AWARD NUMBER: W81XWH-18-1-0594

TITLE: Discovery of Novel Therapeutics for Disordered Sleep in Fragile X Syndrome

PRINCIPAL INVESTIGATOR: Ravi Allada

CONTRACTING ORGANIZATION: Northwestern University

REPORT DATE: June 2021

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE		*Form Approved OMB No. 0704-0188*
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**		
1. REPORT DATE June 2021	**2. REPORT TYPE** Final	**3. DATES COVERED** 9/1/18-2/28/21
4. TITLE AND SUBTITLE. Discovery of Novel Therapeutics for Disordered Sleep in Fragile X Syndrome		**5a. CONTRACT NUMBER** **5b. GRANT NUMBER** W81XWH-18-1-0594 **5c. PROGRAM ELEMENT NUMBER**
6. AUTHOR(S) Ravi Allada (PI) E-Mail: r-allada@northwestern.edu		**5d. PROJECT NUMBER** **5e. TASK NUMBER** **5f. WORK UNIT NUMBER**
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Northwestern Univ., 633 Clark, Evanston, IL 60208		**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 NUMBER(S)**
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		
13. SUPPLEMENTARY NOTES		

14. ABSTRACT

Here we employed a large-scale screen of FDA approved/clinical compounds to identify compounds that can modify disrupted sleep or circadian rhythms deficits in a validated fruit fly (Drosophila)model of Fragile X. We identify and validate a high value compound penfluridol that can address a subset of circadian and sleep phenotypes. Penfluridol is a dopamine D2 receptor and T-type calcium channel blocker highlighting a potential role for these molecular targets for therapeutic strategies. The identification of a potential therapeutic for Fragile X is a major step forward in the development of new treatments for this neurodevelopmental disorder.

15. SUBJECT TERMS

Fragile X Syndrome, Autism Spectrum Disorders, Drug Screen, FDA small molecule compounds

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	8	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. **INTRODUCTION:** Fragile X syndrome is a neurodevelopmental autism spectrum disorder characterized by cognitive impairments as well as poor sleep architecture and circadian deficits that may aggravate behavioral symptoms. Yet there are no effective therapies. Our goal here
2. **KEYWORDS:** Fragile X Syndrome (FXS), Drosophila, FDA Drug Discovery, Penfluridol
3. **ACCOMPLISHMENTS:**
    - **What were the major goals of the project?**
        - Major Task 1: Primary drug screen – End Date 30 Sep 2019
            - Screening of Library 1 (FDA ENZO) – 8 Months
                - Milestones achieved – ~300 preliminary candidates were identified for 6 different parameters that altered Sleep architecture, Anticipation and rhythmicity.
        - Major Task 2: Follow up testing of drugs (Retesting of Hits, Drug safety and Toxicity, Drug Specificity)– End date 15 August 2020
            - Retesting of 300 drug candidates
                - Milestones achieved – 300 Candidates were tested, and 14 hits were selected for second round of retesting
            - Re-retesting and Assessment of Toxicity and Specificity
                - Milestone achieved – 14 drugs were tested in $dfmr^{B55}$ and wildtype flies to ascertain toxicity and specificity. Potential hits were established.
            - Confirmation and validation using an updated behavioral software suite
    - **What was accomplished under these goals?**
        - major activities; FDA approved Library of 1280 compounds was successfully screened, and multiple drug candidate leads were identified for each of these parameters – Total Sleep (24Hrs), Sleep Bouts, Sleep Length, Morning and Evening Anticipation, and Rhythmicity, Fig1. 2) 300 preliminary candidates were retested (`60 for each behavioral paradigm). Drugs which showed same direction of response and strong effects were chosen for further evaluation, for e.g. Fig2, Drugs which showed changes of +/- 100mins of sleep in both the screen and retest were categorized as “hits” and chosen for further evaluation. 3) Total of 14 drug hits were selected and reevaluated figure 3. 4) 14 candidates were selected after the first and second round and tested again alongside wildtype flies to ascertain specificity and toxicity. 3) Drug safety and toxicity was evaluated for each of those compounds by estimating the activity counts per minute (figure4). 5) Drug hits were also tested on wild type flies to ascertain drug specificity to the dfmrB55 flies. Hits included reserpine which have previously been shown to increase sleep and showed similar results in all our retests thus confirming the validity of our screen. The data from activity counts per minutes ruled out any overt toxic effects of the drugs. Results from wild type flies established certain drugs that were only specific to $dfmr^{B55}$, Figure 5.

