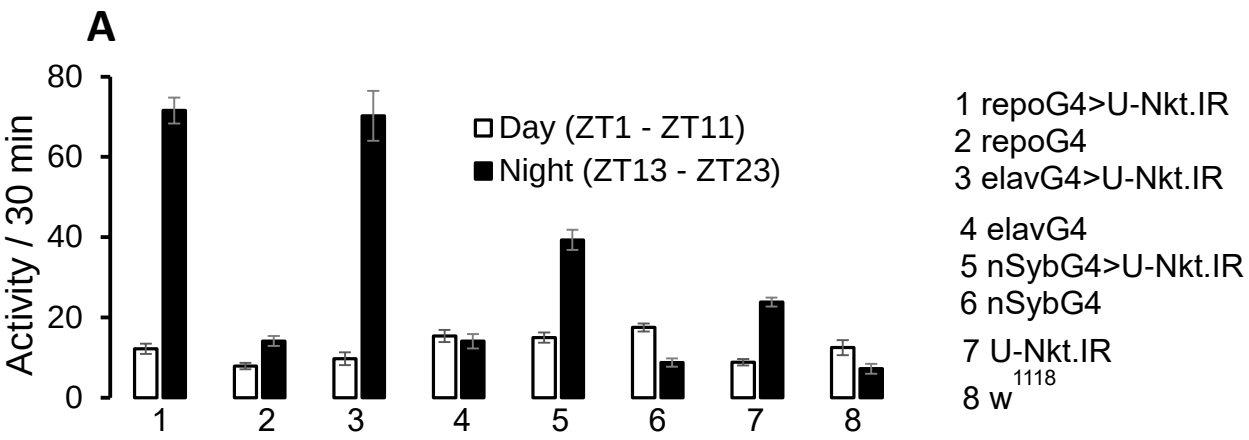
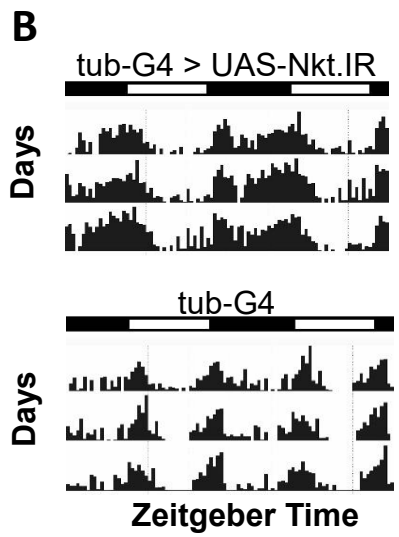
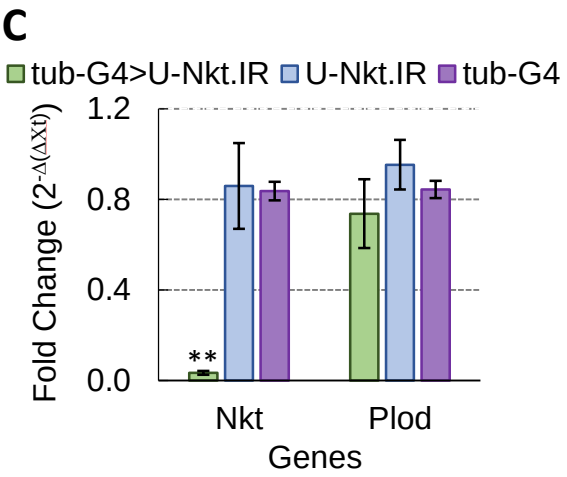

A
Activity / 30 min
80
60
40
20
0
1
2
3
4
5
6
7
8
Day (ZT1 - ZT11)
Night (ZT13 - ZT23)
1 repoG4>U-Nkt.IR
2 repoG4
3 elavG4>U-Nkt.IR
4 elavG4
5 nSybG4>U-Nkt.IR
6 nSybG4
7 U-Nkt.IR
8 w1118


B
tub-G4 > UAS-Nkt.IR
Days
tub-G4
Days
Zeitgeber Time


C
tub-G4>U-Nkt.IR
U-Nkt.IR
tub-G4
Fold Change (2-Δ(ΔXt))
1.2
0.8
0.4
0.0
**
Nkt
Plod
Genes

