Neurobiological Mechanisms of Memory Formation in the Auditory Forebrain of Adult Male Zebra Finches

By

Basilio Furest Cataldo

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And approved by

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ABSTRACT OF THE THESIS

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By BASILIO FUREST CATALDO

Thesis Director:

Dr. David S. Vicario

Songbirds provide a powerful model for studying adult neuroplasticity in the auditory cortex as a function of recent auditory experience due to many parallels with the human auditory system, which is similarly tasked with processing complex conspecific vocalizations. As in human speech processing, lateralized auditory responses are evident in an area of the songbird's higher auditory cortex, NCM (*caudomedial nidopallium*), that encodes specific auditory memories through a process of adaptation that leads to reduced responses to familiar sounds. The right NCM typically shows larger auditory responses than the left, suggesting lateral differences in auditory representations and memory. Furthermore, the songbird brain incorporates new neurons in adulthood, including in NCM. In this study, Zebra finches (*Taeniopygia guttata*) were continuously exposed to a novel heterospecific acoustic environment to confirm a previous report wherein NCM multi-unit activity undergoes dynamic shifts in lateralized activity and assess whether these transient shifts in activity are correlated with shifts in incorporation of new neurons. Bilateral NCM electrophysiology confirmed previous reports wherein left-hemisphere activity was elevated relative to the right after 9 days of exposure to a novel

heterospecific acoustic environment, an effect that was not observed in a cohort exposed to the same environment for 30 days. A novel longitudinal measuring approach, via epidural recordings, revealed the timeline during which these transient shifts in lateralized activity occur. Finally, preliminary data suggests that there is an inverse relationship between the asymmetric electrophysiology and lateralized new neuron incorporation.

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Introduction

As animals interact with their environment, they generate memories through experiences that in turn shape their subsequent behaviors. Conceptually, these memories can be encoded in dynamic perceptual filters that modulate responses to subsequent exposures with the same or similar stimuli. These putative filters are formed throughout development based on genetic programs in interaction with experience, and can then parse incoming stimuli by sifting them on the basis of their ethological relevance (Miller and Knudsen, 2001; Bao et al., 2013; Amin et al, 2013). For example, one can easily pick out one's own name in a room full of noise ("Cocktail party effect"; Arons, 1992; Cohen, 1992; Mullins, 1993). Perceptual filters, in the form of auditory memories and categories, are believed to be stored in higher auditory areas, although organizational changes in response to early acoustic experience are also present in the ascending auditory pathway: cochlear nucleus (Tierney et al., 1997; Mostafapour et al., 2002), inferior colliculus (Knudsen and Brainard, 2003; Gao and Suga, 2000), thalamus, (Speechley et al., 2007), and primary auditory cortex (Zhang et al., 2002; Woolley et al., 2010; Barket et al., 2011; De Villers-Sidani et al., 2007). Similarly, sound-consequence associations formed in adulthood can induce changes in the cortical mapping of a rodent's primary auditory cortex to under- or over-represent sound frequencies associated with the test stimulus (Bieszczad and Weinberger, 2010, Polley et al., 2006). In the context of how experience conditions behavioral outputs, information theory suggests that the underlying key for learning lies in the acknowledgement of stimulus properties, and their regularities, that

can be extracted from a vast and complex stream of potential associations (Ward, Gallistel, & Balsam, 2013).

The acquisition of perceptual filters may rely on the statistical learning of underlying stimulus statistics that are present during development and modified via experience of novel environments with changing stimulus regularities. The putative mechanisms that drive statistical learning are proposed to be engaged during language acquisition (for a review, see Armstrong, Frost, & Christiansen, 2017), have been reported to operate in human infants tasked with segmenting linguistic and non-linguistic stimuli (Saffran, Aslin, & Newport, 1996), and can occur across modalities independently in a stimulus-specific manner (Conway & Christiansen, 2005). Another feature of statistical learning is that can occur implicitly in a passive context, whereby a subject can extract patterns from their environment in the absence of explicit tasks and or demands that require such extraction (Saffran et al., 1999; Fiser & Aslin, 2001, 2002). This type of learning is typically researched in the domain of non-language grammar in humans (e.g. Conway & Christiansen, 2005) and is described as occurring simply as a function of "mere exposure" (Saffran et al., 1999). According to this line of research, and in the context of perceptual filters, auditory representations of one's environment are not static and remain are subject to expansion as a function of exposure to different distributions of stimulus statistics.

Unpublished data from our lab suggests that perceptual filters, composed of auditory memories and classified into acoustic categories (Lu & Vicario, 2017; Yang, *Dissertation*), are present in the songbird's higher auditory area NCM (*Caudomedial Nidopallium*). Additionally, since perception and production of communication signals in

songbirds (Nottebohm et al., 1976; Phan and Vicario, 2010; Floody and Arnold, 1997; Wild et al., 2000; Voss et al., 2007; Poirier et al., 2009), and humans (Marcotte and Morere, 1990; Leybaert and D'Hondt, 2003; Dehaene-Lambertz et al., 2002), elicit lateralized patterns of activity, it is possible that each hemisphere responds selectively to, and, by implication, filters information from different features of communication signals. Therefore, the neurobiological changes that subserve auditory memory formation may differ, or occur at different rates and levels, between hemispheres.

Songbird as an Animal Model for Perception and Production of Communication Signals

Vocal learning refers to the ability to shape vocal motor outputs by using auditory feedback, which allows animals to acquire complex conspecific and ethologically relevant vocalizations; a feature that is not unique to humans and is studied in a broad family of species (Wilbrecht & Nottebohm, 2003; Doupe & Kuhl, 1999). While songbirds are the most widely studied animal model of vocal learning, the act of learning conspecific communication outputs from a tutor model has been reported in cetaceans (McCowan & Reiss, 1997), bats (Boughman, 1998) and songbirds (Doupe & Kuhl, 1999), which suggests that vocal learning engages neural circuitry that presumably precedes human language in evolution (Nottebohm, 1972); that is, human language is a complex use of the vocal learning machinery (high vocal learners; Petkov & Jarvis, 2012), with the inclusion of higher order features such as grammar and recursion (Hauser, Chomsky, & Fitch, 2012). Similarities between human language acquisition and vocal learning include: 1) a critical period wherein conspecific vocalizations are learned from a tutor model; 2) a period during which juveniles practice their species'

vocalizations; and 3) analogous neural substrates for auditory processing and vocal motor learning (Doupe & Kuhl, 1999); 4) Lateralized processing for both perception and production of complex sounds. Thus, the Zebra Finch, a species of songbird, offers an accessible model for studying the neural substrates of vocal learning and the perception of communication signals.

Vocal learners' brains, compared to non-learners (Jarvis, 2007), possess discrete neural systems, primarily in the telencephalon, that are engaged during the acquisition, perception, and production of communication outputs (Jarvis, 2007; Petkov & Jarvis, 2012; Bolhuis & Moorman, 2015). The anterior forebrain pathway, involved in the production of song and the sensorimotor feedback involved in shaping a bird's own song (BOS) to a target tutor song template, is analogous to the anterior-frontal pathway (AFP) in humans, and includes: HVC (High Vocal Center, where motoric outputs are generated), Area X (a basal ganglia-like structure; Doupe, Perkel, Reiner, Stern, 2015), DLM (thalamic nucleus dorsolateral anterior, pars medialis), and LMAN (lateral magnocellular nucleus of the anterior nidopallium) which projects to the remainder of the motor pathway through the RA nucleus (Robust Nucleus of the Arcopallium; as described in Figure 1 in Bolhuis & Moorman, 2015). These neural pathways are known to be functionally lateralized for both sensory and motor processes and are necessary for normal song acquisition (Nottebohm et al., 1976; Floody and Arnold, 1997; Wild et al., 2000; Voss et al., 2007; Poirier et al., 2009; Phan and Vicario, 2010). For the remainder of this proposal, the focus will be on the complementary neural pathways of the vocal learning system that involve the auditory pathway (**Figure 1**), which is primarily comprised of: cochlear nucleus, midbrain (MLD), Ov (nucleus ovoidalis), Field L (a

region that contains discrete subdivisions, functionally analogous to the mammalian primary auditory cortex; Bolhuis & Moorman, 2015), NCM (a higher auditory cortex, functionally analogous to Wernicke's area in humans; Chirarhivat, Raja, & Gobes, 2015), and CMM (*Caudomedial Mesopallium*, functionally analogous to the superior temporal cortex regions in humans; Chirarhivat, Raja, & Gobes, 2015). Due to the vocal learning similarities and presence of functionally analogous structures in vocal learners' brains, songbirds offer a relevant and powerful model for studying neurobiological substrates that are implicated in the acquisition, production, and processing of communication signals.

Chapter 1: Lateralization and the Perception of Sound

Lateralized Functionality

The study of hemispheric asymmetries in the brain originated from the Broca's observation that patients with difficulties in speech production had lesions in their left hemisphere inferior frontal gyrus (Broca, 1865). Shortly thereafter, Wernicke (1874) made a similar hemisphere-dependent association with speech production (i.e. Wernickes Aphasia), whereby subjects with damage to the left posterior section of the superior temporal gyrus experience difficulties speech comprehension and the production of sentences with proper lexicon. By now, lateralization phenomena have been shown to be present in a variety of species (vertebrates: Rogers, Vallortigara & Andrew, 2013; invertebrates: Frasnelli, 2013) and develops both in a gene- (Gunturkun & Ocklenburg, 2017) and experience-dependent (Phan & Vicario, 2010; Rogers, 2004) manner; the evolutionary origin of the phenotype remains unknown (Gunturkun & Ocklenburg, 2017). Whether a lateralized brain is a universal feature in the animal kingdom or is a

favorable trait, studies involving humans and non-humans suggest that each hemisphere subserves different functions during the production of behavioral outputs and perception of stimuli (Rogers, 2002, 2004; Gotts, Joon Jo, Wallace, Saad, Cox & Martin, 2013). The division of labor between the two hemispheres offers a conceptual model for describing how the brain can process information efficiently.

The precise function of a hemisphere, in any species and for any given sensory modality, is a topic that should be addressed holistically if one is to consider that each hemisphere is specialized to fulfill a purpose regardless of the behavioral context. For example, in the context of human language, left and right hemisphere selectivity is present for phonemes and prosody (respectively; Telkemeyer, Rossi, Nierhaus, Steinbrink, Obrig, & Wantenburger, 2011). While language is unique to humans, the fundamental features of speech, to which a hemisphere is selective, could be considered as 'stimulus' features (not in the context of language) to understand the function of each hemisphere in a broader sense. Supporting evidence for the notion that each hemisphere is specialized to serve general functions irrespective of sensory modality has been presented in a comparative analysis based on chick's behavioral, emotional, and cognitive processes (Andrew, 1991). Integrating these observations with the operation of memory systems and cognitive function, one is able to elaborate on the broader functions that each hemisphere undertakes. Vallortigara, Rogers and Biezza (1999) proposed that each hemisphere's function is to process stimuli by categorizing them in terms of novelty and the context in which they are experienced, two analytical processes that are, without the presence of two hemispheres, functionally incompatible (Sherry & Schachter, 1987). According to this conceptual framework (here described in terms of local and global

analyses, derived from Vallortigara, Rogers & Biezza, 1999), one hemisphere is tasked with allocating/comparing an event (or stimulus) to a categorical referent (e.g. speech variants: the "same" word, as spoken by two different individuals, is a variant, or categorical referent, of the same word/category) that is formed through experience (local analysis). In parallel, the other hemisphere assesses whether the context in which the event (or stimulus) occurs has been previously encountered or if it should be considered under the circumstances of the current circumstances (global analysis; **Figure 2**). The local process specializes in detecting variance amongst invariance, while the global process analyzes invariance amongst variance. The two simultaneous analytical tools that a functionally lateralized brain offers, allows animals to efficiently interact with their environment by facilitating the extraction of more information from events, while categorizing them, thus allowing animals to respond appropriately and make predictions based on contextual contingencies, familiarity, and novelty (Gunturkun, Strockens, & Ocklenburg, 2020).

The manner in which information is processed differentially across hemispheres may be via a filtering system that parses incoming stimuli based on the features for which the hemisphere is selective. The location of such perceptual filters is thought to be in higher cortical areas that are comprised of neural circuitry that encodes for the stimulus statistics of the environment; that is, stimuli are filtered on the basis of their probability of occurrence and ethological relevance. Therefore, the perception of a given stimulus can be optimized as a function of experience and presumably by the formation of perceptual filters (through *changes* in neural circuitry) that accommodate for the stimulus' features. Studies show that this may occur through dynamic changes in neuronal receptive fields

(Lesica & Grothe, 2008), which suggests that a neural circuit can encode for a multitude of stimuli; however, this model would be limited by the degrees of freedom of a circuit with a restricted number of processing nodes (Pytte, 2016). A proposed mechanism through which neural circuitry can accommodate for the vast array of possible inputs is by modulating the circuit's ability to be amended, potentially through the introduction of new neurons (see Chapter 4).

