Neural Activity During Introductory Vocalisations in Zebra Finches

A Thesis submitted to Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

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INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled **Neural Activity During Introductory Vocalisations in Zebra Finches** 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 **Abir Dutta** at **Indian Institute of Science Education and Research, Pune** under the supervision of **Dr. Raghav Rajan, Assistant Professor, Department of Biology**, during the academic year 2023-2024.

Dr. Raghav Rajan

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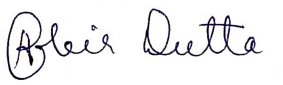
Committee:

Name of your Guide: Dr Raghav Rajan

Name of Your TAC: Dr Collins Assisi

DECLARATION

I hereby declare that the matter embodied in the report entitled “ Neural Activity During Introductory Vocalisations in Zebra Finches ” 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 acknowledgement of collaborative research and discussions.



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ABSTRACT

Communication, especially vocal communication, is essential in the animal kingdom. It consists of the ordering of acoustic elements into a meaningful signal. These rules are collectively referred to as vocal syntax. An important aspect of vocal communication in the songbird Zebra Finch is the song they sing in different social contexts. These songbirds sing a variable number of Introductory Notes (INs) at the beginning of the song. The syntax (and the underlying neural mechanisms) for IN vocalisation, especially in different social contexts, remains poorly understood. HVC is a premotor nucleus in songbirds, and it has been found to play a role in encoding the syntax in other species of songbirds. To examine whether HVC activity encoded information about INs in different social contexts, male zebra finches were implanted with tungsten electrodes to record singing-related activity from HVC neurons. Additionally, previously recorded neurons during directed song (song directed at a female) and undirected song (song sung in social isolation) were analysed to see if these neurons encoded information about social context or if the neural circuitry for these two kinds of song differed in HVC. My analysis revealed 2 out of 11 neurons in which there were differences in activity for directed and undirected songs. My results suggest a small number of HVC neurons might encode differences in activity for different social contexts, although more neurons need to be recorded to further solidify this result.

ACKNOWLEDGEMENTS

I cannot start my acknowledgements without first thanking my supervisor, Dr. Raghav Rajan, for his constant guidance throughout the duration of my thesis. The one-on-one meetings and random discussions we had on our way to lunch played a huge role in shaping my thesis. He had many useful insights into the work that I did, which eventually made their way into this thesis. Apart from the academic contributions, he has also been a very kind and caring mentor, and he played a significant role in uplifting my spirits when the experiments were tough, and the results were hard to come by.

I would also like to thank my TAC member, Dr. Collins Assisi, for his suggestions and counsel in the few discussions that we had regarding my thesis.

My thesis would be incomplete without thanking Manali, with whom I did most of my experiments with. I was extremely lucky to have such a capable member of the lab as my partner, as I was incompetent at a lot of experimental protocols (especially those which required steady hands). I am extremely grateful to the other members of my lab for helping me at some point or another during my stay in the lab. I would like to extend a special thanks to Dr. Shikha Kalra and Dr. Sonam Chorol for guiding me with my experiments and analysis. Half of my work (or half the work in the lab, more likely) wouldn't have been possible without Shikha helping me with surgery, bugs in my code, or small tweaks in my experimental protocols. Special thanks to Shouvik for improving my mood with silly dad jokes, Anand for letting me annoy him with my stupid jokes whenever I was bored, and Gayatri for playing basketball with me whenever I was stressed about work. I would like to extend my thanks to Prakash Raut for taking care of my birds.

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CONTRIBUTIONS

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

LIST OF ABBREVIATIONS

Abbreviated Name	Full Name
HVC	HVC (proper name)
RA	Robust nucleus of arcopallium
Uva	Nucleus uvaeformis
nXIIts	Hypoglossal motor nucleus
LMAN	Lateral Magnocellular nucleus of the anterior neostriatum
AFP	Anterior Forebrain Pathway
DM	Dorsomedial nucleus of the Intercollicular complex
DLM	Medial portion of dorsolateral thalamus
RAm/PAm	Retroambigualis/Paraambigualis
POMMA	Partially observable Markov model with adaptation
IN	Introductory Notes

Table 1: Different abbreviations used throughout the report

INTRODUCTION

Vocal communication involves using a certain sequence of acoustic elements to convey meaning to a receiver or through a signal. The vocal repertoire of any organism consists of a finite number of acoustic elements, but the potential range of signals can be expanded by using syntactic rules. Syntax refers to the rules used by organisms to organise these acoustic elements into meaningful signals. Human language has two levels of syntactic organisation: the organization of meaningless vocal elements into words (phonology) and the organization of words into meaningful sentences (compositional syntax) (Hockett, 1960; Hurford, 2011; Marler, 1998). Animal communication shares many of these properties of human language and can serve as a good model system for human language. In primates, Putty Nosed Monkeys combine two acoustic elements (which have specific meanings on their own) into different call sequences, which convey different meanings (Arnold & Zuberbühler, 2006) (Figure 1). Japanese great tits combine calls (which are also used separately) to extract a compound meaning from the call combination (Suzuki et al., 2016).

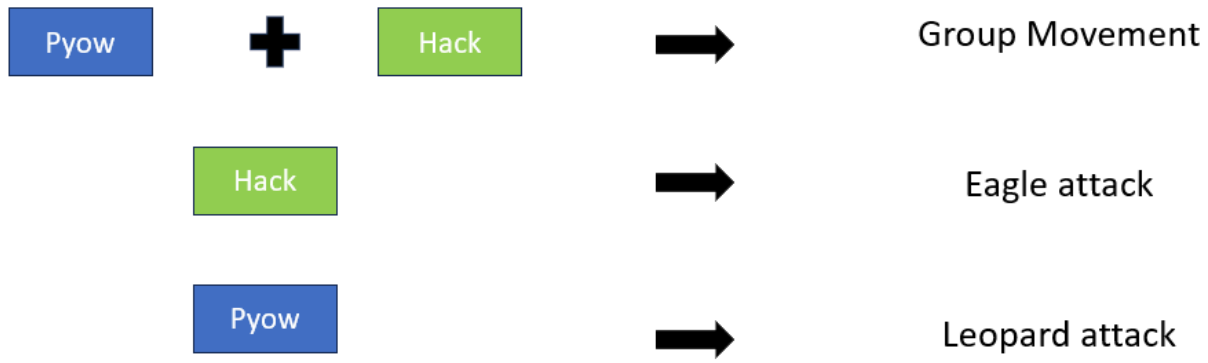


Figure 1: Putty nose monkeys combine vocalisations into different call sequences.

The songs of songbirds are a good example of a communication system in the animal kingdom. Songbirds usually use their songs for courtship displays. These songs are usually made up of a combination of notes and syllables arranged in different sequences. The exact sequence of syllables in the song is decided by the syntax. I am interested in the underlying neural mechanism of the syntax of the songs of a particular songbird, the Zebra Finch.

The songbird song system (Figure 2) is a set of nuclei in the brain of songbirds that are responsible for song production (Nottebohm et al., 1976; Wild, 1997). The song system

has two primary pathways: The anterior forebrain pathway (AFP) and the song motor pathway. The song motor pathway is involved in the vocalisation of the normal song throughout life, while the AFP is responsible for juvenile song learning and adult song plasticity (reviewed in Fee & Scharff, 2010). The premotor nucleus HVC (used as a proper name) is a part of the song motor pathway, and it projects to the robust nucleus of arcopallium (RA) (motor nucleus) (Nottebohm et al., 1976). RA then transmits information to syringeal motor neurons in the brainstem nucleus nXIIts (Vicario, 1991; Wild, 1993). RA also projects to other brainstem and midbrain nuclei, such as DM (Dorsomedial nucleus of the Intercollicular complex) and RAm/PAm (Retroambigualis/Paraambigualis) that control respiratory muscles (Simpson & Vicario, 1990; Gurney, 1981; Nottebohm et al., 1982). The nXIIts nucleus has motor neurons innervating the syringeal muscles (the vocal organ) (Vicario & Nottebohm, 1988). DM is responsible for the production of calls (a type of non-song vocalization used for communication) (Simpson & Vicario, 1990). Midbrain nucleus Uva receives projections from DM and RAm/PAm and projects back to HVC (Nottebohm et al., 1982) (Striedter & Vu, 1998). The AFP is a cortical-basal ganglia-thalamocortical loop, which consists of brain nuclei Area X (basal ganglia homologue) that receives inputs from HVC, a thalamic nucleus DLM (medial portion of dorsolateral thalamus), and a cortical nucleus LMAN (lateral magnocellular nucleus of the anterior nidopallium) (output nucleus of AFP) arranged in a sequence. LMAN projects to RA (reviewed in Fee & Scharff, 2010). The telencephalic nucleus HVC (a premotor area analogous to the mammalian premotor cortex), a part of both pathways, is one of the primary regions in the brain responsible for the production of these songs (Vu et al., 1994) and the nucleus I have focused on during my thesis.

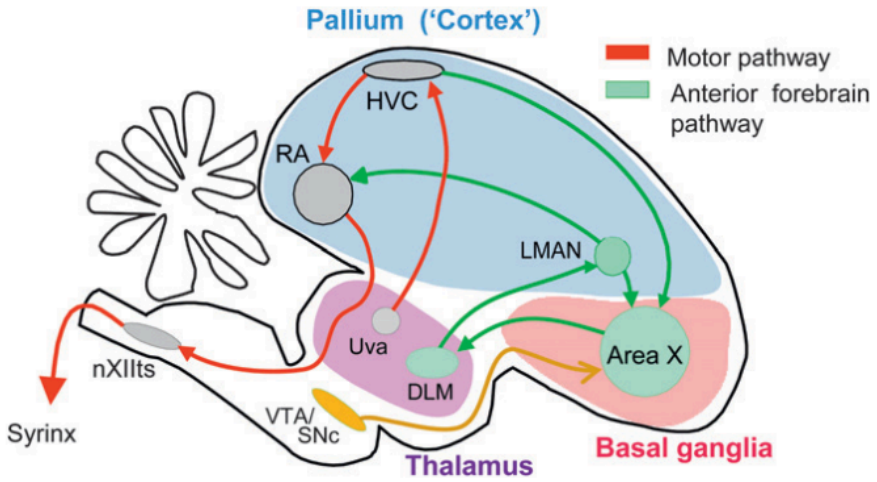


Figure 2: Brain nuclei in songbirds, which are responsible for the control of song (reviewed in Fee & Scharff, 2010)

There are four types of neurons in HVC: Interneurons, RA projecting neurons (HVC_{RA} neurons), Area X projecting neurons (HVC_X neurons) (Mooney and Prather, 2005), and Avalanche (auditory nucleus) projecting neurons (Akutagawa and Konishi, 2010). Both HVC_{RA} and HVC_X neurons form excitatory connections (Glutamatergic) with interneurons, while interneurons form inhibitory connections (GABAergic) to the projection neurons (Mooney and Prather, 2005) (Figure 3). HVC_{Av} projecting neurons are very few in number, and very little is known about the type of connections they have in HVC.

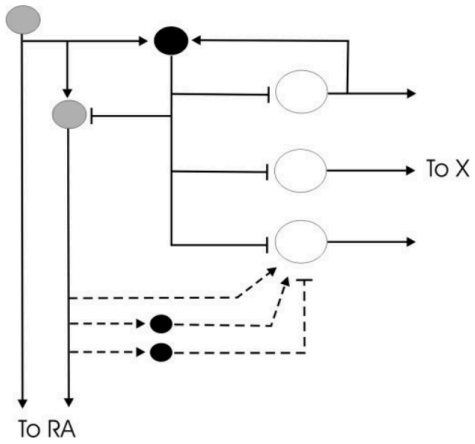


Figure 3: Major features of the HVC microcircuitry (Mooney & Prather, 2005). The grey, black and white circles represent HVC_{RA} neurons, interneurons and HVC_X neurons, respectively. Arrows show excitatory synaptic connections, while t-endings show inhibitory connections. The dashed lines show other polysynaptic (and additionally monosynaptic) excitatory connections and polysynaptic inhibitory connections between HVC_X and HVC_{RA} neurons.

The behaviour of these different kinds of neurons differs during song production (Figure 4). Projection neurons display phasic and sparse firing patterns. HVC_X neurons can burst up to 4 times per motif. HVC_{RA} neurons burst only once per motif, if at all. HVC interneurons display tonic activity patterns and are active during the entire song, reflecting their high connectivity with projection neurons (Kosche et al., 2015; Mooney & Prather, 2005). Bursts of both the projection neurons are extremely stereotyped and time-locked to the song (Hahnloser et al., 2002; Kozhevnikov & Fee, 2007).

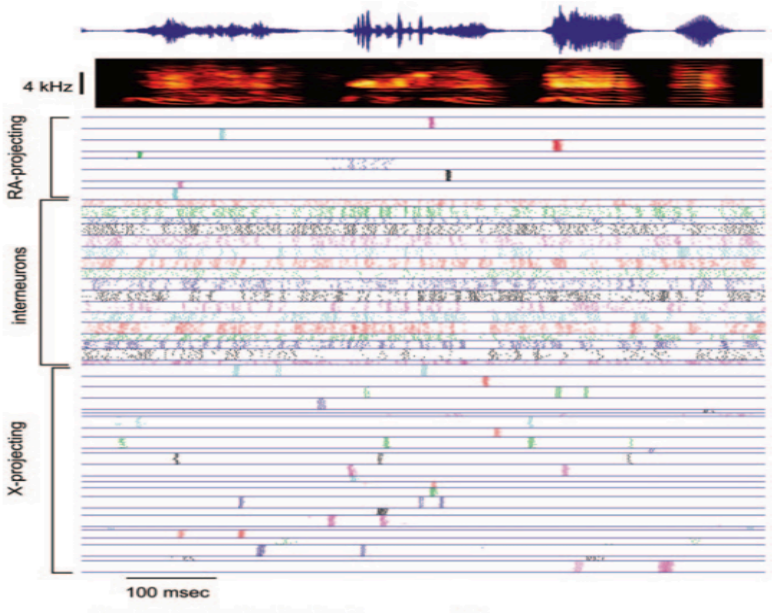


Figure 4: Singing-related activity of different types of HVC neurons (Kozhevnikov & Fee, 2007). The acoustic signal of a song motif, the spectrogram of a song, and the rasters showing the neural activity of 24 HVC_X neurons, 17 interneurons, and 8 HVC_{RA} neurons aligned to the start of the song motif.

Evidence suggests that neuronal sequence generation in HVC during song follows the synaptic chain model (Figure 5). This model suggests that there is a chain of synaptically connected HVC neurons, which burst sequentially as the bird progresses through its song. Thus, this sequential firing chain of neurons can form the basic clock that underlies song timing in zebra finches (Long & Fee, 2008). This model is supported by the activity of HVC_{RA} neurons during singing. These neurons fire extremely sparsely at specific times during every repetition of the song motif, and different neurons fire at different times during the motif (Figure 4) (Kozhevnikov & Fee, 2007). It can be hypothesized that these neurons form a chain-like network structure, which controls the timing of the song. HVC cooling experiments which led to a reduction in song speed, suggest that the dynamics which lead to the sequential activation of these neurons lie in HVC and not in any upstream area (Long & Fee, 2008).
Additional support also comes from intracellular recordings of HVC neurons in singing birds. There was a rapid depolarisation of the membrane potential of HVC neurons just before each burst, consistent with the synaptic chain model. There was no evidence for slow membrane potential modulation suggested by models in which subthreshold dynamics control burst timings (Long et al., 2010).

The aforementioned synaptic chain model talks about how syllables are produced. The mechanism by which the syllables are linked together is unclear, which is where the syntax is important. The syntax allows the individual to order acoustic elements into

multiple possible sequences, and it is not known how the motor system achieves this. To achieve this, the individual must first extract sequence information from auditory information and then establish the syntax to generate motor commands that result in vocalisations. Therefore, to examine the coding of syntax in vocal sequencing, it will be best to start in a brain area that is involved in both sensory processing and the production of vocal signals. The premotor area HVC in songbirds is one such brain nucleus (Nottebohm et al., 1976).

