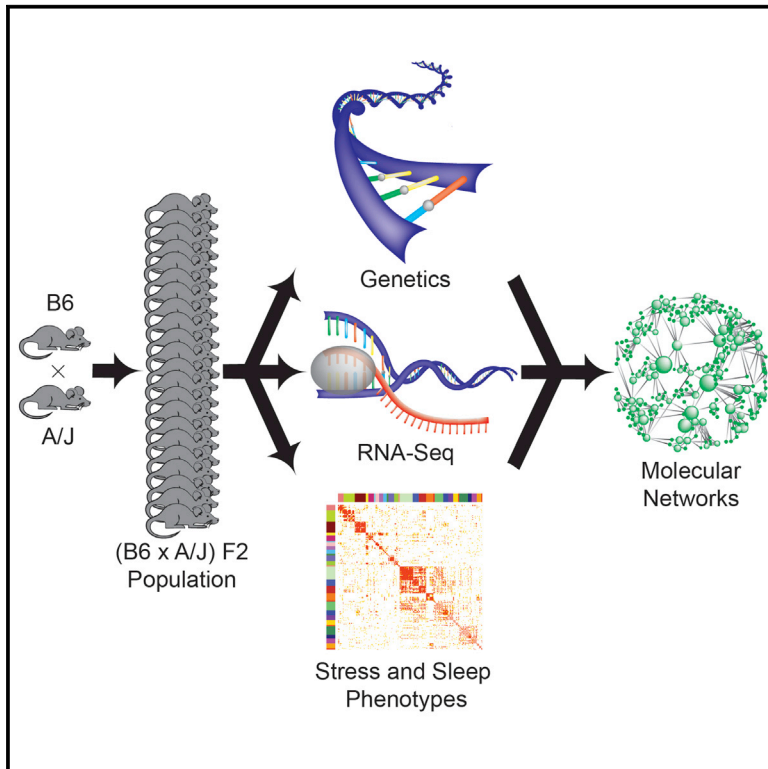# Cell Reports

A Systems Approach Identifies Networks and Genes Linking Sleep and Stress: Implications for Neuropsychiatric Disorders

Graphical Abstract

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In Brief

Jiang et al. utilize a systems approach in a chronically stressed mouse population and identify key genes and networks important for stress and sleep, providing a framework for future investigations of the mechanisms underlying stress, sleep, and related neuropsychiatric disorders.

Highlights

- A large, multiscale dataset to model interactions between stress and sleep in mice

- Reveals a dynamic genetic landscape and striatal gene networks for stress and sleep

- Highlights a mitochondria/synapse network linking stress and sleep

- Key regulators in sleep-stress networks implicated in neuropathology

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A Systems Approach Identifies Networks and Genes Linking Sleep and Stress: Implications for Neuropsychiatric Disorders

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SUMMARY

Sleep dysfunction and stress susceptibility are comorbid complex traits that often precede and predispose patients to a variety of neuropsychiatric diseases. Here, we demonstrate multilevel organizations of genetic landscape, candidate genes, and molecular networks associated with 328 stress and sleep traits in a chronically stressed population of 338 (C57BL/6J × A/J) F2 mice. We constructed striatal gene co-expression networks, revealing functionally and cell-type-specific gene co-regulations important for stress and sleep. Using a composite ranking system, we identified network modules most relevant for 15 independent phenotypic categories, highlighting a mitochondria/synaptic module that links sleep and stress. The key network regulators of this module are overrepresented with genes implicated in neuropsychiatric diseases. Our work suggests that the interplay among sleep, stress, and neuropathology emerges from genetic influences on gene expression and their collective organization through complex molecular networks, providing a framework for interrogating the mechanisms underlying sleep, stress susceptibility, and related neuropsychiatric disorders.

INTRODUCTION

Both acute and chronic stress modulate many aspects of brain function, including cognition, emotion, behavior, and sleep (Lupien et al., 2009). At the same time, stress-susceptible neurobehavioral functions also interact with one another, exerting a complex influence on an organism's responses to stress (Martinez-Gonzalez et al., 2004; Minkel et al., 2012). In humans, stress susceptibility is characteristic of a range of neurological and psychiatric disorders (Lupien et al., 2009), many of which are also comorbid with sleep disturbances (Goldstein and Walker, 2014). In addition, sleep loss during stressful periods exacerbates the risk of neurobehavioral impairment, psychiatric distress, and the development of depression later in life (Breslau et al., 1996; Chang et al., 1997). Despite the breadth of evidence documenting the interactions between stress and sleep, the genetic and molecular mechanisms underlying these interactions remain largely unclear. Both stress responses and sleep regulation are under strong genetic control (Feder et al., 2009; O'Hara et al., 2007), and a number of genes regulating sleep also contribute to stress adaptation and related psychiatric disorders (Chen et al., 2006; Turek, 2007; Yu et al., 2012). Although these findings point toward common molecular mechanisms underlying stress susceptibility and sleep, a comprehensive understanding of the molecular and genetic basis for these overlapping phenotypes remains lacking.

A systems approach is necessary to understand how multiple genetic factors interact in networks and contribute to the emergence of complex traits, including stress and sleep. Previously, such approaches have helped provide insights into both fundamental biological processes (Archer et al., 2014; Millstein et al., 2011; Zhu et al., 2012) and complex diseases (Chen et al., 2008; Emilsson et al., 2008; Wang et al., 2012; Zhang et al., 2013). A comprehensive analysis describing the interactions between stress and sleep has not previously been reported, and there are only a few examples demonstrating molecular mechanisms common to stress and sleep. Here, we address these issues by presenting a large dataset comprising 328 stress- and sleep-related phenotypes measured in a chronically stressed F2 mouse population (n = 338) derived from C57BL/6J (B6) and A/J. To interrogate the possible common genetic factors underlying these stress and sleep phenotypes, we collected genotypes at 781 informative SNP markers throughout the genome. In a randomly selected subpopulation of 100 F2 mice, we performed RNA-Seq gene expression profiling of the striatum, a brain region particularly important for stress adaptation (Ahmad et al.,
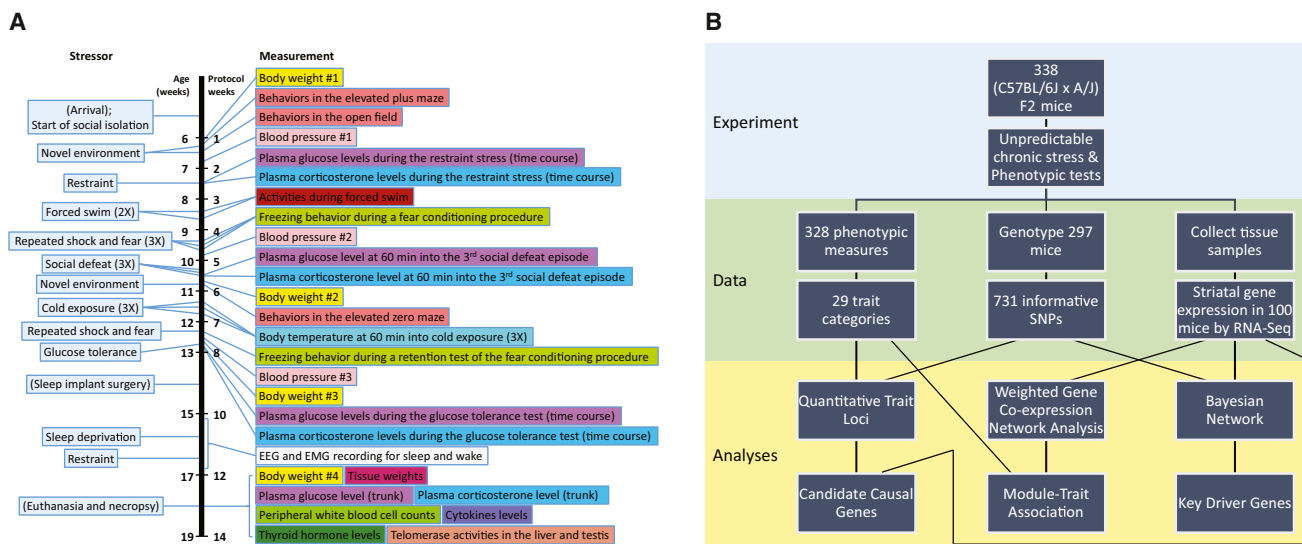
Figure 1. Experimental Design and Analytic Approach

A population of (B6 × A/J) F2 mice underwent a chronic unpredictable stress protocol, data collected during which enables modeling of genetic and molecular networks underlying responses to these stresses.

