PNAS
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Supplementary Information for

Maternal GABAergic and GnRH/corazonin pathway modulates egg diapause phenotype of the silkworm *Bombyx mori*

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Materials and Methods

Silkworms

Larvae were reared on an artificial diet (Kuwano-hana, JA Zennoh Gunma) at 25–27 °C under a 13-h light/11-h dark cycle (13L:11D) and relative humidity of 30–50%. Pupae used in this experiment were collected within 1 h after ecdysis (referred to as day 0) to synchronize their subsequent development. Pupae were kept at 25°C to allow adult development. The percentage of diapause eggs was estimated by counting the numbers of eggs in diapause and those not in diapause after the non-diapause eggs hatched. The results are expressed as the average percentage of diapause in each egg batch (1, 2).

Injection of chemicals and peptides

GABA, PTX, and L-2,4-diamino-*n*-butyric acid (DABA) were purchased from Sigma Aldrich (St. Louis, MI, USA), and trans-aminocrotonic acid (TACA) and Saclofen hydrochloride was purchased from Tocris Bioscience, Ellisville, MO, USA. (−)-Bicuculline Methobromide was purchased from Wako, Osaka, Japan. In addition, synthetic Crz peptide (pQTFQYSRGWTNa) was purchased from ABBIOTEC (San Diego, CA, USA). Each DH and Crz/7_8insD of 95% purity (HPLC area percentage) was obtained from Operon Biotechnologies, Tokyo, Japan.

TALEN and CRISPR-Cas9 systems

TALEN targets were searched using TAL Effector Nucleotide Targeter 2.0 (https://tale-nt.cac.cornell.edu) in the coding regions of target genes. DNA constructs containing the TAL segments were prepared using a Platinum Gate TALEN kit (Addgene, Cambridge, MA, USA). TALEN mRNAs were then synthesized using mMessage mMachine T7 Ultra kit (Ambion, Carlsbad, CA, USA); mRNA of each TALEN was mixed at a concentration of 0.5 μg/μL for microinjection. Non-diapause eggs of the N4 and Kosetsu strains were collected within 1 h after

oviposition during the syncytial blastoderm stage; the TALEN mRNA mixture was injected into the eggs using a glass needle (uMPm-02; Daiwa Union) attached to a manipulator (kaikopuchu-STDU1; Daiwa Union, Iida, Japan) and FemtoJet (Eppendorf).

For screening of germline mutagenesis, the G_0 adults were mated with *wt*. The oviposited G_1 eggs were collected, and approximately 10 eggs from each brood were pooled for genomic DNA extraction using DNAzol® reagent (Thermo Fisher Scientific, Tokyo, Japan). The DNA fragment containing the targeted region of interest was amplified by PCR using Takara Ex Taq (Takara). To test for mutagenesis, the PCR products of *DH-PBAN*, *DHR*, *Crz*, *CrzR*, and *GAT* were digested with restriction enzymes *Psp*1406I (Takara), FastDigest *Mnl*I (Thermo), *Sac*II (Takara), FastDiges *Mbo*II (Thermo), and *Stu*I (Takara), respectively. The presence of an undigested PCR product would suggest that the restriction site in each spacer region was disrupted by TALENs. The PCR product of *8916* was treated with Surveyor Mutation Detection Kit (IDT, Tokyo, Japan). These mutated PCR products were checked by sequencing. The broods containing mutated sequences were reared, and mutated G_1 adults were crossed with the siblings that carried the same mutation. Homozygous mutants were obtained after confirmation by sequencing of the target region in the G_2 or G_3 egg genome.

DH levels in brain–SG complex and hemolymph

The brain–SG complexes of five animals were dissected in cold phosphate buffered saline (PBS) on ice. Tissues were pooled in 250 µl of ice-cold extraction buffer (supernatant after centrifugation of 5% skim milk solution in PBS at 21,130 × *g* for 10 min). After homogenization for 2 min, the tissue suspension was boiled for 5 min and cooled on ice for 10 min. Aliquots of the supernatant after centrifugation at 21,130 × *g* were used for the assay. DH was extracted from the hemolymph as previously described (2). DH extraction was performed at ZT6 (ZT = zeitgeber

time, ZT = 0 corresponds to lights on). DH levels were measured using time-resolved fluoroimmunoassay (TR-FIA) as described previously (2).

cDNA cloning

The partial cDNA was amplified by RT-PCR using the first-strand DNA derived from the pupal brain–SG complex as a template in each N4 and Kosetsu strain, and each primer set (Supplementary Table S2). In five ionotropic GABA receptor subunits, the full-length sequence of each cDNA was determined by SMARTer® RACE 5′/3′ Kit (Takara Bio., Tokyo, Japan).

RT-PCR and quantitative PCR analysis

Eggs were collected 2 h and 3, 5, and 9 days after oviposition. Various tissues were dissected from day 4 fifth instar larvae and day 3 pupae. Total RNAs were extracted from eggs and various tissues using TRIzol reagent (Invitrogen) and then subjected to poly(A)$^+$ RNA purification using Dynabeads oligo(dT)$_{25}$ (Thermo Fisher Scientific). Poly(A)$^+$ RNA from the brain–SG complex was directly purified using Dynabeads Oligo (dT)$_{25}$. First-strand DNA was synthesized using a SMART RACE amplification kit (Takara Bio.). PCR amplification was carried out on mRNAs for BmRDL1, BmRDL2, BmRDL3, BmLCCH3, Bm8916, and actin A3 using each primer set (Supplementary Table S2).

