FINDING THE ENGRAM: A PATHWAY FOR SONG MEMORY IN ZEBRA FINCHES

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FINDING THE ENGRAM: A PATHWAY FOR SONG MEMORY IN ZEBRA FINCHES

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FINDING THE ENGRAM: A PATHWAY FOR SONG MEMORY IN ZEBRA FINCHES

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Finding memory traces, also called engrams, has been a major goal in the neuroscience field for decades. Although episodic memory - the memory of autobiographical events - is known to rely on the hippocampus for its formation, procedural memory – the memory of motor skills – does not require hippocampus and the exact nature and mechanism of it has remained largely unknown. Vocal learning is a form of procedural learning of a sequence of vocal movements from a social model, a rare trait detected in only few animal species including songbirds and humans. The learning of vocal production is guided by the retention of the memory of the social model's vocal behavior. In this dissertation, I used song learning in zebra finches as the animal model to study the neural basis of song memory. I used a newly

developed spatiotemporally specific optogenetic method combined with neuron population-specific genetic lesion to target a neural pathway of zebra finches and examined its role in song memory.

Through this series of experiments, I showed that 1) imposing artificial activity in this pathway results in birds singing songs with temporal structure conforming to the imposed activity, suggesting a mechanism for encoding the temporal structure of song; 2) imposing activity paired with live bird tutoring cause the birds to learn only from the imposed activity, but not from the live bird tutor, suggesting this pathway is either able to override other pathways for acquiring song memory, or a non-redundant pathway for encoding the temporal structure of song; 3) genetic lesioning of cells in this pathway precludes birds from learning from a tutor, but does not affect song learning if birds received tutoring before lesioning, suggesting this pathway is necessary for acquiring song memory and that memory transmitted via this pathway is not stored within but downstream of it.

This study is the first case showing artificial activity imposed in a neural pathway implants memories that subsequently guide the learning of a motor skill.

In Part I of this dissertation, I introduce memory and strategies that can be used to find engram, song learning of zebra finches and previous work in search of the engram of song memory and discuss the rationale of my design of experiments.

In Part II, I present in three separate chapters experiments I conducted to examine of the role of a neural pathway of zebra finches in song memory.

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NIF – the nucleus interfacialis of the nidopallium

HVC – a neural circuit required for zebra finch song production (proper name)

Av – the nucleus avalanche

RA – the robust nucleus of the arcopallium

LMAN – the lateral magnocellular nucleus of the anterior neostriatum

CM– caudal mesopallium

NCM – caudomedial nidopallium

VTA – the ventral tegmental area

scAAV-NX-ChR2 – Neurexin-tagged Channelrhodopsin-2 in a self-complementary AAV

PART I

CHAPTER ONE

MEMORY AND HOW TO FIND IT

Introduction

Memory is the faculty of a system to acquire, store and retrieve information from experience for guiding behavior. Memory is hypothesized to physically exist in the form of memory traces, or engrams – lasting changes in the brain[1]. It is widely accepted by neuroscientists that alteration of synaptic connections between neuronal populations is the basis of engram formation[2]. For example, long-term memory is often categorized into episodic memory and procedural memory. Episodic memory is the memory of autobiographical events - a memory of 'who did what when and where'. Episodic memory is thought to be formed initially by rapid synaptic activity in the hippocampus but gradually consolidated in cortical regions for permanent storage. On the other hand, procedural memory is the memory of motor skills - a memory of how to do things, such as speech, tool use or catching a prey. Procedural memory is thought to involve a different set of brain regions for its formation and storage, including the cerebellum, motor cortex and parts of the basal ganglia system [3-6].

Animals learn extensive motor skills throughout their lifetime, ranging from a simple picking up an object to more complicated tasks like vocal learning. Vocal learning is a rare trait detected in only few species including songbirds and humans. It is a task of imitating a

sequence of vocal movements from a social model. For example, a juvenile male zebra finch learns a courtship song from his father. During the interaction, the juvenile forms an accurate, lasting memory of his father's song, and the retention of this song memory guides the motor learning of his own song production. Finding the engram of the song memory has been a key question in the songbird field, and also has great potential in revealing the mechanism of human speech learning.

Empirically, locating the engram of a certain memory requires either observing or manipulating a neuron population to examine its exact role in the acquisition or storage of this memory. For example, a putative locus of the 'engram' would be expected to display memory-related activity in electrophysiological recording or neural imaging and damaging the locus or disrupting its activity would be expected to cause memory loss. However, in practice, due to the spatially widely distributed and temporally dynamic nature of memory representation, the locus of 'engram' has to be distinguished from loci that are involved in transmitting the sensory input from experience or the motor effectors of the memory. For example, for a dog who learned to sit on hearing the command 'sit', its auditory regions would be expected to be active as well as the engram of the command 'sit'. Similarly, disrupting the motor pathways for controlling the dog's limbs would result in inability for the dog to sit as well as disrupting the engram itself. Therefore, although historically many attempts have been made in many different parts of the brain in attempt of finding engrams of many different kinds of memory across many different species, the exact nature and

location of engram remains elusive[2], largely due to the lack of an empirical strategy that is spatiotemporally specific to the engram itself.

Recently, genetic methods have been developed in rodents to tag and manipulate the 'engram' neurons in the hippocampus for episodic memory[7], allowing the exact hippocampal neurons recruited at the time of learning to be later excited to trigger the behavior. Likewise, in order to find the engram for procedural memory, one would hope to use a method by which the putative 'engram' cells are manipulated exactly at the time of memory acquisition. Since the contents of procedural memory are motor tasks, it will also be ideal if the 'engram' cells of the motor task are manipulated in a way that memory 'model' can be 'shaped' and result in a model-conforming behavior. For example, a juvenile male zebra finch imitates his father's song for courtship. If the 'engram' of this model song can be manipulated in a way that gets the juvenile to learn a song that complies with the imposed pattern, it will greatly help understand about the neural mechanism and location of this type of procedural memory.

Song memory

In this study, I used song learning in zebra finches as the animal model to study the neural basis of the song memory. Zebra finch song is a behavior performed by only the male members for courtship[8]. A juvenile male is tutored by a bird tutor (a senior male conspecific, usually his own father) and eventually learns to sing a highly accurate copy of the tutor song [Figure 1A], a process typical of procedural learning. A zebra finch song is a

highly stereotyped piece of sound of 500-1000ms long, usually consisting of 3-7 ‘syllables’ spaced by silent gaps [Figure 1A]. Sound analysis tools have been developed for automatic analysis of zebra finch song, enabling the quantification of it[9]. Additionally, the ‘song system’ in zebra finches is the most delineated motor control system in vertebrates[8]. These combined make the zebra finch one of the most amenable animal models for both scientific analysis and experimental manipulations.

Unlike humans who are capable of learning many kinds of motor skills throughout their lifetime, zebra finches learn one song for their entire life and the ‘critical period’ for their song learning is around 20-90 dph (days-post-hatch). Male zebra finches receive active tutoring at the age of 20-60 dph, a phase termed ‘sensory learning’ during which the memory of the tutor song is formed [Figure 1A]. After this age frame, birds become insensitive to tutor song input, marking the end of sensory learning. They then rigorously practice the song (45-90 dph) and gradually an accurate copy is achieved, a phase termed ‘sensorimotor learning’, for they use auditory feedback to evaluate their vocal production [Figure 1A]. Around 90 dph, as a result of sexual maturation, their song becomes highly stereotyped and less sensitive to auditory feedback, a phase termed ‘crystallization’. Then they would sing this learned song to court females. It is worth clarifying that conceptually the birds should also have a type of ‘motor’ song memory of how to vocally produce their own song, but in this dissertation the term ‘song memory’ is used to denote the memory of the song that birds acquire from their tutors early in their life when they have not yet learned how to produce their own song.

Note that in zebra finches the ‘sensory’ and ‘sensorimotor’ learning phases overlap [Figure 1A]. Zebra finches start producing rudimentary ‘song-like’ sounds at around 45 dph as a way of vocal exploration, a type of vocalization termed ‘subsong’ which is soft, noisy and lack of a consistent temporal structure (like a serial order of syllables and the timing for individual syllables during the series) [Figure 13]. However, their ability to acquire song memory from a tutor remains until around 60 dph. In the current study, I took advantage of this phenomenon and provided the birds with a source of song memory when they started producing ‘subsongs’. As a result, the effect of experimental manipulations on song learning, manifested as the development of vocal production, can be systematically tracked.

One remarkable feature of sensory learning in zebra finches is that a long-lasting, highly accurate memory of song can be rapidly acquired through very brief interaction with a tutor. It is intriguing what neural circuitry and mechanism are engaged in the effective encoding of a memory for guiding behavioral learning such as this. In this study, I aimed to identify neural circuits in the zebra finch brain for serving this role. I targeted a neural pathway at the interface of the sensory and motor systems and examined its role in song memory acquisition through a series of experiments [Figure 1B].

Finding song memory

Most avian species produce only innate vocalizations – sounds that do not require learning[10]. Oscines, a category of birds who 'learn' to sing contain distinct neural circuits for learning from a social model and producing the learned sounds [see Figure 1B for pathways for song learning in songbirds] [[10]], representing three interconnected networks involved in the perception, production and plasticity of sounds, respectively. The auditory pathways, including key nuclei like the secondary auditory regions NCM (caudomedial nidopallium) and CM (caudal mesopallium), are involved in the processing and recognition of sounds[8]. The song motor pathway (SMP), including nuclei HVC (proper name), RA (the robust nucleus of the arcopallium) and downstream nerves controlling syringeal and respiratory muscles, are involved in vocal production. The anterior forebrain pathway (AFP) originates also in HVC and includes nuclei Area X and LMAN, forming a cortical-basal ganglia-thalamic loop. The AFP connects with the SMP in RA, providing a source of vocal plasticity [11]. The SMP and the AFP are the necessary components for song learning in oscines, commonly referred to as the 'song system'[10].

Historically, the 'template theory' has been central to the studies of song memory[12]. Learning the motor skill of song is considered a template-guided process where an auditory memory of the tutor song is acquired first (sensory learning) and re-visited repeatedly during practice for evaluating the vocal performance (sensorimotor learning). An 'error' signal is thought to be generated as a result of the evaluation and fed into the song plasticity circuits (the AFP), leading to the improvement of performance[12]. Thus, hypothetical questions

about the template can be proposed such as ‘what neural circuits are involved in encoding the template?’ and ‘is the representation of the template localized or distributed?’.

In the history of template searching, two candidate regions are the auditory forebrain and the song system [12].

The auditory forebrain. In the auditory forebrain, research revolves the secondary auditory nuclei NCM and CM[13-15]. Their role as (part of) the neural substrate for song memory is suggested mainly by a series of IEG (immediate early gene) expression-based studies[12]. First, increased IEG is observed to be correlated with bird hearing song in the NCM and the CM but with singing song in the song system[13, 16], suggesting a possibility that the song memory, inherently a memory of auditory information, is more likely stored in the auditory regions, than the song system. Second, one unique feature of the NCM is its ‘habituation’ to song – repeated exposure to the same song decreases the IEG level in the NCM, suggesting NCM activity is an indicator of the ‘familiarity’ with a song [14, 17]. Similarly, there is a significant correlation between the IEG level in the NCM and the number of syllables a bird has copied from the tutor song[15, 18, 19]. Experiments along these lines are suggestive of a correlation of the NCM with the ‘content’ of the song memory, such as identity-related aspects of a song (familiarity) and the amount of information a song contains (number of syllables). Third, transient inhibition of IEG signaling in the NCM during tutoring prevented the birds from learning song while control birds who heard the tutor song when the drug washed out learned the song [20], suggesting the NCM is necessary for tutor song acquisition. Fourth, a series of experiments using female birds, who do not sing but exhibit

preference for familiar songs (like their own father's song), show a tendency for the IEG level in the CM to increase in response to familiar songs [14, 15, 21-25].

Overall, these findings suggest the NCM and the CM are associated with processing the auditory information of a song [12], and that their role in acquisition of the song memory could be in the continuum of these two scenarios: 1) the song memory is stored in the NCM or the CM, which is consistent with the above experimental results; 2) they are actively recruited while processing the auditory information of a song but do not necessarily contain specific information regarding the song, which is also consistent with the above experimental results. For example, theoretically, the 'quantitative' property of the NCM (the correlation between the IEG level in the NCM and the number of syllables a bird has copied from the tutor song), which is usually interpreted as the NCM being involved in the storage of song complexity, could also be interpreted as more cells or higher-level neuronal activation in this auditory nucleus being recruited to represent more syllables yet within it there is no 'temporal structure' of the song, a key, unique feature required to organize different syllables to constitute a song. Thus the quantitative property of the NCM does not provide insights regarding whether such the temporal structure exists within the NCM, or in another nucleus that actively communicates with the NCM when hearing song. For example, the NCM might represent a variety of acoustic features of song syllables in a 'scattered' manner, but to encode an actual song with syllables organized in a fixed serial order it is provided with instructive information about the temporal structure from an external source. On the other hand, any manipulation in the auditory area that causes learning deficits is subject to scruple

that, it is the disrupted auditory processing that prevents the song memory from being acquired, rather than the damaged or erased engram itself. In practice, to rule out this possibility, a 'behavioral preference' test is usually conducted in parallel to show that the auditory region-manipulated birds are able to distinguish one song from another. However, the ability to distinguish two different songs is hardly sufficient to encode the entire information in a song. Just like a person who never learns to read can still distinguish one written word from another, the auditory region-manipulated birds, such as the birds with IEG signaling-inhibited in the NCM, might have not acquired the song memory due to severely compromised auditory processing, but could still use the residue auditory processing to tell a difference. Overall, it can be concluded that the neural mechanism of song memory might be a lot more complicated than a 'localized', 'static' template, which has been previously hypothesized to exist either here or there in the song-related brain regions. On the contrary, it can be distributed spatially, dynamic temporally, or both. Therefore, examining the role of a putative locus in song memory calls for experimental methods of observation or manipulation with higher-level spatiotemporal resolution, as well as experimental paradigms that are more stringent.

