Title: *Pdm3* directs sleep circuit development to control sleep maturation

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Summary: Across species, sleep in young animals is critical for normal brain maturation. In contrast to mature adult sleep, the molecular determinants of early life sleep remain unknown. Through an RNAi-based screen, we identified a gene, *pdm3*, required for sleep maturation in *Drosophila*. *Pdm3*, a transcription factor, acts during nervous system development to coordinate the ingrowth of wake-promoting dopaminergic neurites to a sleep-promoting region. Loss of PDM3 prematurely increases dopaminergic inhibition of the sleep center, abolishing the juvenile sleep state. RNA-Seq/ChIP-Seq and a subsequent modifier screen reveal that *pdm3* regulates expression of the synaptogenesis gene *Msp300* to control sleep ontogeny. These studies define the molecular cues governing sleep behavioral and circuit development, and suggest sleep disorders may be of neurodevelopmental origin.

Introduction

Across species, sleep amounts are highest in early life and decrease as animals mature (Kayser and Biron, 2016; Roffwarg et al., 1966). Increasing evidence suggests early life sleep may represent a distinct behavioral state, uniquely evolved for the needs of a developing nervous system (Blumberg, 2015; Clawson et al., 2016; Dilley et al., 2018; Frank, 2011). In humans, childhood sleep disturbances portend later neurocognitive deficits, possibly because sleep loss impinges on neural circuit formation (Kotagal, 2015; O'Brien, 2009). Although mechanisms controlling mature adult sleep have been uncovered (Allada et al., 2017), the regulation of early life sleep remains poorly understood.

Like other animals, the fruit fly, *Drosophila melanogaster*, exhibits increased sleep duration in young adulthood that tapers with maturity (Dilley et al., 2018; Kayser et al., 2014; Shaw et al., 2000). At the circuit level, activity of wake-promoting dopaminergic (DA) neurons increases as flies mature, exerting greater inhibitory influence on the sleep-promoting dorsal fan shaped body (dFSB) (Donlea et al., 2014; Kayser et al., 2014; Liu et al., 2012; Ueno et al., 2012). The mechanistic underpinnings of how this central sleep circuit develops are not defined. More broadly, there are no known genes that regulate sleep ontogenetic change. We previously found that all studied short and long-sleeping *Drosophila* mutants sleep more when young (Dilley et al., 2018). Thus, genes regulating sleep ontogenetic change are likely distinct from those that control sleep duration.

From an RNAi-based screen, we identified the transcription factor *pdm3* as a genetic regulator of sleep ontogeny. In contrast to all other characterized sleep mutants,

flies lacking PDM3 do not attain appropriately high juvenile sleep amounts. *Pdm3* knockdown specifically during pupal development prematurely increases wake-promoting DA input to the dFSB. Blocking DA signaling to the dFSB rescues sleep ontogeny in flies lacking PDM3, demonstrating that greater inhibitory DA signaling to the sleep center prevents young flies from achieving high sleep amounts. Transcriptional profiling of mid-pupal brains and a subsequent genetic modifier screen reveal that *pdm3* regulates expression of the synapse assembly gene, *Msp300*, to control sleep ontogeny. Thus, miswiring during early development leads to a brain structural abnormality that disrupts the normal ontogeny of sleep behavior.

Results

Pdm3* controls sleep ontogeny in *Drosophila

To identify genes that specifically regulate sleep ontogeny, we performed an RNAi-based screen to search for factors that, when knocked down, abolish normal ontogenetic change in sleep duration. We used Elav-GAL4 to pan-neuronally knockdown >1000 individual genes with predicted neuronal expression (www.flybase.org), and compared sleep amount in adult flies at day 1 post-eclosion (young adults) to flies at day 4-5 post-eclosion (mature adults) (Fig 1A). We calculated an “ontogeny ratio” (OR) as the ratio of sleep amount in young/mature flies of each genotype, and screened for ratios close to 1. After identifying 53 hits from the primary screen, we then conducted a secondary screen with independent RNAi lines (Fig 1C)(Dietzl et al., 2007).

Knockdown of the gene *pdm3* with either one of two RNAi lines, each targeting a different part of the gene, resulted in a consistent loss of sleep ontogenetic change (Fig 1B,C). Following outcrossing to a uniform genetic background, we found that while genetic controls had robust ontogenetic change in total sleep time, pan-neuronal *pdm3* knockdown abolished this transition (Fig 1D,E). This phenotype was driven by attenuation of daytime sleep ontogeny (Fig 1F) and less night sleep in young compared to mature flies with *pdm3* knockdown (Fig 1G). *Pdm3* is part of the POU domain transcription factor family, a gene group with essential roles in nervous system patterning across species (Badea et al., 2012; Dominguez et al., 2013; Olsson-Carter and Slack, 2011). In *Drosophila*, PDM3 is a broadly expressed neuronal protein that coordinates axon targeting in several areas of the brain (Chen et al., 2012; Tichy et al., 2008). Staining with an anti-PDM3 antibody confirmed a drastic reduction in brain protein levels with knockdown (Fig S1A)(Chen et al., 2012). Restoring PDM3 in the setting of *pdm3* knockdown rescued sleep ontogeny (Fig 1H, S1A), indicating that the phenotype is specific to PDM3 reduction. Thus, *pdm3* regulates sleep ontogenetic change.

Increased daytime sleep in young flies typically stems from prolonged duration of sleep bouts (Fig 1I, genetic controls)(Kayser et al., 2014); *pdm3* knockdown disrupted this consolidation of daytime sleep in early life (Fig 1I). Further, at both ages, flies had more fragmented day and night sleep than controls, with greater numbers of short sleep bouts (Fig 1I,J, S1B,C), as well as redistributed sleep across the day and night (Fig 1D, S1D). Mature flies lacking *pdm3* also exhibited reduced locomotor rhythmicity in constant darkness (Fig S1E), though the core molecular clock remained intact (Fig

S1F), suggesting a problem with clock output (Taghert and Shafer, 2006). Importantly, these changes in sleep architecture and rhythmicity were fully dissociable from deficits in sleep ontogeny (demonstrated below, Fig 2E-G, Fig S4D-F) indicating a mechanistically distinct role for *pdm3* in juvenile sleep.

