1. FUNCTIONAL MAGNETIC RESONANCE IMAGING IN THE ZEBRA FINCH

1.1. Why use brain imaging?

Non-invasive Magnetic Resonance Imaging (MRI) and functional Magnetic Resonance Imaging (fMRI) have transformed human brain research by providing high resolution images of brain anatomy and brain activation in time scales of several seconds (Huettel, Song, McCarthy, 2004). In animal research there are many more options for studying neural activity and physiological responses in the brain, but fMRI is now being widely used also in non-human primates to complement traditional electrophysiology, and to study neural representation at a global level in the visual, auditory and limbic systems (Huettel, Song, McCarthy, 2004; Van der Linden et al. 2007). The current study focused on area of activation and intensity features of the hemodynamic BOLD response to biologically relevant auditory stimuli. FMRI does not provide an alternative to traditional techniques (microelectrodes for multi-cell recordings) of studying song representation in the songbird brain, but, as in the monkey, it can provide an additional scale of investigating spatial representation of perceptual encoding by looking at the entire brain.

1.1.1. *Spatial scales of brain activation*

Single and multiunit electrophysiology has been widely used in songbirds to study how neurons in different parts of the song system are involved in hearing, singing, and learning song (Solis and Doupe, 1997; Woolley and Casseday, 2004). These methods have provided a basis for understanding neural activity and neural interactions, projections between nuclei, and general characteristics of song system nuclei (i.e. “sparse-firing” motor nucleus, secondary auditory processing area) (Hahloser, Kozhevnikov, Fee, 2002; Mooney 2000). But the activity of single neurons does not tell the entire story of how the brain responds to a stimulus or how the bird perceives the stimulus. Simultaneous recordings from populations of neurons indicate that sensory percepts might be encoded in a highly distributed manner and across a broad spatial scale that cannot be captured by single and multiunit recordings (Logothetis, 2002). However, simultaneous recording from many neurons is expensive and often problematic in terms of sampling and recording quality.

Recent advances in molecular biology in the past quarter century include technology and techniques that allow tracking and visualization of gene expression throughout the brain. These methods have made it possible to look globally at changes in gene expression triggered by behaviors, perception, or learning (review Mello, Tarciso, Velho, Pinaud, 2004). Several genes were identified that showed a rapid and brief increase, in specific brain areas, in response to certain behaviors or sensory stimulation (and corresponding neural activity). Several of these genes, so-called Immediate-Early Genes (IEGs), were identified independently in different species (including mice, frogs, and invertebrates); and although given different names (Zif268, Egr1, NGFIA, Krox24) they

were found to be homologous (Mello, Nottebohm, Clayton, 1995). IEGs are closely related to neural activity: expression is dependent on membrane depolarization that leads to calcium intake and triggers upregulation of gene-expression (Matthews, 1986). Changes in IEG expression are not merely an outcome of neural responses, but often an indication of plasticity (i.e. song learning) (Mello, et al., 2004).

The first IEG studied in the songbird brain (Mello, et al., 1995) was given the acronym ***zenk*** to acknowledge previous names given to the same IEG. Studying *zenk* expression has led to several discoveries that demonstrate the importance of looking at brain activation at larger spatial scales. *Zenk* expression after song playback was very strong in a large portion of the most posterior medial part of the forebrain hemispheres, an area that had been previously uninvestigated and subsequently was named NCM (caudal nidopallium). Further studies found that NCM represents songs and vocal sounds in a unique way: it has a high-capacity for memories of individual songs (Chew, Vicario, Nottebohm, 1996), it represents the novelty of each song (Mello, et al., 2004) and it has a special representation of songs that the bird uses as a template for imitation (Phan, Pytte, Vicario, 2006). *Zenk* induction to song exposure also confirmed electrophysiological observations of hierarchical processing in primary and secondary auditory regions, for example: neurons in primary auditory areas (Field L) fire to a range of sounds, and neurons in secondary auditory nuclei, NCM and CMM fire selectively to more complex stimuli. Differences in *zenk* expression in these regions also reflect complex stimuli (familiar vs. unfamiliar song) and subtle differences in stimuli (directed vs. undirected song) in (Woolley and Doupe, 2008).

