The role of inhibitory interneurons in song learning in zebra finches

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Certificate

This is to certify that this dissertation entitled 'The role of inhibitory interneurons in song learning 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 Avani Prasad Koparkar at Max Planck Institute for Ornithology, Seewiesen under the supervision of Dr. Daniela Vallentin, Research Group Leader, Max Planck Institute for Ornithology during the academic year 2019-2020

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This thesis is dedicated to my family:

Aai, Baba, Aarya and Ajji.

Without you, I am nothing.

Declaration

I hereby declare that the matter embodied in the report entitled 'The role of inhibitory Interneurons in song learning in zebra finches' are the results of the work carried out by me at Max Planck Institute for Ornithology, Seewiesen under the supervision of Dr. Daniela Vallentin and the same has not been submitted elsewhere for any other degree.

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Contents

Abstract

Inhibition has been shown to play an important role in determining the critical period of development of sensory systems but its role in motor system is unknown. Here, I study the zebra finch, a bird that develops a motor skill during a critical developmental period. In juvenile zebra finches, learning of song syllables during critical period is accompanied by a corresponding stepwise increase in inhibition in HVC (used as proper name). Adult zebra finches have crystallized songs which are protected from further change influenced by external auditory playback by the inhibitory interneuron network in HVC. In this study, I show that pharmacological inactivation of the inhibitory interneuron network in adults leads to reopening of the critical period and induces plasticity in song. Since HVC is a relay center between the auditory and motor systems, I provided an additional auditory playback during pharmacological interneuron inactivation and observed its effect on the changes in song. I quantified changes in spectral features of song syllables and changes in linearity, consistency and stereotypy of syllable sequencing. My results show that the limitation of HVC interneuron efficacy leads to a window of opportunity for zebra finches to learn a new song which seems to be guided by imitation learning. Reopening of the critical period in later stages of development by limiting interneuron signaling opens possible avenues for fine-tuning learned motor sequences in a guided manner.

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This year has been full of challenges and I will always be grateful for my strong support system back in India who helped me overcome all obstacles. I am truly indebted to my family, Aradhana, Prasad, Aarya and Rekha Koparkar for their unwavering confidence in my abilities. I would also like to thank Jay Panchpor, for his picking me up when times were tough and sticking with me through my highs and lows. Lastly, this thesis would not have been possible without the constant love and support from Sayantika Mondal, Meher Kantroo and all my friends and cousins.

Introduction

The role of inhibition in shaping circuits of different sensory modalities has been the focus of several studies. Inhibitory signals play an important role in determining the critical period in the development of neuronal networks. The 'critical period' is a time window in which plastic neuronal networks are especially receptive to sensory information and rely on this acquired information for their continued development (Hensch, 2004). Studies in primary visual cortex of kittens show the presence of neurons that preferred inputs from one eye (contralateral or ipsilateral) grouped into columns. These were termed as 'ocular dominance columns' (Hubel and Wiesel, 1963). Deprivation of visual input by rearing the kittens in the dark unearthed the critical period of development of these ocular dominance columns (Hubel and Wiesel 1962; Hubel and Wiesel 1963; Swindale 1981). Experiments involving deprivation of visual input from one eye along with pharmacological suppression of inhibition show that the columnar structure favours the eye receiving less sensory input. This demonstrates that post-synaptic inhibition plays a role in shaping the neuronal circuitry (Hata Y. and Stryker, 1994). It was also shown that transplantation of inhibitory neurons after the closure of critical period can bring back ocular dominance plasticity in kittens (Southwell D.G. et al, 2010). This relationship between critical period and inhibition is also observed in the mouse auditory cortex. The cortex shows clustered populations of neurons that respond to a range of frequencies but show high response to a 'best-tuned' frequency. These are called frequency specific tonotopic maps (Barkat et al, 2011). Formation of these tonotopic maps during critical period of development is dependent on L1 inhibitory interneurons in the auditory cortex. Silencing of these neurons eliminates plasticity in the circuit during the critical period. (Hensch et al, 2018). Inactivation of parvalbumin positive interneurons has been shown to affect tonotopic plasticity in adult mice even after the closure of critical period (Cisneros-Franco, De Villers-Sidani, 2019).

Although a lot is known about the role of inhibition in the closure of critical period in sensory systems, it is not clear whether neural circuits within motor systems have similar underlying synaptic changes. One challenge to investigate this question if to find an animal model that undergoes a defined critical period of motor learning. Here,

I propose using zebra finches since male zebra finches learn their songs by listening to and imitating a tutor (Immelmann, 1969) and have a critical period of song learning. After hatching, the juvenile bird enters a sensory phase where it is exposed to the songs of its conspecifics. It then incorporates a template of the song that is to be learnt. In the sensorimotor phase, which overlaps with the sensory phase juvenile zebra finches begin to produce initial vocalizations that are akin to babbling in human infants. Between days 28 to 35 post-hatching, the bird produces an unstructured 'subsong'. The song begins to become more stereotyped by day 50. By day 70 post-hatching approximately, the bird manages to copy the tutor's song. The plastic phase of the bird's song can continue up to 90 days post-hatching (Fig. 1) (Doupe and Kuhl, 1999; Brainard and Doupe, 2000; Tchernichovski et al, 2001). The bird learns to imitate the template of the tutor's song, after which the song crystallizes and remains largely invariant for the rest of the bird's life. Auditory feedback is especially important during the critical phase of learning as birds raised in isolation produce abnormal, simplistic songs as compared to normal birds (Nordeen and Nordeen, 1992).

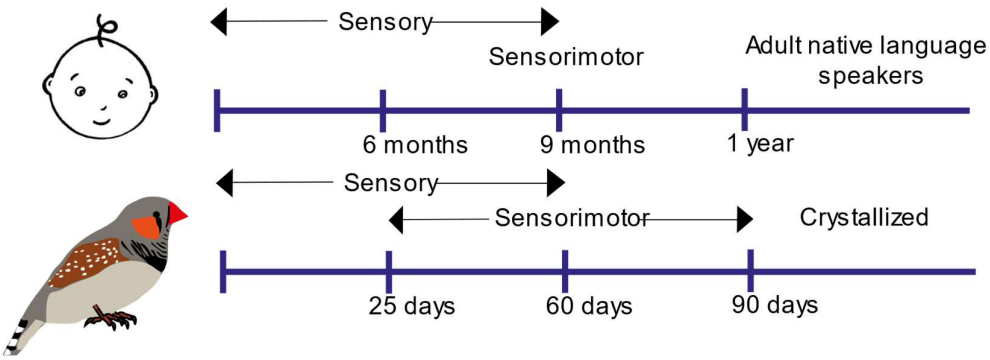

Figure 1: Critical periods of vocal learning in humans and birds. (This figure has been made based on data in Doupe and Kuhl, 1999)

The neural circuitry underlying song learning and production in zebra finches has been the topic of extensive research. There are several nuclei involved in this circuitry, of which the nucleus HVC (used as a proper name) has garnered most attention. HVC is located in the forebrain and is a part of both auditory as well as motor pathways (Lewandowski et al, 2013). Neurons in HVC respond selectively to bird's own song (BOS) in the anesthetized condition (Margoliash, 1986; Margoliash and Fortune, 1992; Lewicki, 1996), but do not respond to the same stimulus in the awake condition (Schmidt and Konishi, 1998). Thus, HVC acts as a gating centre for auditory signals

from its unique junctional position between auditory and motor pathways (Schmidt and Konishi, 1998). Lesion studies and studies in canaries involving pharmacological inactivation of HVC demonstrate that HVC is necessary for maintaining the rhythm of the song as well as spectral features of the song syllables (Nottebohm, 1976). Bilateral lesioning of HVC in adults leads to degradation of the structure and stereotypy of song and reversion to subsong (Aronov, Andalman, Fee, 2008) whereas cooling down of HVC slows down the song overall (Long and Fee, 2008). Electrical stimulation of HVC leads to altered sequencing of syllables in the ongoing song (Vu et al, 1994).

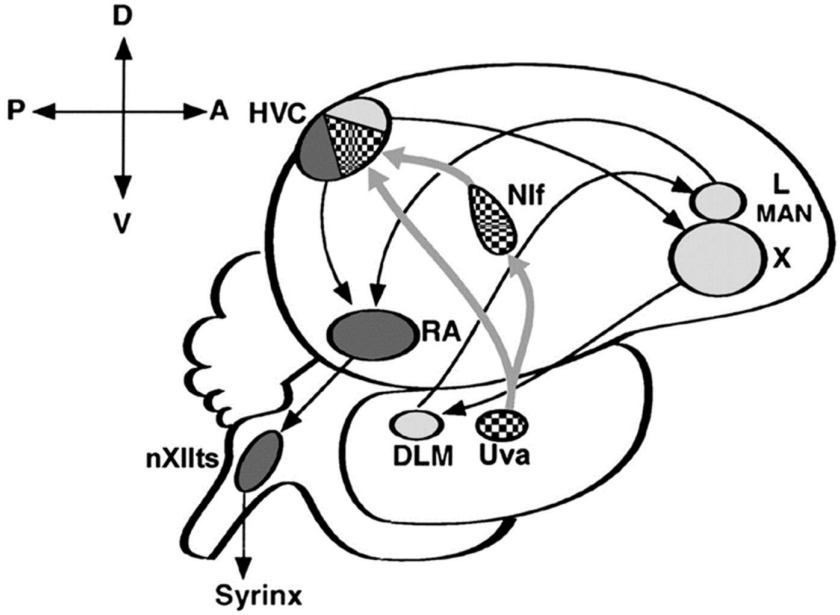

Figure 2: Schematic representation of neuronal projections to and from HVC (Akutagawa and Konishi, 2005) Abbreviations: NIf: *nucleus interfacialis of the nidopallium*, Uva: *nucleus uvaeformis*, LMAN: *lateral magnocellular nucleus of the nidopallium*, RA: *robust nucleus of archipallium*, DLM: *dorsolateral nucleus of thalamus,pars medialis* , nXIIts: *hypoglossal nucleus, tracheosyringeal part*. Arrows representing nuclei connections: Anterior forebrain circuit (light gray), Motor pathway (dark gray), Patterened: efferent projections from both the Uva and NIf

HVC receives inputs from higher order nuclei NIf (nucleus interfacialis of the nidopallium) and Uva (nucleus uvaeformis). NIf is the primary afferent of auditory information to HVC (Lewandowski et al, 2013) while Uva, in addition to direct projections to HVC, also indirectly sends connections via NIf and Av (nucleus

avalanche), thus forming a relay centre of information during ongoing song (Fig.2) (Akutagawa and Konishi, 2005; Danish, Aronov, Fee 2017).

