DIMORPHIC DIFFERENTIATION OF FEMALE-SPECIFIC NEURONAL POPULATIONS AND BEHAVIOR IN *Drosophila*

by
MONICA CECILIA CASTELLANOS KOTKOFF

B.Sc., Universidad Autónoma de Baja California, 2004
M.S., Instituto de Biotecnología UNAM, 2008

A THESIS SUBMITTED IN PARTIAL FULLFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Cell and Developmental Biology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

October 2014

ABSTRACT

Over the past years, numerous studies have advanced our understanding of the generation and function of the sex-specific neuronal populations that control sex-specific behaviours. Prior to the work presented in this thesis, no female-specific subsets of neurons had been identified in *Drosophila*; thus, all models and studies of sex-specific neurons have had a male bias.

This thesis describes the first identification and characterization of a female-specific neuronal population in the central nervous system of *Drosophila*, the Ilp7-motoneurons. These neurons innervate the oviduct and are required for egg-laying. We further identified cellular and genetic mechanisms that direct the dimorphic generation of these female-specific neurons. Programmed cell death of post-mitotic nascent Ilp7-motoneurons in males accounts for their female-specific generation in a process regulated by a non-canonical and dosage-sensitive pro-apoptotic role for the male *fruitless* isoform (*fruM*). Thus, we find that analysis of female-specific neuron generation unveils novel mechanisms of dimorphic nervous system construction.

Our characterization of Ilp7-motoneurons led to a collaboration with Eric Lai (Sloan Kettering, USA), to study the neuronal basis of the female sterility phenotype of the Δ*mir* mutant, a deficiency in the bidirectional *mir-iab-4* and *mir-iab-8* miRNA locus of the Bithorax-Complex. We find that female sterility arises from derepression of *mir-iab-4/8* targets, Ultrabithorax and homothorax, in *fru*-expressing neuronal populations of the posterior abdominal segments of the ventral nerve cord. This results in numerous phenotypes that each likely contribute to sterility. Δ*mir* females have reduced Ilp7-motor innervation of the oviduct. Δ*mir* virgin females are constitutively unreceptive to males; however, if mated, they fail to increase egg production. Our data suggests a novel mechanism that may explain this phenotype; after mating, sex peptide from the male seminal fluid is retained in the female reproductive tract, rather than being transferred to the hemolymph, where it is believed to effect the increase in egg production. Ongoing work aims to identify the neuronal populations that are disrupted in Δ*mir* mutants.

Taken together, this thesis provides novel insight and models to further our understanding of female-specific neuronal differentiation, a field that has long been under-represented in the literature.

PREFACE

Chapter 2: **"Female-biased dimorphism underlies a female-specific role for post-embryonic Ilp7-neurons in *Drosophila* fertility**"

Work in this chapter has produced a manuscript published as: Castellanos, MC., Tang, JC. and Allan, DW. (2013). Female-biased dimorphism underlies a female-specific role for post-embryonic Ilp7-neurons in *Drosophila* fertility. *Development*. Sep;140 (18):3915-26.

For this publication I performed all the experiments and analysis presented. Douglas Allan and I conceived the experiments and methods of analysis, as well as wrote the manuscript. Jonathan Tang aided in the construction of the *Ilp7-GAL4* transgene strain utilized in this chapter and throughout this thesis.

Chapter 3: "**Male-specific programmed cell death mediated by *fru* locus dosage underlies female-specific generation of a motoneuron population in *Drosophila"***

For this chapter, Douglas Allan and I conceived the experiments and methods of analysis, and I performed all the experiments and analysis presented.

Chapter 4: "**Homeotic function of Drosophila Bithorax-Complex miRNAs mediates fertility by restricting multiple Hox genes and TALE cofactors in the central nervous system".**

This chapter was a collaborative effort with Dr. Eric Lai's group at the Sloan-Kettering Institute in New York.

This work produced a manuscript published as: Garaulet, DL., Castellanos, MC., Bejarano, F., Sanfilippo, P., Tyler, DM., Allan, DW., Sánchez-Herrero, E. & Lai EC. (2014). *Developmental Cell*. Jun23; 29(6):635-48.

For this publication I performed the experiments and analysis presented in this chapter. Douglas Allan and I conceived the experiments and methods of analysis. My contribution to the manuscript was limited to the section pertaining to the results, methods and figures of my personal work, while Douglas Allan aided the authors in writing the manuscript.

The introduction and discussion of this chapter are reproduced from the published manuscript with the permission of our collaborators.

Chapter 5: "**The *Drosophila* Bithorax-Complex miRNAs *mir-iab-4* and *mir-iab-8* are required for female receptivity to male courtship and post-mating responses".**

This chapter is a continuing collaborative effort with Dr. Eric Lai's group at the Sloan-Kettering Institute in New York.

For this chapter, Douglas Allan and I conceived the experiments and methods of analysis, and I performed all the experiments and analysis presented.

The results in this chapter are presented with the permission of our collaborators.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

♀ Female

♂ Male

⚥ Intersex

LIST OF ABBREVIATIONS

5-HT	5-Hydroxytryptamine (Serotonin)
Abd-A	Abdominal-A
Abd-B	Abdominal-B
AbNvTr	Abdominal median nerve trunk
ACP	Accessory gland proteins and peptides
AD	Alzheimer disease
ADD	Attention deficit disorder
ANT-C	Antennapedia-Complex
AVPV	Anteroventral periventricular nucleus
Bcl-2	B-cell lymphoma-2
BNSTp	Bed nucleus of the stria terminalis
BrdU	Bromodeoxyuridine
Brp	Bruchpilot
BTB	Broad-complex, tramtrack and bric a brack
BX-C	Bithorax-Complex
CadN	Cadherin-N
Ceh-30	C. elegans homeobox-30
CEM	Cephalic companion sensory neurons
CI	Courtship Index
CNS	Central Nervous System
Df	Deficiency
Dlg	Discs-large
Dmrt	*doublesex* and *mab-3* related/transcription factor genes
DNA	Deoxyribonucleic acid
Dpr	Defective proboscis extension response
Dsf	Dissatisfaction
dsRNAi	Double stranded RNA interference
Dsx	Doublesex
DUP99B	Ductus ejaculatorius peptide 99B

EGFP	Enhanced Green Flourescent Protein
Egl-1	Egg-laying defective-1
ELAV	Embryonic lethal abnormal vision
Exd	Extra-denticle
Flp	Flipasse
Fkh	Forkhead
Fru	Fruitless
GABA	Gamma-aminobutyric acid
GFP	Green Flourescent Protein
GluR	Glutamate Receptor
GRASP	GFP reconstitution across synaptic partners
GRN	Gustatory receptor neuron
Her	Hermaphrodite
Hid	Head Involution defective
Hox	Homeobox
HRP	Horseradish Peroxidase
HS	Heat-shock
HSN	Hermaphrodite-specific neuron
Hth	Homothorax
iab	Infra-abdominal
ILP	Insulin-like peptide
Ix	Intersex
Lola	Longitudinal lacking
meA	Medial-Amygdala
miRNA	micro RNA
MOL	Muscle of Lawrence
NFAT	Nuclear factor of activated T-cells
NIH	National Institutes of Health
NMJ	Neuromuscular junction
ORN	Olfactory receptor neurons
PFA	Paraformaldehyde

ppk	Pickpocket
PTSD	Post-traumatic stress disorder
RNA	Ribonucleic acid
SNB	Spinal nucleus of the bulbocavernous
SP	Sex peptide
SPR	Sex peptide receptor
SRY	Sex determining region Y
SS	Splicing site
SSO	Sperm storage organs
Syb	Synaptobrevin
Syt	Synaptotagmin
Sxl	Sex-lethal
TALE	Three amino acid loop extension
TβH	Tyramine beta-hydroxylase
td	Tandem dimeric
TDC	Tyrosine decarboxylase
TrpA	Transient receptor potential cation channel, subfamily A
Tra	Transformer
UAS	Upstream activating sequence
Ubx	Ultrabithorax
VGlut	Vesicular transporter of glutamate
VNC	Ventral Nerve Cord
xo-l	XO-lethal
yp	Yolk protein

ACKNOWLEDGEMENTS

I would like to specially thank Dr. Douglas Allan for taking the chance, and accepting me as his student. I have learned so much from you; you taught me how to be a better scientist, how to always be passionate about the science we did, and for betting a donair when it matters.

I want to thank my committee, Dr. Vanessa Auld, Dr. James Johnson and Dr. Francis Lynn for all your input into my work, and for your support through out my PhD.

To Jacob, I was really fortunate that you were around, thanks for being the amazing asset you are.

To all my lab mates, past and present, thank you so much for your help, your friendship, and for the good times. You guys made my Phd a great experience.

To my friends that have become my Vancouver family, thank you for being so supportive and for cheering me on when I needed it.

To my parents: Alejandro and Maña; and to all my family, thank you for always being there, for believing in me and for encouraging me to follow my dreams.

DEDICATION

ESTA TESIS ES PARA MI FAMILIA, EN ESPECIAL PARA TÍ PAPÁ, GRACIAS POR SU APOYO INCONDICIONAL A LO LARGO DE ESTE CAMINO.
LOS QUIERO MUCHO
LA TERCERA ES LA VENCIDA!

1 INTRODUCTION

Sexual dimorphism is found throughout the animal kingdom and includes morphological, anatomical, physiological and behavioral differences between both sexes (Williams and Carroll, 2009; Williams et al., 2008). Underlying such differences in behaviour is the dimorphic central nervous system. Neuronal dimorphism includes differences in the presence, structure and function of specific circuits in the male and female brain, that directs differences in sexually dimorphic behaviors such as courtship, communication, copulatory behaviors, as well as the processing of opposite-sex sensory cues (Forger and De Vries, 2010). The importance of understanding neuronal sex differences has been highlighted in recent years by the ongoing discoveries that the state of several neurological disorders varies depending on the sex of the patient (Cahill, 2006). However, despite sex acting as an important variable, most neurobiologists either fail to report the sex of the animals in which they did their studies, or only use one sex assuming that the mechanism holds true in the other sex (McCarthy and A., 2011). Thus, to this day, there is still much to be learned about the differences between the male and female nervous system, and how these differences arise.

How is neuronal dimorphism generated? In mammals, a growing body of evidence suggests that apart from hormonal factors, genetic and epigenetic mechanisms act in parallel to generate dimorphic neuronal circuits (Abel et al., 2011; De Vries et al., 2002; Dewing et al., 2006; Gatewood et al., 2006; McCarthy and A., 2011; Reisert and Pilgrim, 1991). It is now clear that dimorphisms in neuronal circuits are largely genetically hard-wired by sex determination cascades that take their initiating cue from the composition of the sex chromosomes. To understand these cascades, neurobiologists have turned to model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* in which male versus female/hermaphrodite neuronal programs rely on a hard-wired genetic program that is experimentally manipulable (Portman, 2007; Villella and Hall, 2008). Central to this effort has been the analysis of *Drosophila* male courtship behavior, and its underlying neuronal circuitry.

Male courtship is a stereotyped, robust and quantifiable behavior. Screening for mutants with defects in male courtship behaviors, as well as those with sexually dimorphic morphological traits, identified the main players in the sex determination cascade, including *transformer* and sex-specific isoforms of *fruitless* and *doublesex*.

The success of analysis of male courtship behaviour is evident in the progress made in

understanding the core cellular and genetic mechanisms that generate sex-specific neuronal circuits. However, as the bulk of the effort details how male-specific neurons and circuits develop and function, this understanding has come with a distinctly male-biased view of neuronal sexual dimorphism.

This has led to the long held notion that neuronal "maleness" in *Drosophila* is considered the default state, while the female state is relegated to being considered 'not-male'. In support of this, comparative studies have shown that males have neuronal populations that are absent, reduced or non-functional in females, often due to female-specific cell death of 'male' neuroblasts and neurons (Dauwalder, 2011; Villella and Hall, 2008). More recently, there has been increasing effort to study female-specific behaviors and the underlying neuronal subsets. To date, numerous female-specific circuits and female-specific neuronal populations have been identified, yet the genetic mechanism by which they form remains largely unaddressed.

This thesis details work that has a deliberate female bias, as a counterpoint to male-biased studies. I highlight the importance of understanding the mechanisms of neuronal dimorphism in females; where the cellular mechanisms may be similar to those that generate male-specific circuits, but their genetic regulation may not simply be the flipside of a single binary sex switch, as it is often envisioned to be. In chapters 2 and 3 of this thesis, I describe our identification of the first documented example of a female-specific CNS neuronal population in *Drosophila* and present genetic evidence for how female-specific circuits may arise. Intriguingly, the generation of female-specific neurons may utilize a mechanism that is not necessarily the 'other side of the coin' of those mechanisms that give rise to male-specific neurons. In Chapters 4 and 5 of this thesis, I describe our collaborative studies of infertile *mir-iab-4* and *mir-iab-8* mutants (termed *Δmir*). Genetic, cellular and behavioral analyses of these female sterile mutants have provided novel insight into the regulatory mechanisms of female mating and post-copulatory responses.

The studies on female-specific neuronal dimorphism presented in this thesis contribute numerous novel contributions to the field of neuronal dimorphism. Thus, in this introduction I provide a brief overview of central aspects of sexual determination, neuronal dimorphism differentiation as well as sex-specific behaviors, with an emphasis on work done in *Drosophila.*

1.1 SOMATIC SEX DETERMINATION

Throughout the animal kingdom, the mechanisms that establish two distinct sexes are

surprisingly diverse (Lalli et al., 2003). In mammals, as well as *Drosophila* and *C. elegans*, the primary mechanisms for determining sex are chromosomal composition and dosage compensation. In mammals, the combination of the X and Y sex chromosomes ultimately defines the sex; XX organisms are female, while XY develop as males (Parkhurst and Meneely, 1994). Once sex has been established by chromosomal ratios, a sex-specific developmental program is induced. Expression of the Sex determining region Y (SRY) protein, found on the Y chromosome, leads to the formation of the testis, which releases testosterone to trigger the male developmental program in non-gonadal tissues (Dulac and Kimchi, 2007). In the absence of a Y chromosome, the female state is induced as default. The molecular mechanisms involved in these developmental programs remain poorly understood (Lalli et al., 2003).

In *Drosophila* and *C. elegans*, sex determination is established by the ratio of X chromosomes to autosomal chromosomes (Hodgkin, 1990). In *C. elegans*, once the ratio has been established, numerator genes or denominator genes converge on the *XO-lethal (xo-l)* gene to control a series of inhibitory interactions that ultimately lead to the activity of the master sex determining factor *transformer-1* (TRA-1). Active TRA-1 directs a hermaphrodite, while inactive TRA-1 determines the male fate in all somatic cells (Hodgkin, 1990; Portman, 2007; Zarkower, 2006).

However, the molecular components of a sex determination pathway and their regulation are best described in *Drosophila* (**Fig. 1.1**). In *Drosophila*, a ratio of X chromosomes to autosomes equal to 1 (2X: 2A) will produce a female, while a ratio equal to 0.5 will produce a male (X: 2A). This ratio signals the expression of the *sex-lethal* gene (*Sxl*). This gene promotes its own expression as well as the expression of downstream sex determination factors such as *transformer* (*tra*). Autoregulation of *Sxl* perpetuates the female developmental program in spite of dosage compensation. In male flies, sex-specific splicing of Sxl introduces an early stop codon that renders the protein non-functional. In females, Sxl protein regulates the sex-specific splicing of *tra*. Tra acts together with Transformer-2 (Tra-2) to regulate the sex-specific mRNA splicing of the sex determination transcription factors *doublsex* (*dsx*) and *fruitless* (*fru*).
Dsx is spliced in a Tra-dependent manner to generate the female-specific isoform *dsxF* in the presence of Tra, and the male-specific *dsxM* isoform in absence of Tra (Baker and Ridge, 1989).

Both isoforms of *dsx* are known to regulate gene expression of downstream targets to induce characteristic male or female morphologies (Williams et al., 2008). The second major sex

determination transcription factor *fruitless* (*fru*) has no described role in somatic differentiation, but is required for neuronal differentiation and male-specific behaviors. The transcription from the P1 promoter (*fru* has 4 promoters) is sex-specifically spliced in a manner that is regulated by Tra, within a sex-differentially spliced exon. In females, the binding of Tra to the 3' end of this exon blocks splicing to allow translation of a stop codon that prematurely terminates the protein. Thus, the *fruF* transcript is a protein null. In males, the absence of Tra allows default splicing of the exon prior to the stop codon, which allows the FruM protein to be translated (Verhulst et al., 2010).

1.2 DIMORPHIC NEURONAL DIFFERENTIATION

1.2.1 Neuronal differentiation and the sex determination pathway

In mammals, sex hormones are required for sexual differentiation of the brain, where they influence the organization and activation of sexually dimorphic neuronal circuits. Testosterone is the main hormone to which these differences are largely attributed. During late gestation and neonatal stages, testosterone is metabolized to estradiol, by the P-450 aromatase. Estradiol is the primary estrogen required for masculinization of the mammalian brain. Males lacking P-450 aromatase show a decrease in sexual behavior and aggression. In females, hormone secretion from the ovaries is dormant during these stages, thus autonomous neuronal differentiation that occurs in the lack of these hormones results in the proper development of female neuronal circuits that underlie female sexual behavior (McCarthy, 2008; Morris et al., 2004; Wu and Shah, 2011). A growing number of studies in mammals have now proposed that neuronal differentiation is also induced through genetic mechanisms as well as sex hormone effects; however these mechanisms are not well understood, although it has been speculated that members of the *doublesex* family are involved (Arnold, 2004; Dulac and Kimchi, 2007; Jazin and Cahill, 2010).

C.elegans and *Drosophila* have not been reported to rely on hormones to establish sexual dimorphism of the brain; these differences are established through hard-wired genetic programs (Portman, 2007; Villella and Hall, 2008).

In *C.elegans,* the downstream effectors of TRA1-1A in the nervous system are not well characterized, a known downstream effector of TRA1-1A are the *male abnormal genes (mab*),

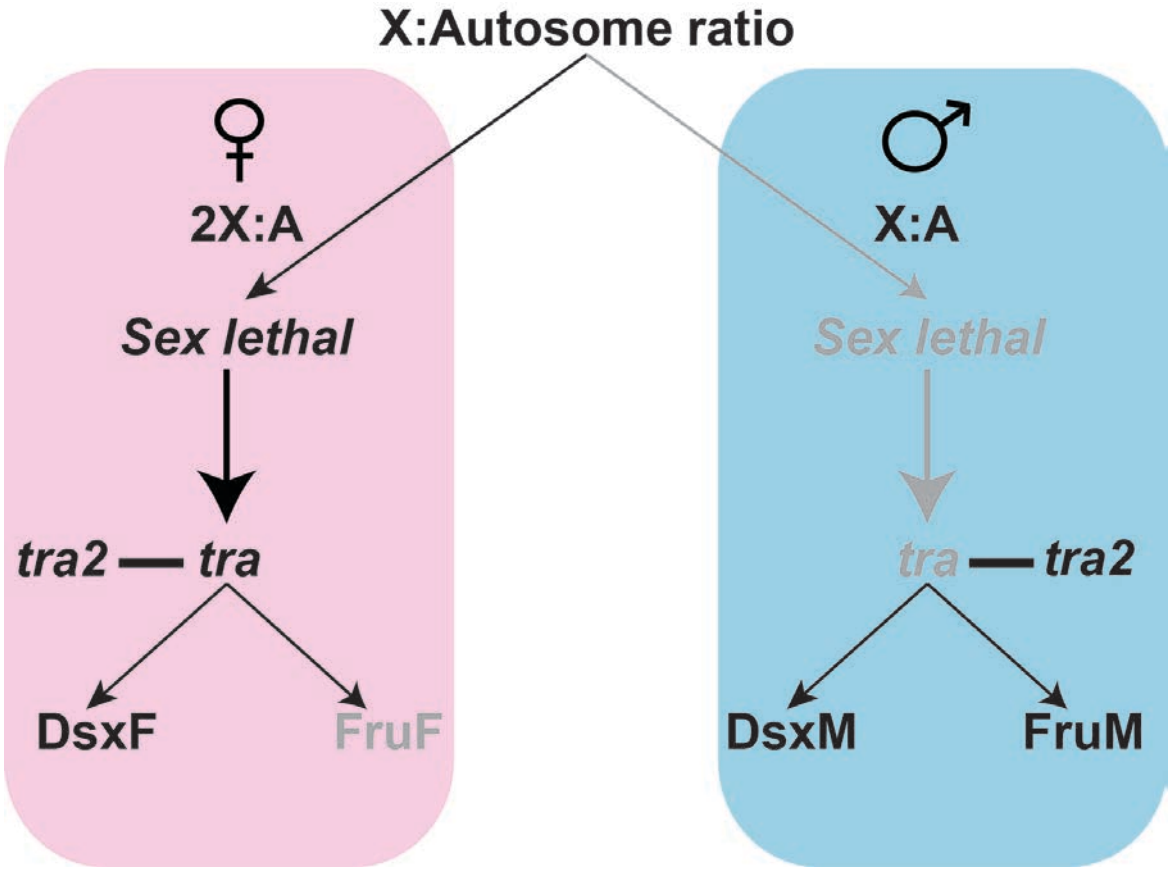

Figure 1.1. Sex determination cascade in *Drosophila*

Schematic of the core sex determination cascade in *Drosophila;* grayed-out genes indicate non-functional proteins within a particular sex, while black bold genes are functional. In females, the 1:1 ratio of X chromosomes to autosomes activates expression of *sex-lethal* (*Sxl*), which directs the female-specific splicing of *transformer* (*tra*) to generate the Tra protein. Tra together with Transformer-*2* (Tra-2) directs the female-specific splicing of *doublesex* (*dsx*) and *fruitless (fru)* into the female-specific isoform DsxF and the non-coding *fruF* transcript. In males, *Sxl* is non-functional, leading to the absence of Tra and the default splicing of *dsx* and *fru* to generate the male-specific protein isoforms DsxM and FruM. Image adapted from (Cachero et al., 2010)

these are well conserved in metazoans and share sequence similarity to *Drosophila doublesex.* These downstream effectors shape dimorphic brains by controlling sex-specific neural cell fate and lineage progression, as well as sex specific cell death and terminal differentiation (Portman, 2007).

Neuronal differentiation in *Drosophila,* is primarily controlled by sex-specific splicing of the *fruitless* locus. Indeed, females expressing constitutive FruM become behaviouraly male in many regards, although it is becoming increasingly apparent that DsxM is required along with FruM for complete masculinization of a female brain (Dauwalder, 2011; Rideout et al., 2007). Both *fruitless* and *doublesex* are known to act on sex-specific cell death, dendritic arbor, neurogenesis, and gene expression in neurons (Dauwalder, 2011). Although *fru* and *dsx* are well characterized to regulate sex differences in the *Drosophila* brain, their downstream targets and the molecular mechanisms by which they generate these differences remain poorly understood.

1.2.2 Establishing sex-specific neuronal differences.

Dimorphism in the nervous system can take many forms, including differences in cell number, connectivity and gene expression (Arbeitman and Winbush, 2010; McCarthy and A., 2011). Perhaps the best conserved mechanism for generating differences in the cell number of neuronal populations between the sexes is programmed cell death of specific neuroblast or neuronal subsets in one sex, and their survival in the other.

In mammals, hormone-dependent cell death regulates differences in cell number of the anteroventral periventricular nucleus (AVPV), where cells expressing the estrogen receptor undergo cell death in response to estradiol (Simerly et al., 1997), Other regions in the mammalian brain such as the spinal nucleus of the bulbocavernous (SNB), and the bed nucleus of the stria terminalis (BNSTp) show sex-specific differences in their cell number that requires the expression of cell death genes of the *Bcl-2* family (Chung et al., 2000; Forger, 2009; Forger and De Vries, 2010).

In *C. elegans*, sex-specific cell death occurs in both sexes, although the responsible mechanism differs for each neuronal population. Two clear examples of neuronal populations that arise in both sexes during embryogenesis and undergo sex-specific cell death are i) the hermaphrodite-specific neurons (HSN) that are specific to hermaphrodites and required for egg-laying, and ii) the cephalic companion sensory neurons (CEM) that are required for the male-

response of secreted chemicals. In males, *egg-laying defective egl-1* activates the caspase cascade to trigger apoptosis of the HSN neurons. In hermaphrodites, TRA1-1A represses *egl-1*, this blocking apoptosis and allowing the HSN to survive. In CEM neurons of hermaphrodite embryos, TRA1-1A represses the expression of C.elegans homeobox (ceh-30), which normally functions to block cell death pathway activation, thus leading to hermaphrodite-specific cell death of CEM neurons (Portman, 2007).

In *Drosophila,* sex-specific cell death has only been described in females and has not been reported to occur in males (Birkholz et al., 2013; Kimura et al., 2005; Sanders and Arbeitman, 2008). During late larval stages, certain subsets of *dsx*-expressing neuroblasts undergo programmed cell death in females, but these survive, proliferate and generate postmitotic neurons in males. As a result, males produce more post-embryonic *dsx*-positive neurons in the A8/A9 posterior region of the abdominal VNC, during metamorphosis. Curiously, this region is known to harbor sex specific neuronal populations innervating the reproductive tracts (Birkholz et al., 2013). Cell death has also been shown to occur in female counterparts for male-specific neuronal clusters required for male courtship, such Female-specific programmed cell death is directed by the balance of a pro-apoptotic role of DsxF and an anti-apoptotic role for DsxM and FruM (Birkholz et al., 2013; Kimura et al., 2008; Sanders and Arbeitman, 2008).

Differences in the morphology and/or connectivity of neuronal populations, that exist in both sexes, have been best described in *Drosophila*. For example, *fruM* is required for male-specific dendritic arborizations of the mAL neurons (Kimura et al., 2005), and for midline crossing of gustatory receptor neuron (GRN) projections in the VNC of males only (Mellert et al., 2010).

Finally, neurons can differ in the gene expression profile that they exhibit; in mammals, a recent study took a genome-wide expression profiling approach to identify sex differences in the expression of genes found in the adult mouse hypothalamus. They confirmed sex-specific expression patterns (by *in-situ* hybridization) for a subset of their candidate genes in the hypothalamus, bed nucleus of the striata terminalis (BNST), and the medial-amygdala (meA), which are regulated by gonadal steroid hormones in males. Genetic analysis for null mutations of a select number of these genes resulted in the loss of specific components of mating, aggression, and maternal care (Manoli et al., 2013; Xu et al., 2012).

In *Drosophila,* a number of potential candidate genes regulated by the sex determination

cascade have been identified in the central nervous system using genomic microarray, RNA-seq and genomic occupancy approaches. The functional role of these genes in establishing neuronal dimorphism has yet to be determined, but has provided relevant candidate genes such as *longitudinal lacking (lola)*, *cadherin-N (CadN)*, and *defective proboscis extension response (dpr)*. RNAi expression to *lola* and *CadN* in fru^{+} expressing neurons leads to defects in male courtship initiation, and mutants for *dpr* show a latency in initiation of wing extension (Dalton et al., 2013; Goldman and Arbeitman, 2007; Neville et al., 2014). Thus, sex-specific differences in gene expression promise a rich avenue for future investigation.

1.3 *Drosophila* AS A MODEL TO STUDY SEX-SPECIFIC NEURONAL DIFFERENTIATION AND CONTROL OF SEX-SPECIFIC BEHAVIORS

Over the past four decades, *Drosophila* has served as a model to study the neuronal control of sex-specific behaviors and the role of the sex determination cascade in the differentiation, and function of sex-specific neuronal populations. *Drosophila* is a genetically tractable organism in which the sufficiency of the sex determination genes can be demonstrated by their capacity to sexually re-assign neuronal subsets, at the molecular, cellular and behavioural level. I will briefly state the molecular mechanisms of the sex determination factors *fruitless* and *doublesex* to provide the basis to understand the genetic approaches that have been used to sexually re-assign neuronal populations. I will then describe the stereotypical, reproducible and quantifiable sex-specific behaviors found in *Drosophila* that allowed the identification of sex-specific neuronal populations that control these behaviors.

1.4 MOLECULAR MECHANISMS OF *fruitless* AND *doublesex* REGULATION

1.4.1 Molecular mechanisms of fruitless transcription and translation

fruitless is a complex gene that spans approximately 130kb (**Fig. 1.2A**). It is transcribed from four different promoters (P1-P4) and undergoes alternative splicing at both the 5' and 3' ends. All transcripts are putative transcription factors of the BTB-Zinc finger family, but the mechanisms that regulate which promoter is utilized, in any neuronal subset at any time, are unknown (Salvemini et al., 2010; Yamamoto, 2007). Alternative splicing at the 3' end generates transcripts with three possible Zinc-finger domains, at the C-terminus of any of the different transcripts from the four promoters. Transcripts from these four promoters have different spatial

and temporal expression patterns, as well as distinct functions. Transcripts from the P3 and P4 promoters are expressed during early embryonic stages and are required for viability through embryonic stages. Transcripts from the P2 promoter are expressed from pupal to adult stages and are implicated in the control of male reproductive behaviors; however, their function remains unknown (Salvemini et al., 2010). Proteins produced from the P2-P4 promoters are common to both sexes, and are generally referred to as the FruCOM proteins (Yamamoto and Koganezawa, 2013).

Transcripts from the P1 promoter are unique in that they undergo Tra-dependent sex-specific splicing in the sex-specific exon (**Fig. 1.2 A, blue/pink box**). In females, the Tra/Tra-2 complex binds to a splicing enhancer, comprised of three 13 nucleotide tandem repeats near the 3' end of the sex-specific exon. This drives female-specific splicing at the 3' end of the sex-specific exon. As a result. the translated fruF transcript encounters an early stop codon and fails to produce a protein. In males, the absence of Tra leads to default male-specific 5' splicing at a site upstream of the stop codon. Thus, male transcripts bypass the stop codon and splice into a downstream exon that allows for coding of full FruM protein isoforms: FruMA, FruMB, and FruMC (depending on how the transcript is spliced at the C-terminus). (**Fig. 1.2 A, blue box**) (Heinrichs et al., 1998; Salvemini et al., 2010; Yamamoto, 2007). These three isoforms are generally all considered together as FruM (Neville et al., 2014; Yamamoto, 2007; Yamamoto and Koganezawa, 2013) and were considered to have similar functions. However, recent studies have found that each FruM isoform has distinct expression patterns in the male CNS, as well as distinct function. FruMB and FruMC are broadly expressed in the CNS, while FruMA has a more restricted pattern. Only FruMC is required for muscle of Lawrence (MOL) formation; expression of FruMA or FruMB cannot induce MOL formation in a *fruMC* mutant. However, males lacking only *fruMB* show a decrease in courtship and copulation levels. *FruMA* mutant males show the least effect on male reproductive behaviors (Neville et al., 2014).

Genomic studies demonstrated that each FruM isoform has a specific DNA-binding motif through which they can regulate the expression of overlapping sets of genes, most of which are involved in neurogenesis (Dalton et al., 2013; Neville et al., 2014). Such studies provide the first insight to uncover the downstream targets of the FruM isoforms in the generation of sexually dimorphic neuronal circuits.

The *fru* locus has been heavily mutagenized and alleles/deficiencies/transposon insertions

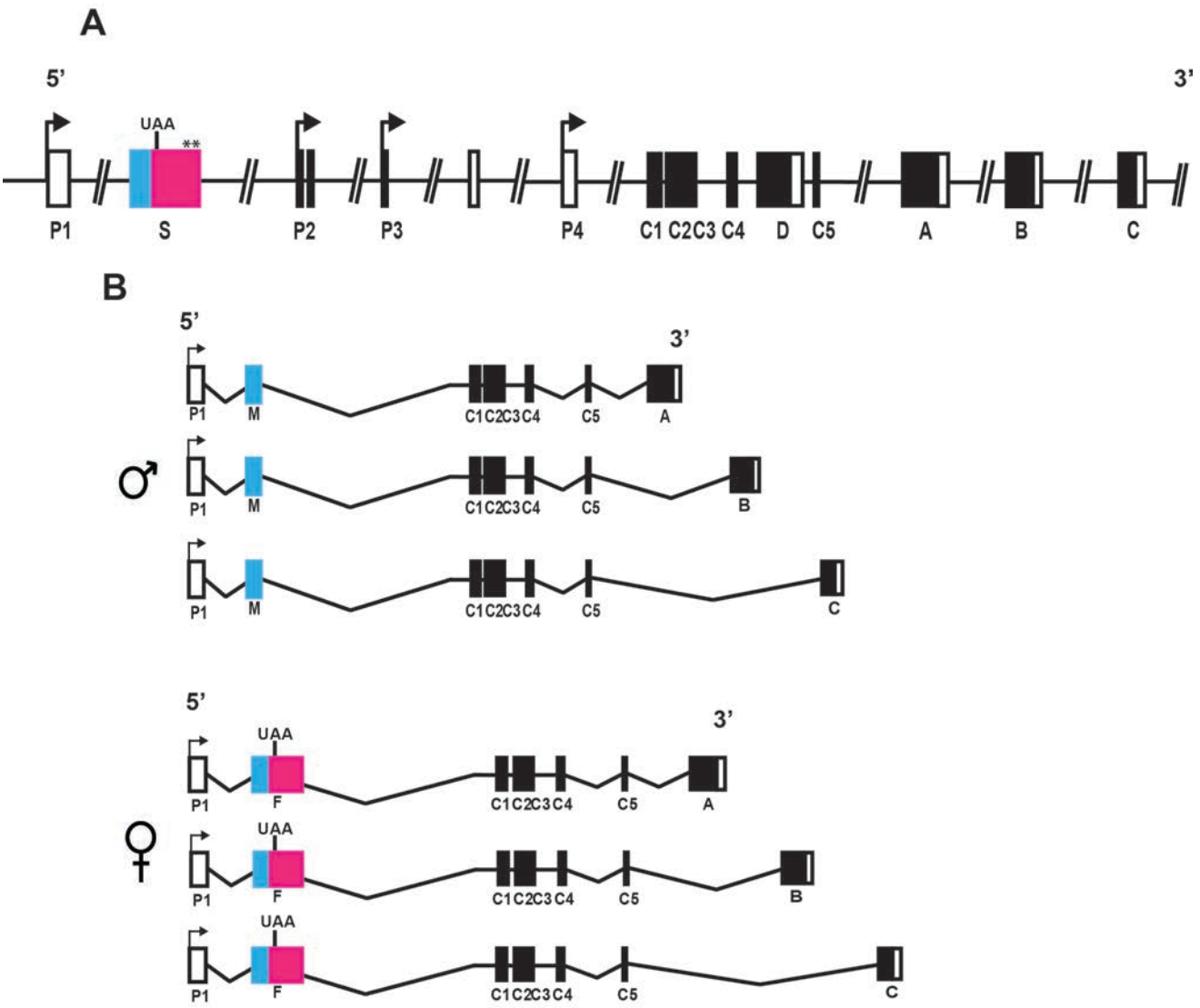

Figure 1.2. Molecular organization of the *fru* locus.

Cartoon schematic representing the structure of the *fru* locus (**A**) and the sex-specific transcripts produced from the P1 promoter (**B**). Image adapted from: (Salvemini et al., 2010)
A) The *fru* locus contains four alternate promoters denoted P1-P4 a sex-specifically spliced exon S (in blue/pink), five common exons (C1-C5), and four alternate 3' exons encoding distinct zinc finger domains (A-D). Transcripts from P2-P4 promoters include all common exons (C1-C5) and differ in their alternative 3' splicing of the different zinc finger domains (A-D). These transcripts are translated into Fru^{COM} proteins that are common to both sexes.
B) Transcripts from the P1 promoter are unique in that they undergo sex-specific splicing. In males (♂), the absence of Tra leads to the utilization of a default splicing site witin the S exon to produce male-specific transcripts. These contain 101 aa from the S exon (blue) in addition to the common exons and different 3' zinc finger domains, which translate into 3 different FruM proteins. In females (♀), Tra binds to a splicing site in the 3' end of the S exon (asterix). This blocks splicing in the S exon and forces translation of a premature in-frame stop codon (UAA) that prevents female-specific transcripts from being translated into functional proteins (Lee et al., 2000; Salvemini et al., 2010).

exist to delete or affect specific promoters and combinations of promoters. Interestingly, detailed analysis of these mutants show that transcripts other than the sex-specific P1 promoter affect specific behaviors of male courtship; and expression of *fru* proteins other than those produced by the P1 promoter rescue the absence of male-specific neurons in in *fru* mutants (Goodwin et al., 2000; Lee and Hall, 2001; Usui-Aoki et al., 2000). Therefore it would be interesting to revisit *fru* alleles and their phenotypes, considering the unknown roles for transcripts from all *fru* promoters.

1.4.2 Molecular mechanisms of doublesex transcription and translation

Doublesex is comparatively less complex than *fruitless*; the gene spans approximately 30kb and undergoes sex-specific splicing, mediated by *tra* in females. Transcripts in both sexes share the first three exons of the locus, but differ in their C-terminus due to alternative sex-specific splicing. In females, the C-terminus is encoded by exon 4, while in males it is encoded by exons 5 and 6 (**Fig. 1.3**). In contrast to the *fru* locus, *dsx* transcripts are translated in females and produce a DsxF protein. Therefore DsxF and DsxM have the same DNA binding N-terminus, but a distinct dimerization C-terminal domain (Yang et al., 2008b). DsxF and DsxM expression is observed broadly in the larval, pupal and adult CNS in a much more restricted pattern than FruM. Many of the sex-specific neurons co-express both Dsx and Fru, and smaller subsets of neurons are observed to express one or the other (Lee et al., 2002; Rideout et al., 2010). *dsx* is required for somatic differentiation in *Drosophila*, and only recently has been implicated in neuronal differentiation. Studies of the role of Dsx in somatic differentiation have shown that, in females, DsxF functions interdependently with the female-specific differentiation factor *intersex (ix)* to regulate the differentiation of the female vaginal teeth, anal plates, foreleg bristles and sixth-tergite pigmentation (Garrett-Engele et al., 2002).

In the nervous system, DsxF can induce post-embryonic female-specific cell death of neuronal populations that arise in both sexes, such as the male-specific TN1 and P1 neurons. These neuronal subsets arise in both sexes, but during early pupal stages DsxF will activate the programmed cell death of the female counterparts of these neurons (Kimura et al., 2008; Sanders and Arbeitman, 2008). DsxF and DsxM have opposing roles in the programmed cell death of sex-specific neuroblasts in the posterior abdominal region of the VNC. Expression of DsxF in these neuroblasts induces apoptosis, while expression of DsxM promotes survival (Birkholz et al., 2013).

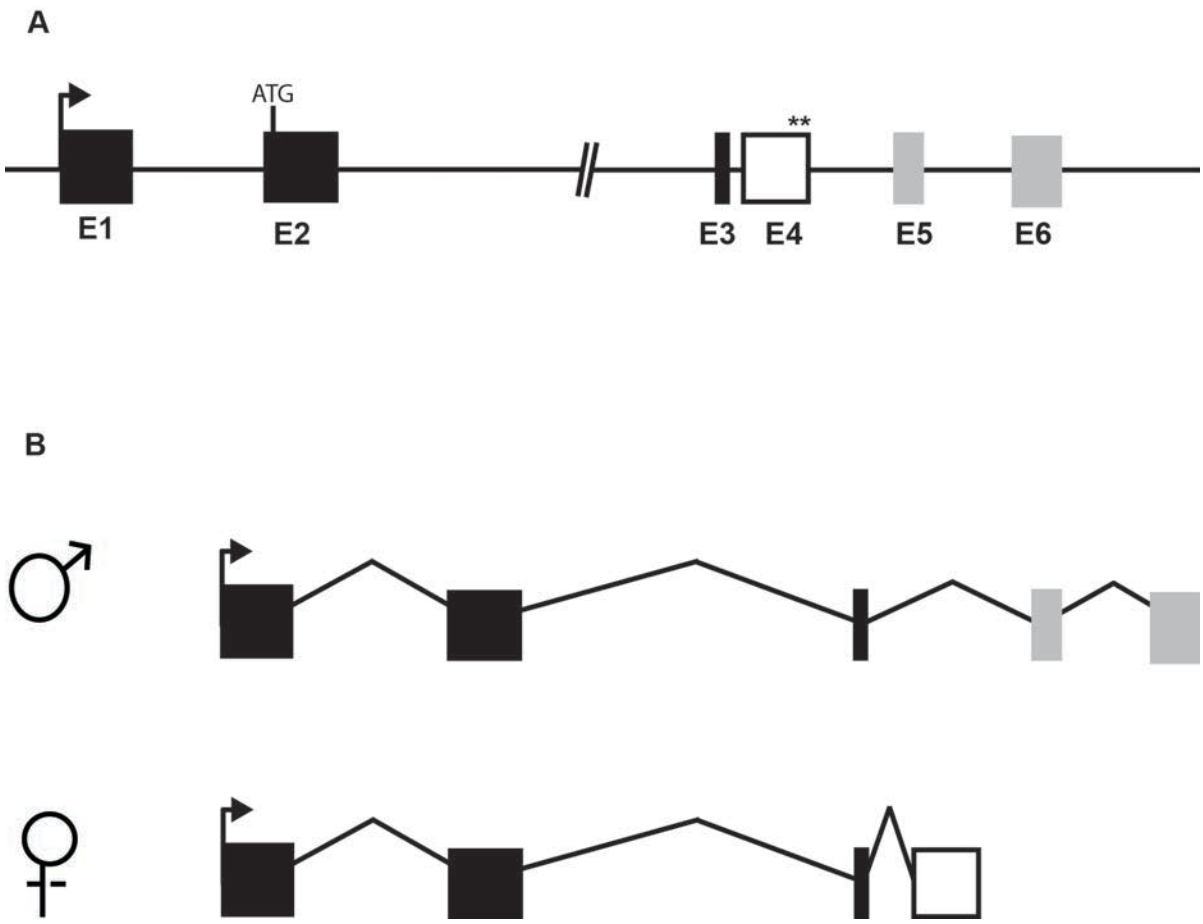

Figure 1.3. Molecular organization of the *dsx* locus

Cartoon schematic representing the structure of the *dsx* locus (**A**) and sex-specific transcripts (**B**). Image adapted from: (Burtis and Baker, 1989)

A) The *dsx* locus contains three common exons (E1-E3, black boxes), a female specific exon (E4, open box) and two male specific exons (E5-E6, gray boxes). Exon 2 contains the common DNA binding domain and the first common oligomerization domain. Exon 3 contains a second common oligorization domain.

B) Sex-specific isoforms of *dsx* mRNA differ in their C-terminal domain. In males (♂), exons 5 and 6 encode the male-specific C-terminal segment. In females (♀), Tra binding sites (asterix) in Exon 4 generates a female-specific C-terminal domain (Burtis and Baker, 1989; Shirangi and McKeown, 2007; Yang et al., 2008b).

DsxF and DsxM can bind to the same DNA motif and therefore are proposed to have opposing effects on the regulation of gene expression in the different sexes; yolk protein genes are direct Dsx targets, where binding of DsxF activates their transcription, and binding of DsxM represses it (Coschigano and Wensink, 1993; Waterbury et al., 1999). Although downstream targets of *dsx* have been described, further studies to uncover downstream targets of *dsx* isoforms in the nervous system and their regulation through *dsx* are required. As *fru* and *dsx* are co-expressed in subsets of sexually dimorphic neurons (Rideout et al., 2010), it would be of interest to determine if they act cooperatively to regulate common genes, perhaps even in a transcriptional complex, or if they act in separable pathways.

Although downstream targets of Dsx have been described, such as the *yolk protein genes* (*yp*), and DsxF and DsxM have been shown to bind to the same region of the *yp* regulatory element to have opposing effects on the regulation of this gene in the different sexes (Coschigano and Wensink, 1993), further studies are required to uncover many of the downstream targets of Dsx isoforms in the nervous system.

1.4.3 Sufficiency of the sex determineation genes in the Drosophila nervous system.

Insertion of GAL4 into the *fru* and *dsx* locus, by homologous recombination, has generated cell-specific drivers that allow for targeted expression in *fru*+ and *dsx*+ expressing neuronal subsets (Rideout et al., 2010; Stockinger et al., 2005). As the *fru* and *dsx*-expressing neuronal populations are believed to exclusively and comprehensively represent all neuroblast lineages and postmitotic neurons that exhibit sexual dimorphism, these GAL4 lines provide exceptionally precise and powerful tools to explore sexual dimorphism of the nervous system. Understanding the sex-specific splicing of *fruitless* and *doublesex* has proven important to understanding the specific roles of *tra* and sex-specific isoforms of *fru* and *dsx* in generating the dimorphic central nervous system. These sex-specific isoforms provide useful tools to sexually re-assign specific cells, in order to examine the molecular and cellular mechanisms of sexual dimorphism.

Expression of *UAS-tra* in males and *UAS-tra-dsRNAi* in females, using a pan-neuronal *ELAV-GAL4* driver, sexually re-assigns the central nervous system of genetic males and females by altering the sex-specific splicing of *fruitless* and *doublesex* in all post-mitotic neurons (Chan

and Kravitz, 2007). Constitutive *fruM* and *fruF* alleles have been generated, that act dominantly in either sex.

There are two fruM alleles, one that eliminates the female-specific region of the sexually spliced S exon, and another that is deleted for Tra-specific binding. This allele generates *fruM* in females and drives male behaviours in those females, including the courting of other females. Equivalently, the *fruF* allele was engineered as a point mutant that abrogates male-specific splicing and is not translated into FruM protein. *fruF* males rarely court virgin females, but actively court other males (Demir and Dickson, 2005). Forced expression of FruM or FruF can be used to determine if FruM is required for the differentiation of a neuronal subset. These alleles are commonly used to determine if *fruM* is required for the differentiation of a neuronal subset. Tools to produce sex-specific Dsx proteins are simpler, and simply involve expression of the sex-specific isoforms *UAS-dsxF* or *UAS-dsxM* (see *section 1.2.2*). These tools were used, for example, to determine how the different isoforms control sex-specific programmed cell death (Birkholz et al., 2013).

As can be seen from these examples, the sufficiency of these regulators to sexually re-assign neurons can be used as a tool to experimentally test how sex modifies neuroblast lineages or/and neuronal differentiation, and defines the role of each sex determination factor in the process.

1.5 NEURONAL CONTROL OF BEHAVIOR

One of the advantages of *Drosophila* as a model to study differentiation and function of sex-specific neuronal populations is the complex series of stereotypical, reproducible and quantifiable sex-specific behaviors it presents.

Drosophila presents a number of sexually dimorphic behaviours such as locomotion, aggression and courtship, which are regulated by sex-specific neuronal populations (Chan and Kravitz, 2007; Dauwalder, 2011; Gatti et al., 2000; Villella and Hall, 2008). Expression of *UAS-tra* in a small subset of neurons form the P1 cluster, is sufficient to feminize locomotor activity patterns in males, while having no affect in the locomotor activity pattern of females(Gatti et al., 2000). Another dimorphic behavior in *Drosophila* is aggression, where fighting patterns in males comprise actions such as “lunge” movements and “boxing” with their forelegs, while females display behaviors such as “headbutts” and “shoves”. Expression of *UAS-tra* in post-mitotic

neurons alters the fighting patterns of males by displaying larger tendencies to "shove" and "headbutt" than control males. On the other hand, females expressing *UAS-traRNAi* will display "lunges" and "boxing" in a male-like aggression pattern (Chan and Kravitz, 2007). For this thesis, I will focus on known sex-specific courtship behaviors and briefly describe the known neuronal populations that regulate these behaviors in males and females.

1.6 NEURONAL CONTROL OF MALE BEHAVIOR IN DROSOPHILA

1.6.1 Male courtship

Courtship in male *Drosophila* follows a complex pattern of behaviors; when a male perceives a female by sensory stimuli such as pheromones and auditory cues, he will orient himself towards her and then start tapping her abdomen and forelegs. Then he extends his wing and "sings" to the female through species-specific wing vibrations. Following this song, the male will lick the female's genitalia and attempt copulation by mounting the female and curling his abdomen to engage the genitalia. The male will continuously display these courtship behaviors in the presence of a female. The length of time a male spends courting a female is termed the courtship index (CI), and can determine the attractiveness of the male to the female (Dauwalder, 2011). If the female is receptive and accepts the male, copulation ensues for approximately 20 minutes. If the female has been mated or is too young, she will reject any attempt of the male to copulate, which in turn leads to a progressive decline in male courtship (Villella and Hall, 2008).

A deficit in any of these steps renders the male sterile. Genetic analysis of male courtship has uncovered a number of male-specific neuronal populations that control specific aspects of male courtship behaviors, as well as central roles for the sex determination factors *fruitless* and *doublesex* in the development and proper connectivity of these neurons.

