Title: Quantifying sleep spindles from overnight EEGs as an Angelman syndrome biomarker

1. Working hypothesis. In the past ten years, basic discoveries in mouse models have identified extremely promising approaches for treating Angelman syndrome (AS) in humans[1-3]. Development of antisense oligonucleotides (ASOs) and gene therapy approaches, and a keen interest from major pharmaceutical companies (Roche, Biogen/Ionis), suggest that clinical trials are on the near horizon for AS. For such trials to succeed, it is critical to develop biomarkers, outcome measures, and measures of target engagement[4]. Biomarkers for AS need not have diagnostic value, as diagnoses are made genetically. Therefore the most important features of a good AS biomarker are that it must be: (a) quantifiable, (b) robust, (c) biologically based, and (d) linked to disease-relevant phenotypes[5]. In a prior ASA-funded study[6], our group quantified delta frequency EEG oscillations as a robust and biologically based biomarker in children with AS. While delta oscillations have great potential as a readout of target engagement, the relationship between delta oscillations and core AS phenotypes remains unknown. Here we seek to quantify a novel biomarker that is robust, biologically based, and directly linked to the abnormal sleep observed in up to 90%[7] of individuals with AS. ***Poor sleep quality*** has a major impact on caregivers and families of children with AS; thus, development of sleep-related AS biomarkers is particularly important. Based on extensive preliminary data taken from clinical EEGs, ***our central hypothesis is that sleep spindles are substantially decreased in children with AS***. Here, we seek to perform the first-ever quantitative study of sleep spindles during overnight polysomnography in individuals with AS and age and sex-matched controls.

We anticipate that our proposed research will define sleep spindle impairments as a robust, quantifiable biomarker with immediate value for clinical trials. Furthermore, insights gained from human studies will enable and inform future studies in AS model mice to understand the mechanisms by which loss of UBE3A disrupts sleep spindles. In this way, a relatively small one-year investment by the ASA has the potential to: a) directly impact the development of clinical trials, and b) generate data that will motivate the NIH to fund a substantial translational research project in AS.

2. Experimental strategy, methods, and objectives. Sleep spindles are thalamocortical oscillations in the sigma band (11-16 Hz) that occur during non-rapid eye movement (NREM) sleep. Spindles are detectable by EEG and play a functional role in memory consolidation[8,9]. To date, there have been no reports of sleep spindle abnormalities from the clinical AS sleep literature, likely for two reasons: (1) pervasive background activity makes manual observation of spindles difficult[10], and (2) few overnight sleep studies have been performed in individuals with AS due to poor tolerance in this population. Our recent work used unbiased automated approaches to show for the first time that ***children with AS have a robust decrease in sleep spindle frequency and spindle duration*** relative to neurotypical children[11] (**Fig. 1**). However, our initial study had major limitations. First, our study was exploratory, and replication studies testing *a priori* hypotheses are extremely important to avoid circular analysis and reduce bias[12]. Second, AS and neurotypical EEGs were not recorded at the same site (UC San Diego and Mass. General Hospital, respectively). Third, and

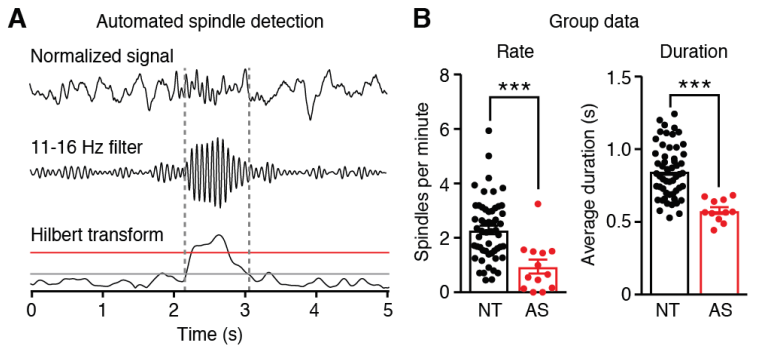

Fig. 1: Spindles are decreased in children with AS in short periods of sleep during clinical EEGs. (A) Steps to automate spindle detection. (B) Spindle rate and duration (red, AS; black, neurotypical).