Zenk is also associated with singing behavior, not just passive listening. Singing induces *zenk* expression in song nuclei in the male brain (Jarvis and Nottebohm, 1997), even more interesting is that *zenk* expression in song nuclei is sensitve to the social context of the singing behavior: forebrain motor-nuclei show strong *zenk* expression during “practice” song (when the male is singing alone) but the same nuclei are silent, showing no *zenk* expression during female-directed singing of the same song (Jarvis, Scharff, Grossman, Ramos, Nottebohm, 1998). These examples illustrate how large-scale brain imaging can capture the most biologically salient response patterns, which could not be detected using single or multiunit recordings in areas of interests.

Visualization of activity-dependent gene-expression is a wonderful tool for functional brain imaging, with one major disadvantage: it is a terminal procedure. IEG detection requires removing and preserving brain tissue (i.e. terminal preparation). *In situ* methods do not allow for probing development of gene-expression related behavior in the same bird, or observing responses to different stimuli or conditions in the same brain (bird). FMRI, in contrast, is a non-invasive, in-vivo brain imaging technique that can provide multiple data points (time and stimuli) in the same subject. We are interested in experience-dependent global changes in the brain; how experience shapes the spatial topography of the brain’s response to (and perhaps the bird’s perception of) different sounds. Therefore we used fMRI to visualize auditory responses *in vivo* on a global scale.

1.1.2. *MRI Basics*

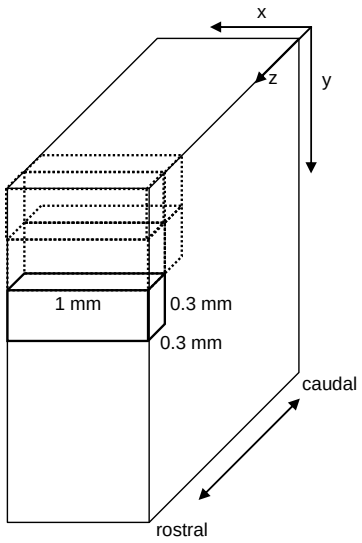
Magnetic Resonance Imaging is used to visualize structures and functions in the body and provides greater contrast between tissue and greater detail than earlier biomedical imaging methods (e.g. Computed Tomography, X-Ray), in particular for soft tissues (Song, Heuttel, McCarthy, 2004). The basic principle of MRI is detection of the response of atomic nuclei in tissue to excitation by electromagnetic radiofrequency waves. In particular, hydrogen atoms are abundant in biological tissue; the hydrogen nucleus (a single proton) emits a weak electromagnetic signal in response to incoming radiofrequency waves if exposed to a strong external magnetic field (Hornak, 2008; Logothetis, 2008). Different tissue types vary in their concentrations of protons and how they interact with surrounding atoms, and therefore in the properties of the signal emitted. This variation accounts for the contrast between different tissues (bone, white matter, gray matter, CSF) and produces a relatively high-quality, high-resolution representation of the tissue being imaged (Logothetis, 2002).

Functional imaging uses MRI technology to look at physiological changes in the brain that are related to neural responses to stimulation (i.e. auditory, visual stimulation). Functional MRI does not observe electrical (neural) activity directly but detects patterns of blood flow and oxygenation that are associated with neural activity via neurovascular coupling, or specifically, the hemodynamic response. Neural activity (induced by sensory stimulation) increases the metabolic demands of cells in the active region, and increased demand is met by increases in cerebral blood flow (CBF), cerebral blood volume (CBV), and oxygen content of the blood (increased oxyhemoglobin) (Logothetis, 2002, 2003; Logothetis and Wandel, 2004). Changes in each of these parameters contribute to the hemodynamic response and can be detected with different fMRI techniques. The current study focused on the blood-oxygen level dependent effect (BOLD) in the hemodynamic response: changes in ratios of oxyghemoglobin and deoxyhemoglobin before and after neural activity induced by sensory stimulation. Unstimulated tissue contains a relatively large percentage of hemoglobin in deoxygenated form (Logothetis, 2002) and deoxyhemoglobin causes protons to “relax”, or cease emitting a signal, very quickly. Highly oxygenated blood (high percentage of oxyhemoglobin), which surrounds active neural tissue, causes slower relaxation times and stronger signal.

