The chromatin remodeler ISWI acts during *Drosophila* development to regulate adult sleep

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Sleep disruptions are among the most commonly reported symptoms across neurodevelopmental disorders (NDDs), but mechanisms linking brain development to normal sleep are largely unknown. From a *Drosophila* screen of human NDD-associated risk genes, we identified the chromatin remodeler *Imitation SWItch/SNF (ISWI)* to be required for adult fly sleep. Loss of *ISWI* also results in disrupted circadian rhythms, memory, and social behavior, but *ISWI* acts in different cells and during distinct developmental times to affect each of these adult behaviors. Specifically, *ISWI* expression in type I neuroblasts is required for both adult sleep and formation of a learning-associated brain region. Expression in flies of the human *ISWI* homologs *SMARCA1* and *SMARCA5* differentially rescues adult phenotypes, while de novo *SMARCA5* patient variants fail to rescue sleep. We propose that sleep deficits are a primary phenotype of early developmental origin in NDDs and point toward chromatin remodeling machinery as critical for sleep circuit formation.

**INTRODUCTION**

Neurodevelopmental disorders (NDDs) are highly prevalent and diverse diseases related to abnormal brain maturation. While numerous behavioral phenotypes are commonly associated with individual genetic mutations in NDDs (1, 2), sleep disturbances are pervasive across NDDs and are a substantial stressor for individuals and caretakers alike (3, 4). Strong clinical associations between disrupted sleep and other NDD symptoms (5, 6) have led to the suggestion that sleep disturbances may be secondary to broader cognitive or behavioral deficits (7–9) and therefore refractory to treatment. Alternatively, sleep dysfunction in NDDs might represent a core phenotype directly related to pathological developmental processes (10). As sleep is important for normal neurodevelopment and function (11), early sleep disturbances might exacerbate other behavioral issues. Given the high prevalence and significant burden of NDD-associated sleep problems, understanding the mechanistic underpinnings of sleep disruptions is crucial for developing therapeutic interventions.

Sleep in the genetically tractable model organism, *Drosophila melanogaster*, has the defining behavioral characteristics of vertebrate sleep and is regulated by evolutionarily conserved signaling pathways (12). These characteristics position *Drosophila* as an ideal, high-throughput model to (i) identify causative NDD risk genes that affect sleep and (ii) investigate how these same risk genes may contribute to behavioral pleiotropy. To identify mechanisms underlying NDD-associated sleep disturbances, we screened for sleep abnormalities using RNA interference (RNAi) targeting *Drosophila* homologs of human NDD risk genes. Constitutive knockdown of *Imitation SWItch/SNF (ISWI)* led to marked sleep disturbances in the adult fly. Across species, ISWI and its homologs are adenosine 5'-triphosphate–dependent chromatin remodelers that regulate the expression of genes important for neural stem cell proliferation and differentiation (13–18). Rare variants in the human homologs of *ISWI*, *SMARCA1* and *SMARCA5*, have been implicated in several NDDs (18–22). Moreover, large-scale genome-wide and exome sequencing studies on patient cohorts have shown that genetic factors contributing to NDDs converge on chromatin regulation pathways (23, 24). Chromatin dynamics are critical for appropriate gene expression during key developmental time points (25). At a behavioral level, disruption to individual chromatin remodeling genes has been associated with abnormal sleep and circadian rhythms (26, 27), memory (28, 29), and social function (30) in animal models. Thus, dysfunction of these important gene regulatory hubs likely results in a multitude of downstream biological effects, contributing to behavioral pleiotropy seen in NDDs. Delineating how chromatin remodelers like *ISWI* control development of neural circuits involved in diverse behaviors will deepen our understanding of behavioral pleiotropy in NDDs.

In addition to sleep deficits, we found that knockdown of *ISWI* leads to circadian abnormalities in the adult fly, as well as memory and social dysfunction. Temporal mapping revealed *ISWI* acts during dissociable preadult stages and spatially distinct circuits to affect these different adult behaviors. At the circuit level, *ISWI* knockdown disrupted the morphology and function of the adult sleep-regulatory dorsal fan-shaped body (dFB) neurons, likely by affecting the cell

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fate of dFB neurons. Expressing either human SMARCA1 or SMARCA5 in the setting of ISWI knockdown differentially rescued adult deficits, while expressing SMARCA5 harboring human patient mutations failed to rescue adult fly sleep. Our results delineate how mutations in a single NDD risk gene give rise to primary disruptions of sleep circuit development in the setting of behavioral pleiotropy.

RESULTS
ISWI is necessary for normal sleep in Drosophila
To identify NDD risk genes with effects on sleep, we took advantage of high-throughput sleep assays in Drosophila (31, 32). We focused on human genes within loci of interest that have been strongly associated with risk for NDD (1, 33–35). These loci included chromosomal copy number variants and individual risk genes. We performed a reverse genetic RNAi-based screen of Drosophila orthologs of NDD-associated human genes (Fig. 1A and data file S1) using the elav-GAL4 enhancer to drive expression of UAS-RNAi lines in the developing and adult nervous system. We individually knocked down 218 genes, comprising a total of 421 unique RNAi lines (including 73 lethal lines) (Fig. 1, B to D, and data file S1). From this screen, we found that knockdown of ISWI markedly decreased total sleep duration (Fig. 1, E and F), with the strongest effect during the night (Fig 1G).

