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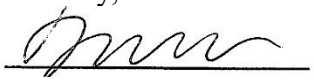
To whom it may concern,

The following study protocol and statistical analysis plan are submitted in reference to NCT03643848 for R01MH116005, Sleep-dependent Negative Overgeneralization in Peripubertal Anxiety.

Aim 3 of this grant was considered a clinical trial at the time it was funded due to random assignment and experimental manipulation. Aims 1 and 2 of the grant were observational and were not considered a clinical trial. The protocol and analytic plan presented here are therefore focused on Aim 3. The primary outcomes for the clinical trial included memory outcomes at 1 week and 12 hours, as detailed in clinicaltrials.gov and the analytic plan. Individual data from the trial and the rest of the project are available via NIMH Data Archive.

Sincerely,



Dana L. McMakin, PhD, ABPP (PI; contact)

Protocol (Aim 3 of R01MH116005)

Objectives.

Our primary objective is to test the malleability of memory processing during sleep in anxious youth. Specifically, we aim to override the selective replay of negative memories during sleep through competitive displacement, utilizing cued audio reactivation of neutral stimuli relative to sham cueing during NREM sleep. We anticipate this will reduce negative overgeneralization in peri-pubertal youth with clinical anxiety.

Design.

We will conduct a double-blind randomized design to examine the effects of Targeted Memory Reactivation (TMR) relative to Sham during NREM sleep on negative overgeneralization in youth with anxiety. Sixty youth diagnosed with Social, Separation, and/or Generalized Anxiety Disorder will first undergo clinical assessment and a 7-10 day baseline evaluation of sleep patterns. Participants will then complete an encoding session (referred to as the 'Study' session) in an MRI scanner. Subsequently, they will travel to the sleep lab at Nicklaus Children's Hospital for polysomnography (PSG) hook-up. Following PSG setup, participants will be randomly assigned to either the TMR (n=30) or sham reactivation (n=30) group by an independent research assistant operating from a control room. To maintain double-blinding, participants and the research assistant directly interacting with them will remain unaware of group assignment for the duration of the study. During PSG-monitored NREM sleep, previously-paired neutral sounds from the encoding session will be replayed in the TMR group. In contrast, for the sham group, guitar strums will be played during NREM sleep. Memory outcomes will be assessed at two post-sleep time points: an initial behavioral assessment approximately 11-12.5 hours after the encoding session, and a final assessment approximately 1 week later (between 11 am and 3 pm) conducted in the MRI. More detailed methods for each of these sessions are provided below. Analyses will compare the effects of neutral TMR (relative to sham) on negative and neutral generalization at both the 12-hour and 1-week delay assessment points.

Methods .

Participants. A total of 60 children between the ages of 10 and 13 years will participate in Aim 3, beginning in the 4th year of the broader project period. Participants will be recruited from specialty child mental health clinics at FIU and Nicklaus Children's Hospital as well as via community advertisements.

The 10-13 year age range is aligned with peri-puberty and allows for the study of sleep-dependent mechanisms of overgeneralization prior to the steep escalation of emotion-related disorders through mid-late adolescence (peaking from 16-18 years). In line with the RDoC framework, we will sample across a continuum of anxiety severity to capture variability on emotion reactivity and sleep neurophysiology during this sensitive period of development, while minimizing developmental variability in the sample. We will focus on symptoms of Separation Anxiety Disorder (SAD), Social Phobia (SP), and Generalized Anxiety Disorder (GAD) based on phenotypic overlap in emotion responding and sleep across these diagnostic categories and comorbidities. The proposed framework has implications for other disorders characterized by

negative emotions and sleep disruptions, such as OCD and Panic Disorder. Given high comorbidity with GAD, SAD, and SP, we do allow for elevated symptoms in these diagnostic categories. However, we exclude those who meet full diagnostic criteria for these disorders because they show somewhat less phenotypic overlap with SAD, GAD and SP in sleep and emotion response profiles and it is important to balance generalizability of findings with precision of investigation at this stage of inquiry. Extensive efforts will be made to prevent attrition and optimize parent and child participation. Parents of children will be compensated up to $300 for completion of the study.

Intake, Screening and Baseline Characteristics. Following a brief phone screen to verify potential inclusion (e.g. no braces, within age range etc.) eligible families will be scheduled for consent and assessment to verify group inclusion/exclusion criteria. Inclusion and exclusion criteria are detailed further in clinicaltrials.gov entry.