1.6.2 Neuronal control of male courtship

Distinct areas in the male central nervous system regulate specific steps of male courtship. Initiation of courtship requires male recognition of a female. These behaviors integrate female pheromones detected by gustatory or olfactory sensing neurons such as the Or67D and Gr32a neurons. These neurons are present in both sexes, but only Or67D has been shown to be required for female receptivity (Kurtovic et al., 2007; Miyamoto and Amrein, 2008). Or67D neurons express *fru* and project to the sexually dimorphic DA1 in the antennal lobe, which also

receives input from other *fru*$^{+}$ olfactory receptor neurons (ORN) (Stockinger et al., 2005). Inactivation of *fru*$^{+}$ ORNs promotes male-male courtship (Stockinger et al., 2005).

Another neuronal population found only in the male central nervous system, that requires *fruM* for its generation, is the P1 cluster. This cluster is male-specific; their counterparts in females undergo programmed cell death, mediated by DsxF (Kimura et al., 2008). These neurons control the first steps of courtship including orientation, tapping, licking and wing song. Activation of P1 neurons using the heat activated TrpA1 channel, promotes wing extension and wing song in males, even in the absence of a female. Also, genetically masculinized females that have the P1 cluster can initiate male-like courtship, including wing extension and wing song, albeit not fully realized (Kimura et al., 2008; Kohatsu et al., 2011; Von Philipsborn et al., 2011). Control of the final steps of male courtship such as curling of the abdomen requires a male-specific muscle called the muscle of Lawrence (MOL). The formation of this muscle requires an unknown signal from a *fruM*-expressing neuronal population in the abdominal ventral nerve cord; the muscle of Lawrence-inducing neurons (Mind) neurons. These Mind neurons undergo cell death in females, resulting in the absence of the muscle of Lawrence in females (Nojima et al., 2010; Usui-Aoki et al., 2000).

1.7 NEURONAL CONTROL OF FEMALE BEHAVIOR IN DROSOPHILA

1.7.1 Overview of female behavior in Drosophila

There are fewer studies of the neuronal control of female-specific behaviors. Mutant analysis of *fru* alleles shows no overt behavioral phenotype in females. A key tool that assisted in the identification of female circuits was the generation of *fru-P1-GAL4*, which faithfully reports the expression pattern of sex-specific *fru* transcripts in males and females (see **Fig. 1.2**). With the help of this *fruP1-GAL4,* it was possible to show that females express *fru* transcripts in approximately 2000 neurons in the CNS. Overall comparative analysis of *fru-P1-GAL4* between males and females did not detect gross differences in the number of *fru*-expressing neurons (Manoli et al., 2005; Stockinger et al., 2005), but did identify numerous neuronal subsets that were specific to males, including the P1 neurons, mAL neurons, as well as the DA1 glomerulus that had been previously identified (see *section 1.6.2*) (Kimura et al., 2008; Manoli et al., 2005; Stockinger et al., 2005). Pertinent to our studies is the apparent lack of female-specific *fru*-expressing neurons in *Drosophila*. Ongoing studies have focused on generating a map of all *fru*-

expressing neurons in the *Drosophila* nervous system to identify regions involved in sexually dimorphic behaviors (Cachero et al., 2010; Yu et al., 2010). In these studies, the overall perception regarding *fru*-expressing neurons in the adult *Drosophila* central nervous system is that of a general similarity between both sexes, with regions of the male brain being expanded relative to the female brain. These studies have yet to report an increase in the number of any neuronal subset in females.

1.7.2 Female behavior in Drosophila: receptivity

Within the past few years, more detailed analysis of female-specific behaviors has started to reveal novel female neurons and circuits. To date, two behaviors are considered quantitative female reproductive behaviors: receptivity and post-mating responses. Receptivity is considered the acceptance of male courtship and copulation. If a female deems a male suitable for mating, she will become stationary and spread her wings to allow the male to mount (Ferveur, 2010; Villella and Hall, 2008).

Where are these neurons situated and what circuits do they participate in? Early gynandromorph studies determined a "receptivity center" located in the female brain, where integration of auditory, gustatory and sensory input is suggested to occur (Tompkins and Hall, 1983).

Mutations with altered female receptivity have been described; *spinster* (*spin*), and *dissatisfaction* (*dsf*) mutants reject male courtship and do not copulate. These genes are expressed in distinct neuronal populations located in a region of the female brain that presumptively overlaps with the proposed receptivity center. These neuronal population have been suggested to regulate female receptivity, however their necessity has not been demonstrated (Finley et al., 1998; Juni and Yamamoto, 2009; Sakurai et al., 2013).

Female receptivity would require the integration of sensory, auditory and gustatory input in order to detect a suitable partner. Recently, a group of fru^{+}/ppk^{+} (*pickpocket*) gustatory neurons found in female forelegs, was described; inactivation of these neurons dramatically reduced female receptivity, and it was suggested that they might be required for detecting male pheromones. Where these gustatory neurons project to in the female CNS and their central targets, is currently not known (Vijayan et al., 2014).

Examples of neuronal populations required for female receptivity are scarce in the literature; the role of the sex-determination pathway in the generation of these neurons or

whether they comprise a subset of female-specific neuronal populations remains to be investigated. Studies of neuronal control of sex-specific behaviors mostly consider receptivity as a post-mating response.

1.7.3 Female behavior in Drosophila: post-mating response

Female post-mating responses refer to the set of behaviors a female adopts after mating. A virgin female is highly receptive to male courtship and lays only very few unfertilized eggs. Once a female is mated, she becomes non-receptive to males and egg production and egg laying increases. These behaviors are induced by male accessory gland proteins and peptides (ACPs), which are transferred within seminal fluid from the male to the female during copulation (Chen et al., 1988; Villella et al., 2006).

Nearly 100 ACP's have been identified; however sex peptide (SP), ductus ejaculatorius peptide 99B (DUP99B) and ovulin are the best characterized ACPs (Kubli, 2003; Rubinstein and Wolfner, 2013). SP is necessary and sufficient for both a short-term and long-term post-mating response, while DUP99B and ovulin have minor roles in short-term responses (Herndon and Wolfner, 1995; Peng et al., 2005). Short-term is defined as the immediate post-mating response; wherein females start to reject males and egg production and egg-laying are increased. The short-term response is mediated once ACPs are transferred into the hemolymph. How ACPs transfer into the hemolymph has not been considered in the literature. Ovulin stimulates the release of older staged eggs, which in turn induces oogenesis (Kubli, 2003). The long-term response involves the ongoing induction of enhanced egg-laying and rejection behavior over the course of several days. This appears to be regulated by the slow release of residual SP into the hemolymph; SP that is not immediately released into the hemolymph during the short-term response is bound to sperm and stored in the female sperm storage organs (SSOs) (Peng et al., 2005). There, SP is cleaved through the action of a male seminase, that allows its release into the hemolymph to maintain rejection behavior and heightened egg production over the period of 3 to 5 days (Peng et al., 2005).

Recently, a neuronal circuit required for the induction of post-mating behaviors in response to SP has been described. This circuit comprises neuronal populations that express *pickpocket* (*ppk)*; a Degenerin/Epithelial Sodium Channel (DEG/ENaC) subunit required for mechanical nociception), and *fruitless*. *ppk*-expressing neurons are found in the periphery of the female reproductive tract and only a subset of them co-express *fruitless* (**Fig. 1.4)**. These neurons

project centrally to terminate in the abdominal VNC and the suboesophageal ganglion, but their specific neuronal targets are unknown (Hasemeyer et al., 2009; Yang et al., 2009).

Inactivation of all female *ppk* or *fru*-expressing neurons, with their respective GAL4 lines driving *UAS-shibire*^{TS} expression, causes virgin females to behave as if mated, with decreased receptivity and increased egg production. Using an intersectional approach to repress *UAS-shibire* expression only in those neurons that co-expressed *fru* and *ppk* demonstrated that activity of the *ppk*$^{+}$/*fru*$^{+}$ sensory neurons in the periphery of the female reproductive is able to restore virgin-like receptivity and egg-laying (Hasemeyer et al., 2009; Yang et al., 2009).

Recent studies have added *dsx*-expressing neurons to post-mating circuit, by demonstrating that expression of *UAS-mSP* (membrane bound sex peptide) in *dsx*-neurons elicits a post-mating response in virgin females. They also show that the previously described *ppk*$^{+}$/*fru*$^{+}$ sensory neurons in the female reproductive are a subset of *dsx*-expressing neurons (Rezával et al., 2012) (**Fig. 1.4)**.

These *ppk*$^{+}$/*fru*$^{+}$/*dsx*$^{+}$ neurons also express the *sex peptide receptor* (SPR), which is the specific receptor to SP. Thus, we term these SP sensory neurons the *ppk-SPR* neurons (Hasemeyer et al., 2009; Rezával et al., 2012; Yang et al., 2009). Interestingly, *ppk*, *fru* and *dsx* neurons behave distinctly in their response to elicit the post-mating behaviors.

While inactivation of *ppk* and *fru* neurons decreases receptivity and increases egg production in virgins (Hasemeyer et al., 2009; Yang et al., 2009), activation of *dsx*-neurons with the heat activated TrpA1 channel, decreases receptivity in virgin females (Rezával et al., 2012). Inactivation of SPR signaling in *fru* neurons allows for a decrease in receptivity, but not an increase in egg laying in response to sex peptide. However, SPR signaling is required in both *ppk* and *dsx* neurons to induce a post-mating response (Haussman et al., 2013).This uncoupling of egg-laying and receptivity in *fru*-expressing neurons, and the unique requirement of function in the *ppk*-SPR neuronal populations, indicates that higher order of *ppk*, *dsx* and *fru* neuronal subsets respond to activity of *ppk*-SPR sensory neurons in the reproductive tract to regulate receptivity and egg production interdependently or independently. One recent study describes a potential target of sensory ppk-SPR neurons in the female reproductive tract. These are female-specific *TDC*$^{+}$/*Dsx*$^{+}$ neurons located in the abdominal VNC, and reduce receptivity and increase egg-laying in virgins when activated (Rezával et al., 2014).

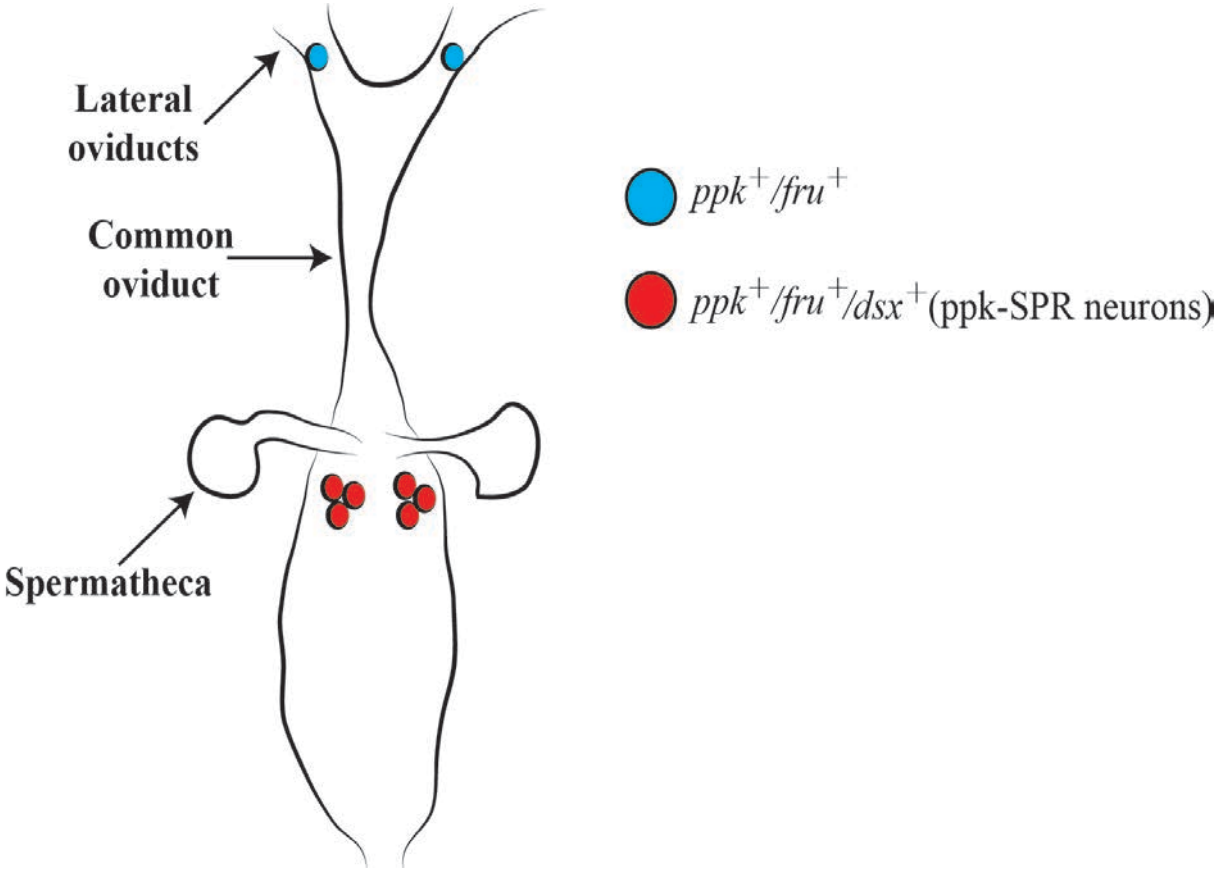

Figure 1.4. Sensory *ppk-SPR* neurons in the female reproductive tract.

Cartoon representation of the location of the sensory *ppk-SPR* neurons in the female reproductive tract. Figure adapted from (Rezával et al., 2012).
Two ppk^{+}/fru^{+} sensory neuron are located on the lateral oviducts (blue). Three $ppk^{+}/fru^{+}/dsx^{+}$ neurons reside in close vicinity to each spermatheca (red) that we term the ppk-SPR neurons throughout because they express the sex peptide receptor (SPR) and pickpocket (ppk). These neurons ramify over the oviduct and uterus and project into the central nervous system to innervate unknown neurons in the posterior abdominal ganglion and suboesophageal ganglion (SOG) in the female brain (Yang et al., 2009).

1.7.4 Female behavior in Drosophila: egg-laying

Drosophila's female reproductive tract is comprised of two ovaries. Each ovary is comprised of 16 to 20 ovarioles, which contain the germarium, and the developing egg chambers. The other structures of the reproductive tract (the oviduct, accessory glands, uterus, vagina and external genitalia) all originate from the genital imaginal discs (Ogienko et al., 2007) (**Fig. 1.5**).

The ovaries are enclosed in a peritoneal sheath that is a network of muscle fibers innervated by TDC^{+} neurons. The oviducts and uterus are lined with super-contractile striated muscles innervated by TDC+ neuromodulatory neurons and glutamatergic type-I motoneurons. The lumen of the oviduct and uterus are lined by an epithelial layer, with microvilli on their apical surfaces. This epithelial layer requires expression of the octopamine receptor OAMB and innervation by TDC+ neurons for ovulation, but the role of octopaminergic input is not known (Lee et al., 2009).

Eggs are released from the ovaries into the lateral oviduct and then into the common oviduct. The contractile activity of the oviduct is regulated by two neuronal populations that are both required for passage of eggs to the uterus, where it will be fertilized and then deposited (Middleton et al., 2006). These are octopaminergic (*TDC*+) neurons and glutamatergic motoneurons. Octopamine is synthesized in octopaminergic neurons from tyrosine, through the activity of two enzymes, *Tyrosine decarboxylase* (*TDC*) and *Tyramine β-hydroxylase (Tβh)* (Cole et al., 2005; Middleton et al., 2006; Monastirioti, 2003).

Drosophila females that lack either TDC or TβH are able to produce eggs, but unable to lay those eggs. Feeding Octopamine-rich food, as well as targeted expression of *UAS-TβH* in *TβH* mutant females restores their ability to lay eggs, indicating the requirement of octopamine innervation in egg-laying (Monastirioti, 2003).

Along the radial muscles of the common oviduct, there are two observable neuronal inputs: the octopaminergic TDC^{+} / $Shaker^{-}$ (potassium channel) neurons and a set of $Shaker^{+}$ fibers that terminate at large boutons apposed by synapses with glutamate receptors, characteristic of type I motoneuron innervation (Middleton et al., 2006). The nature of the neuronal population responsible for the observed glutamatergic motor innervation in the oviduct was unknown prior to the work of this thesis.

Although female-specific reproductive behaviors have been gaining interest, and a variety of

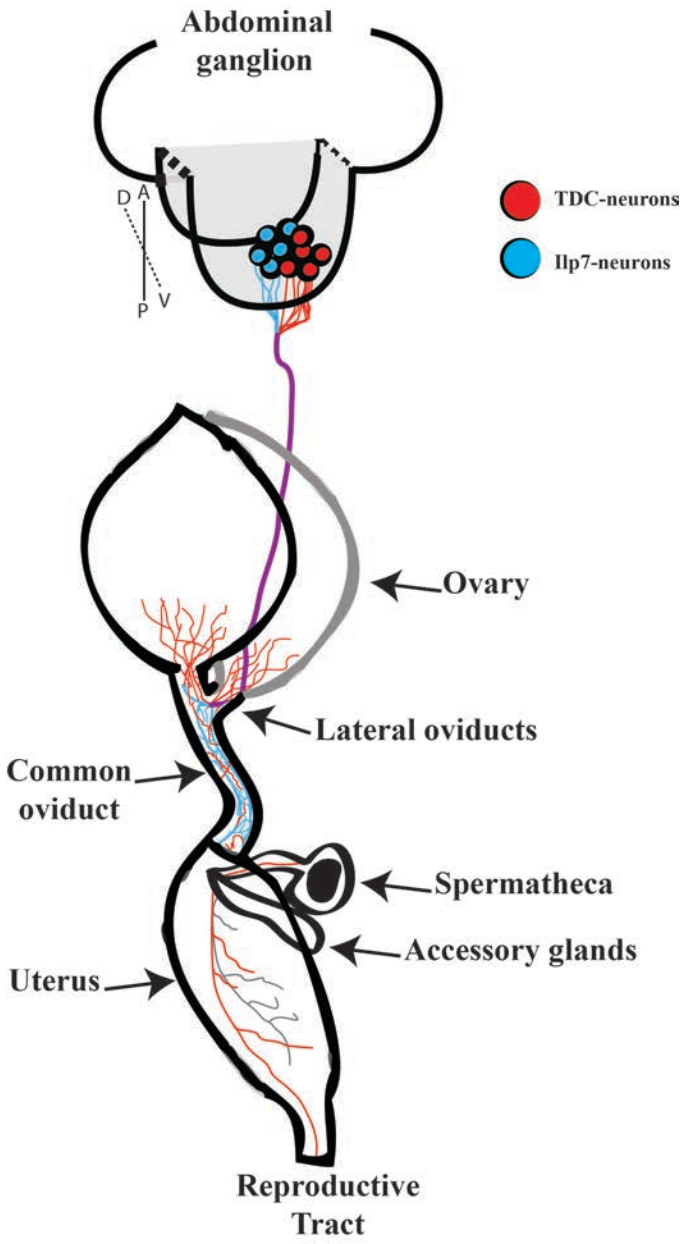

Figure 1.5. Efferent innervation of the female-reproductive tract

Cartoon representation of the female reproductive tract and its efferent neuronal innervation.
Ilp7-motoneurons and TDC-neurons reside in segments A8/A9 of the abdomominal VNC and exit the abdominal ganglion in the Abdominal median nerve trunk (AbNvTr, purple). From here they branch bilaterally to innervate the two ovaries, the two lateral oviducts, the common oviduct and the uterus. The TDC+-neurons (red) innervate the peritoneal sheath of the ovaries, the lateral and common oviduct, as well as the uterus. Ilp7-motoneuron (blue) innervation is restricted to the common oviduct. An unknown glutamatergic neuronal population (gray) is responsible for the innervation observed in the uterus.

neuronal populations have been described, we still lack an understanding of the mechanisms by which the sex determination pathway specifies these neurons in females and regulates their function. Further studies will allow us to compare female vs male mechanisms, determine similarities and differences between the sexes and provide a more complete picture of how neural differentiation is generated in *Drosophila*.

1.8 CONSERVED MECHANISMS OF NEURONAL DIFFERENTIATION IN MAMMALS

This thesis focuses on the mechanisms of female-biased sexual dimorphism in *Drosophila*. We do not aim our studies directly at providing model organism-based insight into mammalian biology or disease. However, we do aim to provide conceptual and mechanistic insight into how female-biased sex-specific characteristics of an organism are generated, as a counterpoint to the better-understood processes in males. This insight may be relevant to mammalian sex determination, because certain core regulators appear to be conserved. Genes that encode for proteins with a DNA-binding domain that appear orthologous to *Drosophila's* Dsx act as terminal effectors of the sex determination cascade in *C.elegans*. These genes are known as *mab* proteins in *C. elegans* and contain a DM domain (named after *dsx*) and are required for several aspects of male-specific development (Portman, 2007). Homologs for *dsx* and *mab* genes have been found in mammals and are classified as *doublesex* and *mab-3* related/transcription factor genes (Dmrt). In mammals, these genes are primarily expressed in gonadal tissue where they are required for gonadal development. Dmrt genes are also expressed in non-gonadal tissues including the brain, but their roles in the brain have not been determined. Mouse mutant for Dmrt4 are viable and fertile, but 25% of males show same-sex copulatory behaviors, suggesting a role for Dmrt4 in the regulation of sexual behavior (Balciuniene et al., 2006; Hong et al., 2007; Kopp, 2012). Increasing number of studies suggest the existence of a molecular mechanism for neuronal differentiation in the mammalian brain that acts independently of hormonal regulation. Neurons taken from the mesencephalon of rats, prior to gonadal secretion, and cultured under similar conditions, will display their female or male developmental programs independently of hormonal instruction (Dulac and Kimchi, 2007). Mice that were gonadally female, but chromosomally male and vice versa, demonstrated that male-specific behaviors are determined genetically without hormonal influence (De Vries et al., 2002). Thus, although sex differentiation in mammals is clearly hormonally influenced, it is becoming

apparent that sex determination is also cell-autonomous and likely driven directly by genetic sex. Thus, work in *Drosophila* and *C.elegans* could be instructive in future studies aimed at unraveling the genetic and cellular mechanisms of sexual dimorphism in mammals.

1.9 SEXUAL DIMORPHISM AND NEUROLOGICAL DISEASES

Many neurological diseases such as Alzheimer's disease (AD), post-traumatic stress disorder (PTSD), anxiety, schizophrenia, stroke, multiple sclerosis, autism, addiction, fibromyalgia, attention deficit disorder (ADD) and Tourette's syndrome show different incidence and disease pathology for both sexes (Abel et al., 2010; Barnes et al., 2005; Becker and Hu, 2008; Bove and Chitnis, 2013; Klein and Corwin, 2002; Zuo et al., 2013). AD affects twice as many women than men. In men, the pathology of AD shows more tau aggregations in the hypothalamus in comparison to women, while the percentage of tau aggregates in the nucleus basalis of Meynert is higher in women than in males (Swaab et al., 2001). Another important feature of sex differences in neurological diseases is functionality. In mice, stimulation of the substantia nigra with GABA produces depolarization in male pups, but hyperpolarization in females (Galanopoulou, 2005). In humans, women progress more rapidly towards drug dependence compared to men due to their sensibility to reinforcement of drugs such as cocaine (Becker and Hu, 2008; Lynch et al., 2002). Interestingly, cocaine decreases the activity of the neurons in right amygdala in women, while in men their activity is increased (Kilts et al., 2004). The influence of sex on the brain is now considered as an important variable in human disease that must be better addressed in the future to tailor treatments for many ailments in the upcoming age of personalized medicine.

2 FEMALE-BIASED DIMORPHISM UNDERLIES A FEMALE-SPECIFIC ROLE FOR POST-EMBRYONIC ILP7-NEURONS IN *DROSOPHILA* FERTILITY.

2.1 SYNOPSIS

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 while it was necessary for eliminating female-specific Ilp7-neurons in males, it was not sufficient for their elimination in females. In 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/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*.

2.2 INTRODUCTION

Behavioral differences between males and females often arise from sexually dimorphic neurons and circuits (Cooke et al., 1998; Paus, 2010; Villella and Hall, 2008; Wade and Arnold, 2004). 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 (Villella and Hall, 2008; Yamamoto, 2007). The sex determination cascade generates dimorphic neuronal populations largely through the sex-specific RNA splicing of the transcription factors *doublesex* (*dsx*) and *fruitless* (*fru*) (Dauwalder, 2011; Salz and Erickson, 2010). 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)* that 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 critical 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 differences in branching and connectivity (Dauwalder, 2011; Villella and Hall, 2008; Yamamoto, 2007). Curiously, only males are reported to have numerically expanded neuronal populations or unique populations not found in females (Cachero et al., 2010; Kimura, 2011; Rideout et al., 2010; Yamamoto, 2007; Yu et al., 2010).

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; Rezával et al., 2012; Rodríguez-Valentín et al., 2006; Yang et al., 2009; Yapici et al., 2008). 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 (Kapelnikov et al., 2008; Middleton et al., 2006; Rodríguez-Valentín et al., 2006). Insulin-like peptide 7 (Ilp7)-expressing neurons are also reported to innervate the oviduct, and their electrical silencing blocks egg-laying (Yang et al., 2008a); 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/fruM-dependent mechanism accounts for the dimorphisms of the shared population of Ilp7-neurons, but that a postmitotic *tra*-dependent and *fru/dsx*-independent mechanism is responsible for generating the female-specific neuronal subset in females.

2.3 MATERIALS AND METHODS

2.3.1 Fly genetics

Flies maintained on standard cornmeal food at 70% humidity at 18°C, 25°C or 29°C. Strains from Bloomington *Drosophila* Stock Centre: *UAS-nEGFP; UAS-mCD8::GFP*^{LL5}*; UAS-hid; UAS-reaper; elav*$^{GAL4\text{-C155}}$; *UAS-Dicer2*; *tubP-GAL80*^{TS}; *w*^{1118} (control strain). Strains obtained as gifts: *Nkx6-GAL4* (Broihier et al., 2004), *VGlut*^{OK371}*-GAL4* (Mahr and Aberle, 2006), *Tdc2-GAL4* (Cole et al., 2005) and *tubP>Gal80>;UAS-CD8::GFP;hs-Flp, MKRS* (Gordon and Scott, 2009). *Act >STOP>nlacZ; UAS-Flp* (Struhl and Basler, 1993). *MHC-CD8-GFP-Shaker* (Zito et al., 1999). UAS-Tra^{F}, UAS-Tra^{dsRNAi} (Chan and Kravitz, 2007). *fruitless-P1-GAL4 (Stockinger et al., 2005)*, *fruF*, *fruM*, *fru4-40* (*fruDf*) (Demir and Dickson, 2005), *dsx-GAL4* (Rideout et al., 2010). *UAS-dsRNAi* strains: *UAS-Ilp7*$^{dsRNAi\text{-KK105024}}$ (VDRC), *UAS-VGluT*$^{dsRNAi\text{-}JF02689}$*; UAS-TβH*$^{dsRNAi\text{-}JF02746}$ (TRiP). To generate *Ilp7-GAL4*, we PCR amplified -1040 to +660 (Ilp7 start codon) relative to the Ilp7 transcriptional start site from *Oregon R*. This was placed upstream of GAL4 within the pC3G4 vector. Fly transformation was performed by Best Gene Inc. To generate Flp-out mosaics in Ilp7-neurons, flies were heat-shocked once (40°C, 55 minutes) as 72 hour pupae or as pharate adults. This produced *GAL80* Flp-outs in 1-4 Ilp7-neurons in 80% of flies.

2.3.2 Immunohistochemistry

Primary antibodies: Rabbit anti-Ilp7 (Yang et al., 2008a) (1:1000, E.Hafen); Guinea Pig anti-Forkhead (Weigel et al., 1989) (1:1000, H. Jäckle); Guinea Pig anti-Odd skipped (Kosman et al., 1998)(1:200, J. Reinitz); Rat anti-TβH (Monastirioti et al., 1996) (1:50, M. Monastirioti); Rat anti-Doublesex (Sanders and Arbeitman, 2008) (1:100, M. Arbeitman). Mouse anti-Abd-A (Kellerman et al., 1990) (1:400, clone D, I. Duncan); Chicken anti–ß-Gal (1:1,000, ab9361, Abcam). Rabbit anti-VGlut, GluRIIC and GluRIIB (Marrus et al., 2004) (1:1000, A. DiAntonio). Rabbit anti-5-HT (1:1000, S5545, Sigma). Goat anti-HRP-Cy5 (1:100, Jackson ImmunoResearch). Rabbit Anti-pMad (1:100, 41D10, Cell Signaling Technology). The

Developmental Studies Hybridoma Bank: provided anti-Dachshund (1:10; clone dac2-3), anti-BrdU (1:10; clone G3G4), anti-Bruchpilot (1:50; clone nc82), anti-Discs large (1:50; clone 4F3), anti-Abd-B (1:20; clone 1A2E9), anti-DGluR-IIA (1:1000; clone 8B4D2). Standard protocols were used (Eade and Allan, 2009), except as follows. For serotonin staining, samples were fixed in 4% Paraformaldehyde with 7.5% Picric Acid for 1 hour. For VGluT and GluR's, we fixed for 5 minutes in Bouin's fixative. For BrdU detection, mid L3 to late L3 larvae were fed 1 mg/ml BrdU (B5002-1G, Sigma) in yeast paste. After standard fixation, adult VNC's were treated with 2N HCl (20 mins) prior to standard immunohistochemical for anti-BrdU. Secondary antibodies: Donkey anti-Mouse, anti-Chicken, anti-Rabbit, anti-Guinea Pig, anti-Rat IgG (H+L) conjugated to DyLight 488, Cy3, Cy5 (1:100, Jackson ImmunoResearch). All images acquired on an Olympus FV1000 confocal microscope. Images were processed using Fluoview FV1000 and Adobe Photoshop CS5.

2.3.3 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 CO_2 when plates were switched. ***6 hr egg-lay assay (*Figs. 2.2G; 2.5B,C; S2.5A,F,G*):*** Details provided in text. Groups of males and females were mated at a ratio of 1 female : 3 males. The egg-lay assays were performed with only 3 females per plate. Eggs were counted and divided by three to give the number of eggs laid per female. ***5 day fertility* (Fig 2.3E,F) *and progeny viability assay* (Fig 2.3G,H*):*** Details provided in text. For female fertility assays, groups of males and females were mated at a ratio of 1 female : 3 males. For male fertility assays, groups of males and females were mated at a ratio of 1 female : 1 male. Egg-lay assays and analysis was performed as for the 6 hr egg-lay. The total number of viable progeny per 6hr plate was counted, (i.e. not divided by 3).

2.3.4 Statistics

GraphPad Prism 5 was used for all analysis and data presentation. Data presented as mean±SD unless otherwise noted. All data underwent D'Agostino and Pearson, Shapiro-Wilk Normality tests. Normally distributed data was compared by parametric unpaired t-test. Non-normally distributed data was compared by non-parametric Mann Whitney test. Statistical tests are shown to the exact P value down to P<.0001.

2.4 RESULTS

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

We generated a transgenic *Ilp7-GAL4* reporter line (Methods) that faithfully reports Ilp7 expression at all ages (detailed in **Fig. S2.1**). In larvae, the posterior Ilp7-subset comprises eight dMP2 neurons in abdominal segments A6 to A9 that innervate the hindgut (Cognigni et al., 2011; Miguel-Aliaga et al., 2008) (**Fig. 2.1 A,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-neuronal clusters by adulthood (**Fig. 2.1 B,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 done 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/ Ilp7-GAL4; tubP-GAL80^{TS}, 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 done using temperature sensitive GAL80^{TS}, which blocks GAL4 activity at 18°C but permits it at 29°C (McGuire et al., 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 mediated 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 (**Fig. S2.2 A**), we examined anti-β-Gal and anti-Ilp7 overlap in adults. β-Gal immunoreactivity was absent from dorsal cluster Ilp7-neurons (**Fig. 2.1 F**).

Instead β-Gal marked subsets of Ilp7-neurons within the ventral cluster; in 2 large cells(~13μm in diameter) with intense Ilp7 immunoreactivity and also in 4-6 small cells (~9μm in diameter) that had extremely low Ilp7 immunoreactivity, that was often undetectable (**Fig. 2.1 F',F"**). In males, these neurons accounted for the entire ventral subset (**Fig. 2.1 F"**).

Unexpectedly, females always had an additional 3-4 Ilp7-neurons in the ventral cluster that were not marked by β-Gal. (**Fig. 2.1 F'**). These female-specific Ilp7-neurons were non-embryonic and were ~9μm in diameter with consistently moderate to high Ilp7 levels.

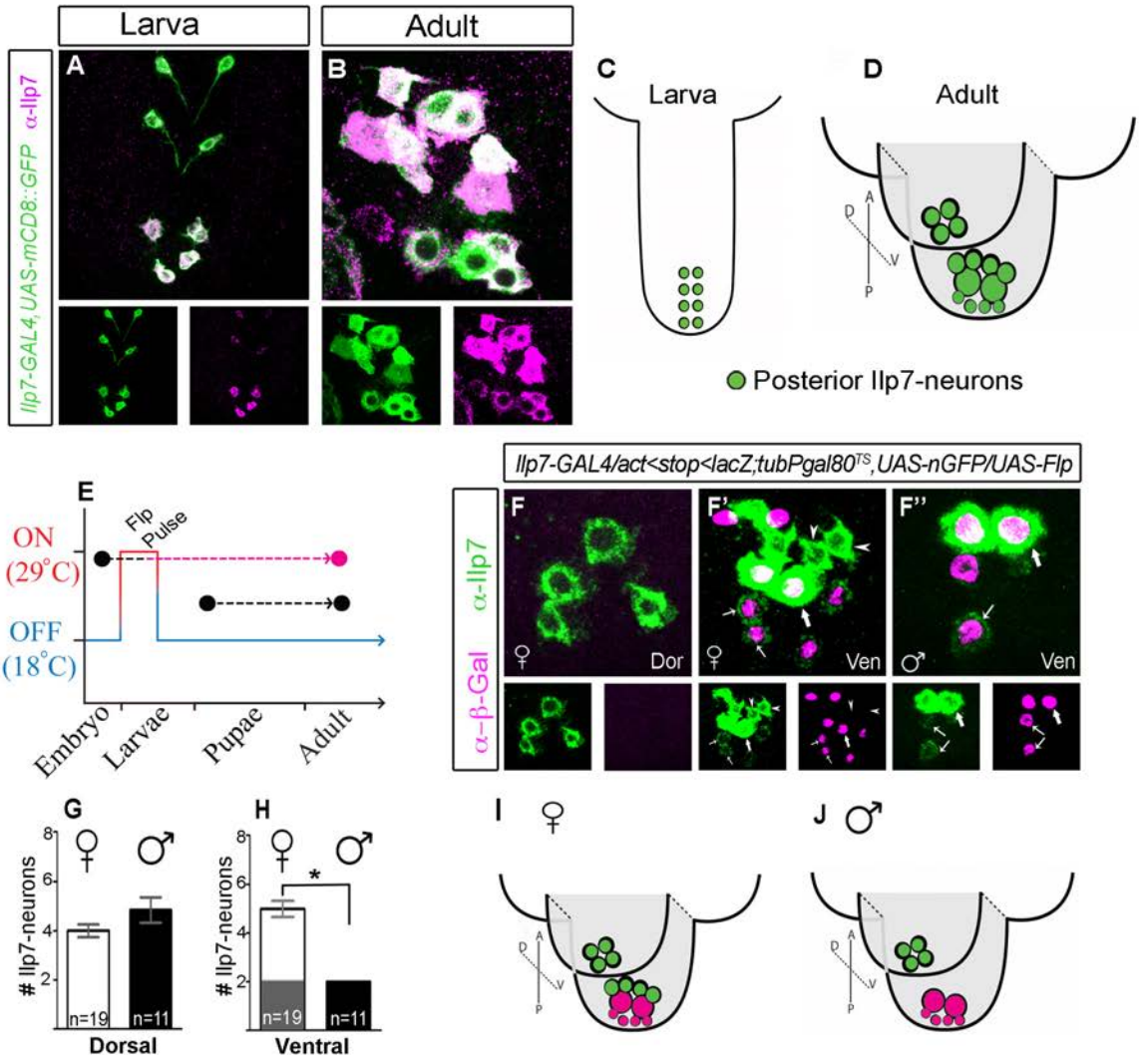

Figure 2.1 . Female-specific posterior Ilp7-neurons in adults

(**A,B**) *Ilp7-GAL4,UAS-mCD8::GFP* and anti-Ilp7 overlap in posterior abdominal VNC Ilp7-neurons in female larvae (**A**) and adults (**B**). (**C,D**) Representation of ventral and dorsal clusters of posterior Ilp7-neurons in larvae (**C**) and adults (**D**). (**E-J**) Fate-tracking of embryonic Ilp7-neurons in adults. (**E**) Schematic of the transient Flp-induction protocol. (**F**) In both sexes, β-Gal immunoreactivity was absent in dorsal (Dor) Ilp7-neurons (shown for females). (**F', F''**) In ventral (Ven) Ilp7-neurons, β-Gal was seen in two large Ilp7-neurons (big arrows) with high Ilp7 and in 4-6 small Ilp7-neurons with very low Ilp7 levels (small arrows). Females had additional β-Gal-negative Ilp7-neurons (arrowhead) in the ventral cluster that were not seen in males. (**G,H**) Numbers of posterior Ilp7-neurons in adults (Mean±SEM), excluding the small embryonic Ilp7-neurons (small arrows in **F',F"**). (**G**) Both sexes have similar numbers in the dorsal cluster (♀ 4.0±0.8; ♂ 4.8±2.2). (**H**) The ventral cluster has 2 embryonic Ilp7-neurons in both sexes (grey region), and also female-specific, non-embryonic Ilp7-neurons (white region) (♀ 5.0±1.1; ♂ 2.0±0.0). (**I,J**) Summary of female and male VNC Ilp7-subsets. * P=0.003. Arrows/arrowheads indicate representative neurons of each Ilp7-subset.

These data are quantitated in **Fig 2.1 G,H** and summarized in **Fig. 2.1 I,J**.

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 (**Fig. S2.2**) (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 2.2 A)**. We use Fkh immunoreactivity hereafter as a marker to discriminate embryonic and non-embryonic Ilp7-neurons.

2.4.2 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 may 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. 2.2 B-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. 2.2 C-E**.

Electrical silencing of all Ilp7-neurons in adults was shown to block egg-laying in females (Yang et al., 2008a). However, our identification of an unanticipated post-embryonic Ilp7-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 GAL80^{TS} to delimit expression of the cell death genes, *hid* and *reaper*, into Ilp7-neurons only in L1 and L2 larvae (**Fig. 2.2 F**). This killed all embryonic Ilp7-neurons but but left post-embryonic Ilp7-neurons intact **(Fig. S2.3**). In spite of this, female egg-laying was

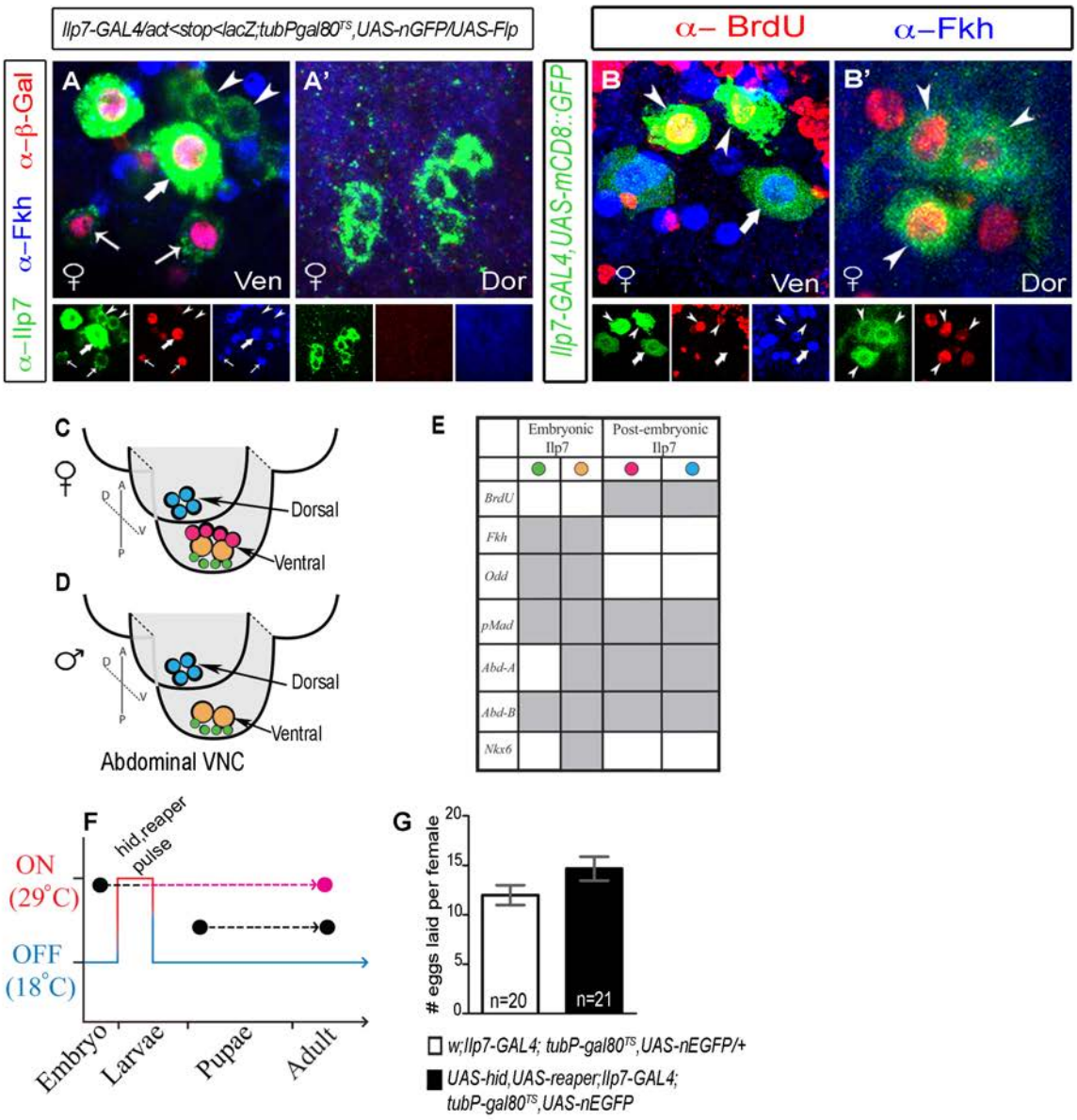

Figure 2.2. Post-embryonic Ilp7-neurons are sufficient for female fertility and can be distinguished from embryonic Ilp7-neurons by lack of Forkhead expression.

(**A-E**) Expression of transcription factors in posterior Ilp7-neurons in adults. (**A,A'**) Transient Flp-induction marked embryonic Ilp7-neurons with β-Gal. Forkhead (Fkh) was only expressed in embryonic Ilp7-neurons (arrows). (**B,B'**) BrdU incorporation into Ilp7-neurons in mid-late L3 larvae was only seen in Fkh-negative Ilp7-neurons (arrowhead) in ventral (Ven) and dorsal (Dor) clusters. (**C-E**) Summary of transcription factor profile in embryonic and post-embryonic posterior Ilp7-neurons in adults (supporting data in **Fig S2.2**). (**F,G**) Selective killing of embryonic Ilp7-neurons does not disrupt female fertility. (**F**) Schematic of transient cell death gene expression in embryonic Ilp7-neurons (hid, reaper pulse). (**G**) We counted the number of eggs laid per female during a 6hr assay following 24hr mating (mean±SEM). Female fertility was not significantly different after killing embryonic Ilp7-neurons (black column), compared to control (white column) (ctrl, 16.5±5.8; exp, 13.4±6.4). *n* = number of egg-laying assays. Arrows/arrowheads indicate representative neurons of each Ilp7-subset.

not affected (**Fig. 2.2 G**); thus, post-embryonic Ilp7-neurons are sufficient for egg-laying.

2.4.3 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., 2008a), 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. 2.3 A,B**) and the seminal vesicles in males (**Fig. 2.3 C,D**). (Bryant, 1978; Kozopas et al., 1998; Sanchez 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., 2008a). 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*) (Veverytsa and Allan, 2012; Zhou et al., 1997). 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 hr mating period, females were removed and placed on an egg-lay plate for 6 hrs and then on a second plate for 18 hrs (3 females per plate).

The numbers of eggs laid, per female, per 24 hrs was quantified from both plates. The total number of viable larvae produced was counted on the 6hr plate and compared to the total egg number on that plate. We found that *Ilp7-KO* and control males fertilized females to the same extent, as egg production and larval viability was not different on most days (**Fig. 2.3 F,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 females to control males for a 24 hr period. Males were then removed from the females and their egg production (per female) and larval viability (per group of 3 females) was tracked over the next 5 days. *Ilp7-KO* females exhibited severely reduced egg-laying throughout the 5 day test period (**Fig. 2.3E**). These females also had distended abdomens and eggs were always found jammed in the lateral oviduct (**Fig. S2.4 A-A''**). Of the small number of eggs laid by *Ilp7-KO* females, only

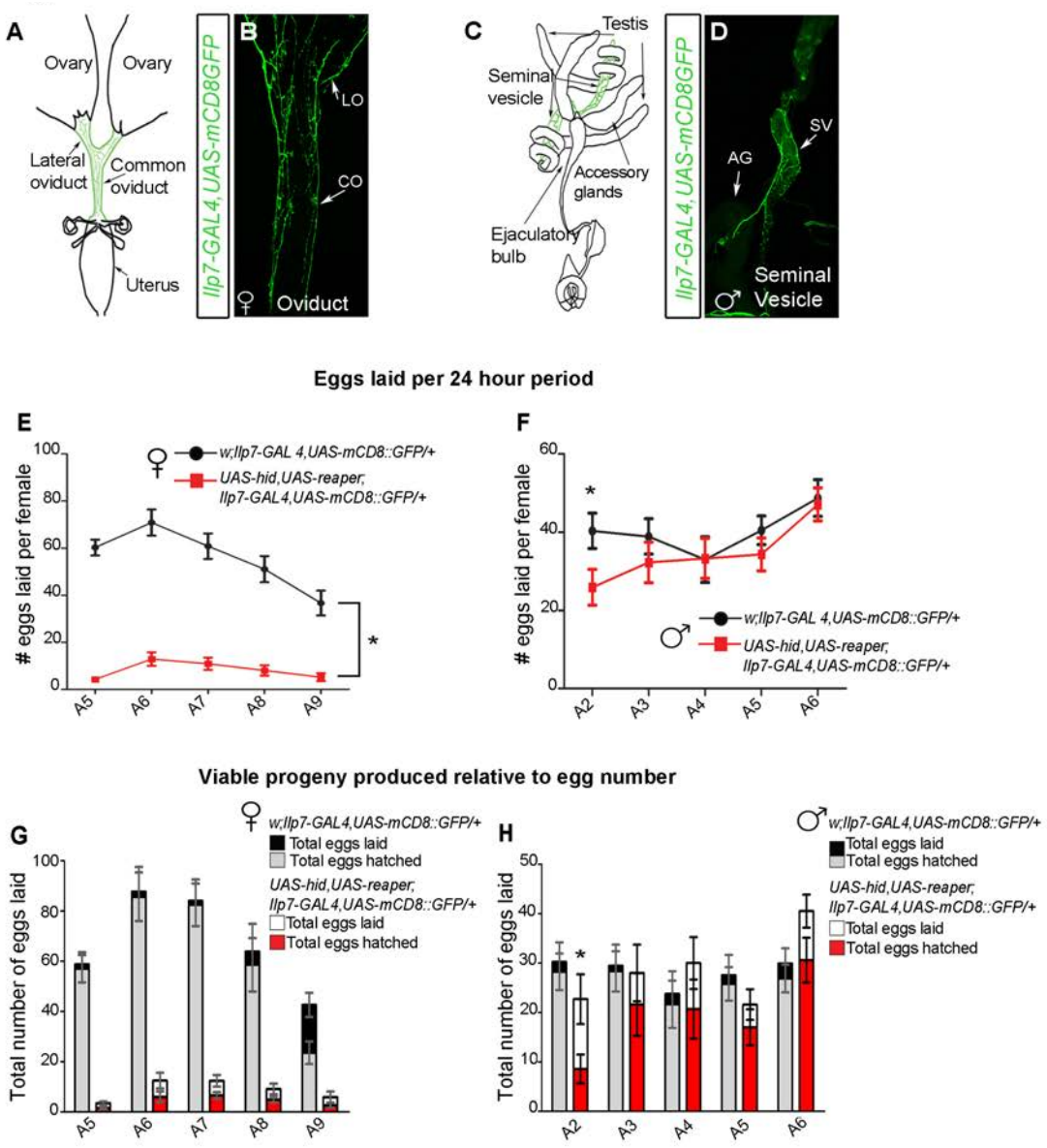

Figure 2.3. Post-embryonic Ilp7-neurons are only required for female fertility.