In the quantitative PCR (qPCR), the RNA extraction and first-strand DNA synthesis were performed using TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA) and ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan), respectively. The qPCR analysis was performed using TB Green Premix DimerEraser with the Thermal Cycler Dice® Real time system (TP800) (Takara Bio., Tokyo, Japan). In both analyses,

the primer sets are represented in Supplementary Table S2. Relative RNA levels were normalized against *Actin A3* mRNA levels.

Thionin-, Immunostaining, and RNAscope®

For thionin staining, the eggs were fixed in the Carnoy's fixative for 1 day at 4 °C. The fixed eggs were treated with a gradient concentration of ethanol for 10 min each. Following 5 min of boiling, the chorion was dissected out, and eggs were transferred into the thionine solution (0.07% of thionine and 0.3% of phenol dissolved in 80% ethanol) for 2 h. The stained eggs were rinsed with 80% ethanol 4 times and dehydrated with a gradient concentration of ethanol for 10 min each. Thereafter, the eggs were soaked in benzene to make the yolk transparent. The embryos were observed by M165 FC (Leica, Wetzlar, Germany).

The immunoreaction procedures were adapted from Hagino. et al. (3). Briefly, the brain–SG complex was dissected in fixative containing 4% paraformaldehyde, 7% picric acid, 10 mM $MgCl_2$, 5 mM EGTA-NaOH, and 0.5 M HEPES-NaOH (pH 6.9) and incubated at 4°C overnight. The fixed tissues were stored in 90% methanol containing 50 mM EGTA at −20°C for storage until used. The fixed and stored tissues were hydrated through decreasing concentrations of methanol and washed with PBS containing 0.2% Tween-20 (PBT). Tissue samples were soaked in PBS containing 2% Tween-20 over two nights, washed with PBT, blocked with PBT containing 5% heat-inactivated goat serum and 2% BSA, and incubated with anti-Crz at 1:1000, or anti-DH[N] at 1:2500 at 4°C overnight. The signal was detected with Cy3-labeled IgG (Jackson ImmunoResearch Lab. West Grove, PA, USA) diluted to 1:1500 using the FV1000-D confocal microscope (Olympus, Tokyo, Japan).

To determine the relative fluorescence intensity of the dorsolateral Crz-producing somata and its axon, we compared the intensity of the mean pixel fluorescence for individual somata and its axon. Confocal scans were performed under the same conditions for each PTX- or DW-

injected silkworm. Using NIH image 1.62 (http://rsb.info.nih.gov/nih-image/), the relative fluorescence intensity of the somata and arbitrarily limited areas of the its axon was determined as the intensity of the individual cells relative to the mean pixel fluorescence for the entire somata or arbitrarily limited areas of the its axon (S) of the brain. Fluorescence images were converted to grayscale and inverted into black and white images. An area adjacent to the area of interest (A) and an area from an image lacking a specimen (N) were scanned as the background signals. When Crz neurons were not visible, the focal plane was adjusted to show faint signals of small cells in the same field. Data are presented as the percentage intensity relative to the mean intensity of the control as follows: Relative fluorescence intensity (%) = 100 × ([(S) − (A) − (N)]/[(A) − (N)]) for PTX-injected silkworm (+PTX) ÷ ([(S) − (A) − (N)]/[(A) − (N)]) for DW-injected silkworm (+DW). Averages were calculated for one silkworm.

We performed the *in situ* hybridization using an RNAscope® Fluorescent Multiplex Reagent kit (Advanced Cell Diagnostics (ACD), Inc., Newark, CA, USA). In brief, the brain–SG complex was fixed and stored as well as in immunostaining described above. Tissues were hydrated with PBS containing 2% Tween-20 (PBT), and treated proteinase III at 1/5 dilution. Thereafter, tissues were washed in PBST and transferred to 0.2-ml PCR tubes incubated at 40°C for hybridization with each RNAscope® probe using a thermal cycler overnight. The hybridized signals were developed according to the manufacturer's instructions for the kit. The signals were observed using the FV1000-D confocal microscope (Olympus). Images were adjusted and assembled in Adobe Photoshop CS6 (Abode Systems, San Jose, CA, USA).

1. O. Yamashita, K. Hasegawa, *Embryonic diapause*. G. A. Kerkut, L. I. Gilbert, Eds., Comprehensive Insect Physiology, Biochemistry and Pharmacology (Pergamon Press, Oxford, 1985), vol. 1, pp. 407-434.
2. K. Shiomi *et al.*, Disruption of diapause induction by TALEN-based gene mutagenesis in relation to a unique neuropeptide signaling pathway in Bombyx. *Sci Rep* **5**, 15566 (2015).
3. A. Hagino, N. Kitagawa, K. Imai, O. Yamashita, K. Shiomi, Immunoreactive intensity of FXPRL amide neuropeptides in response to environmental conditions in the silkworm, Bombyx mori. *Cell Tissue Res* **342**, 459-469 (2010).

