Cellular/Molecular

Drosophila Neuroligin 4 Regulates Sleep through Modulating GABA Transmission

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Sleep is an essential and evolutionarily conserved behavior that is closely related to synaptic function. However, whether neuroligins (Nlgs), which are cell adhesion molecules involved in synapse formation and synaptic transmission, are involved in sleep is not clear. Here, we show that *Drosophila* Nlg4 (DNlg4) is highly expressed in large ventral lateral clock neurons (l-LNvs) and that l-LNv-derived DNlg4 is essential for sleep regulation. GABA transmission is impaired in mutant l-LNv, and sleep defects in *dnlg4* mutant flies can be rescued by genetic manipulation of GABA transmission. Furthermore, *dnlg4* mutant flies exhibit a severe reduction in $GABA_A$ receptor RDL clustering, and DNlg4 associates with RDLs in *vivo*. These results demonstrate that DNlg4 regulates sleep through modulating GABA transmission in l-LNvs, which provides the first known link between a synaptic adhesion molecule and sleep in *Drosophila*.

Introduction

Sleep is an essential and evolutionarily conserved behavior exhibited by animals ranging from worm to human (Cirelli, 2009; Crocker and Sehgal, 2010), yet it remains one of the least understood biological phenomena. *Drosophila*, an advantageous animal for genetic investigation, also exhibits sleep-like behavior (Nitz et al., 2002; van Swinderen et al., 2004). Thus, this species has been used to dissect the molecular underpinnings of sleep, leading to the identification of genes, circuits, and biological processes that are involved in sleep (Shaw et al., 2000; Cirelli et al., 2005; Joiner et al., 2006; Pitman et al., 2006). As in mammals, sleep in *Drosophila* is governed by both circadian and homeostatic regulation (Hendricks et al., 2000; Shaw et al., 2000). In particular, large ventral lateral neurons (l-LNvs) mediate light-driven arousal through the release of pigment dispersing factor (PDF; Parisky et al., 2008; Shang et al., 2008; Sheeba et al., 2008b). The electrical activity of l-LNvs is circadian regulated and suppressed at night by inhibitory neurotransmitters such as GABA (Shang et al., 2008; Sheeba et al., 2008b; Sehgal and Mignot, 2011). Therefore, the inhibition of l-LNvs by GABAergic inputs serves to promote sleep (Agosto et al., 2008; Parisky et al., 2008; Chung et al., 2009).

Neuroligins (Nlgs) are synaptic adhesion molecules involved in synapse formation and synaptic transmission (Scheiffele et al., 2000; Varoqueaux et al., 2006). Four Nlg homologs (Nlg1–Nlg4) have been identified in mammals (Ichtchenko et al., 1996), with different homologs located in different classes of synapses (Song et al., 1999; Budreck and Scheiffele, 2007; Hoon et al., 2011). All Nlgs are expressed in the hypothalamus (Varoqueaux et al., 2004, 2006; Mungenast and Ojeda, 2005), which is a brain region that is essential for sleep regulation. A recent study showed that *nlg1* knock-out mice are not able to sustain wakefulness and spend more time in nonrapid eye movement sleep than wild-type mice (El Helou et al., 2013). However, the potential roles of other Nlg homologs in sleep, especially those that regulate inhibitory synapses, are largely unknown.

Four *Drosophila* Nlgs (DNlgs) have been identified or are predicted to exist: DNlg1 (CG31146), DNlg2 (CG13772), DNlg3 (CG34127), and DNlg4 (CG34139; Banovic et al., 2010). Phylogenetic analysis of Nlg sequences from multiple species suggests that Nlg homologs diversified independently during evolution in both vertebrates and insects (Banovic et al., 2010; Knight et al., 2011). As individual DNlgs are difficult to match with specific human orthologs (Banovic et al., 2010), their nomenclature is based on their similarity to honeybee Nlgs (Banovic et al., 2010). So far, only DNlg1 and DNlg2 have been functionally characterized (Banovic et al., 2010; Sun et al., 2011; Chen et al., 2012). In the present study, we show that DNlg4 promotes sleep through mediating $GABA_A$ receptor RDL clustering and modulating GABA transmission in l-LNvs, thereby providing a direct link between a synaptic adhesion molecule and sleep in *Drosophila*.

Materials and Methods

Animals. Flies were raised at 25°C (except those used in the experiments shown in Fig. 5) with a 12 h light/dark (LD) cycle. *pBac{RB}cic*^{e01254} and

Received Feb. 21, 2013; revised Aug. 14, 2013; accepted Aug. 14, 2013.

Author contributions: J.H. designed research; Y.L., Z.Z., and P.L. performed research; X.Z. and W.X. contributed unpublished reagents/analytic tools; Y.L., Z.Z., H.T., and Z.J. analyzed data; Z.C.Z., W.X., and J.H. wrote the paper.

This work was supported by the National Natural Science Foundation of China (91132706) to J.H., the National Basic Research Program (973 Program) Grant (2012CB517903) and the National Natural Science Foundation of China (31171041 and 30930051) to W.X., and the Scientific Research Foundation of Graduate School of Southeast University (YBJJ1123) to Y.L. We thank Dr. Julie H. Simpson for providing rdl-Gal4 flies, Dr. Li Liu for providing 104y-Gal4 flies, the Bloomington stock center and Vienna Drosophila RNAi stock center for providing flies, and members of the Han laboratory for critical comments on this manuscript.

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The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.0819-13.2013

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pBac{WH}f01735 flies were purchased from the Exelixis collection at Harvard Medical School. *pBac{SAstopDsRed}*^{LL01874} flies were obtained from the Drosophila Genomics Research Center. *UAS-nlg4-RNAi* flies correspond to Vienna Drosophila RNAi Center stocks 6792. Wild-type flies were w^{1118}. Other types of flies were obtained from the Bloomington Stock Center.

Generation of p*[UAS-DNlg4] transgenic flies.* To generate *p[UAS-DNlg4]* flies, *dnlg4* cDNA was subcloned into a *pUAST* vector and injected into w^{1118} flies. The transgene was subsequently crossed into a w^{1118}*;dnlg4* background.

Sleep and circadian analysis. Three- to five-day-old male flies raised in LD-entrained cultures were placed in 65 × 5 mm glass tubes containing 5% sucrose/2% agarose. Locomotor activity was measured for 5–7 d at 25°C during LD cycles using DAM5 monitors (Trikinetics) in a DigiTherm CircKinetics incubator (Tritech Research). Data were collected in 1 min bins, and a sliding window was applied. Sleep was defined as 5 consecutive minutes of inactivity as previously described (Huber et al., 2004). Sleep latency was measured from the time of lights off to the onset of the first sleep episode. Data were analyzed with ClockLab software (Actimetrics).

Antibodies. Anti-DNlg4 antibody was generated in rabbits against a purified glutathione *S*-transferase fusion fragment (aa912–1089) of DNlg4 protein, which is encoded by GH07829 cDNA (GenBank Accession Number BT050584). The antibody was purified using an affinity column generated by coupling a DNlg4 fragment (aa912–1089) to Sepharose 4B. Other antibodies were obtained from Millipore (GFP, MAB3580), Cell Signaling Technology (hemagglutinin (HA)-tag, C29F4), and Developmental Studies Hybridoma Bank (PDF, C7, and tubulin, E7).

Immunostaining. Whole-head staining was performed as previously described (Cao et al., 2011; Tian et al., 2013). Briefly, after dissection and fixation, fly heads were stained with anti-PDF (1:200) and anti-DNlg4 (1:40) antibodies. Samples were imaged on an LSM 510 confocal microscope (Zeiss).

