LATERAL INHIBITION IN ZEBRA FINCH AUDITORY PROCESSING USING A NOVEL APPARATUS FOR ELECTROPHYSIOLOGY

An Honors Thesis

Presented by

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Contents

ABSTRACT

Title: Lateral Inhibition in Zebra Finch Auditory Processing Using a Novel Apparatus for Electrophysiology

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The zebra finch (*Taeniopygia guttata*) provides an opportunity to investigate how complex auditory communication such as that in humans is processed in the brain at a cellular level, as opposed to region-wide resolutions afforded by fMRI. The songbird auditory cortex, known as the caudomedial nidopallium (NCM), shows neural selectivity for birdsong, and is involved in high-level processing of acoustic stimuli. Two putative neural subtypes in the NCM have been observed, known as broad- and narrow-spiking (BS and NS) cells due to the shape of their waveforms. NS cells have been hypothesized to be inhibitory interneurons while broad spiking cells may be excitatory projection neurons. Given this characterization, these neurons might be involved in sound discrimination using lateral inhibition. Understanding the role of BS and NS cells may shed light on the zebra finch brain, as well as the mammalian auditory cortex, in which broad and narrow cells have also been observed.

Unfortunately, exploring behavior and neural activity simultaneously presents technical difficulties, previously limiting investigations to either anesthetized electrophysiology or external observations of awake behavior. Portable and drivable electrode arrays (microdrives) enable unrestrained awake recordings, allowing for neural recordings to accompany behavioral observations. However, current devices are prohibitively expensive, and/or are too heavy for songbirds. We designed a light-weight, inexpensive microdrive with which we obtained high quality extracellular neural recordings in the NCM of anesthetized and awake zebra finches. These microdrives represent an inexpensive platform for electrophysiology in awake and anesthetized zebra finches, in addition to other small animals.

INTRODUCTION

Learned vocal communication

Vocal communication is crucial for successful social behavior in many animals, including humans. Much of the vocal communication found in the animal kingdom is innate, or unlearned, such as that of monkeys. Human language and songbird song represent two of a few examples of learned vocalizations in the animal kingdom. In humans, this capacity can fall victim to the pathologies of communication disorders, such as autism and specific language impairment (SLI), in which affected individuals have difficulty acquiring, producing, and comprehending language, making the phenomenon of learned vocal communication of interest to the scientific community (Haesler et al., 2007; Newbury et al., 2002). Songbirds share several aspects of learned vocal communication with humans and are thus a useful organism of study for investigating the basic biological mechanisms of learned vocal communication.

Songbirds are uniquely suited for studying vocal communication analogous to that of humans. One clear connection between humans and songbirds is their common expression of the FoxP2 gene, which is crucial for the development of proper vocal communication in both humans and songbirds. FoxP2 is a transcription factor responsible for the regulation of myriad genes across the body in both humans and songbirds (Fischer & Hammerschmidt, 2011). Its misexpression in humans leads to developmental verbal dyspraxia, in which affected individuals are unable to properly coordinate facial muscles when speaking (Haesler et al., 2007). FoxP2 expression in the zebra finch (T*aeniopygia guttatae*) has been linked with neural circuits for vocalization. During song acquisition, Area X, one striatal component of the songbird basal ganglia, recruits cells from the proliferative ventricular zone with high FoxP2 expression. As song crystallizes, the number of cells in Area X with high FoxP2 expression declines,

while low FoxP2 expression rises (Thompson et al., 2013). For birds with FoxP2-knockdown localized to Area X, song acquisition is severely impeded (Haesler et al., 2007).

The processes of learning language in humans and song in songbirds have a tutor-tutee dynamic, in which an adult, usually the father in the case of songbirds, produces template vocalizations for the young tutee to imitate (Immelman, 1969; Marler, Konishi, Lutjen, & Waser, 1973; Marler & Tamura, 1964). Whereas human language comprises phonemes, morphemes, syntax, semantics, and pragmatics, our understanding of songbird song is simpler. Fully mature song is composed of a variable number of preparatory stereotyped syllables, followed by an ordered sequence of different syllables, and terminates in another variably long sequence of stereotyped syllables.

The short, 120 day, song-learning period of zebra finches makes them a tractable system for studying song learning, audition, and production (Adkins-Regan, 2011), but also marks a distinction between songbirds and humans. Only male songbirds produce song, and reproductive success is dependent on song production. As such, male songbirds practice their song during and after development. Though their short learning period makes them attractive for large-scale studies of language acquisition, this feature of zebra finch life history distances them from humans, as humans take years to acquire language.

Song in zebra finches is inherited experientially and genetically. Like most songbirds, zebra finch pair bonds are often lifelong, with both parents participating in parental behavior (Tomaszycki, Banerjee, & Adkins-Regan, 2006). Fathers take on an unusual parenting role in that they provision offspring while also usually acting as song tutors to their offspring. Juvenile males end up developing song that closely resembles that of their tutor. However, each animal's song is unique, as the young zebra finch cannot exactly copy its tutor's song before its own song crystallizes. Genetic constraints on zebra finch song can be seen in that juveniles raised in isolation (i.e., without a tutor), spontaneously

produce song with similar frequency modulation but long or stuttered syllable timing (Feher, Wang, Saar, Mitra, & Tchernichovski, 2009). Additionally, cross-fostering juvenile zebra finches with Bengalese finches results in song with the temporal characteristics of zebra finch song and frequency modulation features of Bengalese finch song (Araki, Bandi, & Yazaki-Sugiyama, 2016). Thus, some subset of both temporal and frequency-related features of zebra finch song appear to be encoded genetically.

Zebra finches are well positioned as an organism of study for learned vocal communication. Zebra finch song parallels human language across several dimensions, but their brains are clearly organized quite differently from those of humans. Since such a different brain structure from that of humans can accommodate learned vocal communication, it is important to understand how it works.

Neurophysiology of zebra finch NCM in song production and audition.

The field has learned a great deal about the interconnectedness of the zebra finch vocal and auditory systems. However, study of songbird functional neuroanatomy is still in its relative infancy; many areas have been identified, but general principles of connectivity and function have yet to be established, especially in behaving animals.

Sexual dimorphism is prevalent in zebra finch neuroanatomy, and leads to the study of males and females for different reasons. Besides sex-specific partner preferences, there is an important behavioral difference between males and females: males are the only ones that learn and produce song, and display distinct neuropeptide staining from females in several motor regions including Area X (analogous to the basal ganglia) and the robust nucleus of the archistriatum (Bottjer, Roselinsky, & Tran, 1997).

Due to its function as a secondary auditory area and its regulation by steroid hormones, the caudomedial nidopallium (NCM) lies at the intersection of reproductive behavior and auditory cognition. The NCM is analogous to the mammalian auditory cortex, being a secondary auditory region which more sparsely encodes and processes higher-order song features in comparison to its main input, Field L. NCM is thought to be involved in memory formation, as song degrades when NCM is lesioned (Yanagihara & Yazaki-Sugiyama, 2016). Additionally, neurons within the NCM are endocrinologically active: they express aromatase, an enzyme that converts testosterone to estrogen, and expresses estrogen receptors, especially in its ventral portion (Krentzel, Macedo-Lima, Ikeda, & Remage-Healey, 2018). NCM estradiol levels are rapidly elevated in the presence of females (Remage-Healey, Maidment, & Schlinger, 2008). Since organization and activation of reproductive behavior is dependent on hormone levels across species (Adkins-Regan, 2011), it follows that a region involved in auditory processing, which is critical to reproductive success, is involved in steroid hormone signaling. These converging lines of evidence identify NCM as a promising region for examination for understanding how songbirds process song.

Distinct features of song may be differentially processed by different classes of auditory neurons. A genetic or intrinsic neural basis for unlearned conspecific song-specific selectivity in zebra finch auditory cognition is partially encoded in the NCM's main source of activation, Field L. In one study by Araki et al. (2016), Field L3 was recorded extracellularly during playback of whole songs and individual syllables. Researchers discerned two classes of neurons based on their firing patterns, high-firing and low-firing neurons, in which high-firing neurons were responsive to zebra finch temporal gaps.

Historically, the function of neural subtypes have been examined with respect to morphology, waveform shape, or pharmacology (Ghaderi, Marateb, & Safari, 2018; Markram et al., 2004). Patch clamp techniques can connect intrinsic firing properties with morphology *in vitro*. However, the "function" of a neural subtype directly relates to how it fires in a behavioral contexts; how it fires *in vivo*.

In the rare case where neural subtypes can be differentiated based on their action potential waveforms, we have the opportunity to study how different types of neurons fire during behavior.

The NCM might present one such opportunity. Broad- and narrow-spiking neurons in the NCM have been described several times in the literature (Ono, Okanoya, & Seki, 2016a; Schneider & Woolley, 2013; Yanagihara & Yazaki-Sugiyama, 2016). These neurons have been proposed to belong to different neural subtypes, although there has been no effort to identify them morphologically. Thus, any attempt to further characterize broad- and narrow-spiking neurons electrophysiologically is in some ways "blind" to their true nature. However, some insights can be gained into their role in the NCM through pure electrophysiology.

The NCM's representation of song is sparser than that of Field L, indicating that its neurons have feature selectivity. The contrast between the sparsity in stimulus representation in NCM versus Field L is a powerful motivator for studying NCM. A neuron in this region may change its firing rate in response to the appearance of a certain pitch, the onset of a stimulus, or even temporal characteristics like an ordering of syllables (Ono et al., 2016a). However, no neural subtypes have been identified in Field L based on spike shape. The pockets of this broad feature space around which NCM neurons modulate their activity are known as spectro-temporal receptive fields (STRFs). However, it is unknown how this selectivity arises.

Lateral inhibition

Inhibition is known for its role in the auditory system in computing interaural time differences (Fujita & Konishi, 1991). Another organizational principle using inhibition in the auditory cortex is known as lateral inhibition. Lateral inhibition is a network motif in the nervous system that can enhance the salience of a signal and is one possible mechanism for the emergence of STRFs in the songbird NCM

(Koyama & Pujala, 2018). Simply, lateral inhibition works by quieting the projection neurons around an activated neuron. The contrast between the activated neurons and non-activated neurons is enhanced by a layer of inactivated neurons. Mechanistically, lateral inhibition as a network motif usually takes the following modular form. A pattern of paired projection neurons and inhibitory interneurons maps the receptive field for a sensory modality, be it touch, sight, or hearing. A lateral inhibition network may spatially map tactile regions of the skin, perceived visual space, or a range of abstract auditory features such as pitch, amplitude, duration, or any number of other characteristics. When a projection neuron at a certain point on the sensory map fires, that neuron's inhibitory neuron fires too, directly inhibiting the surrounding projection neurons and other inhibitory neurons as well.