Supplementary Table S1

	[mg/pupa]	N4	Kosetsu (15DD)	Kosetsu (25DD)	Ascoli-Koken
DW	-	0.0 ± 0.0	0.0 ± 0.0	100 ± 0.0	100 ± 0.0
GABA	1	0.0 ± 0.0	0.0 ± 0.0	100 ± 0.0	100 ± 0.0
DABA	5	0.0 ± 0.0	0.0 ± 0.0	100 ± 0.0	100 ± 0.0
TACA	0.2	0.0 ± 0.0	0.0 ± 0.0	100 ± 0.0	100 ± 0.0
PTX	0.05	80.3 ± 19.6	86.6 ± 15.0	100 ± 0.0	100 ± 0.0
Bicuculline	0.036	0.0 ± 0.0	0.0 ± 0.0	100 ± 0.0	100 ± 0.0
Saclofen	0.29	0.0 ± 0.0	0.0 ± 0.0	100 ± 0.0	100 ± 0.0

Effect of chemicals with respect to GABAergic neuron on diapause-egg-inducing activity. The distilled water (DW), GABA, L-2,4-diamino-n-butyric acid (DABA), trans-aminocrotonic acid (TACA), plant alkaloid picrotoxin (PTX), (−)-Bicuculline, and Saclofen were injected at representative doses into pupae of N4, Kosetsu (15DD), Kosetsu (25DD), and Ascoli-Koken strains. The diapause-egg-inducing activities represent the mean ± SD of 5–10 animals.

Supplementary Table S2

List of primers*

a. For qPCR

1. DH-PBAN	F: 5′-GGATGAAAGCGACAGAGGAG-3′
	R: 5′-ATCTTCAGTGGATGGCTTCA-3′
2. Crz	F: 5′-GACGAATGGAAAACGAGACG-3′
	R: 5′-TAATCACACGGCACGAACAG-3′
3. BmGAT	F: 5′-TCTTCAATCTCGTGCAGTGG-3′
	R: 5′-GTGACACGCCACAGGTACAC-3′
4. BmRDL1	F: 5′-TCCAGAATGCCCTCCAG-3′
	R: 5′-AAAACGGACTTCAGATGGTCT-3′
5. BmRDL2	F: 5′-TACCACCTAGCCGATCTTCG-3′
	R: 5′-TTGTCCTCCTGCTTCTTCGT-3′
6. BmRDL3	F: 5′-TTCGCTACAAGGTCCGAGAT-3′
	R: 5′-GTTCATCCTGCTCCTGCTG-3′
7. BmLCCH3	F: 5′-ATCCACTCGACAGCCAGAAC-3′
	R: 5′-TGGGGTAGTTCAGCGTCTTC-3′
8. Bm8916	F: 5′-TCAATCGTGCCCTCTTATCC-3′
	R: 5′-AAAGTTGACGCTCTGCGAGT-3′
9. BmGAD	F: 5′-AGAAGGGTCTCACCAGCATC-3′
	R: 5′-AAGGCACGCACACACAGTAG-3′
10. BmVGAT	F: 5′-GGATGGAATTGCTCCAAGAA-3′
	R: 5′-TTTTAGCTCCTGGCTTTCCA-3′
11. BmGABAT	F: 5′-CAAGACCGGAAAGGTTTTGA-3′
	R: 5′-TCGGCCACGAACACTATGTA-3′
12. BmSsadh	F: 5′-TGAATTTGGCATGGTTGCTA-3′
	R: 5′-CCTTCACGCCCTATACCAGA-3′
13. BmActA3	F: 5′-ATCACCATCGGAAACGAAAG-3′
	R: 5′-GGTGTTGGCGTACAAGTCCT-3′

