Journal of Visualized Experiments

Measuring Neural Mechanisms Underlying Sleep-Dependent Memory Consolidation During Naps in Early Childhood

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60200R1
Full Title:	Measuring Neural Mechanisms Underlying Sleep-Dependent Memory Consolidation During Naps in Early Childhood
Keywords:	polysomnography, memory, consolidation, actigraphy, sleep spindles, visuospatial memory, early childhood, nap, sleep
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	College Park, MD, USA

TITLE:
Measuring Neural Mechanisms Underlying Sleep-Dependent Memory Consolidation During Naps in Early Childhood

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SUMMARY:
This protocol describes methods used to examine neural mechanisms underlying sleep-dependent memory consolidation during naps in early childhood. It includes procedures for examining the effect of sleep on behavioral memory performance, as well as the application and recording of both polysomnography and actigraphy.

ABSTRACT:
Sleep is critical for daily functioning. One important function of sleep is the consolidation of memories, a process that makes them stronger and less vulnerable to interference. The neural mechanisms underlying the benefit of sleep for memory can be investigated using polysomnography (PSG). PSG is a combination of physiological recordings including signals from the brain (EEG), eyes (EOG), and muscles (EMG) that are used to classify sleep stages. In this protocol, we describe how PSG can be used in conjunction with behavioral memory assessments, actigraphy, and parent-report to examine sleep-dependent memory consolidation. The focus of this protocol is on early childhood, a period of significance as children transition from biphasic

sleep (consisting of a nap and overnight sleep) to monophasic sleep (overnight sleep only). The effects of sleep on memory performance are measured using a visuospatial memory assessment across periods of sleep and wakeful-rest. A combination of actigraphy and parent report is used to assess sleep rhythms (i.e., characterizing children as habitual or non-habitual nappers). Finally, PSG is used to characterize sleep stages and qualities of those stages (such as frequencies and the presence of spindles) during naps. The advantage of using PSG is that it is the only tool currently available to assess sleep quality and sleep architecture, pointing to the relevant brain state that supports memory consolidation. The main limitations of PSG are the length of time it takes to prepare the recording montage and that recordings are typically taken over one sleep bought. These limitations can be overcome by engaging young participants in distracting tasks during application and combining PSG with actigraphy and self/parent-report measures to characterize sleep cycles. Together, this unique combination of methods allows for investigations into *how* naps support learning in preschool children.

INTRODUCTION:

Given sleep's prevalence in our daily routine, it is important to understand its function. Studies with this objective require precise measurement of sleep. Polysomnography (PSG) is the gold-standard measure of sleep. PSG allows for objective, quantitative measurement of sleep with high temporal resolution and can be useful for both research and clinical purposes. PSG is a combination of physiological recordings. At minimum, a PSG montage includes the following measures: electroencephalography (EEG), electrooculography (EOG), and electromyography (EMG). These measures assess electrical potentials from the brain, the eyes, and muscles respectively, and allow for classification of sleep stages (see **Figure 1**). Other measures, such as electrocardiography (ECG), respiration, and pulse oximetry may be included to identify the presence of disordered sleep.

[Place **Figure 1** here]

PSG allows sleep to be characterized into four distinct sleep stages: non-rapid eye movement (non-REM) stage 1 (nREM1; 4−7 Hz), non-REM stage 2 (nREM2; 12−15 Hz), and non-REM stage 3 (more commonly known as slow wave sleep [SWS]; 0.5−4 Hz), and rapid-eye movement (REM) sleep. nREM1 marks sleep onset, and is identified based on reduced muscle tone in the EMG recoding and mixed amplitude EEG oscillations relative to the alpha observed in resting wake. This is followed by nREM2, which can be distinguished by the presence of sleep spindles (short bursts of sigma frequency activity; 11−16 Hz) and K-complexes (single slow-waves that stand out from the surrounding activity) in the EEG. SWS is characterized by distinct slow-frequency high-amplitude EEG oscillations. REM sleep is characterized by fast low-amplitude oscillatory brain activity very similar to that observed during wake. However, what distinguishes REM sleep from wake is that it is also characterized by phasic rapid eye movements (hence the moniker REM) and muscle atonia. Over the course of a sleep bout, sleep stages are experienced cyclically, at a rate of about 90 min/cycle.

Sleep also follows the circadian rhythm, with sleep bouts taking place in 24-h cycles. Sleep timing and consistency may influence sleep function and are also important to assess. Although PSG is necessary to characterize sleep stages, it is time-consuming to apply and therefore not ideal for assessing multiple sleep bouts (e.g., multiple nights of sleep, naps and overnight sleep). For this, actigraphy is beneficial. Actigraphy uses a tri-axial accelerometer, typically on the wrist, to estimate sleep based on the absence of movement. Although actigraphy cannot be used to characterize sleep stages, it has been shown to be reliable at detecting sleep onset and wake onset (including sleep fragmentation or wake after sleep onset) in a range of populations from infants[1] to older adults[2]. Both PSG and actigraphy are preferred methods over self/parent-report measures. Self/parent-report measures are easy to administer and relatively inexpensive, however, they are also subject to bias and non-compliance. Finally, it is worth noting that these methods can be used in combination to capitalize on the strengths of each. For example, PSG can be combined with actigraphy and/or self/parent-report to obtain both overnight sleep quality as well as verification of sleep quantities or sleep-wake cycles, especially over long durations (e.g., weeks or months).

One function of sleep that has garnered particular interest is sleep-dependent memory consolidation, the processing of memories that leaves them stronger and less vulnerable to interference[3]. Although memory consolidation can take place during wake in children[4] and adults[5]**,** there is substantial evidence that consolidation is enhanced during sleep. Past research has provided behavioral examples of sleep-dependent memory consolidation by comparing changes in memory performance following an interval of sleep (e.g., 8 pm−8 am) to changes following an equivalent interval spent awake (e.g., 8 am−8 pm). In adults, memories are protected[6] or even enhanced[7] following an interval of sleep while memories typically decay over an equivalent interval of wake. Controls have been employed that dissociate performance changes from circadian influences[8-10] For example, similar benefits of sleep are observed when comparing performance over a mid-day nap to an equivalent mid-day wake period[9].

Although sleep was once thought to reflect a passive process, simply protecting memories from decay or interference, modern theories suggest sleep plays a more active role and actually promotes memory through reactivations[11-13]. Support for this comes from observed correlations between behavioral measures of memory consolidation over sleep (change in memory recall after sleep compared to before sleep) and specific aspects of sleep physiology. For many declarative memory tasks, memory consolidation is associated with aspects of non-REM sleep, specifically measures of SWS or sleep spindles found in nREM2 and SWS. If sleep's role was passive protection from interference, such a correlation would not be expected; rather a correlation between time asleep (regardless of sleep stage) and performance would be expected, as more time asleep would provide more protection from interference[14].

Additional support for the active role of SWS in memory consolidation is evident in studies of targeted memory reactivation. In these studies, a memory is learned in the context of a perceptual cue, for instance an odor, and recall of the memory is greater following sleep if the cue is re-presented during sleep, SWS in particular[15]. Although the underlying mechanism is debated[16,17], one prominent theory, systems consolidation theory, contends that memories encoded in the hippocampus are stabilized in the cortex though hippocampal-neocortical dialogue. Specifically, cortical slow waves and sleep spindles, occurring in conjunction with hippocampal ripples with memory reactivation, support the memory transfer[3].

The role of sleep in memory consolidation during development is less clear. Early childhood is a period of particular interest as children begin to transition from a biphasic (consisting of a mid-day nap and an overnight sleep bout) to a monophasic sleep pattern. Recent research suggests that this transition may reflect brain maturation[18]. This argument is consistent with empirical data showing developmental changes in overnight sleep (i.e., topography of slow wave activity) mirrors that of cortical maturation[19].

Although there are several behavioral demonstrations of overnight sleep-dependent consolidation in children[20,21] and infants[22], research on the neural underpinnings of memory consolidation with mid-day sleep are just beginning to be investigated. In ground-breaking work examining the role of mid-day naps on memory in preschool children, naps were shown to protect memories of recently learned information, whereas memory was reduced (by ~12%) when children stayed awake during the nap interval[23]. This "nap benefit" was greatest in children who napped habitually (i.e., 5 or more times per week as measured with actigraphy) regardless of their age. By recording PSG during the nap, the change in memory performance across the nap period was found to be specifically associated with sleep spindle density (the number of sleep spindles per minute of nREM), suggesting nap quality (not quantity) was a critical factor in promoting memory retention (see the representative results section).

This study highlights the significance of PSG in exploring relations between sleep and memory during development. It points to the importance of characterizing sleep macro- (sleep stages) and micro- (qualities of those stages such as frequencies and the presence of spindles) structures during naps for memory consolidation. It also highlights the importance of assessing sleep rhythms (characterizing children as habitual or non-habitual nappers). Although our work has characterized the function of naps in visuospatial learning (and more recently emotional[24] and procedural[25] learning), many questions remain. For instance, it will be important to examine other declarative memory tasks to assess the generalizability of these findings and to assess tasks used in preschool classrooms to understand specific parameters (e.g., amount of nap benefit relative to learning) for ecologically valid tasks. Additional work will also be necessary to understand when wake is sufficient for memory consolidation. Thus, our objective is to demystify the process of measuring sleep and sleep-dependent memory consolidation in children. We provide practical tips for examining the benefit of an afternoon nap on declarative memory in typically developing preschoolers (approximately 3 to 4 years of age) using a computerized visuospatial memory task as well as methods for assessing nap habituality using actigraphy, parent-report, and nap physiology using PSG. Although these methods were developed for preschool age children who nap with varying frequency, these methods could be adapted to any age group.