The loss of sleep ontogenetic change can arise in two main ways: 1) early life sleep is disrupted, such that young flies do not achieve high sleep amounts or 2) mature flies exhibit a persistent juvenile sleep state. Comparison of sleep duration at each age showed that while mature flies lacking *pdm3* exhibited a mild reduction in sleep duration (Fig S1I,J), young flies had a more substantial loss of sleep (Fig S1G,H). Taken together, our data show that with *pdm3* knockdown, young flies are not able to attain appropriately high sleep amounts or increased sleep consolidation, indicating a particular disruption of early life sleep.

We next wondered whether *pdm3* controls maturation of other behaviors, as opposed to sleep behaviors specifically. Male courtship of females is a robust, innate behavior that also undergoes ontogenetic change during early adulthood (Eastwood and Burnet, 1977). In contrast to its effects on sleep ontogeny, *pdm3* knockdown did not affect ontogenetic change in courtship (Fig S1K). Therefore, *pdm3* is unlikely to be broadly required for behavioral maturation, but plays a critical role in regulating sleep ontogeny.

Pdm3 acts during the mid-pupal stage of development to control sleep ontogeny

Pdm3 could play an active role in regulating sleep amount in young adult flies, or it could act during earlier development to influence how sleep circuits form. We used an

inducible knockdown system (McGuire et al., 2004) to examine when during development *pdm3* is required for normal sleep ontogeny. First, we tested whether *pdm3* acts prior to or following eclosion. Beginning knockdown immediately following eclosion had no effect on sleep duration (Fig 2B), fragmentation (Fig S2A), or day-night distribution (Fig S2B) in mature adults, indicating that *pdm3* does not actively regulate sleep in adulthood. In contrast, knockdown of *pdm3* for the entire pre-eclosion period (starting at the embryonic stage) abolished sleep ontogenetic change (Fig 2C). We further restricted knockdown to narrower pre-eclosion developmental windows. Loss of *pdm3* from embryonic stages until the beginning of the 3rd instar larval stage had no effect on sleep ontogeny (Fig 2D), indicating that *pdm3* acts during later stages of development. Knockdown from the beginning of the 3rd instar larval stage to the middle of pupation likewise did not affect sleep ontogeny (Fig 2E, left panel), but did lead to sleep fragmentation (Fig 2E, right panel) and day-night redistribution (Fig S2C). The disruption of sleep architecture while ontogeny remained intact indicates that these phenotypes are temporally dissociable. Finally, in addition to effects on sleep architecture (Fig 2F, right and Fig S2D), extending knockdown from the beginning of 3rd instar up to the late pupal stage abolished sleep ontogeny (Fig 2F, left). Since sleep ontogeny remained intact until knockdown extended through the mid-pupal stage, we concluded that *pdm3* acts during mid-pupal development to control sleep ontogeny (Fig 2G).

***Pdm3* controls dopaminergic synapse formation in the sleep-promoting dorsal fan shaped body**

Given that *pdm3* acts during early development, we hypothesized that loss of PDM3 may disrupt patterning of ontogeny-relevant sleep circuits. The dorsal fan shaped body (dFSB), part of the central complex (CCX), is a synaptic neuropil in which projections from wake-promoting DA neurons inhibit sleep-promoting ExFI2 neurons (Donlea et al., 2011, 2014; Liu et al., 2012; Pimentel et al., 2016). These DA inputs are less active in young flies, reducing inhibition of the sleep-promoting neurons and facilitating more sleep (Kayser et al., 2014). Previous work showed that *pdm3* null mutants, which do not survive as adults, have grossly disrupted DA innervation of the CCX and intrinsic CCX structural abnormalities (Chen et al., 2012). We investigated whether the decrease in juvenile sleep seen with *pdm3* knockdown could be explained by changes in DA innervation of the dFSB, or aberrant development of the CCX itself.

We first examined whether *pdm3* knockdown leads to structural changes in the sleep-promoting ExFI2 projections to the dFSB, labeled by the 23E10 driver (Donlea et al., 2014). We did not see gross morphological changes in the neurites (Fig 3A), but quantification revealed an increase in innervation with *pdm3* knockdown (Fig S3A). An increase in ExFI2 projections to the dFSB could reflect the sleep-promoting neurons receiving more inhibitory DA input, thereby preventing young flies from achieving high sleep amounts. Indeed, labeling of DA neurites using a tyrosine hydroxylase promoter (TH-LexA) showed a two-fold increase in the density of DA innervation to the dFSB (Fig 3B,C). We also noticed that DA inputs to the ventral FSB (vFSB) were grossly disorganized (Fig 3B). However, loss of PDM3 did not diffusely disrupt DA wiring, as TH+ innervation of the mushroom body was unchanged (Fig S3B). Thus, *pdm3*

knockdown alters DA inputs to the FSB, leading to greater innervation density in the dFSB and disorganization of inputs to the vFSB.

We next wanted to determine whether the increased density of DA-dFSB neurites reflects more pre-synaptic sites, as these projections may not actually form synapses. We expressed a fluorescently tagged form of the pre-synaptic active zone protein, Bruchpilot (Brp-short^{mCherry}) (Fouquet et al., 2009; Wagh et al., 2006) specifically in TH+ neurons (Fig 3D, S3C). In control flies, the density of DA synapses to the dFSB increased as flies matured (Fig 3E). Thus, DA inputs to the dFSB are both more active (Kayser et al., 2014) and more numerous in mature flies. By contrast, in a young fly lacking *pdm3*, the number of DA synapses to the dFSB was elevated (Fig 3E), and ontogenetic changes were abolished (Fig 3E). In the vFSB, *pdm3* knockdown led to a reduction in TH+ synapses at both ages, and ontogenetic change in synapse number was still preserved (Fig S3D). Thus, with *pdm3* knockdown, we observed a particular disruption to developmentally regulated synapse addition in the dFSB but not vFSB. The number of 23E10+ and TH+ neurons projecting to the FSB was unchanged with *pdm3* knockdown when compared to controls, indicating that *pdm3* does not affect early cell fate decisions in this circuit (Fig S3E-G). Together, these data suggest that a premature increase in DA synapses to the dFSB underlies loss of behavioral sleep ontogeny.

Reducing dopaminergic signaling in the dFSB rescues sleep ontogenetic change

We hypothesized that with *pdm3* knockdown, greater DA synaptic input increases inhibition of 23E10+ dFSB neurons, preventing high dFSB activity normally seen in

190 young flies. To measure 23E10+ neuron activity, we used the CaLexA (Calcium-
dependent nuclear import of LexA) system (Masuyama et al., 2012), which relies on
activity-dependent nuclear import of a chimeric transcription factor to drive GFP
expression. We generated *pdm3* RNAi in the QUAS system for compatibility with
CaLexA reagents (Fig S4A-C), and found *pdm3* knockdown led to a decrease in the
195 23E10+ dFSB CaLexA signal in day 1 flies (Fig 3F,G). Thus, increased TH+ innervation
of the dFSB reduces activity of 23E10+ sleep-promoting neurons in young flies.

