Figure S1

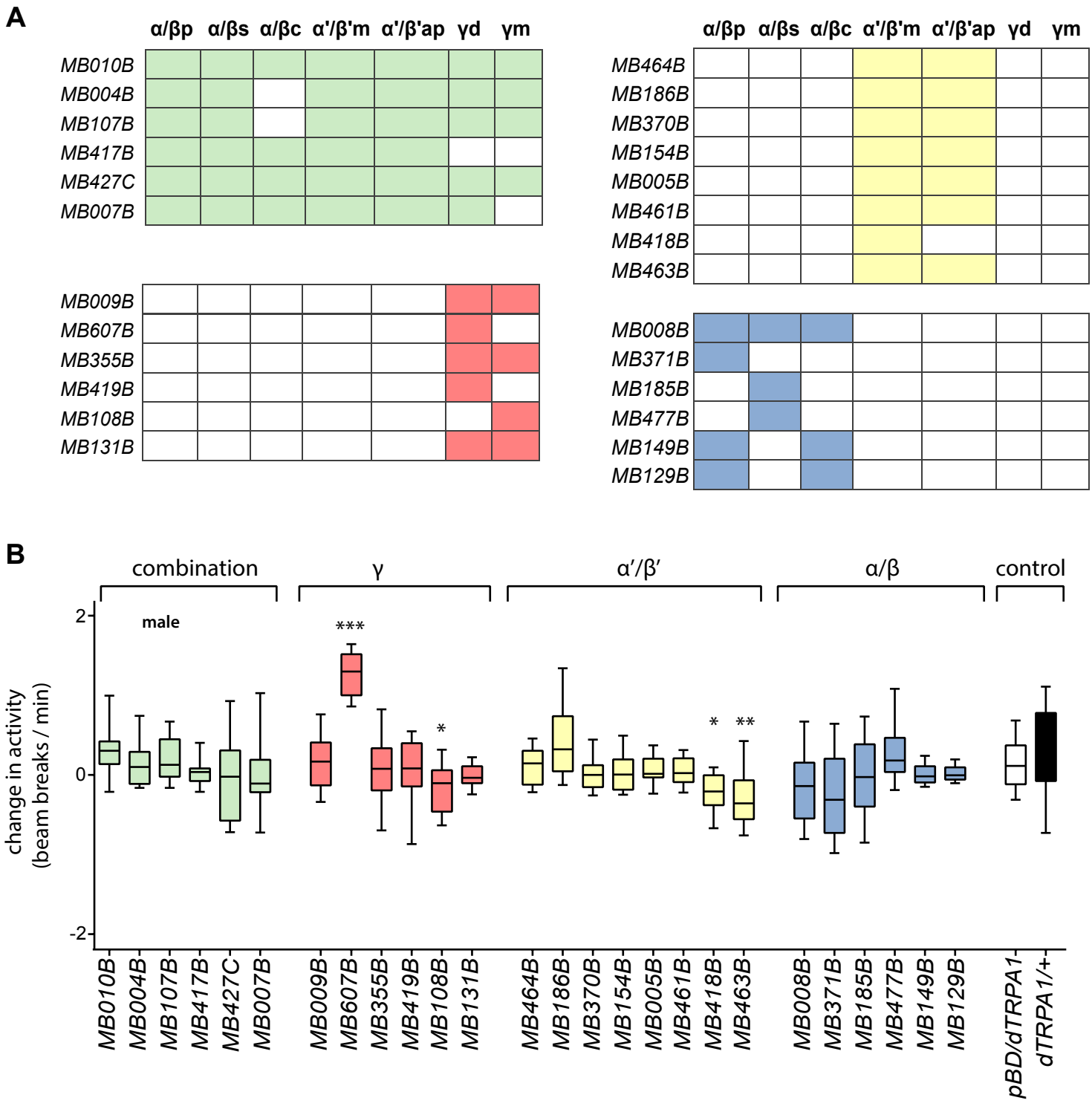
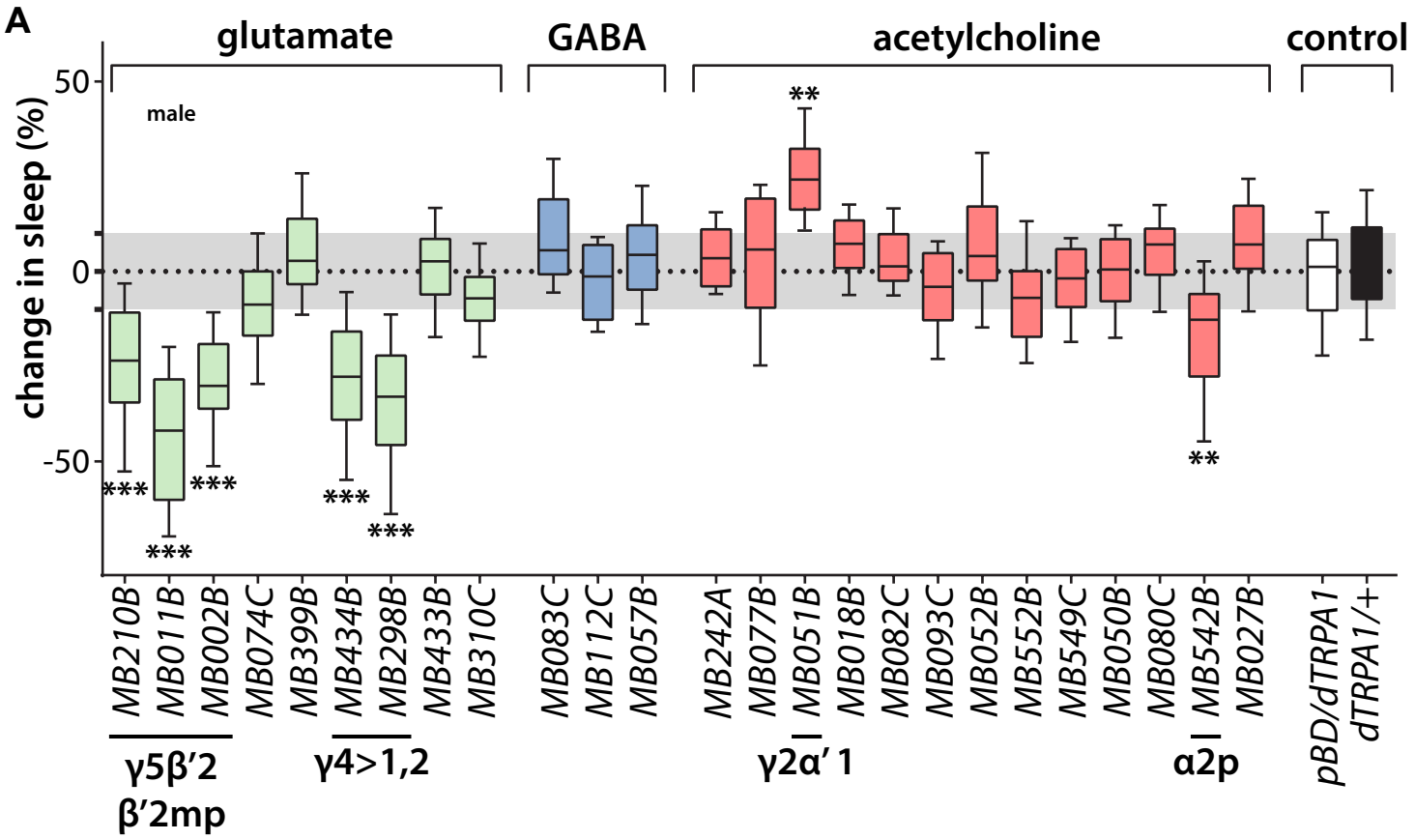