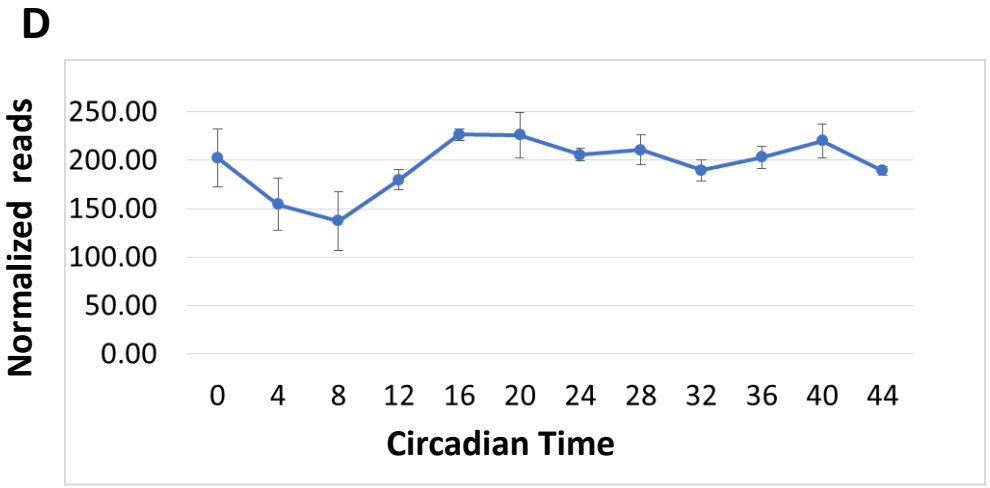

D
Normalized reads
250.00
200.00
150.00
100.00
50.00
0.00
0
4
8
12
16
20
24
28
32
36
40
44
Circadian Time

**Figure S1. *Nkt* locomotor activity and molecular phenotypes. Related to Figure 1 and 2.** **A)** Gal4-regulated knockdown of CG14141 (*Noktochor*, *Nkt*) in either glia or neurons results in high nocturnal activity with no effect on day activity. Histograms depict day and night activity for the various genotypes. In this and other supplemental figures, G4 denotes GAL4. repoG4, pan-glial driver; elavG4, pan-neuronal driver; nSybG4, pan-neuronal driver; U-Nkt.IR, UAS-Nkt.RNAi; $w^{1118}$, a background control strain. Intervals from ZT1 – ZT11 and ZT13 – ZT23 were used to calculate day and night activity, respectively, to exclude lights-on and lights-off effects. **B)** Actograms showing activity for tub-GAL4>U-Nkt.IR and tub-GAL4 control flies in LD 12:12 conditions. All knockdown flies exhibited increased nocturnal activity compared to controls. **C)** qRT-PCR using fly head tissues demonstrates a significant reduction in *Nkt* RNA abundance in knockdown flies, relative to genetic background controls and the Plod gene. **, $p<0.01$ compared to controls. **D)** Abundance of *Nkt* RNA over two days of constant darkness. The Y axis shows quantile-normalized sequence read numbers. Three biological replicates for each timepoint were averaged to produce the plot. Cycling parameters were calculated using the 2010 version of JTK.cycle [S1]. P=0.18 for the curve, indicating that *Nkt* RNA does not show circadian changes in abundance. In the same experiment, *Per* and *Tim* RNAs robustly cycled.