The song system. In the song system, evidences of the nuclei involved in song memory are focused on their 'song selectiveness' [26-28]. Basically, HVC and its downstream nuclei in the song system, including LMAN, Area X and RA, respond with neural activity of higher amplitude to some songs than others. Particularly, they generally show higher responsiveness to the tutor song (TS) and the bird's own song (BOS) than other types of sound stimuli.

Another tendency is for them to prefer the TS earlier during learning (when the resemblance of BOS and TS is low)[29], and the BOS later (when the resemblance is high). First of all, it is questionable whether the level of responsiveness alone is sufficient to encode a song containing multiple features, such as the timing and serial order of the syllables and the various acoustic features of the syllables. As a matter of fact, the level of responsiveness seems more likely a read-out of a more 'generic' property of a song, like familiarity. Second of all, song selectiveness throughout the song system originates in the HVC and it is now known that the HVC selectiveness can be largely accounted for by its two sources of auditory input, Av and NIf [30-34]. Therefore, a convenient interpretation of the selectiveness in the song system can be that the song memory is stored in Av or NIf (for song selectiveness is not found in the auditory regions except for Av and NIf), and the song selectiveness merely passively flows through the song system when the bird hears the song and the song memory in Av or NIf is activated. In this sense, it is worthy to explore the involvement of Av or NIf in song memory.

Why the NIf-HVC pathway?

In summary, previous studies have not yet provided direct evidences of a locus in the song-related brain regions being unambiguously involved in song memory. In order to further validate whether the song memory is stored in the auditory area, questions remained to be answered include whether the 'temporal structure' exists also in the auditory area or elsewhere. Evidences from research regarding the song system point to a promising scenario

that an upstream region of the song system might be the locus of (or involved in) the song memory and the origin of the song selectiveness observed in the song system. Meanwhile, an ideal experimental strategy to search the brain for song memory would be one able to precisely target a putative locus, not affect adjacent areas. An ideal experimental strategy should also be able to have an effect within the time frame of song memory acquisition, and not cause physical changes to the brain circuits, leading to lasting influence on its functioning.

In this dissertation, I set out to utilize an optogenetic method that specifically targets a neural pathway, paired with sound-detection technique [6] that allows neural activity to be precisely manipulated only during tutoring. In collaboration with other pathway-specific techniques, I aim to explore the role of a neural pathway upstream of the song system – the NIf-HVC pathway, the role of which has been suggested by several previous studies to be associated with controlling the temporal aspects of song and song memory acquisition.

The NIf-Av-HVC loop. NIf and HVC, along with Av, are three of the sensorimotor nuclei at the interface of the auditory and vocal motor regions [Figure 1B] [35]. Since auditory regions are in general upstream of motor regions, the NIf-Av-HVC loop is where the majority of the communication from the auditory system to the vocal motor system comes through [30-32].

HVC and its role in song. HVC is the center of the song system and drives the motor production of song [36, 37]. With HVC lesioned, birds lose their ability to produce the

learned song, but reserve the ability to produce innate vocalizations like calls, and the unstructured, noisy pre-learning vocalizations (subsongs), suggesting HVC specifically drives the production of learned vocalizations [38]. The most prominent studies regarding the function of HVC in song production includes one that showed that local cooling in HVC slows the speed of song by up to 45% but affects only slightly the acoustic features of song while cooling in HVC's downstream motor nucleus RA has no effect on speed, leading to a view that HVC contains chains of activity corresponding to song timing and uses them to organize the production of song elements or sub-elements [39].

HVC is connected to the motor nucleus of song RA via two separate pathways, the direct song motor pathway (SMP) and the indirect anterior forebrain pathway (AFP) [11]. In the former, HVC provides precise timing signals to control temporal features of song. For example, when singing, HVC neurons that project to RA (HVC_{RA}) produce a burst at a single, consistent timing during the song, and drive a stereotyped sequence of activity in RA [40]. In the latter, the output nucleus of the AFP, LMAN, is a source of vocal variability and thus responsible for the auditory-feedback-based vocal plasticity during sensorimotor learning [41]. It also drives the production of the pre-learning unstructured vocalizations (subsongs), in contrast with HVC which drives the production of learned vocalizations. In an earlier study, it was shown that the AFP is necessary for learning spectral, but not temporal aspects of song, indicating a strategy of how the brain employs separate circuits to learn different aspects of song. A current view is that the timing signals provided by HVC and the

plasticity signals provided by LMAN converge in RA and lead to the motor learning of song [11].

NIf and its role in song. NIf is the primary source of auditory input and spontaneous activity to HVC [35]. In previous studies, it has been reported that inactivation of NIf eliminates most-to-nearly-all (variation exists across different research groups) of the auditory response and spontaneous activity in HVC, and that NIf's auditory and spontaneous activity co-vary with those of HVC [30, 32]. During singing, NIf displays motor-related rather than auditory-related activity; it exhibits a premotor activity pattern that bears striking synchronization with HVC [35, 42]. Besides, the premotor activity in NIf is correlated with the temporal aspects of song. For example, NIf neurons that project to HVC (NIf_{HVC}) were found to increase activity prior to syllable onset and decrease activity prior to syllable offset, suggesting a mechanism of marking key timing points in a song [42]. Although functionally associated with HVC, NIf is not required for song production – NIf inactivation transiently disrupts singing but songs can recover within a day due to the ability of the song system to compensate for the absence of NIf [43, 44].

NIf is necessary for song learning – permanent lesion of NIf before tutoring impairs song imitation [6]. Brief and reversible inactivation of NIf in juvenile birds singing plastic songs cause the birds to lose learned song features and degenerate toward producing subsong-like vocalizations, suggesting a role of NIf as either related to the representation or production of the learned features of song [43]. However, being a major link of the auditory and vocal

motor systems, NIf is strikingly not involved in processing auditory feedback (NIf displays premotor activity, not auditory activity during singing) [42]. If NIf communicates crucial auditory information to the song system but the information is not the self-generated auditory feedback during sensorimotor learning, could NIf's role in song learning be processing the auditory input from the tutor during sensory learning?

The NIf - HVC pathway and song memory. First evidences of HVC being involved in song memory acquisition during sensory learning include 1) exposure to tutor song rapidly alters the HVC network, 2) tutoring-contingent optogenetic manipulation, which only disrupts HVC activity in juvenile birds exactly when their tutor sings, blocks song learning, suggesting HVC may be directly involved in the sensory acquisition of the song memory . In the same series of experiments, NIf is identified as a critical conduit for conveying auditory information into HVC during tutoring.

NIf, HVC, temporal structure, and song memory. In summary of the discussion in this section, I concluded that if

1) the contribution of HVC in song learning and production suggest that HVC could be an element specialized for the temporal structure of song,
2) NIf drives and exhibits co-varying activity with HVC in many ways, including auditory, spontaneous activity and premotor activity, suggesting NIf could be the source of information regarding temporal structure into HVC,

3) HVC is directly involved in song memory acquisition during sensory learning,

4) NIf conveys crucial auditory input into HVC during sensory learning,

these findings point to a possibility that the NIf-HVC pathway might be a conduit specialized for relaying the temporal structure of song memory - an aspect of song memory that cannot be accounted for by previous studies in search of the song memory in the auditory regions. In the following chapters, I conducted a series of experiments to examine the role of the NIf-HVC pathway in song memory, hypothesizing that the NIf-HVC pathway is involved in encoding song memory regarding the temporal structure.

PART II

CHAPTER TWO

SELECTIVE MANIPULATION OF NEURAL ACTIVITY IN ZEBRA FINCH

Introduction

I seek to establish methods to selectively manipulate NIf's inputs to HVC in juvenile male zebra finches. In this chapter, I present experiments in which I tested the efficacy of an optogenetic construct - an axon-targeted Channlerhodopsin-2 construct [45] delivered with a self-complementary AAV (scAAV) - in the NIf-HVC pathway of zebra finches, and performed neural circuit tracing to evaluate the functional dissociation of the NIf-HVC pathway and the NIf-Av-HVC pathway which might indirectly transmits information from NIf to HVC.

Optogenetics is a tool developed in the last ~10 years, capable of manipulating neural activity of selective neuron populations [46]. The Neurexin-tagged Channelrhodopsin-2 in a self-complementary AAV (scAAV-NX-ChR2) is an excitatory channel guided by the axon-terminal-specific protein Neurexin to express at axon targets. This is a viral construct newly developed and never tested in zebra finches. I injected it into NIf of male zebra finches and recorded optical responses in its efferent target HVC.

Another concern regarding the spatial specificity of optogenetically activating the NIf-HVC is that NIf is connected to HVC via the NIf-HVC pathway and the indirect NIf-Av-HVC pathway. I traced retrogradely the projection neuron types in NIf into Av or HVC to evaluate the overlapping of origins of output. I also recorded in NIf while optically exciting HVC in order to see if NIf activity is antidromically affected, for an antidromically excited NIf might also potentially complicate the pathway-specific optogenetic manipulation.

Methods

Animals. Experiments described in this study were conducted using juvenile and adult male zebra finches (35-120 days-post-hatch (dph)). All procedures were performed in accordance with protocols approved by Animal Care and Use Committee at UT Southwestern Medical Center.

In vivo electrophysiological recording and analysis. Multi-unit neural activity was recorded using a custom LabVIEW software (National Instruments). In vivo recording data from live animals were analyzed using Matlab. Slice recording data were analyzed using SigmaPlot.

In-vitro slice preparation. Zebra finches were anesthetized with isoflurane. Once the animal was no longer responsive to a toe pinch, it was quickly decapitated. The brain was removed from the skull and submerged in cold (1-4°C) oxygenated dissection buffer. Acute sagittal 250 to 300 μm brain slices were cut in dissection buffer at 4°C containing (in mM): 225 sucrose, 3 KCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose. Individual

slices were gently stored at a temperature of 32°C in normal artificial cerebrospinal fluid (aCSF) for at least 1 hour before recording. Normal aCSF was similar to the dissection buffer except the sucrose was replaced by 126 mM NaCl. Both the dissection buffer and normal aCSF were adjusted to 310 mOsm, pH 7.3-7.4, and saturated with a 95%/5% O2/CO2 mix.

Slice electrophysiological recording. Slices were constantly perfused in a submersion chamber with 32°C oxygenated normal aCSF. Patch pipettes were pulled to a final resistance of 3-6 MΩ from filamented borosilicate glass on a sutter P-1000 horizontal puller. Neurons were visualized with video-assisted infrared differential interference contrast imaging and fluorescent neurons were identified by epifluorescence imaging under a water immersion objective (X40, 0.8 numerical aperture) on an upright Olympus BX51 WI microscope with an infrared CCD camera (Q-Imaging Rolera). Data were low-pass filtered at 4 kHz and acquired at 10 kHz with an Axon MultiClamp 700B amplifier and an Axon Digidata 1550B Data Acquisition system under the control of Clampex 10.6 (Molecular Devices). Data and collected and analyzed using Clampex 10.5 software (Clampex 10.6, Molecular Devices, Inc.).

Light evoked EPSCs. For recordings of light evoked excitatory postsynaptic currents (oEPSCs), the internal solution contained the following (in mM): 130 K-gluconate, 4 MgCl2, 4 Na2ATP, 0.3 Na3GTP, 10 Na2-phosphocreatine, 10 HEPES, 0.2 EGTA and brought to pH 7.25 and 295mOsm. To isolate oEPSCs, SR-95531 10 µM was added to the extracellular solution and QX-314 6mM (lidocaine N-ethyl bromide) was added to the intracellular

solution. Membrane potentials were not corrected for liquid junction potential (experimentally measured as 11.4 mV for the K+-based pipette solution). For the photostimulation of ChR2-expressing NIf to HVC glutamatergic terminals, oEPSCs were evoked with 20-50ms blue light pulses. Blue light was emitted from a collimated light-emitting diode (470 nm) driven by an LED driver (CoolLED, pE-300) under the control of an Axon Digidata 1550B Data Acquisition system and Clampex 10.6. Light was delivered through the reflected light fluorescence illuminator port and the X40 objective. oEPSCs were recorded in the whole-cell voltage clamp mode. Only cells with holding currents ≤ 100pA at Vh = -80 mV, series resistance ≤ 20 MΩ were included in the study.

Intrinsic excitability. Neuronal intrinsic excitability was examined with the potassium gluconate-based pipette solution. After whole-cell current clamp mode was achieved and the bridge was balanced, resting membrane potentials were recorded within the first minute after break-in and maintained at -80 mV. Input resistances were measured by injecting a 150-ms hyperpolarizing current (40pA) to generate a small membrane potential hyperpolarization from the resting membrane potentials. Firing rate represents the average value measured from 2 to 3 cycles (700ms duration at 0.1 Hz, –100 to +250pA range with a 50pA step increment, every 12 s).

Tracer injection. Birds were anaesthetized using isoflurane inhalation (0.8-1.5%) and placed in a stereotaxic surgical apparatus. All surgical procedures were performed under aseptic conditions. For neural circuit tracing, we injected 135nl of differently conjugated tracers

(Dextran, Alexa Fluor 488 or 594, 10,000MW, Invitrogen) bilaterally into birds' Av and HVC, respectively. Av was located using stereotaxic coordinates (head angle: 45°, AP: 1.25mm, ML: 2.0mm, DL: 1.0mm, relative to the center of Y-sinus). Birds were perfused7 days after their injections and their brains were sectioned, mounted and inspected under fluorescent microscope (Leica DM 5500B).

Results

In-vitro optical response in HVC. Activation of NIf axon terminals elicited monosynaptic excitatory input to HVC neurons mediated by AMPA/NMDA receptors [Figure 2A-C, +ChR2, n = 10 cells before and after application of DNQX (20um) and DL-AP5 (100uM); -ChR2 n = 10 cells from birds not injected with ChR2 in NIf].