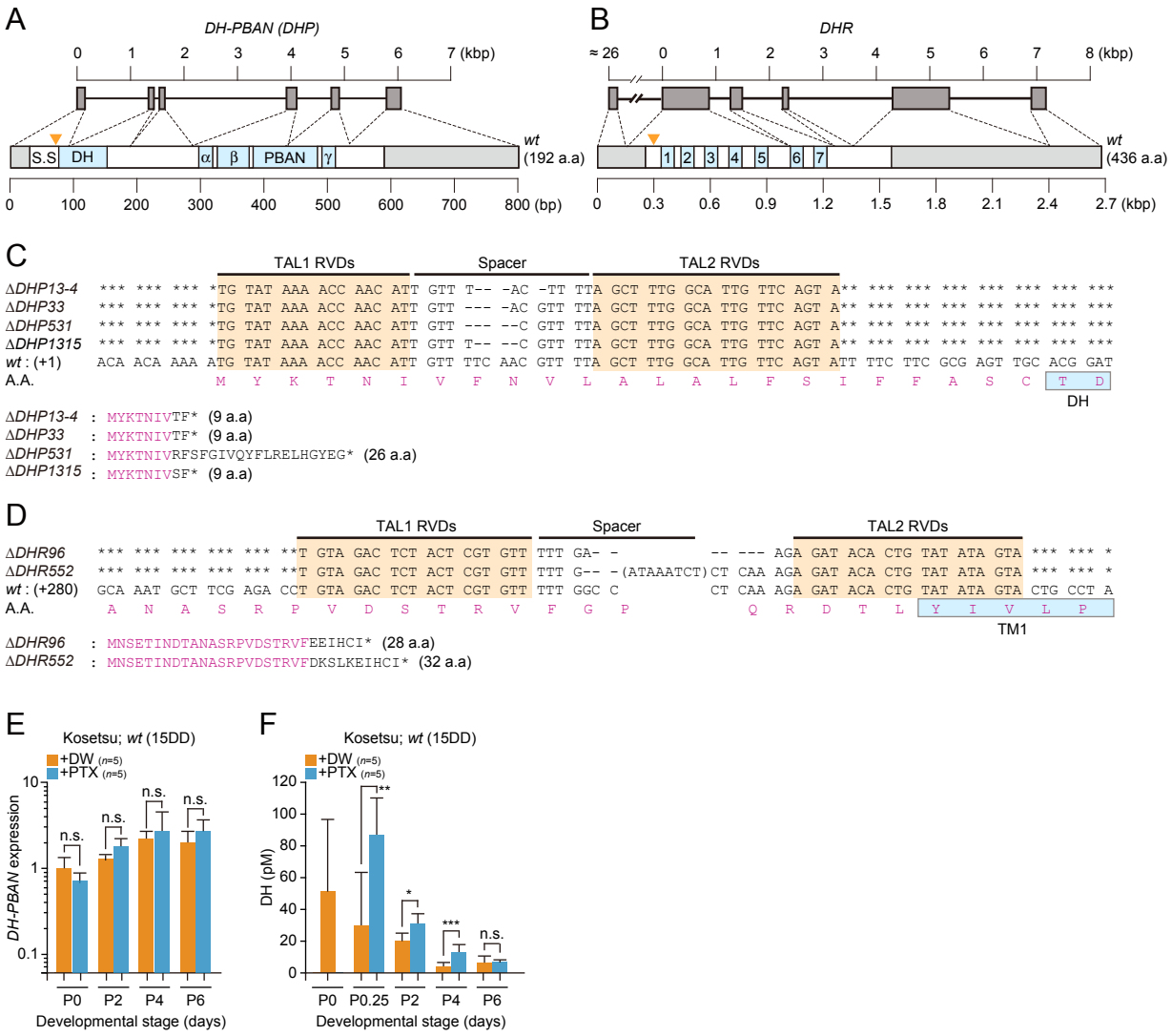
b. For RT-PCR analysis

1. BmRDL1	F: 5′-CGCCGGCGGCGGTGGAGGTGGAATGTTCGG-3′
	R: 5′-TGTAAAATTAGTGGATAATACATAACGCCC-3′
2. BmRDL2	F: 5′-ATGCATAACACGAGCCGTACTCGC-3′
	R: 5′-TCAATTCTCTTCGCCAAGGAGTAC-3′
3. BmRDL3	F: 5′-ATGAGCGCCGTGCGTCACGCCGCG-3′
	R: 5′-TCACTTGTCCTCGAGCAGTTCCAG-3′
4. BmLCCH3	F: 5′-CGACTAGAGAACGTGACGCATACCGTGTCA-3′
	R: 5′-GTTATAGGAAGGAATATTGTTTGTCTAGTT-3′
5. Bm8916	F: 5′-TGGATTGCTACTTCAGGCAATATTGGCGTG-3′
	R: 5′-TCATCCAGTAGAATAGATTGAGCAGTGCAA-3′
6. BmActA3	F: 5′-ATGTGCGACGAAGAAGTTGCCGCGTTG-3′
	R: 5′-CAGCGAGAGCACGGCTTGGATGGCGAC-3′

