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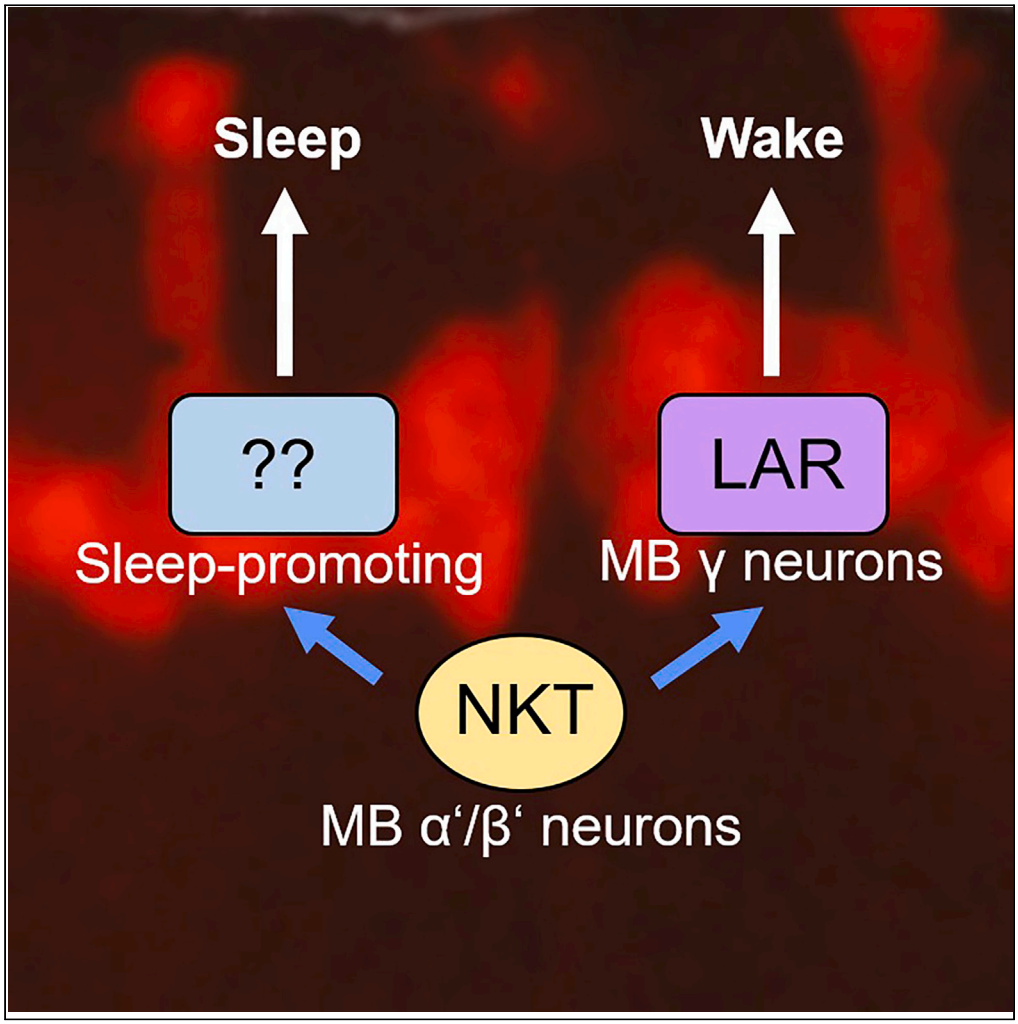

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Highlights

Ig-domain protein NKT is secreted from MB α′/β′ cells to regulate night sleep

Broad membrane-tethered NKT expression reduces sleep, similar to NKT knockout

Lar is a candidate receptor for NKT, based on behavioral results

NKT may promote both sleep and wakefulness, the latter via the MB γ neurons

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Drosophila noktochor regulates night sleep via a local mushroom body circuit

Isabelle R. Draper,[1,2,*] Mary A. Roberts,[1] Matthew Gailloud,[1,3] and F. Rob Jackson[1,4,*]

SUMMARY

We show that a sleep-regulating, Ig-domain protein (NKT) is secreted from *Drosophila* mushroom body (MB) α′/β′ neurons to act locally on other MB cell types. Pan-neuronal or broad MB expression of membrane-tethered NKT (tNkt) protein reduced sleep, like that of an NKT null mutant, suggesting blockade of a receptor mediating endogenous NKT action. In contrast, expression in neurons requiring NKT (the MB α′/β′ cells), or non-MB sleep-regulating centers, did not reduce night sleep, indicating the presence of a local MB sleep-regulating circuit consisting of communicating neural subtypes. We suggest that the leucocyte-antigen-related like (Lar) transmembrane receptor may mediate NKT action. Knockdown or overexpression of Lar in the MB increased or decreased sleep, respectively, indicating the receptor promotes wakefulness. Surprisingly, selective expression of tNkt or knockdown of *Lar* in MB wake-promoting cells increased rather than decreased sleep, suggesting that NKT acts on wake- as well as sleep-promoting cell types to regulate sleep.

INTRODUCTION

In both mammals and insects, neuronal circuits, glial cells, and distinct molecular mechanisms cooperate to determine the timing, amount, and quality of sleep.[1–5] In all species, including the fruit fly *Drosophila melanogaster*, circadian and homeostatic mechanisms shape the rhythmic sleep profile. In flies and other insects, specific neural circuits including those of the mushroom body (MB),[6–9] central complex (ellipsoid/fan-shaped bodies),[10–14] and others[3] regulate fly sleep, wakefulness, and homeostasis, with an important contribution of dopaminergic circuits in the control of arousal.[12,15,16] Importantly, there is conservation of sleep properties and mechanisms in flies and mammals. Similar to mammals, flies exhibit rebound sleep (exhibit homeostasis) after deprivation,[17,18] there is hormonal modulation of sleep (involving endocrine tissues, insulin-like peptides, and ecdysone),[19–22] glial cells modulate sleep,[2,5] there are sleep-dependent effects on metabolism,[23] and neuronal homeostatic synaptic downscaling (reduced synaptic proteins and connectivity) is observed during fly sleep.[24,25] Multiple sleep states (lighter and deeper sleep)—once thought to be a property only of vertebrate sleep—have also been observed in *Drosophila* using local field potential (LFP) recording.[26] LFP recording has also been used to document slow-wave oscillations in the fly brain during sleep,[27] analogous to those seen by electroencephalogram in mammals; these oscillations can be induced by genetic activation of the fly fan-shaped body and are required for normal sleep quality.

In recent studies, circuits regulating fly sleep have been described. It has been shown, for example, that diurnal changes in slow-wave oscillations occur in sleep-regulating R5 neurons of the ellipsoid body (EB) with peak power correlated with sleep onset.[28] These single-unit oscillations are synchronized by activation of circadian circuits via secondary tubercular-bulbar (TuBu) neurons and NMDA receptor-mediated mechanisms, and their synchronization is necessary for normal sleep.[28] Other groups have shown that DN1 clock neurons project to TuBu neurons which, in turn, innervate the EB to modulate sleep.[29,30] In addition, circadian control of daytime wakefulness is mediated by neuronal modulation of dopaminergic neurons (PPM3 cells) via pigment-dispersing hormone (PDF)-dependent mechanisms.[31] Of related interest, it was recently shown that a similar circuit composed of "morning" and "evening" clock neurons upstream of PPM3 and EB neurons drives neuronal excitability rhythms that are essential for normal circadian bouts of morning and evening locomotor activity.[32]

Although some of the circuits and intercellular signaling mechanisms regulating sleep have been elucidated, there is still much to be learned about the circuits that modulate the timing and properties of sleep (e.g., day versus night sleep, sleep duration, etc.). We recently described a gene called *Noktochor* (*Nkt*) that is predicted to encode a small, secreted Ig domain-containing protein that selectively regulates night sleep. *Nkt* is required in both astrocytes and a specific class of MB wake-promoting neurons (the α′/β′ neurons) for normal sleep.[33] In this report, we demonstrate that NKT protein can be secreted from the MB neurons that require its function. To identify neurons with putative receptors that respond to NKT (secreted from either neurons or astrocytes), we expressed membrane-tethered forms of NKT (tNkt)[34] in different groups of sleep-regulating neurons. Pan-neuronal expression of tNkt resulted in a night sleep phenotype similar to that of *Nkt*

