Published in final edited form as:

Methods. 2006 March ; 38(3): 221–226.

Using PET H₂O¹⁵ Brain Imaging to study the functional-anatomical correlates of non-human primate communication

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Abstract

In this article we describe methods for using oxygen-15 water (H₂O¹⁵) positron emission tomography (PET) to explore the functional neuroanatomy of cognition in awake, non-human primates. The discussion is based on a recent study designed to identify regions in the monkey brain associated with perceiving auditory stimuli, and species-specific calls, in particular [1]. Details are provided concerning critical aspects of the experimental paradigm, including pre-scanning habituation sessions to acclimate the animals to the PET scanner environment, and details of a pilot study to determine the auditory stimulus parameters necessary to produce robust activity in brain regions known to process auditory information (belt and parabelt regions of monkey auditory cortex). Methods for acquiring and analyzing PET data in order to identify significant regions of brain activity in single animals are also presented.

Keywords

PET; Functional Brain-imaging; rhesus macaque; species-specific calls; communication; auditory processing; neurophysiology

Introduction

We recently reported a brain imaging study that was designed to identify the neural substrate active in rhesus macaques when presented with meaningful vocalizations (species-specific calls) [1]. We were particularly interested in determining whether the study of species-specific vocalizations in a non-human primates would provide us with some insight into the neural system upon which human conceptual representation, and ultimately language, may have evolved. We used the rhesus macaque as our non-human primate model because they have a well-documented and extensively studied vocal repertoire consisting of meaningful (semantic) calls. These species-specific vocalizations include referential calls (representing an object or condition in the animal's environment) as well as affective calls (containing information about the animal's emotional status) [2-8]. This work, coupled with detailed information on rhesus macaque audiovocal pathways and knowledge about their social structure, makes these non-human primates a valuable species for studying the neural substrates of a communication system that may have provided a foundation for the development of human language. Like

other old world monkeys, rhesus live in matrilineal social groups consisting of 15-75 individuals. The social group revolves around complex kinship networks defined largely by the matrilineal hierarchy but also including friendships, struggles for dominance, and shifting alliances [9,10]. As field studies have shown, and as laboratory studies have confirmed, the macaque repertoire encompasses various types of vocalizations that contain information about their socioecology [8,11,12]. It has been demonstrated that macaques can acoustically recognize individual members of their social group, as well as alert other group members about predators and food through a variety of calls.

In order to identify the functional anatomical correlates of these calls we used two brain imaging techniques: structural magnetic resonance imaging (MRI) and positron emission tomography (PET). Structural MRI is an imaging technique that can be used to create anatomical maps of the brain. PET is an imaging technique that permits the *in vivo* measurement of physiological and biochemical parameters in a quantitative, regional fashion [13]. While the vast majority of PET studies have been done in humans, this technology has recently been extended to the study of cognition in awake non-human primates [14-19]. The PET camera measures the distribution of positron-emitting radiopharmaceuticals in the brain. Mathematical models are used to obtain the desired measurements from these images. The H_2O^{15} isotope method was chosen because it measures regional cerebral blood flow, which, in turn, reflects local neuronal activity. Moreover, unlike other methods used in previous studies such as FDG [18], the H_2O^{15} isotope has a fast uptake (approximately 12 seconds in humans) and short half-life (approximately 2 min), permitting acquisition of multiple scans during a single test session (one scan every ~ 5 minutes). With this design, scans can be acquired during different behavioral states (i.e. different experimental conditions) and compared quantitatively, thus allowing for the generation of statistical parametric brain maps (SPMs) of neuronal activity associated with these behaviors. The co-registration of these “functional” scans with the structural MRI scans provides a means for identifying the anatomical location of each activation.

Research methodology

Subjects

Four adult rhesus macaques, *Macaca mulatta* (two male and two females) were trained according to the method described below and used as subjects in various studies. Only one animal (adult male) was used in the pilot study described here. All procedures and animal care were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Each animal served as its own control. Our design minimizes pain and distress by using the relatively non-invasive PET procedure and by carefully acclimating the animals to each procedure in the course of training.

