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fMRI in alert, behaving monkeys: An adaptation of the human infant familiarization novelty preference procedure

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Abstract

Functional magnetic resonance imaging (fMRI) is increasingly used in non-human primate research. In the present study, we adapt the familiarization-novelty preference (FNP) procedure used in human infant research to examine visual behavior in alert, unanaesthetized rhesus monkeys that were acclimated to the fMRI environment, but did not undergo behavioral training. In keeping with the typical FNP paradigm, we recorded eye movements (looking time and number of fixations) while monkeys viewed a series of four identical pictures (familiarization phase) followed by two different pictures (novelty phase). Number of fixations and looking time both increased during the novelty phase, thereby demonstrating visual discrimination of the new from the old picture. Importantly, discrimination did not occur on catch trials in which six identical pictures were presented. Moreover, brain activation in the amygdala was more strongly associated with the novelty phase than with the familiarization phase. In addition, magnitude of brain activation in the amygdala was correlated with the behavioral effect of visual discrimination. These findings demonstrate the feasibility of using eye movements as an index of visual discrimination in untrained monkeys during fMRI scanning. This methodological approach helps to extend the repertoire of research tools for fMRI in non-human primates.

Keywords: fMRI; Non-human primates; Visual discrimination; Novelty detection

1. Introduction

Functional magnetic resonance imaging (fMRI) has quickly become a widely used brain imaging technique for human research. Its non-invasive nature makes it appealing for a broad range of research questions and its relatively wide availability has made it more accessible than some other brain imaging methodologies. Whereas the number of fMRI studies in humans has grown exponentially since the feasibility of the technique was first demonstrated in the early 1990s, the number of fMRI studies in non-human primates has not grown as rapidly. One barrier to the growth of the technique in alert, unanesthetized monkeys is the significant time investment for training the non-human primates to tolerate the fMRI environment and to be able to perform a meaningful task in the MRI scanner. In the present study, we propose an alternative to the extensive behavioral training normally employed for non-human primate research. Rather than use fMRI on monkeys who have been trained on a specific task or behavior, we use fMRI on untrained monkeys with an adaptation of the familiarization-novelty preference procedure (FNP) used widely in human infant research. We suggest that this approach is less costly, more efficient and ultimately more compatible with the fMRI environment because it involves only eye movements rather than limb movements. This latter feature is important because fMRI is highly sensitive to head motion,

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which is more likely to occur with limb movements than with eye movements.

The FNP procedure (Fagan, 1970; Fantz, 1964) is widely used in human infant research (see Bhatt et al., 2005; Quinn and Bhatt, 2005, for recent applications). The basic protocol involves repeatedly exposing infants to a single stimulus and subsequently presenting a novel stimulus. Typically, there is a decline in looking with the repeated presentation of the familiar stimulus (i.e. habituation) and a rebound in looking with the presentation of the novel stimulus (if participants can discriminate between the two stimuli). Thus, a reliable increase in looking when the novel stimulus is presented is taken as evidence that the participant has represented the familiar stimulus and can differentiate between it and the novel stimulus. Consequently, familiarization to the repeated stimulus is a critical component for determining whether the infant detects the sameness of the stimulus and novelty detection is critical for showing that the infant detects the difference between the repeated and the new stimulus. The FNP procedure is ideal for infant research because infants cannot understand instructions, cannot communicate verbally and are not easily trained to make motor responses. For these same reasons, the FNP procedure is ideal for non-human primates. Spontaneous tests of novelty detection and visual discrimination behavior, like the FNP paradigm, exploit the natural tendency of an organism to orient to novel stimuli. Because eye movements are monitored to infer novelty detection, this task requires minimal training by capitalizing on a natural affinity to attend to novel events and stimuli.

Although previous research has used similar FNP paradigms in behaviorally naïve non-human primates both in the laboratory (Gunderson and Swartz, 1986; Myowa-Yamakoshi et al., 2003; Oden et al., 1990; Ramus et al., 2000; Wilson and Goldman-Rakic, 1994; Zola et al., 2000), and in the field (Hauser et al., 1996; Munakata et al., 2001), to our knowledge these paradigms have not been adapted for fMRI in alert monkeys. fMRI studies of visual behavior in monkeys have typically trained monkeys to visually fixate a particular location on the presentation screen (Denys et al., 2004; Dubowitz et al., 1998; Dubowitz et al., 2001a,b; Logothetis et al., 1999; Orban et al., 2003; Pinsk et al., 2005; c.f. Stefanacci et al., 1998; Tsao et al., 2003; Vanduffel et al., 2001, 2002). Training to fixation, however, is not desirable when using the FNP paradigm because eye movements are critical for inferring novelty detection and familiarization responses. If a monkey is trained to fixate, then the relevant behavioral responses of longer looking times to novel stimuli and looking away during familiarization cannot be detected. Looking away is a critical component of the behavior because it indexes habituation to a familiar stimulus. Therefore, in using the FNP paradigm in monkeys we necessarily expect that the monkeys will not fixate the stimulus for a significant amount of time. The behavior of fixating is not natural and only emerges with a significant amount of training.

Our first goal was to demonstrate that minimally trained rhesus monkeys exhibit novelty detection in an fMRI-scanning environment. We will present behavioral data to illustrate this. Our second goal was to identify the neural circuitry associated with novelty detection and to show that brain activation in those regions significantly correlates with novelty detection behavior. Hence, we present data implicating the amygdala in novelty detection behavior, which corroborates other findings of novelty detection behavior as measured in other species and in the same species with other techniques. Finally, we discuss the feasibility of the approach and make recommendations for future implementations of this methodology.

2. Materials and methods

2.1. Participants

Two female Rhesus monkeys (*Macaca mulatta*), ages 7 (4.3 kg) and 7.5 (4.9 kg) years old, were obtained from a commercial supplier (Covance, Alice, TX). Throughout the study, they were maintained on a 12-h light:12-h dark cycle in individual cages with water available ad libitum. All testing and training were conducted in the Laboratory Animal Facilities of the University of Kentucky, which are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All protocols used in this study were approved by the University of Kentucky's Animal Use Committee.

2.2. Stimuli

A corpus of stimuli representing a range of visual categories was used: photographs of unfamiliar human faces ($n = 29$), familiar human faces ($n = 7$), natural objects ($n = 33$), manufactured objects ($n = 30$), photographs of monkeys ($n = 13$), and letters from the Roman alphabet ($n = 26$). Stimuli were minimally repeated within a session (one to three repetitions), except in the case of familiar faces (seven exemplars total) and monkey photographs (13 exemplars total). Attempts were made to equalize the frequency of different stimuli across sessions, but (as described below) not all sessions were usable; therefore, the stimuli were not counterbalanced in the final analysis. Stimuli were randomly chosen to be either familiarization or novel pictures. Monkeys were lying prone in the magnet with pictures presented on a translucent screen placed in front of the animal using an MRI-compatible Avotec SilentVision SV-6011 (Avotec Inc., Stuart, FL) LCD projection system. Each picture was standardized to be 50 mm high and 45 mm wide on the screen, subtended a visual angle of 8°, and appeared on a black background. Only the black background appeared on the screen during the "resting" phase. An average of 0.4 footcandles (0.1 standard deviation) reached the monkey's eye when the pictures were displayed and less than 0.01 footcandles reached the monkeys eye when the blank screen was displayed. EPrime software (Psychology Software Tools, Pittsburgh, PA) was used to present stimuli.