Pan-neuronal ISWI knockdown also led to more fragmented sleep, as indicated by a reduction in sleep bout length and increase in bout number during the day and the night (Fig. 1, H and I). Although knockdown of several other genes also resulted in increased sleep fragmentation (Fig. 1, C and D), we chose to focus on ISWI given its additional involvement in total sleep duration. Knockdown with an independent RNAi line for ISWI recapitulated the observed sleep deficits (Fig. 1, E and F, and fig. S1, A to C). We validated that both tested RNAi constructs decreased ISWI mRNA levels (fig. S2A) and found that coexpression of a FLAG- and HA-tagged RNAi-resistant UAS-ISWI (UAS-ISWIRec–FH) in the setting of ISWI knockdown rescued sleep deficits (fig. S2, B to F). These results demonstrate that sleep deficits are specific to the effects of ISWI RNAi-based knockdown.

Sleep homeostasis was also impaired in ISWI RNAi flies: elav-GAL4 > UAS-ISWI RNAi flies exhibited ~300 min of sleep loss in response to overnight mechanical deprivation (fig. S1, D and E) but, in contrast to genetic controls, failed to exhibit sleep rebound (Fig. 1] and fig. S1, D and F). Thus, ISWI knockdown results in decreased and fragmented sleep, as well as deficits in homeostatic rebound.

ISWI knockdown impairs circadian rhythmicity, memory, and courtship behaviors
Patients with NDDs exhibit myriad behavioral disruptions in addition to sleep problems, such as circadian disturbances (36), intellectual disability (ID) (37), and social deficits (5). We found that, in addition to sleep disruptions, adult flies exhibited circadian arrhythmicity in the setting of pan-neuronal ISWI knockdown (Fig 2, A to C). ISWI knockdown led to significantly decreased rest/activity rhythm strength (Fig. 2B), as well as an increase in the percentage of arrhythmic flies (Fig. 2C and table S1). The core molecular clock remained intact (fig. S3, A and B), suggesting a disruption of clock output mechanisms.

Since memory disruption is a key characteristic of ID (29, 38–42), we next asked whether ISWI knockdown leads to memory deficits in adult flies. We assessed aversive taste conditioning using the proboscis extension reflex (PER) assay (43) (Fig. 2D). Flies with pan-neuronal ISWI knockdown exhibited intact learning and gustatory responses, as seen by suppressed PER across sequential training sessions; these responses emphasize that not all behaviors are disrupted in the setting of ISWI knockdown. However, elav-GAL4 > UAS-ISWI RNAi flies erroneously extended their proboscis upon fructose presentation during testing (Fig. 2E), indicating memory deficits. The mushroom body (MB) is an associative center in the insect brain that is important for normal memory, including conditioning responses seen with PER (43). We therefore examined whether ISWI knockdown affects MB structure and found severe...
**ISWI is required during the third instar larval stage for normal adult sleep**

ISWI and its homologs are involved in neural development and differentiation across species (13, 16–18). We asked whether ISWI is required during preadult developmental stages or in an ongoing manner in the adult fly to regulate adult behaviors. We leveraged the TARGET system (49) to restrict ISWI knockdown to pre-eclosion (Fig. 3A). Pan-neuronal knockdown only during pre-eczlosion significantly decreased total sleep and resulted in sleep fragmentation, recapitulating the phenotype seen in constitutive ISWI knockdown (Fig. 3B and fig. S4, A to D). More refined temporal mapping revealed that pan-neuronal ISWI loss from embryonic stages through the middle-third instar period leads to decreased total sleep duration and sleep fragmentation similar to constitutive ISWI knockdown (Fig. 3, E to G, and fig. S4, A to D), whereas knockdown only through earlier stages does not (Fig. 3, C, D, and G, and fig. S3, A to D).

Given the pre-eczlosion role of ISWI in determining adult sleep, we wondered whether sleep deficits arise before adulthood. We previously characterized sleep behaviors during the second instar larval stage (50) and found here that ISWI knockdown has no effect on sleep (fig. S3, D to G) but disrupted MB morphology (fig. S3, H and I), suggesting that MB dysfunction is not likely to underlie sleep deficits seen in the setting of ISWI knockdown.

Social dysfunction is another prevalent symptom in NDDs (5). In the male fly, courtship is a social behavior that can be assayed on the basis of a series of stereotyped behaviors. Pan-neuronal ISWI knockdown in male flies using the elav-GAL4 driver was lethal, but restricting knockdown to pre-eczlosion using the temporal and regional gene expression (TARGET) system (47) (Fig. 3A) led to viable males. Male flies with ISWI knockdown limited to the preadult stage exhibited significantly decreased courtship index and copulation success compared to genetic controls (Fig. 2G), suggesting compromised social function. To provide further evidence that ISWI knockdown affects social rather than only reproductive behaviors, we used a social space behavioral assay in which distance between individual flies is measured in a two-dimensional space (48). Female elav-GAL4 > UAS-ISWI RNAi flies exhibited increased social space in relation to genetic controls (Fig. 2H and fig. S3, J and K). This result supports the conclusion that social behaviors are disrupted with ISWI knockdown, independent of reproductive function. Thus, a *Drosophila* homolog of an NDD-associated human gene is required for normal sleep, circadian rhythmicity, memory, and social behaviors.

