Context-Dependent Changes in Pre-Bout Activity in Adult Male Zebra Finch HVC

A Thesis

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by

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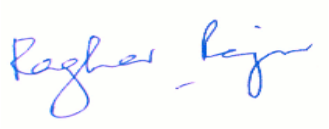
INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled Context-Dependent Changes in Pre-Bout Activity in Adult Male Zebra Finch HVC towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Manali Upadhyaya at 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:

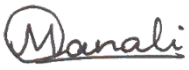
Dr. Raghav Rajan

Dr. Collins Assisi

This thesis is dedicated to curiosity, creativity, and HVC activity.

Declaration

I hereby declare that the matter embodied in the report entitled “Context-dependent changes in pre-bout activity in adult male zebra finch HVC” 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.



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Abstract

Motor preparation is essential for the efficient execution of any motor activity. Prior studies mostly focused on motor preparation before artificially trained motor movements, but the nature of motor preparation before natural, ethologically relevant animal movements remains poorly understood. The zebra finch song is a more ethologically relevant, naturally learned motor sequence consisting of a stereotyped pattern of vocalizations. A song-bout often starts with short vocalizations called introductory notes (INs), followed by a group of syllables called a song motif, which can be repeated one or more times. Two distinct pathways in the brain primarily control song- 1) the Song motor pathway consisting of the song premotor region HVC, downstream nucleus RA, which connects to the avian vocal organ and respiratory muscles, and 2) the Anterior forebrain pathway, which consists of the indirect projection from HVC to RA via the basal ganglia homolog Area-X. Previous studies have shown pre-song-bout activity in HVC hundreds of milliseconds prior to undirected songs, which are songs produced by the bird in isolation. This was suggested to have a role in motor preparation. Zebra finches also produce directed songs in the presence of a female bird. Directed songs are faster and more stereotyped than undirected songs, and directed song-bouts begin with more INs. Whether there are changes in activity before directed song-bouts remains unclear. My thesis focused on examining the context-dependent differences (directed vs. undirected song) in the pre-bout activity. I did not find differences in the average pre-bout firing rate between the two contexts, but I found differences in the latency of change in pre-bout firing. Relative to song-bout onset, the change in pre-bout firing occurred slightly later before directed songs in comparison to the undirected songs. Additionally, directed songs contain more INs, which are suggested to have a preparatory role. My findings suggest a continuum between pre-bout activity and INs, suggesting they may have a shared preparatory role before song onset. Another possibility is that there is a difference in state that causes a sudden abrupt increase in pre-bout activity before directed songs in comparison to the slowly increasing activity before undirected songs.

Acknowledgments

Firstly, I would like to extend my deepest gratitude to Dr. Raghav Rajan for his mentorship and constant support throughout my journey in this lab. I am indebted to him for giving me an opportunity to be a part of the lab, which not only helped me to learn how to work in a lab but also helped me explore my creativity. I feel extremely lucky to have had him as my thesis supervisor.

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Finally, I would like to thank my parents, my brother, and my friends for keeping my morale high throughout the course of my MS project.

All the experimental work was done in collaboration with Abir Dutta. The neural data analyzed was acquired by Raghav Rajan at UCSF. The neural recording of a bird implanted by Raghav Rajan was done in collaboration with Dhanya Raj.

Contributions

Contributor name	Contributor role
Raghav Rajan, Manali Upadhyaya	Conceptualization Ideas
Raghav Rajan, Manali Upadhyaya, Abir Dutta	Methodology
Raghav Rajan, Manali Upadhyaya	Software
Raghav Rajan, Manali Upadhyaya	Validation
Manali Upadhyaya, Raghav Rajan	Formal analysis
Manali Upadhyaya, Abir Dutta, Raghav Rajan	Investigation
Raghav Rajan	Resources
Manali Upadhyaya, Abir Dutta, Raghav Rajan	Data Curation
Manali Upadhyaya	Writing - original draft preparation
Manali Upadhyaya, Raghav Rajan, Shikha Kalra	Writing - review and editing
Manali Upadhyaya, Raghav Rajan	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

Introduction

"Spectacular achievement is always preceded by unspectacular preparation." (*Robert H. Schuller)*

Preparation, by definition, is the act of getting something/someone ready for something in the future. For example, preparation before an important presentation involves things such as noting important points to be conveyed, self-repetition and structuring it to complete on time, which helps build confidence and, thus, enhances the quality of the presentation. Similarly, preparation before any motor activity, such as walking, running, speaking, or writing, is thought to be essential for timely and accurate execution (DAY *et al.*, 1989; Churchland and Shenoy, 2007).

1.1 Pre-movement Neural Dynamics

Studies suggest that preparation at the neuronal level in certain regions of the brain before the start of any motor movement is important (DAY *et al.*, 1989; Ghez *et al.*, 1991; Ackermann and Ziegler, 2013). In the early nineteenth century, it was believed that an excited nerve could instantaneously cause muscle contraction; but later, it was found that in the motor nerve of frogs, the conduction velocity was about 100 feet per second, i.e., the duration became measurable (Donders, 1969; reviewed in Brunia and Van Boxtel, 2012). Then came the concept of 'reaction experiments' (a term coined by Exner, 1873) by Donders and Jaager, where the idea was that the reaction time for a complicated motor activity was equal to the sum of that for a simple motor activity and the additional time for central processing. However, this makes an assumption that only one subprocess is changed between a simple and a complicated movement. Later came the concept of associations, wherein an instruction was given to the subject to start a thinking process before performing an action. This instruction was suggested to invoke preparedness in the subject before the future situation (Watt, 1904; reviewed in Brunia and Van Boxtel, 2012). The work by Walter and his colleagues unraveled the Contingent Negative Variation (CNV) occurring in the one-second gap after the instruction stimulus and before the imperative stimulus. The sustained negative potential shift of roughly -20μV before the imperative stimulus was called CNV (Walter *et al.*, 1964). Meanwhile, Kornhuber and Deecke recorded the electrical activity from the scalp on magnetic tape before

the button press responses that human subjects had to perform at intervals of their own choice. They observed a slowly increasing negative potential shift more than a second before the button was pressed, which they called the readiness potential or the Bereitschaftspotential (Reviewed in Brunia and Van Boxtel, 2012).

If the readiness potentials were preparatory, it was expected that the properties of the anticipated movement would influence the amplitude of the potential. In an experiment where the participants were supposed to squeeze an electronic dynamometer with varying force, there was an amplitude change in the readiness potential on the contralateral hemisphere that correlated with the force (Kutas and Donchin, 1974). In another experiment, it was found that the readiness potential showed a steady increase as the performance of the motor task improved with trials (Taylor, 1978). Hence, the readiness potential was shown to have a preparatory function specific to movement. Later, in a different experiment, the participants were supposed to flex/extend their wrists rapidly after they heard an auditory tone. Here, it was discovered that interrupting brain activity by providing a single brain stimulus in the form of electrical or magnetic stimulation at the scalp, before the expected time of reaction and after the auditory tone, could delay the execution of voluntary movements by up to 150 ms (DAY *et al.*, 1989). The stimulation must have disrupted the motor preparation activity and caused the delay in the movement onset.

1.2 Motor Preparation

Some evidence of preparation before motor activity was also found in studies involving tasks with a temporal delay between the instruction signal and the go-cue. At the behavioral level, before cue-triggered movements, it was found that reaction times from the go-cue to movement onset were shorter when the delay period was longer. The shorter reaction time shows that the delay gives the preparation process a head start (Rosenbaum, 1980; Riehle and Requin, 1989; Riehle *et al.*, 1997; Crammond and Kalaska, 2000). Also, there have been changes in neural activity found in multiple brain regions, such as the premotor cortex and the motor cortex, just before motor initiation (Tanji and Evarts, 1976; WEINRICH *et al.*, 1984; Godschalk *et al.*, 1985; Kurata, 1989; Riehle and Requin, 1989; Snyder *et al.*, 1997). This change in the neural activity was found to be time-locked to the instruction, and its state could predict the reaction time. Moreover, earlier studies have shown that disrupting this neural activity causes a delay in movement (DAY *et al.*, 1989; Ghez *et al.*, 1991). Therefore, this change in neural activity occuring hundreds of milliseconds before the motor initiation was termed motor preparation.

Previous studies on primates have suggested the presence of preparatory activity in the monkey dorsal premotor cortex and the primary motor cortex before movement onset (Churchland *et al.*, 2006). For the experiment, the monkey had to look at a central dot on a screen during the instruction time, followed by a delay period. After this, another second dot appeared elsewhere on the screen during the go-cue time (the central dot disappeared), and the monkey was supposed to move its hand toward the second dot (Fig. 1A). The dot's color indicated whether the monkey was supposed to perform a slow or a fast reach, and the monkey was expected to prepare itself for a slow or a fast reach based on the color of the central dot during the delay period. Churchland and colleagues showed a trial-by-trial correlation between the preparatory activity in the monkey motor cortex and the peak hand speed during a delayed reach task (Fig. 1B; Churchland *et al.*, 2006). This supported the hypothesis that variability in movement, each time it is performed, is due to the differences in the firing rate during the preparatory activity.

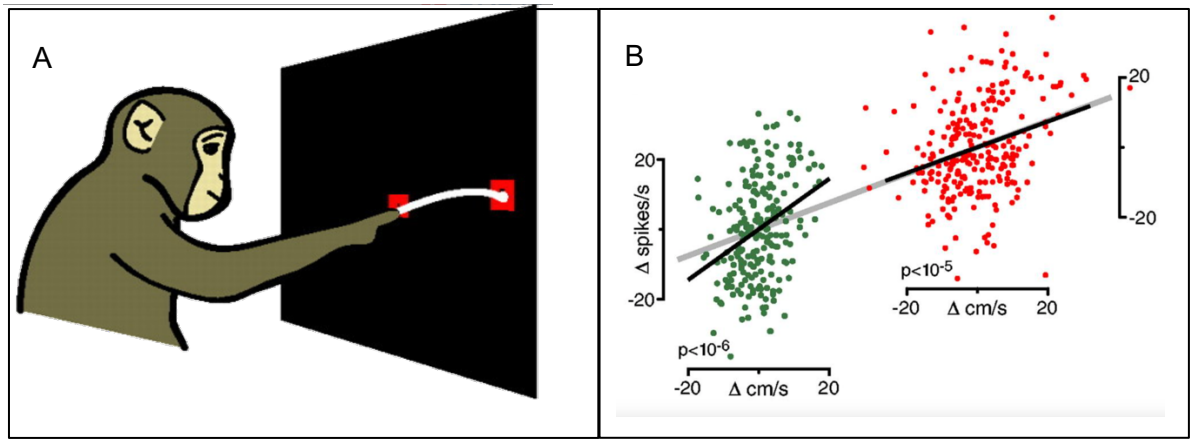

Figure 1: **A: Illustration of delayed reach task** (Churchland *et al.*, 2006). **B: Correlation between firing rate and peak hand velocity.** They are expressed as the difference from their mean. Green dots represent slow reaches and red dots represent fast reaches (Churchland *et al.*, 2006).

Movements can be initiated either as a result of reaction to an external cue, called 'cued movements', or can be self-initiated without the presence of any cue from outside, called 'self-timed movements'. A study on the monkey basal ganglia-cortical circuit showed differences in the timing of the increase in the neuronal firing before a cued movement compared to a self-timed movement (Fig. 2; Lee and Assad, 2003). They found that for a self-timed movement, the firing rate started to increase ~600 ms before movement onset, which is much earlier than before a cued movement; even though, both reached the same peak value of firing rate (Fig. 2).

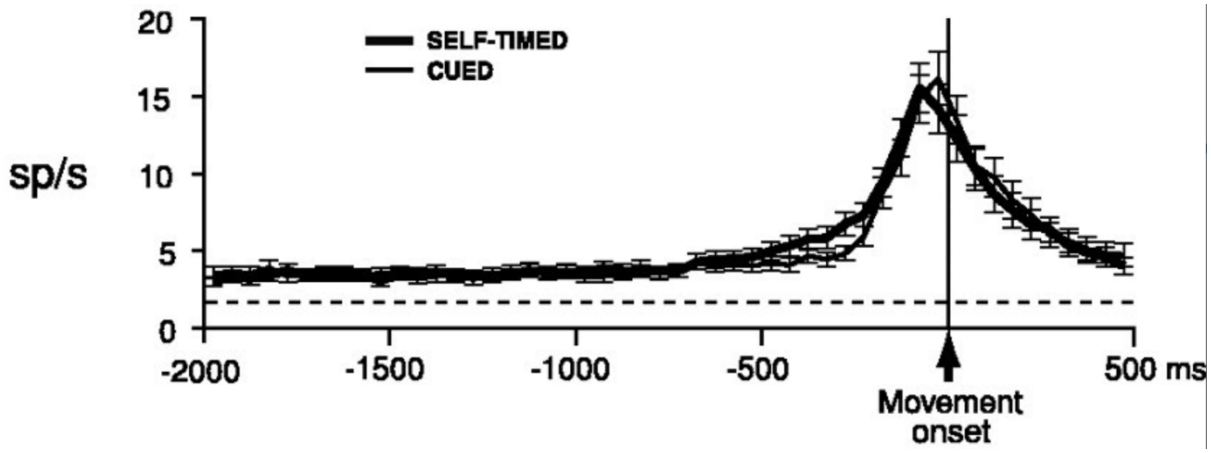

Figure 2: **Population averaged activity before self-timed and cued movements** for phasically active neurons (PANs) in putamen of monkey, aligned to movement initiation (Lee and Assad, 2003)5/15/24 6:12:00 PM (Lee and Assad, 2003). Dashed line: mean background firing rate.

Apart from the studies done on primates, some work has also been done on mice to determine the preparatory role of the anterior lateral motor cortex (ALM) before the action of licking in a particular direction (Guo *et al.*, 2014). Here, the mouse had to report the position of a pole presented to it as anterior or posterior by an artificially trained action of licking left or right. However, these artificially trained behaviors may not be ethologically relevant, so the neural activity patterns during these behaviors may never be seen during naturally occurring behaviors (Krakauer *et al.*, 2017).

1.3 Motor Preparation Prior To Ethologically Relevant Movements

The knowledge of preparation before ethologically relevant movements is limited, thus making it interesting to study. Some of the systems studied are song in songbirds and walking in crayfish. In crayfish, internally generated neuronal activities were found to precede the behavioral onset of self-initiated walking (Kagaya and Takahata, 2010). The descending neurons from the circumesophageal commissure were active at least 1s before walking. These neuronal activities were called readiness discharges. These represented when to produce the action and were not related to the direction of walking or speed.

1.4 Zebra finch as a model

1.4.1 Zebra finch song and the song-control circuitry

A great model organism to study naturally learned movements is the male zebra finch, an oscine songbird native to Australia and the Lesser Sunda Islands. It is sexually dimorphic, and both sexes produce calls frequently, but only the males produce songs to attract the opposite sex. Young male birds learn the song from their father or a tutor, like human speech learning (Zann, 1996; Doupe and Kuhl, 1999; Bolhuis *et al.*, 2010). The zebra finch song has a consistent sequence of sounds interspersed with silent gaps, making it a good model for studying naturally learned movements (Fee and Scharff, 2010). Song bouts begin with a variable number of short repeating vocalizations called introductory notes (INs- marked by 'i' in Fig. 3; Price, 1979). Following the INs, the bird produces a consistent sequence of syllables (sounds marked by 'a', 'b', 'c', and 'd' in Fig. 3), called a song motif (Fig. 3).

