Ecdysone signaling regulates specification of neurons with a male-specific neurite in Drosophila

Binglong Zhang, Kosei Sato and Daisuke Yamamoto*

ABSTRACT

Some mAL neurons in the male brain form the ipsilateral neurite (ILN[+]) in a manner dependent on FruBM, a male-specific transcription factor. FruBM represses robo1 transcription, allowing the ILN to form. We found that the proportion of ILN[+]-mALs in all observed single cell clones dropped from ~90% to ~30% by changing the heat-shock timing for clone induction from 4-5 days after egg laying (AEL) to 6-7 days AEL, suggesting that the ILN[+]-mALs are produced predominantly by young neuroblasts. Upon EcR-A knockdown, ILN[+]-mALs were produced at a high rate (~60%), even when heat shocked at 6-7 days AEL, yet EcR-B1 knockdown reduced the proportion of ILN[+]-mALs to ~30%. Immunoprecipitation assays in S2 cells demonstrated that EcR-A and EcR-B1 form a complex with FruBM. robo1 reporter transcription was repressed by FruBM and ecdysone counteracted FruBM. We suggest that ecdysone signaling modulates the FruBM action to produce an appropriate number of male-type neurons.

KEY WORDS: Sexual dimorphism, Courtship behavior, Circuit remodeling, Metamorphosis, The fruitless gene

INTRODUCTION

It is widely thought that the sex-determination mechanism is distinctly different between vertebrates and insects; in vertebrates, systemic androgens and estrogens primarily determine the sex of an entire body (Sekido and Lovell-Badge, 2009), whereas in insects, every cell adopts a sexual fate according to its own chromosomal composition, without any involvement of androgens and estrogens (Salz, 2011). The only exception to this rule is the presence of a low level of 17β-estradiol in silkworms (Ohnishi et al., 1985), which has been suggested to promote synthesis of vitellogenin, a female-specific protein (Shen et al., 2015). Rather than using them for sex determination, insects use steroids to realize their unique determination, insects use steroids to realize their unique sexual differences (Billeter et al., 2006; Neville et al., 2014; von Philipsborn et al., 2014). FruAM, FruBM and FruEM share the N-terminal BTB domain, with the distinct zinc finger motifs at their C-terminus (Ryner et al., 1996; Usui-Aoki et al., 2000). The male-specific proteins thus produced are collectively called FruM (where ‘M’ stands for male). FruM is composed of five isoforms, three of which (FruAM, FruBM and FruEM) have been demonstrated to contribute to neural sexual differences (Billetter et al., 2006; Neville et al., 2014; von Phillipsborn et al., 2014). FruAM, FruBM and FruEM share the N-terminal BTB domain, with the distinct zinc finger motifs at their C-terminus (Ryner et al., 1996; Usui-Aoki et al., 2000). The FruBM isoform recruits chromatin remodeling factors such as Bonus (Bon), Histone deacetylase 1 (HDAC1) and Heterochromatin protein 1a (HAT; Kirilly et al., 2011). This invites speculation that crosstalk between ecdysone signaling and the sex-determination pathway might provide a means for the organism to create sex differences in an otherwise unisexual neural circuitry (Ito et al., 2013).

