Serotonin and Song: neuronal and behavioral effects in the zebra finch

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Abstract

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Serotonin (5-HT) is a neuromodulator important in a vast array of neurological processes, including sensory and motor processing. Dysfunction of the 5-HT system is associated with depression and other clinically important mood disorders, and has been linked with learning deficits. Song production and song learning in the songbird provide a unique system for studying how the actions of neuromodulators on individual neurons combine to eventually change the dynamics of neural circuits, resulting in alterations of complex behaviors. The results described here are the first identification of a specific action of 5-HT in neurons of the song system in songbirds. I identify the receptors via which 5-HT act in a population of neurons crucial for song learning and production, and describe how 5-HT modulates excitability in these cells. I use *in vivo* electrophysiology combined with playback of the bird's own song to identify how 5-HT modulates network activity in these cells, and I use serotonergic lesions to connect the actions of 5-HT with a concrete change in a complex learned behavior- a decrease in the fundamental frequency of harmonic stacks in learned songs. These results show for the first time that 5-HT is

involved in modulating spectral properties of song. My results follow an action of 5-HT from the level of individual receptors on specific neurons through to a change in a complex learned behavior. The conserved nature of the serotonergic system indicates the findings here are likely to generalize across vertebrates. This greater mechanistic understanding of particular actions of 5-HT on song production in songbirds will lead to improved knowledge of how 5-HT modulates neural activity underlying perception and behavior in mammals.

TABLE OF CONTENTS

LIST OF FIGURES

Figure Number Page

LIST OF TABLES

Table Page

Dedication

To whomever actually reads this: you know who you are.

Introduction

Humans have an unparalleled ability to learn, and the neural mechanisms of complex learning remain a tantalizing goal of modern neuroscience. Among the myriad of learned behaviors exhibited by humans is vocal learning- the ability to learn to shape the spectral properties (such as shifting the pitch) of one's vocalizations[1]. This seemingly effortless task (exemplified by an infant's struggle to form her first words) is extremely rare in the animal kingdom: only humans, cetaceans, and three orders of birds are proven to engage in vocal learning (Garland et al., 2011; Matsunaga & Okanoya, 2009)[2]. The oscine songbirds rely almost entirely on learned song for attracting mates and repelling conspecifics. The neural substrate of vocal learning is thus highly developed and experimentally tractable in songbirds, which has enabled rapid advances in understanding how their brains learn to produce complex vocalizations, such as song. Importantly, we now know that the pathway for song learning shares many similarities with learning in other animals, humans included (Doupe and Kuhl, 1999; Farries et al., 2005). In order to understand how a task is learned one must first understand how that task is carried out. We are beginning to arrive at such an understanding of song production in the zebra finch (Taeniopygia guttata), and this PhD thesis serves as one more piece in that puzzle.

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[1] Note that vocal learning is not merely the ability to associate a meaning with a vocalization, which is indeed widespread among animals.

[2] While more study is needed, bats seem likely to be vocal learners as well (Knörnschild et al., 2010). Elephants may learn vocalizations but the evidence is sparse (Poole et al., 2005), and there is active research into vocal learning in mice (Hoffmann et al., 2012), with the current evidence pointing against them being vocal learners (Kikusui et al., 2011; Hammerschmidt et al., 2012).

Understanding vocal learning in songbirds will inform our understanding of complex learning- vocal and otherwise- in related species, humans included.

Song learning in songbirds shares many characteristics with human speech acquisition, including an innate predisposition to species-specific sounds and a sensitive (or critical) period for song acquisition (Doupe and Kuhl, 1999). Juvenile Zebra Finches learn to sing one highly stereotyped song consisting of ~5 syllables, which they will sing for the entirety of their lives. Juvenile males are capable of learning to sing only during a small time window, lasting for approximately the second and third months of the animal's life (Tchernichovski et al., 2001). In the initial sensory learning phase the zebra finch memorizes the song of a tutor (generally his father), and then during the motor learning phase sings many renditions of his developing song, using sensorimotor feedback to sculpt his song to match that of the memorized tutor template. This learning process has particular benefits to experimental design. First, as song is necessary for mate attraction (and thus also for successful reproduction) juveniles are inherently motivated to learn to sing, they sing thousands of repetitions that can be easily recorded and analyzed without any prompting. This allows for minimum artificial manipulations by the researcher, ensuring a natural learning process and reducing possible confounds that may be present in other common learning paradigms. Second, it is easy to separate the effects of manipulation on song production from song learning simply by studying either juveniles or adults.

The neural substrate of song learning and production in songbirds is a well-defined set of highly discrete forebrain nuclei, collectively termed the song control system (SCS). Forebrain nuclei of the SCS converge on the robust nucleus of the arcopallium (RA), which projects directly to the hypoglossal nucleus (nXII, which innervates the syrinx) and to brainstem respiratory control nuclei (Fig. 0.1). Projection neurons in RA, which are primarily premotor and carry the entire SCS signal out of the forebrain, are hypothesized to be analogous to layer V pyramidal neurons in mammalian premotor cortex (Karten, 1969, 1991), and are discussed in length in chapters 2 and 3 of this thesis. The SCS is composed of two parallel pathways- the direct motor pathway (DMP) and the anterior forebrain pathway (AFP); nucleus HVC (an outdated abbreviation now used as a proper name) is the start of both the DMP and the AFP (Fig. 0.1).

The DMP (regions in red in Fig. 0.1) is necessary and sufficient for the production of normal adult song (Nottebohm et al., 1976). One population of neurons in HVC projects to RA and fires sparse bursts of action potentials time-locked to individual syllables (Hahnloser et al., 2002). These bursts determine the ordering and timing of syllables, and studies where HVC was cooled with a reverse-peltier device showed that song slowed in a direct manner relative to amount of HVC cooling (Long and Fee, 2008). In RA, this sparse timing signal is converted into a more complex signal that is thought to encode the spectral features of song (Abarbanel et al., 2004; Leonardo and Fee, 2005; Fiete et al., 2007). RA projection neurons still fire bursts of action potentials time-locked to syllables, but neurons fire more bursts, often time-locked to multiple syllables per song motif (Leonardo and Fee, 2005). The firing patterns of the hypoglossal nucleus (innervated by RA) have yet to be described during singing.

The AFP (Fig. 0.1, blue) is necessary for song learning in juveniles and for song maintenance in adults, but is not necessary for stereotyped production of adult song (Nottebohm et al., 1976). A population of HVC neurons project to the AFP. These neurons appear to fire in a less stereotyped pattern than the HVC→RA neurons. A series of lesion and inactivation studies unequivocally show the AFP is necessary for song learning, but the details of that process remain elusive. As the activity of midbrain dopaminergic neurons and their projection to the basal ganglia is thought to play a critical role in the acquisition of motor skills, it is noteworthy that the AFP is a “cortical” thalamic basal-ganglia network with an indirect projection to dopaminergic cells in the VTA (ventral tegmental area). These dopaminergic cells exhibit selective auditory responses to the bird's own song (BOS), and in turn project back to area X in the AFP (Gale et al., 2008; Gale and Perkel, 2010). Furthermore, it has recently been shown that DA can act on D1 receptors in Area X to modulate the BG output signal (Leblois et al., 2010; Leblois and Perkel, 2012). Regardless of how learning progresses in the AFP, the learning is eventually consolidated into the DMP, very likely in RA, perhaps involving known long-term depression (LTD) in collateral synapses (Sizemore and Perkel, 2011) or acetylcholine related long-term depression (LTP) (Salgado-Commissariat et al., 2004)□. While DA is clearly implicated in the AFP, DA levels are low in the DMP, whereas 5-HT levels are relatively higher. As 5-HT is involved in many forms of learning it seemed pertinent to understand the role of 5-HT in RA, which forms the bulk of this thesis.

5-HT is implicated in a number of learned tasks across the animal kingdom. For instance, 5-HT is crucial to coincidence detection central to the learned siphon withdrawal reflex in *Aplysia* (2000 Nobel Prize in Physiology and Medicine). More recently, a growing body of work has established that the serotonergic system affects multiple types of associative learning in mammals (including: visual discrimination, verbal memory, conditioned avoidance, eye blink conditioning), with 5-HT$_{2A}$ receptors seeming to play a dominant role (Williams et al., 2002; Harvey, 2003). Associative conditioning of the eye blink response has proven particularly fruitful, revealing the 5-

HT_{2A} agonists enhance the learning of conditioned response (CR) under certain conditions (and in the dose range that produces perceptual changes in humans). Additionally, 5-HT_{2A} neutral antagonists had no effect on CR learning, while 5-HT_{2A} inverse agonists retarded learning (Williams et al. 2002). These findings are provocative and important, and while the neural substrate of these effects is broadly known (the hippocampus and prefrontal cortex), the complexity of these areas (in terms of heterogeneity, multi-processing, and interconnectedness) make it quite difficult to identify the specific mechanisms of 5-HT modulation, from synapse to circuit, that ultimately produce the observed changes in learning. This thesis was designed primarily to address the role 5-HT plays in the acquisition and production of a complex learned behavior- song production in the zebra finch.

Serotonin has been known to be present in the nuclei of the SCS for some time (Barclay and Harding, 1988), but until I began the present work nearly nothing was known about the role 5-HT played in either song production or song learning. For this thesis I focused on RA projection neurons, which seem to be primarily responsible for determining spectral features of song. RA projection neurons are also spontaneously active in acute tissue slices, firing rhythmically around 5 Hz. While recording the firing of RA projection neurons I applied 1 μM 5-HT to the bath and observed that firing rate reliably doubles- a significant increase in firing rate occurred in every projection neuron I tested. I went on to use specific agonists and antagonists, along with *in situ* hybridizations, to determine whether this action was due to a 5-HT_2 class of receptors. Adding the serotonin selective reuptake inhibitor fluvoxamine revealed that endogenous 5-HT was sufficient to mediate this response- these experiments are discussed in detail in chapter two. I then asked how this observed increase in excitability might affect circuit level activity and, eventually, song. Playing the bird's own song (BOS) while the animal is maintained in a low-level of anesthesia evokes firing patterns in RA projection neurons that are similar to those observed during singing and which are propagated along the DMP. Using this methodology along with the puff-application of nanoliter amounts of 5-HT allowed me to probe how individual RA projection neurons change song-related firing patterns in response to 5-HT application. I drastically reduced 5-HT in RA by lesioning the serotonergic system with application of a toxin that selectively kills serotonergic neurons, 5,7-DHT. This caused a decrease in the fundamental frequency of harmonic syllables, and could be explained by the observed effects of 5-HT acting via 5-HT_{2A} receptors on RA projection neurons. These results show for the first time that 5-HT is involved in modulating spectral properties of song, and they are discussed in length in Chapter 3. My results follow an action of 5-HT from the level of individual receptors on specific neurons through to a change in a complex learned behavior. Chapter Four details how I used the information I learned in chapters 2 and 3 to investigate a daily oscillation in song features that I helped discover. Finally, I describe a set of experiments I carried out which are tangential to serotonergic modulation of song but I believe are crucial for the field in general- a careful analysis of the behavioral effects of severing the tract between HVC and RA: these experiments are described in chapter five.

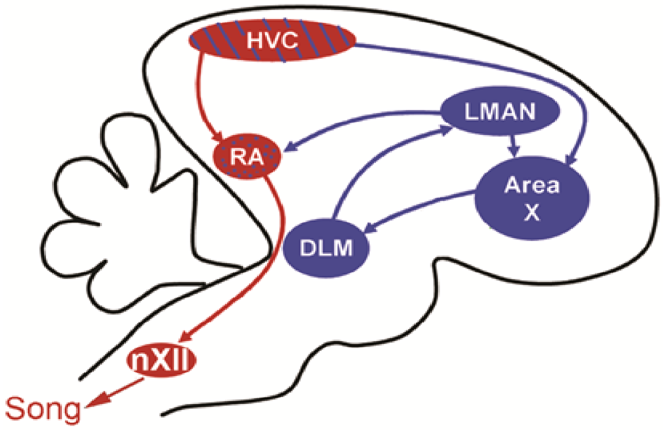

Figure 0.1. The song control system. The direct motor pathway (DMP) is in red and the anterior forebrain pathway (AFP) is in blue. HVC projects to both pathways and thus is both blue and red. RA receives a major projection from both pathways and is thus also blue and red.

Chapter I: Serotonin, via 5-HT_2 receptors, excites neurons in a cortical-like pre-motor nucleus necessary for song learning and production

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All data was collected and analyzed by me, except for Figure 1.3, which was done with the help of a collaborator. I wrote the initial and final versions of the manuscript.

Title:

Serotonin, via HTR2 receptors, excites neurons in a cortical-like pre-motor nucleus necessary for song learning and production

Abbreviated Title: **Serotonergic Modulation of RA**

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Abstract

Serotonin (5-HT) is a neuromodulator that is important for neural development, learning and memory, mood, and perception. Dysfunction of the serotonin system is central to depression and other clinically important mood disorders and has been linked with learning deficits. In mammals, 5-HT release from the raphe nuclei in the brainstem can modulate the functional properties of cortical neurons, influencing sensory and motor processing. Birds also have serotonergic neurons in the dorsal raphe, suggesting that 5-HT plays similar roles in sensory and motor processing, perhaps modulating brain circuitry underlying birdsong. To investigate this possibility we measured the effects of 5-HT on spontaneous firing of projection neurons in the premotor robust nucleus of the arcopallium in brain slices from male zebra finches. These neurons are thought be akin to cortical layer V pyramidal neurons. 5-HT dramatically and reversibly enhanced the endogenous firing of RA neurons. Using pharmacological agonists and antagonists *in vitro*, we determined this action is mediated via HTR2 receptors, which we verified are expressed by *in situ* hybridization. Finally, focal administration of the serotonin selective reuptake inhibitor (SSRI) fluvoxamine revealed that endogenous 5-HT is sufficient to mediate this effect *in vivo*. These findings reveal a modulatory action of serotonin on the physiology of the song system circuitry and suggest a novel role of serotonin in regulating song production and/or learning; further understanding of the role of 5-HT in this system may help illuminate the complex role of this neuromodulator in social interactions and motor plasticity in humans.

Introduction

The biogenic amine serotonin (5-HT) is involved in modulating an array of complex behaviors, including sleep, anxiety, and mood (Dubovsky and Thomas, 1995; Monti, 2010). Perhaps it is not surprising, then, that serotonin deficiencies have been closely linked to mental illnesses such as anorexia, obsessive-compulsive disorders, and clinical depression. A primary treatment for these disorders has been use of antidepressants like the serotonin reuptake inhibitors (SSRIs) which are now the most prescribed class of drug in the US, with use nearly doubling between 1996 and 2005 (Olfson and Marcus, 2009); understanding the myriad ways in which serotonin affects brain function is thus a crucial goal from both healthcare and basic neuroscience viewpoints.

5-HT is important for learning and memory function. For example, 5-HT is involved in perhaps the most comprehensively understood form of learning, sensitization of the gill- and siphon-withdrawal reflexes in *Aplysia* (Glanzman et al., 1989). More recently, a growing body of work has established that the serotonergic system affects multiple types of telencephalic associative learning in rodents (including: visual discrimination, conditioned avoidance, eye blink conditioning), with 5-HT_{2A} receptors seeming to play a dominant role (Williams et al., 2002; Harvey, 2003). Although the neural substrate of these neuromodulatory effects of serotonin is broadly known (i.e., the hippocampus and prefrontal cortex), the complexity of these areas in terms of their heterogeneity, multi-sensory processing, and interconnectedness makes it difficult to identify the specific loci or mechanisms of serotonergic action, from synapse to circuit, that ultimately produce the observed changes in learning.

Song production and vocal learning in zebra finches may be a particularly fruitful system in this context, as it consists in a complex but easily quantifiable learned skill (song) that is subserved by a set of discrete telencephalic nuclei, the song control system (SCS; Zeigler and Marler, 2004; Nottebohm, 2005). The SCS can be divided into two pathways – an anterior forebrain pathway (AFP) necessary for learning but not for production of adult song, and a direct motor pathway (DMP) that is both necessary and sufficient for normal adult song production (Fig. 1.1). These pathways converge on the final telencephalic song nucleus, the robust nucleus of the arcopallium (RA), which projects directly to the hypoglossal nucleus (nXII) and to brainstem respiratory control nuclei. All models of song sensorimotor learning involve some lasting synaptic changes that occur in RA (Abarbanel et al., 2004; Fiete et al., 2007; Gibb et al., 2009; Mooney, 2009).

Here, using *in vitro* single-unit and whole-cell electrophysiology, we show that 5-HT exerts a strong excitatory action on projection neurons of nucleus RA, which contains 5-HT (Barclay and Harding, 1988). Pharmacological manipulations and *in situ* hybridization point to HTR2 receptors mediating this action. While the exact channel mediating this action remains undetermined, we have ruled out the participation of HCN channels, L-type Ca^{2+} channels, and T-type Ca^{2+} channels. Blockade of 5-HT uptake revealed that endogenous 5-HT is sufficient to mediate this same increase in excitability. These findings establish serotonin as a potent modulator of the song control circuitry in the zebra finch.

Methods

Birds

Adult zebra finches (*Taeniopygia guttata*) were obtained from breeding colonies at the University of Washington or OHSU, or purchased from an outside supplier. As only male zebra finches learn to sing and there is marked sexual dimorphism in the SCS we restricted our study to males. Juvenile zebra finches in the sensorimotor period of song learning (day 38-54) were acquired from our breeding colony where they were housed with their family units until the experiment day. All birds were housed in groups of up to five individuals and experienced 12-13 hours light per day. Food and water were provided *ad libitum*. All animal use protocols were approved by IACUC committees at OHSU and University of Washington and are in accordance with NIH guidelines.

Preparation and application of drugs for electrophysiology experiments

All drugs were purchased from either Sigma (kynurenic acis, picrotoxin) or Tocris (DOI, Ketanserin, fluvoxamine). All drugs for *in vitro* experiments were dissolved in water, with the exception of ketanserin, which was dissolved in DMSO. Final concentration of DMSO never exceeded 0.01%, and controls were conclusive that this concentration had no effect on firing rate in these neurons. All drugs for *in vivo* experiments were dissolved in saline.

Preparation of brain slices for in vitro electrophysiological experiments

Methods for preparing slices have been described previously (Solis and Perkel, 2005). Briefly, each animal was anesthetized with isoflurane and euthanized by decapitation, and the brain was dissected rapidly into ice-cold, oxygenated artificial CSF (ACSF) containing the following (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 16.2 $NaHCO_3$, 11 D-glucose, and 10 HEPES, osmolarity adjusted to 310–320 mOsm with sucrose. Parasagittal or coronal brain slices (300 µm thick) were prepared using a Vibratome (Vibratome, St. Louis, MO), and slices were stored at room temperature submerged in bubbled ACSF in which HEPES was replaced with equiosmolar $NaHCO_3$.

In vitro electrophysiological slice recording.

RA neurons fire rhythmically both *in vivo* and *in vitro*, although the firing rate *in vitro* (at 30°) is slightly less than half of *in vivo* rates (4.784 ±2.1 Hz n=97 vs 14.03 ±4.927 n=34, in this study). Previous studies (Mooney, 1992; Park et al., 2005; Solis and Perkel, 2006; Sizemore and Perkel, 2008) have determined the overwhelming majority of spontaneously active cells in RA are projections neurons. Recording methods were similar to those described previously (Meitzen et al., 2007). We allowed the slices to rest for 1 h after collection before recording. For recording, a slice was submerged in a small chamber perfused with ACSF containing the fast glutamatergic and gabaergic transmission blockers kynurenic acid (1 mM) and picrotoxin (150 µM) and maintained at 30°C (±1°). Single-unit extracellular recordings were obtained from neurons within a region that could be reliably identified as RA using transillumination. Only well-isolated spikes with high signal-to-noise ratios were studied. Recording electrodes were made from pulled borosilicate glass pipettes (World Precision Instruments, Sarasota, FL) with tips broken to a resistance of 4–10 MΩ and filled with 0.9% NaCl. Extracellular potentials were amplified 1000x with an Axon MultiClamp 700B amplifier (Molecular Devices, Foster City, CA) and digitized with a Digidata 1322A (Molecular Devices) and stored on a personal computer using pClamp 9 (Molecular Devices).

