Examining the role of dorsomedial nucleus of the intercollicular complex (DM) in the context of song production in zebra finches

A thesis submitted towards partial fulfilment of BS-MS dual degree program

by

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For the study conducted under the guidance of

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Certificate

This is to certify that this dissertation entitled **"Examining the role of dorsomedial nucleus of the intercollicular complex in the context of song production 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 work carried out by **"Aditi Agarwal"** at the **"Indian Institute of Science Education and Research, Pune"** under the supervision of **"Dr. Raghav Rajan, Ramalingaswami Fellow, Department of Biology"** during the academic year 2019-2020.

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Declaration

I hereby declare that the matter embodied in the report entitled “Examining the role of dorsomedial nucleus of the intercollicular complex in the context of song production in zebra finches” are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Raghav Rajan and the same has not been submitted elsewhere for any other degree.

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Contents

Abstract

Communication is vital for survival in the animal kingdom. Communication through vocal learning is an ability possessed by very few taxa. Zebra finch serves as a good model system to study evolution of vocal learning. Akin to humans, juvenile finches memorise the song from an adult tutor in the plastic phase and ultimately produce the same song for life. Song is a stereotyped sequence of sounds consisting of various elements called syllables. The underlying neural circuitry is highly specialised and involves both telencephalic and midbrain nuclei. DM (dorsomedial nucleus of the intercollicular complex), a midbrain nucleus is primarily known for producing unlearned vocalisations- 'calls'. DM, however, is also anatomically a part of the circuitry for producing song but it's role in song production remains elusive. The role of DM was assessed by lesioning. Lesions were not specific to DM and caused damage in the surrounding area ICo (intercollicular complex). Songs were altered in a few birds which led to the production of unrecognizable syllables and loss of song tempo. Upon correlating changes in song with sites of lesions, changes could be attributed to lesions in central part of ICo lying in the medial portion of the brain. Lesions in some other parts of ICo did not affect song. The role of DM was further assessed by recording neural activity in DM during song. Preliminary data shows changes in neural activity during song. Effect of DM lesions was different from what is seen for lesions of other song nuclei. My results suggest that certain parts of ICo are important for normal song production. More generally, my study suggests that the song circuitry is highly decentralised with telencephalic and midbrain areas contributing to normal song production.

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List of abbreviations of different brain nuclei

Area (Abbreviated name)	Full name
HVC	HVC (Proper name)
RA	Robust nucleus of the arcopallium
ICo	Intercollicular complex
Uva	Nucleus uvaeformis
nXIIts	Hypoglossal motor nucleus
DM	Dorsomedial nucleus of the intercollicular complex
LMAN	Lateral Magnocellular nucleus of the anterior neostriatum
DLM	Dorsolateral nucleus of the thalamus
RAm/PAm	Retroambigualis/Paraambigualis
PAG	Periaqueductal gray

Table 1: Abbreviations for various brain areas in zebra finches and mammals

Introduction

Communication, in the simplest of terms, is the transfer of information from the sender to the receiver. Communication serves an important purpose in the animal kingdom especially when animals live in close proximity to each other. The need to communicate could arise if one member of the group had to intimate the others in the herd about the availability of food at a particular location; to alarm others about the danger of a potential predator lurking around; or simply to attract a mate. Animals can communicate through visual signals, olfactory signals, or through vocal exchanges (Bradbury, J W, and Sandra L. Vehrencamp. Principles of Animal Communication. Sunderland, MA: Sinauer Associates, 1998).

In the animal kingdom, many groups of organisms have shown the ability to vocalize but very few learn their vocalizations (reviewed in Petkov and Jarvis, 2012) -in essence, the act of listening to particular sounds, modifying and learning them through vocal imitation. A few such groups which are capable of producing learned vocalizations include humans, bats (Knorschild M, 2014), songbirds (Wilbrecht M, Nottebohm F, 2003) and elephants (Janik VM, Slater PJ, 1997; reviewed in Petkov, Jarvis, 2012). Given the limited taxa capable of producing learned vocalizations, it is interesting to study how social communication evolved through learning of vocalizations. The ability to produce learned vocalizations requires more specialized neural circuits and this makes it even more interesting to study such vocalizations (Reviewed in Petkov and Jarvis, 2012).

People often turn to other model systems, primarily owing to the ease of breeding lower organisms in captivity and maintaining them, for studying the evolution of communication and speech production. Much like human speech, song birds like zebra finches are capable of producing rhythmic learned vocalizations called ‘song’ (Doupe AJ and Kuhl PK, 1999 and Bolhuis JJ, Okanoya K, Scharff C, 2010). Some of the early evidence for vocal learning in songbirds comes from William Thorpe’s work on

chaffinches (W.H. Thorpe, 1958). He showed that juveniles which had no exposure to the song of an adult bird, sang highly abnormal songs as adults. Juvenile chaffinches reared in social isolation but exposed to playbacks of an adult song, sang songs similar to the template they were provided with through playbacks. His experiments suggested that songbirds learn their vocalisations. Further, experiments by Peter Marler showed that songs sung by white-crowned sparrows at different geographical locations had little similarity and that song from a particular locality appeared like a local dialect (Marler, 1970). He hypothesized that the song sung by all individuals in an area could be similar because of auditory exposure followed by vocal imitation of only a particular song dialect. Young individuals (juveniles) raised with other adult males (tutor) produced a song similar to that of the tutor and not of their biological father, suggesting that the ability to vocalize is innate but they learn vocalizations. Many such elegant experiments have been carried out in zebra finches as well (Price, 1979). Juvenile zebra finches raised in social isolation but exposed to songs through playbacks produced a song similar to the ones they were exposed to, suggesting that zebra finches like white-crowned sparrows and chaffinches have the ability to learn their vocalizations (Price 1979). Once the song crystallizes, zebra finches sing the same song over multiple renditions and such a song is termed as a stereotyped song.

Zebra finches serve as an excellent model system to study the neural mechanisms for the production of learned vocalisations (Fischer and Scharff, 2009; reviewed in Fee and Scharff 2010). Vocal learning in both humans and finches requires auditory exposure during the sensory phase followed by vocal imitation for the speech/song to get stereotyped (Konishi 1965; Immelman, 1969). Traditionally, it was believed that song was a way to communicate with conspecifics, particularly to attract mates (Catchpole and Slater, 1995; Kroodsma and Miller, 1996). However, zebra finches sing in social isolation as well. The song therefore can be produced in two contexts (Immelman and Butterfield, 1979; Immelman; Sossinka and Bohner, 1980). Songs sung in the presence

of a female are termed as ‘directed’. Self-initiated songs produced in the absence of conspecifics are referred to as being 'undirected'.

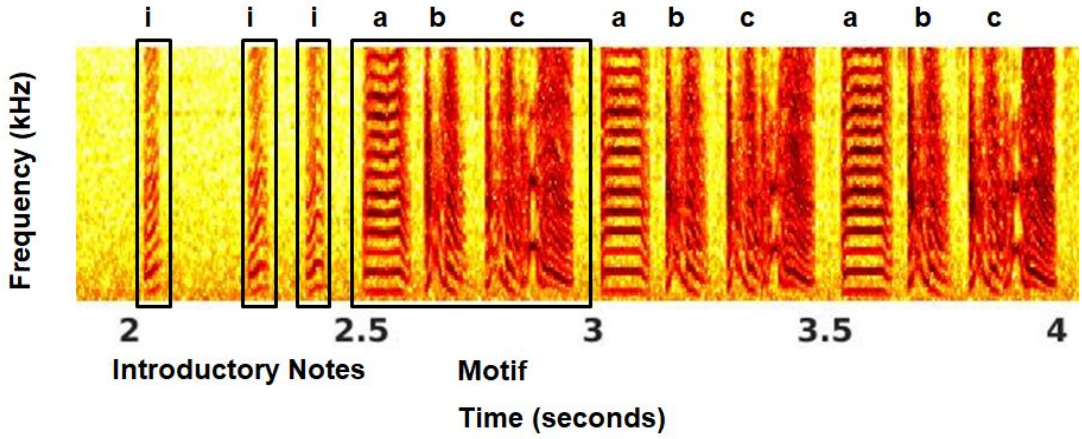

Fig 1: Visual representation of one instance of a zebra finch song- ’spectrogram’. Shades of red indicate the intensity in a particular frequency content. Yellow indicates silence. The song starts with introductory notes that progress to a stereotyped motif. Top: labels given to individual elements of the song. ‘i’ represents introductory notes. ‘a’,’b’,’c’ are the motif syllables. Time interval between the syllables and syllable ordering within a motif is stereotyped across bouts.

The song of a zebra finch starts with a set of short repeating vocalisations called ‘introductory notes’ (INs) (Sossinka and Bohner 1980, Price 1979). The number of these introductory notes differs from one rendition to the other. However, the variability in the mean number of introductory notes across days is negligible (Divya Rao, personal communication). Introductory notes are followed by stereotyped ‘motifs’ (Figure 1). Each motif consists of song syllables occurring in a particular order. Further, each syllable in a motif is produced at specific latencies after the previous syllable implying that the gap durations are also highly consistent across renditions. Although the motif is highly stereotyped, the number of motifs produced per rendition varies. Every rendition of the song which has two seconds silence before and after is termed as a song bout (Sossinka and Bohner, 1980).

Zebra finches have a specialised neural circuitry for learning and producing songs (Nottebohm et al 1976, Simpson and Vicario 1990). The song has characteristic acoustic and temporal features which are learned and controlled independently (Marler

and Peters 1977). A zebra finch brain has many song circuits which are either involved in song learning or maintaining the song or both. It is the interaction at the level of these brain wide circuits which gives rise to such an ordered song. However, the way the order in a song is generated remains poorly understood. Certain brain areas like LMAN (lateral magnocellular nucleus of the anterior nidopallium) and DLM (dorsolateral nucleus of the thalamus) are essential for learning the song, but they do not play a role in producing the song (Fee MS 2005). Lesions in these areas, once the song has crystallized have no effect on the song.

The circuitry for producing the song involves telencephalic areas, HVC (proper name) and RA (robust nucleus of arcopallium). HVC projects to RA (Nottebohm, 1976) which provides motor commands to areas downstream for the production of a stereotyped song (Nottebohm, 1976; Hahnloser, 2002; Figure 2a and 2b). RA then projects to various midbrain and brainstem nuclei- RAm/PAm (Retroambigualis/Paraambigualis), DM (Dorsomedial nucleus of the Intercollicular complex) and nXIIts (hypoglossal motor nucleus) respectively (Figure 2b; Gurney 1981, Nottebohm et al 1982, Simpson and Vicario 1990). RAm/PAm and nXIIts control the respiratory and syringeal muscles. DM is responsible for yet another type of unlearned vocalisation- 'calls' (Simpson and Vicario, 1990). DM and RAm/PAm both project to Uva which then projects to HVC and completes the loop (Fig 2b; Nottebohm, 1982; Striedter and Vu, 1998).

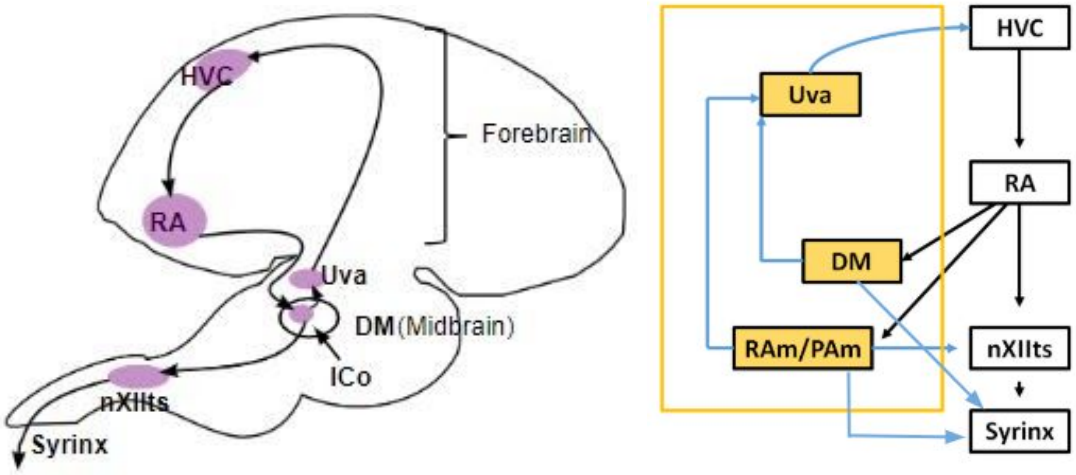

Fig 2a: Brain areas involved in song production in zebra finches. Image represents a cartoon figure of the sagittal section of a zebra finch brain containing a few areas important for song production. HVC, RA lie in the forebrain. DM and the associated nucleus ICo form the midbrain nuclei. Uva in the midbrain is equivalent to the mammalian thalamus. HVC projects to RA, RA to DM, which then via Uva projects to HVC and completes the loop. RA and DM also project to the syrinx. (Image credits: Made with Shikha Kalra)

2b: Block diagram of neural circuitry. RA projects to respiratory nuclei RAm/PAm and nXIIts which project to syrinx. RA also connects to syrinx via DM. Areas in yellow indicate midbrain areas.

Previous studies demonstrate that complete HVC lesions alter the crystallised adult song and make it more like a juvenile bird's song in it's learning phase (Aronov et al, 2008). Similarly, complete RA lesions abolish the song completely whereas partial RA lesions alter only the fast frequency modulations of the song and not the temporal features (Simpson and Vicario, 1990). Lesions in thalamic area-Uva have slightly different effects on the song. While unilateral Uva lesions lead to temporary effects on the syllable structure, bilateral lesions alter the song permanently (Coleman and Vu, 2005). Both unilateral and bilateral Uva lesions lead to the production of syllables which have no similarity with the syllables produced before lesions (Danish HH, Aronov D, Fee MS, 2017). The gap durations post Uva lesions have a greater spread around the mean. Characteristic peaks corresponding to stereotyped gap durations disappear as a result of Uva lesions (Danish HH, Aronov D, Fee MS, 2017). While lesions in all three-HVC, Uva and RA lead to songs which have unrecognizable syllables, HVC lesions result in syllable duration histograms that follow an exponential trend; Uva lesions lead to syllable duration histograms that have a peak at around 50-100 Hz and thereafter follow an exponential trend (Danish HH, Aronov D, Fee MS, 2017); partial RA lesions alter only the syllable structure (fast frequency modulations) but do not have an effect on syllable durations or gap durations (Ashmore, Bourjaily et al, 2007). This shows that each nucleus plays a different role in maintaining and producing the song and the song circuitry is decentralised. While the role of these different nuclei have been explored to a great extent, it is still not well understood what drives activity in Uva. Possible candidates are midbrain nuclei RAm/PAm or DM that may drive activity in Uva.

DM is a potential candidate and is of particular interest since DM is known to produce unlearned vocalisations (Simpson and Vicario, 1990) but is a part of the circuit for the production of learned vocalisations as well. RA, HVC which control learned vocalisations (song), also control the production of unlearned vocalisations-calls, in concert with DM (Simpson and Vicario, 1990). Lesions in either HVC or RA disrupt the frequency modulations of calls and complete bilateral DM lesions abolish long calls (Simpson and Vicario, 1990). This then begs the question, can DM, like HVC and Uva control both learned and unlearned vocalisations?

DM lies in the dorsomedial portion of a bigger nucleus ICo (Intercollicular complex). What is already known about DM (and ICo) in zebra finches is that it is analogous to the mammalian midbrain nucleus PAG (periaqueductal gray; MA Kingsbury, 2011), which is responsible for the production of unlearned vocalizations. Elaborate studies by several people (TG Brown, 1915; Magoun, 1937; reviewed in Jurgen, 1994) state that electrical stimulation in PAG in different organisms like Rhesus Macaques, cats, squirrel monkeys, bats, guinea pigs, etc elicits unlearned vocalisations (reviewed in Jurgens, 1994). PAG lesions in various organisms lead to mutism (Jurgens and Pratt, Adametz and O'Leary, Randall) and single unit recordings in PAG suggest that it is involved in vocal motor control. Similar to the role played by PAG, DM has been extensively studied in the context of call production (Seller, 1981; Fukushima Aoki 2000; Simpson and vicario,1990). A large number of studies show that electrical stimulation in DM in both the awake and the anesthetized state elicits calls similar to bird's unlearned vocalisations (Seller 1981; Brown 1965; Simpson and Vicario, 1990; Fukushima and Aoki, 2000). There are no published studies which look at the role of DM in song production. Most of the studies which examine the role of Uva and RA only speculate the involvement of DM. Only a few computational models like the one proposed by Gibb and Abarbanel (Gibb and Abarbanel, 2009; Gibb and Abarbanel, 2011) explore the role of DM in this context. The model predicts that input from DM is important for regulating neural activity at the level of HVC. The findings of the model were that DM maintains

excitation at the level of Uva which in turn feeds back to HVC. Although the role of DM in the context of call production is well explored, its role in song production remains elusive and through this study I aim to examine its role in this context.

Specific aims and hypotheses

The aim of this study is to examine the role of DM in the context of song production by examining the effects of DM lesions on the features of the song post-surgery. The following could be the outcomes of DM lesions:

1) DM lesions may alter the features of the song. Effects of lesions could be similar to that observed for Uva lesions, given that DM projects to Uva.
2) Alternatively, DM may not play a role in song production at all. DM may only be responsible for the production of calls. During song production, RA controls vocal production by means of its projections to other nuclei like RAm/PAm and nXIIts.

Methods

All protocols were approved by IAEC (Institutional animal ethics committee at IISER Pune) under the guidelines of CPCSEA, New Delhi (committee for the purpose of control and supervision of experiments on animals).

n=20 adult zebra finches (>90 days post hatch, 18 males and 2 females) were used for all the experiments. Few of them were acquired through a local pet dealer (n=8), the rest were bred in the lab (n=12). Birds were housed in cages with ad-libitum access to food and water. Nutritional supplements were provided in the form of cuttlebone, boiled egg and egg shells, sprouts, coriander leaves, cucumber and apple slices. A 14/10 hr light/dark cycle was maintained in the bird housing colony. Temperature in the facility was kept constant at 25 °C.

Song recording and data acquisition

Birds were socially isolated from their conspecifics for 2-4 days in an acoustic enclosure called a 'sound box' (Newtech Engineering systems, Bangalore, India). An omnidirectional condenser microphone (AKG Acoustics C417PP) was placed on the cage through which vocalisations were recorded. Microphone signals were amplified using a mixer (Behringer XENYX 802) digitized and stored on a computer (sampling rate: 44100 Hz). A visual representation of such a wave is called a 'spectrogram' (frequency vs time depiction of a soundwave- Figure 1) Spectrograms were plotted for each rendition and used later for visual examination (See appendix).

Zebra finches vocalise both in the presence as well as the absence of a conspecific. Songs sung in social isolation were saved as 'undirected' songs. To elicit 'directed' songs, a female was exposed to the male at random times during the day for a duration of 5-10 mins. Songs sung during this interval were saved as female-directed or simply as directed songs.

Surgery

All surgery related equipment like scalpel blade, forceps, scissors, etc were sterilized by placing them in a hot bead sterilizer (Fine Science Tools) for a few minutes and then cleaning thoroughly with 99.9% ethanol.

An oral analgesic (meloxicam, 12.5 mg/kg) was administered 2 hrs before the surgery. Birds were anesthetized by injecting a combination of ketamine (30mg/kg), xylazine (3mg/kg) and diazepam (7mg/kg), intramuscularly. Effectiveness of the anesthesia was determined by response to toe press. No response after 5-10 minutes after injecting anesthesia indicated that the bird was deeply anesthetized. In a few cases where the bird responded even after 30 minutes of administering anesthetics, a second dose of anesthesia was injected (1/4th dosage of ketamine with 1/4th dosage of diazepam). The bird was placed under a heat lamp and wrapped in a warm 'bird jacket' to help regulate body temperature during the anesthetized state. Feathers over the ear pinnae were

trimmed using a pair of scissors for ease of fixing the bird to the ear bars on the stereotaxic apparatus (Figure 3, Narishige group, Japan). The bird's head was held in place with the help of a beak bar and ear bars. Further, the bird's body was held in place by taping the jacket onto the stereotaxic apparatus. Feathers on the scalp were plucked out for accessing the skull. The layer of skin on the skull was anesthetized by injecting a local anesthetic, lignocaine (75-100 µL). A tiny incision was made in this layer and it was cut open, exposing the skull. The skull was cleaned using NaCl solution (0.9% w/v). A 'Y-shaped' blood vessel lining the ridge between the two hemispheres was spotted and the centre of this 'Y' was taken as the reference for locating brain areas. Stereotaxic coordinates for locating different brain areas were obtained from stereotaxic atlas for zebra finches (Nixdorf-Bergweiler BE, Bischof HJ, 2007; https://www.ncbi.nlm.nih.gov/books/NBK2348/). A craniotomy was made over DM (A/P: 0.8-1.3, M/L: 2.5, depth: 4.8-5.1 mm, beak angle: 45°) using a scalpel. The layer of dura above the brain was removed using a scalpel blade and saline (0.9% w/v NaCl) was sprayed over the exposed areas to prevent it from drying.