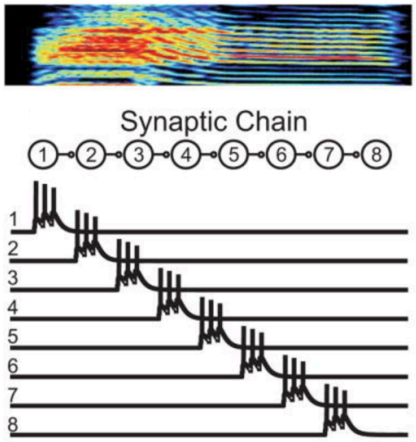


Figure 5: Synaptic chain model for neuronal sequence generation in HVC (Long et al., 2010)

There are various models which hypothesise different ways in which the HVC circuitry controls the song syntax of songbirds. One of them proposes that variable birdsong syllable sequences are generated by branching chain networks in HVC (Jin, 2009). The model says that chains of HVC projection neurons (which code for syllables) are connected in a branching chain network. The end of a syllable chain is connected to the beginning of several other syllable chains at branch points. The selection of the next syllable chain is probabilistic and is the result of a winner-take-all mechanism moderated by noise and inhibition by interneurons.
Another model considers the idea that the transition between syllables is Markovian. However, the first-order Markov model, which says every syllable is linked with one state and state transition probabilities are based on only on the previous transition, could not explain the transition between syllable chains. Instead, a partially observable Markov model with adaptation (POMMA) (a modified version of the Markov model) was shown to be able to explain this (Jin & Kozhevnikov, 2011). In POMMA, many neural states (often called hidden states) can correspond to a single syllable (instead of a single one in the Markov Model). The transition probabilities between states during repeats can also change based on the song sequence prior to the transition. This model suggests the many-to-one mapping of neural populations to syllables of the song.

Experimental data for neural mechanisms of syntax control comes from limited studies on songs of other songbird species. Canaries are songbirds whose songs show long-range syntax rules (Markowitz et al., 2013). Canary songs consist of phrases, which are trilled repetitions of syllables. Phrases are around 1 second long, and the song is a sequence of phrases, typically about 20-40 seconds long. Some particular phrase transitions depend upon the prior order of 2-5 phrases, i.e. the identity of the phrase following a phrase transition depends on the identity of preceding non-adjacent phrases. Thus, there are long-range correlations in the canary song, which can span over multiple seconds. Studies have shown that HVC projection neuron activity keeps track of the identity of preceding 2-5 phrases in a canary song sequence (Cohen et al., 2020). These results provide support for the existence of 'hidden states' in the activity of HVC. These are states in the dynamics of HVC activity that aren't indicated by ongoing behaviour but carry the memory of the history of the song sequence (Jin & Kozhevnikov, 2011). This suggests that different neuronal populations can code for the same syllable (many-to-one mapping of neuronal populations to vocal sequences).

Bengalese finches are another species of songbird. Their songs also consist of a fixed number of syllables. However, the sequence of syllables making up the song is variable. However, the song is still governed by a few syntactical rules, like specific transition probabilities at syllable junctions (Figure 6) (Okanoya, 2004). Fujimoto et al., 2011 checked for neural representation of syntax in the activity of HVC_X. They found that HVC_X neurons have transition-selective and syllable-selective bursts. Bengalese finch songs have repeat sequences with a variable number of syllable repeats in the middle of the song. Some HVC_X neurons were found to code for the start or the end of a repeat sequence. A few HVC_X neurons coded for the progression of a repeat sequence by progressively increasing or decreasing the rate of firing as the bird moved through the syllable repeats. Syllable-specific neurons expectedly fired each time a syllable was repeated.

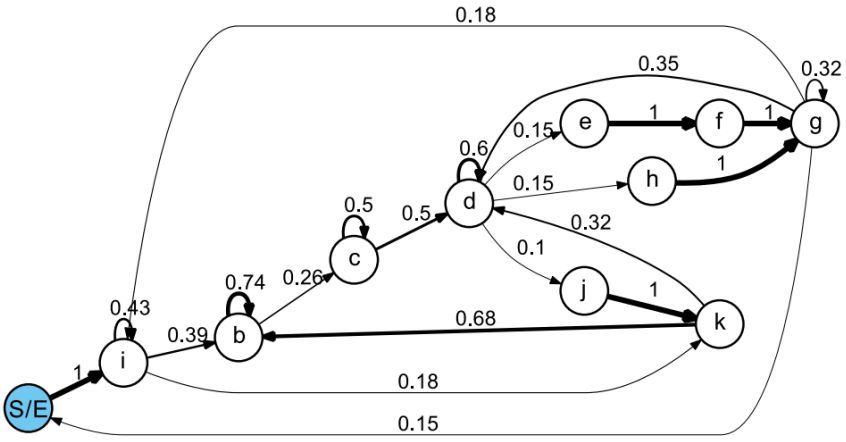


Figure 6: Bengalese finch song syntax as visualized using a transition diagram. The numbers are transition probabilities, and the arrows represent allowed transitions between syllables (Zhang et al., 2017). The thickness of the arrows also represents transition probabilities.

Zhang et al., 2017 further validated HVC's role in encoding for song syntax in Bengalese finches by using a cooling experiment. They reversibly cooled HVC and found that it alters the transition probabilities between syllables. There was also a reduction in the number of repetitions of long-repeated syllables (which was not a simple by-product of the reduction of song tempo because of HVC cooling) and an increase in the randomness of the song sequence as a result of HVC cooling. They also cooled RA, but there was no effect on song syntax. RA is otherwise considered important for controlling the acoustic properties (pitch, amplitude, spectral entropy) of specific syllables in the song (Sober et al., 2008; Yazaki-Sugiyama et al., 2015). A separate study also ruled out any role of AFP in determining the song syntax as they lesioned the lateral magnocellular nucleus of the anterior nidopallium (the main output of AFP) and found that it didn't affect the song syntax (Hampton et al., 2009). Thus, these two results show that changes in song syntax caused by HVC cooling are a result of changes in HVC circuitry and not a result of changes in downstream regions (RA or the AFP).
These results support the model that the neural circuitry of HVC is in control of the probabilistic transitions between syllables in Bengalese finches (Jin, 2009). This model also allows song syntax to be influenced by external inputs from upstream areas like Nif or Uva.

The zebra finch (Taeniopygia guttata) is a species of passerine songbird that is native to Australia (Dunn & Zann, 1996). These finches are easy to maintain in a lab due to them being opportunistic breeders and requiring a short time span (3 months) to reach maturity. The songs of zebra finches are ethologically relevant; their songs are part of their mating rituals. These songs are an example of learned motor sequences in the animal kingdom, as they are learnt from tutors. Zebra finch songs are made up of acoustic elements called syllables. A song usually starts with a number of repeating syllables, called Introductory Notes (INs) (Price, 1979; Sossinka & Böhner, 1980). It is then followed by multiple repetitions of a stereotyped combination of syllables, called a motif (Figure 7). The gap durations between two syllables in a motif are fairly consistent across renditions. The number of motifs in a song rendition is variable in a bird. A song bout is defined as a song rendition with two seconds of silence before and after it (Sossinka & Böhner, 1980).

There are two contexts in which zebra finches sing (Sossinka & Böhner, 1980). Male zebra finches can sing in social isolation, or they can sing in the presence of females to attract mates (Catchpole & Slater, 2003; Kroodsma & Miller, 1996). First, self-initiated

songs that are sung in the absence of any females are termed undirected songs. Songs which are sung in the presence of females are categorized as directed songs. Directed songs usually have a higher number of INs, faster tempo and more number of motifs compared to undirected songs (Sossinka & Böhner, 1980). Song performance is more variable when the bird is singing undirected songs compared to directed songs (Kao et al., 2005).

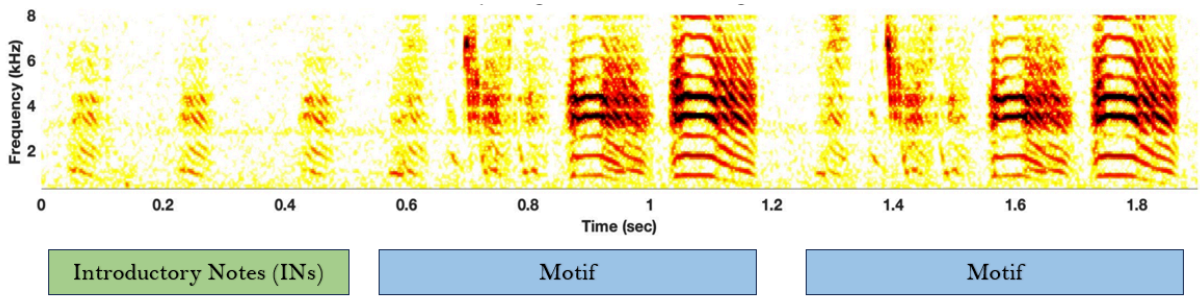


Figure 7: Spectrogram of a zebra finch song. The syllables at the beginning are Introductory Notes (INs), and the repeating set of syllables after the INs is collectively called the motif.

Zebra finches have a stereotyped song, but the number of Introductory Notes at the beginning of the song is variable. I am specifically interested in the syntax which governs the vocalisation of the IN sequence in the zebra finch. I want to study the neural mechanisms that decide the number of INs and the transition from IN to song. A few studies have been done on this topic. The activity of HVC_X neurons during INs has been studied, and it was found that there are some neurons which fire specifically for the last IN, while others fire for all the other INs except the last (Rajan & Doupe, 2013). Studies on interneuron firing during INs have also been conducted. Interneurons weren't specific for different INs based on the magnitude of the firing rate. However, the firing pattern of interneurons was specific to either the first, middle or last INs (Divya Rao, PhD thesis, 2022). Such kind of specificity hasn't been studied for HVC_{RA} neurons. This is partly because it is difficult to isolate HVC_{RA} neurons owing to the absence of spontaneous activity in them (Kozhevnikov & Fee, 2007). HVC_{RA} neurons are also very small in size, so it is difficult to isolate their signals for long or short durations (Benezra et al., 2018). Also, based on an earlier study, a very small proportion of HVC_{RA} neurons actually fire during INs (2 out of 28 recorded cells) (Kozhevnikov & Fee, 2007).

The activity of RA neurons has also been looked at during INs. The timing of spike bursts of RA projection neurons was irregular and imprecise during the vocalisation of INs (Yu & Margoliash, 1996). Multi-unit activity in RA was looked at during IN vocalisation, and it was found that there was consistent activity for all INs (Divya Rao, PhD thesis, 2022). This suggested a lack of representation of IN progression in RA.

Hypotheses

In my project, I want to study the activity of HVC_{RA} neurons during INs and the IN-to-song transition. I consider the activity of HVC_{RA} to be important as it is a part of the song motor pathway directly responsible for the song. Considering that HVC_X and interneuron firing are specific to the IN position, I also expect some IN-specific activity in HVC_{RA} neurons. However, the HVC microcircuitry is very complicated (Figure 3), and the relationship might not be as straightforward.

Specific aims

1. Record activity of HVC_{RA} neurons when the bird is singing INs.

Additional aims

Zebra finches can sing two variations of songs: Undirected and Directed (Sossinka & Böhner, 1980). The directed song of a bird, although almost identical to its undirected song, has a greater number of INs. It is not clear if the same brain circuitry is responsible for directed and undirected songs. A mid-brain cell group A11, which projects to HVC, has been shown to specifically affect directed songs. Lesioning A11 terminals in HVC (or lesioning A11 cell bodies) abolished directed singing, while undirected singing remained unaffected (Ben-Tov et al., 2023). This suggests that atleast part of the brain circuitry active during the directed song is distinct from that of the undirected song. Therefore, another objective I have in my project is to check for the activity of HVC projection neurons and interneurons during the INs of directed songs, as compared to their activity during undirected songs. The previously obtained results for the activity of interneurons and HVC_X neurons during INs (Rajan & Doupe, 2013) were during undirected songs. I want to see if the bird uses the same neurons to track INs during directed songs. Thus, another aim I have is to,

1. Record activity of HVC projection neurons and interneurons when the bird is singing INs in response to a female (directed song).
2. Analyse previously collected data (Rajan and Doupe, 2013) for neurons (interneurons and projection neurons) recorded during both directed and undirected songs and compare their activity.

MATERIALS AND METHODS

All experimental procedures performed were pre-approved by the Institute Animal Ethical Committee, with additional guidelines from the Committee for the Control and Supervision of Experiments on Animals (CCSEA, New Delhi, India). All experimental procedures were done in collaboration with Manali Upadhyaya.

Birds

All the birds used for the experiments were adult male zebra finches (i.e. >90 dph), which were either bred in IISER Pune or bought from local vendors. The birds were kept in custom-made soundboxes under a 14h (light):10h (dark) cycle during the entirety of the experiments. Food and water were provided as much as necessary. The pre-surgery songs of all the birds were recorded.

A total of 22 birds were used for surgery and implantation. The details of the surgery and subsequent recordings are given in Table 2. HVC-like singing-related activity was recorded from 2 birds (either single-unit or multi-unit). In a few of the birds, vocalisations of the bird appeared on the neural channels whenever the bird sang, which made it difficult to detect isolatable neural signals. In one bird vocalizations were small enough for me to record neural activity. One bird didn't sing at all, even after 3-4 weeks post-surgery. There were 7 birds from which neural activity was recorded, but none of the neurons showed singing-related activity. 4 birds died during surgery/immediately after surgery. 3 birds were implanted with a microdrive, but no activity was recorded due to damage to electrodes during surgery.

Sr No.	Bird Name	Recording Details	Comments
1	orange71orange72	No activity	
2	black157black159	Bird died	
3	black106blue72	Implant orientation incorrect	Bird is unusable
4	blue64	No activity	
5	blue62	Bird died after surgery	
6	black84blue55	No 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	
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 during singing	There was movement-related activity but no HVC-like activity
15	green039white096	Recorded activity during singing	HVC-like activity
16	green153white161	Couldn't record activity	Vocalisations were present in the neural channels, so can't use this bird
17	yellow108pink161	Recorded activity	Bird song was damaged after surgery, with no clear INs or motifs
18	green035white024	Couldn't record activity	Vocalisations were present in the neural channels
19	green175white185	Recorded activity	No HVC like activity
20	green112white115	Recorded activity	HVC-like activity was not seen. Bird didn't sing for most trials, so I can't tell if I was in HVC. The shuttle stopped moving after a couple of days
21	brown133brown134	Recorded activity	Vocalisations were present in neural channels.
22	blue190pink121	Recorded activity	HVC-like activity recorded

Table 2: Birds used for surgery

Microdrive construction for extracellular recording

Microdrives were constructed for the purpose of extracellular recordings. The body of the custom-made microdrives and shuttles was designed such that they could hold 2-3 high-impedance tungsten electrodes. The shuttle was connected to a screw with a pitch of 212 µm (000-120 pitch), which was used to move it in the vertical direction. The electrodes were glued to the shuttle using dental cement, and the screw could be used to change the length of the electrode sticking out of the microdrive. Before glueing, the screw head was covered with a lubricant (WD40) to ensure that the screw could turn freely after it was glued to the shuttle. The microdrive allowed the shuttle to move approximately 2 mm in the vertical direction. In our later microdrives, we also started putting a lateral screw to allow the lateral movement of the electrodes. The electrodes were put through polyimide tubes, which were glued to the microdrive. The polyimide tubes were flexible enough to allow lateral movement of the electrodes.

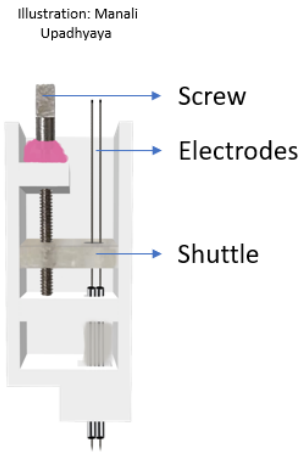


Figure 8: Cartoon of the microdrive, which is implanted on the head of the bird such that the recording electrodes are in HVC.

The electrodes were arranged in a mediolateral direction in the microdrive. The lateral screw was positioned such that the electrodes could be moved in the anterior direction after implantation. After loading the electrodes into the microdrive, the insulating coating (epoxy/glass) at the ends of the electrodes was removed and connected to copper wires. The copper wires were then connected to the pins of a connector. The connector was for plugging in the headstage during recordings. The connector also had one (or two) silver wires connected for grounding purposes.

Single electrodes were prepared for anaesthetised neural recordings. The tungsten electrodes were put inside polyimide tubes, which were then inserted into metal tubes made from 18 gauge needles. The electrode was stripped, and a copper wire

(connected to a connector) was wrapped around its end. The connector also had a silver wire attached to act as the electrical ground.