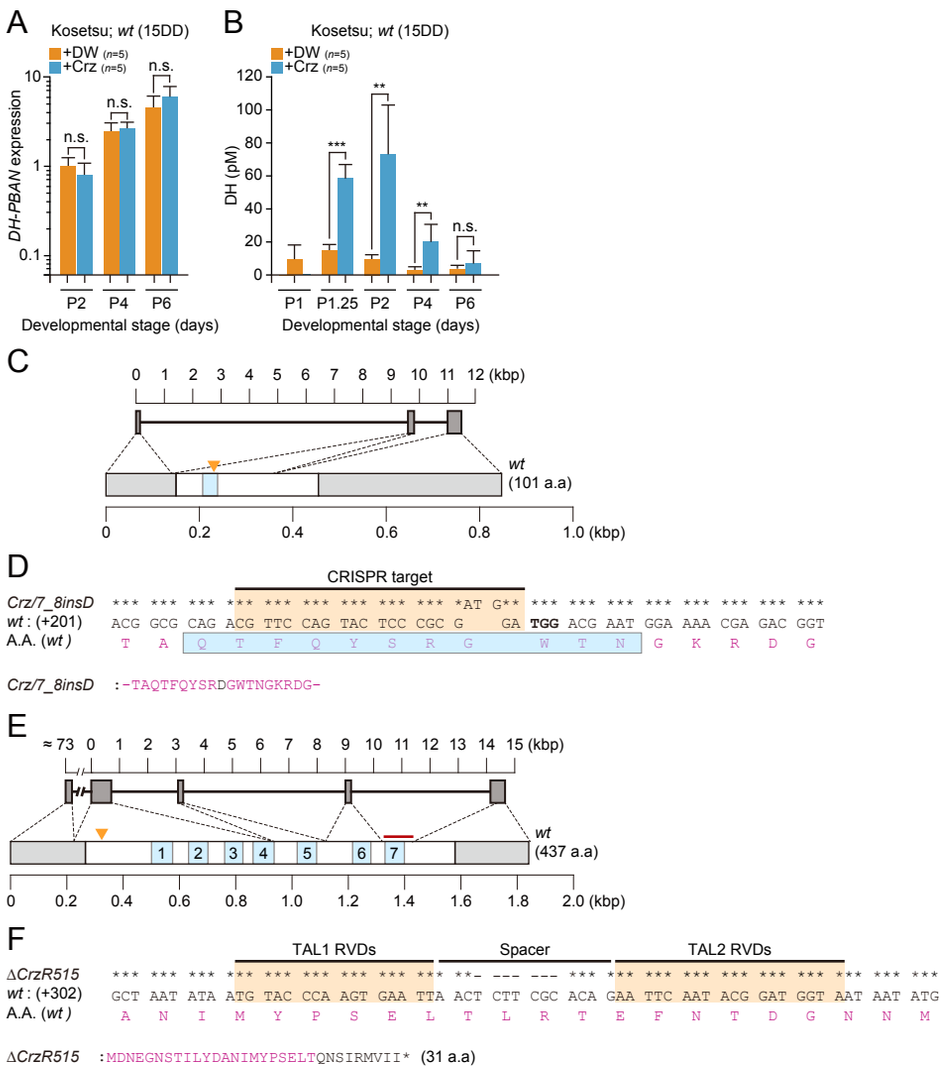
c. For cDNA cloning

1. BmRDL1	F: 5′-GAGTCGAGTCGGTAATTAGGAGGTGAGTAC-3′
	R: 5′-ACAGTTCCAAATTATAAATTCATATTCACG-3′
2. BmRDL2	F: 5′-ATGCATAACACGAGCCGTACTCGC-3′
	R: 5′-TCAATTCTCTTCGCCAAGGAGTAC-3′
3. BmRDL3	F: 5′-ATGAGCGCCGTGCGTCACGCCGCG-3′
	R: 5′-CTGGAACTGCTCGAGGACAAGTGA-3′
4. BmLCCH3	F: 5′-ACACTTCACGAGACCCCACTGCTGATCGCG-3′
	R: 5′-GTTATAGGAAGGAATATTGTTTGTCTAGTT-3′
5. Bm8916	F: 5′-GAACGAAACCTTCACCGTGTCTGCCGCACG -3′
	R: 5′-CACTACATAATATTAATGTAATTTTCCTAAC-3′

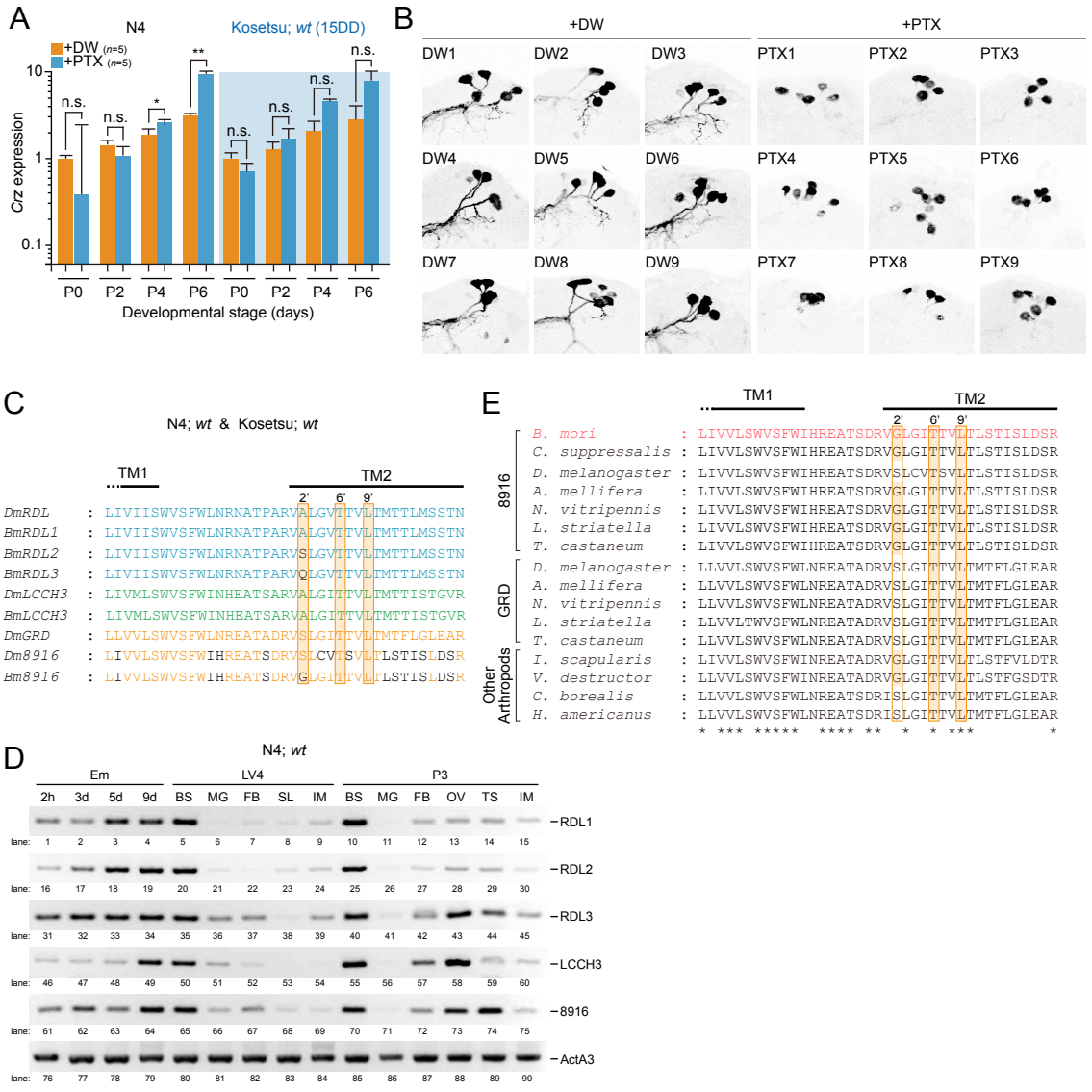
*F; Forward primer, R; Reverse primer.