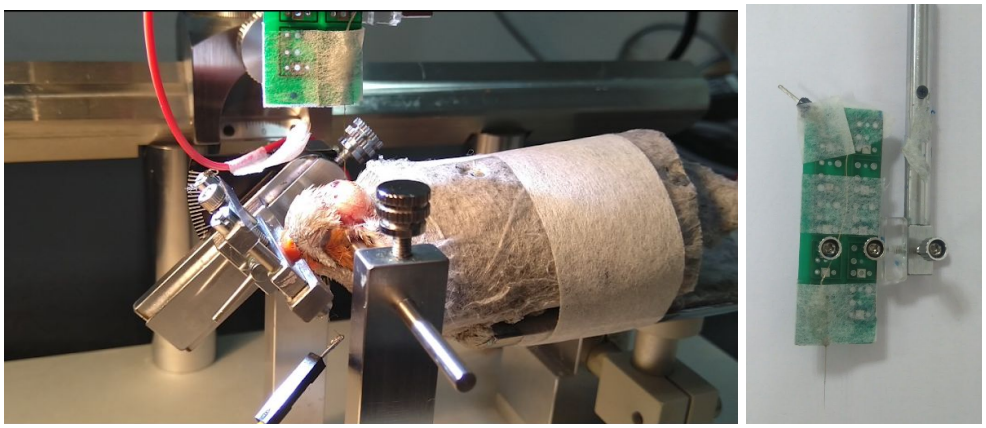

Fig 3: Left: Stereotaxic apparatus.An anesthetized bird is held in place with the help of ear bars and beak holder. Right: Electrode attached to the micro-manipulator

Pilot experiments

Extensive studies have been carried out which show that electrical stimulation of DM elicits calls even in the anesthetized state (Fukushima and Aoki, 2000; Vicario and

Simpson, 1990). This was used to locate DM reliably in the anesthetized state. DM was located using a set of stereotaxic coordinates as described above (Nixdorf-Bergweiler BE, Bischof HJ, 2007) in 3 zebra finches (2 females and 1 male). Bipolar electrodes (stainless steel, diameter 110 µm, pitch: ~0.75 mm, impedance ~1 MΩ) were used to identify coordinates where calls were elicited upon electrical stimulation (biphasic pulse, 200 Hz for 2 seconds) in the anesthetized state (n=1). The pulses were delivered using an arduino coupled with a flexible stimulus isolator (ISO-flex A.M.P.I instruments). Different sites along the anterior-posterior (A/P) and medial-lateral (M/L) axes were stimulated until calls were elicited (A/P: 0.85-1.3, M/L: 2.45-2.63, depth: 4.5-5.6, beak angle: 45°). Since a bipolar electrode led to stimulation of two sites close to each other, it was difficult to assess which one of them was in DM. Once the coordinates were identified, monopolar electrode (Tungsten microprobe WE3PT15.0F3, impedance: 0.5-6 MΩ) was used in this radius to narrow down the coordinates further. Keeping the M/L fixed at 2.5 mm, all the sites from A/P: 0.85 to 0.85+0.75mm (distance between the two electrodes) were stimulated (depth: 4.5 to 5.6 mm). Silver wire was tucked under the second layer of the skull and acted as the reference electrode. DM was located further away in the anterior direction from the reference (Y) than the coordinates prescribed in the stereotaxic atlas (DM located at A/P: 1.7, M/L: 2.5, depth: 4.9 -5.4; coordinates in stereotaxic atlas: A/P: 0.85-1.3, M/L: 2.46-2.63, depth: 4.8-5.2 mm). Sites where calls were elicited upon stimulation were noted for future experiments and lesioned by passing monophasic current (anodal and cathodal) to verify during histology. Different sites were lesioned with different duration (10-30 s) and current amplitude (30-80 µA) parameters to standardize the size of lesions.

Lesion experiments

Seventeen adult male zebra finches were used for lesion experiments. In two zebra finches, current was passed to elicit calls but those sites were not lesioned (red67pink43 and brown36orange36). One zebra finch (black05white16) was used for recording neural activity; the electrode was implanted in DM however no lesions were

made. These three birds have been included for analysis to assess if multiple penetrations destroy DM and hence affect the song. A few zebra finches died during surgery (n=3) or immediately after surgery (n=1). However, the calls elicited during surgery were recorded and used later for further analysis. Data for one bird (green43blue26) was excluded from all the analyses as the bird sang only 'directed' songs. Upon electrical stimulation in DM in the anesthetized state, calls were elicited in a few cases (n=6), ICo typical behaviours like tail quivering and eye opening in a few others (n=10) and none of the above in a few cases (n=2). Sometimes ICo typical behaviours were elicited along with calls (n=6) (Table 2). Different sites in the vicinity of coordinates standardized through pilot experiments were stimulated. Once calls were elicited, the variation in the kind of calls as a consequence of different stimulation parameters (frequency: 50-400 Hz, current amplitude: 30-100 μA, duration: 0.5-4 s) was documented and recorded on a personal mobile phone (Nokia 6.1; n=6). To assess the effect of various stimulation parameters on the features of calls elicited, multivariate linear regression was carried out (predictor variables: current amplitude, duration of stimulus and frequency of stimulus; response variable: duration of elicited call and frequency of elicited call). On the basis of auditory examination alone, elicited vocalisations varied with the stimulation parameters. Production of multiple calls after stimulation with currents as low as 30 μA was taken to be DM based on previous literature (Simpson and Vicario 1990). After locating DM, multiple lesions were made (M/L: 2.5, A/P: multiple sites from 1.4-1.8, depth: multiple depths from 5-5.6) by passing a monophasic pulse (50-200 μA, 10-30 s). In a few cases where no calls were elicited, lesions were made at locations where ICo typical behaviour was elicited. Since DM lies in ICo, it was assumed that lesioning a bigger volume of ICo would lesion a part of DM as well. The exposed brain area was covered by mineral oil. The skull was covered by dental cement and the bird was left to recover under a heat lamp. Once the bird was active again, songs were recorded for 3-5 days or till sufficient number of song bouts were obtained (~25 bouts).

Various behaviours evoked upon electrical stimulation in the anesthetized state

Bird name	Tail flicking, eye opening	Calls elicited
Red 67pink43	Yes	Yes
Green16black84	Yes	Yes
Black05white16	Yes	Yes
Green43blue26	Yes	Yes
Green54pink90	Yes	Yes
Brown74orange74	Yes	Yes
Brown72orange72	Yes	No
Green68orange95	Yes	No
Red02white02	Yes	No
Green41green44	yes	No
Brown53orange62	Yes	No
Green20orange40	No	No
Brown36orange36	No	No

Table 2: Summary of behaviours observed upon electrical stimulation in the anesthetized state. Tail flicking and eye opening are ICo typical behaviours. Given the vicinity of DM to ICo, it is likely that both the behaviours are elicited together in a few cases. It is possible that no behaviour was elicited in a few cases as it may depend on the state of anesthesia.

Neural recording in DM

n=1 male zebra finch (black05white16) was used for implanting electrodes in DM to record neural activity. Customised parts were 3-D printed and assembled together to make a movable implant as described previously (Rajan and Doupe, 2013; Rajan 2018). Electrodes (FHC-tungsten microelectrodes, lot # 2322756, impedance: 9 MΩ) were inserted and soldered onto a connector (Figure 4). DM was located as described above. During surgery, the stereotaxic apparatus was placed in a Faraday cage (bird cage used as a Faraday cage) to prevent other electromagnetic radiations adding to the noise. Further, all the electronic equipment was connected to a common ground for denoising. Once DM was identified by electrical stimulation, the implant was impaled and fixed into place on the skull with dental cement. The electrodes were kept at 1 mm above the actual location of DM. The bird was left to recover. After recovery, the electrodes were lowered slowly each day (about 50-100 μm in every session). Only 2-3

sessions were conducted in a day. During each session, signal was amplified using a head-stage amplifier (Intan technologies). Baseline neural activity was recorded through Intan RHD 2000 software for 2-3 minutes after which a female was exposed, in response to which the bird vocalised. The song and the neural activity were recorded simultaneously. This was analysed later using custom MATLAB scripts.

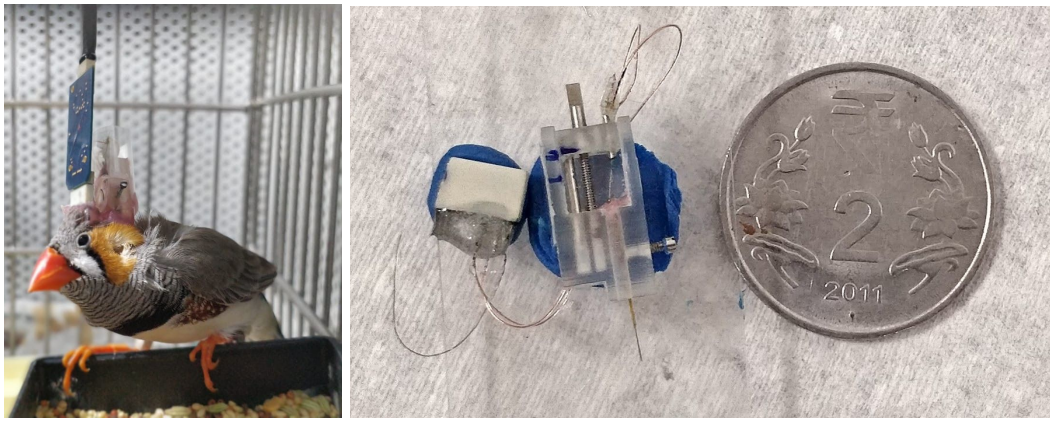

Fig 4: left: Adult male zebra finch with implant in DM. Blue chip represents the head-stage which feeds into the intan software. Right: Implant with connector placed next to a two rupee coin for comparison of size. (Picture credits: Divya Rao)

Identifying DM through anatomical connectivity

As calls were elicited in only a few cases, in order to verify that the coordinates obtained from pilot experiments indeed corresponded to DM, a few other methods were used to locate DM as outlined below.

1) **Electrical stimulation in RA and recording responses in DM**

In n=1 zebra finch, DM was located as described above (green26blue12). A stimulating electrode (microprobe) was held at a site where calls were elicited. RA was located using a set of stereotaxic coordinates (A/P: -0.25-0, M/L: 2.43, beak angle: 45°, depth: 2.5-3.1 mm) and also by recording baseline neural activity (experiment done with Divya Rao). RA has distinct baseline firing activity even in the anesthetized state. Once coordinates for RA were identified, RA was electrically stimulated and the response was noted in ipsilateral DM. This was done to verify that the site that evoked calls was

indeed DM (RA projects to DM). Further, different sites near DM were recorded. Some of these sites in DM were lesioned for further verification through histology.

Due to noise, RA could not be located reliably. More of such experiments need to be done to verify that the site where calls are elicited upon electrical stimulation is indeed DM.

2) Tracer Injections to mark the tract from RA to ipsilateral DM

Motor nucleus RA projects to DM (Simpson and Vicario,1990). Anterograde tracers in RA were used for tract tracing experiments (n=1; green70blue31). RA was located as described above. Glass capillary (diameter ~20 µm; Aurnab Ghose Lab, IISER Pune) was loaded with 0.2% w/v KCl solution and 10% w/v tetramethylrhodamine dye (invitrogen, dextran conjugated, 3000 mW, anionic, lysine fixable) in 25 mM PB (phosphate buffer) using a picospritzer (Harvard apparatus; Aurnab Ghose lab, IISER Pune). Glass capillary was lowered in RA and the dye was iontophoresed by passing negative monophasic pulses (5 µA, 7 seconds on/ 7 seconds off) for 10 minutes (Akutagawa and Konishi, 2005). This was done to expel the negatively charged dye out of the capillary. The capillary was removed and the skull was covered with dental cement. The bird was left to recover for 3-4 days after which it was sacrificed. The brain was used to identify sites where fluorescence was visible.

3) DiI tracing to mark electrode tracts

In n=1 birds (green26blue12), craniotomy was made over DM as described above. Electrodes were coated with DiI (Aurnab Ghose Lab, IISER Pune) before impaling in order to visualize their path. DiI belongs to the broad category of lipophilic dyes called carbocyanines. As the electrode is impaled, all the neurons in contact with the dye take it up through their plasma membrane. As a result of this all the neurons in the path of the electrode turn magenta in colour which shows up distinctly during histology. Bird was sacrificed right after surgery. Although DiI could be visualised in many sections,

due to delay in imaging, most DiI had moved to adjacent areas and hence the tracts could not be imaged.

Histology

After the experiments were over, birds were sacrificed by injecting a lethal dose of anesthesia (25 µL of 50 mg/mL ketamine and 20 mg/mL xylazine each, 50 µL of 5 mg/mL diazepam). Feathers over the abdomen were plucked. A small slit was made to expose the abdominal cavity. The cavity was then cut open using a pair of scissors. Heart was located and the connective tissue around it was cut using a scalpel blade. A tiny incision was made in the right atrium and a needle was inserted in the left ventricle. The bird was then perfused intracardially by first clearing out blood using saline (0.9%w/v NaCl-100 mL). As saline was injected through the left ventricle, blood started flowing out through the right atrium. Once the clear saline started coming out of the right atrium, 100-150 mL of 4% w/v PFA (paraformaldehyde) was injected to fix the tissue. The brain was taken out of the skull and stored in 4% w/v PFA for a few days for post fixing. It was then transferred to 30% w/v sucrose solution in PBS (cryopreservative). Once the brain sank to the bottom implying that all the liquid was out, it was sectioned in a cryostat apparatus (Leica biosystems). The resulting sagittal sections-40 µm thick, (Figure 5) were stored in 24-well plates containing 1x PBS solution. They were either mounted on poly-L-lysine coated slides or gelatin coated slides (0.5g/L chromium potassium sulphate, 5g/L gelatin, 0.75g/L thymol; Aurnab Ghose Lab; Siddhesh Kamat Lab).

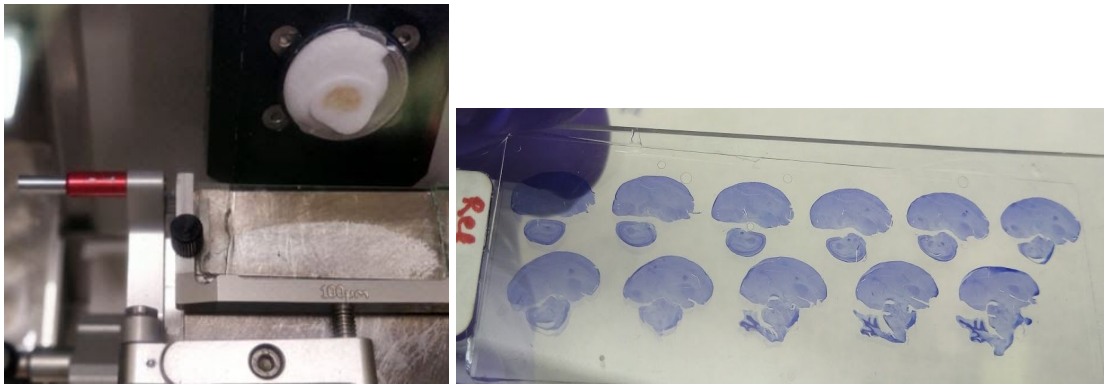

Fig 5: Left: Cryosectioning. Every rotation of the blade cuts sections which are 40 μm thick (Image credits: Divya Rao). The sections are then collected and mounted on slides. Right: sagittal sections stained with cresyl violet and mounted on a poly-L-lysine coated slide.

Staining

Every third section was mounted on a glass slide (Figure 5) coated with poly-L-Lysine or gelatin. Sections were left to dry overnight. Slides were placed in xylene for 5 minutes to remove traces of freezing media. Sections were then slowly hydrated by passing through ethanol solutions of various concentrations (100% w/v ethanol for 5 minutes, followed by 70% w/v ethanol for 2-3 minutes, followed by 20% w/v ethanol for 2-3 minutes). Before immersing in cresyl violet, slides were placed in distilled water for 1-2 minutes. They were then stained using 1% w/v Cresyl Violet solution (Sigma aldrich). Slides were taken out every 2 minutes to assess how well the sections had taken up the stain. Once the sections had evenly absorbed the stain (~5 minutes) and various brain areas could be visualised as dark purple spots, sections were passed through distilled water for removing excess stain. Differentiator solutions were used to improve the contrast of dye uptake by further removing excess stain. These solutions are a combination of ethanol and acetic acid in different proportions (270 mL of 70% w/v ethanol+ 3 mL acetic acid; 270 mL 100% w/v ethanol+ 3 mL acetic acid). Sections were then dehydrated by placing in 100% w/v ethanol solution (2 dips) and in xylene (5 minutes). Slides were preserved by mounting with DPX. Slides were then examined under a brightfield microscope to spot the sites of lesion. A few slides were imaged under a brightfield microscope (Leica microscopes-DM6B; camera: Hamamatsu C11440-22CU; Microscopy facility, IISER Pune), 10x magnification (exposure:12-18, intensity: 120-135). Multiple images of DM were taken and stitched (40% overlap) together using image-tiling features in the microscope (software: Leica Application Suite X). Resulting images were analysed using ImageJ (Fiji) to quantify the volume of DM and ICo and the lesions (Figure 6). Boundaries were marked around ICo and the area was measured using ImageJ. Each area measurement was multiplied by 40 μm x 3 (since every third section was taken) to compute volume. All volume measurements

were then added to compute the net volume of ICo. Volume of area lesioned was calculated in a similar fashion.

In a few birds (n=4), sections were stained using silver stain (Girish Ratnaparkhi Lab, IISER Pune). This was done to label the projections. However, the protocol did not work very well and silver staining was not attempted for more sections.

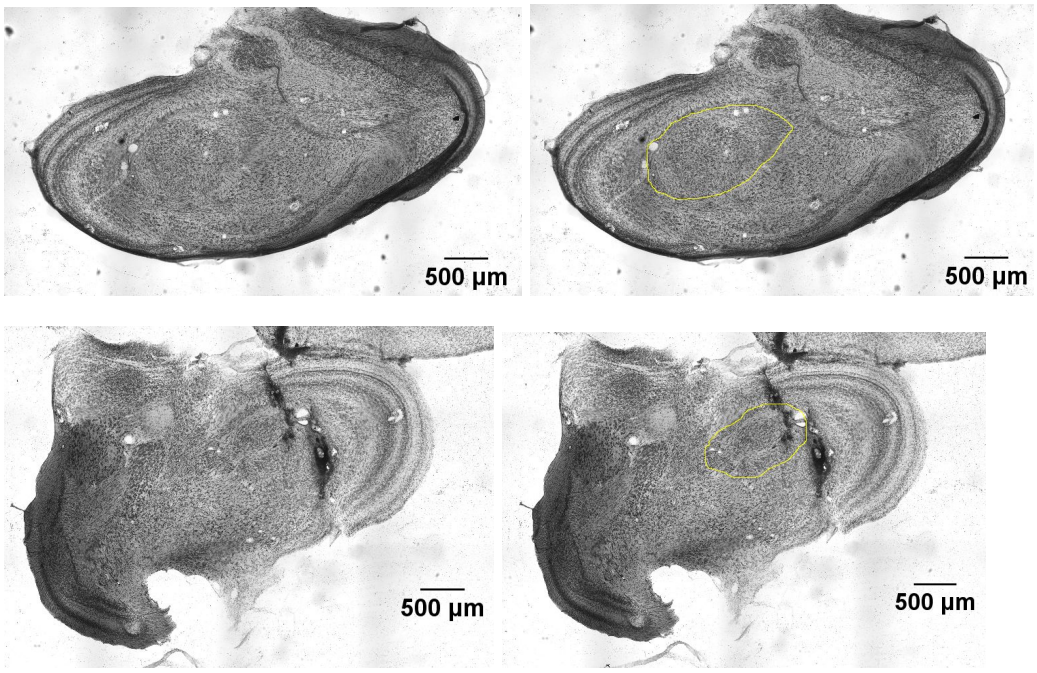

Fig 6: Reference midbrain sections for quantifying the volume of ICo and lesions. Yellow boundary encircles ICo. Images on the left and right in each row are the same images without and with the yellow boundary of ICo marked using ImageJ. .

