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How to detect amygdala activity with magnetoencephalography using source imaging.

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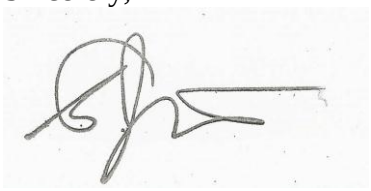
To the editorial board:

Please consider our enclosed manuscript "How to recover amygdala activity with magnetoencephalography using source imaging." for publication in The Journal of Visualized Experiments. In this protocol we show the readers how to design, carry out, and analyze an experiment to record amygdala activity using magnetoencephalography. We feel that this manuscript is suitable for publication in JoVE for two reasons:

1. Our protocol combines structural MRI and MEG, and uses novel source imaging techniques to recover MEG signal from the amygdala.
2. The techniques described in this manuscript can also be applied to recover signal from other subcortical structures, like the hippocampus.

We hope that you share our enthusiasm for the enclosed manuscript, and look forward to your feedback.

Sincerely,



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Title: How to detect amygdala activity with magnetoencephalography using source imaging.

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Short Abstract: This article describes how to record amygdala activity with magnetoencephalography (MEG). In addition this article will describe how to conduct trace fear conditioning without awareness, a task that activates the amygdala. It will cover 3 topics: 1) Designing a trace conditioning paradigm using backward masking to manipulate awareness. 2) Recording brain activity during the task using magnetoencephalography. 3) Using source imaging to recover signal from subcortical structures.

Long Abstract: In trace fear conditioning a conditional stimulus (CS) predicts the occurrence of the unconditional stimulus (UCS), which is presented after a brief stimulus free period (trace interval)[1]. Because the CS and UCS do not co-occur temporally, the subject must maintain a representation of that CS during the trace interval. In humans, this type of learning requires awareness of the stimulus contingencies in order to bridge the trace interval[2–4]. However when a face is used as a CS, subjects can implicitly learn to fear the face even in the absence of explicit awareness*. This suggests that there may be additional neural mechanisms capable of maintaining certain types of “biologically-relevant” stimuli during a brief trace interval. Given

Replace [*] with reference from results paper.

that the amygdala is involved in trace conditioning, and is sensitive to faces, it is possible that this structure can maintain a representation of a face CS during a brief trace interval.

It is challenging to understand how the brain can associate an unperceived face with an aversive outcome, even though the two stimuli are separated in time. Furthermore investigations of this phenomenon are made difficult by two specific challenges. First, it is difficult to manipulate the subject's awareness of the visual stimuli. One common way to manipulate visual awareness is to use backward masking. In backward masking, a target stimulus is briefly presented (< 30 msec) and immediately followed by a presentation of an overlapping masking stimulus[5]. The presentation of the mask renders the target invisible[6–8]. Second, masking requires very rapid and precise timing making it difficult to investigate neural responses evoked by masked stimuli using many common approaches. Blood-oxygenation level dependent (BOLD) responses resolve at a timescale too slow for this type of methodology, and real time recording techniques like electroencephalography (EEG) and magnetoencephalography (MEG) have difficulties recovering signal from deep sources.

However, there have been recent advances in the methods used to localize the neural sources of the MEG signal[9–11]. By collecting high-resolution MRI images of the subject's brain, it is possible to create a source model based on individual neural anatomy. Using this model to "image" the sources of the MEG signal, it is possible to recover signal from deep subcortical structures, like the amygdala and the hippocampus*.

Replace [*] with reference from results paper.

Protocol Text: (Black text to be included in script)

Designing a trace conditioning paradigm using backward masking to block awareness.

1. Design stimuli

1.1. Design the stimuli for the two groups.

1.2. Choose 4 neutral expressions from different individuals.

1.3. Align the faces so that the eye region of each face is in the same location.

1.4. Crop the faces using an oval so that the hair, ears, and other peripheral features are no longer visible.

1.5. Use the signal processing toolbox in Matlab (See Table 1 for software necessary to run the experiment) to create high-pass filtered images, by removing all information that is fewer than 5 cycles per degree[12].

1.6. Create the mask by merging several neutral expressions together, and adding high spatial frequency noise to the image.

1.7. Normalize all images so that they have equal luminance.

2. Program the experiment using Presentation.

2.1. Program the training and testing phases in Presentation using the parameters described below (See Figure 1).

2.2. In addition, program a separate file (PCC) that will be used by the PSYLAB data acquisition software package during training and testing, in order to deliver the shock triggered by Presentation.

2.3. For the training session program 4 blocks of differential trace fear conditioning with 15 trials per CS, per block.

2.4. On each trial present the CSs for 30 msec.

2.5. On each trial present the mask for 970 msec.

2.6. On each CS+ trial present the shock UCS for 100 msec, so that it coterminates with the mask.

2.7. Vary the location of the CS/mask combination so that it randomly appears in 1 of 4 quadrants.

2.8. Present 1 trial every 6±2 seconds using a variable intertrial interval.

2.9. For the testing session program 1 block of reacquisition with 5 trials of each face CS, and 5 trials each of two new face CSs.

Replace [*] with reference from results paper.

2.10. In order to maximize your ability to record skin conductance responses (SCRs) during the testing trials, present the CS for 8 seconds.

2.11. On each CS+ trial present the shock UCS for 100 msec, so that it coterminates with the CS+.

2.12. Present 1 trial every 20±4 seconds using a variable intertrial interval.

2.13. Instruct subjects to report UCS expectancy during both sessions, and record their responses using an MRI/MEG compatible axis device (joystick, slider, dial; See Section 7).

2.14. Record SCRs during the testing session using electrodes attached to the bottom of the participants left foot (See Section 9).

Recording brain activity during the task using magnetoencephalography.

3. Setup equipment for training at MEG suite (See Figure 2).

3.1. Connect the stimulus presentation computer to the MEG acquisition system using a standard DB25 multi connector ribbon cable (See Table 2 for equipment necessary to conduct the experiment.).

3.2. Connect the stimulus presentation computer to the PSYLAB stand-alone monitor (SAM) using the 8-bit to 2-bit isolation adapter and the synchronization cable.

3.2.1. The transistor –transistor logic (TTL) pulses used to mark the stimulus presentations can cause artifacts in the MEG data if they are sent to the SAM. To avoid these artifacts, mark the onset of the stimuli using only the bits blocked by the isolation adapter.

3.3. Connect the shock stimulator (SHK1) to the SAM using the cable provided with the unit.

3.4. Pass the shielded extension cable through the wave guide and connect it to the shock stimulator.

3.5. Connect the SAM to a computer running the PSYLAB data acquisition software using a standard USB cable.

3.6. Connect the rotary dial to the stimulus presentation computer (USB) and the MEG acquisition system (BNC) using the gameport-to-gameport/BNC splitter and the gameport-to-USB adapter.

3.7. Record two minutes of sensor data without the subject in the room.

4. Setup equipment for testing at MRI suite.

4.1. Connect the stimulus presentation computer to the SAM using the synchronization cable.

Replace [*] with reference from results paper.

4.2. Connect the shock stimulator (SHK1) and the skin conductance amplifier (SC5) to the SAM using the cables provided with the unit.

4.3. Pass the shielded extension cables for SCR and shock through the wave guide and connect them to their respective units.

4.4. Connect the SAM to a computer running the PSYLAB data acquisition software using a standard USB cable.

4.5. Connect the rotary dial to the stimulus presentation computer using the gameport to USB adapter.

5. Setup subject for training at MEG suite (See Figure 3).

5.1. Attach electrodes and sensors to the subject using the schematic in Figure 3 as a guide.

5.2. Attach disposable electrodes to monitor eyeblinks above and below the subject's right eye.

5.3. Attach disposable electrodes to monitor heart rate to the subject's left side just below the heart and to the right chest just below the collarbone.

5.4. Attach one disposable electrode as a reference to the back of the subject's left shoulder.

5.5. Attach two cup electrodes to the subject's right leg over the right tibial nerve above the medial malleolus to administer the shock.

