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Noninvasive diffusive optical imaging of the auditory response to birdsong in the zebra finch

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Abstract

Songbirds communicate by learned vocalizations with concomitant changes in neurophysiological and genomic activities in discrete parts of the brain. Here we tested a novel implementation of diffusive optical imaging (also known as diffuse optical imaging, DOI) for monitoring brain physiology associated with vocal signal perception. DOI noninvasively measures brain activity using red and near-infrared light delivered through optic fibers (optodes) resting on the scalp. DOI does not harm subjects, so it raises the possibility of repeatedly measuring brain activity and the effects of accumulated experience in the same subject over an entire life span, all while leaving tissue intact for further study. We developed a custom-made apparatus for interfacing optodes to the zebra finch (*Taeniopygia guttata*) head using 3D modeling software and rapid prototyping technology, and applied it to record responses to presentations of birdsong in isoflurane-anesthetized zebra finches. We discovered a subtle but significant difference between the hemoglobin spectra of zebra finches and mammals which has a major impact in how hemodynamic responses are interpreted in the zebra finch. Our measured responses to birdsong playback were robust, highly repeatable, and readily observed in single trials. Responses were complex in shape and closely paralleled responses described in mammals. They were localized to the caudal medial portion of the brain, consistent with response localization from prior gene expression, electrophysiological, and functional magnetic resonance imaging studies. These results define an approach for collecting neurophysiological data from songbirds that should be applicable to diverse species and adaptable for studies in awake behaving animals.

Keywords

songbird; near-infrared spectroscopy (NIRS); absorption spectra; anesthesia; isoflurane

Introduction

The zebra finch (*Taeniopygia guttata*) is an important animal model in neuroscience for studying relationships between brain and behavior. Like other songbirds, zebra finches

communicate through complex learned vocalizations (songs). Exposure to recorded songs triggers robust changes in gene expression and neurophysiological activity in the brain, especially in the caudomedial nidopallium (NCM) and mesopallium of the forebrain (Mello et al. 1992; Mello and Clayton 1994; Chew et al. 1995; Stripling et al. 1997; Huesmann and Clayton 2006; Dong et al. 2009; Drnevich et al. 2012). The study of these responses has not only broadened our understanding of the neurobiology of sensory processing, it has also informed us about the molecular basis of sociality and social behavior (Robinson et al. 2008; Clayton et al. 2009).

Much of this progress has been made using highly invasive methods or ones that require sacrifice of the animal, which limit their utility for longitudinal studies of how brain physiology changes with organismal behavior, development and experience. Recently, technologies for non-invasive functional imaging have begun to be applied in songbird research (Van Meir et al. 2005; Voss et al. 2007; Boumans et al. 2007; Boumans et al. 2008a; Poirier et al. 2009; Voss et al. 2010; Poirier et al. 2011). Among these technologies is diffusive optical imaging (DOI), which uses optic fiber optodes resting non-invasively on intact scalp (Kato et al. 1993; Gratton and Corballis 1995; Villringer and Chance 1997; Strangman et al. 2002). It measures brain activity by detecting changes in the optical properties of the brain using light wavelengths in the red and near-infrared range. Using a Near Infrared Spectroscopy (NIRS) approach to data interpretation it is possible to estimate blood flow and oxygenation (Jobsis 1977; Frostig et al. 1990; Malonek and Grinvald 1996). Temporal resolution is excellent, and sampling rates of 50 Hz or more can be achieved. The spatial resolution is constrained by the number of optodes used and by the scattering properties of the medium but can in principle be sub-centimeter (Gratton and Fabiani 2007).

For work with songbirds and other experimental animals, DOI has several practical advantages. It is relatively inexpensive to acquire and maintain and is therefore less susceptible to constraints common to large, institutionally-shared devices. It is also relatively portable and may be adapted and applied in diverse laboratory or animal housing environments. It safely measures intrinsic optical properties without requiring harmful injections or powerful magnetic fields (Gratton and Fabiani 2007). Finally, with respect to measuring auditory tasks in songbirds, DOI runs silently and can be relatively motion tolerant if the optodes are properly coupled to the head (Strangman et al. 2002).