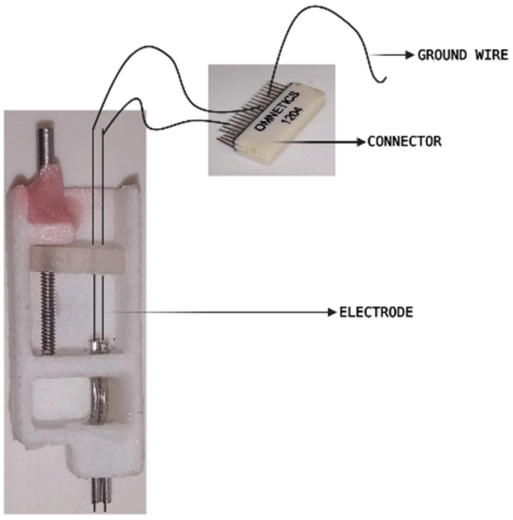


Figure 9: Microdrive with electrodes connected to the connector using copper wires

Surgery and microdrive implantation

Surgery was done on male zebra finches to implant the microdrives at HVC coordinates. An analgesic (meloxicam 0.25 mg/kg) was given orally to the bird atleast one hour before the surgery. The analgesic dosage was calculated based on the bird's weight. Isofluorane was used as gas anaesthesia for the surgery. Initially, 4% isofluorane was used for 5 minutes for induction. After induction, the legs of the bird were tied together with surgery tape and the bird was wrapped in a warm jacket. The ear feathers were removed with a micro scissor. The bird was then loaded onto the stereotaxic apparatus (custom-designed by Narshige), with the head fixed using earbars and a beak holder. The beak angle was fixed at 45° for all the surgeries. Surgery was mostly done under the application of the heat lamp. The feathers on the head were plucked, and local anaesthesia was injected subcutaneously (lignocaine). The skin on the head was then cut using forceps and micro scissors.

I used the bifurcation point of blood vessel Y on the top of the head of the bird as the origin for the stereotaxic coordinates of HVC. The blood vessel is usually visible, but we still opened up a window in the first layer of the skull at that position to get a better view of Y. I also started opening up the first layer near the same blood vessel but more anterior for later surgeries. This was to check if the orientation of the bird on the stereotax was completely straight. A glass pipette was put on the hydraulic micromanipulator (Narshige), which was used to get the coordinates of HVC relative to

Y. I have used a range of coordinates for microdrive implantation at HVC, but I finally fixed the coordinates at 0.1 mm anterior and 2.1 mm lateral. A window in the skull was then opened at the HVC coordinates. The two layers of the skull were removed with the help of a scalpel and fine forceps. The dura was then carefully incised and removed using the tip of an 18 gauge needle. The implant was then mounted on the manipulator, and the lateralmost electrode was aligned to the HVC coordinates. The implant was lowered such that the electrodes were approximately 200 µm inside the surface of the brain. Then, the area around the electrodes and the polyimide tubes was covered with vaseline (to prevent dental cement from touching the electrodes and brain surface). A silver wire was used as electrical ground, which was inserted between the skull and the dura on the contralateral side of the brain. Afterwards, the microdrive, the ground wire and the connector were fixed to the skull using dental cement. To help fix the dental cement, 2-3 insect pins were inserted between the first and the second layer near the HVC window. The windows for these were made at the beginning of the surgery itself. The microdrive was also covered with a transparent sheet to protect it from damage by the bird once it was awake and active. After the end of surgery, the bird was monitored for pain and discomfort until it was fully active and comfortable (eating/drinking).

The bird was allowed to rest for two days before it was plugged in for neural recording.

Stereotaxic coordinate standardisation for HVC

A few surgeries were done for the purpose of standardising the coordinates of HVC before implant surgeries. The start of the surgery was similar to the ones described in the previous section. A large enough window in the skull was made for HVC according to coordinates described in the zebra finch brain atlas (reference). The single electrode prepared for recording neural activity was then mounted in the hydraulic micromanipulator (Narshige). The electrode was lowered at multiple coordinates, and activity was recorded. A range of coordinates was checked in 7 birds (AP [+0.4 to -0.1] and ML [+1.5 to +2.2]). At each coordinate, activity was recorded from 100 µm to approximately 1 mm depth. The activity at each location was checked for its similarity to the characteristic HVC-like bursty activity in anaesthetised birds (reference). After recording, the brain was lesioned at one of the locations used for recording (usually one where we got HVC-like activity). The lesion parameters used were a current magnitude of 30 µA and a duration of 100 seconds. The birds were subsequently perfused, and histological procedures were performed to confirm if the lesions corresponded to the position of HVC.

Song and Neural Recording

During the period when the birds were allowed to rest after surgery (minimum of 2 days post-surgery), the song of the birds was recorded to check their post-surgery song. The

birds were housed in soundproof Sound-Boxes. This was to check if the song of the bird had changed after surgery, as a changed song indicates a damaged HVC. It also indicates when the bird is comfortable enough to sing.

Two days post-surgery, the bird was plugged into the neural recording set-up. The implant was connected to a 16/32 channel headstage. The headstage was connected to a Commutator via flexible cable, which was in turn connected to the Intan Data Acquisition Board. The Commutator was put on the roof of the cage to avoid tangling of the wires and to allow the bird to move freely. This was done even if the bird hadn't begun singing after two days so that the bird would get used to the additional weight of the headstage and cables. Initially, the bird was only plugged in for short periods, which was increased later to cover the entire experiment duration (experiment durations ranged from a couple of days to around 2 weeks). The bird was still being recorded at this point. The neural recordings got started once the bird got comfortable enough to sing undirected songs with the headstage on. In some cases, if the bird took too long to sing undirected songs, females were presented and directed songs were taken as a proxy for the comfort of the bird instead of undirected songs. To record electrical activity and birdsong at the same time, another microphone (Adafruit MAX4466) was directly connected to the Intan Acquisition Board.

Figure 10: Bird connected to the Intan amplifier for neural recording after surgery.

During recording, the amplifier was set at a bandwidth of 500 Hz to 7500 Hz and a sampling rate of 30 kHz. Electrical and audio were set for recording in the 'continuous mode'. Neural recording for both directed and undirected songs was done. In addition to the audio recording in the Intan system, the audio was also recorded via PyCBS (because the quality of the audio recording of the microphone connected to Intan wasn't

up to the mark). Directed recording duration was 2-5 minutes. The lights were switched off, and the female bird was put inside the soundbox and then the lights were switched on. The PyCBS recording was monitored for songs from the male bird. The female bird was removed once the male sang or after 5 minutes of recording. For undirected recordings, the soundbox was empty except for the male, but other males and females were kept just outside the soundbox outside its field of view. This was done to encourage it to sing as it could hear the vocalizations of other birds. Sometimes, an audio recording of colony noise was also played through a speaker for the same purpose. Recording was done at various depths, starting from the implanted position to approximately 1.5 mm below the brain surface. At each location, the neural trace was checked for the singing-related activity that is typical of HVC when the bird is singing. Once singing-related activity is seen, multiple trials were done at that coordinates (atleast 10). If the neural signal was too small to be isolatable, I tried to improve the signal by moving the electrode or by tapping on the screw.

Once the entire depth was checked for HVC-like activity, the electrode was pulled back up, and all the electrodes were moved anteriorly by using the lateral screw. The entire depth at that position was then explored for HVC-like activity. Similarly, multiple electrode penetrations were done for each bird in search of HVC. After the recordings, the brain was lesioned using the same lesion parameters (30 μA for 100 seconds). The bird was then perfused, and the brain was sectioned, stained and imaged using standard histological procedures to visualise the lesion.

Constructing Zebra Finch Brain Atlas

I was using HVC coordinates according to the zebra finch brain atlas (Nixdorf-Bergweiler et al., 2007), but I was not encountering HVC reliably. This might be because the beak angle at which the finch is put on our stereotaxis is different from the one used for the zebra finch brain atlas. This might also happen when the bird isn't put on the stereotaxis properly and is slightly tilted with respect to the orientation of the stereotaxis. To counter this, I carried out a few experiments to build a zebra finch brain atlas for our lab, using lesions at different coordinates.

I also wanted to standardise electrolytic lesion parameters (current magnitude and duration) to optimise the lesion size. This is because the lesion should be big enough to be visualised on the stained sections but smaller than the size of HVC. I also wanted to test a different lesioning technique based on the deposition of tungsten oxide (Oikawa et al., 2023). In this method, a biphasic current is passed through a tungsten electrode in the brain, resulting in the deposition of tungsten oxide at that location due to an

electrochemical reaction. The tungsten oxide deposition can be reliably detected using dark field microscopy.

I used DiI, a lipophilic dye, to mark electrode tracts. A DiI solution with a concentration of 50 mg/ml was made by dissolving 13.7 mg of DiI crystals in 274 uL of ethanol. The crystals of DiI were not fully dissolved even after ~ 20 minutes of shaking. To solve this, the solution was centrifuged, and the liquid at the top was removed and put in another tube (this liquid will be used later for coating the electrode). The dye is light-sensitive, so all of the above was done in darkness.

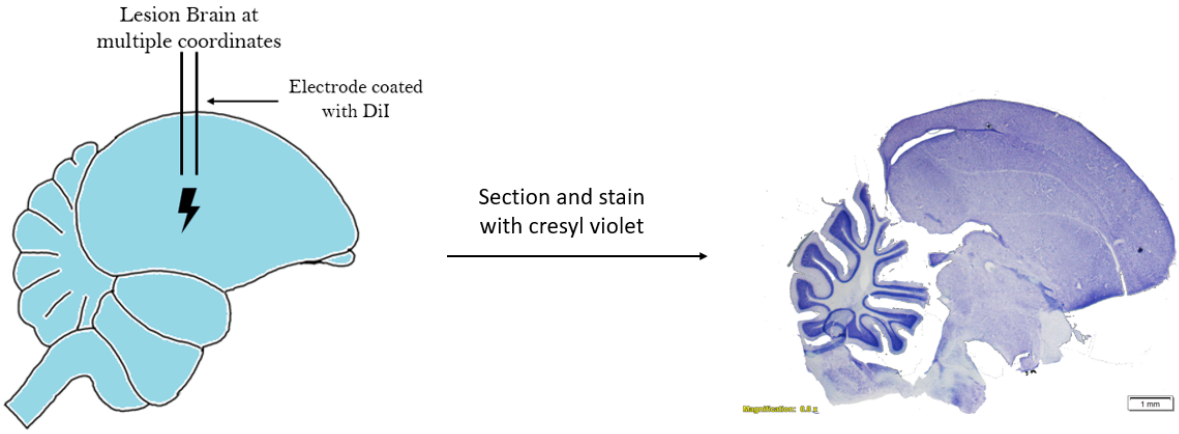


Figure 11: Workflow for the lesioning circuit. The brain is lesioned at multiple places using different combinations of current durations and magnitudes. The brain is sectioned and stained using cresyl violet to visualize the lesions

A surgery was performed with the initial steps similar to the ones used for microdrive implantation. 6 windows were opened on the zebra finch skull, three on each hemisphere. The electrode shank to be used for lesioning was dipped 10 times (with 5-second gaps between consecutive dips) in the DiI solution. This process was performed in the dark. The ground wire in the lesioning circuit was put between the skin and the skull. Lesions were made at two different depths at each location. The details of the lesioning parameters at each location are given in table 3.

Location No.	Coordinate (AP, ML)	Depth	Lesion parameters
1	(0.10, 2.00)	0.7	30 uA for 100 sec
2	(0.10, 2.00)	3.0	30 uA for 100 sec

3	(0.10, -2.00)	0.7	30 uA for 100 sec
4	(0.10, -2.00)	3.0	60 uA for 50 sec
5	(4.40,-1.38)	1.0	60 uA for 50 sec
6	(4.40,-1.38)	3.0	60 uA for 50 sec
7	(4.40, 1.8)	1.0	60 uA for 100 sec
8	(4.40, 1.8)	3.0	60 uA for 100 sec
9	(2.03, 2.00)	1.0	60 uA for 100 sec
10	(2.03, 2.00)	3.0	Biphasic 40 uA current
11	(2.17, -1.6)	3.0	Biphasic 40 uA current
12	(2.17, -1.6)	1.0	Biphasic 40 uA current

Table 3: Details of lesion parameters at different coordinates and depths. The biphasic current had a total of 36000 pulses, with an interpulse interval of 4 ms.

Perfusion and Histology

The birds were euthanised using excess isofluorane. Immediately after death, the feathers at the front of the body (over the abdomen) were removed. A nick was made to expose the abdominal cavity. Scissors were then used to cut open the cavity. A scalpel blade was used to cut the connective tissue around the heart. A tiny nick was made at the right atrium using the scalpel, and a needle was inserted into the left ventricle of the heart. Saline (0.9%w/v NaCl-100 mL) was then pumped into the heart to clear out the blood. The saline was pumped into the left ventricle and was allowed to come out of the right atrium. This was done until clear saline started coming out of the atrium. Immediately afterwards, 100-150 mL of 4% w/v PFA (paraformaldehyde) was injected into the left ventricle to fix the tissue. The fixing of the tissue was checked using the stiffness of the neck. After finishing, the head of the bird was cut using a scissor, and the hair, skin and eye socket were removed using forceps. The skull (after making a hole at the ventral side) was kept in 4% w/v PFA for a day. The skull and the dura were then removed, and the brain was further kept in the same solution for atleast a day. It was kept for a longer period of time if the perfusing wasn't perfect. Finally, the brain was transferred to 30% w/v sucrose solution in PBS (cryopreservative). The brain sinking to the bottom of the solution indicated that it was ready for sectioning.

A cryostat apparatus (Leica Biosystems) was used to section the brains. The brains were sectioned as 40 μm slices. The blade and specimen temperatures were kept at

-20 °C and 12°C, respectively. 5-10 slices are discarded in the beginning. The slices for one hemisphere were kept in two 24-well plates filled with 1x PBS solution. The sections were then mounted on glass slides (the slides were coated with poly-L-lysine to make the sections stick to the slides). The slices were then stained with cresyl violet and imaged under a light microscope.

Staining

Every third section was mounted on glass slides (poly-L-lysine coated). They were left overnight to dry. The slides were then kept for 10 minutes in 1% w/v Cresyl Violet solution (Sigma Aldrich). At the end of 10 minutes, the slides were taken out and dipped three times in MilliQ H_2O. They were then passed through ethanol solutions of varying concentrations (50% ethanol for 2 minutes, followed by 70% ethanol for 2 minutes, followed by 95% ethanol for 1-2 minutes, finally followed by two fast dips in 100% ethanol). When dipping the slides in 95% ethanol, the slides were checked after 30 seconds (and subsequently after every 10 seconds) to see if different nuclei were visible or not (the different brain nuclei were usually visible as dark purple spots). The slides were then placed in xylene for 30 seconds. DePeX was then put on the slides, and the slides were covered with coverslips. The covered slides were then visualised using a brightfield microscope (Microscopy Facility, IISER Pune). The resulting images were analysed using ImageJ.

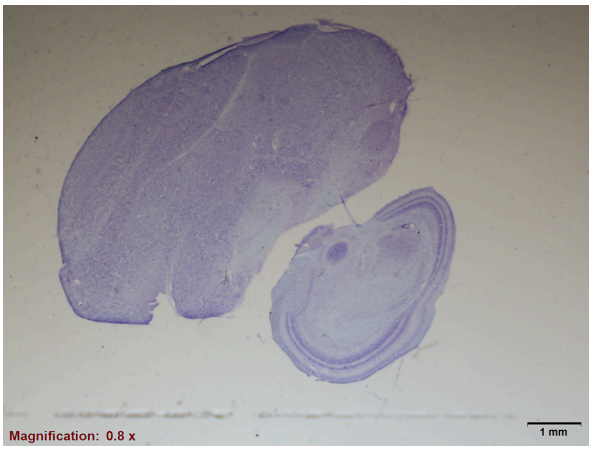


Figure 12: Brain section stained using cresyl violet

Data Analysis

All data analysis was done using custom-written MATLAB/Python scripts.

Spike Sorting

To separate single-unit activity from noise, a program called KlustaKwik (Kadir et al., 2014) was used to do spike sorting. The features used for the separation of clusters were the peak and valley of the waveform. Threshold multipliers of the range 3-5 were used for spike detection. The spike refractory period was kept at 0.5 ms, such that once a spike was detected, another spike was not detected for the next 0.5 ms to avoid detecting the same spike multiple times. KlustaKwik overclassifies waveforms, so clusters containing the same waveform were merged into a single cluster (neuron) based on visual examination. The noise was clustered separately. 1-2 neurons were isolated from each channel at a time. At the end of spike sorting, the program gave me the neuronal spike times corresponding to spikes from each cluster.

IN classification

For analysis of the activity of HVC_X neurons during INs, they were classified into Last and Other INs. The last INs were the INs immediately preceding the first syllable of the motif. All the other INs were classified as Other INs. This was because a previous study had shown that HVC_X neuron activity could separate these two classes of INs (Rajan & Doupe, 2013). A group of HVC_X neurons was shown to fire only for the last IN, while another group of the same class of neurons was shown to be specific for all INs except the last IN. Only the INs at the start of a song bout were considered for the analysis (INs produced before a call were not considered part of the IN-song bout and thus not considered for analysis). In the case of neurons whose firing was not perfectly stereotyped for Other INs, the INs were further classified into INs at different positions relative to the start of the motif to see if the neuron was specific for INs at a particular position.

