Female-biased dimorphism underlies a female-specific role for post-embryonic Ilp7 neurons in Drosophila fertility

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SUMMARY

In Drosophila melanogaster, much of our understanding of sexually dimorphic neuronal development and function comes from the study of male behavior, leaving female behavior less well understood. Here, we identify a post-embryonic population of insulin-like peptide 7 (Ilp7)-expressing neurons in the posterior ventral nerve cord that innervate the reproductive tracts and exhibit a female bias in their function. They form two distinct dorsal and ventral subsets in females, but only a single dorsal subset in males, signifying a rare example of a female-specific neuronal subset. Female post-embryonic Ilp7 neurons are glutamatergic motoneurons innervating the oviduct and are required for female fertility. In males, they are serotonergic/glutamatergic neuromodulatory neurons innervating the seminal vesicle but are not required for male fertility. In both sexes, these neurons express the sex-differentially spliced fruitless-P1 transcript but not doublesex. The male fruitless-P1 isoform (fruM) was necessary and sufficient for serotonin expression in the shared dorsal Ilp7 subset, but although it was necessary for eliminating female-specific Ilp7 neurons in males, it was not sufficient for their elimination in females. By contrast, sex-specific RNA-splicing by female-specific transformer is necessary for female-type Ilp7 neurons in females and is sufficient for their induction in males. Thus, the emergence of female-biased post-embryonic Ilp7 neurons is mediated in a subset-specific manner by a tra- and fru-dependent mechanism in the shared dorsal subset, and a tra-dependent, fru-independent mechanism in the female-specific subset. These studies provide an important counterpoint to studies of the development and function of male-biased neuronal dimorphism in Drosophila.

KEY WORDS: Female behavior, Motoneuron, Neuronal identity, Neuronal lineage

INTRODUCTION

Behavioral differences between males and females often arise from sexually dimorphic neurons and circuits (Cooke et al., 1998; Wade and Arnold, 2004; Villella and Hall, 2008; Paus, 2010). Stereotyped male behaviors in Drosophila melanogaster provide the basis for our current understanding of the genetic mechanisms and neural substrates that generate sexually dimorphic behaviors (Yamamoto, 2007; Villella and Hall, 2008). The sex determination cascade generates dimorphic neuronal populations largely through the sex-specific RNA splicing of the transcription factor genes doublesex (dxsx) and fruitless (fru) (Salz and Erickson, 2010; Dauwalder, 2011). In males, dsx and a fru transcript driven from its P1 promoter (fru-P1) undergo default RNA splicing into coding dsxM and fruM isoforms. Females express the RNA splicing factor transformer (tra), which drives female-specific splicing of dsx and fru-P1 into a coding dsxF isoform and a non-coding fruF isoform. Both Fru and Dsx are expressed in a largely overlapping set of ∼2000 neurons that play crucial roles in sexually dimorphic behaviors (Cachero et al., 2010; Rideout et al., 2010; Robinett et al., 2010; Yu et al., 2010), in which Fru and Dsx direct sexual dimorphic neuronal gene expression and functional properties, as well as differences in branching and connectivity (Yamamoto, 2007; Villella and Hall, 2008; Dauwalder, 2011). Curiously, only males are reported to have numerically expanded neuronal populations or unique populations not found in females (Yamamoto, 2007; Cachero et al., 2010; Rideout et al., 2010; Yu et al., 2010; Kimura, 2011).

Much of our understanding of the genetic and neural substrates of sexually dimorphic behavior comes from analysis of males, with comparatively less work having been performed on female behavior (Ferveur, 2010). Egg laying in females is under tight neuronal control and its regulatory circuitry is one of the best understood female behaviors (Middleton et al., 2006; Rodriguez-Valentín et al., 2006; Yapici et al., 2008; Yang et al., 2009; Rezával et al., 2012). After eggs exit the ovary, they are propelled through the oviduct by somatic-like muscles that ring the oviduct (Hudson et al., 2008). Peristaltic contraction/relaxation activity of these muscles is directed by unidentified excitatory glutamatergic motoneurons and inhibitory octopaminergic neurons (Middleton et al., 2006; Rodriguez-Valentín et al., 2006; Kapelnikov et al., 2008). Insulin-like peptide 7 (Ilp7)-expressing neurons are also reported to innervate the oviduct, and their electrical silencing blocks egg laying (Yang et al., 2008); yet, as Ilp7 mutants have no egg-laying phenotype (Grönke et al., 2010), the function of these neurons is uncertain.

Here, we identify a post-embryonic population of Ilp7-expressing neurons in the posterior adult ventral nerve cord that innervates the female oviduct and the male seminal vesicles. This population exhibits a functionally biased role in females as well as a rare phenomenon in Drosophila: a female-specific subset of CNS neurons. Examination of the role of the sex determination cascade in the dimorphisms displayed by these neurons indicates that a postmitotic tra- and fruM-dependent mechanism accounts for the dimorphisms of the shared population of Ilp7 neurons, but that a postmitotic tra-dependent and fru- and dsx-independent mechanism is responsible for generating the female-specific neuronal subset in females.
MATERIALS AND METHODS

Fly stocks
Flies were maintained on standard cornmeal food at 70% humidity at 18°C, 25°C or 29°C. Strains from Bloomington Drosophila Stock Center were: UAS-nEGFP; UAS-mCD8:GFP::L1; UAS-hid; UAS-reaper; elasGAL4; C155; UAS-Dicer2; tub>Gal80Ts; w1118 (control strain). Strains obtained as gifts were: Nkx6-GAL4 (Brohier et al., 2004), VGlutOK515::GAL4 (Mahr and Aberle, 2006), Tdc2-GAL4 (Cole et al., 2005) and tub>Gal80 >; UAS-CDS::GFP; hs-Flp, MRK-SC (Gordon and Scott, 2009), Act>STOP >; n lacZ; UAS-Flp (Struhl and Basler, 1993), MHC-CDS::GFP::Shaker (Zito et al., 1999), UAS-Tra, UAS-Tra dsRNAi (Chan and Kravitz, 2007), fruitless-P1::GAL4 (Stockinger et al., 2005), fru, fru, fru, fru-40 (fruD) (Demir and Dickson, 2005), dsx-GAL4 (Rideout et al., 2010). UAS-dsRNAi strains were: UAS-Ilp7 RNAi (EPSN, Vienna Drosophila RNAi Center), UAS-VGlut RNAi, UAS-Tra RNAi, UAS-Ilp7 dsRNAi (TRIP). To generate gfp-GAL4 reporter line (Materials and methods) that faithfully reports Ilp7 expression at all ages (detailed in supplementary material Fig. S1). In larvae, the posterior Ilp7 subset comprises eight dMP2 neurons in abdominal segments A6 to A9 that innervate the hindgut (Miguel-Aliaga et al., 2008; Cognigni et al., 2011) (Fig. 1A,C). They are born and differentiate in the embryo, thus we term them ‘embryonic’ Ilp7 neurons. The posterior Ilp7 neuronal population had not been examined in detail. Our analysis showed that it reorganizes into distinct dorsal and ventral Ilp7-expressing neuronal clusters by adulthood (Fig. 1B,D).