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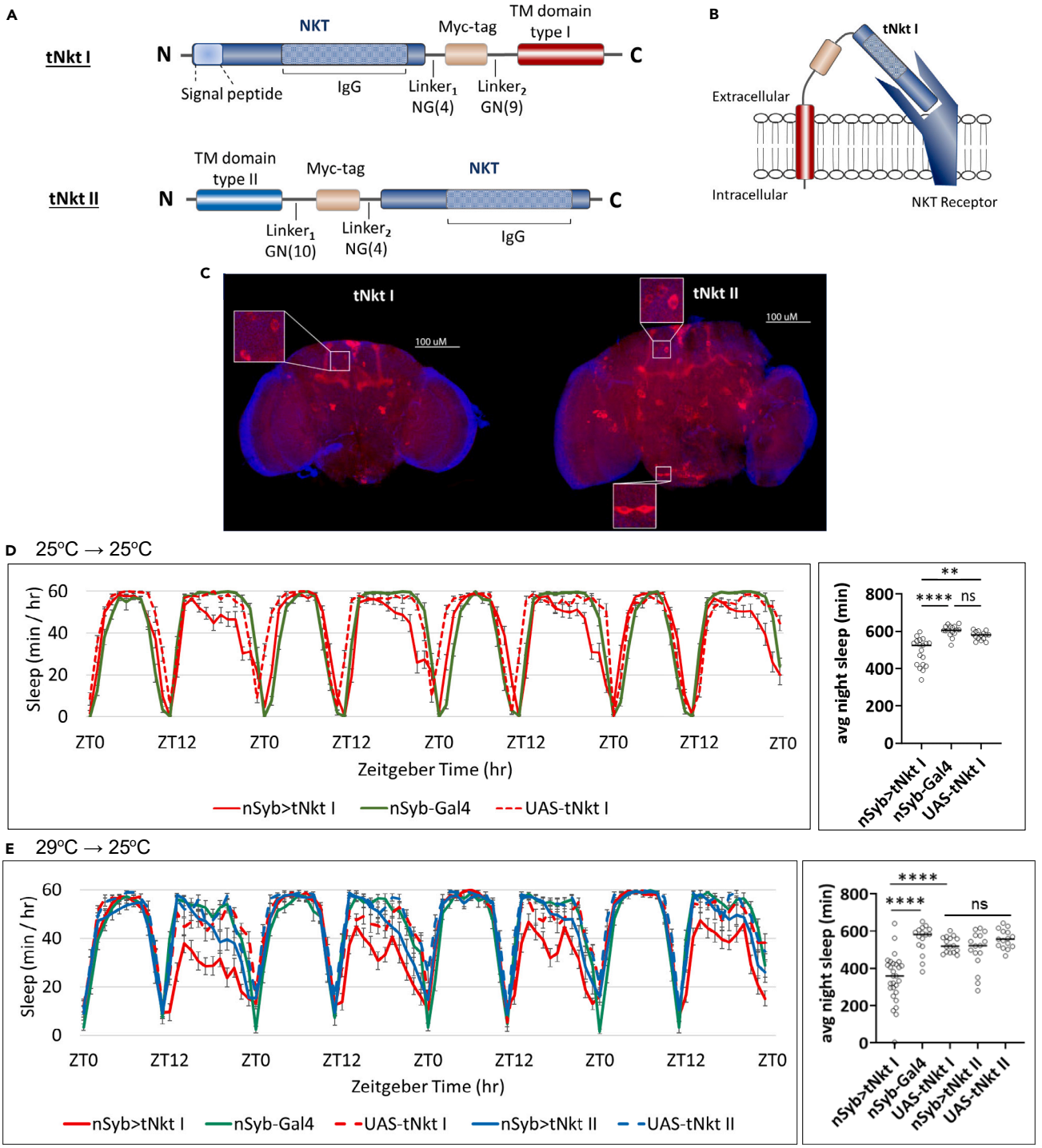

Figure 1. Pan-neuronal expression of tNkt I leads to reduced night sleep

(A) Cartoon representing the membrane-tethered Nkts (tNkt). In the type I conformation (tNkt I), the tethered ligand transgene encodes the full-length NKT (including the signal peptide MQVKCLIVCLALGLLMLATPVC, and the IgG domain), two GN-repeat linker peptides, one Myc tag, and the transmembrane (TM) domain type I from VHS: ALCWVGIGIGVLAAGVLVVTAIVYVV. In the type II conformation (tNkt II), the protein product lacks the signal peptide and includes the TM domain from TNF-α amino acid 1–56.

(B) tNkt is expected to act on cells that express a cognate receptor.

(C) Immunohistochemical analysis of *Drosophila* brains that express the UAS-tNkt I, or UAS-tNkt II, transgenes under the control of the pan-neuronal Gal4 driver nSyb-Gal4. Monoclonal anti-Myc antibody (1:100 dilution, Santa Cruz Biotechnology) was employed to detect the Myc-tagged transgenes.

Figure 1. *Continued*

(D) Population sleep profiles (days 1–4) for flies in light-dark (LD) 12:12 at 25°C using pan-neuronal nSyb-Gal4 (nSyb) to drive expression of tNkt I (genotype: nSyb>tNkt I vs. control siblings nSyb-Gal4 single transgene), with corresponding night sleep parameter: the scatterplots represent averages for 4 days of LD data. (E) The sleep phenotype of nSyb >tNkt I flies was exacerbated when flies are raised at 29°C (the efficacy of the Gal4/UAS binary system is enhanced). Control flies were UAS-, or Gal4-, single transgene-bearing flies. Scatterplots represent averages for 4 days of LD data. The data in D and E were not normally distributed and statistics were performed using the Kruskal-Wallis nonparametric ANOVA with Dunn's multiple comparisons test. ns, not significant, ****, $p < 0.0001$. n = 5 replicates at 25°C and one at 29°C.

knockdown flies or null mutants. Similarly, broad expression in the fly MB reduced night sleep whereas expression in the MB neurons that require endogenous NKT (the α′/β′ neurons) or sleep-regulating cell types in other brain regions had no effect on night sleep. These results suggest that both astrocyte and neuronal NKT act with the MB and that MB neurons communicate with one another via a local circuit composed of NKT-secreting cells and NKT-responding neurons. We demonstrate that leucocyte-antigen-related-like (Lar)—a transmembrane receptor sharing Ig-domain homology with NKT—is required in MB γ neurons for normal sleep, suggesting it may serve as a receptor for NKT.

RESULTS

Generation of transgenic flies expressing tethered NKTs

In order to express a membrane-bound form of NKT (tethered Nkt or tNkt), we generated two recombinant transgenes encoding NKT protein that contained either a type I or a type II membrane-spanning domain (Figure 1A). The two constructs encode flipped orientations of NKT, i.e., either N terminus-free in the type I orientation or C terminus-free in the type II orientation.[35] The complete NKT protein sequence was included in either orientation, with the exception that a signal peptide was missing in tNkt II (Figure 1A; see STAR Methods). For each construct, five independent transgenic *Drosophila* lines were obtained. Expression of the tNkt *in vivo* is predicted to result in a membrane-bound ligand that will act on the same cell if a cognate receptor is expressed (Figure 1B). Such cell-autonomous effects of tethered peptide ligands have previously been reported.[34,36]

Neuronal expression of tNkt results in a night-sleep phenotype

We have reported that CRISPR null mutants of NKT have a selective decrease in night sleep due to changes in sleep bout duration and number (i.e., sleep is fragmented); an identical phenotype is observed with knockdown in astrocytes or neurons.[33] To identify the cell type(s) that modulate sleep in response to secreted NKT, we used the Gal4/UAS bipartite system to drive expression of tNkt I and II in multiple well-established sleep-regulating centers of the brain. Each of the two different tNkts contains sequences encoding a Myc epitope tag (see STAR Methods and Figures 1A and 1B), and expression of either using the nSyb-Gal4 driver resulted in pan-neuronal tNkt-Myc staining (Figure 1C). We note that Myc immunoreactivity was seen in the membranes of certain cell bodies, but it was largely observed as punctate signal throughout the brain, consistent with trafficking into neuronal processes. Indeed, signal was observed in the MB processes but not in Kenyon cell bodies. Of note, nSyb-Gal4-dependent expression of tNkt I resulted in decreased sleep during the second half of the subjective night (Figure 1D). Analysis of sleep parameters obtained with three biological replicates of nSyb-Gal4>UAS-tNkt I progeny indicated that night sleep was reduced and fragmented (i.e., shorter and more frequent sleep bouts) as seen in *Nkt* mutants (Figures 1D and S1A). nSyb-Gal4>UAS-tNkt II progeny that expressed a type II membrane-tethered Nkt also exhibited slightly decreased night sleep (Figure S2A). When reared at 29°C to enhance the activity of Gal4, nSyb-Gal4>tNkt I flies displayed a more pronounced sleep phenotype (Figure 1E), very much like that of *Nkt* knockdown or CRISPR mutant flies.[33] At higher temperature, the tNkt orientation-specific effects were also more pronounced; the type I orientation (tNkt I) had a large effect whereas the type II orientation (tNkt II) had a minimal effect on sleep (Figure 1E). This is an expected result as there are often orientation-specific effects with tethered proteins.[35] At 25°C, the nighttime waking activity of nSyb>tNkt I flies was in the normal range, similar to *Nkt* null flies,[33] excluding effects on locomotion that might resemble sleep (nSyb>tNkt I: 2.6 counts/min, nSyb-Gal4: 2.9 counts/min, UAS-tNkt I: 2.7 counts/min).

