Supplementary Materials for

A sleep-inducing gene, *nemuri*, links sleep and immune function in *Drosophila*

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Tables S1 to S3 (Excel format)

Materials and Methods:

Genetic screen

elav-GS (*60*) (BL #43642 on the third chromosome, back-crossed into iso31 (*39*) for 5 generations) was used to induce expression of genes inserted downstream of UAS sequences in the following collections of flies: EP, EPg, EPgy2, Mae-UAS, XP, WH, UAS-*mir* and *mir* sponge lines from the Bloomington Stock center (USA) and UAS-cDNA libraries made by FlyORF (Swiss) and the Centre for Cellular and Molecular Platforms (India) (Supplemental Table 1). Flies were raised on a standard molasses/cornmeal food medium and maintained at 25 ^{o}C in a 12 hour light:12 hour dark cycle. Flies aged 3-7 days were loaded into locomotor tubes containing 5% sucrose/2% agar food medium with 500 μM of RU486 (Sigma#M8046). Control groups were fed food containing equivalent concentrations of ethanol as vehicle. Sleep assays were carried out in a 12:12 LD cycle at 25 ^{o}C using the DAM system (Trikinetics: http://www.trikinetics.com/). Sleep was analyzed from the day after the flies were loaded (~24 hours later) for 6 days. Four males and four females per line were tested. Flies were considered asleep when they did not move for at least five minutes (*61*). Sleep time was analyzed by pySolo (*61*). Data were analyzed using Prism6.

Sleep deprivation assay

Monitors were placed in an attachment (Vortexer Mounting Plate, Trikinetics #VMP) on the Multi-Tube Vortexer (VWR-2500). The speed of the vortex was set between 3 and 4". Sleep deprivation was carried out using DAMSystem308 software in the following sets: shaking for 2 seconds repeated every ~20 seconds with a randomized protocol for a total of 6 hours between ZT18-ZT24. The sleep rebound was calculated as the difference in sleep between the day after

sleep deprivation and the day before the deprivation at each ZT time. Calculation of sleep time was done by Insomniac3 (*37*). Data were analyzed using Prism6.

Circadian assay

Flies were entrained to a 12:12 LD cycle for several days before being moved into a constant dark (DD) incubator to measure free-running activity. Data were collected after 5 days of DD and analyzed using Clocklab (MatLab). Data were analyzed using Prism6.

Arousal threshold assay

For the mechanical stimulation used in Figure 1D and Figure S3A, a hammer (Plastic Mallet, 12 oz. Head Weight) was dropped onto the incubator shelf housing monitors or 24-well chambers from a height of 4.5 inch at ZT20. Data were analyzed using Mann–Whitney U test using Prism6. For the weak light stimulus used in Figure 4D-F and Figure S12A, a light resource (LightPad® 930) was controlled by DAMsystem308 software to emit for 10 seconds at ZT18. For experiments with an olfactory stimulus, 3MM paper (1 cm x 0.5 cm) absorbed with 20 µl of 3-octanol was placed in a 10 ml syringe for a few minutes. The odor was gently delivered from the syringe to each fly in a DAM monitor via a MAN2 Gas Distribution Manifold (TriKinetics) at ZT15 or ZT16 for 5 minutes. For all stimuli, flies that had been sleeping at the time of the stimulus were considered aroused if they woke up, indicated by movement during the minute immediately after the stimulus. Data were analyzed using Prism6.

Feeding assay

FD&C Blue#1 (Sigma# 3844-45-9) was dissolved in 5% sucrose/ 2% agar to achieve a final dye

concentration of 2.5% and distributed in vials. 10 males and 10 females were kept inside each

vial for one night. At ZT0, single female flies were collected in 1.5 ml tubes, and homogenized

in 200 µl of PBS. Samples were spun at 13,000 rpm for 15 minutes at 4 °C in a table top

centrifuge and supernatants were saved. Standard curves were generated by preparing 0.08% of

FD&C Blue in PBS (0-5%). Each sample was read at 630 nm for absorbance and relative

concentration was estimated by using the standard curve. Data were analyzed using Prism6.

Video tracking assay

Video capture of flies and tracking analysis was carried out as described previously (*28*). Briefly,

Ethovision XT9 software was used for video tracking with an Ikegami Digital Video Camera

(5C46). *elav*-GS/UAS-*nemuri* (#DP2629 at BFRC) flies were briefly anesthetized with CO_2 and

then moved individually onto 24-well plates filled with 2% agar/5% sucrose food. Each well

contained RU486 or an equivalent concentration of ethanol as a vehicle control for the

experiment. Recordings were carried out at 25 °C in a 12:12 LD cycle. Recording was started the

day after the flies were loaded (~24 hours later) and continued for up to 72 hours. Time spent not

moving, velocity (in cm/s) and distance (in cm) were extracted from the video data. The

detection settings were optimized for each experiment so that flies that did not track well were

excluded from subsequent analysis. We set Ethovision to collect data from the video every

1.0335 seconds (one frame). Ethovision compares each frame with the last and calculates how

far each fly moved. Flies that traveled at least 0.125 cm between frames, which is ~50% of their

full body length (as described (*28*)), were considered moving. Velocity was required to go below

0.05cm/s to mark cessation of movement. If a fly did not move for at least 5 minutes, then the fly

was considered to be asleep. Data were analyzed with Microsoft Excel, specifically with an Excel macro (as described (*28*)), and using Prism6.

CRISPR/Cas9 gene targeting to generate *nur* mutants

pCFD4-*nur* gRNAx2 vector: two gRNAs (5'gtttcgatatcgatgctgtg3' and 5'ggcaaactttcaacagcttc3') were cloned into pCFD4 (Addgene#49411) (*62*) following the protocol—http://www.crisprflydesign.org/wp-content/uploads/2014/06/Cloning-with-pCFD4.pdf. See Supplemental Information Table 3 for the primer sequences. HT109 and HT110 primers were used for PCR amplification. Sequence was confirmed by HT18 and HT159 primers.

pHD-DsRed-attp-*nur* vector: Approximately 1 kb upstream and downstream of the *nur* gene (CG31813) were PCR amplified using genomic DNA as a template with the following primers. The 5' *nur* arm was amplified by HT127 and HT130 primers, and the PAM sequence CGG inside the 5' arm was changed to CGC by HT128 and HT129 primers through PCR to prevent potential cutting by Cas9. The 3' *nur* arm was amplified by HT131 and HT134 and the PAM sequence inside 3' arm was changed to TGC by HT132 and HT133 primers. PCR products of the 5' *nur* arm and 3' *nur* arm were cloned into a SmaI site in the pBS-KS vector. After the construct was confirmed by sequencing with T7 and T3 primers, 5' and 3' arms were processed with AarI and SapI restriction enzymes, respectively and inserted into AarI and SapI sites in pDsRed-attP (Addgene#51019).

The pCFD4-*nur* gRNAx2 vector and pHD-DsRed-attp-*nur* vector were mixed to achieve final concentrations of 0.1 µg/ul and 0.5 µg/ul respectively and injected into *vas*-Cas9 (on X chromosome: BL# 51323) embryos by the Rainbow transgenic service. A single G0 male was

crossed with second balancer virgin females to establish the line. Only G1 flies expressing

DsRed in the eye were tested by extraction of gDNA followed by PCR. Further confirmation was

done by southern blotting assay. The correct gene targeting lines were saved for testing in

behavior assays. *nur* mutant flies (*nur*^{attp}) were back-crossed with the iso31 strain for four times

and tested for behavior.