A
α/βp α/βs α/βc α'/β'm α'/β'ap γd γm
MB010B
MB004B
MB107B
MB417B
MB427C
MB007B
MB009B
MB607B
MB355B
MB419B
MB108B
MB131B
α/βp α/βs α/βc α'/β'm α'/β'ap γd γm
MB464B
MB186B
MB370B
MB154B
MB005B
MB461B
MB418B
MB463B
MB008B
MB371B
MB185B
MB477B
MB149B
MB129B
B
combination
γ
α′/β′
α/β
control
male
change in activity (beam breaks / min)
2
0
-2
***
*
*
**
MB010B
MB004B
MB107B
MB417B
MB427C
MB007B
MB009B
MB607B
MB355B
MB419B
MB108B
MB131B
MB464B
MB186B
MB370B
MB154B
MB005B
MB461B
MB418B
MB463B
MB008B
MB371B
MB185B
MB477B
MB149B
MB129B
pBD/dTRPA1
dTRPA1/+

Figure S2


A
glutamate
GABA
acetylcholine
control
male
change in sleep (%)
50
0
-50
MB210B
MB011B
MB002B
MB074C
MB399B
MB434B
MB298B
MB433B
MB310C
MB083C
MB112C
MB057B
MB242A
MB077B
MB051B
MB018B
MB082C
MB093C
MB052B
MB552B
MB549C
MB050B
MB080C
MB542B
MB027B
pBD/dTRPA1
dTRPA1/+
γ5β′2
β′2mp
γ4>1,2
γ2α′1
α2p

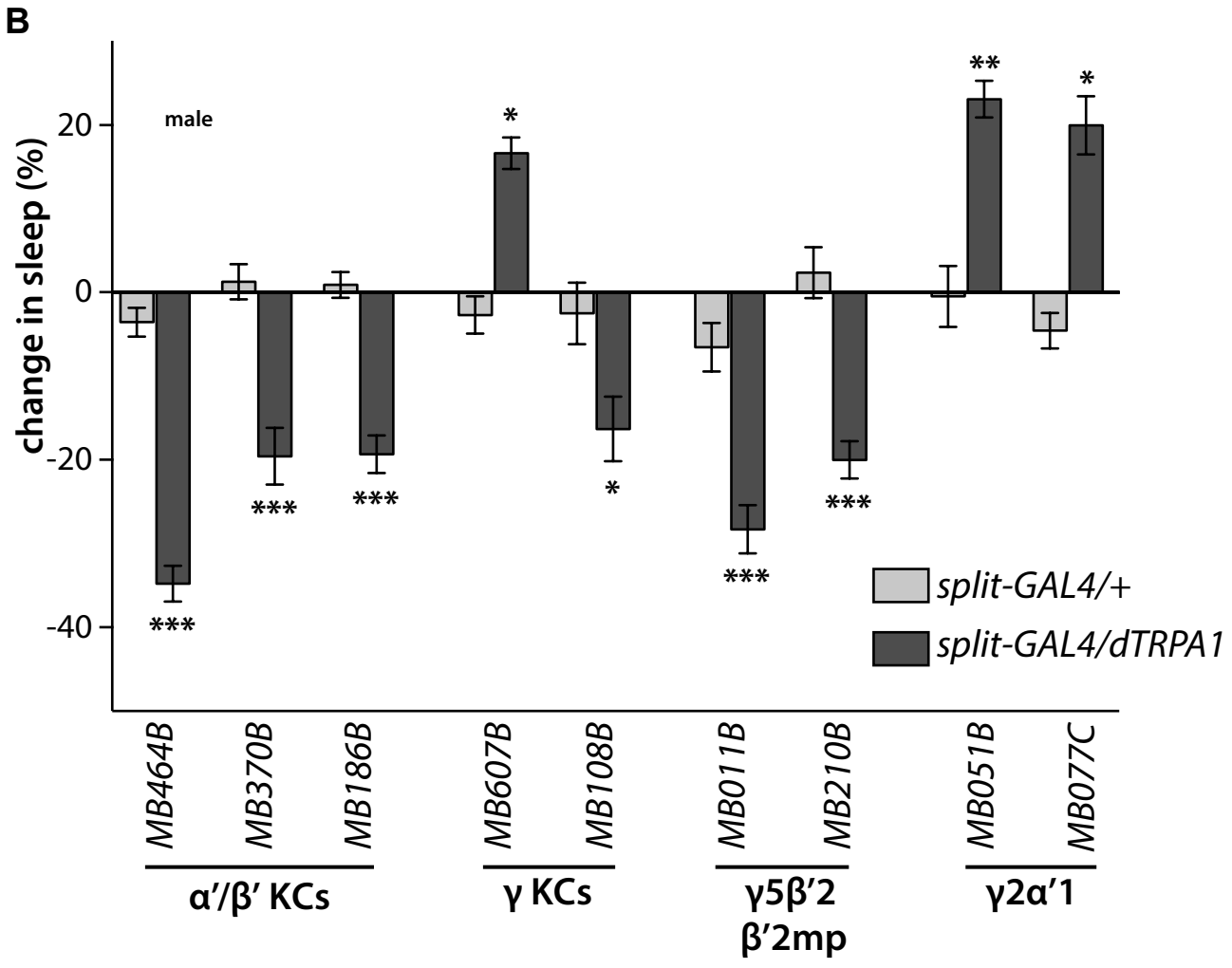

B
male
change in sleep (%)
20
0
-20
-40
split-GAL4/+
split-GAL4/dTRPA1
MB464B
MB370B
MB186B
MB607B
MB108B
MB011B
MB210B
MB051B
MB077C
α′/β′ KCs
γ KCs
γ5β′2
β′2mp
γ2α′1

