

Understanding the vocal learning basal ganglia circuit in zebra finches: Is there co-release of glutamate from midbrain dopamine neurons terminating in area X?

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**Abstract**

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Vocal learning is the process of acquiring and maintaining one of the many complex motor skills that begins to develop from human infancy, during which the brain relies on auditory input. In this study, zebra finches, a species of songbirds, were used as experimental models to study the underlying neural circuitry that facilitates auditory-guided vocal learning and production of song. After the sensory learning stage and during the sensorimotor learning stage of learning and producing song, zebra finches use their own auditory feedback to approximate their tutor birds' song as close as they are able to. The avian anterior forebrain pathway, which supports vocal learning, contains a structure called area X (a portion of the avian basal ganglia), which receives dopaminergic input from the ventral tegmental area (VTA). Dopaminergic input from the VTA to area X allows the birds to learn song through reinforcement/trial and error learning using

auditory feedback. However, dopamine signaling alone may not be sufficiently rapid or temporally precise to guide changes based on auditory feedback during vocal learning as it is classified as a slow-acting neurotransmitter. It was hypothesized that another fast-acting neurotransmitter, glutamate, may be co-released with dopamine from VTA to area X. Two birds (juvenile and adult) were used to detect the presence of markers for both dopamine and glutamate at the terminal boutons of the neuronal axons extending from VTA to area X. In the juvenile bird, 66% of the axon terminals and in the adult bird, 14% of the axon terminals were labeled with markers of both dopamine and glutamate, suggesting a high possibility of the co-release of both these neurotransmitters from VTA to area X. The juvenile bird also had more double-labeled axon terminals when compared to the adult bird (66% vs. 14%), possibly suggesting that the juvenile bird is at a highly variable stage of learning and therefore requiring more temporally precise reinforcement signaling with the co-release of both dopamine and glutamate.

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## **I. Introduction**

### ***What is vocal learning?***

The exact neural circuits underlying many human behaviors throughout the lifespan are still unknown. One such process using these neural circuits that is yet to be understood is learning, which begins at infancy as infants gain both sensory and motor experiences from their environment, activating and further developing the corresponding neural circuits. Vocal learning, in particular, is defined as the act of imitation of sounds to acquire and maintain a complex motor skill for communication throughout which the brain is reliant on various sensory information from the environment (Carouso-Peck & Goldstein, 2019; Hoffmann, Saravanan, Wood, He, & Sober, 2016). Vocal learning develops as humans use their own and others' auditory information as they modify their vocal production to reach a target goal. Their ability to modify their vocal output based on auditory information from the environment allows them to imitate and improvise sounds and participate in the process of trial and error learning. This phenomenon of vocal learning is not unique to humans and has been demonstrated in other mammals as well including whales, dolphins and bats (Prat, Taub, & Yovel, 2015), although isolation experiments to robustly confirm vocal learning in these mammals are sparse. In addition, 5000 different species of birds are known to learn and produce vocalizations (Mischler, Congdon, Scully, Campbell, & Sturdy, 2017). These 5000 species of birds are distributed into three groups: parrots, hummingbirds and oscine songbirds. Of these groups of birds, oscine songbirds are widely used

as models to understand the neural circuitry for auditory-guided vocal learning and production. In particular, a species of oscine songbirds known as the zebra finch (*Taeniopygia guttata*) is commonly used in research and accounts for approximately half of all studies of the neural basis of vocal learning (Mischler et al., 2017).

### ***Why use songbirds as models to understand human vocal learning?***

Infants learn to produce adult-like sounds and eventually begin to converse through assimilating the auditory information from themselves and their environment. Similarly, songbirds, more specifically male songbirds participate in vocal learning with the end goal of producing a matured, stereotyped song as they transition into adulthood. Juvenile songbirds follow and learn from the matured, stereotyped song template provided by their adult, male tutor. Similarly, infants listen to the sounds and words of their caretakers, memorize these sounds and words, targeting productions that are similar to the caretakers (Carouso-Peck & Goldstein, 2019). While many species communicate with their kind using sounds, these sounds are not a product of vocal learning. These sounds or calls are present from a young age and do not undergo a period of gradual trial and error learning process and development of communication as seen in to humans and songbirds (Prat et al., 2015). For example, vervet monkeys from a young age use meaningful vocalizations using different calls to indicate different categories of predators. Young vervet monkeys focus on developing the correct associations of calls to predators rather than the calls themselves (Price et al., 2015). Although songbirds also produce vocal communicative sounds that were present from a young age, these sounds are called “calls” and are characteristically different than their learned songs (Mischler et al., 2017). While calls are associated with particular vocalizations or actions (e.g., alarm, indicating predators) and are fully acquired at a young age, bird song is learned through a trial and error process from a tutor adult

songbird and matures or crystallizes at the age of 90 days after hatching. Bird songs are also longer in duration than bird calls and consist of ordered strings of sounds separated by brief silent intervals, similar to human speech. Several aspects of bird song can be compared with speech. Song note or element is the smallest level of song and may be analogous to phonemes, which are the smallest units of speech. Syllables, or units of sound separated by silent intervals, are formed when song notes are grouped. Syllables are then grouped into motifs or phrases consisting of a series of identical or different syllables, which are strung together following a set of rules with regards to timing and sequencing (Kuhl & Doupe, 2008).

Male zebra finches, when compared to other species such as the vervet monkey, participate in experience-dependent learning of song through memorizing and practicing the tutor bird's song to produce a matured, crystallized song. While zebra finches have the neural circuitry and substrates to support trial and error, experience dependent learning of song, vervet monkeys do not. Overall, zebra finches learn their vocalizations similar to humans, are relatively easy to maintain in laboratories, sing readily for behavioral analysis, and are diurnal, meaning that they are active during the day and sleep during the night. These factors make their species a practical model to understand the process of human learning, its underlying neural circuitries and the process of neural plasticity in various social contexts.

### ***How do songbirds produce song?***

The peripheral motor mechanisms of song production in zebra finches are similar to the peripheral motor mechanisms of speech production in humans. However, more information is known regarding the organs and systems used for speech production in humans when compared to song production in zebra finches. Generally, both song production and human speech involve translating the motor plan or program from the central nervous system into coordinated

peripheral muscle activity of the various motor systems. The two main motor systems of sound generation in song birds are the respiratory system and the muscles of the syrinx, a structure in songbirds that is analogous to vocal folds in humans (Goller & Cooper, 2004). The songbird syrinx contains two sets of six syringeal muscles on each side. These muscles pull on the lateral tympaniform membranes (LTM), which vibrate during expiration creating fundamental frequencies thus resulting in sound production. The coordination between syringeal motor tasks and respiratory movements as well as between the two syringeal sound generators all contribute to song production in birds (Goller & Cooper, 2004). Once sound is produced through the syrinx, the structures and motor systems of the upper vocal tract play a crucial role in modifying the generated sound functioning paralleling the functions of the vocal tract for the production of human speech. The movements of the beak, tongue, syrinx, and/or trachea all may cause changes in the resonance properties of the upper vocal tract or change the acoustic impedance for the radiation of sound.

### ***How do songbirds learn song?***

The learning of song is a sensory and experience-dependent process as it requires the presence of the adult tutor bird and for the juvenile bird to imitate the adult tutor bird. Zebra finches learn song through several stages of development comparable to the increasingly complex speech and language milestones during human development (Doupe & Kuhl, 1999). *See Figure 1.* The first and most crucial stage is known as the sensory learning period during which the young male bird listens to and memorizes the song of a socially salient adult model. The duration of this stage can be anywhere from 25 to 60 days post-hatch (Doupe & Kuhl, 1999). Once the adult tutor song is memorized, zebra finches begin to participate in sensorimotor learning in the second stage, which includes the subsong and plastic song periods of learning.