(**A-D**) *Ilp7-GAL4,UAS-mCD8::GFP* expressing neurons project to the female lateral oviducts (LO) and common oviduct (CO) and the male seminal vesicle (SV). (**E,F**) Control and Ilp7-KO females were mated to control males for 24 hrs; then males were removed. Thereafter, we counted egg numbers laid per female per 24 hr period, for 5 days (see **table S2.1**). (**E**) *Ilp7-KO* females (red) had severely reduced egg-laying compared to controls (black). (**F**) Control (black) or *Ilp7-KO* (red) males were mated to control virgin females for 24 hrs. Then, mated females were removed and males were provided new virgin females for another 24hrs. This was repeated for 5 days (A2-A5). After females were removed, we counted egg numbers per female over 24 hrs. 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 mating protocols used in E,F, we counted the total number of viable larvae produced per plate (not per female) within 6hr assay periods, over 5 days (see **table S2.2**). (**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 timepoint (**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±SEM for egg number per female. *n* = number of egg-lay assays. Arrows/arrowheads indicate representative neurons of each Ilp7-subset.

40% produced viable larvae, compared to 90% of control females (**Fig 2.3 G**).

The sufficiency of post-embryonic Ilp7-neurons for egg-laying (see **Fig 2.1 G**) and the hindgut innervation of embryonic Ilp7-neurons in larvae (Cognigni et al., 2011), led us to test whether the post-embryonic Ilp7-neurons selectively innervate the oviduct. We took a genetic mosaic strategy (Gordon and Scott, 2009) to visualize individual Ilp7-neurons in flies of genotype [*tubP>GAL80> /+; Ilp7-GAL4, UAS-mCD8::GFP/+; hs-Flp/+*]. Transient heat-shock of a *Flp* transgene causes stochastic, mosaic excision of *FRT* (>)-flanked *GAL80* from cells, which permits cell-autonomous *UAS-mCD8::GFP* expression in any neuron that expresses *GAL4*. Based on soma position and Fkh expression, we assigned labeled Ilp7-neurons a subset identity and examined their projections.

The two large ventral-cluster embryonic Ilp7-neurons exclusively innervated the hindgut and rectum but not the oviduct (n=6 mosaic animals) (**Fig. 2.4 A-B''**) while all labeled post-embryonic Ilp7-neurons exclusively innervated the oviduct (n=11 mosaic animals) (**Fig. 2.4 C-F''**).

2.4.4 Post-embryonic Ilp7-neuronal phenotype is sexually-dimorphic.

Targeted mutants of the Ilp7 gene do not exhibit female fertility defects (Grönke et al., 2010), so why does elimination of Ilp7-neurons produce a profound female fertility defect? We confirmed that Ilp7 peptide plays no essential role in egg-laying, by expressing *UAS-ILP7^{dsRNAi}* from *Ilp7-GAL4* and showed that females laid similar numbers of eggs to controls (**Fig. S2.5 A,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 if 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) (**Fig. S2.5 C-D')**. Moreover, TβH knockdown (*UAS-TβH^{dsRNAi}*) blocked egg-laying when expressed in octopaminergic neurons (using *TDC2-GAL4*) but not in Ilp7-neurons (*Ilp7-GAL4*) (**Fig. S2.5 F,G**). To identify alternate modes of neurotransmission, we screened through neurotransmitter markers and found that all post-embryonic Ilp7-neurons expressed *OK371-GAL4* (**Fig. 2.5 A,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

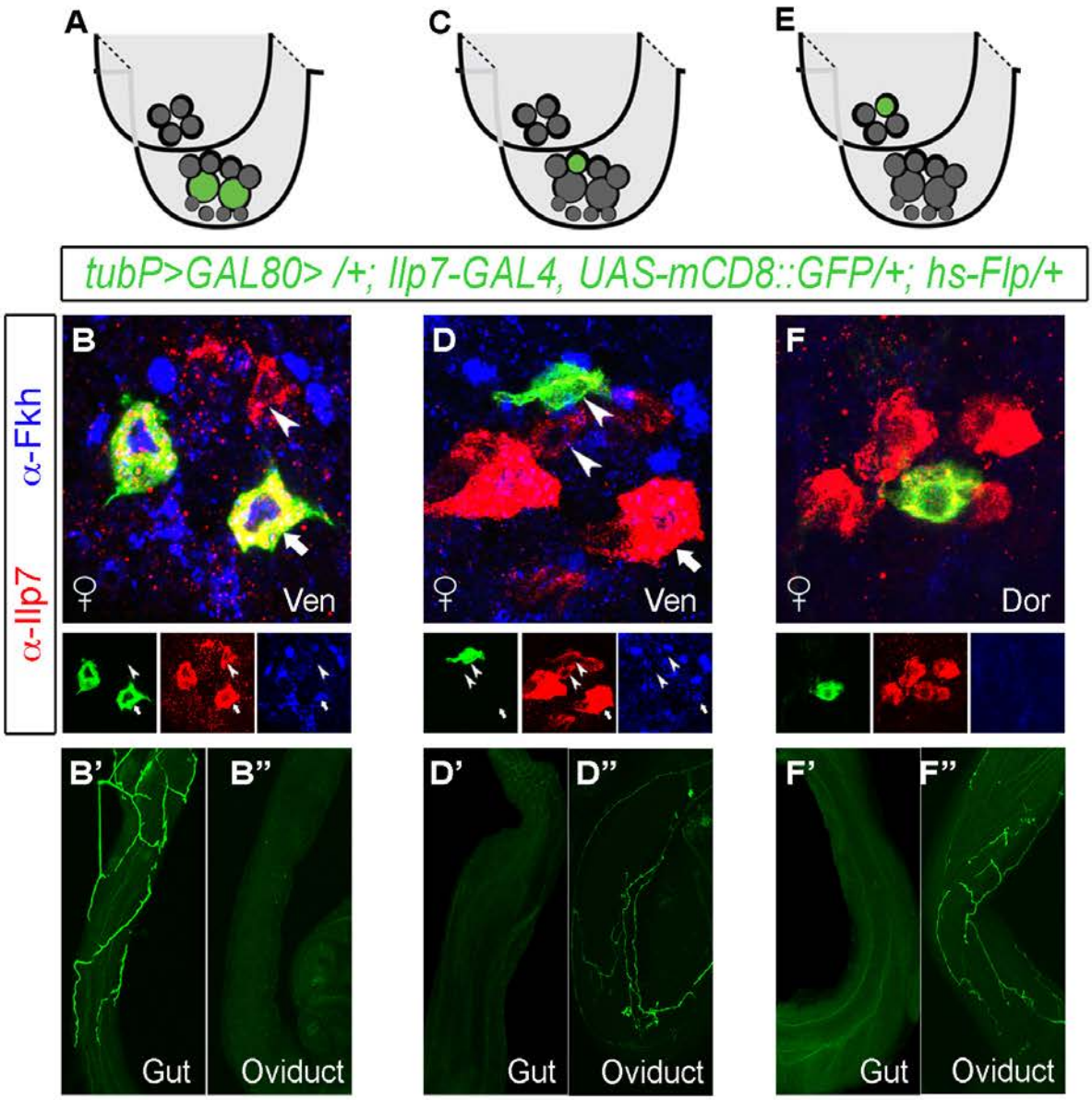

Figure 2.4. Post-embryonic Ilp7-neurons selectively innervate reproductive tracts.

Using stochastic GAL80 Flp-out to generate mosaic *Ilp7-GAL4,UAS-mCD8::GFP* expression, we imaged individual neurons from each Ilp7-subset **(A,C,E)** Cartoons depict the mosaically labeled neurons in the images below. (**B,D,E**) Images of labeled neurons within ventral (Ven) and dorsal (Dor) subsets. Fkh is expressed in embryonic Ilp7-neurons (arrows), but not post-embryonic Ilp7-neurons (arrowheads). (**B-B"**) The large embryonic Ilp7-neurons innervate the gut only. (**D-D"**) Female-specific post-embryonic Ilp7-neurons innervate the oviduct only. (**F-F"**) Post-embryonic Ilp7-neurons of the dorsal cluster innervate the oviduct only in females. Arrows/arrowheads indicate representative neurons of each Ilp7-subset.

exogenous glutamate (Kapelnikov et al., 2008; Middleton et al., 2006; Rodríguez-Valentín et al., 2006).

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. 2.5 E,F**). Moreover, these boutons were apposed to synaptic accumulations of CD8-GFP-Shaker and Discs large (Dlg) (**Fig. 2.5 G,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 (**Fig. S2.6 A).**

We examined 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. 2.5 H-K**). Is glutamatergic transmission of Ilp7 neurons essential for oviduct function? We expressed *UAS-VGluT*^{dsRNAi} in Ilp7-neurons and confirmed that VGluT was efficiently knocked down (**Fig. S2.6 B,B'**). These females had a severe reduction in egg-laying (**Fig. 2.5B**), and an egg-jam phenotype in the lateral oviduct (**Fig. S2.6 B''**), that phenocopies *Ilp7-KO* females (**Fig. S2.4 A'')**. As a control, we expressed *UAS-VGluT*^{dsRNAi} in octopaminergic neurons (*TDC2-GAL4*) and found that this did not disrupt egg-laying (**Fig. 2.5 C**).

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 (Billeter and Goodwin, 2004; Lee and Hall, 2001). We tested the neurotransmitter identity of male postmitotic Ilp7-neurons, and found that they expressed both the vesicular glutamate transporter and also serotonin (**Fig. 2.6 A-B'**). Serotonin is expressed in a posterior neuronal cluster in males, the SAbg, which innervates numerous male reproductive tract structures (Billeter and Goodwin, 2004; Lee and Hall, 2001). We found that Ilp7/serotonin neurons were a small subset of this serotonergic cluster (**Fig. 2.6 B'**), 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-

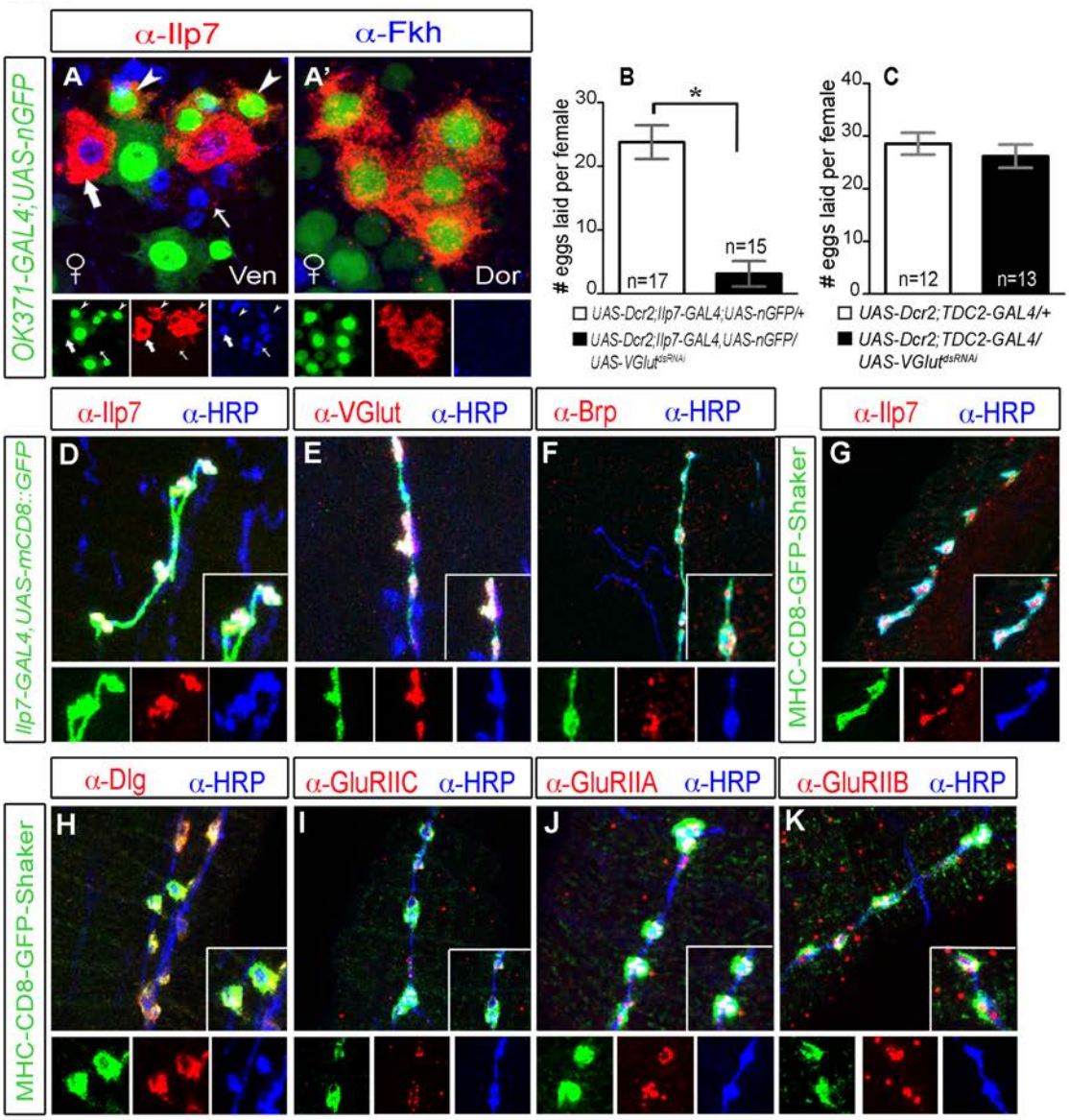

Figure 2.5. Female post-embryonic Ilp7-neurons are glutamatergic motoneurons that terminate at type I-like NMJs on oviduct muscle.

(**A-A'**) *OK371-GAL4,UAS-nEGFP* (VGlut reporter) was expressed in Fkh-negative post-embryonic Ilp7-neurons (arrowheads) but not Fkh-positive embryonic Ilp7-neurons (arrows). **(B,C)** Graphs of eggs laid per female (mean±SEM) over 6hrs (*n* = number of egg-lay assays). (**B**) $VGluT^{dsRNAi}$ expression in Ilp7-neurons reduced egg-laying (ctrl 23.8± 10.9; exp 3.2± 7.7, * P<0.0001). (**C**) $VGluT^{dsRNAi}$ expression in octopaminergic neurons (*TDC2-GAL4*) did not affect egg-laying (ctrl 28.5± 7.2; exp 26.2± 7.9). (**D-K**) Images of Ilp7-neuronal synapses on the oviduct. (***D-F***) *Ilp7-GAL4,UAS-mCD8::GFP*-labeled axons and synaptic boutons co-labeled with anti-HRP (general neuronal marker) and anti-Ilp7 (**D**), anti-VGlut (**E**), and anti-Bruchpilot (Brp) (**F**). (**G**) CD8-GFP-Shaker (type I NMJ marker) is localized to Ilp7-synaptic boutons. (**H-K**) The following NMJ markers are localized to CD8-GFP-Shaker -labeled Ilp7-NMJs: Anti-Dlg (type I NMJ marker) (**H**), GluRIIC (**I**), GluRIIA (**J**) and GluRIIB (**K**). Arrows/arrowheads indicate representative neurons of each Ilp7-subset.

HRP. We then confirmed that all Ilp7-projections were strongly immunoreactive for serotonin (**Fig. 2.6 D)** and for VGluT **(Fig 2.6 E)**, and also found that boutons were immunoreactive for Brp **(Fig 2.6 F).** We note here that female Ilp7-neurons did not express serotonin (**Fig. S2.6 C-C'**).

Although male post-embryonic Ilp7 neurons express glutamatergic markers, we did not expect them to function as motoneurons, due 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 form the seminal vesicle and Ilp7-boutons (**Fig. 2.6 G**). Moreover, we found only weak and infrequent postsynaptic GluRIIC and GluRIIA immunoreactivity apposed to Ilp7 boutons, and no GluRIIB immunoreactivity was observed (**Fig. 2.6 H-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 IIC (Marrus et al., 2004; Monastirioti, 2003; Prokop, 2006; Zito et al., 1997). This analysis demonstrates sexual dimorphism in the transmitter phenotype and NMJs of Ilp7-neurons innervating the reproductive tracts of males and females, with a functional bias to females.

2.4.5 Genetic regulation of Ilp7-neuron dimorphism.

We tested the role of the sex determination cascade is 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 *tra* alternately splices *fru-P1* into a non-coding *fruF* isoform and dsx into a coding *dsxF* isoform. This cascade generates male-specific neurons, but female-specific neuronal subsets has not been tested (Ferveur, 2010; Kimura, 2011). We first examined *fru-P1* and *dsx* expression in Ilp7-neurons, using a GAL4 reporter for the *fru-P1* promoter (that 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. 2.7 A-B', Fig. S2.7 A-B'**). Dsx may be transiently expressed at an earlier stage, but as shown in **Fig S2.7 C-D'**, we could not detect Dsx expression at any time in the lineage of post-embryonic Ilp7-neurons, even by

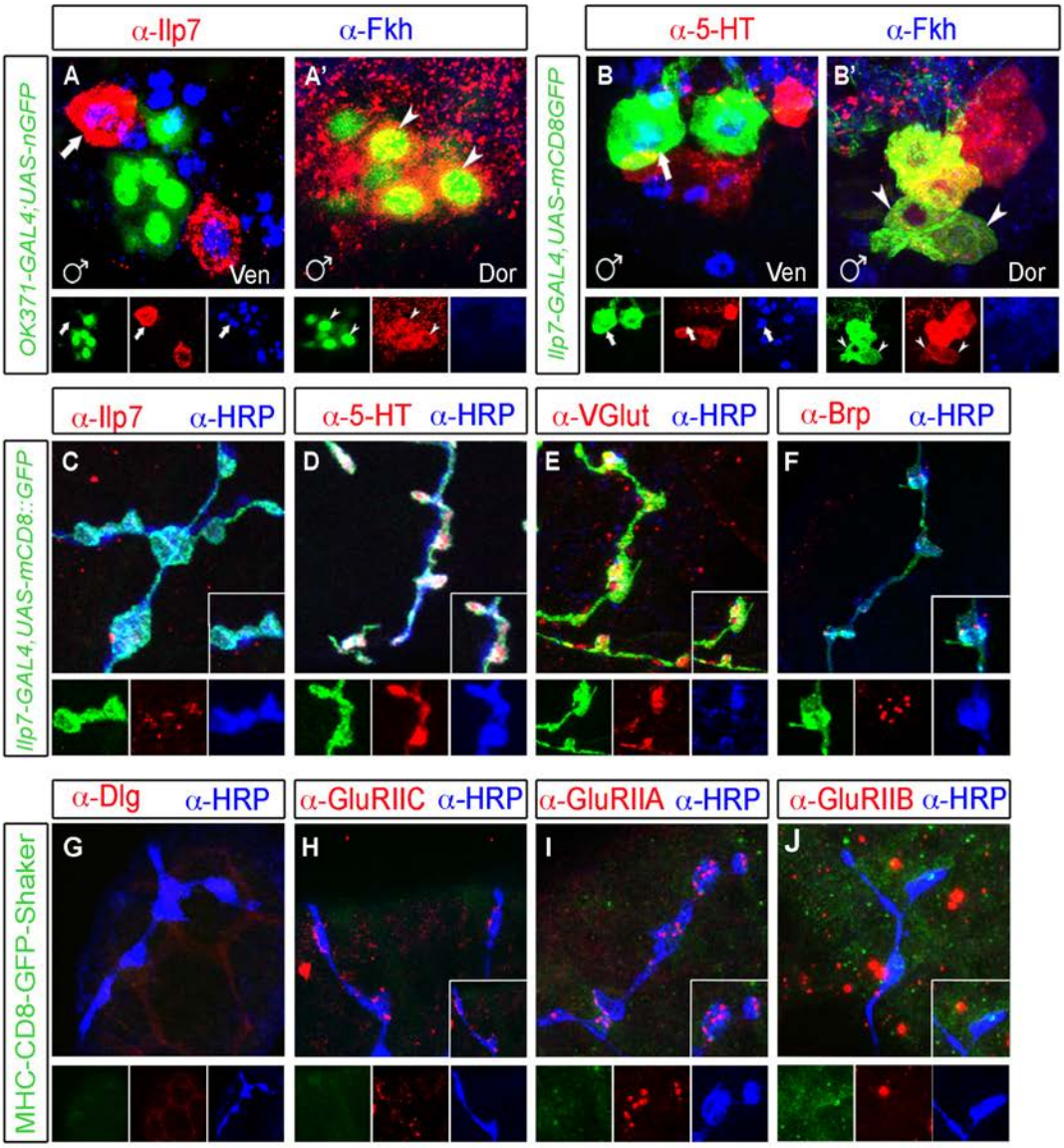

Figure 2.6. Male post-embryonic Ilp7-neurons are serotonergic and glutamatergic and innervate the seminal vesicle with type II-like NMJs.

(**A-A'**) In males, *OK371-GAL4,UAS-nEGFP* (VGlut reporter) was not expressed in Fkh-positive embryonic Ilp7-neurons of the ventral (Ven, arrows)) cluster but was expressed in Fkh-negative post-embryonic Ilp7-neurons of the dorsal (Dor) cluster. (***B,B'***) Serotonin (5-HT) is expressed by post-embryonic (B') but not embryonic Ilp7-neurons (**B**). (**C-J**) Images of Ilp7-neuronal synapses on the seminal vesicle. *Ilp7-GAL4*,*UAS-mCD8::GFP*-labeled axons and synaptic boutons co-labeled with anti-HRP (general neuronal marker) and anti-Ilp7 (**C**), anti-serotonin (5-HT) (**D**), and anti-VGluT (**E**), anti-Bruchpilot (Brp) (**F**). (**G**) Neither CD8-GFP-Shaker not Dlg (type I NMJ markers) were localized to Ilp7-synaptic boutons. (**H-J**) Low level GluRIIC (**H**) and GluRIIA (**I**) were observed at most Ilp7 synapses, but GluRIIB expression was not (**J**). Arrows/arrowheads indicate representative neurons of each Ilp7-subset.

lineage tracing using *dsx-GAL4* to Flp-in permanent *lacZ* expression. Thus, *dsx* is most likely 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* , that do not express FruM) and also in hemizygous *fruM* females (*fruM/fru-Df*, that express FruM protein).In adult *fruF*-males, post-embryonic Ilp7-neurons were feminized; we observed the generation of the ventral subset of female-specific Ilp7-neurons (3-4 Ilp7-positive/Fkh-negative neurons) adjacent to the embryonic Ilp7-neurons (**Fig 2.7 C-C'**).

The shared dorsal cluster Ilp7-neurons is retained (as in females) but serotonin expression is lost from this region (**Fig. 2.7 D,D'**), as was observed in *fru*-null males (Billeter et al., 2006b; Lee and Hall, 2001). Thus, *fruM* is necessary in males for serotonin expression in dorsal Ilp7-neurons and for the loss of the female-specific Ilp7-neurons.

In contrast, post-embryonic Ilp7-neurons were not entirely masculinized in *fruM* females. Notably, the ventral cluster female-specific Ilp7-neurons was not affected by *fruM* expression (**Fig. 2.7 E,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 (**Fig. S2.8 A,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 (**Fig. S2.8 A',B'**) (Billeter et al., 2006b). 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. 2.7 F-F'**). In these oviducts, we find 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

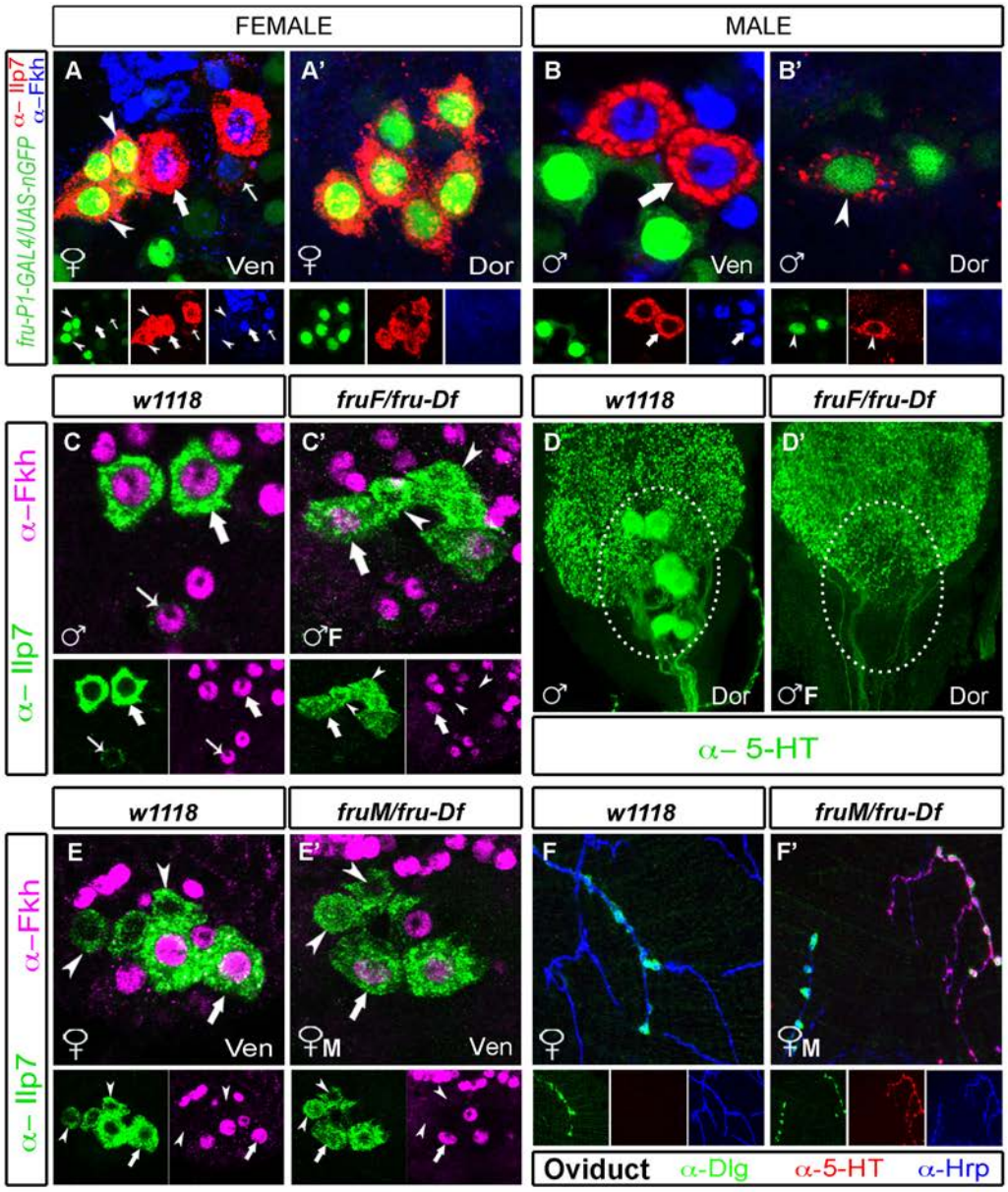

Figure 2.7. FruM is necessary/sufficient for dorsal Ilp7-serotonergic fate, and is necessary, but not sufficient, for loss of female-specific Ilp7-neurons.

(**A-B'**) In both sexes, *fru-P1-GAL4, UAS-nEGFP* is expressed in all post-embryonic Ilp7-neurons (arrowhead; Fkh-negative) but not in embryonic Ilp7-neurons (large and small arrows; Fkh-positive). (**C-D**') In *fruF/fru-Df* males wherein *fruM* is absent (♂F), post-embryonic female-specific Ilp7-neurons are generated in the ventral cluster (C', arrowhead, 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 (♀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 (see **Fig S8B**). Here, we show ectopic serotonin expression at Ilp7-projections on the oviduct. (**F,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 (likely dorsal subset Ilp7-neurons). Arrows/ arrowheads indicate representative neurons of each Ilp7-subset.

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 can 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*^{dsRNAi}) in females, or to express *tra* (*UAS-tra*) in males. In *tra*^{dsRNAi} females (in which *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. 2.8 A,A'**) whereas embryonic Ilp7-neurons were unaffected. We also observed ectopic serotonin in a posterior cluster of dorsal neurons (**Fig. S2.8 C,C'**).

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. 2.8 B-B'**). 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 (through 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. 28 C-C'**). Moreover, serotonin immunoreactivity was lost in the posterior VNC and at the seminal vesicle, even though the seminal vesicle retains its innervation (**Fig. 28 D,D', S2.8 D,D'**). We tested *Ilp7-GAL4* to drive *UAS-tra*^{dsRNAi} 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 Ilp7 expression itself.

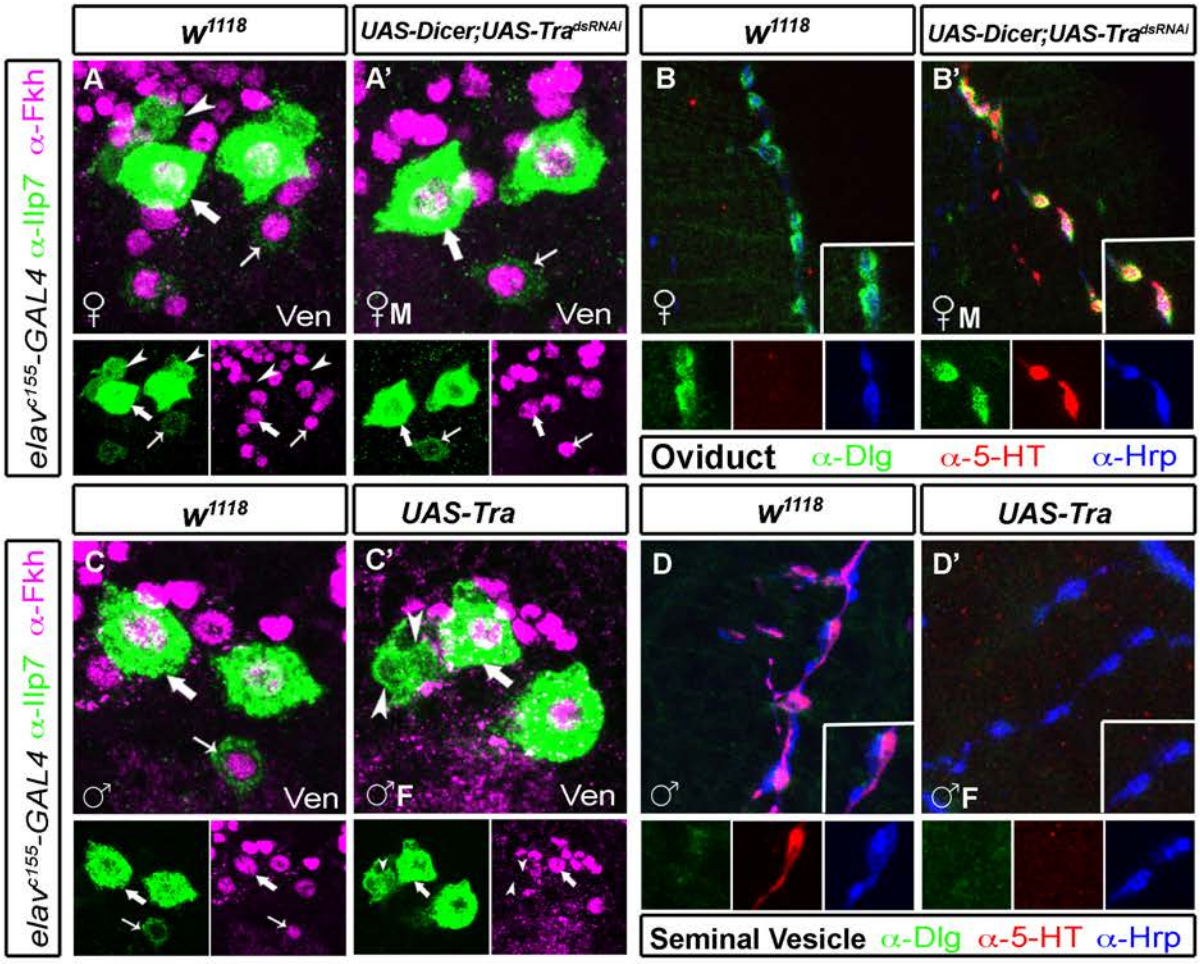

Figure 2.8. transformer is necessary/sufficient for feminizing post-embryonic Ilp7-neurons.

(**A-B'**) Pan-neuronal, postmitotic *UAS-tra*^{dsRNAi} masculinized Ilp7-neurons in females. (**A-A'**) In controls, female-specific Ilp7-neurons (arrowheads, Ilp7-positive/Fkh-negative) are present in the ventral (Ven) cluster (A). These are not seen in *UAS-tra*^{dsRNAi}-females (♀M) (A'). (**B-B'**) In *UAS-tra*^{dsRNAi}-females, Ilp7-neuronal innervation to the oviduct is much reduced, and serotonin is expressed in all Ilp7-processes apposed to Dlg-positive NMJs. Oviducts of control females do not have serotonin in the oviduct (**B**). (**C-D'**) Pan-neuronal, postmitotic *UAS-Tra* feminized Ilp7-neurons in males. (**C-C'**) *UAS-Tra* males (♂F) have female-specific post-embryonic Ilp7 neurons in the ventral (Ven) cluster (arrowheads, Ilp7-positive/Fkh-negative) that are not seen in controls. (**D-D'**) Dorsal cluster (Dor) post-embryonic Ilp7-neurons are feminized and lose serotonin expression (shown in **Fig. S8D-D'**). Here, we show that *UAS-Tra* males retain innervation of the seminal vesicle (D') but have lost serotonin expression in those neurons. Arrows/ arrowheads indicate representative neurons of each Ilp7-subset.

2.5 DISCUSSION

2.5.1 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). In 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 peptides (ILP) expression in motoneurons has only been described in *C.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 its knockdown. Ilp7 functions in the selection of appropriate substrates for egg laying (Yang et al., 2008a), however the circuitry and function of Ilp7 underlying this behavior are unknown.

2.5.2 Sexual dimorphism of post-embryonic Ilp7-neurons.

There are approximately 10 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 (Billeter et al., 2006b; Lee and Hall, 2001; Lee et al., 2001). Our results now show that a subset of these neurons (~4) co-express Ilp7 and serotonin, and selectively innervate the seminal vesicle. Comparing our data to that of previous reports, we can now propose that the generation of dimorphic SAbg neurons is different for Ilp7/serotonergic subset and the other SAbg neurons. In males, the expression of serotonin in all SAbg neurons

requires *fruM* (Lee and Hall, 2001), but *UAS-fruM* only generates a reduced subset of ~4 SAbg neurons in females (Billeter et al., 2006b). We show here that these are Ilp7-neurons, since 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., 2006b). 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 (Billeter et al., 2006b; Birkholz et al., 2013; Taylor and Truman, 1992).

The function of *fruM* is thereafter limited to activating serotonin expression in the remaining neurons (Billeter et al., 2006b). We propose that the absence of *dsx* expression in the lineages of post-embryonic Ilp7-neurons may 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) (Kimura, 2011; Rideout et al., 2010). Female-specific loss of P1 neurons is solely mediated by *dsxF* acting in a pro-apoptotic manner (Kimura et al., 2008). Female-specific loss of TN1 neurons requires a pro-apoptotic role for *dsxF* but this can be partially counteracted by co-expression of pro-survival *dsxM* (Sanders and Arbeitman, 2008). Female loss of the moL motoneuron is due to a necessary and sufficient role for *fruM* in promoting motoneuron survival (Billeter et al., 2006b; Usui-Aoki et al., 2000). 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 *tra*, the expression of *fru-P1* and the absence of *dsx*, held that the presence of ventral female-specific Ilp7-neurons is a default state that was masculinized by *fruM*. However, while *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 *tra*-dependent factor(s) that functions selectively in females and is sufficient for the generation of female-specific Ilp7-neurons, independently of *fru* and *dsx*. 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, 1998b) and *dissatisfaction* (Finley et al., 1998), the latter of which has been demonstrated to function in a *tra*-dependent, *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 whose sex-specific expression is *tra*-dependent but neither *dsx* 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 'fate' of female-specific Ilp7-neurons in males.

In this light, it is interesting that *tra* manipulation fully re-assigned the sexual identity of Ilp7-neurons when manipulated from *elav-GAL4* but not from *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.

2.5.3 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., 2008a), and *dsx*-expressing neurons (Rideout et al., 2010). Regulatory circuits into these reproductive tract efferents are likely complex. One candidate population is the *pickpocket/doublesex*-expressing reproductive tract sensory neurons. Upon mating, these neurons relay a signal to the suboesophageal ganglion and the posterior abdominal VNC, to decrease receptivity to male courtship and increase egg-laying (Hasemeyer et al., 2009; Rezával et al., 2012; Yang et al., 2009). The projection of these sensory neurons into the posterior VNC is particularly intriguing, since Ilp7, octopaminergic and other *fru* and/or *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 that will lead to a more full understanding of how sex-specific circuits are built and function.

3 MALE-SPECIFIC PROGRAMMED CELL DEATH MEDIATED BY *FRU* DOSAGE UNDERLIES FEMALE-SPECIFIC GENERATION OF A MOTONEURON POPULATION IN *DROSOPHILA*

3.1 SYNOPSIS

Over the past few decades, a wealth of studies in *Drosophila* has advanced our understanding of the generation of sex-specific neuronal populations. Male-specific neuronal populations controlling male courtship have been described and the genetic mechanisms required for their differentiation have been addressed. In all these studies, the generation of female-specific neuronal populations has been considered to reflect, or be the direct flipside, of genetic mechanisms required for male-specific neuronal differentiation. In this chapter, we examine the cellular and genetic mechanisms that generate the female-specific Ilp7-motoneurons that innervate the oviduct.

Previously, we found that the loss of Ilp7-motoneurons in males is due to the absence of *tra*, and is *fruM* dependent. However, we showed in females that Ilp7-motoneuron generation is *tra*-dependent but that *fruF* is not necessary for their generation, and also that *fruM* hemizygozity is not sufficient for loss of Ilp7-motoneurons in females. Here, we find that nascent Ilp7-motoneurons undergo programmed cell death in the male. Further, we find that the generation of female-specific Ilp7-motoneurons in females is independent of the sex determination factors *dissatisfaction, hermaphrodite and intersex*. Rather, our genetic data strongly suggest that programmed death in males is driven by a non-canonical pro-apoptotic role for *fruM*. However, in contrast to *fruM* function in most male-specific roles, we find that its function in male-specific neuronal death exhibits haploinsufficiency, thus revealing an unexpected dosage effect that our evidence suggests is mediated through transcripts derived from the *fru-P2* promoter. This chapter provides evidence to show that the generation of female-specific neurons is not a mere flipside of the mechanisms understood to male-specific neurons; instead, it attests to the importance of studying female-specific neuronal generation in order to fully appreciate how sex-specific nervous systems are generated.

3.2 INTRODUCTION

Genetic studies of courtship behaviors of males in *Drosophila* have provided the basis of

our understanding of the mechanisms by which sex-specific neuronal circuits, that drive sex-specific behaviors, are generated developmentally (Villella and Hall, 2008; Yamamoto, 2007). Sexually dimorphic neuronal circuits can vary in the number of neurons in a specific population, the expression of certain genes in those neurons, and/or neuronal morphology or connectivity. These differences are largely believed to arise from sex-specific RNA splicing of the transcription factors, *fruitless* and *doublesex*, and their subsequent isoform-specific regulation of neuronal survival, morphology and gene expression (Dauwalder, 2011; Kimura, 2011; Yamamoto and Koganezawa, 2013). In females, Transformer (Tra) binds to Tra-specific binding sites at the 3' end of the sex-specific exon of *fru* RNA. When bound, Tra blocks male-specific splicing of this exon, resulting in the generation of female-specific transcripts that all share the same short open reading frame that terminates with a premature stop codon to prevent the translation of Fru proteins (Goodwin et al., 2000) (see **figure 1.5**). In males, the absence of Tra leads to default splicing within the sex-specific *fru* exon to generate male-specific transcripts that can be translated into the 3 FruM protein isoforms (Goodwin et al., 2000). Tra also sex-specifically splices *doublesex* RNA into transcripts that are translated into the sex-specific isoforms DsxF and DsxM. These proteins share an N-terminal DNA binding domain and a C-terminal dimerization domain; they differ however in their C-terminal domain where females contain approximately 120 aminoacids less than males (Yang et al., 2008b).

A common mechanism for generating differences between male and female brains is the female-specific programmed cell death of specific neuroblasts or postmitotic neurons in the female. This results in the generation of male-specific or male-expanded neuronal populations in males, and is regulated by the sex-specific isoforms of both Fru and Dsx (Birkholz et al., 2013; Kimura et al., 2008; Kimura et al., 2005; Sanders and Arbeitman, 2008). For example, the male-specific P1 neuronal cluster undergoes female-specific programmed cell death during early pupal stages, and the expression of FruM is postulated to have a protective role in the P1 male counterparts (Kimura et al., 2005). In the case of male-specific neuronal populations, such as the mAL neurons, and the TN1 region, they arise initially in both sexes, but expression of DsxF induces apoptosis in their post-mitotic female counterparts during metamorphosis (Kimura et al., 2008; Sanders and Arbeitman, 2008). It has also been shown that sex-specific isoforms of Dsx can have opposing roles in the programmed cell death of sex-specific neuroblast found in the abdominal region of the larval VNC. Expression of DsxF leads to apoptosis while DsxM is

necessary and sufficient to prevent cell death when expressed in dsx^{+} sex-specific neuroblasts (Birkholz et al., 2013).

Thus, regardless of the relative roles of DsxF/M and FruM, neurons are only spared in males (Kimura et al., 2005; Sanders and Arbeitman, 2008). As this model was formulated with the assumption that sex-specific circuits are only formed in males, it is not surprising that it fails to account for those instances in which a female-specific neuronal population might form. How might a female-specific neuronal subset emerge? How similar, or distinct, are the cellular mechanisms that generate male-specific neurons? Finally, do sex-specific isoforms of Dsx and Fru play similar roles, but in reverse, or are novel mechanisms invoked?

Female-specific neuronal populations have rarely been described and the mechanisms by which they form has not been addressed (Ferveur, 2010). However, females do have robust, stereotyped sex-specific behaviors that are presumably regulated by female-specific neurons and/or circuits, such as egg-laying and male receptivity. Recently, neuronal circuits associated with the control of female receptivity and post-mating rejection have been described. The neuronal components of these circuits express the sex determination factors *fruitless* and/or *doublesex,* although their role in the formation of these circuits is not known (Hasemeyer et al., 2009; Rezával et al., 2012; Yang et al., 2009). Very recently, neuronal populations that seem to be specific to, or expanded in, females have been described: the $TDC^{+}/Dsx^{+}/Fru^{-}$ population (Rezával et al., 2014) and the $Ilp7^{+}/Fru^{+}/Dsx^{-}$ neurons, described in Chapter 2 (Castellanos et al., 2013). These female-specific neuronal populations represent ideal models to study the mechanisms by which female-specific neurons arise.

Our previous work demonstrated that generation of female-specific Ilp7-neurons requires *transformer,* but the role of *fruitless* or *doublesex* in the female-specific emergence of these neurons was not supported by our genetic studies (Castellanos et al., 2013). This led us to examine possible roles for the lesser understood sex determination factors *hermaphrodite* (*her*), *intersex* (*ix*), and *dissatisfaction* (*dsf*), due to their reported roles in female differentiation. Expression of *her* and *ix* is independent of the sex determination cascade, but are known to act together with sex determination factors, such as *dsx*, to repress male differentiation and promote female differentiation (Chase and Baker, 1995; Li and Baker, 1998a; Pultz and Baker, 1995). Epistatic genetic studies suggest that *dsf* is *tra*-regulated but represents an independent branch from *fru* and *dsx*. *dsf* has been implicated in male and female behaviors and is known to be

required for the formation of motoneurons innervating the female uterus reproductive tract (innervation of the oviduct was not reported) and male abdominal muscles (Finley et al., 1998; Finley et al., 1997). However, studies addressing the female-specific mechanisms of *her*, *ix* and *dsf* function are sparse.

In this chapter, we show that *Ilp7*$^{+}$/*fru*$^{+}$/*dsx*$^{-}$ neurons are born in males, but undergo programmed cell death soon thereafter. We eliminate the potential of essential roles for *her*, *ix* and *dsf* in the formation of female-specific Ilp7-neurons. Instead, we demonstrate a non-canonical role for *fruitless*; 1) FruM acts as a pro-apoptotic factor in males that kills newly borm female-specific neurons, and 2) transcripts from the P1 and P2 promoters are required in a dose dependent manner for cell death to occur in male *Ilp7*$^{+}$/*Fru*$^{+}$/*Dsx*$^{-}$ neurons. Taken together, these data provide the first insight into the genetic and developmental mechanisms underlying the generation of female-specific neurons.

3.3 MATERIALS AND METHODS

3.3.1 Fly genetics

Flies were maintained on standard cornmeal food at 70% humidity at 22°C or 25°C. Strains from Bloomington *Drosophila* Stock Centre: *w; Df(2L)cl7,pr*^{1}*cn*^{1}*/Cyo* (referred to as *dsf*^{Df}). *pr*^{1}*cn*^{1} ***ix***^{1}*/SM5.Cy* (referred to as *ix*^{1}). *ab*^{2} ***ix***^{2} *bw*^{1} *sp*^{2}*/In(2L)Cy,Cy dplvl sp*^{2} (referred to as *ix*^{2}). *y*^{1}*; her3/Cyo.* *y*^{1}*w***; P(UAS-dsxM).* *y*^{1}*w***;;P(UAS-dsx F)*$^{24\text{-}3}$ *w;UAS-p35.* *w*^{1118} (control strain). Strains obtained as gifts: *w; dsf1/Cyo 5 (Finley et al., 1997), fruitless-P1-GAL4 (Stockinger et al., 2005)*, *dsx-GAL4* (Rideout et al., 2010), *pJFRC81 10XUAS-IVS-Syn21-GFP-p10* (referred to as *UAS-10x-GFP*) *(Pfeiffer et al., 2012), fruF*, *fruM*, *fru*$^{4\text{-}40}$ (*fru*^{Df}) (Demir and Dickson, 2005). *Ilp7-GAL4 (Castellanos et al., 2013).*

3.3.2 Immunohistochemistry

Primary antibodies: Rabbit anti-Ilp7 (Yang et al., 2008a)(1:1000, E.Hafen); Guinea Pig anti-Fork head (Weigel et al., 1989) (1:1000, H. Jäckle); Rat anti-Doublesex (Sanders and Arbeitman, 2008)(1:100, M. Arbeitman). Rabbit anti-5-HT (1:1000, S5545, Sigma). Tissue and immunohistochemistry were carried out as described in (Castellanos et al., 2013). Secondary antibodies: Donkey anti-Mouse, anti-Chicken, anti-Rabbit, anti-Guinea Pig, anti-Rat IgG (H+L) conjugated to DyLight 488, Cy3, Cy5 (1:100, Jackson ImmunoResearch). All images were

acquired on an Olympus FV1000 confocal microscope. Images were processed using Fluoview FV1000, FIJI and ImageJ, and Adobe Photoshop CS5.

3.4 RESULTS

3.4.1 Generation of Ilp7-neurons is transformer dependent, but independent of canonical sex determination mechanisms.

In the adult, posterior VNC Ilp7-neurons are comprised by an embryonic and post-embryonic subset of neurons. The post-embryonic population is marked by $Ilp7^{+}/fru^{+}/dsx^{-}$ and includes a female-specific and a common subset (**Fig. 3.1 A-A'**). The female-specific subset requires *tra* for their generation. Our previous studies (Chapter 2) demonstrated that generation of female-specific neurons requires *tra* but is apparently *fru* and *dsx* independent (Castellanos et al., 2013). A third regulatory branch downstream of *tra* acts via *dsf*, and is independent of *fru* and *dsx* (Finley et al., 1997), *dsf* is required for motoneuron innervation of the female uterus (Finley et al., 1998; O'Kane and Asztalos, 1999). Do female-specific Ilp7-motoneurons also require *dsf*? To test this, we examined the fate of Ilp7-neurons in null *dsf* mutants. Homozygous dsf^{1} and dsf^{1}/dsf^{Df} are viable and have no morphological intersex phenotypes; however, females are sterile(Finley et al., 1997). We found that both female-specific and common dorsal Ilp7-neurons were still present in dsf^{1}/dsf^{1} or dsf^{1}/dsf^{Df} females (**Fig. 3.1 B-C'**). This rules out an essential role for *dsf.*

3.4.2 Female-specific differentiation factors hermaphrodite and intersex are not required for generation of female-specific Ilp7-neurons.

