Epigenetic Regulation of Axonal Growth of *Drosophila* Pacemaker Cells by Histone Acetyltransferase Tip60 Controls Sleep

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ABSTRACT Tip60 is a histone acetyltransferase (HAT) enzyme that epigenetically regulates genes enriched for neuronal functions through interaction with the amyloid precursor protein (APP) intracellular domain. However, whether Tip60-mediated epigenetic dysregulation affects specific neuronal processes *in vivo* and contributes to neurodegeneration remains unclear. Here, we show that Tip60 HAT activity mediates axonal growth of the *Drosophila* pacemaker cells, termed “small ventrolateral neurons” (sLNvs), and their production of the neuropeptide pigment-dispersing factor (PDF) that functions to stabilize *Drosophila* sleep–wake cycles. Using genetic approaches, we show that loss of Tip60 HAT activity in the presence of the Alzheimer’s disease-associated APP affects PDF expression and causes retraction of the sLNv synaptic arbor required for presynaptic release of PDF. Functional consequence of these effects is evidenced by disruption of the sleep–wake cycle in these flies. Notably, overexpression of Tip60 in conjunction with APP rescues these sleep–wake disturbances by inducing overelaboration of the sLNv synaptic terminals and increasing PDF levels, supporting a neuroprotective role for dTip60 in sLNv growth and function under APP-induced neurodegenerative conditions. Our findings reveal a novel mechanism for Tip60 mediated sleep–wake regulation via control of axonal growth and PDF levels within the sLNv-encompassing neural network and provide insight into epigenetic-based regulation of sleep disturbances observed in neurodegenerative diseases like Alzheimer’s disease.

CHRROMATIN remodeling through histone-tail acetylation is critical for epigenetic regulation of transcription and has been recently identified as an essential mechanism for normal cognitive function (Fischer *et al.* 2007). Altered levels of global histone acetylation have been observed in several *in vivo* models of neurodegenerative diseases and are thought to be involved in the pathogenesis of various memory-related disorders (Stilling and Fischer 2011). Chromatin acetylation status can become impaired during the lifetime of neurons through loss of function of specific histone acetyltransferases (HATs) with negative consequences on neuronal function (Selvi *et al.* 2010). In this regard, the HAT Tip60 is a multifunctional enzyme involved in a variety of chromatin-mediated processes that include transcriptional regulation, apoptosis,

and cell-cycle control, with recently reported roles in nervous system function (Sapountzi *et al.* 2006; Squatrito *et al.* 2006). Work from our laboratory demonstrated that Tip60 HAT activity is required for nervous system development via the transcriptional control of genes enriched for neuronal function (Lorbeck *et al.* 2011). We have also shown that Tip60 HAT activity controls synaptic plasticity and growth (Sarathi and Elefant 2011) as well as apoptosis in the developing *Drosophila* central nervous system (CNS) (Pirooznia *et al.* 2012). Consistent with our findings, studies have implicated Tip60 in pathogenesis associated with different neurodegenerative diseases. The interaction of Tip60 with ataxin 1 protein has been reported to contribute to cerebellar degeneration associated with spinocerebellar ataxia (SCA1), a neurodegenerative disease caused by polyglutamine tract expansion (Gehrking *et al.* 2011). Tip60 is also implicated in Alzheimer’s disease (AD) via its formation of a transcriptionally active complex with the AD-associated amyloid precursor protein (APP) intracellular domain (AICD) (Cao and Sudhof 2001; Slomnicki and Lesniak 2008). This complex increases histone acetylation (Kim *et al.* 2004)

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and co-activates gene promoters linked to apoptosis and neurotoxicity associated with AD (Kinoshita *et al.* 2002). Additionally, misregulation of certain putative target genes of the Tip60/AICD complex has been linked to AD-related pathology (Baek *et al.* 2002; Hernandez *et al.* 2009). These findings support the concept that inappropriate Tip60/AICD complex formation and/or recruitment early in development may contribute or lead to AD pathology via epigenetic misregulation of target genes that have critical neuronal functions. In support of this concept, we recently reported that Tip60 HAT activity exhibits neuroprotective functions in a *Drosophila* model for AD by repressing AD-linked proapoptotic genes while loss of Tip60 HAT activity exacerbates AD-linked neurodegeneration (Pirooznia *et al.* 2012). However, whether misregulation of Tip60 HAT activity directly disrupts selective neuronal processes that are also affected by APP *in vivo* and the nature of such processes remains to be elucidated.

In *Drosophila*, the small and large ventrolateral neurons (henceforth referred to as sLNv and lLNv, respectively) are part of the well-characterized fly circadian circuitry (Nitabach and Taghert 2008). Recent studies have implicated the lLNvs and sLNvs as part of the “core” sleep circuitry in the fly, an effect that is predominantly coordinated via the neuropeptide pigment-dispersing factor (PDF) (Parisky *et al.* 2008; Sheeba *et al.* 2008) that serves as the clock output, mediating coordination of downstream neurons (Lin *et al.* 2004; Lear *et al.* 2009). PDF is thought to be the fly equivalent of the mammalian neurotransmitter orexin/hypocretin because of its role in promoting wakefulness and thus stabilizing sleep–wake cycles in the fly (Crocker and Sehgal 2010). Within this circuit, the sLNvs are a key subset of clock neurons that exhibit a simple and stereotypical axonal pattern that allows high-resolution studies of axonal phenotypes using specific expression of an axonally transported reporter gene controlled by the Pdf-Gal4 driver or by immunostaining for the Pdf neuropeptide that is distributed throughout the sLNv axons (Leyssen *et al.* 2005). These features make the sLNvs an excellent and highly characterized model neural circuit to study as sLNvs are amenable to cell-type-specific manipulation of gene activity to gain molecular insight into factors and mechanisms involved in CNS axonal regeneration as well as those that mediate behavioral outputs such as the sleep–wake cycle. Importantly, the *Drosophila* ventrolateral neurons (LNvs) have been previously used as a well-characterized axonal growth model system to demonstrate that the AD-linked APP functions in mediating the axonal arborization outgrowth pattern of the sLNv (Leyssen *et al.* 2005). Based on these results, and our previous studies reporting that Tip60 HAT activity itself is required for neural function (Lorbeck *et al.* 2011; Sarthi and Elefant 2011) and mediates APP-induced lethality and CNS neurodegeneration in an AD fly model (Pirooznia *et al.* 2012), we hypothesized that both APP and Tip60 are required to mediate selective neuronal processes such as sLNv morphology and function that, when misregulated, are linked to AD pathology. In the

present study, we test this hypothesis by utilizing the sLNvs as a model system to examine whether Tip60-mediated epigenetic dysregulation under neurodegenerative conditions such as that induced by APP overexpression leads to axonal outgrowth defects and if there is a corresponding effect on sLNv function in sleep regulation, a process that is also affected in neurodegenerative diseases like AD.

In this report, we show that Tip60 is endogenously expressed in both the sLNvs and the lLNvs. Specific loss of Tip60 or its HAT activity causes reduction of PDF expression selectively in the sLNvs and not the lLNvs and shortening of the sLNv distal synaptic arbors, which are essential for the presynaptic release of PDF from these cells. The functional consequence of these effects is evidenced by the disruption of the normal sleep–wake cycle in these flies, possibly through disruption of PDF-mediated signaling to downstream neurons. By using transgenic fly lines that co-express full-length APP or APP lacking the Tip60-interacting C terminus with a dominant negative HAT defective version of Tip60, we demonstrate that the APP C terminus enhances the susceptibility of the sLNvs and exacerbates the deleterious effects that the loss of Tip60 HAT activity has on axon outgrowth and PDF expression. Importantly, our studies identify the neuropeptide PDF as a novel target of Tip60 and APP, which, when misregulated, results in sleep disturbances reminiscent to those observed in AD. Remarkably, overexpression of wild-type Tip60 with APP rescues these sleep defects by increasing PDF expression and inducing overelaboration of the sLNv synaptic arbor area. Taken together, our findings support a neuroprotective role for Tip60 on sLNv growth and function under APP-induced neurodegenerative conditions. Our data also reveal a novel mechanism for PDF control via Tip60 and APP that provides insight into understanding aspects of sleep-dependent mechanisms that contribute to early pathophysiology of AD.

