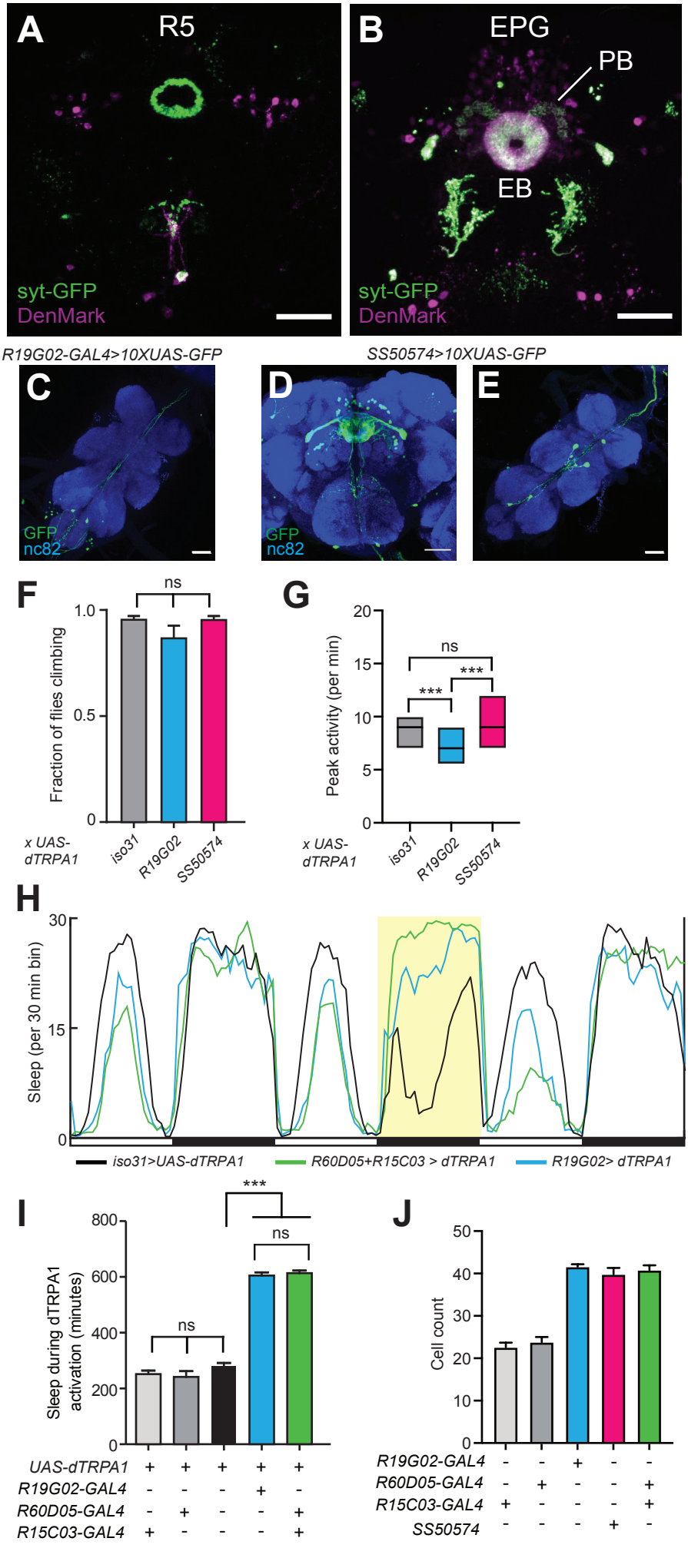
A
R5
syt-GFP
DenMark
B
EPG
PB
EB
syt-GFP
DenMark
R19G02-GAL4>10XUAS-GFP
SS50574>10XUAS-GFP
C
GFP
nc82
D
GFP
nc82
E
F
ns
Fraction of flies climbing
1.0
0.5
0
x UAS-dTRPA1
iso31
R19G02
SS50574
G
Peak activity (per min)
20
15
10
5
0
ns
***
***
x UAS-dTRPA1
iso31
R19G02
SS50574
H
Sleep (per 30 min bin)
30
15
0
iso31>UAS-dTRPA1
R60D05+R15C03 > dTRPA1
R19G02> dTRPA1
I
Sleep during dTRPA1 activation (minutes)
800
600
400
200
0
***
ns
ns
UAS-dTRPA1 + + + + +
R19G02-GAL4 - - - + -
R60D05-GAL4 - + - - +
R15C03-GAL4 + - - - +
J
Cell count
50
40
30
20
10
0
R19G02-GAL4 - - + - -
R60D05-GAL4 - + - - +
R15C03-GAL4 + - - - +
SS50574 - - - + -

