Multi-day rTMS exerts site-specific effects on functional connectivity but does not influence associative memory performance

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Abstract

Transcranial magnetic stimulation (TMS) is a non-invasive brain stimulation technique with the capacity to modulate brain network connectivity and cognitive function. Recent studies have demonstrated long-lasting improvements in associative memory and resting-state connectivity following multi-day repetitive TMS (rTMS) to individualised parietal-hippocampal networks. We aimed to assess the reproducibility and network- and cognitive-specificity of these effects following multi-day rTMS. Participants received four days of 20 Hz rTMS to a subject-specific region of left lateral parietal cortex exhibiting peak functional connectivity to the left hippocampus. In a separate week, the same stimulation protocol was applied to a subject-specific region of pre-supplementary motor area (pre-SMA) exhibiting peak functional connectivity to the left putamen. We assessed changes to associative memory before and after each week of stimulation (N = 39), and changes to resting-state functional connectivity before and after stimulation in week one (N = 36). We found no evidence of long-lasting enhancement of associative memory or increased parieto-hippocampal connectivity following multi-day rTMS to the parietal cortex, nor increased pre-SMA-putamen connectivity following multi-day rTMS to pre-SMA. Instead, we observed some evidence of site-specific modulations of functional connectivity lasting ~24 hours, with reduced connectivity within targeted networks and increased connectivity across distinct non-targeted networks. Our findings suggest a complex interplay between multi-day rTMS and network connectivity. Further work is required to develop reliable rTMS paradigms for driving changes in functional connectivity between cortical and subcortical regions.

Keywords

rTMS, hippocampus, functional connectivity, memory, neuroplasticity

1. **Introduction**

Transcranial magnetic stimulation (TMS) is a non-invasive brain stimulation technique with the capacity to modulate brain network connectivity and cognitive function (Fox, Halko, Eldaief, & Pascual-Leone, 2012). A single session of repetitive TMS (rTMS) can induce short-term changes in cortical excitability (Matsunaga et al., 2005) and memory function (Luber et al., 2007) typically lasting less than an hour. Interestingly, recent studies have shown that multiple doses of rTMS delivered over consecutive days may confer longer-lasting (>24 hrs) cumulative effects on brain function and cognitive performance in healthy individuals (Wang et al., 2014; Wang & Voss, 2015). For example, Wang et al. (2014) reported sustained enhancement of associative memory in a sample of 16 healthy individuals (~24 hours) following five sessions of rTMS applied to subject-specific regions of lateral parietal cortex displaying intrinsic functional connectivity (FC) to left hippocampus. Improvements in memory performance were associated with highly specific increases in FC across the targeted cortico-hippocampal network. These findings suggest that multi-day rTMS may be an effective method of modulating distributed networks critical to learning and memory and commonly implicated in psychiatric (Heckers, 2001; MacQueen et al., 2003) and degenerative disease (Fox et al., 1996). Indeed, such multi-day approaches are closer in design to treatment protocols used in psychiatric disorders such as depression (George & Short, 2014) and therefore may inform future therapeutic applications of stimulation for memory disorders.

The ability to induce long-lasting memory improvement with multi-day stimulation is a prime example of the exciting potential of rTMS. However, the physiological response to non-invasive brain stimulation methods is known to be highly variable between individuals (López-Alonso, Cheeran, Río-Rodríguez, & Fernández-Del-Olmo, 2014), and experimental findings have often proven difficult to replicate (Héroux, Taylor, & Gandevia, 2015). Initial demonstrations of large effects with moderate sample sizes are also prone to effect size overestimation (i.e. ‘The Winner’s Curse’, see Button et al., 2013). Encouragingly, the

declarative memory and FC effects initially reported by Wang et al. (2014) have recently been replicated in similar moderate sample sizes (N = 15 - 16) (Freedberg et al., 2019b; Hermiller et al., 2019). In light of these findings, investigating the reproducibility of these effects in a larger sample remains a crucial step in achieving reliable effect size estimates, and assessing corresponding therapeutic potential.

The capacity of rTMS to modulate the activity of targeted brain networks is central to cognitive and clinical applications of stimulation (Shafi, Westover, Fox, & Pascual-Leone, 2012). Whilst the direct effects of TMS are confined to a small area of cortex adjacent to the TMS coil position, indirect effects on FC may be induced across distributed regions on the basis of shared intrinsic FC (Fox et al., 2012). However, recent evidence suggests that the effects of rTMS may vary substantially across different brain regions (Cocchi et al., 2016; Castrillon et al., 2020). Castrillon and colleagues (2020) demonstrated opposing effects of 1 Hz stimulation between sensory and cognitive networks. Stimulation of a frontal cognitive network resulted in widespread decreases in FC, while stimulation of an occipital sensory network produced a paradoxical increase in FC. Further, applying continuous theta burst stimulation to frontal and occipital brain regions also induced opposing effects on FC (Cocchi et al., 2016). Considering that rTMS is applied to different brain networks to treat different psychopathologies (i.e. dorsolateral prefrontal cortex in depression versus orbitofrontal cortex in obsessive-compulsive disorder), investigating the site-specificity of rTMS-induced effects may be an important step in improving treatment efficacy.

The aims of this study were twofold. The first aim was to investigate the reproducibility of long-lasting improvements in associative memory and resting-state connectivity following multi-day 20 Hz rTMS to individualised parietal-hippocampal networks. The second aim was to investigate the site-specificity of changes to FC and associative memory following multi-day rTMS by also targeting a spatially distinct network. We utilised a multi-day 20 Hz rTMS protocol similar to Wang et al. (2014), adopting the same resting-state fMRI target

localisation approach and outcome measures (i.e. change in functional connectivity and the face-cued word recall task). Further, we extended the approach of Wang et al. (2014) by using a comparison site, instead of sham rTMS. To do this we applied multi-day 20 Hz rTMS to the pre-supplementary motor area (pre-SMA) to modulate a pre-SMA-putamen network. We chose pre-SMA as a comparison site as it corresponds to a distinct brain network supporting other facets of memory function, namely procedural memory (Doyon et al., 2009).

We expected improvements in associative memory performance, as indexed by the percentage of correctly recalled items on face-cued word recall task, following multi-day parietal stimulation. Further, following multi-day parietal stimulation, we expected an increase in FC across the targeted cortico-hippocampal network consistent with the findings of Wang et al. (2014). We also hypothesised a significant positive correlation between associative memory and FC changes following parietal stimulation. In contrast, following multi-day pre-SMA- stimulation, we expected an increase in FC across the targeted pre-SMA-putamen network with no corresponding improvement in associative memory.

2. **Methods**

2.1 Ethics approval

The study was approved by the Monash University Human Research Ethics Committee and all participants provided informed consent prior to their participation. The study conformed to the standards set by the *Declaration of Helsinki* and subjects were remunerated for their participation.

2.2 Participants

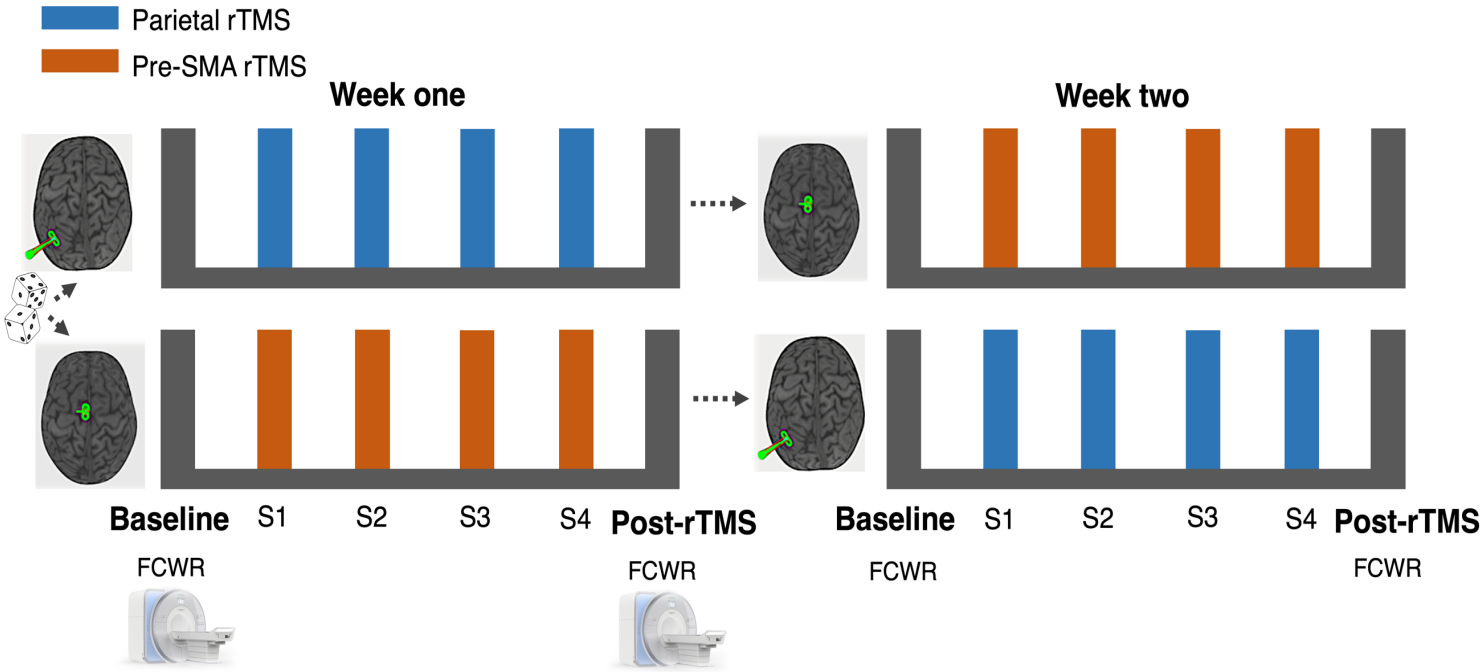
The sample size was determined from the results of a previous study demonstrating long-lasting changes to cortico-hippocampal functional connectivity and memory performance following multi-day rTMS to left parietal cortex (Wang et al., 2014). The effect size reported by Wang et al. (2014) for memory improvement (Cohen's d = 0.75; derived from differences

in raw scores between stimulation and sham conditions), suggests a minimum sample size of 21 for a desired power of 90% at α = .05. Given the tendency for effect sizes to be inflated in the first instance (Button et al., 2013), and the inclusion of an active control condition, we recruited a sample of 40 right-handed healthy adults (52.5% female), aged 25.48 ± 9.35 years (mean ± SD; range 18 – 55). This sample size affords 90% power to detect a moderate effect (η^2_p = 0.06). Participants reported no contraindications to magnetic resonance imaging or transcranial magnetic stimulation and reported no history of psychological or neurological disorders.

2.3 Experimental design

A within-subject cross-over design was used to investigate the effects of multi-day rTMS on associative memory function. Each participant completed two rTMS conditions over the course of two separate weeks; an experimental condition targeting left lateral parietal cortex and another targeting pre-SMA as a comparison site. The stimulation site order was counterbalanced across participants. For comparisons of resting-state network connectivity, MRI scanning was conducted for the first stimulation condition only. Therefore, changes in network connectivity following stimulation to different sites were compared using a between-subjects design (see Figure 1 for overview of experimental design).