Two additional factors that are reportedly essential for female differentiation are *intersex* and *hermaphrodite*. *intersex* (*ix*) is not known to be regulated by *tra* (Chase and Baker, 1995), but acts as a obligatory female-specific cofactor with *dsxF* to repress male somatic differentiation in females, as well as activate the female-specific expression of *yolk proteins* (*yp*) genes (Garrett-Engele et al., 2002). *hermaphrodite* (*her*) has both a maternal and a zygotic role in female differentiation.

The zygotic role of *her* regulates female differentiation in both a *dsx* dependent and independent manner (Li and Baker, 1998a, b). We tested *ix* and *her* mutants for female-specific

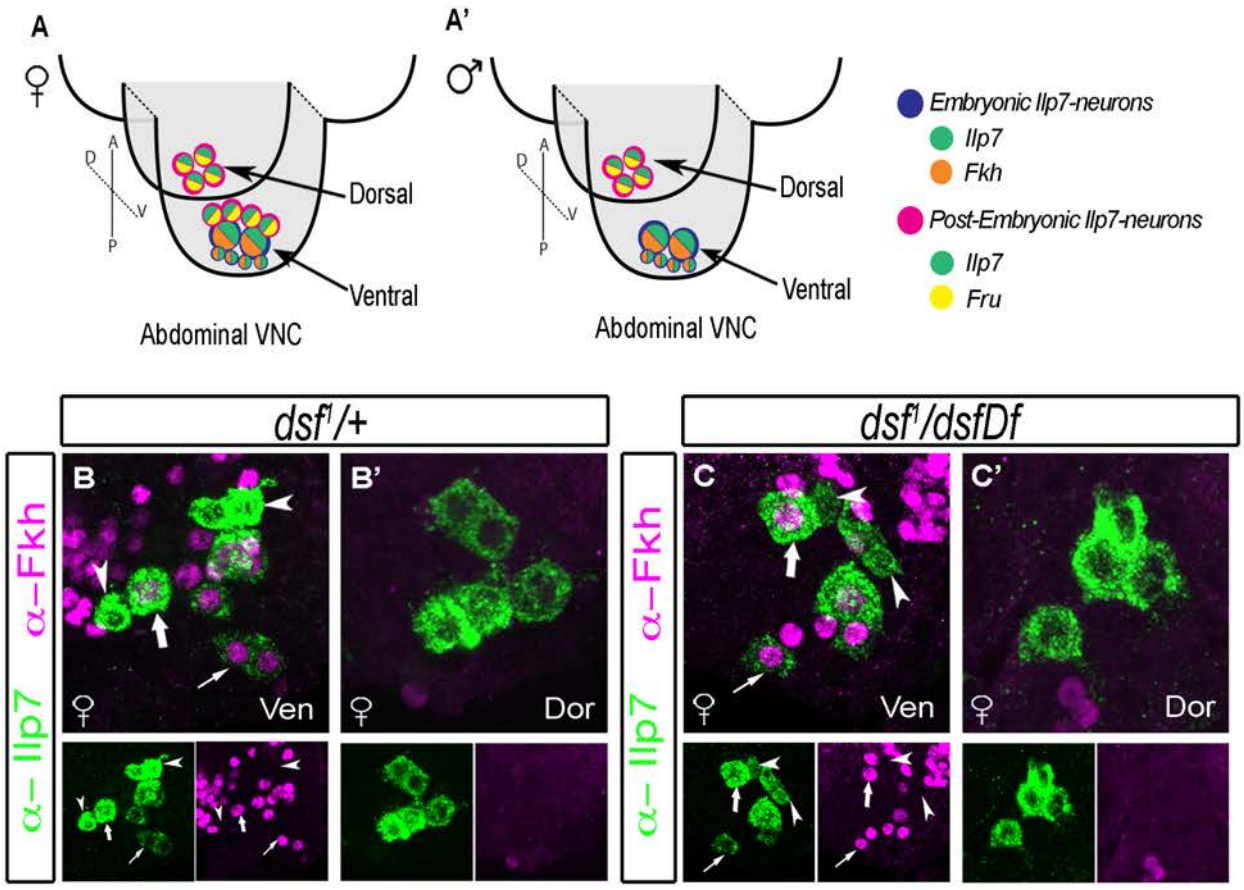

Figure 3.1. Generation of female-specific Ilp7-motoneurons is independent of the *dsf* sex determination branch.

(A-A') Cartoon representation of Ilp7-neuronal subsets in the abdominal VNC of adult females (**A**) and males (**A'**). Ilp7-neurons are comprised of: i) a ventral embryonic subset (blue) expressing Ilp7-peptide and the transcription factor *fkh* (used as a marker for this subset) that is common to both sexes. ii) a ventral and dorsal post-embryonic subset (pink) that express Ilp7-peptide and the sex determination factor *fru*. This subset includes the ventral female-specific Ilp7-motoneurons and a common dorsal Ilp7-neuronal subset (pink/green/yellow)
(B-C') In dsf^{1}/+ controls, female-specific Ilp7-motoneurons ($Ilp7^{+}/fkh^{-}$) are present in the ventral (ven) cluster (arrowheads) in close proximity to embryonic Ilp7-neurons (arrows; *fkh* positive) (B) as well as the common dorsal (dor) Ilp7-motoneurons (B'). In *dsf*-null females ($dsf^{1}/dsfDf$), female-specific Ilp7-motoneurons (arrowheads) and the common dorsal Ilp7-neurons are still generated (C-C'). Arrows and arrowheads indicate representative neurons of each Ilp7 subset.

Ilp7-neuron generation. Loss of function *ix* alleles: ix^1 and ix^2 have strong intersexual phenotypes that vary according to temperature (Chase and Baker, 1995). Intersexual phenotypes are also temperature dependent for *her* alleles, appearing stronger at lower temperatures in her^3, a strong hypomorphic *her* allele (Pultz et al., 1994). We verified phenotypes for *ix* and *her* allelic combinations used (ix^1/ ix^1 and ix^2/ ix^2 and her^3/her^3). We observed lethality for all genotypes at temperatures lower than 22°C. At room temperatures, females of all genotypes had intersexual phenotypes, including male-like pigmentation and genitalia with both male and female characteristics (**Fig. 3.2 A-B'**). Examination of female-specific and dorsal Ilp7-neruons in ix^- and her^- flies showed that these neurons were not affected (**Fig. 3.2 C-F'**), suggesting that neither *ix* nor *her* are essential for female-specific Ilp7-neuron generation.

Thus, using the field-standard genetic approaches, we find that *tra*, but not *dsx*, *fru*, *dsf*, *ix* or *her* are essential for female-specific Ilp7-neuron generation. To shed additional light on the mechanisms of female-specific Ilp7-neuron generation, we decided to determine the fate of female-specific Ilp7-neurons in males. We asked whether female-specific Ilp7-motoneurons undergo programmed cell death in males, or whether they are transfated?

3.4.3 Female-specific Ilp7-motoneurons undergo programmed cell death in males.

Cell death in males has not been implicated in the formation of a sexually dimorphic neuronal circuit. In order to investigate if Ilp7-neurons are present in males and undergo cell death, we over-expressed the cell death inhibitor *UAS-p35* (Hay et al., 1994) from *fruP1-GAL4,* which is expressed in all dimorphic Ilp7-neurons. $Ilp7^+/Fru^+/Fkh^-$ neurons are never observed in the ventral Ilp7-neuornal cluster in control males (Castellanos et al., 2013) However, *fruP1-GAL4,UAS-10xGFP/ UAS-p35* males had one to three additional $Ilp7^+/Fru^+/Fkh^-$ neurons in the Ilp7-neuronal ventral cluster (**Fig. 3.3 A-A'**). In females, *UAS-p35* overexpression did not increase the number of $Ilp7^+/Fru^+/Fkh^-$ neurons in the Ilp7-neuronal ventral cluster (**Fig. 3.3 B-B'**). To control for subtype-specificity, we also expressed *UAS-p35* using *dsx-GAL4,UAS-10xGFP*. This failed to generate $Ilp7^+/Fru^+/Fkh^-$ neurons in males (**Fig. 3.3 C-D'**). These data strongly suggest that female-specific Ilp7-motoneurons undergo cell death in males.

We postulate that this death occurs post-embryonically in post-mitotic neurons for two reasons; 1) *fruP1-GAL4* expression commences post-embryonically (Stockinger et al., 2005), and 2) Our

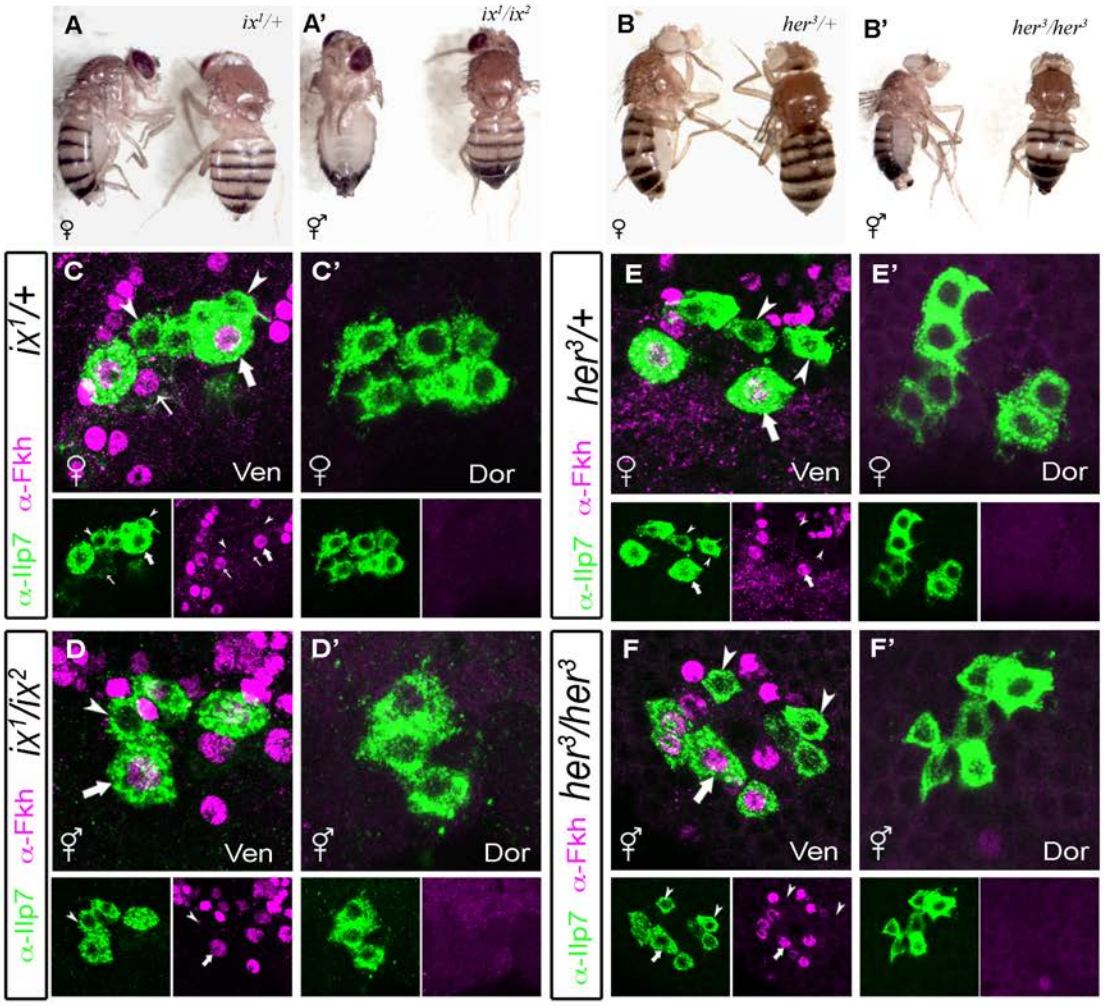

Figure 3.2. *her* and *ix* are not necessary for generating female-specific Ilp7-motoneurons.

(A-B') Intersexual morphology of the genitalia and analia, as well as male-like pigmentation patterns in the abdomen in loss of function alleles for *her* and *ix*. Females for the heteroallelic combination, ix^1/ix^2, exhibit intersexual phenotypes (⚥) not observed in heterozygotes for $ix^1/+$ (A-A'). Intersexual somatic differentiation is observed in females that are homozygote for her^3/her^3 alleles (⚥) but not $her^3/+$ (B-B').
(C-D') Genetic analysis of *ix* heteroallelic genotype. Post-embryonic Ilp7 susbets are generated in both female (♀) $ix^1/+$ (C-C') and intersexual (⚥) ix^1/ix^2 (D-D'). Female-specific Ilp7-motoneurons (arrowheads) are observed in the ventral cluster (ven) and the common Ilp7-neurons in the dorsal cluster (dor).
(E-F') Genetic analysis of *her* loss of function alleles. Female (♀) $her^3/+$ (E-E') and instersexual females (⚥) her^3/her^3 generate both female-specific Ilp7-motoneurons (arrowheads) in the ventral cluster (ven), and the common Ilp7-neurons in the dorsal cluster (dor). Arrows and arrowheads indicate representative neurons of each Ilp7 subset.

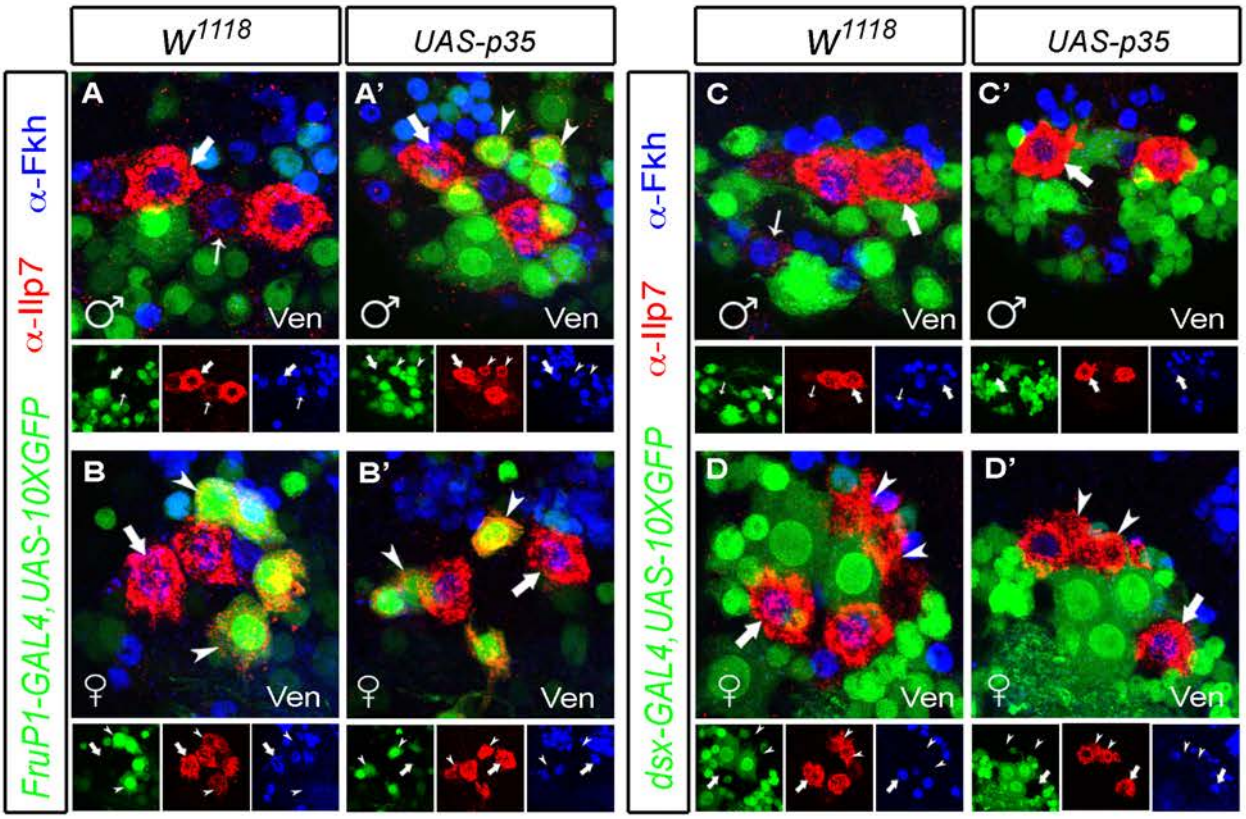

Figure 3.3. Male-specific cell death of female-specific Ilp7-motoneurons.

(A-B') Targeted expression of *UAS-p35* using *fru-P1-GAL4,UAS-10XGFP*. (A-A') Expression of *UAS-p35* in males (♂) results in survival of the female-specific-like *Ilp7*$^{+}$/*Fru*$^{+}$/*Fkh*$^{-}$ neurons (arrowheads) in the ventral cluster (ven); this additional subset is found in close proximity to embryonic Ilp7-neurons (arrows). (A) Female-specific-like *Ilp7*$^{+}$/*Fru*$^{+}$/*Fkh*$^{-}$ neuronal subsets are never observed in controls; control males only have embryonic Ilp7-neurons (arrows) in the ventral cluster (ven) (A'). (B-B') This increase in the number of *Ilp7*$^{+}$/*Fru*$^{+}$/*Fkh*$^{-}$ neurons was specific to males, as expression of *UAS-p35* in females (♀) did not result in additional female-specific Ilp7-motoneurons (B-B'). This increases our confidence that the extra Ilp7-neurons in females are truly the rescued female-specific ILp7-neurons, as opposed to another population of 'spared' neuorns that become Ilp7-expressing in both males and females.
(C-D') Targeted expression of *UAS-p35* using *dsx-GAL4,UAS-10XGFP*. Additional female-specific-like *Ilp7*$^{+}$/*Fru*$^{+}$/*Fkh*$^{-}$ neurons are never observed when *UAS-p35* is expressed with this alternative sex-determining transcription factor GAL4 driver. *UAS-p35*/*dsx-GAL4,UAS-10XGFP* males (♂) (C') and females (♀) (D') display an equal number of Ilp7-motoneurons as their controls (C, D). Arrows and arrowheads indicate representative neurons of each Ilp7 subset.

previous studies (**Fig. 2.8**) showed that post-mitotic expression of *UAS-tra* in male neurons (using *elav-GAL4*) led to the generation of female-specific Ilp7-neurons in males, thus the death of female-specific neurons in males does not occur during lineage progression.

3.4.4 The absence of dsx in the postembryonic Ilp7-neuron lineage is consequential in normal terminal differentiation, but not in lineage specification.

In certain dimorphic lineages, DsxF induces cell death of neuroblasts and neurons that give rise to male-specific neuronal populations, while in males DsxM can promote their survival (Birkholz et al., 2013; Kimura et al., 2008; Sanders and Arbeitman, 2008). The absence of *dsx* in postembryonic Ilp7-motoneurons led us to ask two related questions; first, does the absence of DsxF serve to spare them from cell death? Second, is their death in males due to the absence (and lack of anti-apoptotic function) of DsxM? To test these, we overexpressed *UAS-dsxF* in females and *UAS-dsxM* in males, from *FruP1-GAL4*. First, expression of *UAS-dsxF* in females (confirmed by antibody staining in (**Fig. S3.1 A-D'**) did not alter the number of female-specific Ilp7-motoneurons (**Fig. 3.4 A, B**); thus, the absence of DsxF is not a pro-survival mechanism. This also did not affect the dorsal cluster Ilp7-motoneurons (**Fig. 3.4 A', B'**). Second, overexpression of *UAS-dsxM* in male Ilp7-neurons (with *FruP1-GAL4*) did not generate extra $Ilp7^{+}/Fru^{+}/Fkh^{-}$ neurons in the ventral cluster (**Fig. 3.4 C,D**).

Dorsal Ilp7-neurons are present in males and females; in females they are motoneurons and in males they are serotonin expressing neuromodulatory neurons of the seminal vesicle (Billeter et al., 2006b; Castellanos et al., 2013; Lee and Hall, 2001). Interestingly, expression of *UAS-dsxM* in males appeared to eliminate Ilp7 expression (**Fig. 3.4 C'-D'**) and serotonin expression (**Fig. S3.1 E-E'**) in the dorsal Ilp7-neuron cluster. However, we believe that the dorsal Ilp7 neurons are in fact retained, but fail to differentiate normally. Our key evidence in support of this is that the normal number and position of fru^{+} neurons is retained in the dorsal region. While this does not formally rule out that the dorsal neurons are transfated or die, we conclude that the absence of *dsx* in post-embryonic Ilp7-neurons is important to allow normal differentiation of male post-embryonic Ilp7-neurons.

3.4.5 Genetic regulation of male-specific programmed cell death in dimorphic Ilp7-neurons.

Ilp7-motoneurons present the first case of a female-biased subset of neurons that are

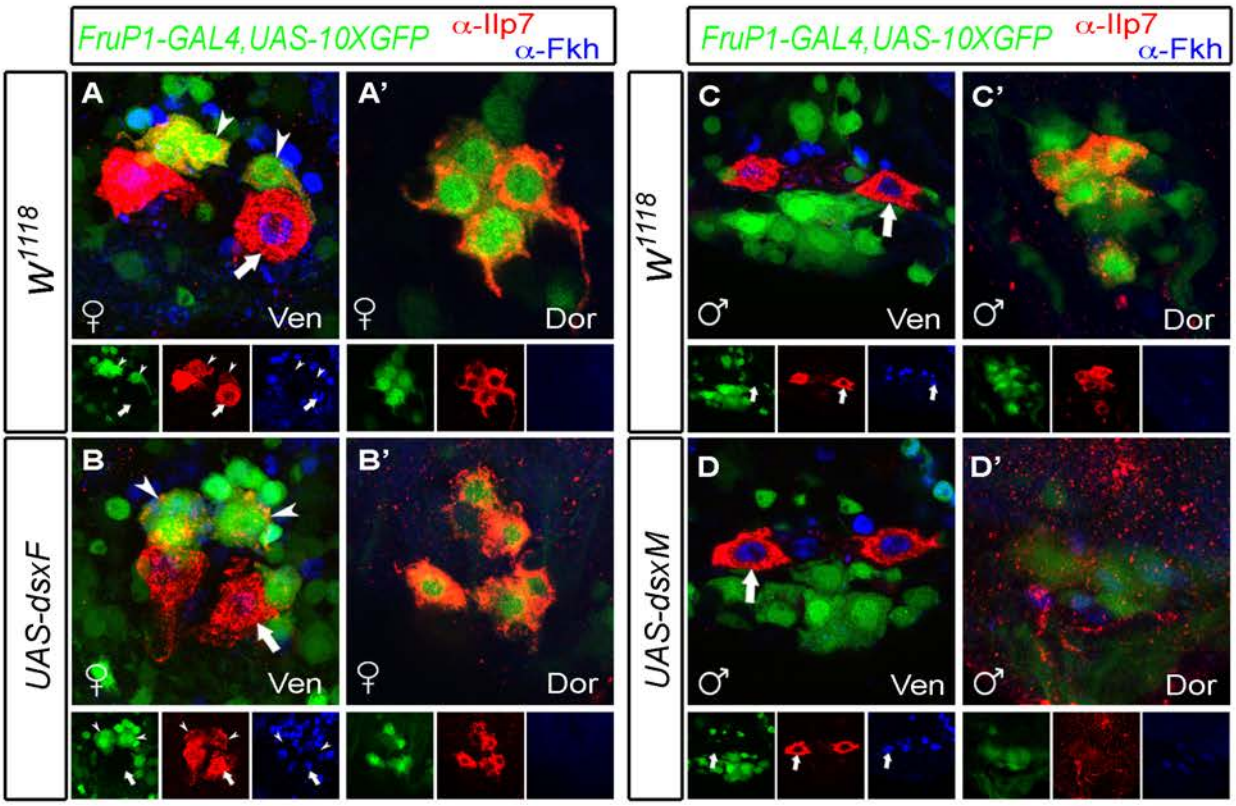

Figure 3.4. The absence of DsxF or DsxM in Ilp7-motoneurons has no role in the survival of female-specific Ilp7-motoneurons, but the absence of DsxM in males dorsal Ilp7-motoneurons is essential for their normal differentiation.

(A-B') *fru-P1-GAL4,UAS-10XGFP* was used to drive expression of *UAS-dsxF* in females (♀). Female-specific Ilp7-motoneurons (arrowheads) in the ventral cluster (ven) do not undergo DsxF induced cell death (B) and the dorsal cluster of Ilp7-neurons (dor) remains unaffected (B') and continue to express Ilp7, as do controls (A-A').
(C-D') Expression of *UAS-dsxM* with *fru-P1-GAL4,UAS-10XGFP* in males (♂). The ventral cluster (ven) in controls (C) and *UAS-dsxM*; *fru-P1-GAL4,UAS-10XGFP* (D) is comprised exclusively of embryonic Ilp7-neurons (arrow). Expression of *UAS-dsxM* does not prevent apoptosis of female-specific Ilp7-motoneurons in males (C,D), but dramatically reduces the expression of Ilp7-peptide in the dorsal Ilp7-neurons (dor) of *UAS-dsxM*; *fru-P1-GAL4,UAS-10XGFP* (D') compared to controls (C). Arrows and arrowheads indicate representative neurons of each Ilp7 subset.

eliminated in males (Castellanos et al., 2013). Studies of male-biased neuronal subsets that are eliminated in females show that FruM can act as a pro-survival factor (in addition to a similar role for dsxM) (Birkholz et al., 2013; Kimura et al., 2005). We previously determined that hemizygous *fruM* (*fruM*/*fruDf*) females do not eliminate female-specific Ilp7-neurons (**Fig.2.7**). This standard approach to determine a sufficient role for FruM led us to conclude that *fru* had no role in the generation of female-specific Ilp7-neurons. However, by testing all available constitutive *fru* allelic combinations, we unexpectedly observed that in *fruM*/*fruDf* males, there was a failure to kill female-specific Ilp7-motoneurons. Thus, hemizygous *fruM* is not sufficient to kill female-specific Ilp7-neurons, even though they are genetic males with regards to *fru* (**Fig. 3.5 A-A'**).

These data led us to re-evaluate the standard genetic methods of using hemizygotes to test *fruM* and *fruF* function (Demir and Dickson, 2005). As we reported, female-specific Ilp7-neurons survive in *fruF*/*fruDf* males (Castellanos et al., 2013). As predicted, we now find that female-specific Ilp7-neurons are generated in *fruF*/*fruDf* and in *fruF*/*fruF* males and females (**Fig. 3.5 B-C'**). Also, as predicted, female-specific Ilp7-neurons are present in *fruC*/*fruDf* females (**Fig. 3.5 D'**); *fruC* is a control allele for *fruM* and *fruF* that splices normally in each sex. Unexpectedly, however, *fruC*/*fruDf* males generate female-specific Ilp7-motoneurons, as do *fruM*/*fruDf* males (**Fig. 3.5 A,D**). Further, wildtype *fru* hemizygotes (*fruDf*/+) also retained female-specific Ilp7-neurons (**Fig. 3.5 E-E'**). Indeed, regardless of the alleleic combination, the presence of *fruDf* led to survival of female-specific Ilp7-motoneurons in males (data is summarized in table 3S1). Of all allelic combinations tested, the only instance where we observed cell death of female-specific Ilp7-neurons in both males and females was in *fruM*/*fruF* (**Fig. 3.5 F-F'**).

We propose that an essential pro-apoptotic factor is absent in *fruDf* that is present in *fruM*, *fruF* and *fruC*. The *fruDf* deficiency removes a region spanning the P1 and P2 promoters, leaving the P3 and P4 promoters intact. We therefore postulate that the pro-apoptotic role of FruM requires two intact copies of the *fru-P2* promoter in order to kill female-specific Ilp7-neurons in males. These results are the first indication that male-specific cell death occurs in sexually dimorphic circuits, that requires a novel essential function for a genetic element derived from a region spanning the *fru-P2* promoter region.

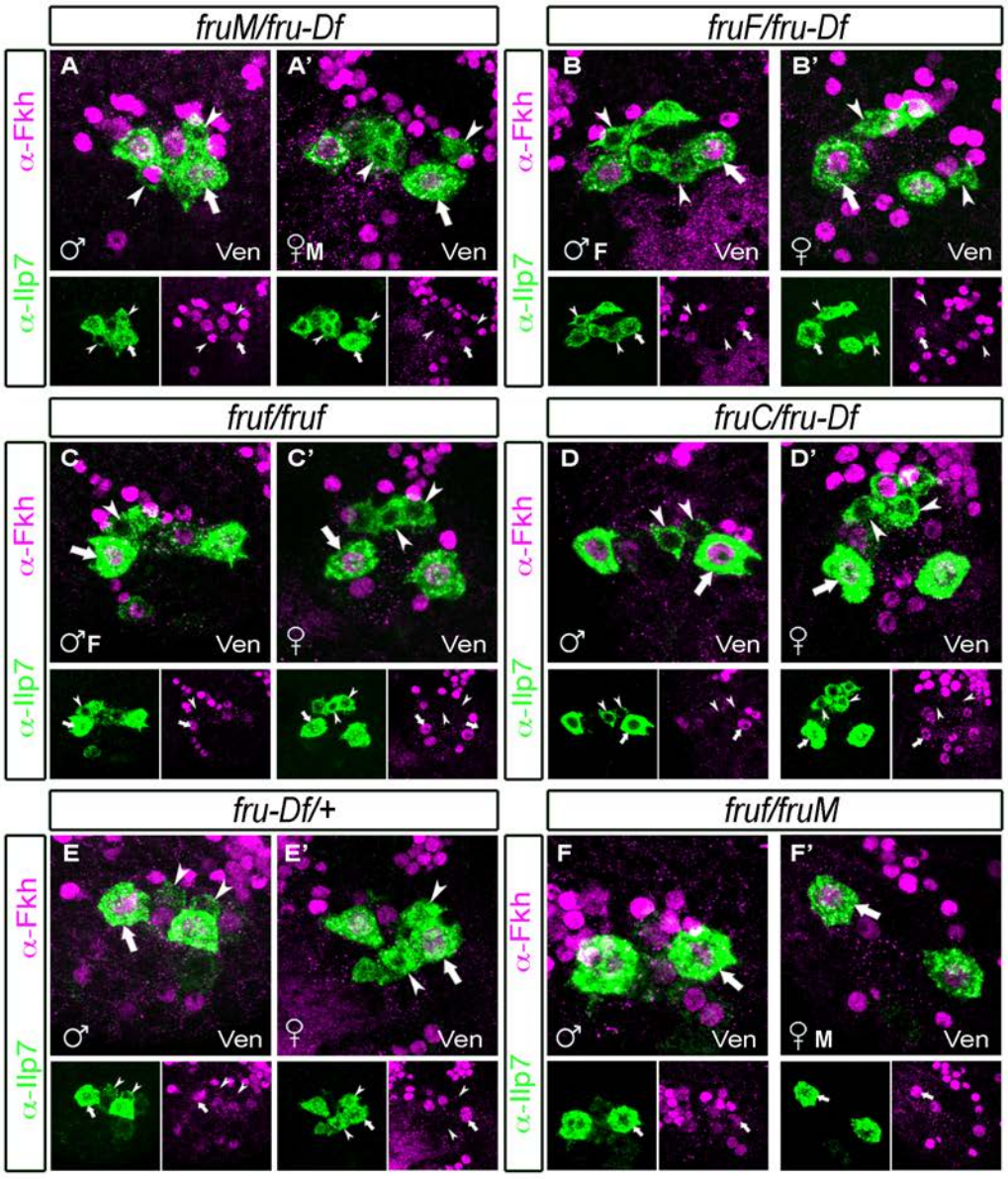

Figure 3.5. Survival of female-specific Ilp7-motoneurons is observed in every allelic combination that includes the *fru*$^{4\text{-}40}$ deficiency.

(A-F) Constitutive *fruM* and *fruF* allelic combinations tested for survival of Ilp7-motoneurons in males and females. **(A-A')** *Ilp7*$^{+}$/*Fkh*$^{-}$ female-specific Ilp7-motoneurons (arrowheads) are observed in masculinized females (♀M) (A') and genetic *fruM/fruDf* males ♂ (A) in close proximity to embryonic Ilp7-neurons (arrows). **(B-C')** Additional *Ilp7*$^{+}$/*Fkh*$^{-}$ neurons (arrowheads) are observed in the ventral cluster of *fruf/fruDf* (B) and *fruf/fruf* (C) feminized males (♂F). Female-specific Ilp7-motoneurons are present in *fruf/fru-Df* (B') and *fruf/fruf* (C') females ♀, as predicted. **(D-E')** In the presence of the *fru*$^{4\text{-}40}$ deficiency, *Ilp7*$^{+}$/*Fkh*$^{-}$ female-specific Ilp7-motoneurons (arrowheads) survive in genetic males ♂ , and females ♀ , that are hemizygous for the *fruC* allele (D-D') and a wild-type allele (E-E'). Both genotypes are controls for the constitutive alleles used in this study.

3.5 DISCUSSION

3.5.1 The sex determination cascade in female-specific Ilp7-motoneuron generation.

Mutant analysis of *fruitless* and *doublesex* show them to be necessary and sufficient for the generation of male-specific neuronal populations in males (Billeter et al., 2006a; Dauwalder, 2011; Villella and Hall, 2008). In this study, we address the genetic mechanisms required for generating female-specific neurons in the *Drosophila* central nervous system. We had previously identified the first example of a female-specific neuronal population in the CNS, which is functionally required for a female-specific behavior, the Ilp7-motoneurons. We showed that their generation in females is *tra*-dependent. However, their generation is independent of *dsx* (not expressed in these neurons), and is not fully dependent upon sex-specific *fru* isoforms (Castellanos et al., 2013).

Here, we rigorously test a role for all major transcriptional regulators of sexual dimorphism. Mutant analysis of *her*, *dsf*, and *ix* show that these factors are not individually essential for generating female-specific Ilp7-neurons; however, we do not exclude a redundant function, which could be resolved by double mutant analysis. In females, neuroblast or neuronal cell death can be induced by DsxF in females and antagonized by dsxM in males (Birkholz et al., 2013; Sanders and Arbeitman, 2008). Thus, its absence in the Ilp7-motoneuron lineage may spare these neurons from death in females, or spare them from survival in males (Castellanos et al., 2013). However, by overexpressing *UAS-dsxF* or *UAS-dsxM* in females and males respectively, we found that the presence of *dsx* does not affect neuronal survival, but does appear to block normal differentiation of Ilp7 neurons in the common dorsal population in males. The activity of *dsx* in females requires its heterodimer partner *ix* (Chase and Baker, 1995; Garrett-Engele et al., 2002). Therefore, it was not unexpected that *ix* mutants exhibit no loss of female-specific Ilp7-neurons. Regardless, the failure of *UAS-dsxF* to kill female-specific Ilp7-neurons in females may reflect a lack of *ix* expression in Ilp7-neurons. This could be tested by co-expression of *UAS-dsxF* and *UAS-ix* in females.

3.5.2 fruitless and male-specific cell death of female-specific Ilp7-neurons.

A prominent mechanism in *Drosophila* for generating super-numerous neurons in males is female-specific apoptosis of neuroblasts or postmitotic neurons that are developmentally

equivalent in both sexes. Such dimorphic neuroblast/neuronal death has only been observed in females (Kimura, 2011). Our studies now provide evidence that nascent female-specific Ilp7-neurons undergo apoptosis in males, providing the first evidence for male-specific neuronal death in *Drosophila*. We show that *Ilp7*$^{+}$/*fru*$^{+}$/*fkh*$^{-}$ neurons are generated in the ventral Ilp7 cluster of *fruP1-GAL4,UAS-10xGFP/UAS-p35* males. In *Drosophila*, the expression of *UAS-p35,* a potent baculovirus caspase inhibitor, is a widely-used method to prevent apoptosis, and the expansion of cellular populations after *UAS-p35* is widely considered to be evidence for the normal loss of those cells through apoptosis (Hay et al., 1994). Regardless, we plan to verify these data using allelic combinations for the cell death effectors *hid*, *reaper* and *grim* that are viable during early pupariation. Trans-heterozygous alleles and deficiencies for combinations of these effectors are viable to pupal stages (Choi et al., 2006; Kimura et al., 2005; Lee et al., 2013; Sanders and Arbeitman, 2008) and evidence for Ilp7-motoneuron survival in males, in these mutant backgrounds, would provide direct evidence for the death of these neurons in males.

What mechanisms could underlie male-specific death? FruM has a pro-survival role in mAL neurons in the male *Drosophila* CNS, but a pro-apoptotic role has not been previously proposed (Kimura et al., 2005). Our previous data suggested that FruM is necessary for the death of female-specific Ilp7-motoneurons in males. However, *fruM* hemizygous females do not lose these neurons, thus FruM is not sufficient for death of these neurons in females. Therefore, it was unclear whether FruM may act as a conditional pro-apoptotic factor in nascent Ilp7-motoneurons, and what those conditions may be. Is FruM a pro-apoptotic factor in male-specific neuronal death, and what additional factors may shape this function? Our data herein indicate that *ix*, *her* and *dsf* do not play a deterministic role in FruM-dependent death. However, we have yet to test the effect of constitutive *fruM* (*fruM/fru*$^{4\text{-}40}$) in female mutant for *ix*, *her* or *dsf*. With regards to *fru* itself, *fru* has a complex genomic locus that produces transcripts from at least four promoters (P1 through P4). All promoters produce putative transcription factors with a common BTB N-terminal dimerization domain, and alternatively spliced DNA-binding zinc-finger domains (Dalton et al., 2013). *fru* transcripts can encode sex-specific and non-sex specific proteins, and exhibit differences in their spatial and temporal expression, as well as distinct functions during development (Lee et al., 2000; Ryner et al., 1996). Transcripts from the P1 promoter are best known as those that undergo sex-specific splicing to produce FruM protein in males and no Fru protein in females (Yamamoto, 2007).

To better understand the role of *fru* in male-specific cell death, we performed a more rigorous genetic analysis of the *fru* locus in female-specific Ilp7-motoneuron generation. As described previously, *fruM*/*fru*$^{4\text{-}40}$ females did not lose female-specific Ilp7-neurons, and thus we considered *fruM* not to be sufficient (Castellanos et al., 2013). Here, we detail an unexpected result showing that *fruM*/*fru*$^{4\text{-}40}$ males also do not kill female-specific Ilp7-neurons. Indeed, any males with a genotype that incorporates *fru*$^{4\text{-}40}$, but is otherwise *fruM* (derived form the constitutive FruM allele, from the control allele FruC, or from a wild type chromosome) failed to kill female-specific Ilp7-neurons. This is particularly curious because a *fruM/fruF* genotype in males, and importantly also females, kill female-specific Ilp7-neurons. Thus, we conclude that *fruM* is required for cell death and this is unaffected by *fruF* (in either sex). We further conclude that inclusion of *fru*$^{4\text{-}40}$ in any genotype is a potent blocker of death. From these data, we propose that the critical missing pro-apoptotic death factor is the *fru*-P2 transcript. All alleles except *fru*$^{4\text{-}40}$ support death in the presence of *fruM* and have the *fru-P2* promoter. Transcripts from the P2-P4 promoters of the *fru* locus encode putative non-sex specific proteins, FruCOMs. The P3 (and possibly P4) promoter produces transcripts that are expressed during embryonic stages and are required for viability. The P2 promoter transcripts are expressed during pupal stages of both sexes and are possibly required for male sexual behavior (Anand et al., 2001). However the relevance of any of these transcripts of sexual dimorphism remains elusive. Polypeptides (FruCOM) encoded from the P2 promoter are similar to FruM, but lack 101 amino acids containing a histidine-rich region specific to FruM proteins (Ryner et al., 1996). Expression of Fru proteins that lack this histidine rich region have been reported to induce muscle of Lawrence formation in *fru* mutant males, as well as in females (Usui-Aoki et al., 2000). Manoli et al. generated a GAL4 insertion into the *fru* locus downstream of the P1 promoter but upstream of P2-P4. *fru-P1-GAL4* males lacked FruM immunoreactivity, but were FruCOM immunoreactive; and showed only a small reduction in male courtship of females (Ferri et al., 2008; Manoli et al., 2005). These data suggests that expression of FruCOM proteins are able to confer a degree of sex-specificity to the neurons in which they are expressed. Here, we further suggest that they play a role in killing female-specific neurons in the male nervous system.

3.5.3 Establishing female-biased sexual dimorphism in the female brain

To date, a wealth of data has described the genetic mechanisms by which sexual

dimorphism is established in the male *Drosophila* nervous system to control male courtship behaviors (Billeter et al., 2006a; Dauwalder, 2011; Kimura, 2011; Villella and Hall, 2008; Yamamoto and Koganezawa, 2013). Comparatively much less has been done to determine how sexual dimorphism is established in the female *Drosophila* nervous system (Ferveur, 2010). Now, numerous neuronal populations have been found to be associated with female-specific behaviors, such as the octopaminergic neurons regulating egg-laying (Monastirioti, 2003; Monastirioti et al., 1995), and the ppk-SPR ($ppk^{+}/fru^{+}/dsx^{+)}$ neurons for receptivity (Hasemeyer et al., 2009; Rezával et al., 2012; Yang et al., 2009). Yet other subsets appear unique to females; the Ilp7-neurons (Castellanos et al., 2013) and TDC^{+}/Dsx^{+} neurons (Rezával et al., 2014).

These neuronal populations now provide models to study the dimorphic development and function of female-biased neuronal populations, following the same approaches as were used to define the development and function of dimorphic male neuronal populations. Our studies have shown that such studies will unveil unexpected mechanisms for generating the dimorphic nervous system. While we showed that *fruM* is essential but not sufficient for killing female-specific neurons in males, the TDC^{+}/Dsx^{+} female-specific neurons do not express *fruitless* (Rezával et al., 2014); therefore, analysis of their female-specific generation may reveal additional novel mechanisms. Establishment of sexual dimorphism in the female *Drosophila* nervous system is an emerging field that will provide a better understanding of the genetic mechanism underlying neuronal sexual dimorphism. Our analysis has shown that we cannot fully understand how neuronal dimorphism emerges by studying the development of the female nervous system from the bias of how it differs from male-specific circuits and behaviors. We now propose that the female nervous system does not develop as a default 'not-male' nervous system. With the characterization of female-specific neuronal populations, and an initial characterization of the underlying genetic pathways, we can now begin to fully study the dimorphic nervous system from the stance of both sides of the story.

4 HOMEOTIC FUNCTION OF DROSOPHILA BITHORAX-COMPLEX MIRNAS MEDIATES FERTILITY BY RESTRICTING MULTIPLE HOX GENES AND TALE COFACTORS IN THE CENTRAL NERVOUS SYSTEM.

4.1 SYNOPSIS

The *Drosophila* Bithorax-Complex (BX-C) Hox cluster contains a bidirectionally-transcribed miRNA locus, and a deletion mutant (Δ*mir*) lays no eggs and is completely sterile. We show these miRNAs are expressed and active in distinct spatial registers along the anterior-posterior axis in the central nervous system. Δ*mir* larvae derepress a network of direct homeobox gene targets in the posterior ventral nerve cord (VNC), including BX-C genes and their TALE cofactors. These are phenotypically critical targets, since sterility of Δ*mir* mutants was substantially rescued by heterozygosity of these genes. The posterior VNC contains Ilp7+ oviduct motoneurons, whose innervation and morphology are defective in Δ*mir* females, and substantially rescued by heterozygosity of Δ*mir* targets, especially within the BX-C. Collectively, we reveal (1) critical roles for Hox miRNAs that determine segment-specific expression of homeotic genes, which are not masked by transcriptional regulation, and (2) that BX-C miRNAs are essential for neural patterning and reproductive behavior.

4.2 INTRODUCTION

Hox genes encode homeodomain proteins that confer positional identities along the antero-posterior axis of bilaterians. These sequence-specific DNA-binding proteins activate or repress particular cohorts of transcriptional targets, in concert with homeodomain cofactors of the TALE (three amino acid loop extension) class, to endow unique characteristics to different organs (Mann et al., 2009; Pearson et al., 2005). Hox genes are best-studied in *Drosophila* and mice, but principles regarding their regulation and function in these model organisms have generally proven broadly conserved.

Hox genes are almost always included in gene complexes, and the *Drosophila* cluster is split into the Antennapedia complex (ANT-C) and the Bithorax complex (BX-C). The latter contains three homeobox genes, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) (Lewis, 1978; Sánchez-Herrero et al., 1985), which specify the posterior thoracic,

abdominal and genital segments. In both *Drosophila* and mouse, the genomic position of genes within the complex correlates with their relative expression domain along the antero-posterior axis, a phenomenon known as colinearity (Lewis, 1978). Thus, in the BX-C, *Ubx*, *abd-A* and *Abd-B* are expressed in progressively more posterior domains that correlate with their 5'→3' order along the genome.

Although Hox genes are deployed in specific spatial domains, their expression overlaps in some settings. However, two mechanisms preserve the major impact of a single Hox gene in a specific region: transcriptional down-regulation (Hafen et al., 1984; Struhl and White, 1985) and phenotypic suppression (Duboule and Morata, 1994; Gonzalez-Reyes and Morata, 1990). Transcriptional down-regulation involves repression of one Hox gene by another Hox gene expressed more posteriorly. Although observed in different tissues, the phenomenon has been most thoroughly studied in the *Drosophila* embryo. Thus, *Ubx* is transcribed in the embryonic ventral cord at high levels in the first abdominal segment and shows reduced expression in more posterior segments. However, when the two more posteriorly-expressed genes of the BX-C (*abd-A* and *Abd-B*) are mutated, *Ubx* is strongly expressed all along the ventral cord (Struhl and White, 1985).

The second mechanism, phenotypic suppression, allows a posteriorly-expressed Hox protein to dictate its own pattern of development even in the presence of more anteriorly-expressed Hox proteins. For instance, forced expression of high levels of *Ubx* in the posterior segments of the embryo do not compromise the formation of structures that are determined by the most posteriorly-expressed gene, *Abd-B* (Gonzalez-Reyes and Morata, 1990). Although observed in several Hox genes, there are some cases where this hierarchy is mildly broken (Heuer and Kaufman, 1992; Lamka et al., 1992). The molecular basis of phenotypic suppression seems to rely, at least for some genes, on the competition of different Hox proteins to bind DNA from common targets with the aid of TALE cofactors (Noro et al., 2011). Phenotypic suppression is also present within the mouse Hox genes, where it is known as posterior prevalence (Duboule and Morata, 1994), although the term posterior prevalence may also include transcriptional down-regulation in vertebrates.

The discovery of miRNAs within Hox complexes adds further complexity. Within the BX-C, two miRNA hairpins are generated from sense and antisense transcription across the same locus: *mir-iab-4* and *mir-iab-8* (Bender, 2008; Ronshaugen et al., 2005; Stark et al., 2008; Tyler

et al., 2008) (Garaulet et al., 2014). A precise deletion of *mir-iab-4*/*mir-iab-8*, which eliminates all BX-C miRNAs, only slightly alters Ubx expression and does not impact Abd-A or Abd-B in embryos (Bender, 2008; Lemons et al., 2012). Therefore, although miRNAs are inferred to contribute to posterior prevalence in *Drosophila* and vertebrates (Ronshaugen et al., 2005; Yekta et al., 2008), functional evidence for this is scant. Nevertheless, flies homozygous for the *mir-iab-4*/*8* deletion are completely sterile (Bender, 2008), indicating critical functions for these miRNAs. This is particularly notable given that most miRNA mutants exhibit mild or no detectable phenotypes (Miska et al., 2007; Smibert and Lai, 2008). Although *mir-iab-4* and *mir-iab-8* are encoded by the same DNA, strand-specific mutant conditions achieved by placing BX-C breakpoint alleles *in trans* to the miRNA deletion revealed that *mir-iab-8* is primarily required for fertility (Bender, 2008). However, the mechanisms that underlie this sterility have not been elucidated.