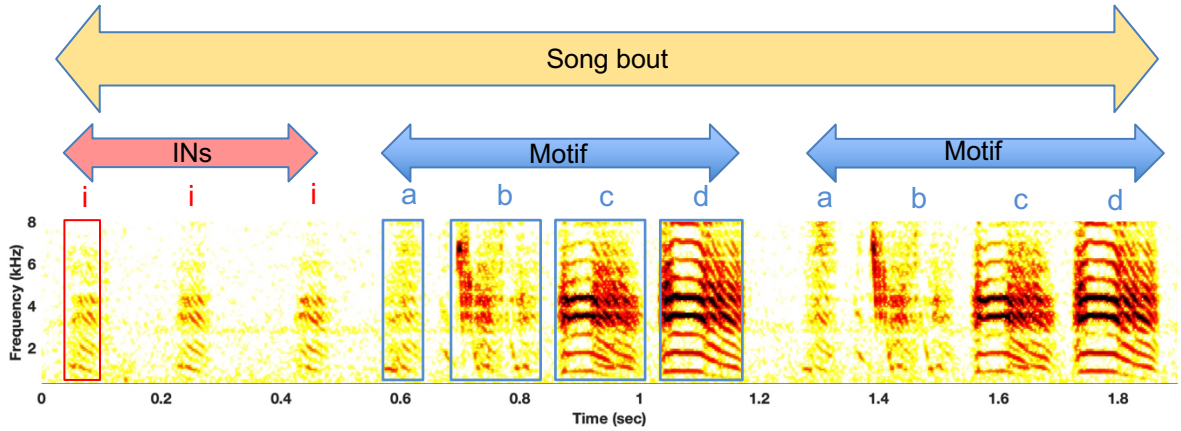

Figure 3: **Spectrogram of a zebra finch song.** It is a plot of frequency on the y-axis and time on the x-axis. The brightness of the color shows the amplitude for a particular frequency at that time. Labels are given to individual song elements called syllables. Here 'i' represents the introductory note (IN) and 'a', 'b', 'c', 'd' represents the syllables in a motif. This song bout starts with three INs and is followed by two motifs.

Different brain regions are known to control song production in the zebra finch. Song is primarily controlled by two distinct pathways in the zebra finch brain– (1) the song motor pathway and (2) the anterior forebrain pathway (AFP) (Fig. 4: Left: Red arrows show the motor pathway, and green arrows show the AFP; Nottebohm *et al.*, 1976; Bolhuis *et al.*, 2010; reviewed in Fee and Scharff, 2010). The song motor pathway consists of the song premotor nucleus called the HVC (proper name), which projects to the song motor nucleus called the RA (robust nucleus of the arcopallium) (Nottebohm *et al.*, 1976; Bottjer *et al.*, 1989). The RA, in

turn, projects to the syringeal (vocal organ) and respiratory muscles via the nuclei nXIIts (tracheosyringeal portion of the hypoglossal nucleus), RAm (nucleus retroambigualis) and PAm (nucleus paraambigualis) (Nottebohm *et al.*, 1976; Simpson and Vicario, 1990; Wild, 1993). This pathway is responsible for song production and is required throughout life to produce normal songs (Nottebohm *et al.*, 1976; Simpson and Vicario, 1990; Vicario, 1991). Meanwhile, the AFP consists of an indirect connection from the HVC to the RA via the basal ganglia homolog called Area-X, the DLM (dorsolateral nucleus of the thalamus), and the LMAN (lateral magnocellular nucleus of the anterior nidopallium). This pathway is essential in juvenile birds for learning the song (Bottjer *et al.*, 1984; Scharff and Nottebohm, 1991; Ölveczky *et al.*, 2005). In adult bird, the AFP was found to have no role in song production but have a role in song plasticity (Brainard and Doupe, 2000). So, damaging of the AFP circuit did not affect the already learned song, but hampered the ability to adaptively alter the learned song.

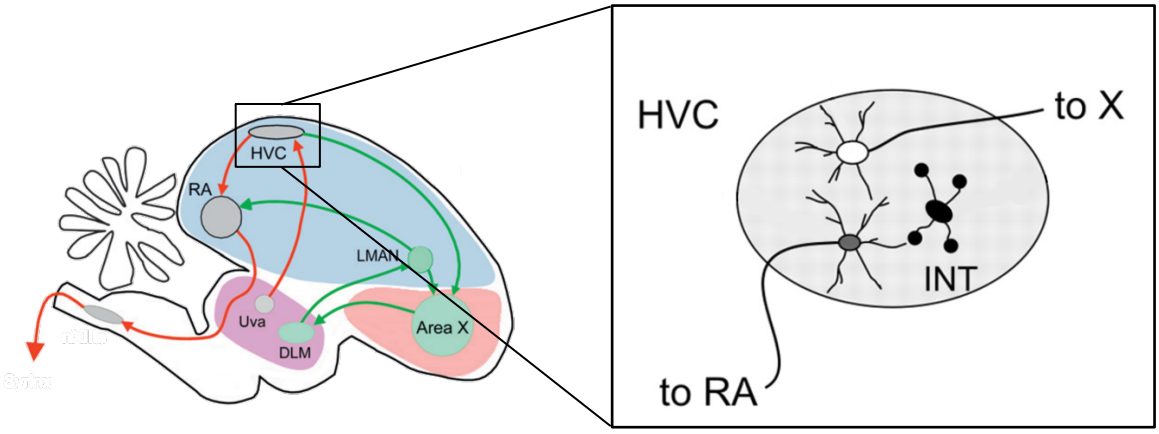

Figure 4: **Lett: Brain regions that control song.** Red arrows show the song motor pathway. Green arrows show the AFP (Fee and Scharff, 2010). **Right: Major types of neurons in premotor nucleus HVC** (Mooney and Prather, 2005).

1.4.2 Production of a Stereotyped Song

The song premotor nucleus HVC is important for song production and shows neural activity patterns stereotyped to the song (McCasland and Konishi, 1981; Yu and Margoliash, 1996; Hahnloser *et al.*, 2002). The HVC mainly consists of three types of neurons– HVC interneurons, Area-X-projecting HVC neurons (HVC_X), and RA-projecting HVC neurons (HVC_{RA}) (Fig. 4: Right; Mooney and Prather, 2005). The HVC_{RA} neurons are a part of the song motor pathway, and these neurons project to downstream nucleus RA, which projects to the syringeal and the respiratory motor neurons. These neurons produce a precise sequential

activity during the song and are important for song production (Scharff *et al.*, 2000; Hahnloser *et al.*, 2002; Long and Fee, 2008; Long *et al.*, 2010). These neurons were found to burst at most once during the song motif and to give the 'time code' to the 'muscle map' in RA (Hahnloser *et al.*, 2002). They are suggested to be active only during vocalizations in awake birds (Hahnloser *et al.*, 2002; Kozhevnikov and Fee, 2007). Nevertheless, about 50% of these neurons are not active during singing, and the function of these neurons is not known (Long *et al.*, 2010). The fact that these neurons have no spontaneous activity and that most do not fire even during singing makes it challenging to find and record activity from these neurons.

1.4.3 Song initiation

It has been demonstrated from intracellular recordings from brain slices that the HVC_{RA} neurons are a primary source of excitatory input to the HVC interneurons (Fig. 5A; Mooney and Prather, 2005; Kosche *et al.*, 2015). Also, previous work from the Rajan laboratory has demonstrated that the HVC interneurons increase their firing rate hundreds of milliseconds before initiating a song bout (Fig. 5B; Raghav Rajan, 2018). The work also showed that some HVC_X neurons that increased their firing before song, and this increase preceded the increased firing of interneurons. Some HVC_X neurons that showed a decrease in firing before the song, showed the decrease just after the interneurons showed the increase. From the studies regarding the connection between the different HVC neurons, it was shown that HVC_{RA} and HVC_X neurons had excitatory connections with HVC interneurons, while the HVC interneurons inhibited the HVC_X neurons (Mooney and Prather, 2005; Kosche *et al.*, 2015). The sequence in which the firing rate of HVC_X and HVC interneurons change before the song, supports the hypothesis that HVC_{RA} neurons might show an increase in the pre-bout firing. Also, since HVC_{RA} neurons have no spontaneous activity, they cannot show a further decrease in their firing.

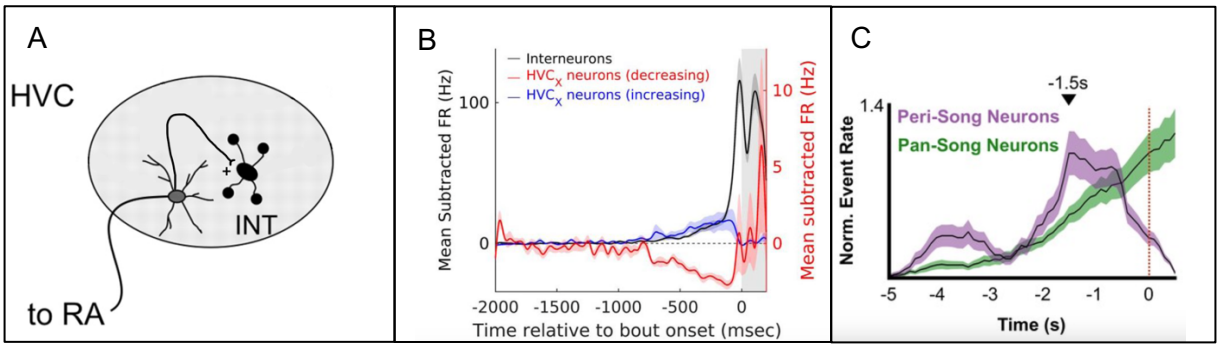

Figure 5: **A: HVC_{RA} neurons excite HVC interneurons** (Mooney and Prather, 2005). **B: Increase in firing rate of HVC interneurons before bout onset and Increase/decrease in firing rate of HVC_X neurons** (Rajan, 2018). **C: Increase in calcium activity before bout onset** (Daliparthi et al. 2019).

A study of the calcium activity of the HVC_{RA} neurons before song initiation has suggested an increase occurring about 1.5 s before song onset (Fig 5C; Daliparthi *et al.*, 2019). However, recording calcium activity has caveats, such as low temporal resolution and not providing individual spike-related information. Thus, looking at the electrophysiological activity of the HVC_{RA} neuron would be important. A comparison study suggested that the correlation of calcium imaging data with electrophysiological data has discrepancies at the single cell and neuron population levels (Wei *et al.*, 2020). Therefore, a calcium activity study would not give the information that electrophysiological data could give. It would be interesting to study the electrophysiological activity of the HVC_{RA} neurons before song initiation.

1.4.4 Introductory Notes and Preparation

Introductory notes (INs) are short repeating vocalizations that occur at the beginning of the song, before the song motif. These notes have been suggested to have a role in motor preparation. Behaviorally, it was found that the IN sequence progressed to a common acoustic state before beginning with the motif. Moreover, it was found that the HVC neurons reached a particular common state before the last IN (Rajan and Doupe, 2013). Some studies have also shown that deafening and disruptions to interhemispheric coordination could cause an increase in the number of INs (Ashmore *et al.*, 2008). However, some studies suggested no change in the IN number immediately after deafening and found that the mean number of INs to be produced in a song was learned by juvenile birds from the father/tutor (Rao *et al.*, 2019; Kalra *et al.*, 2021). Considering that both the pre-song-bout changes and the INs are preparatory, I anticipated that the pre-bout activity would form a continuum with the neural activity during INs in the HVC. That is, the higher number of INs would be preceded by pre-bout activity change occurring slightly later in comparison to when the bird sings a lower number of INs.

1.4.5 Directed and Undirected Song

Zebra finch songs can be directed or undirected (Sossinka and Böhner, 1980). A directed song is produced as a part of the courtship display and sung to a female bird. It can be vaguely compared to a cued movement, where the sight of the female bird is the cue. An undirected or solitary song would be similar to a self-timed movement, where it is not directed to any particular object, and the bird can sing this in a variety of situations– when alone, when kept in monosexual or mixed groups, when separated from mates, and so forth (Sossinka and Böhner,

1980). Earlier studies have shown that there are subtle differences in the song when they sing to a female in comparison to when they sing in isolation. Directed song is suggested to be a state of 'performance' in comparison to the undirected song, which is suggested to be a state of 'practice' (Jarvis *et al.*, 1998). During directed singing, the song was found to be slightly faster and have slightly less syllable variability than during undirected singing (Kao *et al.*, 2005). Studies in the AFP pathway have shown more variable firing when the bird sang in isolation than when it sang in the presence of the female bird (Kao and Brainard, 2006; Kao *et al.*, 2008). If the neurons are at two different states during the two contexts, there could also be changes in the preparatory activity before two types of songs. Therefore, if the pre-bout neural activity changes are observed in HVC before undirected songs, there is a possibility that the pre-bout firing shown by the same neurons might be different in the case of directed songs.

1.5 Specific Aims and Hypotheses

The aim of this thesis is to compare HVC activity hundreds of milliseconds before a directed song bout and an undirected song bout. Based on the results obtained from the monkey basal ganglia cortical circuit, I expect the timing of the increase in firing rate to be earlier before an undirected song bout, compared to a directed song bout (Lee and Assad, 2003).

Another objective of the thesis is to determine whether the variation in the number of INs produced in the bird correlates with the pre-song bout activity in the HVC neurons. Since both are hypothesized to be preparatory, I expect there to be some variation in the firing rate before song initiation, resulting in the IN number variation. Further, since birds produce a higher number of INs during directed songs, I also anticipate that the change in the pre-bout firing might occur a little later before directed song bouts.

Another aim is to electrophysiologically characterize the HVC_{RA} activity hundreds of milliseconds before the initiation of the song in the adult male zebra finch. Based on previous studies, I hypothesize that there will be an increase in the firing rate hundreds of milliseconds prior to song initiation.

Materials and Methods

Prior to start of experiments, Institute Animal Ethical Committee (IAEC) at IISER Pune approved all the experimental protocols in accordance with the Committee for the Control and Supervision of Experiments on Animals (CCSEA), New Delhi. Experiments (n=2 birds) that were performed at UCSF (California, USA) were approved by the UCSF Institutional Animal Care and Use Committee in accordance with the NIH guidelines. Data from the experiments carried out at USCF have been part of other publications (Rajan and Doupe, 2013; Woolley *et al.*, 2014; Raghav Rajan, 2018). Here, I analyzed and compared neural activity before the start of directed song bouts with neural activity before the start of undirected song bouts. This aspect of the activity has not been analyzed earlier.

The experiments were done in collaboration with Abir Dutta.