Downstream, HVC sends projections to RA (robust nucleus of archipallium), Area X (Fig.2) and Avalanche (Fortune and Margoliash, 1995; Foster and Bottjer, 1998; Roberts et al, 2017). It is a part of the posterior descending pathway (PDP) which contains connections from HVC to RA. RA neurons show unique firing patterns for individual notes within syllables of the bird's song (Yu and Margoliash, 1996). HVC_{RA} projectors generate single, sparse bursting patterns during the specific timepoints in the song and thus form an explicit representation of time in the song (Okubo et al, 2015; Picardo et al, 2016). They drive the RA sequence by sequential bursting at a population level. (Hahnloser et al, 2002, Kozhevnikov and Fee, 2007). Neurons from RA project to the hypoglossal nucleus (nXIIts) and respiratory motor neurons and which ultimately innervate the vocal and respiratory muscles necessary for vocal production (Nottebohm, 2005, Sturdy, Wild and Mooney, 2003). The anterior forebrain pathway (AFP) contains connections between HVC to Area X to LMAN (lateral magnocellular nucleus of the nidopallium). This pathway feeds back into the motor pathway via RA and is necessary for song learning in juvenile zebra finches (Andalman and Fee, 2009).

In addition to the three populations of projection neurons (HVC_X, HVC_{RA} and HVC_{Av}), HVC also has a population of heterogenous interneurons (Dutar, Vu and Perkel, 1998). Inhibition in HVC mediated by GABA-ergic interneurons is shown to be important in the control of the timing of calls during vocal turn-taking (Benichov and Vallentin, 2020). Additionally, inhibition also plays an important role in the closure of the critical period i.e. inhibitory interneurons in HVC are thought to protect the memory of the learnt song (Vallentin et al, 2016). During intracellular recordings in juveniles, it was observed that HVC_{RA} neurons showed spiking activity evoked by tutor song playback, which was absent in adult birds. To check whether GABA-ergic inhibitory networks were involved in suppression of this activity in adults, $GABA_A$ receptor antagonist gabazine was locally applied to HVC, which brought back tutor-song evoked spiking in HVC premotor neurons. The precision of firing of inhibitory interneurons correlated with the similarity of the bird's song to the tutor song. Syllable-specific inhibition increased in parallel to the degree of learning of the syllable till the bird incorporated the syllable into its crystallized song in adulthood.

In this project, I examine the effect of perturbation of the inhibitory circuit in HVC on the song of adult zebra finches in order to reopen the critical period for song learning. For this purpose, I apply the $GABA_A$ receptor antagonist gabazine on to HVC and quantify the effect on the spectral features and sequence of syllables of the bird's song. Our hypothesis states that disrupting inhibition in HVC will affect the memory of syllables that have been mastered by the bird. After gabazine application, I record directed song of the bird for one hour. Application of phosphate buffer saline (PBS) is used as control as it does not affect HVC interneurons.

HVC receives direct inputs from the auditory system (Bottjer and Halsema, 1989; Lewandowski et al, 2013). It is possible that modifications in song observed due to pharmacological manipulations of HVC can also be influenced by auditory input. Hence, I am also investigating the effect of auditory playback during drug application on the changes observed in song production. For this purpose, I use a modified playback of the BOS with interchanged syllables. During drug application, the bird also received this playback for two minutes at 15-minute intervals, for a total of four times in an hour. I record directed song of the bird and quantify effect of playback on the changes observed in the song under gabazine treatment. Application of PBS along with the same playback is used as a control for this experiment. As we open the window of plasticity in HVC with pharmacological manipulations, I expect an increase in the magnitude of effects observed due to the influence of auditory playback. I also expect this effect to be directed according to the playback given.

Methods

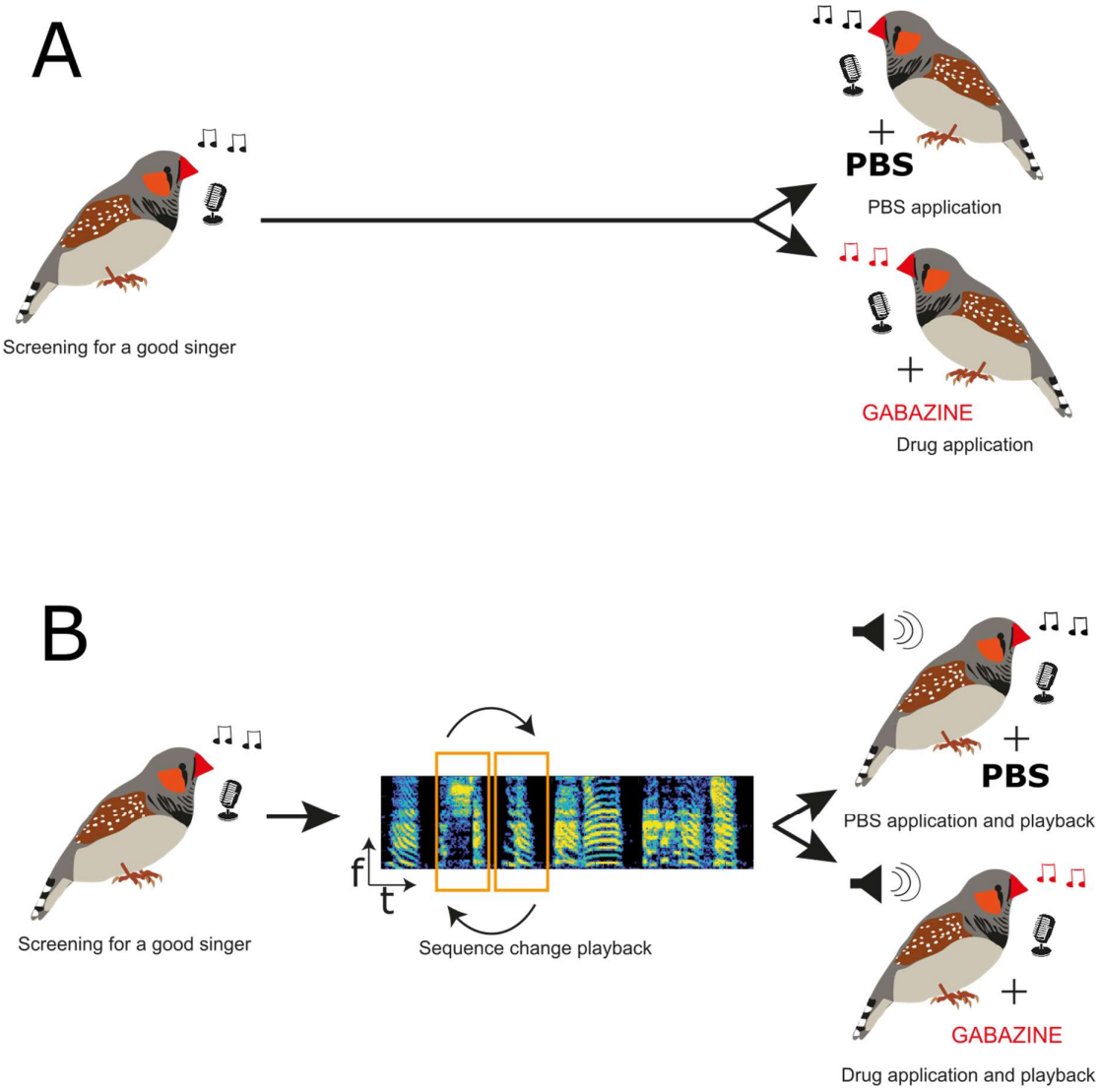

Figure 3: **Experimental pipeline schematic** Birds were screened to be good singers. Song was recorded and syllable positions were switched to create a new song playback. Then Gabazine was applied bilaterally on HVC (A). The experimental pool was divided into two groups. In one group, only gabazine was applied (A) while in the other group, playback was presented along with gabazine application (B) and the bird's own song was recorded

Animals

Animals were bred and acquired from the Max Planck Institute for Ornithology, Seewiesen. Adult males aged > 90 days post hatching were used for all experiments. Birds were screened for their ability to sing under stressed conditions and only those

individuals who immediately produced song upon presenting a female bird were selected for experimentation. Handling of birds and transportation from the aviary to the experimental room was taken as stressed condition during screening. During experimentation, the bird was under additional stress due to application of drug before recording of song. Birds that produced more than 20 motifs under PBS condition as well as under gabazine condition were considered for analysis. The total number of experimental birds analysed was 6 for Gabazine + playback group and 3 for Gabazine group.

Song Recording and Playback

Pre-surgery and post-surgery recordings of directed song were acquired in closed sound boxes using AcquisitionGui software (Fee lab) in MATLAB 2013b. Recording sampling rate was 40 kHz.

Sequence change playbacks were generated using Audacity 2.3.0 software and interchanging the positions of song syllables at positions 2 and 3.

Location of HVC

Antidromic stimulation and recording was used to confirm the location of HVC. (Hahnloser et al, 2002). Downstream nucleus RA was located based on characteristic firing pattern of bursts. A regular stimulus was sent from RA and was detected by a recording electrode placed in the hypothesized location of HVC. The location at which the stimulus from RA could be detected were taken as the confirmed coordinates of HVC.

Surgery

Before surgery, zebra finches were anesthetized using isoflurane (1–3% in oxygen). The location of HVC (confirmed with antidromic recording) was taken as 0.3 mm anterior, 2.3 mm lateral of the bifurcation of the midsagittal sinus (lambda). Two bilateral, rectangular craniotomies were made at ~45 degrees angle away from the midline (Basista et al, 2014). Dimensions of craniotomies were 1mm x 0.5mm. After removing the dura using PBS, the craniotomies were covered using a silicone elastomer (Kwik-Cast; WPI). Additionally, a custom-made stainless-steel head plate was implanted on the skull using dental cement (Paladur, Kulzer International) for head-fixing the bird during experimentation (Benichov and Vallentin, 2020).

Pharmacological experiments

For experiments, $GABA_A$ receptor antagonist Gabazine (Sigma, SR-95531) (0.005mM-0.01mM in PBS) was applied bilaterally onto HVC via saturated gel foam sponges (Avitene Ultrafoam, Bard) in head-fixed birds. The solution was warmed to 40 °C before application. For control experiments, PBS was used instead of Gabazine. In order to restrict the area of application, wells of silicone elastomer were created around the craniotomies before application of sponge. The craniotomies with sponge were then covered with silicone elastomer to ensure that they stayed in place during experimentation (Kosche, Vallentin, Long, 2015).

Songs were recorded for one hour after the application of the drug. After every 15 minutes starting from t=0, the birds were presented with a playback of modified BOS for two minutes, for a total of four times in one hour. A female zebra finch was also presented briefly to motivate the male zebra finch to sing.