PROTOCOL:

Prior to beginning any research procedures, written consent should be obtained from the parent and verbal consent should be obtained from the child for all study procedures.

NOTE: See **Figure 2** for an overview of all procedures.

[Place **Figure 2** here]

1. Nap promotion condition

1.1. Ensure that the nap promotion condition is counterbalanced with the wake promotion condition across participants as discussed below.

1.2. Schedule the nap promotion condition to begin approximately one hour before the child's typical nap period to allow time to apply PSG and to conduct the visuospatial memory task. Ensure that the time between immediate and delayed recall are the same between the wake promotion and nap promotion conditions.

1.3. Explain the procedures for this session to children and parents using age-appropriate materials.

NOTE: Age appropriate materials include story books or short videos of another child undergoing the same procedures.

1.4. Apply polysomnography equipment (see section 3).

1.5. Conduct the encoding and the immediate memory assessment for the visuospatial memory task (see section 4).

1.6. Have the child use the restroom and then initiate the child's typical pre-nap routine.

1.6.1. Allow the parent/caregiver to put the child to sleep as they usually would. Interfere as little as possible because most children fall asleep faster when provided with their normal routine.

1.6.2. Allow the child to nap utilizing their typical nap location.

1.6.3. Utilize nap promotion techniques, but only when necessary as these have proven less successful in the home if they deviate too far from the child's normal routine.

NOTE: Nap promotion techniques include using a weighted blanket, rubbing the participant's back, wrapping the child in a blanket (similar to swaddling), progressive muscle relaxation, and playing soothing music.

1.7. Ensure that the amount of time the child sleeps in the nap promotion condition matches the amount of time they play in the wake promotion condition.

NOTE: If the nap promotion condition is first, allow the child to wake up naturally and use this duration to set the duration of the awake session. If the wake promotion condition is first, use this duration to determine the length of the nap. If the nap exceeds this time, wake the child as naturally as possible by opening the door, walking around outside the bedroom, and gradually speaking louder.

1.8. Conduct the delayed recall assessment for the visuospatial memory task, approximately 15−30 min after the child wakes to avoid sleep inertia.

1.9. Collect child and experimenter rating for Visual Sleepiness Scale (VSS)[26] and Visual Mood Scale (VMS)[27].

1.10. Remove PSG electrodes.

2. Wake promotion condition

2.1. Ensure that the wake promotion condition is counterbalanced with the nap promotion condition discussed above.

2.2. Schedule the wake promotion condition to begin approximately one hour before the child's typical nap period to equate time of day across conditions. Ensure that the time between immediate and delayed recall are approximate the same between the wake promotion and nap promotion conditions.

2.3. Explain the procedures for this session to children and parents using age-appropriate materials.

2.4. Apply PSG electrodes (see section 3) in order to equate the wake and nap promotion conditions.

NOTE: Although sleep is not expected, this equates conditions and can be used to verify the absence of sleep if in doubt.

2.5. Conduct the encoding and the immediate memory assessment for the visuospatial memory task (see section 4).

2.6. Have the child use the restroom and then proceed to the location that they typically nap.

2.6.1. Do not allow the child to nap, instead, have the child play quietly with non-stimulating toys in the same location as their typical nap.

NOTE: Acceptable non-stimulating toys include small sensorimotor toys such as wax sticks and age-appropriate interlocking plastic bricks.

2.6.2. Have the child play for their typical nap length or for the time they slept during the nap promotion condition (see step 1.7 for additional information).

2.6.3. Record any unusual activity such as talking, leaving the room, and playing with toys that are not provided.

2.7. Ensure that the amount of time the child sleeps in the nap promotion condition matches the amount of time they play in the wake promotion condition.

2.8. Conduct the delayed recall assessment for the visuospatial memory task, approximately 15−30 min after the child is finished playing in order to keep delay time similar between conditions.

2.9. Collect child and experimenter rating for VSS[26] and VMS[27].

2.10. Remove PSG electrodes.

3. Polysomnography (PSG)

3.1. Preparation

3.1.1. Facilitate PSG electrode application by having the child engage in a quiet activity such as reading a book, playing with playdough, eating a snack if they are hungry, or watching a short movie.

NOTE: If a movie is used, ensure that the movie is age appropriate but does not elicit rowdiness in the child (e.g., popular child-friendly animated films or shows).

3.1.2. Accessibility to a parent or guardian is not required. However, for shy and tentative children ensure that trusted caregivers are available.

NOTE: For a small number of children, parents and guardians may be distracting instead of helpful. If this is the case, ask the parent if they would be willing to step out of the child’s sight.

3.2. Collect head measurements.

3.2.1. Use a flexible tape measure and china marker to mark locations for subsequent electrode application.

3.2.2. Measure the distance from the inion to nasion and place a mark at the halfway point. Measure the distance from preauricular to preauricular and place another mark at the halfway point. The intersection of these two marks is the “Reference” point (CZ).

3.2.3. Measure 10% of the inion to nasion distance up from the inion. Then measure out 10% of the preauricular to preauricular measurement from this point on either side. Make two marks, one on each side (O3 and O4).

3.2.4. Measure 20% of the preauricular to preauricular measurement from the reference point on either side of the head. Make two marks, one on each side (C3 and C4).

3.2.5. Measure 20% of the inion to nasion distance up from the reference point. Then measure out 20% of the preauricular to preauricular measurement from this point on either side. Make two marks, one on each side (F3 and F4).

3.3. Prepare one electrode at a time for placement.

3.3.1. Clean each electrode location using an alcohol swab. Exfoliate using a slightly abrasive gel and then remove any residual cleaning material.

3.3.2. Fill each electrode using electrode cream.

3.3.2.1. For electrodes placed where hair is present, apply an additional drop of electrode cream to a gauze square and place it on the back of the electrode.

3.3.2.2. For electrodes placed on the face, use medical tape to adhere the electrode to the skin.

3.4. Place an electrode on each corresponding EEG, EOG, and EMG location.

3.4.1. Place an electrode on each marked location on the scalp (CZ, O3, O4, C3, C4, F3, and F4).

3.4.2. Place one electrode on each mastoid (small bony process behind the ear) and one in the center of the forehead.

3.4.3. Place one EOG electrode adjacent to each eye. Place one of these electrodes slightly superior to the outside of the right eye (termed ROC) and one to the outside and slightly inferior to the left eye (termed LOC).

3.4.4. Place two EMG electrodes around the chin area. Place one electrode on the right cheek just above the smile line. Place the other on the left side just above where the chin meets the neck, adjacent to the esophagus. Find the second location by having the participant say the word “milk” out loud while feeling for the location where muscle contractions in the neck and chin are maximal.

3.5. Attach electrodes to the recording device and initiate recording.

3.6 Record impedance readings for all electrodes. Ensure all electrodes pass the impedance test.

NOTE: Some devices may note a 'Pass' or 'Fail', while other devices may give numeric values. In the latter, impedances under 25 kΩ are acceptable. If an impedance fails or is too high, remove and replace the batteries. If this does not amend the issue, reapply that electrode.

3.7. At the completion of each conditions, remove the PSG electrodes.

3.7.1. For electrodes applied in the hair, soak the location of the electrode with a water-based spray. Allow the spray to sit for about one minute then remove the electrode.

NOTE: Detangling hair spray is highly effective for the purpose of removing hair electrodes.

3.7.2. For electrodes applied with tape, typically on the face and mastoids, use a cotton pad with baby oil applied to it to saturate the tape. When the tape is completely covered in baby oil, gently pull the tape up from the corners.

4. Visuospatial memory task

4.7. Administer nine to-be-remembered stimuli arranged in a 3 x 3 matrix to children younger than 44 months of age. Administer the 12 to-be-remembered stimuli arranged in a 3 x 4 matrix to children older than 44 months of age.

NOTE: If a child assigned to the 12-item matrix is too challenged, the 9-item matrix can be used. Likewise, if it is evident that the 9-item matrix is too easy, the 12-item matrix can be used to avoid ceiling effects. This is justified because within-subject accuracy is of the variable of interest and not raw scores. Stimuli are typically cartoon-like pictures of common images (e.g., bear, car, scissors) arranged in a matrix and presented on a laptop screen. There are two sets of stimuli. This allows the task to be counterbalanced across the two conditions (i.e., nap versus wake promotion) so that children do not receive the same pictures in both conditions.

4.8. Administer the task in three phases: encoding, immediate recall, and delayed recall. For each phase allow the child to answer each question at their own pace.

NOTE: Typical durations are: 6 min for the encoding phase, 2 min for the immediate recall phase, and 2 min for the delayed recall phase.

4.8.1. In the encoding phase, direct the child to identify each image by name, then instruct the child to remember their location of each item on the grid. Following encoding, the cards are replaced with 'blank' images and the child must then locate the position of each image until they reach an encoding score ≥70%.

NOTE: A threshold of 70% was chosen based on studies in young adults[28-30] and reflects a point when learning is clearly reached but not at ceiling.

4.8.1.1. During this block, participants receive visual feedback from the task after each response.

After the child selects an image location, reveal the associated image, informing the child whether that was the correct or incorrect location.

4.8.1.2. Provide verbal feedback on performance to motivate the child but ensure that the amount of feedback is consistent across both conditions. When the child succeeds at locating an image use language like "Great job, you got that one!" When a child fails use language that highlights the child's effort (e.g., "Whoops! Not quite but good try! Let's see if you can get the next one.").

4.8.1.3. Provide children who are assigned to the 12-item matrix that cannot pass encoding after 4 rounds with an opportunity to stretch, do jumping jacks, and move for about 5 min. If the child still cannot pass encoding after an additional 2 rounds, restart encoding with the 9-item matrix.