A putative NKT receptor is present in non-α′/β′ neurons of the MB

To identify the specific neurons mediating tNkt action, we used driver fly lines that express Gal4 in most of the known sleep-regulating neurons (Table 1; Figure 2A). Our screen led to the identification of the MB as the main locus underlying the tNkt I-induced sleep phenotype. Although expression with 238Y-Gal4 did not affect sleep, decreased sleep during the subjective night was obtained with two broad MB drivers: dnc-Gal4 (R13F02-Gal4) and OK107-Gal4 (Figure 2; data not shown for OK107-Gal4). Dnc-Gal4>UAS-tNkt I progeny exhibited broad expression of Myc-tagged tNkt I in MB processes (Figure 2B) and displayed a sleep phenotype similar to that observed with NKT knockdown or knockout (Figures 2C and 2D), although sleep fragmentation was not obvious with this driver (Figure S1C). Dnc-Gal4-dependent expression of tNkt II (i.e., NKT in a flipped orientation, Figure 1A) had no effect on night sleep (Figures 2D and S2B). The similar phenotypes of tNkt I-expressing and *Nkt* null flies are consistent with the idea that the tether blocks activation of sleep-promoting neurons by endogenous NKT, perhaps by interfering with ligand binding or downstream receptor-signaling activity.

For the most part, expression of tNkt I in other known sleep-regulating neurons did not affect the amount of night sleep (Table 1; Figure 2C). For example, expression of tNkt I in PDF neurons, the fan-shaped body or neurons innervating the MB (DPM, APL, and dopaminergic neurons) did not influence night sleep (Table 1). We note, however, that expression of tNkt I with an EB driver (c346) did cause increased day

Table 1. Night sleep analysis of *Drosophila* expressing tethered NKT I (tNkt I) in distinct neuronal populations

Gal4 driver	Cell Expression of tNkt I	Night Sleep
nSyb	Pan neuronal	**Decreased**
PDF	PDF secreting	Normal
OK107	MB, broad	**Decreased**
R13F02/*dnc*	MB, broad	**Decreased**
238Y	MB, strong in α/β	Normal
R35B12	MB α′β′ wake-promoting neurons	Normal
C305	MB α′β′ wake-promoting neurons	Normal
R55C10/*DAT*	MB α′β′ wake-promoting and dopaminergic neurons	Normal
104Y	Fan-shaped body	Normal
Crc929	Dimm neurosecretory cells	Normal
AstA	Allatostatin A neurons (regulate AKH and DILP)	Normal
VT64246	Dorsal paired medium (DPM) GABAergic neurons	Normal
C316	Dorsal paired medium (DPM) GABAergic neurons	Normal
C346	Ellipsoid body	**Increased**
GH146	Anterior paired lateral (APL) GABAergic neurons	Normal

A series of Gal4 drivers was used to target transgene expression in a range of established sleep-regulating neuronal loci.

sleep and statistically increased night sleep (Figures 2C and S1B). Although night sleep was only slightly increased with this driver, night bout number was decreased and night bout length was increased, the opposite of what we observed with pan-neuronal expression of tNkt I. While this may indicate a requirement for NKT in the EB, we know from a previous study that pan-neuronal, Gal4-driven Nkt RNAi expression results in significantly decreased night sleep that can be rescued by inhibiting Gal4 only in the MB.[33] In contrast, that result suggests a MB-specific function for the secreted protein. Thus, we have not followed up on effects with the c346 driver.

Notably, expression using c35B12-Gal4, c305-Gal4, or R55C10-Gal4, all of which express in α′/β′ cells of the MB did not affect sleep (Table 1; Figure 2C). This is of interest as we previously showed that *Nkt* is required in the MB α′/β′ cells[33] using the same Gal4 drivers. Altogether, our results are consistent with a non-cell-autonomous action of NKT; i.e., the protein is released from the α′/β′ neurons to act on a different cell type of the MB.

Endogenous *Nkt* expression is required in the adult brain for normal night sleep.[33] Thus, we asked whether tethered NKT acted in the adult brain to regulate sleep. Using a tub-Gal80^{ts} transgene along with the dnc-Gal4 driver, we performed a conditional expression experiment with tNkt I. Experimental and control flies were reared at 25°C and then shifted to 29°C to monitor sleep. The results showed clearly that tNkt I expression only in adulthood results in reduced night sleep, compared to genotypic controls, predominantly in the second half of the night (Figure S3A). Note that two replicates are shown for experimental populations. In contrast, there was no significant effect on night sleep when flies remained at 25°C after being reared at that temperature (Figure S3B). This is an important result as it suggests that the tether is acting in adulthood to regulate sleep, similar to endogenous *Nkt*.

In additional experiments, we attempted to map NKT-responding cells with better resolution using the Janelia HHMI split Gal4 collection,[37] with priority on lines in which the transcription activation domain of human p65 (i.e., Zip+p65AD) is under the control of the *dnc* gene promoter (Table 2). Although none of the split Gal4 lines led to an abnormal sleep profile that phenocopied that observed in dnc>tNkt I flies (i.e., loss of night sleep), MB009B-Gal4-driven expression of tNkt I resulted in a gain of sleep phenotype (Figure S4A). We note that MB009B-Gal4 alone reduced night sleep, but in two replicates, night sleep was increased in MB009B > tNkt I flies relative to the Gal4 control or MB009B-Gal4>tNkt II flies. It is of interest that the MB009B-Gal4 line targets the MB γ main (γm) and γ dorsal lobes,[38] and that γm is known to promote wakefulness.[8] An increased sleep phenotype with γ-neuron, tNkt I expression—which we postulate blocks endogenous NKT action—suggests that NKT may normally activate both wake- and sleep-promoting neurons to fine-tune the sleep profile. The absence of additional phenotypes (e.g., reduced night sleep) when using these split Gal4s may be due to an insufficient level of expression; alternatively, tNkt I action may need to occur in multiple cell types of the MB to produce an overall loss of night sleep.

NKT can be secreted *in vitro* and from MB α′/β′ neurons

Given the requirement for NKT-secreting and -responding cells in the MB, we wished to directly demonstrate that the protein can be secreted from the MB α′/β′ neurons. Initially, we showed that NKT can be secreted from cultured HEK293 cells. For those studies, we transfected plasmid-encoding FLAG-tagged NKT into HEK293 cells and used anti-FLAG immunoprecipitation methods to demonstrate secretion into the medium (see STAR Methods). As shown in Figure 3A, the majority of FLAG-tagged NKT, of the expected size class (~19 kDa), was present in culture medium (lane 1), consistent with secretion of the protein from the cells. Some tagged NKT remained in the cell lysate (lane 2), as

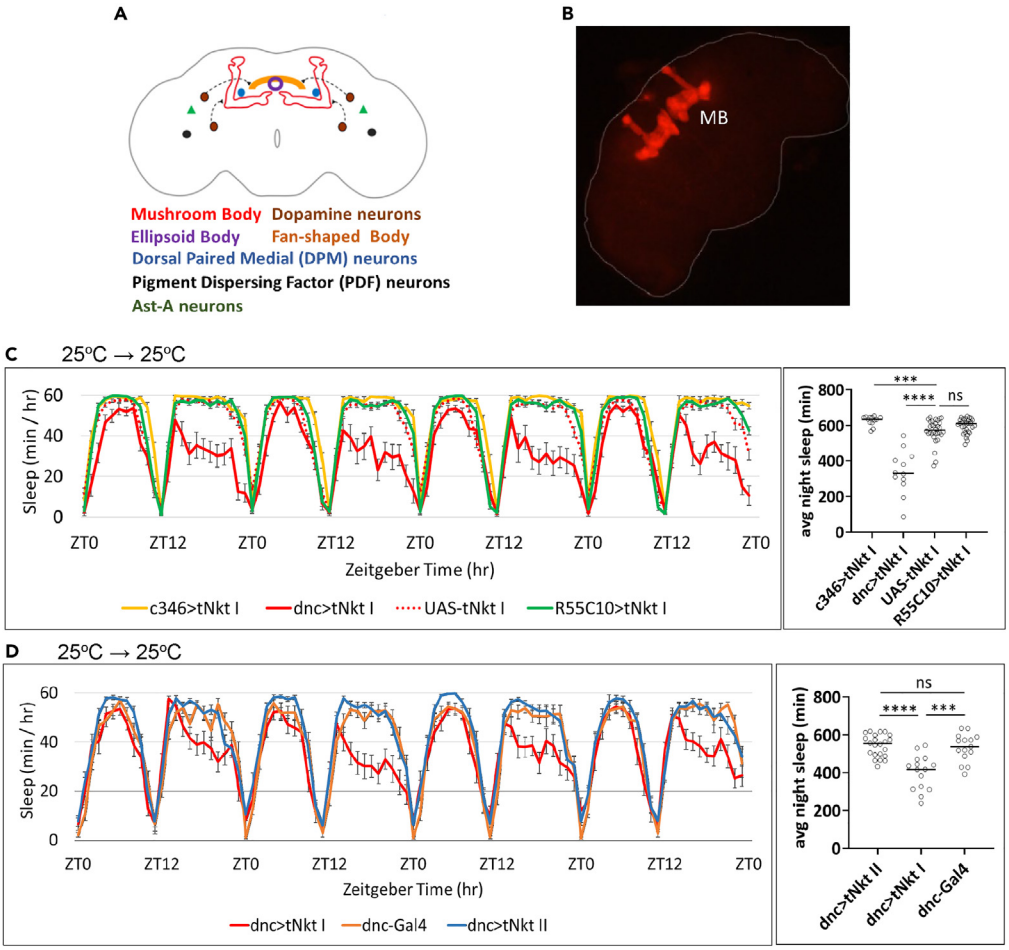

Figure 2. Expression of tNkt I under the control of the mushroom body driver dnc-Gal4 leads to reduced night sleep

(A) Schematic representation of *Drosophila* sleep-regulating neurons of the adult brain.