Figure S1. Further characterization of EPG neuron identity and sleep behavior, Related to Figure 1

(A) Whole-mount brain immunostaining of *R30G03-AD, R58H05-DBD>UAS-syt-GFP, UAS-Denmark* with anti-GFP (green) and anti-dsRed (magenta) antibody staining. Maximal intensity projection of the central brain is shown. Scale bar indicates 50 μm.

(B) Whole-mount brain immunostaining of *R19G02-GAL4>UAS-syt-GFP, UAS-Denmark* with anti-GFP (green) and anti-dsRed (magenta) antibody staining. Maximal intensity projection of the central brain is shown. Protocerebral bridge (PB) and ellipsoid body (EB) are indicated. Scale bar indicates 50 μm.

(C-E) Ventral nerve cord (VNC) immunostaining of *R19G02-GAL4 >10X-UAS-IVS-syn21-GFP-p10* (C) and whole-mount brain (D) and VNC (E) immunostaining of *SS50574>10X-UAS-IVS-syn21-GFP-p10* with anti-GFP (green) and anti-Bruchpilot (BRP, nc82, blue) antibodies. Maximal intensity projections of the central brain and VNC are shown. Scale bars indicate 50 μm.

(F) Proportion of flies climbing above a 10 cm mark following 2 hr heat treatment at 29°C for *iso31* (n=13), *R19G02-GAL4* (n=14), *SS50574* (n=13), crossed to *UAS-dTRPA1*; one-way ANOVA with post-hoc Tukey's test.

(G) Peak activity in the 5 min interval following strong mechanical stimuli for the flies shown in **Figures 1D** and **1E**; Kruskall-Wallis with post hoc Dunn's test.

(H) Sleep profiles of *iso31>UAS-dTRPA1* (black), *R19G02-GAL4>UAS-dTRPA1* (light blue), and *R60D05-GAL4 + R15C03-GAL4>UAS-dTRPA1* (green) flies. Sleep time plotted in 30 min bins. White and black bars indicate 12hr light and dark periods, respectively. The period of 12hr dTRPA1 activation at 29°C is indicated using a yellow background.

(I) Sleep amount during ZT12-24 dTRPA1 activation of single and combination EPG drivers. Data are shown for *R15C03-GAL4>UAS-dTRPA1* (n=73), *R60D05-GAL4>UAS-dTRPA1* (n=86), *iso31>UAS-dTRPA1* (n=117), *R19G02-GAL4>UAS-dTRPA1* (n=86), and *R60D05-GAL4 + R15C03-GAL4>UAS-dTRPA1* (n=41) animals. Data are from the same flies as in **Figure S1H**; one-way ANOVA with post-hoc Tukey's test. Mean ± SEM is shown.

(J) Number of putative EPG neurons labeled in flies expressing mCD8::GFP under control of *R15C03-GAL4* (n=5), *R60D05-GAL4* (n=5), *R19G02-GAL4* (n=5), *SS50574* (n=5), and *R15C03-GAL4+R60D05-GAL4* (n=5) driver lines.