Are embryonic Ilp7 neurons retained within the posterior Ilp7 population in adults? To test this, we fate-tracked embryonic Ilp7 neurons into adulthood by permanently marking them in young larvae, and examining marker expression in adults. This was achieved by temporally delimited Flp-in of a permanent lacZ reporter during larval stages L1 and L2, using animals of genotype [Act5C > STOP > n LacZ, UAS-Flp/Ilp7-GAL4; tub>Gal80Ts; UAS-nEGFP]. Ilp7-GAL4 was used to target UAS-Flp recombinase expression to Ilp7 neurons. Delimitation of Flp expression to an L1/L2 window was achieved using temperature-sensitive Gal80Ts, which blocks GAL4 activity at 18°C but permits it at 29°C (McGuire, 2004). Animals were kept at 18°C throughout life, except for during L1/L2, when they were placed at 29°C. The resulting transient Flp expression allowed Flp-in of lacZ to be expressed permanently from a ubiquitous promoter (Struhl and Basler, 1993).

After confirming that lacZ Flp-in robustly and selectively marked all embryonic Ilp7 neurons (supplementary material Fig. S2A), we examined anti-β-galactosidase and anti-Ilp7 overlap in adults. β-Galactosidase (β-Gal) immunoreactivity was absent from dorsal cluster Ilp7 neurons (Fig. 1F). Instead, β-Gal marked subsets of Ilp7 neurons within the ventral cluster: in two large cells (~13 μm in diameter) with intense Ilp7 immunoreactivity and also in four to six small cells (~9 μm in diameter) that had extremely low Ilp7 immunoreactivity, which was often undetectable (Fig. 1F,F'). In males, these neurons accounted for the entire ventral subset (Fig. 1F'). Unexpectedly, females always had an additional three or four Ilp7 neurons in the ventral cluster that were not marked by β-Gal. (Fig. 1F'). These female-specific Ilp7 neurons were non-embryonic and were ~9 μm in diameter with consistently moderate to high Ilp7 levels. These data are quantified in Fig. 1G,H and summarized in 1J,1J.

We wished to identify useful discriminatory markers between embryonic and non-embryonic subsets. Thus, in adult Ilp7 neurons, we tested the expression of transcription factors reported to be expressed by Ilp7 neurons in the embryo (supplementary material Fig. S2) (Miguel-Aliaga and Thor, 2004; Miguel-Aliaga et al., 2008). Notably, we found that Fork head (Fkh) and Odd-skipped (Odd) were expressed in all β-Gal-positive (embryonic) Ilp7 neurons but were absent in all non-embryonic β-Gal-negative Ilp7 neurons (Fkh shown in Fig. 2A). We use Fkh immunoreactivity hereafter as a marker to discriminate between embryonic and non-embryonic Ilp7 neurons.

RESULTS

Adult females have a unique subset of posterior Ilp7 neurons not present in males

We generated a transgenic Ilp7-GAL4 reporter line (Materials and methods) that faithfully reports Ilp7 expression at all ages (detailed in supplementary material Fig. S1). In larvae, the posterior Ilp7 subset comprises eight dMP2 neurons in abdominal segments A6 to A9 that innervate the hindgut (Miguel-Aliaga et al., 2008; Cognigni et al., 2011) (Fig. 1A,C). They are born and differentiate in the embryo, thus we term them ‘embryonic’ Ilp7 neurons. The posterior Ilp7 neuronal population had not been examined in detail. Our analysis showed that it reorganizes into distinct dorsal and ventral Ilp7-expressing neuronal clusters by adulthood (Fig. 1B,D).

Immunohistochemistry

Primary antibodies used were: rabbit anti-Ilp7 (1:1000; E. Hafen, ETH, Zurich, Switzerland); guinea pig anti-5-HT head (1:1000; H. Jäckle, Max Planck Institute, Göttingen, Germany); guinea pig anti-odd skipped (1:200; J. Reinitz, University of Chicago, IL, USA); rat anti-TJH (1:50; M. Monastirioti, FORTH, Greece); rat anti-Doublesex (1:100; M. Arteltman, USC, CA, USA); mouse anti-Abd-A (1:400; clone D.1, Duncan, Washington University, St Louis, MO, USA); chicken anti-β-Gal (1:1000; ab9361; Abcam); rabbit anti-VGlut, anti-GlurR and anti-GlurRIB (1:1000; A. DiAntonio, Washington University, St Louis, MO, USA). rabbit anti-5-HT (1:1000; S5545; Sigma); goat anti-5-HT (1:100; Jackson ImmunoResearch); rabbit anti-pMad (1:100; 41D10; Cell Signaling Technology). The Developmental Studies Hybridoma Bank provided anti-Dachshund (1:10; clone dach-2); anti-BrdU (1:10; clone G34G), anti-Bruichplot (1:50; clone ne82); anti-DisC large (1:50; clone 4F3), anti-Abd-B (1:20; clone 1A2E9) and anti-DGlurR-IA (1:1000; clone SB4D2). Standard protocols were used (Eade and Allan, 2009), except as follows. For serotonin, samples were fixed in 4% paraformaldehyde with 7.5% picric acid for 1 hour. For VGlut1 and GlurRs, we fixed for 5 minutes in Bouin’s fixative. For bromodeoxyuridine (BrdU) detection, mid L3 to late L3 larvae were fed 1 mg/ml BrdU (B5002-1G, Sigma) in yeast paste. After standard fixation, adult ventral nerve cords (VNCs) were treated with 2 N HCl (20 minutes) prior to standard immunohistochemistry for anti-BrdU. Secondary antibodies used were: donkey anti-mouse, anti-chicken, anti-rabbit, anti-guinea pig and anti-rat IgG (H+L) conjugated to DyLight 488, Cy3 or Cy5 (1:100, Jackson ImmunoResearch). All images acquired on an Olympus FX1000 confocal microscope. Images were processed using Fluoview FV1000 and Adobe Photoshop CS5.

