Visualization of Neural Activity in Insect Brains Using a Conserved Immediate Early Gene, Hr38

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Supplemental Inventory

Figure S1, related to Figure 1. (A) A list of candidate genes identified by microarray screening. (B, C) Results of secondary screening of candidate genes. (D, E, F) Expression analysis of *BmHr38* in response to female odor exposure of different durations (D, E) or to each sex pheromone component (F). (G, H) The tissue distribution analysis and developmental profiling of *BmHr38*. Panels are cited in the Results section.

Figure S2, related to Figure 2. Control data for Figure 2. (A-C) *BmHr38* expression pattern in response to female odor stimulation was similar to that to bombykol stimulation (Figure 2). (D, E) Confirmation of probe specificity.

Figure S3, related to Figure 4. Supplemental data for Figure 4. (A, B) Quantitative analysis of *Dhr38*-positive cells in response to female odor stimulation using different lines of mutant strains. (C-E) Double *in situ* hybridization of *Dhr38* and *Or47b* in the male antennae.

Supplemental Experimental Procedures

Supplemental References

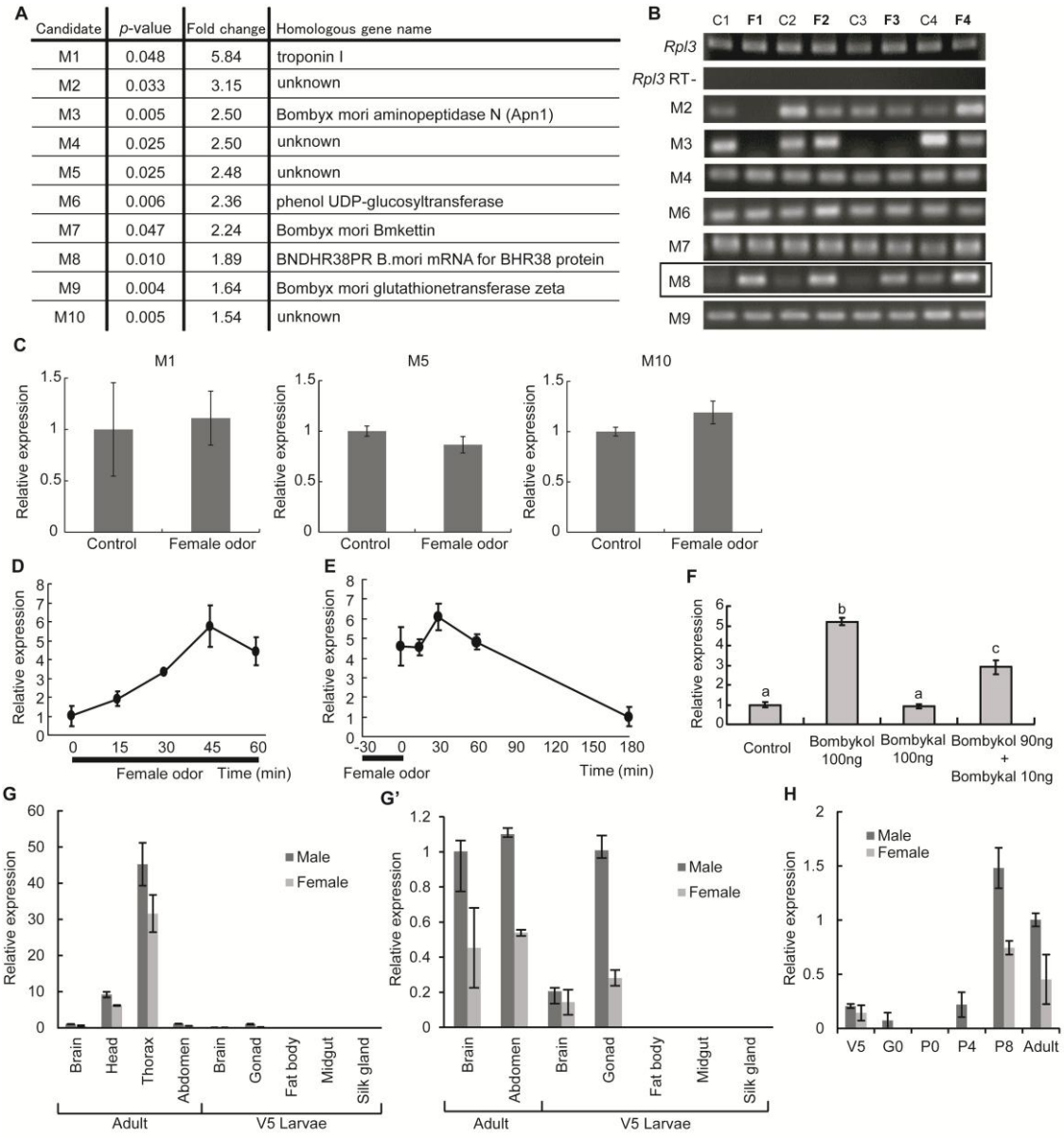

Figure S1: Identification and expression analysis of *BmHr38*. (A) A list of candidate genes identified by microarray screening. Genes that fulfilled the criteria ($P < 0.05$ and Fold change > 2, or $P < 0.01$ and Fold change > 1.5) were selected as candidates. (B, C) Results of secondary screening of candidate genes by semi-quantitative RT-PCR (B) and qRT-PCR (C). Only M8 (*BmHr38*) showed a reproducible increase in expression in response to female odor stimulation. C, control (no stimulation) male