In this study, we provide evidence for specific, colinear expression of *mir-iab-4* and *mir-iab-8* within the posterior larval ventral nerve cord (VNC). Elimination of *mir-iab-8* derepresses its known targets *Ubx* and *abd-A* in the posterior VNC, and we assign both Hox TALE cofactors Extradenticle and Homothorax as new functional targets of the BX-C miRNAs. In contrast to the demonstrated essential role of *abd-A* and *Abd-B* in the embryo to down-regulate the expression of anterior Hox genes in the epidermis, we find they lack a substantial role in the larval VNC. Instead, the BX-C miRNAs execute a critical role in the Hox regulatory hierarchy in this setting, since multiple Hox proteins and their cofactors accumulate inappropriately in the posterior VNC regions of BX-C miRNA mutant larvae. These changes in the spatial accumulation of BX-C miRNA targets are responsible for female sterility, since mild reduction in the expression of these genes substantially rescues fertility. Finally, we identify innervation and bouton defects in miRNA-mutant posterior VNC neurons that project to the oviduct. Altogether, we identify essential roles of miRNAs to restrict the spatial domains of Hox genes and cofactors, which determines normal CNS patterning and reproductive capacity.

4.3 MATERIALS AND METHODS

4.3.1 Drosophila genetics

The deletion of the BX-C miRNA locus (Δ*mir*) was described (Bender, 2008). We

generated new sensor transgenes by amplifying 3' UTR segments of *exd* and *hth-RA* and inserting into *tub-GFP*, followed by P-element transgenesis using Δ2-3 helper transposase. A detailed description of other published mutants, transgenes, and stocks for generating clones, and primary antibodies used for immunostaining experiments are found in (Garaulet et al., 2014).

4.3.2 Fertility assays

Newly eclosed females were mated individually with three Canton-S males in a single vial for three days at 25 ºC, and then checked for the number and viability of eggs. Flies laying viable eggs were considered fully fertile. Those flies unable to lay any egg were considered sterile and egg-laying defective. Flies that produced at least 3 inviable eggs were considered sterile, but capable of egg-laying.

4.3.3 Immunostaining and image analysis

Ventral nerve cords and oviducts from larvae or mated females were dissected in cold 1XPBS and fixed in 4% PFA with 0.1% Triton. Images showing target derepression in third instar larvae VNCs correspond to Z-projections of the ventral half of nerve cords, except those images used in clonal analysis or when specified. To analyze oviduct innervation, we imaged a 210μm span of the oviduct posterior of the point of lateral oviduct fusion to the common oviduct. Total neuronal arbor length within this region, expressing *Ilp7-GAL4,UAS-mCD8::GFP* or *TDC2-GAL4,UAS-mCD8::GFP,* were quantified using the Simple Neurite Tracer plugin of FIJI (Longair et al., 2011). In this software, we 3-D rendered oviduct innervation and report the total length of all arbors in contact with the oviduct. Dlg bouton number: Total number of Dlg-positive boutons was counted in a 200μm span of the common oviduct using mouse anti-Dlg (1:50). A maximum-intensity Z-stack projection was generated in FIJI (ImageJ) and selections were created for each individual bouton in this image. Total number of boutons was quantified using the Analyze Particles plugin of FIJI; boutons were defined as any selection that measured between 2 to infinity mm with a circularity of 0 to 1.

4.3.4 Statistical analysis

Image data were subjected to D'Agostino and Pearson as well as a Shapiro-Wilk

Normality tests. Normally distributed data sets were compared using a parametric unpaired t-test and non-normally distributed groups were compared using a non-parametric Mann Whitney test. One-way ANOVA and Tukey's tests were conducted for multiple comparisons. All statistical analysis and graph data were performed using GraphPad Prism 5. Data were presented as mean±SD.

4.4 RESULTS

4.4.1 Derepression of BX-C miRNA targets causes female sterility in Δmir mutants

Welcome Bender generated a 45nt precise deletion of the region containing the BX-C miRNAs *mir-iab-4* and *mir-iab-8*. Homozygous flies for the *mir-iab-4/8* deletion are adult viable but sterile, indicating a requirement of these miRNAs for fertility (Bender, 2008) (**Fig. 4.1 A**).

To better understand the mechanisms underlying the sterility observed in *mir-iab-4/*8 mutants (termed Δ*mir),* Garaulet et al. (2014) characterized the expression of BX-C miRNAs *mir-iab4* and *mir-iab8*, in the larval VNC. Similar to the homeobox genes of Hox clusters, BX-C miRNAs are restricted to specific domains along the anterior-posterior axis and follow the colinearity rule. The promoter of *mir-iab-4* is located proximally to that of *mir-iab-8* and therefore transcripts of *mir-iab-4* are expressed more anteriorly than those of *mir-iab-8* in the larval VNC.

To provide a foundation for understanding BX-C miRNA function Garaulet et al. (2014) examined the regulatory relationships between *mir-iab-4* and *mir-iab-8* and their targets *Ubx, Abd-A,* as well as the TALE co-factors *hth* and *exd*. Garaulet et al. (2014) tested the capacity of these BX-C miRNAs to repress 3'UTR sensors of these targets in the imaginal disc of third instar larvae. Our collaborators observed a week repression of *hth, Ubx,* and *Abd-A,* but a strong repression of *exd* by *mir-iab-4,* while *mir-iab-8*, strongly repressed all targets mentioned above. Given this strong relationship of BX-C miRNAs and their targets, our collaborators determined if these targets where affected in Δ*mir* mutants. They found a strong derepression of *Ubx* within the *mir-iab-4* domain in the larval VNC, as well as, an expansion of *Ubx* and *Abd-A* expression into the most posterior segments of the VNC, overlapping the expression domain of *mir-iab-8.* The strongest phenotype our collaborators observed was the derepression of *hth*; *hth* is normally absent from the abdominal segments of the VNC, but in Δ*mir hth* expression invades throughout

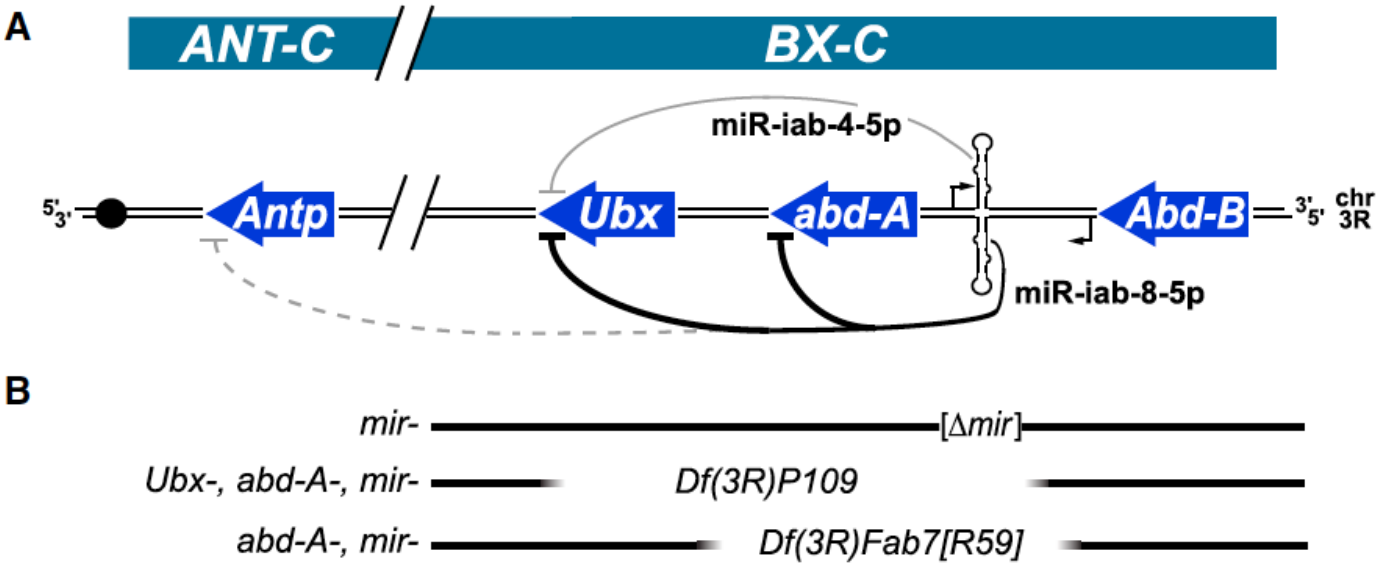

Figure 4.1. Organization of Bithorax-Complex miRNAs.

(A) Organization of Hox protein-coding and miRNA in the Bithorax-Complex (BX-C) and the Antennapedia-Complex (ANT-C). Both miRNAs have conserved seed matches to the 3'UTRs of genomically anterior Hox genes; the relative thickness of the repression lines reflects their targeting capacity. (B) BX-C aberrations used in this thesis, Δ*mir* is 45nt deletion of the *mir-iab-4/8* hairpin (Bender, 2008). *Df(3R)P109* deletes *Ubx, Abd-A* and *mir-iab-4/8. Df(3R)Fab7[R59]* deletes only *Abd-A* and *mir-iab-4/8.* The breakpoints of *Df(3R)P109* and *Df(3R)Fab7[R59]* have not been defined molecularly.

the VNC overlapping both *mir-iab-4* and *mir-iab-8* domains.

In order to determine if the derepression of BX-C miRNA targets observed was casual to Δ*mir* sterility our collaborators determined the sterility of BX-C aberrations that remove the BX-C miRNAs and their genomically adjacent target (**Fig. 4.1 B**). Females where *Ubx* and *Abd-A* is reduced and *mir-iab-4/8* are not present, exhibited demonstrable fertility and rescue of egg-laying compared to the sterility observed in Δ*mir* mutants alone. To distinguish the relative contribution of *Ubx* and *Abd-A* to Δ*mir* sterility, our collaborators determined the fertility of females lacking *Abd-A* and *mir-iab-4/8,* but retaining *Ubx*. *Df(3R)Fab7[R59]/* Δ*mir* females were completely sterile and failed to lay eggs, indicating that heterozygosity of *Abd-A* alone was not sufficient to rescue Δ*mir.* Our collaborators also tested the ability of *hth* to rescue the sterility observed in Δ*mir* by analyzing the strong loss of function allele *hth[P2]*. Heterozygosity for *hth[P2]* produced a strong fertility rescue, and double heterozygosity of the BX-C and *hth* exhibited the most robust rescue of fertility observed.

These data demonstrate that deletion of BX-C miRNA causes failure of the normal transcriptional repression hierarchy, as well as posterior prevalence that leads to the sterility observed in Δ*mir.*

4.4.2 Evidence for a neural basis for Δ*mir sterility*

The misexpression of BX-C miRNA targets was manifest in the VNC, but this did not rule out a spatially broader contribution of target deregulation. We therefore sought a neural basis for the sterility phenotype. We first asked if neural knockdown of any target genes could rescue Δ*mir*. We combined RNAi transgenes against *abd-A*, *hth*, and *exd* with the pan-neuronal driver *elav-Gal4* into the Δ*mir* background. Unfortunately, all of these genotypes were inviable, precluding assessments of female fertility. We attempted to restrict RNAi transgene expression by using *tub-Gal80^{ts}* to temporally restrict *elav-Gal4* driven *UAS-dsRNAi* constructs in the Δ*mir* background. We shifted flies from restrictive (17ºC) to permissive (29ºC) temperature at different timepoints, but all regimens tested were lethal except for shifts to 29ºC in late pupal stages, for which all genotypes maintained sterility. These tests did not distinguish if the knockdowns were insufficient, or involved an earlier phenocritical period. However, Δ*mir* animals carrying *elav-Gal4>UAS-Ubx[RNAi]* transgenes were viable, which allowed us to assess its potential modification of Δ*mir* sterility. Neuronal knockdown of *Ubx* yielded rescue of egg-

laying in 16.7% of sterile Δ*mir* females, and an additional 7.1% were actually fertile (Garaulet et al., 2014). As *elav-Gal4* is expressed postmitotically, this implied a post-developmental role of Hox miRNAs in neurons to regulate fertility.

With this in mind, we sought a physical basis for Δ*mir* fertility defects. Bender described that Δ*mir* mutants have normal ovaries and can mate (Bender, 2008), indicating that failure of egg-laying is not due to the absence of eggs. We confirmed that Δ*mir* females exhibit normal ovary morphology (Garaulet et al., 2014). We also obtained evidence that Δ*mir* females can be fertilized by males bearing Don Juan-GFP, which allow direct visualization of fluorescent transferred sperm (**Fig. 4.2 A-A'**).

On the other hand, Δ*mir* females exhibited defective passage of eggs from the ovary through the reproductive tract. This led us to search for potential defects in the innervation of the genitalia. We attempted to trace the total innervation of the uterus using pan-neural *elav-Gal4* to drive *UAS-CD8-GFP*; however, this revealed a dense axon patterning that precluded quantitative analysis (Garaulet et al., 2014). Moreover, the identity and function of neurons that innervate the uterus are currently poorly-defined. We subsequently focused on the oviduct, whose innervation is simpler and functionally understood. The oviduct is innervated by two efferent subsets required for oviduct function and egg-laying; a dedicated excitatory motoneuron subset that expresses insulin-like peptide 7 (Ilp7) that terminates on the radial muscles of the oviduct (Castellanos et al., 2013; Yang et al., 2008a), and an inhibitory neuromodulatory subset of octopaminergic neurons that ramify over the oviduct muscle and inner epithelial lining, as well as along other reproductive tract structures (Rodríguez-Valentín et al., 2006). Both of these neuronal populations are located in the posterior VNC, where target gene expression is altered the most in Δ*mir*.

We quantified the numbers of adult Ilp7-motoneurons (*Ilp7-Gal4, UAS-CD8::GFP* activity) and octopaminergic neurons (*tyrosine decarboxylase 2* (*TDC2*)*-Gal4, UAS-CD8::GFP* activity). We observed no change in the number of Ilp7+ neurons in Δ*mir* homozygotes (**Fig. 4.2 B-C'**), and only a mild (although statistically significant) reduction in TDC2+ neurons (**Fig. S4.1 A-C**). These neurons retained their transmitter identity; Δ*mir* Ilp7-motoneurons expressed Ilp7 protein and the vesicular glutamate transporter (VGluT) (Castellanos et al., 2013) (**Fig. S4.2 A-C'**) and Δ*mir* octopaminergic neurons expressed the octopamine biosynthetic enzyme tyramine β-hydroxylase (**Fig. S4.1 A-A'**). Moreover, expression of the vesicular glutamate transporter

(VGluT), which is essential for glutamatergic transmission by Ilp7-motoneurons (Castellanos et al., 2013), was normal in Δ*mir* mutants (**Fig. S4.2 A-A'**).

We next examined the capacity of these neuronal populations to innervate the oviduct. To do so, we confocal imaged Ilp7 axons (*Ilp7-GAL4,UAS-CD8::GFP*) and octopaminergic axons (*TDC2-GAL4,UAS-CD8::GFP*) within the anterior 210μm of the common oviduct (the oviduct is evenly innervated through its 450-500μm length in all genotypes examined herein), and quantified the extent of innervation using Simple Neurite Tracer (Longair et al., 2011). Octopaminergic neurons showed no change in innervation (**Fig. S4.1 D-F**). However, total Ilp7 innervation was reduced by 50% in Δ*mir* (**Fig. 4.2 D-E, D'-E'** and quantified in **Fig. 4.2 H**), indicating a specific defect in this neuronal population. This was paralleled by a 50% reduction of anti-Discs large (Dlg)-labeled neuromuscular junction (NMJ) boutons made by Ilp7-motoneurons onto oviduct muscle (Castellanos et al., 2013), in Δ*mir* compared to controls (**Fig. 4.2 D''-E''** and quantified in **Fig. 4.2 I**). Therefore, motoneuron innervation of the oviduct is compromised in Δ*mir* females. Despite this morphological defect, Δ*mir* oviduct neuromuscular junctions (NMJs) still clustered post-synaptic glutamate receptors (GluR), GluRIIB and GluRIIC (**Fig. S4.2 B-C'**).

4.4.3 Contribution of Ubx and hth to defective oviduct innervation in Δmir females

In light of these neural phenotypes, we assessed whether the rescues of Δ*mir* infertility obtained in *Df(3R)P109* and *hth[P2]* heterozygotes were associated with any alterations of Ilp7-neuron innervation or NMJ number. Heterozygosity for *hth[P2]* did not improve Ilp7 innervation in Δ*mir* homozygotes (**Fig. 4.2 F,F'** and quantified in **Fig. 4.2 H**), but partially rescued NMJ bouton numbers (**Fig. 4.2 F''** and quantified in **Fig. 4.2 I**). More notably, fertile *Df(3R)P109/*Δ*mir* females exhibited restoration of oviduct innervation by Ilp7 neurons (**Fig. 4.2 G,G'** and quantified in **Fig. 4.2 H)** and Dlg-labeled NMJs (**Fig. 4.2 G''** and quantified in **Fig. 4.2 I)**. These data directly connect the derepression of these specific BX-C miRNA targets to functional defects in a specific population of posterior VNC neurons that are essential for oviposition and fertility.

In reciprocal tests, we attempted to phenocopy Δ*mir* mutants by forcing the expression of *Ubx* or *hth* in Ilp7 neurons. Heterozygous Δ*mir* females that carried *Ilp7-Gal4* and *UAS-Ubx* transgenes exhibited strongly decreased oviduct innervation by Ilp7+ neurons and of Dlg+

boutons (Garaulet et al., 2014), similar to the effect observed in Δ*mir* homozygotes; this was accompanied by ~12% sterility (Garaulet et al., 2014). On the other hand, ectopic Hth did not substantially affect oviduct innervation and only weakly induced sterility (5%). These findings are consistent with the rescue of Ilp7-motoneuron morphology in *Df(3R)P109/*Δ*mir* that reduces Ubx, but not in *hth[P2],* Δ*mir/*Δ*mir*. These data also support the notion that Ilp7-motoneurons comprise a subset of a larger neuronal population whose function is disrupted in Δ*mir* and that contribute combinatorially to the sterility phenotype. We attempted to test if more general neural misexpression of these Hox/homeotic targets could affect fertility. However, all combinations of *UAS-Ubx, -abd-A, -hth, or -exd* crossed to *elav-Gal4* were inviable, attesting to adverse consequences of broadly ectopic Hox genes and their cofactors in neurons.

We subsequently assessed *fru-Gal4*, which directs restricted CNS expression to neurons with sexually dimorphic function (Stockinger et al., 2005). Within the posterior nerve cord, its expression domain includes Ilp7 oviduct motoneurons (Castellanos et al., 2013), within a larger set of mostly undefined *fru*+ posterior nerve cord interneurons and efferents (Stockinger et al., 2005). Within the reproductive tract, *fru-Gal4>UAS-CD8-GFP* identified extensive innervation of *fru*+ neurons on the uterus, and on the oviduct (Garaulet et al., 2014).

The identity of the *fru*+ neurons innervating the uterus is unknown, but we confirmed that *fru*+ innervation of the oviduct only comes from Ilp7-motoneurons, in part shown by the perfect congruence of *fru*+ innervation and of *Dlg*+ staining that only marks Ilp7-motoneuron NMJs (Castellanos et al., 2013; Garaulet et al., 2014; Kapelnikov et al., 2008).

Interestingly, 22% of *fru-Gal4>UAS-hth* females (n=50) and nearly 90% of *fru-Gal4>UAS-Ubx* females (n=50) were sterile (Garaulet et al., 2014). This was not merely the consequence of expressing a homeotic factor in *fru-Gal4* neurons, since ectopic *abd-A* induced only minor effects on sterility and oviposition by comparison (Garaulet et al., 2014). Currently, specific *fru*+ neurons in the posterior nerve cord that impact female fertility and egg-laying, aside from Ilp7 neurons, are not defined. However, our observations motivate future efforts to identify such neurons and dissect their wildtype functions, and potentially examine how they are affected in the miRNA mutant.

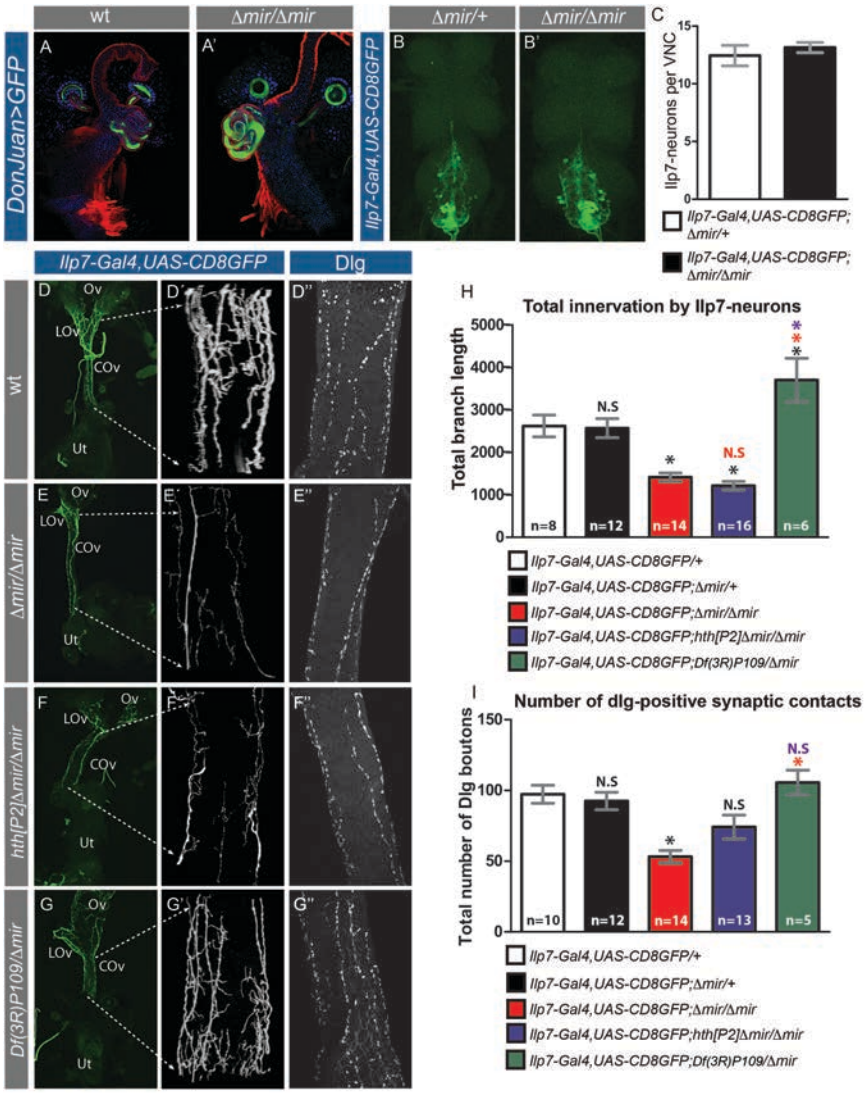

Figure 4.2. Reduced innervation of oviduct by Ilp7-motoneurons in *Δmir*.

(A, A') Δ*mir* females can receive and store male sperm. (A) Control or (A') Δ*mir* homozygous females were crossed to *DonJuan>GFP* males, and their inner genitalia were dissected and stained after 3 days. GFP (green); Phalloidin (red), DAPI (blue). As in controls, Δ*mir* females can receive and store GFP+ sperm. (B-C) Numbers of oviduct Ilp7-motoneurons are not affected in *Δmir*. Whole VNCs showing GFP expression from *Ilp7-Gal4>UAS-CD8::GFP* transgenes in control (B) and *Δmir* (B') adult. (C) Quantification of posterior Ilp7 oviduct motoneurons *(*p=0.44). (D-I) Innervation by Ilp7-motoneurons and NMJ numbers on the oviduct are reduced in *Δmir* and rescued by heterozygosity of its targets. (D,E,F,G) Whole reproductive tract innervation by *Ilp7>CD8::GFP* neurons. (D',E',F',G') 3-D renderings of Ilp7 innervation used to quantify total Ilp7 neuronal branch lengths on the common oviduct. Ov, ovary; LO, lateral oviduct; CO, common oviduct, Ut, uterus. (D'',E'',F'',G'') Maximum sum projection through the common oviduct showing all Dlg-labeled Ilp7-motoneuron NMJs. (H) Quantification of total innervation by Ilp7-axonal arbors shows no difference between control and Δ*mir* heterozygotes, but a 50% reduction in Δ*mir* homozygotes. This was not altered in *hth[P2]*, Δ*mir*/Δ*mir*, but was significantly rescued in *Df(3R)P109/*Δ*mir*. (I) Quantification of the total number of discrete Dlg+ synaptic contacts made between Ilp7-neurons and the oviduct muscle. No difference was observed between control and Δ*mir* heterozygotes, but Δ*mir* homozygotes exhibited 50% reduction in NMJs. This was restored to control by *Df(3R)P109/*Δ*mir*. The *hth[P2],* Δ*mir*/Δ*mir* oviducts exhibited partial restoration of NMJ bouton numbers, which were neither significantly different from controls nor from mutants. Quantifications are shown ±S.E.M.; Tukey tests: **, p<0.01, ***, p<0.001, N.S.=not significant.

4.5 DISCUSSION

4.5.1 Essential, not fail-safe, function of Hox miRNAs in the Hox regulatory hierarchy

Although Hox miRNAs are documented to target other Hox genes, such regulation was suggested to be mostly secondary to transcriptional mechanisms (Bender, 2008; Hornstein et al., 2005; Lemons et al., 2012). The present work documents broadly ectopic expression of multiple homeotic factors in Δ*mir* nerve cords. In particular, the terminal VNC segments, which express the most posterior Hox protein Abd-B, misexpress multiple anterior Hox genes in Δ*mir*. Moreover, their deregulation is causal to Δ*mir* sterility. These data explicitly document that lack of Hox miRNAs causes failure not only of the Hox transcriptional hierarchy, but also of the posterior prevalence rule.

Consideration of the literature alongside our present work identifies an unexpected temporal transition in the mechanisms involved in the Hox regulatory hierarchy. Studies of a P-element insertion (*HCJ199*) that specifically disrupts *Abd-B* (Bender and Hudson, 2000) showed that loss of *Abd-B* derepresses *Ubx* in A8 of the embryonic VNC into a pattern similar to that of anterior abdominal segments (Bender, 2008). By contrast, embryos homozygous for Δ*mir* show only very weak *Ubx* derepression (Bender, 2008). Our results in the larval VNC show the opposite effect: in *Abd-B* mutants there is weak ectopic Ubx expression in the larval VNC, whereas Δ*mir* mutants exhibit substantial Ubx derepression. Therefore, there is a temporal switch from *Abd-B* to *mir-iab-8* as the main *Ubx* repressor in the posterior ventral cord of embryos and larvae, respectively. The situation is slightly different for *abd-A*, since neither *Abd-B* nor BX-C miRNAs alone repress this gene in the posterior embryonic VNC (Gummalla et al., 2012), whereas we now show that Δ*mir* mutants exhibit substantial ectopic expression of Abd-A protein in the larval VNC.

4.5.2 BX-C miRNAs are critical for CNS patterning and reproductive behavior

The essential functions of BX-C miRNAs in neural segmental patterning and organismal fertility are striking, given that most miRNA mutants seem to have subtle phenotypes. Moreover, even though BX-C miRNAs are typical in having hundreds of conserved targets, mere heterozygosity of specific critical targets could rescue female egg-laying and fertility defects. The critical BX-C miRNA targets form protein complexes (i.e., Abd-A/Hth/Exd and

Ubx/Hth/Exd) (Slattery et al., 2011), such that their coordinate deregulation may amplify the effects of miRNA loss.

The CNS region most profoundly affected by BX-C miRNAs comprises the posterior VNC, including segments A8-A9 that are the domain of *mir-iab-8*. Ilp7+ motoneurons in these segments are functionally aberrant in Δ*mir* mutants, since they exhibited substantially reduced oviduct innervation and bouton numbers. This was at least partly due to derepression of specific BX-C miRNA targets, since these defects were partially rescued by heterozygosity for *hth* and more substantially rescued by heterozygosity for a deficiency of *Ubx* and *abd-A*. However, as homeotic genes are derepressed broadly throughout Δ*mir* segments, we expect other neurons may potentially be affected. In fact, whereas the forced expression of *Ubx* or *hth* results in a modest percentage of sterile females, these numbers raise dramatically when they are misexpressed in the *fru*+ domain, encompassing a broader set of neurons that includes populations that innervate the uterus. These data imply that other populations of *fru*+ posterior VNC neurons contribute to the egg-laying program and thus to Δ*mir* sterility. It will be prerequisite in the future to first refine the identity and functional properties of such neurons in wildtype, before we can understand how they may be affected by loss of BX-C miRNAs.

Finally, we highlight the importance of genetics in deciphering miRNA functions. We not only identified phenotypically critical targets of the BX-C miRNAs, we also distinguished their relative dose-sensitivity for the Δ*mir* phenotype. For key targets such as *hth* and *exd*, their target sites were masked by incomplete 3' UTR annotations and poor genome-wide alignments, yet we identified them due to their functional relationship to Hox genes. Moreover, while expression profiling indicated that BX-C miRNAs are expressed in segmental patterns in the early embryo, this proved not to be the location of their most substantial function. Instead, it was necessary to chase their functions inside the CNS, a location that in retrospect was hinted at by the behavioral phenotypes of Δ*mir* flies. Thus evidence from genetics and mutant phenotypes can continue to be crucial for interpreting the biological roles of miRNAs.

5 THE *DROSOPHILA* BITHORAX-COMPLEX MIRNAS *MIR-IAB-4* AND *MIR-IAB-8* ARE REQUIRED FOR FEMALE RECEPTIVITY TO MALE COURTSHIP AND POST-MATING RESPONSES

5.1 SYNOPSIS

The *Drosophila* female post-mating response involves reduced receptivity to male courtship and an increase in egg production and egg-laying. These responses are generally considered to be coordinately regulated in triggered, by the sex peptide receptor-expressing pickpocket sensory (ppk-SPR) neurons that line the reproductive tract and signal the completion of copulation to the central nervous system. -Here, we find that the sterile *Δmir* mutant females exhibit severe disruption of mating and post-mating behaviors. We show that *Δmir* virgin females are precociously unreceptive to male courtship, presenting stereotypical rejection behaviors as if mated. In direct contradiction to this however, *Δmir* females fail to increase egg production or lay eggs, even if mating occurs. We initiated an investigation of the underlying cause of the low receptivity observed in *Δmir* mutants but could not find any gross defect in the ppk-SPR sensory neurons, nor the activity of the essential Ilp7-motoneuorns, that may explain precocious and constitutive rejection behavior. By contrast, we find that mated *Δmir* females aberrantly retain sex peptide (SP) in the reproductive tract. As SP release into the hemolymph is believed to be required for the post-mating response in females, including enhanced egg production, we suggest that a lack of fruitful SP signaling in mated *Δmir* females is an important contributor to *Δmir* female sterility. The uncoupling of post-mating responses in *Δmir* mutants provides a platform from which we can examine novel female-specific neuronal populations controlling receptivity, egg-laying and sex-peptide retention.

5.2 INTRODUCTION

Understanding the mechanisms underlying sex-specific reproductive behaviors has relied on forward genetics to identify essential genes and their mechanisms of action. One notable example was a male-sterile mutant strain that courted both males and females, but did not copulate with either. This mutant was *fruitless (fru),* and to this day analysis of *fru* continues to provide a wealth of information on the specification of male-specific neuronal populations and

behavior (Yamamoto, 2008). Over the past decades, studies of the allelic series and chromosomal lesions of the *fru* locus have revealed defects in male-specific behaviors that have then provided the basis for neurogenetic studies to reveal male-specific neuronal populations and the genetic mechanisms regulating their differentiation (Villella and Hall, 2008). The focus on males has largely arisen due to the numerous stereotyped and elaborate courtship behaviors associated with the genetically tractable *fru* mutant.

Females present robust, stereotypical reproductive behaviors that are amenable to genetic analysis when altered. Recently, female-specific behaviors have become increasingly studied but relevant neuronal types and their organization into functional circuits to regulate female-specific behavior is still in its early stages. Although behavioral mutants for female-specific behaviors do exist, such as *spinster*, *dissatisfaction* and *chaste* (Finley et al., 1998; Juni and Yamamoto, 2009; Sakurai et al., 2013), neurogenetic studies have just recently begun to reveal female-specific neuronal populations required for female behavior (Castellanos et al., 2013; Rezával et al., 2014; Rezával et al., 2012).

In 2008, Welcome Bender generated a precise mutant for the *Δmir* miRNA found in the Bithorax-complex (BX-C) of *Drosophila* Hox genes *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*). Homozygous *Δmir* flies show no evidence for segmental transformation, but both males and females are sterile. He predicted that their sterility was behavioral, due to muscle or neuronal defects, since females did not lay eggs and males cannot bend their abdomens for proper copulation (Bender, 2008).

Our previous characterization of *Δmir* mutants determined that Ilp7-motoneurons, a population of glutamatergic oviduct motoneurons required for egg-laying (Castellanos et al., 2013), show reduced innervation of the female reproductive tract in *Δmir* mutants (Chapter 4). However, we do not believe that this fully accounts for the complete lack of egg-laying in *Δmir* mutants (detailed in chapter 4 discussion). Certain observations prompted us to postulate that additional neuronal populations and/or reproductive processes are affected; first, we had previously found that killing or electrically blocking Ilp7- or octopaminergic- neurons leads to a swollen abdomen due to the typical mating-induced increase in egg production, but an inability to passage those eggs through the oviduct (chapter 2). However, although *Δmir* mutants do not lay eggs, I saw no accumulation of older staged eggs in their ovaries (unpublished). Thus, a simple defect in efferent neurons that controls the passage of eggs cannot fully explain *Δmir*

sterility. Second, Bender had observed normal ovaries in *Δmir* females (Bender, 2008) thus defective oogenesis does not appear to be the underlying cause. Third, overexpression of the *iab-4/8 miRNAs* target, *Ubx*, in *fru*-expressing neurons recapitulates the sterile phenotype (*section 4.4.2*).

In this chapter, we further characterize the sterility of *Δmir* mutant females, in an attempt to understand the underlying defect. Specifically, we focus on potential defects in the female sensory circuits that detect that mating has occurred and promote a post-mating response in females. Central to these circuits are the ppk-SPR ($ppk^+/fru^+/dsx^+$) sensory neurons found in the periphery of the female reproductive tract; these neurons express the sex peptide receptor (*SPR*) and project to unknown targets in the abdominal ganglion of the female VNC. $ppk^+/fru^+/dsx^+$ sensory neurons, that we term ppk-SPR neurons here, induce an increase in egg-laying and a decrease in female receptivity in response to sex peptide (Hasemeyer et al., 2009; Haussman et al., 2013; Rezával et al., 2012; Yang et al., 2009). The ppk-SPR neurons were of interest in relation to the *Δmir* phenotype because inhibition of synaptic transmission in *ppk* or *fru* neuronal subsets leads to an increase in egg-laying and a decrease in receptivity of virgin females; while SPR signaling in fru^+ neurons is required for egg-laying (Hasemeyer et al., 2009{Haussman, 2013 #176; Yang et al., 2009)}. Defects in neuronal connectivity or function of the ppk-SPR neurons could underlie the observed inability of *Δmir* mutants to induce egg production.

Here, we examined putative deficits in sex peptide circuitry and physiology and made key findings that will form the basis of our ongoing studies. We found that gross morphology of ppk-SPR neurons was largely unaffected but that *Δmir* females exhibit two strong phenotypes related to the sex peptide circuitry; 1) female virgins are unusually highly unreceptive towards male courtship, and 2) if mated, abnormally retain sex peptide in their reproductive tracts. Precocious male rejection suggests that the sex peptide circuitry may be constitutively inactive, which we postulate may arise due to a lack of normal connectivity or neurotransmission.

The retention of sex-peptide in the reproductive tract is an intriguing phenotype that has not been reported previously. First, it suggests that sex peptide transfer to the hemolymph is a regulated rather than passive process. Second, failure of sex peptide transfer into the hemolymph suggests a mechanism for the lack of the post-mating increase in oogenesis, and may provide the first direct evidence that sex peptide must exit the reproductive tract to trigger the post-mating response. The mechanism by which sex peptide is released into the hemolymph is currently

unknown, as well as the neuronal targets in the VNC required for receptivity. Therefore the *Δmir* phenotype provides a unique setting from which to uncover novel neuronal populations regulating female-specific behaviors such as receptivity and sex peptide retention.

5.3 MATERIALS AND METHODS

5.3.1 Fly genetics

Flies were maintained on standard cornmeal food at 70% humidity at 25°C. Strains from Bloomington *Drosophila* Stock Centre: *UAS-Syt-eGFP. UAS-nSyb.eGFP. ppk-CD4-td-GFP. ppk-GAL4.* w^{1118} and *CantonS* (control strains). Strains obtained as gifts: BX-C miRNA mutant (*Δmir*)(Garaulet et al., 2014); *SP-GFP* (Villella et al., 2006); *LexAop-CD8::GFP-2A-CD8::GFP. UAS-mLexA::VP16::NFAT Cdel (H-2). LexAop-CD2GFP/TM6B, UAS-mLexA::VP16::NFAT Cdel (31-2). LexAop-CD2::GFP;LexAop-CD2::GFP/TM6B, Pin/Cyo. LexAop-CD2::GFP (Masuyama et al., 2012). Ilp7-GAL4* (see Chapter 2).

5.3.2 Immunohistochemistry

Primary antibodies: Rabbit anti-Ilp7 (Yang et al., 2008a)(1:1000, E.Hafen); Guinea Pig anti-Fork head (Weigel et al., 1989) (1:1000, H. Jäckle); Chicken anti–ß-Galactosidase (1:1,000, ab13970 Abcam). Standard protocols were used (Eade and Allan, 2009). Secondary antibodies: Donkey anti-Chicken, anti-Rabbit, anti-Guinea Pig, conjugated to DyLight 488, Cy3, Cy5 (1:100, Jackson ImmunoResearch). All images acquired on an Olympus FV1000 confocal microscope. Images were processed using Fluoview FV1000 and Adobe Photoshop CS5.

5.3.3 Receptivity assays

Females and males were collected and placed individually on fresh food and aged for 3 days (females) and 5 days (males). For receptivity assays, male and females are placed without CO_2 in custom-made acrylic plastic arenas 1.3mm in diameter, and 0.5 mm in depth. Flies are allowed to acclimate to their surroundings while separated by a sliding divider for 5 mins. The divider is then removed and flies are recorded for 30 minutes during which courtship behavior is scored. After each test, we rinse the arenas with 70% Ethanol and distilled water before placing in the next mating pair.

5.3.4 Female receptivity behavioral video analysis.

Videos were recorded with a Moticam 2300 mounted on a Motic stereomicroscope at 18 frames per second. All parameters: Courtship index, time female spent moving during courtship, duration of copulation and time female spent moving during copulation were assessed by watching the videos on VLC media player, a free open source media player.

5.3.5 Intensity measurements of GFP immunoreactivty in Ilp7-neurons

Images were acquired on an Olympus FV1000 confocal microscope; raw files were imported into FIJI (Schindelin et al., 2012) for analysis. For each Ilp7-neuron, we measured the pixel intensity on the single Z-slice representing the center of the neuron. We define the center of the neuron where the radius of the nucleus reaches its maximum length and is well defined. Each Ilp7-neuron was outlined and the mean of the pixel intensity for each neuron was measured. Background fluorescence intensity was corrected by subtracting a region of equal size from tissue nearby with background fluorescence intensity. The data was normalized and represented as relative intensity.

5.3.6 Statistical analysis

Image data were subjected to D'Agostino and Pearson as well as a Shapiro-Wilk Normality tests. Normally distributed data sets were compared using a parametric unpaired t-test and non-normally distributed groups were compared using a non-parametric Mann Whitney test. One-way ANOVA and Tukey's post hoc tests were conducted for multiple comparisons. All statistical analysis and graph data were performed using GraphPad Prism 5. Data are presented as mean±SD for t-test comparisons.

5.4 RESULTS

5.4.1 Δmir mutant virgins are unreceptive to male courtship

Our previous work (chapter 4) demonstrated that *fru*$^{+}$ neuronal populations regulating female behavior are disrupted in Δ*mir* mutants (Chapter 4, (Garaulet et al., 2014)). Our work and that of others have further shown that subsets of *fru*$^{+}$ neurons are required for the postmating

change in receptivity to male courtship and enhanced egg production (Castellanos et al., 2013; Hasemeyer et al., 2009; Rezával et al., 2014; Rezával et al., 2012; Yang et al., 2009).

Therefore, we tested female receptivity in Δ*mir* mutants to determine if *fru*+ neurons and their function are disrupted. Three-day old naïve virgin Δ*mir* mutants (and control *Δmir/+* and w^{1118} females) were paired with five-day old naïve CantonS males in a mating arena (see methods). We measured the percentage of females that were receptive to male courtship in a 30-minute period. Virgin Δ*mir*/Δ*mir* females exhibited low receptivity and high rejection to male courtship, compared to virgin Δ*mir/+* or CantonS females (**Fig. 5.1 A,B**). These virgin Δ*mir*/Δ*mir* are observed to actively and continuously reject males during courtship by constantly moving, kicking and extruding their ovipositor (**Fig. 5.1 D**). Regardless, males continue to show a high courtship index towards these Δ*mir*/Δ*mir* virgins, as evidenced by their ongoing courtship efforts during the 30 min period (**Fig. 5.1 C**). Occasionally, males are able to mount Δ*mir*/Δ*mir* virgins after 4-7 minutes of courtship; however, this resulted in aberrant copulation because the female does not become stationary or spread her wings to accept mating.

Instead, these females are continuously moving and persistently kicking in an apparent attempt to eject the mounted male (**Fig. 5.1 E**). In these instances, the length of copulation was similar to that seen with Δ*mir*/+ and CantonS virgins, as the males are mostly able to hang on (**Fig. 5.1 F**).

5.4.2 Decreased receptivity of Δmir mutants is not due to gross disruption of ppk-SPR neuron central projections

As Δ*mir*/Δ*mir* virgins are precociously and constitutively non-receptive to males, behaving as mated females, we wished to identify the neuronal circuits that might be disrupted and lead to this phenotype. Inhibition of synaptic transmission in ppk-SPR neurons causes precocious and constitutive non-receptivity of virgin females (Yang et al., 2009{Hasemeyer, 2009 #167). This raises the possibility that the ppk-SPR neurons, or the circuits through which they function, may be dysfunctional in Δ*mir* mutants. Given genetic evidence presented in Chapter 4 showing that the Δ*mir* mutant is most likely a defect of the posterior VNC, we postulate that ppk-SPR neurons are not directly affected in Δ*mir* mutants. Instead, we postulate that the neurons that ppk-SPR neurons synaptically connect to are deficient in some way.

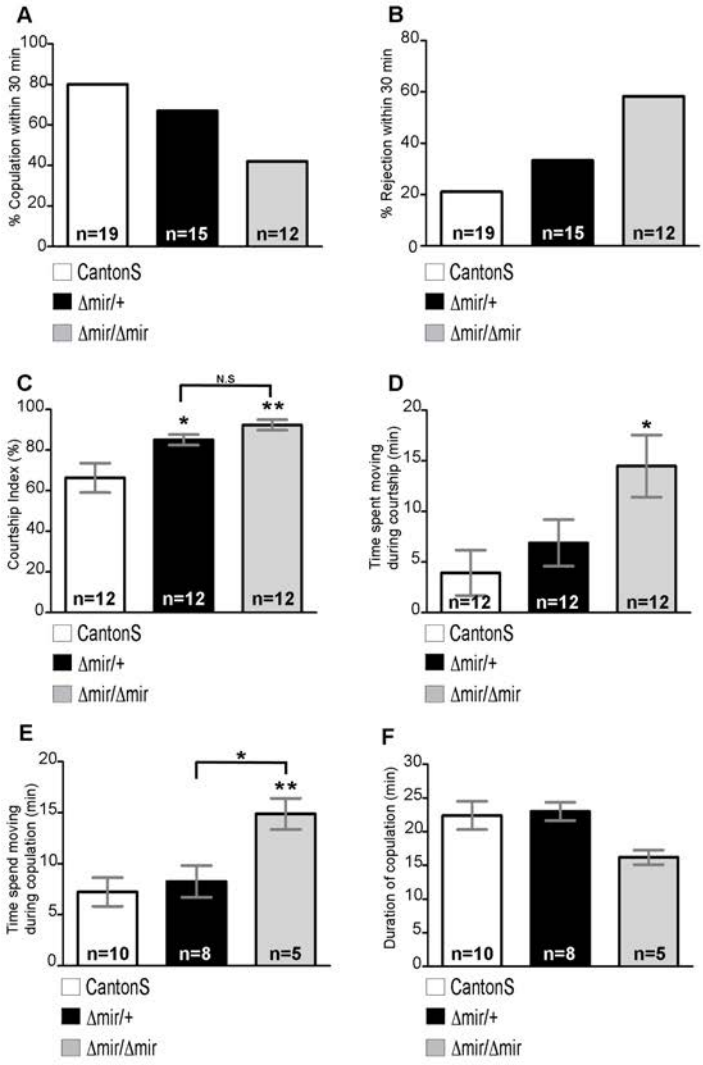

Figure 5.1. Δ*mir*/Δ*mir* virgins are precociously unreceptive to male courtship and actively reject males during copulation.

(A-B) Percentage of virgin females that were receptive to, or rejected, male courtship during the 30 min assay period. (A) 70-80% of Canton S and Δ*mir*/+ virgin females were receptive and mated to Canton S male courtship during the 30 min period. In contrast, 40% of Δ*mir*/Δ*mir* virgins are mated by Canton S males, even though they activley reject. (B) Also, 50% of Δ*mir*/Δ*mir* virgins never mate to Canton S males, compared to only 20-30 % of control females.

(C-D) The courtship activity of Canton S males is equivalent towards control and Δ*mir*/Δ*mir* virgins. (C) Males court females of all genotypes equivalently, as indicated by their courtship indexes, but will spend more time courting Δ*mir*/+ and Δ*mir*/Δ*mir* females [F(2,33)=8.284, p=0.0012] because these females are more unreceptive to male courtship. (D) Males will actively continue to court Δ*mir*/Δ*mir* virgins, even when the female actively rejects the male, by kicking and constantly moving ANOVA:[F(2,33)=4.510, p=0.0186].

(E-F) Length of copulation, although aberrant, is not altered in Δ*mir* mutants. (E) Canton S females will remain stationary during copulation, while Δ*mir*/Δ*mir* females spend significantly more time kicking and walking. Δ*mir*/+ heterozygotes present an intermediate phenotype in which their time spent moving during copulation is greater than Canton S females, but not comparable to Δ*mir*/Δ*mir* [F(2,20)=5.733, p=0.0108]. (F) There is no difference in the duration of copulation between Canton S, Δ*mir*/+ and Δ*mir*/Δ*mir* females [F (2,22)=2.623, p=0.0951]. Graphs show mean±SEM. (Tukey test p=0.01, *, p= 0.001, ** , N.S. not significant). n= recorded single mating pairs.

Perhaps they are not generated properly, do not form properly, or are unable to form synaptic contacts with incoming ppk-SPR axonal input. We deem it likely that these phenotypes would be due to aberrant Ubx and/or hth expression in posterior VNC neurons. Unfortunately, none of these centrally targeted neurons are known at this time. However, any of these phenotypes may be observed as a ppk-SPR bypass phenotype, in which ppk-SPR neurons may continue to grow past their neuronal target in search of its appropriate target We therefore set out to investigate if ppk-SPR projections to the posterior VNC and subesophageal ganglia are disrupted. We visualized ppk-SPR neuronal projections to the female CNS in *ppk-CD4-td-GFP*;Δ*mir*/Δ*mir* mutants and *ppk-CD4-td-GFP*;Δ*mir*/+. Visualizing the gross morphology of ppk-SPR projections in the whole adult female CNS (**Fig. 5.2 A-B'**), and the abdominal ganglion (**Fig. 5.2 A'', B''**), we could not detect any gross disruption in Δ*mir*/Δ*mir*. However, given the complexity of these projections, it is clear that we will be missing finer details of ppk-SPR projections to appropriate central circuits.

Thus, without knowing the targets of ppk-SPR neurons to test for specific defects in synaptic connectivity, we are currently unable to determine if there are specific defects in axonal targeting. Regardless, the lack of overt projection phenotype does suggest that a lack of proper central connectivity may not be the primary defect in Δ*mir* mutants.

