Role of LMAN in Introductory Notes learning in the male zebra finch

A collaborative project with Anand C Krishnan (20191097)

A Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfillment of the requirements for the BS-MS Dual Degree Programme

by

Indian Institute of Science Education and Research Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, INDIA.

Date: March 15, 2024

Under the guidance of

Dr. Raghav Rajan,

Assistant Professor, IISER Pune

From May 2023 to Mar 2024

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled “Role of LMAN in Introductory Notes learning in the male zebra finch” towards the partial fulfillment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Vidushi Sharma at the Indian Institute of Science Education and Research under the supervision of Dr. Raghav Rajan, Assistant Professor, Department of Biology, during the academic year 2023-2024.

Dr. Raghav Rajan

Committee:

Dr. Raghav Rajan

Dr. Nixon Abraham

This thesis is dedicated to finches and their symphonies.

Declaration

I hereby declare that the matter embodied in the report entitled " Role of LMAN in Introductory Notes Learning in the male zebra finch" are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education & Research (IISER) Pune, under the supervision of Dr. Raghav Rajan and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgment of collaborative research and discussions.

I declare that this work is part of a collaborative project executed together with Anand C Krishnan and Dr. Shikha Kalra, building upon the foundation laid by Shikha's previous work.

Vidushi Sharma

20191072

Table of Contents

List of Tables

List of Figures

Abstract

Understanding the neural mechanisms underlying vocal learning in songbirds is crucial for decoding the fundamental principles of motor skill acquisition. In this collaborative study, I investigated the role of the lateral magnocellular nucleus of the anterior nidopallium (LMAN) in IN (Introductory Notes) learning in zebra finches. Building on the previous results from the lab, I examined the impact of LMAN lesions on the learning of song motifs and introductory notes (INs) in juvenile birds. Our preliminary results reveal that LMAN-lesioned birds exhibit impaired learning of IN structures and number compared to socially tutored birds, suggesting a potential role of LMAN in controlling IN learning. Histological examinations confirm the absence or reduced volume of LMAN in lesioned birds compared to sham lesioned counterparts. These findings direct us toward understanding the neural circuitry underlying vocal learning and highlight the importance of LMAN in modulating specific aspects of INs acquisition.

Acknowledgments

This project would not have been possible without the support and guidance of some (actually, many) humans. I would like to take this opportunity to express my gratitude to them. I am grateful to my supervisor Dr. Raghav Rajan for creating a learning opportunity for me under his guidance and support. Thank you, Raghav (sir) for devoting your time and energy to our (so many) one-on-one meetings, patiently addressing our problems, and being ready to arrange whatever resources that aid our research.

I extend my gratitude to my thesis expert Dr. Nixon Abraham for his support and guidance. I am grateful to Prakash Bhaiya for maintaining the good health and well-being of the birds in the colony. I admire his dedication to providing a good living environment for the birds. Along the same lines, I am thankful to Dr. Swapnil Banger for providing his guidance in maintaining the good health of birds in the colony.

I thank Dr. Mimi Kao for her support and for providing us with reagents and protocols for Immunohistochemistry. I also thank Dr. Asheesh Dhawale and his student Rishika Sharma for providing us with a working Cresyl Violet Acetate staining protocol. I am thankful to Dr. Deepa Subramanyam for her continuous support and to her student Dr. Surya Bansi Singh for helping us out with the imaging.

I extend my gratitude to the Imaging Facility at IISER Pune, and Mr. Vijay Vittal for teaching and helping in imaging. I also thank Biology UG Lab at IISER Pune, and Mrs. Shabnam Patil for providing us facilities required for Immunohistochemistry. I would also like to thank Shruthi for helping us with DAPI staining, Sanyukta for pulling glass capillaries for tracer injection, and Nived for helping with imaging.

A heartfelt thanks to Shikha didi for taking responsibility for being my mentor, always being ready to walk the extra mile to help me, and constantly guiding me through the new walk of research life. Thank you Didi for devoting so much time and energy to my growth and well-being (academic, mental, and emotional). It is hard to imagine the smooth flow of this project without her help. I take this opportunity to thank my colleague Anand for his help and support during this time. I am grateful to have the opportunity to work with him and learn from him, and the best part is his attempt to maintain a comfortable working environment. It was fun working with and learning from Shikha didi and Anand. I also thank other friends at Rajan lab for their support and for creating a nice environment. Thank you, Manali, Dhanya, Devika, Gayatri, Sonam didi, Titir didi, Sheetal didi, Shouvik, Abir!

I am grateful for the opportunity to live with and forge friendships with my wonderful pals: Prati, Sujal, Loki, and Manish. Their companionship has been a source of strength and encouragement throughout my college journey. Sharing experiences, laughter, and growth with them has enriched my life in countless ways. I cherish the memories we've created together and am thankful for their unwavering support and camaraderie.

I am grateful to have my dadi, mummy, papa, and bhaiya by my side, showering me with their unconditional love and support. Their guidance and vision have been integral to my journey up to this point. Their unwavering encouragement and belief in me have fueled my determination and aspirations. I am deeply indebted to them for their constant presence and invaluable contributions to my life.

I am grateful for the opportunity to know and interact with a diverse array of individuals on campus, whose presence has enriched my life with positivity and valuable lessons at different junctures. Each encounter has offered unique insights and perspectives, contributing to my personal growth and understanding of the world around me. I extend my sincere appreciation to all those who have crossed my path, for their kindness, wisdom, and the meaningful connections we have shared.

I conclude my acknowledgments by extending gratitude to all the finches in the lab who have contributed to our understanding of the world and will continue to.

Thank you!

Contributions

Contributor name	Contributor role
Raghav Rajan, Shikha Kalra, Vidushi Sharma, Anand C Krishnan	Conceptualization Ideas
Raghav Rajan, Shikha Kalra, Vidushi Sharma, Anand C Krishnan	Methodology
Raghav Rajan, Shikha Kalra, Vidushi Sharma, Anand C Krishnan	Software
Raghav Rajan, Shikha Kalra, Vidushi Sharma, Anand C Krishnan	Validation
Raghav Rajan, Shikha Kalra,Vidushi Sharma, Anand C Krishnan	Formal analysis
Raghav Rajan, Vidushi Sharma, Anand C Krishnan	Investigation
Raghav Rajan	Resources
Vidushi Sharma, Anand C Krishnan	Data Curation
Vidushi Sharma	Writing - original draft preparation
Vidushi Sharma	Writing - review and editing
Vidushi Sharma	Visualization
Raghav Rajan	Supervision
Raghav Rajan	Project administration
Raghav Rajan	Funding acquisition

This contributor syntax is based on the Journal of Cell Science CRediT Taxonomy[1].

[1] https://journals.biologists.com/jcs/pages/author-contributions

Chapter 1
Introduction

1. Introduction

A large repertoire of behaviors/actions is exhibited across different organisms. The examples range from the common (usually taken for granted) phenomena like breathing, blinking of eyes, and effortless walking by healthy adult humans to stereotyped songs by songbirds (zebra finches), nest building by birds, etc. This endless set of behaviors/actions found across organisms has a few things in common across all its elements: genetically they are provided with basic components that make certain actions/behaviors possible (e.g., humans have legs that aid walking, birds have wings that aid flying, zebra finches have syrinx to sing, and so on), some actions/behaviors don't require learning by the organism (breathing, smelling, etc.) while some require active learning by the organism (language learning in humans, learning to walk by humans, song learning in zebra finches, and so on). The genetic makeup ensures the development of all requisite systems within the organism to manifest the desired action. This encompasses the physical organs or structures within the body responsible for executing specific behaviors or actions, as well as the components facilitating effective transmission of information from command centers to execution centers. Additionally, it encompasses the development of fully functional brain regions responsible for issuing commands. Furthermore, it encompasses the development of all other chemical or physical entities essential for the transfer of information from one point to another, which are necessary for the expression of the desired behavior or action. An intriguing aspect arises at the level of neural circuits, as they are not static but exhibit changes based on the context, a phenomenon known as neural plasticity. This dynamic adjustment of neural connectivity allows for adaptation and responsiveness to varying environmental and behavioral demands. So all the neural connectomes in the organism act very smartly: strengthening the more useful connections and discarding the less used connections over time. This flexibility gives rise to learning in the organisms. [1]

Learned behaviors, such as motor skills, language, and predatory/prey behaviors, are found widely across living organisms [2]. My focus is to study the song learning by the songbirds called zebra finches. Certain model organisms are studied to answer particular biological questions. Zebra finches are used as a model system for studying songbirds. Originally, they are native to hot-arid regions of Australia. Their diet naturally comprises dry seeds, so these birds are easy to rear and maintain in laboratory conditions. Also, these are not seasonal breeders but breed all around the year and don't have particular diet requirements for breeding (like a high protein diet, etc. in other birds). This makes it easy to rear them and study them across generations. In addition, their maturation time is also less: around 120 days of age, they become adults and ready to breed, and song learning is also usually completed in male zebra finches by that time. Due to their low maintenance and fast development, zebra finches are a good potential model system. They are used as a model system to study neural mechanisms underlying song vocalizations. Just like any human language has a fixed set of letters from which all the words are created, each adult male zebra finch has a fixed set of vocalizations (called notes/syllables)

from which its song is created. Along with structure, stereotypy is also an important aspect of the zebra finch song. Another important factor that makes zebra finches a model system to study production and learning of song vocalization is that each male zebra finch is not born with a final song but they learn the song from other males in their surroundings (typically their fathers). [3-6]