5.6. Attach 4 head position indicator (HPI) coils to the subject, one above each eye and one behind each ear.

5.7. Digitize the position of the subject's head relative to the HPI coils using fiducial points.

5.8. Using the Polhemus system, map the position of the subject's nasion, and left and right tragi.

5.9. Align the subject's digital head position relative to the fiducial points, making sure that the points are symmetrical.

5.10. Next map the position of the subject's HPI coils.

5.11. Finally, digitize 50-100 points along the subject's scalp.

5.12. Escort the subject to the MEG system and connect the electrodes and sensors to the appropriate interface.

5.13. Plug the disposable electrode leads into the MEG system amplifier.

5.14. Plug the HPI wiring harness into the MEG system.

5.15. Plug the shock electrode leads into the shielded extension cable.

Replace [*] with reference from results paper.

5.16. Raise the chair so that the subject's head is touching the top of the MEG helmet.

5.17. Position the screen so that the projected image is in focus.

6. Shock workup

6.1. Set the shock to a level that the subject reports as painful but tolerable.

6.2. Arm the shock stimulator by turning the dial from the 0 mA position the 5 mA position.

6.3. Administer several presentations of the shock using the stimulus control window from the PSYLAB data acquisition software package.

6.4. After each presentation have the subject rate the intensity of the shock on a scale from 0 (not at all painful) to 10 (painful but tolerable).

6.5. Gradually increase the intensity of the shock until the subject rates it as a 10.

6.6. Record the value from the scale in the parameter value box in the subject details window; shocks will be administered during the experiment at the value indicated in this box.

7. Response device

7.1. Instruct the subject on the proper use of the dial using an example Presentation scenario.

7.2. Instructions: "Move the cursor all the way to the right (100) if you are absolutely sure that you will receive a presentation of the stimulation in the near future. Move the cursor all the way to the left (0) if you are sure that you will not receive a stimulation in the near future. Move the cursor to the middle (50) if you are unsure whether or not you will receive the stimulation in the near future."

8. Record MEG during training.

8.1. Record two minutes of raw data at 2 kHz, while the subject rests with their eyes open.

8.2. Prior to training begin recording event codes and shock delivery using the PSYLAB data acquisition software.

8.3. Be sure that PSYLAB is running the proper PCC code so that it sends the shock when triggered by the computer.

8.4. Record raw data at 2 kHz during each of the four training runs.

8.5. Record online averages as a way to visually inspect the data in realtime for systematic sources of noise.

8.6. Ask the subject to rate the intensity of the shock after each run to assess habituation.

9. Setup subject for testing at MRI suite.

Replace [*] with reference from results paper.

9.1. Escort the subject from the MEG suite to the MRI suite.

9.2. Reattach the shock electrodes and recalibrate the intensity of the shock.

9.3. Attach two cup electrodes to the bottom of the subject's left foot to monitor SCRs.

9.4. Make sure that the subject still understands how to use the response device.

9.5. Position the subject on the MRI table, secure their head, and connect the SCR and shock electrode leads to the corresponding shielded cables.

9.6. Position the mirror attached to the head coil so that the participant can see the screen placed behind the head coil.

10. Record fMRI during testing.

10.1. Collect high-resolution anatomical images (SPGR).

10.2. Record blood-oxygenation level dependent responses during the testing session using standard imaging parameters (TR = 2 sec; TE = 25 msec; field of view = 24 cm; flip angle = 90°).

10.3. After testing have the subject complete a post experimental questionnaire.

Using source imaging to recover signal from subcortical structures.

11. Analyze behavioral and fMRI data.

11.1. Use UCS expectancy to determine whether subjects were able to discriminate between the stimuli.

11.2. Average the UCS expectancy data for the 900 msec trace interval and the preceding 900 msec baseline period for each trial.

11.3. Subtract the value for the baseline period from the value for the trace interval to determine how the subject moved the dial after the stimulus presentation.

11.4. Perform a CS type by trial repeated measures ANOVA across subjects.

11.5. Analyze behavioral and fMRI data from the testing session using previously published standards[5,13–15].

12. Preprocess MRI volume.

12.1. Use Freesurfer[16] to create a segmented subcortical volume, and surfaces of the cortex, outer skin, and outer skull.

12.2. Convert volumes and surfaces to AFNI readable format.

Replace [*] with reference from results paper.

12.3. Run importsurfaces.csh - the first time you run the program it will copy all the files you need into a new 'MODEL' folder in each subject's segmentation folder. It will also create an 'importsurface.mrml' file that is used to create the surface models of the amygdala and hippocampus.

12.4. Create and convert the amygdala and hippocampus volumes into surfaces using Slicer3 and Paraview.

12.4.1. Run Slicer3 importsurface.mrml from the subject's 'MODEL' directory. This will load the surfaces and volumes into 3dslicer.

12.4.2. Generate models of amygdala and hippocampus, save models as {structure}.vtk.

12.4.3. Import .vtk files into paraview.

12.4.4. Run filter "generate surface normals."

12.4.5. Export surface normals for amy and hipp as {structure}.ply (ascii) files.

12.5. Import the surfaces and MRI volume into Brainstorm.

12.6. Run importsurfaces.csh again - this will convert the surfaces into files that can be read by matlab and will copy all of the tess_{structure}.mat files into the Brainstorm database directory.

12.7. Make sure that you have already created the subject in Brainstorm before copying tess_{structure}.mat files to Brainstorm folder (See Step 14.1).

12.8. Once you get the surfaces into Brainstorm be sure to refresh the database.

12.9. Warp the MRI volume into standard space by identifying the fiducial points.

12.10. Manually align scalp surface with MRI, then apply the warp to all other surfaces.

12.11. Merge the two pial surfaces and reduce the total number of vertices to 15,000.

12.12. Merge the two hippocampal surfaces and reduce the total number of vertices to 2,000.

12.13. Merge the two amygdala surfaces and reduce the total number of vertices to 1,000.

12.14. Merge the pial, hippocampal, and amygdala surfaces.

12.15. Create regions of interest (scouts) for the amygdala and hippocampus.

13. Preprocess MEG recordings using Brainstorm[11].

13.1. Create new subject in the Brainstorm database.

13.2. Import the MEG recording file for each training session.

Replace [*] with reference from results paper.

13.3. Remove artifacts caused by sources outside the magnetically-shielded room (MSR) using signal space separation[17].

13.4. Remove artifacts caused by heart beats and eye movements using signal space projections from events identified on the electrocardiography (ECG) and electrooculography (EOG) channels.

13.5. Inspect the recordings to ensure that Brainstorm correctly identified heart beat and eyeblinks events.

13.6. Inspect the recordings for other possible sources of noise.

13.7. Inspect the evoked data created from the online averages for systematic sources of artifact.

13.7.1. Note that the TTL pulses used to mark the onset of the stimuli can cause artifacts in the recordings if sent to PSYLAB SAM unit.

13.7.2. Send only TTL pulses needed to administer the shock to the SAM unit and isolate the unit from the remaining pulses using the 8-bit to 2-bit isolation adapter.

14. Analyze evoked responses using Brainstorm.

14.1. Use the event channel to identify epochs (-200 msec to 900 msec) corresponding to each of the experimental trials.

14.2. Refine MRI registration using head points.

14.3. Compute noise covariance from recordings.

14.4. Compute head model using overlapping spheres method with cortex as input.

14.5. Compute sources using the minimum-norm estimate method[10].

14.6. Continue analysis on sources.

14.7. Band-pass filter sources for individual trials (1 Hz to 20 Hz).

14.8. Take the absolute value of the band-pass filtered sources and convert those values to z-scores based on baseline variability.

14.9. Spatially smooth the sources (sigma = 5 mm).

14.10. Average sources across trials.

14.11. Project the averages onto the default anatomy for the experiment.

14.12. Compute t-tests on the sources across the different conditions.

Replace [*] with reference from results paper.

14.13. Filter significant t-test results using spatial and temporal thresholds to correct for family-wise error.

14.14. Identify significantly activated regions and export the time course of activation for each subject.

14.15. Compute the mean and standard error of the mean across subjects at each time point.

15. Perform time-frequency decompositions on ROI using Brainstorm.

15.1. Project the raw data from the individual trials onto the default anatomy for the experiment.

15.2. Identify and create regions of interest from the analysis of evoked response or from anatomo-functional *a priori* hypotheses.