Materials and Methods

Drosophila stocks

The generation and characterization of fly lines carrying the GAL4-responsive dTip60^{RNAi} or the dTip60^{RNAi} Control construct is described in Zhu *et al.* (2007). Fly lines carrying the dominant negative HAT mutant dTip60^{E431Q} (UAS-dTip60^{E431Q}, line B), wild-type dTip60^{WT} (UAS-dTip60^{WT}, line C) or dTip60^{Rescue} (UAS-dTip60^{Rescue}, line B) construct are described in Lorbeck *et al.* (2011). Fly lines expressing dTip60^{E431Q} or dTip60^{WT} with UAS-APP (UAS-APP; dTip60^{E431Q}, UAS-APP; dTip60^{WT}, line C in both cases) or UAS-APP dCT (UAS-APP dCT; dTip60^{E431Q}, UAS-APP dCT; dTip60^{WT}, line C in both cases) are described in Pirooznia *et al.* (2012). Transgenic UAS lines carrying human APP 695 isoform (UAS-APP) and APP 695 lacking the C terminus (UAS-APP dCT) were obtained from *Drosophila* Stock Center (Bloomington, IN). Stocks carrying both Pdf-Gal4 and UAS-mCD8-GFP were obtained from B. Hassan (University of Leuven, Belgium). R6-Gal4, Mai 179-Gal4, and UAS-Pdfrnai lines were obtained from O. Shafer

(University of Michigan). The *w¹¹¹⁸* line served as the genetic background control. Experimental crosses were carried out at the normal physiological temperature of 25° as higher temperature changes have been reported to induce nonspecific physiological and developmental alterations (Peng *et al.* 2007).

Immunohistochemistry

Third instar larvae or adult brains were dissected in PBS, fixed in 4% paraformaldehyde in PBS, washed three times in PBS containing 0.1% Triton X-100, blocked for 1 hr at RT in PBT containing 5% normal goat serum, and incubated with primary anti-GFP (Millipore), anti-Tip60 (Open Biosystems, Rockford, IL), and anti-PDF (Developmental Studies Hybridoma Bank, University of Iowa) antibodies in blocking solution overnight at 4°. Samples were washed three times in phosphate-buffered Tris (PBT) at room temperature (RT), and secondary antibodies (Jackson ImmunoResearch) were applied in blocking solution for 2 hr at RT. After washing three times in PBS, samples were mounted in Vectashield (Vector Laboratories).

Imaging and quantifications

Larval and adult brain preparations were imaged using GFP, Tip60, or PDF antibodies. Anti-GFP immunostaining was visualized using Alexa-Fluor 488. Alexa-Fluor 568 and Alexa-Fluor 647 was used for anti-PDF and anti-Tip60, respectively. Imaging experiments were performed at Drexel University's Cell Imaging Center. Confocal microscopy was performed using an Olympus microscope with fluoview acquisition software (Olympus, Center Valley, PA). Images were displayed as projections of 1- μ M serial Z-sections. Quantitative analysis of sLNv axon length was performed using National Institutes of Health (NIH) ImageJ software by measuring axon length from the base of the cell body to the distal tip of the axon in the different genotypes. Quantification of the two-dimensional area of the sLNv terminal axonal arbor was done as described in Leyssen *et al.* (2005) using NIH ImageJ software. Briefly, the sLNv axon stem on either half of the brain hemisphere was marked by a straight vertical line followed by a horizontal line between the points that mark the sLNv axon. The outline of the axonal processes dorsal to the horizontal line was traced, and the area inside was measured. The distance between the vertical lines was used as a measure for brain size (Supporting Information, Figure S2). Area measurements were normalized for brain size by scaling the distance between the dorsal projections to the median of the distance as a correction factor. The resulting corrected area is represented in the graphs. Student's *t*-tests were used to calculate the significance in difference between the mean axon lengths and arbor areas, as indicated by the *P*-values in the graphs.

Confocal imaging of whole-CNS PDF expression was done by determining PMT voltage, offset, and laser power settings for the control line and maintaining the same for the experimental genotype, making sure that there was no saturation effect in either the sLNvs or lLNvs. PMT voltage

was always maintained at 1.0 $\times$. The mean pixel intensity of cytoplasmic PDF was quantified using Fluoview software (Olympus), with the cytoplasmic region of interest determined by GFP expression. Sequential scans were used to avoid bleed-through. Mean background pixel intensity was also measured in a region surrounding each neuron, and this value was subtracted from each cytoplasmic value. To compare somatic PDF expression in the different genotypes, the background-subtracted mean pixel intensity of PDF immunoreactivity (IR) was calculated from 10–15 brains for each genotype, with four lLNvs and sLNvs measured in each brain.

Behavioral recording and analysis

Activity assay: Locomotor activity of individual flies was recorded at 25° using the *Drosophila* Activity Monitoring (DAM) system (Trikinetics, Waltham, MA) as per manufacturer's instructions. Briefly, individual F₁ female progeny in each case were collected upon eclosion and allowed to acclimate to a 12 hr:12 hr light/dark cycle at 25° for 4 days after eclosion. The significant difference observed between the control and each of the different experimental groups was determined using a Student's *t*-test for each time point (*n* = 24).

Digital video monitoring: Individual F₁ female progeny (*n* = 28) in each case were collected upon eclosion and allowed to acclimate to a 12 hr:12 hr light/dark cycle at 25° for 4 days after eclosion. Video recording of sleep in these flies was done on day 5 (after eclosion). On day 4, individual flies were anesthetized and transferred to Corning Pyrex Glass tubes (65 mm length, 5 mm diameter) containing *Drosophila* media at one end and capped with a cotton plug at the other end. Movements were monitored at 25° and recorded every 5 sec by use of digital video recording. Total sleep, sleep bout number, and mean sleep bout duration were calculated from video data using custom software as previously described (Zimmerman *et al.* 2008).

Results

Tip60 immunolocalization in the *Drosophila* LNvs

Immunostaining using anti-Tip60 antisera was used to determine whether Tip60 is endogenously expressed in the *Drosophila* Tip60 LNvs and to examine the pattern of dTip60 expression in these cells in the third instar larval and adult brains. In the adult brains, the large and small subset of LNvs revealed different patterns of dTip60 localization. While strong dTip60 immunoreactivity was observed in the lLNv, relatively weaker expression was observed in the sLNvs (Figure 1A'). Tip60 expression was not detected in the larval sLNvs, the only LNv subgroup found in larvae. Experiments examining Tip60 levels at Zeitgeber time 2 and 14, corresponding to 2 and 14 hr after lights on, respectively, suggest that the protein levels do not undergo circadian oscillation (data not shown).

HAT-defective Tip60 negatively affects axonal growth of sLNV in the *Drosophila* brain

To determine whether Tip60 has an effect on sLNV axon growth and morphology, we specifically knocked down Tip60 in these cells by utilizing the GAL4/UAS-targeted gene expression system. Flies carrying the LNV-specific Pdf-Gal4 driver were crossed to our previously characterized UAS-dTip60^{RNAi} lines (Zhu *et al.* 2007) to induce the RNA interference (RNAi) response. LNV-specific knockdown of Tip60 was confirmed by lack of Tip60 expression in the ILNV and sLNV as assessed by Tip60 immunostaining (Figure 1, D and E). The effects on sLNV axonal outgrowth were then examined by confocal microscopy using specific expression of the UAS-mCD8-GFP membrane marker transgene. Structurally, the sLNV axons display a well-characterized and stereotypical migration pattern. During larval and pupal development, the axon stem that projects from the sLNV cell body grows dorsally, bends toward the center of the brain, and sprouts into branches forming the terminal synaptic arbor in the adult animals (Figure 2, A and A'). Induction of the Tip60 RNAi response in the LNV did not have any effect on the early development of the axonal pattern of these cells as evident from the intact axonal pattern seen in the third instar larva (Figure 2B). In contrast, in the adult brains, expression

of dTip60^{RNAi} results in shortening of the sLNV terminal synaptic arbor, evident in the complete lack of the medially projecting axonal branches (Figure 2B'). As a control for the Tip60 RNAi experiments, we used a corresponding UAS-dTip60^{RNAi} control construct (Zhu *et al.* 2007). As expected, LNV-specific expression of dTip60^{RNAi} control did not affect Tip60 levels in the ILNVs and sLNVs (Figure 1, D and E) and had no effect on sLNV axon morphology in the third instar larval or adult brains (Figure 2, C and C'), confirming the specificity of the dTip60^{RNAi} induction.