2.1 Experimental birds

Birds used for the purpose of this study were either bought from a local pet dealer (23 birds) or bred in the lab (7 birds). The birds were kept in cages with ad-libitum access to water and food. For the duration of the experiments, birds were individually housed with a 14-hour (light): 10-hour (dark) cycle in acoustically isolated enclosures (Newtech Engineering Systems, Bangalore, India) called soundboxes. Temperature was maintained at 25˚C at the facility. Nutritional supplements were provided in the form of boiled eggs and egg shells (twice per week), cucumber (once per week), coriander leaves (once per week), and sprouts (once per week).

2.2 Song recording

An omnidirectional polar pattern lavalier microphone (AKG Acoustics C417PP) was placed on top of the cage to record the vocalizations of the bird. The audio signals were amplified using a mixer (Behringer XENYX 802) and stored in the computer (sampling rate: 44100 Hz). Spectrograms, a visual representation of the sound wave in the form of a frequency vs time plot, were used to visualize the data (See Fig. 6 for an example).

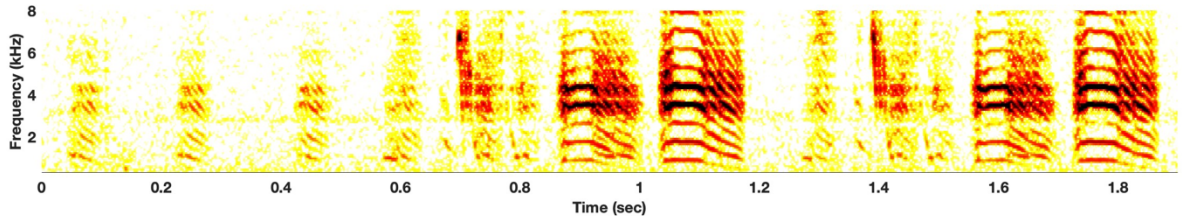

Figure 6: **Spectrogram of a zebra finch song.** The x-axis shows time (in seconds), the y-axis shows frequency (in Hz) and the intensity of the color shows the amplitude of vocalization for a particular frequency.

2.3 Designing Microdrive and Making Implants

2.3.1 Designing Microdrive Parts

To record neural activity in the awake bird, it was necessary to implant the electrodes on the bird's head, targeting the coordinates of HVC. A custom-made microdrive was designed to be lightweight and allow for vertical and lateral movement of the electrodes once implanted on the bird's head. The movement of the electrodes was necessary to record activity from multiple neurons in the same bird. Tinkercad (https://www.tinkercad.com), a 3D modeling software, was used to design the parts of the microdrive. These designed microdrive parts were sent for 3D printing to an online 3D printing service company called Shapeways (https://www.shapeways.com). These designs were largely similar to designs used in earlier studies (Rajan and Doupe, 2013; Rao, 2022). An important difference was the use of a 3D printed shuttle.

2.3.2 Making the Implant

A tap (000-120 threading) was used to make threads on the shuttle hole, and the shuttle was then fit into the microdrive body (Fig. 7A). A small screw similar to the one shown in Fig. 7B was screwed into the microdrive body and the threaded hole of the shuttle, as shown in Fig. 7C. The shank of the screw was lubricated with WD40 spray to enable the turning of the screw while it was fixed in place using dental cement, as shown in Fig. 7D. In order to put the lateral screw, a hole was made on the side of the microdrive body using a needle (21-gauge) and threaded using an M1.4 tap. A tiny M1.4 screw was put in through that hole (Fig. 7E).

Two small pieces of a polyimide tube (Inner diameter=250.4µm, Outer diameter=353.0µm, Polymicro Technologies) were cut to obtain two tubes of about 9mm in length. The two tubes were glued together using Fevikwik, as shown in Fig. 7F, inserted through the square hole on the microdrive body, and glued to it.

Connector: A 18-pin Omnetics connector was used. A silver wire was connected to the ground pin corresponding to either ‘GND’ or ‘REF’ on holding pins (See Fig 7H: Right). Two copper wires were connected to two other pins on the connector (Pins numbers 8 to 23) (Pin numbers on holding pin are preceded by ‘in’ in Fig 7H: Right). Pin numbers were chosen based on the connections with the recording headstage (INTAN 16 channel headstage). The wires were soldered onto the respective pins, and then epoxy was applied over the connections.

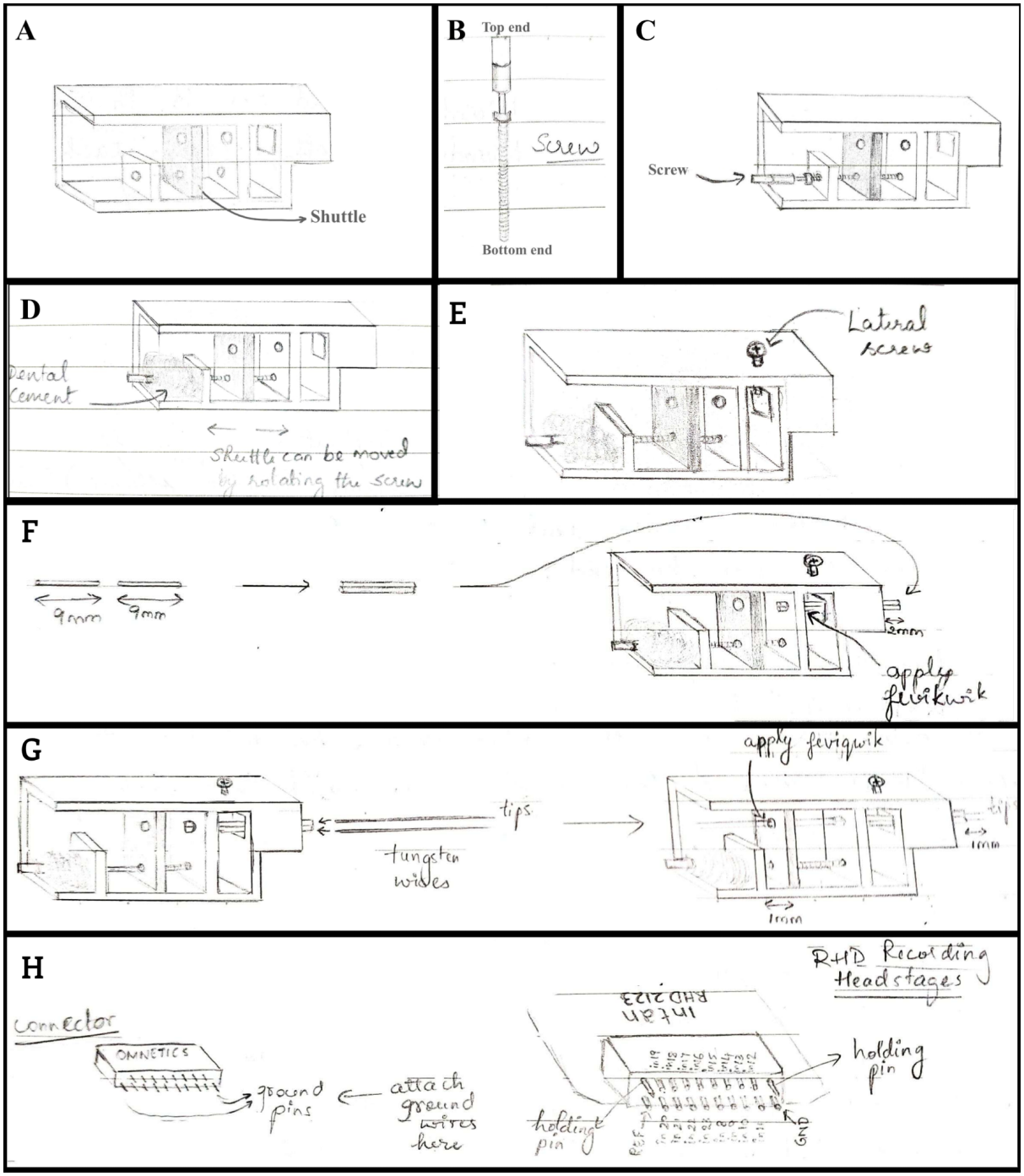

Figure 7: **A-H: Process of making an implant.** A-G: Assembling the microdrive parts. H: Attachment of copper wires to connector.

Two electrodes were inserted, one each, into the two polyimide tubes on the microdrive body, such that about 500 µm of the electrodes remained exposed on the tip side (Fig. 7G). The non-tip side of the electrodes was stripped of its insulation, and the stripped portion of each electrode was connected to one of the copper wires on the connector. The connections were soldered, silver paint was applied (to ensure more robust connections), and then epoxy was

applied over it to prevent cross-connection between the two electrodes. The custom-made electrode implant was then ready to be implanted into the bird's head (Fig. 8).

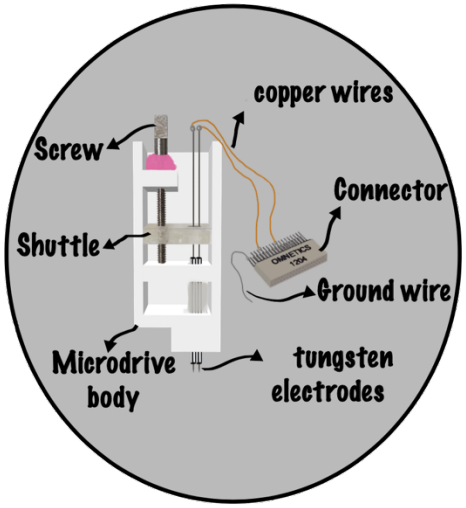

Figure 8: **Assembled custom-made microdrive implant that can be used for implantation.**

2.4 Surgery

All the surgery-related equipment, such as scalpels, forceps, surgical microscissors, etc., were cleaned using 99.9% ethanol and then heat sterilized by placing the equipment in a hot bead sterilizer (Fine Science Tools).

The bird was weighed and meloxicam, an analgesic (dosage: 0.25 mg/kg) was orally administered at least an hour before the surgery. The bird was anesthetized using isoflurane kept at 4% concentration for induction and 0.5-4% for maintenance (isoflurane was mixed with air with a flow rate of 1L/min). The bird was wrapped in a bird jacket, fixed on a custom stereotaxic apparatus, and placed under a heat lamp. Beak holder and ear bars were used to keep the bird's head in place. The beak bar was kept at an angle of 45˚. The head feathers were then plucked using forceps to access the skull. Local anesthetic, Lignocaine (1 unit or 25 µL), was injected subcutaneously above the skull to reduce pain associated with surgical procedures. The skin above the head was cut open using microscissors to expose the skull. A Y-shaped blood vessel was spotted lining the ridge between the right and left hemispheres and the branch-point of this 'Y' was taken as the reference to locate different brain regions. A craniotomy was performed above the left HVC using a scalpel. The coordinates of the HVC for the initial few

surgeries were obtained from the stereotaxic atlas of the zebra finch brain (Nixdorf-Bergweiler *et al.*, 2007) (https://www.ncbi.nlm.nih.gov/books/NBK2348/). Coordinates for later surgeries were obtained from a set of standardization surgeries described below. The layer of dura was removed above the region using a needle, and the region was periodically sprayed with saline (0.9% w/v NaCl) to prevent it from drying.

2.4.1 Pilot experiment to locate HVC in anesthetized birds

For the purpose of this experiment, a total of 8 male zebra finches were used. A tungsten electrode with glass (Impedance- 1-4MΩ)/epoxy (FHC- Impedance- 9MΩ) insulation was mounted on the hydraulic manipulator and positioned at the approximate HVC coordinates using the Y blood vessel as the reference. The electrode was inserted into the brain at the region where the craniotomy was made. The signal was passed through a head-stage amplifier (Intan technologies) to amplify the neural signal. This was connected to the Intan data-acquisition board, which was connected to a laptop for the visualization of neural activity. The HVC coordinates from the zebra finch brain atlas were used in the initial few surgeries. In successive surgeries, the electrode position was slightly varied in both anterior-posterior (A/P) and medial-lateral (M/L) directions. The coordinates where HVC-like bursty activity was observed were noted down for future reference. Here, HVC-like activity refers to bursts that had a duration of about 10-20 ms, with 2-5 ms gap between consecutive spikes in a burst (Hahnloser *et al.*, 2002). The HVC-like activity was obtained in 6 out of 9 birds, as shown in Table 1.

Table 1: Details regarding birds used for anesthetized recordings during surgery.

Bird Name	HVC-like bursty activity	Number of coordinates where bursty activity was seen
Green039White098	Yes	3 out of 7
Green153White161	Yes	3 out of 4
Yellow108Pink161	Yes	3 out of 9
Yellow117Pink162	No	-
Yellow118Pink111	No	-
Yellow148Pink118	Yes	2 out of 5
Yellow149Pink119	No	-
Yellow150Pink120	Yes	2 out of 7
Green179White018	Yes	6 out of 6

2.4.2 HVC implant surgery

The custom-made microdrive was mounted on the manipulator and positioned at the left HVC coordinates using the Y blood vessel as the reference. Every effort was made to align both

electrodes to the HVC coordinates so as to increase the chances of recording HVC activity. The electrodes were inserted into the brain, Vaseline was applied around the area where the electrodes went in, and then it was fixed in place using dental cement. Vaseline was used to prevent the dental cement from coming in contact with the electrodes and the brain surface. Another craniotomy was made elsewhere in the right hemisphere, and the ground wire was inserted through this. The connector was also appropriately placed and fixed onto the skull of the bird using dental cement. The bird was then allowed to recover for two days.

2.4.3 Surgery to Standardize Lesion Parameters and Make a Brain Atlas

This was done on one male bird (Green190White153). In addition to the left HVC (0.10 mm A/P, 2.00 mm M/L), craniotomy was performed at the right HVC coordinate (0.10 mm A/P, -2.00 mm M/L), and four other coordinates– left LMAN/Area-X (1.80 mm A/P, -4.40 mm M/L), right LMAN/Area-X (1.80 mm A/P, -4.40 mm M/L), (2.20 mm A/P, 2.00 mm M/L), and (2.20 mm A/P, -2.00 mm M/L). Lesions/markings were made at two different depths at each of the six coordinates. Different lesioning parameters were– 30 µA for 100 s, 60 µA for 50 s, and 60 µA for 100 s. A different tungsten oxide marking technique was also tried (Oikawa *et al.*, 2023). For this technique, a biphasic current pulse of 40 µA was passed. Each pulse consisted of a positive current for 0.5 ms followed by a negative current for 0.5 ms. There was an inter-pulse interval of 4 ms duration. A total of 36,000 pulses were passed at each coordinate. Since the electrode was made of tungsten, a series of positive and negative currents was supposed to deposit tungsten oxide at the site close to the tip of the electrode.

Prior to two of the electrode insertions into the brain, the electrodes were dipped in DiI to make sure that the electrode tracks were visible in the histology. DiI is a fluorescent lipophilic dye that is used to label the cell membrane.

Table 2 gives the details regarding the lesion parameters used.

Table 2: Details regarding the surgery to standardize the lesion parameters. A/P refers to the anterior-posterior coordinates. Positive A/P means that the location is anterior to Y, and negative means it is posterior to Y. M/L refers to the mediolateral coordinates. A positive M/L refers to a location on the left hemisphere, and a negative value refers to a location on the right hemisphere. Depth refers to how deep the location is from the brain's surface.

