study, in sagittal sections and not coronal sections. Therefore, the orientation of the sections may also have contributed to the differences in fiber density.

The pattern of DSP-4-induced destruction of DBH-ir fibers in finches was very similar to that observed in rats (Fritschy and Grzanna, 1989; Fritschy et al., 1990; Fritschy and Grzanna, 1991a; Fritschy and Grzanna, 1991b). In rats, DSP-4 depleted cortical NA levels, in areas like the olfactory bulb, HP and the neocortex but the hypothalamus was not significantly affected (Lookingland et al., 1986). However, in another rat strain and in other animal

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species, DSP-4 had different effects on the central noradrenergic system. In Long-Evans rats, DSP-4 did not significantly affect DBH-ir fibers anywhere in the brain (Schuerger and Balaban, 1995). The rats used most often in DSP-4 studies were albino Sprague-Dawleys and apparently, they are more susceptible to the neurotoxic effects of this drug.

In Japanese quail and goldfish, in addition to its effects on the telencephalon, DSP-4 also destroyed noradrenergic innervation in the hypothalamus (Balthazart et al., 1988; Villani et al., 1996). DSP-4's effect on noradrenergic neurons in rats was long lasting but this was not the case in goldfish, which demonstrated remarkable plasticity (Villani et al., 1996). Their central noradrenergic innervation was restored 40 days after drug treatment. However, DSP-4 treatment in goldfish did not affect the number of DBH-ir cells as it did in finches and in rats. This difference may account for the observed plasticity in goldfish. If the neurotoxin only affects the axon projections but not the cell body, then regeneration of the axons from the cell body is very likely. In comparison to rats and goldfish, DSP-4 treatment in finches significantly decreased DBH-ir cells in the SCv cell groups examined. However, because noradrenergic innervation as defined by quantitative measurements of DBH labeling in the diencephalon wasn't affected, this may indicate that in finches, the major source of NA to the diencephalon is coming from the remaining NA-cell groups, i.e. A1 and A2. Of course testing this hypothesis is beyond the scope of this study and can only be addressed by a study which combines DBH immunocytochemistry with tract-tracing.

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In a previous study, intracerebroventricular administration of DSP-4 significantly lowered NA levels in the hypothalamus, NIf, Area X and IMAN (Barclay et al., 1996). The hypothalamus was probably affected in that study because the neurotoxin was infused directly into the third ventricle that runs through the medial portions of the hypothalamus. Compared to NIf, Area X and IMAN, both hypothalamic nuclei examined, PVN and POA, had much stronger DBH-ir fibers and terminals, and thus infusing the drug in this region may have overridden DSP-4's predilection for axon terminals that originated from the LoC. Alternatively, the decreased NA levels in PVN and POA in DSP-4-treated males are congruent with data in rats, which found that dorsal LoC neurons send projections to the hypothalamus (Loughlin et al., 1986b). DSP-4-treatment decreased the number of putative NA neurons in the dorsal LoC in finches by 50%.

In another study, when finches were given a single peripheral injection of DSP-4, there were non-significant reductions of NA levels in Area X, RA, DM, PVN and POA (Barclay et al., 1992). Therefore, it would appear that DSP-4 had different neurotoxic effects depending upon the route of administration. Of the two administration routes, central administration of DSP-4 should have produced more neurotoxic effects because the drug was delivered directly to the target tissue. Additionally, the time it took to form DSP-4's inactive azridinium derivative was less of a factor since the drug was injected directly into the brain. In peripheral administration, the drug has to travel through the circulatory system and then cross the blood-brain barrier.

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Clearly, it did accomplish this goal, but the neurotoxic effects must have diminished en route to the brain.

In rats, DSP-4's differential effect on central noradrenergic fibers in the cortex versus the hypothalamus suggested there were some neurons that were more susceptible to the drug's neurotoxic effects. Moreover, DSP-4 treatment in rats did not destroy descending projections from the A5 and A7 groups of the LT to the ventral horn of the spinal cord (Lyons et al., 1989). Since LoC projections were destroyed but the LT system was spared, it was suggested that the primary noradrenergic innervation to the hypothalamus originated from the LT system (Lookingland et al., 1986). However, in rats Loughlin et al. (1986b) found that some LoC neurons projected to the hypothalamus. Both Zaczek et al. (1990) and Fritschy and Grzanna (1991a) reported that in comparison to hypothalamic transport sites, cortical noradrenergic transport sites had a higher affinity for DSP-4. In rats, noradrenergic axons that originated from the LoC were thinner than those from the LT system (Moore and Card, 1984; Fritschy and Grzanna, 1989; Zaczek et al., 1990). The structural and functional differences observed between LoC and LT projection neurons in rats may account for the differential effects of DSP-4 on central noradrenergic neurons in this species. However, this difference in the sizes of noradrenergic axons in different regions was never observed in zebra finches.