4.8.1.4. Provide children assigned to the 9-item matrix who receive an encoding score of 100% on the first round with the encoding task for the 12-item matrix. If they do not go through all the necessary steps to drop back to the 9-item matrix, use the 12-item matrix for the following two phases.

4.8.2. During the immediate recall phase, present the images again, one at a time, and ask the child to recall the corresponding location. Do not provide visual or verbal feedback, and only probe each item once. However, do provide feedback on effort (i.e., "Good job giving your best effort").

4.8.3. Conduct the delayed recall phase immediately after the wake or sleep condition.

NOTE: This phase is identical to the immediate recall phase.

4.8.3.1. At times children will become fussy during the delayed recall phase. If this happens, entice the child to complete the task with a prize or by offering more time to watch their movie during PSG removal. During this time do not allow the child to play with toys or engage in other tasks until the memory task is complete.

[Place **Figure 3** here]

5. Actigraphy

5.7. Program the activity watch.

NOTE: The activity watch is sampled at 32 Hz, with a sensitivity of <0.01 *g*. Activity is stored in 15-s epochs.

5.8. Provide each participant with a pre-programmed activity watch and instructions. Tell the parent that the watch should always be worn. Highlight that it is waterproof so there is no reason to remove the device.

5.8.1. Instruct the child to wear the watch on their non-dominant hand continuously.

5.8.2. Instruct the parent to press the button on the side of the watch face every time their child attempts to sleep, and then again when they wake.

NOTE: This generates an event marker in the data which assists with scoring actigraphy.

5.9. Provide the parent with a sleep diary (similar to a log or spreadsheet) on which they can record sleep times and watch removal.

NOTE: This also assists with scoring actigraphy.

5.9.1. In the sleep diary, ask the parent to provide a complete log of all sleep for the number of days that the activity watch will be worn, including the time that the child goes to bed and when the child wakes up. The parent should provide this information for both naps and regular overnight sleep. Additionally, ask the parent to provide information about any time when the watch is removed.

6. Data analysis

6.7. Visuospatial memory task

6.7.1. Calculate accuracy for each recall phase as the percent of items recalled.

6.7.2. Calculate change in recall over the nap and wake intervals as follows.

6.7.2.1. Calculate change in recall_{nap} by subtracting immediate recall accuracy (before nap) from delayed recall accuracy (after nap).

6.7.2.2. Calculate change in recall_{wake} by subtracting immediate recall accuracy (before wake) from delayed recall accuracy (after wake).

6.8. PSG

6.8.1. Characterize sleep stages in accordance with the standard scoring criteria (e.g., The AASM Manual for the Scoring of Sleep and Associated Events v. 2.5).

6.8.2. Detect sleep spindles at C3 using specialized software by marking spindle onsets and offsets.

6.8.3. Verify sleep stages and spindle onsets and offsets with second trained researcher. In the event the scorings are not concordant, have a third trained scorer make the consensus decision.

6.8.4. Analyze spindle density using specialized software and an in-house MATLAB code based on previous studies[31]. In brief, filter EEG data from 0.5−35 Hz. Consider the maximum voltage between the identified spindle onset and offset the peak spindle amplitude. Use a fast Fourier transform of each spindle to identify the peak spectral frequency between 9−15 Hz[24,32].

6.9. Actigraphy

6.9.1. Score activity watch data using specialized software following standardized protocols[20].

NOTE: Multiple days and nights of data are required to ensure reliability of the data. At minimum, participants need at least three days and three nights of actigraphy data (days and nights do not need to be consecutive); however, 5 nights is preferable, particularly when these data are of primary interest[33].

6.9.2. Use sleep diary information and event markers (button presses) to verify sleep onset and offset.

NOTE: These two items must be within 20 min of each other in order to score the start and end of a rest interval.

6.9.2.1. If a participant is missing sleep diary information, event markers, or the diary and event markers are not within 20 min of each other, determine sleep onset and offset manually[32]: determine sleep onset by the first three minutes of continuous sleep[33] and determine sleep offset by the last five minutes of continuous sleep[34].

REPRESENTATIVE RESULTS:

Using the procedures described here, Kurdziel and colleagues[23] examined sleep-dependent memory consolidation during naps in preschool children. Results showed children's recall accuracy on the visuospatial memory task after a nap was better than their recall accuracy after a similar period during which they remained awake (i.e., signifying a "nap benefit", **Figure 4**). Moreover, those who spent the prior day in the wake condition did not recover memories during overnight sleep. Finally, the actigraphy and parent reported sleep measures were used to examine whether the nap benefit was apparent in both habitual and non-habitual nappers. Findings revealed the nap benefit was only significant in children who napped regularly (i.e., habitual nappers, **Figure 5**).

[Place **Figure 4** and **Figure 5** here]

PSG was used to examine relations between sleep physiology and nap-dependent memory consolidation in both habitually and non-habitually napping children. There was a significant negative correlation between immediate recall accuracy and with sleep spindle density. The better a child performed at immediate recall, the fewer sleep spindles they displayed during nREM2 sleep (**Figure 6A**). This is consistent with previous studies which report a negative correlation between sleep spindles and IQ[35]. Importantly, there was a positive correlation between change in $recall_{nap}$ and sleep spindle density during nREM2 (**Figure 6B**). However, no other measure of sleep physiology (i.e., spindle amplitude, spindle frequency, etc.) was related to memory performance.

[Place **Figure 6** here]

In sum, these results show that napping is associated with improved memory consolidation, especially in habitual nappers. Nap-related improvement in memory performance is related to sleep physiology assessed by PSG in early childhood. Therefore, PSG is an important method for understanding the mechanisms that underlie relations between sleep and memory consolidation in early childhood. Together, these results suggest that naps are critical for long-term memory consolidation in children.

FIGURE LEGENDS:

Figure 1: Example electrode placement and description of activity recorded via PSG.

Figure 2: Overview of protocol. Each square represents one day.

Figure 3: Examples of screen displays during the visuospatial memory task.

Figure 4: Recall accuracy on the visuospatial memory task was tested immediately following encoding ("Immediate"), following the nap opportunity ("Delayed"), and again the following day ("24-hour") across two conditions: a nap-promoted condition (gray bars) and wake-promoted condition (white bars). Error bars represent ± 1 SE. This figure is reprinted with permission from Kurdziel et al.[23].

Figure 5: Change in recall accuracy (delayed recall minus immediate recall) across the nap (gray bars) and wake (white bars) intervals for habitual nappers (who took five to seven naps per week) and non-habitual nappers (those who took zero to two naps per week). Error bars represent ± 1 SE. This figure is reprinted with permission from Kurdziel et al.[23].

Figure 6: Sleep spindle density (spindles per minute of non-REM stage 2 sleep) associations with (A) immediate recall accuracy and (B) the change in recall accuracy from the immediate to delayed recall phase. This figure is reprinted with permission from Kurdziel et al.[23].

DISCUSSION:

This article describes how to investigate sleep-dependent consolidation of declarative memory during naps in early childhood. Methods include behavioral assessment of memory across nap and awake conditions, actigraphy and parent-report to assess sleep cycles, and PSG to assess sleep architecture. This unique combination is critical for assessing memory, characterizing sleep cycles, and examining the neural mechanisms underlying the benefit of sleep on memory. Representative results indicate that learning and memory were dependent on mid-day sleep, particularly for habitual nappers. Specifically, habitual nappers showed a greater benefit from napping compared to staying awake (i.e., nap benefit score). In addition, across all children, better retention across the nap period (i.e., nap change score) was related to sleep spindles recorded during nREM2; greater retention over the nap was associated with more sleep spindles. Although the combination of methods described is critical for full characterization of the impact of sleep on memory, perhaps the most important aspect of this method is identification of underlying neural mechanisms associated with this effect using PSG. At present, PSG is the only methodological tool that allows for characterization of sleep quality via measurement of sleep stages. Thus, it is the only method that allows for insight into neurobiological mechanisms underlying sleep-dependent effects, such as memory consolidation.

Major advantages of PSG include the fact that it is non-invasive and allows for characterization of four sleep stages, including sleep stages nREM 1−3 and REM. The most critical step in acquiring PSG is thoroughly cleaning electrode sites before application in order to achieve low impedances and subsequent high-quality data to realize this advantage. Another advantage is that PSG is portable and easy to administer, even in young children. Furthermore, the technique can be modified to increase resolution. Although we describe a low-density montage of 7 EEG electrodes, higher density EEG montages using specialized caps in order to examine the topographic distribution of sleep-related activity such as sleep spindles may also be used. This can be useful as topography changes developmentally[14]; however, these systems are not ambulatory and can be less comfortable. Finally, although we describe how to record PSG during mid-day sleep, the same method can be applied overnight to examine sleep at other periods, including overnight sleep. It can also be modified for clinical use to assess sleep disturbances (i.e., inclusion of ECG, respiration, pulse ox). We describe how data obtained during PSG can be related to sleep-dependent consolidation of declarative memories (i.e., visuospatial memory). However, other types of memory (e.g., procedural memory, emotional memory, language, etc.) and their relation to sleep components can also be examined[23,28,25,36-38].

The main limitation of PSG is the time it takes to apply the electrodes. In children this can be especially important as they are prone to boredom and limited attention. These effects can be mitigated by providing subjects with distractors during administration (e.g., toys, books, videos). Additionally, PSG typically records activity during one sleep bout. However, it can be combined with self-report and/or actigraphy to obtain insight into sleep quantities or sleep-wake cycles over longer durations (e.g., weeks or months). Finally, PSG can be uncomfortable, and disturb sleep at times. Note that for this reason, an adaptation sleep bout can be considered. This must be weighed against the additional burden placed on the participant and challenges recruiting to the study.