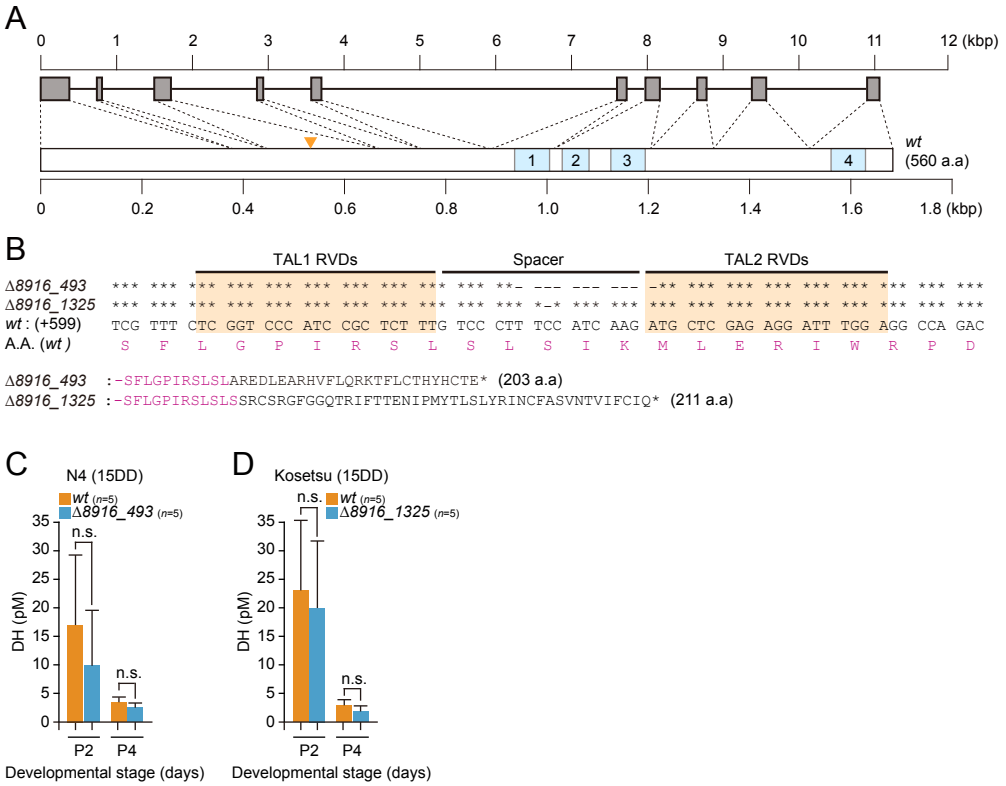
Supplementary Figure S1. (**A**, **B**) Schematic representations of the genes (top) and cDNA structures (bottom) of the *DH-PBAN* (**A**) and *DHR* (**B**). Shaded boxes and lines represent exons and introns, respectively. The cDNA of *DH-PBAN* encodes a signal sequence (S.S), diapause hormone (DH), PBAN, and α-, β-, and γ-SGNPs, which carry the 5′ and 3′ untranslated regions (light-grey boxes on left and right sides, respectively). The cDNA of *DHR* consists of seven transmembrane domains (1–7), which carry the 5′ and 3′ untranslated regions (light-grey boxes on left and right sides, respectively). The FXPRLa and transmembrane domains are indicated by blue boxes. Orange triangles represent the TALEN binding sites. The sizes of exons and introns (in bp) are indicated by scales in each map. (**C**, **D**) The sequences of the TALEN target sites are indicated by orange boxes. Partial coding sequences corresponding to the DH N-terminus and transmembrane domain 1 of DHR are indicated by blue boxes. The deleted bases in spacer regions of each mutant are indicated by hyphens, and identical bases are indicated by asterisks. The Δ*DHP13-4*, Δ*DHP33*, Δ*DHP531*, Δ*DHP1315*, Δ*DHR96*, and Δ*DHR552* are truncated proteins, which encode 9, 9, 26, 9, 28, and 32 amino acids, respectively. (**E**) Effect of PTX injection on *DH-PBAN* expression in brain–SG complex during pupal–adult development of Kosetsu (15DD). The PTX (50 µg) or DW was injected on just after pupation (+PTX or +DW). Each bar represents the mean ± SD of five samples. (**F**) Effect of PTX injection on DH titer in hemolymph during pupal–adult development of Kosetsu (15DD). PTX (50 µg) was injected on just after pupation (P0). Each bar represents the mean ± SD of five samples. n.s., non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.