brain; F, female odor-stimulated brain. n = 4 each. (D) Continuous exposure to the female odor increased *BmHr38* expression in the male silkmoth brain by 45 min. Further exposure (60 min) had no additional increasing effect on *BmHr38* expression, probably due to desensitization to the female odor. n = 3, each. (E) *BmHr38* expression in the male silkmoth brain increased until 30 min after cessation of female odor exposure, but decreased to the basal level after

180 min. n = 3, each. (F) *BmHr38* mRNA expression in response to the sex pheromone components was analyzed by qRT-PCR. Statistically different groups are indicated by different letters ($P < 0.05$, *Tukey-Kramer*'s HSD test after *ANOVA*. n = 4, each). (G) *BmHr38* is predominantly expressed in the adult thorax. The tissue distribution analysis was conducted using various tissues of the male and female moth (adult), and five-instar day-5 (V5) larvae. Relative value to the male moth brain with no stimulation is shown. n = 3, each. To show the lower level of expression in other tissues, expression without the head and thorax is shown in (G'). Because the adult female moth does not have pheromone receptor expression in the antenna, there is no pheromone-induced sexual behavior or resulting *BmHr38* expression. (H) Developmental expression profile in the brains. The level of *BmHr38* expression was analyzed in V5, G0 (gut purge day-0), P0 (pupae day-0), P4, P8, and adult. n = 3, each. The expression profile of *BmHr38* was similar to that of *Dhr38*, the fly homolog of *BmHr38*, which is predominantly expressed in the epidermis and leg joints of pupae and adults [1, 2], suggesting that *Hr38* function is conserved among these insects.

