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Generalized Psychophysiological Interaction (PPI) Analysis of Memory Related Connectivity in Individuals at Genetic Risk for Alzheimer's Disease

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Abstract:	In neuroimaging, functional magnetic resonance imaging (fMRI) measures the blood-oxygenation-level dependent (BOLD) signal in the brain. The degree of correlation of the BOLD signal in spatially independent regions of the brain defines the functional connectivity of those regions. During a cognitive fMRI task, a psychophysiological interaction (PPI) analysis can be used to examine changes in functional connectivity during specific contexts defined by the cognitive task. An example of such a task is one that engages the memory system, asking participants to learn pairs of unrelated words (encoding) and recall the second word in a pair when presented with the first word (retrieval). In the present study, we used this type of associative memory task and a generalized PPI analysis to compare changes in hippocampal connectivity in older adults who are carriers of the Alzheimer's disease (AD) genetic risk factor APOEε4. Specifically, we show that the functional connectivity of subregions of the hippocampus changes during encoding and retrieval, the two active phases of the associative memory task. Context-dependent changes in functional connectivity of the hippocampus were significantly different in carriers of APOEε4 compared to non-carriers. PPI analyses make it possible to examine changes in functional connectivity, distinct from univariate main effects, and to compare these changes across groups. Thus, a PPI analysis may reveal complex task effects in specific cohorts that traditional univariate methods do not capture. PPI analyses cannot, however, determine

	directionality or causality between functionally connected regions. Nevertheless, PPI analyses provide powerful means for generating specific hypothesis regarding functional relationships, which can be tested using causal models. As the brain is increasingly described in terms of connectivity and networks, PPI is an important method for analyzing fMRI task data that is in line with the current conception of the human brain.
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February 14, 2017

Dear Dr. Mani,

I am pleased to resubmit our manuscript entitled “Generalized Psychophysiological Interaction (PPI) Analysis of Memory Related Connectivity in Individuals at Genetic Risk for Alzheimer’s Disease” which we prepared according to the stated guidelines in the Standard Template for the *Journal of Visualized Experiments (JoVE)*.

Our revised manuscript is now more clear and specific, thanks to feedback from reviewers. This paper and the corresponding video will help others implement the PPI approach in fMRI in their own data. Furthermore, we believe our manuscript will help others to avoid common mistakes and misconceptions. Our specific study design, a PPI within the context of an imaging genetics experiment, will expose *JoVE* readers to preclinical genetic risk groups.

All of the listed authors have contributed to the manuscript and agree to it being resubmitted for publication. We warrant that the content of this manuscript represents original work, has not been published elsewhere, and is not under consideration for publication elsewhere. All relevant funding sources are listed in the “Acknowledgements” section. Author DGM is an employee of Biospective, Inc. Biospective, Inc was not involved in the processing of these data or in the preparation of the manuscript. The other authors declare no conflicts of interest.

We looking forward to hearing from you. Thank you for your consideration.

Sincerely,

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TITLE:
Generalized Psychophysiological Interaction (PPI) Analysis of Memory Related Connectivity in Individuals at Genetic Risk for Alzheimer's Disease

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KEYWORDS:
Functional connectivity, hippocampus, functional magnetic resonance imaging, fMRI preprocessing, fMRI statistical analysis, MRI, genetic risk; APOE; psychophysiological interaction (PPI); generalized psychophysiological interaction (gPPI)

SHORT ABSTRACT:
This manuscript describes how to implement a psychophysiological interaction analysis to reveal task-dependent changes in functional connectivity between a selected seed region and voxels in other regions of the brain. Psychophysiological interaction analysis is a popular method to examine task effects on brain connectivity, distinct from traditional univariate activation effects.

LONG ABSTRACT:
In neuroimaging, functional magnetic resonance imaging (fMRI) measures the blood-oxygenation-level dependent (BOLD) signal in the brain. The degree of correlation of the BOLD signal in spatially independent regions of the brain defines the functional connectivity of those

regions. During a cognitive fMRI task, a psychophysiological interaction (PPI) analysis can be used to examine changes in the functional connectivity during specific contexts defined by the cognitive task. An example of such a task is one that engages the memory system, asking participants to learn pairs of unrelated words (encoding) and recall the second word in a pair when presented with the first word (retrieval). In the present study, we used this type of associative memory task and a generalized PPI (gPPI) analysis to compare changes in hippocampal connectivity in older adults who are carriers of the Alzheimer's disease (AD) genetic risk factor apolipoprotein-E epsilon-4 (APOEε4). Specifically, we show that the functional connectivity of subregions of the hippocampus changes during encoding and retrieval, the two active phases of the associative memory task. Context-dependent changes in functional connectivity of the hippocampus were significantly different in carriers of APOEε4 compared to non-carriers. PPI analyses make it possible to examine changes in functional connectivity, distinct from univariate main effects, and to compare these changes across groups. Thus, a PPI analysis may reveal complex task effects in specific cohorts that traditional univariate methods do not capture. PPI analyses cannot, however, determine directionality or causality between functionally connected regions. Nevertheless, PPI analyses provide powerful means for generating specific hypotheses regarding functional relationships, which can be tested using causal models. As the brain is increasingly described in terms of connectivity and networks, PPI is an important method for analyzing fMRI task data that is in line with the current conception of the human brain.

INTRODUCTION:

The term "connectome" was coined in 2005 marking a paradigm shift in neuroscience that continues to this day[1]. The brain is increasingly described in terms of functional networks, connectivity and interactions between and among regions on a large scale. Nevertheless, the delineation of regional functional specialization and associations between fMRI-measured activity and task demands are still valid and useful approaches. In light of the growing interest in connectomics, functional connectivity approaches to task fMRI analysis are growing in popularity. One approach to measuring functional connectivity changes dependent on task demands makes use of the concept of PPI. A PPI is the interaction of an active task phase or particular task demand ("psycho") with the functional connectivity ("physio") of a region of interest or "seed" in the brain. PPI differs from bivariate, correlation-based analysis of functional connectivity, which generally measures the degree of correlation between the activity in two regions without any constraints related to task demands.

The concept and framework of a PPI analysis was originally described by Friston and colleagues in 1997[2]. The authors asserted that their approach was important because it would allow the investigation of connectivity to be more functionally specific and allow for inferences that activity in a distal seed could be modulating activity resulting from a task demand. In 2012, McLaren and colleagues added to this original framework and described a gPPI approach in which all task phases and their interactions are included in a single model[3]. This approach leads to results that are more sensitive and specific to the task phase and interaction being investigated. It is this updated gPPI approach that we employ in the present study (see step 6.2.2 in **Protocol**). The gPPI approach has now been cited in over 200 studies. For clarity,

hereafter we use 'PPI' to describe common features of both the standard and generalized version. 'gPPI' will be used to discuss specific advances associated with the newer framework.

The overall goal of a PPI analysis is to understand how the demands of a cognitive task influence or modulate the functional connectivity of a seed region. A PPI analysis requires a strong *a priori* hypothesis. Activity in the seed region must be modulated by the task in order for the PPI approach to work effectively[4]. For example, in the present study, we based our seed selection on the strong evidence that hippocampal activity is modulated by the cognitive demands of a memory task. Using PPI, regions that are significantly more or less functionally connected to the hippocampus during specific task phases can be identified. In short, we ask the question, "in which regions is activity more correlated with the seed during context A as compared with baseline?" We can also ask the logical opposite (as it is important to understand the difference): "in which regions is activity less correlated with the seed during context A as compared to baseline?" When interpreting group differences in PPI effects, it is important to examine the data and whether positive or negative change in functional connectivity, or both, is driving group differences.

The PPI approach has been used to study dynamic task control hubs in healthy controls, how modulation of functional connectivity is related to cognitive performance in Alzheimer's disease (AD), intelligence in individuals with autism, motor network connectivity in individuals with Parkinson's disease, face processing in individuals with body dysmorphic disorder and anorexia, emotion regulation, memory and many other specific questions related to connectivity[5–11]. In the present study, we compare changes in functional connectivity of subregions of the hippocampus during memory encoding and retrieval between a group of individuals at increased genetic risk for AD to a group without the risk factor[12]. The following describes the protocol that we used, applying the gPPI approach, to allow us to test if task-elicited changes in functional connectivity differ in association with the presence of APOEε4, a genetic risk factor for AD.

PROTOCOL:

The present study was performed in compliance with the UCLA Institutional Review Board (IRB) protocols and approved by the UCLA Human Subjects Protection Committee. All participants gave written informed consent in order to enroll in this study.

1. Participant Selection

1.1. Obtain IRB approval to perform the study.

1.2. Screen individuals aged 55 and older for cognitive decline using a standardized neuropsychological battery. Include tests of General Intelligence (Subtests of the WAIS-III)[13], Fluency (Fruits and Vegetables)[14], Attention (Digits Forward and Backward)[13], Language (Boston Naming Test)[15], Verbal Memory (Buschke-Fuld Selective Reminding Task)[16], WMS-III Logical

Memory and Verbal Paired Associates learning[13], and Visual Memory (Rey-Osterrieth Figure test)[17].

