A Complex Mania Model: The Behavior, Molecules, and Genotype of the Madison Mouse Strain

By

Michael C. Saul

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The dissertation is approved by the following members of the Final Oral Committee:

Stephen C. Gammie, Professor, Zoology

Lauren V. Riters, Professor, Zoology

Antony A.O. Stretton, Professor, Zoology

Anthony P. Auger, Professor, Psychology

Vaishali P. Bakshi, Associate Professor, Psychiatry

Dedication

To my two sisters, who gave me the atlas that showed me the world is not so small.

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Dissertation Abstract

Bipolar spectrum disorders are complex psychiatric phenotypes typified by episodes of mania and depression. Animal modeling for these disorders usually breaks them into complimentary behavioral endophenotypes of mania and depression. One model for mania is the recently characterized Madison inbred mouse strain. These animals show a variety of mania-like behaviors relative to related strains. The work contained in this dissertation seeks to characterize this strain more fully at the behavioral, neuromolecular, and genomic levels. In the first study, we performed a gene expression microarray comparing hippocampus transcriptomes of Madison mice and the outbred Hsd:ICR strain. We found multiple differentially expressed genes, gene networks, and predicted genomic loci in Madison animals in systems previously implicated in bipolar spectrum disorders including purinergic reception and chromatin remodeling. In the second study, we performed an extended ethological phenotyping to better define this strain's complement of behaviors relevant to bipolar spectrum disorders. While we did not find that Madison animals spontaneously cycle from their normally mania-like phenotype to a depression-like phenotype, we did find evidence that the Madison phenotype is sexually dimorphic, developmentally stable, and related to alterations in diurnal rhythm and seasonal response. In the third study, we resequenced the Madison exome and compared it with relevant control strains. We found variants in genes related to chromatin structure, endocannabinoid reception, and chronobiology. These variants included loss-of-function alleles in *Smarca4*, a gene whose product remodels chromatin, and *Polr3c*, a gene coding for a subunit of RNA Polymerase III.

These variants statistically account for much variance in the behavioral phenotype of the Madison strain. Altogether, these results show that the Madison strain's biology includes chronobiological, translational regulation, purinergic, and cannabinoid perturbations, supporting the face and construct validity of the Madison strain as a mania model.

CHAPTER 1: GENERAL INTRODUCTION

The subject of this work is the behavioral, molecular, and genomic characterization of a mouse strain called Madison. These animals show a number of phenotypic features similar to the manic pole of bipolar spectrum disorders. In introducing this work, I will discuss bipolar spectrum disorders, their biology, and some animal modeling approaches undertaken toward the understanding of these disorders. I will cover the history of Madison mice and their characterization as a mania model prior to the time I started working in Stephen Gammie's laboratory. I will then introduce the major research questions remaining on the Madison strain examined in this dissertation before defining the working hypotheses driving the original research contained herein.

ON BIPOLAR SPECTRUM DISORDERS

Characteristics of Bipolar Spectrum Disorders

The DSM-5 definition of bipolar spectrum disorders (BSDs, alternatively called bipolar disorder and bipolar disorders in this dissertation) encompasses affective psychopathologies characterized by episodes of mania, an unusual elevation in mood, and depression, an unusual diminishment in mood [1]. BSDs vary in their clinical presentation by the relative severity of both their manic and depressive phases. They are generally divided into three classical diagnoses based upon this presentation. The most extreme presentation of bipolar spectrum disorders, bipolar I disorder, is characterized by fully manic episodes, episodes of mania accompanied by psychosis, along with episodes of major depression. These episodes usually last for timescales measured in weeks to months. Bipolar II disorder, characterized by hypomanic episodes without psychotic features along with episodes of major depression, is often thought

less severe than bipolar I disorder, though the current diagnostic criteria caution against a hierarchical view of disease severity [1]. Cyclothymia, the least severe disorder in the bipolar spectrum, is characterized by an unusual dysfunctional dysthymia varying between relatively mild manic and depressive episodes. Though these disease subcategories are useful for clinical diagnosis, BSDs are heterogeneous and likely vary on a continuum of severity, making them a complex subject of study [2].

Adding to this complexity, BSDs often present with multiple related clinical features. Though the onset of BSDs was traditionally believed as adolescent or later, recent revisions to the diagnostic criteria allow for juvenile onset BSDs [1,3]. BSDs vary in their cyclicity; some show very rapid cycling between mania and depression that can occur over timescales of days or even hours [1]. These disorders have unusually high clinical comorbidities with other psychiatric disorders like substance abuse and anxiety [2,4]. They show very high comorbidity with pathological cannabis use [5]. BSDs often present with comorbid seasonal affective disorder [6]. Further, while BSDs occur with equal prevalence in males and females [7], the nature of these disorders' presentation differs based upon sex; females are more likely to experience rapid cycling and mixed states [8,9], but women and men have a differing complement of comorbid psychiatric disorders [10]. Though the DSM acts as a guide for disentangling these various psychopathologies in differential diagnosis, it is ultimately the discretion of the clinician that decides which of the BSDs a patient has and what their comorbid illnesses are. While necessary to account for the full repertoire of dysfunctionality psychiatric patients display, such flexibility creates imprecision in a biological definition for BSDs.

At a humanistic level, bipolar disorders are extraordinarily detrimental psychiatric illnesses, causing significant pain, suffering, and financial hardship to the afflicted, their families, and those who rely upon them. The lifetime prevalence for bipolar I and bipolar II is somewhere between 2% and 3%, making it one of the more common mental health diagnoses [2]. Between 20% and 25% of patients with bipolar I or bipolar II will attempt suicide [2], and a recent study of suicidality in psychiatric patients predicted that within 20 years of an initial psychiatric diagnosis, 6-10% of patients with bipolar spectrum disorders will succeed in their suicide attempts [11]. Patients with BSDs experience excess mortality in multiple populations above and beyond their increased suicidality [12,13]. Even when managed pharmacologically, BSDs create problems for patients; the current lines of mood-stabilizing drugs such as lithium and atypical antipsychotics used to treat bipolar have myriad unpleasant side effects and may even shorten lifespans [14,15].

The economic costs of BSDs are very high and growing higher with increasing costs of healthcare. BSDs may even be the most expensive mental health diagnoses [16]. In Australia, a country with much lower per-capita healthcare costs than the United States [17], recent estimates of the per-patient lifetime cost of bipolar I and bipolar II disorders were $76,821-$134,318 2012 AUD ($78,304-$136,910 2012 USD) [18]. The direct and indirect US economic burden of bipolar disorders went from $45 billion in 1991 ($69 billion in 2012 dollars) to an estimated $151 billion in 2009 ($159 billion in 2012 dollars), a more than doubling in costs that is far above the expected increase in

disease cost due to epidemiological factors like better diagnoses and population growth alone [19,20].

For such a diverse and costly group of mental health disorders, better treatment alternatives would save lives, pain, and money. To design better treatments, the etiology of these disorders must be clarified and understood at a deep level.

The Biology of Bipolar Spectrum Disorders

BSDs are among the most highly heritable of psychiatric illnesses, implying a strong genetic constituent of this diverse spectrum of diseases [21]. Consequently, these disorders are the subject of intense study by geneticists. A search for “bipolar disorder genetics” on PubMed yields over 5,600 results, yet despite this strong effort from the research community, much remains unknown about the genetics of bipolar disorders [22]. There are many reasons these disorders’ etiologies remains elusive. First, the difficulty in defining BSDs with precision likely increases the noise in rigorous studies of this spectrum of illnesses. Second, BSDs have complex molecular correlates involving differential regulation of not just single genes, but of gene systems [23]. Third, these pathologies may have multiple heterogeneous molecular bases [23,24], a hypothesis consistent with the disparate findings in genome-wide studies of patients with BSDs from separate populations [25]. Fourth, human genetics research is difficult. Deriving a mechanistic model of human psychiatric disorders from the techniques used on humans presents problems due to methodological constraints on power and to the possible epigenetic constituents of these disorders [22,26-28]. Finally, humans have

highly variable genomes and experiences, and as a long-lived species, gene-by-environment interactions produce a wide variety of potential outcomes.

The best conceptual models for the etiological underpinnings of BSDs admit that genotypes and phenotypes for psychiatric disorders are inherently complex and multivalent [25,29]. The human genome is a system containing tens of thousands of individual genes, and it is highly unlikely that a few discrete genomic loci lead to complex illnesses like psychiatric disorders. The strong heritability of BSDs establishes that the study of the genome provides the highest likelihood for apprehending the biological underpinnings of BSDs [21], but it should be studied as a complex disease.

BSDs have been the subject of a multitude of human genome-wide linkage scans. A recent review of multiple scans found a number of regions implicated in BSDs including human genome regions 4p16, 6q21, 8q24, 12q24, and 17q25 [25]. A few single genes candidates believed to have strong roles in BSDs include CACNA1C, a calcium channel subunit; ANK3, a cytoskeletal protein often concentrated at the nodes of Ranvier in myelinated axons; and P2RX7, a purine-gated ionotropic receptor [30-32]. While promising, the work on these single gene candidates is strongly reductive. A number of loci associated with other genes may also contribute [25]. Further, the genomic correlates of bipolar disorders vary depending upon the population studied [25]. While people often conclude that only one or a few genes are involved, genome-wide association studies and linkage scans often show multiple peaks. These peaks are often interpreted with biases; the people undertaking these studies are often

neuroscientists, so the genes under peaks that interest them are often ion channels or other neural signaling related genes.

Relative to simpler molecular etiologies linked to single genes, this complexity presents difficulties in studying BSDs that must be resolved by advances in biological theory. Recent work attempts to study BSDs using systems biology, the holistic study of complex and highly interconnected biological systems using massive datasets and computational tools. A systems meta-analysis of bipolar disorder gene expression studies found that a system of interacting genes generally related to chromatin packaging are correlated with susceptibility to bipolar disorders [32]. Recent human genomic research has found that the same genomic loci may confer susceptibility to BSDs and other major psychiatric illnesses including ADHD, schizophrenia, autism, and major depressive disorder [33]. This suggests that BSDs may be one resultant disorder in an array of nosologically-related mental health disorders whose origins lay in similar gene systems, a finding consistent with the high comorbidities of these diseases seen in the diagnosis of BSDs [1]. Though these advances show the great ability of systems biology, much remains unknown about the precise gene systems involved in bipolar disorders and how they modulate neural activity. Computational tools used to understand the datasets generated by emergent technologies such as next-generation sequencing show significant power to elucidate causative molecules behind these disorders, and a query of NIMH R01s on BSDs will show that such investigations are underway. However, no published work presents a magisterial causative argument for the genetic origins of BSDs.

Modeling the Bipolar Spectrum

Modeling an illness as complex as a BSD in a rodent is either impossible [34] or very difficult [35,36] depending upon which researcher is asked. Though arguments for the impossibility of understanding these diseases using animal models have merit, they often suffer from an understanding of biology that privileges humans above non-human animals. For over a decade now, we have known just how similar the genomes of humans and non-human mammals are [37]. It follows that they should display similar neural and behavioral phenotypes in the right contexts.

The animal modeling community has approached BSDs by breaking them into pieces, using the complementary behaviorally-defined endophenotypes of mania and depression [29,34,38]. To our knowledge, no animal model cycling between manic-like and depressed-like states has ever been observed [29,34]. Multiple methods for modeling each of these two affective states have been advanced. Models for depression have been highly successful [35]. One of the most successful genetically distinct mouse strains modeling the depressed pole, a naturally-occurring depressed phenotype in a rat model called the Flinders-sensitive line, has aided in the elucidation of much about depression since the mid 1980s [39]. Other non-genetic depression models have included learned helplessness, pharmacological manipulations, various social and non-social stressors, and even brain lesions [35].

Models for mania have had more limited success. A classic method for modeling mania is an induced manic state. Often, the state is induced by administration of the stimulant drugs dextroamphetamine or cocaine. In rodents, this method for inducing

mania in animals first appears in the literature in the 1970s, with treated mice showing a hyperactive and hyperaggressive behavioral phenotype that can be moderated by lithium treatment [40]. This approach to modeling has been used as a method for achieving reproducible mania-like states in humans [41]. Pharmacological manipulations have shown great utility in studying potential neurochemical alterations seen in mania, providing good face and predictive validity [42]. However, because pharmacological manipulations do not change the genome, they cannot show construct validity as mania models. Further, there is no good evidence that these pharmacological models alter the same neural systems altered in BSDs [29]. A variety of other inducible approaches for modeling mania, including environmental manipulations and sleep deprivation, have also been proposed [42], and though these show similar face validity to pharmacological treatments, they also lack in predictive and construct validity. Additionally, the use of an acute inducible phenotype has been attacked as overly reductive for a chronic disease [29].

More recently, engineered transgenic animals bearing relevant genetic manipulations have been advanced as potential mania models. One of the first transgenic manic mice was the dopamine transporter knockout. These mice show locomotor hyperactivity and deficits in social behaviors, though they lack responsiveness to amphetamines [43,44]. They seem to show construct validity for some BSDs [45], though other BSDs are likely genetically unrelated to dopamine transporters [25]. Molecular clock knockouts have also been advanced as mania models [46]. Since BSDs are often associated with perturbations in chronobiology [6,47], these

models show significant construct validity for certain BSDs. However, they fall short on subtlety and complexity. Recent models for mania have utilized subtle mitochondrial dysfunction variants [48], a gene system perturbation consistent with newer molecular and cytological information about BSDs [49-51]. This approach has even been called a method for creating a bipolar animal, though that claim does not hold up to rigorous examination of the data; the supposed depressed phase was observed in a single animal for a short period [48]. Mitochondrial variant transgenics appear to be among the most promising engineered chronic models for BSDs, though they fail to show the same genetic complexity as the human disorders they model.

Within the past decade, a genetic model taking advantage of natural genetic variation in different strains of animals has been advanced as an interesting and novel method for modeling mania. The use of mouse strains naturally displaying a manic phenotype has many advantages, and the Black Swiss strain of mice has seen interest as a possible naturally occurring manic model relative to other strains [52,53]. Compared to the control C57BL/6J strain, Black Swiss mice show heightened sucrose preference and amphetamine responsivity [52]. Compared with multiple other strains, the Black Swiss strain shows increased locomotor activity, increased aggression, decreased immobility in forced swimming, increased amphetamine-induced hyperactivity, decreased anxiety-like behavior, and the predicted attenuation in behavioral phenotype in response to lithium chloride and valproic acid treatment, results consistent with a face and predictively valid mania model [54]. They have decreased expression of the Wnt signaling molecule beta-catenin [53], a finding consistent with

proposed differences in Wnt signaling in patients with BSDs [55]. Altogether, these results suggest that Black Swiss animals are an interesting and genetically valid way of modeling mania. However, Black Swiss animals from different vendors show marked differences in their behavioral phenotype; Charles River Black Swiss animals show decidedly non-manic behaviors and different response to lithium relative to Black Swiss animals from Taconic [56]. This suggests that results seen on the Black Swiss strain may be resultant from a phenocopy effect, a phenotype arising simply from differences in environmental factors.

Altogether, while many good models for mania have been advanced, there is significant room for improvement in contemporary mania models. Further, the promise of strain differences for mania models should not be undercut by the possible failure of the Black Swiss strain.

THE MADISON MOUSE STRAIN

The Madison Strain as a Mania Model

The Madison (MSN) strain is an inbred mouse strain that was observed to display a collection of mania-like traits in the late 2000s. The first observations of MSN as a manic model occurred by chance. When animals are on corncob bedding, their movement produces a rustling sound. The room in which MSN animals were kept was full of near constant rustling while related strains made much less noise.

From this simple behavioral obsesrvation, the Gammie lab successfully characterized the strain as manic using a battery of behavioral tests designed to provide evidence supporting MSN's validity as a mania model. MSN mice displayed elevated

spontaneous in-cage locomotor activity relative to control strains. They showed higher forced swimming than control strains. They engaged in more sex mounts relative to control strains. Critically, they showed an attenuation in manic behavior in response to treatments of two pharmacological interventions often used to treat bipolar disorders, lithium chloride and the atypical antipsychotic olanzapine [57].

The history of the MSN mouse strain suggests that the most important events leading to the manic phenotype were unintended. At no point were MSN mice selectively bred for mania.

A History of the Madison Mouse Strain

In the mid 1990s, Ted Garland, then working in the University of Wisconsin–Madison Department of Zoology, began a selective breeding project on eight separate lines of outbred albino Hsd:ICR (ICR) mice. Four of these lines were bred randomly as a set of control strains while the other four strains were bred with selection for high voluntary wheel running. In the time elapsed since that original selective breeding project, individuals from Line 6 (L6), one of the original selectively bred lines of mice, became the inbred strain that we now call MSN. The following is a history of the breeding events leading to the genesis of the MSN strain.

In the original selective breeding experiment the progenitors of the MSN strain underwent selection for high voluntary wheel running alongside three other selectively bred lines. After the first 10 generations, these four selectively bred strains including L6 already showed significantly heighted voluntary wheel running relative to the control strains [58]. The goal of this experiment was to look at exercise physiology, but few

exercise physiological changes were observed in any selectively bred strains following the selection process [59]. Selection for high voluntary wheel running lasted for a total of 30 generations, though significant increases in wheel running relative to control strains were not seen past approximately generation 15 in any of these strains. The selectively bred strains including L6 were studied around generation 30 for differences in brain physiology correlating with the heightened voluntary wheel running, where they were found to display differential neural activation in areas of the brain associated with motivation in response to blocked wheel running [59]. Additionally, a microarray experiment on the hippocampal gene expression profiles of the high wheel running strains including L6 found some gene expression changes in relation to the selection that included genes related to dopaminergic signaling [60].

The MSN progenitors also displayed significantly heightened maternal defense at generation 30 of selective breeding. These L6 mice were a sharp contrast to the other 3 selected lines, which displayed no significant elevation in maternal defense behavior relative to the control strains [61]. This observation suggests that during the first 30 generations of selective breeding, genetic drift also contributed to the phenotype displayed by these animals. Following identification of high defense, the MSN progenitors were bred for an additional 22 generations with selection for the high maternal defense phenotype. At this point, these progenitors were temporarily called maternal defense line 1 (MaD1). Curiously, though these animals displayed heightened maternal defense, a large subset of females displayed significant maternal neglect correlating with alterations in neural dopaminergic markers [62].

The MSN strain was then maintained using random breeding for colony maintenance without selection for any trait. During the time since the last selection event, they have likely experienced genetic drift, fixing what little variation remained in their genomes. At present, the estimated inbreeding coefficient of the MSN strain is approximately 0.95 [57].

NEW WORK ON MADISON MICE

On Validity

A primary goal of animal modeling is to establish validity, a model that replicates a human disease state. A predictively valid model allows for the successful testing of novel interventions to ameliorate a disease state in both the animal and in humans. Predictive validity in animal models is the ultimate goal for translational research. In the Flinders-sensitive line of rats, one of the best validated animal model for depression, predictive validity for depression was established over a period of decades [39]. Face validity, a simple reiteration of a human phenotype in an animal, is faster and easier to establish than predictive validity. However, face validity can be misleading; there are often many paths to certain phenotypes [29]. Ultimately, construct validity is of the highest interest biologically. Studying the same genes or gene systems as are involved in a disease state is the goal of animal modeling. It is what is truly helpful to understand a disease.

Behavioral batteries such as the one used to validate the MSN strain initially in the Gammie Lab's 2011 paper on MSN animals are used to establish a base level of each of these kinds of validity [63]. Though these batteries are highly useful, they need

occasional updating as more is found out about human diseases [54]. Previous research on the MSN strain established basic face validity via phenotypic similarity and some predictive validity via responses to drugs primarily used to treat BSDs [57]. However, much work remains necessary to establish and enhance all forms of validity for the MSN strain. Construct validity, showing that similar biological systems are perturbed in MSN animals as they are in patients with BSDs, remained unestablished at the beginning of this dissertation work. Further, recent advances in the study of BSDs make new behavioral predictions for a face-valid mania model [64].

Taking all of these concerns into account, the objective of this dissertation is a deeper study of the MSN mouse strain with the goal of providing evidence for each of these types of validity for MSN as a mania model.

Research Approach

Many compelling questions remain about the MSN phenotype. To us, the most interesting of these questions fall generally under the three broad aims of this dissertation project. We aimed to gain more information about their behavior by more carefully crafting the ethological description of these animals' phenotype. We aimed to broaden our knowledge of the molecular correlates of this behavioral phenotype by examining gene expression in these animals' brains. Above all, we aimed to find information about the genotype that drives this phenotype and just how similar that genotype is to the one seen in humans with BSDs.

We believed that the best way to accomplish these aims was to design experiments through the lens of validity. To enhance face validity, we hoped to extend

the previous behavioral phenotyping with a fuller range of assays designed to assess traits like onset of mania during development, intersexual differences in mania presentation, and circadian and seasonal-like differences between MSN mice and control strains. To establish construct validity, we hoped to investigate the gene systems involved in conferring the MSN strain's phenotype and evaluate the degree to which these systems are similar to those involved in human bipolar disorders. Ultimately, it is only through strengthening these validities that the MSN strain can pay dividends in translational research on BSDs.

Dissertation Hypotheses

In testing the validity of the MSN mouse strain models mania, we formed the following three working hypotheses:

1. The MSN strain displays neural gene expression correlates consistent with those observed in humans with BSDs.
2. The MSN strain reiterates behaviors from human BSDs in ways outside those included in traditional behavioral batteries.
3. The MSN strain's genotype is consistent with genomic correlates of BSDs in humans.

In this order, these three working hypotheses form the backbone of the original research chapters in this dissertation.

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CHAPTER 2: HIPPOCAMPUS MICROARRAY

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ABSTRACT

Bipolar disorder (BPD) is a debilitating heritable psychiatric disorder. Contemporary rodent models for the manic pole of BPD have primarily utilized either single locus transgenics or treatment with psychostimulants. Our lab recently characterized a mouse strain termed Madison (MSN) that naturally displays a manic phenotype, exhibiting elevated locomotor activity, increased sexual behavior, and higher forced swimming relative to control strains. Lithium chloride and olanzapine treatments attenuate this phenotype. In this study, we replicated our locomotor activity experiment, showing that MSN mice display generationally-stable mania relative to their outbred ancestral strain, hsd:ICR (ICR). We then performed a gene expression microarray experiment to compare hippocampus of MSN and ICR mice. We found dysregulation of multiple transcripts whose human orthologs are associated with BPD and other psychiatric disorders including schizophrenia and ADHD, including: Epor, Smarca4, Cmklr1, Cat, Tac1, Npsr1, Fhit, and P2rx7. RT-qPCR confirmed dysregulation for all of seven transcripts tested. Using a novel genome enrichment algorithm, we found enrichment in genome regions homologous to human loci implicated in BPD in replicated linkage studies including homologs of human cytobands 1p36, 3p14, 3q29, 6p21-22, 12q24, 16q24, and 17q25. Using a functional network analysis, we found dysregulation of a gene system related to chromatin packaging, a result convergent with recent human findings on BPD. Our findings suggest that MSN mice represent a polygenic model for the manic pole of BPD showing much of the genetic systems complexity of the corresponding human disorder. Further, the high degree of

convergence between our findings and the human literature on BPD brings up novel questions about evolution by analogy in mammalian genomes.