most importantly, we extracted short periods of sleep from clinical EEGs, which were not designed explicitly as a sleep study. Such sleep, which occurred in only 13 of 28 EEGs from children with AS and averaged only ~20 minutes, is not likely to be representative of typical sleep. Thus, we will quantify sleep spindles ***during overnight sleep*** using polysomnography and data analysis resources unique to our cross-disciplinary group at UNC. We propose two Aims:

Aim 1: Test the hypothesis that sleep spindles are impaired in individuals with AS using overnight polysomnography. We will compare sleep spindles in individuals with AS with two age- and sex-matched control groups: (a) neurotypical individuals and (b) individuals with Down syndrome. All data have already been gathered by the Laboratory at UNC's Sleep Disorders Center for clinical purposes; our study will analyze these high-quality EEGs retrospectively.

Aim 2: Explore sleep architecture and sleep quality in individuals with AS. We will leverage the expertise of the UNC Sleep Disorders Center to conduct an exploratory study of sleep architecture and sleep quality in EEGs from individuals with AS. We anticipate that this analysis will both replicate prior findings and be hypothesis-generating.

The completion of these Aims will quantify a biomarker that is biologically-based and linked to sleep dysfunction. We anticipate that sleep spindles may be used as an outcome measure for upcoming clinical trials, particularly in trials where sleep quality is a primary endpoint.

Aim 1: Test the hypothesis that sleep spindles are impaired in individuals with AS using overnight polysomnography. *Methods:* We will analyze retrospective polysomnography data collected by the UNC Sleep Disorders laboratory using quantitative analysis tools developed by the Philpot laboratory. Spindle detection will use methods identical to those used for analysis of clinical EEGs[11] (**Fig. 1**). Briefly, we will use a detection algorithm originally developed by Kim and colleagues[13] and adapted by the Philpot Lab to work in a custom MATLAB environment. Automated spindle detection can be summarized in four steps: (1) To set the impedance levels of electrodes to similar levels, the detector normalizes each pre-processed signal to the average power of the 90-100 Hz frequency range of that signal (**Fig. 1A**, top). (2) The data are band-pass filtered between 11-16 Hz using a 10th order Butterworth filter (**Fig. 1A**, middle). (3) The instantaneous amplitude is computed using a Hilbert transform and smoothed using a Gaussian kernel of 40 ms (**Fig. 1A**, bottom). (4) A spindle is detected if the instantaneous amplitude of the filtered signal crosses a threshold of 5.5x the mean amplitude of the signal (red line in **Fig. 1A**, bottom). When a spindle is detected, its duration is defined by when the signal crosses a lower threshold, (2.5x; grey line in **Fig. 1A**, bottom). All data have been sleep staged by clinical experts at the UNC Sleep Disorders lab (**Fig. 2**). We will quantify spindles during N2 sleep stages, as defined by clinical experts.

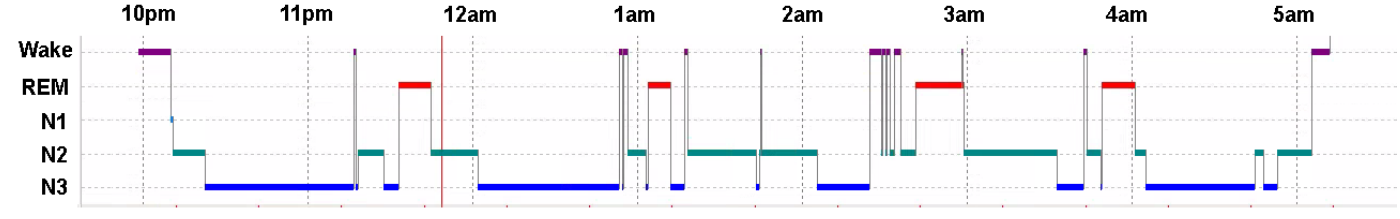

Fig. 2: Sleep staging in a 4-year old child with AS. Sleep staging has already been performed for all overnight EEGs.