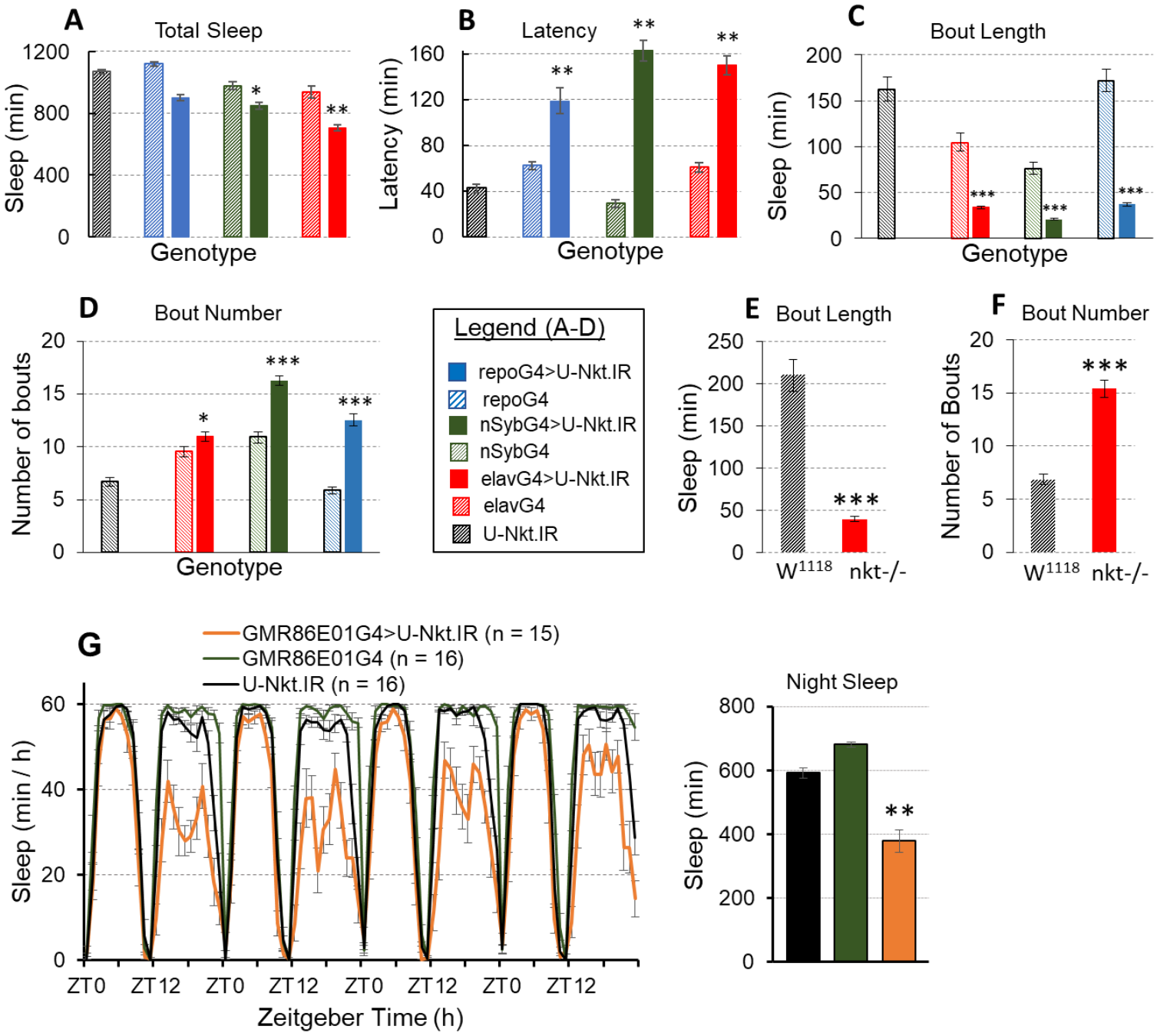



**Figure S2. Sleep parameters in control, *Nkt* knockdown and *Nkt* null flies. Related to Figures 1 and 2. A-D)** total sleep, sleep latency, sleep bout duration and sleep bout number for *Nkt* knockdown flies. **E-F)** sleep bout duration and number for the *Nkt* null mutant. **G)**

GMR86E01-GAL4>Nkt.IR flies and control populations. n=16-23 for all genotypes. GMR86E01-GAL4 is a good reference line for astrocyte expression [S2]. *, p <0.05; **, p < 0.01; ***, p < 0.001 compared to controls.