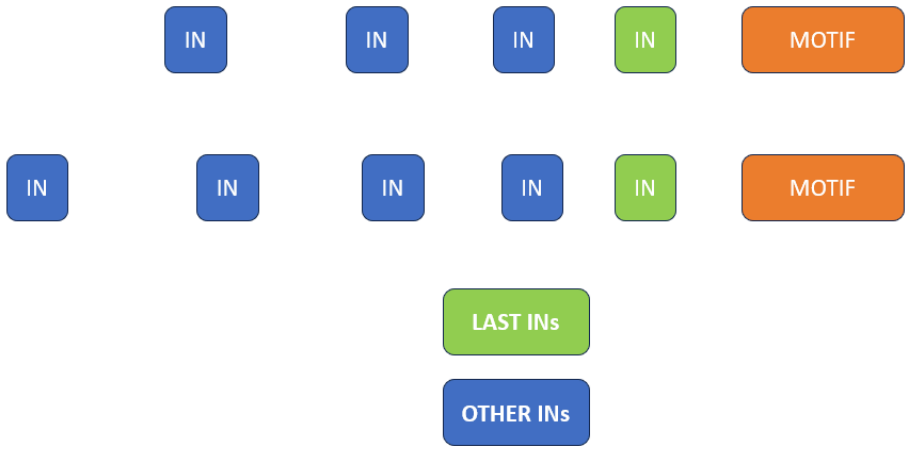


Figure 13: IN classification for HVC_X analysis. INs are classified as either Last INs (INs just before the motif) or Other INs (all the other INs except Last INs).

For analysis of interneuron activity, INs were classified based on their position relative to the start of the motif. This is because it has been shown that interneuron activity is highly stereotyped at each position relative to the start of the motif (Rajan & Doupe, 2013). The activity was analysed till IN6 (IN at the 6th position relative to the start of motif) for all interneurons because there weren't enough trials for further IN positions for most of the neurons.

Figure 14: IN classification for interneuron analysis. In this notation, the number of IN are counted backwards starting from the start of the motif.

For both HVC_X and interneuron analysis, INs before a call weren't considered in the analysis. All other INs were included, even the ones which occur in the middle of a bout. In one bird where HVC was damaged during surgery, the post-surgery song was affected, and there were no clear motifs. There were 2-3 distinct IN-like syllables at the beginning of the song. They were labelled separately, and neuron activity was checked separately for each of them.

Visualisation of neural activity

Raster plots and Peri-Stimulus Time Histograms (PSTHs) were used to visualise the neural activity. In raster plots, the neuronal spike time series' were aligned relative to the onset times of the INs (the onset and offset times of INs in the song were obtained separately using custom-made MATLAB scripts). This was done by subtracting the onset times of the syllables from the spike times for the alignment.

To plot PSTH, the time window in which the activity has to be visualised was binned. The binning was done by dividing the time window into a bin of 10 ms was moved from one end of the window to the other in steps of 1 ms. The reason for using 10 ms bin is mentioned below. The average firing rate for the neuron across all trials was plotted for each bin.

The activity at each IN position was majorly examined in a 100 ms time window centred at the IN onset time (50 ms before and after the IN onset). 50 ms is the offset time considered as pre-motor latency based on earlier studies (Katz & Gurney, 1981; McCasland & Konishi, 1981).

Analysing single unit activity in HVC

The data from some of the neurons included in this study was collected as a part of a previous study (Rajan & Doupe, 2013). These were HVC_X neurons and interneurons, and spike sorting for these neurons was already carried out as a part of the previous study. In the previous study (Rajan and Doupe, 2013), only IN-related activity during undirected songs was examined. In my study, I analysed IN-related activity during the directed song and compared this with IN-related activity during the undirected song. I included only interneurons that had atleast 10 bouts of directed song and HVC_X neurons which had atleast 5 bouts of directed song. The threshold number of bouts for HVC_X neurons was kept lower because spontaneous HVC_X firing is very sparse, and it fires in very stereotyped bursts, and fewer trials are required to detect significant differences in the firing of these neurons. (Kozhevnikov & Fee, 2007). 5 HVC_X neurons had more than 5 directed bouts beginning with INs. 4 HVC interneurons had more than 10 directed bouts, which began with INs. These were included in the analysis of this study. The single HVC interneuron, which was isolatable from my chronic implant recordings, was clubbed with the other interneurons for the overall analysis.

Differences in activity between Directed and Undirected songs

HVC_X neurons with IN specificity during undirected songs were analysed for the same during directed songs. The activity of the neuron during both cases was visualised with raster and Peri-Stimulus Time Histograms (PSTHs). The firing rates for individual neurons were calculated in a 100 ms window centred around the onset of the IN for directed and undirected trials, and the Mann-Whitney U test was used to test for significant differences between the neuronal firing rates during the two contexts.

For further analysis, a 10 ms time window was chosen, and the average and standard error of the mean of the firing rate across all trials was calculated for this window (for both undirected and directed trials). The rationale for choosing a 10 ms window is that the burst duration of the average HVC_X neuron is 6.3 ± 3.3 ms (Kozhevnikov & Fee, 2007). I wanted a time window that was approximately of a similar duration so that changes in activity due to bursts such as these can be captured properly. The Mann-Whitney U test was then used to test if there were significant differences between the firing rates of the neuron during directed and undirected trials. Since multiple tests were being performed, the Bonferroni correction was used, and the adjusted p-value was used for significance testing. Steps of 1 ms moved the 10 ms window from -50 ms to 50 ms relative to the onset time of the IN. Tests for significance were performed for each of these windows. The number of windows at which there were significant differences was calculated.

Activity of neurons at specific IN positions

For HVC_X neurons that fired for all INs except the last, their activity at each IN position was analysed for:

1. The number of spikes in the burst

This was to check if the position of the IN in the IN repeat sequence was represented by this property.

Statistical Testing

The Mann-Whitney U test was used to detect changes in the firing rates of neurons. It is a non-parametric used to test the difference between two independent groups of samples. A non-parametric test was chosen as the sample sizes were too small to use a parametric test in most cases. The neuron firing rates that I compared (i.e., between directed and undirected trials) were also independent of each other. The test was run at a 5% significance level ($\alpha=0.05$).

For detecting changes in paired data, the Wilcoxon signed rank test was used. It is a non-parametric test used to test differences between 2 groups of data with paired data points. The test was run at the same significance level as the Mann-Whitney Test.

RESULTS

Recording of Pre-surgery and Post-surgery songs

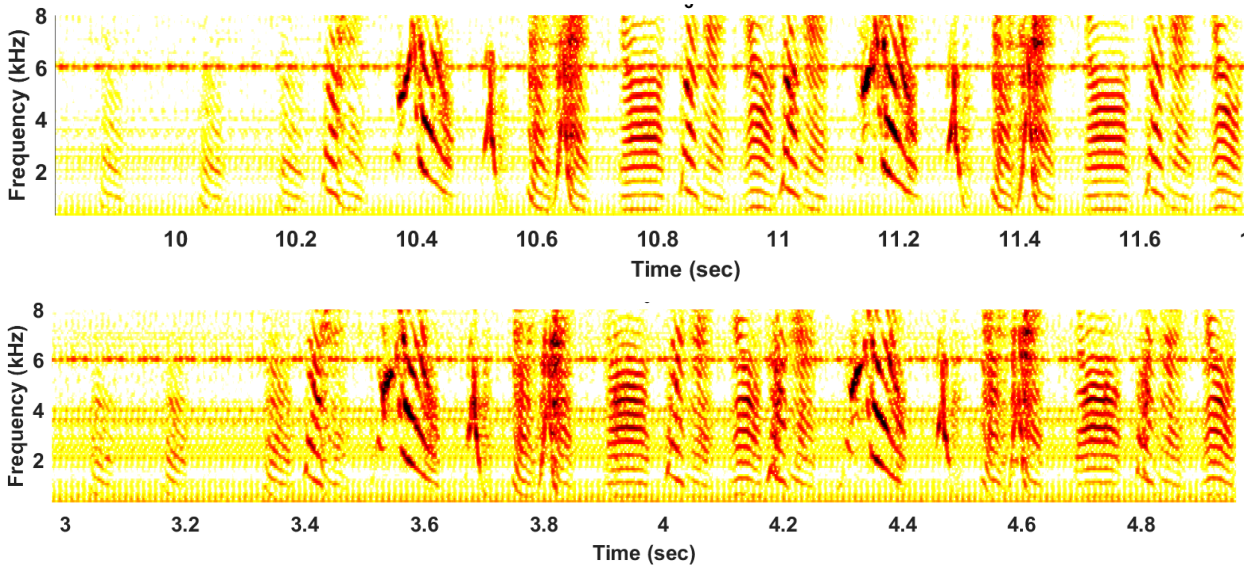
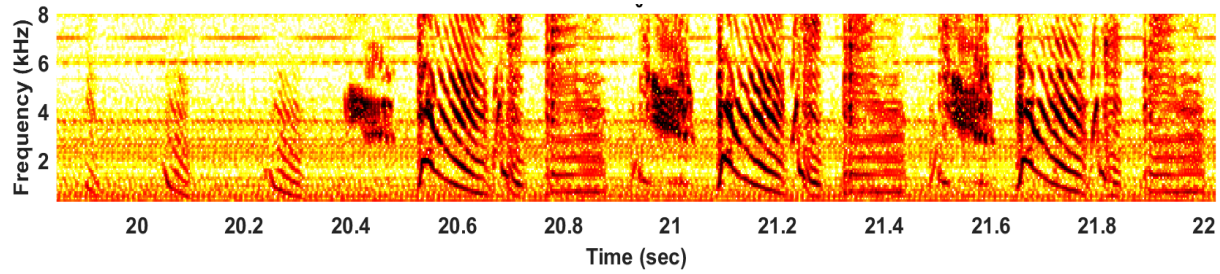


Figure 15: Spectrogram of pre-surgery (upper panel) and post-surgery (lower panel) song of bird green173white183. The song had not changed post-surgery, implying HVC wasn't damaged during surgery.

The pre-surgery songs and post-surgery songs of birds were recorded. This was to check whether I had damaged HVC during the surgery, as previous studies show that the bird's song becomes damaged when HVC is damaged (Thompson & Johnson, 2007). Figure 15 shows the two spectrograms of a bird whose song wasn't damaged post-surgery. Figure 16 shows both the spectrograms of a bird whose song got damaged post-surgery. Out of 22 birds, 2 had damaged HVC, which resulted in altered post-surgery songs.



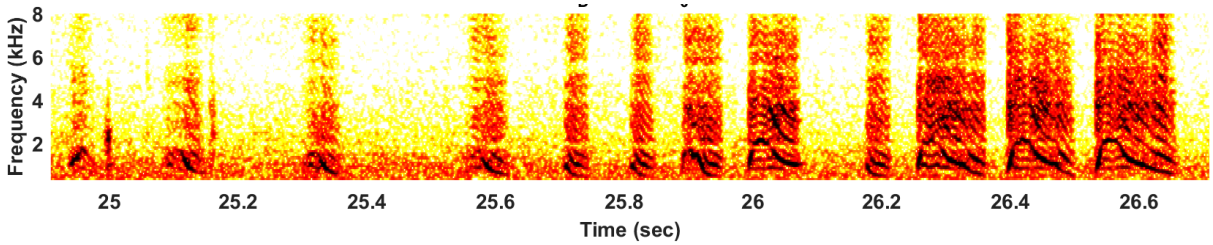


Figure 16: Spectrogram of pre-surgery (upper panel) and post-surgery (lower panel) song of bird green175white185. The song has changed post-surgery, implying HVC was damaged during surgery.

Pilot experiments for standardising the coordinates of HVC

As mentioned in the methods, I recorded from different brain locations to reliably obtain the coordinates of HVC relative to the blood vessel Y. Previous research has shown that the activity in HVC, when the bird is in its anaesthetised state, consists of bursts of 15-20 ms duration, with interspike intervals of 2-5 ms (Cardin & Schmidt, 2004) (Figure 17 bottom). Seven birds were used for this purpose (Table 4). The positive anterior-posterior (AP) coordinate indicates the anterior direction compared to Y, and the positive Mediolateral (ML) coordinate indicates the lateral (left) direction compared to Y. Figure 17 (top) shows the coordinates at which we got HVC-like activity.

Sr. No.	Bird ID	No. of locations recorded	No. of locations at which HVC-like activity was found	Coordinates at which HVC-like activity was found (AP, ML)
1	green039white098	7	3	(0.0, 2.0), (0.1, 2.2) (0.2, 2.0)
2	green153white161	4	1	(0.3, 1.9)
3	yellow148pink118	5	2	(0.1, 1.9), (0.1, 2.1)
4	green179white018	6	6	(0.1, 1.9), (0.2, 1.8), (0.4, 1.7), (0.1, 2.1), (0.3, 2.1), (0.4, 2.1)
5	yellow108pink161	9	3	(0.3, 1.5), (0.4, 1.8)

				(0.4, 1.5)
6	yellow117pink162	10	0	N/A
7	yellow150pink120	7	2	(0.2, 1.8), (0.4, 1.7)

Table 4: Results of the pilot experiments for standardising the HVC coordinates (with respect to the position of Y).

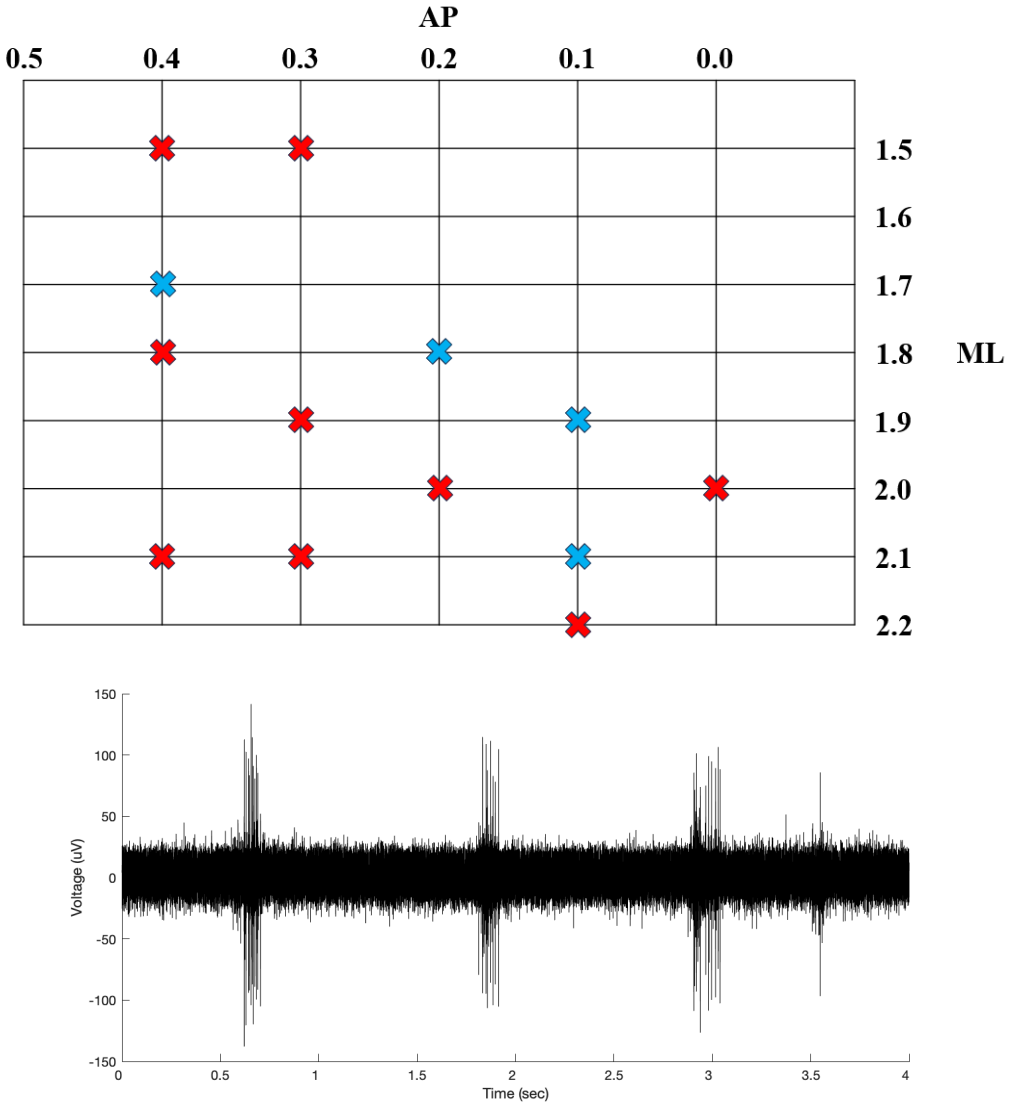


Figure 17: Results of pilot experiments. **Top:** Coordinates at which HVC-like activity was recorded (coordinates are with respect to Y). 1 unit equals 1 mm. **Red crosses** are coordinates at which HVC-like activity has been recorded once, and **blue crosses** indicate HVC-like activity has been recorded twice at that location. **Bottom:** Example of a neural trace showing HVC-like bursty activity. Data obtained in collaboration with Manali Upadhyaya

Group statistics of neurons recorded

There were a total of 22 birds which were implanted with microdrives. We could not record neural activity from 11 birds due to various reasons such as damage to electrodes, death of bird after surgery etc (more details in table 2). Of the birds from which the activity was recorded, 1 didn't sing any songs, and 1 had a damaged song such that there were no clear INs or motifs. Activity was recorded from a total of 9 birds while they were singing. Out of these, HVC-like activity was recorded from 2 birds.