INTRODUCTION

Bipolar disorder (BPD) is a psychiatric disorder characterized by episodic mania and depression [1]. It is a common mental health problem, with an estimated lifetime prevalence of approximately 1-5% [2,3]. A meta-analysis of family, twin, and adoption studies found that relatives of BPD patients have a 10-fold higher risk of the disorder than those without relatives with BPD [4], demonstrating that BPD has a strong heritable constituent. Though ongoing efforts to elucidate the genetic basis of BPD using varied approaches have yielded promising results, a convincing molecular etiology of BPD remains elusive [5]. There are at least a few good reasons for this difficulty in finding a genetic basis for BPD. First, BPD is a complex disorder at the molecular level, involving perturbations of not just single genes, but of systems of genes [6]. Second, it may be more proper to speak of bipolar disorders in the plural; the pathology may have multiple heterogeneous molecular bases [6,7], a hypothesis consistent with the multiple heterogeneous findings in different genome-wide studies of BPD [8]. Third, deriving mechanistic explanations of human psychiatric disorders using classical genetics presents difficulties due to practical constraints on experimental power and the possibility of epigenetic components of these disorders [5,9-11].

Because a convincing BPD molecular etiology poses significant technical and theoretical challenges to human geneticists, animal models for BPD have a strong potential to extend understanding of this disorder. The main animal modeling approach

to date has been the use of separate rodent models for mania and depression [12]. Models for the manic pole of BPD have primarily utilized treatment with psychostimulants [13,14] or single locus transgenic approaches like dopamine transporter knockouts [15,16] and various molecular clock gene knockouts [17,18], though the Black Swiss strain has recently been proposed as a tentative naturally-occurring mania model [19,20]. Importantly, there exists at least one strain of rodent, the Flinders Sensitive Line (FSL) of rat, which shows a well-validated depressed phenotype relative to control strains [21]. This strain of rat has been used in multiple studies to examine several molecular aspects of depression [22-24], and it has been useful in conceptualizing depression as a disorder with a complex molecular etiology [12,25].

Our lab recently characterized a tentative model for the manic pole of BPD [26]. This model, an inbred mouse strain termed Madison (MSN), displays a naturally manic phenotype. Relative to control strains, MSN mice show increased locomotor activity, increased forced swimming, decreased sleeping, and increased sexual activity. Further, treatments with both lithium chloride and olanzapine moderate the MSN manic phenotype, a necessary condition for a predictively valid model for the manic pole of BPD [27].

Our initial behavioral and pharmacological characterization of the MSN strain showed promise, but without molecular correlates, the model lacked construct validity. Consequently, we performed a gene expression microarray study with RT-qPCR confirmation to extend the phenotype of the MSN mouse relative to their ancestral outbred hsd:ICR (ICR) strain. When choosing which brain region to interrogate, we

decided to look at gene expression in hippocampus. In humans, hippocampus shows microstructural and functional differences in BPD patients as assayed by MRI [28,29]. Further, post-mortem analyses of hippocampal tissue from BPD patients show multiple histological and gene expression differences relative to hippocampus from psychiatrically normal controls [29-32]. Prior to the microarray experiment, we decided to replicate the most robust measure from our previous behavioral work, total locomotor activity, to confirm that MSN mice stably display a manic phenotype.

RESULTS

Total locomotor activity

Locomotor activity defined by total distance traveled (Fig. 2.1.A) was significantly higher in MSN mice than in ICR mice (P-value = 7×10^{-7}, Monte Carlo permutation test, $n_{MSN} = n_{ICR} = 19$, $Z = -4.24$, $B = 1 \times 10^{7}$). The probability density distribution for total distance travelled for MSN mice was bimodal whereas the probability density distribution for ICR mice was unimodal (Fig. 2.1.B). Since the MSN strain is almost completely inbred, we do not believe this bimodality is evidence of two separate populations within the MSN strain.

Single gene microarray results

The hundred best annotated genes with the most significant P-values along with a heatmap showing expression in each of the tested samples are listed in Fig. 2.2. MSN mice showed significant differences in gene expression in multiple genes whose orthologs are associated with BPD and the related mental health disorders

schizophrenia, depression, and ADHD in the human literature. Significantly-dysregulated genes (P-value < $1x10^{-3}$) whose human orthologs have been associated with these disorders in at least two separate studies include: Cp (P-value = $1.25x10^{-5}$, t = –7.421) [33-35], Epor (P-value = $2.73x10^{-5}$, t = 6.823) [36-38], Pdgfra (P-value = $4.38x10^{-5}$, t = 6.476) [39,40], Tac1 (P-value = $9.03x10^{-6}$, t = –7.684) [41-43], P2rx7 (P-value = $6.72x10^{-5}$, t = –6.170) [44-49], Fhit (P-value = $5.20x10^{-5}$, t = –6.352) [50,51], and Cat (P-value = $6.56x10^{-6}$, t = 7.946) [52,53]. Significantly-dysregulated genes whose human orthologs have been associated with BPD or a related mental health disorder in one study include: Smarca4 (P-value = $7.58x10^{-7}$, t = –9.897) [54], Mut (P-value = $3.44x10^{-6}$, t = –8.495) [55], Git1 (P-value = $6.24x10^{-6}$, t = 7.988) [56], and Cmklr1 (P-value = $1.12x10^{-4}$, t = 5.818) [57]. An additional dysregulated gene of interest we identified whose human ortholog has not been associated with BPD or related mental health disorders as far as we know is Npsr1 (P-value = $9.48x10^{-4}$, t = 4.455). This gene's product is a G-protein coupled receptor generally involved with arousal and activity [58,59]. The names of genes discussed here are highlighted in grey in Fig. 2.2. All reported values use an empirical Bayesian t-test with 10 degrees of freedom.

RT-qPCR confirmation of microarray results

We confirmed the results of seven genes from our microarray experiments using RT-qPCR. We chose genes for confirmation with an emphasis on gene products that we thought were either related to neural signaling pathways it would be possible to target pharmacologically or genes we could use as dependent variables in the future. We tested Cat (P-value = 0.001, expression ratio = 1.202), Cmklr1 (P-value < 0.001,

expression ratio = 1.404), Epor (P-value < 0.001, expression ratio = 1.370), Fhit (P-value < 0.001, expression ratio = 0.446), Npsr1 (P-value = 0.021, expression ratio = 2.426), P2rx7 (P-value = 0.006, expression ratio = 0.681), and Tac1 (P-value = 0.001, expression ratio = 0.696). The results of the RT-qPCR confirmation are shown in Fig. 2.3. All seven genes we chose to confirm were found significantly dysregulated in the same direction and at the same approximate magnitude as in the results of the microarray experiment. Altogether, our RT-qPCR confirmation provides evidence that our microarray data are fundamentally sound.

Genome enrichment analysis

NIAID DAVID functional annotation analysis by cytoband of all genes dysregulated at $P < 0.01$ found a significant enrichment in murine cytoband 5qF (3.789-fold enrichment, Bonferroni-corrected P-value = 7.84×10^{-7}) in MSN mice. We found this result intriguing, but we believed this cytoband-style enrichment analysis utilized genome regions too wide to allow the assumption of classical genetic linkage. We created a new algorithm for genome enrichment analysis with much narrower partitions of the genome queried. Our novel genome enrichment analysis yields results that look very similar to a conventional genome-wide linkage or association study, and we find it useful for generating predictions for broad chromosomal regions potentially related to a given population's phenotype. We found significant enrichment in a total of fifteen genome regions (Fig. 2.4.A).

When looking at the significantly enriched genome regions, we decided to examine their relationship to the human genome, so we qualitatively looked at shared

synteny, a similar clustering of orthologous genes between species that generally demarcates genome homology. We looked for linkage and association literature implicating the enriched regions' human homologs in BPD and related mental health disorders like schizophrenia and ADHD. We classified a human genome region implicated in BPD in at least two linkage studies with at least one study showing a LOD or NPL score (linkage score) greater than 3 as a region with a strong relationship to BPD. Using this criterion, we found that eight of the enriched genome regions in MSN mice are homologous to seven human genome regions displaying strong relationships to BPD. Shared synteny for these regions is shown in Fig. 2.4.B. These regions include the following cytobands: murine 4qE, homologous to human 1p36, implicated in BPD in two studies with linkage scores of 3.97 [60] and 3.1 [61] and a region in which SNPs predict BPD susceptibility [62]; murine 5qF, homologous to human 12q24, implicated in BPD in multiple studies with linkage scores of 4.91 [63], 3.63 [64], 3.37 [65], 2.8 [61], and 2.08 [66] and a region in which SNPs and allele variants predict BPD susceptibility [44,46,67]; 8qE1, homologous to human 16q24, implicated in BPD in two studies with linkage scores of 3.51 [68] and 2.29 [69]; murine 11qE2, homologous to human 17q25, implicated in BPD in five studies with linkage scores of 3.11 [70], 2.4 [71], 2.4 [72], 2.1 [73], and 2.08 [74]; murine 13qA3 and 17qA3-17qB1, two cytobands with homology to human 6p21-22, implicated in BPD in multiple studies with linkage scores of 3.19 [68], 2.60 [75], 2.26 [72], and 1.91 [69]; murine 14qA1, homologous to human 3p14, implicated in BPD in two studies with linkage scores of 3.51 [76] and 2.31 [77]; and murine 16qB2-B3, homologous to human 3q29, implicated in BPD in two studies with

linkage scores of 3.74 [78] and 2.0 [61]. Additional enriched genome regions showing weaker previous relationships to BPD included: murine 2qE, homologous to human 11p13, implicated in BPD in one study with a linkage score of 1.95 [79] and a region in which SNPs and allele variants predict BPD susceptibility [80,81]; murine 8qB2-B3.1, homologous to human 4q34, implicated in BPD in a study with a linkage score of 3.28 [82]; murine 8qB3.3 and 9qA3, homologous to human 19p13, implicated in BPD in three studies with linkage scores of 2.37 [83], 1.8 [66], and 1.55 [84]; and 15qE3, homologous to 22q13, implicated in BPD in one study with a linkage score of 2.22 [85]. One cluster at murine cytoband 13qD1 shared synteny with human 5q13-14, a region with no significant linkage to BPD in any human literature we could find, but implicated in schizophrenia in a study with a linkage score of 3.20 [86] and implicated in ADHD in a study with a linkage score of 4.16 [87]. A cluster on the X chromosome consisted of mostly predicted genes with no obvious orthology to known human genes. We were not able to find shared synteny between this region and any human region.

Functional network analysis

NIAID DAVID analysis of all genes with FDR-adjusted P-values less than 0.25 found a significant gene cluster generally related to chromatin packaging, so we decided to pursue this finding using Cytoscape to visualize this cluster. Using a gene list generated from this DAVID analysis, we found a network of genes generally related to chromatin packaging that was significantly dysregulated in our microarray study. These genes included a few histone-related genes as well as Smarca4, a gene we found to be one of the most highly-dysregulated in the microarray results that has helicase and

chromatin remodeling activities. The chromatin packaging gene network is shown in Fig. 2.5, with nodes in red representing those genes found dysregulated in our microarray results and nodes in blue representing linking genes previously found related to chromatin packaging not significantly dysregulated in our microarray. These results suggest that differential chromatin packaging is part of the MSN phenotype, which is convergent with the findings of a recent systems biology meta-analysis of BPD [54].

DISCUSSION

A complex phenotype

As BPD is a genetic disorder involving systems of genes [6,8,54], models for either pole of BPD representing single genes, while valuable for many purposes, are necessarily limited. A mammalian genome containing upwards of 30,000 genes is a complex system, and while understanding the effects of single genes and their products will always be necessary, their results should be understood in a systems biology context. We believe that the MSN strain represents among the first true systems biology models for the manic pole of BPD characterized. The polygenic nature of this model presents both challenges and promise. The quantity of loci involved in the phenotype makes inference difficult relative to single locus models, but it reproduces the physiology of the disorder more completely and more subtly. We believe the MSN mouse strain will enable us to glean new insights into the biology of BPD based upon not only face validity, but on fundamental biological construct validity.

The basis of this polygenic phenotype is the MSN strain's ~15 year history of multifaceted inbreeding. The ancestral strain of these mice was one of four replicate

strains originally bred for high wheel running in a study on exercise physiology [88]. The ancestors showed few notable exercise physiological changes; the most significant changes observed in this strain displayed were neurological [89]. These MSN ancestors were part of a 2003 hippocampus microarray paper comparing selectively bred strains with control strains [90]. None of the 53 genes found significantly dysregulated in the 2003 microarray experiment overlap with the significantly dysregulated genes from the present study, and most of these 53 genes are quite far from significance in the current array. While methodological differences prevent easy comparisons between the 2003 microarray and the current microarray, we believe the fundamental divergence of the two arrays implies that the chief physiological changes we see in the MSN strain likely emerged after the MSN progenitors were selectively-bred for high wheel running. The sole selection event occurring after the original microarray experiment was ~30 generations of selection to maintain a trait already observed in the MSN progenitors, high maternal defense [91]. After this maintenance selection, the MSN strain's ancestors were maintained in our lab without selective breeding. Given the lack of breeding toward a novel phenotype, we believe many of the important genetic changes contributing to the MSN phenotype are likely attributable to genetic drift. This is not to deemphasize the effect of the original selective breeding for high wheel running, which provided the genetic foundation of the MSN strain. We suggest that the MSN strain's manic phenotype emerged through a series of random events acting upon an already constrained gene pool.

The MSN mouse strain's locomotor activity displays a bimodal distribution while the outbred mouse strain's locomotor activity shows a unimodal distribution. Because the MSN strain is highly inbred, we believe this bimodality does not suggest divergent populations of mice within the MSN strain. Instead, we speculate that the MSN strain may show a phenotype with true behavioral cyclicity, a finding that has never been seen in a rodent model as far as we know. We are currently working on a behavioral project to examine this possibility in greater detail.

Implications of the genome findings

The genome enrichment algorithm we developed is a new and useful method extending the suite of systems biology tools for high-throughput gene expression data. This algorithm can be applied to gene expression datasets old and new to enhance the biological understanding of the genome as a substrate for the organization of gene systems. Importantly, we believe it predicts both potential perturbations of the genome and of the epigenome, which would provide a more complete accounting of the mechanistic underpinnings of differences on the genome than classical genetics can. When used in concert with classical genetic techniques, we believe this technique has the potential to inform biologists not only about where to look for differences on the genome, but for which types of differences they should interrogate each genome regions.

While this genome enrichment analysis does not substitute for a true genome-wide linkage scan, we believe it provides a strong prediction that the MSN genome experiences perturbations in areas homologous to human genome regions linked to

BPD in some populations. Why human populations with BPD should show differences in the genome relative to psychiatrically normal comparisons is an interesting question. Why MSN mice might share some of the same genomic perturbations as some human BPD populations is a compelling extension of this question. We propose that because the structural and functional components of mammalian genomes do not differ significantly, these genomes, given analogous evolutionary events, display analogous changes. Put more simply, similar genomes experiencing similar forces react similarly. In this case, we believe we may have found a conserved genomic signature observable even after the some 75 million years since the divergence of the mouse and human lineages [92]. We speculate that this signature is related generally to neural activation and organismal arousal. Further, our findings suggest that conserved genomic signatures may exist for other disorders and traits.

Conclusions

Though these results show promise, we must include caveats based upon the complex nature of the phenotype and the limitations of the techniques we used. MSN mice weigh significantly less than ICR controls ($t_{27.957} = -3.986$, P-value = 4.369×10^{-4}, Welch's two-sample t-test), which shows that the MSN background includes other characteristics potentially unrelated to mania. While the microarray platform we used was designed to be robust against the effects of polymorphisms in the probes, ultimately, we cannot preclude the possibility of coding changes in the probe binding sites of some genes of interest affecting our results. Similarly, while we made all practical efforts to design qPCR oligonucleotides on monomorphic sites, we cannot say

with certainty that our primer binding sites do not contain novel polymorphisms. Additionally, as we noted in the results, some of the evidence from the human literature we utilized to contextualize our results is unreplicated.

Despite these complexities, when we look at the rich suite of systems biology differences present in MSN strain relative to the closely related ICR strain, we believe we have found a strong phenotype that models mania with high construct validity. We have demonstrated that MSN mice reiterate a substantial amount of work done on human BPD genetics using three levels of analysis. At the single gene level, MSN mice display dysregulation of multiple transcripts whose human orthologs are related to BPD and related mental health disorders [8,33-57]. At the systems level, MSN mice display dysregulation of a gene network similar to one found conserved across multiple human studies on BPD in a recent systems meta-analysis [54]. At the chromosomal level, MSN mice display perturbations in eight murine genome regions homologous to seven human genome regions with strong relationships to BPD in the human literature [60-78]. We believe that the argument for analogy between MSN mice and human BPD is strong. The genetic, systems, and genome findings we present here imply profound physiological similarities.

Just as the etiology of human BPD remains unresolved [5], so do the mechanistic underpinnings of the MSN strain's phenotype. This study is an extension of our effort to characterize the phenotype and a preliminary step in the process of finding a genotype. While we believe we have fully utilized a strong dataset to glean an interesting picture of these mice, until we understand more about the genomics behind the MSN phenotype,

the scope of our work remains limited. We believe the loci from our novel genome enrichment analysis give us a set of targets relevant to a potential deep sequencing project. Comparing the MSN genome with the outbred ICR genome will be an important next step and will contribute much to our understanding of BPD.

MATERIALS AND METHODS

Ethics statement

Animal use was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the University of Wisconsin–Madison IACUC (protocol #: L00405-0-05-09), and all reasonable efforts were made to minimize animal suffering.

Animals

MSN is an inbred strain of mouse derived over the course of approximately 15 years from the outbred hsd:ICR (ICR) mouse strain (Harlan Laboratories, Madison, WI, USA), making the ICR strain a natural control. The ancestors of MSN mice were one of four replicate strains selected over a period of ~30 generations for high wheel running behavior [88]. This high wheel running ancestral strain was observed to display high maternal defense behavior compared to both control lines and the other three lines selected for high wheel running from the original selective breeding experiment [91]. The ancestral mice were then bred for an additional ~30 generations to maintain high maternal defense behavior. This progenitor strain was also characterized as showing maternal neglect [93]. The mice were maintained in a breeding colony in our lab without

selection for multiple generations, likely experiencing genetic drift and fixation before our lab eventually observed them to display a manic phenotype relative to control strains [26]. The MSN strain is now highly inbred; we estimate its current inbreeding coefficient at 0.95. The MSN and ICR strains were kept in separate breeding colonies in our laboratory under similar conditions for multiple generations prior to this study. Mice from this study were adult males from the same generation singly housed in the same room, and all mice were approximately 10 weeks old during testing.

In-cage locomotor activity observations

Total in-cage locomotor activity observations during portions of both the light and dark periods were made using a camera mounted above mouse home cages with online analysis done by the TopScan 2.0 software (CleverSys, Reston, VA, USA) as described previously [26], though for this experiment, we observed MSN mice during one day and not two. In total, 19 MSN mice and 19 ICR mice were observed. Statistical inference for total distance traveled over time tested was done using the Monte Carlo permutation test implementation in the R package coin. Probability density plots were made using the R package sm, a non-parametric smoothing algorithm for histograms.

Tissue collection

The day after observation of total locomotor activity, mice were weighed, anesthetized with isofluorane gas, and decapitated. After whole brains were removed, hippocampal tissue was quickly dissected out and the hippocampi were flash-frozen over dry ice. Hippocampal tissue was kept frozen at –80°C for no longer than 12 weeks

prior to use in downstream molecular biology applications. Statistical inference on animal weight was performed using the t.test function in R.

RNA extraction

Total RNA was extracted using an Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, CA, USA) to manufacturer specifications. Briefly, tissue was disrupted in a low pH phenol-chloroform-guanidium thiocyanate solution and spun down. The aqueous phase was combined with ethanol and passed through a spin column to bind RNA. The RNA was cleaned and DNase treated on-column, purified total RNA was eluted in nuclease-free elution buffer, and samples were frozen at –80°C prior to use in downstream applications.

Microarray target preparation, hybridization, and scanning

Using the true random number generation service random.org, 6 mice from the MSN group and 6 mice from the ICR group were chosen at random from the mice used in behavioral observations. Prior to the microarray experiment, all total RNA samples were checked for purity, integrity, and concentration using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher, Waltham, MA, USA) in concert with RNA 6000 Pico Chips in a 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). The microarray experiment utilized the GeneChip Mouse Gene 1.0 ST platform (Affymetrix, Santa Clara, CA, USA), a platform with probesets consisting approximately 27 probes spaced along the length of each gene interrogated to minimize the effects of polymorphic sites on the expression results for the gene as a whole, with biotinylated targets derived from total RNA. Briefly, cDNA for hybridization was synthesized from

400ng of total RNA using a GeneChip WT Expression Kit (Ambion, Austin, TX, USA) according to the manufacturer's specifications. The cDNA was fragmented, then biotinylated using a WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's specifications. Biotin-labeled cDNA was hybridized with microarrays at 45°C for 16 hours. The hybridized arrays were washed, stained, and scanned on a GC3000 G7 Scanner (Affymetrix, Austin, TX, USA). Data were extracted and processed from the scanner using the Affymetrix Command Console software, v. 3.1.1.1229. Microarray target preparation, hybridization, and scanning were performed by the Gene Expression Center, a microarray core lab at the University of Wisconsin–Madison.

Probeset level normalization, summarization, and statistical inference

Probeset-level normalization and summarization were performed with the PLIER algorithm with GC-bin background correction using the Affymetrix Power Tools software, v. 1.12.0 and revision 4 of the Affymetrix library for the Mouse Gene 1.0 ST version 1 array platform. The raw and summarized microarray data discussed in this publication have been deposited in the NCBI's Gene Expression Omnibus [94] and are available through the GEO website, accession number GSE29417. Inferential statistics for differential expression between MSN and ICR samples were calculated using the microarray-specific empirical Bayesian t-test implementation in the Bioconductor package limma, v. 3.6.9 [95], to calculate nominal P-values and Microsoft Excel 2010 to calculate linear fold-change differences. A tab-delimited spreadsheet containing exhaustive microarray statistical results with annotations from both Affymetrix, a reliable

annotation source, and fill-ins for transcript clusters unannotated by Affymetrix from Ensembl's BioMart, a less reliable annotation source, is posted as supplementary Table S2.1.

RT-qPCR validation of microarray results

We have included an RT-qPCR supplement, Table S2.2, which contains all of the information about the RT-qPCR methods from this study required by the MIQE [96].