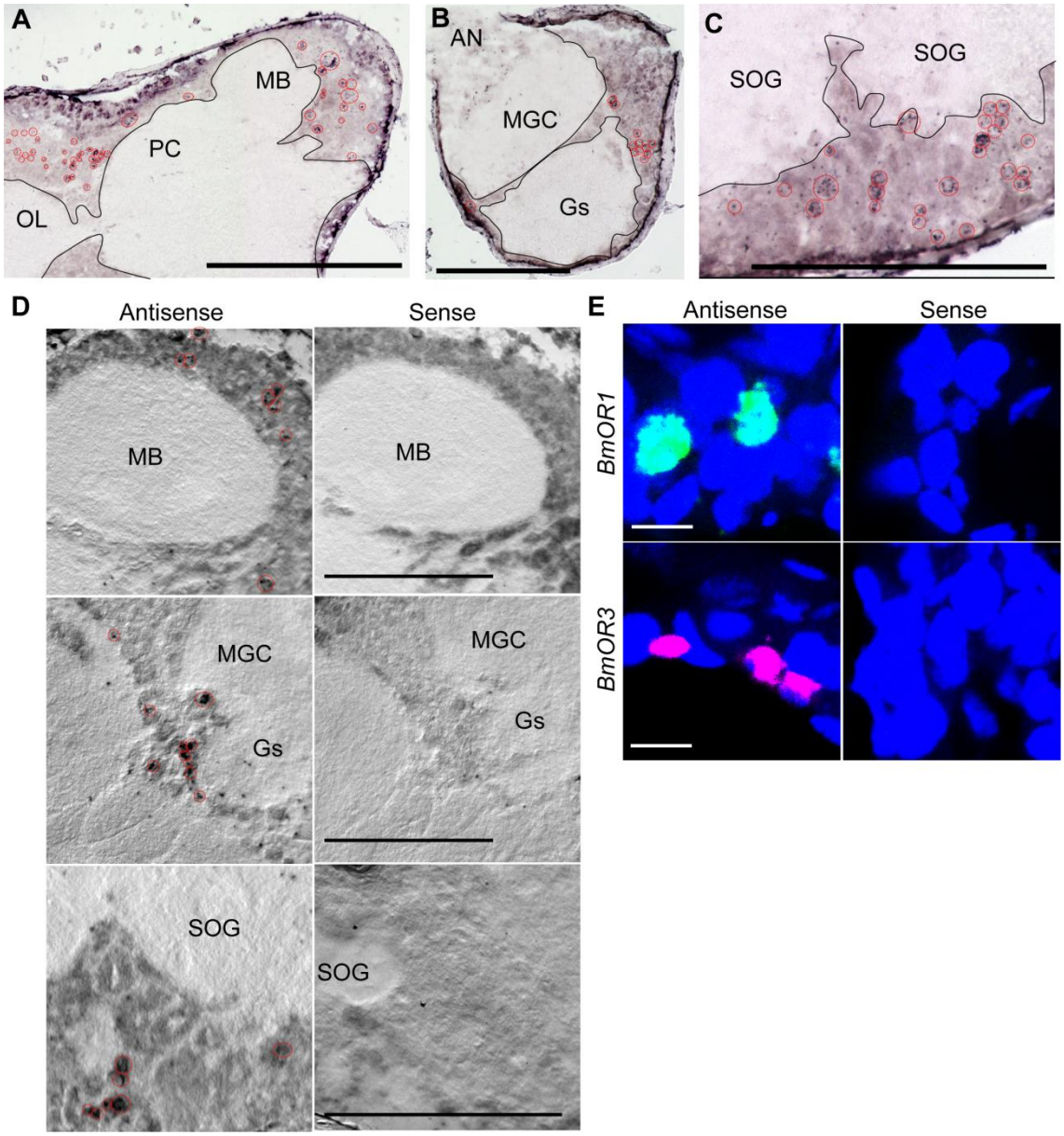

Figure S2: *BmHr38* expression pattern in the male silkmoth brain in response to female odor stimulation. (A-C) Male silkmoths were stimulated with female odor for 30 min, and then placed in normal air for 30 min. The distribution pattern of *BmHr38*-expressing cells was similar to that of the bombykol-stimulated brain. *BmHr38*-expressing cells are indicated by red circles. The neuropil structures are depicted by the black lines. n = 4. (D) Using consecutive sections of bombykol-stimulated male brain, *BmHr38* probe specificity was verified by comparing the staining pattern between neighboring sections hybridized with antisense or sense probes. The signal was detected only when the antisense probe was used for hybridization. Cells stained by antisense probes for *BmHr38* are indicated by red circles (D). (E) Probe specificity for *BmOR1* and *BmOR3* was verified by antennal sections.

The signal was detected only when the antisense probe was used for hybridization. Nuclei of antennal cells were visualized with DAPI staining. Scale bar, 100 μm (A-D) and 10 μm (E).