Although PSG is critical for examining the neurobiological mechanisms underlying sleep-dependent effects, proper administration of all other aspects of the protocol described (i.e., behavioral assessment of memory across nap and awake conditions, actigraphy and parent-report of sleep cycles), are paramount to realizing its full potential. The most critical step in administering the nap and wake promotion conditions is to ensure that the time between immediate and delayed recall is the same between conditions and that the interference is minimized during the wake promotion condition. The former can be achieved by adhering to clear protocols and proper documentation of time for each session for each participant. The latter can be achieved by monitoring of the child's activity during the wake condition and providing them, only when necessary, activities that are least likely to interfere (e.g., for the visuospatial memory task that taps declarative memory avoiding activities that engage declarative systems such as books or verbal material).

In conclusion, PSG is the gold-standard assessment of sleep quality. It allows for objective, quantitative measurement of sleep with high temporal resolution that which can be useful to better understand sleep function. When paired with other tools (e.g., behavioral assessment of memory, actigraphy, and parent-report of sleep) it can yield important and interesting findings regarding how sleep contributes to healthy cognitive development of young children.

ACKNOWLEDGMENTS:
The authors would like to thank the Neurocognitive Development Lab at the University of Maryland, College Park and the Somneuro Lab at the University of Massachusetts, Amherst for assistance with this project. Funding was provided by NIH (HD094758) and NSF (BCS 1749280) to TR and RS. Representative results were funded by NIH HL111695.

DISCLOSURES:
The authors have nothing to disclose.

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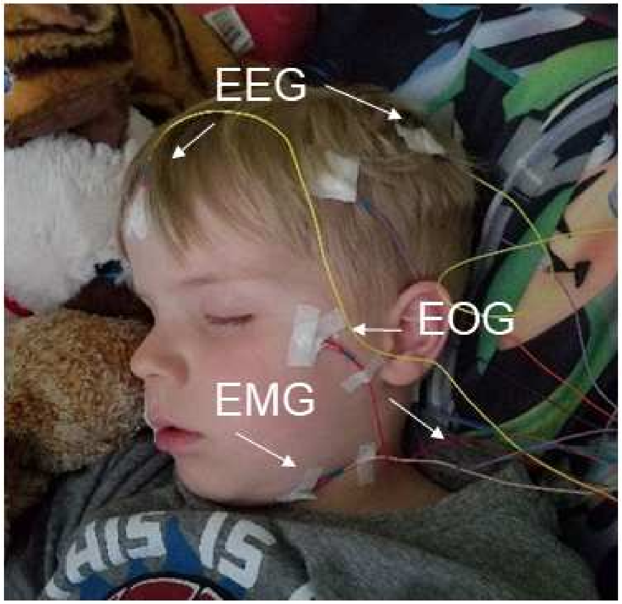
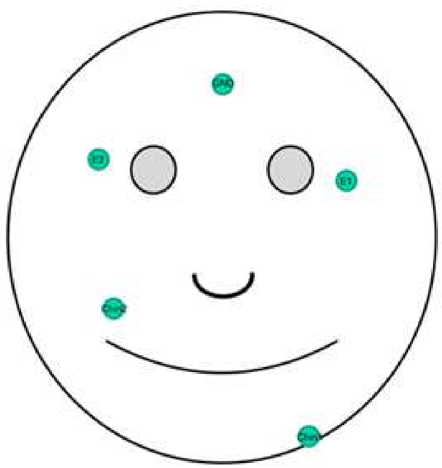


	brain waves (recorded with EEG)	muscle activity (recorded with EMG)	eye activity (recorded with EOG)
wake	very high frequency	low-high	low-high (high/random)
REM	very high frequency (similar to wake)	none	high (slow/rolling)
nREM1	low amplitude/ high frequency	low	none-low
nREM2	low amplitude/high frequency with sleep spindles and K-complexes	low	none-low
SWS	high amplitude/ low frequency	low	none-low

Electrode locations on the face (frontal view)



Electrode locations on the head (top view)

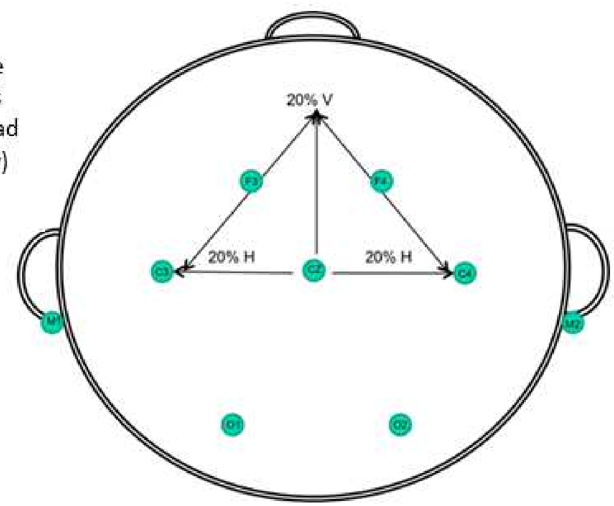
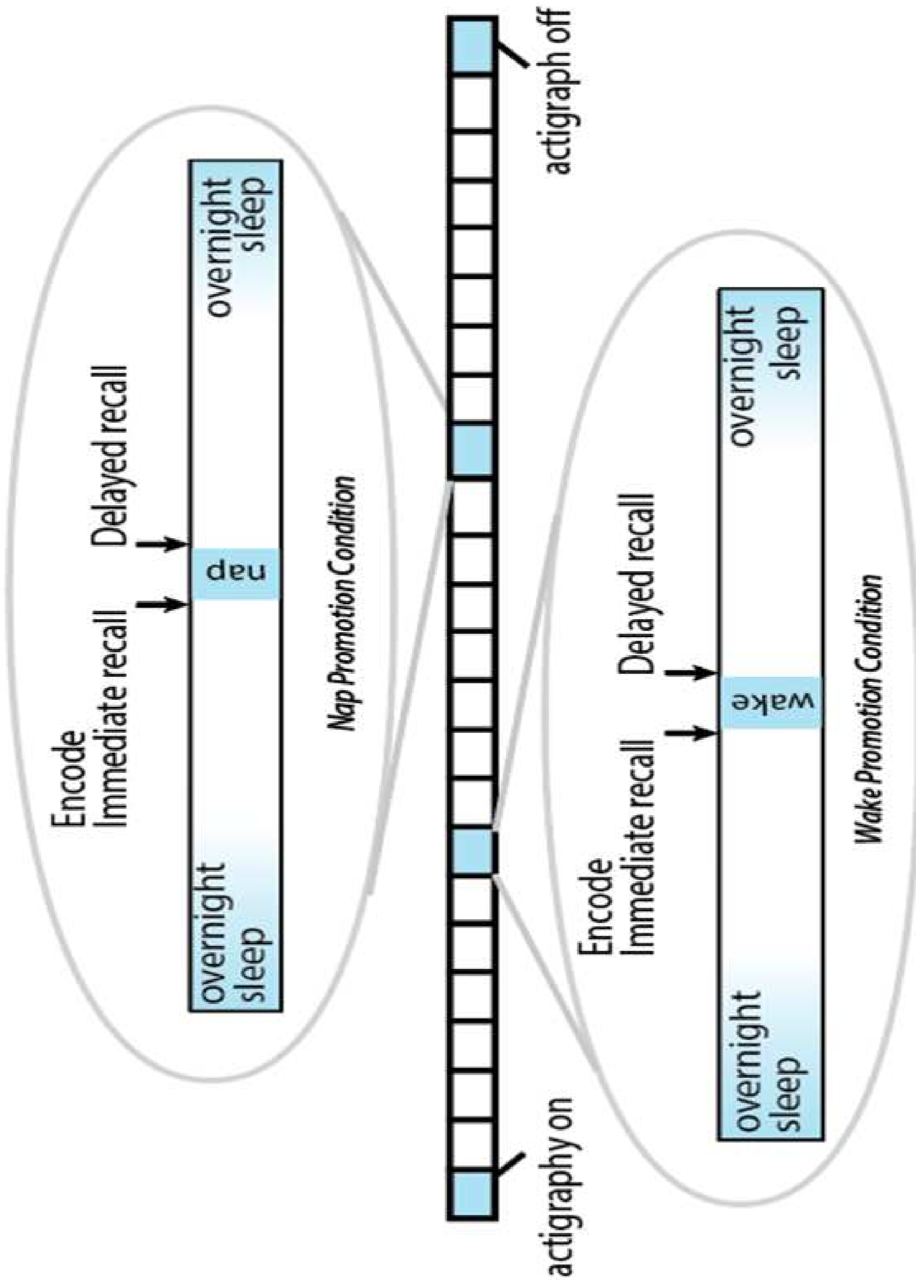


Figure 2


actigraph off
actigraphy on
Encode
Immediate recall
Delayed recall
overnight sleep
nap
overnight sleep
Nap Promotion Condition
Encode
Immediate recall
Delayed recall
overnight sleep
wake
overnight sleep
Wake Promotion Condition