1.2.1. Have the participants complete mood questionnaires such as the Hamilton Depression and Anxiety Inventories[18,19] as well as the Mini Mental State Exam (MMSE)[20].

1.3. Include participants that score 26 or above on the MMSE and perform better than two standard deviations below normal for their age on cognitive tests. Exclude participants with clinical anxiety, depression or any other neuropsychiatric or neurological illness. Exclude participants who do not meet MRI safety criteria or who do not consent to a blood draw.

Note: In the present study, 93 participants met these criteria (mean age = 67.4 years, 31M/49F).

2. Genotyping

2.1. Have a trained phlebotomist or other medical professional draw blood from each participant.

2.2. Isolate 200 µg genomic DNA from 10 mL of the sample as described[21].

2.3. Carry out single nucleotide polymorphism (SNP) genotyping using Real Time PCR at two loci, rs429358 and rs7412 to discriminate *APOE* alleles[22].

2.3.1. Incorporate reporter dyes for rs429358 and rs7412 into a SNP genotyping assay. After each PCR amplification cycle is completed, plot fluorescent signals on a graph showing distribution of reporter and quencher dyes. Perform the experiment in duplicate to confirm results.

2.4. Analyze the SNP genotyping data using a software package developed for the Real Time PCR procedure output[23].

Note: The program used in the present study calculates the affinity of the sample to one of the two reporter dyes that, in turn, represents one *APOE* SNP over the other. In the present study, 34 carriers of the AD risk allele, APOEε4 (heterozygous ε3/ε4) and 46 non-carriers (homozygous ε3/ε3) were enrolled for a total of 80 study participants. Exclude carriers of the APOEε2 allele because there is evidence that this allele may have a protective effect related to AD.

3. Functional and Structural Imaging Data Collection

3.1. Use a 3 Tesla (3T) MRI system to acquire whole-brain imaging data.

3.1.1. For functional imaging, collect axial slices using an echo planar imaging (EPI) sequence. To facilitate registration of the functional images, acquire axial slices of T2-weighted, co-planar

structural images. For high-resolution structural imaging, collect axial slices using a 3D T1-weighted sequence.

Note: In the present study, a 3T magnet was used with a 12-channel head coil. The parameters below were designed for a specific scanner and coil. See **Table of Materials** for more information.

3.1.1.1. Acquire functional imaging data using the following sequence parameters: repetition time (TR) = 2,500 ms, echo time (TE) = 21 ms, field of view (FOV) = 200 mm x 200 mm, flip angle = 75 °, matrix = 64 x 64, 33 slices, slice thickness = 3 mm, interslice gap = 0.75 mm, voxel size = 3.125 x 3.125 x 3.75 mm.

3.1.1.2. Trigger the unrelated words associative memory task to begin with the third volume of the functional imaging sequence. To account for steady-state equilibrium, exclude the first two volumes of each functional scan from analyses.

Note: The unrelated words associative memory task has been described elsewhere[12,24]. Briefly, it is a block-design functional task with encoding and retrieval blocks. Participants are instructed to learn pairs of unrelated words.

3.1.1.3. Acquire T2-weighted, co-planar structural imaging data using the following sequence parameters: TR = 5,000 ms, TE = 34 ms, FOV = 200 mm x 200 mm, flip angle = 90 °, matrix = 128 x 128, 28 slices, slice thickness = 3 mm, interslice gap = 1 mm and voxel size = 1.56 x 1.56 x 4 mm.

3.1.1.4. Acquire high-resolution structural (anatomical) imaging using the following Magnetization Prepared Rapid Gradient Echo (MPRAGE) sequence parameters: TR = 1,900 ms, TE = 2.26 ms, TI = 900 ms, FOV = 250 mm x 218 mm, flip angle = 9 °, matrix = 256 x 215, 176 slices, slice thickness = 1 mm, zero-filled to a matrix of 256 x 224 resulting in a voxel size = 1 x 0.976 x 0.976 mm.

4. fMRI BOLD Data Preprocessing

4.1. Preprocess the functional data using Functional MRI of the Brain (FMRIB) Software Library (FSL) version 6.0 (http://fsl.fmrib.ox.ac.uk) as follows:

4.1.1. For each participant's dataset, remove head motion artifact from the data using the Motion Correction FMRIB's Linear Image Registration Tool (MCFLIRT)[25].

4.1.2. Remove non-brain tissue from the images using brain extraction tool (BET) with the optional -F flag[26].

4.1.3. Use the FSL Motion Outliers tool to identify any volumes in the functional data where there is excessive motion based on frame displacement between volumes. Flag volumes where

motion is measured as an outlier (above the 75th percentile + 1.5 times the inter-quartile range) compared to the rest of the scan and use the output of this program to downweight those volumes in analyses.

Note: Before running group comparisons, check that average motion, as measured by FSL Motion Outliers, does not differ across the two groups. This will help ensure that findings are not driven by group-related differences in motion.

4.2. Set up the preprocessing and first-level general linear model (GLM) using the graphical user interface (GUI) for FSL fMRI Expert Analysis Tool (FEAT) for the first participant.

Note: Repeat this step for each study participant. To save time, after setting up one run for one participant, write a script to run preprocessing for the remaining study participants' data by altering the "design.fsf" file (FSL FEAT output) for each participant to reference that participant's specific data.

4.2.1. In the data tab, click on "add 4D data" and navigate to the motion-corrected and brain-extracted file. Set the TR to 2.5 s (corresponding to the TR of the functional sequence acquired). Use the default high pass filter (set to 100 s).

Note: High pass filtering will remove low frequency signals of no interest.

4.2.2. In the pre-stats tab, click "none" under "motion correction" (as it was already performed in step 4.1). Uncheck "BET brain extraction" (as it was already completed in step 4.1). Type "5" in the box to set 5 mm full-width half-maximum (FWHM) Gaussian kernel for spatial smoothing.

Note: The FWHM for the smoothing kernel should generally be set at about twice the size of the functional scan voxel size.

4.2.3. Use the output (6 columns, rows = # of TRs in the scan) of MCFLIRT to create 6 single-column text files that describe the motion correction performed at each volume within the dataset. These will be added to the model as regressors in the next step.

4.2.3.1. In the stats tab under "full model setup", add the 6 motion parameters and their temporal derivatives as regressors or explanatory variables (EVs) in the GLM. For each motion EV choose "custom" (1 entry per volume) for basic shape, "none" for convolution and check "apply temporal filtering."

Note: Motion parameters do not need to be convolved by any function because they reference the realignment performed at each functional volume during motion correction and thus do not need to be adjusted.

4.2.4. In the stats tab, select the output of FSL Motion Outliers from step 4.1 under the "add additional confound EVs".

Note: This output is a matrix denoting each volume that was flagged for excessive motion and, by adding the confound file, will be deweighted in the GLM.

4.2.5. In the stats tab, click "full model set-up". Create the task timing text files denoting the onset and offset of different task phases and add these as EVs in the GLM by choosing 1 column format and navigating to the relevant text file (include one for the encoding phase of the task and one for the retrieval phase). For "convolution" choose the "double-gamma HRF" option from the drop down list for both of them. Do not model the baseline or non-active portions of the task in the GLM.

Note: HRF stands for hemodynamic response function. Convolving the task EV by the HRF shifts the timing of the task EV to be more consistent with expected task-induced BOLD signal changes in the brain.

4.2.6. In the registration tab, check "expanded functional image" and "main structural image" for a two-step registration.

4.2.6.1. Select the participant's co-planar T2-weighted structural scan for the first step, in which functional data is registered to the co-planar structural data. Choose 6 degrees of freedom (DOF) for this step by clicking on the second drop down box under this step and choosing "6 DOF".

4.2.6.2. For the next step, in which the T2-weighted image is registered to the high resolution T1-weighted MPRAGE, select boundary based registration (BBR) from the drop down box[27].

Note: BBR uses intensity differences between white matter and gray matter to register structural and functional scans and has been shown to perform better than FLIRT and other alternative methods.

4.2.6.3. For the final step, in which the high-resolution structural data is registered to the standard MNI152 template, choose 12 degrees of freedom and a linear transformation by choosing "12 DOF".

Note: When all the steps in section 4 are complete the functional data are preprocessed and ready for further analysis.

5. Hippocampal Seeds

5.1. Generate a mask of the left hippocampus in each participant's high resolution structural space using FSL's FMRIB Integrated Registration and Segmentation Tool (FIRST) segmentation algorithm[28].

Note: Other regions, including right hippocampus, would be interesting and valid seeds for

further analyses.