If DA-driven inhibition of 23E10+ neurons underlies the lack of sleep ontogenetic
change with *pdm3* knockdown, blocking DA signal transmission should rescue sleep
ontogeny. DA signaling promotes wake by inhibiting sleep-promoting 23E10+ neurons
200 via two D1-like receptors, Dop1R1 and Dop1R2 (Liu et al., 2012; Pimentel et al., 2016;
Ueno et al., 2012). We knocked down *pdm3* pan-neuronally in a *Dop1R1* and *Dop1R2*
double null mutant background (hereafter referred to as *Dop1R1/R2* *-/-*) (Keleman et al.,
2012). *Dop1R1/R2* *-/-* mutants exhibited increased sleep amounts overall and retained
normal sleep ontogenetic change (Fig 3H, genotypes i & ii). In keeping with the reported
205 haplosufficiency of this mutant, knockdown of *pdm3* in a *Dop1R1/R2* *+/-* background
mimicked knockdown in an otherwise wild-type background, showing loss of sleep
ontogenetic change (Fig 3H, iii). However, complete loss of these receptors
(*Dop1R1/R2* *-/-*) rescued sleep ontogeny in the setting of *pdm3* knockdown (Fig 3H, iv).

The *Dop1R1/R2* *-/-* mutant did not rescue sleep fragmentation, day-night sleep
210 redistribution, or circadian arrhythmicity, demonstrating again that these phenotypes are
mechanistically independent from loss of sleep ontogenetic change with *pdm3*
knockdown (Fig S4D-F). Further, comparison of sleep amounts at each age in the

setting of *pdm3* knockdown showed that *Dop1R1/2* $-/-$ specifically increases sleep in young flies (Fig S4G), but does not alter mature sleep amounts (Fig S4H). Thus, loss of the two dFSB DA receptors, *Dop1R1* and *Dop1R2*, specifically restores sleep ontogenetic change in a manner dissociable from other *pdm3*-related sleep behaviors.

Lastly, to test the prediction that the loss of sleep ontogeny stems from heightened DA signaling specifically through the dFSB, we knocked down *Dop1R1* using *23E10-GAL4* in the setting of *pdm3* RNAi. Indeed, reduction of dopaminergic influence in the dFSB rescued sleep ontogenetic change (Fig 3I) but, like the null mutant, did not affect sleep architecture (Fig S4I). Together, these results indicate that a developmental wiring error in flies lacking PDM3 prematurely increases wake-promoting DA-dFSB signaling in young adult flies, abolishing juvenile sleep.

***Pdm3* acts in primordial central complex neurons to influence TH+ dFSB innervation**

We next sought to determine which cell population *pdm3* acts in to control sleep ontogeny, and how this wiring error occurs. *Pdm3* could either act in pre-synaptic TH+ cells or in CCX neurons that are targeted by TH+ inputs. Knockdown of *pdm3* using TH-GAL4, which targets the majority of DA neurons and is expressed in the CCX-projecting DA neurons during pupal development (Hartenstein et al., 2017), did not affect sleep ontogeny (Fig S5A). *Pdm3* knockdown using *23E10-GAL4* also did not affect sleep ontogeny (Fig S5A); however, during the mid-pupal stage when *pdm3* is acting, *23E10-GAL4* is not expressed in neurons that project to the FSB (Fig S5B). To address whether *pdm3* acts in neurons that are targets of TH+ inputs, we needed to test GAL4

drivers that express in CCX neurons at the mid-pupal developmental stage. We reasoned that at least some adult-defined drivers might have similar expression patterns in the pupal brain, and screened a subset of GAL4 lines with adult CCX expression from the FlyLight collection (Fig 4A) (Jenett et al., 2012). We identified one driver, R93F07-GAL4, which produced a loss of sleep ontogenetic change and was expressed in the CCX during pupal development. During the mid-pupal stage, R93F07-GAL4 targets both the primordial FSB and ellipsoid body (Fig 4C). These R93F07+ neurons are not TH+ (Fig S5C), further supporting the idea that *pdm3* is not acting in TH+ neurons themselves. Importantly, knockdown of *pdm3* using other developmentally expressed sleep/circadian drivers did not affect sleep ontogeny (Fig S5A). Additionally, glial-specific knockdown did not affect sleep ontogeny, confirming that our phenotype stems from loss of *pdm3* in neurons (Fig S5A). Thus, *pdm3* appears to act in primordial CCX target neurons to coordinate DA innervation of the dFSB.

How does DA innervation of the dFSB go awry during development? Little is known about when and how DA inputs normally integrate into CCX circuitry. To characterize this process, we tracked the ingrowth of TH+ neurites to the FSB during pupation. In controls at 24h after puparium formation (APF), TH+ neurites were rarely observed in the dFSB, but we observed an early phase of TH+ innervation in the vFSB (Fig 4D, upper left and Fig 4E/F, left). By 48h APF, DA innervation in both the dFSB and vFSB closely resembled the adult brain (Fig 4D, lower left). By contrast, *pdm3* knockdown led to a striking increase in TH+ innervation of the dFSB at 24h APF, while vFSB innervation proceeded unchanged (Fig 4D, upper right and Fig 4E/F, right). As with controls, TH+ innervation in the dFSB and vFSB with *pdm3* knockdown resembled

adult brains at 48h APF (Fig 4D, lower right). Thus, TH⁺ neurites normally innervate the vFSB first, and by 48h APF, layered TH⁺ innervation of the entire FSB appears largely complete. Loss of PDM3 leads to early ingrowth of TH⁺ neurites to the dFSB, likely increasing the amount of dFSB TH⁺ innervation in the adult brain.