15.3. Compute time-frequency decompositions of the data from your ROI for each trial using standard parameters (central frequency = 1 Hz; time resolution [FWHM] = 3 sec; frequency range = 10:90 Hz; frequency resolution = 1 Hz).

15.4. Convert resulting time-frequency decomposition maps to z-scores.

15.5. Average the resulting maps across trials for each subject.

15.6. Perform t-tests on the maps across the different conditions.

Representative Results:

Using the methods described here, our investigations have led to two major findings: 1) It is possible to manipulate awareness of visual CSs during trace conditioning, and still show evidence of learning. 2) It is possible to recover MEG signals from the amygdala using source imaging*.

In Section 2, we described how to manipulate awareness of visual CSs with backward masking. When exposed to a masked stimulus that is displayed for ~30 msec, the subjects are generally unaware of the stimulus presentation[5,6,8]*. One way to verify the success of this manipulation is to measure the subjects' ability to predict the occurrence of the UCS. If the masking manipulation is successful, subjects should be unable to accurately predict the occurrence of the UCS based on the CS type (See Figure 4).

Although the timing in this type of training makes it difficult to directly measure learning during the training session. It is possible to indirectly measure learning by exposing them to a subsequent unmasked reacquisition testing session with new and old stimuli[5]*. If subjects are able to learn about the contingencies during the training phase, they should show larger magnitude differential (CS+ > CS-) SCRs to the old stimuli relative to the new stimuli. This effect is apparent in the Unfiltered group when we look at testing phase trials after the subjects have been re-exposed to the CS-UCS contingencies (ie. Trials 2-5; See Figure 4).

In Section 8, we described how to record MEG during the masked trace conditioning session. Using source imaging to process these recordings, it is possible to recover MEG signal from subcortical structures like the amygdala[18]*. Subjects shown unfiltered face ($N = 9$) CSs exhibit

Replace [*] with reference from results paper.

larger amygdala responses (Figure 5) and gamma oscillations (Figure 6) than subjects shown high-pass filtered faces (*N = 9*). In addition, these subjects also show larger responses in a network of face processing regions like the occipital face area (Figure 7 and Supplemental Video).

Discussion:

In this paper we describe methods 1) to manipulate subjects' awareness of target CSs during a trace fear conditioning paradigm. 2) and to recover MEG signal from the amygdala during trace fear conditioning without awareness. Using these methodologies, we were able to show that trace conditioning without awareness is possible when faces are used to predict the UCS. This result suggests that faces receive special processing even when presented below the perceptual detection threshold*. Consistent with this conclusion we found that broad spectrum faces evoke robust amygdala responses and bursts of gamma oscillations during the trace interval. This result suggests that the amygdala is capable of maintaining a representation of a face CS during a brief trace interval.

Although presented together, these two methods can be used independently as well. For instance it is possible to use backward masking to manipulate target visibility in other paradigms where behavior may be affected by emotional cues processed below the level of conscious awareness[5,6,8]*. In addition, using the source imaging approach described here it is possible to create 3d models of other subcortical structures, and it may be possible to recover signal from these structures during other region specific tasks. For instance, by using source imaging to model hippocampal activity, it may be possible recover MEG signal from hippocampal sources during tasks like spatial navigation.

The methods described here were designed with two goals in mind: 1) block awareness of the target stimuli, 2) and maximize the ability to detect stimulus evoked amygdala responses using MEG. These design constraints make it difficult to measure the subjects' implicit knowledge of the stimulus contingencies. For instance, SCRs resolve over the course of several seconds[5,13]; however, the CSs are only presented for ~30 msec during training, and the shock is presented shortly after (~900 msec). Given these time constraints, CR expression will be inevitably confounded by UCR expression during training. Because of this colinearity, it is necessary to test the subjects' knowledge of the stimulus contingencies using a subsequent unmasked testing session. However a testing session at the end of the experiment is not optimal because SCRs tend to habituate over the course of the experiment[1]. Given the number of trials needed to show reliable evoked responses with MEG, this SCR habituation will decrease considerably the power to detect a behavioral effect of the training. Future studies should focus on finding better ways to index implicit learning during fear conditioning with masked CSs. This could be done by either finding an alternative index of fear during the training (ie. pupil dilation[19,20]) or find a more sensitive measure of fear that can be administered after the training session.

Figure captions:

Figure 1. Schematic depicting a typical training session. Present 60 trials of a CS+ and 60 trials of a CS-, in pseudorandom order, such that there are 4 blocks of 15 trials each. Present the CSs for 30 msec, immediately followed by an 970 msec mask that coterminates with the shock UCS on CS+ trials.

Replace [*] with reference from results paper.

Figure 2. Schematic depicting the equipment used in a typical conditioning experiment. This setup makes it possible to: 1) present visual stimuli via the Presentation software, 2) administer an electrical stimulation UCS via the Psylab hardware (SAM), 3) record UCS expectancy using an axis device (dial) attached to the presentation computer, and 4) synchronize the stimulus presentations and responses with the MEG recordings via the MEG acquisition system interface.

Figure 3. Illustration showing the location of each of the sensors and fiducial points described in Section 5. Dots with attached lines correspond to the labeled sensors and leads. Blue arrows represent the fiducial points used to register the MEG recordings with the MRI anatomical volume. Purple points represent digitized scalp points used to further refine the MEG-MRI coregistration.

Figure 4. Behavioral results from a typical conditioning study. The graph on the left shows UCS expectancy across the training session, collapsed across the Unfiltered and Filtered groups. Notice that subjects are showing similar levels of UCS expectancy for the CS+ and CS- across the 60 trials, suggesting that the masking procedure blocked their ability to discriminate between the CSs ($F(1,17) = 2.19$; $p = 0.16$). The graph on the right shows the differential SCRs during the testing session. Notice that the Unfiltered, but not the Filtered group seems to be showing larger differential SCRs to the Old stimuli than the New stimuli (Unfiltered New/Old x CS+/CS- interaction: $F(1,7) = 5.94$; $p = 0.045$; Filtered New/Old x CS+/CS- interaction: $F(1,7) = 1.13$; $p = 0.32$), suggesting that the training leads to better reacquisition of the CS-UCS associations for these subjects. ($^*p < 0.05$)

Figure 5. MEG results from a typical conditioning experiment. The figure on the left shows the 3d models of the amygdala (orange), hippocampus (green), and cerebral cortex used to model the sources of the MEG signal. The graph on the right represents activity from an amygdala cluster modeled from the MEG recordings. The light colored line represents the activity evoked by Unfiltered faces, while the dark colored line represents the activity evoked by Filtered faces. Vertical gray shaded sections represent time intervals where Unfiltered faces evoke significantly larger responses than Filtered faces ($F(1,17) > 3.44$; $p < 0.05$).

Figure 6. Amygdala time frequency results from a typical conditioning experiment. The figure on the left shows the 3d models of the amygdala (orange), hippocampus (green), and cerebral cortex used to model the sources of the MEG signal. The graph on the right represents the MEG signal recorded from the amygdala broken down by time and frequency. Warm colors represent regions in the spectrograph that show significantly more power for unfiltered faces than for filtered faces. Cool colors represent the opposite. Regions with the striped overlay represent significant differences across the groups.

Figure 7. Figure showing occipital face area activation in a typical conditioning experiment. Colors represent the magnitude of the Unfiltered > Filtered t-test at the corresponding dipole. Warm colors represent larger responses to Unfiltered faces than to Filtered faces. Cool colors represent larger responses to Filtered faces than to Unfiltered faces.

Replace [*] with reference from results paper.

Supplemental Video. Video showing cortical responses in a typical conditioning experiment. Colors represent the magnitude of the Unfiltered > Filtered t-test at the corresponding dipole. Warm colors represent larger responses to Unfiltered faces than to Filtered faces. Cool colors represent larger responses to Filtered faces than to Unfiltered faces.

Tables:

Table 1. Software used

Table 2. Equipment used

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Disclosures: The authors have nothing to disclose.

