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**How Neural Activity Shapes Decision Making:
An Optogenetic Investigation of the Neural Basis of Mate Choice in Female Songbirds**

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Physiology, Spanish

Senior Honors Project

With Dr. Jonathan F. Prather

May 5, 2017

Abstract

The intent of this project is to gain new insight into the neural circuitry that underlies decision making through understanding the connection between sensory perception and motor action. This investigation further seeks to identify whether optogenetic manipulation of specific neurons results in a change in mate choice. In defining this connection, the end goal is to combat deleterious decision making behavior in humans, such as drug addiction. Female Bengalese finches (BFs) provide an accessible model in which to investigate the neural mechanisms of behavior. Previous studies reveal that activity in the caudal portion of the mesopallium (CM) in the female BF brain may play an important role in mate preference, and we hypothesize that increased activity in CM will increase female preference for male song. Through viral-mediated expression of light-sensitive channels within CM, we can stimulate action potentials within the region. After optogenetic manipulation, we expected that increased activity in CM through light stimulation would result in increased preference for songs with which the light was paired. We used a well-established protocol¹⁰ to identify each female's baseline mate preference and compared it with each individual's preference post-surgery and after optogenetic manipulation. Results identify CM as a major contributor to the decision making pathway that underlies mate preference and bring to light novel projections from CM which may also play a role. Once this pathway is fully mapped, parallels may be drawn in the human nervous system, and these pathways may be targeted with specific pharmaceutical therapies to treat behavioral anomalies.

Introduction

Where should we go to dinner? Do you want pancakes or waffles? Do I need to bring a coat? Human beings make countless decisions every day. Some sources say even over 35,000 conscious choices face adults during their waking hours—not even including the numerous decisions we make with little to no thought or as a force of habit ²⁰. Whether we make a snap decision, or act out of habit, or spend hours weighing pros and cons, the decision making process is the same. The environment constantly bombards our sensory system with information, which then needs to be integrated and evaluated, and ultimately acted upon—one outcome is selected out of a multitude of possibilities. For instance, if a parking spot is open close to the door of a building, but it is marked “Reserved,” we must analyze the associated risks and benefits of choosing to park there before executing an action. Will a person risk receiving a parking ticket in light of the potential benefit of a close spot? A person may also need to navigate ethical dilemmas as well—who is the spot reserved for? Will parking there cause harm to another individual? Decisions like this are essential to daily functioning, behavior, and future plans, and the results of these daily decisions impact human interaction with each other and our environment. On an individual level, decisions may be categorized as advantageous or deleterious. The choice to dedicate significant time to studying before an exam, or commit to a new workout routine results in additive, positive effects. Conversely, the outcome of a deleterious decision may be undesirable, dangerous, or harmful, as in the case of the parking lot, where a ticket would be issued or the individual would meet the individual for whom the parking space was reserved, and then be forced to act further. More significant consequences loom as a result of poor decision making, such as drug addiction, financial repercussions, or irreparable damage to relationships.

Copious factors influence decisions: previous experience, pressure or support from peers, dedication to a goal or dream, personality, mood, influence of controlled substances, urgency, need, or simple desire, among many others. Each individual utilizes a unique toolbox of sorts to evaluate the options present before them, assign value to each factor which may influence an outcome, and finally come to a decision to act. A mechanistic understanding of this decision making pathway would be very beneficial in developing new therapies to combat pathologies resulting from negative decisions. Once identified, specific regions within the pathway could be targeted therapeutically to alter or prevent deleterious or harmful decision making processes. Currently, our understanding lies solely within the scope of how sensory signals are assessed for meaning and how motor action, as a result of the signal assessment, is initiated. What is not known is how perception and action initiation are linked. If we are to advance our understanding of sensory-guided decision making, it is imperative to define the bridge that connects sensory perception and evaluation to motor activation. It is logical to first study this connection and define it in an animal model, so as to gain insight to the activity of individual neurons and to work with a more simply organized nervous system in comparison to humans.

Use of songbirds

Female songbirds are an excellent animal model for studies that can eventually provide insight into human neuroanatomy and neural processing. First, Bengalese finches thrive in a laboratory setting: our set-up allows individual birds ample space for daily activities, and with the installation of a free-flight colony, we created an environment in which finches could experience essentially normal functioning. Additionally, we can explore the avian nervous system with greater ease than the human nervous system, as it is more simply organized. Finches possess structures that are

analogous to structures in the human brain, allowing comparison of the structure and function of those analogous sites. All our experimental procedures were approved by the University of Wyoming Animal Care and Use Committee in compliance with state and federal regulations regarding care and use of songbirds.

