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TITLE: Investigating Striatal Attentional Circuits to Understand and Mitigate Deficits in Cognitive Flexibility Due to Sleep Loss

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Sleep loss compromises specific cognitive abilities that are both critical to real-world performance and dissociable from impairments in vigilant attention. Specifically, sleep loss impairs cognitive flexibility, which is the ability to adapt to changing events and environmental contingencies. We hypothesize that sleep loss-induced adenosinergic disruption of striatal dopaminergic circuits explains reduced attentional flexibility. We aim to identify dopaminergic and adenosinergic neural circuits responsible for sleep loss-induced deficits in cognitive flexibility using transgenic rats and optogenetic techniques, and performance measures that parallel task requirements for human cognitive flexibility. We seek to obtain converging evidence for the role of these circuits in humans by analyzing genotype differences in the effectiveness of wake-promoting agents during sleep deprivation.
Year 1 of this ongoing project has focused on obtaining the necessary approvals for animal and human subject data collection (WSU IACUC/IRB, DoD ACURO/HRPO, NMRU-D IRB). Equipment for the animal studies has been obtained and tested. Cognitive testing procedures for the transgenic rats have been developed, and a breeding colony has been established. Procedures for the human study have been developed as well.

15. SUBJECT TERMS
Sleep deprivation, performance impairment, attentional control, cognitive flexibility, resilience, striatum, caffeine, modafinil, optogenetic stimulation

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1. INTRODUCTION

This project aims to investigate whether and how adenosinergic disruption of striatal dopaminergic circuits explains the reduced attentional flexibility caused by sleep deprivation. In animal studies, we optimize behavioral techniques that model the effects of sleep loss on cognitive flexibility observed in humans. Next, we use transgenic rats that express Cre and Flp recombinase-dependent viral DNA constructs in striatopallidal medium spiny neurons of the striatum that express both the Adora2a and DrD2 receptors. We use optogenetic methods to either activate these neurons, mimicking the effects of sleep deprivation on task performance in rats injected with flox/Frt -ChR2-GFP, or inactivate these neurons to recover normal task performance in sleep-deprived rats. For human subjects, we compare the effectiveness of standardized doses of modafinil and caffeine during total sleep deprivation in promoting cognitive flexibility based on dopaminergic and adenosinergic genotype. Beyond demonstrating that our animal model of attentional circuitry compromised by sleep loss generalizes to humans, these studies will shed light on the effectiveness of pharmacological agents countering the effects of sleep loss in settings that require the ability to rapidly adapt to changing circumstances. Thus, our research will have immediate real-world relevance for health and safety in industrial settings, emergency occupation, and people engaged in military operations.

2. KEYWORDS

Sleep deprivation, performance impairment, attentional control, cognitive flexibility, resilience, striatum, caffeine, modafinil, optogenetic stimulation

3. ACCOMPLISHMENTS

What were the major goals of the project?

Specific Aim 1: Develop behavioral model of sleep loss and cognitive flexibility in rodents.	**Timeline**	**Completed**
Study Preparations	Months	
Milestone(s) Achieved: Behavior techniques are validated and properties of transgenic rats have been verified for Aim 1.	14	90%
Data Analysis		
Milestone(s) Achieved: Aim 1 analyses completed.	14	n/a
Specific Aim 2: Perform optogenetic experiments with transgenic rats.		
Data Collection		
Milestone(s) Achieved: Completion of data collection for Aim 2.	36	n/a
Data Analysis		
Milestone(s) Achieved: Aim 2 analyses completed.	36	n/a
Specific Aim 3: Demonstrate genotype differences in wake-promoting agents' effect on cognitive flexibility during sleep deprivation.		
Study Preparations	Months	
Milestone(s) Achieved: Procedures documented and IRB/HRPO approvals obtained.	6 (delays incurred due to contracting delays at NMRU-D; no major impact on study)	WSU IRB approval: 4 Jan 2019 HRPO approval: 2 April 2019 NMRU-D IRB approval: Pending
Data Collection		
Milestone(s) Achieved: Aim 3 data collection completed from 90 subjects (3 groups of 30 subjects in sleep deprivation condition with caffeine, modafinil, placebo).	36	n/a
Data Analysis		
Milestone(s) Achieved: Aim 3 analyses completed.	36	n/a
Final Report Preparation		
Compilation of analyses from aims 1–3 and drafting of report and briefing	30-36	n/a
Presentation of study results to the DoD	36	n/a
Milestone(s) Achieved: study completed	**36**	In progress

What was accomplished under these goals?

During Year 1, the major activities to be completed to achieve the goal milestones were:

- Obtain WSU IACUC approval for study protocol for Aims 1 and 2, followed by USAMRMC ACURO approval - completed
- Obtain and validate transgenic rats and establish breeding colony for subsequent experiments – completed
- Optimize parameters of behavioral tasks assessing sleep loss and cognitive flexibility – in progress
- Optimize the dose and localization of viral injections for Aim 2 – in progress
- Procure, assemble, and test/calibrate capital equipment for Aims 1 and 2 – completed
- Ethical review of the Aim 3 study procedures by the Washington State University IRB, DoD's HRPO, and Naval Medical Research Unit Dayton IRB:
    - WSU IRB approval was received on 4 Jan 2019 - completed
    - DoD HRPO approval was received on 2 April 2019 - completed
    - NMRU-D IRB approval is pending – in progress

  A delay incurred at NMRU-D in getting Dr. Lynn Caldwell under contract as a consultant for the project slowed us down in obtaining the regulatory approvals for the human work. However, as this delay allowed us to complete another laboratory study ahead of schedule, freeing up available time slots in the laboratory, the impact on the overall study timeline is expected to be negligible.
- Aim 3 kick-off meeting with DoD consultant, Senior Research Psychologist Dr. Lynn Caldwell, to discuss study design, drug administration, and cognitive testing – completed
- Development of standard operating procedures for participant screening and enrollment, a detailed study protocol, and standard operating procedures for cognitive tasks (Aim 3) – in progress

During Year 1, the specific objectives were to:

- Order, assemble, and test/calibrate capital equipment: auto-sleep deprivation system, Bussey-Saksida operant chambers, and Pinnacle biopotential recording systems.
- Develop the NRAT reversal behavioral paradigm, a rat cognitive testing paradigm needed to investigate cognitive flexibility and its neurobiological substrates.
- Develop the 5-CRT reversal task, our second rat cognitive testing paradigm needed to investigate cognitive flexibility and its neurobiological substrates.

- Optimize expression and injection coordinates, volumes and concentration of viral constructs in transgenic rats.
- Determine the Aim 3 drug (caffeine and modafinil) administration design (timing, dose, etc.).

During Year 1, key outcomes included the following:

Figures 1–4 below describe key outcomes on the rat reversal behavioral paradigms we developed and our work establishing a transgenic rat colony.

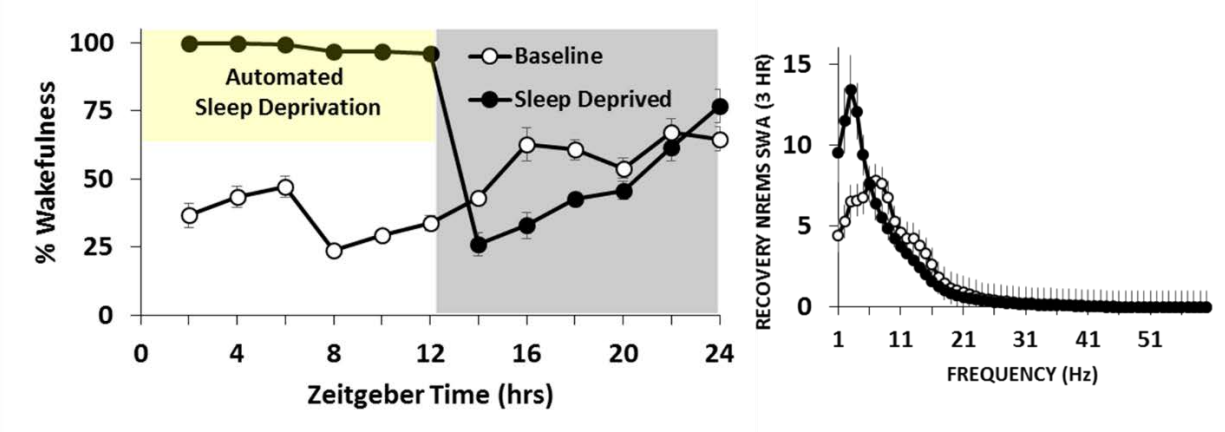


Figure 1. Four male Long Evans rats aged 13-18 weeks were surgically instrumented with EEG electrodes and following recovery habituated to the automated sleep deprivation chambers for at least 48 hrs prior to baseline recordings. To deprive rats of sleep a rotating bar was activated for 4 sec every 16 sec over the 12 hr light period when the propensity to sleep is high. The direction of the bar randomly changed every 10-40 sec. Rats exhibited over 96% wakefulness during sleep deprivation (left panel) indicating that rotating bar is an effective means of sleep deprivation and also demonstrates the biopotential recording unit and analysis software is working properly. The decrease in wakefulness during the subsequent dark period in the sleep deprived condition indicated a sleep rebound (left panel). Further, the increased sleep intensity is evidenced by greater slow wave activity (SWA) during NREM sleep compared with time matched baseline recordings (right panel).

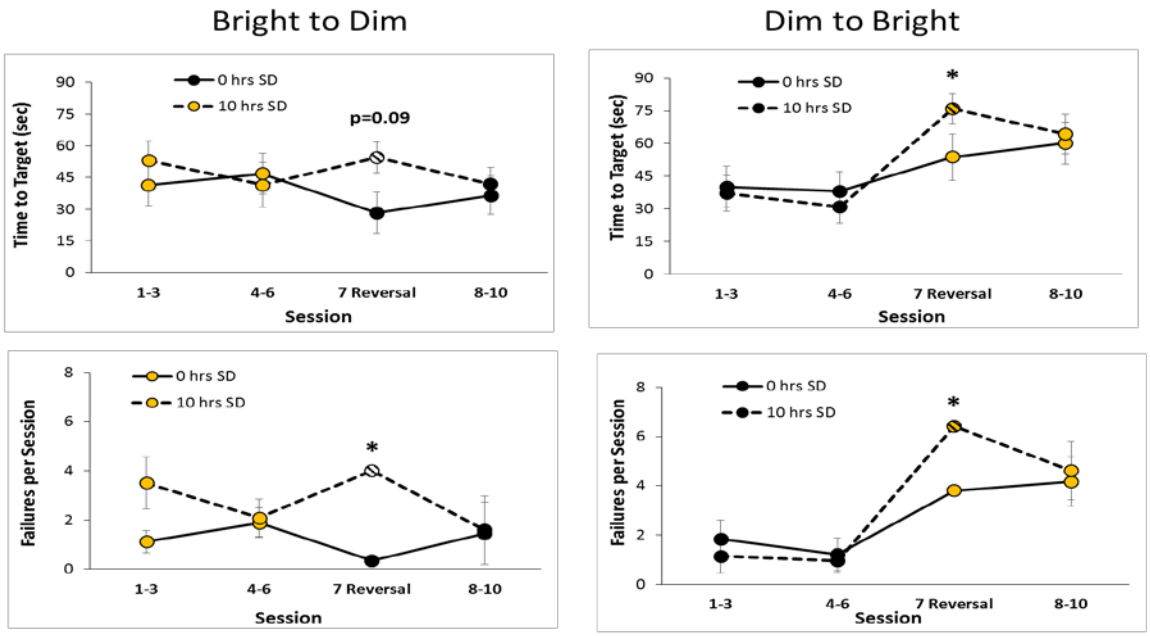


Figure 2. NRAT reversal performance in male Long Evans (n=4-10/group) rats aged 8-12 weeks indicates that sleep deprivation for 10 hr from light onset disrupted reversal (session 7) irrespective of the direction of light cue intensity. This paradigm was adopted because reversal of the vibration intensity (*viz*. increased frequency of vibrations guiding rats to the target destination) was too aversive for rats to effectively demonstrate cognitive flexibility. With the light cued version of the task, a constant intensity vibration stimulus motivated the rats to engage in target seeking behaviors, but graded light intensity guided ambulation to the unmarked target destination. Both light and vibration stimuli ceased when the target was reached indicating the end of a trial. As can be seen in the figure above, it was more ethologically relevant when the maze darkened as a rat approached the target (Dim) as compared to when ambient lighting in the maze became brighter (Bright) with target approximation.

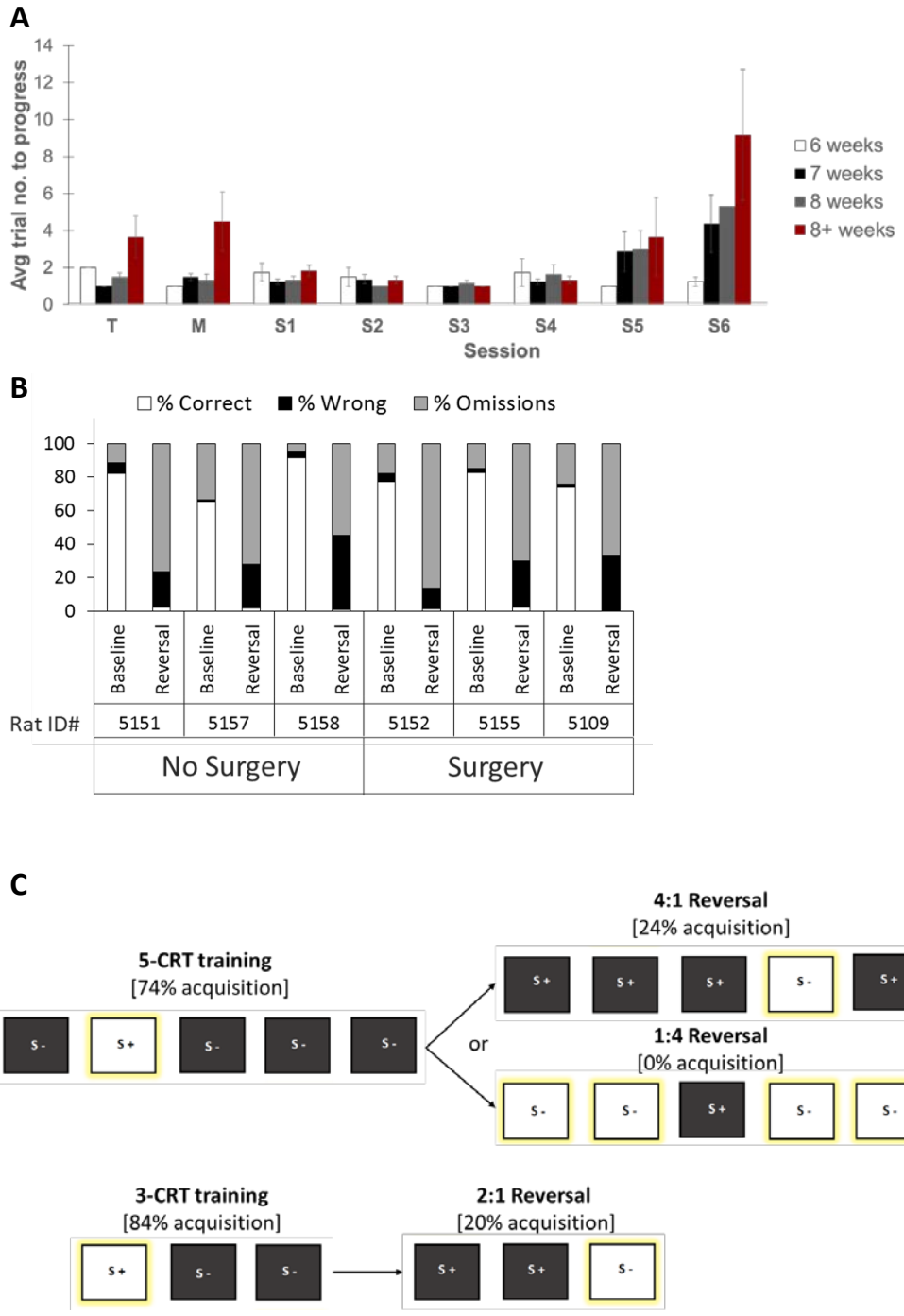


Figure 3. 5-CRT reversal task optimization in male Long Evans rats. The 5-choice reversal task was developed and optimized for (A) age of initial training, (B) surgical recovery, (C) the number of distractor choices and reversal protocol. Starting rats at 6 weeks of age has a marked performance advantage with fewer number of trials to progress to session 5 and 6 as compared with older rats. Moreover, instrumenting the rats with electrodes and head stages and allowing

10-14 days recovery yielded no performance decrements. Next, while changing from a 5-choice serial reaction time to a 3-choice paradigm increased acquisition rates by 10%, reversal performance was not approved. Finally, the 1:4 reversal paradigm was too difficult to acquire, while fewer than a quarter of the rats could successfully learn the 4:1 reversal and took 15 sessions to do so. We are currently testing longer stimulus presentation times and the possibility of a two-choice discrimination task to investigate cognitive flexibility.

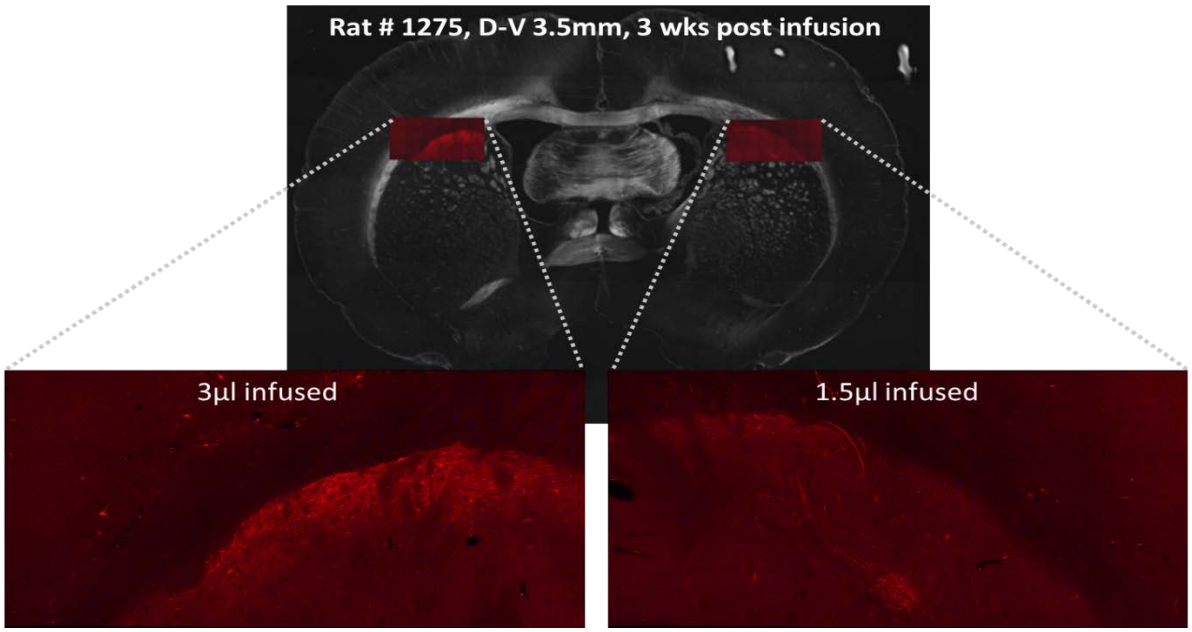


Figure 4. Expression of mCherry reporter in striatal neurons of Drd2::iCre rats 3 weeks following bilateral microinfusion 1.5 or 3 µl of pAAV-EF1a-cDIO-hChR2(H134R)-mCherry. Seven male Long Evans-Tg(Drd2-iCre)1ottc rats age 8-9 weeks at surgery were tested to optimize: infusion volume (3 µl left hemisphere; 1.5 µl right hemisphere), cannula depth (3.5 mm vs. 4.5 mm) and recovery/transfection time (2 weeks vs. 3 weeks). From the current data we conclude effective viral transfection at a cannula depth of 3.5 mm after 3 weeks at a 3 µl volume. This protocol will be executed again once the bigenic strain is successfully colonized using an intersect virus to isolate striatal indirect pathway medium spiny neurons.

During Year 1, other achievements included:

- Publication of three manuscripts (see appendices)

What opportunities for training and professional development has the project provided?

The project provides significant opportunities for undergraduate and graduate education and professional development:

- Undergraduate interns Callum Foakes and Thandi Dralega, under the mentorship of Co-PI Chris Davis, constructed their first two research posters and presented them at two separate interstate symposia (see Appendices).

- A Ph.D. student in the Experimental Psychology program at Washington State University, Amanda Hudson, is involved in dissertation research integrated with the project. This includes programming of cognitive performance tasks in E-Prime software, data reduction and statistical analysis. The graduate student is involved in the project under the direct mentorship of the PI (Van Dongen) and Co-PI (Honn) of the project. During Year 1 of the project, she published a manuscript based on her work on the project (see Appendices).

- Two post-baccalaureate research assistants, Julie Erwin and Myles Finlay, are undergoing training to become Registered Polysomnographic Technicians (RPSGTs). They will be responsible for scoring the human sleep recordings of the project. They are also training the undergraduate research assistants on sleep recording procedures that will be used in the laboratory.

- Two additional post-baccalaureate research assistants, Lilly Skeiky and Rachael Muck, are involved in the project in preparing SOPs for the human subject screening and laboratory data collection procedures. Based in part on the research experience she gained in our group, Ms. Skeiky has just been accepted into the Experimental Psychology Ph.D. program at Washington State University.

- More than a dozen undergraduate students are involved in the project. They provide around-the-clock staffing and constant behavioral monitoring during the 24/7 laboratory experimentation of the project. Our records over the last 15 years show that the experience gained in the laboratory helps these students significantly with their applications for graduate and medical school.

How were the results disseminated to communities of interest?

Preliminary data were presented via posters entitled, *Optimizing performance on an operant rat reversal task* and *Rat strain performance differences on the light attenuated vibration task reversal after spontaneous or disrupted sleep* to the 50+ audience at the Sleep and Performance Center's annual retreat in Coeur d'Alene, ID in March, 2019, and again in April at the regional Inland Northwest Research Symposium in Spokane, WA.

What do you plan to do during the next reporting period to accomplish the goals?