Ganglion cells in the retina illustrate how the structure and function of lateral inhibition networks connect quite well. When the retina takes in the image of a black and a white rectangle arranged horizontally next each other, it enhances the edge between the rectangles. Ganglion cells take advantage of laterally interconnected amacrine cells to dampen activity in neighboring ganglion cells. The differential activation of these cells is conveyed through the visual nerve to early visual processing areas where it is represented as contrast, and this contrast is interpreted as an edge (Barnes & Werblin, 1987). Many optical illusions take advantage of this effect.

Lateral inhibition is an established mechanism for pulling out features in auditory processing in mammalian auditory cortex (Moore & Wehr, 2013), and is thought to be present in the songbird caudomedial mesopallium (CMM), another secondary auditory cortical region (Meliza, Chi, & Margoliash, 2010).

Broad and narrow neurons in NCM

Broad and narrow neurons have been described to a limited extent in the literature, only being mentioned in a handful of publications in NCM. BS and NS neurons are commonly speculated to predict the excitatory or inhibitory identity of neurons, with BS neurons thought to correspond to excitatory neurons and NS neurons thought to correspond to inhibitory neurons (Meliza & Margoliash, 2012; Ono et al., 2016a; Schneider & Woolley, 2013). Since the study of these cells is still in its infancy, it is worthwhile to consider the approach each paper has taken to characterizing these cells.

The first paper to mention broad- and narrow-spiking neurons in zebra finches described them in the context of song signal extraction from a noisy environment (Schneider & Woolley, 2013). Broad-spiking (BS) neurons demonstrated more selectivity for specific elements of song than narrow-spiking (NS) neurons. A few important properties of NS and BS neurons emerged here, which would be confirmed later in future papers. Firstly, the selectivity of BS neurons could be decreased by increasing noise, inhibiting GABA signaling, and spacing out notes. Thus, BS firing selectivity is likely driven by GABA, and this GABA signaling is mediated physiologically by the quality of auditory signal. NS cells were mostly non-selective.

The second paper to mention BS and NS neurons investigated how selectivity of a fraction of BS neurons reflects learning of a tutor song in juvenile animals (Yanagihara & Yazaki-Sugiyama, 2016). They identified a selective and non-selective BS neuron population, as well as a NS population, and found that the population of selective BS neurons grew dramatically after tutoring. They also noticed a few properties of BS neurons independent of development: inhibition of GABA signaling decreases and sleep selectivity for selective BS neurons. It should be noted that at least 50% of all BS cells were non-selective throughout the learning process, and that nearly all NS neurons were unselective as well, and that these data were taken from awake-behaving juvenile males.

Ono, Okanoya, & Seki (2016b) discusses BS and NS neurons in the context of another songbird, the Bengalese finch (Ono, Okanoya, & Seki, 2016b). This paper highlighted the degree to which NCM processes auditory information at a higher level than Field L, its main input. For various deviance tasks, NCM showed higher deviance preference, an indication that unexpected elements in auditory stimuli led to increased activity. Additionally, this paper investigated latency, a crucial parameter of NS and BS firing. Depending on whether NS or BS neurons tend to fire before or after one another can help elucidate the structure of the putative lateral inhibition network that connects them. Unfortunately, the latency relationship between NS and BS neurons described here was not very strong. NS cells fired 81±73.7 ms after the beginning of a stimulus, and BS cells fired 98.0±76 ms after the beginning of a stimulus.

Classifying broad and narrow waveforms

One of the first papers to mention the existence of BS and NS neurons in European starlings (Meliza & Margoliash, 2012) used a unique method for classifying them: an unsupervised clustering algorithm called affinity propagation was deployed on the first two principle components of each unit's peak-aligned average waveform. In later papers, authors set thresholds for measuring spike width. Wooley & Schneider (2013) found NS neurons to be between 0.1 and 0.4 ms peak to peak (p2p), and BS neurons as being greater than 0.4 ms. Both Yanagihara & Yazaki-Sugiyama (2016) and Ono, Okanoya, & Seki (2016b) categorized BS neurons and NS neurons as above and below 0.350 ms p2p.

The technology for extracellular electrophysiology is always evolving, and it is possible that minor capacitances in electrodes can alter temporal properties of spike shapes. Therefore, each data set likely requires its own discretion with respect to the method for classification of BS and NS neuron waveforms.

An alternative way of discriminating between BS and NS neurons is to use genetic markers for inhibitory neurons to confirm that NS neurons are different genetic neural subtype from BS neurons (Moore & Wehr, 2013). Parvalbumin (PV) is a marker for inhibitory neurons expressed by a subset of inhibitory neurons in the brain (Markram et al., 2004). By driving channelrhodopsin-2 expression in PV-positive neurons, PV-positive neurons will fire in response to optogenetic stimulation and PV-negative neurons will not. This check could confirm that most BS neurons are excitatory (PV-negative) and that some NS neurons are PV-positive.

Microdrives

In behavioral neuroscience, there are a few dimensions along which experimentalists can maximize the value of their results: data throughput, versatility of methods (e.g., anesthetized, awake, *in vitro*), and cost. Since Hodgkin and Huxley's experiments with giant squid axons, electrophysiology has expanded to fit an immense number of experimental paradigms. The number of neurons that can be recorded at the same time has simultaneously grown to number in the thousands. Finally, the cost of each experimental subject's setup can become astronomical. Thus, an experimentalist would want to minimize the cost of recording devices.

Devices that allow for mobility while recording from a drivable array electrodes are called microdrives (Korshunov, 1995). Due to their chronic nature, microdrives can record from a single neuron for far longer than traditional acute setups, since the restraint involved in anesthetized or awake recordings prevents subjects from properly eating or sleeping. Many available microdrive designs are quite sophisticated, but are heavy, and as such are suited to primates and rodents, which are both relatively large and strong compared to zebra finches, and do not typically attempt to get airborne (Voigts, Siegle, Pritchett, & Moore, 2013). Our first prototypes were too similar to these traditional

designs and ended up preventing animals from maneuvering their heads properly. However, we successfully implanted and recorded from two animals with devices light enough to allow for proper head positioning, feeding, drinking, and flying. Additionally, commercially available microdrives are quite expensive, even when fabricated by hand. Our goal for this project was to develop a lightweight, low cost microdrive suited to deployment in zebra finches, which are on average about 15 g. Though they were originally designed for large animals, small and light designs for microdrives are feasible (Fee & Leonardo, 2001). Menardy et al. (2014) devised a fully wireless microdrive (Menardy, Giret, & Del Negro, 2014) that used radio to send analog electrophysiological data to an oscilloscope. Several high-quality microdrives on the market are highly expensive and quite delicate (Warm 16 Drive 8mm, Neuralynx, Boseman MT; Nano Drive, Ronal, PA), rendering it financially difficult to do studies in multiple animals. For the current project, we adapted a lightweight, low-cost microdrive design originally meant for rats for use in songbirds. Unlike Menardy et al., our design was not wireless.

Anesthesia adversely affects an animal's sensory and cognitive state, making awake behaving preparations the gold standard for validating neural activity. A powerful example of the distortive effect of anesthesia can be found in zebra finches: HVC's anesthetized neural preference for conspecific song (CON) switches to birds own song (BOS) in awake animals (Nick & Konishi, 2005). In European starlings, urethane anesthesia decreases the precision of spike timing in CMM, another secondary auditory area, compared to awake states (Meliza et al., 2010). However, the most important effect anesthesia has on NCM is on its capacity for song recognition memory. NCM's stimulus-specific adaptation is thought to reflect the formation of memories for song (Stripling, Volman, & Clayton, 1997; Yoder & Vicario, 2012), and this property is absent in anesthetized NCM. These findings challenge the field to reassess NCM's physiology in awake behaving animals, as much of the avian electrophysiology literature relies heavily on anesthesia.

Going forward, the validity of future experiments hinges on the availability of awake behaving setups such as those afforded by a chronically implanted microdrive. This emphasis on wakefulness is somewhat novel for recording from the NCM. Others have devised and used microdrives for zebra finches in the past (Menardy et al., 2012; Okubo, Mackevicius, & Fee, 2014; Sarah W. Bottjer, Andrew A. Ronald, 2018; Terleph, Mello, & Vicario, 2007; Yanagihara & Yazaki-Sugiyama, 2016) but either at higher cost per unit than our design (~$10 per unit) or have recorded from different brain regions. In zebra finches, the only awake behaving recordings that have been done in NCM has been in juveniles (Yanagihara & Yazaki-Sugiyama, 2016) and adult females (Menardy et al., 2012), and all others have been in anesthetized or restrained awake animals; the activity of NCM in awake behaving adult male zebra finches has not yet been explored. Therefore, NCM may have a similar preference switching to HVC, which is best investigated in an awake behaving setup. Microdrives present an avenue for investigating the NCM in a new way. The role of the NCM is nebulous at best, since much of work done to characterize it electrophysiologically and anatomically has been done in acute studies with restrained and anesthetized animals.

A secondary goal for the microdrive was to develop it in such a way that could be used beyond the current project. We wanted it to be flexible enough to be readily modified in the future, incorporating technologies such as microdialysis and optogenetics to accompany electrophysiology. The electrode shaft has enough space to fit an optical fiber and/or a microdialysis cannula.

Present study

The present study is divided in two parts: we hypothesize that BS and NS neurons provide the organizational structure for lateral inhibition, and to address this hypothesis, we set out to design a methodology for robustly investigating this question in the NCM of awake, behaving subjects. Recording

from the NCM in awake behaving animals has only been done in one study with two tetrodes, and never in adults (Yanagihara & Yazaki-Sugiyama, 2016). We adapted a design for a low-cost, lightweight, drivable array of chronically implanted electrodes, known as a microdrive, to suit songbirds.