In vivo electrophysiological recording

Methods were similar to those described previously (Gale and Perkel, 2010; Leblois et al., 2010). Birds were deprived of food and water for 1 h and then anesthetized by intramuscular injection of 20% urethane. Recording coordinates relative to the bifurcation of the midsagittal sinus were (in mm): (anterior, -1:-.5; lateral, 1.6:2.0; depth, 2.0:2.5). Small, iontophoretic injections of the tracer 10 kDa dextran amine conjugated to Alexa 488 (Invitrogen) were made to mark recording locations, which were subsequently verified using standard histological methods previously described (Gale and Perkel, 2010).

Single-unit extracellular recordings were made using glass electrodes with tips broken to 0.5–3 μm diameter. Electrodes were filled with 0.5 M NaCl. Voltage signals were amplified 10x with an Axoclamp 2B amplifier (Molecular Devices) in bridge mode, and then filtered (0.1 kHz high pass; 7 kHz low pass) and amplified an additional 100x with a model 440 amplifier (Brownlee Precision). Signals were sampled at 25 kHz with a Micro 1401 data acquisition unit and Spike2 software (Cambridge Electronic Design).

Electrophysiological Data analysis

Spikes from both the *in vitro* and *in vivo* expts. were sorted using Spike2 (CED) software and analyzed using custom software written in Matlab (MathWorks). Unit isolation was verified based on examination of overlaid spike waveforms and a plot of the principal components of the spike waveform calculated in Spike2 for consistency of spike shape, and evidence of a refractory period in a histogram of interspike intervals. Signal to noise of single units was generally greater than 3:1.

Tissue preparation for in situ hybridization

Six adult male zebra finches were isolated overnight under 12:12-hour light-dark cycle (same as breeding aviary) in custom-built acoustic isolation chambers. The following morning birds were monitored for at least two hours to ensure they were not singing, and were then sacrificed by decapitation. Their brains were quickly removed, blocked in the parasagittal plane and frozen in tissue-tek O.C.T. Compound (Sakura Finetek; Torrance, CA) in a dry ice/isopropanol bath. Parasagittal (10 µm) brain sections were cut on a cryostat, melted onto microscope slides (Superfrost plus; Fisher Scientific, Pittsburg, PA), and stored at -80°C for *in situ* hybridization.

In situ Hybridization for Serotonin receptor 2A, 2B and 2C (HTR2A/HTR2B/HTR2C)

Digoxygenin-(DIG)-labeled riboprobes were synthesized using established protocols (Lovell and Mello, 2011). Briefly, clones corresponding to HTR2A (GenBank accession code: CK306989), HTR2B (FE732340) and HTR2C (DV953355) were obtained from the ESTIMA zebra finch brain cDNA collection (http://titan.biotec.uiuc.edu/cgi-bin/ESTWebsite/estima_start?seqSet=songbird3). Plasmid DNA was extracted (GeneJET Plasmid Miniprep kit; Fermentas, Glen Burnie, MD), restriction enzyme digested (BSSHII; New England Biolabs; Ipswich, MA) to release the insert template, and twice purified with a PCR purification kit (GeneJET PCR Purification Kit). Sense and antisense strand probes were then synthesized at 37°C for 5 hours using the appropriate T3 or T7 RNA polymerase (Promega Inc., Madison, WI) and nucleotide label mix, and purified by Sephadex G-50 columns.

All of the methods for *in situ* hybridization were essentially as described in (Lovell and Mello, 2011). Brain sections were post-fixed in a 3% buffered paraformaldehyde solution for 5 min at room temperature (RT), rinsed twice in 0.1 M PBS, and dehydrated through an alcohol series. Sections were acetylated for 10 min in a solution of 1.35% triethanolamine and 0.25% acetic anhydride in water, and rinsed three times with 2x SSPE containing in mM: 300 NaCl, 20 NaH_2PO_4-H_2O, and 2.5 EDTA (pH 7.4). Each section was then hybridized with a solution (16 µl) containing 50% deionized formamide, 2x SSPE, 1 µg/µl tRNA, 1 µg/µl BSA, 1 µg/µl poly-A in DEPC-treated water, and 1 µl of DIG-labeled riboprobe. Slides were coverslipped, sealed by immersion in mineral oil, and incubated overnight at 65°C. The following day sections were rinsed in chloroform, de-coverslipped in 2x SSPE, and washed by incubating serially for 1 hr at RT in 2x SSPE, 1 hr at 65°C in 2x SSPE containing 50% formamide, and twice in 0.1x SSPE for 30 min at 65°C.

Sections were blocked for 30 min at RT in TNB buffer (100 mM Tris-HCl pH 7.4, 150 mM NaCl, 20 µg/µl bovine serum albumin, 0.3% Triton X-100), and then washed 3 times in TNT (100 mM Tris-HCl pH7.4, 150 mM NaCl, 0.3% Triton-X100). Sections were then incubated for 2 hr in TNB with an alkaline phosphatase conjugated anti-DIG antibody (anti-DIG-AP; 1:300 dil., Roche Applied Science, Mannheim, Germany), washed 3 times for 5 min in TNT, and incubated for 1-3 days in a ready-to-use tris-buffered solution containing the alkaline phosphatase substrates Nitro-Blue Tetrazolium Chloride (NBT; 0.42 g/L) and 5-Bromo-4-Chloro-3-Indolyl-phosphate p-Toluidine Salt (BCIP/NBT; 0.21 g/L; Substrate Solution NEL937, Perkin-Elmer, Waltham, MA). Slides were washed 3 times for 5 min at RT in TNT, rinsed briefly in distilled water to remove salts, and coverslipped with Vectamount AQ (Vector labs; Burlingame, CA).

Results

5-HT increases the spontaneous firing rate of RA neurons in vitro

To investigate a possible serotonergic modulation of the firing properties of RA projection neurons we recorded intrinsic spontaneous activity using the relatively noninvasive (compared with whole cell techniques) single-unit extracellular recording configuration. All cells recorded were RA projection neurons. Bath application of 5-HT (1-100 µM) increased the intrinsic spontaneous firing rate of RA neurons (Fig. 1.2a,b), often by a factor of two ($p<0.0001$, $\Delta FR=5.4Hz$, $n=13$, for 1µM 5-HT) . This effect was reversible to baseline levels in 5-20 minutes upon washout of 5-HT. All units recorded during application of 1 µM 5-HT showed an increase in firing rate (Fig. 1.2b). All *in vitro* experiments were performed in the presence of the ionotropic glutamatergic and GABAergic transmission blockers, kynurenic acid and picrotoxin, respectively. We determined that the half-maximal effect (EC_{50}) for 5-HT was 3.85 µM (Fig. 1.2b), similar to that found in a previous study looking at depolarizing responses of HTR2 receptors in rodent layer V pyramidal neurons in association cortex (Araneda and Andrade, 1991). We therefore focused our experiments on this class of receptors.

HTR2 receptor mRNAs are expressed in RA

A comprehensive search of the zebra finch genome (taeGut3.2.4) revealed three distinct loci encoding the zebra finch orthologs of HTR2 serotonin receptors (*HTR2A*, *HTR2B* and *HTR2C*). Here we use the human gene nomenclature convention (Bruford et al., 2008; uppercase italics for gene and mRNA, uppercase for protein). Based on a comparison of predicted amino acid residues within protein coding domains *HTR2A* showed 90%, 74% and 76% identity at the amino acid level with chicken, mouse and human, respectively, whereas *HTR2B* had 93%, 72% and 75% identity, and *HTR2C* had

97%, 54% and 55% identity. Despite the apparent low conservation among full-length avian and mammalian protein coding regions, the 4 core regions or elements that define a protein fingerprint for all members of the serotonin receptor superfamily (Attwood and Findlay, 1994) showed 92, 86, and 81% identity at the amino acid level with human HTR2A, HTR2B, and HTR2C, respectively. Moreover, most of the elements that identify specific HTR2 receptor subtypes were also recognized by a BLAST-PRINTS search (Wright et al., 1999), including 6/7 elements for HTR2A, 6/8 for HTR2B, and 4/8 for HTR2C. Thus, at the predicted protein level all three members of the HTR2 gene family appear to encode functional serotonin receptors.

We next identified cDNA clones from the Songbird Neurogenomics Initiative consortium database (ESTIMA: Songbird3) corresponding to zebra finch transcripts of *HTR2A* (GenBank Accession code: CK306989), *HTR2B* (FE732340) and *HTR2C* (DV953355). Based on their positions relative to predicted protein coding regions, the clones for *HTR2A* and *HTR2C* represent the 3' untranslated regions, while the clone for *HTR2B* includes both some coding and non-coding 3' regions. To address the sequence specificity of these clones for their respective HTR2s we aligned each of them with the zebra finch genome using UCSC's Zebra Finch Blat Search (http://genome.ucsc.edu/cgi-bin/hgBlat). The results revealed high alignment scores of each clone to their respective single loci, and a lack of significant cross-alignments to other loci, indicating a high degree of sequence specificity for each of these clones.

We next conducted *in situ* hybridizations using riboprobes. Hybridizations with antisense probes yielded expression patterns that were unique for each probe, qualitatively consistent across animals (N=6; Fig.1.1A, B and C), and exclusively cellular (Fig. 1.1A', B', and C'). The signals were particularly strong for *HTR2A* and *HTR2C*.

Within the arcopallium, we observed *HTR2A*-labeled cells almost exclusively contained within nucleus RA (Fig 1.3A; RA boundaries indicated by the arrowheads). Similarly, *HTR2C* labeled many cells in RA, but showed a more widespread distribution throughout the arcopallium than *HTR2A* (Fig. 1.3C). In contrast, for *HTR2B* we found relatively few and weakly-labeled cells within RA, as well as throughout the rest of the brain (Fig. 1.3B). Within RA, both *HTR2A* (Fig. 1.3A') and *HTR2C* (Fig. 1.3B') were highly expressed in a discrete population of relatively large neurons. Given their large soma size it is highly likely that these correspond to projection neurons, as RA projection neurons are 1.5x larger than RA interneurons with little or no overlap in distributions (Spiro et al., 1999). Hybridizations with sense strand riboprobes did not yield detectable signal (inset panels in A', B' and C'), further supporting probe specificity.

HTR2 receptors mediate the increased firing rate of RA projection neurons

Bath application to the slices of the selective HTR2 receptor agonist, (±)-2,5-Dimethoxy-4-iodoamphetamine hydrochloride (DOI) mimicked the increased firing rate following application of 5-HT, with both 1 and 10 μM resulting in approximately a doubling of the firing rate (Fig. 1.4a, p=0.001, ΔFR=3.8 Hz, n=6, for 1 μM DOI). This effect followed a similar timescale to that of 5-HT, but was not reversible in the time periods tested (~30 minutes). DOI occluded the effects of 5-HT: following initial application of 1 μM DOI, the addition of 1-10 μM 5-HT (in conjunction with 1 μM DOI) resulted in no further increase in firing rate (p=0.17, ΔFR=0.17 Hz, n=4). Furthermore, the selective HTR2 receptor antagonist ketanserin blocked the 5-HT mediated increase in firing rate, either when applied after 5-HT (Fig. 1.4b, p=0.3, ΔFR=-1.28 Hz, n=4), or when applied for 3-5 min before 5-HT (Fig. 1.4d, p=0.23, ΔFR=0.35Hz, n=5).

Interestingly, despite the fact that firing rates of RA projection neurons in juveniles during song learning (~35 to 47 days post-hatch) are generally slower than in adults (Adret and Margoliash, 2002), we found that responses to 5-HT and DOI in juveniles were indistinguishable from those in adults (Fig. 1.5, n=10 and n=7 for 5-HT and DOI, respectively). Ketanserin and DOI are quite selective for the HTR2 class of receptors, and are also fairly selective for the HTR2A and 2C receptor types over the 2B. Because this is the first published report of their use on zebra finch serotonergic receptors, and as the mRNA for all three HTR2 receptor types was found in RA, we are uncomfortable pinpointing this effect of 5-HT to a particular receptor of the HTR2 class at this time.

To verify the observed increase in firing rate in RA is specifically associated with an action of 5-HT, we next applied either dopamine (DA) or the D1 dopaminergic agonist SKF 38393 hydrobromide (SKF). Compared to 5-HT, application of 20 μM DA (n=4 cells) to our slice preparation did not influence firing rates of recorded RA neurons, suggesting our observed effects are likely specifically linked to a neuromodulatory action of 5-HT. Interestingly, application of 10 μM SKF did cause a slight increase in firing rate ($p<0.01$, $\Delta FR=1.8Hz$, n=11), indicating the presence of D1 receptors in RA projection neurons, in agreement with recent reports of mRNA signal there (Kubikova et al., 2010). The change in firing rate, however, was not comparable to that evoked by 5-HT nor did it occlude the effects of 5-HT (n=7 cells, data not shown), indicating that the observed effect of 5-HT was not due to a non-specific action of 5-HT on dopaminergic receptors in RA.

In vivo recordings reveal an effect of 5-HT on cell excitability in RA

Application of between ~25 and 500nl of 1 mM 5-HT (via puffer pipette) *in vivo* resulted in consistent increases in firing rates of RA projection neurons ($p<0.0001$, $\Delta FR=28.54$ Hz, $n=8$; Fig. 1.6). As in the *in vitro* preparation, firing rate approximately doubled with 5-HT application, despite the higher baseline spontaneous firing rate of the *in vivo* preparation (12.47 ± 4.18 Hz). The timescale of this effect varied, presumably dependent upon the proximity of the puffer pipette to the neuron being recorded, but was always faster than observed *in vitro*, often occurring in <30 seconds. Consistent with the *in vitro* data, the specific HTR2 agonist DOI mimicked the effect of 5-HT *in vivo* ($p=0.01$, $\Delta FR=19.2$ Hz; $n=5$; Fig. 1.6B). Puffing saline onto neurons had no effect on firing rate (Fig. 1.7C; $p=0.9$, $\Delta FR=-0.03$ Hz; $n=6$

Importantly, when a specific SSRI (~25-500 nl of 10 mM fluvoxamine) was applied we observed a marked increase in firing rate reminiscent of the application of 5-HT ($p=0.02$, $\Delta FR=15.9$ Hz, $n=5$; Fig. 1.7). These results indicate that serotonin reuptake transporters are present, as expected, and suggests 5-HT levels may normally be high within RA. The timescale of the rise in firing rate was slower than that following application of 5-HT or DOI, presumably due to the slower process of building up endogenous 5-HT via decreased re-uptake (Fig. 1.7A).

Ionic mechanisms

Since HTR2's are G-protein coupled receptors known to have downstream effects on HCN and Ca^{2+} channels in other systems (Gerhardt and van Heerikhuizen, 1997; Liu et al., 2003; Cussac et al., 2008)□ we next investigated the possibility that either HCN, L-type Ca^{2+}, or T-type Ca^{2+} channels mediate the observed effect. We utilized the same extracellular recording technique in acute tissue slices as above, in the presence of KA and Ptx to block fast ionotropic neurotransmitter receptors. We used both the selective

HCN channel blocker ZD-7288 and a non-specific blocker of HCN channels, Cs^+, to determine if HCN channels were involved. While both drugs reduced the baseline spontaneous firing rate (Table 1), consistent with the presence of HCN channels in RA projection neurons, neither drug blocked the effects of 5-HT (Fig. 1.8). Of note, Cs^+ application during intracellular recordings confirmed the presence of HCN channels in RA projection neurons (data not shown). Application of specific L-type and T-type Ca^{2+} channel blockers, nifedipine and NNC 55-0396 respectively, failed to alter baseline firing rate or the effects of 5-HT in RA (Figs. 1.9 and 1.10, Table 1.1). These data indicate that HCN, L-type Ca^{2+}, and T-type Ca^{2+} channels are unlikely to mediate the effects of HTR2 receptor-driven increases in firing rate.

Discussion

We describe a novel action of 5-HT: a strong, direct (post-synaptic) excitatory effect on the intrinsic spontaneous neuronal firing rate in song nucleus RA of both adults and juvenile zebra finches. The relatively high 5-HT concentration needed to achieve a half-maximal effect (3.85 μM), along with the fact that this is an excitatory action, predicted that HTR2 receptors mediate this action. The observations that the HTR2 agonist DOI mimics and occludes this effect, and that the HTR2 antagonist ketanserin blocks it, supports this prediction. *In situ* hybridization confirmed the expression of HTR2A, HTR2B, and HTR2C receptor mRNA in large cells in RA, with HTR2B mRNA being expressed at a much lower level than A or C. In an anesthetized *in vivo* preparation, we found that the effects of focally applied 5-HT (or HTR2 agonist) were qualitatively very similar to those observed *in vitro*. Importantly, increasing endogenous 5-HT levels by inhibiting the re-uptake of natively released 5-HT in RA was sufficient to cause a similar increase in excitability (Fig. 1.7), indicating that 5-HT is released locally and highly suggestive that its modulatory action in RA is physiologically relevant.

Although birds lack a laminar cortex, song nucleus RA may be considered analogous, if not homologous, to portions of mammalian motor cortex. Specifically, RA projection neurons may be akin to layer V pyramidal output neurons (Karten, 1969, 1991; Reiner et al., 2004). Consistent with this view, HTR2A receptors are also strongly expressed in layer V pyramidal cells in the mammalian cortex, where they are thought to mediate the effects of many anti-psychotic medications and hallucinogenic drugs (Jakab, 1998; Lambe et al., 2000)□. In particular, HTR2A receptor activation in layer V pyramidal neurons of prefrontal cortex leads to a striking increase in excitatory post-synaptic currents (EPSCs). Here we have reported an increase in electrical excitability, mediated through the same receptors, and present in analogous regions. The source of HTR2A driven increase in EPSCs in mammalian cortex is under some debate (Zhou and Hablitz, 1999; Lambe and Aghajanian, 2001; Marek et al., 2001)□, although a recent study discovered a subset of neurons that fire spontaneously in response to the addition of 5-HT, which would be consistent with the present study (Béïque et al., 2007)□. The function of these receptors under natural conditions is unknown. 5-HT acts at multiple sites throughout the mammalian CNS, and can cause modulation of auditory responses. For example 5-HT plays a role in modulating auditory responses in the inferior colliculus during different social contexts (Hurley and Hall, 2010). Because the avian song control circuit shows similar sorts of modulation, it is possible that 5-HT also participates in neural modulation at multiple sites.

Given that 100% of the neurons we recorded in RA responded to 5-HT, endogenous 5-HT is present in RA (Barclay and Harding, 1988; Fig. 1.7), and mRNAs encoding HTR2 receptors, particularly HTR2A/2C, are abundant, serotonergic mechanisms appear highly suited for modulating vocal-motor activity within zebra finch nucleus RA. The observed change in spontaneous firing rate is likely intrinsic to RA projection neurons (post-synaptic), as the presence of KA and Ptx do not appear to change the results qualitatively (*in vitro vs. in vivo* expts.). We hypothesize that 5-HT acts here as a permissive signal, perhaps even as a sort of attention-related modulator, exciting neurons (and the entire RA circuitry) to a heightened state. This increase in excitability and spontaneous firing would appear to add noise to the system, not allowing for more precise coding and vocal output but rather might be a mechanism necessary for sensorimotor learning, perhaps allowing neurons to attain a more plastic state. This view suggests a number of testable hypotheses, such as a) 5-HT is necessary for sensorimotor song learning and b) 5-HT levels should be higher during song learning.

In adulthood, norepinephrine (NE) decreases and often ceases spontaneous activity in RA projection neurons (Solis and Perkel, 2006), leading to an increase in the signal to noise ratio of activity propagated along the DMP. Acetylcholine content in RA increases during the critical period (Sakaguchi and Saito, 1991; Sakaguchi, 1995) and has been reported to be involved in LTP of field potentials within RA (Salgado-Commissariat et al., 2004). Additionally, the presence of mRNA encoding D1 dopamine receptors has recently been demonstrated in RA (Kubikova et al., 2010), a finding that we extend here by showing a functional effect. The fact that 5-HT causes such a marked increase in the firing rate of RA neurons, with opposite sign from the effects of NE, suggests neuromodulators may fine-tune the properties of RA to function optimally under a variety of behavioral situations. This would include the production of undirected vs. directed singing, conditions that are known to be associated with the differential activation of catecholaminergic systems. It will be intriguing to investigate whether serotonergic activity is also differentially associated with different singing modes.