Generation of *nur*^{Gal4}

For construction of the scarless *nur*^{Gal4}, gRNA vector, pBF-U6.2-*nemuri* gRNA, and targeting

vector, pHD-ScarlessDsRed-Gal4, were made as follows.

pBFv-U6.2-*nemuri* gRNA: pBFv-U6.2 (NIG) was cleaved by BbsI restriction enzyme and gel

purified. HT281 and HT282 Oligos (10uM each) were treated with PNK at 37 degrees for 30

minutes, and 95 degrees for 5 minutes, which was then ramped to 25 degree at a rate of -0.1

degree/second. This mixture was cloned into pBFv-U6.2 and sequence verified with HT283 and

HT284.

pHD-ScarlessDsRed-Gal4: The 5' arm was made with the following three amplified fragments:

1) with HT303 and HT304 primers using genomic DNA as a template, 2) with HT305 and

HT306 primers using pBPGW (Addgene# 17574) as a template and 3) with HT307 and HT286

primers using gDNA as a template. Each PCR product was purified and templates (1)+(2) were

amplified with HT303 and HT306 primers and templates (2)+(3) were amplified with HT305

and HT286. The two PCR products thus generated were purified and then used as template for

amplification with HT303 and HT286 primers to generate the larger fragment. The 3'arm for

pHD-ScarlessDsRed-Gal4 was amplified using genomic DNA as a template with HT287 and

HT288 primers. 5' arm and 3' arms were cloned into a SmaI site in the pBS-KS vector. After the

construct was confirmed by sequencing with T7 and T3 primers, 5' and 3' arms were processed

with AarI and SapI restriction enzymes, respectively, and inserted into AarI and SapI sites in

pHD-ScarlessDsRed (DGRC# 1364) and sequence was confirmed again with HT296, HT297,

HT298 and HT299 primers.

pBFv-U6.2-*nemuri* gRNA and pHD-ScarlessDsRed-Gal4 were mixed to achieve final

concentrations of 0.1 µg/ul and 0.5 µg/ul respectively and injected into *vas*-Cas9 (on X

chromosome: BL# 51323) embryos by the Rainbow transgenic service. A single G0 male was

crossed with iso31 and back-crossed to second chromosome balancer virgin females to establish

a line. Only G1 flies expressing DsRed in the eye were tested by extraction of gDNA and PCR

followed by sequencing with HT296 and HT313 for the 5' side and HT327 and HT326 for the 3'

side to verify the correct targeting of Gal4. These lines were then crossed to iso31 containing

tub-pBac (BL# 8285) to obtain scarless *nur*^{Gal4}. The precise excision of pBac sequence was

confirmed by PCR using HT332 and HT85 primers.

 gDNA extraction for PCR

Single flies expressing DsRed in eyes, or iso31 as negative control, were anesthetized under CO_2

and transferred to 1.5 ml tubes. Each fly was homogenized in 50 µl of buffer (10mM Tris

pH8/1mM EDTA/25mM NaCl/200ug/ml Protease) using a pestle. After incubating at 37 °C for

30 minutes followed by heat inactivating at 90 °C for 5 minutes, samples were spun at 13,000

rpm in a table top centrifuge for 1 minute. Supernatants were saved and 1 µl of sample was used

for the following 25 µl PCR reactions. PCR reactions were carried out using primers: HT187 and

HT50 for the 5' side and HT31 and HT188 for the 3' side (Figure S9B) followed by gel

electrophoresis in 0.8% agar, gel purification and sequence analysis. For Figure S9D, PCRs were carried out using HT80 and HT81 primers for *nur* and HT223 and HT340 for the *actin5c* control.

Southern blotting

Probe synthesis: DIG labeled probe was generated using the PCR DIG Probe Synthesis Kit (SigmaAldrich #11636090910) and the corresponding protocol. The DsRed probe was amplified by HT229 and HT230 primers using pDsRed-attP (Addgene# 51019) as a template.

Genomic DNA preparation: Around 20 flies from each line were collected in 1.5ml tubes. After homogenizing them with a pestle in 400 μl of buffer (100mM TrispH7.5/100mM EDTA/100mM NaCl/0.5% SDS), samples were incubated at 65 °C for 30 minutes, treated with 800 μl of LiCl/KAc (70%/30%) on ice for 10 minutes and then spun at 13,000 rpm in a table top centrifuge for 15 minutes. Supernatants were saved and spun down again. After supernatants were mixed with 600 μl of isopropanol, they were spun down at top speed for 15 minutes. The pellets were washed with 1 ml of cold 70% EtOH. After drying the pellets at room temperature, pellets were dissolved in 50 μl of TE buffer and the concentration of genomic DNA was measured. 4~5 μg of genomic DNA was digested by 20 units of Hind III (NEB# R0104) restriction enzyme at 37°C overnight.

Southern blot: Genomic DNA samples processed by restriction enzyme were loaded in 0.8% agarose gel and run at 80V for 2 hours. The procedure followed a normal southern blotting protocol. Briefly, the gel was incubated with gentle shaking in a denaturation solution (1.5 mM NaCl/ 0.5M NaOH) for 30 minutes and later in neutralization solution (1.5 mM NaCl/0.5 M Tris pH7.4/ 1 mM EDTA) for 30 minutes. Gel transfer to a nylon membrane (ThermoFisher#AM10102) was done by sandwich transfer method overnight. The membrane

was washed with 2x SSC once and dried at room temperature. Genomic DNAs on the membrane were UV cross-linked (1200 mJ/cm^2). The membrane was incubated with pre-hybridization buffer, Ultrasensitive hybridization buffer (ThermoFisher #AM8670) including 0.1 mg/ml Salmon Sperm DNA (ThermoFisher #AM9680), at 42 °C for 3 hours. After discarding pre-hybridization buffer, DIG labeled DsRed probe in Ultrasensitive hybridization buffer was incubated with the membrane at 42 °C overnight. After the membrane was washed twice in 2xSSC/0.1%SDS at room temperature for 10minutes and twice in 0.1xSSC/0.1%SDS at 65°C for 10 minutes, the membrane was treated with DIG wash and block buffer set kit (SigmaAldrich # 11585762001) and DIG Luminescent Detection Kit (SigmaAldrich #11363514910) and signal was detected using X-ray film.

in situ hybridization

Probe synthesis: The *nur* cDNA coding region was amplified by primer sets: HT198 and HT199 using genomic DNA as a template and cloned into a SmaI site in a pBS-KS(-) vector. The sequence was confirmed by T7 and T3 primers and cut to linearize DNA by either EcoRI to generate the anti-sense probe or BamHI to generate sense-probe. Anti-sense probe and sense probe were synthesized in 10 μl volume using T3 or T7 RNA polymerase respectively and a DIG RNA Labeling Mix (SigmaAldrich #11277073910).