The neural basis for sexual behavior has been extensively analyzed in a genetic model organism, Drosophila melanogaster, in which fruitless (fru) and doublesex (dsx), two major transcription factor genes with the sex-determination function, are key players in the construction of the sexually dimorphic circuitry underlying mating behavior (Dickson, 2008; Pavlou and Goodwin, 2013; Yamamoto and Koganezawa, 2013). Whereas dsx is widely involved in the development of sexual traits in a variety of tissues, fru-dependent sexual differentiation is strictly restricted to the nervous system (Dickson, 2008; Pavlou and Goodwin, 2013; Yamamoto and Koganezawa, 2013). Among the four promoters of the fru gene, the most distal promoter (the P1 promoter) is dedicated to sexual function (Ryner et al., 1996), producing multiple transcripts that are translated only in the male nervous system (Lee et al., 2000; Usui-Aoki et al., 2000). The male-specific proteins thus produced are collectively called FruM (where ‘M’ stands for male). FruM is composed of five isoforms, three of which (FruAM, FruBM and FruEM) have been demonstrated to contribute to neural sexual differences (Billetter et al., 2006; Neville et al., 2014; von Phillipsborn et al., 2014). FruAM, FruBM and FruEM share the N-terminal BTB domain, with the distinct zinc finger motifs at their C-terminus (Ryner et al., 1996; Usui-Aoki et al., 2000). The FruBM isoform recruits chromatin remodeling factors such as Bonus (Bon), Histone deacetylase 1 (HDAC1) and Heterochromatin protein 1a (HAT; Kirilly et al., 2011). This invites speculation that crosstalk between ecdysone signaling and the sex-determination pathway might provide a means for the organism to create sex differences in an otherwise unisexual neural circuitry (Ito et al., 2013).
(HP1a) to target sites on the genome, thereby regulating the transcription of genes involved in sex-specific neural development (Ito et al., 2012). Thus, FruBM seems to use an epigenetic mechanism in neural sex fate induction, just as ecdysone signaling does (Sedkov et al., 2003) in neural remodeling during metamorphosis.

In this study, we show that EcR and FruBM indeed interact at the molecular level to produce an appropriate number of neurons that are equipped with the male-specific neurite. Based on this observation, we suggest that the insect molting hormone ecdysone modulates the transcriptional activities of FruBM to induce sex-specific neurobehavioural characteristics. Thus, ecdysone may exert a sex-specific function comparable to that of vertebrate sex steroids, when it operates through the ecdysone receptor complex containing FruBM as a constituent.

RESULTS

EcR is a genetic modifier of fru

In searches for genes that interact with fru, we took advantage of a visible phenotype induced by overexpression of the normal form of FruB in the compound eye (Goto et al., 2011). In contrast to the regular array of ommatidia in the wild-type eye (Fig. 1A), the eye with fruB overexpression exhibited a broad range of abnormalities: the ommatidium was disrupted in shape, the border between neighboring ommatidia became shallow with a melted appearance, bristles were lost with a few remnants, and the entire compound eye was reduced in size (Fig. 1D). When the fly carried a copy of either EcRM554fs or EcRF559fs, null alleles of EcR, the effects of overexpressed FruB were markedly mitigated, except for the reduced size of the compound eye (Fig. 1E,F). The EcR mutant heterozygosity induced only a moderate roughness of the compound eye (Fig. 1B,C), which cannot explain the observed suppression of FruB-induced eye phenotypes by a mutant copy of EcR, suggesting that EcR genetically interacts with fru, at least when FruB is ectopically expressed in the eye.

EcR increases mAL neurons with the male-specific neurite

Because both fru and EcR are primarily involved in the control of development, we presumed that these genes cooperate to organize the neural circuitry for male courtship behavior during development. The male-specific FruM proteins are expressed in ~2000 neurons (Lee et al., 2000; Usui-Aoki et al., 2000), some of which manifest structural sex differences as a result of FruM effects to promote male-typical differentiation (Kimura et al., 2005; Kohl et al., 2013), while others are present only in either sex due to FruM-dependent survival or death (Kimura et al., 2008; Ren et al., 2016). These sex differences at the single neuron level ultimately lead to sexual dimorphisms in neural circuitries and their behavioral outputs (Pavlou and Goodwin, 2013). To unravel the possible roles of EcR for modulating FruM effects to produce sex differences in single neurons, we focused on a particular subset of fru-expressing interneurons called mAL, because they display striking sex differences (Cachero et al., 2010; Kimura et al., 2005; Yu et al., 2010), and because we know some of the molecular mechanisms underlying these sex differences (Ito et al., 2012, 2016). mAL neurons exhibit sexual dimorphism in three respects: the number of cells composing the mAL cluster is five in females versus 29 in males; the ipsilateral neurite is not present in any mAL neurons in females, whereas it is present in some mAL neurons in males; and the tip of the contralateral neurite in the subesophageal ganglion bifurcates in females, whereas there is no branching at the tip in males (Kimura et al., 2005). Functionally, mAL neurons represent second-order interneurons in the processing of contact-chemical sex pheromones, and they control alternate wing motion during courtship song generation in males (Cohn et al., 2015; Koganezawa et al., 2010; Kallman et al., 2015).

