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Investigating Vocal Deficits in Songbirds with Neurotoxin Induced Dopamine Depletion in the Basal Ganglia

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Investigating Vocal Deficits in Songbirds with Neurotoxin Induced Dopamine Depletion in the Basal Ganglia

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Physiological Science

by

George Wagdi Hafzalla

2014

ABSTRACT OF THE THESIS

Investigating Vocal Deficits in Songbirds with

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by

George Wagdi Hafzalla

Master of Science in Physiological Science

University of California, Los Angeles, 2014

Professor Stephanie A. White, Chair

Dopamine (DA) input from the midbrain modulates the activity of basal ganglia (BG) circuitry important for motor learning and control in a variety of taxa. DA loss is associated with movement disorders in humans. In songbirds, DA is important for motivational behavior underlying reproductive drive. Within the zebra finch species, DA modulates social-context dependent behavior when the bird is vocally practicing alone versus performing to a potential female mate. During these singing behaviors, there are differences in DA levels within Area X, the specialized sub-region of the zebra finch BG dedicated to song learning and ongoing adult song maintenance. These natural differences in DA levels are associated with quantifiable changes in features of song, suggesting that the songbird may be a suitable model for

investigating DA-driven changes in voice associated with early stages of Parkinson's Disease. In the present study, I used Western blotting to characterize natural changes in protein biomarkers of DA, such as tyrosine hydroxylase, across non-singing and singing behaviors. In a separate group of birds, I injected the neurotoxin 6-hydroxydopamine (6-OHDA) into Area X and assessed the effects of DA depletion on these biomarkers and on features of song in different behavioral contexts. With 6-OHDA administration, measurable decreases in DA biomarkers were detected, and select acoustic features of song became more stereotyped. Ongoing investigations will determine how this DA loss impacts receptor-mediated changes in the underlying neural circuitry.

The thesis of George Wagdi Hafzalla is approved.

Walter Metzner

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University of California, Los Angeles

2014

DEDICATION

This thesis is dedicated to the memory of my cousin, Nader Hafzalla.

I miss him every day.

His courage and strength I carry with me.

This thesis is also dedicated to…

… My father, Wagdi Hafzalla, for pushing me to realize my dreams

… My mother, Amal Hafzalla, for her love and endless support of my pursuit to happiness

… My sister, Mary Hafzalla, for always being there

… My sister, Michelle Hafzalla, for being my safe place to pour out my emotions

… My brother, John Hafzalla, for always caring and teaching me warmth and kindness

… My cousin, Amir Hafzalla, for being an older brother to me

… My cousins, Mark and Genevieve Yousef, for providing me a home away from home

… My extended family that never stops believing in me

Finally, this thesis is dedicated to all the scientists whom I call friends.

Thank you Stephanie White, Julie Miller, Nancy Day

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…Debora Lee, and Jingwen Yao

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INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder caused by preferential loss of mesencephalic (midbrain) dopamine (DA) neurons located in the substantia nigra (compact pigmented part; SNc) and ventral tegmental area (VTA). These nuclei project axons to the basal ganglia (BG), a group of structures situated at the base of the prosencephalon (forebrain). The BG are strongly connected with the cerebral cortex, thalamus, and brainstem and function to initiate voluntary movements, integrate motor and sensory information, as well as information about cognition and emotions. For that reason, the loss of DA neurotransmission to the BG results in a variety of symptoms.

PD is characterized by a collection of movement-related symptoms including resting tremor, rigid muscles, bradykinesia, impaired posture and balance, as well as vocal deficits[1]. PD is the most frequent neurodegenerative condition of the aging brain following Alzheimer's disease. According to the World Health Organization, the number of cases worldwide is expected to rise dramatically as life expectancy in industrialized countries increases. In the United States, epidemiological studies estimate 1,200,000 individuals affected by PD and 50,000 new cases reported each year[2].

Current diagnosis of PD is based on non-vocal motor symptoms that are present once the disease has reached an advanced state. The first symptoms appear when 50-80% of dopaminergic neurons have already been lost[3]. For this reason, researchers have made efforts to identify biological markers (biomarkers) for PD. In 2011, the U.S. Food and Drug Administration (FDA) approved DaTscan (Ioflupane I 123 injection, also known as phenyltropane), a diagnostic test to aid in PD diagnostics. This radiopharmaceutical agent

is injected into a patient's veins and activity of DA active transporter (DAT) is viewed with single-photon emission computed tomography (SPECT). However, this test does not diagnose PD and is instead used by clinicians to help differentiate between essential tremor, PD or another Parkinsonism (i.e. different etiologies resulting in a similar set of symptoms as found in PD). Furthermore, there is currently no biomarker capable of definitively diagnosing PD, which results in under- or mis-diagnosis of PD[4]. Clinicians commonly use the Unified PD Rating Scale (UPDRS) to follow the progression of a patient's PD. Additionally, they rely on the presence of a 4-6 Hz resting tremor and/or favorable patient responses to the DA precursor, levodopa, to diagnose PD[4].

Between 70-90% of patients with PD show some form of vocal impairment[5], with only 3-4% of people with PD receiving speech treatment[6]. Coordination of speech is highly complex, involving motor subsystems that control respiration, phonation, and articulation; therefore, it has been widely hypothesized that vocal deficits in PD may precede non-vocal motor symptoms as a result of slight degenerative changes in BG circuitry[4, 7]. The identification of an early biomarker for PD has several implications, which are discussed later in this section.

In summary, the link between the loss of DA input to the BG, the primary culprit in PD, and speech problems is not well-understood, thereby providing the impetus for this study. In my research, I have used the zebra finch songbird, a specialized champion of vocal learning and production, to model vocal deficits in PD and investigate how DA loss affects song and through what molecular pathways.

Characteristics of speech

Several components comprise speech, including phonation, respiration, resonance, articulation, and prosody. Phonation is the vibration of the vocal cords to create sound. This requires flexible vocal folds and adequate respiratory expiration[5]. Some commonly used tests of phonation in PD include measurement of the fundamental frequency or pitch (FF or F_0: lowest frequency of a periodic signal), the extent of variation of voice range (jitter), the extent of variation of expiratory flow (shimmer), and the amplitude of noise relative to tonal components in the speech (NHR ratios)[5]. On the other hand, resonance arises from vibrations in the chest, pharynx, and head that selectively amplify specific component frequencies[5]. Articulation of speech is defined as the formation of consonants and vowels by controlling and coordinating the lips, tongue, palate, and pharynx[5]. Finally, the variation in loudness, pitch, and timing accompanying natural speech is termed prosody[5].

Hypokinetic dysarthria in PD

Dysarthria is a motor speech disorder in which the muscles of the mouth, face, and respiratory system become impaired as a result of neurological injury. It may be classified into six major types: 1) flaccid, 2) spastic, 3) ataxic, 4) hyperkinetic 5) hypokinetic, and 6) mixed dysarthrias. Dysarthria results from abnormalities in speed, strength, range, steadiness, tone, or accuracy of movements that can affect the control of the phonatory, respiratory, resonatory, articulatory, and prosodic aspects of speech[8].

Vocal deficits in PD are characterized by hypokinetic dysarthria, which results directly from the loss of DA producing cells in the SNc and VTA. This causes muscle rigidity, altering muscular control of the larynx (phonatory subsystem), increasing laryngeal tension, which may lead to decreased F_0 range and variability of speech[4]. Some characteristics of hypokinetic dysarthria include: reduced voice quality, reduced loudness (or quietness), breathy voice quality, monopitch, voice tremor, inappropriate silences, speech intelligibility, pitch level low, variable rate, imprecise consonants, reduced stress, monoloudness, and short rushes[9].

Progress towards using speech assessment as a biomarker for PD

In the early 1960s, two studies noted a decrease in F_0 range during syllable production of speech in PD patients[10, 11]. Later in the 1980s and 1990s, other researchers observed a decrease in F_0 range and variability during reading tasks (Flint 1992, Metter and Hanson, 1986). More evidence suggested that early stage PD patients exhibited at least two characteristics of dysarthria unbeknownst to them (Stewart 1995). Later, a retrospective analysis proposed that changes in pitch variability could be detected as early as five years prior to diagnosis (Harel 2004). Taken together, it is plausible that acoustic parameters of speech may be used as an early biomarker for PD, which may have several ramifications. For instance, recruiting patients into clinical PD trials may be improved if a speech assessment could be performed remotely rather than relying on the UPRDS, which must be done in person and takes at least 15 minutes[5]. Indeed, mathematical analyses of voice patterns are being performed to determine the feasibility of this approach. The Parkinson's Voice Initiative (PVI) is a new study that allows people with, and without,

PD to contribute data collected by their smartphones for the investigation of voice measurements and the onset of PD. One of the main goals of PVI is to find the signs of the disease before the damage done is irreparable.

Voice treatment in advanced stage PD patients

In advanced stage PD patients who suffer from hypokinetic dysarthria and are resistant to pharmacological treatment (e.g. L-DOPA), a targeted voice therapy called Lee Silverman Voice Treatment (LSVT) was shown to be effective. LSVT targets motor output during speech production by training increased vocal effort and loudness, while also training individuals to monitor their own vocal output[5]. Moreover, deep brain stimulation of the subthalamic nucleus (DBS-STN) dramatically improves non-vocal motor function, however, its effects on speech are inconsistent.

DA function, biosynthesis, and signaling pathways

In the brain, DA functions as a neurotransmitter and plays a role in many distinct systems, including motor control, motivation, arousal, cognition, and reward. For example, DA producing cells in the SNc form a component of the BG and are involved in motor control whereas the VTA is a part of the limbic sector of the BG and plays a role in reward and aspects of motivation[12]. Outside the nervous system, DA has specific functions in blood vessels, in the kidneys, in the digestive system, in the immune system, and the pancreas.

The pathway of DA biosynthesis is summarized schematically in **Figure 1**. The enzyme tyrosine hydroxylase (TH) is involved in the rate-limiting step for catecholamine biosynthesis, including DA. Notably, DA is the first catecholamine synthesized from L-3,4-dihydroxyphenylalanine (L-DOPA or levodopa).

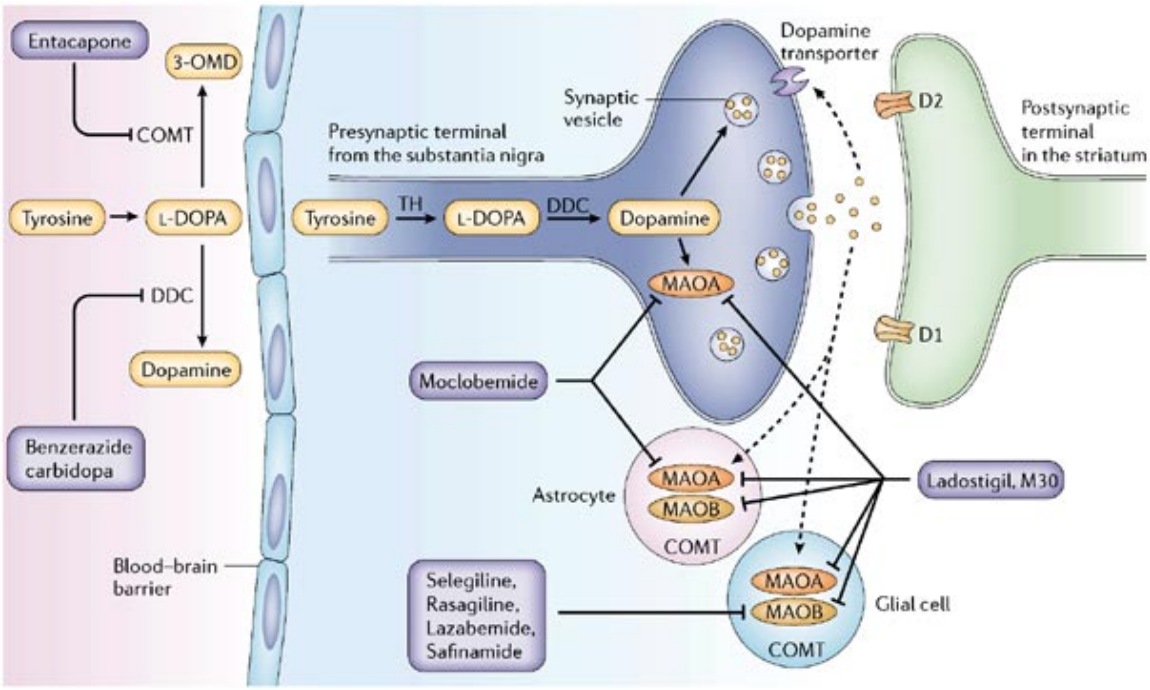

Figure 1: Pathway of DA synthesis: Tyrosine catalysis by tyrosine hydroxylase to L-DOPA, which is further modified to DA decarboxylase (DDC) to DA. *Reprinted by permission from Nature Reviews Neuroscience: Youdim et al., 2006.*

As shown in **Figure 1**, the striatum is strongly modulated by DA input from the SNc (as well as VTA, not shown). DA neurotransmitter acts on DA Receptor 1 (D1R) and DA Receptor 2 (D2R), which are located on medium spiny neurons (MSNs) and have antagonistic postsynaptic effects. In mammals, D1R and D2R are found on separate

MSNs, forming direct and indirect pathways. In contrast, in the zebra finch songbird species (and possibly all birds), MSNs may express either D1R or D2R, but may also express both on the same cell. Thus, the opposing effects of D1R and D2R can occur in the same cell in the songbird BG[13].

Activation of D1R enhances cAMP formation in D1R-expressing MSNs resulting in increased phosphorylation of DA- and cAMP-regulated neuronal phosphoprotein (DARPP-32; also known as protein phosphatase 1 regulatory subunit 1B, PPP1R1B) at the threonine-34 site. Activation of D2R decreases cAMP formation in D2R-expressing neurons resulting in decreased phosphorylation at the threonine-75 site. Phosphorylated DARPP-32 is a potent inhibitor of protein-phosphatase-1 (PPP1CA) and activation of N-methyl-D-aspartate receptors (NMDARs) reduces PPP1CA inhibitory activity of DARPP-32. NMDARs are found in all BG nuclei, with the highest density being found in the striatum and they function to integrate signaling by many transmitter systems[14].

The Lewy Body in Parkinson's disease

Many neurodegenerative diseases are associated with histopathological hallmarks, including protein aggregation and inclusion body formation. In Alzheimer's disease, misfolded β-amyloid proteins aggregate and form plaques, which are found throughout the brain. Similarly, in PD, harmful deposits of protein termed Lewy bodies are found in nerve cells. More than 70 molecules have been identified in Lewy bodies, but the main constituent is a presynaptic protein called α-synuclein[15]. The misfolding of this protein overwhelms normal quality-control systems, such as molecular chaperones, ubiquitin-

proteasomes and phagosome-lysosome systems leading to oxidative stress, protein sequestration, disruption of axonal transport, synaptic dysfunction, and mitochondrial dysfunction[16]. This is summarized schematically in **Figure 2**.