Hybridization: Brains were dissected in ice cold PBS in a dish and immediately transferred to ice cold 4% formaldehyde/PBS and stored at 4 °C for fixation. The solution was replaced with methanol at -20 °C twice and stored for overnight at -20 °C for further fixation. Brains were then washed twice with 100% ethanol, followed by treatment with 50% xylene/ethanol, and incubated for 30 minutes at room temperature. This was followed by an additional two treatments with

ethanol and subsequently a series of 75%, 50% and 25% ethanol /milliQ treatments on ice. The ethanol was replaced with 80% acetone /H2O at -20 $^{\circ}$C and brains were placed on ice for 10 minutes. Following two washes with PBS/0.1% Tween20, 4% formaldehyde in PBS was used to fix the brains for 30 minutes at room temperature. After this, brains were washed three times for 10 minutes each in PBS/0.1% Tween20, treated with a series of 50%, 75% and 100% HB solution (50% formamide/5xSSC/0.1%Tween20/1xDenhardt (Sigma# D2532)/1mg/ml tRNA, 50µg/ml heparin (Sigma# H4784)/0.1mg/ml Salmon Sperm DNA) in PBS/0.1% Tween20 and washed with 100% HB solution twice. Pre-hybridization was in HB solution at 60°C for 1~2 hours, followed by hybridization (50µl HB solution + 1µl RNA probe) at 60°C for 16~20 hours. Wash was with WB solution (50% formamide/ 5xSSC/ 0.1 %Tween20) twice, followed by a series of 1x, 1/2, 1/4x, 1/8x, 1/16x WB diluted with 0.1 %Tween20 /H2O at 60 $^{\circ}$C for 30 minutes each. Subsequently, brains were washed three times, 10 minutes each time, with PBS/0.1% Tween20, blocked in 10% Normal Serum/ PBT at room temperature for 60 minutes and then treated with anti-DIG-AP antibody (1: 2,000, SigmaAldrich#11093274910) in 1% Normal Serum in PBS/0.1% Tween20 at 4 $^{\circ}$C overnight. Following four 10 minute washes in PBT, brains were washed in fresh Staining Buffer (100mM TrisHCl (pH9.5)/100mM NaCl/ 50mM MgCl$_2$/ 0.1%Tween20) twice. Color was developed in 1.5 µl NBT (Sigma# 11383213001) + 1.1 µl BCIP (Sigma# 11383221001) in 333 µl Staining Buffer at room temperature for 30~60 minutes in the dark until the color showed up. The reaction was stopped with PBS/0.1% Tween20, which was replaced with PBS before mounting in 80% glycerol/PBS. Brain samples were mounted on slides and pictures were taken by EVOS FL Auto Cell Imaging System (ThermoFisher# AMAFD1000)

Western blotting

Two micrograms of each plasmid DNA was transfected using effectene (Qiagen # 301425) into S2R+ cells ($1.0x\ 10^6$) maintained in 2 ml of Schneider's Drosophila Medium (ThermoFisher # 21720001) with 10% FBS (ThermoFisher # 10438026) per well of a 6-well plate . After 24 hours, the medium was aspirated and cells were washed carefully once with 2 ml of Express Five® SFM (ThermoFisher #10486025) and incubated with 1.5 ml of SFM for 24 hours. SFM was collected and spun down at 5,000 rpm for 5 minutes. Supernatants were concentrated using Amicon® Ultra-2 mL Centrifugal Filters (EMD Millipore #UFC200324) at 2,000 g for 1 hour at 4ºC. The supernatants were concentrated ~13 times. Supernatants were mixed with SDS loading buffer, boiled for 5 minutes and stored on ice. After 24 hours of incubation with SFM, cells were collected in 150 µl of SDS loading buffer per well, boiled for 5 minutes and stored on ice. Up to 20 µl of sample per lane was loaded in 4-12 % pre-cast gels (ThermoFisher #NP0335PK2) and run in MES Running Buffer (ThermoFisher #NP0002). Samples were transferred to nitrocellulose membrane (ThermoFisher #LC2000) at 20V at 4 ºC overnight. The membrane was washed with milliQ water once and blocked in 5% milk/1% BSA in PBS for 1 hour at room temperature. Guinea Pig anti-NUR (1:1,000, pre-absorbed with a membrane carrying brain extracts from *nur* mutants), rabbit anti-HA (1:5000, Abcam #ab9110) or rabbit anti-GFP (1:500, ThermoFisher#A11122) were incubated with the membrane for 1 hour at room temperature. After washing with PBS-0.1%Tween20 for 5 minutes three times, the membrane was treated with anti-Guinea Pig HRP (1:5,000, Jackson ImmunoResearch #106-035-003), or anti-rabbit HRP (1:5,000, Jackson ImmunoResearch #711-035-152) for 1 hour at room temperature. The membrane was then washed in PBS-0.1%Tween20 for 10 minutes three times, and signal was detected with ECL (ThermoFisher #32106) treatment for 1 minute followed by exposure to film.

Generation of anti-NUR antibody

The *nur* (CG31813) coding region excluding the signal peptide sequence was amplified with primers HT252 and HT250. PCR products were processed with EcoRI and NotI to clone into EcoRI/NoTI sites in a pGEX-4T-1 vector (GE Healthcare # 28-9545-49). After the sequence was confirmed using HT15 and HT10 primers, pGEX-4T-1-*nur* was transformed into BL21 (DE3) cells and spread over an LB (Amp+) plate. A single colony was isolated and inoculated in a small culture shaken at 250 rpm at 37 ^{o}C overnight. One ml of this culture was introduced into 100 ml of LB (Amp+) and incubated at 250 rpm at 37 ^{o}C for 2~3 hours till OD_{600} reached 0.6. After addition of 500 µM of IPTG, the culture was incubated at 150 rpm at 28 ^{o}C overnight. The culture was then spun down and pellets were resuspended in NETN buffer (20mM Tris pH8/150mM NaCl/1mM EDTA/0.5% NP-40/1% Sarcosyl /Protease inhibitor cocktail [Sigma# 11836170001]). Sonication was carried out on ice at output "5" setting for 10 seconds at 20 second intervals for a total of 20 minutes. After spinning down the samples at 12,000 g for 10 minutes, they were mixed with pre-washed Glutathione Sepharose 4B beads (GE healthcare# 17075601) in NETN buffer at 4°C for 1 hour. Beads were spun at 1,500 g for 3 minutes, and washed with NETN buffer 3 times. Beads were then washed with PBS 5 times and 5 units of Thrombin were mixed for several hours-overnight at room temperature. After spinning down beads at 5,000 rpm for 5 minutes, the supernatant including NUR protein was concentrated with Amicon™ Ultra-15 Centrifugal Filter Units (Fisher# UFC901008). NUR protein was injected into Guinea Pigs by Cocalico Biologicals, Inc to raise antibodies. Serum from the animals was used for immunohistochemistry (1:200) or western blotting (1:1,000) as described in other sections.

Plasmid DNAs for SEAP assay

pMTa-AP: PCR amplification was carried out using primers HT334 and HT333 with pECIA (Addgene# 47051) as a template. PCR products were digested with EcoRI and NotI and cloned into EcoRI/NoTI sites in a pMT/V5-His A vector (ThermoFisher# V412020). The sequence was verified with the primers HT293 and HT294.

pMTa-NUR::AP: PCR amplifications were carried out using primers HT318 and HT319 with gDNA as a template and HT320 and HT333 using pECIA as a template, respectively. Each PCR product was purified to use as template with HT318 and HT333 primers to amplify NUR::AP. The PCR product was digested with EcoRI and NotI and cloned into EcoRI/NoTI sites in a pMT/V5-His A vector. The sequence was verified with the HT293 and HT294 primers.

pMTa-BiP::AP: PCR amplifications were carried out with HT316 and HT333 primers using pECIA as a template. PCR products were digested with Bgl II and Not I and cloned into BglII/NoTI sites in a pMT/BiP/V5-His/GFP vector (ThermoFisher #V413020) and sequence was verified with the HT293 and HT294 primers.