A total of 8 MSN mice and 8 ICR mice were selected for RT-qPCR confirmation. An additional 2 MSN mice and 2 ICR mice were selected using random.org from the same set of mice for RT-qPCR confirmation to add to the 6 MSN and 6 ICR mice used in the microarray experiment. RNA extraction from hippocampi of the additional animals was done at the same time as extraction for the animals used in the microarray experiment. Fresh aliquots of RNA were used for RT-qPCR confirmation to avoid samples differentially exposed to freeze-thaw cycles. Prior to RT-qPCR confirmation, total RNA was checked for purity, integrity, and concentration using both a NanoDrop ND-1000 spectrophotometer and RNA 6000 Nano Chips in an Agilent 2100 BioAnalyzer. Results of quality control are reported in table S2.2.10.

Seven gene transcripts found significantly dysregulated in the microarray were chosen for validation by RT-qPCR: catalase (Cat); chemokine-like receptor 1 (Cmklr1); erythropoietin receptor (Epor); fragile histidine triad (Fhit); neuropeptide S receptor 1 (Npsr1); purinergic 2X receptor 7 (P2rx7); and tachykinin, precursor 1 (Tac1). As they were recently demonstrated among the most stable gene transcripts of the widely used RT-qPCR reference genes in the rodent brain [97,98], the transcripts for tyrosine 3-

monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (Ywhaz) and succinate dehydrogenase complex, subunit A, flavoprotein (Sdha) were chosen as reference genes for this study. Primers for all transcripts were designed in NCBI's online Primer-BLAST software suite set to use strict *in silico* specificity requirements and to preclude primer binding sites on known polymorphic sites. Primer sequences are reported in table S2.2.11.

We used a two-step protocol for RT-qPCR. Reverse transcription was done using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications. 2μg of total RNA were used as template for each sample, a poly-T 20mer priming strategy was used, and reaction volumes were scaled up to 26.25μL. Reverse transcription reactions were done in a MasterCycler Personal PCR Machine (Eppendorf, Hamburg, Germany). The resultant cDNA was diluted 1:5 with nuclease-free water to minimize the effects of any PCR inhibitors. The specifics of cDNA synthesis are listed in table S2.2.4.

Real-time quantitative PCR was done in a StepOnePlus real-time thermal cycler (Applied Biosystems, Foster City, CA, USA) using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) according to manufacturer recommendations. Total reaction volumes of 20μL were used; each reaction contained 10μL of 2X Supermix, 2μL diluted template cDNA, 500nM forward and reverse primers, and nuclease-free water up to the final reaction volume. All reactions were performed in triplicate. We utilized a three-step thermal cycling protocol that included a 30s hot start at 95^{o}C, then 40 cycles of a denaturation step at 95^{o}C for 5s, an annealing step at a temperature empirically-

determined for each primer set for 20s, and an elongation step at 72ºC for 20s. Annealing temperatures for each primer set are available in table S2.4. Fluorescence data were collected at the annealing step of each cycle. All experimental qPCR runs were accompanied by a dilution series to calibrate PCRs for empirical efficiency and by a dissociation curve to determine *in vitro* primer specificity. All consumables are listed in table S2.2.7.

C_q values were determined using the StepOnePlus software, v. 2.1, with the same fluorescence threshold for every qPCR run. Descriptive and inferential statistics were calculated using the Relative Expression Software Tool (REST), v. 2009, which corrects for empirical PCR efficiency, allows for the use of multiple reference genes, and utilizes a Monte Carlo style permutation test for significance [99]. We set the REST software to perform 10,000 iterations for the permutation test, and we also used it to find expression ratio of MSN:ICR.

Genome enrichment analysis and shared synteny analysis

To map genome regions with significant enrichment, we built a novel enrichment algorithm that looks at clustering of dysregulated genes along the length of a chromosome. This algorithm works by walking along the length of a chromosome at 1.25Mb intervals, a distance corresponding to a little less than 2.5 centimorgans in mice. The algorithm bins all genes assayed in our microarray platform within 1.25Mb of the center of each interval and counts them. The bins are staggered to prevent bias against clustering at bin breakpoints. The algorithm counts all genes dysregulated at a nominal P-value of 0.01 or less. The amount of genes significantly dysregulated within

each interval should, under a null hypothesis of no significant enrichment of that genome region, display a binomial distribution with a probability of any given gene being significantly dysregulated at no more than 0.01. Our algorithm calculates the binomial probability of the amount of the amount of dysregulated genes within each interval over the entire genome. The probabilities are Bonferroni-corrected for multiple comparisons, then the $\log_{10}$ of the inverse of the probabilities are graphed. A $\log_{10}(p^{-1})$ of 3 indicates a Bonferroni-corrected probability of a cluster occurring 1 in 1,000 times under the null hypothesis of no significant clustering in any particular genome region. This corresponds to the LOD score of 3 commonly used as the cutoff criterion for strong evidence of linkage in linkage scans. Any $\log_{10}(p^{-1})$ value greater than or equal to 9 is collapsed to 9 for ease of visualization. The resulting graphic examines clusters over the whole genome, and spikes indicate clusters highly unlikely to have occurred by chance. The Excel file used to create this analysis is included as supplementary Table S2.3.

This method for calculating the probability of a gene cluster occurring by chance is vulnerable to statistical artifacts introduced by redundancy in annotation sources, so it is necessary to use an annotation source looking at gene-level and not exon-level information. Additionally, it is necessary to systematically curate any gene-level probeset redundancy out of the annotation source used. We used release 32 of the Affymetrix annotation for our array platform, which contains no exon-level information. Further, we chose the probesets with the lowest P-values to represent their genes, then deleted all other probesets representing each individual gene to get rid of any remaining

gene-level redundancy. We manually inspected the data contributing to each significant finding to confirm that they were not redundant or otherwise problematic.

Shared synteny analysis utilized the homology map on the NCBI's website, which has a map showing homologous genome regions between humans and mice. Graphics were generated using the positional information present in the homology map.

Functional network analysis

We utilized a network analysis methodology similar to that used in a recent biological systems meta-analysis done on human BPD datasets [54]. Using a list of every gene with an FDR-adjusted P-value less than or equal to 0.25 from the gene-level microarray inferential statistics, we utilized the functional annotation clustering tool of NIAID's DAVID software [100,101]. We set the functional annotation tool to default settings with medium stringency and a background matching the array platform we used. The most significantly enriched cluster was related to ribosomes. Many of the genes in this cluster were pseudogenes, so we decided to ignore this cluster for the purposes of gene network analysis. The second most significant cluster we found had gene components generally related to chromatin packaging. We took the genes from this cluster and put them into the MiMI plugin v. 3.11 [102] in Cytoscape v. 2.8.0, setting the software to query genes with shared nearest neighbors in order to get rid of genes not closely related to the chromatin packaging network. The resulting network was large, with 183 nodes and 6234 edges. To reduce this network to the genes most closely related to chromatin packaging, we used the Glay plugin v. 2.0 [103] to find

subnetworks. We found the subnetwork most closely related to chromatin remodeling, which is what we report in this study.

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SUPPLEMENTARY INFORMATION

Supplementary Table S2.1: Tab-delimited text file containing exhaustive microarray statistical analysis for all transcript clusters organized by P-value. Annotation sources from build 32 of the Affymetrix annotation for the Mouse Gene 1.0 ST platform with presumptive annotation filled in by Ensembl's BioMart extension.

Supplementary Table S2.2: Portable document file containing all information on RT-qPCR assays considered essential by the MIQE.

Supplementary Table S2.3: Excel file containing results from the genome enrichment analysis broken down by chromosome.

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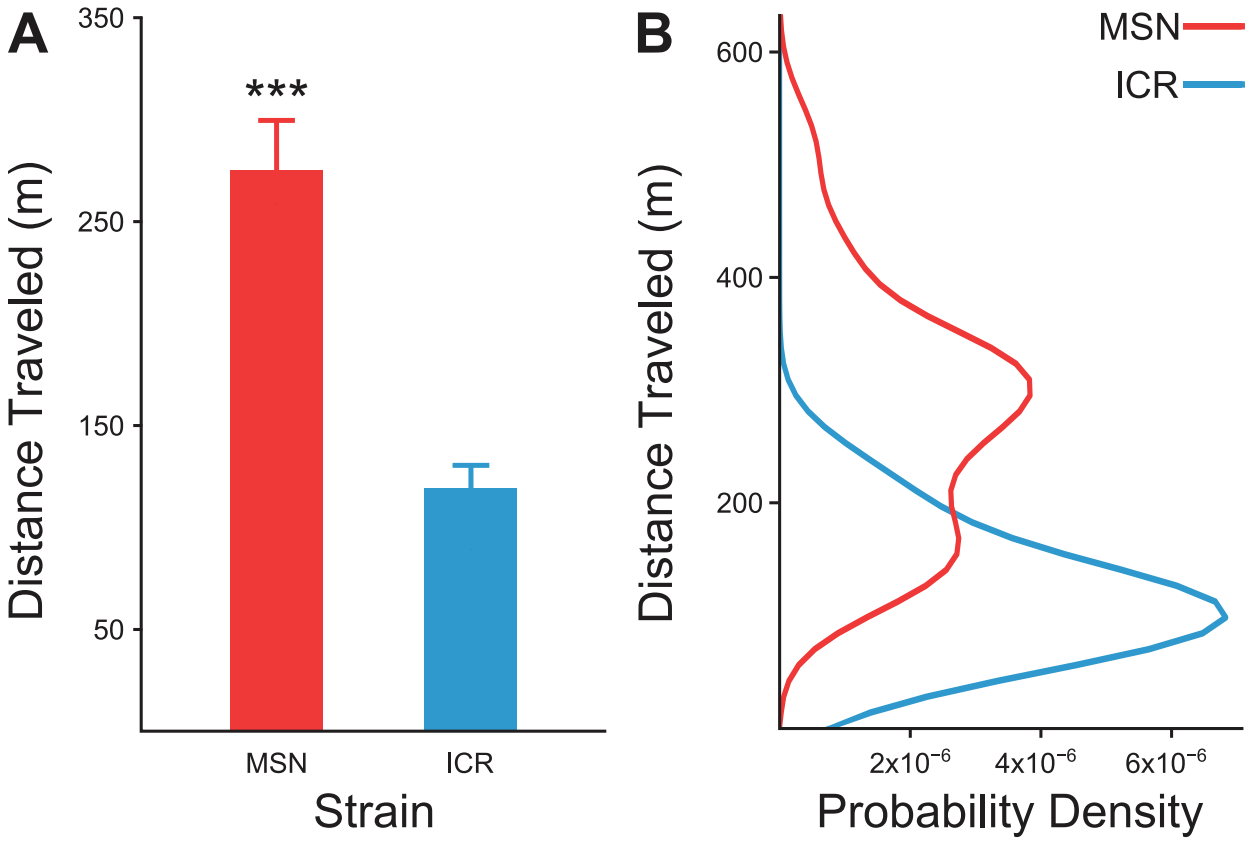
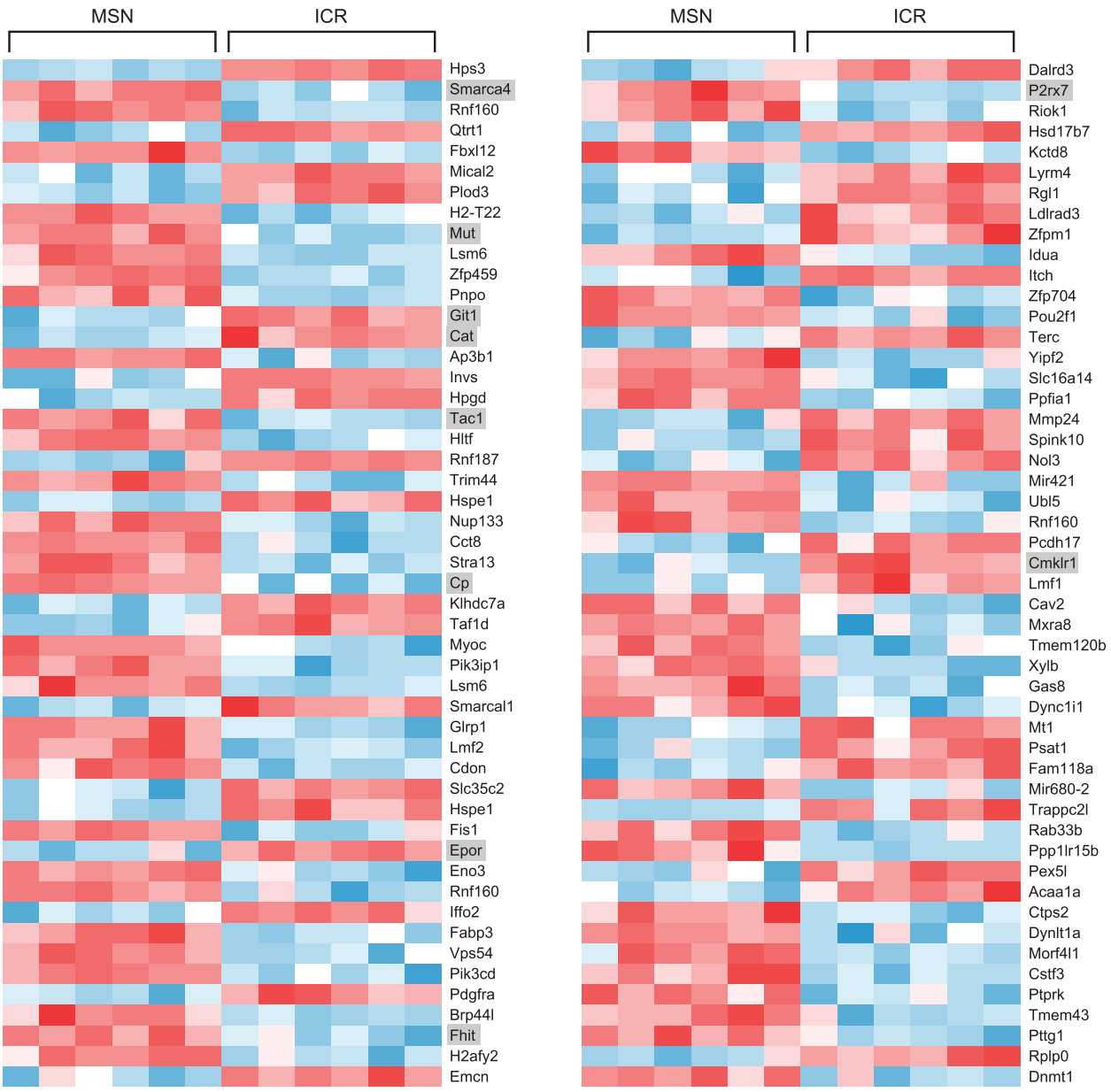
FIGURES

Figure 2.1



Confirmation of the MSN manic phenotype using an experimental replication of the most robust behavioral measure from previous research on this mouse strain, total locomotor activity. **A)** MSN mice display stable heightened locomotor activity relative the outbred strain. *** $P < 0.001$. **B)** The probability density for MSN mouse total locomotor activity is bimodal, while the probability density for the control strain is unimodal. This leads us to the hypothesis that MSN mice may display behavioral bipolarism, a hypothesis that will be examined in future work.

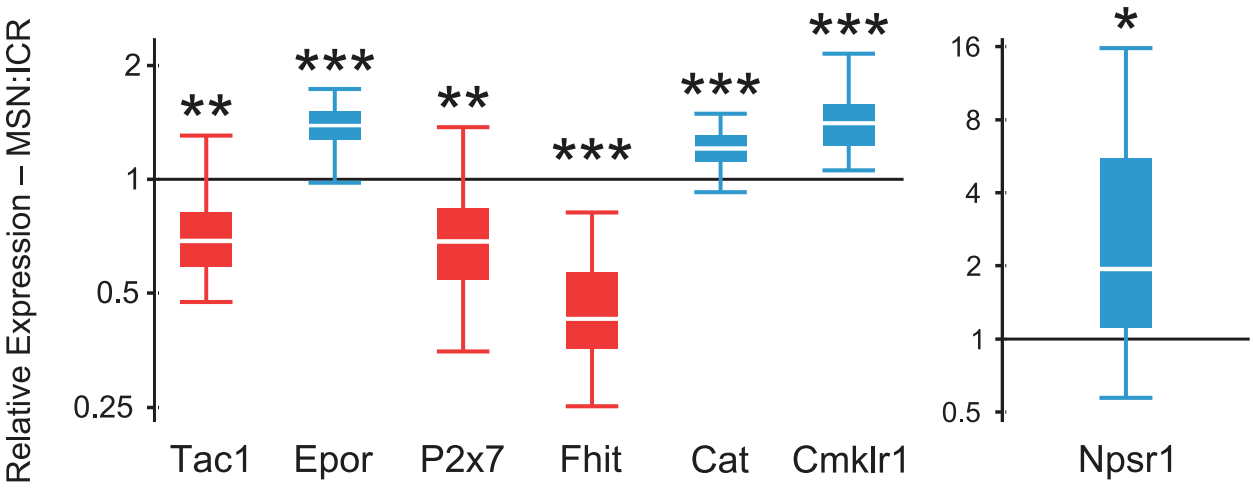
Figure 2.2



Heatmap of normalized values for the top 100 well-annotated genes from the microarray experiment listed by P-value in order from top to bottom, then left to right. The lowest P-values are at the top left corner, and the highest are at the bottom right. Expression values are plotted as a color continuum with red values representing decreased expression, white values representing intermediate expression, and blue values

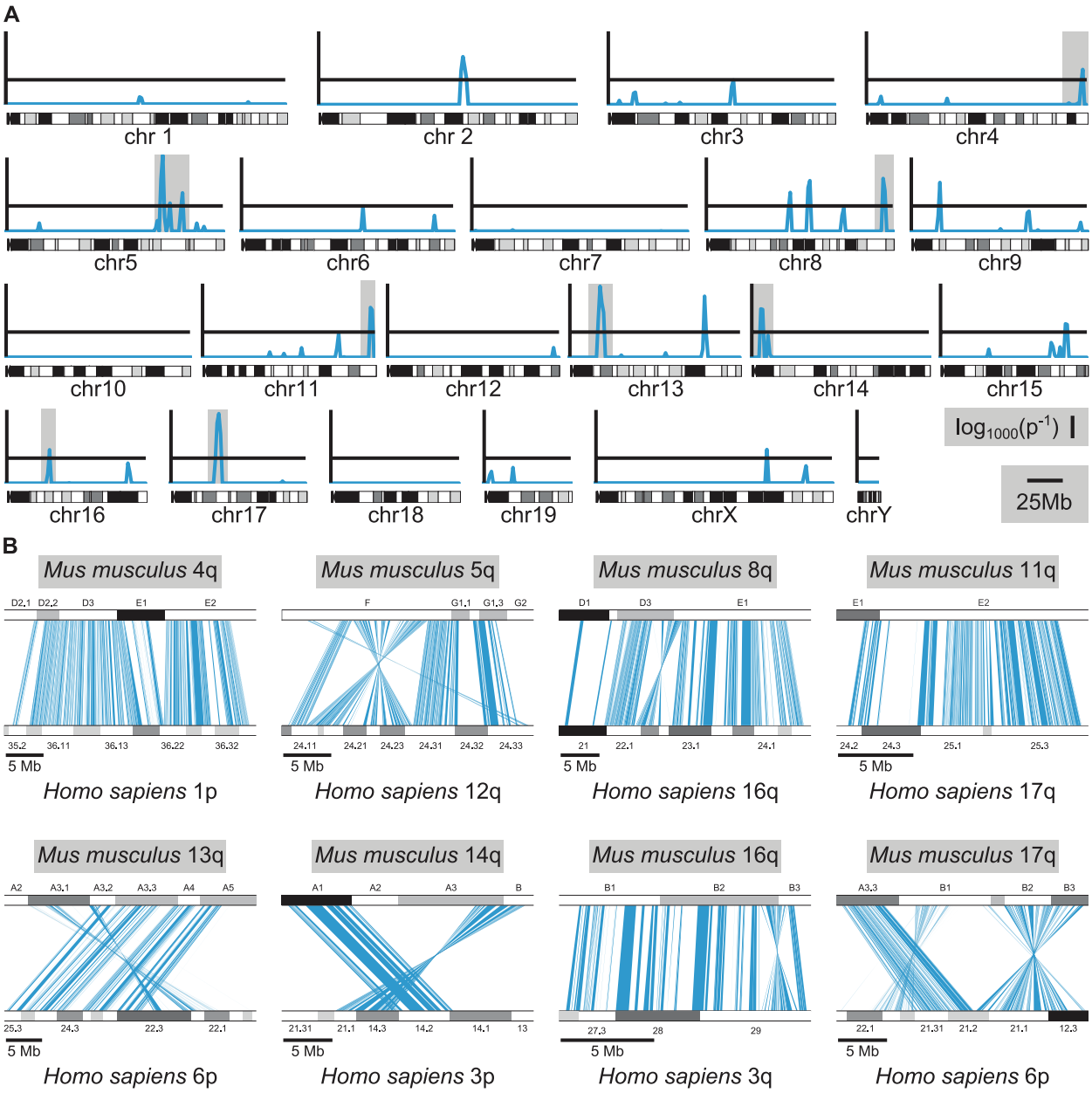
representing higher expression. The names of the individual genes discussed in the text of this manuscript are highlighted in gray.