Egg-lay assays

Egg-lay assays were performed on yeast paste-supplemented grape juice/agar plates at 25°C and 70% humidity. Flies were not exposed to CO2 when plates were switched. For the 6-hour egg-lay assay, groups of males and females were mated at a ratio of one female to three males. The egg-lay assays were performed with only three females per plate. Eggs were counted and divided by three to give the number of eggs laid per female. For female fertility assays, groups of males and females were mated at a ratio of one female to three males. For male fertility assays, groups of males and females were mated at a ratio of one female to one male. Egg-lay assays and analysis was performed as for the 6-hour egg-lay assay. The total number of viable progeny per 6-hour plate was counted (i.e. not divided by three).

Statistics

GraphPad Prism 5 was used for all analysis and data presentation. Data are presented as means±s.d. unless otherwise noted. All data underwent D’Agostino and Pearson, Shapiro-Wilk Normality tests. Normally distributed data were compared using a parametric unpaired t-test. Non-normally distributed data were compared using a non-parametric Mann–Whitney test. Statistical results are shown to the exact P-value down to P<0.0001.
Post-embryonic Ilp7 neurons innervate the reproductive tracts but are only necessary for female fertility

The additional posterior Ilp7 neurons that appear after larval development might be born through post-embryonic neurogenesis in larvae (Truman, 1990), or are perhaps developmentally frozen to express Ilp7 only after metamorphosis (Veverytsa and Allan, 2012). To discriminate between these possibilities, larvae were fed BrdU between mid L3 and pupariation; BrdU incorporation into Ilp7 neurons was examined at adult day A1 (Fig. 2B,B'). In females, we detected BrdU in Ilp7 neurons that did not express Fkh, including the dorsal Ilp7 neurons shared by both sexes and all female-specific ventral Ilp7 neurons. Thus, the non-embryonic Ilp7 neurons are generated by post-embryonic neurogenesis in late L3 larvae (hereafter termed post-embryonic Ilp7 neurons). The position and transcription factor profile of each Ilp7 neuronal subset is summarized in Fig. 2C-E.

Electrical silencing of all Ilp7 neurons in adults was shown to block egg laying in females (Yang et al., 2008). However, our identification of an unanticipated post-embryonic Ilp7-expressing neuronal population raised the question of which Ilp7 neuronal subset is required for egg laying. To test this, we adapted the temporally delimited Flp protocol (Fig. 1E) to temporally delimit the cell death of embryonic Ilp7 neurons. In animals of genotype \[ UAS-hid, UAS-reaper/+; Ilp7-GAL4; tubP-GAL80TS, UAS-nEGFP \], we used GAL80TS to delimit expression of the cell death genes \( \text{hid} \) (Wrinkled – FlyBase) and \( \text{reaper} \) into Ilp7 neurons only in L1 and L2 larvae (Fig. 2F). This killed all embryonic Ilp7 neurons but left post-embryonic Ilp7 neurons intact (supplementary material Fig. S3). In spite of this, female egg laying was not affected (Fig. 2G); thus, post-embryonic Ilp7 neurons are sufficient for egg laying.

Post-embryonic Ilp7 neurons selectively innervate the reproductive tracts

Ilp7 immunoreactivity has been reported at the oviduct, as has the requirement of Ilp7 neuronal activity for female fertility (Yang et al., 2008), but it was not clear which Ilp7 neurons innervate the oviduct nor whether Ilp7 neurons innervate and regulate male reproductive tract function. In Ilp7-GAL4, UAS-mCD8::GFP adults, we found that Ilp7 innervation of the reproductive tracts was restricted to developmentally analogous tissues: the oviduct in females (Fig. 3A,B) and the seminal vesicles in males (Fig. 3C,D) (Bryant,
female) and larval viability (per group of three females) was tracked then removed from the females and their egg production (per control females to control males for a 24-hour period. Males were 2012). To test male fertility, we mated 1-day-old (A1) 2 days after eclosion. Itl7-KO males have reduced fertility. This suggests that newly eclosed, 1-day-old males have reduced fertility. This suggests that newly eclosed, 1-day-old Ilp7-KO females to the same extent, as egg production and larval viability were not different on most days (Fig. 3F,H). Only on the first day of mating did Ilp7-KO males have reduced fertility. This suggests that newly eclosed, 1-day-old Ilp7-KO males exhibit a slight delay in achieving full reproductive capacity, but this is quickly resolved to full fertility by 2 days after eclosion.

To test Ilp7-KO female fertility, we mated adult day A4 Ilp7-KO or control males to control females for a 24-hour period. Males were then removed from the females and their egg production (per female) and larval viability (per group of three females) was tracked over the next 5 days. Ilp7-KO females exhibited severely reduced egg laying throughout the 5-day test period (Fig. 3E). These females also had distended abdomens and eggs were always found jammed over the next 5 days. Ilp7-KO females exhibited severely reduced egg laying throughout the 5-day test period (Fig. 3E). These females also had distended abdomens and eggs were always found jammed on the lateral oviduct (supplementary material Fig. S4A-A'). Of the small number of eggs laid by Ilp7-KO females, only 40% produced viable larvae, compared with 90% of control females (Fig. 3G).