Bengalese finches display a readily identifiable behavioral model of decision making: mate choice. The decision to pursue a specific individual is a fundamental decision to species proliferation and survival and, in the case of female Bengalese finches, requires evaluation of and differentiation between stimuli received from multiple potential mates. Current understanding acknowledges that female Bengalese finches acquire initial preference for male song through early life experience, and use this to guide their preferences in later life^{19,21}. They express that preference by soliciting copulation directly and/or by calling many times in response to hearing the song that



Figure 1. Female white-crowned sparrow demonstrating a CSD²⁵.

they prefer^{10, 30}. Copulation solicitation displays (CSDs) are considered the “gold standard” of female preference for a certain male (Figure 1). However, CSDs are relatively uncommon in a laboratory setting, and research in our lab has demonstrated that calls are an accurate representation of an individual’s preference. A

previous study by our group compared the number of calls performed in response to each song versus the number of times that the female solicited copulation in response to those songs¹⁰. That study revealed that calls are a reliable measure of mate preference¹⁰. Therefore, by counting calls produced in response to each song, we are able to define the mate preference of each experimental subject. Songs performed by male Bengalese finches are a primary method through which females

evaluate the fitness and desirability of a potential mate ²⁶. The processing pathway of auditory stimuli, known as the auditory stream, in finches is analogous to that of humans in that it contains similar structures and patterns: an ascending auditory stream, a primary auditory region, and secondary auditory regions, such as the caudal mesopallium (CM) ⁶.

Experimental focus

Our primary focus is one specific analogous structure: the caudal portion of the mesopallium (CM), which is known to process behaviorally relevant auditory information in female songbirds ³⁷. Parallel to the A2 region in mammals ³, CM is selective only for behaviorally relevant auditory information, not all auditory stimuli ¹⁵. Meaning, CM processes auditory stimuli that may influence a subsequent behavior, as a result of a conscious choice. This differs from innate behaviors which do not result in calculated behavioral changes. In addition, if activity within CM disrupted, for instance if it is damaged or lesioned, female songbirds change their mate preference ²³. Therefore, it functions ideally as locus of this project, as we

are able to isolate auditory stimuli from other factors which may influence a female BF's decision whether a certain male bird is attractive. Our data indicate that cells in CM are selective for songs that an individual female prefers most ¹¹. Previous studies identify increased activity within CM upon exposure to a song a female finds attractive ^{27, 28, 36, 14}. Therefore, it follows that activity of

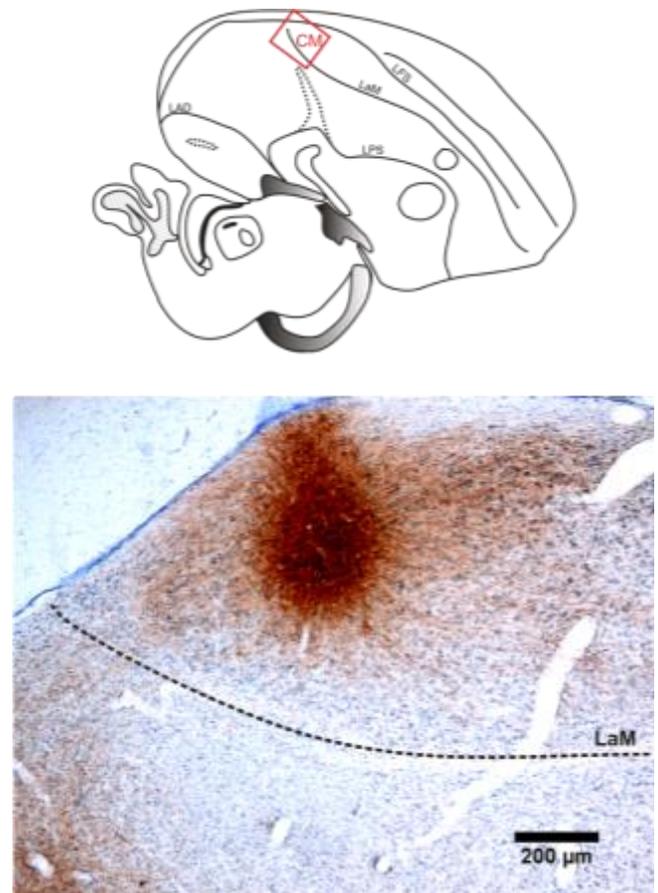


Figure 2. Labeling of CM with BDA¹¹.