The HTR2 class of receptors comprises three distinct receptor subtypes (A, B, and C), which based on comparative genomic data are highly conserved among humans, rodents, chicken, and zebra finch. The HTR2A and HTR2C receptors are much more similar to each other than to HTR2B, and the specific agonist (DOI) and antagonist (ketanserin) that we used here, although not separating between 2A and 2C subtypes, are generally considered sufficiently selective to rule out an HTR2B receptor action. Consistent with this view our *in situ* hybridization data indicate that mRNAs encoding HTR2A and HTR2C are highly expressed within neurons in RA. In contrast, HTR2B mRNA is expressed at low levels and possibly present in relatively few RA neurons. It thus seems unlikely that the HTR2B receptor exerts major actions within RA. Development of more specific ligands and further studies focusing on other neuron and receptor types, as well as synaptic strength and circuit properties, will be critical to a more full understanding of the effects of 5-HT on song production. The basic anatomy of the serotonergic system is conserved across a wide variety of vertebrates, including mammalian and avian lineages. While a projection from the raphe to RA has not been shown directly in the zebra finch, the high homology of this system across vertebrates makes it highly likely the raphe nuclei are the source of 5-HT in RA.

Understanding how 5-HT modulates neural circuits and affects behavior is crucial from a healthcare perspective, but the complexity of both the serotonergic system and the behaviors it modulates has made progress difficult. We have identified a clear effect of 5-HT in a population of neurons necessary for song production - an increase in excitability of RA projection neurons via HTR2 receptors. Intriguingly, these same receptors are known to modulate associative learning in a number of rodent systems and also to underlie the actions of many hallucinogenic drugs in analogous brain regions. Deciphering how 5-HT modulates song production and learning in the zebra finch will inform our understanding of how 5-HT modulates behavior in humans.

Figures

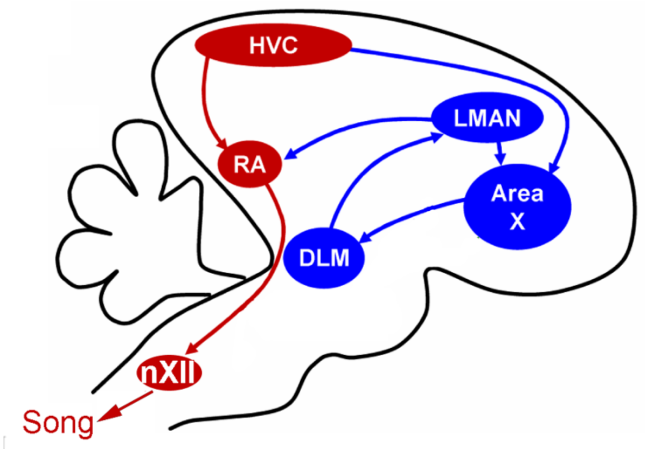

Figure 1.1. Simplified schematic view of the song-control system. The Direct Motor Pathway is in red and the Anterior Forebrain Pathway is in blue.

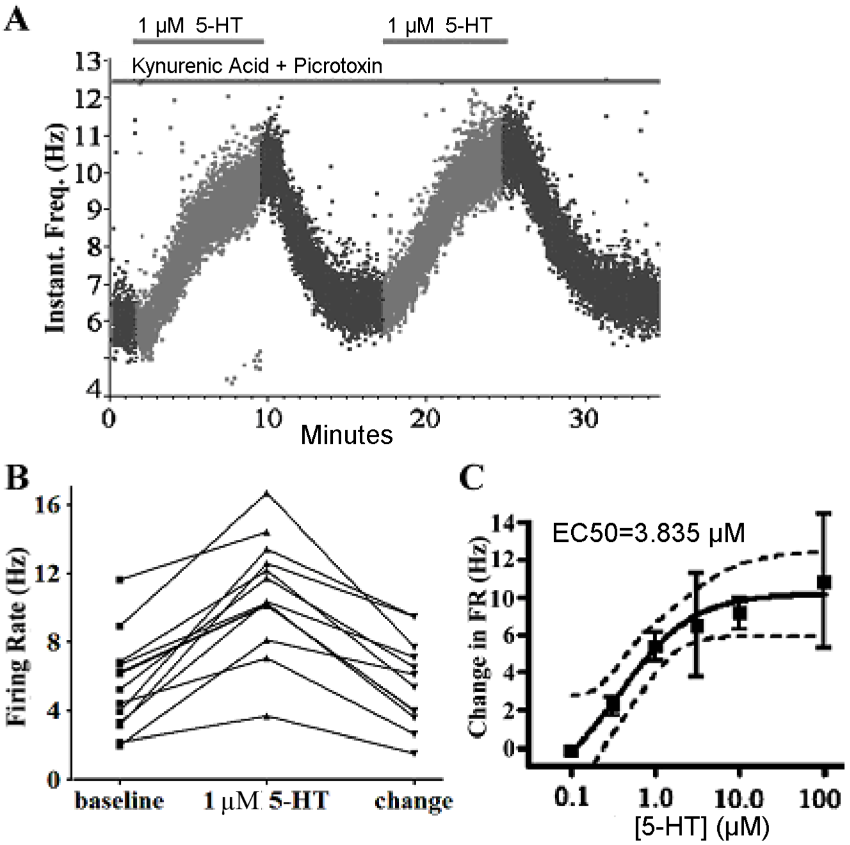

Figure 1.2. 5-HT causes an increase in the spontaneous firing rate of RA projection neurons. A. Plot of instantaneous firing rate vs. time for one example neuron. B. 5-HT increased spontaneous activity in every RA projection neuron tested. C) A dose-response curve indicates that half maximal effects occur with approximately 3.8 μM 5-HT. All experiments were done in the presence of KA and Ptx.

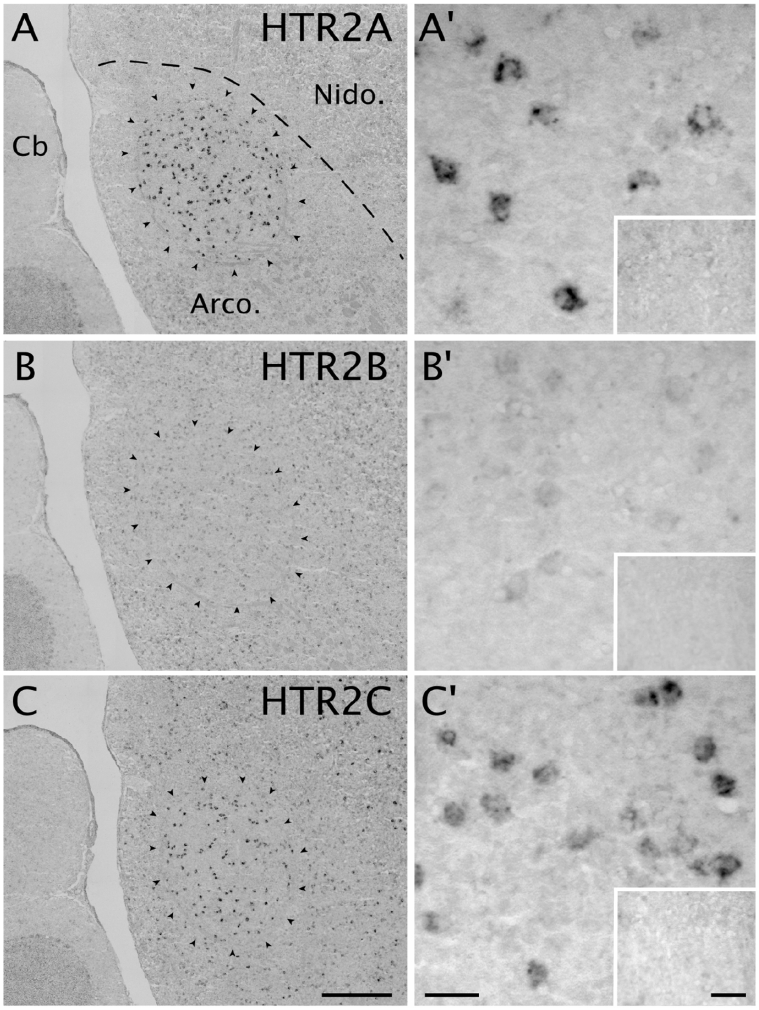

Figure 1.3. *HTR2A, HTR2B* and *HTR2C* expression in the arcopallium of an adult male zebra finch. A-C. High resolution photo-montaged images of *HTR2A* (A), *HTR2B* (B) and *HTR2C* (C) *in situ* hybridization data in parasagittal brain sections ~1.8 mm from the midline. The location of the robust nucleus of the arcopallium (RA) is indicated in each panel by arrowheads. A'-C'. High magnification views of *HTR2A* (A'), HTR2B (B'), and *HTR2C* (C') labeled cells in RA. Based on soma size these likely correspond to RA projecting neurons. Inset panels show high magnification views of sense strand hybridizations; no labeling was detected, supporting demonstrating probe specificity. Scalebar: A-C = 250µm; A'-C' = 25µm. Anatomical abbreviations: Arco., arcopallium; Nido., nidopallium; Cb, cerebellum.

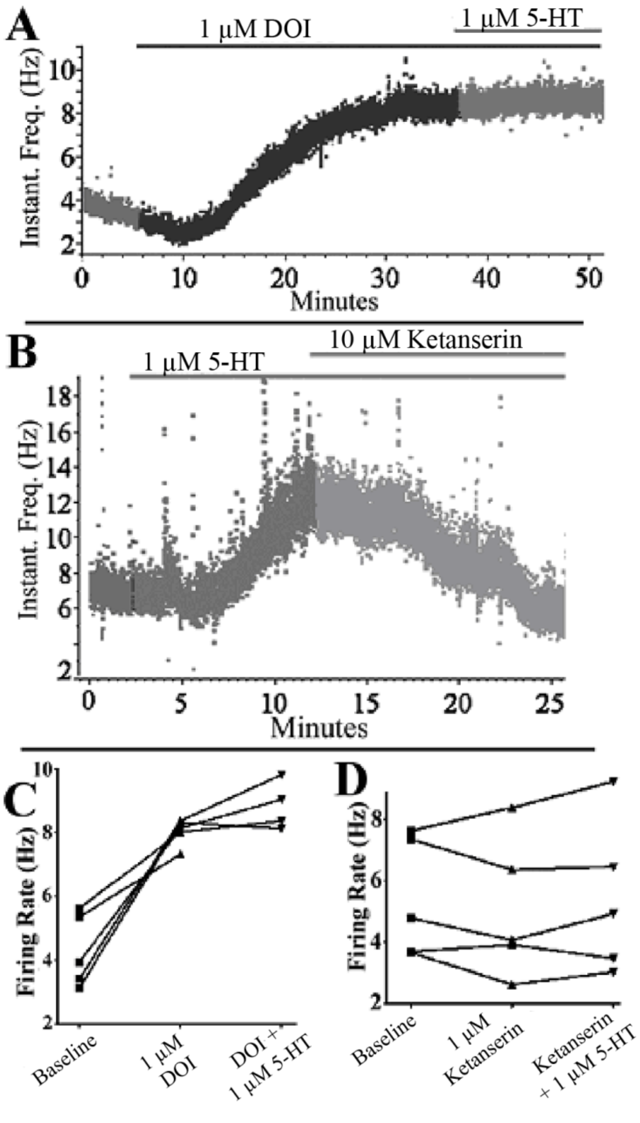

Figure 1.4. 5-HT2 receptors mediate the increase in spontaneous firing rate. A. The 5-HT2A/C specific agonist DOI both mimics and occludes the effect of 5-HT. B. The 5-HT2A/C specific antagonist ketanserin blocks the effect of 5-HT. Ketanserin can be applied either before 5-HT (not shown), or after (shown here). C. Group data of firing rate before and after DOI. D. Group data showing that ketanserin, when applied before 5-HT, blocks the increase in firing rate. All experiments were done in the presence of KA and Ptx.

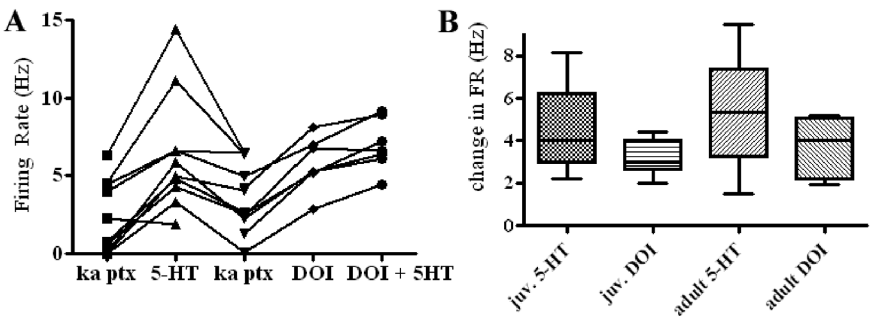

Figure 1.5. HTR2 receptors are present and active in juvenile zebra finches (day 38-54), just as in adults. A. 5-HT causes an increase in firing rate of RA projection neurons in juvenile zebra finches, which is mimicked and occluded by the HTR2 specific agonist, DOI. B. The change in firing rate induced by 5-HT and/or DOI is indistinguishable between juveniles and adults. All experiments were done in the presence of KA and Ptx.

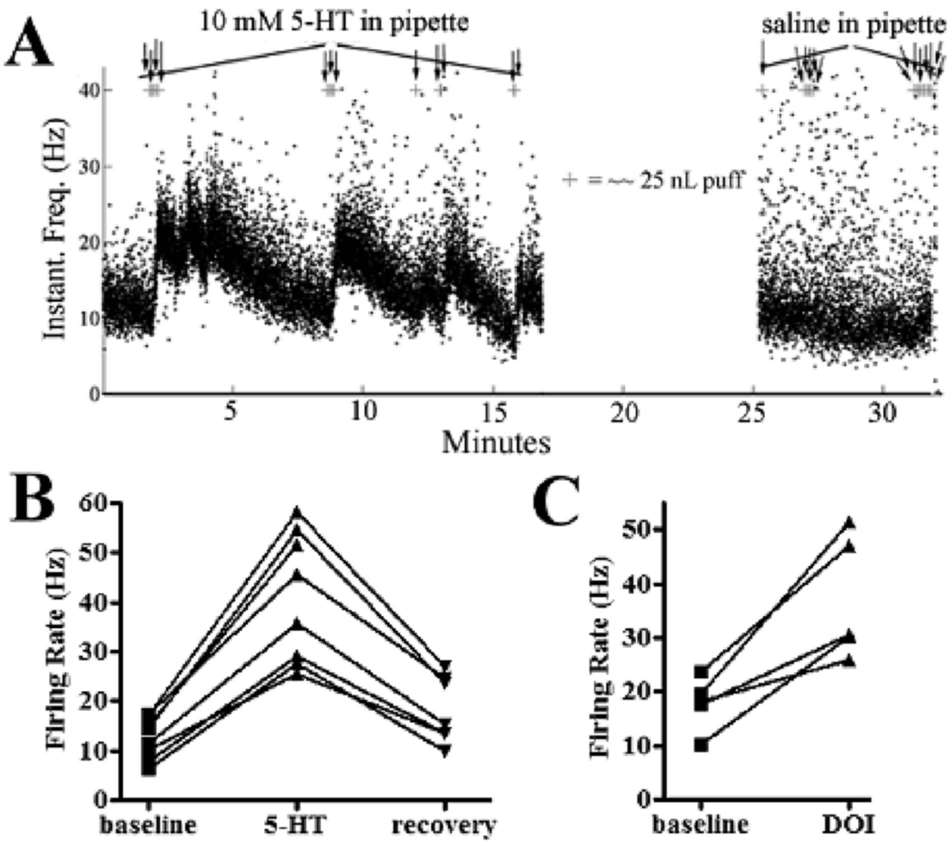

Figure 1.6. 5-HT, via HTR2 receptors, also causes an increase in spontaneous activity *in vivo*. A. Plot of instantaneous firing rate (IFR) vs. time for one example neuron. IFR plot subsequent to the break indicates that following several applications of 5-HT, the injection pipette was removed and replaced with one containing saline. B. In all neurons recorded *in vivo*, 5-HT caused a rapid and drastic increase in firing. C. The specific HTR2 agonist mimicked the effect of 5-HT.

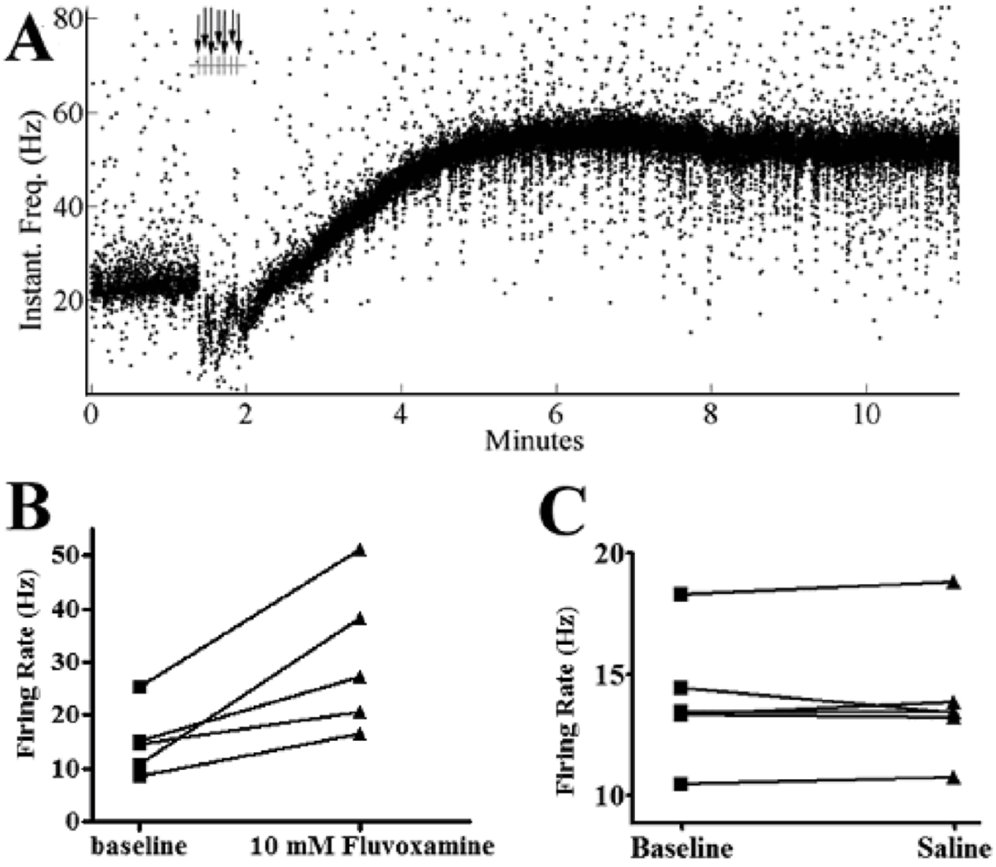

Figure 1.7. Endogenous 5-HT is sufficient to increase the firing of RA projection neurons *in vivo*. A. Plot of instantaneous firing rate vs. time for one example neuron. Fluvoxamine was puffed on at the arrows. There is a fast movement artifact resulting from the force of the puff followed by a slow rise in firing rate. The movement artifact did not occur consistently in other traces. B. Group data showing firing rate before and after fluvoxamine application. C. Puffing saline onto neurons had no effect on firing rate.