Fig. 1. The fru dominant eye phenotype is suppressed by a copy of loss-of-function EcR alleles. (A-F) The compound eyes of a wild-type fly (A), an EcRM554f heterozygote (B), an EcRF559f heterozygote (C) and flies expressing fruB+ via GMR-GAL4 without (D) or with a copy of the EcRM554f (E) or EcRF559f (F) allele, shown at low (left-hand panels) and high (right-hand panels) magnifications. All eyes were from female flies. Scale bars: 100 μm (left) and 15 μm (right).
To selectively visualize and manipulate mAL neurons, we adopted the Mosaic Analysis with a Repressible Cell Marker (MARCM) technique (Lee and Luo, 1999), in which fru-specific GAL4 (expressed via fruNP21-GAL4 in this study) is activated only in cells that stochastically lose a GAL4-repressing transgene GAL80 by chromosomal recombination in response to heat shock-induced Flippase expression (via hs-FLP). The GAL4 protein thus produced in a small subset of fru-positive cells drives expression from UAS-mCD8::GFP for fluorescent marking of the entire structure of these cells as well as expression from UAS-EcR-RNAi for knocking down EcR in these cells. We used UAS-EcR-A-RNAi to knock down the EcR-A isoform and UAS-EcR-B1-RNAi to knock down EcR-B1. Both RNAi constructs significantly reduced the respective mRNA expression (Fig. S1). We were unable to obtain a tool for EcR-B2 knockdown, however. We optimized the timing to apply the heat shock for inducing chromosomal recombination so that mAL neurons were predominantly labeled (and manipulated) and the EcR knockdown effect was maximized. We found that MARCM neuroblast clones that label all constituent cells of a single mAL cluster are not adequate for this analysis, because practically all neurites of the entire set of mAL neurons from both brain hemispheres overlap one another, making it difficult to observe single neuron structures with no ambiguity. We therefore relied on an analysis with single cell MARCM clones (Fig. 2A-D). We quantified the proportion of mAL neurons with the male-specific ipsilateral neurite (mAL with ILN, ILN[+]) in all mAL single cell

![Image](image-url)

**Fig. 2.** EcR-A knockdown increases, whereas EcR-B1 knockdown decreases the proportion of mAL neurons devoid of the male-specific ipsilateral neurite in the male brain. (A-D) Single-cell clones of mAL neurons expressing RNAi against the EcR-A (A) or EcR-B1 (B) isoform and respective control clones (C,D) are shown. Drawings of visualized single mAL clones are illustrated in the right-hand column of each image. Scale bar: 50 µm. All clones shown were obtained in flies heat-shocked at 5-6 days AEL. (E) The proportion of mAL neurons with the ipsilateral neurite (ordinate) is compared between the control genotype (fruNP21/+, left-hand bars) and the cells with knockdown (right-hand bars) of EcR-A (green) or EcR-B1 (red) in flies heat-shocked at four different time points as indicated in the abscissa. The number of clones obtained is shown in parentheses. Statistical differences were evaluated by the Fisher’s exact test (**P<0.01; ***P<0.001; NS, not significant).
clones obtained and used this value as an estimate of the level of masculinization of mAL neurons, based on the knowledge that reductions in functional FruBM result in a small proportion of ILN[+], without producing neurons that have a shorter or longer ILN (Ito et al., 2012). We found that, in control flies, the proportion of ILN[+] varied widely depending on when the heat-shock treatment for clone induction was administered to an animal. The heat-shock treatments at 3-4 days after egg laying (AEL) or 4-5 days AEL invariably yielded a high level of ILN[+] induction, i.e. ~90% (Fig. 2E). Heat shock applied at 5-6 days AEL also resulted in a high ILN[+] rate, ~60% (Fig. 2E). In contrast, when heat shock was given at 6-7 days AEL, the proportion of ILN[+] was only ~30% in control flies (Fig. 2E). This observation is consistent with the notion that the neuroblast produces predominantly ILN[+] during the larval stage, and then generates mainly ILN[−] after pupariation. Notably, when EcR-A was knocked down, the proportion of ILN[+] was always high irrespective of the heat-shock timing; the proportion was ~100% at 5-6 days AEL and ~60% at 6-7 days AEL (Fig. 2E). Remarkably, EcR-B1 knockdown had a contrasting effect; the proportion of ILN[+] declined to ~30% for the fly group heat-shocked at 5-6 days AEL (Fig. 2E). We propose that EcR-A and EcR-B1 function in an inverse manner for the fate switching between ILN[+] and ILN[−], which occurs at pupariation.