We next wanted to examine if the observed effects on sLNV axon growth due to loss of Tip60 were specifically mediated by Tip60 HAT activity. To determine whether Tip60 HAT activity affects sLNV axonal growth, we misregulated *Drosophila* Tip60 (dTip60) in these cells by utilizing well-characterized transgenic flies (Lorbeck *et al.* 2011) that carry Gal4-responsive transgenes for either a dominant negative HAT defective version of dTip60 (dTip60^{E431Q}) or wild-type dTip60 (dTip60^{WT}). Quantification of Tip60 levels in flies overexpressing either dTip60^{WT} or dTip60^{E431Q} in the LNVs revealed a significant increase in Tip60 compared to the control flies (Pdf-Gal4/UAS-mCD8-GFP/+) (Figure 1, A', B', C', D, and E). Tip60 levels in the ILNV were significantly higher than the sLNV (Figure 1, D and E), likely due

Figure 2 Loss of dTip60 HAT activity decreases axonal arborization in the adult sLNv. Representative confocal images of sLNv axon morphology in the control (Pdf-Gal4/UAS-mCD8-GFP/+) third instar larval brain (A) and in the adult brain (A'), showing dorsally projecting axons (arrowhead) and terminal synaptic arbor (arrow). LNV-specific induction of Tip60 RNAi response (dTip60^{RNAi}) or expression of the HAT-defective dominant negative dTip60 (dTip60^{E431Q}) has no effect on sLNv morphology in third instar larva (B and D) but leads to collapse of synaptic arbor in the adult brain (B' and D'). Expression of corresponding Tip60 RNAi control construct (dTip60^{RNAi} Control) or overexpression of wild-type dTip60 (dTip60^{WT}) in the LNvs has no significant effect on sLNv axonal growth in the third instar larva (C and E) or in the adult (C' and E'). Expression of equivalent levels of wild-type dTip60 in the dTip60^{E431Q} background does not affect the sLNv axons in the third instar larva (F) but rescues the HAT-mutant-induced retraction of sLNv axons in the adult brains (F'). Quantification of sLNv axon length (G) (***) $P < 0.001$ compared to control) and synaptic arbor area (H) was done using NIH ImageJ software. ND, not determined. Error bars represent the 95% confidence interval.

to the higher levels of endogenous dTip60 expressed in the ILNvs compared to the sLNvs. However, and importantly, comparison of Tip60 levels in the ILNv and sLNv between dTip60^{WT} and dTip60^{E431Q} flies revealed equivalent levels of exogenous dTip60 in the respective neurons (Figure 1, D and E). Similar to the effects that we observed in the dTip60^{RNAi} flies, targeted expression of dTip60^{E431Q} in the LNV leads to shortening of the outward-projecting sLNv axon terminals in the adult brains without a marked effect on the larval sLNv

axon morphology (Figure 2, D, D', and G). In contrast, overexpression of wild-type dTip60 (dTip60^{WT}) showed no significant effect on either the larval or adult sLNv axonal architecture compared to the control flies (Figure 2, E and E'). To confirm these results, we measured the sLNv axon length and also quantified the synaptic arbor area as described in Leyssen *et al.* (2005) (Figure S2; Figure 2, G and H). Since the effects that we observed in the dTip60^{E431Q} flies indicate that the HAT activity of Tip60 is crucial for establishing the

normal sLNv axon morphology, we wanted to examine if additional levels of HAT-competent Tip60 could rescue dTip60^{E431Q}-mediated effects on sLNv axonal growth to confirm that such defects were specifically caused by loss of dTip60 function. For this purpose, we utilized our previously characterized UAS-dTip60^{Rescue} line (Lorbeck *et al.* 2011) that allows overexpression of equivalent levels of wild-type dTip60 in the dTip60^{E431Q} background. GFP analysis revealed normal sLNv axon morphology in the dTip60^{Rescue} flies similar to the control flies (Pdf-Gal4/UAS-mCD8-GFP/+) (Figure 2, F and F'), indicating that the axonal defects induced by the mutant dTip60^{E431Q} can be counteracted by the presence of additional levels of HAT-competent Tip60. Taken together, these results further demonstrate that dTip60 HAT activity is crucial for establishing appropriate sLNv axon morphology.

Co-expression of dTip60 modulates APP-mediated effect on sLNv axonal growth that is dependent on the APP C terminus

Expression of the neuronal isoform of human APP (APP695) in the sLNv at 28° using the LNV-specific Pdf-Gal4 driver has been shown to induce increased axonal extension and extensive arborization of the sLNv axon terminals (Leyssen *et al.* 2005). Our observation that the dTip60 HAT mutant affects sLNv axon growth prompted us to examine how depletion of Tip60 HAT activity affects sLNv morphology under APP-overexpressing conditions. Although expression of human APP in the sLNv at 25° (Figure 3, B, B', H, and I) did not have the drastic effect that has been reported at 28°, co-expression of APP along with the Tip60 mutant construct (APP; dTip60^{E431Q}) at 25° was found to exacerbate the negative effect that dTip60^{E431Q} alone had on the sLNv axonal growth, resulting in drastic shortening of the axons (Figure 3, D, D', and H). Since the APP C terminus is required for interaction with Tip60, we also examined the effects of expressing a truncated version of APP lacking the C terminus alone (APP dCT) as well as with the HAT-defective Tip60 mutant (APP dCT; dTip60^{E431Q}). Expression of APP dCT alone did not affect the sLNv axon morphology and was not different from that seen in the control (Pdf-Gal4/UAS-mCD8-GFP/+) (Figure 3, C, C', H, and I). In contrast, expression of APP dCT; dTip60^{E431Q} in the sLNv exhibited a less severe effect than that induced by APP; dTip60^{E431Q} in that the axon length was almost identical to that seen when dTip60^{E431Q} was expressed alone (compare Figure 2D' and Figure 3E'). Since co-expression of APP with dTip60^{E431Q} resulted in a phenotypic enhancement of the dTip60^{E431Q}-induced shortening of the sLNv axon, we examined the effect of overexpressing wild-type dTip60 along with APP or APP lacking the C terminus to gain insight into the nature of the functional interaction under these conditions. Targeted overexpression of dTip60 along with APP (APP; dTip60^{WT}) in the LNV resulted in a large increase in the area of the sLNv axonal arbor in the adult flies although there was no significant effect on the early development of the sLNv axonal pattern (Figure 3,

F, F', H, and I). Most of the sLNv axons grew along the right path, but further extended and arborized over a larger area than those seen in control flies or in flies that overexpressed dTip60 alone (compare Figure 2E' and Figure 3F'). In contrast, overexpression of dTip60^{WT} along with APP lacking a C terminus (APP dCT; dTip60^{WT}) did not have any significant effect and resulted in the normal axonal pattern seen in control flies (Figure 3, G, G', H, and I). Thus, co-expression of APP with dTip60 enhances the normal sLNv axonal arborization phenotype observed for overexpression of dTip60 alone, further supporting a synergistic interaction between APP and dTip60 that is dependent on the APP C terminus.

LNV-specific expression of dTip60 or APP leads to selective decrease in PDF immunoreactivity in sLNvs, but not in lLNvs

The LNV-specific neuropeptide PDF is required for circadian behavioral rhythmicity and is expressed in both the large LNV and small LNV subset of cells. Typically, there are four to five PDF-positive lLNvs and four sLNvs in wild-type flies. PDF is also periodically released from the lLNv varicosities and the sLNv terminal synaptic arbor in the dorsal brain (Fernandez *et al.* 2008; Sheeba 2008). Our observation that loss of dTip60 or expression of the HAT-defective dTip60 mutant abolished the formation of these sLNv axon terminals prompted us to examine whether PDF expression and/or transport along the axons was also affected. Anti-PDF immunocytochemical analysis was performed on whole brains dissected from 4- to 7-day-old flies resulting from a cross between Pdf-Gal4 driver and either *w¹¹¹⁸* (Pdf-Gal4/UAS-mCD8-GFP/+), dTip60^{RNAi} (Pdf-Gal4/UAS-mCD8-GFP/UAS-dTip60^{RNAi}), or dTip60^{E431Q} (Pdf-Gal4/UAS-mCD8-GFP/UAS-dTip60^{E431Q}) flies that were maintained under standard light/dark (LD) conditions. GFP expression was used as a marker to locate the lLNvs and s-LNvs. Examination of the lLNv and sLNv soma for PDF IR in flies expressing either dTip60^{RNAi} or dTip60^{E431Q} revealed a partial loss of PDF IR in the sLNvs of adult flies (Figure 4, B' and D') although PDF could still be detected in the soma as well as along the axons, indicating that dTip60 specifically affects PDF expression although its transport along the axons is unaffected. Quantification of PDF intensity also revealed a significant reduction of PDF IR in the sLNv in the dTip60^{RNAi} and dTip60^{E431Q} flies (Figure 4I). However, PDF expression was unaffected in the larval sLNvs in both cases. PDF expression in the lLNv soma and varicosities was also unaffected (Figure 4J). GFP expression in both cell types was unaffected, indicating that the observed effects are specifically on PDF expression (Figure 4, B and D). The persistence of similar effects due to loss of dTip60 protein and expression of the HAT-defective dTip60^{E431Q} indicate that the observed effects on PDF are primarily mediated by Tip60's HAT activity. On the other hand, while targeted overexpression of dTip60^{WT} in the LNVs did not affect PDF IR in the lLNvs (Figure 4, E' and J), it resulted in significant increase ($P < 0.05$) in PDF IR in the sLNvs, compared to the control flies (Pdf-Gal4/UAS-mCD8-