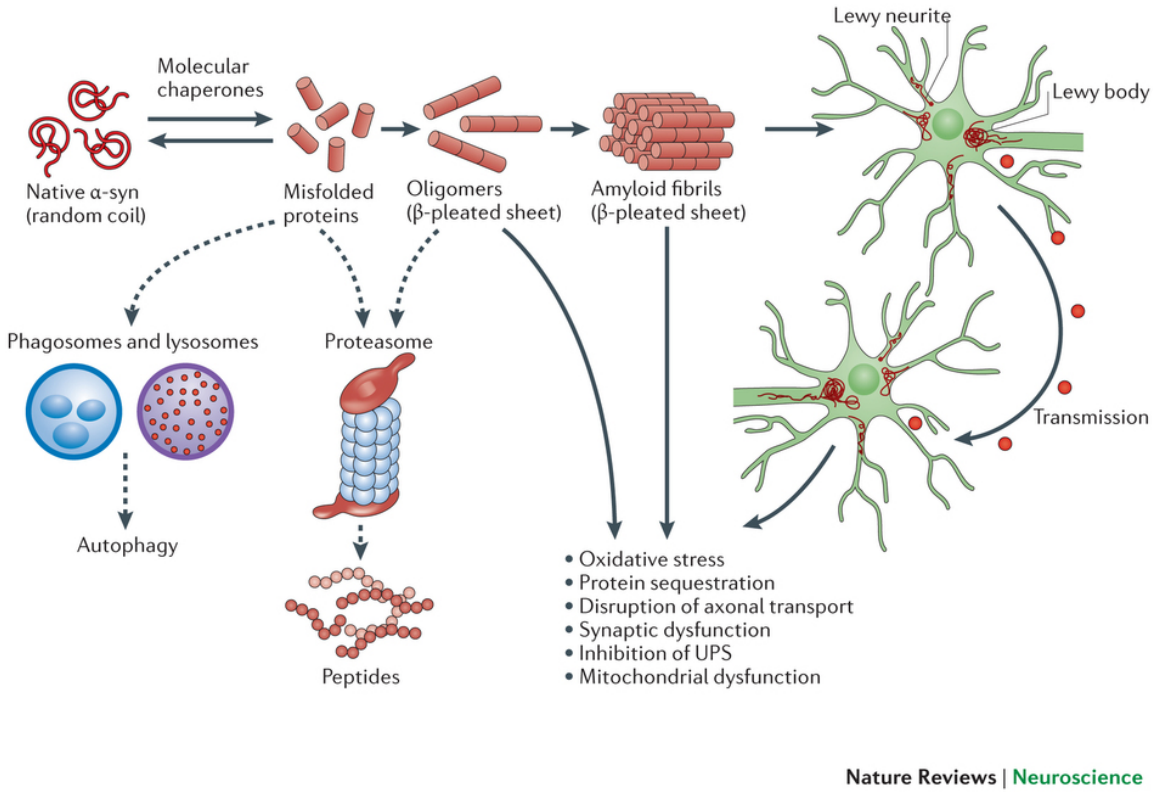

Figure 2. Hypothetical model of α-synuclein toxicity and spread of pathology in PD. α-synuclein exists in a random coil state under physiological conditions, however in PD this protein undergoes misfolding into pathogenic species of α-synuclein (dimers, trimers, oligomers) that further aggregate into higher order structures (protofibrils, other intermediates and amyloid fibrils). These higher-order structures lead to pathological inclusions of α-synuclein (i.e. Lewy bodies and Lewy neurites). *Reprinted by permission from Nature Reviews Neuroscience: Irwin, Lee, & Trojanowski, 2013*.

Animal Models of Parkinson's Disease

Despite several significant breakthroughs in understanding PD, its enigmatic etiology and pathology have perplexed researchers for decades. Several animal models have been employed including, neurotoxin-based, pesticide/herbicide exposure, and genetic models—each offering certain advantages and disadvantages. Here, I only discuss leading neurotoxic models of PD.

Neurotoxin Models:

In the brain, the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is taken up by astrocytes and then metabolized by monoamine oxidase-B (MAO-B) to form MPP+. Evidence suggests that astrocytic OCT-3 transporters release this active metabolite into the extracellular space[17]. MPP+ is then taken up by dopaminergic neurons via the DA transporter and stored in vesicles. Due to limited storage capacity of vesicles, DA is pushed into the intracellular space and is metabolized into a number of toxic compounds (e.g. dihydroxyphenylacetaldehyde also known as DOPAL) or generates reactive oxygen species, such as superoxide radical (5-cysteinyl-DA).

Although far from a true model of PD, the MPTP model exhibits some of its hallmarks, including oxidative stress, reactive oxygen species, energy failure, and inflammation, as tested in monkeys and other higher mammals[18]. Moreover, a number of these hallmarks occur in mouse models; however, rats are impervious to this toxin. Another shortcoming of this model is the absence of Lewy body formation.

6-hydroxydopamine (6-OHDA) is a different compound used to model PD, and like the MPTP model, has its limitations. This compound does not cross the blood-brain barrier and must be directly injected into the SNc/VTA or the striatum in order to initiate DA loss. TH-positive nerve terminals break down prior to the death of TH-positive neurons in the SNc and therefore several researchers inject this compound into the striatum to investigate retrograde degeneration[18, 19]. 6-OHDA is thought to enter nerve terminals via DA and noradrenaline (norepinephrine) reuptake transporters. With respect to its mode of action, it is well accepted that 6-OHDA forms free radicals and is a potent inhibitor of mitochondrial respiratory chain complexes I and IV. These modes of action are thought to be independent[20]. 6-OHDA is produced endogenously as a product of DA metabolism[21] and thus serves as an attractive model of PD for several reasons: 1) it leads to partial damage of DA terminals, 2) it leads to delayed and progressive loss of nigral DA neurons, 3) it mimics many biochemical features of PD including reduced levels of striatal DA and TH **(Figure 1**) and 4) it interacts with α-synuclein[22]. Like many other neurotoxic models, no Lewy bodies are observed as a result of 6-OHDA administration.

To date, only one study has been published using 6-OHDA in songbirds, despite the fact that, like humans and unlike rodents, they are vocal learners. As detailed below, in species such as the zebra finch, young birds learn to copy the song syllables of their adult tutors, and later sing these songs to court mates. Hara et al. performed unilateral injections of 6-OHDA into male zebra finches VTA-SNc. Relative to sham controls; no changes were observed in syllable structure. However, the rate of males' song directed

towards females was reduced. In addition, in three birds that had undergone both VTA-SNc and locus coeruleus lesions, syllable changes were observed.

Further, no research has yet been published on injections of 6-OHDA into the BG song control region of the songbird brain, known as Area X. This is surprising because it is traditionally thought that 6-OHDA enters dopaminergic neurons through DAT terminals, which are heavily expressed in Area X. Given the role of Area X in the modulation of song (see below), I hypothesized that 6-OHDA administration would result in detectable changes in song syllables.

BACKGROUND

Birds and Vocalizations

Vocal learning, the ability to modify or imitate the acoustic structure and syntax of vocalizations, is a rare trait that has only been found in a handful of mammalian groups (e.g. humans, cetaceans, bats, and elephants) and three of some 30 avian orders (parrots, hummingbirds, and songbirds)[23]. In songbirds (order of Passeriformes) there are about 4,000 species; helping make songbirds the most extensively studied model organism for vocal communication. Moreover, bird vocalizations can be separated into two groups: 1) unlearned calls, such as alarm calls, contact calls, begging calls, etc. and 2) bird songs, associated with courtship and territorial defense.

Songbirds and Vocal Learning:

The study of birdsong exemplifies a neuroethological approach to understanding brain function, and because songbirds possess a rare trait, vocal learning, they are advantageous models for uncovering the neural basis for human vocal communication. As in humans, vocal learning in songbirds is restricted to a sensitive or critical period in development**.** Song learning occurs in two stages, sensory and sensorimotor learning **(Figure 3)**. During sensory learning, a juvenile bird listens to its tutor and memorizes the spectral and temporal components of the song. On the other hand, sensorimotor learning involves the juvenile bird producing its own vocalizations and practicing its song until it matches with the tutor song template. This exemplifies that song is not an innate behavior but instead is a learned behavior. Initial vocalizations in the sensorimotor stage are highly variable and called "sub-song," which is analogous to babbling in human infants. Certain songbird species such as the canary are able to imitate and spontaneously combine

learned sounds during all periods of their life, but the zebra finch is limited to song learning during a critical period in their development**.** In zebra finches, sensory and sensorimotor stages are overlapping and song becomes much more stereotyped, or 'crystallized' after about 2 months of practicing.

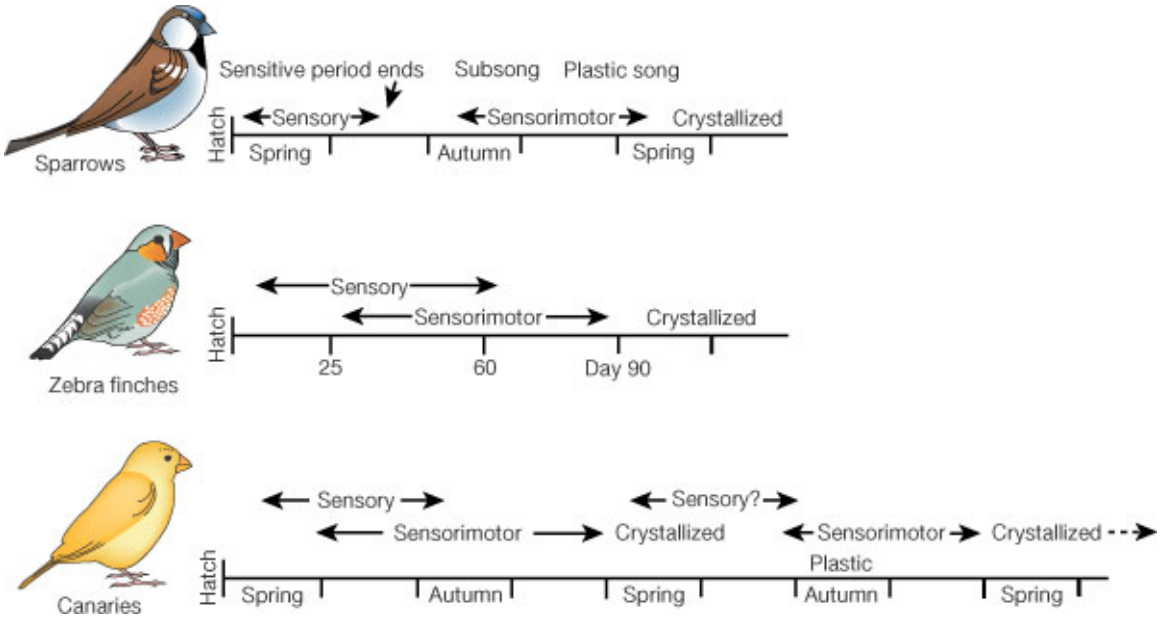

Figure 3. Timelines for song learning. Top: Seasonal "closed learners," such as white-crowned sparrows must learn their song during a critical period in the early weeks of their first breeding season. It is noteworthy that the sensory and sensorimotor phases can be separated in time. **Middle:** Zebra finches are "age-limited learners" and learn their song during a critical period in development. Here, the sensory and sensorimotor stages overlap. **Bottom:** Canaries are "open-ended learners," whereby new songs can be learned during a critical period in the early weeks of each breeding season. *Reprinted by permission from Nature insight review articles: Brainard and Doupe, 2002.*

Zebra finches are sexually dimorphic

Zebra finches (Taeniopygia *guttata*) exhibit a high degree of sexual dimorphism. Differences in specific physical attributes define males and females, such as the orange cheek patch found in males **(Figure 4)**. Brains of zebra finches contain several sexually dimorphic nuclei which are involved in the song control system[24]. For example, males receive denser dopaminergic input to nuclei involved in the song system than do females[25]. Most notably, song is sexually dimorphic in zebra finches, as males learn to sing and females only produce unlearned vocalizations.

Figure 4. **Sexual dimorphism in physical attributes.** Males have distinguishing features including orange cheek patches, stripes on the throat, a black bar on the breast,

and a chestnut colored flank with white spots. Females lack these features and are gray in those aforementioned areas. *Reprinted by permission from Digital Illustration: c2009 by Ken Gilliland.*

Zebra Finch as a Model Species

As mentioned above, there are numerous parallels between human speech and birdsong. As in humans, vocal learning in songbirds is restricted to a sensitive or critical period in development where complex vocalizations are learned—with a greater ability to learn early in life. Both humans and songbirds rely on auditory feedback to imitate adults, as well as themselves when they practice. Hence, these vocalizations are both spontaneously and socially learned. Beyond behavior, both have evolved a complex hierarchy of specialized forebrain areas in which motor and auditory centers interact closely, and which control the lower vocal motor areas[26] (**Figure 5**). The neuroanatomical circuits, including a cortical-basal-thalamic loop, are similar between humans and songbirds and are used during learning and production of vocalizations. Additionally, language related genes, such as *FoxP1* and *FoxP2*, are expressed in similar patterns in human and zebra finch brains[27]. Besides these similarities, zebra finches are an ideal model species because their song is quantifiable. Although other songbirds in which both males and females learn their songs and have full song control circuitry (e.g. duetting birds), zebra finches are an ideal model species because they breed well in captivity with short intergenerational times. Moreover, because song learning is sexually dimorphic in zebra finches, females offer a helpful 'negative control' for many aspects of experiments on the

neural basis of vocal learning. Finally, the crystallized song of adult male zebra finches provides an avenue to investigate vocal deficits in older human beings who develop PD.