5.2. Using a statistical software platform, write code in to calculate the length of the anterior and posterior thirds of the structure[29]. Specifically, use the length of the volumetric hippocampal mask in the anterior-posterior plane to find the coordinates demarking the anterior and posterior thirds of this plane.

Note: A recently published method of segmenting the hippocampus along the longitudinal axis might be an alternative seed creation approach[30].

5.3. Based on these coordinates, create anterior and posterior hippocampal mask images. Register the anterior and posterior hippocampal masks into native functional space using the "example_func2highres" matrix in the registration directory of the FEAT output.

Note: Using the anterior and posterior thirds prevented signal blurring across the two hippocampal seeds after registration to functional space. There is evidence of functional specialization along the longitudinal axis of the hippocampus[31–34]. Anterior regions are input regions and associated with encoding, while the posterior hippocampus is an output region associated with memory retrieval and consolidation[35–37]. Thus, using these regions allows assessment of functional involvement of anterior versus posterior hippocampus in encoding versus retrieval phases of the memory task.

5.4. Use FSL mean timeseries (fslmeants) to extract the denoised average timeseries from the anterior and posterior hippocampal seeds (**Figure 1**). Follow the program instructions and use either the anterior or posterior hippocampal seed as the mask and the denoised, preprocessed functional data as the main image.

[Insert Figure 1 here]

6. PPI Model

6.1. Use the GUI for FSL FEAT to load the preprocessed functional data.

6.1.1. In the data tab, choose the "filtered_func_data" denoised image (output from the steps completed in section 4) as the input file. In the pre-stats tab, set motion correction and brain extraction to "none." Unclick boxes to perform temporal filtering and spatial smoothing.

6.2. PPI Model Set-Up (**Table 1**).

6.2.1. In the stats tab, select "full model set-up". In the EVs tab, add all the EVs from the first level model: 6 motion correction EVs, confound EV matrix from FSL Motion Outliers and task timing EVs. Click the up arrow to add EVs. Include in this model an EV for the physiological timecourse from the seed (the text file output of fslmeants in step 5.4) as a covariate of no interest by clicking on the up arrow.

6.2.2. Create the PPI terms.

6.2.2.1. Choose “interaction” in the basic shape menu and select the seed timecourse EV and one task EV. For the “make zero” option, choose “mean” for the seed timecourse EV and “centre” for the task EV. Repeat this procedure for the other task phase(s). Run a separate model for each seed region.

Note: These new EVs are the PPI terms for the phase of the task selected (psycho) and the seed (physio). In the present study, a PPI term for the encoding phase and a second PPI term for the retrieval phase were included in each PPI model. The “centre” option ensures that the “on” and “off” phases of the block design task are treated equally. The “mean” option is always applied to the seed timecourse and results in the mean being subtracted from this regressor.

6.2.3. In the contrasts and F-tests tab, model the following specific effects by entering “1” in the corresponding EV cell: psych_enc (encoding task phase), psych_ret (retrieval task phase), phys (seed timecourse), PPI_enc (PPI of seed and encoding), PPI_ret (PPI of seed and retrieval). Lastly, enter a “-1” to model negative PPIs for each task phase.

[Insert Table 1 here]

7. Group Comparisons

7.1. Select “higher-level analysis” in FSL FEAT to run a simple group model comparing APOEε4 carriers to non-carriers for each task-seed combination.

Note: These comparisons are run to generate the relevant group 4D residuals images (“res4d”) needed to estimate the smoothness of the dataset. Statistically significant results from this group comparison are valid, but in the steps below another thresholding approach using AFNI and SPM8 to set a significant cluster minimum based on Monte Carlo simulations is described.

7.2. Use Analysis of Functional Neuroimaging (AFNI; https://afni.nimh.nih.gov/) to determine the voxel-level threshold and the cluster size minimum.

7.2.1. Use AFNI’s 3dFWHMx (any version after December 2015) at the command line to estimate the smoothness of the group 4D residuals images generated using FSL.

Note: A bug was discovered in AFNI’s 3dClustSim and corrected in May 2015. In December 2015, AFNI’s 3dFWHMx was updated to more accurately model auto-correlations. Thus, versions of these tools released in December 2015 or later should be used.

7.2.2. Use AFNI’s 3dClustSim (any version after December 2015) to determine cluster extent minimums reaching significance at different voxel-level thresholds. Include the smoothness estimates from the previous step in the command line invocation of 3dClustSim. From the table

generated by 3dClustSim, based on the study hypotheses regarding the expected effects' height and extent, choose a voxel-level threshold and corresponding cluster minimum size.

Note: In general, larger clusters minimize false positives.

7.3. Use Statistical Parametric Mapping (SPM8, http://www.fil.ion.ucl.ac.uk/spm/software/spm8/) tools to run the group level comparisons.

7.3.1. Using the SPM8 GUI, select "specify 2nd-level". The batch editor will open. Select "two sample t-test" under design. Navigate to the directory with the parameter estimate images for group 1 (APOEε4 carriers) and select by clicking on them. Next, add group 2 (APOEε4 non-carriers) images. Run this comparison by clicking on the green play button.

7.3.2. Return to the SPM GUI, select "estimate", and navigate to the SPM.mat file created in the previous step to run the model estimation process.

7.3.3. Select "results" and run group comparison contrasts: APOEε4 carriers > APOEε4 non-carriers, APOEε4 non-carriers > APOEε4 carriers.

7.3.3.1. Click on "define a new contrast", choose "T-contrast" under "type" and enter "1 -1" in the "contrast" box for APOEε4 carriers > APOEε4 non-carriers. Click "done". Choose "none" for Apply Masking, and manually set the voxel-level threshold and the cluster size minimum according to the determination made in step 7.2.2. Enter "-1 1" for APOEε4 non-carriers > APOEε4 carriers.

Note: In the present study, a voxelwise threshold of $p<0.005$ was used and clusters thresholded at alpha <0.05.

REPRESENTATIVE RESULTS:

With two different active task phases (encoding and retrieval) and two seed regions (anterior and posterior hippocampus) there are four conditions to report results for each group. The within-group task activation maps (not shown here, see Harrison *et al.*, 2016[12]) show that the occipital lobe, auditory cortex, large regions of parietal lobe, frontal language areas, superior temporal gyrus, and caudate (more pronounced during retrieval) have significant BOLD signal increases during encoding and retrieval in both experimental groups. Within-group PPI analyses revealed that there are no significant increases in functional connectivity with either anterior or posterior hippocampal seeds for either APOEε4 carriers or non-carriers. Within-group PPI analyses revealed significant decreases in functional connectivity in APOEε4 carriers for both task conditions and hippocampal subregions (**Figure 2**). In APOEε4 non-carriers, significant decreases in functional connectivity were only observed with posterior hippocampus during encoding (**Figure 2**). The positive and negative PPI maps show a divergence between APOEε4 carriers and non-carriers in how hippocampal functional connectivity changes during a memory task. To determine if the divergence is statistically significant, it is necessary to directly compare the groups for each of the four results[38].

For the sake of brevity, group comparison results showing *APOE*-mediated differences only for one region and task phase, anterior hippocampus during retrieval, are presented here (non-carriers > carriers, **Figure 3**). During retrieval, the divergence of anterior hippocampus connectivity changes observed within group (**Figure 2**) results in significant between group differences in bilateral supramarginal gyrus, right angular gyrus and right precuneus.

[Insert Figures 2 and 3 here]

FIGURE LEGENDS:

Figure 1: Hippocampal Seeds. In native space, a single participant's anterior hippocampus seed is shown in yellow. The posterior hippocampus seed for the same participant is shown in pink. Seeds are defined in each participant's unique structural image and then registered to their functional scan. Seeds are never in a standardized space, which improves the accuracy of the hippocampal segmentation. This figure has been reprinted with permission[12].

Figure 2: Hippocampal seeds task-dependent *negative* functional connectivity change maps. Coronal and axial views of group average task-dependent negative functional connectivity change of hippocampal subregions in APOEε4 non-carriers and carriers separately, within group. Task-dependent connectivity decreases with the anterior hippocampus seed are shown in the upper panels. The lower panels show task-dependent connectivity decreases with the posterior hippocampus. Maps were thresholded at $z = 2.3$, cluster corrected at $p<0.05$. Voxels meeting threshold in APOEε4 non-carriers (in red) and carriers (in green) are overlaid. This figure has been reprinted with permission[12].

Figure 3: Anterior hippocampal seed connectivity change differences between APOEε4 carriers and non-carriers during retrieval. During retrieval, significant differences between APOEε4 carriers and non-carriers were found in left supramarginal gyrus (dark blue), right supramarginal/angular junction (orange) as well as right precuneus (purple). The results from this two-sample t-test were thresholded to reveal clusters significant at alpha <0.05 with a voxelwise threshold of $p<0.005$. The peak coordinate for each cluster is reported in MNI space, in x, y, z planes (mm). For illustration of the direction and magnitude of the difference between groups, contrasts of parameter estimates from each cluster are plotted by group. The red horizontal lines indicate zero and highlight that carriers have decreased (negative) functional connectivity to anterior hippocampus in these regions during retrieval. The band within the boxes represents the median while the upper and lower edges of the boxes represent the first and third quartiles, respectively. The whiskers extend up to 1.5 times the interquartile range. Data points outside this range are plotted as outliers. This figure has been reprinted with permission[12].