In the realm of avian communication, the term "song" denotes vocalizations that are distinguished by specific characteristics, setting them apart from other forms of bird vocalizations. These characteristics often include complex sequences of sounds, repeated patterns, and sometimes melodic or rhythmic elements. Unlike simple calls, which serve immediate functions like signaling danger or maintaining contact, songs are more elaborate and often associated with courtship, territory defense, or individual recognition within bird species. To delve into the historical perspective of bird songs, we can trace the fascination with avian vocalizations back to ancient cultures. For example, in Greek mythology, birds were believed to possess special knowledge and powers, with their songs interpreted as messages from the gods or omens of future events. Similarly, indigenous cultures around the world have long revered bird songs, attributing spiritual or symbolic significance to their melodies. In the scientific realm, the study of bird songs has evolved over centuries. Early naturalists like John James Audubon and Charles Darwin recognized the diversity and complexity of bird vocalizations, laying the groundwork for further research. The modern study of bird song started when William Thorpe found out that juvenile birds must listen to their adult conspecific song to learn and produce songs similar to them. He carried out this study by rearing chaffinches in the laboratory: if juveniles were reared in isolation from adult conspecifics they developed an abnormal song, and if they could listen to the tape recordings of adult song they developed a song similar to that. Later on, other scientists in the field showed that juveniles have an innate tendency to learn conspecific songs and they should be capable of hearing themselves singing for the development of a normal song. The timing of song development varies across species: one extreme is species where song memorization is limited to the early period of life and new song development doesn’t happen in adulthood (e.g. zebra finch, white-crowned sparrow), another extreme is species where song development can be developed in adulthood (e.g. canary, European starling), and many species have patterns of song development in between these two extremes. Over time, the discovery of neural circuits in avian forebrain that control song-related behavior directed enthusiasts to study more about these circuits and their functions. The ethology studies have shown that only male birds sing in most of the species and it has two main functions: territory marking and attracting a mate. There is a large diversity in types of sound and their syntactical arrangement across different species, but all of them have well-defined acoustic structures that are characteristic of their species. The various structural components of the bird song are described as elements or notes (simplest individual sounds produced by the bird), syllables (individual elements that occur in a particular sequence in the song), motifs or phrases (sequence of syllables that repeat in the song), bouts (sequence of one or multiple motifs separated from

other motif sequences by a defined minimum silent interval). Fig. 1 shows the song spectrogram of an adult male zebra finch, highlighting the different elements of the song. [7]

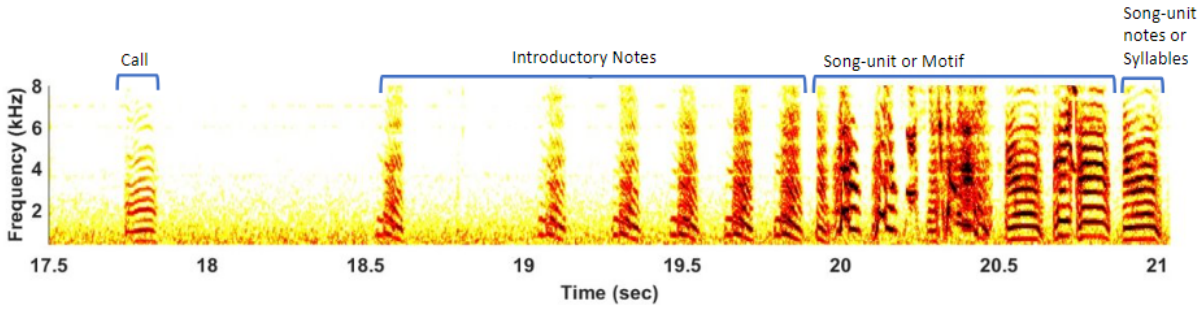

Fig. 1.1: Spectrogram of an adult male zebra finch song.
In this spectrogram of an adult male zebra finch, the X-axis represents time in seconds, while the Y-axis indicates frequency in Hertz. Color intensity reflects the amplitude of sound, with darker colors indicating higher amplitudes and lighter colors representing lower amplitudes. Different components of the song are marked as well.

By adulthood, male zebra finches usually have one kind of motif that they develop as juveniles. Like humans, they are not born with a language but learn it. Unlike humans, who learn new languages throughout their lives, male zebra finches learn the song as juveniles which remains the same for life (under natural conditions). In their song-learning process, there is a sensitive phase where they memorize a song template from other available males in the environment. Under normal conditions, neural pathways for song learning and production development start as early as 15 days of age. Since they have the most physical and paternal interactions with their father, young birds memorize their song templates from their father. So in the case of foster father (during laboratory experiments), they make their songs as templates for learning. If they are kept in isolation from males, the onset of this sensitive period is also delayed. In such cases, they produce a song comprised of call-like elements making up the motif until they are re-introduced to a male song (which they learn finally). So the onset of this sensitive period is dependent on exposure to other male songs. Laboratory studies show that the sensitive period of male zebra finches can begin as early as phd (post-hatch day) 25 and last up to phd 65. This onset embarks major developments in neural circuitry involved in song acquisition. In this period, they generally need 10 days of exposure to an adult tutor to make a song template with fair accuracy. During this sensitive period, the motor abilities of the juveniles are also developing for singing. They start singing (more like human child blabbers) to match their vocal output to the template they have memorized. This vocal practice matches their vocal output with their song template over time through the development of both neural and motor circuits. During song learning, the song of a juvenile evolves through three stages. The timeline of song development in a normally reared juvenile is: “subsong” (when the juvenile starts babbling and produces screechy sounds without any clear elements and the least stereotypy across renditions) from phd 35 to 45, “plastic song” (the amplitude of vocalizations increase and gradually distinct and identifiable elements emerge, but still there is variability in the structure of elements across

renditions) from phd 45 to 80, “adult song” (the plastic song is gradually transformed into stereotyped motif structures) by phd 80 to 90. [8-11]

The memory of the song is stored as a template but is produced successfully by the proper control of sensory and motor organs by the brain. This efficient control of motor organs to achieve the desired vocalizations is not a default skill that a male zebra finch juvenile is born with but is obtained through exploration. For example, baby animals learn the relationship between their actions and the effects they produce by performing very variable behaviors like infant stepping, play, early vocalizations, etc. An important question arises from here: where are exploratory behaviors generated in the brain, is it the same circuit involved in producing that behavior in adulthood or some other specialized brain regions? In zebra finches, HVC (high vocal center) is the premotor nucleus, and RA (robust nucleus of the arcopallium) is the motor nucleus. RA forms synapses with brainstem-motor neurons and respiratory motor neurons, that innervate the syrinx and respiratory muscles that are responsible for song production. HVC and RA make up the motor pathway which is essential for producing stereotyped, learned vocalizations, and the firing pattern of the neurons in these nuclei are precisely time-locked to the song output. To check if the primitive subsong vocalizations are driven by HVC (premotor nuclei), HVC was either bilaterally eliminated or its projections to RA were eliminated. Consequently, all juveniles who were producing subsongs before intervention continued producing subsongs and older birds (in plastic song and adult song stages) lost structure and stereotypy in their songs (started producing subsongs-like vocalizations). This means that HVC is not required for subsongs in juveniles and its absence in adult changes their songs to subsongs. After this discovery, another possibility was tested whether subsong is entirely produced by midbrain or brainstem circuitry and doesn’t require a forebrain song system. RA lesions stopped singing in juveniles, meaning forebrain inputs are essential for subsong production. Then another possibility was tested whether subsong is driven by the intrinsic circuits of RA, without any influence from other known brain regions. Apart from HVC, LMAN (lateral magnocellular nucleus of nidopallium) also projects to RA. On abolishing inputs from LMAN and HVC to RA, birds stopped singing. This means that RA (without any afferent inputs) is not sufficient to produce song in the zebra finches. After this revelation, another hypothesis was put forth that subsong requires inputs from LMAN to RA. LMAN inactivation in juveniles younger than 45 days of age completely stopped their singing. This means that RA is essential for singing while inputs from LMAN to RA are essential for subsong production. In conclusion, the singing-related exploratory vocal practice in male zebra finches is not generated in the same circuit involved in producing the song in adulthood but in other brain regions. [12-26]

LMAN, a forebrain nucleus, is the output nucleus of AFP (anterior forebrain pathway) to RA. AFP comprises three nuclei: Area X, DLM (dorsolateral anterior thalamic nucleus), and LMAN. Area X projects to DLM, DLM projects to LMAN, LMAN projects to Area X & RA, and Area X receives input from HVC. AFP is homologous to mammalian basal ganglia. Since mammalian

basal ganglia are implicated in many motor learning and maintenance functions, AFP is considered a rightful candidate for song learning and maintenance in male zebra finches. Also, the physiological similarity between AFP and mammalian basal ganglia suggests that song learning in birds and motor learning in mammals may use similar physiological mechanisms. [20,27] Studies even show that like cortical-basal ganglia circuits, AFP is essential for motor learning and control. It has been shown that this circuit is essential for the learning of songs in juveniles and vocal plasticity in adults but not required for the production of already learned songs. [28] The output nucleus of AFP "LMAN" is studied age-dependent to understand its role in different stages of song learning and production by males. The lesion of LMAN at different ages produces different behavioral effects on singing. Birds between the ages of 35 and 50 days with no or small LMAN lesion show normal song development: their adulthood song has short stereotyped phrases, high-frequency modulation in individual notes, and even spacing in harmonics. The birds with the lesion show very abnormal songs in adulthood: one or two highly abnormal notes (usually lower amplitude), low-frequency modulation in individual notes, and extremely long bouts without normal phrasing. These abnormalities in song appeared within 72 hours of the lesion. Birds between the ages of 55 and 65 days with LMAN lesions show changes depending on their song development at the time of lesion: if no recognizable song pattern was developed then a highly abnormal song was produced post-lesion, if a recognizable song pattern was developed then the song had no immediate disruption. For former birds, the song in adulthood remains abnormal while for latter birds, the song gradually simplified, and the song in adulthood had only slight abnormalities. Adult birds with LMAN lesions produce completely normal song post-lesion. [29] Like LMAN lesions, LMAN inactivation doesn't stop singing in older birds but produces a significant reduction in song variability. While singing if the LMAN is stimulated, it induces acute and certain changes in the learned parameters of the song. It shows that LMAN is capable of bringing change in the primary motor areas that generate songs via its neural outputs. Additionally, naturally and under experimental conditions, the changes in the structure of the song is related to the changes in the pattern of neural activity. This naturally occurring modulation of song variability stops when LMAN, the output nucleus of AFP, is lesioned. These studies in adults suggest that AFP aids in motor learning by favoring motor output that is closer to the desired target and/or by creating variability required for reinforcement learning. [28] Similar studies in juveniles were conducted by pharmacologically inactivating LMAN and its synaptic inputs to target the premotor area rapidly and reversibly. This dramatically reduced sequence and acoustic variability in their songs. The neurons projecting from LMAN to the motor pathway have highly variable spiking activity from one rendition to another, which shows LMAN as a source of variability in song. [30] Given all that we know about LMAN, it is clear that it aids the exploratory motor behavior in zebra finches which is essential for learning the specific motor sequence of "song" by instructing or modulating the motor pathway during learning to create the output vocalizations (in structure and sequence) similar to the "stored template" with each practice.