Panel 1



Panel 2

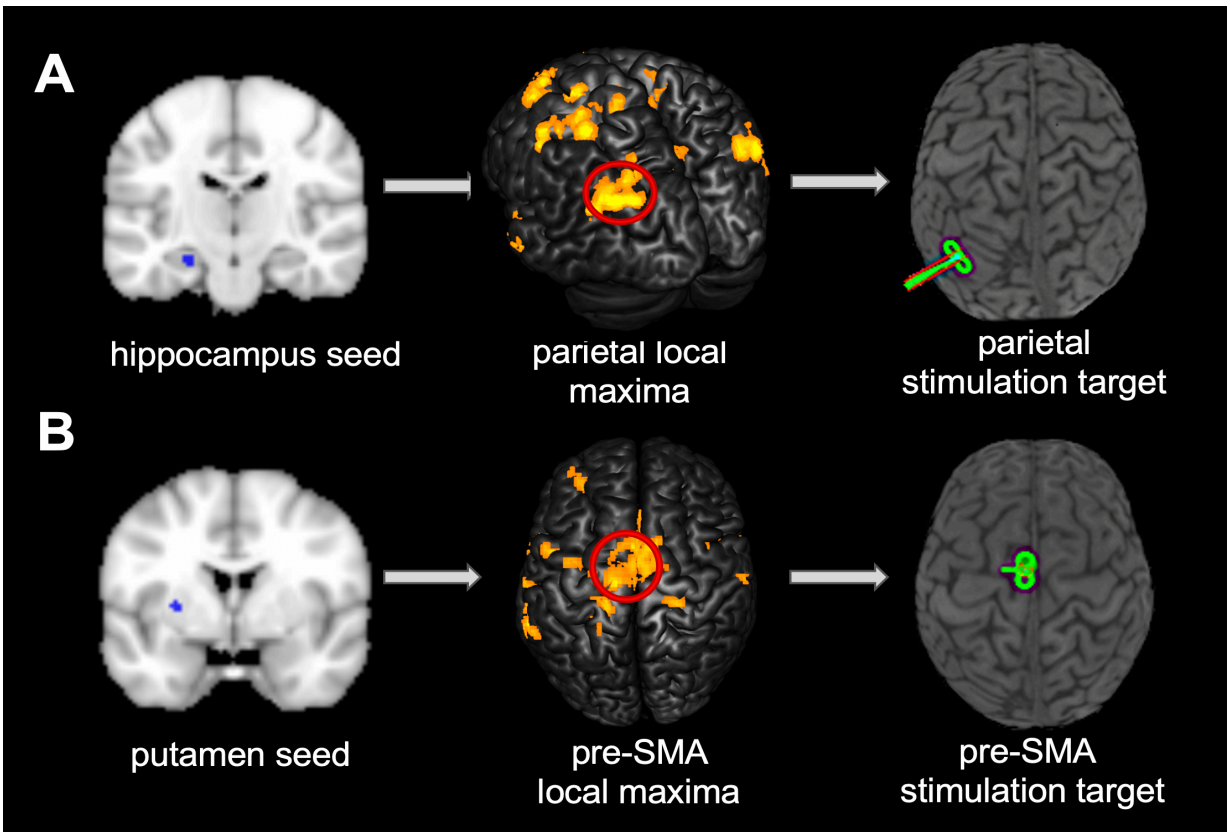


Figure 1. Experimental overview.

The multi-day rTMS protocol is represented in panel 1. Subjects completed two rTMS conditions across two separate weeks. Each condition included four sessions of personalised rTMS (S1-S4) delivered daily. Stimulation was applied to left lateral parietal cortex functionally integrated within a cortico-hippocampal network in one week, and pre-supplementary motor cortex functionally integrated within a cortico-putamen network in the other week. The order of these conditions was counterbalanced and pseudo-randomised across participants. A minimum one-week break separated the two conditions (range 1-5 weeks). Associative memory performance (face-cued word recall; FCWR) was assessed at baseline (~1 hour prior to S1) and post-rTMS (~24 hours following S4). MRI assessments of resting-state functional connectivity were completed in week one at baseline approximately 72 hours prior to S1 and post-rTMS approximately 24 hours after S4.

The rTMS target localisation process is represented in panel 2.
Cortical stimulation sites were determined for each subject on the basis of individual resting-state functional MRI connectivity maps.

(A) Parietal stimulation sites were derived from a cortico-hippocampal network implicated in associative memory performance. The left hippocampal seed time course was extracted from a 3mm sphere corresponding to the middle of the hippocampus proper, closest to MNI coordinate x = -24, y = -18, z =- 18. Parietal targets were defined as the cluster of voxels displaying peak local connectivity within a 15mm radius of MNI coordinate x = -47, y = -68, z = 36 (Wang et al., 2014).

(B) Pre-SMA stimulation sites were derived from a functionally and spatially distinct cortico-striatal pre-motor network**.** The left dorso-caudal putamen seed time course was extracted from a 3mm sphere corresponding to MNI coordinate x = -28, y = 1, z = 3. Pre-SMA targets were localised to an area on the cortical surface within left hemisphere, anterior to the anterior commissure (i.e. a positive y MNI coordinate), displaying peak connectivity to the putamen seed.

A stereotactic neuronavigation system was used throughout each session to ensure accurate target localisation relative to each subject's neuroanatomy.

Each condition included daily rTMS sessions delivered over four consecutive days (i.e. Monday – Thursday), with baseline and post rTMS assessments of associative memory performance. Baseline assessments were conducted ~1.5 hours prior to the first rTMS session (i.e. Monday) and post-rTMS assessments were conducted on Friday, one day after the final rTMS session (parietal condition mean delay = 24.3 ± 2.73 hrs, (mean ± SD; range = 16 - 28.5); pre-SMA condition mean delay = 24.2 ± 2.74 hrs, (mean ± SD; range = 18 - 29; p = .67). Consistent with the procedure of Wang et al. (2014), each condition was separated

by an interval of at least one week (mean = 1.78, range 1 - 5 weeks). The order of experimental versus control weeks was counterbalanced across subjects, and pseudo-randomised to minimise variance associated with gender, age, level of education, self-reported physical activity (IPAQ), and preferred time of day for rTMS administration (i.e. afternoon or morning), using an online algorithm (www.rando.la). Pseudo-randomisation was performed to increase the reliability of between-group comparisons conducted on week one MRI and cognitive data.

To investigate functional connectivity following stimulation, resting-state functional MRI scans were conducted on Fridays whenever possible i.e. three days prior to the first rTMS session (interval between baseline MRI and first rTMS session = 75.38 ± 11.03 hrs, (mean ± SD; range = 66.5 - 148) and one day after the final rTMS session (interval between follow-up MRI and final rTMS session = 23.2 ± 3.21 hrs (mean ± SD; range = 18 - 30)).

2.4 Associative memory task - Face-cued word recall

To assess the effects of rTMS on associative memory function, we employed the same face-cued word recall task as reported by Wang et al. (2014). Subjects studied a set of 20 human face photographs derived from a database of amateur model headshots (Althoff & Cohen, 1999), presented in greyscale on printed cards. Each card was placed on a table in front of the participant for three seconds, and a unique common English word was read aloud by the experimenter when each card was shown. The words were nouns between 3-8 letters in length, with Kucera-Francis written frequencies between 200 to 2000, and concreteness ratings of 300 to 700 (MRC Psycholinguistic Database; www.psych.rl.ac.uk). Subjects were instructed to memorise the association between the face and word. Following presentation of the 20 face-word pairs, there was a filled delay of approximately one minute involving a procedural task with low cognitive demand (i.e. retrieving an online link to a questionnaire from a personal email inbox). Following this, the same set of cards was represented to subjects individually in a different randomised order, and subjects were instructed to try and

recall the word that accompanied each face. Word recall was scored as correct or incorrect, forgiving errors relating to pronunciation, including plurality.

There were six alternative versions of the task (matched for word concreteness and frequency). Alternative versions were used for baseline and post-rTMS assessments for each stimulation condition (and for a follow-up assessment ~1.5 weeks following the second condition) and the order of each set was randomised across subjects using a Latin square. To familiarise subjects with the task and minimise practice effects, the remaining sixth version of the task was employed as a practice set, whereby a smaller sub-set of 10 face-word pairs was administered prior to the baseline assessment of the first condition.

2.5 Magnetic resonance imaging (MRI)

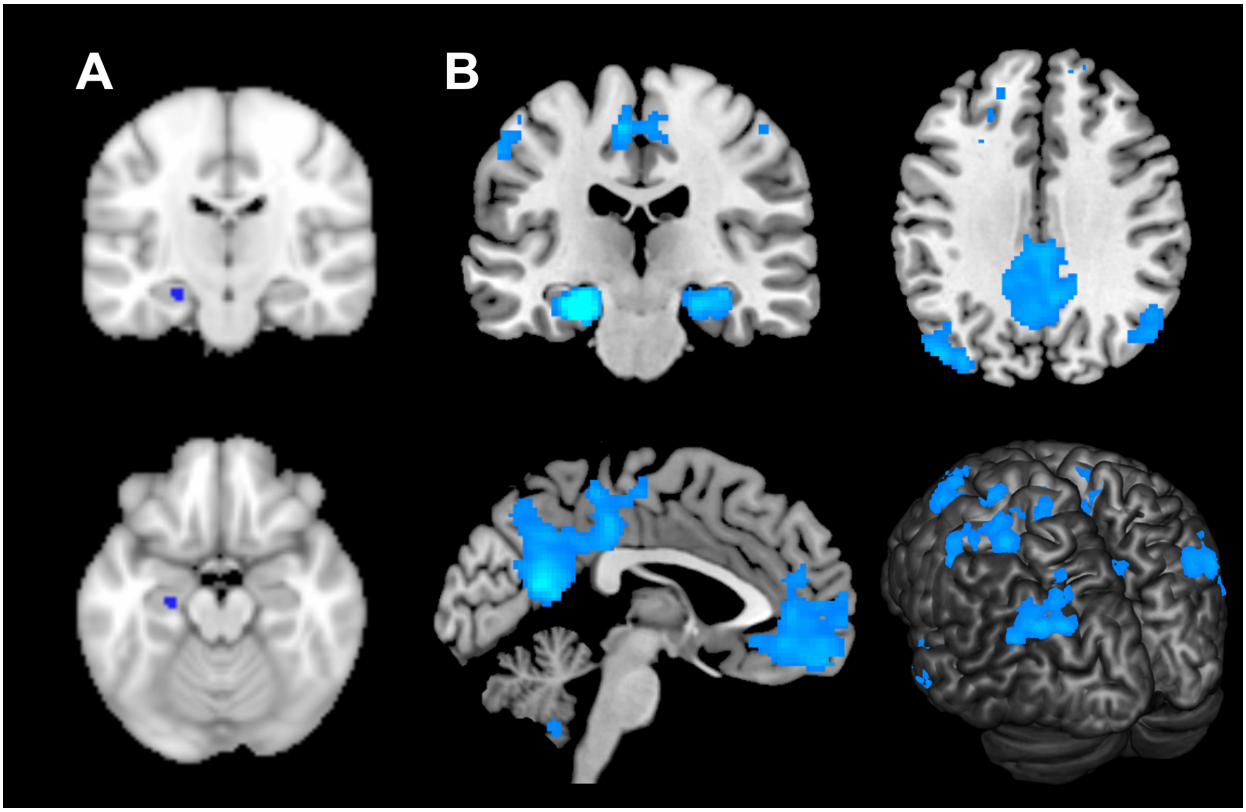
MRI data were collected from a Siemens 3T Skyra scanner with 32-channel head coil. For both baseline and post rTMS scans, T1-weighted structural images (Magnetization Prepared Rapid Gradient Echo, TR = 2.3 s, TE = 2.07 ms, voxel size 1mm^3, flip angle 9°, 192 slices) and resting-state Echo Planar Images (TR = 2.46 s, TE = 30 ms, voxel size 3mm^3, flip angle 90°, 44 slices) with whole brain coverage were acquired. During resting-state scans, subjects were instructed to keep their eyes open and focus on a black fixation cross presented on a white background, whilst trying not to think of anything in particular. MRI scanning was conducted prior to cognitive assessments to ensure that resting-state fMRI measurements were not influenced by completion of cognitive tasks (e.g. to minimise the potential that engaging certain regions during cognitive assessments, such as the hippocampus during associative memory tasks, may alter functional connectivity estimates).