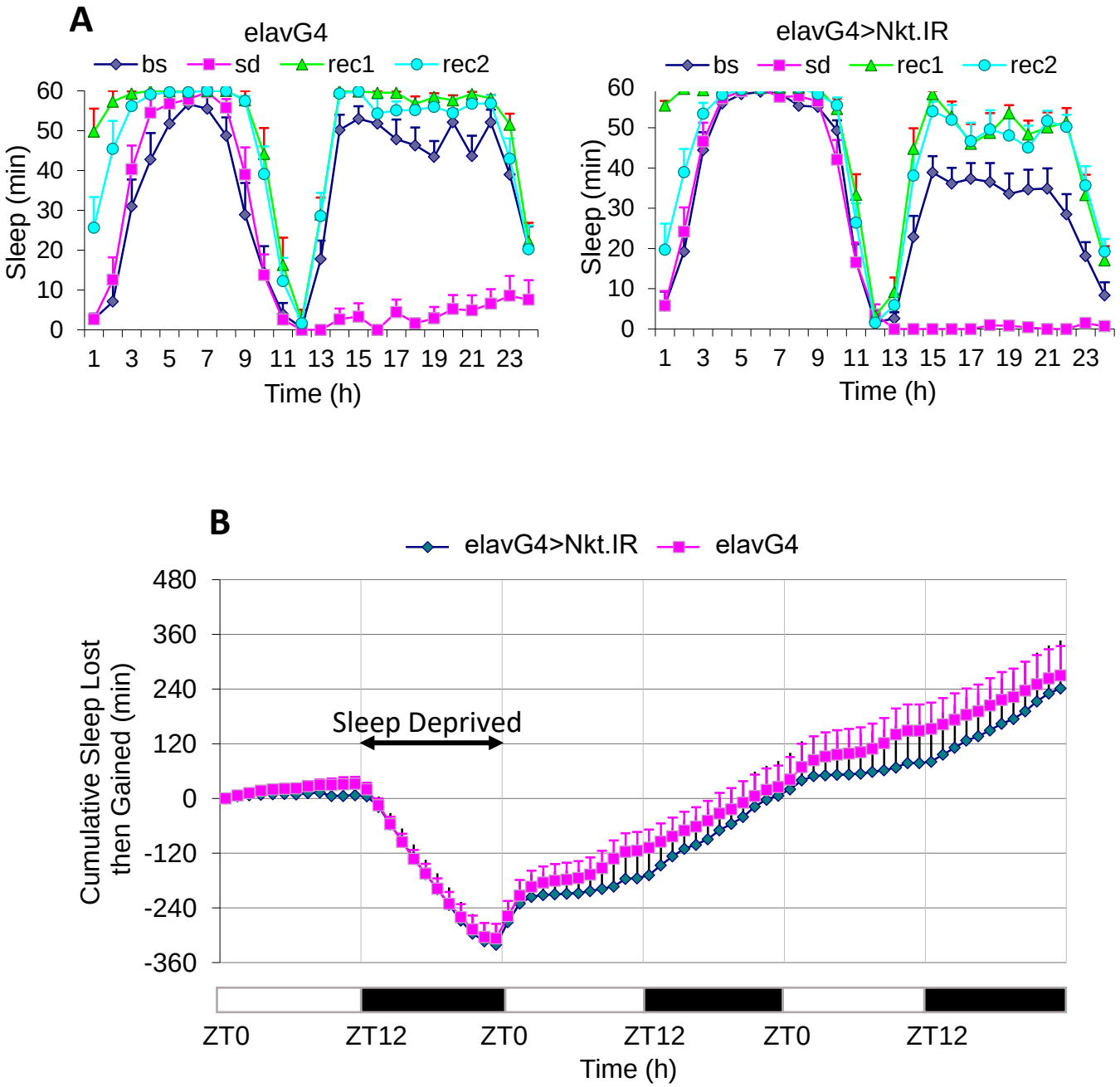


**Figure S3. Sleep loss and recovery (rebound) in neuronal knockdown and control populations. Related to Figure 1.** **A)** Sleep loss and recovery in elav-G4>Nkt.IR (experimental) and elavG4 (control) flies. bs, baseline sleep; sd, sleep during deprivation; rec1, recovery sleep during day 1; rec2, recovery sleep during day 2. **B)** Sleep loss and cumulative recovery sleep in experimental and control flies. Flies were sleep deprived for 12 h, as indicated on the panel. *Nkt* knockdown flies had less baseline night sleep, as expected, but normal sleep loss and recovery. n=16 for both genotypes. This experiment has been replicated once with similar results.