Coordinates with respect to Y			Lesioning parameters/ Tungsten-oxide marking
A/P (mm)	M/L (mm)	Depth (mm)	

0.10	2.00	0.7	Lesioning- 30µA for 100s
0.10	2.00	3.0	Lesioning- 30µA for 100s
0.10	-2.00	0.7	Lesioning- 30µA for 100s
0.10	-2.00	3.0	Lesioning- 60µA for 50s
4.40	1.80	1.0	Lesioning- 60µA for 50s
4.40	1.80	3.0	Lesioning- 60µA for 50s
4.40	-1.80	1.0	Lesioning- 60µA for 100s
4.40	-1.80	3.0	Lesioning- 60µA for 100s
2.20	2.00	1.0	Lesioning- 60µA for 100s
2.20	2.00	3.0	Tungsten oxide marking
2.20	-2.00	3.0	Tungsten oxide marking
2.20	-2.00	1.0	Tungsten oxide marking

2.5 Recording Neural Activity

Once implanted birds recovered and started singing, they were connected to the head-stage amplifier (Intan Technologies – 16 channel). This was connected to the Intan data acquisition board via a commutator (Crist Instruments or Doric Lenses). This was connected to the computer for visualization and recording of neural activity. Throughout the process, the bird was housed inside the sound box. The Intan microphone was placed just above the bird's cage to record the bird's vocalizations. A female bird was presented in the soundbox for about 3-10 minutes for the directed song recording. After recording neural activity from one penetration till a certain depth beyond HVC (~1.1-1.2 mm depth from the brain's surface), the electrodes were retracted, and the lateral screw was turned to enable a new penetration at a slightly different coordinate (Nixdorf-Bergweiler *et al.*, 2007). The process was repeated multiple times to enable neural activity from multiple locations in the same bird to be recorded.

2.6 Lesioning

When no neural activity could be recorded, either because the shuttle stopped moving or the electrode impedances dropped below ~900kΩ, or vocalizations came up on the neural channels, electrolytical lesions were made at the coordinate where HVC-like activity or singing-related activity was obtained. The lesioning parameters were used in accordance with the results obtained from a surgery done to standardize the lesioning parameters and nuclei positions, i.e., 30 μA current was passed for 100 s.

2.7 Perfusion

The lesioned bird was euthanized about 2-3 days after it was lesioned, using a lethal dose of Isoflurane. The feathers over the abdomen were plucked. A small cut using the surgical blade was made to expose the abdominal cavity. The cavity was then opened with the help of scissors, such that the organs, such as the heart and the liver, were visible. A small nick was made on the right atrium using the surgical blade. A 23-gauge needle connected to a peristaltic pump was injected into the left ventricle. The bird was intracardially perfused with ~100 mL saline (0.9% w/v NaCl). Saline was used to clear out the blood from the body. Subsequently, the bird was perfused with about 100 mL of 4% paraformaldehyde (PFA). PFA was used as a fixative to preserve tissue. All protective measures were followed while working with PFA. The bird was then decapitated, and the brain (along with the skull) was put in a 50 mL falcon tube containing 4% PFA for a day. Then the skull and the dura were removed, and the brain was put back in the PFA solution for another day. Thereafter, the brain was transferred into a 30% sucrose solution for cryoprotection.

2.8 Histology

2.8.1 Sectioning

Once the brain was in sucrose for a day and had sunk to the bottom of the falcon tube, it was split into two hemispheres using a blade. Each hemisphere was then sectioned into sagittal sections using the cryostat (CryoStarNX10). During sectioning, the knife and brain temperatures were set at -20˚C and -12˚C, respectively. The thickness of the sections was set to 40 μm. The sections were put into 24-well plates, each well having 2 mL of 1X PBS.

2.8.2 Mounting and Staining

Every third brain section from the 24-well plate was mounted on Poly-L-Lysine coated slides. Poly-L-Lysine helps in the adhesion of the brain sections onto the glass slide. The mounted sections were stained with 1% Cresyl Violet acetate. Cresyl violet stains the Nissl granules in the neuronal cell body and dendrites, thus resulting in the darker staining of certain nuclei, such as the HVC, due to increased density of neurons relative to surrounding areas. The protocol used for staining was as follows. The glass slides containing the sections were transferred to Coplin jars containing:

1) 1% Cresyl Violet for 10 minutes.
2) Dipped 3 times slowly in MilliQ water.
3) 50% ethanol for 1 minute.
4) 50% ethanol for another minute.
5) 70% ethanol for 1 minute.
6) 70% ethanol for another 1 minute.
7) 95% ethanol for variable duration varying from 30 seconds to 4 minutes. During this step, the slides were removed every 15-20 seconds after the initial 40 seconds to check for the amount of de-staining.
8) Two fast dips in 100% ethanol.
9) Xylene for 30 seconds.

Following this, a few drops of DPX were applied to the glass slides, and a cover slip was gently placed on the sections. The glass slide was left to dry for a day.

2.8.3 Bright-field imaging

The stained brain sections were imaged using a Bright-field microscope (Olympus MVX10 microscope, Microscopy facility, IISER Pune). The magnification used was usually 0.8X or 0.63X. The software used to view and take the images on the computer was Olympus cellSens Dimension.

2.9 Designing Microdrive For Silicon Probes

Silicon probes are high-channel count probes that enable the simultaneous recording of multiple neurons. The major drawback when it comes to using these probes is that they are

very expensive. Hence, there was a need to reuse these probes for multiple surgeries. Thus, a new microdrive was designed to be lightweight and ensure the efficient usage of the probes on multiple birds. Tinkercad (https://www.tinkercad.com), a 3D modeling software, was used for designing the microdrive, and the design was sent for 3D printing to Shapeways, an online 3D printing company (https://www.shapeways.com) (See Fig. 9 for the latest design). These microdrives will be used in future experiments in the lab.

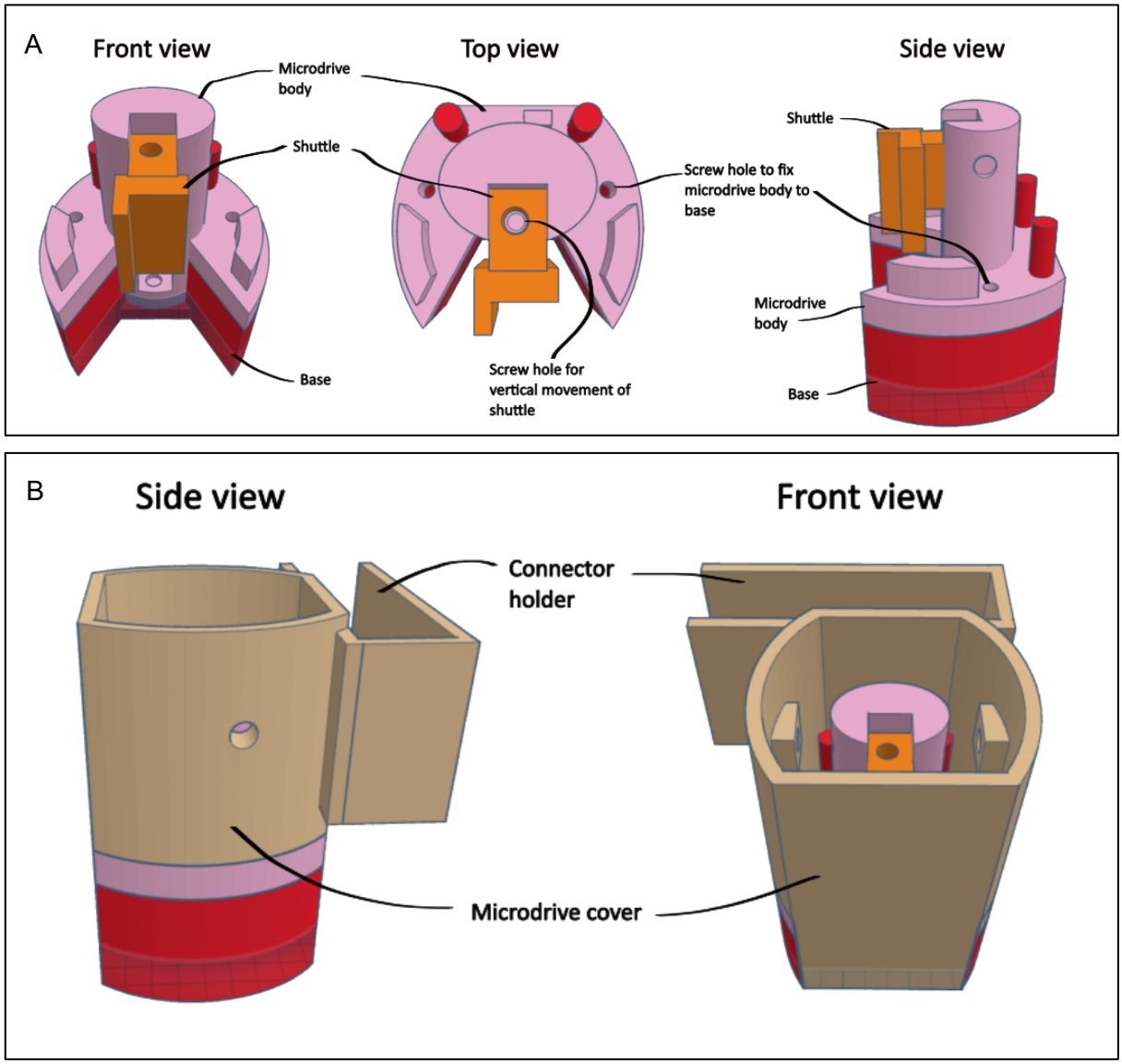

Figure 9: **Latest design of the microdrive for silicon probes**. A: Microdrive body (in pink) attached to the microdrive base (in red). The shuttle (in orange) can be moved in the vertical direction when attached to the microdive body with a screw. The silicon probe can be glued onto the shuttle. Left: Front view, Middle: Top view, Right: Side view. B: Microdrive cover attached to the microdrive body, to protect the silicon probe and the microdrive body. It has a connector holder where the silicon probe's connector can be attached with a screw. Left: Side view, Right: Front view.

2.10 Data Analysis

All the analyses were performed using custom-written Python and MATLAB scripts.

2.10.1 Song Analysis

2.10.1.1 Song Labeling and Bout Identification

Audio (.wav) files were automatically divided into syllables based on a user-defined amplitude threshold and then manually verified/modified based on visual analysis. Individual syllables were manually given labels based on their appearance on the spectrogram, i.e., similar looking syllables were given the same label.

A sequence of syllables was called a song bout if it had at least one motif. The sequence of the labeled syllables was classified into different bouts if there was a silent gap of more than 2 s between them. If the bout began with one or more INs and immediately proceeded to a motif, it was classified as an IN-song bout, and if it began with one or more calls before the IN, it was classified as a call-IN-song bout. Calls are more individual vocalizations that have longer and variable duration of silent gaps in comparison to the song syllables (Price, 1979). All bouts that began with an IN but were interrupted with calls before proceeding to the song were omitted and all solitary INs were omitted for the analysis.

2.10.1.2 Pre- and Post-Surgery Song

For all the birds, pre- and post-surgery songs were compared qualitatively to see if the song had any changes after surgery by visual inspection (See Fig. 10 for an example of damaged post-surgery song). This aided in getting an idea of whether HVC was damaged during surgery, as previous studies show that small damage to HVC results in changes in song (Thompson and Johnson, 2007).

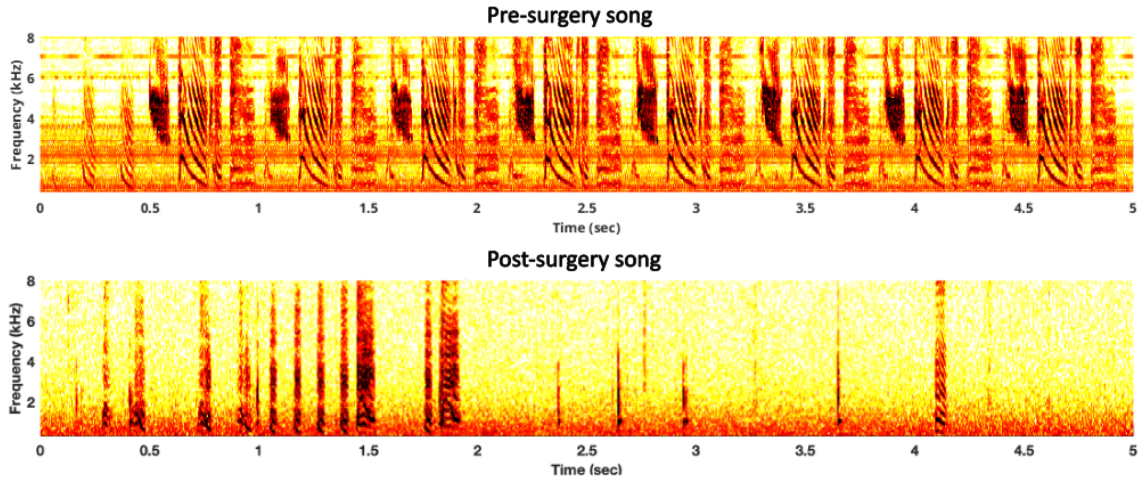

Figure 10: **Spectrogram of pre-surgery and post-surgery songs of the bird where post-surgery song was damaged**. Top: Pre-surgery song; Bottom: post-surgery song. Bird Name: Green175White185.

2.10.2 Neural data analysis

Of the data that were analyzed, 22 HVC neurons (from 2 birds) were recorded in UCSF (California, USA) by Raghav Rajan, and 1 HVC interneuron (from 1 bird) was recorded by me (in collaboration with Abir Dutta and Dhanya Raj) at IISER Pune.

During the recording session, the live spiking of the neurons during singing was observed for any singing-related activity changes. The neural raw trace, along with the song spectrogram, was also plotted using MATLAB to qualitatively observe any singing-related activity.

2.10.2.1 Spike sorting

Spike sorting was performed on the recorded neural activity, where some singing-related changes were found from the neural raw trace. The spikes were sorted based on the crossing of a certain user-defined threshold and based on various spike features such as peak, valley, the first three principal components, and in some cases, the spike width as well. Using these features, the spikes were automatically grouped into different clusters using a program called KlustaKwik (Kadir *et al.*, 2014) (K.D. Harris: https://sourceforge.net/projects/klustakwik/). Through visual inspection, some of the clusters were merged together if they had similar waveforms. The spike times obtained for the spike cluster of the specific neuron were saved for later analysis.