In-vivo optical response in HVC. Optical excitation of NIf terminals in vivo produced reliable yet complex polysynaptic responses in HVC, with most neurons exhibiting strong increases in activity and a small percentage exhibiting strong suppression [Figure 2D-F, excitation = 257/341 recording sites, inhibition = 18/341 recordings sites, non-responsive = 66/341 recordings sites].

Neural tracing of NIf_{Av} and NIf_{HVC} neurons. We mapped efferent projections from NIf using anatomical and physiological methods [Figure 2H-J, Figure 7, 11 hemispheres from 7 birds; Figure 8, NIf-Av, n = 10 cells; NIf-HVC, n = 10 cells]. Approximately ~70% of NIf

projection neurons exclusively innervate HVC, ~30% exclusively innervate Av and only <5% project to both HVC and Av.

Test of antidromic activation in NIf. We made in vivo extracellular recordings in NIf while optogenetically exciting its terminals in HVC to examine if terminal stimulation antidromically excites NIf neurons [Figure 2K-L, n = 3 hemispheres from 2 birds]. Although optogenetic activation of axon terminals reliably evoked postsynaptic responses in HVC, they failed to drive antidromic responses in NIf (9 recordings sites from 3 hemispheres).

Test of the range of optically affected area. To examine if optogenetic excitation of HVC might also directly excite NIf terminals innervating Av, which is located ~1.5 mm from the end of optic fibers over HVC, we measured the depth from the surface of the brain at which we could elicit excitatory responses. Compiling data from all of our in vivo recordings (257 recordings exhibiting excitatory responses), we found that we could only optogenetically excite cells within the first 500μm from the surface of the brain, indicating our optogenetic manipulations are unlikely to directly excite NIf axon terminals innervating Av.

Discussion

In summary, in this chapter I showed that 1) the scAAV-NX-ChR2 construct is capable of effectively exciting the NIf terminals in HVC, 2) two largely non-overlapping groups of

projection neurons in NIf project to Av and HVC, exhibiting a physical dissociation of the NIf-HVC pathway and the NIf-Av-HVC pathway, 3) no antidromic activity was found in NIf when optically exciting HVC, suggesting our axon terminal targeted optogenetic excitation is unlikely to affect NIf activity itself.

Together, these findings indicate that using the optogenetic tool neural activity in the NIf-HVC pathway can be selectively manipulated in juvenile birds.

CHAPTER THREE

INCEPTION OF THE MEMORY OF TEMPORAL STRUCTURE

Introduction

In the last chapter, I showed that using the optogenetic method the neural activity in the NIf-HVC pathway can be specifically manipulated without affecting other neural circuits in the song-related brain areas. In order to examine its role regarding song memory and particularly, the temporal aspects of it, my strategy is to deliver artificial activity patterns in this pathway. If the memory of song – a procedural motor task – is acquired via this pathway, I would predict that artificial activity patterns seed a memory of the task and result in birds singing a song with a temporal structure that conforms to the artificial pattern. Since the manipulation is conducted in a task-specific manner, such experimental results would suggest the role of the NIf-HVC pathway to be associated with encoding the song memory, rather than processing auditory information in general.

We raised young males without any social or auditory experience of adult song tutors, then optically tutored them using light pulses designed to mimic short or long syllables near the end of their song sensory learning phase. Light stimulation was delivered at time intervals derived from natural song tutoring patterns while experimental birds were alone in acoustic chambers [Figure 3A, Figure 9].

We also explored whether opto-tutored birds used their vocalizations appropriately during social interactions. Zebra finches use their song to court female birds in a behavior commonly referred to as directed singing [47], and they spend extended periods of time practicing their song when alone. If opto-tutoring implants a memory of song, it should result in learning of a song which the birds would use to perform the social function of courtship.

Methods

Viral injection. Birds were anaesthetized using isoflurane inhalation (0.8-1.5%) and placed in a stereotaxic surgical apparatus. All surgical procedures were performed under aseptic conditions. For optogenetic experiments, we injected (Drummond, NanojectII or III) 700nl of self-complementary neurexin-tagged Channelrhodopsin2 (scAAV2/9-NX-hChR2-YFP, custom prep from UNC Vector Core or IDDRC Neuroconnectivity Core, Baylor College of Medicine) bilaterally into NIf of juvenile male zebra finches (35-45 days-post-hatch (dph)). NIf was located using stereotaxic coordinates (head angle: 45°, AP: 1.75mm, ML: 1.75mm, DV: 2.1-2.3mm, relative to the center of Y-sinus) and multi-unit recordings. After 2-3 weeks, birds were tested for in vivo light-evoked responses (473nm LED, Prizmatix) in HVC using multi-unit electrophysiological recordings. HVC was located using stereotaxic coordinates (head angle: 45°, AP: 0mm, ML: 2.4mm, DV: 0.4mm, relative to the center of Y-sinus) and multi-unit recordings. A separate set of injected birds were used for slice electrophysiological tests for single cell light-evoked response.

Optogenetic stimulation in behaving birds. Juvenile male zebra finches were isolated from male parents prior to 12dph and raised by foster mothers. At age of 35-45 dph, birds were injected with scAAV2/9-NX-hChR2-YFP. After 2-3 weeks, birds were tested for multi-unit light-evoked response in HVC. Birds with strong optogenetic responses (light-evoked responses recorded from at least 3 sites/hemisphere spaced by at least 100μm) were implanted using dental acrylic with guiding sleeves for receiving optic fibers. 2-3 days before the implantation surgery, birds were put into isolate experiment chambers to habituate and their vocalizations recorded. Vocalizations were confirmed to be subsongs before proceeding into the following optogenetic stimulation procedures. Birds were given 1-2 days of rest before the optogenetic stimulation. A stimulation course was a total of 5 days within the following 10 days. After the 5 days of stimulation birds' implanted sleeves were removed and birds were kept in isolation until adulthood. For birds who received only optogenetic stimulation, a 90-minute session of natural zebra finch tutoring was previously recorded using the LabVIEW application and the timing of songs produced within was extracted to trigger the optogenetic stimulation in the pupil birds to simulate a naturalistic manner of tutoring. Pulses corresponding to 300-370 song motifs were given throughout the 90-minute session to the implanted pupil birds.

Data analysis of song. All birds' songs were viewed and processed using Sound Analysis Pro 2011 (SAP2011) or Audacity. The rest of the analysis was performed with MATLAB. Song analysis was based on one day of singing as an adult. The dominant motif for each light-stimulated bird was determined by visual inspection. 40 repeats of the motif of each

bird were used for subsequent syllable duration analysis. Syllable segmentation and feature analysis. Songs were segmented using Segmentation in SAP2011 by thresholding sound amplitude. To accommodate some of the 50ms opto-tutored birds who sing fast trill-like songs, wherein the gaps between elements could not be defined conventionally, we used Adaptive Threshold in SAP2011, in which the threshold systematically varies with the mean amplitude. A constant Adaptive Threshold was manually chosen and applied to segment all birds' songs in this study. Accuracy and validity of segmentation were verified by visual inspection and tracing development of syllables from subsong to stereotyped adult song. We used Mann-Whitney U test for comparison of syllable duration distributions.

Results

Optogenetic manipulation shapes temporal structure of song. Adult zebra finch song typically contains ~3-6 unique syllables lasting ~100ms each separated by brief periods of silence (73 unique syllables measured from 15 birds, average 4.9 elements/bird, median duration = 106ms). When birds are raised without any social or auditory exposure to a song tutor, their adult song, referred to as isolate song, contains ~4-7 syllables that are significantly longer than normally reared birds with free access to a tutor [42, 48] [Figure 3B-C, 61 unique syllables measured from 10 isolate birds, average 6.1 elements/bird, median duration = 171ms; Mann-Whitney U test comparing duration of syllables from zebra finch tutored birds (n = 73) to syllables from isolate birds(n = 61), p = 2.83e-05, or comparing average syllable durations from zebra finch tutored birds (n = 15 birds) to isolates (n = 10

birds), p = .0025; we should note that isolate birds in this context are best described as untutored birds and differ from 'true' isolate birds that are hand-raised by people and never exposed to other conspecifics]. Birds opto-tutored as juveniles with repeated 50ms light pulses produced adult vocalizations with significantly shorter syllables than normally reared or isolate birds [Figure 3B-C, Figure 10, n = 7 birds, 25 elements; Mann-Whitney U test comparing duration of syllables (n = 25) from 50ms opto-tutored birds to syllables from zebra finch tutored birds(n = 73), p = 3.86e-04 or to duration of syllables from isolate birds(n = 61), p = 4.67e-09; Mann-Whitney U test comparing average syllable duration from 50ms opto-tutored birds (n = 7 birds) to average duration of zebra finch tutored birds (n = 15 birds), p = .0031 or with isolates (n = 10 birds), p = 2.06e-04]. We found that the majority of 50ms opto-tutored birds produced simple songs with only 1-3 unique elements that were repeated or trilled at a high rate. Syllable durations in opto-tutored birds clustered near 50ms (median = 62.3ms), while both normally reared and isolate birds produced syllables spanning a significantly broader distribution of durations [Figure 3D].

To further test if opto-tutoring implants memories that guide learning of syllable duration, we next tutored juvenile birds with long duration light pulses instead of short duration pulses [Figure 3E]. Birds opto-tutored with 300ms duration excitation of NIf-HVC synapses produced adult vocalizations with 1-3 syllables. The duration of these syllables were significantly longer than 50ms opto-tutored birds [Figure 3F-G, Figure 11, median duration = 323ms, Mann-Whitney U test comparing syllable durations between 300ms vs. 50ms opto-tutored by syllable (300ms, n = 7 syllables, 50ms, n = 25 syllables), p = 2.54e-06; or by bird (300ms, n = 4 birds, 50ms, n = 7 birds), p = .0061).

To examine if 50ms or 300ms opto-tutor birds learned vocal parameters other than syllable duration from manipulation of NIf-HVC axon terminals, we measured acoustic features typically imitated during song learning, including pitch, mean frequency, goodness of pitch and entropy [9, 49, 50]. We found that opto-tutored songs followed trends similar to isolate songs and did not systematically differ from songs of normally reared or isolate zebra finches [Figure 12].

Likewise, opto-tutored birds practiced their song when alone and produced a range of other call types typically produced by zebra finches [51]. When presented with female birds, opto-tutored birds readily performed directed singing behavior using the short or long vocal elements shaped by opto-tutoring. Together, these findings indicate that opto-tutoring in juvenile birds selectively shapes the temporal structure of their adult courtship song.

Opto-tutoring implants a memory that guides song learning. Opto-tutoring could shape adult song by implanting a memory that guides developmental learning of temporal structure of song. Alternatively, opto-tutoring might directly imprint or entrain patterns of activity on the HVC network, thereby constraining the production of vocalizations to those with a specific temporal structure. To help discern these possibilities, we examined the developmental trajectory of syllables of our opto-tutored birds [Figure 4, 13]. We found that opto-tutored birds exhibited complex learning trajectories similar to those observed in normally tutored birds [50, 52]. Opto-tutored birds showed initial changes in vocal elements

within 2-3 days of opto-tutoring, similar to birds that are first song-tutored near the end of their sensitive period for sensory learning [53, 54]. Similar to normal song learning, many changes in syllables also slowly accrued over the month of sensorimotor learning that followed the opto-tutoring experience. Birds began to modulate the amplitude of the initially noisy, long, unstructured subsongs in response to the duration of light pulses they received. Birds opto-tutored with 50ms light pulses increased the amplitude modulation of their long vocal elements, eventually learning to produce trilled, short-duration syllables and in some instances learning to produce gaps between these vocal elements [Figure 4A, 13]. Birds opto-tutored with 300ms light pulses, on the other hand, slowly learned to decrease the amplitude modulation across vocal elements, leading to the gradual emergence of longer and more harmonic vocal elements [Figure 4B, 13]. Opto-tutored birds also crystallized their songs starting 85–90dph. Their songs before 80dph exhibited variable vocal durations and acoustic features, while songs after 90dph were increasingly stereotyped, like song crystallization in normally reared birds.

These results suggest that opto-tutoring implants memories that guide learning, rather than directly entraining a specific motor program in young animals. However, it is also possible that opto-tutoring simply biases or selects amongst precursor or innate vocalizations to specify the production of vocal elements with certain durations in adulthood. For example, optical stimulation of NIf axon terminals in HVC could bias the 50ms birds to only sing the short introductory notes that typically precede the bird's normal song motif and bias the 300ms birds to only sing long isolate-like vocal elements or calls. Such a scenario could

point to circuit mechanisms for how innate vocal repertoires are selected or reinforced by activity during development. However, we found that both 50ms and 300ms opto-tutored birds produced songs with distinct introductory notes and song motifs, with the primary difference between them being the duration of the vocal elements within their song motif [Figure 4A-B, 13].

Discussion

In summary, in this chapter I showed that 1) optogenetic manipulation of neural activity in the NIf-HVC pathway implanted memory of temporal structure of song, evidenced by two groups of birds who learned to produce either shorter-than-normal or longer-than normal syllables, 2) from optogenetic manipulation birds learned songs with abnormal temporal structure conforming to the artificial pattern they received, and sang the abnormal songs for courtship. These results support the view that the NIf-HVC pathway is a conduit through which the temporal structure of the memory of the courtship song can be acquired.

To our knowledge, these results provide the first evidence that manipulation of a circuit in the brain can bypass learning from sensory input and social interaction with a behavioral model presumed to be necessary, and directly implant memory that help guide behavioral imitation.

CHAPTER FOUR

EXPLORING THE STORAGE OF THE MEMORY OF TEMPORAL STRUCTURE

Introduction

In the last chapter, I showed that the NIf-HVC pathway is a pathway through which the temporal structure of the memory of the courtship song can be acquired. Even though this suggests a strong association of the NIf-HVC pathway with song memory, questions remain such as ‘are there other pathways that can transmit song memory?’ ‘are the different pathways functionally mutually exclusive?’ ‘are some of them specialized for certain features or even redundant?’. Moreover, knowing that a pathway can input information for song memory acquisition does not provide insights about where the song memory is finally stored. Therefore in this chapter, I seek to evaluate ideas like these in order to further clarify the role of the NIf-HVC pathway in song memory.