Zebra finches typically initiate their songs with a sequence of standardized introductory notes (INs), characterized by brevity and simplicity in spectral composition. [9, 31, 32, 33] Despite the uncertain purpose of INs, recent research on songbirds has revealed significant diversity in both the quantity of IN repetitions and the acoustic characteristics of INs among individuals within a species. [33-37] This variability in song elements is attributed to the process of learning, [9, 31, 38, 39] suggesting that differences in IN number and structure may reflect varying learning experiences under different tutors. An alternative explanation is proposed by the motor preparation hypothesis, drawing parallels with preparatory motor activities observed in primates and how they have different motor preparatory activities before executing distinct movements. [40] If INs serve to prime the brain of the songbird for producing the song, it is fair to anticipate changes in the structure and number of INs corresponding to subsequent songs. Such a hypothesis implies a potential correlation between the variability in IN structure and/or number and the ensuing song across individual birds. Furthermore, disparities in IN attributes among birds could also stem from inherent biological tendencies akin to the ones implicated in producing other elements of the song. [41, 42] Like the song, the juveniles also learn the acoustic structure and repetitions of Introductory Notes (that are sung before the song motif) from the tutor [43]

LMAN's role in song learning has been established clearly, but whether and how LMAN affects the learning of the short, introductory notes that precede the song remains poorly understood. This inspired me to look at whether LMAN affects the learning of INs. I exposed juveniles to an adult male tutor starting from their sensory acquisition phase for their complete song development phase (up to phd 90), and after 10 days when they have successfully memorized the song template perform bilateral electrolytic lesion of LMAN in their very early days of the sensorimotor phase. To capture the role of LMAN in IN learning, I further checked the correlation between the efficiency of IN learned from the tutor (in terms of IN structure and number) and the amount of LMAN lesioned in the bird.

Chapter 2

Methods

2. Materials and methods

All the experimental protocols for the project were conducted after receiving approval from the Institute Animal Ethical Committee, aligning with the guidelines set by the Committee for Control and Supervision of Experiments on Animals (CCSEA, New Delhi, India).

2.1 Experimental birds

The male birds used in the lesion, recording, and antidromic stimulation surgeries, and designated for breeding or tutoring were either lab-bred or bought from vendors. Amongst adult male birds (n = 19) used for surgeries, 11 were bred internally at IISER Pune, while the remaining 8 were procured from vendors. All juvenile zebra finches employed for the surgeries (n = 34 male juveniles originating from 10 separate nests) were exclusively bred within the facilities of IISER Pune. LMAN lesions were performed on 23 male birds (2 adult birds and 21 juveniles), sham LMAN lesions were performed on 10 male juveniles, and antidromic stimulation in RA to identify LMAN was tried in 2 juvenile and 3 adult male birds. Like all other birds maintained at the IISER Pune facility, experimental avian subjects were subjected to a controlled environmental setting, characterized by a 14-hour light and 10-hour dark cycle.

2.2 Experimental design

To check if LMAN affects the learning of IN (structure and number) in male juvenile finches, LMAN (or not LMAN, but any other part of the brain where no known brain region is present) is electrolytically lesioned in juveniles bilaterally, and their final INs are compared to their tutors. I will now describe this experimental paradigm in detail.

The previous study in our lab shows that male zebra finches have an innate tendency to produce songs with mean IN No. 3 [43]. So, to check if juveniles learn mean IN No. from the tutor, they are kept with a social tutor with mean IN No. <2 or >4. By recording undirected songs of various adults from the colony, 13 birds (no IN birds: n = 6, low IN birds: n = 2, high IN birds: n = 5) were selected as tutors.

To mitigate any impact of the father's song on juveniles, fathers were separated from nests (except 2 nests where juveniles were completely tutored by the father) when juveniles (n = 32) were around 10 days old (range: 7 to 15 days, median: 11 days). Until phd 35 (range: 30-42, median: 35), they were kept with their mothers, and their social tutoring starts afterward. Throughout their whole tutoring period (up to phd 120), they were kept in visual isolation from other birds in the colony (but heard them) except their tutor because visual isolation (even without acoustic isolation) prevents learning of song from that bird (Eales, 1989). Roughly after a week of tutoring with the social tutor (range: 5-14, median: 7), surgeries were performed on the juveniles (n = 32). Juveniles (n = 2) tutored by fathers had surgeries at a similar age. The bilateral electrolytic lesions (LMAN: n = 21, sham: n = 10) were performed on the juveniles around phd 43 (range: 38-48, median: 43). Song of the juveniles were recorded for 2-3 days before (along with tutor) and after (without tutor) the surgery. After recording the post-lesion songs, juveniles were returned to tutors for social tutoring until phd 120 (n = 22) except for 2

juveniles whose tutors died before the completion of phd 120 (tutors died at phd 39-41 and phd 54-55, respectively). When the final song is recorded after phd 120, juveniles are returned to the colony (visual and acoustic contact with other conspecifics). Thereafter the birds were euthanized (between phd 127 to 218, for n = 9 birds) to extract the brain for histology to quantify the extent of LMAN lesioned. Fig. 2.1 shows the overview of the experimental design.

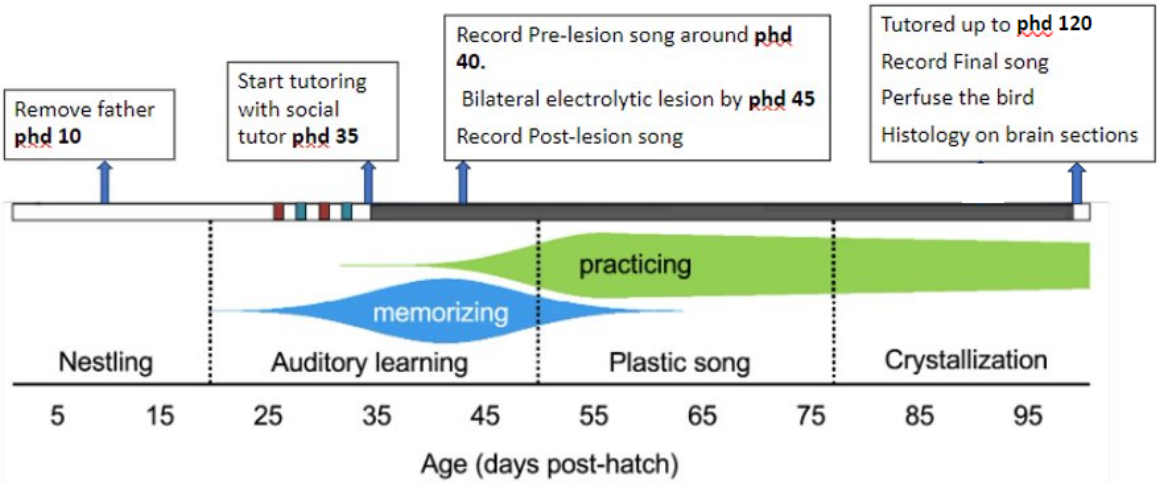

Fig. 2.1: Overview of the experimental design.

2.3 Song Recordings

Birds were kept in individual cages inside the custom-made sound attenuation box, sourced either from NewTech Engineering Systems, Bangalore, India, or constructed from repurposed ice coolers for song recordings. Throughout the recording, the microphone (C417PP omnidirectional condenser microphone by AKG) was placed on the bird's cage facing downwards (placed roughly in the middle of the cage's roof). A custom-written Python script was used to record audio (at a sampling rate of 44,100 Hz) in the form of .wav files. These digitized audio recordings underwent screening for songs, followed by band-pass filtering and labeling, all facilitated by custom MATLAB scripts tailored for this purpose.

2.4 Surgery

All Surgical interventions were conducted under anesthesia. Before surgery (at least one hour), birds were given meloxicam as an analgesic at a dosage of 0.25mg/kg. Anesthesia was induced as isoflurane (flow rate: 1ltr/min) with concentration maintained between 0.25-4%. Given that the bird is unable to regulate its body temperature under anesthesia, a customized jacket was fitted onto each bird, accompanied by the heat lamp nearby throughout the surgery. Before placing the bird onto the stereotaxic instrument (Narshige, custom-designed), ear feathers were trimmed to expose the ear. To prevent head movement during surgery, the head was firmly positioned between ear bars, with the beak secured in the beak bar of the instrument. A subcutaneous injection of lignocaine, a local anesthetic drug (approx. 25 µl), was administered,

followed by a surgical incision to expose the skull. Post-surgery, birds were carefully monitored to ensure successful recovery.

In all the surgeries (antidromic stimulations: n = 5, electrolytic bilateral LMAN/sham lesion: n = 33, retrograde tracer injection: n = 3), the initial step involved the removal of the first layer (usually second as well) of the skull over the 'y' coordinate, which corresponds to a major blood vessel crucial for accurate stereotaxic coordinates, ensuring optimal visualization. For antidromic stimulations, after aligning the electrode to the center of the branch point of ‘y’, it was carefully moved to LMAN and RA coordinates as described by the zebra finch stereotaxic atlas. [44] Since LMAN projects to RA, antidromic stimulation was performed to reliably determine LMAN coordinates in our stereotaxic instrument. A stimulating electrode was inserted into RA and the recording electrode was into the expected LMAN location. These stimulations were performed using a monophasic pulse for 200 μsecs (200 μAmp - 1 mA). However, no significant activity was observed in LMAN coordinates. Neural activity similar to LMAN neurons was recorded in some locations. These coordinates were lesioned and consequently, the brain sections were stained with Cresyl Violet to visualize our lesion coordinates for the location of LMAN. Table 2.1 summarizes all the antidromic stimulation surgeries.

For electrolytic bilateral LMAN/sham lesions, after aligning the electrode to the center of the branch point of ‘y’, it was carefully moved to LMAN coordinates (initially, based on results of neural recordings and staining from preceding surgeries and after a few surgeries these coordinates were standardized). After all the standardization, 100 μA current is passed for 50 sec (6 coordinates in each hemisphere) by carefully moving an electrode aligned to the center of branch point of ‘y’ to LMAN (Anterior-posterior (AP): 4.4, 4.65, 4.9, medial-lateral (ML): 1.5, 1.8 mm) and sham (Anterior-posterior (AP): 5.1, 5.35, 5.6, medial-lateral (ML): 2.0, 2.3 mm) coordinates. And the current is passed at a depth of 2.3 mm for LMAN lesions, and 1.3 mm for sham lesions. Lesions were performed using FHC tungsten microelectrodes either with a fine tip (n = 2) or a blunt tip (n = 31). After the lesion procedure, bone wax and dental cement were employed to securely close the exposed brain area. Tables 2.2 & 2.3 provide Information regarding LMAN coordinates, current parameters, number of lesions for all the birds where LMAN and sham lesions were performed, respectively.