Figure 2.3



RT-qPCR confirmation results for seven genes from the microarray. Ratio distribution is graphed as a box-and-whiskers plot. Ratios greater than 1 represent genes with higher expression in the MSN strain and ratios less than 1 represent genes with lower expression in the MSN strain relative to the ICR strain. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

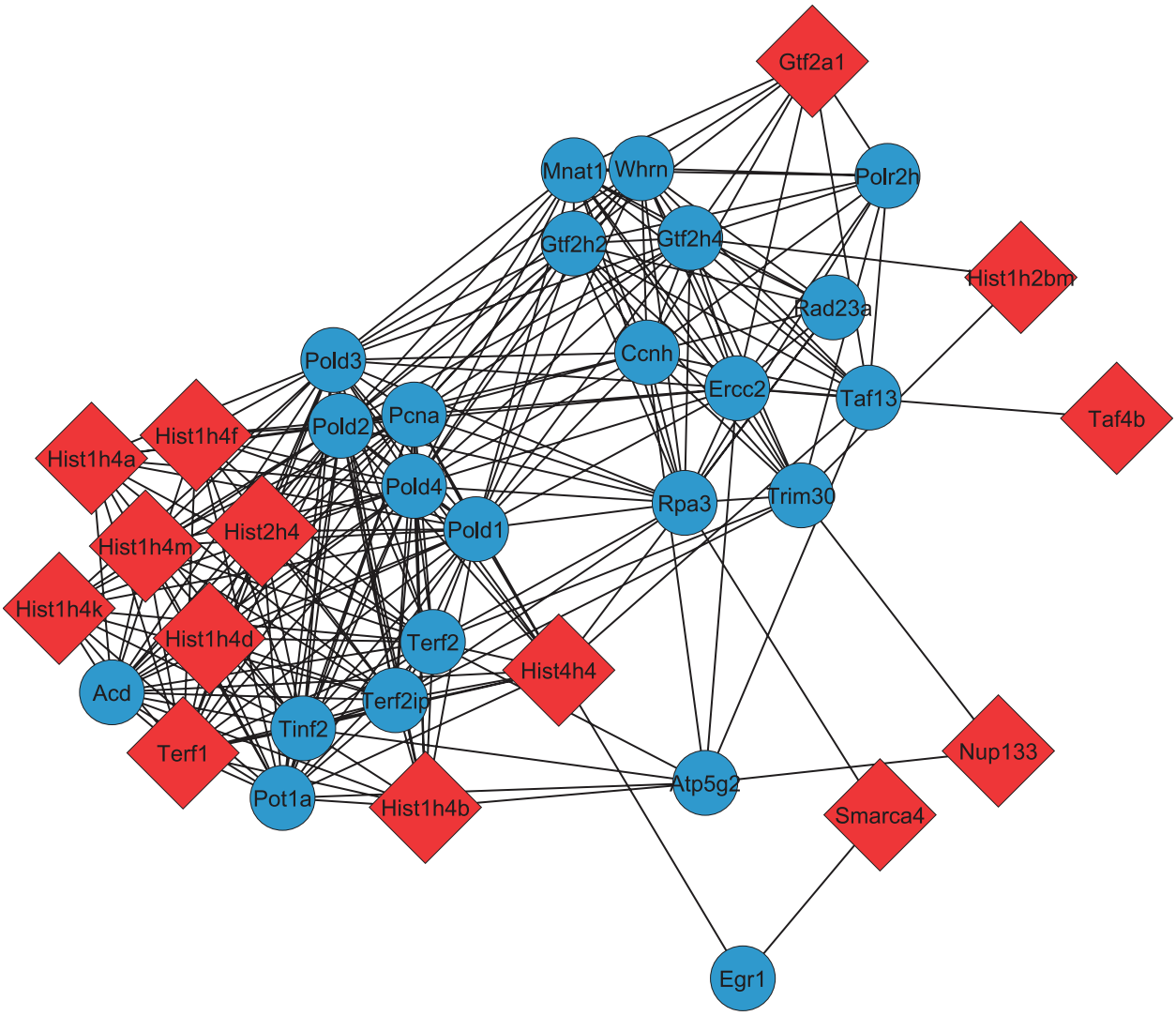
Figure 2.4



Genome enrichment analysis and homology of highlighted enriched clusters to the human genome. **A)** Genome enrichment analysis of the MSN phenotype using a novel enrichment algorithm we created for this study (see Materials and Methods). The y-axis represents the $\log_{10}$ inverse of the corrected binomial probability that a cluster of dysregulated genes would occur by chance. The black horizontal lines demarcate a

cluster occurring by chance with a 0.001 corrected probability, consistent with a LOD or NPL score of 3 in a linkage study. Spikes above the black lines indicate dysregulated gene clusters highly unlikely to occur by chance, indicating that the genome region is significantly enriched. Corrected probabilities less than $1x10^{-9}$ are collapsed to $1x10^{-9}$. **B)** Shared synteny, a similar clustering of orthologous genes, between the clusters on the murine genome highlighted in gray in Fig. 2.4.A and human genome regions strongly implicated in BPD (see Results for details). Blue lines represent orthologous genes and their positions in the murine (upper) and human (lower) genomes.

Figure 2.5



Network analysis of a dysregulated gene network related generally to chromatin packaging. The diamond-shaped red nodes indicate genes found significantly dysregulated MSN mice while the circular blue nodes indicate related genes called by the MiMI plugin for Cytoscape. A recent systems meta-analysis of human BPD genome and transcriptome studies found that a significant chromatin packaging effect is seen across multiple human BPD populations [54].

CHAPTER 3: EXTENDED BEHAVIORAL CHARACTERIZATION

Individual manuscript has been published in *PLoS ONE*.

Saul MC, Stevenson SA, Gammie SC (2013) Sexually Dimorphic, Developmental, and Chronobiological Behavioral Profiles of a Mouse Mania Model. PLoS ONE 8: e72125.

ABSTRACT

Bipolar disorders are heritable psychiatric conditions often abstracted by separate animal models for mania and depression. The principal mania models involve transgenic manipulations or treatment with stimulants. An additional approach involves analysis of naturally occurring mania models including an inbred strain our lab has recently characterized, the Madison (MSN) mouse strain. These mice show a suite of behavioral and neural genetic alterations analogous to manic aspects of bipolar disorders. In the current study, we extended the MSN strain's behavioral phenotype in new directions by examining in-cage locomotor activity. We found that MSN activity presentation is sexually dimorphic, with MSN females showing higher in-cage activity than MSN males. When investigating development, we found that MSN mice display stable locomotor hyperactivity already observable when first assayed at 28 days postnatal. Using continuous monitoring and analysis for 1 month, we did not find evidence of spontaneous bipolarism in MSN mice. However, we did find that the MSN strain displayed an altered diurnal activity profile, getting up earlier and going to sleep earlier than control mice. Long photoperiods were associated with increased in-cage activity in MSN, but not in the control strain. The results of these experiments reinforce the face validity of the MSN strain as a complex mania model, adding sexual dimorphism, an altered diurnal activity profile, and seasonality to the suite of interesting dispositional phenomena related to mania seen in MSN mice.

INTRODUCTION

Bipolar disorders (BPDs) are heritable psychiatric disorders characterized by episodes of mania and depression [1,2]. They are common mental health problems, exhibiting an estimated prevalence between 1% and 5% [3,4]. These behavioral pathologies cause pain and suffering to those afflicted, including the affective oscillations typifying BPDs, side effects from mood stabilizers [5,6], disruption of daily rhythms [7], social dysfunction [8,9], comorbid illicit drug abuse [10,11], psychosis [12], and excess mortality [13,14]. Economically, BPDs have been called the most expensive behavioral health diagnoses [15]. A recent estimate of the per-patient lifetime costs of BPDs in Australia was $76,821-$134,318 AUD ($78,304-$136,910 USD) [16]. The high costs of BPDs may even be increasing; the direct and indirect US economic burden of BPDs more than doubled over 18 years from $45 billion in 1991 ($69 billion in 2012 dollars) to an estimated $151 billion in 2009 ($159 billion in 2012 dollars), a growth in costs well above that expected due to epidemiological factors [17,18]. Though prevalence, heritability, humanistic burdens, and economic costs have made BPDs the subject of intense study by human geneticists, a convincing mechanistic molecular etiology for BPDs remains elusive due to the high likelihood of a polyvalent genotype and to the many technical challenges inherent in working with humans [19-21].

Animal modeling has the potential to elucidate much about BPDs and their mechanistic underpinnings. Models for BPDs typically splits these disorders into the complimentary endophenotypes of mania and depression [22,23]. Single gene transgenics [24-26] and treatment with stimulant drugs [27] are the most frequent

approaches to modeling mania, though for disorders as phenotypically and genetically complex as BPDs, these approaches have limitations. More recently, inbred strains naturally displaying desired endophenotypes have shown utility as models for both poles. The Flinders-sensitive line of rats, an inbred rodent model of depression, has successfully aided in the elucidation of many aspects of depression [28-30]. Valid inbred mania models have only recently been characterized, with the Black Swiss line advanced as a potential inbred mania model [31]. Studies on the Black Swiss strain of mice have shown that while these mice are promising, their use is subject to limitations [32,33].

Our lab has worked to characterize an inbred mouse strain as a model for mania. The Madison (MSN) mouse strain is an inbred strain derived over a period of approximately 15 years via multiple rounds of selection from the outbred hsd:ICR (ICR) strain. A full description of the MSN breeding history appears in our previous work [34]. MSN mice are highly inbred; we have estimated their inbreeding coefficient at 0.95 [35]. MSN mice show increased in-cage activity, decreased sleeping, increased sexual behavior, and increased forced swimming relative to control mouse strains. These mania-like behaviors are not associated with an increase in anxiety measures. Further, the MSN manic phenotype is moderated by lithium chloride and olanzapine (Zyprexa) treatments [35]. The MSN strain shows a suite of brain gene expression differences consistent with BPDs. These gene expression differences imply probable genomic correlates relative to the ICR strain that include genomic loci homologous to human positions implicated in BPDs, schizophrenia, and ADHD, psychiatric disorders with

related molecular correlates [36]. Together, these characteristics suggest that MSN mice share many physiological characteristics with manic aspects of human BPDs [34]. We believe the MSN strain represents a naturally occurring mania model with significant face and construct validity. Further, because our work with the MSN strain utilizes the outbred ICR strain as a natural control, experimentation with MSN mice is methodologically straightforward.

Our previous work elucidated some aspects of the MSN strain's behavior and genetics, but we have yet to answer some essential questions about the phenotype displayed by these mice. Thus far, we have concentrated on MSN males; we have little information on correlates of mania in females from this strain. We do not know the timing of the phenotype's onset during development. Though we have characterized the strain as a primarily manic model, some of our evidence suggests that MSN mice display spontaneous behavioral bipolarism, an interesting finding we have yet to investigate fully. Previous work on these animals' diurnal activity pattern has been rudimentary, and we have not examined the role of seasonality in the MSN phenotype.

The current study seeks to address these limitations of our previous behavioral phenotyping of MSN mice in four experiments. The first experiment characterizes females, seeking both to replicate our previous findings from males in female mice and to describe any sexual dimorphism. The second experiment looks for the age-of-onset of the MSN phenotype from shortly after weaning until early adulthood. The third experiment, a full 28 days of continuous video data collection on the same mice, provides information on both spontaneous behavioral bipolarism and strain diurnal

activity. The fourth experiment examines seasonality as a component of the MSN phenotype, measuring behavior in different photoperiods. In each of these experiments, we use spontaneous in-cage locomotor activity as the dependent behavioral measure. We have found this to be a robust, ecologically-valid measure for observing the MSN phenotype; with modest sample sizes, we have observed that MSN mice consistently show double the in-cage activity of multiple control strains [34,35].

RESULTS

Females

We first tested whether estrous state explained variance in female in-cage activity. We found no significant effect of estrous state on in-cage activity ($F_{3, 28} = 1.67$, $p = 0.20$, $\eta_p^2 = 0.152$) in a one-way ANOVA on transformed in-cage activity and no significant interaction effect of strain and estrous state on in-cage activity ($F_{2, 25} = 2.37$, $p = 0.11$, $\eta_p^2 = 0.159$) in a two-way ANOVA on transformed in-cage activity. Consequently, we excluded estrous state from subsequent ANOVA models.

We found a highly significant strain effect ($F_{1, 60} = 96.28$, $p = 4.4 \times 10^{-14}$, $\eta_p^2 = 0.616$), a significant sex effect ($F_{1, 60} = 11.44$, $p = 0.0013$, $\eta_p^2 = 0.160$), and no significant interaction effect ($F_{1, 60} = 0.04$, $p = 0.84$, $\eta_p^2 = 0.001$) using a two-way ANOVA on transformed in-cage activity. The results of pairwise post-hoc tests are reported in Supplementary Table S3.1a. All results are back-transformed and summarized in Figure 3.1. MSN females displayed locomotor hyperactivity relative to outbred females. Females from both strains displayed heightened in-cage activity compared to males.

A few MSN females showed 24-hour in-cage activity nearly half an order of magnitude higher than other MSN females. After inspecting the raw ethometry traces for errors, we confirmed that six MSN females were extraordinarily hyperactive, travelling over 1km over the course of 24 hours. In all our data on males, we can confirm only a handful of isolated instances of a male animal traveling over 1km in 24 hours and no instances of males exhibiting this extraordinary locomotor hyperactivity in the same photoperiod as the females tested here. One female travelled over 6km in 24 hours while within a cage measuring 30.5cm by 17.7cm.

Development

To observe developmental time course, we recorded 24 hours of behavior each week for 8 MSN and 8 ICR mice between postnatal weeks 4 and 7. We found a highly significant strain effect ($F_{1, 56} = 102.07$, $p = 3.2 \times 10^{-14}$, $\eta_p^2 = 0.646$), no significant effect of week studied ($F_{3, 56} = 1.72$, $p = 0.17$, $\eta_p^2 = 0.084$), and a modestly significant interaction effect ($F_{3, 56} = 2.96$, $p = 0.040$, $\eta_p^2 = 0.137$) using a two-way ANOVA on transformed in-cage activity data. The results of pairwise post-hoc tests are reported in Supplementary Table S3.1b. Back-transformed data are summarized in Figure 3.2. These results suggest that ICR in-cage activity may start higher and attenuate somewhat between weeks 4 and 7. These results also indicate that MSN mice already display hyperactivity after weaning and appear to display relatively stable locomotor hyperactivity over time.

Month Long Observations

To investigate spontaneous behavioral bipolarism in MSN mice, we observed 8 MSN and 8 ICR males continuously over 28 days between postnatal weeks 8 and 12. Figure 3.3 shows the probability density functions for all uninterrupted 24-hour periods for which we have data. While the MSN probability density functions are generally elevated from the ICR probability density functions, these individual probability density functions do not show the strong spontaneous bipolarism we saw in the MSN group probability density functions in our previous work [34].

We broke the month long measurements into half-hour increments for a complete and high-resolution portrait of diurnal activity profile. These observations are displayed by strain in Figure 3.4A, and all formal tests are available in Supplementary Table S3.1c. We found that the MSN activity profile is very different from the ICR activity profile. MSN mice showed significantly higher in-cage activity during the second half of the light period while ICR mice were mostly still asleep (at 1500: $t_{10.64} = 3.51$, FDR-adjusted $p = 0.019$) and they continued to display significantly higher in-cage activity in the first half of the dark period compared to ICR mice (at 2100: $t_{13.73} = 3.99$, FDR-adjusted $p = 0.013$). Their in-cage activity levels fell dramatically during the second half of the dark period, showing no statistically significant difference from ICR mice starting an hour after midnight (at 0100: $t_{10.22} = 1.81$, FDR-adjusted $p = 0.155$), and between 4am and lights on at 6am, MSN mice displayed a trend toward lower in-cage activity than ICR mice (at 0530: $t_{7.57} = -2.47$, FDR-adjusted p = 0.077). These results clearly demonstrate that MSN mice display a response to transitions between light and dark

periods, but their overall diurnal activity profile appears to display an advanced angle of photoentrainment. The MSN activity profile also appears more stereotyped than the ICR activity profile; in Figure 3.4B, the 8 individual MSN mice display activity profiles very similar to one another while the 8 ICR mice display idiosyncratic activity profiles that only become one general activity profile when averaged together.

Photoperiod

After observing the refined diurnal activity profile in the month long activity study, we evaluated whether MSN mice display alterations in in-cage activity in photoperiods associated with different seasons. We raised groups of 8 MSN and 8 ICR males from weaning to 12 weeks in 3 photoperiods: 18h (18:6 L:D), 12h (12:12 L:D), and 6h (6:18 L:D). Activity profiles when housed in these different photoperiods are displayed in Figure 3.5.

In the 18h photoperiod, MSN mice exhibited very high in-cage activity during the dark period and an apparent increased in-cage activity during the light period close to the transitions (Figure 3.5A). This suggested a general elevation of MSN in-cage activity in long photoperiods. Formal testing of this hypothesis found a highly significant strain effect ($F_{1, 41} = 105.72$, $p = 6.5 \times 10^{-13}$, $\eta_p^2 = 0.721$), a highly significant photoperiod effect ($F_{2, 41} = 13.86$, $p = 2.5 \times 10^{-5}$, $\eta_p^2 = 0.403$), and no significant interaction effect ($F_{2, 41} = 1.59$, $p = 0.22$, $\eta_p^2 = 0.001$) in a two-way ANOVA on transformed 24-hour in-cage activity data. The results of pairwise post-hoc tests are reported in Supplementary Table S3.1d. Back-transformed data are summarized in Figure 3.5B. Together, these results show that MSN mice display heightened activity when in long photoperiods, a finding

not seen in the outbred control strain whose in-cage activity appears to be a stable baseline.

To better characterize how MSN mice display heightened activity in long photoperiods, we examined the relative amount of activity occurring in the light period versus the dark period. The dark period results mostly mirrored the 24-hour activity results. We found a highly significant strain effect ($F_{1, 41} = 95.76$, $p = 2.8 \times 10^{-12}$, $\eta_p^2 = 0.700$), a highly significant photoperiod effect ($F_{2, 41} = 33.39$, $p = 2.5 \times 10^{-9}$, $\eta_p^2 = 0.620$), and no significant interaction effect ($F_{2, 41} = 1.59$, $p = 0.22$, $\eta_p^2 = 0.001$) in a two-way ANOVA on inverse square root transformed in-cage velocity for the dark period. Pairwise tests are summarized in Supplementary Table S3.1e, and back-transformed data are summarized in Figure 3.5C. The light period results strongly contrast with the full 24-hour in-cage activity data. Here, there was a highly significant strain effect ($F_{1, 41} = 19.85$, $p = 6.3 \times 10^{-5}$, $\eta_p^2 = 0.326$), a highly significant photoperiod effect ($F_{2, 41} = 25.55$, $p = 6.2 \times 10^{-8}$, $\eta_p^2 = 0.555$), and a significant interaction effect ($F_{2, 41} = 5.19$, $p = 0.0098$, $\eta_p^2 = 0.202$) in a two-way ANOVA on square root transformed in-cage velocity. Pairwise tests are summarized in Supplementary Table S3.1f, and back-transformed data are summarized in Figure 3.5D.

Altogether, these results suggest that while photoperiod alters the ratio of light period to dark period in-cage activity in ICR mice, that strain's 24-hour in-cage activity remains constant in all photoperiods. In MSN mice, the light to dark in-cage activity ratio is similarly altered, but 24-hour in-cage activity significantly increases in the long photoperiod. As an increase not seen in the control strain, this long photoperiod

augmentation of locomotor hyperactivity suggests that MSN have a mania with a comorbid seasonal component. Because mice are nocturnal animals, higher in-cage activity under a shorter dark period is a curious and seemingly paradoxical finding.

DISCUSSION

Females

The presentation of the MSN phenotype is sexually dimorphic. Both MSN sexes showed in-cage locomotor hyperactivity relative to outbred control mice, but MSN females displayed significantly higher hyperactivity than their male cohorts. Further, some MSN females displayed total in-cage activity in excess of 1km, and one female displayed total in-cage activity of greater than 6km. The origin of this enhanced hyperactivity occurring only in females is unclear. In a previous study on in-cage activity in ICR mice, outbred female mice displayed higher baseline in-cage activity than males [37]. Median female to male activity ratios do not differ significantly between the MSN and ICR strains as studied here (Monte Carlo permutation test, $p = 0.88$, B = 1000), so it appears this normal female-to-male ratio persists in MSN mice. However, the distribution of female MSN mice appears to skew toward the high end much more than the female ICR mice we studied. This sex difference may need more characterization in the future.

In humans, though BPDs show equal prevalence in males and females [38], the outcomes are sexually dimorphic. Relative to men, women with BPDs display later disorder onset [39], tend to cycle more rapidly [40], experience a different subset of comorbid psychiatric disorders [41], and are more prone to mixed manic episodes [42].

Additionally, female reproductive state is associated with disorder presentation [43]. Equal prevalence but differential presentation between the sexes implies that though the genetic basis of BPDs remains constant between the sexes, these heritable underpinnings interact with female physiology differently than they do with male physiology to cause a sexually dimorphic phenotype. Dimorphism in both humans and MSN mice may enhance the face validity of the MSN strain as a mania model, though a more complete phenotyping of female MSN mice will be necessary to examine this hypothesis. Helping characterize the nature of sexual dimorphism in mania presentation may prove an important role for MSN mice.

Development

From the earliest time point we can reliably record in-cage activity, MSN mice display an observable hyperactive phenotype. The phenotype appears to be stable, affecting mice equally at the full range of dates tested in this study from 4 weeks old to 13 weeks old. In humans, BPDs are often diagnosed in late adolescence to early adulthood [44], though this age of onset is highly-variable [45] and earlier age of disorder onset is a predictor of the severity of BPDs [46]. There has been a recent trend toward the controversial diagnosis of juvenile BPDs [47,48]. Still, the current consensus on the onset of BPDs appears at odds with our MSN results, a possible caveat to the face validity of the MSN strain.

A contemporary evidence-based theory on the staging of BPDs posits that affective disruptions are rarely observed until early adolescence, but non-affective disruptions predictive of BPDs including hyperactivity, sleep disruptions, and anxiety are

observable at very young ages [49]. Additionally, since human BPDs are highly heritable [2], we would argue that these disorders exist latent in humans even at early stages of development. Thus, it is possible that the high in-cage activity we see in even young MSN mice is consistent with pre-bipolar hyperactivity and sleep disruption in humans. In these experiments, we measured locomotion, and spontaneous locomotor activity may not be synonymous with affect. Thus, correlating hyperactivity in emerging human BPDs and in-cage activity in young MSN mice may still be of interest.

A Unipolar Mania Model

The current evidence suggests that MSN mice do not display a true bipolar phenotype. Instead, they appear stably manic as measured by in-cage locomotor activity. If bipolarism exists in MSN mice, it is difficult to detect. This result is consistent with a previous study on bipolarism in a transgenic mouse line in which only one strong depressed phase was observed in a single mouse as assayed by wheel running [50]. MSN mice may similarly display behavioral bipolarism, but if they do, it is not on any timescale we can reasonably observe.

Altered Diurnal Activity Profile

BPDs have a high comorbiditiy with altered diurnal preference in humans [51,52]. Further, total sleep deprivation has antidepressant effects [53,54], sometimes even throwing patients with BPDs from depression into a manic state [55]. Social rhythm therapy, a new, successful, and non-pharmacological treatment for BPDs, is primarily a chronobiological intervention, implying a strong diurnal constituent to these disorders [56-58].

These chronobiological alterations in patients with BPDs may reflect an altered diurnal entrainment mechanism in the brain that is only partially-characterized [59]. There is an association between molecular clock genes and mood disorders in humans [60-62], and alterations of molecular clock genes have been used to model BPDs in mice [25]. However, as many molecular clock genes are orphaned receptors [63], the mechanistic nature of altered diurnal preference in BPDs remains unresolved. Nonetheless, there is strong evidence that chronobiology and BPDs are deeply intertwined.

MSN mice display a diurnal activity pattern marked by earlier rising than outbred controls, very high in-cage activity early in the dark period, and a precipitous drop in in-cage activity midway through the dark period. This diurnal activity pattern, an advanced angle of photoentraiment, is potentially analogous to morningness, a tendency to rise earlier and sleep earlier, in humans [64], though the precise physiological correlation to humans is unknown. Because mice are nocturnal and humans are diurnal, precisely determining the correspondence between murine and human diurnal activity patterns is difficult. Regardless, the advantage of having a mania model with a comorbid altered diurnal activity pattern is readily apparent. Chronobiological interventions can be tested to see how altering diurnal patterns might affect mania. The MSN diurnal activity pattern may provide additional predictive validity for these animals in the future, a move toward translation that could represent a fruitful new direction for this strain.