Development of the NIRS approach to study songbirds began with measurements of the optical properties of the zebra finch head by Ramstein et al. (2005), and a comparison of optical and fMRI responses to hypercapnia (Vignal et al. 2008). Responses to songs and calls have been demonstrated recently using a method termed Picosecond Optical Tomography, which affords picosecond and submillimeter resolution along a strip of tissue (Mottin et al. 2011). Further improvements in methods are still needed, however, to advance NIRS as a standard technique for recording neurophysiological activities, potentially even in awake behaving birds.

Here we describe the design, construction and application of an apparatus for performing multichannel DOI using 3D computer modeling and rapid prototyping (Fig. 1), an approach

that is both inexpensive and versatile. We also empirically measured extinction coefficients for the zebra finch across the red and near-infrared range for the chromophore species oxy- and deoxy-hemoglobin (HbO and HbR, respectively). Using this new information and our apparatus, we imaged wavelength intensity data from gas-anesthetized birds during playback of song stimuli and derived the corresponding temporal profile of song-induced changes in [HbO] and [HbR].

Materials and methods

Animals

All experiments were performed under protocols approved by the University of Illinois Laboratory Animal Care Advisory Committee. Adult male zebra finches (at least 90 days old) were bred and raised in an aviary of the Beckman Institute animal facility or purchased from vendors. Birds were housed in pairs or three to a cage (35.6 cm long $\times$ 40.6 cm wide $\times$ 45.7 cm high) prior to use. All birds were kept on a 14/10 hour light/dark cycle.

Stimuli

All presentations of song stimuli consisted of a 15s “triple-song” consisting of 3 different 5s songs played back-to-back without pause. All songs were novel to this cohort. This stimulus was chosen because it was expected to elicit a strong response; past research found this stimulus to be particularly powerful for inducing a ZENK immediate early gene response (Mello et al. 1992; Kruse et al. 2000; Cheng and Clayton 2004). Songs were played at an average sound intensity of 80 decibels.

Task and general procedures

Nine adult male zebra finches were individually exposed to 3 min silence baseline, followed by 7 trials of song stimuli consisting of the same 15s “triple-song” stimulus separated by variable interstimulus intervals. Interstimulus intervals varied between 180s and 210s to avoid conflation of responses with pre-existing intrinsic rhythmicity (Elwell et al. 1999) or entrainment to regular stimulus presentation. Interstimulus intervals were intentionally lengthened to ensure recovery of the hemodynamic response before the next stimulus onset. Approximately 3 more minutes of silence followed the last stimulus, for a total session duration of about 26 minutes. All measurements took place in a darkened room. Recording began once intensity measures were stable for 30 seconds.

Recording methods

Placement of the recording device and anatomical co-registration—A male zebra finch head was scanned using SkyScan 1172 MicroCT (SkyScan, Kontich, Belgium), reconstructed into a digital volume using Amira software (Visage Imaging, Carlsbad, CA), and imported into a 3D modeling program, Maya (Autodesk, San Rafael, CA). A slice of this “virtual head” was chosen as the area to be imaged (Fig. 1g). This slice contains the major auditory areas of the forebrain including field L, the avian analog of primary auditory cortex in mammals (Muller and Leppelsack 1985); and the caudomedial nidopallium (NCM), the analog to secondary auditory cortex in mammals (Mello and Clayton 1994). The

slice also contains elements of the circuit responsible for song learning and production, including the magnocellular nucleus of the anterior nidopallium, a region important during juvenile learning of song; the robust nucleus of archipallium, an important area for the motor production of song which receives input from field L or the area around it (specifically the robust nucleus of archipallium cup; Kelley and Nottebohm 1979; Mello and Ribeiro 1998); and the hyperpallium apicale, the origin of the avian pyramidal (a.k.a. corticospinal) tract (Wild and Williams 2000). In this plane, eight evenly spaced spokes were drawn radially, extending from the center of this area. Their intersections with the virtual head surface marked the optode contact points. Virtual optodes were placed at these intersections and adjusted to be normal to the head surface (Fig. 1a).