To fluorescently label and quantify the volume of LMAN, a retrograde tracer (Dextran Alexa Fluor 594) was bilaterally injected into the RA on adult male zebra finches using ‘Drummond Nanoject III’. After aligning the electrode to the center of the branch point of ‘y’, it was carefully moved to RA coordinates, and a tracer (10% Dextran, Alexa Fluor 594 in 0.01 M PB) was injected after recording neural activity from RA (n = 2). Roughly 300 nl of tracer (rate: 10 nl/sec) was injected into each hemisphere. After the surgery, bone wax and dental cement were employed to securely close the exposed brain area. Table 4.1 provides information regarding the sites of injections, and the quantity of tracer injections.

Table 2.1: Summary of all the antidromic stimulation surgeries performed. The first row and column are AP and ML of the coordinates where LMAN was recorded. Different alphabets

denote different birds. Red: LMAN activity not recorded, green: LMAN-like activity recorded (L): left hemisphere, (R): right hemisphere, underlined: some activity in LMAN in response to antidromic stimulation.

	AP= 5.2	AP= 5.0	AP= 4.8	AP= 4.7	AP= 4.6	AP= 4.5	AP= 4.4	AP= 4.2
ML= 1.9			B (L) D (L) (4.875)		D (L)			
ML= 1.8	A (L) A (R)	**E (L)** **E (R)**	A (R) **E (L)** **E (R)**		A (R) **E (L)** **E (R)**			
ML= 1.7		D (L)	B (L) D (L) (4.875)	C (R) D (L)		C (R)	B (L)	C (R)
ML= 1.6		C (R) **E (L)** **E (R)**	**E (L)** **E (R)**		**E (L)** **E (R)**			C (R)
ML= 1.5	A (L) A (R) B (L) D (L)	A (L) A (R) B (L) C (R) D (L) (4.9)	A (L) A (R) B (L) D (L) (4.875)	D (L) **D (R)**	A (R) B (L)		B (L)	

Table 2.2: Information regarding LMAN coordinates, current parameters, and number of lesions for all the birds where LMAN was lesioned

Current parameters	Location in (AP,ML)mm	depth	# total coordinates lesioned	No. of birds with these lesion parameters
100 µA for 100s	(4.2,1.4), (4.2,1.8), (4.4,1.4), (4.4,1.6), (4.6,1.5), (4.6,1.8)	1.9,2.4 mm	12 (unilateral)	1 juvenile
60 µA for 100s	(4.2,1.5), (4.2,1.8), (4.4,1.5), (4.6,1.8), (4.6,1.5),(4.2,1.8)	1.9,2.3 mm	24 (bilateral)	1 juvenile
100 µA for 50s	(4.2,1.5), (4.2,1.8), (4.4,1.5), (4.6,1.8), (4.6,1.5),(4.2,1.8)	1.9,2.3 mm	24 (bilateral)	2 juveniles
100 µA for 50s	(4.2,1.5), (4.2,1.8), (4.4,1.5), (4.6,1.8), (4.6,1.5),(4.2,1.8)	1.9,2.3 mm	24 (bilateral)	1 juvenile
100 µA for 50s	(4.4,1.5), (4.4,1.8), (4.65,1.5), (4.65,1.8), (4.9,1.5), (4.9,1.8)	2.3 mm	12 (bilateral)	16 juveniles
100 µA for 50s	(4.4,1.6), (4.4,1.9), (4.65,1.6), (4.65,1.9), (4.9,1.6), (4.9,1.9)	2.4 mm	12 (bilateral)	1 adult bird
100 µA for 50s	(4.4,1.5), (4.4,1.8), (4.65,1.5), (4.65,1.8), (4.9,1.5), (4.9,1.8)	2.3 mm	12 (bilateral)	1 adult bird

Table 2.3: Information regarding LMAN coordinates, current parameters, number of lesions for all the birds where sham lesions were performed

Current parameters	Location in (AP,ML)mm	depth	# total coordinates lesioned	No. of birds with these lesion parameters
100 µA for 50s	(4.4,1.5), (4.4,1.8), (4.65,1.5), (4.65,1.8), (4.9,1.5), (4.9,1.8)	1.3 mm	12 (bilateral)	4 juveniles
100 µA for 50s	(5.1,2.0), (5.1,2.3), (5.35,2.0), (5.35,2.3), (5.6,2.0), (5.6,2.3)	1.3 mm	12 (bilateral)	6 juveniles

Table 2.4: Information regarding sites of injections, and quantity of dye for tracer injections

Name of the bird	Coordinates (AP,ML) for dye injections (targeting LMAN)	depth	Volume injected in each hemisphere (nl)
green141white141	Left hemisphere: (0,2.43), (-0.15,2.43) Right hemisphere: (0,2.43), (-0.2,2.43)	3.12 mm	Roughly 300 nl
green185white158	(-0.1,2.43), (+0.1,2.43)	2.8 mm (LH) 2.6 mm (RH)	Roughly 300 nl
yellow38	(-0.1,2.43), (+0.1,2.43)	2.7 mm	Roughly 300 nl

2.5 Histology

All procedures for visualizing LMAN started with perfusing the birds and fixing their brains with a 4% paraformaldehyde (PFA) solution prepared in 1X PBS. The brains were then immersed in PFA for at least two days before being transferred to sucrose solution (30% in PBS) for an additional day. Subsequently, the brains were sectioned in a sagittal plane using a cryostat (Cryostar NX70 cryostat) to obtain 40 µm thick slices. These sections were initially placed in 24-well plates filled with 1X PBS. Then sections were stained using different methods.

2.5.1 Cresyl violet acetate

LMAN, Area X and RA are typically visible in unstained wet brain sections. Amongst the sections containing at least RA or LMAN, every third section was mounted on a poly L Lysine-coated slide, one day before the staining. The first protocol didn't work reliably (sometimes not clear contrast, and sometimes not staining LMAN) in showing a clear LMAN so tried and switched to the second protocol. For both protocols, 1% cresyl violet (cresyl violet acetate, Sigma, C5042-10G) was prepared by stirring 1g cresyl violet acetate in 100 ml Milli-Q, 0.136g sodium acetate and 0.539 ml of 100% acetic acid overnight in the dark. The next day, the solution was filtered with Whatman filter # 1 in a dark environment.

• Protocol-1

Slides were immersed in 1% cresyl violet solution for 10 minutes, followed by 2-3 dips in Milli-Q water. Subsequently, the slides underwent sequential immersion in 50%, 50%, 70%, and 70% ethanol solutions for 1 minute each. Following this, the slides were transferred to 95% ethanol and monitored at regular intervals of either 1 minute or 30 seconds. Once the LMAN region was clearly distinguished from the background, the slide was quickly dipped twice in 100% ethanol and then given 3 dips in xylene. Then DPX (mounting media) was applied and a cover slip was placed over the sections. The next day, the slides were cleansed with 70% ethanol and observed under a bright field microscope (Olympus MVX10) for imaging purposes.

• Protocol-2

The slides were initially placed in Xylene for 5 minutes. Subsequently, they underwent sequential immersion in 95% ethanol, 70% ethanol, and Milli-Q solutions, with each immersion lasting for 3 minutes. Following this, the slides were immersed in a 1% cresyl violet solution for 10 minutes. Next, they were sequentially immersed in Milli-Q water and 70% ethanol solutions for 3 minutes each, followed by a 2-minute immersion in a 95% ethanol solution. A quick dip in 100% ethanol solution was then performed, followed by a 2-minute immersion in Xylene. Similar to 'Protocol-1', DPX mounting media was applied, and a cover slip was placed over the sections. The sections were observed under the microscope (after cleaning) on a subsequent day. ‘Protocol-2’ has been working reliably.

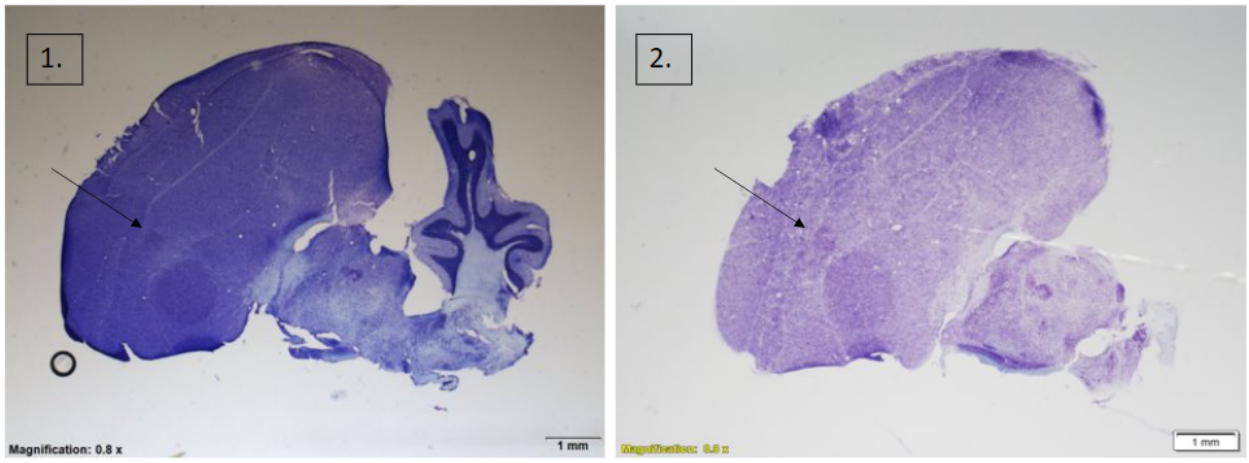

Fig. 2.2: Cresyl violet stained sagittal sections of zebra finch brain. The small circular region towards which the arrow points is the LMAN. The image on the left was obtained for Protocol-1, while the right one was for Protocol-2.

