Award Number: W81XWH-18-1-0594

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

PRINCIPAL INVESTIGATOR: Ravi Allada, M.D.

CONTRACTING ORGANIZATION:
Northwestern University
633 Clark St.
Evanston, IL 60208

REPORT DATE: SEPTEMBER 2020

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
SEPTEMBER 2020	Annual	1SEPT2019 - 31AUG2020

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)
Dr. Ravi Allada (PI), Dr. Sumit Saurabh (Postdoctoral Fellow)

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 University, 633 Clark St, Evanston, IL 60208

8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Materiel 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
Fragile X syndrome is the single gene disorder that cause sleep and circadian deficits in both humans and Drosophila melanogaster. In this project we employed the enormous benefits of this model system to perform a large-scale drug screen. Here we identify from a large scale in vivo drug screen compounds that replicably alter sleep and circadian rhythms in Fragile X mutants. Initial studies indicate that some of these effects may be evident in Fragile X flies but not in wild-type.

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
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	13	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. **INTRODUCTION:** Fragile X Syndrome (FXS) is a single gene disorder that results from the silencing of Fragile Mental Retardation (FMR) gene. FMR gene deletion is also the single most contributing factor to the severity of Autism Spectrum Disorders, leading to cognitive impairments, poor sleep architecture and circadian deficits thus aggravating behavioral symptoms. Current research on therapeutics intervention for sleep deficits is limited to Melatonin and has given mixed results. Hence, the need for alternative drug molecules is high. The goal of this study was to screen 1280 FDA approved small molecules against Drosophila model of FXS to discover drug candidates that can be repurposed as a therapy for the disease. The scope of the study was to screen, identify the "lead" candidates, replicate and confirm the "hits" and carry behavioral assessment for sleep and cognitive improvements. Since these compounds have already been studied, gone through clinical trials and have been FDA approved, bringing them from bench to bedside will pose fewer challenges.
2. **KEYWORDS:** Fragile X Syndrome (FXS), Fragile Mental Retardation (FMR), Autism Spectrum Disorders, Drosophila, Drug Screening
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.
    - **What was accomplished under these goals?**

        1) 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).

- **What opportunities for training and professional development has the project provided?**
  - This project employed a Postdoctoral Fellow, A Master's Student and an undergrad for training purposes. During the project the assigned individuals were trained on crucial aspects of scientific research such as experimental design, Animal husbandry, data collection and analysis
  - The personnel were trained in the field of Sleep and Circadian Biology. Those on the project were required to complete the data analysis and present results regularly during lab meetings. To ensure professional development trainees were encouraged to read literature and contribute to the project. They were routinely assessed during one-on-one meetings and encouraged to attend scientific journal clubs about the relevant topics.

- **How were the results disseminated to communities of interest?**

- The results and methodology of this study were discussed and shared within the PI's own group as well as presented by the master's and undergraduate student during poster presentations to their peers and mentors at Northwestern University.
-

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - Assess the effects of these drugs in alleviating the other behavioral deficits such as learning and memory, Social behaviors – Courtship and Aggression.