(B) Expression of Myc-tagged tNkt I in the MB of dnc-Gal4>UAS tNkt I flies.

(C) Population sleep profiles (days 1–4) for flies in light-dark (LD) 12:12 at 25°C. Loss of night sleep was seen when using the MB dnc driver, but not the ellipsoid body (EB) driver, or the R55C10 driver. tNkt I was backcrossed into w^{1118} background to generate control flies w^{1118}, tNkt I. Night sleep parameter of the corresponding genotypes, showing decreased night sleep when using the MB dnc driver; the dnc>tNkt I flies also displayed fragmented sleep, as reflected by the decreased bout length observed for this genotype (Figure S1).

(D) Population sleep profiles (days 1–4) for dnc>tNkt I, dnc>tNkt II and Gal4 control flies in LD 12:12 at 25°C, with corresponding night sleep scatterplots representing averages for 4 days of LD data. Statistical analysis of data shown in C and D, respectively, utilized a Dunn's multiple comparisons test and Tukey-Kramer multiple comparisons test. ns, not significant, ***, $p < 0.001$, ****, $p < 0.0001$. c346-Gal4 and R55C10-Gal4 drivers, n = 2 and 1 replicate, respectively. Dnc-Gal4 driver, n = 5 replicates (additional examples are shown in Figure S2B).

expected, but none was present in medium from cells lacking the NKT-FLAG plasmid (PCD media, lane 3). As expected, anti-FLAG signal was present in lane 4 (indicated by *), a positive control lane containing FLAG-tagged bacterial alkaline phosphatase. An independent replicate yielded similar results (not shown).

To demonstrate secretion from MB neurons, *in vivo*, we utilized a previously described UAS-Nkt-GFP strain and the R35B12-Gal4 driver, which expresses predominantly in the α′/β′ neurons of the MB, the cells that require NKT function for normal sleep. As in previous studies of secreted *Drosophila* proteins,[33,39–42] decreased fluorescence in these neurons was interpreted as secretion of NKT-GFP. As a negative control, we expressed soluble GFP in the same neurons, which is not expected to be secreted. As shown in Figures 3B and 3C, 50 mM KCl treatment of brains expressing NKT-GFP in these cells resulted in significantly reduced GFP fluorescence, relative to untreated brains, consistent with secretion of the protein. In contrast, there was no significant difference between treated and control brains that expressed soluble GFP alone. An independent replicate with 50 mM KCl showed similar results ($p = 0.003$ for experimental versus $p = 0.376$ for control). In addition, a replicate using 200 mM KCl (Figure 3D) also indicated significant secretion of the NKT-GFP. Given that tNkt expression in the α′/β′ neurons has no effect on sleep, these results support the hypothesis that NKT is secreted from the MB α′/β′ neurons to act non-autonomously on a different MB cell type.

Table 2. Results of sleep analysis with flies expressing tethered NKT I (tNkt I) in selected subsets of MB neurons, or MB output neurons, that can be targeted using split Gal4 drivers

Split Gal4 BL stock	Janelia Line	Expression pattern	Sleep analysis/tNkt I
68255	MB594B	MB αβc	Normal
68256	MB607B	MB γd	Normal
68265	MB131B	MB γ main	Normal
68267	MB185B	MB αβs	Normal
68383	MB371B	MB αβ	Normal
68292	MB009B	MB γ d, γ main	**Gain of sleep**
68293	MB010B	MB, all Kc, lower in γ main	Normal
68306	MB005B	MB α′β′ap, α′β′m	Normal
68319	MB370B	MB α′β′ap (mild), α′β′m	Normal
68283	MB077B	MBON α′1γ2	Normal
68294	MB011B	MBON 2α	Normal
68296	MB018B	MBON α′2	Normal
68301	MB027B	MBON α′3m3ap	Normal
68305	MB002B	MBON β′2mp	Normal

The transmembrane protein Plum is not required in dnc-expressing cells of the MB for sleep regulation

The Plum protein, a transmembrane Ig-domain protein was identified as a candidate interactor of NKT,[43] and it is expressed in the MB and other regions of the brain.[44] Thus, we wondered whether Plum might serve as a receptor for NKT within the MB. We employed the dnc-Gal4 driver to trigger either RNAi-mediated knockdown, or overexpression, of Plum, in MB neurons. However, Plum knockdown, using two different RNAi lines (see STAR Methods) had no significant effect on sleep (Figure 4A and data not shown). Similarly, Plum overexpression (not shown) in dnc-positive neurons did not recapitulate the loss of night sleep displayed by dnc-Gal4>UAS-tNkt I progeny. Thus, Plum is likely not required in the MB for sleep regulation. Conversely, RNAi-mediated knockdown of *Nkt* in dnc-Gal4-expressing neurons resulted in the expected night-sleep phenotype (Figure 4A).

Expression of tNkt I in Plum-positive cells phenocopies a Plum loss-of-function sleep phenotype

Although perhaps not relevant for the function of NKT in the MB, we nonetheless pursued analysis of Plum and a possible role for NKT as a ligand of Plum. Of interest, a Plum loss-of-function mutant, $Plum^{\Delta 1}$, exhibited an increased sleep phenotype (Figure 4B). To assess the extent to which tNkt might recapitulate or antagonize the Plum-mediated regulation of sleep, we used a Plum-Gal4 driver line available from a public repository. Plum-Gal4>UAS-tNkt I progeny displayed a significant increase in sleep, most notably at ZT12, when compared to control siblings (Figure 4C). In contrast, expression of tNkt II in the same cells had no effect (Figure 4C). The phenotypes of Plum-Gal4>UAS-tNkt I and $Plum^{\Delta 1}$ flies are comparable, suggesting that NKT may act in Plum-expressing cells to regulate sleep. However, the relevant Plum-containing cells must be distinct from the dnc-Gal4-expressing population as neither knockdown nor overexpression of Plum with this driver affected sleep. We postulate that Plum signaling normally positively regulates wakefulness, and that downregulation of Plum function (in mutants or by tNkt I) leads to a signaling deficiency and reduced wakefulness (i.e., increased sleep).

Lar is a candidate receptor for NKT in the MB

We identified Lar as a potential candidate receptor based on the use of the FlyBase CV term report function[45] and cross referencing of all plasma membrane proteins with expression in the adult male and female brains. This search identified 101 proteins; among these are seven immunoglobulin (Ig)-like domain proteins, including the synaptic adhesion molecule Lar). Lar has three extracellular N-terminal Ig domains that are evolutionarily conserved, and all have sequence similarity to the single Ig domain of NKT. This is significant as proteins with homologous Ig domains often serve as ligand-receptor pairs at synapses and other locations.[46,47] Also of interest, *Lar* is expressed in the α/β and γ lobes of the adult MB, with no detectable expression in the α′/β′ lobes.[48] This expression pattern is complementary to the known cellular requirement for NKT in the α′/β′ neurons of the MB and suggests that Lar might serve as a receptor for NKT. Notably, genome-wide association studies and mutant analysis have shown that both *Drosophila Lar* and its close mammalian ortholog *PTPRD* are associated with variations in sleep pattern.[49–52]

To examine a role for *Lar* in sleep regulation, we generated gene knockdown and overexpression flies. Knockdown of *Lar* using either the dnc or OK107 MB Gal4 driver resulted in an increase in night sleep (Figures 4E and S2C), a phenotype opposite to that observed with NKT deficits, but similar to that seen in MB009B-Gal4>tNkt I flies (Figure S4A). Indeed, OK107>Lar IR flies slept almost continuously at the end of night/beginning of morning (Figure S2C), such that locomotor activity was nearly absent during this interval; at ZT 0, for example, there were 28.0 ± 4.0 and 127.8 ± 13.0 activity events for OK107>Lar IR (n = 16) and UAS-Lar IR (n = 24) populations, respectively ($p < 0.0001$). Similarly,