morphic abnormalities in this brain region: In 100% of elav-GAL4 > UAS-ISWI RNAi brains, we observed bilateral ablation of the vertical α/β lobes and thinning of the horizontal γ lobes using Fasciclin II (FasII) immunostaining (Fig. 2F). MB morphologic deficits were not the result of altered FasII levels, as coexpression of ISWI RNAi with mCD8::green fluorescent protein (GFP) using MB-specific GAL4 lines revealed similar MB abnormalities (fig. S3C). In addition, RNA sequencing (RNA-seq) in the setting of ISWI knockdown confirmed that FasII mRNA levels were unchanged. MB structural changes were specific to loss of ISWI, as coexpressing UAS-ISWI-RFP-FH with UAS-ISWI RNAi was sufficient to rescue the phenotype (fig. S2G). Together, these results indicate that ISWI knockdown disrupts MB morphology and memory function. Although the MB is known to be involved in adult fly sleep (44–46), we found that MB morphologic deficits were dissociable from sleep abnormalities: Expression of ISWI RNAi using the MB driver OK107-GAL4 did not alter sleep (fig. S3, D to G) but disrupted MB morphology (fig. S3, H and I), suggesting that MB dysfunction is not likely to underlie sleep deficits seen in the setting of ISWI knockdown.
that pre-eclosion ISWI knockdown also led to adult arrhythmicity (Fig. 4, A to C). However, in contrast to sleep, knockdown through only the first instar stage led to adult rest-activity arrhythmicity comparable to constitutive ISWI knockdown (Fig. 4A, fig. S5, and table S2). This result indicates that ISWI knockdown through earlier larval stages primarily results in adult rhythmic deficits, associated redistribution of sleep across the 24-hour day, and sleep fragmentation. Together, our findings suggest that primary sleep disruptions and behavioral arrhythmicity are temporally separable to distinct developmental windows of ISWI knockdown.

**ISWI acts during separable preadult stages for adult fly memory and social functions**

We next investigated the temporal window of ISWI action for adult memory and courtship behaviors. In contrast to sleep, knockdown through only the mid-second instar stage led to MB morphologic abnormalities and deficits in aversive taste conditioning (Fig. 4, D and E). These results suggest that the ISWI-dependent sleep phenotypes do not arise from MB disruptions, as sleep and memory deficits are temporally dissociable. This conclusion is further supported by our finding that ISWI knockdown in the MB (using OK107-GAL4) is associated with MB but not sleep deficits (fig. S3, C to H), underscoring ISWI functions in distinct circuits and developmental times for sleep and memory. Last, male flies exhibited disrupted courtship behavior with ISWI knockdown through early pupation but not through mid-third instar (Fig. 4, F and G), dissociating sleep and
courtship behaviors. Together, these results demonstrate that ISWI acts in different developmental windows to coordinate distinct adult behaviors (Fig. 4H).

**ISWI function in type I neuroblasts is necessary for normal adult sleep and MB morphology**

How does ISWI affect development of adult fly sleep circuits? Since elav is expressed pan-neuronally (51), we reasoned that ISWI loss in specific neurons might result in adult fly sleep deficits. We performed a neuronal GAL4 screen (>400 lines) but found none of the tested lines recapitulated sleep loss seen with elav-GAL4 > UAS-ISWI RNAi. These negative results led us to wonder whether the sleep phenotype seen with elav-driven ISWI knockdown is unrelated to ISWI function in neurons. Elav is also expressed in larval glial cells and neuroblasts (52), but restricting ISWI knockdown to glia using repo-GAL4 also had no effect on sleep (fig. S6, A and B), arguing against a glial role. ISWI is necessary for maintaining chromatin structure in larval neuroblasts (13, 14) and normal progenitor cell proliferation across species (15, 18). To test whether ISWI RNAi in neuroblasts leads to sleep deficits, we knocked down ISWI in type I neuroblasts (Fig. 5, A and B). In addition, wnoru-GAL4, a pan-neuroblast driver (53), and observed a reduction in sleep duration (Fig. 5, A and B). Knockdown ISWI in all neuroblasts also led to disruptions in MB morphology (100% of MBs were abnormal in wnoru-GAL4 > UAS-ISWI RNAi flies) similar to that seen with elav-GAL4-driven knockdown (Fig. 5E). Consistent with the hypothesis that ISWI functions in neuroblasts, OK107-GAL4 expresses in MB neuroblasts (54) and also disrupts MB morphology with ISWI knockdown (Fig. 5, G and H).

We next asked whether ISWI Knockdown in specific neuroblast lineages is responsible for adult sleep and MB deficits. In the developing *Drosophila* nervous system, type I and II neuroblasts undergo asymmetric cell divisions; type II divide into intermediate progenitor cells (INPs), which are capable of several rounds of cell division before differentiating into neurons (55, 56). ISWI knockdown in type I neuroblasts using asense-GAL4 significantly decreased sleep duration (Fig. 5, F to H) and increased sleep fragmentation (Fig. S6, C and D). Asense is also expressed in type II lineage INPs (56), so the sleep phenotype with asense-GAL4 could result from ISWI knockdown in INPs rather than type I neuroblasts. However, knockdown in INPs using R9D11-GAL4 or R16B06-GAL4 (57) did not impair sleep duration or continuity (Fig. 5, F and H, and fig. S6, C and D), suggesting that ISWI acts in type I neuroblasts for adult fly sleep behaviors. Similarly, ISWI knockdown in type I neuroblasts, but not in INPs, resulted in MB morphologic deficits (Fig. 5, I and J). Thus, ISWI function in type I neuroblasts during development is required for normal adult sleep and MB structure.