Replace [*] with reference from results paper.

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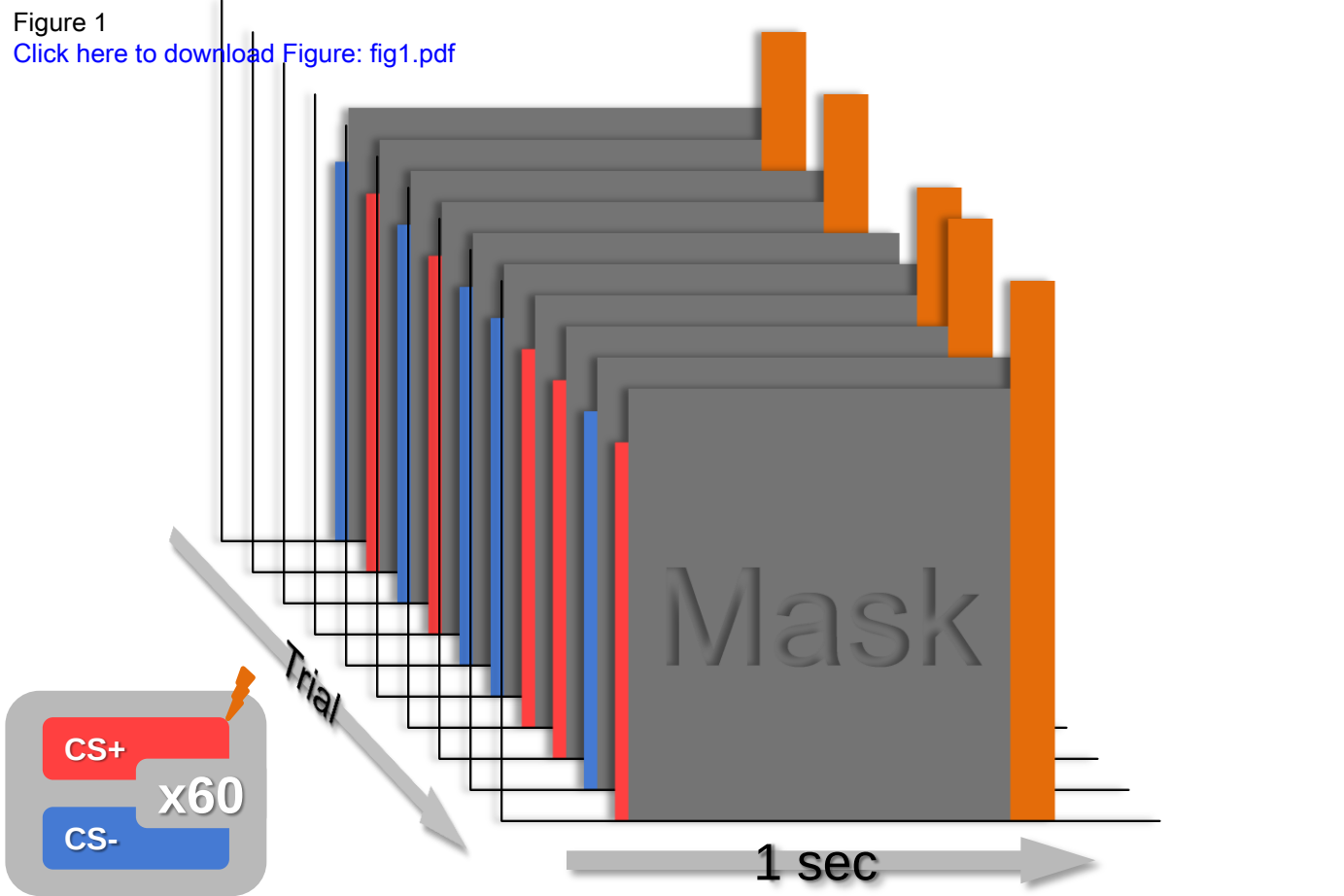

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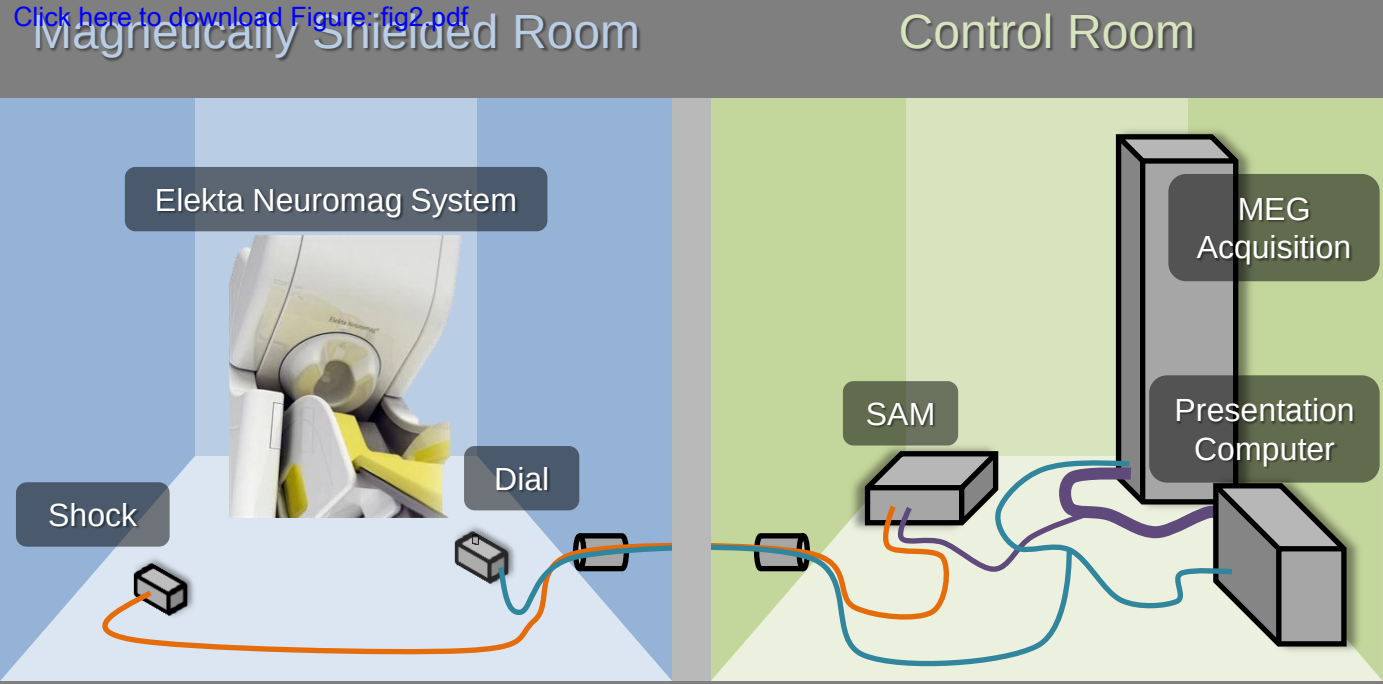