Optode placement was designed to intersect NCM; its location relative to the virtual head was estimated based on an overlay of microCT X-ray images and camera-lucida images depicting the NCM (Terpstra et al. 2004). Intersection of the source-detector plane with the NCM was later verified by warping the 3D MRI atlas (Poirier et al. 2008) to the brain-case of our virtual head (Fig. 1b) using Amira software (a current version of the tutorial can be found here: <http://walnut.zib.de/documentation/521/amira/usersguide/tutlandmarks.html>). The warping allowed us to approximate neuroanatomical areas present in the MRI atlas in the skull we used for our 3D modeling. The composite brain was fitted to our virtual skull (Fig. 1c, 1d), allowing us to demonstrate that the source-detector plane intersects with our region of interest (Fig. 1e, 1f, 1g).

A frequency domain oximeter (Imagent™, ISS Inc., Champaign, IL) recorded intensity produced by laser diode sources carrying 690 nm and 830 nm light modulated at 100 MHz and multiplexed through each light source every 16 ms on four photomultiplier tube detectors. DC (direct current, i.e., overall light intensity) and AC (alternating current, i.e., modulated light intensity) were recorded. Four source pairs (each composed of a 690 nm and an 830 nm light source) and four detectors were placed on opposite sides of the head to maximize light diffusion; imaging was therefore based on transmission of light through the head, and was sensitive to absorption and scattering changes in tissue. The eight individual sources were time-multiplexed so as to allow for the separate measurement of each source-detector pair.

Animal interfaces

The anesthetized animal interface (Fig. 2) allows gas anesthesia delivery while keeping the head available for imaging. It uses an optode guide-crown to hold relatively stiff glass-core optic fibers. Guide-crown height is adjusted by turning a knob on a precision leadscrew. Source optodes consisted of 0.4 mm (when jacket is stripped) optic fibers physically paired to form a 0.8 mm double-tipped end to allow presentation of both 690 nm and 830 nm wavelengths at each source position. 0.3 optical density Kodak Wratten filters were fitted to the ends of source fibers to reduce overall light intensity. The detector fibers also were 0.4mm in diameter (stripped of jacket), and number 29 deep red Kodak Wratten filters were placed at the ends of detector fibers to protect detectors from room light during set-up and take-down of the experiment.

Interface designs were physically realized using a Viper si Stereo Lithography Apparatus (3D Systems, Rock Hill, SC), a FDM 3000 Fused Deposition Modeling 3D printer (Stratasys, Eden Prairie, MN), and custom-made parts.

Subject preparation

Subjects were isolated at least 1 hour prior to recording. Room temperature was adjusted to 80°F to protect subjects from hypothermia during anesthesia. Subjects were placed in an anesthesia induction chamber and exposed to 3% isoflurane carried on medical air (79% nitrogen, 21% oxygen) at 1 L/min (Boumans et al. 2007). Subjects were wrapped in a custom made restraining jacket of woven cotton which covers the entire body below the neck and secured in the anesthetized animal interface. No warming blanket was used and body temperature was not monitored. The head was lightly restrained on both sides by moveable L-shaped arms which slide medially and laterally on rails. The base of the 'L' contacts the head. On the base of the 'L' is a 3 mm diameter circular hole corresponding to the ears. The hole is aligned with the opening of the bird's ear and each arm is moved the same distance to center the head. The line of the ventral border of the upper beak was adjusted to 57 degrees relative to horizontal. The height of the fiber-optic-holding crown was adjusted relative to beak length. Head feathers were gently brushed aside using a wet cotton swab to expose the scalp.

The optodes were gently lowered until they contacted the scalp. Laboratory labeling tape affixed to the guide-crown was crossed over the fiber to secure them in place. Room lights were turned off to check for stable light intensities. Optodes were readjusted by the preceding procedure until stable light intensity (AC) measures could be maintained. Isoflurane level was adjusted to 1.5% at 0.4L/min maintenance level and five minutes were given for the subject to stabilize.

Upon completion of recording, subjects were removed from anesthesia immediately and monitored until they were spontaneously perching. Once perching, they were checked every ten minutes for the next half hour and subsequently monitored once per hour for the next three hours by our animal care staff per our animal use protocol.

Absorption spectra determinations

The optical responses are expected to be dominated by changes in light absorption by oxygenated hemoglobin (HbO) and deoxygenated hemoglobin (HbR), and therefore the intensity values can be converted to estimates of changes in [HbO] and [HbR] using the modified Beer-Lambert law (Wray et al. 1988). For this conversion we used differential path lengths measured from the zebra finch head (Ramstein et al. 2005) and extinction coefficients measured from zebra finch hemoglobin rather than those reported for mammalian species to account for species-specific idiosyncrasies. The extinction coefficients were obtained for both HbO and HbR (Wray et al. 1988; Hanash and Shapiro 1981).