Units of fMRI are based on three-dimensional volume-elements, “voxels”, determined by the brain slice activated in a plane (e.g. sagittal, coronal) and a two-dimensional grid within each slice. Protons in each voxel of the grid (coordinates in row and column of brain slice) provide a unique signal resulting from the phase and frequency at which they spin about their axes. Voxel size reflects spatial resolution of the scan; th experiment used a voxel size of 1 mm (sagittal slice thickness) x 0.3 mm x 0.3 mm (Figure 2-1).

Figure 1-1

Schematic of voxels in one slice in the sagittal plane.



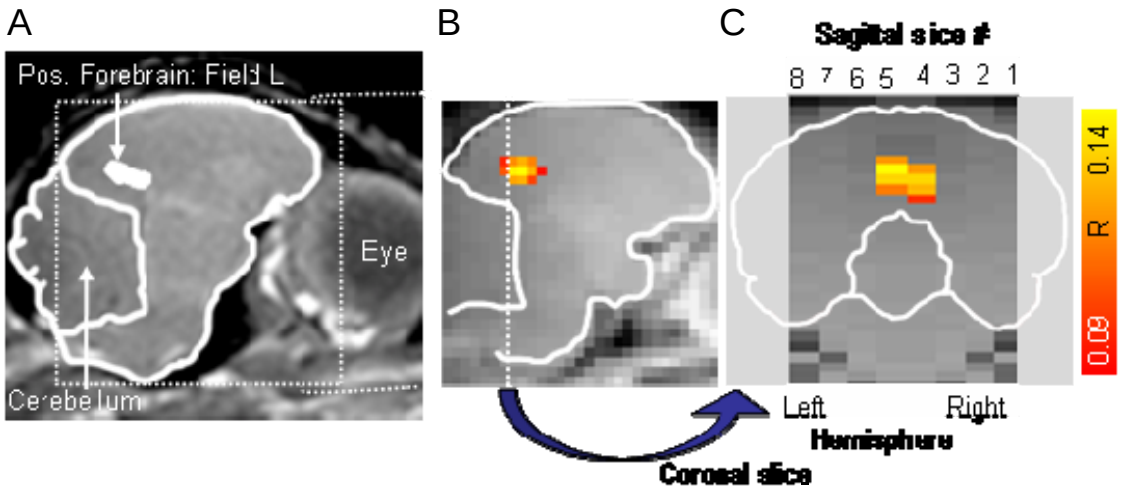
In the current study, for each bird we formed a parametric activation plot overlaid on the anatomical scan (MRI image); this representation shows general location of **volume of activation** (area or spatial extent) and **intensity of activation** (strength of BOLD response). Figure 2-2 shows an example parametric activation plot, with activity centered in the posterior forebrain.

Volume of activation: statistical significance is defined voxel-wise by correlating the signal intensity with the "on-off" block stimulus indicator function (i.e. stimulus-on; silence). Area of activation is the number of voxels with an intensity of activation corresponding to $p < 0.005$ (multiple test corrected significance).

Intensity of activation: Average percent of BOLD signal change from stimulus-off (silence) block to stimulus-on (stimulation). The strength of BOLD effect is measured as the significantly activated number of voxels (voxelwise $p < 0.005$) in a region of interest against overall signal intensity (baseline). This is summarized as the percentage change in signal over baseline (e.g. mean BOLD effect 3%).