**ISWI knockdown disrupts morphology and function of sleep-regulatory dFB neurons**

To understand how ISWI knockdown affects sleep regulatory circuits, we focused on the adult sleep-promoting dFB neurons that are defined by the R23E10 enhancer. dFB neurons are involved in the homeostatic sleep response: Sleep deprivation increases activity of dFB neurons, and activation of these neurons induces sleep (58–60). Since ISWI knockdown results in sleep rebound deficits following mechanical deprivation (Fig. 1J and fig. S1, D to F), we hypothesized that the sleep-promoting function of dFB neurons might be impaired with ISWI knockdown. In the setting of pan-neuronal ISWI knockdown, R23E10 adult neurons exhibited abnormal neurite morphology, with aberrant projections to brain regions outside of the dFB; we also observed abnormal cell body location of R23E10 adult neurons in the context of ISWI knockdown compared to genetic controls (Fig. 6, A and B). Quantification of R23E10 axonal innervation of the dFB showed decreased innervation volume in the setting of ISWI knockdown (Fig. 6C). In addition, there was a significant increase in the total number of R23E10 neurons as measured by...
ISWI knockdown disrupts the morphology and function of the sleep-promoting dFB. (A) Representative images of R23E10 neuron morphology as visualized by GFP staining (middle) in genetic controls (top) and in the setting of ISWI knockdown (bottom), with FasII counterstaining (left). White arrowheads point to abnormal R23E10 neuron projections, yellow arrowheads indicate cell body locations. Scale bar, 100 µm. (B) Magnification of denoted areas in (A). Scale bar, 25 µm. Quantification of (C) dFB volume and (D) number of R23E10 soma in genetic controls (black, n = 10) and in the setting of pan-neuronal ISWI knockdown (red, n = 15) as measured by GFP immunostaining. (E) Thermogenic activation of R23E10 neurons, with experimental design showing temperature shifts (top) and representative sleep traces (bottom). Quantification of day (top) and night (bottom) sleep at 22°C baseline and 31°C activation (bottom). Genotype: +/+ = UAS-ISWI RNAi; UAS-TrpA1
e1av-OF2; R23E10-GAL4 > +, UAS-TrpA1
e1av-OP2; R23E10-GAL4 > +, UAS-TrpA1
e1av-OF2; R23E10-GAL4 > UAS-ISWI RNAI
e1av-OP2; R23E10-GAL4 > UAS-ISWI RNAI
e1av-OF2; R23E10-GAL4 > +, UAS-TrpA1 +/+; QUAS-ISWI RNAI; UAS-TrpA1.

Fig. 6. ISWI knockdown disrupts the morphology and function of the sleep-promoting dFB.
The human ISWI homologs SMARCA1 and SMARCA5 differentially rescue sleep and memory. (A) Representative sleep traces with ISWI knockdown (red) compared to SMARCA1WT (blue) and SMARCA5WT (green) expression in the setting of ISWI knockdown or overexpression alone (black) with (B) quantification of total sleep and (C) activity index across experimental and control groups (n = 104, 48, 29, 102, and 55 from left to right). (D) Representative images of Fasll immunostaining activity index across experimental and control groups. (E) Quantification of mushroom body volume across groups (n = 5, 21, 16, and 14 from left to right). (F) Quantification of proboscis extensions in ISWI knockdown (red), compared to genetic control (black, n = 37), SMARCA1WT rescue (blue, n = 29), and SMARCA5WT rescue (n = 26) (two-way ANOVA with post hoc multiple comparison test). (G) Representative sleep traces of pan-neuronal ISWI knockdown (red) compared to rescue with SMARCA5WT (green) or SMARCA5del268-319 (purple) and overexpression of WT or mutant constructs alone, with (H) quantification of total sleep duration (left) and night sleep duration (right) (n = 104, 102, 55, 41, and 31 from left to right). For graphs in this figure, letters represent statistically similar groups.

DISCUSSION

Despite clinical heterogeneity among and even within NDDs, sleep disturbances are highly prevalent (3, 4). Clinical evidence points toward a link between sleep dysfunction and other behavioral symptoms in NDDs (5, 6, 65). Whether sleep disturbances are a by-product of other NDD-related deficits or directly result from developmental disruptions remains a point of debate (3, 7–9). From a sleep-focused screen of Drosophila orthologs of human NDD-associated genes, we found that the chromatin remodeler ISWI is important for adult fly sleep. Knockdown in distinct developmental windows and circuits resulted in dissociable adult deficits in sleep, circadian, memory, or social behaviors. Notably, along with other behavioral deficits, our findings demonstrate that sleep disruptions represent a primary phenotype arising directly from ISWI knockdown during preadult development.

Mutations in chromatin remodelers are strongly associated with NDDs. For example, de novo mutations in the chromatin remodeler CHD8 have the strongest overall association with autism spectrum disorders (23). In addition to its role in growth-regulatory pathways (66, 67), CHD8 has been shown to interact with and control the expression of other autism risk genes (68, 69). These lines of evidence suggest that chromatin remodelers are ideal candidates to identify the mechanisms by which behavioral pleiotropy arises in NDDs. Our results trace how disruptions in a single NDD risk gene affects development of neural circuits controlling distinct behaviors and identify a genetic etiology underlying NDD-associated sleep disruption.

ISWI and its homologs have been implicated in neural stem cell fate decision, and ISWI is necessary for proper chromatin regulation in larval neuroblasts (13–15). Mouse models with mutations in Smarca1 and Smarca5 exhibit abnormal neural progenitor cell proliferation (16–18). In our present study, we have begun to parse out the stem cell lineage and timing of events that lead to specific disruptions in adult behaviors. ISWI knockdown in type I neuroblasts disrupted adult sleep and resulted in disrupted morphology and function of sleep-promoting R23E10 neurons, suggestive of abnormalities in neural stem cell proliferation and differentiation. These significantly enhanced connectivity to human homologs of SMARCA5 and human sleep-related genes were involved in neurogenesis, nervous system development, and Wnt signaling pathways. These results underscore the relevance of ISWI in sleep function and neurodevelopment and implicate a developmental role for SMARCA5 in human sleep gene networks.

