Science Advances
AAAS

advances.sciencemag.org/cgi/content/full/4/7/eaat1294/DC1

Supplementary Materials for

Cross-species systems analysis identifies gene networks differentially altered by sleep loss and depression

Joseph R. Scarpa, Peng Jiang, Vance D. Gao, Karrie Fitzpatrick, Joshua Millstein, Christopher Olker, Anthony Gotter, Christopher J. Winrow, John J. Renger, Andrew Kasarskis*, Fred W. Turek*, Martha H. Vitaterna*

*Corresponding author. Email: m-vitaterna@northwestern.edu (M.H.V.); fturek@northwestern.edu (F.W.T.); andrew.kasarskis@mssm.edu (A.K.)

Published 25 July 2018, *Sci. Adv.* **4**, eaat1294 (2018)
DOI: 10.1126/sciadv.aat1294

The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at advances.sciencemag.org/cgi/content/full/4/7/eaat1294/DC1)

Data file S1 (Microsoft Excel format). Phenotypic categories and descriptions.
Data file S2 (Microsoft Excel format). eQTL and phenotypic associations.
Data file S3 (Microsoft Excel format). Coexpression network modules.
Data file S4 (Microsoft Excel format). SD signatures.
Data file S5 (Microsoft Excel format). MDD signatures.
Data file S6 (Microsoft Excel format). Bayesian network and key drivers.

