Video Article

Functional Magnetic Resonance Imaging (fMRI) with Auditory Stimulation in Songbirds

Lisbeth Van Ruijssevelt[1], Geert De Groof[1], Anne Van der Kant[1], Colline Poirier[1], Johan Van Audekerke[1], Marleen Verhoye[1], Annemie Van der Linden[1]

[1]Bio-Imaging Lab, University of Antwerp

Correspondence to: Annemie Van der Linden at annemie.vanderlinden@ua.ac.be

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Abstract

The neurobiology of birdsong, as a model for human speech, is a pronounced area of research in behavioral neuroscience. Whereas electrophysiology and molecular approaches allow the investigation of either different stimuli on few neurons, or one stimulus in large parts of the brain, blood oxygenation level dependent (BOLD) functional Magnetic Resonance Imaging (fMRI) allows combining both advantages, *i.e.* compare the neural activation induced by different stimuli in the entire brain at once. fMRI in songbirds is challenging because of the small size of their brains and because their bones and especially their skull comprise numerous air cavities, inducing important susceptibility artifacts. Gradient-echo (GE) BOLD fMRI has been successfully applied to songbirds [1-5] (for a review, see [6]). These studies focused on the primary and secondary auditory brain areas, which are regions free of susceptibility artifacts. However, because processes of interest may occur beyond these regions, whole brain BOLD fMRI is required using an MRI sequence less susceptible to these artifacts. This can be achieved by using spin-echo (SE) BOLD fMRI [7,8]. In this article, we describe how to use this technique in zebra finches (*Taeniopygia guttata*), which are small songbirds with a bodyweight of 15-25 g extensively studied in behavioral neurosciences of birdsong. The main topic of fMRI studies on songbirds is song perception and song learning. The auditory nature of the stimuli combined with the weak BOLD sensitivity of SE (compared to GE) based fMRI sequences makes the implementation of this technique very challenging.

Video Link

The video component of this article can be found at https://www.jove.com/video/4369/

Protocol

1. Preparation of the Auditory Stimuli

1. First record the sound-stimuli while being played inside the bore of the 7T MR system. The bore is a confined space that can distort the auditory stimuli resulting in enhancement of certain auditory frequencies. **Figure 1** shows the frequencies enhanced and suppressed as shown by our recordings of white noise made at the location of the bird's head within the magnet bore using a fiber-optic microphone (Optimic 1160, Optoacoustics). To compensate this artificial enhancement, an equalizer function is applied to each stimulus using WaveLab software. For our particular setup, the function consists of a Gaussian kernel with the following parameters: maximum amplitude: -20dB, centered on 3,750 Hz, width: 0.05 octaves (corresponding to the range 2,500-5,000 Hz for our system).
2. The song stimuli are composed of several individual song motifs of each bird interleaved with periods of silence. The duration of these silent periods is adjusted to keep the total amount of sound and silence identical over all stimuli. This construction conserves the natural intra-individual and inter-individual variability of song length. The total length of each stimulus is 16 sec. The intensity of each song is normalized in terms of matched root-mean-square and high-pass filtered at 400 Hz before being integrated into the complete stimulus (song and silent periods). These manipulations are done using Praat software.
3. The experiment consists of an ON/OFF block design alternating auditory stimulation periods (ON blocks) with resting periods (OFF blocks) (**Figure 2**). Each block (ON and OFF) lasts 16 sec, which corresponds to the acquisition time of 2 images (see below for acquisition). Each stimulus type is presented 25 times, resulting in the acquisition of 50 images per stimulus and per subject. The presentation order of the conditions should be randomized within and between subjects. This randomized order of the stimuli can be coded into Presentation software.

2. Subject Preparation

2.1 Subject and group size

Here we present a protocol specifically adapted to the use of (adult) zebra finches. The choice of the species depends on the scientific question. However, other considerations like bird robustness to anaesthesia may also be taken into account. Zebra finches (*Taeniopygia guttata*) should be housed in aviaries under a 12 hr light: 12 hr dark photoperiod and have access to food and water ad libitum throughout the study. The minimal number of individuals per experiment is 15. This number takes into account the sensitivity of spin-echo fMRI and the natural inter-individual variability of biological phenomena measured in the experiment.

