AWARD NUMBER: HT9425-23-1-0200

TITLE: Dual Orexin Antagonist Treatment to Prevent the Neurobehavioral Sequelae of TBI

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CONTRACTING ORGANIZATION: University of Wisconsin, Madison, WI

REPORT DATE: May 2024

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
May 2024	Annual	01Apr2023-31Mar2024

4. TITLE AND SUBTITLE

Dual Orexin Antagonist Treatment to Prevent the Neurobehavioral Sequelae of TBI

5a. CONTRACT NUMBER
HT9425-23-1-0200

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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Madison, WI 53715-1218

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The central hypothesis here is that following TBI, treatment with a sleep aid namely a dual orexin antagonist will mitigate the development of neurobehavioral sequelae of TBI by: a) normalizing sleep and its homeostatic drive/sleep pressure, and b) enhancing GABAergic inhibition in brain regions relevant for sleep disturbances, seizures and mood/anxiety disorders namely the thalamus, hippocampus and amygdala.
Specific Aims: Aim 1: To perform CCI or sham injury followed by 2-month long EEG recordings for sleep and epileptiform activity during treatment with a dual orexin antagonist (DORA) ACT462206 or a vehicle. Aim 2: To perform behavioral testing including tail suspension for a depression phenotype, fear conditioning for PTSD phenotype and Morris Water Maze for spatial learning and memory in CCI or sham injury groups towards the end of 2-month-long daily treatment with ACT-462206 or a respective vehicle. Aim 3: To perform patch-clamp electrophysiology to record cellular and network excitability measures focusing on thalamic reticular neurons (important for sleep slow-wave or delta activity generation), dentate granule cells and CA1 pyramidal neurons (important for epilepsy) and pyramidal neurons in basolateral amygdala (mood disorders) at the end of 2-month long DORA (ACT462206) or vehicle treatment. Aim 4: To perform EEG recordings and behavioral experiments as in Aim 1&2 following CCI after treatment with an orexin receptor-2 agonist YNT185 as a comparative study.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	17	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction: Traumatic brain injury (TBI) is common in civilians and military personnel with ~5.3 million Americans living with its consequences[1]. The chronic consequences of TBI include sleep disturbances, posttraumatic epilepsy (PTE), mood disorders including depression and posttraumatic stress disorder (PTSD) as well as cognitive problems[2] which we collectively termed neurobehavioral sequelae. The sequelae of TBI not only require long-term medical care but also cause a huge economic toll on patients, their families, and the society. The neurobehavioral sequelae of TBI are much more common in military personnel compared to civilians[3]. Moreover, sleep disturbances, seizures or depression/PTSD can all occur in the same person following TBI. A gap in understanding of biomarkers and molecular targets for the neurobehavioral sequelae of TBI has hindered the translation of experimental TBI work to the clinic. The long-term vision of this proposal is to address the gaps and to foster preventive therapies for the neurobehavioral sequelae of TBI.
This PRMRP Expansion
Award is relevant to the *FY22 Topic area* of *Sleep Disorders*. We will perform a *confirmatory preclinical study*

building on the data from a prior PRMRP award (PR161864). In a mouse model of controlled cortical impact (CCI), TBI resulted in: a) sleep disturbances including increases in non-rapid eye movement (NREM) sleep suggestive of a "hypersomnia" acutely and reduced time in NREM and REM sleep or "insomnia" chronically and b) increased NREM delta power which is a marker of sleep homeostatic drive or sleep pressure[4]. TBI also resulted in: a) posttraumatic epilepsy (PTE) in ~23% of animals but also in interictal spikes and absence-like spike wave discharges[4]; and b) depression and posttraumatic stress disorder phenotypes. Finally, a month-long treatment with a sleep aid (a dual orexin antagonist-DORA22) following CCI, restored the sleep homeostatic drive, enhanced GABAergic inhibition in dentate granule cells and suppressed PTE[5]. Here we will take a holistic approach to foster preventive therapeutics for neurological and psychiatric consequences of TBI.

Experimental Aims:

1. Aim 1: To perform CCI or sham injury followed by 2-month long EEG recordings for sleep and epileptiform activity during treatment with a *dual orexin antagonist* (DORA) ACT462206 or a vehicle.
2. Aim 2: To perform behavioral testing including tail suspension for a depression phenotype, fear conditioning for PTSD phenotype and Morris Water Maze for spatial learning and memory in CCI or sham injury groups towards the end of 2-month-long daily treatment with ACT-462206 or a respective vehicle.
3. Aim 3: To perform patch-clamp electrophysiology to record cellular and network excitability measures focusing on thalamic reticular neurons (important for sleep slow-wave or delta activity generation), dentate granule cells and CA1 pyramidal neurons (important for epilepsy) and pyramidal neurons in basolateral amygdala (mood disorders) at the end of 2-month long DORA (ACT462206) or vehicle treatment.
4. Aim 4: To perform EEG recordings and behavioral experiments as in Aim 1&2 following CCI after treatment with an orexin receptor-2 agonist YNT185 as a comparative study.

Study End points:

1. Does treatment with a dual orexin antagonist immediately and chronically following TBI prevent the development of posttraumatic epilepsy?
2. Does treatment with a dual orexin antagonist immediately and chronically following TBI prevent the development of chronic sleep disturbances and sleep homeostasis changes?
3. Does treatment with a dual orexin antagonist immediately and chronically following TBI prevent the development of depression, PTSD and attenuate memory deficits associated with TBI?
4. Does treatment with a dual orexin antagonist mechanistically enhance GABAergic tonic or phasic inhibition in brain regions such as hippocampus, thalamus and amygdala to prevent the neurobehavioral sequelae of TBI?
5. In a comparative study, does treatment with orexin receptor-2 agonist NOT prevent posttraumatic epilepsy or behavioral consequences such as depression and PTSD and NOT attenuate memory deficits associated with TBI?