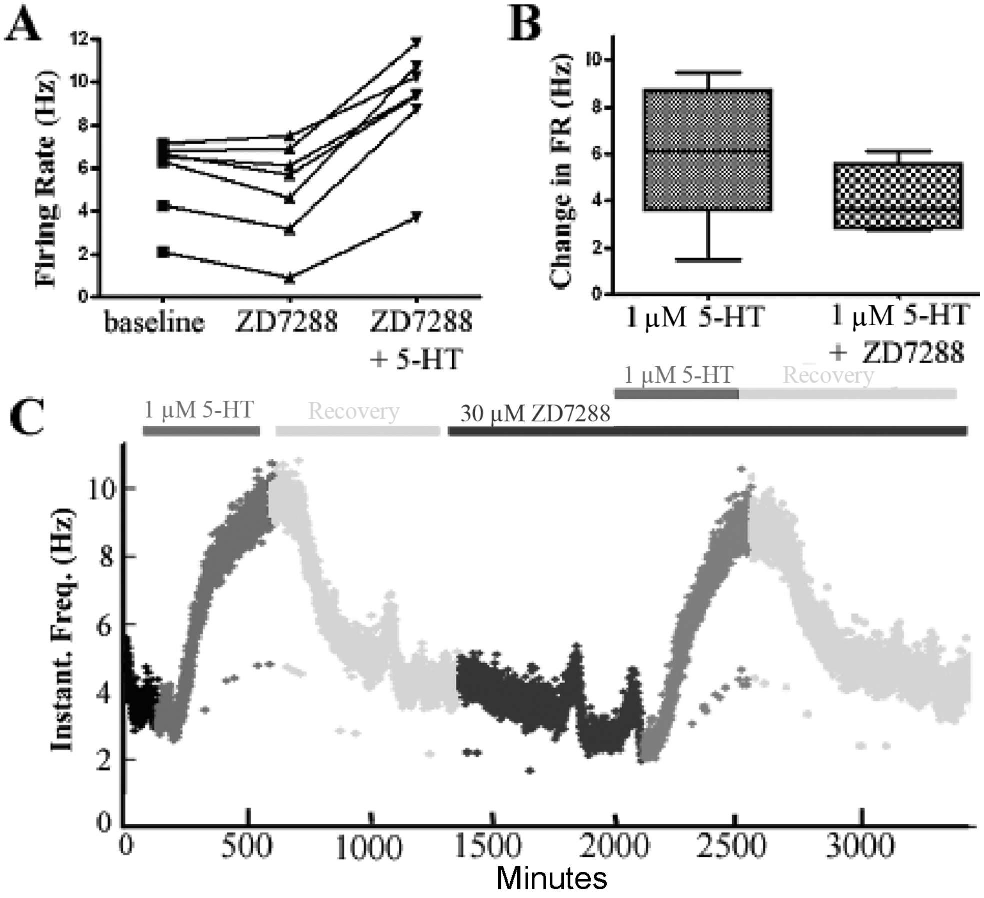

Figure 1.8. The specific HCN channel blocker ZD7288 did not inhibit the effects of 5-HT on RA projection neurons. A. While ZD7288 did cause a significant decrease in firing rate (p=0.0452) indicating that HCN channels are present on RA projection neurons, it did not block the effect of 5-HT. B. The change in firing rate elicited by 5-HT was not significantly different from that in the presence of ZD7288. C. Plot of firing rate v. time through an example experiment from one neuron.

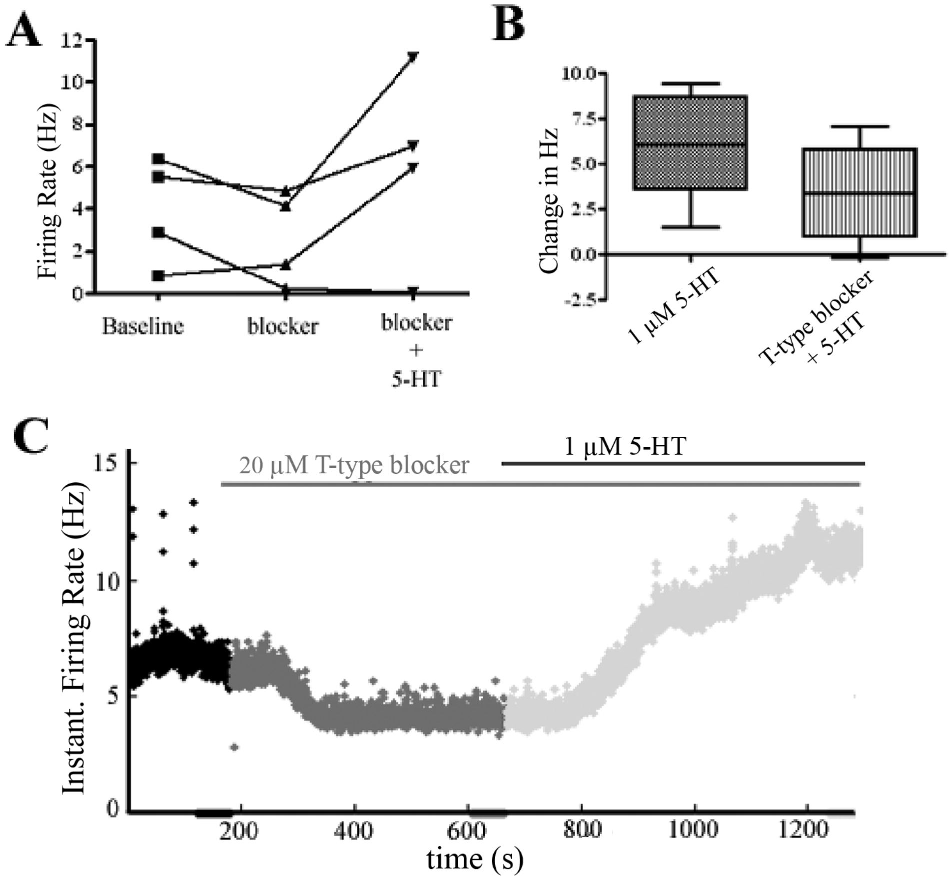

Figure 1.9. The specific L-type Ca^{2+} channel blocker nifedipine did not inhibit the effects of 5-HT on RA projection neurons. A. Firing rate at baseline (KA + Ptx), after application of 10-20 µM nifedipine, nifedipine + 1µM 5-HT, and recovery. B. The change in firing rate elicited by 5-HT was not significantly different from that in the presence of nifedipine. C. Plot of firing rate vs. time through an example experiment from one neuron.

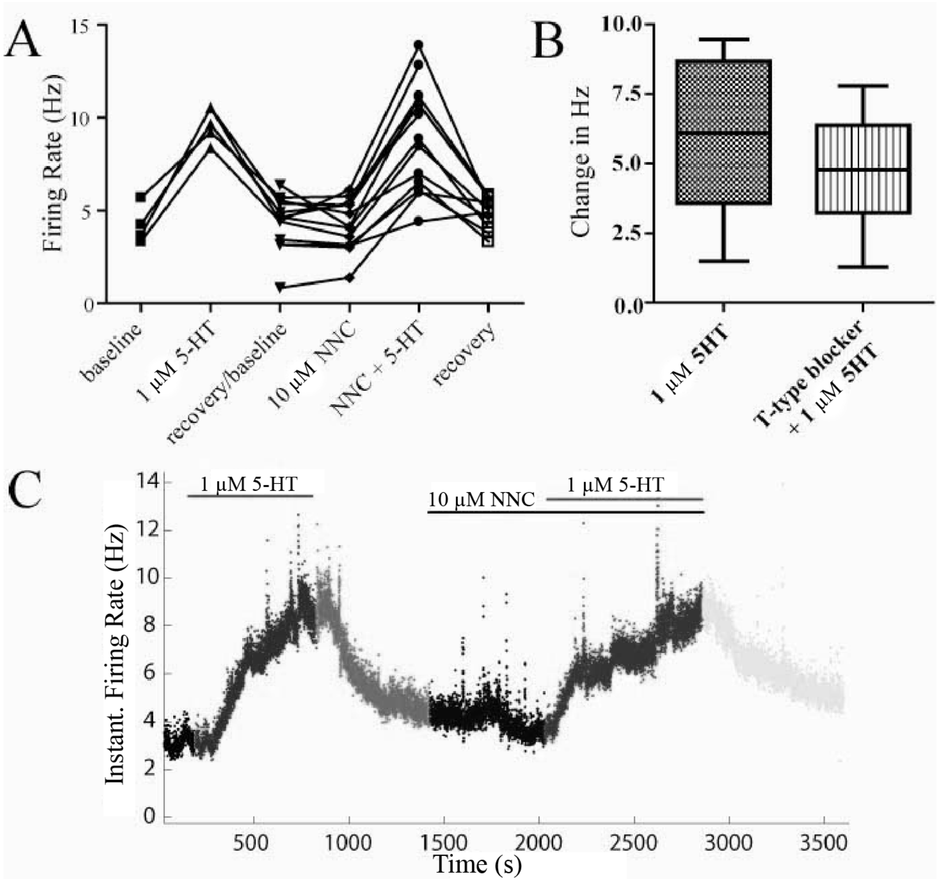

Figure 1.10. The specific T-type Ca^{2+} channel blocker NNC 55-0396 did not inhibit the effects of 5-HT on RA projection neurons. A. NNC 55-0396 did not cause a significant decrease in baseline firing rate (p=0.688), nor did it block the effect of 5-HT (p=0.2346). B. The change in firing rate elicited by 5-HT was not significantly different from that in the presence of NNC 55-0396. C. Plot of firing rate v. time through an example experiment from one neuron.

drug	channel	change from baseline	p value	change in 5-HT induced increase	p value
ZD-7288	HCN	-0.69 Hz., stdv = 0.7346	**0.0452**	-1.723 Hz., SEM = 1.095	0.1313
Cesium	HCN	-1.17 Hz., stdv = 1.081	0.0738	2.988 Hz., SEM = 1.466	0.0565
Nifedipine	L-type Ca^{2+}	-0.153 Hz., stdv = 1.223	0.7718	-0.369 Hz., SEM = 1.282	0.7761
NNC 55-0396	T-type Ca^{2+}	-0.286 Hz, stdv = 1.1193	0.688	-1.109 Hz., SEM = 0.9117	0.2346

Table 1.1: The effects of blockers of HCN, L-type Ca^{2+}, and T-type Ca^{2+} channel blockers on firing rates of RA projection neurons.

Chapter 2: HTR2 receptors in a songbird premotor cortical-like area modulate spectral characteristics of zebra finch song

After initial review at the Journal of Neuroscience, this chapter has been judged potentially suitable for publication. I have made changes suggested by two anonymous reviewers and is currently under secondary review, and is reproduced in that form.

I did all surgeries and song recordings for this paper, and was involved in the making of all figures. Tom Roseberry contributed to the analysis of fundamental frequency (FF).

Title:

HTR2 receptors in a songbird premotor cortical-like area modulate spectral characteristics of zebra finch song

Abbreviated Title:

Serotonergic Modulation of Song

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Figures: 6 Tables: 1

Words-
Abstract 226
Introduction 447
Discussion 1443

So supplemental, No conflicts

Acknowledgments: Gregg D. Stanwood, John F. Neumaier

Abstract

Serotonin (5-HT) is involved in modulating an array of complex behaviors including learning, depression, and circadian rhythms. Additionally, HTR2 receptors on layer V pyramidal neurons are thought to mediate the actions of psychedelic drugs; the native function of these receptors at this site, however, remains unknown. Previously, we found that activation of HTR2 receptors in the zebra finch forebrain song premotor structure the robust nucleus of the arcopallium (RA) led to increased excitation, and that endogenous 5-HT could roughly double spontaneous firing rate. Here, using *in vivo* single unit recordings we found that direct application of 5-HT to these same RA projection neurons (analogous to layer V cortical pyramidal neurons) caused a significant increase in the number of action potentials per song-related burst, and a dramatic decrease in signal-to-noise ratio. Injection of serotonergic neurotoxin 5,7-DHT into the third ventricle greatly reduced telencephalic 5-HT and resulted in decreased fundamental frequency (FF) of harmonic syllables as well as increased goodness of pitch. Both of these results can be explained by the observed actions of 5-HT on RA projection neurons, and both effects recovered to baseline within two weeks following the toxin injection. These results show that 5-HT is involved in modulating spectral properties of song, likely via effects on RA projection neurons, but that adult zebra finches are able to compensate for this deficit after approximately 10-14 days.

Introduction

The biogenic amine serotonin (5-HT) is involved in modulating an array of complex behaviors, including learning, anxiety, and mood (Dubovsky and Thomas, 1995; Monti, 2010). 5-HT deficiencies have been closely linked to mental illnesses such as anorexia, obsessive-compulsive disorders, and clinical depression (Ordway et al., 2002; Schatzberg et al., 2002). Understanding the precise loci and mechanisms of action of the serotonergic system- from synapse to behavior- has been difficult due to the complexity of the behaviors and of the system itself. One attractive focus, however, is the action of HTR2 receptors on layer V pyramidal neurons, where activation by specific agonists (such as LSD, lysergic acid diethylamide) leads to profound perceptual and behavioral changes (Jakab, 1998; Nichols, 2004; Béïque et al., 2007).

Songbirds and mammals share an evolutionarily conserved overall brain architecture, with the major exception being that avians lack an obviously layered cortex, instead having a pallium with analogous cortical-like areas in distinct regions (Reiner et al., 2004). The song-control system is a set of discrete forebrain nuclei that converge on the robust nucleus of the arcopallium (RA, Fig. 2.1), which projects directly to the hypoglossal nucleus (nXII) and to brainstem respiratory control nuclei. Projection neurons in RA are hypothesized to be analogous to layer V pyramidal neurons in premotor cortex (Karten, 1969, 1991)□, and indeed can be similarly excited by 5-HT via HTR2 receptor activation (Wood et al., 2011)□. In addition to being anatomically accessible, network dynamics within RA are believed to determine spectral characteristics of song, a complex but easily quantifiable learned behavior.

Here, we use *in vivo* single unit recordings from RA projection neurons, coupled with playback of bird's own song (BOS) and focal application of 5-HT and HTR2 specific agonists into RA to reveal how HTR2 receptor activity alters song-related firing in these neurons. We then reduced telencephalic 5-HT by injecting 5,7-dihydroxytryptamine (5,7-DHT) into the 3rd ventricle and recorded song production continuously for > 2 weeks. Acoustic analysis of song syllables revealed specific changes in song production, including decreased fundamental frequency (FF) of harmonic syllables as well as increased “goodness of pitch” (Tchernichovski et al., 2000), a measure of the presence of harmonics. Both of these results can be explained by direct actions of 5-HT on RA projection neurons, which we observed electrophysiologically, and both effects recovered to baseline within 2 weeks following surgery. These results show that 5-HT is involved in modulating spectral properties of song, likely via effects on RA projection neurons, but that adult zebra finches are able to compensate for this deficit after approximately 10-14 days. We thus demonstrate how 5-HT can act at HTR2 receptors on cortical-like premotor projection neurons to finely modify a complex learned behavior.

Materials and Methods

Animals

Adult male zebra finches were obtained from a commercial supplier or reared in our colony. Animals were housed in groups of 4-10 males, on a 13:10 hr light:dark cycle, with food and water available *ad libitum*. All procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Electrophysiology- auditory presentations

Animals were placed in a sound attenuation chamber one - two days before experiments began and BOS was recorded with Sound Analysis Pro software (SAP, Tchernichovski et al., 2000). Songs were sorted from other cage noises by hand, bandpass filtered (0.1-10 kHz) and digitized at 22 kHz using GoldWave Audio Editor (GoldWave). The auditory stimuli for electrophysiology experiments were BOS, BOS played in reverse, conspecific song (the song of another zebra finch), and bursts of noise. The BOS stimulus was the first two motifs from a bout of singing (a motif is a stereotyped sequence of acoustic elements that is repeated during a song bout). Introductory notes preceding the first motif were not included. The duration of BOS averaged ~1.5s. Conspecific song was chosen to match approximately the duration of BOS and hence varied for each experiment. Noise stimuli were generated from a normal distribution and had the same duration, sampling frequency, and bandpass filter setting as the BOS stimulus. Unmodulated noise had 20 ms linear onset and offset ramps and the same total power as BOS. Auditory stimuli were presented from a speaker 35 cm in front of the bird at a peak sound level of 68–72 dB SPL and randomly interleaved with variable inter-trial intervals of 10–15 s. Songs were triggered using Spike2 software (Cambridge Electronic Design).

Electrophysiology- surgery and recordings

Each animal was given three intramuscular (IM) injections totaling 5-6.6 ml/kg of 20% urethane over 1 hour. Local anesthetic (1% lidocaine) was injected under the scalp before the animal was placed in a stereotaxic apparatus with the beak at an angle of 64^o downward from the horizontal. Small craniotomies were made above the midline reference point, the bifurcation of the midsagittal sinus, and above RA. Lidocaine gel was then applied to the incision at ~ 1 hour intervals, or as necessary. For auditory playback experiments, ear bars were removed after cementing the skull to a metal post and fixing the head via the post.

Glass pipettes (TW100F-3, World Precision Instruments) were pulled using a micropipette puller (Model P97, Sutter Instrument Co.), the tips were blunted to impedances of 10–25 MΩ, and saline was used as the pipette recording solution. A ground electrode was placed in the cerebellum posterior to the midline reference point. The recording electrode signal was amplified 10× and low pass filtered at 3 kHz (Axoclamp 2B amplifier, Molecular Devices), amplified further by 100× and high pass filtered at 300 Hz (Model 440, Brownlee Precision). The signal was digitized at 20 kHz and spike times and raw traces were stored for further analysis (Spike2, Cambridge Electronic Design). Principal components analysis of the spike shapes allowed clear separation from noise, although in practice this was mostly unnecessary as we find that with these techniques units in RA generally have a signal to noise ratio > 3:1. All extracted units obeyed a refractory period of 1 ms., and spike waveforms were automatically overlaid following recording to ensure there were no changes in spikewaveform, and we are confident that recordings were not contaminated by the recruitment of additional neurons. Following successful recordings, 10 kDa dextran amine conjugated to Alexa 488 (Invitrogen) was iontophoretically injected at recording sites for post-hoc histological verification of their locations.

All drugs were diluted in a 0.9% saline solution and pressure ejected from glass pipettes (10–20 µm tip diameter) using a Pressure System IIe (Toohey; 50 ms pulses at 10 psi). Injected volumes were 20–100 nl. We aimed to place the tip of the injection pipette 200–300 µm from the tip of the recording pipette.

Electrophysiology- histology

At the end of each experiment, birds were given a lethal dose of sodium pentobarbital via intramuscular injection and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose dissolved in 0.1 M phosphate buffer (PB) and cut on a freezing microtome into 50 µm sections.

Electrophysiology- Analysis

Spike times were determined with Spike2 and exported to Matlab (MathWorks) using a custom implementation of the SON library. Firing rates were thenanalyzed in the Matlab environment as follows. For each cell, we calculated spontaneous firing rate, inter-spike interval (ISI) distribution, and peri-stimulus time histogram (PSTH) of the response to auditory playback. The average firing rate during song playback was calculated, and spontaneous firing rate was calculated during a window of the same duration as the song playback preceding the playback. We subtracted the firing rate prior to song playback from that during playback for each trial, and averaged that value across trials to give a mean song response strength (RS, Solis and Doupe, 1997). Song-response strength measurements were used to calculate the discriminability statistic *d'*, which is used to quantify the selectivity of a neuron for a given stimulus over another (Solis and Doupe, 1997), where the difference between the average song response strength (RS) to two songs was normalized by the square root of the average of the variances of the song response strength (σ) measurements for the two songs. Song evoked bursts were defined as 2 or more spikes firing above 125 Hz during auditory presentations. We chose 125 Hz based on a valley in our firing rate histogram, and because it agreed with a previously published criterion (Leonardo and Fee, 2005)□.