Before and after all experiments, craniotomies were cleaned of any overlying tissue and flushed with PBS warmed to ~40 °C. The craniotomies were subsequently sealed with fresh silicone elastomer.

Controlling for the spread of drug to HVC

The experimental protocol involves application of the drug onto HVC using a sponge. It was necessary to ensure that the effect of the drug was limited to the targeted area. To control for the spread of the drug limited to HVC, fluorescence conjugated Muscimol (Invitrogen, M23400) (0.01mM in PBS) was applied onto HVC using the above saturated sponge approach (Allen et al, 2008). After one hour of application, the bird was perfused after achieving a deep anesthetized state using isoflurane. The brain was fixed using PFA and subsequently immersed in 15% sucrose solution followed by 30% sucrose solution. The brain was then sliced (52µm thickness) using cryomicrotome. The slices were mounted on slides and stained using DAPI. Images were generated using Leica Fluorescent Microscope.

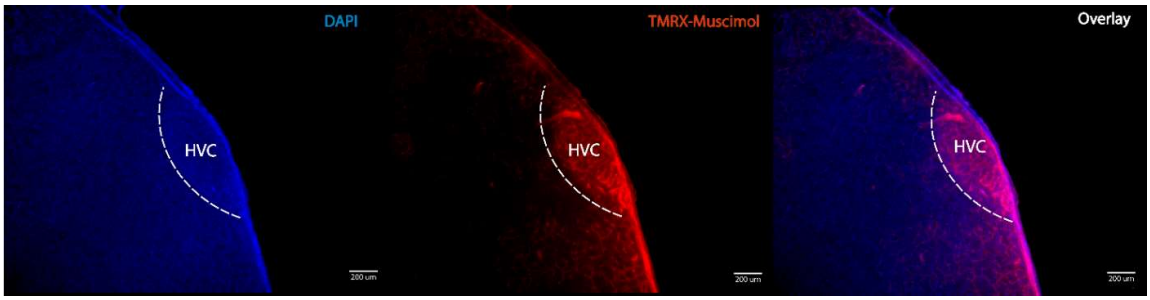

Figure 4: **Localization of fluorescence conjugated Muscimol in HVC labelled by DAPI** (From left to right) Fluorescent microscopy image of HVC cells labelled by DAPI, fluorescent microscopy image of HVC cells labelled by fluorescence conjugated muscimol drug applied onto HVC for one hour using sponge method, Overlay image to show colocalization.

Data analysis

Recordings were labeled and analyzed using ElectroGui software in MATLAB (Fee Lab). The syllables were segmented using automated threshold and were labelled manually. Before labelling, each experimental condition was renamed as a number by another lab member to ensure blinding of the data for unbiased analysis.

Changes in sequence (if any) were analyzed using a custom MATLAB script (R2019a), which calculates the probability of one syllable transitioning to the next. It creates a graph of the sequence the bird sings along with the probability of the syllable transition, known as '**transition probability graph**' for each individual bird. The code calculates the total incidences of paired syllable transitions in a hour-long experimental session using labelled data and divides it with the number of total syllable transitions in the session.

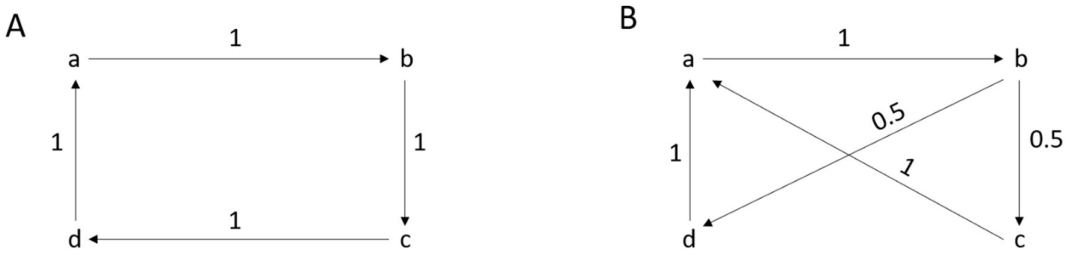

Figure 5: **Transition probability graphs** A: Completely linear song B: Branching-transition probability of syllables <1

To objectively check whether gabazine leads to the occurrence of new or modified syllables, Song Analysis Pro (SAP) (Tchernichovski, O., Nottebohm, F., et al, 2000) was used. Individual syllables from the bird's control song were compared to the 'new' syllables using the software's percentage similarity score (Symmetric, time-courses, p-value = 0.05, interval = 70ms) which determines the possibility that the given syllables are from the same bird. In order to classify two syllables as similar or dissimilar, a similarity threshold was determined. Since the percentage similarity of syllables in the control song of the bird with syllables from the control song fell in the range 93-100%, I set a conservative similarity threshold at 90%.

Measures of changes in song with PBS and Gabazine treatment:

Three measures of changes in the bird's song with PBS and Gabazine treatment (Scharff and Nottebohm, 1991) were used:

1. Sequence Linearity (Slin) measures the degree of branching of the song i.e. whether the bird's song linearly follows a stereotypical sequence.

$$Slin = \frac{\#different\ notes\ per\ song}{\#transition\ types\ per\ song}$$

   For a highly linear song, Slin = 1.

2. Sequence consistency (Scon) measures the number of times times the song follows the stereotypical original path.

$$Scon = \frac{\sum\ typical\ transitions\ per\ song}{\sum\ total\ transitions\ per\ song}$$

   For a highly consistent song, Scon = 1.

   Typical transition is defined as a transition which occurs under the control (PBS) condition.

3. Sequence stereotypy (Sster) is a score calculated by taking the average of Slin and Scon.

$$Scon = \frac{(Slin\ + Scon)}{2}$$

Results

To establish a baseline of normal sequencing of song in birds I applied PBS on HVC which has been shown to leave the singing behaviour of zebra finches unaffected.

To test whether inhibition in HVC guides the sequence of song production I limited the impact of inhibition by applying the $GABA_A$ antagonist gabazine onto HVC.

I then quantified the changes in transition probability under experimental and control condition.

In total, I performed surgery on 20 experimental birds. Out of those, I only selected birds which produced more than 20 motifs each (predetermined threshold required for analysis of data) under control and gabazine conditions.

Gabazine + playback experimental group

For the following birds, the experimental protocol included application of gabazine along with presentation of playback. The birds' songs were recorded in the experimental condition for a duration of one hour.

Bird 1

The control song of the bird had 4 syllables under normal conditions with repetitions of 'b-c-d' sequence in motifs (Fig. 6A).

No. of motifs under PBS condition: 20, No. of motifs under gabazine condition: 113

Effects under Gabazine + Playback condition

The bird shows repetition of syllable 'a' in the song in place of syllable 'b' (Fig. 6B). New syllables 'n' and 'p' are observed. 'n' occurs before the beginning of the motif (Fig. 6C) and is not similar to any syllables present in the control song. Syllable 'p' occurs between two syllables 'd's (Fig. 6D). Syllables 'p' is not similar to syllable 'd' (57%) but are 97% and 90% similar respectively to sequence 'b-c' of the control song. Based on the transition probability graphs (Fig. 6E, F), sequence linearity and sequence stereotypy decrease while sequence consistency remains unchanged (Table 1).

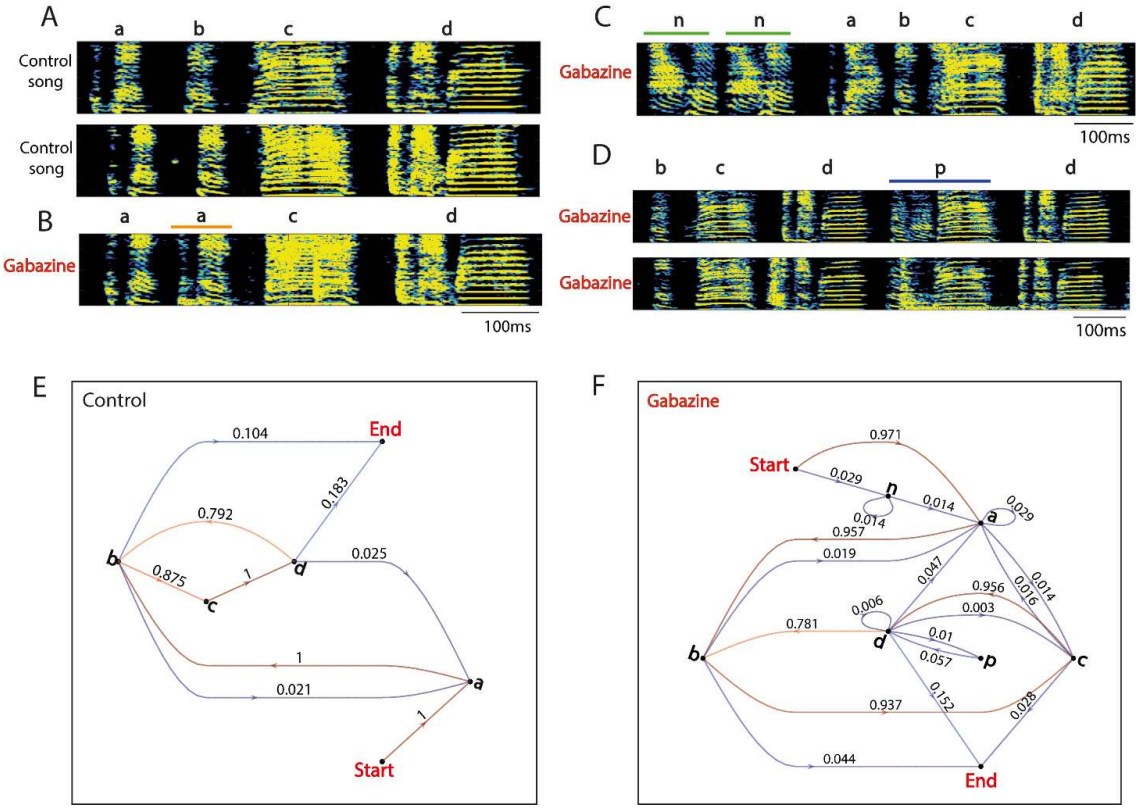

Figure 6: **Effects of gabazine + playback for bird 1** A: control song syllables B: repeated syllable ‘a’ (orange) C: new syllable ‘n’ (green) D: new syllable ‘p’ (blue) E,F: transition probability diagrams for control and gabazine conditions

	Control	Gabazine
Sequence Linearity	0.6667	0.4000
Sequence Consistency	0.5556	0.5556
Sequence Stereotypy	0.6111	0.4778

Table 1: Measures of change for bird 1

Bird 2

The control song of the bird had 7 syllables under normal conditions (Fig. 7A)

No. of motifs under PBS condition: 24, No. of motifs under gabazine condition: 66