2.5.2 Immunohistochemistry

A subset of sections were placed in a 24-well plate for staining. Initially, the sections were fixed in 4% paraformaldehyde (PFA) for 15 minutes. Following this, they underwent three 10-minute washes in Phosphate buffer Triton X-100 (PBT; 0.3% in 0.02M Phosphate buffer) on a shaker for

permeabilization. Next, the sections were treated with 0.5% H2O2 for 10 minutes in the dark to quench endogenous peroxidase, followed by a single 5-minute wash in PBT on the shaker. Blocking was performed using 5% normal goat serum in 0.3% PBT for 1.5 hours on the shaker. The sections were then incubated with primary antibody (CGRP by Sigma-Aldrich - C7113) at room temperature for 4 hours and then overnight at 4°C. Various primary antibody dilutions (ranging from 1:1000 to 1:5000) in 0.3% PBT and 2% goat serum were attempted. The following day, the sections underwent three 10-minute washes in 0.3% PBT, followed by amplification using biotinylated secondary and HRP conjugated tertiary antibodies (VectaStain ABC-HRP kit, PK-4002 by Vector Laboratories). Excess antibody was removed by four 10-minute washes in 0.3% PBT on a shaker. Visualization was achieved using the 3,3' diaminobenzidine (DAB) reaction (3,3'-diaminobenzidine by Vector Laboratories, SK-4100), followed by washes with Milli-Q water to stop the reaction. The sections were then washed three times for 10 minutes each in 0.02M Phosphate buffer. Subsequently, they were stored in 0.02M Phosphate buffer. The next day, the sections were mounted on poly-L-lysine-coated slides (sigma, P8920-100ml) and air-dried for 2.5 hours. They were then sequentially immersed in 70% ethanol for 1 minute, 100% ethanol (twice, 1 minute each), and xylene for 1 minute. DPX (mounting media) was applied to the slides, and coverslips were carefully placed to prevent air bubbles. Excess DPX was removed the following day, and slides/coverslips were cleaned with 70% ethanol before visualizing the sections under a bright field microscope (Olympus MVX10) at 0.8x magnification.

No reliable staining was achieved using this protocol. It only worked once to show LMAN clearly but never after that.

Fig. 2.3 shows the images of sections (containing LMAN) on which this IHC protocol was applied.

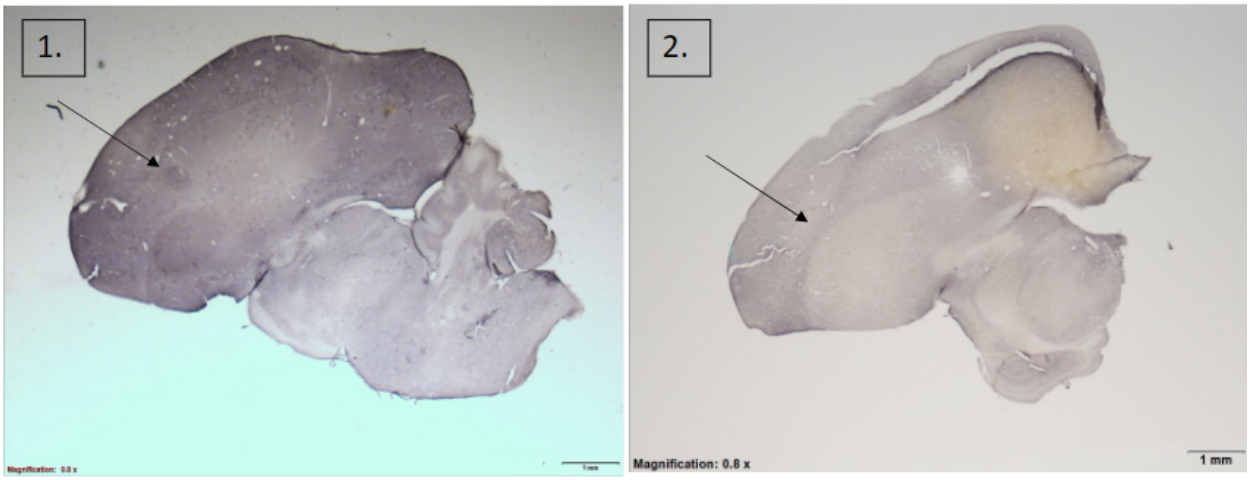

Fig. 2.3: Images of sections (containing LMAN) on which IHC protocol was applied at two different times. Both sections had clear LMAN when seen as wet sections, but only one section showed up LMAN (the circular area towards which the arrow points) after IHC.

2.5.3 Tracer

Sectioning and mounting of the tracer-injected birds was done in the dark. Amongst the sections containing at least RA or LMAN, every third section was mounted on a poly L Lysine-coated slide. Then Vectashield (Vector Laboratories) was applied as mounting media before putting on the coverslips. Typically after 5 minutes (when Vectashield was dried), the edges of coverslips were sealed with nail polish. After mounting, slides were stored in the dark at 4°C. The sections were observed under a confocal microscope (Nikon Eclipse Ti).

2.6 Data analysis

Song data was analyzed in MATLAB (Mathworks) using custom-written scripts.
Image analysis (volume/area of LMAN left quantifications) was performed using Fiji.

2.6.1 Song labeling and Song elements

The '.wav' files were obtained from continuous recording (all day long) of the birds and were screened to obtain a list of files containing the song. These song files were segmented into 'elements' through an amplitude threshold which has the criterion that a syllable should have a minimum duration of 10 ms and an inter-syllable gap should be at least 5 ms. Using custom-built algorithms, all identified 'elements' could be grouped into different clusters based on their properties. So these clusters were labeled as different syllables initially and then manually checked by going through each song file.
Zebra finches produce stereotyped songs using units called syllables, these syllables make up the stereotyped sequence called motif, and renditions of motifs make up a song 'bouts'. The song 'Bout' is a group of syllables (Introductory Notes and Motif Syllables) that has a minimum of 2s gap in the beginning and end as defined by *Sossinka and Böhner, 1980.* [33] For each bird, a motif was defined individually as a syllable sequence that was found consistently in most of the bouts. Introductory Notes were the syllables that repeated (variable number of times) before the first motif of the song bout.

2.6.2 Calculation of Mean IN Number

20 '.wav' files containing bouts were labeled to calculate the mean IN Number per bout. The average was calculated using a custom-made MATLAB script. For calculating the number of INs in each bout, the calculation starts from IN just preceding the first motif and continues calculating backward up to the point where either the silence between the syllables exceeded 500 ms or any syllable other than the IN was encountered. There were certain birds with various kinds of INs, for them IN number was calculated by considering all the types of INs.

4.2.6.5 Similarity

Sound Analysis Pro was used for calculating similarity (http://soundanalysispro.com/) (Tchernichovski et al., 2000). In the birds where the INs and motifs were identified, 10 random first motifs (of song bouts) were selected from 20 random song files as '.wav' files (with 10 ms

silence added at the beginning and end) using a custom-made script on MATLAB. From the same 20 random song files, 10 random last INs (preceding the first motif) were selected as '.wav' files (with 10 ms silence added at the beginning and end) using a similar script. In birds where INs and motifs were not identified, 10 random song bouts were trimmed either in the initial 2.5 seconds or trimmed up to the syllable where the next syllable is at a gap of more than 500 ms (with 10 ms silence added at the beginning and end). Since there was no repetitive syllable at the beginning of the bout, they were considered as No IN birds. Using the 10 '.wav' files created by trimming, the average similarity was calculated between the last IN of the tutor and the tutee. Similarly, between the motifs of tutor and tutee.

Chapter 3

Results

3. Results

The results presented in this section stem from a collaborative work between myself and Anand, building upon the foundation laid by Shikha's previous work. Shikha's ongoing guidance and support throughout this project have been invaluable in advancing our research.
All the results discussed are based on the data collected from a subset of the experimental birds (motif data for 10 birds, IN data for 3 birds, histology data for 9 birds) in either of the two age groups: phd 90-100 or phd 120-130. Results will be further updated as more experimental juveniles become adults.

3.1: In comparison to socially tutored birds (Shikha's data), by phd 90-100 LMAN lesioned birds don't learn IN structures significantly from the tutor (see Fig. 3.1). This data suggests that IN structure learning could be controlled LMAN. But the sham lesion bird shows an IN Similarity value less than almost all the socially tutored birds. This means either surgery is traumatic and affecting IN learning, or some projections were damaged which led to poor learning. Out of 10 birds, INs could be identified for 3 juveniles for phd 90-100 song. But INs could be identified in 11 out of 12 bids for phd 120-130 songs. Since INs could be identified for phd 120-130 songs, performed all further analysis for that age group.

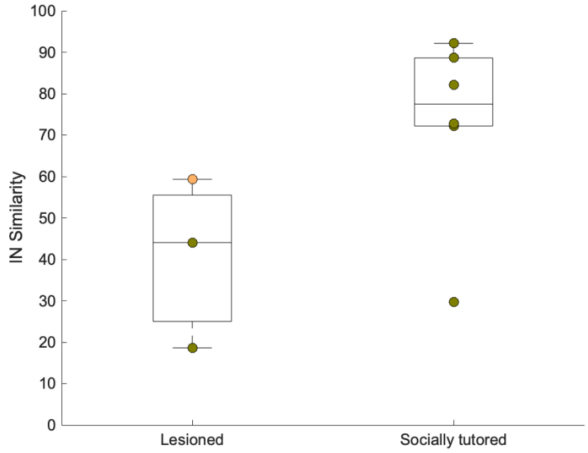

Fig. 3.1: Similarity of IN between tutor and tutee in lesioned and socially tutored birds. The Y-axis is the percentage of IN similarity of the tutee to its tutor. Each dot represents one bird. The left box plot is obtained from lesioned birds (orange color dot for the sham lesioned bird) by calculating SAP similarity of INs of phd 90-100 songs of tutee to tutor. The right box plot is obtained by calculating the SAP similarity of socially tutored birds' INs to their tutee's INs in the same age ranges. Amongst the 10 experimental birds (whose phd 90-100 day song data is

available), only 3 birds were singing syllables that could be identified as INs. So IN Similarity could only be done for 3 birds.

3.2: The juveniles were divided into 2 categories based on the percentage of LMAN lesioned: >=60%, and <60% (see Fig. 3.2).