2.10.2.2 Plotting Raster and PSTH

The spike times obtained after spike sorting was used to plot rasters during the time period of -2000 ms to +200 ms relative to the bout onset, with each row in the plot corresponding to one song bout (or trial) and each tick corresponding to one spike. For neurons that had both undirected and directed trials, the undirected trials were plotted in blue and the directed trials in black. For most of the plots, the number of INs was also mentioned on the right y-axis.
The peri-stimulus time histogram, or PSTH, was plotted along with the rasters to show the average firing rate across trials within 100 ms bins of the neural data. The baseline activity, along with the 95% confidence interval, was also plotted along with the average firing rate on the PSTH. The baseline activity was obtained from 500 ms spontaneous activity (no singing) window of -2000 ms to -1500 ms relative to the bout onset. The 95% confidence interval for the baseline activity was obtained by resampling 10,000 times from the 1 ms bins in the spontaneous activity window (convolved with a Gaussian kernel of σ=20 ms and length=3σ). The method for obtaining the baseline activity is similar to that used in a previous study (Raghav Rajan, 2018).

2.10.2.3 Obtaining Average Firing Rate for Neurons

The Wilcoxon signed-rank test was used to compare the average pre-bout activity between undirected and directed song bouts over 500 ms (i.e., -600 to -100 ms relative to song onset) windows. For this, all the HVC neurons that had more than 3 bouts of both directed and undirected songs was used.

On the basis of the pattern of spontaneous activity and the neuronal firing during the song, the HVC neurons were classified as HVC interneurons and HVC_X neurons. Among the HVC_X neurons, some showed a pre-bout increase in firing, and others showed a pre-bout decrease in firing. These neurons were classified as HVC_X (increasing) and HVC_X (decreasing), respectively, similar to the classification done in Rajan (2018), based on previous literature (Hahnloser *et al.*, 2002; Raghav Rajan, 2018). For each category of neurons, the bouts were classified into directed and undirected bouts based on the presence or absence of the female bird during the singing. The average firing rate was obtained for 100 ms bins over all trials for each neuron during the time period of -2000 ms to +200 ms from the bout onset. Each of the bins was subtracted by the mean baseline activity for that neuron (baseline activity was obtained as mentioned in section 2.9.2.2). This baseline subtracted activity was averaged over all the neurons of the same type in 100 ms bins.

2.10.2.4 Latency of change in pre-bout firing

The method for calculating the latency of change in firing was taken from an earlier study (Raghav Rajan, 2018). Prior to calculating the latency, the trials were separated into directed and undirected bouts, and then the above-mentioned method was used for each neuron. The spike times, obtained after spike sorting, from each trial were divided into 1 ms bins. These were converted into binary lists of 0 and 1 depending on the presence or absence of spikes. The spike trains in the period of -2000 ms to the bout onset were convolved with a Gaussian kernel of σ=20 ms and length=3σ for each trial. The mean for each bin was taken over all trials for a neuron. The time when the mean value went outside the baseline 95% confidence interval values and remained outside till 100 ms before bout onset was considered the latency of change in pre-bout firing. To get the standard error, an artificial dataset was created with the same number of trials as the original dataset, obtained from the original dataset by resampling with replacement. Then the latency for the artificial dataset was calculated as mentioned above. 100 such artificial datasets were created, and the process was repeated. The standard error for the original latency measurement of each neuron was determined by taking the standard deviation of the latency estimations derived from this resampling technique.

2.10.3 Histological analysis

The bright-field images of the stained brain sections were analyzed using the image analysis program called ImageJ (Schneider *et al.*, 2012).

2.10.3.1 Standardizing Lesion Parameters and Customized Zebra Finch Brain Atlas

The area of the lesions was quantified using ImageJ.

For the purpose of making the brain atlas, the original images were modified by making the image background white and rotating the images to align all the sections with each other. The images of the sections were rotated in such a way that the electrode tracks labeled by DiI were vertical. The dimensions/sizes of the images were not changed. The approximate A/P coordinate of Y (reference point; blood vessel) was marked in all the sections. Each of the sections was of 50µm thickness. This was used to calculate the approximate M/L coordinate of each section by matching the lesion coordinates used during surgery with the sections where the lesions were visible.

Results

3.1 Pilot experiment to standardize HVC coordinates

A bursty pattern of spontaneous activity seen during anesthetized recording was used as an indicator that the electrodes were at HVC coordinates. These bursts were typically ~10-20 ms long, with the gap between the spikes within the burst roughly 2-5 ms (Hahnloser *et al.*, 2002). Multiple such coordinates in and around the HVC coordinates mentioned in the zebra finch brain atlas were recorded. But the most reliable coordinates from where such a bursty pattern of activity was observed were noted down. Fig. 11 shows the points where HVC-like bursty activity was found in anesthetized birds. It includes observations from 6 out of 9 birds.

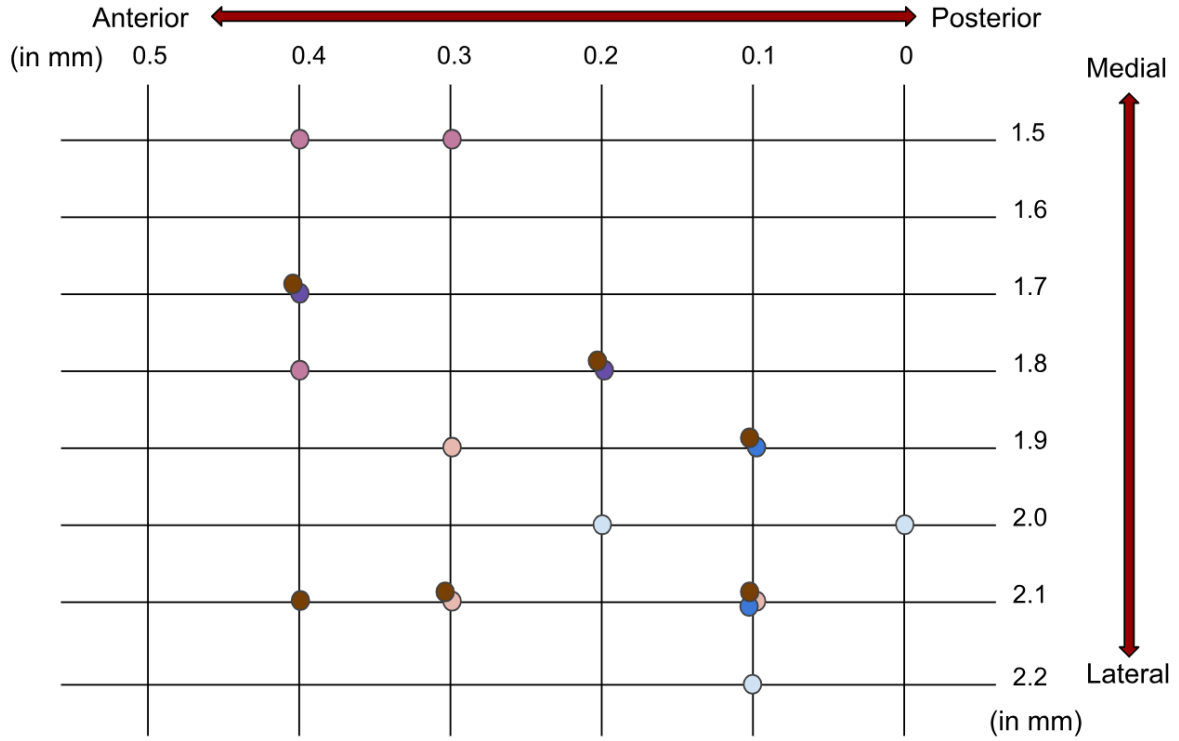

Figure 11: **Coordinates** (with respect to Y) on the left hemisphere of the brain where **HVC-like bursty activity was observed in anesthetized male zebra finches**. Each color represents a bird.

The neural raw trace shown in Fig. 12 was recorded from an anesthetized zebra finch HVC. The activity was recorded from the left hemisphere at the following location: 0.4 mm A/P, 1.8 mm M/L, and 470 µm depth, with respect to Y. Finally, the range of coordinates roughly obtained were 0 to 0.4 mm A/P and 1.5 to 2.2 mm M/L.

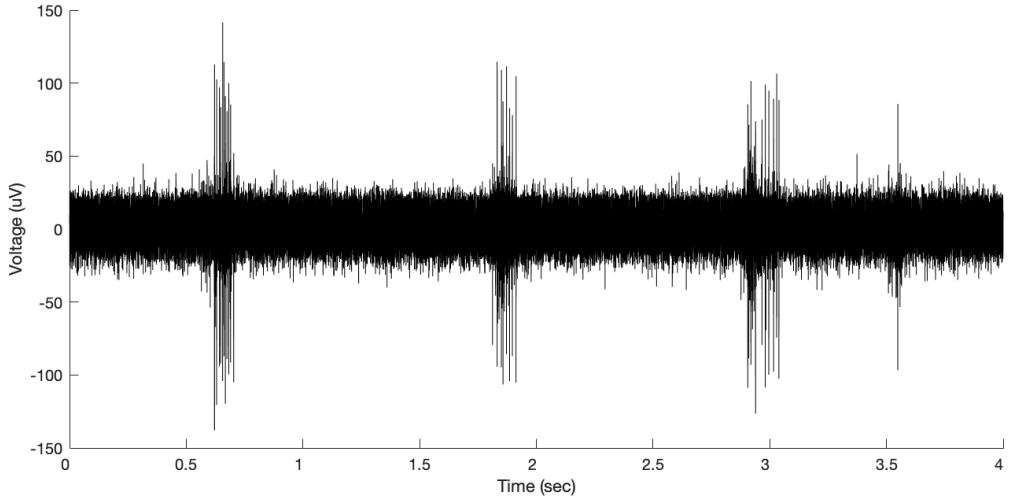

Figure 12: **Bursty activity recorded from anesthetized zebra finch HVC.** Bird Name: Yellow108Pink161.

3.2 Experiment to standardize lesion parameters

Since the HVC lesions that were made previously were not visible in the stained brain sections, surgery was done to standardize the lesion parameters (Refer Materials and Methods, section 2.3.3). The lesions that showed up reliably on the brain sections had the following parameters:

1) 30 µA for 100 s (Fig. 13A-B (i. and ii.), Arrows point to lesions)
2) 60 µA for 100 s (Fig. 13C, Arrow points to the lesion)

The lesions done with 30 µA for 50 s and the tungsten oxide markings did not show up on the sections. Since, the parameter of 30 µA for 100 s consistently seemed to show the lesion marks, it was used for lesions in the subsequent birds.

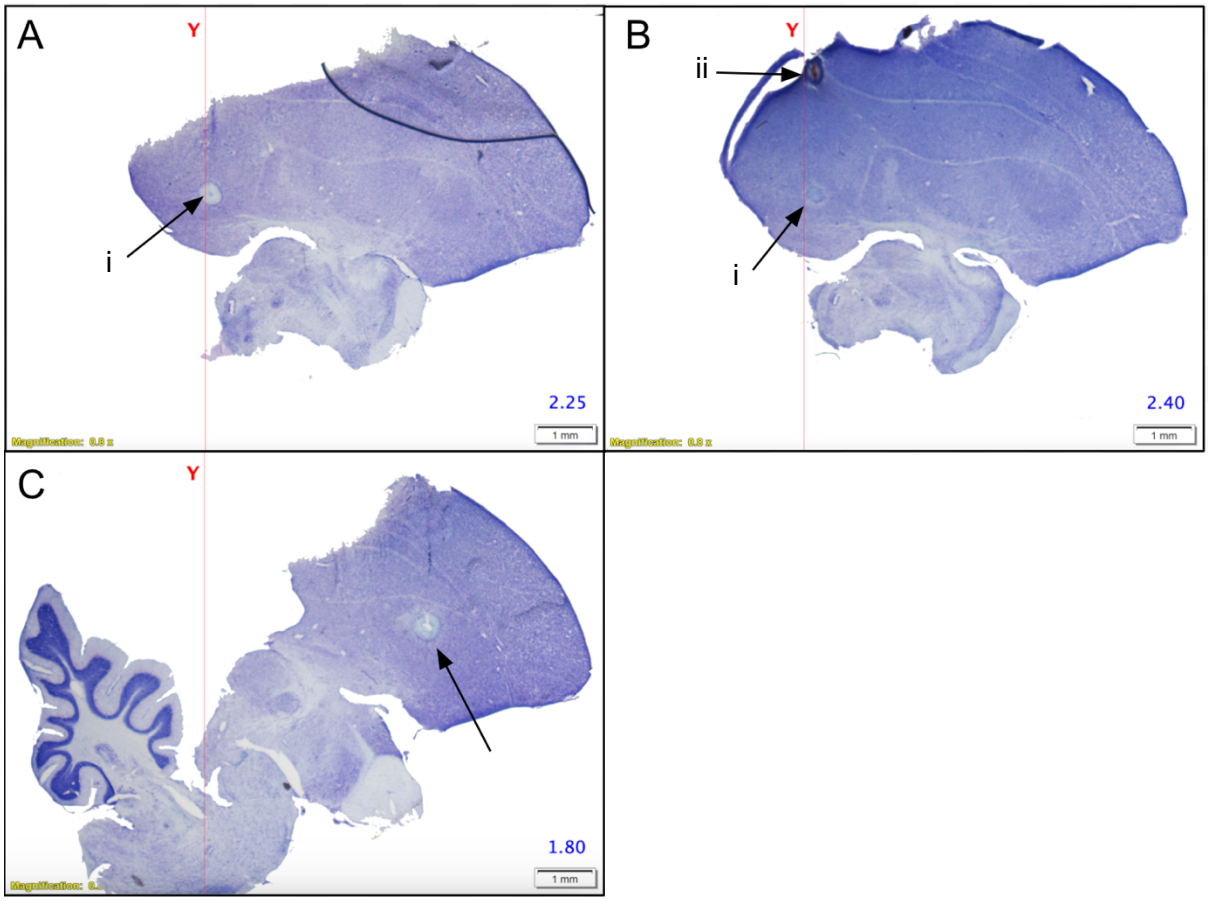

Figure 13: **A-C: Left hemisphere brain sections with lesions** of bird Green190White153. Arrows are pointing towards the lesions. The red vertical line shows the A/P coordinate of Y. The numbers in blue on the right-bottom corner show the approximate M/L coordinate of the sections. **A:** Lesion at 0.1 mm A/P, 2.0 mm M/L, 3.0 mm depth. **B:** Lesions at two coordinates: i) 0.1 mm A/P, 2.0 mm M/L, 3.0 mm depth (~0.051 mm^3 lesion), and ii) 0.1 mm A/P, 2.0 mm M/L, 0.7 mm depth (~0.023 mm^3 lesion). A-B: Lesion parameter=30 µA for 100 s. **C:** Lesion at 2.2 mm A/P, 2.0 mm M/L, and 1.0 mm depth (0.059 mm^3 lesion). Lesion parameter=60 µA for 100 s.

Table 3: Lesion sizes obtained at the coordinates for the respective lesion parameters used. Since every third section was mounted on the slide that was imaged, and the thickness of the sections was 50 µm, the area of lesion in each section was multiplied by 3 and $50*10^{-3}$ and a summed over the number of sections having the lesions, to obtain the lesion volume.

