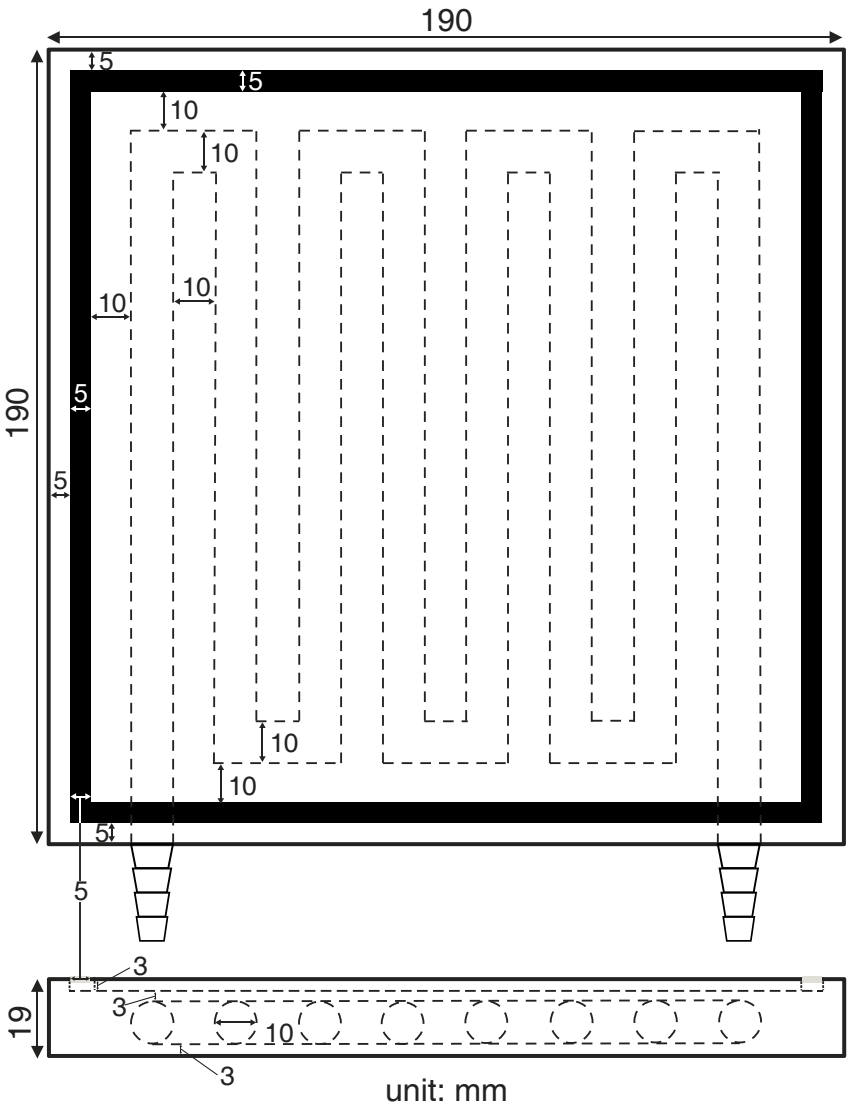
A



B

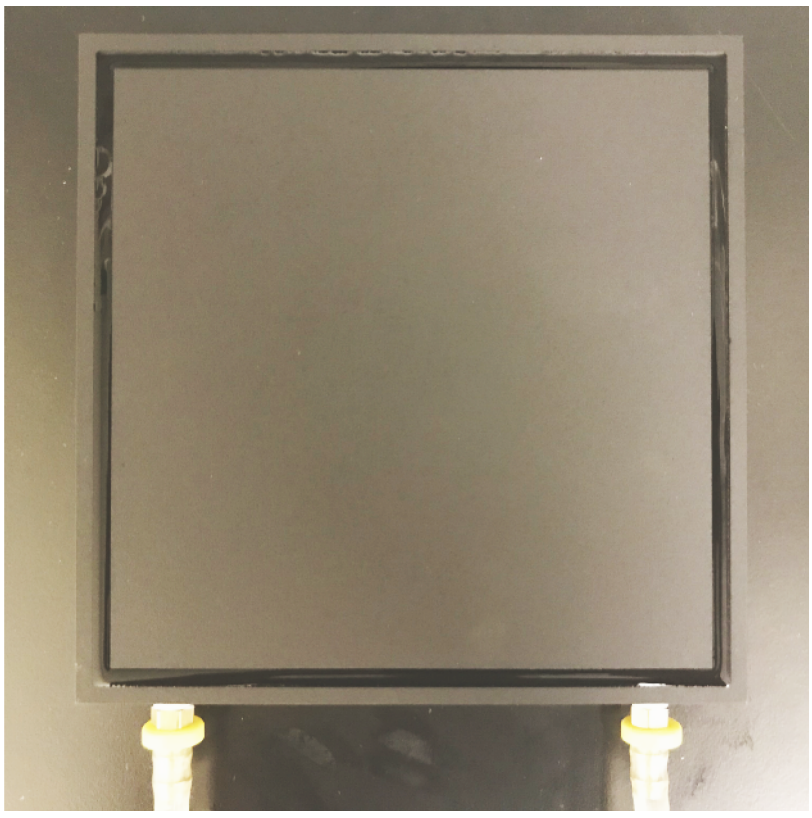


Figure S1. Dimensions of the hot plate used to assay thermal nociception, Related to Figure 1.

(A) Design and parameters of the hot plate for the jump assay. The dashed lines indicate the water tunnels inside the copper plate. The gray shading near the periphery represents the moat, which is 3 mm deep.

(B) Photo of a hot plate. The hot plate is connected to a circulating bath. Water circulating in the tunnels control the surface temperature of the hot plate.