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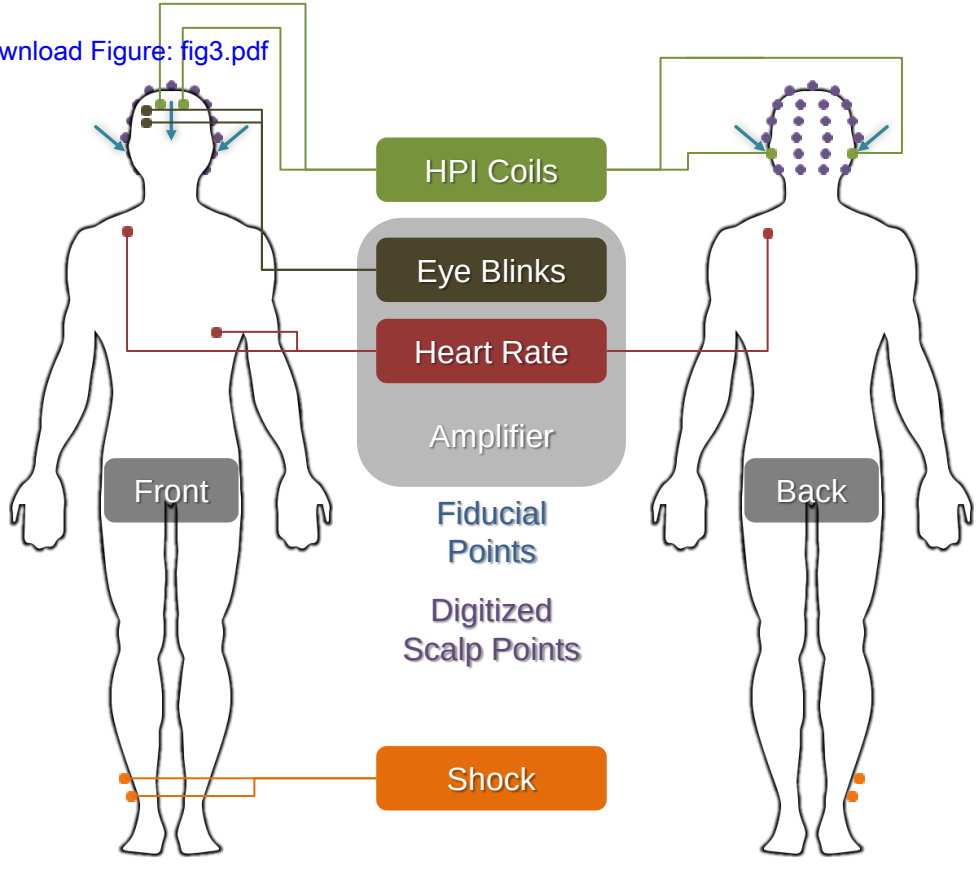

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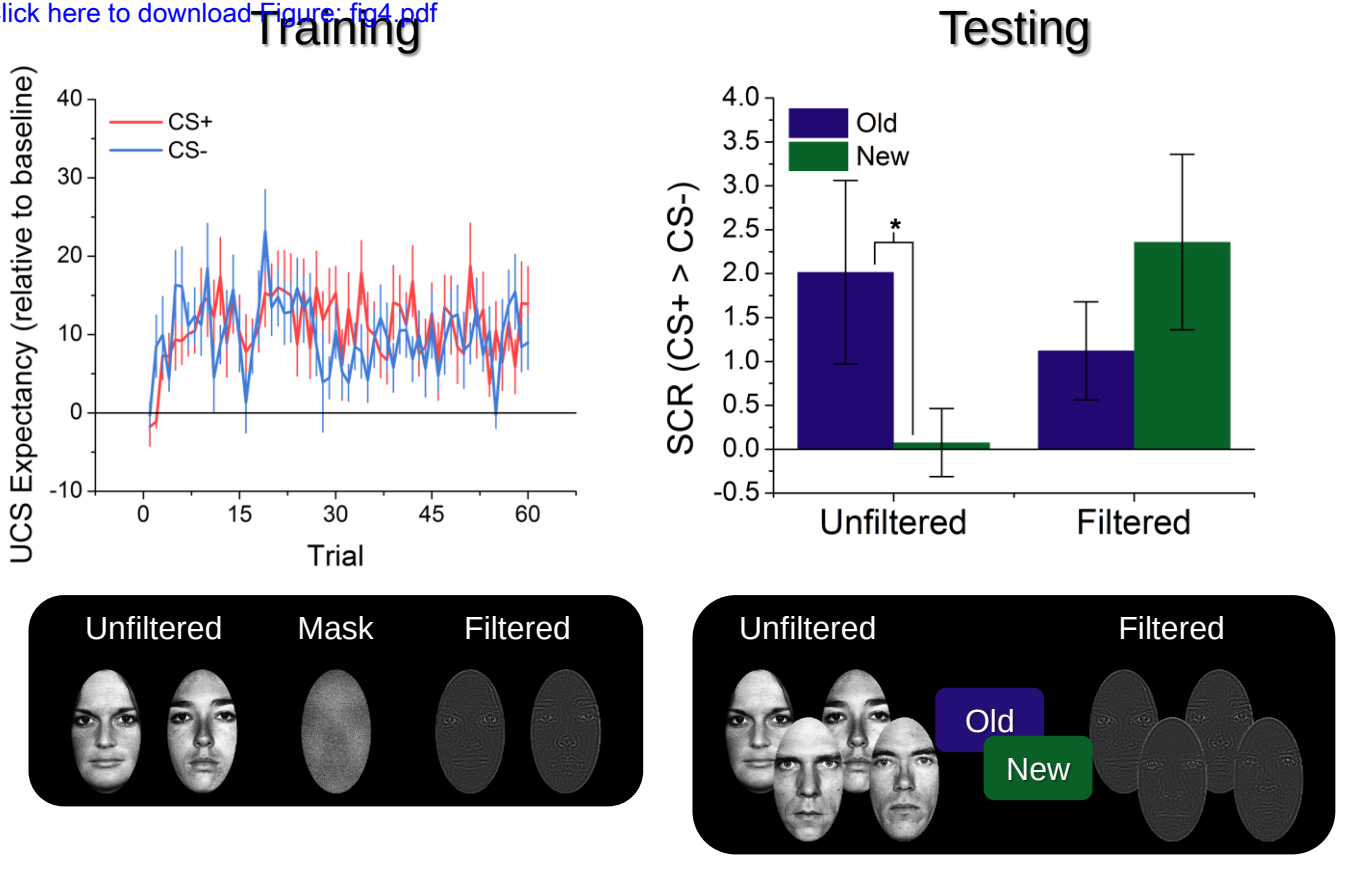

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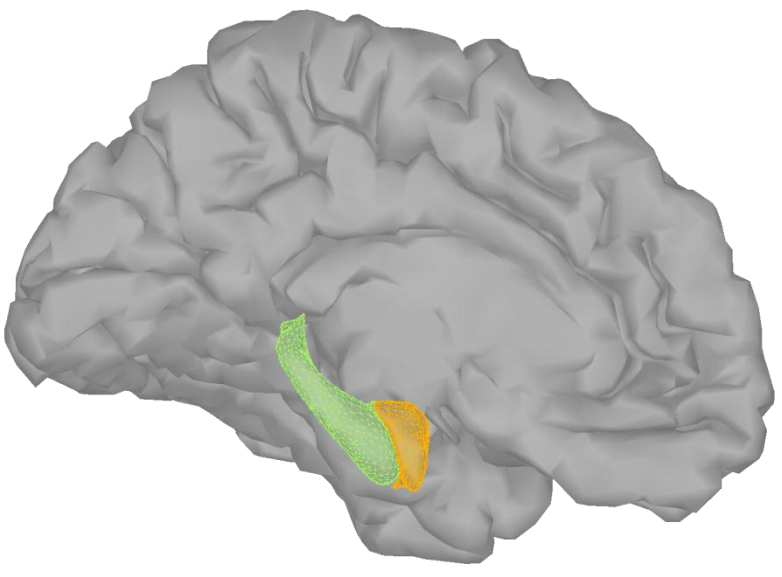