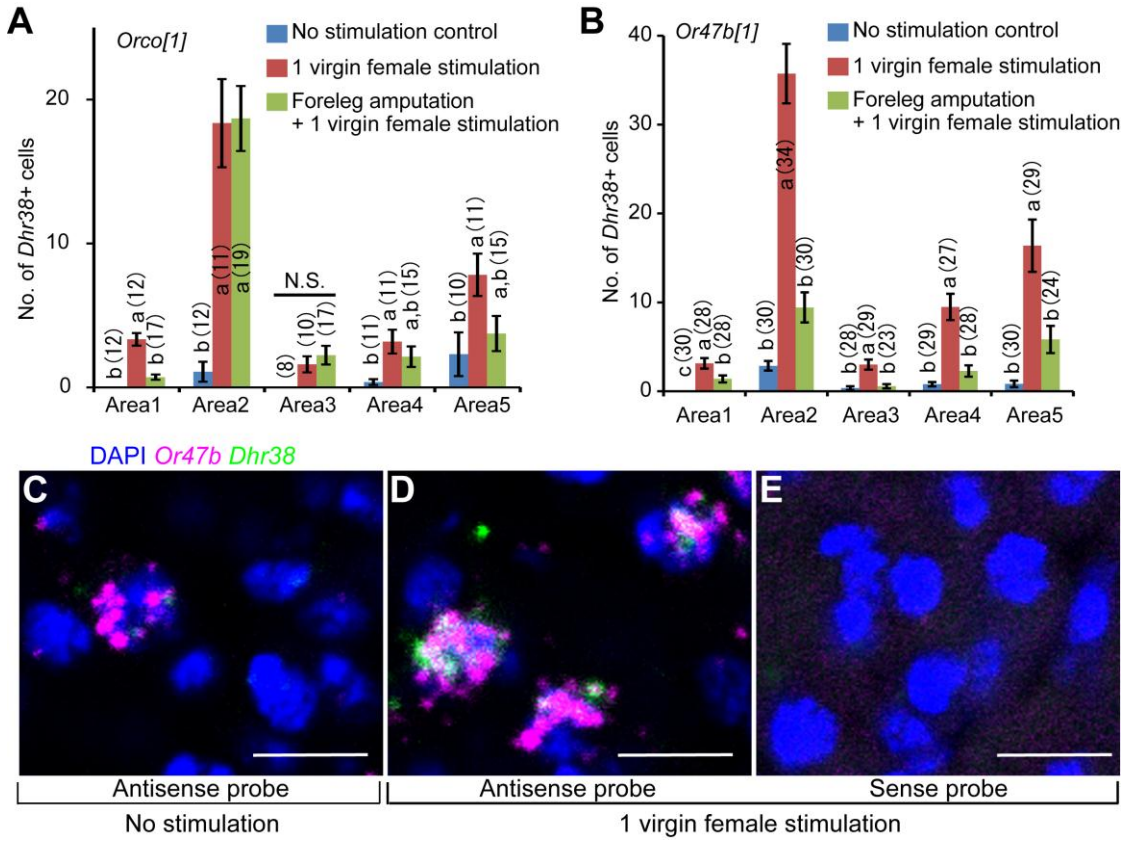

Figure S3: *Dhr38* expression in the brain and antennae of male flies. (A, B) Quantitative analysis of *Dhr38*-positive cells in the male brain of the *Orco* mutant (A) or *Or47b* mutant (B) under various stimulus conditions. Essentially similar data to Figure 4 were observed in these strains. (C-E) Double *in situ* hybridization of *Dhr38* and *Or47b* using antennal sections of CS males revealed that *Dhr38* is expressed in *Or47b*-positive cells in response to female stimulation. Signals were detected only when antisense probes were used for hybridization. Scale bars, 5 μm.

Supplemental Experimental Procedures

Silkmoths and sex pheromone

Eggs of a racial hybrid of *Bombyx mori*, Kinshu × Showa, were purchased from a local dealer (Ueda Sanshu, Nagano, Japan). Larvae were reared on an artificial diet (Silkmate II, Nihon Nosan Kogyo, Yokohama, Japan) at 25°C under a 12-h light/12-h dark photoperiod cycle. Adult moths were used within 0 to 4 days after eclosion. The sex pheromone components, bombykol [(*E*,*Z*)-10,12-hexadecadien-1-ol] and bombykal [(*E*,*Z*)-10,12-hexadecadien-1-al], were synthesized by a local dealer (Sumika Techno Serves, Takarazuka, Japan). The amount of sex pheromone loaded on the paper filter is described in the legends.