Photoperiod-Dependent Elevated Hyperactivity and Seasonality

Seasonal affective disorder (SAD) and BPDs have a high comorbidity [65]. Seasonal disruptions in sleep are associated with BPDs [65]. Seasonal decreases in performance on neuropsychological tasks are unusually high in both patients with BPDs and their psychiatrically healthy relatives [66], implying a strongly genetic component to this comorbid seasonality. Additionally, BPDs occur with higher frequency in people born in the winter and in the early spring, though the causality and relevance of this winter-spring birth excess of patients with BPDs are heavily disputed [67].

SAD has few well-validated animal models, and most working models show depression in short days [68]. MSN mice are different, displaying long-day increases of in-cage activity consistent with a seasonally dependent elevation in hyperactivity, the opposite effect seen in other seasonally variable animals. Further, this elevation in activity is not seen in the outbred strain from which MSN mice were derived. Mice are nocturnal, and nocturnal animals moving more in a shortened dark period appears contradictory. This paradoxical long photoperiod increased hyperactivity necessitates some underlying physiological correlate, though we can only speculate about the identity of this component at present. A seasonal constituent unique to the MSN strain could be the result of selection for altered melatonin or kisspeptin systems, which have effects on the GnRH axis and lead to seasonal breeding patterns in other species [69-71]. This represents an ethologically based, testable, and parsimonious hypothesis for the physiological basis of the seasonal-like alterations of in-cage activity seen in MSN mice.

Conclusions and Future Directions

MSN mice are a complex model for the manic pole of BPDs displaying multiple aspects of the human disorders it replicates. Our previous research shows that these mice display a rich suite of behavioral and neural gene expression correlates consistent with a face- and construct-valid mania model. We have also demonstrated that MSN mice display probable genomic perturbations at loci homologous to human loci linked to BPDs, schizophrenia, ADHD, and related disorders. Here, we have added to the corpus of dispositional traits consistent with human BPDs seen in MSN mice. They display sexually dimorphic hyperactivity, an altered chronotype, and photoperiod-dependent increases in hyperactivity. However, unlike humans, the MSN phenotype appears stable during development, and they do not display bipolarism.

The utility of the MSN mouse strain will come from its use as a tool for the translation of basic psychogenetics to human health interventions. Like human BPDs, the MSN phenotype is a complicated one. Understanding the physiological and genetic underpinnings of BPDs is as essential as it is difficult, and we hope that the MSN mouse strain aids in this genetic study. We are currently investigating some of the possible genomic perturbations in these mice seen in the neurogenetic paper we published recently [34]. A partial sequence of the MSN genome is the likeliest path toward novel insights into this phenotype. We are interested in correlating these genotypic differences to human disorders.

MATERIALS AND METHODS

Ethics Statement

Animal use was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the IACUC for the University of Wisconsin–Madison College of Letters and Sciences (protocol #L00405-0-05-09), and all reasonable efforts were made to minimize animal suffering.

Animals

The Madison (MSN) mouse strain is a mostly inbred strain derived over a period of approximately 15 years from the outbred hsd:ICR (ICR) strain (Harlan Laboratories, Madison, WI, USA). We estimate the current inbreeding coefficient of the MSN strain at approximately 0.90-0.95. Full details of the inbreeding are described previously [34,35]. As the genetic background for the MSN strain, the ICR strain is a natural control. We keep breeding colonies of each strain in our laboratory to eliminate as many environmental differences as possible, though we breed new males ordered from Harlan into our ICR colony to prevent this population from experiencing genetic drift. The MSN mouse strain has been submitted to the NIH Mutant Mouse Regional Resource Centers as stock number 036809-MU.

Behavioral Apparatus

We created a custom experimental apparatus for the behavioral experiments contained in this paper. The apparatus was a 4x4 matrix of clear acrylic home cages in

which mice were individually housed modeled after the apparatus used in Zombeck et al. [37]. Cages were manufactured from clear 3/8” acrylic by the shop in the University of Wisconsin–Madison’s Department of Zoology. They measured 30.5cm by 17.7cm and had steel mesh inserts in the end walls to enhance ventilation. The cages were placed on a black tabletop and purpose-built steel camera rig was placed over the table. Four low-light sensitive security cameras (Panasonic WV-CP284) were placed overhead to capture video.

Since the video ethometry suite we use needs high contrast between the animal and its background in order to analyze position, we used a 1:1 mixture of the bedding materials Cellu-Dri Soft and PAPERCHIP and added the bedding/enrichment material EnviroDri (Shepherd Specialty Papers, Kalamazoo, MI, USA). These materials are dark grey or brown and are easily distinguished by our software from a white mouse under even the lowest light. To keep glare from disrupting the analysis software, cages were lit from below the table. Compact fluorescent red lights were kept on 24h a day so that we could collect video during the dark period. Compact fluorescent white lights were put on timers appropriate to the photoperiod chosen for the specific group of mice under study, generally 12h light and 12h dark unless otherwise noted. This behavioral apparatus was inspired by the one used by Zombeck et al. [37].

Software and Statistics

We used TopScan v. 2.00 (CleverSys, Reston, VA, USA) for data analysis as described previously [34,35] with some important modifications. Instead of using the software’s online video analysis capabilities as we did previously, we collected 48 half-

hour mpeg files per 24 hours and analyzed them offline from a separate computer networked to the computer collecting the data. Our new behavioral apparatus allowed for us to measure activity continuously for all 24 hours each day instead of 5.5 hours during the light period and 9.5 hours during the dark period as we were doing previously. Additionally, by collecting videos in half-hour increments, we could analyze each half-hour separately and compare behavior at different time points throughout the day. This allowed both continuous data collection and high-resolution analysis of diurnal activity profile.

All statistical analyses were performed using R v. 3.0.1 (x86_64) in OS X v. 10.8.4. Remedial transformations for datasets were chosen using the Box-Cox power transformation method implemented in the R package MASS v. 7.3-23. Type II ANOVAs and effect sizes were calculated using the R package heplots v. 1.0-5. Plots were generated using the R package lattice v. 0.20-13. Welch's two-sample t-tests were generated using the t.test() function in R. All *p*-values are from two-sided tests, and FDR adjustment was done using the p.adjust() function in R.

Upon examination of all 24-hour in-cage activity data contained in this paper, we determined that they were problematically non-normal and heteroscedactic. We found that an inverse square root transformation adequately remediated this problem for 24-hour in-cage activity data. All inferential statistics are performed on data thus transformed unless otherwise noted.

Because any statistical correction is conservative, when presenting pairwise tests, we chose a cutoff for our Tukey HSD *p*-values of 0.10. We note that this is a

deviation from the customary *p*-value significance cutoff of 0.05. We checked the nominal significance of each of these tests using two-tailed t-tests, and each was nominally significant at $p < 0.05$. We believe that because these data are exploratory, type I errors are more acceptable than type II errors.

Experiment 1: Female Observations

We were concerned primarily with presence or absence of a phenotype in females. Because of estrous cycling, we believed we would see greater variance in females, so we doubled the replicates we generally used for testing the males' in-cage activity, looking at 16 females from each strain from a single generation. Shortly after behavioral testing, we determined estrous state in females by vaginal lavage. We also looked for sexual dimorphism, comparing these females to equal numbers of age-matched males from each strain.

We used animals aged between postnatal weeks 12 and 13 for this experiment. When we previously looked at 2 days' data for the simple presence or absence of the MSN phenotype, we found the same results for the first day as the second. This is evidence that habituation is unnecessary to observe the effect, so we measured in-cage for the 24 hours directly after putting the animals in the experimental apparatus. All animals used in this experiment were group housed prior to behavioral data collection in a 12h photoperiod and had ad libitum access to water and food.

Experiment 2: Developmental Observations

To assess whether the MSN phenotype is present as soon as in-cage activity is easily observable, we designed an experiment that looked at in-cage activity in juvenile

male mice shortly after weaning until they reached adulthood. These mice were group housed at 12h photoperiods and had ad libitum access to food and water. We took 24 hours of behavioral data without habituation each week between postnatal weeks 4 and 7 from 8 MSN mice and 8 ICR mice from the same groups over the course of four weeks. Since the mice were group housed, it was not practical to keep track of individuals. Consequently, we do not have repeated measures for these animals.

Experiment 3: Month Long Observations

8 MSN and 8 ICR males from the same generation were group housed from weaning to postnatal week 8. The animals were raised under 12h photoperiods and had ad libitum access to water and food. At 8 weeks, the mice were individually housed in experimental cages. Since a primary purpose for this experiment was to explore bipolarism, we wanted to get stable data for in-cage activity with as few outside influences as possible. Consequently, animals were allowed to habituate for 24 hours prior to collection of in-cage activity data. We collected behavioral data for 28 days in total. We cleaned cages during the light period once every 7 days and excluded the data from that light period and the subsequent dark period from our analysis.

We experienced occasional difficulties during this experiment. In one case (ICR 13), the bedding materials were distributed in an unusual manner, causing a failure in tracking for most of one light period and one dark period. We excluded the affected light and dark periods for this animal. Additionally, the computer collecting the video experienced a blue screen event and crashed for 27 hours at days 21 and 22 of the experiment. We have no data for any animal during this time period.

When looking at probability density for 24-hour activity, we excluded all days for which we did not have a dark period and subsequent light period from our density plots. All observations in these plots contain a full 24 hours of in-cage activity from lights off to lights off. When looking at the diurnal activity pattern, we first found the mean in-cage activity expressed as a velocity for each animal at each time point, averaging all available data from each time point. We used these averages to find group means for both MSN and ICR. These data are double plotted with standard errors of the mean as ribbons around the lines.

Experiment 4: Photoperiod Observations

To assay effects of photoperiod, we took mice directly from weaning and group housed them in rooms with 3 different photoperiods. A group of 16 male mice, 8 MSN and 8 ICR each, was housed in each the following photoperiods: 6h of light (6:18 L:D), 12h of light (12:12 L:D), and 18h of light (18:6 L:D). Mice were group housed in these photoperiods until they were aged 12-13 weeks. Before the experiment, one mouse from the MSN 6h photoperiod group died, so we only ended up testing 7 MSN mice and 8 ICR mice from that photoperiod. We collected behavioral data in the same photoperiod in which the mice were raised for 24 hours after an hour of habituation.

ACKNOWLEDGEMENTS

We would like to thank Scott Timme for his invaluable work on fabricating the behavioral apparatus.

SUPPLEMENTARY INFORMATION

S3.1: Supplementary tables containing statistical test results (.pdf).

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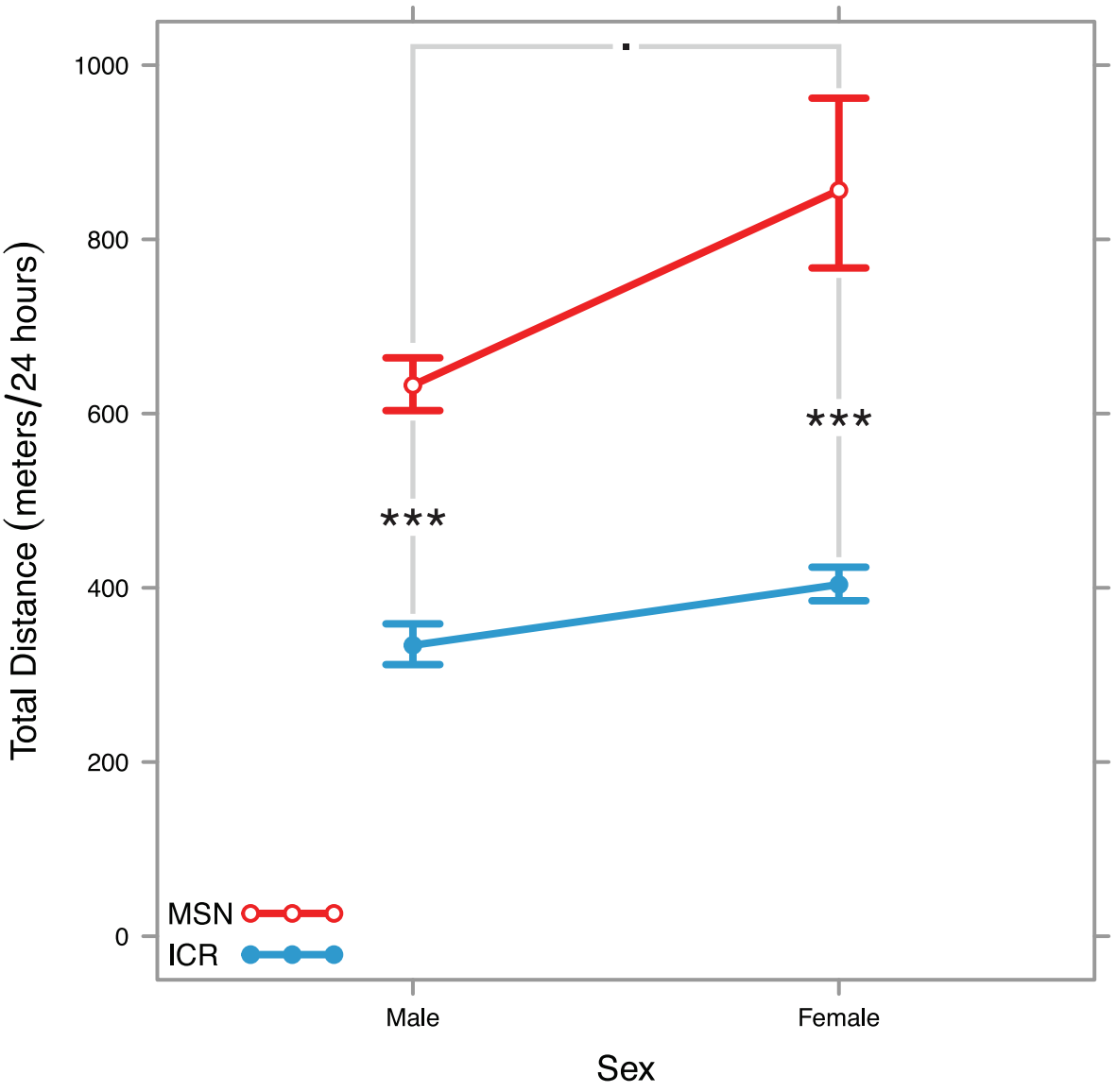
FIGURES

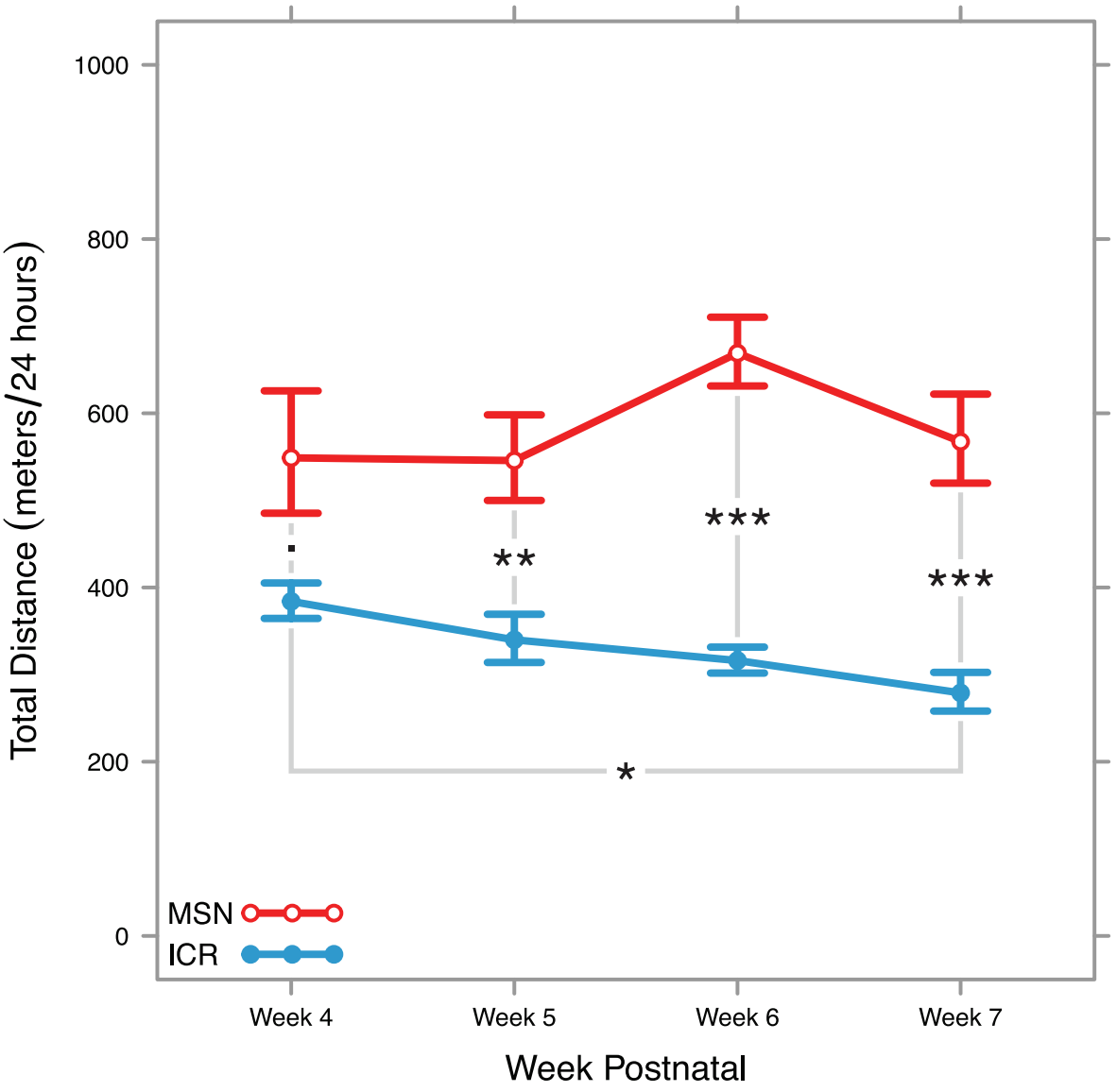
Figure 3.1



Interaction plot showing strain differences between back-transformed means and standard errors of MSN and ICR males and females. A two-way ANOVA found a highly significant strain effect, a significant sex effect, and no significant interaction effect. All

significant pairwise tests are summarized in the plot (Tukey HSD: *** < $p = 0.001$ ≤ ** < $p = 0.01$ ≤ * < $p = 0.05$ ≤ . < $p = 0.10$).

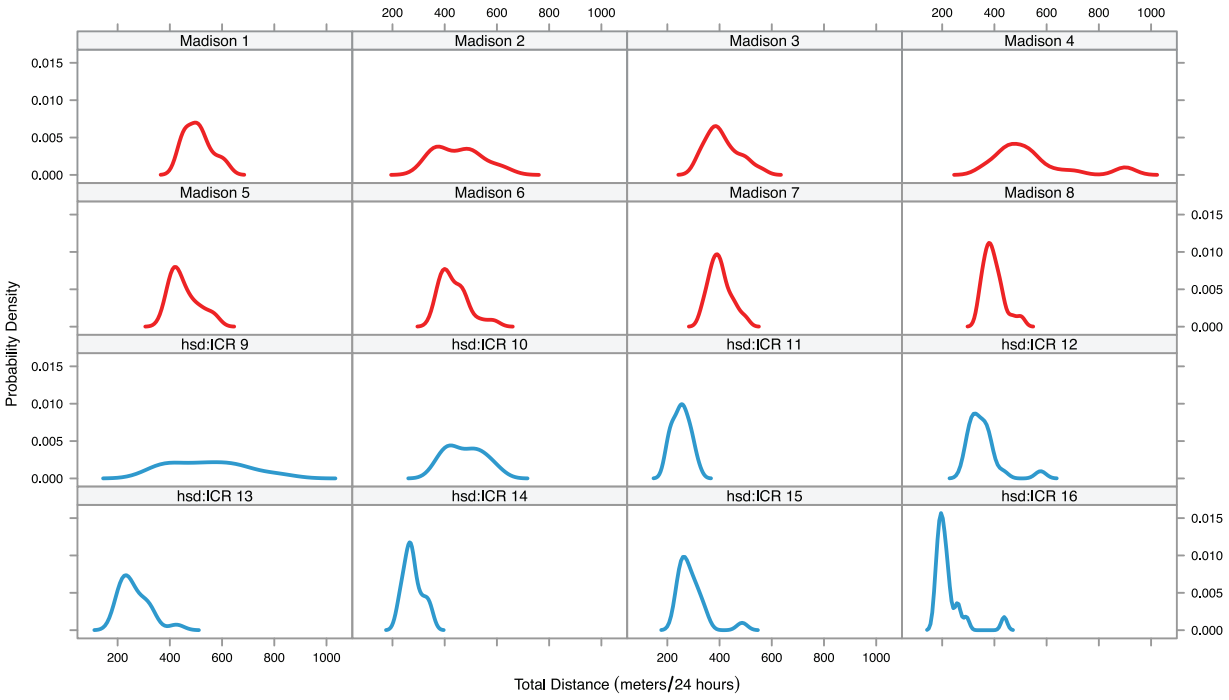
Figure 3.2



Interaction plot showing back-transformed means and standard errors of MSN and ICR mice at four different stages of early development. A two-way ANOVA found a highly significant strain effect, no significant effect of developmental stage, and a mildly significant interaction effect that may be resultant from statistical noise. All significant

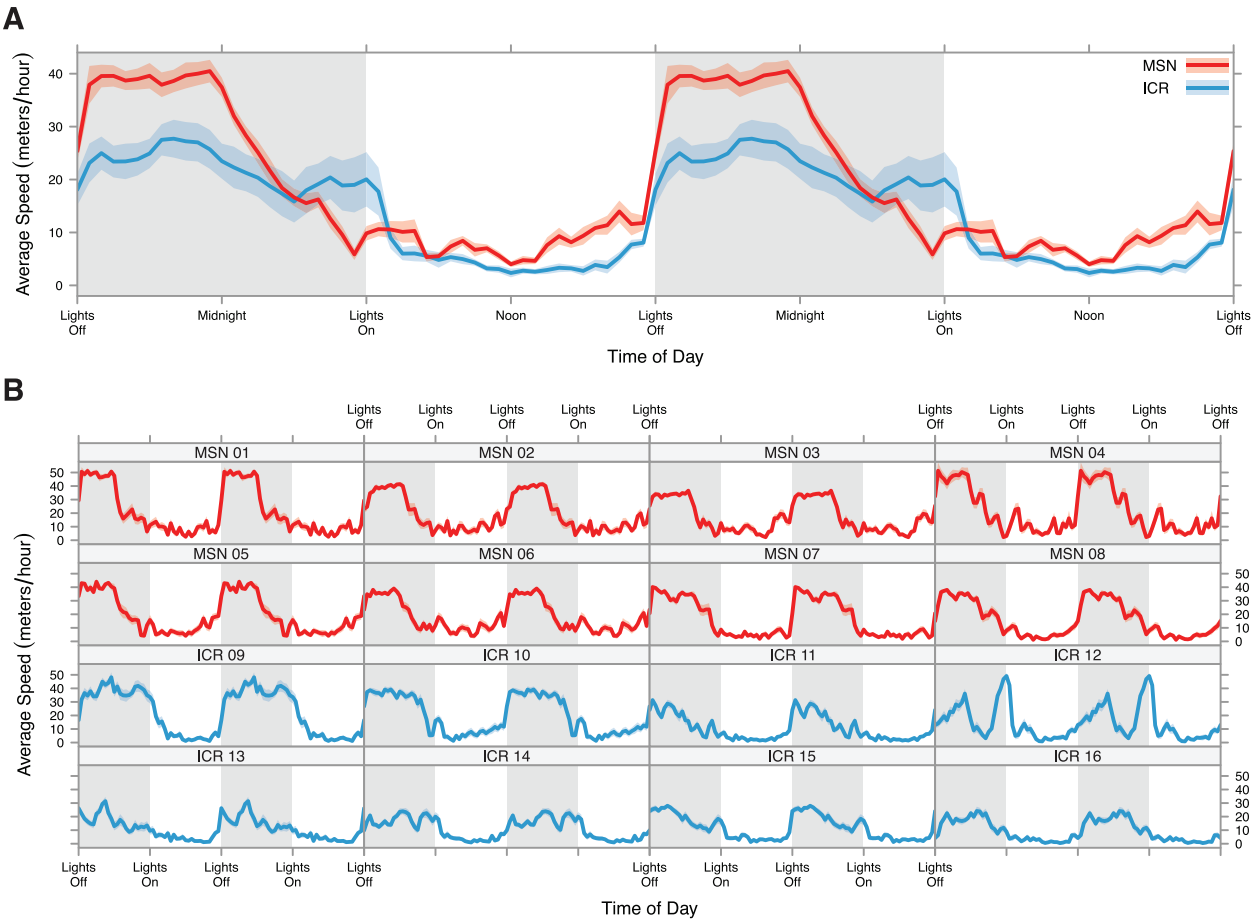
pairwise tests are summarized in the plot (Tukey HSD: *** < $p = 0.001$ ≤ ** < $p = 0.01$ ≤ * < $p = 0.05$ ≤ . < $p = 0.10$).