The involvement of lateralized functionality in both sensory and motoric processes is apparent across species and the processing power that two hemispheres afford is, in theory, ethologically advantageous. Following the tradition from early studies that assigned "dominance" to a given hemisphere for an observed function, it is often considered and or assumed that lateralization is a static trait, or feature, of the bi-hemispheric brain. This conclusion is incomplete, as any observation of lateralized activity that is performed under experiment-specific circumstances can only reflect a function that is imposed by the current conditions (Hickok & Poeppel, 2015). For example, in a dichotic listening task wherein human subjects were tasked to make decisions based on the presentation of word pairs, it was observed that a majority of the subjects displayed a left hemisphere dominance in language processing (Bethmann et al., 2007) based on the decision pattern and the elevated fMRI activity in the left hemisphere relative to the right. This interpretation can only be made in the context of the experiment's methodology and the hemispheric dominance assertion cannot be generalized to language processing as a whole, since the perception of communication is complex, involves elements that appear to elevate hemispheric activity differentially (e.g. prosody; Telkemeyer et al., 2011), and consists of components that appear to equally

recruit both hemispheres' functions (Lindell, 2006). Additionally, the elevated activity that is often reported in one hemisphere over the other is not necessarily indicative of "dominance", it can also reflect aspects of the stimuli currently being processed: in language processing, it is plausible to argue that phonemes occur at a higher rate in speech which leads to elevated levels of activity in the human's left hemisphere, and prosody, occurring at longer time scales, do not elicit higher right-hemisphere relative to its counterpart at any given time point when hemispheric activity is compared. As such, the observation of lateralized activity at any given time can be considered as a snapshot of the brain's activity as a function of external, internal, stimulus-feature influences, and, contrary to traditional views, is not a static state. Together with published and unpublished accounts, the present thesis aims to challenge the notion of lateralization as a static state by proposing the conception that hemispheric activity is labile and dynamically reactive to dramatic, possibly salient, changes in the environment.

Lateralization in the Zebra Finch Model

The pioneering insights into lateralized brain processes in songbirds closely emulated Broca's work with humans, whereby vocal bilateral motor centers and organs were susceptible to producing behavioral deficits upon lesioning, subsequently leading to the discovery of differences in the hemispheric control of vocal outputs. Specifically, lesions to left HVC and hypoglossal nerve resulted in more profound vocal production deficits (Nottebohm 1976, 1977). Similarly, auditory perception of sounds and communication signals also appears to be lateralized (Phan & Vicario; 2010), although each hemisphere's specific role in such tasks remains elusive and/or largely unknown. The putative function of each hemisphere is difficult to ascertain due to the multi-

dimensional characteristics of communication that lead to different contributions in perception by each hemisphere depending on the type of experimental task (e.g. Voss et al., 2007), stimulus-feature control (e.g. zebra finches: spectral vs. temporal filtering; Van Ruijssevelt, et. al., 2017), and local estrogen availability (Ramage-Healey et al., 2010). While unilateral lesions of the songbird auditory forebrain NCM are seldom performed to study the potential contributions of each hemisphere during auditory perception, the lateralization phenomenon is often studied in relation to tutor-song memories, conspecific biases, early experience, and changes in acoustic environments (Mello et al., 1992; Voss et al.; 2007; Phan & Vicario; 2010; Yang & Vicario, 2015).

As in humans, songbirds, specifically Zebra finches, exposed to sounds of their own species display different activation patterns between hemispheres in higher auditory areas, although the direction of lateralization depends on the playback stimuli, behavioral state, and experimental method (for a review, see Moorman, 2015). For example, immediate early gene *ZENK* expression is typically observed to be left-lateralized in the Zebra finch NCM (Avery et al., 2005; Moorman et al., 2012), while fMRI studies suggest that NCM is right-lateralized (Voss et al., 2007) with further observations suggesting that the right-bias is evident at the level of the auditory mesencephalic nucleus DLM (Poirier et al., 2009). Previous studies employing electrophysiology in Zebra finches suggest that juveniles exposed to tutors during their critical period for song acquisition (i.e. typical rearing conditions) develop right-biased activity in NCM (Phan & Vicario, 2010), and that this pattern of higher right-side activity persists if Zebra finches are maintained in a conspecific acoustic environment (Yang & Vicario 2015; Yang *Dissertation*).

Chapter 2: Dynamic Lateralization and Neuroplasticity in NCM

Sensory cortices are shaped through experience to have an increased affinity for processing sensory inputs that are part of the known stimulus statistics and/or important in their immediate environment. Such changes in sensory areas, denoted by changes in cortical mapping and neuronal receptive fields, were initially shown in by Merzenich and colleagues (1983) in owl and squirrel monkeys wherein cortical somatosensory representations of hand digits were modified as a function of physical changes to the monkeys' hand. Similar changes have been demonstrated in the rat's primary auditory cortex (A1) as a function of alterations to developmental acoustic environments (Zhang et al., 2001) and instrumental learning (Bieszczad & Weinberger, 2010). Dramatic changes in A1 tonotopic mapping can facilitate the navigation within an environment by facilitating the access of sensory inputs into A1 neural networks and or memories related to them (Bieszczad & Weinberger, 2010). In other words, incoming stimuli may be filtered differently depending on their stimulus statistics within an environment and their related contingencies, leading to the rise of perceptual filters (Polley et al., 2006) and the reduction of redundant processes (Barlow, 1972).

If each hemisphere subserves a different function in sensory processing, it is conceivable that they would each filter inputs based on environmental changes and their function, as to increase encoding efficiency via complimentary routines. Classical lateralization patterns, wherein one hemisphere exhibits elevated activity relative to its counterpart, can be interpreted in terms of hemisphere-dependent function which can be defined by the distribution of inputs for which it is selective. When faced with a completely novel set of stimulus statistics, hemispheric function may not be optimal since

sensory areas have yet to adapt to the novel changes. The resulting bilateral activation (e.g. Newman-Norlund et al., 2006) could be a product of cortical areas undergoing adjustments to the new sensory environment. As a function of exposure and learning, sensory representations are more efficiently encoded and subsequently lateralized patterns emerge.

As previously introduced, a lateralized brain provides, in theory, a more efficient method of completing cognitive tasks by dividing them into *parallel* processing streams for which each hemisphere is specialized, however the conventional tools for assessing lateralized brain activity often depict a snapshot of hemispheric activity, are dependent on the experimental methodology, and generally suggest that lateralization is a static feature of brain function. Recent observations suggest that novel experiences may exert influence on the lateralized responses. For example, humans being trained on a novel artificial (non-language) grammar initially do not display any hemisphere-biased activity (i.e. bilateral activation), however, as the same cohort became proficient with the new grammar, typical left-biased activity was observed (Newman-Norlund et al., 2006). This observation has been replicated (Plante et al., 2015) and conforms with other reports that language lateralization is more directionally pronounced as a function of age and depends on maturational processes (for a review, see Herve et al., 2013); potentially related to language acquisition critical periods and language proficiency. Bilateral brain activation during novel-language acquisition, suggests that while each hemisphere reserves its own putative function, they are no yet specialized to process the new language. In such a case, bilateral activation may reflect the current state of lateralized activity as a function of learning the new language's features and or processing inputs that are, at the current time

point, novel. Thus, it is plausible that lateralization is more dynamic than previously thought and that the apparent changes in hemispheric activity reflect a state of new-information encoding.

Zebra finches are closed-ended learners and are unable to learn more than one song within their lifetime, potentially due to the onset of neurobiological constraints that close their critical period for vocal learning and hinder the ability to seasonally change their vocal repertoire (e.g. development of perineuronal nets in HVC; Balmer et al., 2009; Cornez et al., 2017), as occurs in some other songbird's groups. Alternatively, NCM retains plasticity into adulthood as indexed by Stimulus-Specific Adaptation—rapid decrements in activity in response to repeated presentations of the same stimulus (SSA; Chew et al., 1995, 1996a, b; Bell et al., 2015; Yang et al., 2015; Yoder et al., 2015; Soyman & Vicario, 2017). The higher auditory area has also been shown to form long-lasting memories as a function of early experience (Phan et al., 2006) and the combination of exposure and pharmacologically-engaged epigenetic mechanisms (Phan et al., 2017). Additionally, processing of auditory inputs by NCM has been shown to be lateralized in response to complex conspecific communication signals in electrophysiological (Phan & Vicario 2010; Yang & Vicario 2015) and IEG studies (Bolhuis et al., 2012; Olson, Maeda, & Gobes, 2017). Similar to humans exposed to a challenging set of novel acoustic categories (e.g. Newman-Norlund et al., 2006), Zebra finches immersed in a novel acoustic environment have been shown to exhibit changes in their typical pattern of lateralization.

Dramatic changes in the acoustic environment of adult male Zebra finches have been shown to alter typical patterns of lateralized activity NCM. Four days of passive

exposure to a novel canary (i.e. heterospecific) aviary recording was sufficient to shift typical patterns of activity from right-biased to left-biased neural response strengths and adaptation rates (Yang & Vicario, 2015). This pattern of responses was also observed in birds after nine days of exposure but not in any group that was exposed to a novel Zebra finch (ie. conspecific) aviary recording for the same durations (Yang & Vicario, 2015). However, results from a prolonged exposure paradigm, showed that other cohorts exposed to the same novel heterospecific environment for 14 or 30 days displayed typical patterns of right-biased measures (Yang, *Dissertation*). Although these data came from different cohorts, they suggest that exposure to the unfamiliar heterospecific environment initially caused a shift in lateralization, but that prolonged exposure produced a reversion to the original pattern. In addition, behavioral experiments were conducted on birds after various exposure durations to conspecific and heterospecific environments. Naïve zebra finches, those exposed to the conspecific environment, and those exposed to the heterospecific environment for up to 9 days were very poor at learning a discrimination between 2 canary vocalizations. In contrast, those exposed to the heterospecific environment for 14 or 30 days were able to acquire the discrimination of heterospecific exemplars rapidly (Yang, *Dissertation*). The dynamic changes in lateralized responses – reversal and then reversion – observed in Yang's work suggest that NCM activity remains labile in adulthood. The behavioral data suggest that lateralization shifts may represent modifications of perceptual filters in NCM driven by novel acoustic statistics, and that those modifications underlie more efficient processing, leading to improved discrimination performance. To assess the underlying processes of neuroplasticity that may be engaged during the shifts in lateralized activity in response to the challenge of a

novel acoustic environment, a new cohort of Zebra finches was initially divided into groups that were exposed to novel heterospecific or conspecific acoustic environments for different durations. After exposure, these birds were assessed both for electrophysiological evidence of dynamic changes in lateralized activity and for the incorporation of new neurons into NCM (described in Chapter 4).

Methods

Subjects

Adult (>120 post-hatch days) male Zebra finches were randomly selected from the Zebra finch breeding aviary at Rutgers University, New Brunswick, NJ; the subjects were either hatched in the colony or acquired from the Rockefeller University Field Research Station, Millbrook, NY. Once Zebra finches were randomly assigned to their groups, they were housed together (4 at a time) and maintained in the main aviary for three days, during which time they received BrdU injections (see *Methods* in Chapter 4). To accommodate the superimposed BrdU protocol, the cohorts remained in the aviary housing until their exposure period commenced. At all times, prior and during exposure, birds received food and water *ad libitum*. All procedures were aligned with laboratory protocols approved by the Animal Care and Use of Committee at Rutgers University.

Acoustic Environments

The passive exposure paradigm employed in these experiments was adopted from the previous study in which Zebra finches were individually isolated and exposed to either a novel conspecific acoustic environment (playback of a 12h recording of an unfamiliar Zebra Finch aviary; CONENV) or a novel heterospecific acoustic environment

(playback of a 12h recording of an unfamiliar canary aviary; HETENV) throughout their 12h day-cycle for different numbers of days (Yang & Vicario, 2015; Yang, *Dissertation*). These same environment recordings were used in the following experiments and simulate a cross-housing scenario first described by Terleph and colleagues (2008). In general, individual sounds in the heterospecific environment were longer in duration and displayed more energy at higher frequencies. (For a depiction of the differences between the two acoustic environments, see **Figure 3**, adapted from Yang & Vicario, 2015). CONENV and HETENV aviary sounds were played back at an amplitude of ~70dB SPL and matched for average sound intensity.

Passive Exposure Manipulation

Zebra finches were randomly assigned to six independent groups defined by exposure type (CONENV or HETENV) and exposure duration in days (9D, 14D, or 30D). Following the superimposed BrdU/exposure protocols, Zebra finches were removed from the main aviary and individually housed in acoustically isolated chambers (Controlled Acoustical Environments, IAC inc.). They then underwent passive exposure to their respective acoustic environemnts.

Head-Fixation Pin Surgery

Forty-eight hours prior to the cessation of passive exposure, Zebra finches were removed from their chamber for ~60min to undergo a procedure to implant a head-fixation pin for subsequent electrophysiology. Following an approved protocol, they were deeply anesthetized with 1.5-2% Isoflurane (Piramal) in Oxygen, and the head was fixed in a stereotaxic apparatus. Bupivicaine (0.5%, 5mg/mL, Auromedics) was used as a local anesthetic prior to making a midline incision in the scalp. Small openings were made in

the outer layer of the rostral portion of the skull to facilitate head-fixation pin adhesion. Next, a craniotomy of the outer layer of skull was performed over the caudal telencephalon and the mid-sagittal sinus. A well was formed around the craniotomy and a head-fixation pin was implanted with dental cement (Anhydrous Polycarboxylate Cement; Tylok Plus, LOT: 161018). After the surgery, Zebra finches were administered Meloxicam (5mg/mL, Covetrus) to facilitate the recovery process. Following 30-60 minutes of full recovery, Zebra finches were returned to their respective chambers to continue their passive exposure period.

Multi-Unit Activity (MUA) Electrophysiology

Upon finishing their respective exposure periods, the birds were removed from their isolation chambers and placed in a soundproof booth equipped with electrophysiology-recording equipment. The birds were placed in a comfortably plastic tube, and their heads were fixed to the stereotaxic apparatus by clamping the head-fixation pin. The brain was then exposed by removing the inner layer of skull. Following previously defined coordinates (Yang & Vicario, 2015), slits were made in the dura mater over left and right NCM . Then a 4X4 shank-by-site fluorescent-dye-coated silicon probes (NeuroNexus, 4x4 A16 silicon probes) was inserted vertically into each hemisphere to target NCM (16 sites per NCM). Neural activity was monitored during probe insertion to identify typical bursting activity at all recording sites. Once both probes were positioned, auditory responses to testing stimuli were acquired. Multi-unit activity was recorded simultaneously from all 32 sites using two synchronized Spike2(v7.01)-operated Power 1401 A/D converters (CED, Cambridge, England) that received amplified (x19,000 gain) and band-pass filtered (.5-5kHz) voltage signals.