Electrophysiology. Whole-cell recording was performed as previously described (Sheeba et al., 2008a). Briefly, PDF neurons of wild-type and *dnlg4* mutant flies were visualized by membrane expression of a GFP-tagged mCD8 using the pdf-Gal4 driver. Flies were maintained under a 12 h LD cycle, and the brains of 2- to 3-d-old flies were quickly dissected under a light intensity of 4000 lux in external solution. The perineural sheath of the brain was gently removed with fine tweezers. The dissected brains were incubated in standard external solution containing 20 U/ml papain and 1 mM[SCAP] L-cysteine at 37°C for 10 min, and then transferred to a submersion chamber and fixed with a platinum holder. The chamber was perfused with extracellular recording solution containing the following (in mM): 101 NaCl, 3 KCl, 1 $CaCl_2$, 4 $MgCl_2$, 5 glucose, 1.25 NaH_2PO_4, and 20.7 $NaHCO_3$. The solution was saturated with 95% O_2/5% CO_2 throughout all recordings. For recordings at ZT13-ZT16, dissections were executed under dark conditions except for brain dissection, which was executed under a light intensity of 400 lux for <90 s.

GFP-expressing l-LNvs were visualized using an Olympus X51 microscope with a 40× water-immersion objective. Recording pipettes (8–10 MΩ) were filled with solution containing the following (in mM): 102 K-gluconate, 0.085 $CaCl_2$, 1.7 $MgCl_2$, 17 NaCl, 0.94 ethylene glycol tetraacetic acid, and 8.5 HEPES adjusted to 7.3 pH and 235 mOsm. Signals were acquired using an Axon-700B amplifier, digitized at 10 kHz by Digidata 1440, and filtered at 2 kHz.

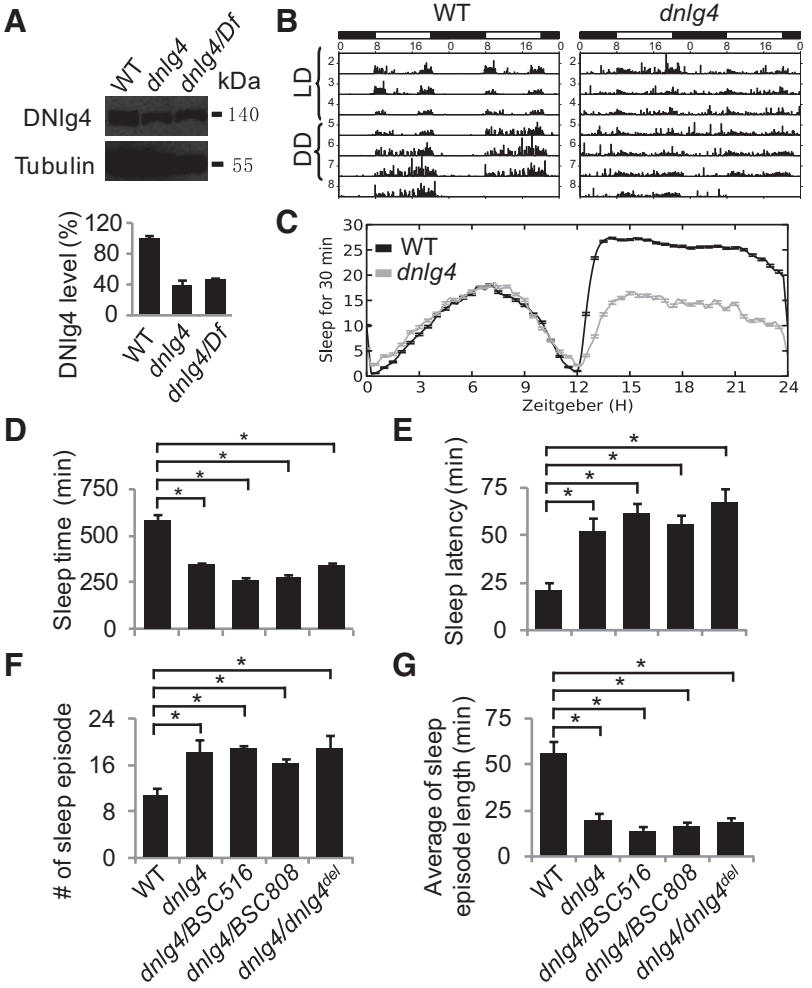

Figure 1. *dnlg4* mutant flies show abnormal sleep behavior. ***A***, Western blot analysis showing the protein level of DNlg4 in wild-type (WT), *dnlg4* mutant, and *dnlg4/BSC516* heterozygous mutant flies. Tubulin was used as a loading control. Quantification of relative DNlg4 protein level for each genotype is presented on the bottom. ***B***, Representative 7-d locomotor traces of WT (left) and *dnlg4* mutant (right) flies. White areas indicate day, and black areas indicate night. ***C***, Average total sleep per night in WT and *dnlg4* mutant flies, plotted as a 30 min moving average. $n = 200$. ***D***, ***E***, Quantification of total sleep time per night and sleep onset latency after lights off for each genotype. $n = 200$. ***F***, ***G***, Quantification of number of sleep episodes per night and average sleep episode length for each genotype; $n = 200$, $^*p < 0.001$.

Spontaneous firing was recorded in I-clamp mode (I = 0), and 30 s traces of each recording were analyzed. For measurement of GABA currents, PDF neurons were clamped at −30 mV, and puffs of 0.1, 1, and 10 mM GABA lasting 100 ms were applied to neurons using a Picospritzer (WPI, PV820). The puffing pipettes were set ~50 μm from the neurons. The pressure applied (4 psi) was sufficient to allow GABA to rapidly reach the neurons without producing artifacts. Two sweeps of each cell were pooled to generate the sample traces. For all electrophysiological experiments, *n* represents the number of neurons, and only one neuron per fly was used.

Drug treatment. For experiments involving carbamazepine (CBZ), a stock solution (20 mg/ml) of CBZ was dissolved in ethanol and mixed into standard agar medium. After three to four baselines were recorded on standard medium, flies were switched to CBZ/sucrose/agar 5 h before the onset of the dark period.

Statistical analysis. Data are presented as mean ± SEM. For statistical analysis of sleep parameters with normal distributions, two-tailed Student's *t* tests were used to compare genotypes. To analyze sleep episode length, which was not normally distributed, Mann–Whitney *U* tests were used. Statistical significance was set at $p < 0.05$.

Results

dnlg4 mutant flies exhibit abnormal sleep behavior

To obtain null mutant *dnlg4* (CG34139) flies, we generated a $dnlg4^{del}$ mutant line using the FRT/FLP-mediated genomic deletion strategy with two FRT-containing Exelixis lines (f01735 and e01254) flanking the *dnlg4* locus. Homozygous $dnlg4^{del}$ deletion is lethal. To get a viable allele for sleep-behavior analysis, we obtained a hypomorphic allele, $p\{FRT\}2A,\ p\{FRT\}82B,\ pBac\{SAstopDsRed\}^{LL01874}$, which contains two FRT transposons and a piggyBac transposon insert in the region near the stop codon of CG34139. The mutant line was out-crossed (based on the DsRed marker) for more than six generations with the w^{1118} strain to delete the extra FRT transposons and standardize the background. From here on, the $pBac\{SAstopDsRed\}^{LL01874}$ mutant is referred to as the *dnlg4* mutant. Western blots showed a marked reduction of DNlg4 protein levels in both homozygous and combination *dnlg4*-deficient flies (Fig. 1*A*).

To examine the role of DNlg4 in sleep, we assessed sleep behavior in *dnlg4* mutant flies during 12 h LD cycles. We found that *dnlg4* mutant flies showed significantly less total night sleep time (582 ± 29.9 min vs 339.8 ± 11.5 min; Fig. 1*B–D*) and longer sleep onset latency (21 ± 3.7 min vs 52 ± 7.2 min; Fig. 1*E*) compared with wild-type flies. Furthermore, compared with wild-type flies, mutants exhibited more night sleep episodes (10.5 ± 1.4 vs 17.9 ± 2.3; Fig. 1*F*) with markedly shorter durations (55.7 ± 6.7 min vs 19.1 ± 3.8 min; Fig. 1*G*), reflecting poorly consolidated night sleep in *dnlg4* mutants. Similar results were observed for $dnlg4/dnlg4^{del}$ combination flies and two additional *dnlg4*-deficient flies (Fig. 1*D–G*) in which the mutant allele was combined with *Df(3R)BSC516* or *Df(3R)BSC808* (both flies lacked the entire *dnlg4* gene). These results indicate that impaired DNlg4 function results in poorly consolidated night sleep, which strongly suggests the involvement of DNlg4 in sleep regulation.