        - Summary-Our results show that penfluridol and reserpine were the strongest candidates that altered sleep in both fragile x flies and wild type flies. The increased and decreased the total sleep respectively and influenced bout no, period of sleeping (bout length) and latency (time from lights off to first sleep bout) accordingly. The compound that were found to be specific for fragile x flies were ethacridine, ketoprofen, etoxybenzamide and alogliptin. These increased the total sleep. L-Glutamine was found to be selectively decreasing morning and evening anticipation only in fragile x flies. Penfluridol strongly affected evening anticipation in fragile x flies but spared the behavior in wild type flies. Penfluridol is a long acting antipschycotic drug which has been widely used for treating schizophrenia and been implicated in alleviating neurological disorders. Alogliptin which is an antidiabetic drug that targets dipeptidyl peptidase was also a strong candidate. Since there have been growing evidence of potential links between autism and diabetes it warrants further investigation and points to common genetics pathways between autism and diabetes(Chen et al., 2016).

- Going through our rigorous therapeutic pipeline, we discovered penfluridol as a the most robust candidate therapeutic for treatment of Fragile X related sleep symptoms.

- During the most recent reporting period, we performed confirmatory behavioral analysis with an updated behavioral software program. These studies highlighted a potential therapeutic role for penfluridol. In addition, we also performed literature searches to ascertain the mechanism of penfluridol and potential links to Fragile X.

- **What opportunities for training and professional development has the project provided?**
  - During the reporting period, 3 postdoctoral fellows, Master's student and undergraduate received training in drug screening, circadian rhythms and sleep analysis.

- **How were the results disseminated to communities of interest?**
  - We plan to publish the results for the scientific community