Furthermore, we aim to use the spatial range provided by an array of tetrodes to test hypotheses about the presence of lateral inhibition. First, we expect broad and narrow neurons to cluster around peak to peak width and peak to peak symmetry as it does in the mammalian literature, suggesting two distinct population of excitatory and inhibitory cell types. Next, we expect broad neurons to be more selective for auditory stimuli than narrow neurons, in that broad neurons will fire more sparsely in response to auditory stimuli. This would support the notion that broad neurons are projection neurons that only relay pertinent information between brain regions. Finally, we expect broad and narrow neurons recorded on the same tetrode to have consistent cross-correlation patterns across tetrodes and across recording days, indicating that broad and narrow neurons might cluster in a repeating network motif.

MATERIALS AND METHODS

Microdrive fabrication

The procedure for assembling the microdrive body is taken from du Hoffman, Kim, and Nicola (2011), with some changes. Since the drive body was shortened, the length of the drive tube was shortened to 9/16 in. We found that the mount tube was not necessary for the overall function of the device and was dispensed with. Silica tubing (TSP Standard FS Tubing, 100µm ID, 164µm OD, BGB Analytik), which housed four individual tetrodes and one reference ground, was cut to a length slightly longer than the drive tube and epoxied in the drive tube (Figure 1).

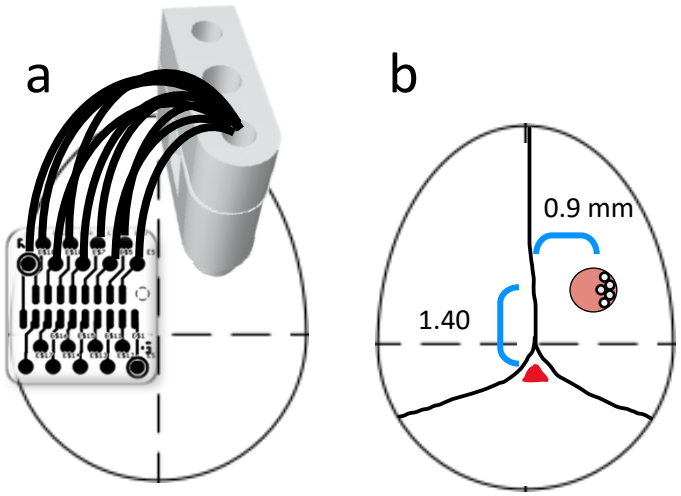

Figure 1. ***Orientation of microdrive and PI tube placement. a*** *Gross placement of the drive on the animal's skull.* ***b*** *Stereotaxic coordinates of NCM and placement of PI tubes in the drive tube.*

An 18 channel omnetics connector surface-mount part was soldered to the electrode interface board (EIB; Omnetics Part No. A79042-001). This soldered connection was reinforced with a layer of epoxy, making sure not to cover the holes.

Electrodes were spun from 12.5 µm diameter nichrome wire using a tetrode spinner from Open Ephys. A 5/16" x 5/16" piece of acrylic was epoxied to the side of the microdrive body to house the EIB (

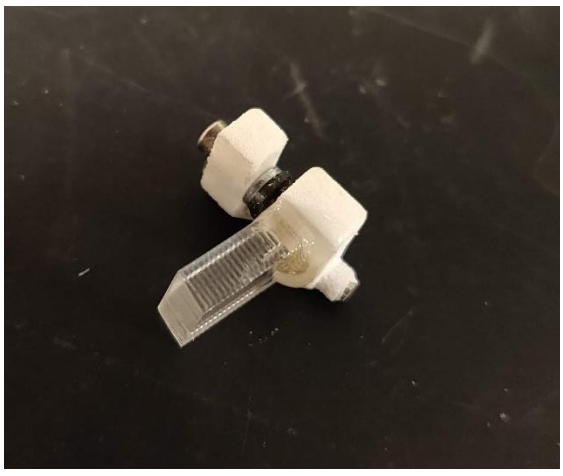

Figure 2) The EIB was affixed with blu-tack to the mount. Electrodes were pinned the EIB with small gold EIB pins from Neuralynx. Contrary to the recommended pinning technique, the EIBs were pinned from the same direction as the electrodes protruded.

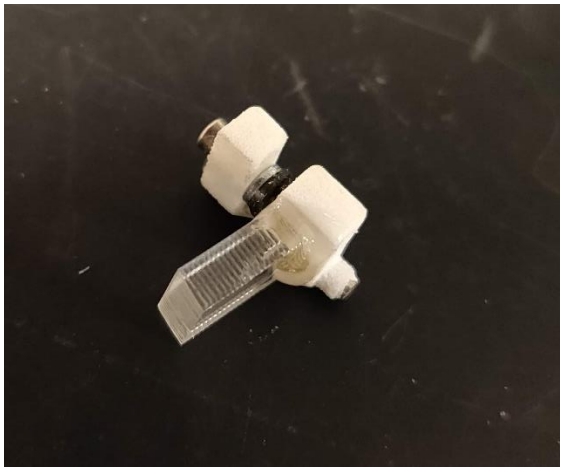

*Figure 2**. Attachment of the acrylic mount for the EIB to the side of the microdrive.***

Electrodes were glued to both ends of the PI tube with Loctite Super Glue Gel Control and cut to a length of 2.6 mm from the end of the PI tube. They were then gold-plated to the range of 200k Ohms to 300k Ohms.

A shield/ground screw splice was made with coated copper wire (Cooner wire PN: C21187). A ground screw (ABE part number: -002MSP188) was soldered to the deinsulated end of coated copper wire. A ½" by 2" piece of aluminum foil electromagnetic (EM) shield was soldered to the deinsulated end of another coated copper wire. The ground screw wire was shortened to a length of 1" and the EM shield wire was shortened to a length of ½". The wires were spliced at a ½", leaving another ½" free to be soldered into the EIB (Figure 3). Due to an error in circuit board design, the hole labeled "R" served as the ground and the hole labeled "G" served as the reference. The EIB and all wires were coated in liquid electrical tape.

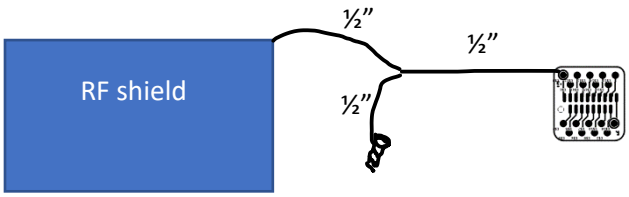

*Figure 3**. RF shield/ground screw splice.** The RF shield is made from aluminum foil and is soldered to the wire by applying flux and solder to a few millimeters of deinsulated wire resting in a valley fold of the aluminum foil.*

Finally, electrodes were coated with DiI. The 50 µL vial of DiI was centrifuged for 30 s. DiI was resuspended in 40 µL absolute ethanol. The vial was vortexed for 1 m. The contents of the vial were added to the cap of a small centrifuge tube vial, labeled, and stored at -20 °C. To coat the electrodes, they were simply dipped in the cap of the centrifuge tubes and allowed to dry for at least 10 min.

Microdrive implantation

Surgical procedures were taken from previously published methods (Pawlisch & Remage-Healey, 2015). Instead of equithesin, we used isofluorane for anesthesia. Birds were induced at 5% isofluorane and 2% O_2 and maintained at 2% isofluorane and 1% O_2. A small craniotomy was made ipsilateral to the NCM coordinates for the ground screw. The ground screw was placed inside of the craniotomy. Keeping away from the coordinates of the NCM, metabond was applied to the skull to prep it for dental cement and to seal the ground screw in place. A larger craniotomy was made at the stereotaxic coordinates for the NCM, +1.4 mm R/C and ±0.9 mm M/L relative to the midsagittal bifurcation (Figure 1b), and the dura resected. Next, the microdrive was lowered 1 mm into the brain, and Kwik-Cast was applied to the space between the microdrive collar and the brain surface. Dental cement was used to connect the microdrive to the skull. Another layer of dental cement was used to keep the EM shield wrapped around the microdrive.

Experimental setup

Animals were housed in a ~6 ¼” wide by ~7 ½” deep by ~7” high clear acrylic cage next to a female companion, with separate food bowls and water bottles for each animal. The acrylic cage was kept in a sound-attenuated chamber with a 12 hour light cycle. During experiments, the companion female was removed, except for trials which stipulated the presence of the female in the adjacent cage within the chamber. Three auxiliary data streams were time-locked with electrophysiological data: Stimulus timestamp, and stimulus identity, and microphone input (Adafruit Electret Microphone Amplifier- MAX4466 with Adjustable Gain, Part No. 1063) recording of the chamber (Figure 4).

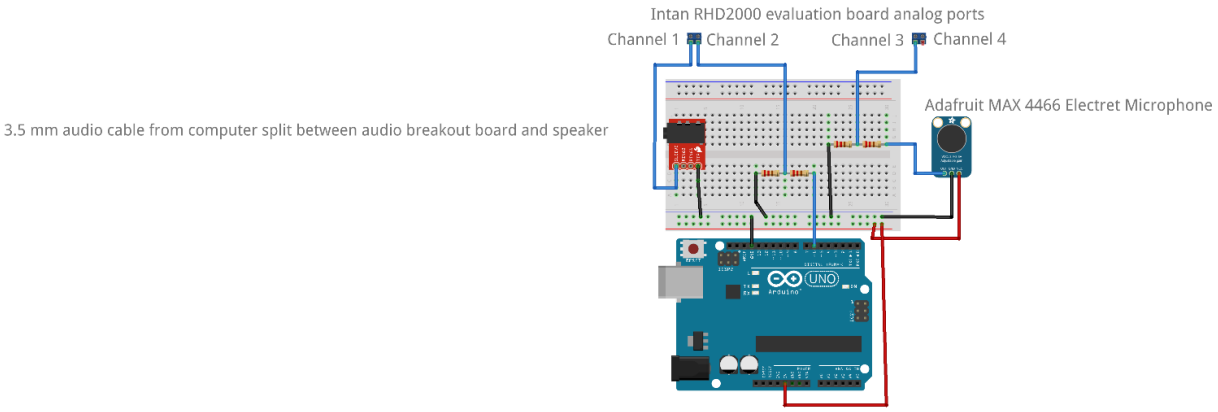

*Figure 4**. Breadboard setup for auxiliary data acquisition.** Image made with Fritzing software.*

Randomized trials, habituation trials (so-called for colloquial accessibility; there were no behavioral outputs of these experiments but there were neural outputs), and social experiments were three types of stimulus presentation experiments to address simple, unanswered questions about the firing properties of the NCM in an awake behaving animal. Randomized trials consisted of blocks of four stimuli, scrambled within each block. Habituation trials consisted of consecutive presentations of a single stimulus for each of the four stimuli, since this kind of consecutive presentation accelerates stimulus-specific adaptation. Stimulus-specific adaptation is the process by which neurons in sensory brain regions decrease their baseline-subtracted evoked firing rate for a specific stimulus logarithmically with the number of stimulus presentations. Stimulus-specific adaptation occurred in random trials too,

but the effect is stronger and faster in consecutively presented stimuli (Bäuerle, Behrens, Kössl, & Gaese, 2011; Ulanovsky, 2004). Auditory stimulus presentations were performed to record the responses of NCM neurons to auditory stimuli. Both randomized and habituation trials were performed to establish that stimulus-specific adaptation of the NCM despite the invasiveness of the microdrive, and to examine the firing properties of BS and NS neurons in awake behaving animals. Specifically, I extracted baseline-subtracted evoked firing rate and latency to fire from each recorded neuron using stimulus identity and timestamp information from random and habituation trials.