The sufficiency of post-embryonic Ilp7 neurons for egg laying (see Fig. 1G) and the hindgut innervation of embryonic Ilp7 neurons in adults (Coginigi et al., 2011) led us to test whether the post-embryonic Ilp7 neurons selectively innervate the oviduct. We took a genetic mosaic strategy (Gordon, 2009) to visualize individual Ilp7 neurons in flies of genotype \[ \text{UAS-mCD8::GFP/>FRT(-/-)-flanked} \] whereas all labeled embryonic Ilp7 neurons did not disrupt female fertility. (F) Schematic of transient cell death gene expression in embryonic Ilp7 neurons (hid, reaper pulse). (G) Quantification of the number of eggs laid per female during a 6-hour assay following a 24-hour mating (mean±s.e.m.). Female fertility was not significantly different after killing embryonic Ilp7 neurons (black column), compared with control (white column) (control, 9.4±3.3; experimental, 11.8±5.7). \( n \) number of egg-lay assays.
neurons produce a profound female fertility defect? We confirmed that Ilp7 peptide plays no essential role in egg laying, by expressing UAS-ILP7dsRNAi from Ilp7-GAL4 and showed that females laid similar numbers of eggs to controls (supplementary material Fig. S5A,B). We reasoned then that Ilp7 neurons use another essential mode of neurotransmission. Octopaminergic innervation of the oviduct is essential for egg laying (Monastirioti, 2003; Rodríguez-Valentín et al., 2006). We tested whether Ilp7 neurons are octopaminergic. Ilp7 neurons did not express reporters for the octopamine enzymes tyrosine decarboxylase 2 (TDC2-GAL4) or tyrosine β-hydroxylase (anti-TβH) (supplementary material Fig. S5C-D). Moreover, TβH knockdown (UAS-TbhRNAi) blocked egg laying when expressed in octopaminergic neurons (using TDC2-GAL4) but not when expressed in Ilp7 neurons (Ilp7-GAL4) (supplementary material Fig. S5F,G). To identify alternative modes of neurotransmission, we screened through neurotransmitter markers and found that all post-embryonic Ilp7 neurons expressed OK371-GAL4 (Fig. 5A, A’), an enhancer-trap that reports Vesicular glutamate transporter (VGlut) gene expression (Mahr and Aberle, 2006). This was intriguing in light of reports of glutamatergic motoneuron innervation of the oviduct; type I-like neuromuscular junctions are present on the oviduct, which contracts vigorously in response to exogenous glutamate (Middleton et al., 2006; Rodríguez-Valentín et al., 2006; Kapelnikov et al., 2008).

In Ilp7-GAL4, UAS-mCD8::GFP females, Ilp7 neurons terminated on the radial muscles of the oviduct with boutons immunoreactive for VGlut and Bruchpilot, a marker for presynaptic active zones (Wagh et al., 2006) (Fig. 5E,F). Moreover, these boutons were apposed to synaptic accumulations of CD8-GFP-Shaker and Discs large (Dlg) (Fig. 5G,H), which together are unique markers for type I neuromuscular junctions (NMJs) of somatic muscle (Guan et al., 1996; Parnas et al., 2001). Indeed, all CD8-GFP-Shaker and Dlg synaptic accumulations were innervated by Ilp7 neurons (supplementary material Fig.

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**Fig. 3. Post-embryonic Ilp7 neurons are required only for female fertility.** (A-D) Ilp7-GAL4, UAS-mCD8::GFP-expressing neurons project to the female lateral (LO) and common (CO) oviducts and the male seminal vesicle (SV). AG, accessory gland. (E,F) Control and Ilp7-KO females were mated to control males for 24 hours, then males were removed. Thereafter, we counted egg numbers laid per female per 24-hour period, for 5 days (supplementary material Table S1). (E) Ilp7-KO females (red) exhibited severely reduced egg laying compared with controls (black). (F) Control (black) or Ilp7-KO (red) males were mated to control virgin females for 24 hours. Then, mated females were removed and males were provided new virgin females for another 24 hours. This was repeated for 5 days (A2-A5). After females were removed, we counted egg numbers per female over 24 hours. Females mated to Ilp7-KO and control males laid similar egg numbers; only females on assay day 1 had reduced egg numbers. (G) Using the mating protocols described for E,F, we counted the total number of viable larvae produced per plate (not per female) within 6-hour assay periods, over 5 days (supplementary material Table S2). (G) Control females produced a high percentage of larvae. The decline in larvae by A9 reflects a lack of mating for 5 days. Ilp7-KO females produced low egg numbers and only ~40% of eggs produced larvae at each time point (H) Ilp7-KO and control males produced similar percentages of viable larvae at most ages, except for during the first day of the assay. Graphs show mean±s.e.m. for egg number per female. n, number of egg-lay assays.
S6A). We examined the localization of glutamate receptor (GluR) subunits to these oviduct NMJs and further confirmed that Ilp7 neurons terminate at type I NMJs. GluRs form heterotetrameric complexes and the subunits GluRIIC (also GluRIII), GluRIIA and GluRIIB are all localized to type I NMJs (DiAntonio, 2006). At the oviduct, GluRIIC, GluRIIA and GluRIIB all cluster exclusively at Ilp7 boutons within Dlg and CD8-GFP-Shaker synaptic accumulations (Fig. 5H-K). Is glutamatergic transmission of Ilp7 neurons essential for oviduct function? We expressed UAS-VGlut1:dsRNAi in Ilp7 neurons and confirmed that VGlut was efficiently knocked down (supplementary material Fig. S6B,B’). These females had a severe reduction in egg laying (Fig. 5B), and an ‘egg-jam’ phenotype in the lateral oviduct (supplementary material Fig. S4A*). As a control, we expressed UAS-VGlut1:dsRNAi in octopaminergic neurons (TDC2-GAL4) and found that this did not disrupt egg laying (Fig. 5C).

A layer of muscle surrounds the male seminal vesicle (Bairati, 1967; Kozopas et al., 1998) that is innervated by serotonergic input from posterior abdominal VNC neurons (Lee et al., 2001; Billette and Goodwin, 2004). We tested the neurotransmitter identity of male postmitotic Ilp7 neurons, and found that they expressed both the vesicular glutamate transporter and serotonin (Fig. 6A-B’). Serotonin is expressed in a posterior neuronal cluster in males, the SAbg, which innervates numerous male reproductive tract structures (Lee et al., 2001; Billette and Goodwin, 2004). We found that Ilp7- and serotonin-expressing neurons were a small subset of this serotonergic cluster (Fig. 6B’), and our data would suggest that this subset exclusively innervates the seminal vesicle. We examined Ilp7 neuron innervation of the seminal vesicle. In Ilp7-GAL4, UAS-mCD8::GFP males, we found that the seminal vesicle was only innervated by Ilp7 neurons, as determined by counterstaining with the pan-neuronal membrane marker anti-HRP. We then confirmed that all Ilp7 projections were strongly immunoreactive for serotonin (Fig. 6D) and for VGlut (Fig. 6E), and also found that boutons were immunoreactive for Bp (Fig. 6F). We note here that female Ilp7 neurons did not express serotonin (supplementary material Fig. S6C,C’).