***Pdm3* regulates the synaptic gene, *Msp300*, to control sleep ontogeny**

How does *pdm3* coordinate appropriate DA innervation of the dFSB at the molecular level? *Pdm3* binds to a TAAT Hox motif 2-3 base pairs upstream of a POU motif (TGCAAT)(Andersen and Rosenfeld, 2001; Jafari and Alenius, 2015). Transcriptional targets of *pdm3* remain largely uncharacterized (Jafari and Alenius, 2015; Tichy et al., 2008). To determine which genes affect sleep ontogeny downstream of *pdm3*, we performed RNA-Seq analysis of central brains in *pdm3* knockdown and controls at the mid-pupal stage (Fig 5A, n = 4 biological replicates). Staining of pupal brains confirmed *pdm3* knockdown (Fig S6). Differential gene expression analysis revealed 35 genes that were downregulated with *pdm3* knockdown and 54 genes that were upregulated (p-adj < 0.05, Fold Change > 1.2; Data S1; Fig 5B). Interestingly, some of the most significant changes in expression occurred in genes known to facilitate synapse formation and plasticity, such as *Arc1* and *Msp300* (Fig 5B)(Ashley et al., 2018; Morel et al., 2014; Packard et al., 2015). Since loss of *pdm3* results in excessive DA synaptic ingrowth in the dFSB, we hypothesized that *pdm3* may regulate synapse dynamics by directing expression of some of these genes in particular.

To identify transcriptional changes relevant to the loss of sleep ontogeny, we performed a modifier RNAi screen (Fig 5A). We focused specifically on the top genes

that were upregulated, and combined RNAi against these genes with *pdm3* RNAi to test if reducing their levels rescued the sleep ontogeny phenotype. Expression of *pdm3* RNAi along with *UAS-Dcr2* (to facilitate knockdown of other genes) resulted in a more drastic sleep ontogeny phenotype, with young flies sleeping less than mature flies and an ontogeny ratio below 1.0 (Fig 5C). Co-expression of most RNAi lines did not result in a significant rescue of sleep ontogeny (Fig 5C). However, in the setting of *pdm3* RNAi, knockdown of *Msp300* using three independent RNAi lines suppressed the *pdm3* sleep ontogeny phenotype (Fig 5C) without affecting PDM3 knockdown level (Fig S6B).

To determine whether PDM3 directly binds and regulates *Msp300*, we took advantage of a pre-existing ChIP-Seq dataset available at the ENCODE database (www.encodeproject.org) (Davis et al., 2018). ChIP-Seq for *pdm3* was performed at the pre-pupal stage, close the critical time window for *pdm3* regulation of sleep ontogeny. Comparison of the top 200 genes by p-value and signal strength in this dataset to differentially expressed genes from our RNA-Seq data showed *Msp300* is one of five genes present in both datasets (Fig 5D). Visualization of *Msp300*-associated peaks revealed a strong binding peak across all replicates located approximately 400 bp upstream from the transcription start site in the putative *Msp300* promoter region of three *Msp300* isoforms, as well as an intronic peak (Fig 5E). Our RNA-Seq analysis with *pdm3* knockdown demonstrated increased reads across exons specific to the *Msp300*-RD, RL and RB isoforms compared to control, immediately downstream of the strongest binding peak (Fig 5E, S6C, shaded boxes), suggesting that *pdm3* may regulate these *Msp300* isoforms. Thus, the observed changes in *Msp300* mRNA level with *pdm3* knockdown are likely due to direct transcriptional regulation.

Finally, in the setting of *pdm3* RNAi, we tested whether *Msp300* knockdown also rescued excessive TH+ dFSB innervation. Indeed, TH+ dFSB innervation with *pdm3* knockdown was significantly reduced upon co-expression of *Msp300* RNAi (Fig 5F), further supporting the idea that exaggerated TH+ input to the dFSB drives the loss of sleep ontogeny. Together, our results suggest *pdm3* acts through suppression of *Msp300* levels during pupal development to control wiring of sleep ontogeny circuitry.

Discussion

Despite a long-standing appreciation for the importance of early life sleep in brain development, its regulation at the molecular level has thus far been unknown. We have identified the transcription factor, *pdm3*, as a genetic regulator of sleep ontogeny. *pdm3* acts during development to coordinate synapse formation in a sleep ontogeny circuit, facilitating increased sleep in young animals. These findings provide novel insight into how a central component of *Drosophila* sleep circuitry is wired, and how disruption of this process impinges on sleep behavior.

At the circuit level, *pdm3* knockdown leads to premature and exaggerated DA input to the dFSB, inhibiting sleep output neurons. Rather than acting in TH+ neurons themselves, *pdm3* functions within CCX neurons (labeled by *R93F07-GAL4*) to pattern DA inputs in this region. CCX intrinsic neurons form rudimentary projections to the FSB neuropil during larval development (Riebli et al., 2013), prior to DA innervation, supporting the idea that DA inputs are directed by pre-existing CCX targets. How does early entrance of TH+ neurites into dFSB territory lead to the eventual increase in innervation in the adult brain? We propose that *pdm3* restricts the timing of a developmental window during which target-derived cues facilitate the guidance and

stabilization of TH⁺ processes in the dFSB. Upon PDM3 reduction, this window opens
 330 early, allowing more time for innervation and an increase in TH⁺ synapse number. Our
 data suggest *pdm3* controls development of this circuit and behavioral sleep ontogeny
 via repression of *Msp300*. *Msp300*, also known as dNesprin-1, is a cytoskeleton-
 associated protein that facilitates delivery of mRNAs to promote synapse maturation
 (Morel et al., 2014; Packard et al., 2015). As such, an increase in *Msp300* may result in
 335 exaggerated or inappropriately-timed delivery of synaptic mRNAs and exuberant
 synaptogenesis. Future studies will further delineate how *pdm3* coordinates expression
 of *Msp300* and other synaptic genes to orchestrate development of this sleep circuit.

POU family proteins have highly conserved roles in nervous system patterning
 across species. In mammals, POU proteins have been linked to neurodevelopmental
 340 diseases that often have comorbid sleep abnormalities (Kotagal, 2015; Robinson-
 Shelton and Malow, 2015) hinting at potential early life sleep-regulatory roles. In
 particular, the human homolog of *pdm3*, *POU6F2*, has been associated with subtypes
 of autism spectrum disorder (ASD)(Anney et al., 2010). Additionally, disrupted function
 of the *Brn-2* gene (also known as *POU3F2*) is associated with ASD in humans and
 345 ASD-like social deficits in mice (Belinson et al., 2016; Marchetto et al., 2017; Salyakina
 et al., 2011). Developmental sleep abnormalities could thus contribute to behavioral
 pathology seen with these lesions.