SUPPLEMENTARY FIGURES

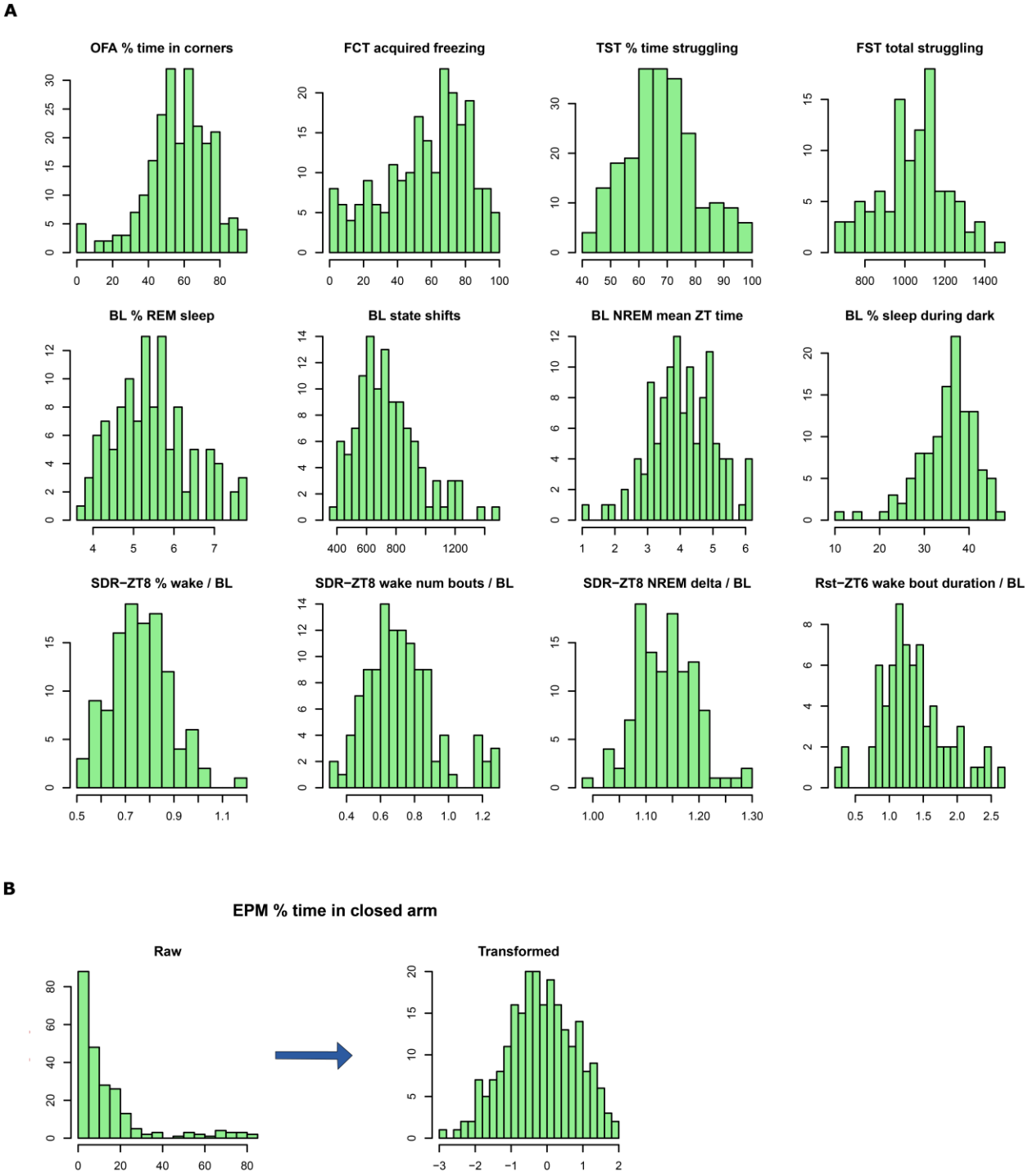

Fig. S1. Distributions of phenotypic measurements. (**A**) Histograms of the distributions of selected phenotypic measurements. These measurements were chosen because they are of particular interest and because they are representative of the phenotypic categories we examined. Descriptions of phenotypes and phenotypic categories are included in Data file S1. (**B**) Illustration of the Yeo-Johnson procedure, which was used to transform skewed data to a normal distribution.

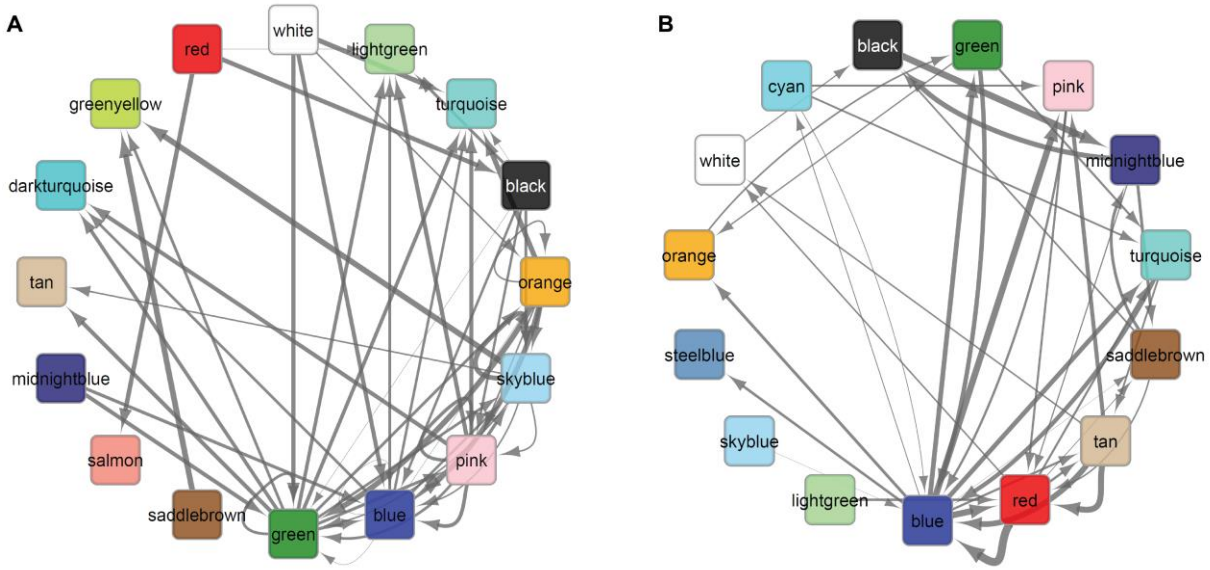

Fig. S2. Regulatory relationships among gene coexpression network modules. (**A**) Inter-modular transcriptional regulatory network. Each node represents a module, and a regulatory edge is present if the source node module is enriched in the transcription factor protein interaction network of the target node module. The width of the regulatory edge is proportional to the odds ratio computed in the Fisher's exact test. (**B**) Module-level Bayesian regulatory network. A global Bayesian network was reconstructed and intersected by coexpression modules. In the module-level network, a node represents a module, and a regulatory edge is present if the gene-level regulatory edges in the Bayesian network occur between genes of the two modules significantly more than random chance. The thickness of the edges in the module level network is proportional to the number of inter-modular edges in the gene-level network normalized by the combined size of two modules. In both (**A**) and (**B**), network nodes were ordered counter-clockwise from the most interconnect node (bottom of the circle) to the least interconnected.