Table 1: gPPI model set-up.

DISCUSSION:

Early task-based fMRI studies were designed to uncover statistical relationships between particular cognitive processes or demands and changes in the BOLD signal relative to a baseline

measurement. This traditional approach is useful for identifying specific regions in the brain where activity is modulated by an experimental task. In contrast, a PPI analysis is chiefly concerned with the modulation of functional connectivity, or synchrony of activity, that results from a task-induced cognitive process. PPI measures context dependent functional connectivity between a defined region of interest (seed) and other regions of the brain, not just activity increases and decreases in localized areas. The selection of the seed region must be hypothesis-driven as PPI analyses will perform optimally when activity in the seed region is modulated, in a univariate framework, by the task-induced cognitive context. Then, the PPI framework can be used to explore how seed region activity becomes more or less synchronized with other regions in response to specific task contexts, such as memory encoding or retrieval. Differences between groups, therefore, are limited to the functional connectivity changes between the seed and other regions that are modulated by a particular task phase.

A thorough understanding of the GLM is essential for implementing a PPI analysis. A complete, group comparison PPI study has three levels of linear modeling: the first level (preprocessing, task and motion modeling), the mid-level PPI model (add seed timecourse and task interaction EVs) and the higher level group comparison model (group contrasts of parameter estimates). At each step, an output image is used as the input for the following step. The gPPI approach proposed in 2012 and employed in the present study utilizes features of the GLM to ensure that contrasts are specific to interactions with the task phase of interest[3]. In the classic PPI, one models two conditions and an assumption is made that the two conditions are on the opposite side of baseline (if there is a baseline condition). gPPI allows one to accurately model all conditions and does not make any assumptions about how the conditions relate to the baseline condition. Another critical component of any PPI analysis is the appropriate selection of a seed region. Seed regions can be chosen based on prior evidence in the literature, such as in the present study in which the hippocampus was used as the seed region for a memory task. Another method of seed selection is to choose a region where activity significantly increases during a particular task phase. With this method, the seed region is defined not anatomically but using a group of suprathreshold voxels in univariate activation maps. With this approach to seed selection, PPI analyses avoid circularity because the main effect of the task is accounted for and the PPI only reveals effects that are distinct from (over and above) the main effect of the task.

Since PPI was first proposed, the concept of functionally connected, spatially distant brain regions has become broadly accepted. Through the use of resting state fMRI, it has been shown that the brain has intrinsic networks, or sets of regions that are functionally connected at rest. Thus, resting state fMRI studies often investigate functional connectivity, the same term used in PPI studies. The interpretation of functional connectivity, however, differs in resting state fMRI and PPI studies. PPI findings are, by definition, explanatory effects of an interaction between task and seed region that cannot be explained by the task design, the seed timecourse or any other confounding variable[4]. In resting state fMRI, differences in network activity might be caused by changes in connectivity between specific regions or by overall changes in network activity. Thus, if the goal of a study is to compare changes in functional connectivity between

two groups, a PPI approach is better. In contrast, if the goal of a study is to describe differences in intrinsic connectivity between two groups, resting state fMRI analyses are better.

One major limitation of the original PPI framework is the lack of statistical power inherent in the approach[4]. Because the PPI term (EV) is created using two EVs also included in the model, it is likely to be correlated to both. In a GLM, the variance that can be explained by more than one predictor or EV is not assigned to a single EV. Thus, the PPI term only has power to detect effects that cannot be explained by the task or the seed timecourse, which are both correlated to the PPI term. Because of this, it is likely that false negatives occur in PPI analyses. gPPI, however, has been shown to minimize the number of false negatives and is more sensitive to small effect size findings[3,39].

PPI can uncover task-dependent changes in functional connectivity between two regions, but it cannot determine whether activity in one region causes a change in activity in the other. In other words, a PPI analysis cannot be used to explore causality in functional connectivity changes. Other methods, such as dynamic causal modeling, are better suited for analyses of causality in functional data[40]. PPI analyses can inform the design of experiments using these techniques. In sum, PPI is a useful approach for examining task-specific changes in functional connectivity of a relevant seed region and comparing these changes between groups. Results from PPI studies can lead to a better understanding of the dynamic nature of functional connectivity in health, disease and risk for disease.

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DGM is an employee of Biospective, Inc. Biospective, Inc. did not process any of the data presented.

REFERENCES:
1. Sporns, O., Tononi, G. & Kötter, R. The Human Connectome: A Structural Description of the Human Brain. *PLoS Comput Biol*. **1** (4), e42, doi:10.1371/journal.pcbi.0010042 (2005).
2. Friston, K. J., Buechel, C., Fink, G. R., Morris, J., Rolls, E. & Dolan, R. J. Psychophysiological and modulatory interactions in neuroimaging. *NeuroImage*. **6** (3), 218–29, doi:10.1006/nimg.1997.0291 (1997).
3. McLaren, D. G., Ries, M. L., Xu, G. & Johnson, S. C. A generalized form of context-dependent psychophysiological interactions (gPPI): a comparison to standard approaches. *NeuroImage*. **61** (4), 1277–86, doi:10.1016/j.neuroimage.2012.03.068 (2012).
4. O'Reilly, J. X., Woolrich, M. W., Behrens, T. E. J., Smith, S. M. & Johansen-Berg, H. Tools of the trade: psychophysiological interactions and functional connectivity. *Soc Cogn Affect Neurosci*. **7** (5), 604–9, doi:10.1093/scan/nss055 (2012).

5. Moody, T. D., Sasaki, M. A., *et al.* Functional connectivity for face processing in individuals with body dysmorphic disorder and anorexia nervosa. *Psychol Med*. **45** (16), 3491–503, doi:10.1017/S0033291715001397 (2015).
6. Simard, I., Luck, D., Mottron, L., Zeffiro, T. A. & Soulières, I. Autistic fluid intelligence: Increased reliance on visual functional connectivity with diminished modulation of coupling by task difficulty. *NeuroImage Clin*. **9**, 467–478, doi:10.1016/j.nicl.2015.09.007 (2015).
7. Yan, L.-R., Wu, Y.-B., Zeng, X.-H. & Gao, L.-C. Dysfunctional putamen modulation during bimanual finger-to-thumb movement in patients with Parkinson's disease. *Front Hum Neurosci*. **9**, 516, doi:10.3389/fnhum.2015.00516 (2015).
8. Cole, M. W., Reynolds, J. R., Power, J. D., Repovs, G., Anticevic, A. & Braver, T. S. Multi-task connectivity reveals flexible hubs for adaptive task control. *Nat Neurosci*. **16** (9), 1348–1355, doi:10.1038/nn.3470 (2013).
9. McLaren, D. G., Sperling, R. A. & Atri, A. Flexible modulation of network connectivity related to cognition in Alzheimer's disease. *NeuroImage*. **100C**, 544–557, doi:10.1016/j.neuroimage.2014.05.032 (2014).
10. Morawetz, C., Bode, S., Baudewig, J. & Heekeren, H. R. Effective amygdala-prefrontal connectivity predicts individual differences in successful emotion regulation. *Soc Cogn Affect Neurosci*. nsw169, doi:10.1093/scan/nsw169 (2016).
11. Takashima, A., Bakker, I., van Hell, J. G., Janzen, G. & McQueen, J. M. Richness of information about novel words influences how episodic and semantic memory networks interact during lexicalization. *NeuroImage*. **84**, 265–278, doi:10.1016/j.neuroimage.2013.08.023 (2014).
12. Harrison, T. M., Burggren, A. C., Small, G. W. & Bookheimer, S. Y. Altered memory-related functional connectivity of the anterior and posterior hippocampus in older adults at increased genetic risk for Alzheimer's disease. *Hum Brain Mapp*. **37** (1), 366–80, doi:10.1002/hbm.23036 (2016).
13. Wechsler, D. *Wecshler Adult Intelligence Scale, 3rd Edition*. Harcourt Assessement. San Antonio, TX. (1997).
14. Cauthen, N. R. Verbal fluency: normative data. *J Clin Psychol***. 34** (1), 126–9 (1978).
15. Goodglass, H. P. & Kaplan, E. P. Boston Naming Test, 3rd Edition. (2001).
16. Buschke, H. & Fuld, P. A. Evaluating storage, retention, and retrieval in disordered memory and learning. *Neurol*. **24** (11), 1019–25 (1974).
17. Osterrieth, P. A. Le test de copie d'une figure complex: Contribution à l'étude de la perception et de la memoir. *Archives de Psychologie*. **30**, 286–356 (1944).
18. Hamilton, M. The assessment of anxiey states by rating. *Br J Med Psychol*. **32** (August), 50–55, doi:10.1111/j.2044-8341.1959.tb00467.x (1959).
19. Hamilton, M. A rating scale for depression. *J Neurol Neurosurg Psychiatry*. **23**, 56–62, doi:10.1136/jnnp.23.1.56 (1960).
20. Folstein, M. F., Robins, L. N. & Helzer, J. E. The Mini-Mental State Examination. *Arch Gen Psychiatry*. **40** (7), 812 (1983).
21. O'Brien, D., Campbell, K. A., Morken, N. W., Bair, R. J. & Heath, E. M. Automated Nucleic Acid Purification for Large Samples. *J Lab Autom*. **6** (2), 67–70, doi:10.1016/S1535-5535(04)00131-5 (2001).
22. Lehmann, M., Ghosh, P. M., *et al.* Greater medial temporal hypometabolism and lower