The subsong stage of song learning is highly variable in regards to syllable duration, duration between syllables (gap), and the acoustic structure of the syllable sound (Goldberg & Fee, 2011). The subsong stage is similar to human infant babbling during which the juvenile zebra finches produce sounds while listening to their own productions and calibrate their vocal instrument through auditory feedback to prepare for the next stage of learning. The duration of the subsong stage can last anywhere from 50 to 60 days post-hatch. The variability of song production continues into the third stage, or the plastic song stage, during which the juvenile male adult adjusts his song to approximate the memorized model. After about 90 days post-hatch, zebra finches enter the last and final stage is known as crystallization. During the crystallization phase, the birdsong becomes fixed in its adult form and the components of the song and the order in which they are sung become stereotyped. Once their song is stereotyped, the adult male zebra finches are less likely to learn from a new tutor bird. The four stages of song learning can be separate and distinct during development or may overlap, particularly the first (sensory learning period) and second (subsong) stages (Takahashi, 2019). During the four stages of song learning, auditory and sensorimotor changes are observed most likely resulting from the changes and development in the associated neural circuits (Ross et al., 2019). In summary, juvenile zebra finches initially produce highly variable song sounds and syllables learned from their adult tutor. They then produce more stereotyped songs using auditory feedback and motor exploration, eventually leading to the production of fully mature song. Zebra finches use their learned, matured and stereotyped song in varying social contexts, including mating with females.

***What are the pathways involved in song production?***

In the songbird brain, two distinct pathways aid in the production and learning of song, respectively (Ziegler & Ackermann, 2017). *See Figure 2.* The motor pathway is involved in the

production of song and begins in the nucleus HVC (proper name, not an abbreviation) (Reiner, Laverghetta, Meade, Cuthbertson, & Bottjer, 2003). First, the neuronal pathways from the HVC innervate the robust nucleus of arcopallium (RA). Then, the RA projects to various brainstem motor control centers that support respiration and vocalization in songbirds. Of these projections to the brainstem motor control centers includes the neuronal projections to the motor neurons that innervate the syrinx, the organ in songbirds crucial for sound production (Ziegler & Marler, 2008).

### ***What are the pathways involved in song learning?***

Song learning is primarily controlled by the anterior forebrain pathway (AFP). Similar to the motor pathway, the AFP begins in the nucleus HVC. The HVC then makes an indirect connection to the RA by first projecting to Area X, a specialized region of the striatum. Area X then projects to the medial part of the dorsolateral anterior thalamic nucleus (DLM). Then, DLM projects to the lateral magnocellular nucleus of the anterior nidopallium (LMAN). Finally, LMAN neurons project to RA and sends axon collaterals to Area X. While the AFP is not required for song production, which relies mainly on the motor pathway, it is necessary for song learning and adult song plasticity (Achiro, Shen, & Bottjer, 2018). Research by Kao, Doupe, & Brainard (2005) suggested that the neurons found in the AFP are often song selective, meaning that during variability in motor output of song production, these neurons selectively reinforce motor activity that resemble the target behavior (Solis & Doupe, 1997). The presence and contribution of these song selective neurons further confirms the role of AFP in the auditory processing of song related information and thus the AFP's primary function being learning of song rather than production (Kao, Doupe, & Brainard, 2005). In addition to supporting auditory processing, the AFP also mediates motor exploration in songbirds allowing them to experience

greater acoustic and song variability. Neuronal projections from LMAN to RA form the last step in the indirect pathway of the AFP. As the direct projections from the RA to the motor control centers mediate song production, projections from indirect pathway play an important role in motor exploration. Motor exploration in turn supports songbirds in their song learning period of development (Kojima, Kao, Doupe, & Brainard, 2018). Studies have demonstrated that stimulating the LMAN increases song variability and disruption to the projection from LMAN to RA reduces song variability, inhibiting possibilities of motor exploration and therefore hindering song learning (Achiro et al., 2018). Another input to area x that is a part of the AFP is from the ventral tegmental area (VTA). Area x receives dopaminergic projections from the VTA, providing positive reinforcements with its reward signals (Leblois & Perkel, 2012).

Dopaminergic neurons are excited and show increased firing in the presence of rewards and are decreased in the absence of rewards. For example, when a male zebra finch is singing in the presence of a female for mating purposes, the dopaminergic projections from the VTA to area x increases thus decreasing song variability producing more stereotyped song (Kubikova, Wada, & Jarvis, 2010; Leblois & Perkel, 2012).

### ***Why do only male zebra finches learn to sing?***

Female zebra finches do not sing and therefore, do not participate in the vocal learning process (Shaughnessy, Hyson, Bertram, Wu, & Johnson, 2018). The regions of the brain necessary for song learning that are found in male zebra finches are smaller in female zebra finches and the presence of area X is yet to be discovered in the female birds. Additionally, many of the neural pathways and projections from cortical structures that facilitate song learning in male zebra finches are not as robust in female zebra finches. The first main efferent target of the HVC neuronal projections is to the RA, for motor production of song (Shaughnessy et al., 2018).

The connection between HVC to RA is incomplete in juvenile female zebra finches, which may be why they do not sing as much as their male counterparts (Holloway & Clayton, 2001). The second main efferent target from HVC is to area X, which is the beginning of the AFP, crucial for song learning (Shaughnessy et al., 2018). Many studies have attempted to quantify the location and size of area X in the female zebra finch brain and have been unsuccessful as it was not clearly discernible (Grisham et al., 2007). The presence of area X, one of the important neural substrates in the AFP, remains unclear in female songbirds. Due to differences in the neural structures and the connections between the neural structures between the male and female zebra finches, males are able to learn and produce song. Male birds not only learn to sing but are able to produce and modify their song based on social situations because of their developed pathways from the HVC for the purpose of attracting female counterparts (Takahashi, 2019).

***What is the role of striatal area x and dopamine?***

In both humans and birds, the basal ganglia form a crucial subcortical structure of the brain that supports acquisition, initiation and selection of motor acts (Xiao et al., 2018). In mammals, the structure and function of the components of the basal ganglia circuitry are well studied and clearly defined. However in songbirds, the primary defined region that is equivalent to the striatum in the mammalian basal ganglia circuitry, is striatal area X (Kojima et al., 2018). Area X and the mammalian basal ganglia are composed of the similar neuron types which facilitate the acquisition and learning of song in songbirds (Brainard & Doupe, 2013; Leblois & Perkel, 2012). Area X, one of the subcortical neural substrates part of the vocal learning circuitry, performs the functions of acquisition, initiation and selection of motor acts in songbirds, receiving primary input projections from the HVC, LMAN, and the VTA. Output projections from area X are to DLM, which then projects to LMAN as part of the indirect AFP (Brainard & Doupe, 2013). The

output functions of area X are largely determined by its constituent neurons and they connect and communicate with the other regions (HVC, LMAN, and VTA) through the neural circuitry (Ross et al., 2019). Neurotransmitters, or the chemical messengers, are transmitted between two neurons that facilitate the complex connections within the neural circuitry (Yoo et al., 2016). Specifically, the connection from VTA to Area X results in the known release of the neurotransmitter striatal dopamine. Striatal dopamine functions to regulate of the acquisition, initiation, and selection of motor acts and also modulates the basal ganglia circuitry to change behavior when necessary (Leblois & Perkel, 2012). The ability to modulate and change behavior is what results in learning, specifically trial and error learning observed in mammals and songbirds. In both mammals and songbirds, the primary source of input of dopaminergic neurons to the basal ganglia is from the VTA, or midbrain. As mentioned previously, excitation and inhibition of dopaminergic neurons in area X guides song learning through positive and negative reinforcements. Hence, it is hypothesized that the VTA is a structure in both humans and songbirds that plays a vital role in reward-seeking behaviors and learning (Xiao et al., 2018). However, through *in vitro* studies, it has been found that the neurons in the VTA are capable of transmitting two more distinct types of neurotransmitters in addition to dopamine to the striatum: glutamate and gamma-aminonutyric acid (GABA) (Barker, Root, Zhang, & Morales, 2016). The functions of the midbrain/VTA neurotransmitters are extremely diverse and are responsible for reward processing, decision making, flexible behaviors, learning, processing aversive outcomes, fear, aggression, cognition, arousal, etc. Because of their diverse signaling abilities that support complex behaviors, it was hypothesized and found that neurons from the VTA are able to signal using more than one neurotransmitter, a process known as co-release and co-transmission (Yoo et al., 2016). While electrophysiological *in vitro* studies in rodents

demonstrate the ability of neurons that release dopamine (dopaminergic neurons) to also release and transmit glutamate, the co-release mechanism of dopamine and glutamate has not been demonstrated in humans or songbirds (Barker et al., 2016). In songbirds, a study conducted by (Xiao et al., 2018), suggested that while dopaminergic input from the VTA to area X supports and guides song learning through positive and negative reinforcement, dopamine signaling alone may not be sufficiently rapid or brief to make fast changes based on auditory feedback during the song learning process. Neurons communicate with each through exchanging neurotransmitters through two different mechanisms: fast or slow synaptic transmission. Dopamine is classified as a slow-acting neurotransmitter and its effects are carried out over hundreds of milliseconds to minutes. In contrast, the glutamate and GABA are classified as fast-acting neurotransmitters, resulting in effects on their target neurons within only one millisecond (Greengard, 2001). Xiao et al. (2018) hypothesized that there must be a faster reacting neurotransmitter such as glutamate that is co-released with dopamine that can modify birdsong syllables that are only 10 milliseconds in duration. Glutamate, when co-released with dopamine, from VTA to area X supports the temporal precision in which songbirds use reinforcement learning to guide their song learning and neuronal plasticity. Here, I carried out an initial test of this hypothesis by examining whether the terminal boutons (i.e., presynaptic ends) of VTA axons in area X express immunoreactivity for markers of both glutamatergic and dopaminergic neurotransmission.