A longstanding view is that song memory is encoded in auditory regions presynaptic to the NIf-HVC pathway, such as NCM, CM and field L [8, 12, 55, 56]. Song memory encoded presynaptic to HVC might be capable of guiding song learning independent of the memory encoded via the NIf-HVC pathway. In addition, auditory information entering HVC via other routes, such as Av projections into HVC, may be capable of encoding song memory. To evaluate these ideas, we paired opto-tutoring with normal song tutoring as juvenile birds socially interacted with live tutors. Optical activation of NIf-HVC synapses was contingent

on the tutors' singing behavior [Figure 5A], providing the juvenile with two simultaneous potential sources of information from which to learn – ascending auditory information from a live zebra finch tutor and light evoked activity at NIf-HVC synapses. Potentially this experiment should provide insights about the role of the NIf-HVC pathway relative to other possible pathways involved in song memory acquisition.

Additionally, if imposing activity at the NIf-HVC pathway implants the temporal structure of song memory, this memory could either be stored at the NIf-HVC synapses or relayed via the NIf-HVC pathway and stored in downstream regions. In an attempt to shed light on this issue, we genetically lesioned neurons in NIf projecting to HVC using an intersectional viral approach for cre-dependent expression of caspase3 [34, 57]. NIf neurons projecting to HVC were lesioned in young isolate birds several days prior to providing them with natural tutoring experiences, and in age matched birds after they had an opportunity to memorize a tutor song [Figure 6A]. I reason that if the NIf-HVC synapses store aspects of the song memory, lesioning before or after tutoring should both cause loss of memory and unsuccessful song learning; if the downstream regions store this memory, then the NIf-HVC pathway serves merely as a conduit of this memory and thus lesioning after tutoring should not impair song learning.

Methods

Optogenetic stimulation in behaving birds. Juvenile male zebra finches were isolated from male parents prior to 12dph and raised by foster mothers. At age of 35-45 dph, birds were injected with scAAV2/9-NX-hChR2-YFP. After 2-3 weeks, birds were tested for multi-unit light-evoked response in HVC. Birds with strong optogenetic responses (light-evoked responses recorded from at least 3 sites/hemisphere spaced by at least 100μm) were implanted using dental acrylic with guiding sleeves for receiving optic fibers. 2-3 days before the implantation surgery, birds were put into isolate experiment chambers to habituate and their vocalizations recorded. Vocalizations were confirmed to be subsongs before proceeding into the following optogenetic stimulation procedures. Birds were given 1-2 days of rest before the optogenetic stimulation. A stimulation course was a total of 5 days within the following 10 days. After the 5 days of stimulation birds' implanted sleeves were removed and birds were kept in isolation until adulthood. For birds who received both zebra finch and optogenetic tutoring, we used a custom LabVIEW application to detect the songs of the tutor and detection of each song motif triggers the light stimulation of the pupil bird.

Tutoring NIf-HVC lesioned birds and song analysis. Isolate and normally reared birds were injected with viruses expressing Cre and an activatable form of caspase3 into HVC and NIf, respectively at 35-40 dph. Isolate birds were then housed with a song tutor between days 55 and 60 days of age. All birds were separated from their tutors at 60 days of age and raised to adulthood. Song similarity was calculated using SAP2011.

Results

The NIf-HVC pathway is likely a necessary, non-redundant pathway for song memory acquisition. When birds were provided with both live tutoring and opto-tutoring, they failed to imitate the songs of their tutors [Figure 5B, Mann-Whitney U test comparing %similarity to tutor between control birds (n = 5 birds) and ZF + opto-tutor birds (n = 3 birds) p = 0.03]. As adults, they exhibited levels of similarity to their tutor song that were indistinguishable from the songs of isolate birds [Mann-Whitney U test comparing %similarity to tutor between isolate birds (n = 4 birds) and ZF + opto-tutor birds (n = 3 birds) p = 0.8]. Instead, these birds learned from the 300ms optical stimulation, displaying syllable durations similar to birds that were opto-tutored, but never tutored by adult zebra finches [Figure 5C-D, Mann-Whitney U test comparing duration of syllable durations between 300ms opto-tutored birds (n = 7 syllables from 4 birds) and ZF+opto-tutored birds (n = 4 syllables from 3 birds) p = 0.9]. Finding that birds learn from opto-tutoring, even when provided with a normal song model, suggests that activity at NIf-HVC synapses either overrides learning from auditory experiences with a social model or that the song memory that might be encoded presynaptic or independent of NIf-HVC synapses are not sufficient to guide song imitation.

Grouping all birds opto-tutored with 300ms light pulses (n = 7 birds producing 11 syllables), we find that their syllable durations are significantly longer than those of isolate or normally reared birds [Figure 5E, Mann-Whitney U test comparing duration of syllables (n = 11) from 300ms opto-tutored birds to syllables (n = 73) from zebra finch tutored birds, p = 3.2549e-06 or to duration of syllables (n = 61) from isolate birds, p = 0.0010; Mann-Whitney U test

comparing average syllable duration from 300ms opto-tutored birds (n = 7 birds) to average durations of zebra finch tutored birds (n = 15 birds), p = 2.4684e-04 or from isolates (n = 10 birds), p = 2.0568e-04].

Temporal structure of song memory is stored downstream of the NIf-HVC pathway. We found that genetic lesions of NIf_{HVC} neurons in isolate birds disrupted their subsequent ability to imitate the song of their tutors [Figue 6B-C, Mann-Whitney U test, lesioned then tutored birds (n = 4) vs control birds (n = 5 birds), p = 0.02] and that they exhibited similarity scores to their tutor that were indistinguishable from isolate birds [Mann-Whitney U test, lesioned then tutored birds (n = 4) vs isolate birds (n = 4 birds), p = 0.3429]. It has previously been shown that non-selective lesions or inactivation of NIf prior to song tutoring disrupts subsequent vocal imitation [6]. Our current findings show that the lesions of only the NIf-HVC pathway is sufficient to disrupt song learning. However, it is not clear if these lesions disrupt acquisition of tutor song memories, or subsequent sensorimotor learning. We found that lesions to this pathway after birds had an opportunity to learn from their tutor did not affect the ability of juvenile birds to accurately imitate the song of their tutor [Figure 6B-C, p = 1.0, Mann-Whitney U test], suggesting that the NIf-HVC circuit plays a specific role during the acquisition of song memory. Together with our opto-tutoring results, this indicates that the NIf-HVC circuit is necessary for forming a memory used to guide learning of song-element-duration, but that this circuit is dispensable once this song memory is acquired. This suggests that at least certain aspects of tutor song memories used to evaluate vocal performances are ultimately stored downstream of NIf-HVC synapses.

Discussion

In summary, in this chapter I showed that, 1) when provided with both a live tutor and artificial activity pattern in the NIf-HVC pathway, birds only learned from the artificial pattern but not the live tutor, suggesting that the NIf-HVC pathway either overrides other pathways involved in learning from a social model or that the song memory that might be encoded presynaptic or independent of the NIf-HVC pathway is not sufficient to guide song imitation, 2) lesioning the NIf-HVC pathway prevented birds from learning from a tutor but lesioning after tutoring experience did not affect learning, suggesting the NIf-HVC pathway is a necessary component for song memory acquisition and that it is a conduit of the temporal structure of song memory, but not the storage site of it.

DISCUSSION

Animals learn extensive skills throughout their lifetime. However the mechanisms of how skills are acquired, represented and executed in the neural system are still poorly understood. Unlike the episodic memory that involves the hippocampus for its initial formation and the cortex for its permanent storage, the memory of motor skills or the procedural memory employs distinct strategies and neural substrates.

Some of the most sophisticated skills animals can maneuver are acquired by imitating from a more experienced conspecific, and vocal learning is a typical example of this kind. The task of vocal learning is to vocally produce a sequence of movements in a fixed or near-fixed order, indicating that the vocal learning animals acquire information regarding what vocal movements to produce at what timepoints from the social model, form a memory of it and use the memory to guide the learning of their own vocal production. Since only few animal species can perform vocal learning, including songbirds and humans, understanding the neural mechanism of vocal learning in zebra finches is potentially beneficial for understanding vocal learning in generalized forms.

In this dissertation, I used song learning in zebra finches as the animal model to identify neural circuits that are involved in the song memory, a memory acquired from a social model for guiding the learning of the production of the courtship song. Historically this type of memory has been theorized as a sensory ‘template’ and massive search for the

template has been done in songbirds' auditory regions. Accordingly, song-related memory has also been distinguished into two types: 1) a sensory template acquired from social experience with the tutor, an episodic memory for guiding the formation of the procedural memory of song production, and 2) a motor memory of song, the actual procedural memory of the bird's own song production. However, unlike the classic acquisition of an episodic memory, song learning does not require the hippocampus. On the contrary, besides the auditory regions for acquiring and processing the auditory input of song, songbirds contain a unique set of neutral circuits – the 'song system'– for the purpose of song learning, production and plasticity, suggesting that the song memory is essentially a different form of representation of experience than a typical episodic memory. In fact, the song memory is more likely a form of memory specialized in a way for serving the role of guiding the learning of the procedural memory of song production, a mixed sensory-motor form of memory that potentially assists translating the auditory experience from the social model to the motor production of the bird's own courtship song.

Previous studies in search of the song memory in the auditory regions suggest that some auditory nuclei in the songbird brain are likely involved in the storage of song memory, but do not provide evidence of whether a key feature of the song memory – the temporal structure of song, referring to the serial organization of the timing of syllables in a song – also exists in these regions. Moreover, confusions often arise in terms of whether a manipulation of a putative locus in the auditory regions actually affects the song memory

itself, or just the auditory processing for the formation of the song memory. Even though previously people have used a song preference test to show that birds with sites manipulated in the auditory regions failed to form the song memory but were still able to distinguish between two different song stimuli, the auditory processing capacitiy to successfully perform a song preference test is hardly sufficient to support the acquisition of the memory of a full song which contains complex and detailed temporal and spectral structures.

On the other hand, other lines of research pointed to another possibility that crucial aspects of the song memory are dependent on the sensorimotor circuits for their acquisition and these aspects are highly likely associated with the temporal structure of song. This suggests a view that the brain can employ separate neural circuits to acquire certain aspects of the memory of a motor skill. However it remains to be evaluated whether it truly is the case, and how these aspects are integrated to form a full song and subsequently guide the learning of song production. In this dissertation, I aimed at identifying the role of a sensorimotor pathway in the zebra finch song system in song memory. The NIf-HVC pathway represents the primary flow of information from the auditory system to the motor system during song learning. I hypothesized that the NIf-HVC pathway has a role in encoding the memory regarding the temporal structure and conducted a series of pathway-specific manipulations to target the NIf-HVC pathway in order to examine its exact role in song memory.

Through these experiments, I showed that 1) imposing artificial activity in this pathway results in birds singing songs with temporal structure conforming to the imposed activity, suggesting a mechanism for encoding the temporal structure of song; 2) imposing activity paired with live bird tutoring cause the birds to learn only from the imposed activity, but not from the live bird tutor, suggesting this pathway is either able to override other pathways for acquiring song memory, or a non-redundant pathway for encoding the temporal structure of song; 3) genetic lesioning of cells in this pathway precludes birds from learning from a tutor, but does not affect song learning if birds received tutoring before lesioning, suggesting this pathway is necessary for acquiring song memory and that memory transmitted via this pathway is not stored within but downstream of it.

These findings suggest that the NIf-HVC pathway is necessary for acquiring the song memory and it serves as a conduit for relaying the temporal structure of song for its storage in downstream regions, supporting a view that the brain can employ separate neural circuits to acquire certain aspects of the memory of a motor skill, such as the temporal features. Therefore in this dissertation, I essentially identified a neural circuitry that is required for the initial formation of a memory but not the permanent storage of it, which is reminiscent of the role of hippocampus in the acquisition of the episodic memory. If the role of the NIf-HVC pathway is to merely relay the temporal aspects of the song memory, promising sites downstream of the NIf-HVC pathway for the permanent storage of memory include: 1) HVC itself, HVC is known to contain a ‘syn-fire’ chain that controls the timing of song production. A role of HVC as the permanent

storage site of the temporal structure would be convenient for HVC it to simply use the temporal structure directly acquired from the social model to guide the learning and production of the temporal structure of the birds' own song. 2) Av, another crucial nucleus at the interface of the auditory and motor system and also embedded in the secondary auditory nucleus CM. Neuronal activation level in the secondary auditory regions have been known to correlate with the number of song syllables birds have copied from the tutor. Since zebra finch song syllables are largely distinguished by their distinct spectral features, this is suggestive of a neutral substrate for representing the amount of distinct spectral features contained in a song but not necessarily the temporal structure that organizes the individual pieces of spectral features into a full song. A role of Av as the permanent storage site of the temporal structure of the song memory would be convenient for it to integrate both the temporal and spectral features to form the memory of a full song.

Using optogenetic tutoring of juvenile songbirds I have shown that the temporal aspects of song memory can be implanted through manipulation of sensorimotor synapses that convey information from a social model to motor circuits. But in this set of experiments I have only implanted using over-simplified single long (a single 300ms) or repeated short (four pulses of 50ms) optogenetic stimulation patterns, and only the temporal structure of song was affected by the manipulation of these synapses. Therefore questions remain whether more naturalistic temporal patterns, spectral features or even a full zebra finch song can also be implanted using similar methods.