(A) The sequence of chronic unpredictable stress treatments and phenotypic data collection (see Supplemental Experimental Procedures for details).

(B) Schematic diagram of the molecular and physiological data collection and integrative analysis.

2010; Rossi et al., 2009), sleep-wake regulation (Earley et al., 2013; Kim et al., 2010; Lazarus et al., 2012; Qiu et al., 2010; Stoffers et al., 2013), and neuropsychiatric diseases (Shepherd, 2013; Tritsch and Sabatini, 2012; van den Heuvel et al., 2010). Given its important roles in sleep, stress, and disease, the striatum is ideal for investigating common molecular networks underlying sleep and stress traits. With extensive genotypic, molecular, and phenotypic assays, we utilize an integrative, multiscale systems approach to characterize the genetic landscape, candidate causal genes, and gene transcriptional networks shared by stress and sleep traits. We also report that genes implicated in neuropsychiatric disorders are overrepresented in key regulators of stress-sleep gene networks, providing a potential molecular basis for the comorbidity of stress, sleep, and neuropathologies. Our systems analysis provides a framework for identifying and prioritizing pathways and therapeutic mechanisms associated with abnormal stress responses and altered sleep and offers biological insights into the roles of stress and sleep in neuropsychiatric pathophysiology.
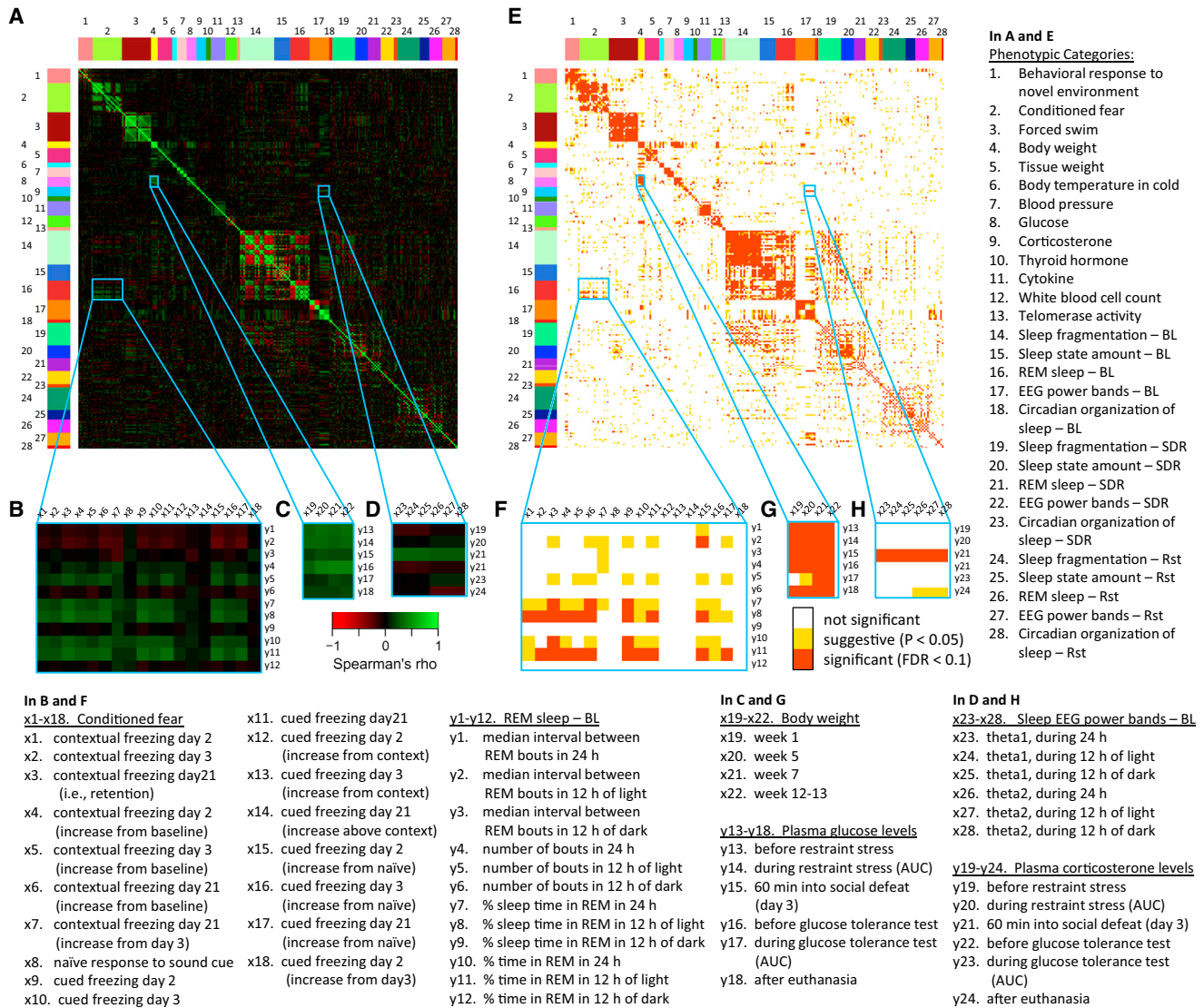
RESULTS

A (B6 × A/J) F2 Mouse Population Models Complex Interactions between Stress and Sleep

A genetically segregating population of 338 (B6 × A/J) F2 male mice was subjected to a chronic, unpredictable stress schedule (Figure 1), during which we measured multiple stress-related behavioral and physiological phenotypes. Mice were then surgically implanted with electroencephalography (EEG) and electromyography (EMG) electrodes to record sleep/wake states. Upon euthanasia, serological parameters and tissue/organ weights were also obtained. We selected 328 phenotypic measurements with large variance in this population for analysis (Figure S1), and we grouped these phenotypes into 29 broad categories (full list in Table S1).

We identified correlations between phenotypes using Spearman's rho and assessed false discovery rates (FDRs) using the Benjamini-Hochberg procedure (Figure 2; Table S2). We observed associations between traits within the same phenotypic categories, as well as across different categories. At FDR < 0.10, we identified 3,491 pairs of significantly correlated phenotypes. As expected, strong correlations between phenotypes within the same category are prevalent, confirming the overall quality of the phenotypic measurements and categorical groupings. In addition, many known interactions between distinct aspects of stress and sleep biology were observed. This includes well-known physiological relationships, such as body weight measurements and plasma glucose levels (Figures 2C and 2G), as well as previously reported associations between sleep and stress traits, such as conditioned fear and REM sleep (Figures 2B and 2F) (Menz et al., 2013; Polta et al., 2013). Other phenotypic relationships were also observed. For instance, corticosterone levels at 60 min into the third (i.e., last) exposure of a 3-day social defeat procedure were specifically correlated with EEG theta I (4–8 Hz) and theta II (8–11 Hz) power densities in REM sleep measured across multiple conditions (Figures 2D and 2H). This comprehensive phenotypic dataset models interactions between stress and sleep, providing an opportunity to study genetic and molecular mechanisms underlying stress, sleep, and their interactions. Phenotypic correlation data are available in its entirety to facilitate future study of additional phenotypic relationships (Table S2).