Figure S3

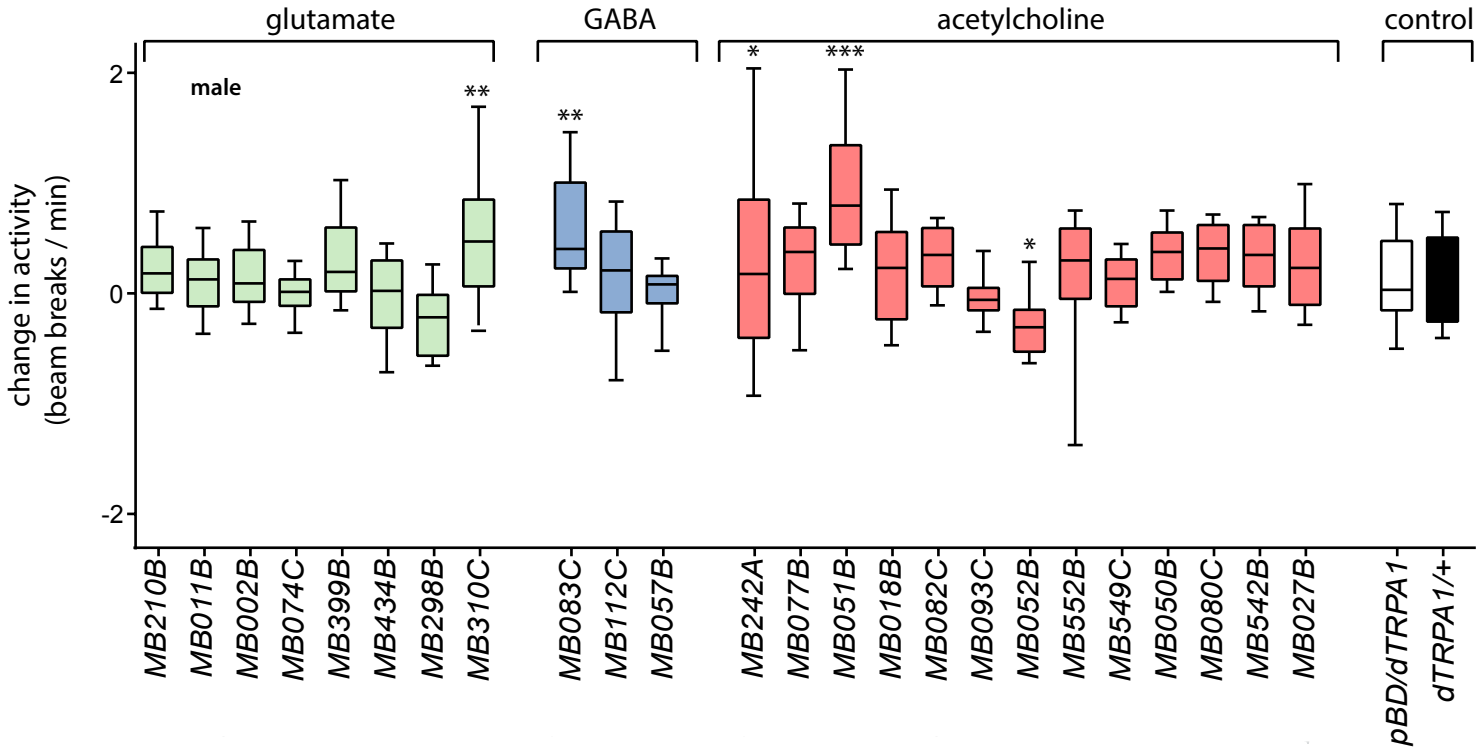

glutamate
GABA
acetylcholine
control
male
change in activity
(beam breaks / min)
2
0
-2
*
***
**
**
*
MB210B
MB011B
MB002B
MB074C
MB399B
MB434B
MB298B
MB310C
MB083C
MB112C
MB057B
MB242A
MB077B
MB051B
MB018B
MB082C
MB093C
MB052B
MB552B
MB549C
MB050B
MB080C
MB542B
MB027B
pBD/dTRPA1
dTRPA1/+