DNlg4 is highly expressed in l-LNvs and required for sleep

To investigate how DNlg4 regulates sleep, we first examined the expression pattern of endogenous DNlg4 in the adult brain using an anti-DNlg4 antibody. In wild-type flies, strong immunostaining was observed in LNvs, identified by double staining with anti-PDF antibody (Renn et al., 1999; Fig. 2*A*). DNlg4 staining was also observed in fan-shaped body neurons and other unidentified neurons (Fig. 2*A*). In LNvs, DNlg4 was located in somata and also appeared as puncta in the terminals of the accessory medulla (Fig. 2*B*). In *dnlg4* mutant flies, both overall and LNv terminal immunostaining was significantly reduced (Fig. 2*A–C*), consistent with Western blot results. Interestingly, somata of small LNvs (s-LNvs) were weakly labeled, but somata of l-LNvs, as identified with the c929-Gal4 driver (Taghert et al., 2001), were strongly labeled by anti-DNlg4 staining (Fig. 2*D*,*E*). To further investigate the subcellular localization of DNlg4 in l-LNv somata, we labeled LNv membranes with mCD8-GFP and stained with an anti-DNlg4 antibody. Surprisingly, DNlg4 was mostly located in intracellular regions (Fig. 2*F*). To further display the organellar distribution of endogenous DNlg4, we stained the LNvs, whose endoplasmic reticulum (ER) or Golgi had been labeled with GFP. The results showed that DNlg4 predominately exists in the Golgi (Fig. 2*G*,*H*).

To map the anatomical requirements of DNlg4 for sleep, we directed RNAi against DNlg4 using the Gal4/UAS system. More than 10 Gal4 drivers with targeted expression in various regions of the nervous system were tested. RNAi against DNlg4 using several Gal4 divers with complicated expression patterns (e.g., pan-neuronal *elav*-Gal4, *Rdl*-Gal4, *c929*-Gal4, *117y*-Gal4, and *cry*-Gal4) led to lethality. However, both *pdf*-Gal4 and *per*-Gal4 drivers recapitulated the sleep defects observed in *dnlg4* mutant flies (Fig. 3*A–E*), with other drivers only slightly affecting sleep (Fig. 3*A*,*B*). Immunostaining showed that *pdf*-Gal4 RNAi specifically repressed DNlg4 expression in PDF neurons (Fig. 3*F*,*G*). Together, these results indicate that PDF neuron-expressed DNlg4 is involved in sleep regulation.

Next, we generated *p*[*UAS-DNlg4*] flies and conducted rescue experiments to validate the anatomical requirements of DNlg4 for sleep. Sleep defects, especially those in early evening sleep, were restored in *dnlg4* mutant flies through expression of DNlg4 in l-LNvs using *pdf*-Gal4 or *c929*-Gal4 drivers (Fig. 4*A–E*). However, we failed to rescue sleep defects using other drivers with more restricted neuronal expression, including *104y*-Gal4 expressed in fan-shaped body neurons (Young and Armstrong, 2010; Fig. 4*A*,*B*). Therefore, DNlg4 is highly expressed in l-LNvs, and l-LNv-derived DNlg4 is essential for early evening sleep.

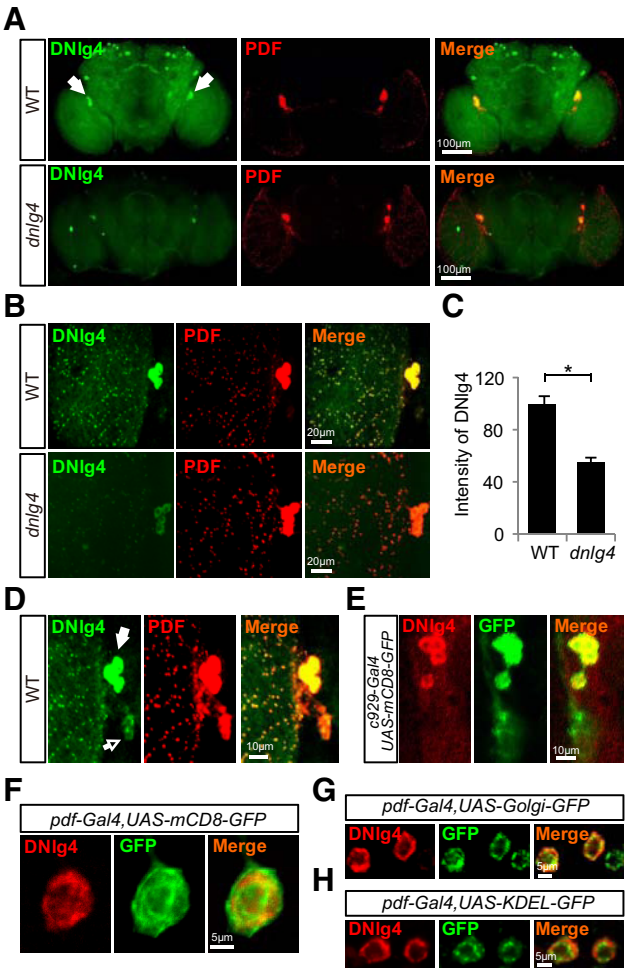

Figure 2. DNlg4 is highly expressed in l-LNvs. ***A***, Immunostaining images indicating the expression pattern of DNlg4. PDF neurons are indicated by arrows. Scale bar, 100 μm. ***B***, Distribution of DNlg4 in LNv terminals. Scale bar, 50 μm. ***A***, ***B***, Dissected whole brains were stained with anti-DNlg4 (green) and anti-PDF (red) antibodies. ***C***, Quantification of relative DNlg4 protein amount in LNvs for each genotype. ***D***, DNlg4 was highly expressed in l-LNvs. LNvs were labeled with mCD8-GFP under the control of the *pdf*-Gal4 driver. l-LNvs are indicated by the closed arrow, and s-LNvs are indicated by the open arrow. Scale bar, 10 μm. ***E***, l-LNvs were labeled with mCD8-GFP under control of the *c929*-Gal4 driver. Scale bar, 10 μm. ***F***, DNlg4 was located in the intracellular region of l-LNv somata. l-LNv somata membranes were labeled with mCD8-GFP under the control of the *pdf*-Gal4 driver. Scale bar, 5 μm. ***G***, In *pdf-Gal4/p*[*UASHgalactosyltransferase-GFP*] flies, DNlg4 colocalized with the GFP signal. Dissected LNvs were costained with DNlg4 antibody (red) and GFP antibody (green, showing Golgi). Scale bar, 5 μm. ***H***, In *pdf-Gal4/p*[*UASHGFP-KDEL*] flies, DNlg4 did not colocalize with GFP-KEDL. Dissected LNvs were costained with DNlg4 antibody (red) and GFP antibody. Scale bar, 5 mm. WT, wild type.