Data Analysis

A few songs were randomly chosen from any two days before the surgery and any one day after the surgery. The number of songs chosen on each day were such that the number of syllables across these three days were similar. Data for these three days was analysed separately. In a few cases where the bird sang very few songs after the surgery, data across multiple days after the surgery was pooled to get a comparable number of syllables as before the surgery. All the syllables were segmented on the basis of amplitude using custom MATLAB scripts. Syllable onsets and offsets were marked using this script and each file was manually checked to make sure that there

were no errors due to the segmenting algorithm. After each syllable was segmented, various features like syllable duration, mean frequency, syllable onsets and offsets were extracted using custom MATLAB scripts for further analyses as mentioned below.

Correlation values for comparison across different days

For this analysis, songs from any two days before the surgery and any one day after the surgery were used. Syllables were segmented as described above. Irrespective of each syllable, all the syllable durations for a particular day were pooled and plotted as a normalized histogram. Similarly, histograms were plotted for the other days as well. Although the number of songs (25-50 bouts) were kept the same for different days, the total number of syllables across days varied slightly. Since the total number of syllables on each day differed, for computing the number of syllables in each bin in the histogram, respective frequencies were divided by the total number of syllables i.e. normalized. Syllable duration histograms for two days before surgery, were compared with the histogram for the day after surgery. Two days before the surgery were picked so as to account for baseline, day to day variability. Syllable duration histograms were compared using Pearson's correlation coefficient computed using 'corr' (in-built function in MATLAB). For visual representation of syllable durations, histograms for two days were overlaid (one day before surgery with another day before surgery and one day before surgery with one day post surgery-Figure 7). Since the correlation values depend on the number of bins used for comparison, bin sizes were varied and correlation values were calculated to assess how bin size affects the correlation coefficient. Further, this was used to choose a bin size appropriate for quantifying differences. The maximum and the minimum syllable duration was calculated for each day for each bird. Based on these values, zebra finches in my study sang syllables between 10 ms - 480 ms (on the basis of data from twelve birds). Keeping this variability in mind, three bin sizes: 5 ms, 15 ms and 30 ms were used to assess the effect of bin size on correlation values. Since the syllables are highly stereotyped the variation in any syllable duration on a day to day basis or from bout to bout would not be as high as 15 ms. It is however possible that

there could be ± 5 ms variation in case of longer syllables. Keeping the bin size as low as 5 ms would mean that only those syllables that differ by 5 ms or less are placed together in a bin. This allows for a better representation of variability in syllable durations and is hence a strict criteria for comparison of syllable durations. Keeping the bin size 15 ms would not take all such tiny variations into consideration and place all such syllables under the same bin. Similarly, 30 ms would represent an even more lenient bin size and account for very little variability (Table 3).

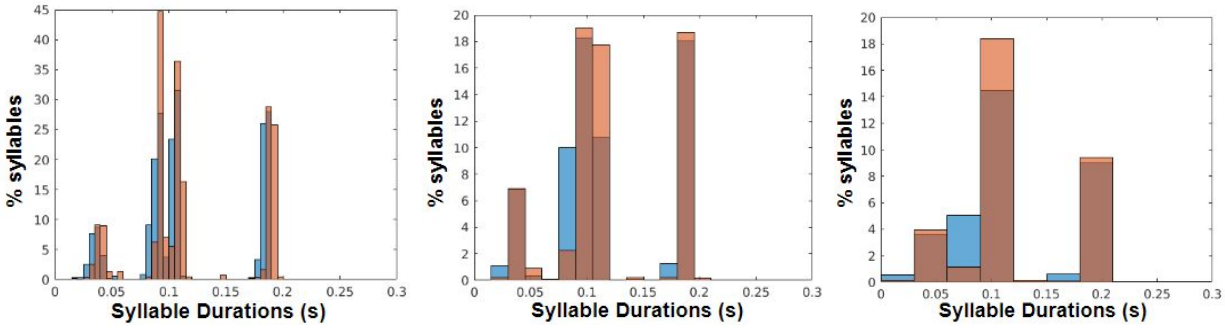

Fig 7: Normalized histograms plotted for one day before surgery and one day after surgery. Blue represents histograms plotted for pre-surgery data and orange represents histograms plotted for post-surgery data. Left: histograms with 5 ms binning i.e. each bin is 5 ms long. Middle: histograms with 15 ms binning. Right: histograms with 30 ms binning.

Correlation coefficients as a function of bin sizes

Bird name	5ms bin (pre-pre)	5ms bin (pre-post)	15ms bin (pre-pre)	15ms bin (pre-post)	30ms bin (pre-pre)	30ms bin (pre-post)
blk05wht16	0.6986	0.2836	0.9186	0.4338	0.9595	0.8834
brn72org72	0.8335	0.9380	0.8964	0.9721	0.9885	0.9880
red02wht02	0.7413	0.5183	0.8976	0.6425	0.9350	0.9012
grn68org95	0.8848	0.7450	0.9532	0.8637	0.9733	0.9025
grn20org40	0.7812	0.5417	0.8819	0.5653	0.9768	0.8007
brn53org62	0.9504	0.4388	0.9662	0.4774	0.9797	0.5750
grn41grn44	-	0.2874	-	0.3297	-	0.3423
red67pnk43	0.8508	0.8000	0.9628	0.9547	0.9816	0.9664

grn54pnk90	**0.5215**	**0.5286**	**0.6475**	**0.4856**	**0.7765**	**0.6665**
grn16blk84	**0.9890**	**0.7270**	**0.9995**	**0.9383**	**0.9996**	**0.9667**
brn36org36	**0.7974**	**0.7263**	**0.9223**	**0.9217**	**0.9759**	**0.9205**
brn74org74	**0.8401**	**-0.0062**	**0.8495**	**-0.0374**	**0.9977**	**-0.1341**

Table 3: Correlation values for one day before and one day after surgery. Different bin sizes were used to see how binning affected the correlation values. Values highlighted in blue represent pre-surgery comparisons for two days for different bin sizes. Values in black represent pre-post comparisons for different bin sizes.

From the above analysis, differences can be seen in the absolute value of the correlation coefficients suggesting that bin size affects the correlation values (Table 3). However, the differences are only in the absolute values and not the interpretation. In all the cases, correlation values increase as the bin size increases. Given that the song of a zebra finch is highly stereotyped, one would expect the correlation coefficients for any two days before the surgery to be close to 1 even after accounting for day to day variability. On choosing 5 ms as the bin size, the correlation values are low even for two days before the surgery in a few cases. Hence it is not a very good criteria to bin syllable durations. It is evident that out of the three bin sizes picked, 30 ms is a very lenient criteria as both pre-pre and pre-post comparisons are similar in terms of their absolute values, even in those cases where differences could be seen in spectrograms. Differences in spectrograms are best represented when the bin size is chosen as 15 ms. On the basis of these results, 15 ms was chosen as the appropriate bin size for further analysis. Upon changing the bin sizes, the shape of the distribution does not change. However, the number of elements in a particular bin changes.

Comparison with stretched / compressed syllable durations

Although no obvious differences could be seen in spectrograms in a few cases, differences in syllable duration histograms for pre-surgery overlaid with post-surgery could be seen. The shape of the distribution appeared the same but shifted in one direction suggesting that the syllable durations changed after surgery. To account for these changes, syllable durations post surgery were increased and decreased by 5%

and 10% using custom MATLAB scripts. Syllable durations before surgery were compared with these new post surgery syllable durations to assess if the correlation values improved. Maximum correlation values across normal, increased and decreased syllable durations were considered for further analysis.

Comparison of syllable intervals before and after surgery

Syllable intervals (silent gaps) were compared before and after surgery by computing correlation coefficients as described above for syllable durations. Since the gaps between the syllables are slightly longer than the duration of a syllable, larger bin sizes (25 ms) were used for computing correlation values and for plotting histograms. Within a bout the syllable gap durations do not exceed 500 ms. All syllable gap durations were computed but only those within 500 ms were saved and used for further analysis.

Lilliefors statistics

Upon visual examination of spectrograms differences in syllable structure and durations could be seen in a few cases. Lesions of Uva and HVC result in the production of syllables whose durations follow a random distribution which can be best represented by an exponential curve (Aranov et al, 2011; Danish and Fee, 2017). Aronov et al (Aronov, Andalman, Fee, 2008; Aranov et al, 2011) have previously used Lilliefors statistics to compare syllable duration distributions to an exponential distribution. Like the KS-test, in Lilliefors test also the empirical cumulative distribution (eCDF) of syllables is compared with the cumulative density function (CDF) for an exponential curve generated from the data itself. This was done to assess how well the observed data fitted an exponential curve. In accordance with their analysis, fit values < 2 indicated that the observed data fitted an exponential curve well. Lilliefors statistic looks for the maximum difference between the two curves and this difference is normalized by dividing by the total number of syllables.

$$\textbf{Test statistic} = |\,\text{eCDF} - \text{CDF}\,| \,/\, \sqrt{\text{total syllables}}$$

Call analysis

DM is responsible for the production of distance calls (Simpson and Vicario, 1990). In order to assess the effects of lesions on calls, a few files containing distance calls were screened and saved. 5 such files for any two days before surgery and one day after surgery each were used for analysis. In case there were multiple calls in one file, only one call from each file was picked for analysis (code written by Shikha Kalra). Similarity between calls produced before surgery and similarity between calls produced before and after surgery was calculated using Sound analysis pro (Tchernichovski et al, 2000). The software uses multiple acoustic features like pitch, wiener entropy, frequency modulation, amplitude modulation etc. It then compares these features over multiple short intervals. A similarity score is calculated on the basis of differences in these features over the entire duration of the call. Each call was compared to all the other calls and a matrix of similarity between calls on two days was generated. Similarity of calls on two days was calculated by computing the average similarity.

Results

Zebra finches produce a stereotyped song which is controlled by various song nuclei. Forebrain nuclei like HVC and RA provide motor commands to downstream motor neurons and are required for producing normal song (Simpson and Vicario, 1990). RA does not directly control song production. RA projects to various other nuclei like nXIIts, RAm-PAm and DM (Simpson and Vicario, 1990). These nuclei then project to syringeal muscles (vocal organ) and respiratory muscles to control vocal production (Figure 2). Since DM projects to Uva, which then provides feedback to HVC, it may play a role in controlling song production. DM lies within a bigger nucleus ICo (intercollicular complex). Although ICo surrounds DM, previous literature (tract tracing experiments) suggest that ICo itself does not project to song controlling nuclei (Vicario, 1991). However, the role played by ICo and DM play in song production is poorly understood. Hence, to assess the role played by DM and the associated nucleus ICo in song

production, DM was first reliably located using stereotaxic coordinates and by electrical stimulation to elicit calls. This was followed by assessing the effects of lesions and verifying the sites of lesions by histological quantification.

Pilot experiments for locating DM in the anesthetized state

Previous studies have shown that electrical stimulation in DM even in the anesthetized state can elicit calls (Simpson and Vicario, 1990). As a first step towards identifying DM reliably in the anesthetized state, I carried out a few pilot experiments using a set of stereotaxic coordinates and electrical stimulation at these coordinates (A/P: 0.85-1.3, M/L: 2.43-2.64, beakangle: 45, depth: 4.8-5.1; https://www.ncbi.nlm.nih.gov/books/NBK2348/) to evoke calls in n=2 zebra finches (brown36orange36, black98black87). DM could not be located using the set of stereotaxic coordinates prescribed in the atlas for zebra finches in both these cases. In n=1 zebra finch (brown56orange65), calls were evoked at a different set of coordinates (A/P: 1.7, M/L: 2.5, beak angle: 45, depth: 5.0-5.25). DM was located further in the anterior direction (A/P: 1.7), the rest of the coordinates (M/L, depth, beak angle) were the same. To further verify if the coordinates indeed corresponded with DM, sites where calls were elicited upon electrical stimulation were lesioned. Histological verification was later carried out by sectioning the brain and staining these sections with cresyl violet. In n=1, no lesions were made (brown36orange36) as the connections in the lesioning apparatus were loose. In n=1 (black98black87), lesions could be located in both the hemispheres (see appendix for images). However, most of the lesions were in the forebrain. Only one lesion could be seen in the midbrain. In n=1 (brown56orange65), lesions could be seen on at least one side in DM and ICo (Figure 8). In this case calls were elicited in the anesthetized state. These coordinates were noted and used for all the future surgeries.

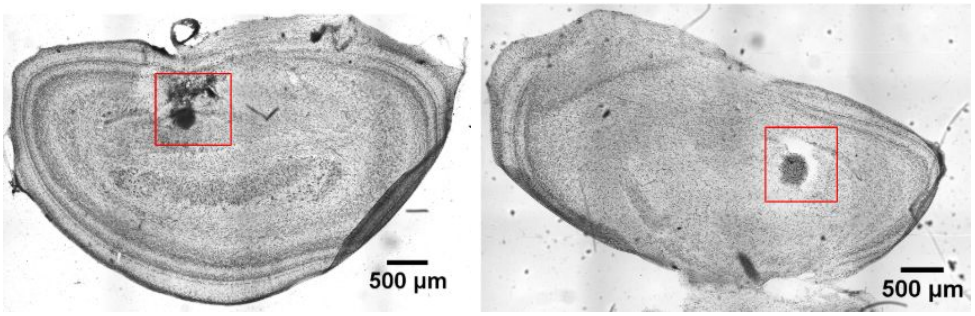

Fig 8: Sagittal sections for brown56orange65. left: left sagittal section of midbrain stained with cresyl violet with lesions in lateral sections containing ICo. Although calls were elicited in this case, no lesions could be seen in DM. Right: right sagittal section of midbrain with lesions in ICo and DM. Calls were elicited upon electrical stimulation at this site. Red boxed part in each figure represents lesions.

Stimulation in DM evoked calls which varied with stimulation parameters

After locating DM reliably, I was interested in analysing how stimulation parameters affected the features (mean frequency, duration, number) of the elicited calls. Zebra finches have a huge repertoire of calls that vary mainly in duration and frequency. I was particularly interested in seeing if different sites were responsible for generating the variation in calls elicited or if manipulating the firing of neurons by changing the stimulation parameters could generate such a variability. Only two studies have been carried out in Bengalese finches (a related songbird) which show that the kind of calls elicited vary with the stimulation parameters like current amplitude, duration of pulse, frequency of pulse (Fukushima and Aoki, 2000, Fukushima and Aoki, 2002). Similar to these studies, calls similar to bird's long calls were evoked in my experiments as well (n=9, Fig. 9; also see appendix).

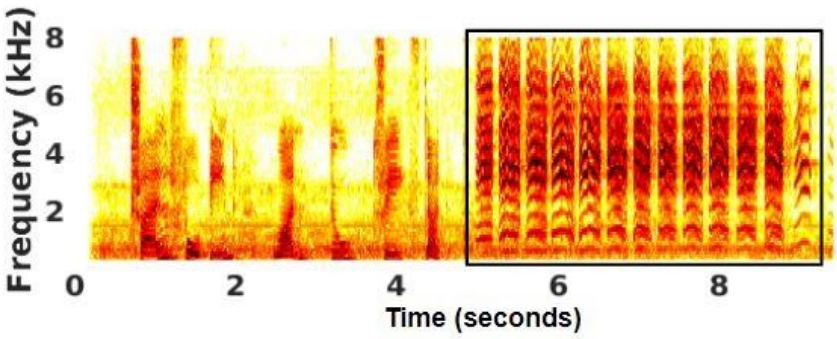

Fig 9: Spectrogram above represents calls elicited (boxed part) upon electrical stimulation in the anesthetized state. The sounds before the elicited calls are the experimenter uttering "Trial# , 1, 2, 3,

start” and the stimulation is started immediately after “start”.

Calls were elicited at current amplitudes as low as 30 μA at a few sites. By auditory examination alone, differences in the loudness, frequency and number of calls elicited could be perceived. However, at a particular site the kind and number of calls elicited remained consistent. This was verified by repeatedly stimulating at a site with a latency of at least 30 seconds. The calls elicited in all the cases had little frequency modulation and were mostly harmonic stacks (Figure 9; also see appendix).

To assess how different stimulation parameters affected the kind of calls elicited (Figure 10), multivariate linear regression was carried out for four zebra finches. The predictor variables were current amplitude (x1), stimulus duration (x2) and frequency of stimulus (x3). The response variable (Y) was either mean frequency of elicited call or duration of first call. To account for interaction effects of predictors on each other, interaction terms were also included in the model:

$$Y \sim x1 + x2 + x3 + x1 * x2 + x2 * x3 + x1 * x3$$

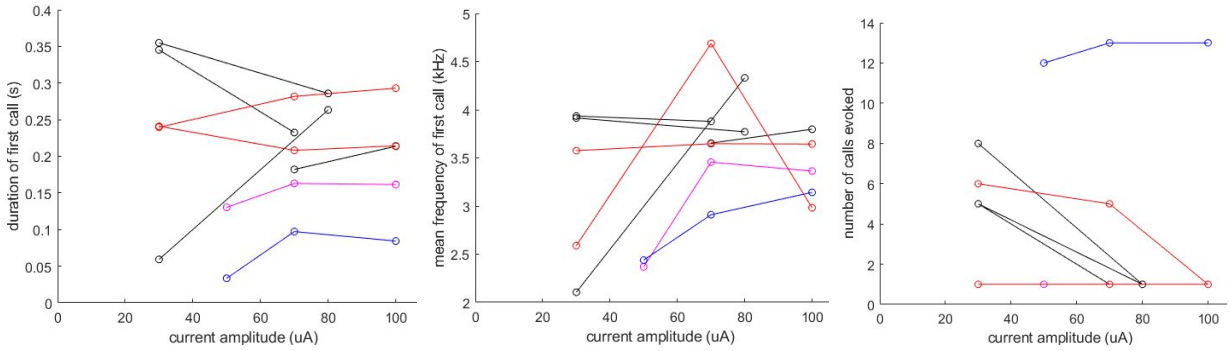

Fig 10: Effect of varying current amplitude on the features of the elicited call. Left: Variation in the duration of first call with variation in current amplitude. Middle: Variation in the mean frequency of the first call with variation in current amplitude. Right: Variation in the number of calls elicited with variation in current amplitude. Black, red, blue and magenta represent different birds. Circles and lines joining them represent stimulation at a particular site. Different lines in the same colour represent different stimulation sites in the same bird.

Multivariate linear regression analysis:

Bird name	Response variable	R^2	F statistic	p-value	variance
Brn74org74 (31 data points)	Mean freq of first call	0.4897	3.8390	0.0080	0.3408

	Duration of first call	0.4602	3.4099	0.0141	0.0026
Blk05wht16 (18 data points)	Mean freq of first call	0.1232	0.3373	0.8807	0.3262
	Duration of first call	0.2499	0.7994	0.5709	0.0125
Brn100org41 (9 data points)	Mean freq of first call	0.4889	0.5739	0.7262	0.2013
	Duration of first call	0.5632	0.7737	0.6269	0.0032
Grn26blu12 (12 data points)	Mean freq of first call	0.6675	1.6727	0.2944	0.1327
	Duration of first call	0.6921	1.8731	0.2539	0.0018

Table 4: Summary of computed statistics for multivariate linear regression. Table provides the R^2, F-statistic, p-value computed at 95% confidence and a measure of variance for each bird. In every case, the effect of three predictors (current amplitude, frequency and duration of the stimulating pulse) was examined for both outcome variables (mean frequency of first call or duration of first call) separately.

The model significantly predicted duration and mean frequency of the first elicited call in only one of the 4 birds (Table 4) showing that elicited calls were largely invariant to these three stimulation parameters. This could also be because data from different sites was pooled due to insufficient data points. This was not taken into consideration in the model.

Tracer injections to locate and quantify the extent of DM

As an alternative way of determining the extent of DM, I also tried anatomical tract tracing experiments. Previous studies have shown that DM receives inputs from RA (Gurney, 1981; Nottebohm et al, 1982). As a first step towards locating DM and quantifying it's extent on the basis of this connectivity, I injected anterograde tracers (dextran conjugated tetramethylrhodamine) in RA in one zebra finch and checked if these tracers could be detected in ipsilateral DM. Upon histological verification, sparse fluorescence could be detected in midbrain areas (Figure 11) which correspond to ICo in the stereotaxic atlas. However, fluorescence in RA was minimal indicating that more experiments needed to be performed to standardize tracer injections. From this experiment DM could not be located reliably.