Figure S4

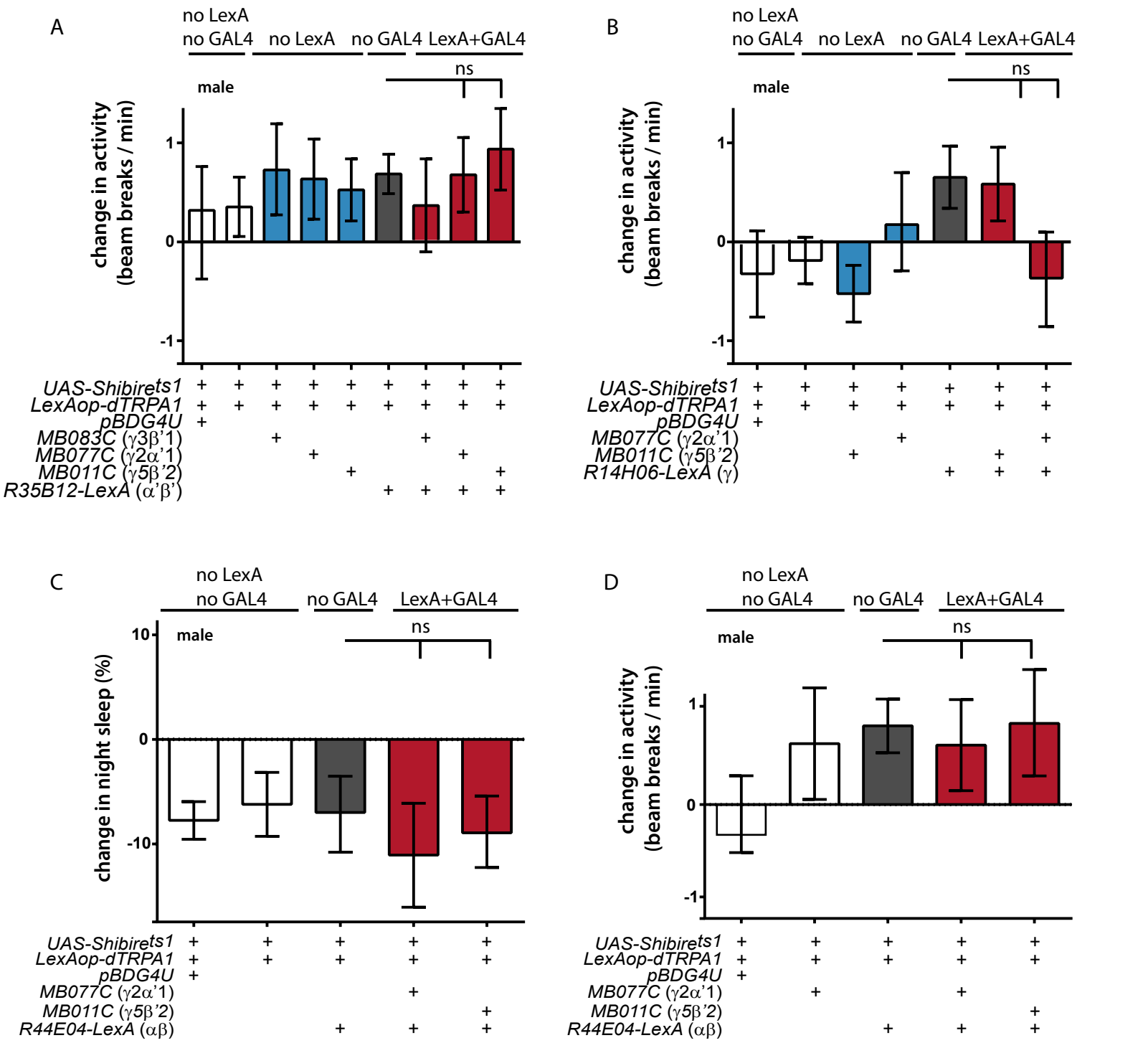

A
no LexA
no GAL4
no LexA
no GAL4
LexA+GAL4
ns
male
change in activity
(beam breaks / min)
1
0
-1
UAS-Shibirets1 + + + + + + + + +
LexAop-dTRPA1 + + + + + + + + +
pBDG4U +
MB083C (γ3β'1) + +
MB077C (γ2α'1) + +
MB011C (γ5β'2) + +
R35B12-LexA (α'β') + + +
B
no LexA
no GAL4
no LexA
no GAL4
LexA+GAL4
ns
male
change in activity
(beam breaks / min)
1
0
-1
UAS-Shibirets1 + + + + + + +
LexAop-dTRPA1 + + + + + + +
pBDG4U +
MB077C (γ2α'1) + +
MB011C (γ5β'2) + +
R14H06-LexA (γ) + + +
C
no LexA
no GAL4
no GAL4
LexA+GAL4
ns
male
change in night sleep (%)
10
0
-10
UAS-Shibirets1 + + + + +
LexAop-dTRPA1 + + + + +
pBDG4U +
MB077C (γ2α'1) +
MB011C (γ5β'2) +
R44E04-LexA (αβ) + + +
D
no LexA
no GAL4
no GAL4
LexA+GAL4
ns
male
change in activity
(beam breaks / min)
1
0
-1
UAS-Shibirets1 + + + + +
LexAop-dTRPA1 + + + + +
pBDG4U +
MB077C (γ2α'1) + +
MB011C (γ5β'2) +
R44E04-LexA (αβ) + + +

Figure S5

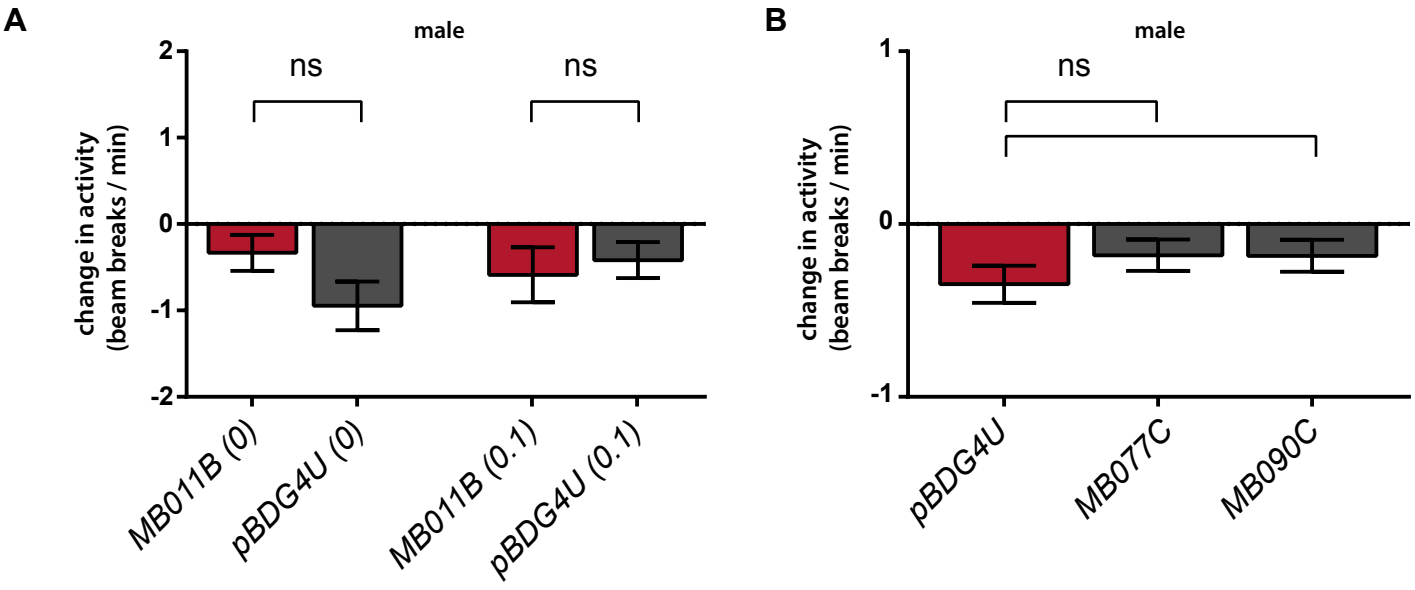
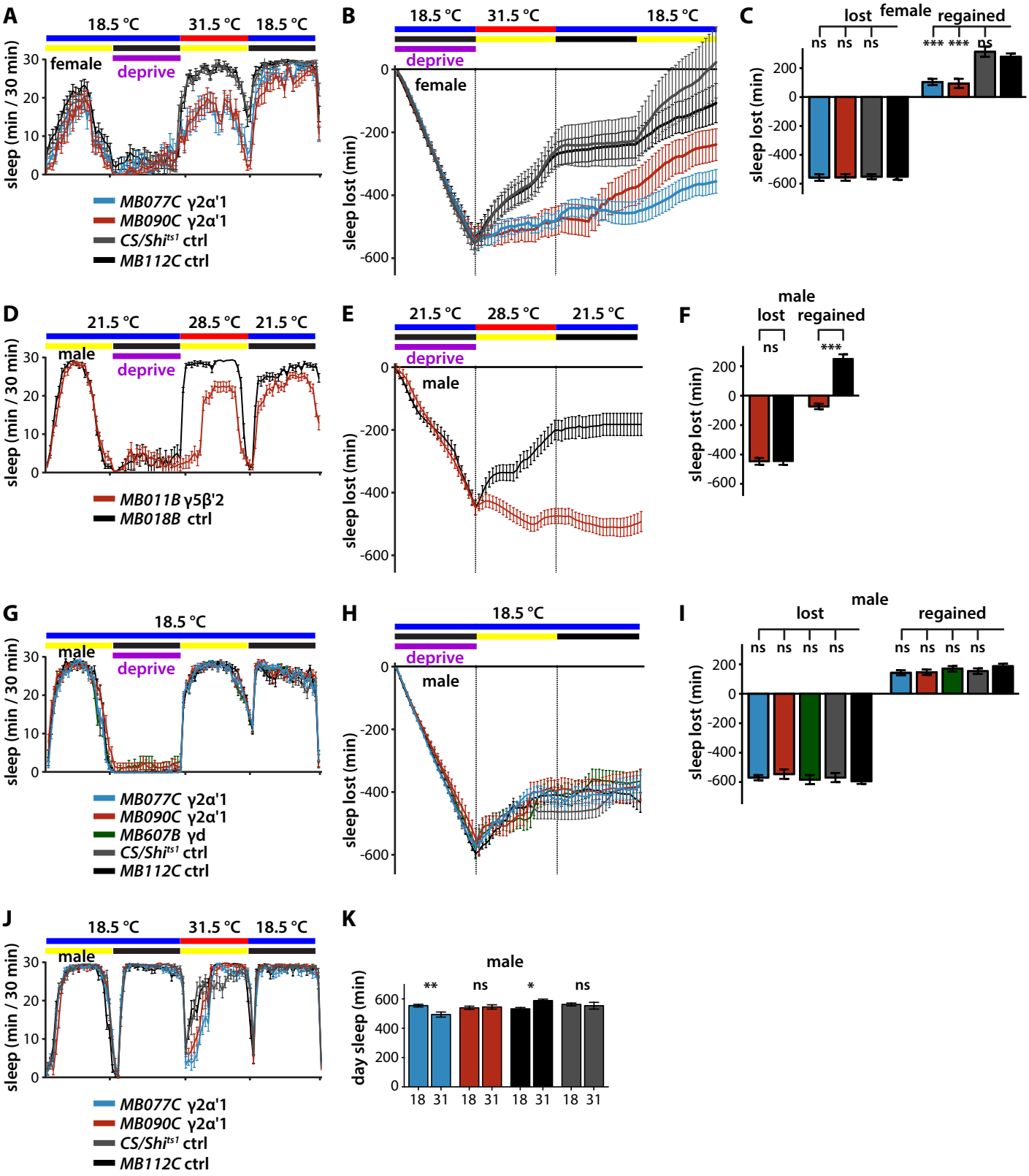