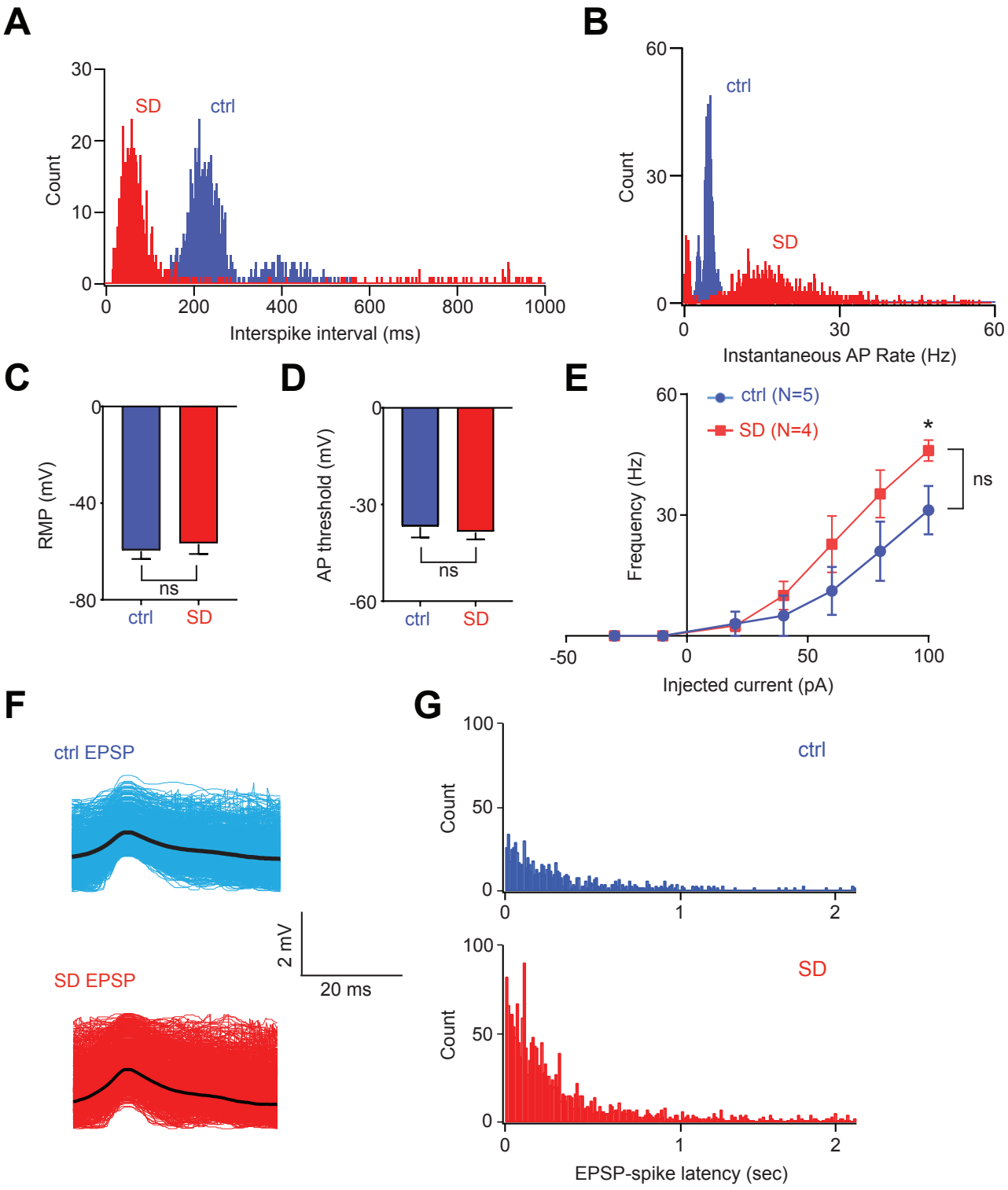

Figure S2. Additional Electrophysiological Data, Related to Figure 2.
(A and B) Interspike interval histograms of spontaneous AP activity (A) and instantaneous AP rate distribution (B) for EPG neurons from *R19G02-GAL4>UAS-CD4::tdGFP* flies with (red) and without SD (blue).
(C and D) Resting membrane potential (RMP) (C) and AP threshold (D) during spontaneous activity of EPG neurons with (red) and without SD (blue) for *R19G02-GAL4>UAS-CD4::tdGFP* flies.
(E) Mean frequency of APs elicited in response to current injections ranging from -40 pA to 100 pA with 20 mV steps for EPG neurons with (red) and without SD (blue).
(F) Superimposed spontaneous excitatory postsynaptic potentials (sEPSPs) sorted from membrane potentials of EPG neurons with (red) and without SD (blue).
(G) Histogram plots of EPSP-spike latencies from EPG neurons, showing an increased fraction of shortened EPSP-spike coupling events in SD flies. Note that data from **Figure S2A-D**, **F**, and **G** are derived from the same dataset used in **Figure 2**.