The absorption spectrum for zebra finch hemoglobin (Fig. 3) was measured from hemoglobin isolated from pooled whole blood collected from 9 (male and female) adult zebra finches (Riggs 1981).

The absorbance spectrum for oxy-hemoglobin (HbO) was measured in a Cary 300 UV-VIS spectrophotometer (Varian Inc., Lake Forest, CA) after Hb was allowed to saturate in air. To obtain values for HbR, purified hemoglobin was placed in an anoxic chamber and gently pipetted for 30 s. The cuvette was sealed with a rubber septum within the anoxic chamber prior to measurement. To double-check hemoglobin reduction, a second reading was taken after returning the cuvette to the anoxic chamber and adding several micrograms of dithionite.

Protein concentration, necessary for calculation of extinction coefficients, was determined by amino acid analysis (Moore and Stein 1948). This involved hydrolysis in hydrochloric acid followed by quantitation by gas chromatography and mass spectrometry. Protein concentration was further verified by bicinchoninic acid assay (Olson and Markwell 2007).

Optical data analysis

Data were processed using programs custom-written using MATLAB (MathWorks, Natick, MA). Signals from 8 sources were received by 4 detectors, giving a total of 32 channels. Raw data were sorted based on channel (source and detector pair) for all 32 channels. Since light sources were paired together, the 32 channels represent 16 light paths.

Sorted data were normalized: intensity data were divided by the mean intensity across the recording period, resulting in proportional change values. The data were then curve-fitted to a third order polynomial, which was then subtracted from the data to remove slow changes and drifts. Data were then filtered using a band-pass Butterworth filter of 0.05 – 0.5 Hz.

AC data is presented because, compared to DC data, they are relatively tolerant to changes in ambient light levels and therefore better represent biological activity. Data were converted to hemodynamic measures using the Modified Beer Lambert Law (Wray et al. 1988), which implicitly assigned all changes to absorption. The Modified Beer Lambert Law requires the use of several constants: the mean reduced scattering coefficient measured from zebra finch (Ramstein et al. 2005) through a path estimated to go through the higher auditory areas NCM and field L is 4.857/mm. This was the best measured estimate we could use for this coefficient, and we used the same coefficient for all light paths although they may have passed through different tissue and geometry. We used the extinction coefficients measured from pooled zebra finch hemoglobin as described above (Fig. 3).

Mapping procedures

Channel data were combined using back-projection from 16 paths to show an approximation of the spatial distribution of activity in two-dimensions. A matrix was weighted based on a model of light diffusion and created using a simplified form of the Boltzmann transfer equation applied to each optode location (Fishkin and Gratton 1993). This weighting matrix is based on the probability that recorded light intensities can be represented in a voxel based

on the voxel's distance to sources, detectors, and the surface of the head. It assumes the head is a homogeneous medium and that the only border exists at the scalp of the imaged slice (Fig. 4b). These maps should be taken to represent approximations of activity location. Maps created for [HbO], [HbR], and total hemoglobin concentration ([HbT]) are shown in (Fig. 6).

Results

Extinction coefficients

Extinction coefficients were measured from zebra finch blood (Fig. 3). These values allow changes in oxy- and deoxyhemoglobin levels to be calculated from changes in light intensity values. The shape of the spectra and the values of these measured coefficients were similar to values found for mammals; however, the wavelength at which the spectra for oxy- and deoxyhemoglobin intersect, the isosbestic point, occurs at a longer wavelength in the zebra finch than it does in mammals. In the zebra finch, the isosbestic point is between 870 nm and 880 nm. While our source wavelengths would straddle the isosbestic point of mammals, for the zebra finch both our source wavelengths are shorter than the isosbestic point.

Optical and hemodynamic response

The time course of the optical response is revealed in the grand mean of all channels, trials, and subjects (Fig. 4). The transmitted 690nm light intensity increased immediately after stimulus onset and peaked at approximately 2s after stimulus onset before reversing direction. This reversal of direction was larger in magnitude in comparison with the initial peak and reached its nadir between 5s and 7s after stimulus onset. A decrease was observed in 690nm intensity that began 0.7 – 0.8s after stimulus offset and reached its nadir between 2.5 and 3s after stimulus offset.