Data sources: The limiting factor for any sleep study in AS is the difficulty in conducting overnight EEG recordings in individuals with AS. We have already collected eight overnight EEGs from a total of five individuals with AS (2 male, 3 female), ranging in age from 1.6 to 27.8 years (**Fig. 3**). We have also identified age and sex-matched controls from two populations: (a) neurotypical individuals, and (b) individuals with Down syndrome (DS), from a database maintained by the UNC Sleep Disorders Laboratory. Thus, we will have three groups for comparison: Angelman syndrome (AS), neurotypical (NT), and Down syndrome (DS).

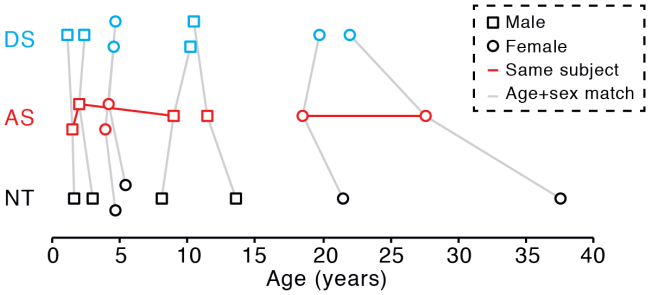

Fig. 3: Overnight polysomnography data. We have obtained 8 EEGs from 5 individuals with AS. Longitudinal data is available for two patients. All EEGs are age and sex-matched with NT and DS EEGs.

Statistical comparisons: While our current sample (n = 8 EEGs) is smaller than the clinical EEG sample we analyzed previously (n = 13)[6], the average length of recordings in the current sample is significantly longer (**Fig. 4**). The current sample contains a total of 29.7 hours of sleep data, compared to just 4.7 hours analyzed from the clinical EEGs. However, we must take into account the relatively small sample of overnight EEGs when designing comparisons that will have sufficient statistical power to detect group differences. Given the large effect size observed in our preliminary clinical EEG data, we anticipate that our dataset is sufficiently well-powered to compare two groups, but not to compare three groups while correcting for multiple comparisons. Therefore, our primary analysis will be a direct comparison between AS and NT groups (Expt. 1.1). Secondarily, we will compare AS to DS groups in an exploratory manner (Expt. 1.2).

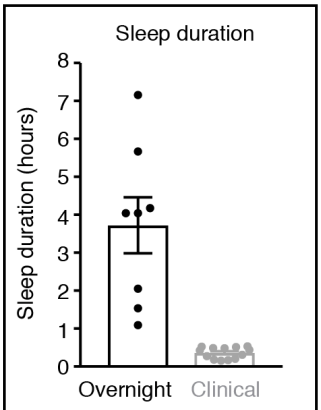

Fig. 4: Sleep averaged 3.7 hr in overnight EEGs and 22 min in

Experiment 1.1: Compare sleep spindles between individuals with AS and age and sex-matched neurotypical controls using established automatic detection methods.

We previously developed a custom data analysis suite in MATLAB that enables pre-processing and rapid analysis of diverse EEGs (PARADE). PARADE was designed specifically for pre-processing and analysis of Angelman EEGs, and enables high-throughput spindle analysis and group comparisons. We will use PARADE to pre-process EEG data and analyze spindles during expert-defined N2 sleep. First, pre-processing steps will include re-referencing, filtering (1-100 Hz band-pass), and artifact removal[6]. ***We will then analyze the number and duration of sleep spindles*** in processed EEG files using methods described above. We will make group comparisons between AS and NT groups. We will also compare the effect sizes of sleep spindle phenotypes and established delta rhythmicity phenotypes[6].

Experiment 1.2: Compare sleep spindles between individuals with AS and age and sex-matched controls with Down Syndrome (DS) using established automatic detection methods.