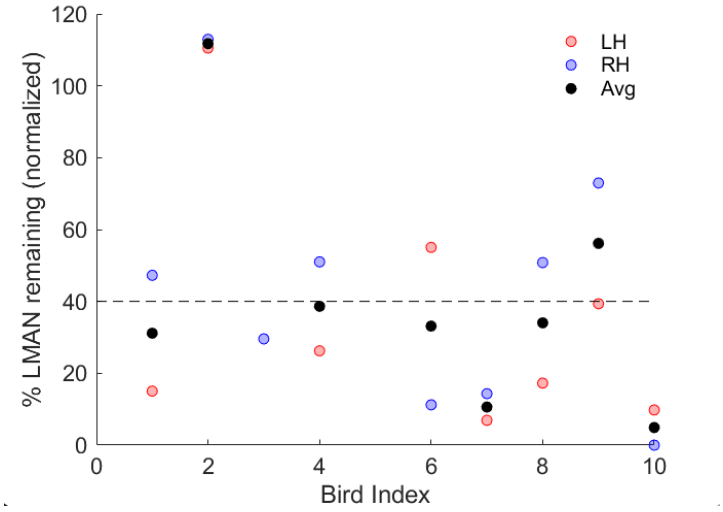

Fig. 3.2: The normalized percentage of LMAN remaining in different LMAN and sham lesioned juveniles. The X-axis is the bird index. Each dot represents one bird. The Y-axis is the percentage of LMAN remaining in a lesioned bird with respect to a non-lesioned bird stained with the same Cresyl violet acetate protocol as other experimental birds. Red, blue, and black circles represent the %LMAN remaining for the left hemisphere, right hemisphere, and the average of both hemispheres, respectively. The dotted horizontal line (passing through 40%) is roughly the mean of % Average LMAN remaining across all birds. Based on this, juveniles are divided into 2 groups: >=60% Average LMAN lesioned, and <60% average LMAN lesioned. Bird 3 has only one hemisphere's data because staining didn't work in the other hemisphere.

3.3: Out of 11 birds where INs are identified in song for phd 120-130, 7 birds are LMAN lesioned, and staining worked only for one hemisphere in one of these 7 birds. Birds with % Average LMAN lesioned >=60% didn't learn Pupil IN number. There is only one bird with <60% lesion and it also didn't learn the Pupil IN number. Projections to LMAN might be damaged in this bird. (see Fig. 3.3)

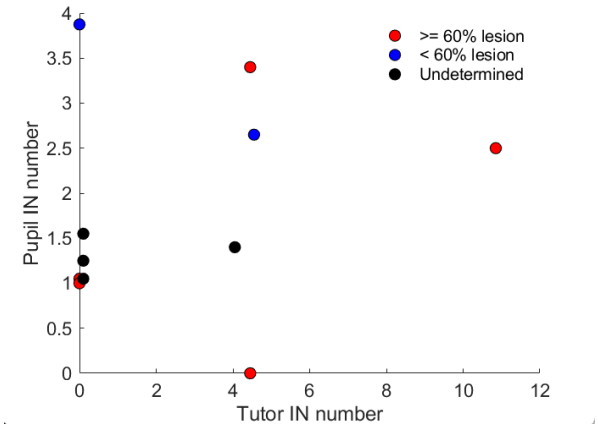

Fig. 3.3: The mean IN number of Pupils and tutors. The Y-axis is the mean IN number of the pupil. Each dot represents one bird. Red, blue, and black color indicates birds with >=60% Average LMAN lesion, <60% Average LMAN lesion, and undetermined LMAN lesion, respectively. The X-axis is the mean IN number of tutors.

3.4: Out of the 10 birds where %Average LMAN lesion is calculated, in 8 birds motif could be determined for phd 120-130 songs. Birds were categorized based on the percentage of LMAN lesions namely greater than 60% and less than 60%. Birds with more than 60% lesion had the least motif similarity with the tutor compared to socially tutored and birds with less than 60 lesions ($p = 0.0432$ in Kruskalwallis, Post hoc Tuckey Kramer). (see Fig. 3.4)

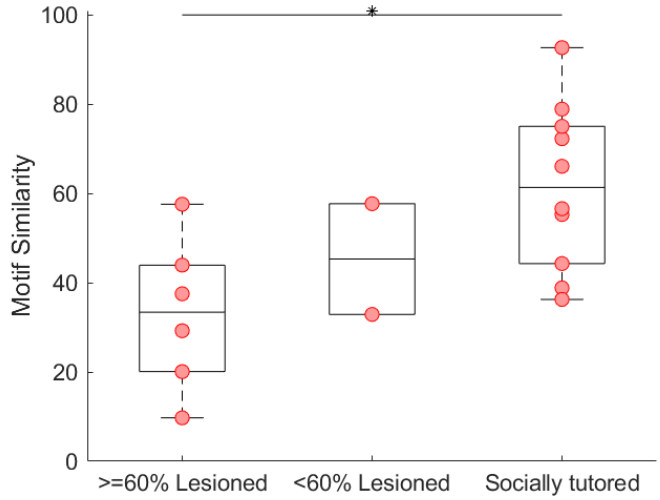

Fig. 3.4: The similarity of motif between tutors and tutees. The Y-axis is the percentage of motif similarity between the tutor and tutee (phd 120-130) song. Each dot indicates a bird. The first box plot from the left is for the birds with an Average LMAN lesion of >=60%, the second one is for the birds with an Average LMAN lesion of <60% and the third one is Shikha's data for Socially tutored birds.

3.5: Out of the 10 birds where the %Average LMAN lesion is calculated, IN similarity could be calculated only in 3 birds because in 6 birds either the tutor was no IN bird or the tutee, due to which IN similarity couldn't be calculated. And IN couldn't be identified for one bird. More data needs to be gathered before making any deductions about the relation between IN similarity (of tutor and tutee) and LMAN lesioned. (see Fig. 3.5)

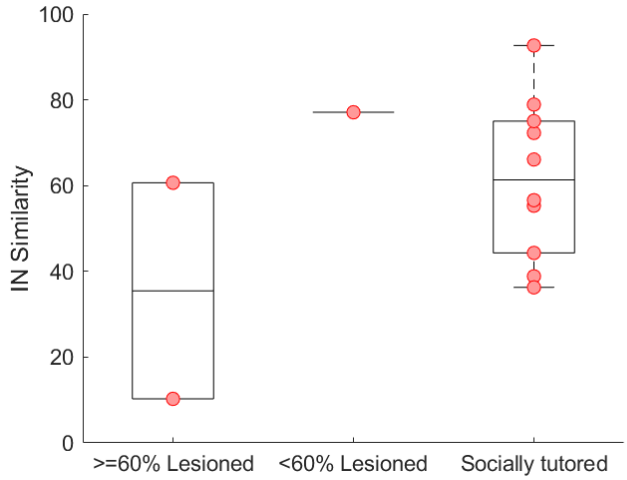

Fig. 3.5: The similarity of IN between tutors and tutees. The Y-axis is the percentage of IN similarity between the tutor and tutee (phd 120-130) song. Each dot indicates a bird. The first box plot from the left is for the birds with an Average LMAN lesion of >=60%, the second one is for the birds with an Average LMAN lesion of <60% and the third one is Shikha's data for Socially tutored birds.

3.6: Looking at the data for the 4 birds in which both the IN similarity and Motif Similarity between the tutor's and tutee's song could be calculated for phd 120-130 song it was found that the lesion of LMAN doesn't always impact badly the learning of one particular component (IN or motif) of the song over the other. (see Fig. 3.6)

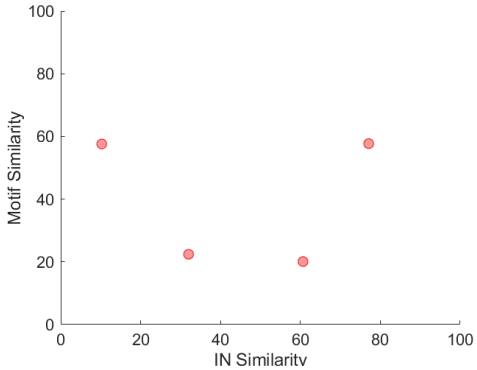

Fig. 3.6: Similarity to Motif and IN of tutor in LMAN lesioned birds. The Y-axis is the percentage of similarity between the motif of the tutor and its tutee. The X-axis is the percentage of similarity between the IN of the tutor and its tutee. Each dot represents one different bird.

3.7: Qualitatively, we have found that LMAN lesion have affected INs and motif learning in juveniles while the sham lesion has not. (see Fig. 3.7 & 3.8)

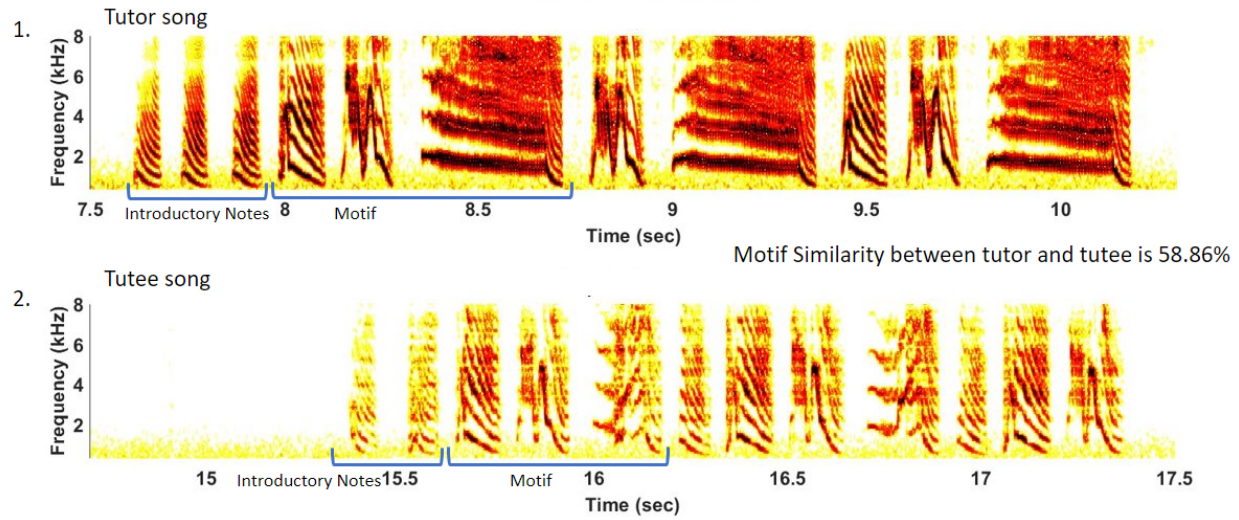

Fig. 3.7: Qualitative comparison of a spectrogram of a tutor-tutee pair where juvenile underwent sham lesion. The top song spectrogram belongs to the tutor and down one belongs to its tutee (on whom sham lesion surgery was performed). Both the spectrograms are plotted as the amplitude of frequencies (represented by the intensity of color) with the time. Tutee's song was recorded at an age more than phd 90. INs and 2 out of 3 syllables of the tutee are very similar to the counterparts in the tutor's song. Since there is quite visible similarity between the song motifs of the tutor and the tutee, tutee has learned the song significantly from his tutor.