Auditory stimulus sets were comprised of 5 novel Zebra finch and 5 novel Canary songs (10 stimuli total) ranging from 900-1500ms in duration) that were not a part of the passive exposure environments. During electrophysiology, the order of stimulus presentation was randomized, each stimulus was presented 25 times (10 stimuli, 25 presentations each, 250 presentation trials in total), and the inter-stimulus-interval (ISI) was fixed at 8s.

Data Analysis

Absolute Response Magnitude

To measure the strength of MUA elicited by auditory stimulation, absolute response magnitude (ARM), an indirect measure of response strength, was used following previously established methods (Phan et al., 2006; Phan & Vicario, 2010; Yang & Vicario…). ARMs are calculated by subtracting the root-mean square (RMS) of the multi-unit waveform during a control period (500ms prior to stimulus playback) from the RMS of the waveform function during the respective stimulus playback period, defined as from stimulus onset to (stimulus offset + 100ms) per protocol. For each recording site, and for each stimulus, the average ARM for trials #2-6 was obtained. This provided a measure of response strength for stimuli when novel. Depending on the type of analysis performed, ARM values were grouped either across stimulus type (Canary or Zebra finch) or across hemisphere.

Adaptation Rate

NCM neurons exhibit attenuated responses following repeated presentation of the same stimulu. Adaptation rate is a measure that indexes decrements in response strength as a function of stimulus repetition while normalizing and accounting for a channel's

average activity (Chew et al, 1995; Phan et al., 2006). Following previously established protocol, adaptation rates were calculated as the quotient between the slope of the regression line of ARM responses to stimulus repetitions for trials 6-25 and the average ARM over the same trials. Adaptation rates were used as an index of novelty or familiarity; stimuli that are familiar elicit lower adaptation rates (less negative slopes), while novel stimuli elicit rates mean, thus the rate indexes the strength of neuronal recognition memory for specific songs (Chew et al., 1996).

MUA ARM Lateralization Index

A lateralization index (LI) was calculated to readily measure the lateralization state of a given bird. To calculate the LI, ARMs were initially collapsed across stimulus-type. To determine ARMs for a given hemisphere, ARMs were also averaged across all electrode sites for each hemisphere independently.; **Equation 1** demonstrates how the LI was calculated to determine activity in one hemisphere relative to the other. For a given bird, LIs were defined as the quotient between the absolute difference in hemisphere-averaged ARMs and their arithmetic mean; the same calculation was employed in a stimulus-type-specific manner. The computations yielded LIs that represented lateralized activity based on responses to Zebra finch and Canary song responses; an overall LI (OLI) was also calculated as an average of the stimulus-specific LIs.

Data Analysis

To assess the effect of exposure type and duration of exposure on hemispheric activity, 2 2x2 ANOVAs (Exposure duration x Hemisphere) were conducted separately for each exposure type yielding results for the effect of duration of exposure to a given exposure type on hemispheric activity. Additionally, for each exposure type subgroup,

two 2x2x2 ANOVAs (Exposure duration x Hemisphere x Stimulus type) were carried out to test the effect of stimulus type on hemispheric activity in birds exposed to a given exposure type for 9 or 30 days. The same pattern of ANOVA analyses were carried out to test the effects, for each exposure type separately, of exposure duration and stimulus type on NCM activity. Lastly, to understand the underlying linear relationship between stimulus type on ARMs, ARM LIs were averaged based on stimulus type. All statistical tests were conducted via OriginPro (2020) with an alpha criterion of .05. All *post-hoc* comparisons were carried out using Bonferroni corrections by dividing the omnibus alpha criterion by the number of comparisons.

Results

To capture the state of lateralization exhibited by groups of birds exposed to novel (HETENV) or familiar (CONENV) acoustic environments, bilateral NCM multi-unit activity was recorded after different periods of exposure (9D, 14D, or 30D). **Figure 4** illustrates the group average ARMs between the hemispheres of birds exposed to HETENV for either 9 days or 30 days. While the hemisphere factor did not significantly load onto the ANOVA ($F(1, 279) = 0.049, p=.82$), there was a significant effect of duration of exposure ($F(1, 279) = 18.17, p=.00002$) as well as a significant interaction between the hemisphere and duration variables ($F(1, 279) = 10.85, p=.001$). A *post-hoc* analysis clarified that the significant differences amongst the compared pairs were only present between left-30/left-9D ($t(1, 279)= -5.25, p=.000001$), left-30D/right-9D ($t(1, 279)= -3.21, p=.009$), and right-30D/left-9D ($t(1, 279)= -2.82, p=.03$). Unexpectedly, there were no significant differences between the hemispheres of the groups within the

same exposure duration, although there was a trend towards significance in the difference between the right-9D/left-9D comparison (*t(1, 279)= -2.59, p=.06)*. Results of a three-way ANOVA suggested that the stimulus type was not a significant factor in driving bilateral NCM activity as it did not load significantly as an independent factor nor as an interaction coefficient.

Figure 5 demonstrates the group average ARMs from birds exposed to CONENV for either 9D or 14D. Only the exposure of duration loaded significantly onto the ANOVA model (*F(1, 293)= 12.99, p= .0003),* an effect that seems to be primarily driven by the significantly different pairs: right-9D/right-30D (*t(1, 293)= 3.06, p= .01)* and right-9D/left-30D ((*t(1, 293)= 3.54, p=.003)*. Relative to the previous analysis based on birds exposed to HETENV, stimulus type loaded significantly as an independent factor *F(1, 289)= 8.23, p= .004)* in a three-way ANOVA assessing the relationship between hemisphere, exposure duration, and stimulus type. As seen in **Figure 6,** and following a *post-hoc* analysis, the results suggested that the significance of the stimulus type factor may have been driven by the significant pair comparisons: left-30D-CAN/right-9D-ZF (*t(1, 298)= -4.46, p= .0003)*, right-30D-CAN/right-9D-ZF (*t(1, 298)= -4.25, p= .0008)*, left-9D-CAN/right-9D-ZF (*t(1, 298)= 4.07, p= .001)*, right-9D-CAN/right-9D-ZF (*t(1, 298)= 3.27, p= .03)*.

The present results partly meet the expected trends observed in Yang & Vicario (2015), specifically for the HETENV group where there was a significant interaction between the duration of exposure and hemispheric activity (*F(1, 12)= 8.66, p= .01*). The overall pattern of results across exposure group types and its interaction with exposure duration are illustrated in **Figure X.** However, the *post-hoc* analysis did not confirm any

expected significant differences among the hemispheres at the different levels of exposure as was observed by Yang (*Dissertation*): while visually evident, there was no statistical confirmation that the left hemisphere activity was more robust relative to the right at 9D of exposure and right hemisphere activity was more robust relative to the left at 30D of exposure. No omnibus test reflected the expected result that birds exposed to familiar environments and or sounds exhibit higher activity in the right hemisphere relative to the right (Phan & Vicario, 2010; Yang & Vicario, 2015). The low effect of stimulus type was similar as previously reported (Yang & Vicario, 2015; Yang, *Dissertation*). As seen in **Figure X**, there is a significant linear relationship ($r = .81$, $p = .0002$, $R^2 = .66$) between the lateralization indices calculated as a function of the lateralized activity elicited by either ZF or CAN stimulus independently, which may explain the relatively low stimulus type effect seen in the omnibus tests.

Chapter 3: Chronic Epidural Electrophysiology

The results reported in Chapter 2 and the findings by Yang & and Vicario (2015; Yang, *Dissertation*) suggest that typical patterns of lateralized activity in NCM may be subject to *reversal* (from right-biased to left-biased) after a period of exposure to a novel acoustic environment and, after a sufficiently prolonged period of exposure, there is a *return* (from left-biased to right-biased) to typical patterns of activity. The striking results from changes in NCM bilateral activity are the correlated improvements in auditory discrimination of novel-environment exemplars (Yang, *Dissertation*) by birds who were exposed to HETENV for prolonged periods and thus presumably underwent the *reversal* and *return* in lateralized activity seen in another cohort exposed for the same period. It is

possible that the apparent shifts in lateralized activity were serendipitous and or occurred by chance, a repeated measures approach may reveal if and how these lateralization changes occur as a function of time of exposure to a novel acoustic environment. Additionally, in the prior and current paradigms, the exposure durations (e.g. 4D, 9D, 14D, and 30D) were conceived *a priori*, therefore the timecourse of lateralized NCM activity remains unknown. To both assess the dynamic lateralization phenomenon and increase its temporal resolution, a novel methodology was developed to longitudinally assess the effects of prolonged exposure to novel acoustic environments on lateralized activity.

Methods

Subjects

Adult (>120 post-hatch days) male Zebra finches were randomly selected from the Zebra finch housing aviary at Rutgers University, New Brunswick, NJ; the subjects were either born in the colony or acquired from the Rockefeller University Field Research Station, Millbrook, NY. At all times, prior and during exposure, birds received food and water *ad libitum*. All procedures were carried out in accordance with laboratory protocols approved by the Animal Care and Use of Committee at Rutgers University.

Acoustic Environments

For information about the acoustic environments used in this experiment, please refer to the subsection of the same name in Chapter 2.

Passive Exposure Manipulation

After Zebra finches were randomly selected and assigned to experience CONENV or HETENV, they were removed from the aviary, surgically implanted with an epidural

electrode array (see below), and individually housed in acoustically isolated chambers (Controlled Acoustical Environments, IAC inc.). The birds remained in silence for a duration of 8-10 days during which time baseline lateralization readings could be recorded. Following baseline recordings, each group was passively exposed to its respective acoustic environment for the remainder of the experiment (up to 22 days following the onset of passive exposure). The birds were removed from their chambers every other day for a ~20min electrophysiology recording to ascertain the current state of lateralized activity, after which they were returned to their housing.

Epidural Electrode Array

The epidural electrode array consisted of a modified 10-position, dual-row, Nano strip connector (NPD-10-DD-GS, Omnetics). This connector mated with the corresponding connector of a miniature 9-channel headstage preamplifier (Neuralynx HS-8-CNR-MDR50) that was used to record neural activity. A short (1.5mm) silver ground wire was soldered to the male pin of the Nano-strip that aligned with the reference pin of the headstage. The remaining 8 male pins (2 rows of 4) of the connector array were shortened to a length of 2mm and were bent outwards (from the base) such that the tips of each row of 4 pins was located 1.1mm from the center (0.55mm lateral on each side). This allowed each row of 4 pins to make contact with the dura when implanted.

Head-Fixation Pin and Epidural Electrode Array Surgery

The steps for implanting the head-fixation pin followed the procedure detailed in Chapter 2 with the exception that the cement well was not built. After securing the pin with cement (as described in Chapter 2), the epidural array, held by a stereotaxic arm was

lowered with its long axis centered, and parallel, to the midsagittal sinus, until it made contact with the dura; the correction positioning was defined by the confirmation that the most-caudal pins of the array flanked the bifurcation of the midsagittal sinus (**Figure 8)**. Once all pins were in contact with the dura without causing damage, the silver ground wire (described above) was secured in between the inner layer of skull and the dura (**Figure 8**). Silastic compound (Silicone Elastomer, Kwik-Cast) was used to secure the position of the pins, to isolate them from each other, and to fill the void in the chamber. After the silicon compound solidified, cement was used to secure the array onto the skull and the head-fixation pin. Anaesthesia was discontinued and the birds were allowed to recover under a heat lamp until perching, feeding, and drinking behaviors ensued (30-60 minutes).

Event-Related Potential (ERP) Electrophysiology

Chronic, bilateral, electrophysiological recordings were carried out in a large soundproof booth (IAC Inc., Bronx, NY). The bird was comfortably restrained in a custom plastic tube, the fixation pin was clamped to a stereotaxic frame, and the headstage pre-amplifier was connected to the implanted epidural electrode array. Electrical signals were amplified with a gain of x1000, filtered (bandpass 1-1000 Hz), and digitized with a Power 1401 A/D converter (Cambridge Electronic Design), using Spike2 v7.01 software and custom scripts to record and further process the waveforms. Recordings were obtained in test sessions lasting ~20 minutes conducted every other day both during the baseline period (8-10 days), when the subjects were isolated in silence, and during the exposure phase (20-22 days) when the subjects passively heard either the novel heterospecific or novel conspecific acoustic environments.

Natural female Zebra finch long calls were used as acoustic stimuli to assess lateralized activity, due to the ethological relevance of the stimuli and the heightened responsiveness seen in Zebra finch males that hear a female call (Vicario, Naqvi & Raksin, 2001). Each stimulus set consisted of 3 different female calls (180-300 ms duration; sampling rate: 44 kHz) each presented 100 times in shuffled order (total 300 stimuli), with an inter-stimulus interval of 5 seconds. Stimuli were played from a midline speaker 0.5m in front of the subject at an intensity of 70 dB SPL. At each test point in time, each subject heard a new set of 3 novel calls. The order of stimulus sets was randomized between subjects to account for order and stimulus-specific effects.