Key words: TBI; Post-traumatic epilepsy; sleep disturbances, depression, PTSD, memory, GABAergic inhibition

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Major Task 1			**Current Status**
1. ACUC and ACURO approvals **2. Practice surgeries and EEG recording testing with wireless transmitters**	**1-2**		**Completed**
Major Task 2			
Specific Aim 1: EEG recordings for sleep and epileptiform activity during treatment with a DORA or a vehicle Surgeries for TBI and EEG electrode implantation followed	**Months**	**PI/Co-Is**	**Number of CD-1 mice**

by EEG recordings will be performed in 12 Cohorts for CD-1 mice			
TBI/Sham injury, Implantation of EEG/EMG electrodes and wireless transmitters followed by continuous Video EEG recordings during treatment with DORA or vehicle (8 sham and 8 TBI; 4 male and 4 female each) x 12 weeks each for 12 cohorts. Cohort 1 Cohort 2 Cohort 3 Interim Analysis to determine efficacy of DORA treatment on seizures, behavioral tests and electrophysiology. We will switch to selective orexin receptor 1 & 2 antagonists if it is determined that DORA treatment is ineffective **Milestone 1; Identify efficacy of dual orexin antagonist treatment in suppressing posttraumatic seizures** Cohort 4 Cohort 5 Cohort 6 Cohort 7 Cohort 8 Interim Analysis of Cohorts 4-8 to determine differences in seizures, behavioral measures and electrophysiology findings in drug and vehicle treated groups. **Milestone 2: Identify efficacy of dual orexin antagonist or selective orexin antagonist treatment in suppressing posttraumatic seizures and posttraumatic epileptiform activity** Cohort 9 Cohort 10 Cohort 11 Cohort 12 **Milestone 3: Identify efficacy of drug treatment in enhancing sleep disturbances and sleep homeostasis after TBI** **Analysis of sleep patterns and seizures (To be done for every group at different time points as sleep analysis is a big bottleneck)**	3-4 5-6 7-8 8-10 11 10-11 13-14 16-17 19-20 22-23 24-25 25-26 25-26 28-30 32-33 36-37 38 8-11; 25-26; 37-38	Maganti and Post doc/Research assistant Bergstrom/Jones/Maganti	16 each x 12 cohorts= 192; **Not completed**
Major Task 3 **Specific Aim 2: To perform behavioral testing including tail suspension for a depression phenotype, fear conditioning for PTSD phenotype and Morris Water Maze for spatial learning and memory in CCI or sham injury groups towards the end of 2-month-long daily treatment with a DORA or a respective vehicle.**			
Behavioral tests: 1. Tail suspension and Fear conditioning during week 10 of treatment 2. Morris water maze during week 12 of treatment Cohort 1 Cohort 2	 4 6 8	Cara Westmark/Pam Westmark	All 16 cohorts: n=8 in each cohort;

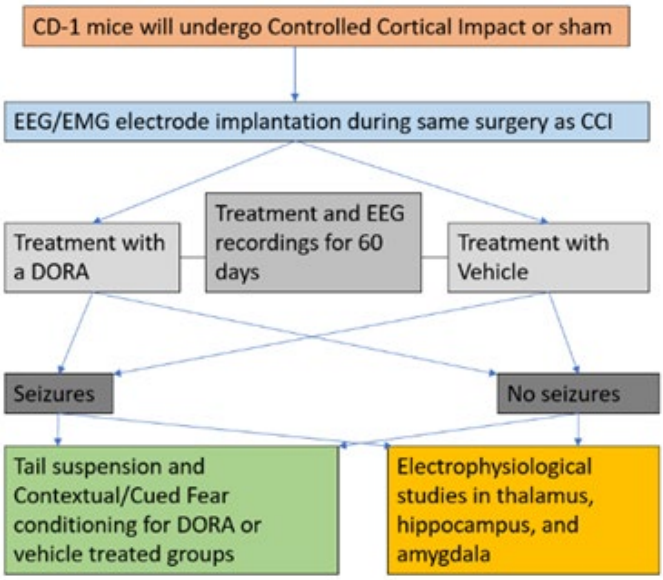
Cohort 3 Interim Analysis of behavioral test measures in drug and vehicle treated groups **Milestone 1: Determine efficacy of drug treatment in suppressing depression and PTSD phenotypes after TBI** Cohort 4 Cohort 5 Cohort 6 Cohort 7 Cohort 8 Interim Analysis of behavioral test measures in drug and vehicle treated groups. **Milestone 2; Determine effects of drug treatment on memory function following TBI** Cohort 9 Cohort 10 Cohort 11 Cohort 12 **Final Analysis of all cohorts with respect to behavioral test measures in drug and vehicle treated groups** **Milestone 3: Determine efficacy of dual orexin antagonist or selective orexin antagonist treatment on all behavioral sequelae of TBI**	9 10 11 14 17 20 23 24-25 26 30 33 37 38 38		**Not completed**
Major Task 3 **Specific Aim 3: To perform patch-clamp electrophysiology to record cellular and network excitability measures focusing on thalamic reticular neurons (important for sleep slow-wave or delta activity generation), dentate granule cells and CA1 pyramidal neurons (important for epilepsy) and pyramidal neurons in basolateral amygdala (mood disorders) at the end of 2-month long DORA or vehicle treatment.**			
Sacrificing animals at the end of 12 weeks treatment and performance of patch-clamp electrophysiology experiments and analysis of data. Patch-clamp experiments will be done on hippocampus in cohorts 1-3; amygdala in cohorts 4-8 and in the thalamus in cohorts 9-12 Cohort 1 Cohort 2 Cohort 3 Interim Analysis of electrophysiology data and trouble shooting **Milestone 1: Identify the neurophysiologic basis of antiseizure effects of dual orexin antagonist treatment, specifically on excitation-inhibition balance in the hippocampus** Cohort 4 Cohort 5 Cohort 6 Cohort 7 Cohort 8 Interim Analysis of electrophysiology data **Milestone 2; Identify of mechanisms of how dual orexin**	5 7 9 10 11-12 12 15 18 21 22 22-24 24 27	Jones/Yuzhen Pan	n=8 animals each (4 male and 4 female) for patch-clamp electrophysiology on hippocampus, thalamus and amygdala for a total of ~24 animals **Not Completed**