Restraining apparatus – customized chair

The monkey chairing apparatus was designed by Crist Instrument Co., Inc. and the authors, in agreement with the NIH Guidelines for the Use of Restraint Chairs with Nonhuman Primates (Figure 1). The design was based on a previous Crist Instrument Co., Inc. model modified to optimize monkey restraining, comfort, and PET scan compatibility. The chairs are non-metallic and designed such that the arms, legs, body, and neck are passively, but securely, restrained. Safety features of the chair include a chest plate and front double locks. For transport of the awake animals between the housing room and the PET suite the animal was chaired and moved inside of a solid transport cover box (Crist Instrument Co., Inc. model VPCK0A). This transport cover box is built on an aluminum frame and covered with opaque white Lucite. It includes filter vents, spring latches, to fixate the chair, and a trolley base. Chairs are sanitized between sessions using Alcide, and are cagewash resistant. Prior to construction, we tested several materials to assure that there would be no, or minimal, interference with the operation

of the PET scanner. Materials chosen were acrylic and graphite. In order to be rolled into the scanner gantry and stabilized in the proper position for scanning, the chair has an attachable base that can be securely fixed to a rolling table. The table was constructed of aluminum, with casters to facilitate the positioning of the animal. The table was equipped with a base with hinge joints over which the chair was fixed, allowing the animal to be tilted backwards for proper placement in the PET scanner. The chair includes an adjustable base to allow for animals of different sizes, and an adjustable back support to increase comfort of the animal when tilted backwards (see Figure 2).

Animal training

Behavioral training was accomplished using incremental training steps reinforced by positive rewards for the animal (e.g. grapes, juice, etc.). Negative reinforcement was never utilized. Training manuals were kept as part of the clinical records. Detailed behavioral observations were recorded at each training session. Animals were carefully monitored for any behavioral abnormalities, such as persistent intense agitation, self-inflicted injuries, or distress vocalizations that would suggest poor adaptation to the training and subsequent testing.

The study required that the animals be maintained in a relaxed state while listening to auditory stimuli in the PET scan environment. Following typical pole and collar training, each animal was habituated to the chair/restraining apparatus and to the PET suite environment using the following training sequence:

1. Chair acclimation: *Phase I*- the animal was transported to an isolated training room. Training consisted of passively sitting in the chair (in the vertical position; no stimuli presented); *Phase II*- Identical to 'phase I' but with the chair tilted 70° backwards (same position as in future scanning sessions).
2. Auditory stimuli acclimation: the animal was transported to an isolated training room. Training consisted of passively sitting in the chair, tilted backwards while broadcasting noise bursts from a hidden speaker.
3. Acclimation to the PET scanner suite: *Phase I*-The animal was transported to the PET suite. Training consisted of passively sitting in the chair tilted backwards (Figure 2a) and rolled into the scanner gantry (Figure 2b). The PET suite environment (including the speaker) was occluded by a canopy of white sheets to avoid distraction and create a more soothing environment for the animal; *Phase II*- Identical to 'phase I' but acoustic stimuli (broadband noise bursts) were played from a hidden speaker. The animal was rewarded with juice between auditory presentations (Figure 2c).

To ensure useable, artifact free PET data, it is critical that the subject's head does not move during scanning. In order to prevent monkey head movement (>1 mm) it is necessary to stabilize the head. Previous behavioral studies have determined that only rigid fixation of the head will provide the necessary immobility. Implantation of head fixation devices in non-human primates is routinely done in various non-human primates laboratories, generally without complication or significant discomfort to the animals. Therefore, after successful completion of the above described training program, each animal was surgically implanted with a head fixation device. The head fixation device, also produced by Crist Instrument Co., Inc., consists of three separate pieces made of non-magnetic, MRI compatible material (graphite). Two of these pieces were small indented hollow tubular structures. One of these structures is implanted in the monkey's skull using dental cement and supported by surrounding head screws. Ideally, these screws should be ceramic, although the titanium screws are within reasonable interference limits for PET scanning. The second tubular structure attaches directly to the top of the chair. The third piece is a long screw that enters the two tubular structures (from the top of the chair) attaching them together.

The full habituation training procedure took approximately between six to nine months to complete. Habituation training was accomplished solely with positive reinforcement (using fruits and juice).

Acoustic stimuli presentation

As stated in the introduction, this setup and animal training procedure aimed to study the neural basis of species-specific vocal communication in awake non-human primates, using blocks of species-specific vocalizations as auditory stimuli. In order to avoid habituation to a given call and present enough call exemplars to elicit a robust activation of the auditory cortex, a large number of stimuli may be necessary. However, high quality recordings of animal vocalizations given in natural settings are very hard to obtain. Also, when we began our studies, no information was available on how best to activate auditory cortex in monkeys using auditory stimuli. In fact, we were aware that that previous attempts to record activity in auditory cortex using functional brain imaging technologies had not been successful. Successful functional brain imaging studies in humans suggest that the duration of stimulus presentation and the inter-stimulus interval (ISI) were important parameters. Thus, prior to beginning our experiment, it was crucial to determine how many unique stimuli would be needed, how long each stimulus should last, and the rate at which they should be presented, in order to elicit a significant brain activation.