Figure 1-2

FMRI activation plot in the zebra finch. A. The posterior forebrain is the region of interest, with expected activation in and around Field L, the primary auditory region. Slices were prescribed sagittally from left to right. **B.** A parametric map of activation and intensity: each colored voxel indicates stimulus-related activity (during stimulus) that is significantly above baseline activity (silence) ($p < 0.005$); colorscale indicates intensity of BOLD response (response amplitude relative to mean signal intensity for all active voxels). Yellow voxel indicates greater signal (higher correlation coefficient between signal intensity and stimulus-on period of trial) and red indicates lower intensity. This example activation plot is cumulative over birds and stimuli (multiple bird groups, multiple stimuli). **C.** Coronal slice through mean activation in the region of interest (posterior forebrain) showing that activity is centered in the medial slices (4 + 5) of each hemisphere and the posterior region of the forebrain (from Maul, Voss, et al. under review).



1.1.3. *The effect of behavioral state on auditory responses in the songbird forebrain*

Functional activity of the cerebral cortex and its coupling with blood flow and metabolism is very sensitive to anesthetics (Van der Linden, et al., 2007). This coupling between cerebral blood flow and metabolism may be significantly reduced by several anesthetics commonly used in birdsong research, such as urethane (Van der Linden, et al., 2007). Neural responses are also sensitive to behavioral state, and changes between natural sleep and waking state have profound effects on auditory responses of single and multiple units in auditory and song learning nuclei of the songbird forebrain. For example, neurons in primary and premotor motor nuclei respond strongly to BOS during sleep, but these auditory responses are eliminated in the wakeful state (Dave, Yu, Margoliash, 1998; Nick and Konishi, 2001). Induced sleep (sedation, anesthesia) also increases selectivity of auditory responses in song nuclei and diazepam specifically has been shown to increase selectivity to the bird's own song in the major premotor song nucleus but appears to have no effect on auditory responses in the primary auditory nucleus, Field L (Cardin and Schmidt, 2003).

We used natural stimuli and light sedation (rather than urethane or other heavy anesthetics) because we are interested in observing responses that are relevant and possibly reflective of the bird's perception of meaningful sounds. Initial attempts to scan fully awake, restrained zebra finches resulted in

noisy and unreadable images due to movement artifacts; birds mildly sedated with Diazepam remained calm and still during the scan and their scans produced useful data.

1.2. fMRI procedure

Birds were brought to the MRI facility and allowed 20 minutes in cages to become accustomed to the new environment. Birds were then sedated with 40 µl Diazepam (Abbott Labs.) i.m. (1.34 mg/ml Diazepam in saline solution) 10 min before MRI scanning and immobilized in a restraining device made of two soft plastic tubes. The head tube was fixed in a custom-made coupled solenoid-type radiofrequency coil (CBIC, Henning Voss), Figure 2-3. The restrained bird was then placed into a custom-made sound-attenuating box that fit within the scanning bore (dimensions: inside box 25 x 18 x 12 cm – height; outside box 40 x 30 x 29cm - height). The walls of the box consisted of multiple layers of acoustical foam and the outside form was wrapped in several layers of acoustical rubber; the chamber provided attenuation of about 20 dB.

Figure 1-3

Sedated bird in MRI radiofrequency coil (surrounding head) and restraint. Bird is immobilized in plastic tygon tube, and additional tube and radiofrequency coil surround head. The coil is a transmit/receive head coil: it transmits radiofrequency signal to excite protons, and receives electrical current (radiofrequency signal) from these protons as they relax after additional RF pulses have been applied (from Voss et al, 2007).

1.2.1. *MRI Parameters*

In this experiment we used an Echo Planar Imaging (EPI) sequence. EPI is a fast MR imaging technique that allows high quality images, reduced imaging time, decreased motion artifact, and ability to image rapid physiologic processes (Poutschi-Amin, Mirowitz, Brown, McKinstrey, Li, 2001). The EPI sequence allows us to obtain all spatial data after 4 radio-frequency excitations per stimulus trial (four-shot gradient echo sequence), rather than the 100 or more RF excitation pulses that would be required using a more conventional pulse sequence (Poutschi-Amin, et al., 2001). But EPI also requires increased gradient strength and durability of hardware.