neurons in CM may play an important role in shaping how females evaluate song quality and use that information to determine potential mates. The downstream connections from CM are not well defined in female BFs, and they do not exactly mirror the structural arrangement or functional processing of their male counterpart, thus necessitating our work in this study. Though we hypothesize that CM may be an integral part of the decision making pathway, it is not the only contributor. This study investigates whether CM is an essential player in decision making, and identify which structures it receives input from and projects to, in order to construct the conjectured “decision making pathway,” a connection between sensory input and motor action.

Experimental objectives and hypothesis

Long-term goal

Ultimately, we are working toward defining the decision making pathway as outlined in female BFs, in order to outline similar connectivity and function within the human nervous system. With this knowledge, therapeutic targeting of specific structures and connections within the pathway can be achieved. This advancement can be utilized in the prevention or treatment of the results of deleterious decision making, such as alcoholism.

Objective 1: Identification and visualization of the decision making pathway

Objective 2: Optogenetic manipulation of the decision making process

In order to visualize the neural circuitry of decision making, we will employ neuronal tract labeling techniques. We will inject both anterograde and retrograde fluorescent tracers to select projections toward and away from the locus of CM. We will then explore the connectivity of

selected neurons. The goal is to map a single auditory stimulus, a single electrical signal, from its entry into the auditory stream, through processing, and to a behavioral response. This response will be the manifestation of a decision made. We hypothesize that increased activity in CM will increase preference for a specific stimulus.

In order to test this hypothesis, we injected neural tracers into CM to identify where it receives stimuli from and to where it projects. Then, we identified an individual female's baseline preference through an established behavioral testing procedure and used this information to conduct manipulation experiments of the conjectured decision making pathway (Figure 3). We employed optogenetic techniques to accomplish this manipulation and look forward to continuing this investigation.

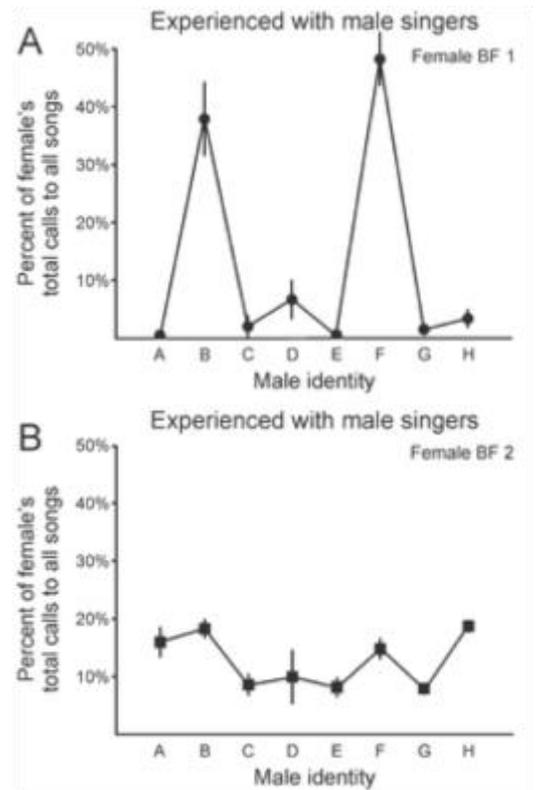


Figure 3. Mate preference profile of two female Bengalese finch (BF) subjects. Peaks indicate greater preference for a specific male song.

A brief history of optogenetics

Optogenetic technology is a developing technology which combines optics and genetics in order to alter or control specific tissues⁹. The technique involves insertion of light-sensitive genes into target cells to ultimately observe and manipulate normal functioning. Optogenetics allows us to target the minutia of cell function and timing in an exceptionally accurate manner. Historically, laser technology has been employed to destroy and thus inhibit specific cell function in order to observe downstream effects, but with the development of optogenetics, destruction of cells is not necessary. This is what contributes to the unprecedented success and necessity of optogenetics—