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - N/A

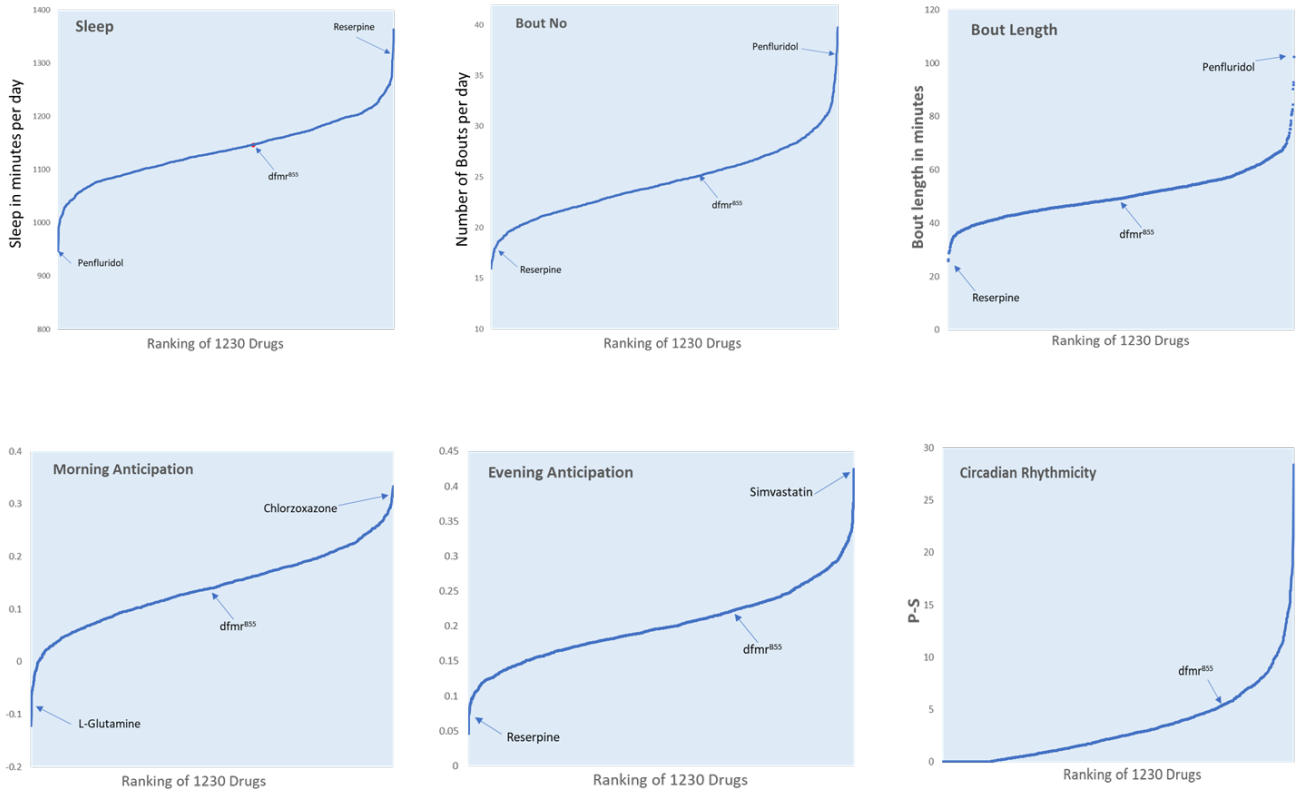
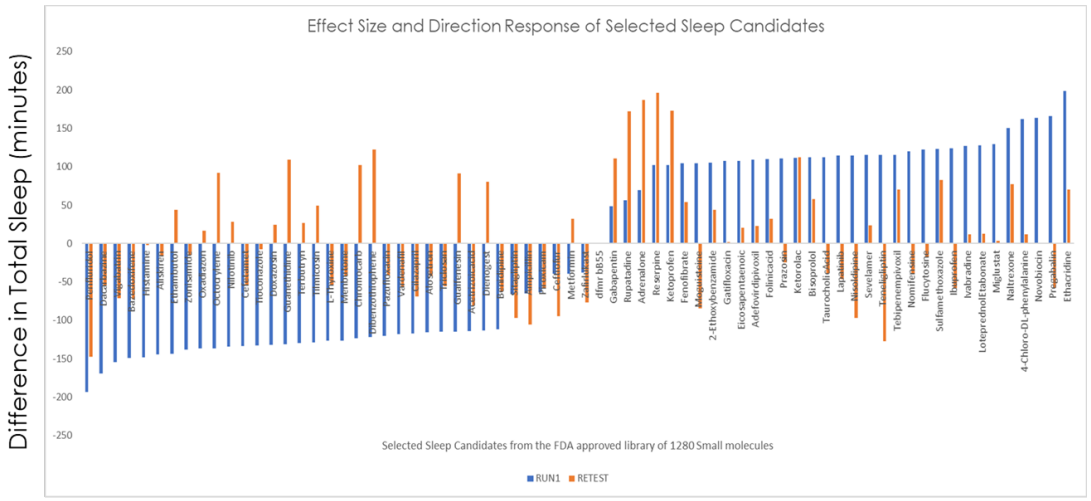


Figure 1. Ranking plots for all 1280 Drugs tested from FDA approved Drug Library. The sigmoidal curve represents the average values over the period of 4 days, N=~8 for each fly. Six different behavioral phenotypes are shown for which the top and bottom 2% drugs were selected. Total of 300 drugs were retested in following experiment.



Values post retesting for candidates are shown side by side in orange. Y-axis represents the difference between *dfmr*^{b55} Total Sleep and Total Sleep of *dfmr*^{B55} on a drug*.

Figure 2. Representative graph - Comparison of Total Sleep for 60 candidates retested. Shown above are graphs comparing initial screen values and after retesting. Plotted are the effect size and direction of effect from dfmr*^{B55}. Drugs with same direction of response and more than +/-100 minutes of sleep were chosen further for validation.*