Effects under Gabazine + Playback condition

The bird introduces new syllable ‘n’ which occurs between syllables ‘e’ and ‘g’. The two instances of ‘n’ had similarity scores below the 90% threshold with all other syllables of the control song (Fig. 7B). Based on the transition probability graphs (Fig. 7C, D), sequence linearity and sequence stereotypy decrease while sequence consistency remains unchanged (Table 2)

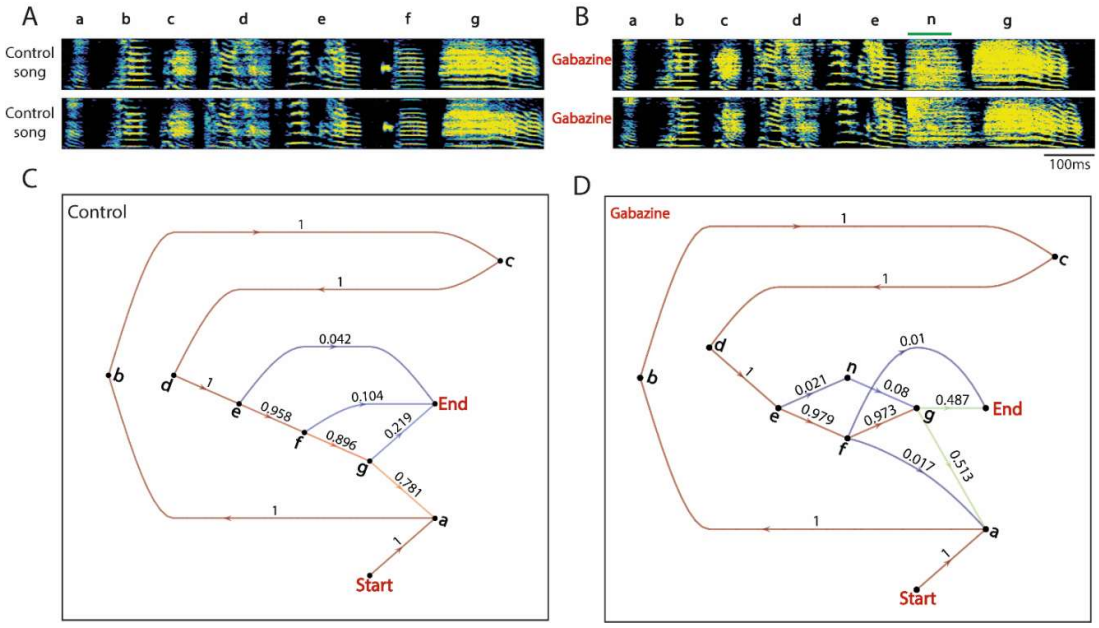

Figure 7: **Effects of gabazine + playback for bird 2** A: control song syllables B: new syllable ‘n’ (green) C,D: transition probability diagrams for control and gabazine conditions

	Control	Gabazine
Sequence Linearity	0.8182	0.7612
Sequence Consistency	0.7273	0.7273

Sequence Stereotypy	0.7727	0.7442

Table 2: Measures of change for bird 2

Bird 3

The control song of the bird had 5 syllables under normal conditions (Fig. 8A)

No. of motifs under PBS condition: 27, No. of motifs under gabazine condition: 59

Effects under Gabazine + Playback condition

The bird produces new syllables ‘o’ and ‘p’ (Fig. 8B). ‘o’ occurs before first syllable ‘a’ and is 89% similar to syllable ‘b’. ‘p’ stacked structure added between ‘b’ and ‘c’ syllables which is not similar to any syllables from control song. The bird also ends song motifs abruptly after ‘a’, ‘b’ or ‘c’ syllables (Fig. 8C). Syllable ‘n’ is produced in place of syllable ‘c’ along with abrupt ending of motif (Fig. 8D). Based on the transition probability graphs (Fig. 8E, F), sequence linearity and sequence stereotypy decrease while sequence consistency remains unchanged (Table 3).

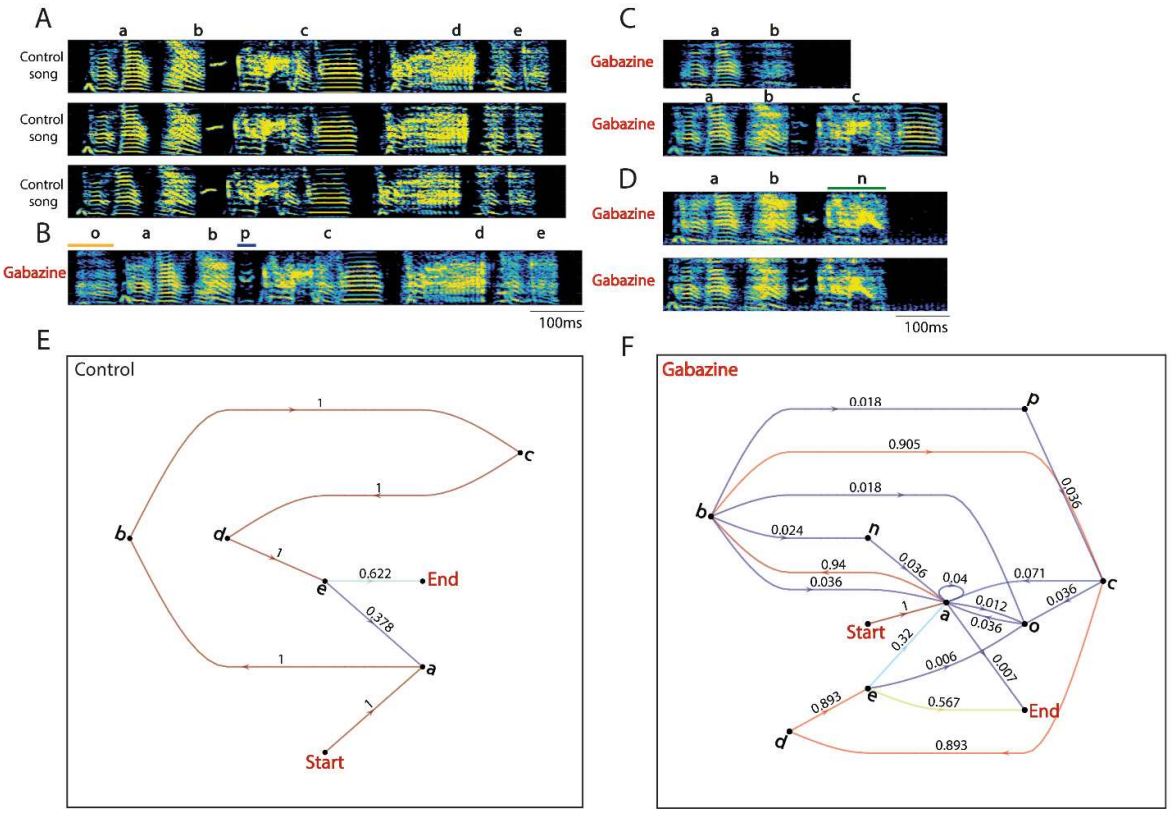

Figure 8: **Effects of gabazine + playback for bird 3** A: control song syllables B: new syllable ‘o’ (orange) and ‘p’ (blue) C: abrupt end of motifs at ‘b’ and ‘c’ D: syllable ‘n’

(green) in place of syllable ‘c’ E,F: transition probability diagrams for control and gabazine conditions

	Control	Gabazine
Sequence linearity	1	0.5
Sequence consistency	0.7143	0.7143
Sequence stereotypy	0.8571	0.6071

Table 3: Measures of change for bird 3

Bird 4

The control song of the bird had 4 syllables under normal conditions (Fig. 9A)

No. of motifs under PBS condition: 148, No. of motifs under gabazine condition: 86

Effects under Gabazine + Playback condition

The bird produces motif which starts with syllable ‘d’ after introductory notes (Fig. 9B). The bird ends motifs abruptly after syllable ‘c’ (Fig. 9C). Changes in structure of syllable ‘b’ are observed. New ‘o’ is 52% similar to syllable ‘b’ in PBS and 82% similar to syllable ‘c’ (Fig. 9D) while ‘n’ is 84% similar to both ‘b’ and ‘c’ (Fig. 9E). Both ‘n’ and ‘o’ could be a mixture of the structures of ‘b’ and ‘c’ syllables. Based on the transition probability graphs (Fig. 9F,G), sequence linearity, sequence consistency and sequence stereotypy decrease (Table 4).

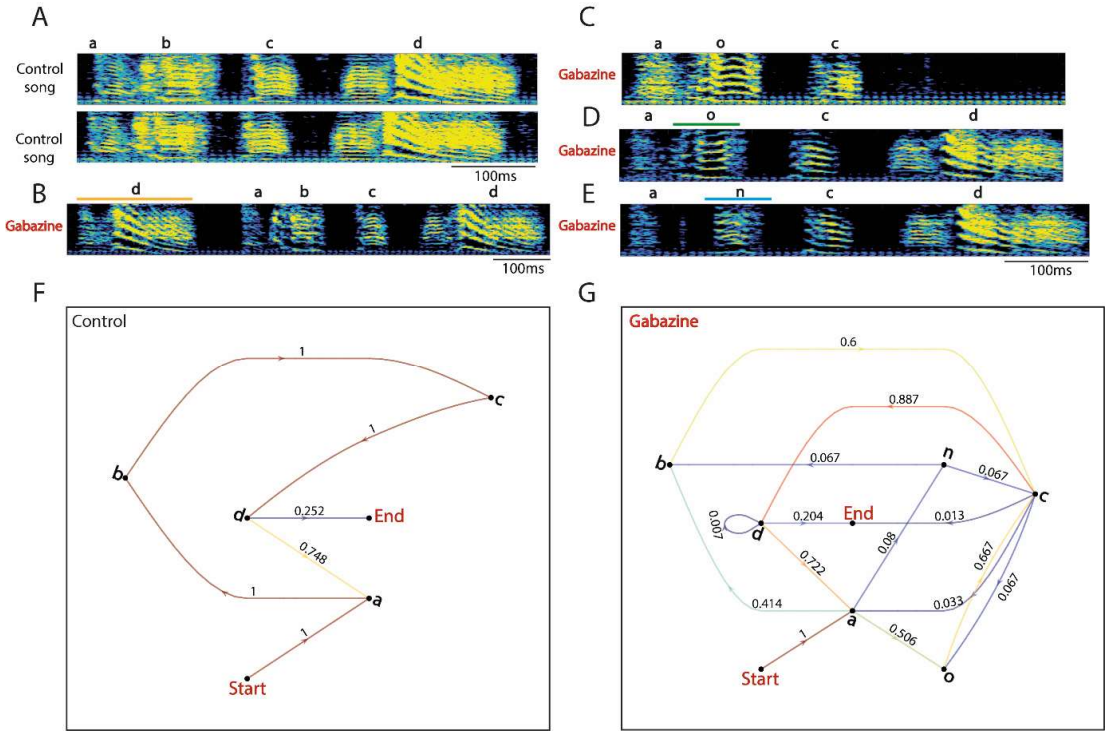

Figure 9: **Effects of gabazine + playback for bird 4** A: control song syllables B: motif begins with syllable ‘d’ (orange) C: abrupt motif end at ‘c’ D: new syllable ‘o’ (green) E: new syllable ‘n’ (blue) F,G: transition probability diagrams for control and gabazine