**EcR forms a complex with FruBM**

Next, we attempted to clarify the molecular basis for the EcR action to switch the neural fate from ILN[+] to ILN[−] across pupariation. Because both EcR and FruM likely act through chromatin remodeling (Ito et al., 2012; Sedkov et al., 2003), we tested the possibility that they form a complex to regulate transcription. We transfected the *Drosophila* cell line S2 with constructs that each encoded a tagged version of EcR isoforms and FruBM to obtain cell lysates for coimmunoprecipitation assays. We chose FruBM as the isoform of FruM to test here, because this isoform is the most prevalently finding (Ito et al., 2016), the reporter transcription was repressed by EcR-B1 mRNA by quantitative polymerase chain reaction (qPCR) in white pupae with or without ECR-B1 mRNA. Indeed, robo1 mRNA was significantly increased upon EcR-B1 knockdown (Fig. 4D). Of note, EcR-A knockdown had a contrasting effect on robo1 transcription, i.e. it decreased the level of robo1 mRNA (Fig. 4E). In view of the fact that EcR forms a complex with FruBM (Fig. 3), it would be conceivable that the effect of EcR on robo1 transcription is, at least in part, mediated through the EcR-FruBM complex. We thus carried out reporter assays in S2 cells with a robo1 promoter-luciferase fusion construct that contained a 1.7 kb fragment with the FruBM-binding site (Ito et al., 2016) and its flanking regions. In support of our previous finding (Ito et al., 2016), the reporter transcription was repressed by fruBM transfection (Fig. 5A). Additional transfection of S2 cells with EcR-B1 enhanced the repressor activity of FruBM on robo1 reporter transcription (Fig. 5A). Interestingly, when ecdysone was added to the culture medium, FruBM was unable to repress transcription from the robo1 promoter (Fig. 5B). In the presence of ecdysone, transfection of EcR-B1 in addition to fruBM did not
increase or decrease the robo1 reporter activity (Fig. 5B). Based on these observations, we suggest that robo1 transcription is repressed by FruBM, and ecdysone impedes the FruBM repressor action. The fact that EcR-B1 overexpression did not affect the FruBM action in the presence of ecdysone implies that EcR-B1 expressed endogenously in S2 cells is sufficient for mediating the ecdysone action to impede FruBM-induced repression of robo1 transcription.

**DISCUSSION**

The present study unraveled a novel role for EcR. Namely, EcR was found to switch the cell type of a group of fru-expressing neurons depending on whether the cells are produced before pupariation when the systemic ecdysone level is low or produced immediately after pupariation when the ecdysone surge occurs. More specifically, we found that mAL neurons with the male-specific ipsilateral neurite (ILN[+]) are preferentially generated before pupariation, whereas those without the ipsilateral neurite (ILN[−]) are generated after pupariation.