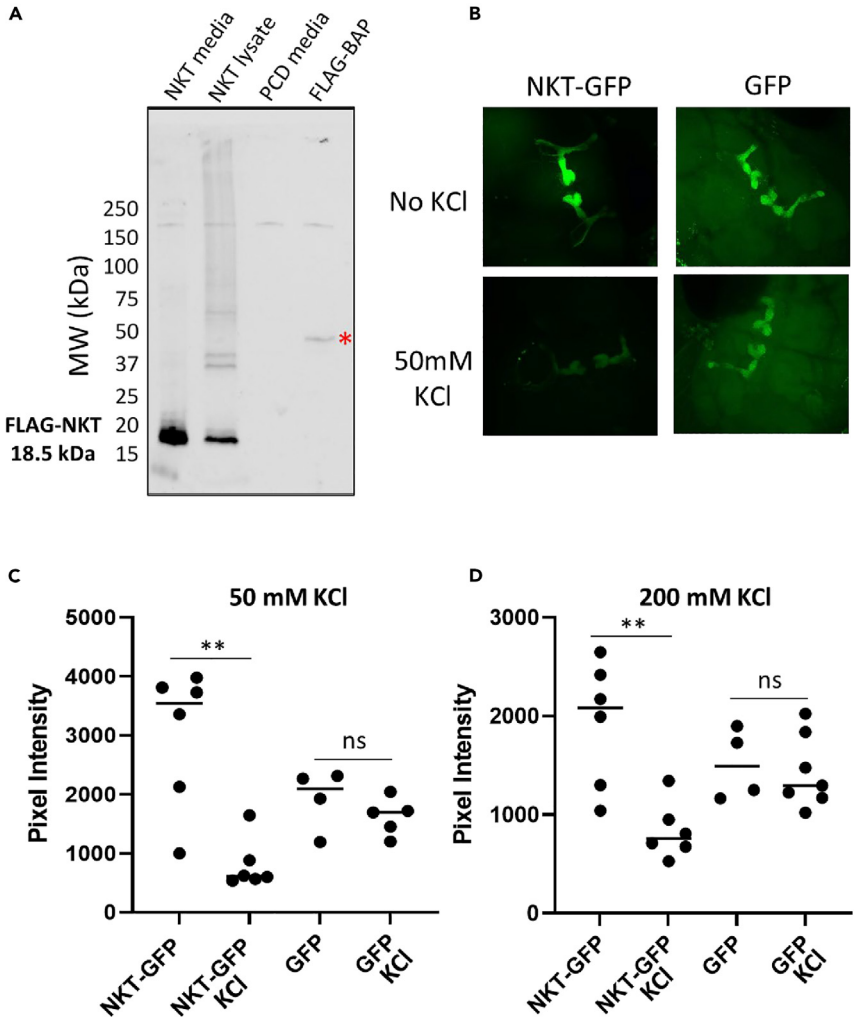

Figure 3. NKT is secreted *in vitro* from cultured cells and *in vivo* from mushroom body α′/β′ neurons

(A) Recombinant HEK293 cells that express FLAG-tagged NKT secrete NKT (~19 kDa) in the medium, as shown using anti-FLAG immunoprecipitation (lane 1). Residual tagged-NKT is detected in the cell lysate (lane 2), but none is present in the medium from cultured cells that do not express the NKT-FLAG plasmid (PCD media, lane 3). As expected, an anti-FLAG signal is present in positive control lane 4 that was loaded with FLAG-tagged bacterial alkaline phosphatase (FLAG-BAP, indicated by *).

(B) RB35B12-Gal4>UAS-NKT-GFP double transgenic flies show expected fluorescence in MB neurons (upper left panel). Decreased NKT-GFP fluorescence, an index of protein secretion, was observed following KCl-induced neuron depolarization (bottom left panel). No significant difference was observed between KCl-treated and non-treated brains that expressed soluble GFP alone (right panels).

(C and D) Quantification of corresponding GFP pixel signal intensity observed in treated, vs. non-treated brains. Student's *t* test was utilized to compare the "No KCl" and "KCl" treatment groups for GFP and NKT-GFP expression. ns, not significant, **, $p < 0.01$.

MB009B-Gal4-driven knockdown of *Lar* in γ neurons also significantly increased sleep, predominantly in the late night, relative to either UAS-Lar IR or MB009-Gal4 controls (Figure S4B), and in a manner very similar to that seen with dnc-Gal4 knockdown of Lar. In contrast, overexpression of *Lar*, using the dnc or OK107 Gal4 driver and a UAS-full length Lar transgene, resulted in reduced night sleep, similar to that seen in flies with NKT deficits (Figures 4D and S2C). All these results indicate a role for Lar in sleep regulation and are consistent with the known wake-promoting function of the MB γm neurons[8] (see Discussion). In addition to activating sleep-promoting cells, we suggest that endogenous NKT may also activate Lar (Figure 4F) in wake-promoting cells, as decreased Lar function—with tNkt I expression—is predicted to increase sleep (see Discussion).

DISCUSSION

In this report, we demonstrate that NKT is secreted, *in vivo*, from the MB cells that require its function. Broad expression of a tNkt protein in MB neurons reduced night sleep, similar to the effects of NKT knockdown or knockout. These results indicate that tNkt I expression is

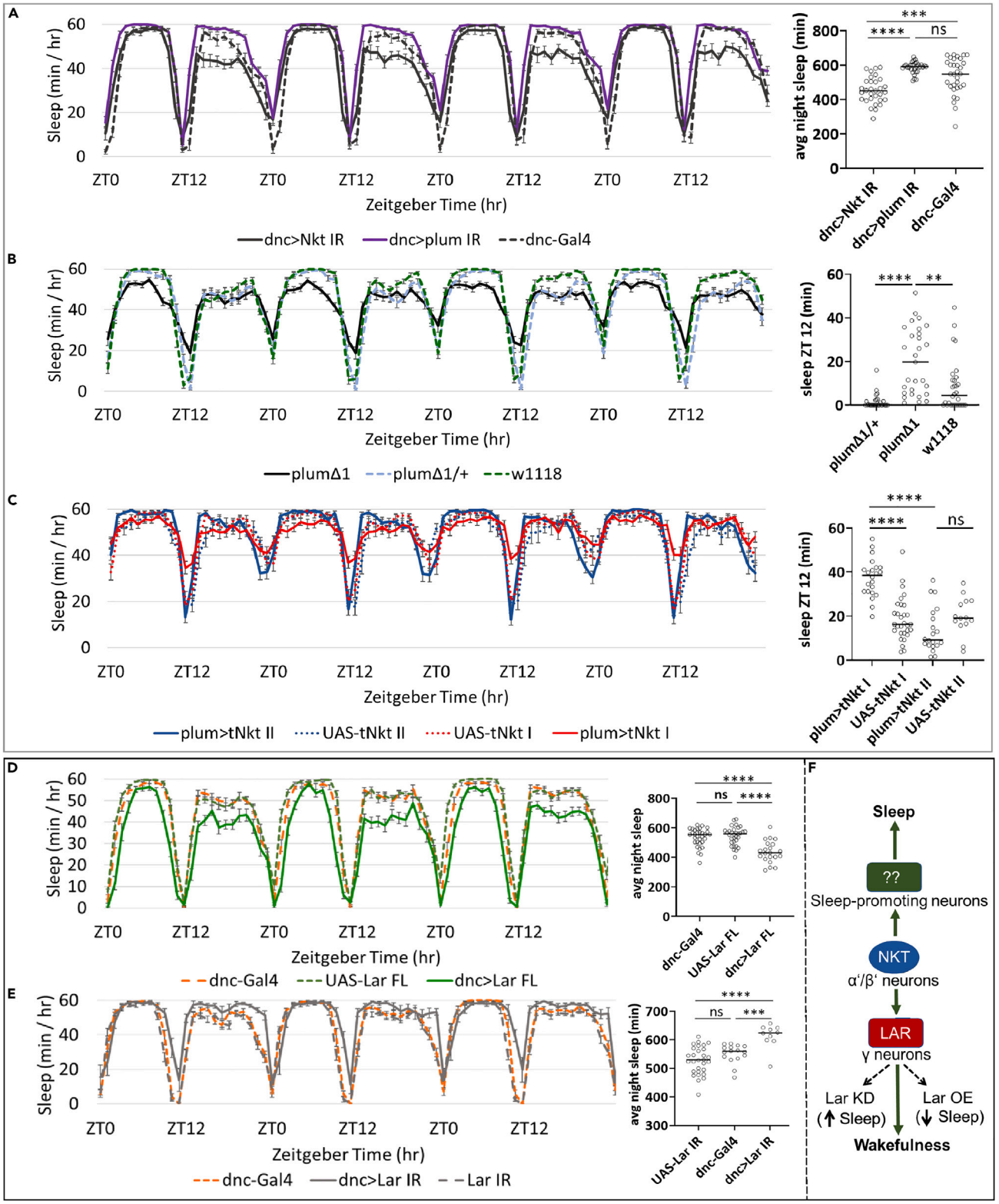

Figure 4. Genetic manipulation of candidate Nkt receptors identifies Lar as a sleep regulator in the MB

(A–E) Population sleep profiles (days 1–4) for flies in light-dark (LD) 12:12 at 25°C. Scatterplots of respective sleep parameters are also shown. (A) *Nkt* knockdown in dnc MB cells led to decreased night sleep. Plum knockdown in the same cells had no effect. Loss of night sleep was seen in flies with decreased Nkt (via RNAi),