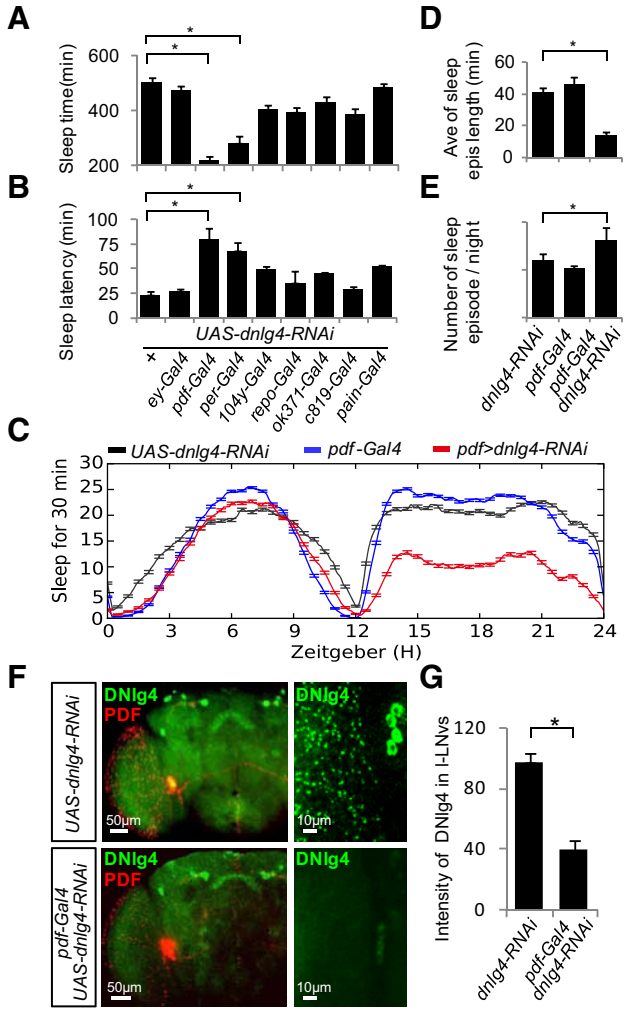

Figure 3. Depletion of DNlg4 in l-LNvs leads to abnormal sleep. ***A***, ***B***, Total night sleep and sleep onset latency in flies with depletion of DNlg4 using UAS-*dnlg4*-RNAi driven by anatomically restricted Gal4 drivers. For each Gal4 line, a single copy of the Gal4 driver was used for the test; $n = 32$, $^*p < 0.001$ for RNAi versus control flies. ***C***, Average sleep traces for *UAS-nlg4-RNAi;pdf-Gal4* and control flies, plotted as a 30 min moving average; $n = 32$. ***D***, ***E***, Quantification of average sleep episode length and number per night in *UAS-nlg4-RNAi;pdf-Gal4* and control flies; $n = 32$, $^*p < 0.001$ for *UAS-nlg4-RNAi;pdf-Gal4* versus control flies. ***F***, Expression of DNlg4 in *UAS-nlg4-RNAi;pdf-Gal4* (bottom) and control (top) flies. Dissected whole brains were stained with anti-DNlg4 (green) and anti-PDF (red) antibodies. Scale bar, 50 μm. Note that DNlg4 distribution in *UAS-nlg4-RNAi;pdf-Gal4* flies is comparable to control flies except in PDF neurons. Enlarged images showing expression of DNlg4 in PDF neurons are presented on the right. Scale bar, 10 μm. ***G***, Quantification of relative DNlg4 protein amount in LNvs for each genotype.

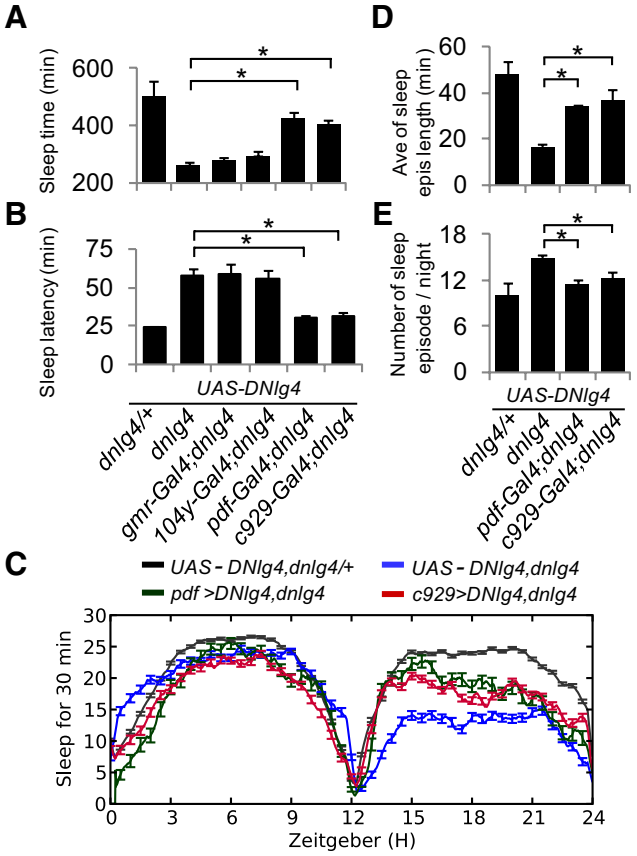

Figure 4. Specific expression of DNlg4 in l-LNvs rescues defective sleep in *dnlg4* mutant flies. ***A***, ***B***, Total night sleep and sleep onset latency in flies with rescued DNlg4 expression using anatomically restricted Gal4 drivers. Flies bear one copy of the indicated drivers; $n = 32$, $^*p < 0.001$ for rescued versus *dnlg4* mutant flies. ***C***, Average sleep traces for rescued and control flies, plotted as a 30 min moving average; $n = 32$. ***D***, ***E***, Quantification of average sleep episode length and number per night in rescued and control flies. $n = 32$. * indicates $p < 0.001$ for rescued *vs* control flies.

Knock-down of DNlg4 expression in adult flies reduces night sleep

The abnormal sleep observed in *dnlg4* mutant flies could be due to developmental deficits in larva or impaired DNlg4 function in adults. To distinguish between these possibilities, we generated *UAS-dnlg4-RNAi;tubulin-Gal80^{ts}/pdf-Gal4* flies and assessed their sleep behavior. In these flies, RNAi was suppressed during development by the ubiquitous expression of temperature-sensitive Gal80^{ts} but selectively induced by exposure to a temperature of 30°C (McGuire et al., 2004). Flies were reared at 21°C for their entire development. As adults, flies were entrained for 3 d of LD at 21°C, switched to 30°C for 2.5 d, and then shifted back to 25°C. Immunostaining revealed that DNlg4 protein levels were reduced after *UAS-dnlg4-RNAi;tubulin-Gal80^{ts}/pdf-Gal4* flies were switched to 30°C for 24 h (Fig. 5*A*,*B*). Compared with control flies, *UAS-dnlg4-RNAi tubulin-Gal80^{ts}/pdf-Gal4* flies exhibited a progressive delay of sleep onset latency after switching to 30°C (Fig. 5*C*,*D*; Fig. 5*C* shows a continuous trace of sleep behavior starting on the last day of entrainment). Two days after shifting back to 25°C, *UAS-dnlg4-RNAi tubulin-Gal80^{ts}/pdf-Gal4* flies still showed longer sleep onset latency and less total night sleep time, whereas control flies exhibited normal sleep behavior (Fig. 5*E–H*). These findings demonstrate that knock-down DNlg4 expression in adult flies is sufficient to reduce night sleep.