2.3. Design and procedure

Each experimental trial consisted of four phases (Fig. 1): an alerting phase, a familiarization phase, a novelty phase and a resting phase. In the alerting phase, a series of colored rectangles

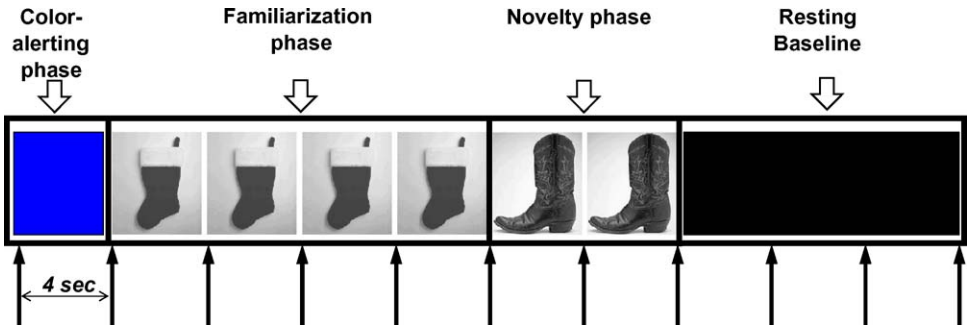

Fig. 1. Structure of a single trial in the present study. Each trial began with a series of flashing colored rectangles (color alerting phase), followed by four familiarization frames in which the same picture was presented for 3 s each followed by a blank for 1 s. Familiarization frames were followed by two novelty frames in which the picture was changed. In the three resting baseline frames, a blank screen was presented for 12 s. The solid arrows indicate the collection of one whole-brain volume.

that covered the entire screen flashed at a rate of 3.3 Hz for 3 s, followed by a blank screen for 1 s. The purpose was to alert the monkey as to the start of a trial, similar to the FNP procedure in infants. In the familiarization phase, the same picture was presented for four frames. In each frame, the picture appeared for 3 s followed by a brief blank screen for 1 s. In the novelty phase, a different picture from the same general category was presented for two frames.[3] The resting phase consisted of a blank screen for 12 s, which also served as the intertrial interval. For the analysis of behavior, we only consider the familiarization and novelty phases (six frames total) as a trial. We do not analyze looking behavior during the alerting and resting phases. The frame rate of the stimulus presentation was matched to the 4-s repetition time of the fMRI data acquisition. A small percentage of trials (14%) were designated as "catch" trials in which the same picture that was presented during the familiarization phase was also presented in the novelty phase. The purpose of "catch" trials was to prevent the monkeys from building an expectation for a change to occur on frame 5. To ensure that collection of brain volumes was well synchronized with presentation of the stimuli, each individual frame depicted in Fig. 1 was triggered by an optical pulse from the MRI scanner, which was then converted into a standard serial pulse and interfaced with EPrime via the Patient Response System with Trigger Interface produced by MRA Inc. (Washington, PA).

The initiation of each experimental frame in EPrime then updated the parallel port, which was continuously monitored by the eye tracking software, to mark the start of the new frame (and the end of the previous frame). A single session consisted of eight trials for the initial sessions we conducted (lasting 8 min each) or 10 trials for the remaining (and majority of) sessions (lasting 6.7 min each). The number of different pictures used in each session depended on the number of catch trials within that session and the stimulus class that was used (there were fewer exemplars of familiar faces and monkey faces). Therefore, 7–16 different pictures would be presented within a session—with fewer pictures in the stimulus class (e.g. familiar faces) the pictures had to be repeated two to four times within a session. Across all sessions, a given picture could appear 1–10 times for Monkey 1 and one to four times for Monkey 2 (the high number of repetitions for Monkey 1 was due to the familiar faces being presented more frequently).

In each experimental session, the monkey was prepared for fMRI scanning (see below). Eye tracking was performed using bright pupil optics and the pupil to corneal reflection method with MR-compatible eye tracking equipment and software (Model 504 LRO, Applied Science Laboratories, Bedford, MA). In several of the initial sessions, we performed a five- to nine-point calibration for each monkey. To calibrate the eye tracker for a monkey, we hung a black curtain at the back of the MRI scanner, which the monkeys were facing. We projected the nine calibration points on the display screen to the black curtain and cut nine 6.4 cm holes, one for each calibration point. Each hole was covered with a flap, which could be lifted in order to present a stimulus to guide the monkeys' attention to that point. During calibration, we removed the display screen to allow the monkeys full view of the nine holes in the curtain. To calibrate a given point, one of the monkey handlers would look through the hole corresponding to that point. The animal handler would verbally indicate when the monkey was looking back at the handler and the calibration point would be marked by a different individual in the control room. The handler continuously repeated "now" while the monkey maintained its gaze. The experimenter in the control room would only mark the calibration point if the handler repeated "now" for approximately 2 s or more. If the monkey did not seem to respond to the animal handler, we also used a small mirror placed in each hole so that the handler could indicate when the monkey viewed herself in the mirror. We initially inserted various small toys through the holes, but we did not know when the monkey was viewing the objects, so the approach of having the animal handler looking through each hole was better for knowing when the monkey was looking at that calibration point.

Once a calibration was established for each monkey, we tested the saved calibration on each scanning day to ensure that the monkey was positioned properly with respect to the screen and eye tracking equipment. If the saved calibration was not optimal, we recalibrated for that day. This occurred one time for Monkey 1 (before Session 12) and one time for Monkey 2 (before Session

[3] Fourteen percent of the trials presented three familiarization frames followed by three novelty frames (3–3 trials), but the behavioral analyses presently exclude these trial types.

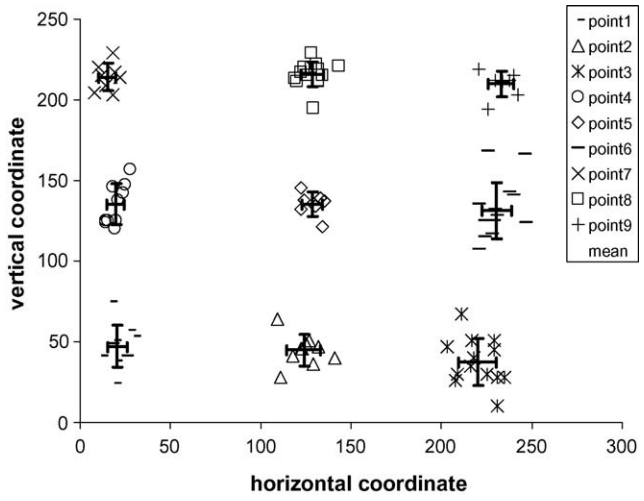

Fig. 2. Results of the calibration in Monkey 1. Each point on the scatter plot represents the location where the monkey was looking at the animal handler through one of nine holes in a curtain that corresponded with one of nine points on the calibration screen. For example, when the handler looked through point 5, the samples taken when the monkey was looking back at the handler are plotted as diamonds. Error bars reflect the standard deviation of the average coordinate in each direction (horizontal and vertical) for each calibration point.

11). Fig. 2 shows the results of the calibration of the equipment for Monkey 1. In this figure, we collected several samples for each of the nine calibration points. Specifically, after the initial calibration, an experimenter in the control room instructed the animal handler to look at the monkey through one of the nine holes. When the monkey was looking back at the animal handler, he stated "now" and the experimenter in the control room marked that point in time when the animal was looking at the calibration point. We collected several samples for each of the nine points in this manner and those results are plotted in Fig. 2. This plot shows excellent calibration—none of the nine points overlaps with each other and the center point (where we presented the visual stimulus) shows the least variability. Following the nine-point calibration procedure, eye tracking is accurate to within 1° over a visual field of 17.7° × 11.4°. We continuously recorded eye movements at 60 Hz during each session. On each testing day, the monkey completed an average of four sessions in which 8–10 experimental trials were presented to the monkey in a session. The time in between sessions on a given day ranged from 1 to 10 min. Monkey 1 completed 22 sessions and Monkey 2 completed 16 sessions over a period of 4 months in which they were tested no more than twice a month. The average time between sessions was 13 days for Monkey 1, and 19 days for Monkey 2. All sessions took place in the MRI scanner.

2.4. fMRI procedure

Prior to exposure to the MRI environment, the monkeys were trained with a series of conditioning steps designed to incrementally habituate the animals to the MRI primate chair and scanning environment using positive reinforcement (see Andersen et al., 2002; Zhang et al., 2000). Training sessions lasted 45–75 min and were conducted at least twice a week per subject until no major head movements were observed in the head frame by technicians in real time and also judged by frame-by-frame video tracking. (We regularly videotape the animals during the initial training sessions.) As acceptance to the training chair varied with each subject, adaptive training usually involved 1–3 months of in-chair training. Food was removed from the subject's home cage approximately 20 h prior to a training or fMRI session to decrease excreta and increased receptivity to positive reinforcements. The training chair, with subject, was lifted onto a MRI compatible cart and placed in a horizontal position. As the training chair was transferred from the floor to the cart, the subjects learned to pivot their body into a prone position. Having the trainer visually present during scanning was important in the initial scanning sessions.

2.5. Chair apparatus

The animals were adapted to the MRI compatible chair constructed from non-ferromagnetic materials and designed to comfortably position a rhesus monkey in a prone, sphinx-like position in a clear acrylic tube within the magnet bore. The monkey rested on a pad within the tube. The head holder was modified from a previous study (Andersen et al., 2002; Zhang et al., 2000) and designed to restrict head motion without having to surgically attach a head holder to the skull. A customized head holder in the form of a rectangular frame (width = 11.5 cm, height = 10 cm, depth = 15 cm) was constructed from Lexan and other MRI-compatible non-ferrous materials. The head frame was placed over the monkey's head and secured to the chair tube using two nylon screws. The monkey's head rested comfortably on a padded chin support that acted as a stabilizer and cushion. Its nose and mouth were positioned outside the head holder for comfort and ease of breathing. Under local anesthesia (1% lidocaine, 2.0 ml on each side), two disposable MRI compatible pins used for gamma-knife surgery in humans (Radionics, Burlington, MA) were inserted through the overlying skin and connective tissue to contact but not penetrate the bony cranium. There was a 2-week period between scanning days to allow the skin to heal. Earbars/earplugs were constructed that followed the natural angle of the rhesus ear canal (10–15° down from the horizontal) and secured firmly to the chair-mounted head holder. Earbars/earplugs were used both for MRI and training sessions to reduce head movement and protect the animals from the high ambient MRI noise levels. Well-trained animals were sufficiently comfortable in the primate chair to sleep when active testing was not underway.