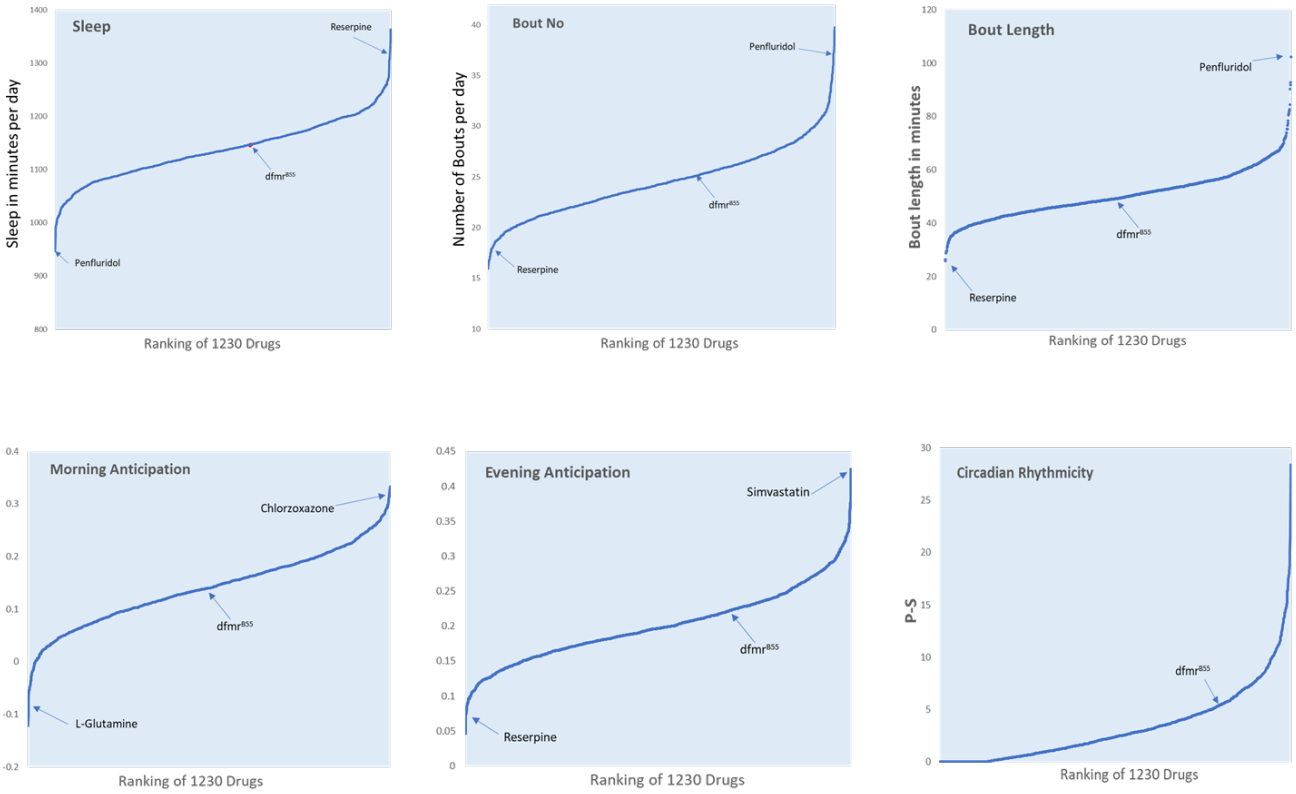
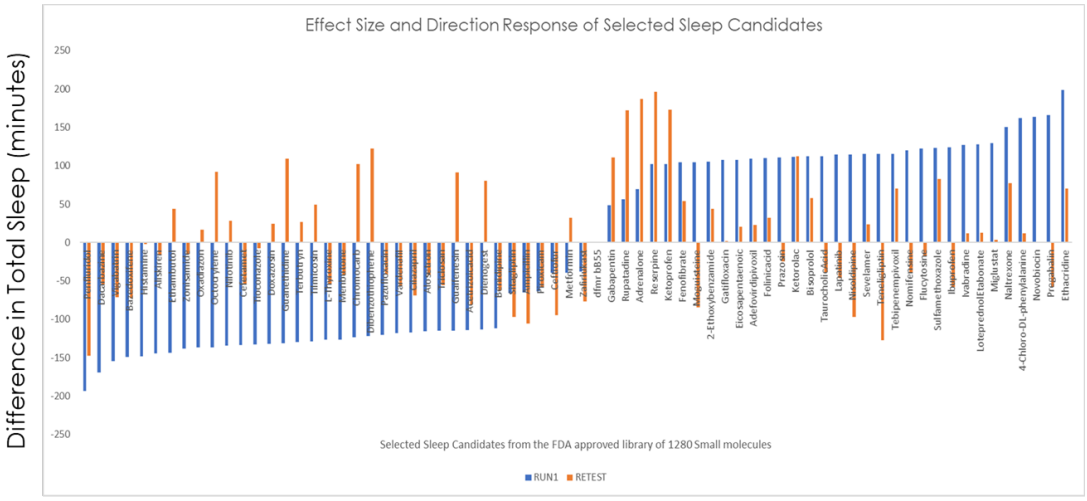


Figure 1. Ranking plots for all 1280 Drugs tested from FDA approved Drug Library. The sigmoidal curve represents the average values over the period of 4 days, N=~8 for each fly. Six different behavioral phenotypes are shown for which the top and bottom 2% drugs were selected. Total of 300 drugs were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