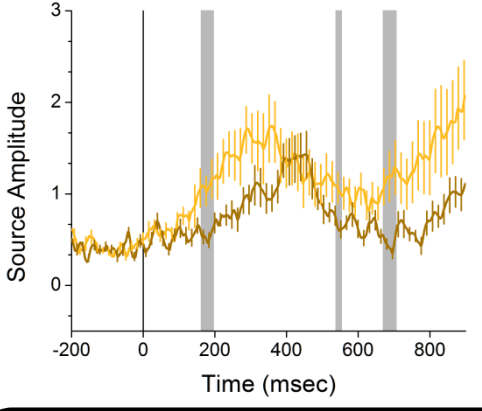

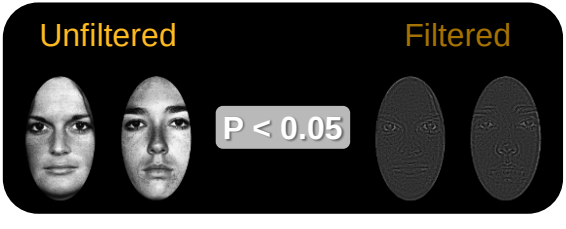

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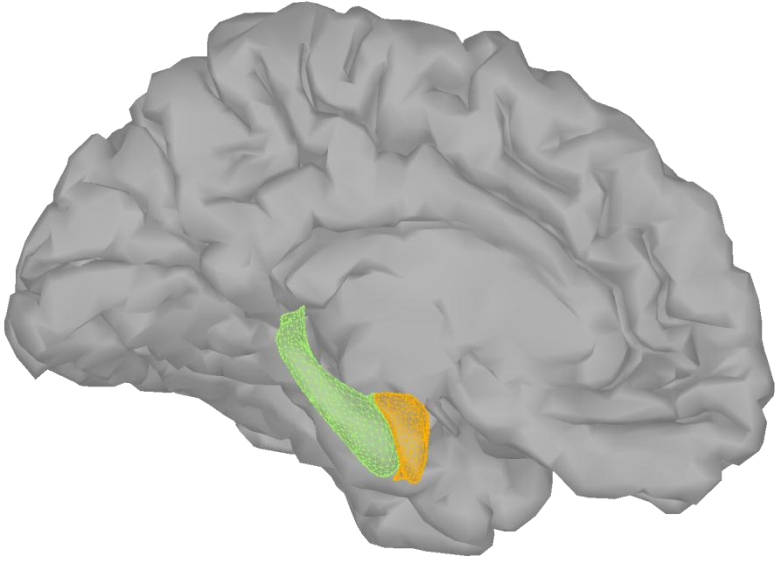

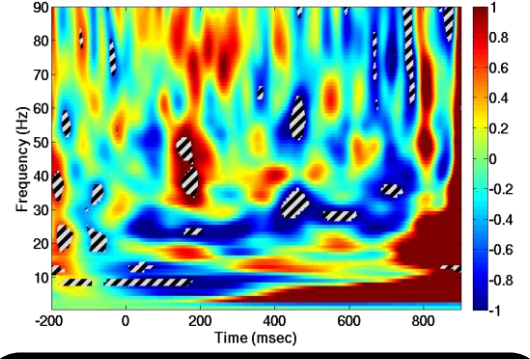

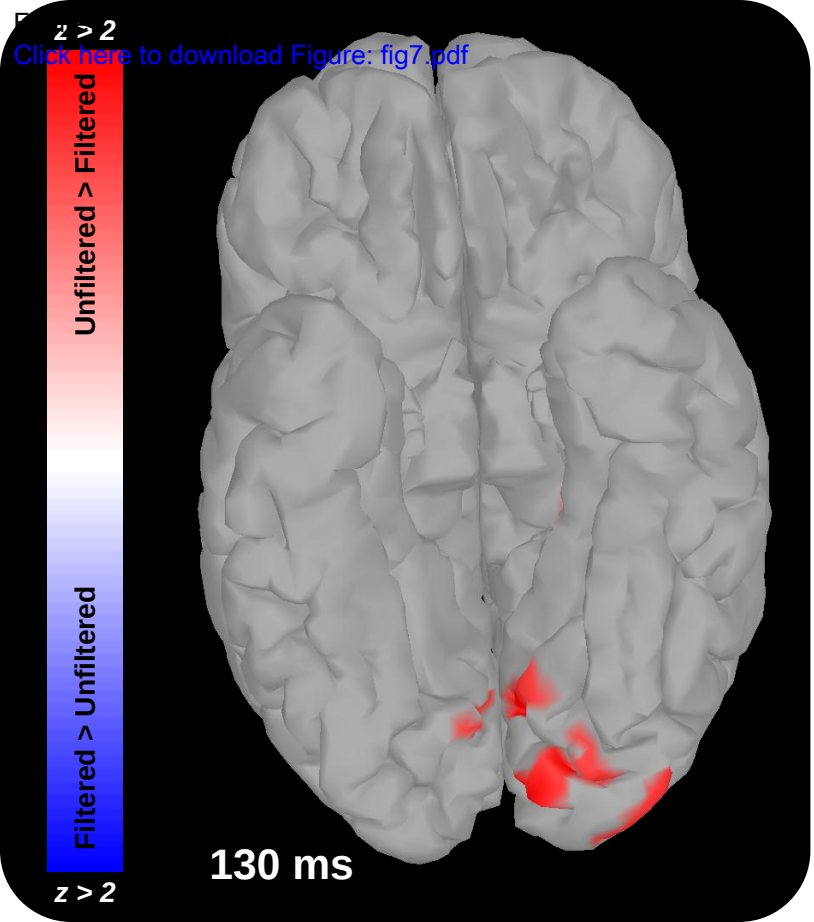

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Program	Provider	URL
	Software	
Matlab	Mathworks	mathworks.com/products/matlab
Presentation	Neurobehavioral Systems	neurobs.com
Psylab	Contact Precision Instruments	psychlab.com
AFNI	NIMH - Scientific and Statistical Computing Core	afni.nimh.nih.gov/afni
Freesurfer	Martinos Center for Biomedical Imaging	surfer.nmr.mgh.harvard.edu/fswiki
MNE	Martinos Center for Biomedical Imaging	nmr.mgh.harvard.edu/martinos/userInfo/data/sofMNE.php
Brainstorm	open-source collaboration	neuroimage.usc.edu/brainstorm
3d Slicer	open-source collaboration	slicer.org
Paraview	Kitware	paraview.org

Table 2 - materials used

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Equipment	Company	Item number
Physiological Monitoring System		
Psylab stand alone monitor (x2)	Contact Precision Instruments	SAM
Skin conductance amplifier	Contact Precision Instruments	SC5
Shock stimulator (x2)	Contact Precision Instruments	SHK1
Additional Components		
8-bit synchronization cable (x2)	Contact Precision Instruments	Included with SAM
8-bit to 2-bit isolation adapter	N/A	Custom
DB25 ribbon cable (x2)	N/A	Standard
Shielded extension cable (x3)	Contact Precision Instruments	CL41
Radiotranslucent cup electrodes for SCR and shock (x6)	Biopac	EL258-RT
Signa Gel	Parker Laboratories	15-250
Response Device		
Rotary dial with gameport connector (x2)	N/A	Custom
Gameport-to-gameport/BNC splitter	N/A	Custom
BNC cable	N/A	Standard
Gameport-to-USB adapter (x2)	Rockfire	RM203U
Additional Components for MEG Setup		
HPI coils and wiring harness	N/A	Custom
HPI positioning system	Inition	Polhemus Isotrak

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Author(s): Nicholas L. Balderston, Doug H. Schultz, Sylvain Baillet, & Fred J. Helmstetter

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Name: Fred J. Helmstetter

Department: Psychology

Institution: University of Wisconsin-Milwaukee

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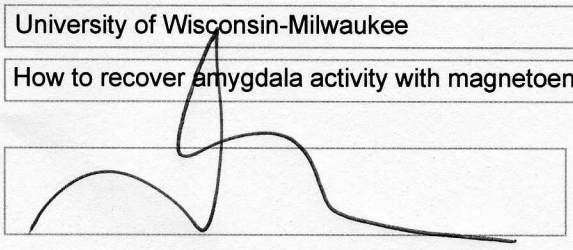
Signature:  Date: 6/25/12

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Overall response to reviewers:
We would like to thank the reviewers for their helpful comments, and thorough review. Having addressed their concerns and clarified points of confusion, we believe that the current draft of the manuscript is much improved. In this initial section, I'd like to summarize our overall response to some of these concerns.

Major Concerns:
Choice of design parameters

Many of the reviewer's comments were related to the experimental design and the choice of fear conditioning as an approach to demonstrate subcortical responses with MEG. Although there may be simpler or more robust ways to demonstrate subcortical responses with MEG, we think that this should not impact the overall value of the manuscript for two reasons. First, we chose to use MEG as a way to investigate an interesting theoretical question regarding the relationship between amygdala function and unconscious learning. Second, we believe that our approach to the analysis of MEG is generalizable to other experimental paradigms, and thus has utility in a methods based journal, like JoVE.