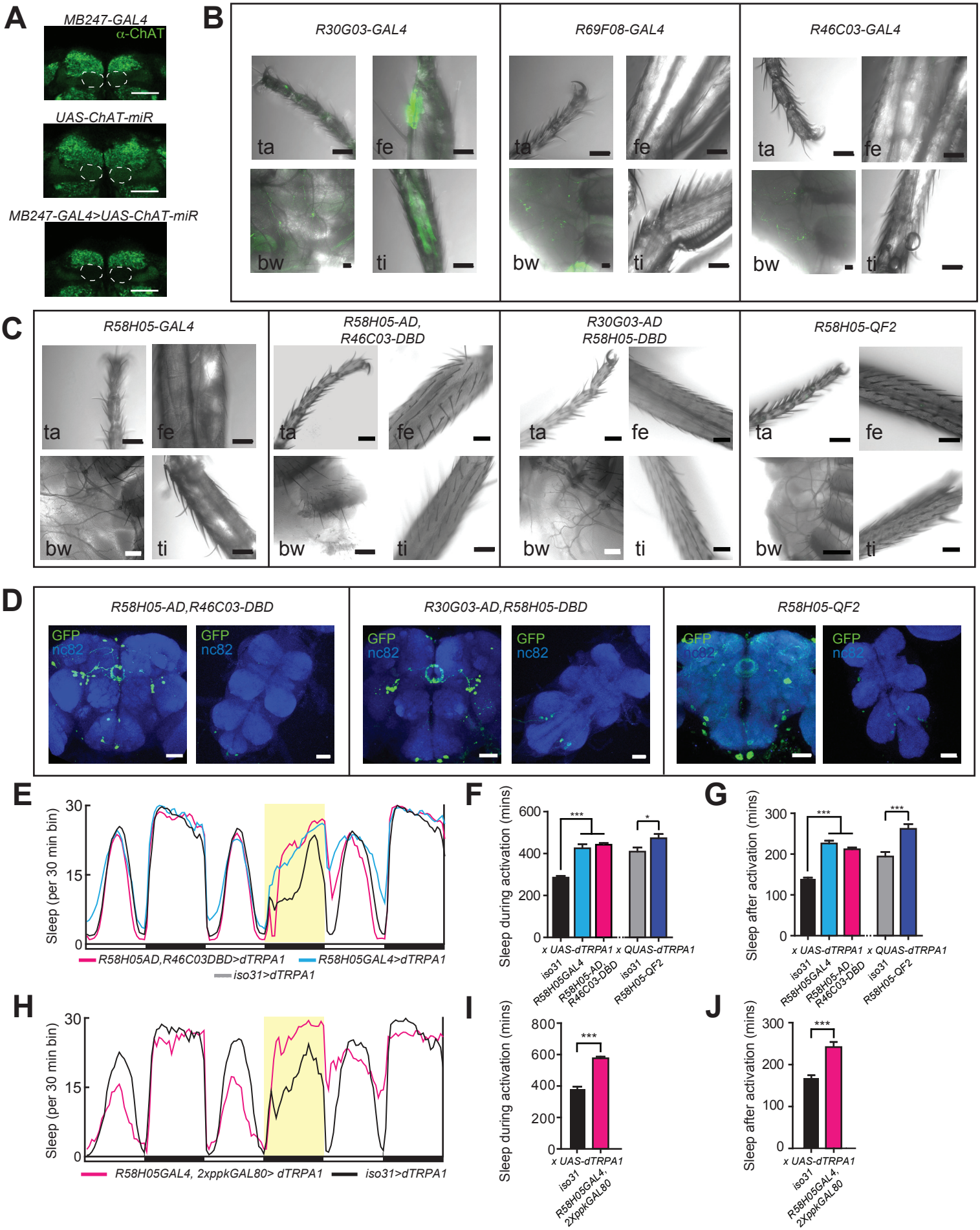
A
MB247-GAL4
α-ChAT
UAS-ChAT-miR
MB247-GAL4>UAS-ChAT-miR
B
R30G03-GAL4
R69F08-GAL4
R46C03-GAL4
ta
fe
bw
ti
C
R58H05-GAL4
R58H05-AD,
R46C03-DBD
R30G03-AD
R58H05-DBD
R58H05-QF2
D
R58H05-AD,R46C03-DBD
R30G03-AD,R58H05-DBD
R58H05-QF2
GFP
nc82
E
Sleep (per 30 min bin)
30
15
0
R58H05AD,R46C03DBD>dTRPA1
R58H05GAL4>dTRPA1
iso31>dTRPA1
F
Sleep during activation (mins)
600
400
200
0
***
*
x UAS-dTRPA1
x QUAS-dTRPA1
iso31
R58H05GAL4
R58H05-AD
R46C03-DBD
iso31
R58H05-QF2
G
Sleep after activation (mins)
300
200
100
0
***
***
x UAS-dTRPA1
x QUAS-dTRPA1
iso31
R58H05GAL4
R58H05-AD,
R46C03-DBD
iso31
R58H05-QF2
H
Sleep (per 30 min bin)
30
15
0
R58H05GAL4, 2xppkGAL80> dTRPA1
iso31>dTRPA1
I
Sleep during activation (mins)
800
600
400
200
0
***
x UAS-dTRPA1
iso31
R58H05GAL4,
2XppkGAL80
J
Sleep after activation (mins)
300
200
100
0
***
x UAS-dTRPA1
iso31
R58H05GAL4,
2XppkGAL80