The EcR-B1 isoform has been demonstrated to recruit the CREB-binding protein (CBP) with the activity of a histone acetyltransferase (HAT) in the presence of ecdysone, in order to activate transcription of sox14 via H3K27 acetylation in this locus for facilitating dendrite pruning of sensory neurons during metamorphosis (Kirilly et al., 2011). In contrast, FruBM is known to recruit HDAC1 as mediated by the Transcriptional Intermediary Factor 1 (TIF1) homolog Bonus to its target sites on the genome, presumably resulting in gene silencing for the induction of male-typical development of neurons (Ito et al., 2012). According to a prevalent bimodal switch model, steroid hormone receptors recruit corepressors in the absence of hormone and coactivators in its presence (Johnston et al., 2011; Sedkov et al., 2003). In the present case, ecdysone seems to turn the repressor role of the EcR/FruBM complex off for switching the type of neurons to be produced, i.e. from the ILN[+]-type mAL to ILN[−]-type mAL. A recent study
revealed a novel mode of EcR action in the absence of ecdysone, wherein dMi-2 replaces Usp in the complex to induce chromatin remodeling for gene silencing (Kreher et al., 2016). This mechanism may be excluded from possible modes of action of the EcR-FruBM complex, because FruBM invariably coprecipitates with Usp.

In this study, we classified male mAL neurons into two groups based solely on the presence or absence of the male-specific ipsilateral neurite. We have previously demonstrated that the male-specific ipsilateral neurite forms when the guidance cue receptor gene robo1 is transcriptionally repressed (the male state), whereas this neurite does not form when robo1 is transcriptionally activated (the female state; Ito et al., 2016). FruBM plays a role in switching the robo1 transcription state; it represses robo1 transcription in males, while robo1 is transcribed in females that lack FruBM (Ito et al., 2016). Our result is consistent with the idea that the observed effects of EcR-B1 knockdown on the ipsilateral neurite formation in the male brain are mediated by altered regulation of robo1 transcription by FruBM. It is plausible that the inclusion of EcR-B1 in the FruBM-containing complex enhances transcriptional repression of robo1 in the absence of ecdysone, and binding of ecdysone to EcR-B1 results in the restoration of robo1 transcription (Fig. 6). robo1 repression in the absence of ecdysone promotes the production of ILN[+], whereas robo1 activation in the presence of ecdysone promotes that of ILN[−]. Thus, EcR-B1 may function as a fate-controlling switch between ILN[+] and ILN[−], either of which is chosen depending on the ecdysone titer.

It appears that EcR-A has an ability to confer the male-fate on fru-expressing neurons other than mAL neurons; decreased EcR-A expression results in a decrease in the volume of male-enlarged glomeruli in the antennal lobe, and a concomitant increase in male-to-male courtship in male flies (Dalton et al., 2009). There is a report describing that female flies of ecdysoneless1 (ecd1) mutants with a decreased ecdysone titer generate courtship songs that are similar to those produced by males when courted by a male (Ganter et al., 2012). These mutant females were also reported to show a reduction in ovipositor-extrusion attempts toward a courting male to express their unwillingness to mate (Ganter et al., 2012). These observations might suggest that EcR plays a role in neural feminization. Alternatively, the male-like behavior observed in Ecd1 mutant females might result from impairments of sex-specific splicing of sex-determination factor transcripts, because Ecd is a component of the U5 snRNP pre-mRNA splicing complex (Claudius et al., 2014).

Our study showed that EcR is an important component in the FruBM-containing protein complex that instructs certain fru-expressing neurons to develop a male-specific structure in the male brain. Thus ecdysone signaling acquires a sex-specific function by crosstalk with a sex-determination pathway component, exhibiting an action comparable to steroid sex hormones in vertebrates on neurons to promote or inhibit the formation of a male-specific structure, depending on the developmental context (Fig. 6).

**MATERIALS AND METHODS**

**Fly strains**

Flies were reared on cornmeal-yeast medium at 25°C. Canton-S served as a wild-type control. The EcRM554fs and EcRV559fs alleles (EMS-induced null alleles; Bender et al., 1997) were generous gifts from Dr T. Kitamoto (University of Iowa, Iowa City, USA). The UAS-EcR-A-RNAi (BL9328) and UAS-EcR-B1-RNAi (BL9329) were obtained from the Bloomington Drosophila Stock Center.