5-HT Lesions

Brain 5-HT levels were drastically reduced by injecting 500 nL of 5,7-dihydroxytryptamine creatinine sulfate salt (5,7-DHT) into the third ventricle (coordinates: 0 lateral, -1 rostral, 3.75 deep, 40^{o} head angle). While 5,7-DHT is a neurotoxin mostly selective for serotonergic terminals (Neumaier et al., 1996)□, dopaminergic neurotoxicity was blocked by pretreatment with .1 mL desipramine (2 mg/mL) given by IM injection. Two weeks after injection of either 5,7-DHT or saline, animals were sacrificed by decapitation during isoflurane anesthesia between 9 AM and 12 PM. Brains were sliced at 300 μM using a vibrating microtome (Oxford) and RA, adjacent arcopallium, HVC, adjacent nidopallium, X, striatum, cerebellum, and brain stem were dissected under light microscopy with micro-dissecting scissors and stored at -80^{o}. Lesion effectiveness and specificity were verified by high pressure liquid chromatography (HPLC, Vanderbilt University core facility) for a variety of monoamines in these tissue punches. Monoamine levels were express as ng/mg of control protein, calculated via the BCA assay (bicinchoninic acid assay). Brains were sent to be analyzed in a number of batches, and not all regions were analyzed in all batches (marked by N.A. “not analyzed” in table 1). While results were generally very consistent, some assays returned obvious outliers and numbers more than 4 standard deviations outside the average were excluded (light grey in table 1).

In two instances 5,7-DHT lesioned animals had to be excluded from song analyses: 1 animal was found to sing extremely rarely, before and after surgery, and was unusable for song analysis. In another instance, SAP failed to record song after surgery due to the hard drive being full (the program continued running and appeared to be functioning normally but no files were created). These two animals thus have HPLC data but no song analysis data. Two control animals underwent a more complicated procedure in which they initially had a control (saline injection) surgery followed two weeks later by an attempted injection of 5,7-DHT. This 5,7-DHT was over 1.5 years old, had experienced multiple freeze-thaw cycles, and had turned a dark yellowish-brown. HPLC analysis revealed no deficit in 5-HT levels following these injections. These animals were therefore used only for analyzing song before and after the saline injection (as controls) and their monoamine levels were excluded from all analyses (although they were within control levels): to be clear- no data was used from these animals following attempted 5,7-DHT injection as it was clear in hindsight the 5,7-DHT had gone bad.

Lesion- Song Analysis

Birds were individually housed in sound attenuation chambers (Acoustic Systems) at least seven days before and 14 days after 5-HT lesion surgery. We continually recorded spontaneous (undirected) vocalizations using Sound Analysis Pro (SAP) software. Songs were sorted and analyzed using custom Matlab software and SAP. Zebra finch songs are highly stereotyped, making them especially well-suited for in-depth analysis. The acoustic structure of song is arranged in a hierarchy, with 25-250 ms vocal units known as syllables strung together in a stereotyped sequence called a motif. Each song consists of one or several motifs, preceeded by introductory notes and separated from each other by <100 ms of silence. We used an updated version of a program (Leblois et al., 2010)□ to sort individual motifs from calls and cage noise. Briefly, after presorting with SAP, the program detected putative motifs based on peaks in the cross-correlation between the spectrogram of the song and that of a clean preselected motif. Such putative motifs were then sorted based on their spectral similarity with the preselected clean motif, using thresholds set by the experimenter. For motifs for which such analysis did not allow unambiguous distinction, an additional PCA analysis on the spectrograms of putative motifs allowed us to sort motifs from other sounds. This analysis allowed us to successfully sort >90% of the motifs sung by a bird on a given day (assessed by comparing hand sorting with the automated sorting by the program). Once clean motifs had been sorted, individual syllables were sorted using custom Matlab software somewhat similar to previously reported methods (Wu et al., 2008). Briefly, we ran clean motifs (following the above sorting) through the SAP batch processing module to separate individual syllables and calculate spectral and temporal features. Amplitude threshold was used to determine proper syllable segmentation, and was determined empirically for each individual animal. These data were imported into Matlab and sorted by a semi-automated procedure in which the user selects spectral and temporal feature values for each syllable, as well as the order in which they occur. In summary: songs were pre-sorted in SAP, motifs were cut into individual .wav files with Matlab software, which were then fed back into SAP where syllables were identified and spectral features were calculated. Spectral data was fed back into Matlab for sorting of syllable types, and final analysis was performed in Matlab, Excel, and Prism Software programs. While tedious, these methods allowed us to sort and analyze the overwhelming majority of syllables produced daily by each animal over the course of the experiment. When calculating changes over time, each syllable was averaged within a type, and the values per syllable were then averaged to arrive at one value per animal. While tedious, these methods allowed us to sort and analyze the overwhelming majority of syllables produced daily by each animal over the course of the experiment. When calculating changes over time, each syllable was averaged within a type, and the values per syllable were then averaged to arrive at one value per animal.

Calculation of Fundamental Frequency

A 23-ms spectrally stable portion of the syllable waveform was windowed from onset of harmonic syllables. The power spectrum of the syllable waveform was calculated with frequency bins of 86 Hz. The fundamental frequency of the syllable was calculated from the frequency bin of greatest spectral power using a weighted average from the four neighboring bins and the inter-harmonic frequency.

Definitions of Baseline, Lesion, Recovery

Baseline for all birds was established using the average of three stable days in the one to five days before surgery. After surgery, birds often sing little or not at all for a few days, and thus we excluded days following surgery when birds sang less than 30% of the pre-surgery average number of songs (generally 2-5 days were excluded). Lesion was defined as the first 3 days following resumption of singing. Recovery was defined as 7-10 days following resumption of singing, unless explicitly stated otherwise.

Drug Sources

All drugs were purchased from Sigma-Aldrich: 5,7-Dihydroxytryptamine creatinine sulfate salt (5,7-DHT); Desipramine hydrochloride (desipramine), and Serotonin creatinine sulfate monohydrate (5-HT).

Results

Serotonin alters song-related electrical activity in premotor nucleus RA

Serotonin application increased spontaneous firing rate of all RA projection neurons *in vivo*, as previously reported (Wood et al., 2011)□. To determine if this change might be of functional significance to motor output (song), we stimulated RA projection neurons using auditory playback of the bird's own song (BOS). We made extracellular single-unit recordings from RA projection neurons in urethane anesthetized animals, in response to BOS playback elicited bursts of firing time-locked to particular syllables and quantitatively similar to those reported during song production in awake animals (Fig. 2.2; Dave, 1998; Hahnloser et al., 2006)□. All neurons showed selectivity for BOS over conspecific song (CON, mean d'=2.97), white noise (d'=3.34), or BOS played backwards (REV, d'=1.91, Fig. 2.3A).

Pressure application of ~5-50 nL of 1 mM 5-HT near the recording site caused a significant increase in the number of spikes per BOS-evoked burst (13.9% increase, Friedman test, p=0.0239, Δ =0.39 spikes per burst, n=5 neurons in 4 animals, Friedman statistic =7.6, Fig. 2.3B), an increase in the length of BOS-evoked bursts (19.4% increase, paired t-test: p=0.0407, Δ =1.483 ms, n=5 neurons in 4 animals, t=2.982, df=4,, Fig. 2.3C). As we previously reported, spontaneous activity (non BOS-evoked) increased dramatically (217% increase, paired t-test: p<0.0001, Δ = 26.47 Hz, n=9, t=7.240, df=8, Wood et al., 2011)□. Spontaneous activity thus shows the most increase by far.

Signal-to-noise ratio can be calculated as the ratio of song-related spikes (bursts) to the number of spikes not related to song activity (spontaneous activity). Spontaneous activity, which nearly doubled, experienced a greater increase in firing rate than song-related bursts, which increased by only about 1 spike per burst (Figs. 2.2, 2.3B) resulting in a robust decrease in signal to-noise ratio (Fig. 2.3D, p=0.0041, Δ =-1.64, n=5 neurons in 4 birds, t=5.925, df=4). There was no similar change in the RS to either REV or CON (paired t-test: p=.72 and .41, respectively).

Since activity in RA is widely held to shape spectral properties of song these results make two predictions for how 5-HT affects adult song spectral characteristics. First, as 5-HT decreases the signal-to-noise ratio of song-related activity in RA, 5-HT may make song noisier and less precise. Second, as 5-HT increases the number of spikes per burst, and since these bursts lead to activity in the syrinx, 5-HT can be predicted to cause increased contractions in the syrinx, resulting in, on average, higher frequency songs. Removing 5-HT from RA would thus be expected to a) lower the frequency of songs and b) decrease syllabic entropy and increase the goodness of pitch. To test these hypotheses we lesioned the 5-HT system in adult male zebra finches.

Monoamine levels following serotonin lesions

Lesioned animals had significantly lower 5-HT levels in all brain regions tested (for RA: 9.537±0.75 ng n=5 vs. 3.2±1.229 ng n=7 following 5,7-DHT injections; unpaired t-test: p=0.0009, t=4.657, df=10 Table 1), while norepinephrine and dopamine levels were unaffected (p=0.25 and 0.65 for RA, respectively, data not shown). All brain regions tested had similar reductions in 5-HT (Table 1), but here we focus on levels in RA.

Spectral Song Analysis

All animals sang relatively normal songs following lesion/sham surgery (Fig. 2.4A), allowing us to compare syllables before and after treatment.

We measured the frequency of song syllables using SAP's “mean frequency” measure, which quantifies the central tendency of syllable frequency. As predicted by *in vivo* electrophysiological data, syllable frequency was consistently and significantly reduced following 5-HT lesions (Fig. 2.4B). This significant reduction was observed immediately after surgery (days 1-3, unpaired t-test: p=0.0126, Δ -189.7 ± 62.04 Hz., t=3.328, df=7) and remained significant two weeks later (Fig. 2.4B, days 13-15, unpaired t-test: p=0.0026, Δ -178.7 ± 29.32 Hz., t=4.301, df=8). Furthermore, the change in mean frequency two weeks post-surgery (when 5-HT levels were measured) was correlated with 5-HT levels in RA (Fig. 2.4C, p=0.0082, R^2=0.6032, F=12.16). One data point appeared to be an outlier, although it is consistent with the trend established without it (p=0.0646, R^2=0.4068, F=4.8).

Similarly, fundamental frequency (FF) of harmonic syllables decreased after lesion surgery (Fig. 2.5A, p=0.035, Δ – 72.5 Hz ± - 36.6 Hz drop in lesion group vs Δ 0.4 Hz ± 6.2 Hz in control animals). Following the initial decline, FF slowly recovered to baseline with a trajectory and timescale suggestive of compensatory learning (Fig. 2.5A, recovery of 11.03 ± 2.102 Hz/day). These findings are in agreement with the generally accepted role of RA in determining spectral properties of song, and suggest that bursts in RA, and serotonin's effects on them, play a role in determining the frequency of syllables.

Electrophysiological data also predicted that animals with depleted 5-HT would produce more exact syllables, due to increased signal to noise ratio. Indeed, we observed a significant increase in “goodness of pitch” (the peak of the derivative-cepstrum calculated for harmonic pitch- SAP manual). This effect was quite strong in the first days following lesion (Fig. 2.5B, Δ 33.5 ± 3.5, p<0.0001) but, similar to FF, goodness of pitch recovered to baseline by days 13-15 (p=.65). No other measures of song structure changed, including motif stereotypy (syllable order), syllable duration, and Wiener entropy.

Daily Patterns in Song

Serotonin is implicated in controlling circadian behaviors, and we investigated the effect of 5-HT lesions on daily changes in song patterns. As birds tend to sing most in the morning, we asked whether 5-HT lesions alter the fraction of songs sung in the morning vs. evening. There was no change in either the total number of songs sung by controls vs. lesioned animals or in the ratio of songs sung in the morning vs. evening (Fig. 2.6, paired t-test, p=.384, df=4). While the amount of singing was quite variable between animals and may obscure slight effects, these findings do indicate that 5-HT is not critically involved in determining when, or how much, a bird sings.

Discussion

HTR2 receptors are present on RA projection neurons, and their activation with either 5-HT or the specific agonist DOI causes an increase in excitability (Wood et al., 2011). Here, we found that activation of these receptors during playback of BOS leads to 1) increased song-related burst activity and 2) increased spontaneous firing, leading to 3) a decreased signal-to-noise ratio. To understand how these actions affect the motor output of this circuit (song), we then lesioned the serotonergic system with 5,7-DHT and analyzed song. Fundamental frequency decreased and goodness of pitch increased, in keeping with findings 1 and 2 above.

The role of 5-HT in BOS selective auditory responses in RA

Application of 5-HT had a relatively weak effect on BOS evoked bursts of activity in RA projection neurons, on average increasing spikes per burst by 0.39. While significant, it is a much smaller effect than the approximate doubling in spontaneous firing rate we consistently observe in all RA projection neurons. BOS related bursts in

RA in adult zebra finches appear to be robust to perturbations- a finding not at all surprising in an animal which sings a single song for the entirety of its adult life. The most likely biophysical mechanism underlying this increase in action potentials is an interaction between a direct increase in excitability of RA projection neurons due to HTR2 mediated depolarization with incoming synaptic inputs from HVC, although increased baseline subthreshold activity and the non-linearity defined by the action potential threshold may also play a role.

There is tonic release of 5-HT in RA. We know this because HPLC reveals 5-HT in RA (Barclay and Harding, 1988) and because direct application of the SSRI fluvoxamine results in increased firing rate in these neurons (Wood et al., 2011). One possible experiment would be to block the actions of 5-HT on these neurons via specific antagonists during BOS playback. A number of cautionary findings, however, caused us to avoid this manipulation. Primary is that application of 5-HT has such a subtle effect on BOS-evoked bursts. Experimentally, application of 5-HT is in a sense its own positive control, as we can be assured the pipette is not blocked and is applying 5-HT since FR increases drastically even as burst activity increases only modestly. Application of antagonists would have no similar internal control, and the expected minimal decrease in burst activity would thus be exceedingly difficult to uncover. It is worth note that 5-HT can affect auditory processing at other levels (Hurley and Hall, 2010).

The role of 5-HT in determining adult song features

Injection of 5,7-DHT into the third ventricle substantially reduced 5-HT levels across the telencephalon. Consistent with studies in other organisms, animals functioned almost completely normally following lesions (Smale et al., 1990; Neumaier et al., 1996), songs were generally indistinguishable from baseline, and animals sang a similar amount during similar times of day compared to baseline periods (Fig. 2.8). Our electrophysiological data indicate that 5-HT increases spikes per song-related burst in RA projection neurons, which are thought to shape spectral properties of song (Fee and Goldberg, 2011). RA has a direct projection to the hypoglossal nucleus, which projects directly to the syrinx. Bursts in RA lead to muscle contractions in the syrinx. Avian song production involves muscle contraction driven apposition of the syringeal labia coincident with expiration (Goller and Suthers, 1996a). Further contraction, increasing tension, produces sounds with higher fundamental frequencies. According to Goller and Suthers, “EMG activity in musculus syringealis ventralis (vS), the largest syringeal muscle, increases exponentially with the fundamental frequency of the ipsilaterally generated sound and closely parallels frequency modulation.” (Goller & Suthers, 1996b, p. 287). Additionally, a dynamical model based on these observations has been shown to reproduce frequency sweeps when run with either simulated functions representing tensions in vocal muscles (Gardner et al., 2001; Laje et al., 2001) or with transformed EMG activity recorded from singing birds (Mindlin et al., 2003). While we would by no means expect to find an exact linear match between large changes in activity in RA and FF, the strong dependence upon excitation in vS and FF does indicate that small changes in RA activity, such as seen in this study, would be expected to change the FF of songs- as observed here. Furthermore, the variation in number of spikes in RA projection neurons has been shown to significantly correlate with syllable FF, and while the authors did not seem to quantify the number of positive correlations versus negative correlations, it appears to be around 2:1 (Sober et al., 2008).

Reducing 5-HT in RA should lead to fewer action potentials per burst and thus to less muscle tone in the syrinx (Vicario and Nottebohm, 1988; Abarbanel et al., 2004; Fiete et al., 2007). This supports the idea that spectral properties of song are primarily determined by RA (Fee et al., 2004; Fiete et al., 2007; Gibb et al., 2009). Removing 5-HT from RA, then, should lead to a decrease in the frequency (~pitch) of syllables. In agreement with this hypothesis, FF of harmonic syllables was significantly reduced following lesions (Figs. 2.4B, 2.5A), and the amount of 5-HT in RA was linearly correlated with change in FF frequency following lesion (Fig. 2.6C). While we consider RA to be the mostly likely locus for 5-HT mediated changes to spectral features of song, there are of course other possibilities. 5-HT receptors are expressed in other areas of the song control system, including HVC and area X (Dutar et al., 1998; Lovell and Mello, 2012), and 5-HT is known to modulate auditory responses in other species (Hurley and Hall, 2010).

“Goodness of pitch”, which is an estimate of harmonic pitch periodicity, was increased following 5-HT lesion. Reduced 5-HT in RA leads to reduced spontaneous activity, which leads to increased signal-to-noise ratios during song-related bursting. One interpretation of this result is that harmonic stacks were less modulated ('cleaner') following lesion (increased goodness of pitch), primarily due to an increased signal-to-noise ratio in RA. We did not, however, observe a concomitant decrease in spectral entropy which might also have been expected.

5-HT is necessary for proper body-temperature regulation in a mouse model involving acute serotonergic neuron inhibition (Ray et al., 2011). If this is true in birds as well, it is possible that some of the effects we observed may be partially explained via a decrease in brain temperature and associated downstream effects. Previous studies cooling the avian brain found the most drastic effects occurred in a lengthening of song tempo, however, and the lack of that finding here argues against this hypothesis (Long and Fee, 2008).

It is perhaps surprising that changes in song following 5-HT lesion recovered to normal, particularly given the known importance of serotonin in learning (Harvey, 2003). It would seem 5-HT is not necessary for song maintenance in adult song zebra finches. Interestingly, the recovery to baseline seemed to depend more on amount of singing then on the time since surgery (data were by necessity, aligned to resumption of singing, which led to a robust time-dependence of recovery of FF, Fig. 2.5A), suggesting an activity-dependent mechanism. This could be due to adult error correction, although it would be slower than that seen in other scenarios (Andalman and Fee, 2009; Sober and Brainard, 2009; Charlesworth et al., 2012). An alternative possibility is the potential for activity-dependent expression of serotonin receptors in the song system, supported by the previous finding that HTR2 binding increases following 5-HT lesions in a rodent model (Compan et al., 1998). We cannot differentiate between these hypotheses at this time. A final point to note is that mean frequency did not recovery during the experiments. Why one measure of syllable frequency did recover while the other did not is initially vexing, although two possibilities seem probable. One is that birds, similarly to humans, may find FF to be a more salient feature than mean frequency, and thus the birds may correct alterations in FF more rapidly than they do alterations to mean frequency. Secondly, we took great pains to ensure that our FF measurement occurred on stable portions of harmonic stacks, which may be more tractable to adult error correction than noisier complex syllables for a variety of reasons that are beyond the scope of this paper.

This study reveals that 5-HT acts in forebrain premotor circuits to alter complex learned behavior. The conserved nature of the serotonergic system (Hen, 1993) indicates the findings here are likely to generalize across vertebrates. This greater mechanistic understanding of particular actions of 5-HT on song production in songbirds will lead to improved knowledge of how 5-HT modulates neural activity underlying perception and behavior in mammals.

Acknowledgments

We thank Gregg Stanwood for help in HPLC and John Neumaier for insights in the methodology of 5,7-DHT lesions. The authors declare no competing financial interests.

Previous Presentation of this data

These data appeared in part as posters at the Society for Neuroscience conference (Wood and Perkel, 2010; Wood et al., 2012).

Figures

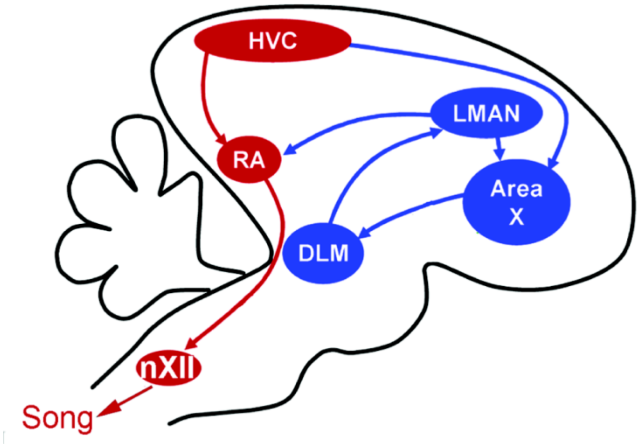


Figure 2.1: **A)** The song-control system is composed of the direct motor pathway (in red) and the anterior forebrain pathway (in blue). HVC may be considered part of both pathways.