2.6 Identification of stimulation locations using resting-state fMRI

Following a similar approach and rationale to Wang et al. (2014), cortical stimulation sites were determined for each subject on the basis of individual resting-state functional MRI connectivity maps calculated from baseline data (Figure. 1). Stimulation sites were chosen on the basis of their superficial location along the cortical surface, allowing direct targeting

with TMS, and previously established functional connectivity to distinct cortico-subcortical networks (Di Martino et al., 2008; Wang et al., 2014). Parietal stimulation sites were derived from a cortico-hippocampal network implicated in associative memory performance (Wang et al., 2014). Pre-SMA stimulation sites were derived from a functionally and spatially distinct cortico-striatal pre-motor network (Di Martino et al., 2008) (see Figure. 2).

Panel 1



Panel 2

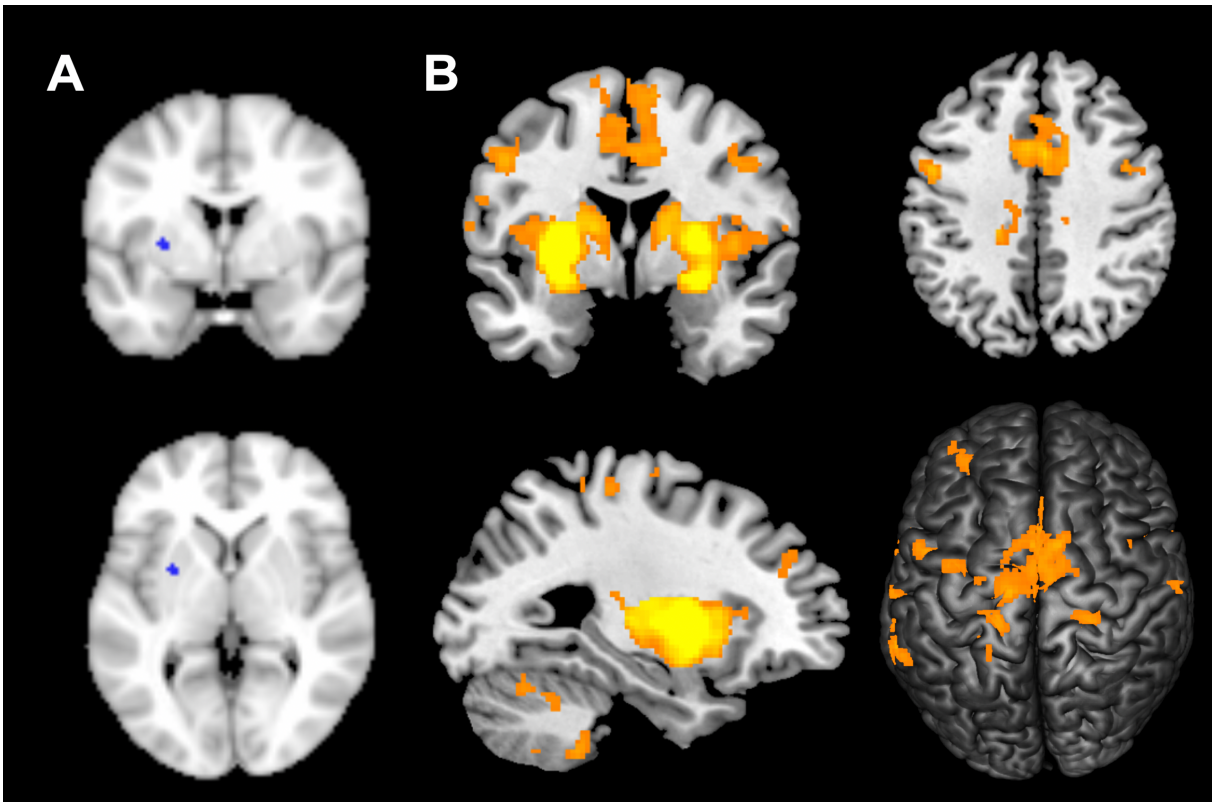


Figure 2. Overview of cortical-hippocampal and cortical-putamen resting-state networks.

Cortical-hippocampal network is represented in panel 1. To generate functional maps **(B)**, the left hippocampal seed time course was extracted from a 3mm sphere corresponding to middle of hippocampus proper (MNI coordinate x = -24, y = -18, z =- 18) **(A)**. This region shows strong functional connectivity to regions within default-mode network and left parietal cortex stimulation target (Wang et al., 2014). Functional maps **(B)** are derived from the group mean of baseline data (FWE $p < .0001$, extent threshold $k > 50$ contiguous voxels).

Cortical-putamen network is represented in panel 2. To generate functional maps **(B)**, the left putamen seed time course was extracted from a 3mm sphere corresponding to dorsal putamen (MNI coordinate x = -28, y = 1, z = 3) **(A)**. This region of the putamen shows distinct patterns of connectivity across a motor/pre-motor network, including positive correlations to primary and supplementary motor cortices. Functional maps **(B)** are derived from the group mean of baseline data (FWE $p < .0001$, extent threshold $k > 50$ contiguous voxels).

A MATLAB-based, in-house pre-processing pipeline was used to determine individualised stimulation targets. Structural and functional images were realigned, normalised to MNI

space (MNI_152 template), and co-registered using SPM8. Functional images were slice-time corrected to the first acquired slice and linearly detrended using REST toolbox (Song et al., 2011). Nuisance regressors based on CSF and white matter time courses were generated using CompCor (Behzadi, Restom, Liau, & Liu, 2007), and the fsl_regfilt function (Jenkinson, Beckmann, Behrens, Woolrich, & Smith, 2012) was used to perform motion correction (using six head motion parameters), and nuisance regression of white matter and CSF signals. Functional images were bandpass filtered (0.008 – 0.08 Hz) using REST toolbox (Song et al., 2011) and spatially smoothed using an 8mm Gaussian kernel.

To generate baseline functional maps, the time course of two sub-cortical seeds were extracted for each subject using fslmeants function. Specifically, the left hippocampal seed time course was extracted from a 3mm sphere corresponding to the middle of the hippocampus proper, closest to MNI coordinate x = -24, y = -18, z =- 18 (Wang et al., 2014). The left dorso-caudal putamen seed time course was extracted from a 3mm sphere corresponding to MNI coordinate x = -28, y = 1, z = 3. This region of the putamen shows distinct patterns of connectivity across a motor/pre-motor network, including positive correlations to primary and supplementary motor cortices (Di Martino et al., 2008). To ensure time series reflected fluctuations in grey matter tissue, the extracted seed time courses were weighted by grey matter probability masks derived from each subject's segmented structural image. The time-series for each seed was used as a regressor to derive individual functional connectivity t maps in SPM8. The group level functional connectivity networks at baseline are shown in Figure 2 (FWE $p < .0001$, extent threshold $k > 50$ contiguous voxels).

Individual functional connectivity t-maps were used to determine the stimulation targets (Figure 3). Parietal targets were identified from the hippocampus seed-based network and defined as the cluster of voxels displaying peak local connectivity within a 15mm radius of MNI coordinate x = -47, y = -68, z = 36 (Wang et al., 2014). Across subjects the average

(±SD) MNI coordinate for parietal stimulation was x = -46.4 (2.9), y = -69.8 (3.7), z = 36.3 (3.8). Consistent with the procedure reported in Wang et al. (2014), stimulation and seed locations were localised to the left hemisphere due to the established role of the left lateral parietal cortex role in associative memory (Wagner, Shannon, Kahn, & Buckner, 2005) and predominant ipsilateral connectivity between the hippocampus and parietal cortex (Kahn, Andrews-Hanna, Vincent, Snyder, & Buckner, 2008). Pre-SMA targets were localised to an area on the cortical surface within left hemisphere, anterior to the anterior commissure (i.e. a positive y MNI coordinate), displaying peak connectivity to the putamen seed. Pre-SMA stimulation was biased towards left hemisphere to ensure consistency between the two stimulation conditions (i.e. ipsilateral relationship between sub-cortical seed and stimulation site). Across subjects the average (±SD) MNI coordinate for pre-SMA stimulation was x = -4.6 (2.7), y = 2.9 (2.4), z = 69.7 (4.1), conferring with past studies that have localised pre-SMA to 1-4cm anterior to the scalp vertex (Hamada, Ugawa, & Tsuji, 2009; Matsunaga et al., 2005) (see supplementary materials for overlay of individualised seed and stimulation locations).

2.7 Repetitive transcranial magnetic stimulation

At the beginning of each rTMS session, resting-motor threshold was determined using electromyography recorded from the right first dorsal interosseous (FDI) muscle. Biphasic pulses were applied to the left primary motor cortex (M1) using a MagVenture MagPro X100 stimulator and B-65 figure-8 cooled coil (75mm outer diameter). The motor 'hotspot' was determined as the area of scalp that produced the largest and most consistent motor-evoked potential in the targeted FDI muscle, and resting motor threshold was defined as the minimum stimulation intensity necessary to evoke a potential with a peak-to-peak amplitude of ≥ 50 μV in the resting FDI in at least 5 out of 10 consecutive trials.

For both stimulation conditions, rTMS was administered at 100% of resting-motor threshold at 20 Hz (2s on, 28s off) for 20 minutes (1600 pulses total) daily for four consecutive days.

These parameters conform to internationally established safety guidelines (Rossi, Hallett, Rossini, & Pascual-Leone, 2009) but as an additional precautionary measure, EMG recordings from the FDI muscle were continuously monitored throughout rTMS for evidence of altered motor activity changes (i.e. kindling). A stereotactic neuronavigation system was used throughout each session to ensure accurate target localisation relative to each subject's neuroanatomy (Brainsight, Rogue Research; with the exception of subjects 1-9 for whom a Zebris system was used). Analysis of associative memory change was performed for the sub-sample that completed the protocol with the Brainsight system (N = 31) (see supplementary materials), however the results were consistent with the complete sample (N = 40), reported herein. Stimulation targets were loaded onto each subject's normalised T1-weighted image and aligned to the middle of the gyral crown to maximise the field size induced in cortical grey matter by the TMS pulse (Thielscher, Opitz, & Windhoff, 2011). For the parietal condition, stimulation was delivered to the target site with the coil handle perpendicular to the long axis of the gyrus to induce posterior/anterior current flow. For the pre-SMA condition, the coil was held with the coil handle pointing left, i.e. perpendicular to the midsagittal plane.

2.8 Data analysis

2.8.1 Face-cued word recall task analysis

For one subject, scheduling conflicts meant that it was not feasible to conduct post-rTMS assessments 24 hours following the final rTMS session. Thus, data from this subject was removed and analyses of associative memory change were conducted on N = 39. Performance on the face-cued word recall task was scored as the percentage of correctly recalled words out of a total of 20. Individual z scores more extreme than 2.58 were labelled as outliers and removed from subsequent analysis. To investigate the effects of parietal stimulation on associative memory performance, a 2 x 2 repeated measures ANOVA was conducted with within-subject factors of stimulation condition (parietal, pre-SMA) and time (baseline, post-rTMS). A paired-sample t-test was also conducted on the follow-up

assessment data to investigate whether stimulation elicited long-lasting (~1.5 week) changes to associative memory performance (Wang & Voss, 2015). In order to account for inter-subject variability in task performance, an additional paired-sample t-test (Wang et al., 2014) was also conducted on Δ values for each stimulation condition expressed as: Δ memory = (post-rTMS % correct – baseline % correct) / baseline % correct.

2.8.2 Resting-state fMRI analysis

Previous lines of evidence have demonstrated that pre-processing methods, in particular denoising methods to minimise motion-related artefact, can exert considerable influence on resting-state fMRI group-level comparisons (Parkes, Fulcher, Yücel, & Fornito, 2018). Therefore, data were pre-processed using two separate pre-processing pipelines, including the previously described MATLAB-based, in-house pipeline, and fMRIprep (version 1.1.1), a 'best-in-breed' workflow developed to ensure high-quality outputs, robust to data idiosyncrasies (Esteban et al., 2019). fMRIprep was run with default parameters, including ICA-AROMA denoising and susceptibility-derived distortion estimation (AFNI 3dqwarp). The choice of pre-processing pipeline did not significantly alter second-level group comparisons, thus fMRIprep was employed for the final reported analysis.