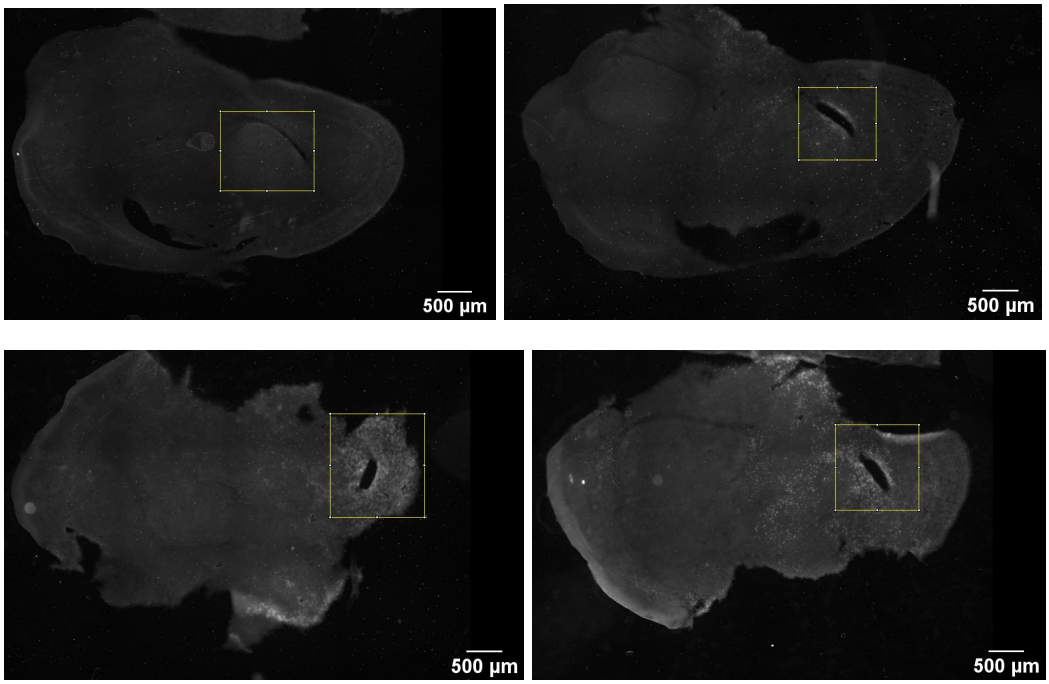

Fig 11: Serial sagittal midbrain sections as observed under a fluorescence microscope. The boxed part indicates ICo.

Lesioning DM and the associated area ICo alters the song in a small subset of birds

After reliably locating DM in the anesthetized state in the pilot experiments, the aim was to lesion DM and assess the effects of these lesions on the consequent song. To achieve this, I located DM based on electrical stimulation and then lesioned it by passing anodal current (50-150 µA for 10-60 s; n=10 males). In order to lesion the entire volume of DM, multiple sites were lesioned (range: 3-6 sites/bird). In a few birds where no calls were elicited, lesions were made at sites where ICo typical behaviour (tail-flicking) was elicited on electrical stimulation. Given the close proximity of DM and ICo, it was assumed that lesions in ICo would damage DM as well. Data for three more birds where DM was not lesioned was included in the analysis as controls (one bird was used for pilot experiments, one was used to record neural activity in DM and in one bird current was passed to elicit calls but lesions were not made). In the songs recorded after surgery, upon visual examination alone, changes in syllables could be seen in

n=5/10 birds (Figure 12). Out of these 5 birds that changed their song, data was excluded for 1 bird as the bird sang only 'directed' songs (see appendix for reference spectrograms). Further analysis was done to assess which features of the song changed.

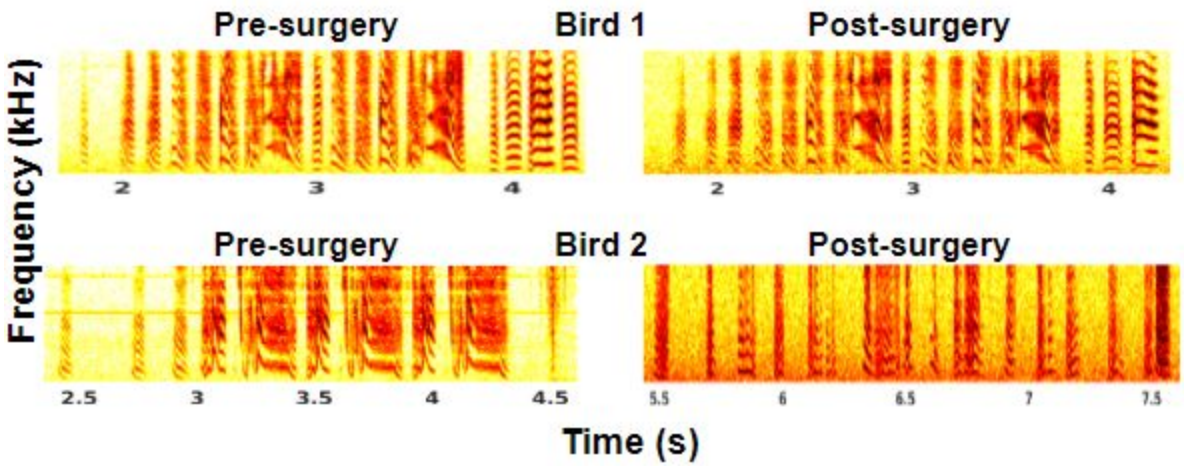

Fig 12: Top Left: spectrogram for a song before surgery. Top Right: spectrogram for the same bird after surgery. Note:In this case no changes in song were observed. Bottom Left: Spectrogram for a song before surgery. Bottom Right: spectrogram for the same song after surgery. Note:the song was altered completely after DM lesions. Individual elements cannot be recognized. The graphs on the left and right have equal timescales.

Temporal features of the song are altered post DM lesions

Upon visual examination alone, changes in syllables could be seen. In n=4/9 birds, syllables were 'unidentifiable' (Figure 12). Zebra finches sing a highly stereotyped song implying that the duration of each syllable is also highly stereotyped over multiple renditions. To assess if DM lesions altered this stereotypy, syllable durations and syllable gap durations were compared for songs before surgery and after surgery. As a first step towards quantifying differences in syllable durations and gap durations due to lesions, mean durations before and after surgery were compared (Figure 13). As a control for the effect of surgery the 3 birds where current was passed to evoke calls but no lesions were made were used. In addition, to control for the effects of anesthesia, I also used data from 6 sham tracheosyringeal nerve cut birds. These birds were anesthetized in exactly the same way as my birds and the tissue overlying their syrinx

was opened and glued back without any stimulation/lesions in their midbrain (data obtained from Divya Rao; Rao et al, 2019)

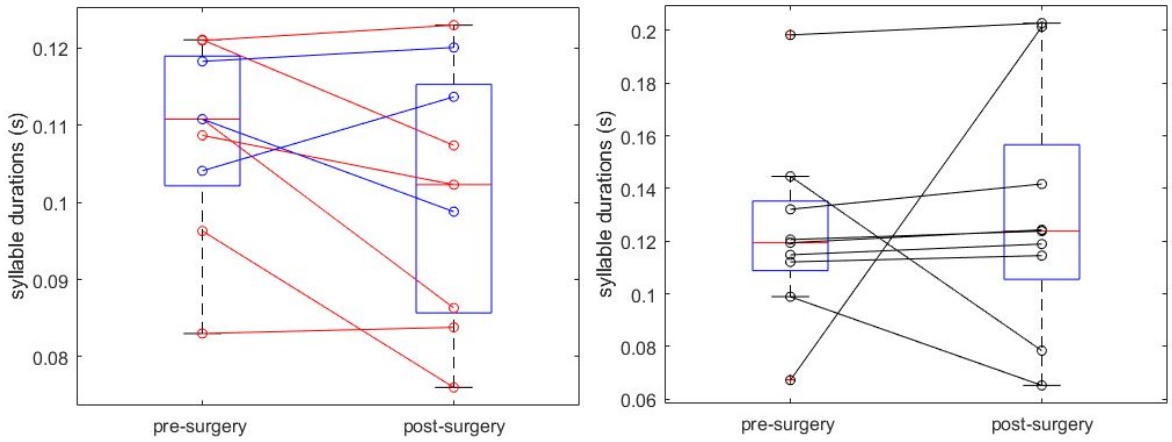

Fig 13: Comparison of mean syllable durations before and after surgery. Left: comparison for control group. Birds in blue indicate those birds where current was passed in DM to evoke calls but no lesions were made. Birds in red indicates those on which sham ts-cut was performed. Right: comparison of mean syllable durations for birds where lesions were made in DM.

Mean syllable duration was not significantly different for both the control group and the DM lesioned birds (Figure 13; p = 0.2031 for control and p = 0.4258 for DM lesioned birds, Wilcoxon sign-rank test). Inter-syllable gap durations were also not significantly different post-surgery for both control and DM lesioned birds (Figure 14; p = 0.2031 for control and p = 0.0742 for DM lesioned birds).

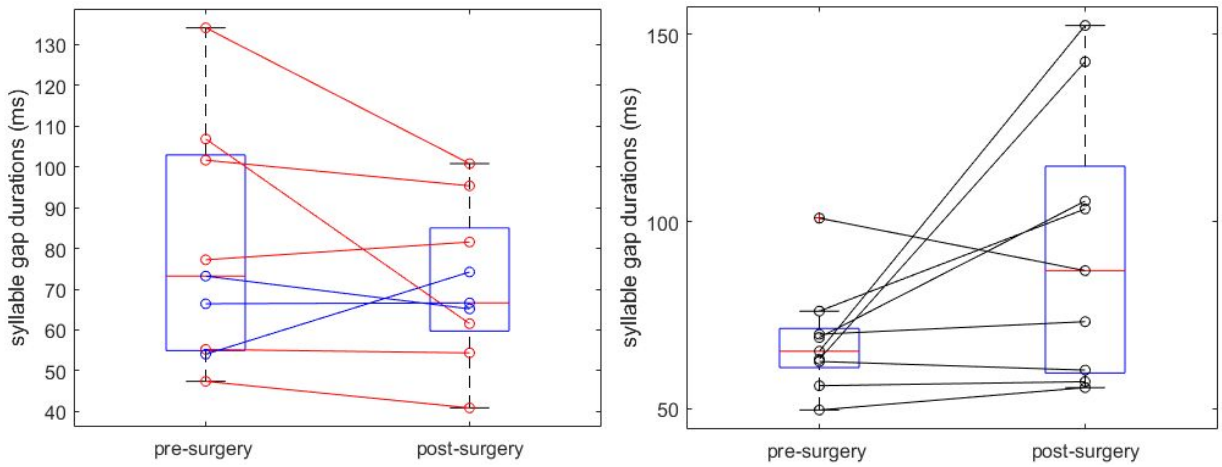

Fig 14: Comparison of mean gap durations before and after surgery. Left: comparison for control group. Birds in blue indicate those birds where current was passed in DM to evoke calls but no lesions were

made. Birds in red indicates those on which sham ts-cut was performed. Right: comparison of mean gap durations for birds where lesions were made in DM.

While as a group, there were no significant differences after surgery, some birds showed much greater change in mean syllable duration and mean gap duration as compared to controls (Figure 15; see outliers highlighted by filled circles). These differences matched the qualitative changes observed in the spectrograms.

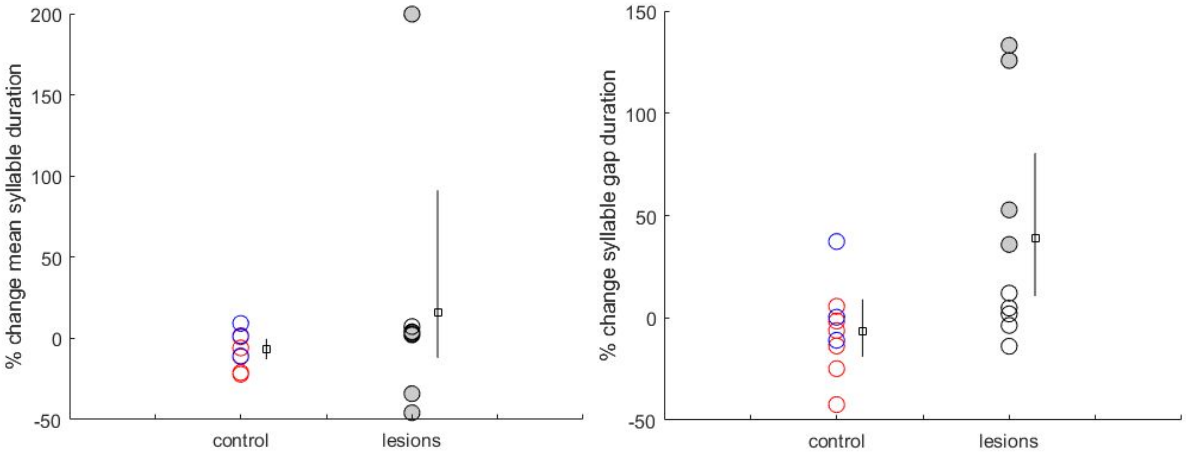

Fig 15: percentage change in durations. Left: percentage change in syllable durations before and after surgery. Right: percentage difference in gap durations before and after surgery. Points in red are for ts-cut sham birds, points in blue are those birds where current was passed in DM but no lesions were made. Black line next to each group indicates 95% confidence intervals around the mean (square).Filled circles represent birds with qualitative changes to songs seen in the spectrograms.

The present analysis only tested for differences in mean syllable duration. While overall mean duration was not significantly different, the distribution of syllable durations could have changed. To further assess how these syllable durations change post surgery, the following comparisons were made taking into account the distribution of syllable durations.

1) Correlation coefficients generated by bin-wise comparison of histograms

Each syllable in a zebra finch song has a stereotyped duration across multiple renditions. The gap durations are also consistent. If syllable durations for all the syllables are pooled, durations corresponding to a particular syllable should always fall

in a specific bin. This should lead to different peaks in a histogram corresponding to different syllables. In case the temporal features are altered, such peaks should disappear and the number of elements in each bin should change. Histograms were plotted with 15 ms wide bins for syllable durations and syllable intervals before and after surgery (Figure 16, Figure 17).

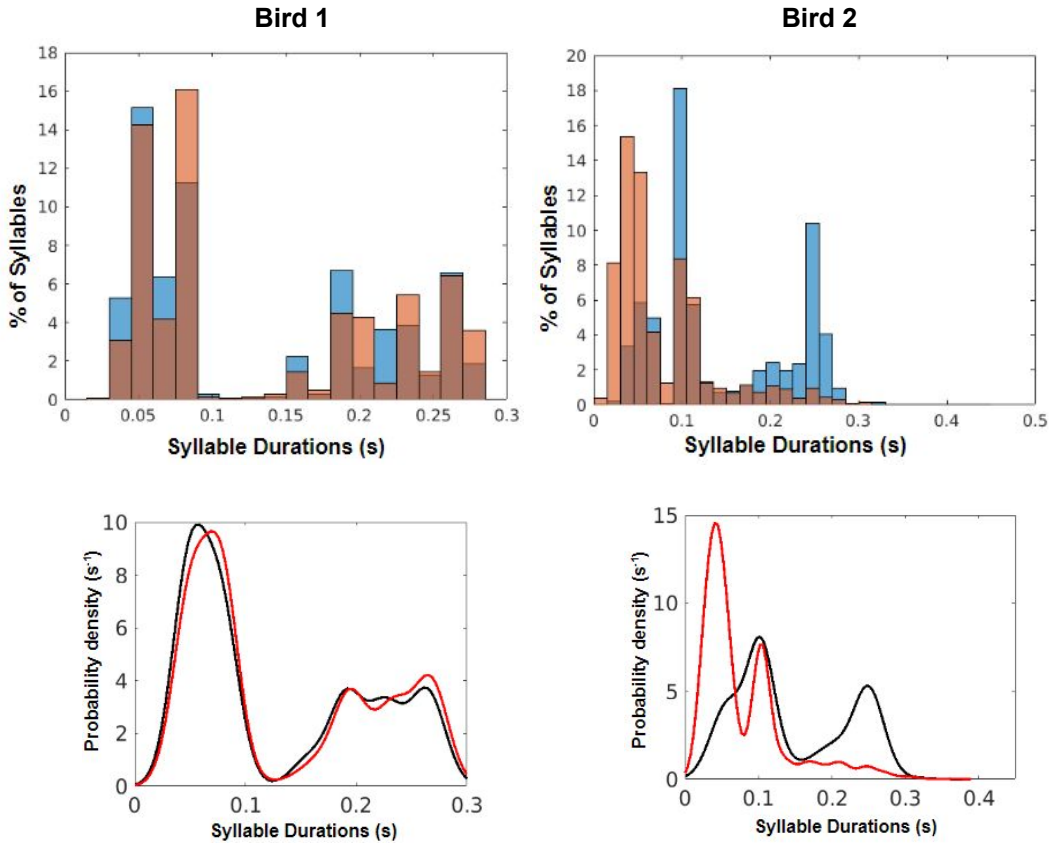

Fig 16: Top left: syllable duration histograms for a bird (bird 1) where the song did not change after surgery. Top right: syllable duration histograms for a bird (bird 2) where the song changed after surgery. Blue represents histograms plotted for data before surgery, orange represents histograms plotted for data after surgery. Bottom left: probability density function for syllable distributions for data on top left (bird 1). Bottom right: pdf for syllable distributions for data on top right (bird 2). Black represents pre-surgery data, red represents post-surgery data.

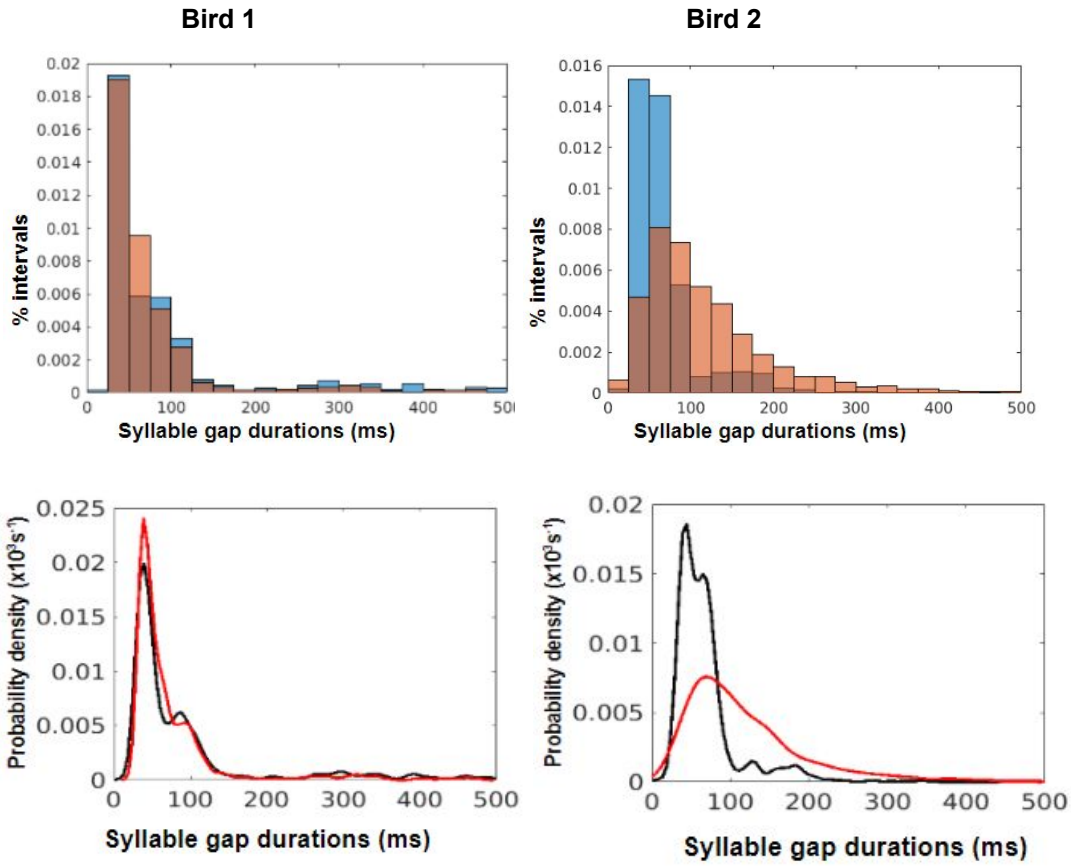

Fig 17: Syllable gap duration comparison. Left: syllable gap duration histogram (normalized) for a bird whose song did not change after surgery. Right: Syllable gap duration histogram (normalized) for a bird where the song changed after surgery. Blue represents histograms for pre-surgery and orange represents histograms for post-surgery. Bottom left: Syllable gap duration probability density function for the histogram on top left. Bottom right: pdf for the histogram on top right. Black trace represents the trend before surgery and red trace represents the trend after surgery.