SEAP assay

Two micrograms of each plasmid DNA were transfected using effectene (Qiagen# 301425) into S2R+ cells (1.0×10^6) maintained in 2 ml of S2 medium with 10% FBS per well of a 6-well plate. After 48 hours, the medium was collected from each well and spun down at 6,000 rpm for 1 minute and supernatants were saved for the assay. Fifty µl of supernatant was mixed with 100 µl of BluePhos® Microwell Phosphatase Substrate System (KPL# 50-88-02, mixture of 50 µl of Phosphatase Substrate Solution A and B solution, respectively) in each well of a 96-well plate. After incubating at room temperature for several hours, measurements were carried out at 595 nm wavelength in a 1420 multi-label counter (PerkinElmer Victor3).

RNA extractions for RT and qPCR

Thirty to sixty female flies with or without SD were collected in 15 ml tubes and frozen on dry ice for 5 minutes. After tapping the 15 ml tube to separate the heads or bodies from the bodies, heads were carefully collected by paint brush into 1.5 ml tubes for extracting RNA by TRIzol reagent (ThermoFisher# 15596026). cDNAs were synthesized using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher# 4374966) with or without Reverse transcriptase as a negative control. qPCR was performed using the SYBR® Green PCR Master Mix (ThermoFisher# 4364346) and the following primer sets: HT205 and HT206 for *nur* and HT223 and HT224 for *actin5c,* which was used to normalize.

Immunohistochemistry and image acquisition

Five to ten day-old adult fly brains were dissected in PBS and fixed in 4% PFA (EMS# 15710) in PBS for 30 minutes at room temperature. After washing with PBS-Triton 0.3% for 20 minutes three times, samples were incubated with 5% Normal Donkey Serum (Jackson ImmunoResearch# 017-000-121) in PBS-Triton 0.3% for 1 hour. This was followed by treatment with guinea Pig anti-NUR (1:200 pre-absorbed in *nur* mutants), rabbit anti-GFP (1:1,000, Lifetech# A-11122), anti-RFP (1:200, Rockland# 600-401-379), anti-HA.C5 (1:1,000, abcam: # ab18181) or nc82 (concentrated mouse anti-Brp, DSHB: 1:1000) in the cold room for one to two overnights. After washing with PBS-Triton 0.3% for 30 minutes six times, brains were incubated with anti-rabbit Alexa488 (1:1,000, ThermoFisher# A-11008), anti-mouse Alexa647 (1:1,000, ThermoFisher# A-21236), anti-mouse Alexa488 (1:1,000, ThermoFisher# A-11001), anti-rat Cy3 (1:1,000, Jackson# 712-165-153), anti-Guinea Pig Alexa 633 (1:1,000, ThermoFisher# A-21105) or Alexa Fluor® 633 Phalloidin (1:100, ThermoFisher# A22284) in the cold room for

one to two overnights. After washing with PBS-Triton 0.3% for 30 minutes 6 times, samples

were washed in PBS once and moved into 50% glycerol before mounting on slides with anti-fade

medium (VECTASHIELD:H-1000). Images were acquired at 0.45 μm thickness using Plan APO

40x/1.3 oil lens in a Leica SP5 microscope. Maximum intensity projections were obtained using

Fiji (ImageJ) and images were further processed for linear adjustments using Adobe Photoshop

CS. For Figure 5B and Supplemental Figure S16D, SD was carried out for 12 hours over three to

five days at ZT12. Caffeine was fed one day before dissections.

Survival assay

To evaluate lifespan, flies fed with or without RU486 for one day were collected up to 20 flies

per vial and raised in a 25°C incubator in an LD: 12-12 cycle. Every two days, flies were flipped

into new vials and the dead flies on the food were counted. The flipping continued till all the

flies were dead. Results were analyzed with the log rank test using Prism6.

Fly husbandry and crosses

Flies were maintained in cornmeal, molasses and yeast medium (Bloomington) food in a 12:12

LD cycle at constant 25 °C. For infection assays, flies were reared on a similar medium, except

substituting molasses with dextrose or light corn syrup as the sugar source. Flies used for

screening are listed in Supplemental information Table 1 and 2.

Infection

Infection procedure was performed as described (*36, 63*). For these experiments, the *nur* mutant

and transgenes for the 23E10-Kir experiment (Figure S18) were crossed into the w^{1118}

background, which shows robust sleep responses in infection assays (*42*). Flies were

anesthetized under CO_2 at ZT 18 and infected with *E.coli* (ML35:ATCC #43827) or *S. marcescens* (ATCC #8100) grown in LB medium and diluted in a mixture of PBS and 1% food coloring (Brilliant Blue FCF) to $O.D._{600}$ of 0.03–0.06 using glass capillary needles. A similar infection protocol was used for *S. pneumoniae* (strain D39; P210, gift of Dr. Michael Sebert), except bacteria were grown in BHI medium in anaerobic conditions. Survival was determined by using activity data derived from the DAM system. Flies were considered dead from the time all activity counts remained at zero for 24 hours or the remainder of the experiment. Custom software (Insomniac3 (*37, 63*)) was used to evaluate survival time in individual flies as well as infection-induced sleep. Sleep induction after infection was calculated as the difference in sleep between the day after infection and the day before infection at each ZT time. Kaplan Meier curves and log rank tests were generated using Prism6.

To evaluate bacterial clearance, flies were infected with *S. marcescens* as described above, and collected immediately (0 hour) or 24 hours afterwards. Flies were homogenized in LB broth in groups of 3-10, serially diluted, spread onto LB plates, and incubated overnight at 37 ˚C. The next day, the number of colony forming units (cfu) per plate was determined and cfu per fly calculated as previously described (*63*).

Alamar Blue Cell Viability Assay

A single colony of *E.coli* (ML35:ATCC #43827) and *S. marcescens* (ATCC #8100) was inoculated in 1 ml of LB culture at 250 rpm in 37 ºC for 4 hours. Bacterial cultures were diluted in PBS to $O.D._{600}$=0.05. Ten μl of diluted bacterial cultures were mixed with 80 μl of indicated final concentration of NUR and incubated at room temperature for two hours. Ten μl of AlamarBlue (Thermofisher: DL1025) was added into each sample in a 96 well plate and further

incubated overnight at 37 ℃. The concentration of NUR used in Fig. 3B and Fig. S6A is 18 μM.

The measurements were carried out at 595 nm wavelength in a 1420 multi-label counter

(PerkinElmer Victor3)

Supplemental Figure Legends:

Fig. S1. Pan-neuronal over-expression of *nur* has profound effects on sleep

(A) and (B) Sleep profile of elav-GS/UAS-*nur* (+RU486) or controls (-RU486) in both males

and females during the day as well as the night. The sleep profile is shown (A) and quantification

(B). n=32 for males or females treated with RU486 and n=31 for each of the controls. (Mann-

Whitney U test, ****P<0.0001). Median ± interquartile is shown. Sleep was assayed using the

DAM system. Main Figure 1 shows results of video analysis. (C) *in situ* hybridization of brains

over-expressing *nur* (*elav*-GS/UAS-*nur* +RU486), using an anti-sense probe from CG31813

(*nur*). *nur* is expressed all throughout the brain. The sense probe (a negative control) confirms

the specificity of the probe for *nur*.