First of all, although the temporal resolution of the optogenetic method is sufficient to reliably deliver light pulses markedly longer or shorter than natural zebra finch syllable durations, I would doubt a more natural temporal pattern with varied durations can be implanted optogenetically using the same optogenetic construct, for example, a four-syllable song with syllable durations of 50ms, 100ms, 150ms and 200ms, respectively (since the range of wild type zebra finch song syllable duration is approximately 70-200ms). The reason is mainly due to the highly variable latency of the optical responses elicited within HVC, a phenomenon that is inevitable because of the complex interacting networks of excitatory and inhibitory neuron types in HVC. If the temporal structure is implanted through manipulating the NIf-HVC synapses by affecting the temporal structure of neural activity received by HVC, a highly variable latency of optical responses would have a much stronger impact on a song with multiple syllable durations in a fixed order, than an over-simplified song with only one syllable duration whether long or short. In both 300ms and 50ms opto-tutored experimental groups, most birds learned songs with very few syllable types. Specifically, the 300ms opto-tutored birds typically sing songs with only either one extremely long syllable or 2-3 syllable types with little difference in spectral features. The 50ms opto-tutored birds sing songs with short syllables also with little difference in spectral features (or trill) but the number of syllables (or the times of fluctuation in the trill) has a relatively wide range of 2-5 and cannot be predicted based on birds or the subsongs they produce before opto-tutoring. However, each bird has a stereotyped song with a relatively constant syllable number,

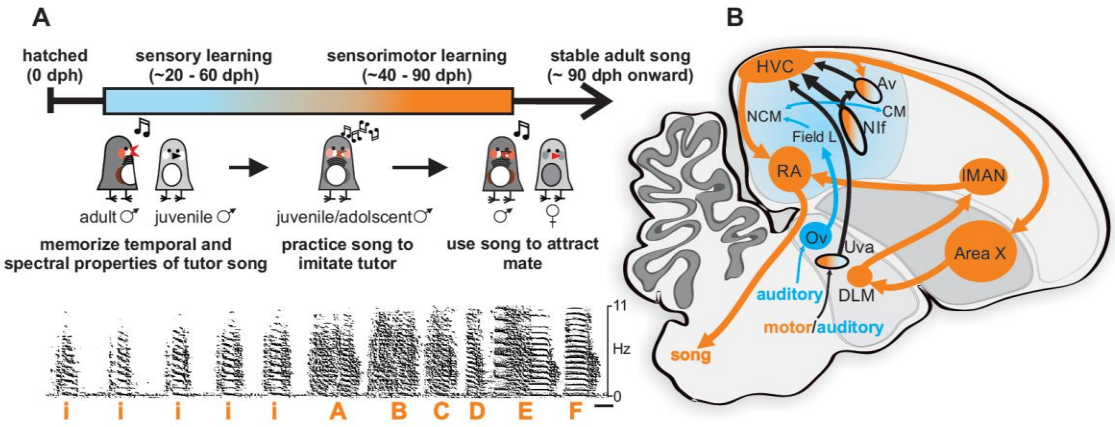
suggesting different yet consistent ‘deciphering’ strategies of the light pulses are utilized by each bird. Therefore, it can be presumed that a complex sequence of light pulse durations delivered with irregular latencies would introduce a chaos in the HVC networks, causing great difficulty or even failure to consistently translate the light patterns into the correct sequence of syllable durations. In conclusion, unless another synapse manipulation method is developed in which the latency of optical response can be controlled down to a satisfying low level, implanting a naturalistic song with multiple syllable durations would be difficult. Regarding implanting the spectral features, it is not yet fully understood where and how exactly the spectral features are stored but previous studies have pointed to the secondary auditory regions as containing those aspects of the song memory. Using similar optogenetic methods to implant spectral features will have to await more knowledge of how spectral features are represented in those brain areas, for example, whether a high-pitch sound is represented by a certain type of activity of a certain zone or neuron group within those areas.

This study has also suggested a critical role of the motor circuits in the acquisition of a memory from social experience, a memory that was previously considered an essentially ‘sensory’ memory and searched for mainly in the auditory regions of the brain. In fact, premotor cortical circuits involved in speech production are activated by listening to speech, even in pre-verbal infants, suggesting that tight coupling between sensory and premotor cortical circuits exists at the earliest stages of learning. This further suggests

that sensorimotor pathways may provide a general substrate for encoding aspects of memory that guides procedural learning during memory acquisition from a social model.

Lastly, towards a model of the full memory of a zebra finch song, previous studies have suggested that song memory encoded in higher order auditory regions function as an 'auditory template' used to guide vocal imitation, and findings in the current study indicate that song memory acquired during social interaction requires transformation of pertinent auditory experiences into the sensorimotor circuits prior to consolidation. Additionally, HVC is known to provide feedback to the auditory system via its projection to Av, and this pathway may facilitate integration of aspects of memory acquired via the NIf-HVC pathway and the aspects stored in the auditory regions. Accordingly, a possible model consistent with these ideas mentioned above would be that, during sensory acquisition, the temporal structure of song is stored in a downstream locus of the NIf-HVC pathway, such as HVC itself, and the spectral features (like the pitch and entropy of each syllable) are stored in the synapses between the sensorimotor nucleus Av and the secondary auditory area nuclei NCM or CM, such that the song complexity (could be related to the various spectral features of different syllables) stored in NCM as suggested by previous studies are serially organized by the temporal structure provided via the HVC-Av pathway. In such manner, a full memory of song would be represented by the synaptic connections revolving the sensorimotor nucleus Av. Considering Av's projection to the reward center VTA (the ventral tegmental area) in the zebra finch song plasticity circuits via the nucleus Aiv (the intermediate arcopallium), the full song

memory of the social model could be readily compared with the auditory feedback processed by the auditory regions during the bird's vocal practice. Mismatch of the bird's own developing vocal production with the song memory of the social model can be signified as 'errors' and fed into the reward center VTA. Subsequently, through VTA's input to the song plasticity circuits (Area X-LMAN) in the zebra finch song system, motor commands can be modified according to the error signals to perfect the bird's own vocal performance towards the song model.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♂
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memorize temporal and spectral properties of tutor song
practice song to imitate tutor
use song to attract mate
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Av
CM
NIf
NCM
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IMAN
Ov
Uva
Area X
auditory
DLM
motor/auditory
song

Figure 1 | Overview of song learning and neural circuits for song. (A) Male zebra finches learn their adult song during a developmental sensitive period that is comprised of a sensory learning phase (~20-60 days post-hatching (dph) when they memorize the temporal and spectral features of their tutors' song and a sensorimotor phase (~40-90 dph) when they use auditory feedback to guide changes in their singing behavior to vocally imitate the memorized song. At the bottom is a spectrogram of an adult zebra finch song illustrating the introductory notes (i) and the individual vocal elements in the song (A-F). Scale bar, 50ms. (B) Parasagittal schematic of auditory (blue) and song motor circuits (orange). The thalamic auditory nucleus ovoidalis (Ov) projects to the pallial region field L which then projects to secondary and tertiary auditory regions in the caudomedial nidopallium (NCM) and caudal mesopallium (CM). The premotor song nucleus HVC (proper name) receives projections from three sensorimotor regions, the thalamic nucleus Uvaeformis (Uva), nucleus interface of the nidopallium (NIf) - a pallial nucleus embedded in field L, and nucleus avalanche (Av) – a pallial nucleus embedded in CM. HVC has three output pathways, 1) a descending pre-motor projection onto the robust nucleus of the arcopallium (RA), which then projects onto brainstem respiratory and vocal motor neurons for the control of song, 2) a projection to the striato-pallidal basal ganglia nucleus Area X, which relays through the medial portion of the dorsolateral thalamus (DLM) and the lateral magnocellular nucleus of the anterior nidopallium (LMAN) and then onto RA, and 3) a projection onto Av.

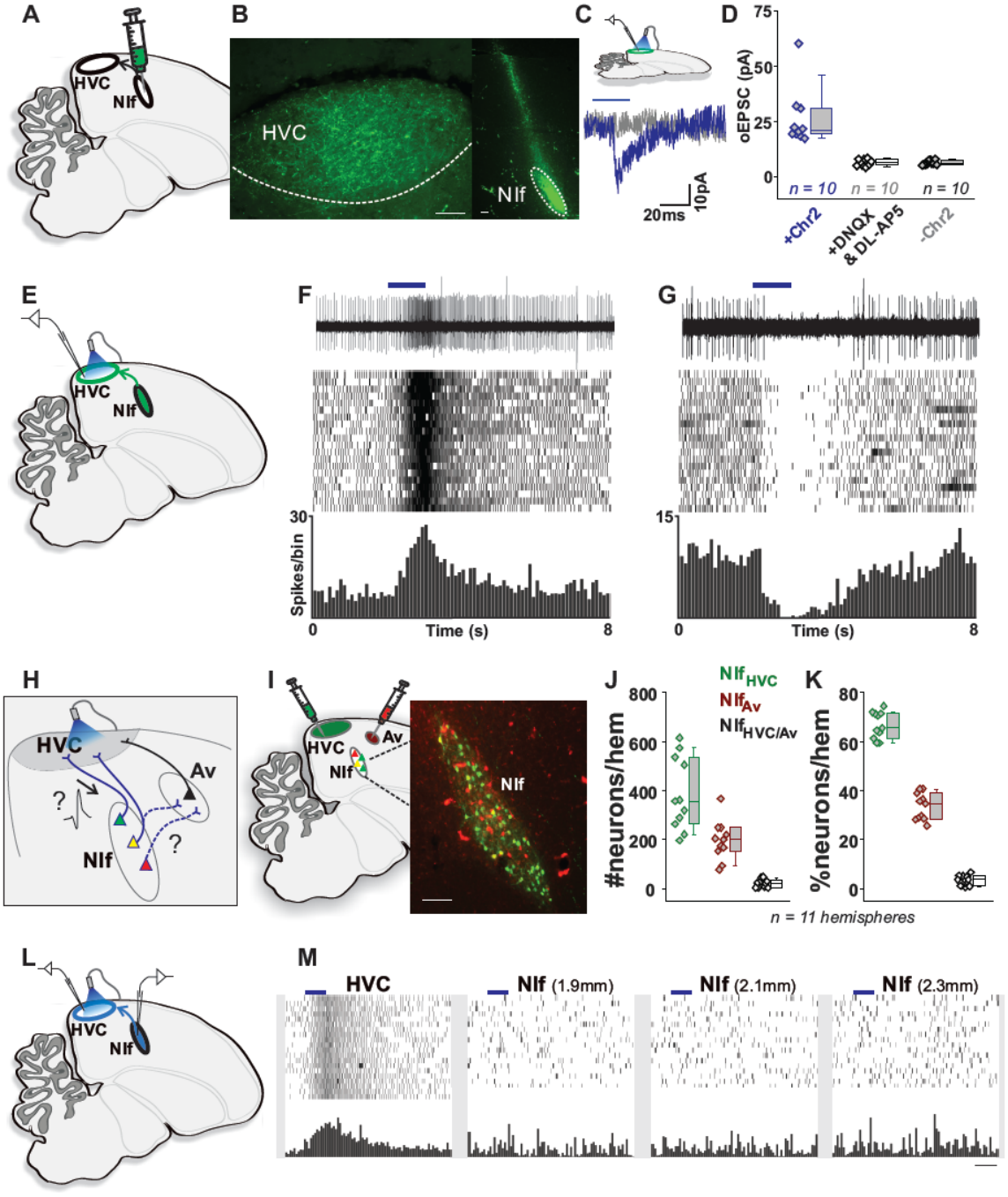

A
HVC
NIf
B
HVC
NIf
C
20ms
10pA
D
oEPSC (pA)
75
50
25
0
n = 10
n = 10
n = 10
+Chr2
+DNQX & DL-AP5
-Chr2
E
HVC
NIf
F
Spikes/bin
30
0
Time (s)
8
G
15
0
Time (s)
8
H
HVC
Av
NIf
?
?
I
HVC
Av
NIf
NIf
J
NIf HVC
NIf Av
NIf HVC/Av
#neurons/hem
800
600
400
200
0
K
%neurons/hem
80
60
40
20
0
n = 11 hemispheres
L
HVC
NIf
M
HVC
NIf (1.9mm)
NIf (2.1mm)
NIf (2.3mm)

Figure 2 | Selective manipulation of the NIf to HVC pathway. (A) Schematic of the viral injections of axon targeted Channelrhodopsin (scAAV-NX-hChR2-YFP) made into NIf. (B) Parasagittal section through HVC showing axon terminals labeled by tracer (Alexa Fluor 488) injected into NIf. Scale bar: 75um. (C) Schematic of slice recording in HVC. Bottom, example traces of light-evoked AMPA receptor mediated optogenetic excitatory postsynaptic currents (oEPSCs) recorded at -80mV (20ms, 1Hz pulse (blue trace)) from an HVC neuron compared to the same neuron when applying glutamate blockers (gray trace). (D) oEPSC recorded from HVC neurons in response to light stimulation (blue, gray fill) of NIf axon terminals. Monosynaptic responses are blocked by DNQX (20µm) and DL-AP5 (100µM) glutamate blockers (black, no fill) and non-existent in birds not injected with ChR2 in NIf (black, gray fill). (E) Schematic of *in vivo* multi-unit recordings in HVC used to assess light-evoked response. (F-G) Multi-unit recordings (top: representative single trials, middle: raster plots of 20 trials, bottom: histograms of 20 trials (100ms bins)) show both types of light response (left: excitatory, right: inhibitory) found in HVC. (H) Schematic illustrating two outstanding questions about NIf-Av-HVC circuitry: 1) whether individual NIf projection neurons send projections to HVC and Av; 2) whether light stimulation at NIf's axon terminals in HVC could antidromically drive activity in NIf. (I) Schematic of two different color tracer injections (Alexa 488 or 594) made into Av and HVC in order to retrogradely label Av- and HVC-projecting neurons in NIf (NIf_{Av} and NIf_{HVC}). Right, parasagittal section through the NIf showing that most labeled NIf projection neurons project to HVC and only a small percentage project to both Av and HVC. (Scale bar: 100um). (J-K)

Quantification of NIf neurons labeled by tracer injections ((J) actual numbers of neurons labeled, (K) percentages of neurons labeled)) (L) Schematic of *in vivo* recording in HVC and NIf used to test antidromic activity while light-stimulating HVC. (M) Raster plots of multi-unit light evoked response from HVC (leftmost) and activity from 3 sites at different depths in NIf in the same hemisphere while the HVC is being light-stimulated (100ms bins).