	Control	Gabazine
Sequence Linearity	1.0000	0.5333
Sequence Consistency	0.6667	0.3330
Sequence Stereotypy	0.8333	0.4332

Table 4: Measures of change for bird 4

Bird 5

The control song of the bird had 5 syllables under normal conditions (Fig. 10A)

No. of motifs under PBS condition: 41, No. of motifs under gabazine condition: 161

Effects under Gabazine + Playback condition

Under gabazine, syllables of the song lose their sharp spectral features (Fig. 10C, D). New syllable structures are produced in place of syllable 'c': syllable 'n' (Fig. 10C) and syllable 'm' (Fig. 10D). The sequence of the bird's song changes (Fig. 10B) to 'a-b-d-b-n'. The syllable labelled d (orange) has a similarity score of 85% with syllable 'd' in control condition. Based on the transition probability graphs (Fig. 10E,F), sequence linearity and sequence stereotypy decrease while sequence consistency remains unchanged (Table 5).

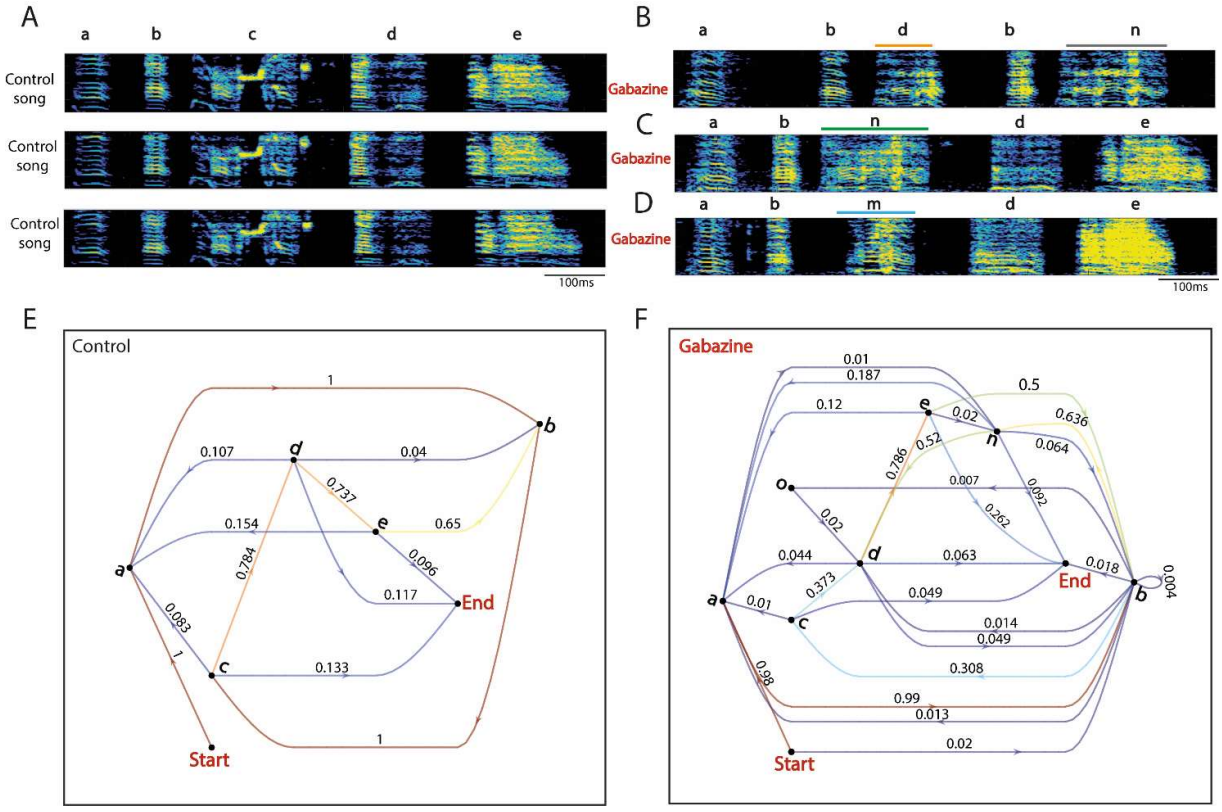

Figure 10: **Effects of gabazine + playback for bird 5** A: control song syllables B: Change in sequence of motif syllables to 'a-b-d(orange)-b-n(grey)' C: new syllable 'n' (green) D: new syllable 'm' (blue) E,F: transition probability diagrams for control and gabazine conditions

	Control	Gabazine
Sequence Linearity	0.5385	0.3333
Sequence Consistency	0.4615	0.4615
Sequence Stereotypy	0.5	0.3974

Table 5: Measures of change for bird 5

Gabazine only experimental group

For the following birds, the experimental protocol included application of gabazine only. The birds' songs were recorded in the experimental condition for a duration of one hour.

Bird 6

The control song of the bird had 6 syllables under normal conditions (Fig. 11A)

The motif can start with the syllable 'a' or syllable 'n' in one instance.

No. of motifs under PBS condition: 133, No. of motifs under gabazine condition: 30

Effects under Gabazine only condition

The bird produces abortive motifs that end at syllables 'c' or 'd' (Fig.11B). Sequence Linearity and Sequence Stereotypy increase under gabazine for this bird (Table 6)

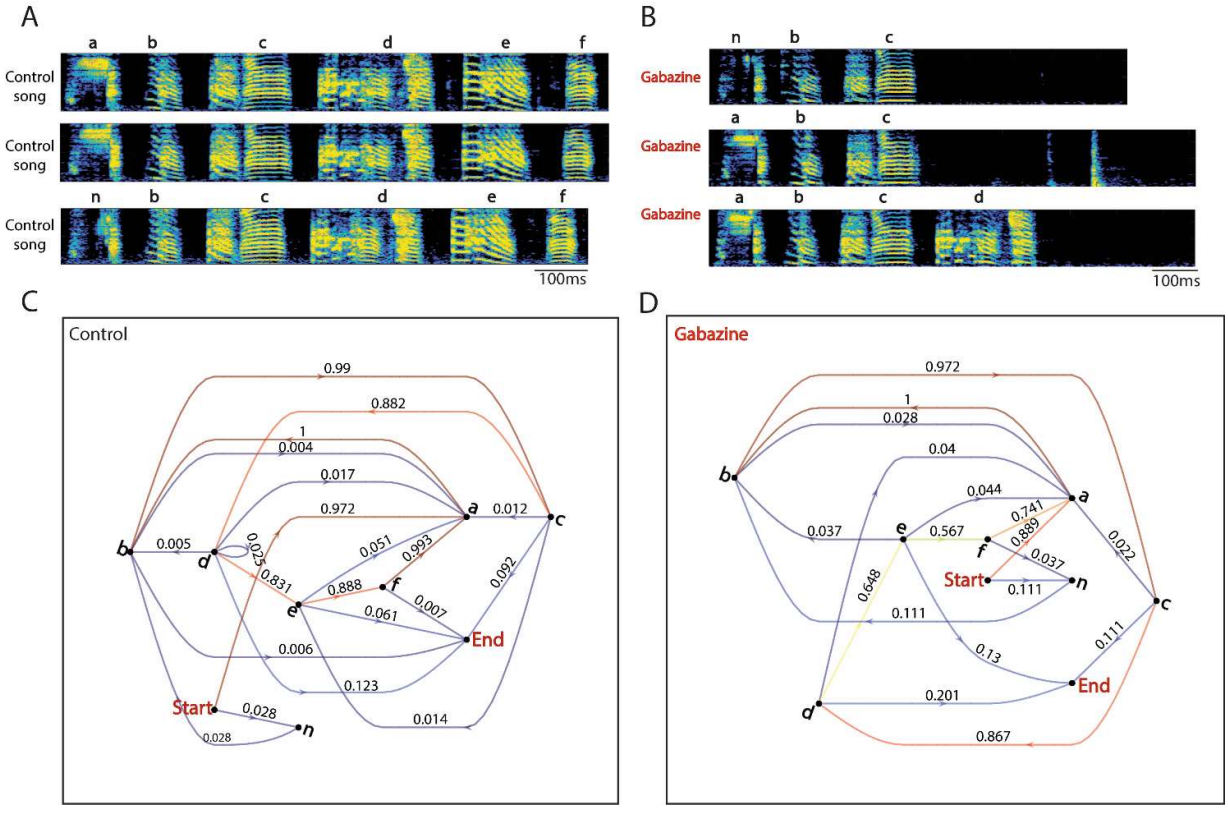

Figure 11: **Effects of gabazine for bird 6** A: control song syllables B: Abrupt ending of motifs C,D: transition probability diagrams for control and gabazine conditions

	Control	Gabazine
Sequence Linearity	0.4286	0.5000
Sequence Consistency	0.3333	0.3333
Sequence Stereotypy	0.3810	0.4167

Table 6: Measures of change for bird 6

Bird 7

The control song of the bird had 5 syllables under normal conditions (Fig. 12A)

The motif can have syllable ‘a’ followed by syllable ‘b’ or syllable ‘a’ followed by syllable ‘n’ in one instance.