Fig. S2. *nur* has limited effect on circadian rhythms.

(A) Circadian behavior was assayed in constant darkness (DD) using the DAM system. A

representative double-plotted activity record of *nur* over-expressing male flies (*elav*-GS/UAS-

nur +RU486) or controls (–RU486). (B) Period (left) in *nur* over-expressing flies (*elav*-GS/UAS-

nur +RU486) or controls (–RU486) (Mann-Whitney U test, n=30 for each, ****P<0.0001). FFT

values (right): 0.127±0.04 in *nur* over-expressing flies versus 0.104±0.05 in controls (Mann-Whitney U test, n.s., n=30 males for each). Median ± interquartile is shown.

Fig. S3. Analysis of speed in *nur* over-expressing flies after a stimulus and at transitions between light and dark

(A) Velocity of each fly, using the video tracking system, after a mechanical arousing stimulus at ZT20. Flies that moved either during the minute the stimulus was given, or during a subsequent five minute period after the stimulus, had their velocities averaged over the entire (6 minute) time period. *elav*-GS/UAS-*nur* flies +RU486 (black, n=5 females) and –RU486 (blue, n=47 females) (Mann-Whitney U test, ** p<0.01). Median ± interquartile. (B) Analysis of speed at environmental transitions. *elav*-GS/UAS-*nur* female flies +RU486 (black) or –RU486 (blue) were maintained in an LD 12:12 hour cycle and analyzed for average speed (top). The difference in speed between flies maintained on RU486 and those on vehicle (bottom). The negative values indicate faster movement in *nur* over-expressing flies. Red arrows highlight movement in these flies at dark-light transitions. (C) Data from (B) were analyzed to examine speed in the 30 minutes flanking light:dark and dark:light transitions. Before (top left), after (top right) lights on at ZT0 or before (bottom left), after (bottom right) lights off at ZT12 in *elav*-GS/UAS-*nur* + RU486 (black) or - RU486 (blue) flies. (Mann-Whitney U test, ** p<0.01, **** p<0.0001, n=24 females, respectively). Median ± interquartile is shown in all cases.

Fig. S4. *nur* over-expressing flies show normal food intake and lifespan.

(A) Blue dye feeding assay: *elav*-GS/UAS-*nur* female flies +RU486 (black) and –RU486 (blue) consumption of blue dye within a 24 hour period (Mann-Whitney U test, n.s., n=10 females each). Median ± interquartile is shown. (B) Survival assay. The survival rate of *elav*-GS/UAS-*nur* female flies +RU486 (black) and –RU486 (blue) was assessed until all died (Log rank test, n.s., n=50 and 51 females, respectively).

Fig. S5. NUR is expressed efficiently following metal induction in cultured cells.

(A) The *nemuri* (CG31813) gene (blue) is located on the left arm of the 2nd chromosome, and consists of a single exon. Two potential mRNA transcripts containing UTRs (gray) and coding sequence (519bp) (red) produce a single protein with a 25 amino acid signal sequence at the N terminus (orange). The rest of the protein (brown) has no obvious functional domains.

(B) NUR was expressed in S2R+ cells to assay secretion (Figure 2). Western blot of cell extracts using anti-HA antibodies show expression of proteins with and without induction by Cu^{2+} (-:left lane and +:right lane, respectively)

Fig. S6. NUR functions as an AMP *in vitro* and *in vivo*

(A) Alamar Blue Cell Viability Assay for AMP function *in vitro*. *E.coli* were incubated in PBS alone (middle), or with kanamycin (left) or NUR protein (right) (upper). Kanamycin was used at a 50 μg/ml concentration, which is standard for antibiotics. The assay was quantified by measuring absorbance at 595nm in a spectrophotometer (bottom) (Student t-test, ***p<0.001, n=3). Note *S. marcescens* was used in Figure 3B. (B) Alamar Blue Cell Viability Assay: NUR

protein mixed with or without *E.coli* in a 96 well plate (top). Quantification of the assay by measuring absorbance in a spectrophotometer at 595nm for each sample (bottom). (C) AMP assay *in vivo*. Wild-type flies and *nur* mutants were tested for bacterial load following infection with *S. marcescens*. Box and whisker plots of colony forming units (cfu)/fly in indicated groups immediately (0 hour) or 24 hours after infection (Mann-Whitney U test, *p< 0.03, n=4). Median ± interquartile is shown.

Fig. S7. NUR over-expression promotes survival after infection.

(A) Kaplan-Meier plot depicting survival of infection with *S. pneumoniae* in *nur* over-expressing flies (+RU486: black) and controls (-RU486: blue); ***p<0.001, log rank test, n=88, 89 females, respectively. (B) Effects of RU486 on survival of control flies: Kaplan-Meier plot depicting survival of w^{1118} flies infected with *S. marcescens* in the presence of RU486 (black) and controls (-RU486: blue) (Log rank test, p>0.05, n=38 and 37 females, respectively). (C) Daytime sleep following *S. pneumoniae* infection in *nur* over-expressing flies (+RU486: black) and controls (-RU486: blue); ****p<0.0001 (Student t-test, n=63 and 40 females, respectively). Mean ± SEM is shown.

Fig. S8. Over-expression of *nur* in the fat body does not promote sleep or survival. (A) Total sleep time of S106-GS/UAS-*nur* flies in the presence of RU486 (black) and controls (-RU486: blue); Mann-Whitney U test, n.s. n=16 females for each. Data show median ± interquartile. (B) Survival of S106-GS/UAS-*nur* flies infected with *S. marcescens* in the presence of RU486 (black) and controls (-RU486: blue) (Log rank test, p=0.21, n=46 and 43 females, respectively).

(C) Bacterial load of S106-GS/UAS-*nur* in (+RU486: black) and controls (-RU486: blue) (Mann-Whitney U test, n=9 for each). Data show median ± interquartile.

Fig. S9. Confirmation of *nur* mutant lines.

(A) The *nur* gene carrying attp-DsRed: The figure indicates primers used for genomic DNA PCR to amplify 5' arms (purple primer sets) or 3' (orange primer sets) arms. Hind III sites are indicated by red arrows. Red bar indicates the location of the probe used for Southern blotting. (B) PCR bands detected using 5' side primers (shown in A in purple). lane1: *nur²*; lane2: *nur³*; lane3: iso31; lane 4: DNA Ladder (left). 3' side primers (shown in A in orange) lane5: DNA ladder; lane6: *nur²*; lane7: *nur³* (right). Each band was extracted from the gel and sequenced to verify the integrity of the product. (C) Southern blot analysis of genomic DNA extract digested by Hind III (shown by the arrow: 1.6 kbp). The probe was prepared as shown in A and specific bands were detected in *nur²* and *nur³* but not in wild-type controls (iso31) as indicated by the orange arrow. lane 1: positive control, anti-sense of the DsRed probe; lanes 2 and 3: DNA Ladders; lane 4: iso31 genomic DNA; lane5: *nur²* genomic DNA; lane 6: *nur³* genomic DNA. (D) Confirmation of the absence of *nur* gene in the *nur* mutant. PCR bands were detected in genomic DNA from wild-type but not from *nur* mutants. lane 1: DNA Ladder; lane 2: wild-type control (iso31); lane 3: *nur²*; lane 4: *nur³*. *actin5c* was amplified from each genotype as a loading control (bottom).