In A and E

Phenotypic Categories:
1. Behavioral response to novel environment
2. Conditioned fear
3. Forced swim
4. Body weight
5. Tissue weight
6. Body temperature in cold
7. Blood pressure
8. Glucose
9. Corticosterone
10. Thyroid hormone
11. Cytokine
12. White blood cell count
13. Telomerase activity
14. Sleep fragmentation – BL
15. Sleep state amount – BL
16. REM sleep – BL
17. EEG power bands – BL
18. Circadian organization of sleep – BL
19. Sleep fragmentation – SDR
20. Sleep state amount – SDR
21. REM sleep – SDR
22. EEG power bands – SDR
23. Circadian organization of sleep – SDR
24. Sleep fragmentation – Rst
25. Sleep state amount – Rst
26. REM sleep – Rst
27. EEG power bands – Rst
28. Circadian organization of sleep – Rst

Spearman's rho: −1 0 1

not significant
suggestive (P < 0.05)
significant (FDR < 0.1)

In B and F

x1–x18. Conditioned fear
x1. contextual freezing day 2
x2. contextual freezing day 3
x3. contextual freezing day21 (i.e., retention)
x4. contextual freezing day 2 (increase from baseline)
x5. contextual freezing day 3 (increase from baseline)
x6. contextual freezing day 21 (increase from baseline)
x7. contextual freezing day 21 (increase from day 3)
x8. naïve response to sound cue
x9. cued freezing day 2
x10. cued freezing day 3
x11. cued freezing day21
x12. cued freezing day 2 (increase from context)
x13. cued freezing day 3 (increase from context)
x14. cued freezing day 21 (increase above context)
x15. cued freezing day 2 (increase from naïve)
x16. cued freezing day 3 (increase from naïve)
x17. cued freezing day 21 (increase from naïve)
x18. cued freezing day 2 (increase from day3)

y1–y12. REM sleep – BL
y1. median interval between REM bouts in 24 h
y2. median interval between REM bouts in 12 h of light
y3. median interval between REM bouts in 12 h of dark
y4. number of bouts in 24 h
y5. number of bouts in 12 h of light
y6. number of bouts in 12 h of dark
y7. % sleep time in REM in 24 h
y8. % sleep time in REM in 12 h of light
y9. % sleep time in REM in 12 h of dark
y10. % time in REM in 24 h
y11. % time in REM in 12 h of light
y12. % time in REM in 12 h of dark

In C and G

x19–x22. Body weight
x19. week 1
x20. week 5
x21. week 7
x22. week 12-13

y13–y18. Plasma glucose levels
y13. before restraint stress
y14. during restraint stress (AUC)
y15. 60 min into social defeat (day 3)
y16. before glucose tolerance test
y17. during glucose tolerance test (AUC)
y18. after euthanasia

In D and H

x23–x28. Sleep EEG power bands – BL
x23. theta1, during 24 h
x24. theta1, during 12 h of light
x25. theta1, during 12 h of dark
x26. theta2, during 24 h
x27. theta2, during 12 h of light
x28. theta2, during 12 h of dark

y19–y24. Plasma corticosterone levels
y19. before restraint stress
y20. during restraint stress (AUC)
y21. 60 min into social defeat (day 3)
y22. before glucose tolerance test
y23. during glucose tolerance test (AUC)
y24. after euthanasia

Figure 2. Phenotypic Interactions Observed in the Chronically Stressed (B6 × A/J) F2 Mice

(A–D) Correlation coefficients between pairs of phenotypes. The phenotypes were ordered according to their phenotypic categories.
(E–H) Statistical significance of observed associations. The phenotypes were ordered exactly the same as in (A–D).
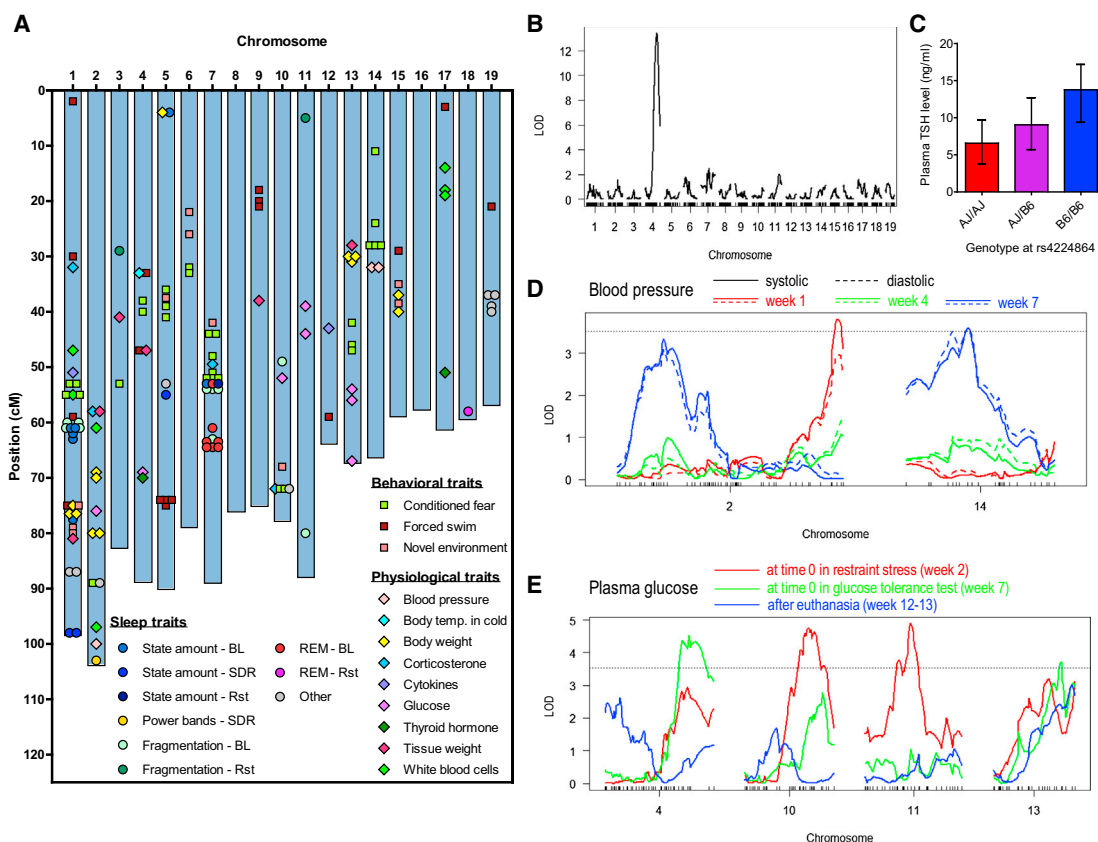BL, baseline; SDR, sleep deprivation recovery; Rst, after restraint stress; AUC, area under the curve. Note that 93 sleep measurements that were not grouped into any of the sleep categories are not presented here, but are included in Table S2.

A Stress-Modulated Dynamic Genetic Landscape Reveals Linked Genetic Control of Stress and Sleep Phenotypes

To reveal the genetic landscape of stress and sleep biology, we mapped quantitative trait loci (QTL) that regulate stress and sleep phenotypes. The dataset was permuted 1,000 times to estimate the FDR. At a permissive FDR < 0.2 (LOD >3.53), we uncovered 143 QTL for the set of 328 stress and sleep phenotypes (Figure 3A; Table S3). We confirmed a number of QTL that were previously identified using genetically diverse mouse populations derived from B6 and A/J. These include a chromosome 1 (Chr.1) QTL (peaking at 70–80 cM) influencing open field activities (Gershenfeld and Paul, 1997), a Chr.1 QTL (peaking at

∼75 cM) for body weight (Zhang and Gershenfeld, 2003), and a Chr.7 QTL (peaking at ∼50 cM) co-localized with the *albino* (*Tyr*) locus influencing conditioned fear (Ponder et al., 2008).

Interestingly, we identified a number of QTL that were not previously detected when the same phenotypes were studied using unstressed B6 × A/J populations. Notably, the most significant QTL (LOD = 13.4) identified in our chronically stressed mice is located at 69.58 cM on Chr.4, which strongly influenced the plasma thyroid-stimulating hormone (TSH) levels measured at euthanasia (Figure 3B). Median TSH levels in mice with the homozygous B6 genotype at the QTL were twice as high as in mice with the homozygous A/J genotype (Figure 3C). This QTL was not detected in a recent study of unstressed B6 × A/J

Figure 3. Identification of QTL that Influence Stress and Sleep Phenotypes

(A) Genetic landscape of stress and sleep. Genomic locations of the 142 identified QTL (FDR < 0.2) are shown (also see Table S3). BL, baseline; SDR, sleep deprivation recovery; Rst, after restraint stress.

(B) A highly significant QTL for plasma TSH level on Chr.4. LOD, log of odds.

(C) Median ± interquartile range of plasma TSH level as a function of genotype at rs4224864, the most strongly associated SNP in the Chr.4 QTL region.

(D) Distinct QTL were linked to blood pressure measured at different times of the chronic stress protocol.