Social trials were performed to investigate the firing properties of neurons in the NCM in the presence of a female. Social experiments consisted of four sets of randomized trials, organized as follows: 1st set, no female, lights on; 2nd set, no female, lights off; 3rd set, with female, lights on, 4th set, with female, lights off. Additionally, social trials were originally designed to find an experimental configuration that would minimize movement and thereby minimize noise in the recording.

The microdrive was driven down in 80 µm and 160 µm increments until the probe reached a new site in the brain with enough actively firing neurons. After driving to a new depth, I waited at least 30 m before recording to allow the tissue to settle.

Experiments were carried out using a set of custom scripts in MATLAB which can be found at www.github.com/zeebie15/ephysSuite, in addition to Intan Technology's RHD2000 Evaluation Interface software. Spike sorting was initially carried out with wave_clus, a fully automated spike sorter, and was eventually ported to phy/Kilosort, which allows for more supervision by the experimenter. Figures were generated in MATLAB using a custom database platform which can be found at www.github.com/zeebie15/KS-analysis.

RESULTS

Microdrive development

I altered the design for a microdrive designed by Saleem Nicola's lab at the Albert Einstein College of Medicine, shortening it significantly so that the center of mass would not sit too high above the head and thus produce less torque on the head. This mechanism included an electrode shaft and simple drivability. From there, I added a custom-printed EIB, a simple PCB that interfaces wire tetrodes with the Intan data acquisition system.

The current microdrive design allows for the deployment of four tetrodes into the NCM, and can be moved up and down throughout the entire length of the region, meaning that multiple units can be independently acquired over the course of multiple days. To investigate the role of NS and BS neurons in auditory processing, our approach was to record from these implanted tetrodes while exposing the animal to multiple blocks of stimuli including BOS, BOS reversed (BOS-REV), CON, and white noise (WN). Then, we analyzed spike waveforms and categorize each unit as broad or narrow. We expected to see differential activation in response to auditory stimuli with respect to neuron type, with NS neurons having a higher basal firing rate than BS neurons. Then, we cross-correlated neuronal spike trains to find possible individual network motif units of lateral inhibition (BS/projection-NS/inhibition pairs). It is difficult to assign lateral inhibition to a region through pure electrophysiological experiments, so we expect that this study will provide the basis of more informed experiments to address questions around how auditory processing computations are performed in the brain.

The microdrive underwent several stages of development to fully address a few key design goals. To be deployed in an awake behaving setup, the microdrive had to be, first and foremost, lightweight. This constraint excluded many commercially available microdrives from the beginning, as they are often bulky. Additionally, the microdrive design had to be inexpensive and relatively easy to fabricate, because we wanted to incorporate it into multiple ongoing and future projects in the lab being spearheaded by investigators with differing skills.

Previously, the lab had only used single electrodes to perform *in vivo* recordings. When it became clear that handmade arrays of tetrodes had advantages in spike sorting over single electrode recordings, the microdrive design split into two separate devices: a microdrive; a drivable array of 4 tetrodes to be implanted chronically, and a larger static array of 8 tetrodes that could be lowered into the brain of an anesthetized or restrained animal using a micromanipulator for acute experiments.

Additionally, both arrays have the potential for integration with techniques such as retrodialysis, microdialysis, and optogenetics. Preliminary tests have confirmed the effectiveness of GABA agonist infusion by retrodialysis.

Prototype 1

Initially we set out to make a microdrive reminiscent of the flexDrive produced by Open Ephys (Voigts et al., 2013), which consists of an EIB mounted above a hollow body. I adapted an EIB design from Open Ephys in Autodesk EAGLE to accommodate a different, more compact body design (Figure 5a). For the body, I lightly adapted a microdrive design for rats by shortening the body (du Hoffmann, Kim, & Nicola, 2011). The microdrive sits on the head perpendicular to the skull surface and as such it acts as a lever that exerts torque on the skull surface. Shrinking the body shortened this lever and decreased torque applied to the skull (Figure 5b,c).

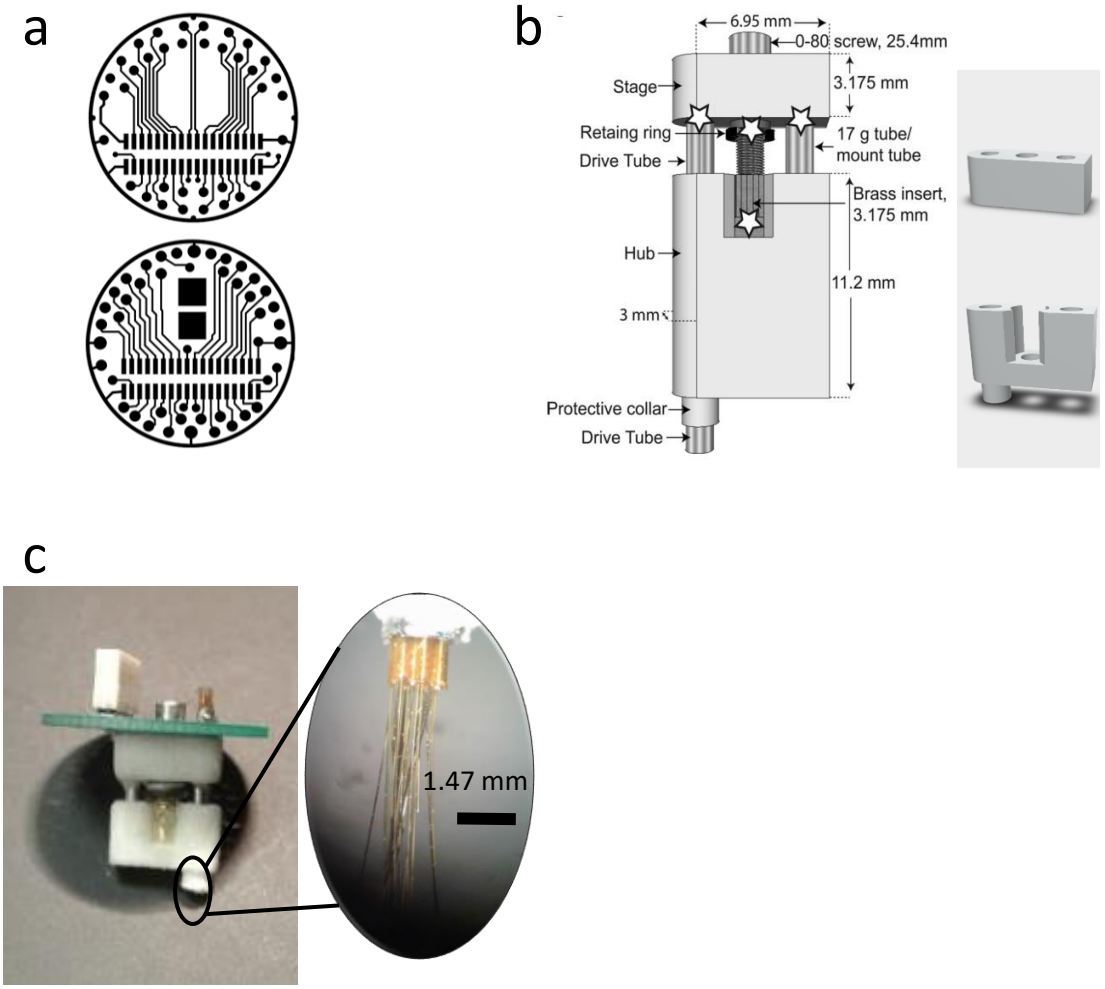

*Figure 5**. Comparison of original and modified microdrive EIBs and bodies. a** Top, the original EIB design from Open Ephys. Below, the modified version of the EIB. The black squares contain holes through which drive tubes and mount tubes can penetrate**. b** Left picture copied from du Hoffmann, J., Kim, J. J., & Nicola, S. M. (2011). On the right, the shortened design for the microdrive body. This change decreased torque applied on the skull. **c** Finished prototype for acute recording.*

Prototype 1 was first deployed in an anesthetized animal, using only single electrodes. Later iterations would incorporate stereotrodes (comprising two electrodes) and eventually tetrodes (comprising four electrodes). Stereotrodes and tetrodes provide a greater spatial range and allow for more accurate spike sorting. The wiring was insulated and grounded well enough that we successfully recorded multiunit activity (Figure 6a) from prototype 1, constituting a preliminary step towards making a microdrive that could be implanted in an awake behaving animal. Figure 6a shows multiunit activity on

one channel, and Figure 6b shows the two neurons extracted from this trace using the wav_clus algorithm.