Data Analysis

ERP ARM (eARM)

The calculation of eARM is a modified version of the ARM calculation (detailed above and established in Phan et al., 2010) that accommodates the nature of the ERP waveform. eARM is calculated by using the processed waveforms obtained by averaging the first 25 stimulus presentations (for each stimulus separately). Following the aforementioned formula, eARM is defined calculated by subtracting the eARM from the control eARM (500ms prior to stimulus playback) from the eARM during the duration of the stimulus.

eARM Lateralization Index (eLI)

To determine the relative difference in response strength between hemispheres, a Lateralization Index (eLI) was calculated as the quotient of the absolute difference between each hemisphere's eARMs and their arithmetic mean. Additionally, to compare

eLIs between groups and across birds, baseline eLIs were averaged and subtracted from each subsequent LI value; this was done for each bird using their own baseline. This correction was made to normalize the data such that each bird's LI throughout passive exposure was controlled by their own levels of lateralized activity during baseline.

Statistical Analysis

Statistical analyses were performed using OriginPro(2020). The time course of the transient shifts in lateralization was assessed by using the eLI measure and by grouping time into 3-day bins. The nature of the eARM LI set of data did not allow for a repeated measures analytical approach due to an unequal number of data points per time bin. Therefore, the eARM LI data was analyzed in three approaches: factorial ANOVA, post-hoc comparisons, and Multi-Level Model (MLM). To compare time, acoustic environment, and their interactive effect on lateralized patterns of activity, a 2x7 ANOVA was employed with time bins and exposure type as fixed factors. Post hoc comparisons, via independent t-tests, between CONENV and HETENV at discrete time bins were carried out following the omnibus test. Additionally, a three-level model (a type of MLM) was employed to analyze the same dataset due to its multi-level nature, specifically to accommodate for the different number (e.g. 0, 1, or 2 observations per time bin) of observations for a given bird in a given time. Three-level models are generally employed in human research to assess the influence of factors on "observations within days within people", do not have a limit on the number of observations per day per individual, and do not require a measure to be present at every level (Kleinman, *MLM tutorial*).

The criterion of significance for all omnibus tests was set to 0.05 and the criterion for *post hoc* comparisons was Bonferroni-corrected for the number of comparisons (3 pair comparisons; Bonferroni-corrected $\alpha = .0167$). One bird from the HETENV group of the eARM LI data set was dropped from the statistical analysis due to highly variable and inconsistent data.

Results

Lateralized brain activity was collected from each subject by recording ERPs bilaterally to assess the effects of exposure to a novel acoustic environment as a function of time. An eLI was calculated for each bird to enable the comparison of relative lateralized activity longitudinally for a given bird as well as between groups that experienced different acoustic environments. Due the order of subtraction, negative LIs indicate left-biased activity and positive LIs indicate right-biased activity at the time of recording. **Figure 9** illustrates an exemplar time course of changes in lateralized activity for one bird exposed to a heterospecific acoustic environment, wherein LIs were negative during the early days of exposure to a novel acoustic environment and became positive after prolonged exposure. Group-averaged data (**Figure 9)** illustrated the expected effect that was observed in the latter figure dynamic shifts in lateralization were only observed in Zebra finches continuously exposed to a novel heterospecific acoustic environment.

An ANOVA that compared groups across all time points showed and revealed a significant effect of acoustic environment on the LI *($F(1, 46) = 4.650$, $p = .036$)*, however time of exposure did not significantly affect LI *($F(9, 46) = 1.540$, $p = .163$, n.s.).* The null interaction result may have been a byproduct of the inability for the employed ANOVA to account for the binned repeated measures nature of the data. When

the data was modeled via a three-level MLM, again, only the acoustic environment factored significantly loaded (estimated $\beta = .27$, $p = .007$; model conditional $R^2 = 0.646$). To further explore whether the environment effect was related to the duration of exposure, post-hoc comparisons between the COENV an HETENV groups were carried out for LI at different times (Figure 1C). Time bins 6-8, 9-11, and 12-14 (**Figure 9**); all of the comparisons of time bins 0-2, 3-5, 6-8, 9-11, and 12-14 yielded significant differences (*t(46) = -3.620, p = .0004; t(46) = -3.755, p = .0002; t(46) = -3.419, p = .0007,* respectively), reflecting the LI reversal in HETENV birds. No differences were seen on earlier and later time points.

The eight epidural electrodes (4 per side) sample a volume of tissue over the caudal telencephalon that includes structures rostral to NCM; in particular, Field L and CMM. The position of the epidural array pins was defined by the relative position of the caudal-most pins to the midsagittal sinus, which were presumably located above NCM. When samples from epidural electrodes at different rostro-caudal levels were compared, an ANOVA confirmed that the caudal-most pins (closest to NCM) displayed higher activity relative to rostral pins, *F(1, 892) = 16.156, p = .000006* **(Figure 9)**. This suggests that the epidural electrodes have some spatial selectivity and that NCM likely contributes to the ERP data recorded.

General Discussion

Neuroplasticity in the Songbird

The retention of neuroplasticity beyond critical periods can play a critical role in enabling an organism to properly adapt to and navigate within a changing world. In songbirds, specifically among open-ended learners, it is generally accepted that higher vocal centers remain labile due to the seasonal turn-over of neurons that are replaced by younger counterparts (Goldman and Nottebohm 1983), which potentially subserve the encoding of new song-memory routines (Nottebohm, 1989). However, Zebra finches only learn one song (i.e. closed-ended learners). Nonetheless, surprisingly, neuronal incorporation also occurs in the Zebra finch HVC throughout the lifespan despite rendering the overall HVC mass unaffected (Walton et al., 2012) and proportionally compensating for cell death in HVC (Walton et al., 2012). One interpretation of this line of results suggests that a potential function of new neuron incorporation is not only to enable new song learning (in open-ended learners) but to replace "burned-out" cells that are no longer firing properly (burnout-replacement; Pytte, 2010); such a framework also emphasizes the notion that there are cells that require functional replacement as well as the existence of cells that are not readily replaced. Although HVC is a vocal motor nucleus, HVC neurons respond to auditory stimuli (Margoliash, 1992; Soyman & Vicario, 2017) and lesions to this the motor area result in decrements in auditory discrimination (Brenowitz 1991; Del Negro et al. 1998). It is possible that, similar to humans (i.e. Broca's area), the motor area subserves some function in the decoding of communication signals. While the neuronal population that is replaced by newer counterparts in HVC is not fully understood (e.g. HVC-X, HVC-RA; Pytte 2010), it is

possible that a portion of the neurons that are replaced, or a group of the newly arriving neurons, are not exclusively vocal-motor neurons.

The Neurobiology of Lateralization and NCM Plasticity

Changes in neuronal function and structure, as a function of experience, indicate that the brain remains labile in adulthood (Fuchs & Flugge, 2014), presumably in order for an animal to adapt to its changing environment. Neurogenesis and the incorporation of new neurons in functional circuits is a mechanism that can contribute to neuroplasticity; new neurons are not only distributed unequally between left and right NCM, but also the degree of inequality is positively correlated with tutee-tutor song similarity index (a metric related to the ability to generate strong acoustic memories during development; Tsoi et al., 2014). Published and unpublished data suggest that passive exposure to a novel acoustic environment is sufficient to trigger neuroplasticity events in the NCM of Zebra finches as suggested by changes in lateralization patterns and behavior (Yang & Vicario, 2015; Yang, 2016). If new neurons in NCM facilitate the formation of auditory memories in adulthood, then the observed changes in lateralized patterns of electrophysiological activity may reflect the incorporation of new neurons in NCM.

Neurogenesis is commonly studied in the context of learning and memory formation in the rodent hippocampus (for review, see Sahay, Wilson, & Hen, 2011), wherein corresponding bilateral regions have been shown to subserve different functions in memory processing (Klur, Muller, Pereira de Vasconcelos, Ballard, & Lopez, 2009; Goto, Kurashima, Gokan, Inoue, Ito, & Watanabe; 2010). The introduction of new

neurons in neural circuitry is theorized to broaden the processing scope of a circuit by providing additional nodes that allow for the encoding of additional information (Pytte, 2016). While cell proliferation is abundant in the hippocampus, and is often studied due to the connection with learning and memory, neurogenesis is also present in the subventricular zone (SVZ) and provides telencephalic regions with new neurons. Of interest, neurons that were mitotically active in SVZ have been found in HVC (Nottebohm, 1989) and NCM (Tsoi et al., 2014). In songbirds, the NCM is believed to store auditory memories, particularly of the tutor song template (Phan, Pytte, & Vicario, 2006), which is correlated to the relatively lateralized influx of new neurons (Tsoi et al., 2014). Additionally, changes in neuron incorporation in HVC reflect the vocal motor plasticity that is observed in canaries during periods that correspond to mating seasons; a process that is mediated by steroid hormone fluctuations (Cohen, Macedo-Lima, Miller, & Brenowitz, 2016). Since canaries do not sing the same song from one mating season to another, new neurons in HVC may subserve this new learning and are not simply part of a mechanism intended to replace pre-existing neurons. Similarly, the introduction of new neurons into NCM may depend on novel acoustic experiences that challenge the current representation of the acoustic space.

In Zebra finches, new neurons migrate from the SVZ to NCM through glial scaffolding (Pytte, 2016); most protocols agree that it takes up to 30 days (e.g. Tsoi et al., 2014). In the rodent hippocampus, immature neurons exhibit high excitability prior to being integrated into a circuit, at which time ion conductivity decreases and reduces the excitability as they mature (Mongiat et al., 2009). If this property is also present in cells that originate from SVZ, then pronounced changes in left hemisphere NCM activity, in

response to prolonged exposure to a novel acoustic environment, may reflect the presence of more immature neurons with high excitability. If these new neurons are more numerous in the left hemisphere (cf. Tsoi et al., 2014), the higher activity in left NCM after exposure to HETENV might reflect their activity.

To characterize the relationship between passive exposure to a novel set of acoustic statistics, the density of new neurons between hemispheres was compared between hemispheres across birds that had experienced a novel but familiar environment (CONENV) or a novel and foreign acoustic environment (HETENV) for different exposure durations (9D or 30D; see *NCM Neurogenesis Methods* in **Appendix**). Preliminary results that test the relationship between the changes in lateralized activity produced by exposure to HETENV and asymmetry new neuron densities are plotted in **Figure 14.** As it stands, the sample size for each group is not sufficiently robust to make meaningful comparisons across groups, however, by using the degree of ARM lateralization as an index of exposure effect, an analysis on the relationship between NAI and ARM LI was conducted. **Figure 14** illustrates the inverse correlation between ARM LI and NAI, which trended towards significance ($r = .45$, $p=.1$), suggesting that higher electrophysiological activity in the left hemisphere may be related to an increased presence of new neurons in the right NCM relative to the left.

Interpretations from Experiment 1 and Experiment 2

To navigate through an environment, animals must learn about the relationship between stimulus regularities and their consequences. These learning mechanisms rely on the meaningful extraction of information (Ward, Gallistel, & Balsam, 2013) based on the context in which external events occur. This is hypothesized to occur via statistical

learning, which posits that an environment can be described in terms of the frequency distribution of stimuli and their properties. This information set, typically established in a species-specific manner due to typical developmental environment and experiences, can be used to identify common signals and associate them with their contextual relevance.

Conceptually, the brain must adapt to discriminate the stimulus statistics (distribution of acoustic features) of the animal's familiar environment. The neural circuit mechanisms that are sensitive to the acoustic parameters that distinguish sounds can be described as perceptual filters, made up of convergences and combinations of simpler receptive fields of individual neurons. Since the external environment is continually changing, it is necessary for the neural circuitry, that underlies perceptual filters, to be able to adapt to changes in the stimulus statistics in order to ensure discrimination of relevant signals. The overarching goal of the experiments described here is to investigate how cellular activity in auditory centers is modulated by the challenge of a novel distribution of acoustic statistics.

Experiment 1 investigated how NCM MUA is modified in Zebra finches exposed to a novel and foreign environment composed of a canary aviary recording (HETENV). Typical right-lateralized responses in NCM were reversed after 9 days of exposure to HETENV, an effect not seen in birds exposed to a novel yet generically familiar acoustic environment (CONENV). In birds exposed to HETENV for prolonged periods, NCM activity returned to the typical right-biased patterns by 30d of exposure but did not determine just when the return occurred. Experiment 2 used a new method of epidural recording for longitudinal assessment of bilateral activity that is sensitive enough to elucidate the time course of dynamic changes in lateralization after initial and prolonged

exposure to HETENV. The epidural results show that the onset of left-biased activity occurs within a few days of HETENV exposure and the return to typical right-biased activity occurs after ~14 days, consistent with previous observations at fixed time points (Yang, *Dissertation*). In Experiment 3, the observed shifts in lateralized NCM activity were investigated by determining the density of new neurons in NCM in both hemispheres under various exposure conditions. Preliminary data suggest that there may an inverse relationship between lateralized MUA and an index of new neuron asymmetry; there was a non-significant but suggestive trend between higher activity in the left NCM and higher new neuron density on the right NCM.

Together with unpublished behavioral data (Yang, *Dissertation*), these results suggest that NCM retains plasticity into adulthood and that the acquisition of novel acoustic categories may depend on changes in lateralized activity. It is possible that the shifts in lateralized activity reflect the development and or reorganization of receptive fields in NCM neurons which do not fire in their typical lateralized patterns when challenged with dramatic changes in the acoustic environment. The higher activity in the left hemisphere could reflect the novelty of HETENV sounds. An alternative, and untested, explanation for the changes in lateralized activity is that the duration of exposure to HETENV has an effect on the firing pattern of one hemisphere over the other. That is, the elevated activity in the left, relative to the right, hemisphere observed in birds exposed to HETENV for 9 days, does not imply that activity in the right hemisphere has changed. It is possible that the activity in the right NCM remained relatively equal across the exposure to HETENV and that only activity in left NCM changed as a function of exposure; the inverse interpretation also applies, whereby the

dynamic changes in activity are altered in NCM and unaltered in left NCM. This explanation may be partially supported by the general decrease in activity after 30 days of exposure to both HETENV or CONENV. However, the overall decrease in activity could also reflect a site-specific, electrode-biased, and or global adaptation/habituation of activity after persistent exposure to the same 12hr environmental stimuli for 30 days.