After informed parental consent and child assent/consent, the *Anxiety Disorders Interview Schedule Child/Parent versions* (ADIS C/P) will be administered by a trained clinician to assess for past and current anxiety diagnoses, and to screen for other psychiatric diagnoses. The *Pediatric Anxiety Rating Scale* (PARS) will be administered by a trained clinician to capture the severity of anxious symptomatology. Youth and parents will also complete symptom severity inventories for anxiety: *Screen for Child Anxiety Related Emotional Disorders* (SCARED) a 41-item child and parent self-report to assess severity of symptoms of separation anxiety, social phobia, general anxiety disorders and panic disorder. These measures will be included in a factor analysis to generate a factor score that can be used for primary analyses (anxiety severity factor). General intelligence will be measured by the *Wechsler Abbreviated Scale of Intelligence* (WASI), significant memory impairments will be assessed using the *Auditory Verbal Learning Test,* sleep-related disorders (e.g. sleep apnea, parasomnias) will be evaluated by *Structured Clinical Interview of Sleep Disorders,* an inventory of habitual sleep-wake times will be measured by *Pittsburgh Sleep Quality Index,* and a history of chronic (>6 months) or current medication that impacts emotion and sleep-wake function (i.e. steroids, psychotropics) will be gathered in a brief interview. At the end of the intake visit, eligible participants will take home an actigraph and sleep diary to assess sleep for 7-10 days prior to the memory task procedures. This assessment is conducted in order to 1) capture a representative, prospective sampling of habitual sleep/wake patterns in the home environment and 2) to use sleep data to ensure the lab protocol is aligned with sleep timing at home (within 3 hours of bedtime/wake time). The use of the internet version of the Pittsburgh Sleep Diary, modified for use with children and adolescents, allows for verification that data is collected within close temporal proximity to sleep. Actigraphy will quantify the level and pattern of activity across the 24-hour period to approximate sleep patterns in correspondence with the sleep diary. The wristwatch-sized device (Actiwatch-2, Philips Respironics) is worn on the non-dominant arm, and records number of movements per minute. It summarizes the frequency of motions into epochs of specified time duration and can later be analyzed to generate various sleep parameters.

Emotional Memory Task Protocol. As in past studies and our preliminary data, negative overgeneralization will be assessed by the administration of an emotional memory task that lasts approximately ~20 minutes (two ~10-minute runs) during the Study session and ~40 minutes (four ~10-minute runs) during Test sessions. BOLD fMRI data will be collected during the Study session and at the Test session following a 1-week delay. During the task, images (negative, neutral) are presented in the center of the screen on a black background for 2.5 seconds. Simultaneously, semantically related auditory cues will be presented and co-terminate with the visual stimulus. A variable duration (4-9 seconds) fixation cross is displayed in the center of the screen on a black background during the inter-trial-interval. During the Study session participants will undergo an incidental encoding task where they are shown images in a randomized order and asked to rate the images for their emotional valence (negative, neutral). Test sessions follow at both a 12-hour delay and 1-week delay. During the Test sessions, participants will see *session specific* stimuli that were presented to them during the encoding phase (targets), new stimuli (foils), and stimuli that are similar to the ones seen at encoding but not identical (lures). Emotional valence and similarity level will be evenly distributed across the targets, lures, and foils. Participants will be asked to indicate whether the image they are currently viewing is "old" or "new" with a button press. Instructions during the retrieval phase will explicitly indicate that only the exact image that was presented during the Study phase can be called "old." Thus, responding to critical lures ("false alarms") will provide a signature of generalization within each participant. A total of 175 images will be presented during the Study phase and 266 images will be presented during the Test phases. Sixteen (8 neg) of the original 175 images presented during the Study phase will be tested at the 12-hour Test session along with 30 lures (15 neg) and 24 foils (12 neg); 44 study images (22 neg) will be tested at the 1-week Test session with 85 lures (43 neg) and 67 foils (31 neg).