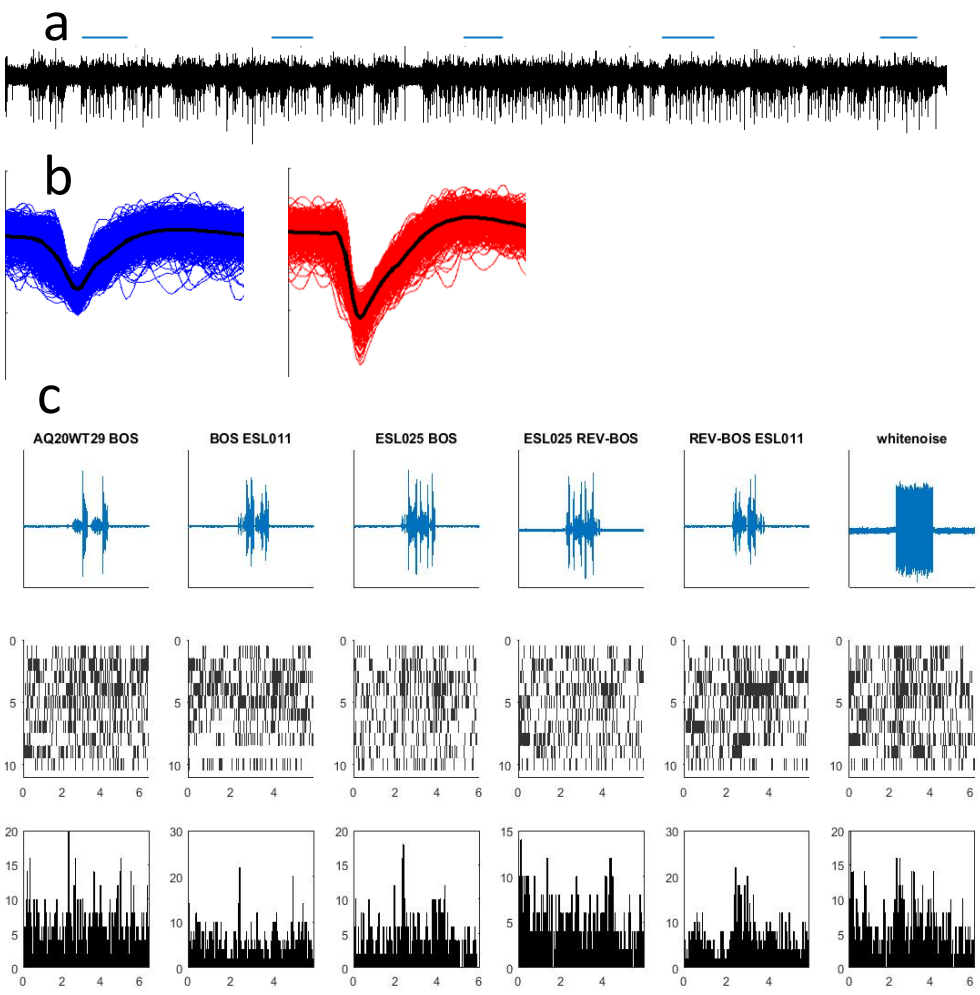

Figure 6. ***Recording from anesthetized zebra finch from prototype 1 of the microdrive. a*** *Raw trace with stimulus times.* ***b*** *Sorting from this raw trace using wav_clus. This channel yielded two units, one broad and one narrow.* ***c*** *PSTHs of each stimulus presented for one of the units in b. The unit showed some degree of stimulus-evoked activity even in the animal's anesthetized state and in spite of 32 electrodes penetrating the brain.*

The microdrive can be modified to integrate existing technologies without harming its capability for electrophysiology. Having successfully recorded from an anesthetized animal, we next attempted to infuse baclofen and muscimol, $GABA_B$ and $GABA_A$ receptor agonists, via retrodialysis. Indeed, spontaneous and evoked activity was fully inactivated following this treatment (Figure 7).

Even though torque on the skull was decreased by shortening the microdrive, the weight of the microdrive body and the EIB presented an issue when we finally implanted the microdrive chronically in a zebra finch. As a result, the animal recovered from surgery very slowly due to the weight on its head, which also prevented it from feeding effectively. The weight also made the bird's movements sluggish and devalued any recordings from the animal because it could not display normal behavior.

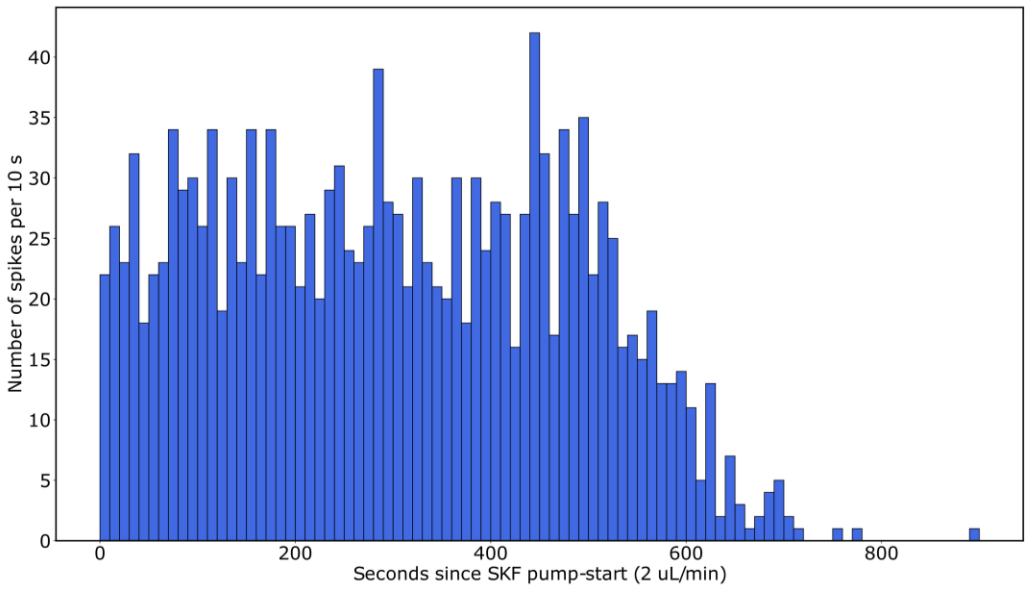

Figure 7. ***Effect of GABA receptors A and B agonists on NCM response to conspecific song.*** *1 mM baclofen ($GABA_B$ agonist) and 0.1 mM muscimol ($GABA_A$ agonist) were used to inactivate NCM. Infusion begins at 0 min and reaches the NCM at ~550 min.*

Prototype 2

For Prototype 1, since the EIB sat on top of the microdrive, it exerted more torque on the skull than it would if it were mounted directly on the skull. To alleviate the torque on the skull from prototype 1, the microdrive underwent a redesign, shrinking the EIB from 32 channels to 16 channels, and moving it from the top to the side of the drive body (Figure 8). This placement lowered the torque by lowering the center of weight, and the smaller form factor decreased the weight of the design. We obtained high signal-to-nosie (SNR) ratios in recordings from Prototype 2, allowing for robust analysis of waveforms

and spiking shape. As a result, the microdrive became a viable tool for awake behaving electrophysiology. Movement-induced artifact is always more prevalent in awake-behaving setups, but spike sorting with phy/Kilosort extracts single units throughout the recording.

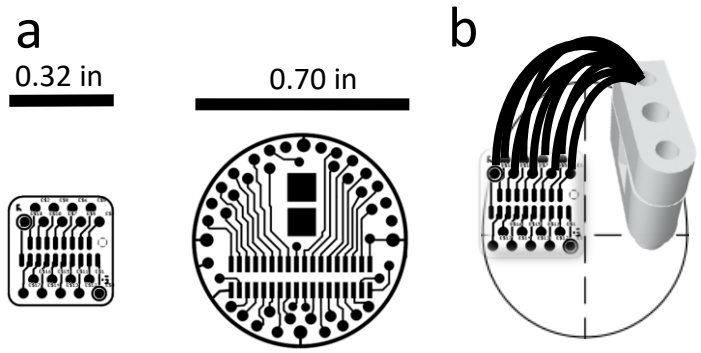

*Figure 8**. Comparison between prototypes 1 and 2. a** Left: new EIB design with 16 channels. Right: old EIB design with 32 channels. **b** New placement of the EIB on the skull as opposed to on top of the microdrive.*

Deployment of microdrive and identifying possible lateral inhibition in NCM

Three outcomes of the microdrive recordings would be consistent with the presence of lateral inhibition in NCM: first, BS and NS neurons should exhibit distinct waveform properties, second, BS neurons should have more selective STRFs than NS neurons, and third, BS and NS neurons should have a consistent pattern of latencies neural activity (Ostojic, Brunel, & Hakim, 2009; Schneider & Woolley, 2013; Yanagihara & Yazaki-Sugiyama, 2016).

In mammals and zebra finches, BS and NS neurons cluster in two dimensions: peak-to-peak spike width and peak-to-peak amplitude ratio (Moore & Wehr, 2013; Yanagihara & Yazaki-Sugiyama, 2016). These measures provide a moderately well-rounded picture of the overall waveform's shape, since neural spikes are relatively simple shapes. If two populations of neuron's waveforms are different, then they will likely form two separate clusters in a scatterplot of these two dimensions. The formation of these clusters would therefore imply the existence of two separate populations of neural subtypes. Neurons clustered clearly in these dimensions (N=2, n=121), which was confirmed by five passes of the

k-means clustering algorithm (Figure 9), which converged on the same final centroids from pseudorandomly seeded initial centroids on each pass. Therefore, we used a peak-to-peak width threshold of 0.43 ms to differentiate between BS and NS neurons for later analyses.