Figure 3.3



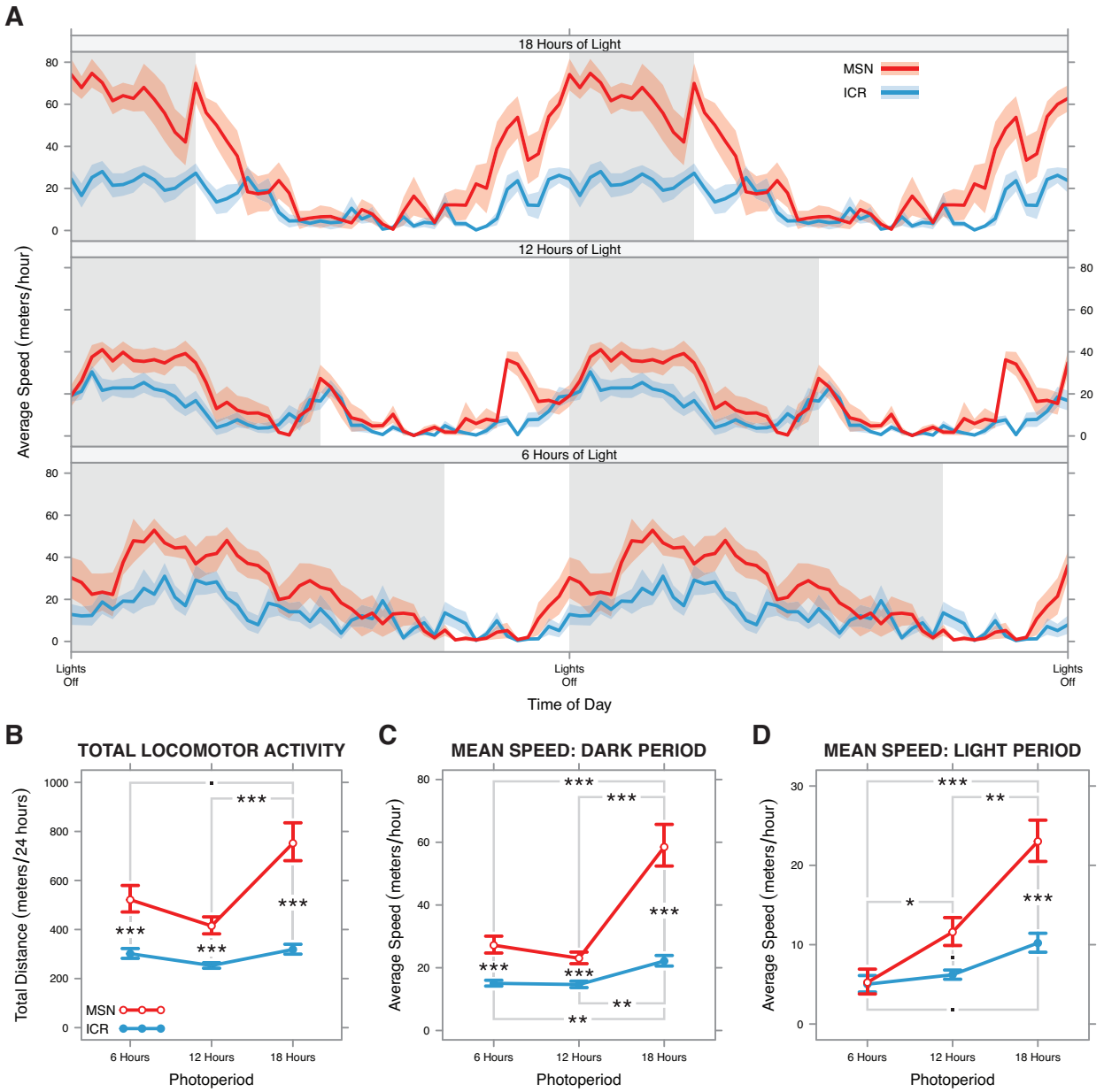
Probability density plots for distance travelled per day from each mouse in the month-long activity experiment. MSN mice showed no noticeable bipolarism relative to ICR mice.

Figure 3.4



Diurnal activity plot of mice examined for a month. **A)** Half-hour in-cage activity averages for each strain with ribbons representing standard error. Diurnal activity panels are double plotted for ease of viewing. MSN mice showed a different diurnal activity pattern than ICR mice, displaying elevated activity just prior to the transition to dark period, highly elevated activity in the early dark period, and a drop in activity midway through the dark period. **B)** Half-hour in-cage activity averages for each mouse studied with ribbons representing standard error. Panels are double plotted for ease of viewing. MSN mice showed less variability in diurnal activity profile than outbred ICR mice.

Figure 3.5



Results of photoperiod experiment. **A)** Diurnal activity plots for each photoperiod examined with lights off times aligned. Diurnal activity panels are double plotted for ease of viewing all time periods. **B-D)** Interaction charts showing back-transformed means and standard errors of: **B)** total 24-hour in-cage activity, **C)** mean speed during the dark period, and **D)** mean speed during the light period. Photoperiod-dependent differences

in total in-cage activity were seen in MSN but not ICR mice (**B**), though photoperiod-dependent alterations to the amount of total activity budgeted in the light and dark periods were seen in both strains (**C**, **D**). All significant pairwise tests are summarized in panels **B-D** (Tukey HSD: *** < $p = 0.001$ ≤ ** < $p = 0.01$ ≤ * < $p = 0.05$ ≤ . < $p = 0.1$

CHAPTER 4: WHOLE EXOME RESEQUENCING

Individual manuscript is in peer review.

Saul MC, Stevenson SA, Zhao C, Driessen TM, Eisinger BE, Gammie SC (in peer review) Exome resequencing of the Madison mouse mania model reveals multiple variants related to bipolar spectrum and related mental health disorders.

ABSTRACT

Bipolar spectrum disorders are modeled using animals with the complementary behavioral endophenotypes of mania and depression. Rodent models for mania typically utilize either single gene transgenics or pharmacological manipulations. Recently, inbred mouse strains have been advanced as potential novel models for mania. While these inbred strain models show great promise, their use up to now has been limited by the lack of available genotypic information necessary to establish construct validity. In this study, we address this lack of genotypic information for one manic mouse strain termed Madison. Using whole exome resequencing, we identified 447 non-synonymous SNPs and short INDELs mostly fixed in the Madison strain relative to multiple control strains. Using multiple filtering criteria and hand annotation, we identified 11 interesting variants we thought likely to alter protein chemistry. We used high-resolution melt curve genotyping to examine population allele ratios at these variants, finding that 6 of them showed population allele frequencies consistent with possible explanatory variants for the Madison strain's phenotype. Those six variants were in *Npas2*, *Cp*, *Polr3c*, *Smarca4*, *Trpv1*, and *Slc5a7*. Many of these genes are in systems implicated in bipolar disorders in humans. A statistical model using sex and the genotypes at the *Smarca4* and *Polr3c* variants – genes involved in nucleosome structure and translation – explained over 40% of the variance in the outbred strain from which Madison animals were derived. Altogether, these results suggest that Madison animals display fundamental construct validity as a mania model at the genotypic level.

INTRODUCTION

Bipolar spectrum disorders (BSDs) are a heterogeneous group of mental health diagnoses marked by episodes of mania and depression [1]. This broad continuum of psychiatric illnesses afflicts 2-3% of the population and has one of the highest suicide rates for known mental health disorders [2,3]. A 2009 estimated US economic impact of $151 billion in direct costs and lost productivity, making it one of the the costliest mental health disorders [4]. Further, the current line of pharmacological interventions used to treat bipolar disorders carry debilitating side effects including metabolic problems and reduced lifespan [5], making development of new treatments a high priority.

BSDs are highly heritable [6]. Development of better treatments for BSDs rooted in these heritable diseases' genetic correlates has been the goal of over a decade's worth of work in genome-wide studies of mental health disorders [7]. Despite the investment of considerable resources into the study of BSDs, a convincing molecular etiology remains elusive [7,8]. The difficulty defining this disease at its molecular level is attributable mainly to its complexity. Genome-wide linkage scans and association studies of BSDs typically contain multiple genomic findings that change depending upon the population examined [9-12], suggesting that the disease originates from heterogeneous alterations of many genes in the biological systems contributing to the BSDs phenotype. Further, BSDs appear genetically related to other mental health disorders like schizophrenia [13]. Because of these complexities, studying BSDs necessitates a systems biology approach [14,15].

Modeling BSDs should similarly utilize a systems approach. In rodents, models for BSDs primarily split these illnesses into two constituent behavioral endophenotypes reflecting mania and depression [16]. As far as we know, nobody has observed an animal model reliably showing cyclicity between manic-like and depressed-like states [17]. Animal models for mania generally fit into three categories: transgenics, pharmacological manipulations, and strain variants [16]. Each of these approaches has its strengths and weaknesses. Though transgenics precisely alter the ortholog of a human gene implicated in a disorder, they are usually knockouts lacking biological nuance, and they are limited to alterations of only one or a few genes. These conceptual difficulties limit transgenic approaches in the study of complex, subtle, or multigenic processes. Pharmacological manipulations may produce face-valid endophenotypes, but these models do not alter the genome and thus lack construct validity at the genetic level. Under the assumption that similar gene systems are perturbed in similar phenotypes, strain differences allow for the use of natural variation to mimic a disease state similar to human populations, but they are difficult to validate at the construct level due to the complexity of both the disease and strain phenotypes.

In this study, we attempt a genomic validation of a strain variant model for mania using whole exome resequencing. The Madison (MSN) mouse strain is an inbred mouse strain derived from the outbred Hsd:ICR (ICR) strain over the course of 15 years. MSN mice are a face-valid mania model, displaying a suite of behavioral phenotypes associated with unipolar mania including locomotor hyperactivity, decreased swim immobility, increased sexual behavior, advanced diurnal rhythm, and seasonal-like

alterations in locomotor hyperactivity relative to control strains [18,19]. MSN animals also fulfill some requirements of a predictively valid mania model, showing the expected attenuation of mania when treated with lithium chloride or the atypical antipsychotic olanzapine [18]. Further, MSN animals show similar hippocampal gene expression perturbations to those seen in post-mortem limbic brain tissue from patients with BSDs including systems-level alterations related to chromatin packaging [20], a finding that establishes provisional construct validity. The MSN phenotype is highly reproducible, showing mania-like behaviors at the same approximate magnitude across multiple generations [18-20]. Altogether, we believe the MSN strain is a novel, unique, and useful mania model that shows many relevant biological similarities to the human disorder it models [20].

Based upon our previous gene expression and behavioral work, we predicted variants unique to the MSN strain in purinergic reception related genes, molecular clock genes, and chromatin remodeling genes [19,20]. Additionally, genomic enrichment of our gene expression work predicted that MSN animals would show perturbations in genomic regions sharing synteny with human loci previously linked to bipolar disorder and related mental health disorders in humans [20]. Though these *a priori* predictions are useful for finding causative variants, exome resequencing allows for querying all protein-coding sites. Thus, we remained open to the possibility of genes and gene systems with structural variants unrelated to these systems.

The experimental design of an exome resequencing experiment defines how much noise can be screened from the results. With any experiment, controls are

important. We utilized a total of 3 control strains. The outbred ICR strain, the strain from which MSN was derived 15 years ago, is the behavioral reference against which we have compared the MSN animals in our previous work. As the source of the genetics leading to the MSN strain, ICR was the natural control strain for both finding new variants and for comparing population allele structure. Additionally, the Maternal Defense 2 (MaD2) mouse strain received parallel selective breeding for high maternal defense alongside the MSN progenitors, but does not show the same manic phenotype as MSN mice [18]. Finally, the C57BL/6J has been used as a control strain in studies of mania in inbred strains by other researchers [17]. As the inbred strain representing the reference mouse genome, the C57BL/6J strain was a tacit control requiring no additional sequencing.

The MSN breeding history and its phenotype relative to related and reference strains allows us to make a number of assumptions that reduce the amount of sequence necessary to find explanatory variants: 1) MSN animals will be nearly fixed mismatching the reference for explanatory variant alleles; 2) ICR animals supplied important genetic variants and will show variability for these alleles, but ICR homozygous variants will be rare at alleles important to the MSN phenotype; 3) MaD2 animals will show a similar allele pattern to ICR animals at important MSN alleles; and 4) important MSN alleles will change the biochemical function of the proteins their genes encode. Starting from these assumptions, we resequenced the whole exomes of a sample of MSN animals and relevant control strains to find interesting protein-coding variants in the MSN genome. Because sequencing is still cost-prohibitive for large numbers of samples, we followed

up on the most interesting variants using a real-time PCR technology, high-resolution melt curve genotyping (HRM) [21], to measure differences in the population allele ratios of the most interesting variants between MSN and outbred ICR animals.

MATERIALS AND METHODS

Animals

Animal use was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The University of Wisconsin–Madison College of Letters and Sciences IACUC approved all animal protocols (protocol #L00405-0-05-09). All reasonable efforts were made to minimize animal suffering.

The animals in this study were randomly selected using a true random number generator (http://random.org) from breeding colonies of three mouse strains: the manic Madison (MSN) strain, the outbred Hsd:ICR (ICR) strain (Harlan Laboratories, Madison, WI, USA), and the Maternal Defense 2 (MaD2) strain. The MSN strain was derived from ICR animals over the course of approximately 15 years, going through 30 generations of selective breeding for high voluntary wheel running [22], then through 25 generations of selective breeding for maintenance of high maternal defense [23], and finally through a number of generations of random breeding before being characterized as manic [18]. MSN mice are available at the Mutant Mouse Regional Resource Centers as stock number 036809-MU. MaD2 is an ICR-derived strain with parallel selective breeding to MSN for high maternal defense [24], but that does not display a mania-like phenotype [18]. All animals from both the MSN and ICR strains were used in a previous behavioral

study examining sexual dimorphism in the MSN phenotype [19], giving us a behavioral dataset of opportunity in this paper. All animals were euthanized by decapitation under deep anesthesia prior to tissue collection.

DNA Extraction, Targeted Exome Capture, and Deep Sequencing

After the animals were euthanized, we dissected and flash froze liver samples over dry ice, storing them at –80° C until DNA extraction. To extract DNA, liver slices were digested at 60° C overnight in a dry block heater in lysis buffer containing 50 mM Tris-HCl (pH 8.5), 5 mM EDTA, 10% SDS, 150 mM NaCl, and Proteinase K at 10 μg/mL (Thermo Fisher, Waltham, MA, USA). RNAse A/T1 mixure (Thermo Fisher, Waltham, MA, USA) was added at 2 μg/mL concentration an hour before organic extraction. DNA was extracted in 3 changes of phenol-chloroform-isoamyl alcohol mixture saturated in pH 8.0 Tris buffer, then cleared in 2 changes of chloroform before being precipitated in 2.5 volumes of ice-cold absolute ethanol. DNA pellets were desalted in two changes of ice-cold 70% ethanol, air dried, and dissolved in nuclease-free water.

We sequenced a library derived from a total of 3 MSN, 3 MaD2, and 5 ICR animals. After checking extracted DNA using a NanoDrop spectrophotometer (Thermo Fisher, Waltham, MA, USA) and gel electrophoresis for purity and quality, a multiplexed sequencing library was constructed using an Agilent SureSelect XT Whole Exome kit (Agilent, Austin, TX, USA) for mouse according to the manufacturer's specifications. Briefly, gDNA was sheared to ~300 bp and ligated to adapters before hybridization to biotinylated ribobaits. Hybridized samples were captured and PCR-amplified with adapters containing sample-specific barcodes. Libraries were purified, quality checked

using real-time PCR, then sequenced on a total of four HiSeq 2000 lanes across two runs. All sequencing procedures were performed by the University of Wisconsin-Madison Biotechnology Center's Next Generation Sequencing Facility.

Bases were called, reads were demultiplexed, and FASTQ files were generated using CASAVA v. 1.8.2.

Alignment, Variant Calling, and Functional Annotation

All analysis was done in OS X v. 10.9. Raw paired-end reads in FASTQ format were aligned to the GRCm38 genome used by the Sanger Mouse Genome Project (MGP) [25] with Bowtie 2 v. 2.1.0 [26]. Alignments were converted to BAM format, then pre-processed to update read groups, fix mate pair information, and mark PCR duplicates using Picard v1.101 (http://picard.sourceforge.net) running on Java v. 1.7.0_25. Reads were locally realigned around INDELs using the Genome Analysis Tool Kit (GATK) v. 2.7-4 [27] using the MGP INDELs VCF file aligned to GRCm38, then quality scores were recalibrated in GATK using the MGP SNPs VCF file aligned to GRCm38. Picard was used to fix mate pair information and merge BAM files from the same biological samples across lanes and then GATK was used to call SNPs and small INDELs as a VCF file. This VCF file was functionally annotated using SnpEff v. 3.3 [28] with the GRCm38.70 annotation.

The variants with the highest likelihood of uniqueness and relevance to the MSN phenotype are 1) mismatches to the C57BL/6J reference genome, 2) homozygous variant in all MSN animals, and 3) not homozygous variant in any ICR or MaD2 animals. We created a custom query in VarSifter v. 1.6, for only variants fitting these criteria. We

filtered the results for functional changes to coding sequences, defined as: missense mutations; insertions or deletions of codons in coding sequences; splice site variants; nonsense-mediated decay (NMD) variants; stop gain or loss variants; and frameshift variants. Variants meeting these criteria were queried with Ensembl's VEP tool to predict likelihood of functional effects using SIFT [29].

Raw reads in FASTQ format are deposited in the NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/traces/sra) under the SRA project accession number SRP040655.

High Resolution Melt Curve Genotyping

To compare interesting alleles and their ratios in populations, we utilized a real-time PCR derived technique called high-resolution melt curve genotyping (HRM) [21]. This technique uses oligonucleotide primers flanking each side of a variant. After PCR amplification, the dissociation temperature of an amplicon is measured using a fluorescent dsDNA binding dye like EvaGreen while increasing temperature at 0.2° C increments. Subtle variations in dissociation temperature are associated with each genotype; HRM is sensitive enough to detect even A/T and T/A transversions [21].

We used HRM on 32 MSN and 32 ICR samples, randomly selected and balanced between males and females. Each HRM assay was calibrated to the sequencing results, allowing us to find genotypes for unsequenced samples. Since 3 MaD2 animals were sequenced, we performed HRM on these samples to aid calibration. PCRs were performed using 10 μL reactions with 4 ng of template, 500 nM concentrations of each primer, and a 1X reaction mixture of SsoFast EvaGreen

Supermix (Bio-Rad, Hercules, CA, USA). PCRs were performed using a CFX-96 Touch real-time thermal cycler with fluorescence collected using CFX Manager v. 2.1 software (Bio-Rad, Hercules, CA, USA). Initial amplifications utilized a two-step hot start protocol with an initial 98° C dissociation step for 30 seconds, then 40 cycles of a 2 second 98° C dissociation step followed by 15 seconds at an empirically-determined oligonucleotide-specific annealing temperature. Fluorescent signal was measured in real-time during PCR, and all PCRs had a Cq between 20 and 30 as recommended by the manufacturer for HRM. After a 1 minute 98° C step and a 1 minute 70° C step, 10 second dissociation (melt) steps occurred at 0.2°C increments between 70° C and 95° C. Flourescent signal was collected at each dissociation step, and dissociation curves were normalized and analyzed by statistical clustering for genotype using Precision Melt Analysis v. 1.2 (Bio-Rad, Hercules, CA, USA).

Though HRM genotyping does not have a published standard like the MIQE [30], we appreciate the need for reproducible assays. Supplementary Table S4.1 contains much of the information required by the MIQE standards including information about oligonucleotide primer sets and their annealing temperature.

RESULTS AND DISCUSSION

We found a total of 447 MSN-unique nsSNPs, short INDELs, or other large-scale protein primary structure alterations. We filtered these variants using SIFT scores, finding 119 candidate MSN-unique variants that included nsSNPs with SIFT scores less than 0.25, short INDELs, or variants predicted to lead to major transcriptional events like nonsense-mediated decay, listed in Supplementary Table S4.2. We exhaustively

queried these variants in UniProt and the UCSC Genome Browser for gene ontology, sequence conservation across species, variant novelty, and possible biochemical effects, annotating each variant with our findings. The 34 variants we believed likely to change protein function based upon this annotation are listed in Table 4.1 and highlighted in grey in Supplementary Table S4.2.

We believed the most interesting variants occurred in *Npas2*, *Hltf* (*Smarca3*), *Slcl5a7*, *Cp*, *Trpv1*, *Cad*, *Lpcat2b*, *Nrac*, *Ces1b*, *Polr3c*, and *Smarca4*. We chose the variants in these genes for downstream analysis of population allele ratios in both MSN and ICR using HRM genotyping. The allele ratios for MSN and ICR at each locus are shown as pie graphs in Figure 4.2 along with FDR-corrected results of Fisher's exact tests examining the differences between MSN and ICR allele ratios. Allele ratios differed significantly for each variant queried, though some showed a higher degree of population fixation in MSN. After FDR correction, there were no significant deviations from Hardy-Weinberg equilibrium in either strain for any variant, indicating that no chosen allele is presently under measurable selection. Because the MSN phenotype is highly reproducible and based upon dozens of generations of inbreeding, the genotypes showing less complete fixation are likely of lower phenotypic relevance than those showing near total population fixation. Raw HRM results, allele and genotype ratios, and statistical tests are included in Supplementary Table S4.3.