Some of the neurons from green175white185 were done in a Bird Own Song (BOS) response assay. In the BOS response assay, the bird's own song is played back when the bird is anaesthetized to check for responses (Lewicki & Konishi, 1995; Margoliash, 1983). The bird was anaesthetized, and various stimuli such as BOS, reverse BOS and conspecific songs were played. These trials have not been analysed, although qualitatively, I did not observe any playback-related activity.

Sr. No.	Bird Name	No. of neurons recorded
1	red011red091	2
2	red122black194	8
3	green111white114	8
4	green173white183	45
5	green039white098	8
6	green112white115	4
7	green175white185	30
8	brown133brown134	20
9	blue190pink121	5

Table 5: Total number of neurons recorded from different birds.

Neurons that reacted to external movements were also recorded from one of the birds. In these trials, various objects were moved in different directions around the vicinity of the bird, and the responses of the neurons were recorded. These neurons were probably outside HVC as no such neurons have been characterised previously in HVC, and they did not have any singing-related activity.

Neuron No.	Bird ID	No. of bouts	HVC-like activity (Y/N)
1	red011red091	13	No
2	red011red091	3	No
3	red122black194	5	No
4	red122black194	3	No
5	green173white183	6	No
6*	green173white183	10	No
7	green173white183	5	No
8**	green173white183	4	No
9**	green173white183	1	No
10	green039white098	9	Yes
11	green039white098	2	Yes
12	green039white098	10	Yes
13	green039white098	3	Yes
14***	green175white185	7	No
15***	green175white185	6	No
16***	green175white185	19	No
17	green175white185	3	No
18	green175white185	4	No
19	green175white185	4	No
20	green175white185	5	No
21	blue190pink121	4	Yes

Table 6: Number of neurons which were recorded for more than 3 bouts during singing.
*: There might be two neurons in these files.
**: Movement-related activity recorded in these neurons
***: Recorded BOS response for these neurons

Singing-related HVC activity

Neurons at different depths were recorded when the bird was singing. One strong indication of recording from an HVC neuron is when it changes its activity when the bird is singing (Figure 18). It was very clear in the figure that there is an increase in the activity of the neuron when the bird is singing. A raster of the same neuron's activity during the motif is shown in Figure 19. The neuron should be an interneuron because it has tonic activity during the motif (Kozhevnikov & Fee, 2007).

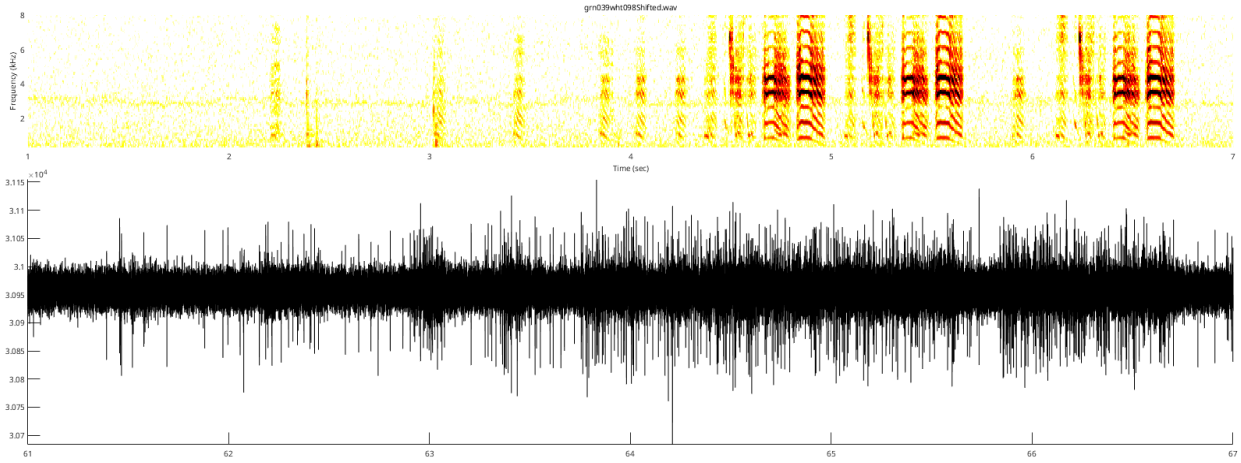


Figure 18: Singing-related increase in activity. This activity indicates that we are recording an HVC neuron. The upper plot is a spectrogram of the song of the bird. The lower plot shows the raw neural trace of the neuron.

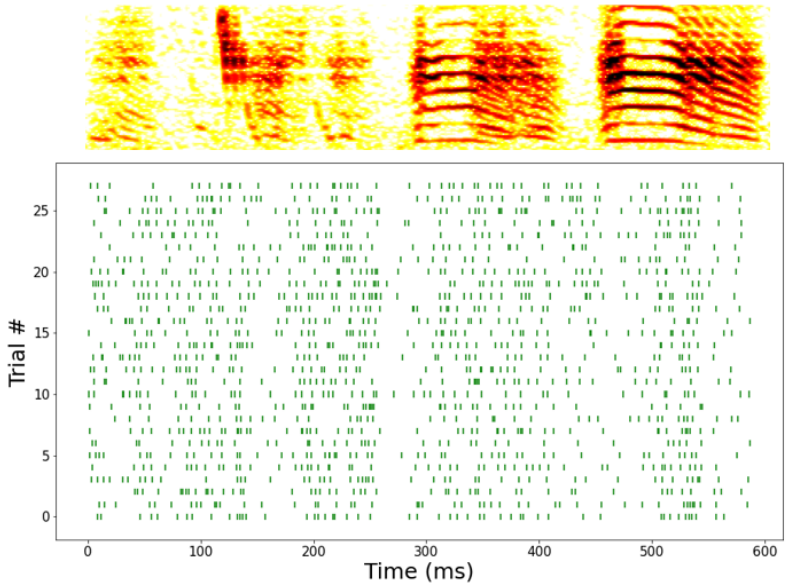


Figure 19: Motif-related activity of an HVC interneuron. The upper panel shows the spectrogram of the motif. The lower panel shows the raster of the neural activity from the same neuron from Figure 2.

Comparison of neural activity between directed and undirected song

HVC_X neurons

HVC_X neurons show specificity for the last INs or other INs during the undirected song (Rajan & Doupe, 2013). As shown in the previous study, out of the 5 HVC_X neurons, 4 fired only for Other INs, while the 5th one fired for the last IN in undirected trials. I wanted to see if this same specificity is present in directed trials as well. All of the 5 HVC_X neurons showed selective firing for the same class of INs as in their undirected trials (compared to baseline firing) ($p < 0.05$, Mann-Whitney test for each neuron).

Next, I checked if the average firing rate in a 100 ms window centred at IN onset differed in neurons between directed and undirected trials. 100 ms window was chosen to include the time of the vocalisation of the IN (approximately 50 ms in duration) and the premotor period of the vocalisation in HVC (50 ms before the vocalisation of the syllable). Only 1 out of the 5 HVC_X neurons showed a difference in firing between the two contexts (Figure 20). The neural activity of this neuron for Other INs during directed trials was significantly higher than that during undirected trials (Figure 21) ($p < 0.05$, Mann-Whitney test).

For this neuron, I wanted to see if there are any finer differences in activity during the directed and undirected -50 ms to 50 ms time window. To this end, I compared the firing rates of all the neurons in different overlapping 10 ms windows between directed and undirected trials (more details in methods). Significant differences in 3 consecutive 10 ms windows were considered biologically significant. I found 10 consecutive windows for which there were significant differences (Figure 21 and Table 7).

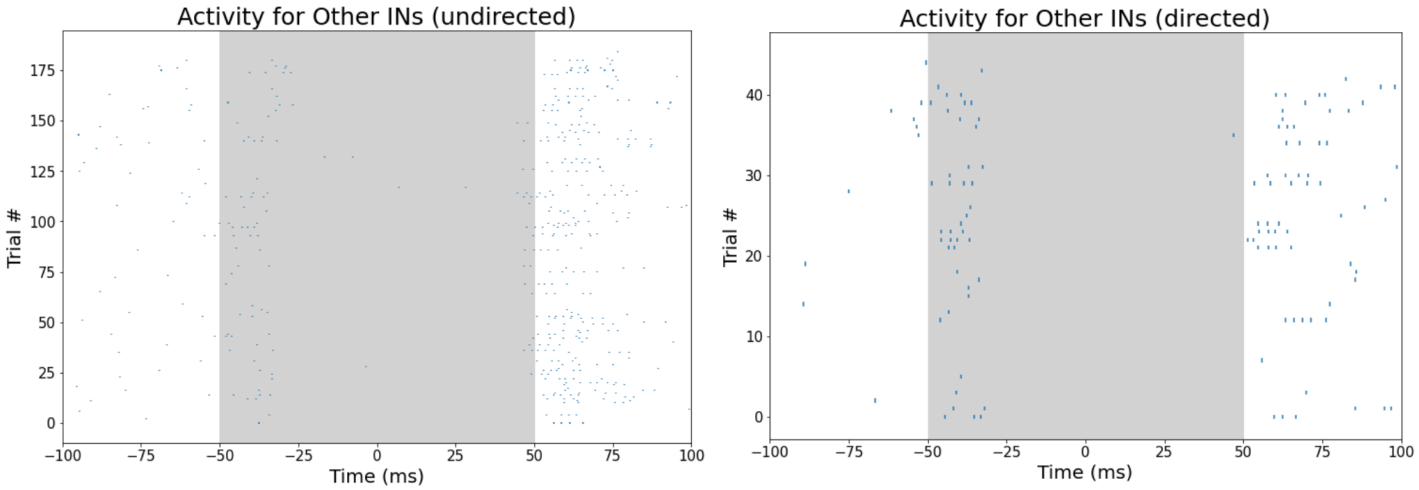


Figure 20: Activity of HVC_X neuron during Other INs for directed and undirected trials. **Left:** Raster showing the neuron's activity for the Other IN during undirected trials. **Right:** Raster showing the neuron's activity for Other INs during directed trials. The x-axis shows time in ms

relative to the onset of the IN. The shaded region is a 100 ms window centred around the IN onset. This is the window I used for further analysis of IN related activity.

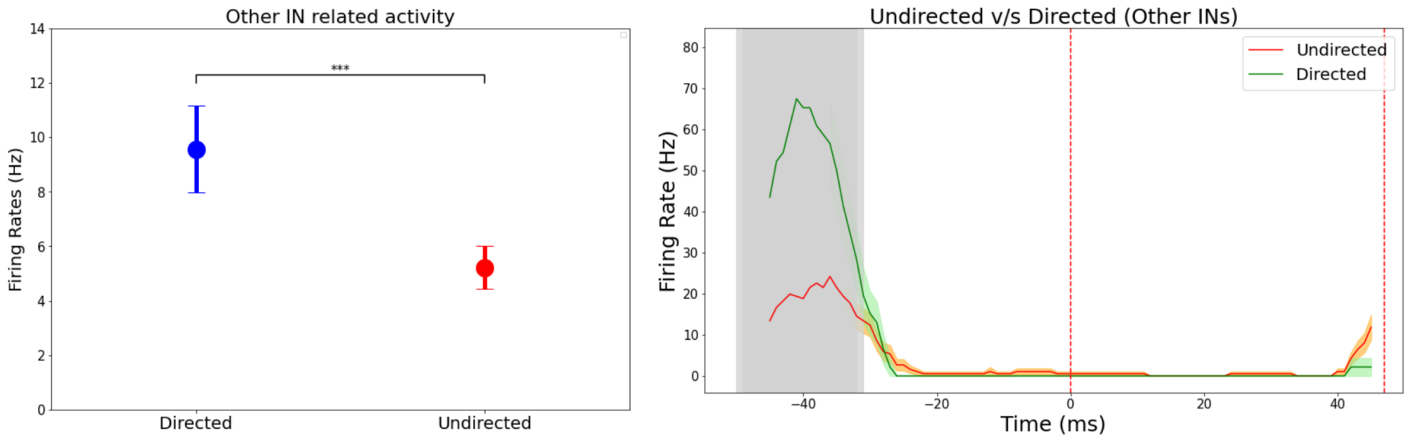


Figure 21: Comparison of the activity of HVC_X neuron (same as in Figure 20) between directed and undirected trials for Other INs. **Left:** Plot showing the differences in the average activity of the neuron in Figure 20 during the Other INs between directed and undirected trials. The blue represents directed, and the red represents undirected firing rates. The whiskers denote the 95% confidence intervals. *** denotes $p < 0.001$. **Right:** PSTH showing the firing rates of the HVC_X neuron for the Other INs for directed (green) and undirected (orange) trials. The red dotted line shows the average onset and offset time of INs. The times are calculated relative to the onset of the IN. The grey shaded areas show the time windows where there are significant differences ($p < 5.49e\text{-}4$. Mann-Whitney Test. Adjusted p-value obtained after applying Bonferroni correction) between the firing rates of the neuron during directed and undirected trials.

Sr. No	Window onset (ms)	Window offset (ms)	p-value
1	-50	-40	1.922e-04
2	-49	-39	3.233e-05
3	-48	-38	6.387e-05
4	-47	-37	3.935e-06
5	-46	-36	2.704e-07
6	-45	-35	9.340e-07
7	-44	-34	2.073e-06

8	-43	-33	8.992e-05
9	-42	-32	2.947e-05
10	-41	-31	4.213e-04

Table 7: Time windows where significant differences between the firing rates of the neuron in Figure 20 were found between directed and undirected trials. The time windows noted here are the same as the shaded regions in Figure 21.

Interneurons

HVC interneurons show stereotyped activity for each IN position relative to the start of the motif (Rajan & Doupe, 2013). I wanted to see if this pattern of activity is similar between directed and undirected trials. The activities of 4 HVC interneurons (which are active during the undirected song) and 1, which was only recorded during the directed song, were analysed during the directed song. All 5 of these were active during all IN positions (IN_1 to IN_6) ($p < 0.05$. Mann-Whitney Test), similar to undirected trials.

The average firing rates of interneurons for directed and undirected trials were compared (Figure 22). This was done for 4 interneurons for which both directed and undirected data were available, one interneuron for just directed trials and one for just undirected trials. There were no significant differences detected for both the paired ($p > 0.05$. Wilcoxon signed rank test) and the unpaired data ($p > 0.05$. Mann-Whitney Test).