Figure 4. *Continued*

using the dnc MB driver. Flies with decreased Plum in dnc cells, however, displayed normal sleep. Sleep parameters of the corresponding genotypes (control progeny is dnc-Gal4), showing decreased and fragmented night sleep in dnc>Nkt RNAi. (B) Expression of tNkt I in Plum-positive cells led to an abnormal sleep phenotype reminiscent of that observed in Plum loss-of-function mutants. Flies that expressed tNkt I under the control of the plum Gal4 driver exhibited increased sleep, most notable at the light on/off transition (quantified in corresponding night sleep plot). Expression of tNkt II in the same cells had no effect. The effect was observed at 25°C and 29°C. (C) $Plum^{\Delta 1}$ null mutant flies also displayed increased sleep (quantified in corresponding night sleep plot). n = 3 replicates. (D) Behavior of flies overexpressing Lar using the Lar.K FL construct described in FlyBase. Expression in the MB (dnc>Lar.K FL) caused decreased night sleep. (E) RNAi-mediated knockdown of Lar caused increased sleep. The UAS-Lar and dnc-Gal4 transgenes were backcrossed into the w^{1118} background to generate the corresponding control flies. The same effect was observed at 29°C. n = 3 replicates. (F) Model depicting the postulated action of secreted NKT on both sleep- and wake-promoting neurons. We suggest that NKT activates both types of neurons. Activation of wake-promoting cells may involve the Lar receptor as we have shown that knockdown (KD) of Lar in γ neurons results in increased sleep whereas overexpression (OE) of Lar in these cells reduces sleep. In addition, as NKT is also required in astrocytes, secretion from these cells must also play a role in the regulation of sleep. Statistics for A and B utilized Tukey-Kramer multiple comparisons test, whereas C, D, and E used Dunn's multiple comparisons test. ns, not significant, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

equivalent to loss of endogenous NKT, suggesting that the tethered construct likely decreases endogenous NKT action, either by blocking binding of the ligand or interfering with downstream receptor-signaling activity. Notably, expression of tNkt in sleep-regulating centers outside the MB did not mimic an *Nkt* null mutant. Given that NKT is required in both neurons and astrocytes,[33] our results suggest that NKT secreted from either cell type acts within the MB to regulate night sleep. Furthermore, tNkt expression in the cells requiring NKT (the MB α′/β′ Kenyon cells) also did not alter sleep, suggesting there is a local MB sleep-regulating circuit in which NKT-secreting neurons communicate with NKT-responding neurons. The mechanism regulating NKT secretion from the α′/β′ lobes is unknown, but it is possible that release of the protein is stimulated by changes in sleep pressure.

NKT acts in Plum-containing cells

As Ig-protein ligand-receptors pairs often share Ig-domain homology,[46,47] we examined two transmembrane proteins that share homology with NKT for roles in sleep regulation. One called Plum was identified in a co-immunoprecipitation screen as a potential interactor of NKT.[43] However, Plum knockdown in the MB, using dnc-Gal4, had no effect on night sleep, indicating that Plum does not regulate sleep in these MB cells. However, the $Plum^{\Delta 1}$ null mutant had increased sleep, as did Plum-Gal4>Nkt I flies. Together, this suggests that NKT may act on Plum-expressing cells that are not components of the dnc-Gal4 population to regulate sleep.

Lar is required in the MB for sleep regulation

Based on behavioral phenotypes, we suggest that a transmembrane receptor protein tyrosine phosphatase called Lar may mediate NKT action in the MB, although we have not shown direct binding between the two proteins. *Lar* is expressed in the α/β and γ lobes of the adult MB but not in the α′/β′ lobes,[48,53] which require NKT, consistent with a ligand-receptor relationship. Importantly, knockdown or overexpression of *Lar* in the MB increased or decreased sleep, respectively (Figures 4D and 4E), indicating that Lar normally promotes wakefulness. Using selective activation of MB subtypes, Sitaraman et al.[8] showed that the α′/β′ and γm lobes promote wakefulness, whereas there was no effect of α/β lobe activation on sleep. Given the expression pattern of *Lar* in the MB, this suggests that Lar-containing γm neurons might be a target of NKT. It will be of interest in a future study to ask whether there is direct binding of NKT to Lar. Additionally, Lar-Gal4 transgenic strains as well as existing split Gal4s can be employed to further dissect the requirement of Lar within the MB.

Loss of endogenous NKT or broad MB neuronal expression of tNkt I leads to decreased night sleep. Surprisingly, however, MB009B Gal4-driven expression of tNkt I or knockdown of Lar either broadly, using OK107-Gal4, or only in MB γ Kenyon cells (KCs), using MB009B-Gal4, actually increased sleep (Figure S4). This suggests that NKT normally activates the γm wake-promoting cells, with loss of ligand activity resulting in decreased wakefulness and increased sleep. We think that NKT must also activate sleep-promoting MB neurons—even though they were not identified using other split-Gal4 drivers—given that knockout of NKT or broad MB expression of tNkt I reduced sleep. Although there are alternative models, our results suggest a complex action of NKT on both sleep- and wake-promoting neurons that fine-tunes the sleep profile. In the complete absence of NKT, loss of sleep-promoting neuronal activity must dominate as a significant sleep deficit is observed. This may be due to interactions between sleep- and wake-promoting KC neurons or downstream processing of information in MB output neurons. Furthermore, NKT may act selectively on wake- and sleep-promoting neurons at different times of day as a consequence of changes in receptor activity or downstream signaling components that are driven by circadian mechanisms or sleep need. One intriguing idea is that NKT activation of Lar-containing neurons does not occur at times of peak sleep, but rather is initiated only at the end of night to suppress sleep and promote wakefulness. Indeed, increased sleep is observed predominantly in the late night with knockdown of *Lar* in the MB or selectively in MB γ neurons (Figures 4E, S2C, and S4B).

Of interest, the MB009B Gal4 driver targets a neuronal population that may express both *dnc* and the Robo3 (roundabout 3) receptor, which belongs to the Ig-domain superfamily of cell adhesion molecules. As seen with Lar, the Robo3 extracellular Ig domains show homology with the NKT Ig domain. Previous studies have demonstrated that Robo3 cell surface expression is regulated by Ptp69D (the fly homolog of human PTPRC) which, like Lar/PTPRD, is a protein tyrosine phosphatase receptor.[54] Moreover, it was shown that Syndecan, an established Lar ligand,[55] is essential to Robo3 signaling induced by its ligand Slit.[56] In combination, these data highlight an intricate network of MB ligand/receptor partners, that together may fine-tune sleep/wakefulness, and likely synchronize the effect to other MB-modulated physiological processes (e.g., energy metabolism[57,58]). Intriguingly, one MB circuit that participates in the regulation of fat content comprises the α′/β′ and γ

cells but excludes the α/β cells. In that study, the γ lobes were targeted with the same MB009B split-Gal4 driver identified in our genetic screen[58] (Figure S4).

How NKT release from the α′/β′ KCs might activate γ or other MB neurons is unknown. However, several studies indicate that there might be communication between the projections of different Kenyon cell types. Dendrites of KC cells overlap in the MB Calyx,[38] and presynaptic active zone (AZ) markers (BRP, DSyd1, and Syt) exist in those dendrites, suggesting that they are both pre- and post-synaptic.[59] Indeed, the complete connectome of the *Drosophila* larval brain indicates that there is significant dendritic output from KC neurons,[60] consistent with dendro-dendritic communication among those cells. While AZs exist in α/β and γ dendrites of the Calyx, they are not present in α′/β′ dendrites.[59] However, we presume that NKT protein is released via dense core vesicle (DCV) machinery or another secretion mechanism, and DCV sites of release are known to be separate from active zones that utilize classical transmitters.[61] Interestingly, DCVs are assumed to be present in the MB Calyx and lobes of the adult brain, based on the presence of neuropeptide staining in those areas;[62] however, to our knowledge, nobody has explicitly studied DCV distribution in the Calyx or other MB regions. Nonetheless, the potential for dendrite-dendrite communication suggests the possibility that NKT release from α′/β′ cell dendrites may act on dendrites of other MB cell types to regulate sleep.

In humans, mutations in PTPRD, the fly homolog of Lar, have been linked to sleep disorders including restless leg syndrome[50,51], and *PTPRD* knockout mice display decreased sleep at the beginning of the subjective sleep period.[63] While other studies also show that PTPRD loss in mammals is associated with decreased sleep,[52] our observations suggest that a deficit for fly Lar in the MB leads to increased sleep. This may be a consequence of differences between a global knockout (in the mouse) and a more specific deficit in a defined fly sleep-regulating circuit. In addition, effects of Lar loss will obviously depend on the cellular microenvironment and/or molecular partners. It will thus be of interest to assess the effect of Lar overexpression, or knockdown, when restricted to other known sleep circuits, outside of the MB. We note that Lar has also been implicated in insulin signaling,[64] and altered *Drosophila* insulin levels or perturbation of insulin-containing cells affect sleep,[19–21] indicating a connection between Lar and insulin in sleep regulation.