BOLD sensitive images were acquired on a GE Excite 3.0 Tesla MRI scanner used for human and animal research. Each voxel measured 1 mm (sagittal slice thickness) x 0.3 mm x 0.3 mm. Slices were

prescribed in right-left direction, covering the forebrain. Additionally, in-plane anatomical images and image correction maps were acquired.

1.2.2. *Auditory Stimuli – syllables and songs*

We took an ethological approach, examining responses to naturally-produced biologically relevant stimuli including: Bird's Own Song (BOS), Conspecific song (CON - same-species song, unfamiliar to the subject bird), Tutor song (TUT). As a reference, we also exposed the bird to a pure-tone of 2 kHz (a simple, unnatural sound well within the hearing range of the bird).

Auditory stimuli were delivered using a pair of stereo headphones with the magnets removed (magnetic field of the MRI scanner is strong enough to replace the field of the headphone magnets). The sound pressure level of the auditory stimuli at the head position was about 100 dB, the background noise during the EPI sequence about 83 dB. Stimuli were normalized with respect to peak amplitude.

1.2.3. *Birds*

Sixteen male zebra finches were raised by an adult male tutor from day 15 post-hatch to day 100 post-hatch. Multiple birds were raised in groups of two to six with a single male tutor. All birds were kept in a common room, thus they could hear each other, but birds in each group had the closest social contact and auditory input from the male in their cage. Birds were scanned in late-adulthood between 24-48 months.

1.3. Results

Our principal motivation in performing fMRI is to be able to characterize features of auditory responses to learned vocal sounds, and (potentially) discover how those evolve and differentiate in an experience dependent manner. The fact that BOLD captures brain activation in response to sound does not necessarily mean that it captures interesting features of those responses. In using fMRI as a potential indicator of perception, it is important to test that the BOLD signal we obtain from the zebra finch brain reveals differences in responses across sounds that should have different biological significance to the bird. The limitation is not only in the spatial discrimination of our 3T machine, which provides a relatively coarse representation of the small auditory centers of the zebra finch brain, but one could imagine that biologically meaningful differences in responses would not express themselves at all in the scales we are observing. With this in mind, we examined two features of the BOLD signal: the area of activation of BOLD response and the BOLD signal intensity (both could show sensitivity to stimulus).

Van Meir (2005) is the first report of BOLD-fMRI in songbirds, with scans of anesthetized starlings. The zebra finch is a more commonly studied songbird in the laboratory, and we succeeded to obtain significant BOLD responses in the awake (but sedated) zebra finch. BOLD activity centered on posterior forebrain auditory areas (medial slices covering NCM), and hemispheric differences in both extent of activity and intensity of BOLD response were sensitive to biological relevance of the stimulus.