We will perform an exploratory comparison of spindles between AS and DS groups. DS is the most common inherited genetic cause of intellectual disability, and is associated with sleep disruptions (including delayed sleep onset, night wakings, and sleep apnea) in ~75% of cases[14-16]. While AS and DS have substantially different clinical presentations, DS does provide an opportunity to assess the degree to which spindle disruption generalizes to other

neurogenetic disorders. We have chosen DS as a comparison group because of the large, easily accessible database of overnight DS polysomnography available at the UNC Sleep Disorders Lab (we recently published a study of sleep apnea from 144 individuals with DS[16]). Because our primary objective is to assess spindles as an AS biomarker, our *a priori* statistical comparison will be limited to AS and NT groups, in order to increase statistical power. Thus, Expt. 1.2 will be an exploratory, hypothesis-generating comparison.

Experiment 1.3: Compare multiple spindle detection algorithms and clinical expert performance.

Here, we seek to develop methods and best practices for quantifying sleep spindles as an AS biomarker. Preliminary Data and Expt. 1.1 will employ a spindle detection algorithm originally developed by Kim and colleagues[13], and adapted into MATLAB by the Philpot lab. Automated spindle detection is a rapidly growing field, and we anticipate that new approaches will become iteratively more accurate. However, despite recent advances, even the best automated detector does not yet perform at the "gold-standard" level of clinical expert consensus[17]. Thus, we will compare the performance of the detector used in Expt. 1.1 with other published spindle detectors[17-19] and with the blinded spindle detection of one clinical expert (Dr. Zheng Fan). The clinical expert will be blind to genotype by high-pass filtering data at 5Hz (to remove delta activity that identifies AS individuals). While prior work has compared the performance of a subset of approaches to detect spindles ***in neurotypical individuals***, no study to date has compared the ability of detection algorithms to detect group differences in a neurodevelopmental disorder.

Aim 2: Explore sleep architecture and sleep quality in individuals with AS.

Overnight polysomnography provides a wealth of data and enables analyses beyond spindle detection. We will leverage the availability of this rare dataset to quantitatively compare other aspects of sleep in AS and NT groups. We anticipate that this Aim will both provide valuable replication and generate new hypotheses.

Experiment 2.1: Test the hypothesis that delta oscillations are increased across all stages of NREM sleep in AS.

We previously demonstrated that delta oscillations are increased in children with AS during both periods of wakefulness and periods of NREM sleep[6]. However, for this study we did not have the resolution of complete sleep staging (**Fig. 5**); rather, sleep was only categorized as REM or NREM. Here we will compare delta power (average of all electrodes) between AS and NT groups as a function of sleep stage using two-way ANOVA. As sleep staging is already complete for all data, this analysis can be easily and rapidly conducted using PARADE.

Experiment 2.2: Replicate the finding that long-range gamma coherence is increased in AS.

EEG coherence provides a measure of how neural activity is correlated between brain areas, and is widely used as a proxy for functional connectivity[20]. We recently made the novel discovery that long-range coherence in the gamma band (30-50 Hz) is significantly increased in children with AS during sleep[11]. The pattern of elevated long-range gamma coherence during sleep in AS children resembles what is typically seen in a wakeful state. This experiment will serve as a replication study, to test whether abnormal coherence during sleep can also be seen during representative overnight sleep. This experiment can be conducted with little additional effort using built-in PARADE functions.

Experiment 2.3: Test the hypothesis that sleep apnea correlates with spindle phenotypes in AS.

There is a high prevalence of sleep breathing disorder in AS[21]. Sleep breathing is typically measured using the

apnea/hypopnea index (AHI), and abnormal sleep breathing is reflected by AHI > 1.5 in children and AHI > 5 in adults. We calculate AHI routinely in the course of overnight polysomnography. Here, we will compare AHI between AS, NT, and DS groups. We will test the relationship between sleep apnea and sleep spindles within subjects. Perhaps disruption in the sleep cycle caused by sleep apnea may underlie group differences in spindle frequency.

Experiment 2.4: Comprehensively compare sleep architecture in AS and neurotypical groups.