Figure 3 dTip60 modulates APP-mediated effects on sLNv axonal growth post developmentally in the *Drosophila* CNS. Representative images of sLNv axons in adult *Drosophila* brains expressing the UAS-mCD8-GFP reporter gene at 25° in conjunction with each of the different GAL4-responsive transgenes under the control of the PDF-Gal4 driver. sLNv axonal arborization pattern in control (Pdf-Gal4/UAS-mCD8-GFP/+) (A) third instar larval brain and (A') adult, respectively. sLNv-specific expression of neuronal isoform of (B and B') APP or (C and C') APP lacking the C terminus (APP dCT) has no observable effect on sLNv axon structure in both third instar larvae and adult. (D) Co-expression of full-length APP with HAT-activity-deficient mutant dTip60 (dTip60^{E431Q}) has no effect in third instar larvae stage but (D') causes severe retraction of the sLNv synaptic arbor in the adult brain, resulting in much shorter axons. (E) Co-expression of APP dCT with dTip60^{E431Q} causes no effects in third instar larvae (E') but causes shortening of the sLNv similar to shortening observed for dTip60^{E431Q} alone. Overexpression of APP with wild-type dTip60 (dTip60^{WT}) causes (F) no effect in third instar larvae but (F') in adult brain causes the sLNv axons to extend farther and arborize over a larger area, an effect that was dependent on the Tip60-interacting APP C terminus as seen from the lack of any significant effect due to expression of APP dCT with dTip60^{WT} (G and G'). Histogram showing quantification of the sLNv axon length shows significant reduction of sLNv axons in flies co-expressing dTip60^{E431Q} with APP or APP dCT (**P* < 0.05 compared to control) (H) and quantification of the two-dimensional area of the terminal axonal arbor using NIH Image J shows a robust increase in flies co-expressing APP and dTip60^{WT} (I) (****P* < 0.001 compared to control). Error bars represent 95% confidence interval.

GFP/+) (Figure 4, E' and I). Since dTip60^{E431Q} leads to a decrease in sLNv PDF and overexpression of wild-type Tip60 had the converse effect and increased PDF levels in the sLNvs,

we hypothesized that co-expression of the HAT-competent Tip60 with dTip60^{E431Q} in the dTip60^{Rescue} flies would counteract the effects of the latter. Consistent with our hypothesis,

Figure 4 dTip60 or APP expression in the LNV subsets selectively affects PDF IR in the sLNV and not the ILNV. Representative images of anti-GFP or anti-PDF staining in ILNV (arrow) and sLNV (arrowhead) soma in adult flies expressing each of the different transgenes (indicated next to each panel) under the control of the Pdf-Gal4 driver. (A–H) Anti-GFP staining used as a marker to localize the ILNVs and sLNVs in the adult brains. Scale bar, 20 μ m. (A') Anti-PDF staining in ILNV (arrow) and sLNV (arrowhead) soma in control flies (Pdf-Gal4/UAS-mCD8-GFP/+). (B') LNV-specific induction of dTip60^{RNAi} results in partial loss of PDF IR in sLNV while (C') expression of the corresponding dTip60^{RNAi} Control has no effect on PDF. (D') Expression of HAT activity defective dTip60^{E431Q} results in partial loss of PDF IR in sLNV while (E') overexpression of wild-type dTip60 (dTip60^{WT}) significantly increases PDF IR in the sLNV. (F') Expressing equivalent amounts of wild-type Tip60 with mutant dTip60^{E431Q} rescues dTip60^{E431Q}-induced loss of PDF IR in sLNVs in the dTip60^{Rescue} flies. (G') Expression of APP also resulted in partial reduction in sLNV PDF IR, an effect dependent on its C terminus as seen from (H') the lack of any observable effect on PDF IR due to expression of APP lacking the C terminus (APP dCT). PDF expression in ILNV was largely unaffected in each of the different genotypes. Quantification of PDF IR in sLNVs (I) and in ILNVs (J). Values represent average of four sLNV and four ILNV PDF IR from 15 brains for each genotype. A Student's *t*-test revealed a significant decrease in sLNV PDF IR in flies expressing dTip60^{E431Q} or APP compared to control (**P* < 0.05). Error bars represent 95% confidence interval.

PDF levels in the sLNVs but not the ILNVs of dTip60^{Rescue} flies was significantly greater (*P* < 0.05) than that observed in the dTip60^{E431Q} flies and the control flies (Figure 4, F', I, and J). However, the sLNV PDF level in this case was much less than that observed in flies overexpressing wild-type dTip60 alone

(*P* < 0.001) (Figure 4I). This indicates that, when co-expressed in equivalent amounts, dTip60^{E431Q} and dTip60^{WT} counteract their respective effect on PDF expression.

Finally, we examined whether LNV-directed expression of APP had any effect on PDF expression. Similar to the

dTip60^{RNAi} and dTip60^{E431Q} flies, APP expression did not have any observable effect on PDF IR in the LLNs (Figure 4J) but specifically affected PDF IR in the sLLNs, resulting in partial reduction of PDF IR in sLLN soma (Figure 4, G' and I). In contrast, APP lacking a C terminus (APP dCT) did not have any significant effect on PDF IR in the sLLN or the LLN (Figure 4, H', I, and J). These observations suggest that dTip60 and APP selectively affect PDF expression in the sLLNs and that the effects are dependent upon the APP C terminus. Moreover, the observed effect on PDF levels due to dTip60^{E431Q} or APP and the lack of any significant effect with the APP-lacking C terminus that is required for interaction with Tip60 suggests that PDF is a potential target of the Tip60/APP-containing complex.

dTip60 and APP functionally interact to regulate PDF expression in sLLNs

Our observation that expression of either dTip60^{E431Q} or APP each affected PDF IR in the sLLNs prompted us to ask whether dTip60 and APP functionally interact to mediate the sLLN-specific effect on PDF expression. We therefore measured PDF IR in the sLLN soma in flies co-expressing APP or APP dCT with either Tip60^{WT} or the HAT-defective mutant Tip60^{E431Q}. Although PDF IR in the LLNs remained largely unaffected (Figure 5G), significant effects on sLLN PDF IR were observed in all fly lines. APP;Tip60^{E431Q}-expressing flies exhibited the most drastic effect, with APP expression exacerbating the effects of Tip60^{E431Q} expression alone, thereby resulting in complete loss of PDF IR in the sLLNs (Figure 5, B' and F). Although PDF IR was absent in the sLLN soma, these cells could still be located using GFP expression (Figure 4B). Importantly, the observed effect on sLLN PDF in the APP; dTip60^{E431Q} flies is similar to the phenotypic enhancement that we observed on the sLLN axon growth in these flies. We therefore examined if the degenerative effects on sLLN axon and PDF expression in the APP; dTip60^{E431Q} flies was due to induction of an apoptotic response. To address this, we performed TUNEL assays using whole-mount brains of 4- to 7-day-old adult flies that resulted from a cross between the Pdf-Gal4 driver and *w¹¹¹⁸* or APP; dTip60^{E431Q} flies. However, we did not detect any TUNEL-specific signal in the sLLNs or in other regions of the adult brain for this age group in either the APP; dTip60^{E431Q} or the control flies (data not shown). This indicates that co-expression of dTip60^{E431Q} with APP leads to neuronal dysfunction, likely via a mechanism distinct from apoptosis.