Fig. S10. Sleep profile in *nur* mutant flies

(A) Sleep pattern of nur^3 mutants (red) or wild-type controls (iso31) (black) in males (left) or females (right). Total sleep time is indicated below. (B) Sleep time during the day, night and 24 hour period in nur^3 mutants (red) and wild-type (black) (Mann-Whitney U test, *p<0.05, n=16,15 for wild-type and nur^3 mutants, respectively). (C) Sleep during the day, night and 24 hour period in nur^2 mutants (red) and wild-type (black) (Mann-Whitney U test, *p<0.05, n=16 for each wild-type and nur^2 mutants, respectively). Median ± interquartile are shown in all cases.

Fig. S11. Effects of *nur* on sleep consolidation

nur mutants were assayed for sleep bout length and sleep bout number, as measures of consolidation. Data are shown for day and night sleep in males and females. (Mann-Whitney U test, *p<0.05, **p<0.01, ***p<0.001, n=16, 16, 15 for wild-type, nur^2 or nur^3 mutants, respectively). Median ± interquartile is shown.

Fig. S12. *nur* mutants show altered response to arousing sensory stimuli

(A) The average speed of aroused flies after a light pulse of 10 seconds. The data are the average speed over 30 minutes for all flies aroused by the initial pulse, while Figure 4E only reports speed of each fly until it falls asleep. $nur^2/+$ (black), $nur^3/+$ (grey) or nur^2/nur^3 (red). Arrows indicate the time of light pulse. (B) Percent flies awakened by a concentrated odor, 3-Octanol (Fisher's exact test, ***p<0.001, **** p<0.0001, n=98, 121, 108 females for $nur^2/+$, $nur^3/+$ or nur^2/nur^3, respectively).

Fig. S13. Response of *nur* mutants to sleep-inducing stimuli.

(A) Duration of recovery sleep from ZT0 to ZT6, assayed as the increase in sleep relative to sleep during this time pre-deprivation, following 6 hours of night-time sleep deprivation. (Mann-Whitney U test, $p>0.05$, n=64, 64, 63 for $nur^2/+$, $nur^3/+$ or nur^2/nur^3 females, respectively). Median ± interquartile is shown. (B-C) Sleep induced between ZT0 and ZT6 following bacterial infection at ZT18. Induced sleep was calculated as the difference in sleep between post-infection and pre-infection days relative to the difference in controls that were handled but not infected. (B) *S. marcescens* was used for infection (Mann-Whitney U test, ***$p<0.001$, n=115, 113 for $nur^2/+$ or nur^2 mutants, respectively). (C) *E.coli* was used for infection (Mann-Whitney U test, **$p<0.01$, n=46, 48 for $nur^2/+$ or nur^2 mutants, respectively. Median ± interquartile is shown.

Fig. S14. Generation and characterization of nur^{Gal4}

(A) Schematic depicting the strategy for generation of nur^{Gal4} by scarless genome engineering using CRISPR. Arrow indicates the region targeted by gRNA. Homology arms (~1kbp: blue) are flanked by pBac recognition sites-3xP3 (eyeless promotor)-DsRed-pBac recognition sites with Gal4 sequences. After the targeting, tub-pBac was crossed in to excise the pBac recognition sites to finally generate nur^{Gal4}. Successful integration and pBac excision were confirmed by PCR using the sets of primers indicated by arrows (blue, green and purple) (B) Expression of nur^{GAL4} in the brain. Scarless nur^{GAL4} was used to drive expression of CD4:tdGFP. Expression of GFP (arrows) in the dFSB is indicated in the boxed region amplified on the right. Neuropil is stained with nc82 (magenta). Scale bar indicates 50µm. (C) Sleep values are plotted against zeitgeber time, following bacterial infection with *S. marcescens* at ZT18. (Two-way ANOVA followed by

Bonferroni's multiple comparison test, **p<0.01, ***p<0.001, ****p<0.0001, n=42, 44 and 38 females for *nur*Gal4/UAS-Kir2.1(red), *nur*Gal4/+(gray), and UAS-Kir2.1/+(black), respectively.) Mean±SEM is shown.

Fig. S15. Induction of *nur* following infection. (A) *nur* expression normalized to actin was assayed via qPCR in whole fly bodies, 6 or 21 hours after infection at ZT18. (Student's t-test, ***p<0.001, n=3). Note that *nur* is essentially undetectable at baseline (Figure 5A). Mean ± standard error is shown. (B) CD4::td-GFP expression driven by *nur*Gal4was assayed in the brain 21h after infection. Expression was detected in 3 out of 10 brains. Neuropil is indicated by nc82 staining.

Fig. S16. Verification of NUR antibodies and localization of NUR in the dFSB region.

(A) Schematic for antibody generation against the NUR protein. Antibodies were generated to recognize NUR (25aa-172aa) (brown). Signal peptide sequence is in orange. (B) Western blot of NUR::GFP transfected into S2R+ cells. The blot was probed with anti-GFP (1:500) (left) and anti-NUR (1:1,000) (right) after stripping. Arrow indicates NUR::GFP (46kDa) -:uninduced,+:induced by Cu^{2+}. Both are 1 minute exposures. (C) Immunostained images of *elav*-GS/UAS-*nur* +RU486 (upper) or –RU486 (lower). The brains were stained with anti-NUR (green) (left) and nc82 (neuropil marker, magenta) (middle); the merged image is on the right. Expression of NUR in the FSB (arrowheads) or peduncles of mushroom body neurons (arrow) and other areas of the brain was observed in RU486-treated flies. Scale bars indicate 50µm. (D) Immunostaining with anti-NUR (top) in unperturbed wild-type controls (left) as well as in the

nur mutant with (right) or without SD (middle). nc82 staining is shown to indicate neuropil structures for each brain (bottom). Arrowheads indicate the location of the FSB. Scale bars indicate 50μm.

Fig. S17. Characterization of NUR expression with different Gal4 drivers

(A) Expression of transgenic NUR in Gal4 lines that do not induce sleep when over-expressing *nur*. Flies over-expressing NUR-HA were assayed for expression of NUR by staining whole brains with an anti-HA antibody (top). Phalloidin was used as a counter-stain to localize the dFSB (middle). Note little expression of NUR in the dFSB region. Scale bars indicate 20μm. (B) Expression of Synaptotagmin::GFP (Syt::GFP) in Gal4 lines that do not induce sleep when over-expressing *nur*. The region shown is that of the FSB. Syt::GFP staining was not detected in or near the FSB for any of these lines. nc82 was stained to detect neuropil (middle). Scale bars indicate 20μm.