2.2 Installation of setup and preparation of the animal

(For specification of the used equipment, we refer to the list of specific reagents and equipment at the end of this article)

1. Install the beak mask on the MRI bed of a 7T MR system and connect it to the gas controller device with plastic tubes. Open both oxygen and nitrogen gas bottles and switch on the gas controller device (flow rate oxygen: 200 cc/min; nitrogen: 400 cc/min).

As mentioned above, a 7T MR system is used in the presented setup. Other MR systems with different field strengths are also possible, but at 7T a good compromise is reached between signal-to-noise ratio and degree of susceptibility artifacts (see discussion). At higher field strengths the signal-to-noise ratio will increase together with the degree of susceptibility artifacts.

2. Switch on the feedback controlled system and warm airflow device.
3. Anaesthetize the zebra finch with 3% isoflurane in a mixture of oxygen and nitrogen by introducing its beak into the mask and holding the head down until the bird is fully anesthetised. This can be verified by pulling the foot softly: when the bird is fully sedated the foot will not be retracted by the bird. In addition, the eyes of the bird will be partly closed.
4. Introduce the cloacal temperature probe to screen the body temperature and monitor the breathing rate by placing a pneumatic sensor underneath the zebra finch belly. Close the jacket to restrain the body of the bird (**Figure 3**).
5. Maintain breathing rate within the range of 40 - 100 breaths per minute and keep body temperature constant within a narrow range of 40 ± 0.5 °C. When the breathing range is too low/high, adjust the level of anaesthesia (% isoflurane) accordingly. When the problem persists, the experiment should be stopped and the animal removed from the setup in order to recover.
6. Position the non-magnetic dynamic speakers on either side of the zebra finch head and connect them to the amplifier. Make sure that the wires of the speakers are led away from the temperature probe, because it can influence the temperature reading when too close.
7. Place the surface RF coil on top of the zebra finch head and position the zebra finch in the center of the magnet (and automatically the center of the transmit coil which is situated in the middle of the magnet).
8. Reduce the anaesthesia level to 1.5% isoflurane mixed with oxygen and nitrogen.

3. Data Acquisition

1. Acquire a set of 1 sagittal, 1 horizontal and 1 coronal gradient-echo (GE) scout image (tri-pilot sequence) and sets of horizontal, coronal and sagittal multi-slice images (piloting T_2-weighted rapid acquisition relaxation-enhanced (RARE) SE sequence) to determine the position of the brain in the magnet (**Figure 4**).
2. Decrease the noise of the gradients by increasing their ramp times to 1,000 µs.
3. Prepare the fMRI sequence: RARE T_2-weighted sequence, effective TE: 60 msec, TR: 2,000 msec, RARE factor: 8, FOV: 16 mm, matrix size: 64 x 32, orientation: sagittal, slice thickness: 0.75 mm, Inter-slice gap thickness: 0.05 mm, 15 slices covering nearly the whole brain (**Figure 4**).
4. Select the auditory protocol (auditory stimuli and timing of stimulus delivery) in the presentation software. This protocol consists of a sequence of commands - for the initiation of specific auditory stimuli - which are executed at a specific scan-number. At every repetition within the fMRI sequence, the scanner software will send a trigger to the auditory presentation software which in turn registers the scan number and executes the corresponding command.
5. To ensure that the auditory presentation software does not miss any trigger from the scanner, the auditory protocol is initiated first. Once the protocol is fully loaded, the fMRI sequence is started.
6. Each fMRI experiment is preceded by the acquisition of 12 dummy images to allow the signal attributed to the scanner noise to reach a steady state before starting auditory stimulation.
7. After acquisition zero-fill the data to 64 x 64.
8. Take a first (preliminary) look at the results using the Functional Tool of Paravision (option Processing/Functional Imaging). Calculate the differential BOLD response between all ON blocks and the baseline (OFF blocks). This analysis gives a first indication of the quality of the experiment. If no activation is seen in the primary auditory areas at this stage, the bird did probably not hear/processed the auditory stimuli due to technical problems with the stimulus presentation, anaesthesia level, etc. The setup should be verified and the measurement repeated.
9. Run an anatomical 3D RARE T2-weighted sequence in the same orientation as the previous fMRI scans and with effective TE: 60 msec, TR: 2,000 msec, RARE factor: 8, FOV: 16 mm, matrix size: 256 x 128 x 64.
10. Zero-fill the data to 256 x 256 x 256.
11. Take the zebra finch from the MRI bed and let it recover from anaesthesia in a cage under a red lamp. Normally, the recovery of a zebra finch after isoflurane anaesthesia goes relatively fast (maximal 5 min). After only a few minutes, the birds will try to stand up and once the bird is fully recovered, it will perch on a branch instead of sitting on the bottom of the cage. The duration of anaesthesia is about 2 hr for the present experiment. The maximum time of isoflurane anaesthesia applied to zebra finches in our lab is 6 hr, after which the birds also recovered within 5 min.