antagonist treatment suppresses depression or PTSD phenotypes after TBI, focusing on excitation-inhibition balance in the amygdala. Cohort 9 Cohort 10 Cohort 11 Cohort 12 **Milestone 3: Identify the mechanism of how dual orexin antagonist treatment enhances sleep and it homeostasis focusing in GABAergic inhibition in the thalamus and the cortex** **Final Analysis of Electrophysiology data**	31 34 38 30-40		
Major Task-4: Dose-response study			
TBI/Sham injury, Implantation of EEG/EMG electrodes and wireless transmitters followed By continuous Video EEG recordings during treatment with DORA or vehicle of 2 doses (low dose and high dose)	38-39	Maganti and Post doc/Research assistant	n= 16 each x2= 32
Behavioral tests: Tail suspension and Fear conditioning during week 10 of treatment	39	Cara Westmark/Pam Westmark	~8 each in high and low doses
Behavioral test: Morris water maze during week 12 of treatment	39	Cara Westmark/Pam Westmark	~8 each in high and low doses
Analysis of EEG and sleep data using automated methods and identify animals with seizures	39	Maganti/Jones/ Bergstrom	
Analysis of Behavioral test data **Milestone: Identify dose dependent effects of dual orexin antagonist treatment in suppressing posttraumatic seizures and behavioral sequelae**	39	Cara Westmark	**Not completed**
Major Task 5: Study with an orexin agonist YNT185 **Specific Aim 4: To perform EEG recordings and behavioral experiments as in Aim 1&2 following CCI after treatment with an orexin receptor-2 agonist YNT185 as a comparative study.**			
TBI/Sham injury, Implantation of EEG/EMG electrodes and wireless transmitters followed By continuous Video EEG recordings during treatment with YNT185	40-41	Maganti and Post doc/Research assistant	16
Behavioral tests: Tail suspension and Fear conditioning during week 10 of treatment	41	Cara Westmark/Pam Westmark	All 16
Behavioral test: Morris water maze during week 12 of treatment	41	Cara Westmark/Pam Westmark	All 16
Analysis of EEG and sleep data using automated methods and identify animals with seizures	41	Maganti/Jones/ Bergstrom	
Analysis of Behavioral test data **Milestone: Identify effects of selective orexin2 agonist on seizures and behavioral sequelae of TBI**	41	Cara Westmark	**Completed**

Total sample size estimate			240
Meetings for Quality assurance of data and Interim Analyses			
Interim analysis meetings will be scheduled between the PI and Co-PIs to review data at the end of completion of all experiments for Cohorts 4, 8 and 12. Samples sizes will be adjusted depending on the outcome measures and to replace lost animals in subsequent cohorts	In Months 13; 25 and 37		
Data Compilation and Final Analyses			
Compilation of all data analyzed from each of the cohorts of DORA/Vehicle treated animals; from dose response study and from study using orexin agonist will be performed	42-44		
Manuscript preparation and Preparation of final reports to submit to DOD	45-48		

Accomplishments/ Research Outcomes:

Schematic of Experimental Design:



1. ACUC and ACURO approvals: Feb 2023
2. DOD funds released April 2023
3. Recruitment of Research Assistant: September 2023
4. Submission of application to Eisai Pharmaceuticals and negotiation of MOA: May-September, 2023
5. Execution of the MTA through UW-Madison Office of Research Sponsored Programs: Sept 2023-April 2024. We finally received the drug (Lemborexant on April 16th, 20240.
6. Initial experiments with the wireless EEG system: August to October, 2023.
7. First set of mice (Cohort 1) (n=16) with TBI (n=8) or sham injury (n=8) recorded with wireless EEG for ~4 weeks Oct, ’23 to Nov’23. These TBI or sham mice had treatment with either treatment with an Orexin agonist (Major task 5 in SOW) or a vehicle (saline) for 4 each of sham and TBI mice (2 male and 2 female). All of these mice had complication of the wireless transmitters which either dislodged or

mice had infections. Therefore, we ended up sacking these mice without having any useful EEG or sleep data. Moreover, we found that the EMG was not reliable for sleep scoring. **Thus, we have no usable data**.

8. Second and Third set of mice (Cohort 2 and 3) n-16 each (of which 8 had TBI and 8 had sham) underwent EEG/EMG recording with EEG headcaps and a tethered recording (as we have done in prior DOD grants). In cohort 2, 5 mice had died of complications after implantation of headcaps in the first 2 weeks of recording. All the remaining 11 mice in cohort 2 [7 TBI of which 4 had drug and 3 had vehicle) and 4 sham (3 drug treated and 1 vehicle treated) of which and all 16 mice in Cohort 3 had 5 weeks of EEG/EMG recordings while they are treated with Orexin-2 agonist (YNT185) or vehicle (8 each of which 4 were mal and 4 female). After completing 5 weeks of EEG/EMG recording, both Cohorts underwent behavioral testing including tail suspension, radial arm water maze followed by fear conditioning. (All of these data pertain in Major Task 5). Cohort 2 experiments were completed between Jan '24 to end of Feb '24 and Cohort 3 experiments were completed between March '24 and end of April '24.

RESULTS to date:

1. **Posttraumatic epilepsy: See below**
2. **Sleep Data: See below**
3. **Behavioral tests data: see below**

Other accomplishments:

1. **Development of an automated algorithm for scoring interictal spikes:**

We have started development of an interictal spike (IIS) detection algorithm in MatLab that will be used on mouse EEG data collected from each cohort of CD1 mice used in this study to evaluate the frequency of interictal spikes and thus make inferences on if an animal is likely to have epilepsy. The algorithm currently employs a two peak threshold method as developed in earlier iterations of this detection algorithm (Pfammatter et. al, 2018). This algorithm takes in the right frontal EEG signal from a 24-hour EEG file filters the signal using a 60 Hz notch filter and a 5 to 35 Hz bandpass filter. The filtered signal is then normalized using a Gaussian Normalization. From here the 2 peak detection is implemented where spikes or event are identified if the signal goes above a threshold for positive amplitude followed by it dipping below a threshold of a negative amplitude. For each event detected only events with a duration between 50 and 100 milliseconds and an amplitude under 20 standard deviations from the baseline are kept.