Fig. S18. NUR does not signal through sleep-promoting dFSB neurons labeled by the 23E10 driver

(A) 23E10+ neurons were labeled with GFP (top) stained with anti-NUR antibody (middle) and merged image (bottom). Images in the box (left) are enlarged (right). NUR was detected in the FSB, but not in 23E10+ cells. Arrowheads indicate NUR that does not co-localize with GFP. Scale bars indicate 20μm. (B) 23E10 neurons were silenced by driving Kir2.1, an inward rectifier potassium channel, with the LexA system. NUR was simultaneously over-expressed by driving

UAS-*nur* expression with an *elav*-GS driver in the presence of RU486 (top). Controls without RU486 are at the bottom. Flies in which *nur* overexpression occuured in conjunction with silencing of 23E10+ dFSB neurons (red) were compared to controls, 23E10-LexA (black) or LexAop-Kir2.1 (gray). Note that *nur* is overexpressed in all flies in the top panel. Number of flies tested is indicated below. (Mann-Whitney U test, $p>0.05$). Median ± interquartile is shown.

Supplemental Tables:

Table S1. Lines assayed in over-expression screen for sleep genes. Each line contains a UAS element, either randomly inserted into the genome or as a transgene driving a specific gene.

Table S2. Gal4 lines assayed for their ability to increase sleep when driving *nur* expression. Those that increased sleep are indicated by plus signs (++) underneath the name of the line.

Table S3. List of PCR primers used in this study.

Fig. S1

A

Male

Female

1178 minutes
1065 minutes

1020 minutes
853 minutes

— +RU486
— -RU486

B

Male

Day   Night   Total

****   ****   ****

Sleep time

+   -   +   -   +   -

Female

****   ****   ****

Sleep time

+   -   +   -   +   -

C

anti-sense   sense
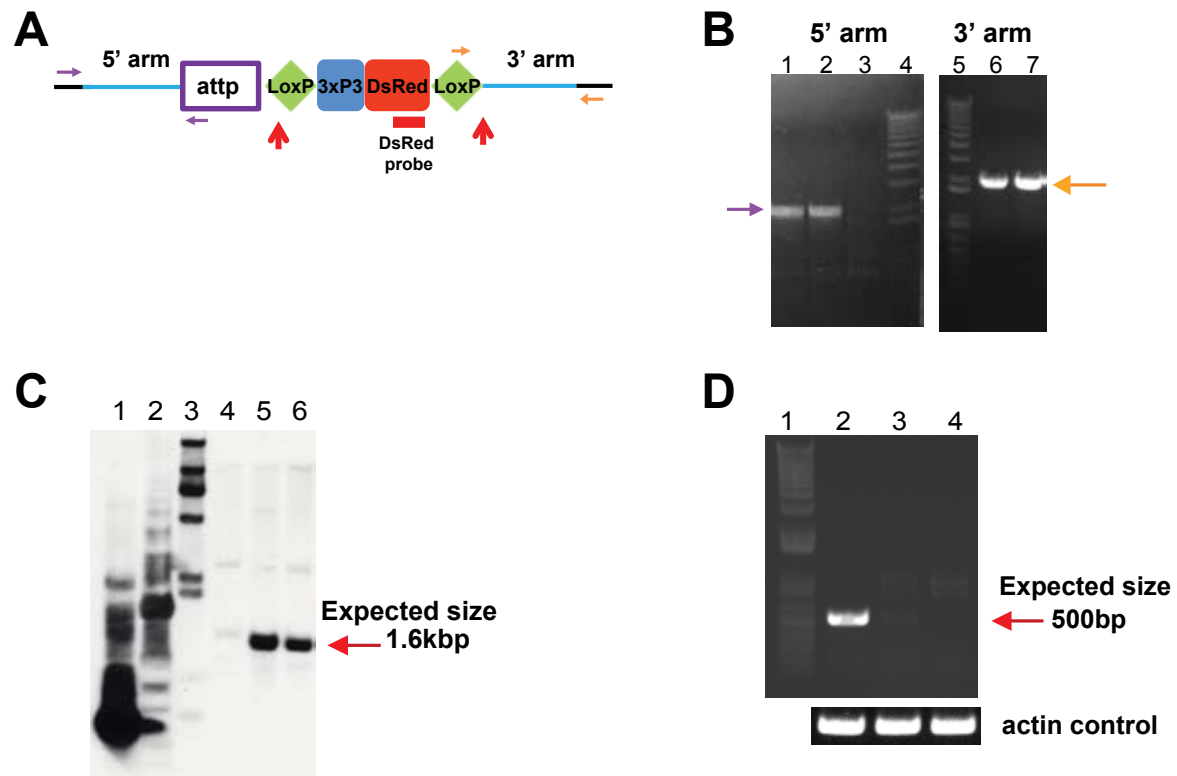
Fig. S2

A

Period / FFT

B

Fig. S3

A

B

C

Fig. S4

A

B

Fig. S5

A

2L   *nemuri* (CG31813)   893bp

500bp

Signal peptide

1aa   25aa   172aa

B

Cell extract

	−	+
AP		
NUR::AP		
BiP::AP		

anti-HA

Fig. S6

A

E. coli

B

NUR protein (uM)

C

Fig. S7

A

S. pneumoniae

***p<0.001

+RU486
-RU486

Survival Probability

Hours post-infection

B

S. marcescens

n.s.

+RU486
-RU486

Survival Probability

Hours post-infection

C

S. pneumoniae

****

(min)

Day time sleep

+   −

Fig. S8

Fig. S9

A

B

5' arm   3' arm

1 2 3 4   5 6 7

C

1 2 3 4 5 6

Expected size
1.6kbp

D

1 2 3 4

Expected size
500bp

actin control

Fig. S10

A

B

C

Fig. S11

A   Male

B   Female

C   Male

D   Female

Fig. S12

A

B

Fig. S13

A

(min)

n.s.

Sleep recovery time

400

300

200

100

0

-100

● nur^2/ +

■ nur^3/ +

▲ nur^2/ nur^3

B

S. marcescens

***

(min)

Sleep induced

100

50

0

nur^2/+ nur^2 mutant

C

E.coli

**

(min)

Sleep induced

100

50

0

nur^2/+ nur^2 mutant

Fig. S14

A

B

C

Fig. S15

A

B

Fig. S16

A

Signal peptide

B

After Strip

Induction   − +   − +   (kDa)
                              —250
                              —150
                              —100
                              —75

NUR::GFP →   —50
(46kDa)
                              —37

                              —25
                              —20
                              —15
                              —10

anti-GFP   anti-NUR

C

+RU486

NUR   nc82   Merge

−RU486

NUR   nc82   Merge

D

Control   *nur* mutant   *nur* mutant after SD

NUR   NUR   NUR

nc82   nc82   nc82

Fig. S17

Fig. S18

A

B

+RU486

Male

n.s

Sleep time (min)

1400
1200
1000
800

n=31   n=32   n=32

23E10-LexA/+   LexAop-Kir2.1/+   23E10-LexA LexAop-Kir2.1

Female

n.s

Sleep time (min)

1000
500
0

n=31   n=32   n=30

23E10-LexA/+   LexAop-Kir2.1/+   23E10-LexA LexAop-Kir2.1

-RU486

n.s

Sleep time (min)

1400
1200
1000
800

n=32   n=32   n=22

23E10-LexA/+   LexAop-Kir2.1/+   23E10-LexA LexAop-Kir2.1

n.s

(min)