Choice of analysis

The reviewers also commented on our application of a surface based source imaging model to recover signal from subcortical structures like the amygdala and hippocampus, suggesting that other approaches like BEM may be better at recovering signal from deeper sources. It's true, we did not directly compare the relative efficacy of these additional models; however, our method did produce statistically reliable results, consistent with current theories of amygdala function. Additionally, because our approach uses the same method to model signals from cortical and subcortical sources, we think that it can be easily integrated into existing processing streams in other laboratories.

Minor Concerns:
Inclusion of stats

In the original draft of the manuscript we provided a narrative description of typical results one might see if they replicated our design, without providing the supporting statistical results. Although we have kept the basic narrative structure, we have also added specific references to the statistical tests conducted. These can be found in the figure captions.

Clarity of results

In the original draft there were also some results that were either not pictured, or difficult to view. We have added two figures corresponding to the requests of the reviewers. We hope that the current draft of the manuscript is clearer.

Specific responses:
In the following sections the reviewers' comments will appear in Times New Roman, and our responses will appear in Arial.

Production comments:
*There are a lot of acronyms in this manuscript. Please make sure that they are all spelled out in full the first time they are used (for example, SCR, TTL and EOG.
We have gone over the manuscript to ensure that all acronyms are spelled out on their first mention.

Reviewers' comments:

Reviewer #1:
Summary:
The manuscript titled "How to record amygdala activity with magnetoencephalography using source imaging" details methods for 1) designing a trace fear conditioning procedure in which awareness is manipulated, 2) recording brain activity using MEG, and 3) analyzing MEG using source imaging to identify signal from subcortical structures like the amygdala. The described methodologies are sound and described in good detail, and the representative results provide good evidence of the effectiveness of the procedures. I believe this manuscript will be a valuable addition to the literature in this field.

Minor Concerns:
In section 2.11 the authors state that the UCS should coterminate with the mask. This section appears to be describing the testing section when the CS is presented for 8 seconds (stated in 2.10). It's not clear if the mask is really being presented in the testing session given CS durations are 8 seconds. Please clarify if the mask is truly presented on test session trials. If it is, more detail regarding the duration of the mask during the test session and why it is still presented given the long duration CS seems warranted.

Reviewer 1 is correct; it would be unnecessary to present the mask during the 8 s testing trials. It appears that there was a typo in section 2.11. There was actually no mask presented during the testing session.

Reviewer #2:
Summary:
This article is well written with extensive details on how to conduct a trace fear conditioning experiment. The authors show that trace conditioning without awareness activates the amygdala when faces are used as the conditional stimulus. This further shows that a careful paradigm design, a careful measurement and removal of confounding signals, and a careful data analysis allows the detection of deep sources from MEG signals. This paper would serve as a good example on how to properly conduct and analyze an MEG experiment in general and a fear trace conditioning experiment in particular.

Major Concerns:
The authors suggest that high-resolution MRI images can help recover MEG signals from deep

sources. While this statement is generally true, whether it translates into a measurable effect has not been demonstrated. The localization problem in MEG is highly dependent on the inverse model used (see for example Wipf, NI 2009). With spatial filtering methods (minimum-norm, beamformers, etc), the localization accuracy of deep sources is rather crude and highly dependent on the number of sources, the source configuration and the head model used. The strong dependence on these factors makes it unlikely that much would be gained from resorting to such an elaborate individual subject anatomy. Furthermore, the number of trials and number of subjects (in the context of group averaging) play a major role in the ability to detect deep sources (see for example Quraan, HBM 2011). Additionally, analysis techniques that rely on subtracting a control condition from an experimental condition prior to applying spatial filtering models (in the context of event related experiments) can substantially improve the reconstruction of signals from deep sources (Mills, Brain Topography 2012).

These are all insightful comments and there is probably room for amelioration of multiple factors in the modeling of the anatomy, electrophysiological response and source estimates from deeper brain structures. However, we could not identify a specific critique in this reviewer's major concern. Overall, our results indicate significant effects with the approach we have used. Very much akin to fMRI studies, most experimental reports using MEG source imaging use a single method for estimating source distribution, without contrasting results against methodological alternatives. If this is what this reviewer is suggesting that we shall do, we think this would be beyond the scope of our study and would not be justified, given the significant effects we are reporting, which directly address our central neuroscience question.

Minor Concerns:
- I would suggest changing the title from "How to record amygdala activity?" to "How to reconstruct amygdale activity?", as well as other occurrences throughout the article.
This has been changed.

- 1.2 "Chose" should be "Choose".
This has been changed.

- 2.8 It is not clear why the trial needs to be so long.
For theoretical reasons we wanted to keep the interstimulus interval longer than 500 msec. With intervals shorter than 500 msec, it is difficult to behaviorally differentiate between delay and trace conditioning.

Also the inter-trial interval range is not specified.
This has been added.

- 2.9 The total number of trials is rather small for reconstructing a deep source.
Conducting fear conditioning while recording MEG requires tradeoffs. As the reviewer mentions, in order to maximize your ability to detect signals from deep regions, it is necessary to include a large number of trials. However, previous fear conditioning studies recording autonomic activity and amygdala activity have shown that these two indices of conditional learning tend to rapidly habituate across repeated trials[1–3].

Therefore it is important to include a sufficient number of trials to localize the neural generators of the MEG data, but it is equally important to limit the number of trials to those that produce a measureable effect in those generators. This balance is one that still needs to be determined empirically. Although it's possible that our protocol may not have included the optimal number of trials, our results suggest that we do seem to have enough trials to recover signal from amygdala generators.

- 2.10 Seems rather long. What is the reason?
In order to show evidence of autonomic conditioning with masked stimuli, it is necessary to include a subsequent testing session during which responses with longer timecourses (ie SCRs and BOLD) can be resolve. In our experiment, we designed this testing session so that it would be consistent with previous fear conditioning studies conducted by our lab and several others [4–15]. Many of the concerns raised by reviewer 2 are related to the parameters of this testing session.

For instance, Reviewer 2 was concerned that the trial length and ITI were quite long (See comments "-2.10"). Fear conditioning studies like ours typically use ~8 s stimulus presentations and ~20 s intertrial intervals to avoid colinearity in BOLD and SCR measures. Although time consuming, these parameters have been shown to produce robust and stable behavioral and neural results[4–15].

- 2.12 Again the trial length is rather long.
See above comment.

- 7.2 This seems to be too complicated. A button press would simplify the experiment and the analysis.
Reviewer 2 also raised an issue regarding our choice of response criterion, suggesting that it may be too complicated. Although this is a valid criticism, there are many benefits to our method. In the training and testing phase we asked the subjects to continuously rate their expectation of receiving an electrical stimulation. To report their ratings they used a dial to move a cursor along a scale from 0 (sure that no shock will occur) to 100 (sure that shock will occur). With this type of a response, we can get a continuous, realtime measure of their level of expectation. With a button press, you can assess whether or not the subject expects to get a shock on a given trial. With our method you can assess whether they expect to get a shock, when they expect that shock to occur, and how confident they are in their expectation. Interestingly, a previous fMRI study in our lab used the timing information from subjects' shock expectancy to show that the hippocampus responded during trace conditioning only in subjects that could accurately predict the timing of the picture shock associations (Knight et al. 2004).

- 10 .2 This is the first mention of fMRI. It is not clear at this point why/if it is needed. Saying this upfront would have been useful.
Reviewer 2 raises a good point here. We did not include the fMRI results in this report, so describing the methodology could be considered unnecessary. Even though we did not include the fMRI results here, we included the methodology in the protocol for two reasons. First, the behavioral results were collected while recording fMRI. Given that

some behavioral effects are context dependent, we felt that it was important to mention that our behavioral testing was conducted in a different context. Second, we recommend that researchers collect anatomical MRIs for each of the subjects, adding an fMRI scan to this session offers many benefits, and only requires an additional ~10 minutes of scan time.

- 2.11 No mask is mentioned in the testing session.
We have addressed this issue. See above comment from Reviewer 1.