Lesion parameter	Coordinate (in mm)	No. of sections having lesion	Area of lesion in each section (A_1,A_2,..) (in mm^2)	Lesion size (in mm^3) = $(\sum A_i)*3*50*10^{-3}$
30µA for 100s	0.1 A/P, 2.0 M/L, 3.0 depth	4	0.076,0.131,0.117,0.017	0.051
30µA for 100s	0.1 A/P, 2.0 M/L, 0.7 depth	1	0.155	0.023
60µA for 100s	2.2 A/P, 2.0 M/L, 1.0 depth	3	0.073,0.193,0.126	0.059

3.3 Customized Zebra Finch Brain Atlas

The coordinates of most brain regions in the laboratory were not consistent with the available zebra finch brain stereotaxic atlas (Nixdorf-Bergweiler *et al.*, 2007) (https://www.ncbi.nlm.nih.gov/books/NBK2348/). Therefore, a customized brain atlas was needed for the lab.

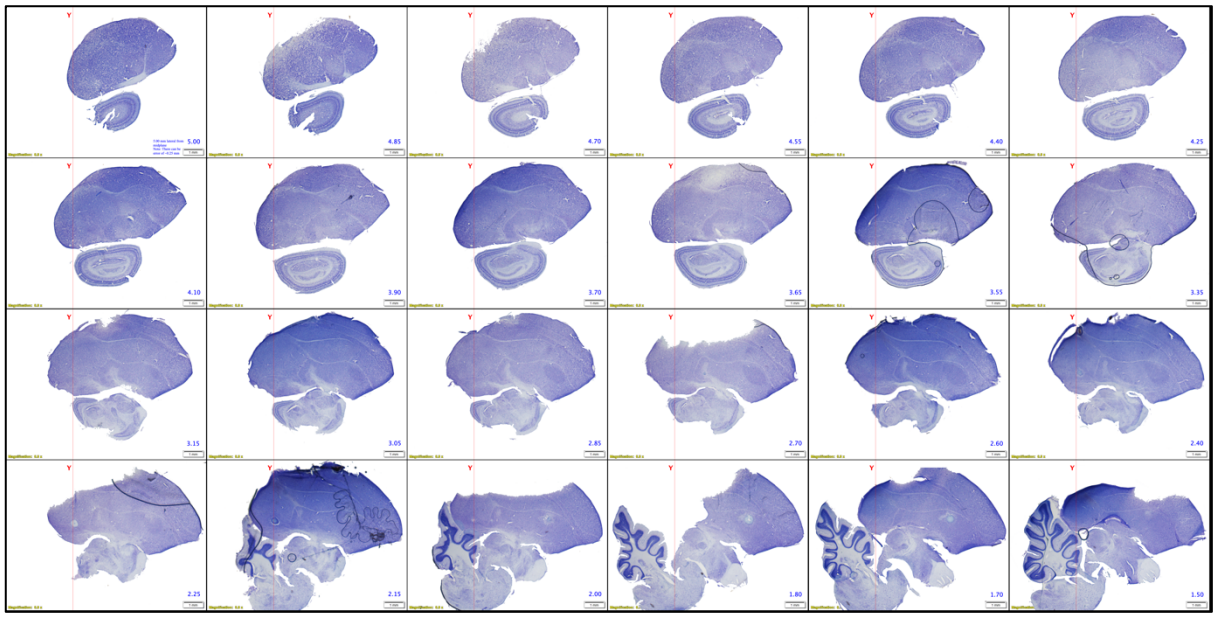

Figure 14: **Left hemisphere brain sections** (Bird name: Green190White153) aligned from the lateral-most section to the medial-most section. In each of the sections, a vertical red line shows the approximate A/P coordinate of Y, and each section has the approximate M/L coordinate written on the right-bottom corner in blue font.

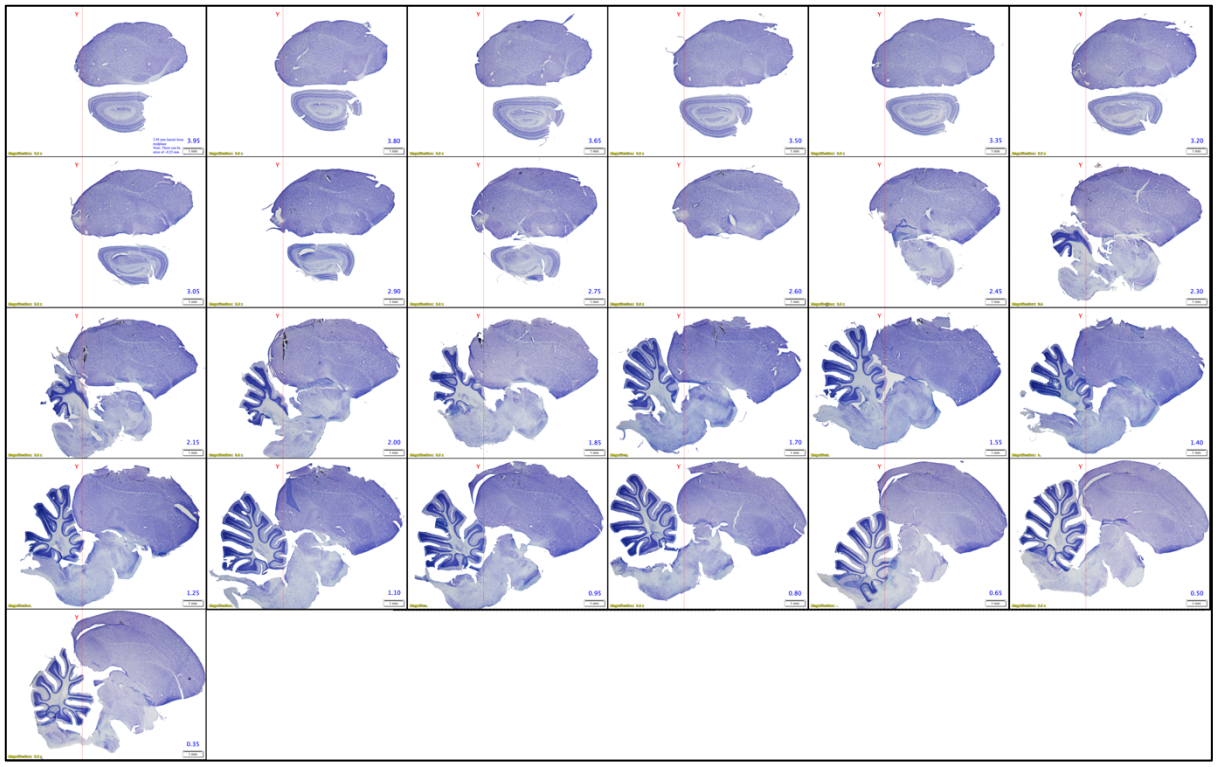

Figure 15: **Right hemisphere brain sections** (Bird name: Green190White153) aligned from the lateral-most section to the medial-most section. In each of the sections, a vertical red line shows the approximate A/P coordinate of Y, and each section has the approximate M/L coordinate written on the right-bottom corner in blue font.

3.4 HVC Activity in Awake Singing Birds

The premotor nucleus HVC majorly consists of three types of neurons. Previous studies found that the HVC_{RA} neurons were found to be completely inactive in non-singing, awake birds and burst very sparsely during the song, that is, they burst only once during a motif (Hahnloser *et al.*, 2002). The HVC_X neurons also burst sparsely, but they fire about 0-5 times during a motif (Hahnloser *et al.*, 2002). HVC interneurons, on the other hand, are active spontaneously when the bird is not vocalizing and show very high firing rates and bursts during vocalizations (Hahnloser *et al.*, 2002).

Similar to previous studies, when recording from HVC in awake singing birds, an overall increase in neuronal firing was observed during the song. The raw trace aligned to the spectrogram of the song qualitatively showed an increase in firing during the song (Example shown in Fig. 16). The rasters of the neuronal spiking aligned to the motif show the stereotypy in the firing during syllables. An example of the spiking of an HVC interneuron during motif is shown in Fig. 17.

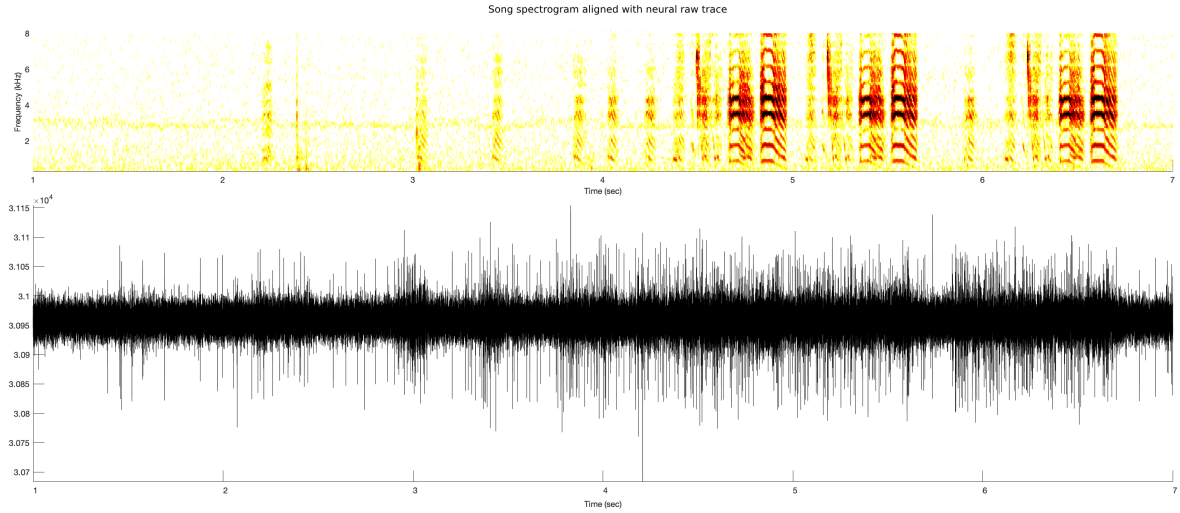

Figure 16: **Song spectrogram aligned with the neural raw trace**. Bird Name: Green039White098. Top: Song spectrogram. The x-axis shows time in seconds and the y-axis represents frequency in Hz. The intensity of color shows the amplitude of the sound. Bottom: Neural raw trace showing the activity of an HVC interneuron. The y-axis shows the Voltage in µV (shifted by 3.095 µV) and x-axis shows time in seconds.

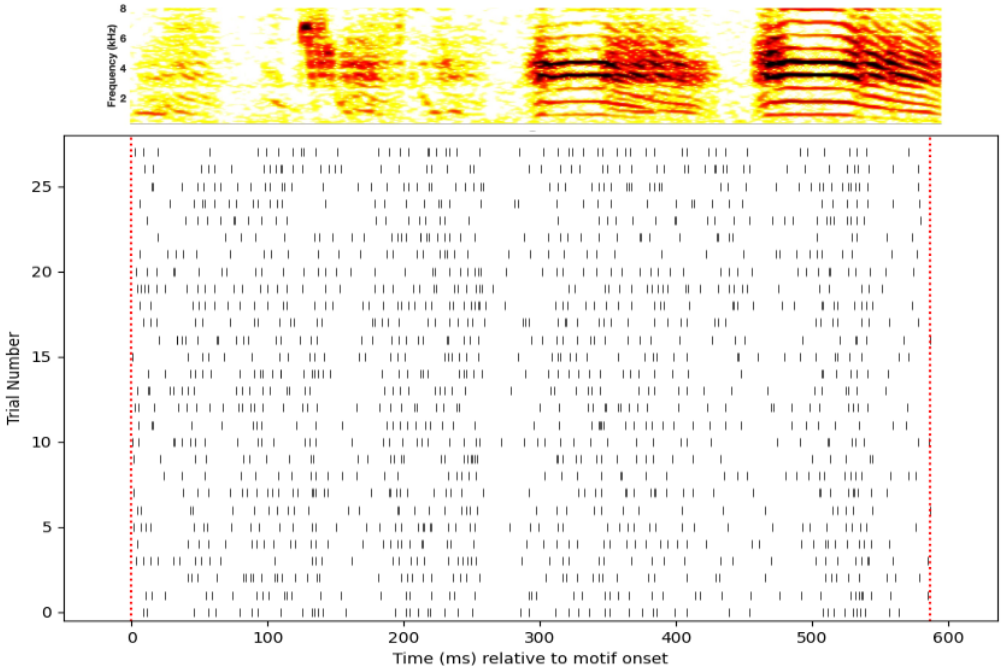

Figure 17: **Neuron raster aligned with the song motif spectrogram.** Bird Name: Green039White098. Top: Spectrogram of the song motif. Bottom: Raster plot showing the spiking of a neuron, with x-axis showing time in milliseconds with respect to motif onset, and each row on the y-axis being a trial. Each tick represents a spike. The dotted red lines show the start and the end of the motif.

Due to various reasons, such as electrodes not reaching the right coordinates, the recordings having a low signal to noise ratio, the bird's vocalizations coming on the neural channels, and

the birds not singing, I could only record one HVC interneuron from my birds when singing. I analyzed a subset of the HVC data recorded at UCSF, that was used in previous publications (Rajan and Doupe, 2013; Woolley *et al.*, 2014; Raghav Rajan, 2018). Out of the neurons that I analyzed, 9 were classified as HVC interneurons, 13 were HVC_X neurons that showed decrease, and 1 that showed increase in pre-bout activity. I examined the activity of these neurons during directed song and compared this with activity during undirected song. The Fig. 18 shows an example of the raster and PSTH of the pre-bout activity from an HVC interneuron.