In contrast to the above, flies that expressed APP lacking the C terminus with dTip60^{E431Q} (APP dCT; dTip60^{E431Q}) resulted in only a partial loss of PDF IR in the sLLNs, identical to that observed when dTip60^{E431Q} was expressed alone (Figure 5, C' and F), indicating that the APP C terminus is required for the Tip60^{E431Q}/APP-mediated negative effects on PDF expression. Remarkably, overexpression of wild-type dTip60 with APP appeared to rescue the APP-mediated negative effects on PDF expression as APP; Tip60^{WT}-expressing flies had significantly increased PDF IR in the sLLNs in com-

parison to control flies (Pdf-Gal4; UAS-mCD8-GFP/+) (Figure 5, D' and F). Quantification of PDF IR, however, revealed a small but significant decrease in PDF in sLLNs in the APP; dTip60^{WT} flies compared to flies expressing dTip60^{WT} alone ($P < 0.05$) (Figure 4I and Figure 5F). Similar to dTip60^{WT} flies, an increase in PDF levels was also observed in the sLLNs of flies co-expressing both Tip60^{WT} and APP lacking the C terminus (Figure 5, E' and F), suggesting that the increase in PDF expression is predominantly mediated by Tip60. Together, these findings suggest that Tip60 and APP functionally interact to regulate PDF expression in the sLLNs. However, the effect on PDF expression seems to be critically dependent upon the HAT activity of Tip60.

dTip60^{E431Q} flies exhibit nighttime sleep deficits with an increase in daytime sleep

The PDF neuropeptide is implicated as the principal transmitter of the LLN group, as flies lacking Pdf function exhibit phenotypes similar to ablation of the PDF-positive LLNs. These phenotypes include loss of morning anticipatory behavior and advanced evening behavior in LD (12 hr light: 12 hr dark condition) and locomotor arrhythmicity in DD (12 hr dark: 12 hr dark, constant darkness) (Renn *et al.* 1999). Our observation that LLN-targeted expression of dTip60^{E431Q} and APP results in selective disruption of PDF levels specifically in the sLLN prompted us to ask whether biphasic locomotor rhythm in these flies was also affected. Toward this end, we first examined locomotor behavior in Pdf-Gal4/UAS-mCD8-GFP/dTip60^{E431Q} flies using the DAM assay in standard LD condition for 2 days followed by constant darkness for 5 days. Pdf-Gal4/UAS-mCD8-GFP/+ and UAS-dTip60^{E431Q}/+ flies were used as controls for the DAM assay. Inspection of averaged locomotor activity of control and experimental dTip60^{E431Q} flies showed similar gradual increases in activity in anticipation of morning and evening, coinciding with lights-on and lights-off in standard 12 hr:12 hr LD cycles (Figure 6A). The dTip60^{E431Q} flies also maintained rhythmicity in constant darkness similar to the control flies (Figure 6B). However, dTip60^{E431Q} flies exhibited significantly less locomotor activity during the day compared to the controls with a concomitant increase in nighttime activity under both LD and DD conditions (Figure 6, A and B), which is suggestive of sleep defects in these flies.

Recent studies have demonstrated that PDF-expressing LLNs are the target of γ -aminobutyric acid (GABA)ergic sleep-promoting cells and that their activation promotes arousal through release of the neuropeptide PDF (Parisky *et al.* 2008; Sheeba 2008; Chung *et al.* 2009). Flies mutant for pdf or its receptor are hypersomnolent and exhibit more daytime sleep (Parisky *et al.* 2008) as well as reduced sleep consolidation at night (Chung *et al.* 2009). Our observation that loss of dTip60 HAT activity in the LLNs reduced sLLN PDF expression prompted us to examine whether there was also a corresponding effect on sleep in these flies. Although the DAM assay is widely used to assess both circadian and sleep behavior (Pfeiffenberger *et al.* 2010), it has certain

Figure 5 dTip60 and APP functionally interact to regulate PDF expression in the sLNv. Representative images of anti-GFP or anti-PDF staining of ILNv (arrow) and sLNv (arrowhead) in adult flies expressing the different transgenes under the control of the Pdf-Gal4 driver as indicated above each column. (A–D) Anti-GFP staining used as marker to localize the ILNvs and sLNvs in the adult brains. Scale bar, 20 μ m. (A') Anti-PDF staining in ILNv (arrow) and sLNv (arrowhead) soma in control flies (Pdf-Gal4/UAS-mCD8-GFP/+). (B') Co-expression of APP with HAT-activity-defective dTip60^{E431Q} resulted in selective loss of PDF IR in the sLNvs although PDF IR in the ILNvs was not affected. (C') dTip60^{E431Q} in the presence of APP dCT resulted in only a partial reduction of sLNv PDF IR, similar to that observed when only dTip60^{E431Q} was expressed in the LNV's. (D') Expressing full-length APP or (E') APP lacking the C terminus with wild-type dTip60^{WT} increased sLNv PDF IR but had no effect on PDF IR in the ILNvs. Quantification of PDF IR in the sLNvs (F) and the ILNvs (G) revealed significant difference in PDF expression in the different genotypes compared to control (**P* < 0.05). Error bars represent 95% confidence interval. Scale bar, 20 μ m.

limitations for specifically studying sleep in some cases wherein it is insensitive to small fly movements that occur outside of the path of the infrared beam, thus affecting the identification of actual quiescent sleep behavior (Zimmerman *et al.* 2008). We therefore used digital video analysis to determine if LNV-specific expression of dTip60^{E431Q} leads to

sleep disturbances using single-staged, 4- to 7-day-old female dTip60^{E431Q} flies (Pdf-Gal4/UAS-GFP/dTip60^{E431Q}). Behavioral recording of fly sleep was carried out for 3 days at 25° in standard LD condition. LNV-specific expression of dTip60^{E431Q} was found to specifically disrupt nocturnal sleep without a marked variation in total sleep within

Figure 6 LNV-specific modulation of dTip60 HAT activity has no effect on generation of biphasic locomotor rhythm but leads to sleep defects in *Drosophila*. Locomotor activity records of control (Pdf-Gal4/UAS-mCD8-GFP/+ and UAS-dTip60^{E431Q}/+) and HAT mutant dTip60^{E431Q} (Pdf-Gal4/UAS-mCD8-GFP/UAS-dTip60^{E431Q}) flies show persistence of morning and evening anticipatory behavior under both (A) LD and (A') DD conditions. Under LD conditions, dTip60^{E431Q} flies are less active during the day but also exhibit increased activity during the night (A). A similar activity pattern persists during the subjective day (SD) and subjective night (SN) in DD (A'). (B) Digital video analysis of sleep in LD in flies expressing dTip60^{RNAi} or HAT mutant dTip60^{E431Q} and flies overexpressing dTip60^{WT} revealed a marked decrease in sleep during the night compared to control flies (Pdf-Gal4/UAS-mCD8-GFP/+ ($n = 28$) while dTip60^{Rescue} flies did not have any effect on nighttime sleep. Sleep assessment was based on behavioral immobility lasting 5 min or longer. Nighttime sleep in dTip60^{RNAi} and dTip60^{E431Q} flies was characterized by increased bout number (B') and decreased bout duration (B''), indicating fragmentation of sleep during the night. dTip60^{WT} flies also exhibited reduced consolidation of nighttime sleep as seen from the decrease in nighttime bout duration (B''). Flies expressing dTip60^{RNAi} or dTip60^{E431Q} displayed an increase in daytime sleep although dTip60^{WT} overexpressing flies and dTip60^{Rescue} flies did not have any observable effect on daytime sleep (C). Daytime sleep in dTip60^{RNAi} and dTip60^{E431Q} flies was characterized by an increase in both bout number (C') and bout duration (C'). *** $P < 0.001$; * $P < 0.05$ compared to control as determined by Student's t test. Error bars represent 95% confidence interval.

a LD cycle compared to control flies (Pdf-Gal4/UAS-mCD8-GFP/+ and UAS-dTip60^{E431Q}/+) (Figure 6B). Similar sleep defects were observed in the dTip60^{RNAi} flies that exhibited a partial reduction in sLNv PDF IR (Figure 6B). Interestingly, the nighttime sleep in both cases was characterized by increased sleep bout number and decreased duration of sleep bout (Figure 6, B' and B''). The number and duration of sleep bout are used to assess consolidation of sleep