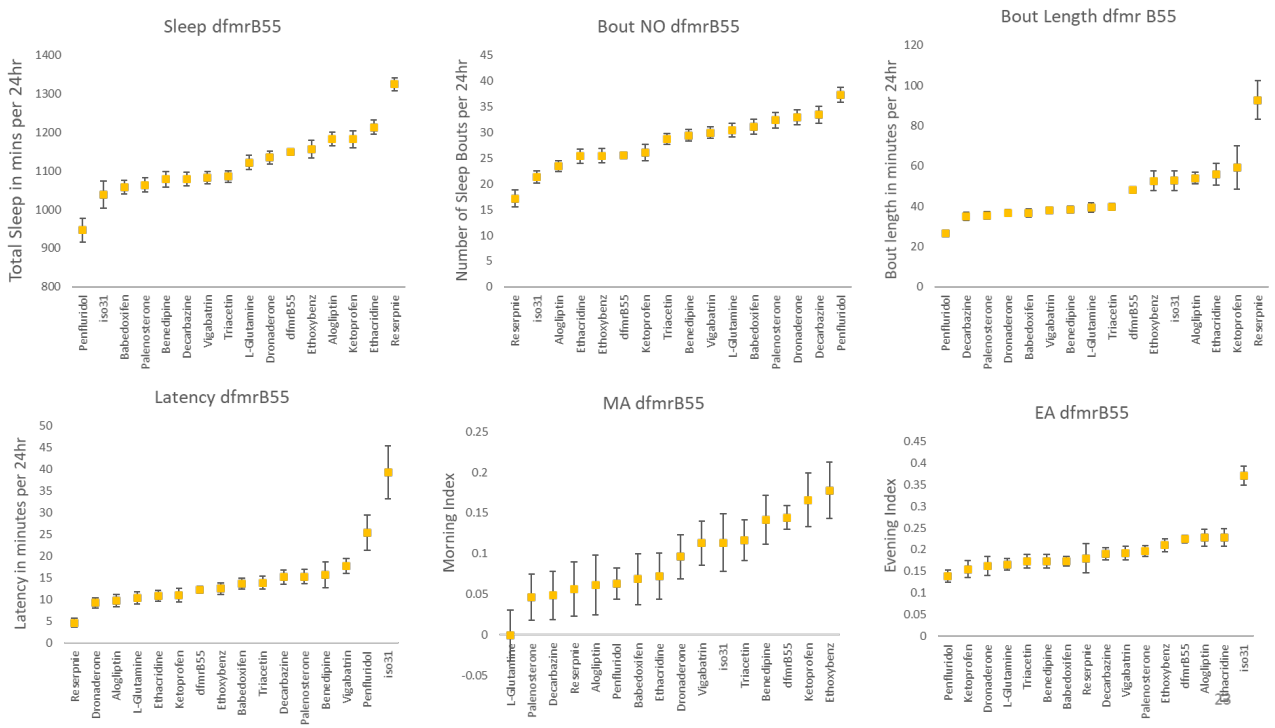


Figure 3. Ranking plots of different behavioral paradigms for selected drug "hits". Total of 14 Drugs were retested belonging to either of the behavioral paradigm (Total Sleep, Bout No, Bout Length, Latency, Morning anticipation and evening anticipation. Reserpine consistently showed similar results as previously documented in other studies. Penfluridol (antipsychotic, Calcium channel blocker) consistently lowered the sleep where as Alogliptin (antidiabetic), Ketoprofen (NSAID) and Ethacridine (Antiseptic) consistently enhanced sleep. L-Glutamine consistently lowered the morning anticipation.