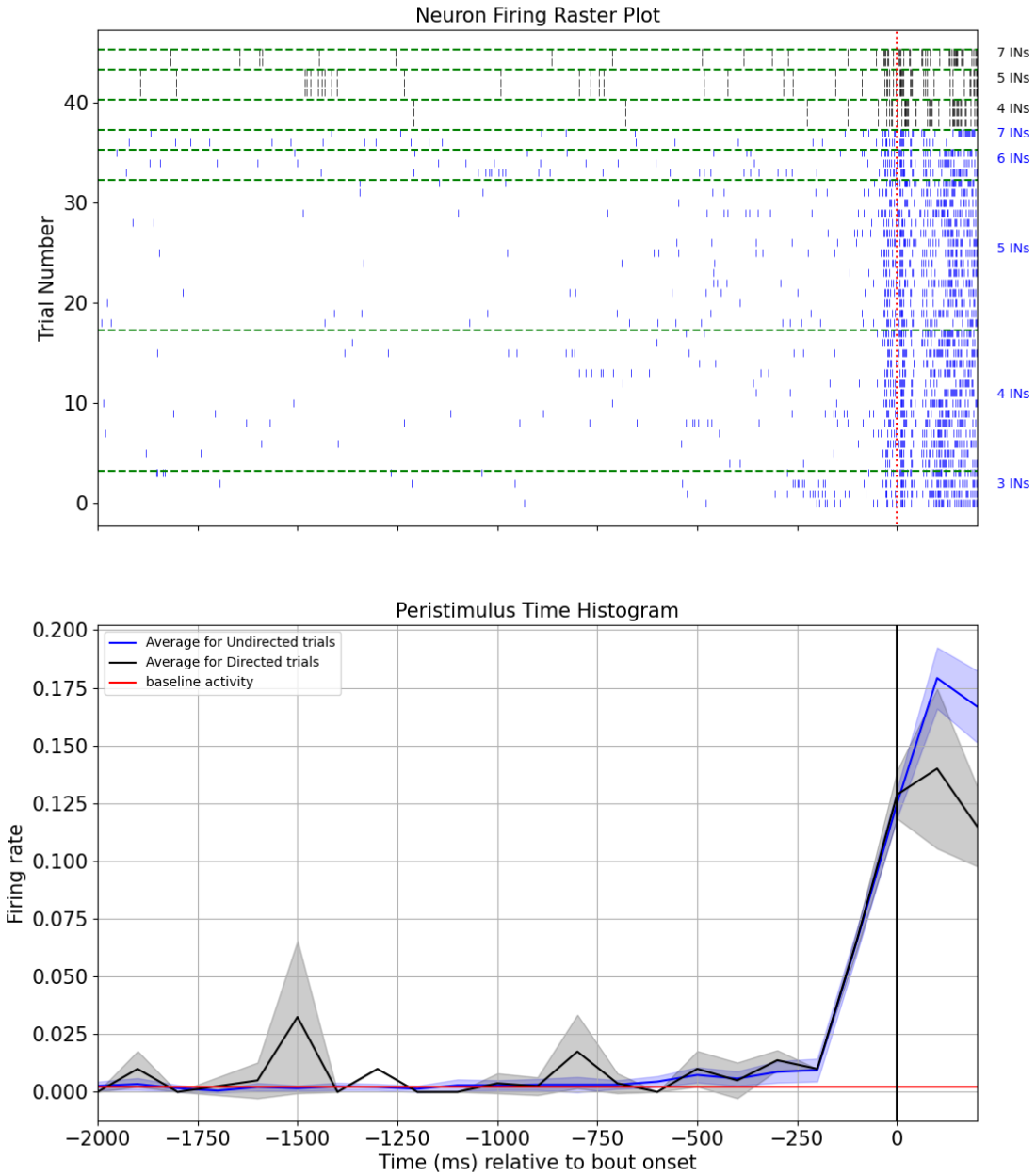

Figure 18: **HVC interneuron pre-bout activity.** Top: Raster plot, with blue ticks corresponding to undirected trials and black ticks corresponding to directed trials. The right-side y-axis shows the number of INs for the group of trials within the horizontal green dotted lines. Bottom: PSTH for the neuron, with the y-axis representing the firing rate across trials. Blue: Undirected trials. Black: Directed trials. Red: Baseline activity. Shaded region: 95% confidence intervals.

3.5 Context-Dependent Pre-Bout Activity Differences

Zebra finches sing two kinds of songs, that is, undirected and directed songs. Undirected songs are mostly sung when the bird is in isolation or surrounded by conspecific males. Directed songs, on the other hand, are directed towards a female bird, and the male birds change the amount of variability and tempo when singing to a female bird (Sossinka and Böhner, 1980; Kao *et al.*, 2005; Cooper and Goller, 2006).

Previous research has already shown that there is pre-bout activity observed in HVC before undirected song bouts (Raghav Rajan, 2018), that is, when the bird is singing when it is alone. Here, I compared the pre-song bout activity between directed and undirected songs. Extracellular recordings from single units in HVC were obtained from 34 neurons. For the analyses, a neuron was included only if it had three or more bouts of both directed and undirected songs. This included 4/9 HVC interneurons and 1/13 HVC_X neuron that had decrease in pre-bout activity.

To quantify the differences, I plotted the average pre-bout activity from -600 to -100 ms with respect to bout onset for directed songs against that for undirected songs (Fig. 19). There were five such neurons recorded, and there was no significant difference in firing rate between the directed and undirected cases (Fig. 19, $p=0.8125$, Wilcoxon signed-rank test). However, more neurons need to be recorded to conclude this.

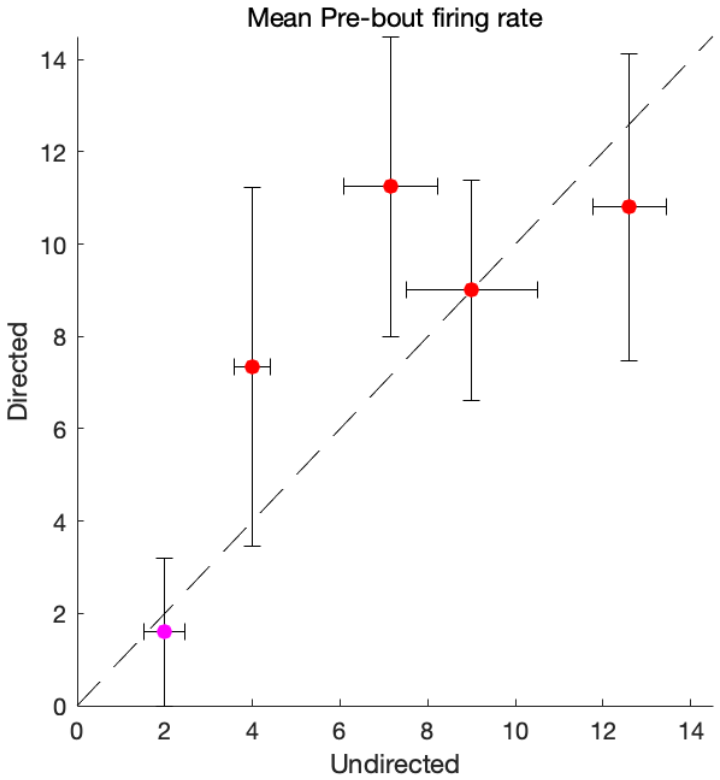

Figure 19: **Comparison of mean pre-bout firing rate between directed and undirected bouts for each neuron**. Each point represents the mean pre-bout firing rate for a neuron in Hz. Red: Interneurons. Magenta: HVC_X (decreasing) neuron. Error bars show the SE.

3.6 HVC Pre-Bout Activity during Directed Singing

Previous literature has shown different types of changes in the pre-bout activity for undirected songs: 1) interneurons showed an increase in activity, 2) some HVC_X neurons showed a decrease in activity, and 3) some HVC_X neurons showed an increase in activity (Raghav Rajan, 2018). My findings in the previous section suggests no significant differences in the pre-bout firing in HVC neurons depending on the context. Hence, I individually plotted the average neural activity for the different types of HVC neurons over 100 ms bins.

I plotted the average neural activity for different neurons over 100 ms bins for HVC interneurons (n=4 neurons) before directed and undirected song bouts (Fig. 20; Blue: Directed song; Red: Undirected song). HVC interneurons were found to show an increase in pre-bout firing before directed songs, similar to undirected songs.

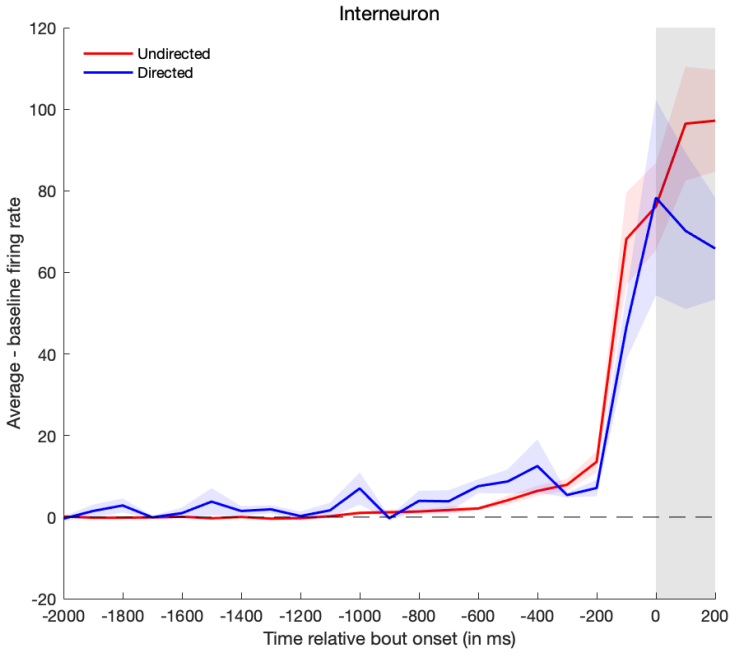

Figure 20: **Average baseline subtracted firing rate for HVC interneurons** (in Hz). Red: Undirected song bouts. Blue: Directed song bouts. Shaded regions: SEM. The x-axis shows the time in milliseconds with respect to the song-bout onset.

Similarly, I plotted the average neuronal firing for HVC_X neurons for undirected and directed songs. However, only one HVC_X neuron was recorded during directed songs, and it showed a decrease hundreds of milliseconds before bout onset. So, the average firing of that neuron before directed songs was plotted with the average firing of different HVC_X (decreasing) neurons before undirected songs (Fig. 21). In the case of directed song bouts, there is a decrease in pre-bout activity like that observed before undirected song bouts.

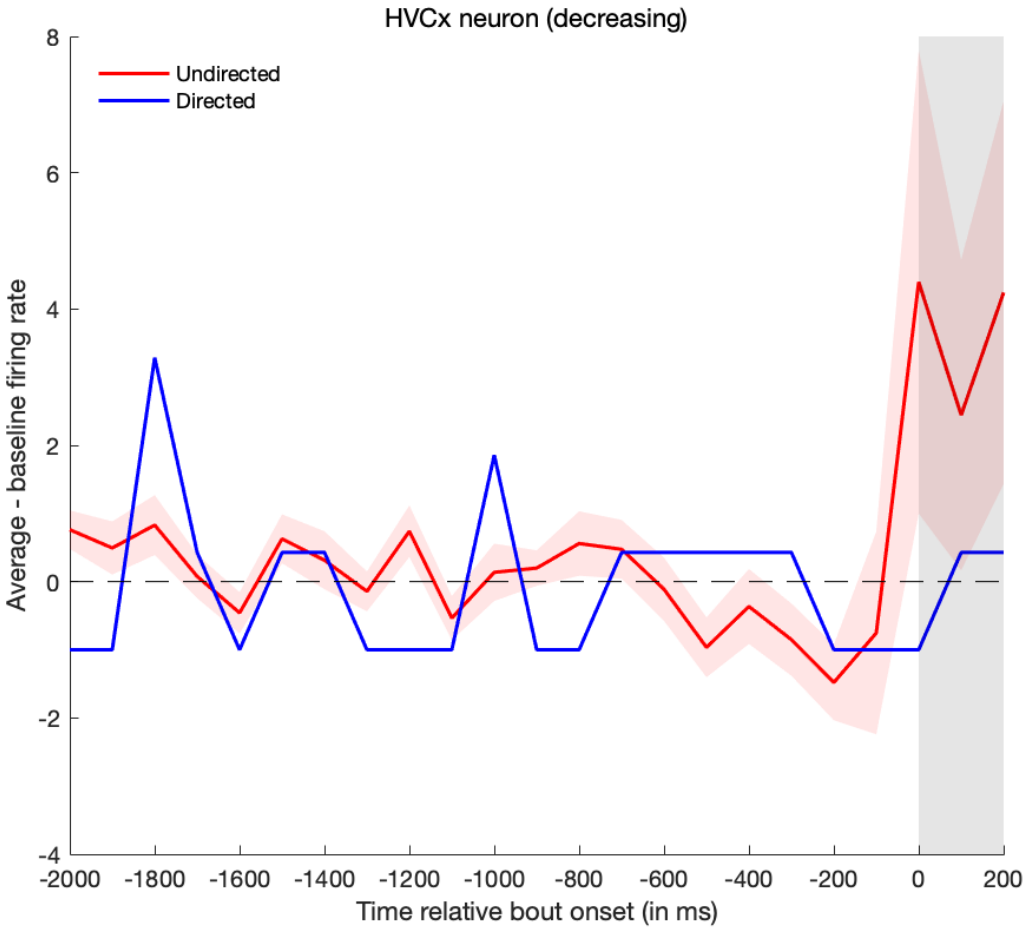

Figure 21: **Average baseline subtracted firing rate for HVC_X (decreasing) neurons** (in Hz). Red: Undirected song bouts; shaded region: SEM. Blue: Directed song bouts; Baseline subtracted firing rate for one neuron.

3.7 Latency of change in firing in different contexts

Previous studies from basal-ganglia cortical circuitry in primates have shown differences in the latency of change in firing, i.e., when firing rates change prior to movement onset, based on whether the movement was self-timed or cue-dependent (Lee and Assad, 2003). The undirected and directed songs sung by male zebra finches could also be vaguely compared to self-timed and cue-dependent movements.

The latency of change in pre-bout firing rate was calculated and plotted for 5/34 neurons before undirected and directed songs (Fig. 22). In the plot, 4/5 neurons showed differences between directed and undirected songs, and in all 4 cases, the firing rates changed later in directed songs.

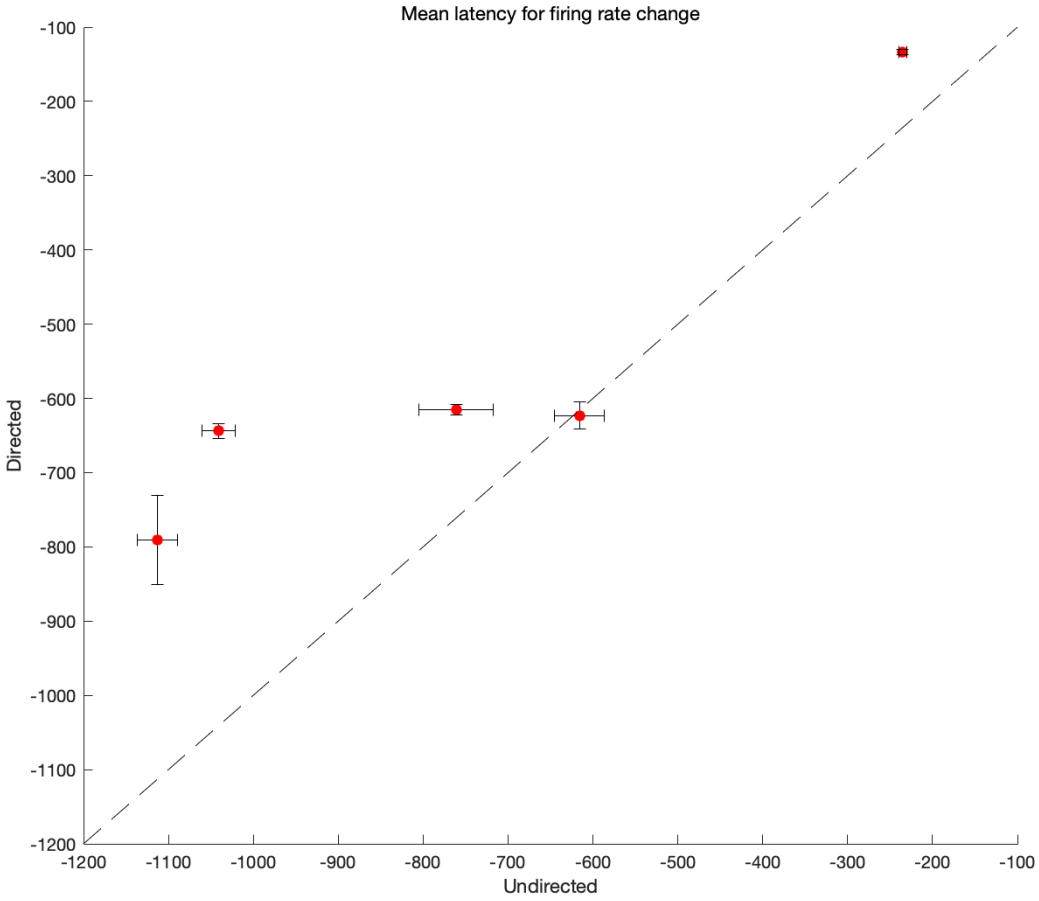

Figure 22: **Comparison of mean latency of change in firing rate for HVC neurons between directed and undirected song bouts.** Each point represents the mean latency for a neuron, and the error bars show the SE. The x and y-axes represent time relative to song-bout onset in milliseconds.