GABA transmission is impaired in mutant l-LNvs

PDF is secreted by LNvs and acts as a major regulator of sleep in *Drosophila* (Parisky et al., 2008). The electrical activity of l-LNvs is circadian-regulated (Sheeba et al., 2008a) and LNv-hyperexcited flies exhibits a PDF-mediated reduction in sleep quantity and quality (Sheeba et al., 2008b). We conducted whole-cell current-clamp recordings of l-LNvs to determine their electrophysiological properties. We dissected flies that were entrained in different conditions and performed whole-cell recording of l-LNvs with various recording buffers. l-LNvs fired spontaneous action potentials (APs), with firing patterns heavily influenced by entrainment condition and recording buffer. Using previously established recording conditions (Sheeba et al., 2008a), we found that l-LNvs from *dnlg4* mutant flies showed similar electrophysiological properties as those from wild-type flies during the day (ZT2-ZT5,

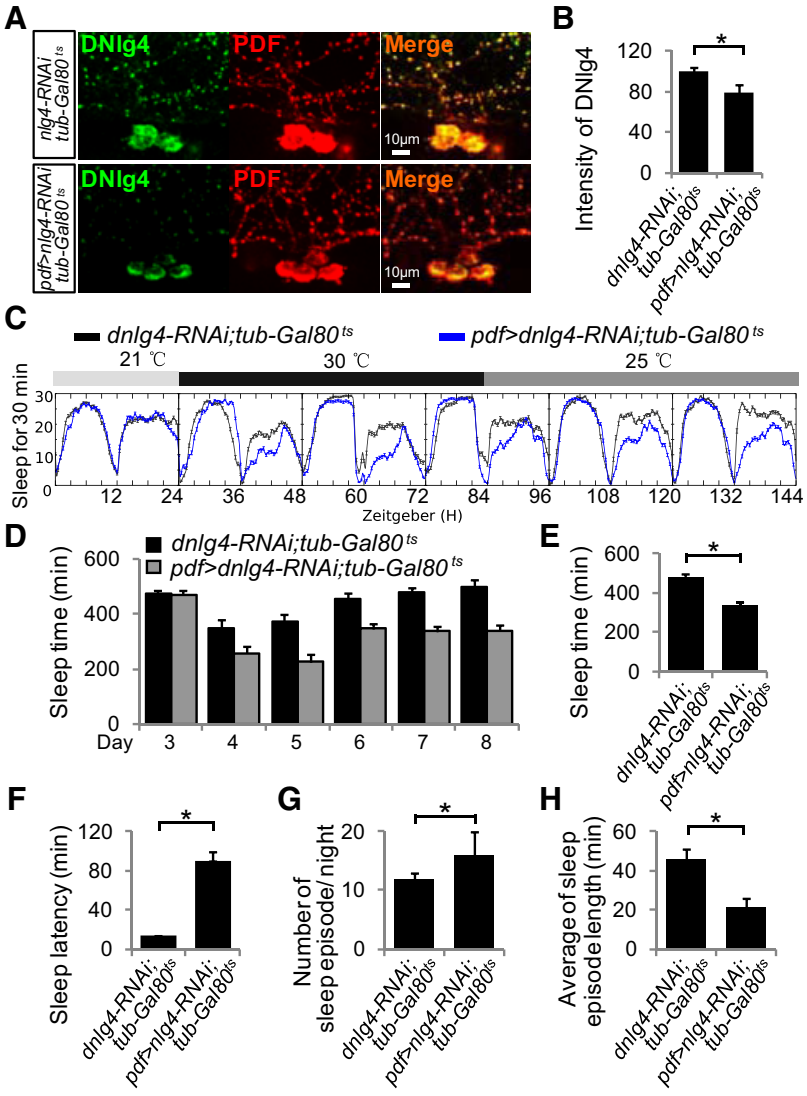

Figure 5. Sleep behavior of *UAS-dnlg4-RNAi;tubulin-Gal80^{ts}/pdf-Gal4* flies. ***A***, Immunostaining images showing DNlg4 protein levels in *UAS-dnlg4-RNAi;tubulin-Gal80^{ts} /pdf-Gal4* (bottom) and control (top) flies. Dissected whole brains were stained with anti-DNlg4 (green) and anti-PDF (red) antibodies. Scale bar, 10 μm. ***B***, Quantification of relative DNlg4 protein amount in LNvs for each genotype is presented on the right. ***C***, Continuous sleep measurements of flies expressing the *dnlg4-RNAi* and temperature-sensitive Gal80^{ts} in PDF neurons. Entrainment temperatures are shown at the top; $n = 32$. ***D***, Quantification of total sleep time for individual nights. ***E***, Average total sleep time per night in each condition. ***F***, Quantification of sleep onset latency after lights off for each condition. ***G***, ***H***, Quantification of sleep episode number and average length. ***D–G***, $n = 32$, and values for day 7 and 8 are quantified and presented. *$p < 0.001$ for *UAS-dnlg4-RNAi;tubulin-Gal80^{ts} /pdf-Gal4* versus *UAS-dnlg4-RNAi /pdf-Gal4* flies.

with ZT0 denoting the time that lights were turned on), with tonic spontaneous AP firing and frequent spike bursts (6/8 wild-type cells and 8/9 mutant cells; Fig. 6*A*). At ZT13–ZT16, wild-type l-LNvs showed reduced activity consisting of tonic spontaneous AP firing and rare bursts (10/10 cells; Fig. 6*A*), whereas most *dnlg4* mutant l-LNvs showed high activity consisting of tonic spontaneous AP firing and frequent bursts (7/9 cells; Fig. 6*A*). At ZT13–ZT16, wild-type and *dnlg4* mutant l-LNvs showed comparable firing amplitudes but significant differences in percentage of spikes fired in bursts, frequency of firing, and resting membrane potential (Fig. 6*B–E*). Therefore, l-LNvs in *dnlg4* mutants are hyperactivated at night.

l-LNv activity is suppressed at night by inhibitory neurotransmitters such as GABA (Shang et al., 2008; Sheeba et al., 2008b; Sehgal and Mignot, 2011). Furthermore, the $GABA_A$ receptor RDL is expressed in l-LNvs and promotes sleep in *Drosophila* (Parisky et al., 2008; Chung et al., 2009). We found that puffing GABA (10 mM, 100 ms) on l-LNvs resulted in a hyperpolarized resting membrane potential and blockade of spontaneous AP firing (Fig. 6*F*), suggesting that l-LNv hyperactivity in *dnlg4* mutants could be due to impaired GABA transmission. To address this possibility, we directly measured GABA receptor-mediated currents in l-LNvs. A Picospritzer was used to puff different concentrations of GABA (0.1, 1, and 10 mM) on l-LNvs that were voltage clamped at −30 mV. Wild-type l-LNvs did not exhibit detectable currents after puffing 0.1 mM GABA (data not shown). GABA currents were slightly increased after puffing 10 mM GABA compared with 1 mM GABA, but this difference was not significant (Fig. 6*G*). By puffing 10 mM GABA, we recorded saturated GABA-gated outward currents that were blocked by $GABA_A$ receptor inhibitor picrotoxin (PTX, 200 μM; Fig. 6*H*). At night, *dnlg4* mutant l-LNvs showed significantly reduced GABA currents compared with wild-type l-LNvs (peaking at 23.16 ± 2.16 pA vs 68.45 ± 8.91 pA; Fig. 6*H*,*I*). These results demonstrate that GABA transmission is largely impaired in *dnlg4* mutant flies.

Impaired GABA transmission in mutant l-LNvs leads to sleep defects

We next investigated whether the sleep defects observed in *dnlg4* mutant flies are due to impaired GABA transmission. If sleep defects are due to impaired GABA transmission, then normal sleep should be restored by increasing RDL channel currents. The hypomorphic mutation RDL^{A302S} specifically decreases the rate of RDL desensitization with little or no effect on other channel properties (Zhang et al., 1994). As a consequence, the mutant receptor has a longer single-channel open duration, which increases RDL channel currents (Agosto et al., 2008). By introducing the mutant RDL^{A302S} channel into the *dnlg4* mutant background, we successfully restored night sleep (Fig. 7*A–C*), indicating that the sleep defects observed in *dnlg4* mutants are due to impaired GABA transmission. To further support this conclusion, we also performed pharmacological experiments with CBZ, which accelerates RDL desensitization and inhibits night sleep in a dose-dependent manner (Agosto et al., 2008). We found that 0.5 mg/ml CBZ significantly inhibited night sleep in wild-type flies but did not further inhibit night sleep in *dnlg4* mutant flies (Fig. 7*D–F*). A higher dose of CBZ (1 mg/ml), however, might further suppress night sleep in *dnlg4* mutants (Fig. 7*D–F*), as GABA transmission was not completely blocked in mutant flies (Fig. 6*H*,*I*). Together, these results indicate that DNlg4 regulates sleep behavior through modulating GABA transmission.