For each network of interest, we examined changes to FC from both the site of stimulation and corresponding sub-cortical target. To investigate changes to cortical-hippocampal FC, we extracted the time course of subject-specific seed coordinates corresponding to the parietal stimulation site and corresponding left hippocampus target. To investigate changes to cortical-putamen FC, we extracted the time course of subject-specific seed coordinates corresponding to the pre-SMA stimulation site and corresponding left putamen target. Time courses were extracted from the pre-processed functional image using fslmeants for baseline and post-rTMS sessions. Time series were weighted by individual's grey matter probability mask and resulting time series were entered into first-level analysis in SPM8 to generate individual contrast maps.

Second-level group comparisons of FC change were conducted using SPM and run separately for each seeded region to eliminate statistical issues relating to multi-collinearity. T maps were converted to Fisher's Z using the SPM8 function imcalc. Similar to the analytical approach of Wang et al. (2014), difference maps were calculated by subtracting the baseline MRI assessment from the post-rTMS assessment and compared between stimulation conditions using an independent-samples t-test design. Additionally, to account for the possibility that stimulation may have altered FC across broader networks, within-subject comparisons were also conducted for each stimulation condition. Specifically, change to hippocampal FC (seed in the hippocampus) was analysed following parietal stimulation, and changes to putamen FC (seed in the putamen) were analysed following pre-SMA stimulation, using a paired-sample t-test design (baseline vs post-stimulation). Group-level connectivity change maps were FWE-corrected at the cluster level with a cluster defining height threshold of $p < .05$ to approximate the approach of Wang et al. (2014). Given subsequent evidence that more stringent primary thresholds are more appropriate (Eklund, Nichols, & Knutsson, 2016), we also report results using a more stringent cluster defining height threshold of $p < .001$, which is commonly used for cluster level FWE inference in SPM.

2.8.3 Parietal-hippocampal functional connectivity and associative memory

Connectivity change between stimulation target and sub-cortical seed was calculated in SPM8, using Marsbar toolbox (Brett, Anton, & Valabregue, 2002). We extracted beta values between subject-specific hippocampal locations (3mm spherical seed) and the stimulation site within left parietal cortex (10 mm spherical seed, approximating the estimated e-field diameter induced by the TMS pulse (De Geeter, Crevecoeur, Leemans, & Dupré, 2015)), centred on MNI coordinate x = -41, y = - 67, z = 36. To examine the relationship between changes in resting-state connectivity and memory performance, Pearson's correlations (two-tailed, $p < .05$) were conducted between change scores calculated from parietal-

hippocampal connectivity beta weights (Δ = post beta– pre beta) and Δ memory. This analysis included only those subjects who received parietal stimulation in the first condition.

3. **Results**

Data were collected from a sample of 40 participants (19 males, 21 females) with an average age of 25.48 (SD ± 9.35) years. Overall, both conditions of TMS were well tolerated by subjects. Two participants reported transient mild headaches following rTMS (one following parietal stimulation and one following pre-SMA stimulation), but no other adverse events were reported.

Due to scheduling conflicts, 2/40 subjects did not complete the MRI protocol. Data were also omitted from an additional two subjects (due to error in MRI console software (n = 1) and excessive head motion (n = 1) during acquisition). Thus MRI analyses were conducted with N =36, whereby comparisons were made between individuals who received parietal stimulation (n = 18) versus pre-SMA stimulation (n = 18) in week one (see figure 3). There were no significant differences between groups in regards to age (parietal stimulation = 24.89 ± 9.05 years; pre-SMA stimulation = 24.83 ± 8.90 years, t(1,34) = 0.02, p = .99, Cohen's d = 0.00), or sex (parietal stimulation = 50.0 % female; pre-SMA = 61.1 % female, X^2(1, N = 36) = 0.45, p = .50, Cramer's V = .11).

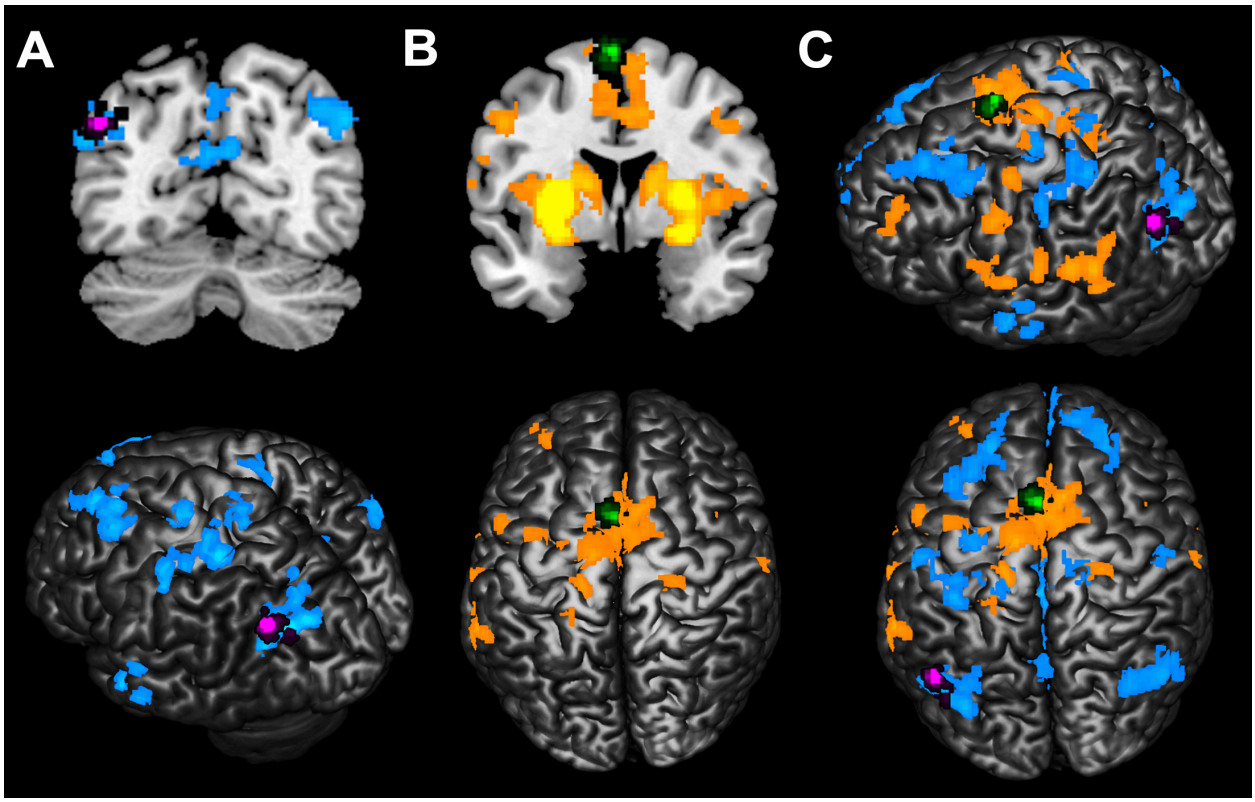


Figure 3. Overlay of individualised stimulation targets on resting-state networks.

(A) Individual parietal stimulation targets & cortico-hippocampal resting-state network. Parietal stimulation targets (represented in magenta) were selected within a 15mm radius of MNI coordinate x = -47, y = -68, z = 36. Stimulation targets are localised to region of the left parietal cortex functionally connected to left hippocampus. The spatial overlap of stimulation targets across subjects is shown, with greater proportion of overlap represented by lighter magenta colouration.

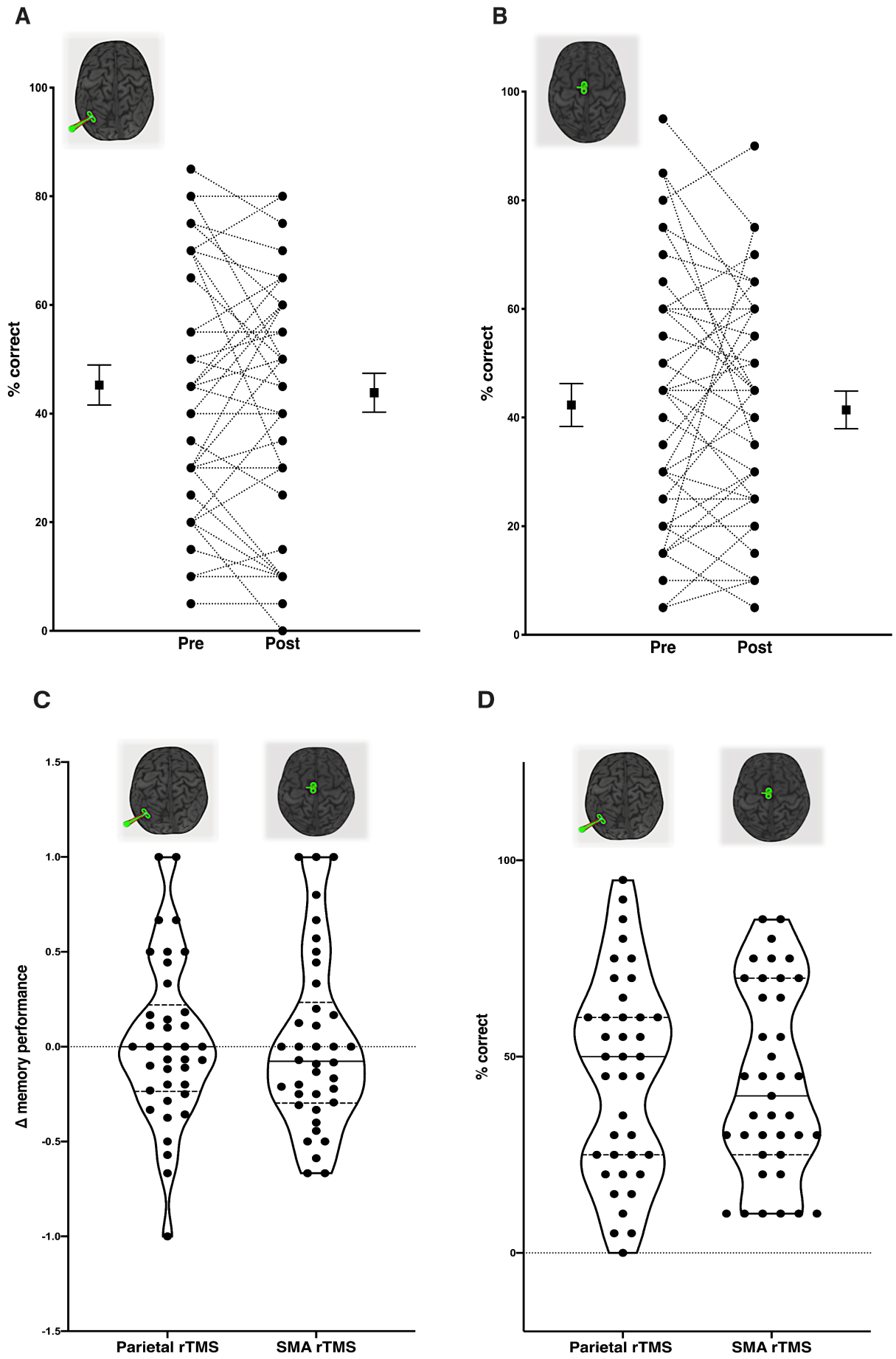
(B) Individual pre-SMA stimulation targets & cortico-putamen resting-state network. Pre-SMA targets (represented in green) were localised to an area on the cortical surface within left hemisphere, anterior to the anterior commissure (i.e. a positive y MNI coordinate). Stimulation targets are localised to region of pre-SMA functionally connected to left putamen. The spatial overlap of stimulation targets across subjects is shown, with greater proportion of overlap represented by lighter green colouration.

(C) Distinct spatial profile of targeted resting-state networks. Cortical-hippocampal network is represented in blue-light blue colour scale and shows functional connectivity across default-mode network. Cortico-putamen network is represented in yellow-orange colour scale and shows distinct pre-motor functional connectivity. Parietal stimulation targets are displayed in magenta and pre-SMA targets are displayed in green (FWE $p < .0001$, extent threshold $k > 50$ contiguous voxels).