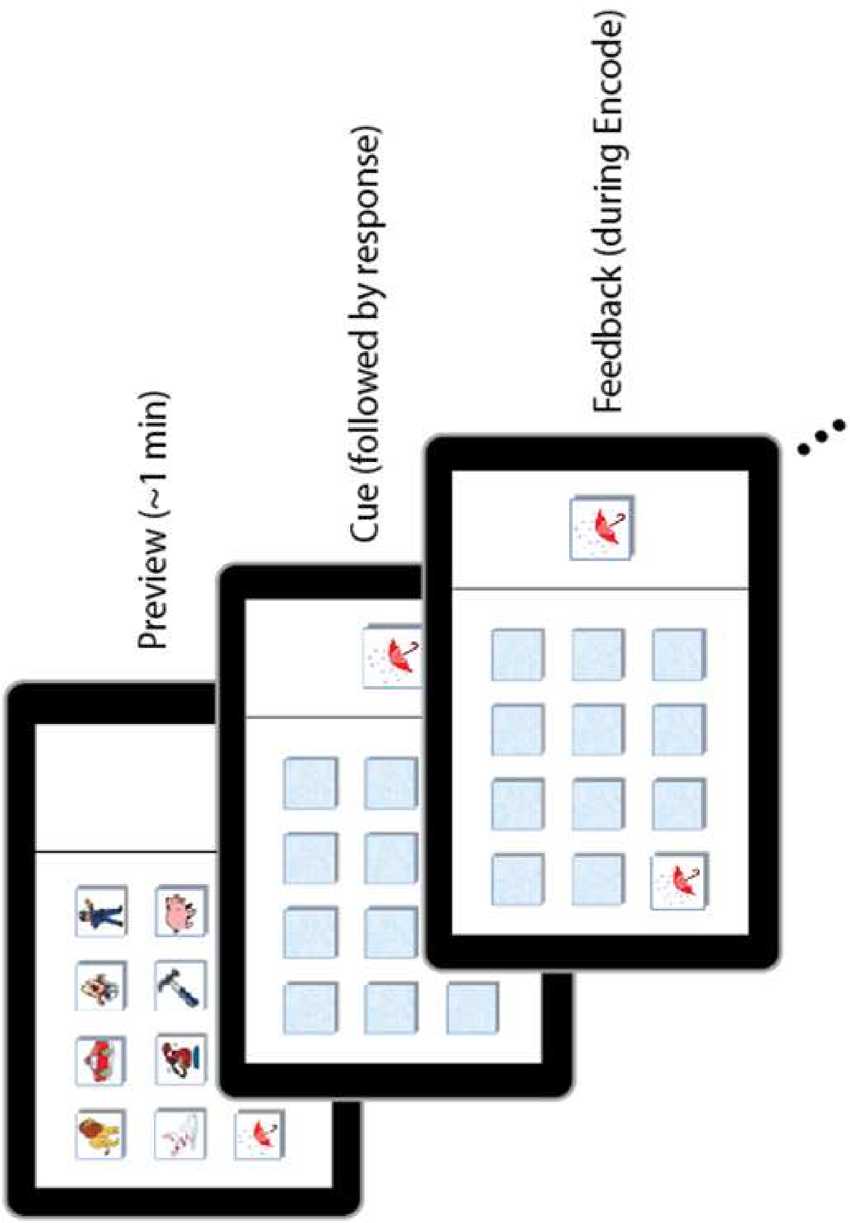

Preview (~1 min)
Cue (followed by response)
Feedback (during Encode)

Figure 4 Click here to access/download;Figure;Figure 4.eps

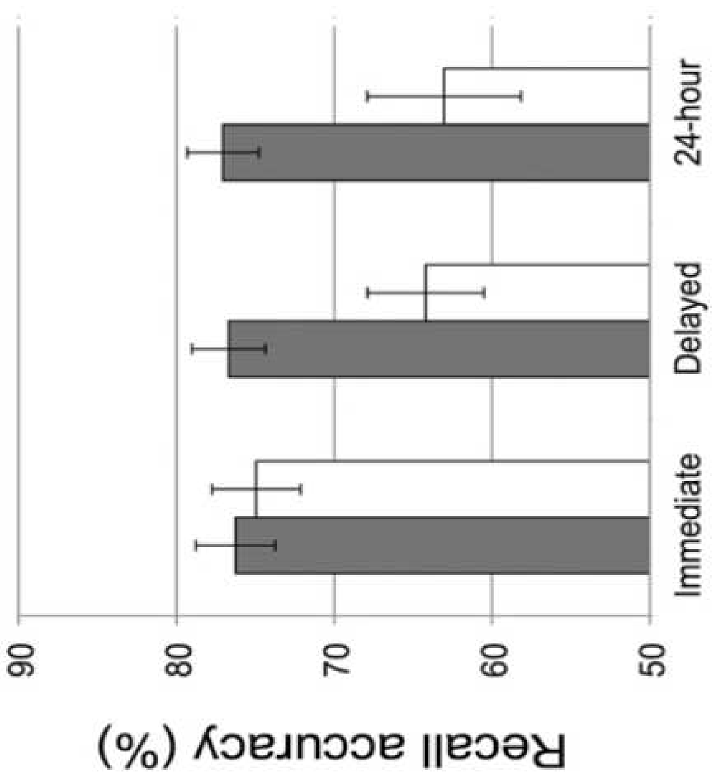


Figure 5

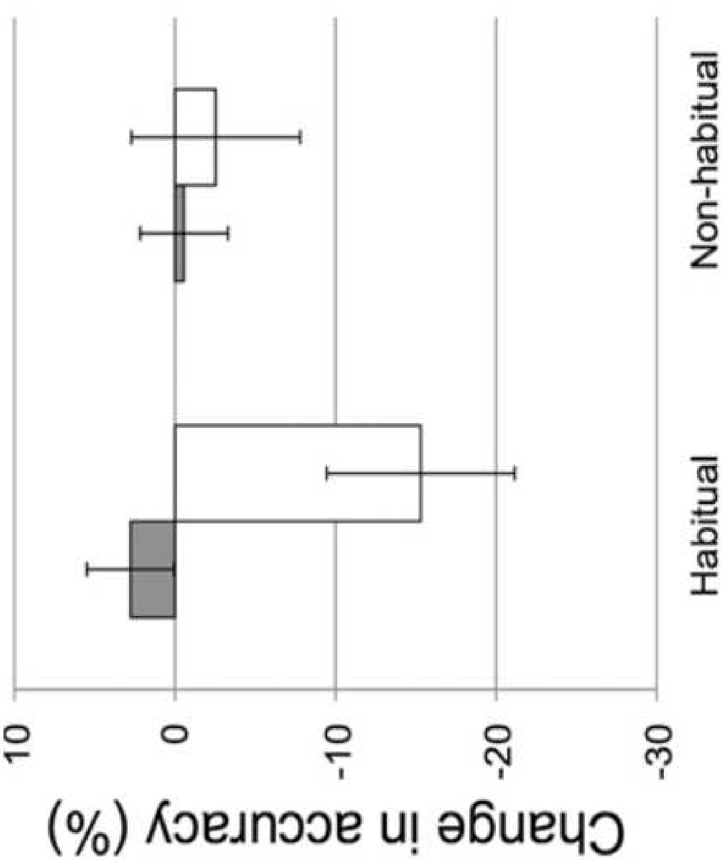
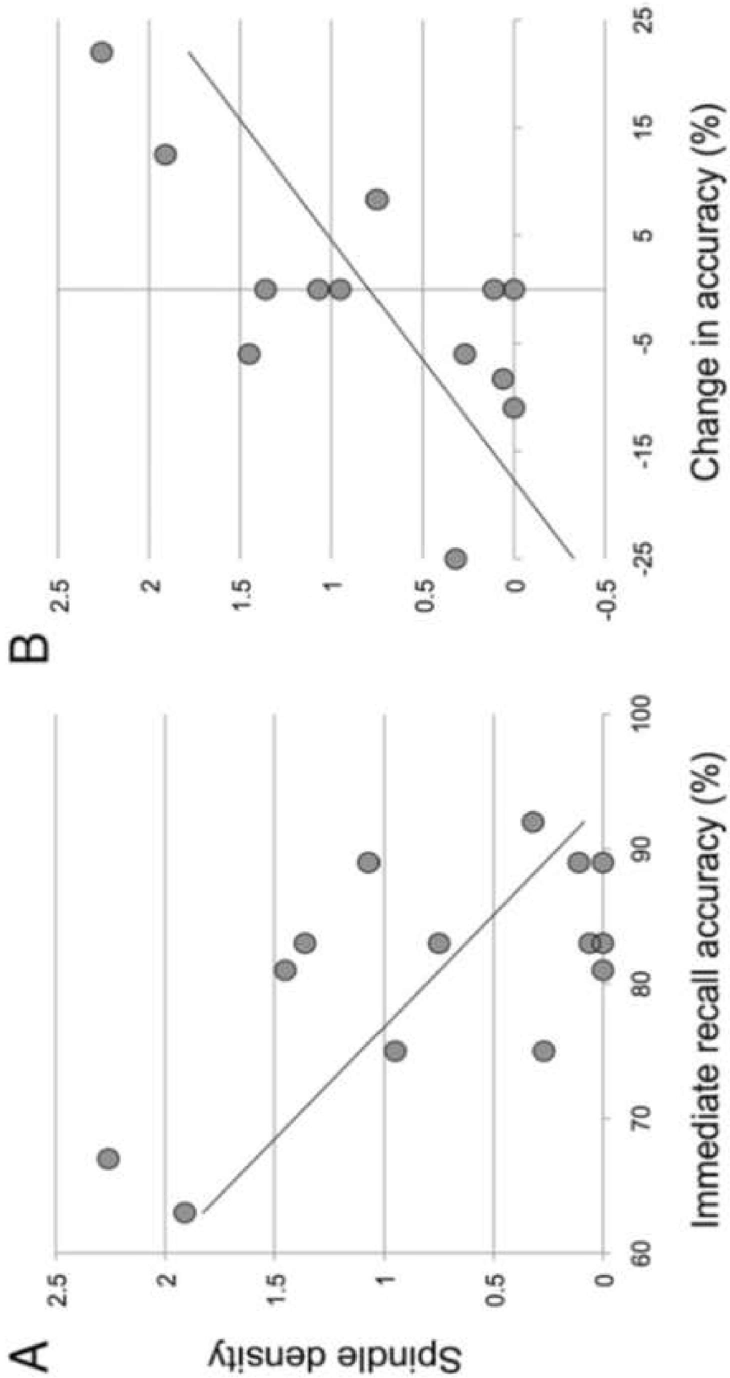


Figure 6


A
B
Spindle density
Immediate recall accuracy (%)
Change in accuracy (%)
2.5
2
1.5
1
0.5
0
-0.5
60
70
80
90
100
-25
-15
-5
5
15
25

Name of Material/ Equipment	Company	Catalog Number
Actiwatch Spectrum Plus Starter Kit	Philips Respironics	1109516
Actiware software	Philips Respironics	1114828
Brain Analyzer	Brain Products	BV-BP-170-1000
Dell Latitude 5580 Laptop	Dell	X5580T [210-AKJR]
EC2 cream	Grass	12643
Embla REMLogic software	Natus Medical Inc.	21475
Embletta MPR PG Sys - XR - US	Natus Medical Inc.	12077
Embletta MPR ST + Proxy Kit	Natus Medical Inc.	12696
Nuprep cleaning solution	Natus Medical Inc.	12643
Sleep Supplies Starter Kit for Embletta MPR ST/ST + Proxy	Natus Medical Inc.	12643

Comments/Description

Includes: Actiwatch Spectrum Plus Device, Actiware Software Licesnce, and manual

Alternatives may be available.