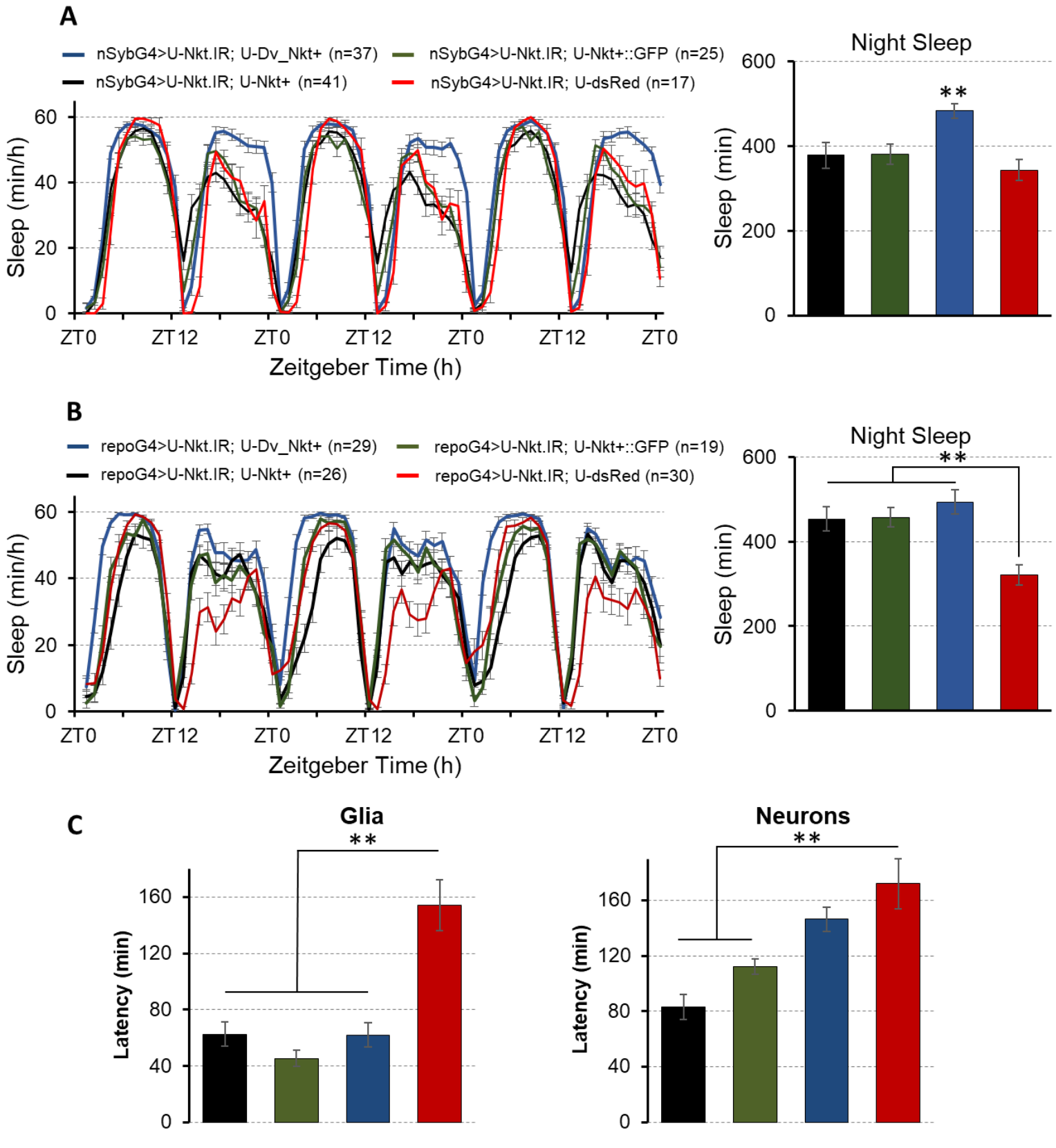

A
nSybG4>U-Nkt.IR; U-Dv_Nkt+ (n=37)
nSybG4>U-Nkt.IR; U-Nkt+::GFP (n=25)
nSybG4>U-Nkt.IR; U-Nkt+ (n=41)
nSybG4>U-Nkt.IR; U-dsRed (n=17)
Sleep (min/h)
Zeitgeber Time (h)
ZT0
ZT12
Night Sleep
Sleep (min)
**
B
repoG4>U-Nkt.IR; U-Dv_Nkt+ (n=29)
repoG4>U-Nkt.IR; U-Nkt+::GFP (n=19)
repoG4>U-Nkt.IR; U-Nkt+ (n=26)
repoG4>U-Nkt.IR; U-dsRed (n=30)
C
Glia
Neurons
Latency (min)