3.1 Face-cued word recall task

Based on the findings of Wang et al. (2014, 2015), we hypothesised significant improvements in face-cued word recall accuracy following parietal stimulation, relative to stimulation of the pre-SMA active control site. Contrary to our expectations, a within-subject 2 x 2 repeated-measures ANOVA revealed no significant interaction between Stimulation Condition and Time ($F_{1,38}$ = 0.02, p = .89, η^2_p = .00), no main effect of Time (baseline, post-rTMS; F(1,38) = 0.35, p = .56, η^2_p= .01), and no main effect of Stimulation Condition (parietal, pre-SMA), F(1,38) = 1.86, p = .18, η^2_p = .05.

Two additional paired-sample t-tests were conducted to assess changes in associative memory performance. Associative memory estimates taken at follow-up (i.e. ~1.5 weeks following final rTMS session) were compared across stimulation conditions. No significant differences were found between parietal stimulation (M = 45.13, SD = 25.10) and pre-SMA stimulation (M = 43.50, SD =23.57), t(1,38) = 0.59, p = .56, Cohen's d = 0.09. A paired-sample t-test was also conducted on Δ values calculated for each stimulation condition. Two outlier values (Z-score > 2.58) were identified from the pre-SMA stimulation condition, and these datapoints were removed from the analysis. No significant differences were found between Δ parietal stimulation (M = 0.03, SD = .43) and Δ pre-SMA stimulation (M = 0.02, SD = 0.46), t(1,36) = 0.09, p = .93, Cohen's d = 0.01.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Δ memory performance
Parietal rTMS
SMA rTMS
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SMA rTMS

Figure 4. Assessments of associative memory performance. No evidence of sustained improvement in associative memory was shown following parietal or pre-SMA active control stimulation. **A**) Associative memory performance following parietal stimulation (circles represent individual scores; squares and error bars depict group mean and standard error); **B)** Associative memory performance following active control pre-SMA stimulation; **C)** Change in associative memory following parietal stimulation (left) and pre-SMA stimulation (right) expressed as change relative to baseline performance. **D)** Comparison of associative memory performance at follow-up (~1.5 after final session) following parietal stimulation (left and pre-SMA stimulation (right).

Given that these results do not align with those previously reported in the literature, we conducted Bayesian paired-sample t-tests on associative memory estimates using JASP (v 0.10.2) to quantify the relative evidence for the alternative vs. the null hypothesis (prior set in support of the alternative over null hypothesis (i.e. BF10) and Cauchy parameter = 0.707). Percentage correct values before and after parietal stimulation were analysed using a model based on a non-directional alternative hypothesis, and results showed moderate evidence in favour of the null hypothesis (BF_{10} = 0.212). The Δ values calculated for each stimulation condition were analysed using a non-directional model and also showed moderate evidence in favour of the null hypothesis (BF_{10} = 0.176). In summary, our data does not support the hypothesis that multi-day parietal stimulation improves associative memory.

3.2 Resting-state fMRI

<u>3.2.1 Parietal stimulation</u>

We first compared changes in connectivity seeded from the hippocampus following parietal stimulation (within-subject comparisons; baseline vs post-rTMS). No significant clusters surviving an FWE-corrected $p < .05$ cluster threshold were observed. However, when directly comparing changes in hippocampal seeded connectivity between the parietal and SMA stimulation groups (between-subject comparisons; post-pre between conditions) we found evidence for divergent effects between sites, with parietal stimulation tending to decrease connectivity between the hippocampus and paracingulate cortex, left putamen,

temporal fusiform cortex and postcentral gyrus, whereas SMA stimulation tended to increase connectivity between these regions (FWE-cluster corrected $p < .05$) (see figure 5). These effects did not survive FWE-cluster correction using a $p < .001$ cluster-forming threshold.

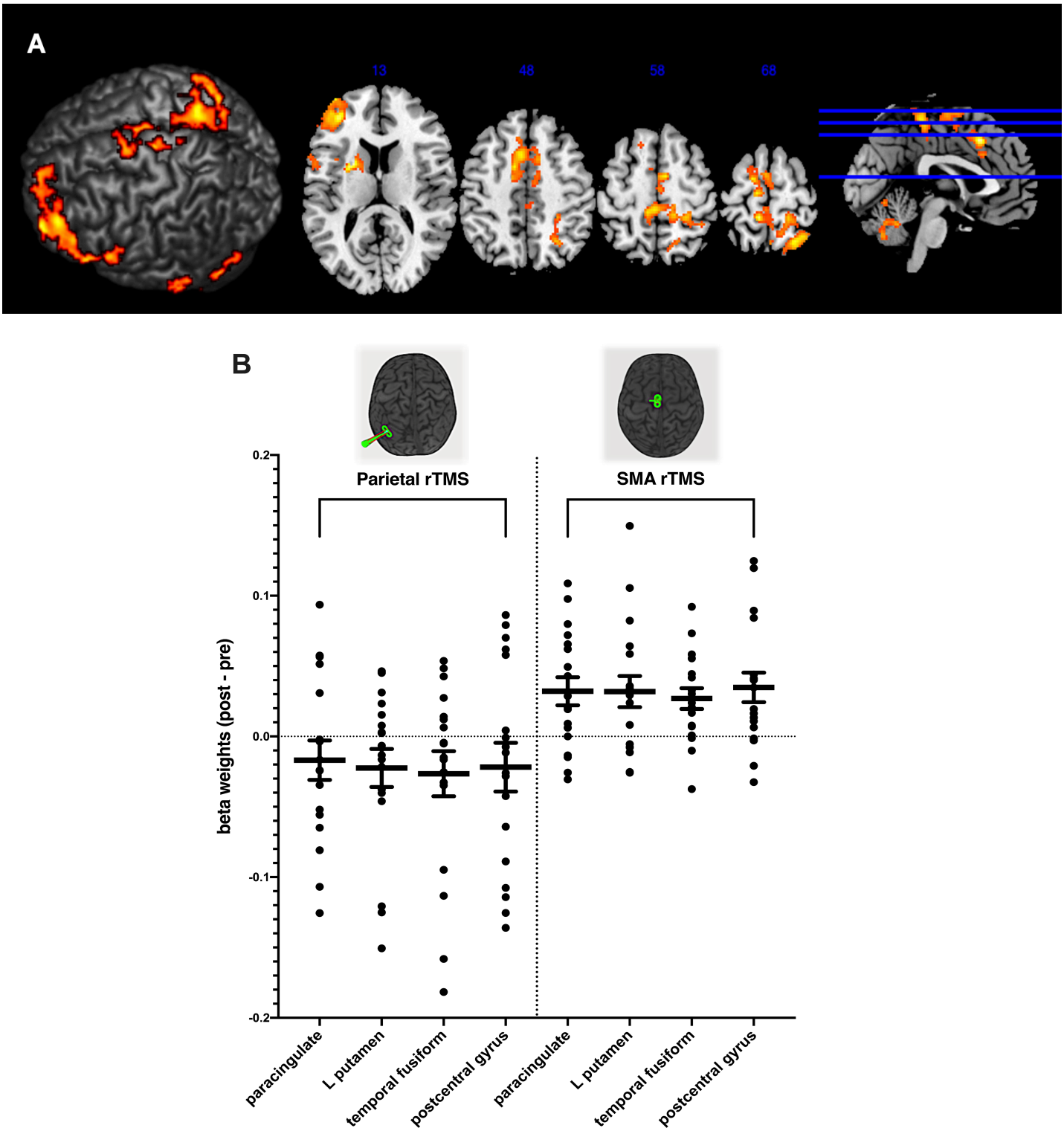
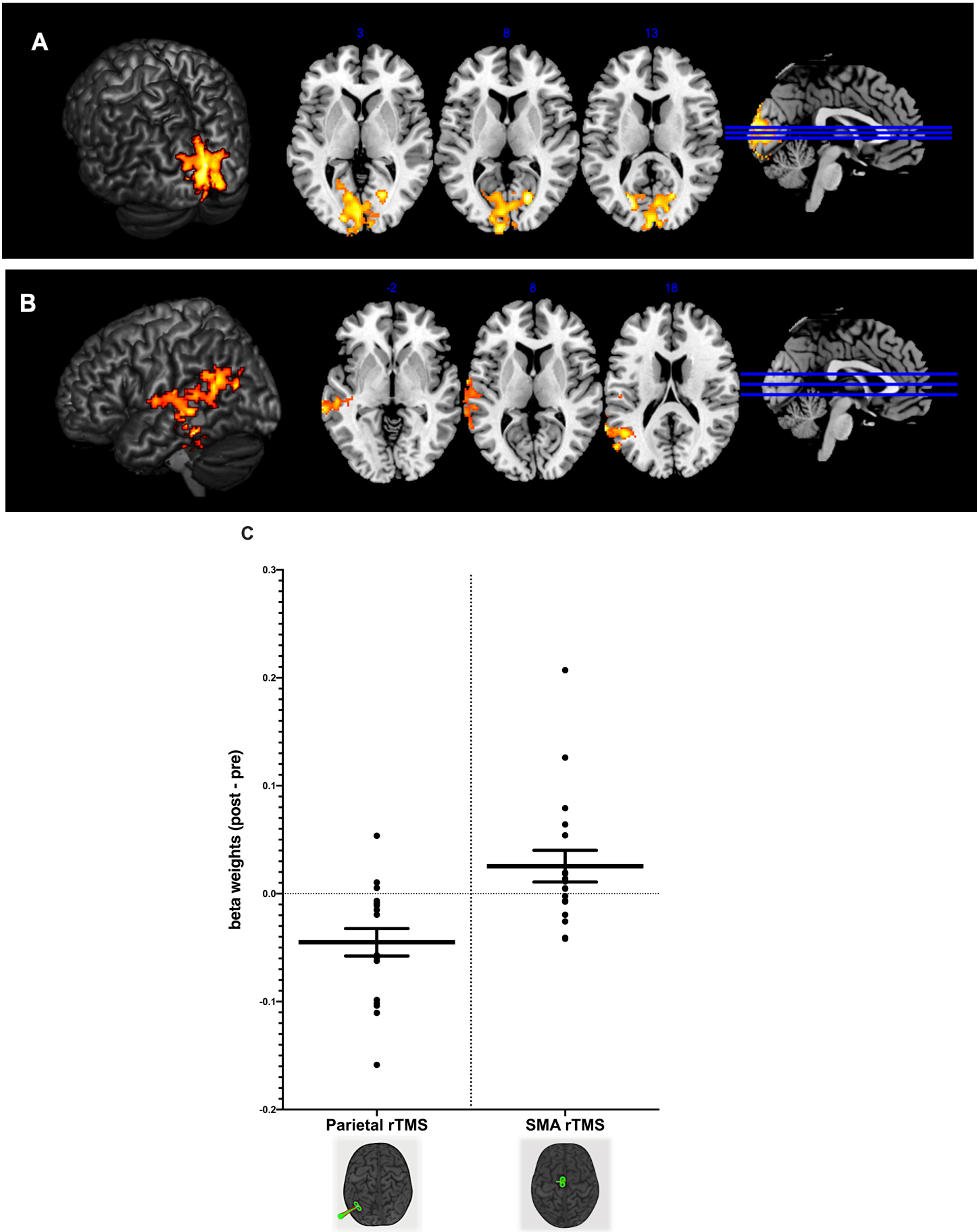


Figure 5. Resting-state connectivity change from hippocampus seed. Evidence of differential effects on hippocampal connectivity between multi-day parietal and pre-SMA stimulation. Subtle evidence of reduced FC following parietal stimulation and increased FC following pre-SMA stimulation between hippocampus seed region and locations outside of *a priori* networks of interest. **A)** Regions showing altered hippocampal FC following stimulation, including paracingulate cortex, left putamen, temporal fusiform cortex and postcentral gyrus, overlaid on anatomical template. **B)** Beta weights obtained from regions showing significant change in FC with the hippocampus seed region following stimulation. Left side of plot depicts changes to beta weight strength following parietal stimulation, with evidence of net reduction in functional connectivity. Right side of plot depicts changes to beta weight strength following pre-SMA stimulation, with evidence of net increase in FC. Error bars reflect mean and standard error.