5.4.3 Ilp7-motoneurons are candidate targets for the ppk-SPR circuit in the CNS

The location and function of Ilp7-motoneurons in the posterior VNC, and their requirement for egg-laying in mated females (Castellanos et al., 2013), led us to consider the possibility that Ilp7-motoneurons may be a direct target of ppk-SPR neurons. We would anticipate direct input to be inhibitory, but indirect input via local interneurons may be excitatory onto an inhibitory interneuron. To begin to address this, we expressed either *UAS-nSyt::EGFP* or *UAS-nSyb::EGFP* (EGFP-tagged synaptotagmin or synaptobrevin) from *ppk-GAL4,* and observed the relationship of ppk-SPR presynaptic sites with the two clusters of Ilp7-motoneurons. Both presynaptic tags provided the same data. Projections of *ppk-GAL4/ UAS-nSyt::EGFP* (or *UAS-nSyb::EGFP*) are found in close association with the soma of the common dorsal cluster of Ilp7-motoneurons (**Fig. 5.3 A', B'**). These projections are not in close

association with the soma of the female-specific Ilp7-ventral cluster motoneurons, but we cannot rule out the possibility of dendrite innervation further from the soma (**Fig. 5.3 A, B**).

5.4.4 The gross projection of ppk-neurons to Ilp7-motoneurons is not affected in Δmir/Δmir mutants and neither is the activity of Ilp7-motoneurons.

Given the observed close association of ppk-SPR presynaptic termini and Ilp7-motoneurons, we considered the possibility that this may represent an opportunity to determine if the close association of ppk-SPR neurons with a specific neuronal subset was disrupted. We also considered the possibility that aberrant input onto Ilp7-motoneurons in Δ*mir* may reduce their activity, which we know, is required for egg-laying.

We visualized projections of ppk-SPR neurons in the vicinity of Ilp7-motoneurons in *ppk-CD4-td-GFP*;Δ*mir*/Δ*mir* mutants and *ppk-CD4-td-GFP*;Δ*mir* /+, but did not detect any major disruptions (**Fig. 5.4 A-B'**). We had not finished constructing the genotype to examine *ppk-GAL4* driving *UAS-nSyt::EGFP* or *UAS-nSyb::EGFP* in Δ*mir* mutants in time to be included here. Thus, at this time, we are still unable to determine if there is any aberration in central projections of ppk-SPR neurons.

We could not detect any major disruptions in *ppk*-neuronal central projections in Δ*mir*/Δ*mir* females (**Fig. 5.4 B-B'**). Although *ppk*-projections to Ilp7-motoneurons do not seem to be grossly affected in Δ*mir*/Δ*mir* mutants, these results do not provide the adequate resolution to disregard the possibility that synaptic contact of *ppk*-neurons to central target neuronal subsets are not disrupted in Δ*mir*/Δ*mir* mutants.

We attempted to examine whether the lack of egg-laying observed in Δ*mir* mutants may be due to loss of Ilp7-motoneuron activity, perhaps as a result of aberrant input. To test this, we took advantage of an NFAT::CaLexA based method for labeling active neurons. This method takes advantage of *Drosophila's LexA*/*LexAop* binary expression system (del Valle Rodriguez et al., 2012), and the calcium-dependent nuclear import of an NFAT (nuclear factor of activated T cells) transcription factor. We generate flies with *Ilp7-GAL4* driving *UAS-mLexA::VP16::NFAT* and 2 to 3 copies of *LexAop-td.CD8::GFP* in control and Δ*mir* mutant backgrounds.

Increased levels of calcium during depolarization shuttles the chimeric mLexA::VP16::NFAT protein into the nucleus of Ilp7-motoneuorns where it can activate

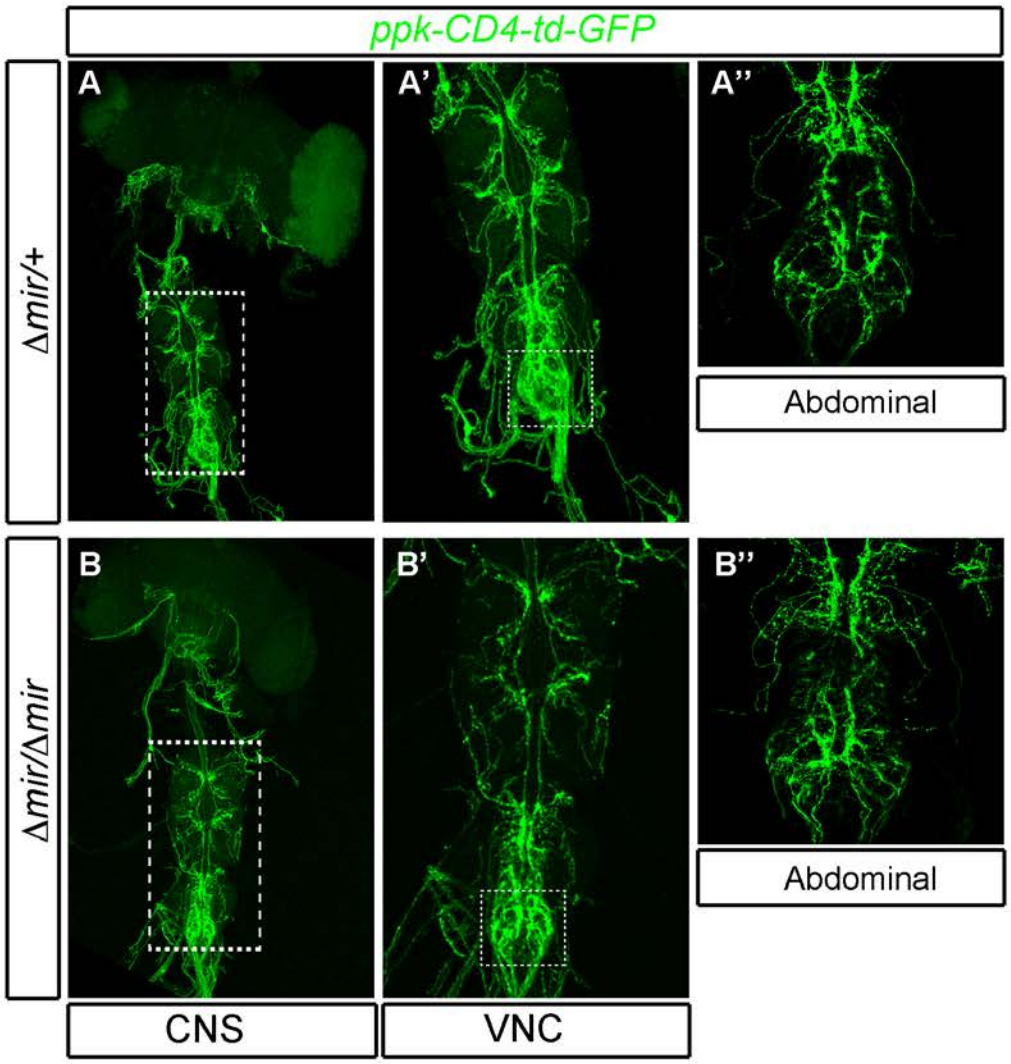

Figure 5.2. Gross morphology of ppk-SPR central projections into the female CNS is not disrupted in Δ*mir*/Δ*mir*.

(A-B'') ppk-SPR neuronal projections visualized with *ppk-CD4-td-GFP* in the female whole CNS, VNC, and abdominal ganglion.
(A-A'') ppk-SPR neurons project primarily to the posterior region of the VNC (A'), including the abdominal ganglion (A''). In the brain, only a few ppk-SPR projections are observed to the suboesophageal ganglion (A).
(B-B'') We observe no major disruption in the gross morphology of ppk-SPR projections in the posterior VNC (B'), abdominal ganglion (B'), and SOG (B) in *ppk-CD4-td-GFP*; Δ*mir*/Δ*mir* females. The resolution of these images is only intended for gross morphological defects; they do not allow for detection of specific axonal targets (see *section 5.5.1*)

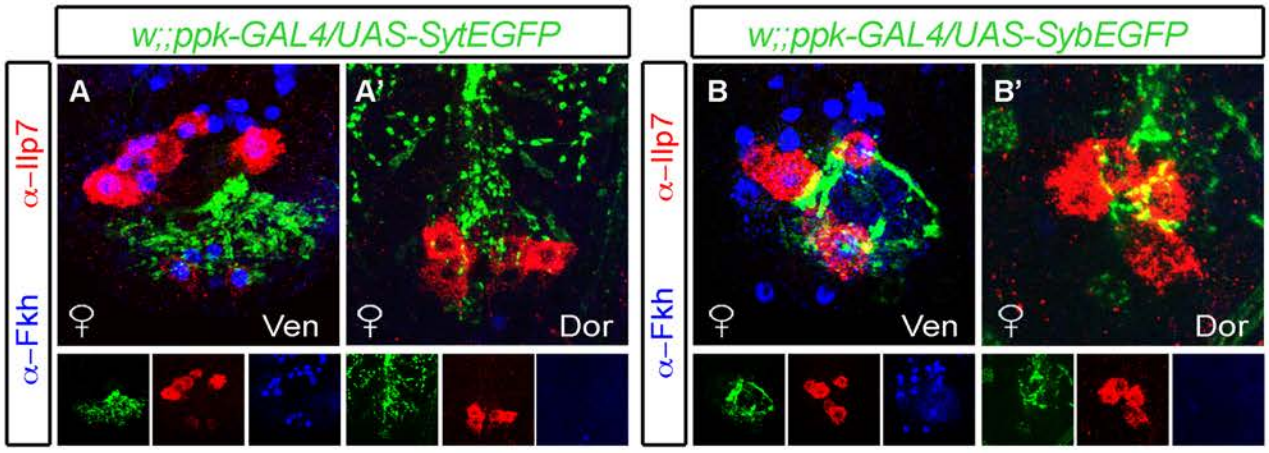

Figure 5.3. Expression of *UAS-Syt::EGFP* and *UAS-Syb::EGFP* in presynaptic termini of ppk-SPR neurons is observed in close proximity to Ilp7-motoneurons.

(A-B') Presynaptic termini of *ppk*-neuronal projections are observed in close proximity to Ilp7-motoneurons in females. (A,B) *ppk-GAL4/UAS-Syt::EGFP* and *ppk-GAL4/UAS-Syb::EGFP* marks regions in close proximity, but not directly adjacent to female-specific Ilp7-motoneurons in the ventral cluster (ven). (A',B') *ppk-GAL4/UAS-Syt::EGFP* and *ppk-GAL4/UAS-Syb::EGFP* marks regions in close proximity to Ilp7-motoneurons of the dorsal cluster (dor).

transcription of the reporter genes through *LexAop-CD8::GFP::CD8::GFP* (Masuyama et al., 2012).

We therefore expect to observe GFP expression only if Ilp7-motoneurons receive stimulatory input (from any neuronal subset). We tested this system in flies of mutant genotype *Ilp7-GAL4 / UAS-mLexA-VP16::NFAT-Cdel (31-2), LexAop-CD2::GFP*; *LexAop-CD2::GFP*, Δ*mir*/Δ*mir* and control genotype *Ilp7-GAL4/ UAS-mLexA-VP16::NFAT-Cdel (31-2), LexAop-CD2::GFP*; *LexAop-CD2::GFP*, Δ*mir*/+. (**Fig. 5.4 C-C'**).

Robust GFP expression was observed in all clusters of Ilp7-motoneurons of both genotypes tested, suggesting that Ilp7-motoneurons remain active in Δ*mir*/Δ*mir* females (**Fig. 5.4 D-E'**). Intensity measurements of GFP expression in these neurons confirm that activity of Ilp7-motoneurons in Δ*mir*/Δ*mir* mutants is comparable to controls (**Fig. 5.4 F**).

These studies lead us to conclude that ppk-SPR central projections are not grossly disrupted in Δ*mir* mutants and that the lack of egg-laying in Δ*mir* is unlikely due to decreased activity of Ilp7-motoneurons. Further, the reduced motor innervation of the oviduct by Ilp7-motoneurons is also unlikely due to reduction in Ilp7-motoneuron activity.

5.4.5 Mated Δmir mutant females exhibit abnormal retention of sex peptide in the reproductive tract.

Successful copulation allows for transfer of sperm and associated accessory gland proteins and peptides (ACP's) into the female reproductive tract, which in turn are required for the induction of post-mating responses in females, such as male rejection behavior and increased egg-production and egg-laying (Kubli, 2003). In spite of poor copulation, sperm is successfully transferred and stored in mated Δ*mir*/Δ*mir* females (Garaulet et al., 2014). However, even though these females cannot lay eggs, they do not exhibit a swelling of the ovaries or the abdomen (MC and DWA, unpublished observation) as occurs when Ilp7-motoneuron activity is blocked (Castellanos et al., 2013).

Thus, Δ*mir* mutants exhibit a failure to enhance egg production after mating. We wished to test if this phenotype was due to any deficit in sex peptide (SP) transmission or function. SP is transferred together with sperm during copulation and is necessary and sufficient for the immediate induction of post-mating responses, such as increased ovulation and rejection of males. These two responses are considered to be coordinately regulated and have only been

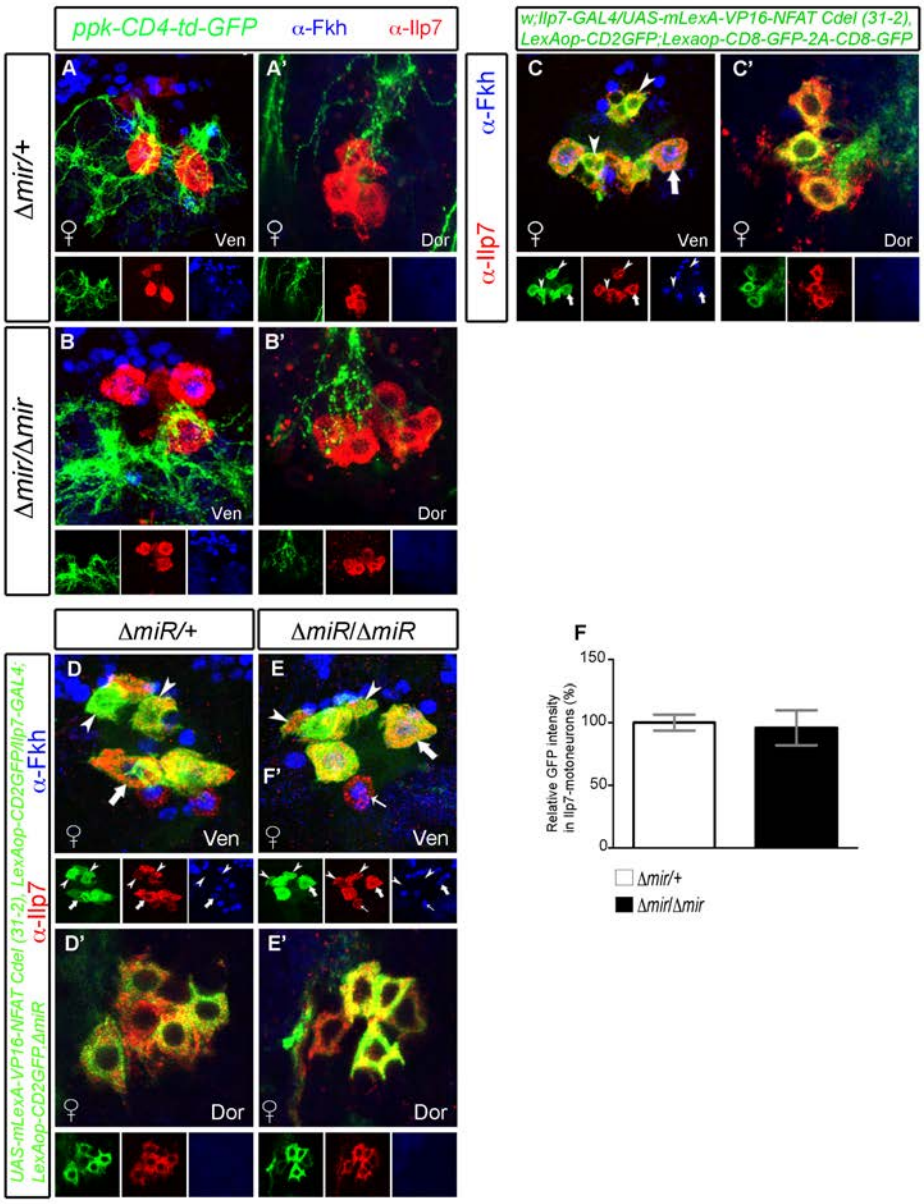

Figure 5.4. ppk-SPR neuronal projections in the vicinity of Ilp7-motoneurons appear unaffected in Δmir mutants, and Ilp7-motoneurons retain activity in Δmir mutants.

(A-B') Gross morphology of ppk-SPR neuronal projections that appear to terminate near Ilp7-motoneurons is not disrupted in *Δmir/Δmir* mutants.
(C-F) Neuronal activity of Ilp7-motoneurons detected with *UAS-mLexA-VP16::NFAT-Cdel (31-2), LexAop-CD2::GFP*. (C-C') GFP expression observed in embryonic (arrows) and post-embryonic (arrowheads) Ilp7-neurons of mated *Ilp7-GAL4/ / UAS-mLexA-VP16::NFAT-Cdel (31-2), LexAop-CD2::GFP; Lexaop-td.CD8::GFP* females indicate neuronal activity in these neurons with this system. **(D-F)** Neuronal activity of Ilp7-motoneurons in *Δmir/Δmir* mutants. (D-D') Induction of EGFP expression is observed in ventral Ilp7-motoneurons (ven, arrowheads) and dorsal Ilp7-motoneurons (dor) of *Δmir/Δmir* mutants (E-E') and *Δmir/+*. (F) Relative intensity of EGFP expression indicates that activity of *Δmir/Δmir* mutants is comparable to *Δmir/+* (ctrl, 100.0±47.86, n=55; exp, 95.94±79.14, n=32; p=0.7658). Graphs show mean±SEM. Arrows and arrowheads indicate representative neurons of each Ilp7 subset.

found to be separable in one study to date. The only observation in the literature that reported such a disconnect was the action of a pertussis-toxin dependent G(o) signaling dependent pathway that is required to activate egg-laying in *dsx*+ neurons that acts independently of a G(o) signaling independent pathway regulating receptivity in fru^{+} neurons (Haussman et al., 2013). Therefore, we postulated that G(o) signaling is somehow not being activated in Δ*mir* mutants. The sex peptide receptor (SPR) expressed by ppk-SPR neurons is a G-protein coupled receptor that utilizes pertussis toxin-dependent G(o) signaling. Thus, we wished to test the possibility that these neurons fail to receive the SP signal. These and our own observations led us to ask if the lack of egg-laying in Δ*mir*/Δ*mir* mutants resulted from improper transfer of sex peptide during the aberrant copulation observed of Δ*mir* virgin females.

To test for aberrant sex peptide transfer, we examined the transfer of GFP-tagged genomic SP (SP-GFP) from males into Δ*mir*/Δ*mir,* Δ*mir*/+ and Canton S virgin females. Virgin females were placed in mating arenas and we verified that mating had occurred; only females that had mated were considered in the following analysis. Females were removed from the arena and the reproductive tract was dissected to determine if SP-GFP was transferred. We found that SP-GFP was successfully transferred into Δ*mir*/Δ*mir,* Δ*mir*/+ and CantonS females immediately post-mating (**Fig. 5.5 B-B''**). Thus, SP transfer from male to female during a single courtship is not affected. Villela et al. 2006 determined that at 6 hours post-mating, approximately only 22% of females showed SP-GFP in their reproductive tract, this timing corresponds to the timing of transfer of ACP's into the reproductive tract (Villella et al., 2006).

In order to determine if SP-GFP was transferred normally to the hemolymph, from where it is believed to control post-mating responses, we examined SP-GFP in the reproductive tract after 24hrs. In Δ*mir*/+ and Canton S mated females, SP-GFP could no longer be seen in the reproductive tract 24hrs post-mating. Dramatically, however, SP-GFP was fully retained in the reproductive tract of mated Δ*mir*/Δ*mir* females 24 hours post-mating (**Fig. 5.5 B-C'', and quantified in A**). These results suggest that the failure to increase oogenesis and egg-laying in mated Δ*mir* females may be due to a lack of SP entering the hemolymph. This phenotype has not been observed previously and may provide the first demonstration that SP release into the hemolymph is a regulated process, and perhaps a neuronally-regulated process, and also the first demonstration that SP release into the hemolymph may be necessary to induce the post-mating

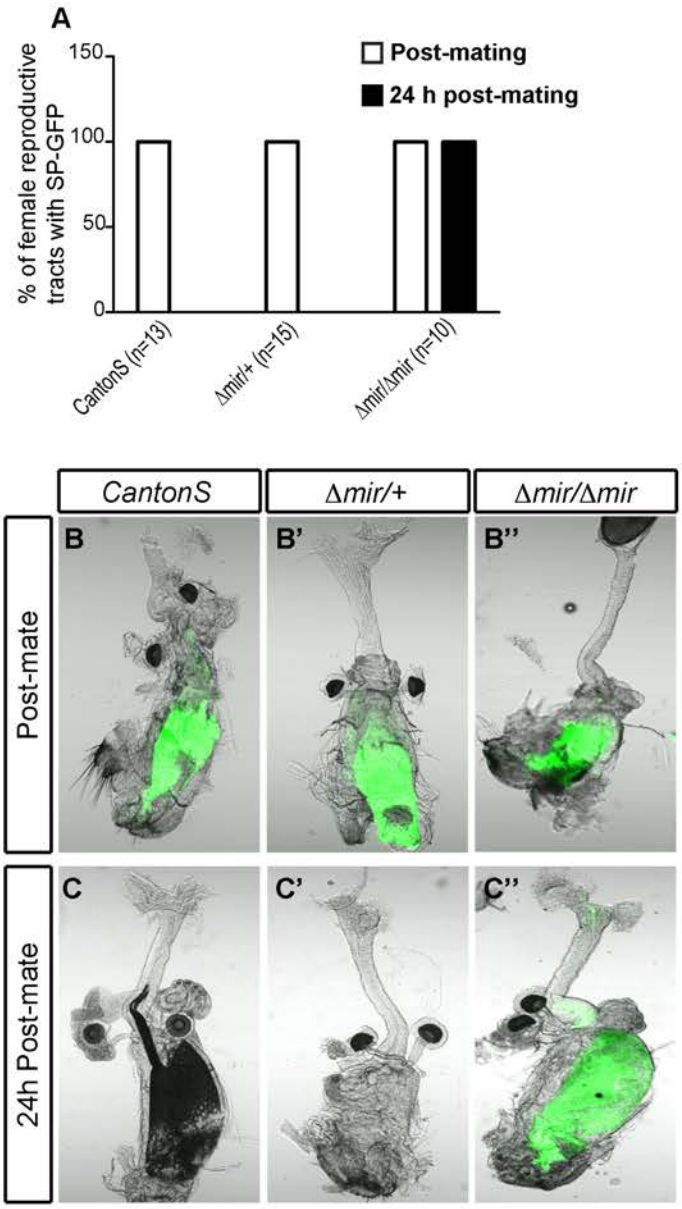

Figure 5.5. Post-mating retention of sex peptide is observed in the female reproductive tract of Δ*mir*/Δ*mir*.

(A) GFP is observed in the abdomen of all Canton S, Δ*mir*/+ and Δ*mir*/Δ*mir* female immediately after completion of copulation with SP-GFP males. However, only mated Δ*mir*/Δ*mir* females retain SP-GFP after 24 hours.
(B-C'') SP-GFP fluorescence after 24hours Canton S, Δ*mir*/+ and Δ*mir*/Δ*mir* mated females. SP-GFP is only retained in Δ*mir*/Δ*mir* mutants, after 24 hours. n= mated females.

response. Future experiments are aimed at testing whether SP retention in the reproductive tract is the primary reason for the lack of enhanced egg production in mated Δ*mir* females.

5.5 DISCUSSION

We have uncovered an uncoupling of two post-mating behaviors generally considered to be coordinately regulated in female *Drosophila*; male rejection and enhanced egg-production (Haussman et al., 2013; Yang et al., 2009). Therefore, we discuss each behavior separately in an attempt to understand how these may be independently disrupted in a way that may contribute to the observed sterility of *Δmir* mutant females.

5.5.1 Δmir mutants constitutively reject male courtship

Virgin *Δmir* mutant females have a strong rejection behavior towards males. This could indicate a defect in recognizing a male as "suitable", or could indicate a precociously activated "mated" state, where receptivity towards male courtship is decreased. Recent studies show that female receptivity is strongly reduced in virgin females of mutants for members of the *ppk*-DEG-ENAC family (*ppk25*, *29* and *23*). These ion channels are expressed in gustatory sensory neurons of the female's front legs, and inhibition of these neurons reduced female receptivity (Vijayan et al., 2014).

Our collaborative genetic analyses of *Δmir* mutants would not suggest a defect in those neurons, but this requires specific testing. Receptivity is dependent upon sex peptide (SP) in male seminal fluids, regulated through a ppk-SPR-neuron driven circuit (Hasemeyer et al., 2009; Yang et al., 2009). It is intriguing that both receptivity-regulating pathways have input via peripheral sensory neurons expressing members of the *pickpocket* DEG/ENAC sodium channel family. However, none of the ppk receptors are themselves SP-responsive and the central circuits that these neurons project to are currently unknown and likely different.

Studies of gynandromorphs had suggested that the anterior dorso-medial protocerebrum was the center for female receptivity (Tompkins and Hall, 1983) and a few neuronal populations that express genes required for receptivity have been mapped to this region (Finley et al., 1998; Sakurai et al., 2013; Soller et al., 2006). However, the SP dependent ppk-SPR neurons project primarily to the posterior abdominal VNC and the suboesophageal ganglion (Hasemeyer et al.,

2009; Yang et al., 2009). Thus, it is likely that there are two distinct receptivity-processing centers in *Drosophila*. The brain could integrate auditory, olfactory and gustatory information to deem a mating partner suitable. In addition, neuronal populations in the abdominal VNC respond to SP through ppk-SPR neurons to induce post-mating responses. Given the expression pattern of *mir-iab-8* miRNA in the posterior VNC we propose that in *Δmir* mutants, the central targets of ppk-SPR circuit are either absent, disconnected or in an altered state of activity. Characterization of the neuronal populations that are affected in *Δmir* mutants will provide insight into the targets required for decreasing receptivity solely as a response to mating.

We suggest that the constitutive rejection behavior of *Δmir* virgin females may be due to a loss of ppk-SPR circuit activity in virgin females. Investigation of the role of ppk-SPR neurons in the post-mating response shows that reducing the activity of ppk-SPR neurons (by expression of *UAS-Kir2.1*) induces constitutive rejection behavior in virgin females (just like *Δmir* females). Thus, it is believed that SP/SPR activity reduces spontaneous ppk-SPR neuronal activity, and it is this that triggers male rejection behavior. Based upon our results, we postulate that this may be recapitulated in *Δmir* mutant females by one of several mechanisms. First, ppk-SPR neurons may be constitutively inactive in *Δmir* mutants. We plan to examine this using the CaLexA reporter system for neuronal activity wherein we would compare the activity of ppk-SPR neurons in *Δmir* and control females. Further, if inactivity of ppk-SPR neurons is the sole reason for male rejection in *Δmir* virgins, we would expect that activation of ppk-SPR neurons by expression of *UAS-NaChBac* (Nitabach et al., 2006) or *UAS-TrpA1* (Pulver et al., 2009) would revert this phenotype to normal receptivity. Second, ppk-SPR neurons may not be connected properly centrally, or the central neurons that normally relay the ppk-SPR signal are not present, or dysfunctional in some way. Unfortunately, these central neuronal circuits are not defined at this time, and our attempts to identify any gross abnormality in the central projections of ppk-SPR neurons showed no such gross defect. Thus, further analysis of this aspect of the phenotype awaits further definition of these pathways.

5.5.2 Δmir mutants and the discovery of novel targets of the post-mating circuit

The complexity of female behaviors has become increasingly apparent over the past few years; a growing number of neuronal populations involved in female-specific behaviors have

been described, and the relationship amongst them is still in the early days of being defined. Our work and that of one other group now shows that receptivity and enhanced egg-production may be mediated through separable pathways downstream of ppk-SPR neurons (Haussman et al., 2013). Therefore, understanding the central targets of these neurons should provide a better understanding of the mechanisms by which the combinatorial action of these neuronal populations leads to a coordinated post-mating response.

The primary targets for ppk-SPR neurons are believed to be located in the abdominal VNC and the suboesophageal ganglion. Apart from a somewhat poorly-resolved population of abdominal TDC^{+}/dsx^{+} neurons that are required for post-mating responses (Rezával et al., 2014), no other central neuronal target has been defined. In chapter 4, we describe our results showing that innervation of *TDC* neurons to the reproductive tracts are not grossly disrupted in *Δmir* mutants, although we did observe a slight increase in the number of *TDC*-expressing neurons (Garaulet et al., 2014). However, we have not yet tested whether ppk-SPR neuronal projections or *TDC* neuronal activity is disrupted in *Δmir* mutants.

The observed sterility of females expressing *UAS-Ubx* in fru^{+} neurons (Garaulet et al., 2014) allows for a potential screen to investigate candidate neuronal populations required for female receptivity in *Drosophila*. *fruitless*, is expressed in approximately 1700 neurons in the CNS (Villella and Hall, 2008),

With the aid of *fru* enhancer fragment-GAL4 lines from the Janellia Farm collection (Pfeiffer et al., 2008), we could select cell-specific *fru* enhancers that are expressed in neuronal subsets of the abdominal VNC. Using these enhancers, we would express *UAS-Ubx* in subsets of fru^{+} neurons, and test for receptivity of virgin females in single-pair matings. Those enhancers that present a precocious decrease in receptivity in virgin females would then be tested to resolve potential synaptic contact with the ppk-SPR neuronal projections by GFP reconstitution across synaptic partners (GRASP). GRASP consists of expressing two complementary fragments of GFP on the outer membrane of the neuronal populations one wishes to study. These complementary fragments will reconstitute GFP expression only if both neuronal populations come in contact (Feinberg et al., 2008). For a screen to determine synaptic contact between candidate *fru*-expressing neuronal subsets and ppk-SPR neurons, we would use the available *fru-lexA* (from the Janelia *fru* enhancer fragments) to drive *LexAop-CD4::spGFP11* and observe if GFP is reconstituted when *UAS-CD4::spGFP1-10* is expressed only in ppk-neurons from the

female reproductive tract, using an intersectional approach to eliminate all *ppk*-projections from the forelegs (Gordon and Scott, 2009; Hasemeyer et al., 2009).

Ultimately, these ppk-SPR central targets could then be tested in *Δmir* mutants to identify a role of *mir-iab-4/mir-iab-8* miRNA in their neuronal function or differentiation.

5.5.3 Sex-peptide release into the haemolymph is under neuronal control and fails in Δmir females.

Wildtype males show a high courtship index towards *Δmir* females; they court vigorously and in a small percentage of instances are able to copulate and transfer sperm and SP (Garaulet et al., 2014). Although sperm and SP are transferred, *Δmir* females do not increase egg-production, as evidenced by a lack of older staged eggs retained in their ovaries. Strikingly, we found that *Δmir* mutants retain SP in their reproductive tracts for at least 24 hours post-mating, long after controls and *Δmir* heterozygotes lose SP-GFP fluorescence in their reproductive tract. This is the first mutant found to have such a phenotype, suggesting that SP release into the hemolymph is a regulated process. SP is released into the hemolymph soon after mating, and it is believed to signal the post-mating response from the hemolymph.

However, while SP in the hemolymph has been shown to be sufficient for post-mating responses, no study had found that SP in the hemolymph is actually necessary (Kubli, 2003). The mechanisms by which SP is released into the hemolymph have not been considered previously, perhaps being assumed to be mediated by passive diffusion. Haussman et al. did propose a SPR mediated transfer of SP from the reproductive tract to the hemolymph; however, SP is still observed in the hemolymph of *SPR* mutant females at concentrations that elicit a post mating response (Haussman et al., 2013). This makes SPR-dependent transport an unlikely mechanism.

The *mir-iab-4/8* miRNA transcripts are neuronally expressed in the abdominal VNC (Garaulet et al., 2014). Thus, the mechanism underlying SP transfer is likely to derive from a central neuronal output from the posterior VNC that is defective in *Δmir* females, although the neuronal populations required for this regulation remain to be determined. Other possibilities exist, however. We postulate that reproductive tract muscle contractions post-mating generate sufficient internal pressure to extrude SP into the hemolymph, perhaps through fenestrations along the reproductive tract.

Δmir mutants provide the first indication that SP release into the hemolymph is a regulated process in female *Drosophila*, and perhaps by a neuronal component. Our first step towards defining the contribution of SP reproductive tract retention to the sterility phenotype will involve raising the SP titre in the hemolymph and/or nervous system. Expression of *UAS-SP* from neuronal GAL4 drivers or fat body GAL4 drivers (which would secrete SP into the hemolymph) can induce a full post-mating response in virgin females (Aigaki et al., 1991; Haussman et al., 2013; Rezával et al., 2012; Yang et al., 2009). We have now constructed appropriate genotypes with which to perform these experiments, and these await testing.

We would also test genotypes that recapitulate or rescue *Δmir* phenotypes for the SP retention phenotype, in order to ascertain whether the SP retention phenotype is seen in any genotype that causes sterility, and not seen in any genotype that rescues sterility. This would serve to tie the SP retention phenotype to *Δmir*. Through all of these experiments, we would be careful to discriminate the difference between sterility without ovary swelling and sterility with ovary swelling. We believe that the inability of mated *Δmir* females to increase egg-laying is probably separable from their inability to increase egg production. Thus, if SP retention in *Δmir* females underlies their lack of increased egg production, but not lack of egg-laying, then the expresison of *UAS-SP* in *Δmir* mutants should result in females with swollen ovaries and abdomen.

6 DISCUSSION

It is becoming more apparent that sex can influence the physiological state of a cell. In response, the NIH recently developed policies to ensure that the sex of the organism/cell must be reported and considered in future applications for funding (Clayton and Collins, 2014). Canadian research funding agencies also require all operating grants to describe whether the research considers gender-related issues. Currently, less than 40% of studies report the sex of the organism on which a study was performed; and even less will report sex, when dealing with cells in culture (Miller, 2012). Of those studies that do report the sex of the organism, the majority will carry out experiments and report findings obtained from males; leaving females greatly underrepresented (McCarthy and A., 2011).

There is increasing interest in the influence of sex on the brain, in part spurred by this modern era of personalized medicine. Areas of specific interest include differential responses to sex hormones and therapeutics, differences in gene expression and epigenetic regulation, and sex differences in neurological disease states (Jazin and Cahill, 2010). However, the mechanisms by which sex can influence neuronal differentiation and development, as well as neuronal function is the subject of ongoing research.

Starting with the identification of behavioral mutants that exhibit aberrant sexual behavior {Hall, 1971 #305}, *Drosophila* has been used for the past four decades as a model to study the genetic mechanisms for dimorphic neuronal differentiation, and dimorphic neuronal circuit formation and function. Studies using *Drosophila* to study neuronal control of behavior have primarily focused on male-specific courtship behaviors, and there has been a distinct male bias to date (Villella and Hall, 2008).

At the time that we initiated our studies: i) Female-specific neuronal populations had not been described, and gross observations of *fru*+ and *dsx*+ neurons had shown that only males have neuronal populations not found in the other sex (Rideout et al., 2010; Stockinger et al., 2005). ii) This male-specific increase in the numbers of numerous neuronal populations was shown to arise from female-specific death of specific neuroblasts or neurons that form equally in both sexes during development. Thus, the female was often considered to be 'not-male" by subtraction from the male state (Kimura et al., 2005; Nojima et al., 2010; Sanders and Arbeitman, 2008). iii) The female behavior of post-mating receptivity was considered difficult to quantitate, and was subject to the variability of male courtship behavior (Ferveur, 2010). Our

work and the recent work of other groups (Rezával et al., 2014; Rezával et al., 2012) has contributed to recent advances that have changed all of these long-held views.

In Chapter 2 of this thesis, we outline the first described example of an expanded female-specific neuronal population in the CNS, the Ilp7-motoneurons. We further show that these serve as the sole motoneurons of the oviduct required for passage of eggs through the reproductive tract. Also, we provide evidence to suggest that a non-canonical genetic mechanism underlies the generation of these female-specific neurons. Very recently, a second female-specific neuronal population, the TDC^{+}/dsx^{+} neurons, has been described. These neurons are also expanded in females and are required for female post-mating responses, receptivity and egg-laying (Rezával et al., 2014). These two examples counter the long-held view that the female nervous system arises primarily by elimination of male circuits and emphasize that the complexity of female reproductive behaviors has the potential for the discovery of other female-specific neuronal populations.

The primary contributions of Chapter 3 are to provide the first evidence for male-specific neuronal cell death in *Drosophila*, and to show that the underlying genetic mechanisms are distinct from those that govern dimorphic neuronal cell death in females. No previous work had addressed the genetic mechanisms underlying the generation of female-specific neuronal populations or male-specific neuronal death.

The work presented in Chapter 4 is a collaborative effort wherein our main contribution was to determine the neuronal basis for the sterility phenotype observed in the *mir-iab4/8* (*Δmir*) mutants, to better understand the biological role of these miRNA's in the nervous system. Our results show that the sterility phenotype of *Δmir* mutants is due to deficits in *fru*-expressing neurons including Ilp7-motoneurons of the posterior VNC. The advantage of the strong behavioral phenotype in *Δmir* mutants allows for ongoing genetic analysis to identify specific neuronal subsets and physiological processes required for female reproductive behaviors, and provides the basis for the results presented in Chapter 5.

In Chapter 5 we describe a novel post-mating response that we postulate is likely to be under neuronal control, the regulated release of SP into the hemolymph. Previously, release of sex peptide from the reproductive tract into the hemolymph was not considered to be regulated. The literature seems to assume that it passively diffuses, and no specific mechanism is suggested.

However, our identification of a mutant that affects neurons in the posterior VNC and that retains SP in the reproductive tract suggests that SP release into the hemolymph is neuronally regulated.

This thesis, and recent studies on female-specific post-mating circuitry now provide the impetus and the models to study the genetic mechanisms that generate female-specific neuronal populations and their integration into female-specific circuits. Our work has provided sufficient evidence to suggest that the generation of female-specific circuits involves genetic mechanisms that do not reflect, and are not necessarily predicted, by our knowledge of mechanisms regulating male-specific circuit generation.

6.1 FEMALE-BIASED NEURONAL DIFFERENTIATION

Chapters 2 and 3 represent our continuing effort to understand how a female-specific neuronal population arises. Here, I discuss certain caveats that we encountered in our studies and ways to resolve these in the future to make progress in this novel field.

6.1.1 Caveats and future directions in our studies for female neuronal differentiation

Here, I discuss certain limitations in the analysis carried out within this thesis and how we plan to address these in the future: i) Identify markers for Ilp7-motoneurons in addition to our current reliance on Ilp7 immunoreactivity to identify Ilp7-motoneurons. ii) Rigorously prove that nascent Ilp7-motoneurons in males undergo sex-specific programmed cell death. iii) Analyze the potential non-canonical pro-apoptotic role for *fruM* and *fru-P2* transcripts in male-specific cell death of neurons.

A primary caveat in our studies of female-specific Ilp7-motoneurons to date has been our inability to identify these neurons in the absence of Ilp7-peptide expression. In Chapter 2 *section 2.4.1,* we carried out a screen to identify transcription factors in these neurons that could act as potential markers; however the only transcription factors that we identified in female-specific Ilp7-motoneurons were *Abd-A*, *Abd-B* and phosphorylated *Mad* (Castellanos et al., 2013). Unfortunately, these transcription factors are too broadly expressed (even combinatorially) to provide good discriminatory markers for female-specific Ilp7-neurons. Ongoing efforts, not included in this thesis, involve screening candidate transcription factors in order to identify a discriminatory set of markers that combinatorially allow for their unambiguous identification.

However, we have been unsuccessful to date. There would be numerous advantages in the discovery of discriminatory transcription factor markers in female-specific Ilp7-motneurons: such markers would help identify these neurons in the absence of Ilp7-peptide. Given our lack of discriminatory markers for Ilp7-neurons, we are unable to determine if the absence of female-specific neurons observed in our genetic analysis of the sex determination factors *tra* and *fru* is due to the nonexistence of the cells, or their lack of Ilp7-peptide expression. Further genetic analysis of the role for the sex determination cascade in the neuronal differentiation of Ilp7-motoneurons would therefore benefit from our ability to discriminate between these two possibilities. And second, these markers could potentially help to identify the neuroblast from which female-specific Ilp7-neurons are derived during late larval neurogenesis. To date, we have not been able to determine the mechanisms by which female-specific Ilp7-motoneurons are generated because we have no discriminatory markers prior to the onset of Ilp7 peptide expression. For our lab and many others, the identification of specific combinations of transcription factors expressed in a neuronal subset serves numerous purposes. First, these transcription factors are often expressed throughout lineage progression (and can be used as discriminatory markers for specific neuroblast lineages) or as soon as the neuron become post-mitotic. This allows for detailed genetic analysis of lineage progression and postmitotic neuronal differentiation. Second, those transcription factors are typically involved in specifying or differentiating the neuron, and mutant analysis of its function can provide important insight into the generation of the neuronal subtype (Birkholz et al., 2013) Recently, a detailed map of all embryonic neuroblast lineages in the posterior abdominal region of the VNC was described (Birkholz et al., 2013). This map provides candidate lineages and transcription factors whose expression can be tested for expression in Ilp7-motoneurons.

A second main caveat regards our analysis of male cell death of Ilp7-motoneurons. Male-specific cell death is an important and novel finding from this work, with significant implications for the field. Therefore, we wish to further verify our findings. We showed that we could generate female-specific Ilp7-motoneurons in males if we expressed *UAS-tra* in post-mitotic neurons, using *elav-GAL4*. However, expression of *UAS-tra* from *Ilp7-GAL4* failed to generate these neurons. Thus, the stage at which these neurons become sexually dimorphic appears to be soon after they become post-mitotic, but before they differentiate to express Ilp7 peptide. Our studies in Chapter 5 showed that expression of *UAS-p35* in *fru*-expressing cells (including

nascent Ilp7-motoneurons) rescues the generation of female-specific-like Ilp7-motoneurons in males. Expression of *UAS-p35* is widely accepted as a specific and potent blocker of caspase-mediated programmed apoptosis in cells (Hay et al., 1994; Nojima et al., 2010; Rideout et al., 2010). Indeed, it has long been used as a tool to identify neuroblasts and postmitotic neurons that undergo programmed cell death; if extra neuroblasts or neurons are observed after *UAS-p35* expression, this is generally seen as proof that programmed cell death would normally have eliminated those cells during development (Zhou et al., 1997) (Choi et al., 2006; Wing et al., 1998). Thus, our finding that *UAS-p35* expression in male *fru*-expressing cells leads to the generation of female-specific-like Ilp7-motoneurons in males provides strong evidence for their programmed cell death, normally.

Regardless, we plan two additional tests to further support our conclusion. First, we will test for the presence of female-specific Ilp7-motoneurons in males in viable allelic combinations that are mutant for the cell death effectors *hid, grim* and/or *reaper.* Deficiencies in which all three genes are eliminated, such as *Df(3L)H99*, are embryonic lethal. However, certain hemizygous combinations are viable at pupal stages with *Df(3L)H99,* including hid^{A206}/ *Df(3L)H99* and *rpr-XR38*/*Df(3L)H99* (there is no reported viable combination at pupal stages to test the effect of *grim*)(Choi et al., 2006; Kimura et al., 2005; Lee et al., 2013; Sanders and Arbeitman, 2008). The survival of female-specific-like Ilp7-motoneurons in males, in these mutants, would provide strong supportive evidence for male-specific death of these neurons. Second, we plan to positively identify dying Ilp7-motoneurons in males, and determine their time of death. Our BrdU birth-dating studies indicate that neuroblast divisions within the Ilp7-motoneuron lineage occur in larvae at mid L3. Thus, we expect that Ilp7-motoneurons become post-mitotic in late L3 to early pupal stages. Our studies indicate that male-specific Ilp7-motoneuron death occurs in post-mitotic neurons, most likely in late larval or early pupal stages (see **Fig 2.8**). To approximately identify the stage of death, we plan to trigger *UAS-p35* expression, in males, at different timepoints during early metamorphosis using *fruP1-GAL4; tubgal80*^{ts}. The time at which this no longer spares Ilp7-motoneurons in males would signify a time immediately after their death. We then plan to utilize TUNEL staining (Choi et al., 2006; Sanders and Arbeitman, 2008) or activated caspase-3 immunorectivity (Fan and Bergmann, 2010) at around that time in an effort to identify neurons undergoing programmed cell death.

As mentioned above, our lack of markers for Ip7-motoneurons prior to the onset of Ilp7

expression stands as a caveat to this analysis. However, any neurons that we identify that express *fru-P1-GAL4* and are positive for cell death in the male nervous system would be of great interest.

A third main caveat of our work regards our analysis of constitutive *fru* alleles that lead us to propose a non-canonical pro-apoptotic role for *fruM* in neurons, as well as a non-canonical obligatory co-regulatory role for transcripts from the *fru-P2* promoter. In males, any function attributed to the *fru* locus is considered the action of FruM protein. In our studies, the constitutive *fruM* allele induced cell death of female-specific Ilp7-neurons in females or males, regardless of whether the other *fru-P1* allele drove *fruF* or control transcripts. However, the constitutive *fruM* allele was incapable of inducing Ilp7-motoneuron death in females, and even males, when placed over the $fru^{4\text{-}40}$ deficiency. Indeed, the $fru^{4\text{-}40}$ deficiency spared Ilp7-motoneurons in males even when placed over a wildtype *fru* locus, as outlined in *section 3.5.2*. The $fru^{4\text{-}40}$ deficiency deletes the *fru* locus through the P1 and P2 promoters. As *fruM* killed nascent Ilp7-motoneurons in males over numerous fru-P1 alleles, we conclude that the fru-P2 promoter may be the missing pro-apoptotic factor that is requires in *trans* to *fruM*.

We concluded from these studies that fruM is essential for cell death of nascent Ilp7-motoneurons, but suggest that the function of FruM requires transcripts from the *fru-P2* promoter (or some other co-regulator missing in $fru^{4\text{-}40}$). To test a role for *fru-P2*, we could overexpress a *UAS-fru-P2* transcript in a $fruM/fru^{4\text{-}40}$ background to determine whether this rescues male death of Ilp7-motoneurons. Alternatively, we also plan to generate a *fru-P2* deletion. This could be achieved through Flp-mediated deletion of genomic sequence between two available FRT-containing transposons that flank *fru-P2* (Parks et al., 2004). Alternatively, our lab uses homologous recombination to delete targeted genomic fragments and replace them with an attP site for integrase-based reconstitution of control or mutant genomic sequence (Huang et al., 2009). Such a *fru-P2* allele would allow us to test an essential role for *fru-P2* in Ilp7-motoneuron cell death in males.

An alternative interpretation of these data could indicate that the function of FruM in the cell death program is affected in the constitutive *fruM* allele used in this thesis, due to the introduction of 1601bp deletion of the sex-specifically spliced exon that includes the female *tra*-binding sites (Demir and Dickson, 2005). We can test a second constitutive FruM expressing allele, fru^{tra}, which was generated by Demir et al. (2005) but only introduces a 261 bp deletion

of the *tra* binding site. If the effects we described in this thesis are in fact due to the requirement of FruM and *fru-P2*-transcripts, we expect to observe the same results in $fru^{\Delta tra}$/ $fru^{4\text{-}40}$ as those presented in this thesis. If $fru^{\Delta tra}$/ $fru^{4\text{-}40}$ is able to induce male-specific cell death, we can conclude that a region in the 1601bp deletion of *fruM* is required for cell death. However, the inability of FruM to induce cell death in hemizygous males for the $fru^{4\text{-}40}$ deficiency strongly suggests that transcripts from the P2 promoter, or two copies of FruM are required for cell death to occur.

A final caveat of our analysis regards the roles of *ix*, *her* or *dsf*. We have not yet determined the egg-laying phenotype, or any phenotype of the numerous properties of Ilp7-motoneurons including oviduct innervation, VGlut expression and formation of mature NMJs with all NMJ markers in mutants for *ix*, *her* or *dsf*. Determining if these neuronal properties develop normally in mutants for these sex determination factors, as well as in double mutants, will allow us to rule out any requirement for these genes in Ilp7-motoneuron development or function. Given that female-specific Ilp7-neurons undergo cell death in males, it would be interesting to study if *ix*, *her* or *dsf,* play a role in FruM-mediated cell death. Examining *fruM*/*FruDf* females in *ix*, *her* or *dsf* mutant backgrounds would allow us to determine if their absence can induce cell death, suggesting a role as protective factors in females.

Our proposed experiments can offer insight into the generation of female-specific Ilp7-motoneurons, however these studies can be expanded to examine how female-specific neurons are generated throughout the nervous system. Since publication of our identification of Ilp7-motoneurons, a second subset of female-specific neurons have been identified in the CNS, the TDC^{+}/dsx^{+} neurons. We expect that there are many neuronal subsets that are specific to females, both central and peripheral. Our long-term intention is to identify such neurons and to examine the genetic regulatory mechanisms that lead to their generation and function. Such studies would provide an interesting counterpoint to studies of male-specific behaviors, and we anticipate that they will also provide a more full description of how sexual dimorphism is genetically regulated.