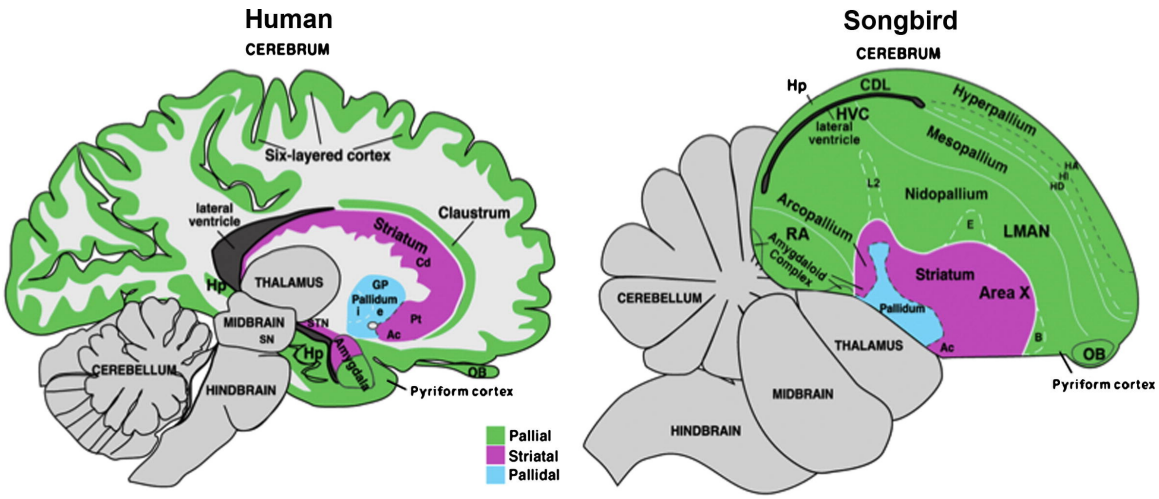

Figure 5: Comparative anatomy of songbird and human brains. Songbirds are able to learn songs similar to how humans learn the speech component of language. The highlighted areas are involved in song/language production. These anatomical correlates serve as an ideal model to study language at the circuit level. *Reprinted by permission from Elsevier, Brain and Language: Simonyan, Horwitz, and Jarvis, 2012.*

Song Production Circuit

Song learning and vocal motor production occur in separate neural pathways[28], as shown in **Figure 6A**. Song learning requires the anterior forebrain pathway (AFP). The AFP is important for the maintenance and modification of song. In this pathway, a subset of premotor cortical neurons in HVc (acronym used as a proper name) project to Area X, which relays this information to the lateral portion of the dorsolateral thalamus (DLM). DLM projects to the lateral portion of the magnocellular nucleus of the anterior

nidopallium (LMAN), which projects to the robust nucleus of the arcopallium (RA) and thereby connects the AFP to the vocal production pathway[29]. The vocal motor production pathway consists of projections from a separate set of HVc neurons to RA, which connects to the motor neurons of the tracheosyringeal portion of the hypoglossal nerve (nXIIts) to innervate the syrinx and respiratory muscles involved in singing[30]. The AFP in male zebra finches closely resembles the BG circuit in humans, as shown in **Figure 6C**.

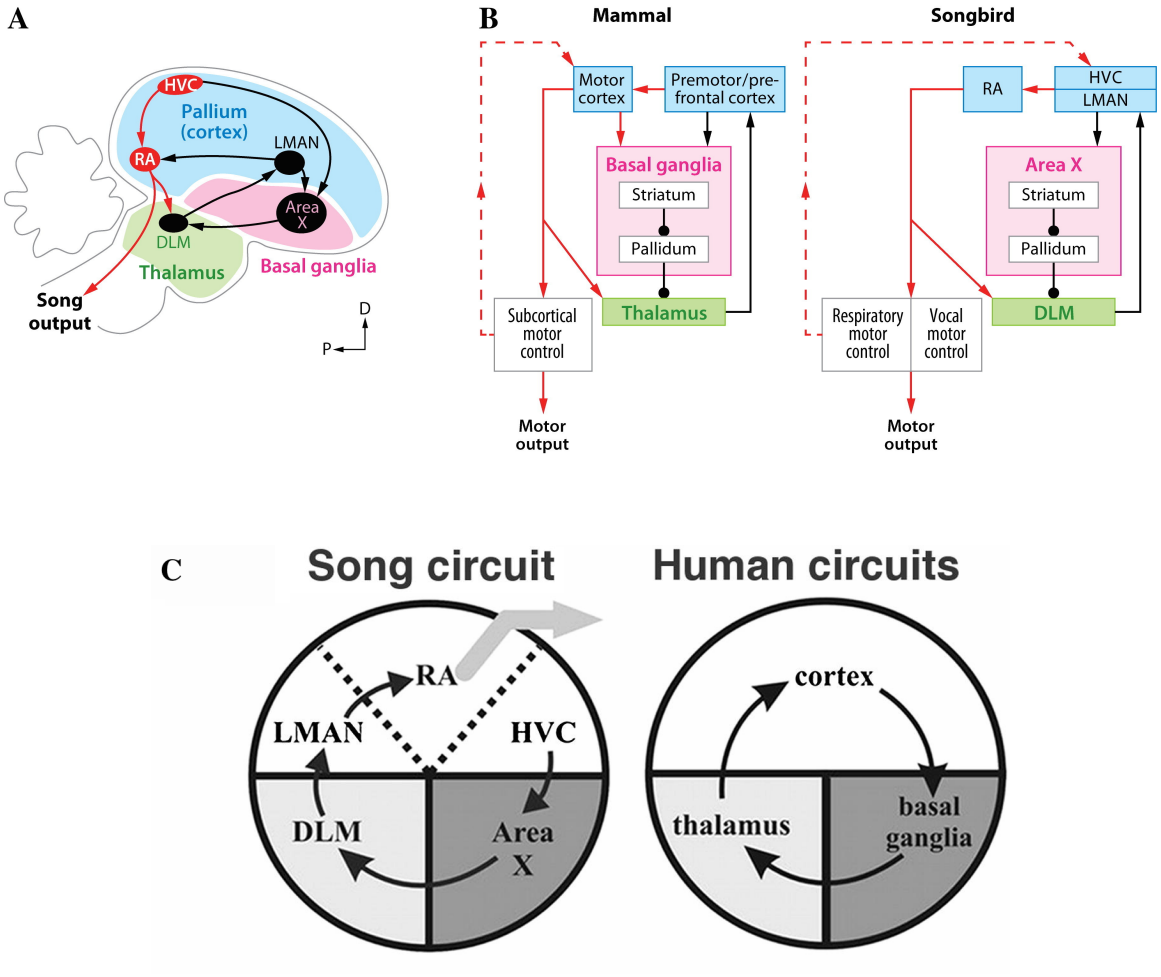

Figure 6: Songbird Song Control Circuit. A. A schematic of the neural substrates critical in the song system of zebra finch. The motor pathway (red arrows) includes HVC (abbreviation used as proper name) and the robust nucleus of the archipallium (RA). RA

projects to the tracheosyringeal portion of the hypoglossal nucleus (nXIIts), which controls the bird's vocal organ or syrinx, and to nuclei involved in control of respiration during song. In the anterior forebrain pathway (AFP; black arrows), which is responsible for song learning, HVc sends projections to Area X, which is analogous to mammalian BG. Area X sends projections to the medial nucleus of the dorsolateral thalamus (DLM), which sends its projections to the lateral magnocellular nucleus of the anterior neostriatum (LMAN). LMAN sends a projection back into the motor pathway at the level of RA. Like BG in other vertebrates, Area X is the target of strong midbrain DA projections. *Reprinted by permission from Annual Review of Neuroscience: Brainard and Doupe, 2013.* **B.** Block diagram highlighting parallels between mammalian and birdsong neural microcircuitry. *Adapted from Brainard and Doupe, 2013.* **C.** Songbird cortico-BG circuitry is simplified to illustrate song-specialized subregions that are embedded within similar brain areas in the human brain. *Reprinted by permission from Neuron: Hilliard et al., 2012.*

Social Contexts

Adult male zebra finches sing in at least two distinct social contexts, alone and to a female. When a male sings alone, referred to as undirected (UD) song, his songs are slightly more variable than when he sings to a female[31, 32]. For this reason, UD singing may be thought of as a form of vocal practice. In contrast, when a male sings towards a female, referred to as female-directed (FD) song, it is considered to be a performance state[33].

Song Structure

Teramitsu and White (2010) describe song structure as follows[34]:

The acoustic structure of birdsong is typically described as being composed of bouts, phrases, motifs, syllables, and notes. Notes are the smallest unit, combining together to form syllables. Syllables are separated from one another by silent intervals. Two or more syllables may group together to form phrases. A motif is a sequence of notes and/or syllables that are repeated in a stereotyped order. One or more motifs or phrases followed by a second or more of silence comprises a bout of song.

Notes and Syllable Types

Zebra finches produce diverse sounds that are acoustically complex, rich in harmonic structure, and variable among individual birds. Notes are elaborate structures that are modulated in amplitude and frequency and are highly variable in fundamental frequency; furthermore most notes include harmonics that vary in amplitude[35]. Remarkably, the maximum amplitude or energy across harmonics does not occur at the FF[36]. Instead, the amplitude increases harmonic by harmonic into the frequency range 2 kHz – 6 kHz, and then decreases. Hence, an amplitude envelope can be drawn enclosing these increasing and decreasing amplitudes. Furthermore, zebra finch syllables may be classified into five types: 1) short slide note, 2) slide note, 3) combination note, 4) flat harmonic stack and 5) high note. This is summarized in **Figure 7**.

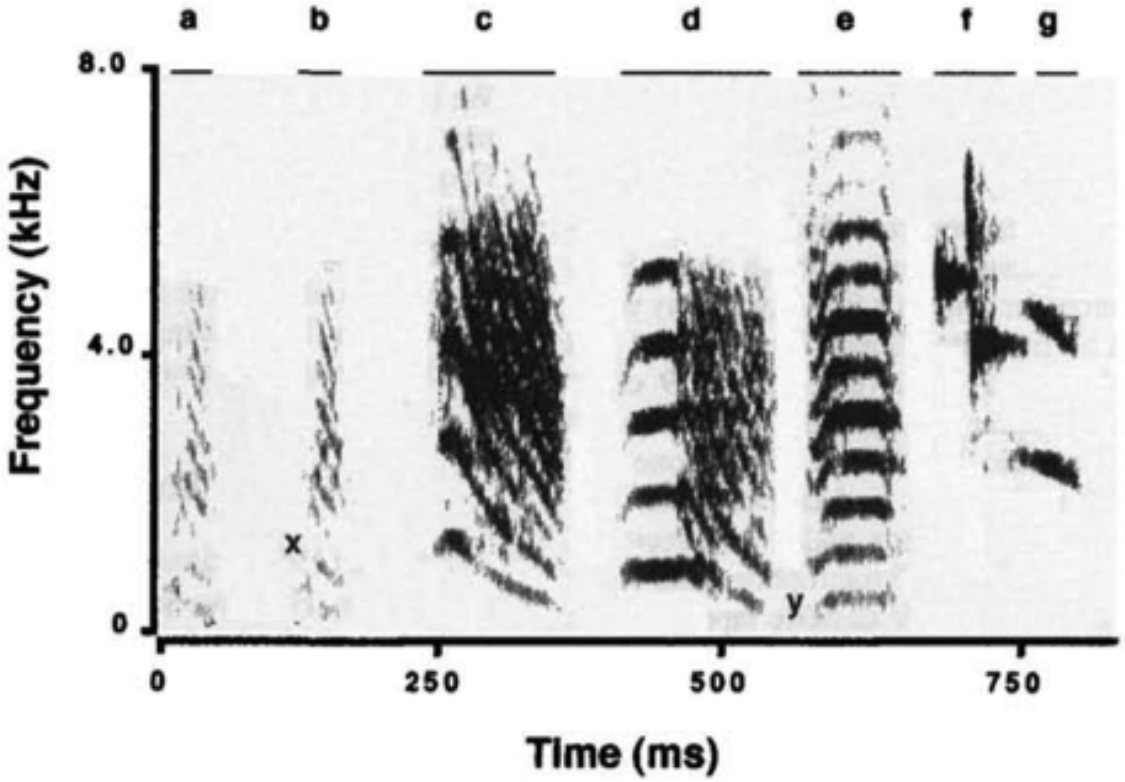

Figure 7. Proposed classification of zebra finch syllable types. A sound spectrogram of a typical zebra finch song, which contains two short slide notes (a, b), a slide note (c), a combination note (d), and a flat note (e). *Reprinted from Journal of Comparative Psychology: Sturdy et al., 1999.*

Moreover, UD song is accompanied by greater variability in the fundamental frequency of flat harmonic syllables compared to FD song[37]. DA may be important in modulating the differences between UD and FD song because higher levels of DA are released into Area X from SNc-VTA during FD song production[38-41].

METHODS

ANIMALS

All animal use was in accordance with NIH guidelines for experiments involving vertebrate animals and approved by the University of California at Los Angeles Chancellor's Institutional Animal Care & Use Committee. Adult male zebra finches 120-400 days of age were moved from our breeding colony to individual sound attenuation chambers (Acoustic Systems; Austin, TX) under a 14:10 hour light/dark cycle. This age range was chosen for two reasons: 1) adult song has crystallized and 2) to facilitate the detection of differences in fundamental frequency variability following 6-OHDA administration because it has previously been shown that birds of this age typically display greater variability in the fundamental frequency of harmonic stacks sung during undirected song relative to directed song[31]. Birds were left undisturbed for at least 2-3 days prior to the behavioral experiments to allow for acclimation to the new environment.

SONG RECORDING

Conditions: Male zebra finches were behaviorally tested in two distinct social contexts, singing alone (undirected, UD) and singing towards a female (female-directed, FD). UD song collection was ongoing and did not require the presence of an experimenter. FD experiments were run both pre-surgery (day 0) and post-surgery (day 4). In the event that insufficient UD or FD song was collected on day 4 post-surgery, song was additionally collected on day 5 post-surgery. This is summarized in schematic form (see **Figure 14** in Results Section).

Behavioral Paradigm: The male zebra finch was allowed at least 30 minutes to sing UD song in his isolation chamber. Immediately following this step, a metal grate divider that allowed visual but not physical contact between a male and female was placed in the center of the cage; song recorded under this context was categorized as FD song. To maximize FD song collection, males were presented with five novel, stimulus females every 3-5 min over 30 minutes[42, 43]. Each female could potentially be rotated in for a maximum of two times (preferred female), however, when a male did not sing to a female, we replaced the female with another bird. This ensured continual female-directed singing by the male zebra finch. For FD collection post-surgery, males were presented with the same set of preferred females in the same order. Moreover, a Logitech Webcam Pro 9000 (Fremont, CA) was mounted in the attenuation chamber in order to collect video recordings of UD and FD song. For FD song, male singing behavior, including body posture and orientation, was monitored. Prior to administration with 6-OHDA, UD and FD songs were tested for the presence of at least 20 motifs sung. Birds that did not meet these criteria in either session were excluded from my study.