As we used individualised cortical targets to determine the stimulation site, we also directly seeded the targeted cortical regions to compare changes in connectivity following stimulation. Contrary to our expectations, parietal stimulation resulted in subtle increases in FC between the stimulation site and occipital cortex (within-subject comparisons; baseline vs post-rTMS). When comparing changes between the parietal and SMA stimulation groups (between-subject comparisons; post-pre between conditions), we again found evidence of divergent effects, with parietal stimulation tending to decrease FC between stimulation site and left medio-temporal cortex close to left hippocampus, whereas SMA stimulation resulted in a net increase in FC between these regions (FWE-cluster corrected $p < .05$) (see figure 6). These effects did not survive FWE-cluster correction using a $p < .001$ cluster-forming threshold.

Taken together, we could not find any evidence that multi-day parietal stimulation increased connectivity in a parieto-hippocampal network. If anything, our data indicates that parietal stimulation tends to reduce connectivity in hippocampal networks, including connectivity with the stimulation site.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13
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-2
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beta weights (post - pre)
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0.2
0.1
0.0
-0.1
-0.2
Parietal rTMS
SMA rTMS

Figure 6. Resting-state connectivity change from parietal stimulation site seed. A) Increased FC between parietal seed region and occipital cortex following parietal stimulation. **B)** Evidence of differential effects on parietal connectivity between parietal and pre-SMA stimulation. Subtle evidence of reduced FC following parietal stimulation and increased FC following pre-SMA stimulation in left temporal regions close to hippocampus. **C)** Beta weights obtained from regions showing significant change in FC with the parietal seed region following stimulation. Left side of plot depicts changes to beta weight strength following parietal stimulation, with evidence of net reduction in functional connectivity. Right side of plot depicts changes to beta weight strength following pre-SMA stimulation, with evidence of net increase in functional connectivity. Error bars reflect mean and standard error.

3.2.2 Pre-SMA stimulation

We next compared changes in connectivity seeded from the putamen following pre-SMA stimulation. We found evidence of increased FC between putamen and left frontal cortex (FWE-corrected $p < .05$; within-subject comparisons; baseline vs post-rTMS; see figure 7), though these effects did not survive FWE-cluster correction using a $p < .001$ cluster-forming threshold. We found no evidence of FC change when directly comparing changes in putamen seeded connectivity between the parietal and SMA stimulation groups (between-subject comparisons; post-pre between conditions).

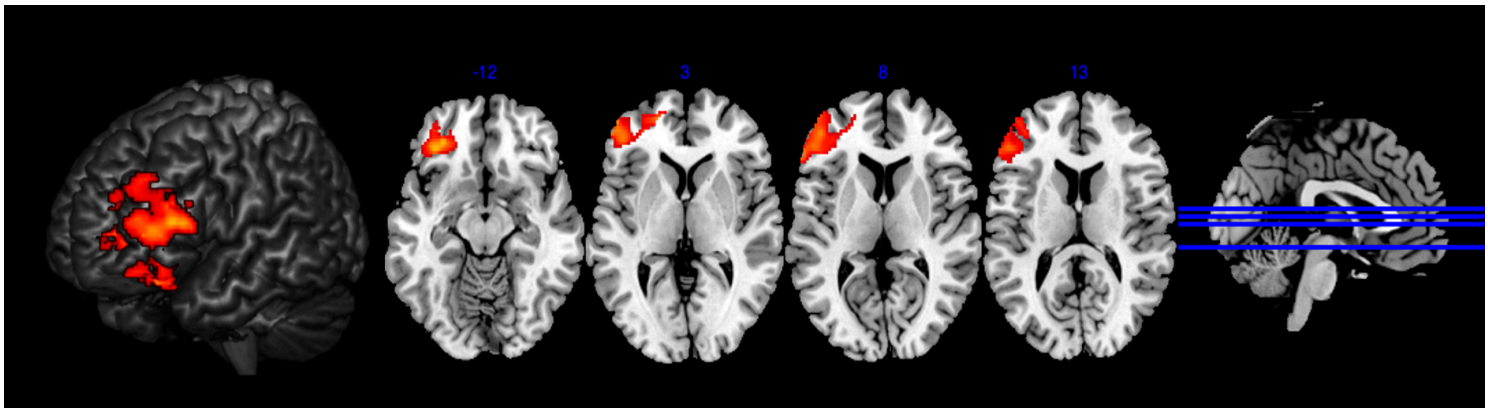
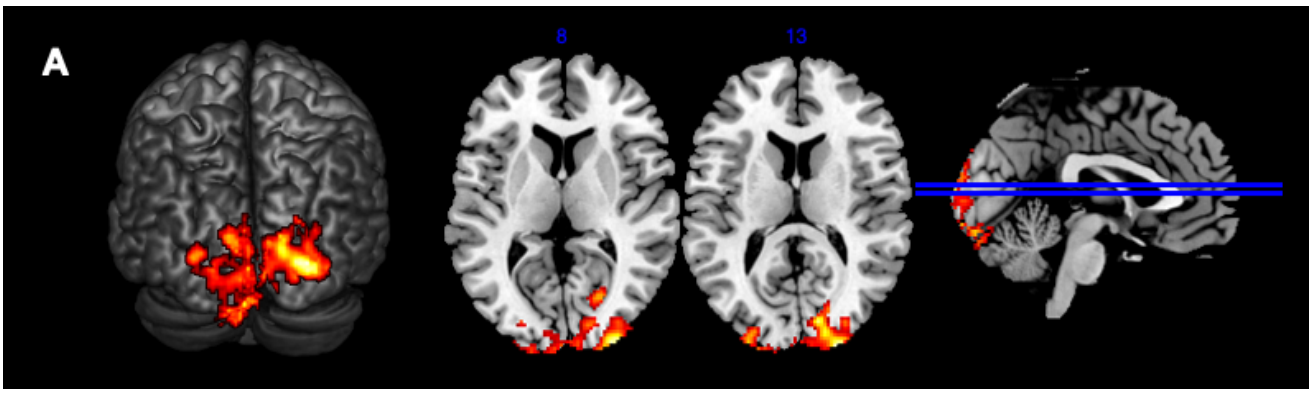


Figure 7. Resting-state connectivity change from putamen seed.
Evidence of increased FC between putamen seed region and left frontal cortex following pre-SMA stimulation. No differences in FC were observed when comparing between parietal and pre-SMA stimulation.

We also directly seeded the individualised pre-SMA stimulation target to compare changes in connectivity following stimulation. Contrary to our expectations, we found no evidence of increased FC following pre-SMA stimulation (FWE-cluster corrected $p < .05$; within-subject comparisons; baseline vs post-rTMS; see figure 8). When comparing changes between the parietal and SMA stimulation groups (between-subject comparisons; post-pre between conditions), we found evidence of decreased FC between stimulation site and occipital cortex following pre-SMA stimulation, and increased FC across these regions following parietal stimulation (FWE-cluster corrected $p < .05$) (see figure 8). These effects did not survive FWE-cluster correction using a $p < .001$ cluster-forming threshold.



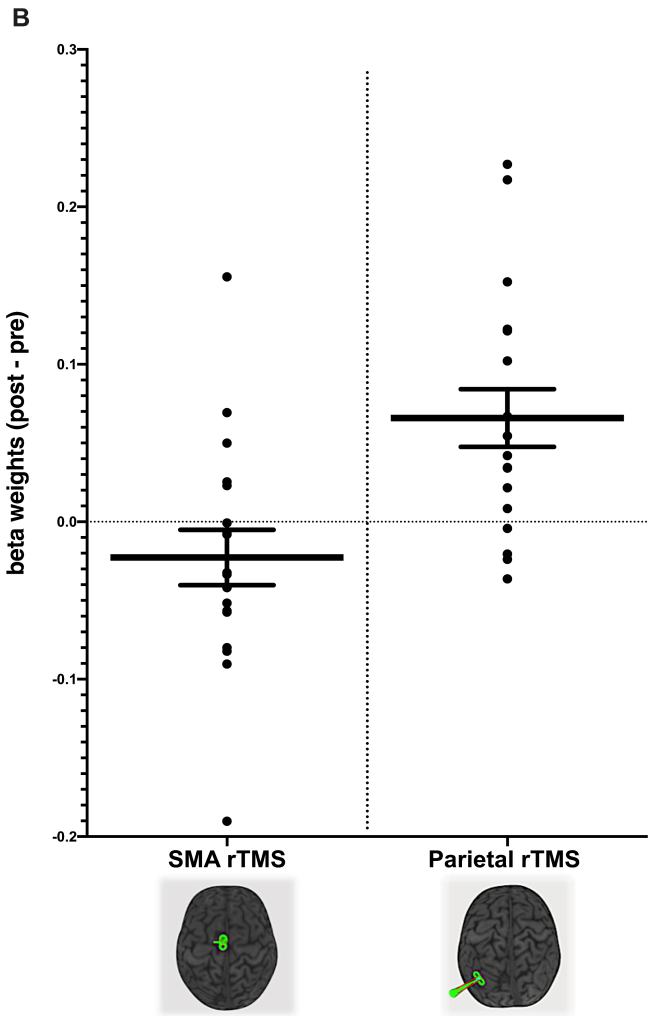


Figure 8. Resting-state connectivity change from pre-SMA stimulation site seed. A) Evidence of decreased FC between pre-SMA seed region and occipital cortex following pre-SMA stimulation, and increased FC following parietal stimulation. **B)** Beta weights obtained from occipital regions showing significant change in FC with the pre-SMA seed region following stimulation. Left side of plot depicts changes to beta weight strength following pre-SMA stimulation, with evidence of net reduction in functional connectivity. Right side of plot depicts changes to beta weight strength following parietal stimulation, with evidence of net increase in functional connectivity. Error bars reflect mean and standard error.

Overall, we provide moderate evidence of increased connectivity between left putamen and left frontal cortex following pre-SMA stimulation. We also provide subtle evidence of differential effects on FC between the stimulation site and occipital cortex across stimulation conditions, suggesting a site-specific effect of stimulation on FC.

3.3 Correlation of changes in memory performance and resting-state fMRI connectivity

Previous studies have reported associations between learning and changes in functional connectivity (Sampaio-Baptista et al., 2015; Wang et al., 2014). We explored whether individual changes to associative memory performance correlated with changes to resting-state network connectivity following parietal stimulation. A Pearson's correlation was conducted between Δ memory and Δ beta weights between stimulation site and corresponding hippocampus target for individuals who received parietal stimulation during the first week of the experiment. We could not detect a significant relationship between change in associative memory performance and change in parietal-hippocampal network connectivity, $r = .25$, $p = .33$, $n = 17$ (see supplementary materials for figure).

4. **Discussion**

Multi-day rTMS has shown promise as a method for altering connectivity in brain networks related to memory. The aims of this study were to: 1) investigate the reproducibility of long-lasting enhancement of associative memory and FC following multi-day rTMS to the parietal cortex; and 2) assess the site specificity of any changes in memory or FC following multi-day rTMS by stimulating different sites. We applied multi-day rTMS to a region of the parietal cortex functionally integrated within a cortico-hippocampal network and to a region of the pre-SMA functionally connected with the putamen. Contrary to our expectations, we did not observe evidence for improvements to associative memory performance, nor increases in cortico-hippocampal FC following parietal stimulation. We did, however, observe some evidence for site-specific modulations of functional connectivity. We found divergent changes in connectivity within seeded networks following stimulation of the different cortical sites, though effects were subtle and will require independent replication.