Changes in spectrograms were reflected in syllable duration/gap duration histograms and from the probability density function graphs in most cases (Figure 16, Figure 17). On the basis of these histograms, a binwise correlation coefficient was calculated. In a few cases, no changes could be observed qualitatively in the spectrograms but the correlation coefficients seemed to drop after surgery. Further, as seen previously in the comparison of mean durations before and after surgery, there are minor insignificant effects on the syllable durations. To account for these variations as a result of surgery, syllable durations before surgery were compared with modified syllable durations as described below.

Syllables may stretch or compress by ~5-10% as a result of surgery

To assess the drop in correlation values, syllables post surgery were modified (stretched/compressed) using custom MATLAB scripts and correlated with pre-surgery syllable durations (Table 5). Syllable durations post surgery were stretched by 5%,10% and compressed by 5%,10%. Histograms were calculated for each of the modified syllable durations and compared with pre-surgery syllable duration histograms. For birds where no qualitative differences in song were seen, correlation values between pre and post surgery were higher when post-surgery syllables were compressed by 5-10% (black traces in Figure 18). For birds where qualitative changes to song were seen, correlation values were lower and did not change after modification of syllable duration (red traces in Figure 18).

Comparison of syllable durations with stretched/compressed syllables

Bird name	Correlation coefficient for pre1-pre2	Correlation coefficient pre1-post (10% compressed)	Correlation coefficient pre1-post (5% compressed)	Correlation coefficient for pre1-post (unaltered)	Correlation coefficient pre1-post (5% stretched)	Correlation coefficient pre1-post (10% stretched)
brn72org72	0.8964	0.8095	0.9037	0.9721	0.8264	0.5345
red02wht02	0.8976	0.7333	0.9412	0.6425	0.3558	0.3309
grn54pnk90	0.6475	0.7101	0.8997	0.4856	0.1408	0.0304
grn68org95	0.9532	0.8927	0.9709	0.8637	0.5914	0.5305
grn16blk84	0.9383	0.5515	0.5330	0.9995	0.8929	0.5200
brn53org62	**0.9662**	**0.3953**	**0.4808**	**0.4774**	**0.3922**	**0.3112**
grn41grn44	**-**	**0.3090**	**0.3298**	**0.3297**	**0.3401**	**0.4107**
brn74org74	**0.8495**	**-0.1326**	**-0.0841**	**-0.0374**	**-0.0285**	**-0.0881**
grn20org40	**0.8819**	**0.6459**	**0.6231**	**0.6204**	**0.6345**	**0.6010**
bird1	-	0.4405	0.5849	0.7621	0.8523	0.8390
bird 2	-	0.3484	0.5632	0.9476	0.8286	0.5453
bird3	-	0.8523	0.9420	0.9683	0.8767	0.7548

bird4	-	0.5653	0.7350	0.7832	0.8170	0.7399
bird5	-	0.3879	0.4607	0.5936	0.8885	0.9813
bird6	-	0.0609	0.2361	0.7133	0.8891	0.9354
blk05wht16	0.9186	0.9497	0.8859	0.4338	0.2630	0.2329
red67pnk43	0.9628	0.8286	0.8774	0.9547	0.5599	0.3491
brn36org36	0.9223	0.7611	0.8973	0.9217	0.8469	0.6890

Table 5: Comparison of correlation values before the surgery and after the surgery. Values were generated keeping the bin size as 15 ms and x-limit as 450 ms. Birds in blue are those where current was passed but no lesiones were made (last three) or birds that had undergone sham ts-cut surgery (Bird1-Bird6). Birds in black are the birds where lesions were made and those highlighted in red are where changes in song could be detected qualitatively by looking at the spectrograms.

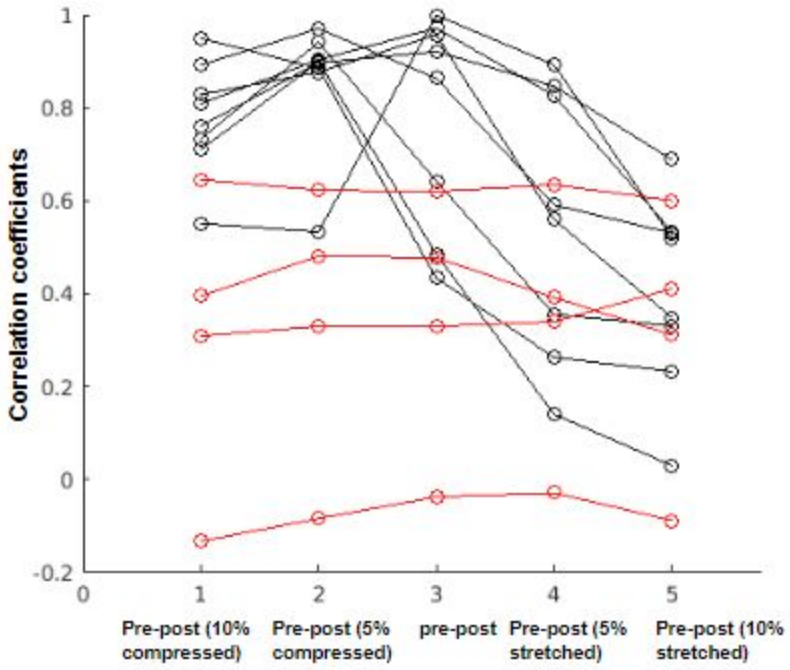

Fig 18: Comparison of correlation values before and after surgery. One day before the surgery (pre) was compared with one day post surgery, with syllables that were 5% stretched and compressed and with syllables that were 10% stretched and compressed.
Birds in red are those for which the song changed completely (as observed in spectrograms). Black series represent birds where no change in the spectrogram was seen.

As a control for the analysis, I also calculated correlation values for any two days before

surgery. Correlation values for any two days before surgery (pre-pre) were high showing that the song is stereotyped in all the cases before surgery (Table 5).

For birds in the control group that had undergone tracheosyringeal cut sham surgery, correlation values were higher for stretched syllables (pre-surgery syllable durations compared with 5-10% stretched post surgery syllable durations) unlike for the DM lesion birds. This suggests that this effect is specific to midbrain surgeries. Such an effect was seen only for syllable durations. Correlation coefficients for syllable intervals did not change when compared with 5-10% stretched/compressed gap durations.

In order to account for the effects of surgery, the best pre-post correlation value was picked for each bird from the above table and the correlation coefficients were re-assessed as shown in the graph below:

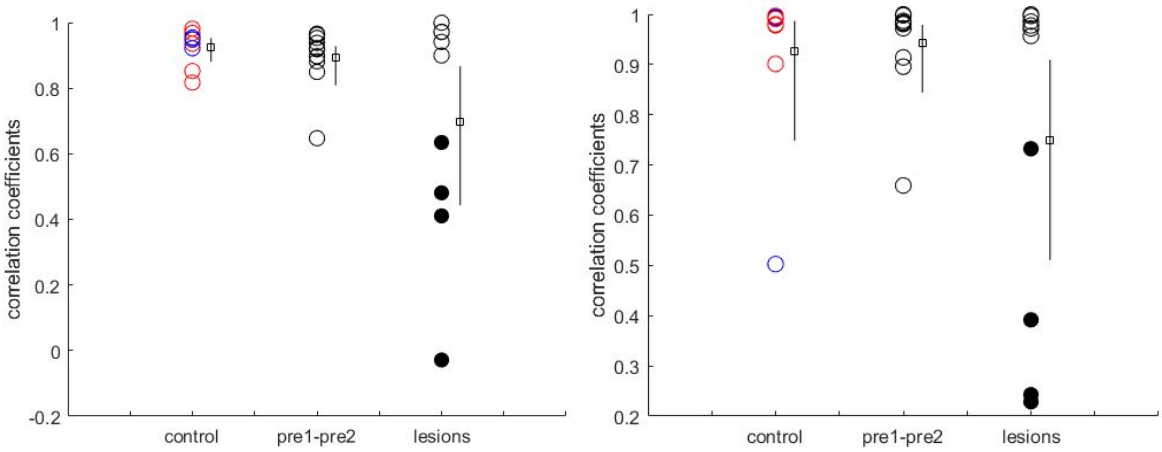

Fig 19: Left: Comparison of correlation coefficients for syllable durations. Right: Comparison of correlation coefficients for syllable intervals. Correlation coefficients (pre-post) for the control group (sham ts-cut and sham DM surgery) are represented in red and blue respectively. Pre1-pre2 represents correlation coefficients for any two days before surgery. Filled circles in lesions group represent those birds where changes in song could be seen upon qualitative examination. 95% Confidence interval is plotted next to each group (mean represented by square) using CI plot (matlab). Highest correlation values have been used for scatter points in the lesioned group to take into account the effect of surgery itself.

Only in those cases where a significant change in the song had occurred as a result of surgery, the best correlation value was still low and significantly different from the pre-surgery and control correlation values. This analysis provided further quantitative

evidence that some birds changed their songs post DM lesions.

2) Lilliefors statistics

Aronov et al and Danish et al (Danish HH, Aronov D, Fee MS, 2017) used Lilliefors statistics for comparison of the HVC and Uva lesion data to an exponential distribution. Normal zebra finches sing the same set of syllables over and over again as part of their song. Therefore, the distribution of syllable durations shows a peaked distribution corresponding to the different syllables in that bird's song (Figure 16). HVC lesions and Uva lesions result in syllable duration distributions which are not as stereotyped (Danish HH, Aronov D, Fee MS, 2017). In case of HVC lesions, syllable durations fit an exponential curve well indicating that the resultant distributions are random. Similarly the shape of the syllable duration histograms post Uva lesions also becomes exponential like. Given that DM projects to Uva, it is likely that DM lesions result in similar changes as seen for Uva lesions. In accordance with Aronov et al. (Danish HH, Aronov D, Fee MS, 2017) Lilliefors statistics were computed for syllable durations before and after surgery (Table 6). Each day (pre-surgery and post-surgery) was compared to an exponential distribution using custom MATLAB scripts (see methods).

Lilliefors statistics for comparison of syllable durations before and after surgery to an exponential distribution

Bird name	Pre-surgery statistic	Post-surgery statistic
blk05wht16	0.6708	0.7015
brn72org72	0.7531	0.8150
red02wht02	0.5631	0.5784
grn68org95	0.7202	0.7886
grn20org40	0.7600	0.8086
brn53org62	0.7190	0.7356
grn41grn44	0.8855	0.9937
red67pnk43	0.6939	0.7444

grn54pnk90	0.7490	0.7679
grn16blk84	0.5748	0.6021
brn36org36	1.0198	0.9929
brn74org74	1.0829	1.2127

Table 6: Lilliefors statistic values for a day before surgery and for a day after surgery. All the values are <2 suggesting that data for each data comes from a population that follows an exponential distribution.

Aronov et al (Danish HH, Aronov D, Fee MS, 2017) used a critical value = 2 to classify data as 'exponential' or 'not exponential'. In my case, values for all test-statistics were < 2 (Table 6), suggesting that data for each day follows an exponential distribution. From the above table it is evident that this method cannot distinguish between syllable duration distributions before and after surgery. Given that zebra finches sing a highly stereotyped song, one would expect to see certain clusters of syllable durations characteristic of each syllable before surgery. The resulting distribution for pre-surgery should hence not follow an exponential curve.

The Lilliefors statistic depends inversely on the root of the total number of syllables. By reducing the total number of syllables used for analysis, the statistic gets closer to 2. However, this results in sub-sampling and hence is not a good method for analysing differences between syllable durations. Further, the number of syllables used in the previous studies was ~10,000. The critical value also depends on the number of syllables used. Since fewer syllables were used in my study (~1000), using the same critical value may lead to erroneous interpretations. Due to the caveats listed above, Lilliefors statistics was not used for further analysis.

Similar effects seen in 'directed' songs

A few directed songs were recorded before and after surgery to assess if DM played a role in controlling both directed and undirected songs in a similar manner. The changes

seen in spectrograms for undirected songs was similar for directed songs in all the birds. One bird did not sing undirected songs post surgery however changes in directed song could be seen. No further analysis was done to compare pre-directed songs with post-directed songs.

Lesioning DM and the associated area ICo alter distance calls but not short calls

DM is responsible for production of distance calls (Fukushima and Aoki, 2000). Lesioning DM alters the distance calls but not short calls (Simpson and Vicario, 1990). Similar to these previous studies, I wanted to assess if DM lesions in my birds had a similar effect on calls. Upon qualitative analysis alone, distance calls were altered in 4/9 birds (Figure 20). Distance calls post lesions were harmonic stacks and had little frequency modulation as compared to the calls before surgery. Differences could be seen in terms of duration of calls as well. No such differences could be seen in short calls.

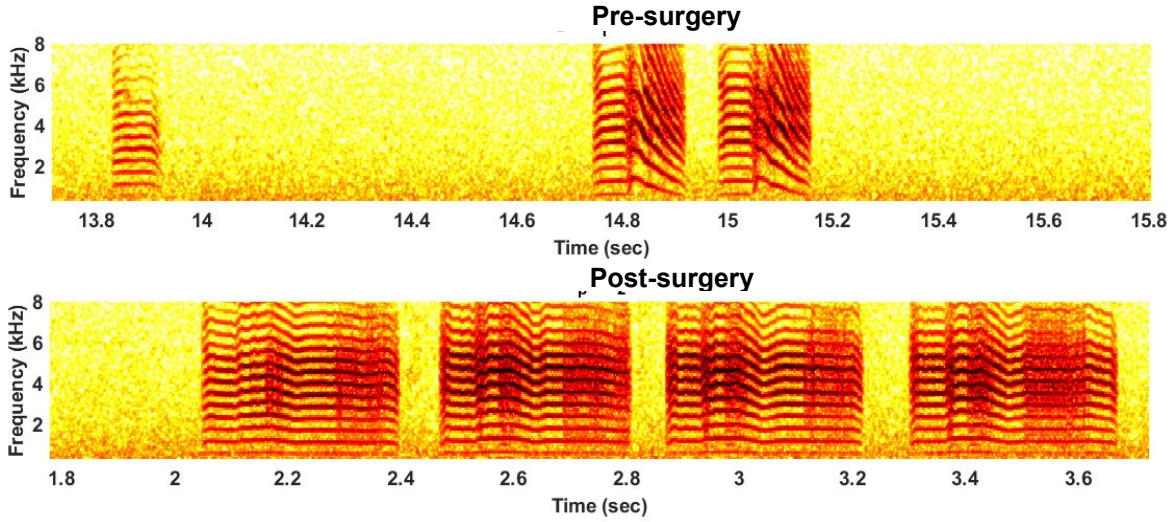

Fig 20: Spectrograms of distance calls before and after surgery. Top: call spectrogram before surgery. Bottom: call spectrogram after lesions in DM and ICo. Calls post lesion are longer and do not have the same frequency modulation.

To assess the effects of lesions on distance calls, similarity between distance calls before and after surgery was computed using SAP (Tchernichovski et al, 2000; see

methods). Significant differences could be seen in distance calls due to ICo and DM lesions in a few birds (Figure 21; p=0.0371, Wilcoxon sign-rank test).

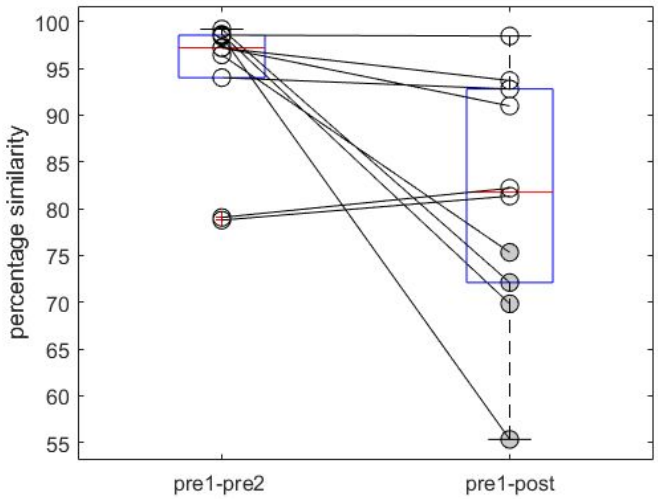

Fig 21: Comparison of calls before and after surgery. Calls for any two days before surgery (pre1-pre2) were compared and a percentage similarity score was computed. Distance calls one day before surgery and one day after surgery were compared and the percentage similarity (pre1-post) was compared with pre1-pre2. Gray filled circles represent those birds where differences could be seen in the calls upon qualitative examination alone.

DM/ICo lesions affect both song and distance calls

Lesions in DM and ICo altered both song and distance calls (Figure 22). Although differences in song and calls both were seen in 5/10 birds, data for 3 birds (song changed in 2 and no change in 1) could not be used in this analysis as either data for calls or data for undirected songs was not available. Data for two birds where no lesions in DM were made, was included as controls.

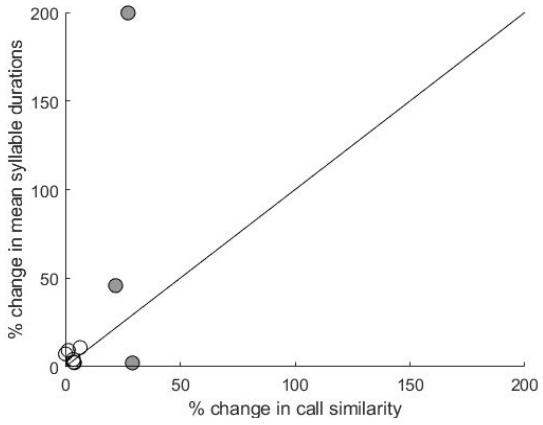

Fig 22 : Lesions alter both calls and song. % change in mean syllable durations (pre-post) is taken as a measure of change in song. % change in similarity of calls (pre-post) is taken as a measure of change in calls. Dark gray circles represent those birds where change in both the features was seen qualitatively.

Correlating changes to the sites of lesions

Histology was done to assess if the changes seen in the song were correlated with the sites of lesions and the extent of lesions. Every third section was mounted and imaged at 10x magnification under a brightfield microscope (Figure 23).

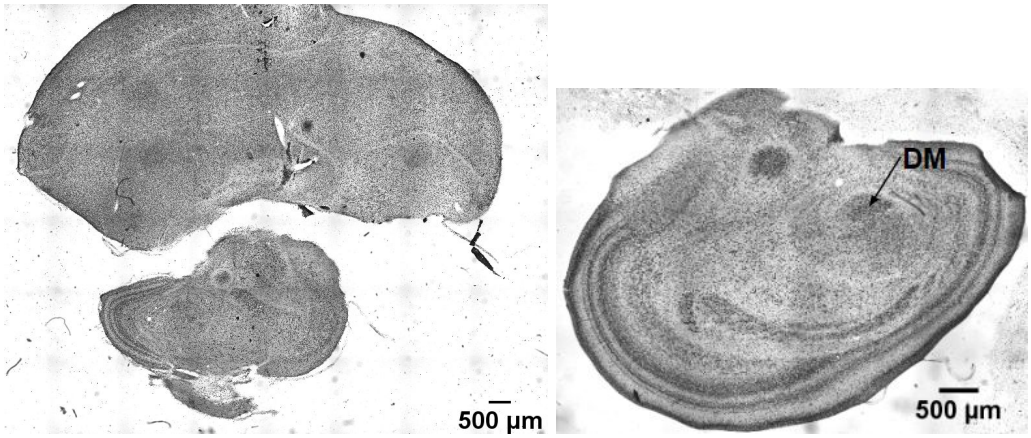

Fig 23: Left: sagittal section of a cresyl violet stained whole brain image generated at 10x magnification. Bigger portion on top is the forebrain, smaller portion at the bottom is midbrain. Right: midbrain section. Arrow points at DM (dark stained area), dark stained oval around DM encompasses the Intercollicular complex (ICo).

The extent of DM and ICo was calculated using ImageJ for 11 birds (Table 7; both the hemispheres for 3 birds, and 2 sets of sections for 1 bird).