A
male
ns
ns
change in activity
(beam breaks / min)
2
1
0
-1
-2
MB011B (0)
pBDG4U (0)
MB011B (0.1)
pBDG4U (0.1)
B
male
ns
change in activity
(beam breaks / min)
1
0
-1
pBDG4U
MB077C
MB090C

Figure S6


A
18.5 °C
31.5 °C
18.5 °C
female
deprive
sleep (min / 30 min)
MB077C γ2α'1
MB090C γ2α'1
CS/Shi^ts1 ctrl
MB112C ctrl
B
18.5 °C
31.5 °C
18.5 °C
deprive
female
sleep lost (min)
C
lost
female
regained
ns ns ns
*** *** ns
sleep lost (min)
D
21.5 °C
28.5 °C
21.5 °C
male
deprive
sleep (min / 30 min)
MB011B γ5β'2
MB018B ctrl
E
21.5 °C
28.5 °C
21.5 °C
deprive
male
sleep lost (min)
F
male
lost
regained
ns
***
sleep lost (min)
G
18.5 °C
male
deprive
sleep (min / 30 min)
MB077C γ2α'1
MB090C γ2α'1
MB607B γd
CS/Shi^ts1 ctrl
MB112C ctrl
H
18.5 °C
deprive
male
sleep lost (min)
I
male
lost
regained
ns ns ns ns
ns ns ns ns
sleep lost (min)
J
18.5 °C
31.5 °C
18.5 °C
male
sleep (min / 30 min)
MB077C γ2α'1
MB090C γ2α'1
CS/Shi^ts1 ctrl
MB112C ctrl
K
male
** ns * ns
day sleep (min)
18 31 18 31 18 31 18 31

SUPPLEMENTAL FIGURE LEGENDS

Figure S1 (related to Figure 1): **Waking locomotor activity and cell type descriptions of KC split-GAL4 lines**

(A) Detailed description of cell classes targeted by each KC split-GAL4 line defined by their lobe-specific projections. Any detectable expression with *pJFRC225-5xUAS-IVS-myr::smGFP-FLAG* reporter in VK00005 in female brains is indicated.

(B) Change in locomotor activity while awake of male flies induced by activation of KC subsets targeted by the indicated split-GAL4 driver lines to express dTRPA1 temperature-gated depolarizing cation channel. Change in activity is defined as the difference between average beam breaks / min during periods of wakefulness on day 2 (28.5°C) and day 1 (21.5°C) of the experiments. Sleep-promoting γd KCs (*MB607B*) exhibit a significant increase in waking activity while some wake-promoting lines targeting α'/β' or γm KCs (*MB418B, MB463B, MB108B*) exhibit reduced waking activity. Midline, box boundaries, and whiskers represent median, quartiles, and 10^{th} and 90^{th} percentiles, respectively. split-GAL4 lines are grouped by the indicated lobe-specific projections of their targeted KC subsets. Statistical comparison of each split-GAL4 line to an enhancerless GAL4 (*pBDG4U*) is by Kruskal-Wallis one-way ANOVA and Dunnett's post-hoc correction for multiple comparisons **(***, $p<0.05$; **, $p<0.01$, ***, $p<0.001$; n=24-46 flies for each genotype).

Figure S2 (related to Figure 2): Screen of MBON split-GAL4 lines in male flies

(A) Change in sleep of male flies induced by activation of KC subsets targeted by the indicated split-GAL4 driver lines to express dTRPA1 temperature-gated depolarizing cation channel.

(B) Independent confirmation of each KC and MBON *split-GAL4*/*UAS-dTRPA1* screen hit in comparison to each *split-GAL4/+* negative control genotype that possesses the two split-GAL4 driver transgenes but lacks the *UAS-dTRPA1* effector transgene. Each *split-GAL4/UAS-dTRPA1* genotype was compared to the corresponding *split-GAL4/+* and to *UAS-dTRPA1/+* by Kruskal-Wallis one-way ANOVA with Dunnett's post-hoc correction. This generated two p-values, and the most conservative/higher p-values are reported on the graph (*, $p<0.05$; **, $p<0.01$, ***, $p<0.001$; n=17-24 flies for each genotype).

Figure S3 (related to Figure 2): Waking locomotor activity of MBON split-GAL4 lines

(A) Change in waking locomotor activity of male flies induced by activation of MBON subsets targeted by the indicated split-GAL4 driver lines. Sleep-promoting lines (*MB242A, MB051B*) exhibit a significant increase in waking locomotor activity. Quantification and statistical analysis as in Figure S1 (n= 23-59 flies for each genotype).