4.1 Multi-day parietal rTMS, associative memory, and hippocampal connectivity

Contrary to our hypotheses, we could not find any evidence for either long-lasting enhancement of associative memory or increased parieto-hippocampal connectivity following mulit-day rTMS to the parietal cortex (see figures 4-6). Instead, we found moderate

evidence that multi-day rTMS did not alter associative memory performance using Bayesian statistics. Furthermore, we found no evidence of increased parieto-hippocampal connectivity in the targeted network, and subtle evidence that parietal stimulation decreased connectivity between the medial temporal lobe and stimulation sites. This finding is in contrast to previous studies following similar multi-day protocols (Wang et al., 2014; Wang & Voss, 2015). The initial study conducted by Wang et al. (2014) with N = 16, reported significant improvements in face-cued word recall accuracy following five days of stimulation to subject-specific regions of left parietal cortex. These behavioural improvements were accompanied by increased functional connectivity between the targeted parieto-hippocampal network. Impressively, a subsequent follow-up study conducted on a smaller sub-sample of the same experiment (N = 8) revealed that these effects persisted for ~15 days following stimulation (Wang & Voss, 2015). Both the improvements in associative memory (Hermiller et al., 2019) and the increases in parieto-hippocampal connectivity (Freedberg et al., 2019b) following multi-day rTMS have recently been replicated in independent samples, although the sample sizes in these studies were moderate.

The reasons for such substantial differences between the results of our study and previous studies are unclear, however several factors may have contributed. It is possible that different sample sizes may have contributed to discrepant study outcomes. Our study was conducted using a sample of 40 healthy individuals, whereas past demonstrations of enhanced associative memory and FC have featured moderate sample sizes (N = 15-16). The response to rTMS protocols is known to be highly variable between individuals (Hamada et al., 2013; López-Alonso et al., 2014). For example, in a sample of 56 healthy individuals, Hamada et al. (2013) demonstrated that only 25% of individuals responded in the expected direction to different forms of theta burst stimulations using motor-evoked potentials as the outcome measure, while 31% responded in the opposite/ paradoxical direction. Therefore, given this pronounced inter-individual variability, it is possible that sample size differences between studies may have impacted the consistency of study

outcomes. The importance of careful sample size calculations / larger samples in managing variability has been underscored by a past reviews (Guerra, López-Alonso, Cheeran, & Suppa, 2017a, 2017b). While we do not seek to question the validity of past findings, we recommend that future studies examining similar effects of multi-day rTMS and associative memory adopt comparable sample sizes to the present study.

Differences in total stimulation dosage may have influenced study outcomes. Wang and colleagues applied five daily sessions of stimulation, while in the current study we applied four sessions of stimulation utilising the same stimulation parameters (i.e. 20 Hz, 2 second trains, 28 seconds inter-trail interval). It is therefore possible that the reduced total dose applied in the current study may have attenuated stimulation-induced effects on FC. A recent study investigated the dosage utilising a Bayesian predictive approach (Freedberg et al., 2019a), concluding that a minimum of five sessions is necessary to induce increases in cortico-hippocampal FC comparable to Wang et al. (2014). However, a subsequent study by the same group found comparable increases in hippocampal FC following 3-4 sessions of parietal stimulation (Freedberg et al., 2019b). Given inconsistencies in dose-response and the absence of a physiological basis for a minimum effective dose (i.e. four vs five sessions of rTMS), further investigation of the minimum session number to elicit reliable FC changes in this context is required.

Additionally, we also acknowledge differences between our protocol and past studies in regard to the number of associative memory assessments. In the current study, associative memory assessments were conducted before and after each week of stimulation. In comparison, Wang et al. (2014) conducted an additional assessment of associative memory at a mid-week time point (i.e. prior to the third session for both active and sham stimulation conditions). This additional exposure to the face-cued word recall task, followed by stimulation shortly after (~1 hour), may have bolstered the consolidation of associative learning, rather than inducing a generalised enhancement of associative memory capacity

per se. The absence of this mid-week task exposure in our study may have attenuated this effect. However, a recent study has successfully replicated these associative memory effects without a mid-week assessment in a sample of 16 healthy adults (Hermiller et al., 2019), suggesting that there may also be other factors influencing our observed outcomes. It is possible that inconsistent effects of rTMS on associative memory may more broadly reflect a variable response to the stimulation. Future studies may investigate whether certain physiological or lifestyle factors (e.g. parietal-hippocampal FC at baseline or concurrent level of physical activity (Hendrikse et al. 2017; Ridding & Ziemann, 2010) are predictive of associative memory changes following rTMS.

4.2 Site specificity of changes in functional connectivity following multi-day rTMS

While rTMS is often applied to different cortical regions, few studies have compared the influence of stimulation site on network connectivity. Our results suggest that the effects of multi-day rTMS on FC are site-specific and differ across targeted and non-targeted networks. When seeding either the parietal stimulation site or hippocampus target within individualised cortico-hippocampal networks, we observed evidence of decreased FC following parietal stimulation and increased FC following pre-SMA stimulation. Similarly, when seeding the stimulation site of the pre-SMA-putamen network, we again observed divergent effects, with decreased FC following pre-SMA stimulation and increased FC following parietal stimulation. Thus, we report some evidence of reduced FC within targeted networks and increased FC across distinct non-targeted networks following multi-day 20 Hz rTMS. These findings of site-specific changes to FC align with the results reported by Castrillon et al. (2020) who demonstrated differential effects of 1 Hz stimulation between a posterior sensory network and a frontal cognitive network, and evidence of widespread changes to FC across non-targeted regions. Furthermore, a recent systematic review of the effects of rTMS on FC concluded that rTMS-induced effects are frequently seen outside of the targeted network and overall do not follow accepted frequency-dependent conventions (i.e. low frequency (< 1 Hz) stimulation reduces FC, high frequency (> 5 Hz) increases FC)

(Beynel, Powers, & Appelbaum, 2020). Indeed, our findings of reduced FC within targeted networks challenges the notion that local changes to cortical excitation/inhibition induced by multi-day 20 Hz rTMS translate to similar effects at the network level. However, it must be acknowledged that our study conducted a between-subject comparison of FC, which may limit the generalisability of our findings. Further, these effects were FWE-corrected with a cluster defining height threshold of $p < .05$ to approximate the approach of Wang et al. (2014). When adopting a more conservative cluster defining height threshold of $p < .001$, these effects are absent. Further research is needed to examine the reliability of site-specific changes to connectivity following multi-day rTMS.

4.3 Conclusion

In summary, we could not find evidence that multi-day 20 Hz rTMS to an individualised parietal-hippocampal network improves associative memory performance, or increases FC within the targeted network. In contrast, we found subtle reductions in FC within targeted parietal networks and increases in FC within non-targeted networks at 24 hrs following stimulation. There was evidence of both increased and decreased FC within targeted pre-SMA networks. Our findings suggest a complex interplay between multi-day rTMS and network connectivity, with changes in both target and non-target networks, often in the opposite direction to those hypothesised. Future work uncovering the factors which determine how rTMS modulates cortical networks is required to develop more reliable paradigms for driving desired changes in functional connectivity between cortical and subcortical regions.

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Supplementary materials

Individualised sub-cortical seeds and stimulation targets

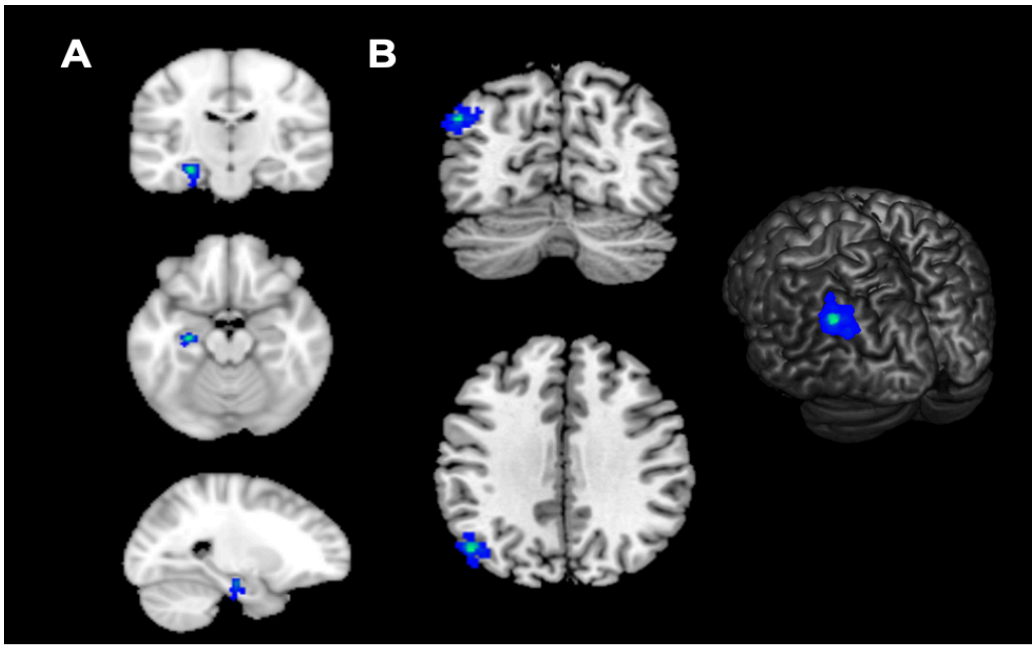

Figure: overlay of individual hippocampus seed locations (A) and corresponding parietal stimulation targets (B), superimposed on template brain. Hippocampus seeds were localised to the middle of the hippocampus proper on each subject's anatomical image. Parietal stimulation targets were individualised on the basis of peak functional connectivity to left hippocampus. Voxels in blue to green represent the average spatial overlap across subjects, with greater proportion of overlap represented in green.

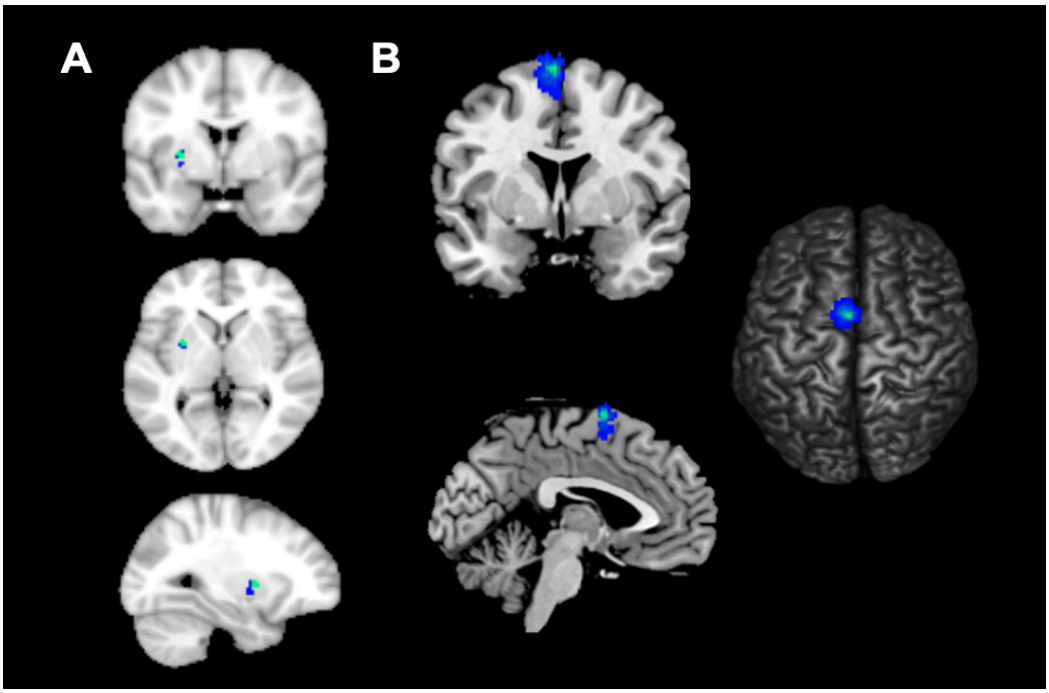

Figure: overlay of individual putamen seed locations (A) and corresponding pre-SMA stimulation targets (B), superimposed on template brain. Putamen seed were localised to MNI coordinate x = -28, y = 1, z = 3. Small transformations in the z and y plane were performed for two subjects to mirror the anatomical locations reported by Di Martino et al. (2008). Pre-SMA stimulation targets were individualised on the basis of peak functional connectivity to left putamen.