For the animal studies (Aims 1 and 2): During the next reporting period we will receive the Adora2a::Flp rats, cryopreserve the specimen, and begin pairing them with the Drd2::Cre rats to derive the desired bigenic strain. We will demonstrate the effectiveness of Chr2 optogenetic stimulation using local field potential leads implanted at the optrode tip to verify electrophysiological changes to blue light stimulation and optimize the stimulation frequency, intensity and duration.

For the human study (Aim 3): During Year 2 of the project, as soon as NMRU-D IRB approval is received (expected within the next month), human subject recruitment for Aim 3 will begin. We plan to intensify our usual recruitment strategies (such as advertising to wider geographic areas, using a heavier advertising presence with more flyers throughout our local area, and implementing ads in new mediums, such as social media) to compensate for the delay in regulatory approvals that occurred in Year 1. At least half of the Aim 3 data is expected to be collected in Year 2, which will put us back on the originally anticipated timeline.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

The field of sleep and performance research will be enhanced by construction of an effective rodent model for cognitive flexibility and the optogenetic dissection of associated neural pathways that are susceptible to insufficient sleep.

What was the impact on other disciplines?

The construction of an effective rodent model for cognitive flexibility has broad implications on the fields of learning and memory, cognitive neuroscience and even the search for treatments for neurodegenerative diseases.

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

- The guiding stimulus of the NRAT was changed from gradient vibration driven to light guided, because the rats did not approach the target during concurrent increases in vibration commensurate with the reversal phase.

- The Drd2::iCre rats were only available in the Long Evans strain. Therefore, the rat strain was changed from Sprague-Dawley to Long Evans. Switching to the light stimulus in the NRAT produced better performance outcomes in this strain.

- Adding a reversal to the 5-CRT has proven difficult for rats to consistently demonstrate learning. We are decreasing the number of possible distractors to mitigate the complexity of this task while still maintaining the goal of modeling cognitive flexibility.

- Per the recommendation of our DoD consultant, Dr. Lynn Caldwell, we are developing custom driving simulator and shooting simulator tasks that were not previously part of the data collection plan, but which will model cognitive flexibility in military-relevant environments. These simulator tasks will replace a computer-based task, which requires a longer learning period and has less clear outcome measures.

Actual or anticipated problems or delays and actions or plans to resolve them

- As described in the quarterly reports, there have been delays in the construction of the Adora2a::Flp rats at Cyagen. They are on the fifth round of vector injections (over 100 pups) and have only a single pup that meets the standard. If Cyagen does not produce at least 2 pups by the end of June, we will pursue specific Aim 2 using the Drd2::iCre monotransgenic strain to accomplish our objective. To compensate for the difference in timeline we have acquired a second holding room with a 2-hour light offset so that we can run experiments in parallel, effectively doubling our throughput.

- As described in the quarterly reports, we incurred a delay in obtaining regulatory approvals for the human study due to delays at NMRU-D in processing the contract for Senior Research Psychologist Dr. Lynn Caldwell to be able to contribute to the project. As this delay allowed us to complete another laboratory study ahead of schedule, which has freed up

available time slots in the laboratory, the impact on the overall study timeline is expected to be negligible.

Changes that had a significant impact on expenditures

Because we incurred a delay in starting the human laboratory study due to delays at NMRU-D in processing Dr. Lynn Caldwell's contract, we moderated our overall spending rate in Year 1, thereby ascertaining that needed funds for the accelerated rate of data collection in Years 2 and 3 are available without requiring any change in overall project funding.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS

Journal publications

Hudson AN, Van Dongen HPA, Honn KA. Sleep deprivation, vigilant attention, and brain function: a review. Neuropsychopharmacology Reviews, in press. Acknowledgement of federal support: yes. See Appendix.

Satterfield BC, Stucky B, Landolt HP, Van Dongen HPA. Unraveling the genetic underpinnings of sleep deprivation-induced impairments in human cognition. Progress in Brain Research, 2019; 246: 127-158. Acknowledgement of federal support: yes. See Appendix.

Vanderheyden WM, Van Dongen HPA, Frank MG, Gerstner JR. Sleep pressure regulates mushroom body neural-glial interactions in Drosophila. Matters Select, 2019; 5(3): e201903000008. Acknowledgement of federal support: yes. See Appendix.

Books or other non-periodical, one-time publications

Nothing to report.

Other publications, conference papers, and presentations

Dralega AT, Foakes C, Schmidt MA, Harvey DO, Davis CJ. Optimizing performance on an operant rat reversal task. Poster presented on March 19, 2019 at the Washington State University Sleep and Performance Research Center Retreat and April 23, 2019 at the Inland Northwest Research Symposium. Acknowledgement of federal support: yes. See Appendix.

Foakes C, Dralega AT, Sidebottom DH, Schmidt MA, Harvey DO, Davis CJ. Strain differences on a rat light actuating search task reversal after spontaneous or disrupted sleep. Poster presented on March 19, 2019 at the Washington State University Sleep and Performance Research Center Retreat and April 23, 2019 at the Inland Northwest Research Symposium. Acknowledgement of federal support: yes. See Appendix.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

We have a manuscript in preparation documenting a behavioral reversal paradigm for rat studies:

Darian H. Sidebottom, Michelle A. Schmidt, Daniel O. Harvey, Hans P.A. Van Dongen, & Christopher J. Davis. The Rat Vibration Actuating Search Task (VAST) and the Effect of Sleep Deprivation on Task Performance.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Hans P.A. Van Dongen, Ph.D.*
Project Role:	*PI*
Researcher Identifier:	*ORCID ID: 0000-0002-4678-2971*
Nearest person month worked:	1
Contribution to Project:	*Dr. Van Dongen has provided general oversight, worked on the human study design (specific aim 3), and co-authored and submitted publications. He has coordinated between the animal and human studies and submitted the IRB/HRPO application.*
Funding Support:	

Name:	*Chris Davis, Ph.D.*
Project Role:	*Co-PI*
Researcher Identifier:	*ORCID ID: 0000-0002-9613-928X*
Nearest person month worked:	4
Contribution to Project:	*Dr. Davis procured the Dopamine receptor 2 (DRD2)-Cre mutant rats, ordered capital equipment, submitted animal use protocols, directed operations and recruited workforce for the rat behavioral research, and developed a prototype version of the behavioral paradigm for specific aim 1.*
Funding Support:	

Name:	*John M. Hinson, Ph.D.*
Project Role:	*Co-PI*
Researcher Identifier:	*ORCID ID: 0000-0002-5012-5974*
Nearest person month worked:	1
Contribution to Project:	*Dr. Hinson contributed to the specification of the operant reversal paradigm for specific aim 1 and the cognitive performance tasks to be used in specific aim 3.*
Funding Support:	

Name:	*Paul Whitney, Ph.D.*
Project Role:	*Co-PI*
Researcher Identifier:	*ORCID ID: 0000-0003-1973-5261*
Nearest person month worked:	1
Contribution to Project:	*Dr. Whitney contributed to the specification of the cognitive performance tasks to be used in specific aim 3.*
Funding Support:	

Name:	*Kimberly A. Honn, Ph.D.*
Project Role:	*Co-PI*
Researcher Identifier:	*ORCID ID: 0000-0001-8911-6277*
Nearest person month worked:	1
Contribution to Project:	*Dr. Honn helped with the IRB/HRPO application and the specification of the research design for specific Aim 3. She co-authored the publication on sleep loss and vigilant attention.*
Funding Support:	

Name:	*Marcos Frank, Ph.D.*
Project Role:	*Co-PI*
Researcher Identifier:	*ORCID ID: 0000-0002-6233-516X*
Nearest person month worked:	1
Contribution to Project:	*Dr. Frank provided general oversight of resources and budget for Aims 1 and 2.*
Funding Support:	

Name:	*Jonathan Wisor, Ph.D.*
Project Role:	*Co-PI*
Researcher Identifier:	*ORCID ID: 0000-0003-4948-4379*
Nearest person month worked:	1
Contribution to Project:	*Dr. Wisor provided scientific advice on genotype-phenotype relationships for a manuscript. He was the*

	primary consult for the viral localization and transfection data collection for specific aim 2.
Funding Support:	

Name:	*Darian Sidebottom*
Project Role:	*Ph.D. Student*
Researcher Identifier:	*Washington State University ID: 11357866*
Nearest person month worked:	1
Contribution to Project:	*Ms. Sidebottom helped with SOP and performance task development for the animal and human studies.*
Funding Support:	

Name:	*Matthew E. Layton, M.D., Ph.D.*
Project Role:	*Physician of Record*
Researcher Identifier:	*ORCID ID: 0000-0002-3287-9203*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Layton helped to specify the pharmacogenetic interventions in the human subjects and began interactions with a compounding pharmacy for later use during study compound administration.*
Funding Support:	

Name:	*Stephen James, Ph.D.*
Project Role:	*Researcher*
Researcher Identifier:	*ORCID ID: 0000-0003-4139-7967*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. James developed the technical implementation of operationally relevant testing in the study design for specific aim 3 and ordered system components.*
Funding Support:	

Name:	*Michelle Schmidt*
Project Role:	*Research Technician*
Researcher Identifier:	*Washington State University ID: 11126756*
Nearest person month worked:	6
Contribution to Project:	*Ms. Schmidt performed rat EEG surgery, viral injections, and optimized user protocols for the 5-choice reaction time task and managed the wild-type and DRD2-Cre rat colonies for specific aims 1 and 2.*
Funding Support:	

Name:	*Daniel Harvey*
Project Role:	*Research Technician*
Researcher Identifier:	*Washington State University ID: 10245277*
Nearest person month worked:	6
Contribution to Project:	*Mr. Harvey completed the setup of the serial reaction time behavioral paradigm and optimized the auto sleep deprivation system, and assembled and calibrated EEG recording systems. He also scored sleep, excluded artifacts and performed FFT analyses for specific aim 1.*
Funding Support:	

Name:	*Callum Foakes*
Project Role:	*Research Assistant*
Researcher Identifier:	*Washington State University ID: 11650093*
Nearest person month worked:	8
Contribution to Project:	*Mr. Foakes continued optimizing the training protocols for rats in specific aim 1.*
Funding Support:	

Name:	*Thandi Dralega*
Project Role:	*Research Assistant*
Researcher Identifier:	*Washington State University ID: 11650094*
Nearest person month worked:	8
Contribution to Project:	*Ms. Dralega continued optimizing the training protocols for rats in specific aim 1.*
Funding Support:	

Name:	*Lynn Caldwell, PhD / NMRU-D (on subcontract)*
Project Role:	*DoD Consultant*
Researcher Identifier:	*ORCID ID: 0000-0002-6461-4023*
Nearest person month worked:	1
Contribution to Project:	*Dr. Caldwell contributed expertise regarding the caffeine and modafinil administration protocols for specific Aim 3.*
Funding Support:	

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

Grants started:

Washington Research Foundation, "Clinical Sleep Research Facility," September 2018–present (PI: Van Dongen HPA). This grant provides construction funding for a new clinical lab in the Sleep and Performance Research Center at Washington State University.

Mars Wrigley Confectionery US, "Effect of Mastication on Sustained Attention," December 2018–present (PI: Hansen DA). This contract seeks to test the efficacy of mastication as a countermeasure for fatigue.

Puyallup Tribe, "Qwibil Evaluation Project," April 2019–present (PI: McDonell). This project establishes a collaboration between Washington State University and the Puyallup Tribe of Indians focused on developing a research and quality improvement infrastructure that will allow the Tribe to evaluate whether medicinal cannabis is associated with reduced opioid use and pain and improved physical and mental health in those who receive care at the Qwibil clinic.

There is no overlap among the new projects and the current project, and effort on the new project has been offset by reduced effort on completed and closed grants (see below).

The new projects did not stem from results obtained in the present project. However, we expect that follow-up funding may come in the future based on results obtained in Years 2 and 3.

Grants closed:

Jazz Pharmaceuticals, “FACT: Flexible Attention Control Task,” September 2017–April 2019 (PI: Van Dongen). This grant involved the development of a clinical test of cognitive flexibility.

Department of Defense (DURIP), “Instrumentation for Physiological and Neurobehavioral Measurement in Around-the-Clock, High-Fidelity Laboratory Studies of the Impact of Fatigue on Warfighter Performance,” September 2017–September 2018 (PI: Van Dongen). This grant provided funds for new equipment in the human sleep and simulation laboratories of the Sleep and Performance Research Center at Washington State University.

Federal Motor Carrier Safety Administration, “Flexible Sleeper Berth Pilot Program,” October 2015–September 2018 (PI: Honn KA). This grant investigated the fatigue mitigation effectiveness of a new working time rule being considered for commercial motor vehicle drivers.

What other organizations were involved as partners?

- Organization Name: Naval Medical Research Unit Dayton
- Location of Organization: Dayton, OH
- Partner's contribution to the project: Dr. Lynn Caldwell is subcontracted as a DoD consultant for the project.

8. SPECIAL REPORTING REQUIREMENTS

Quad Chart

Investigating Striatal Attentional Circuits to Understand and Mitigate Deficits in Cognitive Flexibility Due to Sleep Loss

BA170226 / W81XWH1810100

PI: Hans P.A. Van Dongen **Org:** Washington State University

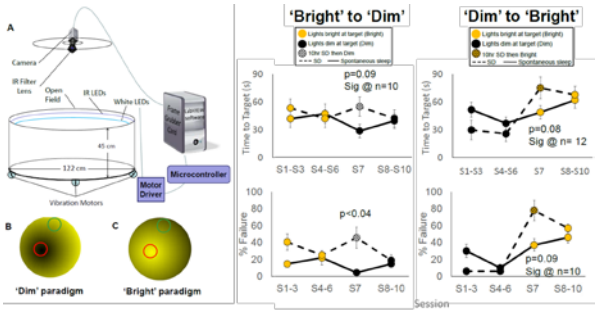
Award Amount: $2,797,841

Study/Product Aims

1. Develop behavioral model of sleep loss and cognitive flexibility in rodents.
2. Perform optogenetic experiments of sleep loss and cognitive flexibility with transgenic rats.
3. Demonstrate genotype differences in wake-promoting agents' effect on cognitive flexibility during sleep deprivation in humans.

Approach

In animal studies, we will optimize behavioral techniques that model the effects of sleep loss on cognitive flexibility observed in humans. Next, we will use transgenic rats that express Cre and Flp recombinase-dependent viral DNA constructs in striatopallidal medium spiny neurons of the striatum that express both the Adora2a and DrD2 receptors. We will use optogenetic methods to either activate these neurons, mimicking the effects of sleep deprivation on task performance in rats injected with flox/Frt -ChR2-GFP, or inactivate these neurons to recover normal task performance in sleep-deprived rats. For human subjects, we will compare the effectiveness of standardized doses of modafinil and caffeine during total sleep deprivation in promoting cognitive flexibility based on dopamine and adenosine genotype.



Accomplishments: Optimized rat 5-CRT and NRAT cognitive flexibility testing paradigms; established transgenic rat breeding colony; obtained WSU IRB approval and HRPO approval for human study.

Timeline and Cost

Activities CY	18	19	20	21
1.Development of rodent model				
2.Optogenetic experiments				
3.Human genotype differences				
Estimated Budget ($K)	**$445K**	**$910K**	**$955K**	**$488K**

Updated: 2019 June 12

Goals/Milestones

CY19 Goals – Complete transgenic rat model establishment; begin cognitive flexibility testing in transgenic rat model; et up optogenetics procedure. Obtain NMRU-D IRB approval; begin human subject laboratory sleep deprivation experiments.

Comments/Challenges/Issues/Concerns

- Experiencing delay in ETA of transgenic rats (manageable).
- Experiencing delay in obtaining NMRU-D IRB approval folllowing delay in NMRU-D contracting of Dr. Lynn Caldwell (manageable).

Budget Expenditure to Date

Projected Expenditure: approx. $890K.
Actual Expenditure: $591K (see delays above for justification).

9. APPENDICES

Dralega AT, Foakes C, Schmidt MA, Harvey DO, Davis CJ. Optimizing performance on an operant rat reversal task. (Poster)

Foakes C, Dralega AT, Sidebottom DH, Schmidt MA, Harvey DO, Davis CJ. Strain differences on a rat light actuating search task reversal after spontaneous or disrupted sleep. (Poster)

Hudson AN, Van Dongen HPA, Honn KA. Sleep deprivation, vigilant attention, and brain function: a review. Neuropsychopharmacology Reviews, in press.

Satterfield BC, Stucky B, Landolt HP, Van Dongen HPA. Unraveling the genetic underpinnings of sleep deprivation-induced impairments in human cognition. Progress in Brain Research, 2019; 246: 127-158.

Vanderheyden WM, Van Dongen HPA, Frank MG, Gerstner JR. Sleep pressure regulates mushroom body neural-glial interactions in Drosophila. Matters Select, 2019; 5(3): e201903000008.

OPTIMIZING PERFORMANCE ON AN OPERANT RAT REVERSAL TASK.

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Introduction

Cognitive flexibility is the ability to adjust decision making strategies when contingencies change, and is impaired by sleep loss[1,2].

There is substantial evidence that cognitive flexibility is dependent on dopamine and adenosine signaling in the prefrontal cortex (PFC) and the striatum [3]. A rodent model is needed to optogenetically test the contributions of these brain areas.

Herein we employed modified versions of the 5-choice serial reaction time task (5-CSRT) which is sensitive to sleep loss, to study cognitive flexibility in rodents [4]. A reversal has not previously been tested with the 5-CSRT operant paradigm, so we set out to confirm that rats are capable of adapting to a reversal in the 5-CSRT task as well as optimizing the parameters of age, training and the reversal paradigms.

Methods

31 male Long Evans rats aged 6-8 weeks were housed 2 to a cage separated by a cage divider and were placed on a 12: 12hr light cycle. Food was restricted 1 week before training commenced to reduce their body weight by ~15% to induce motivation for the sugar pellet reward. To maintain them at a lowered % body weight, rats were given 6g of food per 100g of body weight each day, after session completion. Water was available *ad libitum* in their home cage.

Rat Touch Screen apparatus for 5-CSRT task

The basic 5-CSRT requires the animal to sustain and divide its attention across a row of five screen locations to detect and respond to a brief visual stimulus - one white square out of five [Fig.3. Top panel]. The touch screen is inside a trapezoidal chamber with a food reward tray at the narrow end [Fig.1].

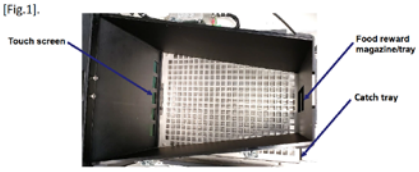

Fig.1. Overhead view of Rat Touch Screen Systems apparatus for 5-CSRT.

Behavioral Training

On the first day rats were habituated to the chamber for 30 mins. 20 food pellets were put in the food reward magazine tray. No visual stimuli was presented. The next day initial training (**IT**) began [Fig.2].

In the **IT**, each session consisted of 100 trials for which a maximum of 60 mins was allowed. Initial criteria for success was ≥90 trials, this was lowered to 60 trials because in the basic (**S**) training for a baseline measurement there was also 60 trial/60 min maximum.

If no touch response was made, the trial was classified as an "omission". If a location was touched during the delay prior to stimulus onset, the trial was deemed as "premature" and was not included in the total trial count for the session. The criteria to progress to the next session was ≥80% accuracy and ≤20% omissions. If criteria were not met, the session was repeated until it was achieved.

Methods (continued)

Fig.2. The session protocol

With each progressive session, the stimulus duration and the time to elicit a response (limited hold) is reduced, according to Table 1.

	Session 01	Session 02	Session 03	Session 04	Session 05	Session 06	Session 07	Session 08
Stimulus Duration	60 s	30 s	20 s	10 s	5 s	2.5 s	1.5 s	1 s
Limited Hold	60 s	30 s	20 s	10 s	5 s	5 s	5 s	5 s

Table.1. Session variations with each progression.

When the rat successfully completed session 5,6,7 or 8, it repeated that session twice more and the average of the 3 sessions was used as the baseline session criteria for the reversal.

After each session, the catch trays were emptied and the chambers were cleaned with 10% ethanol solution. Rats completed one session a day (Mon-Fri).

5-CSRT task Reversal

We tested 2 variations of the reversal task. In one reversal paradigm a "correct trial" was achieved by touching one of the 4 dark squares instead of the white one - L-reversal. In the second reversal paradigm 4 of the squares were white and one was dark/blank. A "correct trial" was achieved by selecting the dark square – D-reversal.

Reversals were carried out with rats on sessions 5, 6, 7 or 8 in each paradigm for 12 sessions or until baseline performance was met.

Fig.3. Diagram depicting rat's view of the touch screen in each paradigm: Normal, L-Reversal and D-reversal (One dark stimulus).

Results

Rats started the training at either 6, 7, 8 or 8+ weeks of age. Out of the 8 rats that started at 8+ weeks 4 did not progress to **S** training. Out of these 2 rats spent 28 days in **IT**, 1 spent 21 and 1 only 6 before dismissal from the task. The other 4 spent an average of 20.25 days in **IT** then moved onto **S** training. Those that started at 6,7 and 8 weeks spent 4 days on average in **IT** before moving on to **S** training.

However, we found that the rats that began training at 6 weeks learned the task faster, repeated sessions less often and progressed further than those who started at 7 or 8 weeks [Fig.4.].

Results (continued)

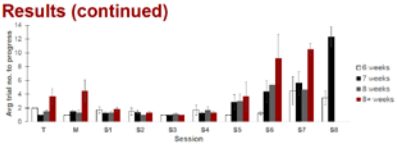

Fig.4. Average number of times a session had to be repeated before they progressed to the next session.

Out of the 31 rats that started **S** training, 20 learned it sufficiently to undergo the reversal of the task with baseline of 5,6,7 or 8. Of the rats that underwent L-Reversal 4/11 learned the reversal [Fig.5A and 5D]. All 9 of the rats that underwent the D-reversal failed to learn it.

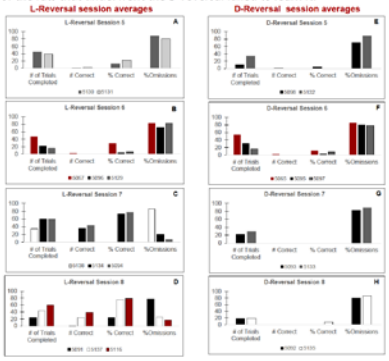

Fig.5. L-reversal (A-D) and D-reversal (E-H) average results of last 3 (out of 12) sessions/last 3 sessions before they achieved ≥80% accuracy and ≤20% omissions (baseline) on sessions 5,6,7 and 8.