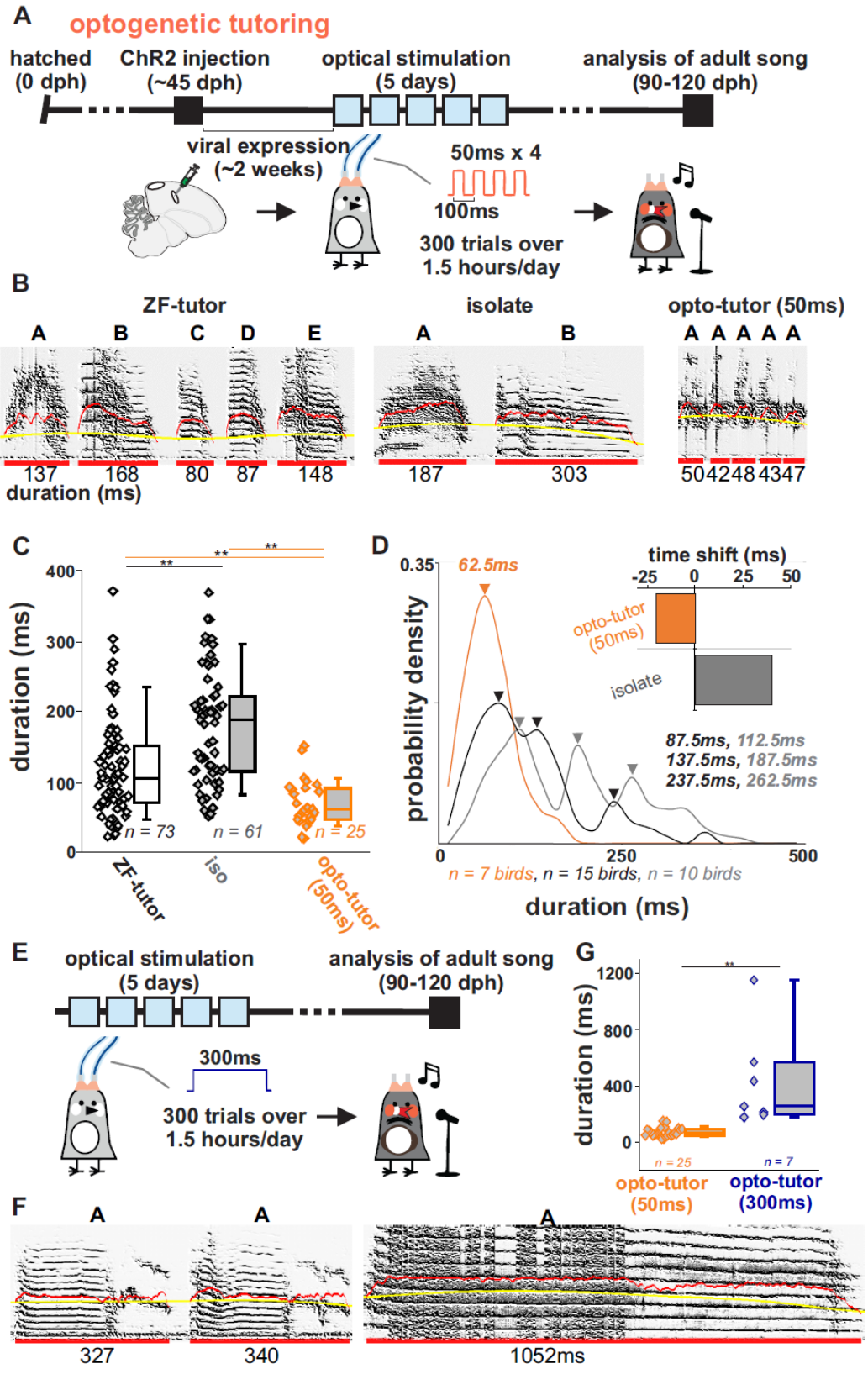

A
optogenetic tutoring
hatched (0 dph)
ChR2 injection (~45 dph)
optical stimulation (5 days)
analysis of adult song (90-120 dph)
viral expression (~2 weeks)
50ms x 4
100ms
300 trials over 1.5 hours/day
B
ZF-tutor
isolate
opto-tutor (50ms)
A B C D E
A B
A A A A A
137 168 80 87 148
187 303
504248 4347
duration (ms)
C
duration (ms)
400
300
200
100
0
n = 73
n = 61
n = 25
ZF-tutor
iso
opto-tutor (50ms)
D
probability density
0.35
62.5ms
time shift (ms)
-25 0 25 50
opto-tutor (50ms)
isolate
87.5ms, 112.5ms
137.5ms, 187.5ms
237.5ms, 262.5ms
0
250
500
n = 7 birds, n = 15 birds, n = 10 birds
duration (ms)
E
optical stimulation (5 days)
analysis of adult song (90-120 dph)
300ms
300 trials over 1.5 hours/day
G
duration (ms)
1200
800
400
0
n = 25
n = 7
opto-tutor (50ms)
opto-tutor (300ms)
F
A
A
A
327
340
1052ms

Figure 3 | Optogenetic tutoring affects learning of song temporal structure. (A) Timeline for the optogenetic tutoring experiment using 50ms light pulse. (B) Spectrograms of representative songs sung by a bird tutored by zebra finch tutor (ZF-tutor, left), a bird reared without a song tutor (isolate, middle), a bird opto-tutored by 50ms light pulse (50ms, right). Song elements were systematically segmented and quantified by thresholding (yellow curves) the amplitude (red curves) of sounds using SAP2011 (see methods). Red solid lines and numbers at the bottom show segmented song elements and their duration. (C) Quantification of song element duration of birds tutored by zebra finch tutor (ZF-tutor, n = 73 elements, 15 birds), isolate birds (iso, n = 61 elements, 10 birds), and birds who were optogenetically tutored using 50ms light pulse (opto-tutor, n = 25 elements, 7 birds). (D) Song element duration distribution of birds shown in (C). Arrows and numbers are showing the peak positions for each curve (black: ZF-tutor, grey: isolate) and the corresponding duration. Inset, time-shift to achieve maximum cross-correlation with normally reared birds are shorter for 50ms-stimulated birds (orange bar) and longer for isolate birds (grey bar). (E) Timeline for the optogenetic tutoring experiment using 300ms light pulses. (F) Spectrograms of representative songs of two birds opto-tutored with 300ms light pulses. (G) Song element durations of birds opto-tutored with 300ms light pulses are significantly longer than birds tutored with 50ms pulses of light.

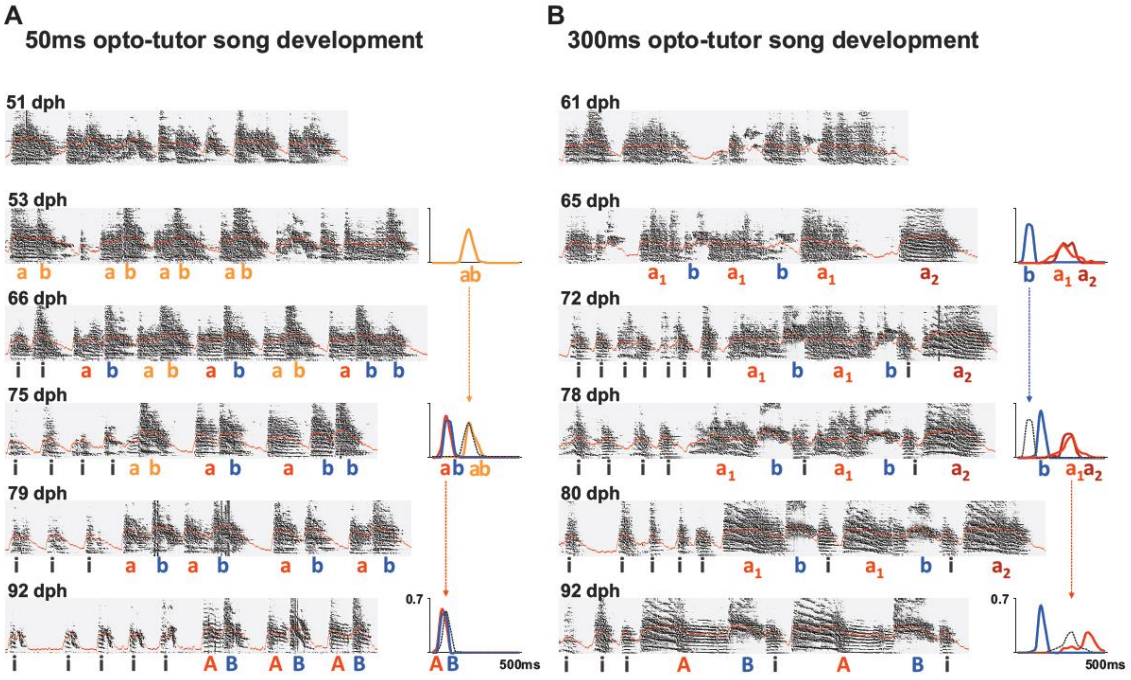

A
50ms opto-tutor song development
51 dph
53 dph
66 dph
75 dph
79 dph
92 dph
B
300ms opto-tutor song development
61 dph
65 dph
72 dph
78 dph
80 dph
92 dph
0.7
500ms

Figure 4 | Optogenetic tutoring implants a memory that guides song learning. Spectrograms of representative vocalizations produced by a 50ms opto-tutored bird (A, opto-tutored on 49-51 and 53-54 dph) and a 300ms opto-tutored bird (B, opto-tutored on 56-57 and 61-63 dph) on different days during song development. Capital letters denote song element types present in the adult songs and lower-case letters the putative precursors of each song element. Red curves on spectrogram show original sound amplitude without segmentation. Plots on the right of the spectrograms show the probability density of song element durations with different colors corresponding to different song elements labeled under the spectrogram. Dotted lines allow for comparison of song element duration from earlier in development. Both 50ms and 300ms birds started with noisy, long and unstructured subsongs with no recognizable element types. The 50ms bird developed the precursor song element ab (yellow) during the days he was opto-tutored. Over development this bird increased the amplitude fluctuations within ab, splitting this long duration vocalization into two shorter elements A and B. The 300ms began by singing noisy and variable versions of song elements A and B and increased the duration of these elements over development. He first increased the duration of song element B between 65 and 78 days and then lengthened the duration of song element A between 78 and 92 dph. The duration of song element A increased as the bird reduced the amplitude modulation of precursor song elements and incorporated a longer harmonic to the end of the song element. Regardless of singing short or long song elements, 50ms and 300ms opto-tutored birds produced introductory notes (i) typical of zebra finch songs prior to producing song bouts.

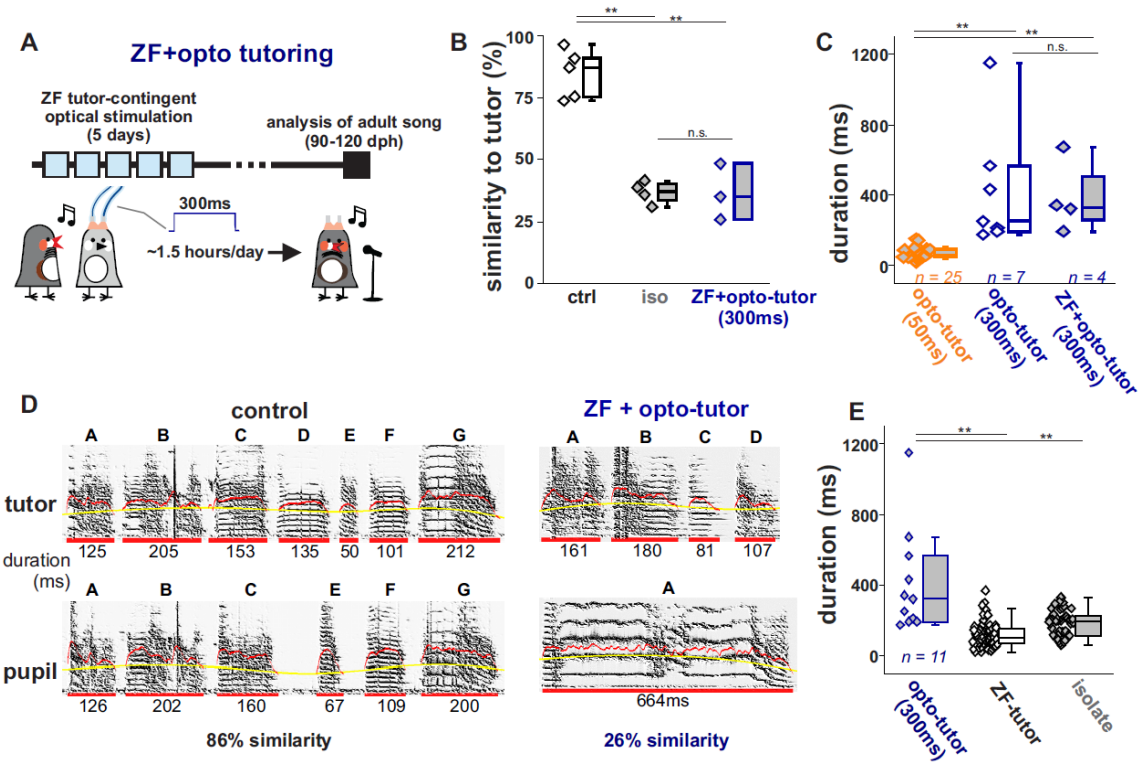

A
ZF+opto tutoring
ZF tutor-contingent optical stimulation (5 days)
analysis of adult song (90-120 dph)
300ms
~1.5 hours/day
B
similarity to tutor (%)
100
75
50
25
0
**
**
n.s.
ctrl
iso
ZF+opto-tutor (300ms)
C
duration (ms)
1200
800
400
0
**
**
n.s.
n = 25
n = 7
n = 4
opto-tutor (50ms)
opto-tutor (300ms)
ZF+opto-tutor (300ms)
D
control
ZF + opto-tutor
tutor
pupil
duration (ms)
A B C D E F G
125 205 153 135 50 101 212
161 180 81 107
126 202 160 67 109 200
664ms
86% similarity
26% similarity
E
duration (ms)
1200
800
400
0
**
**
n = 11
opto-tutor (300ms)
ZF-tutor
isolate

Figure 5 | Optogenetic tutoring overrides natural tutoring. (A) Timeline for the experiments in which birds received both zebra finch and optogenetic tutoring (ZF+opto tutoring). (B) Birds naturally tutored by zebra finch tutors (black, no fill) show high similarity to the tutor song. ZF+opto-tutored birds (blue, gray fill) do not imitate the song of their tutor and are on par with isolate birds (black, gray fill). (C) Song element duration shows no difference for ZF+opto-tutored birds (blue, no fill) and birds only receiving opto-tutoring (blue, gray fill). (D) Spectrograms of representative tutor-pupil songs of control birds (good copy, 86% similarity) and a ZF+opto tutoring pair using 300ms pulse (poor copy, 26% similarity). (E) Summary data of song element durations for birds opto-tutored with 300ms (blue, gray fill) and comparisons with song element durations from normally reared (black, no fill) and isolate birds (black, gray fill).