Computed volume of ICo in different birds

Bird name	Volume (mm^3)	Bird name	Volume (mm^3)
Blk05wht16	0.7926	grn16blk84	0.6285
Brn72org72	0.7122	red02wht02(left)	0.8589
grn41grn44	0.6000	red02wht02(right)	0.7869
Grn43blu26 (left)	0.9258	reference	0.9453
Grn43blu26 (right)	0.5586	grn68org95	0.7542
grn54pnk90(left)	0.7545	grn20org40	0.6858

grn54pnk90(right_2)	0.7233	brn74org74	0.7344
grn54pnk90(right_4)	0.7122	**Average=0.7293**	**std=0.1077**

Table 7: computed volume of ICo (in mm^3) for different birds. Right side bottom: average volume and standard deviation.

Similarly the extent of lesions was estimated and a percentage of total area lesioned was calculated. Since DM is a small nucleus in ICo, it is difficult to electrolytically lesion only DM. Also, as previously mentioned, birds where calls were not elicited upon electrical stimulation, lesions were made in ICo. Hence the whole of ICo was considered for lesion analysis. Lesions could be located in 7/10 birds. Serial sections for one such bird are shown below.

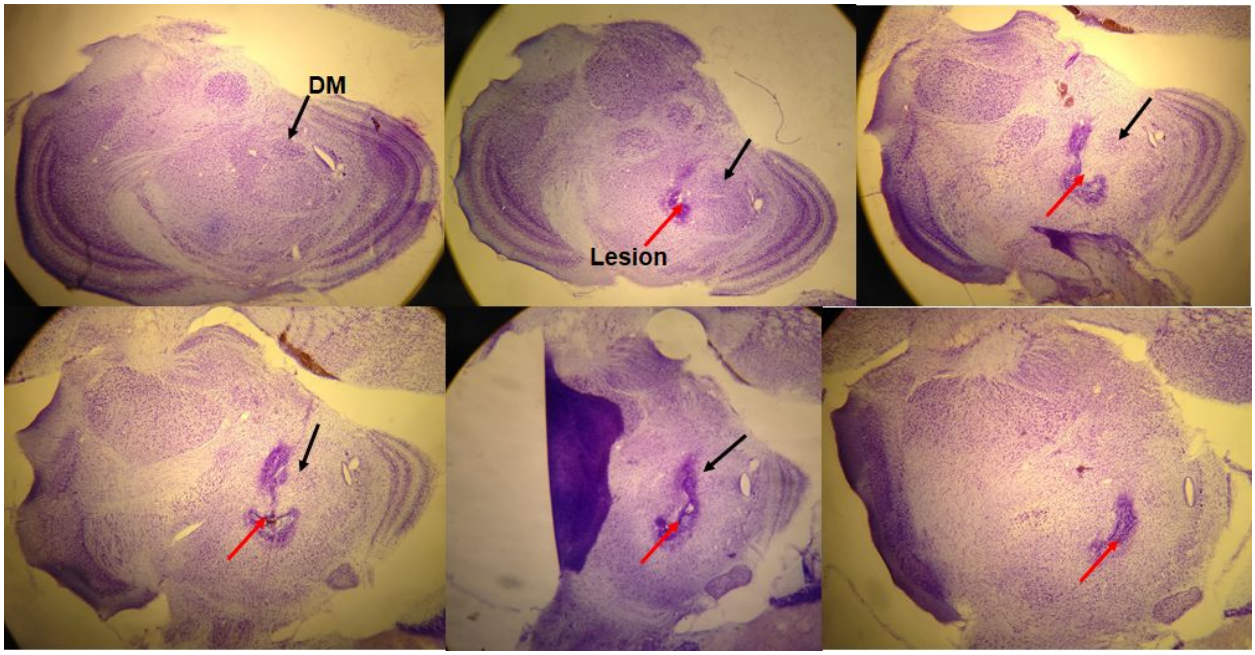

Fig 24: serial lesions in ICo and DM. Black arrow indicates the position of DM. Red arrow indicates lesions. Sections appear purple as they have been stained with cresyl violet.

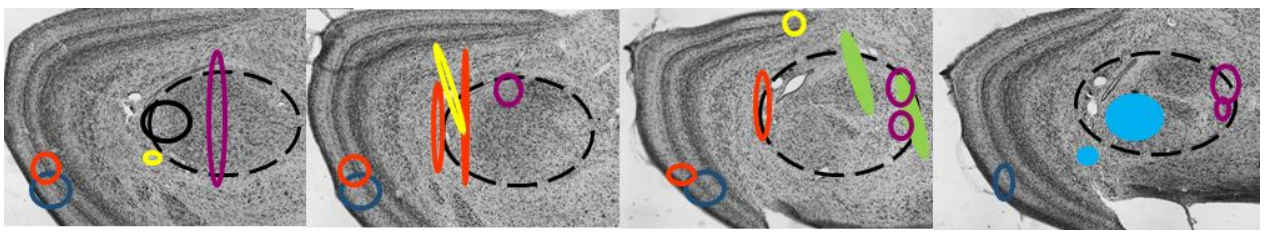

Fig 25: Approximate sites and extent of lesions in various birds. Different colours represent different birds. Filled shapes indicate change in song observed. Area marked with broken lines encloses ICo. Colour code same as in table 8

Volume of ICo lesioned

Bird name	Site of lesion	Volume of ICo lesioned	Change in song observed (as observed in spectrograms)
Blk05wht16	None (used for recording neural activity-no lesions made)	-	no
Brn72org72	Posterior border of midbrain	-	no
red02wht02	Posterior border of ICo and Posterior border of midbrain	2.94%	no
grn68org95	Dorsal- posterior border of ICo	1.25%	no
grn20org40	Lesions could not be located	-	yes
brn53org62	Lesions could not be located	-	yes
grn41grn44	Dorsal-central ICo	2%	yes
red67pnk43	None (did not lesion)	-	no
grn54pnk90	Anterior ICo, covering a part of DM	27.65%	no
grn16blk84	Lateral Posterior ICo	18.13%	no
brn36org36	None (did not lesion)	-	no
brn74org74	Lesions could not be located	-	yes
grn43blu26	Center of ICo	10.20%	yes

Table 8: Summary of sites of lesions across different birds and the percentage of ICo lesioned. Names of birds have been highlighted in the same colour used for marking the sites of lesions in Figure 25.

Lesions could be located in only 2/5 cases where a change in song was observed upon qualitative examining of spectrograms. From this analysis, it can be said that lesions in central-medial ICo altered the song and may play a role in controlling song production (Table 8). Lesions in other parts of ICo did not alter the song and may not be important for producing song.

Neural recording data analysis

Although electrolytic lesions proved to be a good method to assess the role of DM and the associated nucleus ICo, the contribution of DM alone in maintaining the song could not be assessed. This was primarily because lesioning DM alone was not possible. Hence, to assess the role of DM in song production, neural activity was recorded in n=1 zebra finch (Figure 26).

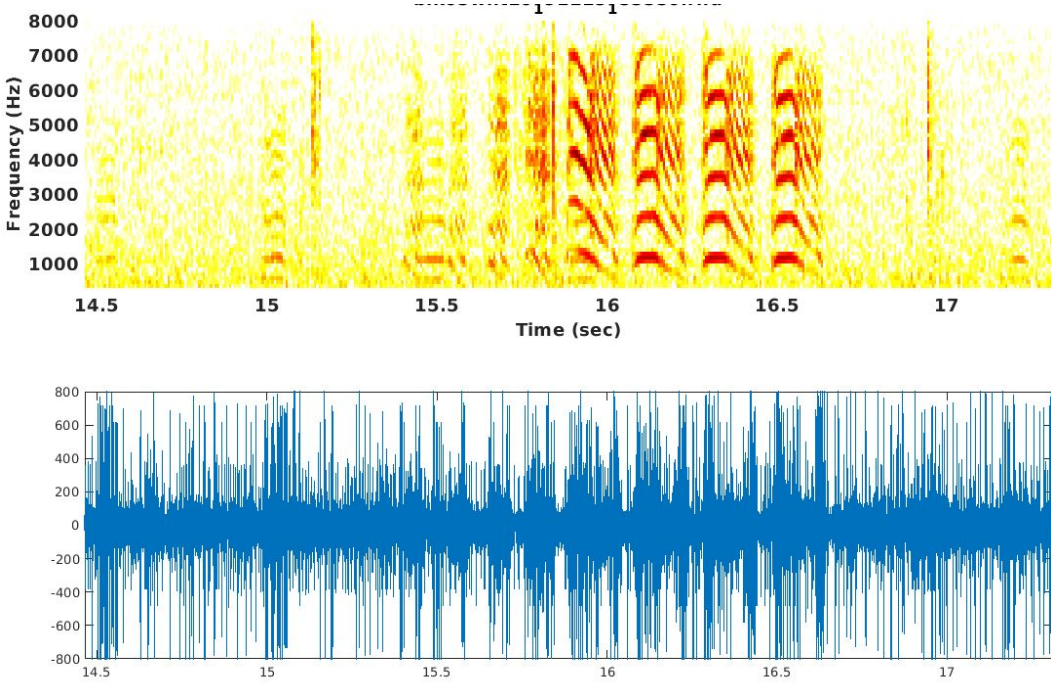

Fig 26: Above: one bout of directed song spectrogram, below: neural activity trace. The graph depicts change in neural activity when the bird sings one bout of directed song.

From preliminary analysis, neural activity does seem to change during song. More analysis is needed to conclusively assess the role of DM.

Discussion

In this study, I have looked at the role of DM in song production in zebra finches. DM is a midbrain nucleus that is a part of ICo (intercollicular complex) and is anatomically a part of the circuitry for producing learned vocalisations (song) as well as the circuitry for

producing unlearned vocalisations (call). While the role of DM has been studied in context of call production, it's role in song production has not been established well.

DM was located using stereotaxic coordinates along with electrical stimulation in DM to elicit calls (n=6) in accordance with Fukushima and Aoki (Fukushima and Aoki, 2000). Duration and mean frequency of the first elicited call did not vary upon varying stimulation parameters (current amplitude, frequency of stimulation, duration of stimulation). Sites where calls were elicited were lesioned. In some birds (n=7), however, no calls were elicited upon stimulation at different sites. Lesions were made either where ICo typical behaviour was elicited assuming that lesioning a big part of ICo would lesion at least a part of DM (DM lies within ICo) or nearby regions. Distance calls were altered as a result of lesions in DM and ICo (n=5/10). Lesions also resulted in the production of unidentifiable syllables and altered song tempo in a fraction of the birds (n=5/10). Birds where differences could be seen in distance calls, differences could also be seen in song. In all the cases where the song was altered, significant differences could be seen in mean syllable durations and the correlation values for syllable distributions before and after surgery were very low. A similar trend was observed for gap durations as well. In other cases, lesions had no effect on the song syllables but tempo was slightly slower post-lesion (n=5). The changes in song were then correlated to the sites of lesions. Song changes were correlated with lesions in central ICo (n=2), while lesions in other parts of ICo were correlated with no changes in song.

Uncertainties associated with locating and identifying the extent of DM and the extent of lesions

My results suggest that parts of ICo are involved in normal song production. However, one important caveat with identifying the exact areas involved is the problem of locating the extent of DM and the extent of the lesion itself. While lesions could be located in most birds, locating lesions was tough in those where the song changed post surgery. This was primarily because all these birds did not sing at all until 15-20 days after

surgery. The damage may have recovered in this time and hence lesions could not be traced by cresyl violet staining alone. Further, it was difficult to tell apart DM from ICo, given their spatial proximity and fused boundaries. An alternative to this problem would be to use tracer injections. Since RA projects to DM and not ICo, (Gurney, 1981; Nottebohm et al, 1982), tracer injections in RA would label neuronal populations in DM. In case of lesions in any other area, tracers would be visible in DM. This would require injecting tracers in RA prior to lesioning DM.

As mentioned previously, calls were not elicited in all the cases. This could be due to the state of anesthesia. Also, calls could be evoked from other sites too. In one bird, a single site lesion was lesioned where calls were elicited. However, upon histological verification, lesion was at a place significantly away from ICo and DM. After comparison with stereotaxic atlas, lesion was found to be close to a projection of RA. However, this was only in one bird. In many other cases calls were elicited in the anesthetized state and the lesions were close to DM suggesting that locating DM in the anesthetized state using electrical stimulation is a good method. Previous studies (Simpson and Vicario, 1990; Fukushima and Aoki, 2000) have used this method to locate DM in the anesthetized state.

Distinguishing between lesions at two different sites

The coordinates where calls were evoked in the two hemispheres differed, possibly due to the placement of head onto the beak bar. Since most lesions were bilateral (8/10 birds), this could have led to lesions in slightly different areas on the two sides. In such cases, ascertaining the cause of change in song to the site of lesions was difficult.

Changes in song syllable durations after midbrain surgeries

Interestingly, it was observed that lesions in ICo led to stretching of syllables post surgery. This was most probably an effect of midbrain surgery. Birds that had

undergone a different type of surgery did not show this trend (tracheosyringeal cut sham surgery). This will be investigated further by obtaining more data points.

Effect of DM/ICo lesion may or may not be similar to Uva lesion

It is known that DM projects to Uva. We would expect to see similar effects of Uva and DM lesions. In all the birds where the song changed post surgery, the effects on song were similar across birds with the syllables being unrecognizable and the song tempo altered. Upon qualitative comparison with Uva lesions (Figure 27), DM/ICo lesions appear similar to Uva lesions in a few cases and were very different in the others. Since the number of data points available for comparison were very less, more of such data points are needed to draw meaningful conclusions.

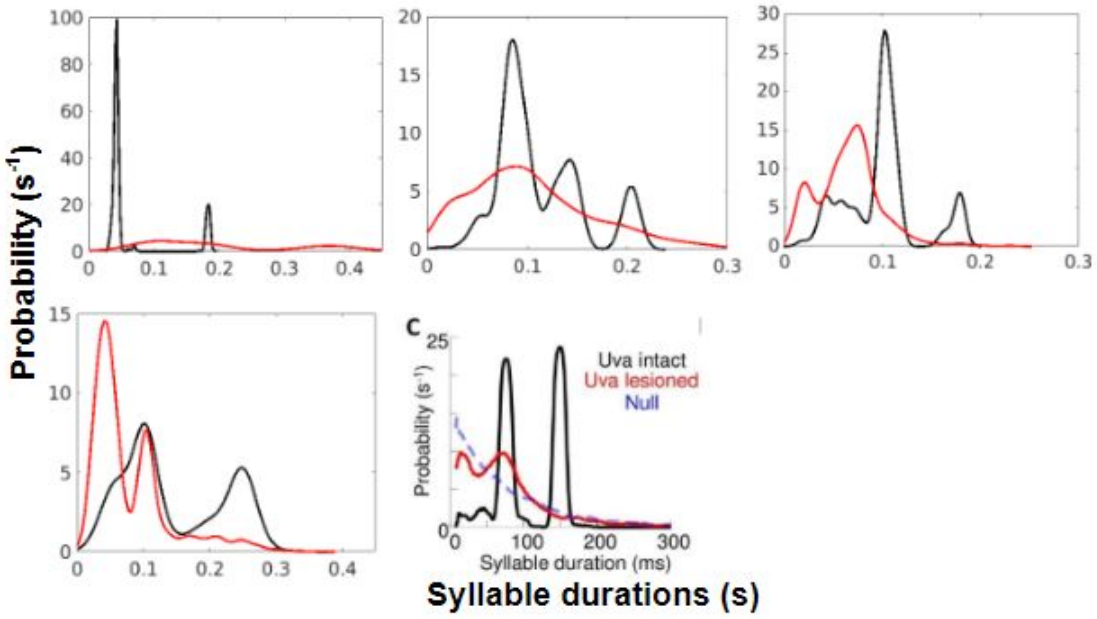

Fig 27: Top panel: Pdf for syllable durations before and after ICo/DM lesions in different birds. Black trace is for syllable durations before surgery and red trace is for syllable durations after surgery. Bottom right: PDF for syllable durations before and after lesions in Uva (Danish HH, Aronov D, Fee MS, 2017)

ICo/DM lesions affects both directed and undirected song similarly, unlike HVC and Uva lesion

Lesions in song nuclei HVC and Uva lead to differences in directed and undirected songs. HVC lesions abolish directed singing completely, but birds continue to sing undirected songs, which have atypical syllables (Aronov et al, 2008). In case of Uva lesions, songs in the directed context mostly consisted of introductory note like syllables (Danish HH, Aronov D, Fee MS, 2017). While, undirected songs had an effect similar to the ones seen in case of HVC lesions (Danish HH, Aronov D, Fee MS, 2017). Unlike the findings in case of HVC and Uva lesions, both directed and undirected songs had the exact same effect in my study, suggesting that lCo/DM modulate songs in a similar manner in both the contexts.

DM might be involved in production of both learned and unlearned vocalisation

Nuclei important for song production (RA and HVC) are also required for generating modulation in calls and maintaining their timing and synchronization (Benichov et al, 2016 a, Benichov and Vallentin, 2020). Similarly, DM plays a role in call production, it may also be required for song production and the circuitry for the two types of vocalisations-learned and unlearned may not be very different. Here, I show that lesions in DM, which has been primarily studied in context of call production, also affect the properties of learned song. This supports the idea that DM also plays a role in both song and call production.

Circuitry for producing song is highly decentralised

The overall implications of this study are that at least a part of lCo plays a role in song production and that the circuitry for production and maintaining the song is highly decentralised since DM/lCo lesions seem to have a different effect on song as compared to HVC and Uva lesions.

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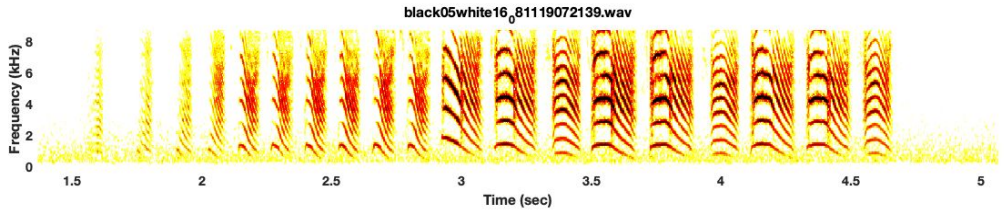
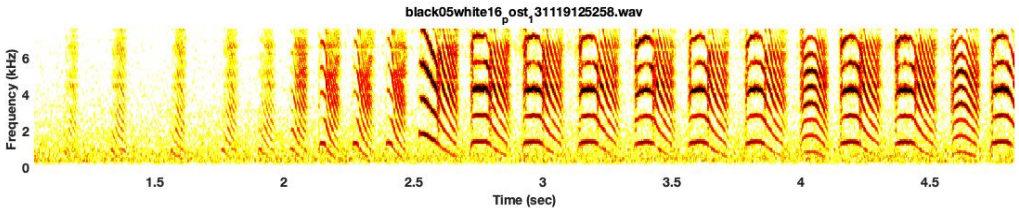
Appendix:

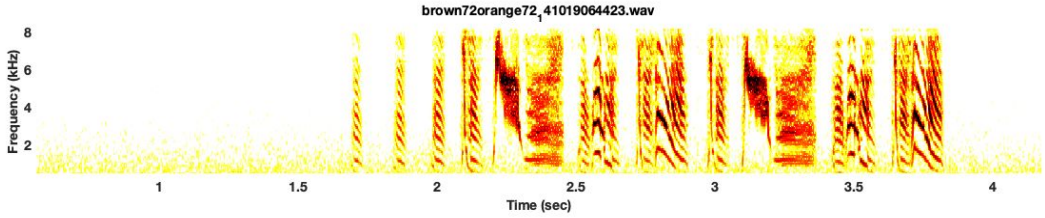
Song spectrograms for all birds (pre-surgery and post-surgery)