Conclusions

The starting age of the rat has a substantial effect on its performance in this task [Fig.4]. The rats that started at 8+ weeks spent above 20 days in **IT** and many did not progress to session 8.

Greater than 90% correct trials was overly stringent due to the fact that in **S** training for baseline there was a 60 trial maximum. This meant they repeated **IT** sessions unnecessarily which may have hindered their progression. The cohorts that started at 6/7 weeks of age also performed better in the reversal tasks. However it appeared that despite starting age, the D-reversal task was too difficult for the rats to grasp [Fig.5E-H].

References

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(3) Klanker, Marianne, Matthijs Feenstra, and Damiaan Denys. "Dopaminergic control of cognitive flexibility in humans and animals." *Frontiers in neuroscience* 7 (2013): 201.

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STRAIN DIFFERENCES ON A RAT LIGHT ACTUATING SEARCH TASK REVERSAL AFTER SPONTANEOUS OR DISRUPTED SLEEP.

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Introduction

Human studies have shown that sleep deprivation (SD) can cause deficits in multiple aspects of cognition [1,2]. Cognitive flexibility - the ability to adapt behaviors in response to a change of conditions – is thought to be especially sensitive to SD [3]. In this study, we examine strain differences in performance using the Light Actuating Search Task (LAST). The LAST is a novel task that uses gradient visual feedback to guide rat navigation to an unmarked target destination. This model successfully measures how SD affects cognitive flexibility through performance decrements in a reversal spatial navigation paradigm, while averting confounds typically associated with appetitive or aversive motivational cues [4].

Methods

23 Male Sprague Dawley (Sp. Daw), and 14 male Long Evans (LE) rats aged 9-12 weeks, were housed 2 or 3 to a cage at 23 ± 1° C and placed on a 12:12hr cycle with access to food and water *ad libitum*. All behavioural tasks (including handling) were performed during the final 2 hours of the light cycle (ZT 10-12) under red light (25-30 lux). All animal procedures were approved by the WSU IACUC.

Apparatus [Fig.1A]

The LAST is an enclosed circular maze with four small vibration motors mounted on the base to create a continuous vibration to motivate ambulation. A LED strip attached to the top of the wall created visual feedback based on the rat's position. To track rat movement, a monochrome camera equipped with an IR filter lens was connected to a CPU, which recorded the X,Y coordinates of the rat. The LAST requires rats to find an unmarked, quasi-randomly presented target point in an open field using visual feedback from the LED lights.

Behavioral Training

Rats began each trial facing the outside of the wall at one of four predetermined entry **[Fig.1B,1C]** locations, each of which were equidistant from the center of the maze. Once a rat is placed inside the apparatus the motors began vibrating at a constant 2500RPM, and the LED's illuminated at an intensity consistent with the rat's position from the target point **[Fig.1B,1C].**

Methods (continued)

The lights either diminished intensity as the rat approached the target, in the 'Dim' paradigm, or intensified as the rat approached the target in the 'Bright' paradigm **[Fig.1B,C,D].** The vibration and LED's stopped once the rat entered the target or 90s passed, the latter resulting in a failure. Rats were tested with 9 trials each on one paradigm for 6 days, then at light onset of day 7 either received a 10 hr gentle handling SD gentle or remained in their home cage for spontaneous sleep.
Rats proceeded to receive the opposite paradigm for the remaining four days. 4 or 5 rats were tested in each cohort with animals being alternated between trials, and the maze cleaned with 10% ethanol between each trial. Animals with >2 failures (<78% success) in session 6 were eliminated from the analysis (Sp. Daw n=6, LE n=10).

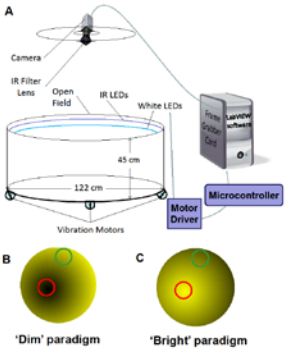

Figure 1: The LAST Apparatus diagram (A), a representation of light intensity vs proximity to target (red circle) in 'Dim' paradigm (B) and 'Bright' paradigm (C), with lux values for 'Dim' (black) and 'Bright' (orange) paradigms (D).

Handling/Habituation Protocol

Days 1-5: Five minutes gentle handling per animal. Day 6: Introduced to the arena with the entire cohort for 300s, then each animal has 300s alone without lights or vibration. Day 7: 150s with no lights or vibration, then 150s with lights only. Day 8: 300s with vibration only when a rat is on the perimeter. Day 9: 300s with lights, with vibration when a rat is on the perimeter. Day 10: 90s probe trial.

Results

'Dim' to 'Bright'

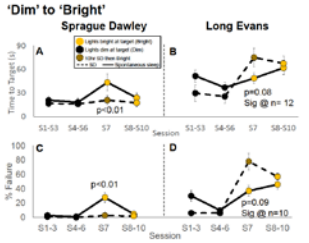

Figure 2: 'Dim' to 'Bright' - Average Time to target (A) and corresponding % failure (C) in Sprague Dawley's, compared to average time to target (B) with corresponding % failure (D) in Long Evans. Dashed line shows Sleep Deprived (SD) group and solid line shows non-SD group. Switch of paradigm shown by change in marker colour, with a striped marker signifying SD preceded that session.

'Bright' to 'Dim'

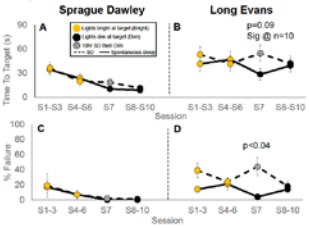

Figure 3: 'Bright' to 'Dim' - Average Time to target (A) and corresponding % failure (C) in Sprague Dawley's, compared to average time to target (B) with corresponding % failure (D) in Long Evans. Dashed line shows Sleep Deprived (SD) group and solid line shows non-SD group. Switch of paradigm shown by change in marker colour, with a striped marker signifying SD preceded that session.

Results (continued)

'Dim' to 'Bright'

- In Sp. Daw, SD significantly improved time to target ($p<0.013$) and success rate ($p<0.014$) **[Fig.2A,C]** compared to the undisturbed sleep group. In LE, however, the opposite is observed, where SD significantly impedes time to target ($p<0.004$) and success rate ($p<0.011$) compared to the spontaneous sleep group **[Fig.2B,D].**

'Bright' to 'Dim'

- In LE, SD trends toward the detriment of both time to target ($p=0.10$) and success rate ($p=0.07$) compared to the undisturbed sleep group, but is not significant **[Fig. 3B,D].** No trend is seen between SD and performance in Sp. Daw's **[Fig. 3A,C].**
- The effects of SD did not carry over to subsequent sessions, with similar performance in sessions 8-10 between those which received SD, and those that did not **[Fig. 2,3].**

Conclusions

- The 'Bright' paradigm appears to be a more challenging task due to the aversive nature of the increased light intensity at the target.
- Anecdotally, it was observed that during the 10hr SD, LE rats required more perturbations to sustain wakefulness in comparison to age matched Sp. Daw. Therefore, LE may be more sensitive to SD, as evident in the performance deficits.
- Surprisingly, SD inhibits the ability of LE rats to adapt to the 'Dim' to 'Bright' reversal paradigm, while actually improving the ability for Sp. Daw's to adapt to the same task.

Future Directions

We are currently working on a continuous trial version of the LAST, whereby the rat is left in the arena for 5 minutes to complete 50 trials. This minimizes animal contact with handlers and therefore fewer opportunities for discrepancies to influence behavior.

References

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Sleep Deprivation, Vigilant Attention, and Brain Function: A Review

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Abstract

Vigilant attention is a major component of a wide range of cognitive performance tasks. Vigilant attention is impaired by sleep deprivation and restored after rest breaks and (more enduringly) after sleep. The temporal dynamics of vigilant attention deficits across hours and days are driven by physiologic, sleep regulatory processes – a sleep homeostatic process and a circadian process. There is also evidence of a slower, allostatic process, which modulates the sleep homeostatic setpoint across days and weeks and is responsible for cumulative deficits in vigilant attention across consecutive days of sleep restriction. There are large inter-individual differences in vulnerability to sleep loss, and these inter-individual differences constitute a pronounced human phenotype. However, this phenotype is multi-dimensional; vulnerability in terms of vigilant attention impairment can be dissociated from vulnerability in terms of other cognitive processes such as attentional control. The vigilance decrement, or time-on-task effect – a decline in performance across the duration of a vigilant attention task – is characterized by progressively increasing response variability, which is exacerbated by sleep loss. This variability, while crucial to understanding the impact of sleep deprivation on performance in safety-critical tasks, is not well explained by top-down regulatory mechanisms such as the homeostatic and circadian processes. A bottom-up, neuronal pathway-dependent mechanism involving use-dependent, local sleep may be the main driver of response variability. This bottom-up mechanism may also explain the dissociation between cognitive processes with regard to trait vulnerability to sleep loss.

Keywords: circadian rhythms, cognitive impairment, individual differences, local sleep, sleep homeostasis, sleep loss, sustained attention, time-on-task effect, vigilance decrement

Introduction

The study of vigilant attention in the context of sleep deprivation has yielded far-reaching insights into the effects of sleep deprivation on cognition and the brain. The primary focus of this review is to provide an overview of inter-related findings on sleep deprivation and vigilant attention and review how these findings have shaped our understanding of the neurocognitive effects of sleep deprivation and, by extension, sleep.

Vigilant attention, also called sustained attention, refers to the ability to maintain stable, focused attention across a time interval [1]. Vigilant attention is typically measured with (computerized) performance tasks requiring responses to target signals. In the psychology literature, deficits in vigilant attention have been studied in the context of Signal Detection Theory [2], which provides a "discriminability" measure of an individual's performance on a stimulus detection task while accounting for the individual's willingness to respond. The concept of discriminability is directly related to the concept of signal-to-noise ratio [3], which may be seen as a measure of the fidelity of information processing in the brain [4]. Deficits in vigilant attention are also commonly described in terms of the time-on-task effect, or vigilance decrement, which is a decline in timely or correct responses across the duration of a vigilant attention performance task [5].

A variety of task paradigms have been used to measure vigilant attention, including simple reaction time tasks, go/no-go tasks, and two-alternative forced choice tasks, all of which typically require subjects to make speeded, accurate responses to visual or auditory stimuli. Historically, performance tasks employed to study vigilant attention required responding to relatively infrequently appearing target signals or events (e.g., occurring less than once a minute) across extended periods of time (an hour or more) [6]. High workload – operationalized as a

relatively high rate of target signals – can increase the vigilance decrement, so that sensitivity to deficits in vigilant attention can be retained in shorter tasks by increasing the stimulus density (although the vigilance decrement may be reduced again if stimuli are presented as often as every few seconds) [7]. As such, shorter tasks (less than an hour in duration) are currently favored in studies of vigilant attention.

Vigilance deficits have previously been posited to be the result of neural habituation to repeated stimulation [8]. However, studies based on event-related potentials have cast doubt on this idea [9]. It has also been proposed that the vigilance decrement arises from under-stimulation or boredom due to the monotonous nature of stereotypical vigilance tasks [10, 11]. At the level of brain functioning, though, it is not evident why under-stimulation would lead to a vigilance decrement. Even more puzzling, from this perspective, is the pivotal observation that sleep deprivation causes significant impairment in vigilant attention [12–15] and acceleration of the vigilant decrement [16–19]. As we shall see, this phenomenon suggests an alternative explanation for the vigilance decrement (and an alternative view of what constitutes "boredom" at the level of brain function).

Central to several of the findings on sleep deprivation and vigilant attention is the psychomotor vigilance test (PVT) [20]. The PVT is a 10-minute, one-choice reaction time task that requires responding – as quickly as possible – to a stimulus appearing at random, 2–10 second inter-trial intervals. Performance on the task can be quantified by means of the number of lapses of attention (traditionally defined as the number of response times greater than 500 milliseconds) [21] and a variety of other outcome measures [22] including the time-on-task effect (vigilance decrement) [15, 23]. The high stimulus load and the varying inter-trial intervals require a level of vigilant attention that appears to be near-optimally sensitive to impairment due

to sleep deprivation. The task has been characterized in depth [24]. It has no learning curve [25], and baseline aptitude for the task varies little among individuals [21]. As such, it is well suited for use in studies pursuing frequent, repeated measurements to probe the temporal dynamics of vigilant attention.

Temporal Dynamics

The timing and duration of periods of sleep and wakefulness are governed by two key physiological processes [26]. A homeostatic process – for which the underlying neurobiology is yet to be elucidated – serves to balance time spent awake with time spent asleep by building up a pressure for sleep across time spent awake and dissipating that pressure across time spent asleep. Simultaneously, a circadian process – originating in the biological clock in the suprachiasmatic nuclei (SCN) of the hypothalamus – produces a physiological drive for wakefulness during the day and sleep at night (in humans and other diurnal species) by generating a pressure for wakefulness during the afternoon and evening and withdrawing that pressure during the night and early morning.

The difference between the pressure for sleep from the homeostatic process and the pressure for wakefulness from the circadian process is a primary determinant of the level of sleepiness experienced while awake [27]. Although this was first shown on the basis of self-reported fatigue [28], the interplay between the homeostatic and circadian processes also drives the temporal dynamics of vigilant attention, as can be readily observed in performance on the PVT across a period of total sleep deprivation [15]. As shown in Fig. 1, vigilant attention deficits increase across time awake due to the build-up of homeostatic pressure for sleep, with strong modulation over time of day due to the waxing and waning of circadian pressure for wakefulness

[29]. As a result of these effects acting in tandem, performance impairment during a period of acute total sleep deprivation is greatest during the early to late morning hours following a night awake, whereas performance is partially restored during the subsequent afternoon in spite of continuing sleep deprivation. While these temporal dynamics can be understood principally as an additive interaction between the homeostatic and circadian processes, there is evidence that the interaction is actually synergistic in nature, with the influence of the circadian process on vigilant attention increasing when homeostatic pressure is high [30, 31].

<figure 1 approximately here>

In comparison with studies of acute total sleep deprivation, studies of sustained sleep restriction (i.e., daily sleep curtailment) have revealed that this two-process understanding of the temporal dynamics of vigilant attention is incomplete. That is, based on the effects of the homeostatic and circadian processes alone (or various other theories of sleep regulation no longer widely considered), it would be predicted that people could adapt to sustained sleep restriction rapidly, reaching a steady state of only mild impairment within a few days and returning to baseline after only one or two nights of extended recovery sleep [32]. Whereas that is indeed what is observed for self-reported sleepiness, data from other performance tasks including the PVT tell a different story [33–36].

Several important observations have stood out. First, there is a steady build-up of vigilant attention deficits (but not self-reported sleepiness) across consecutive days of sleep restriction [35]. Second, this build-up is sleep dose-dependent, such that the balance between wakefulness and prior sleep determines the build-up rate [37, 38] – see Fig. 2. Third, the deficits are

expressed primarily in the morning hours, while performance in the afternoon and evening is comparatively little affected (like what is seen during total sleep deprivation, cf. Fig. 1) [39] – see Fig. 3. Fourth, recuperation across consecutive days of recovery sleep following a period of sustained sleep restriction can be slow [34] – see Fig. 4 – compared to recuperation from much greater vigilant attention deficits due to acute total sleep deprivation (cf. Fig. 1). Fifth, the build-up rate of impairment across consecutive days of sleep restriction [40], or across a period of total sleep deprivation [41, 42], and the recuperation rate across subsequent days of recovery sleep [40, 42] depends on prior sleep/wake history – see Fig. 4.

<figures 2–4 approximately here>

These observations point to the involvement of another key physiological process regulating vigilant attention, for which the temporal dynamics play out more slowly than the homeostatic and circadian processes (i.e., over days and weeks instead of hours and days). Mathematical modeling [37] has indicated that this third process can be seen as an allostatic process [43], which serves to preserve homeostatic and circadian regulation in the face of chronic sleep insufficiency. Another, equivalent way to understand the third process is that it shifts the setpoint of the homeostatic process around which the interplay of the pressures for sleep and wakefulness takes place. Thus, sustained sleep restriction gradually shifts the homeostatic setpoint, while consecutive days of extended recovery sleep gradually shift it back. Interestingly, this means that people's sleep/wake history may enduringly influence their baseline state and, as such, their vulnerability to vigilant attention deficits due to sleep loss [40, 42, 44].

Vigilant attention is affected by a spectrum of other, predominantly transient, factors, including ambient temperature, light exposure, physical activity and posture, hunger, and environmental noise and other distractions [29]. The influences of these and other factors are integrated with homeostatic, circadian, and allostatic processes; recent evidence suggests that this may occur through orexinergic/hypocretinergic neurons in the lateral hypothalamus [45]. Vigilant attention is also significantly affected by sleep inertia, a brief period of disorientation and cognitive impairment immediately after awakening from (deep, non-REM) sleep [46]. The temporal dynamics and underlying mechanisms of these factors are generally poorly understood, and they are outside the scope of this paper. They have considerable practical relevance, though, especially in work settings where managing vigilant attention is critically important for productivity and safety.

Inter-Individual Differences

There is wide variability among people in how much vigilant attention performance impairment they exhibit while deprived of sleep [15, 47–50]. The same inter-individual differences in vigilant attention performance impairment are seen under conditions of total sleep deprivation and sustained sleep restriction [51], and they are quite substantial – see Fig. 5. Importantly, these inter-individual differences in vulnerability to sleep loss are highly replicable and stable within individuals [41, 51], indicating that they are a trait, or phenotype. Furthermore, a study in twins demonstrated that vulnerability to sleep loss is heritable [52].

<figure 5 approximately here>

Extensive attempts have been made to find predictors of phenotypic vulnerability to sleep loss. Baseline levels of vigilant attention performance are to some (limited) extent predictive of performance impairment during total sleep deprivation [53]; as are several aspects of brain functioning as characterized through functional neuroimaging [54], which continue to be the subject of investigation [55].

Much attention has been paid to genetic predictors of phenotypic vulnerability to sleep loss, of which several have been discovered – predominantly genetic variants of genes associated with adenosinergic mechanisms that may underlie the homeostatic process [56, 57] and genetic variants of clock genes involved in circadian rhythmicity [58, 59]. Complex biobehavioral traits tend to involve multitudes of genes, generally making it difficult to explain even a small percentage of the observed phenotypic variance [60]. Nonetheless, vigilant attention impairment due to sleep loss is a strongly expressed phenotype [61] (see Fig. 5), and at least one gene has been identified that predicts a relatively substantial portion of inter-individual variability in psychomotor vigilance impairment due to sleep loss. This concerns a single nucleotide polymorphism of the *TNFα* gene, which was found to explain 6.4% of the variance in PVT performance during total sleep deprivation [62]. Moreover, two genetic variants of the dopaminergic system – a variable number tandem repeat polymorphism of the *DAT1* gene and a single nucleotide polymorphism of the *DRD2* gene – together explained 15% of the variance in PVT performance during total sleep deprivation [63].

However, the phenotype of cognitive vulnerability due to sleep deprivation is not a unitary phenomenon. A surprise finding in the study that first established inter-individual differences in vulnerability to sleep loss as a phenotype [41] was the task-dependence of the trait. That is, trait inter-individual differences in vigilant attention deficits as measured on the PVT

were not congruent with inter-individual differences in performance deficits on a number of other cognitive tasks, and also not with inter-individual differences in self-reported sleepiness. This paradoxical result exposed the issue that overall performance impairment on a cognitive task may be caused by deficits in any of the underlying cognitive processes – a conundrum known as the "task impurity problem" [64]. This highlighted the importance of decomposing task performance into the constituent cognitive processes and examining the effects of sleep deprivation on these processes separately [65, 66].

Efforts to explore this issue further revealed that various cognitive processes, such as working memory scanning, resisting proactive interference, semantic encoding, and motor action planning, may be resilient to degradation caused by sleep deprivation [67–70] – suggesting that performance impairment from sleep loss on a range of cognitive tasks may be driven primarily by underlying deficits in vigilant attention [71]. Interestingly, a recent study of human gene expression during total sleep deprivation suggested a distinction between a large number of genes for which the expression profiles were non-specifically influenced by time awake, and a smaller group of genes for which the expression profiles appeared to be more closely related to the level of performance impairment on the PVT in particular [72]. It is currently unknown whether similar gene expression patterns would emerge with respect to other performance tasks for which performance impairment reflects deficits in vigilant attention.

These intriguing results aside, at least one other cognitive process is also greatly affected by sleep deprivation, independently from vigilant attention: top-down attentional control [73]. Sleep deprivation-induced impairments in this process are believed to underlie deficits in cognitive flexibility, which are not linked to deficits in vigilant attention and not predicted by the same genes [74, 75]. More research is underway to better understand what is unique about

attentional control and the impact of sleep deprivation thereon [76]. At the same time, though, the question may be asked: what is unique about vigilant attention?

Top-Down and Bottom-Up Regulation

Crucial for a deeper understanding of the effects of sleep deprivation on vigilant attention, and on the brain mechanisms that subserve vigilant attention performance, is the observation that sleep loss-induced deficits in performance on vigilant attention tasks such as the PVT are characterized by increased moment-to-moment variability. This was first recognized as an increase in exceptionally slow responses (of, say, more than twice the mean response time) interspersed among otherwise normal, fast responses on reaction time tasks performed during sleep deprivation. This observation led to the "lapse hypothesis," which posited that performance during sleep deprivation is disrupted by brief moments of reduced arousal that prevent timely responding to the task [77]. The lapse hypothesis provides a simple heuristic for how sleep deprivation leads to increased errors and accidents in real-world settings [78]. This useful feature notwithstanding, careful inspection of the distribution of response times on the PVT (and other vigilant attention tasks) shows a more nuanced picture. That is, during sleep deprivation the whole response time distribution skews to the right – see Fig. 6. As such, all response times are affected by sleep deprivation, with the right tail of the distribution showing the greatest change [14].

<figure 6 approximately here>

This finding gave rise to the "state instability hypothesis," which purported that sleep deprivation makes cognitive performance progressively more variable due to the interaction of escalating homeostatic pressure for sleep with the waxing and waning of circadian pressure for wakefulness and a person's compensatory effort to continue to perform [15]. This hypothesis is consistent with the documented neurobiology of global, top-down sleep/wake regulation. Notable components of this neurobiology include the ascending arousal system, which mediates the brain's arousal and, as such, reflects the homeostatic sleep drive and the prevailing amount of compensatory effort; the SCN, which orchestrates circadian rhythmicity and the associated wake drive; and the ventrolateral preoptic (VLPO) nucleus or "sleep switch," which blocks arousal from the ascending arousal system to initiate sleep [79]. When homeostatic sleep drive is high due to sleep deprivation, the interaction of these components may result in rapid fluctuations between wake and sleep states (i.e., state instability) [80], which could explain the observed skewing of response time distributions.