- 8.4 Online averaging is Neuromag specific. They can be reconstructed more accurately after data cleaning.
In this study we used online averaging as a method to visually inspect the data in realtime, but not for further analyses. We suggest that other researchers also use these online averages if available because it can help to identify potential sources of noise that are systematically related to the stimulus presentations, such as voltage leaks from TTL pulses. In agreement with Reviewer 2, we do not recommend using these online averages for analyses. We have edited the referenced statement to make that point more clear.

- 13.3 & 13.4 These steps (SSS and SSP) are specific to the Neuromag system. Alternatives for noise subtraction and artifact rejection for other systems include the use of reference channels (CTF and 4D) and ICA decomposition.
SSP is not Neuromag-specific: it is featured in platform-independent software such as Brainstorm. SSS was mandatory to compensate for the light shielded room that was used with the MEG instrument. Hence, results should be replicable with conventional, multilayer magnetically-shielded rooms, and other instrument technology because the other processing steps were instrument-independent.

- 13.6 No instructions are given here as to what would constitute a significant head movement and what to do in this case. Would the authors advice running SSS to correct for head motion?
Real-time monitoring (visual or with activation of heap-position indicators) of head movements is important during acquisition. We do not recommend that offline head-movement correction is applied because in our experience and it yielded strong artifacts and signal aberrations on our system. We do however recommend visually inspecting the raw data for additional sources of noise.

- 13.7 Not clear if this step provides a lot of help as the data has not been cleaned in these averages. It seems more logical to reconstruct the data averages from cleaned data and inspect those instead.
See above (Comment 8.4).

- 14.4 Why not a BEM model. Is it not available in Brainstorm? This might make a significant difference for deep sources.
Yes, it is available. However, the overlapping sphere model has been shown to be an excellent trade-off between numerical accuracy and stability [16]. We do not anticipate that much difference be found by using a BEM head model vs an OS model as both

take into account the actual head shape of each individual participant. Plus the benefits of BEM in MEG are marginal to the major influence they have in EEG source modeling.

- 14.7 If amygdale activity contains a fair amount of gamma bursts, would filtering at 20 Hz reduce the observed activity in the amygdala (i.e. are these oscillations random with respect to the stimulus onset?).
In this experiment we performed two separate group level analyses. In the analysis of the evoked response we performed a 20 Hz low-pass filter. However Reviewer 2 is correct, this will filter out oscillations above 20 Hz, including gamma bursts (low: 30-60 Hz; high 60-90 Hz). To address this concern we also performed time frequency decompositions on data that had not been passed through the 20 Hz low-pass filter. This allowed us to determine power changes across the time and frequency spectra. We feel that this is a more appropriate method to investigate high frequency oscillations because, as the reviewer points out, these are often random with respect to stimulus onset. Therefore, by analyzing both the evoked responses and the time frequency decompositions we can investigate response dynamics across the entire frequency spectrum. We have added an example graph from our time frequency analysis to clarify this issue (Figure 6).

- 14.9 Please add units.
This has been added.

Representative Results:
- How many subjects were used in this analysis?
This has been added.

- A time frequency plot showing the bursts of gamma oscillations would be useful.
Added as Figure 6.

- It is not clear from the online video that the FA in particular has been activated and whether this occurs within the expected time range (140-180 ms). A still shot would make it easier to see.
Added as Figure 7.

Discussion:
It seems that the long time it takes to resolve the SCR presented a major constraint in terms of timing resulting in long epochs and reduced number of trials. An alternative measure would certainly provide a substantial improvement.
This is definitely a valid criticism. One way to address this issue would be to simultaneously record pupil dilation as a measure of autonomic arousal during the MEG training session[17,18]. Pupil dilation resolves at a more rapid time scale than SCRs (~2 s) during conditioning. Unfortunately we were unable to record pupil dilation during our experiment. Future studies should be conducted to address this gap in the literature.

Mentioning the SCR upfront would help better understand the timing constraints as the reader goes through the paradigm. The language in section 2.10 has been clarified to address this concern.

Figure captions:
Figure 1: 870 msec should be 970 msec?
This has been corrected.

Figure 5: "hippoocampus (hippocampus)" should be "hippocampus (green)".
This has been corrected.

Reviewer #3:
Summary:
This study investigates how activity from amygdala generators can be estimated from MEG measurements. This is accomplished by using a trace conditioning paradigm and a source model composed by the neocortex and two subcortical structures (the amygdala and the hippocampus). Both approaches are challenging for MEG and the authors demonstrate here that these approaches are promising.

Major Concerns:
Playing with the experimental paradigm is one of the best way to increase the signal to noise ratio associated with subcortical evoked responses, as it was shown in recent studies as Quraan et al., HBM 2011 or Mills et al. Brain Topo, 2012. These two studies use less complex protocols than backward masking to manipulate awareness but are still in the same line and the authors should mention these studies to introduce their work.
Reviewer 3 is correct, there are more simple ways of activating subcortical regions, and these designs may be preferred if the intention of the study is a proof of concept, designed simply to show MEG activation in a subcortical region, like the amygdala or hippocampus. We chose the methodology of trace fear conditioning without awareness because it generated theoretically interesting questions about the amygdala and the timing of amygdala responses. One of the strengths of this submission is that the design of the experiment and the source imaging analyses described are somewhat independent of one another. Our hope is that researchers interested in fear conditioning will be able to use this manuscript to replicate and extend our findings whether or not they have access to an MEG system. Likewise, we hope that researchers interested in other behavioral phenomena may be able to use this manuscript as a framework for the analysis of their subcortical MEG data, even if the design does not involve fear conditioning.

On another hand, some points need to be clarified.

The authors must define precisely, the numbers of subjects and associated statistics.
In order to maintain the overall instructive tone of the manuscript, we've used the results section to briefly describe typical results that other researchers might expect if they intend to replicate or extend our findings. However, Reviewer 3's point that we should explicitly describe the number of subjects and statistics is well taken. Therefore, we

have added the corresponding statistical results to the figure captions where appropriate.

Analyze of evoked responses using Brainstorm must be more detailed. The following choices are not clear:
- Why amygdala and hippocampal sources are defined on the surface whereas there are considered, for the amygdala, as a volume in the study of Dumas et al. The same question about dipolar orientation? These points are well discussed in a recent review; Attal et al. 2012 Rev in Neurosci. Please clarify and discuss these points according to the previous mentioned studies.
The models in the studies cited used more detailed/realistic source distribution in these structures. The amygdala is indeed a nucleus with no preferred current flow *a priori*, which can be modeled with a volumic grid of orthogonal current dipole triplets. Here we constrained currents to flow perpendicularly to the nucleus' surface. Given the relatively small size of this structure, we had no objective to resolve activation in different subparts of the amygdala but rather, to account for global variations of current amplitude generated from the structure. hence, instead of using triplets of dipoles, we used a dense array of dipoles radiated from around the surface. From a forward modeling strandpoint, both models are similar in the sense they span the subspace of possible signals generated by this structure.

For the hippocampus, we used the same current models as in Attal et al., 2009[19]

- How was defined ROIs in the amygdala (size and location)? And what about the nearest sources of hippocampus estimations? Is that possible to differentiate them according to your data?
We defined the ROIs of the amygdala and hippocampus using freesurfer. This allowed us to use an objective method that can also be replicated across labs. Although we did not directly analyze the adjacent sources for the amygdala and hippocampus, we agree with Reviewer 3 that this would be an interesting analysis, given an *a priori* hypothesis about differential activity across these structures. Unfortunately given that this experiment involved a manipulation of awareness, we did not have any *a priori* hypothesis about what the hippocampus should be doing[20]. We included the hippocampus as a region of interest here because we wanted to create a general pipeline that we could use to analyze MEG data from fear conditioning studies that we might conduct in the future.

Finally, it could be interesting to study hippocampus-estimated sources as well. Indeed, in this context, learning and episodic processing could engage hippocampal or parahippocampal areas. The authors should also discuss this point. See comment above.

Minor Concerns:
In the caption of the figure 1; "Present the CSs for 30 msec, immediately followed by an 870 msec mask". Is that 870 or 970 ms?
This has been corrected.

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