Currently to validate and improve this algorithm we first tested the algorithm in other mouse models. We ran a total of 25 EDF files including C57 and DBA mice each with a group being treated either with kainic acid (KA) or saline (SA). For each file run the algorithm would clip out a section of the normalized right frontal EEG signal where each event was detected saving one second of EEG before and after the start of each event. This matrix of all detected waveforms from all baseline files was then run through principal component analysis (PCA) in which the matrix of principal component scores was used to project each waveform into 3D based on the first 3 principal components. Each point in this space represents a detected waveform and the distance between point represents the variance between the shapes of the corresponding waveforms. After identifying which points came from each of the four treatment groups (i.e. C57 KA, C57 SA, DBA KA and DBA SA) it was clear there is a separation between waveforms detected from kainic acid treated animals and saline treated animals as seen in figure 1. To further investigate of the differences in shape between waveforms from saline treated animals and kainic treated animals which we would consider epileptic the 3D space was dived into voxels and groups of waveforms to represent each treatment group were extracted from different voxels where the probability for that treatment group was the highest and the density of points in the voxel was at least over 1% of the total number of points. These waveforms were then averaged and plotted by treatment group as seen in figure 2, where overall the traces from waveforms that represented kainic acid treated animals had a higher amplitude and longer total duration until thee signal returned to baseline. It is seen from this that there are visual

differences in the shape of spikes or waveforms from kainic acid treated or epileptic animals versus those from saline treated animals.

We plan to more thoroughly determine the differences between strains and treatment analyzing the projected PCA data using principals from Bayesian statistics to eventually predict if a waveform is the result of the animal having epilepsy based on its shape. From there we will be to improve the spike detection algorithm and predict which animals have epilepsy and also investigate the inter-spike interval to better determine epileptic activity in mice just from the EEG. We will use a similar algorithm for TBI or sham mice.

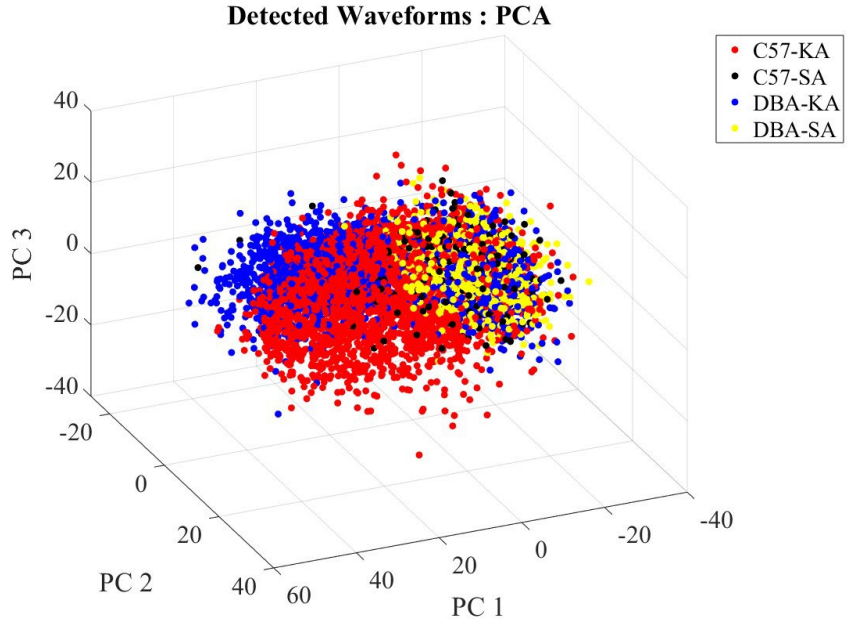


Figure 1. Points representing each detected waveforms projected into 3D space based on first 3 principal components from PCA. Red points represent waveforms recorded from C57 mice treated with kainic acid treated animals, black points represent waveforms recorded from C57 mice treated with saline, blue points represent waveforms recorded from DBA mice treated with kainic acid and yellow points represent waveforms recorded from DBA mice treated with saline.