Results of neuroimaging studies of the effects of sleep deprivation on PVT performance support a global (albeit regionally distributed), top-down perspective on state instability and the skewing of response time distributions in vigilant attention performance [54, 81–86]. The restorative effects of wake-promoting drugs (stimulants) that target neurotransmitter systems in the ascending arousal system, such as modafinil and amphetamine [87], are also consistent with this view. However, the well-known restorative effect of caffeine [88], which at typical doses (in the 100–200 mg range) acts predominantly as an adenosine antagonist, is not readily explained in terms of global, top-down sleep/wake regulation.

Moreover, if the effects of sleep deprivation are solely globally mediated, the impact of sleep deprivation would not be expected to differ fundamentally between cognitive processes,

nor should there be any profound task-dependence of inter-individual differences in vulnerability to sleep loss. A global, top-down perspective on vigilant attention also does not provide a parsimonious explanation for one of the most prominent features of impaired vigilant attention, i.e., the vigilance decrement or time-on-task effect. The key to understanding these issues may lie in a more detailed investigation of the time-on-task effect during sleep deprivation and adapting a bottom-up perspective on vigilant attention.

Vigilance Decrement and Local, Use-Dependent Sleep

The vigilance decrement involves a gradual degradation of performance across the duration of a vigilant attention performance task [5]. The homeostatic and circadian processes that drive the temporal dynamics of vigilant attention interact with the time-on-task effect, amplifying the time-on-task effect when the homeostatic drive for sleep is high and the circadian drive for wakefulness is low [15, 16, 18, 89, 90] and as a function of consecutive days of sleep restriction [19] – see Fig. 7. Rest breaks provide recuperation from the time-on-task effect [91, 92], as do brief periods of engagement in a different task [92, 93]. The temporal dynamics of such breaks have not been well characterized – e.g., it is unclear how much time is needed to reset the time-on-task effect – but even relatively short breaks can be quite effective. The underlying mechanisms are a topic of debate [94, 95].

<figure 7 approximately here>

Although often described as a gradual decline in the mean level of performance across the duration of a vigilant attention task, the vigilance decrement actually entails an increase in

response variability as a function of time-on-task [96]. Under conditions of sleep deprivation, the increase in response variability over time-on-task is accelerated [15]. The effects of sleep deprivation and time-on-task on response variability are thus similar and interacting – and also involve overlapping brain areas as seen in functional neuroimaging experiments [97] – which has been interpreted as evidence that these effects may essentially be the same [19]. However, whereas the time-on-task effect can be overcome by a mere rest break, the effect of sleep deprivation can only be undone by a period of sleep.

This paradox may be resolved by considering "local sleep theory," which posits that sleep may be expressed locally in the brain – at the level of neuronal/glial assemblies, such as cortical columns – in a bottom-up, use-dependent manner [98]. In the local sleep state, neuronal/glial assemblies show synchronized firing patterns typical of the sleeping brain, with short bursts of high activity followed by brief periods of inactivity that are characteristic of slow wave sleep [99–101]. Furthermore, they show altered neuronal input-output relationships resulting in high-amplitude evoked responses typical of the sleeping brain [102], which are reversible and show homeostatic and use-dependent properties that are also typical of the sleeping brain [102–104]. However, they can do so independent of the states of neighboring neuronal/glial assemblies and independent of the global brain state [102]. For a review of local sleep theory, see [98].

Local sleep theory's relevance for vigilant attention has been illustrated in a whisker-twitching experiment in rats, in which specific whisker barrels (i.e., cortical columns) exhibited evoked responses characteristic of sleep, while other whisker barrels simultaneously exhibited evoked responses characteristic of wakefulness and while the whole organism was functionally awake. The probability of a whisker barrel entering the local sleep state increased with time spent in the wake state and the intensity of stimulation of the associated whisker, indicating a

homeostatic, use-dependent process [102]. Notably, rats trained to monitor and respond to stimulation of a specific whisker showed greater performance impairment (more failures to respond) when the corresponding whisker barrel had been driven into the local sleep state [103]. Similarly, in humans implanted with intracranial electrodes to record single neurons, local changes in neuronal activity were observed immediately prior to lapses of attention on the PVT [105]. Collectively, these results suggest that repeated stimulation of the same neuronal circuitry produces local sleep in that circuitry, which results in degraded information processing and increased variability in task performance [19].

This bottom-up perspective on performance impairment in vigilant attention tasks may explain a number of otherwise unexplained phenomena at the intersection of human vigilant attention and sleep deprivation [106]. Although task performance in humans is unlikely to rely critically on information processing by only a single cortical column, there are components of cognitive pathways with relatively sparse circuitry – such as visuospatial mental operations in the precuneus and posterior cingulate cortex [107] – which represent a potential bottleneck for cognitive processing. Extended and/or intensive use of such circuitry through sleep deprivation and/or task performance may result in expression of local sleep and consequent degradation of information processing, leading to a steady increase in performance instability [19]. This would explain the pervasive link between the vigilance decrement and monotony (i.e., persistent use of the same brain circuitry) [108, 109], and suggests that what is commonly experienced as "boredom" during monotonous tasks may actually be an epiphenomenon of the occurrence of local sleep.

During a rest break (or after switching to another task not critically affected by the same bottleneck for cognitive processing), the specific circuitry involved in the prior task can recover

through local sleep (without any overt impact, since that circuitry is no longer being relied upon). Thus, a rest break may be conceptualized as an opportunity for local sleep to overcome the time-on-task effect, functionally equivalent to how global sleep overcomes the overall effect of sleep deprivation on cognitive performance.

People may vary in the degree of sparsity of circuits that are potential bottlenecks for cognitive processing. That is, they may differ from each other in how much or how little redundancy there is in the capacity to process information – and this level of redundancy (or "cognitive capacity") may be dissimilar across distinct circuits in a given individual's brain. This would provide a plausible explanation for why different cognitive processes are found to be differentially affected by sleep deprivation [66] and why there is considerable task-dependence in the inter-individual differences in vulnerability to sleep deprivation [55]. It has also been suggested as an explanation for developmental changes in vulnerability to vigilant attention performance impairment in adolescents [110].

Furthermore, local sleep theory predicts that the effect of sleep deprivation on cognitive performance is modulated by information processing load (or "task load"). For example, it has been shown that performance deficits on a visual short-term memory task occur especially in individuals who fail to maintain baseline levels of brain activation in the precuneus and posterior cingulate cortex (as observed through functional neuroimaging) while sleep deprived *and* under high processing load [111] – presumably because these individuals have less redundancy in that task-critical neuronal circuitry and thus suffer the consequences of local sleep induced by sleep deprivation and information processing load more strongly [55].

This same line of reasoning may provide an explanation for the counterintuitive observation that performance on the relatively brief (i.e., 10-minute) PVT is more sensitive to

sleep deprivation than performance on classical vigilant attention performance tasks with infrequent critical signals and much longer task duration. Specifically, the PVT has a much higher stimulus density and thus presents a greater information processing load, persistently engaging the same neuronal circuitry [19]. Intriguingly, it would follow that the PVT has been such a useful tool for research on sleep deprivation and vigilant attention [24] because it probes the susceptibility of a key attentional network in the brain to local sleep by very effectively inducing local sleep in that network, with definite, readily interpretable consequences for the response time distribution.

This theoretical account of sleep deprivation and vigilant attention has been supported by research based on cognitive modeling [112, 113]. Additionally, a (simplified) model for the neurobiological underpinnings, which combines bottom-up and top-down views on vigilant attention, has been proposed – see Fig. 8.

<figure 8 approximately here>

Future Research Directions

The conceptual model shown in Fig. 8 of the neurobiological underpinnings of vigilant attention deficits and the impact of sleep deprivation, time-on-task, rest breaks, and recovery sleep is, of course, an oversimplification of the mechanisms underlying sleep regulation and cognition. Modern experimental techniques such as optogenetics and chemogenetics are sketching an increasingly complex picture of the regulatory mechanisms involved [45, 114]. Nonetheless, evidence has been accumulating for many of the individual components of the model in Fig. 8, such as the top-down regulation of neuronal activity by the homeostatic and

circadian processes [115], the bottom-up, use-dependent drive for local sleep in neuronal/glial assemblies [102], the variability in cognitive processing due to local sleep [103], the binding of extracellular ATP to purine type 2 (X7) receptors [116], key aspects of the signaling mechanisms involving sleep regulatory substances [117, 118], and the upregulation of purine type 1 (A_1) receptors in response to prior sleep loss [119].

Even so, the proposed neurobiological integration of the bottom-up and top-down aspects depicted in Fig. 8 awaits experimental confirmation. This facet of the model could be investigated, for example, by measuring performance on a sequence of alternating cognitive tasks, one being a vigilant attention task such as the PVT and another being a task associated with intense use of a different neuronal pathway, during sleep deprivation. By comparing the result with sleep-deprived performance on just the PVT, extended to match for total duration of performance testing, the interaction between bottom-up, use-dependent regulation of the time-on-task effect and top-down, homeostatic regulation of the sleep deprivation effect would be exposed.

The model of Fig. 8 would predict that continued testing on the extended PVT results in progressively worsening performance (per the time-on-task effect), as has been found [120]. However, even if the other task exhibits a time-on-task effect also, the model would *not* predict that performance would continue to worsen across the sequence of alternating tasks. Rather, it would predict that temporarily engaging in the other task allows the neurons specifically used intensively in the PVT to recover so as to reset the time-on-task effect. Thus, the other task would effectively serve as a “rest break” for the PVT. If this prediction does not hold true, then the model needs to be revised.

Several other important implications to the specifics of the interplay between local and global processes remain to be investigated. For example, if performance of a vigilant attention task, such as the PVT, induces the local sleep state, does that then also influence the global homeostatic process, such that repeated performance of the PVT across a period of sleep deprivation would accelerate the build-up of sleep pressure (as compared to not performing the task during sleep deprivation)? And if so, what is the signaling mechanism for this local-to-global interaction? Also, would it work the same way for performance tasks that do not require much vigilant attention, but rather rely critically on, say, attentional control or emotional control? Might this explain why a day full of social interaction can make one feel particularly sleepy? And could addressing these issues shed any light on why sustained sleep restriction leads to cumulative deficits in cognitive performance, but not in homeostatic sleep markers (e.g., delta power in the non-REM EEG or theta power in the waking EEG [32, 35]) – suggesting a fundamental distinction between the regulation of sleep and wakefulness versus the regulation of waking alertness and performance?

Sleep deprivation represents a powerful, reversible intervention that allows for the probing of vigilant attention and other aspects of cognition, as well as the underlying mechanisms [61, 73]. Ultimately, research on sleep deprivation, vigilant attention, and brain function may help to determine fixed and/or malleable connections between specific neuronal pathways with specific cognitive processes, which may yield new insights with respect to the elusive mind-body problem [121].

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Figure Captions

Figure 1. Effects of acute total sleep deprivation and recovery sleep on vigilant attention. Twenty-six healthy young adults were randomized to a total sleep deprivation condition (n=13) or a control condition (n=13) in a laboratory study. Subjects in the sleep deprivation condition had two baseline days with 10-hour sleep opportunities, a 62-hour period of total sleep deprivation, and two recovery days with 10-hour sleep opportunities. Subjects in the control condition had 10-hour sleep opportunities every night. A 10-minute PVT was administered repeatedly during scheduled waking periods to measure vigilant attention. Data show the mean (± standard error) number of lapses (defined as response times greater than 500 ms) on the PVT. Performance was stable across days in the control group (blue line). In contrast, during the 62-hour sleep deprivation period, subjects had significantly impaired performance, with deficits increasing across days of total sleep deprivation – modulated by circadian rhythmicity, such that the number of lapses was highest during the morning hours; and performance was quickly recuperated following recovery sleep (red line). Tall gray bars represent sleep opportunities (22:00–08:00) in both conditions; short gray bars represent sleep opportunities in the control condition only. Figure adapted from Whitney et al. [122] with permission.

Figure 2. Vigilant attention and self-reported sleepiness under conditions of sustained sleep restriction. Forty-eight healthy young adults were assigned to 3 days of acute total sleep deprivation (0 hours time in bed [TIB]; n=13; black) or 14 days of sustained sleep restriction with randomization to 4 hours TIB per day (n=13; red), 6 hours TIB per day (n=13; yellow), or 8 hours TIB per day (n=9; green), in the laboratory. The 10-minute PVT and the Stanford

Sleepiness Scale (SSS) [123] were administered repeatedly during scheduled waking periods to measure vigilant attention and subjective sleepiness, respectively. Data show the daily means (± standard error) for the number of lapses (defined as response times greater than 500 ms) on the PVT and the self-reported sleepiness score on the SSS, relative to baseline. The horizontal gray bands represent the mean (± standard error) in the total sleep deprivation condition following 1 night and 2 nights with 0 hours TIB. In the 8-hour TIB condition, lapses of attention were relatively rare and subjective sleepiness was stable and low across the study duration. In the 6-hour and 4-hour TIB conditions, there was a steady build-up of vigilant attention deficits on the PVT across the 14 days of sleep restriction, in a sleep dose-response manner – such that impairment in the 4-hour TIB condition reached levels equivalent to 2 to 3 days of acute total sleep deprivation. However, there was no steady build-up of self-reported sleepiness on the SSS, and no systematic dose-response effect – such that subjective sleepiness in the 4-hour and 6-hour TIB conditions stabilized at a much lower level than seen in the total sleep deprivation condition. Figure adapted from Van Dongen et al. [35] with permission.

Figure 3. Vigilant attention deficits after sustained sleep restriction as a function of sleep dose and time of day. In a laboratory study, ninety healthy adults were randomized to one of 18 sustained nocturnal sleep restriction conditions with or without daytime naps of various durations, with the total sleep opportunity ranging from 4.2 to 8.2 hours per day. A 10-minute PVT was administered repeatedly during scheduled waking periods to measure vigilant attention. Data show the estimated means for the number of PVT lapses (defined as response times greater than 500 ms) after 8 days of sleep restriction, relative to baseline, for daily total time in bed (TIB) of 4.2 hours (red), 5.2 hours (orange), 6.2 hours (yellow), and 8.2 hours (green). The data

reveal that the dose-response effect of sleep restriction is most pronounced in the morning hours, while the circadian drive for wakefulness provides a degree of protected against vigilant attention deficits in the afternoon (the “wake maintenance zone” [124]). Figure adapted from Mollicone et al. [39] with permission.

Figure 4. Effects of sustained sleep restriction and prior wake extension on vigilant attention. In a laboratory study, twenty-four healthy young adults were assigned to 7 days of sustained sleep restriction to 3 hours time in bed (TIB) per day (SR1–7), followed by 5 days of recovery sleep at 8 hours TIB per day (R1–5). In the days prior to the laboratory study, they were randomized to a week of sleep extension to 10 hours TIB per day (n=12; black) or keeping their habitual sleep schedule (n=12; red). A 5-minute PVT was administered repeatedly during scheduled waking periods in the laboratory to measure vigilant attention. Data show the daily means (± standard error) for the number of PVT lapses (defined as response times greater than 500 ms). In the prior habitual sleep condition, there was a steady build-up of vigilant attention deficits across days of sleep restriction, and a gradual recuperation across recovery days. In the prior sleep extension condition, however, the build-up of deficits across days of sleep restriction was attenuated, and recuperation across recovery days was accelerated. These results show long-term effects of sleep restriction and extension indicative of an allostatic process modulating the setpoint of the sleep homeostatic process. Figure adapted from Rupp et al. [40] with permission.

Figure 5. Inter-individual differences in vigilant attention deficits during periods of total sleep deprivation following two different conditions for prior sleep/wake history. Twenty-one healthy young adults were each exposed to 36 hours of acute total sleep deprivation in the laboratory on

three separate occasions. In the week prior to each laboratory sleep deprivation session, subjects were randomized to a week of sleep restriction to 6 hours time in bed (TIB) per day (one session) or sleep extension to 12 hours TIB per day (two sessions). A 20-minute PVT was administered repeatedly during scheduled waking periods in the laboratory to measure vigilant attention. Data show mean (± standard error) lapses of attention (response times ≥500 ms), averaged across the final 24 hours of each 36-hour sleep deprivation period. While the effect of prior sleep restriction was evident (red), and consistent with the idea of a shifting homeostatic setpoint due to prior sleep/wake history, the effect was small compared to idiosyncratic, trait inter-individual differences in vulnerability to sleep deprivation (green; 95% range). Trait inter-individual differences in vulnerability to vigilant impairment due to sleep deprivation dominated the data set, explaining 67.5% of the variance [41].

Figure 6. Effect of sleep deprivation on the PVT response time distribution. Sixteen healthy young adults were each exposed to 38 hours of acute total sleep deprivation in the laboratory. A 10-minute PVT was administered repeatedly during the sleep deprivation period to measure vigilant attention. A histogram of the response times, in bins of 10 ms each, is shown for daytime performance test times at baseline (blue) and for the same daytime performance test times 24 hours later during sleep deprivation (red). The data show that the main effect of sleep deprivation is a skewing of the response time distribution to the right, such that many more response times end up in the right tail of the distribution. The dashed line denotes the commonly used threshold defining lapses of attention (i.e., response times greater than 500 ms). The graph illustrates that the skewing of the distribution due to sleep deprivation lengthens the slowest response times and increases the number of lapses considerably. In contrast, the effects of sleep deprivation on the

peak of the distribution and the fastest response times is much more modest, and the majority of responses remains in the baseline range (approximately 200–300 ms for well-rested, healthy young adults). Figure adapted from Grant et al. [90] with permission.

Figure 7. Effect of sustained sleep restriction on the time-on-task effect. Following a baseline period, sixty-six healthy young adults were assigned to 7 days of sustained sleep restriction or extension, with daily time in bed (TIB) randomized to 3 hours (n=18; red), 5 hours (n=16; orange), 7 hours (n=16; green), or 9 hours (n=16; blue), which was followed by 3 days of recovery sleep at 8 hours TIB daily. A 10-minute PVT was administered repeatedly during scheduled waking periods to measure vigilant attention. Data show average response times per 1-minute bin (not drawn to scale on the time axis) during a baseline day (BL), during the 7 days of experimental sleep restriction or extension (E1–7), and during the 3 recovery days (R1–3). In each of the conditions, there was a general increase of the 1-min average response time across the 10-minute task duration, which was reset by the rest breaks between the test bouts. This time-on-task effect was exacerbated as a function of consecutive days of sleep restriction, with shorter sleep durations corresponding to greater time-on-task effects in a dose-response manner. The time-on-task effect was diminished across consecutive recovery days. Figure adapted from Van Dongen et al. [19] with permission.

Figure 8. Conceptual model pertaining to the effects of sleep deprivation on vigilant attention. *Left:* The ascending arousal system (AAS) promotes global arousal throughout the cortex by means of wide-ranging projections (red pathways). These projections originate from cholinergic structures in the brainstem (PPT, pedunculopontine tegmental nucleus; LDT, laterodorsal

tegmental nucleus) and basal forebrain (BF), monoaminergic structures in the BF and hypothalamus (e.g., LC, locus coeruleus; TMN, tuberomammillary nucleus; raphe nuclei), and orexinergic/hypocretinergic neurons in the lateral hypothalamus (LH), modulated by circadian rhythmicity generated in the suprachiasmatic nucleus (SCN). The strength of arousal from these projections, through interaction between cortical glutamatergic excitatory neurons and GABAergic inhibitory neurons in the cortex (not shown), instantiates the homeostatic and circadian processes reflecting prior wakefulness and time of day and mediates the *propensity* for local sleep at the level of neuronal/glial assemblies. This propensity is *manifested* as a consequence of intense neuronal use in support of information processing during vigilant attention task performance, in task-activated cortical areas such as the precuneus (magnifying glass). *Right:* Information processing in a neuronal/glial assembly triggers a series of biochemical processes that induce the local sleep state. Synaptic transmission is associated with increased local metabolic activity and energy transfer and release of adenosine triphosphate (ATP) from presynaptic neurons and glial cells into the extracellular space. Breakdown of extracellular ATP to recover the energy captured in the phosphoryl groups results in use-dependent accumulation of adenosine. Binding of adenosine at postsynaptic adenosine receptors (P1R, purine type 1 receptors) promotes local sleep, thereby fundamentally altering the neuronal assembly's synaptic transmission. As a consequence, the fidelity of task-relevant information processing is degraded in a use-dependent manner, modulated by the strength of subcortical arousal from the AAS. This gives rise to the time-on-task effect (vigilance decrement) in interaction with the homeostatic and circadian processes. A rest break (or switching to a task that does not intensively use the same neuronal/glial assemblies) allows adenosine levels to decay (e.g., through the enzymatic action of ADA, adenosine deaminase), thereby resetting the time-

on-task effect. Binding of ATP, prior to breakdown, to purine type 2X7 receptors (P2X7R) leads to release of a cascade of sleep regulatory substances (SRSs) such as interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), and brain-derived neurotrophic factor (BDNF). Sustained wakefulness allows SRSs to accumulate and, via their receptors and nuclear factor κB (NF-κB), causes the density of postsynaptic adenosine receptors to increase. This leads to a build-up of the propensity for use-dependent local sleep across consecutive days of wake extension. Across days of recovery sleep, adenosine receptors downregulate and the baseline propensity for local sleep is gradually restored. *Left and right:* Through mechanisms yet to be elucidated, accumulation of SRSs across the cortex reflecting the collective states of neuronal/glial assemblies is signaled to subcortical circuits (transparent downward arrows), influencing in particular the ventrolateral preoptic nucleus (VLPO). In response, the VLPO blocks the AAS and induces local sleep across the whole cortex (i.e., global sleep), which enables restoration of baseline SRS concentrations and allows recuperation from prior information processing deficits across neuronal/glial assemblies. Figure adapted from a schematic in Van Dongen et al. [19], with visual elements derived from Saper et al. [79] and Davis et al. [125].