6.2 NEURONAL CONTROL OF FEMALE SPECIFIC BEHAVIORS IN *Δmir* MUTANTS

Chapters 4 and 5 report our continuing effort to understand the female sterility phenotype of *Δmir* mutants with the anticipation that this will further our understanding of the neuronal

circuits and physiological processes that underlie female reproductive behavior. Here, I discuss certain caveats and open questions that we encountered during these studies and the ways in which we plan to resolve them in order to proceed with future studies.

6.2.1 Caveats and future directions for our analysis of female-specific reproductive behaviors in the Δmir mutant.

Here, I will discuss certain limitations in the interpretation of our results, the analysis carried out within this thesis, and how we plan to address these in the future towards: i) Analysis of neuronal control for decreased egg-laying in *Δmir* mutants and its connection to the female post-mating response. ii) Analysis of defects in neuronal connectivity and activity of the post-mating neuronal circuit in *Δmir* mutants. iii) Uncoupling of receptivity and egg-laying in *Δmir* mutants. iv) Determination of whether the retention of sex peptide underlies the sterility phenotype observed in *Δmir* mutants. v) Potential to uncover novel neuronal populations regulating female-specific behaviors.

In Chapter 4, we started to examine the two best understood neuronal candidates that may underlie the sterility observed in *Δmir* mutants, the essential oviduct efferents, Octopaminergic neurons and glutamatergic Ilp7-motoneurons (Castellanos et al., 2013; Monastirioti, 2003; Rezával et al., 2014). The only notable phenotype we observed was a 50% reduction in overall oviduct innervation and neuromuscular synaptic contact by Ilp7-motoneurons. We have not determined how this may affect oviduct contractility but we do not believe that this would explain the total lack of egg-laying. While one may propose that some other defect in either of these neuronal populations may underlie the *Δmir* phenotype, we believe that this is not the case because selectively killing or electrically silencing those neurons causes infertility that is accompanied by a distended abdomen and ovaries full with mature eggs. Notably, *Δmir* females have no increase in mature eggs in their ovaries and do not have distended abdomens (data not shown), thus other major defects remained to be explored, and we turned our focus to those. This inability to increase egg-production led us to investigate the *ppk-SPR* circuit, which regulates the decrease in receptivity and increase in egg-production / egg-laying that occurs as part of the post-mating response in females (Hasemeyer et al., 2009; Rezával et al., 2012; Yang et al., 2009).

Our behavioral analysis of *Δmir* virgin females showed that they precociously and constitutively reject male courtship, just as mated control females do. Such a phenotype points to potential disruption of the *ppk-SPR* circuit, which regulates female receptivity and post-mating behavioral changes. This circuit comprises peripheral sensory ppk-SPR neurons, that project into unknown central neurons of the abdominal VNC and suboesophageal ganglion, that express *fru* and/or *dsx*, and then perhaps into additional unknown circuits. The ppk-SPR neurons themselves also express *fru* and *dsx*, although the role of these factors in the generation of these neurons remains to be tested. Either genetic electrical inactivation of *fru*+/*ppk*+ neuronal subsets (using *ppk-GAL4* or *fru-P1-GAL4*), or genetic activation of dsx^{+} neurons (*dsx-GAL4*) yields virgins with decreased receptivity (Hasemeyer et al., 2009; Rezával et al., 2012; Yang et al., 2009). This suggests that SPR-mediated deactivation of ppk-SPR neuron activity is required to trigger the post-mating response and at some point further down the circuit this leads to activation of fru^{-}/dsx^{+} expressing neurons. Based on these findings, we postulate that the *Δmir* phenotype may arise due to perpetual inactivation of ppk-SPR neurons (or downstream *fru*+ neurons), or due to disconnection of these neurons from their appropriate targets. Therefore we wish to further address key aspects of the *ppk-SPR* circuit function that would include: a) connectivity and b) circuit activity.

To begin to address possible defects in the connectivity of the *ppk-SPR* circuit, we here show that the gross morphology of *ppk* neuronal projections to the abdominal ganglion remains intact in *Δmir* mutants. For these studies we used a 3kb *ppk* enhancer region-GAL4 that expresses in all *ppk*-expressing neurons. However, Hasemeyer et al (2009) showed that GAL4 can be selectively expressed in only the reproductive tract *ppk* neurons, using an intersectional strategy. We plan to use this method to solely visualize only ppk-SPR neuron projections within the CNS. We will use this to drive *UAS-syb::EGFP* to map specific regions of synaptic contact of these *ppk*-neurons in *Δmir* mutants.

In *section 5.5.2* we address the possibility of Ilp7-motoneurons as potential targets of ppk-SPR circuit given their requirement for egg-laying; utilization of the GFP reconstitution across synaptic partners (GRASP) system would allow us to define Ilp7-motoneurons or Octopaminergic neurons as synaptic partners of the ppk-SPR neurons. GRASP requires the expression of two complementary fragments of GFP on the outer membrane of two distinct neuronal populations; if these two neuronal populations make synaptic contact, GFP expression

is reconstituted (Feinberg et al., 2008). In order to determine if ppk-SPR neurons come in contact with Ilp7-motoneurons or TDC (octopaminergic)-neurons we would build an Ilp7-LexA transgene (TDC-LexA is publicly available) to activate LexAop-CD4::spGFP11 and utilize the intersectional approach described in Hasemeyer et al. 2009 to only express UAS-CD4::spGFP1-10 in ppk-SPR neurons from the female reproductive tract (Gordon and Scott, 2009; Hasemeyer et al., 2009).

Investigation of whether a perpetual inactivated state of ppk-SPR neurons is a primary deficit in *Δmir* mutants presents a technologically more complex problem. However, given the location of ppk^{+} neurons in the periphery, they are not likely to be affected in *Δmir* mutants, which we postulate only affects abdomnal VNC neurons. Therefore, we would have to determine if potential downstream central ppk-SPR neurons are constitutively inactive, disconnected or absent. However, these are unknown at present. In *section 5.4.4* we address the possibility of Ilp7-motoneurons as potential targets of the *ppk-SPR* circuit and we addressed whether these neurons are active or not by using activity monitor systems such as the NFAT-LexA system (Masuyama et al., 2012). However, this tool is quite a blunt instrument and does not allow us to determine whether normal activity patterns are disrupted in *Δmir* mutants.

A question that emerges is, how do we identify neurons with the ppk-SPR circuit that may underlie the constitutive rejection behavior of *Δmir* virgins. The primary mechanism of action of *Δmir* is via the derepression of Ubx and/or hth in posterior abdominal neurons of segments A8 and A9. Further, we found that overexpresison of *UAS-Ubx* using *fru-P1-GAL4* recapitulates the mating phenotype. First, we would test to ensure that these virgin females also have constitutive rejection behavior. If this is the case, we would then screen a series of *fru*-enhancer GAL4 lines (we have obtained all those that express in the abdominal VNC from the Janelia GAL4 collection) for those that recapitulate the phenotype but are expressed in fewer neurons. This would start to restrict the number of neurons that we would examine for a role in the ppk-SPR circuit. Thereafter, we could use intersectional genetics in an effort to continue to restrict the population of neurons that may underlie this phenotype. We would then examine the effect of activation or silencing of these neurons in receptivity behavior, and also test their activity, development, morphology and connectivity in controls and *Δmir* mutants.

Typically, the decrease in receptivity that we see in *Δmir* mutants is accompanied by an increase in egg-production and egg-laying. For example, increased egg-laying and rejection of

males is observed in virgin females with inactivated ppk-SPR neurons even if the females have not been mated (Hasemeyer et al., 2009; Yang et al., 2009). However, this concomitant increase in egg production does not occur in *Δmir* females. Although highly unreceptive, a small percentage of *Δmir* mutants will mate; however, even once mated *Δmir* females do not increase egg-production, therefore remaining in a virgin-like state of oogenesis. This phenotype indicates an uncoupling of the post-mating response in *Δmir* mutants, where we observe precocious rejection in virgins (as if mated) and decreased egg-laying in mated females (as if virgin). However, at least a partial resolution of this dichotomy may be found in our result showing that SP is retained in the reproductive tract of *Δmir* females. This raises the possibility that oogenesis is not induced by sex peptide in these females as a secondary effect of sex peptide retention. In order to test this hypothesis, we will first verify whether any sex peptide enters the hemolymph; the easiest approach will be to mate SP-GFP males to *Δmir* females and collect their hemolymph to perform anti-GFP ELISA. We will also genetically bypass SP failure to enter the hemolymph by over-expressing *UAS-SP* in neurons using *ELAV-GAL4* (Hasemeyer et al., 2009; Rezával et al., 2012; Yang et al., 2009) or in the fat body, using *Lsp2-GAL4* (Lazareva et al., 2007). Both methods are known to induce a post-mating response in virgins (Aigaki et al., 1991; Haussman et al., 2013). If SP retention in the reproductive tract is pertinent to the *Δmir* phenotype, we would expect this to increase egg production. It would then be intriguing to determine whether these females could then lay eggs (showing that neuronal control of egg passage through the reproductive tract is not seriously affected) or exhibit abdomen extension and a build up of mature eggs (indicating a second, independent phenotype in reproductive tract neuro-regulation).

This analysis would first benefit from determining if the rescue of fertility (*section 4.4.6*) for heterozygotes of *Δmir* targets: *Df(3R)P109/*Δ*mir* and *hth[P2]/*Δ*mir*, as well as the induction of infertility (*section 4.4.8*) through the expression of *UAS-Ubx* in *fru*-expressing neurons (using *fru-P1-GAL4*) correspond to a rescue or induction of SP retention. Single mating of females of either genotype (*Df(3R)P109/*Δ*mir* and *hth[P2]/*Δ*mir*) to males expressing SP-GFP and determining loss of GFP, through the cuticle of the abdomen, 24hours post-mating would suffice us to determine if SP retention is regulated through *Ubx* derepression in *fru*-neurons. These studies would test whether all phenotypes that rescue or recapitulate the *Δmir* phenotype correlate with the extent of SP retention in the reproductive tract.

Both phenotypes observed in *Δmir* mutants suggest defects in neuronal populations controlling receptivity and SP retention; however these populations remain unidentified. In *section 5.5.2* we propose a screen to identify novel neuronal populations controlling female receptivity, this screen would take advantage of the Janelia Farm enhancer fragments collection (Pfeiffer et al., 2008) to drive *UAS-Ubx* expression in distinct, sometimes overlapping, subsets of fru^{+} neurons. This screen could be expanded to identify neuronal subsets that control sex peptide release into the hemolymph. Candidates would be chosen based on either defects in receptivity determined by single pair mattings; or by the observation of SP-GFP retention through the cuticle of mated females. Of the Janelia enhancer fragments tiling the entirety of the *fru* locus approximately 13 have discrete expression patterns in the abdominal ganglion that reflect endogenous *fru* expression and are considered candidate enhancers for this proposed screen.

Although many unresolved questions remain from this work, I believe it provides a solid and highly novel position from which to examine female-specific neuronal circuits and their dimorphic emergence. The ultimate goal is to understand the intrinsic influence that sex has on the development and function of the nervous system. As studies in females have been underrepresented in the literature to date, the work presented in this thesis now provides the data and models to fill that gap in the future, and very importantly, shows that analysis of what is NOT a male circuit or behavior in the female is not a proxy for understanding what IS a female circuit or behavior.

BIBLIOGRAPHY

Abel, J., Witt, D., Rissman, E., 2011. Sex differences in the cerebellum and frontal cortex: Roles of estrogen receptor alpha and sex chromosome genes. Neuroendocrinology 93, 230-240.

Abel, K., Drake, R., Goldstein, J., 2010. Sex differences in schizophrenia. International review of Psychiatry 22, 417-428.

Aigaki, T., Fleischmann, I., Chen, P., Kubli, E., 1991. Ectopic expression of sex peptide alters reproductive behavior of female D. melanogaster. Neuron 7, 557-563.

Anand, A., Villella, A., Ryner, L., Carlo, T., Goodwin, S., Song, H., Gailey, D., Morales, A., Hall, J., Baker, B., Taylor, B., 2001. Molecular genetic dissection of the sex-specific and vital functions of the *Drosophila melanogaster* sex determination gene *fruitless*. Genetics 158, 1569-1595.

Arbeitman, M., Winbush, A., 2010. Neurogenetics: Short-Circuiting Sexually Dimorphic Behaviors. Current Biology 20, R804-R806.

Arnold, A., 2004. Sex chromosomes and brain gender. Nature Reviews: Neuroscience 5, 1-8.
Bairati, A., 1967. The structure and ultrastructure of the male genital apparatus of the *Drosophila melanogaster* Meig. 1 The testis. Z Xellforsch Mikrosk Anat 76, 56-99.

Baker, B., Ridge, K., 1989. Sex in flies: the splice of life. Nature 340, 521-524.

Balciuniene, J., Bardwell, V., Zarkower, D., 2006. Mice mutant in the DM domain gene *Dmrt4* are viable and fertile but have polyovular follicles. Molecular and Cellular Biology 26, 8984-8991.

Barnes, L., Wilson, R., Bienias, J., Schneider, J., Evans, D., Bennett, D., 2005. Sex differences in the clinical manifestations of alzheimer disease pathology. Archives of general psychiatry 62, 685-691.

Becker, J., Hu, M., 2008. Sex differences in drug abuse. Frontiers in Neuroendocrinology 29, 36-47.

Bender, W., 2008. MicroRNAs in the *Drosophila* bithorax complex. Genes and Development 22, 14-19.

Bender, W., Hudson, A., 2000. P element homing to the *Drosophila* bithorax complex. Development 127, 3981-3992.

Billeter, J., Goodwin, S., 2004. Characterization of *Drosophila fruitless-gal4* transgenes reveals expression in male-specific *fruitless* neurons and innervation of male reproductive structures. The Journal of Comparative Neurology 475, 270-287.

Billeter, J., Rideout, E., Dornan, A., Goodwin, S., 2006a. Control of male sexual behavior in *Drosophila* by the sex determination pathway. Current Biology 16, R766-R776.

Billeter, J., Villella, A., Allendorfer, J., Dornan, A., Richardson, M., Gailey, D., Goodwin, S., 2006b. Isoform-specific control of male neuronal differentiation and behavior in *Drosophila* by the *fruitless* gene. . Current Biology 16, 1063-1076.

Birkholz, O., Rickert, C., Berger, C., Urbach, R., Technau, G.M., 2013. Neuroblast pattern and identity in the Drosophila tail region and role of doublesex in the survival of sex-specific precursors. Development 140, 1830-1842.

Bove, R., Chitnis, T., 2013. Sexual disparities in the incidence and course of MS. Clinical Immunology 149, 201-210.

Broihier, H., Kuzin, A., Zhu, Y., Odenwald, W., Skeath, J., 2004. *Drosophila* homeodomain protein Nkx6 coordinates motorneuron subtype identity and axonogenesis. Development 131, 5233-5242.

Bryant, P.J., 1978. Pattern formation in imaginal discs. . London: Academic Press.

Burtis, K., Baker, B., 1989. Drosophila *doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell 56, 997-1010.

Cachero, S., Ostrovsky, A., Yu, J., Dickson, B., Jefferis, G., 2010. Sexual dimorphism in the fly brain. Current Biology 20, 1589-1601.

Cahill, L., 2006. Why sex matters for neuroscience? Nature Reviews: Neuroscience 6, 477-484.

Castellanos, M.C., Tang, J.C., Allan, D.W., 2013. Female-biased dimorphism underlies a female-specific role for post-embryonic Ilp7 neurons in Drosophila fertility. Development 140, 3915-3926.

Chan, Y., Kravitz, E., 2007. Specific subgroups of FruM neurons control sexually dimorphic patterns of aggression in *Drosophila melanogaster*. Proceedings of the National Academy of Sciences 104, 19577-19582.

Chase, B., Baker, B., 1995. A genetic analysis of *intersex*, a gene regulating sexual differentiation in *Drosophila melanogaster* females. Genetics 139, 1649-1661.

Chen, P., Stumm-Zollinger, E.A., T., Balmer, J., Blenz, M., Böhlen, P., 1988. A male accessory gland peptide that regulates reproductive behavior of female D. melanogaster. Cell 54, 291-298.

Choi, Y., Lee, G., Park, J., 2006. Programmed cell death mechanisms of identifiable peptidergic neurons in *Drosphila melanogaster*. Development 133, 2223-2232.

Chung, W., Swaab, D., De Vries, G., 2000. Apoptosis during sexual differentiation of the bed nucleus of the stria terminalis in the rat brain. J Neurobiol 43, 234-243.

Clayton, J., Collins, F., 2014. NIH to balance sex in cell and animal studies. Nature 509, 282-283.

Cognigni, P., Bailey, A., Miguel-Aliaga, I., 2011. Enteric neurons and systemic signals couple nutritional and reproductive status with intestinal homeostasis. Cell Metabolism 13, 92-104.

Cole, S., Carney, G., McClung, C., Willard, S., Taylor, B., Hirsh, J., 2005. Two functional but noncomplementing *Drosophila* tyrosine decarboxylase genes. The Journal of Biological Chemistry 280, 14948-14955.

Cooke, B., Hegstrom, C., Villeneuve, L. , Breedlove, S., 1998. Sexual differentiation of the vertebrate brain: principles and mechanisms. Frontiers in Neuroendocrinology 19, 323-362.

Coschigano, K., Wensink, P., 1993. Sex-specific transcriptional regulation by male and female doublesex proteins of *Drosophila*. Genes and Development 7, 42-54.

Dalton, J., Fear, J., Knott, S., Baker, B., McIntyre, L., Arbeitman, M., 2013. Male-specific fruitless isoforms have different regulatory roles conferred by distinct zinc finger DNA binding domains. BMC Genomics 14, 1-15.

Dauwalder, B., 2011. The roles of *fruitless* and *doublesex* in the control of male courtship. International review of Neurobiology 99, 87-105.

De Vries, G., Rissman, E., Simerly, R., Yang, L., Scordalakes, E., Auger, C., Swain, A., Lovell-Badge, R., Burgoyne, P., Arnold, A., 2002. A model system for study of sex chromosomes effects on sexually dimorphic neural and behavioral traits. The Journal of Neuroscience 22, 9005-9014.

del Valle Rodriguez, A., Didiano, D., Desplan, C., 2012. Power tools for gene expression and clonal analysis in *Drosophila*. Nature Methods 9, 47-55.

Demir, E., Dickson, B., 2005. *fruitless* splicing specifies male courtship behavior in *Drosophila*. Cell 121, 785-794.

Dewing, P., Chiang, C., Sinchak, K., Sim, H., Fernagut, P., Kelly, S., Chesselet, M., Micevych, P., Albrecht, K., Harley, V., Vilain, E., 2006. Direct regulation of adult brain function by male-specific factor SRY. Current Biology 16, 415-420.

DiAntonio, A., 2006. Glutamate receptors at the Drosophila neuromuscular junction. Int Rev Neurobiol 75, 165-179.

Duboule, D., Morata, G., 1994. Colinearity and functional hierarchy among genes of the homeotic complexes. Trends in genetics : TIG 10, 358-364.

Dulac, C., Kimchi, T., 2007. Neural mechanisms underlying sex-specific behaviors in vertebrates. Current Opinion in Neurobiology 17, 675-683.

Eade, K., Allan, D., 2009. Neuronal phenotype in the mature nervous system is maintained by persisten retrograde bone morphogenetic protein signaling. Journal of Neuroscience 29, 3852-3864.

Fan, Y., Bergmann, A., 2010. The cleaved-Caspase-3 antibody is a marker of Caspase-9-like DRONC acitivity in *Drosophila*. Cell death and differentiation 17, 534-539.

Feinberg, E., VanHoven, M.B., A., Wang, G., Fetter, R., Shen, K., Bargmann, C., 2008. GFP reconstitution across synaptic partners (GRASP) defines cell contacts and synapses in living nervous systems. Neuron 57, 353-363.

Ferri, S., Bohm, R., Lincicome, H., Hall, J., Villella, A., 2008. *fruitless* gene products truncated of their male-like qualities promote neural and behavioral maleness in *Drosophila* if these proteins are produced in the right places at the right times. Journal of Neurogenetics 22, 17-55.

Ferveur, J., 2010. Drosophila female courthsip and mating behaviors: sensory signals, genes, neural structures and evolution. Current Opinion in Neurobiology 20, 1-6.

Finley, K., Edeen, P., Foss, M., Gross, E., Ghbeish, N., Palmer, R., Taylor, B., McKeown, M., 1998. *dissatisfaction* encodes a Tailles-like nuclear receptor expressed in a subset of CNS nuerons controlling *Drosophila* sexual behavior. Neuron 21, 1363-1374.

Finley, K., Taylor, B., Milstein, M., McKeown, M., 1997. *dissatisfaction,* a gene involved in sex-specific behavior and neural development of *Drosophila melanogaster*. Proceedings of the National Academy of Sciences 94, 913-918.

Forger, N., 2009. Control of cell number in the sexually dimorphic brain and spinal cord. Journal of Neuroendocrinology 21, 393-399.

Forger, N., De Vries, G., 2010. Cell death and sexual differentiation of behavior: worms flies, and mammals. Current Opinion in Neurobiology 20, 776-783.

Galanopoulou, A., 2005. GABA A Receptors as broadcasters of sexually differentiating signals in the brain. Epilepsia 46, 107-112.

Garaulet, D., Castellanos, M., Bejarano, F., Sanfilippo, P., Tyler, D., Allan, D., Sánchez-Herrero, E., Lai, E., 2014. Homeotic function of *Drosophila* bithorax-complex miRNAs mediates fertility by restricting multiple hox genes and TALE cofactors in the CNS. Developmental Cell 29, 635-648.

Garrett-Engele, C., Siegal, M., Manoli, D., Williams, B., Li, H., Baker, B., 2002. *intersex*, a gene required for female sexual development in *Drosophila,* is expressed in both sexes and functions together with *doublesex* to regulate terminal differentiation. Development 129, 4661-4675.

Gatewood, J., Wills, A., Shetty, S., Xu, J., Arnold, A., Burgoyne, P., Rissman, E., 2006. Sex chromosome complement and gonadal sex influence aggresive and parental behaviors in mice. The Journal of Neuroscience 26, 2335-2342.

Gatti, S., Ferveur, J., Martin, J., 2000. Genetic identification of neurons controlling a sexually dimorphic behaviour. Current Biology 10.

Goldman, T., Arbeitman, M., 2007. Genomic and functional studies of *Drosophila* sex hierarchy regulated gene expression in the adult head and nervous system tissue. PLOS Genetics 3, e216.

Gonzalez-Reyes, A., Morata, G., 1990. The developmental effect of overexpressing a Ubx product in Drosophila embryos is dependent on its interactions with other homeotic products. Cell 61, 515-522.

Goodwin, S., Taylor, B., Villella, A., Foss, M., Ryner, L., Baker, B., Hall, J., 2000. Aberrant splicing and altered spatial expression patterns in *fruitless* mutants of *Drosophila melanogaster*. Genetics 154, 725-745.

Gordon, M., Scott, K., 2009. Motor Control in a *Drosophila* Taste Circuit. Neuron 61, 373-384.

Grönke, S., Clarke, D., Broughton, S., Andrews, D., Patridge, L., 2010. Molecular evolution and functional characterization of *Drosophila* Insulin-like peptides. PLOS Genetics 6, 1-18.

Guan, B., Hartmann, B., Kho, Y.H., Gorczyca, M., Budnik, V., 1996. The Drosophila tumor suppressor gene, dlg, is involved in structural plasticity at a glutamatergic synapse. Curr Biol 6, 695-706.

Gummalla, M., Maeda, R.K., Castro Alvarez, J.J., Gyurkovics, H., Singari, S., Edwards, K.A., Karch, F., Bender, W., 2012. abd-A Regulation by the iab-8 Noncoding RNA. PLoS Genet 8, e1002720.

Hafen, E., Levine, M., Gehring, W.J., 1984. Regulation of Antennapedia transcript distribution by the bithorax complex in Drosophila. Nature 307, 287-289.

Hasemeyer, M., Yapici, N., Heberlein, U., Dickson, B., 2009. Sensory neurons in the *Drosophila* genital tract regulate female reproductive behavior. Neuron 61, 511-518.

Haussman, I., Hemani, Y., Wijesekera, T., Dauwalder, B., Soller, M., 2013. Multiple pathways mediate the sex-peptide regulated switch in female *Drosophila* reproductive behaviors. Proceedings of the Royal society B 280.

Hay, B., Wolff, T., Rubin, G., 1994. Expression of baculovirus P35 prevents cell death in *Drosophila*. Development 120, 2121-2129.

Heinrichs, V., Ryner, L., Baker, B., 1998. Regulation of sex-specific selection of *fruitless* 5' splice sites by *transformer* and *transformer-2*. Molecular and Cellular Biology 18, 450-458.

Herndon, L., Wolfner, M., 1995. A *Drosophila* seminal fluid protein, Acp26Aa, stimulates egg laying in females for 1 day after mating. Proceedings of the National Academy of Sciences 92, 10114-10118.

Heuer, J.G., Kaufman, T.C., 1992. Homeotic genes have specific functional roles in the establishment of the Drosophila embryonic peripheral nervous system. Development 115, 35-47.

Hodgkin, J., 1990. Sex determination compared in *Drosophila* and *Caenorhabditis*. Nature 344, 721-728.

Hong, C., Park, B., Saint-Jeannet, J., 2007. The function of *Dmrt* genes in vertebrate development: it is not just about sex. Developmental Biology 310, 1-9.

Hornstein, E., Mansfield, J.H., Yekta, S., Hu, J.K., Harfe, B.D., McManus, M.T., Baskerville, S., Bartel, D.P., Tabin, C.J., 2005. The microRNA miR-196 acts upstream of Hoxb8 and Shh in limb development. Nature 438, 671-674.

Huang, J., Zhou, W., Dong, W., Watson, A., Hong, Y., 2009. Directed, efficient, and versatile modifications of the *Drosophila* genome by genomic engineering. Proceedings of the National Academy of Sciences 106, 8284-8289.

Hudson, A., Petrella, L., Tanaka, A., Cooley, L., 2008. Mononuclear muscle cells in Drosophila ovaries revealed by GFP protein traps. Developmental Biology 314, 329-340.

Jazin, E., Cahill, L., 2010. Sex differences in molecular neuroscience: from fruit flies to humans. Nature Reviews: Neuroscience 11, 9-17.

Jia, X.X., Gorczyca, M., Budnik, V., 1993. Ultrastructure of neuromuscular junctions in Drosophila: comparison of wild type and mutants with increased excitability. J Neurobiol 24, 1025-1044.

Juni, N., Yamamoto, D., 2009. Genetic analysis of *chaste*, a new mutation of *Drosophila melanogaster* characterized by extremely low female sexual receptivity. Journal of Neurogenetics 23, 329-340.

Kapelnikov, A., Rivlin, P., Hoy, R., Heifetz, Y., 2008. Tissue remodeling: a mating-induced differentiation program for the *Drosophila* oviduct. BMC Developmental Biology 8.

Kellerman, K., Mattson, D., Duncan, I., 1990. Mutations affecting the stability of the *fushi tarazu* protein of *Drosophila*. Genes and Development 4, 1936-1950.

Kilts, C., Gross, R., Ely, T., Drexler, K., 2004. The neural correlates of cue-induced craving in cocaine-dependent women. The American Journal of Psychiatry 161, 233-241.

Kimura, K., 2011. Role of cell death in the formation of sexual dimorphism in the *Drosophila* central nervous system. Development, Growth & Differentiation 53, 236-244.

Kimura, K., Hachiya, T., Koganezawa, M., Tazawa, T., Yamamoto, D., 2008. Fruitless and Doublesex coordinate to generate male-specific neurons that can initiate courtship. Neuron 59.

Kimura, K., Ote, M., Tazawa, T., Yamamoto, D., 2005. Fruitless specifies sexually dimorphic neural circuitry in the *Drosophila* brain. Nature 438, 229-233.

Klein, L., Corwin, E., 2002. Seeing the unexpected: how sex differences in stress responses may provide a new perspective of psychiatric disorders. Current Psychiatry Reports 4, 441-448.

Kohatsu, S., Koganezawa, M., Yamamoto, D., 2011. Female contact activates male-specific interneurons that trigger stereotypic courtship behavior in *Drosophila*. Neuron 69, 498-508.

Kopp, A., 2012. *Dmrt* genes in the development and evolution of sexual dimorphism. Trends in Genetics 28, 175-184.

Kosman, D., Small, S., Reinitz, J., 1998. Rapid preparation of a panel of polyclonal antibodies to *Drosophila* segmentation proteins. Develoment Genes and Evolution 208, 290-294.

Kozopas, K.M., Samos, C.H., Nusse, R., 1998. DWnt-2, a Drosophila Wnt gene required for the development of the male reproductive tract, specifies a sexually dimorphic cell fate. Genes Dev 12, 1155-1165.

Kubli, E., 2003. Sex-peptides: seminal peptides of the *Drosophila* male*. Cellular and Molecular Life Sciences 60, 1689-1704.

Kurtovic, A., Widmer, A., Dickson, B., 2007. A single class of olfactory neurons mediates behavioural responses to a *Drosophila* sex pheromone. Nature 446, 542-546.

Lalli, E., Ohe, K., Latorre, E., Bianchi, M., Sassone-Corsi, P., 2003. Sexy splicing: regulatory interplays governing sex determination from *Drosophila* to mammals. Journal of Cell Science 116, 441-445.

Lamka, M.L., Boulet, A.M., Sakonju, S., 1992. Ectopic expression of UBX and ABD-B proteins during Drosophila embryogenesis: competition, not a functional hierarchy, explains phenotypic suppression. Development 116, 841-854.

Lazareva, A., Roman, G., Mattox, W., Hardin, P., Dauwalder, B., 2007. A role for the adult fat body in *Drosophila* male courtship behavior. PLOS Genetics 3, 0115-0122.

Lee, G., Foss, M., Goodwin, S., Carlo, T., Taylor, B., Hall, J., 2000. Spatial, temporal, and sexually dimorphic expression patterns of the *fruitless* gene in the *Drosophila* central nervous system. Journal of Neurobiology 43, 404-426.

Lee, G., Hall, J., 2001. Abnormalities of male-specific FRU proteins and serotonin expression in the CNS of *fruitless* mutants in *Drosophila*. Journal of Neuroscience 21, 513-526.

Lee, G., Hall, J., Park, J., 2002. *Doublesex* gene expression in the central nervous system of *Drosophila melanogaster*. Journal of Neurogenetics 16, 229-248.

Lee, G., Sehgal, R., Wang, Z., Nair, S., Kikuno, K., Chen, C., Hay, B., Park, J., 2013. Essential role of *grim*-led programmed cell death for the establishment of corazonin-producing peptidergic nervous system during embryogenesis and metamorphosis in *Drosophila melanogaster*. Biology Open 2, 283-294.

Lee, G., Villella, A., Taylor, B., Hall, J., 2001. New reproductive anomalies in *fruitless*-mutant *Drosophila* males: extreme lengthening of mating durations and infertility correlated with defective serotonergic innervation of reproductive organs. Journal of Neurobiology 47, 121-149.

Lee, H., Rohila, S., Han, K., 2009. The Octopamine receptor OAMB mediates ovularion via Ca+2/Calmpdulin-dependent protein kinase II in the *Drosophila* oviduct epithelium. PLOS One 4, 1-9.

Lemons, D., Pare, A., McGinnis, W., 2012. Three Drosophila Hox complex microRNAs do not have major effects on expression of evolutionarily conserved Hox gene targets during embryogenesis. PLoS ONE 7, e31365.

Lewis, E.B., 1978. A gene complex controlling segmentation in Drosophila. Nature 276, 565-570.

Li, H., Baker, B., 1998a. her, a gene required for sexual differentiation in *Drosophila,* encodes a zinc finger protein with characteristics of ZFY-like proteins and is expressed independently of the sex determination hierarchy. Development 125, 225-235.

Li, H., Baker, B., 1998b. *hermaphrodite* and *doublesex* function both dependently and independently to control various aspects of sexual differentiation in *Drosophila*. Development 125, 2641-2651.

Longair, M.H., Baker, D.A., Armstrong, J.D., 2011. Simple Neurite Tracer: open source software for reconstruction, visualization and analysis of neuronal processes. Bioinformatics 27, 2453-2454.

Lynch, W., Roth, M., Carroll, M., 2002. Biological basis of sex differences in drug abuse: preclinical and clinical studies. Psychopharmacology 164, 121-137.

Mahr, A., Aberle, H., 2006. The expression pattern of the *Drosophila* vesicular glutamate transporter: A marker protein for motorneurons and glutamatergic centers in the brain. Gene and Expression Patterns 6, 299-309.

Mann, R.S., Lelli, K.M., Joshi, R., 2009. Hox specificity unique roles for cofactors and collaborators. Curr Top Dev Biol 88, 63-101.

Manoli, D., Fan, P., Fraser, E., Shah, N., 2013. Neural control of sexually dimorphic behaviors. Current Opinion in Neurobiology 23, 330-338.

Manoli, D., Foss, M., Villella, A., Taylor, B., Hall, J., Baker, B., 2005. Male-specific *fruitless* specifies the neural substrates of *Drosophila* courtship behaviour. Nature 436, 395-400.

Marrus, S.B., Portman, S.L., Allen, M.J., Moffat, K.G., DiAntonio, A., 2004. Differential localization of glutamate receptor subunits at the Drosophila neuromuscular junction. J Neurosci 24, 1406-1415.

Masuyama, K., Zhang, Y., Rao, Y., Wang, J., 2012. Mapping neural circuits with activity-dependent nuclear import of a transcription factor. Journal of Neurogenetics 26, 89-102.

McCarthy, M., 2008. Estradiol and the developing brain. Physiological reviews 88, 91-124.

McCarthy, M., A., A., 2011. Reframing sexual differentiation of the brain. Nature Neuroscience 14, 677-683.

McGuire, S., Mao, Z., Davis, R., 2004. Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in *Drosophila*. Science STKE 220, pl6.

Mellert, D., Knapp, J., Manoli, D., Meissner, G., Baker, B., 2010. Midline crossing by gustatory receptor neuron axons is regulated by *fruitless, doublesex* and the Roundabout receptors. Development 137, 323-332.

Middleton, C.N., U., Parry, K., Sweeny, S., Sparrow, J., Elliott, C., 2006. Neuromuscular organization and aminergic modulation of contractions in the *Drosophila* ovary. BMC Biology 4.

Miguel-Aliaga, I., Thor, S., 2004. Segment-specific prevention of pioneer neuron apoptosis by cell-autonomous, postmitotic hox gene activity. Development 131, 6093-6105.

Miguel-Aliaga, I., Thor, S., Gould, A., 2008. Postmitotic specification of *Drosophila* Insulingergic neurons from pioneer neurons. PLOS Biology 6, 0001-0014.

Miller, V., 2012. In pursuit of scientific excellence: sex matters. Advances in Physiological Education 36, 83-84.

Miska, E.A., Alvarez-Saavedra, E., Abbott, A.L., Lau, N.C., Hellman, A.B., McGonagle, S.M., Bartel, D.P., Ambros, V.R., Horvitz, H.R., 2007. Most Caenorhabditis elegans microRNAs Are Individually Not Essential for Development or Viability. PLoS Genet 3, e215.

Miyamoto, T., Amrein, H., 2008. Suppression of male courtship by a *Drosophila* pheromone receptor. Nature Neuroscience 11, 874-876.

Monastirioti, M., 2003. Distinct octopamine cell population residing in the CNS abdominal ganglion controls ovulation in *Drosophila melanogaster*. Developmental Biology 264, 38-49.

Monastirioti, M., Gorczyca, M., Rapus, J., Eckert, M., White, K., Budnik, V., 1995. Octopamine Immunoreactivity in the Fruit Fly *Drosophila melanogaster*. The Journal of Comparative Neurology 356, 275-287.

Monastirioti, M., Linn, C., White, K., 1996. Characterization of *Drosophila Tyramine β-Hydroxylase* Gene and Isolation of Mutant flies lacking Octopamine. The Journal of Neuroscience 16, 3900-3911.

Morris, J., Jordan, C., Breedlove, M., 2004. Sexual differentiation of the vertebrate nervous system. Nature Neuroscience 7, 1035-1039.

Neville, M., Nojima, T., Ashley, E., Parker, D., Walker, J., Southall, T., Van de Sande, B., Marques, A., Fischer, B., Brand, A., Russel, S., Ritchie, M., Aerts, S., Goodwin, S., 2014. Male-specific fruitless isoforms target neurodevelopmental genes to specify a sexually dimorphic nervous system. Current Biology 24, 229-241.

Nitabach, M., Wu, Y., Sheeba, V., Lemon, W., Strumbos, J., Zelensky, P., White, B., Holmes, T., 2006. Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and includes mutliple behavioral periods. The Journal of Neuroscience 26, 479-489.

Nojima, T., Kimura, K., Koganezawa, M., Yamamoto, D., 2010. Neuronal synaptic outputs determine the sexual fate of postsynaptic targets. Current Biology 20, 836-840.

Noro, B., Lelli, K., Sun, L., Mann, R.S., 2011. Competition for cofactor-dependent DNA binding underlies Hox phenotypic suppression. Genes Dev 25, 2327-2332.

O'Kane, C., Asztalos, Z., 1999. Sexual behaviour: Courting dissatisfaction. Current Biology 9, R289-R292.

Ogienko, A., Fedorova, S., Baricheva, E., 2007. Basic aspects of ovarian development in *Drosophila melanogaster*. Russian Journal of Genetics 43, 1120-1134.

Parkhurst, S., Meneely, P., 1994. Sex determination and dossage compensation: lessons from flies and worms. Science 264, 924-932.

Parks, A., Cook, K., Belvin, M., Dompe, N., Fawcett, R., Huppert, K., Tan, L., Winter, C., Bogart, K., Deal, J., Deal-Herr, M., Grant, D., Marcinko, M., Miyazaki, W., Robertson, S., Shaw, K., Tabios, M., Vysotskaia, V., Zhao, L., Andrade, R., Edgar, K., Howie, E., Killpack, K., Milash, B., Norton, A., Thao, D., Whittaker, K., Winner, M., Friedman, L., Margolis, J., Singer, M., Kopczynski, C., Curtis, D., Kaufman, T., Plowman, G., Duyk, G., Francis-Lang, H., 2004. Systematic generation of high-resolution deletion coverage of the *Drosophila melanogaster* genome. Nature Genetics 36, 288-292.

Parnas, D., Haghighi, A.P., Fetter, R.D., Kim, S.W., Goodman, C.S., 2001. Regulation of postsynaptic structure and protein localization by the Rho-type guanine nucleotide exchange factor dPix. Neuron 32, 415-424.

Paus, T., 2010. Sex differences in the human brain: a developmental perspective. Progress in Brain Research 186.

Pearson, J.C., Lemons, D., McGinnis, W., 2005. Modulating Hox gene functions during animal body patterning. Nat Rev Genet 6, 893-904.

Peng, J., Chen, S., Büsser, S., Liu, H., Honegger, T., Kubli, E., 2005. Gradual release of sperm bound sex-peptide controls female postmating behavior in *Drosophila*. Current Biology 15, 207-213.

Pfeiffer, B., Jenett, A., Hammonds, A., Ngo, T., Misra, S., Murphy, C., Scully, A., Carlson, J., Wan, K., Laverty, T., Mungall, C., Svirskas, R., Kadonaga, J., Doe, C., Eisen, M., Celniker, S., Rubin, G., 2008. Tools for neuroanatomy and neurogenetics in *Drosophila*. Proceedings of the National Academy of Sciences of the United States of America 105, 9715-9720.

Pfeiffer, B., Truman, J., Rubin, G., 2012. Using translational enhancers to increase transgene expression in *Drosophila*. Proceedings of the National Academy of Sciences 109, 6626-6631.

Pierce, S.B., Costa, M., Wisotzkey, R., Devadhar, S., Homburger, S.A., Buchman, A.R., Ferguson, K.C., Heller, J., Platt, D.M., Pasquinelli, A.A., Liu, L.X., Doberstein, S.K., Ruvkun, G., 2001. Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse C. elegans insulin gene family. Genes Dev 15, 672-686.

Portman, D., 2007. Genetic control of sex differences in *C. elegans* neurobiology and behavior. Advances in Genetics 59, 1-37.

Prokop, A., 2006. Organization of the efferent system and structure of neuromuscular junctions in Drosophila. Int Rev Neurobiol 75, 71-90.

Pultz, M., Baker, B., 1995. The dual role of *hermaphrodite* in the *Drosophila* sex determination regulatory hierarchy. Development 121, 99-111.

Pultz, M., Carson, G., Baker, B., 1994. A genetic analysis of *hermaphrodite,* a pleiotropic sex determination gene in *Drosophila melanogaster*. Genetics 136, 195-207.

Pulver, S., Pashkovski, S., Hornstein, N., Garrity, P., Griffith, L., 2009. Temporal dynamics of neuronal activation by channelrhodopsin-2 and TRPA1 determine behavioral output in *Drosophila* larvae. Journal of Neurophysiology 101, 3075-3088.

Reisert, I., Pilgrim, C., 1991. Sexual differentiation of monoaminergic neurons-genetic or epigenetic? TRENDS in Neurosciences 14, 468-473.

Rezával, C., Nojima, T., Neville, M., Lin, A., Goodwin, S., 2014. Sexually dimorphic octopaminergic neurons modulate female specific postmating behaviors in *Drosophila*. Current Biology 24, 725-730.

Rezával, C., Pavlou, H., Dornan, A., Chan, Y., Kravitz, E., Goodwin, S., 2012. Neural circuitry underlying *Drosophila* female postmating behavioral responses. Current Biology 22, 1-11.

Rideout, E., Billeter, J., Goodwin, S., 2007. The sex-determination genes *fruitless* and *doublesex* specify a neural substrate required for courtship song. Current Biology 04, 1473-1478.

Rideout, E., Dornan, A., Neville, M., Eadie, S., Goodwin, S., 2010. Control of sexual differentiation and behavior by the *doublesex* gene in *Drosophila melanogaster*. Nature Neuroscience 13, 458-467.

Robinett, C., Vaughan, A., Knapp, J., Baker, B., 2010. Sex and the single cell. II There is a time and a place for sex. PLOS Biology 8, 1-16.

Rodríguez-Valentín, R., López-González, I., Jorquera, R., Labarca, P., Zurita, M., Reynaud, E., 2006. Oviduct contraction in *Drosophila* is modulated by a neural network that is both, octopaminergic and glutamatergic. Journal of Cellular Physiology 209, 183-193.

Ronshaugen, M., Biemar, F., Piel, J., Levine, M., Lai, E.C., 2005. The Drosophila microRNA iab-4 causes a dominant homeotic transformation of halteres to wings. Genes Dev 19, 2947-2952.

Rubinstein, C., Wolfner, M., 2013. *Drosophila* seminal protein ovulin mediates ovulation through female octopamine neuronal signaling. Proceedings of the National Academy of Sciences 110, 17420-17425.

Ryner, L., Goodwin, S., Castrillon, D., Anand, A., Villella, A., Baker, B., Hall, J., Taylor, B., Wasserman, S., 1996. Control of male sexual behavior and sexual orientation in Drosophila by the *fruitless gene*. Cell 87, 1079-1089.

Sakurai, A., Koganezawa, M., Yasunaga, K., Emoto, K., Yamamoto, D., 2013. Select interneuron clusters determine female sexual receptivity in *Drosophila*. Nature Communications 4, 1-9.

Salvemini, M., Polito, C., Saccone, G., 2010. *fruitless* alternative splicing and sex behaiour in insects: an ancient and unforgettable love story? Journal of Genetics 89, 287-299.

Salz, H.K., Erickson, J.W., 2010. Sex determination in Drosophila: The view from the top. Fly (Austin) 4, 60-70.

Sanchez, L., Gorfinkiel, N., Guerrero, I., 2001. Sex determination genes control the development of the Drosophila genital disc, modulating the response to Hedgehog, Wingless and Decapentaplegic signals. Development 128, 1033-1043.

Sánchez-Herrero, E., Vernos, I., Marco, R., Morata, G., 1985. Genetic organization of Drosophila bithorax complex. Nature 313, 108-113.

Sanders, L., Arbeitman, M., 2008. Doublesex establishes sexual dimorphism in the *Drosophila* central nervous system in an isoform-dependent manner by directing cell number. Develomental Biology 320, 378-390.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J., White, D., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open source plataform for biological-image analysis. Nature Methods 9, 676-682.

Shirangi, T., McKeown, M., 2007. Sex in flies: What 'body-mind' dichotomy? Developmental Biology 306, 10-19.

Sieburth, D., Ch'ng, Q., Dybbs, M., Tavazoie, M., Kennedy, S., Wang, D., Dupuy, D., Rual, J.F., Hill, D.E., Vidal, M., Ruvkun, G., Kaplan, J.M., 2005. Systematic analysis of genes required for synapse structure and function. Nature 436, 510-517.

Siegal, M., Baker, B., 2005. Functional conservation and divergence of *intersex*, a gene required for female differentiation in *Drosophila melanogaster*. Develoment Genes and Evolution 215, 1-12.

Simerly, R., Zee, M., Pendleton, J., Lubahn, D., Korach, K., 1997. Estrogen receptor-dependent sexual differentiation of dopaminergic neurons in the preoptic region of the mouse. . Proceedings of the National Academy of Sciences of the United States of America 94, 14077-14082.

Slattery, M., Riley, T., Liu, P., Abe, N., Gomez-Alcala, P., Dror, I., Zhou, T., Rohs, R., Honig, B., Bussemaker, H.J., Mann, R.S., 2011. Cofactor Binding Evokes Latent Differences in DNA Binding Specificity between Hox Proteins. Cell 147, 1270-1282.

Smibert, P., Lai, E.C., 2008. Lessons from microRNA mutants in worms, flies and mice. Cell Cycle 7, 2500-2508.

Soller, M., Haussman, I., Hollmann, M., Choffat, Y., White, K., Kubli, E., Schäfer, M., 2006. Sex-peptide-regulated female sexual behavior requires a subset of ascending ventral nerve cord neurons. . Current Biology 16, 1771-1782.

Stark, A., Bushati, N., Jan, C.H., Kheradpour, P., Hodges, E., Brennecke, J., Bartel, D.P., Cohen, S.M., Kellis, M., 2008. A single Hox locus in Drosophila produces functional microRNAs from opposite DNA strands. Genes Dev 22, 8-13.

Stockinger, P., Kvitsiani, D., Rotkopf, S., Tirian, L., Dickson, B., 2005. Neural circuitry that governs *Drosophila* male courtship behavior. Cell 121, 795-807.

Struhl, G., Basler, K., 1993. Organizing Activity of Wingless Protein in Drosophila. Cell 72, 527-540.

Struhl, G., White, R.A., 1985. Regulation of the Ultrabithorax gene of Drosophila by other bithorax complex genes. Cell 43, 507-519.

Susic-Jung, L., Hornbruch-Freitag, C., Kuckwa, J., Rexer, K.H., Lammel, U., Renkawitz-Pohl, R., 2012. Multinucleated smooth muscles and mononucleated as well as multinucleated striated muscles develop during establishment of the male reproductive organs of Drosophila melanogaster. Dev Biol 370, 86-97.

Swaab, D., Ching, W., Kruijver, F., Hofman, M., Ishunina, T., 2001. Structural and functional differences in the human hypothalamus. Hormones and behavior 40, 93-98.

Taylor, B.J., Truman, J.W., 1992. Commitment of abdominal neuroblasts in Drosophila to a male or female fate is dependent on genes of the sex-determining hierarchy. Development 114, 625-642.

Tompkins, L., Hall, J., 1983. Identification of brain sites controlling female receptivity in mosaics of *Drosophila melanogaster* Genetics 103, 179-195.

Truman, J., 1990. Metamorphosis of the central nervous system of *Drosophila*. Journal of Neurobiology 21, 1072-1084.

Tyler, D.M., Okamura, K., Chung, W.J., Hagen, J.W., Berezikov, E., Hannon, G.J., Lai, E.C., 2008. Functionally distinct regulatory RNAs generated by bidirectional transcription and processing of microRNA loci. Genes Dev 22, 26-36.

Usui-Aoki, K., Ito, H., Ui-Tei, K., Takahashi, K., Lukacsovich, T., Awano, W., Nakata, H., Piao, Z., Nilsson, E., Tomida, J., Yamamoto, D., 2000. Formation of the male-specific muscle in female *Drosophila* by ectopic *fruitless* expression. Nature Cell Biology 2, 500-506.