Sleep Experimental Protocol. For participants randomized to the TMR condition auditory cues that were previously paired with neutral images during Study will be presented during NREM sleep. In contrast, sham auditory stimuli – random guitar strums – will be presented during NREM sleep to participants randomized to the sham group. Specifically, during post study session periods of NREM sleep, sounds that had been paired with a neutral image during learning (or sham sounds) will be presented via loudspeaker (with a 55 dB sound pressure level). Randomly ordered sounds will be presented with an interstimulus interval of 6 seconds with a random jitter of 0 to 0.4 seconds. Each of the sounds will be cued a maximum of 12 times. A trained research assistant will inspect the EEG in real time to determine sleep states and to detect any indicator of an arousal, while an AASM certified sleep technologist will supervise weekly and review all records. Cueing will begin when a participant has spent more than 10 minutes in NREM stage N3 sleep and will be immediately stopped whenever any sign of an arousal, waking up, or any other change in sleep states is observed by the experimenter. In line with prior work

showing beneficial effects of cueing of emotional stimuli during NREM but not REM sleep, and our focus on peripuberty as a developmental cusp of dynamic changes in related neuromaturational processes, we focus only on NREM reactivation in this study

Polysomnography data acquisition parameters. Participants in the sleep condition will be wired for polysomnography following the Study session using Alice Respironic Sleepware G3 or Natus SleepWorks System. The LD_E Headbox EEG will be placed on the scalp for recording of electroencephalographic (EEG) signals (i.e., C3, C4, F3, F4, O1, O2, FCZ, FZ, each referenced to linked mastoids A1/A2) in combination with two electrooculographic (EOG) and two electromyographic (EMG) leads. Signals will be sampled at 500 Hz. These procedures allow for sleep staging according to standard criteria. Quantitative EEG (qEEG) will also be calculated using our team's established methods. Briefly, artifacts will be rejected in 4-second epochs using an automated algorithm for EMG-twitches, and manually to remove eye-movement and pulse artifacts. Artifact-free NREM and REM epochs will be subjected to spectral analysis using a fast Fourier transform model.

MRI acquisition and preprocessing parameters (NOT relevant for clinical trial outcomes but included here for procedural reference). Neuroimaging data will be collected on a Siemens Prisma 3T scanner equipped with a 32-channel head coil at Florida International University. BOLD (TR/TE = 2000/25 ms, flip angle = 75°, resolution = 2.5 x 2.5 x 3 mm, FOV = 240 x 240 mm^2, 42 axial slices) and structural (T1-weighted magnetization-prepared rapid gradient-echo [MP RAGE], TR/TE = 9.184/3.68 ms, flip angle = 90°, resolution = 1 x 1 x 1 mm, and FOV = 256 x 256 mm) MRI data will be collected during the Study session and Test session following a 1-week delay. Neuroimaging data will be preprocessed and analyzed using Nipype[99] and standard preprocessing pipelines from Nipy[100], artifact detection toolbox (ART – as implemented in Nipype) and custom in-house software. Briefly, the following preprocessing steps will be implemented: simultaneous slice-timing and motion correction[105], registered to structural scans, normalized to a study specific template, temporally high-pass filtered (0.008 Hz) and spatially smoothed with a 5 mm FWHM Gaussian kernel. Data will be reviewed for motion related artifacts. Data points (TRs) that exceed 3 z-normalized standard deviations away from the global brain activation or a composite frame-wise displacement of 0.5 mm from the preceding scan will be flagged as outliers. Motion parameters and their first and second derivatives as well as outlier time points will be regressed out of the first level general linear models. The amygdala and subfields of the hippocampus will be automatically segmented using a multi-atlas segmentation, similarity-weighted voting, and learning-based bias correction technique implemented in the Advanced Normalization Tools (ANTs) package. Automatic segmentation will be followed by manual verification and corrections.

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Statistical Plan

Primary Hypothesis (1 week outcomes): We expect audio cueing of neutral memories relative to sham cueing will result in decreased Lure Generalization Index (LGI) for negative stimuli and increased LGI for neutral stimuli at one week. To test these hypotheses, we will employ a mixed-effects model. The dependent variable will be the Lure Generalization Index (LGI) at 12 hours. The model will include fixed effects for valence (neutral vs. negative stimuli), experimental condition (neutral Targeted Memory Reactivation [TMR] vs. sham), and their interaction term (valence x condition). A random subject effect will be included to account for individual variability. Age (months) and gender will also be included as fixed effects to control for known gender and developmental differences in sleep, memory and anxiety.
Should the valence by condition interaction prove statistically significant, we will conduct planned post-hoc comparisons (using t-tests with the Welch-Satterwaite equation) to thoroughly investigate the directionality and magnitude of the effects for LGI across the different valence categories and experimental conditions.

Secondary Hypothesis (12 hour outcomes): We will apply the same analytical approach as described for the 1-week outcomes to the 12-hour outcomes. We expect to observe a similar pattern of results, though with a smaller effect size.