Results from Experiment 3 present interesting observations that were contrary to what was expected based on previous knowledge about new neurons and NCM function. Normally- reared adult male Zebra finches have a higher new neuron density in left NCM relative to right NCM, and the resulting left-right ratio is correlated with a Zebra finch's ability to closely imitate the song of their tutor (Tsoi et al., 2014). If the relative neurogenesis rate in NCM between hemispheres is a constant feature that results in individual variability, it is possible that it may indirectly or directly impact a bird's ability to form memories in development and, perhaps, in adulthood. The latter hypothesis, which posits that NCM new neuron asymmetry is correlated with the ability to form acoustic memories has not yet been tested. Acoustic learning and new neuron migrations are constantly taking place occur at different time scales. The former may depend on the difficulty of the learning task and the latter presents a limiting factor since, with the current new neuron visualization methods, new neurons are not detectable in NCM until 30d post mitosis. It also would be very difficult to ascertain whether new neurons come to represent novel features of the acoustic environment. The passive exposure environment presented an opportunity to study the relationship between asymmetric new neuron densities and lateralized activity in NCM. The experimental paradigm involves

acoustic learning on large time scales, and there are detectable changes in physiological activity that corresponds with the type of exposure and its related behavioral outcomes.

The preliminary analysis between new neuron densities and lateralized NCM activity suggested the higher activity one hemisphere's NCM is perhaps correlated with higher new neuron density in the opposite hemisphere. While the sample size is not sufficient to make appropriate analyses across groups to determine the effect of passive exposure on new neuron asymmetries, given the pattern of lateralized activity, it is possible that the introduction of processing nodes to a circuit transiently increases and then attenuates activity in the global circuit rendering activity to be lower in the left hemisphere NCM. However, this depends on the idea that different cellular changes are occurring in the two hemispheres.

It was initially hypothesized that the introduction of new neurons would elevate neural activity since *in vitro* hippocampal electrophysiology demonstrated that immature neurons actively fire with no specific pattern (Mongiat et al., 2009). However, these observations may have been due to the experimental method and or the circuit in which the immature neurons were migrating. In Experiment 3, new neurons were visualized in a relatively mature state once they had arrived to NCM, therefore the level of maturity was not a tested variable in terms of how it affected overall lateralized NCM activity. Furthermore, it is possible that while the visualized neurons were relatively mature, not all of them were destined to survive nor join NCM circuitry. Accordingly, new neurons that were not going to be integrated into NCM might display different firing properties relative to those that were to be incorporated. If new neuron excitability lowers as they mature (Mongiat et al., 2009), this may explain why there was an inverse relationship

between lateralized response strengths and new neuron density. Specifically, lowered relative activity in the right NCM might be explained by the number of mature neurons being integrated into NCM and the higher relative activity in left NCM may be explained by the presence of more excitable new neurons that are not maturing and or incorporating into NCM neuronal circuits.

The current set of studies suggest that NCM retains plasticity in adult, male Zebra finches. The lateralization changes in NCM, observed in birds exposed to HETENV, and not CONENV, indicate that lateralized processes can be modulated by exposure to a novel set of stimulus statistics, are dynamic, and depend on the duration of exposure. These dynamic changes in NCM activity suggest that higher auditory areas exhibit experience-dependent neuroplasticity and an ability to adapt to dramatic changes of the auditory environment. Considering the preliminary results of Experiment 3 and the interrelationship between new neuron asymmetry and NCM memory formation, it is proposed that new neuron densities in NCM may reflect a neurobiological mechanism of neuroplasticity through which NCM is able to accommodate to dramatic changes in the acoustic regularities of the environment.

Future Directions

Neuroplasticity

Neuroplasticity mechanisms are marked by the ability of a neuronal circuit to change through experience (Fuchs & Flugge, 2014). Potential regulators of neuroplasticity include perineuronal nets (PNNs), which are specialized extracellular matrices (ECMs) found throughout the CNS and are first detected in later stages of

development once synaptic circuitry is established and stabilized (Wang & Faweett, 2012). They are mainly composed of hyaluronan (HA), link proteins, chondroitin sulfate proteoglycans (CSPGs) and tenascin-R (Tn-R) (Know et al., 2010). Expression of the PNN components is dependent on normal patterns of activity during development and in some cases correlates to a delay in the closing of sensory critical periods (Sur et al., 1988; Guimaraes et al., 1990; Cornez et al., 2018). Breaking down CSPGs (i.e. dissolving PNNs) with the enzyme chondroitinase ABC (ChABC) has been shown to re-open the critical period for the organization of visual circuitry: ocular dominance plasticity was observed in adult animals who had been raised with monocular deprivation. The decrease in PNN density also resulted in elevated visual acuity and increased dendritic spine density (Pizzorusso et al., 2002). PNNs are not fixed structures and are subject to change in adulthood, suggesting that they may play a role in adult neuroplasticity. Accordingly, adult rats with amblyopia showed a decrease in PNN densities when exposed to a visually-rich environment which partially restored ocular dominance (Sale et al., 2007). Changes in PNN density have also been observed in studies that do not involve sensory-deprivation interventions during critical periods. In adult rats, and not in juveniles, fear conditioning and subsequent extinction resulted in a stronger conditioned response that was correlated to the PNN densities in the basolateral amygdala; juveniles did not produce a large conditioned response suggesting that PNNs protect fear memories in adults (Gogolla et al., 2009). The modulation of PNN densities may reflect a neurobiological mechanism through which juveniles and adults are able to encode new information into a neural circuit by facilitating the proliferation/reorganization of synaptic connectivity and subsequently preserving the connections.

Songbirds also display similar PNN patterns during critical periods for vocal learning in regions associated with the bird's song system. During development, HVC, RA, and Area X undergo changes in morphology, synaptic connectivity, long distance connections, and volume (Bottjer & Arnold, 1997). In zebra finches, higher PNN densities in HVC and RA are related to greater song stereotypy (Balmer et al., 2009). The roles of PNN densities in NCM, CMM and Field L are less understood, although their density relative to motor nuclei is lower (Cornez et al., 2018). The lower PNN densities are suggested to be related to the flexibility that auditory regions (i.e. NCM and CMM) must have to adapt to changing acoustic environments, as shown by the rapid decline in ZENK expression after the first presentation of a stimulus; subsequent repetitions did not elicit ZENK expression levels comparable to the ZENK activity induced by the initial presentation (Mello, Nottebohm & Clayton, 1995). The ability for these auditory nuclei to remain labile suggests that they may play a role in learning novel patterns of acoustic stimuli in adulthood. While the function of PNNs is not well understood in the NCM and CMM, it is possible that changes in PNN densities in these nuclei are related to the formation of new acoustic memories.

Excitation/Inhibition

Experiment 1 investigated NCM response strengths between hemispheres. The origin of the changes in typical lateralized activity remains unknown. A secondary set of analyses could determine, based on the already obtained data, whether a given birds experience and resulting lateralized activity is in part explained by changes in neuronal-type activity. Specifically, the intended analysis will extract single-unit activity from MUA. The goal will be to compare data from narrow- and broad-spike neurons which are

putatively inhibitory and excitatory, respectively. Data based on *in vitro* recording and computational models suggest that, in the context of short-term plasticity, the excitatory/inhibitory (E/I) balance of feed-forward inhibitory circuits can modulate spiking probability and precision (Wahlstrom-Helgren & Klyachko, 2016). The passive exposure paradigm employed in the present experiments occurs on a much longer time scale. If E/I balance is a dynamic source of plasticity in NCM circuitry, it is possible that changes in lateralized activity could be explained by a asymmetry in E/I balance indexed by differential activity displayed by narrow- and wide-spiking neurons.

Serial and Transient Shifts in Lateralization

Experiment 2 demonstrated that the newly developed epidural electrode array can be used to track changes in auditory-related lateralized activity in longitudinal contexts. Additionally, all of the experiments used only one type of HETENV. To test if the dynamic lateralization phenomenon occurs in response to other types of acoustic environments, another HETENV environment would have to be introduced to the subjects. With the novel epidural array, it is hypothesized that the transient shifts in activity could be captured when a bird is exposed to a candidate $HETENV_1$. To test the potential capacity that NCM has to adapt to dramatic and chronic changes to acoustic environments, a future direction would be to track lateralization in birds that sequentially experience different HETENVs: $HETENV_1$, $HETENV_2$, $HETENV_3$… $HETENV_n$. The results of such a study would test the feasibility of conducting long-term experiments with the array, elucidate whether lateralized activity reverses every time the acoustic environment changes dramatically, and potentially explain whether there is a capacity

limit in the songbird's NCM in terms of the ability to represent various novel and foreign acoustic environments.

Appendix

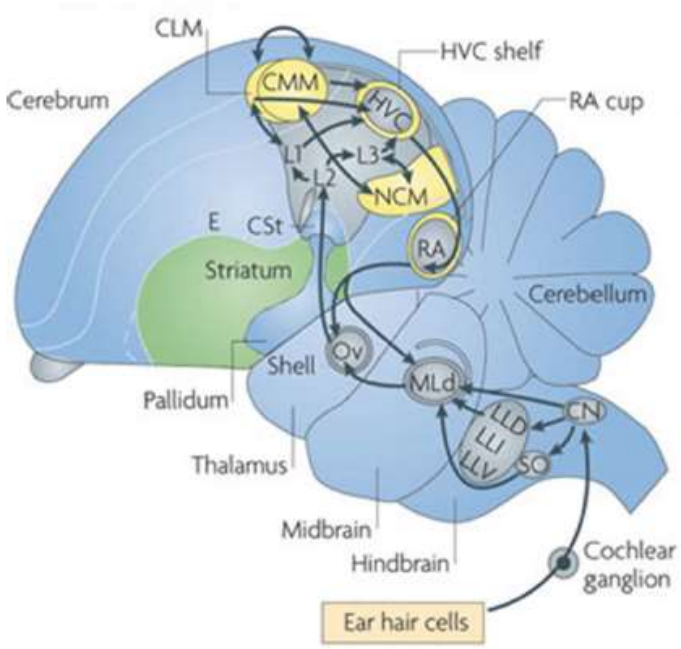

Figure 1. Songbird ascending auditory pathway. Auditory inputs are transduced by cochlear hair cells and their respective ganglia, travel via the avian homologue of the mammalian inferior colliculus (MLD), through the auditory thalamus, nucleus Ovoidalis (OV; homologous to the mammalian medial geniculate nucleus), and project to the primary auditory area Field L2 (avian homologue of mammalian A1). Field L2 then projects to complimentary regions L1 and L3, which subsequently innervate into the avian higher auditory areas caudomedial nidopallium (NCM) caudolateral mesopallium (CLM), and caudomedial mesopallium (CMM). Image adopted from Bolhuis and colleagues (2010).

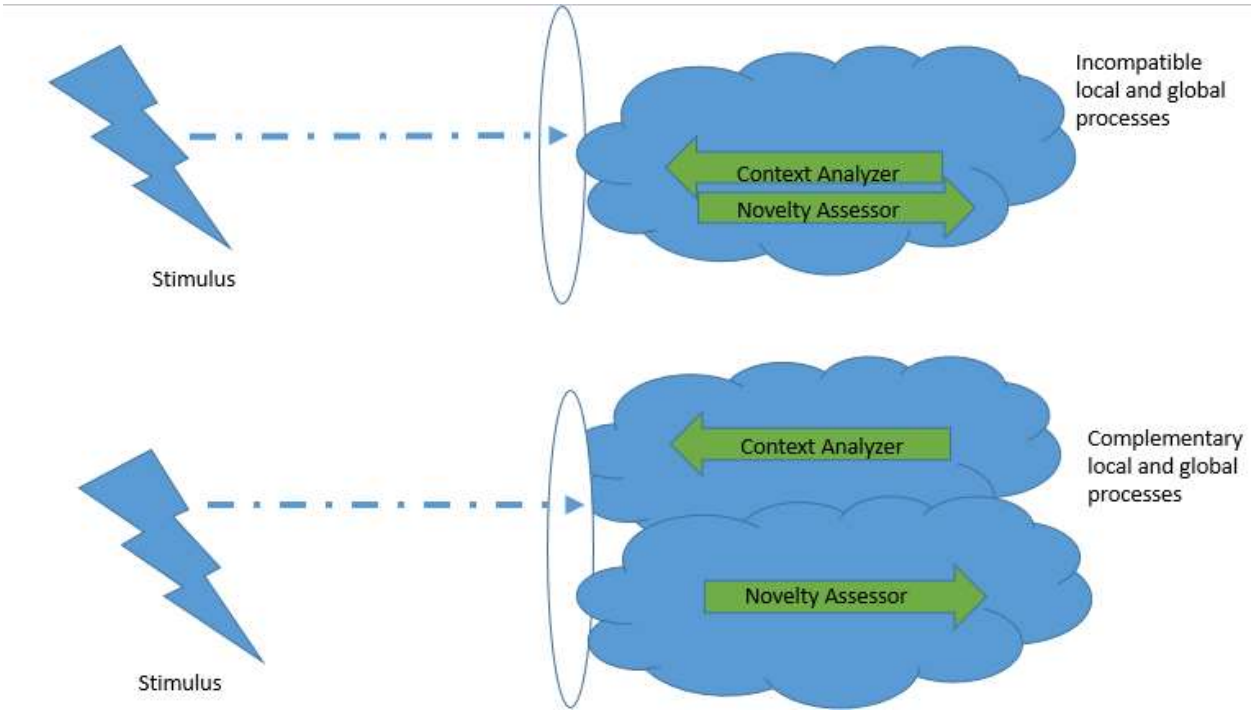

Figure 2. Proposed processing mechanisms subserved by a functionally lateralized brain with two processing streams (below) relative to a brain that processes stimulus features based on a single processing stream (above). Stimuli can be generally processed in terms of the novelty of its event (local) and the context in which the event is experienced (global). Procedurally, these processes are thought to be incompatible in the context of a single-stream processing stream (Sherry Schachter, 1987).