Song Recording and Analysis:

Sounds were recorded using either a Countryman EMW omnidirectional lavalier microphone (Countryman Associates, Menlo Park, CA) or a Shure SM58 microphone (Nile, IL) and digitized using a PreSonus Firepod (44.1 kHz sampling rate, 24 bit depth; Baton Rouge, LA). Recordings were acquired and song features quantified using Sound Analysis Pro (SAP) 2011 software[44].

Vocalizations were recorded continuously at least one day prior to surgery through post-surgery day 5 or 6. For each bird, song data was collected in the same sound attenuation chamber (Acoustic Systems, Austin, TX) with the same microphone.

Song was hand-segmented on both the motif- and syllable-level. Motifs ranged from 3-8 syllables long and were identified as a repeated order of multiple notes, excluding introductory notes. Syllables were identified as sound envelopes that could be separated from other syllables by silence or local minima in amplitude. Motifs and syllables were analyzed as described below:

At the level of motifs, 25 renditions (unless otherwise indicated) were analyzed using asymmetric pairwise comparisons of time course, which enables comparison of the most similar sound elements in the two motifs, independent of their position (Sound Analysis Pro Manual). At the level of syllables, 25 consecutive renditions (unless otherwise indicated) were analyzed using symmetric pairwise comparisons of time course, which enables comparison of a single frame of one sound element or syllable to another (Sound Analysis Pro Manual). 25 renditions of motifs/syllables yielded 625 unique comparisons. SAP computes the mean absolute deviation of pitch, frequency modulation (FM), Wiener entropy, pitch goodness, amplitude modulation (AM) and computes difference measurements by taking the mean Euclidian distance between samples. Motifs and syllables were compared pre- and post-surgery and for self-similarity. 25 renditions of motifs/syllables were selected as the minimum song needed to evaluate song pre- and post-surgery based on Miller et al 2010.

Syllables were additionally assessed by SAP for the following mean features: duration, amplitude, FM, AM^2, entropy, pitch goodness, mean frequency. Mean values were obtained and coefficient of variation (CV) values reported. For flat harmonic syllables, fundamental frequency variability was measured using a custom written MATLAB code provided by Dr. Michael Brainard at the University of California San Francisco.

STATISTICS

Syllable independence

To determine whether syllables within a bird should be evaluated independently, or collapsed within one bird, SAP was utilized to compute similarity scores between groups of syllables in a pre-surgery bird. 25 renditions of one syllable were scored against themselves as well as 7 other syllables in the bird's repertoire. This generated 600 self-comparisons and 625 cross-syllable comparisons for each of the 7 additional syllables **(Figure 8)**. When a syllable is more similar to itself than other syllables, it is considered independent. Therefore a one-way analysis of variance (ANOVA) was performed on the similarity scores and a p-value of $p < 10^{-4}$ was achieved. This indicates that the syllables are independent of one another and therefore syllables were not collapsed within birds for the purpose of mean features and similarity scores. However, when multiple renditions were present in a motif, only the first occurrence of that syllable within each motif was used. This was done in order to prevent placing unequal emphasis on one unique element of a bird's repertoire.

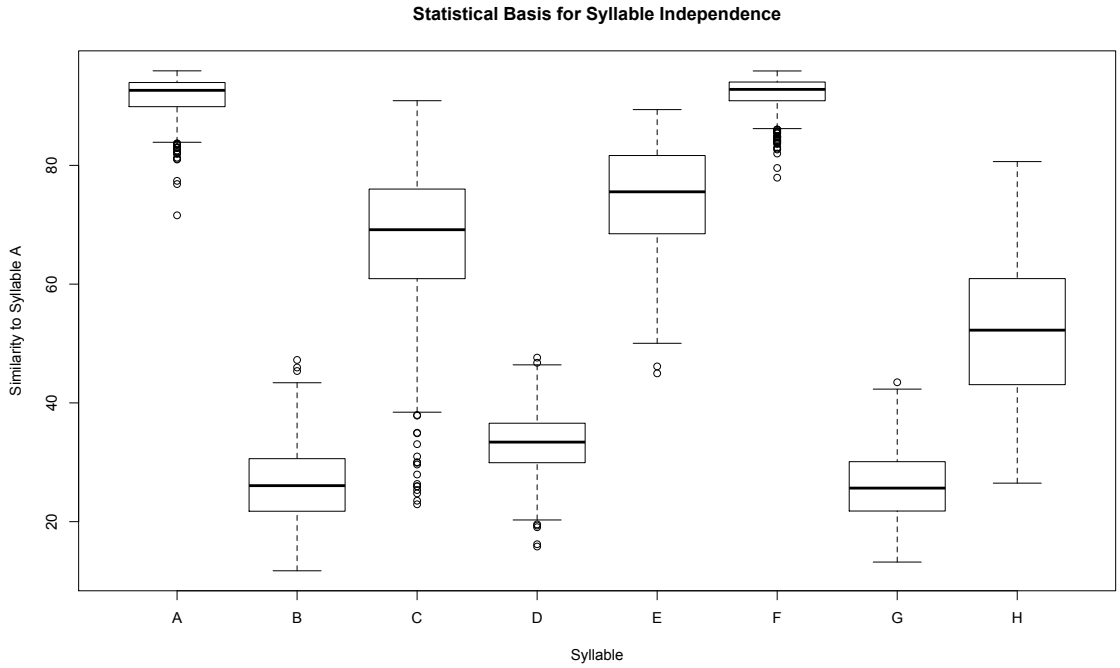

Figure 8. Acoustic similarity relationships between syllables from a zebra finch. A series of boxplots depicting similarity scores based on comparisons made between 25 renditions of syllable 'A' with 25 renditions of each other syllable in his motif (i.e. 600 self-comparisons and 25 cross-syllable comparisons). See **Figure 16** in Results Section for spectrogram of motif.

Independence of syllable fundamental frequency variability

To determine whether variability in fundamental frequency differed across syllables, a resampling test was performed between the most variable and least variable syllable from one bird. The actual pitch scores from 25 renditions of each syllable were used to generate a CV and the difference between these CVs was used as a test statistic. Due to the large difference in mean pitch for these syllables, actual pitch scores were 'de-meaned' by the group mean for each syllable. This allows preservation of variance while

setting the means to be equal across these syllables. The de-meaned observations were combined into a resampling pool and groups equivalent to the number of original observations were drawn from this pool with replacement. CVs were calculated for each resampled group and the difference in CV was calculated and stored. This was repeated 10,000 times to generate a distribution of CV differences. The actual test statistic was then compared to this distribution to generate a p-value of 0.0026, which indicates a statistically significant variability between these two syllables was observed **(Figure 9)**. Therefore, for the purposes of variability analyses, all syllables were treated as independent of each other. Because significant differences in variability were observed for one syllable feature, all syllables were treated independently for all other acoustic feature variability analyses.

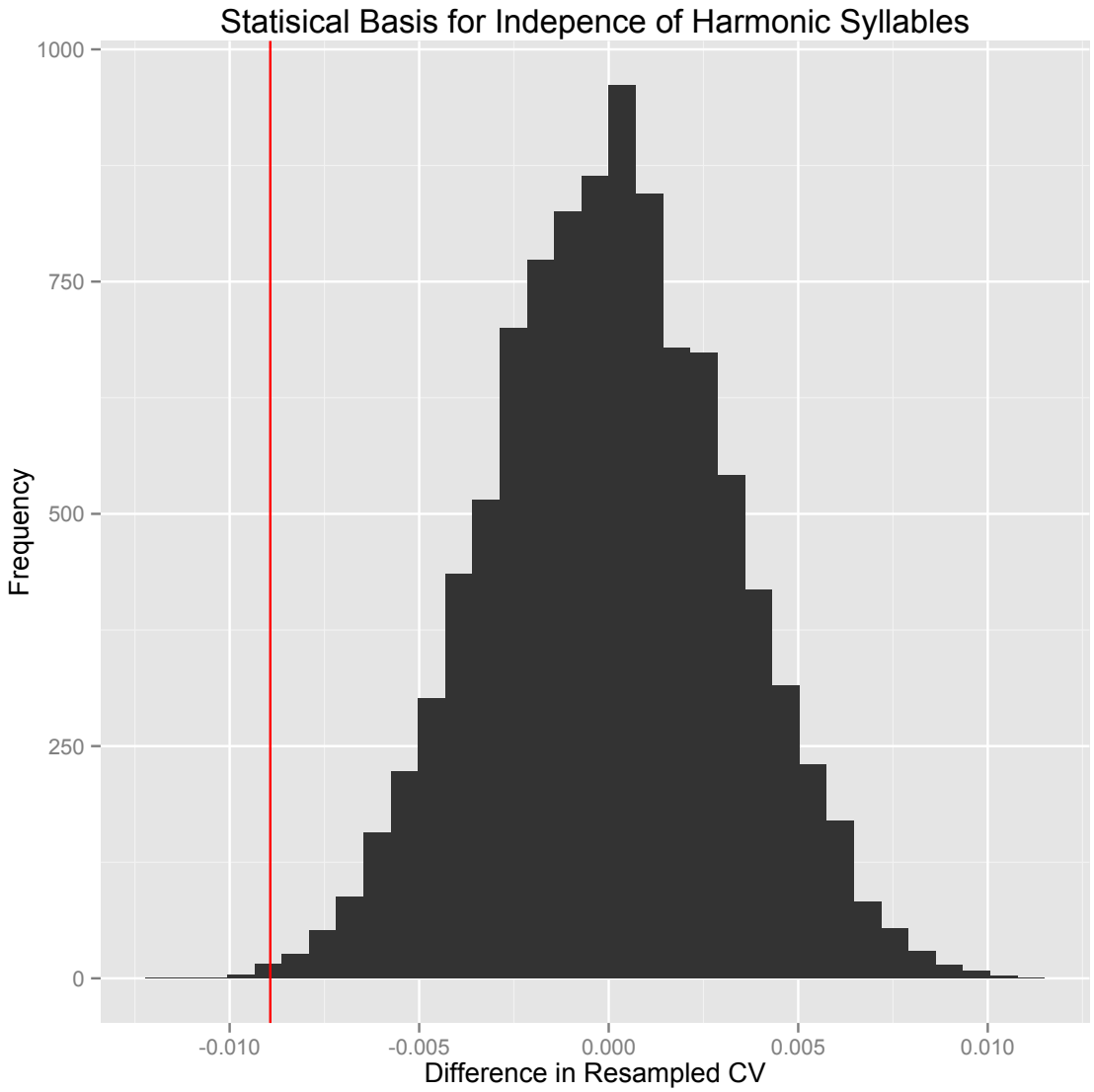

Figure 9. Resampling statistics to test for syllable independence. The actual test statistic (in red) was compared to the distribution of resampled CV differences. This generated a p-value of 0.0026, which indicates a statistically significant variability between the two observed syllables. Therefore, all syllables were treated as independent of each other for the purposes of CV analyses.

Assessment of vehicle and 6-OHDA administration effects

To assess administration effects on UD and FD song, two-tailed paired t-tests were performed. Paired tests were used as the two dependent variables (UD and FD) were obtained from the same bird. Significance was determined at a confidence level of α=0.05.

Quantifying TH depletion by Western blotting

The intensities of immunological signals ('bands') were quantified using Quantity One software (Bio-Rad, Hercules, CA) with each lane normalized to loading protein control, GAPDH (Millipore #MAB374). Quantification was confirmed further using ImageJ software (U.S. National Institutes of Health, Bethseda, Maryland, USA). Mann-Whitney U-test determined statistical significance.

STEREOTAXIC NEUROSURGERY:

All surgical instruments were sterilized preoperatively while the vaporizer for isoflurane anesthesia was prepared and the homeothermic blanket was allowed time to become warm for the bird. Males were anaesthetized with 2% isoflorane and placed in a custom built avian stereotax (Herb Adams Engineering, Glendale CA). Small scissors were used to cut the feathers to expose the ear holes. Ear bars secured the head while a beak holder secured the beak in order to immobilize the head. The head was held at a 45-degree angle relative to the vertical axis. Blunt forceps were used to pluck the feathers from the head, which exposed the scalp. The scalp was sanitized with iodine and kept moistened with saline solution. Subcutaneous injections of 0.5% (at 2 mg/kg body weight) bupivicaine, a

local anesthetic, were then administered bilaterally around the planned incision site. The skin was cut in a U-shape whereby it would attach caudally to the scalp in order to preserve vasculature. The skin was folded back over the head using a small sterile moistened sponge to reveal the midsagittal sinus (Y-shaped). A targeting electrode was moved to the posterior end of the bifurcation marking 0 rostrocaudal and 0 mediolateral coordinates (origin). To target Area X, the tip of the electrode was moved to these coordinates: 5.15 mm rostro-caudal, 1.5-6 mm ventro-lateral. A small window was cut into the dura mater layer of the scalp to reveal the cerebrospinal fluid at these coordinates. A nanoject device (Drummond Nanoject II, Drummond Scientific Inc., Broomall, PA) containing a glass electrode pipette (inner diameter 30-50 μm) for the injection site was back-filled halfway with mineral oil then with freshly made 6-OHDA solution just prior to the injection. To target Area X, the injecting electrode was moved 3.1-3.15 mm depth (z-axis). At the desired depth, 55.2 nL aliquots were delivered by hitting the inject button once every 15 seconds for a total injected volume of 0.5μl unless otherwise indicated, then the pipette was slowly retracted after a 5 minute diffusion period. Once completely retracted, the electrode tip was checked to ensure that it had not become clogged during the injection process. The scalp flap was replaced and the incision closed using Vetbond tissue adhesive (Santa Cruz Animal Health, Santa Cruz, CA). Then the bird was removed from the stereotaxic apparatus and placed in a small transport cage until awakening. The bird was returned to its sound attenuation chamber and monitored for general health and vital signs.

SACRIFICING CONDITION

For comparisons between vehicle- and 6-OHDA administered groups, birds were sacrificed at lights-on using an overdose of inhalation anesthesia (isoflurane) for euthanasia. This was done in order to prevent social context dependent related and/or circadian changes from influencing the amounts of protein present for each group. For other experiments, the sacrificing condition(s) are indicated in the Results Section.

HISTOLOGY

Tissue collection:

Tissue punches were obtained using methods described in Miller et al., 2008. Sections of 30 μm thickness were collected and thionin Nissl stained for visualization of Area X. Bilateral punches were then obtained for Area X, ventral striato-pallidum (VSP) as well as nidopallium (NP) to serve as a negative control **(Figure 10)**. Punches were obtained at a depth of 1 mm using a 20-gauge Luer adaptor (Becton Dickinson, Sparks, MD) attached to a 1 mL syringe and combined in 40 uL of protease inhibitor containing RIPA lysis buffer. Tissue was homogenized and 4 aliquots were made with 2x Laemmli loading buffer (Bio-Rad) with 0.1% beta-mercaptoethanol. Tissue was stored at -80°C until use.