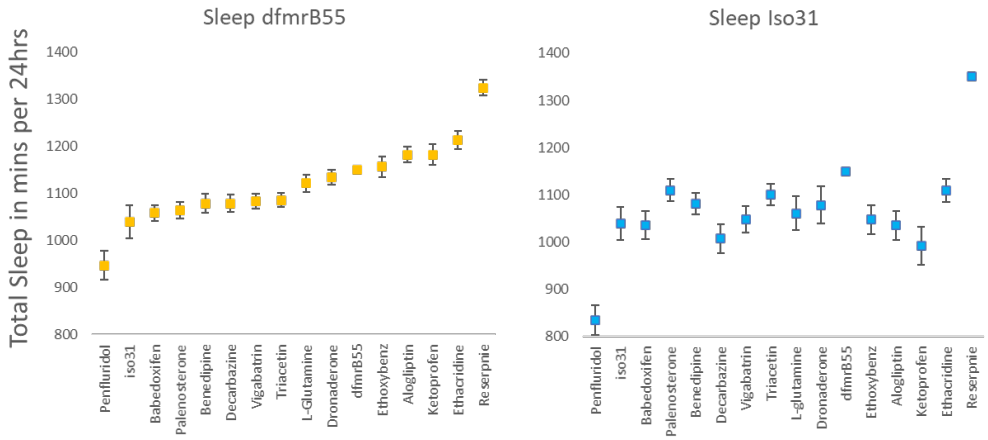
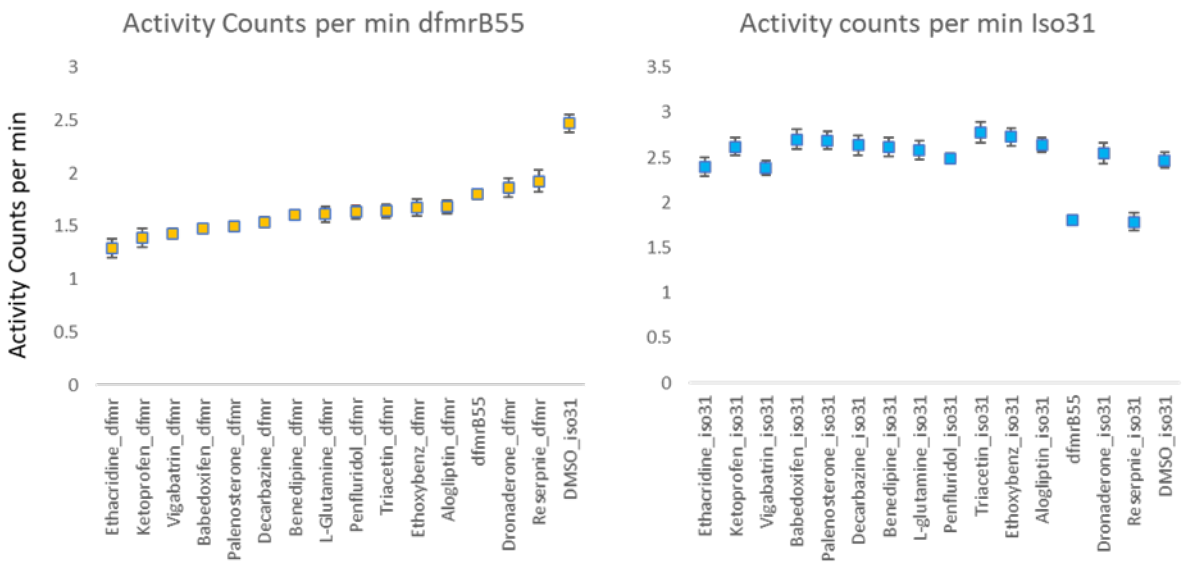
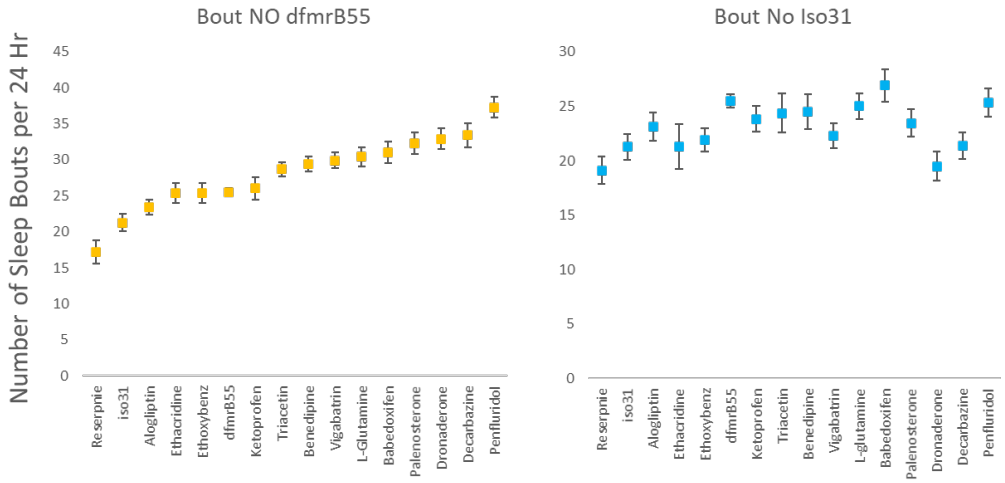
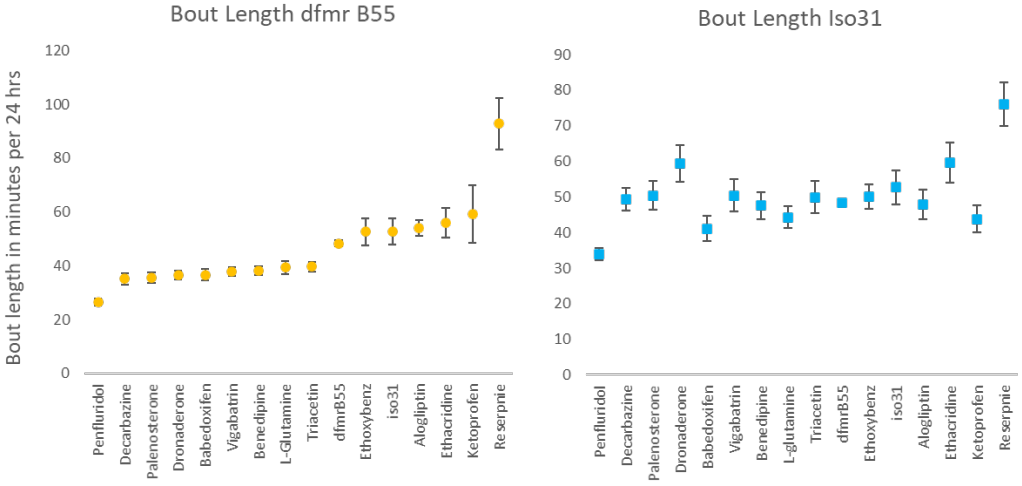