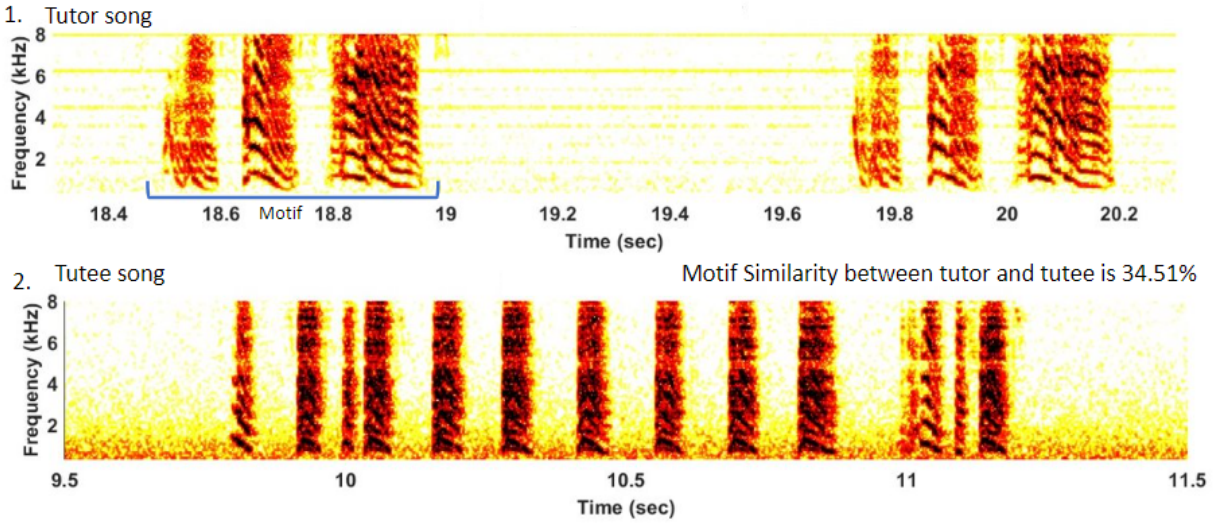

Fig. 3.8: Qualitative comparison of a spectrogram of a tutor-tutee pair where juvenile underwent LMAN lesion. The top song spectrogram belongs to the tutor and down one belongs to its tutee

(on whom LMAN lesion surgery was performed). Both the spectrograms are plotted as the amplitude of frequencies (represented by the intensity of color) with the time. Tutee's song was recorded at an age more than phd 90. The tutor is a no-IN bird. Since there is no visible similarity between the song motifs of the tutor and the tutee, tutee has not significantly learned the song from his tutor.

3.8: If LMAN is lesioned significantly in one hemisphere and is intact in the other hemisphere, juveniles can still learn motifs significantly similar to their tutors (see Fig. 3.9).

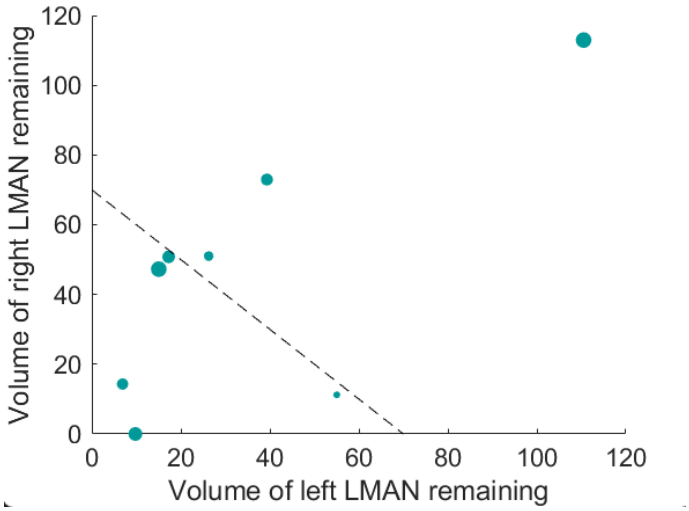

Fig. 3.9: The relation of motif similarity (of tutee to its tutor) with Volume of LMAN remaining in the left and right hemisphere after lesion. The X-axis indicates the percentage volume of LMAN remaining post-lesion (in the left hemisphere), Y-axis indicates the percentage volume of LMAN remaining post-lesion (in the right hemisphere). Each dot represents one bird, with its area indicating the SAP similarity between the motif (phd 120-130 song) of that bird and its tutor. The volume of LMAN of a non-lesioned bird (stained with the same Cresyl violet acetate protocol as other experimental birds) is used to calculate the normalised percentage of LMAN left.

3.9: Histology shows that LMAN is absent (or left in lesser volume) in LMAN lesioned birds while it is present in completely in sham lesion birds (see Fig. 3.10 & 3.11).

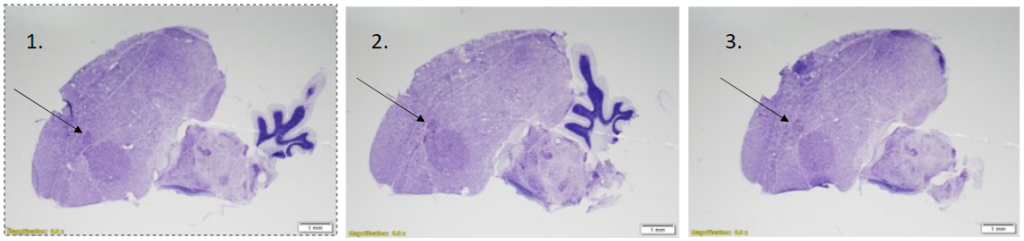

Fig. 3.10: Cresyl violet stained sagittal sections of zebra finch brain where LMAN is intact. The circular region towards which the arrow points is the LMAN. All sections belong to one bird

which had undergone a sham lesion surgery. In these sections, LMAN is present in complete areas/volumes.

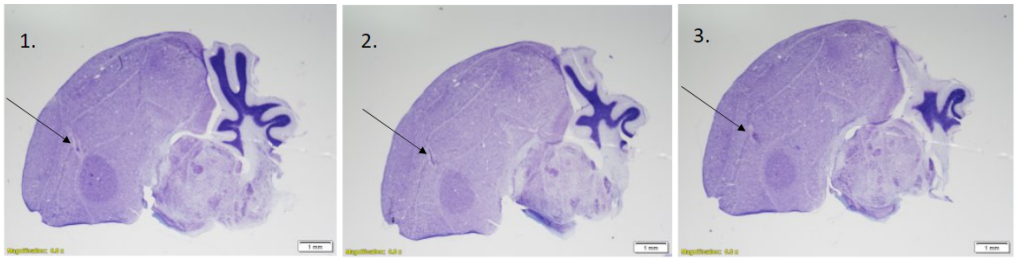

Fig. 3.11: Cresyl violet stained sagittal sections of zebra finch brain where LMAN is lesioned. The region towards which the arrow points is the most probable location of LMAN in these sections. All sections belong to one bird that had undergone an LMAN lesion surgery. In these sections, LMAN is left in very less areas/volumes.

3.10: The dye of tracer injections is successfully going into RA and fluorescently labeled RA. It is retrogradely transferred to LMAN and fluorescently labeled LMAN as well (see Fig. 3.12).

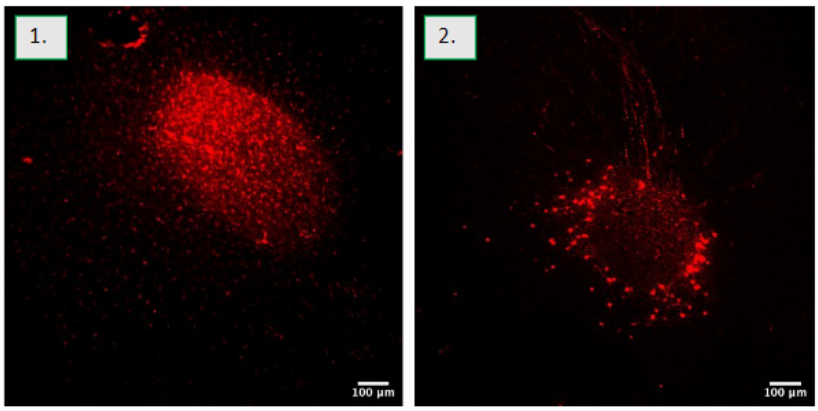

Fig. 3.12: Confocal images of sagittal brain sections of the tracer-injected bird. Both the images were taken at 10X magnification. The left image shows the fluorescently labeled RA and the right image shows the fluorescently labeled LMAN.

Chapter 4

Discussion

We have been successfully locating and lesioning LMAN. Histology data shows 6/7 LMAN lesioned birds have <40% LMAN remaining. And histology of one sham lesioned birds shows that LMAN is not being damaged, which means LMAN is intact in our control birds.

For some birds, syllables are not stereotyped after LMAN lesion when they reach the age of phd 90-100. They don't produce any stereotyped repetitive syllables at the beginning of the first motif by phd 90-100 but start producing by phd 120. This means their songs became more stereotyped over this period. So, we started labeling phd 120 songs and checking the learning of INs and songs by that age. Probably, LMAN lesions not only hinder the learning of IN structure but also slow down the process of stereotyping the IN structure. We will check this hypothesis by systematically comparing our data with Socially tutored birds' data (from Dr. Shikha Kalra).

Another observation is that juveniles with LMAN lesioned and tutored from 0 or low IN tutors were producing some variable elements (in place of INs) by phd 90-100 which later became INs by phd 120. Probably, birds have an innate tendency to produce INs and this is not influenced by the absence or presence of LMAN. Even though the process of attaining an IN could be slowed down in LMAN lesioned birds.