Figure 1

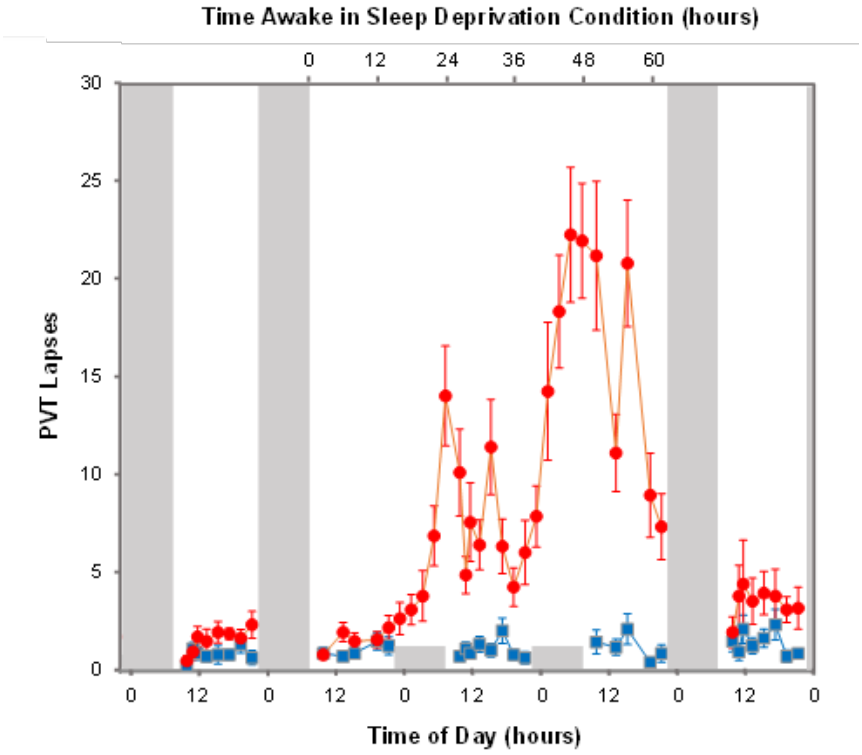

Time Awake in Sleep Deprivation Condition (hours)
0
12
24
36
48
60
30
25
20
15
10
5
0
PVT Lapses
0
12
0
12
0
12
0
12
0
12
0
Time of Day (hours)

Figure 2

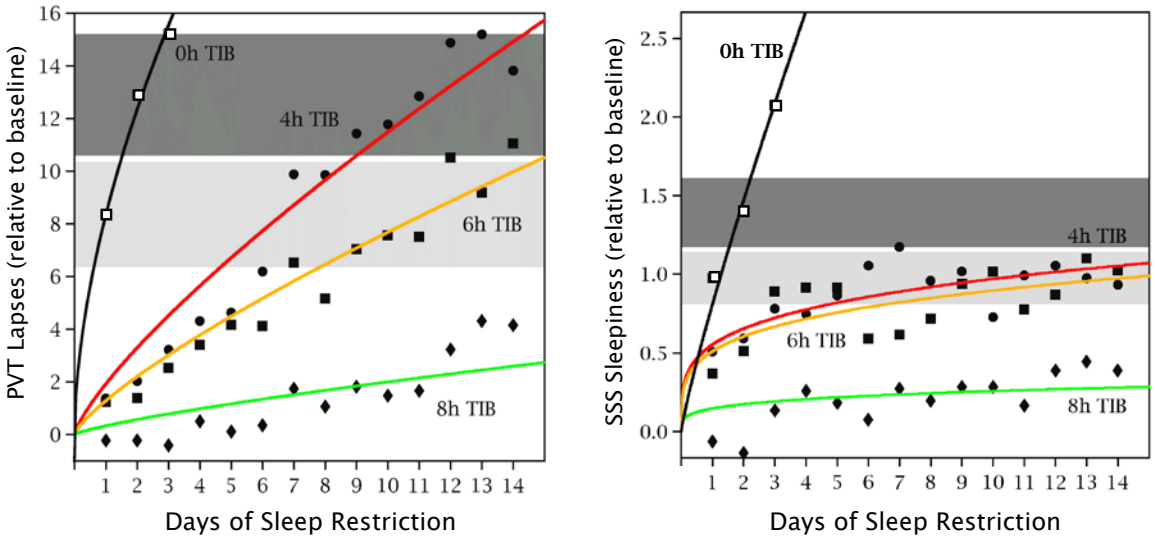

PVT Lapses (relative to baseline)
0h TIB
4h TIB
6h TIB
8h TIB
Days of Sleep Restriction
SSS Sleepiness (relative to baseline)
0h TIB
4h TIB
6h TIB
8h TIB
Days of Sleep Restriction

Figure 3

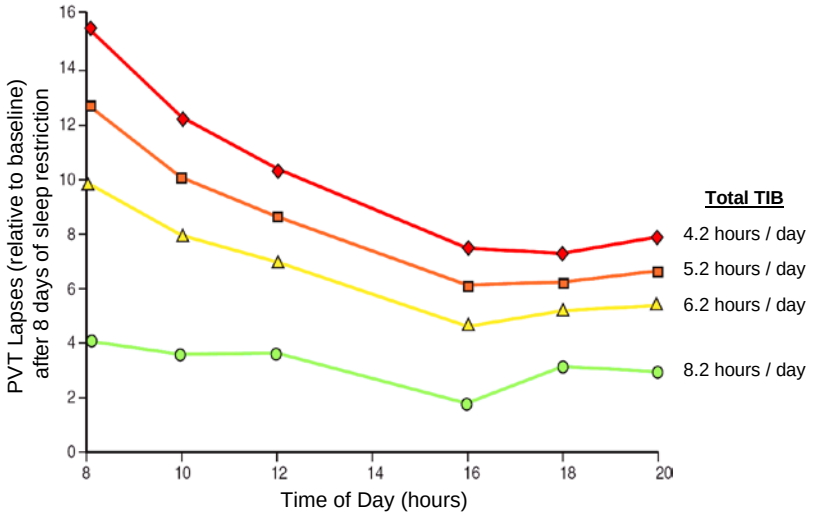

PVT Lapses (relative to baseline)
after 8 days of sleep restriction
16
14
12
10
8
6
4
2
0
8
10
12
14
16
18
20
Time of Day (hours)
Total TIB
4.2 hours / day
5.2 hours / day
6.2 hours / day
8.2 hours / day

Figure 4

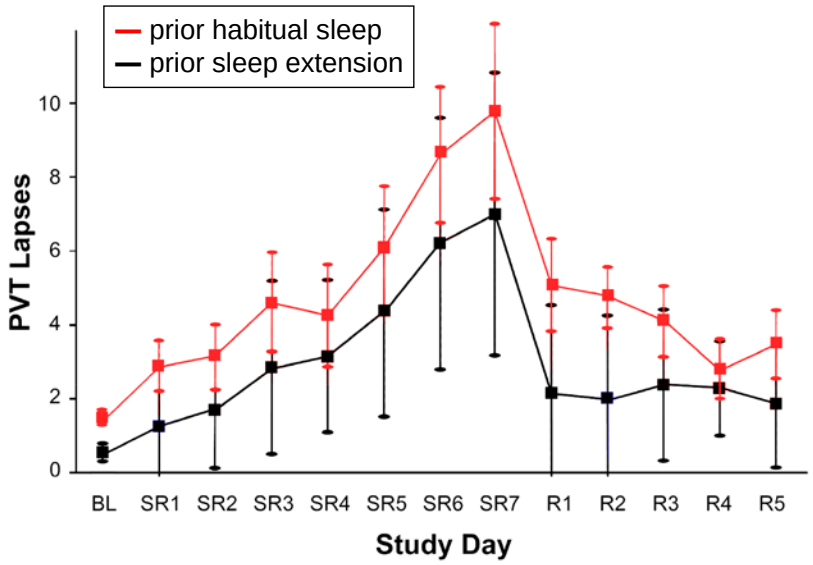

prior habitual sleep
prior sleep extension
PVT Lapses
0
2
4
6
8
10
BL
SR1
SR2
SR3
SR4
SR5
SR6
SR7
R1
R2
R3
R4
R5
Study Day

Figure 5

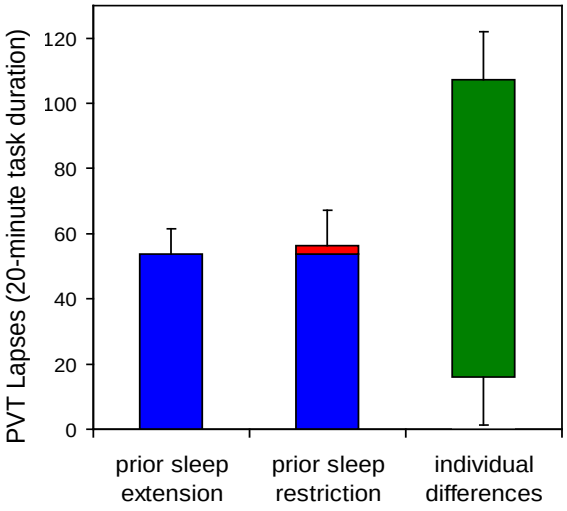

PVT Lapses (20-minute task duration)
120
100
80
60
40
20
0
prior sleep extension
prior sleep restriction
individual differences

Figure 6

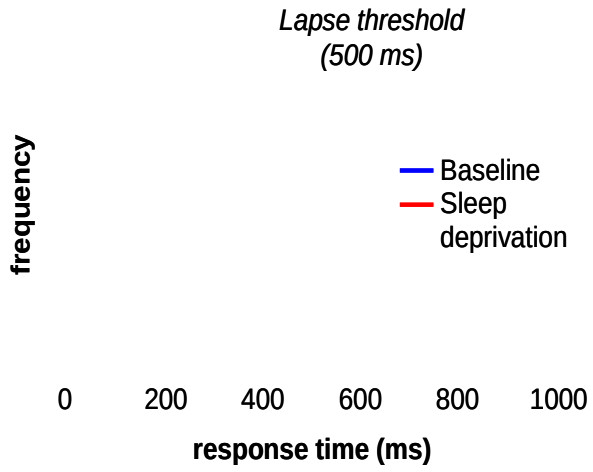

Lapse threshold
(500 ms)
frequency
Baseline
Sleep deprivation
0
200
400
600
800
1000
response time (ms)

Figure 7

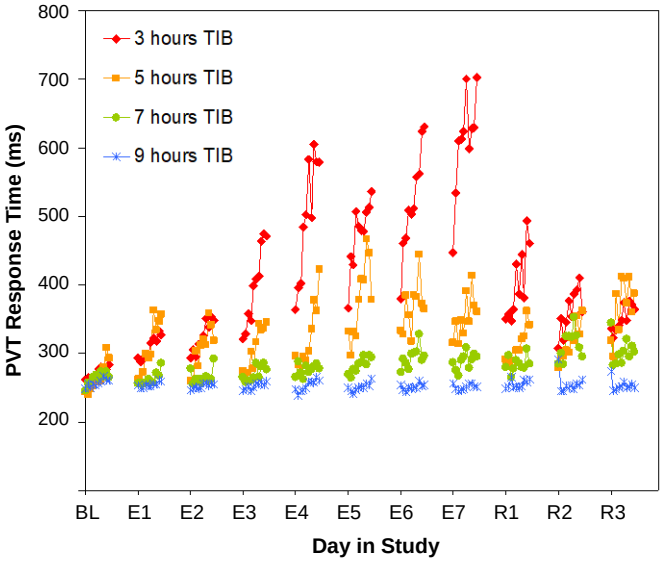
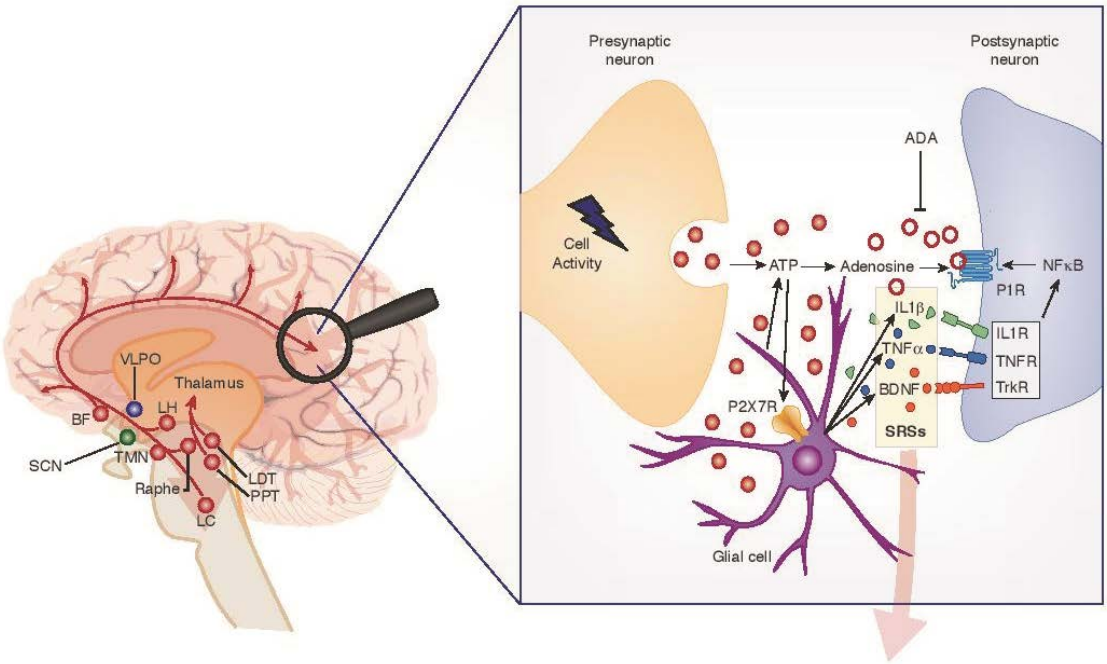

800
700
600
500
400
300
200
PVT Response Time (ms)
3 hours TIB
5 hours TIB
7 hours TIB
9 hours TIB
BL
E1
E2
E3
E4
E5
E6
E7
R1
R2
R3
Day in Study

Figure 8


Presynaptic neuron
Postsynaptic neuron
ADA
Cell Activity
ATP
Adenosine
NFκB
P1R
IL1β
TNFα
BDNF
SRSs
IL1R
TNFR
TrkR
P2X7R
Glial cell
VLPO
Thalamus
LH
BF
SCN
TMN
Raphe
LDT
PPT
LC

CHAPTER

6

Unraveling the genetic underpinnings of sleep deprivation-induced impairments in human cognition

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Abstract

The biobehavioral phenomena of sleep and cognition involve complex phenotype-genotype associations, i.e., complex relationships between observable traits and the genetic variants that contribute to the expression of those traits. There is a general belief that investigating such relationships requires large sample sizes. However, sleep- and cognition-related phenotype-genotype associations may be strengthened through carefully controlled laboratory studies that amplify a given cognitive phenotype by perturbing the biobehavioral system through sleep deprivation and/or pharmacogenetic interventions. Utilization of performance tasks that dissociate cognitive processes allows for cognitive endophenotyping, that is, making precise measurements that capture the essence of a cognitive phenotype. This enables assessment of the genetic underpinnings of cognitive impairment due to sleep deprivation without necessarily requiring large samples. Theory-driven gene selection, selective population sampling techniques to avoid underrepresentation of rare genetic variants, and modern statistical techniques informed by prior knowledge further enhance statistical power. Here we illustrate these approaches on the basis of recent findings, supplemented with some new results, as well as a discussion of modern regression methods for statistical analysis. Ongoing research employing these methods is driving advancements in the understanding of the genetic underpinnings of cognitive impairment associated with sleep loss.

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Keywords

Inter-individual differences, Gene polymorphisms, Gene expression, Cognitive impairment, Sleep loss, Endophenotyping, Pharmacogenetics, Phenotype-genotype relationships

1 Introduction

Even in normal, healthy human populations, inter-individual differences in complex biobehavioral phenomena such as sleep and cognition can be surprisingly trait-like (Deary, 2012; Kane and Engle, 2002; Landolt, 2011; Van Dongen et al., 2005). While this suggests profound phenotypic variability with strong genetic influence, complex traits typically involve multitudes of genes, most of which have variants that are too rare to contribute substantially to the population variance and/or represent very small effect sizes (Gibson, 2012). This is a problem for phenotype-genotype association studies, which aim to assess the relationship between the expression of a particular behavior or trait (phenotype) and the underlying genetic information that contributes to the manifestation of that behavior or trait (genotype). In such studies, it is generally difficult to explain much of the observed phenotypic variance, which has led to the conventional wisdom that phenotype-genotype association studies require very large sample sizes to reach statistical significance (e.g., Gottlieb et al., 2015).

Studying large samples addresses at least three critical issues:

- *Rare variants*. Many genetic mutations such as single nucleotide polymorphisms (SNPs) and other genetic variants occur infrequently, such that individuals homozygous (or even heterozygous) for these variants may be quite uncommon. Studying large samples increases the likelihood of capturing these individuals.
- *Missed associations*. Genetic variants that are rare in the sample at hand and/or that have weak influence on the biobehavioral phenomenon of interest produce small effect sizes. Studying large samples increases statistical power to detect them, which is especially important if adjustment of the statistical significance level is needed when multiple genes are being considered (multiple comparisons problem).
- *Spurious findings*. In the context of small effect sizes, there is considerable potential for spurious phenotype-genotype associations when the sample size is small, either because the sample is not sufficiently representative of the larger population from which it is drawn (generalizability problem) or because of random chance (replicability problem). Studying larger samples helps to statistically mitigate these problems.

For studies of sleep and cognition, where phenotyping may require elaborate measurement procedures in the laboratory and the research is usually labor-intensive, studying large samples is a slow and costly process. Also, if there is large heterogeneity across the population, then studying large samples does not adequately address

the generalizability problem, potentially turning it into a missed associations problem instead (as the differential effects in distinct subsamples may simply cancel each other out across the larger sample). The advent of genome-wide association studies (GWASs) has revealed that even in studies involving very large sample sizes, the genetic underpinnings of complex traits often remain elusive (Manolio et al., 2009). However, with such large-scale studies, the precision of phenotyping often suffers (e.g., Oexle, 2018).

In this paper we focus on alternative approaches to investigating phenotype-genotype associations in sleep and cognition that do not necessarily require large samples. Specifically, we focus on studies of the impairing effects of sleep deprivation on cognitive performance, which have been shown to be highly trait-like (Van Dongen et al., 2004a), to illustrate a number of complementary research methods. These research methods include:

Systems perturbation: The typically small effect sizes in phenotype-genotype associations may be increased by amplifying the expression of the phenotype and/or the effect of the genotype. Either or both could be achieved by perturbing the biobehavioral system through experimental intervention. Sleep deprivation is a good example of this, representing a powerful perturbation of sleep homeostasis with strong effects on cognitive performance and on post-deprivation recovery sleep (e.g., Bodenmann et al., 2012). Cognitive challenges can further amplify the effects of sleep deprivation to expose phenotype-genotype relationships (Satterfield et al., 2017; Whitney et al., 2017). Pharmacogenetic approaches involving pharmacological interventions on the neurotransmitter systems underlying sleep regulation provide additional means of perturbing the system, producing large effect sizes in phenotype observations and manifestly revealing genotype relationships with relatively small sample sizes (Urry and Landolt, 2015).

Theory-driven genotyping: The multiple comparisons problem of investigating phenotype-genotype associations may be mitigated by limiting the focus on a small number of genes of interest, selected on the basis of their a priori expected involvement in the biobehavioral phenomenon of interest (e.g., Holst et al., 2017). This approach is particularly powerful in the context of pharmacogenetics (i.e., the study of drug responses in relation to genetic factors). Pharmacogenetics provides a basis for the use of pharmacological agents to perturb systems for which the underlying mechanisms are already partially known or suspected, thereby providing a strong justification for a priori selection of specific target genes (e.g., Bodenmann et al., 2009b). Theoretical considerations pertaining to the involvement of specific genes can also be used to inform statistical regression models used to estimate phenotype-genotype relationships (Huang et al., 2011; Wang et al., 2013).

Selective sampling: The challenge of dealing with rare genetic variants may be circumvented by selective sampling. That is, the risk of undersampling of uncommon genotypes can be addressed directly by prospectively selecting

subjects from each of the different genotypes (i.e., homozygous mutant, heterozygous, and homozygous common) of the gene of interest (e.g., Viola et al., 2007). It could also be addressed indirectly by oversampling of portions of the distribution of inter-individual differences in a given biobehavioral phenomenon (e.g., Allebrandt et al., 2010). Care should be taken not to sample exclusively from the extremes of the genotype distribution (e.g., only homozygotes) or the phenotype distribution (e.g., only the phenotypic extremes), as the phenotype-genotype association may be nonlinear and could be obscured or misinterpreted this way. Although selective sampling precludes assessment of the amount of population variance explained by a particular gene, it can be particularly effective for studying underlying mechanisms (e.g., Rétey et al., 2007).

Cognitive endophenotyping: Paradoxically, inter-individual differences in the phenotypic effects of sleep deprivation on cognitive performance have been found to be outcome-specific (e.g., Van Dongen et al., 2011a,b). Research into this issue has revealed the importance of considering distinct components of cognition (Jackson et al., 2013), which are differentially vulnerable to impairment due to sleep deprivation (Honn et al., 2018). This points to the importance of cognitive endophenotyping, by which we mean the dissociation of phenotypic outcomes that are more neurocognitively specific than the overall biobehavioral phenomenon (i.e., performance impairment) and may therefore have more well-defined genetic connections. Cognitive performance tasks designed to assess such endophenotypic outcomes or "cognitive endophenotypes" may help to establish strong phenotype-genotype relationships (Grant et al., 2018; Satterfield et al., 2018; Whitney et al., 2017).

Here, we illustrate these approaches to studying phenotype-genotype associations using data from laboratory studies of the effects of sleep deprivation on cognitive performance. We show that robust findings can be achieved with relatively small samples by (1) using theory-driven selection of gene targets and pharmacogenetic perturbations to amplify phenotype-genotype associations, (2) outcome-specific endophenotyping of cognitive responses to sleep deprivation, and (3) application of cutting-edge statistical techniques informed in part by prior knowledge.

2 Pharmacogenetics to elucidate molecular-genetic contributions to sleep deprivation and cognition

Both sleep and cognition can be modulated with pharmacological interventions, and an experimental pharmacogenetic approach is a powerful tool to examine phenotype-genotype relationships between sleep deprivation and cognition. By examining the interactions of extended wakefulness and pharmacologically induced network modulations at different time scales, distinct molecular-genetic mechanisms underlying the consequences of sleep loss on cognitive functioning can be elucidated.