Limitations of the study

Our study suggests that tNkt can regulate both sleep and wakefulness. Results indicate the MB γ neurons respond to NKT to regulate wakefulness; however, we have not identified specific classes of NKT-responding, sleep-promoting cells. Although we demonstrate effects of membrane tNKT in the MB, we have not definitively identified a receptor for the secreted protein. Based on behavioral results, we suggest that the Lar receptor may serve this purpose.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

I.R.D., M.A.R., and F.R.J. collaborated to design experiments, I.R.D. and M.A.R. carried out behavioral experiments, M.G. carried out the experiments summarized in Figure 3, and I.R.D. performed the immunostaining experiments of Figures 1 and 2. M.A.R. performed all statistical analyses, and I.R.D., M.A.R., and F.R.J. created figures for the paper. I.R.D. and F.R.J. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
ANTI-FLAG M2 affinity gel	Sigma Aldrich	Cat # FLAGIPT1
Mouse M2 monoclonal anti-FLAG primary antibody	Sigma Aldrich	Cat # F3165-.2MG
Mouse monoclonal IgG1 anti-Myc antibody (9E10)	Santa Cruz Biotechnology	Cat# SC-40
Cy™3 AffiniPure Goat anti-Rabbit IgG	Jackson ImmunoResearch	Cat# 111-165-144
Chemicals, peptides, and recombinant proteins		
Opti-MEM™ Medium	ThermoFisher	Cat # 31985062
Lipofectamine 2000	ThermoFisher	Cat # 11668030
NKT-6XHis	Generated in our lab	N/A
Experimental models: Cell lines		
HEK-293 cells	Gift of Ben Harwood; Tufts Medical Center	N/A
Experimental models: Organisms/strains		
D. melanogaster: nSyb-Gal4 driver of y[1] w[*]; P{w[+m*]=nSyb-GAL4.S}3	Bloomington Drosophila Stock Center	BDSC:51635
D. melanogaster: Pdf-Gal4 driver of w[*]; P{w[+mW.hs]=Pdf-Gal4/CyO	Dr. Paul Taghert (Washington University)[39]	BDSC:80939
D. melanogaster: OK107-Gal4 driver of w[*]; P{w[+mW.hs]=GawB}OK107 ey[OK107]/In(4)ci[D], ci[D] pan[ciD] sv[spa-pol]	Dr. Ron Davis, Scripps Research Institute, Jupiter, Florida	BDSC:854
D. melanogaster: 13F02/*dnc*-Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=GMR13F02-GAL4}attP2	Dr. Ron Davis, Scripps Research Institute, Jupiter, Florida	BDSC:48571
D. melanogaster: 238Y-Gal4 driver of w[1118]; P{w[+mW.hs]=GawB}238Y	Dr. Ron Davis, Scripps Research Institute, Jupiter, Florida	BDSC:81009
D. melanogaster: 35B12-Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=GMR35B12-GAL4}attP2	Bloomington Drosophila Stock Center	BDSC:49822
D. melanogaster: R55C10-Gal4 driver of w*; P{R55C10-GAL4.DBD}attP2/TM6C, Sb1	Dr. Ron Davis, Scripps Research Institute, Jupiter, Florida	BDSC:95083
D. melanogaster: c305a-Gal4 driver of w[*]; P{w[+mW.hs]=GawB}Cka[c305a]	Bloomington Drosophila Stock Center	BDSC:30829
D. melanogaster: 104Y-Gal4 driver of w[1118]; P{w[+mW.hs]=GawB}104y	Bloomington Drosophila Stock Center	BDSC:81014
D. melanogaster: crc929-Gal4 driver of w[*]; P{w[+mW.hs]=GawB}dimm[929] crc[929]	Bloomington Drosophila Stock Center	BDSC:25373
D. melanogaster: AstA-Gal4 driver of w[1118]; wg[Sp-1]/CyO; P{w[+mC]=AstA-GAL4.2.74}4	Bloomington Drosophila Stock Center	BDSC:80160
D. melanogaster: VT64246-Gal4 driver of P{VT064246-GAL4}attP2	Vienna Drosophila Resource Center	VDRC:204311
D. melanogaster: c316-Gal4 driver of w[*]; P{w[+mW.hs]=GawB}c316	Bloomington Drosophila Stock Center	BDSC:30830
D. melanogaster: c346-Gal4 driver of P{w[+mW.hs]=GawB}c346, w[*]	Bloomington Drosophila Stock Center	BDSC:30831
D. melanogaster: GH146-Gal4 driver of y[1] w[1118]; P{w[+mW.hs]=GawB}GH146	Bloomington Drosophila Stock Center	BDSC:30026

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
D. melanogaster: Split Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=R13F02-p65.AD}attP40; P{y[+t7.7] w[+mC]=R58F02-GAL4.DBD}attP2	Bloomington Drosophila Stock Center	BDSC:68255
D. melanogaster: Split Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=R19B03-p65.AD}attP40; P{y[+t7.7] w[+mC]=R39A11-GAL4.DBD}attP2	Bloomington Drosophila Stock Center	BDSC:68256
D. melanogaster: Split Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=R13F02-p65.AD}attP40/CyO; P{y[+t7.7] w[+mC]=R89B01-GAL4.DBD}attP2	Bloomington Drosophila Stock Center	BDSC:68265
D. melanogaster: Split Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=R52H09-p65.AD}attP40; P{y[+t7.7] w[+mC]=R18F09-GAL4.DBD}attP2	Bloomington Drosophila Stock Center	BDSC:68267
D. melanogaster: Split Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=R13F02-p65.AD}attP40; P{y[+t7.7] w[+mC]=R85D07-GAL4.DBD}attP2	Bloomington Drosophila Stock Center	BDSC:68383;
D. melanogaster: Split Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=R13F02-p65.AD}attP40/CyO; P{y[+t7.7] w[+mC]=R45H04-GAL4.DBD}attP2	Bloomington Drosophila Stock Center	BDSC:68292
D. melanogaster: Split Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=R13F02-p65.AD}attP40; P{y[+t7.7] w[+mC]=R52H09-GAL4.DBD}attP2	Bloomington Drosophila Stock Center	BDSC:68293
D. melanogaster: Split Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=R13F02-p65.AD}attP40/CyO; P{y[+t7.7] w[+mC]=R34A03-GAL4.DBD}attP2	Bloomington Drosophila Stock Center	BDSC:68306
D. melanogaster: Split Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=R13F02-p65.AD}attP40; P{y[+t7.7] w[+mC]=R41C07-GAL4.DBD}attP2	Bloomington Drosophila Stock Center	BDSC:68319
D. melanogaster: Split Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=R25D01-p65.AD}attP40; P{y[+t7.7] w[+mC]=R19F09-GAL4.DBD}attP2	Bloomington Drosophila Stock Center	BDSC:68283
D. melanogaster: Split Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=R14C08-p65.AD}attP40/CyO; P{y[+t7.7] w[+mC]=R15B01-GAL4.DBD}attP2/TM6B, Tb[1]	Bloomington Drosophila Stock Center	BDSC:68294
D. melanogaster: Split Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=R20G03-p65.AD}attP40; P{y[+t7.7] w[+mC]=R19F09-GAL4.DBD}attP2	Bloomington Drosophila Stock Center	BDSC:68296
D. melanogaster: Split Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=R24H08-p65.AD}attP40/CyO; P{y[+t7.7] w[+mC]=R53F03-GAL4.DBD}attP2	Bloomington Drosophila Stock Center	BDSC:68301
D. melanogaster: Split Gal4 driver of w[1118]; P{y[+t7.7] w[+mC]=R12C11-p65.AD}attP40; P{y[+t7.7] w[+mC]=R14C08-GAL4.DBD}attP2/TM6B, Tb[1]	Bloomington Drosophila Stock Center	BDSC:68305
D. melanogaster: UAS-Lar expression of w[*]; P{w[+mC]=UAS-Lar.K}P4B	Bloomington Drosophila Stock Center	BDSC:9149
D. melanogaster: UAS-Lar RNAi of y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02186}attP40	Bloomington Drosophila Stock Center	BDSC:40938
D. melanogaster: w[1118]; P{w+w[+mC]=UAS-tNkt I} attP2, #1 or #2	This manuscript	Line 37999C
D. melanogaster: w[1118]; P{w+w[+mC]=UAS-tNkt II} attP2, # 4 or #5	This manuscript	Line 38000C

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
D. melanogaster: UAS-Nkt RNAi of w[1118]; P{GD8239}v43017	Vienna Drosophila Resource Center	VDRC:43017
D. melanogaster: plum$^{\Delta 1}$ (null mutant)	Gift of Oren Schuldiner; Weizmann Inst. Science; Yu et al.[44]	N/A
D.melanogaster: UAS-plum RNAi	Vienna Drosophila Resource Center	VDRC:6683-GD
D. melanogaster UAS-plum RNAi (plum^{HMC05055})	Bloomington Drosophila Stock Center	BDSC:60062
D. melanogaster: tub-Gal80 of w[*]; sna[Sco]/CyO; P{w[+mC]=tubPGAL80[ts]}ncd[GAL80ts-7]	Bloomington Drosophila Stock Center	BDSC:7018
D. melanogaster: tub-Gal80 of w[*]; P{w[+mC]=tubP-GAL80[ts]}20; TM2/TM6B, Tb[1]	Bloomington Drosophila Stock Center	BDSC:7019
D. melanogaster: plum-Gal4 driver of y[1] w[*]Ti{GFP{3XP3.cla}=CRIMIC.TG4.1} plum{CR01114-TG4.1}/TM3, Sb{1} Ser{1}.	Bloomington Drosophila Stock Center	BDSC:81177

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by F. Rob Jackson (rob.jackson@tufts.edu).