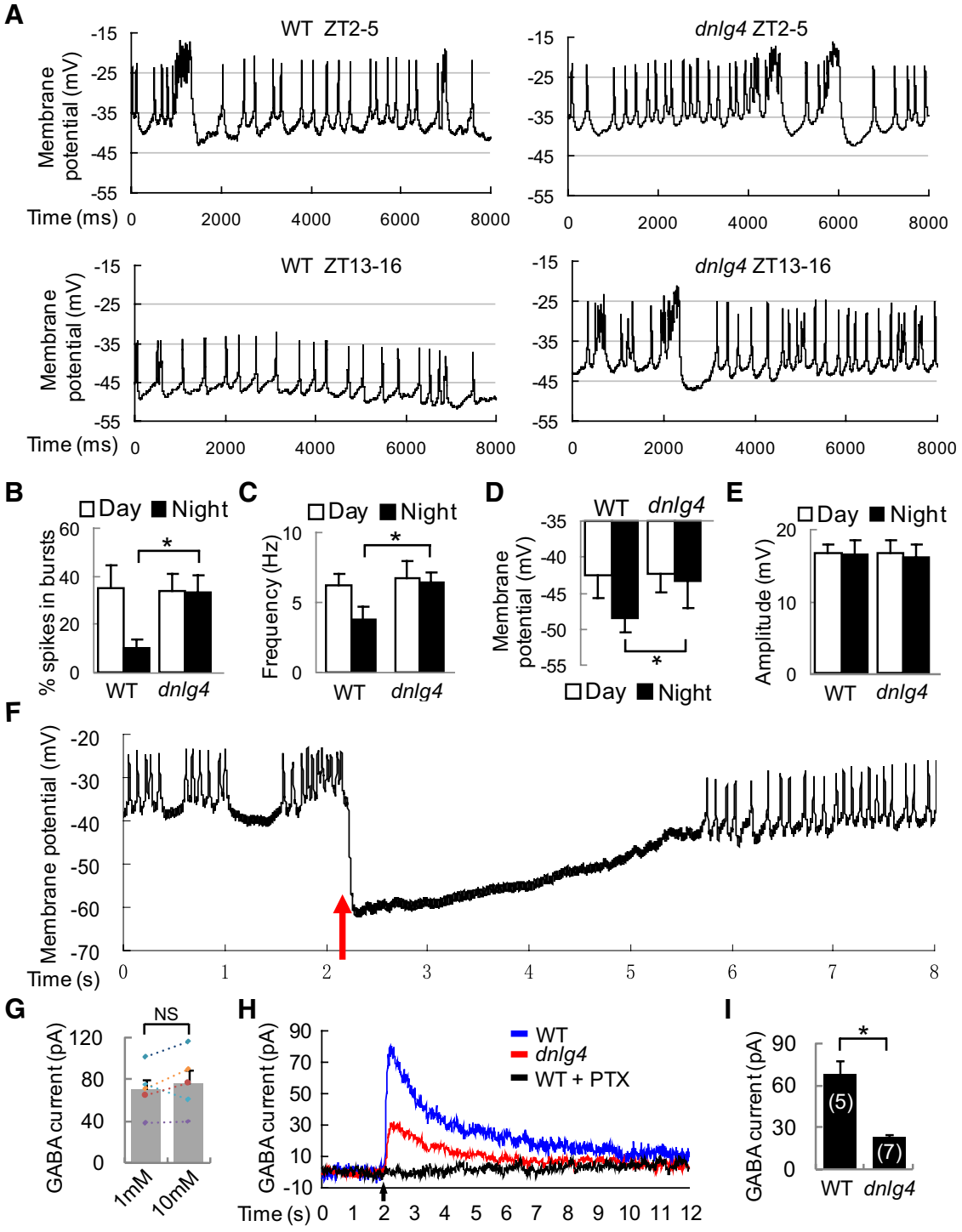

Figure 6. GABA transmission is impaired in *dnlg4* mutant l-LNvs. ***A***, Representative whole-cell recording traces from wild-type (WT) and *dnlg4* mutant l-LNvs at day and night. ***B***, Percentage of spikes fired in bursts in WT and *dnlg4* mutant l-LNvs. ***C***, Average frequency of spontaneous AP firing. ***D***, Average resting membrane potentials for each genotype and condition. ***E***, Average spike amplitude for each genotype and condition. ***B–E***, $n = 9$ for WT daytime, $n = 10$ for WT nighttime, $n = 9$ for *dnlg4* daytime, $n = 8$ for *dnlg4* nighttime, $^*p < 0.05$ for WT versus *dnlg4* mutant l-LNvs. ***F***, GABA puffing on l-LNvs hyperpolarizes resting membrane potential and blocks spontaneous AP firing. PDF neurons were held in I-clamp (I = 0) and puffs of 10 mM GABA lasting 100 ms were applied. Arrow indicates the time point of GABA puffing. $n = 6$. ***G***, GABA-gated currents with puffing of 1 and 10 mM GABA on five individual WT l-LNvs. Averaged GABA currents with puffing of 1 and 10 mM are also presented. ***H***, Representative responses of WT (blue) and mutant (red) l-LNvs to applications of 10 mM GABA puffs in the presence (black) and absence of 200 μM PTX. The time point of GABA puffing is indicated by an arrow. ***I***, The averaged GABA currents in WT and *dnlg4* mutant l-LNvs. The number of l-LNvs for each genotype and condition are indicated. $^*p < 0.001$ for WT versus *dnlg4* mutant l-LNvs.

DNlg4 regulates RDL clustering through associations with RDL *in vivo*

RDL puncta in l-LNv varicosities represent synaptic inputs to l-LNvs (Chung et al., 2009). To further reveal how DNlg4 regulates GABA transmission in l-LNvs, we first examined the number of varicosities formed by l-LNvs in the optic lobe. Dendrite branches of LNvs were labeled with mCD8-GFP (membrane-tethered GFP), and varicosities were marked using anti-PDF staining. In wild-type flies, dendrite branches of l-LNvs regularly covered the optic lobe, with varicosities formed along each

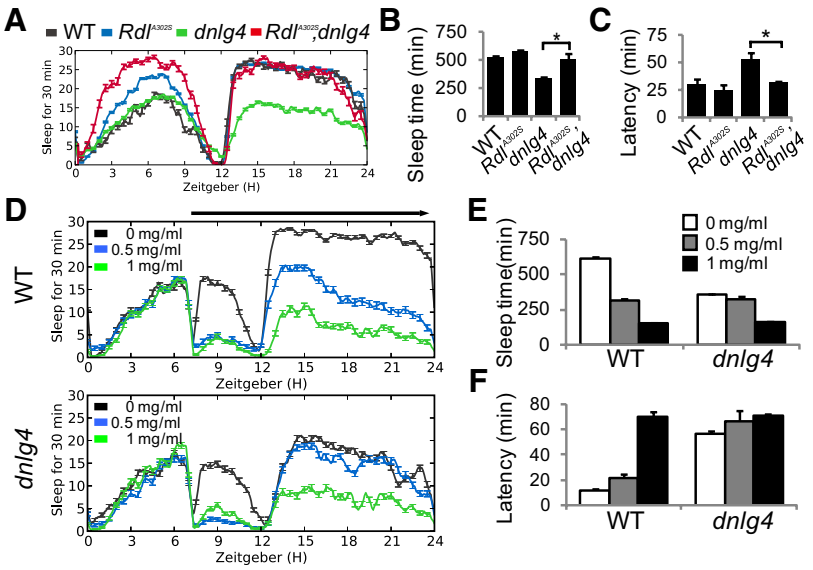

Figure 7. Impaired GABA transmission leads to defective sleep in *dnlg4* mutant flies. ***A***, Average sleep traces for wild-type (WT), *dnlg4*, Rdl^{A302S}, and Rdl^{A302S},*dnlg4* flies plotted as a 30 min moving average, $n = 32$. ***B***, ***C***, Quantification of total sleep time and sleep onset latency after lights off for each genotype; $n = 32$, $^*p < 0.001$ for *dnlg4* mutant versus Rdl^{A302S},*dnlg4* double mutant flies. ***D***, Effect of different CBZ concentrations on the sleep pattern of WT (top) and *dnlg4* mutant (bottom) flies during the first day of drug treatment: $n = 32$; blue, 0.5 mg/ml; green, 1 mg/ml. Black arrow at top of graph indicates CBZ application. ***E***, ***F***, Quantification of total sleep time and sleep onset latency after treatment with different CBZ concentrations, $n = 32$.

branch (Fig. 8*A*). In contrast, in *dnlg4* mutant flies, l-LNv dendrite branches were distributed in a disordered fashion (Fig. 8*A*). Furthermore, *dnlg4* mutant flies had a fewer varicosities in the optic lobe compared with wild-type flies (Fig. 8*A*,*B*).