## **II. Materials and Methods**

### ***Animals***

Two male zebra finches (*Taeniopygia guttata*), post hatch were used in this study. The adult bird was raised in our breeding colony or purchased from a commercial supplier and the juvenile bird was hatched in our colony. The juvenile bird was 55 days post-hatch, in the

subsong stage (sensorimotor stage) of learning and the adult bird was over 90 days post-hatch, past the crystallization stage of learning. Food and water were available *ad libitum*. All procedures and experiments were conducted in accordance with an animal protocol approved by the University of Washington Institutional Animal Care and Use Committee.

### ***Surgical Procedure***

Retrograde tracers were injected into VTA, surgically on the adult and juvenile male zebra finches ( $n = 2$  birds). The animals were isolated from food and water for 30 minutes prior to surgery, and then anesthetized with 2–3% isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane). Once anesthetized, feathers on the head and neck were removed with scissors and the head was fixed into the stereotaxic apparatus using both a beak clamp and ear bars. The scalp was cleaned using a sterile betadine swab (Purdue Products L.P., Stamford, CT), and a small bolus of 1% lidocaine (APP Pharmaceuticals, Schaumburg, IL) was injected subcutaneously. After a midline scalp incision, small craniotomies were performed above the brain region of interest. The stereotaxic coordinates used to inject VTA for the adult bird were (relative to the anastomosis of the sinuses): 0.5 mm anterior; 0.6 mm lateral; 6.4 mm deep from the brain surface; 64° head angle (Adapted from Gale et al., 2008). The same parameters for the juvenile bird were (relative to the anastomosis of the sinuses): 0.3 mm anterior; 1.0 mm lateral; 6.4 mm deep from the brain surface; 64° head angle (Adapted from Gale et al., 2008). Head angle was defined as the measure between horizontal plane of ear bar to tip of beak. 10% fluororuby (D1817, Invitrogen) tracer was injected via iontophoresis for 10–15 min with an alternating current of +5  $\mu$ A and a 50:50 on/off duty cycle of 7 s. In some animals, two injections were made to increase the size of the injection. In such cases, the electrode was lowered to 4.2 mm for 10 min then lowered another 0.2 mm (to 4.4 mm) for a second 10-min injection. After 6–

7 days survival time, the animals were euthanized, transcardially perfused and processed as described below. Meloxicam and bupivacaine were used as the postoperative analgesics.

### ***Immunohistochemical Procedure***

Immunohistochemical methods were used in this experiment to localize antibodies, which were used to stain and view two proteins of interest in this study: tyrosine hydroxylase (TH) as marker for dopamine and VGLUT2, the vesicular transporter for glutamate. The animals were first euthanized with an overdose of isoflurane and transcardially perfused with 0.9% saline for 8-9 minutes followed by 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) with 0.1% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M PB for 30 minutes using a peristaltic pump. Brains were removed from the skull and stored overnight in 0.1 M PBS solution. Using a freezing microtome (American Optical), the brains were sectioned parasagittally at 40 micrometers. Sections were washed with 0.1 M PB and then blocked with 3% normal goat serum for an hour to reduce non-specific labeling/binding. Then, tissue was incubated in primary antibody for 2-3 days at 4 degrees Celsius on a horizontal shaker. The following primary antibodies were used to initially bind to TH and VGLUT2 on the tissue: mouse anti-VGLUT2 at 1:200 concentration and rabbit anti-TH at 1:1000 concentration. The tissue was again rinsed for 3 times, 10 minutes each to remove unbound antigens. To increase specificity of binding and to label with fluorescent for easier viewing, tissue was incubated in secondary antibodies for 2 hours. The following secondary antibodies were used: goat anti-mouse conjugated to Alexa (Thermo Fisher) fluorophore emitting light at 488 nm and goat anti-rabbit conjugated to Alexa fluorophore emitting at 647 nm. Tissue was washed with 0.1 M PBS for 3 times, 10 minute each and mounted immediately on microscope slides and cover-slipped with mounting medium.

### ***Confocal Microscopy***

Olympus Fluoview-1000, a confocal laser scanning microscope, was used to obtain the images from the tissue sections for qualitative and quantitative data analysis. Confocal images allow for in depth analysis of the processed tissue sections through the reduction of background noise and by providing multiple optical sections of the 40 micrometers thick tissue specimen. *See Table 1* for the number of optical sections analyzed for each image. Additionally, each image contained three output channels. Channel 1 displayed only the VGLUT2 positive terminal boutons (green, 647 nm), channel 2 displayed the axons from VTA to area X (red), and channel 3 displayed the TH positive terminal boutons (blue, 488 nm). The parameters for the images from the adult bird were 105.6 microns (width) x 105.6 microns (height) and depth ranging from 20-50 microns with an average of 73 images in each sample image. The parameters for the juvenile bird were 212 microns (width) x 212 microns (height) and depth ranging from 15-16 microns with an average of 62 images in each sample image.

### ***Data Analysis***

Fiji and Image J were used to obtain data for qualitative and quantitative data from the confocal images. Each of the 8 images (4 from each bird) was thoroughly scanned to identify the presence of terminal boutons positive for TH, VGLUT and both TH and VGLUT2. Brightness and contrast for each image was adjusted for efficient scanning. Three channels of each image were overlaid to visually identify terminal boutons positive for TH and VGLUT2. A yellow color suggested when terminal boutons were positive for TH and VGLUT2 immunoreactivity. The tracer dye (fluororuby), red in color, which was injected during the surgical procedure identified the axons projecting from VTA to area X, ending at the terminal boutons. To obtain quantitative data, a threshold was set and adjusted for each image to further remove any residual

background noise to increase specificity of terminal bouton identification following which the images were processed as binary images. Then, the images were split into their three channels (VGLUT2 channel 1, tracer channel 2, and TH channel 3). The “analyze particle” command in ImageJ was used for channel 1 and channel 3 to obtain the number of VGLUT2 positive terminal boutons and the number of TH positive terminal bouton, respectively. The size, measured in pixel<sup>2</sup>, was adjusted to 1-infinity (i.e., not including particles less than 1 pixel<sup>2</sup> in data) and the circularity was adjusted to 0.20-1.00 (a value of 1.0 indicating a perfect circle). The “image calculator” command was then used to identify the percent of TH positive terminal boutons that also was positive for VGLUT2. Using the “AND” function from the image calculator command with the analyzed particles data from channel 1 VGLUT2 and channel 3 TH, the numerical data of TH and VGLUT2 positive terminal boutons was obtained. Paired t-test was used to compare quantitative data.