1.3.1. *Robust BOLD responses to songs can be measured in lightly sedated birds*

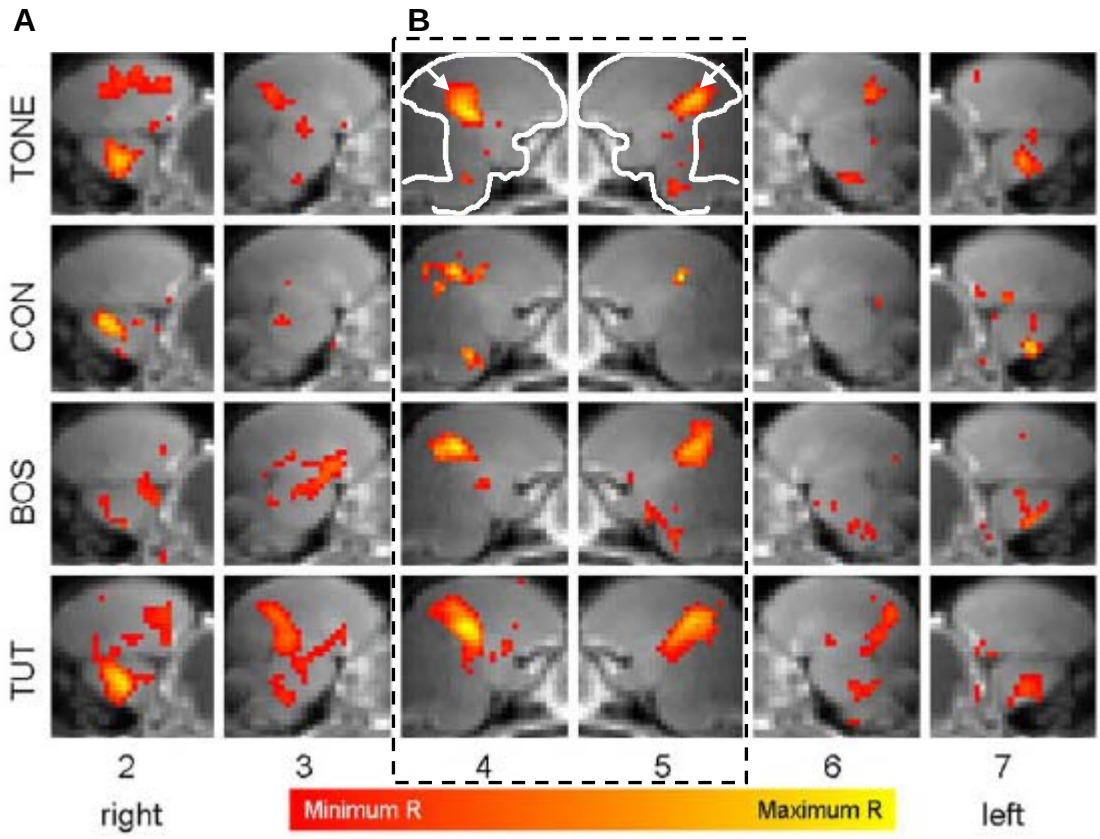
We scanned sixteen adult males and in each bird we could clearly see activation in response to each auditory stimulus. In addition, the correlation coefficient between the signal (BOLD levels) during stimulus presentation and silence shows clear stimulus-evoked activation. Stimulus-dependent activation is concentrated in the posterior forebrain in a region corresponding to the primary auditory areas of the zebra finch. In both hemispheres the medial slices showed the most consistent and reproducible activation (Figure 2-4, B); the medial-most slice in each hemisphere covers from the midline to 1 mm lateral encompassing approximately one-half to two-thirds of the primary auditory region Field L and presumably all of the secondary auditory region NCM (Fortune and Margoliash, 1992, Vates, et al., 1996). The lateral brain slices (1-3 mm lateral to midline) encompass the lateral-most portion of Field L and nuclei active in song production (RA and HVC) and song learning (Area X and lman).

1.3.2. *Stimulus dependent response activation in auditory areas.*

Activation of response reflects the spatial extent of the response and is measured by the number of voxels with activity significantly above baseline (silence). In the medial slices the largest contiguous area of activation was seen in response to tutor song (TUT); the activated area covered the auditory nuclei NCM and Field L (Vates et al., 1996; Nixdorf-Bergweiler and Bischof, 2007). The distribution of active voxels in these slices depended on the stimulus, with more activation (i.e. greater number of voxels significantly active above threshold) to TUT and the smallest area of activation to the unfamiliar song (CON), see Figure 2-4, B. Compared to TUT, activation to BOS was condensed and the center of activation shifted caudally, more-likely reflecting activity in NCM. Conspecific song elicited the smallest area of activation. In addition distribution of active voxels shifted to the posterior, medial region when BOS was played as compared to TONE (not shown). These results indicate that the bird brain distinguished between biologically relevant and less relevant stimuli, and that this discrimination can be observed in the hemodynamic responses, using a method that is non-invasive and can be repeated in the same bird.

Figure 1-4

Average functional activation depends on stimulus and slice position A. Functional activation is consistently seen in the posterior forebrain (white arrows) in Medial slices (4 + 5) of left and right hemisphere but not lateral slices (2 + 7). Rows and columns of activation plots indicate responses to different stimuli across sagittal slices: columns 2, 3, 4 right hemisphere slices, lateral to medial; columns 5, 6, 7 left hemisphere slices, medial to lateral. **B.** In the medial slices (4 + 5) area of activation is sensitive to stimulus, with familiar TUT song eliciting the largest contiguous area of activation and unfamiliar song (CON) eliciting the smallest area of activation.