Analysis of associative memory changes in participations who received rTMS using the Brainsight neuronavigation system

To assess whether the use of different neuronavigation systems influenced the effects of stimulation on memory, an additional analysis was performed on the sub-sample that completed the protocol with the Brainsight neuronavigation system (N=31). In keeping with the statistical approach presented in the manuscript, a 2 x 2 repeated-measures ANOVA (within-subject factors of Stimulation Condition and Time) was conducted on percentage correct values on the face-cued word recall task. The results revealed no significant interaction between Stimulation Condition and Time, F(1,30) = 0.03, p = .86, $\eta^2{}_p$ = .00, main effect of Time (baseline, post-rTMS), F(1,30) = 0.23, p = .63, $\eta^2{}_p$ = .01, or main effect of Stimulation Condition (parietal, pre-SMA), F(1,30) = 0.92, p = .35, $\eta^2{}_p$ = .03. In summary, this analysis with neuronavigation system held constant yields the same conclusion as the main analysis i.e. multi-day rTMS had no effect on associative memory performance.

Comparison of associative memory performance following parietal stimulation in week one

To account for possible carryover effects of pre-SMA stimulation on the response to parietal stimulation or associative memory estimates, a paired-samples t-test was conducted on pre-post- associative memory scores in subjects who received parietal stimulation in the first week of the study (N = 20) (i.e. subjects who had not received prior pre-SMA stimulation). There were no significant differences in associative memory present in this sub-sample with similar task performance pre (M = 40.00 SD = 21.76) to post ((M = 40.00, SD =24.87) stimulation, t(1,19) = 0.00, p = 1.00, Cohen's d = 0.00.

Analysis of network-specific connectivity between stimulation site and sub-cortical targets

Parietal – hippocampal connectivity

To investigate the possibility of rTMS exerting network-specific, rather than brain-wide effects, we conducted a spatially constrained analysis of functional connectivity change between *a priori* regions of interest. We expected a significant increase in beta weights representing the correlation between parietal cortex and left hippocampus following stimulation of the parietal cortex, but not following stimulation of the active control region in pre-SMA.

An independent-samples t-test of the difference in beta weight strength between parietal cortex and hippocampus following stimulation to either parietal cortex (M = -0.02, SD = 0.08) or pre-SMA active control site (M = -0.005, SD = 0.05), was not significant $t(34) = -0.47$, $p = .64$, Cohen's d = 0.16. Thus, analysis of our data revealed no evidence of a change to functional connectivity between the parietal cortex stimulation site and the corresponding hippocampal target.

Pre-SMA – putamen connectivity

Conversely, a significant increase in beta weights between pre-SMA and left putamen were expected following pre-SMA stimulation, but not parietal stimulation.

An independent-samples t-test was also conducted to assess the effects of pre-SMA stimulation on functional connectivity between the stimulated region of the cortex and left putamen target. There was no significant difference observed in beta weight strength between pre-SMA and putamen following stimulation to either pre-SMA (M = 0.004, SD = 0.07) or parietal (M = -0.01, SD = 0.09) targets, $t(34) = 0.69$, $p = .49$, Cohen's d = 0.23. Thus, consistent with the cortico-hippocampal network results, we report no evidence of sustained changes to pre-SMA – putamen functional connectivity following stimulation.

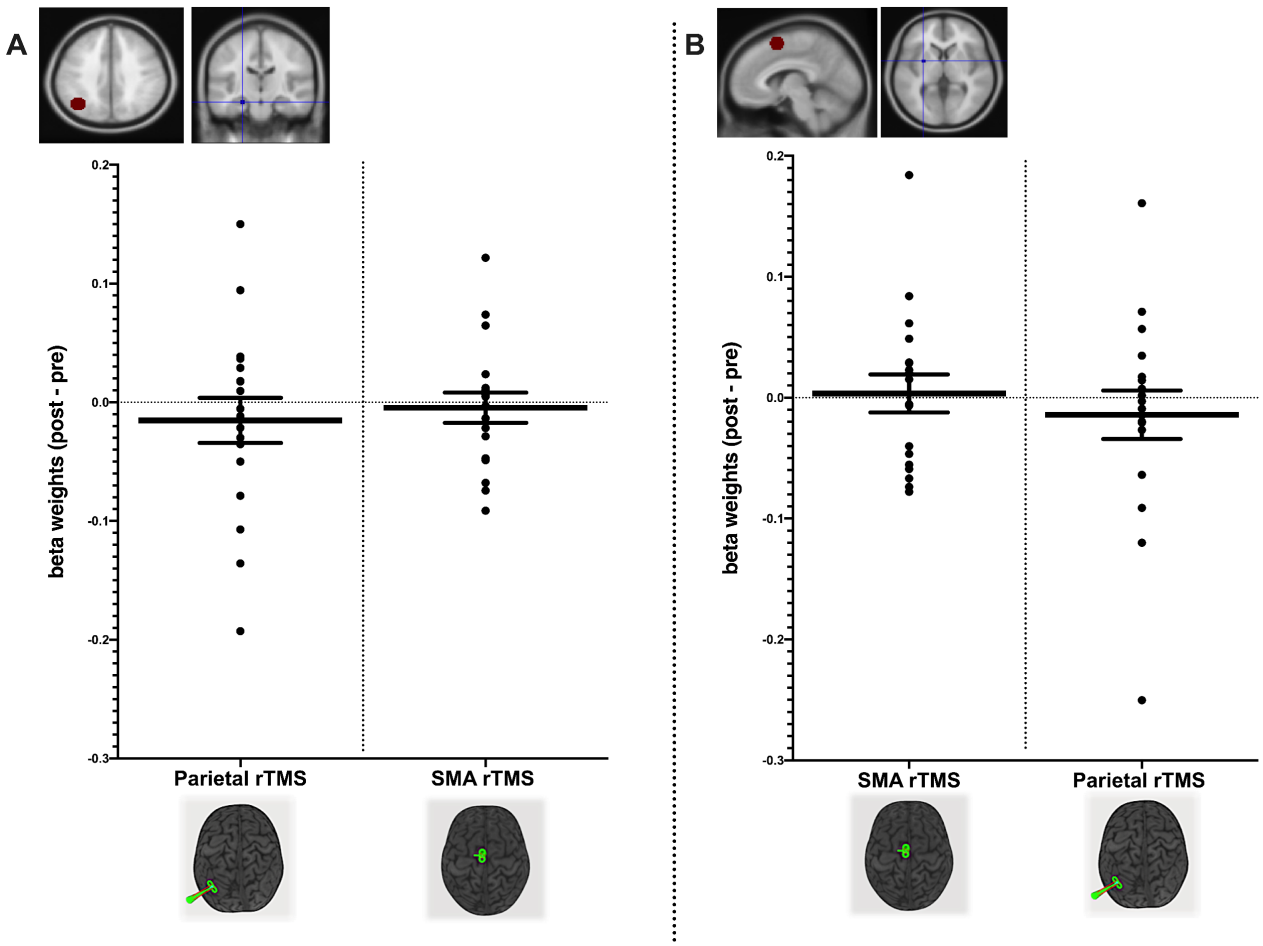

Figure: network-specific analysis of functional connectivity change. No significant changes between stimulation site and subcortical targets. **A)** Changes to beta weight strength between left parietal cortex and left hippocampus seed following parietal stimulation (left) and pre-SMA active control stimulation (right). **B)** Changes to beta weights strength between pre-SMA and left putamen seed following pre-SMA stimulation (left) and parietal stimulation (right).

<u>Correlation of changes in memory performance and resting-state fMRI connectivity</u>

We explored whether individual changes to associative memory performance correlated with changes to resting-state network connectivity following parietal stimulation. There was no significant relationship between change in associative memory performance and change in parietal-hippocampal network connectivity, $r = .25$, $p = .33$, $n = 17$.

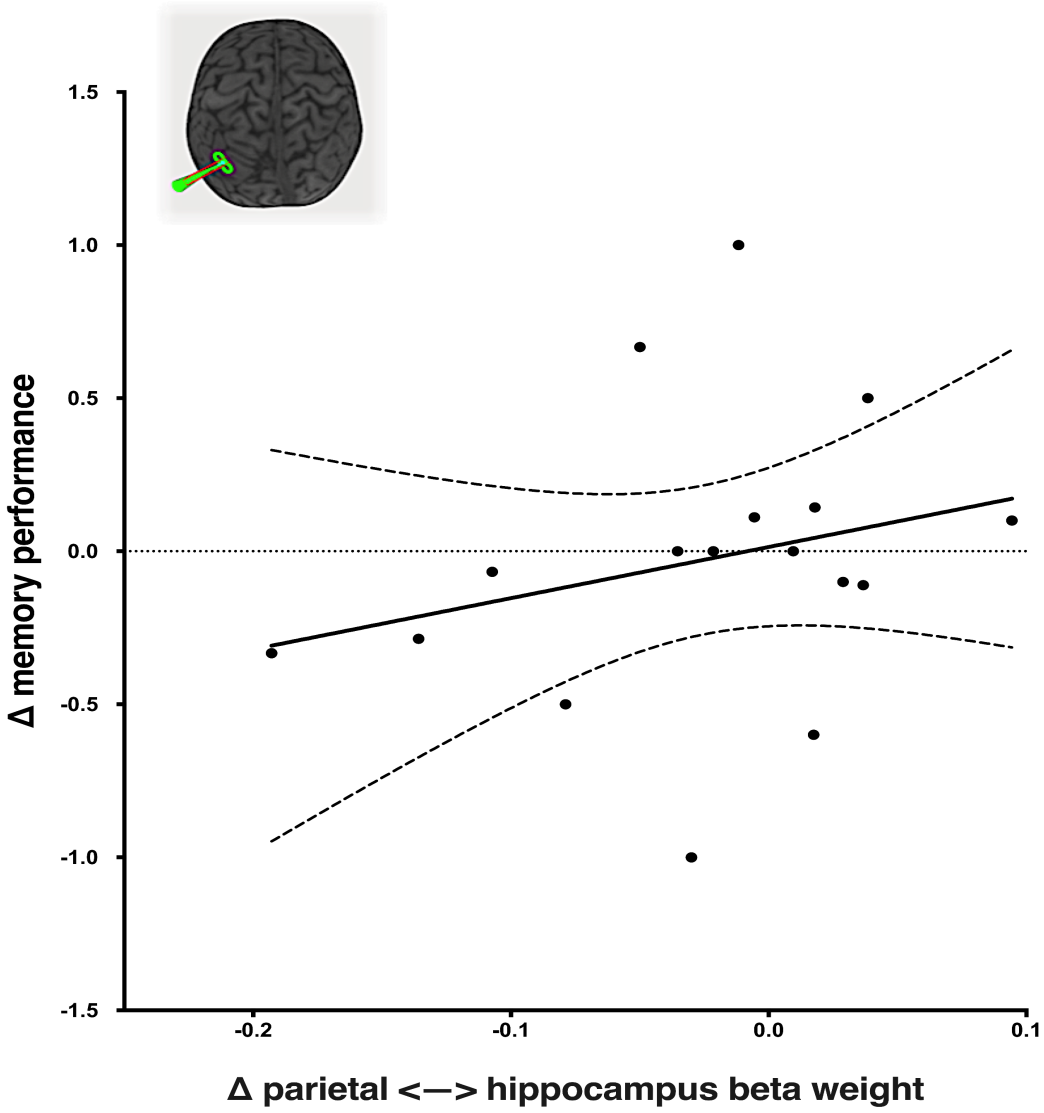

Figure: **association between changes in memory performance and resting-state fMRI connectivity.** Change in associative memory following parietal stimulation (expressed as change relative to baseline performance) is shown on the y-axis and change in parietal-hippocampal connectivity (post beta weights – pre beta weights) is represented on the x-axis. Solid line shows regression line of best fit, dotted lines depict 95% confidence interval.