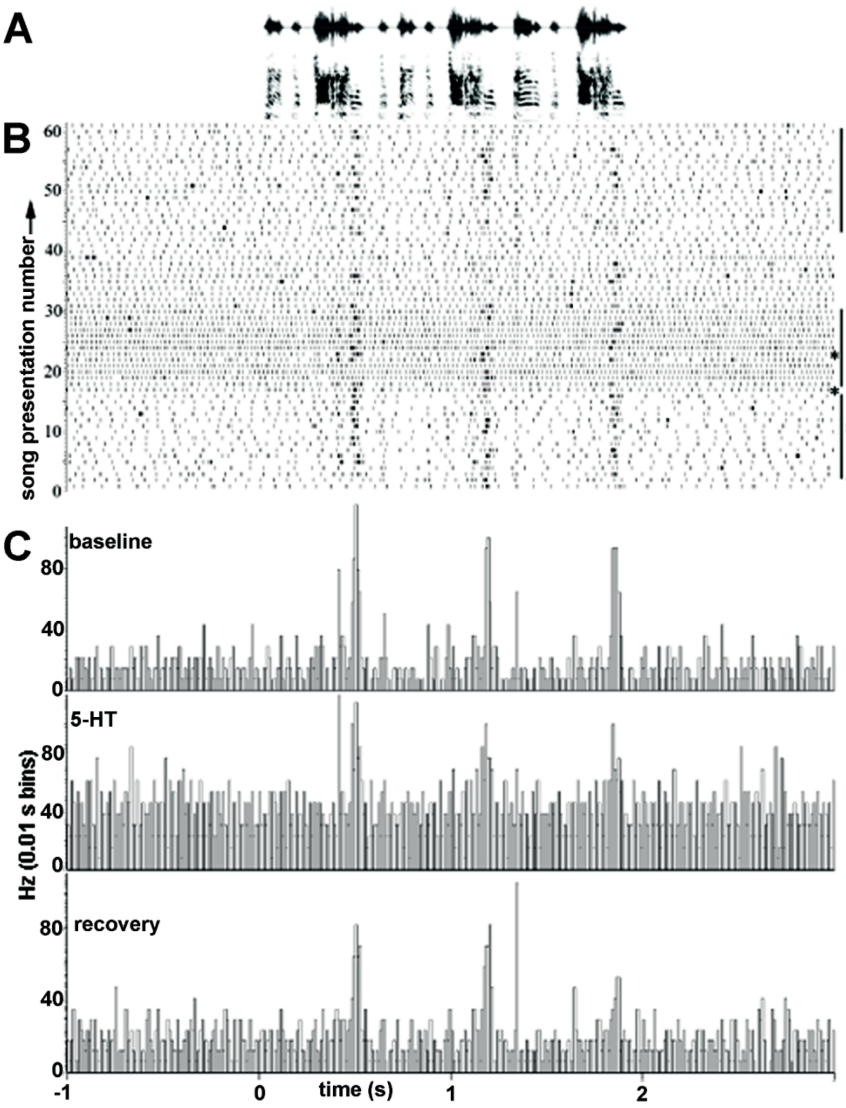


Figure 2.2: Example of *in vivo* spiking patterns in RA projection neurons with 5-HT application and BOS presentation. Neurons fired more in general, and also fired more song-related spikes during bursts. **A)** Waveform and Spectrogram of BOS- all panels are aligned to A. **B)** Raster of spikes from an RA projection neuron selective for BOS. 5-HT was applied at the 2 asterisks. **C)** histogram of spike activity from the regions identified by bars on the rights side of panel B.

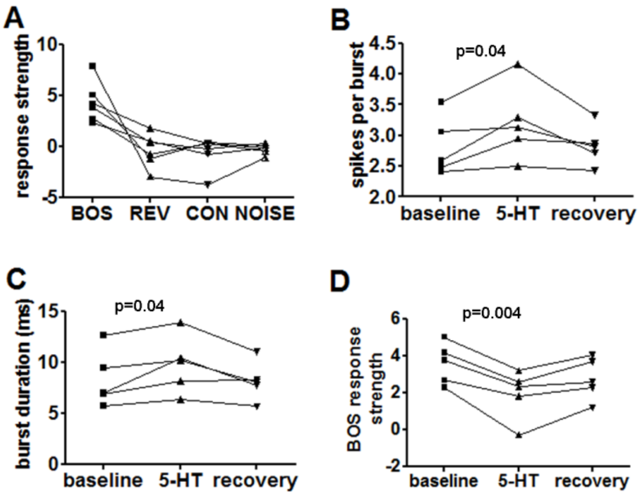


Figure 2.3: Quantification of song evoked spiking patterns in RA projection neurons with 5-HT application. Neurons fired more in general, and also fired more song-related spikes during bursts, n=5 neurons in 4 animals. **A)** RA neurons were selective for BOS over reverse BOS, conspecific song, or white noise. **B)** Application of 5-HT significantly increased the spikes per burst evoked by BOS. **C)** 5-HT also significantly increased the duration of bursts evoked by BOS. **D)** Response strength decreased with application of 5-HT, primarily due to the increase in spontaneous firing rate that 5-HT causes.

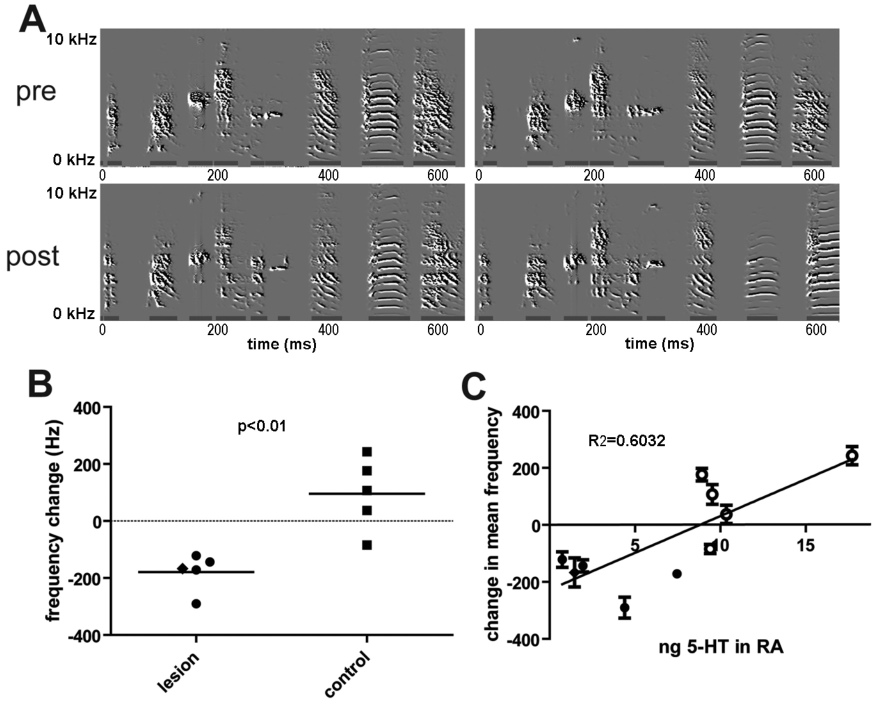


Figure 2.4: A) Example spectrograms before (top) and after (bottom) 5-HT lesion surgery. Song was left largely intact. **B)** Mean frequency of syllables was significantly reduced 2 weeks after lesion (days 13-15, $p=0.0026$, Δ -178.7 ± 29.32 Hz., n=5 animals per group). **C)** The change in mean frequency at two weeks post lesion was significantly related to the amount of 5-HT in RA measured the following day ($p=0.0082$, $R^2=0.6032$). Closed circles are lesioned animals, open circles are controls. Each data point in B and C corresponds to to the mean of syllables for one animal, with each syllable type sorted and analyzed separately.

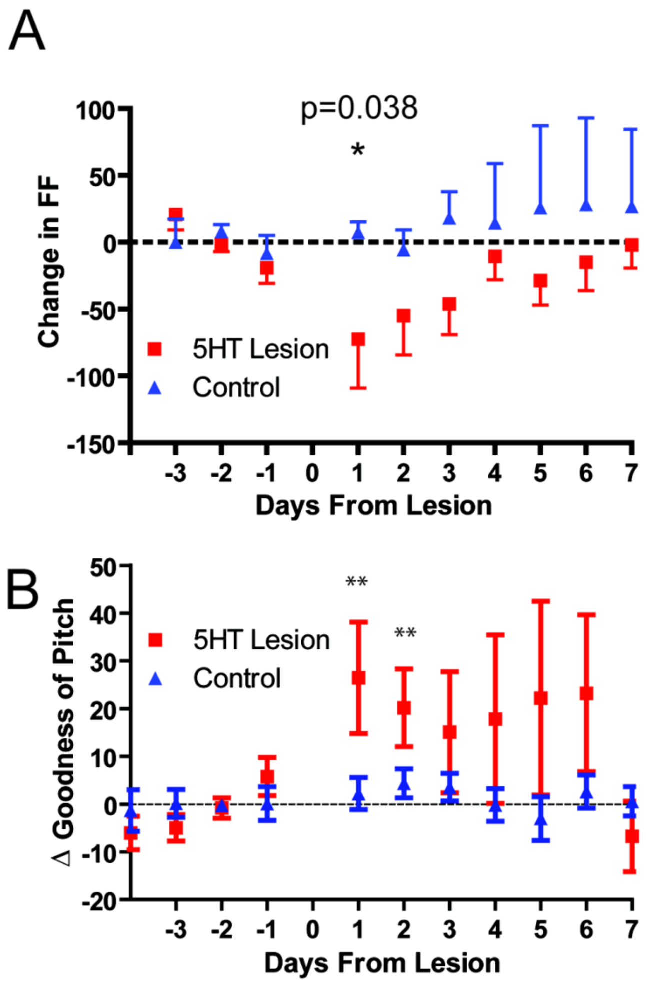


Figure 2.5: Fundamental frequency and goodness of pitch of syllables were transiently altered in 5-HT lesioned animals (n=5), but not controls (n=5). **A)** Lesioned animals had reduced FF immediately following surgery, while control animals did not (p=0.035, Δ – 72.5 Hz ± - 36.6 Hz immediately following surgery). This effect recovered to baseline within a week, with a trajectory and timescale suggestive of learning. **B)** Goodness of pitch was significantly increased in lesioned animals in the first days following surgery but not in control animals.

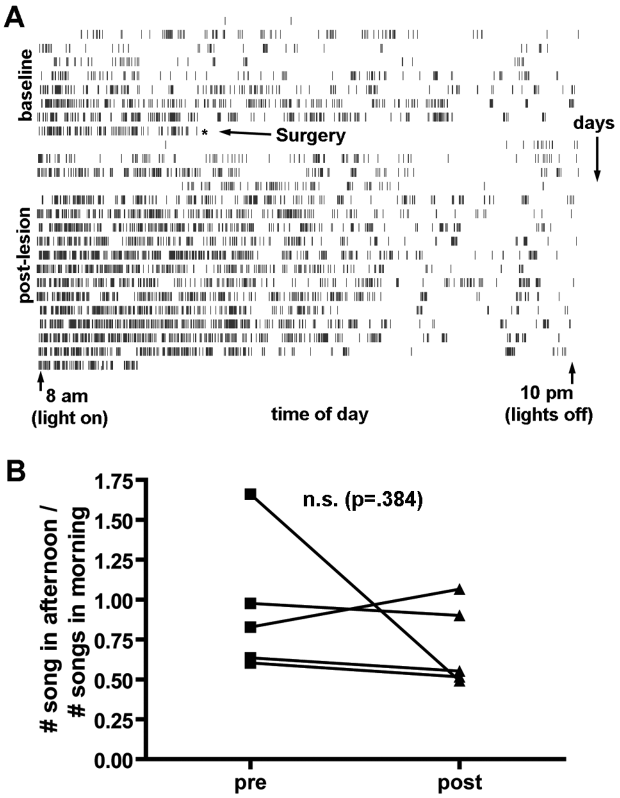
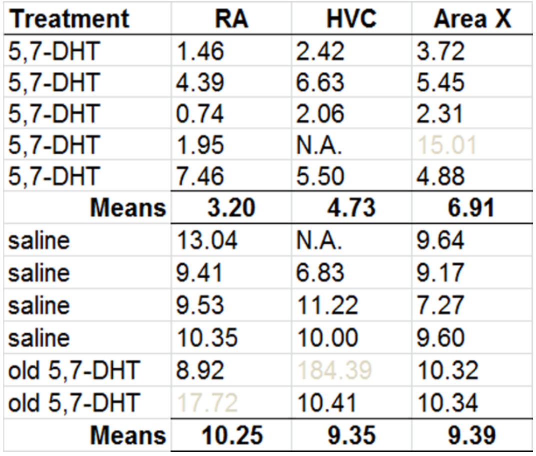


Figure 2.6: Birds tended to sing more in the morning than in the afternoon, and 5-HT lesions did not interfere with this pattern. A) A representative animal's song production data. Each tick mark represents 1 motif. This bird did not sing for ~1 day following surgery but song production was back to normal after ~3 days. The missing songs on the morning of the third day following surgery are an instance of song recording software crashing and were excluded from analysis. B) There were no significant differences in when birds sang following the 5-HT lesion (n=5); here we plot the ratio of afternoon to morning songs (2PM-4PM vs. 8AM-10AM) for the 3 days before surgery and after 2 weeks following surgery. No effects were apparent at any time following acute recovery from surgery.



Treatment	RA	HVC	Area X
5,7-DHT	1.46	2.42	3.72
5,7-DHT	4.39	6.63	5.45
5,7-DHT	0.74	2.06	2.31
5,7-DHT	1.95	N.A.	15.01
5,7-DHT	7.46	5.50	4.88
Means	**3.20**	**4.73**	**6.91**
saline	13.04	N.A.	9.64
saline	9.41	6.83	9.17
saline	9.53	11.22	7.27
saline	10.35	10.00	9.60
old 5,7-DHT	8.92	184.39	10.32
old 5,7-DHT	17.72	10.41	10.34
Means	**10.25**	**9.35**	**9.39**

Table 2.1: HPLC analysis of 5-HT levels following control and 5,7-DHT surgeries. Levels are expressed as ng of 5-HT per mg of control protein. Not all samples were analyzed in every brain region (N.A.). Values in grey font were >4 standard deviations from the mean, and were excluded as outliers.

Chapter 3: A Daily Oscillation in Fundamental Frequency of Harmonic Syllables in the Zebra Finch

I performed all experimental work in this chapter and worked extensively with Thomas Roseberry in analyzing the data- I wrote the manuscript.

Title:

A Daily Oscillation in Fundamental Frequency of Harmonic Syllables

Abbreviated Title:

Daily Oscillation in FF

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Figures: 3 Tables: 0

So supplemental, No conflicts

Acknowledgments: Dina Popovkina

Abstract

Complex motor skills are universally known to be more difficult shortly after waking up, but the daily trajectory of motor skill errors is poorly understood. By undertaking a careful analysis of the fundamental frequency (FF; ~'pitch') of thousands of zebra finch syllables per animal per day we find zebra finches also appear to have difficulty singing in the morning. More interestingly, however, we find that not only is there an effect upon waking, but that there is a reproducible daily rhythm to song, such that the FF of harmonic syllables starts low in the morning, rises across the morning and then falls again in the late afternoon. This oscillation is consistent across days and across animals. Additionally, this oscillation appears to be influenced by adult song maintenance, such that mornings when birds begin singing syllables with fundamental frequencies further from their average the slope of this daily oscillation tends to be steeper. To test this correlation we artificially lowered FF by reducing 5-HT in the telencephalon, a manipulation which otherwise leaves song unaffected. Compensation for the lowered FF occurred most dramatically in the later afternoon, where the late afternoon fall in FF was replaced with a rise. We have therefore found 1) a hitherto undescribed daily oscillation in song wherein 2) FF starts lower (~'worse') in the morning and 3) has a component of error correction, or song maintenance. Song production in zebra finches is a model system of complex learned behavior because of the ease of gathering good behavioral data and the tractability of the underlying neural circuitry- the presently discovered daily oscillation, then, promises to reveal new insights into how time of day may influence our ability to accomplish a variety of complex learned motor skills.

Introduction

Complex motor skills are universally known to be more error-prone shortly after waking up, and perhaps to a lesser degree, also before going to sleep. This colloquial knowledge, however, has been largely ignored by the neuroscience community, perhaps primarily because of the lack of a model system of study. We find that zebra finches sing syllables with more deviant fundamental frequencies (FFs) in the morning. Interestingly, while the morning error (and subsequent correction) may be the most obvious daily effect of FF, we also find consistent trends across the day which may be present, but overlooked, in a variety of complex motor skills across the animal kingdom.

Oscine songbirds, such as the zebra finch, must learn to sing their songs- it is a process requiring tens of thousands of repetitions (Tchernichovski et al., 2001). The zebra finch learns to sing between the first and third month of his life (only the males sing), after which period he sings the same song, with very little variation, for the rest of his life. During the learning period birds sing poor songs immediately after waking (Derégnaucourt et al., 2005), which was hypothesized to be a byproduct of sleep learning, perhaps of the consolidation of memory during sleep. No similar effect was reported in adults.

We have undertaken a careful analysis of the fundamental frequency (FF; ~'pitch') of thousands of zebra finch syllables per animal per day. We find adult zebra finches have a similar deviance in their morning songs, although the time-course of this effect appears to be slower than that reported before. More interestingly, however, we find that not only is there an effect upon waking, but that there is a reproducibly daily rhythm to song, such that the FF of harmonic syllables starts low in the morning, rises across the morning and then falls again in the late afternoon. This oscillation is consistent across days and across animals. We theorize this oscillation is driven by underlying physiological factors which are likely shared across the animal kingdom and may be present in humans as well.

Additionally, this oscillation appears to be influenced by adult song maintenance, such that mornings when birds begin singing syllables with fundamental frequencies further from their average the slope of this daily oscillation tends to be steeper. To test this correlation we artificially lowered FF by reducing 5-HT in the telencephalon, a manipulation which otherwise leaves song unaffected. Compensation for the lowered FF occurred most dramatically in the later afternoon, where the late afternoon fall in FF was replaced with a rise. We have therefore found 1) a hitherto undescribed daily oscillation in song wherein 2) FF starts lower (~'worse') in the morning and 3) has a component of error correction, or song maintenance. Song production in zebra finches is a model system of complex learned behavior because of the ease of gathering good behavioral data and the tractability of the underlying neural circuitry- the presently discovered daily oscillation, then, promises to reveal new insights into how time of day may influence our ability to accomplish complex learned motor tasks.

Methods

Animals

Adult male zebra finches were obtained from a commercial supplier or reared in our colony. Animals were housed in groups of 4-10 males, on a 13:10 hr. light:dark cycle, with food and water available *ad libitum*. All procedures were approved by the University of Washington Institutional Animal Care and Use Committee. All animals were part of a previous study investigating the actions of 5-HT in song nucleus RA (Chapter 3).