### **III. Results**

#### **Qualitative**

Qualitative examination of the images revealed that the terminal boutons of the VTA axons projecting to area X did express immunoreactivity for the markers of both glutamatergic and dopaminergic neurotransmission. Both images contain stained terminal boutons within area X. *See Figure 3.*

#### **Quantitative**

Four tissue sections in each bird (total of 8 samples) were analyzed. We found that during the critical learning period of zebra finches, which can be anywhere from 25-60 days post hatch, and after crystallization of song (90 or more days post hatch), the terminal boutons of the VTA axons projecting to area X quantitatively expressed immunoreactivity for markers of both

glutamatergic and dopaminergic neurotransmission. To ensure adequate penetration of antibodies through tissue to label the proteins of interest TH and VGLUT2, two image stacks were analyzed (one from juvenile bird and one from adult bird). In each of the stacks, the number of TH positive terminal boutons vs. TH and VGLUT2 positive terminal boutons were analyzed in the top 10, middle 10, and bottom 10 images. In all images of the two sample stacks antibody penetrated through the entire tissue and as expected, there were more TH positive terminal boutons compared to TH and VGLUT2 positive terminal boutons. *See Figure 4.*

#### ***Percentage of VGLUT2 and TH Positive Terminal Boutons***

For the tissue samples of the adult bird, there were on average  $73 \pm 25$  images in each stack with  $2040 \pm 1248$  TH positive terminal boutons. Of these TH positive terminal boutons,  $277 \pm 142$  or  $19.5 \pm 17.8\%$  were also positive for VGLUT2. For the tissue samples of the juvenile bird, there were on average  $62 \pm 1.5$  images in each stack with  $27054 \pm 4213$  TH positive terminal boutons. Of these TH positive terminal boutons,  $13999 \pm 427$  or  $52.75 \pm 9.63\%$  were also positive for VGLUT2.

#### ***Frequency of TH and VGLUT2 Positive Images in Stacks***

In all sample stacks from both birds, most if not all images had at least one terminal bouton that expressed immunoreactivity for TH and VGLUT2. In each of the sample stacks from the adult bird which had stereotyped and crystallized song, at least one image did not have VTA terminal boutons that positively expressed both TH and VGLUT2. In contrast, all images from each of the sample stacks from the juvenile bird had at least one VTA terminal bouton positive for TH and VGLUT2. *See Figure 5.*

#### ***Particle Analysis of TH Positive vs. TH and VGLUT2 Positive Terminal Boutons***

The size of individual particles of TH positive and TH and VGLUT2 positive terminal boutons were calculated for each stack for each image in all eight sample stacks (4 from juvenile and 4 from adult). The average individual particle size of the TH positive terminal boutons of the juvenile bird was  $1.7 \pm 0.10 \mu\text{m}^2$ . The average individual particle size of TH and VGLUT2 positive terminal boutons of the juvenile bird was  $1.6 \pm 0.10 \mu\text{m}^2$ . The average individual particle size of the TH positive terminal boutons of the adult bird was  $1.4 \pm 0.05 \mu\text{m}^2$ . The average individual particle size of TH and VGLUT2 positive terminal boutons of the adult bird was  $1.1 \pm 0.18 \mu\text{m}^2$ .

There were no significant differences between the individual particle sizes between TH positive terminal boutons vs. TH and VGLUT2 positive terminal boutons in both birds. In other words, the size of the terminal bouton was the same regardless of the presence of one vs. two neurotransmitters. *See Table 2.*

#### **IV. Discussion**

The main findings of this study indicate that in both the juvenile- and adult-male zebra finch, some VTA axon terminals in area X expressed immunoreactivity for both glutamatergic and dopaminergic neurotransmission. The fraction of TH positive neurons that were VGLUT2 positive (i.e., confirming the presence of co-labeled axon terminals) was higher in the juvenile bird than the adult bird. Although this finding cannot confirm the co-release of both the neurotransmitters dopamine and glutamate, the presence of co-labeled axon terminals in both birds demonstrated in this study indicates a strong possibility of co-release.

The cortico-basal ganglia circuitry, or the AFP, is critical for mediating song learning in zebra finches by allowing the birds to constantly evaluate their performance through auditory feedback to select appropriate motor actions that matches the memorized song learned from their

adult tutor bird (Achiro et al., 2018). Human infants, in the sensory learning phase of development, also participate in goal directed learning during development as they initially listen to vocal sounds from their parents/caregivers. Once they start engaging in vocal play, beginning to explore their own communicative behaviors, they make comparisons between the feedback of their own productions and the target production. However, one difference between goal-directed learning in infants and songbirds is the type of feedback they receive while they participate in goal-directed learning of their way of communication. In songbirds, once the critical learning period closes, they can rely solely on their auditory feedback to adjust their next performance of their song to match their tutor bird's song, which they have listened to and memorized in the sensory learning stage. However, infants use and receive more than just their own auditory feedback while imitating their adult models. Maternal responses to their vocal exploration also reinforces their vocal behaviors, thus activating the cortico-basal ganglia circuitry promoting further vocal learning (Levickis, Reilly, Girolametto, Ukoumunne, & Wake, 2014). Infants that received a higher maternal responsiveness to their vocal explorative behaviors actually reached speech-language milestones earlier at nine months of age compared to the infants who received a lower maternal responsiveness rate (Tamis-LeMonda, Bornstein, & Baumwell, 2001). As infants mature, their vision and proprioception, or their perception and awareness of the position and movement of articulators also improve, two more feedback mechanisms that advance learning of their communication (Guenther & Vladusich, 2012). In comparison, songbirds solely rely on their own auditory feedback during the sensorimotor learning stage to eventually produce crystallized song, 90-days post-hatch. Zebra finches primarily use auditory feedback, which not only activates the AFP but also the signaling between the VTA and area X, providing positive and negative reinforcement signaling to learn to produce the appropriate motor act (Budzillo,

Duffy, Miller, Fairhall, & Perkel, 2017; Xiao et al., 2018). While the presence of dopaminergic signaling from VTA to area X has been studied and demonstrated to regulate vocal learning behaviors, how this neurotransmitter modulates the AFP circuitry to change the behaviors is not yet clear (Budzillo et al., 2017). This present study's findings suggest that one method dopamine could be changing behaviors in song-birds is by recruiting the co-release of glutamate from axons extending from the VTA to area X.

After the crystallization stage, zebra finches produce fixed, stable and stereotyped song and remains unchanged throughout their life span (Doupe & Kuhl, 1999). Motor learning through auditory feedback is sparse in birds after the crystallization stage, when compared to a bird in the sensorimotor learning stage. More production of syllables of song, auditory feedback from each iteration, and adjusting the next production based on this feedback is an ongoing process in a juvenile bird in the sensorimotor learning stage, particularly the subsong stage, which is highly variable and is comparable to infant babbling (Goldberg & Fee, 2011), when compared to an adult bird producing a stable song. It is possible, although further studies are warranted, that the juvenile bird may be reliant on more reinforcement signaling through dopaminergic and glutamatergic projections from VTA to area X to facilitate temporally precise learning compared to the adult bird. However, the small fraction of co-labeled dopaminergic and glutamatergic axon terminals found in the adult bird in this study could be attributed to the plasticity of song, depending on social-context. Male zebra finches use their mature, crystallized song, which was learned through trial and error, to attract and court females. Although it is unlikely they will learn a new song after reaching crystallization, the variability of their song production is highly dependent on social context. Their stereotyped song is found to be less variable and therefore more attractive in the presence of a female bird than song produced in

isolation (Budzillo et al., 2017). It is probable that some, although not as much as in the juvenile bird, co-release of dopamine and glutamate to area X is responsible for the less variable song production in the presence of a female bird.

This initial step in identifying the possibility of co-release of the neurotransmitters, dopamine and glutamate from the VTA to area X, provides one out of the many possible neural signaling mechanisms modulating the AFP, making changes to the vocal behavior during song learning. This particular co-release mechanism has not yet been identified in humans yet (Barker et al., 2016), but has been detected in rodents (Higley et al., 2011). Further studies that explore neural circuitries involved in learning skilled behaviors is warranted as the loop between the basal ganglia (area X in songbirds), cortical and thalamic structures is complex both in humans and songbirds (Caligiore, Mannella, Arbib, & Baldassarre, 2017). Alterations in this circuitry or the signaling between the circuitry leads to many disorders of neural motor control and therefore severely impacting communication such as Parkinson's Disease, Huntington's Disease, attention deficit hyperactivity disorder (ADHD), Tourette's, and stuttering (Alm, 2004; Caligiore et al., 2017; Kalkhoven, Sennel, Peeters, & Van Den Bos, 2014; McGregor & Nelson, 2019). Understanding the unique underlying signaling mechanisms in each of these disorders as well as disorders of learning may provide the potential to develop clinical drugs which correct the altered, atypical signaling.