The optical response recorded at 830nm was smaller in magnitude than the response recorded at 690nm. The initial increase in transmitted 830nm light intensity occurs just after the stimulus and then begins decreasing 5s after stimulus onset (Fig. 4). Conversion of these data to represent change in [HbO], [HbR], and [HbT] reveals the time course of the hemodynamic response. In both awake and anesthetized birds we observe three distinct phases in the response as it develops after stimulus onset (labeled I, II, and III on the right side of Fig. 4). During phase I there is a large initial increase in [HbO] with a small decrease in [HbR] resulting in an overall increase in [HbT]. This initial response shares features of hemodynamic (BOLD, blood-oxygen level dependent) responses measured in other species, including humans (Hoshi and Tamura 1993; Villringer and Chance 1997; Malonek et al. 1997; Berwick et al. 2002). Phase II immediately follows phase I and is marked by a dramatic reversal, with [HbO] and [HbT] now decreasing below baseline and [HbR] increasing. Phase III emerges 16–17 sec after stimulus onset and may represent a response to stimulus offset (at 15 sec). The optical responses to stimuli were robust and consistently elicited in single trials (Fig. 5). In each trial the three phases of the response can be seen.

Response localization

We created spatial representations (maps) of recorded activity using back-projection of channel data. The responses in the first trial of the first tested subject are shown as representative responses (Fig. 6). Colors represent relative change: positive changes are depicted in red tones, negative changes in blue tones, and levels close to baseline in green tones. Separate maps are shown for change (relative to baseline) in [HbO], [HbR], and [HbT], as measured at 17 different time points before and after onset of the song stimulus and corresponding to the peaks and valleys from Fig. 4. The anatomical orientation of the maps is depicted in Fig 6b, which is derived from merged microCT and MRI data as described in Methods. It is important to stress that this image serves only to inform the reader of the anatomy underlying the areas directly between the light sources and detectors rather than to suggest precise correspondence between Fig. 6a and the anatomy shown in the MRI image of Fig. 6b. Further work will be necessary to determine the degree of registration between our spatial representations of optical data versus a standard reference such as one produced using fMRI, ideally by performing DOI simultaneously. Activity maps in Fig. 6 localize the greatest activity to the caudal and medial portions of the brain, consistent with prior evidence from gene expression, electrophysiological recordings and fMRI. However the anatomical resolution appears coarse, and does not sharply outline an anatomical boundary or any internal structure for the song responsive region. For example, the maps do not resolve Field L from NCM, adjacent subareas where different auditory responses have been extensively demonstrated. Nevertheless, these maps demonstrate sensitivity to spatial change.

Discussion

This study demonstrates a strategy for using non-invasive diffusive optical imaging to monitor broad regional brain activities in the zebra finch. We used 3D modeling software and rapid prototyping to achieve a high level of control and flexibility over the design of the physical interface. This interface is compatible with repeated measurements over time from the same animal and should be readily adaptable to recording from awake birds (ms. in prep) and potentially even from behaving animals. The time course of the hemodynamic response revealed in this study may be helpful for designing future experiments using fMRI, as alluded to by (Poirier et al. 2009). Image resolution using fMRI may also be improved by timing acquisition to specific phases of the response (Zhao et al. 2007a; Chen-Bee et al. 2007; Zhao et al. 2007b). Our results corroborate several previously reported results in zebra finch (Mottin et al. 2011), but with some key differences.

Response

The optical response to auditory stimuli is robust, reliable, and complex for lightly isoflurane anesthetized subjects (Fig. 4 and 5) and the response can be seen in single trials (Fig. 5). The hemodynamic response follows a recognizable pattern in Phase I (Fig. 4) in comparison to those found in anesthetized mammals (Malonek and Grinvald 1996; Malonek et al. 1997). The increased oxygenation of phase I is similar in appearance to the BOLD response recorded using fMRI reported by Voss et al. (2007), and is consistent with the

finding in highly vascularized brain areas such as the neo-cortices of mammals demonstrating a relative overabundance of oxygenated hemoglobin (Fox and Raichle 1986; Maloney and Grinvald 1996; Ekstrom 2010). The shape of the optical HbR response is expected to be inverted compared to the BOLD fMRI response since BOLD-contrast originates from paramagnetic HbR formed when oxygen is lost from diamagnetic HbO (Ogawa et al. 1990).