Song Analysis

Birds were individually housed in sound attenuation chambers (Acoustic Systems) at least seven days before and 14 days after 5-HT lesion surgery. We continually recorded spontaneous (undirected) vocalizations using Sound Analysis Pro (SAP) software (Tchernichovski et al., 2000). Songs were sorted and analyzed using custom Matlab software and SAP. Zebra finch songs are highly stereotyped, making them especially well-suited for in-depth analysis. The acoustic structure of song is arranged in a hierarchy, with 25-250 ms vocal units known as syllables strung together in a stereotyped sequence called a motif. Each song consists of one or several motifs, preceded by introductory notes and separated from each other by <100 ms of silence. We used an updated version of a program (Leblois et al., 2010) to sort individual motifs from unlearned calls and cage noise. Briefly, the program detected putative motifs based on peaks in the cross-correlation between the spectrogram of the song and that of a clean preselected motif. Such putative motifs were then sorted based on their spectral similarity with the preselected clean motif, using thresholds set by the experimenter. For motifs for which such analysis did not allow unambiguous distinction, an additional PCA analysis on the spectrograms of putative motifs allowed us to sort motifs from other sounds. This analysis allowed us to successfully sort >90% of the motifs sung by a bird on a given day (assessed by comparing hand sorting with the automated sorting by the program). Once clean motifs had been sorted, individual syllables were sorted using custom Matlab software somewhat similar to previously reported methods (Wu et al., 2008). Briefly, we ran clean motifs through the SAP batch processing module to separate individual syllables and calculate spectral and temporal features. These data were imported into Matlab and sorted by a semi-automated procedure in which the user selects spectral and temporal feature values for each syllable (as well as the order in which they occur). Using these methods we sorted and analyzed the overwhelming majority of syllables produced daily by each animal over the course of the experiment.

Calculation of Fundamental Frequency

A 23-ms spectrally stable portion of the syllable waveform was windowed from onset of harmonic syllables. The power spectrum of the syllable waveform was calculated. The fundamental frequency of the syllable was calculated from the frequency bin of greatest spectral power using a weighted average from the four neighboring bins and the inter-harmonic frequency.

Calculation of Fundamental Frequency Derivatives

The daily time course of the mean fundamental frequency of each syllable was calculated using the MATLAB smoothing curve function rloess windowed every hour with a smoothing value of 0.7 and a second degree polynomial. The average morning and evening derivative for each day was calculated using the rloess curve values in the first and last three hours of lights on (respectively) divided by the time between points.

The difference from mean FF of a period was defined as the difference between the daily average FF and the FF of the first hour of that period. Sporadic singing occasionally resulted in erratic derivative values, and values 3 standard deviations outside of the mean were excluded.

Definitions of Baseline, Lesion, Recovery

Baseline for all birds was established using the average of three stable days in the one to five days before surgery. After surgery, birds often sing little or not at all for a few days, and thus we excluded days following surgery when birds sang less than 30% of the pre-surgery average number of songs (generally 2-5 days were excluded). Lesion was defined as the first 3 days following resumption of singing. Recovery was defined as 7-10 days following resumption of singing, unless explicitly stated otherwise.

Surgery

See chapter 3 for a comprehensive discussion of the surgery and effects. Briefly, FF of syllables was lowered by drastically reducing 5-HT across the telencephalon via 5-HT lesion. We injected 500 nL of 5,7-dihydroxytryptamine creatinine sulfate salt (5,7-DHT) into the third ventricle (coordinates: 0 lateral, -1 rostral, 3.75 deep, 40^o head angle). While 5,7-DHT is a neurotoxin mostly selective for serotonergic terminals (Neumaier et al., 1996), noradrenergic and dopaminergic neurotoxicity was blocked by pretreatment with .1 mL desipramine (2 mg/mL) given by IM injection. Two weeks after injection of either 5,7-DHT or saline, animals were sacrificed by decapitation during isoflurane anesthesia between 9 AM and 12 PM. Brains were sliced at 300 μM using a vibrating microtome (Oxford) and RA, adjacent arcopallium, HVC, adjacent nidopallium, X, striatum, cerebellum, and brain stem were dissected under light microscopy with micro-dissecting scissors and stored at -80^o. Lesion effectiveness and specificity were verified by high pressure liquid chromatography (HPLC, Vanderbilt University core facility) for a variety of monoamines in these tissue punches.

Results

Daily Patterns in Song

We found that FF of harmonic syllables followed a diurnal pattern (Figs. 3.1, 3.2A). FF started slightly lower in the morning than average (-1.448 ± 35.5 Hz, $p=0.0261$). FF then rose over the course of the morning, which we quantified as the slope of FF for the first 3 hours of the day (Figs. 3.1, 3.2A; mean morning slope = 9.584 ± 21.584 Hz/hr, $p<0.0001$). FF then fell during the last three hours of the evening (Figs. 3.1, 2A; mean evening slope=-18.25±34.68 Hz/hr, $p<0.0001$). This pattern was clearly observable via the rloess smoothing curve (Fig. 3.1, red lines).

We asked whether these changes in FF might be related to maintenance of FF at the bird's ideal value. We plotted the slope of FF versus the difference between the current and the daily FF, where current FF was the FF of the first hour of the morning or evening period being plotted. Morning FF slope was significantly correlated with difference in FF such that if the syllable started lower than average the slope of FF rise tended to be steeper than average (Fig. 3.2C cyan 'x', $p<0.0001$, slope=-0.3765, $R^2=0.404$). The slope of FF in the evening was not significantly correlated with the difference in FF at the beginning of the period (Fig 3.2C purple 'o', $p=0.109$, slope=-0.1524, $R^2=0.035$,).

Lowering and Recovery of FF

To further clarify if these daily oscillations were related to maintaining FF at the bird's ideal value we artificially lowered the FF of song by reducing 5-HT levels across the telencephalon. We have previously shown that this manipulation leaves song intact while selectively lowering the FF of syllables (chapter 3), likely via decreasing intrinsic excitability in RA projection neurons (chapter 2) which leads to decreased tension on the syringeal labia (chapter 3). Following 5-HT lesion, the FF of songs was significantly reduced ($p=0.035$, Δ – 72.5 Hz ± - 36.6 Hz drop in lesion group vs Δ 0.4 Hz ± 6.2 Hz in control animals). Following the initial decline, FF recovered to baseline with a trajectory and timescale suggestive of compensatory learning (Fig. 3.1, recovery of 11.03 ± 2.102 Hz/day).

During this recovery period the evening fall in FF, became, in many cases, an evening rise (Figs. 3.1, 3.2B). Whereas during baseline conditions we observe a positive slope in only 20% of the time, after surgery (during the period when animals were correcting their songs) we observed a positive slope 64.7% of the time ($p<0.05$); average evening slope during baseline was -23.83 Hz/hr, whereas after lesion it increased to -8.75 Hz/hr. While not nearly as striking (or significant), morning slope did also increase, going from 1.5 Hz/hr baseline to 5.38 Hz/hr during the corrective period.

As we found the steepness of the morning rise was correlated with the deviation from mean FF of the syllable, we asked whether lowering the FF would lead to a steeper correlation. The correlation between morning FF slope and difference in mean FF became strikingly more robust following the 5-HT lesion, with the slope of correlation increasing from -.382 (blue exes) to -0.698 (red circles) and the R^2 going from 0.4037 to 0.5073 (Fig. 3.2D).

As we reported previously, 5-HT lesion did not alter the fraction of songs sung in the morning vs. evening- there was no change in either the total number of songs sung by controls vs. lesioned animals or in the ratio of songs sung in the morning vs. evening (Chapter 3), indicating the changes in slope of FF observed are not due to a shift in the timing or amount of singing.

Discussion

Daily Oscillations in FF

We found a previously unreported daily oscillation in FF of harmonic syllables that is consistent within and between animals. FF of harmonic syllables rises in the morning and then descends again in the evening (Figs. 3.1, 3.2A). One explanation for this oscillation is that circadian 5-HT levels drive the oscillation in FF. The presence of dense serotonergic innervation to the suprachiasmatic nucleus (SCN, Meyer-Bernstein and Morin, 1996; Pickard and Rea, 1997) supports this hypothesis. The fact that the oscillation re-asserts itself after ~1 week, when 5-HT levels are still drastically reduced, argues strongly against this interpretation. Serotonergic lesions have been shown to disrupt circadian rhythms in other systems, although unmasking the effect generally involves altering the photoperiod (Morin and Blanchard, 1991; Mistlberger et al., 1998; Paulus and Mintz, 2012), which we did not do here.

Alternatively, it is known that adult finches maintain the FF of harmonic syllables, and the observed daily oscillations in FF may involve this process (Nordeen and Nordeen, 1992; Kao and Brainard, 2006; Andalman and Fee, 2009). Specifically, we hypothesize that there is some physiological pressure that causes FF to start lower in the morning. It could be body temperature or a consequence of sleep-related memory reconsolidation. The bird may begin correcting its song over the course of the morning until the desired FF is reached. If this were true we would expect the slope of changing FF in the morning to be directly proportional to the extent of the difference in current FF from the idealized FF- if the syllable starts at a particularly low FF one morning we would expect the rise in FF that day to be particularly severe. Conversely, on days when the FF was higher than average (which happens less commonly but does happen) we would expect the trend of FF to be smaller, or even in the opposite direction. Indeed, this is exactly what we find; when FF is higher than average FF tends to drop, not rise, in the morning. The magnitude of FF slope in the morning is significantly, and highly associated with the degree of deviancy from average daily FF (Fig. 3.2C).

To more directly test this correlation we artificially lowered FF by reducing 5-HT in the telencephalon- a manipulation which leaves songs and song patterns otherwise unaffected. Birds corrected the altered FF of song over the course of a few days of singing (during a time when 5-HT levels where still extremely depleted), allowing us to examine how daily oscillations in FF were involved in this correction. Both morning and evening slopes in FF become more positive. The evening was the most striking- in baseline periods FF generally falls in the evening, but during correction it often rose instead (Fig. 3.2B). The correlation between morning slope and FF deviancy also became higher during this period (Fig. 3.2D), suggestive of error correction maintaining stable FF.

The evening fall in FF is also a consistent aspect of the oscillation, although we are uncertain how to explain it. One possibility is that the oscillation in FF may be the result of a corrective mechanism involved in song maintenance. Perhaps birds tend to over-correct for the morning low FF, overshooting their ideal FF as a rising physiological mechanism (possibly temperate) and their own error correction coincide, and are then forced to re-correct FF in the evening. What drives this oscillation, and whether it is involved in the deterioration following sleep during the learning period (juvenile song is less similar to tutor song during the morning- 'morning breath', Derégnaucourt et al., 2005), remains undetermined.

While we find strong evidence for correction towards the mean in FF, it is not necessary that this correction acts through the same mechanism as that driving the basic oscillation in FF. It could be that there is a daily oscillation and that the correction acts on top of that- it will be difficult to differentiate between these possibilities until we know what the source of the daily oscillation is. Birds across the world sing more in the morning- a phenomenon referred to as the dawn chorus. We originally thought this increase in song rate may be associated with the daily oscillation, but we have been unable to uncover a relationship (data not shown).

We find that zebra finches sing syllables with more deviant fundamental frequencies (FFs) in the morning, and while the morning error (and subsequent correction) may be the most obvious daily effect of FF, we also find consistent trends across the day which may be present, but overlooked, in a variety of complex motor skills across the animal kingdom. This novel oscillation in a tractable model organism may well lead to insights into numerous complex behaviors across the animal kingdom, perhaps it will even lead to a better understanding of how to avoid pouring your coffee in your cereal bowl.

Figures

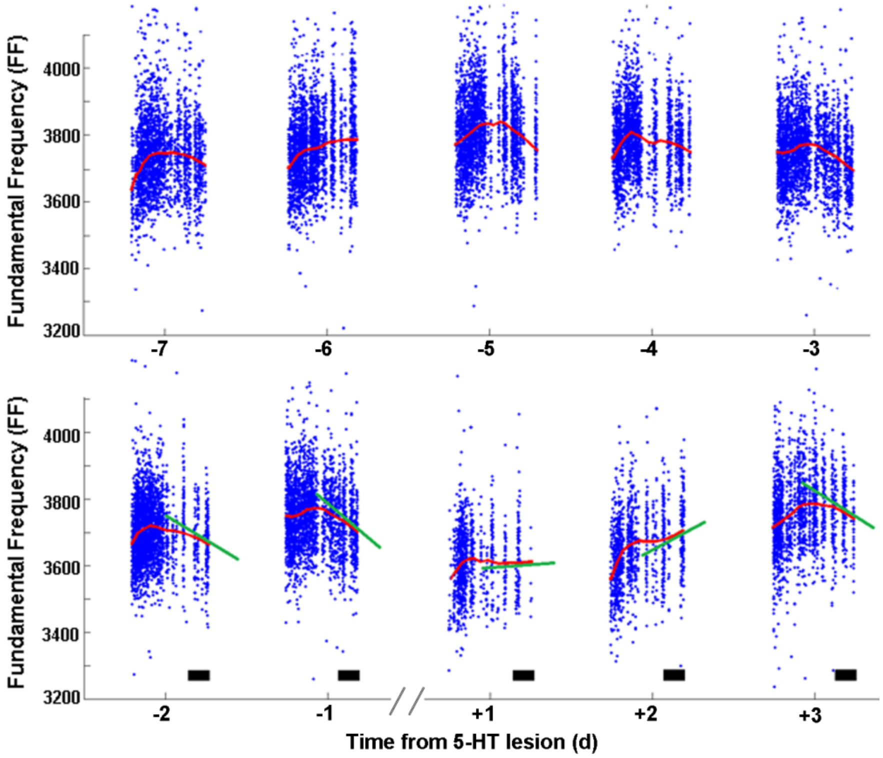

Figure 3.1: Daily patterns of FF in a typical harmonic syllable across seven days of baseline and three days post lesion (after the bird began singing again). The red line is a second degree polynomial fit. In the morning FF is low, rises during the early day to a peak, and then falls again in the evening. 5-HT lesion disrupted the evening fall, and in fact FF instead rose in the evening on Lesion+1 day. The green line is the slope of the polynomial fit during the last 3 hours of lights on, marked by the black bars at the base. A similar polynomial fit was calculated for the first 3 hours of lights on (the 'morning rise') but is not shown on this graph. Also visible on this graph is the overall decrease in FF following lesion (all dots shifted downward after lesion).

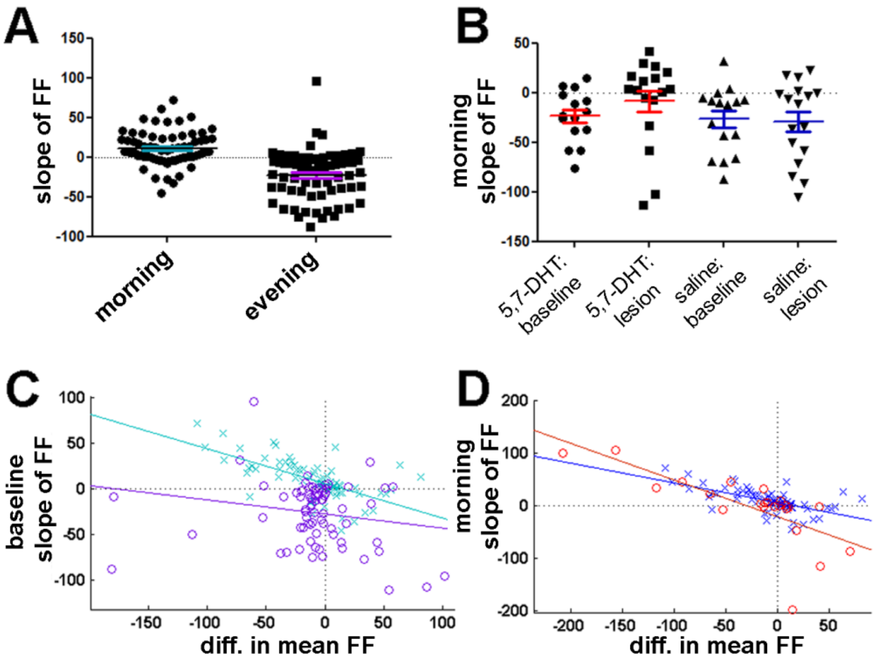

Figure 3.2: Analysis of daily patterns in FF. A) Baseline data of all syllables analyzed. In the morning FF tends to start lower than average and climb for the beginning of the day, resulting in a positive slope in the first 3 hours of the day. In the evening FF tends to fall, and the slope is generally negative. B) Following 5-HT lesion there was no significant change in the slope of FF in the last 3 hours of the day, although the mean did shift from -23.8 to -8.75. Saline controls showed no changes. C) Morning (cyan x) and evening (purple o) slopes of all syllables analyzed during baseline period plotted versus the mean difference in FF from the daily FF average. The slope of a syllable's FF on a given morning is associated with the starting difference from daily average FF of that syllable ($p<0.0001$, $R^2=0.404$, slope=-0.3765). Evening slope is not significantly correlated with diff. from mean FF ($p=0.109$, $R^2=0.035$, slope=-0.1524). D) The correlation between the morning slope of FF and the difference in mean FF was significant in the lesioned animals before (blue x, slope=-0.63, $R^2=0.32$) and after (red circles, slope=-0.7614, $R^2=0.74$) surgery.

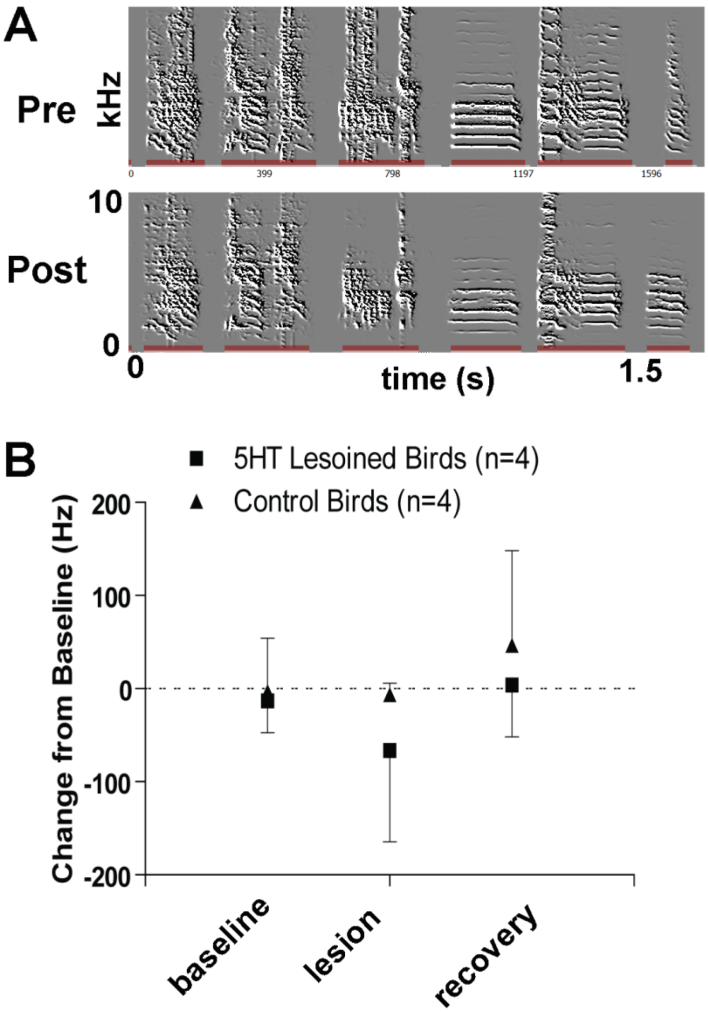

Figure 3.3: FF of syllables was temporarily lowered by 5-HT lesion while keeping song intact. **A)** Spectrograms of one animal's song before (top) and after (bottom) 5-HT lesion [Get a new bird's specs for this]. Song was primarily unaffected by 5-HT lesion and sounded and looked identical to human observers. **B)** The FF of syllables was lowered following 5-HT lesion but rapidly recovered.

Chapter Four: Bilateral Transections of the HVC-RA Fiber Tract Reveals a High Level of Redundancy in the HVC-RA Projection

I collected and analyzed all data and wrote the manuscript. [I am not planning on submitting this article for publication at this time.]

Title:

Bilateral Transection of the HVC-RA Fiber Tract Reveals a High Level of Redundancy in the HVC-RA Projection

Abbreviated Title: **HVC-RA Tract Cut**

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Figures: 4 Tables: 0

Abstract

Song production in adult songbirds is produced primarily along the direct motor pathway, comprising in part the HVC to RA projection, with bilateral lesions of either HVC or RA resulting in a cessation of singing. HVC also projects to RA, however, via a more circuitous route through the anterior forebrain pathway (AFP)- a pathway which drives song production in early stages of song learning. Here, we analyze to what extent the AFP is able to drive song in the adult zebra finch by cutting the fiber-tract from HVC to RA in adulthood, thereby functionally lesioning the DMP while leaving the AFP entirely intact. Complete transections of the DMP entirely abolish song, not even leaving sub-song like activity, indicating the AFP is entirely unable to drive song in the adult zebra finch in the absence of DMP input. Surprisingly, we find large bilateral transections spanning greater than 70% of the fibers can leave song largely intact, indicating a previously under-appreciated level of redundancy in this circuit.