The daily alternation between wakefulness and sleep constitutes a fundamental temporal rhythm, which is tightly regulated by the fine-tuned interplay between homeostatic and circadian processes (Achermann and Borbély, 2016). Sleep need accumulates during wakefulness and dissipates during sleep (homeostatic process), whereas the circadian clock determines when sleep occurs as a function of time of day (circadian process). The interaction between the two processes determines daily fluctuations in subjective state, mood, alertness, and cognition (Boivin et al., 1997; Schmidt et al., 2007). Both homeostatic and circadian influences also affect brain electrical activity in a vigilance state-specific manner. Power (energy) in the electroencephalogram (EEG), especially in the theta range (~4–8 Hz), provides a reliable biomarker of accumulated sleep need induced by sustained wakefulness (Bodenmann and Landolt, 2010; Finelli et al., 2001; Holst et al., 2017).

Conscious decisions and optimal interactions with the environment require alert wakefulness, and sleep is essential for maintaining optimal cognitive functioning. Extended wakefulness characteristically alters long-range temporal correlations (LRTCs) in the EEG alpha band (~8–12 Hz), where overall signal power is virtually unchanged as a function of time awake (Holst et al., 2017; Meisel et al., 2017). The LRTCs slowly decay over seconds and reflect cortico-cortical connectivity and balanced cortical excitation and inhibition (E-I balance) (Meisel et al., 2017). The maintenance of an optimal E-I balance allows brain networks to function properly, and normal LRTCs are associated with optimal decision making and working memory task performance.

It has been proposed that sleep deprivation disrupts information processing by intrusions of sleep-like events into wakefulness (Doran et al., 2001; Nir et al., 2017; Van Dongen et al., 2011a,b; Vyazovskiy et al., 2011). LRTCs decline across prolonged wakefulness and normalize following recovery sleep, suggesting that sleep maintains LRTCs in the human brain and reorganizes cortical networks for optimal cognitive functioning (Meisel et al., 2017). Genetic factors contribute up to 60% to the strength of LRTC (Linkenkaer-Hansen et al., 2007) and can be pharmacologically modulated (Meisel et al., 2015; Yang et al., 2012). The monitoring of spontaneous brain activity and its modulation by pharmacological interventions in genetically characterized individuals may establish sleep-wake dependent timescales of information processing in cortical networks with link to cognition and reveal biomarkers of E-I balance and cortical dynamics during prolonged wakefulness.

2.1 System perturbations with pharmacological interventions and sleep deprivation

Pharmacogenetic approaches can be used to probe the causality of phenotype-genotype associations. The use of careful behavioral phenotyping and sleep deprivation opens the possibility for a closer examination of the effects of different pharmacological interventions on neurobehavioral performance. Stimulants have been of particular interest in the study of sleep deprivation and cognition. Stimulants are unique from a pure cognitive perspective because they offer direct

manipulation of specific signaling pathways that may underlie different aspects of cognition, and thus can offer insight into neurocognitive mechanisms that are impacted by sleep loss. For example, caffeine's main mode of action is adenosinergic, whereas modafinil and dextroamphetamine act upon different aspects of the dopaminergic system. Killgore et al. (2009) found that caffeine, modafinil, and dextroamphetamine had differential effects on three executive function tasks following 45–50 h of sleep deprivation. Both modafinil and dextroamphetamine reduced the total number of moves required to complete the Tower of London, a task that involves planning and visuospatial working memory (Shallice, 1982), whereas caffeine was not found to be effective. Conversely, only caffeine significantly improved performance on the Tower of Hanoi, a task that involves planning, strategy, and response inhibition (Welsh et al., 1999). Individuals who received modafinil had significantly better performance on the Wisconsin Card Sorting Task (WCST), purportedly a measure of mental set-shifting (Killgore et al., 2009; Puente, 1985), although the WCST is complex and known to recruit several neurocognitive systems (Berman et al., 1995).

These results suggest that different stimulants may have different effects on various aspects of executive function, allowing a better understanding of the underlying cognitive processes based on the pharmacological effects of each (Killgore et al., 2009). In a follow-up study, none of the stimulants were found to significantly improve decision-making performance on the Iowa Gambling Task (Killgore et al., 2012). Similarly, Wesensten et al. (2005) found little effect of stimulants on a range of executive function tasks. Lack of significant findings may be contributable to the "task impurity" problem that any performance task involves a number of interrelated cognitive processes, which must be distinguished to understand the causal factors determining performance in any given circumstance (Whitney and Hinson, 2010). This highlights the importance of employing neurocognitive assessment batteries that offer distinct, and dissociable, measures of cognitive functioning, as will be discussed later.

2.2 Theory-driven selection of gene targets: Proof of concept

A pharmacogenetic approach has been successfully applied to deconstruct a role for a distinct adenosine receptor subtype and adenosinergic-dopaminergic mechanisms in regulating cerebral activity and neurobehavioral performance during sleep deprivation. Consistent with a long-proposed role for adenosine and its binding sites in sleep-wake regulation (Porkka-Heiskanen et al., 1997; Radulovacki, 2005), caffeine, which is an adenosine antagonist, attenuates EEG markers of sleep homeostasis in wakefulness and sleep (Landolt et al., 2004). Some individuals are very sensitive to caffeine's stimulant effects, whereas others are not. In part these individual differences are genetically determined (Yang et al., 2010).

Caffeine blocks adenosine A_1 and A_{2A} receptors in the central nervous system without preferred specificity (Lazarus et al., 2017). However, basic pharmacogenetic work in transgenic mice demonstrated that functional A_{2A} receptors are indispensable for the promotion of wakefulness by the stimulant (Huang et al., 2005).

Translating these insights to humans, complementary observational and laboratory studies confirmed that common variants of the A_{2A} receptor gene (*ADORA2A*) modulate the effectiveness of caffeine to counteract the consequences of sleep loss on EEG activity and neurobehavioral performance. Table 1 summarizes a step-wise, pharmacogenetic approach to further elucidating a role for adenosine A_{2A} receptors in regulating neurophysiological and neurobehavioral markers of sleep homeostasis after sleep deprivation.

2.3 Selective sampling: Proof of concept

First, 20,343 university students were addressed with a brief internet questionnaire about self-rated caffeine sensitivity and sleep, and 4329 individuals responded. Habitual caffeine consumption was associated with subjectively reduced sleep quality in caffeine sensitive respondents, but not in caffeine insensitive respondents (Rétey et al., 2007). Intriguingly, in a laboratory study of 22 individuals from this sample, a night of sleep deprivation increased frontal EEG theta activity and impaired neurobehavioral function more in self-rated caffeine-sensitive than in caffeine-insensitive men (Rétey et al., 2006). Double-blind administration of the stimulant (2 times 200 mg across a 12-h interval) during prolonged wakefulness confirmed the classification of caffeine sensitivity based on the questionnaire. Thus, caffeine counteracted the differences between the phenotypic groups, although the caffeine concentration in saliva did not differ (Rétey et al., 2006, 2007).

The genetic sequence of *ADORA2A* was not the same in caffeine sensitive versus insensitive individuals. More specifically, the C allele of the functional c.1976T > C SNP of *ADORA2A* (SNP-ID: rs5751876) appeared to be more frequent in sensitive individuals ($n = 58$), whereas the T allele of this polymorphism occurred more frequently in insensitive individuals ($n = 84$) (Rétey et al., 2007). By contrast, the prevalence of the C/T genotype was the same in sensitive and insensitive subjects. These findings were further corroborated in the laboratory (Bodenmann et al., 2012) and confirmed by an independent GWAS (Byrne et al., 2012). Together, these findings suggest an important role for A_{2A} receptors in sleep-wake regulation and sleep-dependent neurobehavioral performance.

Conceptually, the experimental roadmap outlined above (Table 1) highlights the usefulness of self-classification to estimate a subjective phenotype as an initial step in large-scale epidemiological studies, followed up by physiological studies aimed to provide insights into the molecular bases of sleep deprivation and cognition. Furthermore, the example of variation in caffeine sensitivity has implications for clinical studies of novel pharmacological interventions targeting A_{2A} receptors, such as in chronic sleep restriction (Doty et al., 2017) and insomnia (Korkutata et al., 2018). The proof of concept presented above suggests that such studies could yield clearer outcomes if participants were subdivided and analyzed by their genotypes for *ADORA2A* and other genes of interest (Chen et al., 2013). These genes may hint to pharmacogenetic markers of individual responses to novel compounds and offer a unique opportunity for personalized sleep-wake medicine (Holst et al., 2016).

Table 1 Step-wise elucidation of a role for adenosine A_{2A} receptors and dopamine transporters in regulating neurophysiological and neurobehavioral markers of sleep homeostasis after sleep deprivation using a translational pharmacogenetic approach.

Conventional method	Additional methods	Sample size	Outcome(s)	Genetic approach	References
Basic pharmacogenetics	Perturbation	Tens/ experiment (mice)	Sleep-wake neurophysiology	Genetic engineering	Wisor et al. (2001), Hayaishi et al. (2004) and Huang et al. (2005)
Observational	Theory-driven genotyping	$n = 94–102$	Caffeine-induced anxiety	N/A	Alsene et al. (2003) and Childs et al. (2008)
	Selective sampling	$n = 20,343$	Sleep-wake habits, self-rated caffeine sensitivity	N/A	Rétey et al. (2007)
	Selective sampling	$n = 4329$	Caffeine consumption and sleep quality in extremes of caffeine sensitivity	N/A	Rétey et al. (2007)
Human pharmacology	Selective sampling, perturbation, deep phenotyping, within- and between-subjects variance	$n = 12–32$	Interaction of sleep deprivation and caffeine in caffeine sensitive and insensitive individuals	N/A	Landolt et al. (2004), Gottselig et al. (2006), Rétey et al. (2006), and Landolt et al. (2012)
Human pharmacogenetics	Theory-driven genotyping, perturbation, deep phenotyping, within- and between-subjects variance	$n = 22–504$	Interaction of sleep deprivation and caffeine in genetically characterized individuals	*ADORA2A* c.1976T > C SNP *ADORA2A* haplotype *DAT1* VNTR	Rétey et al. (2007), Bodenmann et al. (2012), and Holst et al. (2014)
Replication, observational		$n = 2402$	Coffee-attributed sleep disturbance	GWAS in twins	Byrne et al. (2012)
Back-translation, pharmacological innovation		Tens/ experiment (mice)	Sleep-wake neurophysiology, body temperature and heart rate measurement	N/A	Korkutata et al. (2018)

Perturbation approaches include sleep deprivation and pharmacological interventions. Deep phenotyping in humans includes subjective state questionnaires combined with neurophysiology (EEG quantification in waking and sleep) and neuropsychological assessment of sustained attention and executive functioning. ADORA2A = human gene encoding the adenosine A_{2A} receptor; DAT1 = human gene encoding the dopamine transporter; GWAS = genome-wide association study; N/A = not applicable; SNP = single nucleotide polymorphism; VNTR = variable number tandem repeats.

2.4 System perturbation, theory-driven genotyping, and deep phenotyping

The A_{2A} receptor is primarily expressed in striatal nuclei (caudate nucleus, putamen, nucleus accumbens, globus pallidus) and the olfactory tubercle, and partly co-localizes in synapses with dopamine D_2 receptors. The D_2 receptors and dopamine transporters (DAT) are important regulators of dopaminergic neurotransmission in striato-pallidal pathways and main target proteins of psychostimulants and wake-promoting medications such as modafinil (Volkow et al., 2009; Wisor et al., 2001). Intriguingly, $Dat^{-/-}$ knock-out mice and human homozygous 10-repeat (10R) carriers of the variable number tandem repeat (VNTR) polymorphisms of the *DAT1* gene encoding DAT (i.e., SNP-ID: rs28363170) are especially sensitive to the wake promoting action of caffeine (Holst et al., 2014; Wisor et al., 2001). Furthermore, compared to 9R-allele carriers, the homozygous 10R/10R variant has an accelerated build-up during extended wakefulness of the theta/alpha-ratio in the waking EEG, reflecting a faster increase of sleep drive during sleep deprivation (Holst et al., 2017). This notion is further corroborated by genotype-dependent differences in the time-on-task effect and performance instability during sleep deprivation and the effects of the DAT inhibitor, modafinil, on markers of sleep need (Holst et al., 2014; Lim et al., 2012; Satterfield et al., 2017). In conclusion, convergent findings in animals and humans suggest that adenosine-dopamine interactions in the striatum are important for regulating the neurobehavioral consequences of sleep deprivation.

The available knowledge accumulated by genetic studies in animals and humans suggests that each aspect of sleep-wake regulation is a complex phenotype with different molecular-genetic underpinnings (Andretic et al., 2008; Landolt and Dijk, 2016). This notion is supported by the lack of clear associations between neurophysiological markers of vigilance and neurobehavioral performance during sleep deprivation (Bachmann et al., 2012; Galliaud et al., 2008; Leproult et al., 2003; Lim and Dinges, 2008; Van Dongen et al., 2004a). For example, recent findings indicate that theta activity or the theta/alpha-ratio in the waking EEG on one hand and attentional lapses on the psychomotor vigilance task on the other hand are governed by distinct mechanisms that may both, yet separately, be influenced by dopaminergic neurotransmission (Holst et al., 2017).

Functional variants in the *ADORA2A* and *DAT1* genes and in *DRD2* (encoding the dopamine D_2 receptor) may modulate sleep-wake mechanisms by affecting striato-pallidal dopaminergic neurotransmission. On the contrary, dopaminergic neurotransmission in the prefrontal cortex (PFC) is primarily regulated by catechol-*O*-methyltransferase (COMT), which has much less influence on striatal dopaminergic neurotransmission (Slifstein et al., 2008). The COMT enzyme degrades catecholamines, such as dopamine, and the Val158Met polymorphism of the *COMT* gene (SNP-ID: rs4680) has a consistent impact on metabolic, neurophysiological, behavioral, and cognitive markers of PFC functioning (Bodenmann et al., 2009a,b; Egan et al., 2001; Tunbridge et al., 2006). By contrast, functional *COMT* variants and pharmacological modulation of PFC dopaminergic neurotransmission do not significantly affect EEG markers of sleep pressure in wakefulness and sleep

(Bodenmann and Landolt, 2010; Bodenmann et al., 2009b; Jawinski et al., 2016). This was confirmed by a pharmacogenetic study of tolcapone, a specific inhibitor of COMT (Farrell et al., 2012) that allows for probing the causal relationships between mesocortical dopaminergic signaling and neurobehavioral performance after sleep deprivation.

Some cognitive differences among genotypes only become apparent when individuals are challenged by sleep deprivation (Whitney et al., 2017). For the *COMT* Val158Met polymorphism, on both a vigilant attention task called the psychomotor vigilance test (PVT; Lim and Dinges, 2008) and a go/no-go reversal learning task (GNGr; Whitney et al., 2015), Val/Val, Val/Met and Met/Met allele carriers performed similarly in a well-rested baseline condition. However, systems perturbation with sleep deprivation consistently revealed that the homozygous Met/Met genotype provides a certain degree of resilience to performance impairment, whereas both heterozygous and homozygous carriers of the Val allele are more vulnerable (Satterfield et al., 2018; Valomon et al., 2018). This result suggests that mesocortical dopaminergic neurotransmission contributes to the regulation of distinct sleep-wake dependent cognitive functions, while neurophysiologic markers of elevated sleep pressure after sleep deprivation are controlled by different underlying mechanisms.

3 Sleep deprivation, individual differences, and cognitive endophenotyping

Further insight into phenotype-genotype relationships can be gained by focusing on the large inter-individual differences observed in studies of sleep deprivation (Tkachenko and Dinges, 2018). These inter-individual differences constitute robust phenotypes (Rupp et al., 2012; Van Dongen et al., 2004a). Given a representative sample of the population, investigation of the amount of variance explained by a genotype provides insight into how much of any given cognitive effect of sleep deprivation may be attributable to the gene in question.

3.1 Genetically explained phenotypic variance

Through theory-driven selection of target genes (e.g., see the previous section) and the use of well-designed tasks performed during sleep deprivation, recent studies have shown that individual SNPs can explain a substantial portion of the variance—much more than has been seen in traditional genotype studies and GWASs of cognition (Barnett et al., 2008; Davies et al., 2015, 2016; Ihne et al., 2015). For example, in what was arguably the first sleep deprivation study to quantify the amount of variance explainable by a genetic polymorphism, a SNP of the tumor necrosis factor alpha gene (*TNFα308*, SNP-ID: rs1800629) explained 6.4% of the variance in PVT performance during sleep deprivation (Satterfield et al., 2015). In another study, the Val158Met SNP of the *COMT* gene explained as much as 8.4% of the variance in GNGr performance during sleep deprivation (Satterfield et al., 2018). Similarly, the combination of two genetic variants of the dopaminergic

system, the above-referenced VNTR of *DAT1* and a SNP of *DRD2* (C957T, SNP-ID: rs6277) explained 15% of the variance in PVT performance during sleep deprivation (Holst et al., 2017). All three studies were conducted under carefully controlled laboratory conditions in samples drawn from a general population of healthy young adults.

In yet another such study (Whitney et al., 2017), a task designed to measure cognitive flexibility (the AX Continuous Performance Task with switching, AX-CPT-s) was administered during sleep deprivation. Cognitive flexibility was probed by changing the requirements of the task approximately halfway the duration of the task. The ability to flexibly adapt to the change was severely degraded by sleep deprivation ($F_{1,47} = 19.0, P < 0.001$; see supplementary information in Whitney et al. (2017). The amount of variance in the cognitive flexibility data that was explained by sleep deprivation in this experiment was 34.6%. Of this amount, a portion of 22.9% (i.e., 7.9% of the total variance) was associated with a SNP in *DRD2* (C957T, SNP-ID: rs6277). Importantly, while sleep deprivation also significantly degraded performance on the PVT, the *DRD2* SNP did not significantly predict the inter-individual differences in responses to sleep deprivation on this task (Whitney et al., 2017), with *DRD2* genotype explaining only 2.8% of the total variance; see Fig. 1.

3.2 Statistical focus on between-subject variance

Another way to look at explained variance is to focus specifically on the between-subjects variance, that is, the variance associated with systematic differences between individuals that could reasonably be expected to have genetic underpinnings. Between-subjects variance can be separated from within-subjects variance

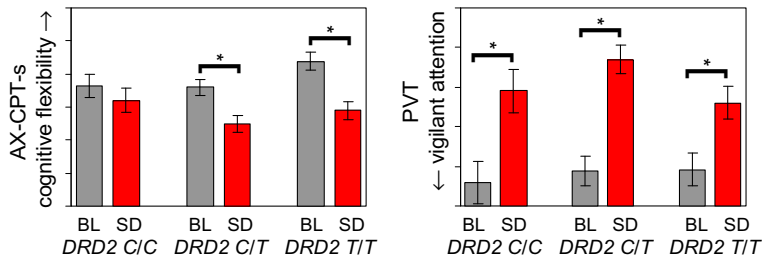

FIG. 1

Performance (mean ± SE) on the AX-CPT-s (left) and the PVT (right) during rested baseline (BL) and after sleep deprivation (SD) for each genotype of the *DRD2* C957T polymorphism. Whereas individuals homozygous for the C allele were relatively resilient to sleep deprivation-induced impairment in cognitive flexibility on the AX-CPT-s, there was no significant difference among the genotypes in vigilant attention on the PVT. Note that performance impairment is downward in the AX-CPT-s graph, but upward in the PVT graph. Brackets with asterisk indicate significant difference between BL and SD ($P < 0.05$).

Graphs adapted from Whitney, P., Hinson, J.M., Satterfield, B.C., Grant, D.A., Honn, K.A., Van Dongen, H.P.A., 2017. Sleep deprivation diminishes attentional control effectiveness and impairs flexible adaptation to changing conditions. Sci. Rep. 7, 16020. doi:10.1038/s41598-017-16165-z, with permission.

(general changes over time and measurement noise) by means of mixed-effects regression techniques, which are explained elsewhere (e.g., Van Dongen et al., 2004b). Analyzed this way, it was found that 23.8% of the between-subjects variance in the cognitive flexibility data (Fig. 1) was associated with *DRD2* C957T. By contrast, only 2.9% of the between-subjects variance in the PVT data was associated with the *DRD2* SNP. Thus, regardless of how explained variance is calculated, the phenotype-genotype relationship was profoundly dependent on the cognitive performance task being investigated.

3.3 Cognitive endophenotyping

Underlying this paradoxical finding is the reality that cognitive performance is not a unitary concept (Whitney and Hinson, 2010). Standard tasks often used to measure performance during sleep deprivation depend on multiple cognitive components simultaneously, which each may be affected differently by an intervention such as sleep deprivation (Jackson et al., 2013)—i.e., the task impurity problem already referenced in the previous section. The task impurity problem reduces the strength of phenotype-genotype relationships. Conversely, performance tasks designed to address the task impurity problem can strengthen and help to expose phenotype-genotype relationships. The PVT, GNGr, and AX-CPT-s used in the studies described above meet this criterion, effectively measuring *cognitive endophenotypes*. The assessment of cognitive endophenotypes contributed to the substantial amounts of variance explained by the genes investigated in these studies.

3.4 Challenges of inferring genetic mechanisms

It should be noted that the amount of variance explained by genotype differences depends on a number of factors only indirectly related to the strength of the genotype to (endo)phenotype relationship per se. These factors include the nature of the population being studied, the specific sample drawn from that population and the inclusion and exclusion criteria being used, and the genotype distributions (i.e., the allele frequencies in the general population and the study sample). They also include the overall study design, the degree of sleep deprivation, the timing and frequency of performance measurements, the level of measurement noise, and the specific outcome measures employed. Any enduring physiological states, such as those that may be induced by a history of chronic sleep restriction or by illness, may further shape genotype findings.

Rapid population growth and mixing of ancestries over the last several hundred years has shifted population genetic equilibriums, resulting in genotypic frequency and diversity variations across racial and ethnic groups (Ortega and Meyers, 2014). This introduces a systematic source of genetic variation that must be considered when interpreting between-subject variances and their generalizability across populations. Thus, while between-subjects variance serves as a useful tool to quantify phenotype-genotype relationships, these factors make it difficult to compare explained variance statistics between studies.

Even when phenotype-genotype associations are well established and quantified, inferring the genetic mechanisms underlying cognitive functioning may nonetheless be difficult, because knowledge of the functional consequences of genetic variants of interest (e.g., changes in protein expression levels, or differences in receptor affinity) is often incomplete. For example, the literature is mixed regarding the effects of the VNTR polymorphism of *DAT1*, with some studies suggesting that the 9R allele results in the downregulation of *DAT1* activity, and others finding the same for the 10R allele (Faraone et al., 2014). Further, individual genes do not act in isolation, and gene-gene interactions may alter or obscure the effects of the individual genes. And there are complex interactions between different neurotransmitter systems, further complicating the interpretation of genotype effects.