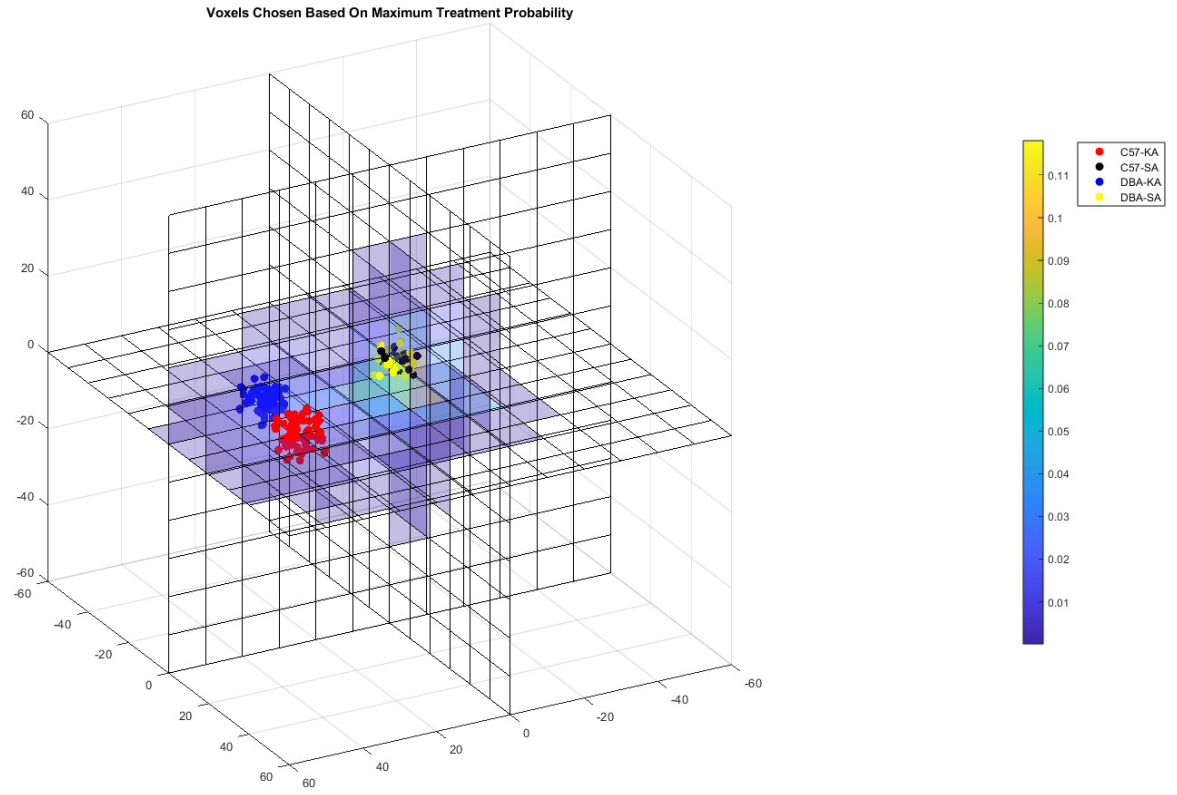


Figure 2. Points in the 3D space from voxels chosen to best represent waveforms from each treatment group. Voxels for each treatment group were chosen with density of points over 1% of the total number of points or waveforms considered in the analysis and the highest probability of containing points representing waveforms for that treatment group. The color map represents the density of points in each voxel that contains points from the original distribution shown in figure 1. For the C57 KA treatment group (red) and the DBA KA treatment group (blue) waveforms were drawn from a different voxel than the other 3 groups but for the C57 and DBA SA (black and yellow respectively) treatment groups waveforms were drawn from the same voxel.

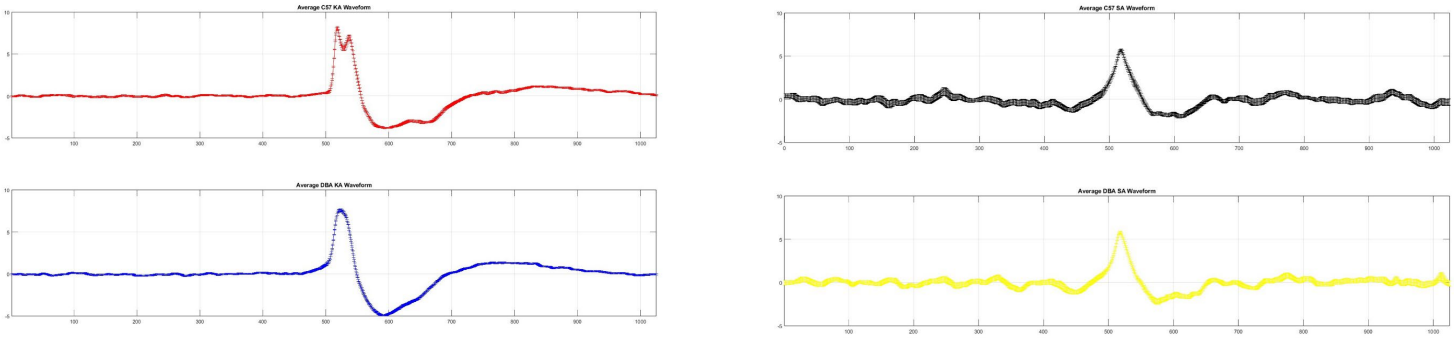


Figure 3. Average waveform with standard error bars for each treatment group pulled from voxels in the 3D space with density of points over 1% of the total number of points and the highest probability of containing points representing waveforms for that treatment group. For the C57 KA treatment group and the DBA KA treatment group waveforms were drawn from a different voxel than the other 3 groups but for the C57 and DBA SA treatment groups waveforms were drawn from the same voxel. The red trace represents the C57 KA treatment group (Top), blue trace represents the DBA KA treatment group (2nd from top), black represents the C57 SA treatment group (2nd from bottom) and yellow represents DBA SA treatment group (bottom).

2. Development of Automated Sleep scoring algorithm using Accu Sleep.

In order to measure the effect of TBI and its treatment on sleep-wake parameters, it is necessary to score a subject's sleep stage at each point in time. Conventionally, sleep scoring is done in 4-second epochs manually into Wake, NREM and REM in mouse EEG recordings. We traditionally used a manual scoring software called Sirenia Sleep (Pinnacle, KS) using EEG and EMG features. Example of conventional manual scoring using Sirenia Sleep is shown in Figure 3.