Although male post-embryonic Ilp7 neurons express glutamatergic markers, we did not expect them to function as motoneurons, owing to their limited functional role in fertility and their co-expression of serotonin. In confirmation, synaptic accumulation of Dlg and CD8-GFP-Shaker was absent from the seminal vesicle and Ilp7 boutons (Fig. 6G). Moreover, we found only weak and infrequent postsynaptic GluRIIC and GluRIIA immunoreactivity apposed to Ilp7 boutons, and no GluRIIB immunoreactivity was observed (Fig. 6H-J). These findings match the marker expression profile of neuromodulatory type-II-like NMJs at somatic muscle, which utilize glutamate and octopamine as co-transmitters, but have no accumulation of Dlg, CD8-GFP-Shaker or GluRIIB, and have faint and infrequent GluR receptor clustering of GluRIIA and GluRIIC (Zito et al., 1997; Monastirioti, 2003; Marrus et al., 2004; Prokop, 2006). This analysis demonstrates sexual dimorphism in the transmitter phenotype and in NMJs of Ilp7 neurons innervating the reproductive tracts of males and females, with a functional bias to females.

**Genetic regulation of Ilp7 neuron dimorphism**

We tested the role of the sex determination cascade in generating the observed Ilp7 neuronal dimorphisms. The output of this cascade is principally mediated through sex-specific splicing of frau-P1 and dsx. In males, they are ‘default’ spliced into coding dsxM and frauM transcripts. In females, the presence of tra splices frau-P1 into a non-coding frauF isoform and dsx into a coding dsxF isoform. This cascade generates male-specific neurons, but female-specific neuronal subsets have not been investigated (Ferveur, 2010; Kimura, 2011). We first examined frau-P1 and dsx expression in Ilp7 neurons, using a GAL4 reporter for the frau-P1 promoter (which drives in both sexes) and Doublesex immunoreactivity (recognizing a common domain in DsxM and DsxF). We found that all post-
embryonic Ilp7 neurons were fru-P1 positive but Dsx negative in adults. Embryonic Ilp7 neurons did not express fru-P1 or Dsx (Fig. 7A–B’; supplementary material Fig. S7A-B’). Dsx may be transiently expressed at an earlier stage but, as shown in supplementary material Fig. S7C-D’, we could not detect Dsx expression at any time in the lineage of post-embryonic Ilp7 neurons, even by lineage tracing using flp-in permanent lacZ expression. Thus, dsx is probably not expressed in the post-embryonic Ilp7 neuron lineage.

We examined fru function in the generation of female-type and male-type Ilp7 neurons. Using constitutive fruM or fruF-expressing alleles (Demir and Dickson, 2005), we examined the fate of post-embryonic Ilp7 neurons in hemizygous fru-F males (fruF/fru-Df, which do not express FruM) and also in hemizygous fruM females (fruM/fru-Df, which express FruM protein). In adult fru-F-males, post-embryonic Ilp7 neurons were feminized; we observed the generation of the ventral subset of female-specific Ilp7 neurons (three or four Ilp7-positive/Fkh-negative neurons) adjacent to the embryonic Ilp7 neurons (Fig. 7C,C’). The shared dorsal cluster of Ilp7 neurons is retained (as in females) but serotonin expression is lost from this region (Fig. 7D,D’), as was observed in fru-null males (Lee and Hall, 2001; Lee et al., 2001; Billeter et al., 2006). Thus, fruM is necessary for males for serotonin expression in dorsal Ilp7 neurons and for the loss of the female-specific Ilp7 neurons.

By contrast, post-embryonic Ilp7 neurons were not entirely masculinized in fruM females. Notably, the ventral cluster of female-specific Ilp7 neurons was not affected by fruM expression (Fig. 7E,E’). Do these female-specific Ilp7 neurons now express serotonin? It was not possible to co-immunostain for Ilp7 and serotonin; however, serotonin is normally expressed in very few neurons in the vicinity of female-specific Ilp7 neurons, and we found in fruM females that there was no apparent increase in the number of serotonin-expressing neurons in the region (supplementary material Fig. S8A,B). Examination of the shared dorsal cluster, however, showed that these post-embryonic Ilp7 neurons were masculinized in fruM females. These fruM females had gained a population of posterior serotonin-expressing neurons similar to that previously reported in females expressing UAS-fruM isofoms (supplementary material Fig. S8A,B) (Billeter et al., 2006). We examined the oviducts of fruM females to confirm that dorsal Ilp7 neurons were indeed masculinized to express serotonin. As expected, a subset of Dlg-stained NMJs was indeed apposed to serotonin-expressing axons (Fig. 7F,F’). In these oviducts, we find serotonin-negative ventral Ilp7 neurons. Thus, although fruM is necessary and sufficient for serotonin expression in dorsal cluster Ilp7 neurons and necessary for loss of female-specific neurons in males, it is not sufficient for the loss of ventral female-specific Ilp7 neurons in females.

The apparent lack of effect of fruM, and presumably of dsx, in the generation of female-specific neurons in females led us to test whether transformer plays a role, which could affect sex-specific gene expression/function beyond that accounted for by dsx or fru (Finley et al., 1997; Goldman and Arbeitman, 2007). We
manipulated \textit{transformer} expression pan-neuronally in postmitotic neurons, using \textit{elav-GAL4} to express \textit{dsRNAi} to \textit{tra} (UAS-\textit{tra}^{\textit{dsRNAi}}) in females, or to express \textit{tra} (UAS-\textit{tra}) in males. In \textit{tra}^{\textit{dsRNAi}} females (in which \textit{fruM} would be expressed post-mitotically in female Ilp7 neurons), we observed a total loss of the female-specific, post-embryonic Ilp7 neurons in the ventral cluster (Fig. 8A,A'\text{H11032}) whereas embryonic Ilp7 neurons were unaffected. We also observed ectopic serotonin in a posterior cluster of dorsal neurons (supplementary material Fig. S8C,C'\text{H11032}). To confirm that dorsal Ilp7 neurons now expressed serotonin, we examined serotonin immunoreactivity at the oviduct. Indeed, serotonin was expressed by axons terminating at Dlg-stained neuromuscular junctions (Fig. 8B,B'\text{H11032}). Notably, there was a large reduction in the number of Dlg-marked NMJs at the oviduct and all innervation of Dlg-positive NMJs was serotonergic. We suggest that this phenotype results from a loss of innervation by the ventral post-embryonic Ilp7 neurons, although the mechanism is unknown; possible mechanisms include mis-targeting, programmed cell death or an inability to induce postsynaptic Dlg accumulation.

Feminization of male neurons by postmitotic, pan-neuronal expression of \textit{UAS-tra} led to the opposite phenotype. Female-specific Ilp7 neurons were observed in the ventral subset, adjacent to the embryonic Ilp7 neurons (Fig. 8C,C'). Moreover, serotonin immunoreactivity was lost in the posterior VNC and at the seminal vesicle, even though the seminal vesicle retains its innervation (Fig. 8D,D'; supplementary material Fig. S8D,D'). We used \textit{Ilp7-GAL4} to drive UAS-\textit{tra}^{\textit{dsRNAi}} in females and \textit{UAS-tra} in males, but found no change in the Ilp7 neuronal population in these animals (data not shown). That total re-sexualizing of Ilp7 neurons is possible post-mitotically but not after Ilp7 expression commences indicates that the ‘decision’ to become female-type or male-type Ilp7 neurons is irreversibly made in young postmitotic Ilp7 neurons prior to expression of Ilp7 itself.

**DISCUSSION**

**Functional bias of female post-embryonic Ilp7 neurons**

Using a standard set of genetic and immunological tools, we demonstrate that female post-embryonic Ilp7 neurons are the sole glutamatergic motoneuron input that terminates at fast excitatory type I-like NMJs on the oviduct, whereas their male counterparts terminate at neuromodulatory type II-like NMJs on the seminal vesicle (Jia et al., 1993; Prokop, 2006). Glutamatergic neurotransmission is required for contraction of oviduct muscle, which comprises super-contractile radial muscle fibers (Middleton et al., 2006; Rodríguez-Valentín et al., 2006). By contrast, the male seminal vesicle is lined by thin striated muscle that receives only Ilp7/serotonergic innervation and has no NMJs characteristic of fast excitatory transmission. Male seminal vesicle contractility has not been examined, but peristaltic activity of the adjacent ejaculatory duct is under serotonergic modulation; however, innervation is not essential for this (Susic-Jung et al., 2012). Together with our data here, it appears that innervation of the seminal vesicle is not a requirement for the passage of sperm.

Our study leaves unresolved the role of Ilp7 at the oviduct. To our knowledge, insulin-like peptide (ILP) expression in
motoneurons has only been described in *Caenorhabditis elegans* (Pierce et al., 2001; Sieburth et al., 2005). The nervous system is a primary locus for ILP expression in *C. elegans* (Pierce et al., 2001), but any specific motoneuron role for ILPs is unknown. In *Drosophila*, *Ilp7* mutants have no overt phenotype in viability, development, lifespan, fecundity or response to starvation (Grönke et al., 2010), and we detected no egg-laying phenotype after knockdown of *Ilp7*. *Ilp7* functions in the selection of appropriate substrates for egg laying (Yang et al., 2008); however, the circuitry and function of *Ilp7* underlying this behavior are unknown.

**Sexual dimorphism of post-embryonic *Ilp7* neurons**

There are approximately ten male-specific serotonergic neurons in the posterior dorsal VNC, termed SAbg, that innervate the seminal vesicle, accessory glands and ejaculatory duct of the male reproductive tract (Lee and Hall, 2001; Lee et al., 2001; Billeter et al., 2006). Our results now show that a subset of these neurons (approximately four) co-express *Ilp7* and serotonin, and selectively innervate the seminal vesicle. Comparing our data with that of previous reports, we can now propose that the generation of dimorphic SAbg neurons is different for the *Ilp7/Lp7* subset and the other SAbg neurons. In males, the expression of serotonin in all SAbg neurons requires *fruM* (Lee et al., 2001), but *UAS-fruM* only generates a reduced subset of approximately four SAbg neurons in females (Billeter et al., 2006). We show here that these are *Ilp7* neurons, as they innervate oviduct NMJs in *fruM* females. Why does *fruM* expression in females only generate serotonin in the *Ilp7* subset of SAbg neurons? The answer lies in the control of neuroblast lineage progression by *dsx*. Many SAbg neurons are lost in *dsx*-null males, and a subset is gained in *dsx*-null females (Billeter et al., 2006). Underlying this is the induction of female-specific programmed cell death of posterior neuroblast lineages in females by *DsxF*, and their survival and lineage progression in males due to *DsxM* (Taylor and Truman, 1992; Billeter et al., 2006; Birkholz et al., 2013). The function of *fruM* is thereafter limited to activating serotonin expression in the remaining neurons (Billeter et al., 2006). We propose that the absence of *dxx* expression in the lineages of post-embryonic *Ilp7* neurons might spare them from *DsxF*-induced programmed cell death in larvae, so that they survive to become oviduct motoneurons in females.

The postmitotic activity of *tra* fully accounts for all dimorphisms observed in post-embryonic *Ilp7* neurons. Aside from regulation of neuroblast progression, postmitotic mechanisms also contribute to the generation of male-specific neurons, including the P1 and TN1 clusters that function in male courtship behavior, and also the motoneurons that innervate the male-specific muscle of Lawrence (moL) (Rideout et al., 2010; Kimura, 2011). Female-specific loss of P1 neurons is solely mediated by *dxx* acting in a pro-apoptotic manner (Kimura et al., 2009).

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**Fig. 7. FruM is necessary and sufficient for dorsal *Ilp7* serotonergic fate, and is necessary, but not sufficient, for loss of female-specific *Ilp7* neurons.** (A-B') In both sexes, *fru-P1-GAL4, UAS-nEGFP* is expressed in all post-embryonic *Ilp7* neurons (arrowheads; Fkh negative) but not in embryonic *Ilp7* neurons (thick and thin arrows; Fkh positive). (C-D') In *fru/Fru-DF* males, in which *fruM* is absent (D F), post-embryonic female-specific *Ilp7* neurons are generated in the ventral cluster (C', arrowheads, Fkh negative, *Ilp7* positive) adjacent to embryonic *Ilp7* neurons (arrows, Fkh positive). Also, posterior serotonin expression is lost (D'). (E-F') In *fruM/Fru-DF* females (F M), post-embryonic female-specific *Ilp7* neurons are not lost (E', arrowheads) but serotonin expression is observed in dorsal (but not ventral) post-embryonic *Ilp7* neurons (supplementary material Fig. S8B). Here, we show ectopic serotonin expression at *Ilp7* projections on the oviduct. (F') In controls, female *Ilp7* neurons lack serotonin expression at the oviduct (F), but serotonin is expressed by about half of the *Ilp7* neurons apposing *Dlg*-marked NMJs in *fruM/FruDF* females (probably dorsal subset *Ilp7* neurons) (F'). Arrows/arrowheads indicate representative neurons of each *Ilp7*-subset.
Female-specific loss of TN1 neurons requires a pro-apoptotic role for 
\(\text{dsxF} \), but this can be partially counteracted by co-expression of pro-survival \(\text{dsxM} \) (Sanders and Arbeitman, 2008). Female loss of the moL motoneuron is due to a necessary and sufficient role for \(\text{fruM} \) in promoting motoneuron survival (Usui-Aoki et al., 2000; Billeter et al., 2006). In the context of these studies, we were interested to uncover how a female-specific set of neurons emerges. Our initial hypothesis, based on the role of \(\text{tra} \), the expression of \(\text{fru-P1} \) and the absence of \(\text{dsx} \), held that the presence of ventral female-specific Ilp7 neurons is a default state that was masculinized by \(\text{fruM} \). However, although \(\text{fruM} \) in males is necessary for the loss of female-specific Ilp7 neurons, it is not sufficient to eliminate them in females.

These data suggest the existence of a \(\text{tra} \)-dependent factor(s) that functions selectively in females and is sufficient for the generation of female-specific Ilp7 neurons, independently of \(\text{fruM} \) and \(\text{dsx} \). Three additional genes that act in the sex determination cascade function in female sexual differentiation: \(\text{intersex} \) (Garrett-Engele et al., 2002; Siegal and Baker, 2005), \(\text{hermaphrodite} \) (Li and Baker, 1998) and \(\text{dissatisfaction} \) (Finley et al., 1998), the latter of which has been demonstrated to function in a \(\text{tra} \)-dependent, \(\text{dsx} \)-independent manner in at least one neuronal population (Finley et al., 1997). Moreover, genomic approaches to identifying sex-differentially expressed genes have identified numerous genes for which sex-specific expression is \(\text{tra} \)-dependent but neither \(\text{dsx} \)- nor \(\text{fru} \)-dependent (Goldman and Arbeitman, 2007). Ongoing work will address any potential role for these genes in the generation of female-specific Ilp7 neurons. Also, it will be interesting to determine the ‘fate’ of female-specific Ilp7 neurons in males. In this light, it is interesting that \(\text{tra} \) manipulation fully re-assigned the sexual identity of Ilp7 neurons when manipulated from \(\text{elav-GAL4} \) but not from \(\text{Ilp7-GAL4} \). This finding suggests that the underlying pathway makes a permanent ‘decision’ soon after the neuron exits the cell cycle but before Ilp7 is expressed. Such studies would provide an intriguing counterpoint to mechanisms that generate male-biased neuronal populations in males.

**Female-specific circuits and female-specific neuronal populations as models for neuronal sexual dimorphism**

Egg laying is a well-characterized, stereotypical, sex-specific behavior. Effector populations regulating this behavior include octopamine- (Monastirioti, 2003), Ilp7- (Yang et al., 2008) and \(\text{dsx} \)-expressing neurons (Rideout et al., 2010). Regulatory circuits into these reproductive tract efferents are likely to be complex. One candidate population is the pickpocket- and doublesex-expressing reproductive tract sensory neurons. Upon mating, these neurons relay a signal to the suboesophageal ganglion and the posterior abdominal VNC, to decrease receptiveness to male courtship and increase egg laying (Häsemeyer et al., 2009; Yang et al., 2009; Rezával et al., 2012). The projection of these sensory neurons into the posterior VNC is particularly intriguing, because octopaminergic, Ilp7-, and other \(\text{fru} \)-and/or \(\text{dsx} \)-positive neurons ramify their dendritic fields in this region. Work in this field will no doubt provide more details of the circuitry between such neurons, to which our identification of the oviduct motoneurons contributes significantly. Ongoing studies of the development and function of neuronal circuits regulating female-specific behavior will provide an important counterpoint to such studies in males and will lead to a more full understanding of how sex-specific circuits are built and function.