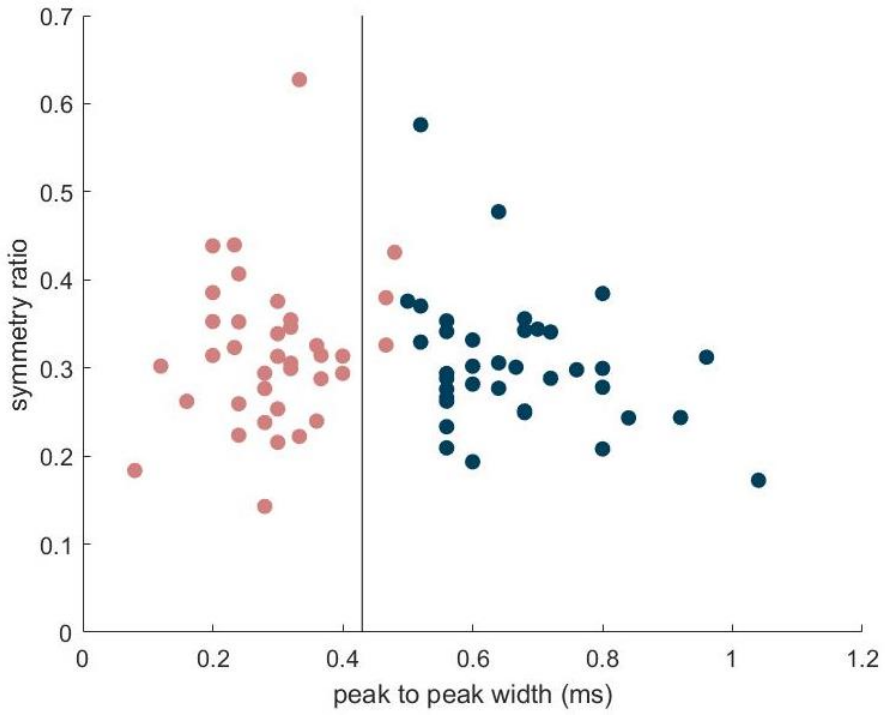

Figure 9. ***Clustering of NS and BS neurons with k-means algorithm and the decision boundary used in later analyses.*** *Based on this clustering, we used a peak to peak width threshold of 0.43 ms to classify neurons as broad- or narrow-spiking. The max sum of differences was 1.3851.*

I hypothesized BS neurons have more selective STRFs than BS neurons and found evidence supporting it. STRF selectivity can be measured in several ways (Meliza & Margoliash, 2012; Yanagihara & Yazaki-Sugiyama, 2016) using various statistical techniques. A simple way of evaluating selectivity is by evoked firing rates instead of selectivity itself. If a neuron has a high evoked firing rate in response to a stimulus (or in response to many stimuli), it can be said to have a non-selective STRF. Thus, I expected BS neurons to have lower evoked firing rates than NS neurons, because in a lateral inhibition network, inhibitory NS interneurons were would be more active than BS neurons to inhibit the surround. Evoked firing rate was measured relative to baseline firing rate, where the baseline firing rate was subtracted

from the evoked firing rate for each stimulus. The resulting differences were then averaged. BS neurons anecdotally appear to have lower firing rates than NS neurons (Figure 10).

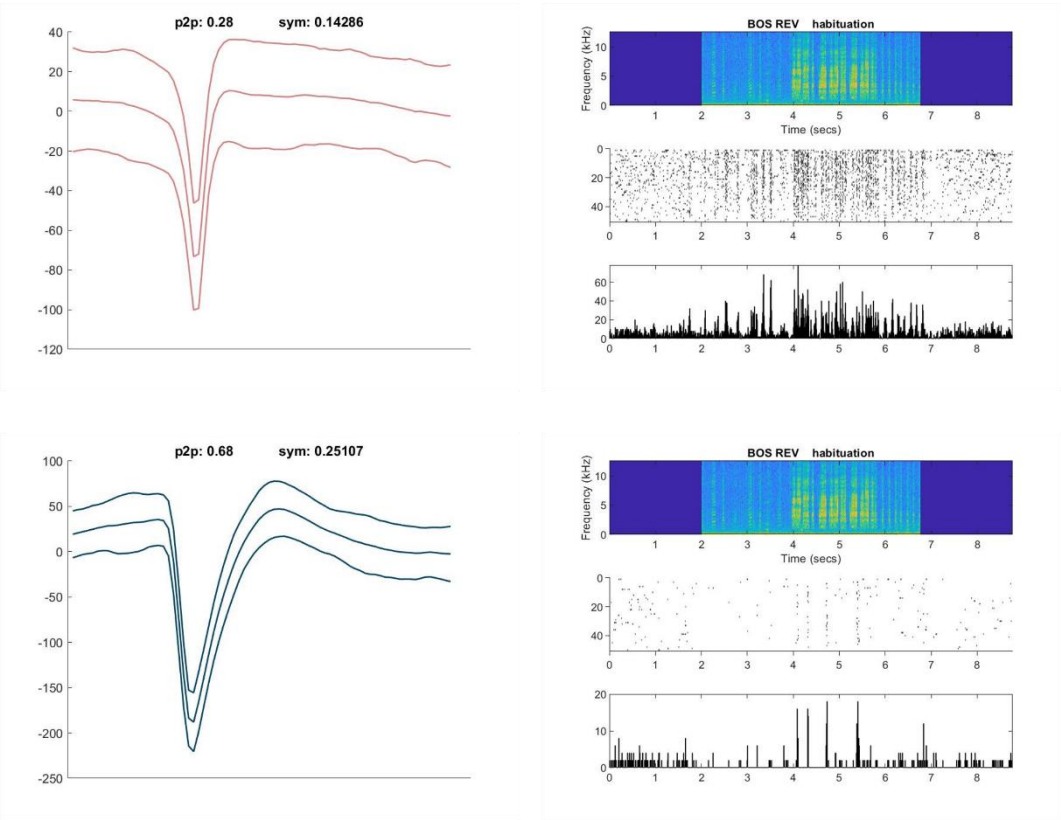

Figure 10. ***Anecdotal comparison of evoked spiking activity in BS and NS neurons.*** *Right: Narrow (top) and broad (bottom) waveforms with peak-to-peak width and symmetry ratio inset. Right: corresponding PSTHs of neurons' responses to bird's own song reversed. These figures were chosen as exemplar units from the same tetrode as that shown in Figure 13.*

BS and NS neurons are hypothesized to have differential selectivity to auditory stimuli (Figure 11) and they were found to display distinct levels of activation (BS: M=2.6737, SD=4.3723; NS: M=9.1506, SD=11.0066) in response to sound on average [t(80)=-3.7820, p=0.0002874]. Across four stimuli, BS neurons consistently had lower average evoked firing rates (BS BOS: M=2.7634, SD=4.5330; BS BOS REV: M=3.1572, SD=4.9761; BS CON: M=2.8419, SD=5.1119; BS WN: M=1.9354, SD=3.3010; NS BOS: M=9.1095 SD=11.7716; NS BOS REV: M=9.3440, SD=10.3298; NS CON: M=11.4836, SD=16.2175; NS WN: M=5.5081, SD=5.8381;). In stimulus-specific adaptation, NS neurons have a higher average

responsiveness to stimuli at each presentation of each stimulus compared to BS neurons and tend to attenuate their evoked firing rate less as the number of stimulus presentations increases [Figure 11; BOS: t(86)=3.6251, p<0.001; BOS REV: t(80)=3.5404, p<0.001; CON: t(80)=3.7001, p<0.001; WN: t(44)=2.6042, p=0.0125].

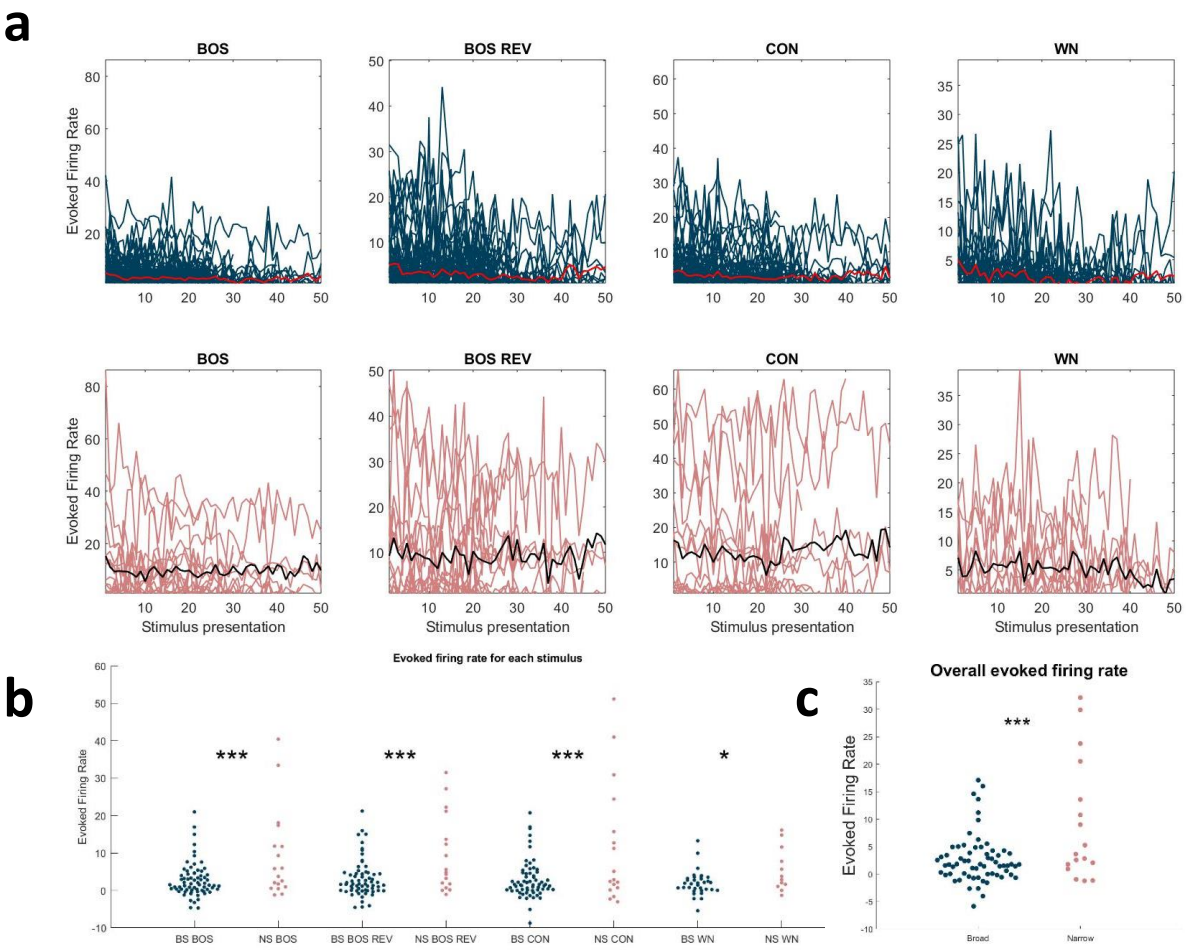

*Figure 11. S**timulus preferences in BS vs NS neurons. a** For each stimulus, each evoked firing rate at each stimulus presentation for each BS and NS neuron. Some neurons were presented with fewer than 50 stimuli. The average firing rate across all neurons at each stimulus presentation is shown by a red line for BS neurons and by a black line for NS neurons. **b** Average evoked firing rate of stimulus specific adaptation for all cells and all stimuli. Asterisks indicate significance in independent samples t-test. **c** Average firing rates for each stimulus in BS and NS neurons, averaged across stimulus type and stimulus presentation.*