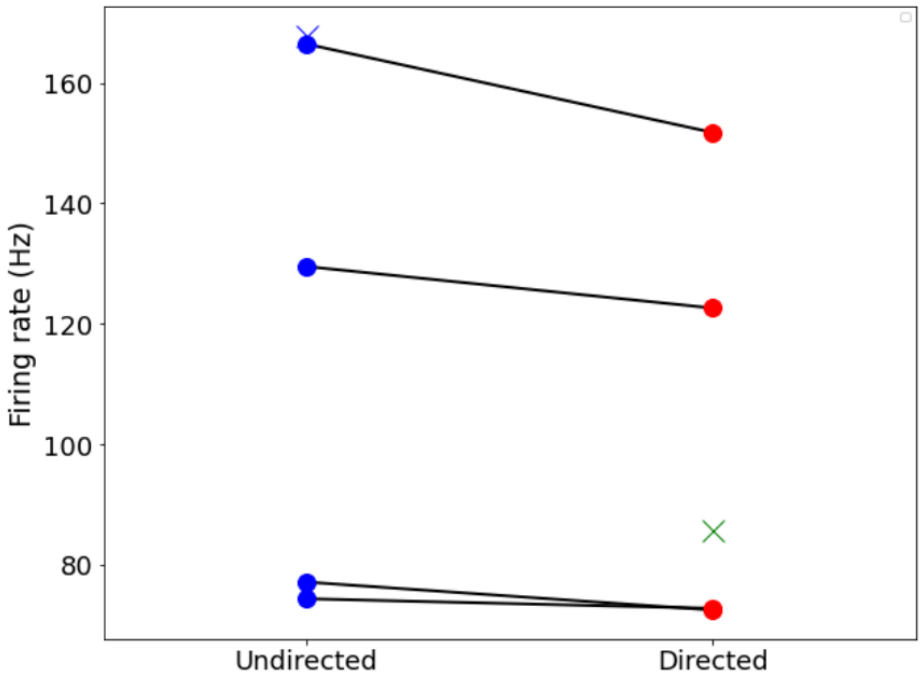
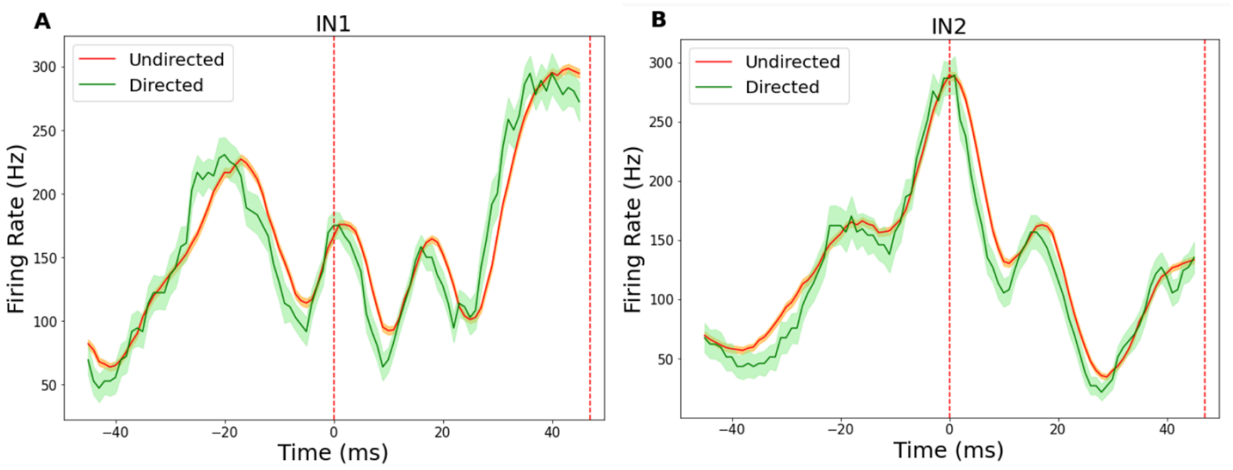


Figure 22: Average firing rates of interneurons during INs for directed and undirected songs. Blue symbols are for undirected and red symbols are for directed songs. Connected points denote paired data. The crosses denote unpaired data. The green cross denotes the neuron that I recorded as part of my experiments.

Next, I checked specific 10 ms time windows in the -50 to 50 ms range to calculate specific time points at which there were differences in neuron activity between directed and undirected trials (similar to HVC_X neuron analysis).

Out of 4 interneurons, 2 of them had no differences in activity between the two contexts at any of the 6 IN positions ($p > 5.49e\text{-}4$ Mann-Whitney U Test after application of Bonferroni correction). One interneuron had 2 windows at the IN2 position where there were differences, but these were not consecutive windows. The remaining interneuron showed differences in firing at the IN6 position (Figure 23F). There were a total of 5 windows where there were significant differences (including 4 consecutive ones).



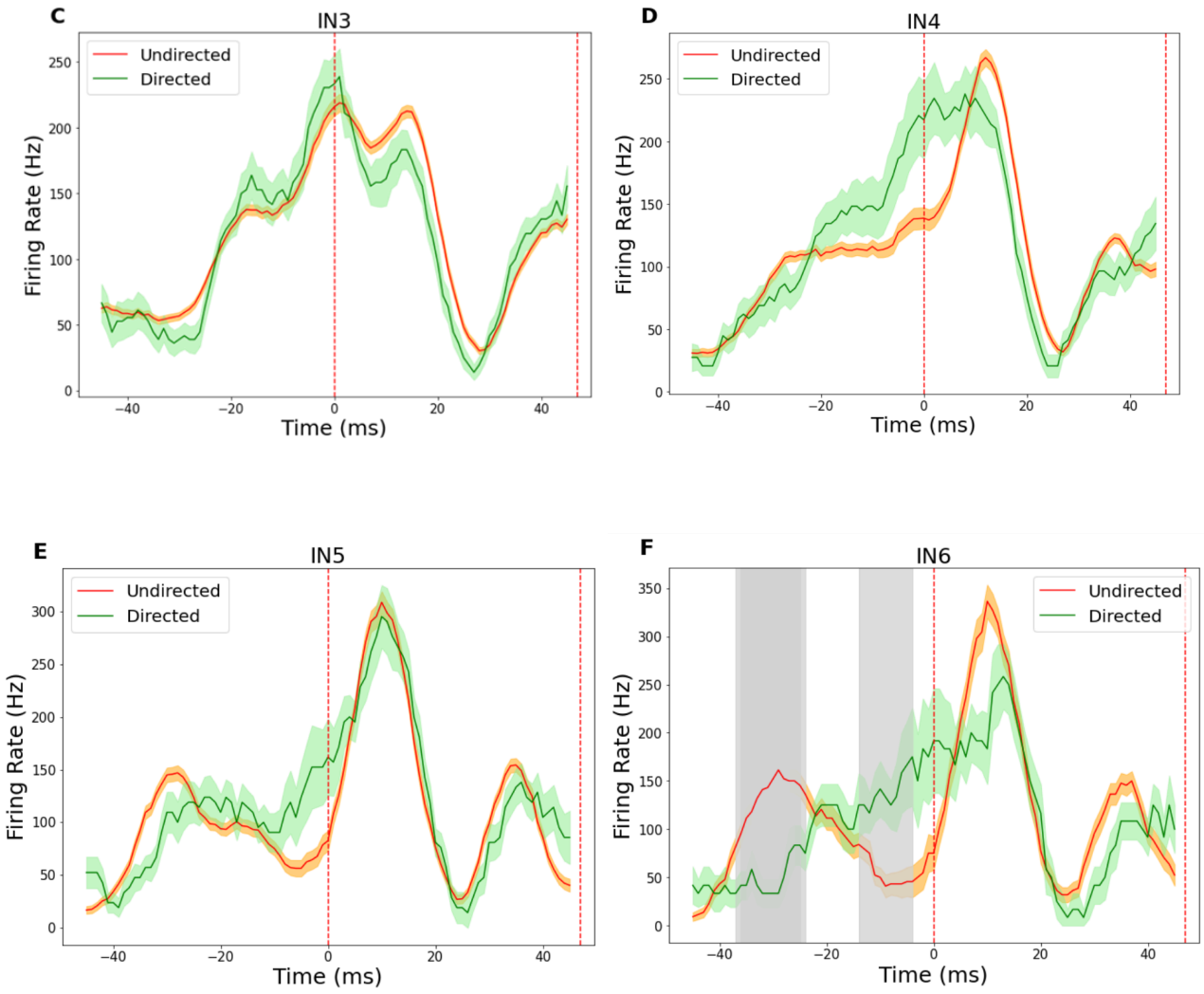


Figure 23: Comparison of interneuron activity between directed and undirected trials for each IN position **A-F:** PSTH showing the activity of an interneuron for IN positions IN1 to IN6 for directed and undirected trials. The red dotted line shows the average onset and offset time of INs. The times are calculated relative to the onset of the IN. In **F**, the grey shaded areas show the time windows where there are significant differences ($p < 5.49e\text{-}4$. Mann-Whitney Test, after application of the Bonferroni correction) between the firing rates of the neuron during directed and undirected trials. The shaded regions denote time windows during which there were significant differences between firing rates during directed and undirected trials

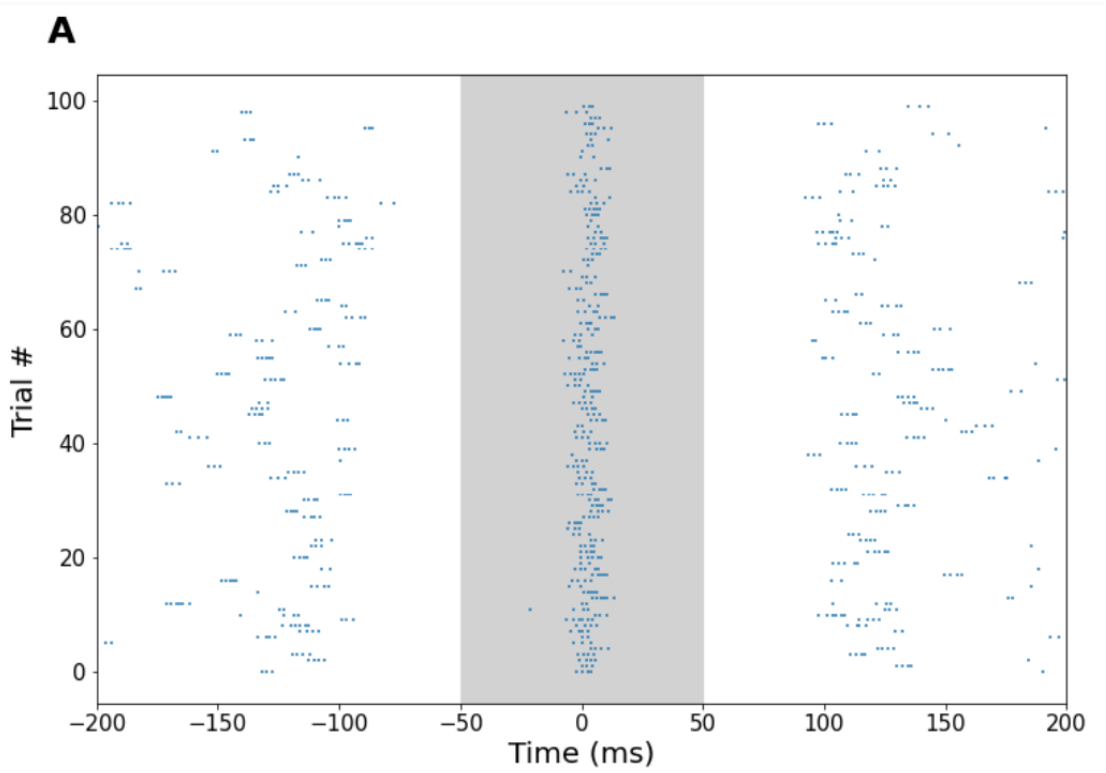
IN position-specific activity of HVC_X neurons

Number of spikes

As mentioned earlier, a certain class of HVC_X neurons fire for every other IN except the last IN (Figure 24A). However, the activity of these neurons with respect to specific IN positions hasn't been looked at. I wanted to see if the number of spikes that this neuron

fires differs between different IN positions. I counted the number of spikes each neuron fires in the time window stretching from -50 ms to 50 ms for each IN position across multiple trials and qualitatively looked at the data for any trends.

3 neurons in undirected trials seem to progressively fire less for later INs (black, blue and yellow neurons), while the other 2 seem to have the opposite trend (Figure 24B). There doesn't seem to be any similar trend across IN positions for directed trials (Figure 24C). All 5 neurons seem to have similar rates of firing between directed and undirected trials, except the yellow neuron. This yellow neuron is the same neuron as in Figure 21, which showed significant differences in firing rates between directed and undirected trials.



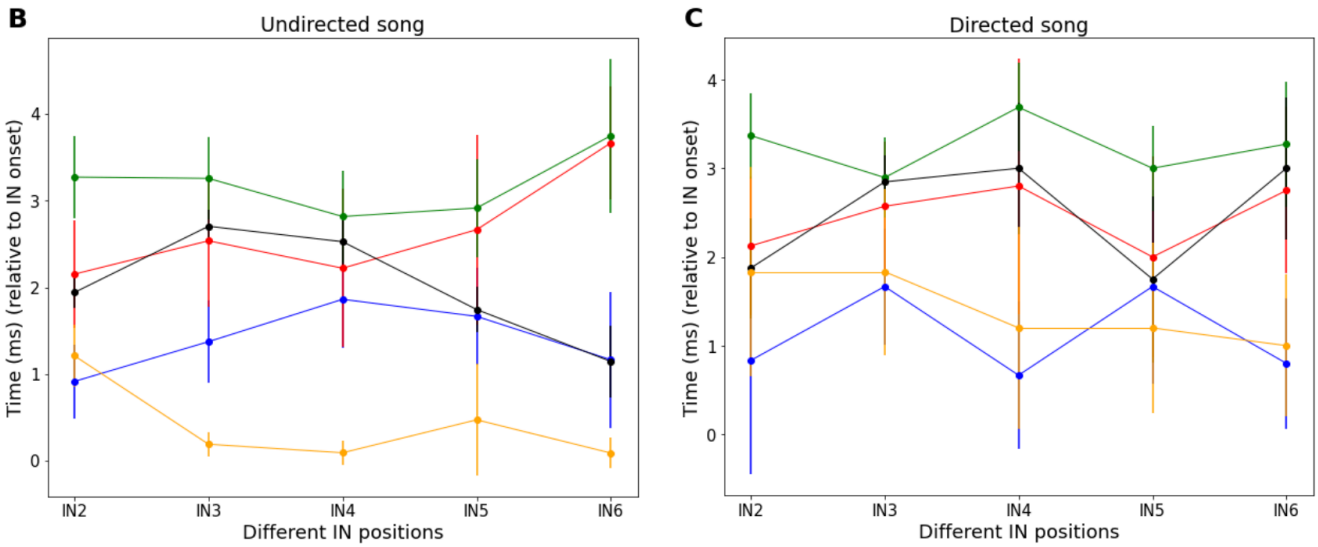


Figure 24: IN position-related activity for HVC_X neuron which fires for Other INs. **A:** Raster showing a neuron which fires for Other INs during directed trials. Plot showing the activity of neurons for different IN positions for undirected (**B**) and directed (**C**) songs. Different colours signify different neurons (the same colour denotes the same neuron for both plots). The points denote the mean number of spikes, and the error bars show the 95% confidence interval. The neuron in **A** is denoted by green colour in **B** and **C**.

Construction of Zebra Finch Brain Atlas for our lab

Aligned images and marked the origin

The images of the stained brain slices were aligned with each other using Adobe Photoshop. The position of the Y blood vessel was marked on each of the brain slices. This was done by back-calculating using the known coordinates of the lesions that we had made earlier (or the dil marked electrode tracts) (table 3) (Figure 25).

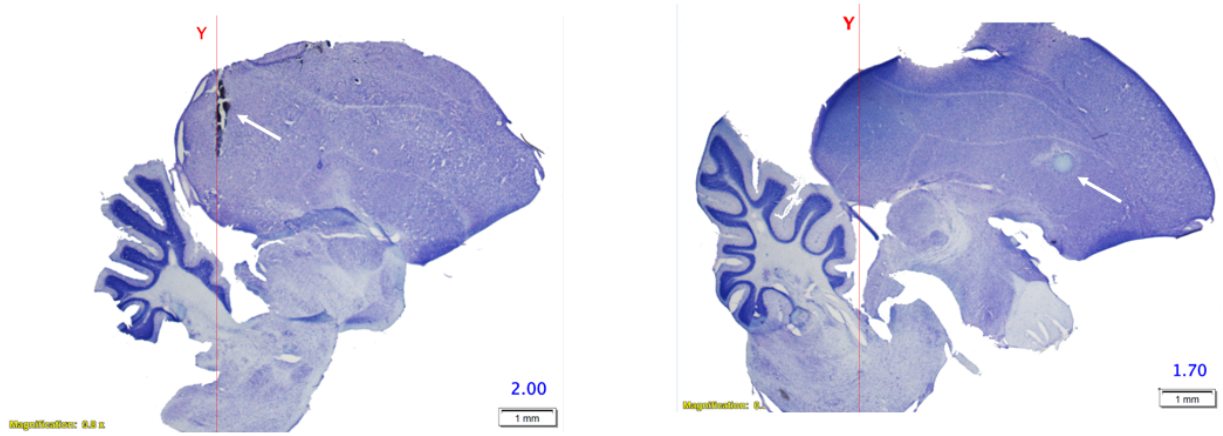


Figure 25: Brain slices stained with cresyl violet, with the position of Y marked. The position of Y has been marked with a red vertical line. The position of Y has been calculated using known

coordinates of DiI marked electrode tract (left) or electrolytic lesion (right). The electrode tract and electrolytic lesion have been marked with white arrows.