Prior work using polysomnography confirmed that children with AS have delayed sleep latency and increased night wakings, as reported by caregiver survey, as well as a lower percentage of time spent in REM sleep[10,21]. Here, we will seek to replicate these findings, as well as report comprehensively on the percentage of time spent at each sleep stage.

Future Directions: In addition to having potential for biomarker development, our work may also provide insights into circuit-level biological mechanisms underlying AS. Mechanisms governing spindle initiation and propagation have been well-characterized[8]. Spindles are driven by the intrinsic properties of, and interactions between, thalamocortical cells and thalamic reticular cells. Thalamocortical circuits, which also drive cortical delta rhythms[22], may be studied in mouse models to better understand how loss of UBE3A disrupts neural circuits. Future work will use mouse models to test the hypothesis that loss of UBE3A from a small population of like neurons is sufficient to disrupt sleep spindles in AS.

3. Timetable, schedule and deliverables. We have already obtained IRB approval for this proposal and have identified all data to be analyzed. Months 1-3: data download, blinding, re-formatting, pre-processing for unbiased analysis in MATLAB. Months 4-6: Completion of Aim 1. Months 7-10: Completion of Aim 2. Months 11-12: Submission, editing, and re-submission of manuscript. Deliverables: We will present this work at the ASA meeting in October 2019 and will submit a manuscript by the end of grant funding.

4. Project budget. The proposed research is feasible on a one-year, €60,000 budget (to support one postdoc salary and benefits), because all data have already been collected and analyses will be done retrospectively. We anticipate no costs for equipment and reagents or other costs.

5. Research institution. Our approach is made possible by a collaborative research environment unique to UNC. The Angelman Syndrome Clinic at the Carolina Institute for Developmental Disabilities (CIDD) helped enable recruitment of individuals for overnight sleep studies, which were conducted by the UNC Sleep Disorders Laboratory. UNC is a top research institution, and the Departments of Neurology (Fan) and Cell Biology and Physiology (Philpot) have an established record of productive research.

6. Collaboration partners. Our group is uniquely positioned to conduct these experiments because it includes the the polysomnography expertise of Dr. Zheng Fan at the UNC Sleep Disorders Laboratory, and the data analysis expertise of the Philpot Laboratory. In her role as Associate Professor of Child Neurology, Sleep Medicine and Clinical Genetics, lead PI Zheng Fan both sees AS patients at the CIDD clinic and manages the UNC Sleep Clinic. Co-investigator Ben Philpot brings expertise in quantitative EEG analysis and biomarker development. The postdoc to be supported by this grant (Michael Sidorov) is a leader in the field of Angelman syndrome biomarker development, with a demonstrated record of productivity in this field from 2017-2019[6,11].

References:

1 Meng, L. *et al.* Towards a therapy for Angelman syndrome by targeting a long non-coding RNA. *Nature* **518**, 409-412, doi:10.1038/nature13975 (2015).

2 Huang, H. S. *et al.* Topoisomerase inhibitors unsilence the dormant allele of Ube3a in neurons. *Nature* **481**, 185-189, doi:10.1038/nature10726 (2011).

3 Daily, J. L. *et al.* Adeno-associated virus-mediated rescue of the cognitive defects in a mouse model for Angelman syndrome. *PLoS One* **6**, e27221, doi:10.1371/journal.pone.0027221 (2011).

4 Berry-Kravis, E. M. *et al.* Drug development for neurodevelopmental disorders: lessons learned from fragile X syndrome. *Nat Rev Drug Discov* **17**, 280-299, doi:10.1038/nrd.2017.221 (2018).

5 Jeste, S. S., Frohlich, J. & Loo, S. K. Electrophysiological biomarkers of diagnosis and outcome in neurodevelopmental disorders. *Curr Opin Neurol* **28**, 110-116, doi:10.1097/WCO.0000000000000181 (2015).

6 Sidorov, M. S., Deck, G. M., Dolatshahi, M., Thibert, R. L., Bird, L. M., Chu C. J., & Philpot, B. D. . Delta rhythmicity is a reliable EEG biomarker in Angelman syndrome: a parallel mouse and human analysis. . *Journal of Neurodevelopmental Disorders* **9** (2017).