Alternatives may be available.

Laptop for running MatLab, Actiware, and RemLogic as well as storing/uploading data

Possible alternatives include Ten20 paste and Lic2 electride cream

Alternatives may be available.

Embletta system for PSG recordings

Attachment to Embletta to record PSG sensors

Possible alternatives may be available.

Started kit for sleeping including guaze, EC2 cream, NuPrep cleaning solution, cotton swabs and more.

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Dear Editor:

Thank you for the opportunity to revise our manuscript [JoVE60200 "Measuring neural mechanisms underlying sleep-dependent memory consolidation during naps in early childhood"]. We have tried our best to address all comments from the Editor and Reviewers. Per your request, we provide a version with track changes. In addition, we reply to each of the Reviewers comments in the letter below.

Best,
Tracy Riggins

Editorial comments:
Changes to be made by the author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: We have done our best to proofread the manuscript to ensure there are no spelling or grammatical errors.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Response: We have obtained explicit copyright permission to reuse Figures 4, 5, and 6 from Kurdziel et al., 2013. We have uploaded a letter from PNAS granting permission. We have also cited the original source of the figure in the legend as requested.

3. Authors and affiliations: Please provide an email address for each author.

Response: We provided emails for each author at the beginning of the document as requested.

4. Please include single line spacing between each numbered step or note in the protocol.

Response: We added a single line space between each section and subsection in the protocol section.

5. Everything in the protocol (except for the introductory ethics statement) should be in a numbered step (in the imperative tense and with no more than 4 sentences), numbered header,

or a “NOTE”. Please move the introductory paragraphs of the protocol to the Introduction, Results, or Discussion (as appropriate) or break into steps.

Response: We removed the introductory portion of the protocol and added it to the numbered steps. We also used imperative language, as requested.

6. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “NOTE.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Response: The Protocol now contains only action items. “NOTES” were used for text that added additional relevant information that could not be written in the imperative tense, as requested.

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Response: More detailed information about PSG removal and distractors were added. More detail about the memory task was added. More details about nap promotion techniques and waking procedures were added. References were added.

8. Please organize the sections/steps properly so that the protocol can be followed in chronological order.

Response: Per the instruction of the editor via email on 6/10/2019 we left the PSG, memory task, and actigraphy separate from the condition due to the fact that these sections are used in both conditions.

9. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

Response: We have highlighted sections as requested.

10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the

next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

Response: We have highlighted sections as requested.

11. References: Please do not abbreviate journal titles; use full journal name.

Response: We have revised the references to provide the full journal names.

12. Table of Materials: Please sort the materials alphabetically by material name.

Response: The materials have been sorted alphabetically by material name.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript presents a useful summary of a novel and state-of-the-art method for acquiring sleep EEG from young populations. There are a number of gaps in this literature and only limited number of researchers in this field; hence, this methodological paper provides important and useful information to initiate new research.

Response: Thank you for the positive feedback regarding our manuscript.

Concerns:
I have no major concerns, but a number of points that could be clarified / expanded upon...
1. Lines 80-82. It would be useful to include mention of the kinds of variables that can be measured using actigraphy. At present, it just states that actigraphy measures "sleep". It would also be useful to say what actigraphy cannot capture, relative to PSG.

Response: Variables that can be measured using actigraphy are now clarified in the revised manuscript.

2. Line 83. "Both PSG and actigraphy are…. Although these methods maybe easy to administer and relatively inexpensive, they are also subject to bias and non-compliance." Clarify that "these methods" refers to the self/parent report measures.

Response: As requested, we have clarified that "these methods" refer to the self/parent report measures.

3. Line 91. "the off-line processing of memories that leaves them stronger and less vulnerable to interference" is not a particularly clear description of memory consolidation (i.e., "off-line processing of memories" is what I found to be vague).

Response: We have replaced the term "off-line" with "during sleep" to enhance clarity.

4. Line 102. "…modern theories suggest that sleep plays a more active role…" The references provided in this section are quite outdated, and a more up-to-date (brief) description of current theories of systems and synaptic consolidation could be referred to here. E.g., see the work of Bernard Staresina and his colleagues in Birmingham, for intracranial evidence of the nesting of spindle - ripple - slow oscillation events in human hippocampi supporting memory consolidation. You could also mention the role of REM, as purported to support synaptic consolidation within neocortical networks.

Response: We now provide newer references in the revised manuscript.

5. Line 113. "The role of sleep during development is less clear." The role of sleep in memory consolidation during development? Or in development more generally?

Response: We have clarified this and now state: "The role of sleep in memory consolidation during development is less clear."

6. Line 130- to the end of the introduction. Relating to the above point, it would be nice if you provided a couple of sentences to provide examples of <u>what's needed in the way of future research into sleep-dependent memory consolidation in preschool children.</u> Also, can you clarify whether the presented protocol is specific to preschoolers or whether it could be applied to school aged children (who are a neglected bunch in the nap domain)?

Response: We have added examples of questions for future research at the end of the Introduction. We have also added a sentence clarifying that our procedure was designed for use with preschoolers but that it can also be used with both younger and older children.

7. Line 160 - We also use short videos documenting each step of the procedure, which can be put on a lab website. This could be useful to mention here.

Response: We added the recommendation that lab-made video clips can be used to facilitate a child friendly explanation of the procedures.

8. Line 173 - Clarify what you mean by "allowing the child to wake up as naturally as possible". Does this mean you only ever let them wake naturally? What's the procedure here?
You refer to the brands of exfoliant and electrode cream that you use and the type of actiwatches / PSG device, but not the type of electrodes. Out of interest, do you really want to be endorsing all of this equipment, or would it be more appropriate to present these as example kit, with other options available? There are also other methods of applying electrodes to the scalp (e.g., Ten20 paste). As a fellow researcher in this field, I'm reading this wondering whether your endorsement of these things comes through trying various alternatives? It would also be particularly useful to know whether you use silver/gold electrodes and the length of the electrode wires you use. Also, how do you decide when electrodes are no longer usable / do you use a single set for a study?

Response: The child is allowed to wake up naturally when the nap promotion condition is first, however, they should be woken up gently (or as naturally as possible) when the wake promotion condition is first so that the time of the two conditions match. This is now reflected in the protocol. As for the products, we clarified this with the Editor during the revision process. JoVE policy states that the manuscript and video are objective and not biased towards a particular product. To this end, we now use generic terms in the manuscript but reference all commercial products in the Table of Materials. When possible, we list alternatives in the Table of Materials in the Comments column.

As for electrodes, in the Kurdziel paper on which this protocol is based, gold cup electrodes were used. Typical length is 48 inches. Electrodes are judged to be usable until (1) we detect a bad lead (impedences do not go down or yield noisy recordings) or (2) we can visibly see when the film starts getting worn or even corroded. We have a couple sets of electrodes ready to use in the event one set is no longer usable. These details go beyond the scope of the manuscript and were not added to the text.

9. Line 264 - I thought you could go into more detail on electrode removal, given this can be a particular source of stress for researchers and children. We've found that water sprays can be as effective as detangling hair spray.

Response: As suggested we provided more details about electrode removal.

Reviewer #2:
Manuscript Summary:
This protocol described a typical experimental procedure to record neural mechanisms of sleep in young children. Techniques used in this procedure involves polysomnography (PSG), actigraphy, and sleep diaries from participants' parents. This protocol is based on a previous publication from the same group. In that study, based on the same/similar procedure, clear nap benefits for declarative memory was shown in 3-5 years' old children. The current protocol provided relatively detailed descriptions for each experimental step, making it easy to follow and replicate. In particular, this protocol also tailored some procedure for young children participants, providing a general comfortable and child-friendly process to motivate young children. For example, the authors considered to prepare a storybook to explain the whole procedure, and to allow experimenters to encourage children for their efforts during memory recall. Furthermore, it also considered the possible PSG's influence to disturb sleep so that a adaptation nap is recommended, even though it needs to be considered not burdening families and children significantly.
I believe this protocol is suited to be published as a Methods Article - JoVE Produced Video to provide the field (children sleep experiments) an important reference, when the following issues are well addressed:

Response: Thank you for the positive review of our manuscript.