After the BOLD-like response of phase I (Fig. 4), [HbO] and [HbT] dipped dramatically from about 2s to 7s after stimulus-onset. There was a correspondingly dramatic increase in [HbR]. Together, these define a distinct second phase of the hemodynamic response (Fig. 4, phase II). An equivalent phase II component of the hemodynamic response has not been specifically reported in fMRI or optical imaging studies using isoflurane on the zebra finch (Boumans et al. 2007; Poirier et al. 2009; Mottin et al. 2011) nor in studies involving anesthetized rats or cats (Berwick et al. 2002; Zhao et al. 2007a; Zhao et al. 2007b). While it logically follows that [HbO] and [HbR] would change in opposite directions, it is interesting to note that [HbT] is decreasing. This apparent decrease could be interpreted at face value as a net relative blood flow out of the area of activation, perhaps due to vasoconstriction. Alternatively, this could be an artifact, perhaps of a change in light scattering which has not been unaccounted for.

Shortly after stimulus offset there is an undershoot (Fig. 4, phase III) corresponding to a decrease in [HbO] and an increase in [HbR]. Similar findings have previously been reported in mammalian studies (Maloney and Grinvald 1996; Maloney et al. 1997; Chen-Bee et al. 2007). This phase of the response may be caused by blood vessel compliance (Mandeville et al. 1999; Chen-Bee et al. 2007).

Our results share important similarities to the results reported by Mottin et al. (Mottin et al. 2011). Specifically, both our findings and theirs for HbR demonstrate highly similar phase I (BOLD) and III (undershoot) behavior.

Our results also differ in a key way. The behavior of the HbO response described in this study is almost the inverse of what is shown by Mottin et al. Our data show an immediate increase in [HbO] after stimulus onset, which peaks at 2 seconds while Mottin et al. shows HbO dropping to low levels around 2 seconds after stimulus onset. Where we find HbO to decrease in phase II, Mottin et al. show an increase. Finally, although our respective findings demonstrate a nearly identical timing of HbO changing at stimulus offset (phase III), the direction of the change reported here (undershoot) is exactly opposite to what Mottin et al. report (overshoot; i.e. - “post-stimulus BOLD”).

The findings reported here, in contrast to those reported by Mottin et al., appear to agree more clearly with precedence from mammalian studies. In mammals, the delivery of oxygenated blood is in excess of what is used. This is thought to happen due to increase of blood flow and volume into tissue (Fox and Raichle 1986; Maloney and Grinvald, 1996; Ekstrom 2010). Likewise, the BOLD response reported here also occurs with an increase, rather than a decrease, in [HbO].

We suggest that the discrepancy between our findings and those of Mottin et al. is due to the different extinction coefficients for HbO and HbR that we used for our respective calculations; we calculated the extinction coefficients from absorbance values measured using oxygenated and deoxygenated zebra finch hemoglobin (see Methods; Fig. 3) while Mottin et al. used extinction coefficient values from mammals (Mottin et al. 2011). Although the difference between zebra finch and mammalian values is subtle, the zebra finch isosbestic point is found at a longer wavelength than it is in mammals. This means, for all wavelengths shorter than approximately 870 nm to 880 nm, which would include both our source wavelengths, 690 nm and 830 nm, HbR is responsible for absorbing a greater proportion of light than HbO. In contrast, with the shorter isosbestic point of mammals, the proportion of light absorbed at 690 nm is more greatly represented by HbR than HbO while the proportion of light absorbed at 830 nm is more greatly represented by HbO than HbR. Hence, the usage of mammalian absorption spectra values for calculating zebra finch hemoglobin concentrations will result in values which are incorrect and opposite in sign whenever a wavelength greater than the mammalian isosbestic point but less than the zebra finch isosbestic point is used.