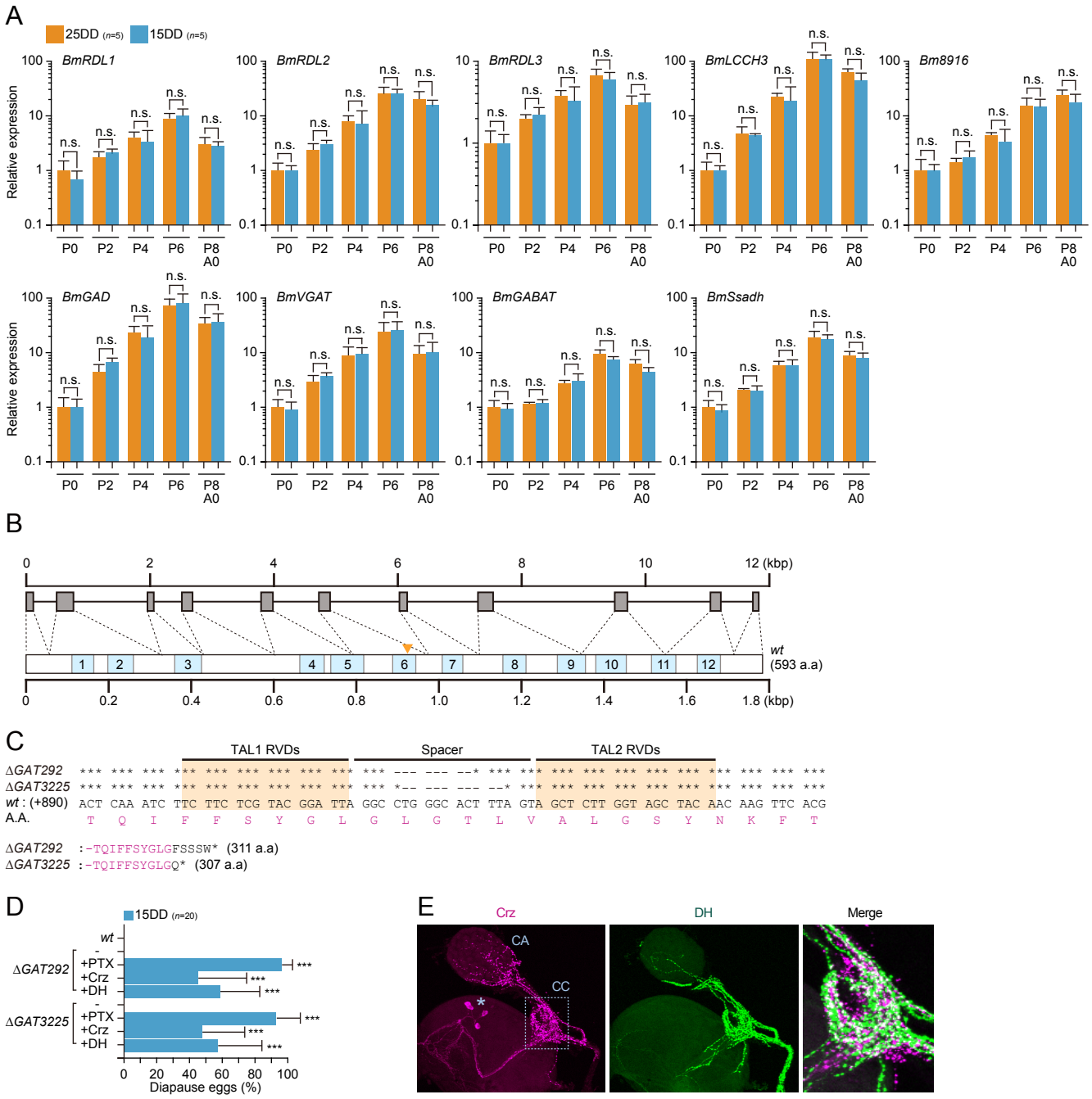
Supplementary Figure S2. (**A**) Effect of corazonin (Crz) injection on *DH-PBAN* expression in brain–SG complex during pupal–adult development of Kosetsu (15DD). The Crz (1 nmol) or distilled water (DW) was injected just after pupation (+Crz or +DW). Each bar represents the mean ± SD of five samples. (**B**) Effect of Crz injection on diapause hormone titer in hemolymph during pupal–adult development of Kosetsu (15DD). Crz (1 nmol) was injected 1 d after pupation (P1). Each bar represents the mean ± SD of five samples. n.s., non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (**C**, **E**) Schematic representations of the genes (top) and cDNA structures (bottom) of the *Crz* (**C**) and *CrzR* (**E**). Shaded boxes and lines represent exons and introns, respectively. The cDNA of *Crz* encodes the mature peptide of Crz (blue box), which carry the 5′ and 3′ untranslated regions (light-gray boxes on left and right sides, respectively). The cDNA of *CrzR* consists of seven transmembrane domains (1–7), which carry the 5′ and 3′ untranslated regions (light-gray boxes on left and right sides, respectively). Orange triangles represent the CRISPR and TALEN binding sites. We could not identify the partly genomic sequence corresponding to the cDNA sequence of *CrzR* using KAIKObase (Red line). The sizes of exons and introns (in bp) are indicated by scales in each map. (**D**, **F**) The sequences of the CRISPR and TALEN target sites are indicated by orange boxes. Partial coding sequence corresponding to the mature peptide of Crz is indicated by blue box. The PAM sequence (TGG) indicated by bold letters. The deleted bases in spacer regions of *CrzR* mutant are indicated by hyphens, and identical bases are indicated by asterisks. The *Crz/7_8insD* is inserted ATG sequence, and Δ *CrzR515* are mutated proteins that encode 31 amino acids.