cortical amyloid burden in ApoE4-positive AD patients. *J Neurol Neurosurg Psychiatry*. **85** (3), 266–73, doi:10.1136/jnnp-2013-305858 (2014).
23. TaqMan® SNP Genotyping Assays User Guide. Thermo Fisher Scientific. at <https://tools.thermofisher.com/content/sfs/manuals/TaqMan_SNP_Genotyping_Assays_man.pdf> (2014).
24. Bookheimer, S. Y., Strojwas, M. H., *et al.* Patterns of brain activation in people at risk for Alzheimer's disease. *N Engl J Med*. **343** (7), 450–6, doi:10.1056/NEJM200008173430701 (2000).
25. Jenkinson, M., Bannister, P., Brady, M. & Smith, S. Improved optimization for the robust and accurate linear registration and motion correction of brain images. *NeuroImage*. **17** (2), 825–41 (2002).
26. Smith, S. M. Fast robust automated brain extraction. *Hum Brain Mapp*. **17** (3), 143–55, doi:10.1002/hbm.10062 (2002).
27. Greve, D. N. & Fischl, B. Accurate and robust brain image alignment using boundary-based registration. *NeuroImage*. **48** (1), 63–72, doi:10.1016/j.neuroimage.2009.06.060 (2009).
28. Patenaude, B., Smith, S. M., Kennedy, D. N. & Jenkinson, M. A Bayesian model of shape and appearance for subcortical brain segmentation. *NeuroImage*. **56** (3), 907–22, doi:10.1016/j.neuroimage.2011.02.046 (2011).
29. Learn MATLAB Basics. at <https://www.mathworks.com/support/learn-with-matlab-tutorials.html?s_tid=hp_ff_l_tutorials>
30. Lerma-Usabiaga, G., Iglesias, J. E., Insausti, R., Greve, D. N. & Paz-Alonso, P. M. Automated segmentation of the human hippocampus along its longitudinal axis. *Hum Brain Mapp*. **37** (9), 3353–3367, doi:10.1002/hbm.23245 (2016).
31. Salami, A., Eriksson, J. & Nyberg, L. Opposing effects of aging on large-scale brain systems for memory encoding and cognitive control. *J Neurosci*. **32** (31), 10749–57, doi:10.1523/JNEUROSCI.0278-12.2012 (2012).
32. Schacter, D. L. & Wagner, A. D. Medial temporal lobe activations in fMRI and PET studies of episodic encoding and retrieval. *Hippocampus*. **9** (1), 7–24, doi:10.1002/(SICI)1098-1063(1999)9:1<7::AID-HIPO2>3.0.CO,2-K (1999).
33. Strange, B. & Dolan, R. Functional segregation within the human hippocampus. *Mol Psychiatry*. **4** (6), 508–11 (1999).
34. Strange, B. A., Fletcher, P. C., Henson, R. N., Friston, K. J. & Dolan, R. J. Segregating the functions of human hippocampus. *Proc Natl Acad Sci U S A*. **96** (7), 4034–9 (1999).
35. Eldridge, L. L., Engel, S. A., Zeineh, M. M., Bookheimer, S. Y. & Knowlton, B. J. A dissociation of encoding and retrieval processes in the human hippocampus. *J Neurosci*. **25** (13), 3280–6, doi:10.1523/JNEUROSCI.3420-04.2005 (2005).
36. Strange, B. A., Witter, M. P., Lein, E. S. & Moser, E. I. Functional organization of the hippocampal longitudinal axis. *Nat Rev Neurosci*. **15** (10), 655–669, doi:10.1038/nrn3785 (2014).
37. Zeineh, M. M., Engel, S. A., Thompson, P. M. & Bookheimer, S. Y. Dynamics of the hippocampus during encoding and retrieval of face-name pairs. *Science*. **299** (5606), 577–80, doi:10.1126/science.1077775 (2003).
38. Nieuwenhuis, S., Forstmann, B. U. & Wagenmakers, E.-J. Erroneous analyses of interactions in neuroscience: a problem of significance. *Nat Neurosci*. **14** (9), 1105–1107, doi:10.1038/nn.2886 (2011).

39. Cisler, J. M., Bush, K. & Steele, J. S. A comparison of statistical methods for detecting context-modulated functional connectivity in fMRI. *NeuroImage*. **84**, 1042–52, doi:10.1016/j.neuroimage.2013.09.018 (2014).
40. Friston, K. J., Harrison, L. & Penny, W. Dynamic causal modelling. *NeuroImage*. **19** (4), 1273–1302, doi:10.1016/S1053-8119(03)00202-7 (2003).

Figure 1

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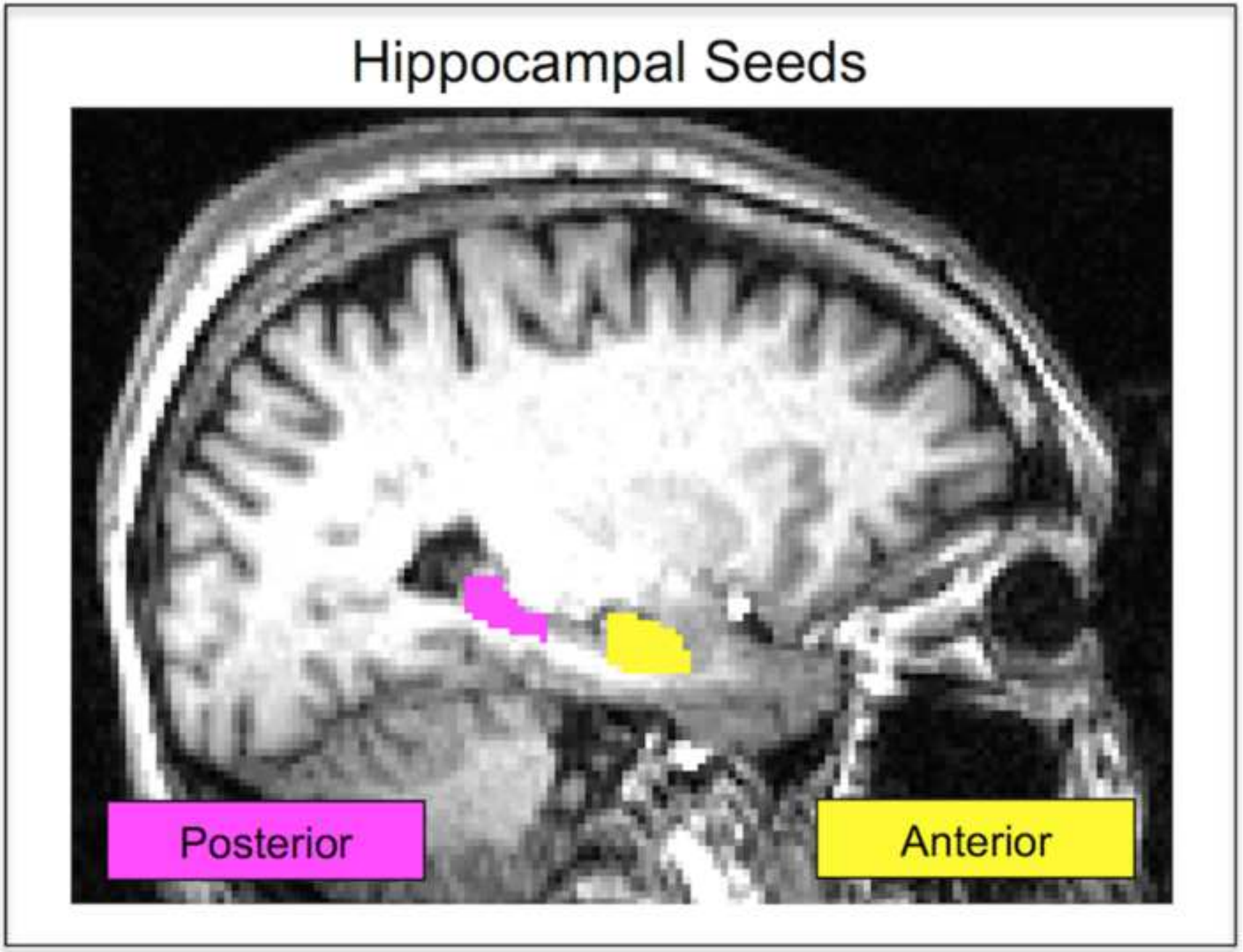