Figure S3. Characterization of R5 neuron drivers, Related to Figure 3

(A) Whole-mount brain immunostaining using anti-ChAT antibody (green) showing mushroom body lobes for *MB247-GAL4>iso31*, *iso31>UAS-ChAT-miR*, and *MB247-GAL4>UAS-ChAT-miR* flies. Dashed lines indicate region of interest in γ-lobes. Scale bar indicates 40 μm.

(B) DIC and native GFP signal measured in tarsus (ta), body wall (bw), femur (fe), and tibia (ti) for *R30G03-GAL4*, *R69F08-GAL4*, and *R46C03-GAL4* lines driving expression of *20XUAS-6XGFP*. Scale bar of leg image (and all following leg images) denotes 50 μm. Scale bar of body wall image (and all following body wall images) indicates 100 μm.

(C) No detectable GFP signal observed in legs or body wall in *R58H05-GAL4* or split-GAL4 strains (*R58H05-AD, R46C03-DBD;* or *R30G03-AD,R58H05-DBD*) driving expression of *20X-UAS6XGFP*. Minimal GFP signal observed in legs or body wall in *R58H05-QF2-A>10XQUAS6XGFP* strain.

(D) Whole-mount immunostaining of central brain (left) and VNC (right) of transgenic drivers labeling R5 neurons. Central brain and VNC immunostaining with anti-GFP (green) and anti-Bruchpilot (BRP, nc82, blue) of split-GAL4 lines (*R58H05-AD, R46C03-DBD* and *R30G03-AD, R58H05-DBD*) driving expression of *10XUAS-syn21-GFP-p10*. Also shown is central brain and VNC immunostaining for *R58H05-QF2-A>QUAS-mCD8::GFP*. Scale bars denote 50 μm.

(E) Sleep profiles of *R58H05AD, R46C03DBD>UAS-dTRPA1* (magenta), *R58H05-GAL4>UAS-dTRPA1* (blue), and *iso31>UAS-dTRPA1* (gray) control flies. Sleep time plotted in 30 min bins. White and black bars indicate 12 hr light and dark periods, respectively. The period of 12hr dTRPA1 activation at 29°C is indicated using a yellow background. Data are from same flies as in (F) and (G).