DESCRIPTION OF SUPPLEMENTARY DATA FILES

Data file S1. Phenotypic categories and descriptions.

Data file S2. eQTL and phenotypic associations. Tabs in the excel file contain significant results (FDR < 0.05) of single-region eQTL analysis, multi-region eQTL analysis, as well as multi-region conditional correlations between eQTL-regulated genes and phenotypes.

Data file S3. Coexpression network modules. This excel file contains 24 tabs. For each brain region, results related to coexpression network analysis are given in 6 tabs in the following order: **1**) module membership and connectivity statistics given by WGCNA; **2**) gene functional annotation for each module (i.e., enrichment statistics from Fisher's exact test); **3**) enrichment of target genes of transcription factors determined by Chip-seq or Chip-on-chip experiments; **4**) enrichment of eQTL-regulated genes; **5**) differential connectivity against other brain regions; and **6**) correlation between module eigengene and phenotypes.

Data file S4. SD signatures. Datasets from three brain regions (cortex, hippocampus, and hypothalamus) were available for differential expression analysis. For each brain region, we provide in tabs **1**) the differential expression (DE) statistics, **2**) gene functional annotation of significantly differentially expressed genes (selected at FDR < 0.05; with the exception that functional annotation analysis was not performed in hypothalamus, since no gene was differentially expressed at FDR < 0.05), and **3**) enrichment of (B6 x 129) F2 coexpression modules determined by GSEA.

Data file S5. MDD signatures. Tabs in this excel file contain information regarding the transcriptomic datasets used in the meta-analysis of differential gene expression in the cortex of MDD patients, differential expression (metaDE) statistics in the cortex, enrichment of cortical network modules, differential expression (DE) statistics in the hippocampal (GSE53987) dataset, and enrichment of hippocampal network modules.

Data file S6. Bayesian network and key drivers. Two tabs of this excel file present the pairs of source and target nodes in the Bayesian regulatory network and the key drivers identified in subnetworks resulting from intersecting the Bayesian network with coexpression modules.

Supplementary Methods

Phenotyping procedures

For all behavioral tests, animals were given at least one hour to acclimate in a holding room adjacent to the testing rooms to reduce possible effects of transportation. All tests were performed by the same experimenter across all animals to eliminate a known source of large variability (*69*). The phenotyping tests were given in the same order for all animals, with the exceptions of adding or discontinuing tests as described in detail below. The test sequence was determined so that tests with mild stress were given first and tests with higher levels of stress were given later. Animals from the same cohort were tested on the same date for each test, and all affective behavior tests were completed within 14 day, although the exact days between some of the tests vary slightly among cohorts. In general, a few days are given between tests to allow for recovery from stress associated with tests so that the subsequent tests were not influenced by the acute stress from the previous test. We did not observe significant carry-9over effects for tests that were applied to some but not all animals.

Elevated Plus Maze

Mouse behavior in the elevated plus maze (EPM) was assessed at seven weeks of age. The EPM testing apparatus was elevated 30.5 cm from the floor. The apparatus consists of 4 arms (57.8 cm long and 5.7 cm wide each) in a shape of a plus. A pair of opposing arms has 14cm high walls (closed arms), and the other pair does not have walls (open). At the beginning of the test, the mouse was placed at the center of the apparatus and allowed to explore freely for 5 minutes. A video camera above the apparatus was used to record the animal's path and was connected to a computer for data processing using a video tracking software (LimeLight, Actimetrics, Evanston, IL). The software tracked a midpoint between the animal's shoulders to determine its path traversed and boundary crossings within the apparatus. Before testing of each animal, the apparatus was cleaned with ethanol and allowed to dry.

Open Field Activity

Open field activity (OFA) was measured 1-5 days after the EPM test. The open field arena (52.7 cm x 52.7 cm) was placed in a dimly lit room. Mice were placed in the center of the arena and allowed to freely explore for 5 minutes. A video camera above the arena was used to record the animal's path and was connected to a computer for data processing using a video tracking software (LimeLight, Actimetrics, Evanston, IL). The software tracked a midpoint between the animal's shoulders to determine its path traversed and boundary crossings within the arena. Using the video tracking software, the area of the arena was subdivided into a grid of 5 x 5 sections of equal sizes. The "center" of the OFA area is defined as the center 3 x 3 sections in the grid (31.62 cm x 31.62 cm), and the "corners" of the arena are the 4 sections (10.54 cm x 10.54 cm each) abutting the corners. Before testing of each animal, the open field arenas were cleaned with ethanol and allowed to dry.