In rats, DSP-4 not only destroyed noradrenergic innervation, but it also affected the physiology of the remaining noradrenergic neurons. Féty et al.

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(1986) reported following drug treatment, DBH activity measured by enzymatic assay decreased. LoC neurons had slower neuronal firing rates 10 and 50 days after drug administration (Olpe et al., 1983). Finally, Magnuson et al. (1993) found that the electrophysiology of LoC neurons also changed after DSP-4 treatment. The conductance of these neurons to calcium was diminished, and the influx of calcium into axon terminals of neurons was necessary for neurotransmitter release (Schoffelmeer and Mulder, 1983).

Overall, the results of the current study are fairly consistent with our earlier studies on the effects of DSP-4 treatment on NA levels in finch brains. The neurochemical data from our earlier reports showed that DSP-4 had differential effects on NA levels in the VCN depending upon the route of administration (Barclay et al., 1992; Barclay et al., 1996). Although NA levels were not significantly depleted in the first study, one peripheral injection of DSP-4 caused minor decreases in NA levels in Area X, RA and DM (Barclay et al., 1992). In the second study, central administration of the neurotoxin caused significant depletions of NA levels in NIf, Area X and IMAN, but not in RA and DM. In the current study, two peripheral injections of the neurotoxin lowered the percentage of the nucleus covered by DBH-ir fibers in RA, DM, IMAN, HVC and MLd.

Despite the fact that we consistently found moderate to high levels of NA in Area X in previous studies (Barclay and Harding, 1988; Barclay and Harding, 1990; Barclay et al., 1992; Barclay et al., 1996; Harding et al., 1998), we found few if any DBH-ir fibers in Area X and the surrounding parolfactory

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lobe (LPO) in the current study. The reason for this discrepancy is not clear. This nucleus is very large and highly visible making it easy to accurately microdissect. Although DBH labeling was very limited or absent in LPO and Area X, adrenoceptors have been localized in those areas in birds, which also suggests that NA plays a role in modulating function in this area. In chicks, Dermon and Kouvelas (1989) found both alpha-1 and beta-adrenoceptors in LPO. However, alpha-2 adrenoceptors were not found in the entire chick telencephalon. In contrast, in starlings, Ball et al. (1994) found alpha-2 adrenoceptors in Area X. Whether the differences in the localization of these two types of alpha-adrenoceptors in these birds were a consequence of their evolutionary history, or the result of differences in methodology are not known. However, an argument for evolutionary differences can be made given the finding that one of the areas that demonstrated adrenoceptor binding was a VCN. Mismatches in the distribution of various neurochemicals involved in neurotransmitter function (e.g., receptors, synthetic enzymes, and neurotransmitters) are fairly common. For instance, one brain region that has very dense DBH fiber labeling, but very few adrenoceptors is the hypothalamus. Herkenham (1991) reviewed this discrepancy with many neurotransmitter systems including NA.

The current methodology allowed us to assess the effects of DSP-4 treatment throughout finch brains, unlike our prior studies which had been limited to measuring NA levels in 10-12 microdissected brain areas. While the current study did not measure NA levels, the significant reduction of DBH fiber

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staining following DSP-4 treatment suggests that multiple systemic administrations of DSP-4 would cause greater depletion of NA in the midbrain and telencephalon than the single-drug administration procedure used in our earlier studies. Finally, in finches as in rats, DSP-4 treatments decreased DBH labeling in telencephalic areas including VCN but left hypothalamic areas relatively unaffected. Although cell loss occurred in both LoC and SCv, other NA cell groups that were not affected by DSP-4 treatments may provide noradrenergic innervation to the hypothalamus. This study also found in zebra finches that the number of DBH-ir cells in SCv significantly decreased following DSP-4 treatment. DSP-4 studies in rats have never identified the SCv cell group as a target.

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CHAPTER 5:

GENERAL DISCUSSION

The three studies presented here utilized dopamine-beta hydroxylase (DBH) immunocytochemistry to investigate the distribution of central noradrenergic cells and fibers in male zebra finches. As expected, the mapping study found that noradrenergic cell groups were limited to the brainstem regions in the metencephalon, but the projections from these cell groups innervated many other brain areas. However, the distribution of DBH-ir fibers was not uniform. The heaviest labeling occurred within two brain regions, the metencephalon and the diencephalon. DBH fiber staining was heaviest in and around the areas that also had DBH-ir cells, i.e. locus coeruleus (LoC) and ventral subcoeruleus (SCv).