4. Data Processing

1. Convert the MR-data into Analyze or Nifti format.
2. Because SPM has been developed to process fMRI data acquired in humans, that is for voxels of around 2 mm. Numerous SPM settings are adapted to this approximate voxel size. If one does not want to change all these settings, the simplest way to proceed is to artificially increase the voxel size of bird fMRI data. Adjust the voxel size in the header by multiplying the real voxel size by 10 using MRIcro. It should be noted, that such adjustment does not influence the data in itself, no resampling or any other modifications to the data is applied.

An alternative to this is the use of 'SPMMouse' which is a toolbox allowing SPM to open and analyze files of any voxel dimension. The tool allows SPM 'glass brains' to be created from any image, and automatically adjusts defaults length scales based on the headers of image files or user entered data. Hence, this toolbox works in the opposite way than what we propose. Instead of changing the voxel size of the images to fit in SPM, the default settings of SPM are changed to use images with different voxel sizes.

3. Realign the fMRI data. Co-register the anatomical 3D dataset to the fMRI time series. Normalize the 3D data (and the co-registered fMRI time series) to the zebra finch brain MRI atlas. Apply the transformation matrix to the fMRI dataset. This can all be done using Statistical Parametric Mapping (SPM) 8 software.
4. Smooth the data with a 0.5-mm width Gaussian kernel using SPM8.
5. Carry out statistical voxel-based analyses using SPM8. Model the data as a box-car (no hemodynamic response function). Estimate model parameters with the classical Restricted Maximum Likelihood algorithm. Compute the mean effect of each auditory stimulus in each subject (fixed-effect analysis) and then compute statistics as wished for group analyses (mixed-effect analyses).
6. Project the statistical parametric map onto the zebra finch atlas (**Figure 5**) [9] in SPM8 to localize the functional activations (**Figure 6**).

Representative Results

We here visually presented an optimized sequence of procedures for successful imaging of neural substrates of auditory stimuli in the zebra finch brain. Firstly, the described procedure for preparation of the auditory stimuli results in stimuli that can be incorporated into an ON/OFF block paradigm (**Figure 2**) and that are normalized to eliminate potential differences in sound pressure level that could evoke a differential response in the brain. After preparing the zebra finch for MRI scanning and positioning it into the bore of the magnet (**Figure 1**), fMRI can be acquired. Additionally, a 3D high-resolution image is taken in order to normalize the data to the zebra finch atlas [9]. Finally, pre-processing and statistical analysis of the data allows visualization of the results obtained (**Figure 6**).