Figure S4 (related to Figures 3 and 4): Waking locomotor activity of flies with simultaneous manipulation of KCs and MBONs

(A) Change in locomotor activity while awake at night of male flies induced by simultaneous activation of α'/β' KCs and inhibition of MBON synaptic outputs after temperature shift from 18.5°C to 31.5°C (mean±sem). Statistical comparisons were by one-way ANOVA followed by Dunnett's pairwise comparisons (n=48-62 flies per genotype).

(B) Change in locomotor activity while awake at night of male flies induced by simultaneous activation of γ KCs and inhibition of MBON synaptic outputs after temperature shift from 18.5°C to 31.5°C (mean±sem). Quantification and statistical analysis as in **(A)** (n=48-79 flies per genotype).

(C) Change in night sleep of male flies induced by simultaneous activation of α/β KCs and inhibition of MBON synaptic outputs after temperature shift from 18.5°C to 31.5°C (mean±sem). Quantification and statistical analysis as in **(A)** (n=24-32 flies per genotype).

(D) Change in locomotor activity while awake at night of male flies induced by simultaneous activation of α/β KCs and inhibition of MBON synaptic outputs after temperature shift from 18.5°C to 31.5°C (mean±sem). Quantification and statistical analysis as in **(A)** (n=24-32 flies per genotype).

Figure S5 (related to Figure 6): Waking locomotor activity with silenced MBONs and altered sleep pressure

(A) Change in locomotor activity while awake at night of male flies induced by inhibition of MBON-γ5β′2a/β′2mp/β′2mp_bilateral synaptic outputs after temperature shift from 18.5°C to 31.5°C in the presence of CBZ. Quantification and statistical analysis as in **Figure S4** (n=46-55 flies per genotype).

(B) Change in locomotor activity while awake during the day of male flies induced by inhibition of MBON-γ2α'1 synaptic outputs after sleep deprivation and temperature shift from 18.5°C to 31.5°C. Quantification and statistical analysis as in (A) (n=62-64 flies per genotype).

Figure S6 (related to Figure 6): Effects of thermogenetic activation/silencing of MBONs on homeostatic sleep rebound

(A) Sleep profiles of female flies expressing Shibire^{ts1} in sleep-promoting MBON-γ2α'1 deprived of sleep for one night at 18.5°C and then shifted to 31.5°C for the following day. Synaptic silencing of MBON-γ2α'1 using either of two split-GAL4 drivers potently suppressed homeostatic rebound sleep induced by sleep deprivation, in comparison to control flies

expressing Shibire^{ts1} in a GABAergic MBON that doesn't influence sleep (*MB112C*) or control flies bearing the *UAS-Shi^{ts1}* transgene but lacking any split-GAL4 driver.

(B) Cumulative sleep lost during sleep deprivation at 18.5°C and regained during subsequent recovery for 12 hours at 31.5°C and then 24 hours 18.5°C (mean±sem). Genotypes are color coded as in (A).

(C) Quantification of sleep lost during deprivation and regained during the 12 hours of recovery at 31.5°C. Homeostatic rebound in female flies was potently suppressed by Shibire^{ts1}-mediated synaptic silencing of sleep-promoting MBON-γ2α'1 using either the *MB077C* or *MB090C* split-GAL4 drivers. Genotypes are color coded as in (A); statistical analysis was by one-way ANOVA and Dunnett's paired-comparison test (n=22-24 flies per genotype).

(D) Sleep profiles of male flies expressing dTRPA1 in wake-promoting MBON-γ5β′2a/β′2mp/β′2mp_bilateral deprived of sleep for one night at 21.5°C and then shifted to 28.5°C for the following day to activate MBON-γ5β′2a/β′2mp/β′2mp_bilateral during homeostatic rebound. Activating MBON-γ5β′2a/β′2mp/β′2mp_bilateral potently suppressed homeostatic rebound sleep induced by sleep deprivation, in comparison to control flies expressing dTRPA1 in a cholinergic MBON that doesn't influence sleep (*MB018B*).

(E) Cumulative sleep lost during sleep deprivation at 21.5°C and regained during subsequent recovery for 12 hours at 28.5°C and then 12 hours 21.5°C (mean±sem). Genotypes are color coded as in (D).

(F) Quantification of sleep lost during deprivation and regained during the 12 hours of recovery at 28.5°C. Homeostatic rebound in male flies was potently suppressed by dTRPA1-mediated activation of wake-promoting MBON-γ5β′2a/β′2mp/β′2mp_bilateral. Genotypes are color coded

as in (D); statistical analysis was by one-way ANOVA and Dunnett's paired-comparison test (n=32 flies per genotype).

(G, H, I) Control experiment in which male flies with the indicated split-GAL4 transgenes and *UAS-Shi^{ts1}* transgene were deprived of sleep for one night, but not temperature shifted during rebound the following day. There was no difference between any of these genotypes in homeostatic rebound sleep. Statistical analysis was by one-way ANOVA and Dunnett's paired-comparison test (n=26-31 flies per genotype).

(J, K) Control experiment in which male flies with the indicated split-GAL4 transgenes and *UAS-Shi^{ts1}* transgenes were not deprived of sleep at night, but temperature shifted for 12 hr during the day. Statistical analysis was by one-way ANOVA and Dunnett's paired-comparison test (n= 24 flies per genotype).

Table S2

** p<0.05, **p<0.01, ***p<0.001*

Male Kenyon Cells

Comparison	***result***
Pooled Control vs. MB010B	ns
Pooled Control vs. MB427C	ns
Pooled Control vs. MB131B	ns
Pooled Control vs. MB417B	ns
Pooled Control vs. MB005B	ns
Pooled Control vs. MB464B	***
Pooled Control vs. MB370B	***
Pooled Control vs. MB461B	ns
Pooled Control vs. MB418B	***
Pooled Control vs. MB463B	***
Pooled Control vs. MB008B	ns
Pooled Control vs. MB371B	ns
Pooled Control vs. MB185B	ns
Pooled Control vs. MB477B	ns
Pooled Control vs. MB465C	ns
Pooled Control vs. MB460B	ns
Pooled Control vs. MB007B	ns
Pooled Control vs. MB154B	ns
Pooled Control vs. MB186B	***
Pooled Control vs. MB149B	ns
Pooled Control vs. MB004B	ns
Pooled Control vs. MB129B	ns
Pooled Control vs. MB107B	ns
Pooled Control vs. MB607B	***
Pooled Control vs. MB009B	ns
Pooled Control vs. MB355B	***
Pooled Control vs. MB419B	ns
Pooled Control vs. MB108B	ns
Pooled Control vs. pBDG4U	ns
Pooled Control vs. dTRPA1/+	ns