(Gonzales and Yin 2010). Changes in these sleep parameters with the dTip60^{RNAi} and dTip60^{E431Q} flies indicate that sleep becomes highly fragmented during the night. Additionally, the dTip60^{RNAi} and dTip60^{E431Q} expressing flies slept more during the day (Figure 6C) with an increase in both sleep bout number and duration of sleep bout (Figure 6, C' and C''). Taken together, these sleep data indicate that flies expressing dTip60^{RNAi} or dTip60^{E431Q} exhibit nighttime

sleep disruption and fragmentation as well as daytime sleepiness, reminiscent of sundown syndrome exhibited by human AD patients. PDF has also been reported to have a wake-promoting effect in the fly and, as a result, is thought to function as a stabilizer of the sleep–wake cycle (Crocker and Sehgal 2010). Since overexpression of wild-type Tip60 in the LNVs increased PDF expression in the sLNVs, we wanted to examine how sleep is affected under these conditions. In contrast to dTip60^{E431Q}- and dTip60^{RNAi}-expressing flies, the dTip60^{WT} flies did not exhibit any significant effect on daytime sleep (Figure 6C), likely due to increased expression of PDF in the sLNVs. However, these flies exhibited reduced consolidation of sleep during the night, resulting in a significant decrease in night sleep (Figure 6, B and B’). We also examined the sleep pattern in the dTip60^{Rescue} flies as the effect on sLNV PDF expression in these flies was different from either the dTip60^{E431Q} or dTip60^{WT} flies. Intriguingly, there was no observable effect on sleep in the dTip60^{Rescue} flies even though these flies exhibited a moderate increase in sLNV PDF level compared to the control flies (Figure 6, B and C). However, the lack of any effect on sleep in the dTip60^{Rescue} flies indicates that the observed sleep defects in the dTip60^{E431Q} and dTip60^{WT} flies are mediated through misregulation of Tip60’s HAT function.

Knockdown of PDF in the sLNVs replicates dTip60^{E431Q}-mediated effects on sleep

Our observations that dTip60^{E431Q} induced selective disruption of PDF expression in the sLNV soma as well as features of sleep interference similar to Pdf null mutants prompted us to ask whether it was the lack of PDF that contributed to these sleep phenotypes. Toward this end, we used a Pdf-RNAi approach to knock down PDF specifically in the sLNVs and monitored how this affects sleep. PDF knockdown was carried out using Mai179-Gal4 and R6-Gal4 drivers, well-characterized drivers that predominantly express Gal4 in the sLNVs (Dubruille and Emery 2008; Shafer and Taghert 2009). Mai179-Gal4-mediated knockdown of PDF in the sLNVs reduced sleep during the night with a concomitant increase in daytime sleep (Figure 7, A and B, respectively) similar to that observed with dTip60^{E431Q} flies. The nighttime sleep was also highly fragmented as evident from the increase in bout number and decreased duration of sleep bout (Figure 7, A’ and A’). Similar effects were observed with R6-Gal4 driven knockdown of PDF (Figure S1, A and B, respectively). Taken together, these data demonstrate that knockdown of PDF in the sLNV affects sleep consolidation and suggests that reduction of PDF is responsible for the sleep disturbances observed in the dTip60^{E431Q} flies.

Tip60 and APP functionally interact to mediate PDF expression and sleep–wake cycles in the fly

Since the selective reduction of PDF IR in the sLNVs by dTip60^{E431Q} was accompanied by sleep defects reminiscent of those seen in AD, we wanted to examine how APP ex-

pression in the LNV affects sleep as sLNV PDF expression was affected in these flies as well. Similar to dTip60^{E431Q} flies, APP expression significantly decreased night time sleep (Figure 8A) with concomitant increase in daytime sleep (Figure 8B). Expression of APP dCT in the LNVs did not have a significant effect on sleep (Figure 8, A and B), consistent with its lack of effect on sLNV PDF expression, indicating that the C terminus of APP mediates the sleep effects seen in the APP flies. Our observation that dTip60 and APP functionally interact to regulate PDF expression in the sLNVs prompted us to ask whether this interaction also mediates the effects that we observed on daytime and nighttime sleep when either of these constructs were expressed alone. Using video monitoring assessment, we found that the APP; dTip60^{E431Q} and APP dCT; dTip60^{E431Q} flies that displayed complete and partial loss of PDF in the sLNV, respectively, also exhibited significant decrease in nighttime sleep with a concomitant increase in daytime sleep (Figure 8, A and B). Taken together, these data suggest that the sleep defects are primarily due to dTip60^{E431Q} and APP-mediated effects on PDF expression in the sLNVs.

Overexpression of wild-type dTip60 rescues APP-induced sleep deficits

The increase in PDF expression in sLNVs due to dTip60^{WT} overexpression prompted us to examine how sleep was affected in APP; dTip60^{WT} and APP dCT; dTip60^{WT} flies as these flies exhibited a similar increase in sLNV PDF. Although overexpression of wild-type dTip60 (dTip60^{WT}) decreased nighttime sleep (Figure 8A), co-expression of dTip60^{WT} with APP rescued the nighttime sleep defects that we observed for dTip60^{WT} or APP alone (Figure 8A). Co-expression of Tip60^{WT} with APP lacking the Tip60 interacting C-terminal domain did not rescue Tip60^{WT}-induced decrease in nighttime sleep, indicating that an interaction between Tip60 and APP is required for the rescue of the nighttime sleep deficits (Figure 8A). In addition, neither of these fly lines exhibited any significant effects on the daytime sleep, and their sleep pattern was similar to wild-type controls (Figure 8B). Taken together, these data indicate that dTip60 and APP functionally interact to mediate sleep in the fly and that the sleep phenotypes that we observe are dependent upon the APP C terminus. Moreover, overexpression of Tip60 appears to rescue both day and nighttime sleep defects that are induced by APP alone, indicating that, under APP-expressing conditions, Tip60 HAT activity alleviates the sleep deficits that are reminiscent of sundown syndrome seen in AD patients.

Discussion

Selective vulnerability of specific neuronal populations to degeneration even before disease symptoms are seen is a characteristic feature of many neurodegenerative diseases. Consistent with these studies, here we show that, when induction of the dTip60 RNAi response or expression of the dTip60 HAT mutant was directed to both the small and large

Figure 7 RNAi knockdown of Pdf in the sLNv recapitulates dTip60^{E431Q}-mediated sleep deficits. (A) Mai-179-Gal4 driven knockdown of Pdf in the sLNv (Mai-179 Gal4/UAS-Pdf RNAi) results in a marked decrease in sleep during the night compared to the controls (Mai-179 Gal4/+ and UAS-Pdf RNAi/+). (A') Nighttime sleep was highly fragmented as inferred from the increase in bout number and (A'') the decrease in bout duration. The nighttime sleep deficit was accompanied by (B) an increase in sleep during the day that was characterized by (B') an increase in bout number and (B'') in bout duration ($n = 28$). * $P < 0.05$ compared to control flies (Mai-179-Gal4/+ and UAS-Pdf RNAi/+). Error bars represent 95% confidence interval.

LNvs, only the sLNvs were susceptible to the mutant effects induced under these conditions while the lLNvs were spared. The lack of any morphological effect on the lLNvs in the dTip60^{E431Q} flies could stem from the fact that, compared to the sLNvs, these neurons express higher levels of endogenous Tip60 that counteracts the mutant dTip60^{E431Q} protein. However, induction of the RNAi response causes complete loss of Tip60 expression in both the lLNvs and the sLNvs (Figure 1), and yet only the sLNvs are affected while the lLNvs are spared, similar to our findings with dTip60^{E431Q} expression. This suggests that the sLNvs may be more susceptible to misregulation of Tip60 or its HAT activity. Notably, the dTip60^{WT} flies did not have any marked effect on the lLNvs either, likely because these neurons are not susceptible to the moderate increase in Tip60 levels in the lLNvs induced under these conditions compared to the sLNvs. Developmentally, the sLNvs are known to differentiate much earlier than the large cells (Helfrich-Forster 1997), and this developmental difference may also in part account for the selective vulnerability of the sLNvs. In many neuro-

degenerative diseases, axon degeneration is known to involve protracted gradual “dying back” of distal synapses and axons that can precede neuron cell-body loss and contribute to the disease symptoms (Saxena and Caroni 2007; Yan *et al.* 2010). Importantly, loss of synapses and dying back of axons are also considered as early events in brain degeneration in AD (Mandelkowitz *et al.* 2003). While APP overexpression in the LNvs did not have any observable effect on the sLNv axon growth at normal physiological temperatures, co-expression of the dTip60 HAT mutant with the APP C terminus appears to cause the sLNv axons in the adult animals to retract. The lack of any effect on the sLNv axon in the third instar larva in this case indicates that the axons grow to their full potential in the larval stage, but undergo degeneration post-mitotically in a process similar to dying back.