Figure 4. Ranking plots of activity profile of dfmr^{B55} and Wild type (iso31) flies on each retested hit drugs. Activity counts per mins were calculated for each drug and evaluated for toxicity. Only drugs with no over toxicity were selected from lead candidates and tested. When compared to Wild type flies the overall activity for dfmr^{B55} flies was lower that Wild type flies but did not pose challenges in evaluation of the drug hits.


Activity Counts per min dfmrB55
Activity Counts per min
3
2.5
2
1.5
1
0.5
0
Ethacridine_dfmr
Ketoprofen_dfmr
Vigabatrin_dfmr
Babedoxifen_dfmr
Palenosterone_dfmr
Decarbazine_dfmr
Benedipine_dfmr
L-Glutamine_dfmr
Penfluridol_dfmr
Triacetin_dfmr
Ethoxybenz_dfmr
Alogliptin_dfmr
dfmrB55
Dronaderone_dfmr
Reserpnie_dfmr
DMSO_iso31
Activity counts per min Iso31
3.5
3
2.5
2
1.5
1
0.5
0
Ethacridine_iso31
Ketoprofen_iso31
Vigabatrin_iso31
Babedoxifen_iso31
Palenosterone_iso31
Decarbazine_iso31
Benedipine_iso31
L-glutamine_iso31
Penfluridol_iso31
Triacetin_iso31
Ethoxybenz_iso31
Alogliptin_iso31
dfmrB55
Dronaderone_iso31
Reserpnie_iso31
DMSO_iso31


Bout NO dfmrB55
Number of Sleep Bouts per 24 Hr
45
40
35
30
25
20
15
10
5
0
Reserpnie
iso31
Alogliptin
Ethacridine
Ethoxybenz
dfmrB55
Ketoprofen
Triacetin
Benedipine
Vigabatrin
L-Glutamine
Babedoxifen
Palenosterone
Dronaderone
Decarbazine
Penfluridol
Bout No Iso31
30
25
20
15
10
5
0
Reserpnie
iso31
Alogliptin
Ethacridine
Ethoxybenz
dfmrB55
Ketoprofen
Triacetin
Benedipine
Vigabatrin
L-glutamine
Babedoxifen
Palenosterone
Dronaderone
Decarbazine
Penfluridol