Constructed Zebra Finch Brain Atlas

Figure 26 shows the brain atlas of the left hemisphere and the right hemisphere. The position of Y has been marked in every section. The AP coordinates of different nuclei can then be calculated by calculating their distance from Y. The different ML coordinates of sections are written at the bottom right corner of every panel.

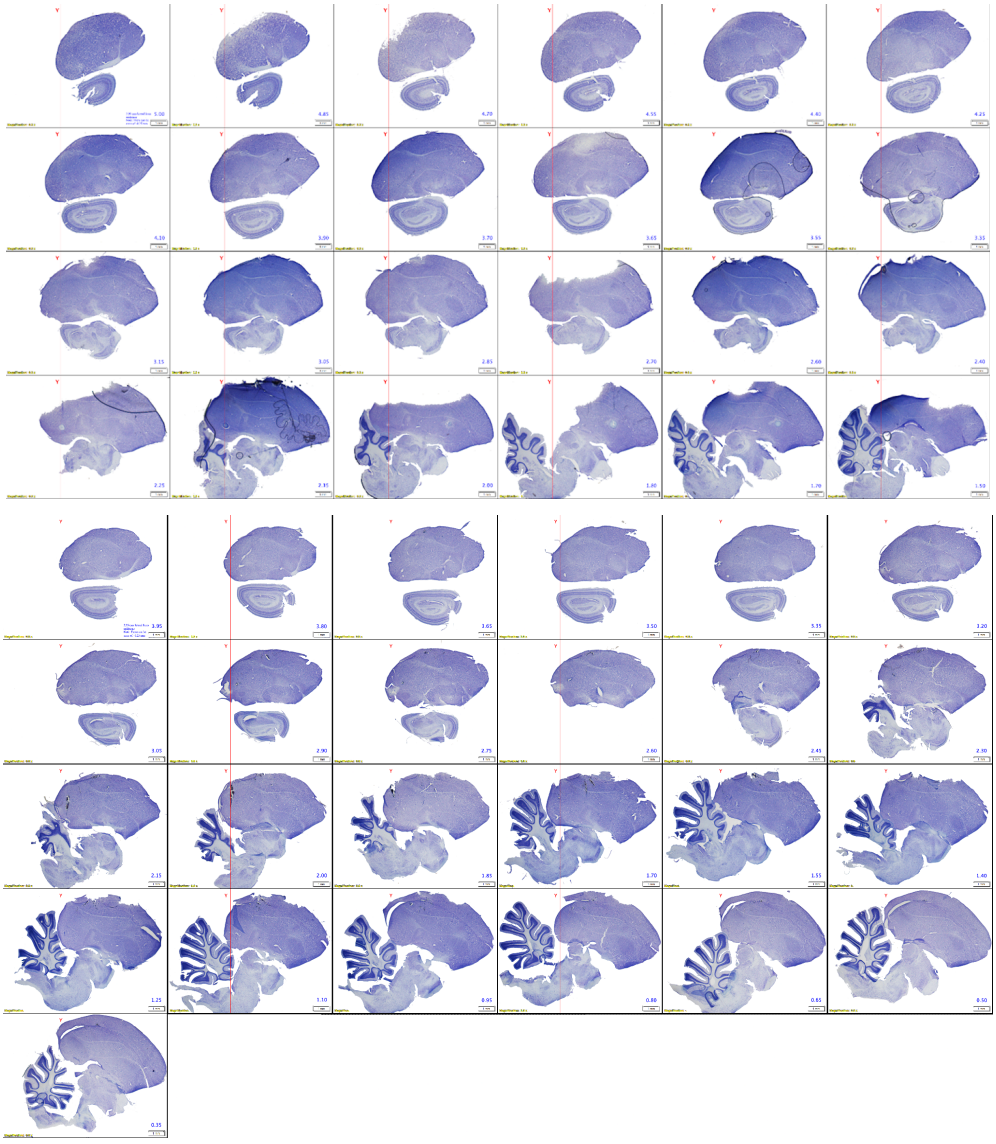
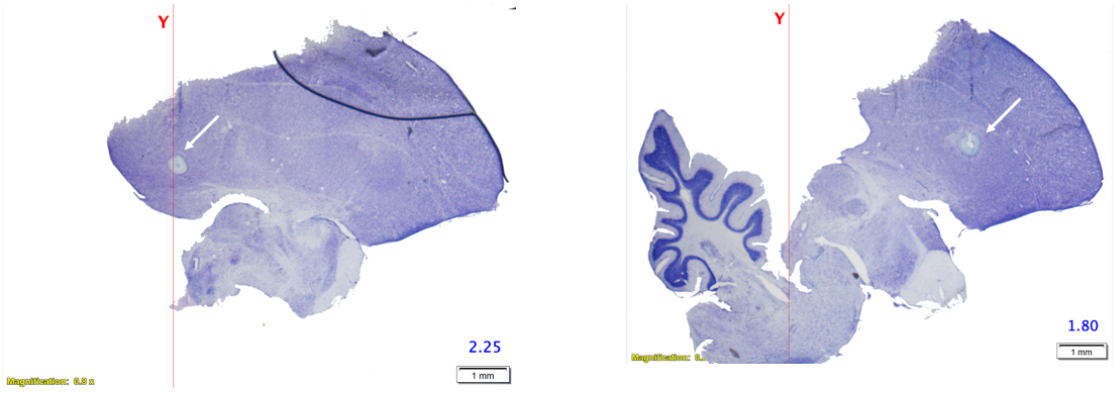


Figure 26: Zebra Finch Brain Atlas. All the sections of the left hemisphere (**Top**) and right hemisphere (**Bottom**) have been aligned with each other, with the position of Y marked in each section.

Standardising lesion parameters

I used 3 different combinations of monophasic current magnitudes and durations;

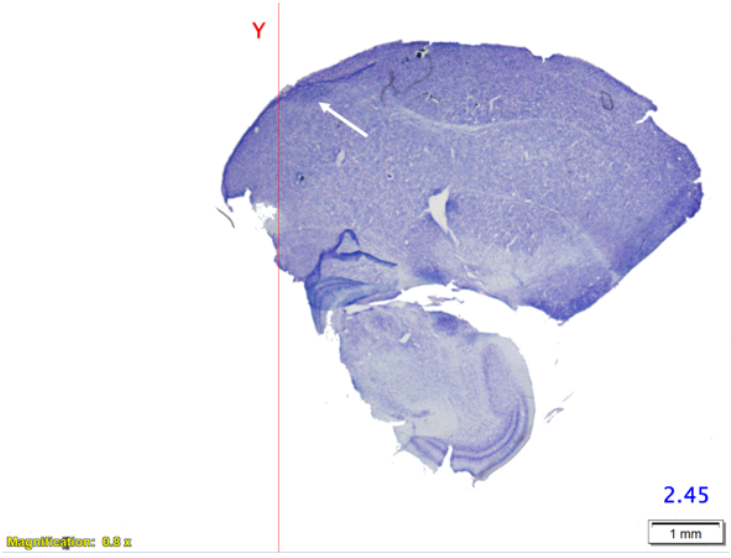
1. 30 uA for 100 sec
2. 60 uA for 50 sec
3. 60 uA for 100 sec



30 µA, 100 sec
Area: 0.155 mm^2

60 µA, 100 sec
Area: 0.210 mm^2

Figure 27: Cresyl Violet stained brain sections showing lesions of different sizes. The section on the left has a lesion made by a monophasic current of 30 uA for 100 seconds. The section on the right has an electrolytic lesion made by a current of magnitude 60 uA applied for 100 seconds. White arrows mark the lesions.



Area: 0.175 mm^2

Figure 28: Cresyl violet stained brain section showing the brain nucleus HVC. HVC appears as a dark patch in the otherwise lightly stained section. A white arrow marks the position of HVC, and the area is given below.

However, I could only see lesions corresponding to the 1st and 3rd lesion parameters. For 30 uA for 100 seconds, the lesion size was 0.155 mm^2. For 60 uA for 100 seconds, the lesion size was 0.210 mm^2 (Figure 27). In contrast, the size of HVC in the same sections was 0.175 mm^2 (Figure 28). Thus, 60 uA for 100 seconds is not a good combination to use for lesioning HVC because the lesion size is considerably larger than the size of HVC itself.

On the other hand, the biphasic current (magnitude 40 uA) did not leave any tungsten oxide deposition, as seen under brightfield microscopy.

DISCUSSION

In this study, I looked at the activity of different HVC neurons during the production of introductory vocalisations (INs) in zebra finches. While extensive studies have been done on the neural mechanisms underlying the song of a zebra finch, very few studies have looked specifically at the INs at the beginning of the song

Surgeries were done on the male zebra finches to implant custom-made microdrives, which contained tungsten electrodes for recording neural activity. HVC was reached using stereotaxic coordinates. Pilot experiments were conducted to fix the stereotaxic coordinates of HVC, as HVC recordings were not being obtained using the standard coordinates from the zebra finch brain atlas (Nixdorf-Bergweiler et al., 2007). Once in HVC, one single-unit interneuron recording and multiple multi-unit recordings dominated by interneuron firing were recorded. At the end of neural recordings, one of the points at which recordings were taken was lesioned, and the brain was sectioned to visualize the brain nuclei and see if the lesion was in HVC.

I also analysed the neuronal data that had been recorded as part of a previous study (Rajan & Doupe, 2013). The previous study looked at the activity of HVC_X neurons and interneurons during IN production in undirected songs (songs sung when the bird is alone). However, this question had not been tackled in the context of directed songs (songs directed at a female). I first looked at HVC_X neurons, which fire for either the last IN or all other INs during the undirected song. I discovered one neuron which had significantly higher firing during other INs during directed songs compared to undirected songs. This neuron only fired for the last INs for undirected trials. The rest of the HVC_X neurons didn't have any differences in firing. Next, I looked at whether interneuron firing differs between directed and undirected contexts for specific IN positions relative to the start of the song. Out of 4 interneurons, 2 of them had scattered points at which there were differences between directed and undirected trials. 1 of those two had differences for a continuous stretch of time for the 6th IN position (IN6), which I considered to be biologically significant. The other two interneurons didn't have any differences between the two contexts at any IN position.

I also looked at whether HVC_X neurons code for the IN position in the IN repeat sequence. I looked at those neurons that fire for every other IN except the last and checked whether the number of times the IN fires differs between IN positions. I did not see any noticeable and consistent trend in the data.

The HVC circuitry for directed and undirected is mostly the same

My study implies that the circuitry in HVC, which is involved in the production of INs during directed and undirected songs, is mostly the same, as most of the neurons have similar activity in both of these contexts. However, a small number of neurons might code for contextual information, as one HVC_X neuron and one interneuron show differences in activity between directed and undirected trials.

Previous studies have looked at the activity of different nuclei in the AFP in different social contexts during the song motif. It has been shown that the AFP output nucleus, LMAN, shows differences in activity for directed and undirected song motifs (Kao et al., 2008). Specifically, they saw that the LMAN neurons in undirected trials show bursty firing and trial-by-trial variability. In contrast, the same neurons in directed trials show time-locked reliable firing during the motif. Another study found that there weren't any differences in the activity of HVC_X neurons during the motif for directed and undirected trials (Woolley et al., 2014). They instead saw context sensitivity in pallidal neurons of Area X (but not in spiny neurons). These changes were still present even after lesioning LMAN, proving that the observed results are not a by-product of the differences in LMAN activity feeding back into the AFP. They concluded that the generation of variability and contextual information occurs in the songbird basal ganglia in multiple steps. My results showing minimal differences in IN-related activity across social contexts suggest that the neural control for INs might be similar to the neural control for motif syllables. Future studies could examine whether context-dependent differences in IN-related activity exist in basal ganglia neurons.

However, differences in the circuitry for directed and undirected songs have been observed upstream of HVC as well. Midbrain cell group A11, which has projections to HVC, has been shown to have a role in directed songs but not in undirected songs. Lesioning A11 abolishes the directed song (and all other courtship behaviours directed to a female) but leaves the undirected song intact (Ben-Tov et al., 2023). This suggests that the inputs to HVC might be different for different social contexts. This leads to two possibilities: either different inputs for directed and undirected songs deploy the same neural circuitry in HVC, or there are two different circuitries in HVC for directed and undirected songs. Based on my results for IN vocalisation and the result that HVC_X neurons do not show contextual differences during motif (Woolley et al., 2014), I would hypothesise that most of the neurons involved in vocalisation during directed and undirected songs are the same, while there might be a small fraction of neurons where there might be differences in activity based on social context. More neurons need to be recorded to support this hypothesis.

Coding of IN position by HVC_X neurons

My data suggests that HVC_X neurons do not code for IN position in zebra finches. This is in contrast to studies in Bengalese finches that show that HVC is responsible for coding for syllable repeats in the middle of the song. It has been shown that there are HVC_X neurons in Bengalese finches which show progressively higher or lower firing as the bird proceeds through syllable repeat sequences (Figure 29). Thus, the firing rate of the neuron codes for syllable repeat progression (Fujimoto et al., 2011). Another study has shown that reversible cooling of HVC in Bengalese finches results in the reduction of repetition of long and variably repeating syllables (Zhang et al., 2017). One straightforward reason for this might be the low number of HVC_X neurons which have been analysed in this study. More neurons have to be recorded and analysed for it to be certain that HVC_X neurons do not code for IN position or number in Zebra Finches.

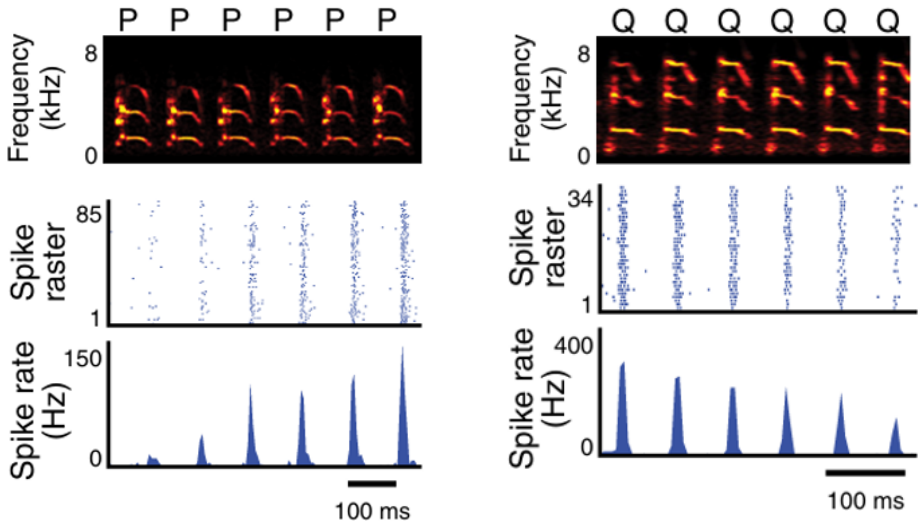


Figure 29: HVC_X activity during variable repeat sequences in Bengalese finches. The HVC_X neuron on the **left** progressively increases its firing while the one on the **right** progressively decreases its activity when the bird is vocalising syllable repeats (Fujimoto et al., 2011).

Difficulty in targeting HVC

There was a lot of difficulty in targetting HVC with electrodes in earlier surgeries. I used standard HVC coordinates according to the zebra finch brain Atlas (Nixdorf-Bergweiler et al., 2007), but I was unsuccessful in recording from HVC after most surgeries. One possible reason the standard HVC coordinates were not working, in this case, could be that the beak angle of the bird on our stereotaxis was not exactly the same as that specified in the zebra finch brain Atlas. The beak angle will affect the stereotaxic coordinates of brain nuclei. Another reason could be the inappropriate positioning of the bird in the stereotaxic apparatus. The ear bars that hold the head of the bird in place

have to be placed properly so that the bird's head is properly oriented with respect to the stereotaxis. I also observed that in multiple earlier implantation surgeries, HVC used to get damaged during the surgical procedure itself. This was because HVC is present very close to the surface of the brain compared to other brain nuclei.

Throughout my project, I standardised how I put the bird on the Stereotax so that it was consistent surgery-to-surgery. Since I could not account for the small changes in beak angle, which added to the error in locating HVC, I did my own pilot experiments to standardise the coordinates of HVC for my own stereotaxic set-up. I also improved my surgery skills so that HVC was not damaged during the implantation of the microdrive.