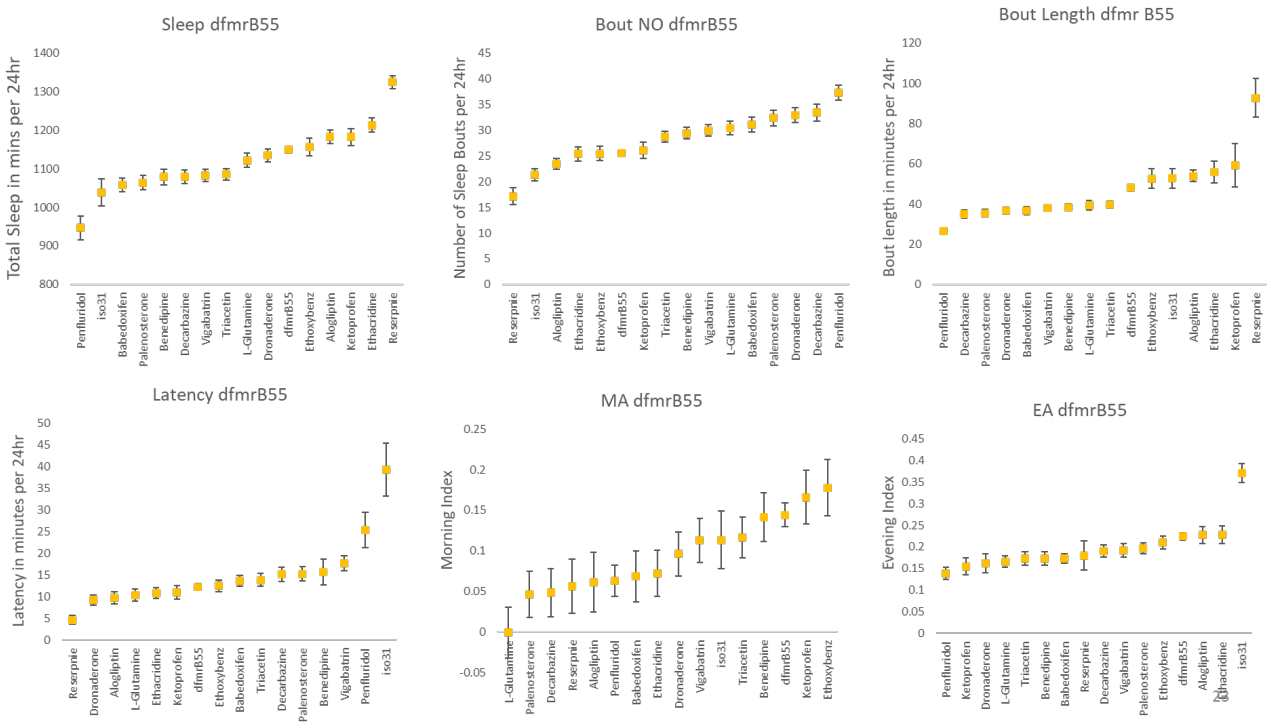


Figure 3. Ranking plots of different behavioral paradigms for selected drug "hits". Total of 14 Drugs were retested belonging to either of the behavioral paradigm (Total Sleep, Bout No, Bout Length, Latency, Morning anticipation and evening anticipation. Reserpine consistently showed similar results 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.