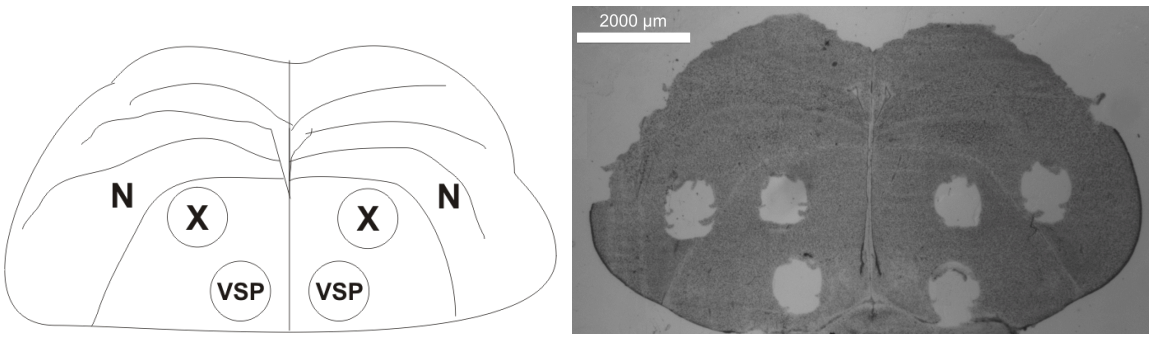

Figure 10. Tissue punches in zebra finch brain. Schematic of a coronal zebra finch brain section (left) and thionin stained section for Nissl bodies (right) show the locations of bilateral tissue punches obtained from Area X, VSP and Nidopallium (N). Schematic modified from Miller et al. 2008 & Hilliard, Miller et al., *Neuron*, 2012.

Thionin Nissl stain:

Brains were sectioned on a cryostat at 30 µm and thaw-mounted on slides, then dehydrated through graded alcohols and stained with 0.1% thionin. This staining procedure was used to estimate the location of Area X and VSP prior to extraction of tissue and was also used to visualize tissue in order to evaluate the accuracy and depth of tissue punches.

Quantification of DA depletion:

Bilateral tissue punches were obtained from Area X, ventral striato-pallidum (VSP) and nidopallium (N). Protein lysates were run on a 10% SDS-PAGE gel followed by conventional Western blotting. Signals were quantified using ImageJ software and normalized to GAPDH (Millipore #MAB374; Miller al al. 2008). DA signaling markers were detected using antibodies to TH (1:1,200 or 1,500 dilution; Millipore #AB152) and DARPP-32 (1:30,000 dilution; Abcam #ab40801; Murugan et al., *Neuron*, 2013). Blots

were then probed with horseradish peroxidase conjugated anti-rabbit IgG (1:2000 for TH, 1:30,000 for DARPP-32) and anti-mouse IgG (1:10,000 dilution; Amersham Pharmacia Biotech).

RESULTS

Stereotaxic targeting and determination of 6-OHDA concentrations

In order to create a songbird model of vocal deficits in PD, both the amount and volume of 6-OHDA neurotoxin injection were evaluated to determine a dose that did not mechanically damage cell bodies of MSNs within Area X while still causing depletion of DA input to these neurons. Stereotaxic coordinates for targeting Area X were previously determined in the White laboratory using injections of tetramethylrhodamine 10,000 MW lysine-fixable dextran (fluororuby dye liquid; Molecular Probes, Eugene, OR; **Figure 11)**. These coordinates yielded consistent bilateral targeting of Area X in the experiments discussed here.

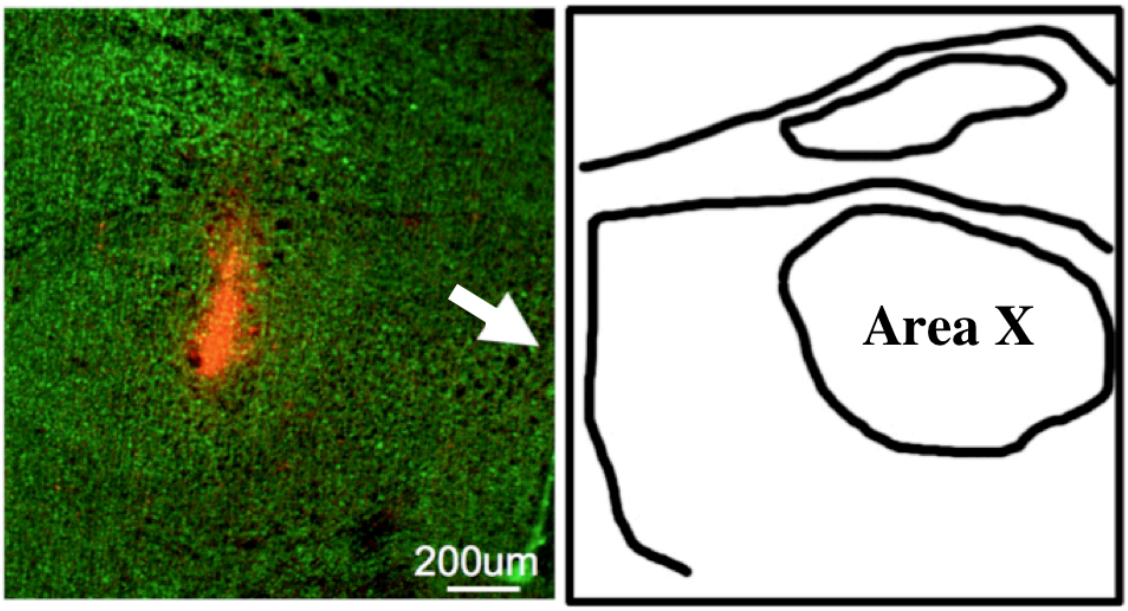

Figure 11: Targeting stereotaxic injections to Area X. A hemicoronal section of fluororuby dye injection to Area X with schematic mirrored opposite. Green fluorescent Nissl stain denotes cytoarchitecture and red fluororuby dye illustrates accurate targeting of Area X. White arrow denotes the midline. *Image obtained from Burkett, MS Thesis, 2011.*

Prior to my study, an effort was made to develop a 6-OHDA songbird model of vocal deficits in PD. In this prior study[45], 8 μg doses of 6-OHDA delivered to Area X in volumes ranging from 0.25 μl to 1 μL in 0.1% sodium ascorbate resulted in bird mortality. In contrast, 4 μg of 6-OHDA in 0.25 μL was not lethal and no mechanical damage to striatal tissue was evident. In addition, there were promising behavioral effects that were specific to song with no evidence of general motor deficits after scoring non-vocal behaviors in 6-OHDA administered animals. However, untimely changes in the apparent potency of 6-OHDA in subsequent drug lots from the manufacturer muddied these preliminary findings and mandated a re-evaluation of the efficiacious dose and volume. This work is extended here in my study.

Because of the new lot of 6-OHDA, and in recognition of its rapid oxidization, a series of surgeries were performed in order to once again determine ideal amounts and volumes that could be effectively administered to Area X. Unlike the prior studies, 4 μg of 6-OHDA in 0.25 μL resulted in mechanical damage to Area X as evidenced by the appearance of holes in the target sites upon tissue collection. This was also true at doses of 2 μg and 3 μg, delivered in the same volume. Based on these outcomes, two important conclusions were reached: 1) Area X can be accurately targeted bilaterally and 2) 0.25 μL was a sufficient volume to affect DA input to Area X. Once the ideal injection volume was determined, a dose response study was conducted to evaluate lower doses. This information is summarized in **Table 1**.

Table 1. Dose Response Surgery Birds

Bird	6-OHDA Dose (µg)	Volume per injection (nL)	Number of Injections	Total Volume (nL)
Bird 1	0.6	27.6	9	248.4
Bird 2	0.6	27.6	9	248.4
Bird 3	0.6	27.6	9	248.4
Bird 4	0.8	27.6	9	248.4
Bird 5	0.8	27.6	9	248.4
Bird 6	1.2	55.2	9	496.8
Bird 7	1.2	55.2	9	496.8

In one unique experiment (not shown), tetramethylrhodamine 10,000 MW lysine-fixable dextran (fluororuby dye liquid; Molecular Probes, Eugene, OR) was mixed with 1.5 µg 6-OHDA in a final volume of 0.25 µL to help determine both the injection site as well as the effect of 6-OHDA in Area X**.** It was determined that a larger injection volume may cover Area X tissue more expansively. The ideal conditions for efficaciously delivering the new lot of 6-OHDA to Area X were determined to be 1.2 µg in either 0.25 µL or 0.5 µL. At this dose, there was no evidence of structural damage to the striatum (i.e. cell body loss) and preliminary behavioral evidence suggested song was affected. Ongoing immunohistochemical experiments will determine whether signals for TH-containing nerve terminals in Area X and TH-containing cell bodies in the SNc and VTA are reduced as a result of 6-OHDA administration which would be consistent with dopaminergic neuron loss. However, the timeline for when this effect occurs remains unclear.

Validation of TH and DARPP-32 antibodies using rat BG tissue

Validation for use of TH (Milllipore #AB152) and DARPP-32 (Abcam #ab40801) antibodies on zebra finch tissue was achieved by Western blotting **(Figure 12)**. Tissue

from rat BG was used as a positive control and zebra finch tissue collected from NP was used as a negative control; because unlike striatum, NP is not enriched in dopaminergic input. As expected, TH and DARPP-32 bands ran near their expected molecular weights, as shown by bands present in rat BG. The faint band present in NP support the specificity of the TH and DARPP-32 antibodies used in this study. An additional specificity test for the TH and DARPP-32 antibodies using their immunizing peptides was not possible because the peptides are not commercially available. Moreover, the GAPDH antibody (Millipore #MAB374) appeared at its expected molecular weight and has been used in previously published studies from our laboratory[43, 46].

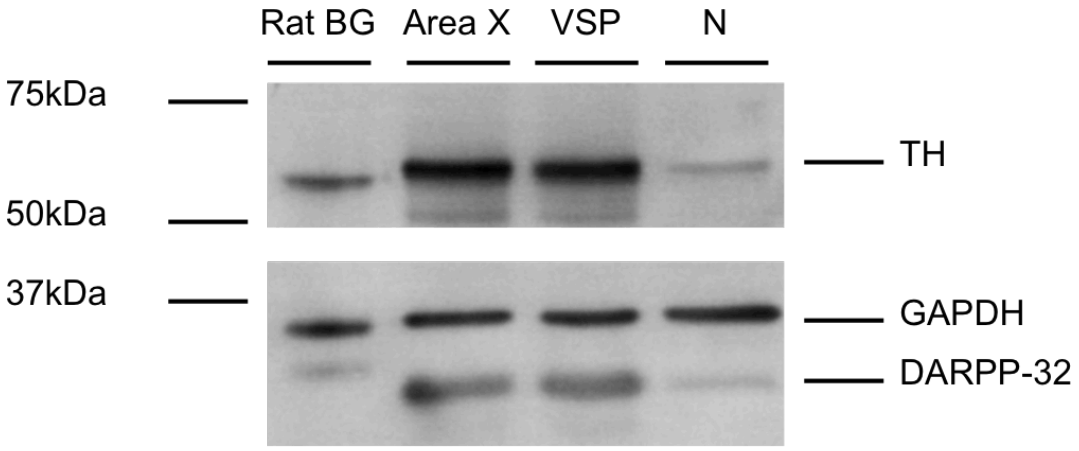

Figure 12. Validation of TH and DARPP-32 antibodies in zebra finch. Immunoblot of 15±3 μg of protein/lane depicting signals for TH (~62kD), DARPP-32 (~32kD for rat BG, ~31kD for Area X, VSP, N), and GAPDH (~35kD, loading control). Molecular mass markers are provided on the left. TH signal is robust at the expected molecular weight in Area X and VSP of zebra finch as compared with rat BG (positive control) with expected reduction in nidopallium (N, negative control).

Dose-Response TH depletion in 6-OHDA administered birds

Tissue extraction of Area X and VSP tissue as well as electrophoresis and Western blotting was performed on one vehicle-injected bird as well as one 6-OHDA administered bird for doses of 0.6 and 1.2 µg. As shown in **Figure 13**, there is an evident gradient of TH levels in Area X, but not in VSP. This visual interpretation was verified after quantification of TH bands using GAPDH as a protein-loading control. These results are summarized in **Table 2**. As a result of this Western blot, 1.2 µg 6-OHDA was determined to be an ideal concentration based on the 66% reduction in TH compared with vehicle.

In order to assess the extent of 6-OHDA's effect in the striatum, TH was also probed using VSP punches. VSP has similar cell types and DA innervations as Area X, as was shown previously for TH and DARPP-32 in **Figure 12**. Hence, changes in TH were not expected because 6-OHDA was specifically targeted to Area X. No changes in TH were observed.

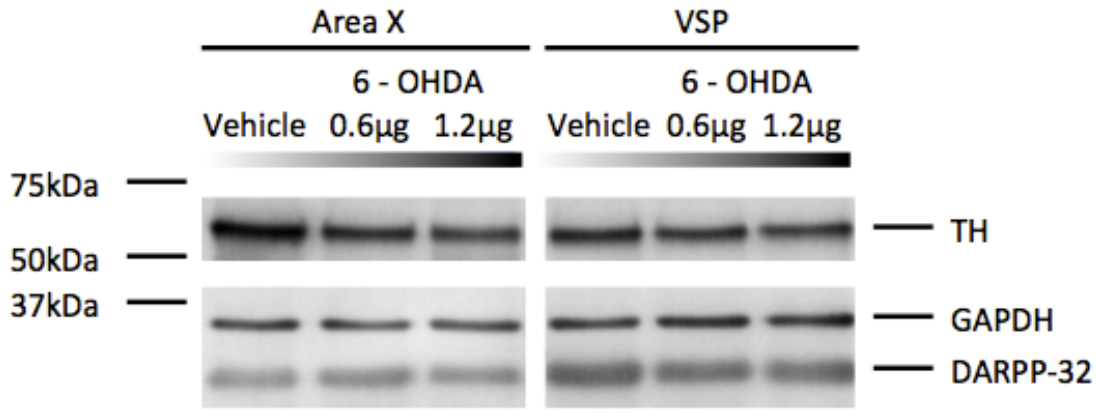

Figure 13. Dose response Western Blot of Area X and VSP punches. Immunoblot of 15±3 µg of protein/lane depicting signals for TH (~62kD), DARPP-32 (~31kD), and

GAPDH (~35kD, loading control). Molecular mass markers are provided on the left. **Top:** In Area X, TH band intensities decrease (from left to right) with increasing 6-OHDA administration. In VSP, TH band intensities are preserved. **Bottom:** GAPDH band intensities are relatively similar in both Area X and VSP lanes, indicating that similar amounts of total protein were loaded in each lane. Based on raw values (not shown here), lane 3 was loaded with ~17% more protein than lanes 1 and 2, and yet, a reduction in TH is still visually apparent showing the dramatic effect of 6-OHDA on this protein.