Given the cross-species conservation and importance of sleep ontogeny for brain
 development, there are likely to be additional genes that control this behavior. In
 350 particular, there may be molecules that actively regulate sleep in young flies or genetic
 lesions that result in a persistent juvenile sleep state. Notably, the developmental role of

pdm3 stands in contrast to most other known *Drosophila* sleep genes (Chakravarti et al., 2017; Dubowy and Sehgal, 2017) and suggests the existence of an entirely separate class of “sleep genes” that orchestrate establishment of sleep circuits. This idea raises the intriguing possibility that primary sleep disorders such as insomnia or hypersomnia may have neurodevelopmental origins. The identification of genes and circuits regulating sleep ontogeny thus deepens our understanding of how sleep matures and its age-specific functions for the nervous system.

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Figure 1: *Pdm3* controls sleep ontogeny in *Drosophila*. (A) Ontogeny screen design (B) Primary sleep ontogeny screen. Ontogeny ratio = (min daytime sleep, young) / (min daytime sleep, mature). (C) Secondary screen of primary hits ($n \geq 8$ flies per genotype/age in B and C). (D) Representative sleep traces of genetic controls (top) and *pdm3* knockdown (bottom). Young flies are shown in blue and mature flies are shown in orange. Comparison of (E) total sleep, (F) day sleep and (G) night sleep in *pdm3* RNAi and controls at day 1 versus day 4-5 ($n = 97, 106, 119, 95, 140, 145$ left to right in E-G and I-J). (H) Total sleep time with re-expression of PDM3 (right) versus a control UAS-GFP construct (middle) ($n = 24, 24, 19, 16, 30, 31$ left to right). (I) Day sleep average bout length and (J) day sleep average bout number in *pdm3* RNAi and controls. Graphs in this figure and all others unless otherwise specified are presented as means $\pm$ SEM. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; multiple Student's *t* tests with Holm-Sidak correction, alpha = 0.05 (E-J).

Figure 2: *Pdm3* acts during mid-pupal development to control sleep ontogeny. (A) Drosophila life cycle. (B) Total sleep in mature adults with *pdm3* knockdown post-eclosion and genetic controls (n = 50, 60, 70 left to right). (C) Total sleep with pre-eclosion *pdm3* knockdown in day 1 (blue) versus day 4-5 (orange) (n = 44, 42, 38, 42, 35, 50 left to right) (D) Total sleep with *pdm3* knockdown from embryo to the 3rd instar larval stage (n = 76, 79, 82, 64, 73, 99 left to right). (E) Total sleep (left) and day sleep bout number (right) with *pdm3* knockdown from the 3rd instar larval stage up to the mid pupal stage (n = 33, 32, 32, 31, 32, 32 left to right). (F) Total sleep (left) and day sleep bout number (right) with *pdm3* knockdown from the 3rd instar larval stage to the late pupal stage (n = 30, 32, 32, 32, 32, 31 left to right). (G) Summary of temporal mapping and dissociation of sleep ontogeny from sleep architecture *****P* < 0.0001, ****P* < 0.001, ***P* < 0.01, **P* < 0.05; multiple Student's *t* tests with Holm-Sidak correction, alpha = 0.05 (C, D, E/F left); ANOVA with Tukey's test (B, E/F right).

Figure 3: Loss of PDM3 increases inhibitory DA input to the sleep-promoting dFSB.

(A) Projections of 23E10+ dFSB neurons in controls (top) and *pdm3* RNAi (bottom). (B) TH+ projections to the FSB in controls (left) and *pdm3* RNAi (right). (C) Innervation density of TH+ neurites in the adult dFSB ($n = 16$ controls, 12 *pdm3* RNAi). (D) Labeling of TH+ pre-synaptic sites in the FSB with Brp-short^{mCherry} (E) TH+ synapse density in the dFSB in controls (left) and *pdm3* RNAi (right) at day 1 and day 4-5 ($n = 14, 14, 12, 12$ left to right). (F) Pseudocolored CaLexA signal in 23E10+ neurons in day 1 controls (top) versus *pdm3* RNAi (bottom), quantified in (G) ($n = 11$ controls, 13 *pdm3* RNAi). (H) *Dop1R1/R2*^{-/-} with *pdm3* RNAi and controls ($n = 55, 92, 72, 96, 58, 97, 45, 75$ left to right). (I) *Dop1R1* knockdown in 23E10+ neurons in the setting of *pdm3* RNAi and controls ($n = 39, 46, 17, 25, 33, 50$ left to right). **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; unpaired two-tailed Student's *t* test plus Welch's correction (C, G), multiple Student's *t* tests with Holm-Sidak correction, $\alpha = 0.05$ (E, H).

Figure 4: *Pdm3* acts in CCX target cells and controls TH+ innervation during pupation. (A) Spatial mapping screen of FlyLight GAL4 lines with adult CCX expression patterns ($n \geq 8$ per genotype/age). (B) Total sleep time in *R93F07-GAL4 > UAS pdm3 RNAi* versus controls ($n = 41, 32, 40, 40, 40, 40$ left to right). (C) At the mid-pupal stage, *R93F07-GAL4* is expressed in the FSB (top) as well as the ellipsoid body (EB, bottom). (D) TH+ innervation in the FSB at 24h after puparium formation (APF) (top) and 48h APF (bottom). (E,F) Percentage of brains with TH+ innervation in the dFSB (E) or vFSB (F) at 24h APF (gray = positive for innervation, black = negative; $n = 10$ controls, 10 *pdm3 RNAi*). **** $P < 0.0001$; multiple Student's t tests with Holm-Sidak correction, $\alpha = 0.05$ (B).

Figure 5: *Pdm3* controls expression of the synaptic gene *Msp300* to regulate sleep ontogeny. (A) Scheme of RNA-Seq and follow up modifier screen. (B) Volcano plot of RNA-Seq data highlighting significant changes in gene expression with *pdm3* knockdown compared to controls in mid-pupal brains. Labeled genes have $-\log_{10}(\text{padj}) > 1.3$ and absolute value of $\log_2(\text{Fold Change}) > 0.5$. Yellow = increased expression, blue = decreased expression upon *pdm3* knockdown (n = 4 biological replicates per genotype, 40 brains per replicate for RNA-Seq). (C) Modifier screen with co-expression of RNAi targeting upregulated genes from RNA-Seq alongside *pdm3* RNAi (n ≥ 16 per genotype/age). (D) Overlap of hits from RNA-Seq and *pdm3* ENCODE ChIP-Seq experiments. (E) Control-normalized peaks of PDM3 binding within the *Msp300* gene. The strongest binding peak occurs upstream of the first exon in the RD, RL and RB transcript isoforms (shaded gray box). (F) Quantification of TH+ staining in the dFSB with *Msp300* RNAi and *pdm3* RNAi (n=7, 10, 13 left to right). RNA-Seq statistical analysis is detailed in Methods. **** $P < 0.0001$, * $P < 0.05$; ANOVA with Tukey's test (F).