(F and G) Sleep amount during (F) and after (G, ZT0-6) activation for *iso31>UAS-dTRPA1* (n=277), *R58H05-GAL4>UAS-dTRPA1* (n=124), *R58H05AD, R46C03DBD>UAS-dTRPA1* (n=216), *iso31>QUAS-dTRPA1* flies (n=29), and *R58H05-QF2-A>QUAS-dTRPA1* (n=25). Mean ± SEM is shown; one way ANOVA with Tukey's post-hoc test for UAS lines and Student's t-test for QUAS lines.

(H) Sleep profiles of *R58H05-GAL4, ppk-GAL80 (2x)>UAS-dTRPA1* (magenta) and *iso31>xUAS-dTRPA1* (black) control flies. Sleep time plotted in 30 min bins. White and black bars indicate 12 hr light and dark periods respectively. The period of 12 hr dTRPA1 activation at 29°C is indicated using a yellow background. Data are from the same flies as in (I) and (J).

(I and J) Sleep amount during (I) and after (J, ZT0-6) activation for *iso31>UAS-dTRPA1* (n=62) and *R58H05-GAL4, ppk-GAL80 (2x)>UAS-dTRPA1* (n=44). Mean ± SEM is shown; Student's t-test.

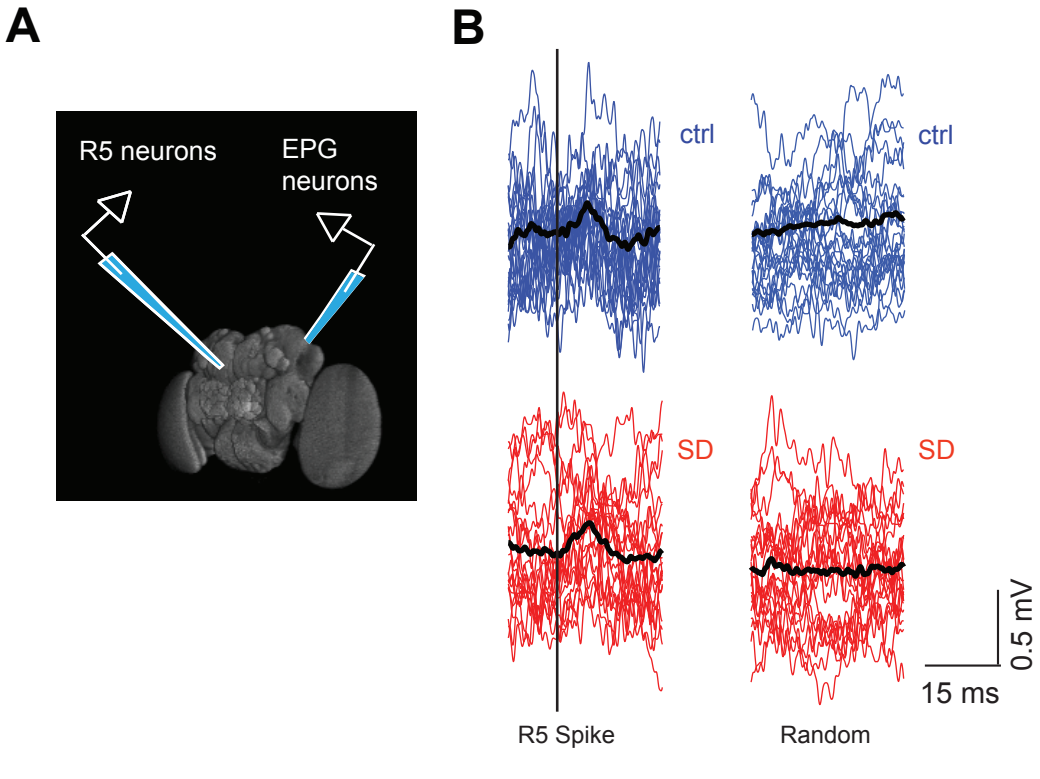

Figure S4. Additional Electrophysiological Data, Related to Figure 4.
(A) Schematic of novel brain preparation for dual patch-clamp recording of R5 and EPG neurons. The brain specimen is mounted in a vertical position to enable dual access, where R5 and EPG neurons are approached from the anterior and posterior directions, respectively.
(B) Superimposed traces of R5 spike-triggered average of EPG neuronal membrane potentials and randomly chosen EPG membrane voltage sorting data in the presence (red, SD) or absence of SD (blue, ctrl). Genotype of the flies used was *R19G02-GAL4, R58H05-GAL4>UAS-CD4-tdGFP*.
(C and D) Average EPG voltage response triggered by R5 spike vs randomly selected in the presence (D) or absence (C) of SD from the dual recording dataset shown in (B).

A	Genotype	Activation Night Bout Length (mins)	Activation Night Bout Number	n
	iso31 > UAS-dTRPA1	27.7 ± 1.5	12.5 ± 0.5	96