This explanation for the discrepancy might also help resolve some of the peculiarities reported from an earlier study using mammalian extinction coefficients to calculate [HbO] and [HbR] (Vignal et al. 2008); in this study total hemoglobin levels were found to slightly decrease in response to hypercapnia – a result which opposite from what has been observed in mammals (Martin et al. 2006; Wegener and Wong 2008).

Substitution of mammalian for zebra finch extinction coefficients might also explain why the novel post-stimulus BOLD responses reported in Mottin et al. have not been reported previously. If these post-stimulus BOLD responses are, instead, actually post-stimulus undershoots as we observe (Fig. 4), then the findings would agree with precedents evident from mammalian and human optical imaging studies (Malonek et al. 1997; Franceschini et al. 2003) as well as findings from zebra finch fMRI (Voss et al. 2007; Boumans et al. 2007).

It is also possible that the discrepancy between the present study and Mottin et al. may be explained by procedural differences such as level of anesthesia or arrangement of light source and detector. For instance, the present study transmitted light across the skull using sources and detectors placed on opposite sides of the head. By using multiple optodes surrounding the head, we sampled the aggregate activity of a large area of the brain. On the other hand, Mottin et al. specifically target the caudal medial area of the brain (including NCM) by illuminating the head using a white laser and detecting the emerging light along a slit with an incredible resolution of 250µm from the same side of the head (Ramstein et al. 2005; Vignal et al. 2008; Mottin et al. 2011). Also, while we did not monitor temperature, Mottin et al. used a feedback-controlled heating pad holding body temperature between 39° and 40° C. Although shivering was not observed in any subjects, this raises the possibility that our different findings may be influenced by body temperature and thermoregulation.

Irrespective of the discrepancy, it is important to note that the application of diffusive optical methods in birds is new and may produce results that cannot be interpreted in the same way

as those in mammalian studies. There exist optical properties that have not yet been accounted for in models based on mammalian data. An important example of this is demonstrated in the avian red blood cell: these contain nuclei and organelles (Stevens 1996) which are absent from mature mammalian red blood cells but are primary contributors to light scattering in tissues (Beauvoit et al. 1995; Mourant et al. 1998).

It is also possible that the DOI technique is unmasking physiological processes that are not captured in a purely hemodynamic interpretation of the data. Changes in neural activity have been reported to alter the light scattering properties of brain tissue (Malonek and Grinvald 1996; Rector et al. 1997; Stepnoski et al. 1991; Gratton et al. 1995). Our calculations also assume constant tissue absorption and scattering coefficients across different brain regions and over the duration of the experiment. As Ramstein et al. (2005) and others (Kocsis et al. 2006) have noted, these coefficients may change with different paths or activity. Eventual incorporation of these variables into our analytical models may allow additional physiological information to be extracted from DOI measurements.

Response localization

The activity maps generated using back-projection show that the response to auditory stimuli is largely localized to the caudal and medial portions of the brain (Fig. 6), very closely corresponding to the NCM and auditory response areas of the avian forebrain. This general area of localization is consistent with those previously shown to be activated using fMRI in response to auditory stimuli (Voss et al. 2007; Boumans et al. 2007; Boumans et al. 2008a; Boumans et al. 2008b; Poirier et al. 2009).

Despite the strong correspondence between our mapped responses and our a priori expectations for localization, our localization model requires further validation. It is possible that our model was incidentally correct in displaying the location of greatest activity, and future studies may focus on fine-tuning our model versus a more established imaging modality such as fMRI or utilizing stimuli of other sensory modalities such as vision which would map to different parts of the avian brain. That being said, the maps of the auditory response demonstrate evolution and lability over time. The most apparent change in location of peak activity can be seen in the images of [HbO] in Fig. 6a. This would suggest that the predominant caudomedial localization of activity using our multi-channel method is not some simple signal processing artifact of signal processing. Future improvement in the quality and spatial resolution of the maps may be obtained by increasing the number of recording channels and their spatial overlap, decreasing optode size, adding more wavelengths, improving our model of photon transport, taking more precise measurements of optical coefficients, and using better image reconstruction algorithms.

Notwithstanding these limitations, the data clearly indicate the feasibility and usefulness of our approach to non-invasive diffusive optical imaging.