Next, we investigated the distribution and amount of RDLs in l-LNv terminals. Due to a lack of specific anti-RDL antibody, we expressed HA-tagged RDL under the *pdf*-Gal4 driver to mimic endogenous RDL. Expression of RDL-HA existed in most l-LNv varicosities in wild-type flies but was present in only ~73% of varicosities in *dnlg4* mutant flies (Fig. 8*C*,*D*). Furthermore, in *dnlg4* mutants, RDL-HA signals were sharply reduced in each varicosity as a result of their accumulation in l-LNv somata (Fig. 8*C*,*E*,*F*). These results suggest that DNlg4 is required for RDL clustering in l-LNvs. As DNlg4 and RDL are similarly distributed in l-LNvs, it is possible that DNlg4 regulates RDL clustering through associations with RDL. To test this possibility, we performed immunoprecipitation experiments and demonstrated that DNlg4 associates with RDL *in vivo* (Fig. 8*G*).

Discussion

Sleep is an essential and evolutionarily conserved behavior. Mounting evidence suggests that sleep in a diverse array of animals is regulated by conserved molecular mechanisms that have not yet been identified. Here, we demonstrated that DNlg4 regulates sleep through modulating $GABA_A$ receptor RDL clustering and GABA transmission. This study provides the first evidence that DNlg4 modulates the function of inhibitory synapses and regulates sleep in *Drosophila*.

Expression of DNlg4 in LNvs

Immunostaining revealed that DNlg4 was highly expressed in l-LNvs. Previous genechip data showed that *dnlg4* mRNA levels oscillate in l-LNvs (Kula-Eversole et al., 2010). As *dnlg4* mRNA levels in l-LNvs are significantly reduced in per^{01} mutant flies compared with wild-type flies (Kula-Eversole et al., 2010), the cycling of *dnlg4* mRNA might be under circadian control. Indeed, we found seven E-box elements in the promoter region of the *dnlg4* gene. Recent research showed that the expression profile of Nlg1 oscillates in mice and that the clock transcription factor, BMAL1 and CLOCK, can bind to one E-box located 653 bp before the Nlg1 transcription start site (El Helou et al., 2013). Considering that *dnlg4* mRNA levels oscillate in l-LNvs, DNlg4 protein in l-LNvs might undergo rapid synthesis and degradation similar to these clock genes. The present study provides two lines of evidence to support this speculation. First, we found that DNlg4 colocalized with ER/Golgi-export machinery in l-LNv somata, suggesting that DNlg4 is constantly synthesized and transported. Second, DNlg4 protein level was reduced in RNAi flies after turning the RNAi effect on for 24 h, suggesting that DNlg4 is not stable and may undergo rapid degradation in l-LNvs. These processes of DNlg4 synthesis and degradation in l-LNvs are worthy of further investigation.

Our immunostaining reveals that DNlg4 was also expressed in fan-shaped body neurons and some unidentified neurons. In our rescue experiments, expression of *UAS-DNlg4* with either *c929*-Gal4 or *pdf*-Gal4 drivers only partially rescued the sleep defects in *dnlg4* mutant flies, especially during early evening sleep. This result is consistent with our observation of a progressive delay in sleep onset after turning on RNAi effects in *UAS-dnlg4-RNAi;tubulin-Gal80^{ts}/pdf-Gal4* flies. A similar increase in wakefulness during the early night has been observed in l-LNv hyperexcited flies (Parisky et al., 2008), suggesting that persistent l-LNv firing increases wakefulness but that the effects are larger at the beginning than at the end of night. Therefore, sleep circuits downstream of l-LNvs and unknown wake-promoting neurons may be gated differentially over the course of the night.

Although fan-shaped body neurons function to promote sleep in *Drosophila* (Joiner et al., 2006; Pitman et al., 2006; Donlea et al., 2011; Ueno et al., 2012), we found no effects of manipulating DNlg4 expression level in fan-shaped body neurons on sleep. One explanation is that GABA signaling in the fan-shaped body may have a minor role in promoting sleep. Indeed, fan-shaped body neurons only moderately express $GABA_A$ receptors (Parisky et al., 2008), and dopaminergic signals to the dorsal fan-shaped body have been found to promote arousal in *Drosophila* (Liu et al., 2012; Ueno et al., 2012). Given that DNlg4 is also expressed in some unidentified neurons, we cannot exclude the possibility that these neurons may have a role in sleep. To fully address these questions, unidentified DNlg4-positive neurons should be characterized and their potential roles in sleep regulation should be directly investigated.

Neuroligins regulate sleep in *Drosophila*

Our anatomically restricted manipulations of DNlg4 lead us to conclude that its expression within l-LNvs is essential for normal sleep, which is further supported by our immunostaining results. l-LNvs but not s-LNvs mediate light-driven arousal through the release of PDF neuropeptide (Parisky et al., 2008; Sheeba et al., 2008b), which acts as an arousal-promoting molecule with a

function similar to orexin in mammals (Chemelli et al., 1999; Lin et al., 1999). PDF release is controlled by l-LNv activity, with higher firing rates during the day and lower firing rates at night (Sheeba et al., 2008a). Electrophysiological recordings revealed that *dnlg4* mutant l-LNvs showed normal firing rates during the day but maintained daytime firing rates at night, consistent with a previous study showing that LNv-hyperexcited flies display increased arousal and decreased sleep, especially at night (Sheeba et al., 2008b). Thus, alterations of l-LNv activity correlate with changes in sleep behavior in *dnlg4* mutant flies.

GABA is an inhibitory neurotransmitter that is thought to promote sleep in mammals (Pace-Schott and Hobson, 2002). Here, we provide solid evidence that GABA transmission in l-LNvs is impaired in *dnlg4* mutant flies. l-LNvs are light-activated neurons (Sheeba et al., 2008a; Fogle et al., 2011), with activity suppressed at night by GABA signaling (Shang et al., 2008; Sheeba et al., 2008b; Sehgal and Mignot, 2011). Therefore, impaired GABA transmission results in l-LNvs exhibiting daytime firing rates at night, leading to defective sleep in *dnlg4* mutant flies. The $GABA_A$ receptor RDL is expressed in l-LNvs (Parisky et al., 2008; Chung et al., 2009), and the number of RDLs appears to be important for sleep regulation. Downregulation of RDLs in LNvs decreases total night sleep time and increases sleep onset latency (Parisky et al., 2008; Chung et al., 2009). Conversely, overexpression of RDLs specifically in PDF neurons increases total sleep time and increases sleep onset latency (Parisky et al., 2008; Chung et al., 2009). Here, we showed that RDL density in l-LNv terminals was reduced in *dnlg4* mutants. Therefore, impaired GABA transmission in *dnlg4* mutants appears to be due to a reduced RDL density in l-LNv terminals.