Finally, NS and BS neurons were hypothesized to fire together in a pattern with each other, for which I found some evidence. Two measures were expected to reveal patterned firing between BS and

NS neurons. First, BS and NS neurons on the same tetrode were expected to be cross-correlated. Second, BS and NS neurons were expected to display some generalizable pattern of latency. A set of cross-correlation matrices such as that in Figure 12a were generated for all individual neurons on each tetrode for all recordings. Various pairings of cell types are represented by the coloring of each correlogram (Figure 12 inset). Figure 12b summarizes all matrices by reporting the timepoints of the maximum and minimum values in each correlogram across all matrices (all tetrodes). A maximum and minimum were taken from each side of each cross-correlogram. Thus, for each neuron, four data points were generated: a maximum and minimum on each side. These maxima and minima represent the expected latency between two neurons. Maxima of BS/BS neuron pairings produced expected values near 25 ms, whereas NS/NS neuron pairings produced expected values nearer to 0 ms. If these expected values approximate inter-spike intervals, then homogenous pairings (BS/BS and NS/NS) were consistent with the firing properties of BS and NS neurons (Figure 11). Maxima of heterogenous (BS/NS and NS/BS) neuron pairings were more varied. Minima for all neuron pairs on each side also stayed around 0 ms. Heterogenous pairings' maxima were more spread out on both sides.

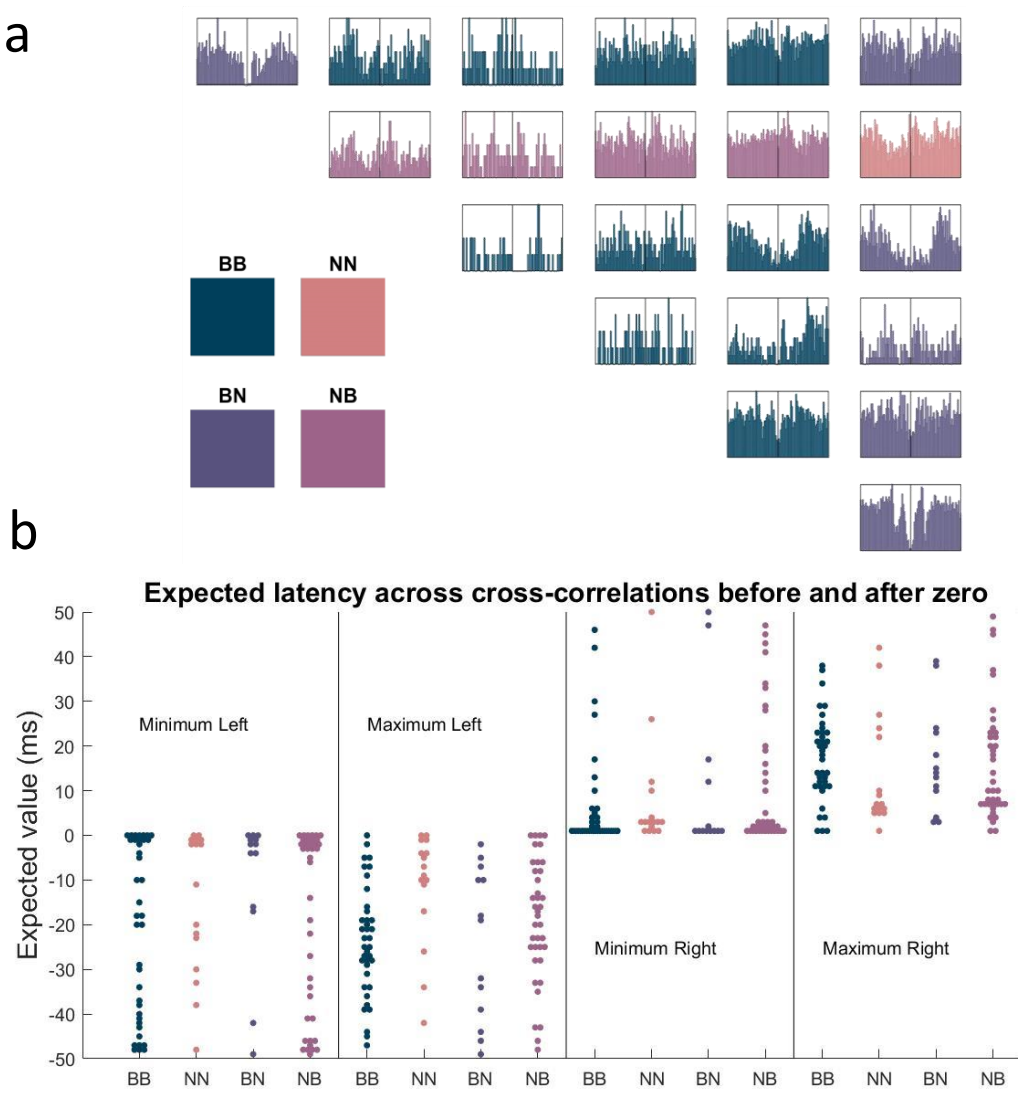

Figure 12 ***Cross correlation analysis shows latency patterns between broad and narrow neurons. a*** *An example cross-correlation matrix in which a column of neurons are cross-correlated with the same row of neurons. Though cross-correlation is non-commutative, less than half of the square is shown, as the inverse cross-correlation does not yield significantly different results. Units were filtered based on whether they crossed a threshold of 2 Hz baseline-subtracted stimulus-evoked firing rate. Inset: color code for cell type pairings.* ***b*** *Expected value of cross-correlation index peaks of all cross-correlograms.* ***c*** *Maximum and minimum peaks on the left and right sides of all cross-correlograms.*

NS and BS neurons were expected to have different latencies to fire after the onset of various stimuli. BS neurons fired after NS neurons when compared across all stimuli (Figure 13) and when averaged across all stimuli (Figure 13, inset).

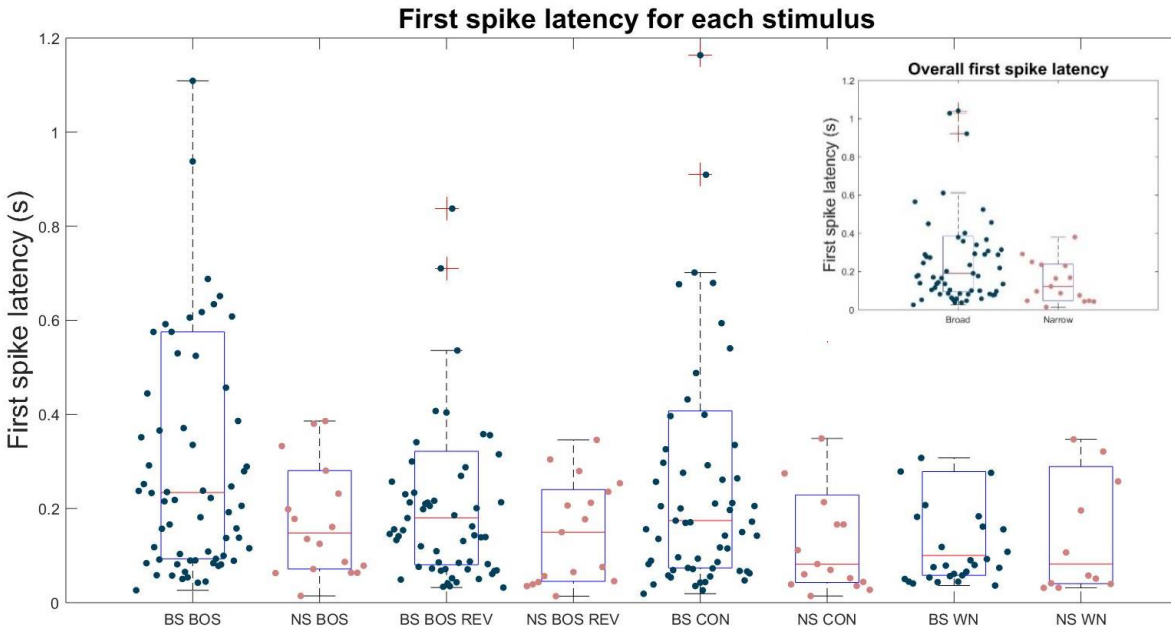

Figure 13. ***First spike latencies for each neuron across each stimulus.*** *Latency to fire between the onset of a stimulus and the first time a neuron fires. This graph excludes some outliers above 1.2 s. Inset: Latency to fire averaged across all stimuli.*

Is NCM really non-laminar?

Depth data were taken at each recording. These data are imprecise due to human error in surgical implantation of the microdrive and due to imprecise driving of the screw with a hex wrench. Nevertheless, there appears to be a lower number of neurons in the range of 1400 µm to 1600 µm below the surface of the brain inside the NCM than there are in areas above and below (Figure 14). The distribution of narrow and broad neurons remains consistent at all depths.

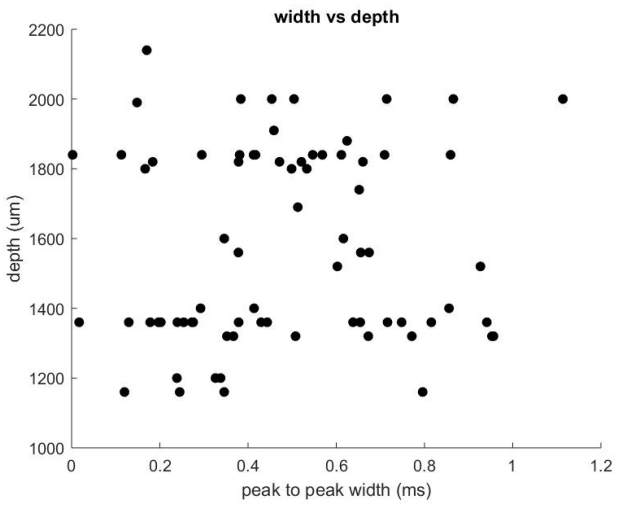

Figure 14. ***Width versus depth of neurons recorded in NCM.***

Discussion

Microdrive

Since the days of Hodgkin and Huxley's experiments in giant squid axons, the technology behind electrophysiology has exploded in power. Probes have grown from single electrodes to stereotrodes, to tetrodes, to current cutting-edge technologies sporting mesh arrays of hundreds or thousands of electrodes (Hong & Lieber, 2019). The first microdrive was invented in 1993 (Korshunov, 1995) for recording neurons during behavior in rats, and had a single electrode that could be driven up and down in the brain. Since then, microdrive and probe technology has progressed to the point of featuring arrays of independently drivable electrodes (Voigts et al., 2013).