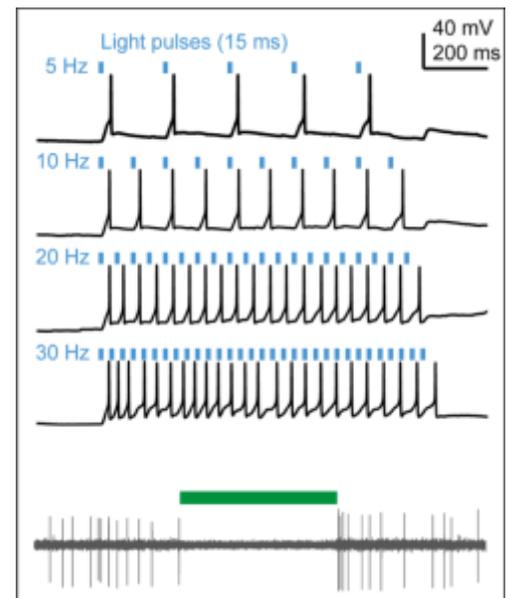
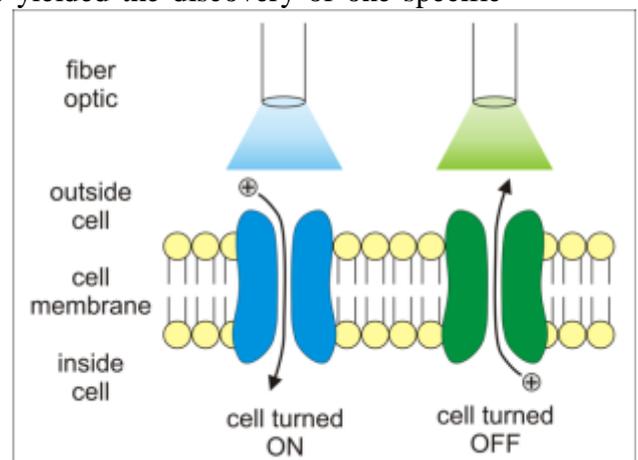
specific cells can be targeted and manipulation with little to no injury with precise temporal and spatial control. Beginning in 1971, light-sensitive microbial transmembrane proteins, called opsins, were discovered to regulate ion flow in response to light ⁹. Bacteriorhodopsins were identified in 1971, followed by halorhodopsins in 1977 and channelrhodopsins 2002. All three later proved to both excite and inhibit (turn on/off) specific cells in response to light ⁵.

A specific channelrhodopsin, channelrhodopsin-1 (or ChR1) was isolated by Peter Hegemann and Georg Nagel in 2002. Their experiments yielded the discovery of one specific single-protein membrane channel which regulated the flow of positive ions when stimulated by blue light—channelrhodopsin-1, ChR1. Another was identified soon after, channelrhodopsin-2, ChR2 ⁹.

Optogenetic technology was first successfully employed to deliver microbial opsins to hippocampal mammalian neurons in 2005, demonstrating that pulses of blue light can induce action potentials in specifically-targeted neurons ⁵. In our experiments, administration of blue light through a surgically implanted fiberoptic causes cells to be excited, or “turned on.” Green light will have the opposite effect (Figures 4 & 5).

Mechanism of optogenetic action

Female BFs were injected with an adeno-associated virus to induce neurons to express trans-membrane, light-sensitive opsin proteins.



Figures 4 and 5. Schematics of proposed optogenetic function.

When illuminated with blue light, this opsin allows positive charges to flow into CM neurons, resulting in generation of action potentials.

Retinal is a necessary protein for opsin function; it isomerizes upon photon absorption, and is readily present in all vertebrate neuronal systems¹⁷. The bound opsin-retinal complex is light-sensitive and will cause a conformational change after photoisomerization, which ultimately leads to a change in cell membrane potential. When the membrane potential is altered, the neuron is either excited or inhibited as a result of the opening or closing of an ion channel or ion pump activation.

Materials and Methods

Subject characteristics

All experiments were performed with female Bengalese finches obtained from a commercial breeding colony or the breeding colony we maintain in the Biological Sciences building at the University of Wyoming. Because Bengalese finches do not possess any sexual dimorphic physical features for simple identification, each female was identified through a series of sexing procedures. First, each bird was isolated and monitored for exhibition of identifiable male or female behavior, i.e. singing. As only male Bengalese finches vocalize through song, we recorded vocalizations 24/7 in order to identify whether a certain subject sings or not. If, after a period of continuous vocal monitoring for four days, the audio recording revealed no singing patterns, it was concluded that subject was a female.