(E) Baseline plasma glucose levels were linked to QTL with consistent effect throughout the chronic stress treatment as well as QTL specific to different stages of the protocol. Note that while a 6-hr fasting procedure preceded the glucose tolerance test and the glucose measurement at time 0 (i.e., week 7), it did not appear to have a significant effect on the genetic control of glucose, as it did not result in presence or absence of a QTL specific to the baseline glucose measurement at week 7. The most distinct genetic regulations of baseline glucose levels were observed between week 2 (i.e., most naive) and weeks 12 and 13 (i.e., most experienced).

recombinant inbred mice (McLachlan et al., 2014). As prior exposures to stress are known to modulate the activity of the hypothalamic-pituitary-thyroid axis and the TSH profile (Armario et al., 1993), our results may suggest that TSH levels are regulated by an interaction between the Chr.4 QTL and chronic stress exposure.

To demonstrate further how prior stress exposure may modulate the genetic control of physiological parameters, we investigated repeated measures of phenotypes across the chronic stress protocol. Blood pressure was measured at weeks 1, 4, and 7 (Figure 1A). Significant phenotypic correlations were observed between week 1 and week 4 measurements as well as between week 4 and week 7 measurements, but not between week 1 and week 7 measurements (Table S2). Interestingly, this observation is accompanied by the involvement of distinct QTL (Figure 3D). At week 1, blood pressure was influenced by a QTL located on the distal portion of Chr.2, while a significant

QTL on Chr.14 and a suggestive QTL on the proximal portion of Chr.2 were identified at week 7. No significant QTL at FDR < 0.2 was found at week 4. We noticed a similar phenomenon for baseline plasma glucose levels in the absence of acute stress (Figure 3E). These data suggest that even basic physiological parameters are regulated by complex genetic architecture and that such regulation is highly susceptible to prior stress experiences.

The genetic landscape of stress and sleep revealed a number of co-localized QTL influencing distinct phenotypic categories (Figure 3A). Among the 143 significant QTL, 83 were mapped to a locus less than 10 cM from a QTL for a phenotype of a different category. For example, a cluster on Chr.1 includes QTL that influence open field activities, body weight, a baseline NREM trait, and a forced swim test measure (Figure 3A; Table S3). Furthermore, QTL for conditioned fear on Chr.7 co-localize not only with the *albino* locus, but also with loci influencing open field activities, corticosterone levels during restraint stress,
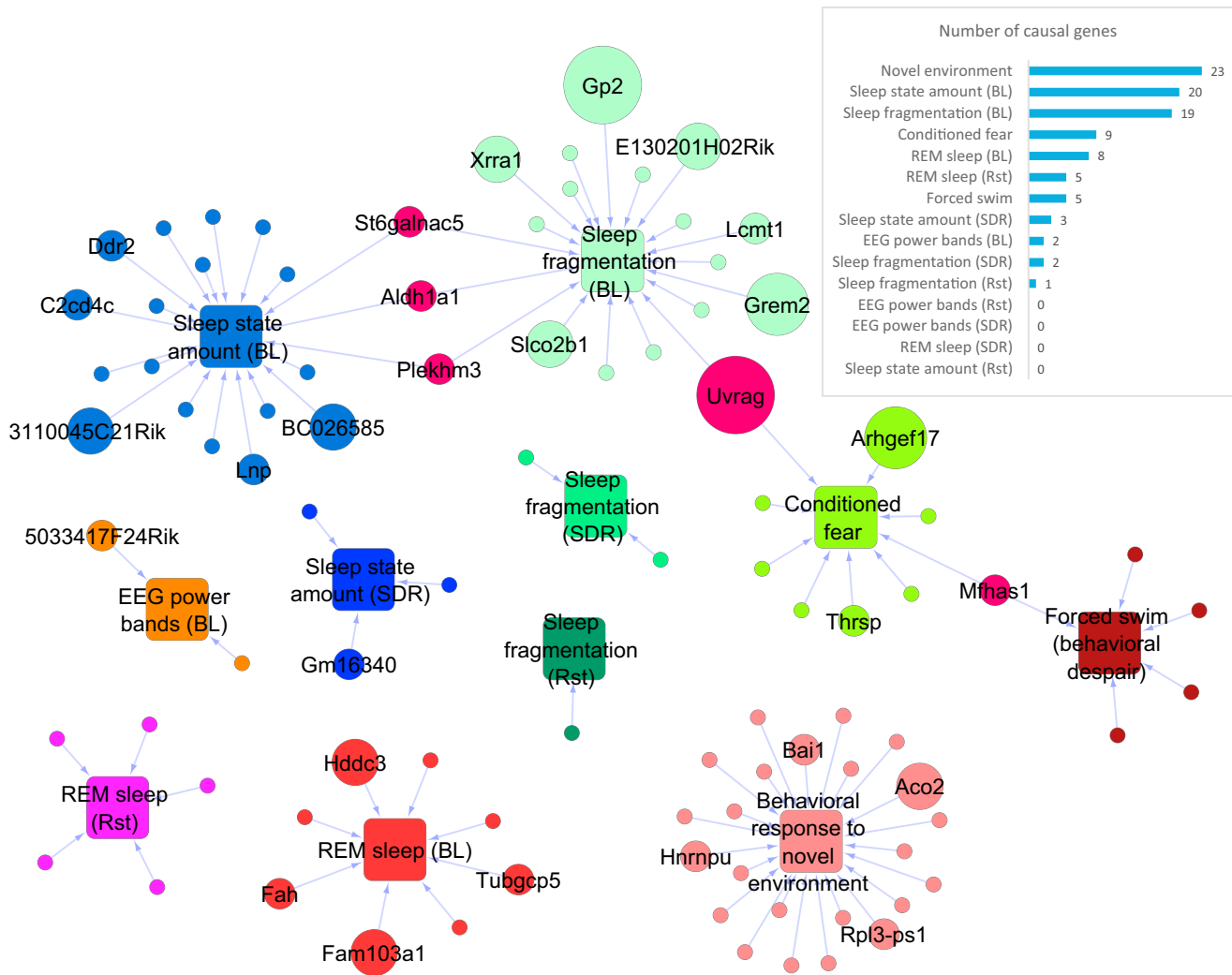
Figure 4. A Network View of Striatal Genes Causal to Selected Behavioral and Sleep Phenotypes

Circles represent genes and squares represent phenotypic categories. The circle is sized in proportion to the number of phenotypes for which the gene tests causal. Circles and squares are colored according to phenotype categories, with colors assigned to genes from the category of the phenotypes for which the genes test causal. The five pink circles represent genes causal to phenotypes of multiple distinct categories. (Insert) The number of genes found causal to at least one phenotype in each category. BL, baseline; SDR, sleep deprivation recovery; Rst, after restraint stress. See Table S4 for the full list.

and sleep parameters both under baseline conditions and after restraint stress. In summary, our analysis characterizes a rich genetic landscape and identifies many co-localized QTL that may underlie the interactions between stress and sleep and their effects on other behavioral and physiological parameters.

Identification of Candidate Causal Genes Underlying Stress and Sleep QTL

Limited by the number of meiotic recombinations, a QTL identified using ~300 F2 mice typically spans a ~20-cM genomic region, harboring hundreds of genes (Li et al., 2005). To identify candidate genes underlying the sleep and stress QTL, we integrated the QTL data with RNA-seq expression data of 26,927 striatal genes and associated these molecular data to the 15 phenotype categories (Figure 4) that are known to be influenced

by the striatum. We focused on phenotypic QTL that overlapped with loci regulating gene expression (i.e., eQTL; see Supplemental Experimental Procedures) and utilized conditional independence models to determine whether expression variation of the gene mediates the QTL effect and causes phenotypic variations (Millstein et al., 2009; Schadt et al., 2005). This causality test has successfully identified causal genes for complex sleep traits (Millstein et al., 2011) that were later pharmacologically validated (Brunner et al., 2011).

In this analysis, we identified 92 causal genes at p < 0.05 (Table S4), several of which have already been well supported. For example, somatostatin signaling is known to modulate anxiety-like behaviors and adaptive responses to stress (Stengel et al., 2013), and we identified *Sstr3* (somatostatin receptor 3) as a causal gene for the number of entries into the center of open field

arena. Our analysis also reveals many other causal candidates, some of which are causal for several traits. For example, *Arhgef17* is one of 28 genes that are relevant to multiple phenotypes (Figure 4), testing causal for four conditioned fear measures. *Arhgef17* encodes a RhoGEF, which regulates Rho GTPase and downstream kinases, a signaling pathway known to affect conditioned fear memory (Lamprecht et al., 2002). Taken together, the causality test revealed a large number of high-profile candidate genes linking genetic variability to functional consequences in sleep and stress-related affective behaviors.