Novel Object Recognition

For mice in the first 5 cohorts (N = 90), novel object recognition (NOR) was performed on the same day of the OFA to reduce effects of the novel environment. Mice were placed in the center of the arena used for OFA with a small plastic object and allowed to investigate the object for 15 minutes. Animals were returned to the arena the next day with the same object in the same location and the addition of a novel object in a different quadrant of the arena. Before each testing episode, the objects and the arena were cleaned with ethanol and allowed to dry. The same video tracking system as in the OFA was used to determine preferential exploration of the novel object, as a measure of novel object recognition. However, since the mice did not demonstrate robust, reproducible preferences for either object under our test settings, the test was only performed on the first 90 mice and discontinued thereafter, and the data was not included for further analysis.

Fear Conditioning Test

At eight weeks of age, all mice were subjected to the fear conditioning test (FCT), which consisted of a two-day protocol, with training on the first day and testing of contextual and cued responses on the second day. The test was done in a closed and lighted fear conditioning chamber located in a dimly lit room. For the training session, a piece of filter paper treated with lemon oil (5ml) was placed in the chamber to provide a scent cue. The experimenter wore white latex gloves and placed the mouse in the chamber for 9 minutes. Animals are given 3 minutes to acclimate without disturbance, followed by a sequence of repeated (4x) treatment of audio tone cues and foot shocks. Each treatment consisted of a 30-second auditory tone, the last two seconds of which coincides with a 0.6 mA foot shock, followed by 60 seconds of no stimulus. After this training session, animals were returned into their home cages, until the testing session the next day. For the contextual response, animals were subjected to the same environment as in the training session but in absence of the tone and shock, with responses recorded for 4 minutes, before being returned to their home cages. The cued response (i.e., response to the auditory tone) was tested 1-2 hours later. For the cued response, the testing environment was altered as the scent cue is no longer present and the chamber grid floor replaced by a solid plastic floor. Additionally, the environment immediately preceding the testing was also altered by increasing the lighting in the testing room and changing the gloves worn by the experimenter to blue nitrile gloves. Mice were left in the chamber for 3 minutes with no stimulus, and then the tone was played for another 3 minutes. Freezing behavior was quantified using a custom software package (FreezeFrame, Actimetrics, Evanston, IL). All cage mates were tested at the same time in separate chambers, and the testing chambers and removable grid floor were cleaned with ethanol and allowed to dry before training or testing of each animal.

Tail Suspension Test

The tail suspension test (TST) was performed 2-6 days after the FCT. Mice were placed in a closed and lighted chamber and were suspended by the end of their tail for 6 minutes using a binder clip customized to prevent tail damage, and their behavior was monitored and recorded using a video camera mounted behind the chamber and connected to a computer. If a mouse successfully climbed its tail, they were immediately placed back into the hanging position, and animals that repeated this behavior more than twice were excluded from data analysis. The testing chambers and clips were cleaned with ethanol and allowed to dry between animals. The recorded digital video files were later viewed and scored in one-second bins for struggling activities.

Forced Swim Test

Animals in the second half of the cohorts (N = 106) were also subjected to the forced swim test (FST). At 8-9 weeks of age, mice were placed in a beaker (25 cm tall, 15 cm diameter) filled with 16 cm of water (25–26°C) for 6 minutes, while struggling activity was recorded by a video camera placed at the side of the beaker. After the test was completed, animals were removed from the water and allowed to dry in a clean cage placed halfway on a heating pad permitting free choice of temperature. The recorded videos were then viewed and scored by an experienced experimenter for struggling activities (defined by intense movement involving 3-4 limbs) in 1-second bins. A custom auto-scoring software was also used to score the animal's activity level in 1-minute bins (*70*). Cage mates were tested in the same water after removal of any fecal boli, while the water was changed between the testing of animals from different cages.