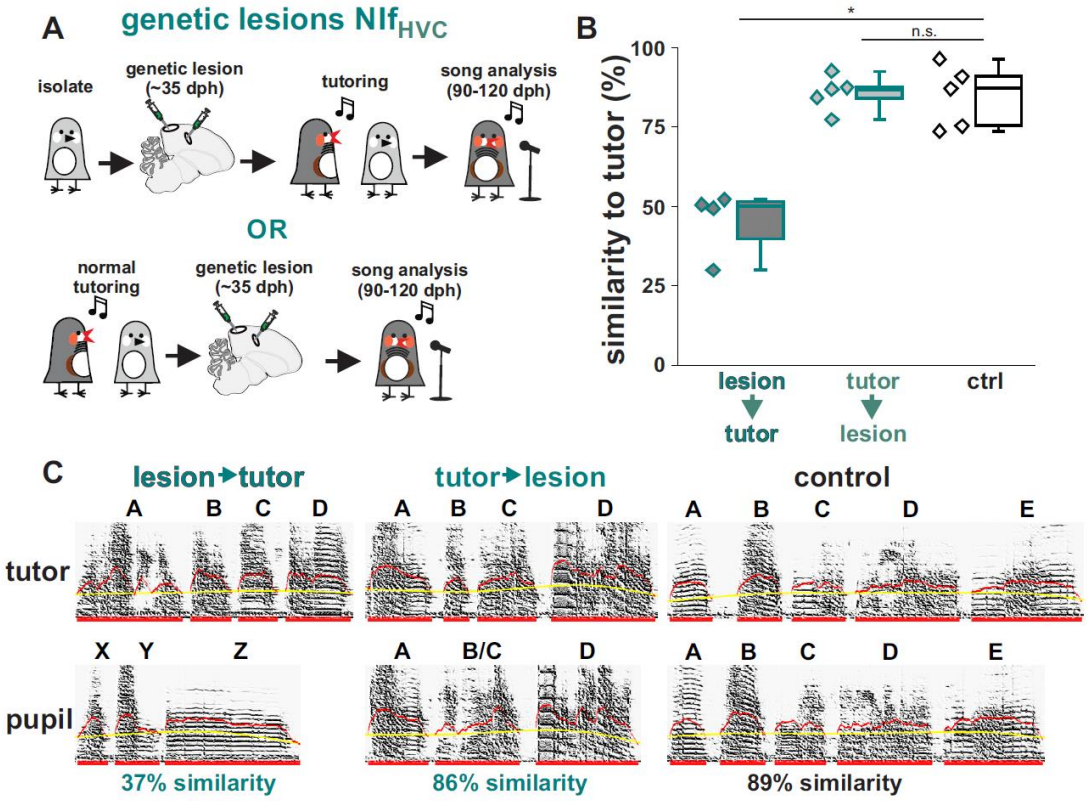

A
genetic lesions NIf$_{HVC}$
isolate
genetic lesion (~35 dph)
tutoring
song analysis (90-120 dph)
OR
normal tutoring
genetic lesion (~35 dph)
song analysis (90-120 dph)
B
*
n.s.
similarity to tutor (%)
100
75
50
25
0
lesion
tutor
ctrl
tutor
lesion
C
lesion→tutor
tutor→lesion
control
tutor
pupil
A B C D
A B C D
A B C D E
X Y Z
A B/C D
A B C D E
37% similarity
86% similarity
89% similarity

Figure 6 | NIf-HVC synapses are necessary for acquisition of a tutor song memory but not for vocal imitation. (A) Schematic of the NIf-HVC lesion experiments. Genetic lesions of NIf neurons projecting to HVC using viral expression of a cre-dependent caspase3 were performed either after birds had memorized the song of their tutor or prior to song tutoring. Song learning outcomes were examined when pupils reached adulthood. (B) Birds with NIf-HVC neurons lesioned before tutoring (lesion→tutor, green with dark gray fill) failed to copy the tutor songs. Birds tutored before having their NIf-HVC neurons lesioned (tutor→lesion, green with light gray fill) copied the tutor songs as well as un-lesioned birds (ctrl, black with no fill). (C) Spectrograms of representative tutor-pupil song comparisons shown in (B).

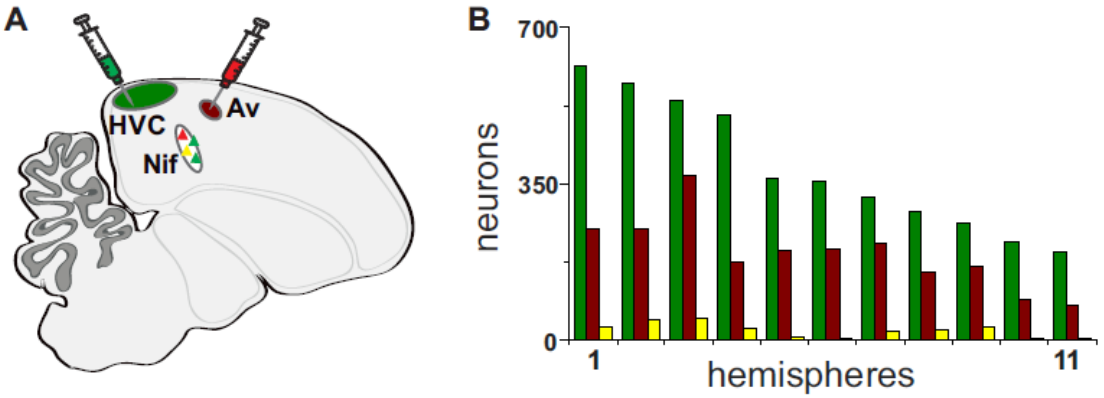

A
HVC
Av
Nif
B
700
350
0
neurons
1
11
hemispheres

Figure 7 | NIf_{Av} and NIf_{HVC} neurons are two largely non-overlapping populations. (A) Schematic of retrograde tracer injections made into HVC (green) and Av (red) in order to label projection neurons in NIf. (B) Cell count of NIf projection neurons labeled as shown in (A) from 11 hemispheres (green: labeled by tracer injected in HVC, red: labeled by tracer injected in Av, yellow: labeled by both).

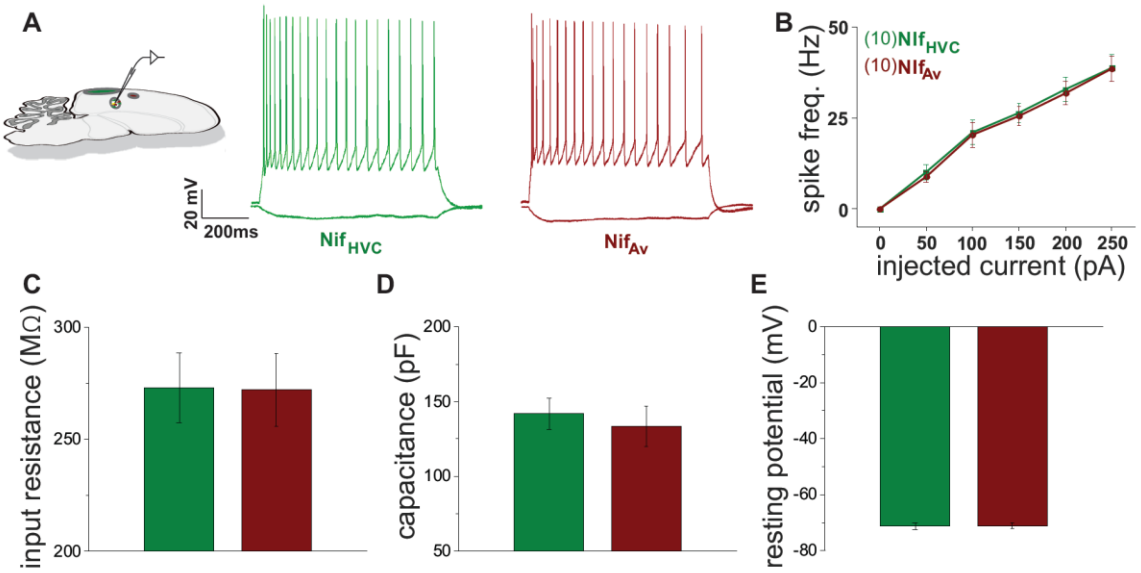

A
20 mV
200ms
Nif HVC
Nif Av
B
(10)NIf HVC
(10)NIf Av
spike freq. (Hz)
injected current (pA)
C
input resistance (MΩ)
D
capacitance (pF)
E
resting potential (mV)

Figure 8 | NIf_{Av} and NIf_{HVC} neurons do not exhibit significant difference in intrinsic properties. (A) Schematic of slice recording in HVC and representative responses of NIf_{HVC} (green) and NIf_{Av} (red) to current injection. (B-E) No significant difference was found between NIf_{Av} and NIf_{HVC} neurons in spike frequency, input resistance, capacitance, and resting potential.

timing for presentation of 'opto-motifs'

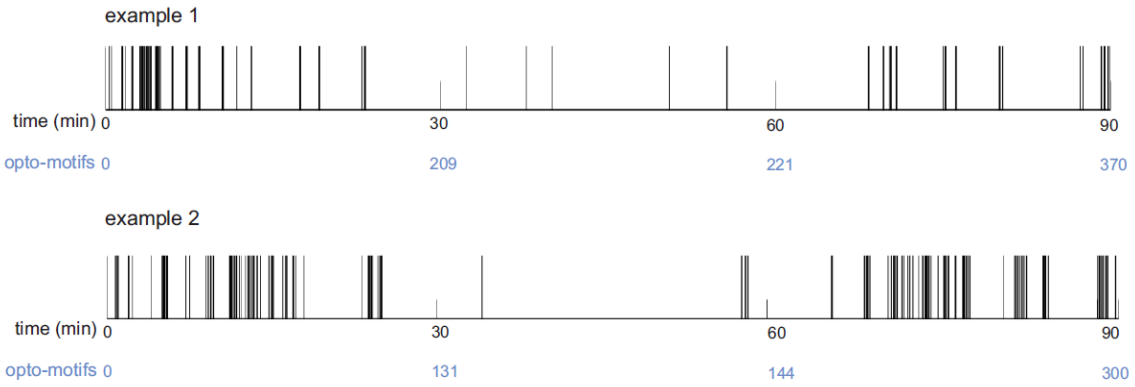
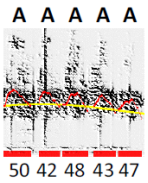
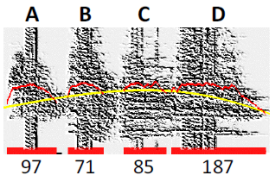
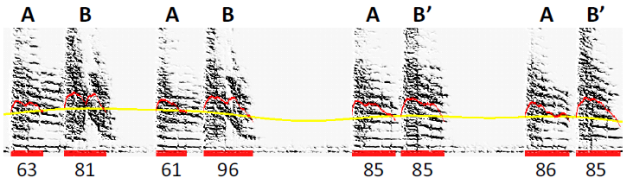
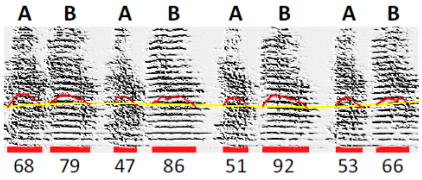


Figure 9 | Timing of optogenetic activation in behaving juvenile birds. Two examples of the timing of optogenetic stimulation in juvenile birds. Each vertical line represents the timing of a single optogenetic stimulation. The temporal organization of stimulations is based on the timing of song motifs produced by two birds as they actively tutored for 90 minutes. In example 1 the tutor sang 370 motifs over 90 minutes and in example 2 the tutor sang 300 song motifs over 90 minutes. Optogenetically tutored birds in our study were randomly assigned to be 'tutored' with the temporal patterns generated from one of these two tutors**.** Elapsed time (black) and the cumulative number motifs produced (blue) are listed on the x-axis.