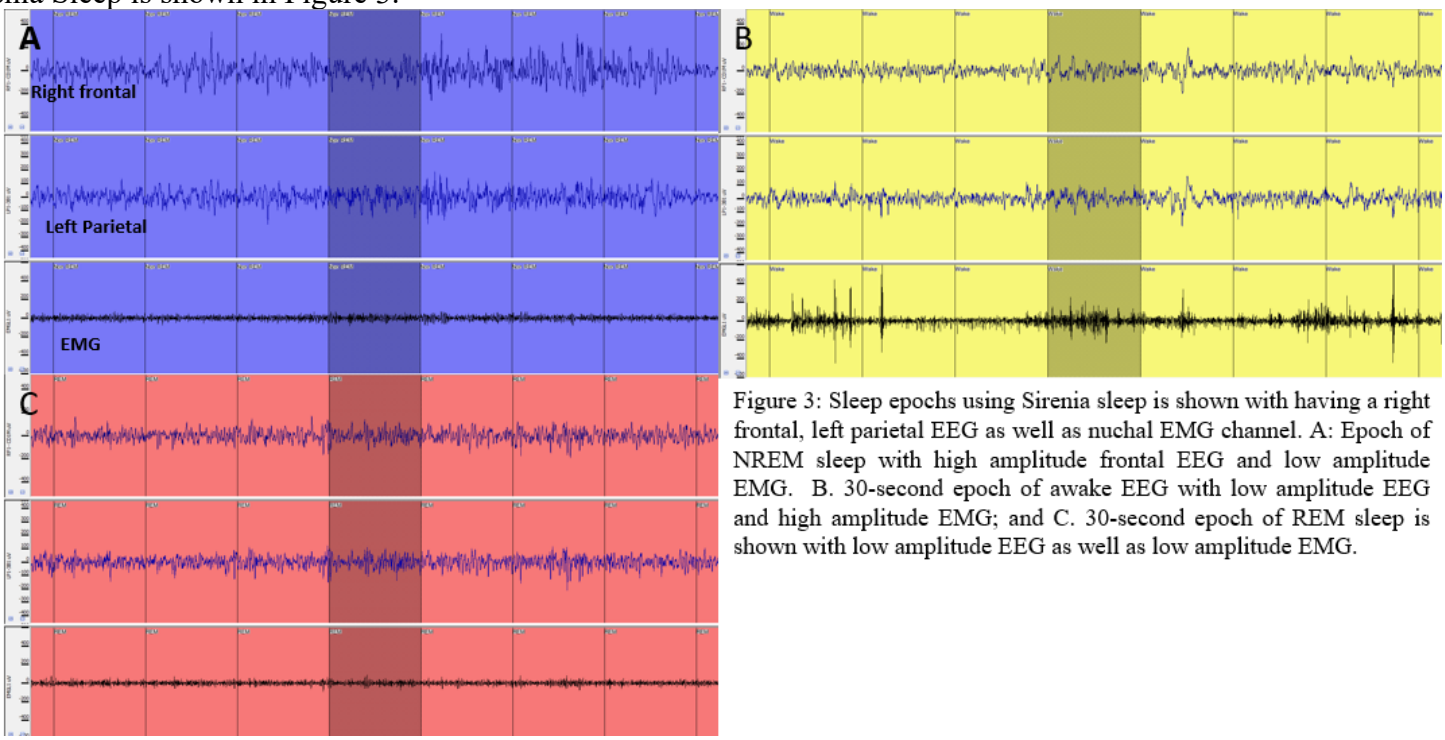


Figure 3: Sleep epochs using Sirenia sleep is shown with having a right frontal, left parietal EEG as well as nuchal EMG channel. A: Epoch of NREM sleep with high amplitude frontal EEG and low amplitude EMG. B. 30-second epoch of awake EEG with low amplitude EEG and high amplitude EMG; and C. 30-second epoch of REM sleep is shown with low amplitude EEG as well as low amplitude EMG.

Manual scoring of sleep wake patterns in mouse EEG is tremendously labor intensive and automated algorithms are desirable for scoring of large number of animals recorded for many weeks. We have 2 cohorts of TBI recorded for 5 weeks (2 animals with all data available so far) (a total of 27 animals) which translates to 945 days of recording.

We utilized a previously published algorithm namely AccuSleep (Ref: Barger Z et al, PLoS One 2019; 14(12): e0224642), which is a set of graphical user interfaces for scoring rodent sleep using EEG and EMG recordings. It comprises of a convolutional neural network (CNN) and a hidden Markov model (HMM). The CNN operates on multi-channel EEG and EMG spectrograms and comprises convolution, and max-pooling followed by two fully-connected layers. It has 6.8M parameters, distributed primarily in the first fully-connected layer. The HMM is used to constrain the transitions between sleep stages predicted by the CNN. AccuSleep is a set of MATLAB graphical user interfaces that allow for manual scoring of EEG/EMG data followed by automated scoring using sleep scoring artificial neural network (SS-ANN).

The acquired EEG is converted to an EDF file. The data is preprocessed by band pass filtering the EEG data with 1-30 Hz and EMG data 5-500 Hz filters. Sleep scoring was performed manually into wake, NREM or REM based on criteria similar to what was performed with Sirenia sleep. To demonstrate the agreement between human scoring and automated scoring, we performed a confusion matrix in mice treated with saline or Kainic acid in C57BL/6 mice and DBA mice. Agreement between human scoring vs automated scoring in each strain is as shown in Figure 4.

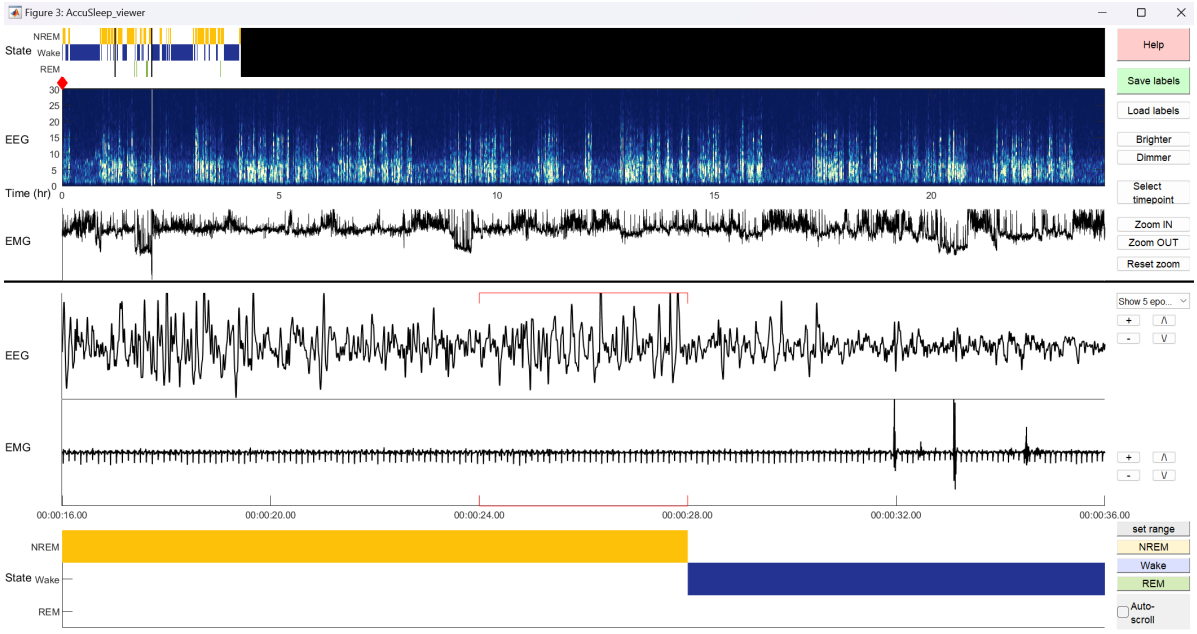


Figure 5: **AccuSleep interface for manual sleep scoring:** The lower three panels display the EEG and EMG signals as well as the sleep stage labels for epochs surrounding the currently selected epoch. The upper three panels provide context by displaying the sleep stages, EEG spectrogram, and EMG power on a longer time scale. The top most panel shows hypnogram of manual scoring.