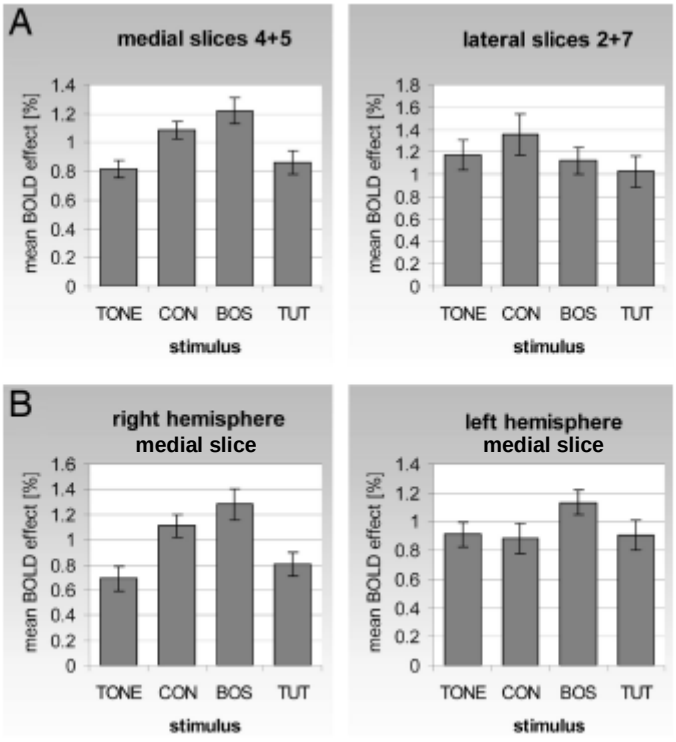
1.3.3. *Stimulus discrimination in BOLD intensity across the forebrain*

We looked at BOLD intensity across the sagittal plane, and compared medial and lateral slices (averaging across hemispheres). Medial slices clearly show differential BOLD response across the stimuli ($p<0.01$) (Figure 2-5, A left panel), whereas lateral activation is not sensitive to stimulus ($p=0.5$). BOLD activity was seen in both right and left hemisphere, but the patterns of activity differed, with right-hemisphere stimulus-discrimination which was not evident in the left hemisphere. Mean BOLD signal amplitude is comparable across right and left hemispheres (Figure 2-5, B), but the right hemisphere shows significant differences in amplitude of the response across stimuli ($p<0.01$), with the strongest responses to Con and BOS. In the left hemisphere, BOS also elicited a greater response than the other stimuli, but this was not significant ($p=0.2$). Overall, consistent and meaningful activation was found in medial (and not lateral slices) in a region covering the primary auditory processing areas. Of the medial slices, the right hemisphere medial slice showed better stimulus discrimination than the left hemisphere (Figure 2-5 B).

Figure 1-5

Mean BOLD effect is greater to BOS in medial slices and in the right hemisphere. **A.** Comparison of activity in medial slices (both hemispheres) vs. lateral slices (both hemispheres) BOLD response amplitude (mean and standard error) over all activated voxels in the medial slices of both hemispheres

(medial 4 + 5, top left panel) and lateral slices of both hemispheres (lateral 2 + 7, top right panel). Response amplitudes are significantly greater to BOS than TUT in medial, but not lateral slices (1-way ANOVA across stimuli: medial slices: $p<0.01$; lateral slices: $p = 0.5$). **B.** Activity of the medial slice in each hemisphere. Responses across stimuli vary significantly in right hemisphere medial slices (left panel) but not left hemisphere medial slices (right panel); $p<0.01$ and $p =0.2$, respectively. Variability in response amplitude across stimuli indicates greater selectivity in the medial vs. lateral parts of the posterior auditory areas and greater sensitivity to song type (BOS, CON, TUT) in the right hemisphere (from Voss et al., 2007).