2.6. fMRI data acquisition

A Siemens Trio 3 Tesla magnet and a CP extremity coil were used to collect whole-brain images. After a standard second-order shim, a $T2^*$-weighted gradient echo sequence (39 or 36 ms echo time, 4 s repetition time, 90° flip angle, 64 × 64 matrix, 90 mm field of view, 40 2-mm slices with no gap acquired in interleaved order) yielded images with 1.4 mm × 1.4 mm × 2 mm resolution. For three of the initial sessions, we collected functional images with 2 mm × 2 mm × 2 mm resolu-

tion and for eight of the initial sessions we used a 6 s repetition time (TR) because we were determining the optimal intertrial interval for recording eye movements. The longer repetition times used in the present study were chosen to allow a long enough window to record looking time on each trial frame (which was triggered by the scanner). The slightly longer than normal TE's did not significantly increase susceptibility artifacts. We also collected anatomical images for each monkey using an MPRAGE sequence (2.93 ms echo time, 12° flip angle, 128 × 112 field of view, 128 1-mm slices acquired sagittally) yielding images with 1 mm × 1 mm × 1 mm resolution.

2.7. Eye tracking data analysis

Previous studies in humans have shown that the eye tracking system used in the present study (Applied Science Laboratories, Bedford, MA) allows for artifact-free collection of eye movements with no introduction of noise in the MR images (Gitelman et al., 2000). The eye tracker recorded the eye position every 17 ms using bright pupil optics, and the pupil to corneal reflection method, which calculates the point of gaze of the eye based on the center of the pupil and the 1st Purkinje image (corneal reflection). This raw data was analyzed using Eyenal software (Applied Science Laboratories, Bedford, MA) to determine the fixations that occurred within each frame. A single fixation was defined as a period of at least six data samples (83 ms) during which the point of gaze did not shift more than 1° of visual angle.[4] From the summarized data, we then further reduced the dataset to reflect the number of fixations and total looking time within each familiarization and novelty frame.

2.8. fMRI data analysis

MEDx software (Medical Numerics, Sterling, VA) was used for fMRI data analysis. Echo-planar images for each time series were registered to the mean intensity image of the time series using a six-parameter rigid body model and the 3D scanline chirp-Z interpolation algorithm with a least-square cost function to correct for within-session head motion (Woods et al., 1992). The motion-corrected data were then submitted to the following preprocessing steps: Gaussian filtering (3 mm^3 or 4 mm^3 filter and kernel size of 9 pixels), intensity normalization and high-pass filtering (cutoff = 120 s for 6 s TR, cutoff = 80 s for 4 s TR). After pre-processing, each time series was submitted to a voxel-wise multiple regression in which each phase of the trial (color alerting, familiarization and novelty phase) was modeled as a separate regressor. In the case of "catch" trials, the six consecutive frames that presented an identical stimulus were modeled as a longer familiarization phase.[5] The model also included a phase shift of 8 s to account for the hemodynamic lag. The multiple regression yielded one z-map for each regressor. Each z-map was registered to the anatomical image for each monkey using linear algorithms as implement in AIR (Woods et al., 1992). These co-registered z-maps were then averaged together sessions in a fixed effects approach for each monkey separately (Lazar et al., 2002; Papoulis, 1965). Based on these averaged z-maps for each trial phase, regions of interest (ROIs) were identified in the medial temporal lobe for each monkey based on previous research suggesting these regions are involved in novelty detection. ROIs defined as clusters of 20 or more spatially contiguous, activated voxels were further explored. Cluster sizes ranged from 21 to 216 voxels. Average percent signal change for color alert, familiarization and novelty phases was submitted to a repeated-measures ANOVA for each monkey and each ROI.

2.9. Head motion

We included a session in the fMRI analysis if there was less than 0.7 mm (i.e. 1/2 voxel size) corrected head motion in all three dimensions. To formalize this criterion, we computed the average and maximum deviation of the center of intensity relative to the first time point over the course of a session. Average and maximum deviations were computed in each dimension (x, y, z) separately for each session both before and after motion correction. We then submitted these values to a repeated measures ANOVA for each monkey. Head motion correction significantly reduced head motion in both monkeys (Fig. 3), and for both monkeys the average maximum deviation of the center of intensity was below the cutoff of 1/2 voxel. Although the y (anterior–posterior) and z (inferior–superior) dimensions had more motion associated with them, motion correction significantly reduced the amount of motion to within an acceptable range.

3. Results

3.1. Establishing the behavioral effect of novelty detection

To determine whether the monkeys were attending to the presented stimuli, we only considered frames in which a pupil was detected more than 10% of the time. This liberal detection threshold was used to allow us to detect adaptation to a repeated stimulus. A much higher threshold would have only included frames in which the monkey was looking at the stimulus or screen for a significant amount of time, but would not have enabled us to detect adaptation to the stimulus. The FNP paradigm not only measures the increase in looking time to a new stimulus, but adaptation to a repeated stimulus as well. The lower

[4] More specifically, a fixation was considered to begin with the first of six sequential samples whose vertical and horizontal standard deviations were not more than 0.5° visual angle. If we assume a normal distribution, this translates to a 95% confidence interval of 1°. Subsequent samples were considered to be part of the fixation if they were not more than 1°, in either axis, from the average position of the first six points. The fixation was considered to end when three consecutive samples were more than 1° from the average position of the first six. The sample preceding these three samples was considered the last sample to be part of the fixation. The final fixation position was computed as the average position of all data samples within the fixation, but excluding any that were more than 1.5° from the average of the first six.

[5] The 3–3 trial type was modeled in these analyses in order to control for the effect of that trial type when considering the 4-2 and catch trial types.

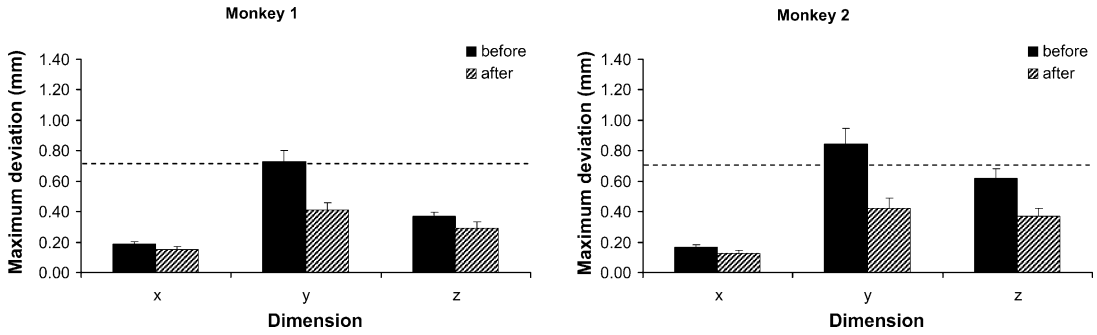

Fig. 3. Head motion results in each monkey. Each graph shows the average maximum deviation in mm relative to the first time point across all 14 sessions for Monkey 1 (left graph) and Monkey 2 (right graph), before motion correction (solid bars) and after motion correction (striped bars). The dotted line in each graph shows the cut-off for acceptable amount of head motion for a voxel size of 1.4 mm. Error bars are standard error of the mean. For both monkeys, motion correction significantly reduced the maximum deviation of head motion ($p < 0.05$ for all F-tests).

detection threshold included those frames in which the monkey was less attentive to a stimulus as an index of adapting to it. We then classified a trial as usable if the monkey was attentive during the presentation of at least one of the four familiarization frames *and* at least one of the two novelty frames; otherwise, the trial was not considered. Given this criterion, 83% of the trials were usable, and the average percent of time a pupil was detected per frame was 65%. For this first set of analyses, we do not consider catch trials (i.e. the control trials). The dependent measures were number of fixations in the area of interest (*fixAOI*), total looking time in the area of interest (*timeAOI*), number of fixations on the screen (*fixSCR*) and total looking time on the screen (*timeSCR*) within each frame of a trial (Table 1). The area of interest was the picture itself, which subtended a visual angle of 8°. We included *fixSCR* and *timeSCR* to allow for detecting new pictures outside of the area of interest.