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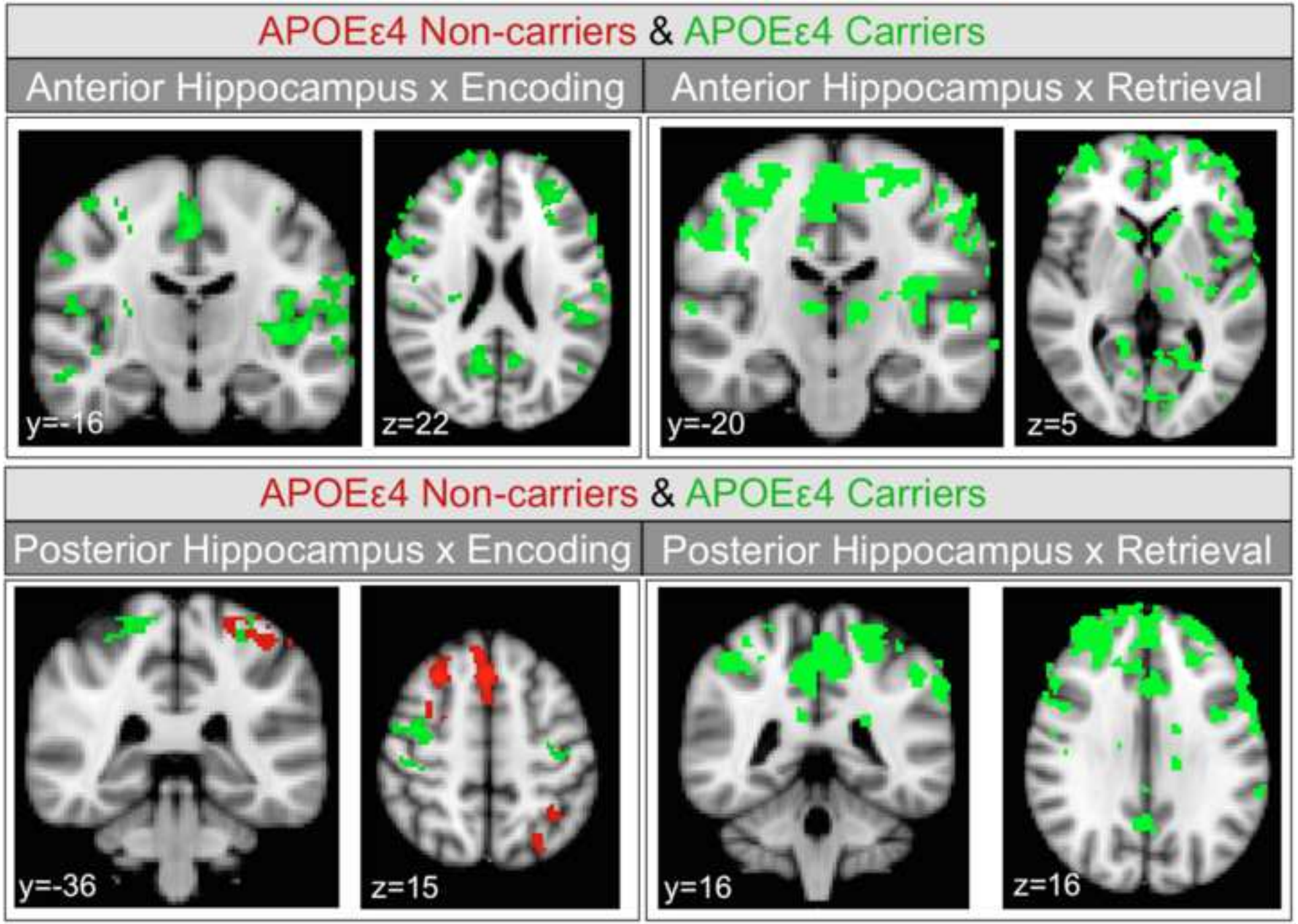

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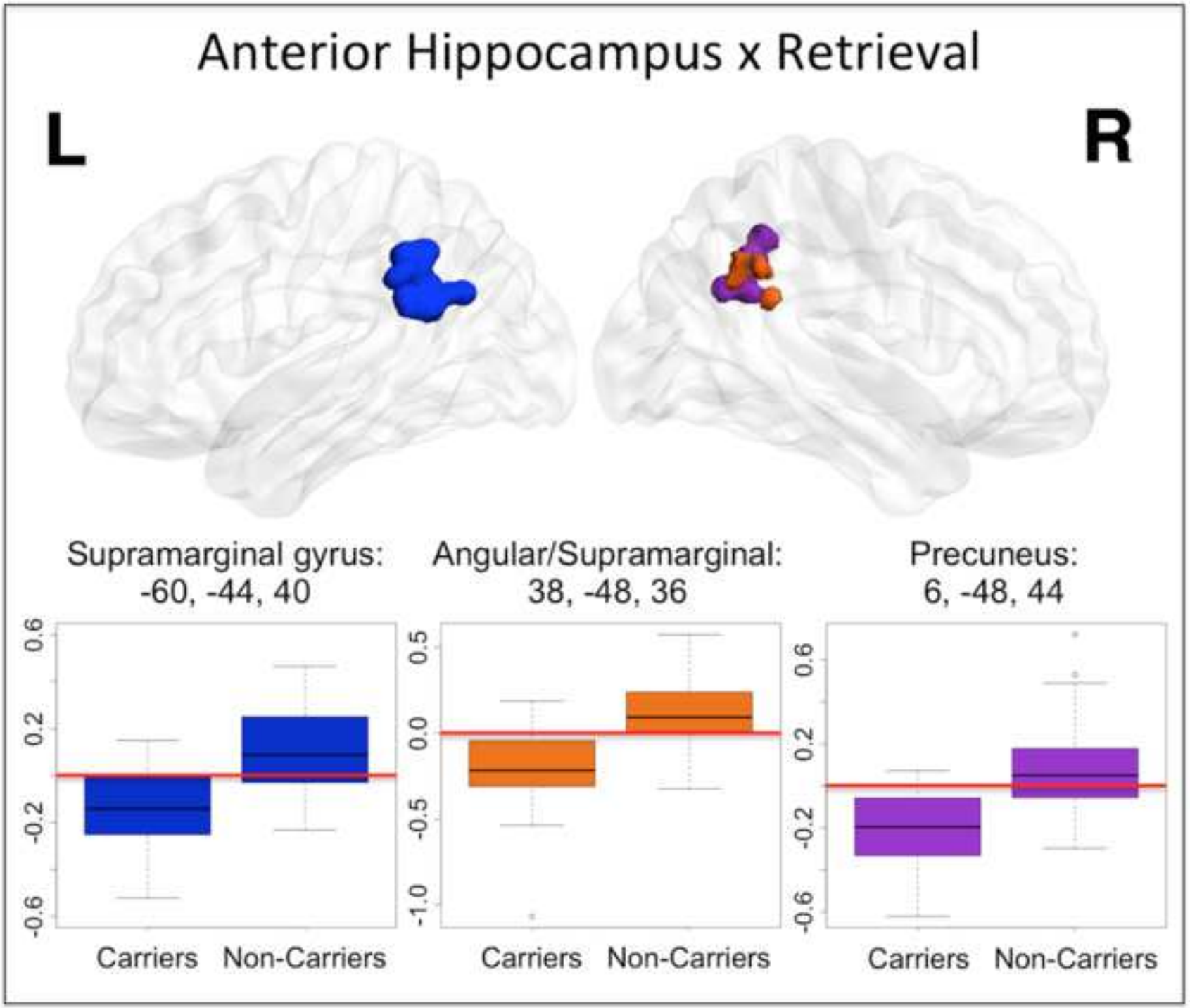

Table 1: gPPI Model Set-Up

Explanatory Variable (EV)	EV Details
Psych- : Task Condition 1	Text file denoting task timing for condition 1 (e.g., encoding)
Psych- : Task Condition 2	Text file denoting task timing for condition 2 (e.g., retrieval)
Phsyio- : Seed Timecourse	Average BOLD signal extracted from seed region using FSL tool fslmeants after preprocessing
Psychophysiological Interaction: Task Condition 1 x Seed	Created in FSL FEAT by choosing "interaction" in EV basic shape menu: choose seed timecourse EV and task condition 1 EV.
Psychophysiological Interaction: Task Condition 2 x Seed	Created in FSL FEAT by choosing "interaction" in EV basic shape menu: choose seed timecourse EV and task condition 2 EV.
Motion Regressors (6 total)	Output from MCFLIRT split into 6 single-column text files. The six columns correspond to 3 directions of translation and 3 axes of rotation and capture the motion correction performed at each volume.
Motion Outliers (# varies)	FSL tool Motion Outliers generates a confound matrix denoting volumes with excessive motion. Rather than loading each column of this matrix as its own EV, add the whole matrix to the GLM by selecting "add additional confound EVs" in the stats tab of the FSL FEAT GUI.

