DEVELOPMENTAL NEUROSCIENCE

# The CHD8/CHD7/Kismet family links blood-brain barrier glia and serotonin to ASD-associated sleep defects

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**Sleep disturbances in autism and neurodevelopmental disorders are common and adversely affect patient's quality of life, yet the underlying mechanisms are understudied. We found that individuals with mutations in *CHD8*, among the highest-confidence autism risk genes, or *CHD7* suffer from disturbed sleep maintenance. These defects are recapitulated in *Drosophila* mutants affecting *kismet*, the sole *CHD8/CHD7* ortholog. We show that Kismet is required in glia for early developmental and adult sleep architecture. This role localizes to subperineurial glia constituting the blood-brain barrier. We demonstrate that Kismet-related sleep disturbances are caused by high serotonin during development, paralleling a well-established but genetically unsolved autism endophenotype. Despite their developmental origin, Kismet's sleep architecture defects can be reversed in adulthood by a behavioral regime resembling human sleep restriction therapy. Our findings provide fundamental insights into glial regulation of sleep and propose a causal mechanistic link between the CHD8/CHD7/Kismet family, developmental hyperserotonemia, and autism-associated sleep disturbances.**

## INTRODUCTION

Sleep is essential for health and cognition. In neurodevelopmental disorders, sleep disturbances are among the most common co-occurring features, observed with an incidence ranging between 50 and 80% (compared to ~20% in typically developing children) (*1*, *2*). Sleep problems adversely affect quality of life in patients and their families, to an extent that can exceed the burden of other physical and cognitive deficits (*1*, *3*). Despite evidence that sleep interventions can improve behavioral and cognitive outcomes (*4*), the mechanisms underlying sleep disturbances in the context of these disorders are unclear and understudied.

We propose that monogenic (Mendelian) neurodevelopmental syndromes that are characterized by highly prevalent and specific sleep disturbances offer unique opportunities to investigate disease pathologies and mechanisms, including those underlying sleep difficulties. At the same time, this work holds the potential to provide novel fundamental insights into the biology of sleep and its mechanistic links to cognitive disorders.

Pathogenic mutations in the *CHD8* (chromodomain helicase DNA binding protein 8) gene are a leading risk factor for autism spectrum disorders (ASDs) and among the most frequent findings revealed by large-scale exome sequencing efforts (*5*–*7*). They define an ASD subtype with distinctive clinical features [Online Mendelian Inheritance in Man (OMIM) 615032], including sleep complaints in most individuals with *CHD8* mutations, while other clinical comorbidities such as intellectual disability (ID) remain variable (*5*, *8*). However, the nature of CHD8-associated sleep problems (i.e., which parameters of sleep are affected) is unclear, and whether *CHD8* mutations increase the risk of sleep complaints compared to other genetic forms of ASD is controversial (*9*). Disruptive de novo mutations in the *CHD8* paralog *CHD7* lead to CHARGE syndrome (OMIM 214800), a rare and phenotypically variable neurodevelopmental disorder characterized predominantly by severe ID (*10*, *11*). Although initially less attention has been paid to behavioral problems, it is now well established that children with CHARGE syndrome also frequently exhibit autistic features and sleep disturbances (*12*, *13*). In these patients, sleep problems are significantly associated with anxiety and autistic-like behaviors, greatly influencing their quality of life (*12*). CHD8 and CHD7 interact with each other and are part of a large multisubunit complex that organizes chromatin assembly (*14*). Their molecular interaction suggests that the overlapping clinical features of both disorders have a common mechanistic basis.

Work in *Drosophila melanogaster*, the fruit fly, has made seminal contributions to our understanding of sleep and neurodevelopmental disorders (*15*, *16*). In *Drosophila*, *kismet* (*kis*) is the sole ortholog of *CHD8* and *CHD7* (*17*). Neuronal Kismet regulates morphology and synaptic physiology of the larval neuromuscular junction, axonal morphology and pruning of Kenyon cells, memory, and motor function (*17*–*19*). Moreover, Kismet is essential in a subset of circadian neurons for light-dependent circadian responses (*20*).

Here, we analyzed the role of the CHD8/CHD7/Kismet family in sleep regulation across species. First, we characterized the nature of sleep complaints in individuals with *CHD8* and *CHD7* mutations. We found specific defects in sleep initiation and maintenance that

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are highly consistent with each other and closely recapitulated in *Drosophila*. Leveraging the genetic tractability of *Drosophila*, we investigated the underlying spatiotemporal mechanisms of these sleep disturbances. Our data demonstrate that Kismet is required in glia, not in neurons, for sleep integrity at both larval and adult stages. We further localize sleep disturbances to a glial subtype constituting the blood-brain barrier (BBB) and to high serotonin levels during development, linking several previously unconnected dots in autism pathology. Last, despite the developmental origin of Kismet sleep defects, we provide a translatable behavioral strategy to ameliorate adult sleep abnormalities.

## RESULTS

### Individuals with mutations in *CHD8* and *CHD7* suffer from sleep initiation and maintenance defects

Previous studies suggest a high prevalence of sleep complaints in individuals with mutations in *CHD8* (*5*, *9*). A more detailed examination of sleep disturbances in this population has not been described. We extracted detailed sleep data from 23 individuals with disruptive *CHD8* variants from the Simons Simplex Collection (SSC) and National Institute of Mental Health-funded TIGER study, two large studies of neurodevelopmental disorder cohorts characterized by rare, ASD-associated single gene etiologies. This cohort showed significantly higher rates of difficulties falling asleep and maintaining asleep (i.e., frequent or prolonged nighttime awakenings) compared to a large sample of SSC individuals with idiopathic ASD (Fig. 1A and table S1). In addition, we observed a higher frequency of daytime complaints; however, this tendency did not withstand correction for multiple testing. There was no significant difference in rates of other sleep difficulties between groups.

Next, we further pursued in-depth sleep phenotyping in a small cohort consisting of one individual with a *CHD8* (individual 01) and three with *CHD7* mutations (individuals 02 to 04). All four individuals presented with sleep initiation and maintenance problems as indicated by increased sleep onset latency (SOL), increased wake after sleep onset (WASO), decreased sleep efficiency (SE; proportion of time spent asleep while lying in bed), and shorter total sleep time (TST) compared to reference values. Hence, all individuals exhibited chronic insomnia disorder as defined by the ICSD (International Classification of Sleep Disorders)–3 criteria (Table 1). Notably, except in one individual with a sleep-related rhythmic movement disorder, a common finding in children, no other sleep disorders [e.g., obstructive sleep apnea, as previously reported in some individuals with CHARGE syndrome (*21*)] were present. Objective measures of sleep, polysomnography (PSG) or actigraphy, were collected in individuals 02 and 04, respectively (fig. S1), validating the results of the sleep diaries. Our results are also consistent with previously reported sleep initiation and maintenance problems in CHARGE syndrome based on questionnaires, albeit these were collected, in part, for individuals lacking a genetic diagnosis (*3*, *13*). Together, our data strongly suggest a specific role for both *CHD8* and *CHD7* in sleep initiation and maintenance.

### *Kismet* mutants recapitulate human sleep maintenance defects

To address the potential role of the *Drosophila* CHD8/CHD7 ortholog Kismet (Fig. 1B) in sleep, we first set out to characterize sleep in $kis^{k13416}$ mutants. While homozygous $kis^{k13416}$ flies are not viable,

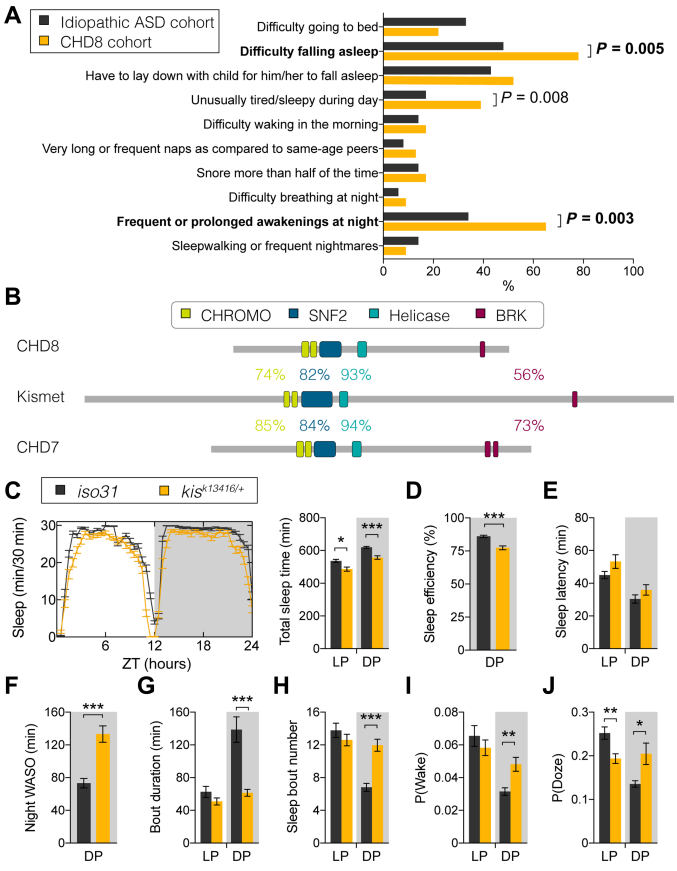


**Fig. 1. Mutations in the conserved CHD8/7/kismet family leads to sleep disturbances.** (**A**) Individuals with disruptive *CHD8* mutations present with higher rates of difficulties falling asleep and frequent/prolonged night awakenings compared to a SSC idiopathic ASD cohort. Groups were compared using Fisher's exact tests. Bonferroni correction for multiple comparisons was applied to the significance level ($P < 0.005$). Values in bold withstand correction for multiple testing. (**B**) High conservation of protein domains between human CHD8 and CHD7 and *Drosophila* Kismet. Percentages indicate similarity of amino acid sequence between Kismet and CHD8 (top) or CHD7 (bottom). CHROMO, chromatin organization modifier. (**C**) Sleep profile and quantification of TST of $kis^{k13416/+}$ flies ($n = 55$) and *iso31* genetic background controls ($n = 61$). $kis^{k13416/+}$ mutants show decreased TST during the light period [LP; Zeitgeber time (ZT) 0 to ZT12, $P = 0.016$, $t = 3.244$, df = 96.44] and dark period (DP; ZT12 to ZT24, $P < 0.0001$, $t = 5.201$, df = 87.4). (**D**) SE is reduced in $kis^{k13416/+}$ ($P < 0.0001$, $t = 5.201$, df = 87.4). (**E**) Latency to the first sleep bout after lights-on or lights-off is unaffected ($P > 0.8$). (**F**) WASO is increased at night ($P < 0.0001$, $t = 5.2$, df = 88.14). (**G**) Average bout duration in $kis^{k13416/+}$ flies is strongly reduced specifically during the DP ($P < 0.0001$, $t = 4.781$, df = 68.78), while (**H**) the number of bouts is increased ($P < 0.0001$, $t = 1.039$, df = 111.1). (**I**) P(Wake) is significantly increased during the DP ($P = 0.0048$, $t = 3.442$, df = 48.07), while (**J**) P(Doze) is decreased in LP ($P = 0.0075$, $t = 3.255$, df = 59.37) but increased in DP ($P = 0.042$, $t = 2.692$, df = 36.62) in $kis^{k13416/+}$ mutants compared to *iso31*. Data are represented as means ± SEM. Two-tailed unpaired Welch's *t* test with Bonferroni correction for multiple testing. *P* values are indicated as follows: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