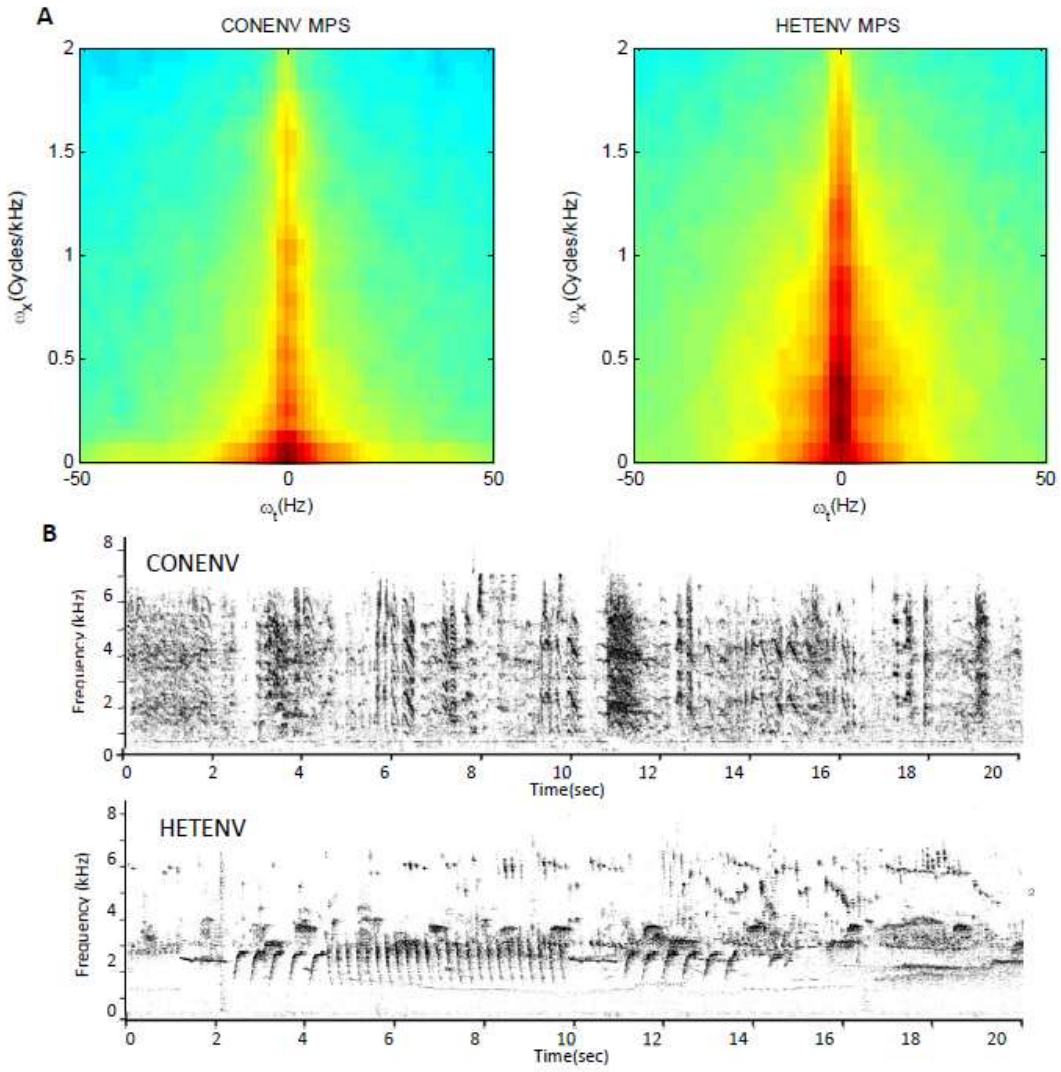

Figure 3. Visual representations of the different heterospecific (HETENV; Canary Aviary Recording) and conspecific (CONENV; Zebra Finch Aviary Recording) acoustic environments. A) Modulation power spectra comparing the temporal and spectral features composed by the cacophonies of each environment type. B) Representative spectrograms obtained from each acoustic environment.

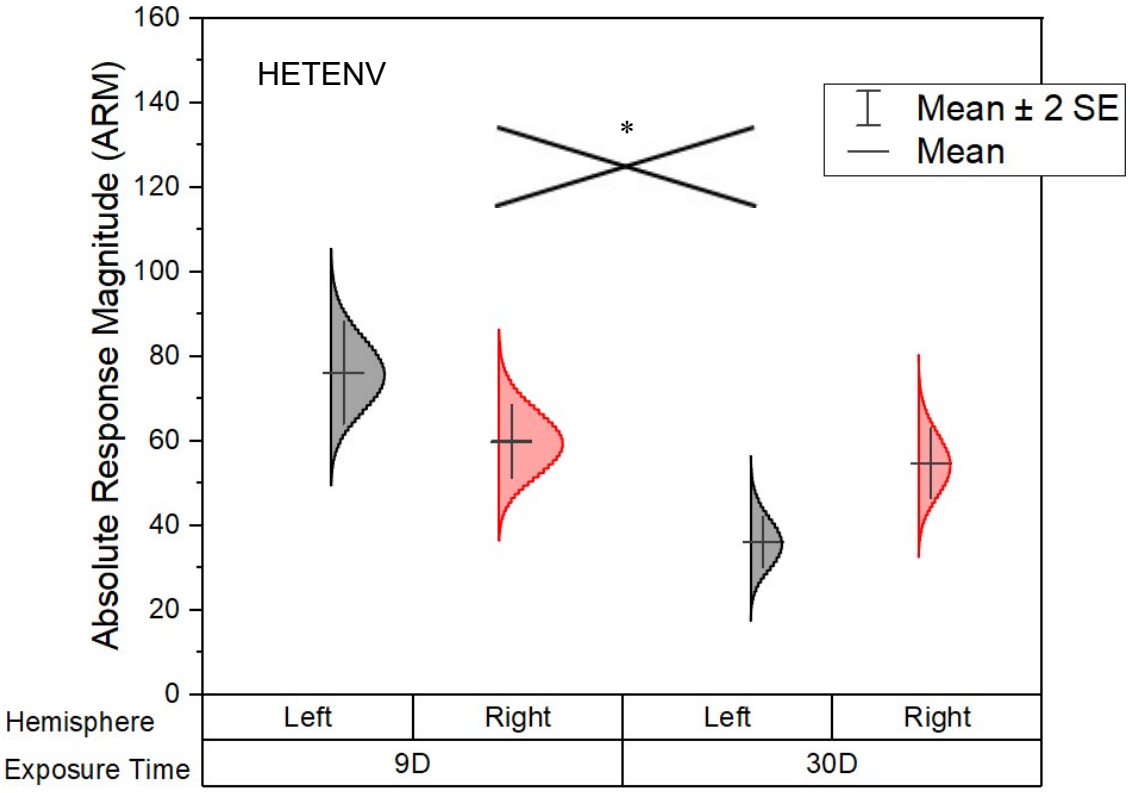

Figure 4. Absolute response magnitudes (ARMs) from groups exposed to HETENV plotted as a function of exposure time and hemisphere. * and crossed lines indicate a significant interaction at the .05 alpha level.

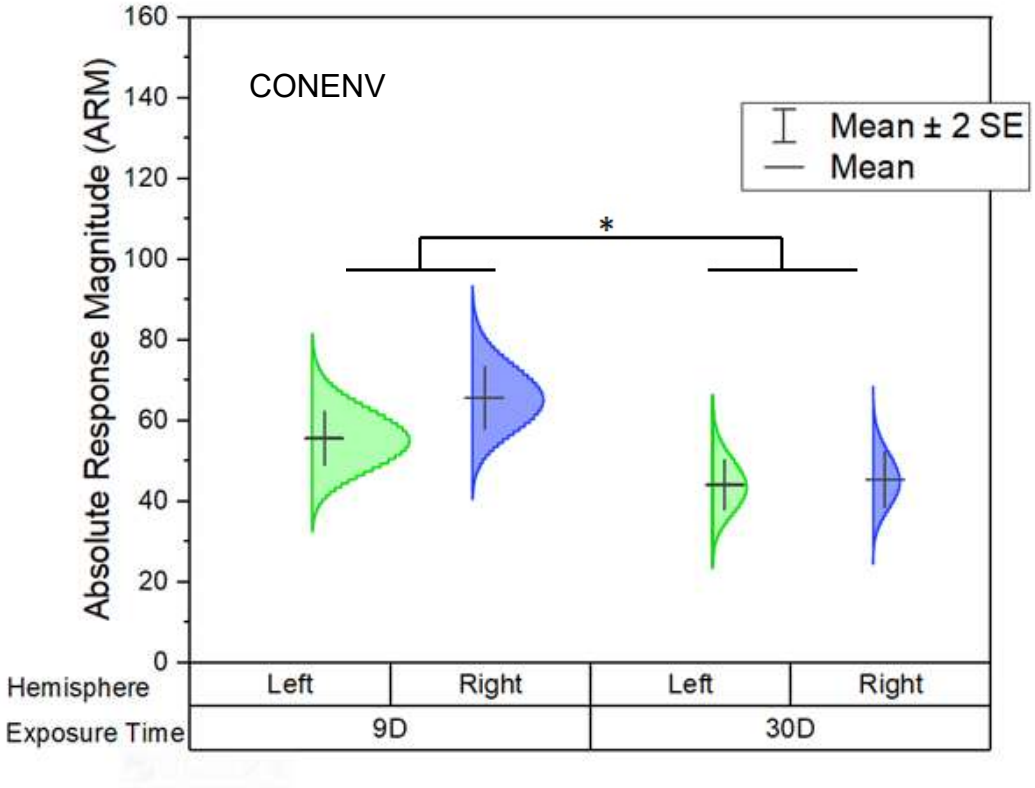

Figure 5. Absolute response magnitudes (ARMs) from groups exposed to HETENV plotted as a function of exposure time and hemisphere. Crossed lines indicate a significant interaction. * denotes significant differences at the alpha level of .05

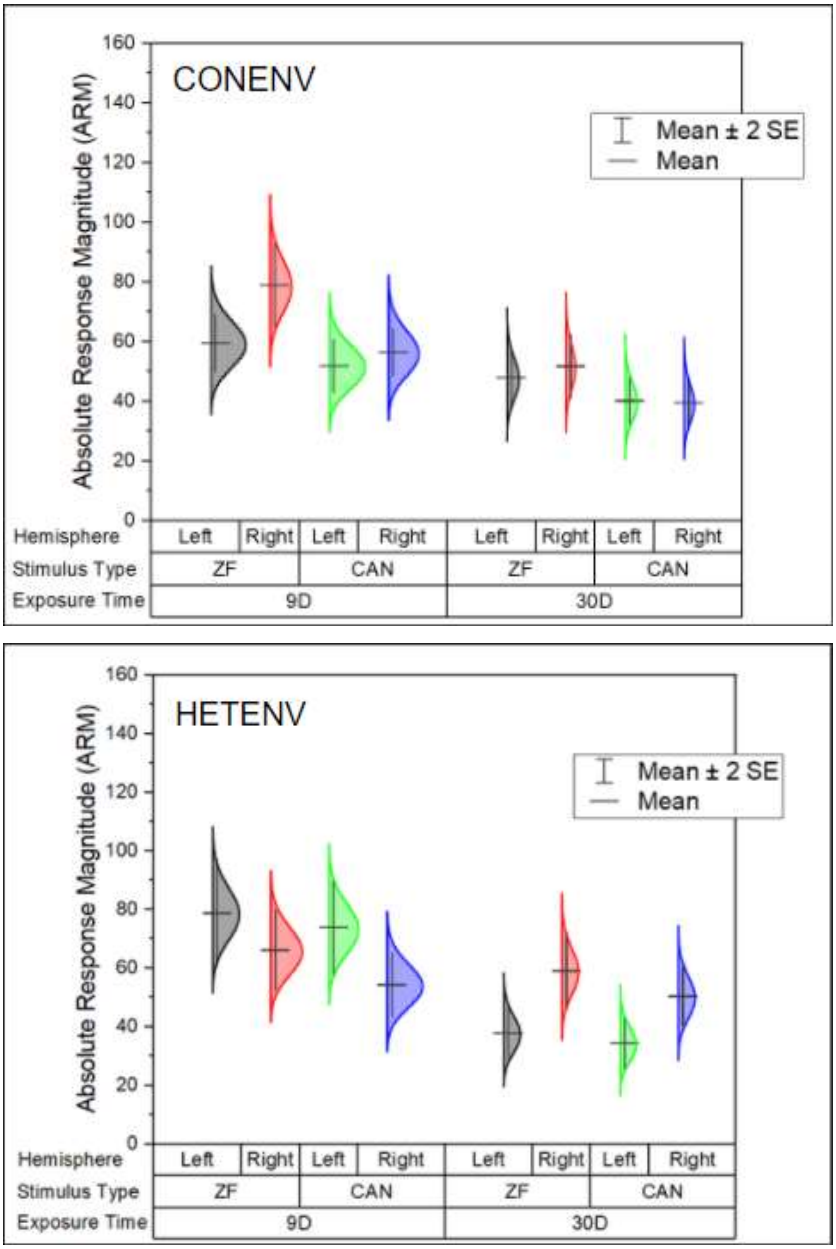

Figure 6. Absolute response magnitudes (ARMs) graphed for HETENV (above) and CONENV (below) groups as a function of exposure time, stimulus type, and hemisphere. There was no significant effect of stimulus type on the level of lateralized activity (left vs right) for any of the groups relative to HETENV, CONENV, or exposure time.