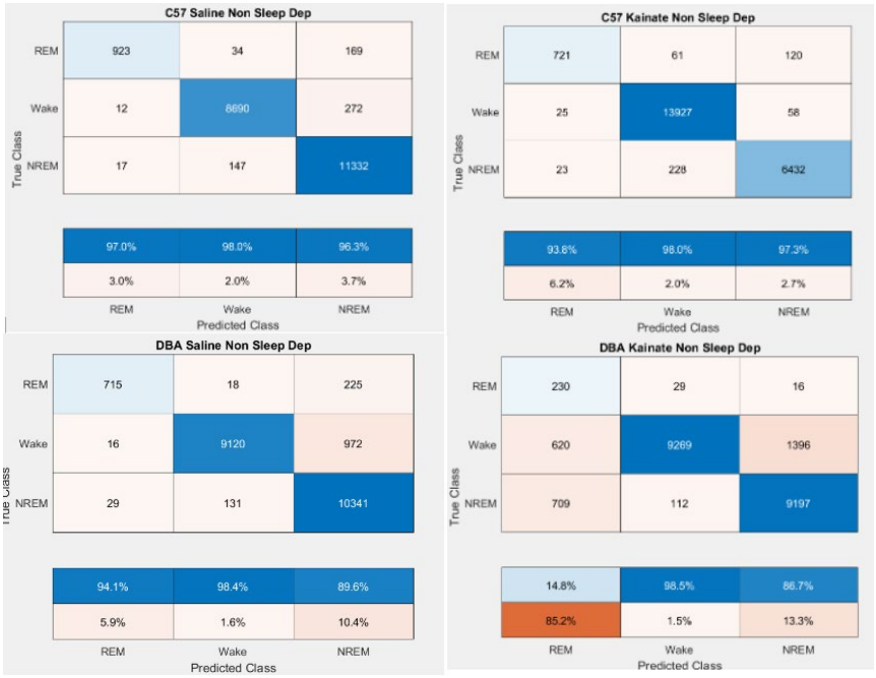


Figure 6: Accuracy of automated scoring shown in Saline or Kainate treated C57 and DBA mice. Note that the accuracy of automated scoring compared to human scoring was between 89-97% in C57 mice whereas it is between 85 and 93%in DBA mice.

Our next steps are to improve the accuracy and precision of the algorithm by further training the network with the CCI model of TBI in CD-1 mice. We will use this algorithm for sleep scoring in batches of mice recorded. We are currently performing the same for Cohorts 2 and 3 that already completed the EEG/EMG recordings with the Orexin-2 agonist or vehicle.

3. Posttraumatic epilepsy: Thus far, analysis of the raw EEG data showed seizure that were acute posttraumatic seizures as well as posttraumatic epilepsy. Examples of the epileptiform activity are shown in Figure 7.

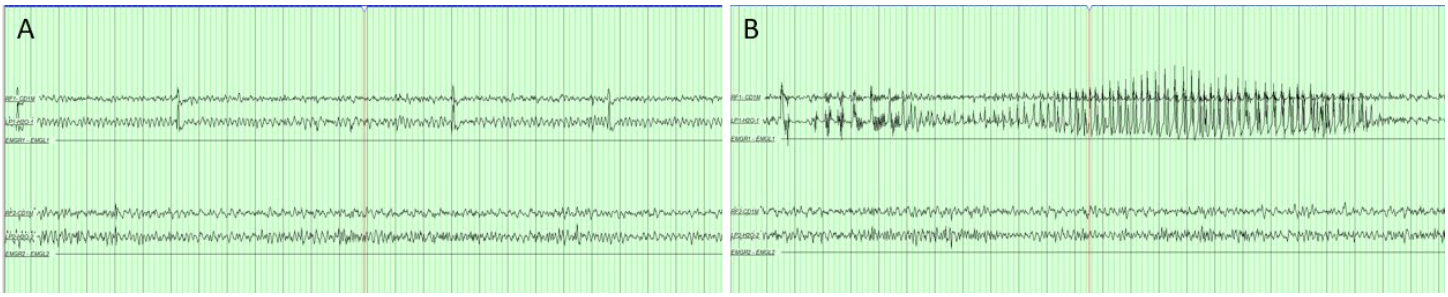


Figure 7: EEGs shown from 2 mice with each having a left frontal, right parietal and an EMG electrode. A shows interictal spikes in a mouse that had CC. B shows a Racine class V seizure in the same mouse which had interictal spikes.

Cohort 1 EEG recordings could not be analyzed. Between cohorts 2 and 3, we have data from 28 mice and of these12 had sham injury 16 mice had CCI. Of these Acute post traumatic seizures (Seizures in the first week after CCI) were seen in 6 of the 16 CCI mice. Of these, 1 mouse had acute posttraumatic seizures on day 1 which progressed to status epilepticus as well as Sudden Unexpected Death (SUDEP). Chronic posttraumatic seizures were seen in 4/16 (25%) mice that had CCI with the seizures seen in week 4 and 5 after CCI. Of the 12 sham injury mice, 1 had seizures in week 4 after CCI.
Interictal spike analysis: This is still ongoing and we do not have any data to present in TBI and Sham groups treated with Orexin-2 agonist or vehicle.

4. **Sleep data:**

We are currently performing sleep analysis in the TBI and Sham groups treated with Oxrein-2 agonist (YNT-185) or Vehicle from cohorts 2 (n=11) and 3 (n=18). We have 5 weeks of EEG recordings from 28 mice in TBI and Sham, drug and vehicle treated groups. The automated sleep analysis using Accu Sleep is ongoing and we do not have any data to report currently.

5. **Behavioral Tests:**

We performed behavioral tests including tail suspension (test of depression), radial arm water maze (test for spatial learning and memory) and fear conditioning (test for posttraumatic stress disorder) among animals in cohort 2 (n=11) and cohort 3 (n=18) which had TBI and Sham groups either treated with Orexin-2 agonist YNT185 or Vehicle.

Tail Suspension Test: No differences were seen latency to, frequency or duration of immobility following tail suspension in TBI and Sham groups treated with Orexin-2 agonist YNT185 or vehicle.