In addition, five genes are causal for phenotypes from multiple categories (Figure 4). For instance, *Aldh1a1* was found causal for baseline sleep fragmentation and state amount measures. *Aldh1a1* encodes an aldehyde dehydrogenase important for the synthesis of retinoic acid (Fan et al., 2003), a molecule involved in sleep/wake regulation (Kitaoka et al., 2011). Interestingly, the causality test identified only one gene, *Uvrag* (UV radiation resistance associated gene), that directly links sleep and stress-related behavioral phenotypes. *Uvrag* tested causal for three conditioned fear traits and two baseline sleep fragmentation phenotypes in our experiment. Though its role in the central nervous system remains unclear, it responds to various cellular stresses, maintains chromosomal stability, and promotes autophagy (Liang et al., 2006; Zhao et al., 2012). While our causality analysis uncovered causal genes relevant to particular traits, it identified few pleotropic causal genes. This suggested that analysis on the level of gene networks rather than individual genes was needed to sufficiently capture the molecular relationship between concomitant stress and sleep phenotypes.

Network Organization of Striatal Gene Expression Exhibits Functional and Cell-type Specificity

We constructed co-expression networks for the striatum of the (B6 × A/J) F2 mice and identified 62 independent transcriptional modules (Figure 5A), each named with an arbitrarily assigned color. Twenty-eight modules are enriched for genes in specific biological pathways and gene ontology (GO) functional groups (Table S5). Transcriptional co-expression of genes suggest similar regulatory control (Zhang and Horvath, 2005), and we identified many co-regulatory relationships between known genes of interest and other gene groups. For example, the Indianred4 module includes four genes commonly associated with the molecular circadian pathway, *Csnk1e*, *Arntl*, *Cry1*, and *Hdac3*. Interestingly, this module is functionally enriched for chaperone (p = 3.36 × 10^{-5}, 3.89x) and stress response (p = 3.6 × 10^{-5}, 7.32x) GO categories and includes a number of heat shock proteins traditionally implicated in disease pathways. These relationships suggest that seemingly diverse molecular pathways may be under similar regulatory control in the striatum. Using cell-type-specific gene signatures from the Allen Brain Atlas (Lein et al., 2007), we also identified several cell-type-specific modules, including an oligodendrocyte-enriched Darkolivegreen module (p = 2.8 × 10^{-64}, 54.3x) and a neuron-enriched Turquoise module (p = 8.1 × 10^{-4}, 2.24x). Previous studies have described the functional and pathophysiological importance of cell-type specific modules in the human brain (Oldham et al., 2008; Zhang et al., 2013), so we considered these modules particularly interesting candidates for downstream analysis.
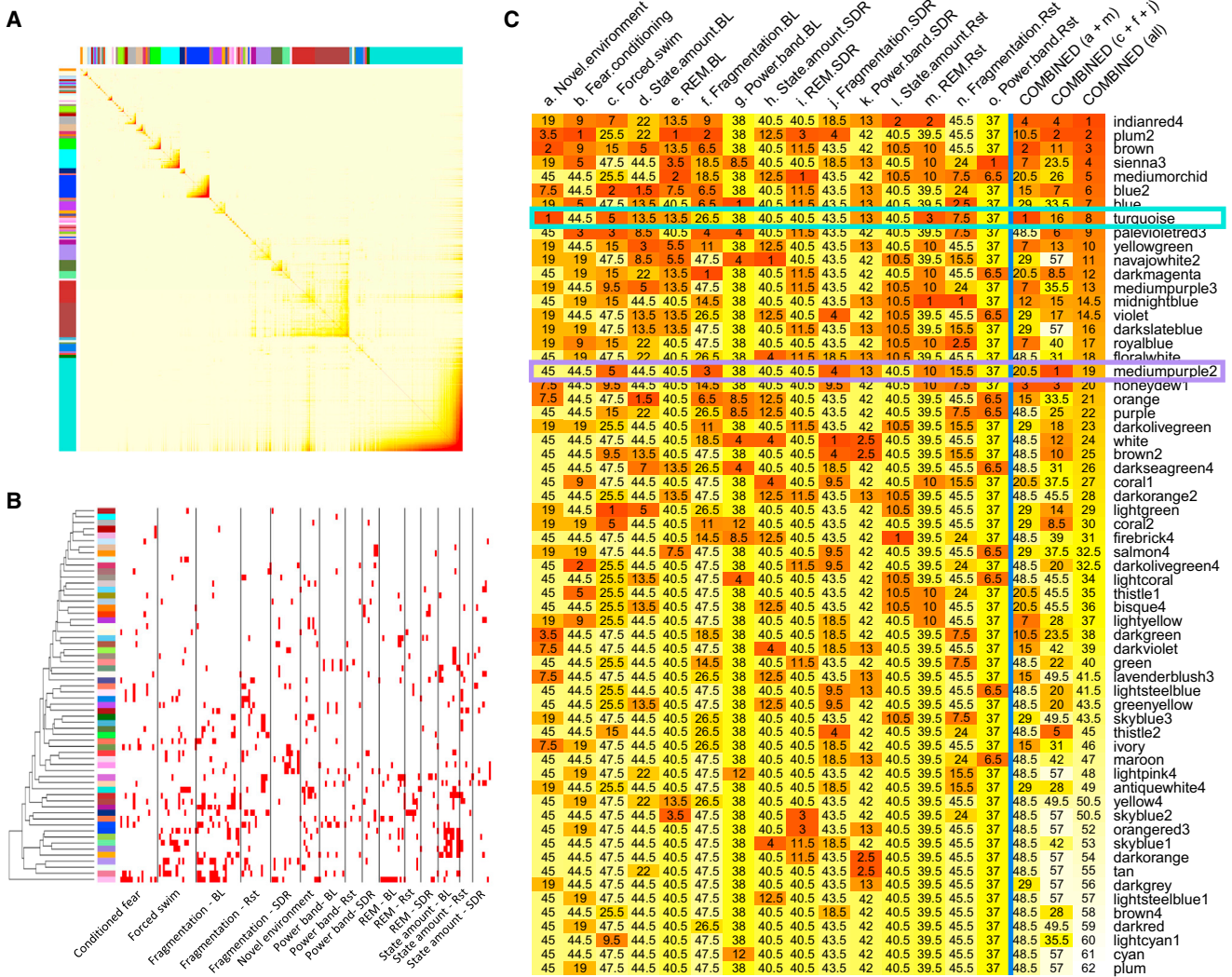
Striatal Gene Co-expression Modules Link Distinct Aspects of Sleep and Stress Biology

Genetic variability and its effect on gene expression contribute to both module organization and phenotypic segregation in our chronically stressed mice. Our causality test results suggest that it is difficult to link complex phenotypes through single pleiotropic genes and that a network-based approach may help better understand common molecular bases linking complex phenotypes. By correlating modules with phenotypes, we identified specific traits most relevant to striatal co-expression modules. Previous efforts have focused on relating modules to a single class of traits (Zhang et al., 2013), but our extensive phenotyping assays enabled us to identify module-phenotype associations in 15 independent trait categories (Figure 5B). We ranked module relevance for each phenotype category and identified modules shared by multiple phenotypic classes (see Supplemental Experimental Procedures and Table S5).

Module ranking across phenotypic categories reveals numerous relationships between distinct aspects of stress responses and sleep. For example, the Turquoise module ranks first for novel environment behavioral responses and third for REM traits after restraint stress, giving it the highest composite ranking for these traits (Figure 5C). Interestingly, it is highly enriched with causal genes for behavioral responses to novel environment (15 of the 23 causal genes; p = 2.9 × 10^{-7}, 3.9x). This result complements the module ranking by providing strong gene-level evidence for its relationship to novel environment stress. The Turquoise module is also enriched with genes in the mitochondrial membrane (p = 1.23 × 10^{-12}, 1.89x) and synaptic (e.g., a variety of neurotransmitter receptors; p = 1.01 × 10^{-9}, 1.85x) GO categories, which is consistent with its neuron-specific gene signature. A growing body of evidence suggests that mitochondria modulate synaptic plasticity, contribute to many CNS diseases, and can serve as an important therapeutic target (Manji et al., 2012). Our results support the hypothesis that mitochondria and synaptic mechanisms are highly integrative and suggest that this relationship fundamentally exists at the level of transcriptional co-expression, at least in the striatum. Because mitochondrial and synaptic impairment is characteristic of CNS diseases, it is particularly interesting that the Turquoise module is most relevant to both behavioral responses to novel environment and REM sleep after acute restraint stress. Specifically, increased module expression correlates with increased anxiety measures, as well as increased REM bouts and decreased median inter-REM interval in the first half of the dark period after restraint stress (Table S5). Transcriptional co-regulation of mitochondrial and synaptic genes may provide a mechanistic basis for the comorbidity of anxiety, stress-related REM sleep disruptions, and neuropathology.