Identification of the decision making pathway

Subjects underwent stereotactic microsurgery which involved administration of anterograde fluorescent tracer, BDA (biotinylated dextran amine). 10,000 MW BDA was chosen because it is a largely anterograde tracer, providing imagery of projections from CM to other neuronal areas. Projections in this case were categorized as axons and varicosities which terminated at target sites outside the injection site ¹¹.

Each bird was first anesthetized with a 3% isoflurane gaseous solution and positioned in a stereotaxic apparatus with a head angle of 45 degrees below horizontal. Bilateral craniotomies were performed and specified stereotax was marked with reference to the Y-zero intersection. Glass micropipettes fashioned in house, with a diameter of 25-35 microns, were filled with BDA and a phosphate buffer solution and lowered into CM at specified coordinates (0.85 mm ventral). BDA was then injected via iontophoresis. After each hemisphere underwent the described procedure, the electrode was withdrawn using a negative holding current, the skull was covered with a silicon elastomer, and a topical analgesic (lidocaine) was applied to the incision sites.

Each subject was isolated for recovery and monitoring post-surgery for a period of 5-7 days to allow full perfusion expansion of BDA into the neural tract. After this period, subjects were exposed to an overdose of isoflurane and perfused through the left ventricle with saline and 4% paraformaldehyde (PFA). Then, the brain was removed and submerged in 4% PFA for a 24-hour period followed by submersion in 30% sucrose PFA cryoprotecting solution for 72 hours. Brain tissue was harvested and treated with immunohistochemical protecting procedures then observed with an Olympus BX51 Fluorescence Microscope.

Labeling neuronal pathways with Fluoro-gold

In comparison with BDA, we also performed similar injection procedures with Fluoro-gold. Fluoro-gold is a very well-known strictly retrograde tracer, which allowed us to visualize projections toward CM. This specific tracer is taken up by axons and fills back to neuron somas, granting us the ability to identify which brain regions project to CM. We hypothesize that CM plays a major role in the decision making pathway, but recognize the possibility of existence of another nucleus of cells which project to CM. We can explore that possibility with use of a retrograde tracer. Furthermore, Fluoro-gold is easier to process than BDA, immunohistochemical procedures are not necessitated and it may provide a more complete image as we can visualize projections with a confocal microscope. With confocal images, we can view projections in 3D.

Optogenetic manipulation

If we are able to successfully reversibly manipulate neurons within CM, we can observe downstream effects on other neural regions in the decision making pathway and observe behavioral changes as a result. To do this, we employed the cutting-edge method of optogenetics involving the use of light-sensitive integral proteins, or opsins, that serve as channels to turn cells “on” or “off.” We injected female BFs with an adeno-associated virus that will transfect neurons to express the specific opsin, channel rhodopsin (ChR2). This opsin is trans membrane protein and is light sensitive. When illuminated with blue light, this opsin will allow positive charges to flow into the cell, depolarizing it, resulting in generation of action potentials. Through the use of an optic fiber chronically implanted into the cranium, this method allows us to address the hypothesis that excitation of neurons in CM will increase preference for selected male songs. Then, in comparison

to the original preference, we can determine if light manipulation alters mate preference using the same behavioral assay method.

In order to promote successful experimentation, the females underwent experiments which assessed “participatory value.” Meaning, each female was for a minimum of 30 minutes prior to playing her an array of eight different male songs: six Bengalese finch songs and two heterospecific songs. We recorded the number of times each female calls in response to the male song stimulus package, compare the data against an established threshold and use this as a measure of relative participation. If the female called 15 or more times to one male, she was considered participatory. If not, that specific individual was not included in the optogenetic study.

Once past this initial phase, we conducted surgery in which we injected female BFs with an adeno-associated virus to induce neurons to express trans-membrane, light-sensitive opsin proteins. The virus requires approximately 30 days to fully express, which we validated with electrophysiological

stimulation. This validation is necessary to ensure that the virus is indeed driving neurons to depolarize at the targeted opsin expression levels, thus driving production of action potentials³⁸. Next, a cannula and fiber optic cable were inserted over CM. At this point, we began behavioral assay testing again. The initial behavioral test post-surgery was used as a baseline of the individual