Last, we recently characterized human mutations in SMARCA5 as a cause of a previously unidentified NDD, identifying 12 individuals with de novo or rare heterozygous variants (22). We generated fly lines of SMARCA5 with two patient mutations under UAS control (UAS-SMARCA5R592Q and UAS-SMARCA5del268-319). While elav-GAL4 > UAS-SMARCA5R592Q flies appeared normal, coexpression of ISWI RNAi and UAS-SMARCA5R592Q resulted in unhealthy flies that died prematurely. UAS-SMARCA5del268-319 were viable when expressed alone or in combination with ISWI RNAi. However, in contrast to UAS-SMARCA5WT, expression of UAS-SMARCA5del268-319 did not fully rescue sleep deficits in the setting of ISWI loss; in particular, nighttime sleep deficits persisted (Fig. 7, G and H). These results provide evidence that pathogenic variants of a NDD-associated gene directly contribute to sleep deficits.
results raise the possibility that ISWI knockdown changes the fate of adult sleep-regulatory neurons, perhaps through dysregulation of temporally expressed transcription factors or cellular signaling important for neuroblast differentiation. We also found ISWI function in type I neuroblasts to be important for normal MB morphology. Notably, knockdown in MB neuroblasts, which are type I neuroblasts, was sufficient to disrupt MB morphology but not sleep, distinguishing the neural substrates underlying ISWI-related sleep and memory deficits. Together with our temporal mapping results, we propose that ISWI affects the fate of neural stem cells contributing to adult circuits responsible for separate adult behaviors during the course of larval development. We note that INP-specific ISWI knockdown was associated with a small increase in day sleep (Fig. 5F), raising the possibility that ISWI knockdown has additional nuanced effects on sleep via different neuroblast lineages. Regardless, our results demonstrate the importance of ISWI chromatin remodelers for development of normal adult behaviors, building on existing evidence that ISWI plays a critical role in neural stem cell differentiation. Determining which populations of stem cells are affected at a given stage of preadulthood, and tracing how ISWI knockdown affects formation of specific circuits, is the next step toward understanding how ISWI loss disrupts adult behaviors.

It remains unknown whether ISWI loss specifically in 23E10 cells is causative for the observed sleep deficits. One limitation of the GAL4/UAS system is the shifting expression patterns of GAL4 drivers across development (61, 70): The 23E10 driver labels sleep-promoting dFB neurons in the adult fly but is expressed in different cells at mid-third instar. Congruent with this, ISWI knockdown with 23E10 has no effect on adult fly sleep. Work is needed to identify and genetically access the relevant primordial sleep cells in the larval nervous system. In addition, using an RNAi-based method relies on effective levels of mRNA knockdown; the absence of a phenotype with ISWI knockdown using a given GAL4 might be related to knockdown inefficiency. Last, it is possible that additional sleep-regulatory regions contribute to the ISWI sleep phenotype. While the MB has been implicated in sleep control (44–46), our findings indicate that this region does not contribute to sleep disruptions with ISWI knockdown. Examination of sleep-regulatory circuits beyond dFB and MB in the setting of ISWI knockdown will yield a deeper mechanistic understanding of how sleep deficits arise.

We found that the human homologs of ISWI, SMARCA1 and SMARCA5, are able to selectively rescue memory or sleep deficits, respectively. Why do SMARCA1 and SMARCA5 differentially rescue these abnormalities? One possible explanation lies in mouse studies that have noted differences in temporal and spatial distributions of Smarca1 and Smarca5 in the developing mouse brain. Differences in protein sequences between SMARCA1 and SMARCA5 may also facilitate differential expression and function. Our results begin to parse the differential functions of SMARCA1 and SMARCA5, an outstanding question in the field. This is an area of major interest, as understanding the differences between paralogs in the setting of disease can inform our knowledge about compensatory effects that paralogs may exert to alter phenotypes.

Mutations in the human ISWI homolog SMARCA1 have been implicated in diverse NDDs (19–21), and we have recently described a novel NDD caused by mutations in SMARCA5 (22). However, there has been no clinical characterization of sleep phenotypes arising from patient mutations in SMARCA1 or SMARCA5. A human brain-specific gene network analysis shows that ISWI and its human homologs interact with a conserved network of genes. Compellingly, SMARCA5, which rescued sleep deficits in the setting of ISWI knockdown, exhibited increased connectivity to human sleep and circadian genes through connector genes broadly involved in development. These results implicate a role for SMARCA5 in the development of normal human sleep regulation. It will be of great interest to assess sleep in patients with SMARCA5 mutations given our findings in flies and humans (22). Moreover, because developmental ISWI knockdown leads to sleep abnormalities in the adult fly, longitudinal patient sleep phenotyping may reveal sleep differences across the lifespan. In sum, our results provide new insight into the etiology of sleep disruptions in NDDs and suggest a mechanism whereby temporally and spatially constrained gene function underlies behavioral pleiotropy. This work supports the idea that sleep is a developmentally programmed behavior; sleep abnormalities in NDDs are not simply a by-product of broad cognitive/behavioral deficits but rather emerge from specific developmental anomalies.

MATERIALS AND METHODS
Fly stocks
Flies were raised and maintained on standard molasses food (8.0% molasses, 0.55% agar, 0.2% Tegosept, and 0.5% propionic acid) at 25°C on a 12-hour:12-hour light:dark (LD) cycle unless otherwise specified. Unless otherwise specified, female flies were used in all experiments.