At last, I would end up the discussion by pointing out that our current results show that LMAN might be involved in learning of INs. After performing histology and song analysis on the juveniles that will be turning 120 days of age soon, we will be able to make more strong conclusions. In total, we will have data from 24 juveniles: 15 LMAN lesioned, and 9 sham lesioned.

References:

1. Tierney, A.J. The evolution of learned and innate behavior: Contributions from genetics and neurobiology to a theory of behavioral evolution. Animal Learning & Behavior 14, 339–348 (1986). https://doi.org/10.3758/BF03200077
2. Bennett G Galef and Kevin N Laland. Social learning in animals: empirical studies and theoretical models. Bioscience, 55(6):489–499, 2005.
3. Simon C. Griffith & Katherine L. Buchanan (2010) The Zebra Finch: the ultimate Australian supermodel, Emu - Austral Ornithology, 110:3, v-xii, DOI: 10.1071/MUv110n3_ED
4. Spierings, M. J., & Ten Cate, C. (2016). Zebra finches as a model species to understand the roots of rhythm. Frontiers in neuroscience, 10, 345.
5. Griffith SC, Ton R, Hurley LL, McDiarmid CS, Pacheco-Fuentes H. The Ecology of the Zebra Finch Makes It a Great Laboratory Model but an Outlier amongst Passerine Birds. Birds. 2021; 2(1):60-76. https://doi.org/10.3390/birds2010004
6. Doupe, A. J., & Konishi, M. (1991). Song-selective auditory circuits in the vocal control system of the zebra finch. Proceedings of the National Academy of Sciences, 88(24), 11339-11343.
7. Brenowitz, E. A., Margoliash, D., & Nordeen, K. W. (1997). An introduction to birdsong and the avian song system. Journal of neurobiology, 33(5), 495-500.
8. Eales, L. A. (1985). Song learning in zebra finches: some effects of song model availability on what is learnt and when. Animal behaviour, 33(4), 1293-1300.
9. Immelmann, K. (1969). Song development in the zebra finch and other estrildid finches. (No Title), 61.
10. Roper, A., & Zann, R. (2006). The onset of song learning and song tutor selection in fledgling zebra finches. Ethology, 112(5), 458-470.
11. Aronov, D., Andalman, A. S., & Fee, M. S. (2008). A specialized forebrain circuit for vocal babbling in the juvenile songbird. Science, 320(5876), 630-634.
12. Wallace PS, Whishaw IQ. Independent digit movements and precision grip patterns in 1-5-month-old human infants: hand-babbling, including vacuous then self-directed hand and digit movements, precedes targeted reaching. Neuropsychologia. 2003;41(14):1912-8. doi: 10.1016/s0028-3932(03)00128-3. PMID: 14572524.
13. Robinson SR, Blumberg MS, Lane MS, Kreber LA. Spontaneous motor activity in fetal and infant rats is organized into discrete multilimb bouts. Behav Neurosci. 2000 Apr;114(2):328-36. doi: 10.1037//0735-7044.114.2.328. PMID: 10832794.
14. Doupe AJ, Kuhl PK. Birdsong and human speech: common themes and mechanisms. Annu Rev Neurosci. 1999;22:567-631. doi: 10.1146/annurev.neuro.22.1.567. PMID: 10202549.
15. Imada T, Zhang Y, Cheour M, Taulu S, Ahonen A, Kuhl PK. Infant speech perception activates Broca's area: a developmental magnetoencephalography study. Neuroreport.

2006 Jul 17;17(10):957-62. doi: 10.1097/01.wnr.0000223387.51704.89. PMID: 16791084.
16. Fagen, R. (1981). Animal play behavior. (No Title).
17. Nottebohm F, Stokes TM, Leonard CM. Central control of song in the canary, Serinus canarius. J Comp Neurol. 1976 Feb 15;165(4):457-86. doi: 10.1002/cne.901650405. PMID: 1262540.
18. Simpson HB, Vicario DS. Brain pathways for learned and unlearned vocalizations differ in zebra finches. J Neurosci. 1990 May;10(5):1541-56. doi: 10.1523/JNEUROSCI.10-05-01541.1990. PMID: 2332796; PMCID: PMC6570078.
19. Farries MA, Perkel DJ. A telencephalic nucleus essential for song learning contains neurons with physiological characteristics of both striatum and globus pallidus. J Neurosci. 2002 May 1;22(9):3776-87. doi: 10.1523/JNEUROSCI.22-09-03776.2002. PMID: 11978853; PMCID: PMC6758399.
20. Bottjer SW, Halsema KA, Brown SA, Miesner EA. Axonal connections of a forebrain nucleus involved with vocal learning in zebra finches. J Comp Neurol. 1989 Jan 8;279(2):312-26. doi: 10.1002/cne.902790211. PMID: 2464011.
21. Leonardo A, Fee MS. Ensemble coding of vocal control in birdsong. J Neurosci. 2005 Jan 19;25(3):652-61. doi: 10.1523/JNEUROSCI.3036-04.2005. PMID: 15659602; PMCID: PMC6725314.
22. Hahnloser RH, Kozhevnikov AA, Fee MS. An ultra-sparse code underlies the generation of neural sequences in a songbird. Nature. 2002 Sep 5;419(6902):65-70. doi: 10.1038/nature00974. Erratum in: Nature. 2003 Jan 16;421(6920):294. PMID: 12214232.
23. Chi Z, Margoliash D. Temporal precision and temporal drift in brain and behavior of zebra finch song. Neuron. 2001 Dec 6;32(5):899-910. doi: 10.1016/s0896-6273(01)00524-4. PMID: 11738034.
24. Yu AC, Margoliash D. Temporal hierarchical control of singing in birds. Science. 1996 Sep 27;273(5283):1871-5. doi: 10.1126/science.273.5283.1871. PMID: 8791594.
25. Nottebohm, F., Stokes, T. M., & Leonard, C. M. (1976). Central control of song in the canary, Serinus canarius. Journal of Comparative Neurology, 165(4), 457-486
26. Dmitriy Aronov et al.,A Specialized Forebrain Circuit for Vocal Babbling in the Juvenile Songbird.Science320,630-634(2008).DOI:10.1126/science.1155140
27. Farries MA, Perkel DJ. A telencephalic nucleus essential for song learning contains neurons with physiological characteristics of both striatum and globus pallidus. J Neurosci. 2002 May 1;22(9):3776-87. doi: 10.1523/JNEUROSCI.22-09-03776.2002. PMID: 11978853; PMCID: PMC6758399.
28. Kao MH, Doupe AJ, Brainard MS. Contributions of an avian basal ganglia-forebrain circuit to real-time modulation of song. Nature. 2005 Feb 10;433(7026):638-43. doi: 10.1038/nature03127. PMID: 15703748.

29. Bottjer SW, Miesner EA, Arnold AP. Forebrain lesions disrupt development but not maintenance of song in passerine birds. Science. 1984 May 25;224(4651):901-3. doi: 10.1126/science.6719123. PMID: 6719123.
30. Olveczky BP, Andalman AS, Fee MS. Vocal experimentation in the juvenile songbird requires a basal ganglia circuit. PLoS Biol. 2005 May;3(5):e153. doi: 10.1371/journal.pbio.0030153. Epub 2005 Mar 29. PMID: 15826219; PMCID: PMC1069649.
31. Price PH. 1979 Developmental determinants of structure in zebra finch song. J. Comp. Physiol. Psychol. 93, 260. (doi:10.1037/h0077553)
32. Zann RA. 1996 The zebra finch: a synthesis of field and laboratory studies. Oxford, UK: Oxford University Press.
33. Sossinka R, Böhner J. 1980 Song types in the zebra finch Poephila guttata castanotis. Z. Für Tierpsychol. 53, 123-132. (doi:10.1111/j.1439-0310.1980.tb01044.x)
34. Rao D, Kojima S, Rajan R. 2019 Sensory feedback independent pre-song vocalizations correlate with time to song initiation. J. Exp. Biol. 222, jeb199042. (doi:10.1242/jeb.199042)
35. Baker MC, Thompson DB. 1985 Song dialects of white-crowned sparrows: historical processes inferred from patterns of geographic variation. Condor 87, 127-141. (doi:10.2307/1367144)
36. Zann R. 1993 Variation in song structure within and among populations of Australian zebra finches. Auk 110, 716-726. (doi:10.2307/4088627)
37. Janes SW, Ryker L. 2013 Rapid change in a type I song dialect of hermit warblers (Setophaga occidentalis). Auk 130, 30-35. (doi:10.1525/auk.2012.11273)
38. Thorpe WH. 1958 The learning of song patterns by birds, with especial reference to the song of the chaffinch Fringilla coelebs. Ibis 100, 535-570. (doi:10.1111/j.1474-919X.1958.tb07960.x)
39. Marler P, Tamura M. 1964 Culturally transmitted patterns of vocal behavior in sparrows. Science 146, 1483-1486. (doi:10.1126/science.146.3650.1483)
40. Shenoy KV, Kaufman MT, Sahani M, Churchland MM. 2011 A dynamical systems view of motor preparation: implications for neural prosthetic system design. Prog. Brain Res. 192, 33-58. (doi:10.1016/B978-0-444-53355-5.00003-8)
41. James LS, Sakata JT. 2017 Learning biases underlie ‘universals’ in avian vocal sequencing. Curr. Biol. CB 27, 3676-3682.e4. (doi:10.1016/j.cub.2017.10.019)
42. James LS, Davies R, Mori C, Wada K, Sakata JT. 2020 Manipulations of sensory experiences during development reveal mechanisms underlying vocal learning biases in zebra finches. Dev. Neurobiol. 80, 132-146. (doi:10.1002/dneu.22754)
43. Kalra, S., Yawatkar, V., James, L. S., Sakata, J. T., & Rajan, R. (2021). Introductory gestures before songbird vocal displays are shaped by learning and biological predispositions. Proceedings of the Royal Society B, 288(1943), 20202796.

44. Nixdorf-Bergweiler BE, Bischof HJ. A Stereotaxic Atlas Of The Brain Of The Zebra Finch, Taeniopygia Guttata: With Special Emphasis On Telencephalic Visual And Song System Nuclei in Transverse and Sagittal Sections [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 2007. Available from: https://www.ncbi.nlm.nih.gov/books/NBK2348/#