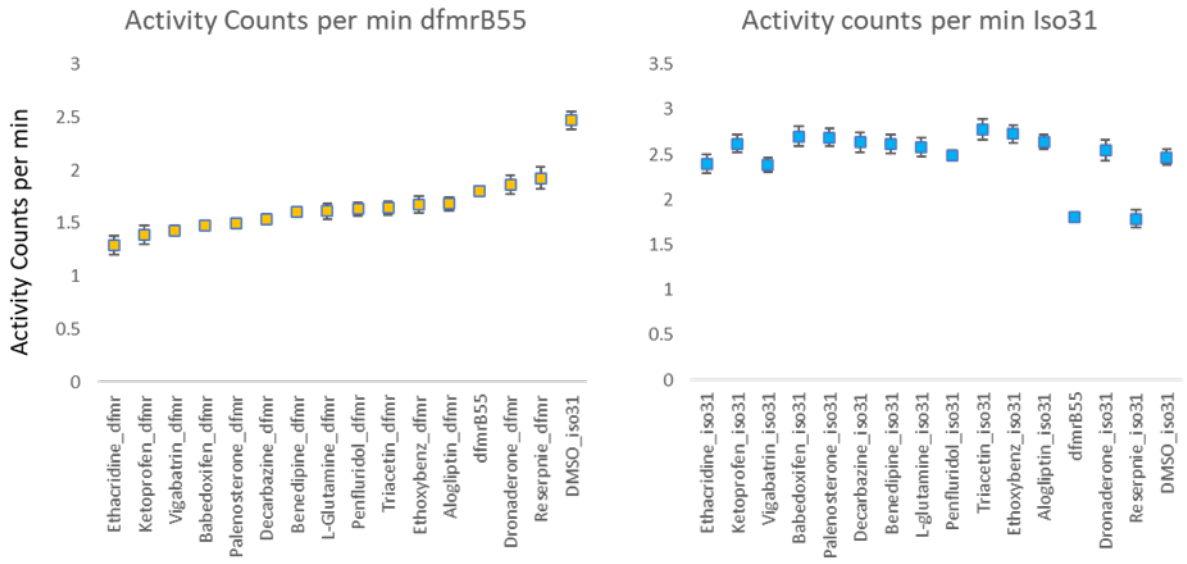
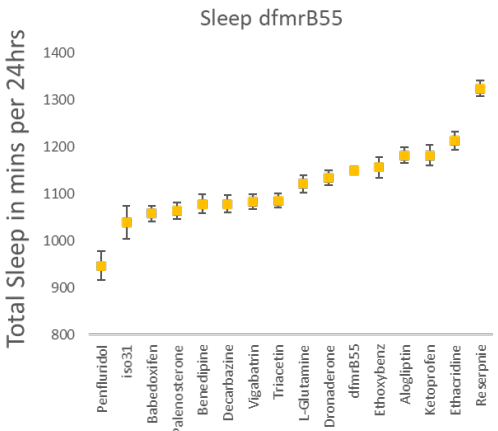
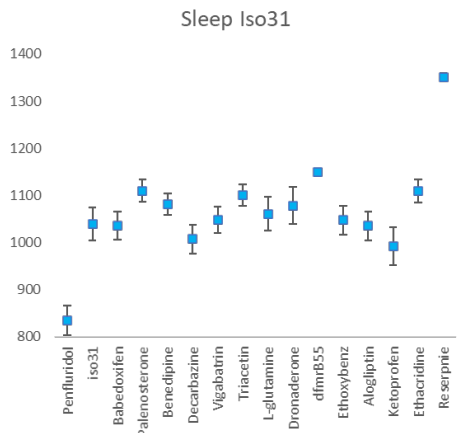
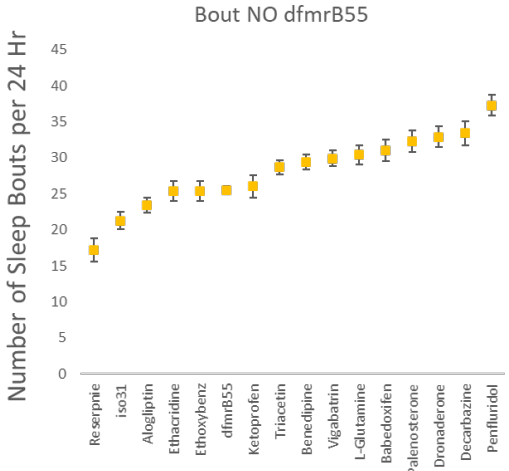
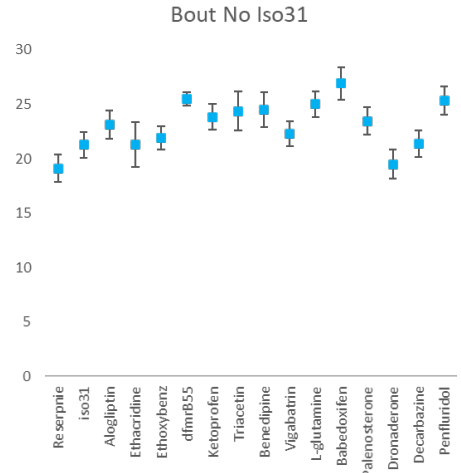