Fly lines, culture, and treatments

For experiments in which artificial neural stimulation by dTrpA1 was performed, flies were maintained on standard culture medium at 18°C on a 12-h light/12-h dark cycle. The night before warmth treatment (neural stimulation), flies were moved to 23°C and maintained at that temperature until use. For experiments in which the female-body stimulation was conducted, adult male flies were caught within 4 h after eclosion and maintained individually in small glass tubes (10 mm in diameter and 75 mm in height, and ~1 ml of standard culture medium) at 25°C in a 12-h light/12-h dark cycle until use. Within 3 to 5 days from eclosion, male flies were stimulated with a decapitated adult female body in the glass tube for 2 h. In all experiments, Canton-S (CS) females 3 to 5 days after eclosion were used for stimulation. When a decapitated male body was used for stimulation, CS males 6 to 7 days after eclosion were used. In experiments using males with foreleg and/or antennae amputation, the foreleg tarsi on both sides and/or antennae were amputated on the day of eclosion. All fly strains were obtained from the Bloomington Stock Center and Drosophila Genetic Resource Center, except for *Or47b* mutant flies (gift from Leslie B. Vosshall).

Microarray analysis

To identify genes in male silkmoths whose expression is increased by female odor stimulation, gene expression levels were compared between the brains of control (no stimulation) and stimulated moths. Male silkmoths were stimulated by exposing them to the female odor for 30 min. The whole brains (10 brains of 5 lots for each condition) were collected and frozen immediately. Five independent total RNA samples were isolated from different groups of control and stimulated moths with TRIzol (Invitrogen, Carlsbad, CA). The quality of the RNA samples was assessed using the RNA 6000

Nano LabChip Kit (Bioanalyzer 2100; Agilent Technologies, Palo Alto, CA). Total RNA was then subjected to microarray experiments using the *Bombyx mori* EST 8 X 15k custom microarray (two arrays were used), which was designed based on Unigene information and eArray service (Agilent Technologies). Microarray analyses were performed according to the Agilent 60-mer Oligo Microarray Processing Protocol (Agilent Technologies). Total RNA samples (400 ng) were used to prepare Cy3-labeled cRNA using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies). Fluorescence-labeled cRNAs were purified using the RNeasy RNA Purification Kit (Qiagen, Valencia, CA). Five independent RNA samples were used to confirm the reproducibility of the microarray analyses. Hybridized and washed arrays were scanned with maximum laser intensity in the Cy3 channel using a microarray scanner (G2565BA, Agilent Technologies). The images were analyzed using Feature Extraction Software (Ver. 9.1.3.1; Agilent Technologies). These data were further analyzed with GeneSpring GX11.5 software (Agilent Technologies). Normalization was performed as follows: (1) intensity-dependent Lowess normalization; (2) data transformation, with measurements set to less than 0.01 or 0.01; (3) per-chip 75th-percentile methods were used to normalize each array; (4) per-gene normalization, wherein data were normalized to control samples. After normalization, gene sets determined to have *P* values lower than the cut-off value of 0.05 were considered statistically significant. Then, up-regulated genes with a greater than 1.5-fold change in the expression ratio (stimulated *vs* control moths) were identified. Thus, a combination of statistical analyses and fold change methods was used to assess changes in gene expression.

Because many spots (23 spots) were identified by the above criteria, the number of candidate genes was further reduced by the following criteria: $P < 0.05$ and Fold-change > 2, or $P < 0.01$ and Fold-change > 1.5. The 10 candidate genes identified with these criteria were further verified by a secondary screening with semi-quantitative RT-PCR or qRT-PCR. The primers used for this screening were as follows; M1, 5’-CGAGGAGAGGTGCGGTAAA-3’ and 5’-GAGCGATGCGTTCATGGTAG-3’; M2, 5’-CGACCCCGCCAAATAGAA-3’ and 5’-CACGAGACCCTGAACCCAAG-3’; M3, 5’-GCTGCACTCCTCATGCTAACTC-3’ and 5’-TGGGTGTTGCCTTCGTTTC-3’; M4, 5’-AAACAGCAGTATGCAGCCAAAA-3’ and 5’-TGAAGGAGTGAGGTGCAACAA-3’; M5, 5’-GCGGTGACACAAGTGCAAA-3’ and 5’-TGGGAGCAAATGGGACAATAC-3’; M6, 5’-GTGGCATTCTACGAGTCCCTATTC-3’ and 5’-TCACAAGCAGCACCGACAC-3’; M7, 5’-TCATTTAGGATCTGCTCACACTTCA-3’ and