Bayesian Network Reconstruction Identifies Key Driver Genes Linking Sleep and Stress

We used Bayesian network reconstruction to calculate causal probabilistic relationships between genes and identify the causal regulators (i.e., key drivers) of transcriptional networks linking stress and sleep traits. We utilized *cis*-eQTL (FDR < 0.1) as causal anchors in our directed probabilistic network and reconstructed a single consensus Bayesian network from 18,460

Figure 5. Identification of Gene Co-expression Modules Relevant to Selected Behavioral and Sleep Phenotypes

(A) The topological overlap matrix (TOM) plot corresponds to the striatal gene co-expression network. Darker color indicate stronger co-regulation between a pair of genes (in rows and columns). Gene modules are identified by hierarchical clustering of the matrix, as labeled by arbitrarily assigned color bars on the top and at the left.

(B) Identification of modules (rows) significantly associated with selected phenotypes (columns) organized in categories. Gene modules are indicated by their assigned color at the left. Red bars indicate significant associations (p < 0.05 and FDR < 0.05).

(C) Ranking of modules (rows) based on relevance to individual phenotypic categories and combined categories of interest (columns). Module rankings for a phenotypic category were determined by the number of significant module-trait associations within the phenotypic category. For a combination of multiple phenotypic categories, rankings for each category were summed to determine a composite ranking for each module. Darker color indicate higher ranking. The actual rankings are also labeled.

BL, baseline sleep; SDR, sleep deprivation recovery; Rst, sleep after restraint stress.

genes with the greatest variance across the mouse population (Supplemental Experimental Procedures). We intersected the full Bayesian network with the Turquoise module and identified the key driver genes that primarily control the expression of the module. Our agnostic approach identified many key drivers that have been supported by other experiments. For instance, we identified *Slc17a7*, *Fmr1*, and *Grm5* as striatal key drivers, which have previously been implicated in regulating anxiety-related behavior (Moy et al., 2009; Tordera et al., 2007; Varty

et al., 2005). We also identified *Syngr1* as a key driver, which is differentially regulated in an animal model of depression (Kroes et al., 2006) and has been genetically linked to panic disorder in humans (Hamilton et al., 2003). Furthermore, another key driver of the module, *Pde10a* (phosphodiesterase 10A), was reported as a candidate genes for conduct disorder (Dick et al., 2011) and has been tested as an antipsychotic target in animal models (Smith et al., 2013). Our results offer tissue-specific resolution, suggesting that the transcriptional actions of these key
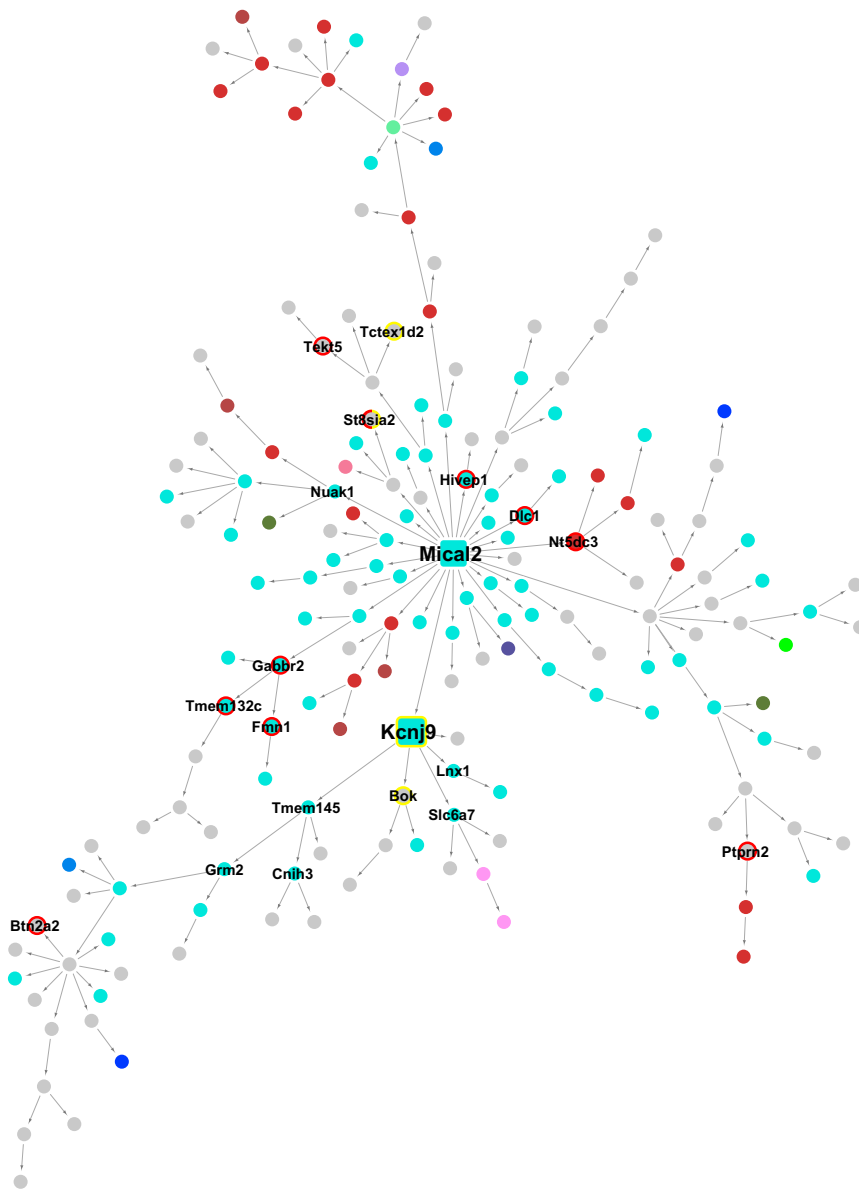
Figure 6. Striatal Bayesian Networks Downstream of *Mical2*

Each node represents a gene and each directed edge indicates a causal link between genes. Nodes are colored according to their module assignments, using the names of the respective modules. Key driver genes are represented by larger square nodes. Nodes with red rims denote homologs of human GWAS candidates for neuropsychiatric disorders, and nodes with yellow rims denote candidate genes identified in this study as causal to stress and sleep phenotypes. One node is labeled with a half red and half yellow rim, as the represented gene (*St8sia2*) is a both reported GWAS candidate for bipolar disorder and tested causal to a REM sleep phenotype in this study.

downstream of another key driver gene, *Mical2* (a microtubule associated monooxygenase), in its transcriptional subnetwork (Figure 6). Molecularly, MICALs link cytoskeletal dynamics, synaptic structure, vesicle trafficking, and redox signaling (Zhou et al., 2011). They also bind CasL, alleles of which are associated with neurological disease (Li et al., 2008). By regulating the expression of *Kcnj9*, *Mical2* drives a metabotropic glutamate receptor (*Grm2*), which has been implicated in anxiety (Galici et al., 2006). *Mical2* is also upstream of a GABA$_B$ receptor (*Gabbr2*), whose physiological properties are intimately linked with those of GIRK receptors (Lüscher and Slesinger, 2010). Furthermore, *Mical2* is immediately upstream of several mitochondrial genes, including *Dlc1*, which is responsible for Bcl-2-activated mitochondrial-mediated apoptosis, and *Nuak1*, which controls synaptic plasticity and axon branching dependent on mitochondria mobilization (Courchet et al., 2013; Sun et al., 2013). Taken together, this subnetwork suggests that *Mical2* may serve as a striatal regulator of synaptic and mitochondrial pathways and contribute to mechanisms fundamental to sleep, stress, and neuropathology.