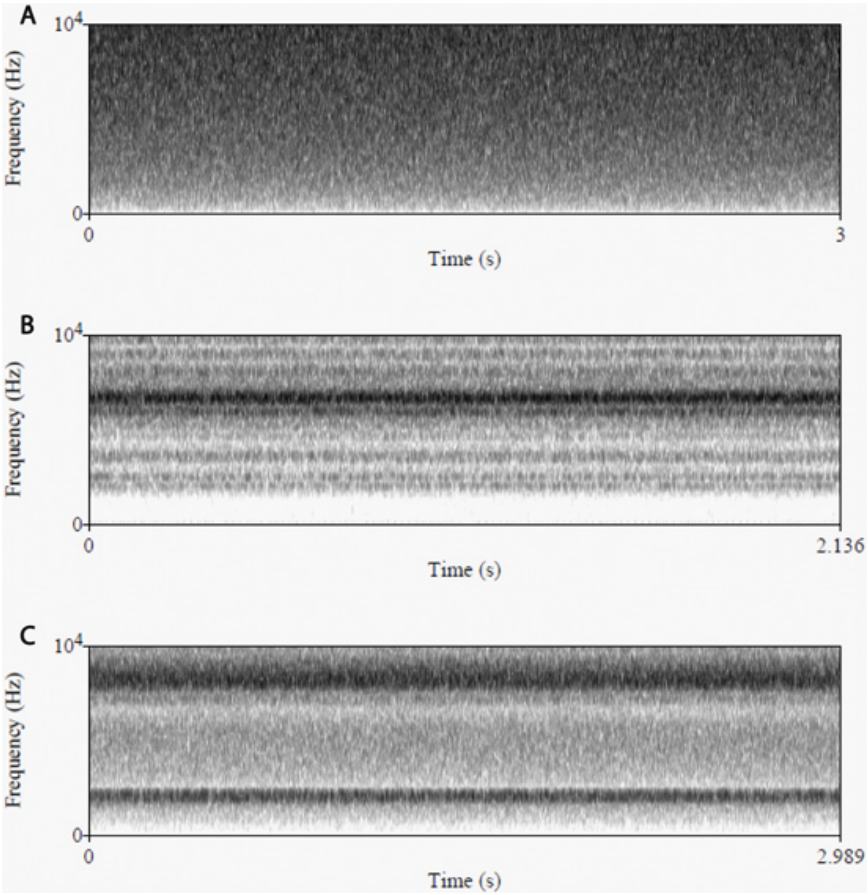

Figure 1. Spectrograms of white noise recorded in order to establish frequency bands that are enhanced/suppressed within the magnet bore. A. White noise outside the magnet bore. **B.** White noise recorded at the location of the bird's head inside the magnet bore. **C.** White noise after application of the equaliser function to correct for enhanced/suppressed frequency bands.

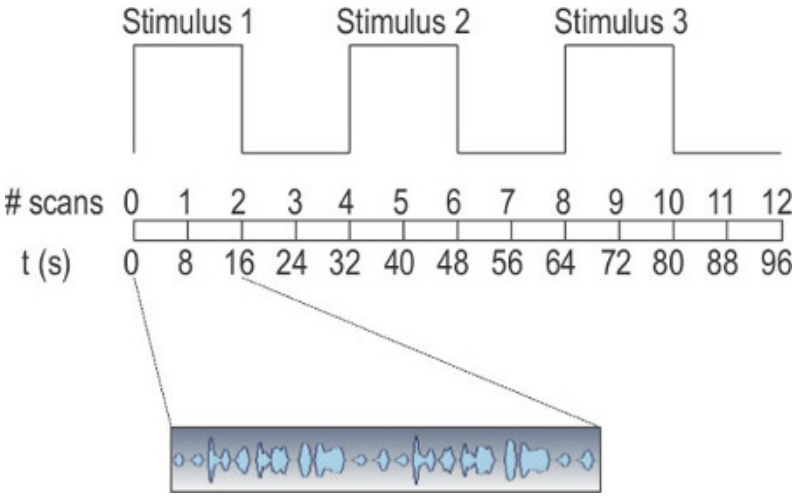

Figure 2. Overview of the ON/OFF block paradigm in which auditory stimulation periods are alternated with rest periods. Each block (stimulus/rest) lasts 16 sec during which 2 images are acquired. The different stimuli consist of representative motifs of birdsong or other types of sound depending on the experiment. These motifs are concatenated and interleaved with silent periods and the duration of the silent periods is adjusted to keep the total amount of sound and silence identical over all stimuli.