Difficulties in recording from HVC_{RA} neurons

My original aim was to record from HVC_{RA} neurons, but I did not isolate any HVC_{RA} neurons from my recordings. It is very difficult to record from HVC_{RA} neurons for multiple reasons. HVC_{RA} neurons have a very sparse spontaneous firing rate (spontaneous firing rate: 0.001spike/s) when the bird is not singing but is awake (Kozhevnikov & Fee, 2007). So, while moving the electrodes in the brain, it is difficult to tell when the electrode is near an HVC_{RA} neuron. This problem doesn't arise for interneurons because they have a sufficiently high spontaneous firing rate to tell when the electrode is recording from an interneuron. Even in situations where the electrode is near an HVC_{RA} neuron, recording from a neuron that is active during INs is rare. In an earlier study, it was found that out of 28 HVC_{RA} neurons recorded, only 2 neurons fired during INs (Kozhevnikov & Fee, 2007).

A common way to work one's way around this problem is to stimulate the nucleus RA while recording from HVC. This is called antidromic stimulation (Hahnloser et al., 2002). Antidromic stimulation stimulates multiple axon terminals in RA, which can be detected from their dendritic terminals in HVC. It is a surefire way of detecting HVC_{RA} neurons and recording their activity. Another solution is to implant silicon probes in HVC instead of single tungsten electrodes. More than 3 electrodes can't be implanted on a zebra finch because of weight restrictions on the implant. Instead, one can implant multi-channel silicon probes that can record data from a greater number of neurons simultaneously than a couple of tungsten electrodes. With the sheer number of channels on a silicon probe (it can go above 100 channels), the probability of recording from HVC_{RA} neurons increases exponentially (Egger et al., 2020; Elmaleh et al., 2021).

Conclusion

The overall inference of this study is that while the neuronal circuitry for producing INs during directed and undirected songs is mostly similar in HVC, a small number of neurons might carry information about the social context in their firing rate or pattern of

firing. More number of HVC neurons (and HVC_{RA} neurons specifically) have to be recorded to further support this hypothesis. Studies for context-related differences in IN-related activity can also be done in basal ganglia neurons, as these neurons have been shown to code for social context in motif-related activity.

REFERENCES

Arnold, K., & Zuberbühler, K. (2006). Semantic combinations in primate calls. *Nature*, *441*(7091), 303–303. https://doi.org/10.1038/441303a

Benezra, S. E., Narayanan, R. T., Egger, R., Oberlaender, M., & Long, M. A. (2018). Morphological characterization of HVC projection neurons in the zebra finch (Taeniopygia guttata). *The Journal of Comparative Neurology*, *526*(10), 1673–1689. https://doi.org/10.1002/cne.24437

Ben-Tov, M., Duarte, F., & Mooney, R. (2023). A neural hub for holistic courtship displays. *Current Biology*, *33*(9), 1640–1653.

Cardin, J. A., & Schmidt, M. F. (2004). Auditory Responses in Multiple Sensorimotor Song System Nuclei Are Co-Modulated by Behavioral State. *Journal of Neurophysiology*, *91*(5), 2148–2163. https://doi.org/10.1152/jn.00918.2003

Catchpole, C. K., & Slater, P. J. (2003). *Bird song: Biological themes and variations*. Cambridge university press. https://books.google.com/books?hl=en&lr=&id=sB24pLg4gywC&oi=fnd&pg=PA1&dq=Catchpole,+C.+K.+and+P.+J.+B.+Slater.+1995.+Bird+song:+biological+themes+and+variations.+Cambridge+University+Press,+New+York,+USA&ots=MdaBCWsNW8&sig=XUZspZg61-DeyQt3QO3KZe_qH3o

Clarke, E., Reichard, U. H., & Zuberbühler, K. (2006). The Syntax and Meaning of Wild Gibbon Songs. *PLoS ONE*, *1*(1), e73. https://doi.org/10.1371/journal.pone.0000073

Cohen, Y., Shen, J., Semu, D., Leman, D. P., Liberti, W. A., Perkins, L. N., Liberti, D. C., Kotton, D. N., & Gardner, T. J. (2020). Hidden neural states underlie canary song syntax. *Nature*, *582*(7813), Article 7813. https://doi.org/10.1038/s41586-020-2397-3

Dunn, A. M., & Zann, R. A. (1996). Undirected Song in Wild Zebra Finch Flocks: Contexts and Effects of Mate Removal. *Ethology*, *102*(4), 529–539. https://doi.org/10.1111/j.1439-0310.1996.tb01145.x

Egger, R., Tupikov, Y., Elmaleh, M., Katlowitz, K. A., Benezra, S. E., Picardo, M. A., Moll, F., Kornfeld, J., Jin, D. Z., & Long, M. A. (2020). Local Axonal Conduction Shapes the Spatiotemporal Properties of Neural Sequences. *Cell*, *183*(2), 537-548.e12. https://doi.org/10.1016/j.cell.2020.09.019

Elmaleh, M., Kranz, D., Asensio, A. C., Moll, F. W., & Long, M. A. (2021). Sleep replay reveals premotor circuit structure for a skilled behavior. *Neuron*, *109*(23), 3851-3861.e4. https://doi.org/10.1016/j.neuron.2021.09.021

Engesser, S., Crane, J. M. S., Savage, J. L., Russell, A. F., & Townsend, S. W. (2015). Experimental Evidence for Phonemic Contrasts in a Nonhuman Vocal System. *PLOS Biology*, *13*(6), e1002171. https://doi.org/10.1371/journal.pbio.1002171

Favaloro, C. (n.d.-a). *EXPLORING PARALLELS BETWEEN LATERALIZED CONTROL OF HUMAN LANGUAGE AND THE NEURAL CONTROL OF BENGALESE FINCH SONG SYNTAX*.

Favaloro, C. (n.d.-b). *EXPLORING PARALLELS BETWEEN LATERALIZED CONTROL OF HUMAN LANGUAGE AND THE NEURAL CONTROL OF BENGALESE FINCH SONG SYNTAX*.

Favaloro, C. (2018). *Exploring Parallels Between Lateralized Control of Human Language and the Neural Control of Bengalese Finch Song Syntax*. https://repository.tcu.edu/handle/116099117/22432

Fee, M. S., & Scharff, C. (2010). The Songbird as a Model for the Generation and Learning of Complex Sequential Behaviors. *ILAR Journal*, *51*(4), 362–377. https://doi.org/10.1093/ilar.51.4.362

Fujimoto, H., Hasegawa, T., & Watanabe, D. (2011). Neural Coding of Syntactic Structure in Learned Vocalizations in the Songbird. *Journal of Neuroscience*, *31*(27), 10023–10033. https://doi.org/10.1523/JNEUROSCI.1606-11.2011

Gurney, M. E. (1981). Hormonal control of cell form and number in the zebra finch song system. *Journal of Neuroscience*, *1*(6), 658–673.

Hahnloser, R. H., Kozhevnikov, A. A., & Fee, M. S. (2002). An ultra-sparse code underliesthe generation of neural sequences in a songbird. *Nature*, *419*(6902), 65–70.

Hahnloser, R. H. R., Kozhevnikov, A. A., & Fee, M. S. (2002). An ultra-sparse code underliesthe generation of neural sequences in a songbird. *Nature*, *419*(6902), Article 6902. https://doi.org/10.1038/nature00974

Hampton, C. M., Sakata, J. T., & Brainard, M. S. (2009). An Avian Basal Ganglia-Forebrain Circuit Contributes Differentially to Syllable Versus Sequence Variability of Adult Bengalese Finch Song. *Journal of Neurophysiology*, *101*(6), 3235–3245. https://doi.org/10.1152/jn.91089.2008

Hockett, C. F. (1960). *Logical considerations in the study of animal communication*. American Institute of Biological Sciences.

Hurford, J. R. (2011). *The Origins of Grammar: Language in the Light of Evolution II*. OUP Oxford.

Jin, D. Z. (2009). Generating variable birdsong syllable sequences with branching chain networks in avian premotor nucleus HVC. *Physical Review E*, *80*(5), 051902. https://doi.org/10.1103/PhysRevE.80.051902

Jin, D. Z., & Kozhevnikov, A. A. (2011). A Compact Statistical Model of the Song Syntax in Bengalese Finch. *PLoS Computational Biology*, *7*(3), e1001108. https://doi.org/10.1371/journal.pcbi.1001108

Kadir, S. N., Goodman, D. F. M., & Harris, K. D. (2014). High-dimensional cluster analysis with the masked EM algorithm. *Neural Computation*, *26*(11), 2379–2394. https://doi.org/10.1162/NECO_a_00661

Kao, M. H., Doupe, A. J., & Brainard, M. S. (2005). Contributions of an avian basal ganglia–forebrain circuit to real-time modulation of song. *Nature*, *433*(7026), 638–643. https://doi.org/10.1038/nature03127

Kao, M. H., Wright, B. D., & Doupe, A. J. (2008). Neurons in a Forebrain Nucleus Required for Vocal Plasticity Rapidly Switch between Precise Firing and Variable Bursting Depending on Social Context. *The Journal of Neuroscience*, *28*(49), 13232–13247. https://doi.org/10.1523/JNEUROSCI.2250-08.2008

Katz, L. C., & Gurney, M. E. (1981). Auditory responses in the zebra finch's motor system for song. *Brain Research*, *221*(1), 192–197.

Kosche, G., Vallentin, D., & Long, M. A. (2015). Interplay of Inhibition and Excitation Shapes a Premotor Neural Sequence. *The Journal of Neuroscience*, *35*(3), 1217–1227. https://doi.org/10.1523/JNEUROSCI.4346-14.2015

Kozhevnikov, A. A., & Fee, M. S. (2007). Singing-Related Activity of Identified HVC Neurons in the Zebra Finch. *Journal of Neurophysiology*, *97*(6), 4271–4283. https://doi.org/10.1152/jn.00952.2006

Kroodsma, D. E., & Miller, E. H. (Eds.). (1996). *Ecology and Evolution of Acoustic Communication in Birds*. Cornell University Press. https://doi.org/10.7591/9781501736957

Lewicki, M. S., & Konishi, M. (1995). Mechanisms underlying the sensitivity of songbird forebrain neurons to temporal order. *Proceedings of the National Academy of Sciences*, *92*(12), 5582–5586. https://doi.org/10.1073/pnas.92.12.5582

Long, M. A., & Fee, M. S. (2008). Using temperature to analyze temporal dynamics in the songbird motor pathway. *Nature*, *456*(7219), 189–194. https://doi.org/10.1038/nature07448

Long, M. A., Jin, D. Z., & Fee, M. S. (2010). Support for a synaptic chain model of neuronal sequence generation. *Nature*, *468*(7322), 394–399. https://doi.org/10.1038/nature09514

Margoliash, D. (1983). Acoustic parameters underlying the responses of song-specific neurons in the white-crowned sparrow. *The Journal of Neuroscience*, *3*(5), 1039–1057. https://doi.org/10.1523/JNEUROSCI.03-05-01039.1983

Markowitz, J. E., Ivie, E., Kligler, L., & Gardner, T. J. (2013). Long-range order in canary song. *PLoS Computational Biology*, *9*(5), e1003052.

Marler, P. (1998). Animal communication and human language. *The Origin and Diversification of Language*, *1*, 19.

Martin, R. D., Jablonski, N. G., & Aiello, L. C. (1998). The origin and diversification of language. *San Francisco, CA: California Academy of Sciences*, 69–98.

McCasland, J. S., & Konishi, M. (1981). Interaction between auditory and motor activities in an avian song control nucleus. *Proceedings of the National Academy of Sciences*, *78*(12), 7815–7819. https://doi.org/10.1073/pnas.78.12.7815

Mooney, R., & Prather, J. F. (2005). The HVC Microcircuit: The Synaptic Basis for Interactions between Song Motor and Vocal Plasticity Pathways. *The Journal of Neuroscience*, *25*(8), 1952–1964. https://doi.org/10.1523/JNEUROSCI.3726-04.2005

Nixdorf-Bergweiler, B. E., Bischof, H.-J., Nixdorf-Bergweiler, B. E., & Bischof, H.-J. (2007). *A Stereotaxic Atlas Of The Brain Of The Zebra Finch, Taeniopygia Guttata*. National Center for Biotechnology Information (US).

Nottebohm, F., Paton, J. A., & Kelley, D. B. (1982). Connections of vocal control nuclei in the canary telencephalon. *Journal of Comparative Neurology*, *207*(4), 344–357. https://doi.org/10.1002/cne.902070406

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. https://doi.org/10.1002/cne.901650405

Oikawa, T., Nomura, K., Hara, T., & Koida, K. (2023). A Fine-Scale and Minimally Invasive Marking Method for Use with Conventional Tungsten Microelectrodes. *Eneuro*, *10*(9), ENEURO.0141-23.2023. https://doi.org/10.1523/ENEURO.0141-23.2023

Price, P. H. (1979). Developmental determinants of structure in zebra finch song. *Journal of Comparative and Physiological Psychology*, *93*(2), 260–277. https://doi.org/10.1037/h0077553

Rajan, R., & Doupe, A. J. (2013). Behavioral and Neural Signatures of Readiness to Initiate a Learned Motor Sequence. *Current Biology*, *23*(1), 87–93. https://doi.org/10.1016/j.cub.2012.11.040

Simpson, H. B., & Vicario, D. S. (1990). Brain pathways for learned and unlearned vocalizations differ in zebra finches. *Journal of Neuroscience*, *10*(5), 1541–1556.

Sober, S. J., Wohlgemuth, M. J., & Brainard, M. S. (2008). Central contributions to acoustic variation in birdsong. *Journal of Neuroscience*, *28*(41), 10370–10379.

Sossinka, R., & Böhner, J. (1980). Song Types in the Zebra Finch *Poephila guttata castanotis* [1]. *Zeitschrift Für Tierpsychologie*, *53*(2), 123–132. https://doi.org/10.1111/j.1439-0310.1980.tb01044.x

Striedter, G. F., & Vu, E. T. (1998). Bilateral feedback projections to the forebrain in the premotor network for singing in zebra finches. *Journal of Neurobiology*, *34*(1), 27–40. https://doi.org/10.1002/(SICI)1097-4695(199801)34:1<27::AID-NEU3>3.0.CO;2-E

Suzuki, T. N., Wheatcroft, D., & Griesser, M. (2016). Experimental evidence for compositional syntax in bird calls. *Nature Communications*, *7*(1), 10986. https://doi.org/10.1038/ncomms10986

Thompson, J. A., & Johnson, F. (2007). HVC microlesions do not destabilize the vocal patterns of adult male zebra finches with prior ablation of LMAN. *Developmental Neurobiology*, *67*(2), 205–218. https://doi.org/10.1002/dneu.20287

Vicario, D. S. (1991). Organization of the zebra finch song control system: Functional organization of outputs from nucleus robustus archistriatalis. *Journal of Comparative Neurology*, *309*(4), 486–494. https://doi.org/10.1002/cne.903090405

Vicario, D. S., & Nottebohm, F. (1988). Organization of the zebra finch song control system: I. Representation of syringeal muscles in the hypoglossal nucleus. *Journal of Comparative Neurology*, *271*(3), 346–354. https://doi.org/10.1002/cne.902710305

Vu, E. T., Mazurek, M. E., & Kuo, Y.-C. (1994). Identification of a forebrain motor programming network for the learned song of zebra finches. *Journal of Neuroscience*, *14*(11), 6924–6934.

Wild, J. M. (1993). Descending projections of the songbird nucleus robustus archistriatalis. *Journal of Comparative Neurology*, *338*(2), 225–241. https://doi.org/10.1002/cne.903380207

Wild, J. M. (1997). Neural pathways for the control of birdsong production. *Journal of Neurobiology*, *33*(5), 653–670. https://doi.org/10.1002/(SICI)1097-4695(19971105)33:5<653::AID-NEU11>3.0.CO;2-A

Woolley, S. C., Rajan, R., Joshua, M., & Doupe, A. J. (2014). Emergence of Context-Dependent Variability across a Basal Ganglia Network. *Neuron*, *82*(1), 208–223. https://doi.org/10.1016/j.neuron.2014.01.039

Yazaki-Sugiyama, Y., Yanagihara, S., Fuller, P. M., & Lazarus, M. (2015). Acute inhibition of a cortical motor area impairs vocal control in singing zebra finches. *European Journal of Neuroscience*, *41*(1), 97–108. https://doi.org/10.1111/ejn.12757

Yu, A. C., & Margoliash, D. (1996). Temporal Hierarchical Control of Singing in Birds. *Science*, *273*(5283), 1871–1875.

Zhang, Y. S., Wittenbach, J. D., Jin, D. Z., & Kozhevnikov, A. A. (2017). Temperature Manipulation in Songbird Brain Implicates the Premotor Nucleus HVC in Birdsong

Syntax. *The Journal of Neuroscience*, *37*(10), 2600–2611.

https://doi.org/10.1523/JNEUROSCI.1827-16.2017