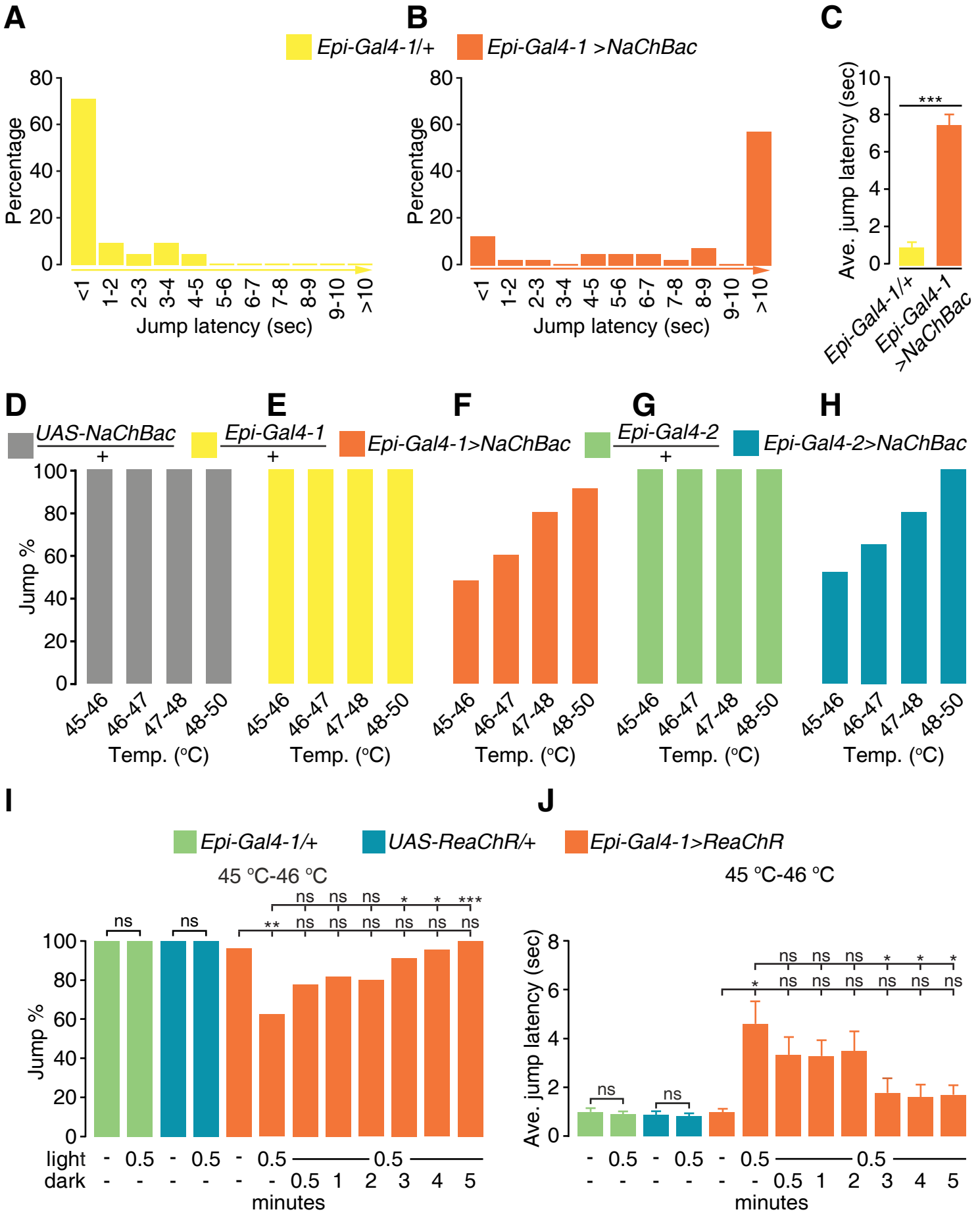

A
B
C
Epi-Gal4-1/+
Epi-Gal4-1 >NaChBac
Percentage
Jump latency (sec)
<1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
>10
Ave. jump latency (sec)
***
Epi-Gal4-1/+
Epi-Gal4-1 >NaChBac
D
E
F
G
H
UAS-NaChBac
Epi-Gal4-1
Epi-Gal4-1>NaChBac
Epi-Gal4-2
Epi-Gal4-2>NaChBac
Jump %
45-46
46-47
47-48
48-50
Temp. (°C)
I
J
Epi-Gal4-1/+
UAS-ReaChR/+
Epi-Gal4-1>ReaChR
45 °C-46 °C
ns
*
**
***
light
dark
minutes
Ave. jump latency (sec)

Figure S2. Nociceptive responses resulting from manipulating Epi neurons, Related to Figure 1.
(A) Percentages of control flies (*Epi-Gal4-1*) showing the indicated jump latencies (sec) when placed on a 45 °C—46 °C hot plate.
(B) Epi neurons were inactivated with NaChBac (*Epi-Gal4-1* and *UAS-NaChBac*), and then these flies were placed on a 45 °C—46 °C hot plate and tested for jump latencies.
(C) Average jump latencies from (A and B). n = 21 (A) and 40 (B). Error bars indicate S.E.M.s. Mann-Whitney test. ***$P < 0.001$.
(D-H) Jump percentages of the indicated flies on hot plates set at the indicated temperatures. n $\geq$ 20.
(I and J) Effects of optogenetically stimulating Epi neurons with ReaChR (*UAS-ReaChR*/+ and *Epi-Gal4-1*/+) on the jump percentages (I) and the average jump latencies (J) after placing the indicated flies on a 45 °C—46 °C hot plate. The Epi neurons were optogenetically activated for 0.5 min and then allowed to recover in the dark for 0.5—5 min. n $\geq$ 20. Error bars indicate S.E.M.s. Fisher's exact test (I). Mann-Whitney test (J). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns, not significant.