1000
500
0

n=30   n=26   n=26
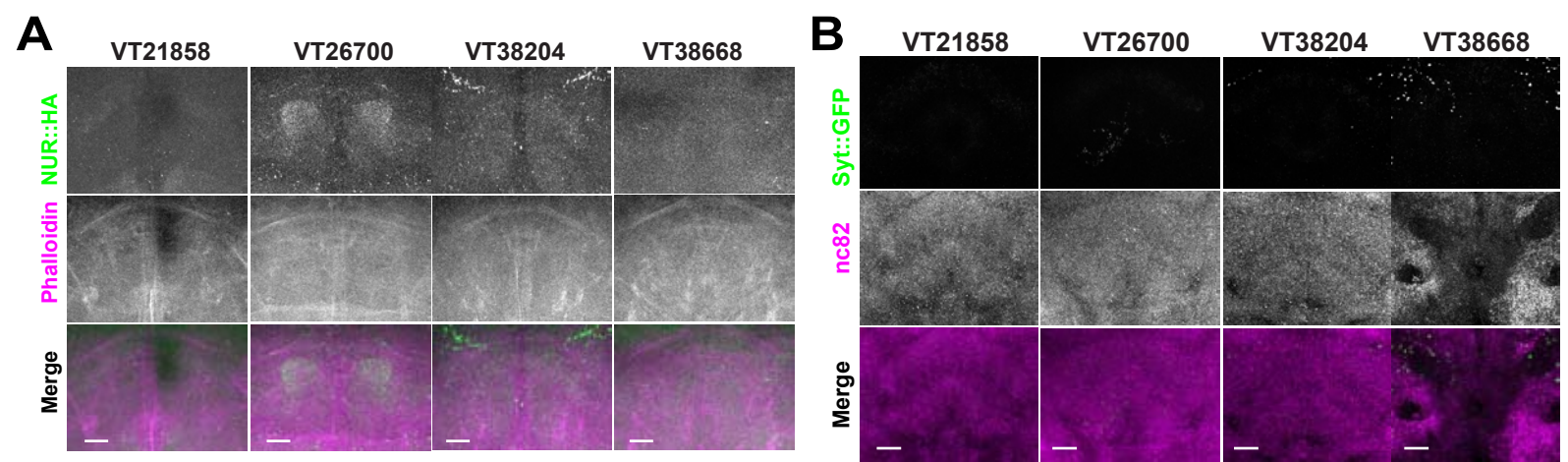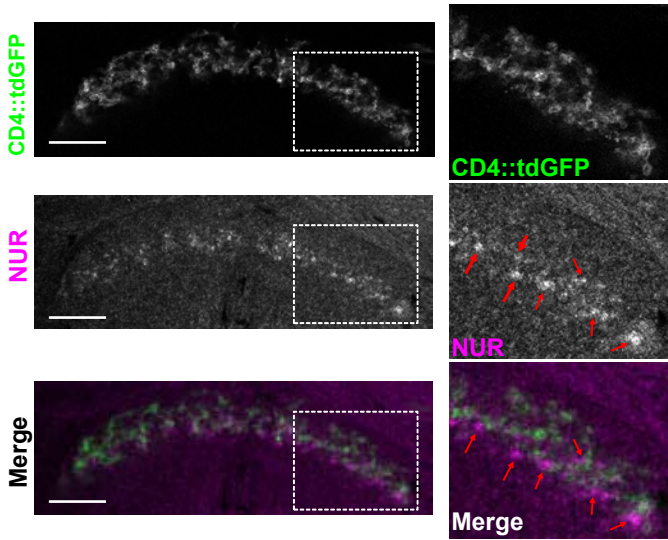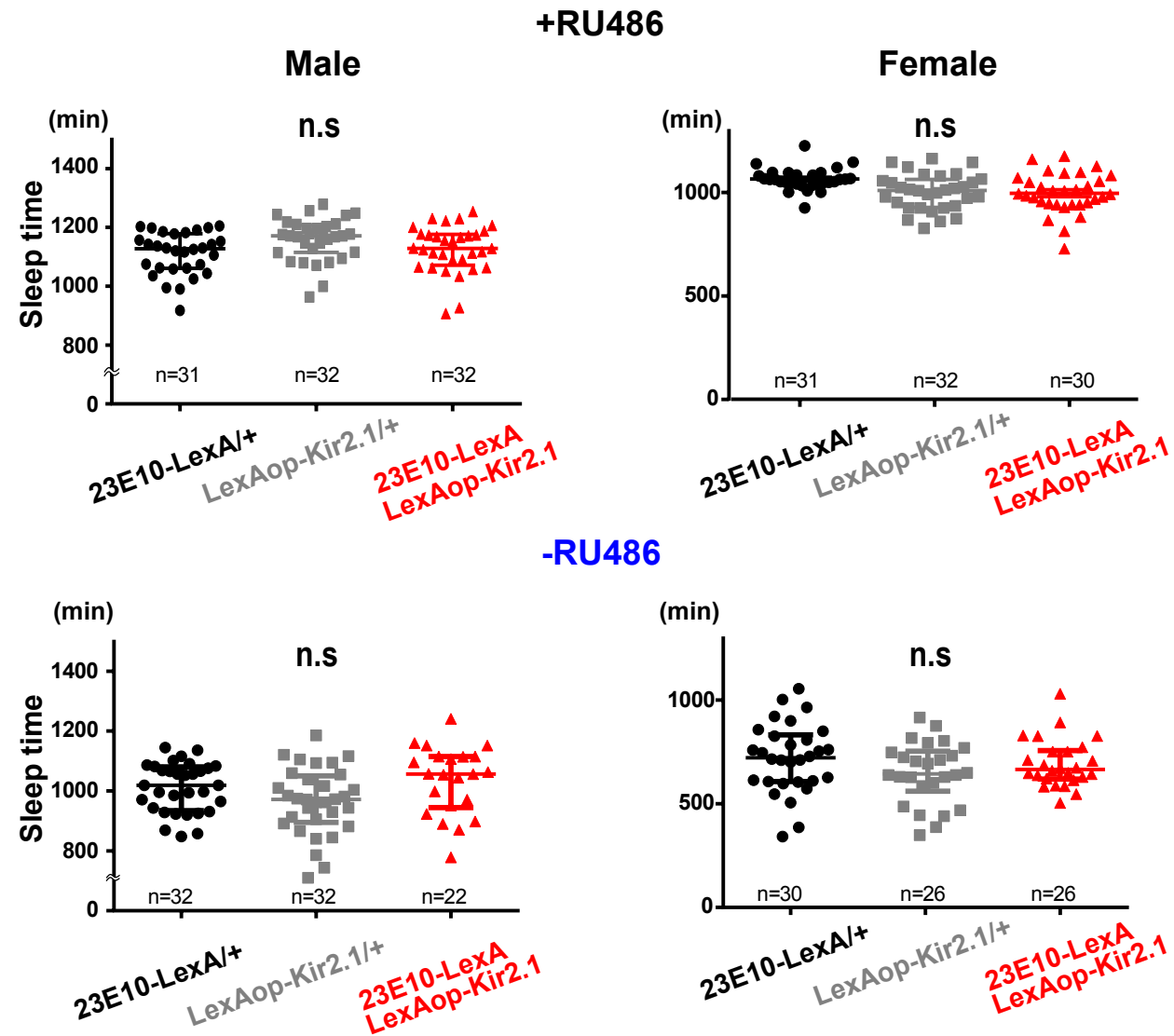
23E10-LexA/+   LexAop-Kir2.1/+   23E10-LexA LexAop-Kir2.1

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