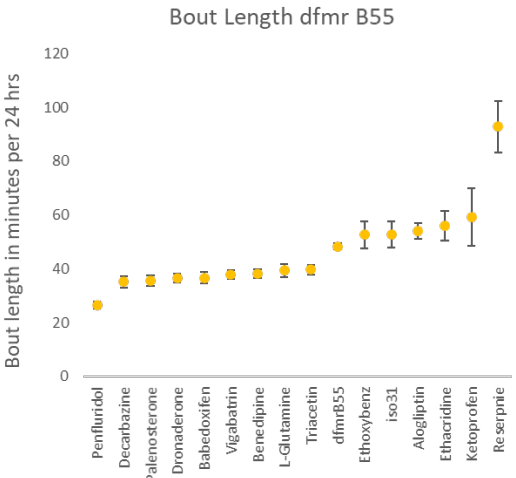
Figure 4. Ranking plots of activity profile of dfmr^{B55} and Wild type (iso31) flies on each retested hit drugs. Activity counts per mins were calculated for each drug and evaluated for toxicity. Only drugs with no over toxicity were selected from lead candidates and tested. When compared to Wild type 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.

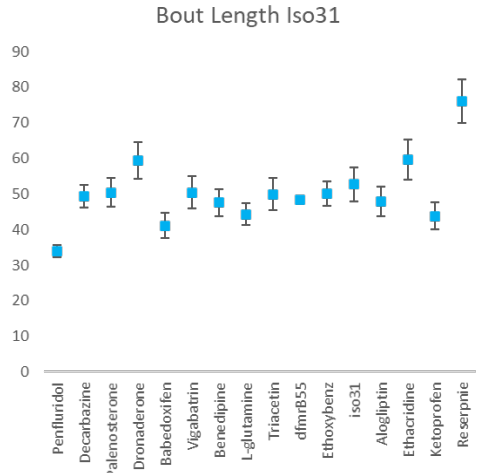

Sleep dfmrB55
Total Sleep in mins per 24hrs
1400
1300
1200
1100
1000
900
800
Penfluridol
iso31
Babedoxifen
Palenosterone
Benedipine
Decarbazine
Vigabatrin
Triacetin
L-Glutamine
Dronaderone
dfmrB55
Ethoxybenz
Alogliptin
Ketoprofen
Ethacridine
Reserpnie


Sleep Iso31
1400
1300
1200
1100
1000
900
800
Penfluridol
iso31
Babedoxifen
Palenosterone
Benedipine
Decarbazine
Vigabatrin
Triacetin
L-glutamine
Dronaderone
dfmrB55
Ethoxybenz
Alogliptin
Ketoprofen
Ethacridine
Reserpnie