Effects under Gabazine only condition

The bird produces abortive motifs with syllables ‘a’ and ‘a-e’ under gabazine (Fig. 12B). It also produces new syllable ‘o’ which looks like syllable ‘d’ of PBS condition but has a similarity score of 70% with syllable ‘d’ (Fig. 12C). It also produces syllable ‘n’ often under gabazine condition (Fig. 12D). Based on the transition probability graphs (Fig. 12E,F), sequence linearity and sequence stereotypy decrease while sequence consistency remains unchanged (Table 7)

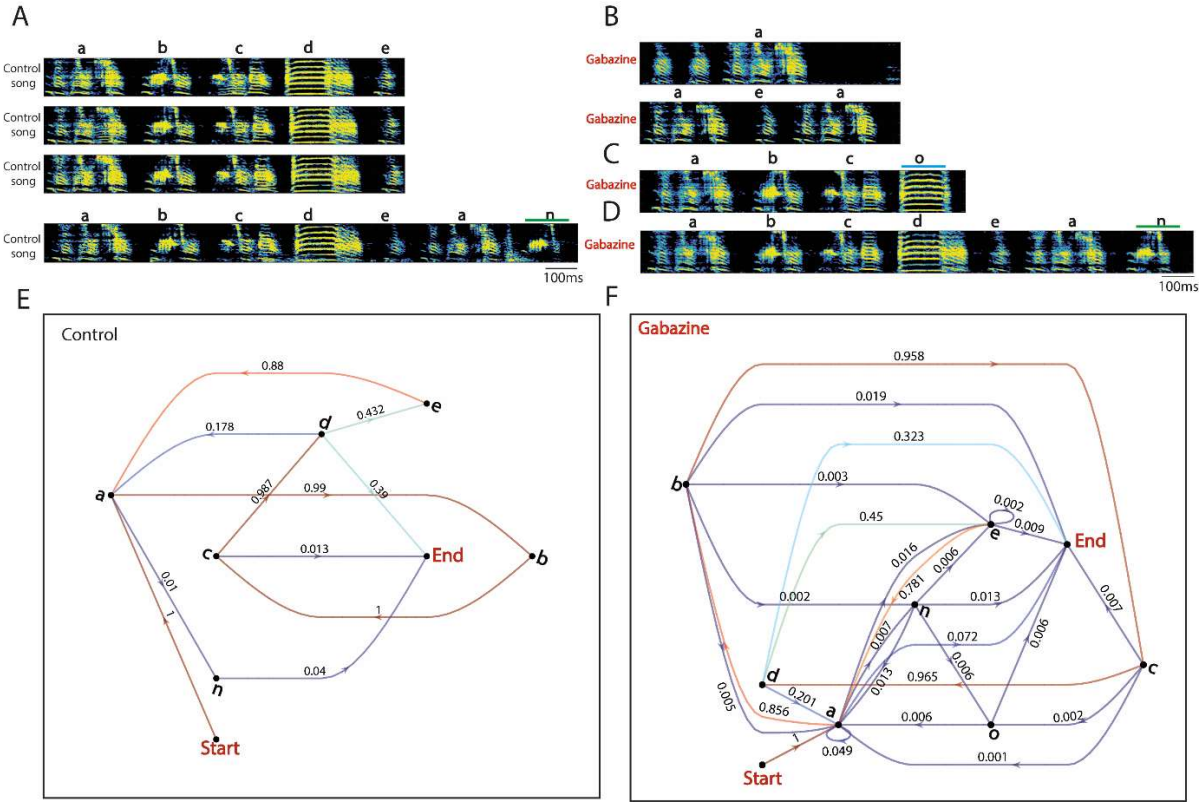

Figure 12: **Effects of gabazine for bird 7** A: control song syllables. Syllable 'n' (green) occurs under PBS condition B: Abrupt ending of motifs under gabazine C: New syllable 'o' (blue) in place of syllable 'd' D: Syllable 'n' (green) occurs often E,F: transition probability diagrams for control and gabazine conditions

	Control	Gabazine
Sequence Linearity	0.7273	0.3333
Sequence Consistency	0.4545	0.4545
Sequence Stereotypy	0.5909	0.3939

Table 7: Measures of change for bird 7

Bird 8

The control song of the bird had 4 syllables under normal conditions (Fig. 13A)

Effects under Gabazine only condition

Song syllable structure does not change under gabazine only condition for this bird (Fig. 13B). Sequence consistency remains unchanged while sequence linearity and sequence stereotypy show a slight increase.

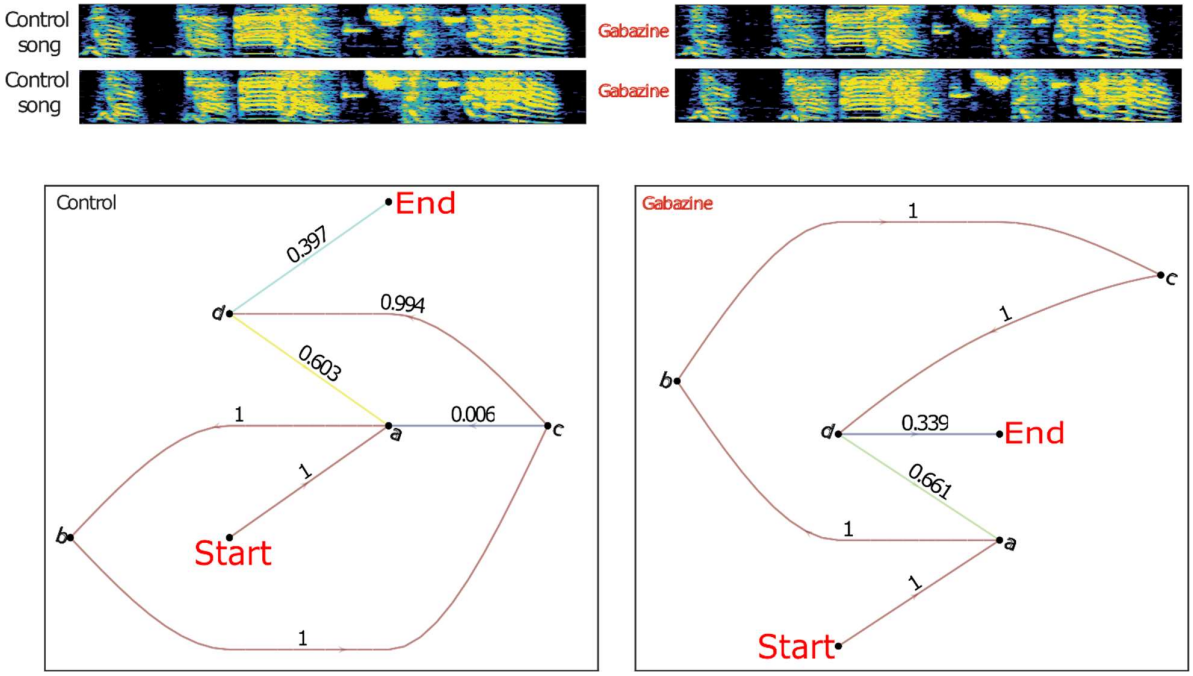

Figure 13: **Effects of gabazine for bird 8** A: control song syllables. B: song under gabazine unchanged C,D: transition probability diagrams for control and gabazine conditions

	Control	Gabazine
Sequence Linearity	0.8571	1
Sequence Consistency	0.5714	0.5714
Sequence Stereotypy	0.7143	0.7857

Table 8: Measures of change for bird 8

Summary Graphs and Statistics

I plotted box and whiskers plots depicting medians and IQR of Sequence Linearity, Sequence Consistency and Sequence Stereotypy under PBS and Gabazine treatments for the two groups. I performed Wilcoxon sign-rank test to check whether the change in the values upon gabazine treatment was statistically significant. The tests were performed at 95% confidence levels.

Gabazine application with playback

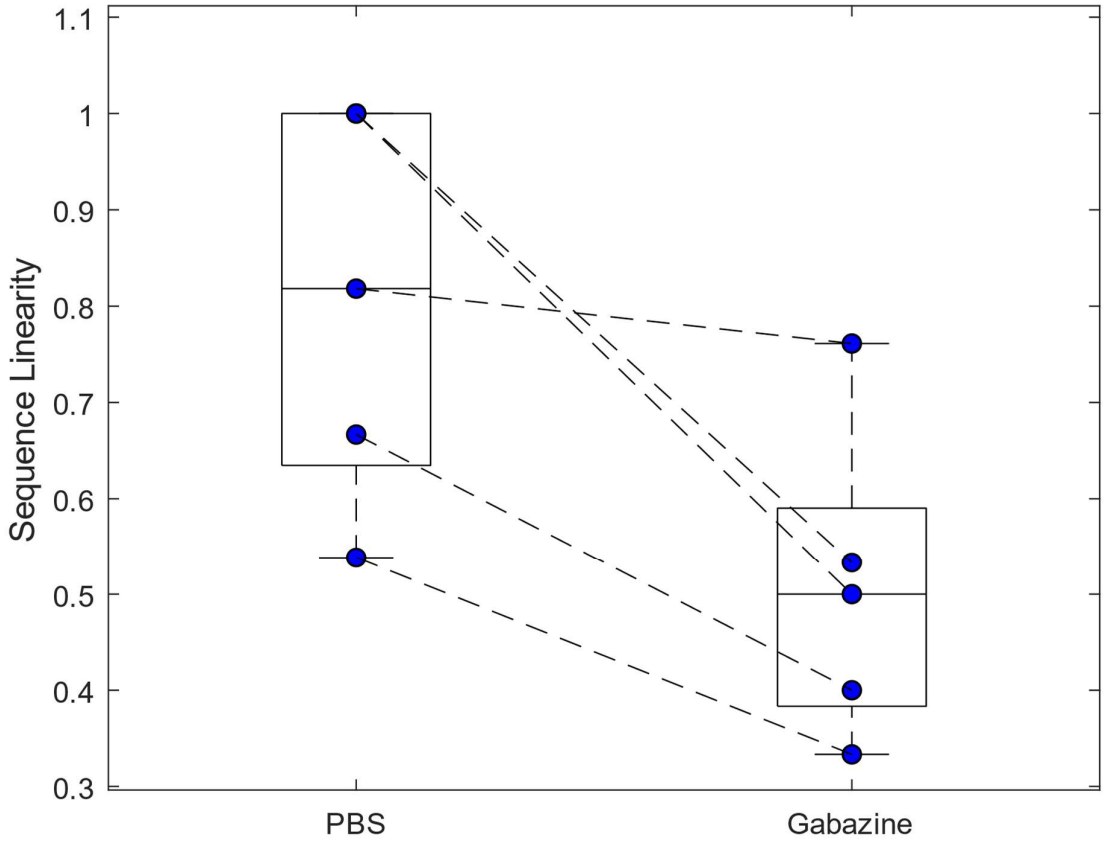

Figure 14: Sequence Linearity under PBS and Gabazine applications for Gabazine+Playback group. Dotted lines indicate data from the same bird (n=5)

The null hypothesis was that sequence linearity does not reduce under gabazine + playback treatment. Using Wilcoxon sign-rank test, the null hypothesis was rejected with a p-value of 0.0313. We can interpret this as sequence linearity reduces under gabazine + playback treatment at a confidence level of 95%.