Sleep-Wake Recordings

Sleep and wake in 121 mice of the latter 9 cohorts were recorded using EEG and EMG, as previously described (*71*). At 10-12 weeks of age and a minimum of five days after the last behavioral test, all mice were surgically implanted with EEG and EMG electrodes for sleep recordings. Surgical procedures were performed using a mouse stereotaxic apparatus (David Kopf Instruments) with standard aseptic techniques in a ventilated, specially-equipped surgical suite. Anesthesia was induced by IP injection of cocktail of Ketamine HCL (98 mg/kg) and Xylazine (10 mg/kg) before surgical implantation of a headmount, which consists of a plastic 4-pin connector (Plastics One, Roanoke, VA) connected to two electroencephalographic (EEG) electrodes and two electromyographic (EMG) electrodes. Two stainless

steel screws (Small Parts, Miami Lakes, FL) serving as the EEG leads were screwed into the skull and placed in the cerebral cortex with one screw located 1 mm anterior to bregma and 2 mm lateral to the central suture, and the other at 1 mm anterior to lambda and 2.5 mm lateral to the central suture. The exposed ends of two stainless steel Teflon-coated wires (0.002 in. in diameter, Medwire, Mt. Vernon, VA) serving as EMG leads were then inserted into the nuchal muscles using a pair of forceps. The headmount was then sealed by dental acrylic (Lang Dental Manufacturing Co., Wheeling, IL), and a single stitch at the front and back of the implant was given using a 5-0 chromic gut (United States Surgical, Norwalk, CT) to close the skin. Subcutaneous injection of analgesic meloxicam (2 mg/kg) was given to the animals immediately after the surgery while the animals were still under anesthesia and once more on the following day.

After the surgery, mice were housed individually and were given a minimum five days of recovery before transferred into a cylindrical sleep recording cages (25.5 cm in diameter) for acclimation for 3 days. The headmount was then connected to the transmission tether. An additional 2 days were allowed for acclimation to the tether connection prior to any data collection. The signals were transmitted to an amplifier which amplifies the EEG signals 10000 times and the EMG signals 5000 times. The signals were pass filtered at 1-30 Hz for EEG and at 30-100 Hz for EMG. Both signals were digitized by an analog-to-digital converter (model DT-01EZ; Data Translation, Marlboro, MA) at 100 Hz/channel, and were transmitted to a computer for storage and analysis using a custom software (Multilevel, Actimetrics, Evanston, IL).

EEG and EMG data were collected from each mouse continuously for 48 hours starting at light onset (ZT0). This recording consisted of 26 hours of undisturbed baseline sleep/wake, 6 hours (ZT2-ZT8) of sleep deprivation administrated via gentle handling, and undisturbed recovery for the remaining 16 hours. The animals were then left undisturbed for 7 days, after which the response to restraint stress was assessed using two separate 24-hour recordings. During the first 24-hour recording (starting at ZT0), mice were subjected to restraint stress for 1 hour starting at ZT5 by being placed in a 35 ml translucent polypropylene tube with a lid (Covidien, Mansfield, MA), which was placed in their home cage. Small holes were made on the tube to permit airflow and a 1 cm x 3.5 cm hole was made on the lid to allow for the sleep recording tether. After 1 hour, mice were removed from the restraint apparatus and allowed for recovery. A minimum of 48 hours after the restraint stress, another 24 hours of recording was done with a sleep deprivation of one hour at the same time of the restraint stress to serve as the handling control.

All recorded EEG and EMG data was then viewed and scored manually as wake, NREM, REM states in 10-second epochs. Scored data were then used to calculate sleep/wake phenotypes using a custom Macro in Microsoft Excel.

Brain dissection procedures

Brain tissue dissection is performed immediately after euthanasia by decapitation. Cuts are performed using forceps, while Petri dishes on a layer of dry ice are used as the dissection surface. Dissected samples are placed in sample tubes and are stored temporarily in dry ice, before being transferred to long-term storage in a -80°C freezer.

Hypothalamus

The extracted brain was placed upside down in a Petri dish. Blood vessels mark the caudal and lateral boundaries, and connective white matter marks the rostral boundary.

Hippocampus

The brain was flipped right side up. The corpus callosum was cut along the centerline, and the left and right cortical lobes were then peeled to the sides to reveal the hippocampus. The hippocampus was rolled caudally and separated from the white matter.

Prefrontal cortex
The cerebral cortex was cut rostral to the striatum.

Thalamus
The thalamus was taken after removal of hypothalamus, hippocampus, and cortex. A cut was made at the caudal boundary to separate from midbrain.