$kis^{k13416/+}$ flies showed a mild but significant decrease in TST both during the light and dark periods (Fig. 1C). To allow for comparison with human sleep measures, we assessed orthologous sleep metrics (*22*) and found SE to be decreased (Fig. 1D). Whereas sleep latency was unaffected (Fig. 1E), mutants spent more time awake at night after their first sleep episode (WASO) (Fig. 1F). The elevated WASO

**Table 1. One individual with a *CHD8* (01) and three individuals with *CHD7* (02 to 04) pathogenic mutations, seen at a sleep clinic, also reported difficulties falling asleep and night awakenings.** Quantitative sleep parameters from parent-reported sleep-wake diaries confirmed these complaints: SOL and WASO were increased, and SE was decreased. Values in bold are deviating from established healthy reference values (*68*, *79*, *80*). See also Fig. 1, fig. S1, and tables S1 and S2.

| **Individuals** | **01** | **02** | **03** | **04** |
|---|---|---|---|---|
| Gender | Male | Female | Female | Female |
| Age of examination (years) | 4 | 3 | 8 | 19 |
| Molecular diagnosis | *CHD8* | *CHD7* | *CHD7* | *CHD7* |
| | c.4062 + 5G > C | c.4493_4506del | c.2643 T > A | c.5181 > G |
| | p.(Ser1350fs) | p.(Ile1498fs) | p.(Tyr881*) | p.(Tyr1727*) |
| Main sleep complaints | Difficulty falling asleep | Difficulty falling asleep | Difficulty falling asleep | Difficulty falling asleep |
| | Night awakenings | Night awakenings | Night awakenings | Night awakenings |
| ICSD-3 diagnosis | Chronic insomnia | Chronic insomnia | Chronic insomnia | Chronic insomnia; Sleep-related rhythmic movement disorder |
| **Sleep-wake diaries characteristics** | | | | |
| Days measured | 14 | 14 | 7 | 16 |
| TIB (min) | 715.7 | 677.1 | 762.9 | 647.8 |
| Total sleep time (min) | **474.6** | **559.3** | **514.3** | **400.3** |
| SOL (min) | 0 | **20.4** | **160.7** | **197.8** |
| WASO (min) | **241.1** | **97.5** | **87.9** | **49.7** |
| SE (%) | **66.7** | 82.5 | **67.6** | **61.7** |

in these animals was due to fragmented nighttime sleep, with a strong reduction in average sleep bout duration (from 140 min in controls to 61 min in $kis^{k13416/+}$) and an increase in the number sleep bouts (Fig. 1, G and H), revealing altered sleep architecture. We assessed circadian rhythms of locomotor activity in *kismet* mutants by means of fast Fourier transform (FFT). They showed normal rhythmicity (fig. S2).

We further determined two recently defined conditional probabilities to measure *Drosophila* sleep depth and pressure (*23*); P(Wake) can be regarded as a behavioral correlate of sleep depth or arousal, whereas P(Doze) reflects sleep pressure. $kis^{k13416/+}$ mutants showed elevated P(Wake) and P(Doze) at night, suggesting that sleep-wake instability drives nighttime sleep fragmentation (Fig. 1, I and J). P(Doze) was also decreased during the day, indicating reduced sleep drive during this time. Together, *kismet* mutant flies show reduced sleep duration with altered sleep architecture, indicating defects in sleep maintenance. These sleep deficits resemble the identified sleep disturbances in individuals with *CHD8/7* mutations.

## Kismet is required in glia for sleep integrity

To collect independent evidence for the role of Kismet in sleep integrity and to identify the cellular substrates through which it exerts its function, we next knocked down its expression in various cell types using the UAS-Gal4 system and inducible RNA interference (RNAi). Unexpectedly, *kismet* knockdown with the pan-neuronal *elav-Gal4* did not recapitulate $kis^{k13416/+}$ sleep defects (fig. S3, A to C). In contrast, pan-glial expression of two nonoverlapping RNAi constructs, $UAS\text{-}kis^{RNAi\text{-}1}$ and $UAS\text{-}kis^{RNAi\text{-}2}$, both gave rise to a significant reduction in TST at night (Fig. 2, A and B). This loss was due to a markedly decreased mean sleep bout duration (Fig. 2, Ai and Bi), partially compensated by an increase in the number of sleep bouts (Fig. 2, Aii and Bii). Thus, pan-glial knockdown recapitulated the reduced and fragmented nighttime sleep, causing even more severe defects than shown by the heterozygous *kismet* mutants. This indicates that Kismet is required in glia for sleep integrity.

## Adult sleep integrity relies on Kismet during development

To investigate whether Kismet is required in glia for sleep integrity during development or acutely in adulthood, we used temporal and regional gene expression targeting (TARGET) to restrict *kismet* knockdown to either before or after eclosion (Fig. 2, C and D). Flies carrying the *repo-Gal4* driver and ubiquitous GAL4 repressor $tub\text{-}Gal80^{ts}$ kept at restrictive temperature (22°C, knockdown OFF) throughout their life span showed comparable TST and architecture to background controls (fig. S4, A, Ai, and Aii). In contrast, when kept at the permissive temperature (28°C, knockdown ON), flies exhibited decreased and fragmented nighttime sleep (fig. S4, B, Bi, and Bii). Pan-glial *kismet* knockdown confined to pre-eclosion stages with either $UAS\text{-}kis^{RNAi}$ line resulted in decreased and fragmented nighttime sleep in the adult, recapitulating the defects in *kismet* knockdown throughout life span (Fig. 2, E, Ei, and Eii, and fig. S4, C, Ci, and Cii). In contrast, *kismet* knockdown exclusively in adult glia did neither alter sleep quantity nor architecture (Fig. 2, F, Fi, and Fii, and fig. S4, D, Di, and Dii). Together, these results demonstrate that Kismet is required during pre-eclosion developmental stages in glia for adult sleep integrity.

## Kismet is required in subperineurial glia of the BBB for sleep integrity

We next asked whether Kismet acts in neuropil-associated glia [astrocyte-like glia (ALG) and ensheathing glia (EG)], cortex glia (CG), or surface glia [perineurial glia (PG) and subperineurial glia (SPG)]. While previous work has implicated almost all glia subtypes in sleep