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Materials and Methods

Fly Stocks:

Stock	Identifier(s)	Source
<i>hs-hid; Elav-GAL4; UAS Dcr2</i>		Dragana Rogulja
<i>UAS-pdm3 RNAi</i>	BSC #53887, TRiP HMJ21205	Bloomington Stock Center
<i>UAS-pdm3 RNAi</i>	VDRC #30538, Construct ID 4312	Vienna Drosophila Resource Center
<i>ElavC155-GAL4</i>		Amita Sehgal
<i>UAS-mCD8::GFP</i>		Bloomington Stock Center
<i>UAS-pdm3</i>		John Carlson
<i>tubGAL80-ts</i>	BSC #7017	Bloomington Stock Center
<i>23E10-LexA</i>	BSC #52693	Bloomington Stock Center
<i>TH-LexA</i>		Ron Davis
<i>LexAOp-GFP</i>	BSC #32203	Bloomington Stock Center
<i>LexAOp-Brp-short-mCherry</i>		Takashi Suzuki
<i>23E10-GAL4</i>	BSC #49032	Bloomington Stock Center
<i>UAS-CaLexA</i>		J. Wang
<i>ElavC155-QF2</i>	BSC #66466	Bloomington Stock Center
<i>QUAS-pdm3 RNAi</i>		Generated in this study
<i>Dop1R1[attP]</i>		Krystyna Keleman
<i>Dop1R2[attP]</i>		Krystyna Keleman
<i>UAS-Dcr2</i>	BSC #24651	Bloomington Stock Center
<i>UAS-Msp300 RNAi</i>	VDRC #105694	Vienna Drosophila Resource Center
<i>UAS-Msp300 RNAi</i>	VDRC #109023	Vienna Drosophila Resource Center
<i>UAS-Msp300 RNAi</i>	VDRC #107183	Vienna Drosophila Resource Center

Sleep Assays:

Flies were raised and maintained in bottles on standard molasses food at 25°C on a 12 hr:12 hr LD cycle. For all ontogeny experiments unless otherwise noted, day 1 females (young adults) were compared to day 4-5 females (mature adults) of the same genotype. For day 4-5 flies, newly eclosed females were collected and aged in group housing on standard food at 25 degrees on a 12hr:12hr LD cycle, and flipped onto new

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food every 3-4 days. For experiments with young flies, newly eclosed females were
 505 group housed until loading into glass tubes containing 5% sucrose and 2% agar.
 Locomotor activity was monitored using the Drosophila Activity Monitoring (DAM)
 system (Trikinetics, Waltham MA). Activity was measured in 1 min bins and sleep was
 identified as 5 minutes of consolidated inactivity(Hendricks et al., 2000; Shaw et al.,
 2000). Data was processed using PySolo(Gilestro and Cirelli, 2009).

Ontogeny Screen:

For the sleep ontogeny primary and secondary screens, the hs-hid; Elav-GAL4, UAS
 Dcr2 (hEGD) fly stock was crossed to each RNAi line from the TRiP, GD and KK
 collections. We screened genes with neuronal expression (www.flybase.org, 1067
 515 genes). For each gene, we screened one RNAi line from the Transgenic RNAi Project
 (TRiP) collection(Ni et al., 2009). Control lines for the primary screen were hEGD x
 TRiP library landing site host strains: P{y[+t7.7]=CaryP}attP2 (Chr3, BSC #36303) or
 P{y[+t7.7]=CaryP}attP40 (Chr2, BSC #36304). Controls for the secondary screen were
 hEGD x Landing site VIE-260B KK library host strain (VDRC #60100) and GD library
 520 host strain (VDRC #60000). Day 1 females were loaded alongside day 4-5 females of
 the same genotype. Since young flies typically sleep about twice as much during the
 day compared to mature flies(Kayser et al., 2014), we focused on daytime sleep for our
 screen. An “ontogeny ratio” (OR) for each genotype was quantified as follows: (minutes
 of daytime sleep in day 1) / (minutes of daytime sleep in day 4-5). Most wild-type flies,
 525 including our genetic controls, had a ratio in between 1.5 and 2.0 (Fig 1B). A lack of
 ontogenetic change would correspond to a ratio of 1.0, and a ratio below 1.0 indicates

that young flies are sleeping less than mature flies. We thus set a ratio of 1.2 and below as our cutoff for defining hits.

Circadian experiments:

Flies were entrained to a 12:12 LD cycle for 3 days and then transferred to constant darkness (DD). Locomotor activity during days 1–7 in DD was analyzed with Clocklab software (Actimetrics, Wilmette, IL). Fast Fourier transform (FFT) values were calculated for all genotypes.

Courtship Assays:

Virgin male flies were collected within 4 hours after eclosion and kept in isolation on regular food until being used in courtship experiments. Female Canton-S virgins (3-7 days post eclosion) were used in all courtship assays. A 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 done in a temperature and humidity-controlled room at 25°C, 40-50% humidity. Courtship index (CI) was determined as the percentage of total amount of time a male was engaged in courtship activity during a period of 10 min or until successful copulation (Siegel et al., 1979). Courtship assays were recorded using a video camera (Sony HDR-CX405) and scored blind to experimental condition.

TARGET system experiments:

For the temporal mapping experiments using the TARGET system, flies were reared at 19°C (restrictive temperature) to prevent GAL80 denaturation, resulting in suppression of RNAi expression. For temporal windows during which *pdm3* knockdown was desired, flies were kept at 28°C (permissive temperature). Because of temperature-related changes in *Drosophila* developmental timing, flies were staged visually based on prior descriptions of developmental stages. Genetic controls were reared alongside experimental flies to control for temperature effects on development. All sleep experiments were conducted at 22°C in 12:12 LD cycles.