Fig. 4 shows the average *fixAOI* and *timeAOI* collapsed across all sessions for both monkeys ($n = 38$ sessions) as a function of trial frame. A repeated measures ANOVA with frame as the repeated factor revealed a significant main effect for frame for both *fixAOI* [$F(5, 33) = 3.6$, $p < 0.05$] and *timeAOI* [$F(5, 33) = 3.5$, $p < 0.05$]. Paired t-tests confirmed that the monkeys habituated to the stimulus during the first four familiarization frames (frame 1 versus frame 4, $p < 0.01$ for both measures) and that the new picture induced more fixations in the novelty phase (frame 4 versus frame 5, $p < 0.05$; frame 4 versus frame 6, $p < 0.05$). The new picture also induced longer looking times but this effect was strongest for the frame 4 versus frame 6 comparison ($p < 0.01$). With respect to the dependent measures *fixSCR* and *timeSCR*, the novelty effect was not as pronounced: the main effect of frame was not significant for either measure ($p > 0.05$).

Both monkeys showed novelty detection, but they differed in terms of the dependent measure that revealed novelty effects (Fig. 5). Monkey 1 showed novelty detection for *fixAOI* and *timeAOI*, whereas Monkey 2 showed novelty detection for *fixSCR* and *timeSCR*. For Monkey 1, the repeated measures ANOVA revealed that the effect of frame was significant for *fixAOI* ($p < 0.009$; frame 4 versus frame 5, $p < 0.05$; frame 4 versus frame 6, $p < 0.01$) and *timeAOI* ($p < 0.007$; frame 4 versus frame 6, $p < 0.01$) but not for *fixSCR* and *timeSCR*. For Monkey 2, the effect of frame did not reach significance for any dependent measure; however, the contrast of frame 4 versus frame 5 was marginally significant for timeAOI ($p = 0.086$).

Average looking time and number of fixations during the familiarization phase (frames 1–4) versus novelty phase (frames 5–6) were not different for any dependent measure for either monkey (all p's > 0.29). Because the average looking time across phases is well equated, any differences in brain activation as a function of trial phase cannot be attributed to greater retinal stimulation as a function of differential looking time. We also examined whether the peak behavioral response during the novelty phase (i.e. the greater of frame 5 or 6) was different from the first familiarization frame (i.e. frame 1) for each dependent measure for each monkey. For example, for Monkey 1 it appears that the peak novelty response does not reach the level of the initial familiarization frame for some dependent measures. However, none of these comparisons was significant (all p's > 0.2). For Monkey 2, it appears that the peak novelty response may be greater than the initial familiarization response for some dependent measures, but again, none of these comparisons was significant (all p's > 0.18). Taken together, this set of comparisons shows that familiarization and novelty phases did not, on average, induce differential looking times or fixations and the peak responses within each phase were also not different. Therefore, differences in brain activation between the two phases will not be confounded by longer looking times or more fixations.

Table 1
Dependent measures of looking behavior used in the present study

Abbreviation	Dependent measure
fixAOI	Number of fixations in the area of interest
timeAOI	Total looking time in the area of interest
fixSCR	Number of fixations anywhere on the screen
timeSCR	Total looking time anywhere on the screen

3.2. Novelty detection or learned response?

An alternative explanation for the novelty effect is that the eye movement patterns we observed simply reflect a learned response to attend to the AOI after four pictures were presented, rather than reflecting discriminative processing of the new stimulus. Consequently, we analyzed "catch" trials in which the same

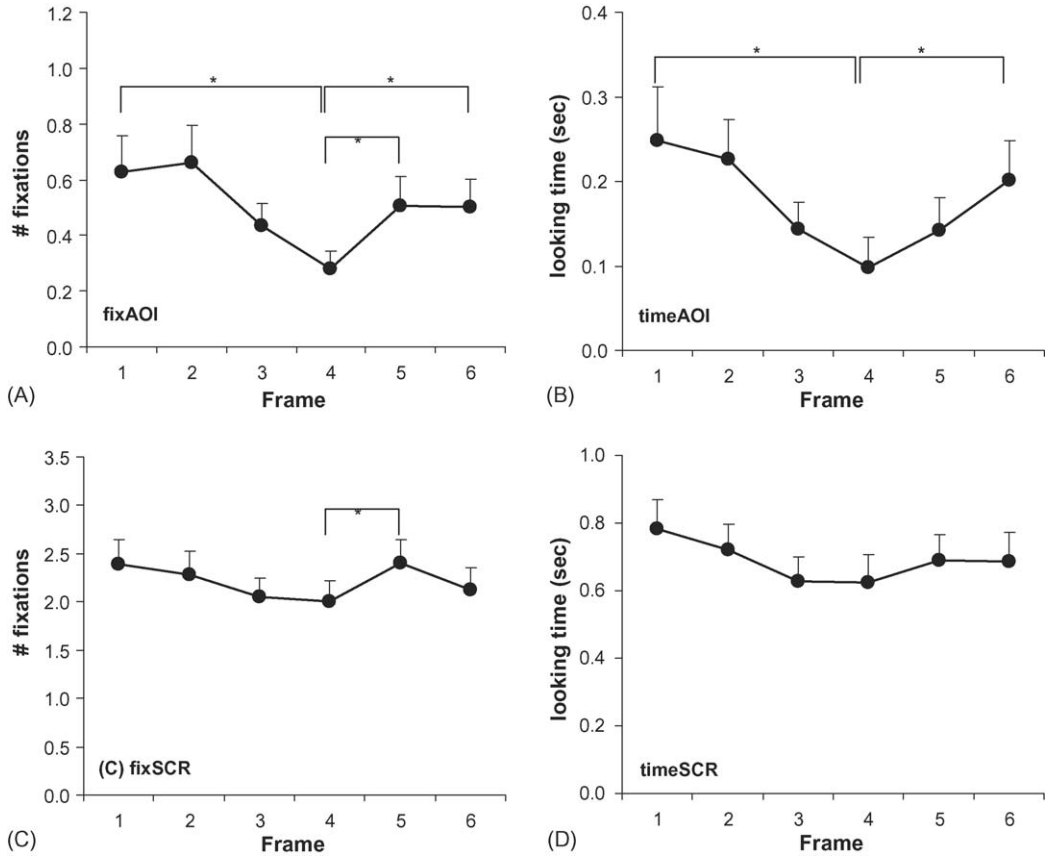

Fig. 4. Average behavioral responses across 38 sessions for two monkeys. On frames 1–4 of each trial, the same picture was presented repeatedly (familiarization phase), whereas on frames 5 and 6 a new picture was presented (novelty phase). Both number of fixations in the area of interest (A, *fixAOI*) and looking time in the area of interest (B, *timeAOI*) increased when the new picture was presented on frame 5. Significant paired *t*-tests ($p < 0.05$) are indicated with a "*". Although a trend emerged for more fixations anywhere on the screen (C, *fixSCR*) and increased looking time anywhere on the screen (D, *timeSCR*) for the new picture, the effect was not significant.

stimulus was presented for all six frames (catch trials occurred in 25 of the 38 sessions because we did not initially include catch trials—the present analysis includes only those 25 sessions). On catch trials, there was no change to detect, so number of fixations or looking time should not increase from the last familiarization frame to the first novelty frame, unless these measures reflect a learned response to look longer or more frequently on frame 5 or 6.

As shown in Fig. 6, none of the four dependent measures increased from the last familiarization frame to the first novelty frame for catch trials. In contrast, number of fixations and looking time increased on the novelty frames for novelty trials. A repeated measures ANOVA analyzing the effect of frame (frames 4 and 5) and trial type (catch trial, no catch trial) revealed marginally significant interactions for *timeAOI* and *fixSCR* ($p = 0.056$) and a significant interaction for *fixAOI* ($p < 0.05$). When each monkey was analyzed separately, the interaction was significant for Monkey 1 for *timeAOI* ($p = 0.057$) and *fixAOI* ($p < 0.05$), whereas Monkey 2 showed the interaction for *fixSCR* and *timeSCR* ($p < 0.05$). Because catch and novelty trials were well equated in terms of fixations and looking time during the familiarization phase (i.e. no main effect of trial type), the absence of a novelty effect on catch trials cannot be explained by lack of attention during familiarization on these trials. In addition, the novelty effect cannot be explained by overall more looking time.