Fly strains
The hs-hid; elav-GAL4; UAS-Dcr2 strain was a gift of D. Rogulja (Harvard University). Elav^{23E10}-GAL4 (elav-GAL4), OK107-GAL4, repo-GAL4, and 23E10-GAL4 were gifts of A. Seghal (University of Pennsylvania). Worniu-GAL4 (asense-GAL4) and worniu-GAL4 (ase-GAL80) were gifts of M. Syed (University of New Mexico). UAS-dTrpA1 was a gift from L. Griffith (Brandeis University). The following strains were obtained from the Bloomington Drosophila Resource Center: UAS-ISWI RNAi^{HMS00628} (UAS-ISWI RNAi^{TRIP}) was used for all experiments unless otherwise specified and from the Harvard Transgenic RNAi Project (TRIP) [Bloomington Stock Center (BSC), #32845]; UAS-mCD8::GFP (BSC #5137); tub-GAL80P (BSC #7019); 23E10-GAL4 (BSC #49032); LexAOp-mCD8::GFP (BSC #32203); R9D11-GAL4 (BSC # 40731); R16B06-GAL4 (BSC # 40731); elav^{23E10}_QF2 (elav-QF2; BSC #66466). All RNAi strains used in the primary screen were obtained from Bloomington Drosophila Resource Center (see table S1 for a full list of lines). UAS-ISWI RNAi^{Drosophila Resource Center (DVRD #24505, construct ID GD1467). The following fly strains were generated as described below.

UAS-ISWi^{G1467-FH construct
A vector containing the ISWI gene sequence with a C-terminal FLAG-HA tag under UAS control (UFO10052) was obtained from the Drosophila Genomics Resource Center [National Institutes of Health (NIH) grant 2P40OD010949]. The gene location targeted by the HMS00628 ISWI RNAi hairpin (5′-ACCCAAGAAGATCAAAGCTGAACA-3′) was identified. The Q5 Site-Directed Mutagenesis Kit (New England Biolabs, catalog no. E0554S) and corresponding primer design tool were used to create RNAi-resistant UAS-ISWI. Primers were as follows: ttaaagattGGACAGAGAAAAAGATGTG (forward) and ttttttttagGCTTACCCCTTACGCGTGG (reverse). DNA injection was prepared with the Midiprep Kit (Qiagen). Injections were
performed by Rainbow Transgenic Flies Inc. for production of transgenic flies at the attP40 landing site.

**QUAS-ISWI RNAi construct**

QUAS-WALIUM20 vector was obtained from J. Zirin at the Fly TRiP (71). The HMS00628 ISWI RNAi hairpin, originally used to generate the UAS-ISWI RNAi construct (BSC #32845), was cloned into the QUAS-WALIUM20 vector using the pWALIUM20 cloning protocol (available at [flyrnai.org](http://flyrnai.org)). Briefly, the following oligonucleotides were synthesized and annealed (21-nt hairpin sequence shown in capital letters): 5’ctagcatACCCAGAGAGATCAAGACGACCTttatattacagaaTTTCTTTTGTATGCTCTGTTGTTGctgc 3’ and 5’attgcAC- CCAAGAGATCAAAGACATattgtgtttactataactTTGTCTTTT- GATCTTTTGTGGTAcctg 3’. The QUAS-WALIUM20 vector was linearized by Nhe I and Eco RI, and the DNA fragment containing the hairpin was ligated into the vector. DNA injection was prepared with the Midiprep Kit (Qiagen). Injections were performed by Rainbow Transgenic Flies Inc. for production of transgenic flies at the attP40 and VK00033 landing sites.

**UAS-SMARCA1 construct**

Flies carrying UAS-SMARCA1WT were generated using human SMARCA1 cloned into the pFastBac Dual vector (Addgene plasmid #102243). Gateway cloning (Invitrogen) was used to generate the pDonr221-20xUAS (a gift from P. Haynes) and pDonrP2rP3-SMARCA1. Primers for pDonrP2rP3-SMARCA1 were designed by fusing Gateway attB2r and attB3 sequences upstream and downstream, respectively, of the SMARCA1 sequence. Primer sequences were as follows (capitalized letters indicate Gateway sequences): attB2r-SMARCA1, GGGGACAACTTTCTTGTACAAATGg- gaacaagacactgctgcc (forward); attB3-SMARCA1, GGGGACAGCTTTCTTGTACAAAGTGGatg- caaatctgctttg (reverse). A modified pBPGUw, pBPGUw-R1R3-p10 (72) (a gift from P. Haynes), was used for gateway recombination. Injections were performed by Rainbow Transgenic Flies Inc. for production of transgenic flies at the attP40 landing site.

**UAS-SMARCA5 constructs**

The patient SMARCA5 mutations studied were p.268-319del (SMARCA5del268-319) and p.(Arg592Gln) (SMARCA5 R592Q). The coding sequences of SMARCA5WT, SMARCA5del268-319, and SMARCA5R592Q were synthesized and cloned into the pACU2 vector (GenScript). Injections were performed by Rainbow Transgenic Flies Inc. for production of transgenic flies at the attP2 landing site.