The primary limitations of this study were its small sample size and the possibilities of imperfect automatic detection of terminals using the ImageJ/Fiji software. Obtaining and analyzing results with a larger sample size, including multiple birds in both of the stages (subsong with juvenile bird and crystallization with adult bird) will further validate this study, providing a clearer understanding regarding the relationship between VTA/area and co-release of

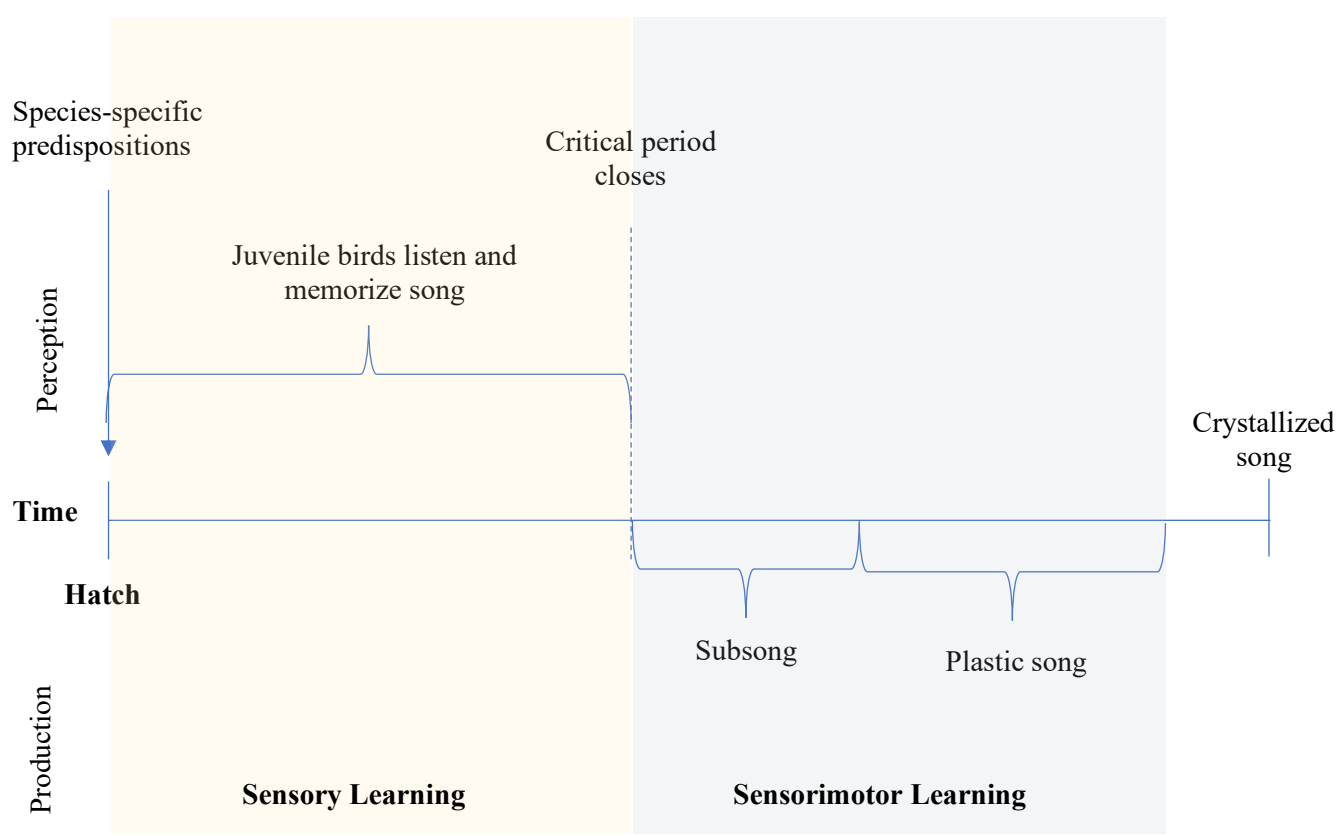
neurotransmitters. Studying the presence of dopamine and glutamate co-release from VTA into area X in other stages of development in the zebra finch is also warranted to better understand the relationship between development and reinforcement learning through performance based evaluation. This study only examined differences between a bird in the subsong stage and a bird past crystallization stage. Future studies can potentially address this same question between birds in the sensory stage vs. sensorimotor stage or subsong stage vs. plastic song stage.

Simultaneously, electrophysiological studies to test for actual co-release, meaning both neurotransmitters are released with a single action potential from one axon, would be another possible direction to take this study further. Lastly, applying this study to behavioral research is the first step to slowly start bridging the gap between cellular research and clinical application. Examining the impact of other sensory feedback mechanisms such as visual and proprioceptive feedback on their auditory performance evaluation based learning of zebra finches and considering not only the possibility of depletion of dopamine but also an excess of glutamate in Parkinson's Disease patients will allow this study to expand into more clinically relevant studies.

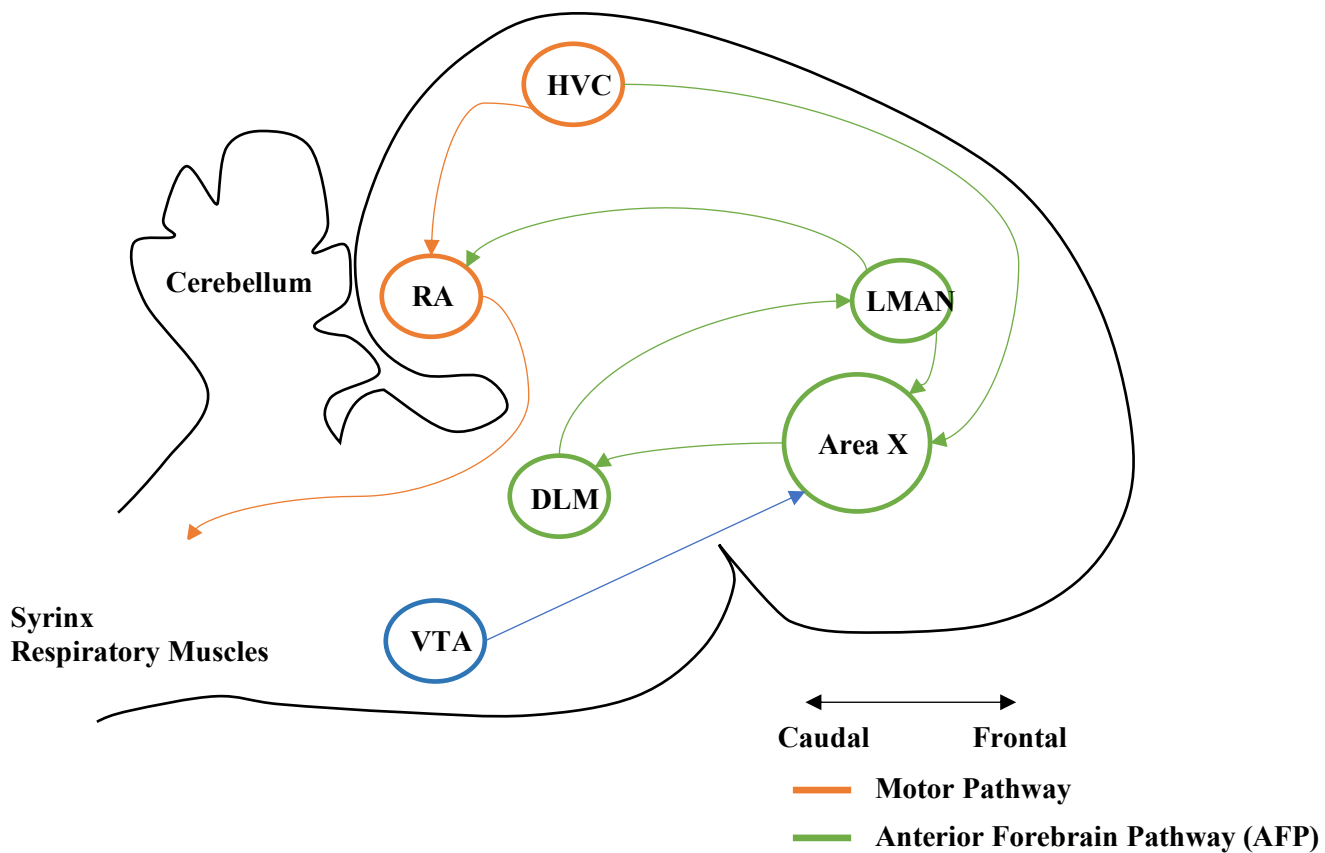
## **V. Conclusion**

This study adds to the growing body of research literature, trying to understand the complex neural circuitries underlying vocal learning in zebra finches and in humans. As the first study to demonstrate the possibility of co-release from VTA to area X in zebra finches, it qualitatively and quantitatively identified the presence of double-labeled terminal boutons positive for dopaminergic and glutamatergic neurotransmission. Although there were limitations to our study, this is one more step towards a clearer understanding of ultimately how speech and language not only develop from infancy but also how both are perturbed if there are alterations to the relevant circuitries.