Supplementary Figure S3. (**A**) Effect of plant alkaloid picrotoxin (PTX) injection on corazonin (Crz) expression in brain–SG complex during pupal–adult development of both N4 and Kosetsu (15DD). The PTX (50 μg) or distilled water (DW) was injected just after pupation (+PTX or +DW). Each bar represents the mean ± SD of five samples. n.s., non-significant; *, $P < 0.05$; **, $P < 0.01$. (**B**) The raw images in Crz immunostaining of DW-injected (DW1-9) or PTX-injected (PTX1-9) pupae. (**C**, **E**) Sequence alignment between TM1 and TM2 of ionotropic GABAR subunit orthologs between *Drosophila melanogaster* and *Bombyx mori* (**C**) and among the arthropods (**E**). GenBank accession numbers: DmRDL (M69057.2), BmRDL1 (AB278155.1), BmRDL2 (GQ890664.1), BmRDL3 (NM_001195701.1), DmLCCH3 (NM_206746.2), BmLCCH3 (AB278156.1), *Ixodes scapularis* (KC710947.1), *Varroa destructor* (KY748054.1), *Cancer borealis* (KU986873.1), *Homarus americanus* (KU986877.1), and others referred to in Fig. 3D. The boxes at the 2′, 6′, and 9′ positions of TM2 residues in each subunit implicated in PTX binding. *, a.a. corresponding among 8916 and GRD. (**D**) Developmental profiles of expression of ionotropic GABAR subunit orthologs. RT-PCR analysis was performed during embryogenesis 2 h and 3, 5, and 9 d after oviposition (Em) and on day 4 in the fifth instar larvae (LV4) as well as on day 3 in pupae (P3). BS, brain–subesophageal ganglion complex; MG, midgut; FB, fat body; SL, silk gland; IM, integument and muscle; OV, ovary; TS, testis.



Supplementary Figure S4. (**A**) Schematic representations of the genes (top) and cDNA structures (bottom) of *8916*. Shaded boxes and lines represent exons and introns, respectively. The cDNA of *8916* consists of four transmembrane domains, which are indicated by blue boxes. Orange triangles represent the TALEN binding sites. The sizes of exons and introns (in bp) are indicated by scales in each map. (**B**) The sequences of the TALEN target sites are indicated by orange boxes. The deleted bases in spacer regions of each mutant are indicated by hyphens, and identical bases are indicated by asterisks. The Δ*8916_493* and Δ*8916_1325* are truncated proteins, which encode 203 and 211 amino acids, respectively. (**C**, **D**) Comparison in DH titer with *wt* and Δ*8916_493* and Δ*8916_1325* during pupal–adult development of N4 (**C**) and Kosetsu (15DD) (**D**). Each bar represents the mean ± SD of five samples. n.s., non-significant.



Supplementary Figure S5. (**A**) Quantitative PCR analysis of genes with respect to GABAergic neurotransmission in brain–SG complex during pupal–adult development between 25DD and 15DD of Kosetsu. Each bar represents the mean ± SD of five samples. n.s., non-significant. (**B**) Schematic representations of the genes (top) and cDNA structures (bottom) of the plasma membrane *GAT*. Shaded boxes and lines represent exons and introns, respectively. The cDNA of *GAT* consists of 12 transmembrane domains, which are indicated by blue boxes. Orange triangles represent the TALEN binding sites. The sizes of exons and introns (in bp) are indicated by scales in each map. (**C**) The sequences of the TALEN target sites are indicated by orange boxes. The deleted bases in spacer regions of each mutant are indicated by hyphens, and identical bases are indicated by asterisks. The Δ*GAT292* and Δ*GAT3225* are truncated proteins, which encode 311 and 307 amino acids, respectively. (**D**) The diapause-egg-inducing activity in KO mutants of *GAT*, Δ*GAT292*, and Δ*GAT3225* in 15DD. The proportions of diapause eggs oviposited from *wt* (*wt*) and KO mutant (−) female moths were measured as well as that of moths injected with plant alkaloid picrotoxin, corazonin, and diapause hormone at 50 μg, 1 nmol, and 100 pmol/pupa, respectively. Each bar represents the mean ± SD of 20 animals. The significant differences vs. (−) were represented. n.s., non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (**E**) Immunostaining of Crz and DH neurons in the pupal brain–corpus cardiacum (CC)/corpus allatum (CA). Magnified merged image shows the CC, indicated by the box, in Crz-immunostaining. The asterisk indicates the somata of Crz neurons.