**Sleep assays**

Adult female flies were collected 2 to 3 days after eclosion and aged in group housing on standard food at 25°C on a 12-hour:12-hour LD cycle (unless otherwise noted). Flies aged 5 to 7 days were anesthetized with CO2 at ZT1, and the posterior thorax and wings were gently glued to microscopy glass slides using nail polish under CO2 anesthesia. Flies were placed in a humidified chamber and allowed to recover for 5 hours before the start of the assay. For experiments, slides were mounted at a 45° angle under a dissecting microscope to observe proboscis extension. A 1-ml syringe was used to present the following solutions to the front tarsi of each fly: ddH2O, 100 mM fructose, 10 mM quinine, or 1000 mM sucrose (Sigma-Aldrich). Flies were satiated with ddH2O before the start of the experiment. Pretraining proboscis extension was tested by presenting 100 mM fructose three times, separated by 10-s intervals. Flies that did not satiate with ddH2O or did not extend to initial fructose presentation (pretraining) were excluded from the remainder of the experiment. For training rounds, fructose was presented to the fly tarsi. Quinine was presented to the extended proboscis of each fly, and flies were allowed to drink for up to 2 s. Quinine presentation occurred in 10-s intervals within each training round. There was a 1-min interval between each training round and a total of three training rounds before testing. For testing, fructose was presented three times to the fly tarsi with a 10-s interval between each presentation. At the end of each experiment, flies were given 1000 mM sucrose to check for intact PER, and nonresponders were excluded from statistical analyses. The number of proboscis extensions was recorded during each training round and during testing and reported as a percentage of the total number of possible extensions.
Courtship assays
Newly eclosed virgin male flies were collected within 4 hours after eclosion, kept in isolation on regular food, and aged to 3 days after eclosion before the start of courtship experiments. Female Canton-S virgins (3 to 7 days after eclosion) were used in all courtship assays. A single male and female were gently aspirated into a well-lit porcelain mating chamber (25-mm diameter and 10-mm depth) covered with a glass slide. Experiments were performed in a temperature and humidity-controlled room at 22°C, 40 to 50% humidity. Courtship index was determined as the percentage of time a male was engaged in courtship activity during a period of 10 min or until successful copulation. Courtship assays were recorded using a video camera (Sony HDR-CX405) and scored blinded to experimental condition.

Social space assays
Newly eclosed virgin female flies were collected within 4 hours after eclosion and housed in groups of 20 in vials with standard fly food. Flies were aged to 5 to 7 days after eclosion before the start of the assay. The social space area was made of two 18 cm by 18 cm square glass plates separated by 0.47-cm acrylic spacers. Two right triangle spacers (8 cm by 16 cm) were placed on opposite sides of the square area and two rectangular spacers (9 cm by 2 cm) were placed at the bottom of the area, resulting in an isosceles triangle-shaped space (base, 15.2 cm; height, 15.2 cm). Flies were gently aspirated into the social space arena by briefly removing the bottom rectangular spacers. Forty flies were included in each assay. After all 40 flies were introduced to the arena, the rectangular spacers were replaced, and the bottom of the arena was firmly tapped down five times. Digital images were captured using a video camera (Sony HDR-CX405) after allowing flies to settle for 20 min. Images were imported into Fiji for analysis. For each image, a body length measurement was taken as the average length in pixels, measured from top of the head to tip of the abdomen, of five randomly selected flies within the arena. The center of each fly was manually selected, and an automated measure of the nearest neighbor to each selection was determined using the Nearest Neighbor Distances Calculation plugin on Fiji (https://icme.hpc.msstate.edu/mediawiki/index.php/Nearest_Neighbor_Distances_Calculation_with_ImageJ.html). Results were binned by body length distances. We calculated a social space index as the difference between the number of flies in the first bin (0 to 2 body lengths) and the number of flies in the second bin (2 to 4 body lengths).

TARGET system experiments
For development temporal mapping experiments using the TARGET system, parental crosses were maintained on standard fly food at 18°C. Timed egg lays were achieved by flipping parental crosses to bottles with standard fly food from ZT1-ZT6 at 28°C or from ZT1-ZT8 at 18°C. To achieve ISWI knockdown at specific developmental stages, flies were kept at 28°C to allow for GAL80 denaturation. To repress RNAi expression, flies were moved to 18°C to prevent GAL80 denaturation. Because of temperature-related changes in Drosophila developmental timing, developmental periods were visually determined. Genetic controls were subject to the same temperature shifts as experimental flies to account for the effect of changing temperature on development. Sleep assays were conducted on 5- to 7-day old female flies at 22°C in 12-hour:12-hour LD light schedule.

Second instar larval sleep experiments
To synchronize developmental stages, adult fly parental crosses were placed in embryo collection cages (Genesee Scientific, catalog no. 59-100) for 24 hours. Eggs were laid on a petri dish containing 3% agar, 2% sucrose, and 2.5% apple juice with yeast paste spread on top. Molting first instar larvae were collected 2 days after egg lay and moved to a separate petri dish with yeast and allowed to molt into second instar. Freshly molted second instar larvae were placed in the LarvaLodge to monitor sleep as previously described. Collected data were analyzed using a custom MATLAB code (50).

Thermogenetic activation experiments
Animals were reared at 18°C to prevent activation of TrpA1 during development. Adult female flies were collected 2 to 3 days after eclosion and aged at 18°C on standard fly food. Five- to 7-day old flies were loaded into the DAM system to monitor sleep and placed at 22°C on a 12-hour:12-hour LD schedule for 3 days. TrpA1 activation was achieved by a temperature shift to 31°C across nonconsecutive 12-hour light or 12-hour dark period. Between increases in temperature, flies remained at 22°C.