Major Concerns:
1. * I think the authors did not specify to which age range of children should this experiment protocol apply appropriately. The authors did mention "early childhood" in the title and abstracts. However, "early childhood" may cover a wide range of age from infancy, toddlers and preschoolers (see Wikipedia with the term "early childhood"). Although the authors did mention in the abstract that this protocol focuses on "early childhood, a period of significance as children transition from biphasic sleep (consisting of a nap and overnight sleep) to monophasic sleep (overnight sleep only)", should we understand that this protocol is purely suited for children whose sleep is only during this transition? This also generates a question, independent on the cognitive task, which should be age-appropriate, would nap in toddlers whose sleep is still in polyphasic or in a transition to biphasic sleep be different from the nap at older children? Would the sleep part (PSG and actigraphy) of this protocol still suit for those younger children? 2. * Maybe the authors should also claim what health status of children this protocol is suited for. For example, I assume that children with special need (disabled) or typical psychiatric issues like ADHD and autism would need to be specially tailored with more care during the whole procedure. So better claim this either at the beginning with subject description or in the Discussion.

Response: We now clarify, at the end of the Introduction, that the protocol was designed for typically developing preschoolers approximately 3 to 4 years of age. We also now clarify that, in general, the methods described in the protocol could be used (or adapted slightly) for use with both younger and older age groups, as well as atypically developing children.

Minor Concerns:
Suggestions:
3. * Would be better to write exact frequency band range for sleep featured waves in the second paragraph of Introduction (e.g., spindle, sigma, slow-wave activities), also the corresponding text in the table of Figure 1.

Response: We have included the frequency bands in the text as requested. However, to maintain a visually appealing Figure, we elected not to include them in Figure 1.

4. * Even though it is generally accepted that sleep is crucial for memory consolidation, there are also evidence that sleep in children might not be exactly the same as adults, namely the wake period could also plays a role in memory consolidation in children1, 2. Although is not the main purpose of the current protocol to study wake consolidation, please at least discuss the wake consolidation in children, and how part of the protocol might apply for wake rest studies, for example, using actigraphy.

Response: We address wake consolidation in the Introduction of the revised manuscript.

Other questions:
5. * What the short movie is about? Should it be emotionless content or child-friendly, like cartoons.

Response: Because the goal of the short movie is to engage the child, it should be child-friendly. However, it should not be overly stimulating. We have added these details to the manuscript.

6. * Is there anything to control pre-nap routines that are probably different from children? Depending on the actual age group, would something like nursing should be avoided?

Response: Nursing is fairly uncommon in this age group (ages 3 to 5 years). Typically if children need to eat, we allow them to do so during PSG application. However, our most critical goal is to get the child to sleep as quickly and effortlessly as possible in order to minimize additional time awake. We have found that in the home the best way to accomplish this is by reducing variation from the child's typical nap routine. This is now reflected in the protocol.

7. * Would be better to shortly introduce parents' involvement, should they always be visible around their children to make them feel safe and conduct the sleep routine together with the experimenter?

Response: Parent involvement is dependent on the child. For some children parent involvement is necessary to make them feel at ease. In other cases parents can hinder the child's focus and can even lead to tantrums that would not happen otherwise. How to treat each situation is often left to the experimenter. We have added these details to the manuscript.

8. * Would the Nap and Wake promotion are randomly balanced across all subjects? And would children and their parents be blind to the condition until they conducted the children's sleep routine?

Response: The Nap and Wake promotion conditions are counterbalanced across all children. Children and parents are not blind to the condition mostly for logistical reasons so that parents are aware of what we are going to be doing when we arrive at their house and can plan their day around the activities (e.g., not planning an outing after the wake condition).

9. * Depends on children's age, I am curious how to comfort a sleep-deprived child and keep them engaged for the subsequence cognitive tests? What if they gets fussy and irritable?

Response: The best way to get the child to finish the cognitive task is to incentivize their participation. For example, offer the child a prize or something rewarding if they complete the task.

10. * I wonder how is the 9 item in 3x3 matrix and 12 in 3x4 matrix are decided? And how 70% criteria is determined? And how fast can children at different age group to reach the criteria?

Response: We now note why the 70% criteria is chosen and how this is implemented. In addition, we also now note in the manuscript that encoding typically takes about 6 minutes.

Finally, we would like to note that we chose this task as difficulty can be adjusted to accommodate this variability. Pilot data prior to the Kurdziel et al. study led to the levels recommended.

11. * Are the test items in the immediate recall and delayed recall are half-half of the total items to avoid testing effect? I am curious, how is the testing effect in such young children group?

Response: All items are probed at each recall phase. No feedback is given at recall so there is no new learning per se. Children's memory could improve via the testing effect. Importantly, this testing effect would be similar across sleep and wake conditions.

12. * Could you provide how motivation verbal feedback differ from succeeded trials and failed trails?

Response: Specific examples of feedback for both types of trials is now provided in the current version of the manuscript.

13. * Is the recall session with/without feedback is self-paced? Should specify it to understand children's possible stress level. And, how long each session lasts on average?

Response: All three phases are self-paced. The protocol now reflects this. The typical durations are: 6 minutes for the Encoding phase, 2 minutes for the immediate recall phase, and 2 minutes for the delayed recall phase.

14. * For electrodes placed on the face, should EC2 cream safe enough? Any alternatives for sensitive skins? Also, some children's skin could be very sensitive to medical tapes. So please provide general recommendations for tapes and electrical creams for sensitive skin.

Response: Per JoVe policy, in the current version of the manuscript, we have omitted name brand information. However, we have found that EC2 cream is safe for application to the skin. However, Ten20 can also be used to apply electrodes to the face and maybe better for sensitive skin.

15. * Line 252 "One electrode is placed on the right cheek just above the smile line and one on the left side just above where the chin meets the neck, adjacent to the esophagus." For me, I cannot easily imagine the location of EMG according to this sentence. Figure 1 illustrated the locations, but I am not sure whether that picture really match this description. Also, Figure 1 showed EMG was only placed one side, is it considered by children's habitual sleep body position? Maybe a drawing graph would be more accurate about electrode locations.

Response: The description has been modified to include additional instructions for placing the electrodes. Specifically, we have included a "tip" in which we ask the participant to say the word "milk" out loud while we look and feel for where the movement in the muscles in the neck and chin are maximal. This clarifies the placement of the EMG recordings. We have also clarified *that Figure 1 is simply an example in the Figure caption. We have added a drawing as well to further clarify EMG where all electrodes are placed.*

16. * Line 257 please specify the value that the impedance test should pass, for different electrodes and different age of children

Response: This information has been added to the protocol.

17. * In Line 259 "impedances under 25 are acceptable", the unit here is missing. I assume should be kOhm

Response: We added the unit, which is indeed kOhm.

18. * Line 269 "with a sensitivity of <0.01g", what's the unit of this "g" means? Could you shortly explain?

Response: Here we are talking about acceleration. Acceleration is often measured in units of "g" for gravity.

19. * Line 274 "Instruct the parent to press the button on the side of the watch face whenever their child is about to fall asleep or wake up." How to judge when is "is about to fall asleep"? or they should press the button as soon as the child lays on bed to calculate Total bed time?

Response: The button should be pressed when the child attempts to sleep and once they wake up. The purpose of the button is to aid with actigraphy scoring by signalling the researcher when a sleep attempt began. For this reason, precision is not required. The protocol now reflects this.

20. * Would you provide an example of the sleep diary? Should parent also record children's sleep behavior beyond the sleep times?

Response: We have provided examples of the necessary questions to be included in the sleep diary.

21. * I see the authors recommend AASM 2007 in this protocol as the standard for children sleep recording, why not a recent version e.g., version 2.3 of AASM?

Response: We have changed the wording and refer to the most recent version. The newer versions have not significantly affected how sleep is scored in these records (changes are largely about sleep disorder identification) and, as long as the same metric is applied to every record, exactly which version is utilized is not likely to influence the results of interest.

23. * Line 302 "however some labs require at least 10 days, particularly when these data are of primary interest." Could you provide the citations?

Response: We have changed this to 5 days and provide a citation.

24. * Line 309-311 "manually determine sleep onset" is according to which citation?

Response: The citation for this has been added.

25. * Regarding the result, how would Figure 4 of this protocol (from Kurdziel 2013) look differently for habitual nappers and non-habitual nappers?

Response: Figure 5 displays the behavioral results separately for habitual and non-habitual nappers. We edited the Results section in order to make this more apparent.

26. * Just curious, is there any known baseline performance difference between habitual and non-habitual nappers?

Response: We have found no baseline differences (immediate recall) in these groups (as reported in Kurdziel et al., 2013).

27. * Just to be clear, does each small grid indicate one day in the overview protocol? Would also be important to provide the possible time of the day for Nap and Wake promotions to understand the circadian rhythm difference.

Response: Yes, each small grid indicates one day. This has been clarified in the figure caption. We agree that it is important to note the time of day for nap and wake promotion. Given this protocol was designed for use with preschool children, we have clarified in the Introduction that the focus is on the benefit of an afternoon nap. In the Method section we stress the importance of keeping all factors identical (except for sleep) between nap and wake promotion conditions. This includes the time of day in order to control for the effects of circadian rhythm and the amount of time between immediate and delayed recall. In the Discussion we also note that similar methods can be used to examine sleep at other times.

Reviewer #3:
Manuscript Summary:
Methods used to examine neural mechanisms underlying sleep-dependent memory consolidation during naps in early childhood. It includes procedures for examining the effect of sleep on behavioral memory performance, as well as the application and recording of both polysomnography and actigraphy.

Major Concerns:
None.

Minor Concerns:
None.

Response: Thank you for the positive review of our manuscript.

Reviewer #4:
Manuscript Summary:
The manuscript describes a thorough protocol to measure sleep and sleep-dependent memory consolidation in a relatively understudied population, i.e. young children. It uses and describes methodologies that adhere to the standards of the field and thus could be a valuable research for other researchers. I do want to point out several instances where further detail or explanation is necessary, though.
Major Concerns:
1. * What is not clearly described in the manuscript is the scheduling/timing of the conditions: is it important to schedule them at the same time of day? How much time should be between conditions/sessions?