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Fig. 6. A model for steroid hormone actions in sex-type specification of a cell. (A) Ecdysone action in *Drosophila*. (B) Testosterone action in vertebrates.
Modifier screens
The female flies with both GMR-GAL4 and UAS-fru-typeB' transgenes were crossed with male flies from mutant stocks reported to have developmental defects in the nervous system (FlyBase: http://flybase.bio.indiana.edu/). In this screen, we overexpressed a FruCOM protein rather than a FruM protein when inducing the rough eye phenotype, as the former yielded more viable offspring. The nomenclature for Fru isoforms is adapted from that used in our previous study (Usui-Aoki et al., 2000) and different from that of other groups (Song et al., 2002). Among the 5 Fru C-terminal variants, TypeB was most effective at rescuing the fru mutant phenotype (Biller et al., 2006; Usui-Aoki et al., 2000; von Philipsborn et al., 2014) and thus was most likely to yield modifiers that were relevant to the in vivo functions of fru. Images of the compound eye surface were obtained with a scanning electron microscope (SU8000; Hitachi High-Technologies, Tokyo, Japan).

Com-imunoprecipitation assays
In the coimmunoprecipitation assays for EcR-A, EcR-B1, Usp and FruBM, constructs encoding each protein were overexpressed in S2 cells. Then, 5 µg of one of the pMT-HA-EcR-A-V5-His, pMT-HA-EcR-B1-V5-His or pMT-MYC-Usp-V5-His plasmid vectors and 5 µg of the pMT-FLAG-fruBM plasmid vector were transfected into S2 cells (5×10^6 cells) using FugeneHD (Roche Diagnostics, Indianapolis, IN, USA), and protein expression was induced by addition of copper sulfate. Lysates were prepared by homogenizing in a cold lysis buffer [50 mM HEPES, pH 7.5, 100 mM NaCl, 50 mM ZnSO₄, 10 mM NaF, 0.2% NP40 and complete Protease Inhibitor (Roche)] for 1 h at 4°C, then incubated with rabbit IgG (10500C, Invitrogen) or rabbit anti-Flag antibody (F7425, Sigma-Aldrich) in the aforementioned lysis buffer for 3 h at 4°C. The immuno-complexes were precipitated with Dynabeads™ Protein G (10040D, Invitrogen) according to the manufacturer’s instructions. Finally, the immuno-complexes were analyzed by western blotting with a primary antibody, anti-V5 (1:5000; 46-0705, Invitrogen), or rabbit anti-Flag antibody (F7425, Sigma-Aldrich), and, as a secondary antibody, with horseradish peroxidase (HRP)-conjugated, anti-rabbit or mouse IgG antibody (1:3000; Sigma-Aldrich).

Reporter assays
Reporter assays were carried out with the robo1 promoter luciferase reporter as described in Ito et al. (2016). The pGL3-promoter vector carrying a 1.7 kb robo1 promoter fragment was used as a reporter construct. The pHLSlv40 Renilla luciferase vector (Promega, Fitchburg, USA) served as an internal control. First, 100 ng of a reporter construct and 10 ng of an internal control were co-transfected into S2 cells (2×10⁶ cells) with either pact-HA-FLAG-fruBM or pact-MCS (Ito et al., 2016) using FugeneHD (Roche Diagnostics). Cells were lysed after 48 h of transfection with a passive lysis buffer (Promega), and luciferase activity was measured using a Dual-Luciferase Assay System (Promega). To standardize the transfection efficiency, the reporter luciferase activity of each sample was normalized to the corresponding control Renilla luciferase activity: the luciferase activity of Renilla was $\frac{\text{Renilla luciferase activity of each sample}}{\text{Renilla luciferase activity of corresponding control}}$.

Clonal analysis of mAL neurons
We used a fru^{np21-GAL4} line to label mAL neurons. The somatic clones were produced using the MARCM method (Lee and Luo, 1999). Flies with the genotype $y hs-flp / Y$ or $w; FRT13 UAS-mCD8::GFP / FRT13 tub-Gal80; fru^{np21}, UAS-Dcr2/+ were used as the control males. The genotype of flies used in clonal EcR knockdown experiments was $y hs-flp / Y$ (for males) or $w; FRT13 UAS-mCD8::GFP / FRT13 tub-Gal80; fru^{np21}, UAS-EcR-RNAi$. For the production of single-cell clones of mAL neurons, larvae (3-4, 4-5 and 5-6 days AEL) or pupae (6-7 days AEL) were heat shocked at 37°C for 20 min (larvae) or for 40 min (pupae). Flies to be tested were reared at 29°C after the heat shock in order to enhance the expression of transgenes.