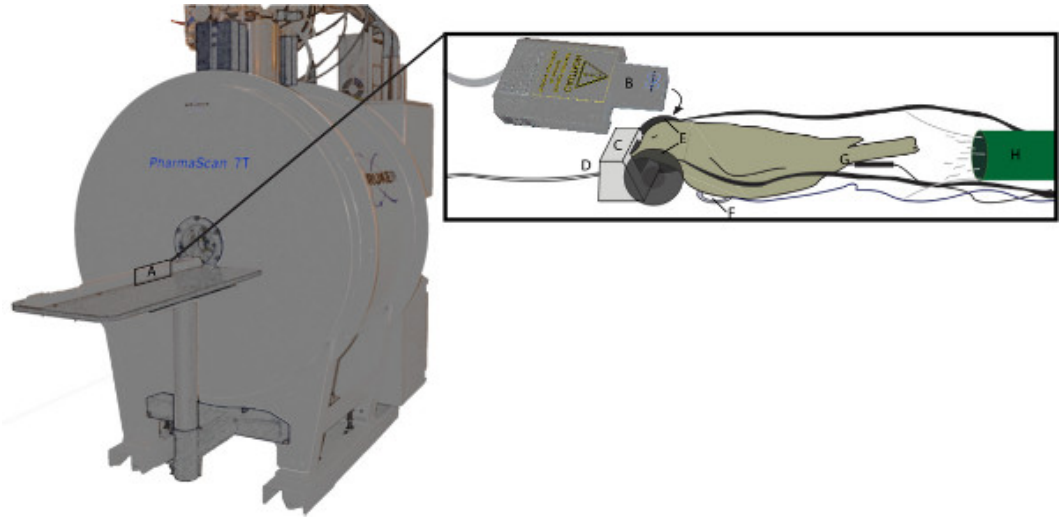

Figure 3. Setup for auditory fMRI in small songbirds. A. Animal bed. *Inset:* Detailed schematic overview of the positioning of the bird in the animal bed of the scanner: **B.** RF head coil, **C.** Beak mask with **D.** supply of anaesthetic gas, **E.** non-magnetic headphones, **F.** pneumatic pillow sensor to monitor the respiration rate, **G.** cloacal temperature probe, **H.** feedback controlled heater system to keep body temperature of the bird stable during the measurement. Click here to view larger figure.