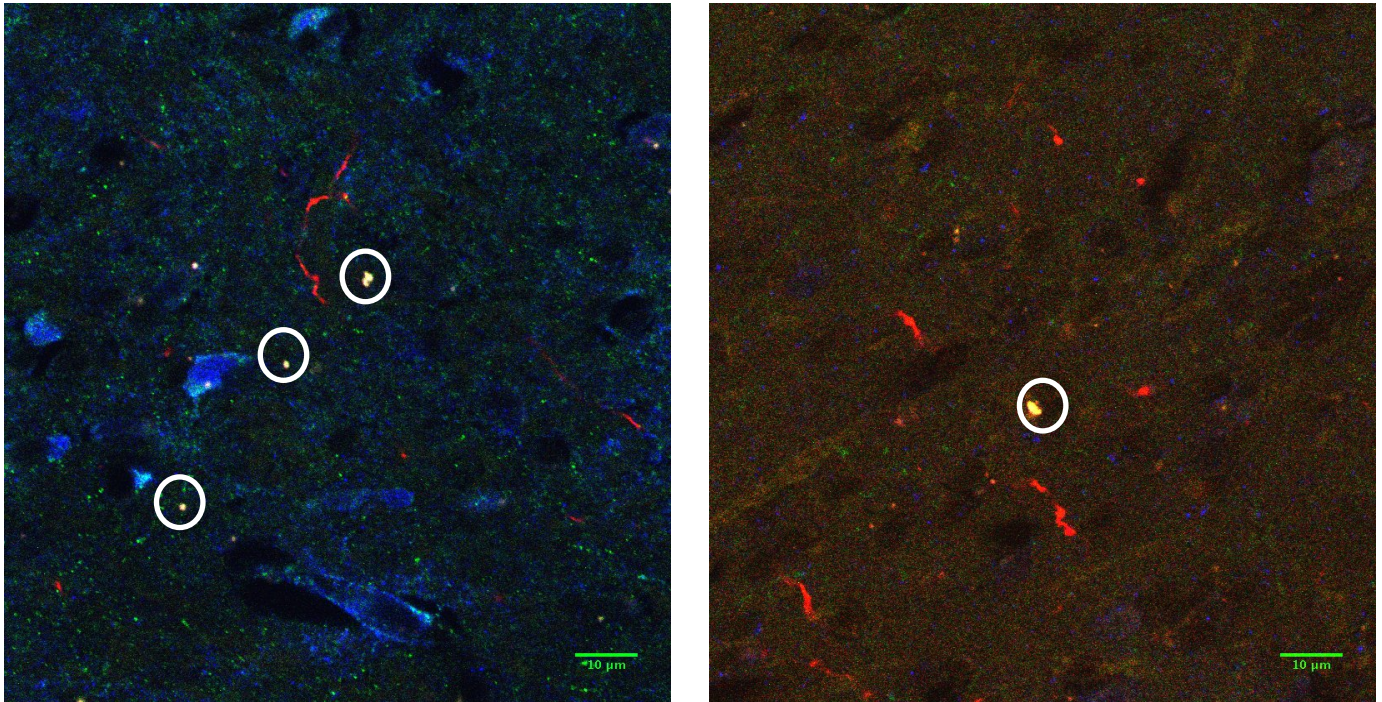
**Figures**



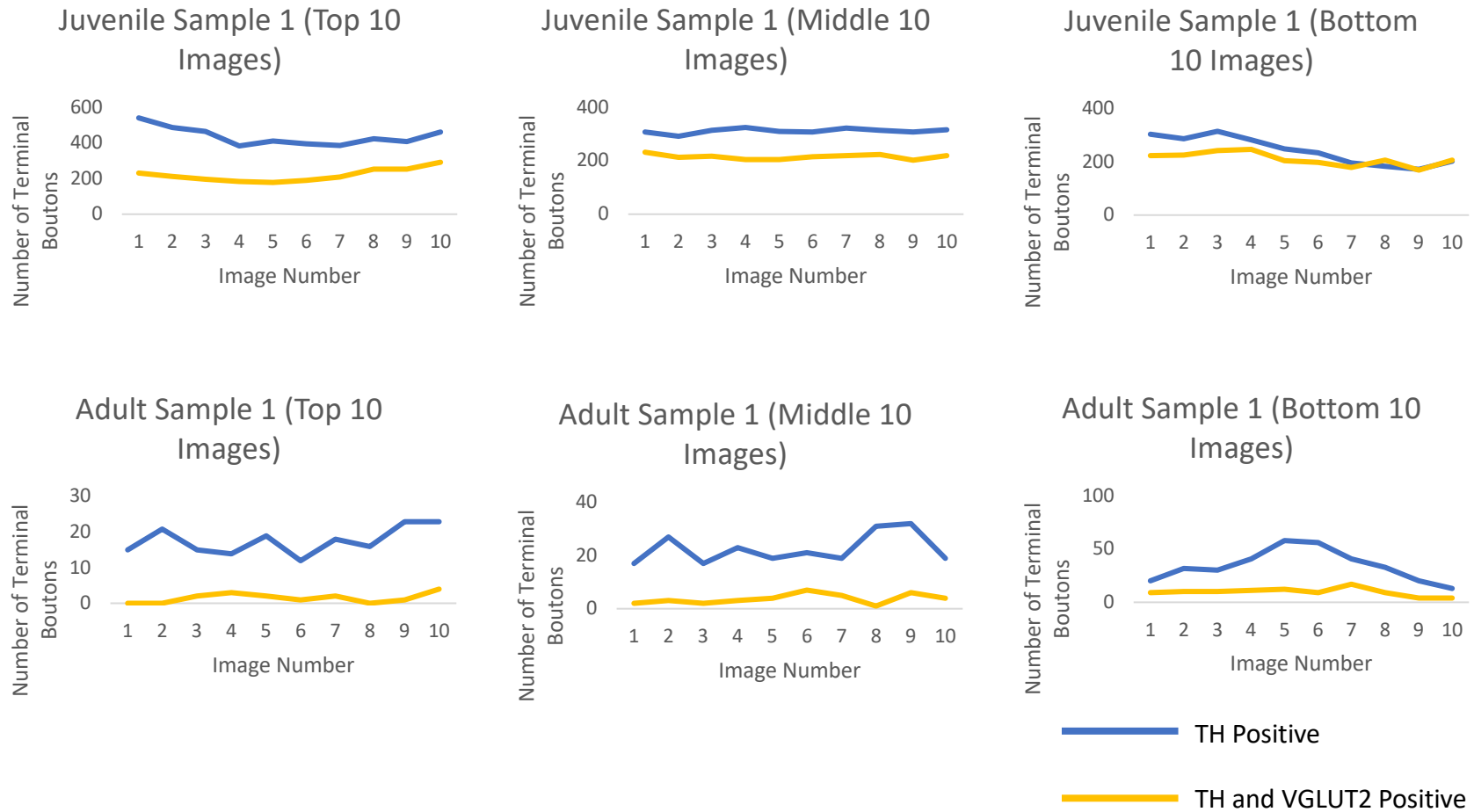
**Figure 1.** Stages of zebra finch song development. Sensory learning period can be anywhere from 25-60 days post hatch. The sensorimotor learning period includes the subsong and plastic song stage. The duration of the sensorimotor learning period can be anywhere from 50-90 days post hatch. After 90 days, the adult zebra finch produces a crystallized song, which is highly stereotyped.