Female KCs

Comparison	***Result***
Pooled Control vs. MB010B	ns
Pooled Control vs. MB009B	ns
Pooled Control vs. MB355B	***
Pooled Control vs. MB419B	***
Pooled Control vs. MB427C	ns
Pooled Control vs. MB131B	ns
Pooled Control vs. MB417B	ns
Pooled Control vs. MB005B	ns
Pooled Control vs. MB370B	***
Pooled Control vs. MB461B	***
Pooled Control vs. MB418B	*
Pooled Control vs. MB463B	ns
Pooled Control vs. MB008B	ns
Pooled Control vs. MB371B	ns
Pooled Control vs. MB185B	ns
Pooled Control vs. MB477B	ns
Pooled Control vs. MB465C	ns
Pooled Control vs. MB460B	ns
Pooled Control vs. MB004B	ns
Pooled Control vs. MB107B	ns
Pooled Control vs. MB464B	***
Pooled Control vs. MB186B	***
Pooled Control vs. MB607B	***
Pooled Control vs. MB007B	ns
Pooled Control vs. MB149B	ns
Pooled Control vs. MB129B	ns
Pooled Control vs. MB108B	*
Pooled Control vs. pBDG4U/dTRPA1	ns
Pooled Control vs. dTRPA1/+	ns

SUPPLEMENTAL TABLE LEGENDS

Table S1: Sleep parameters (sleep amount, average bout length, average bout frequency, and latency) of male and female flies expressing dTRPA1 in KCs and MBONs at 21.5°C (baseline) and 28.5°C (activation). Total sleep amount, bout length, and bout frequency were calculated over the entire 24 hr day. Daytime sleep, daytime bout length, and daytime bout frequency were calculated during the 12 hr light period. Nighttime sleep, nighttime bout length, and nighttime bout frequency were calculated during the 12 hr dark period. Sleep latency was calculated as the time (in minutes) between lights-off and the start of the first sleep bout. Data were quantified as mean and sem for each of the above sleep parameters.

Table S2: KC screening results reanalyzed by statistical comparison to aggregate of all *split-GAL4/UAS-dTRPA1* genotypes. Change in total 24 hour sleep for all *split-GAL4/UAS-dTRPA1* genotypes in the KC screen of Figure 1 were aggregated into a pooled control group, and then compared to each individual *split-GAL4/UAS-dTRPA1* genotype using the Kruskal-Wallis ANOVA on ranks with Dunn's post-hoc correction for multiple comparisons.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Molecular and genetic methods/fly stocks

Genomic enhancers used in split-GAL4 and LexA transgenic lines were selected based on expression patterns of GAL4 lines using those same enhancers [S1] and constructed as previously described [S2]. Flies were maintained on conventional cornmeal-agar-dextrose medium in 12 hr light: 12 hr dark conditions at 21°C (for dTRPA1 and *Shibire*^{ts1} experiments) or 25°C (for immunohistochemistry), with ambient humidity of 60-70%. Flies were collected and tested for sleep 3-7 days after eclosion. LexA lines used in the experiments—*R35B12-LexA, R14H06-LexA,* and *R44E04-LexA*—are described in [S1, S3]. Detailed genotype information of split-GAL4 strains—including p65ADZp-ZpGAL4DBD combinations—is as described [S4]. p65ADZp split-half transgenes were inserted at attP40 or VK00027 while ZpGAL4DBD split-half transgenes were inserted at attP2. Split-GAL4 lines with the terminal letter "B", such as MB077B, contain p65ADZp in attP40 and ZpGAL4DBD in attP2, while "C" lines, such as MB112C, contain the p65ADZp in attVK00027 and ZpGAL4DBD in attP2, recombined on the third chromosome. This recombination approach was necessary to allow other chromosomes to house additional transgenes required for simultaneous orthogonal manipulation of two distinct sets of neurons driven by a split-GAL4 and LexA driver. The key genetic controls for all sleep experiments employing split-GAL4 drivers are otherwise identical genotypes, but with different split-GAL4 transgene pairs ("B" or "C" lines, as appropriate) that induce no or different phenotypes. This is because such strains differ solely in the regulatory DNA fragments cloned into the two split-GAL4 transgenes, and are otherwise identical with respect to genetic background and number, identity, and chromosomal location of all transgene insertions.

Sleep assays

Split-GAL4 and LexA flies were crossed to flies bearing either *10X UAS-dTRPA1* (in attP16) [S5], *20X UAS- Shibire^{ts1}* (pJFRC100-20XUAS-TTS-Shibire-ts1-p10 in VK00005 and attP2) [S4], or *LexAop2-dTRPA1* (in attP40 and VK00005) [S6] individually or in combination and maintained at 21-22°C. Genomic sites were chosen to avoid transvection, as described [S7]. Male progeny, 3-7 d post-eclosion, were placed in 65 mm x 5 mm transparent plastic tubes with standard cornmeal dextrose agar media, placed in a Drosophila Activity Monitoring system (Trikinetics), and locomotor activity data were collected in one minute bins. Activity monitors were maintained in a 12 h:12 h light-dark cycle at 65% relative humidity. Total 24-hour sleep quantity (daytime plus nighttime sleep) was extracted from locomotor activity data as described [S8]: sleep is defined as a contiguous period of inactivity lasting five minutes or more [S9, S10]. Sleep profiles were generated depicting average sleep (minutes per 30 minutes) for day 1 (baseline), days 2 and 3 (activation), and day 4 (recovery). In addition to permissive temperature controls other genotypic controls were used for hit detection as indicated. Because of the large number of genotypes to be screened, genotypes were tested in batches, with all batches containing appropriate internal positive and negative controls. For all screen hits, waking activity was calculated as the number of beam crossings/min when the fly was awake. Temperature changes to activate and silence neurons are as indicated in Fig.1-3. Statistical comparisons between experimental and control genotypes were performed using Prism (Graphpad Inc, CA) by Kruskal-Wallis one-way ANOVA followed by Dunn's post-hoc test or one-way ANOVA followed by Dunnett's pairwise comparison test.

Carbamazepine feeding

CBZ was dissolved in 45% (2-hydroxypropyl)-beta-cyclodextrin (Sigma) as described in [S11] to prepare a stock solution. For CBZ experiments, flies were loaded in tubes containing 5% agarose and 2% sucrose with 0.1mg/ml CBZ.