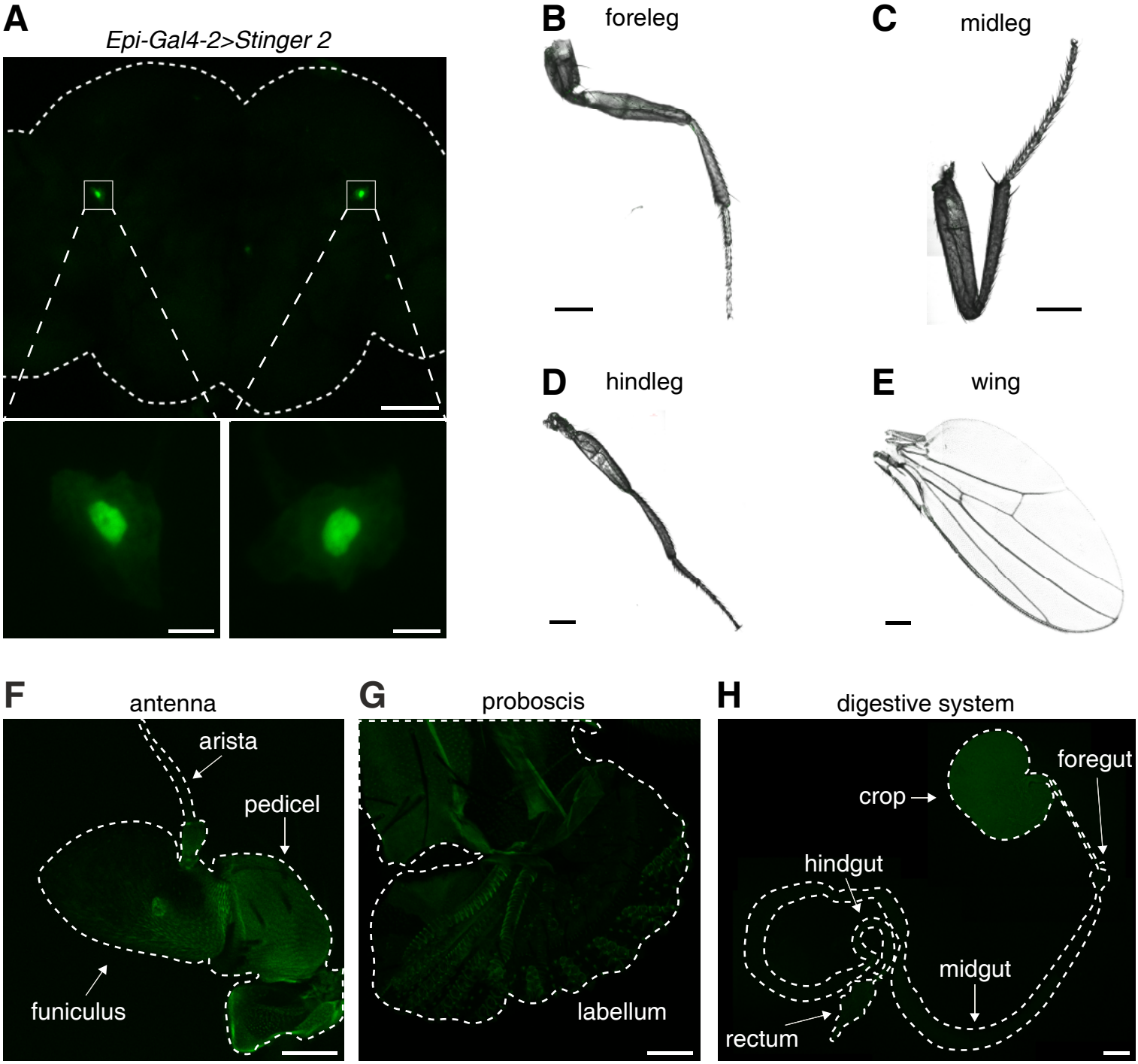


Figure S3. Expression pattern of *Epi-Gal4-2*, Related to Figure 2.
(A) Fluorescence exhibited by nuclei of Epi neurons expressing *UAS-Stinger 2* under control of the *Epi-Gal4-2*. The boundary of the brain tissue is indicated with the short dashes. Scale bar indicates 50 μm. The framed areas are enlarged below, and include 5 μm scale bars.
(B-H) Testing for expression of *UAS-GFP* under control of the *Epi-Gal4-2* in the indicated tissues: (B) foreleg; (C) midleg; (D) hindleg; (E) wing; (F) antenna; (G) proboscis; and (H) digestion system. The boundaries of the tissues in F-H are indicated by the dashes. Scale bars indicate 0.5 mm.