Table 2. Dose Response Western Blot Area X Quantification of TH and DARPP-32

Area X	TH Relative Density (%)	DARPP-32 Relative Density (%)
Vehicle	2.47	0.78
0.6µg 6-OHDA	1.85	1.19
1.2µg 6-OHDA	0.85	0.84

Table 3. Dose Response Western Blot VSP Quantification of DARPP

VSP	TH Relative Density (%)	DARPP-32 Relative Density (%)
Vehicle	1.58	1.23
0.6µg 6-OHDA	1.94	1.84
1.2µg 6-OHDA	1.51	1.32

Vehicle- versus 6-OHDA injected birds:

A total of 7 birds received bilateral Area X injections of 1.2 µg 6-OHDA in 0.1% sodium ascorbate whereas a vehicle group of the same size receiving only 0.1% sodium ascorbate (NaAsc). The experimental paradigm is schematized in **Figure 14.** For more detailed information on drug delivery for each bird, refer to **Tables 4 and 5**.

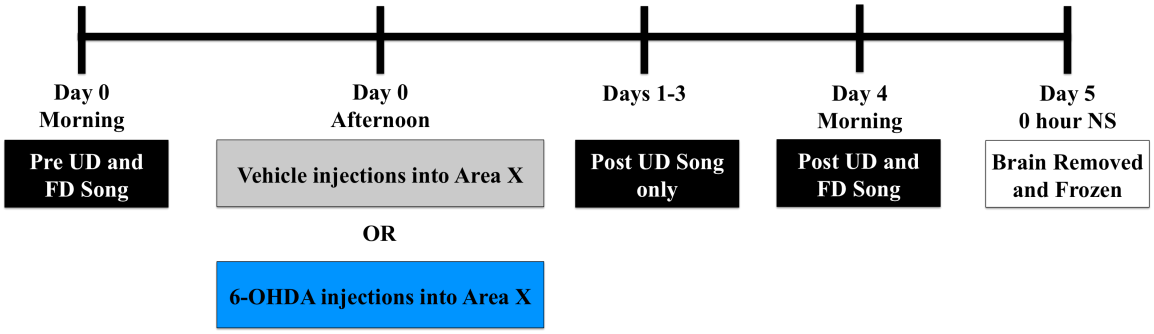

Figure 14: Timeline for vehicle and 6-OHDA administered birds. Pre-surgery undirected (UD) song was collected immediately preceding female-directed (FD) song. Provided that sufficient song was recorded, surgery was performed with either vehicle or 6-OHDA injections. Following surgery, birds were carefully monitored for health. Non-vocal motor symptoms were not observed. UD song was collected continuously until day 4, when FD song was collected at approximately the same times as pre-surgery. In the event that insufficient UD or FD song was collected on day 4, recordings were repeated the following day, shifting the timeline for that individual bird. These time points were chosen based on preliminary evidence suggesting recovery after only 14 days[45]. Finally, on day 5, birds were sacrificed at lights-on using anesthesia for euthanasia. Brains were removed and frozen in liquid nitrogen to be processed later for tissue punches and Western Blotting.

Table 4: Information on drug delivery for vehicle birds

Bird	NaAsc Dose (µg)	Volume per injection (nL)	Number of Injections	Total Volume (nL)
Bird 1	0.1%	27.6	9	248.4
Bird 2	0.1%	27.6	9	248.4
Bird 3	0.1%	27.6	18	496.8
Bird 4	0.1%	55.2	9	496.8
Bird 5	0.1%	55.2	9	496.8
Bird 6	0.1%	55.2	9	496.8
Bird 7	0.1%	55.2	9	496.8

Table 5: Information on drug delivery for 6-OHDA birds

Bird	6-OHDA Dose (µg)	Volume per injection (nL)	Number of Injections	Total Volume (nL)
Bird 1	1.2	55.2	9	496.8
Bird 2	1.2	55.2	9	496.8
Bird 3	1.2	55.2	9	496.8
Bird 4	1.2	55.2	9	496.8
Bird 5	1.2	55.2	9	496.8
Bird 6	1.2	55.2	9	496.8
Bird 7	1.2	55.2	9	496.8

TH depletion in 6-OHDA administered birds relative to control

Bilateral tissue extraction from Area X and VSP was obtained with electrophoresis and Western blotting performed on two groups of birds, a vehicle group receiving 0.1% sodium ascorbate and a neurotoxin administered group receiving 1.2 µg 6-OHDA (n=4 per group). As shown in **Figure 15**, sterotaxic targeting of 6-OHDA into Area X consistently decreases TH signal in Area X only, with no detectable depletion in VSP. This was confirmed following quantification. This led to two important interpretations about 6-OHDA: 1) it was accurately targeted to Area X during surgery and 2) it effectively depletes TH fibers in Area X. Moreover, there were no detectable changes in

cAMP regulated phosphoprotein, DARPP-32, in either Area X or VSP. Preserved levels of this postsynaptic marker suggest a preservation of a key type of MSNs in Area X.

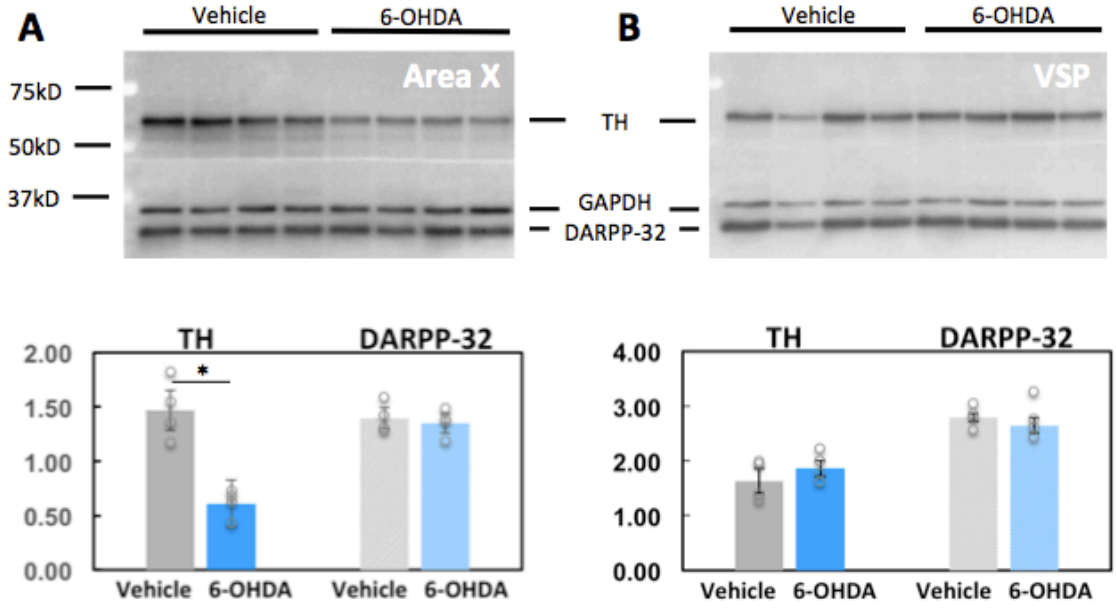

Figure 15. 6-OHDA injections into Area X decrease TH signals in Area X but preserves those in VSP. Immunoblots of 15±3 µg of protein/lane depicting signals for TH (~62kD), DARPP-32 (~31kD), and GAPDH (~35kD, loading control). Molecular mass markers are provided on the left. **A.** Top: Immunoblot shows decreased TH signal in 0NS birds which received 6-OHDA compared to vehicle-injected birds in Area X (n=4/group; mean±SE: vehicle 1.47±0.19 vs. 6-OHDA 0.61±0.21; Mann-Whitney U test, U=0, *P<0.025). No change in DARPP-32 signal is detected (mean 1.40±0.1 vs. 1.35±0.1). Bottom, Bar graphs show means (top of the bar), standard errors and individual values (circles) per bird plotted for TH and DARPP-32 signals. **B.** Top: Immunoblot and bar graph underneath show no significant change in TH and DARPP-32 signals in the VSP for the same group of birds as in **(A)**, validating the accuracy of the

injection (n=4/group; mean vehicle TH 1.63±0.22 vs.1.86±0.14; DARPP-32, 2.79±0.07 vs. 2.64±0.14).

6-OHDA administration does not affect natural fundamental frequency difference in variability between social-contexts

Birds were isolated in sound attenuation chambers prior to receiving vehicle or 6-OHDA administration. Prior to surgery, songs were screened in order to select birds who met two conditions: 1) their songs contained at least one harmonic syllable 2) at least one harmonic stack exhibited a reduction in the variability of the fundamental frequency (FF CV) in FD conditions relative to the UD conditions as previously published[31]. An exemplar of an ideal motif is shown in **Figure 16**.

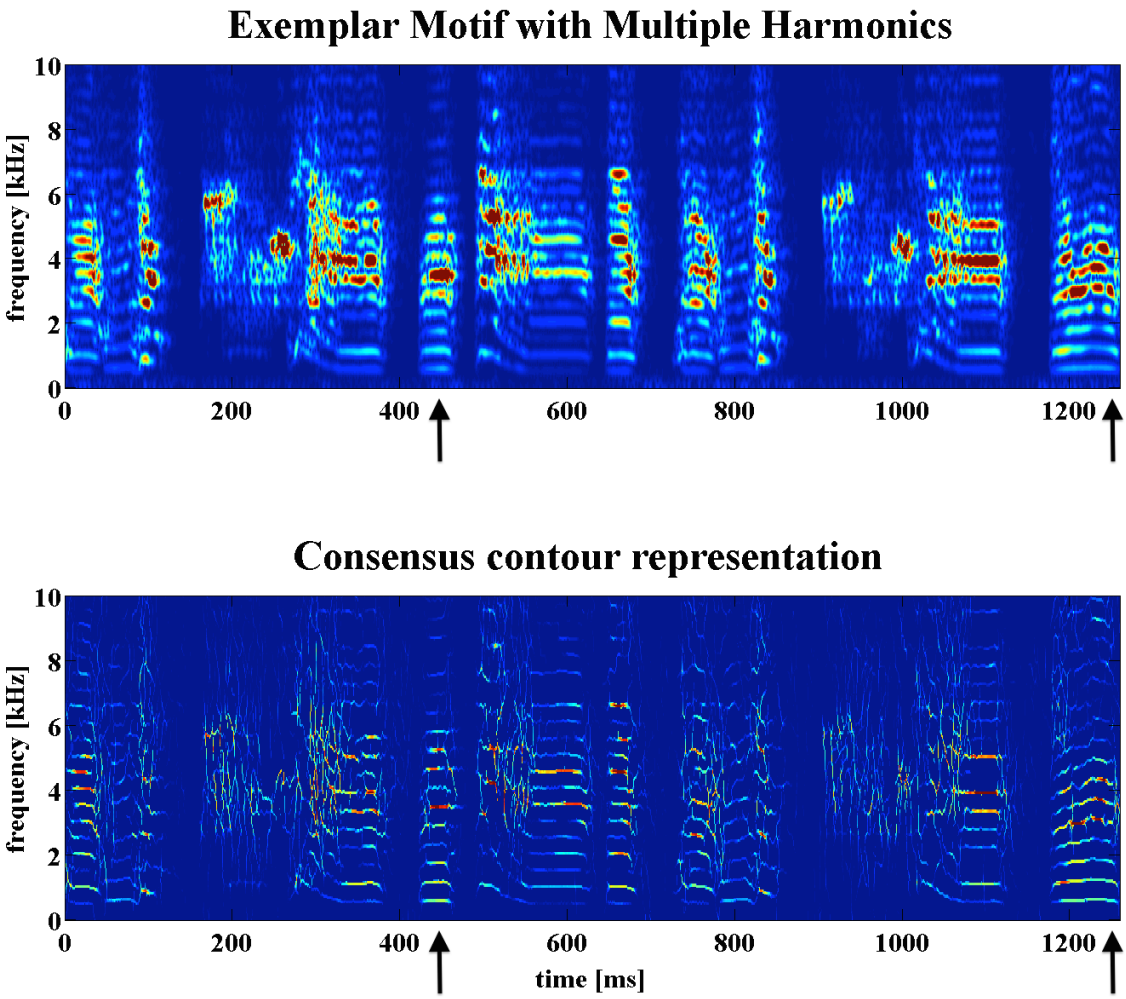

Figure 16. Spectrogram of a single motif. Top: Exemplar motif containing several harmonic notes, including an entirely harmonic syllable. Bottom: Harmonic notes are more clearly visualized in the consensus contour representation. Representative harmonic syllables are denoted by black arrows.

Prior to surgery, both the vehicle (n=5) and 6-OHDA groups (n=7) showed a significant effect of social context (**Table 6**, $P<10^{-5}$, two-tailed paired t-test) in FF variability, that is, a decrease in FF CVs of FD relative to UD song **(Figure 17)**. As expected, following surgery, the vehicle injected group continued to show a significant social context-dependent difference (**Table 6**, $P<10^{-5}$, two-tailed paired t-test) in FF variability. However, following surgery in the 6-OHDA group, this effect was unexpectedly preserved (**Table 6**, $P<10^{-4}$, two-tailed paired t-test).