A functional interaction between Tip60 and the APP AICD has been shown by us and others to epigenetically regulate genes essential for neurogenesis (Baek *et al.* 2002; Kinoshita *et al.* 2002; Pirooznia *et al.* 2012). Such an effect is thought to be mediated by recruitment of the Tip60/AICD-containing

Figure 8 dTip60 and APP functionally interact to mediate daytime and nighttime sleep. (A) dTip60 and APP functionally interact to mediate nighttime sleep deficits. Adult flies were entrained to cycles of 12 hr LD, and their sleep was monitored for 3 days in 12 hr LD. Shown is a histogram depicting the average sleep during the dark period in control (Pdf-Gal4/UAS-mCD8-GFP/+) and flies expressing each of the different transgenes under the control of Pdf-Gal4 driver (dark blue bars). Light blue bars show the average sleep during the nighttime in the respective UAS controls for each transgenic line (UAS-transgene/+). Asterisks indicate the values that are statistically different from those of control flies (Pdf-Gal4/UAS-mCD8-GFP/+ and the respective UAS control): $*P < 0.05$. All data bars represent mean $\pm$ SEM. (B) Nighttime sleep deficits in dTip60^{RNAi}, dTip60^{E431Q}, and APP flies are accompanied by an increase in daytime sleep. Digital video analysis of sleep for 3 days in adult flies entrained to cycles of 12 hr LD. Shown is a histogram depicting the average sleep during the light period in flies expressing each of the different transgenes under the control of Pdf-Gal4 driver (dark blue bars) and the respective UAS control (light blue bars). Asterisks indicate statistically significant values compared to control flies (Pdf-Gal4/UAS-mCD8-GFP/+ and the respective UAS control): $*P < 0.05$. All data bars represent mean $\pm$ SEM.

complex to certain gene promoters in the nervous system that are then epigenetically modified by Tip60 via site-specific acetylation and accordingly activated or repressed. While the E431Q mutation in our dominant negative HAT-defective version of Tip60 (dTip60^{E431Q}) reduces Tip60 HAT activity, it should not interfere with its ability to assemble into a protein complex (Yan *et al.* 2000; Lorbeck *et al.* 2011). Thus, dTip60^{E431Q} likely exerts its dominant negative action over endogenous wild-type Tip60 via competition with the endogenous wild-type Tip60 protein for access to the Tip60/AICD complex and/or additional Tip60 complexes, with subsequent negative consequences on chromatin histone acetylation and gene regulation critical for nervous system function. Here, we show that co-expression of HAT-defective Tip60 (dTip60^{E431Q}) with APP in the APP; dTip60^{E431Q} flies exacerbates the mutant effects that either of these interacting partners has on the sLNv axon growth and Pdf expression when expressed alone. In contrast, co-expression of additional dTip60^{WT} with APP alleviates these effects, and this rescue is dependent upon the presence of the AICD region of APP. Thus, Tip60 HAT activity appears to display a neuroprotective effect on axonal outgrowth and Pdf expression, with concomitant alleviation of sleep defects under APP-expressing neurodegenerative conditions. We propose that Tip60 might exert this neuroprotective function either by itself or by complexing with other peptides such as AICD for its recruitment and site-specific acetylation of specific neuronal gene promoters to redirect their expression and function in selective neuronal processes such as sLNv morphology and function. Such a neuroprotective role for Tip60 is consistent with our previous work demonstrating that excess dTip60^{WT} production under APP-expressing neurodegenerative conditions in the fly rescues APP-induced lethality and CNS neurodegeneration and that dTip60 regulation of genes linked to AD is altered in the presence of excess APP (Pirooznia *et al.* 2012). We speculate that the degenerative effects that we observe in the APP; dTip60^{E431Q} flies may result from formation of Tip60^{E431Q}/AICD complexes that ultimately cause activation or derepression of factors that promote axonal degeneration while excess Tip60/AICD complex formation in the APP; dTip60^{WT}-expressing flies promotes gene regulation conducive to sLNv outgrowth and Pdf expression.

Sleep- or wake-promoting neurons in the hypothalamus or brainstem are known to undergo degeneration in a number of neurodegenerative diseases, resulting in sleep dysregulation (Chokroverty 2009). In AD, such sleep disturbances are characterized by excessive daytime sleepiness and disruption of sleep during the night. These features resemble the symptoms of narcolepsy, a sleep disorder caused by general loss of the neurotransmitter hypocretin/orexin (Thannickal *et al.* 2009). Hypocretin is involved in consolidation of both nocturnal sleep and diurnal wakefulness (Ohno and Sakurai 2008), and loss of hypocretin levels have been correlated with sleep disturbances observed in AD (Fronczek *et al.* 2012). While the neuropathological changes in AD may contribute to hypocretin disturbances, a direct and causative role for APP in regu-

lating hypocretin expression is not yet known. The LNV-specific neuropeptide PDF is postulated to be the fly equivalent of hypocretin (Crocker and Sehgal 2010) and has been shown to promote wakefulness in the fly. Consistent with these reports, our data demonstrating somnolence during the light phase due to knockdown of PDF in the sLNvs further supports a wake-promoting role for PDF. Accordingly, we observed that overexpression of APP in the LNVs results in reduction of sLNv PDF expression as well as sleep disturbances that, intriguingly, have been associated with AD pathology. The presence of similar effects on PDF and sleep due to loss of dTip60 HAT activity supports a role for both APP and Tip60 in controlling the PDF-mediated sleep-wake regulation pathway. Previous studies have reported that the circadian modulators CLOCK and CYCLE regulate PDF expression in the sLNvs but not in the lLNvs (Park *et al.* 2000). We also observe a similar sLNv-specific regulation of PDF by dTip60 in the adult flies. However, there was no effect on PDF expression in sLNvs in the larvae when Tip60 levels are undetectable. This is also consistent with the sLNv axonal defects that persist only in the adult flies. This suggests that the sLNvs may be subject to differential regulation during development as well as a temporal requirement for Tip60 in these cells in the adult flies. A recent study reported persistence of morning anticipation and morning startle response in LD in the absence of functional sLNvs that were ablated due to expression of the pathogenic Huntington protein with polyglutamine repeats (Q128) (Sheeba *et al.* 2010). Consistent with this study, we did not observe any marked effect on the morning and evening anticipatory behavior in LD in the dTip60^{E431Q} flies that exhibit a partial reduction in sLNv PDF. However, while the Q128-expressing flies were arrhythmic under constant darkness, dTip60^{E431Q} flies maintained rhythmicity in DD, indicating that the sLNvs are still functional in these flies. The remarkable cell specificity of PDF regulation indicates the presence of additional as-yet-unidentified clock-relevant elements or developmental events that distinguish between the two cell types.

Recent evidence indicates that LNVs are light responsive and that their activation promotes arousal through release of PDF. Furthermore, PDF signaling to PDF receptor (PDFR)-expressing neurons outside the clock, such as those found in the ellipsoid body that directly control activity, is thought to be important in translating such arousal signals into wakefulness (Parisky *et al.* 2008). Since PDF is released from the sLNv axon terminals, the retraction of the sLNv axon terminals induced by the Tip60 HAT mutant can interfere with PDF-mediated interaction of the sLNvs with downstream circuits. In the case of APP overexpression, while sLNv axon structure is unaffected, PDF expression is reduced; we speculate that the decrease in PDF under these conditions is responsible for the abnormal sleep phenotype observed. In support of this theory, we find that expression of APP lacking the C terminus that also has no observable effect on the sLNv axon growth or PDF expression did not have any effect on sleep behavior. Thus our results indicate that the degenerative effect on the sLNv axons and/or the effect on

PDF expression could contribute to the observed sleep disturbances. Likewise, co-expression of the dTip60 HAT mutant with full-length APP or APP lacking the C terminus affected both the sLNv axon growth and PDF expression and, consequently, resulted in similar sleep disturbances.