3.3. fMRI results

Having established novelty detection behavior in both monkeys, we were next interested in establishing neural correlates for novelty detection using fMRI. In 37 of 38 experimental sessions, we also collected functional brain images; however, not all of the collected data were usable due to excessive head motion that was not well corrected (five sessions), equipment malfunctions (three sessions), or extremely inattentive behavior (one session). This yielded 14 sessions for Monkey 1 and 14 sessions for Monkey 2. The average behavioral response across the 28 fMRI sessions showed the same pattern of novelty detection as in the more inclusive set of 38 sessions, with the overall effect of frame significant for *timeAOI* ($p < 0.047$) and *fixAOI* ($p < 0.043$) and the difference between the last familiarization frame and one of the novelty frames significant for *timeAOI* (frame 4 versus frame 6; $p < 0.015$), marginally significant for *fixAOI* (frame 4 versus frame 6; $p < 0.055$) and significant for *timeSCR* (frame 4 versus frame 5; $p < 0.032$).

The voxel-wise multiple regression analysis allowed us to examine brain activation associated with the different phases of

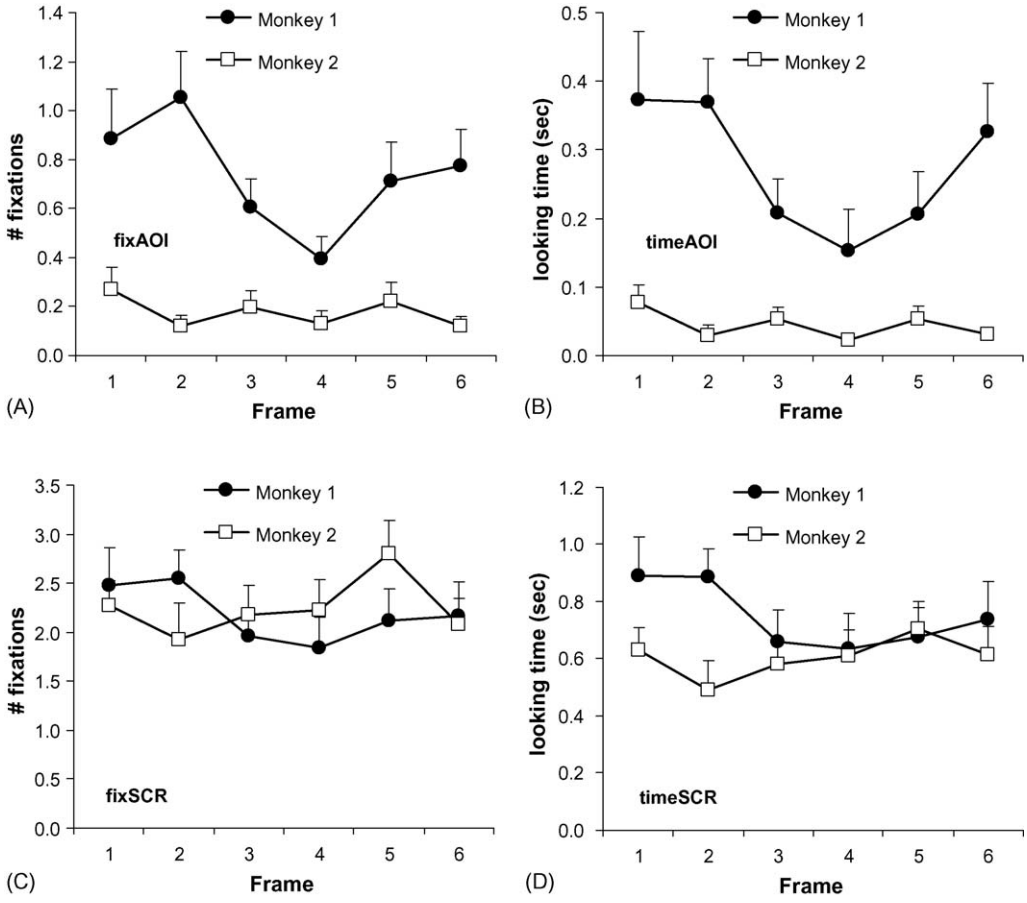

Fig. 5. Average behavioral responses shown separately for each monkey. See Fig. 4 caption for details.

an experimental trial (color alerting, familiarization and novelty phases). Fig. 7 shows the results for Monkey 1 averaged across 14 sessions. In this monkey, activation in the amygdala and the extended amygdala was associated with the novelty phase (red voxels). fMRI signal in each region was greatest for the novelty phase of each trial when compared with the alerting and familiarization phases. The main effect of trial phase was significant for the right amygdala and right extended amygdala ($p < 0.05$) and marginally significant for the left amygdala and left extended amygdala ($p < 0.087$). fMRI signal associated with the novelty phase was greater than the familiarization phase, according to planned contrasts in three of the regions ($p < 0.05$) and greater than the color alerting phase in three of the regions ($p < 0.05$). Consequently, in each region the fMRI signal for the novelty phase was greater than at least one other trial phase. Extensive frontal activation also emerged in this monkey, but this was not specific to novelty detection because the familiarization phase also induced frontal cortex activation. Nevertheless, the novelty phase produced more widespread frontal activation than did the familiarization phase. Additional activation during the novelty phase was found in a region in visual cortex that bordered V1 and V2. Activation associated with the familiarization phase and color alerting phase emerged in area V2.

Fig. 8 shows the fMRI results for Monkey 2 averaged across 14 sessions. Novelty detection induced activation in the amygdala bilaterally (red voxels). fMRI signal was, in general, greater for the novelty phase of each trial when compared with the alerting and familiarization phases; however, the main effect of trial phase did not reach significance for either region and planned contrasts revealed only marginally significant differences between the novelty and color alerting phases in each region ($p < 0.098$). Consequently, although there was a trend for greater signal in the novelty phase, these results did not reach significance, unlike Monkey 1. Additional novelty-related activation was found in V1. The familiarization phase was associated with activation in V1, V2, and V3v. The color-alerting phase was associated with activation in frontal cortex as well as V1 and V2.

Visual cortex activation was more extensive for the familiarization phase than the other two phases for both monkeys. This more extensive activation during familiarization could reflect the fact that there were four familiarization frames versus only two novelty frames. Hence, cumulative visual stimulation was likely greater during familiarization than during the novelty phase. Importantly, though, more extensive visual cortex activation cannot explain novelty effects in other brain regions because (1) average looking time was not different between familiarization and novelty frames, and (2) cumulative stimulation was not greater during the novelty phase than the familiarization phase.

An important concern is whether the novelty-related activation in the amygdala is replicable across sessions within each monkey. Given that previous studies have demonstrated rapid

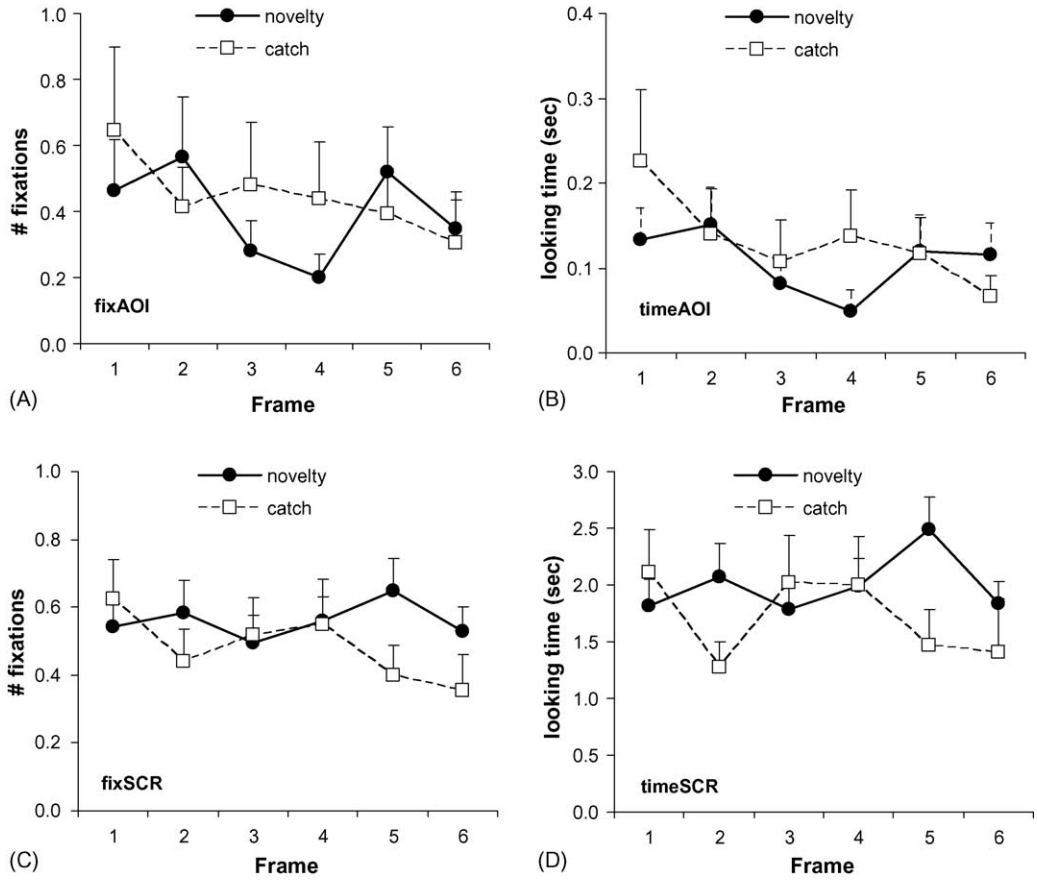

Fig. 6. Average behavioral response across 25 fMRI sessions for two monkeys. The solid line represents those trials in which a new picture was presented on frame 5. The dotted line represents catch trials in which the picture did not change from frames 4 to 5. Number of fixations (A and C) and looking time (B and D) increased from frames 4 to 5 only on the "novelty" trials in which a new picture was presented, but these measures did not increase when the same picture was repeated on frame 5.