In the diencephalon, the strongest labeling (percent area calculations) of DBH-ir fibers was seen in two hypothalamic regions (paraventricular nucleus - PVN and preoptic area - POA) and one vocal control area, nucleus uvaeformis (Uva). In the hypothalamus, noradrenergic innervation was more concentrated in nuclei that surrounded ventricular regions. Innervation to lateral hypothalamic regions, though present, was not as strong.

In the vocal control system (VCS), the strongest labeling was in nucleus Uva, which is believed to be the first nucleus in the motor circuit controlling the patterning of song (Nottebohm et al., 1982; Williams and Vicario, 1993). There were differential intensities of DBH-ir fibers throughout the VCS which

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could not be attributed to function or location alone. The VCS is comprised of two pathways, the song learning pathway and the motor output pathway. The song learning pathway as its name implies plays an important role in juvenile males during the acquisition of song (Sohrabji et al., 1990; Scharff and Nottebohm, 1991). The motor output pathway is important for the production of learned vocalizations (Nottebohm et al., 1976). The nuclei of the remaining motor output pathway that were analyzed had heavy to light staining, e.g. dorsomedial portion of the intercollicular nucleus (DM) – heavy, robust nucleus of the archistriatum (RA) – moderate, and high vocal center (HVC) – light. Nucleus interfacialis (NIf) is also part of the motor output pathway but it was not analyzed because its borders were hard to delineate in coronal sections and DBH-ir staining in that area was very weak.

Only one nucleus in the song learning pathway was analyzed. DBH fiber staining ranged from heavy to moderate in the lateral magnocellular nucleus of the anterior neostriatum (lMAN). This was in direct contrast to the nearby song nucleus, Area X of the parolfactory lobe (Area X), which was practically devoid of DBH labeling. In fact, the whole parolfactory lobe did not demonstrate any appreciable DBH staining. It is interesting to note that both lMAN and HVC which are both part of the neostriatum, differed in their staining intensities. The staining intensity of the DBH-ir fibers was not simply related to the distance of a nucleus from the noradrenergic cell groups. In rats and birds, the olfactory bulb is the most anterior point in the brain, yet in rats, it receives strong fiber innervation from noradrenergic cells, especially the LoC

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(Moore and Card, 1984; Fritschy and Grzanna, 1989). The olfactory bulb was not analyzed in finches, but moderate DBH labeling was observed.

In the sensory nuclei, DBH fiber staining ranged from moderate to strong. Again, noradrenaline (NA) has been implicated in tasks that require attention and arousal. Most of the primary sensory processing areas of the brain are located within the hindbrain regions. The sensory information from these primary areas reaches the thalamus and then is sent to the specialized telencephalic areas for further processing (Martin, 1991). In the primary sensory processing brain areas, the optic tectum (TeO) and an auditory area, dorsal part of the lateral mesencephalic nucleus (MLd) had strong to moderate fiber staining. In the TeO, DBH-ir fibers formed a layered arrangement similar to that seen in cresyl violet staining. Although other fiber tracts have been identified in the avian brain (Kitt and Brauth, 1986), noradrenergic fibers only appeared to course through the following fiber tracts in the lower brainstem, brachium conjunctivum ascendens - BCA, brachium conjunctivum descendens - BCD, and posterior commissure - CP.

The next two studies took an immunocytochemical approach to complement earlier work which employed high performance liquid chromatography with electrochemical detection (HPLC-EC) techniques (Barclay and Harding, 1988; Barclay et al., 1992; Barclay et al., 1996). In the first of these two studies, castrates were treated with either androstenedione (AE)-silastic implants or empty-silastic implants. In castrated zebra finches,

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only hormones that provide both androgenic and estrogenic metabolites, e.g. AE, can restore the full complement of sexual behavior (Harding et al., 1983).

As expected, hormone-treated castrates had higher levels of courtship behavior than sham-treated castrates. However, while the HPLC-EC studies showed that hormone-treated castrates had an upregulation of noradrenergic functioning within some nuclei of the motor output pathway and downregulation of noradrenergic functioning within some nuclei of the song learning pathway (Barclay and Harding, 1988; Barclay and Harding, 1990), no such changes of AE treatment on DBH staining in these nuclei were evident in the present study. Only the hypothalamic brain areas (PVN and POA) demonstrated evidence of a hormonal effect on the areas of DBH-ir fibers labeling there.