In addition to the wake-promoting role, the LNvs also express GABA_A receptors (Parisky *et al.* 2008; Chung *et al.* 2009) and are thus subject to inhibition by sleep promoting GABAergic inputs, analogous to those from the mammalian basal forebrain that regulate hypocretin neurons (Henny and Jones 2006). The current consensus view is that sleep regulation is mediated by mutually inhibitory interactions between sleep and arousal-promoting centers in the brain (Sakurai 2007; Schwartz and Roth 2008). The normal release of PDF from LNvs is part of the arousal circuitry in the fly and determines the duration of the morning and evening activity peaks (Nitabach and Taghert 2008; Lear *et al.* 2009) while inhibition of these neurons, and thus reduction in PDF release, is necessary for normal sleep (Chung *et al.* 2009). Current models of sleep regulation suggest that the drive to sleep has two components: The first component is driven by the circadian clock. The second component is homeostatic in nature, and the strength of this drive is based upon the amount of time previously awake (Franken and Dijk 2009). PDF release from sLNv axon terminals exhibits diurnal variation (Park *et al.* 2000), and its release increases the probability of wakefulness by activating arousal-promoting centers (Parisky *et al.* 2008). However, the homeostatic drive for sleep that accumulates during the wake period eventually inhibits such arousal centers to promote sleep (Carter *et al.* 2009). Consistent with these reports, the reduction of PDF that we observe due to either dTip60^{E431Q} expression alone or co-expression of dTip60^{E431Q} with APP that leads to flies sleeping more during the day may also lead to a decrease in their homeostatic drive for sleep, thus resulting in the less-consolidated sleep patterns that we observe for these flies during the night. Conversely, we found that overexpression of sLNv PDF due to dTip60 overexpression induces wakefulness and arousal. Additionally, these flies exhibit impaired ability to maintain sleep at night that may be mediated through inappropriate activation of arousal circuits due to PDF overexpression. Similar effects have been reported in a zebrafish model due to hypocretin overexpression that results in hyperarousal and dramatic reduction in ability to initiate and maintain a sleep-like state at night (Prober *et al.* 2006). Despite the moderate increase in sLNv PDF levels in the dTip60^{Rescue} flies, we did not observe a marked effect on the sleep–wake cycle in these flies. Extracellular levels of PDF and its signaling at synapses is thought to be regulated by neuropeptidases like neprilysin. In fact, neprilysin-mediated cleavage of PDF has been shown to generate metabolites that have greatly reduced receptor-mediated signaling (Isaac *et al.* 2007). Thus, we speculate that the lack of any corresponding effect on sleep in the Tip60^{Rescue} flies could be because such small increases in PDF might be regulated by endopeptidases like neprilysin.

Although overexpression of wild-type dTip60 with full-length APP increased PDF expression in the sLNvs compared to the normal levels that persist in the control flies, it did not result in the sleep defects that were observed when Tip60^{WT} was overexpressed alone or with APP lacking the C terminus. The absence of any observable effects on sleep under these conditions suggests the presence of other sleep-promoting compensatory mechanisms that counteract the sleep defects mediated by PDF overexpression. Intriguingly, significant exacerbation of axonal arborization was only observed as a result of co-expression of APP and Tip60^{WT}, and not when Tip60^{WT} was expressed alone or with APP lacking the C terminus; this may account for the differences in sleep phenotypes between these two genotypes. Consistent with this notion, recent electron microscopy studies indicate the presence of sparsely distributed input synapses at the sLNv axon terminals in addition to the PDF-positive output synapses. This indicates that the sLNvs may also receive additional neural inputs directly through such synaptic connections in the dorsal protocerebrum (Yasuyama and Meinertzhagen 2010). The sLNv axon terminals have also been reported to express postsynaptic GABA_B receptors and thus receive slow inhibitory GABAergic input through the dorsal terminals. Incidentally, GABAergic neurons have also been observed in the vicinity of the sLNv axon terminals in the adult CNS (Hamasaka *et al.* 2005). These observations suggest that the sLNvs can also integrate signals from GABAergic or other sleep-promoting neurons via their axon terminals. Indeed, the firing rate of sLNvs is thought to be dependent on a finely balanced interaction of cholinergic, GABAergic, and glutamate signaling (McCarthy *et al.* 2011). Based on these studies, we propose a model by which the overelaborated sLNv synaptic arbors observed in flies co-expressing Tip60^{WT} and APP may provide additional input sites for signals from sleep-promoting neurons in the vicinity that counteract the arousing effect of PDF overexpression on nocturnal sleep (Figure 9).

Light-mediated release of PDF from the lLNvs has been reported to modulate arousal and wakeful behavior as well as sleep stability. Thus, it has been suggested that the lLNvs may be part of an arousal circuit that is physiologically activated by light and borders with, but is distinct from, the sLNv and downstream sleep circuits (Sheeba *et al.* 2008). However, other studies have suggested that both lLNv subgroups promote wakeful behavior and that the lLNvs act upstream of the sLNvs (Parisky *et al.* 2008; Shang *et al.* 2008). Our observation of sLNv-directed effects on PDF expression and the persistence of sleep–wake disturbances suggest that the sLNvs may be part of the neural circuitry that regulates sleep downstream of the lLNvs via a PDF-dependent mechanism. In this regard, the sLNvs may participate in the communication between the lLNvs and other brain regions to promote light-mediated arousal. It has been proposed by Shang *et al.* (2008) that the lLNvs may promote neural activity of the ellipsoid body (EB) in the central complex (CC), a higher center for locomotor behavior that

Figure 9 Schematic representation of proposed events for sleep-wake cycle rescue by Tip60 under APP-overexpressing conditions. Significant exacerbation of axonal arborization in APP;dTip60^{WT} flies may create sites for neural inputs that counteract PDF-mediated sleep disruption through activation of compensatory sleep-promoting mechanisms. Since sLNv axon terminals in the protocerebrum express the post-synaptic GABA_B receptor, neural input from sleep-promoting GABAergic cells in the vicinity may be such a compensatory mechanism.

expresses the PDF receptor (Lear *et al.* 2009). However, we observe disruption of sleep-wake cycles even in the absence of any marked effect on the ILNv morphology or PDF expression. While no direct projections from the ILNvs to the EB have been detected, the sLNv axonal projections are relatively closer to the CC and thus may promote PDF receptor-mediated signaling in such regions that control activity.

Sleep disturbances, while prominent in many neurodegenerative diseases, are also thought to further exacerbate the effects of a fundamental process leading to neurodegeneration (Kang *et al.* 2009). For these reasons, optimization of the sleep-wake pattern could help alleviate the disease symptoms and slow the disease progression. In this regard, the modulatory effects that Tip60 HAT activity (dTip60^{E431Q} vs. dTip60^{WT}) has on the sLNvs, the fly counterpart of the mammalian pacemaker cells, under APP-overexpressing conditions may provide novel mechanistic insights into epigenetic regulation of neural circuits that underlie behavioral symptoms such as the “sundowner’s syndrome” in AD. Future investigation into the downstream mechanism by which Tip60 regulates the sleep-wake cycle may provide further insight into the utility of specific HAT activators as therapeutic strategies for the sleep disturbances observed in AD.

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Supporting Information

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Epigenetic Regulation of Axonal Growth of *Drosophila* Pacemaker Cells by Histone Acetyltransferase Tip60 Controls Sleep

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Figure S1 R6-Gal4 driven RNAi knock down of Pdf in the sLNv also results in sleep defects similar to dTip60E431Q. (A) Pdf knock down flies exhibit significant reduction in sleep during the night with (A') marked decrease in bout duration, indicative of defects in sleep maintenance. This was accompanied by (B) increase in daytime sleepiness with (B') significant increase in both bout number and (B'') bout duration. Sample size n=28. * P<0.05 compared to control flies (R6-Gal4/+ and UAS-Pdf RNAi/+). Error bars represent 95% confidence interval.

Figure S2 Quantification of sLNv axonal arborization. Quantification of two dimensional area of sLNv terminal axonal arbor was done as described in Leyssen et al (2005). The axonal stem on either half of the brain hemisphere was marked by straight vertical lines followed by a horizontal line between them (blue vertical and horizontal lines). The outline of the axonal processes dorsal to the horizontal line was traced (shown in red), and the area inside was measured using ImageJ. The distance between the vertical lines was used as a measure for brain size. Area measurements were normalized for brain size by scaling the distance between the dorsal projections to the median of the distance as a correction factor.