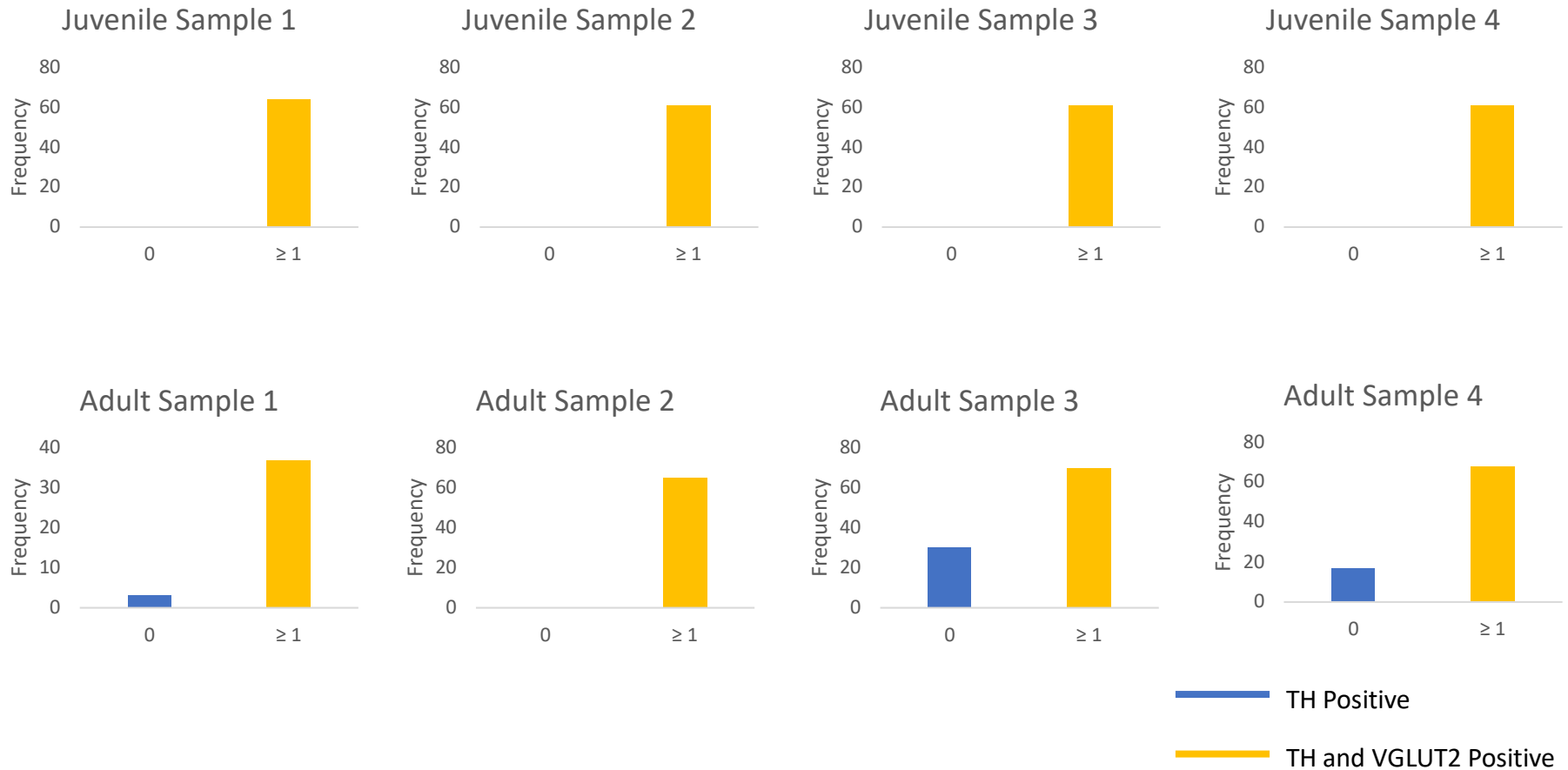
**Figure 2.** Neural pathways in the zebra finch that support the learning (AFP) and production (motor pathway) of song. The ventral tegmental area (VTA) is the primary source of dopaminergic input to area X involved in reinforcement learning of song.



**Figure 3.** Terminal boutons in area X. Blue is a marker for tyrosine hydroxylase (TH), an indicating dopaminergic terminal boutons. Green is a marker for VGLUT2, indicating glutamatergic terminal boutons. Yellow terminal boutons enclosed in white circles suggest the colocalization of both glutamate and dopamine in the same terminal bouton.



**Figure 4.** Confirming antibody penetration through tissue. In the top, middle, and bottom images of both stacks, antibody penetration was demonstrated.



**Figure 5.** Frequency of TH and VGLUT2 positive images in stacks. All images from both birds (adult and juvenile) had at least one terminal bouton that expressed immunoreactivity for TH and VGLUT2.

	# of images in stack	TH Positive Terminal Boutons	TH and VGLUT2 Positive Terminal Boutons	Percentage
JUVENILE				
Sample 1	64	20738	14085	67%
Sample 2	61	29067	14410	50%
Sample 3	61	29361	13399	46%
Sample 4	61	29050	14103	48%
<i>Average</i>	$62 \pm 1.5$	$27054 \pm 4213$	$13999 \pm 427$	$52.75 \pm 9.63^*$
ADULT				
Sample 1	40	991	189	19%
Sample 2	65	987	448	45%
Sample 3	100	3445	338	10%
Sample 4	85	2740	136	5%
<i>Average</i>	$73 \pm 25$	$2040 \pm 1248$	$277 \pm 142$	$19.75 \pm 17.8^*$

**Table 1.** Percentages of co-labeled terminal boutons. A higher percentage of co-labeled terminal boutons were found in the juvenile bird compared to the adult bird. \* $p < 0.0$

	Juvenile Bird (Individual Particle Size, $\mu\text{m}^2$ )		Adult Bird (Individual Particle Size, $\mu\text{m}^2$ )	
	TH	TH + VGLUT2	TH	TH + VGLUT2
Sample 1	$1.5 \pm 0.08$	$1.5 \pm 0.08$	$1.4 \pm 0.09$	$1.3 \pm 0.47$
Sample 2	$1.7 \pm 1.10$	$1.5 \pm 0.09$	$1.5 \pm 0.36$	$1.3 \pm 0.25$
Sample 3	$1.7 \pm 0.10$	$1.5 \pm 0.08$	$1.4 \pm 0.12$	$0.97 \pm 0.73$
Sample 4	$1.7 \pm 0.08$	$1.7 \pm 0.09$	$1.4 \pm 0.08$	$1.0 \pm 0.55$
<i>Average</i>	$1.7 \pm 0.1$	$1.6 \pm 0.1$	$1.4 \pm 0.05$	$1.1 \pm 0.18$

**Table 2.** Individual particle analysis of TH vs. TH and VGLUT2 positive terminal boutons. No significant difference in individual particle size was found between TH positive and TH and VGLUT2 positive terminal boutons.

## References

- Achiro, J. M., Shen, J., & Bottjer, S. W. (2018). Neural activity in cortico-basal ganglia circuits of juvenile songbirds encodes performance during goal-directed learning. *eLife*, 1–25. <http://doi.org/10.7554/eLife.26973.001>
- Alm, P. A. (2004). Stuttering and the basal ganglia circuits: a critical review of possible relations. *Journal of Communication Disorders*, 37(4), 325–369. <http://doi.org/10.1016/j.jcomdis.2004.03.001>
- Barker, D. J., Root, D. H., Zhang, S., & Morales, M. (2016). Multiplexed neurochemical signaling by neurons of the ventral tegmental area. *Journal of Chemical Neuroanatomy*, 73, 33–42. <http://doi.org/10.1016/j.jchemneu.2015.12.016>
- Brainard, M. S., & Doupe, A. J. (2013). Translating Birdsong: Songbirds as a Model for Basic and Applied Medical Research. *Annual Review of Neuroscience*, 36(1), 489–517. <http://doi.org/10.1146/annurev-neuro-060909-152826>
- Budzillo, A., Duffy, A., Miller, K. E., Fairhall, A. L., & Perkel, D. J. (2017). Dopaminergic modulation of basal ganglia output through coupled excitation–inhibition. *Proceedings of the National Academy of Sciences*, 114(22), 5713–5718. <http://doi.org/10.1073/pnas.1611146114>
- Caligiore, D., Mannella, F., Arbib, M. A., & Baldassarre, G. (2017). Dysfunctions of the basal ganglia-cerebellar-thalamo-cortical system produce motor tics in Tourette syndrome. *PLoS Computational Biology*, 13(3), e1005395–34. <http://doi.org/10.1371/journal.pcbi.1005395>
- Carouso-Peck, S., & Goldstein, M. H. (2019). Female Social Feedback Reveals Non-imitative Mechanisms of Vocal Learning in Zebra Finches. *Current Biology*, 29(4), 631–636.e3. <http://doi.org/10.1016/j.cub.2018.12.026>
- Doupe, A. J., & Kuhl, P. K. (1999). BIRDSONG AND HUMAN SPEECH: Common Themes and Mechanisms. *Annual Review of Neuroscience*, (22), 567–631.
- Goldberg, J. H., & Fee, M. S. (2011). Vocal babbling in songbirds requires the basal ganglia-recipient motor thalamus but not the basal ganglia. *Journal of Neurophysiology*, 105(6), 2729–2739. <http://doi.org/10.1152/jn.00823.2010>
- Goller, F., & Cooper, B. G. (2004). Peripheral Motor Dynamics of Song Production in the Zebra Finch. *New York Academy of Sciences*, (1016), 130–152.
- Greengard, P. (2001). The Neurobiology of Slow Synaptic Transmission. *Science*, 294(5544), 1024–1030. <http://doi.org/10.1126/science.294.5544.1024>