Bout Length dfmr B55
Bout length in minutes per 24 hrs
120
100
80
60
40
20
0
Penfluridol
Decarbazine
Palenosterone
Dronaderone
Babedoxifen
Vigabatrin
Benedipine
L-Glutamine
Triacetin
dfmrB55
Ethoxybenz
iso31
Alogliptin
Ethacridine
Ketoprofen
Reserpnie
Bout Length Iso31
90
80
70
60
50
40
30
20
10
0
Penfluridol
Decarbazine
Palenosterone
Dronaderone
Babedoxifen
Vigabatrin
Benedipine
L-glutamine
Triacetin
dfmrB55
Ethoxybenz
iso31
Alogliptin
Ethacridine
Ketoprofen
Reserpnie

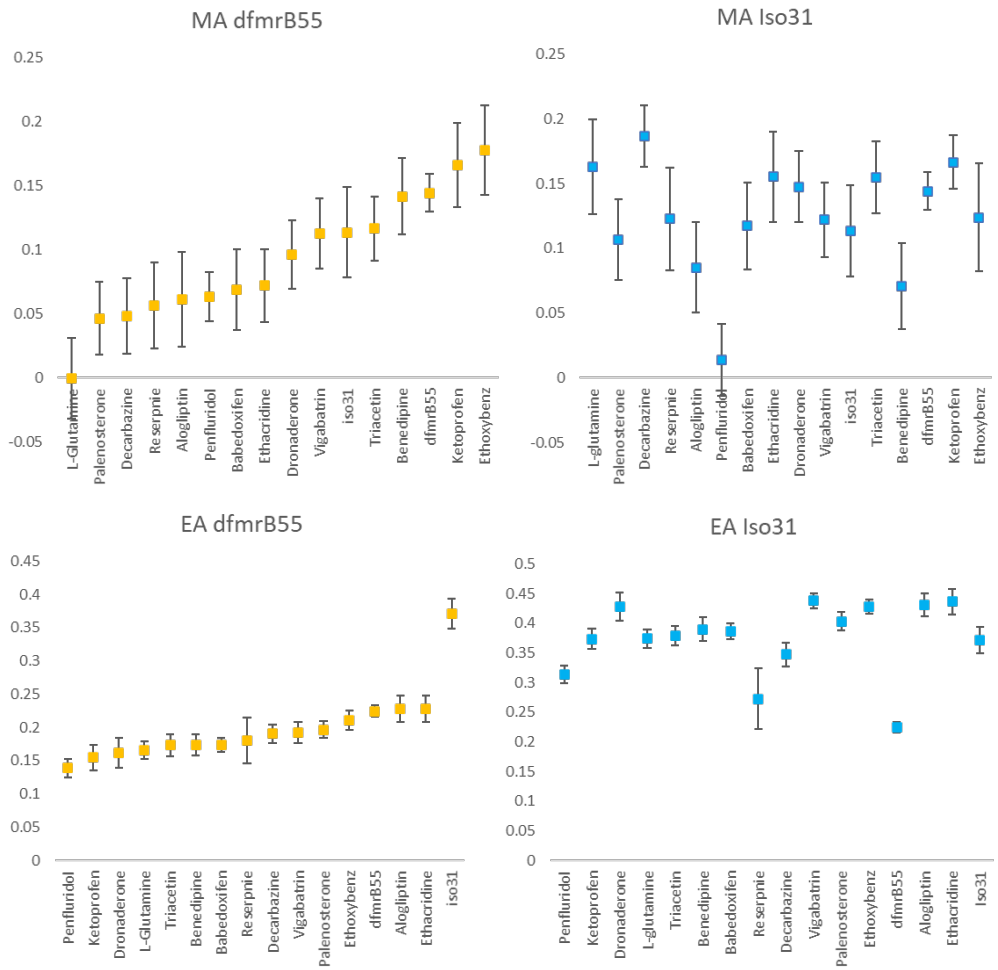


Figure 5. Ranking Plots comparing behavioral phenotypes for dfmr^{B55} and Wild type (Iso^{31}) on drug hits (Sleep Architecture – Total Sleep, Bout NO, Bout Length, Latency), Morning anticipation, Evening anticipation). Penfluridol and reserpine showed similar effects in both dfmr^{B55} and wild type flies. However, ethacridine, ketoprofen, Ethoxybenzamide and alogliptin altered sleep profiles for dfmrB55 flies only.