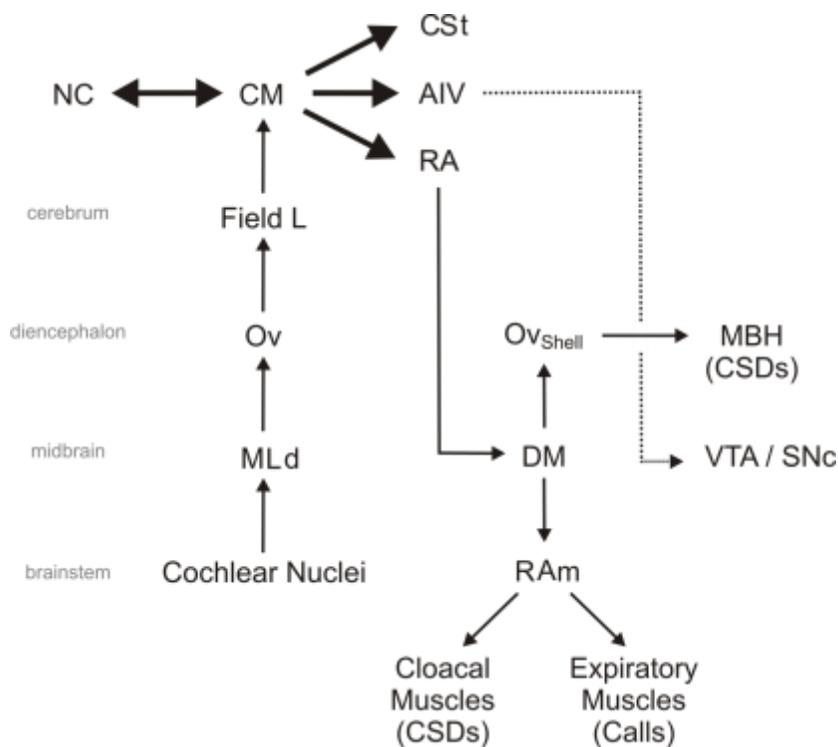


Figure 6 CM Projections. Decision making pathway as currently understood. Novel connections identified through our studies are shown as bold arrows.

female's preference. Experiments continued with light administration in efforts to manipulate mate preference.

Results

Identification and labeling of auditory tract from CM

CM projections were identified by pinpointing BDA-labeled somas and processes outside the injection site, as BDA works in anterograde fashion. These BDA-labeled axons and projections were interpreted as terminal projections from CM. Previously described projections were visible with dense projections from CM to auditory regions within the nidopallium, including the caudal nidopallium (NC), ventricular border of the caudal nidopallium (cNC), the caudolateral nidopallium (NCL), and the caudal medial nidopallium (NCM) ³⁴. Further injection analysis reveals reciprocal interconnectivity between both CM and NC and between CM and Field L, which is the main source of auditory input to CM ^{4,34}. Field L is considered analogous to the primary auditory cortex in humans, meaning that it is the first site which receives auditory stimuli for processing.

Additional projections from CM were found to the arcopallium, specifically the robust nucleus of the arcopallium (RA) and the ventral intermediate arcopallium (AIV). AIV then projects to the ventral tegmental area (VTA). Further projections from CM were observed to terminate within the caudal striatum (CSt).

In male songbirds, VTA is implicated in reward and incentive behaviors, and projections here have proven to drive motivational learning, likely causing an individual to perform a specific behavior again ²⁴. Reinforcement learning and motivation are important considerations within the

realm of mate choice, and further study investigating the connectivity between CM, AIV, and VTA would provide great insight^{2, 18, 31}.

As the projection from CM to CSt is a novel finding, current understanding as to its role in the decision making pathway is unknown. Further study into this connection should be a priority in future studies. Figure 5 displays CM projections and interconnectivity as currently understood.

Preliminary optogenetic manipulation results

We currently have preliminary data from four subjects. Each female underwent bilateral viral injection surgery, including the permanent placement of a fiberoptic within a cannula placed in CM. Two weeks after the procedure, each individual was evaluated for behavioral baseline preference testing. This testing is considered Phase 1, in which we identified each subject's least preferred song. Next, in Phase 2, each individual was subjected to the same song selection with bilateral stimulation of CM during the playing of the least preferred song. Phase 3 is the same procedure as Phase 1 in order to investigate in behavior after optogenetic stimulation. Preliminarily, our hypothesis proved true. During stimulation of CM, the percent calls almost doubled for an individual's least preferred song between