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

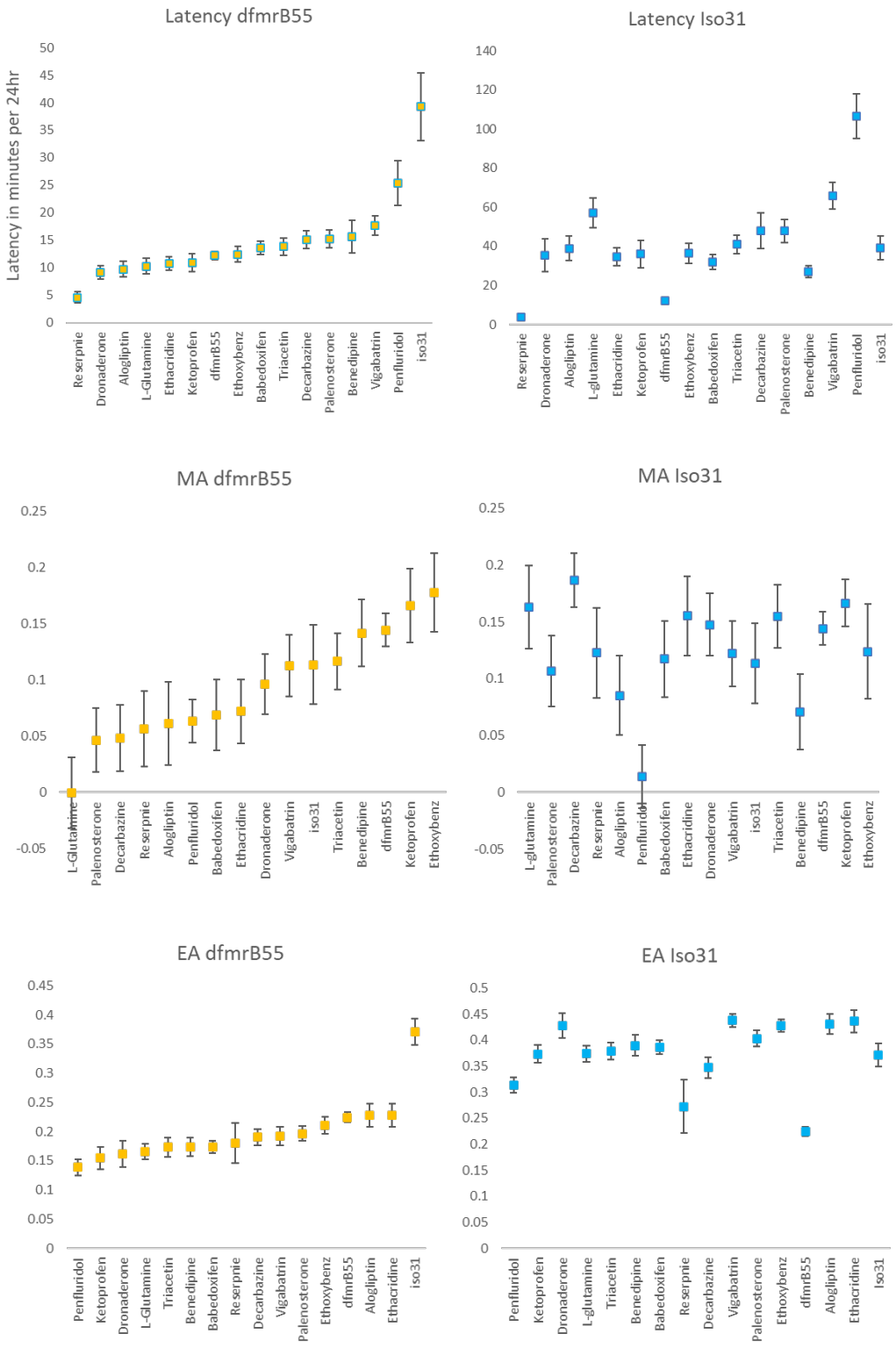


Figure 5. Ranking Plots comparing behavioral phenotypes for dfmr^{B55} and Wild type (Iso^{31}) on drug hits (Sleep Architecture – Total Sleep, Bout NO, Bout Length, Latency), Morning anticipation, Evening anticipation). Penfluridol and reserpine showed similar effects in both dfmr^{B55} and wild type 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?**
        - Nothing to report
    - **What was the impact on other disciplines?**
        - Nothing to report
    - **What was the impact on technology transfer?**
        - Nothing to report
    - **What was the impact on society beyond science and technology?**
        - Nothing to report
5. **CHANGES/PROBLEMS:**
    - **Changes in approach and reasons for change**
        - We initially proposed to screen two commercial libraries of FDA/clinical compounds. We were able to obtain an existing FDA library through our core facility. We have already identified 300 candidate regulator and thus we are choosing to prioritize these candidates over screening s. Given the large number, we are opting on focusing on replicating these potential results
    - **Actual or anticipated problems or delays and actions or plans to resolve them**
    - **Changes that had a significant impact on expenditures**
    - **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:	*7*
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*

- **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

  5U41HG007355-06 (PI: Waterston), University of Washington/NHGRI, Creating Comprehensive Maps of Worm and Fly Transcription Factor Binding Site (pending to active)

  PR190010, U.S. Army Medical Research and Material Command, Uncovering New Therapeutics and Neuroprotective Mechanisms for TBI (pending to active)

- **What other organizations were involved as partners?**
  - Nothing to Report

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

9. APPENDICES

- Not applicable