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Abbreviations

AC	alternating current
BOLD	blood-oxygen level dependent
DC	direct current
DOI	diffusive optical imaging
fMRI	functional magnetic resonance imaging
HbO	oxyhemoglobin
HbR	deoxyhemoglobin
HbT	total hemoglobin
MicroCT	X-ray microtomography
MRI	magnetic resonance imaging
NCM	caudomedial nidopallium
NIRS	near-infrared spectroscopy

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Fig 1.

Optic fibers were coupled to the zebra finch scalp by designing and building interfaces around a scanned and digitized head. **a** Several views of the optode arrangement relative to the zebra finch head. **b** Optode arrangement is based on location of NCM. Visualization of NCM relative to the skull was accomplished by warping (in blue) the MRI zebra finch atlas (Poirier et al., 2008) with (in red) the brain-case of our digitized head to form a composite brain (yellow). See Methods for details. **c** The composite brain is fitted to the skull of our virtual head. The top part of the virtual head is removed to reveal the composite brain. **d** Mid-sagittal cut-away view of Fig. 1c showing the fitted MRI atlas. **e** A closer parasagittal and **f** horizontal view reveal brain areas relative to the optodes. The primary auditory area, Field L, serves as a landmark (dark area indicated by red arrow). **g** Closer view of the anatomical areas interrogated by the light paths from optodes to detectors. NCM = caudomedial nidopallium; L = field L; Cb = cerebellum; LMAN = lateral magnocellular nucleus of the anterior nidopallium; HA = apical part of the hyperpallium; and RA = robust nucleus of the arcopallium (Poirier et al., 2008; Reiner et al., 2004; Nixdorf-Bergweiler and Bischof, 2007 [cited 24 Mar 2010]).

Fig 2.

a The apparatus for anesthetized-birds allows delivery of gas anesthesia while leaving the head free. Optodes are held in a crown that can be precisely lowered using a leadscrew. **b** A different angle of the apparatus showing a bird under gas anesthesia prior to having the guide-crown lowered.

Fig 3.

Absorption spectra for zebra finch HbO (red) and HbR (blue). Extinction values are highlighted for 690nm and 830nm by the vertical double lines. The isosbestic point for zebra finch (*Taeniopygia guttata*) hemoglobin is between 870nm and 880nm, which is at a longer wavelength than it is for mammalian hemoglobin.

Fig 4.

Grand mean AC intensity and hemodynamic responses of all subjects, channels, and trials during 15s song playback (yellow area shows when song was played). Bandpass filtered AC intensity values are shown on the left. Red lines are intensity values for 830nm; blue are for 690nm. These intensity values were used to estimate hemodynamic changes (on the right) by using the modified Beer-Lambert Law. Red, blue, and green lines show relative change from baseline in oxy-, deoxy-, and total hemoglobin concentrations, respectively. Dotted lines show respective standard errors for each time point calculated from the variance of all channels, subjects, and trials. The y-axis shows change relative to baseline in arbitrary units. Three distinct phases of the response are indicated by I, II, and III.

Fig 5.

Mean hemodynamic response of all subjects and channels, displayed by trial. Red, blue, and green lines show relative change from baseline in oxy-, deoxy-, and total hemoglobin concentrations, respectively. Dotted lines show respective standard errors for each time point. Standard errors are small so they are difficult to see but are present in the above plot. Y-axis is in arbitrary units of change in concentration relative to baseline. Responses to the same stimulus were repeatable across trials and consistent in showing the three phases mentioned in Fig. 4.

Fig 6.

Localization of responses: Hemodynamic activity maps showing spatiotemporal development of the response to the first presentation of the song stimulus for one subject (we arbitrarily chose to show the first subject). **a** Each map represents activity in the area shown in Fig. 1g. Greener colors correspond to baseline levels, redder colors indicate increases, and bluer colors indicate decreases. Time points are relative to stimulus onset and roughly trace the evolution of the response shown in Fig. 4. Brackets on the left side of the figure correspond to the approximate timing of response phases. Time is in seconds relative to stimulus onset (Note: the stimulus is 15s in duration), the gray box in the background represents time when stimulus was playing. **b** Enlarged versions of the boxes highlighted in red from Fig. 6a are shown below an anatomical slice of brain from the zebra finch MRI atlas. Maps crudely approximate areas of changes relative (and not absolute) to baseline. The color scale for these images was adjusted to prevent clipping.