Materials availability

Newly generated *Drosophila* strains expressing tethered NKTs (tNkt I and II) will be made available to other researchers upon reasonable request to the lead author.

Data and code availability

This paper does not report any standardized data. Data reported in the paper will be shared with other investigators upon request to the lead contact.

No original code is reported in the paper.

Additional information required to reanalyze the data reported in this paper is available from the lead contact, F. Rob Jackson, upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Drosophila melanogaster

Flies were reared, and crosses performed using standard procedures and media (see Method details). The study used adult male flies and thus we cannot necessarily generalize to comparable phenotypes in female flies. The genotypes of strains used in the study are shown in the key resources table.

METHOD DETAILS

Generation of tNkt transgenic flies

For the construction of tethered Nkts (tNkts), we employed Genscript Inc. services for synthesis of transgenes. The tNkt I transgene contained in-frame sequences (5′ to 3′) encoding the following peptides: NKT with the signal peptide, an 8 aa NG spacer, a myc tag, a 18 aa GN spacer, and a 26-aa type I VHS transmembrane domain. tNkt II contained sequences (5′ to 3′) encoding a 56-aa TNFα transmembrane domain, a 20-aa GN spacer, a myc tag, an 8-aa GN spacer, and NKT minus the signal peptide. Eco RI and Xba I sites, respectively, were added to the 5′ and 3′ ends of the tNkt transgenes for cloning into the pUAST attB vector, downstream of UAS sequences. Transgenic flies carrying the UAS constructs were generated by Rainbow Transgenic Flies Inc. by insertion into an attP40 site on the second chromosome. Microinjections yielded multiple independent transgenic lines, each carrying one genomic insertion of either construct.

Fly stocks and maintenance

A standard cornmeal-agar medium, with added wheatgerm, was used for crosses and maintenance of Drosophila cultures. Crosses were reared at 25°C, or 29°C, in a light/dark cycle consisting of 12 hours of light and 12 hours of dark (LD 12:12) unless otherwise stated. The Gal4 driver stocks (including all split-Gal4s) listed in Tables 1 and 2 were obtained from the Bloomington Drosophila Stock Center at Indiana

University, except the OK107, 238Y and R55C10 Gal4 drivers that were a gift from Dr. Ron Davis (Scripps Research Institute, Jupiter, Florida). These and all other fly stocks employed in these studies are listed in the key resources table.

NKT secretion from HEK-293 cells

A pcDNA3 plasmid expressing NKT-FLAG was generated by Dr. Lauren Crowe and used for expression of tagged NKT in HEK-293 cells. It was transfected into 10 ml of HEK-293 cells in Opti-MEM™ Medium (ThermoFisher Scientific) containing 2 μl Lipofectamine (ThermoFisher Scientific) transfection reagent. pcDNA3 control plasmid was transfected in the same way. Cells and conditioned media from both types of cells (1.4 x 10^6 cells per aliquot) were stored in 1 mL aliquots at -80C. To obtain lysate, cells were washed with Phosphate-Buffered Saline (PBS) and 1 ml of lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl) was added.

Immunoprecipitation experiments were performed with one aliquot of each sample (experimental medium or lysate) using the FLAGIPT1 kit (Sigma Aldrich). For each sample, 40 μl of ANTI-FLAG M2 affinity gel was transferred to centrifuge tubes and washed four times with 0.5 mL of wash buffer. Lysate or conditioned medium (800 μl) was added to the centrifuge tubes to bring the total volume to 1 m and then left overnight. Tubes were then centrifuged at 8,200 X g for 30 seconds and supernatant was removed; the remaining resin was washed three times with 0.5 ml wash buffer. In addition to the negative control samples, a FLAG-BAP fusion protein was used as a positive control for antibody reactivity. Four μl of 50 ng/μl FLAG-BAP fusion protein was added to the washed resin for this control.

Twenty μl of 2X sample buffer (125 mM Tris HCl, pH 6.8, 4% SDS, 20% glycerol, 0.004% bromophenol blue) was added to samples and controls, boiled for 3 minutes, and all tubes were then centrifuged at 8,200 X g for 30 seconds. Supernatants were transferred to gel using a Bio-Rad Mini-PROTEAN Tetra Vertical Electrophoresis Cell and run at 120 mV for approximately 10 minutes. The gel was transferred to PVDF membrane using a Bio-Rad Trans-Blot Transfer System. Mouse M2 monoclonal anti-FLAG primary antibody purchased from Millipore Sigma was added to 5% milk in TBST for a final dilution of 1:250 and incubated overnight. The PVFD membrane was washed 3X for 30 minutes in TBST and left in TBST overnight. HRP-conjugated goat anti-mouse secondary antibody (Millipore Sigma) was diluted 1:2,000 in 5% milk in TBST.

In vivo secretion studies

The R35B12-Gal4 driver, which is selective for the mushroom body α′/β′ neurons, was used to express UAS-NKT-GFP.[33] Live fly brains were dissected in ice cold 1X PBS. To stimulate secretion, brains were placed in a KCl solution (50mM or 200 mM) for 90 seconds and then fixed in 4% paraformaldehyde (PFA) in Phosphate-Buffered Saline with 0.1% Tween 20 (PBST) at room temperature for 20 minutes. After washing 3X with 0.5 ml PBST, brains were mounted on imaging slides with mounting media and stored at 4°C until imaging. Three independent biological replicates were completed, twice with 50 mM KCl concentration and once with 200 mM KCl. Confocal imaging utilized a Nikon A1R confocal microscope with an excitation wavelength of 488 nm; all acquisition parameters were identical for the different genotypes. Fiji, an image processing package that includes the image processing program ImageJ, was used for analysis of images. Each image was transferred to Fiji in a hyper-stack of z-axis images and then converted to a z-projection image with maximum fluorescence at each x-y axis point.

Immunohistochemistry

To examine expression of tNkt proteins, whole mount preparations of brains isolated from larvae and adult nSyb-Gal4>UAS-tNkt (I or II), dnc-Gal4> tNkt (I or II), and w^{1118} (control) Drosophila were fixed in 4% paraformaldehyde. Immunohistochemistry was performed according to a standard protocol.[65] The following antibodies were utilized: primary monoclonal anti-Myc antibody (Santa Cruz Biotechnology; 1:100 dilution and 1:300 dilution for nSyb-Gal4 and dnc-Gal4 experiments, respectively); secondary fluorochrome-conjugated goat anti-mouse (Jackson ImmunoResearch; 1:200 dilution and 1:400 dilution for nSyb-Gal4 and dnc-Gal4 experiments, respectively). Confocal imaging was performed using a Leica SP8 microscope within the Tufts CNR Imaging Facility. The mushroom body image shown in Figure 2B was captured using a Nikon E800 (Eclipse) epifluorescence microscope.

Behavior assays

Locomotor activity was assayed using monitors and software from Trikinetics, Inc. Activity was collected in 1-min bins. For sleep data analysis, the activity data was collected 48 h after CO2 administration. Two to three-day old male flies were loaded in activity tubes and entrained to LD 12:12 at either 25°C, or 29°C (as indicated in figure legends). Analyses of sleep and activity were performed using an Excel-based package obtained from Paul Shaw[66] and a MATLAB-based package (Fly Toolbox) from Dr. Joel Levine.[67] For Figures 1, 2, 4A–4C, and S2, four cycles of data were used for calculations of sleep parameters. Due to a computer/data collection error on day 4, for Figures 4D, 4E, and S4, three cycles of data were collected and used to calculate sleep parameters.

QUANTIFICATION AND STATISTICAL ANALYSIS

For the *in vivo* secretion studies (Figure 3), Fiji, an image processing package that includes the image processing program ImageJ, was used for analysis and quantification of images. Quantification of sleep and activity data were performed using an Excel-based package from Dr. Paul Shaw (Washington University) and the Fly Toolbox package, as described under Behavior Assays. Replicates for both types of studies are mentioned in the text or relevant figure legends.

For statistical analysis, the D'Agostino & Pearson normality test was used to assess normality in datasets. A one-way Tukey-Kramer Multiple Comparisons test was used to assess statistical significance if data were normally distributed. In the absence of normality, we used the Kruskal-Wallis nonparametric ANOVA with Dunn's Multiple Comparison test, which yields adjusted p values. For experiments with two genotypes, a two-tailed, unpaired Student's t test was used. Significance was determined as $p < 0.05$.