Key Driver Nodes of Stress and Sleep Subnetworks Are Implicated in Neuropsychiatric Diseases

Since the Turquoise module is strongly associated with anxiety-related traits in mice, we investigated whether its key drivers have been previously implicated in neuropsychiatric disease in humans. We queried the National Human Genome Research Institute Catalog of Published Genome-Wide Association Studies (GWAS) (Welter et al., 2014) for candidate genes associated with neuropsychiatric disorders (Table S5). Interestingly, mouse homologs of these GWAS genes are overrepresented in

drivers in the striatum may contribute to the emergence of these anxiety-related traits in animal models and relevant psychiatric disorders in humans.

Since the Turquoise module is enriched with causal genes for anxiety-like behaviors, we investigated the organization of these causal genes in the subnetwork and found that two causal candidates in the Turquoise module, *Cadm2* and *Kcnj9*, are also key drivers. *Kcnj9* encodes GIRK3, a subunit of G-protein-dependent inwardly rectifying K$^+$ channels. GIRK channels have been implicated in a variety of diseases, including anxiety (Pravetoni and Wickman, 2008) and addiction (Morgan et al., 2003). GIRK3 in particular is thought to modulate the availability of all GIRK channels on the plasma membrane through lysosomal trafficking and thus may be key to GIRK-related disease mechanisms (Lüscher and Slesinger, 2010). Interestingly, GIRK3 (*Kcnj9*) is immediately

Figure 7. Striatal Bayesian Networks Downstream of *Htt and Bsn*
Each node represents a gene and each directed edge indicates a causal link between genes. Nodes are colored according to their module assignments, using the names of the respective modules. Key driver genes are represented by larger square nodes.

the Turquoise module (p = 2.98 × 10^{-5}, 1.3x) and are more likely to overlap with the key drivers of the module than with downstream module genes (p = 0.02, 1.8x). Since GWAS data lack mechanistic information, our results contextualize human gene-phenotype associations within a causal probabilistic model with tissue-specific resolution. Interestingly, key drivers of the Turquoise module are not exclusively related to neuropsychiatric diseases. Its key drivers are also overrepresented for genes involved in Parkinson's disease (p = 0.01, 6.6x) and Huntington's disease (p = 0.069, 4.8x) according to the KEGG (Kyoto Encyclopedia of Genes and Genomes) database. Taken together, these results suggest that genes associated with neurological and psychiatric diseases are important regulators of subnetworks linking stress and sleep traits.

The Mediumpurple2 Module Reveals Links between Stress, Sleep, and Neurodegeneration

Links between neurodegeneration and key drivers of stress/sleep gene networks are not limited to the Turquoise module.

The Mediumpurple2 module also links neurodegeneration to stress and sleep. It has the highest composite ranking for forced swim measures (fifth), sleep fragmentation traits at baseline (third), and after sleep deprivation (fourth) (Figure 5C). This module is enriched for the chromatin-modification GO category (p = 9.78 × 10^{-7}, 5.7x), suggesting transcriptional control of chromatin-modifying genes may link depressive behaviors with sleep fragmentation. While the Mediumpurple2 module ranks highly for sleep fragmentation measures at baseline and after sleep deprivation, it ranks poorly for sleep fragmentation after restraint stress (15th). Such stressor-specific module-trait relationships are also observed in other modules, including the White module, which is specifically associated with sleep fragmentation after sleep deprivation, and the Midnightblue module, which is specifically associated with sleep fragmentation after the restraint stress. It is well known that different stressors correlate with different physiological sleep changes (Suchecki et al., 2012), and these stressor-specific sleep modules, which include Mediumpurple2, suggest that this phenomenon is also apparent on the gene network level.

In addition to its stressor-specific relationships to sleep phenotypes, the Mediumpulple2 module is also linked with neurodegeneration. Key driver genes of the Mediumpurple2 subnetwork include *Htt*, whose polyQ expansion causes Huntington's disease. Other Mediumpurple2 key drivers such as *Bsn*, *Mll1*, and *Celsr3* have also been associated with ataxia, epilepsy, and neurodegeneration (Altrock et al., 2003; Lim et al., 2009; Tissir et al., 2005). The *Htt* and *Bsn* subnetworks converge to drive the expression of the gene encoding heavy-chain of cytoplasmic dynein, *Dync1h1* (Figure 7), whose mutations have been linked to Huntington's disease-like striatal atrophy and metabolic defects (Braunstein et al., 2010; Eschbach et al., 2011). We found that 37% of the key drivers in this module are genes that cause motor abnormalities when disrupted, as cataloged by the Mouse Genome Database (Blake et al., 2014). This network enrichment (p = 8.4 × 10^{-4}, 6.56x) suggests that genes classically associated with neurological disease drive subnetworks shared by stress and sleep traits. Patients of neurodegenerative diseases concomitantly suffer from psychiatric and sleep disorders (Morton, 2013; Sauerbier and Ray Chaudhuri, 2014), which often precede the disease onset (Postuma et al., 2012; Shirbin et al., 2013). While links between stress and neurodegeneration have been hypothesized (Kibel and Drenjancević-Perić, 2008), studies on their connections at the genetic and molecular level are limited. Our results thus suggests that biological mechanisms linking stress, sleep, and neurodegeneration may reside fundamentally in the network organization of striatal gene expression.

DISCUSSION

Sleep-wake and stress traits are controlled by complex genetic architectures (Feder et al., 2009; O'Hara et al., 2007). Here, we combined a multiscale systems approach with extensive phenotyping to investigate how genetic variation and transcriptional networks contribute to the emergence of multiple sleep and stress phenotypes. We uncovered candidate genes underlying the associations between genetic and phenotypic variations

using a causality test, but noted that this gene-level approach was limited. Consequently, we demonstrated that network-level analysis better captures the effect of numerous loci on the organization of transcriptional networks and the emergence of complex interacting phenotypes. Since stress maladaptation and sleep disturbance may precede and predispose patients to neuropsychiatric disease, understanding their molecular intersections is critical for developing a more sophisticated and nuanced conception of disease mechanism, progression, and therapeutic intervention (Goldstein and Walker, 2014). Our analysis indeed revealed that key drivers regulating sleep- and stress-related transcriptional networks are functionally important and significantly overlap with genetics associated with human neuropsychiatric diseases. This result not only contextualizes GWAS findings, but also suggests that the seemingly discrete GWAS genes can be functionally linked via gene regulatory networks important for stress and sleep. Furthermore, the broad overlaps between neuropsychiatric GWAS candidates and key drivers of stress/sleep gene networks support the concept that gene network structure can be used to predict functional consequences produced by molecular perturbations.

We have highlighted several module-trait relationships that link sleep, stress, and neuropsychiatric disease. In this article, we highlight the co-expression of mitochondrial and synaptic genes and their relationship to anxiety-related behaviors and REM sleep traits after restraint stress. Since both sleep and stress disorders are common in many psychiatric diseases, these molecular networks can provide insights into the onset and maintenance of neuropsychopathology. We also provide a full catalog of all module-traits relationships (Table S5) as a resource that can facilitate in silico hypothesis testing and in vivo validation of potential molecular mechanisms and therapeutic candidates relevant for chronic stress, sleep, and neuropathology.

Impaired mitochondrial and synaptic functions, similar to sleep dysfunction and stress susceptibility, are commonly linked with many neurological and psychiatric diseases. However, studies of the interaction between mitochondrial and synaptic pathways and its role in disease have produced conflicting and sometimes tenuous evidence, which speaks to the complexity of the biology and its consequent pathophysiology. Studying individual genes and pathways insufficiently explains this complex relationship, so a systems approach is ideal for identifying the basis of this interaction (Manji et al., 2012). In the present study, the Bayesian network reconstruction revealed many key driver genes that regulate both mitochondrial and synaptic pathways and may serve as potential therapeutic targets for human neuropsychiatric disorders. *Mical2* is one particularly interesting example since it links multiple pathways related to CNS diseases. Although the molecular function of *Mical2* in mammals has not been extensively characterized, several independent lines of evidence support the role of *Mical2* in mediating anxiety behaviors and neuropathology. The expression of *Mical2* is downregulated in patients of major depressive disorder (Tochigi et al., 2008), in stress-susceptible rats after chronic restraint stress (Crews et al., 2012), and in offspring of prenatally stressed rats (Mychasiuk et al., 2011). *Mical2* was also identified as a hub gene in a co-expression module implicated in human autism

spectrum disorder (Parikshak et al., 2013), which has a significant anxiety/phobia component. Our analyses converge with these previous studies and provide strong evidence for the role of *Mical2* in neuropsychiatric disease. Importantly, therapeutics targeting mitochondrial and synaptic pathways have been successful for treating CNS diseases and may represent an important direction for developing treatment for a range of neurological and psychiatric disorders (Manji et al., 2012). Therefore, the key drivers of the mitochondria/synaptic subnetwork represent strong therapeutic candidates, as exemplified by *Pde10a*, a pharmacological target for psychotic disorders (Smith et al., 2013).