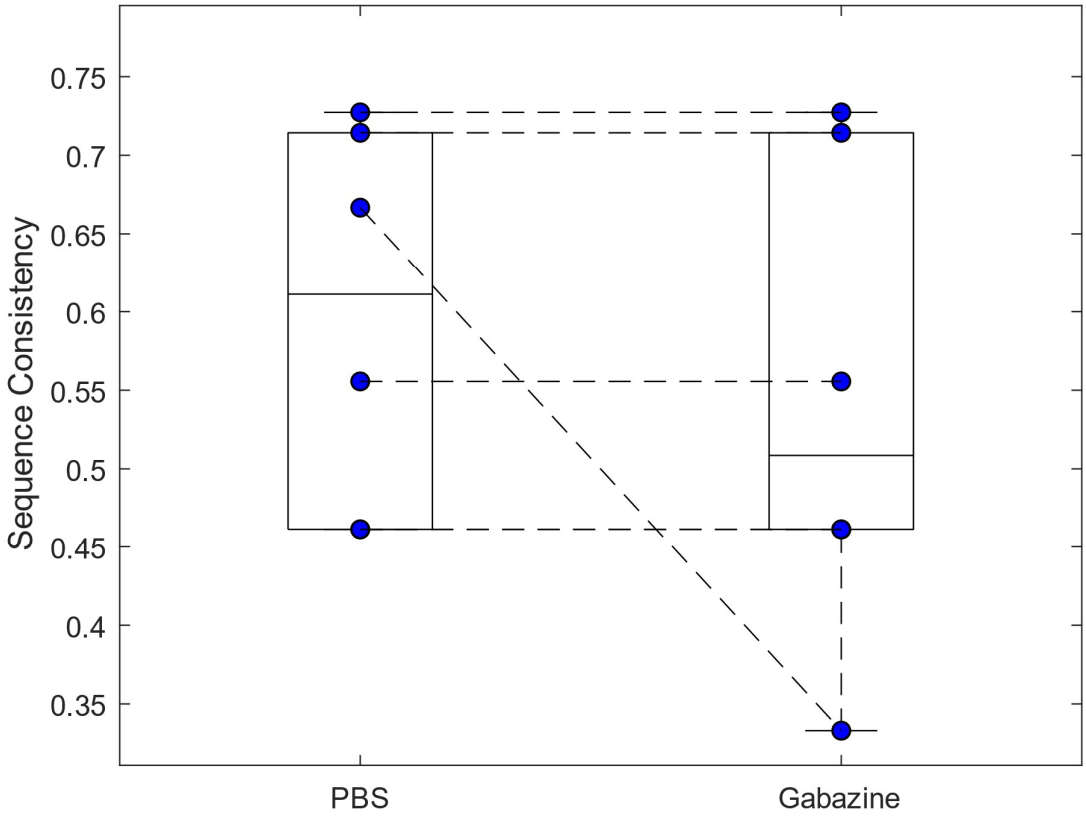

Figure 15: Sequence Consistency under PBS and Gabazine applications for Gabazine+Playback group. Dotted lines indicate data from the same bird (n=5)

The null hypothesis was that sequence consistency does not reduce under gabazine + playback treatment. Using Wilcoxon sign-rank test, the null hypothesis was not rejected with a p-value of 0.500. At 95% confidence level, sequence consistency does not reduce under gabazine + playback treatment.

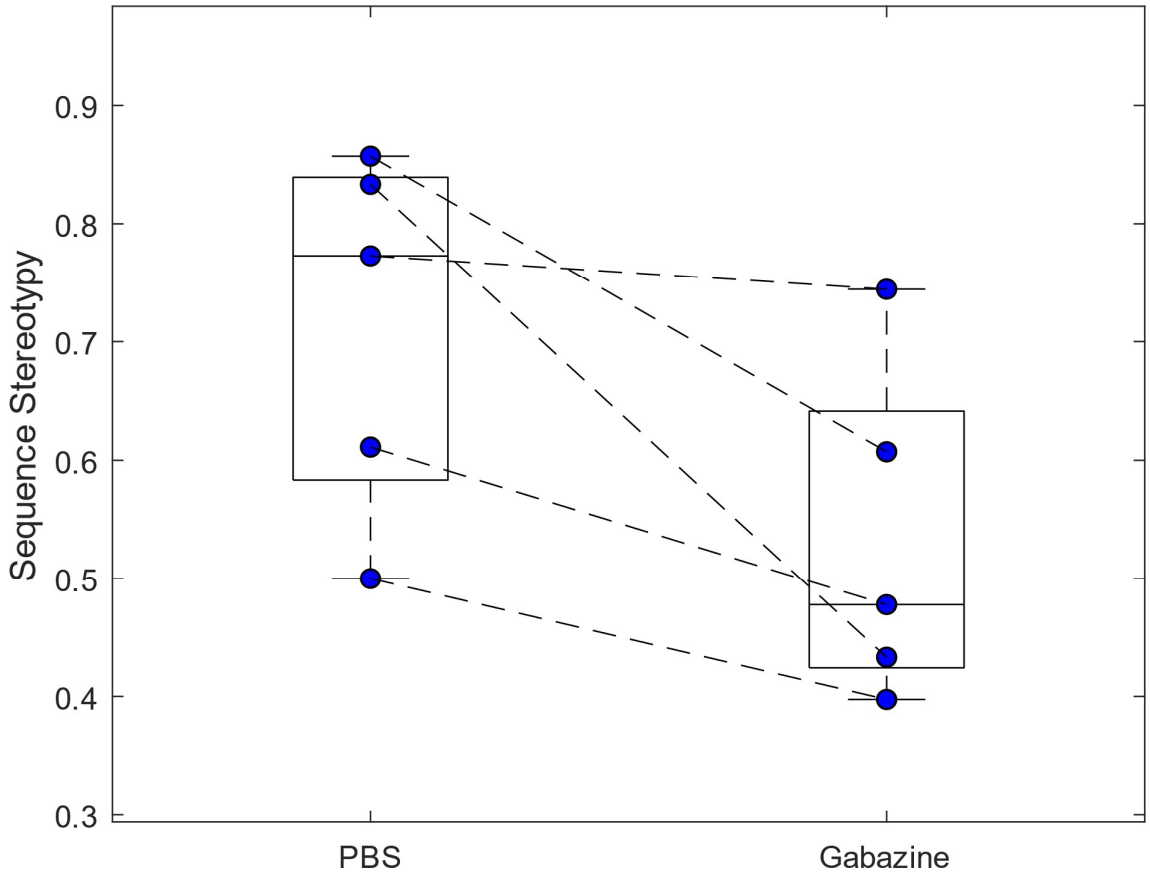

Figure 16: Sequence Stereotypy under PBS and Gabazine applications for Gabazine+Playback group. Dotted lines indicate data from the same bird (n=5)

The null hypothesis was that sequence stereotypy does not reduce under gabazine + playback treatment. Using Wilcoxon sign-rank test, the null hypothesis was rejected with a p-value of 0.0313. We can interpret this as sequence stereotypy reduces under gabazine + playback treatment at a confidence level of 95%.

Gabazine application only

For this group, the total number of experimental birds was 3. The minimum sample size was not reached and hence statistical power for testing the change under gabazine only treatment was insufficient. Box and whiskers plots were plotted for better visualization of the three measures of change.

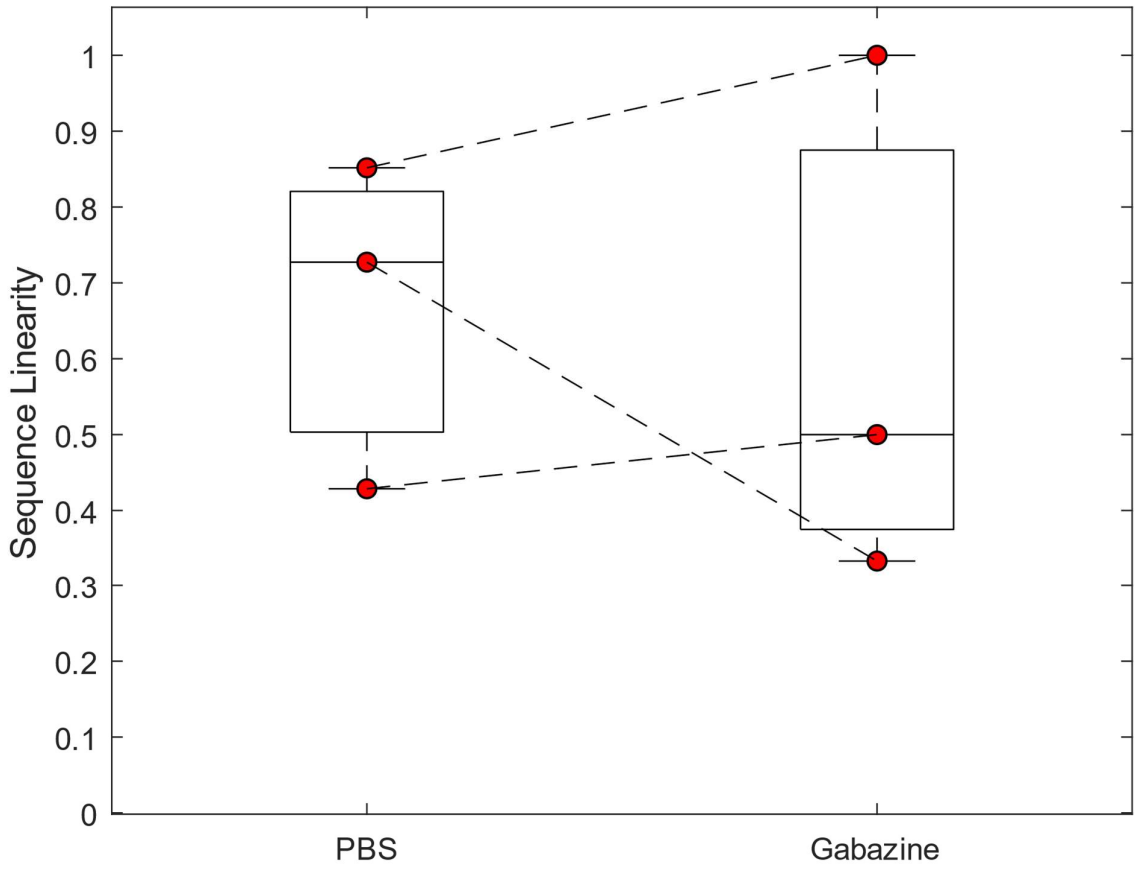

Figure 17: Sequence Linearity under PBS and Gabazine applications for Gabazine only group. Dotted lines indicate data from the same bird (n=3)

The data shows mixed trends with syllable sequencing becoming more linear in 2 out of 3 birds under gabazine treatment.