Response: It is important to schedule the sessions at the same time of day. We have added this information to the manuscript. We have also included details about timing within the session.

2. * More details for parts of the procedure is necessary for other researchers to be able to reproduce the procedure. I'm not exactly sure what the policy of JoVE is on that but I would suggest to include more detail and/or references or links to the specific materials and where to find more info:
○ Visuspatial memory task
○ Spindle analyses
○ Actiwatch scoring

Response: Additional details were added on spindle analysis, actigraphy data, and the visual spatial memory task.

3. * What did not become clear in the description of the visuospatial memory task was the reasoning for the two different versions for children younger and older than 44 months. Where did that cut-off come from? Piloting? Pretesting? A larger study?

Response: The preschool age range is broad and heterogeneous. We chose this task as difficulty can be adjusted to accommodate this variability. Pilot data prior to the Kurdziel et al., study led to the levels recommended.

4. * What about controlling possible issues of sleepiness, especially in the wake promoting conditions where children are "deprived" of their usual nap? Do the authors have any measures taken for that? Would be necessary to discuss at least.

Response: We collect measures of both sleep and mood using the Visual Sleepiness Scale (VSS) and the Visual Mood Scale. The protocol now includes this step.

5. * Is 15-30 minutes after awakening really enough to overcome sleep inertia? I am aware of other studies using at least 30 or 45 minutes. Is there some kind of standardization of what to do/not to do after awakening? e.g. eating, arousing activities

Response: Unfortunately, to our knowledge, there is little research on sleep inertia and what has been done is in atypical populations and, importantly, involving overnight sleep. Therefore we make this recommendation based on our current practices.

6. * The representative results on sleep physiology and memory-consolidation are somewhat confusing. First of all, I recommend carefully revising the wording of the correlational results presented in lines 328 and the following. The order of the variables and especially the phrase "was a positive predictor of sleep spindle density" implies a direction of causality. It could well be the other way around (sleep spindles predicting the change in recall. Additionally - and I am aware the this is not the main focus of the paper - the presentation of these correlations is so shortened that it may be misleading to read. For example, the somewhat unexpected (at least to me) NEGATIVE association between sleep spindles and immediate recall performance should be explained and put into context more, possibly other research finding similar or contrary associations between sleep spindles and performance. I see the same need for further explanation for the correlation with change in recall. Could the correlation with change in recall mainly result from a ceiling effect in the high performers and therefore only present itself because of the negative correlation of sleep spindle density with immediate performance? Those with high immediate performance (& low spindle density) could not improve much more, those who started out worse (& high spindle density) had room to change/improve?

Response: This issue was addressed in Kurdziel et al and we now summarize it in the revised manuscript. Notably, high performers are removed from the data – which is why we avoid ceiling behavior. In conjunction with adult data, we believe that this provides evidence in support of both trait-like (association with IQ as described now in the manuscript) and state-like (association with acute memory change). See discussion in Schabus M, et al. (2008) Interindividual sleep spindle differences and their relation to learning-related enhancements. Brain Res 1191:127–135.

Minor Concerns:

7. - Maybe it would be helpful for the reader to point out some more potential applications of the procedure/research questions?

Response: We have added examples of questions for future research at the end of the Introduction.

8. - l. 329: delete "during"

Response: The typo has been corrected.

Reviewer #5:
Manuscript Summary:
1. This paper reports on a protocol for measuring memory in very young children, within a sleep paradigm. This is an exciting area of research, where there is much still to be learned. As such, the topic broadly is of current interest to the field.
2. The abstract is well-written.

Response: Thank you for noting these positive aspects of the manuscript.

3. The introduction is heavily under-referenced. There are numerous instances where statements about aspects of sleep, sleep recording, and the role of sleep for memory consolidation should be referenced.

Response: We added additional references to the manuscript.

4. Some of the statements in the introduction are not supported or are contentious. For example the authors state that if the role of sleep for memory was simply passive protection from interference, we would not observe correlations between features of sleep and memory performance. However, such processes may be acting as a "passive guard" rather than be part of an active consolidation process, and the correlations still be apparent. Similarly, in one sentence the author mentions the nested occurrence of spindles, slow waves and hippocampal ripples to support memory formation. However nothing is said regarding how these processes are achieving stabilization of reactivated memories…again more depth is required for the concepts core to the topic of the article.

Response: We have revised this section. We now provide additional evidence for an active role of sleep and additional discussion of the mechanism. However, a full review of these topics is outside the scope of this paper.

5. While the topic is of interest, the Protocol lacks the level of detail that will make this article attractive and useful. Primary concerns are listed below, however in sum the article states the obvious and known aspects of this type of research. There is no additional practical knowledge to be gained, which I think would be the primary motivation for reading such as article.

Response: We have added additional details to the manuscript. When these details are paired with the video produced by JOVE we anticipate this being a useful tool for researchers new to the field.

Major Concerns:

The objective is then stated to be the provision of practical advice for measuring sleep and sleep-dependant memory in young children. This is problematic for two primary reasons:
6. The advice on sleep is simply a reiteration of standard pediatric PSG and actigraphy recommendations. In other words, there is really nothing new this article provides in this regard.

Response: We have added details to the manuscript that provide information on how to assess sleep-dependent memory consolidation in preschoolers. Although the method is not new, we are aware of no other published protocols that bring together the methods we have described for this purpose.

7. The memory paradigm has the potential to inform researchers interested in investigating memory in young children. However, the level of detail does not allow for replication. There is arguably as much detail in the methods of the representative results cited.

Response: We have additional details to the manuscript to allow for replication of the methods.

Minor Concerns:

8. There are some smaller concerns, which are also simply examples of the points above: 'Distractors' are said to be useful during PSG set-up - and every pediatric sleep researcher or technician will tell you the same thing. If this is to be useful then more explicit examples and recommendations are required.

Response: Explicit examples of age appropriate PSG distractors are now described in the revised manuscript.

9. Similarly, the conclusion focuses only on sleep recording, but wasn't the article to cover sleep-dependent memory processes? So, what of the memory aspect in the conclusions?

Response: We agree that the focus of the article is on sleep-dependent memory consolidation. As such, the full combination of methods that are described is warranted and appropriate. However, at present, PSG is the only methodological tool that allows for characterization of sleep quality via measurement of sleep stages. Thus, it is the only method that allows for insight into neurobiological mechanisms underlying sleep-dependent effects, such as memory consolidation. Per the requirements of JoVE we elaborate on its major advantages, most critical steps, and limitations of PSG specifically. However, in response to this comment, we have revised the Discussion to include critical steps of the protocol in general and include the suggestion that the memory protocol can be altered to examine other forms of memory and/or cognition.

10. A number of studies investigating sleep and memory in young children are completely absent (e.g. the work of Sabine Seehagen and Carolin Konrad).

Response: We have added additional references, including some from Seehagen and Konrad, to the manuscript. Thank you for this suggestion.

UNIVERSITY OF MARYLAND

Tracy Riggins <riggins@umd.edu>

Permission Inquiry - Kurdziel et al 2013

PNAS Permissions <PNASPermissions@nas.edu> Wed, Jun 5, 2019 at 10:53 AM
To: Tracy Riggins <riggins@umd.edu>, PNAS Permissions <PNASPermissions@nas.edu>

Dear Dr. Riggins,

Permission is granted for your use of the material as described in your request. Please include a complete citation for the original PNAS article when reusing the material.

Best regards,

Jennifer Nguyen for

Diane Sullenberger

PNAS Executive Editor

https://www.pnas.org/content/110/43/17267

From: Tracy Riggins <riggins@umd.edu>
Sent: Monday, June 3, 2019 9:50 PM
To: PNAS Permissions <PNASPermissions@nas.edu>
Subject: Re: Permission Inquiry - Kurdziel et al 2013

Dear Delaney

As indicated below, you previously granted us permission to reuse Figures 1-3 from: Kurdziel, L., Duclos, K. & Spencer, R. M. C. Sleep spindles in midday naps enhance learning in preschool children. Proc. Natl. Acad. Sci. U. S. A. 110, 17267–17272 (2013).

I am writing to request permission to also use Figure 4 from this same manuscript for the same new paper we are writing for JoVE.

Would this be possible?

Thank you, in advance,

Tracy

Tracy Riggins, Ph.D.

Associate Professor

Secretary, Cognitive Development Society

Department of Psychology

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Pronouns: She/Her/Hers (What's this?)

On Mon, Apr 15, 2019 at 3:52 PM Tracy Riggins <riggins@umd.edu> wrote:

> Great, thank you very much!
>
> Best,
>
> Tracy Riggins, Ph.D.
>
> Associate Professor
>
> Secretary, Cognitive Development Society
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> Department of Psychology
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>>
>> Best regards,

Delaney Cruickshank for

Diane Sullenberger

PNAS Executive Editor

From: Tracy Riggins <riggins@umd.edu>
Sent: Monday, April 15, 2019 11:01 AM
To: PNAS Permissions <PNASPermissions@nas.edu>
Subject: Permission Inquiry - Kurdziel et al 2013

Good morning

I am writing to clarify if re-using a figure from an article in PNAS for an article in Jove - the Journal of Visualized Experiments - requires permission from PNAS.

Specifically, I am seeking to re-use Figures 1-3 from Kurdziel, L., Duclos, K. & Spencer, R. M. C. Sleep spindles in midday naps enhance learning in preschool children. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 17267–17272 (2013).

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The example given is for a review article. Hence, my question is whether a different type of publication - such as a methods paper in Jove also falls into this category.

Your advice would be greatly appreciated.

Tracy

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