Name of Reagent/ Equipment	Company
3T manetic resonance imaging scanner	Siemens Medical Solutions
FSL (FMRIB Software Library)	Oxford University
AFNI (Analysis of Functional Neuroimaging)	National Institute of Mental Health, National Institutes of Health
SPM8 (Statistical Parametric Mapping)	University College of London
Matlab Software	The Mathworks, Inc
SDS Software	Applied Biosystems, Inc
Taqman Assays	ThermoFisher Scientific

Catalog Number	Comments/Description
MAGNETOM Trio, A Tim System	3T MRI Scanner
Version 6.0	Functional Imaging Processing Software
Any version after May 2015	Functional Imaging Processing Software
SPM8	Functional Imaging Processing Software
Version R2012a	Computing Software
7900HT Fast Real-Time PCR System	Real Time PCR
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Signature: Theresa M Harrison Date: 08/21/2016

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February 15, 2017

Re: Resubmission of 55394R1

This is a resubmission of our manuscript entitled “Generalized Psychophysiological Interaction (PPI) Analysis of Memory Related Connectivity in Individuals at Genetic Risk for Alzheimer's Disease”. After peer review, the reviewers agree that the steps required to perform a PPI analysis with functional MRI data are well-described and the study is methodologically and scientifically sound. Their main concerns were focused on the addition of specific details, clarification and adjustments to the way the findings are presented and suggestions to improve clarity in the step-by-step protocol. In the text that follows we address each comment from the reviewers. We thank the reviewers for their time and effort and believe that their comments and our subsequent revisions have results in an improved paper. We have also responded to all Editorial Comments that were not already directly addressed during the initial Editorial Review process.

Reviewer #1:

Provide further details of the memory task.

We added a note to the protocol briefly describing the task and included citations of published articles in which the task is described in detail.

Provide information on the mean age, the gender distribution, and the MRI machine used.

We added the mean age and the sex distribution for the participants in our study to step 1.3. We were instructed to remove the specific MRI scanner information from the manuscript during the Editorial Review process that precedes peer review. Instead, following JoVE guidelines, this information is available alongside the article in a special “Materials” table that is submitted with the manuscript. In this table we included the following information about the scanner: Siemens MAGNETOM Trio, A Tim System. We added a note to the main text referring to the Materials list after the general description of the scanner.

Mention, before the point number 5, that the data is preprocessed at that point.

We thank the reviewer for this suggestion. We added a note at the end of section 4 of the protocol to ensure that this is clear.

Add additional notes; in 6.2.2, clarify what "make zero" and "centre" option means; justify the choice of the BBR registration; explain the use of (an updated) version of AlphaSim.

We followed this reviewer’s suggestions and added notes addressing each of these points to the manuscript.

Report the 3dFWHMx version that should be used.

A bug in 3dClustSim was identified and fixed in the AFNI software code in May 2015. In December 2015, AFNI released an updated version of 3dFWHMx to address the issues that Eklund et al. (2016). Since these tools are used together, we have updated the manuscript to reflect that versions of both tools after released in December 2015 or later are required for valid analyses.

Add a schematical figure of the PPI model (i.e. with the different psy* psycho* and PP* conditions).

We thank the reviewer for this suggestion and agree that a visual summary of the PPI model was missing from the manuscript. After several attempts we decided that this summary was best provided as a table with room for the title, details and notes for each EV to be provided. We have added Table 1: gPPI Model Set Up to this revision.

Specify the direction of the group-comparison analysis in the last paragraph of the representative results section.
We thank the reviewer for pointing out this omission. We have added a note that the representative results in Figure 3 arose from a non-carriers > carriers contrast.

Can the authors discuss the use of the PPI approach in a data-driven analysis as opposed to a hypothesis driven approach?
PPI seeds can be identified in a number of different ways. PPI is still hypothesis driven as it tests whether the connectivity with a region differs between conditions. Whether the seed is defined anatomically, based on the prior literature, or based on the task activity maps is independent. In the case of choosing the seed based on task activity, we are assessing whether the connectivity with regions that are activated or differentially activated by conditions or groups also has altered connectivity.

Further discuss the correct convolution/deconvolution settings of the different regressors.
We have added notes to the manuscript expanding on the convolution settings. Specifically, convolving the task EV by the HRF shifts the timing of the task EV to be more consistent with expected task-induced BOLD signal changes in the brain. Motion parameters do not need to be convolved by any function because they reference the realignment done at each functional volume during motion correction and thus don't need to be adjusted by the HRF.

Provide some rule-of-thumb limits of what can and cannot be done with PPI (i.e. subjects or signal change needed to detect changes) in terms of power limitations.
We have not performed rigorous, iterative studies to determine rules of thumb regarding PPI analysis and statistical power. This type of analysis would be interesting to the community, but it is outside the scope of this manuscript. For a thorough discussion on power in PPI please see O'Reilly et al. (2012), which is cited in the present manuscript in the text discussing power limitations. For additional discussions on the differences in gPPI and PPI please see McLaren et al. (2012) and Cisler et al. (2013).

Comment on the TE and why it was chosen.
The functional scan used in the present study was designed by Dr. Mark Cohen, an experienced neuroimager and engineer at UCLA. The low TE was in part the result of optimizing against signal dropout in the medial temporal lobes, an area of great interest in this population.

Can smoothing be problematic when carried before the time series extraction? For example, the groups used (APOE carriers/non-carriers) might differ in hippocampal volume thus differentially affecting the amount of white-matter/CSF signal introduced in the voxels of interest?
To ensure that group differences in white matter and CSF signal introduced to the hippocampal seeds timeseries were not driving our results, we compared hippocampal seed volumes between APOEε4 carriers and non-carriers. There were no differences between groups in anterior ($p=0.96$) or posterior ($p=0.86$) seeds volumes.

In regards to the hippocampal segmentation, Lerma-Usabiaga HBM et al., 2016 might be considered a useful alternative to writing Matlab code.
We thank the reviewer for alerting us to this study. We have cited it in the manuscript as a potential alternative approach to writing custom code.

Expand the section on the relevance (and the differences) of gPPI analysis vs. classic PPI.
PPI analyses aim to investigate how different conditions or contexts alter the connectivity in the brain.

Based on the work to date, there is little evidence to support the classic PPI analysis over a gPPI analysis. As shown in McLaren et al. 2012, there is no disadvantage to gPPI, but there is a high risk of false negative and false positives in the classic PPI. The difference in the two approaches is the ability to model the data. In the classic PPI, you are only modeling 2 of the conditions and are assuming that the two conditions are on the opposite side of baseline (if you have a baseline condition). gPPI allows you to accurately model all conditions and does not make any assumptions about how the conditions relate to the baseline condition. We have added a couple sentences to the discussion expanding on this topic.

Reviewer #2:

Use right hemisphere anterior and posterior hippocampus as seeds as well as left hemisphere.

We thank the reviewer for this suggestion and agree that it is not necessarily true that the right hippocampus is not involved in a language-based memory task. The scope of this manuscript, however, allows for only the presentation of a few "representative" results. We have already had to select carefully what we will include. Adding additional seed regions, while we agree would not be too complicated for an original research article, is not in keeping with JoVE guidelines and we would not be able to present those results. To this reviewer's point, we added a note in the protocol indicating that right anterior and posterior hippocampus would be interesting seed regions for further analysis.

Add a better description of the rationale behind the selection of the seed(s).

We added a rationale for examining the anterior and posterior hippocampus separately as well as supporting citations. There is evidence of functional specialization along the longitudinal axis of the hippocampus. Anterior regions are input regions and associated with encoding, while posterior hippocampus is an output region associated with memory retrieval and consolidation. Thus, using these regions allows assessment of functional involvement of anterior versus posterior hippocampus in encoding vs retrieval phases of the memory task. Given the focus of this manuscript on implementation of the PPI, we have kept the background and discussion of hippocampus and memory brief.

Justify the use of different software packages for different steps in the analysis.

We believe that the best available tools should be used at every step of analysis and consideration of software package should be limited only to concerns of compatibility. The benefits of using a permutation-based cluster extent thresholding method include allowing for hypotheses about extent as well as height to be considered. In FSL, the threshold is set without considering data smoothness and cluster size, and therefore users often apply the default settings without reference to their specific data. 3dClustSim is related to the family-wise error method standard in the FSL software in that it takes into account the spatial correlation in the data, unlike the false discovery rate. This approach has been used in many published studies, including those that proposed and implemented the gPPI approach (McLaren et al., 2012; McLaren et al., 2014). We have not examined the results of our PPI analyses using the FSL standard thresholding approach. This analysis would be exploratory as it was not part of our original data processing stream.