Dissection, immunohistochemistry and imaging of the central nervous system (CNS)
For dissection of the CNS, 3-5 day-old files were dissected in cold phosphate buffered saline (PBS) with sharp forceps (Dumont #5). After dissection, the CNS was fixed in 4% paraformaldehyde for 1 h followed by two 30-min washings in 0.2% PBS and Tween 20 (PBT). Then the CNS was kept in blocking buffer containing normal goat serum and 0.2% PBT overnight at 4°C. Immunostaining was performed using a rabbit anti-GFP antibody (at a dilution of 1:500) and a mouse anti-nc82 antibody (Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Iowa City, IA: 1:50 dilution). Tissues were incubated with the primary antibody for 2 days, then subjected to 20-min washings in 0.2% PBT twice. Alexa Fluor 488 anti-rabbit IgG antibody and Alexa Fluor 546 anti-mouse IgG antibody (Invitrogen; 1:200) were used as secondary antibodies. The CNS was stained for 1 day with the secondary antibody, washed for 30 min in 0.2% PBT twice, and then washed for 30 min with 50% (v/v) glycerol in PBS. Finally, the CNS was mounted on a slide glass with 80% (v/v) glycerol in PBS. Images were acquired with a LSM 510 META confocal microscope (Zeiss, Oberkochen, Germany) using LSM Image Browser software (Zeiss). All images were acquired with either 20× Plan-Apo 0.8 or 40× Plan-Apo 0.95 lenses at a resolution of 512 µm × 512 µm with 1 µm intervals.

qPCR
qPCR was performed using a LightCycler 1.0 system (Roche). Total RNA was extracted from the CNS of white pupae using an RNeasy Mini Kit (74104, Qiagen). To quantify robo1 expression levels (Fig. 4D,E), equal amounts of cDNA were synthesized from the extracted RNA using a ReverTra Ace qPCR RT kit (FSQ-101, TOYOBO, Osaka, Japan). Each cDNA was mixed with SYBR Premix Ex Taq II (RR820A, TAKARA, Kusatsu, Japan) and 5 pmol of both forward (5'-CCACGCTCAACTGCAAATGGAG-3') and reverse (5'-AAGTGGACGCGTGATTTCTT-3') primers. RpL32 (rp49) was amplified as an internal control using the primer pair 5'-AGATGTCGAAAGCAGCAGA-3' (forward) and 5'-CACCAGAACTTCTTGAAATCGC-3' (reverse). qPCR was conducted at 95°C for 30 s (initial denaturation), followed by 50 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s. Data processing was performed using LightCycler Software Ver. 3.5 (Roche).

Acknowledgements
We thank N. Hamada-Kawaguchi, M. Ote, H. Ito, Y. Miwa and Z. S. Chowdhury for technical guidance; Y. Hara for valuable comments on our manuscript; T. Kitamoto, the Bloomington Drosophila Stock Center, the Drosophila Genome Resource Center at Kyoto, and the Vienna Drosophila RNAi Stock Center for fly stocks; and A. Utsumi for secretarial assistance.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: D.Y.; Methodology: K.S.; Investigation: B.Z.; Writing - original draft: D.Y.; Writing - review & editing: D.Y.; Visualization: K.S.; Supervision: D.Y.; Project administration: D.Y.; Funding acquisition: D.Y.

Funding
This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (16H06371, 17H05935, 17K19371 to D.Y.; 17K07040 to K.S.), and the Tohoku Kaihatsu Memorial Foundation (Uehara Memorial Research Fellowship and Scholarship to B.Z.).

Supplementary information
Supplementary information available online at http://bio.biologists.orglookup doi:10.1242/bio.029744.supplemental
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