	R19G02-GAL4 > UAS-dTRPA1	126.5 ± 14.0 ***	9.1 ± 0.6 **	96
	SS50574 > UAS-dTRPA1	405.2 ± 29.4 ***	5.9 ± 0.8 ***	93

B	Genotype	Sleep During dTRPA1 Activation (mins)	Activation Night Bout Length (mins)	Activation Night Bout Number	n
	iso31>20XUAS-dTRPA1	377 ± 10.4	31.7 ± 4.4	14.5 ± 0.4	156
	R19G02-GAL4>20XUAS-dTRPA1	630.8 ± 4.8 ***	77.1 ± 5.1 ***	12.7 ± 0.6 ns	145
	SS50574>20XUAS-dTRPA1	638.8 ± 8.5 ***	6.9 ± 0.7 ***	6.9 ± 0.7 ***	94
	R60D05-GAL4>20XUAS-dTRPA1	181.9 ± 12.5 ***	13.0 ± 0. 9 ***	13.0 ±0.7 ns	173
	R15C03-GAL4>20XUAS-dTRPA1	284 ± 12.8 ***	24.4 ± 1. 3 ns	12.2 ±0.5 ns	142

C	Genotype	Activation Night Bout Length (mins)	Activation Night Bout Number	N
	iso31 > UAS-dTRPA1	24.9 ± 1.4	13.0 ± 0. 6	117
	R19G02-GAL4 > UAS-dTRPA1	106.0 ± 14.0 ***	10.4 ± 0.6 ns	86
	R60D05GAL4 > UAS-dTRPA1	16.2 ± 2.6 ***	18.0 ± 1.3 ***	86
	R15C03GAL4 > UAS-dTRPA1	23.8 ± 1.5 ns	11.7 ± 0.7 ns	73
	R60D05GAL4 + R15C03 GAL4 > UAS-dTRPA1	201.7 ± 29.6 ***	7.3 ± 1.0 **	41

D	Genotype	Baseline TST (mins)	Day Sleep (mins)	Night Sleep (mins)	Night Bout Length (mins)	Night Bout Number	n
	R19G02-GAL4 > UAS-TNT	727.4 ± 23.2 ns	229.3 ± 13.4 ***	498.1 ± 14.9 ***	87.0 ± 10.6 ns	9.1 ± 0.5 ns	81
	iso31 > UAS-TNT	793.8 ± 20.1	309.3 ± 10.9	484.5 ± 12.9	62.8 ± 5.2	11.3 ± 0.7	90
	R19G02-GAL4 > iso31	897.8 ± 18.3 **	321.4 ± 10.7 ns	576.3 ± 11.8 ns	71.5 ± 9.0 ns	13.6 ± 0.9 ns	62

Table S1. Additional Sleep Behavior Measurements, Related to Figure 1

(A). Activation Night Bout Length and Number for Sleep-Promoting EPG drivers.

(B). Sleep during dTRPA1 activation, Activation Night Bout Length and Number for all EPG drivers tested with *20XUAS-dTRPA1.*

(C). Activation Night Bout Duration and Number for all EPG drivers tested with *UAS-dTRPA1* and control.

(D). Baseline Total Sleep Time (TST), Day and Night Sleep, Night Bout Length and Number for EPG neuron inhibition experiments.