Genotype effects may also be brain region-specific. This is well illustrated by differential mechanisms for the clearing of dopamine from the extracellular space. Within the prefrontal cortex, extracellular dopamine is metabolized, with the *COMT* gene modulating the clearance rate (Savitz et al., 2006). Within the striatum, cellular reuptake is the dominant mechanism for dopamine clearance, with *DAT* genes modulating the reuptake rate (Ettinger et al., 2016). Striatal-dependent probabilistic learning and PFC-dependent cognitive flexibility are both aspects of reward-based learning, but mediated by different dopaminergic gene polymorphisms which offer different perspectives on how sleep deprivation may impact various aspects of cognition that utilize fronto-striatal neural pathways.

4 Biomarker panels and statistical tools

Whereas genotypes modulate the effects of sleep deprivation, the actual profiles of gene expression—i.e., the conversion of genetic information into functional products such as proteins—are an essential part of the biomolecular pathways underlying the impact of sleep deprivation on cognition. Indeed, gene expression profiles change dynamically with increasing levels of sleep deprivation (Möller-Levet et al., 2013; Pellegrino et al., 2014; Uyhelji et al., 2018), and may one day serve as biomarkers of individuals' cognitive responses to sleep loss (Mullington et al., 2016; Uyhelji et al., 2018). Given the involvement of multiple genes, the search for biomarkers involves the identification of biomarker panels that can act like a fingerprint, a so-called biosignature (Laing et al., 2018; McDermott et al., 2013). Recent advances in high-throughput technologies have enabled the rapid production of large gene expression data sets. These data sets tend to have many more gene-related predictors than phenotypic outcome data, which presents a serious challenge for data analysis but is well-suited for the utilization of modern, multivariate statistical approaches.

4.1 Data-driven and theory-driven statistical approaches

The search for meaningful biomarkers can either be knowledge-driven (also termed theory-driven) or data-driven. Knowledge-driven approaches focus on a carefully chosen set of (potentially) biologically relevant variables, whereas data-driven

strategies explore patterns in the data that are not readily apparent from theory or human reasoning. Both these approaches have advantages and disadvantages. The theory-driven approach is often concerned with testing of an a-priori stated hypothesis, where mitigating type I error (the probability of reporting false positive findings) becomes important. This can be achieved by limiting the number of hypotheses (e.g., by investigating only one or a small number of carefully selected genes), and/or by applying a type I error adjustment for multiple comparisons during statistical testing. The latter may involve adjusting the significance level of each test, such that the Family-Wise Error Rate (FWER) is controlled. The FWER seeks to limit the probability of any false positives among a set of tests. While statistically powerful, the theory-driven approach has a fundamental limitation, captured by the words of Einstein (quoted in Salam et al., 1990): "It is the theory which decides what can be observed." In other words, this approach may miss important features of the data set that are not in the focus of investigation.

By contrast, data-driven investigations attempt to identify the potentially interesting features of the data set (Jaeger and Halliday, 1998). This approach emphasizes controlling the type II error (the probability of failure to report true positive findings). Strict control of type I error (like in theory-driven research) would be too restrictive in this context, and is therefore not commonly applied in data-driven research. However, the type I error cannot be entirely neglected, as that could lead to too many false positives in the results, rendering the data analysis uninformative. To address this issue, alternative type I error control methods have been developed (Benjamini and Hochberg, 1995). These include false discovery rate (FDR) methods, which seek to curb the expected proportion of false positive findings among all reported findings from a study; and Per-Family Error Rate (PFER) methods, which aim to limit the expected total number of false positive findings being reported. While poised to yield rich findings, the data-driven approach has a fundamental limitation as well, in that the results may not reflect any concrete biological mechanisms or lead to significant new insights.

4.2 Exploratory statistical techniques: Proof of concept

Modern statistical methods allow the theory- and data-driven approaches to be combined by including prior knowledge into exploratory statistical techniques (McDermott et al., 2013). Here we discuss a set of modern, multivariate statistical tools called penalized regression, and review its contributions and pitfalls in the identification of biomarker panels. For illustration purposes, we show different ways to apply these methods in the statistical environment "R" (version 3.5.1), using a gene expression data set that is publicly available (NCBI GEO online repository; accession number GSE98582). The data were collected in the laboratory in a relatively small sample of 17 healthy young adults, 11 of which were subjected to 62 h of total sleep deprivation and 6 of which served as well-rested controls. To measure cognitive impairment due to sleep deprivation, subjects performed a PVT at intervals of 2–4 h during scheduled wakefulness. Blood samples were taken and gene

expression was quantified, using microarrays, for specific times of day—08:00, 12:00, 16:00 and 20:00—at baseline, after one night of sleep deprivation (or well-rested control), and after recovery sleep. In the publication where these data were introduced (Uyhelji et al., 2018), the gene expression data were filtered for low expression levels, and data from 8397 genes were retained (where some genes that would a priori be potentially relevant, such as *DAT1* or *BDNF*, were not included). Further details can be found in the original publication (Uyhelji et al., 2018).

The gene expression data set considered here is characterized by "high-dimensionality," which means that there are many more variables (i.e., 8397 genes) than samples (i.e., 17 subjects times 12 samples per subject = 204 samples). This situation entails several challenges that have been coined "the curse of dimensionality" (Bellman, 1961). An attempt to include all 8397 genes in a single analysis would normally result in an underdetermined regression model, and application of standard estimation methods such as least-squares fitting would lead to overfitting—such that not only the gene expression signal but also the noise in the data is fitted and predictions derived from the model would be unreliable. Therefore, some form of data processing or dimensionality reduction must occur, which may be achieved by optimization algorithms applied in advance of, or embedded in, the regression analysis. For example, the data could first be subjected to principal component analysis (PCA), which organizes the data in a number of clusters that show similar behavior (i.e., gene expression levels increasing and decreasing in tandem over time and/or across subjects). Together these clusters capture the bulk of the variance in the data set. A drawback of PCA and related methods is that they transform the data set, which may make it difficult to interpret the results they yield.

Alternatively, the data could be submitted to penalized regression, which as part of the regression algorithm selects only the most predictive subset of the genes in the data set. The published literature provides detailed introductions into penalized regression and related statistical methods (Bühlmann and van de Geer, 2011; James et al., 2013) and their application (McDermott et al., 2013; Price et al., 2006, 2010; Wang and Huang, 2014)—here we provide just a brief introduction. A common approach to fitting a regression equation to data is the ordinary least squares method. This method estimates the parameters of the regression model (here reflecting the estimated impact of each of the genes) by minimizing the squared differences between the model predictions and the actual data points. This optimization scheme ensures a good fit, especially if the noise in the data is approximately normally distributed. Penalized regression adds to this approach the concept of "sparsity," or the idea that not all potentially explanatory variables (genes) are relevant for the phenotype of interest (Candès, 2006).

4.3 LASSO and cvLASSO

The best-known implementation of penalized regression is the "least absolute shrinkage and selection operator" (LASSO; Tibshirani, 1996). This method balances finding a good fit of the phenotype data (PVT performance) with only including a

parsimonious set of influential variables (a small panel of genes). It does this by adding a penalty for each gene used in the regression equation. Thus LASSO sets the model parameters for genes that are not likely to be predictive of the observations to zero, thereby effectively removing them from consideration in the regression model. Achieving a compromise between goodness-of-fit (i.e., how well the model fits the data) and parsimony (i.e., how many parameters are retained in the model) becomes of central importance. Overemphasizing goodness-of-fit would lead to overfitting, whereas overemphasizing parsimony (by applying a penalty that is too harsh) would lead to not including enough genes and thereby compromising the fit. In the LASSO algorithm, the balance between goodness-of-fit and parsimony is mediated by a tuning parameter, which determines the magnitude of the penalty for including additional genes—the higher the tuning parameter, the greater the penalty, and the fewer variables (genes) are included in the model.

In LASSO regression, the tuning parameter (denoted by λ) is usually chosen by a cross-validation (CV) scheme (Bühlmann and van de Geer, 2011). First, a grid of λ values is selected to try out. Then for each λ, the data set is randomly divided into a training subset and a test subset. The model with tuning parameter λ is fit to the training subset, and goodness-of-fit (quantified as the mean squared error; MSE) is calculated for the test subset. The λ value that produces the greatest goodness-of-fit is picked as the tuning parameter to be used in the LASSO regression on the whole data set. A variation on this theme is n-fold CV LASSO (cvLASSO), in which the tuning parameter is determined by dividing the data set into n subsets with similar number of data points, each of which is once used as a test subset. This thus yields an average of n estimates of goodness-of-fit for each λ in the grid, and the λ with the best average goodness-of-fit (MSE) is picked as the tuning parameter for use on the whole data set.

Other implementations of penalized regression are based on variations on the same theme; they include Ridge Regression (Hoerl and Kennard, 1970), group LASSO (Yuan and Lin, 2005), and elastic net regression (Zou and Hastie, 2005). While these methods are inherently linear, generalized versions exist, which allow for non-linear predictor effects while retaining high interpretability of results (Meier et al., 2009; Petersen and Witten, 2019).

We applied a few different variations on the LASSO method to the gene expression data set described above. LASSO regressions assume that the distribution of the error (noise) in the dataset is normal, but the method can be extended to a generalized linear model (GLM) in which the error distribution is assumed to be non-normal. This is relevant here, as PVT performance outcomes in the data set were measured in terms of "lapses," defined as the number of response times longer than 500 ms (Doran et al., 2001). Noise in this discrete outcome variable would be expected to have a distribution that is approximately Poisson-like, and therefore we implemented a Poisson error distribution. The first analysis we performed in this framework was a 10-fold cvLASSO (R package glmnet version 2.0-16). Fixed regression terms (covariates) were included for study condition (2 levels), day (3 levels), time of day (4 levels), and their interactions, as well as sex and age. The selected value of the tuning parameter was 0.075. The results of the analysis are discussed later.

4.4 Stability selection LASSO

One problem with the cvLASSO method is that the presence of noisy (i.e., biologically unrelated) genes in the data set may lead to less meaningful results (Wang and Huang, 2014). This is an issue of stability, which entails that findings in one part of the data set should also be found in the remaining part of the data set (Bühlmann and van de Geer, 2018). Stability Selection LASSO is a recently developed approach to identifying stable results in LASSO regression analysis (Meinshausen and Bühlmann, 2010). This method considers the selection probabilities of the different variables, essentially asking how often the different genes are selected in subsets of the data set. Genes are retained in the model based on a pre-specified selection probability cutoff. This strategy naturally allows for control of type I error in terms of PFER (Meinshausen and Bühlmann, 2010). Alternatively, confidence intervals can be used for gene selection (Stucky and van de Geer, 2018; van de Geer et al., 2014). The second analysis of the gene expression data set we performed was a Stability Selection LASSO regression (R package stabs version 0.6-3). In addition to a Poisson error distribution, fixed regression terms were included for study condition, day, time of day, and their interactions, as well as sex and age. The PFER threshold was set to 1 (i.e., allowing no more than 1 false positive), and a pre-specified selection probability cutoff of 0.6 was used. The selection probabilities of the top 30 genes are shown in Fig. 2; only genes to the right of the gray vertical line were retained. The results of this analysis are further discussed below.

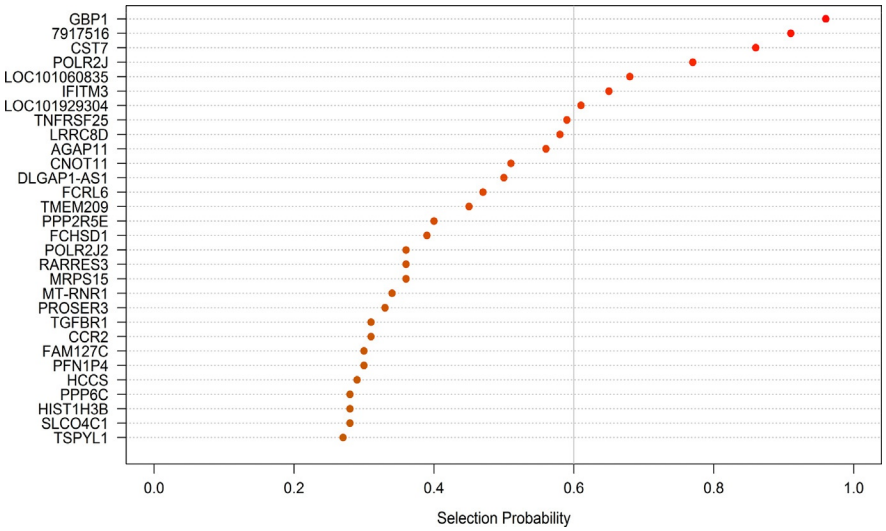

FIG. 2

Illustration of Stability Selection LASSO regression results. The selection probabilities for the top 30 genes are shown; the selection probability cutoff of 0.6 used in the analysis is indicated by the gray vertical line. The genes starting with LOC are unnamed and are identified here by their gene id number.

4.5 Hierarchical inference

Another problem in high-dimensional settings is the fact that single genes cannot be unambiguously identified, because the effects of different genes are often highly correlated and/or because there are not enough data in the sample to uniquely tell the genes apart (Renaux et al., 2018). Various generalizations of the LASSO method have tried to circumvent this problem by either only testing for group variables (clusters) or including some prior biological knowledge. The recently introduced Hierarchical Inference method (Buzdugan et al., 2016; Renaux et al., 2018) clusters correlated genes in an adaptive way, such that the size of the clusters is as small as possible while still contributing significantly to the overall model. We applied the Hierarchical Inference method as the third analysis of the gene expression data set (R package hierinf version 1.1.0 using the standard function "cluster_position()" together with Bioconductor version 3.7.4). Fixed regression terms were included for study condition, day, time of day, and their interactions, as well as sex and age. No Poisson GLM implementation is currently available in the R package hierinf—therefore, PVT lapses were log-transformed before analysis. The results of the Hierarchical Inference analysis can be found in Table 2 (left side).

4.6 Group LASSO

Several of the clusters in the Hierarchical Inference analysis retain many genes. To help with interpretation of such abundant results, functional gene networks can be plotted using the R package FGNet (version 3.14.0), which performs functional enrichment analysis and clustering through the GeneTerm Linker online tool (https://omictools.com/geneterm-linker-tool) based on reciprocal linkage (Fontanillo et al., 2011). See Fig. 3 for an illustration, focused on the 16th cluster (see Table 2). This gene expression cluster contains many adenosine- and dopamine-related genes (e.g., *ADA*, *ADORA2A*, *COMT*), which is consistent with the phenotype-genotype associations discussed earlier in this paper.

Instead of using a purely data-driven approach, it is also possible to incorporate prior knowledge to guide the regression algorithms. General frameworks and examples for such "structured sparsity" methods can be found elsewhere (Morales et al., 2010; Stucky and van de Geer, 2017; van de Geer, 2014). Signaling pathways, functional groupings and gene ontological groupings are available from online tools such as the meta-database consensuspathDB (http://cpdb.molgen.mpg.de/CPDB). A simple extension of the LASSO method that can make use of this information is the group LASSO (Yuan and Lin, 2005). This structured sparsity method can select groups of genes instead of individual genes, which is useful to identify genetic pathways. One drawback of this method is that it does not consider that genes may be members of more than one group. Therefore, the group LASSO with overlaps was introduced (Jacob et al., 2009).

Another useful approach, termed bi-level selection, seeks sparsity at the level of genes and at the level of groups of genes simultaneously (Park et al., 2015), so that

Table 2 Hierarchical inference results for the relationship between gene expression profiles and PVT performance, and comparison with cvLASSO, stability selection LASSO, and oGEL results.

Hierarchical inference results					Shared members		
Number	***P* value**	**R^2**	**Cluster**	**Potentially interesting members**	**cvLASSO**	**Stability selection**	**oGEL**
1	0.001	0.13	FCRL3 [1]	FCRL3	FCRL3	No element	No element
2	0.001	0.15	MT-TA [1]		No element	No element	No element
3	0.005	0.24	USP24,…,[62]	SLC17A7	LINC01356, GBP1	GBP1	RPS26P15, GBP1, LINC01356
4	0.007	0.23	PRKAB1,…, [252]	TNFRSF1A, UBE2N	NUP37, ACRBP	No element	Unknown.393
5	0.008	0.14	KIAA1715 [1]		No element	No element	No element
6	0.011	0.30	ABI1,…,[505]	COMTD1, GLUD2, AMPD3, TNFAIP3, PIK3AP1, UBE4A, PIK3C2A, UBE2L6	EIF3F, KMT2A, RARRES3, TMEM138, WDR74, OSBP, IFITM3, HBB, DKFZp779M0652, KRTAP5-7, VPS37C	IFITM3	NLRP6, RARRES3, KMT2A, TPT1-AS1, unknown.186, KBTBD4, VPS37C
7	0.012	0.13	PI3 [1]		No element	No element	No element
8	0.013	0.21	TMUB1,…, [126]	GRINA, ERICH1, TNFRSF10C	PROSC	No element	PTP4A3
9	0.014	0.14	Unknown.9 [1]	Unknown.9	No element	No element	Unknown.9
10	0.015	0.15	SNORD56B [1]		No element	No element	No element
11	0.016	0.17	CDK11A,…, [31]	TNFRSF14, TNFRSF25	TNFRSF25	TNFRSF25	MAD2L2
12	0.019	0.20	UBR5,…,[62]		KRT18	No element	KRT18
13	0.024	0.23	ADSL,…,[505]	PIK3IP1, CYP2D6, UBE2E2, UBE2E1, PIK3CA, PIK3R4, PIK3CB	ZNF654, PPP4R2, GLT8D1, NEK4, RASSF1, SLC9A9, EIF4ENIF1, RABL2B, HSPBAP1, GHRL	Unknown	Unknown.550, GPR27, TFG, RNU6-736P, unknown, unknown.492, DVL3, NICN1, GLT8D1
14	0.025	0.17	SNORD116-2, SNORD116-6 [2]		No element	No element	No element

Continued

Table 2 Hierarchical inference results for the relationship between gene expression profiles and PVT performance, and comparison with cvLASSO, stability selection LASSO, and oGEL results.—cont'd

Hierarchical inference results					Shared members		
Number	***P* value**	**R^2**	**Cluster**	**Potentially interesting members**	**cvLASSO**	**Stability selection**	**oGEL**
15	0.029	0.24	EDC4,…,[252]	ADAT1	TERF2IP, TXNL4B, VPS4A, PSMB10, LOC642533, EDC4	No element	TERF2IP, FAM192A, C16orf80, FAM96B
16	0.029	0.19	FKBP1A,…, [252]	ADA, ADARB1, COMT, SPECC1L-ADORA2A, ADORA2A, UBE2D3P1, UBE2V1, UBE2G2, UBE2L3	CCT8, SNORA80A, APOL6, RNA5SP474, CABIN1, C21orf33, TMEM191C, SEPT5-GP1BB	No element	RNA5SP474, RN7SKP111, MAP3K7CL, CCT8, KRTAP12-4, SEPT5-GP1BB.1, TMEM191C, CABIN1, APOL6
17	0.029	0.13	CD7,…,[63]	PIK3C3	DLGAP1-AS1	No element	No element
18	0.031	0.18	ACO1,…,[63]	UBE2R2	No element	No element	Unknown.488, TGFBR1
19	0.033	0.19	GDAP2,…,[31]		No element	No element	No element
20	0.033	0.33	NRF1,…,[252]	POLR2J, UBE3C, UBE2H	ZNF394, NSUN5, TMEM209, FAM185A, POLR2J2, POLR2J3	POLR2J	RASA4CP, STYXL1, ZNF394, TMEM209, CNOT4
21	0.034	0.26	GSTM4,…,[3]	GSTM4	No element	No element	GSTM4
22	0.034	0.22	GPR171,…, [252]	TNFSF10, UBE2K, CYP4V2, TNIP2	RUFY3, CCR2, MIR4800, TMEM41A	No element	CCR2, RUFY3, unknown.212, unknown.37
23	0.049	0.20	MAK,…,[125]	GABBR1, GCLC	DNPH1, OARD1, HIST1H3B, HIST1H1C, NEU1	No element	HIST1H4I, unknown.136, TAF11, SYNCRIP

Rows correspond to significant clusters from Hierarchical Inference. On the left side of the table, cluster numbers, P *values, aggregated explained variances (R^2), cluster descriptors and the number of cluster members (in square brackets), and some potentially interesting members are shown for illustration purposes. On the right side of the table, the clusters are compared to results from the other methods used for analysis of the data set.*

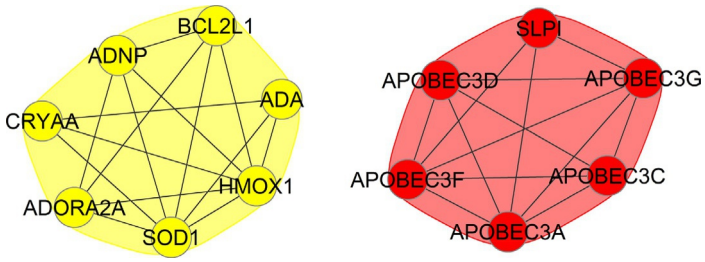

FIG. 3

Illustration of a functional gene network plot for the Hierarchical Inference method. Results are shown for the 16th significant cluster (see Table 2). Different colors (*yellow and red*) indicate different biological processes found in the cluster based on the R package FGNet (version 3.14.0) using the GeneTerm Linker tool.

sparse pathways with relatively few members can be identified. One implementation thereof is Group Exponential LASSO (GEL; Breheny, 2015). Again a version with overlaps exists, called the overlapping Group Exponential LASSO (oGEL). The fourth (and last) analysis of the gene expression data set we performed here was an oGEL analysis based on 10-fold cvLASSO regression, as implemented in the R package grpregOverlap (version 2.2). It is not yet possible to include fixed regression terms within this implementation; therefore, study condition, time of day, sex, and age were not controlled for in this analysis. Table 3 shows the gene groups (pathways) selected by the analysis.