habituation to novel stimuli in medial temporal lobe (MTL) structures (e.g. Martin, 1999; Yamaguchi et al., 2004), it is important to consider whether the results shown in Figs. 5 and 6 reflect only the initial scanning sessions in which most of the stimuli were new or only the initial sessions on a given day. Novelty-related MTL activation was replicable across sessions for both monkeys. Monkey 1 showed novelty-related MTL activation in 12 of 14 sessions and Monkey 2 showed novelty-related MTL activation in 10 of the 14 sessions. The extent and magnitude of novelty-related MTL activation did not systematically increase or decrease across testing sessions.

We also examined whether the fMRI novelty response was associated with any of the behavioral indices of novelty detection. To that end, we conducted bivariate Pearson correlations between the percent change in fMRI signal from frame 4 to frame 5 (or from frame 4 to frame 6) and the percent change in looking time (*timeAOI*, *timeSCR*) or number of fixations (*fixAOI*, *fixSCR*) from frame 4 to frame 5 (or from frame 4 to frame 6). In both monkeys, fMRI signal increases in the left amygdala were associated with increased looking time or number of fixations on novelty trials. In Monkey 1, fMRI signal increase in the left amygdala from frame 4 to frame 5 was marginally correlated with the increase in number of fixations (*fixAOI*) from frame 4 to frame 6 ($r = 0.52$, $p = 0.055$, Fig. 9A). The same correlation emerged in the left extended amygdala ($r = 0.52$, $p = 0.059$, Fig. 9B). In Monkey 2, fMRI signal increase in the left amygdala from frame 4 to frame 5 was correlated with the increase in looking time (*timeAOI*) from frame 4 to frame 5 ($r = 0.54$, $p < 0.05$, Fig. 9C). These correlations provide further evidence that the fMRI response in the amygdala is associated with the ability to detect recent changes in visual stimuli.

Although the correlations just described are quite clear in establishing a link between behavior and brain activation, an alternative explanation is that a longer looking time that is not related to novelty detection is actually driving the BOLD response in the amygdala. In other words, could the BOLD response to novelty be driven by number of fixations and looking time anywhere on the screen over the entire trial, potentially as an index of a general attentional mechanism at work? To address this, we performed bivariate Pearson correlations between fMRI signal during the novelty phase and looking time and number of fixations anywhere on the screen (*fixSCR*, *timeSCR*) across the entire trial in each of the medial temporal lobe structures shown in Figs. 7 and 8. None of these correlations was significant, with the exception of a marginally significant correlation ($r = 0.46$, $p = 0.097$) for Monkey 1 in the right amygdala. Consequently, the fMRI activation observed in the amygdalae – especially in the left hemisphere – reflects an ability to visually discriminate

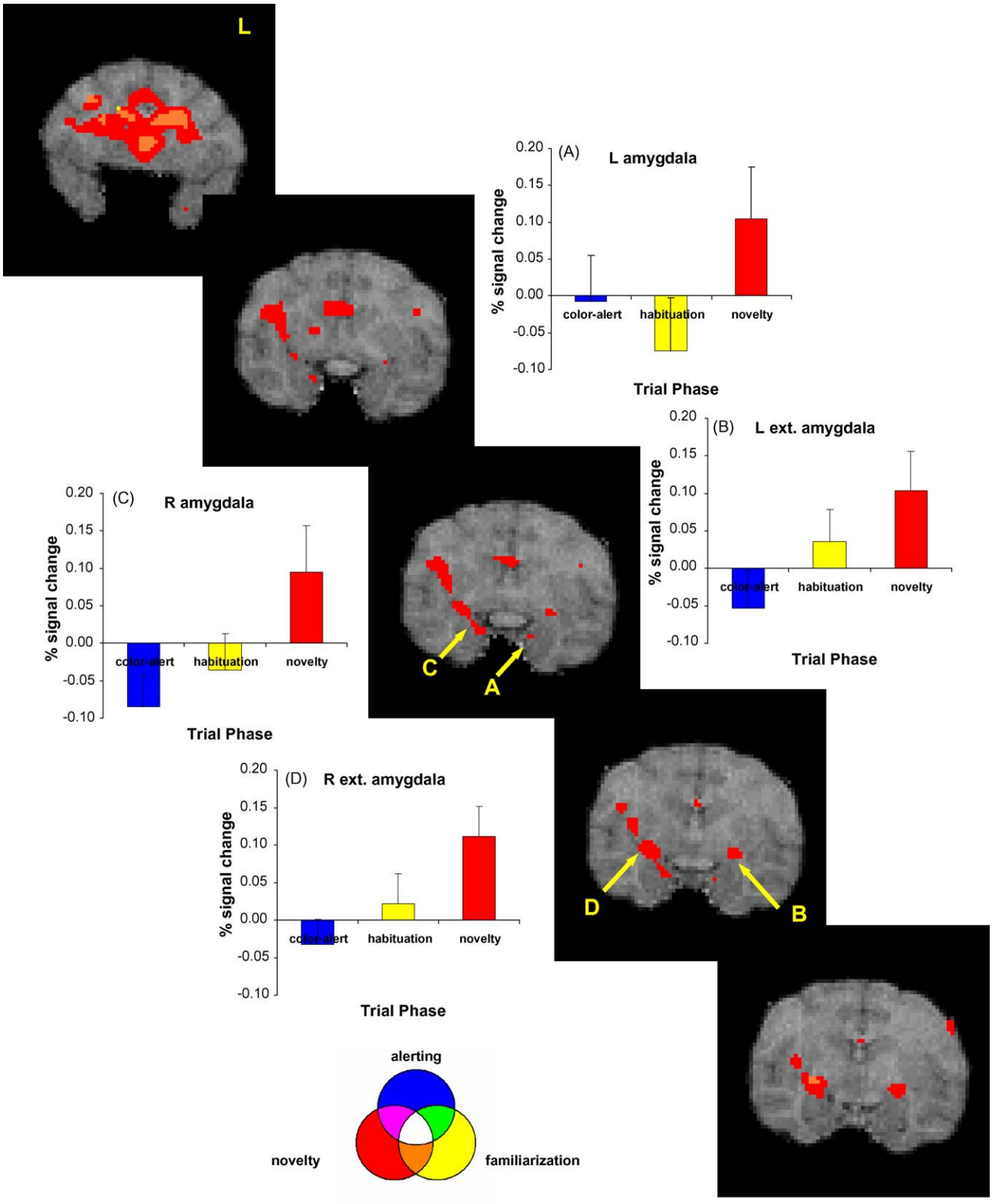

Fig. 7. Brain activation associated with different phases of a trial for Monkey 1. Activation associated with the color-alerting phase is rendered in blue, activation associated with familiarization is rendered in yellow, and activation associated with the novelty phase is rendered in red ($p < 0.05$, uncorrected). Each graph shows the fMRI signal (percent change) in the designated region as a function of trial phase.