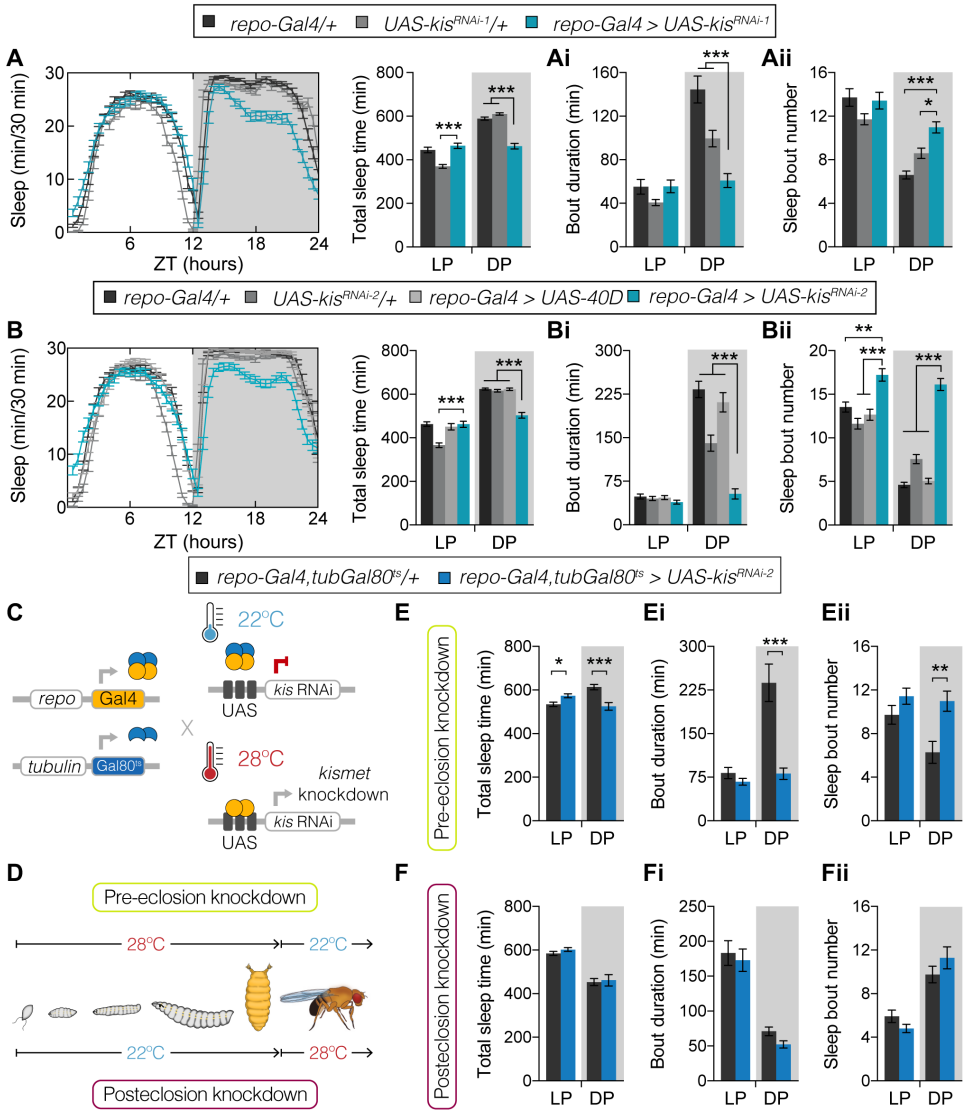


**Fig. 2. Pan-glial *kismet* knockdown leads to decreased and fragmented nighttime sleep, a phenotype of developmental origin.** (**A**, **B**, **E**, and **F**) Sleep profiles and/or quantification. (Ai, Bi, Ei, and Fi) Average duration and (Aii, Bii, Eii, and Fii) number of sleep bouts during the LP (ZT0 to ZT12) and DP (ZT12 to ZT24). (A, Ai, and Aii) Pan-glial *kismet* knockdown flies (*repo-Gal4,UAS-Dcr2 > UAS-kis*$^{RNAi-1}$, in turquois, $n = 65$) compared to background controls (*repo-Gal4,UAS-Dcr2/+* in black, $n = 74$; *UAS-kis*$^{RNAi-1}$*/+* in gray, $n = 61$) show reduced sleep time ($P < 0.0001$) and sleep fragmentation, characterized by reduced bout length ($P < 0.0001$), accompanied by increased bout number ($P < 0.0001$ and $P = 0.011$, respectively) exclusively during DP. (B, Bi, and Bii) Pan-glial *kismet* knockdown (*repo-Gal4,UAS-Dcr2 > UAS-kis*$^{RNAi-2}$, in turquois, $n = 77$) compared to background (*repo-Gal4,UAS-Dcr2/+* in black, $n = 90$; *UAS-kis*$^{RNAi-2}$*/+* in dark gray, $n = 65$) and UAS-40D controls (*repo-Gal4,UAS-Dcr2 > UAS-40D*, in light gray, $n = 67$), causes reduced sleep time ($P < 0.0001$) and reduced bout length ($P < 0.0001$), accompanied by increased bout number ($P = 0.0027$ and $P < 0.0001$, respectively), during DP. (**C**) Schematic representation of the TARGET system and (**D**) performed temperature shifts to achieve pre- and posteclosion knockdown. (E, Ei, and Eii) Pre-eclosion pan-glial *kismet* knockdown (*repo-Gal4,tubGal80*$^{ts}$ *> UAS-kis*$^{RNAi-2}$, in blue, $n = 44$) show decreased and fragmented sleep ($P = 0.0006$, $P = 0.0003$, and $P = 0.006$, respectively) during DP compared to controls (*repo-Gal4,tubGal80*$^{ts}$*/+*, in black, $n = 31$). (F, Fi, and Fii) Posteclosion pan-glial *kismet* knockdown (*repo-Gal4,tubGal80*$^{ts}$ *> UAS-kis*$^{RNAi-2}$, in blue, $n = 51$) does not alter sleep compared to controls (*repo-Gal4,tubGal80*$^{ts}$*/+*, in black, $n = 34$). Data are represented as means ± SEM. (A, Ai, Aii, B, Bi, and Bii): Kruskal-Wallis test with Dunn's correction. (E, Ei, Eii, F, Fi, and Fii): Two-tailed unpaired Welch's *t* test. Bonferroni correction for multiple testing. *P* values are indicated as follows: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. See also fig. S4.

regulation (*24*–*26*), sleep loss and fragmentation have exclusively been linked to ALG (*27*, *28*). Using specific Gal4 drivers (*29*, *30*), we noted that *kismet* knockdown in ALG (*NP3233-Gal4*) showed mild sleep fragmentation during both day and night (fig. S5, A, Ai, and Aii). Knockdown in EG (*NP6520-Gal4*) or CG (*NP2222-Gal4*) did not cause nighttime sleep fragmentation (fig. S5, B, Bi, Bii, C, Ci, and Cii).

Notably, *kismet* knockdown using the well-characterized *R54C07*-Gal4 driver targeting SPG presented with the combination of decreased nighttime sleep time and reduction of average sleep bouts observed in *kis*$^{k13416/+}$ and pan-glial knockdown, with both *UAS-kis*$^{RNAi}$ lines (Fig. 3, A, Ai, Aii, B, Bi, and Bii). Furthermore, kismet knockdown with an independent SPG driver line (*NP2276*-Gal4) resulted in decreased total sleep and bout duration and increased

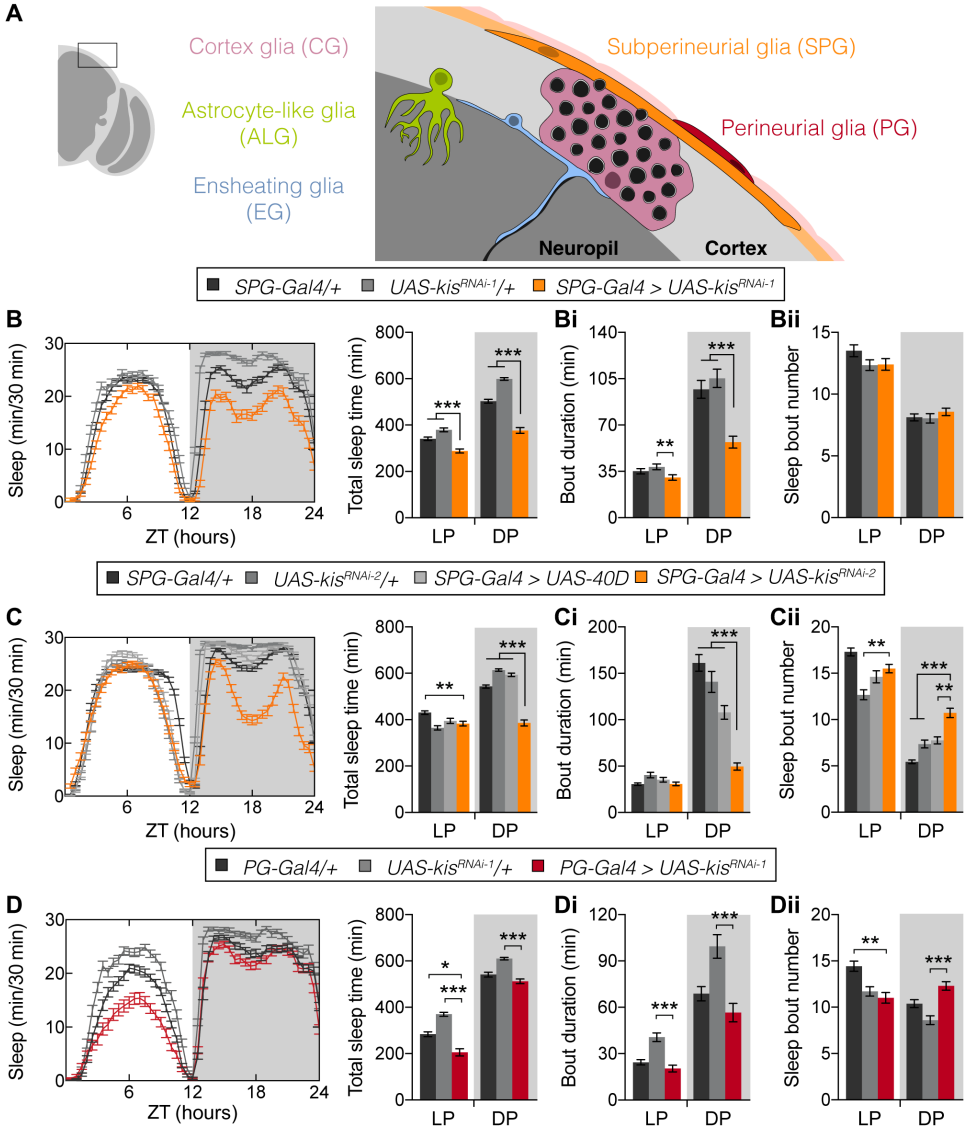


**Fig. 3. Kismet function in sleep integrity maps to SPG of the BBB.** (**A**) Schematic representation of glia subtypes in the adult *Drosophila* brain. (**B** to **D**) Sleep profiles and quantification. (Bi, Ci, and Di) Average duration and (Bi, Ci, and Di) number of sleep bouts in the LP (ZT0 to ZT12) and DP (ZT12 to ZT24). (B, Bi, and Bii) *Kismet* knockdown in SPG with the *R54C07*-Gal4 driver (*SPG-Gal4 > UAS-kis*$^{RNAi-1}$, in orange, $n = 104$) compared to background controls (*SPG-Gal4/+*, in black, n = 134; *UAS-kis*$^{RNAi-1}$*/+* in gray, $n = 85$) shows reduced sleep time during both LP ($P = 0.0006$) and DP ($P < 0.0001$), due to shorter sleep bouts ($P < 0.0001$). (C, Ci, and Cii) *Kismet* knockdown in SPG with the *R54C07*-Gal4 driver (*SPG-Gal4 > UAS-kis*$^{RNAi-2}$, in orange, $n = 108$) compared to background controls (*SPG-Gal4/+*, in black, $n = 150$; *UAS-kis*$^{RNAi-2}$*/+* in dark gray, $n = 86$) and UAS-40D control (*SPG-Gal4 > UAS-40D*, in light gray, $n = 66$) shows reduced sleep time during DP ($P < 0.0001$). Knockdown flies also display shorter nighttime bouts ($P < 0.0001$) accompanied by an increase in their number ($P < 0.0001$ and $P = 0.0024$, respectively). (D, Di, and Dii) *Kismet* knockdown in PG with the *R85G01-Gal4* driver (*PG-Gal4 > UAS-kis*$^{RNAi-1}$, in red, $n = 80$) compared to background controls (*PG-Gal4/+* controls in black, $n = 84$; *UAS-kis*$^{RNAi-1}$*/+* in gray, $n = 62$) shows reduced sleep time at LP ($P = 0.013$ and $P < 0.0001$, respectively). Nighttime sleep is unaffected. Data are represented as means ± SEM. Kruskal-Wallis test with Dunn's correction. Bonferroni correction for multiple testing. *P* values are indicated as follows: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. See also figs. S5 and S6.

bout number (fig. S5, E, Ei, and Eii). SPG are a group of about 300 cells that, together with the PG, form the *Drosophila* BBB (*29*). The inner SPG layer forms the paracellular barrier that separates the brain from the hemolymph, whereas PG provide physical and metabolic support and harbor a peripheral molecular clock that modulates circadian changes in BBB permeability (*31*). However, we found that *kismet* knockdown in PG (*R85G01*-Gal4) did not alter nighttime sleep (Fig. 3, C, Ci, and Cii). It has also been shown that inhibition of endocytosis of the surface glia increases baseline sleep and induces resistance to mechanical sleep deprivation (*26*). In contrast, SPG *kismet* knockdown did not affect homeostatic sleep rebound after mechanical deprivation (fig. S6, A and B). Together, these findings demonstrate that loss of *kismet* in SPG underlies reduced sleep and severe sleep fragmentation.

## Loss of Kismet in SPG disrupts sleep architecture in developing larvae

Given that *kismet* knockdown in glia before eclosion is sufficient to alter sleep architecture in the adult fly, we wondered whether sleep problems may already manifest during development. Second instar

*Drosophila* larvae have recently been shown to exhibit quiescent periods that meet the behavioral criteria of sleep (*32*). *Kismet* knockdown in SPG did not alter the total amount of larval sleep (Fig. 4A) but disrupted sleep architecture, resulting in decreased bout length (Fig. 4Ai) accompanied by an increase in bout number (Fig. 4Aii). As observed for adult sleep, larval sleep remained unaffected by *kismet* knockdown in PG (Fig. 4, B, Bi, and Bii). Together, Kismet is required in second instar larvae for proper sleep architecture, and this role too localizes to SPG.

## Glial *Kismet* loss leads to hyperserotonemia

To shed light onto the mechanisms by which loss of Kismet affects sleep, we first considered whether established sleep regulatory mechanisms may mediate Kismet function. Extensive research has highlighted the crucial role of dopamine in sleep and arousal and how dysfunctions of the dopaminergic system can lead to reduced and fragmented sleep (*33*). Therefore, we performed high-performance liquid chromatography (HPLC) to measure dopamine, using a column that simultaneously allowed us to assess serotonin (5-hydroxytryptamine) levels. Fly heads from pan-glial *kismet* knockdown and controls were collected at Zeitgeber time 6 (ZT6) and 18 (ZT18), without disturbing the light:dark (LD) cycle. Dopamine levels at ZT6 were unaltered, whereas we observed a significant, although mild, decrease of total dopamine levels at nighttime (ZT18) (Fig. 5A). In contrast, serotonin showed a notable 2-fold (ZT6) and 2.4-fold (ZT18) increase (Fig. 5B).