Of the variants examined in HRM, we believe six are of the highest biological interest based upon their near total fixation in MSN, consistency with previous gene expression and behavioral results, and neurobiological relevance. These variants are

NPAS2 L481P, CP C712G, POLR3C T268M, SMARCA4 R351Q, TRPV1 P14A, and SLC5A7 R38H. Since many gene products lack biochemical characterization, the simplest way to assess functional consequences for a given coding variant is to visualize its alignment to peptide sequences of multiple species. Highly conserved amino acid residues likely have biochemical function. Sequence homology to selected vertebrates from the UCSC Genome Browser's Multiz alignment for each of these variants is shown in Figure 4.3. For each variant allele, we generated a functional hypothesis for its effects on the mature protein's biochemistry, listed in Table 4.4.

These genes' products are diverse in their ontology and functional properties. NPAS2 is a transcription factor and canonical molecular clock gene, CP is involved in copper transport and iron metabolism, POLR3C is a subunit of RNA polymerase III, SMARCA4 is an ATP-dependent DNA helicase with chromatin remodeling properties, TRPV1 is the transient receptor potential channel responding to excessive heat and capsaicin that has found recent acceptance as an ionotropic endocannabinoid receptor [31], and SLC5A7 is a neuronal choline transporter. These variants are subtle, indicating that the MSN mania model's phenotype likely results from a complex genotype involving small changes in multiple genes. Locomotor hyperactivity is difficult to selectively breed into ICR mice [32] and these variant alleles show low but measurable population allele frequencies in the outbred ICR strain, so we believe there are interactions between multiple loci to cause the MSN genotype.

Many of these genes belong to systems previously linked to BSDs in humans. *Smarca4* is among a network of genes predicted to play a role in the differential

chromatin dynamics of BSDs based upon a meta-analysis of gene expression data from postmortem brain tissue of patients with BSDs [14]. Both the genome region and a network that contain *Smarca4* were predicted to have perturbations in our previous work [20]. Further, *Smarca4*'s human ortholog sits between D19S714 and D19S586, markers with significant linkage to BSDs in two independent studies [12,33]. Variants in the human ortholog in the vicinity of NPAS2 L481 are correlated with seasonal affective disorder [34], and BSDs have high comorbidity with seasonal affective disorder [35]. Not only are cannabinoid receptors associated with mood and anxiety disorders [36], BSDs have an unusually high comorbidity with pathological cannabis abuse [37], and TRPV1 is an endocannabinoid receptor. SLC5A7 is a neuronal choline transporter, and there is suggestive evidence from a meta-analysis of human genomics studies on BSDs that cholinergic transmission is potentially altered in patients with BSDs [7]. Levels of the human ortholog of CP are elevated in patients with schizophrenia, a related mental health disorder [38], and patients with Wilson's Disease, a genetic disorder caused by dysfunction of an enzyme upstream of ceruloplasmin, have a higher incidence of BSDs than the general population [39]. Copy number variants in the human ortholog of *Polr3c* have been reported in schizophrenic patients [40], and *Polr3c* was proposed as a schizophrenia candidate gene relative to a number of metabolites altered in plasma samples from schizophrenic patients [41]. Altogether, we believe these variants imply fundamental systems-level construct validity for MSN as a mania model.

Of all variants seen in this study, the two that best explain behavior are in *Smarca4* and *Polr3c*. In two-way ANOVAs on transformed behavioral data from

previous work [19], the interaction between *Smarca4* genotype and sex significantly accounts for variance in the ICR strain's locomotor behavior ($F_{1,28} = 7.126$, $p = 0.0125$, model multiple $R^2 = 0.337$, model adjusted $R^2 = 0.266$) and the genotype at the *Polr3c* variant alone significantly accounts for variance in the ICR strain's locomotor activity ($F_{1,28} = 9.854$, $p = 0.00397$, model multiple $R^2 = 0.299$, model adjusted $R^2 = 0.224$). Further, an ANOVA model adding in effects of both *Smarca4* and *Polr3c* genotypes boosts the model multiple R^2 to 0.419 and the model adjusted R^2 to 0.307, indicating that the two genotypes together explain more variance better than they do in isolation. That these results explain variance in the outbred strain's behavior provides a correlative basis for a cause-and-effect prediction. Summaries of all ANOVA models appear in Supplementary File S4.4.

SMARCA4 R351 is a highly conserved residue in the protein two amino acid residues away from T353, a threonine residue that was found phosphorylated in a high-throughput phosphoproteomic screening of human cells [42]. Using GPS 2.1 and NetPhos 2.0, phosphorylation prediction software packages with divergent prediction algorithms [43,44], we found that the subtle change of an arginine to a glutamine residue in the variant is predicted to abolish T353 phosphorylation. The POLR3C variant is at a conserved residue in the middle of an alpha helix, likely affecting the secondary structure of this protein. Since each of these proteins plays a role in recruitment of molecular machinery for restructuring the nucleosome and transcribing DNA, we speculate that these variants together constitute causative genomic perturbations

behind the chromatin structure system found differentially regulated in our previous work.

While genomic tools can find variants, validation is a more difficult, costly, and low throughput process. Nonetheless, functional characterization of these results is the next step in understanding the biological relevance of each variant. New nuclease-based transgenic techniques like CRISPR allow easy transgenic animal models to be made. Knocking these MSN variants into an unrelated strain like C57BL/6 animals shows much potential for functional assessment of each allele. This would allow for behavioral characterization of each variant in isolation from possible cis factors in unqueried parts of the genome. It would also provide a useful tool to measure the effects of these variants on protein chemistry using assays specific to each gene of interest.

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CONFLICTS OF INTEREST

The authors declare that no conflicts of interest exist.

SUPPLEMENTARY INFORMATION

Supplementary Table S4.1: MIQE-inspired HRM genotyping table including primers, primer annealing temperatures, and reaction conditions necessary to reproduce HRM analysis (PDF).

Supplementary Table S4.2: Exhaustive table of variants meeting the criteria of being mostly fixed in MSN, variable in ICR and MaD2, and likely to change protein coding or splice variation (PDF).

Supplementary Table S4.3: Raw HRM genotyping results, genotype and allele counts for each mouse strain of interest, and relevant statistical tests (Excel spreadsheet}.

Supplementary File S4.4: Results of two-way ANOVAs using genotype information and sex as factors explaining inverse of square root transformed data on locomotor activity (PDF).

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TABLES AND FIGURES

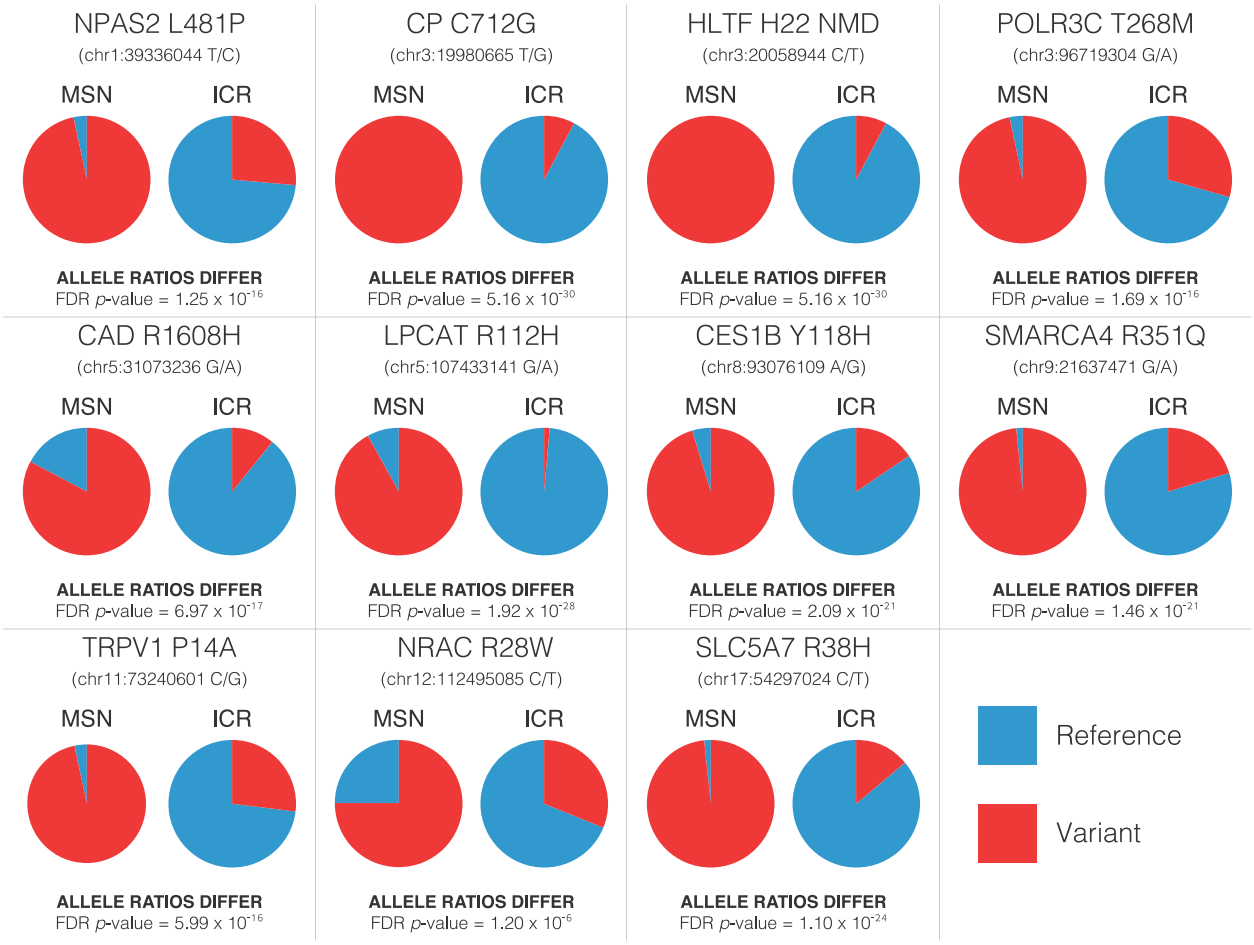
Table 4.1

Location and Allele	Gene ID	UniProt	Consequence	Amino Acid	SIFT
chr1:39336044 T/C	*Npas2*	P97460	Missense	L481P	0.23
chr2:25363944 C/T	*Uap1l1*	Q3TW96	Missense	G300D	0.13
chr2:167036853 C/T	*Znfx1*	Q8R151	Missense	G1868S	0.00
chr3:19980665 T/G	*Cp*	G3X8Q5	Missense	C712G	0.01
chr3:19987423 G/A	*Cp*	G3X9T8	Missense	S903N	0.22
chr3:20005467 T/C	*Cp*	G3UXD2	Missense, Splice	V23A	---
chr3:20012808 C/A	*Hps3*	E9PZY1	Missense	K531N	0.16
chr3:20012845 T/C	*Hps3*	Q91VB4	Missense	N651S	0.14
chr3:20058944 C/T	*Hltf*	G3UVU1	NMD	H22	---
chr3:20076540 G/C	*Hltf*	Q6PCN7	Missense	K369N	0.20
chr3:96719304 G/A	*Polr3c*	Q9D483	Missense, NMD	T268M	0.00
chr5:31073236 G/A	*Cad*	B2RQC6	Missense	R1608H	0.00
chr5:107433141 G/A	*Lpcat2b*	Q9D5U0	Missense	R112H	0.04
chr5:110127696 C/T	*Zfp605*	E9QAH2	Missense	P227S	0.01
chr5:117555266-117555277 Deleted	*Ksr2*	M0QW59	Long Deletion	VRTPP259-263V	---
chr8:89032312 G/T	*Sall1*	Q6P5E3	Missense	A388E	0.02
chr8:93076109 A/G	*Ces1b*	D3Z5G7	Missense	Y118H	0.01
chr8:121916500 G/A	*Car5a*	P23589	Missense	T255M	0.14
chr9:13826880 C/A	*Cep57*	D6RH89	NMD	S7	---
chr9:21637471 G/A	*Smarca4*	Q3TKT4	Missense	R351Q	0.04
chr9:21835572 G/A	*Gm6484*	Q8R1L8	Missense	A19T	0.11
chr11:59000521 C/CTGG	*Obscn*	F6TJX7	Insertion	V1583PV	---
chr11:59000884 C/T	*Obscn*	H7BX05	Missense	V6941M	0.12
chr11:59076137 G/A	*Obscn*	J9JIB2	Missense	H586Y	0.02
chr11:73240601 C/G	*Trpv1*	Q704Y3	Missense	P14A	0.00
chr11:73254291 C/A	*Trpv1*	Q704Y3	Missense	D734E	0.05
chr12:112495085 C/T	*Nrac*	Q8BNX7	Missense	R28W	0.05
chr12:112497893 C/T	*Nrac*	Q8BNX7	Missense	R110C	0.12
chr12:112498037 T/C	*Nrac*	Q8BNX7	Missense	C158R	0.23
chr12:118190825 T/C	*Dnahc11*	E9Q7N9	Missense	E240G	0.18
chr12:118198712 G/A	*Dnahc11*	E9Q7N9	Missense	R41C	0.01
chr17:7772146 A/T	*Fndc1*	E9Q043	Missense	L906H	0.06
chr17:54297024 C/T	*Slc5a7*	Q8BGY9	Missense	R38H	0.08
chr17:81054224 C/CTAT	*Thumpd2*	Q9CZB3	Insertion	S191NS	---

Significant protein-coding variants unique to MSN. Of the variants of interest including short INDELs or SNPs with SIFT scores less than 0.25, these 34 variants were the ones

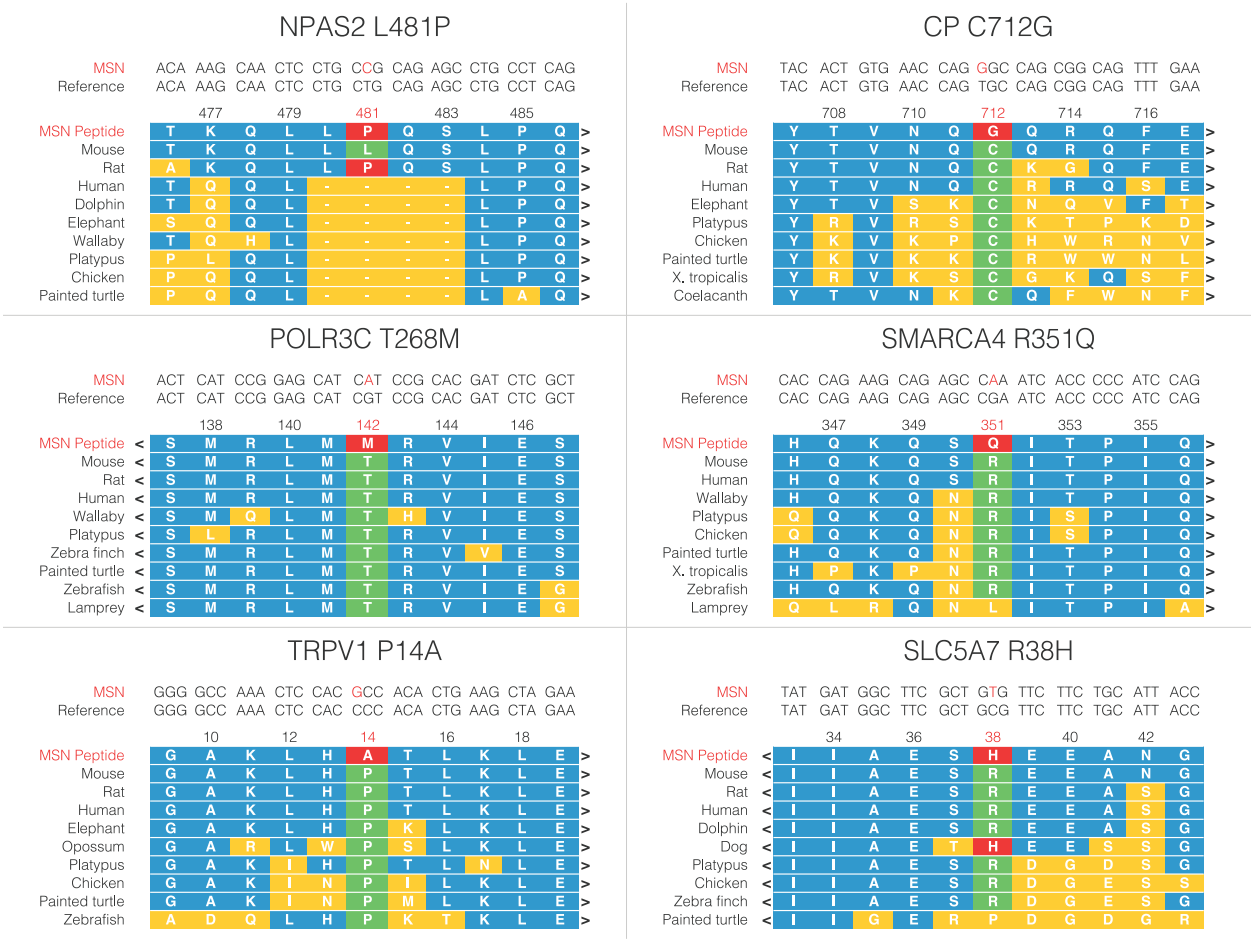
we believed most likely to change protein chemistry based upon exhaustive hand annotation of each variant using UniProt. All short INDELs and SNPs with SIFT scores less than 0.25 are included in Supplementary Table S3.

Figure 4.2



HRM population allele ratios for 11 variants of interest. The allele ratios for each these variants was assessed using HRM genotyping. Allele ratios were compared between MSN and ICR using a Fisher's Exact Test and corrected for multiple comparisons using the FDR method. All 11 comparisons were highly statistically significant, though not all allele ratios showed the near fixation necessary for biological significance.

Figure 4.3



Selected interspecific alignments for six high-interest variants. Using the UCSC Genome Browser's Multiz Alignments for selected vertebrate genomes, we visualized homology between a diverse sampling of aligned genomes for each coding variant, adding 5 amino acids on each side for context. The MSN sequence is highlighted in red while the reference at each genome is highlighted in green. The context is highlighted in blue, and any variation away from reference that is not the same as the MSN variant is highlighted in yellow. More highly conserved residues are more likely to change protein chemistry and have biological effects.

Table 4.4

Variant	Functional Hypothesis
NPAS2 L481P	L481P is very close to human variant S471L, associated with seasonal affective disorder.
CP C712G	From structural biology on the human peptide, C712G breaks a disulfide bond.
POLR3C T268M	T268M is in a conserved site in an alpha helix. It likely changes secondary structure.
SMARCA4 R351Q	R351Q likely abolishes phosphorylation of T353.
TRPV1 P14A	P14A changes a conserved residue in an ankyrin domain of the mature channel.
SLC5A7 R38H	R38H is in the cytoplasmic domain of the mature neuronal choline transporter.

Six high interest variants with functional hypotheses. For these variants, we advanced mechanistic ideas about how they change the proteins of interest. Where possible, these hypotheses are testable, allowing for further characterization of protein chemistry in the future.

CHAPTER 5: DISCUSSION AND CONCLUSIONS

ON THE BIOLOGY OF MADISON MICE

The biological knowledge gained in this work on the MSN strain aids in its utility as a valid model for mania. We took multiple biological stories away from this dissertation work, many of them related to bipolar spectrum disorders (BSDs).

Chromatin Structure and Translational Regulation

A systems meta-analysis of gene expression studies of BSDs predicted a chromatin structure system to be differentially expressed in patients [1]. Schizophrenic and BSD patients treated with the anticonvulsant valproic acid, a drug often used to treat these psychiatric illnesses, showed measurable increases in acetylated histones in lymphocytes [2], implying that the pharmacology of this drug may affect chromatin structure. Based upon this association between chromatin structure and BSDs, many have speculated that histone deacetylase (HDAC) inhibitors (HDIs) may act as a novel treatment for BSDs. This hypothesis has recently been tested in mice; an inhibitor of HDACs significantly decreased hyperlocomotion in amphetamine-challenged animals while reducing time spend immobile in forced swimming [3].

The MSN strain shows both differential expression of and polymorphisms in genes and gene systems related to chromatin structure and general translational regulation. In the gene expression microarray work, we found differential expression in the brain of multiple histone subunit genes and many transcripts whose products are involved in transcriptional regulation and the remodeling of chromatin including *Smarca4*, *Hltf*, *Smarcal1*, and multiple RNA polymerase subunit related genes. In the exome resequencing work, we found non-synonymous polymorphisms that likely

change protein chemistry in *Smarca4*, a gene encoding an ATP-dependent DNA helicase with chromatin remodeling properties, and *Polr3c*, a gene encoding a subunit of RNA Polymerase III. Taken together, these lines of evidence strongly imply that MSN animals show differences in chromatin structure and transcriptional regulation relative to control animals. Further, the *Smarca4* gene was predicted to be related to BSDs in the systems meta-analysis of gene expression studies we cited many times [1]. We believe this is the most promising biological story for future work on the MSN strain.

Purinergic Reception

The human ortholog of *P2rx7* has been implicated in BSDs and in major depressive disorders in multiple studies [4-7]. Gene expression of *P2RX7* in human blood mononuclear cells is significantly increased in rapid cycling bipolar I patients and in sleep-deprived healthy patients [8]. Polymorphisms in *P2RX7* explain some variation in depression severity [9], though rigorous studies are somewhat conflicted on a causal association between P2RX7 polymorphisms and mood disorders [5,10].

These human gene expression and genomic results partially correspond with our results from MSN mice. In the gene expression work, we found neural expression differences in the *P2rx7* transcript between MSN animals and the control ICR strain; *P2rx7* was expressed at approximately 2/3 the level in MSN mice as it is in the outbred strain. Though we predicted we would see non-synonymous SNPs in the *P2rx7* gene or other purinergic signaling genes, we did not see any such polymorphisms in the exome resequencing work. We are thus led to believe that *P2rx7* gene expression differences are associated with the phenotype and may even partially drive the phenotype, but we

believe they do so by gene expression differences and not through coding changes to the gene itself.

P2rx7 and its human ortholog have an interesting neurobiology. P2X receptors have diverse functions in modulating neural signaling throughout the nervous system [11]. The P2RX7 receptor regulates glutamatergic signaling, likely regulates GABAergic signaling, and has been immunolocalized to presynaptic termini of excitatory neurons [12]. As a calcium-permeable channel active on presynapses, it is well positioned to regulate neurotransmitter release. Given this regulatory action on the two major CNS neural transmitter systems alongside the dual nature of BSDs, decreased expression of this receptor implies a mechanism for bipolarism. If P2RX7 receptors modulate both major neural transmission systems and there are fewer of these receptors available for that modulation, it follows that a neural system thus affected will have less ability to modulate itself in either direction. When the dominant signaling molecule that cannot be modulated is glutamate, we speculate that brains go through runaway excitatory signaling resulting in mania. When the dominant signaling molecule that cannot be modulated is GABA, we postulate that a runaway inhibitory effect occurs associated with depression.