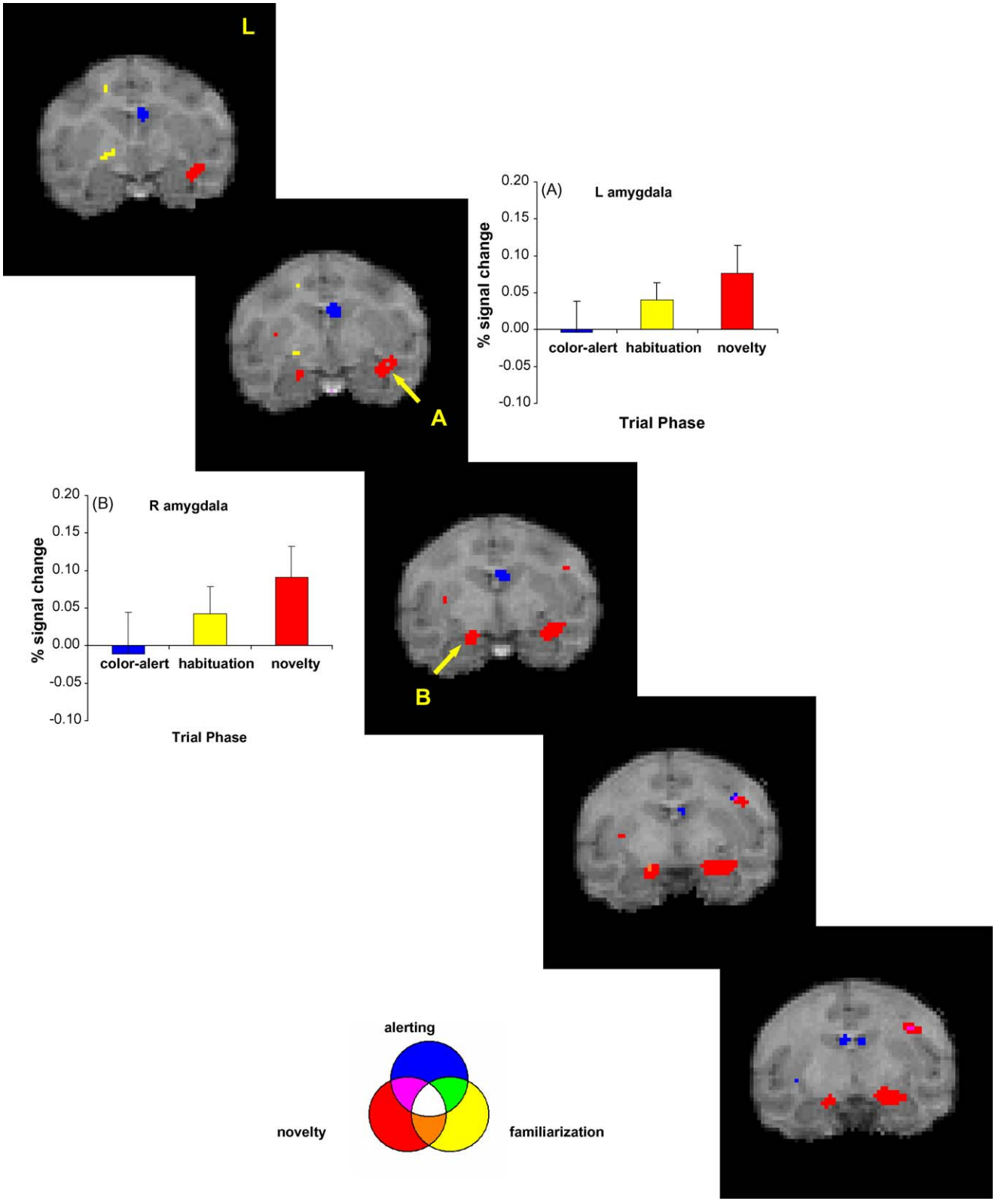

Fig. 8. Brain activation associated with different phases of a trial for Monkey 2. See Fig. 7 caption for details.

a new from an old stimulus and is not driven by an overall attentional mechanism. However, the right amygdala may be involved in such an attentional mechanism, based on the marginally significant correlation reported above.

A final concern in interpreting the amygdala activation as related to detecting novelty is whether head motion might explain that activation. For each session in each monkey, we correlated the time series of head motion in each dimension

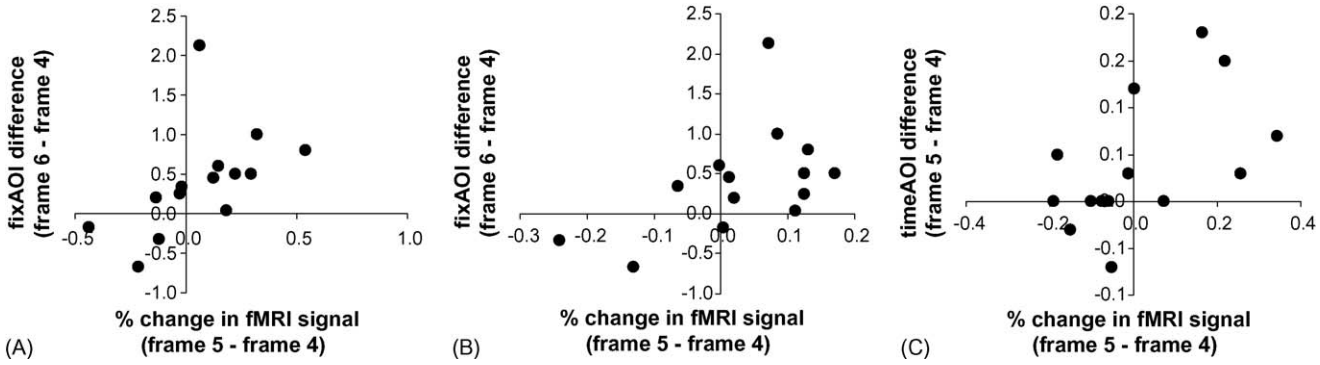

Fig. 9. Correlations between change in fMRI signal and change in behavior (i.e. the novelty effect) for (A) Monkey 1 in the left amygdala, (B) Monkey 1 in the left extended amygdala, and (C) Monkey 2 in the left amygdala.

with a reference waveform that reflected the expected hemodynamic response during the novelty phase (i.e. a repeating gamma function associated with each novelty phase over the time course of the experiment). If head motion can explain the novelty-related amygdala activation, then these correlations (one for each session) should be significant and positive. In Monkey 1, head motion was *negatively* correlated with the novelty phase in only one of the 14 sessions (Session 3, $r = -0.25$, $p < 0.02$). Interestingly, Session 3 did not show replicable MTL activation. In keeping with the fact that this was a negative correlation, there was no amygdala activation in response to novelty in this particular session, so head motion was working to diminish detectable effects, rather than to induce artifactual activation. In Monkey 2, head motion was not correlated with the novelty phase in any of the 14 sessions. Therefore, it seems unlikely that the novelty-related activation we observed in both monkeys in the amygdala can be explained by head motion artifacts.

4. Discussion

In the present study, we have described the apparatus and procedures for fMRI methods in alert, behaving monkeys with behavioral assessment from in-magnet eye tracking measurements. We showed that untrained, behaviorally naïve rhesus monkeys exhibit novelty detection behavior in an fMRI experimental setting. The new and original contribution of the present work is that the monkeys were not trained on any particular task. Rather, we exploited the natural tendency of an organism to orient to novel stimuli and adapted the FNP paradigm from human infant research to capture this behavior. The present study demonstrated that this response can be measured both in terms of eye movements and in terms of brain activation using fMRI, and that a significant correlation existed between behavior and brain activation measured by fMRI. The behavioral data showed that both monkeys increased looking time and number of fixations when a new stimulus was presented, but did not exhibit the same behavior when the stimulus did not change (i.e. catch trials). Consequently, the novelty detection response reported here is related to discriminative processing of the new stimulus rather than reflecting a learned response to orient at a given point in time (i.e. on frame 5 or frame 6).

Because these were freely viewing monkeys who did not undergo behavioral training, eye movements reflected very active visual exploration of the environment and relatively little time spent processing the stimuli. However, such behavior is expected based on studies that use untrained animals. As an example, Clark et al. (2000) used an adaptation of the visual paired comparison task for rats. On average, in order to accumulate 30 s of object exploration, the animals were exposed the stimuli for 158 s. In other words, untrained rats only explored the presented stimuli for about 19% of the time they were exposed to those stimuli during familiarization. Similarly, in the present study, the overall looking time within a 4 s time window was on the order of 10–20% (see Figs. 2 and 3). The important finding was that when the animals were looking at the screen or picture itself, they showed novelty detection behavior.

Although both monkeys showed novelty detection behavior, the two animals used different strategies. Monkey 1 exhibited novelty detection within the area of interest (i.e. within the confines of the picture itself), whereas Monkey 2 showed novelty detection behavior only when number of fixations and looking time were measured anywhere on the screen. In addition, Monkey 1 showed familiarization to the repeated stimulus by exhibiting reduced fixations and looking time (*timeAOI* and *fixAOI*) as the stimulus was repeated during the familiarization phase, whereas Monkey 2 did not show familiarization for any dependent measure. Individual differences in delayed match to sample (Hampson et al., 2004), visual preference (Wilson and Goldman-Rakic, 1994) and novelty seeking (see Bardo et al., 1996) have been reported elsewhere, and a recent study suggests that prolonged looking responses in rhesus monkeys may not emerge if the animal is being observed while looking (Kruger and Hauser, 2004). In the present study, the animals were not directly observed while they were viewing the visual stimuli, but using both human and rhesus faces as stimuli may have provided an artificial social context that affected the eye gaze direction of Monkey 2.