A A A A A
50 42 48 43 47


A B C D
97 71 85 187


A B A B A B' A B'
63 81 61 96 85 85 86 85


A B A B A B A B
68 79 47 86 51 92 53 66

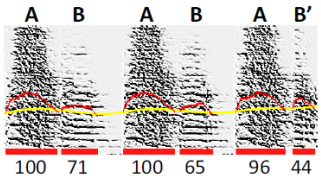
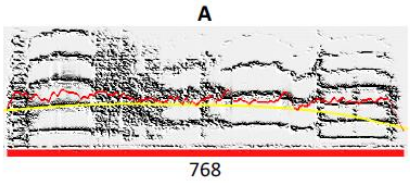
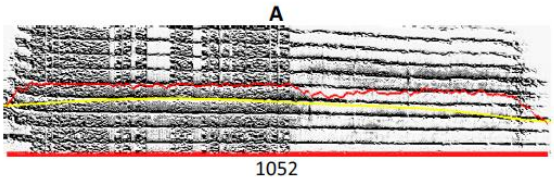
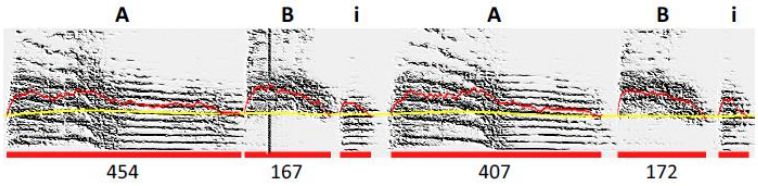
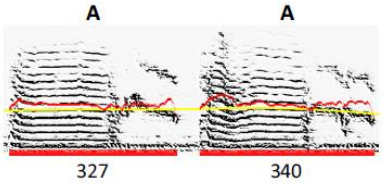

A B A B A B'
100 71 100 65 96 44

Figure 10 | Spectrograms of birds opto-tutored using 50ms light pulse. More examples of songs of birds opto-tutored using 50ms light pulse. Birds typically trill or sing songs with introductory note-like elements. Song elements were systematically segmented and quantified by thresholding (yellow curves) the amplitude (red curves) of sounds using SAP2011 (see methods). Red solid lines and numbers at the bottom show segmented song elements and their duration.


A
768


A
1052


A
B
i
A
B
i
454
167
407
172


A
A
327
340

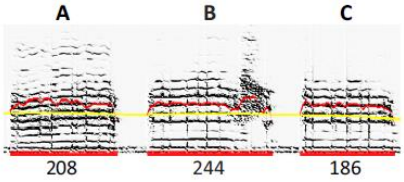

A
B
C
208
244
186

Figure 11 | Spectrograms of birds opto-tutored using 300ms light pulse. More examples of songs of birds opto-tutored using the 300ms light pulse. Birds typically sing songs with few but long, harmonic elements. Song elements were systematically segmented and quantified by thresholding (yellow curves) the amplitude (red curves) of sounds using SAP2011 (see methods). Red solid lines and numbers at the bottom show segmented song elements and their duration.

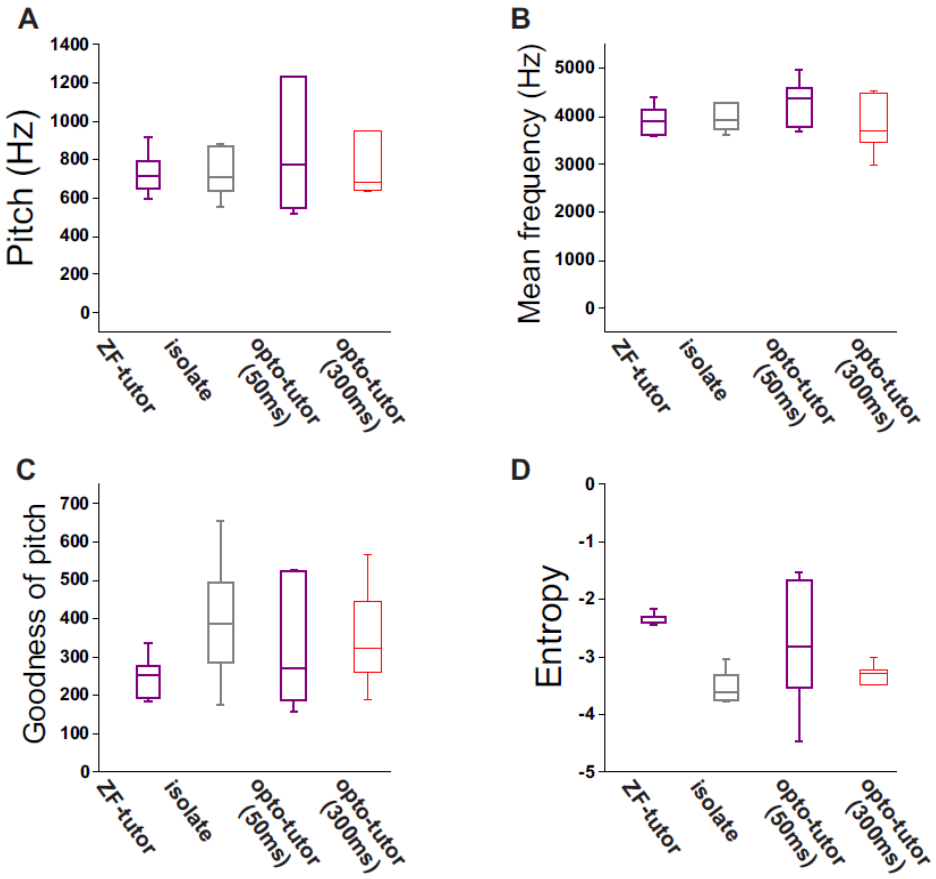

A
Pitch (Hz)
1400
1200
1000
800
600
400
200
0
ZF-tutor
isolate
opto-tutor (50ms)
opto-tutor (300ms)
B
Mean frequency (Hz)
5000
4000
3000
2000
1000
0
ZF-tutor
isolate
opto-tutor (50ms)
opto-tutor (300ms)
C
Goodness of pitch
700
600
500
400
300
200
100
0
ZF-tutor
isolate
opto-tutor (50ms)
opto-tutor (300ms)
D
Entropy
0
-1
-2
-3
-4
-5
ZF-tutor
isolate
opto-tutor (50ms)
opto-tutor (300ms)

Figure 12 | Optical tutoring at NIF-HVC synapses does not drive systematic changes in song spectral features. 4 acoustic features typical of zebra finch songs, pitch, mean frequency, goodness of pitch and entropy, were examined for opto-tutored birds compared with normally tutored birds (ZF-tutor) and isolate birds. No significant difference was seen for pitch, mean frequency, or goodness of pitch. However, normally tutored birds have a higher entropy ($p = 6.24e-04$, one-way ANOVA), a feature that represents the amount of information carried in the communication and is dependent on being transmitted from a social model. Entropy for normally tutored birds is higher than isolate ($p = 0.002$, Bonferroni test) and 300ms opto-tutored birds ($p = 0.004$, Bonferroni test), but not 50ms opto-tutored birds presumably due to the high variance of song element morphology of the trill or introductory note-like songs.

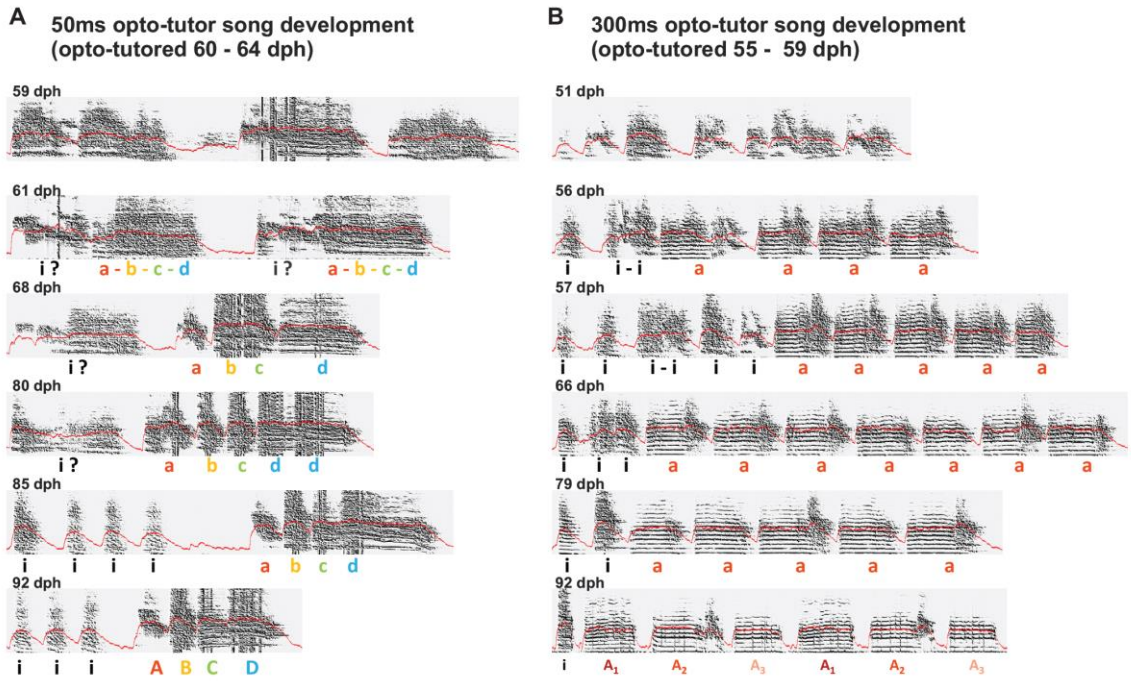

A
50ms opto-tutor song development
(opto-tutored 60 - 64 dph)
59 dph
61 dph
i ? a - b - c - d i ? a - b - c - d
68 dph
i ? a b c d
80 dph
i ? a b c d d
85 dph
i i i i a b c d
92 dph
i i i A B C D
B
300ms opto-tutor song development
(opto-tutored 55 - 59 dph)
51 dph
56 dph
i i - i a a a a
57 dph
i i i - i i i a a a a a
66 dph
i i i a a a a a a a
79 dph
i i a a a a a
92 dph
i A1 A2 A3 A1 A2 A3

Figure 13 | Song Development in Opto-Tutored Birds. Examples of 50ms and 300ms opto-tutored birds' song development. Capital letters denote song element types present in the adult songs and lower-case letters the putative precursors of each type. Red curves show original sound amplitude without segmentation. The bird tutored with 50ms light stimulus intensified the fluctuation of sound amplitude of the original noisy, long and unstructured subsong sounds. This resulted in the bird breaking a long vocalization into four different short types of song elements through development. Much of this occurred after opto-tutoring was finished. The bird tutor with 300ms light stimulus rapidly began to sing a harmonic song element during opto-tutoring. Over development this bird refined this harmonic into three separate harmonic sounds elements, with this the majority of this refinement occurring after opto-tutoring was finished. Both birds produced introductory song elements in addition to the elements that made up their song motif. In fact, 12/14 opto-tutored birds produced recognizable introductory elements in addition to their song motif. One opto-tutored sibling pair sang continuous yet soft and noisy introductory 'sounds' instead of separate 'notes', but nonetheless sang a clearly discernable song motif.

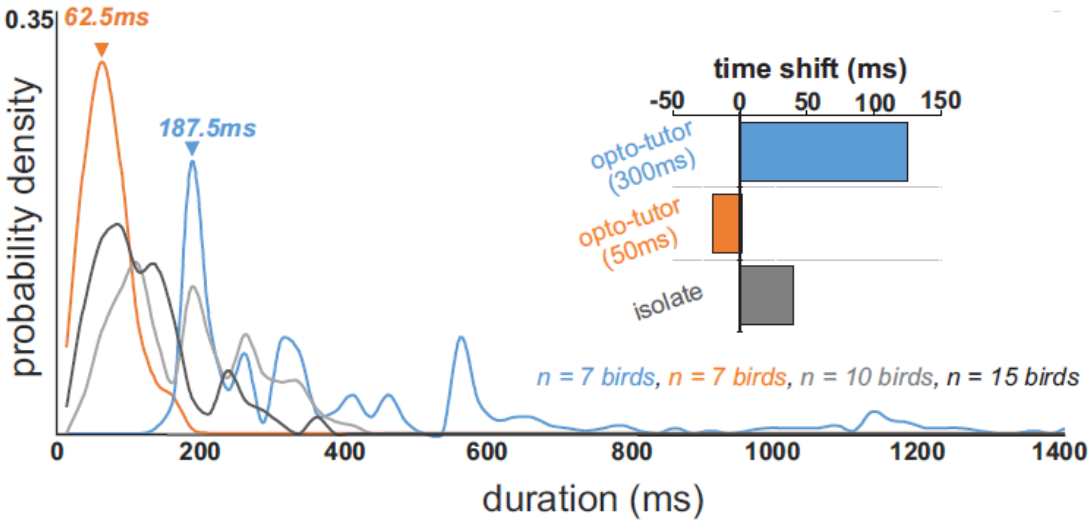

0.35
62.5ms
187.5ms
probability density
time shift (ms)
-50
0
50
100
150
opto-tutor (300ms)
opto-tutor (50ms)
isolate
n = 7 birds, n = 7 birds, n = 10 birds, n = 15 birds
0
200
400
600
800
1000
1200
1400
duration (ms)

Figure 14 | Comparison of song element duration distribution of opto-tutored birds to ZF-tutored and Isolate birds. Distribution shifts towards shorter duration for 50ms opto-tutored birds (orange), longer duration for 300ms opto-tutored birds (blue) compared to birds normally tutored by zebra finch tutors (black) and isolate birds (grey).

Table 1. Opto-tutored Bird Syllable Durations (ms)

	Mean Duration and SD for Each Syllable (ms)																													Average
50ms opto-tutored	20.4	22.2	37.7	40.8	45.0	45.5	47.0	47.2	50.6	51.6	54.5	56.9	59.2	62.3	64.6	69.9	83.0	86.9	90.4	91.9	93.1	96.8	101.4	105.7	145.3	152.0				70.1
SD	2.3	3.1	3.5	9.1	4.3	5.9	5.7	9.3	6.2	5.9	4.1	6.4	3.3	9.8	5.2	8.9	8.6	6.4	5.0	4.7	18.7	7.0	6.9	5.1	8.3	17.4				
300ms opto-tutored	176.1	194.2	194.4	213.4	253.0	323.5	342.9	434.3	568.1	674.2	1152.7																			411.5
SD	13.0	6.4	11.9	18.6	7.9	9.3	39.7	33.2	6.7	101.3	109.6																			

red = median duration

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