In the present study, we examined the striatum because it is critical for regulating motivation, stress susceptibility, and sleep and is dysfunctional in many neuropsychiatric diseases. Our integrative analysis revealed that striatal gene networks are extensively shared by sleep and stress phenotypes and that these points of intersection are relevant to neuropsychiatric disease. However, many other brain regions are known to be involved in the regulation of stress and/or sleep. It is likely that coordinated organization and functionality of gene networks in multiple brain regions are required for appropriate stress adaptation and sleep regulation. Limited to only one brain region, our current analysis does not address tissue specificity or multi-tissue coordination of gene networks relevant to stress and sleep. Since we collected other brain regions and peripheral organs in our chronically stressed and extensively phenotyped (B6 × A/J) F2 mouse population, we are in a position to extend our systems approach to multiple tissues in future studies.

Finally, our data-driven systems approach has revealed a number of intriguing but unexpected results. For example, we found that key drivers of multiple networks linking sleep and stress are enriched with neurodegenerative genes, suggesting a molecular mechanism linking stress, sleep, and neurodegeneration. The frequency of this association in our networks suggests that neurodegenerative properties may be a general attribute of key driver nodes important for both stress and sleep, at least in the striatum, a brain region known to be prone to neurodegenerative diseases such as Huntington's and Parkinson's diseases. This hypothesis requires further investigation but proposes that identifying common networks relevant to stress and sleep can reveal molecular mechanisms and therapeutic targets of neuropathology. Furthermore, although the main text of this paper focuses on 15 categories of phenotypes most relevant to the striatum, our data-driven approach can also be applied to the other 14 categories of phenotypes that are not classically associated with striatal function. As expected, the neuronal-specific module Turquoise is almost exclusively associated with sleep and behavioral phenotypes and is not associated with phenotypes that do not directly involve the CNS, confirming the functional specificity of gene network organization. However, a number of associations between other striatal gene modules and non-CNS phenotypes were also observed. For instance, the modules enriched for cellular for stress response genes (Indianred4) and chromatin-modification genes (Mediumpurple2) ranked the highest for peripheral white blood cell measurements, whose association with the striatum is unknown. Some

evidence suggests that the peripheral immune system plays an important role in stress resilience, cognition, and other central nervous system functions (Cohen et al., 2006; Kipnis et al., 2012; Schwartz and Kipnis, 2011), and our unbiased analyses support this link by showing that serological measurements of the peripheral immune system correlate with striatal transcriptional networks that are also associated with stress-related behavioral and sleep phenotypes. The precise relationship between the striatum and peripheral immune system is beyond the scope of this study, but this analysis can serve as complementary evidence for future investigations regarding the interactions between the functions of multiple organ systems involved in stress and sleep.

Overall, our analysis provides the foundation for a data-driven approach that links diverse phenotypes through common molecular networks, and our strategy considers the complex symptomatology of neuropsychiatric disease as a guide, rather than a hindrance, to our molecular analysis. We provide all the data and analysis results as a resource to the biomedical research community, and we expect it will guide future investigations into the biological mechanisms underlying stress, sleep, and related neuropathology.

EXPERIMENTAL PROCEDURES

Animals and Housing

All mice were housed and handled according to the Federal Animal Welfare guidelines, and all studies were approved in advance by the Institutional Animal Care and Use Committee at Northwestern University. This study utilized 338 male (B6 × A/J) F2 mice, bred at the Jackson Laboratory. Animals arrived at Northwestern at 4 to 5 weeks of age and were individually housed in opaque cages without enrichment items for the duration of the study to create social isolation stress. Mice were maintained on a 12-hr light/12-hr dark cycle at a room temperature of 23°C ± 2°C with food and water available ad libitum (except during test procedures).

Stress Procedures and Phenotypes

Mice were divided into 12 consecutively run cohorts of 10–40 animals each, which were all subjected to the same chronic unpredictable stress protocol (Figure 1A) with accompanying phenotypic measurements (see Supplemental Experimental Procedures). The stressors included social isolation, novel exposed environments (elevated plus maze, open field, elevated zero maze), restraint, forced swimming, fear conditioning, social defeat, cold exposure, a metabolic stressor (6-hr fast and glucose tolerance test), and the sleep behavior response to sleep deprivation and restraint. Sleep/wake behavior was recorded from each mouse by surgically implanting EEG and EMG electrodes. Following all stress and sleep behavior tests, all animals were euthanized by decapitation, and blood and tissue samples were collected for additional analyses. Phenotypes were functionally grouped into 29 categories; for sleep traits, factor analysis was used to confirm categories (Table S1).

Genotyping

Genotypes of all animals were determined from DNA extracted from tail-tip biopsies by using the Illumina medium-density single-nucleotide polymorphism (SNP) panel. A complete set of the genotypic data is provided in Table S1.

RNA-Seq

The striatum brain region was rapidly dissected from each mouse after euthanasia and frozen in liquid nitrogen. Gene expression from the striatum was evaluated using RNA sequencing; 100-bp single-end sequencing reads were aligned against the Ensembl NCBIM37 mouse reference genome for gene-level expression profiling.

Analysis Procedures

Details of analysis procedures are described in the Supplemental Experimental Procedures. Briefly, for the genome-wide QTL and eQTL scan, we used Haley-Knott (HK) regression in the r/qtl package. The sample order of the genotypic data was randomly permuted 1,000 times to estimate FDRs for phenotypic QTL. FDRs for cis-eQTL and trans-eQTL were separately estimated based on 500 permutations.

In the causality test, we consider combinations of a trait (T), expression of a gene (G), and a locus (L) regulating both T and G. G tests causal to T if the following four conditions are met: (1) L is associated with T, (2) L is associated with G|T, (3) G is associated with T|L, and (4) L is independent of T|G.

To construct co-expression networks, we used a weighted gene co-expression network analysis (WGCNA) framework, in which gene expression correlations were weighted with a positive power in order to satisfy a "scale-free" power law connectivity distribution in the resulting network. Genes were then grouped into modules using hierarchical clustering based on their topological overlap. Gene expression in a module was reduced to their first principal component and correlated with phenotypes to identify module-trait relationships.

Bayesian network reconstruction was used to decipher regulatory relationships among genes. To break Markov equivalent structures and infer causality, cis-eQTL data were used as causal anchors. We used a Monte Carlo Markov Chain (MCMC) simulation to reconstruct 1,000 gene networks, evaluating the fit of each network with Bayesian Information Criterion (BIC). A single consensus network was constructed from these simulations and was used to identify key regulators of modules relevant to sleep and stress traits. We calculated the size of the h-layer neighborhood (HLN) downstream of each gene in the subnetwork resulting from the intersection between Bayesian network and the module. Genes were identified as causal network regulators (i.e., key drivers) if their HLNs are greater than $mean(\mu) + \sigma(\mu)$, where μ is the size of the respective HLN of each gene in the subnetwork.

ACCESSION NUMBERS

Raw and processed RNA-Seq gene expression data are available via the Gene Expression Omnibus database (accession number GEO:GSE60312).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, one figure, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.04.003.

AUTHOR CONTRIBUTIONS

P.J. and J.R.S. performed all data analyses and wrote the paper. F.W.T., A.K., and M.H.V. conceived the project and designed the studies. H.S.Y. also contributed to the study design. K.F., V.D.G., K.C.S., and M.H.V. performed the chronic stress treatment and collected phenotypic data. B.L. prepared the RNA-Seq data. K.H., B.Z., and A.K. supervised the genetic and gene network analyses. K.F., K.C.S., M.H.V., R.A., F.W.T., and A.K. also contributed significantly to the preparation of the paper.

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