Figure 2 is difficult to follow and and does not show the main finding. Report these data in a Table.

JoVE requests that the figures represent possible outcomes from the protocol so that readers should know what to expect and look for in their own results. We have chosen to include the within-group results in Figure 2 even though they are busy for this reason, so that readers can see what they may expect to see at this stage in their own data. Reporting these clusters in a table would be less helpful in familiarizing readers with how voxelwise PPI results might look. We feel it is especially important to keep Figure 2 in its current form since we show the between group results in Figure 3 in a so-called

‘glass brain’.

Report or refer to published regional effects (i.e. not the connectivity) within the manuscript. It usually helps reporting them as they might help the interpretation of the data.
We have added a brief description of these maps in *Representative Results* and a reference to where more information can be found.

Please report more details about the MRI scanner in the methods section.
We were instructed to remove this information from the manuscript during the Editorial Review process that precedes peer review. Instead, following JoVE guidelines, this information is available alongside the article in a special “Materials” table that is submitted with the manuscript. In this table we included the following information about the scanner: Siemens MAGNETOM Trio, A Tim System. We added a note to the main text referring to the Materials list after the general description of the scanner.

Add a brief discussion of the results.
JoVE manuscript preparation guidelines indicate that the Discussion should be focused on the method and protocol, which is why we did not include a thorough discussion of the results. However, if the reviewer is interested, a thorough discussion of these results can be found in the original article in which they first appeared: Harrison et al. (2016) Human Brain Mapping 37:366-380

Add to the *Introduction* by including studies that have reported PPI connectivity effects related to individual differences in memory and emotional functions.
We have added citations to studies of memory and emotion regulation to the *Introduction* for more thorough coverage of published applications of the PPI approach.

Editorial comments:
Editorial comments are italicized in bullet points below. Our responses follow each point.

• *Please note that protocol sections 1 (participant screening process) and 5 (algorithms/coding) cannot be filmed. If sections 2 is to be filmed, please provide step wise detail on how to draw blood, isolate genomic DNA, perform SNP, etc. Please note that steps that refer to manufacturer's protocol or cite a reference on how to perform a particular method cannot be filmed.*

We note that sections 1, 2 and 5 will not be filmed.

• *Steps 3.1.1.1-3.1.1.4: Please add step wise detail on what is clicked on in the MRI software to set the parameters and acquire functional images.*

Every scanner system is different and a different order of operations would be used to achieve the same result. Also, a sequence must be designed and loaded onto a scanner before it can be run on individual subjects. This is a separate process that is outside the scope of this manuscript.

• *Step 3.1.12: How are the unrelated words, associative memory task triggered? Please mention how to exclude the first to volumes.*

MacStim is the specific program we used to administer the task. This program allows the user to trigger the task to the scanner and start on a specific TR. Since we are not able to mention specific commercial tools and software we did not include this information in the manuscript.

• *4.1: Please add details on how to preprocess the functional data. What is done here?*

This is covered under this point in steps 4.1.#. We have a note to the manuscript indicating this.

• *4.1.1: Please add details on how to remove the head motion artifact from the data. What is clicked on in the MCFLIRT tool to achieve this.*

MCFLIRT, like many image processing tools, is a command line program. Therefore, nothing is "clicked on" but rather the user types the command into the command line based on the instructions associated with the tool. It is not practical to include verbatim commands in JoVE articles, but we can film the user running the program from the command line as easily as we can film the user clicking on a button in a GUI.

• *4.1.2: Please add details on how to remove non-brain tissue from the images using BET. What is clicked on in the software to achieve this.*

As with MCFLIRT above, BET is a command line tool. The user types the command into the terminal window according to the instructions provided with the program.

• *4.1.3: Please add details on how to use the FSL motion outliers tool to identify volumes in the functional data. How are the volumes flagged? How is the output of the program used to downweight the volumes?*

FSL Motion Outliers is a command line tool. The output of this tool is a file a confound file (which the user is required to name when submitting the command so it does not have a standardized name). This file is a matrix with 1s indicating volumes that need to be downweighted. This matrix is added using the FSL FEAT GUI during processing (see step 4.2.4).

• *4.2: Please add details on how to set up the preprocessing and GLM using GUI. What is clicked on in the software to set up the model? Please add details on what is repeated. Please provide a reference for writing a script to run the preprocessing.*

We have added more details to Step 4.2. The details of whatt is clicked on is detailed in the substeps of this step (4.2.#).

• *4.2.1: How are the motion-corrected and brain-extracted files selected as inputs?*

This is one data file, specifically the imaging data file that has already been processed through the steps preceding this one. To select it as an input the user will select it from its location on their computer or server using a typical user interface for file navigation on a computer. To launch that window, click on "Add 4D data". We have added this step to the manuscript.

• *4.2.3.1: How are the 6 motion parameters added? Manually? Please mention the 6 motion parameters used in your studies.*

The motion parameters are added by selecting the files from their location on the user's computer or server using a typical user interface for file navigation on a computer. They are text files. They are mentioned in Step 4.2.3. The output of MCFLIRT, the motion correction program, is a file with 6 columns and a row for each TR in the image (named by the user). The motion regressors are each one column of this output. We added some text to Step 4.2.3 to help make this more clear.

• *4.2.5: Please add details on how to create the task timing text files? What is clicked on in the software? How are these files added as EVs in GLM? Please add details on how to include one for the encoding and retrieval phase. What is done here on the software? Where can one find the double-gamma HRF option? Data tab/stats tab/pre-stats tab?*

The task timing files are created using a text editor based on the design of the task. They are saved as text files. They are added as EVs in the GLM in the Stats tab under "Full Model Set Up". This is also where the convolution options can be found.

• *4.2.6.1/4.2.6.3: Please add details on what is clicked on in the software to choose the 6/12 degrees of freedom and linear transformation.*

In the registration tab there is a drop down box where "Full Search" should be left as the default. A second drop down box where the user chooses the 6/12 DOF or BBR options. We have added this to the protocol.

• *5.3: This step can be filmed only if performed using GUI. Please add details on how to create the anterior and posterior mask images if using GUI. Please add details on how to register. Alternatively, please remove the highlight and provide a reference.*

We have removed the highlighting and provided useful references in the Note following this step.

• *5.4: This step cannot be filmed if it is performed using codes or algorithms.*

We have removed the highlighting from this section and others that involve command line processes.

• *6.1.1: What is selected to exclude temporal filtering or spatial smoothing?*

The user should unclick the boxes for these options in FSL FEAT. We have added text to this step to make it more clear.

• *6.2.1: Please add details on how to include an EV for physiological timecourse from the seed? Is the text file selected from a file location?*

This file is created by the user in Step 5.4. It will be named whatever the user names the output when running fslmeants and it will be saved where the user specifies on their computer or server. The user should save it to a location where they can access it while running FSL FEAT.

• *6.2.2: Please mention the step numbers that describe the seed time course and one "task EV". Please mention how to run separate models for each seed region.*

The seed timecourse is extracted in Step 5.4. The task EVs are created in Step 4.2.5. Multiple models are run by performing the steps in the manuscript and then repeating them with another seed region (since only one is included in a PPI model) if needed.

• *7.2: What is clicked on in the AFNI software to set the voxel-level threshold?*

In Step 7.3.3, a box appears asking for the voxel-level threshold and the user types in the desired thresholding level. Step 7.2 and substeps refer to the process/programs used to determine the thresholds to use. We have changed the wording of this step to reflect that.

• *7.2.1 an 7.2.2: These steps cannot be filmed if performed using commands/coding. Please remove the highlighting.*

We have removed the highlighting from these sections and others that involve command line processes (MCFLIRT, BET, fslmeants etc). Much of the processes are completed at the command line and it would be better if the video could include these steps (e.g., how to access the "help" or instructions for each command and then type and run the correct command).

• *7.3.1: Please add details on how to add the parameter estimate images. How are the images chosen? What is clicked on in the SPM8 software to run the comparison?*

We have added more detail to Step 7.3.1 to make these points more clear.

• *7.3.2: Where does the "estimate" option appear? Does is appear after the comparison is completed? What is clicked on in the SPM8 software to run the estimation process?*

Estimate is a button in the SPM8 GUI. It is always there but can only be used once an SPM.mat file has been created in Step 7.3.1

• *7.3.3: What is clicked on in the SPM8 software to run the group comparisons? Is the voxel-level threshold set manually?*

We have updated 7.3.3 with more detail of how to run the group comparisons.

• *After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3-page limit for filmable content. Please highlight (in yellow) 2.75 pages or less of text (which includes*

headings and spaces) to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

We have followed these instructions.

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