For this purpose, a pilot study was performed in one animal. Two variables were tested: i) *Inter-stimulus interval (ISI)*, that was either 1) short: a mean of 0.875 seconds, randomly jittered between 0.5 and 1.25 seconds or, 2) long: a mean of 1.75 seconds, randomly jittered between 1 and 2.5 seconds; and ii) *Presentation-block duration*, that was either 15 or 30 seconds (Figure 3). These two variables were combined into three test conditions (long ISI/long block; short ISI/short block; short ISI/long block). Broadband noise bursts were used as acoustic stimuli (Figure 4), since it is a neutral stimulus (meaningless, with no emotional valence) and has been shown to elicit robust activation of auditory cortex based on previous neurophysiological studies [20,21]. The sounds were amplitude normalized and the stimulus-block sequences were created and presented using 'SIGNAL[®]' software (Engineering Design, Inc.). Sounds were broadcast using a DAQCard-6062E (National Instruments[®]) and an Advent (Powered partners AV570) speaker (frequency response: 40 Hz-30 kHz with a broadcast value of 67.93 dB SPL at 1.5 m).

Brain images acquisition

Magnetic Resonance Imaging (MRI) images—Animals were previously anesthetized with ketamine and transported under anesthesia to the MRI facilities. MRI was performed, on anesthetized animals, in a 1.5 T Signa unit (GE Medical Systems, Milwaukee, WI), using a 5-inch GP surface coil. T1-weighted MR images were obtained using 3-D volume SPGR pulse sequence (TE 6, TR 25, flip angle 30), field of view (FOV) was 11 cm, and slice thickness 1 mm.

Positron Emission Tomography (PET) images—We have acquired PET images from fully awake animals. The monkey was rolled into the scanner gantry. The PET suite had the lights dimmed and, as during training, all objects in the room were hidden from view by a canopy constructed of white sheets. PET images were acquired in 2-D mode with a GE Advance scanner (GE Medical Systems, Waukesha, WI). For each scan, stimulus presentation was initiated simultaneously with the beginning of intravenous injection of 50 mCi of [¹⁵O]water diluted in 13 ml of saline. Each injection was performed slowly (~ 20 sec from start to finish). There was a 5-min period between injections (scans). The beginning of the 60-sec scan period was triggered automatically following a step increase in the scanner count rate reflecting the arrival of radioactivity in the brain. 35 slices were acquired. The voxel size was 2mm × 2mm

× 2mm, with a resolution 6.5mm FWHM (Full Width Half Maximum) in x, y and z axes. The transverse field of view was 55cm, and the axial field of view was 15cm. Images were reconstructed using 8 minute transmission scans for attenuation correction obtained at the beginning and end of each scanning session. During the PET session, we monitored heart rate and saturated oxygen by pulse oximetry. We also monitored vocalizations (i.e. screams and pant threats rather than coos, serve as indices of distress) to measure stress levels. If high stress levels were observed, and/or if the monkey emits vocalizations that are continuous, the experimental session for that monkey would have been aborted. This never happened, neither during the pilot study described here, nor during the subsequent auditory studies.

Data analysis

PET brain image analyses were performed using software incorporated in MEDx[®] (Sensor Systems Inc. Sterling, VA). MEDx[®], as well as other brain imaging analysis programs such as SPM[®] or AFNI[®], are designed to analyze human brain data. Thus, customized scripts were needed in order to analyze the monkey PET data. The scripts were created by Sensor Systems Inc. Sterling, VA in collaboration with the authors. Realignment of the multiple PET images was accomplished using the FLIRT (FMRIB's Linear Image Registration Tool – v3.1) function – intra-modality routine (Model/DOF: Rigid Body (6 parameter model)). Analyses of whole brain activity and creation of statistical maps (t-maps) were calculated using Statistical Parametric Mapping (SPM99[®] Wellcome Department of Cognitive Neurology, London, UK). Separate analyses were performed on the data acquired from each animal. We identified regions that showed a significantly enhanced response to the presented acoustic stimuli (broadband noise bursts) versus silence (no acoustic broadcast, only the PET suite environmental noise) ($P < 0.01$). The functional activation t-maps were transformed and realigned with FLIRT – inter-modality routine and shadow transform functions – using the animal's own MRI image as reference. For each animal, functional render statistics were used to fuse the functional activation maps with the respective high resolution structural MRI's so that the anatomical location of each active area could be identified. The neuroanatomical structure was defined using “The rhesus monkey brain in stereotaxic coordinates” by G. Paxinos et al. (Academic Press; 2000) as reference [22].