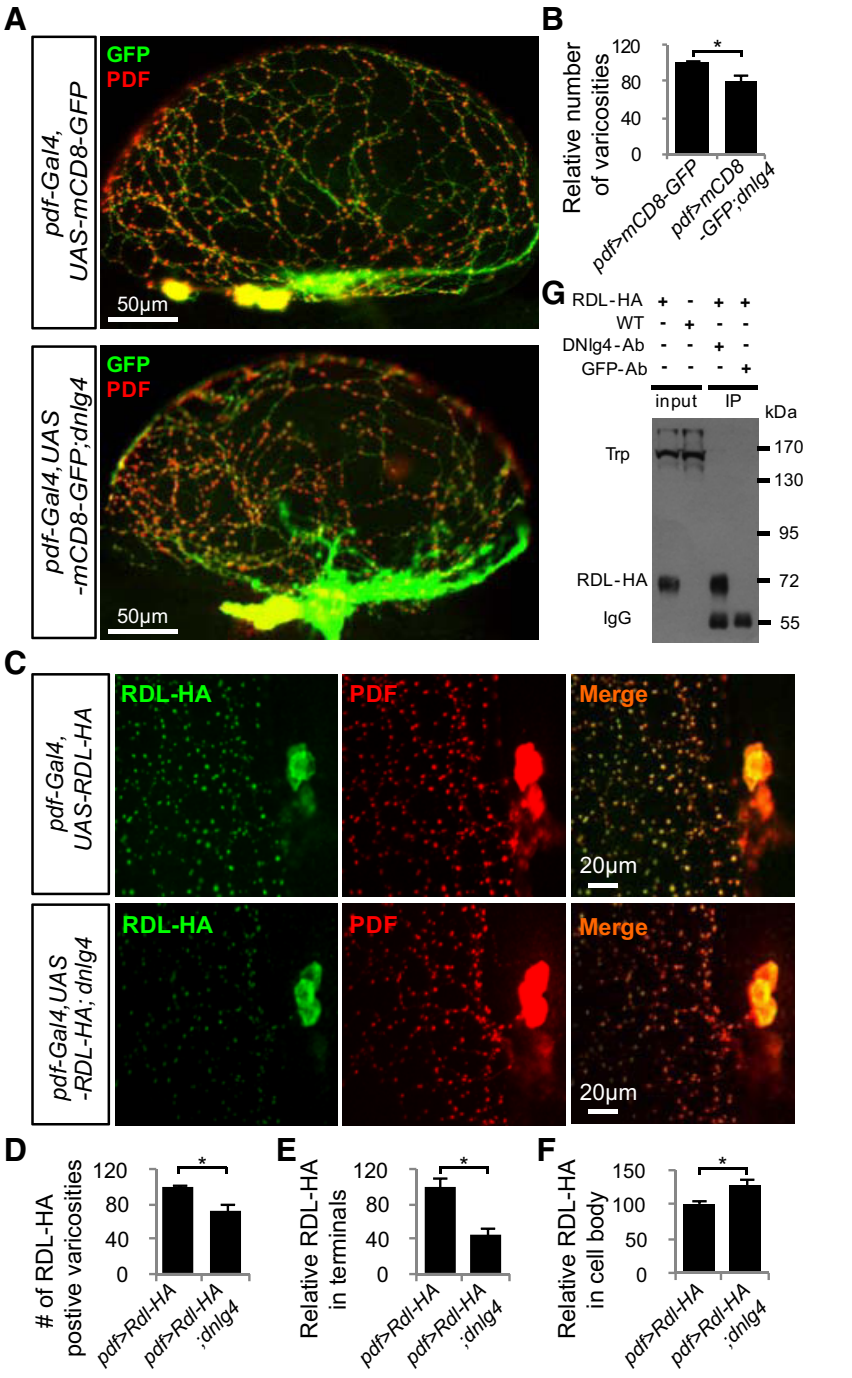

Figure 8. *dnlg4* mutant flies exhibit reduced RDL clustering in l-LNvs, and DNlg4 associates with RDLs. ***A***, Representative images showing the distribution of dendrite branches and number of varicosities in l-LNvs for wild-type (WT) and *dnlg4* mutant flies. l-LNvs membranes were labeled with mCD8-GFP under the control of the *pdf*-Gal4 driver. Dissected whole brains were stained with anti-GFP (green) and anti-PDF (red) antibodies. Scale bar, 50 μm. ***B***, Quantification of the number of l-LNv varicosities. Eight images were used for quantification for WT and *dnlg4* mutant flies. Each image was taken from a different fly. ***C***, Distribution of RDL-HA in l-LNvs neurons. RDL-HA was expressed in PDF neurons under the control of the pdf-Gal4 driver. Dissected whole brains were stained with anti-HA (green) and anti-PDF (red) antibodies. Scale bar, 20 μm. ***D***, Quantification of the number of RDL-HA-positive varicosities in l-LNv neurons. ***E***, Quantification of average RDL-HA protein amount in RDL-HA-positive varicosities in l-LNvs. ***F***, Quantification of RDL-HA protein amount in l-LNv somata. ***C–F***, Six images were used for quantification for WT and *dnlg4* mutant flies. Each image was taken from a different fly. ***G***, Co-immunoprecipitation of DNlg4 and Rdl *in vivo*. The precipitates and a portion (1% of the input) of the head extracts were subjected to Western blotting with anti-HA or anti-TRP (negative control) antibodies.

All Nlg homologs are expressed in the hypothalamus of rodents and primates (Varoqueaux et al., 2004, 2006; Mungenast and Ojeda, 2005). The evolutionarily conserved sleep regulatory machinery (Sehgal and Mignot, 2011) and molecular functions of Nlgs across different species (Varoqueaux et al., 2006; Szatmari et al., 2007; Banovic et al., 2010; Sun et al., 2011) suggest that mammalian Nlgs may play an important role in sleep regulation. A recent study showed that *nlg1* knock-out mice are not able to sustain wakefulness and spend more time in nonrapid eye movement sleep than wild-type mice (El Helou et al., 2013). Our studies further demonstrate that DNlg4 regulates night sleep through modulating GABA transmission. In mammals, inhibitory GABAergic neurons, which are located in the ventrolateral preoptic area of the hypothalamus, are involved in sleep (Sehgal and Mignot, 2011). Thus, we suspect that Nlg2 may also regulate sleep in mammals. Furthermore, based on its role in

regulating $GABA_A$ receptor clustering, DNlg4 may be functionally equivalent to Nlg2 in mammals.

DNlg4 modulates RDL clustering

Nlgs are postsynaptic cell adhesion molecules that are thought to function in synaptogenesis. Both *dnlg1* and *dnlg2* mutants exhibit a severe reduction in bouton number at neuromuscular junctions (Banovic et al., 2010; Sun et al., 2011; Chen et al., 2012). Using mCD8-GFP and anti-PDF labeling, we showed that the number of LNv varicosities was reduced in *dnlg4* mutant flies compared with wild-type flies. RDL puncta in l-LNv varicosities has been shown to represent synaptic inputs to LNv dendrites (Chung et al., 2009). Here, we found that HA-tagged RDL puncta appeared in most l-LNv varicosities in wild-type flies but in only some varicosities in *dnlg4* mutant flies. These results provide solid evidence that DNlg4 is essential for the number of GABAergic synapse in l-LNvs.

In addition to synaptogenesis, Nlgs also play essential roles in synapse maturation through regulating postsynaptic protein assembly. In mammals, Nlg1 determines the number of functional NMDARs at glutamatergic synapses (Wittenmayer et al., 2009). In the mouse retina, the absence of Nlg2 results in a severe reduction of $GABA_A$ receptor clustering (Hoon et al., 2009). In *Drosophila*, both DNlg1 and DNlg2 promote the accumulation of postsynaptic GluRs at neuromuscular terminals (Banovic et al., 2010; Sun et al., 2011; Chen et al., 2012). The present study shows that RDL clustering is impaired in *dnlg4* mutant flies and that DNlg4 associates with RDL *in vivo*, suggesting that DNlg4 modulates RDL clustering through associating with RDLs.

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