Introduction

Song production in the oscine song birds is controlled by 2 main telencephalic pathways, together composing the song control system (SCS, Figure 4.1a). The direct motor pathway (DMP) is both necessary and sufficient for normal adult song, and is composed of the nuclei HVC (proper name) and RA (robust nucleus of the arcopallium). The anterior forebrain pathway (AFP) is composed of a cortical basal ganglia loop and is necessary for song learning and plasticity, but not for production of normal adult song. The evidence for the previous statements is substantial, although lesion experiments have been particularly informative (Nottebohm et al., 1976; Simpson and Vicario, 1990). Bilateral lesions or inactivations of either HVC or RA result in a cessation of song production, while lesions or inactivations to the nuclei of the AFP interfere with the ability to learn or correct song, but not the ability to produce normal adult song (Miller-Sims and Bottjer, 2012).

Early in song acquisition (during the critical period), the AFP has an active role in driving song production (Aronov et al., 2008). As learning progresses, however, the DMP plays an increasing role in driving song until, after song becomes crystallized (~90 days post hatch- dph), lesions of the output nucleus of the AFP (nucleus lMAN) result in no immediate changes to song structure, and in fact generally result in more stereotyped song (deafening, inactivation). It is clear from these and other studies the DMP becomes the primary pathway for song production, while the AFP is involved in adult error correction, possibly via sending an instructive or biasing signal to RA.

To assess whether the AFP can drive any type of song in the adult zebra finch we severed the HVC-RA tract bilaterally. We find that bilateral tract cuts comprising between ~70-95% of fibers result in song that looks superficially sub-song like. Additionally, we find large bilateral transections spanning greater than 70% of fibers can leave song relatively intact, indicating a previously under-appreciated level of redundancy in this circuit, but in agreement with at least one other study currently in progress (Basista et al., 2012).

Methods

Animals

Adult male zebra finches (>90 dph, many > 120 dph) were obtained from a commercial supplier or reared in our colony. Animals were housed in groups of 4-10 males, on a 13:10 hr light:dark cycle, with food and water available ad libitum. All procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Surgery

Animals were anesthetized with 1-4% isofluorane and placed in a stereotaxic apparatus at the steepest angle available (90^o). Lidocaine was injected subcutaneously over the scalp and the skin was retracted over the caudal portion of the skull. Animals then received 1 of 2 types of complimentary surgeries, both aimed at transecting the entire HVC-RA tract while leaving the sinus vein intact. As the exact location of the sinus vein is variable slight adjustments to coordinates were often necessary.

Small craniotomies were made over both hemispheres as follows. A mark was made 1.00 mm caudal of the zero point (sinus bifurcation). Approximately 1 mm lateral to that mark the outer layer of skull was removed to find the sinus vein. Craniotomies began at the lateral edge of the sinus vein, at the point at which it was 1.00 mm caudal of the bifurcation, and extended 2 mm further lateral. After removing both layers of skull and carefully cutting the dura along a lateral line, a blade (an insulin syringe flattened with pliers) was inserted into the brain and moved laterally with the aid of the stereotaxis. Two sweeps were made per cut- one at 1.00 mm deep, and another at 1.50 mm deep. As we found these coordinates tended to leave the medial most portion of the HVC-RA tract intact, we used 2 equally viable solutions to sever the medial portion, which lies under the sinus vein and is difficult to reach without causing mortal bleeding. In some animals we made additional craniotomies on the medial side of the sinus vein, slightly caudal to the initial cuts. In others, we angled the blade at a 45^o angle (towards the rostral-caudal midline), enabling us to cut under the sinus vein without cutting the vein itself. In some animals injections of BDA were made bilaterally into HVC immediately after cutting the HVC-RA fiber tracts. See Fig. 4.2A for a schematic of craniotomies.

One week following surgery animals were sacrificed with an overdose of nembutal and transcardial perfusion. Brains were sliced on a freezing microtome at 50 microns in the sagittal plane and every third section was mounted and NISSL stained for analyzing the extent of fiber transection. Sections were captured using ImageJ and a QImage plugin. We defined the fiber tract as extending between any sections which contained any amount of both HVC and RA. While most fibers are fairly superficial, some do run deeper, and thus we considered the fiber tract to possibly extend from the caudal surface of the brain to a line drawn between the midpoints of HVC and RA (Fig. 4.2B).

Song Recordings and Analysis

A few days before surgery animals were isolated in sound boxes equipped with microphones attached to a preamp and recorded on an computer (intel processor) using Sound Analysis Pro (SAP) software. Animals never changed sound boxes or microphones and no recording settings were changed over the course of experiments. Song recordings were made constantly until animals were sacrificed 6-8 days after surgery.

Songs were analyzed using the SAP Explore and Score module. After determining the song motif, recognizable syllables were selected by hand and spectral features were calculated automatically and exported to Excel and Prism V software for analysis. Approximately 100 syllables were analyzed per bird at baseline and post-surgery time-points. Changes in syllable duration, amplitude, pitch (cepstrum calculation method), mean frequency, goodness of pitch, and entropy were analyzed. The only exception to this were the 3 birds which had extremely limited amount of vocal activity post-surgery and in whom it was not possible to compare post-surgery with pre-surgery song in a quantitative way.

In addition to calculating spectral features with SAP, three observers blind to the amount of tract cut were asked to score the songs on a scale of 0-5 for amount of degradation, with 0 being no degradation (perfect adult song) and 5 being absolute degradation (comparable to cuts of the tracheosyringeal nerve). They were given a set of spectrograms of at least 3 motifs from before and a set of at least 5 motifs after surgery which were temporally aligned with respect to each other as well as possible. Post-surgery spectrograms were chosen to be among the more recognizable motifs sung by the birds. Observers were instructed to use their own intuition as well as the following guidelines in assigning scores to songs: 5- perfect adult song; 4- syllables variable; 3-syllables spectrally degraded but motifs present; 2-syllables unidentifiable but somewhat present; 1-syllables entirely unrecognizable; 0-completely degraded, like XII nerve cut vocalizations.

Results

The extent of bilateral HVC-RA tract cut correlated positively with the extent of song degradation, although serious degradation was not observed until > ~75% of tracts were cut (Figs. 4.2-4.4). When less than ~75% of the HVC-RA tract is cut bilaterally syllables begin to show marked spectral abnormalities but remain easily identifiable and the ordering of syllables remains unperturbed (Figs. 4.2,4.3). Bilateral cuts of between 75-90% caused substantial degradation of song (Figs. 4.2,4.4) with syllables becoming increasingly difficult to identify and syllable ordering eventually deteriorating. Numerous measurements showed a steep decline across this range including blinded human observers (Fig.4.2A), amplitude (Fig.4.2A), FF (Fig.4.2C), and goodness of pitch (Fig.4.2D).

Discussion

The primary finding of this study is that this pathway appears to have a surprising amount of redundancy. Syllables maintained enough spectral characteristics to be recognizable even after substantial tract cuts, and multiple automated measures (amplitude, pitch, goodness of pitch) confirmed the birds were able to sing nearly normal song with over half of the HVC-RA tract severed bilaterally. This redundancy could be present by a number of non-exclusive mechanisms. The simplest hypothesis is that the HVC to RA tract is extremely redundant. The recent finding that lesioning approximately one third of HVC bilaterally can leave syllables intact supports this interpretation (Basista et al., 2012)□. Alternatively, there could be rapid compensation for the loss of HVC inputs into RA, perhaps with the help of the AFP. Network dynamics in RA may also, after song is crystallized, become something of a central pattern generator, capable of transforming an extremely degraded signal into the bird's own song.

Figures:

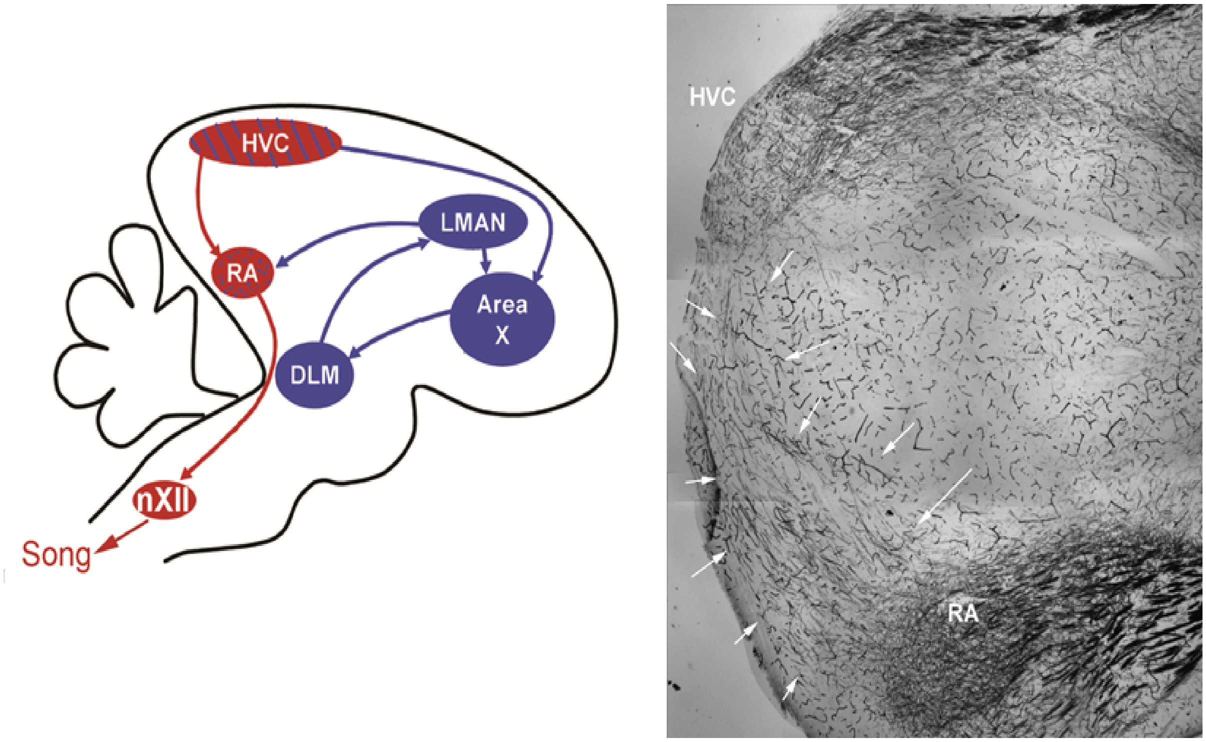

Figure 4.1: The song control system. A) HVC is part of both the DMP (red) and the AFP (blue). The AFP synapses onto RA. B) Myelin stain of the HVC to RA projection.

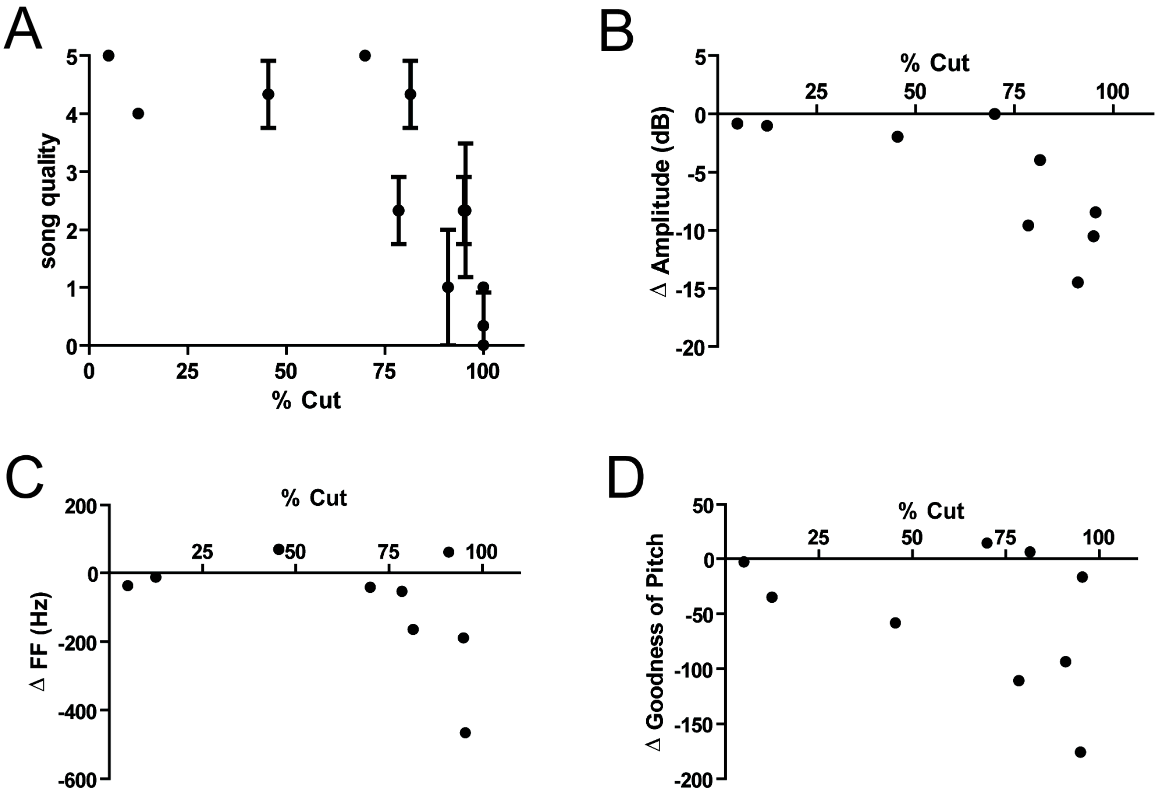

Figure 4.2: Changes in spectral features of syllables as measured by SAP. Cuts of less than 75% had minimal effects on a variety of song characteristics. **A)** Song quality was assessed by 3 blind observers with 5 being no change to song and 0 being unrecognizable (see examples in Figs. 3-5). **B)** Amplitude shows a steep reduction for cuts > 75% but not for those that are less. **C)** Change in FF showed a similar trend. **D)** Goodness of pitch also follows the general trend, albeit with a bit more noise.

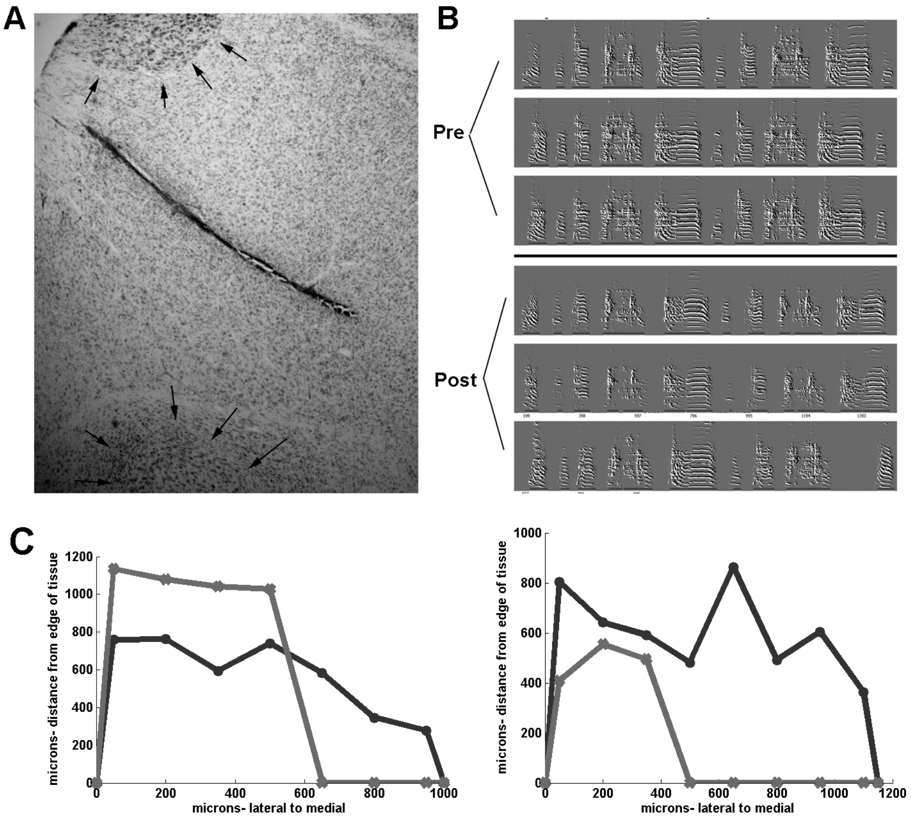

Figure 4.3: An example of a partial (45.5% overall) tract cut. **A)** This animal had a second cut made at a 45° angle towards the midline, and thus in 2 sections in the left hemisphere the cut does not reach the tissue surface (shown). **B)** Despite the partial tract cut, song was graded by blind observers as being a 4.33 out of 5. **C)** The left hemisphere had a 63% cut. The dark line is the approximate maximum depth of the HVC-RA tract, and the lighter line is the depth of the cut. The right hemisphere had an 28% cut. Variations in thickness of tract are likely due to slight abnormalities in tissue processing.

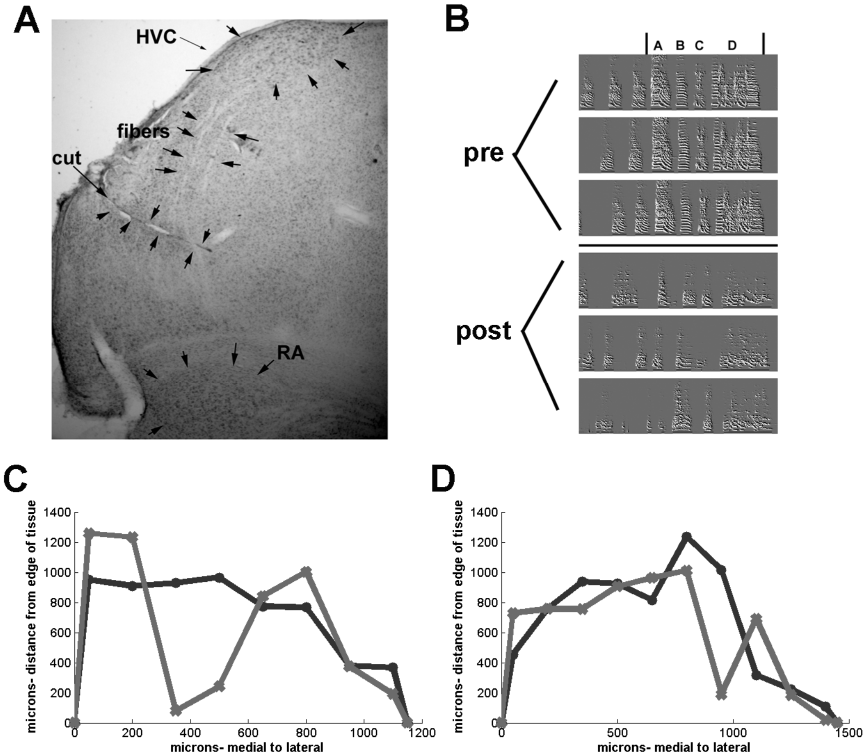

Figure 4.4: An example of a partial (78.5% overall) tract cut **A)** Sagittal NISSL section from one hemisphere showing a successful cut at this laterality. HVC-RA fibers appear as white lines in the tissue and the tract cut is clearly visible. **B)** Example spectrograms from before and after tract cut. This animal was scored as having a song quality of 2.333 out of 5 by blind observers. **C)** Extent of cut at different sagittal planes throughout the fiber tract. The dark line is the approximate maximum depth of the HVC-RA tract, and the lighter line is the depth of the cut. This animal received a surgery composed of two separate cut sites per hemisphere, thus the two peaks in cut extent. **D)** Right hemisphere, as in C.

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VITA

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