1.4. Discussion

We confirmed earlier reports of meaningful fMRI BOLD responses to natural sounds in a small avian species of songbird. We designed hardware and software for scanning zebra finches in a 3.0 T GT scanner used for human and small animal research to obtain consistent and reproducible activation in auditory forebrain regions in the sedated zebra finch. We found widespread forebrain and midbrain activation; BOLD responses were identified in forebrain regions that correspond to the auditory nuclei Field L, NCM, and CM (medial slices in sagittal slice prescription). BOLD response area of activation varied by stimulus type, with greater responses to stimuli that are presumably familiar and meaningful to the bird than a less relevant stimulus, i.e. Bird's Own Song and Tutor song vs. an unfamiliar conspecific song. Such a difference in overall areas and centers of activation between stimuli with varying degrees of significance to the bird could reflect general salience of the stimulus, i.e. more neurons fire throughout the region; or specific representation of the stimulus in the auditory areas, i.e. BOS center of activation in NCM. Although TUT and CON are both conspecific song, the bird has presumably actively attended to

and compared the TUT “auditory template” to his own song during song development. Activation to TUT in both Field L and NCM could reflect a wider representation of TUT song throughout the auditory region.

Response activation had an interesting relationship to BOLD effect, or the intensity of activation. Whereas TUT appeared to be the most salient stimulus in terms of number of active voxels (i.e. per voxel a significant change from baseline), BOS elicited the greatest magnitude response, in medial slices. Presumably, fewer neurons fired in response to BOS (compared to TUT), but these neurons consumed more oxygen, perhaps due to higher firing rate or longer duration of firing to stimulus. Note, however, that the relationship between neural activity and hemodynamic response is complex and under active investigation (for detailed review see Logothetis, 2002, 2003, 2008). This intensity difference between BOS and TUT was significant; and interestingly BOLD intensity in response to CON song was comparable to intensity of BOS (both significantly different from TUT). These results viewed together suggest that BOLD activation and intensity potentially reflect stimulus saliency, in different ways: salient in terms of many neurons in multiple nuclei responding to the song, as is the case in TUT; or salient in terms of a stronger response in fewer neurons (the case in BOS and CON).

In medial slices Bird’s Own Song also elicited the greatest BOLD effect (% change in BOLD intensity), interestingly only the right hemisphere showed significant differences across all stimuli tested. This brain lateralization to species-specific vocalizations is reminiscent of left-hemisphere language dominance in humans, and suggests that perhaps in the zebra finch right-hemisphere is specialized for processing and perceiving vocalizations (Poirier, Boumans, Verhoye, Balthazart, Van der Linden, 2009).

Despite wide use of fMRI in human and non-human primate research for the last decade, this technique is only currently coming into focus as a technique to use with small-animals for non-invasive studies in a range of fields: animal cognition, models of neurodegenerative disease, high-resolution sensory brain maps. In animal models of human disease and brain function, MRI has the advantage of comparability to human studies; which is not possible with other optical imaging methods (e.g Flourescent Immunohistochemistry).

FMRI has great potential for studies of song learning and perception but technical challenges still exist. For example, in the current study we used a 3.0 T scanner magnet and obtained; greater magnet strength (e.g. 7 T) could provide better spatial resolution (0.08 mm) and higher-quality images. In addition, a three-dimensional MRI atlas of the zebra finch with established landmarks and automatic anatomic registration methods would greatly improve the anatomic image and as well as functional localization. Such a brain atlas has very recently been made available by C. Poirier et al. 2008 (after the completion of the experiment described here).

We have demonstrated that fMRI is a feasible research tool to use in zebra finches. This method has the advantage of within-subject longitudinal studies of the development of neural correlates of song perception. We obtained normative data on adult male birds raised in a colony with a rich auditory and social environment. We will further explore auditory responses (in the adult bird) by manipulating developmental auditory experience and observing brain responses to a wider range of natural stimuli.