However, a serious limitation to this approach has always been cost. As the technology progressed, the cost of adopting new technology has progressed as well, leading to inequities in science where only the most highly funded labs can adopt the newest technologies and limiting the questions less well-funded labs and institutions can ask. Alongside pushing the technological frontier forward, there is a complementary imperative to make near-cutting-edge technology open source.

A dearth of inexpensive, lightweight microdrive designs has presented a challenge in the songbird field. Open-sourced microdrives such as the flexDrive (Voigts et al., 2013) are too heavy for zebra finches, and commercially available microdrives are prohibitively expensive. Indeed, others have developed chronic implantations and microdrives for songbirds, but these designs were either not drivable or functionally extensible (allowing for a variable number of electrodes and for the inclusion of optic fibers and microdialysis probes). I adapted a design for a microdrive in rats (du Hoffmann et al., 2011) to make a drivable microdrive with an extensible design, allowing for multiple configurations (acute, chronic, 16-channel, 32-channel, etc) and for integration with optogenetics and microdialysis. It is compatible the Intan Technologies RHD2000 series evaluation board and software suite, and I wrote a set of scripts in MATLAB for sorting and storing electrophysiology data. The components of each microdrive cost less than $10, and a microdrive can be assembled in two to four days after an initial training period.

The value being able to record from myriad neurons from multiple sites in the brain is immediately obvious when compared to the labor-intensiveness of acute setups. However, the value of recording from hundreds of neurons compared to recording from five or ten must be carefully examined. Without careful analysis, analyzing spike train data from hundreds as opposed to a few neurons will likely introduce enough noise into the data set to obscure any real effects, since a greater number of neurons comes with a higher likelihood of including poor quality units. Another trade-off of having many as opposed to few neurons is in depth vs breadth of analysis: the fewer neurons, the more deeply tuned the analysis can be to the idiosyncrasies of each cell. My project errs on the side of many neurons and as such my analyses dive less deep.

In order to establish a region-wide pattern of connectivity such as lateral inhibition, I have decided that the price of a greater number of neurons is worth it. Since the putative network motif of

excitatory/inhibitory clusters are prevalent throughout the NCM, I needed to observe as many putative network motif units as possible to begin to draw conclusions about the region-wide network as a whole.

These drives were successfully deployed in two animals, recording with a high signal-to-noise ratio for months at a time. Units could not be kept for more than one day, since plugging the microdrive into the computer disturbs the tissue. Nonetheless, this device provides an opportunity merge behavior, electrophysiology, optogenetics, and even microdialysis in the context of songbird neuroscience, representing fertile ground for scientific inquiry.

This microdrive is a work in progress, and there are several design considerations that have yet to be realized. The current configuration has a significant drawback: vertical and rotational movement of the animal is restricted. Other microdrive systems have a pully and commutator, which allow for vertical and rotational movement, respectively. A pully contains a weight that counterbalances that of the bird, the microdrive, and the cables, allowing vertical movement. Such vertical movement involves overcoming inertia of both the microdrive and cables and the counterweight, which slightly slows the movement, but its functionality outweighs such costs.

I have tried to replicate a commutator setup from William Liberti (https://github.com/gardner-lab/FinchScope/wiki/Commutators) using an Arduino, a hall sensor, and magnets to drive a gear system to move with the animal. This proved to be infeasible, but others have used a simple passive commutator design using cheap commercial commutators. This is likely the approach I will take in the future.

Lateral inhibition

In this study, I developed a novel microdrive for data collection in songbirds and showed that it could be used to answer questions about the NCM in awake behaving animals. I decided to investigate whether the NCM uses a lateral inhibition network used to recognize stimuli in birdsong (tones,

syllables, or whole songs) comprising broad-spiking projection neurons and narrow-spiking interneurons. Excitatory and inhibitory neurons are thought to cluster in pairs in the NCM (Ikeda, Krentzel, Oliver, Scarpa, & Remage-Healey, 2017). This clustering implies that the NCM's architecture comprises repeating structural units known as a network motif (Koyama & Pujala, 2018). Lateral inhibition comprises one such motif, and this premise drove my analysis. If lateral inhibition was present in the NCM, I predicted the presence of three indicators.

My first predicted indicator was that there would be clear clustering of waveform shapes along width and symmetry. This clustering has been observed in the mammalian and avian literature (Moore & Wehr, 2013; Yanagihara & Yazaki-Sugiyama, 2016). Moore and Wehr (2013) demonstrate properties of lateral inhibition in the mammalian auditory system, as well as clustering of mammalian neurons in the auditory cortex based on these measures. They found symmetry to be a more powerful predictor of spike class than width. I was able to replicate findings from the literature to show clustering; however, this clustering (N=2, n=121) was better predicted by peak to peak width than symmetry (Figure 9), which follows from the avian literature (Yanagihara & Yazaki-Sugiyama, 2016).

My second hypothesis was that in order to support lateral inhibition BS neurons, which cluster with NS neurons, would have more selective STRFs than NS neurons. In a lateral inhibition network, I predicted that inhibitory interneurons would fire more frequently than broad-spiking neurons, which was confirmed by evoked firing rates (Figure 11a). BS and NS cells clearly exist independently of each other, having different waveform shapes and different firing rates. If lateral inhibition is present in these two subtypes, they are synaptically connected in some way.

Investigating the selectivity of STRFs helps distinguish BS and NS neurons as distinct subtypes, but to test whether NS and BS neurons are arranged in a network motif, I needed to see if they tend to

fire together. There are two analyses I used to investigate this possibility: cross-correlation (Ostojic et al., 2009) and latency to fire.

Cross-correlogram shapes can reveal excitatory and inhibitory connectivity between neurons (Ostojic et al., 2009), with dips indicating inhibition and peaks indicating excitation. Cross-correlation revealed hints about a possible difference between heterogenous and homogenous synaptic pairings (Figure 12). I extracted the index of the maximum and minimum of each side of each cross-correlogram. On either side, BS/BS pairings yielded maxima around 10 ms from the reference spike and NS/NS pairings yielded maxima around 5 ms from the reference spike, though there were enough outliers in both groups of heterogenous pairings to constitute a multiple clusters. Heterogenous pairings yielded maxima between 5 and 10 ms from the reference spike. While this latency signature is different from the homogenous pairings, it is also right between the homogenous pairings and may simply reflect an average between 5 and 10 ms.

Minima did not reveal any clear insights into inhibition between neural subtypes because this analysis was impacted by imperfect spike sorting (Figure 12). When cells are correlated with themselves, they create a symmetrical bimodal cross-correlogram, which are well-evidenced in the cross-correlation matrices (Figure 12a). Thus, some of the cross-correlograms may not reflect inhibition with minima, but imperfectly separated units. This impure sorting likely led to a skewing of the minima values in Figure 12 toward zero.

To further examine the firing properties of NS and BS neurons relative to each other, I computed a latency to fire, which is the time interval between the start of a stimulus and the first spike of a neuron. Neurons were not expected to fire in response to the start of the stimulus, but if BS and NS neurons are synaptically connected in clusters, there should be a repeatable pattern of latencies in which one subtype responds slightly faster than the other. The average latency to fire of NS neurons was

lower than that of BS neurons regardless of stimulus (Figure 13). This finding implies that in heterogenous clusters, NS neurons receive afferent information first, which upon first inspection is not consistent with the canonical lateral inhibition motif: excitatory neurons are innervated, which in turn innervate inhibitory neurons to inhibit surrounding neurons. In the case of the NCM, BS neurons should fire first. Though NS and BS neurons seem to fire together (Figure 12), the specific ordering of this firing may invalidate the possibility of lateral inhibition in NCM.

Further analysis is required to fully invalidate my third hypothesis. Firstly, a larger sample size is necessary to draw strong conclusions about BS and NS neurons, as I only analyzed 70 BS and 18 NS neurons for most analyses. Cross-correlations in particular would benefit from a larger sample size. Additionally, raw latency to fire (Figure 13) does not necessarily capture relative firing latencies between neuron pairs on individual electrodes. Latency to fire on the level of individual tetrodes would more powerfully compare NS and BS neurons' relative firing patterns.

The NCM must not only contain circuitry for lateral inhibition, so the ratio of BS and NS neurons should not be even, nor should NCM recordings always reflect to a 1 BS -to- 1 NS neuron pattern. BS neuron would be expected to be accompanied by a NS neuron on the same electrode given Ikeda et al. 2017, yet this was not observed during spike sorting. If lateral inhibition exists in this region, some or most neurons are expected not to participate in it. Thus, the lower latency to fire of NS neurons need not completely invalidate my overall hypothesis. There were significantly fewer NS neurons in this analysis than BS neurons, and a larger sample size will be needed to make any sort of confirmation or rejection.

Hypothesis generation

This project was driven by methods rather than hypotheses, per se. The experimental design of sending auditory stimuli was not tuned particularly to the lateral inhibition question, it was instead

designed to test the methodology and to generate new hypotheses. Thus, research questions have serendipitously emerged from testing the microdrive.

Anecdotal evidence for stratification of the NCM in terms of cell density emerged (Figure 14). However, there is no evidence for this stratification from anatomical studies, but further deployment of the microdrive while keeping track of approximate depth will reveal in time any underlying patterns of neural activity in awake animals.

The addition of a microphone to the setup enables the investigation of NCM activity during the production of song. Extracellular recordings have not yet been taken during song production in awake behaving adult zebra finches. The microdrive allows for this highly specialized behavior despite invasive extracellular electrophysiology, which awake-restrained electrophysiology setups for birds cannot produce.

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