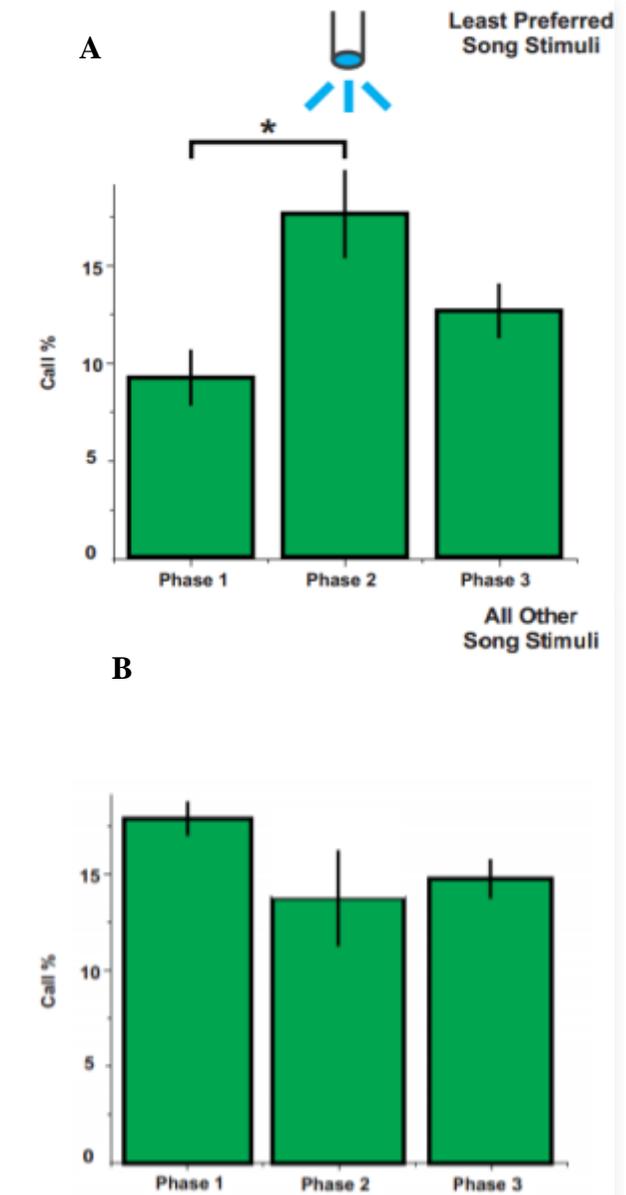


Figure 7. Preliminary results from optogenetic manipulation of four subjects.

Phases 1 and 2 (Figure 7, A). In addition, there was no significant difference observed when light was not paired with song (Figure 7, B).

Discussion

In addition to previously identified connections, our study identified two novel connections from CM—to the RA and to the CSt. Projections from RA are related to voluntary muscle movement. RA projects to dorsomedial nucleus of the intercollicular complex (DM) in the midbrain, which further connects to three areas which may provide a link between integration of sensory input and motor output³³. Indirectly, through the auditory thalamic nucleus, ovoidlalis (OV_{shell}) and ventromedial nucleus of the hypothalamus, DM is connected to the mediobasal hypothalamus (MBH)^{8, 12}. Current understanding recognizes MBH as a driver behind performing CSDs^{7, 28}.

Experiments in female canaries have identified connections between DM and a medullary respiratory center (nucleus retroambigualis, RAm) which is further connected to spinal motor neurons in the cloaca, a female reproductive organ where copulatory contact with the male occurs³⁵.