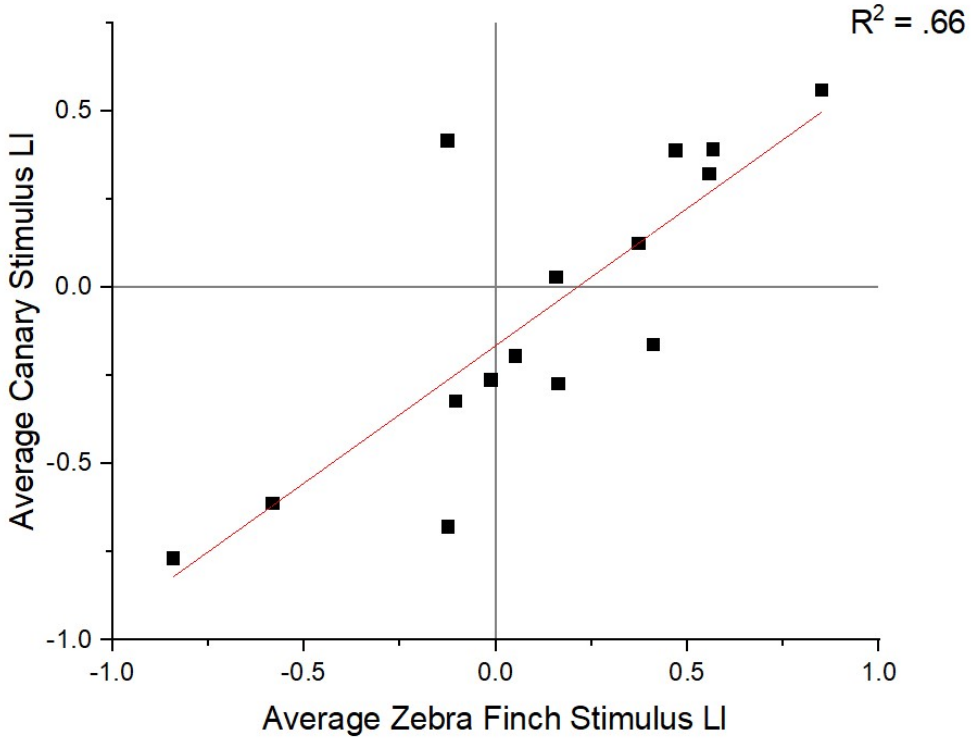

Figure 8. Correlation analysis comparing average lateralization indices (LI) of activity elicited by either Canary (Can; y-axis) or Zebra finch (Zf; x-axis) stimuli. Both stimuli, Can and Zf, elicited, on average, the same pattern of lateralized activity regardless of environmental exposure and exposure time.

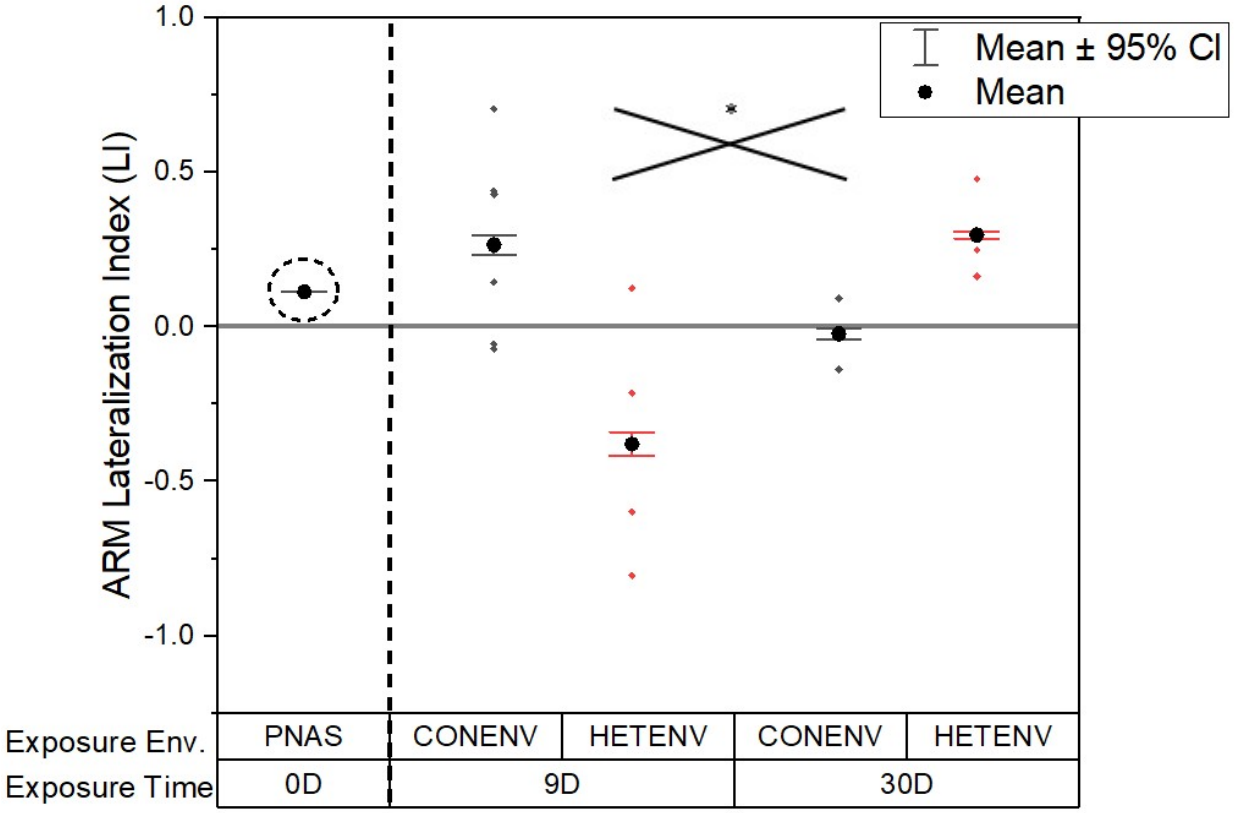

Figure 7. Lateralization Indices plotted as a function of exposure environment and exposure duration. In the left portion of the panel, an LI is plotted from normally reared typical right-lateralized adult male Zebra finches (Phan & Vicario, 2010). * denotes significant differences and the elongated cross describes an interaction at alpha level of .05.

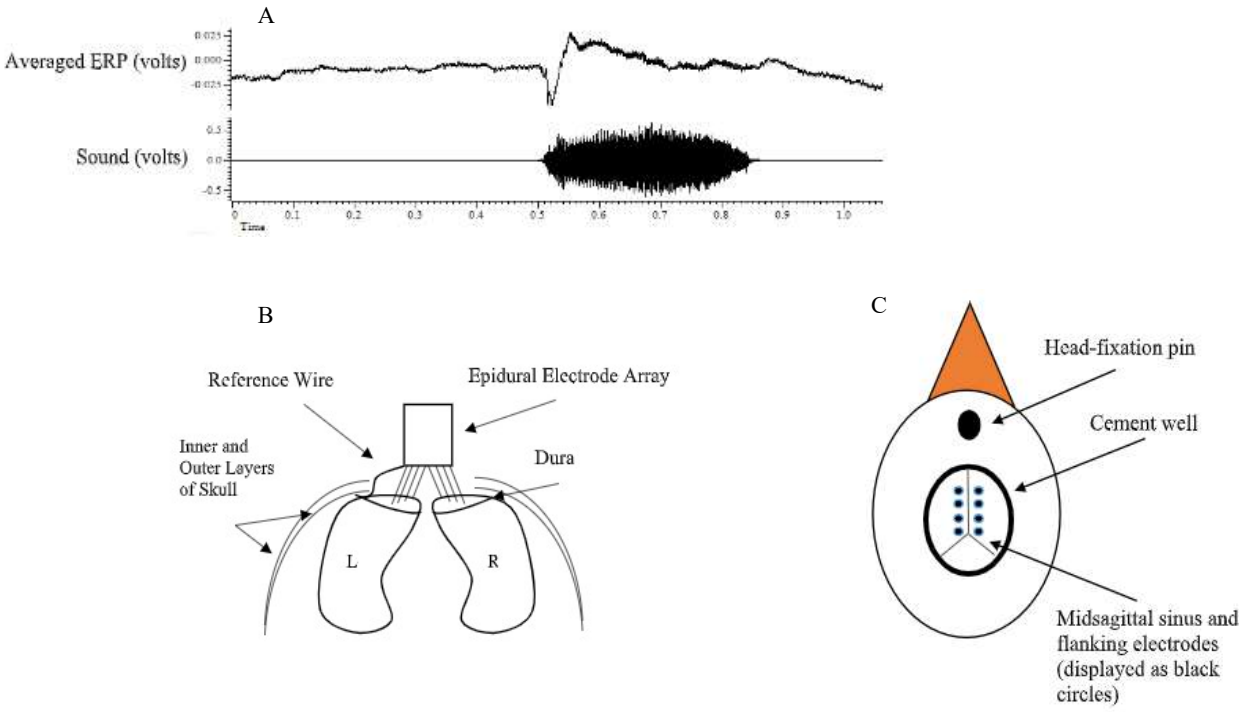

Figure 9. Epidural electrode array positioning and the corresponding Event-related Potential (ERP) activity that is captured at the level of the dura. A) ERP in response to a female call. B, C) Visual representation of how the epidural microarray was positioned on the dura in relation to the midsagittal sinus. The caudal-most pins were positioned in such a way that they were close to auditory region NCM. A silver wire, connected to the array, was tucked in between the dura and the inner-layer of the skull to serve as a reference for the epidural activity that was captured by the recording array-pins.

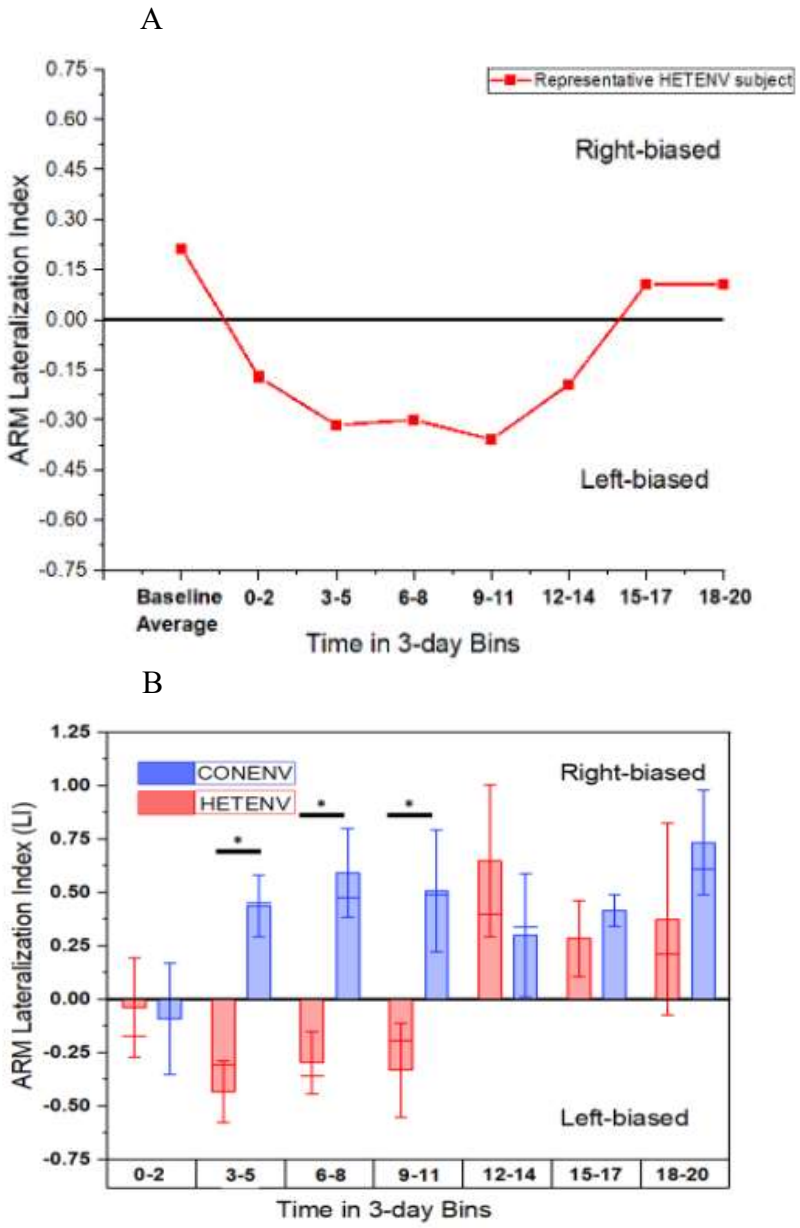

Figure 10. Lateralization Indices graphed as a function of exposure time**.** A) Representative subject exposed to HETENV that displays the dynamic shifts in lateralization as a function of exposure duration, B) Absolute response magnitude (ARM)-derived lateralization indices (LIs) plotted as group averages for HETENV (Red) and CONENV (blue) groups as a function of time of exposure in 3-day bins. Dynamic shifts in lateralization was only observed in HETENV group as reflected by negative LI values from 3-11 days to positive