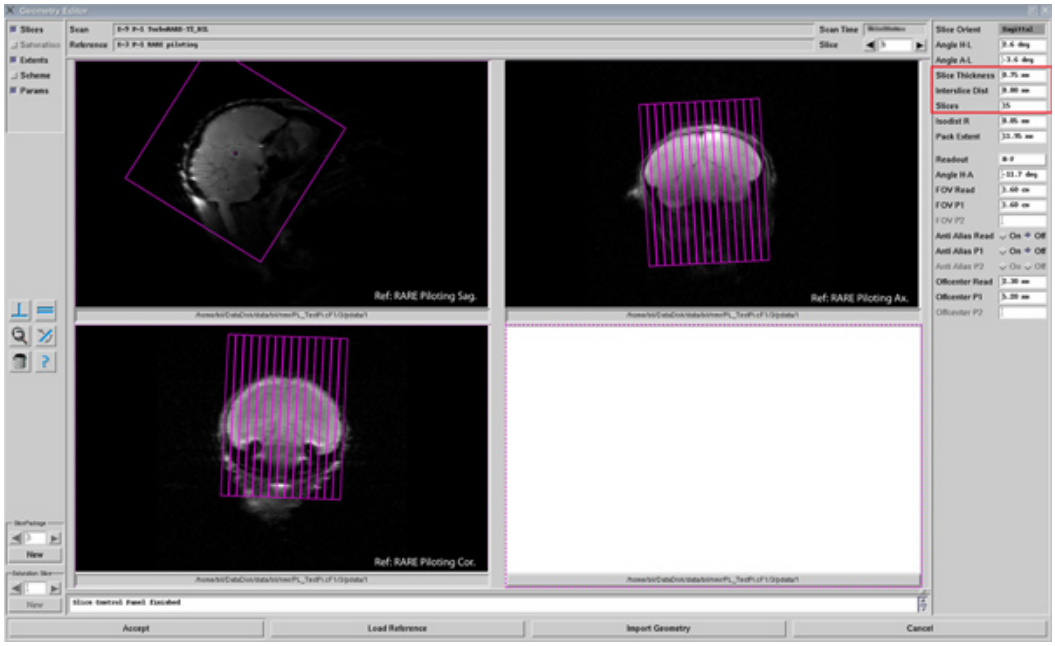

Figure 4. Slice geometry for whole-brain fMRI imaging. Composition of screenshots from geometry editor in ParaVision software. Previously acquired axial, sagittal and coronal RARE piloting images are used to define the slice orientation for the fMRI scan.

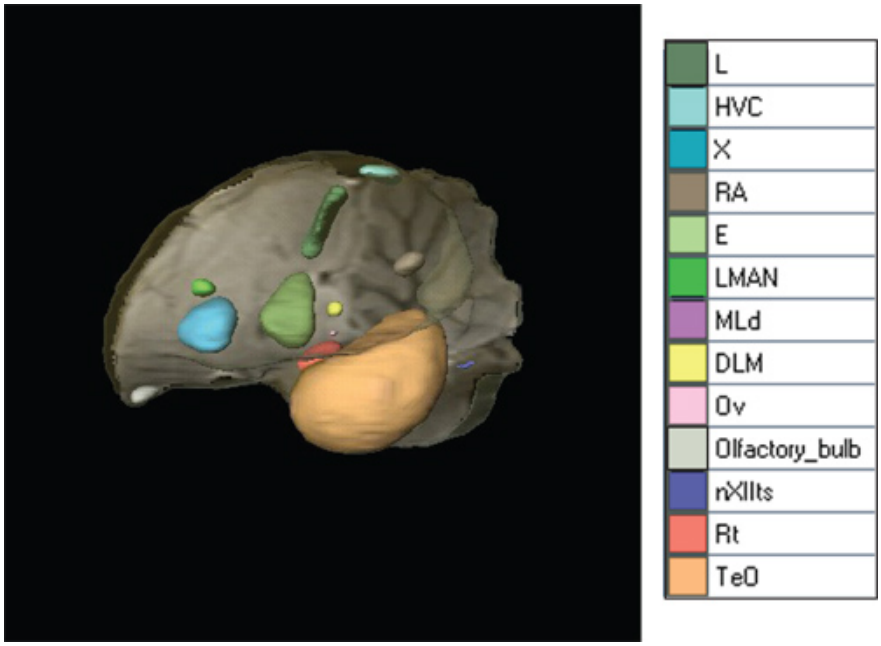

Figure 5. Lateral view of a 3D representation of the left hemisphere with delineated structures from the zebra finch atlas [9], projected on its mid-sagittal slice. The color code of the delineated nuclei is presented on the right. These delineated structures are part of the vocal motor pathway: HVC, nucleus robustus arcopallii (RA), nXII pars tracheosyringealis (nXIIts); the anterior forebrain pathway: nucleus lateralis magnocellularis pars lateralis (LMAN), area X (X); the auditory system: field L, nucleus ovoidalis (Ov), nucleus mesencephalicus lateralis pars dorsalis (MLd); the olfactory system: olfactory bulb (OB); and the visual system: nucleus entopalliallis (E), tectum opticum (TeO).