We have recently completed another study with the same two monkeys (unpublished data) and they continue to exhibit the same behaviors that they demonstrated in the present study. In this more recent study, we also tested human subjects on the same paradigm, but they were instructed to press a button each time the stimulus changed and we measured reaction time to

make the same-different response rather than looking time and number of fixations. The human subjects essentially fall into two different groups. Four of the human subjects showed adaptation to the repeated stimulus (i.e. decreased RT to the repeated stimulus), similar to the behavior exhibited by Monkey 1 for looking time in both the present and more recent study. Eight of the human subjects did not show adaptation to the repeated stimulus, similar to the behavior exhibited by Monkey 2 for looking time in both the present study and the more recent study. The latter behavior (i.e. lack of adaptation to a repeated stimulus) may reflect an expectancy strategy in that the observer is attempting to predict or anticipate the change in stimulus. The observers remain vigilant during frames in which a change is likely to occur and this anticipation washes out (or interferes with) the adaptation effect. We are continuing to explore these behaviors in both humans and monkeys as well as the underlying neural substrates of such process. Nevertheless, the parallel human and monkey study reveals individual differences in strategies for detecting a new stimulus. Such individual differences are present in both species.

In addition to showing that novelty detection behavior could be measured with eye movements in an MRI scanner, the present study also showed that the novelty phase of a trial was associated with brain activation in the amygdala in both monkeys. Novelty-related activation was more extensive than activation associated either with the familiarization or color alerting phases, which is somewhat surprising given that the novelty phase was associated with fewer time points than the other two phases of a trial. Nevertheless, the finding of amygdala and MTL involvement in this task is not surprising when other literature is considered. In non-human primates, MTL structures seem to be important for making discriminations between novel and familiar stimuli. For example, lesions that involve the amygdala either in combination with the hippocampus (Murray and Mishkin, 1984) or in combination with rhinal cortex (Murray and Mishkin, 1986) lead to impaired delayed non-match to sample performance. Human functional neuroimaging studies have also implicated MTL structures, including the hippocampus, entorhinal cortex and the amygdala, in some aspects of novelty detection (Daselaar et al., 2004; c.f. Eichenbaum, 1999; Fischer et al., 2002; Fried et al., 1997; Grunwald et al., 1998; Henke et al., 1999; Hunkin et al., 2002; Jessen et al., 2002; Martin, 1999; Opitz et al., 1999; Strange and Dolan, 2001; Wright et al., 2003; Yamaguchi et al., 2004). In addition, a recent computational model of infant habituation (Sirois and Mareschal, 2004) includes the hippocampus and entorhinal cortex as critical functional units.

In the present study, the amygdala in particular, was strongly implicated in novelty detection. First, amygdala activation was associated almost exclusively with the novelty phase of a trial and not with the familiarization or alerting phases. Second, amygdala activation was replicated across several scanning sessions and across both monkeys. Third, the left amygdala showed fMRI signal increases in the novelty phase that were associated with increased fixations (Monkey 1) and increased looking time (Monkey 2) in the novelty phase. Taken together, these findings indicate that the amygdala was consistently involved in the novelty phase of a trial and fMRI signal in this structure was associated with novelty detection.

The small percentage of time that the monkeys spent attending to the stimuli may raise concerns about interpreting novelty-related brain activation. If the animals are only engaged in novelty-detection behavior a small percentage of the time, then what behavior does the brain activation actually reflect for the remaining percentage of the time? Although we do not characterize the behavior of the monkeys when they are not attending to the stimuli, visual exploration of the environment occurred during all phases of the trial rather than during the novelty phase exclusively. Consequently, brain activation associated with visual exploration would necessarily be present in all trial phases and would not be reflected systematically in the brain activation maps for each separate trial phase. Moreover, the left amygdala activation was clearly associated with novelty-detection behavior, as reflected by the correlations between increase in fMRI response from familiarization to novelty frames and increase in looking time from familiarization to novelty frames (Fig. 9). Therefore, even if the amount of time spent looking at the novel stimulus was fairly minimal, this behavior was most meaningful in terms of novelty detection and visual discrimination. As we stated in the introduction, in order for the FNP procedure to work, the monkey must look away from area of interest, so we would not expect the monkey to fixate the screen or the stimulus the entire time.

The involvement of the amygdala, rather than the hippocampus, in the present novelty-detection task may seem puzzling given that studies in humans and other species implicate the hippocampus and/or entorhinal and perirhinal cortex in novelty detection (e.g. Brown and Xiang, 1998; Clark et al., 2000; Jessen et al., 2002). However, in many of those studies, the novelty-detection task involved maintaining a memory representation across a delay. In the present task, memory demands were minimal given that the delay between stimuli was only 500 ms. Some studies show that damage to the hippocampal region in monkeys and rats does not impair recognition memory involving very short delays of 1–10 s, but recognition memory with longer delays is impaired (Clark et al., 2000; Zola et al., 2000). Hence, novelty-detection that is nearly immediate, as in the present study, may not depend on the hippocampal formation. Consequently, the present novelty-related activation reflects the capacity for the animal to detect a recent change in stimulus, which corroborates findings from other non-human primate and human studies that implicate MTL structures, including the amygdala in some cases, in this form of novelty detection (Guillem et al., 1996; Martin, 1999; Murray and Mishkin, 1984; Strange and Dolan, 2001; Wilson and Rolls, 1990; Wilson and Rolls, 1993; Wright et al., 2003). For example, Wright et al. (2003) showed that in humans the left amygdala showed the greatest response to novel faces that followed repeated faces rather than vice versa similar to the sequence of events in the present paradigm. Hence, the amygdala seems to play a role in detecting recent changes in stimuli over the context of an experiment, rather than detecting novelty as defined by the meaningfulness of a stimulus or novelty as defined by experiences with stimuli over a larger time scale (see Martin, 1999).

The present study showed consistent and replicable brain activation in two monkeys that were acclimated to the fMRI environment but were not trained on any particular behavioral task. Given that the monkeys were not trained, there was necessarily more variability in behavior than would be expected in an experiment with trained animals. In spite of this expected variability, reliable behavior was detected in 14 fMRI sessions conducted over the course of 4 months with no more than four sessions completed on each testing day. This modest level of testing is less taxing than some behavioral protocols with monkeys, but the present procedure could be compressed into a shorter time period. We recommend testing no more frequently than once every 2 weeks to allow the animals to recover from the insertion of the MR-compatible pins into the overlying skin of the cranium. In fact, our observation was that more frequent testing led to less data loss. If the monkey had not been tested for several weeks, she tended to be less compliant and we lost some of the sessions on that day. However, we only lost one entire testing day in one monkey, on the other testing days at least one session was usable.

The greater challenge for data retention was to minimize head motion. Five sessions (across both monkeys) were lost due to excessive head motion. We did not use a surgically implanted head holder as in many other fMRI studies of non-human primates, but this approach could further minimize data loss. Importantly, however, when amount of head motion was deemed acceptable in the present study (i.e. less 1/2 voxel size in all three dimensions of space), the remaining minimal head motion did not lead to spurious BOLD signals. Another way to control for effects of head motion is statistically. Amount of head motion can be used as a covariate in multiple regression analyzes to control for any effects of head motion on the variables of interest.

Another important aspect of the present methodology is calibrating the equipment properly for each individual monkey. Without a proper calibration, the eye movement data are not easily interpreted. We have described an innovative procedure that works well. Specifically, in order to attract the monkey's attention to a certain calibration point, we recommend that an animal handler look through one of the holes in the curtain associated with a calibration point. Monkeys tend to be interested in the animal handlers, and the animal handlers, in turn, can easily determine when the monkey is looking back at him or her and can verbally signal to a person in the control room who is marking the calibration point. We have found that this procedure works better than presenting small objects or toys through one of the holes in the curtain because it is difficult to determine whether the monkey is actually looking at that object.

In conclusion, we have demonstrated that reliable and meaningful visual behavior can be detected in untrained monkeys in an fMRI setting. The need to train monkeys to centrally fixate for the duration of an fMRI study may not be necessary for some experimental questions. Whereas the present approach would not be useful for visual field experiments in which maintaining central fixation is critical, the present approach can be used for studying higher level visual processing such as object and face perception. In addition, novelty detection paradigms are widely used for studying learning and memory with broader applications for studying specific health issues, such as addiction and anxiety disorders.

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