Table 6. P-values for comparisons between vehicle and 6-OHDA groups for social-context dependent differences in FF variability

Vehicle		OHDA	
Pre UD vs. Pre FD		Post UD vs. Post FD	
Before Surgery	After Surgery	Before Surgery	After Surgery
1.52×10^{-5}	7.33×10^{-5}	4.71×10^{-5}	1.70×10^{-4}

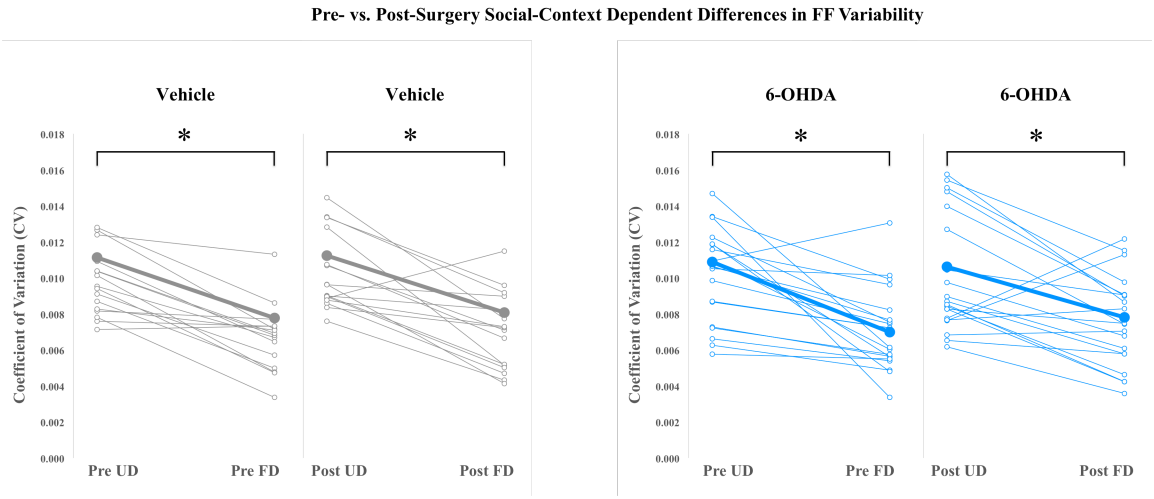

Figure 17: Natural drop in FF CVs between UD and FD song is preserved in both vehicle and 6-OHDA groups. Paired plots of FF CVs in vehicle injected birds (gray, n=18 harmonic syllables) display a significant social context-dependent effect pre- and post-surgery. Similarly, paired plots of FF CVs in 6-OHDA injected birds (blue, n=23 syllables) display a significant social context-dependent effect pre- and post-surgery. For each plot, thin lines represent syllables and heavy weighted lines represent averages of all syllables. P-values for each comparison may be found in **Table 6**.

6-OHDA administration increases syllable accuracy in UD

Further song analysis was carried out at both the motif and syllable levels. No significant changes were observed on the motif level, however, this does not preclude other macroscopic vocal deficits from being present (e.g. stuttering, shortened bout lengths). On a syllable level, there was an overall increase in syllable accuracy in UD song in 6-OHDA administered, but not vehicle birds **(Figure 18)**. This suggests that syllables became more stereotyped as a result of 6-OHDA administration.

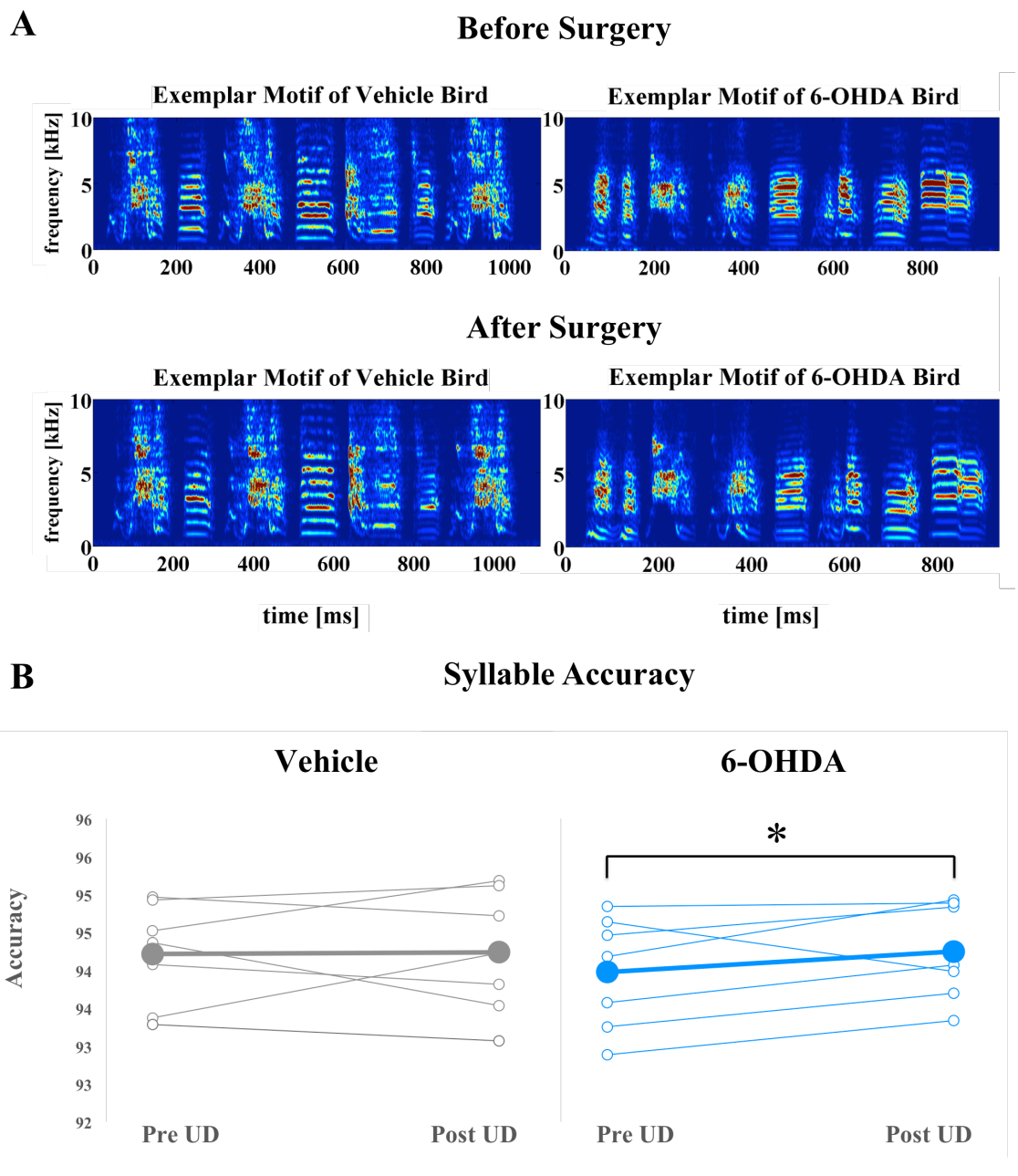

Figure 18. Birds administered with 6-OHDA, but not vehicle, show more stereotyped syllable accuracy. A. Top: Exemplar motifs before surgery from vehicle (left) and 6-OHDA administered (right) birds, respectively. Bottom: Exemplar motifs after surgery from the same birds shown above. **B.** No change in syllable accuracy detected in vehicle birds (n=7; paired t-test, P=0.617). Increase in syllable accuracy

detected in 6-OHDA administered birds (n=7; paired t-test, *P=0.011). Syllable level scores were obtained from 25 consecutive renditions using symmetric comparisons in SAP. For each paired-plot, the weighted line (filled circles) represents the average of the birds in that respective group.

6-OHDA administration increases UD syllable stereotypy and decreases mean amplitude

Spectral acoustic features were analyzed for each syllable in 6-OHDA and vehicle birds. In only 6-OHDA birds, significant differences ($P<0.05$) in CVs for mean amplitude and mean Wiener entropy were present, but not for mean pitch or mean frequency modulation (refer to Song recording and analysis in Methods Section for an overview on these measures). In vehicle-injected birds, there were no significant changes observed in these same measures. It is worth mentioning that amplitude measurements depend heavily on the distance the zebra finch is from the microphone while singing. Because song is collected from 25 consecutive motifs, this may be inconsistent from one time point to the next. Overall, 6-OHDA administration increased variability on a syllable level in UD song. These results are summarized in **Figure 19**.

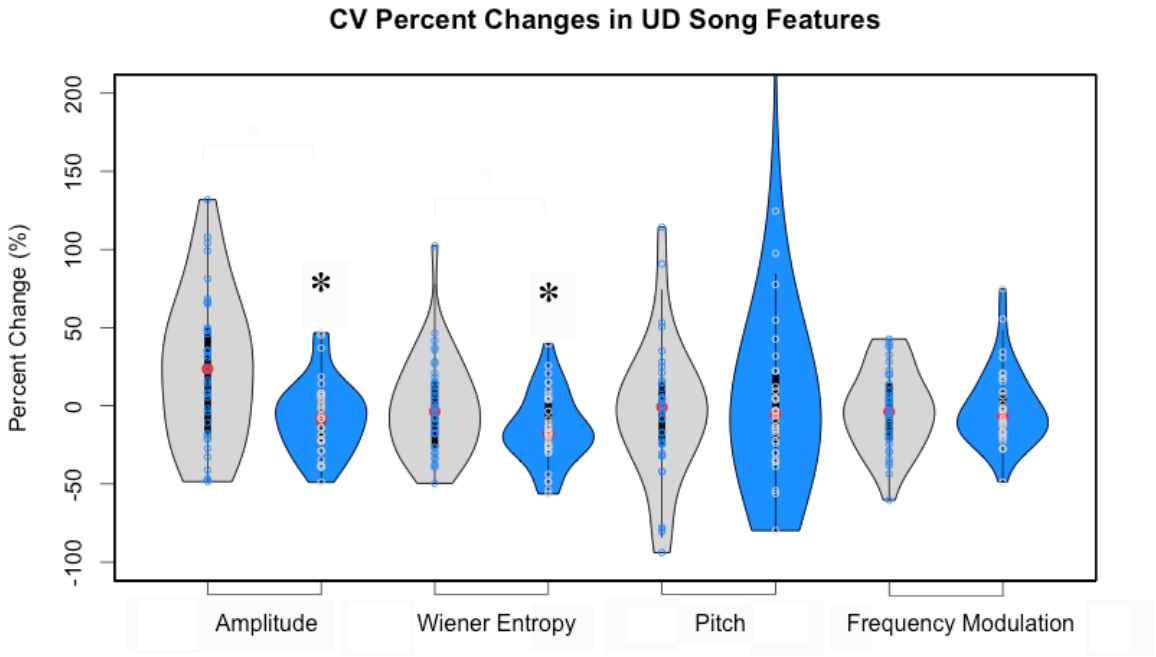

Figure 19. Decrease in mean amplitude CVs and increase in syllable stereotypy in UD song. Violin plots display percent changes in song feature CVs in vehicle (gray) and 6-OHDA (blue) groups. Each open circle represents one syllable from a bird. The limits of the black rectangle represents the 1st and 3rd quartiles, while the filled red circle represents the median of the distribution. A significant decrease (*P=0.039) in UD mean amplitude CVs was observed in 6-OHDA birds, but not vehicle birds (P=0.121). Additionally, a significant decrease in UD Weiner entropy CVs was observed in 6-OHDA birds (*$P<10^{-4}$), but not vehicle birds (P=0.350). No significant changes were observed in pitch (6-OHDA group, P=0.347; vehicle group, P=0.480) or frequency modulation (6-OHDA group, P=0.352; vehicle group, P=0.279) CVs for either group. For visual purposes, two data points are not shown for pitch CV in 6-OHDA birds (367%, 501%), but were included in the analysis.

6-OHDA administration increases FD syllable variability and decreases mean amplitude

In FD song, significant differences (P<0.05) in CVs were observed in only 6-OHDA birds in mean amplitude and mean entropy, but not mean pitch or mean frequency modulation. In vehicle birds, no significant CV differences were observed in these features. Overall, 6-OHDA administration increased variability on a syllable level in FD song. These results are summarized in **Figure 20.**

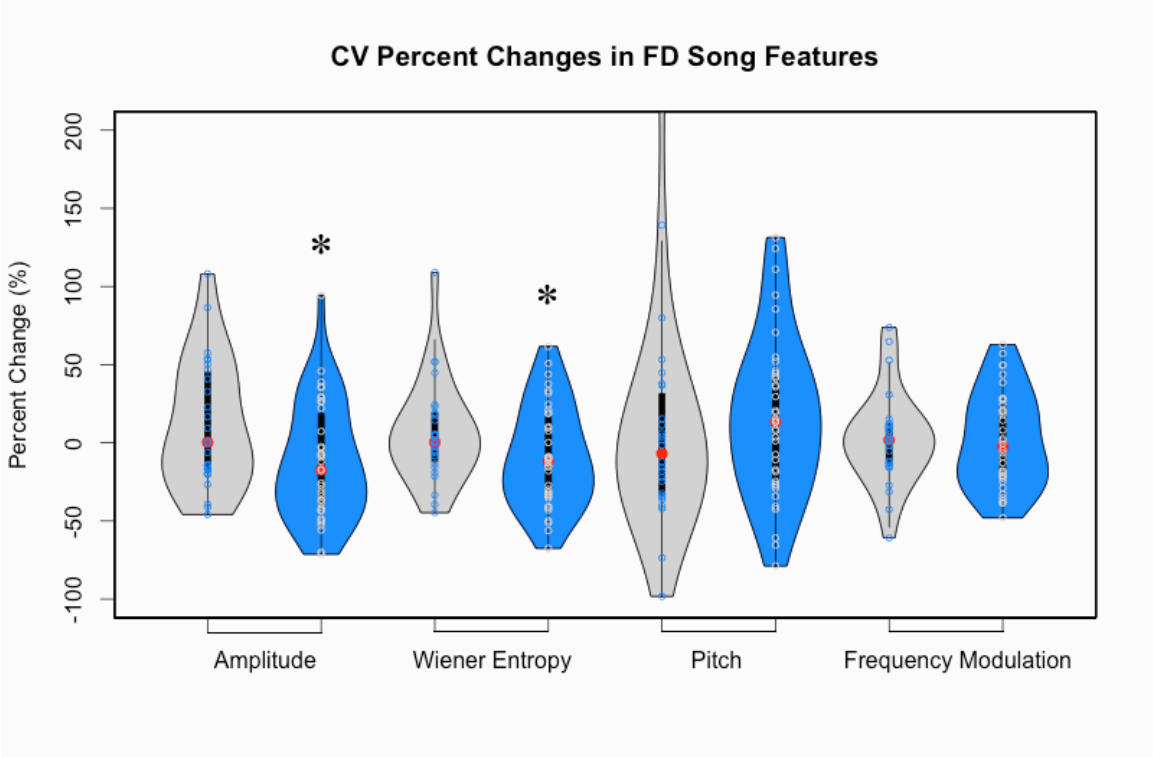

Figure 20. Decrease in mean amplitude CVs and increase in syllable variability in FD song. Violin plots display percent changes in mean amplitude CVs due to vehicle (left) and 6-OHDA (right) administrations. Each open circle represents one syllable from a bird. The limits of the black rectangle represents the 1st and 3rd quartiles, while the filled red circle represents the median of the distribution. A significant decrease (*P=0.003) in

FD mean amplitude CVs was observed in 6-OHDA birds, but not vehicle birds (P=0.256). Additionally, a significant decrease in FD Weiner entropy CVs was observed in 6-OHDA birds (*P=0.039), but not vehicle birds (P=0.657). No significant changes were observed in pitch (6-OHDA group, P=0.941; vehicle group, P=0.243) or frequency modulation (6-OHDA group, P=0.573; vehicle group, P=0.766) CVs for either group. For visual purposes, two data points are not shown for pitch CV in vehicle birds (139%, 242%), but were included in the analysis.