5’-TCCCGCACTTGTTTGGTTC-3’; M8, 5’-CGTGGGTATGGTGAAAGAAGTG-3’ and 5’-AGAGATGGGTGGGCTTGGT-3’; M9, 5’-CAACTTTTCCTTAGTGGCTTTTGG-3’ and 5’-ACGGAGTAAGCAGACATAAATTGGA-3’; M10, 5’-AAGTTTGCGGACCCCTCTC-3’ and 5’-ACGACTCTCTCGCCCGTTT-3’.

Quantification of gene expression

The level of gene expression was quantified by qRT-PCR. For the silkmoth experiments, total RNA of brains or various tissues was isolated from three to five moths or larvae for each sample, and three to six samples were analyzed for each data point. For the fly experiments, total RNA was isolated from ca. 20 heads for each sample, and three to four samples were analyzed for each data point. The total RNA was treated with DNase I (Invitrogen) and reverse-transcribed with PrimeScript RT reagent kit (Takara-Bio, Otsu, Japan), according to the manufacturer’s protocol. Real-time RT-PCR was performed with *Premix Ex Taq*™ for probe qPCR (Takara-Bio), using gene-specific primers [*Bombyx ribosomal protein L3* (*Brpl3*); 5’-AAGAGATCGTGGAGGCTGTCA-3’ and 5’-CGTAGTCCATGAGGGGTCTCA-3’, *BmHr38*; 5’-GGCTGCCCTCAAAGCCTAA-3’ and 5’-TCGAAGAAGGGCTGTTATCAGA-3’, *Drosophila rpl3* (*Drpl3*); 5’-AAGAAGCGCTCAGCTCGCCA-3’ and 5’-ATGCCGGCCTTGTAGCCGAT-3’, *Dhr38*; 5’-ATTCGGCGGCAAGTTCGCCA-3’ and 5’-TGTCGCCACAAACGGCACACA-3’] and gene-specific Taqman probes [*Brpl3*; 5’-FAM-TCATCGAGACTCCTCCGATGGTTTGTG-BHQ-3’, *BmHr38*; 5’-FAM-TGTCCACAAGAATCTCCACCAAGCCC-BHQ-3’, *Drpl3*; 5’-FAM-CCCCAAGGATGACGCCAGCAAGCCA-BHQ-3’, *Dhr38*; 5’-FAM-CCGTTTGCTCCAGGCTCCGTCGCAG-BHQ-3’]. The fluorescent signals from samples were obtained using ABI7900HT (Applied Biosystems, Foster City, CA) with default settings. The amount of gene expression was determined using the quantification standards. PCR products amplified by each set of primers were purified using a PCR purification kit (Qiagen), serially diluted (10-fold dilutions for a dynamic range of 10^6), and used as quantification standards. The determined value of each sample was normalized by that of *rpl3*. The normalized values were again normalized to the mean value of control samples, and shown as the relative expression. As a negative control, real-time PCR without reverse transcription was performed and confirmed no genomic contamination. Statistical analyses were conducted using the *F*-test and *Student*’s *t*-test or *Welch*’s *t*-test when the data exhibited normal distribution or using a

U-test when the data did not exhibit a normal distribution, using Microsoft Excel (Microsoft) or JMP (SAS) software. Multiple comparisons were conducted using a *one-way analysis of variance* (*ANOVA*) and *Tukey-Kramer*'s HSD test. All data are presented as mean ± standard error.