RNA sequencing
Dissection and RNA extraction. Forty brains per sample at the mid-third instar stage were dissected in cold artificial hemolymph (AHL) (108 mM NaCl, 5 mM KCl, 2 mM CaCl2, 8.2 mM MgCl2, 4 mM NaHCO3, 1 mM NaH2PO4·H2O, 5 mM trehalose, 10 mM sucrose, and 5 mM Hepes). Three biological replicates for the control group and four for the experimental, each with 40 brains, were dissected. Brains were transferred to 1 ml of TRizol and incubated for 5 min at room temperature (RT). Chloroform (0.2 ml) was added, and samples were inverted. Samples were incubated 2 to 3 min at RT and then centrifuged at 12,000 g for 15 min at 4°C. Genomic DNA was removed using a genomic DNA elevator column (RNeasy Plus Micro Kit, Qiagen). RNA was then extracted using the RNeasy MinElute Cleanup Kit (Qiagen).

RNA library preparation and sequencing. Sequence libraries for each sample were synthesized using the NEBNext Ultra II Directional RNA kit following supplier recommendations and were sequenced on Illumina HiSeq-4000 sequencer as single reads of 100 base reads following Illumina’s instructions.

Differential gene expression analysis. RNA-seq reads were mapped to the D. melanogaster assembly BDGP6 preindexed with transcript models from Ensembl 87 using STAR 2.5.0b with default parameters except --alignIntronMax set to 10,000. Aligned reads were assigned to gene models using the summarizeOverlaps function of the GenomicRanges R package. Reads per kilobase per million were calculated with a slight modification, whereby only reads assigned to annotated protein-coding genes were used in the denominator, to minimize batch variability due to different amounts of contaminant ribosomal RNA. Differential expression was determined using the DESeq2 package. The annotated genes exhibiting an adjusted P value > 0.1 were considered to be differentially expressed compared to control. Visualization of DEGs was done using R package ggplot2 v3.2.0.

Gene interaction network analysis
Human homologs of Drosophila DEG in the setting of ISWI knockdown were identified using DIOPT (DRSC Integrative Ortholog Prediction Tool) v8.0 (76). We assessed the connectivity of SMARCA5 and SMARCA1 with these DEG homologs in the context of a brainspecific gene interaction network (63, 64). This network was constructed using a Bayesian classifier trained on gene coexpression data, which predicts the likelihood of interactions between pairs of genes in the brain. We generated a subnetwork containing all interactions.
Immunochemistry

Fly brains were dissected in 1× phosphate-buffered saline with 0.1% Triton X-100 (PBST) and fixed in 4% paraformaldehyde (PFA) for 15 min at RT. Following three 10-min washes in PBST, brains were incubated with primary antibody at 4°C overnight. Brains were washed three times for 10 min in PBST and incubated with secondary antibody for 2 hours at RT. After three 10-min PBST washes, brains were cleared in 50% glycerol and mounted in VECTASHIELD. The following primary antibodies were used at 1:1000 dilutions: mouse 1D4 anti-FasII (Developmental Studies Hybridoma Bank), rabbit anti-hemagglutinin (HA)-Tag (Cell Signaling Technology), guinea pig anti-γ-PER (a gift from A. Seghal), and rabbit anti-GFP (Thermo Fisher Scientific). The following secondary antibodies were used at 1:1000 dilutions: Alexa Fluor 488 donkey anti-mouse, Alexa Fluor 488 donkey anti-rabbit, Alexa Fluor 488 donkey anti–guinea pig, and Alexa Fluor 647 donkey anti–mouse (Thermo Fisher Scientific).

Imaging and analysis

Microscopy images were taken using a Leica TCS SP8 confocal microscope. Images were processed in NIH Fiji. All settings were kept constant between conditions within a given experiment. Images were taken in 1.0-μm steps unless otherwise noted.

PER quantification

To investigate PER expression in small ventral lateral neurons (sLNvs) and large ventral lateral neurons (lLNVs), brains were costained with anti-FasII staining and prior description of normal MB and large ventral lateral neurons (lLNVs), brains were costained with anti-pigment dispersing factor (PDF) (to label relevant cells) and anti-PER antibodies. Brains were dissected at CT0, CT4, CT12, and CT20. We defined the area of each sLNV or lLNv cell body by PDF staining. Area, mean gray value, and integrated density of the PER signal were measured for each cell body. Corrected total cell fluorescence (CTCF) of the cell body was calculated with the formula: CTCF = Integrated densitycell − (Area_cell × Mean background fluorescence). All cells per brain were averaged and compared across genotypes.

MB quantification

For temporal mapping, spatial mapping (OK107-GAL4 > UAS-ISWI RNAi), and neuroblast experiments, a maximum projection image of all Z-slices was generated. MB morphology was manually quantified from the maximum projection as a binary normal versus abnormal based on anti-FasII staining and prior description of normal MB morphology. For SMARCA1WT and SMARCAS4WT rescue experiments, for each Z-slice, the vertical or horizontal lobe on one hemisphere was manually outlined. The full volume of the vertical or horizontal lobe was measured using the 3D Objects Counter function in Fiji with the following settings: threshold = 1 and minimum puncta size = 100.

dFB volume

For each Z-slice, the dFB was selected on the basis of anti-GFP staining for R23E10 dFB projections. The full volume of the dFB was measured using the 3D Objects Counter function in Fiji with the following settings: threshold = 1 and minimum puncta size = 10,000.

dFB cell body

GFP+ soma were counted across the entire brain based on anti-GFP staining for R23E10 neurons.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 8.4.1). Sample size, specific tests, and significance values are denoted in figure legends.

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The chromatin remodeler ISWI acts during Drosophila development to regulate adult sleep

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