Following up on this finding, we investigated serotonin levels during development. We performed antiserotonin fluorescence immunohistochemistry and quantitative imaging (via photon counting, see Materials and Methods), in pan-glial and SPG-specific *kismet* knockdown wandering third instar larvae. We focused on the projections of serotonergic neurons of the subesophagal ganglion that innervate the proventriculus and anterior midgut of the larval digestive tract (Fig. 5C) (*34*). In controls, serotonin was visible in the proventricular nerve and at the level of proventricular innervations but absent or hard to discern in the distal projections that innervate the anterior midgut. In contrast, loss of glial Kismet led to elevated serotonin signal (Fig. 5D and fig. S7A), with serotonergic midgut innervations strongly labeled. As the innervated tissues appeared to show slightly higher levels of staining too, we quantified total signal intensities of α-serotonin signal across the proventriculus and in the anterior midgut, containing both the serotonergic terminals and the innervated tissues. We observed significantly increased serotonin at the midgut region of pan-glial *kismet* knockdown larvae (fig. S7, B, Bi, Bii, C, Ci, and Cii). Similarly, serotonin was significantly increased at the level of the proventriculus and midgut of SPG-specific *kismet* knockdown larvae (Fig. 5Di and fig. S7, E and Ei). Together, these results demonstrate that Kismet loss in glia leads to increased serotonin during development. The finding that glial loss of Kismet leads to increased serotonin is of considerable interest, as elevated serotonin (hyperserotonemia) is one of the most replicated, yet genetically unsolved, biomarkers in ASD (*35*, *36*).

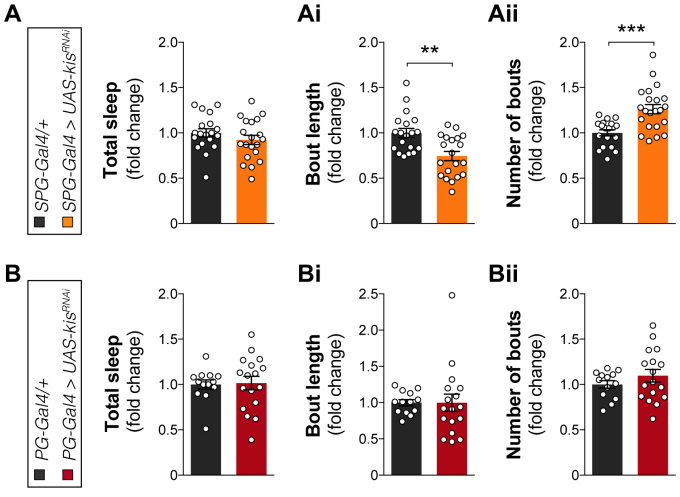


**Fig. 4. Loss of Kismet in SPG leads to fragmented sleep in the larval stage.** *Kismet* knockdown in SPG with the *R54C07*-Gal4 driver (*SPG-Gal4 > UAS-kis*$^{RNAi\text{-}2}$, in orange, $n = 20$) compared to background controls (*SPG-Gal4/+*, in black, $n = 19$) does not alter the (**A**) total amount of larval (second instar) sleep (quiescence bouts) ($P = 0.7$, $t = 1.149$, df = 36.78) but causes (Ai) decreased duration ($P = 0.003$, $t = 3.585$, df = 36.98) and (Aii) increased number ($P = 0.0003$, $t = 4.32$, df = 35.14) of sleep bouts. (**B**) *Kismet* knockdown in PG with the *R85G01*-Gal4 driver (*PG-Gal4 > UAS-kis*$^{RNAi\text{-}2}$, in red, $n = 17$) compared to background controls (*PG-Gal4/+*, in black, $n = 13$) does not alter the total amount of larval (second instar) sleep ($P > 0.9$, $t = 0.178$, df = 26.72), (Bi) average sleep bout duration ($P > 0.9$, $t = 0.0186$, df = 20.04) and (Bii) number ($P = 0.7$, $t = 1.183$, df = 25.44). Data are represented as means ± SEM. Two-tailed unpaired Welch's *t* test with Bonferroni correction for multiple testing. *P* values are indicated as follows: $^{**}P < 0.01$ and $^{***}P < 0.001$.

## Developmental hyperserotonemia underlies Kismet's glia-dependent sleep defects

To investigate whether restoring dopamine signaling can reverse the sleep defects of pan-glial *kismet* knockdown, we supplemented the food with methylphenidate (MPH; 1 mg/ml a dopamine transporter blocker that increases extracellular dopamine levels by blocking its reuptake), a dose that we and others has previously been shown to rescue sleep and other behavioral deficits of dopamine-related mutant conditions (*33*, *37*). However, MPH did not rescue sleep duration or architecture in the setting of *kismet* knockdown (fig. S8, A, Ai, and Aii).

Next, we addressed the hypothesis that the observed high serotonin levels underlie the reduced and fragmented sleep in the setting of glial *kismet* knockdown, by testing the effect of pharmacological serotonin depletion. Adult administration of 20 mM α-methyl-DL-tryptophan (αMTP), a specific *Trh* (*CG9122*) inhibitor (Fig. 5E) that has been show to induce a ~2-fold decrease in serotonin levels in fly heads (*38*), failed to improve sleep deficits of *kismet* pan-glial knockdown (Fig. 5, F and Fi). Administration of the same dose throughout development led to high lethality in control flies but notably allowed pan-glial *kismet* knockdown flies to survive. In this population, αMTP supplementation fully averted the adult sleep fragmentation (Fig. 5, F and Fi). Thus, elevated serotonin levels during development underlie, at least partially, the sleep fragmentation observed in adult *kismet* pan-glial knockdown flies.

Next, we sought to determine whether increased serotonin levels are sufficient to induce decreased and fragmented sleep. First, we attempted to pharmacologically increase serotonin levels by feeding its precursor 5-hydroxytryptophan (5-HTP; Fig. 5E). Administration of 5 mM 5-HTP specifically during development led to decreased sleep time and shorter sleep bouts in adults (Fig. 5, G and Gi). Second, recent work has shown that acute opto- or thermogenetic activation of serotonergic neurons in *Drosophila* adults leads to a decrease in sleep bout length and increase in sleep bout number, indicating that increased release of serotonin can fragment sleep (*39*). As Kismet's