Acoustic stimulus-block structure

Analyses of the data from the pilot study indicated that the short ISI (0.5 to 1.25 seconds) and the stimulus-block duration of 30 seconds, elicited the strongest activation of auditory cortex (Superior Temporal Gyrus) (Figure 5). This stimulus-block structure was used for all acoustic stimulus-blocks presented in our subsequent studies [1].

Concluding Comment

During the past two decades, the advent of non-invasive, functional brain imaging technologies has revolutionized the study of the human brain. The need for these procedures (PET, functional MRI, and magnetoencephalography, MEG) was self-evident. Without non-invasive imaging techniques, the only means of studying brain/behavior and brain/mind relationships in humans was by investigating cognitive and emotional dysfunction in brain damaged patients. In contrast, the advantage of using imaging to address questions about functional neuroanatomy in non-human primates was not as readily apparent. After all, the monkey brain could be studied directly by recording from single neurons during behavior, and by evaluating the effects of precisely placed, surgical lesions. However, both of these time-honored approaches have their limitations. Single cell recordings are severely limited in view. Only small regions of cortex can be evaluated at any given time. Lesions produce irreversible damage and, although the location of damage is known, the potential disruptive effects of this damage on distal brain regions is not. In contrast, functional brain imaging techniques reveal the location of all regions

involved in performing a particular task and provide a non-invasive means for probing the functional characteristics of these areas. As a result, functional imaging technologies are now increasing recognized as an important addition to the armamentarium of investigators interested in the non-human primate brain [23].

It is likely that future studies of non-human primates will most commonly use fMRI rather than PET; especially if standard MRI machines prove to be adequate for this purpose [24]. MRI provides better spatial and temporal resolution than PET and does not require producing and injecting a radioisotope. However, PET will continue to be the modality of choice for investigating the chemical basis of the neurophysiological events via ligand-binding studies. PET will also continue to be used in the investigation of nonhuman primate cognition because of two main advantages over the MRI environment. First, only the top of the animal's head is inside the apparatus, whereas MR requires that the animal's entire body being encase in a relatively small diameter cylinder. As a result, with PET the animal can be tested while restrained in its customary testing chair, thus greatly reducing habituation and training time. Second, the PET environment is nearly silent, whereas fMRI environment is extremely noisy. The banging noise generated during scan acquisition would be expected to be particularly disruptive for studies of auditory cognition as described in this report.

Acknowledgments

We thank Steve Suomi for providing the animals; Marco Lopes, James O'Malley, Shawn Milleville, Lucinda Prevost, Sam Antonio, Bonny Forrest, Steve Wise, Jim Fellows, Richard Carson, Peter Herscovitch, Jerry Jacobs, Tonya Howe, Jeff Bacon, Jay Chincuanco, Terence San Juan, Shielah Conant, Jonathan Fritz, Richard Saunders, Marc Hauser, Jeff Solomon and Jeff Quinlivan, for assistance on various phases of this research; This work was supported by the National Institute on Deafness and Other Communication Disorders –Division of Intramural Research Programs (DIRP), the National Institute of Mental Health–DIRP, and a grant from Fundação para a Ciência e Tecnologia.

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Figure 1.
Customized monkey chair (Crist Instrument Co., Inc.)

Figure 2. Animal in the PET suite. **a.** subject chaired and tilted in the PET suite. **b.** Final position inside the scanner gantry. **c.** Subject being rewarded (juice) between scans, during a session.

Figure 3.

Illustration of the two variables (ISI and block duration) manipulated during the pilot study to determine an adequate stimulus-block structure.

Figure 4. Waveforms and spectrograms of the acoustic stimulus (broadband noise bursts) presented during the pilot study. **a.** Long ISI. **b.** Short ISI.

Figure 5. Selective activation of the STG ($P < 0.01$) during the three different conditions (i.e. different stimulus-block structures) of 'broadband noise bursts' broadcast. The black areas correspond to clusters of significant activations.