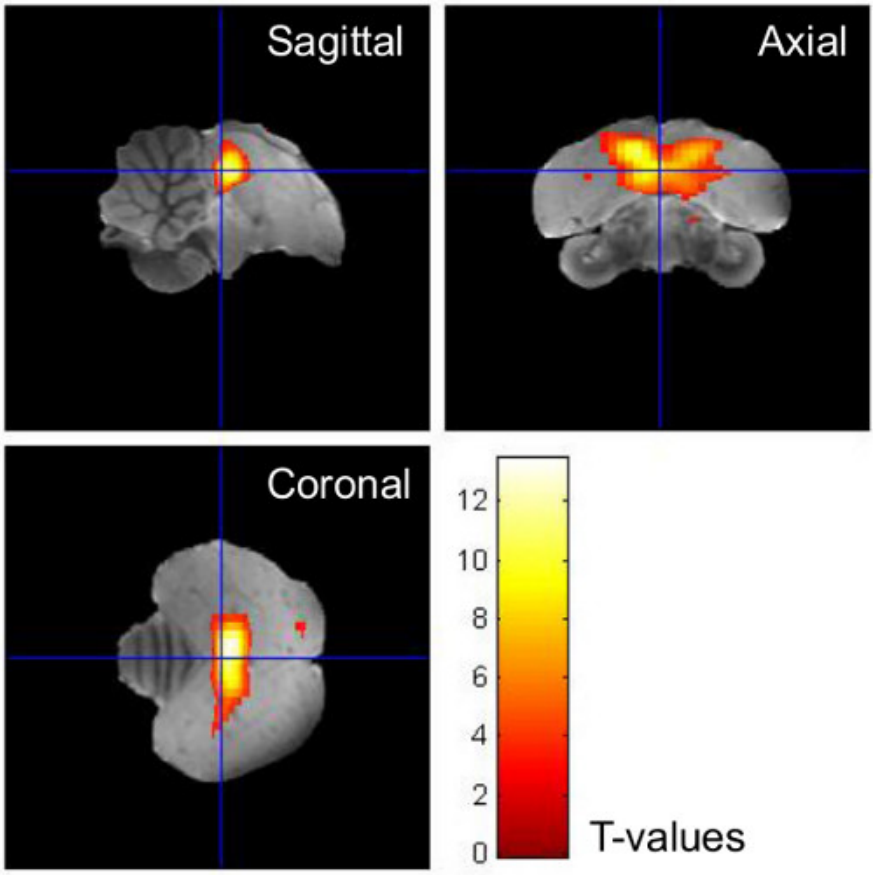

Figure 6. Example of an fMRI BOLD response in the primary auditory region, Field L, and adjacent secondary auditory regions evoked by different auditory stimuli compared to the rest condition. The images consist of statistical parametric maps superimposed on high-resolution anatomical images from the zebra finch brain atlas [9]. T-values are colour coded according to the scale displayed in the figure and only voxels in which the t-test was found to be significant ($p<0.001$) are displayed.

Discussion

In this report, we describe an optimized protocol for the detailed *in vivo* characterization of neural substrates of auditory stimulation in anaesthetized zebra finches.

In line with the presented protocol, the majority of functional brain activation studies in animals using BOLD fMRI, anaesthetize the animals during the acquisition. Training animals to accustom them to the magnet environment and the scanner noise during the study periods is also possible but rather time-consuming and challenging and therefore rarely employed.

Although anaesthesia minimizes stress-induced effects on the physiological responses of interest and facilitates animal handling, its effect both on the neural response and on the transfer function between the neural activity and the BOLD response measured in fMRI is an on-going and important research topic. Therefore, the effects of anaesthesia on the BOLD response during auditory stimulation in zebra finches were investigated in our lab [2]. Accordingly, three widely used anaesthetics in zebra finches - medetomodine, isoflurane and urethane - acting on different neurotransmitter systems, were studied. The results indicated that auditory stimulation resulted in clear BOLD responses with all three anaesthetics, but that slight differences occurred between the three reagents in relation to *e.g.* extension of the area of activation. Based on the results of this study and on the fact that isoflurane is the most common anaesthetic in clinical applications as it has the great advantage of having relatively rapid recovery and minor side effects and thus has the highest potential for use in longitudinal studies, isoflurane became the anaesthetic of choice for zebra finch fMRI at our lab.