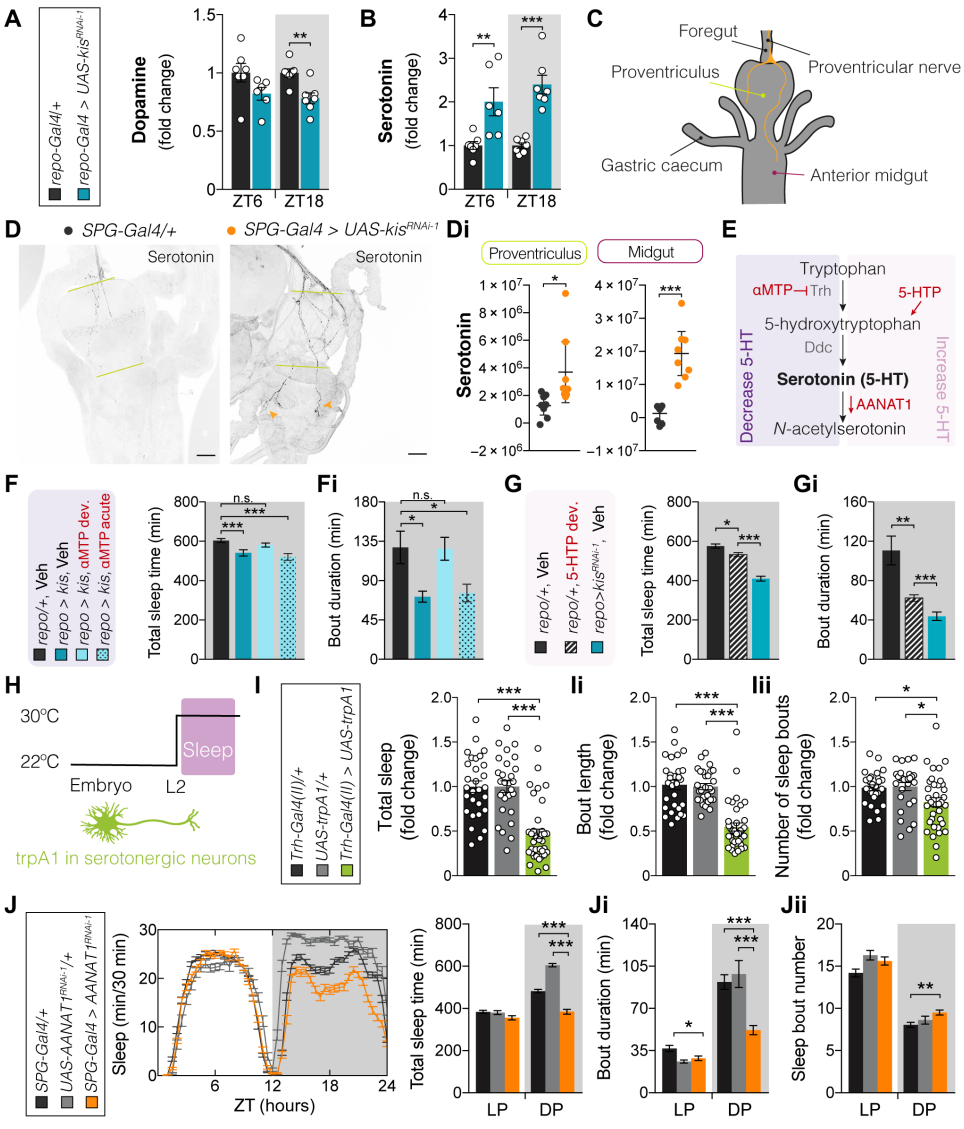


**Fig. 5. Sleep defects upon glial *kismet* loss are caused by developmental hyperserotonemia.** (**A** and **B**) *repo-Gal4,UAS-Dcr2 > UAS-kis*$^{RNAi-1}$ (blue, $n = 6$ to 7) exhibit decreased dopamine at ZT18 ($P = 0.0028$) and ≥2-fold increased serotonin at ZT6 ($P = 0.0077$) and ZT18 ($P = 0.0004$) compared to controls (black, $n = 7$). (**C**) Scheme of the larval digestive tract and serotonergic innervations. (**D**) Representative images and (Di) quantification of α-serotonin immunolabeling. SPG *kismet* knockdown (*R54C07-Gal4 > UAS-kis*$^{RNAi-1}$, orange, $n = 8$) causes increased serotonin in distal innervations (arrowheads) and diffuses signal in the proventriculus ($P = 0.037$) and anterior midgut ($P = 0.0002$) compared to controls (black, $n = 8$). Scale bars, 50 μm. (**E**) Serotonin metabolism in *Drosophila* and used approaches to manipulating it. (**F**, **G**, and **J**) Sleep profiles and/or quantification. (Fi, Gi, and Ji) Average duration and (Jii) number of sleep bouts during LP (ZT0 to ZT12) and DP (ZT12 to ZT24). (F and Fi) Pan-glial knockdown (*repo-Gal4,UAS-Dcr2 > UAS-kis*$^{RNAi-1}$, dark blue, $n = 32$) fed with αMTP during development (light blue, $n = 49$), but not adulthood (light blue dotted, $n = 30$), shows restored TST and sleep bout duration to vehicle-reared controls (black, $n = 41$). (G and Gi) Increasing serotonin in wild type (hatched black, $n = 72$) by developmental 5-hydroxytryptophan (5-HTP) feeding leads to decreased sleep due to significantly reduced sleep bout length compared to vehicle-fed animals (black, $n = 70$), phenocopying *kismet* knockdown (blue, $n = 62$). (**H**) Temperature program for thermogenetically controlled larval sleep recordings: (**I** to Iii) Thermogenetic activation of serotonergic neurons [*Trh-Gal4(II) > UAS-dTrpA1*, green, $n = 37$] leads to decreased sleep amount ($P < 0.0001$), bout length ($P < 0.0001$), and number ($P = 0.035$ and $P = 0.02$, respectively) compared to parental controls (*Trh-Gal4(II)/+*, $n = 29$; *UAS-dTripA1/+*, $n = 28$). (J to Jii) SPG *AANAT1* knockdown (*SPG-Gal4 > UAS-AANAT1*$^{RNAi-1}$, $n = 97$) leads to decreased total sleep ($P < 0.0001$) and bout length ($P < 0.0001$) compared to controls (*SPG-Gal4/+*, $n = 125$; *UAS-AANAT1*$^{RNAi-1}$*/+*, $n = 37$). Data are represented as means ± SEM, except (Di) represented as means ± 95% confidence interval. (A, B, and Di): Two-tailed unpaired Welch's *t* test. (F, Fi, G, Gi, H, I, Ii, and Iii): Kruskal-Wallis test with Dunn's correction. (J, Ji, and Jii): One-way analysis of variance (ANOVA). Bonferroni correction for multiple testing. *P* values: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. n.s., not significant. See fig. S7.

sleep phenotype is due to increased serotonin during development, we evaluated the effect of activating serotonergic neurons at this time. We expressed the heat-activated channel dTrpA1 using the *Trh-Gal4* driver and activated these neurons by raising the temperature while monitoring sleep in second instar larvae (Fig. 5H). Activation of serotonergic neurons with two different drivers led to a large reduction in sleep bout length and total sleep amount (Fig. 5, I, Ii, and Iii, and fig. S8, B, Bi, and Bii).

Last, we increased serotonin by reducing its metabolization. Serotonin acetylation, catalyzed by the *arylalkylamine N-acetyltransferase 1*

(*AANAT1*) enzyme (Fig. 5E), is the main mechanism of neurotransmitter inactivation in *Drosophila*, which stands in contrast to mammals where oxidation plays a central role (*40*). We therefore knocked down *AANAT1* in all glia and in SPG. Whereas pan-glial *AANAT1*$^{RNAi\text{-}1}$ knockdown was lethal, *AANAT1* knockdown with a second RNAi line (*AANAT1*$^{RNAi\text{-}2}$) resulted in fragmented nighttime sleep (fig. S8, C, Ci, and Cii). Moreover, *AANAT1*$^{RNAi\text{-}1}$ induction in SPG was viable and phenocopied the reduced and fragmented nighttime sleep (Fig. 5, J, Ji, and Jii). The latter finding was replicated with a third independent RNAi line targeting *AANAT1* (*UAS-AANAT1*$^{RNAi\text{-}3}$) (fig. S8, D, Di, and Dii). Together, our data provide strong evidence for loss of glial Kismet causing sleep loss and fragmentation through increased serotonin levels during development.

## Sleep restriction therapy restores sleep architecture in glial Kismet models

We aimed to identify approaches to improving sleep quality in our preclinical Kismet models. Approaches suitable for direct translation into the clinic should (i) require only postdevelopmental intervention and (ii) should be noninvasive and nonhazardous. Human sleep restriction therapy (SRT) is a key component of cognitive behavioral therapy for insomnia (CBT-I), the first-line intervention to treat chronic insomnia in humans (*41*). By restricting the sleep opportunity window, SRT enhances sleep drive leading to a more efficient and consolidated sleep. We recently established a model for SRT in flies and demonstrated that SRT in flies improves SE and continuity in short-sleeping *Drosophila* mutants and a fly model for Alzheimer's disease (*22*). Whether SRT can reverse sleep defects in neurodevelopmental disorders models is unknown.

We first restricted sleep opportunity in pan-glial *kismet* knockdown flies by reducing dark period duration. An SRT regime of 14:10 LD did not increase TST compared with pan-glial or SPG *kismet* knockdown flies in 12:12 LD (fig. S9, A and Ai); however, SE was fully rescued to control levels (Fig. 6, A, Ai, B, and Bi). Furthermore, SRT rescued sleep bout duration (Fig. 6, C and Ci) and bout number (Fig. 6, D and Di). Most notably, both pan-glial and SPG *kismet* knockdown flies showed a significant decrease in WASO, back to control at 12:12 LD levels (Fig. 6, E and Ei). SRT further reduced latency to sleep at night compared to flies in a 12:12 LD regime (fig. S9, B and Bi). Together, our data show that a behavioral regime can rescue sleep defects of Kismet models despite their developmental origin, including disturbed sleep architecture, decreased efficiency and increased WASO, The critical sleep metrics affected in individuals with *CHD8* and *CHD7* mutations.

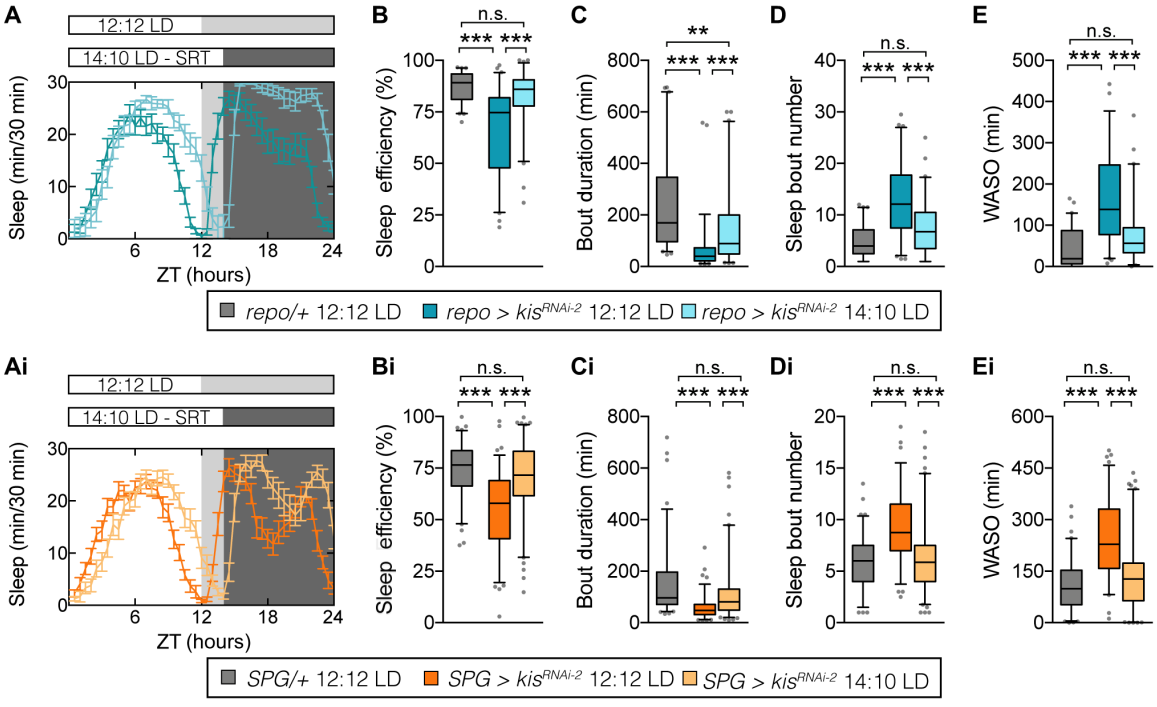


**Fig. 6. Sleep restriction therapy rescues sleep architecture in *Drosophila* glia Kismet models.** (**A**) Representative sleep profiles of pan-glial *kismet* knockdown flies entrained either at 12:12 LD (*repo-Gal4,UAS-Dcr2 > UAS-kis*$^{RNAi\text{-}2}$, in dark blue, $n = 13$) or at 14:10 LD (*repo-Gal4,UAS-Dcr2 > UAS-kis*$^{RNAi\text{-}2}$, in light blue, shortened DP ZT14 to ZT24, $n = 15$). (Ai) Representative sleep profiles of SPG *kismet* knockdown flies entrain either at 12:12 LD (*SPG-Gal4 > UAS-kis*$^{RNAi\text{-}2}$, in dark orange, $n = 16$) or at 14:10 LD (*SPG-Gal4 > UAS-kis*$^{RNAi\text{-}2}$, in light orange, $n = 16$). (**B** to **E**) Quantification of sleep parameters of control flies entrained at 12:12 LD (*repo-Gal4,UAS-Dcr2/+*, in gray, $n = 61$), and pan-glial *kismet* knockdown flies entrain either at 12:12 LD (*repo-Gal4,UAS-Dcr2 > UAS-kis*$^{RNAi\text{-}2}$, in dark blue, $n = 62$) or at 14:10 LD (*repo-Gal4,UAS-Dcr2 > UAS-kis*$^{RNAi\text{-}2}$, in light blue, $n = 63$). (Bi, Ci, Di, and Ei) Quantification of sleep parameters of control flies entrained at 12:12 LD (*SPG-Gal4/+*, in gray, $n = 85$), and SPG *kismet* knockdown flies entrain either at 12:12 LD (*SPG-Gal4 > UAS-kis*$^{RNAi\text{-}2}$, in dark orange, $n = 97$) or at 14:10 LD (*SPG-Gal4 > UAS-kis*$^{RNAi\text{-}2}$, in light orange, $n = 100$). (B and Bi) SE, (C and Ci) average duration, and (D and Di) number of sleep bouts in the DP. (E and Ei) WASO; minutes spent awake after first sleep episode during DP. SRT successfully improves SE and architecture in pan-glial and SPG *kismet* knockdown flies. Data are represented as a boxplot that extends from the 25th to the 75th percentiles with the median indicated. Whiskers indicate the 5th and 95th percentiles. Kruskal-Wallis test with Dunn's correction. Bonferroni correction for multiple testing. *P* values are indicated as follows: $^{**}P < 0.01$ and $^{***}P < 0.001$. See also fig. S9.