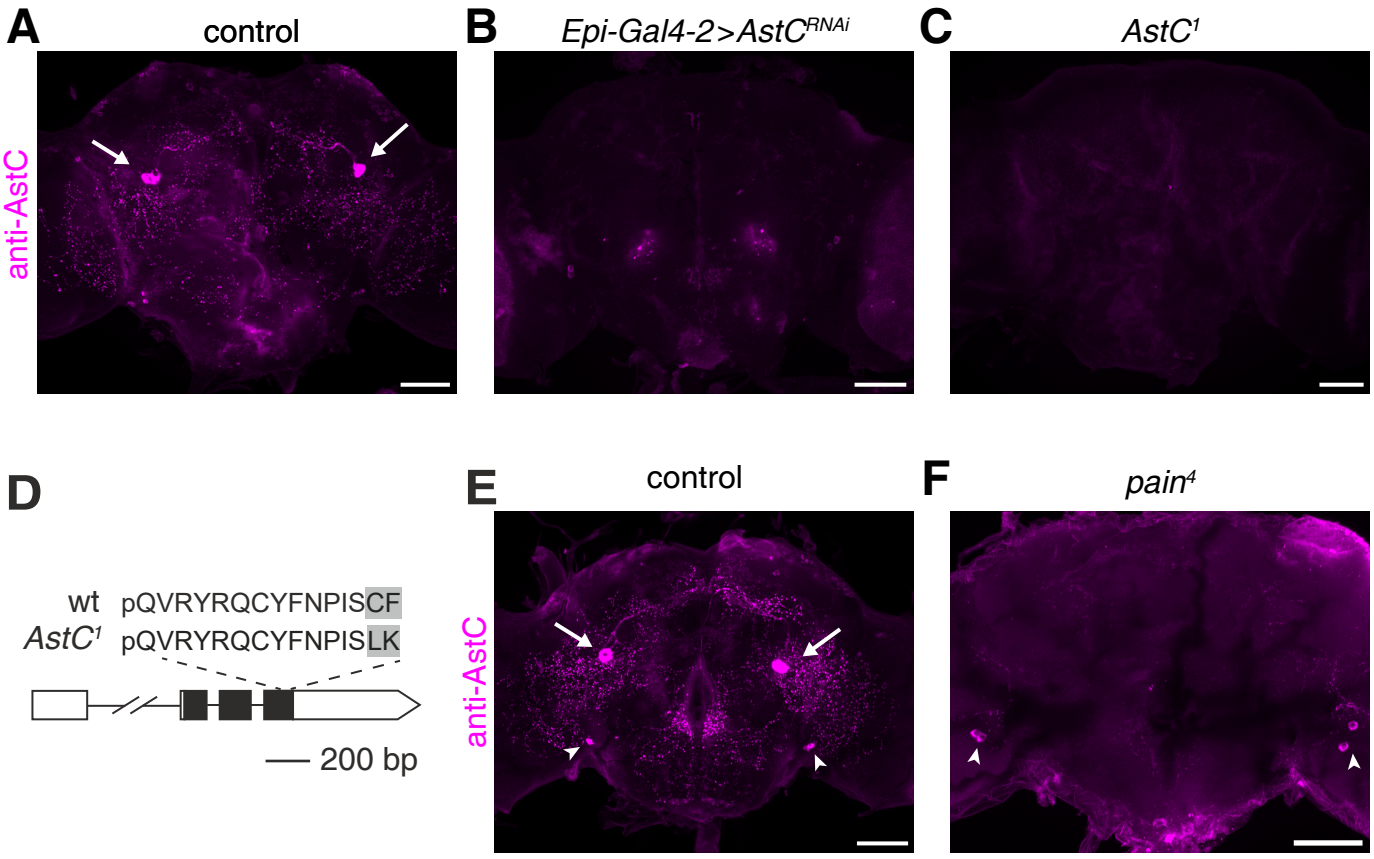


Figure S4. Loss of expression of AstC in the *AstC*^{1} mutant or due to RNAi knockdown of *AstC*, Related to Figure 3.

(A-C, E and F) Brains were stained with anti-AstC (magenta). Scale bars indicate 50 μm.

(A) Control (*w*^{1118}). The white arrows point to Epi neurons.

(B) AstC knockdown (*Epi-Gal4-2* and *UAS-AstC*^{RNAi}).

(C) *AstC*^{1}.

(D) Schematic illustration of the *AstC*^{1} mutant generated by CRISPR-Cas9. The coding region is indicated in black, the introns are indicated with horizontal lines, and the parallel diagonal lines with the adjacent horizontal lines indicate a 3915 bp intron. The primary AstC translation product is 122 amino acids, and the AstC peptide is 15 residues. The pQ is pyroglutamate.[S1] The last two amino acids of the AstC peptide are changed from cysteine and phenylalanine to leucine and lysine in *AstC*^{1}.

(E) Control (*w*^{1118}). The arrows point to Epi neurons. The arrowheads point to two pairs of AstC-positive neurons proximal to the optic lobes.

(F) *pain*^{4}. The arrowheads point to two pairs of AstC-positive neurons proximal to the optic lobes.