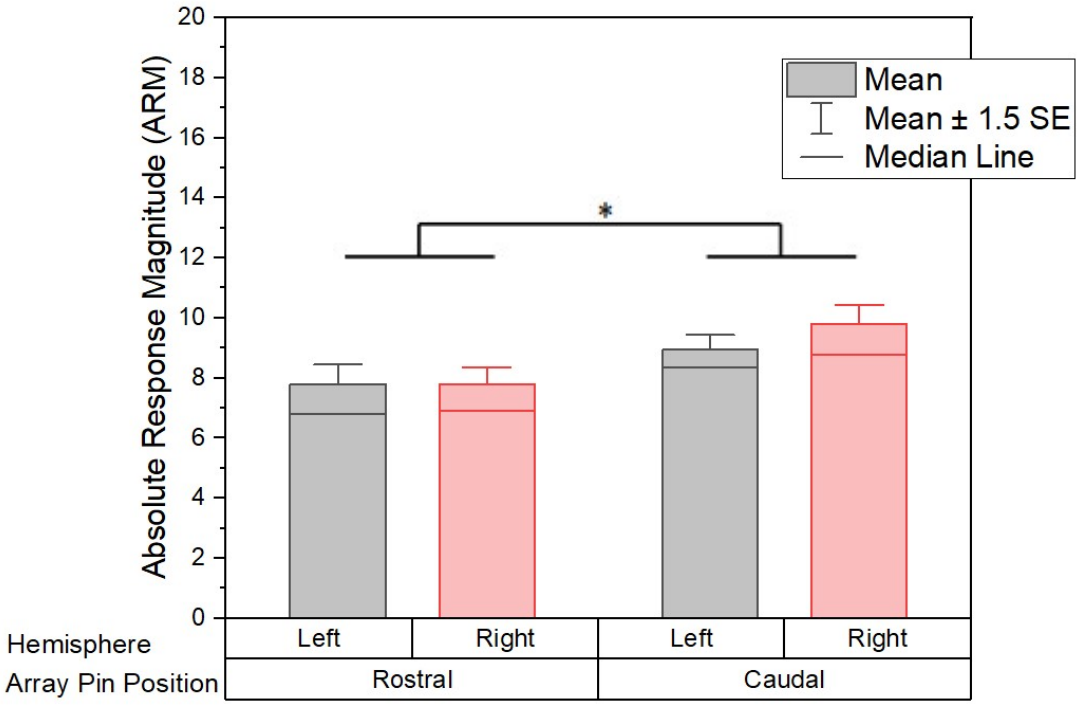

Figure 11. Epidural absolute response magnitudes (ARMs) compared across hemispheres and array pin position (rostral vs caudal). Caudal electrodes captured a higher level of activity than the rostral counterparts. * denotes a significant difference at the .05 alpha level.

Figure 12. Passive exposure paradigm superimposed on the BrdU+ cell migration timecourse. Colored lines refer to the duration of passive exposure to either HETENV or CONENV and the bold black-line illustrates when BrdU injections were administered. All exposure periods end after 30 days from the first BrdU injection, the time at which BrdU-tagged cells can be visualized in NCM.

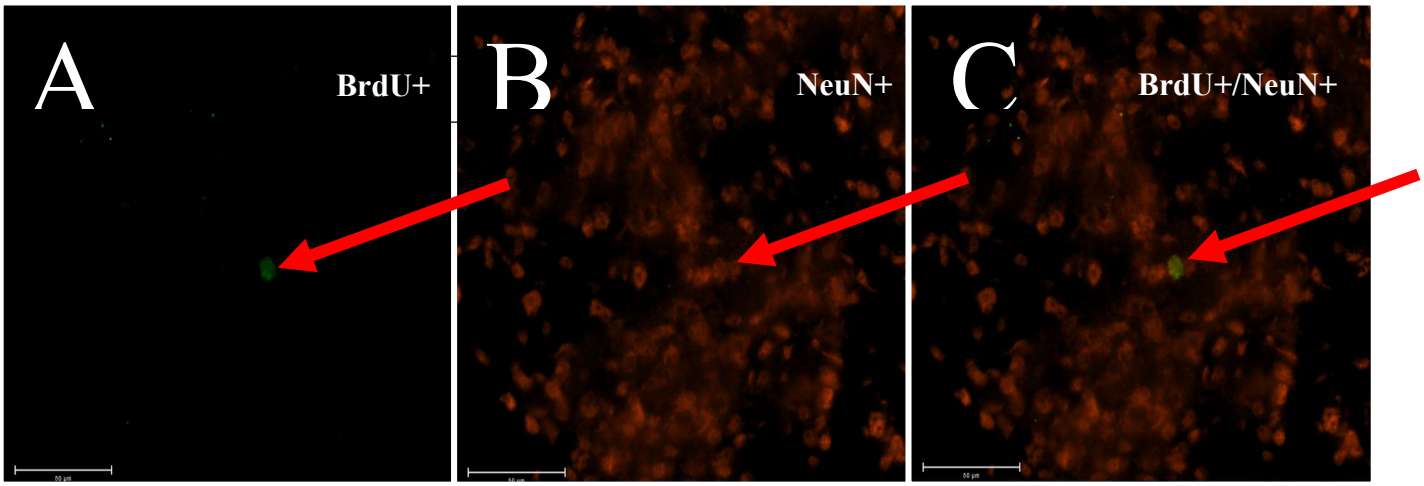

Figure 13. Illustration of how cells were determined to be new neurons. A) Green fluorescence was utilized to visualize new cells (BrdU+), B) red fluorescence enabled the visualization of neurons (NeuN+), C) the collocalization of BrdU+/NeuN+ cells was used to determine if a cell was a new neuron.

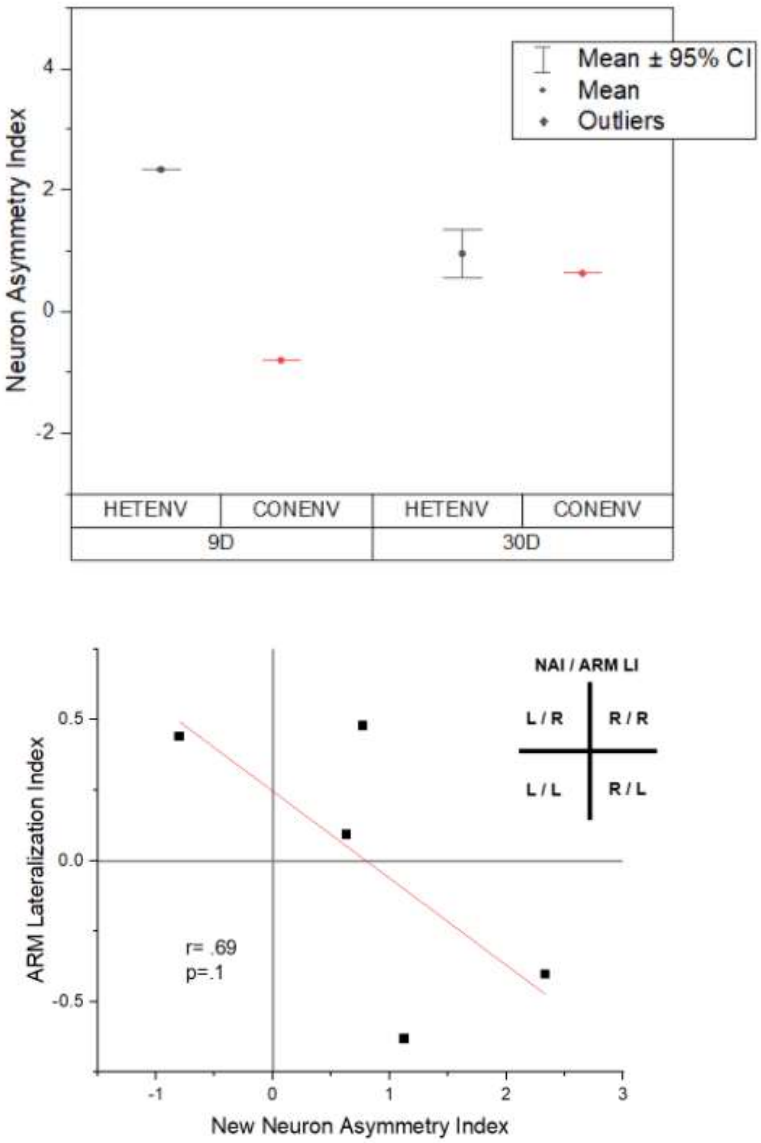

Figure 14. Preliminary results from new neuron data plotted. New neuron Asymmetry index (NAI) graphed as a function of exposure duration and exposure environment (above). A potential relationship between absolute response magnitude (ARM) lateralization index and NAI (below), in the top-right of the graph there is a quadrant explaining the relationship between the two variable in relation to right and left differences (based on LI and NAI calculations).

NCM Neurogenesis Methods

Subjects and Passive Exposure

The subjects utilized in this chapter were the same as described in Chapter 2. Following microelectrode electrophysiology, the birds' brains were prepared in order to visualize the effects of passive exposure on NCM neurogenesis.

BrdU Injections

Bromodeoxyuridine (BrdU; 78 µl of a 10 mg/ml solution in 0.1 M Tris buffered saline, pH 7.4) injections were administered intramuscularly 3 times a day for 3 consecutive days, with the first injection being given 30 days prior to electrophysiology and brain extraction. BrdU solution and injection administration followed a protocol used by Tsoi and colleagues (2014).

Histology

After the electrophysiology, deeply anesthetized birds were transcardially perfused with a 9% saline solution (~30mL), followed by cold 4% paraformaldehyde (~30mL) and de-braining. The brains were placed in 4% paraformaldehyde solution for one hour, followed by three consecutive 7.4 pH PBS washes with one hour in between. After the last PBS wash, the brains were placed in a 15% sucrose in PBS solution until they sank to the bottom of the container and the process was repeated in a 30% sucrose in PBS solution. The brains were then frozen in OCT at -20 °C, cut into 8-12 µm (for BrdU IHC) sections, and stored at -20 °C until reacted.

Immunohistochemistry

To enable the visualization of new neurons in NCM, brain sections underwent a series of immunostaining procedures that were intended to target cellular DNA that

contains BrdU (BrdU+) and NeuN expressing cells (NeuN is a neuronal marker). This protocol was adapted from a previously established protocol (Tsoi et al., 2014), with the exception that the Citrate Buffer procedure was not utilized (as suggested by antibody vendors) as it damaged and or consistently yielded sections that were poorly stained, rendering images unusable. Immunohistochemistry was initiated by removing frozen brain sections and allowing them to reach room temperature (~25C) in PB and placing them in an incubator until they reached a temperature of 37C. Next, they were submerged in 2.5% pepsin in 0.1 N HCl (maintained at 37°C) for 5 minutes, washed in a series of three 3 minute PB washes, blocked in a PB-based 10% normal donkey serum (NDS) and 0.3% Triton-X 100 solution for 1 hour at room temperature, and incubated overnight at 4 °C with sheep anti-BrdU blocking solution. Next, brain sections were reacted with an avidin/biotin blocking kit and blocked overnight with a biotinylated donkey anti-sheep solution. Following PB rinses, the slides were reacted overnight with a streptavidin conjugated Alexa 488 solution. Next, neurons were labeled by reacting the brain sections initially with a PB-based 10% normal donkey serum (NDS) and 0.3% Triton-X 100 solution for 1 hour followed by an overnight incubation period with a blocking solution with monoclonal anti-mouse NeuN. The fluorescent label was added the following day by reacting the slides with CY-3 conjugated donkey anti-mouse solution for 1 hour. Finally, sections were cover-slipped with Vectashield+DAPI to preserve label fluorescence and provide nuclear staining.

Fluorescent Microscopy and New Neuron Quantification

Fluorescence microscopy was carried out with an EVOS FL Auto 2 microscope (ThermoFischer) on brain sections to visualize cells that were mitotically active (BrdU+)

and expressed neuronal protein (NeuN+). Five to seven histological sections were imaged at regular intervals from ~200 µm to ~500 µm from the midline of each hemisphere and were matched across subjects and conditions according to depth. Neurons were visualized using an RFP filter for NeuN label, and new cells were visualized using a GFP filter for BrdU label. Cell profiles that showed co-registration of the two labels were counted as new neurons.

Imaging and counting of co-labeled neurons was undertaken by two separate groups of experimenters, both blind to condition or condition and hemisphere respectively. The surface area of NCM that was visually scanned was maintained as a constant across all sections. The examined area was defined as a rectangle, of a known length (3,200µm) and width (1,200µm), inscribed within NCM, as determined by a Zebra Finch Histology Atlas (http://www.zebrafinchatlas.org/gene_display/histological-atlas). Prior to imaging, the microscope operator defined the aforementioned rectangle and, starting from the top-left corner, visually scanned NCM frame-by-frame with a 40x objective by moving 250µm laterally and or ~196 µm longitudinally (area of frame at 40x = 196µm X 250µm). Photomicrographs were only captured when BrdU+ cells were visible via the GFP-filter (**Figure 13a**), at which point the RFP-filter was used to capture an image of the NeuN+ cells present in the same frame (**Figure 13b**); the corresponding pictures were co-registered via ImageJ (Schneider, Rasband, & Eliceiri, 2012) or Evos AutoFL2 Imaging software (Thermofischer; **Figure 13c**) for counting.

Data Analysis

Based on a predefined counting system, new neuron quantification was undertaken by a group that was blind to hemisphere and condition. The proportion of new

neurons in NCM for a given hemisphere was calculated based on the total sum of new neuron observations divided by the total surveyed volume (i.e. 3,840,000μm^2 X sections of the assayed hemisphere). To determine the relative new-neuron counts between hemispheres for a given bird, a Neuron Asymmetry Index (NAI) was calculated by calculating the difference between new neuron densities from each hemisphere's NCMs and diving it by the combined neuron density of both hemisphere's NCMs (cf. Tsoi et al., 2014).

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