The results of the four analyses of the gene expression data set are compared in Table 2. There are large differences in the findings, which is not unusual in case of high-dimensionality. However, some of the genes in the data set were identified consistently across the different methods. For example, all four analyses identified the guanylate binding protein 1 (*GBP1*) gene. A SNP of *GBP1* (SNP-ID: rs10801703) was found to be associated with caffeine-induced insomnia in a GWAS study (Byrne et al., 2012), suggesting it is a candidate for further study. Tumor necrosis factor receptor superfamily member 25 (*TNFRSF25*), which is part of the tumor necrosis factor-mediated signaling pathway, was also identified repeatedly. Related genes were shown to be affected by sleep deprivation (Maret et al., 2007) and have been proposed as biomarkers for acute total sleep deprivation (Laing et al., 2018; Satterfield et al., 2015). Histone-lysine *N*-methyltransferase 2A (*KMT2A*) is another notable finding, as it might play a crucial role in prefrontal synaptic plasticity and working memory (Jakovcevski et al., 2015), which could have negative downstream consequences on cognitive performance during sleep deprivation.

In the original analysis of the gene expression data set (Uyhelji et al., 2018), 28 genes were identified for which the expression was specifically associated with PVT performance impairment due to sleep deprivation (and not just sleep deprivation per se). Of these, 13 were identified by cvLASSO (*POTEKP*, *SPDYE2*, and *WWC3*) or as members in Hierarchical Inference clusters (*ODF2L*, *IPMK*, *LPCAT2*, *LITAF*, *AP1G1*, *KCNJ15*, *ETS2*, *RAB7A*, *FLOT1*, *ELOVL5*, *STEAP4*, *SPDYE8P*, and *SDCBP*).

Table 3 Results of the oGEL model analysis. Gene groupings were extracted from the consensuspathDB tool with the setting "pathways."

Apoptosis	Influenza A	Rho GTPase effectors
Beta-catenin independent Wnt signaling	JAK STAT pathway and regulation	Rho GTPases activate formins
Bisphosphonate pathway, pharmacodynamics	Kaposi's sarcoma-associated herpesvirus infection	Ribosome
Cargo recognition for clathrin-mediated endocytosis	Late phase of HIV life cycle	RNA degradation
Cell cycle	M phase	RNA polymerase II pre-transcription events
Cellular responses to external stimuli	Membrane trafficking	RNA polymerase II transcribes snRNA genes
Cellular senescence	Metabolism	RNA polymerase II transcription
Chromatin modifying enzymes	Metabolism of proteins	Role of calcineurin-dependent NFAT signaling in lymphocytes
Chromosomal and microsatellite instability in colorectal cancer	Metabolism of RNA	Role of MEF2D in T-cell apoptosis
Control of skeletal myogenesis by HDAC and calcium	miR-targeted genes in leukocytes—TarBase	Shigellosis
C-type lectin receptor signaling pathway	miR-targeted genes in lymphocytes—TarBase	Signaling by Rho GTPases
Deadenylation of mRNA	Mitochondrial translation	Spliceosome
Disease	Mitotic prometaphase	TCF dependent signaling in response to Wnt
DNA damage response (only ATM dependent)	mRNA processing	TCR
EGF-core	mTOR signaling pathway	TGFbeta receptor
EGFR1	NOD-like receptor signaling pathway	Thermogenesis
Endocytosis	Pathogenic *Escherichia coli* infection	Transcriptional regulation by TP53
Gene expression (transcription)	Pathways in cancer	Tuberculosis
Generic transcription pathway	Phagosome	Ub-specific processing proteases
Hepatocellular carcinoma	Post-translational protein modification	Validated targets of C-MYC transcriptional repression
HTLV-I infection	Processing of capped intron-containing pre-mRNA	Vesicle-mediated transport
Immune system	Protein processing in endoplasmic reticulum	Viral carcinogenesis
Infectious disease	Regulation of TP53 activity	Wnt signaling pathway

The oGEL model selected a sparse set of these consensuspathDB grouping (pathways) to model PVT performance. The resulting groupings are (arbitrarily) shown here in alphabetical order.

This convergence across different analysis methods makes these genes strong candidates for further study. This could include investigation of new data from different samples, and it may involve inclusion of genes previously determined to be potentially relevant as fixed (unpenalized) regression terms for specific hypothesis testing and further discovery.

5 Conclusion

In this paper we outlined a roadmap for the investigation of phenotype-genotype relationships in sleep deprivation and its effects on cognitive performance showed the power of cognitive endophenotyping by focusing on specific cognitive functions and illustrated modern statistical tools to address the high-dimensionality of gene expression data sets that is typical in this domain. Application of the methods described here does not obviate the need for replication studies (or rather, extension studies; see Zatz, 2000) and investigations of generalizability to other populations (e.g., children or older adults or patients with sleep disorders or other medical conditions) to further unravel the genetic underpinnings of human sleep deprivation and its effects on cognition. We also did not cover twin study paradigms, which provide another powerful strategy for studying phenotype-genotype associations (e.g., Pellegrino et al., 2014).

Systems biology approaches beyond the scope of this paper will be needed to further clarify the mechanisms implicated in the results of phenotype-genotype analyses, as genetic, neural, cognitive, and behavioral systems do not work in isolation, but rather in complex interacting frameworks. Also, translational research will be needed to understand how results apply to real-world settings and real-world performance tasks, where sleep deprivation and cognitive functioning interact with circumstantial and environmental factors that may profoundly shape performance outcomes (Riggs et al., 2018). This, in turn, may yield new insights into the underlying mechanisms.

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Sleep pressure regulates mushroom body neural-glial interactions in Drosophila

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Abstract

Sleep is a behavior that exists broadly across animal phyla, from flies to humans, and is necessary for normal brain function. Recent studies in both vertebrates and invertebrates have suggested a role for glial cells in sleep regulatory processes. Changes in neural-glial interactions have been shown to be critical for synaptic plasticity and circuit function. Here, we wanted to test the hypothesis that changes in sleep pressure alters neural-glial interactions. In the fruit fly, *Drosophila melanogaster*, sleep is known to be regulated by mushroom body (MB) circuits. We used the technique GFP Reconstitution Across Synaptic Partners (GRASP) to test whether changes in sleep pressure affect neural-glial interactions between MB neurons and astrocytes, a specialized glial cell type known to regulate sleep in flies and mammals. The MB-astrocyte GRASP signal was reduced after 24 h of sleep deprivation, whereas the signal returned to baseline levels following 72 h of recovery. Social enrichment, which increases sleep drive, similarly reduced the MB-astrocyte GRASP signal. We did not observe any changes in the MB-astrocyte GRASP signal over time-of-day, or following paraquat exposure or starvation. These data suggest that changes in sleep pressure are linked to dynamic changes in neural-glial interactions between astrocytes and neuronal sleep circuits, which are not caused by normal rest-activity cycles or stressors.

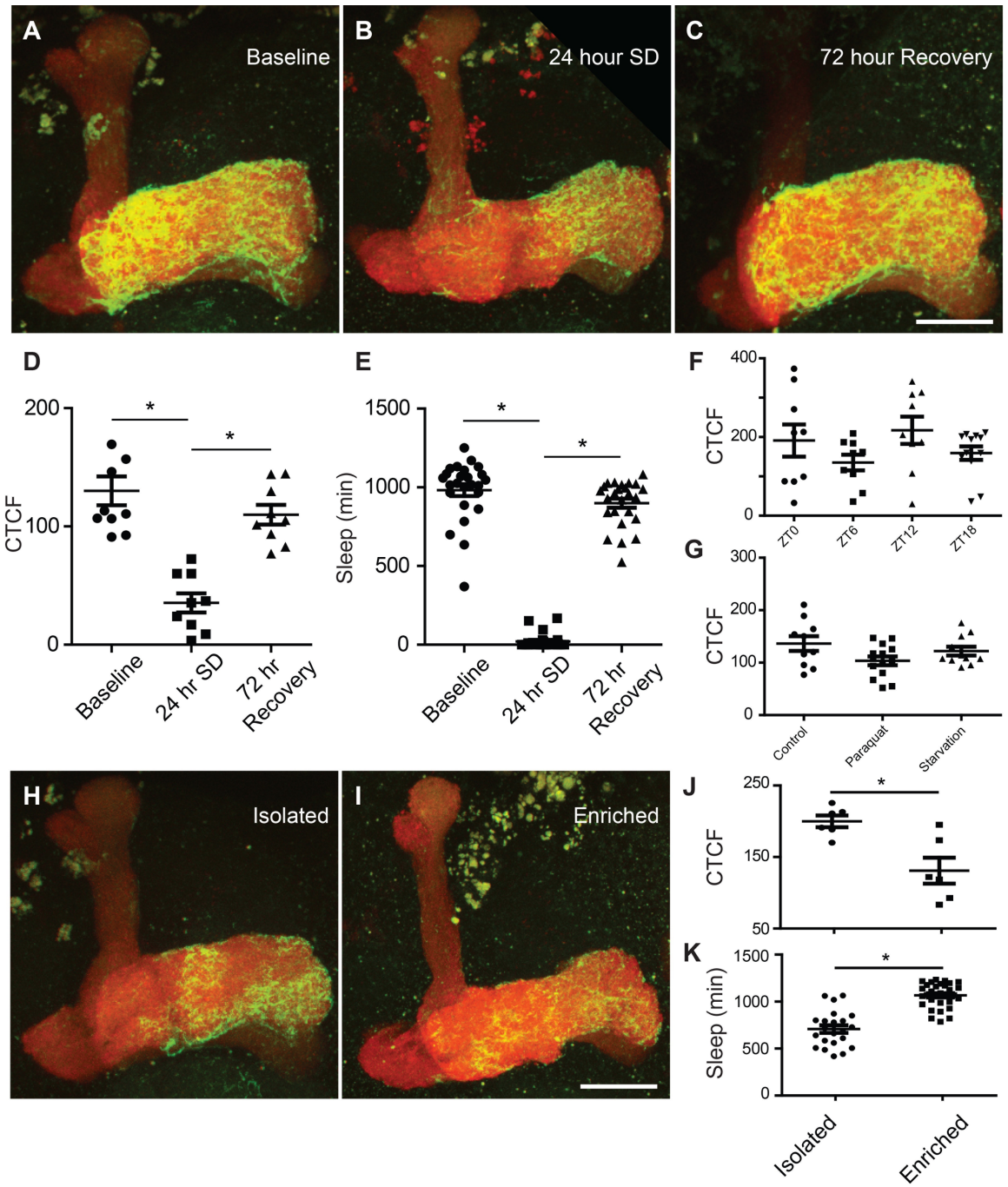
Introduction

Sleep is a ubiquitous behavior exhibited broadly throughout animal phyla, yet its functions remain enigmatic. Fundamental properties of sleep function may be revealed by determining phylogenetically conserved mechanisms associated with sleep behavior across evolutionarily distant species. Sleep is affected both by the amount of time spent awake, as well as experience-dependent changes in synaptic plasticity. Sleep regulatory processes have largely focused on the role of neurons; however, glial cells also play an important role in sleep, across invertebrates and vertebrates alike, including humans [1] [2] [3] [4] [5] [6] [7].

The fruit fly *Drosophila melanogaster* is a powerful model for exploring neural and glial processes that regulate sleep. For example, sleep in *Drosophila* has been shown to be regulated independently by mushroom body (MB) neural circuits [8] [9] as well as glial cells [1] [4] [6] [10] [11]. However, it is currently unclear how MB neurons and glial cells physically interact as a result of changes in sleep pressure. The GFP Reconstitution Across Synaptic Partners (GRASP) technique is a method that labels the membrane contact of two cell types by complementary fragments of the green fluorescent protein (GFP) molecule [12]. Here we use the GRASP technique to label interactions between MB neurons and astrocytes, a specific glial cell type known to regulate sleep in flies and mammals [1] [2] [5]. Determining that changes in neural-glial interactions are associated with sleep pressure would provide a new model system to test functional aspects of sleep-regulatory molecular events that may be conserved across species.

Objective

To determine whether changes in sleep pressure regulate mushroom body neural-glial interactions in flies.



a

Figure Legend

Figure 1. Increased sleep pressure decreases neural-glial interactions between mushroom body neurons and astrocytes in *Drosophila melanogaster*.

(A) Representative image of MB-astrocyte GRASP signal in baseline (non-sleep deprived) condition.

(B) Representative image of MB-astrocyte GRASP signal after 24 h of sleep deprivation (SD).

(C) Representative image of MB-astrocyte GRASP signal after 72 h of recovery following 24 h of SD. Scale bar 25 μm.

(D) Average CTCF signal in groups from the 24 h of baseline (non-sleep deprived) condition, after 24 h of sleep deprivation (SD), and after 72 h of recovery following 24 h of SD. One-way ANOVA revealed a significant effect of sleep loss on GRASP signal (P<0.0001). Tukey multiple comparisons test showed a significant reduction in GRASP signal after 24 h of SD when compared to both baseline (*P<0.001) and after 72 h of recovery following 24 h of SD (*P<0.001).

(E) Average sleep amount (min) per 24 h day in groups from the 24 h of baseline (non-sleep deprived) condition, during 24 h of sleep deprivation (SD), and for the final 24 h period after 72 h of recovery following 24 h of SD. One-way ANOVA confirmed a significant effect of sleep loss (P<0.0001). Tukey multiple comparisons test showed a significant reduction in sleep during SD when compared to baseline (*P<0.001) and during 72 h of recovery following 24 h of SD (*P<0.001).

(F) Diurnal measures of MB-astrocyte GRASP signal in flies on a 12:12 light-dark cycle. One-way ANOVA indicated no significant changes across time-of-day (P=0.22). ZT0 (zeitgeber time 0) = lights on, ZT12 = lights off.

(G) Stress induced by 24 h of paraquat treatment (0.1 mM) or by 24 h of starvation did not affect MB-astrocyte GRASP signal compared to control flies. One-way ANOVA (P=0.08); Tukey multiple comparisons tests among conditions (n.s.).

(H) Representative image of MB-astrocyte GRASP signal in control (isolated flies).

(I) Representative image of MB-astrocyte GRASP signal in flies following social enrichment (enriched flies). Scale bar 25 μm.

(J) Effect of social enrichment (enriched flies) on MB-astrocyte GRASP signal compared to control (isolated flies). T-test (*P<0.01).

(K) Total sleep amount (min) in the 24 h day from MB-astrocyte GRASP flies following social enrichment (enriched flies) compared to control (isolated flies). T-test (*P<0.0001).

Results & Discussion

Our GRASP studies utilized flies harboring mb247-splitGFP11 (specific to MB neurons) [13] [14] and the UAS-splitGFP1-10 expressed in glia, using the astrocyte-directed Alrm-GAL4 driver [15]. MB neural-glial interactions are quantified by the presence of a GFP fluorescent signal, which is triggered upon contact of the individual GRASP components [12]. MB-astrocyte GRASP flies also express Ds-Red under control of the mb247 MB neuronal promoter, which labels the whole MB region of the fly brain. MB-astrocyte GRASP flies were subjected to 24 h of mechanical sleep deprivation [16] and brains were harvested and examined for changes in GFP signal compared to control (undisturbed) flies using confocal microscopy (Fig. 1A–C). All flies were examined at the same time-of-day (ZT0, lights-on). MB-astrocyte GRASP flies showed a significant reduction in GFP signal (Corrected Total Cell Fluorescence, see methods) following sleep deprivation compared to control flies (Fig. 1D). Following 72 h of recovery after 24 h of sleep deprivation, we observed that the GFP signal returned to control levels (Fig. 1D). This MB-astrocyte GRASP response to sleep deprivation and recovery closely resembled the amount of sleep observed under these conditions (Fig. 1E).

To determine whether the changes in MB-astrocyte GRASP signal may have been influenced by the normal sleep-wake cycle, we performed brain dissections on MB-astrocyte GRASP flies at multiple times-of-day. We did not find any differences in the diurnal profile of GFP signal (Fig. 1F), indicating that the observed differences in MB-astrocyte GRASP signal were due to changes in sleep pressure, and not normal sleep-wake cycles. To rule out the possibility that the differences in GFP signal were caused by stress effects, we examined MB-astrocyte GRASP flies for changes in GFP signal following paraquat treatment and starvation stressors. We did not observe any differences in GFP signal in stressor-treated MB-astrocyte GRASP flies compared to control flies (Fig. 1G). To determine whether the decreases in MB-astrocyte GRASP signal were due to increases in sleep pressure, and not a nonspecific artifact of mechanical sleep deprivation, we tested

flies following a social enrichment paradigm, which is known to increase sleep drive in flies [17]. Compared to control (isolated flies), MB-astrocyte GRASP flies subjected to social enrichment (enriched flies) showed a significant reduction in GFP signal (Fig. 1H–J) in tandem with a significant increase in sleep (Fig. 1K), pointing to sleep pressure as the specific driver of the changes in MB-astrocyte GRASP signal.

Sleep deprivation is detrimental to cognitive functioning [18], including learning and memory [19], as well as overall health [20]. Yet, we lack a fundamental understanding of sleep function [21]. The fly model is a powerful tool to understand sleep regulatory processes and functions [22]. Here, we used the GRASP technique to show that increases in sleep pressure decrease neural-glial interactions between MB neurons and astrocytes. Dynamic changes in neural-glial interactions may influence sleep homeostasis through glial uptake of neurotransmitters, efficiency of gliotransmission, and glial processes underlying metabolic support of neurons. Future studies examining phylogenetically conserved molecular pathways in regulating the dynamic relationship of sleep pressure with sleep deprivation- and experience-dependent changes in neural-glial interactions will help to better understand the functional roles for sleep behavior.

Conclusions

This study shows that changes in sleep pressure regulate MB neural-glial interactions in flies.

Limitations

We used *Drosophila melanogaster* to test whether changes in sleep pressure regulate changes in neural-glial interactions. The current studies are limited to MB neurons and astrocytes, and we have not determined whether other sleep circuits or other glial cells are responsive to changes in sleep pressure. Our studies are also limited to the GRASP technique, and alternative strategies, such as immunohistochemistry against neural-glial extra-cellular matrix proteins or cell adhesion molecules [23] or electron microscopy-based morphological studies [24] may provide alternative means to examine sleep-dependent changes in neural-glial interactions. More research is needed to fully understand the time constants of the changes at the neural-glial interface. These changes may take place at time scales ranging from milliseconds to hours, and perhaps even days. Our study was limited to assessing the effects of acute (24 h) sleep deprivation. Our studies are also limited to the fly model, and testing vertebrate models is desirable in order to determine whether the observed changes in neural-glial interactions are phylogenetically conserved. Recent studies demonstrated sleep-dependent changes in the astrocyte glutamate transporter, GLT1, apposition to hypothalamic neurons in mice [25]. However, these studies did not conclude whether the changes were due to a redistribution of GLT1 protein in astrocytes, or to structural changes of astrocyte processes (e.g., extension/retraction) onto these neurons.

Alternative Explanations

Conjectures

Future studies using, e.g., RNAi-based strategies to knock down cell type-specific target proteins involved in cell growth or morphology or the maintenance of the extracellular matrix in our model will be needed to determine mechanisms underlying sleep-dependent changes in neural-glial interactions. In addition, since the MB is an important brain region for learning and memory processes in flies [26], testing whether changes in neural-glial interactions are important for cognitive function would be a worthwhile direction for future studies.

Additional Information

Methods

Fly stocks

Alrm-Gal4 stocks were obtained from the Bloomington Drosophila Stock Center (Indiana University). The mb247-DsRed; mb247-splitGFP11, UAS-splitGFP1-10/TM3sb flies were obtained from T. Riemensperger and A. Fiala (University of Göttingen).

Fly husbandry

Flies were cultured at 25℃, 60% humidity, maintained on a 12:12 Light:Dark cycle, on Nutri-fly Bloomington Formulation fly food (Genesee Scientific, San Diego, CA). Newly eclosed virgin female flies were collected from culture vials daily under CO_2 anesthesia and housed in groups of approximately 30 prior to experimentation.

Sleep analysis

Female flies 4–7 days after eclosion were used for all sleep studies. Flies were mouth aspirated into 5 mm × 65 mm (outside diameter × length) polycarbonate recording tubes (Trikinetics, Waltham, MA) with food (Bloomington Nutri-fly formula) on one end and yarn plugs on the other. Sleep parameters were continuously evaluated using the Trikinetics Drosophila activity monitoring system (DAMS; Trikinetics, Waltham, MA) as described previously [27]. One acclimatization day was followed by 2 days of baseline sleep recording, one 24 h period of mechanical sleep deprivation, and 72 h of recovery sleep. Sleep deprivation was performed using a Sleep Nullifying Apparatus (SNAP), which produces waking without nonspecifically activating stress responses [16], as described previously [27].

Imaging

Drosophila brains were dissected in phosphate-buffered saline (0.9% NaCl, 10 mm NaH_2PO_4, pH 7.2) containing 0.3% Triton X-100 (PBS-T) and fixed in 4% paraformaldehyde, washed, and mounted on cover slips. Optical sections were collected with a Leica DMi8 laser scanning confocal microscope. For each experiment, calibration on the microscope was held constant by establishing a signal threshold value for the control group. GRASP intensity levels were measured using Corrected Total Cell Fluorescence (CTCF). The corrected total fluorescence = Integrated Fluorescence density – (Area of ROI multiplied by Mean Fluorescence of background) and was calculated in max projected image stacks with the region of interest (ROI) around the mushroom bodies.

Stress and starvation

Virgin female flies were collected as described above. 4–7 days after eclosion, animals were mouth aspirated into 5 mm × 65 mm (outside diameter × length) polycarbonate recording tubes (Trikinetics, Waltham, MA) containing 0.1 mM Paraquat in minimal media (2% agar, 5% sucrose in ddH_2O) or starvation food (2% agar in ddH_2O). Animals were housed for 24 h on these media and afterwards were rapidly dissected at ZTo for imaging of GRASP signal.

Social enrichment

To standardize the environmental conditions during critical periods of brain development, virgin female flies were collected upon eclosion and maintained in same-sex vials containing approximately 30 flies for 2 days. This protocol kept environmental conditions constant between subsequently isolated and enriched flies for the first 2 days of adult life. 3 day old flies were then divided into a socially isolated group, in which flies were individually housed in 5 mm × 65 mm plastic tubes, and a socially enriched group, consisting of 50 female flies housed in a single vial. After 5 days of social enrichment/isolation, flies were placed into clean 5 mm × 65 mm plastic tubes and sleep was recorded for 3 days using the Trikinetics DAMS.

Data analysis

Statistics were calculated using Graphpad Prism software. Student's t-test, one-way ANOVA, two-way ANOVA, and Tukey post-hoc analysis were used for analyses. Sleep data were analyzed by averaging across multiple experiments. Flies that did not survive the entire experimental paradigm were removed from data analysis.

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Ethics Statement

Not Applicable.

Citations

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