In this protocol we use spin-echo (SE) fMRI instead of the more traditional gradient-echo (GE) fMRI. Compared to GE fMRI, SE fMRI has the great advantage of providing signal to the whole brain as there is no signal dropout in the images. Another advantage of SE BOLD fMRI is its better spatial specificity [10,11]. Indeed, at high magnetic field, the intravascular component of the SE BOLD signal is reduced (because of a long TE) and the extravascular component from large vessels is suppressed (by the 180 ° refocusing pulse of the SE MRI sequence). The SE BOLD signal is thus dominated by an accurate extravascular signal originating from small vessels [12-14]. The main limitation of SE fMRI is its relatively weak sensitivity, requiring optimized sequences and optimized stimulation paradigms. The contrast to noise ratio (CNR) increases with field strength [15]. A long TE also increases the CNR, but compromises the signal-to-noise ratio [12,13,15]. The optimal TE usually corresponds to a time equal to or longer than the T_2 value of the tissues. We have shown that, at 7T, a TE value of 60 msec provides a CNR and a signal-to-noise ratio sufficient to detect significant differences in BOLD responses triggered by different stimuli (Poirier, 2010).

Compared to GE T2*-weighted contrast, SE T2-weighted contrast requires a long TR (1,500-2,000 msec at 7T). To be able to image 15 slices, we used a TR of 2,000 msec. To keep the acquisition time at a reasonable limit, SE MRI sequences need to be speeded up. This is usually achieved using the echo planar imaging (EPI) sampling scheme [10,16-19]. However, EPI induces image distortions that increase with the magnitude of the magnetic field, and contaminates the BOLD signal with T2* effects (making the signal stronger but less specific). EPI also produces a very intense acoustic noise, making it less relevant for use in investigating auditory stimuli. We thus used a RARE sequence with a matrix size of 64 x 32, which resulted in an acquisition time of 8 sec. This temporal resolution is still compatible with the sluggish BOLD response induced by block designs, but too slow to accurately sample the time course of the BOLD response or to use event-related designs. With this sequence, we thus obtained a pure T2-weighted SE signal, which is characterized by a very good spatial specificity, a sensitivity sufficiently high to detect differential BOLD responses and a temporal resolution compatible with the used stimulus paradigm [20,21].

Advantages and Limitations of the use of fMRI in Songbirds

During the last decades, fMRI has become one of the most popular neuroimaging techniques in clinical cognitive neuroscience for the study of brain activity during various tasks ranging from simple sensory-motor to highly cognitive tasks. In preclinical research, this method is, however, still only scarcely used. The scarcity of fMRI experiments completed in small animals and particularly songbirds to date possibly relates to the fact that anaesthesia or sedation is required to achieve complete immobilization of the subjects (see discussion above). Hence, this is considered the major drawback of the technique and restricts the type of questions that can be addressed. However, although fMRI requires anaesthesia and the BOLD signal mainly reflects local field potentials and thus differs from the action potentials measured in electrophysiological and Immediate early gene (IEG) studies (*e.g.* [22]), BOLD fMRI has confirmed many results obtained by these techniques.

To date, the most popular techniques in songbird neuroscience are still activity-dependent expression of IEG and electrophysiological recordings of single- or multi-unit activity. These techniques benefit from a very high spatial resolution (5-30 µm; cellular level). However, they are highly invasive or even lethal. Additionally, electrophysiological techniques are limited by the number of locations that can be sampled in one experiment and thus require a priori hypotheses about the localization of the neuronal substrate involved in the investigated process. In contrast, BOLD fMRI allows a whole-brain approach - with a spatial resolution of 250 µm - and can thus be used to perform assumption-free experiments. Finally and most importantly, the non-invasiveness of MRI enables repeated longitudinal measures on the same subjects, which opens a large range of new possibilities.

Disclosures

No conflicts of interest declared.

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