Sleep deprivation

Flies were sleep deprived by the intermittent mechanical perturbation method for 12 hours at night while housed in TriKinetics DAM monitors. Flies received mechanical perturbations on a horizontal shaker with a total duty cycle of 15 seconds per minute, delivered in 8 pulses of 1-3 seconds each occurring intermittently at random times. Intensity of perturbation was calibrated to the minimum required to result in complete or nearly complete loss of sleep during the night, with only some minimal "breakthrough" sleep late at night as sleep need accumulates (see **Figure 6D**). Sleep lost during deprivation and regained afterwards was calculated as described [S12]. Sleep loss was calculated by subtracting the amount of sleep occurring during each thirty minute period of sleep deprivation from the amount of sleep occurring during the corresponding period of the previous unperturbed day:night cycle. Sleep rebound was calculated by subtracting the amount of sleep occurring during each thirty minute period following mechanical deprivation from the amount of sleep occurring during the corresponding period of the previous unperturbed day:night cycle. Total sleep lost and regained was compared by one-way ANOVA followed by Dunnett's pairwise comparison test using Prism (Graphpad Inc, CA).

Stimulation of KCs by ATP/P2X2 and simultaneous GCaMP6m imaging of MBONs

LexAop2-dsRed in attp18 (X); *LexAop2-P2X2* in su(Hw)attp5(II); *UAS-GCaMP6m* in VK0005(III) flies were generated using standard molecular and genetic methods, with the

original transgenes as described [S2, S13-S15]. These flies were crossed to flies bearing appropriate LexA and split-GAL4 driver transgenes. To gain access to the KCs for ATP application whole brain explants were placed on 8mm diameter coverslips and placed in a recording chamber containing external solution (103mM NaCl, 3mM KCl, 5mM N-tris(hydroxymethyl) methyl-2-aminoethane-sulfonic acid, 8mM trehalose, 10mM glucose, 26mM NaHCO3, 1mM NaH2PO4, 2mM CaCl2 and 4mM MgCl2, pH 7.4). The ATP ejection electrode was filled with freshly prepared 10mM ATP solution and placed close to the KCs using a micromanipulator. The KCs were visually identified by their dsRed fluorescence. ATP was ejected by applying a 25 ms pressure pulse at 20 psi using a picospritzer (Parker Hannifin, Precision Fluidics Division, NH). The picospritizer was triggered by Zeiss image acquisition and processing software Zen pro 2012. Calcium imaging was performed with a Zeiss Axio Examiner Z1 upright microscope with W Plan Apochromat 40× water immersion objective. GCaMP6m was excited with a 470 nm LED light source (Colibiri, Zeiss) and images were acquired using ORCA-R2 C10600-10B digital CCD camera (Hamamastu, Japan) at 3-10Hz. For simultaneous imaging of spatially identical frames of dsRed (KCs) and GCaMP6m (MBONs) fluorescence we used a DV2 emission splitting system (Photometrics Inc). As a negative control, we imaged MBONs in brains of flies that lacked P2X2 expression in the KCs and confirmed absence of Ca^{2+} increases in MBONs following ATP application (data not shown). The microscope was focused on the MB lobes, and the region of interest (ROI) for quantification was defined to encompass the dendrites of the MBONs in the MB lobes, and was kept constant between flies for the MBON-γ5β′2a/β′2mp/β′2mp_bilateral and MBON-γ2α'1, respectively. The average fluorescence of all pixels for each time point in the ROI was subtracted from the average background fluorescence of an identically sized ROI elsewhere within the brain. The resulting pixel

fluorescence value for each time point was defined as F_t. Changes in fluorescence were computed as $\%\Delta F/F_o = ((F_t-F_o)/F_o) \times 100$, where F_o is defined as the average background-subtracted baseline fluorescence for the ten frames preceding ATP application. All images were processed and quantified using Zen and Fiji (Image J).

Arclight optical electrophysiology

Imaging was performed on a Zeiss Axio Examiner upright microscope using a W Plan Apochromat 40x N.A. 1.0 water immersion objective (Zeiss, Germany). ArcLight was excited with a 470 nm LED (Zeiss, Germany). The objective C-mount image was projected onto the 80 × 80 pixel chip of a NeuroCCD-SM camera and acquired using NeuroPlex software (RedShirtImaging, GA). The LED was turned on and images were acquired in a single trial for each brain for 11 s at a frame rate of 125 Hz, with the first second of images discarded to exclude the transient phase of very rapid ArcLight photobleaching [see S16, S17]. Optical traces were obtained by spatial averaging of intensity of all pixels within the ROI, with signals processed as previously reported [S16, S17]. The objective was focused on the MB lobes of the right hemisphere of each explanted fly brain and ROIs were defined manually to encompass the MBON dendrites as for the GCaMP6m imaging described above, or the axons of the KCs in the appropriate MB lobes, and were identical for each brain within the respective cell type. Fly brains were acutely dissected from flies after twelve hours of sleep deprivation at night or from non-deprived flies at the same time of day and imaged within five minutes of dissection. Statistical analysis and plotting of the data were performed using Prism and R, with standard deviations compared with unpaired t-test and power spectra compared with repeated measures ANOVA.

Immunohistochemistry

Dissection and immunohistochemistry of fly brains were performed as previously described with minor modifications [S1]. Brains of 3-10 day old female flies were dissected in Schneider's Insect medium and fixed in 2% paraformaldehyde in Schneider's medium for 50 min at room temperature (RT). After washing in PBT (0.5% Triton X-100 in PBS), brains were blocked in 3% normal goat serum (or normal donkey serum, depending on the secondary antibody) for 90 min. Brains were then incubated in primary antibodies diluted in PBT for 2-4 d on a nutator at 4°C, washed three times in PBT for 30 min or longer, then incubated in secondary antibodies diluted in PBT for 2-4 days on a nutator at 4°C. Brains were washed thoroughly in PBT four times for 30 min or longer, and mounted in Vectashield (Vector laboratories, CA) for imaging. The following antibodies were used: anti-GFP (1:1000; Invitrogen; #AB124), mouse anti-nc82 (1:50; Developmental Studies Hybridoma Bank, Univ. Iowa), and cross-adsorbed secondary antibodies to IgG (H+L): goat Alexa Fluor 488 anti-rabbit (1:800; Invitrogen A11034) and goat Alexa Fluor 568 (1:400; Invitrogen A11031).

Tap-response Arousal Assay

We probed the quiescence induced by activation of sleep promoting neuron by dTRPA1 by tapping vials housing flies as previously described [S18]. Groups of fifteen flies (8 males and 7 females) expressing dTRPA1 in γd KCs, MBON-γ2α'1, or empty split-Gal4 *pBDG4U* controls were sorted under CO_2 anesthesia and maintained in vials containing standard dextrose fly growth medium at 20-22°C for 48 hr. These vials were then incubated at 29-31°C for 2 hr, and flies were then transferred to empty vials and held at 29-31°C for 2 hr prior to video recording using a Microscoft Lifecam Cinema Camera (http://www.microsoft.com/hardware/en-us/p/lifecam-cinema). Vials were gently tapped on the table 5 seconds after the start of the

recording, and response/recovery was recorded for 30 seconds. Genotypes were hidden with tape, and recordings were thus carried out blind to which vial contained experimental and which contained control flies.

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