Natural, social-context-driven changes of DA markers

As described in the Background Section, differences in the social context of singing produce different levels of DA in Area X as measured by microdialysis and HPLC[41]. FD song is associated with greater DA levels than is UD song. We hypothesized that these natural differences might correspond to subtle differences in biomarkers for DA, including the biosynthetic enzyme, TH. Thus, to test for these potentially subtle changes and to optimize detection of changes in TH, tissue was extracted from Area X and VSP in non-operated birds sacrificed subsequent to the following social contexts and/or conditions: 0 hours of non-singing or 2 hours of either non-singing, undirected singing, or female directed singing. Electrophoresis and Western blotting was then performed to evaluate differences in baseline levels of TH and DARPP-32 in any behavioral context. The results are summarized in **Figure 21**.

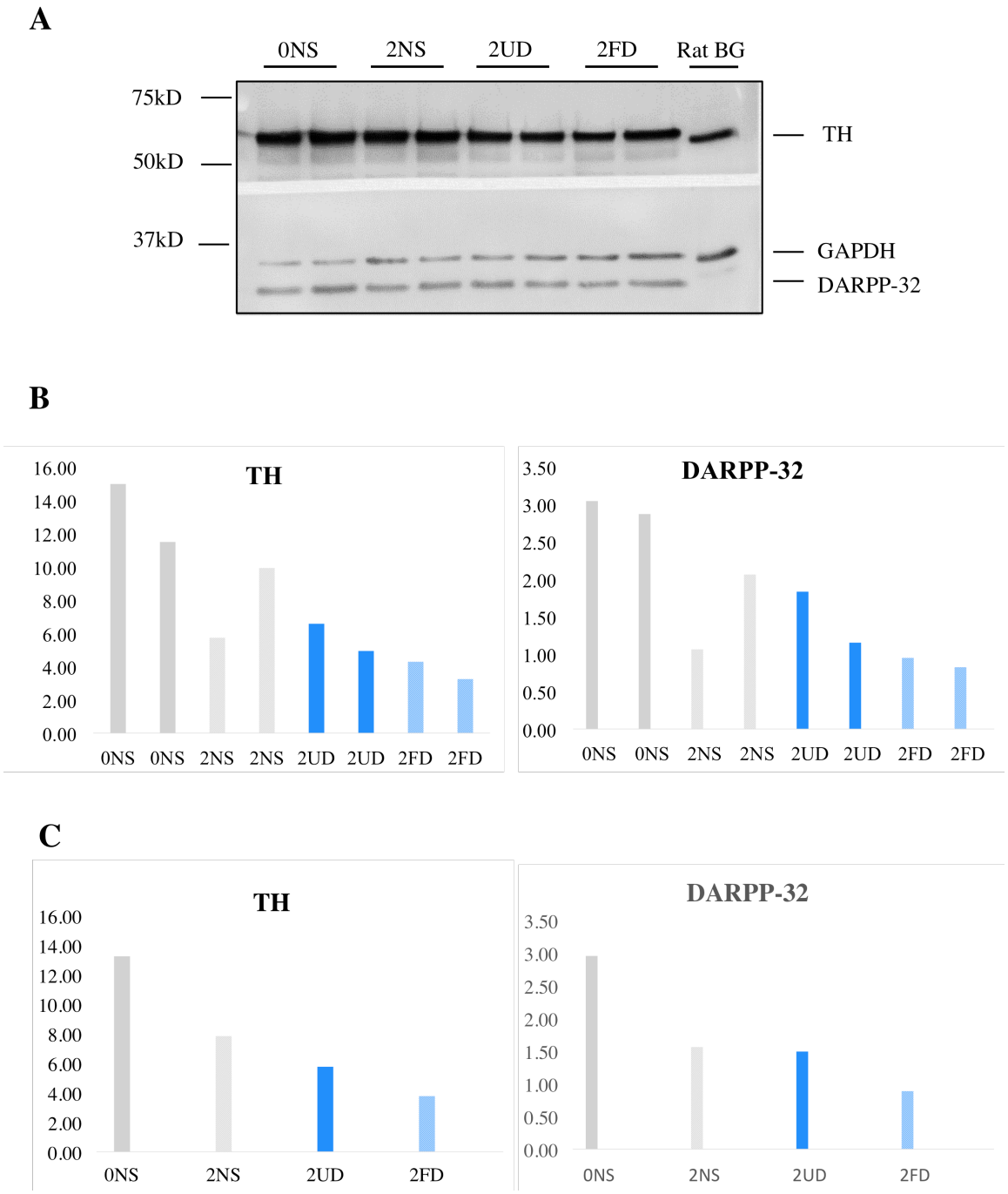

Figure 21. A trend for social-context regulation of TH and DA and DARPP-32 in Area X. Immunoblot of 15µg of protein/lane depicting signals for TH (~62kD), DARPP-32 (~31kD), and GAPDH (~35kD, loading control). Molecular mass markers are provided on the left. **A.** Area X punches were obtained from birds following lights-on in

the morning (0NS), 2 hours of non-singing (2NS), and either 2 hours of singing undirected (2UD) or female-directed (2FD) song. The last lane contains lysate from rat BG. **B.** Bar graphs displaying TH and DARPP-32 levels for each bird. **C.** Bar graphs displaying averages for each condition shown in **(B)** depict trend for higher TH and DARPP-32 levels in Area X of non-singers compared to singers (n=2/group).

In this pilot study, TH levels appeared highest in the 0 hour non-singing condition, raising the possibility of circadian, as well as social-context dependent effects. This condition was selected as the sacrificing time for subsequent experiments in order to quantifiably detect TH depletion in Area X of 6-OHDA administered birds, compared to vehicle injected control birds. The rationale behind this selection was that depletion of TH may be more evident when compared to a high, versus to low, baseline levels. Moreover, the 0 hour time point removed the influence of any circadian or behaviorally induced variability.

DISCUSSION

In songbirds, several studies have implicated the midbrain dopaminergic system in social context-dependent vocal production[13, 33, 38, 39, 41, 47-51]. As described above, the underlying similarities between human and songbird systems, such as the neural circuitry for vocal production, advocates further investigation in songbirds to help elucidate how learned vocalizations, such as speech, are maintained, and regarding vocal deficits in relevant human diseases. Furthermore, songbirds have the potential to be informative about vocal rehabilitation strategies that may be effective in humans. In this study, I have investigated vocal deficits associated with DA depletion in the songbird to uncover its potential use as a model for vocal deficits in PD. Vocal production is often overlooked when considering motor impairments in patients affected with PD.

I here demonstrate that depletion of DA in the song dedicated region of the zebra finch striato-pallidum increases syllable stereotypy in UD song. This result was initially puzzling because our initial hypothesis was that DA depletion would cause FD song, previously associated with high Area X DA levels, to become more similar to UD song which is associated with lower DA levels[41]. However, this result is less surprising following discussion with collaborators in the PD motor speech community who work with patients and train clinicians with behavioral therapy. Dr. Cynthia Fox, co-founder of LSVT, conveyed that patients' vocal deficits (soft, hoarse, monotonous voice, loss of prosody) manifest more clearly in the absence of an external cue such as from a therapist prompting them to overcome their vocal deficits. This notion holds true in zebra finches with DA-depleted BG. Syllables become more centralized, as demonstrated by increased stereotypy in UD song (**Figure 19**). If vocal deficits in humans and zebra finches are

juxtaposed, we can see that DA depletion in the BG leads to decreased vocal modulation in the absence of an external cue. In the case of zebra finch, the external cue is a female conspecific. For example, syllables becoming more similar following 6-OHDA administration (**Figure 18B**), which is in line with patients whose speech becomes more flattened or monotonous in the dysarthria accompanying the disease. This further supports the notion that zebra finches administered with 6-OHDA comprise an appropriate model for vocal deficits observed in PD.

Moreover, these results are relevant to our curent understanding of song regulation in zebra finches. Consistent with several studies[31, 37, 52], the results presented here show that the fundamental frequency of harmonic components of syllables (known as F_0, the comparable measure in humans) is more variable when a male sings alone than when he sings to a female. In a recent study, interference with D1 receptor-mediated signaling by infusing the antagonist SCH23390 into Area X eliminated differences in variability of the fundamental frequency due to social context[32]. Specifically, SCH23390 caused FF CVs in the FD social-context to resemble FF CVs in the UD social context. Here, I have shown that 6-OHDA does not achieve this same effect: interference with DA transmission by depleting TH, has no effect on social context-dependent changes in FF CVs of UD song and FD song. To explain this result, it is important to consider both D1R and D2R expression in the avian brain as well as its proportional distribution, as discussed below.

Unlike in mammals, D1R and D2R can be expressed on the same MSNs in the zebra finch striato-pallidum[13]. Hence, depletion of presynaptic DA in Area X may affect postsynaptic activity levels in three MSN types: 1) in D1R-only containing neurons 2) in D2R-only containing neurons or 3) in D1- and D2-receptor containing neurons. Because it is traditionally thought that 6-OHDA is taken up by DA active transporters, all three scenarios may be in effect simultaneousy. Moreover, previous studies and reviews indicate that the D1:D2 receptor ratio is different in the avian striatum as compared with mammals[25, 51, 53-55]. While these differences may imply important differences in BG function between birds and mammals, they also help reconcile the results of this study with recently published results using a D1R antagonist[32]. Taken together, I propose that 6-OHDA depletion of DA active transporters exerts greater effects via D2Rs than D1Rs, based on the greater prevalence of the former. To more fully understand and appreciate unique aspects of avian neural microcircuitry, future studies would benefit from investigating social-context dependent changes using D2R selective ligands in Area X (e.g. L-741,626; highly selective D2 antagonist).

Changes in song can also be accounted for when considering the role of DA active transporters in synaptic transmission. These membrane-spanning proteins pump DA out of the synapse back into the cytosol where it is repackaged into vesicles for later storage and release. With destruction of DA active transporters, as caused by 6-OHDA, elevated extracellular DA levels may persist, leading to increased D1R activity levels in MSNs. A similar effect is observed in mice administered with dopaminergic drugs, such as cocaine, which elevates extracellular DA levels by blocking DA active transporters[56]. Increased

levels of DA are present in Area X during FD song as compared with decreased levels during UD song[41]. Hence, increased extracellular DA levels, and greater, rather than less, dopaminergic signaling may be caused by 6-OHDA and its destruction of DA active transporters. In mutant mice lacking norepinephrine or DA transporters, the lack of a reuptake mechanism leads to large increases in extracellular concentrations of catecholamines and decreased levels of TH[57]. Hence, this leakage of DA may lead to chronic activation of D1 and D2 receptors, which is similar to the consequences of cocaine use. This alternative explanation may account for the increase in syllable self accuracy observed in **Figure 18B** and may also reconcile the results of this study with those obtained using a D1R antagonist[32].

To further shed light on 6-OHDA and its mechanism of action, Western blotting allowed for quantification of Area X proteins that have been widely researched in mammals where their roles in molecular signaling are well-understood. Here, I have shown that 6-OHDA decreases levels of TH in Area X, but not VSP, which are likely indicative of overall lower levels of DA synthesis in the striatal region of the songbird brain that is dedicated to song. Because TH is not completely lost, the zebra finch 6-OHDA model may elucidate molecular mechanisms associated with early-onset symptoms observed in PD.

DARPP-32, a target for the action of DA expressed in MSNs, was not affected in 6-OHDA administered birds relative to control birds. Moreover, preliminary evidence using Western blotting indicates phospho-Thr 34 of DARPP-32 was unaffected in 6-OHDA

administered birds relative to control. Taken together, postsynaptic changes are not observed 4-5 days following 6-OHDA administration. However, future work may unveil changes that could occur over a longer timecourse. Additionally, if D2Rs are indeed affected in higher proportion than D1Rs, then measurement of phospho-Thr 75 of DARPP-32 levels may provide further information on the effects of 6-OHDA in Area X.

Finally, in seeking to determine whether TH and/or DARPP-32 were regulated by social context, I incidentally came upon the result that both TH and DARPP-32 may be regulated by the circadian rhythm. The relevant immunoblot **(Figure 21)** showed a trend toward increased TH/DARPP-32 levels for the 0 hour non-singing condition whereas the 2 hour NS condition showed a trend toward decreased levels. However, for both proteins, the 2 hour FD condition yielded the lowest levels of each respective protein. Based on these initial impressions, their may be dual-regulation of TH and DARPP-32, both circadian and social-context dependent. Further work using an increased number of animals may resolve these potential effects.

In summary, the work contained in this thesis advocates for future studies to further investigate the molecular consequences of DA-depletion in the avian BG to better understand dopaminergic modulation of vocalizations. In PD, dopaminergic neurons in the SNc die, which results in the loss of DA input to the BG. To model early-stage vocal symptoms of PD, I have utilized a neuroethological approach in zebra finches. The zebra finch AFP is analogous to the cortico-BG-thalamocortical loop in mammals. To deplete DA in Area X, a specialized subregion of the avian BG devoted to song learning and

vocal production, I employed 6-OHDA, a neurotoxin, which selectively depletes TH fibers. I have shown here that delivery of this drug depletes DA input to Area X in zebra finches, as shown by quantitative differences in TH levels using Western blotting. Despite an approximately 60-70% loss in TH, male zebra finches maintain social context-dependent differences in FF variability of harmonic syllables, dissimilar to previous reports that block the actions of DA using D1 antagonists[32].

Further song analysis revealed that 6-OHDA differentially affected acoustic properties on a syllable level based on social context. In UD song, a slightly variable singing state, syllables became more stereotyped following TH fiber depletion (**Figure 18B** and **Figure 19**). However, in FD song, which is known to be more stable, syllables became more variable (**Figure 20**). This suggests a convergence between UD and FD song. Additionally, birds administered with 6-OHDA showed a decrease in amplitude in both UD and FD contexts. Taken together these phenotypes are reminiscent of vocal deficits observed in PD speech, which is characterized by quietness and a lack of vocal range.

I have shown that this powerful, established model for speech and language provides an avenue for investigating vocal deficits that may occur in an early Parkinsonian state. This provides a foundation for future studies to determine whether vocal deficits may serve an early biomarker for this devastating neurodegenerative disease.

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