**Figure S4. *Nkt+* transgenes rescue neuronal and glial knockdown of the gene. Related to Figures 1 and 2.** **(A-B)** Average night sleep for knockdown flies expressing either *D. melanogaster* (U-Nkt or U-Nkt+::GFP) or *D. virilis* (U-Dv_Nkt+) *Nkt+* transgenes. *D. virilis Nkt+* expresses an mRNA that is predicted not to be targeted by *D. melanogaster* U-Nkt.IR RNAi. The pan-neuronal *Nkt* knockdown was significantly rescued only by *D. virilis Nkt+* expression whereas pan-glial knockdown was rescued by all three *Nkt+* transgenes, perhaps because of differences in RNAi efficacy between the two cell types. **C)** Sleep latency of knockdown flies was rescued (shortened) to varying degrees by all *Nkt+* transgenes. A dsRed transgene was expressed in knockdown flies as a control for any GAL4 dilution effects that might occur with the addition of another UAS transgene. Histograms represent averages for 3 days of LD data.

**, p<0.01; ***, p<0.001 compared to controls.

| Glia Class | Genotype | n | Activity/30 min ± SEM (ZT13 – ZT23) |
|---|---|---|---|
| Astrocyte | Eaat1G4>U-Nkt.IR; elavGAL80 | 47 | **30.4 ± 2.0** |
| | Eaat1G4;elav-GAL80 | 31 | 10.6 ± 1.1 |
| | | | |
| Astrocyte | NP3233G4>U-Nkt.IR | 15 | **42.0 ± 2.3** |
| | NP3233G4 | 16 | 29.5 ± 2.5 |
| | | | |
| Cortex | NP2222G4>U-Nkt.IR | 12 | 16.6 ± 1.6 |
| | NP2222G4 | 8 | 15.6 ± 3.4 |
| | | | |
| Ensheathing | MZ0709G4>U-Nkt.IR | 17 | 21.5 ± 1.9 |
| | MZ0709G4 | 10 | 16.3 ± 4.2 |

**Table S1**. **Levels of night activity with *Nkt* knockdown in astrocyte, cortex and ensheathing glia. Related to Figure 1.**

| Oligo type | Sequence |
|---|---|
| Primers for cloning *Nkt* cDNA | *Nkt*-FP - 5’ TACTTAGAATTCGCAGCATTATCCATTATCCGG 3’<br>*Nkt*-RP - 5' TCTACACTCGAGAGTCGAAGGCAATGGAGAAATC 3' |
| Guide RNAs and primers for CRISPR mutagenesis | *Nkt* sgRNA 1 - 5’ GCGCGCTTCGCAAACGGGCG 3’<br>*Nkt* sgRNA 2 - 5’ GACGTGGACGAACCAAGTCC 3’<br>*w* sgRNA - 5’ ATACCATTCCTGCTCTTTGG 3’<br>*Nkt* fwd genotyping primer - 5’ CATCTCCGTCAGCAGCATTA 3’<br>*Nkt* rev genotyping primer - 5’ CCGGTAATTGGCAATCCGAT 3’<br>*Nkt* sequencing primer - 5’ CGAGGCTTGTAGGATTTTGG 3’ |
| Primers for Q-PCR | *Nkt* forward primer (FP) - 5' AGATCGGATTGCCAATTACC 3'<br>*Nkt* reverse primer (RP) - 5' TTGGCGCCATTATTATTATTGTA 3'<br>*Plod*-FP - 5' ACGGATCGATGAAGGAAATC 3'<br>*Plod* -RP - 5' ACCGATACGAAGAAGGGATG 3'<br>*rp49*-FP - 5’ GCCCAAGATCGTGAAGAAGC 3'<br>*rp49*-RP - 5' CGACGCACTCTGTTGTCG 3' |

**Table S2. Primers and guide RNAs (sgRNAs) employed in cloning, CRISPR mutagenesis and quantitative PCR (Q-PCR). Related to Star*Methods.** All oligos were obtained from GenScript, Inc. (Piscataway, NJ, USA). *Nkt, Noctochor; w, white; Plod, procollagen lysyl hydroxylase; rp49, ribosomal protein 49.*

## SUPPLEMENTAL REFERENCES


S1. Hughes M.E., Hogenesch J.B., and Kornacker K. (2010). JTK_CYCLE: an efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets**.** J. Biol. Rhythms *25***:** 372-380.

S2. Kremer M.C., Jung C., Batelli S., Rubin G.M., and Gaul U. (2017). The glia of the adult Drosophila nervous system**.** Glia *65***:** 606-638.