Immunohistochemistry:

Fly brains were dissected in 1X PBS, fixed in 4% PFA for 20 minutes at room temperature, and cleaned of remaining tissue in 1X PBS with 0.1% Triton-X 100 (PBS-T). Following 3x10 minutes washes in PBS-T, brains were incubated with primary antibody at 4°C overnight. Following 3x10 minutes washes in PBS-T, brains were incubated with secondary antibody for 2 hours at room temperature. After 3x10 minutes washes in PBST, brains were cleared in 50-70% glycerol and mounted in Vectashield.

Antibodies used:

Primary antibodies	Dilution used	Source
Guinea pig anti-PDM3	1:500	Cheng-Ting Chien
Rabbit anti-GFP	1:500	Molecular Probes
Mouse anti-GFP	1:500	Sigma
Guinea pig anti-PER	1:1000	Amita Sehgal
Mouse anti-PDF C7	1:500	Developmental Studies Hybridoma Bank
Mouse anti-nc82 (Brp)	1:100	Developmental Studies Hybridoma Bank

Secondary antibodies	Dilution used	Source
Alexa Fluor 488 Donkey anti-Rabbit	1:1000	Thermo Fisher
Alexa Fluor 555 Donkey anti-GP	1:1000	Thermo Fisher
Alexa Fluor 488 Donkey anti-Mouse	1:1000	Thermo Fisher
Alexa Fluor 647 Donkey anti-Mouse	1:1000	Thermo Fisher

Imaging and analysis quantification:

Brains were visualized with a TCS SP8 confocal microscope and images processed in NIH Fiji (Schindelin et al., 2012). All settings were kept constant between experimental conditions. Images were taken in 0.5μm steps unless otherwise specified.

1. PER quantification

To investigate PER expression in sLN_vs, sLN_vs were labeled using an anti-PDF antibody and brains were co-stained with anti-PER. Flies were dissected at ZT0, 4, 6, 8, 12, 16 and 20. Area, mean gray value and integrated density of the PER signal was measured for each of the 4 sLN_vs per hemisphere, defined by PDF staining. Corrected total cell fluorescence (CTCF) of the cell body was calculated using the formula: $CTCF_{Cell} = Integrated\ density_{Cell} - (Area_{Cell} \times Mean\ fluorescence_{Background})$ (Dusik et al., 2014).

2. TH+ innervation density

Images were thresholded with the same settings across all images, and the thresholded area of TH+ neurites in the dFSB was measured. dFSB location was defined by use of anti-nc82 as a general anatomical stain. Innervation density was calculated as (area occupied by TH+ neurites)/(area of dFSB).

3. 3D synapse counting (Brp-short^{mCherry})

For each Z-slice, the FSB was selected and the surrounding signal was cleared. The full volume of the dFSB or vFSB was measured using the 3D Objects Counter function in Fiji with the settings: threshold=1 min. puncta size=1 Brp-short^{mCherry} puncta were counted using the 3D Objects Counter function with the settings: threshold=52 min.=2 max.=80.

4. CaLexA analysis:

GFP fluorescence was quantified in a region of interest (ROI) based on single optical sections from whole-mount fly brains; mean GFP signal was normalized to fluorescent signal in an adjacent background ROI.

5. TH+ immunofluorescence:

GFP fluorescence was quantified in a region of interest (ROI) based on single optical sections from whole-mount fly brains; mean GFP signal was normalized to anti-nc82 signal in the same ROI.

RNA-Seq Experiments:

Dissection/RNA extraction:

40 brains per sample at the mid-pupal stage were dissected in cold AHL (108 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 8.2 mM MgCl₂, 4 mM NaHCO₃, 1 mM NaH₂PO₄-H₂O, 5mM trehalose, 10 mM sucrose, 5 mM HEPES). Four biological replicates (each with 40 brains) were dissected per genotype. Brains were transferred to 1ml of Trizol and incubated for 5 min at room temperature (RT). 0.2 ml of chloroform was added and samples were inverted. Samples were incubated 2-3 min at RT, then centrifuged at

12000g for 15 min at 4°C. Genomic DNA was removed using a gDNA eliminator 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. The quality of the data was analyzed using fastqc v0.11.8 and multiqc v1.0.dev0.

Differential gene expression analysis:

The sequenced reads were mapped to the *Drosophila melanogaster* genome assembly dm6 using STAR v2.7.0f(Dobin et al., 2013). STAR was run with the default parameters with the following exceptions: --outFilterMultimapNmax 1 and --twopassMode Basic.

The aligned reads were assigned to genes and counted using featureCounts (v1.6.4) run with default options on the dmel-all-r6.27 version of the *Drosophila melanogaster* annotation. Differential gene expression was performed on the gene count data in R v3.4.2 using DEseq2 v1.22.2(Love et al., 2014). The annotated genes exhibiting an adjusted-P value < 0.05 and |log2FC|>1.0 were considered differentially expressed compared to control. Visualization of differentially expressed genes was done using the R-package ggplot2 v3.2.0.

Gene set enrichment analysis:

Gene set collections for Gene Ontology annotations were downloaded from public sources (<http://www.go2msig.org/cgi-bin/prebuilt.cgi?taxid=7227>). For comparison

between the experimental and control groups, a gene signature was generated by ranking all expressed genes according to the DEseq2-derived test statistics. Enrichment analysis was performed with GSEA v3.0 (Subramanian et al., 2005) using weighted statistical analysis.

ChIP-Seq Analysis:

We accessed the ENCODE database eGFP-pdm3 ChIP-Seq experiment (ID ENCSR518FUJ, contributed by K. White) and downloaded the ENCFF757XFT.bed file (optimal IDR thresholded peaks) (Davis et al., 2018; ENCODE Project Consortium, 2012). ChIP-Seq protocol and analysis workflow is detailed at the ENCODE website (<https://www.encodeproject.org/experiments/ENCSR518FUJ/>). Peaks were sorted by 1) p-value and 2) Signal value. Peaks were annotated using the HOMER (Heinz et al., 2010) annotatePeaks function with version dm6 of the *Drosophila melanogaster* genome.

Generation of QUAS constructs:

QUAS-WALIUM20 vector was obtained from J. Zirin at the Fly Transgenic RNAi Project (Perkins et al., 2015). The HMJ21205 pdm3 RNAi hairpin, originally used to generate the UAS-pdm3 RNAi construct (BSC #53887), was cloned into the QUAS-WALIUM20 vector using the pWALIUM20 cloning protocol (available at www.flyrnai.org). Briefly, the following oligonucleotides were synthesized and annealed (21 NT hairpin sequence shown in capital letters):

5'ctagcagtCAGCAACATTGTGAAGCGAGAtagttatattcaagcataTCTCGCTTCACAATGTT
GCTGgcg 3' and

5'aattcgcCAGCAACATTGTGAAGCGAGAtatgcttgaatataactaTCTCGCTTCACAATGTTG

655 CTGactg-3'. The QUAS-WALIUM20 vector was linearized by NheI and EcoRI, 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.