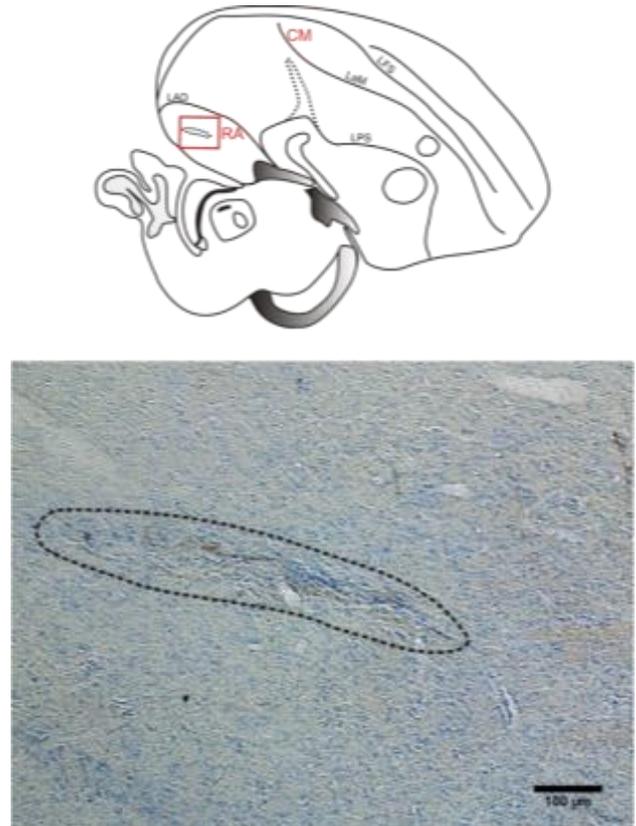
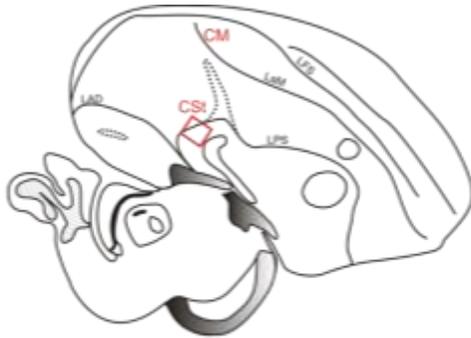


Figure 8. Projections from CM to RA¹¹.

A third route through which CM may influence female preference behaviors connects calling behavior with DM stimulation. Lesions within DM eradicate calling behaviors and electrical stimulation of DM drives call production, which together implicate DM essential for call production^{13,32}. This links CM with neurons that drive call production.

The newly identified projections from the CM to the CSt are important in the comparison of avian brains to those of mammals. CM is analogous to neurons in layers two and three of the human auditory association cortex. Field L is most analogous to the primary auditory cortex in humans. Its projection to CM relates to the projection from the primary auditory cortex to layers 2



and 3 of the human neocortex, and CM's further projection to CSt has been postulated to function similarly to higher level functioning areas. This novel connection identified between CM and CSt therefore provides further evidence of similarities between avian and human nervous systems and auditory processing systems¹¹.

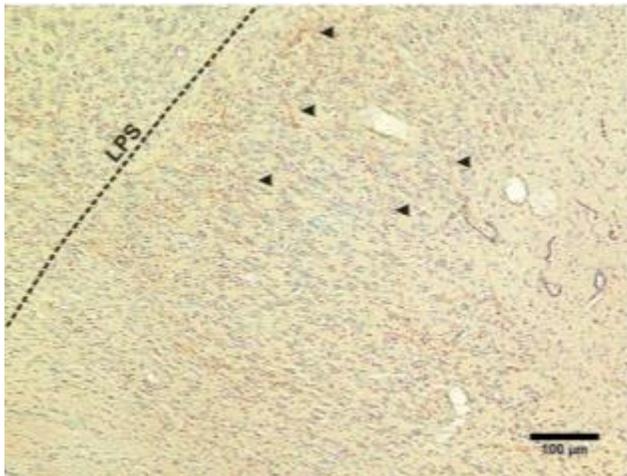


Figure 9. Novel projections identified from CM to CSt¹¹.

Future Directions

We look forward to continuing research into decision making and further developing the decision making pathway. Additionally, we anticipate valuable results from continuing optogenetic manipulation. We further look forward to supporting those in pursuit of similar research. Though optogenetics is the most spatially and temporally precise approach, additional avenues may be explored in the pursuit

of understanding decision making. Alternate methods could be employed to stimulate brain activity and evaluate mate preference. One of these methods is to activate the cells through electrical stimulation. Additional lesion studies could shed light on the functions of specific brain region and their interconnectivity. Finally, pharmacological activation or inactivation of targeted cells or brain regions could provide similar excitation of the neurons and produce intriguing experimental results.

Though our experiments identify CM as a major contributor to the decision of mate choice, other regions and structures have influence. Further exploration into the role these additional regions play would provide ever deeper understanding into a decision making pathway. Using optogenetic technology, we can explore stimulation during the most preferred songs or even during heterospecific songs to observe effects. We can investigate whether administering light during the most preferred song would have the opposite effect as previously observed with the least preferred song and CM.

In time, a true pathway will be proved in songbirds and preferences may be manipulated with optogenetic techniques. Upon successful alteration of decision outcome, research may advance to human clinical trials.

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