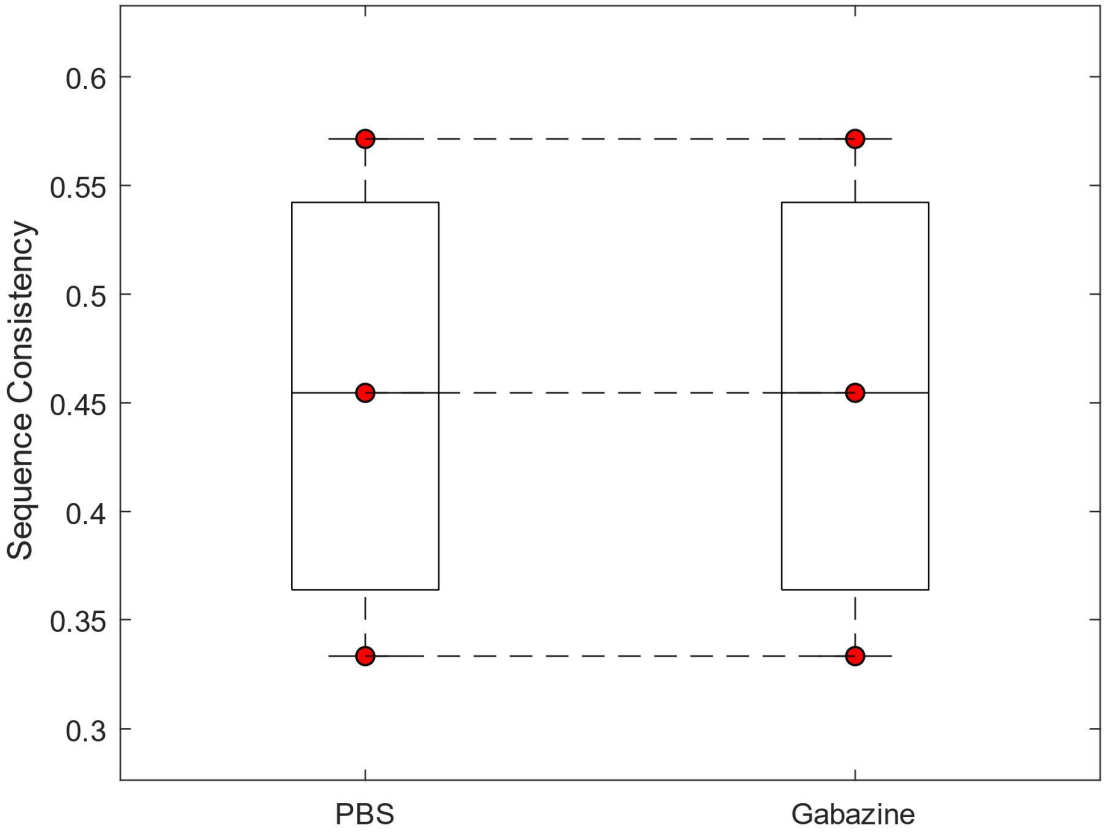

Figure 18: Sequence Consistency under PBS and Gabazine applications for Gabazine only group. Dotted lines indicate data from the same bird (n=3)

Sequence consistency does not deviate for all three birds under gabazine treatment.

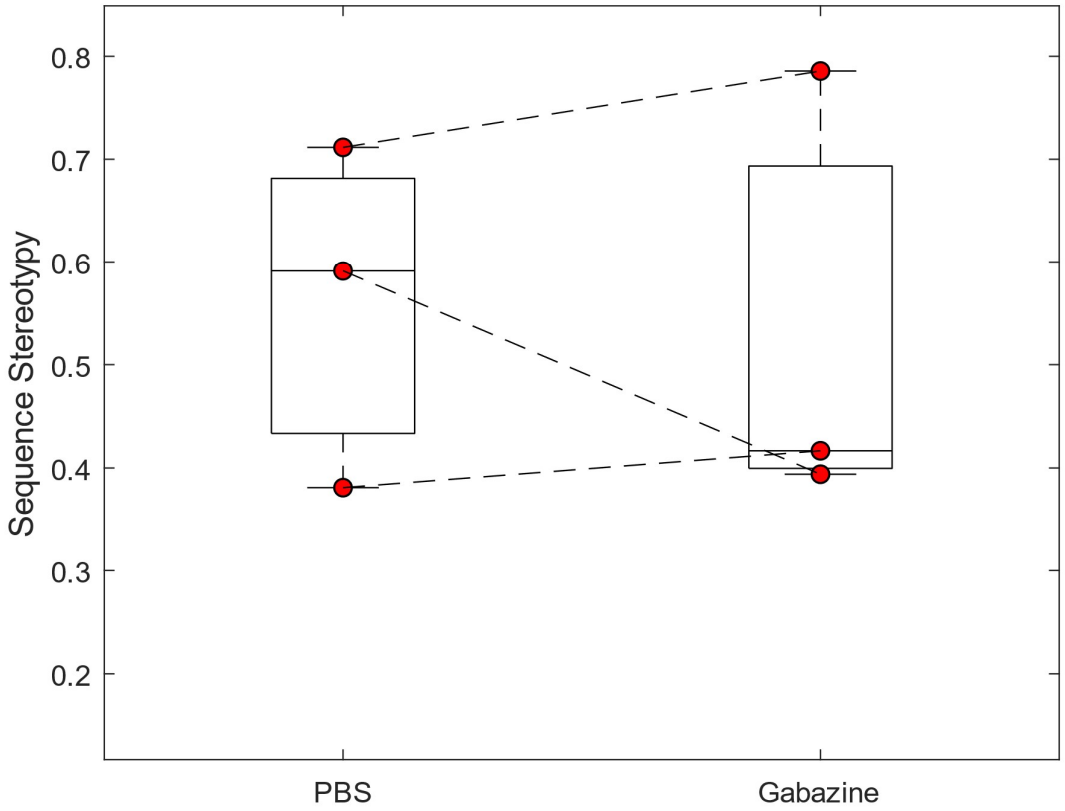

Figure 19: Sequence Stereotypy under PBS and Gabazine applications for Gabazine only group. Dotted lines indicate data from the same bird (n=3)

Sequence stereotypy also shows a mixed trend as a consequence of sequence linearity. The sequence stereotypy increases for 2 out of 3 birds under gabazine treatment.

Discussion

In the sensory-motor system of the zebra finch, HVC is the primary control center which contains a population of inhibitory interneurons. These interneurons show gradual increase in inhibition specific to well-learnt song segments as the bird acquires its tutor's song during the critical period of song learning (Vallentin et al, 2016)

In this project, I studied the effect of perturbing inhibition after the closure of the critical period on the zebra finch song using gabazine, which is a $GABA_A$ receptor antagonist. When applied to HVC, gabazine affects the population of GABA-ergic inhibitory interneurons in HVC and disturbs signaling in the inhibitory network.

My results show that perturbing inhibition in HVC of adult zebra finches affects spectral features as well as sequence of syllables in the bird's song. In 2 out of 3 birds tested in gabazine only condition, I observe abrupt ending of motifs after a few syllables. This effect is also observed in 2 out of 5 birds in gabazine+playback condition. Sequence generation in HVC is determined by an intricate chain of excitation and inhibition (Cannon et al, 2015). Hence it is plausible that perturbation of local inhibition using gabazine affects the syllable sequence and causes the motifs to be ended abruptly. It leads to increased branching of the song as the linearity of the syllable sequence reduces. 3 out of 5 birds which received sequence change playback with gabazine application showed a change in syllable sequence of their song.

Inhibition in HVC is involved in determining the structure and stereotypy of the song. Pharmacological inactivation of HVC using muscimol, a $GABA_A$ receptor agonist lead to conversion of adult zebra finch song to juvenile-like subsong without clear spectral features (Aronov et al, 2008). My results show that application of gabazine on HVC leads to change in spectral features of the syllables. In 6 out of 8 birds tested, new syllable structures were introduced in the song. Birds that were provided with a guiding playback during gabazine application produced a greater number of syllables that were different from those in the control song. In 4 out of 5 birds of this group, the new syllables produced were similar to syllables in positions 2 or 3 in the control song of the bird. It should be noted that the playback provided was also BOS with modifications at syllable positions 2 and 3. Under gabazine only condition, 1 out of 3 birds produced spectrally different syllables from those in the control condition, but their structures were incomplete versions of structures of syllables in the condition. Additionally, these altered structures were present at the end of the motif.

My results show that perturbing inhibition in adult zebra finches brings back plasticity in their song, which can be influenced by external playback. The disruption of inhibition weakens the protection of the learnt syllable and increases plasticity of the adult song. As HVC is a nucleus that receives inputs from the auditory pathway also, providing a guiding playback during this plastic condition can influence the direction of change seen in the bird's song. In order to pinpoint precise effects of playback, I would require more birds tested under the gabazine only condition. With a larger sample size, it would be possible to identify patterns of change and classify the effects of gabazine and playback. The changes observed under gabazine are infrequent, as evidenced by the nearly unchanging sequence consistency. I think this does not depend on the area

affected by gabazine or by its concentration. My imaging experiment using fluorescent conjugated muscimol shows gabazine can be applied to almost the entire HVC using the sponge method. I do not think the effects depend much on the gabazine concentration but have more to do with the interneurons network itself. Within a given range of concentration of gabazine, we suppress the signaling in this network enough to perturb their effect on the RA projecting neurons which play a role in syllable sequencing. Anything below 0.005mM does not elicit enough perturbation to affect this, while anything above 0.01mM sends the bird into seizure-like vocalizations.

Recent research in Bengalese finches shows that perturbing inhibition using pharmacological methods leads to change in syllable sequencing (Isola, Sakata, Vochin, 2019). Adding to these, my results show that perturbing inhibition in HVC of adult zebra finches not only changes the sequencing of the stereotyped song but also opens the critical period of song learning. This study highlights the role of inhibition in HVC in determining the critical period of song learning.

I was able to demonstrate that limitation of HVC interneuron efficacy leads to opening of a window of opportunity for zebra finches to learn a new song which seems to be guided by imitation learning.

In other songbirds, the critical period of song acquisition is not limited to juvenile developmental stage. For example, male canaries change their song with every breeding season. In canaries, the HVC undergoes seasonal neurogenesis that can be temporally correlated with changes in song (B O'Loughlin et al, 1994). In Bengalese finches, auditory feedback is required throughout life for maintenance of song. Absence of auditory feedback in deafened birds leads to changes in song structure within a week of deafening (Okanoya and Yamaguchi, 1997). The methods used here can be applied in other songbirds to study their neural determinants of critical period.

Pharmacological drug application is a quick inactivation method with possible side effects. Building on this work, it would be possible to get a detailed insight into this phenomenon using methods cell type specific (de-) activation by techniques like optogenetics and implantation of interneurons in adulthood. The extent of perineuronal nets in zebra finches is correlated with maturation of song (Balmer et al, 2009). It is possible to study the relation between inhibition and critical period with manipulation and destruction of perineuronal nets.

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