Sexual Dimorphism

BSDs show an equal prevalence in males and in females [13,14]. However, the presentation of these disorders is very different between the sexes. Females are more likely to experience rapid cycling than males [15]. Their reproductive state is associated with disease presentation [16]. They are more likely to experience seasonal affective

disorder comorbid with bipolar disorders than males [14]. Males are more likely to experience bipolar I than females while females are more likely to experience bipolar II than males [17]. Males experience earlier disease onset and a different cluster of comorbid medical and psychiatric diseases in disorder presentation [14]. BSDs may occur with equal frequency in both sexes, but how they occur is highly dependent on sex.

The MSN behavioral phenotype is seen in both sexes, but its presentation is similarly sexually dimorphic. Sexual dimorphism in behavior has been shown among Hsd:ICR-derived mice in the past [18]. Our data suggest that this generalized behavioral phenotype in females has been exacerbated in MSN animals; MSN females show locomotor hyperactivity so much above males from their strain, they appear almost a different strain altogether. When we look at our results, we do not believe our experimental design was powerful enough to resolve whether the sexual dimorphism we observed in MSN was a general trait of ICR-derived animals or whether it was increased above expectations in MSN animals. Addressing this issue may allow us to ask important molecular questions about differences in behavioral phenotype based upon sex.

Chronobiology and Seasonality

Chronobiological factors strongly affect those who suffer from BSDs. Patients with BSDs are unusually influenced by seasonal effects [19]. Among patients reporting seasonality, those with BSDs tested during the spring scored better on measures of cognitive function than those tested during the winter [20]. This seasonality has a

genetic component; people with family histories of affective disorders are at a higher likelihood of subclinical seasonal differences in affect than those with no family history of affective disorders [21]. Schizophrenia and BSD frequency is higher amongst those born during the winter and early spring months, an observation that can be explained by the speculation that those with a genetic predisposition toward bipolarism are manic during the spring and choose that time to conceive [22], though other explanations, including heightened *in utero* exposure to winter illnesses, are possible [23]. Seasonal affective disorder is often associated with a delayed sleep onset chronotype [24], establishing a fundamental connection between seasonality and disruptions in sleep. Patients with BSDs have a tendency toward a delayed sleep onset chronotype colloquially known as eveningness [25]. These sleep disruptions are among the earliest signs of bipolar disorder onset [26]. Sleep disruption is intimately connected with affective state; total sleep deprivation switches bipolar patients from depressed to manic states at a rate comparable to treatment with antidepressants [27]. The seasonal and sleep disruptions observed in affective disorders are associated with multiple non-synonymous SNPs observed within the molecular clock genes [28].

The difference in sleep schedule and the seasonal-like behavioral phenotype seen in the MSN animals are a strong contrast with the ICR animals, who sleep through most of the day and display no measurable differences in locomotion across different photoperiods. Seasonally breeding animals must modulate their behavior in response to circannual cues like duration of photoperiod. While this seasonality would be advantageous to wild populations, laboratory mice have undoubtedly undergone

selection for fecundity under very general conditions. Thus, we are not surprised that ICR mice do not show a seasonal-like phenotype. Some of the genetic variants associated with seasonality are probably present in any population of animals with enough genetic variation. We believe there are strong connections between the nsSNP in the *Npas2* gene and the MSN strain's chronotype and seasonality. We speculate that the MSN strain's seasonality is a reversion to an ancestral seasonal phenotype that existed in some wild mice; the specific *Npas2* variant we see in MSN matches the rat reference, leading us to believe this variant occurs throughout the rodent taxon.

Cannabinoid Reception

Cannabinoid receptors play diverse roles throughout the nervous system and in affective disorders. This neuromodulator system is understudied in part because compounds that agonize them remain on the DEA's Schedule I. Still, much is known about cannabinoids. Though *Trpv1* is best known as a receptor sensitive to noxious heat in peripheral and hypothalamic neurons, in many central nervous system contexts it receives anterograde transmission of endocannabinoid signaling and aid in long-term potentiation [29]. Other cannabinoid receptors, notably CB1, have well-known responses to the cannabinoid drug Δ^9-tetrahydrocannabinol (THC), the primary psychoactive component of marijuana. This receptor likely play a role in schizophrenia and related disorders like BSDs [30]; schizophrenic patients show higher binding of radiolabeled cannabinoid ligands in the pontine nuclei, a negative correlation between CB1 receptor binding the prefrontal cortex and a measure of psychotic withdrawal, and a general downregulation of CB1 receptors in the dorsolateral prefrontal cortex [31,32].

Cannabinoid-derived drugs have been proposed as novel therapeutic interventions against BSDs [33]. Pathological cannabis use disorder occurs at a much higher rate in patients with BSDs [34], suggesting that cannabinoids already see use as self-medication for these illnesses.

The *Trpv1* variant seen in the MSN strain likely alters the subcellular localization of the TRPV1 channel by changing its ankyrin domain, the motif in the protein responsible for anchoring the channel to the cytoskeleton. Though *Trpv1* is the only cannabinoid receptor gene discussed in the exome resequencing work, it may not be the only cannabinoid receptor gene altered in MSN animals. We used a strict cutoff of a SIFT score below 0.25 for nsSNPs associated with MSN animals. Among the SNPs fixed in MSN that did not make it below that cutoff value was an nsSNP with a SIFT value of 0.27 in *Gpr55*, a G-protein coupled anandamide receptor gene whose translated product was an orphan until recently. Though this specific nsSNP, which encodes GPR55 R19S, may not be significant from an evaluation with SIFT scores, SIFT is designed to find profound and usually deleterious SNPs. R19 is at the very border between the first extracellular and first transmembrane domain in the primary structure of the GPR55 receptor, and if this SNP has functional consequences for GPR55, we believe they are subtle. Further evidence for a cannabinoid story in the MSN strain are seen in the gene expression results. Aside from those discussed, some transcripts found differentially expressed in the microarray work on MSN were *Pik3ip1* and *Pik3cd*, genes related to phosphatidylinositol 3-kinase (PI3K) activity. The G-protein coupled cannabinoid receptors work upstream of the PI3K pathway and have

neuroprotective properties through this system [35], leading us to believe the effect we saw in the PI3K pathway is driven by the coding changes in cannabinoid receptors. Thus, we see a strong likelihood of *Gpr55* and *Trpv1* playing a role in the MSN phenotype. Two different cannabinoid-related receptors showing genotypic differences at possible functional sites in MSN animals along with expression changes in a related functional system implies differences in cannabinoid reception in the MSN phenotype.

Other Results

For brevity, not all interesting results made it into the publishable data chapters on the MSN strain contained in this work. There are unresolved questions about these animals' biology that seem unrelated to BSDs. In the gene expression chapter, we replicated a finding from previous work [36], showing that MSN animals have lower average body weight than ICR animals. This effect is weak but measurable. In the exome resequencing chapter, we briefly mentioned one of the non-synonymous SNP we found in a gene called *Nrac*, a poorly characterized gene whose product encodes a protein likely to have a transmembrane domain. *Nrac* stands for "nutritionally regulated, adipose and cardiac enriched", and as this name suggests, the *Nrac* transcript is differentially-expressed in adipose and cardiac tissue in response to differences in diet composition [37]. Because of its involvement in metabolism, we believe the MSN nsSNPs in the *Nrac* gene suggest that variants in this gene are likely explanatory variant for body weight. Further, the allele ratio of *Nrac* approximately corresponds with the effect size of the strain difference in weight between MSN and ICR animals. When we look at the genotype at rs4967734, the *Nrac* SNP examined in the HRM results from

the exome resequencing study, we can build an ANOVA model with sex and genotype explaining variance in the square root of MSN body weight even when sex is brought into the model first. We find a mildly significant effect of *Nrac* genotype on the square root MSN body weight ($F_{2,27}$ = 3.49, p = 0.0446). We find no such effect in the ICR animals ($F_{2,27}$ = 0.34, p = 0.716), and we note that no homozygote variant animals are seen in the ICR population. These statistical results suggest that the *Nrac* allele may be a recessive allele driving the differences in body weight seen in MSN animals. We find no significant effect of activity on body weight in MSN mice when sex is brought into the model before activity ($F_{1,28}$ = 0.50, p = 0.484), implying that the differences seen in MSN mice are independent of activity.

The MSN strain was originally selectively bred in an experiment designed to assess exercise physiology [38]. Though successive generations of these animals provide strong evidence that their neural and behavioral phenotypes are among their more interesting traits, this original breeding as an exercise model should not be ignored. We believe this unpublished *Nrac* result shows that the MSN strain biology includes metabolic effects that may be distinct from the neural phenotype.

Difficulties in Genotype and Phenotype

The gene expression results predicted perturbations in multiple genomic positions whose orthologs were implicated in BSDs in the human work. The exome resequencing confirmed only a single result, that of the variant in *Smarca4*, as a spot where significant genomic perturbations leading to protein-coding changes with functional effects were observed. While we do not believe the apparent contradiction

between these results is problematic, we do believe it merits discussion. There are two probable explanations for this failure to find functional variants in the predicted regions: 1) Exome resequencing only sequences the 60 Mb of the protein-coding genome and not the 3 Gb of the full mouse genome. Unsequenced genomic changes in nearby regulatory elements contribute to the phenotype in the regions with negative findings. 2) An epigenetic component like differential chromatin availability that drives the MSN neuromolecular phenotype cannot be differentiated from genomic perturbations by measuring gene expression. We believe that both of these explanations are likely. The strongest molecular phenotype we see in the MSN gene expression results is a chromatin structure network that experiences strong differential expression. Because epigenetics is a constituent of many gene expression differences, this provides a partial explanation for some missing findings. However, in the exome resequencing results, we found some fixed SNPs without strong predicted functional consequences in areas predicted by the enriched gene expression results, such as a 10 Mb region of the qF cytoband of chromosome 5 that contained 20 non-synonymous SNPs. Disentangling epigenomic and genomic contributions will be an area of intense interest in the future the MSN strain and of genomic sciences in general.

We were excited about the possibility of seeing true behavioral bipolarism in MSN mice, but we must admit that we have no evidence for this. There is still a possibility that these animals, under the right contexts, show true behavioral bipolarism. It is also possible that their seasonality is an example of a specific type of bipolar behavior; the hyperlocomotion that MSN animals show in long photoperiods may be an

enhancement of mania. However, we cannot show any evidence of spontaneous cycling between different affective states. We do not believe this lack of a finding negates the utility of the MSN model as a partial model for bipolar disorder, but we must note that our understanding of these mice continues to be as a model for mania only.

MADISON MICE AS A VALID MANIA MODEL

The three chapters of this dissertation have presented the MSN mouse strain as a valid mania model with important caveats. The validity of models for psychiatric disorders is typically divided into three parts: face validity, construct validity, and predictive validity [39,40]. The following is an outline of how the work done in this dissertation contributed to these different measures of validity.

Face Validity

The new behavioral work done on the MSN strain extends the strain's face validity as a mania model, but it does not provide evidence for MSN mice as a face valid model for a bipolar phenotype. Three generations of MSN animals have shown locomotor hyperactivity, making this the most robust measure for the MSN phenotype. The phenotype is seen in both males and females. It is seen from early in development up until 12 weeks of age, the latest time we chose to measure the phenotype. The MSN strain also shows face validity for some of the comorbidities associated with BSDs. Seasonal affective disorders and disruptions in daily rhythm are often seen in BSDs, and MSN animals show both. Just as there are differences between males and females with BSDs, there are differences between male and female behavioral phenotypes in MSN animals.

Construct Validity

The new genomic and gene expression work done on the Madison strain establishes the strain's construct validity as a model for mania. While the genomic and gene expression work on BSDs and related disorders like schizophrenia are heterogeneous and often contradictory, consensus exists for a few things. Chromatin remodeling, purinergic reception, cannabinoid signaling, and chronobiological and seasonal-like changes are all well supported biological changes seen in patients with BSDs. Changes to the same systems are seen in MSN animals. We have highlighted where the changes overlap in our discussion of the biology of these animals. Though it is difficult to show construct validity for a disease whose etiology is ill defined in many human populations, we believe our results allow for the cautious use of MSN animals as a construct valid model for some BSDs.

Predictive Validity

Many researchers discuss predictive validity as something established when similar pharmacological interventions moderate a phenotype in a similar way. Lithium salt treatment was used to establish provisional predictive validity for the Black Swiss strain as a potential mania model [41]. Previous work on MSN animals used this same approach to provide preliminary predictive validity for the MSN strain, using treatments with lithium chloride and olanzapine to establish that they responded as a manic bipolar patient would to these drugs [36].

This approach to predictive validity has its merits, but it is a limited view of predictive validity. The true goal of translational research is to use animal models in the

creation of novel treatments that outperform the current pharmacological interventions. Successful use of a model in the development of new drugs that work well on humans is something that might be called ultimate predictive validity. The only way to establish this sort of predictive validity is to create these new interventions and test them in humans. Since it costs on average over a billion dollars and takes many years to bring a new molecule to market [42], ultimate predictive validity is a difficult prospect. While ultimate predictive validity is inaccessible to us without extending the Madison findings to novel pharmacological manipulations, the new results on MSN mice serve as a broad framework for future advances on pharmacological and behavioral interventions. We now have predictions for systems that, when manipulated, may moderate the MSN phenotype. After validating these predictions in MSN mice, these interventions could prove useful in humans with BSDs and major depression. In time, this could provide the predictive validity necessary for MSN mice to be a true translational model.

PREDICTIONS FOR FUTURE WORK

As has been discussed previously, MSN animals show perturbations in behavior and in a number of gene systems possibly related to organismal arousal, diurnal rhythm, chromatin packaging, purinergic signaling, and cannabinoid signaling. The disruptions in these gene systems provide predictions for both new biological and pharmacological studies on these animals.

Smarca4 and Chromatin Structure

The *Smarca4* and *Polr3c* results suggest that chromatin structure and translational regulation play a strong role in the MSN strain's behavioral phenotype.

Functional assays for these variants using transgenic animals generated using CRISPR technology would be the simplest and most linear way to assess these variants' effects on biochemistry, chromatin structure, gene expression, and behavior. Relative to traditional transgenic techniques that take years, cost tens of thousands of dollars, and target single genes at a time, CRISPR and other nuclease-based transgenic technologies allow for the generation of transgenic mice in months, cost thousands of dollars, and have been used to target up to five genes at a time [43]. This makes CRISPR techniques ideal for the study of complex genotypes.

Creation of a transgenic animal carrying the variant coding for SMARCA4 R351Q using CRISPR would be a potentially fruitful new direction. Specifically, it would allow for functional testing of this protein for the prediction of decreased phosphorylation at T353 from the exome resequencing work using immunoprecipitation coupled with mass spectrometry. Further, because SMARCA4 is a DNA binding protein, chromatin immunoprecipitation (ChIP) of SMARCA4 in both variants could prove a useful assay of the functional differences between these variants' functions at the whole genome level. We would predict that the specific consequences of the R351Q allozyme explain gene expression results seen in our first paper that did not show corresponding genomic changes. Specifically, we would predict that in neural tissue, ChIP-Seq or ChIP-qPCR of SMARCA4 would show differences in its binding to the *P2rx7* gene in MSN mice. We believe SMARCA4 would bind less to this gene in MSN animals, a causal prediction for how the MSN genotype leads to lower expression of *P2rx7*.

Further, we are aware of multiple more generic ways of assaying global chromatin structure involving both ChIP-Seq on other targets such as acetylated and methylated histones, DNAse-Seq, and FAIRE-Seq. The state-of-the-art seems to embrace ChIP-Seq with carefully selected markers like acetyl-histone H3; these methods seem to need less sequencing depth to show significant results. We predict that in neural tissue of MSN animals, there would be a global decrease in heterochromatin associated with acetylated histones and a global increase in euchromatin associated with methylated histones. Before sequencing, these predictions could be tested preliminarily with relative simplicity using western blotting or EIA techniques to compare acetyl-histone H3, methyl-histone H3, and general histone H3 expression.

Trpv1 and Cannabinoid Signaling

The *Trpv1* result suggests that aberrant cannabinoid signaling may be related to the manic phenotype seen in the Madison mouse model. We have predicted that the variant in *Trpv1* alters the subcellular localization of the protein it encodes. The TRPV1 channel has been found expressed in the cortex of animals at the pre- and postsynaptic termini of excitatory synapses [44]. We predict that the TRPV1 channel would follow a diffuse and ectopic expression pattern on the neurons of variant animals relative to channels bearing the reference allele. This hypothesis could be tested using confocal microscopy with immunofluorescent labeling of TRPV1. Should this pattern of ectopic expression be confirmed in MSN animals, it makes strong predictions about the ability of MSN brains to undergo long-term potentiation in response to cannabinoid signaling.

Further, it implies that the variant may even change developmental influences on the balance of excitatory and inhibitory neurotransmission.

Npas2, Diurnal Rhythm, and Seasonality

Npas2 is a well-characterized molecular clock gene that binds to DNA and regulates transcription. The relationship between the MSN *Npas2* polymorphism and the other molecular clock genes is hard to resolve. Adding more difficulty to this problem is the diurnal preference of mice. To extend chronobiological genetics from mice, a nocturnal animal, to humans, a diurnal animal, requires a theoretical framework for these genes that explains how they vary between diurnal and nocturnal animals that does not yet exist. Meaningfully characterizing this *Npas2* variant and any circadian gene at a molecular level sufficient to create the data necessary for such a framework appears unwieldy. Chronobiological genes are difficult to study with rigor; they necessitate multiplying sample sizes by 8 to 12 to get samples at different distances to lights-on and lights-off. This multiplication of samples dilutes the power at any given timepoint, necessitating the multiplication of sample sizes by another 1.5 to 2. In total, a rigorous examination of circadian genes necessitates collection of up to 24 times the samples as a straight pairwise gene expression comparison. Further, these systems operate in a circle governed by the clock, meaning that any inferential work necessitates boutique statistical modeling built for circular datasets. While such work would be difficult, it is tractable, and we have designed a panel of 13 circadian gene real-time PCR primers to examine differences in how *Npas2* and other genes are regulated in response to lights-on and lights-off in MSN animals relative to outbred ICR animals. This

experiment requires the collection of 288 samples of suprachiasmatic nuclei from both the MSN and ICR strains. It only makes sense with a large grant.

Additional work on dextroamphetamine sensitization work is generally merited in the MSN strain. This would be an effective addition to the corpus of behavioral phenotypes already characterized in this strain, and we believe the dextroamphetamine sensitization paradigm could add significant interest when used in concert with the already characterized differences in locomotor hyperactivity observed in MSN animals under different photoperiods. If amphetamine sensitization increases in animals exposed to a long, summer-like photoperiod, it would show that they are inducibly more mania-like than they are under 12-hour photoperiods. Further such behavioral work could be coupled to anxiety measures, forced swimming, and lithium chloride treatment to observe multiple behavioral measures of mania, depression, and other traits for the long photoperiod animals. This might allow for the MSN animals to stand in for a truly bipolar mouse strain; a mouse that can be induced to more or less manic depending upon seasonal cues begins to look like a face valid model for bipolar disorder with comorbid seasonal affective disorder.

Pharmacological Predictions for Bipolar Spectrum Disorders Treatment

The biology of these animals makes very specific predictions about systems that we could affect to moderate the MSN phenotype. Specifically, purinergic reception, cannabinoid reception, and chromatin structure represent three systems that can be targeted with relative specificity using some off-the-shelf molecules.

We believe the variant in *Smarca4* is a subtle loss-of-function allele, causing a less open chromatin state than is present under the reference allele. If this is truly the driver of the chromatin structure network seen differentially expressed in the gene expression results, we believe the primary chromatin story is one of a greater ratio of euchromatin to heterochromatin. This euchromatin should be associated with histones that are not acetylated and possibly methylated. There are specific drugs that promote the acetylation of histones, a class known as histone deacetylase inhibitors (HDIs). One HDI already approved by the FDA is suberanilohydroxamic acid, also known as vorinostat or its trade name Zolinza. The approved usage for this drug is to treat cutaneous T cell lymphoma, an immune cancer that causes skin lesions [45]. This drug has also been tested in a pair of mood-related behavioral tasks in C57BL/6 mice and was found to suppress amphetamine-induced locomotor hyperactivity and reduce time immobile in forced swimming [3]. We predict that MSN mice chronically treated with vorinostat would show decreases in mania-like behaviors. Use of this compound in the MSN strain represents among the more interesting pharmacological predictions we have; if the drug works to moderate MSN mania, the possibility that it could moderate BSDs in humans could be tested with relative speed and ease due to the molecule's status as an FDA-approved drug.

Purinergic reception represents another opportunity for novel pharmacological intervention on the MSN strain. We have argued that the downregulation of the P2RX7 receptor – the *P2rx7* transcript is expressed at approximately 2/3 the level in MSN as it is in ICR animals – appears related to an inability of the neural system to regulate

glutamatergic transmission at the presynaptic terminal, causing runaway excitatory neural transmission and mania in these animals. P2RX7 receptors are expressed all throughout the body and are generally related to inflammation and immunity [46], making specific agonists and antagonists of these receptors too messy for a functional pharmacological intervention on the nervous system and behavior. There are drugs that act mostly in the nervous system that would increase the availability of ATP as a ligand in neural contexts. These molecules are ecto-ATPase inhibitors like ARL 67156, BGO136, and PV4, a class of drugs shown to increase the concentration of available neural ATP by inhibiting the inactivation mechanism at the synapse, thus increasing purinergic neural transmission [47,48]. The use of these drugs represents a novel way of increasing ATP availability in the brain, and we believe treatment of MSN mice with these drugs may moderate the phenotype.

Cannabinoid receptors represent another class of specific drugs we predict would moderate mania in MSN animals. However, because this gene system contains variants in MSN mice, this strain may not be an ideal system to study these drugs and their relation to mania. Instead, the use of an inducible mania model such as dextroamphetamine-treated C57BL/6 mice may work better. Direct manipulations of the TRPV1 receptor would prove nearly as messy as direct manipulations of purinergic receptors; TRPV1 is conventionally known as an excessive heat and capsaicin receptor, and it is likely that direct systemic treatment with an agonist of this receptor would cause an animal the same pain and discomfort as eating spicy peppers. However, cannabinoid manipulations have been increasing in sophistication [33]. While we have no specific

predictions for how manipulating the cannabinoid system might change affective state in induced manic mice, we nonetheless believe this is a promising lead.

CONCLUSIONS

Madison animals are a complex model for the manic pole of bipolar spectrum disorders. Their biology includes perturbations of chronobiology, chromatin structure and translational regulation, purinergic reception, and cannabinoid signaling. The known perturbations to these systems provide face validity and construct validity for the MSN strain as a mania model. Further, predictions for pharmacological manipulations of some of these systems may provide predictive validity for these animals in the future.

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