### Human *CHD8* and *CHD7* are expressed in glia and components of the BBB

Last, to address whether a conserved role in glia or other cells forming the BBB can underlie the sleep disturbances shared by *Drosophila* Kismet mutants and individuals with mutations in *CHD8* or *CHD7*, we revisited recent high-resolution single-cell RNA sequencing (RNA-seq) datasets of human cortex at prenatal and postnatal stages. We found *CHD8* and *CHD7* to be modestly to highly expressed in all cells forming the human BBB, both during development (radial glia, astrocytes, endothelial, and mural cells) and in adulthood (astrocytes, endothelial cells, and pericytes) (Fig. 7, A, Ai, and Aii). At adult stage, *CHD7* shows a notable enrichment in glia compared to neuronal populations, whereas *CHD8* is widely expressed across the brain (Fig. 7, B, Bi, and Bii). In conclusion, cutting edge single-cell data support a role for both *kismet* orthologs in glia and other cell types constituting the human BBB, both during development and in adulthood.

## DISCUSSION

Sleep problems in ASD-associated neurodevelopmental syndromes and idiopathic ASD are a common complaint. The negative impact of sleep loss on these vulnerable patient groups and their families is particularly high, and therapeutic options are urgently needed. Whether sleep is under direct control of genes implicated in ASD and related neurodevelopmental disorders is unknown for the vast majority of disorders. Motivated by reports on frequent and severe sleep disturbances in individuals with mutations in the chromatin remodeler *CHD8* and its paralog *CHD7*, we here characterized in-depth their sleep deficits and dissected the underlying mechanisms using *Drosophila* as a model. Our study provides previously unknown fundamental insights into the regulation of sleep, linking autism-related *CHD8* and *CHD7* genes, BBB glia, and developmental hyperserotonemia with sleep maintenance defects, and identifies a safe, validated, and translatable behavioral strategy to reduce sleep challenges for patients.

### Specific and highly conserved sleep deficits caused by mutations in the CHD8/CHD7/Kismet chromatin remodeling family

By extracting sleep data from two large and comprehensive ASD cohorts, combined with more detailed sleep phenotyping in a smaller cohort, we found that individuals with disruptive *CHD8* and *CHD7* mutations show difficulties falling asleep and frequent and/or prolonged night awakenings, identifying specific problems in sleep maintenance. Despite sleep disturbances being one of the most prevalent complaints reported in *CHD8* individuals (*5*, *8*), it has been questioned whether the frequency of sleep defects is elevated over the already high prevalence associated with ASD (*42*) and thus whether CHD8 does play a specific role in sleep (*9*). Resolving various aspects of sleep disorders, we found sleep initiation and maintenance to be specifically enriched compared to idiopathic ASD, highlighting the importance of examining sleep defects with the greatest possible resolution. Moreover, using objective sleep assessment shows that the same complaints—short TST, low SE, and increased SOL and WASO—are present in individuals with mutations in its paralog *CHD7*, in accordance with previous CHARGE syndrome literature based on sleep questionnaires (*3*, *12*, *13*). These results must be interpreted in the context of the limited cohort size that was available to us. Because of this limitation, it also remains to be determined whether sleep disturbance severity in individuals with *CHD7* compared to *CHD8* mutations correlate with the overall more severe and broader clinical manifestation of CHARGE syndrome. However, the shared sleep characteristics of both disorders made them highly suitable for sleep studies in *Drosophila* with its single orthologous gene.

Similar to humans, we identified decreased and fragmented nighttime sleep in *Drosophila* $kis^{k13416/+}$ mutants, denotating disturbed sleep maintenance. Although flies also sleep during the day, longer

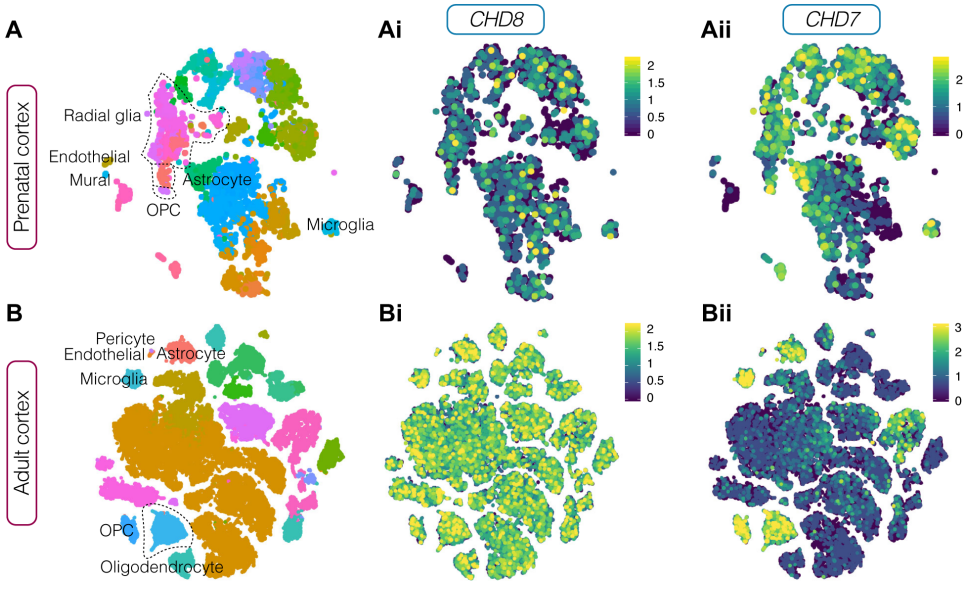


**Fig. 7. Human *CHD8* and *CHD7* are highly expressed in glia and components of the BBB.** (**A** and **B**) (Ai and Bi) *CHD8* and (Aii and Bii) *CHD7* expression in single-cell RNA-seq data of (A, Ai, and Aii) prenatal and (B to Bii) adult human cortex support a role for both *kismet* orthologs in glia throughout life span. Scatterplots after principal components analysis and t-stochastic neighbor embedding from left to right: colored by cluster, *CHD8*, and *CHD7* expression.

sleep episodes preferentially occurring during the night are thought to reflect a crucial, deep sleep state (*39*). In *kismet* mutants, this disruption in sleep architecture results in decreased SE and increased night WASO, paralleling the sleep defects identified in individuals with mutations in *CHD8*/*CHD7*. Although sleep differences in the *kismet* mutant primarily rely on glial Kismet function, the absence of sleep phenotypes in pan-neuronal knockdown flies does not exclude that Kismet may also modulate sleep through executing similar functions in neuronal entities regulating sleep. Considerable evidence suggests key roles for sleep in synaptic development and plasticity (*43*). Kismet has previously been shown to regulate synapse assembly and synaptic transmission (*18*), and our findings establish a role for Kismet in sleep. Recent work in flies has likewise found that insomniac controls synaptic homeostasis in addition to its role in adult sleep (*44*). These results set the stage for investigating how sleep and synaptic functions are coupled at a genetic level. Our study reveals specific, highly conserved, and consistent defects in sleep maintenance caused by mutations in the CHD8/CHD7/Kismet chromatin remodeling gene family.

### Kismet and BBB glia play a role in sleep integrity, potential implications for individuals with *CHD8*/*CHD7* mutations

Despite Kismet's predominant expression in neurons (*17*, *18*), we mapped its role in sleep to glia and more specifically to SPG, a key component of the *Drosophila* BBB. Thus, our data raise the intriguing possibility that glia and the BBB are involved in the pathophysiology of the disorder. Despite only representing 2% of the glia population (*29*), *kismet* knockdown in SPG fully recapitulated pan-glial manipulations. Notably, this does not rule out that Kismet also operates in other glial subtypes to affect sleep. We observed mild but significant sleep fragmentation upon *kismet* knockdown in ALG and a decrease in sleep time upon EG manipulation. These additional roles of Kismet likely contribute to minor differences in phenotype between mutant, pan-glial, and SPG-specific phenotypes. We focused on the most marked and innovative phenotype and revealed that SPG dysregulation can give rise to reduced and fragmented sleep.

Inhibition of endocytosis in SPG and PG has recently been found to increase and consolidate sleep and to confer resistance to sleep deprivation (*26*). Our finding that sleep rebound remains intact upon *kismet* knockdown and that its function is confined to SPG, not PG, suggests that SPG regulate different sleep processes through multiple mechanisms. Apart from sleep, it has been shown that the permeability of the *Drosophila* BBB is rhythmically controlled by a molecular clock residing in PG (*31*). Whether Kismet loss also leads to changes in BBB permeability and how this relates to loss of CHD8 and/or CHD7 are exciting questions for future research, as emerging data suggest a potential connection of BBB changes to other dysregulated states, including epilepsy (*45*).

Notably, Kismet loss in SPG already disrupts sleep architecture in *Drosophila* larvae, resulting in shorter and more frequent bouts. Thus, our work reveals the importance of glia cells, specifically of SPG, in larval sleep and shows that Kismet's function in sleep persists from early development to adulthood. In contrast, classic short-sleeping mutants do not show larval sleep defects, and even a key regulator of arousal such as dopamine does not show the same waking effects in larvae as in adults (*32*). It is conceivable that Kismet's function in glia and its persistent role in sleep throughout life span are directly related. SPG are specified during embryogenesis and maintain numbers and function from early larval specification to adulthood (*30*), while neurons undergo extensive remodeling during metamorphosis (*46*). Hence, SPG-encoded regulation of sleep may provide a mechanism for persistent sleep control across the life span.

Could dysregulation of the BBB contribute to CHD8- and CHD7-associated pathologies? Our single-cell expression analyses revealed that both genes are expressed in glia in prenatal and adult human cortex. Although the mature mammalian BBB is not exclusively constituted by glia cells, it shares many commonalities with the *Drosophila* BBB, including structural features, function, and regulatory mechanisms (*45*). However, in mammalian embryogenesis, the first cellular barrier separating the nervous system from the vascular elements is constituted by radial glia (*47*). Most relevantly, both *CHD8* and *CHD7* are expressed in prenatal endothelial and mural cells and in radial glia. Both genes are also highly expressed in the components of the mature BBB (astrocytes, endothelial cells, and pericytes). They are also highly expressed in oligodendrocyte precursor cells (OPCs) and oligodendrocytes, together supporting a sustained role of CHD8/7 in glia and previous findings in mouse that have revealed a key role for both genes in OPC survival and oligodendrocyte differentiation, myelination, and postinjury remyelination (*48*–*50*). On the basis of their expression and in light of the highly specific and conserved aspects of sleep that are affected in both species, it appears plausible that glia and/or other BBB-contributing cells, and a function of CHD8 and CHD7 therein, are relevant to the pathophysiology of these neurodevelopmental disorders.