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Statistical Analysis:

All analysis was done in GraphPad (Prism). Individual tests and significance are
detailed in figure legends.

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Fig. S1.

(A) Staining with anti-PDM3 antibody to confirm *pdm3* knockdown and rescue. Co-expression of a control construct, *UAS-GFP*, in the setting of *pdm3* RNAi does not affect PDM3 protein levels in the brain. (B) Night bout length and (C) night average bout number at day 1 (blue) versus day 4-5 (orange) in *pdm3* RNAi and controls ($n = 97, 106, 119, 95, 140, 145$ left to right in B, C). (D) Quantification of sleep redistribution as ratio of day/night sleep in mature flies ($n = 106, 95, 145$ left to right). (E) FFT amplitude of free-running rhythms in constant darkness (mature flies) ($n = 29, 25, 28$ left to right). (F) Readout for integrity of molecular clock: intensity of PER protein levels in sLNv core clock cells across the circadian day in *pdm3* knockdown and controls ($n = 7-9$ brains per condition). (G) Total sleep time at day 1, *pdm3* RNAi versus controls ($n = 97, 119, 140$ left to right) (H) Minutes of sleep lost in *Elav-GAL4 > UAS pdm3 RNAi* at day 1 compared to each genetic control. (I) Total sleep time at day 4-5, *pdm3* RNAi versus controls ($n = 106, 95, 145$ left to right). (J) Minutes of sleep lost in *Elav-GAL4 > UAS pdm3 RNAi* at day 4-5 compared to each genetic control. (K) Courtship Index (time spent engaged in courtship behavior / total assay time) at day 1 versus day 4-5 in *pdm3* RNAi and controls (darker bars, right) ($n = 28, 27, 21, 28, 23, 28$ left to right). $****P < 0.0001$, $***P < 0.001$, $**P < 0.01$, $*P < 0.05$; multiple Student's *t* tests with Holm-Sidak correction, $\alpha = 0.05$ (B,C,K); ANOVA with Tukey's test (D,E,G,I).

Fig. S2.

(A) Day average sleep bout number with *pdm3* knockdown post-eclosion. (B) Ratio of day/night sleep distribution with *pdm3* knockdown post-eclosion (n = 50, 60, 70 left to right in A,B). Ratios of day-night sleep distribution with *pdm3* knockdown from (C) 3rd instar larval stage to mid-pupal stage and (D) 3rd instar larval stage to late pupal stage (C: n = 32, 31, 32 left to right, D: n = 32, 32, 31 left to right). *****P* < 0.0001, **P* < 0.05; ANOVA with Tukey's test (A-D).

Fig. S3.

(A) Innervation density of 23E10+ neurites in the adult dFSB (n=9 controls, 10 *pdm3* RNAi). (B) TH+ innervation in the mushroom body (MB) (n=10 controls, 9 *pdm3* RNAi). (C) Representative processed images used for 3D counting of Brp-short^{mCherry} synaptic puncta. (D) TH+ synapse density in the vFSB at day 1 versus day 4-5 in *pdm3* RNAi and controls (n = 14, 14, 12, 12 left to right). (E-G) Numbers of 23E10+ (E), TH+ PPL1 (F) and TH+ PPM3 (G) cells per hemisphere (n = 11 controls, 12 *pdm3* RNAi in E, n = 9 controls, 13 *pdm3* RNAi in F,G). *****P* < 0.0001, ****P* < 0.001, ***P* < 0.01, **P* < 0.05; unpaired two-tailed Student's *t* test plus Welch's correction (A,B, E-G), multiple Student's *t* tests with Holm-Sidak correction, alpha = 0.05 (D).

Fig. S4:

(A) Representative sleep trace of controls (top) and *pdm3* knockdown using the QF2-QUAS system. (B) Total sleep time with *pdm3* RNAi and controls in the QF2-QUAS system at day 1 versus day 4-5 ($n = 16, 22, 15, 11, 16, 24$ left to right). (C) Anti-PDM3 staining to confirm *pdm3* knockdown with *Elav-QF2* > *QUAS-pdm3 RNAi*. (D-F) *Dop1R1/2* null mutation with *pdm3* RNAi: (D) day sleep bout number, (E) ratio of day/night sleep, and (F) FFT amplitude are shown in mature flies ($n = 92, 96, 97, 75$ left to right in D-F). *Dop1R1/2* null mutation in the setting of *pdm3* knockdown: (G) total sleep time in day 1 flies compared to controls, (H) total sleep time in day 4-5 flies compared to controls. (I) *23E10-GAL4* > *Dop1R1 RNAi* in the setting of *pdm3* RNAi: ratio of day/night sleep ($n = 52, 33, 51$ left to right) **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; multiple Student's *t* tests with Holm-Sidak correction, $\alpha = 0.05$ (B), ANOVA with Tukey's test (D-F, I), unpaired two-tailed Student's *t* test plus Welch's correction (G, H).

Fig. S5:

(A) Total sleep time: *pdm3* knockdown with spatially restricted GAL4 drivers with expression in sleep/circadian circuitry or glial expression ($n \geq 8$ per genotype/age). (B) Expression pattern of 23E10-GAL4 in the mid-pupal brain. (C) Co-labeling of TH+ neurons (labeled by anti-TH) and R93F07+ neurons (labeled by anti-GFP). While the merged Z-stack shows some apparent overlap in TH+ and R93F07-GFP signal, this stems from overlap of Z planes rather than true co-staining. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; multiple Student's t tests with Holm-Sidak correction, $\alpha = 0.05$ (A).

Fig. S6:

(A) Confirmation of PDM3 protein reduction at the mid-pupal developmental timepoint used for RNA-Seq experiments. (B) Confirmation of PDM3 protein reduction with co-expression of *Msp300* RNAi line with strongest effect on modifying sleep ontogeny (VDRG #105694). (C) Visualization of reads from RNA-Seq in *pdm3* RNAi (top, dark pink) versus controls (bottom, light pink), indicating a greater read pileup in *pdm3* RNAi in the first exon common to *Msp300* isoforms RD, RL and RB (gray box).

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