Methodology: - 6-8 days old Drosophila dfmr^{B55} male flies were given 50um of each drug mixed with food (2%Agar + 5% Sugar) and kept in Drosophila Activity Monitors for 5LD and 7DD to assess activity. Data was collected after two weeks and analyzed for different parameters – Total Sleep (more than 5 minutes of inactivity), Sleep bouts (average no of periods of sleep), Sleep length (average duration of periods of Sleep). Data was also analyzed for anticipation (morning and evening). Data was pooled from initial screen and 2 rescreen with selected leads and hits were established. For each drug data was pooled for ~32 flies (except reserpine=21).

4. **IMPACT:**
    - **What was the impact on the development of the principal discipline(s) of the project?**
        - The discovery of penfluridol could potentially transform therapeutics for Fragile X
    - **What was the impact on other disciplines?**
        - These studies may also highlight novel treatments for sleep and circadian disorders
    - **What was the impact on technology transfer?**
        - Confirmatory studies may lead to patent submission
    - **What was the impact on society beyond science and technology?**
        - If successful, new treatments for Fragile X could improve the lives of affected children and their families.

5. **CHANGES/PROBLEMS:**
    - **Changes in approach and reasons for change**
        - We proposed screening two commercial libraries of FDA/clinical compounds but were able to obtain an existing FDA library through an NU core facility. We identified 300 candidate compounds and given the large number focused on retesting and validating leading to the discovery of penfluridol
        - We had technical issues with testing of courtship behavior in Fragile X mutants and thus were unable test our compounds in this assay
    - **Actual or anticipated problems or delays and actions or plans to resolve them**
        - As this is the final report we will be unable to resolve them under this award
    - **Changes that had a significant impact on expenditures**
        - Nothing to report
    - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
        - Not applicable
6. **PRODUCTS:**
    - **Publications, conference papers, and presentations**
        - **Journal publications.** Nothing to report
        - **Books or other non-periodical, one-time publications.** Nothing to report
        - **Other publications, conference papers, and presentations.** Nothing to report
    - **Website(s) or other Internet site(s)** Nothing to report
    - **Technologies or techniques** Nothing to report
    - **Inventions, patent applications, and/or licenses** Nothing to report
    - **Other Products** Nothing to report

7. **PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS**
    - **What individuals have worked on the project?**

Name:	*Shiju Sisobhan*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	9
Contribution to Project:	*Dr. Sisobhan has developed software to analyze drug data*
Funding Support:	*This award*

Name:	*Bart Van Alphen*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*4*
Contribution to Project:	*Dr. Van Alphen developed protocols to analyze drug effects on behavior*
Funding Support:	*This award*

Name:	*Ravi Allada*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	3
Contribution to Project:	*He supervised this project*
Funding Support:	*This award*

Name:	*Sumit Saurabh*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	6
Contribution to Project:	*He started the project and was responsible for collection of data and analysis. He was also involved in training the Master's and undergraduate students on the project*
Funding Support:	*This award*

Name:	*Timothy David Earl*
Project Role:	*Master's Trainee*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	8
Contribution to Project:	*Mr. Earl was responsible for maintenance of animals for the*

	project and helped with executing the screen and carried out data collection and analysis
Funding Support:	*This award*

Name:	*Brandon Cho*
Project Role:	*Undergraduate Trainee*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	11
Contribution to Project:	*Mr. Cho was responsible for maintenance of animals for the project and helped with executing the screen and carried out data collection and analysis*
Funding Support:	*This award*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** Yes

  735135(PI: Allada), Simons Foundation(SFARI), Defining Behavioral Gene networks for Autism Spectrum Disorder Genes Using Sleep and Circadian Rhythms (pending to active)

  AARG-17-532626 (PI: Allada), Alzheimer's Association, Discovery of Novel Mechanisms by which Sleep Modulates AB Toxicity (active to past funding)

  1R21NS110420-01 (PI: Allada), NIH/NINDS, Discovery of Novel Pathways Mediating Huntingtin Neurotoxicity (active to past funding)

- **What other organizations were involved as partners?**

  - Nothing to Report

8. **SPECIAL REPORTING REQUIREMENTS**
   - Not applicable
9. **APPENDICES**
   - Not applicable