### Origin and consequences of hyperserotonemia caused by glial loss of Kismet

We found that glial loss of Kismet leads to increased levels of serotonin, both in adults and in third instar larvae. Multiple studies have demonstrated elevated platelet serotonin in idiopathic ASD cohorts; this finding, referred to as hyperserotonemia, has proven to be one of the most replicated and consistent biomarkers in ASD (*35*, *36*). However, despite ~60 years since the initial study reporting hyperserotonemia, its origin and role in ASD-associated clinical features have remained largely unsolved. Our study provides several important steps forward in this field. First, the genetic basis of hyperserotonemia in ASD has remained largely unknown (*51*). Our findings link hyperserotonemia to the evolutionary conserved CHD8/CHD7/Kismet family, highly implicated in ASD (*6*, *7*). Second, the etiology of sleep problems associated with autism is unclear; they have classically been regarded as secondary to other behavioral deficits (*52*). Moreover, whereas the involvement of serotonin in sleep regulation has been known for many years, it has remained unclear whether serotonin plays a sleep- or a wake-promoting effect (*34*, *53*). Recent work has shown that acute activation of serotonergic neurons in *Drosophila* adults leads to sleep fragmentation without changing sleep duration (*39*), whereas we observed that serotonergic neuronal activation during development (in second instar larvae) leads to a decrease in sleep amount and shorter sleep bouts. Together, these data suggest that the serotonergic regulation of sleep is neuronally mediated but context dependent (i.e., dependent on developmental stage and/or duration of serotonin dysregulation), which contributes to the complexity of serotonin's role in sleep. Moreover, not unexpectedly, it appears to depend on the cell type of origin. Whereas we did not (yet) observe a change in total sleep amount in *kismet* knockdown L2 larvae, activation of serotonergic neurons at the same stage diminished total sleep. It is conceivable that this difference is

due to a stronger activation of serotonergic neurons in the latter case. Despite this complexity, our multiple lines of evidence obtained through genetic, thermogenetic, and pharmacological manipulations demonstrate that high serotonin levels during development cause sleep disturbances later in life and suggest hyperserotonemia as a sleep-relevant mechanism in individuals with mutations in *CHD8/7*.

Last, increased serotonin has been associated with various ASD-relevant behaviors, including social behavior, in both patients and animal models (*54*, *55*). Thus, our findings may account for other ASD-associated clinical features than just the sleep disturbances in individuals with *CHD8/7* mutations. As we observed increased serotonin levels in the projections to the proventriculum and anterior midgut, serotonin imbalance in these organs could contribute to other physiological and/or behavioral problems [e.g., gastrointestinal problems reported in individuals with *CHD8* mutations (*5*)]. In conclusion, our study establishes a causal relationship between BBB glia, hyperserotonemia, sleep, and one of the most frequent risk factors of ASD, providing substantial progress in autism research.

How does Kismet loss lead to hyperserotonemia? Although direct target genes of Kismet in glia remain to be determined, a recent targeted DNA adenine methyltransferase identification (DamID) analysis in intestinal stem cells (*56*) identified four genes associated with the serotonergic system: the receptors *5-HT2A*, *5-HT2B*, and *5-HT7* and *AANAT1*. Since we showed that increasing serotonin levels via *AANAT1* knockdown in glia and SPG phenocopied the fragmented nighttime sleep observed in *kismet* knockdown, glial *AANAT1* deregulation may account for or contribute to the observed sleep defects in the *Drosophila* models and participate in CHD8/CHD7 pathophysiology. Human *AANAT* (homolog of *AANAT1*) is required for melatonin synthesis, and its activity is reduced in ASD cohorts (*51*), which additionally present with low plasma and urine melatonin levels (*57*). Previous work has shown that *AANAT1* mutants exhibit increased rebound upon sleep deprivation (*58*), and that astrocytic AANAT1 is crucial for proper homeostatic rebound, while its loss in neurons leads to a more consolidated sleep (*59*). Our work shows yet another previously unidentified and cell-specific role of AANAT1 in sleep regulation and is consistent with evidence for serotonergic regulation of sleep in *Drosophila*. Further work is needed to understand which dysregulated Kismet target genes in SPG lead to hyperserotonemia and how these findings may relate to human CHD8 and CHD7 pathology.

How does hyperserotonemia arising from Kismet dysfunction lead to sleep disturbances? Our data show that pharmacologically reducing serotonin levels in pan-glial *kismet* knockdown flies restores adult sleep integrity when applied during development. The fact that αMTP treatment causes lethality in control animals with normal serotonin levels, but is not lethal in *kismet* knockdown larvae, further supports a link between Kismet and developmentally increased serotonin. Reversely, pharmacologically increasing serotonin levels with 5-HTP in the same critical window lead to a decreased and fragmented sleep in wild-type flies. The effect of 5-HTP may not be as notable as *kismet* knockdown due to the light sensitivity of this compound as treated animals were kept at 12:12 LD. Moreover, it has been reported that during development, serotonin restricts the growth of serotonergic neurons via a negative feedback loop (*60*). Thus, increased serotonin levels upon Kismet loss during critical periods for circuit formation and maturation may lead to loss of serotonergic terminals and miswiring of neural networks, in analogy to existing prominent hypotheses how hyperserontonemia can cause ASD-associated clinical features (*55*).

### Applicability of SRT in CHD8/7 syndromes and other neurodevelopmental disorders

The developmental trajectories of sleep disturbances in young children with neurodevelopmental disorders suggest that we should instigate intervention as early as possible, yet there is an urgent need to remediate sleep problems in older children and adults as well. We show here that SRT improved sleep quality in both pan-glial and SPG *kismet* knockdown flies. With this behavioral approach, we effectively rescued shortened sleep bout duration and increased bout number, restored SE and WASO to control levels, and significantly improved sleep latency. Notably, these aberrancies in sleep metrics in *Drosophila* reflect those perturbed in *CHD8* and *CHD7* individuals. It is remarkable that sleep opportunity restriction, applied only in adulthood, can override sleep defects that are, as we demonstrated, of developmental origin. Hence, we propose that SRT provides a viable strategy to improve sleep in patients with *CHD8/CHD7* mutations and perhaps other forms of autism and related neurodevelopmental disorders. Although not quantitatively assessed, components of CBT-I including sleep opportunity restriction (i.e., shortening bedtimes) were advised to CHD8/7 individuals 01 to 04 seen in our sleep center and showed positive effects on the insomnia, as documented in the patient files (table S2).

In this study, we shed light onto mechanisms and cellular substrates regulating sleep, linking the evolutionary CHD8/CHD7/Kismet family to BBB glia, hyperserotonemia, and ASD-associated sleep defects. With our work, we also aim to contribute to a paradigm shift away from the commonly held view that sleep deficits are coupled inevitably to the neurodevelopmental disorders in which they occur and are therefore refractory to therapy.

## MATERIALS AND METHODS

### Study design: Sleep assessment of *CHD8* and *CHD7* individuals

In neurodevelopmental disorder cohorts, the prevalence of *CHD8* and *CHD7* loss-of-function mutations lies in the range of 0.15 to 0.40% and 0.28%, respectively (*5*, *61*, *62*). We assessed sleep characteristics in a cohort of individuals with *CHD8* mutations using structured questionnaires. Probands included 23 individuals with disruptive mutations to *CHD8*. Six individuals were drawn from the SSC (*63*, *64*), and 17 probands were drawn from an ongoing study of individuals with mutations to ASD-associated genes (NIMH TIGER) (*9*). Pathogenic or likely pathogenic mutations to *CHD8* were confirmed through review of clinical or research genetic testing results; individuals with other pathogenic mutations to neurodevelopmental disorder and/or sleep related genes were excluded from analyses. To establish a comparison group of individuals with ASD but no known genetic etiology ("idiopathic" ASD), 2305 probands without pathogenic ASD-associated single-gene variants or deleterious copy number variants as confirmed by whole-exome sequencing were drawn from the SSC (*64*, *65*). Age and sex for the *CHD8* group and the idiopathic comparison group are presented in table S3. Written consent was obtained from participants, and all procedures were approved by the University of Washington Institutional Review Board.

A comprehensive, structured medical history interview initially designed for the large-scale SSC was completed with primary caregivers

of all participants in SSC and NIMH TIGER, which included questions about history of sleep problems. Caregivers were asked whether their child had ever experienced the sleep problems listed in table S1, which included bedtime problems, excessive daytime sleepiness, sleep-disordered breathing, and nighttime awakenings. The same sleep history questions were used in both SSC and TIGER. Fisher's exact tests were used to determine whether rates of specific sleep problems in individuals with disruptive *CHD8* mutations differed from a large, idiopathic ASD group obtained through the SSC. Bonferroni correction for multiple comparisons was applied to the significance level ($P < 0.005$). Groups differed by gender [$\chi^2(1, n = 2311 = 9.84, P = 0.002)$], with the *CHD8* sample containing a significantly larger proportion of females than the SSC group. Groups did not differ by age [$t(22.19) = -1.52, P > 0.05$]. Analyses were completed in SPSS version 26 (IBM Corp., 2019).

Second, to further address the clinical significance of *CHD7/8*-associated sleep problems, we searched for individuals with *CHD7* or *CHD8* pathogenic mutations that have been seen in a tertiary sleep medicine center upon referral due to sleep complaints. Therefore, we screened all referrals between 2012 and 2019 for individuals with mutations in those genes, identifying one additional individual with a pathogenic *CHD8* mutation and three patients with *CHD7* mutations. All four patients underwent a basic clinical workup including a full history taking, physical examination, and a consensus sleep diary (*66*), based on which time in bed (TIB), TST, SOL, WASO, and SE were calculated per individual with a 15-min bin resolution provided by the parent-reported sleep-wake calendars. SE was calculated as TST divided by TIB and multiplied by 100. SOL was calculated as the time between going to bed and sleep onset. WASO was calculated as the total time awake between sleep onset until the end of TIB. When appropriate, wrist actigraphy (MotionWatch 8, CamNtech, Cambridgeshire, UK) or video-PSG was performed. A sleep disorder diagnosis was made according to the ICSD-3 criteria (*66*, *67*). Mean sleep duration was compared to values typically characterizing a pediatric population (*68*). Effective and ineffective treatments (pharmacological and behavioral) either received in the past or applied after consultation in the sleep clinic are reported in table S2. Informed consent was according to local ethical procedures.