Verhulst, E., van de Zande, L., Beukeboom, 2010. Insect sex determination: it all evolves around *transformer*. Current Opinion in Genetics and Development 20, 376-383.

Veverytsa, L., Allan, D., 2012. Temporally tuned neuronal differentiation supports the funcitional remodeling of a neuronal network in *Drosophila*. PNAS.

Vijayan, V., Thistle, R., Liu, T., Starostina, E., Pikielny, C., 2014. *Drosophila* pheromone-sensing neurons expressing the *ppk25* ion channel subunit stimulate male courtship and female receptivity. PLOS Genetics 10, 1-11.

Villella, A., Hall, J., 2008. Neurogenetics of courtship and mating in *Drosophila*. Advances in Genetics 62, 67-184.

Villella, A., Peyre, J., Aigaki, T., Hall, J., 2006. Defective transfer of seminal-fluid materials during matings of semi-fertile *fruitless* mutants in *Drosophila*. Journal of Comparative Physiology A 192, 1253-1269.

Von Philipsborn, A., Liu, T., Yu, J., Masser, C., Bidaye, S., Dickson, B., 2011. Neuronal control of *Drosophila* courtship song. Neuron 69, 509-522.

Wade, J., Arnold, A., 2004. Sexual differentiation of the zebra finch song system. Annals of the New York Academy of Sciences 1016, 540-559.

Wagh, D.A., Rasse, T.M., Asan, E., Hofbauer, A., Schwenkert, I., Durrbeck, H., Buchner, S., Dabauvalle, M.C., Schmidt, M., Qin, G., Wichmann, C., Kittel, R., Sigrist, S.J., Buchner, E., 2006. Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in Drosophila. Neuron 49, 833-844.

Waterbury, J., Jackson, L., Schedl, P., 1999. Analysis of the doublesex female protein in *Drosophila melanogaster*: role in sexual differentiation and behavior and dependence on intersex. . Genetics 152, 1653-1667.

Weigel, D., Jürgens, G., Küttner, F., Seifert, E., Jäcke, H., 1989. The homeotic gene *fork head* encodes a nuclear protein and is expressed in the terminal regions of the *Drosophila* embryo. Cell 57.

Williams, T., Carroll, S., 2009. Genetic and molecular insights into the development and evolution of sexual dimorphism. Nature Reviews: Genetics 10, 797-804.

Williams, T., Selegue, J., Werner, T., Gompel, N., Kopp, A., Carroll, S., 2008. The regulation and evolution of a genetic switch controlling sexually dimorphic traits in *Drosophila*. Cell 134, 610-623.

Wing, J., Zhou, L., Schwartz, L., Nambu, J., 1998. Distinct cell killing properties of the *Drosophila reaper, head involution defective,* and *grim* genes. Cell death and differentiation 5, 930-939.

Wu, M., Shah, N., 2011. Control of masculinization of the brain and behavior. Current Opinion in Neurobiology 21, 116-123.

Xu, X., Coats, J., Yang, C., Wang, A., Ahmed, O., Alvarado, M., Izumi, T., Shah, N., 2012. Modular genetic control of sexually dimorphic behaviors. Cell 148, 596-607.

Yamamoto, D., 2007. The neuronal and genetic substrates of sexual behavior in *Drosophila*. Advances in Genetics 59, 39-66.

Yamamoto, D., 2008. Brain sex differences and function of the *fruitless* gene in *Drosophila* Journal of Neurogenetics 22, 309-332.

Yamamoto, D., Koganezawa, M., 2013. Genes and circuits of courtship behavior in *Drosophila* males. Nature Reviews: Neuroscience 14, 681-692.

Yang, C., Belawat, P., Hafen, E., Jan, L., Jan, Y., 2008a. *Drosophila* egg-laying site selection as a system to study simple decision-making processes. Science 319, 1679-1683.

Yang, C., Rumpf, S., Xiang, Y., Gordon, M., Song, W., Jan, L., Jan, Y., 2009. Control of the postmating behavioral switch in *Drosophila* females by internal sensory neurons. Neuron 61, 519-526.

Yang, Y., Zhang, W., Bayrer, J., Weiss, M., 2008b. Doublesex and the regulation of sexual dimorphism in *Drosophila melanogaster.* Structure, function, and mutagenesis of a female-specific domain. The Journal of Biological Chemistry 283, 7280-7292.

Yapici, N., Kim, Y., Ribeiro, C., Dickson, B., 2008. A receptor that mediates the post-mating switch in *Drosophila* reproductive behaiour. Nature 451, 33-38.

Yekta, S., Tabin, C.J., Bartel, D.P., 2008. MicroRNAs in the Hox network: an apparent link to posterior prevalence. Nat Rev Genet 9, 789-796.

Yu, J., Kanai, M., Demir, E., Jefferis, G., Dickson, B., 2010. Cellular organization of the neural circuit that drives *Drosophila* courtship behavior. Current Biology 20, 1602-1614.

Zarkower, D., 2006. Somatic sex determination.

Zhou, L., Schnitzler, A., Agapite, J., Schwartz, L., Steller, H., Nambu, J., 1997. Cooperative functions of the reaper and head involution defective genes in the programmed cell death of *Drosophila* central nervous system midline cells. Proceedings of the National Academy of Sciences 94, 5131-5136.

Zito, K., Fetter, R.D., Goodman, C.S., Isacoff, E.Y., 1997. Synaptic clustering of Fascilin II and Shaker: essential targeting sequences and role of Dlg. Neuron 19, 1007-1016.

Zuo, W., Zhang, W., Chen, N., 2013. Sexual dimorphism in cerebral ischemia injury. European Journal of Pharmacology 711, 73-79.

APPENDIX

A.1 Supplementary data for chapter 2

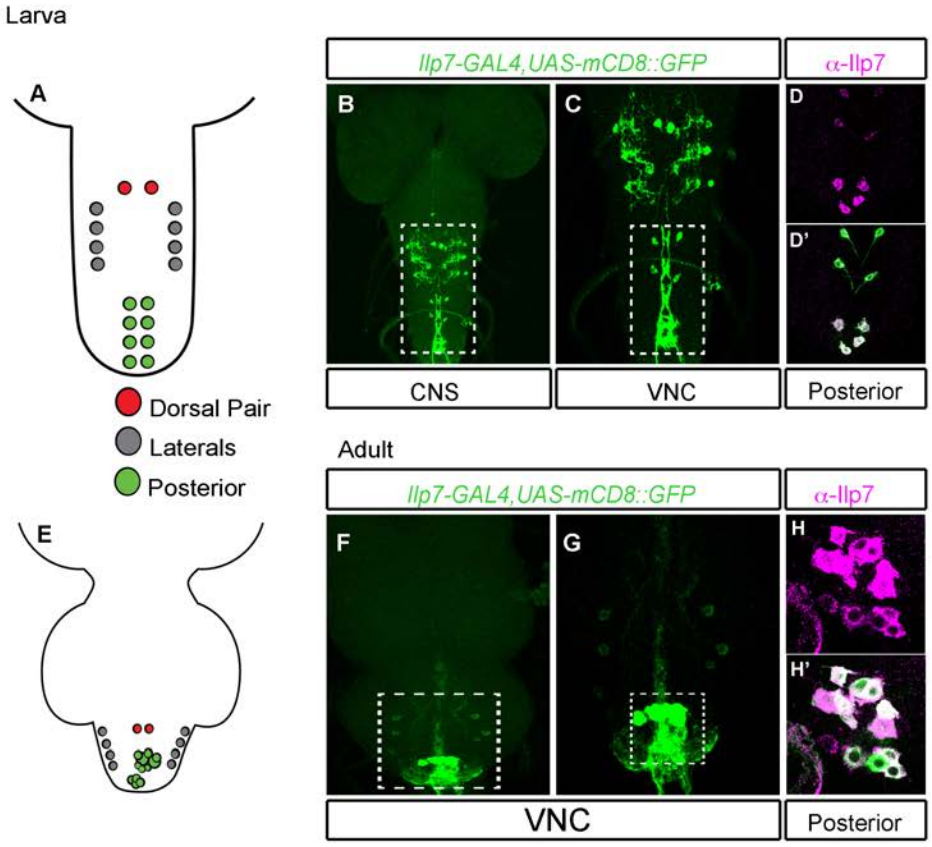

Figure S2.1. Specificity of *Ilp7-GAL4* transgene expression to Ilp7-expressing neurons in the larval and adult VNC.

(**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 (Cognigni et al., 2011; Miguel-Aliaga et al., 2008). (**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 of adults (**F,G**). (**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.

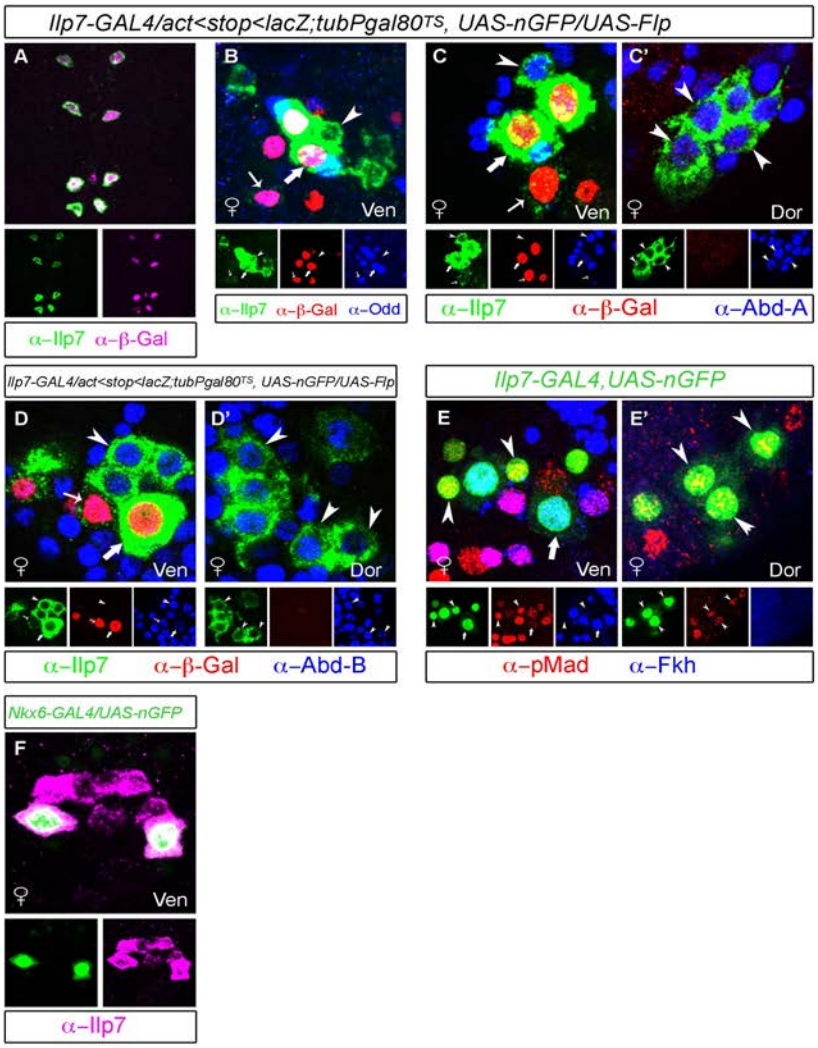

Figure S2.2. Transcription factor expression in embryonic and post-embryonic Ilp7-neurons (A-D')

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 large arrow, and the small embryonic Ilp7- neurons by small arrow. (**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 (*Nkx6-GAL4;UAS-nEGFP*) is expressed in only the large embryonic Ilp7-neurons in adults. Previous reports had indicated that Nkx6 is expressed in dMP2/Ilp7-neurons in the embryo, but had not specified in which segments (Broihier et al., 2004). Here, we find that Nkx6 is expressed only in A6 dMP2/Ilp7-neurons in L3 larvae (**G**). 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**).

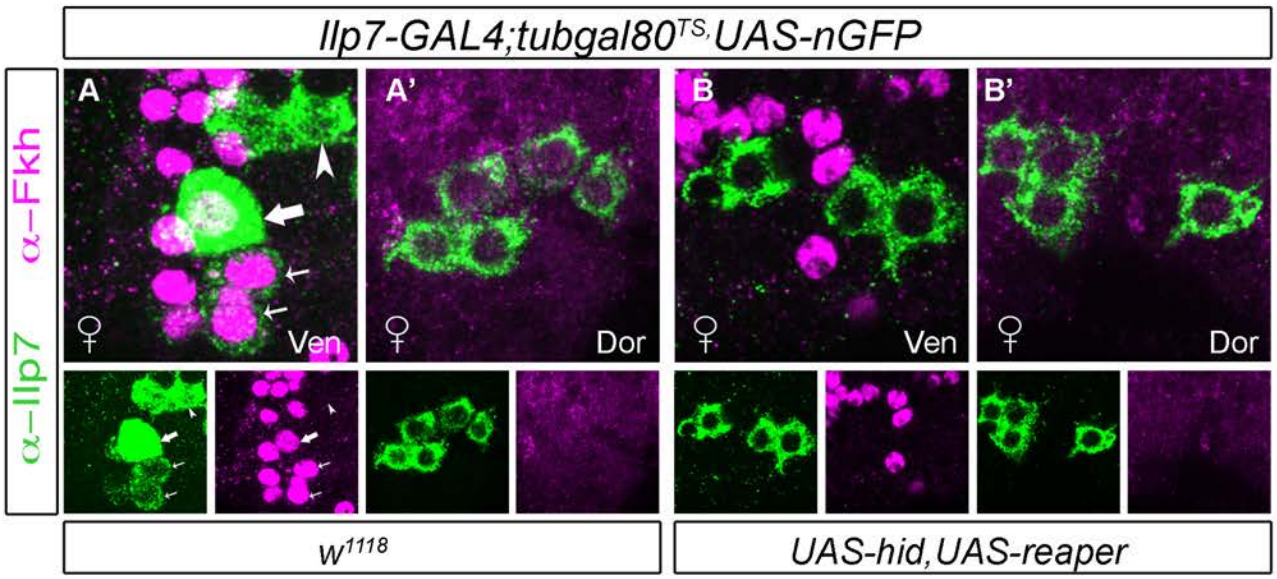

Figure S2.3. Confirmation that only embryonic Ilp7-neurons are killed by pulsing hid and reaper expression in embryonic Ilp7-neurons in early larval stages.

(**A,A'**) Controls in which *hid* and *reaper* were not expressed. Anti-Fkh identifies the embryonic Ilp7-neurons in the ventral cluster (Ven) (large arrow and small arrow); 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 subset of Ilp7-neuronal

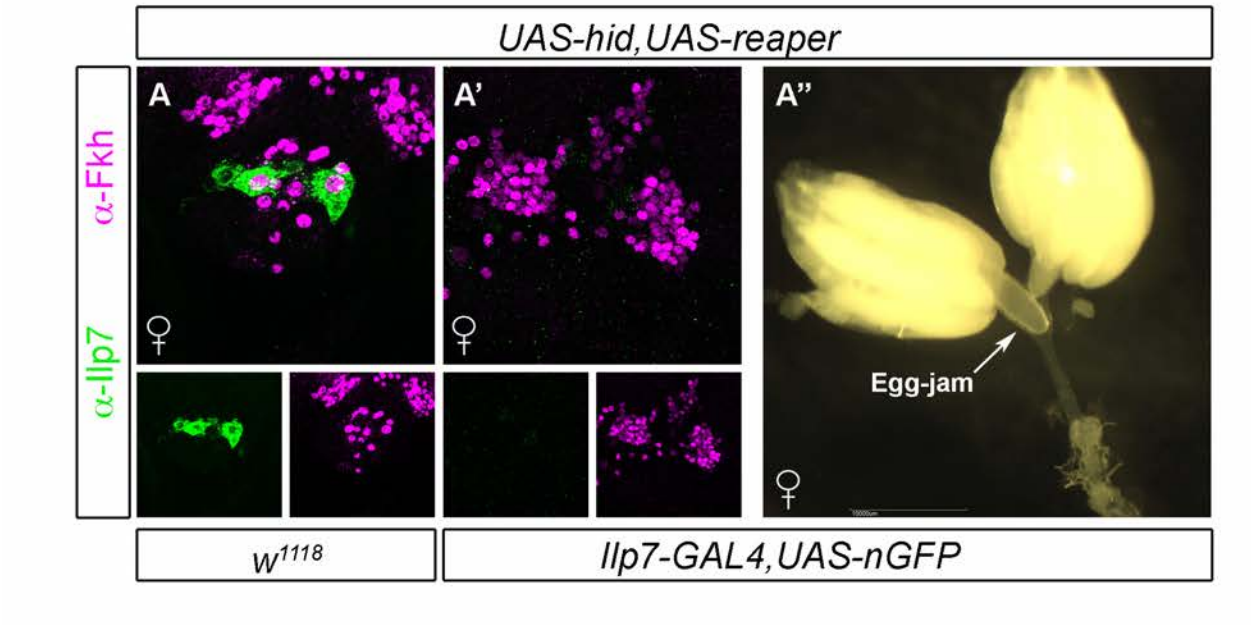

Figure S2.4. Expression of *hid* and *reaper* causes an egg-jam phenotype in the lateral oviduct.

(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.

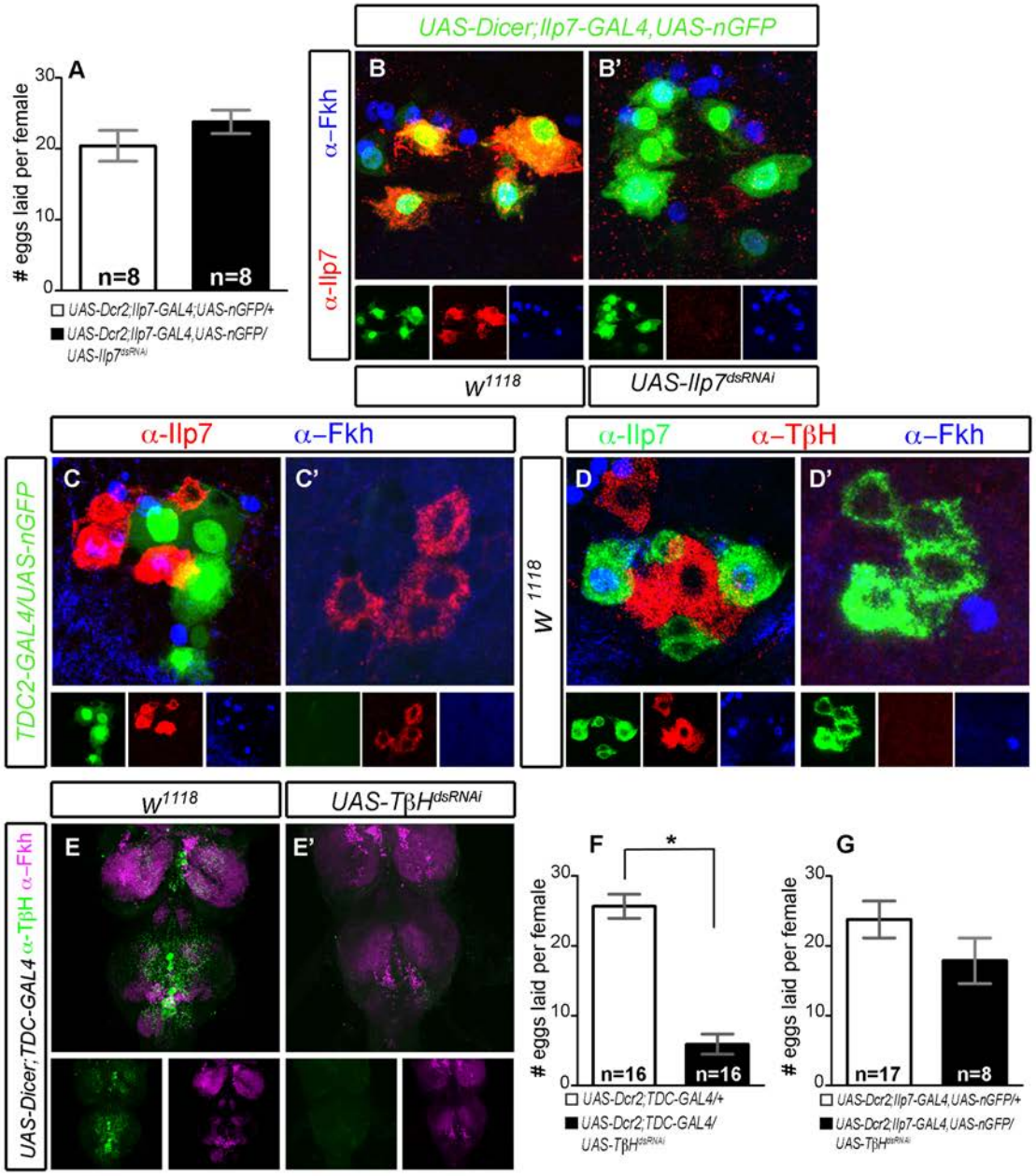

Figure S2.5. Ilp7 neurons are not octopaminergic

(**A-B**') Elimination of Ilp7 in Ilp7-neurons using *UAS-Ilp7*^{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*^{dsRNAi}. (**F,G**) Octopaminergic neurons are required for egg-laying (Rodríguez-Valentín et al., 2006). Expression of *UAS-TβH*^{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).

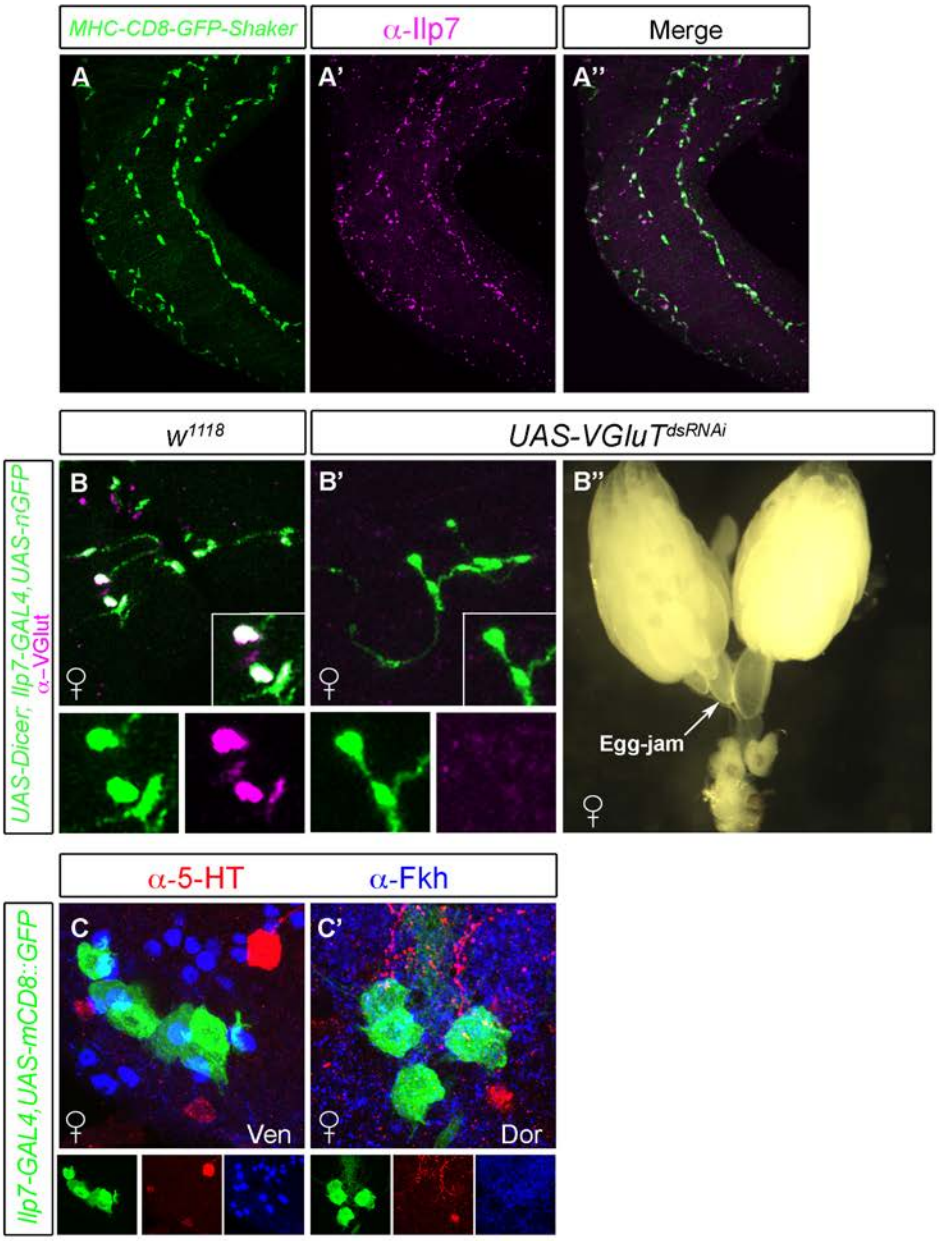

Figure S2.6. Female Ilp7-neurons are glutamatergic but not serotonergic.

(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.

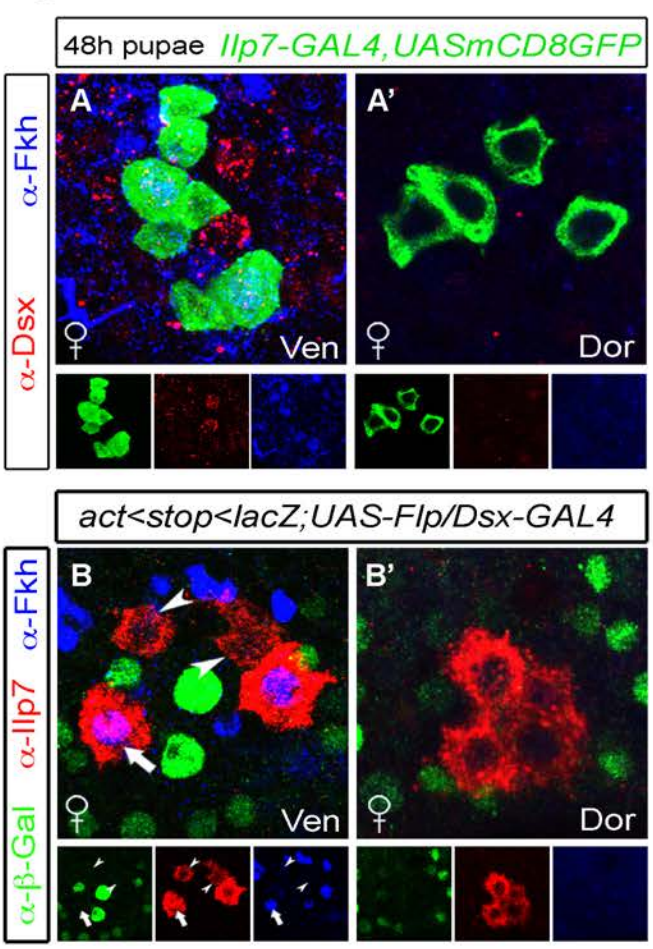

Figure S2.7. Post-embryonic Ilp7-neurons do not express *dsx*.

(**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. 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 (48h 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.

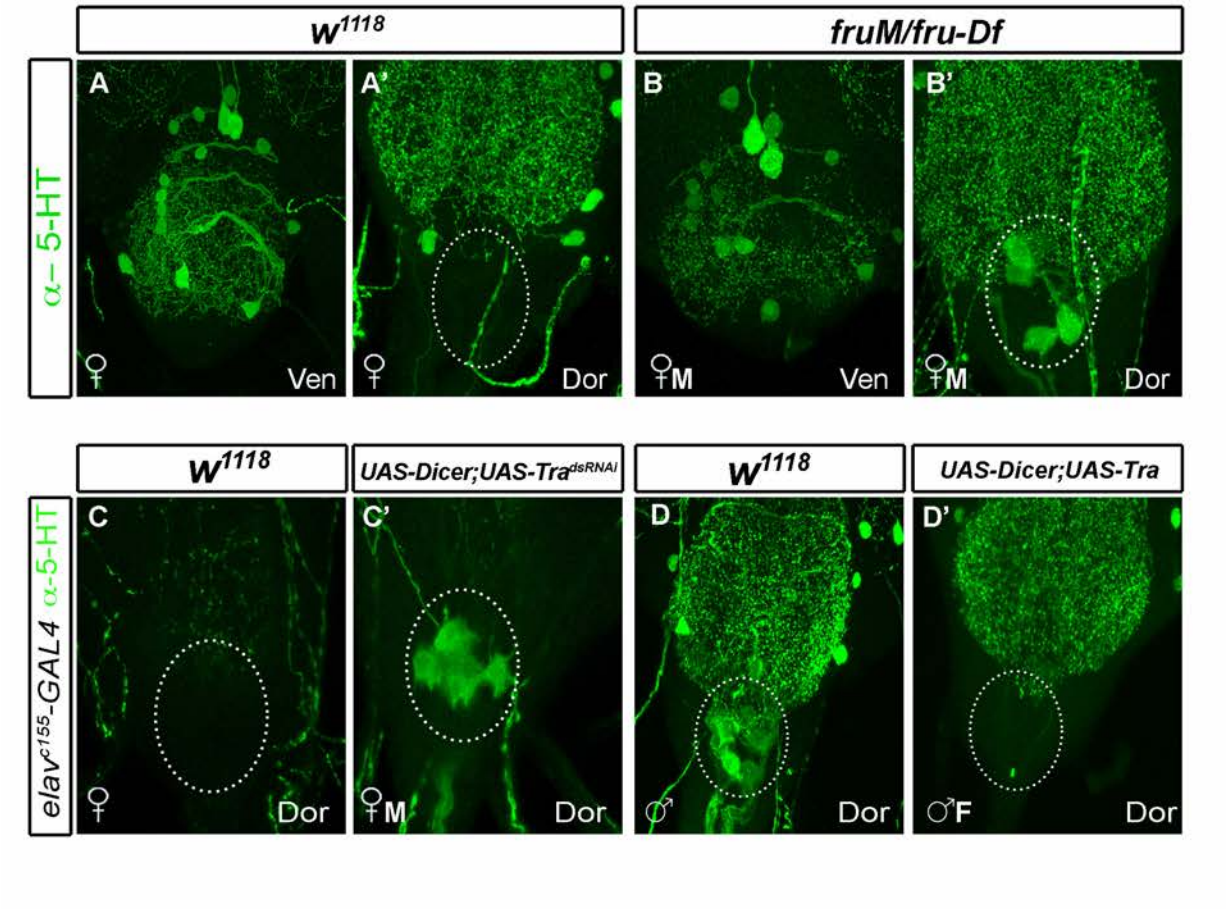

Figure S2.8. Post-embryonic dorsal Ilp7-neurons lose serotonin expression in feminized males (♂F) or gain a serotonergic fate in masculinized females (♀M).

(**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 eurons 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).

Table S2.1. Quantification of the number of eggs laid per female per 24hr period over a 5 day timespan.

2S1 A ♀ *Ilp7-KO*

Day	Genotype	Eggs laid per female	N	
A5	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	60.3± 10.2	9	
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	4.3± 3.3	9	P<0.0001
A6	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	70.9± 16.7	9	
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	12.9± 8.5	9	P<0.0001
A7	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	60.8± 16.1	9	
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	10.9± 7.8	9	P<0.0001
A8	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	51.1± 16.6	9	
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	8.0± 6.6	9	P<0.0001
A9	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	36.7± 15.8	9	
	UAS-hid,UAS-R\reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	5.2± 4.9	9	P<0.0001

Eggs laid per female: total number of eggs counted after a 24 hour lay and divided by the number of females per assay, represented as mean± S.D. n = number of egg-lay assays. Standard t-test performed at each timepoint.

S2.1 B ♂ *Ilp7-KO*

Day	Genotype	Eggs laid per female	N	
A2	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	40.4± 13.6	9	
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	25.9± 14.5	10	P=0.04
A3	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	38.9± 13.6	9	
	UAS-Hid,UAS-Reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	32.3± 16.5	10	P=0.4
A4	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	32.9± 17.5	9	
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	33.3± 16.2	10	P=0.9
A5	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	40.5± 10.9	9	
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	34.33± 13.3	10	P=0.3
A6	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	48.7± 14.1	9	
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	47.1± 13.3	10	P=0.8

Eggs laid per female: total number of eggs counted after a 24 hour lay and divided by the number of females per assay, represented as mean± S.D. n = number of egg-lay assays. Standard t-test performed at each timepoint.

Table S2.2. Numbers of viable progeny produced in relation to the number of eggs laid within 6hr assay periods over 5 days.

2S2 A ♀ *Ilp7-KO*

Day	Genotype	Eggs laid Eggs hatched (%)	N	
A5	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	58.8± 14.3 57.0± 16.5 (95.8± 9.9)	9	P<0.0001
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	3.4± 2.1 1.6± 1.7 (40.7± 37.4)	9	
A6	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	87.7± 29.5 85.7± 28.9 (97.7± 1.4)	9	P<0.0001
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	12.4± 9.3 6.0± 6.5 (43.7± 22.0)	9	
A7	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	84.0± 25.7 82.4± 25.6 (98.0± 1.8)	9	P<0.0001
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	12.3± 6.9 6.6± 3.9 (53.1± 16.9)	9	
A8	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	63.8± 33.2 58.6± 31.9 (87.3± 15.8)	9	P=0.0001
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	9.1± 6.4 4.9± 3.6 (47.8± 30.8)	9	
A9	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	42.6± 14.4 23.6± 13.6 (54.1± 21.8)	9	P=0.0003
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	5.9± 6.6 2.0± 2.3 (37.2± 44.5)	9	

Eggs laid: total number of eggs counted after a 6 hour lay. Eggs hatched: total number of eggs hatched after 24 hours (percent of eggs hatched relative to total number of eggs laid), represented as mean± S.D. n = number of egg-lay assays. Standard t-test was performed at each timepoint for the normalized percentage of eggs hatched.

S2.2 B ♂ *Ilp7-KO*

Day	Genotype	Eggs laid Eggs hatched (%)	N	
A2	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	30.2± 11.9 28.2± 11.1 (93.3± 6.3)	9	P=0.0003
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	22.7± 15.9 8.6± 9.3 (49.7± 45.3)	9	
A3	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	29.4± 12.8 28.3± 12.2 (96.6± 2.8)	9	P=0.66
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	28.0± 18.1 21.6± 19.9 (69.4± 40.3)	9	
A4	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	23.8± 13.7 21.7± 14.3 (88.5± 19.5)	9	P=0.78
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	30.0± 16.6 20.7± 18.9 (59.7± 45.6)	9	
A5	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	27.6± 12.2 25.8± 10.2 (94.9± 5.0)	9	P=0.13
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	21.6± 9.8 17.0± 11.5 (72.9± 37.8)	9	
A6	*w;Ilp7-GAL4,UAS-mCD8::GFP/+*	29.9± 9.3 26.9± 8.4 (90.3± 7.7)	9	P=0.61
	UAS-hid,UAS-reaper;Ilp7-GAL4,UAS-mCD8::GFP/+	40.5± 10.5 30.6± 14.4 (68.4± 29.4)	9	

Eggs laid: total number of eggs counted after a 6 hour lay. Eggs hatched: total number of eggs hatched after 24 hours (percent of eggs hatched relative to total number of eggs laid), represented as mean± S.D. n = number of egg-lay assays. Standard t-test was performed at each timepoint for the normalized percentage of eggs hatched.

A.2 Supplementary data for chapter 3

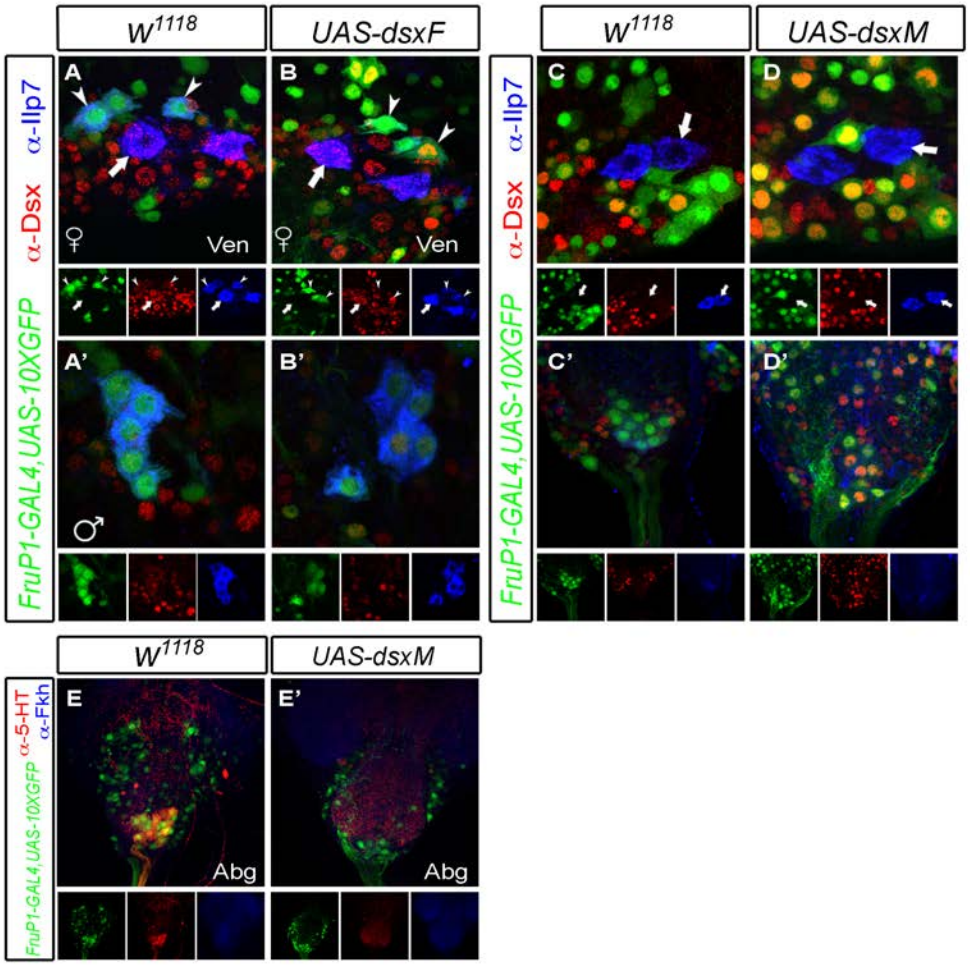

Figure S3.1. Absence of DsxM in males is necessary for terminal differentiation of Ilp7-neurons in males.

(**A-D'**) Sex-specific *dsx* isoforms are present in Ilp7-neurons when *UAS-dsxM* and *UAS-dsxF* are expressed from FruP1-GAL4. **(A-B')** Embryonic (arrows) and post-embryonic (arrowheads) ventral (ven), and dorsal Ilp7-neurons (dor) never express *dsx* (A-A'). In females expressing *UAS-dsx*F from the *FruP1-GAL4*, *dsx* immunoreactivity can be observed in both female-specific (arrowheads) and dorsal (dor) Ilp7-motoneurons, but not embryonic Ilp7-neruons due to their lack of *fru* (B-B'). **(C-D')** Only dorsal Ilp7-neurons (dor) in males are post-embryonic and express fru but not dsx (C-C'). Therefore expression of UAS-dsxM;FruP1-GAL4, UAS-10XGFP will lead to dsx immunoreactivity in the male dorsal (dor) Ilp7-neurons (D-D').
E-E') Expression of *UAS-dsxM* abolishes serotonin expression. In males, dorsal Ilp7-neurons can co-express Ilp7-peptide (see **Fig. 3.4 D)** and serotonin (E). Expression of *UAS-dsxM* in these neurons abolishes expression of Ilp7-peptide (see **Fig. 3.4 D'**) and serotonin (E'), while *FruP1-GAL4, UAS-10XGFP* neurons are still observed in the region, raising the possibility that these are in fact undifferentiated dorsal Ilp7-neurons in males.

Table S3.1. Summary of the phenotypes observed in relation to survival of female-specific Ilp7-motoneurons in the genetic analysis of the allelic combinations of constitutive FruM and FruF alleles.

Genotype	♀	♂
fruM/fruDf	$Ilp7^{+}/Fru^{+}/Fkh^{-}$ survive	$Ilp7^{+}/Fru^{+}/Fkh^{-}$ survive
fruF/fruDf	$Ilp7^{+}/Fru^{+}/Fkh^{-}$ survive	$Ilp7^{+}/Fru^{+}/Fkh^{-}$ survive
fruF/fruF	$Ilp7^{+}/Fru^{+}/Fkh^{-}$ survive	$Ilp7^{+}/Fru^{+}/Fkh^{-}$ survive
fruC/fruDf	$Ilp7^{+}/Fru^{+}/Fkh^{-}$ survive	$Ilp7^{+}/Fru^{+}/Fkh^{-}$ survive
fruDf/+	$Ilp7^{+}/Fru^{+}/Fkh^{-}$ survive	$Ilp7^{+}/Fru^{+}/Fkh^{-}$ survive
fruM/fruF	$Ilp7^{+}/Fru^{+}/Fkh^{-}$ die	$Ilp7^{+}/Fru^{+}/Fkh^{-}$ die

A.3 Supplementary data for chapter 4

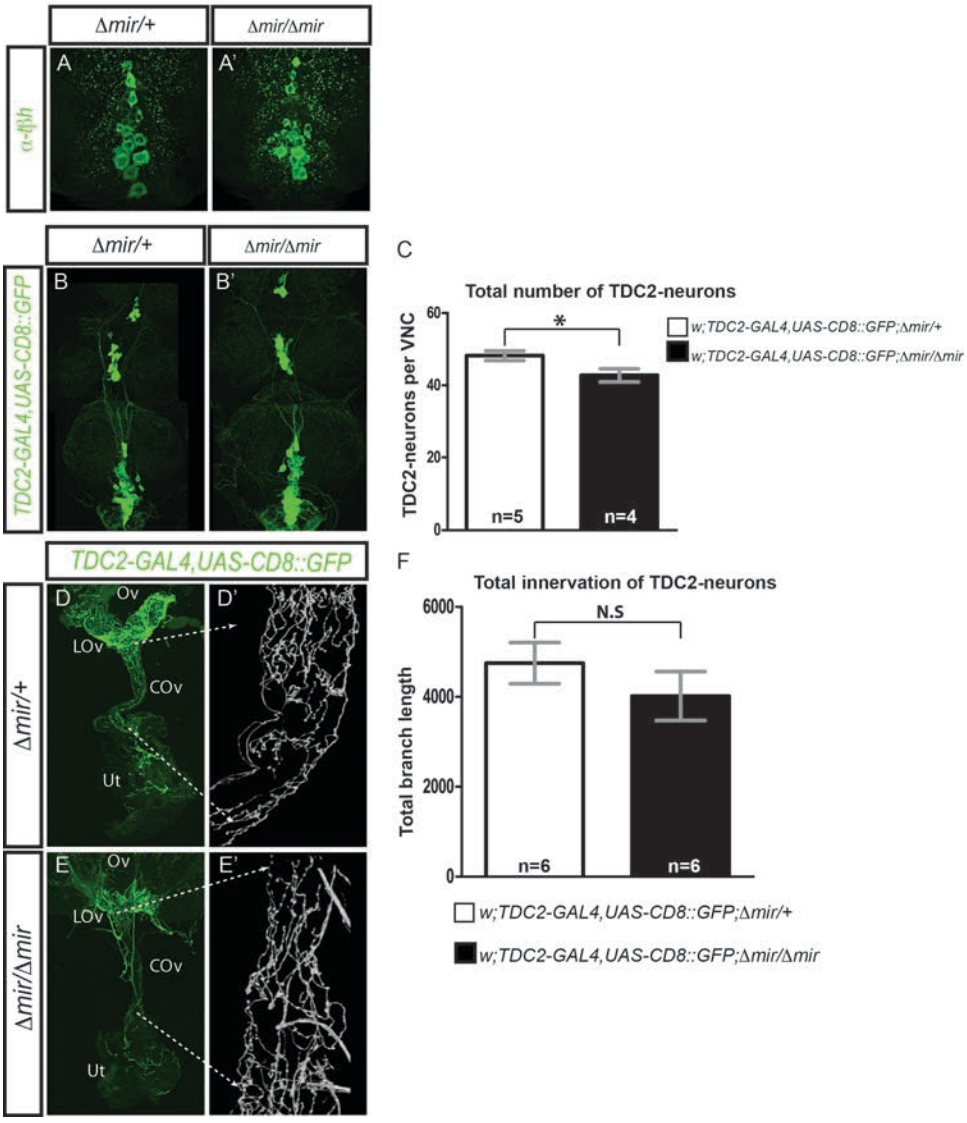

Figure S4.1. Analysis of octopaminergic neurons and their projections.

(**A-B'**) Δ*mir* homozygotes express Tyrosine beta-hydroxylase (TßH) protein and Tyrosine decarboxylase (TDC2). (**C**) Quantification of neurons expressing *TDC2-GAL4/UAS-CD8::GFP* reporter activity show a moderate decrease in the number of TDC2-positive cells (p=0.043). Innervation by TDC2-neurons on the oviduct is not affected in *Δmir* homozygotes.
(D,E) Show whole reproductive tract and TDC2 innervation of the oviduct (*TDC2-GAL4*, *UAS-CD8::GFP*) for control and Δ*mir* homozygotes. **(D',E')** 3-D rendering of TDC2 innervation using Simple Neurite Tracer to trace and quantify the length of all TDC2 neuronal branches on the common oviduct. (**F**) Quantification of total oviduct innervation length shows no substantial difference between these genotypes (p=0.33). Graphs show quantifications ±S.E.M. *p=0.043, N.S=not significant.

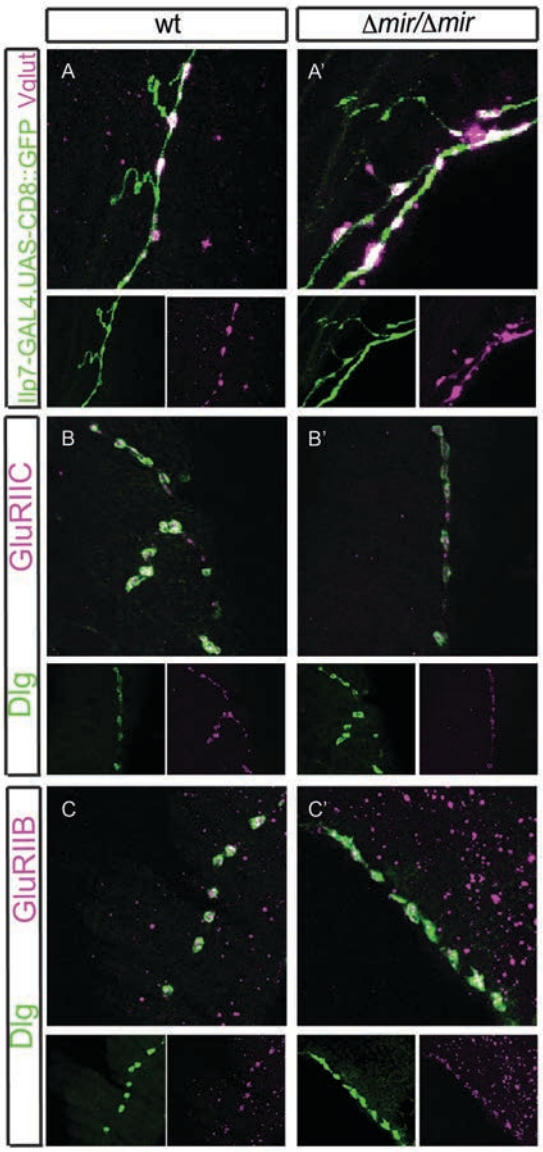

Figure S4.2. Ilp7-neurons maintain their glutamatergic identity in Δ*mir* homozygotes.

(**A-A'**) Termini of Ilp7-neurons at the oviduct in Δ*mir* homozygotes maintain the expression of unique markers for type I- glutamatergic neuromuscular junctions. Δ*mir* homozygotes express the vesicular glutamate transporter (VGluT) at their presynaptic zones at the oviduct. VGluT immunoreactivity (magenta) is observed at the presynaptic zone of Ilp7-termini (green) in both control (**A)** and Δ*mir* homozygotes (**A'**).
(**B-C'**) Δ*mir* homozygotes localize glutamate receptor subunits (magenta) to Dlg-positive (green) neuromuscular junction. Accumulation of GluRIIC (**B-B'**) and GluRIIB (**C-C'**) is observed in both controls and Δ*mir* homozygotes.