- Grisham, W., Park, S. H., Hsia, J. K., Kim, C., Leung, M. C., Kim, L., & Arnold, A. P. (2007). Effects of long-term flutamide treatment during development in zebra finches. *Neuroscience Letters*, *418*(1), 92–96.
- Guenther, F. H., & Vladusich, T. (2012). A neural theory of speech acquisition and production. *Journal of Neurolinguistics*, *25*(5), 408–422. <http://doi.org/10.1016/j.jneuroling.2009.08.006>
- Higley, M. J., Gittis, A. H., Oldenburg, I. A., Balthasar, N., Seal, R. P., Edwards, R. H., et al. (2011). Cholinergic Interneurons Mediate Fast VGluT3-Dependent Glutamatergic Transmission in the Striatum. *PLoS ONE*, *6*(4), e19155–7. <http://doi.org/10.1371/journal.pone.0019155>
- Hoffmann, L. A., Saravanan, V., Wood, A. N., He, L., & Sober, S. J. (2016). Dopaminergic Contributions to Vocal Learning. *Annual Review of Neuroscience*, *36*(7), 2176–2189. <http://doi.org/10.1523/JNEUROSCI.3883-15.2016>
- Holloway, C. C., & Clayton, D. F. (2001). Estrogen synthesis in the male brain triggers development of the avian song control pathway. *Nature Publishing Group*, *4*(2), 170–175.
- Kalkhoven, C., Sennef, C., Peeters, A., & Van Den Bos, R. (2014). Risk-taking and pathological gambling behavior in Huntington's disease. *Frontiers in Behavioral Neuroscience*, *8*(103), 1–12. <http://doi.org/10.3389/fnbeh.2014.00103/abstract>
- Kao, M. H., Doupe, A. J., & Brainard, M. S. (2005). Contributions of an avian basal ganglia–forebrain circuit to real-time modulation of song. *Nature Publishing Group*, *433*(7026), 638–643.
- Kojima, S., Kao, M. H., Doupe, A. J., & Brainard, M. S. (2018). The Avian Basal Ganglia Are a Source of Rapid Behavioral Variation That Enables Vocal Motor Exploration. *Annual Review of Neuroscience*, *38*(45), 9635–9647. <http://doi.org/10.1523/JNEUROSCI.2915-17.2018>
- Kubikova, L., Wada, K., & Jarvis, E. D. (2010). Dopamine receptors in a songbird brain. *The Journal of Comparative Neurology*, *518*(6), 741–769. <http://doi.org/10.1002/cne.22255>
- Kuhl, P. K., & Doupe, A. J. (2008). Birdsong and human speech: common themes and mechanisms. In P. Zeigler & P. Marler (Eds.), *Neuroscience of Birdsong*. Cambridge.
- Leblois, A., & Perkel, D. J. (2012). Striatal dopamine modulates song spectral but not temporal features through D1 receptors. *European Journal of Neuroscience*, *35*(11), 1771–1781. <http://doi.org/10.1111/j.1460-9568.2012.08095.x>
- Levickis, P., Reilly, S., Girolametto, L., Ukoumunne, O. C., & Wake, M. (2014). Maternal Behaviors Promoting Language Acquisition in Slow-to-Talk Toddlers: Prospective Community-based Study. *Journal of Developmental and Behavioral Pediatrics*, *35*, 274–281.

- McGregor, M. M., & Nelson, A. B. (2019). Circuit Mechanisms of Parkinson's Disease. *Neuron*, 101(6), 1042–1056. <http://doi.org/10.1016/j.neuron.2019.03.004>
- Mischler, S. K., Congdon, J. V., Scully, E. N., Campbell, K. A., & Sturdy, C. B. (2017). Passerine Vocal Communication. In J. Vonk & T. Shackelford (Eds.), *Encyclopedia of Animal Cognition and Behavior* (Vol. 99, pp. 1–7). Cham: Springer International Publishing. [http://doi.org/10.1007/978-3-319-47829-6\\_892-1](http://doi.org/10.1007/978-3-319-47829-6_892-1)
- Prat, Y., Taub, M., & Yovel, Y. (2015). Vocal learning in a social mammal: Demonstrated by isolation and playback experiments in bats. *Science Advances*, 1(2), e1500019–6. <http://doi.org/10.1126/sciadv.1500019>
- Price, T., Wadewitz, P., Cheney, D., Seyfarth, R., Hammerschmidt, K., & Fischer, J. (2015). Vervets revisited: A quantitative analysis of alarm call structure and context specificity. *Nature Publishing Group*, 1–11. <http://doi.org/10.1038/srep13220>
- Reiner, A., Laverghetta, A. V., Meade, C. A., Cuthbertson, S. L., & Bottjer, S. W. (2003). An immunohistochemical and pathway tracing study of the striatopallidal organization of area X in the male zebra finch. *The Journal of Comparative Neurology*, 469(2), 239–261. <http://doi.org/10.1002/cne.11012>
- Ross, M. T., Flores, D., Bertram, R., Johnson, F., Wu, W., & Hyson, R. L. (2019). Experience-Dependent Intrinsic Plasticity During Auditory Learning. *Annual Review of Neuroscience*, 39(7), 1206–1221. <http://doi.org/10.1523/JNEUROSCI.1036-18.2018>
- Shaughnessy, D. W., Hyson, R. L., Bertram, R., Wu, W., & Johnson, F. (2018). Female zebra finches do not sing yet share neural pathways necessary for singing in males. *The Journal of Comparative Neurology*, 527(4), 843–855. <http://doi.org/10.1002/cne.24569>
- Solis, M. M., & Doupe, A. J. (1997). Anterior Forebrain Neurons Develop Selectivity by an Intermediate Stage of Birdsong Learning. *Annual Review of Neuroscience*, 17(16), 6447–6462.
- Takahashi, D. Y. (2019). Vocal Learning: Shaping by Social Reinforcement. *Current Biology*, 29(4), R125–R127. <http://doi.org/10.1016/j.cub.2019.01.001>
- Tamis-LeMonda, C. S., Bornstein, M. H., & Baumwell, L. (2001). Maternal Responsiveness and Children's Achievement of Language Milestones. *Child Development*, 72(3), 748–767.
- Xiao, L., Chattree, G., Oscos, F. G., Cao, M., Wanat, M. J., & Roberts, T. F. (2018). A Basal Ganglia Circuit Sufficient to Guide Birdsong Learning. *Neuron*, 98(1), 208–221.e5. <http://doi.org/10.1016/j.neuron.2018.02.020>
- Yoo, J. H., Zell, V., Gutierrez-Reed, N., Wu, J., Ressler, R., Shenasa, M. A., et al. (2016). Ventral tegmental area glutamate neurons co-release GABA and promote positive reinforcement. *Nature Communications*, 7, 1–13. <http://doi.org/10.1038/ncomms13697>

Ziegler, W., & Ackermann, H. (2017). Subcortical Contributions to Motor Speech: Phylogenetic, Developmental, Clinical. *Trends in Neurosciences*, 40(8), 458–468.  
<http://doi.org/10.1016/j.tins.2017.06.005>