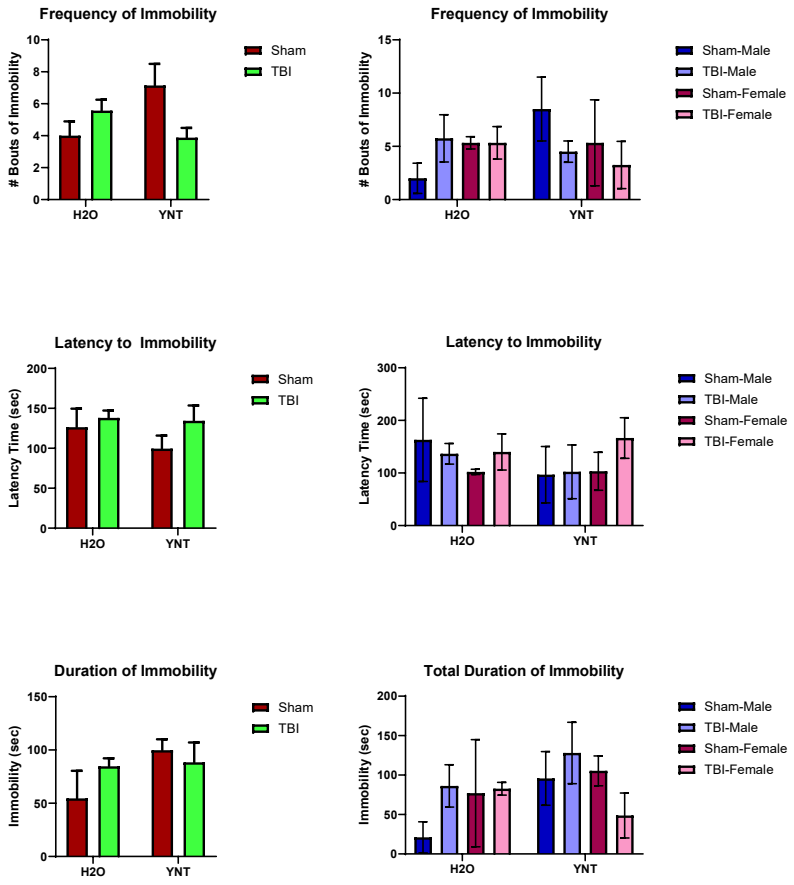


Figure 8: No differences seen in frequency, duration or latency to immobility in tail suspension test between different groups.

Fear conditioning and Radial arm water maze: TBI mice treated with YNT185 showed no improvements in the contextual fear conditioning. In the Radial arm water maze however, TBI group treated with YNT185 did better with less number of errors (Figure 9)

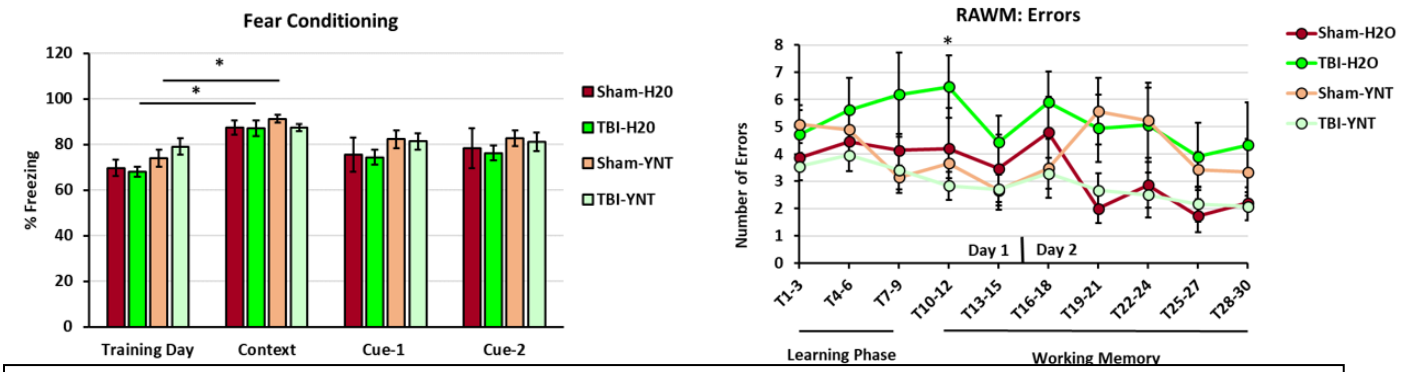


Figure 9: Left is fear conditioning result whereas number of errors in the Radial arm water maze is shown on the right between different treatment groups.

Next Experimental steps:

1. Complete data analysis of Major task 5. Once analysis is complete, we will make a decision on increasing the sample size.

2. Now that we have finally received the dual orexin antagonist (Lemborexant) from Eisai Pharmaceuticals, we will begin work om Major tasks 2-4.

Training/Professional Development: The Staff scientist Sue Osting had been trained in performing the TBI and EEG implantation surgeries. The Research Assistant Anna Goforth is learning analysis in MATLAB for sleep, interictal spike scoring. The figures presented in Data 1 and 2 were obtained by Anna. Four undergraduates are being trained in automated sleep analysis and were trained in oral gavage of different drugs or vehicle treatment.

Impact: Nothing to report

Problems and Changes:

1. The first problem we faced was with the wireless implants. We found that after implantation, scruffing the mice for oral gavage resulted in sutures breaking and wires exposed. Some mice had infection on the incision due to poor healing. This happened for most mice in cohort 1 and some died because of this. Hence we temporarily abandoned the wireless implants and resorted to conventional tethered recordings for Cohorts 2 and 3.
2. We negotiated with Eisai Pharmaceuticals to obtain the dual orexin antagonist Lemborexant for our DORA experiments. This was done for 2 reasons. First is that if an FDA approved drug can show positive findings in a preclinic study, transitioning the findings to a clinic trial would be easier. Second is a cost issue. DORAs are extremely expensive when they are obtained from vendors. Once an agreement was reached with Eisai, and MTA was executed, it was about 7 months. Then the drug had be shipped from UK which requires additional DEA authorizations and that was another delay. We finally received the drug and plan to start the experiments in Major Tasks 2-4.

Products: Nothing to report.

Participants and collaborating Organizations:

1. University of Wisconsin Madison: Rama Maganti, MD (PI); Mathew Jones, PhD (Co-I);
   Staff scientists: Yuzhen Pan PhD; Sue Osting MS
   Research assistant: Anna Goforth BS
   Undergraduate students: Students perform maintenance of animals during EEG recordings, Analyzing sleep and EEG data; performance of oral gavage to treat animals daily with a drug or vehicle.

2. Beloit College: Rachel Bergstrom PhD (Collaborator)

Special Reporting Requirements: None

Appendices: None