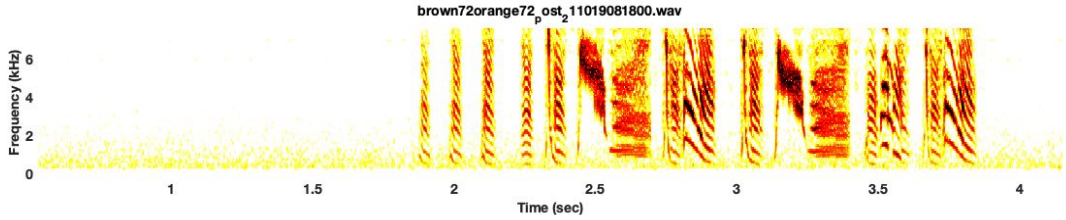
1. **Black05white16**



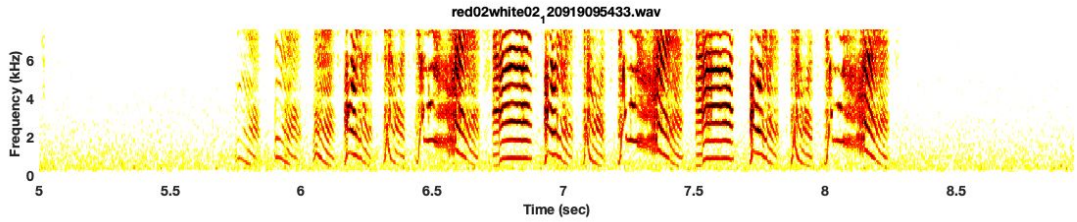


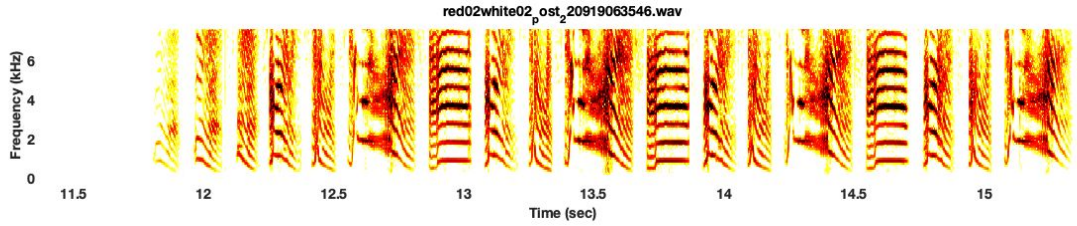
2. Brown72orange72



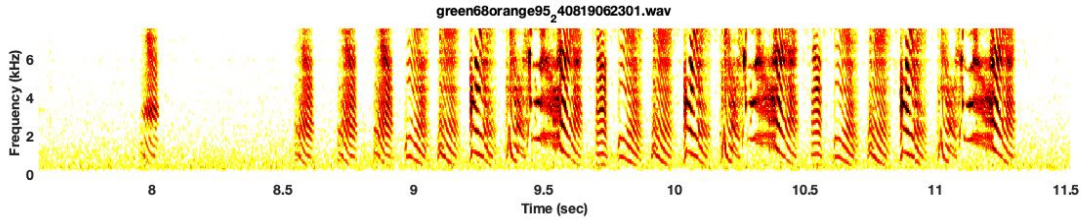


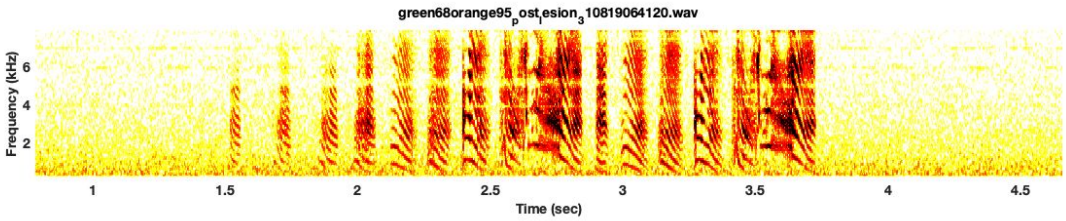
3. Red02white02



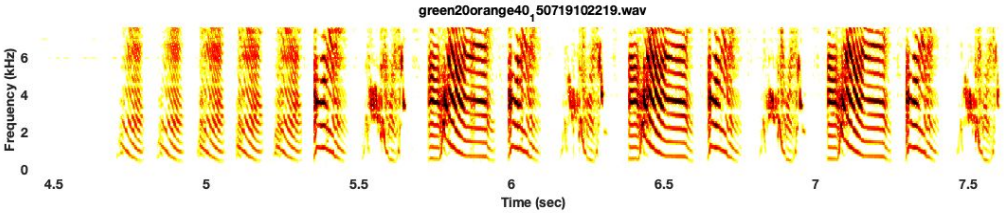


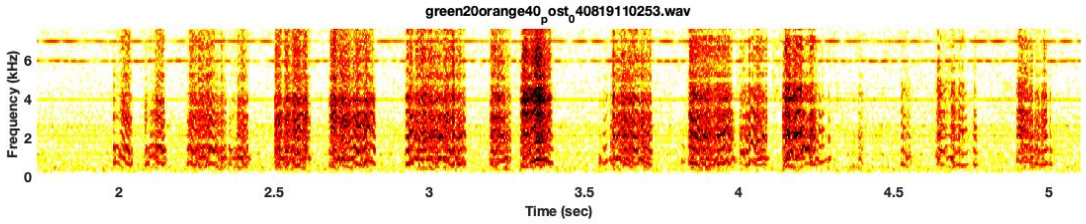
4. Green68orange95





5. Green20orange40





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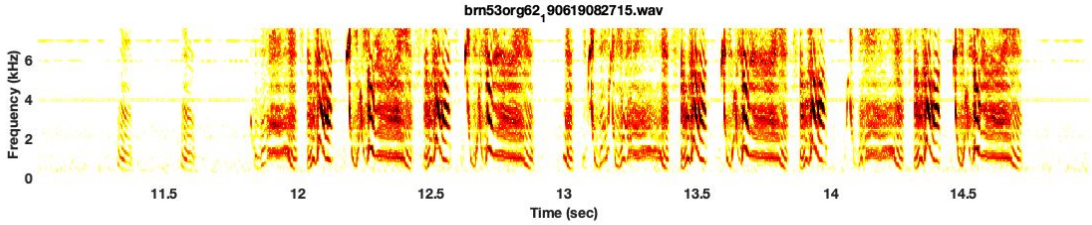
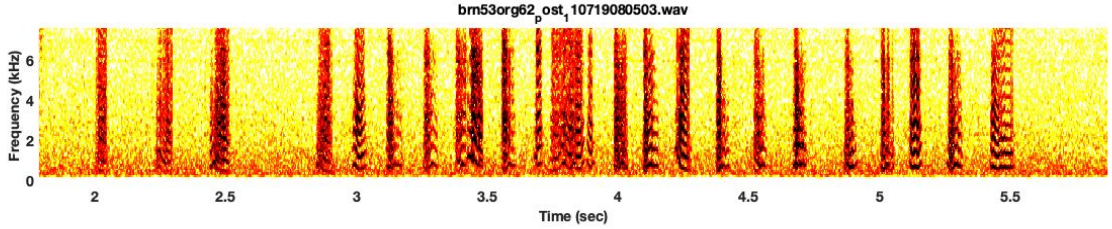
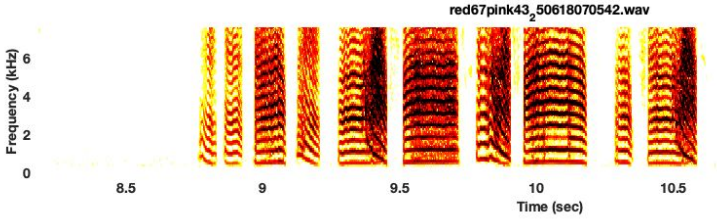
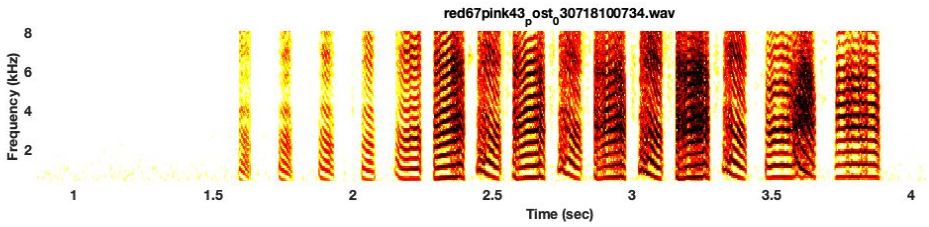




7. Red67pink43





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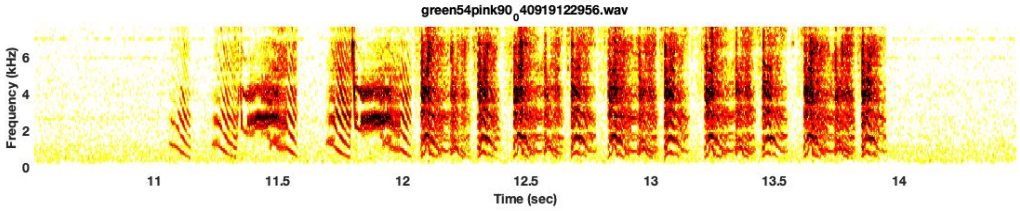
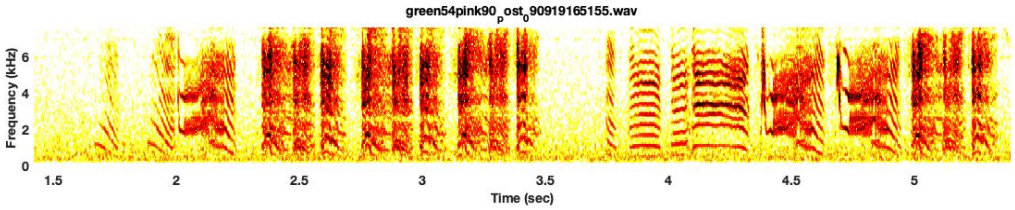
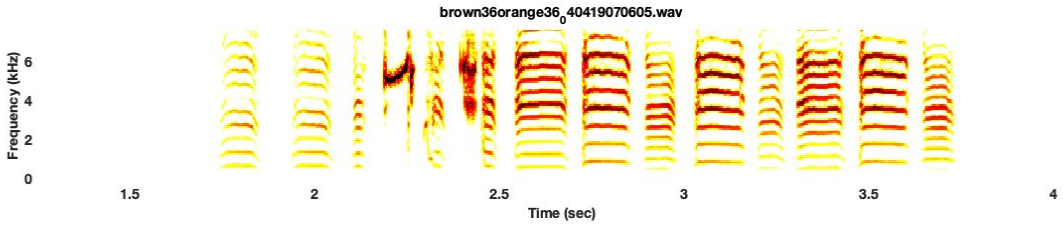
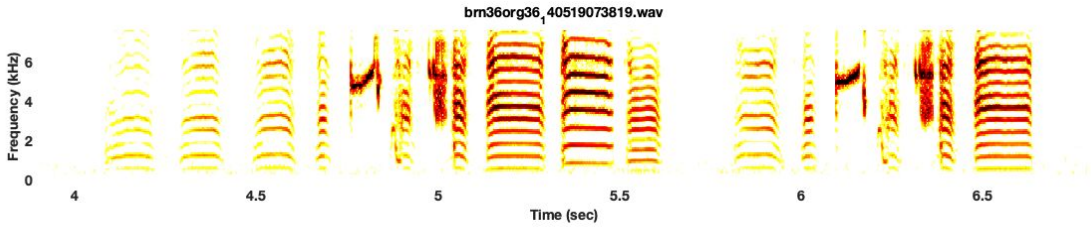