7 Trickett, J., Heald, M. & Oliver, C. Sleep in children with Angelman syndrome: Parental concerns and priorities. *Res Dev Disabil* **69**, 105-115, doi:10.1016/j.ridd.2017.07.017 (2017).

8 McCormick, D. A. & Bal, T. Sleep and arousal: thalamocortical mechanisms. *Annu Rev Neurosci* **20**, 185-215, doi:10.1146/annurev.neuro.20.1.185 (1997).

9 Ulrich, D. Sleep Spindles as Facilitators of Memory Formation and Learning. *Neural Plast* **2016**, 1796715, doi:10.1155/2016/1796715 (2016).

10 Miano, S., Bruni, O., Leuzzi, V., Elia, M., Verrillo, E. & Ferri, R. Sleep polygraphy in Angelman syndrome. *Clinical Neurophysiology* **115**, 938–945 (2004).

11 den Bakker, H. *et al.* Abnormal coherence and sleep composition in children with Angelman syndrome: a retrospective EEG study. *Mol Autism* **9**, 32, doi:10.1186/s13229-018-0214-8 (2018).

12 Kriegeskorte, N., Simmons, W. K., Bellgowan, P. S. & Baker, C. I. Circular analysis in systems neuroscience: the dangers of double dipping. *Nat Neurosci* **12**, 535-540, doi:10.1038/nn.2303 (2009).

13 Kim, D., Hwang, E., Lee, M., Sung, H. & Choi, J. H. Characterization of Topographically Specific Sleep Spindles in Mice. *SLEEP* **38**, 85-96 (2015).

14 Maris, M., Verhulst, S., Wojciechowski, M., Van de Heyning, P. & Boudewyns, A. Sleep problems and obstructive sleep apnea in children with down syndrome, an overwiew. *Int J Pediatr Otorhinolaryngol* **82**, 12-15, doi:10.1016/j.ijporl.2015.12.014 (2016).

15 Dosier, L. B. M., Vaughn, B. V. & Fan, Z. Sleep Disorders in Childhood Neurogenetic Disorders. *Children (Basel)* **4**, doi:10.3390/children4090082 (2017).

16 Fan, Z., Ahn, M., Roth, H. L., Li, L. & Vaughn, B. V. Sleep Apnea and Hypoventilation in Patients with Down Syndrome: Analysis of 144 Polysomnogram Studies. *Children (Basel)* **4**, doi:10.3390/children4070055 (2017).

17 Warby, S. C. *et al.* Sleep-spindle detection: crowdsourcing and evaluating performance of experts, non-experts and automated methods. *Nat Methods* **11**, 385-392, doi:10.1038/nmeth.2855 (2014).

18 't Wallant, D. C., Maquet, P. & Phillips, C. Sleep Spindles as an Electrographic Element: Description and Automatic Detection Methods. *Neural Plasticity* (2016).

19 Ray, L. B., Sockeel, S., Soon, M., Bore, A., Myhr, A., Stojanoski, B., Cusack, R., Owen, A. M., Doyon, J. & Fogel, S. M. Expert and crowd-sourced validation of an individualized sleep spindle detection method employing complex demodulation and individualized normalization. *Frontiers in Human Neuroscience* **9** (2015).

20 Srinivasan, R., Winter, W. R., Ding, J. & Nunez, P. L. EEG and MEG coherence: measures of functional connectivity at distinct spatial scales of neocortical dynamics. *J Neurosci Methods* **166**, 41-52, doi:10.1016/j.jneumeth.2007.06.026 (2007).

21 Miano, S. *et al.* Sleep breathing and periodic leg movement pattern in Angelman Syndrome: a polysomnographic study. *Clin Neurophysiol* **116**, 2685-2692, doi:10.1016/j.clinph.2005.08.005 (2005).

22 Lewis, L. D. *et al.* Thalamic reticular nucleus induces fast and local modulation of arousal state. *Elife* **4**, e08760, doi:10.7554/eLife.08760 (2015).