Research in rats and birds suggests that these two hypothalamic brain areas control various aspects in activating male sexual behaviors (Chen et al., 1997; Arendash and Gorski, 1983; Balthazart and Surlemont, 1990; Bailhache et al., 1993). In songbirds, (Foster et al., 1997) found the lateral hypothalamus sends input to the VCS via the thalamus. More recently, Riters and Ball (1999) lesioned medial preoptic area (POM) in male European starlings and observed decreases in their courtship singing. These lesions did not affect other factors known to affect this hormone-dependent behavior, i.e. testis mass, volume and GnRH immunoreactivity in the hypothalamus. Males with lesions confined to rostral POM, which we define as POA in our studies were never observed to sing. These authors also suggested a general role for the

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POM in sexual arousal or anticipation of sexual activity. The POA had high levels of NA in previous studies (Barclay and Harding, 1988; Barclay and Harding, 1990) and high levels of DBH labeling in the current study. It is believed that NA plays a role in state-dependent behaviors (Marshall and Finlayson, 1988; Aston-Jones et al., 1991; Aston-Jones et al., 1994, Delini-Stula et al., 1984; Harro et al., 1995). In particular, the activity of LoC neurons appears to be sensitive to changes in the environment (Marshall and Finlayson, 1988; Waterhouse et al., 1988). Our data suggest that the noradrenergic system may activate or bring about some chain of events that activate the singing component of courtship behavior in males.

The lack of effect of AE treatment on both the area covered by DBH staining and the intensity of DBH staining was somewhat surprising given the robust effects of this hormone treatment on noradrenaline levels and turnover in the VCS and hypothalamic nuclei (Barclay and Harding, 1988). Although hormone treatment significantly affected NA levels and turnover in many brain areas, it did not have much of an effect upon the area and intensity of DBH staining. However, Kritzer (2000) demonstrated that in the cortices of male rats, TH staining was sensitive to hormone treatments while DBH staining was not. Since TH is the rate-limiting step in the synthesis of all catecholamines, it is possible that hormonal regulation of central NA occurs at that level and not so much at the final synthesis step which involves DBH.

Finally, treatments with the noradrenergic neurotoxin, DSP-4 significantly decreased DBH staining in many brain areas. Following DSP-4

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administration, central NA levels were also lowered (Barclay et al., 1992; Barclay et al., 1996). In contrast to the hormone treatment study, DBH-ir and HPLC-EC data were in agreement. Although DSP-4 treatment decreased the number of DBH-ir cells in both the LoC and SCv, the area covered by DBH-ir fibers in the two hypothalamic nuclei examined was not significantly affected. Therefore, in finches as in rats, DSP-4 decreased noradrenergic innervation in the telencephalon but left innervation to the hypothalamus relatively unaffected. In rats, DSP-4 selectively destroys noradrenergic innervation that originates from the LoC (Ross, 1976; Ross and Renyi, 1976; Jaim-Etcheverry and Zieher, 1980; Jonsson et al., 1981; Hallman and Jonsson, 1984; Hallman et al., 1984). However, in finches unlike in rats, DSP-4 treatment significantly decreased the number of DBH-ir cells in the SCv, in addition to the LoC. When DSP-4's effects were measured by the same immunocytochemical methodology, its overall effect appeared to be consistent across species.

From the DBH-ir data, it was evident that this neurotoxin significantly decreased the number of DBH-ir cells in the metencephalon. Central noradrenergic innervation originates from these brainstem cell groups; therefore DSP-4 treatment significantly decreased the distribution of noradrenergic fibers throughout the mesencephalon and telencephalon. However, immunocytochemical techniques revealed that unlike rats, DSP-4 treatment targeted cell bodies in both the LoC and in the SCv.

The onset of DBH-ir cell loss following DSP-4 treatment was much faster in finches than in rats, but this may be the result of a combination of

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factors. In this study, these birds received double treatments because single administration of the standard dosage in rats failed to significantly deplete NA levels in a previous study (Barclay and Harding, 1992). Rat brains are much bigger and more elongated than finch brains and so the neurotoxin most likely had a greater distance to travel from the axon terminal to the soma. In rats, the toxicity of the compound may have decreased by the time it reached the soma. Finally, the higher metabolic rate in finches may have contributed to the faster onset of DSP-4's neurotoxic effects.

In conclusion, with the exception of the telencephalic VCN, DBH labeling in zebra finches was in agreement with that reported in rats and in other birds. In the second study, hormone treatments that significantly increased courtship singing also increased the area of DBH labeling in both hypothalamic nuclei. However, previous HPLC-EC studies by Barclay and Harding (1988, 1990) demonstrated that noradrenergic functioning in both the VCN and hypothalamic nuclei were most responsive to hormone treatment.

Finally, in the DSP-4 study, DBH labeling revealed the widespread and rapid destruction of noradrenergic innervation in finch brains. The decreased number of LoC and SCv neurons also indicated that this neurotoxin targeted noradrenergic cells in the LT system. This was in contrast to research in rats which found that DSP-4 selectively destroys projections that originate from the LoC but leaves those projections from the LT system relatively intact. Therefore, in finches and in rats, although the location of noradrenergic cell bodies and the fiber projections from these cells are highly conserved, there

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were differences observed in the population of noradrenergic neurons that were affected by DSP-4 treatment.

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