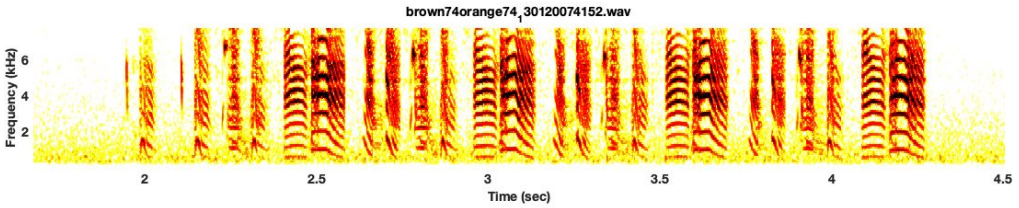


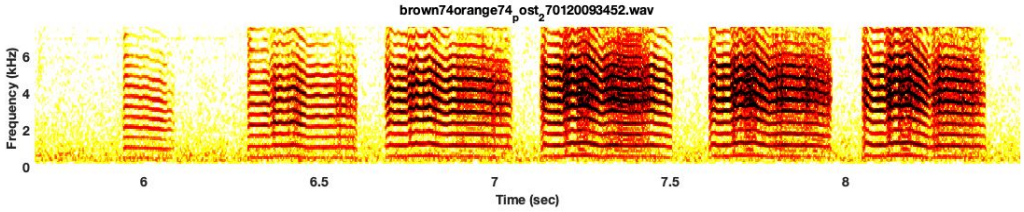
9. Brown36orange36



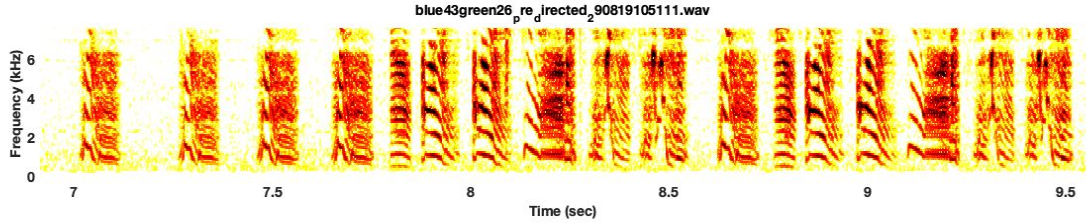


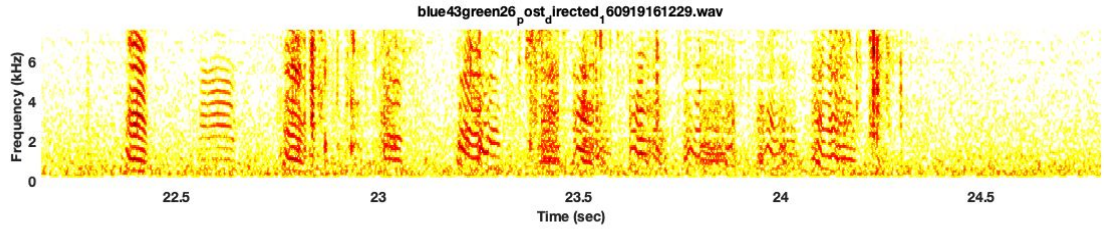
10. Brown74orange74



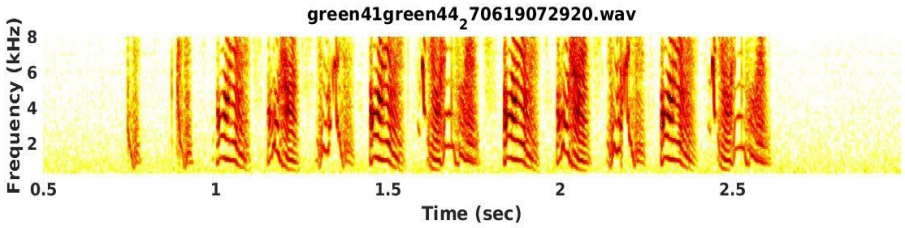


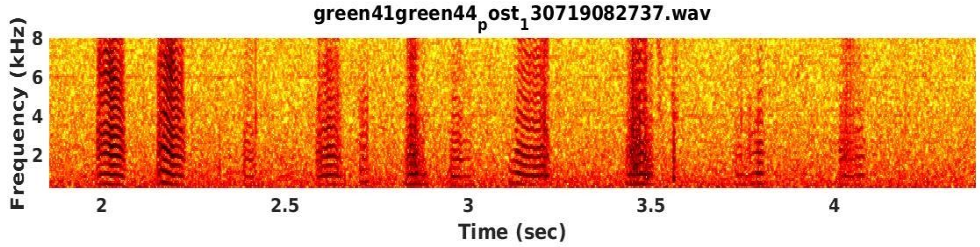
11. Green43blue26



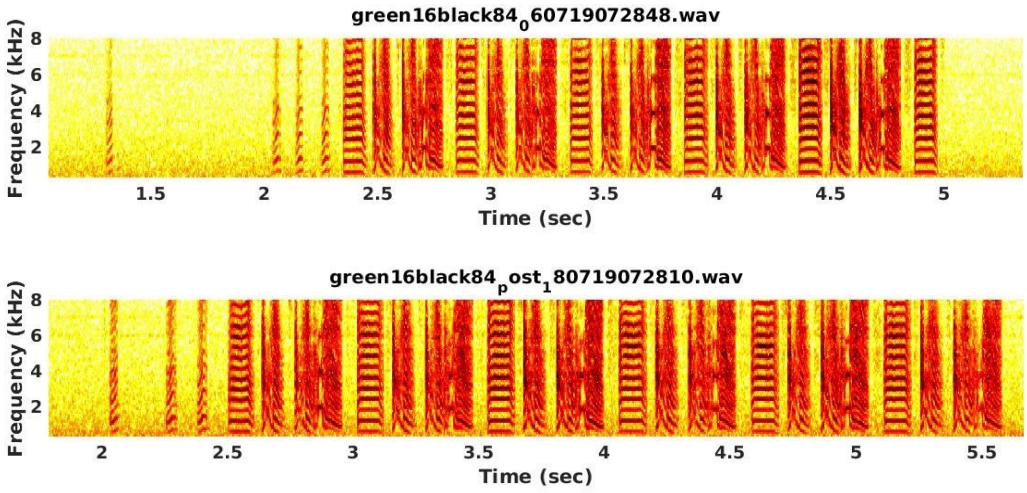


12. Green41green44





13. Green16black84



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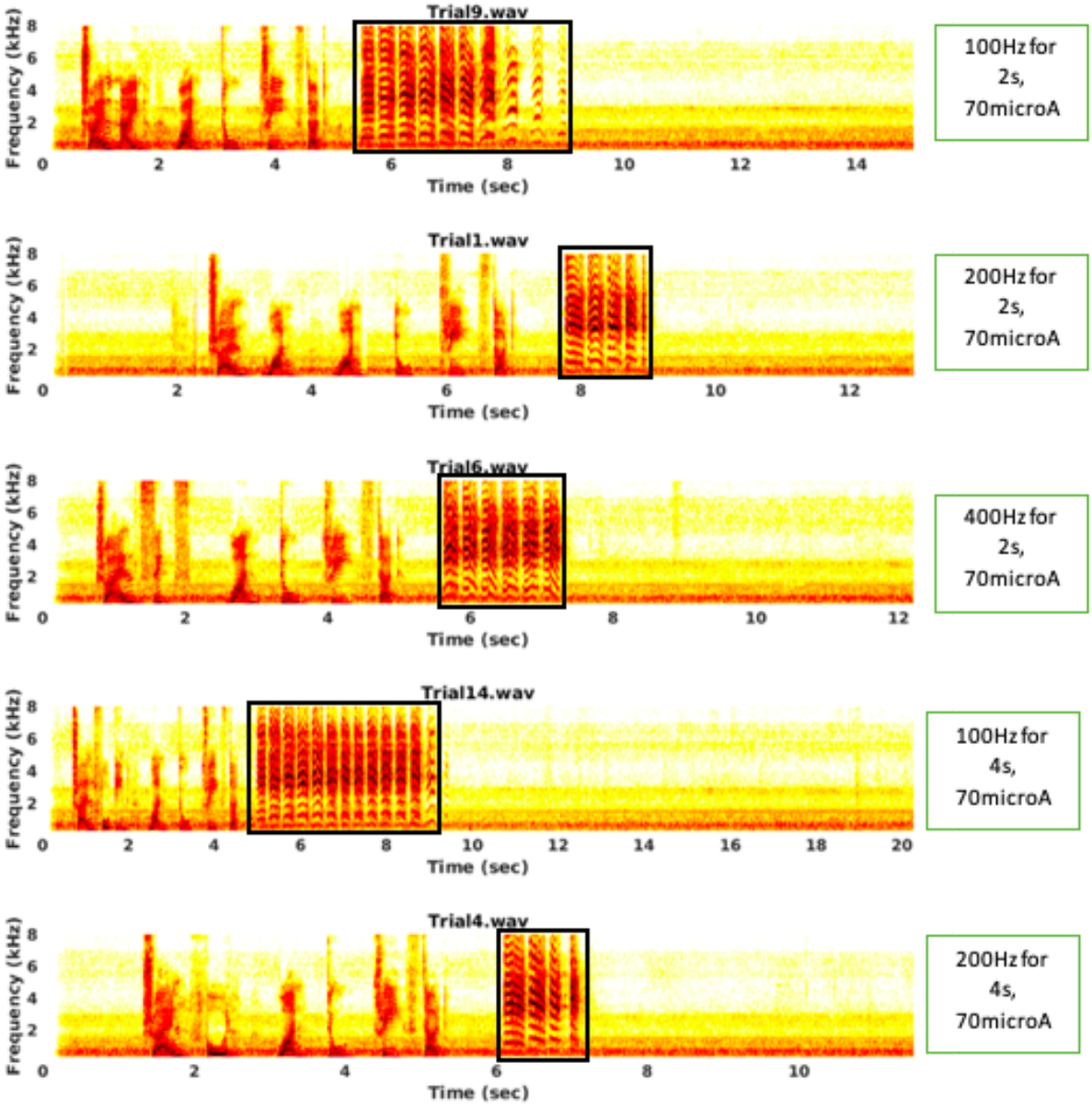
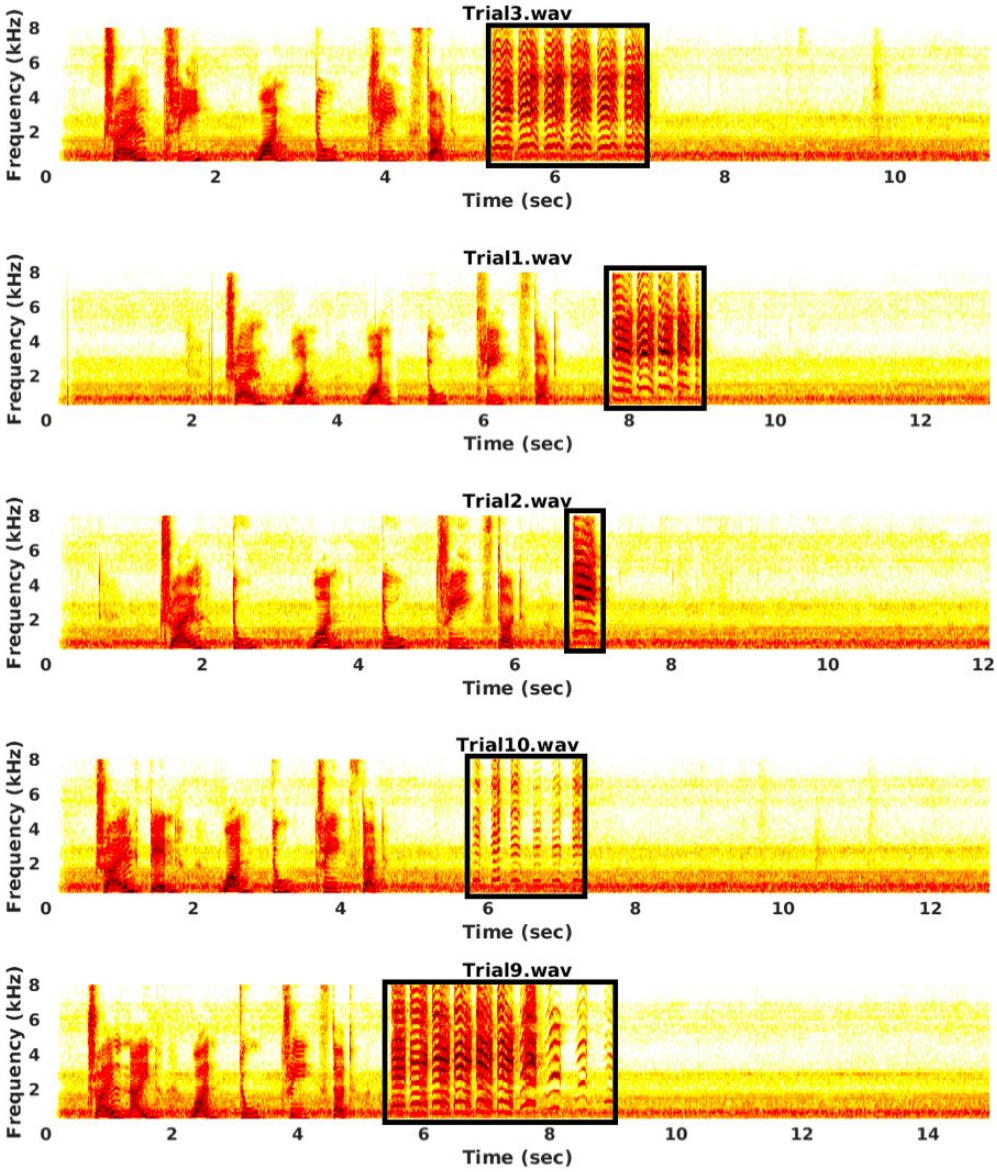
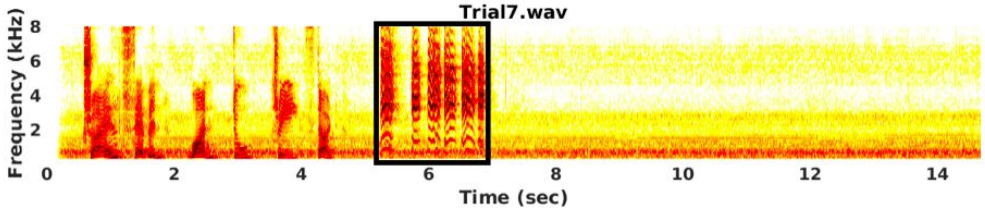


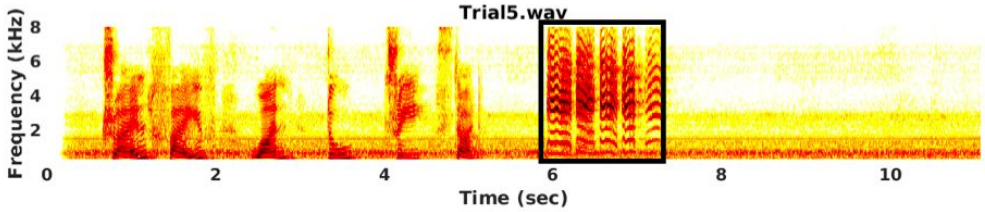
Variation on the basis of current amplitude:



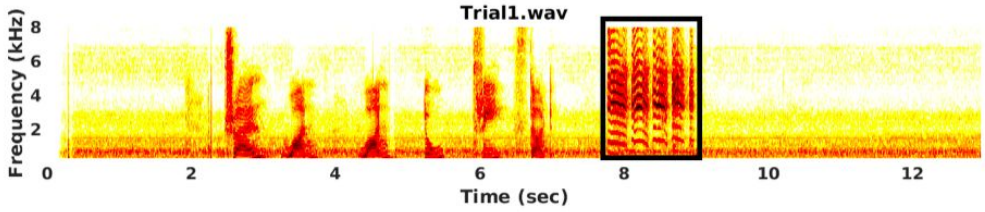


400Hz for 2s, 100microA

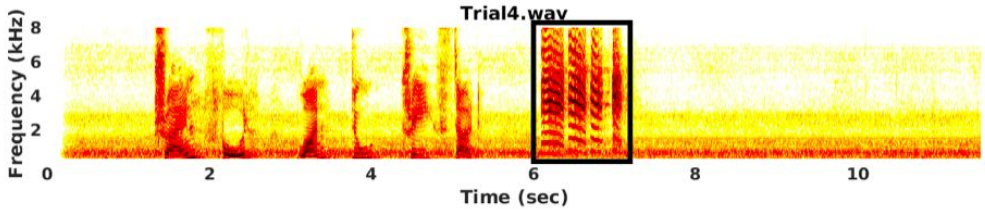
Variation on the basis of duration:



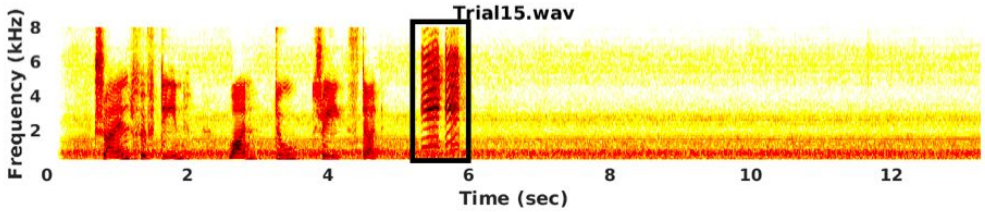
200Hz for 1s, 70microA



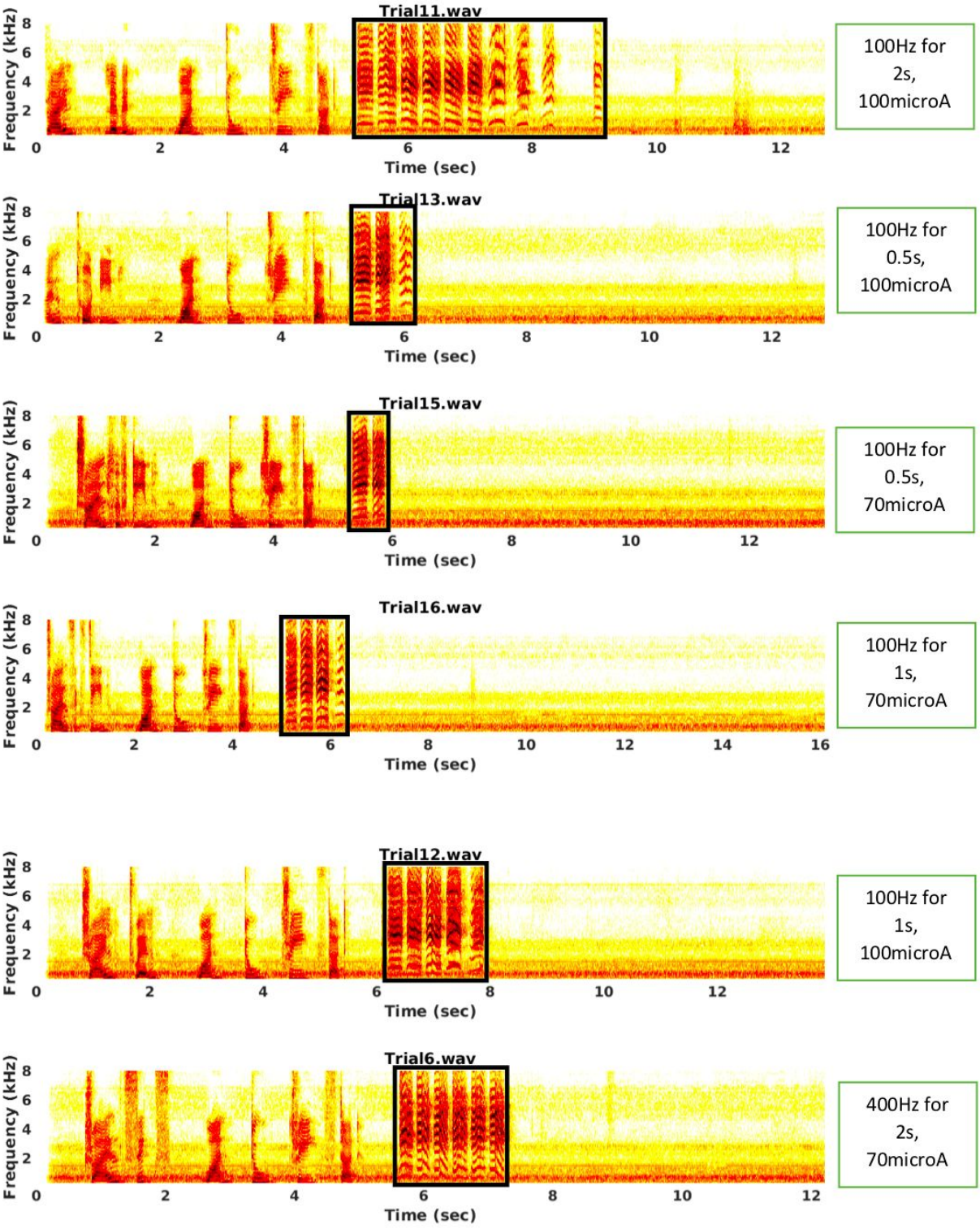
200Hz for 2s, 70microA

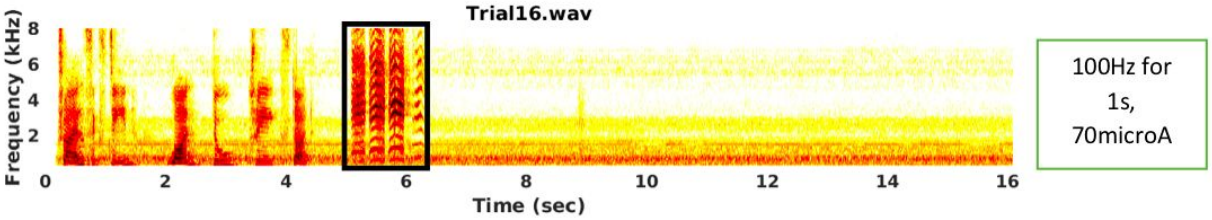


200Hz for 4s, 70microA

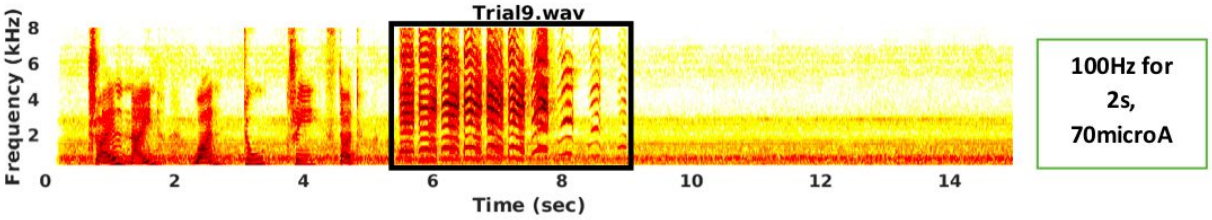


100Hz for 0.5s, 70microA

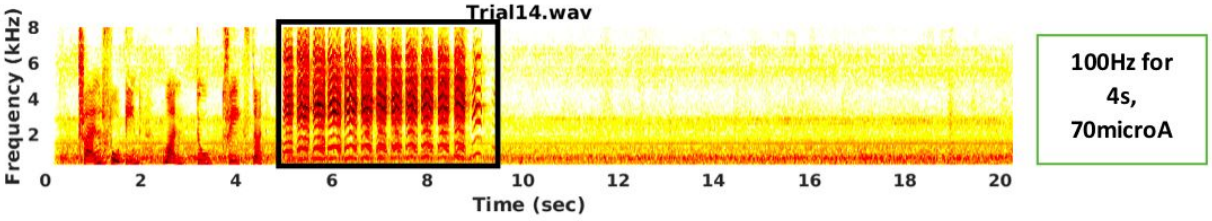

Trial11.wav
Frequency (kHz)
Time (sec)
100Hz for 2s, 100microA
Trial13.wav
Frequency (kHz)
Time (sec)
100Hz for 0.5s, 100microA
Trial15.wav
Frequency (kHz)
Time (sec)
100Hz for 0.5s, 70microA
Trial16.wav
Frequency (kHz)
Time (sec)
100Hz for 1s, 70microA
Trial12.wav
Frequency (kHz)
Time (sec)
100Hz for 1s, 100microA
Trial6.wav
Frequency (kHz)
Time (sec)
400Hz for 2s, 70microA



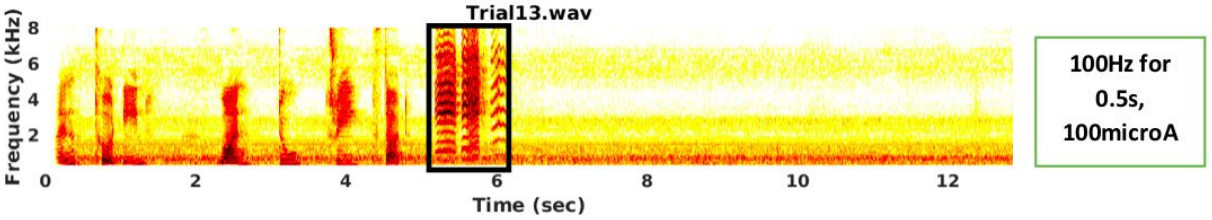
100Hz for 1s, 70microA



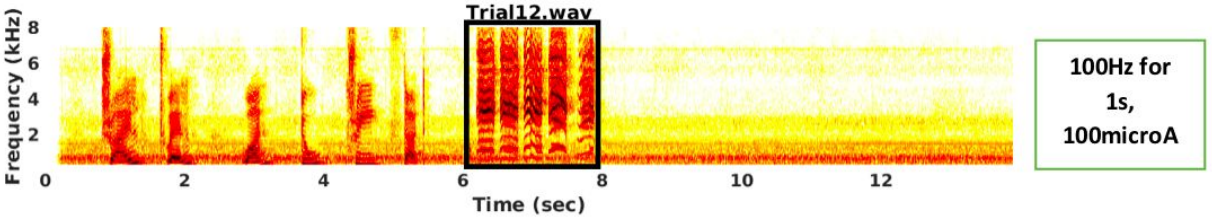
100Hz for 2s, 70microA



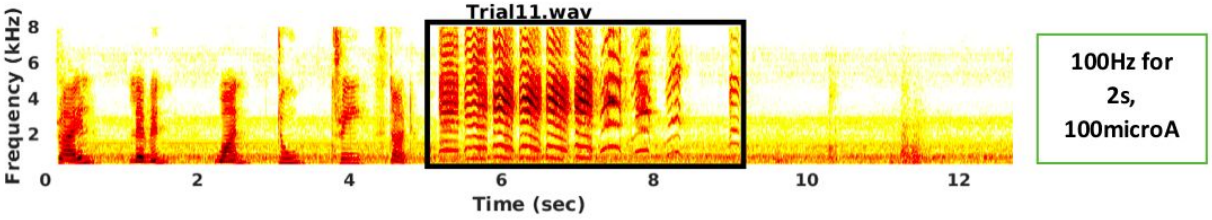
100Hz for 4s, 70microA



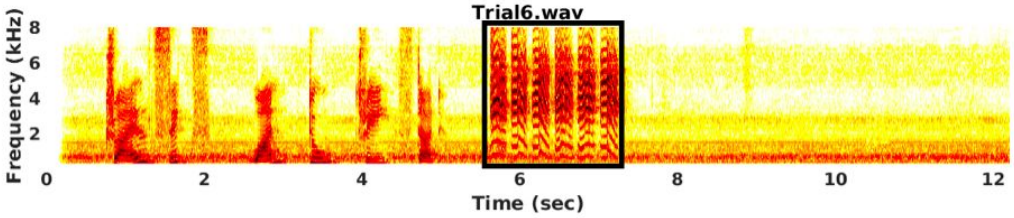
100Hz for 0.5s, 100microA



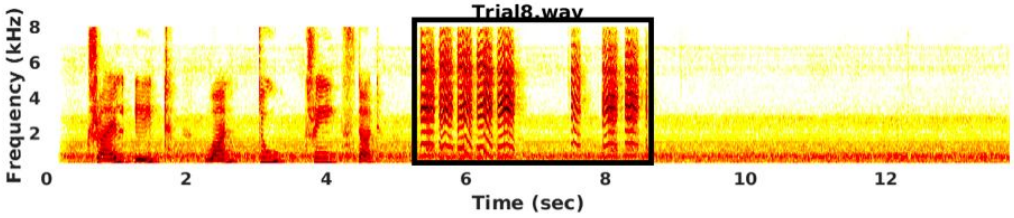
100Hz for 1s, 100microA



100Hz for 2s, 100microA



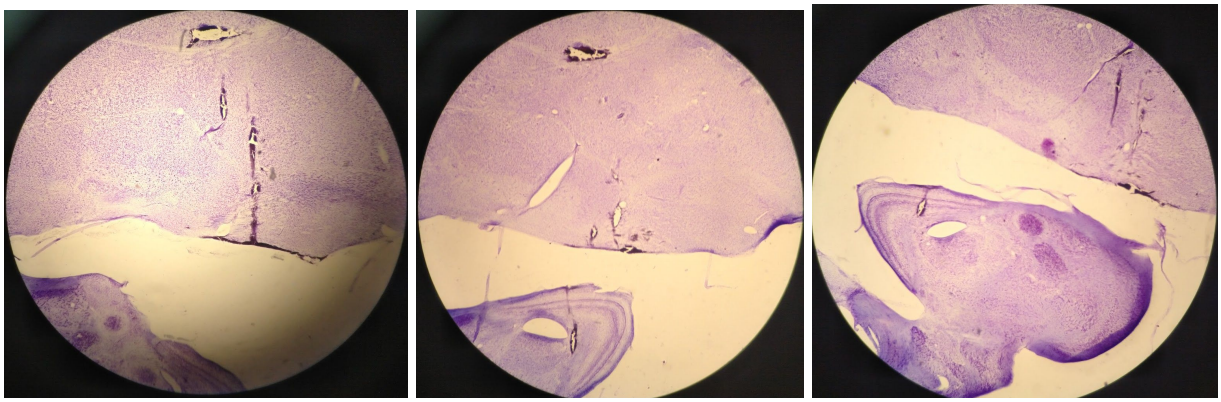
400Hz for 2s, 70microA



400Hz for 4s, 70microA

Histology- Pilot experiments:

Black98black87-right hemisphere:



Black98black87-left hemisphere: