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-spooling 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 (dxxs) and fruitless (fru) (Salz and Erickson, 2010; Dauwalder, 2011). In males, dxxs and fru transcript driven from its P1 promoter (fru-P1) undergo default RNA splicing into coding dxxsM and fruM isoforms. Females express the RNA splicing factor transformer (tra), which drives female-specific splicing of dxxs and fru-P1 into a coding dxxsF isoform and a non-coding fruF isoform. Both Fru and Dxs 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 Dxs 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-Valentin 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-Valentin et al., 2006; Kapelinkov 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 dxxs-independent mechanism is responsible for generating the female-specific neuronal subset in females.

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Fy 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*-mdCD8::GFP-L; *UAS*-hid; *UAS*-reaper; elavGal4;UAS-Dicer2; tubp-GAL80TS; w1118 (control strain). Strains obtained as gifts were: *Nkx6-GAL4* (Brohieter et al., 2004), *VGlutOK37; GAL4* (Mahr and Aberle, 2006), *Tdc2-GAL4* (Cole et al., 2005) and *tubp > GAL80 > UAS-CDS*: *GFP*, hs-Flp, *Mkrs* (Gordon and Scott, 2009), Act > STOP > nlac2; *UAS-Flp* (Struhl and Basler, 1993), *MHC-CDS-GFP-Shaker* (Zito et al., 1999), *UAS-Traf*; *UAS-Traf*; *UAS*; *UAS-TradsRNAi*; (Chan and Kravitz, 2007), fruitless-P1-GAL4* (Stoeger et al., 2005), fru1; fru2; fru4-40 (fru2) (Demir and Dickson, 2005), *dsx-GAL4* (Rideout et al., 2010). *UAS-*dsRNAi strains were: *UAS-Ilp7* (RNAi); *UAS-FrU* (Vienna *Drosophila* RNAi Center), *UAS-VGlut* (RNAi); *UAS-TBP*; *UAS-FP* (TRIP). To generate *Ipl7-GAL4*, we PCR amplified 1040 to +660 (*Ipl7* start codon) relative to the *Ipl7* transcriptional start site from *D. reinitzi*. This was placed upstream of GAL4 within the pC3GA vector. Fly transformation was performed by Best Gene Inc. To generate *Flp-out* mosaics in *Ipl7* neurons, flies were heat-shocked once (40°C, 55 minutes) as 72-hour pupae or as pharate adults. This produced *GAL80* Flp-outs in one to four *Ipl7* neurons in 80% of flies.

**Immunohistochemistry**

Primary antibodies used were: rabbit anti-Ilp7 (1:1000; E. Hafen, ETH, Zurich, Switzerland); guinea pig anti-Ilp7 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-TBP (1:50; M. Monastirioti, FORTH, Greece); rat anti-Doublesex (1:100; M. Arbeitman, University, St Louis, MO, USA); chicken anti-β-H (1:50; M. Basler, 1993); goat anti-HRP-Cy5 (1:100; Jackson ImmunoResearch). All images acquired on an Olympus FV1000 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 male fertility assays, groups of males and females were mated at a ratio of one male to three females. 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.

**RESULTS**

**Adult females have a unique subset of posterior Ipl7 neurons not present in males**

We generated a transgenic *Ipl7-GAL4* reporter line (Materials and methods) that faithfully reports Ilp7 expression at all ages (detailed in supplementary material Fig. S1). In larvae, the posterior Ipl7 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’ Ipl7 neurons. The posterior Ipl7 neuronal population had not been examined in detail. Our analysis showed that it reorganizes into distinct dorsal and ventral Ipl7-expressing neuronal clusters by adulthood (Fig. 1B,D).

Are embryonic Ipl7 neurons retained within the posterior Ipl7 population in adults? To test this, we fate-tracked embryonic Ipl7 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 < nLacZ, UAS-Flp/ Ipl7-GAL4; tubp-GAL80TS, UAS-nEFGP*. *Ipl7-GAL4* was used to target *UAS-Flp* recombination expression to Ipl7 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 Ipl7 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 Ipl7 neurons (Fig. 1F). Instead, β-Gal marked subsets of Ipl7 neurons within the ventral cluster: in two large cells (~13 μm in diameter) with intense Ipl7 immunoreactivity and also in four to six small cells (~9 μm in diameter) that had extremely low Ipl7 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 Ipl7 neurons in the ventral cluster that were not marked by β-Gal (Fig. 1F′). These female-specific Ipl7 neurons were non-embryonic and were ~9 μm in diameter with consistently moderate to high Ipl7 levels. These data are quantified in Fig. 1G,H and summarized in 1I,J.

We wished to identify useful discriminatory markers between embryonic and non-embryonic subsets. Thus, in adult Ipl7 neurons, we tested the expression of transcription factors reported to be expressed by Ipl7 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) Ipl7 neurons but were absent in all non-embryonic β-Gal-negative Ipl7 neurons (Fkh shown in Fig. 2A). We use Fkh immunoreactivity hereafter as a marker to discriminate between embryonic and non-embryonic Ipl7 neurons.
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 (Veverysa 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’/H11032). 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-GAL80 TS, UAS-nEGFP], we used GAL80TS to delimit expression of the cell death genes hid (Wrinkled – FlyBase) and 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,
1978; Kozopas et al., 1998; Sánchez et al., 2001). We also observed hindgut innervation in both sexes, as previously shown (Cognigni et al., 2011).

Is Ilp7 innervation of the male seminal vesicle required for male fertility, as it appears to be for female fertility (Yang et al., 2008)? We compared the effects of killing Ilp7 neurons on male and female fertility, using Ilp7-GAL4 to drive the cell death genes UAS-hid and UAS-reaper (Ilp7-KO) (Zhou et al., 1997; Veverysa and Allan, 2012). To test male fertility, we mated 1-day-old (A1) Ilp7-KO and control males to new groups of virgin control females each day for 5 days. After each 24-hour mating period, females were removed and placed on an egg-lay plate for 6 hours and then on a second plate for 18 hours (three females per plate). The numbers of eggs laid, per female, per 24 hours was quantified from both plates. The total number of viable larvae produced was counted on the 6-hour plate and compared with the total egg number on that plate. We found that 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 females to control males 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 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 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 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 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 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 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 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
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-TβHmRNA) 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).

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,H) Using the mating protocols described for EF, 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.
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 GluR1C (also GluRII), 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-VGluT1HSVAI 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. S6B’), which phenocopies Ilp7-KO females (supplementary material Fig. S4A’). As a control, we expressed UAS-VGlut1HSVAI 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 also 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 fru-P1 and dsx. In males, they are ‘default’ spliced into coding dsxM and fruM transcripts. In females, the presence of fra splices fru-P1 into a non-coding fraF isofrom and dsx into a coding dsxF isofrom. This cascade generates male-specific neurons, but female-specific neuronal subsets have not been investigated (Ferveur, 2010; Kimura, 2011). We first examined fru-P1 and dsx expression in Ilp7 neurons, using a GAL4 reporter for the fra-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 dsx-GAL4 to 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 fru-F-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 in 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 isoforms (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 expression in octopaminergic neurons (TDC2-GAL4) that approximately half of all Ilp7 projections are serotonin positive, probably reflecting innervation by serotonergic dorsal Ilp7 neurons and 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 *transformer* expression pan-neuronally in postmitotic neurons, using *elav-GAL4* to express dsRNAi to *tra* (*UAS-tra<sub>dRNAi</sub>*) in females, or to express *tra* (*UAS-tra*) in males. In *tra<sub>dRNAi</sub>* females (in which *fraM* 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 (V) whereas embryonic Ilp7 neurons were unaffected. We also observed ectopic serotonin in a posterior cluster of dorsal neurons (supplementary material Fig. S8C). 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). 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 *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). Moreover, serotonin immunoreactivity was lost in the posterior VNC and at the seminal vesicle, even though the seminal vesicle retains its innervation (Fig. 8D). We used *Ilp7-GAL4* to drive *UAS-tra<sub>dRNAi</sub>* in females and *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; Rodriguez-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 serotoninergic 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/serotonergic subset and the other SAbg neurons. In males, the expression of serotonin in all SAbg neurons requires fraM (Lee et al., 2001), but UAS-fraM 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 fraM females. Why does fraM expression in females only generate serotonin in the Ilp7 subset of SAbg neurons? The answer lies in the control of neuroblast lineage progression by dxx. Many SAbg neurons are lost in dxx-null males, and a subset is gained in dxx-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 fraM 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 dxxF acting in a pro-apoptotic manner (Kimura et al., 2011).
Female-specific loss of TN1 neurons requires a pro-apoptotic role for dxf, but this can be partially counteracted by co-expression of pro-survival dsm (Sanders and Arbeitman, 2008). Female loss of themoil motoneuron is due to a necessary and sufficient role for fru 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 oftra, the expression offru-P1 and the absence ofdsm, held that the presence of ventral female-specific Ilp7 neurons is a default state that was masculinized byfruM. However, althoughfruM 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 atra-dependent factor(s) that functions selectively in females and is sufficient for the generation of female-specific Ilp7 neurons, independently offru anddsm. Three additional genes that act in the sex determination cascade function in female sexual differentiation: intersex (Garrett-Engele et al., 2002; Siegal and Baker, 2005), hermaphrodite (Li and Baker, 1998) and dissatisfection (Finley et al., 1998), the latter of which has been demonstrated to function in atra-dependent, dsm-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 tra-dependent but neither dsm- nor 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 thattra manipulation fully re-assigned the sexual identity ofIlp7 neurons when manipulated fromelav-GAL4 but not fromIlp7-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. Efferent populations regulating this behavior include octopamine- (Monastirioti, 2003), Ilp7- (Yang et al., 2008) and dsm-expressing neurons (Rideout et al., 2010). Regulatory circuits into these reproductive tract efferents are likely to be complex. One candidate population is thepickpocket- 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; Rezäval et al., 2012). The projection of these sensory neurons into the posterior VNC is particularly intriguing, because octopaminergic, Ilp7-, and otherfru-and/or dsm-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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References


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;UAS-nEGFP 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, the 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 the adult. 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\textsubscript{dsRNAi} 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\textsubscript{dsRNAi}. (F,G) Octopaminergic neurons are required for egg laying (Rodriguez-Valentin et al., 2006). Expression of UAS-TβH\textsubscript{dsRNAi} 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 fraM 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 fraM females (fraM/fra-Df) have ectopic serotonin-positive neurons in the dorsal region (Dor), similar to that seen in control males; however, no additional serotonergic eurons are seen in the ventral region (Ven). Thus, fraM 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).