Fig. 23 shows the latency of change in firing for all neurons, even for neurons that had only directed trials or only undirected trials (Fig. 23; Red: Undirected songs; Blue: Directed songs). Similar to the observation made from Fig. 22, 4/5 neurons that had both undirected and directed trials showed a change in pre-bout firing rate earlier for undirected bouts in comparison to the corresponding directed bouts.

Moreover, previous work showed that in case of undirected songs, the HVC_X (increasing) neurons changed their activity first, followed by HVC interneurons, after which the HVC_X (decreasing) neurons changed their pre-bout activity (Raghav Rajan, 2018). Consistent with the result for undirected songs, HVC interneurons were found to change their firing before HVC_X (decreasing) neurons before directed songs as well (Fig. 23).

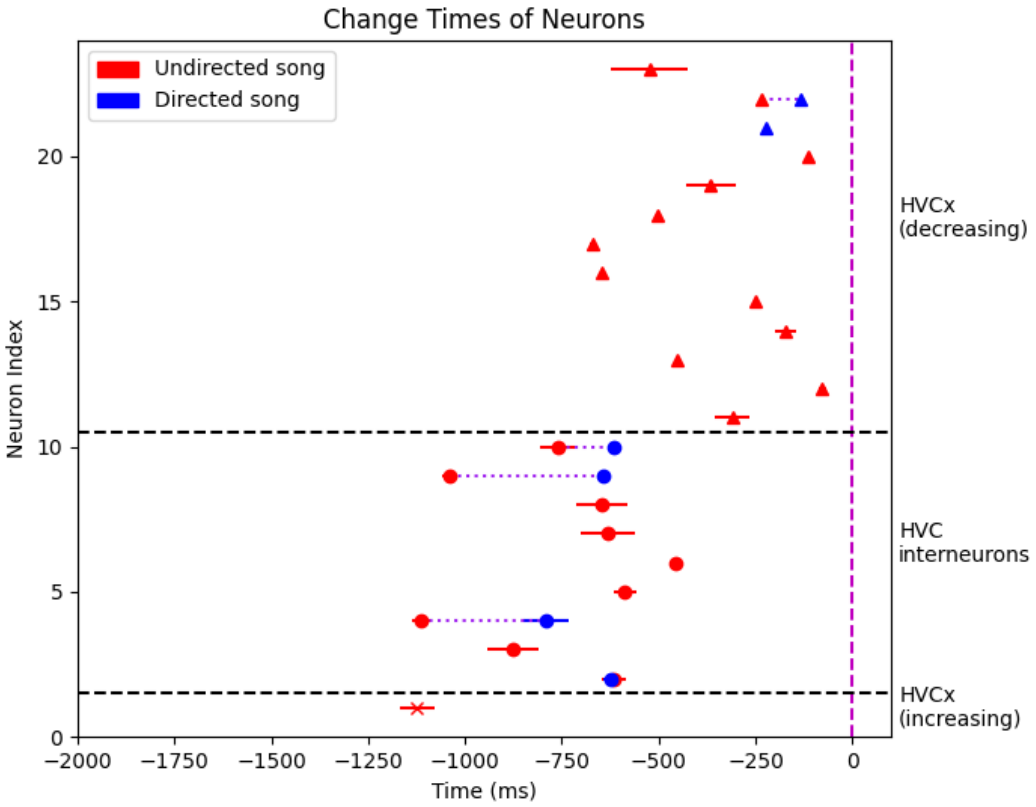

Figure 23: **Neuron-wise latency of change in pre-bout firing rate**. The x-axis represents time in milliseconds with respect to the song-bout onset. Each row represents a neuron, and neuron indices are arranged in an order starting with HVC_X (increasing), followed by HVC interneurons, and then HVC_X (decreasing) neurons. Red: Undirected trials; Blue: Directed trials. Horizontal black dotted line separates the different types of HVC neurons. X marker: HVC_X (increasing) neuron; Circle marker: HVC interneuron; Triangle marker: HVC_X (decreasing) neuron. Purple dotted line joins the directed and undirected points for the same neuron. Whiskers on each marker represents the SEM (Refer Materials and Methods section 2.9.2.4).

Discussion

The accurate execution of any motor movement is suggested to be preceded by appropriate preparatory activity at the neuronal level hundreds of milliseconds before the movement onset (Svoboda and Li, 2018; Churchland and Shenoy, 2024). Previous studies have shown that the song of a zebra finch (a natural motor movement) was found to be preceded by a change in neural firing in the song premotor nucleus HVC hundreds of milliseconds before the bout onset (Raghav Rajan, 2018). This pre-bout firing was suggested to have a preparatory role before song onset (Raghav Rajan, 2018). Zebra finches sing two kinds of songs, 1) internally generated undirected song, which is sung by the male bird in isolation, and 2) externally triggered directed song, sung by the male bird to a female bird. In this thesis, I examined the context-dependent differences in properties of the pre-bout activity. To achieve this, I designed the parts of the microdrive implant, and assembled them to make the implant. To assist the process of obtaining the HVC coordinates, I constructed a custom-stereotaxic zebra finch brain atlas appropriate for the stereotaxic setup in the laboratory and standardized the lesion parameters. Besides, neural recording was done in the anesthetized birds to obtain an approximate range of HVC coordinates, that could be used during the implantation surgeries.
Subsequently, I investigated the presence of context-dependent differences in properties of the pre-bout activity, such as the pre-bout firing rate and the latency of change in the pre-bout firing rate. There was no difference in the average pre-bout firing rate of HVC interneurons (n=4 neurons) and HVC_X (decreasing) neurons (n=1 neurons). However, since the number of HVC neurons recorded during both undirected and directed trials is low, it is difficult to conclude this statistically. Further, I looked at differences in the time of change in the pre-bout firing, depending on the context. I found that the change in pre-bout firing before directed songs occurred later relative to the change in firing before undirected song bouts. However, since the number of HVC neurons recorded during both directed and undirected trials was low (n=5 neurons), my results are preliminary.

4.1 Complexities in Recording HVC Activity

There were many caveats that prevented me from obtaining HVC activity in awake singing birds. Since the coordinates mentioned for the brain region in the stereotaxic atlas of the zebra finch brain (Nixdorf-Bergweiler *et al.*, 2007)

(https://www.ncbi.nlm.nih.gov/books/NBK2348/) were not similar to the coordinates of HVC with our stereotaxic setup in the laboratory, it took a significant amount of time to obtain the coordinates of HVC with our setup.

Once HVC coordinates were obtained from the various anesthetized recordings, HVC recordings were obtained in 1/4 (i.e., 25%) implanted birds in comparison to 1/18 (i.e., 5.6%) birds before the anesthetized recordings. The other caveats in getting HVC recordings after this was with respect to the microdrive implantation procedure itself. The microdrive holder (3D printed) attached to the manipulator on the stereotax was not sturdy, and thus led to the movement of the implant during the surgery, leading to errors in the coordinates where the microdrive was implanted. So as to solve this issue, subsequently, an aluminium holder was made. Following this, in 1/1 (i.e., 100%) implanted birds, HVC-like activity was obtained.
A different problem throughout the period was that the birds would sing very little with the implants on (Refer Table 4; 3/22 birds did not sing at all; most other birds sang very few bouts). Another issue was that the 3D printed microdrive shuttles stopped moving after a while because the threading for the screw went bad. This caveat did not allow long-term recordings from the same bird. To resolve this issue, new aluminum shuttles were made, and this would be used for future experiments in the lab. Therefore, the number of HVC neurons recorded was very low. One of my objectives was also to characterize the pre-bout activity of HVC_{RA} neurons. Since it took a lot of time to obtain HVC activity in awake singing birds, I could not perform the surgeries to record HVC_{RA} activity by antidromically stimulating at RA.
The following table gives information regarding the birds used in this experiment.

Table 4: Details regarding birds used for implantation surgery and recording.

Sl. No.	Bird Name	Neural recording details	Other comments
1	Orange71Orange72	No activity	
2	Black157Black159	Bird died	
3	Black106Blue72	Incorrect implant orientation	Cannot use the bird
4	Blue64	No activity	
5	Blue62	Bird died after surgery	
6	Black84Blue55	Recorded activity	No HVC-like activity

7	Red011Red091	Recorded activity	No HVC-like activity
8	Pink069Orange98	Bird died during surgery	
9	Green100Green101	Bird died after surgery	
10	Green113White116	Bird didn't sing after surgery	No HVC-like activity
11	Green115White118	No activity	
12	Red122Black194	Recorded activity during singing	No HVC-like activity
13	Green111White114	Recorded activity	No HVC-like activity
14	Green173White183	Recorded activity related to movement done in front of the bird	Not HVC-like activity
15	Green039White096	Recorded activity during singing	HVC like activity
16	Green153White161	Recorded activity during singing	Vocalizations came on neural channels. Cannot use this bird
17	Yellow108Pink161	Recorded activity	Song was damaged after surgery, no clear motifs or INs.
18	Green035White024	Couldn't record activity	Vocalizations on neural channels
19	Green175White185	Recorded activity during singing	No stereotyped singing-related activity
20	Green112White115	Recorded activity	No HVC-like activity seen. Bird didn't sing for most trials. Later, microdrive shuttle stopped moving
21	Brown133Brown134	Recorded activity during singing	Vocalizations came on the neural channels.
22	Blue190Pink121	Recorded activity during singing	Multiunit HVC like activity

4.2 HVC Pre-Bout Activity and Motor Preparation

In this thesis, I observed the time of change in pre-bout firing rate to be occurring slightly later in the case of directed songs relative to undirected. This result seems to be suggesting a lesser amount of motor preparation before directed songs in comparison to undirected songs. I have mentioned two different interpretations below that could explain this result.

Prior studies have hypothesized that INs might have a preparatory role before the start of the song and it is known that the male zebra finches sing a higher number of INs when directing the song towards a female bird than when in isolation (Sossinka and Böhner, 1980; Rajan and Doupe, 2013). It was also hypothesized that the HVC pre-bout activity before the song might also be motor preparation (Raghav Rajan, 2018). Previous literature suggests that the pre-bout activity might form a continuum with the IN-related activity, and my data also supports this (Raghav Rajan, 2018; Rao *et al.*, 2019). That means, if the preparation starts earlier, a more ready state would be reached and would require a lesser amount of preparation to reach the final state required for starting the motor activity, and vice versa. This could be one of the explanations for my result that the pre-bout firing rate changes later in case of directed bouts. Additionally, to strengthen this hypothesis, one would have to investigate whether there is a difference in the latency of change in pre-bout firing correlated with the variation in the IN number among undirected bouts (or among directed bouts).

Conversely, my results could also be explained as the changes occurring due to differences in state between the two contexts. Earlier research on another region in the zebra finch brain, LMAN had shown context-dependent changes in firing. They found syllable-locked, single spikes during directed songs, while the same neurons showed bursty firing with variability over trials during undirected songs (Kao *et al.*, 2008). In addition to this, they also found pre-bout activity before directed songs in LMAN, but no pre-bout activity before undirected songs (Kao *et al.*, 2008). Moreover, the differences in the latency of change in firing before directed and undirected songs is also similar to that before cued and self-timed movements seen in monkey basal-ganglia cortical circuitry (Lee and Assad, 2003). The study in the case of monkeys observed that the peak firing rate before the movement for cued and self-timed movements. Similarly, irrespective of the context in which the song is produced, I found the average pre-bout firing rates for both directed and undirected songs to be similar. Further, to initiate movement, the neurons might need to cross a certain threshold of firing. In the case of externally triggered movements, such as the directed song of the zebra finch, the neurons might

be abruptly reaching that threshold, in comparison to internally generated movements like the undirected song, where the neurons gradually reach that threshold.

4.3 Mechanism of Pre-Bout Activity Generation

My findings regarding the context-dependent differences in the latency of change in pre-bout firing rate raises the question regarding the possible mechanisms that could be causing the differences between the two contexts. It is possible that the differences arise due to external inputs (maybe neuromodulator or neurotransmitter) to HVC during the two contexts, or it could arise within HVC itself.

It is known that when all the dopamine inputs to HVC are removed, by lesioning the dopamine terminals in HVC using 6-OHDA, the birds tend to sing only a stretch of INs instead of the directed song (Ben-Tov *et al.*, 2023). A midbrain region called A11, which is one of the regions that gives dopaminergic inputs to the HVC, was found to send signals to the HVC pertaining to directed songs (Appeltants *et al.*, 2000; Ben-Tov *et al.*, 2023). A11 lesions caused the abolition of directed songs, but the undirected song recovered 5-10 days after the lesion (Ben-Tov *et al.*, 2023). A different study also showed that there was increase in the VTA activity (another dopaminergic input to HVC) about 10 to 150 ms before the undirected song onset in juvenile male zebra finches (Yanagihara *et al.*, 2021). The results from these studies suggest that the HVC might be receiving dopaminergic inputs from different sources during the different contexts. It is possible that the dopamine levels in the HVC might be higher during directed songs. Additionally, it is possible that higher dopamine levels might lead to a higher or a lower neuronal excitability in the different HVC neuron types. If these speculations are true, then it could explain the later increase of the pre-bout activity before directed songs, because during the directed song the HVC neurons would get excited or inhibited faster.

The above-mentioned possibilities regarding the effect of dopamine on the HVC could be tested by injecting dopamine agonists and antagonists in the HVC, and observing the variation it causes to the reaction time. Here, reaction time would refer to the time between the presentation of a female bird and the onset of the directed song.

4.4 Function of Pre-Bout Activity before Directed Song

My current results, along with earlier studies, can only suggest that pre-bout activity seen in the HVC might have a role in motor preparation. In order to prove that this change in firing before the song is indeed motor preparation, experiments where the pre-bout activity is

interrupted, maybe through electrical stimulation experiments, would be required. In primates and rodents, the neural population is thought to reach an optimal state or a subspace, wherein it can enable the start of a movement (Shenoy *et al.*, 2011). An interruption of this preparatory activity has been shown to cause a delay in the movement onset, as it would take time to reach back to the optimal state to begin the movement (Shenoy *et al.*, 2011; Li *et al.*, 2015).

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