In situ hybridization

In situ hybridization was conducted as described previously [3, 4]. The digoxigenin (DIG)-labeled RNA probes corresponding to entire *BmHr38* cDNA (1.7 kb) and exon 4 of *Dhr38* (1 kb) were synthesized using a DIG RNA labeling mix (Roche Diagnostics, Basel, Switzerland). The biotin-labeled RNA probes corresponding to +160/+1150 of *BmOR1* cDNA and +154/+1298 of *BmOR3* cDNA were synthesized using a biotin RNA labeling mix (Roche Diagnostics). In *in situ* hybridization using the moth brain sections, DIG-labeled probes were detected immunocytochemically with peroxidase-conjugated anti-DIG antibody (1:500; Roche Diagnostics) and TSA Biotin System (Perkin Elmer, Waltham, MA). In double-*in situ* hybridization using the moth antennae, DIG-labeled probes and biotin-labeled probes were serially detected. TSA Plus Fluorescein System (Perkin Elmer) was used to detect biotin-labeled probes.

In fluorescent whole-mount *in situ* hybridization of fly brains, the brains of control (23°C, overnight) or warmth-stimulated (31°C, 2 h after 23°C, overnight) were dissected, fixed in 4% formaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, and dehydrated with methanol overnight at 4°C. The brains were further dehydrated with ethanol, permeabilized with 50% xylene in ethanol for 30 min, and then rehydrated using a descending series of ethanol in H_2O washes. The brains were again permeabilized with 80% acetone for 10 min, washed with H_2O, fixed in 4% formaldehyde in PBS for 30 min, and hybridized with RNA probes overnight at 60°C, after several washes in PBS with 0.1% Tween 20 and prehybridization for 1 h in hybridization buffer (50% formamide, 5x saline sodium citrate [SSC], 0.1% Tween 20, 1x Denhardt's solution, 1 mg/ml tRNA, 50 μg/ml heparin, and 100 μg/ml salmon sperm DNA). After several washes in serial concentration of wash buffer (50% formamide, 5x SSC, and 0.1% Tween 20), the brains were blocked in 7% normal donkey serum for 1 h, and then incubated in peroxidase-conjugated anti-DIG antibody (1/2000), rabbit anti-green fluorescent protein (GFP) antibody (1/200; Clontech, Mountain View, CA), mouse anti-ELAV antibody (1/20; Developmental Studies Hybridoma Bank [DSHB]) or mouse anti-nc82 antibody (1/20; DSHB), and 1% normal donkey serum overnight at 4°C. After several washes in PBS with 0.3% TritonX100, signals were developed with fluorescein isothiocyanate-conjugated anti-rabbit IgG (1/200; Cappel, Aurora, OH) or Alexa688-conjugated anti-mouse IgG (1/200; Molecular Probes-Invitrogen, Carlsbad,

CA) and TSA Plus Cy3 System (Perkin Elmer). Pictures were obtained using the confocal microscope LSM5 (Carl Zeiss, Germany).

For quantification, the numbers of *BmHr38* or *Dhr38*-positive cells in defined area were manually counted. The area of each brain region was measured using ImageJ analysis software (NIH, http://rsb.info.nih.gov/ij). The number of *BmHr38*-positive cells was divided by the analyzed area and the density was shown as a value relative to 10,000 μm^2.

Antibody development and Western blot analysis

Rabbit antiserum was raised against a synthetic peptide mixture designed to recognize the homologous region between BmHR38 and DHR38 (NH2-TDSLKGRRGRLPSKPKC-COOH and NH2-C+ANLDYSQYREPSPLE-COOH). The antibody was then affinity-purified using affinity columns conjugated with the synthetic peptides. Proteins from three adult male brains (silkmoth) or 10 heads (flies) in each treatment group were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membrane, and incubated with affinity-purified anti-HR38 antibody. The bound antibody was detected by horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling Technology, Japan) and ECL Advance (GE Healthcare) according to the manufacturers' protocols. The membrane was deprobed and used for loading control detection. Mouse monoclonal antibody against Synapsin was obtained from the DSHB.

Accession number

The microarray data was deposited on GEO according to MIAME guideline (Accession# GSE39306).

Supplemental References

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