### *Drosophila* strains and husbandry

Fly stocks were reared on standard medium containing cornmeal, yeast, sugar, and agar at 25°C and 60% humidity in a 12:12 LD cycle. The following fly stocks were used for this study: *iso31*, $kis^{k13416}$ mutants [(Bloomington Drosophila Stock Center (BDSC) #10442)] backcrossed 6× to *iso31*, *UAS-*$kis^{RNAi1}$ (VDRC #46685), *UAS-*$kis^{RNAi2}$ (VDRC #109414), *UAS-AANAT1*$^{RNAi1}$ (VDRC #47906), *UAS-AANAT1*$^{RNAi2}$ (VDRC #105064), *UAS-AANAT1*$^{RNAi3}$ (BDSC #36726), background control GD library [Vienna Drosophila Resource Center (VDRC#60000 )], background control KK library (VDRC #60.100), background control Transgenic RNAi Project (TRiP) library attP40 (BDSC #36304), *repo-Gal4* (*UAS-Dcr2/CyO; repo-Gal4/TM3*, *Sb*), *repo-2-Gal4* (*repo-Gal4/TM6b, Tb, Sb*), *repo-Gal4,tubGal80*$^{ts}$ (*repo-Gal4,tubGal80*$^{ts}$*/TM3, Sb*), *elav-Gal4* (*UAS-Dcr2; elav-Gal4*), *SPG-Gal4* (*R54C07-Gal4*, BDSC #50472), *PG-Gal4* (*R85G01-Gal4*, BDSC #40436), *ALG-Gal4* [*NP3233-Gal4*, [Drosophila Genomics Resource Center (DGRC) #113173], *EG-Gal4* (*NP6520-Gal4*, DGRC #105240), *CG-Gal4* (*NP2222-Gal4*, DGRC #112830), *SPG-2-Gal4* (*NP2276-Gal4*, DGRC #112853), *Trh-Gal4(II)* (BDSC #38388), *Trh-Gal4(III)* (BDSC #38389), and *UAS-dTrpA1* backcrossed 6× to *iso31*. The *UAS-*$kis^{RNAi1}$ line has previously been shown to induce a strong knockdown (≈90%) (*17*, *20*). The pKC43 insertion sites of the KK RNAi lines used in this study were verified by diagnostic polymerase chain reaction according to the findings of Green and colleagues (*69*). The hairpin construct of the *UAS-*$kis^{RNAi-2}$ line (VDRC #109414) was found to be inserted in both 40D and 30B landing sites, in agreement with information meanwhile available at VDRC. Thus, an additional control for the effect of the UAS insertion into the 40D locus was used (VDRC #60101), referred to as UAS-40D control.

### Adult sleep analysis

Locomotor activity and sleep were recorded with the *Drosophila* Activity Monitor (DAM2) system from TriKinetics (Waltham, MA, USA). Briefly, 3- to 5-day-old male flies were individually transferred without $CO_2$ anesthesia to glass tubes (65 mm by 5 mm) containing standard food, loaded into the DAM systems, allowed to acclimatize to activity monitors and food for at least 12 hours, and monitored for 4 days at 25°C in a 12:12 LD cycle. For SRT experiments, the acclimation and monitoring were performed simultaneously in two different incubators at 12:12 LD and 14:10 LD. Motion was detected via the monitors' infrared light beams, and activity and sleep (defined in *Drosophila* as 5 or more minutes of inactivity) (*58*, *70*) parameters were extracted from the beam breaks using the publicly available Sleep and Circadian Analysis MATLAB Program (*71*) for MATLAB. P(Wake) and P(Doze) were calculated from the two first days of activity monitoring recordings with the MATLAB scripts deposited in GitHub (*23*, *39*). SE in *Drosophila* is depicted as time spent asleep divided by the opportunity window (ZT12 to ZT24 for flies entrained at 12:12 LD or ZT14 to ZT24 for flies entrained at 14:10 LD). WASO was calculated by subtracting the latency to the first sleep episode from the total time spent awake during the dark period (ZT12 to ZT24 for flies entrained at 12:12 LD or ZT14 to ZT24 for flies entrained at 14:10 LD). All sleep data are the average over the 4 days of data acquisition, except for SRT where data are the average of days 3 and 4 to allow entrainment to the new light regime.

### Circadian analysis

Three- to five-day-old male flies were loaded into the DAM system as described above and entrained to a 12:12 LD cycle for 3 days before being transferred to constant darkness (DD). Locomotor activity during days 2 to 7 in DD was analyzed in ClockLab software (Actimetrics, Wilmette, IL). FFT (*72*) was performed, and the maximum amplitude of the FFT was calculated and compared across genotypes. According to their FFT, flies were categorized as strongly rhythmic (FFT ≥ 0.05), moderately rhythmic (0.05 > FFT ≥ 0.03), weakly rhythmic (0.03 > FFT ≥ 0.01), or arrhythmic (FFT < 0.01).

### TARGET system experiments

The temperature-dependent TARGET system (*73*) was used for temporal mapping experiments. Flies were reared at either 22°C (restrictive temperature) or 28°C (permissive temperature) and, after eclosion (day 0), were either kept at the same temperature or swapped as indicated to confine gene knockdown to pre- or posteclosion periods. Genetic background controls were reared in parallel with the experimental flies. All experiments were conducted in a 12:12 LD cycle.

### Drug supplementation experiments

MPH (Brocacef, Maarssen) was supplemented in fly food at a final concentration of 1 mg/ml (*33*, *37*) only during sleep monitoring.

αMTP (Sigma-Aldrich, M8377) was diluted in fly food to achieve a final concentration of 20 mM (*38*). αMTP was administered only during sleep monitoring for acute treatment, or only throughout development in 5 ml of food containing the drug (until DAM on vehicle food). 5-HTP (Sigma-Aldrich, H9772) was diluted in fly food to achieve a final concentration of 5 mM and administered only during development in 5 ml of food containing the drug, and adults after eclosion were reared and monitored on regular food. All drugs were dissolved in Milli-Q water.

### Sleep deprivation

Flies in DAM2 monitors were placed on a TriKinetics vortexer mounting plate (Waltham, MA, USA), and their activity was recorded for three consecutive days. On the second day, they were shaken for 2 s randomly within every 20-s window for 12 hours during the dark period (ZT12 to ZT24). The capability of each tested genotype to rebound was assessed by comparing the total sleep during ZT0 to ZT3 in the preceding day under unperturbed conditions (baseline) and the same time period following sleep deprivation (recovery).

### Larval rearing and sleep assay

Experiments were carried out as previously described (*32*, *74*). Briefly, to collect developmentally synchronized second instar larvae, adult flies were placed in an embryo collection chamber, kept at 12:12 LD and 25°C, and allowed to lay eggs on a petri dish containing 3% agar, 2% sucrose, and 2.5% apple juice with yeast paste on top for 24 hours. Animals developed on this medium for 48 hours, after which late first instar larvae near ecdysis (based on the presence of double mouth hooks and double vertical plates) were collected. After they finished molting into second instars, single larvae were carefully transferred into LarvaLodge wells containing 100 ml of 3% agar and 2% sucrose medium covered with a thin layer of yeast paste. The LarvaLodge was covered with a transparent acrylic sheet and placed into a behavior room at 25°C and DD for imaging via infrared light. Notably, larval sleep is neither light- nor circadian-regulated (*32*). Time-lapse images were acquired every 6 s with the software IC Capture (The Imaging Source). Images were analyzed using custom-written MATLAB software (*74*). The first hour of data acquisition was discarded to allow acclimation. All data shown are the average of the 4 hours following acclimation. For dTrpA1 activation experiments, embryo collection chambers were kept instead at 22°C and 12:12 LD, and temperature was raised to 30°C during sleep recordings.

### High-performance liquid chromatography

HPLC was used to quantify dopamine and serotonin levels in fly head extracts. Three- to 5-day-old male flies entrained in a 12:12 LD cycle were frozen by immersion in liquid nitrogen at ZT6 and ZT18 after at least 24-hour recovery from $CO_2$. Samples were acquired without disturbing the LD cycle. Fly heads were detached from their bodies by gentle vortexing. Per sample, three heads were transferred to an Eppendorf tube containing 100 µl of ice-chilled 0.1 M perchloric acid (pH 3.0) and homogenized using a hand tissue grinder. Homogenates were kept on ice for 10 min to denaturalize the proteins, followed by centrifugation at 14,000 rpm for 15 min at 4°C. The supernatant was transferred to an Ultrafree-MC filter with a pore size of 0.45 µm (Merck Millipore), filtered by centrifugation for 1 hour at 14,000 rpm at 4°C, and stored at −80°C until HPLC analysis. Ten microliters of the filtrate was analyzed using HPLC with electrochemical detection (EICOMPAK SC-30DS, EICOM) using a mobile phase consisting of 80% citrate-acetate buffer (0.1 M) (pH 3.5), methanol (20%) with sodium octane sulfonate (220 mg/liter) and EDTA-2Na (5 mg/liter), pumped at a rate of 360 µl/min through a 3.0 mm by 100 mm column (EICOMPAK SC-30DS, EICOM), as described by the manufacturer.

### Immunohistochemistry, imaging, and quantification

Proventriculus and anterior midgut of male from wandering third instar larvae were dissected in phosphate-buffered saline (PBS) and immediately fixed in 4% paraformaldehyde dissolved in PBS for 30 min at room temperature. Tissues were washed for 30 min with PBS with 0.6% Triton X-100 (PBST) and blocked for 2 hours at room temperature with 3% goat serum in PBST. Tissues were incubated overnight at 4°C with primary antibody against serotonin (1:500; Sigma-Aldrich, S5545). Secondary donkey anti-rabbit antibody conjugated with Alexa Fluor 488 (1:1000; Thermo Fisher Scientific, A-21206) was incubated overnight at 4°C. Samples were then washed again five times in PBST and mounted in ProLong Gold Antifade Mountant (Thermo Fisher Scientific, P36930).

Samples were imaged using a Leica SP8 laser scanning confocal microscope with Hybrid Detector technology for quantitative imaging through single-photon counting. Images were analyzed using ImageJ to calculate the corrected total cell fluorescence (CTCF): CTCF = integrated density [corresponding to the sum of all pixel values the region of interest (ROI)] – (area ROI × mean fluorescence of background readings), with ROIs being per image (i) the entire proventriculus and (ii) region of the anterior midgut. Two regions per image in the proventriculus not containing the proventricular ganglion nor serotonergic innervation were selected, averaged, and used to determine the mean fluorescence of background readings.

### *CHD8* and *CHD7* expression in human cortex

The t-stochastic neighbor embedding projections along with the cluster annotations were retained from the original body of work for both the developing (*75*) and the adult cortex (*76*). The single-cell RNA-seq counts per million for both datasets were processed into Unique molecular identifier (UMI) counts using quantile normalization (*77*) with the shape parameter set to "2.3." SCTransform (*78*) was applied on the post quantile normalized count matrix. The plots for expression are the log-normalized expression corrected through SCTransform.

### Statistical analysis

Adult and larval *Drosophila* sleep data statistical analysis was carried out in GraphPad Prism version 7 for Macbook (GraphPad Software, San Diego, CA, USA). Datasets of only two groups that followed a Gaussian distribution but showed differences in their standard deviation with the controls were analyzed with a two-tailed unpaired Welch's *t* test. For groups of more than two genotypes, Kruskal-Wallis test with Dunn's multiple comparisons test was performed to assess significance between the tested groups. Post hoc Bonferroni correction for multiple testing was further applied for the number of tests performed on a dataset per genotype to determine the corrected two-sided significance level. Only *P* values that pass the corrected significance level are indicated in the figures. All data shown are from at least three independent experiments ($N = 3$) unless specified otherwise. For sleep deprivation experiments, statistical significance was determined with a two-tailed paired *t* test between

baseline and recovery day per each genotype. Post hoc Bonferroni correction for multiple testing was further applied to correct for the number of tests performed on a dataset per genotype. Only *P* values that withstand multiple testing correction are indicated in the figures. At least three independent biological replicates were performed per genotype. Statistical significance of dopamine and serotonin levels was determined with a two-tailed unpaired *t* test between the tested groups of three independent biological replicates.

## SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/23/eabe2626/DC1

View/request a protocol for this paper from *Bio-protocol*.

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