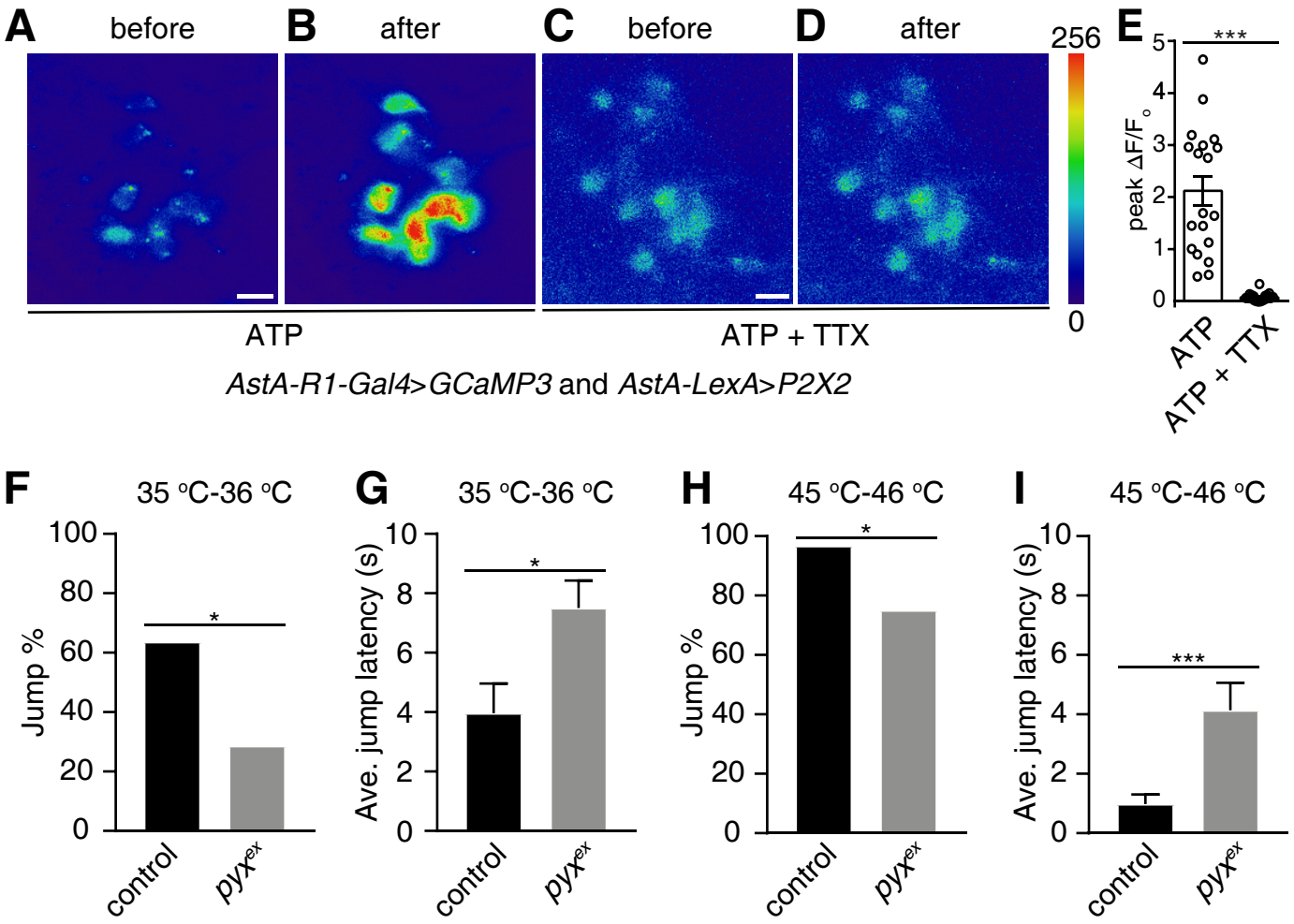


Figure S5. TTX blocks signaling between AstA and AstA-R1 neurons, and effects of the *pyx* mutation on the jump response, Related to Figures 4 and 5.

(A-B) GCaMP3 signals exhibited by AstA-R1 neurons before (A) and after (B) application of 200 µl of 50 mM ATP.

(C-D) GCaMP3 signals exhibited by AstA-R1 neurons before (C) and after (D) application of 200 µl 50 mM ATP in the presence of 1 µM TTX.

(E) Average peak $\Delta F/F_0$ of AstA-R1 neurons in (A-D). n = 19-21 neurons from 3 dissected brains.(F and G) Jump percentages (F) and average jump latencies (G) of control and pyx^{ex} flies on a 35 °—36 °C hot plate.

(H and I) Jump percentages (H) and average jump latencies (I) of control and pyx^{ex} flies on a 45 °—46 °C hot-plate.

Error bars indicate S.E.M.s. Mann-Whitney test. ***$P < 0.001$. Scale bars indicate 10 µm.

SUPPLEMENTAL REFERENCE

S1. Veenstra, J.A. (2009). Allatostatin C and its paralog allatostatin double C: the arthropod somatostatins. Insect Biochem Mol Biol *39*, 161-170.