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Fig. S1. Specificity of Ilp7-GAL4 transgene expression to Ilp7-expressing neurons in the larval and adult VNC. (A,E) Cartoon schematic of Ilp7 neuronal subsets: dorsal pair (red), laterals (gray) and posterior Ilp7 neurons (green). All neuronal subsets labeled match those described previously (Miguel-Aliaga et al., 2008; Cognigni et al., 2011). (B,C,F,G) Confocal images showing specific Ilp7-GAL4,UAS-mCD8::GFP expression in the CNS and VNC of larva (B,C) and VNC (F,G) of adults. (D,D’,H,H’) Confocal images of overlap of Ilp7-GAL4,UAS-mCD8::GFP expression with that of anti-Ilp7 immunoreactivity in the posterior Ilp7 neurons of larval and adult VNCs.
Fig. S2. Transcription factor expression in embryonic and post-embryonic Ilp7-neurons. (A-D’) Expression of numerous transcription factors in Ilp7 neurons in the adult, using the temporally delimited Flp-in of lacZ to selectively mark embryonic Ilp7 neurons. The transcription factors tested are all expressed in embryonic Ilp7 neurons in the embryo. (A) Verification that lacZ Flp-in robustly and selectively activates β-Gal expression in embryonic Ilp7 neurons. Representative image of A6-A9 abdominal VNC showing Ilp7-GAL4/act<stop>lacZ,tubPgal80Ts, UAS-nGFP/UAS-Flp co-localized with anti-β-Gal immunoreactivity at late L3 larval stages. All embryonic Ilp7 neurons are labeled robustly. (B-D’) In adults, we used anti-Ilp7 to identify posterior Ilp7 neurons and β-Gal immunoreactivity to identify the embryonic Ilp7 neuronal subset. Post-embryonic Ilp7-neurons denoted by arrowheads; large embryonic Ilp7-neurons by thick arrows, and the small embryonic Ilp7-neurons by thin arrows. (B) Odd-skipped (Odd) was only expressed in embryonic Ilp7 neurons. (C,C’) Abdominal-A (Abd-A) was expressed in all Ilp7 neurons except for the small embryonic neurons that typically express low level Ilp7 in the adult. In the embryo, Abd-A is expressed in A6 and A7 Ilp7-neurons (Miguel-Aliaga et al., 2008), suggesting that the two large Ilp7 neurons are either the A6 or the A7 Ilp7 neurons. (D,D’) Abdominal-B (Abd-B) was expressed in all subsets of Ilp7 neurons. Interestingly, there was a distinct difference in Abdominal B levels in large Ilp7 neurons (E,E’) Fkh is expressed only in the embryonic subset of Ilp7 neurons (Fig. 2A) and can thus be used as a marker for embryonic versus post-embryonic Ilp7 neurons. We found phosphoMad (pMad) accumulated in the nuclei of all Ilp7 neurons. This indicates that all Ilp7 neurons exit the VNC to access the BMP ligand and activate BMP signaling (Miguel-Aliaga et al., 2008). (F) Nkx6 (also known as HGTX) (Nkx6-GAL4,UAS-nEGFP) is expressed in only the large embryonic Ilp7 neurons in L3 larvae (not shown). Thus, we postulate that the large embryonic Ilp7-neurons in the adult are from segment A6. This also agrees with their expression of Abd-A (C).
Fig. S3. Confirmation that only embryonic Ilp7 neurons are killed by pulsing hid and reaper expression in embryonic Ilp7 neurons in early larval stages. (A,A') Controls in which hid and reaper were not expressed. Anti-Fkh identifies the embryonic Ilp7 neurons in the ventral cluster (Ven) (thick arrows and thin arrows); Fkh-negative/Ilp7-positive (arrowheads) neurons indicate post-embryonic female specific Ilp7 neurons in the ventral cluster. (B,B') Expression of hid and reaper in early larvae, using Ilp7-GAL4, results in an absence of all Fkh-expressing Ilp7 neurons in the adult. These data show that the protocol is highly effective in killing embryonic Ilp7 neurons and also that this appears to have no effect on the differentiation of post-embryonic Ilp7 neurons. Arrows and arrowheads in all figures indicate representative neurons of each Ilp7 subset.

Fig. S4. Expression of hid and reaper causes an egg-jam phenotype in the lateral oviduct. (A) Ilp7 neurons in control animals labeled by anti-Ilp7. (A') Killing all Ilp7 neurons results in an absence of Ilp7 immunoreactivity in the posterior VNC. (A'') Killing Ilp7 neurons results in an egg-jam phenotype in the lateral oviduct wherein eggs are unable to pass through the common oviduct.
**Fig. S5. Ilp7 neurons are not octopaminergic.** (A-B’) Elimination of Ilp7 in Ilp7 neurons using UAS-Ilp7<sup>dsoRNAi</sup> did not disrupt egg-laying in females (ctrl 20.4± 6.2; exp 23.8± 4.6) in spite of efficient knock down of Ilp7 (B’). (C-D’) Ilp7 neurons do not express markers for the octopaminergic neurons TDC2 (C,C’) and TβH (D,D’). (E,E’) TβH expression is eliminated in octopaminergic neurons using the TDC2-GAL4 driver to express UAS-TβH<sup>dsoRNAi</sup>. (F,G) Octopaminergic neurons are required for egg laying (Rodriguez-Valentin et al., 2006). Expression of UAS-TβH<sup>dsoRNAi</sup> in octopaminergic neurons significantly reduced egg laying (ctrl 25.7± 6.9; exp 5.9± 5.8; *P<0.0001) (F), but its expression in Ilp7 neurons did not significantly reduce egg laying (ctrl 23.8± 10.9; exp 17.9± 9.2; P=0.2) (G).
Fig. S6. Female Ilp7 neurons are glutamatergic but not serotonergic. (A) Postsynaptic accumulation of CD8-GFP-Shaker (green) in the oviduct is present exclusively at synapses with Ilp7 boutons (magenta). (B) Ilp7 neuronal boutons label strongly with anti-VGluT. (B’,B”) Expression of UAS-VGluT^{dsRNAi} in Ilp7 neurons eliminated VGluT immunoreactivity and causes an egg-jam phenotype. (C,C’) Unlike male post-embryonic Ilp7 neurons, female post-embryonic Ilp7-neurons do not express serotonin.
Fig. S7. Post-embryonic Ilp7 neurons do not express dsx. (A-B') In adults, embryonic and post-embryonic Ilp7 neurons do not express Dsx. We used an antibody that recognizes both sex-specific variants of Dsx. (A,A') In adult A1 females, female-specific Dsx immunoreactivity was not observed in female-specific neurons (arrowheads) or dorsal Ilp7 neurons (Dor). (B,B') Post-embryonic Ilp7 neurons in males did not express the male sex-specific variant of dsx (dsxM). Embryonic Ilp7 neurons do not express Dsx in the adult. (C-D') To rule out the possibility of transient Dsx expression in the lineage of Ilp7 neurons, we examined postmitotic Ilp7 neurons at the onset of Ilp7 expression (48 hour pupal stages; C,C') and lineage tracked dsx-GAL4 expression, by using it to Flp-in permanent lacZ expression (D,D'). In both instances we could not detect the presence of dsx gene expression in the Ilp7 neuronal lineage.
Fig S8. Post-embryonic dorsal Ilp7 neurons lose serotonin expression in feminized males (♂ F) or gain a serotonergic fate in masculinized females (♀ M). (A-B') Constitutive fruM females show additional serotonin-positive cells only in the dorsal region of abdominal segments. (A,A') In control females (♀ ), a few serotonin-positive neurons are present in the ventral region (Ven) but never in the dorsal region (Dor) of abdominal segments. (B,B') Constitutive fruM females (fruM/fru-Df) have ectopic serotonin-positive neurons in the dorsal region (Dor), similar to that seen in control males; however, no additional serotonergic neurons are seen in the ventral region (Ven). Thus, fruM does not make female-specific Ilp7 neurons adopt a serotonergic fate. (C-D') Genetic manipulation of transformer induces sexual re-assignment of serotonergic neurons in males and females. (C,C') Masculinized females (♀ M), have extra serotonergic neurons in the dorsal region (Dor), but not in the ventral region (not shown). (D,D') Feminized males (♂ F) lose expression of serotonin in the dorsal region (Dor).