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Mapping novel expression and mechanisms of octopamine signaling in the female Drosophila reproductive system



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# Mapping novel expression and mechanisms of octopamine signaling in the female Drosophila reproductive system

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Neuroscience

by

Ethan William Rohrbach

2023

ABSTRACT OF THE DISSERTATION

Mapping novel expression and mechanisms
of octopamine signaling
in the female Drosophila reproductive system

by

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Doctor of Philosophy in Neuroscience
University of California, Los Angeles, 2023
Professor David E Krantz, Chair

Throughout the animal kingdom, female fertility is orchestrated by a vast number of signaling pathways. These include both direct neural regulation of reproductive tissues as well as CNS computations that indirectly affect reproductive behavior. Noradrenergic signaling in particular is known to play a critical role in regulating a variety of reproductive processes in both mammals and insects. Despite this established role, knowledge of noradrenergic mechanisms in fertility lacks detailed descriptions of the complex neural circuitry regulating required behaviors. The model organism *Drosophila Melanogaster* offers a unique opportunity to establish a tractable system for studying conserved principles underlying the neuromodulation of fertility with

single cell resolution. In *Drosophila*, the ortholog of noradrenaline, octopamine (Oa), is required for egg laying as well as several other reproductive subprocesses. The goals for this dissertation were to 1) comprehensibly map octopaminergic circuitry innervating the female fly reproductive tract and 2) prove the utility of such circuitry as a model for uncovering both translationally relevant mechanisms in fertility and fundamental principles of adrenergic neuromodulation. This study provides detailed reproductive tract expression pattern analyses for all six known octopamine receptors using recently made tools for capturing genetic expression profiles with unprecedented completeness. Novel Oa receptor expression is noted among extensive networks of peripheral neurons and several other reproductive organ cell types. Stimulation of octopaminergic pathways is found to induce specific reproductive muscle cell behaviors, and an assay has been established for comparing rhythmic contractions of the lateral oviducts that are regulated by Oa. Gain of function experiments increasing activity in Oa receptor expressing neurons (OaRNs) are found to reduce egg laying, suggesting for the first time a conserved mechanism between mammals and insects where increased noradrenergic tone is associated with impaired fertility.

Novel roles for Oa receptors are also provided with an updated analysis of presynaptic Oa neurons. Neurons of the central octopaminergic cluster required for ovulation have been mapped with single cell resolution. Heterogeneity between neighboring Oa neuron projection targets pairs with heterogeneity in roles stimulating reproductive organ behavior, showing that each individual neuron in this model aminergic cluster may have a unique role in conducting egg laying. Future studies will

be able to use the information in this dissertation as a basis for understanding the complexity of aminergic neuromodulation in fertility and other systems.

The dissertation of Ethan William Rohrbach is approved.

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2023

This dissertation is dedicated to my family.
To my mother, Joan, my father, John, and my fiancé, Liz;
I feel your love in every moment of life.
You give me confidence to pursue my dreams.

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Ethan W. Rohrbach; Elizabeth M. Knapp; Sonali A. Deshpande; Pei-Tseng Lee; David E Krantz (2023 *in Submission***)** Drosophila cells that express octopamine receptors can either inhibit or promote oviposition. Plos Genetics.

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# CHAPTER 1: INTRODUCTION

No single organism can live forever. To avoid species extinction, life must reproduce. There exists a vast array of biological mechanisms that facilitate this characteristic of life across different species, but there are also several common principles.

## Unresolved questions in female fertility

In all sexually reproducing organisms, meiosis produces gametes that are stored and specifically released so that gametes from the opposite sex may bind, fuse, and fertilize new diploid cells with unique genetic code. In humans, the regulation of male and female gametes (sperm and eggs respectively) is a complexly orchestrated process requiring many diverse cell types and chemical signals. Failure in the function of such reproductive systems is a major societal health concern, and the freedom of reproductive choice granted by a properly functioning reproductive tract can have profound implications for an individual's mental health ("Cost of Mental Health Disorders Linked with Polycystic Ovary Syndrome Almost $6 Billion in 2021", 2022). Understanding reproductive system function and uncovering therapeutic targets that affect fertility is therefore an important objective of biomedical research. The goal of this dissertation is to describe a newly expanded model of female fertility in the fruit fly, *Drosophila melanogaster*, ideal for such investigations.

Female infertility can be a devastating problem, particularly for those who are unresponsive to infertility treatment. Approximately 12.7% of reproductive age American women seek treatment for infertility each year (Carson and Kallen 2021). Polycystic

ovary syndrome (PCOS) is a common condition where excess androgen hormone is associated with irregular egg development and ovulation. This problem costs the US health care system >$4 billion annually, but the mechanisms underlying its causes and symptoms remain poorly understood (Riestenberg et al. 2022). It has also been estimated that >10% of women display bouts of anovulation despite having normal oogenesis and hormonal fluctuations (Qublan et al. 2006). Such anovulation can be detected during fertility treatment when increases in the hormone that signals ovulation, rise but fail to correspond with an ovulation event (Lemaire 1987). These symptoms characterize luteinized unruptured follicle syndrome (LUFS), another type of anovulatory disease.

To better understand and treat these diseases, many questions still need to be answered about ovulation, such as the case of a follicle not responding to its ovulatory hormone highlighted in LUFS. One approach to answering such questions is to utilize the conserved nature of ovulation as a fundamental reproductive process in not only humans but also rats, mice, fish, and insects (Curry and Osteen 2003; Curry and Smith 2006; Takahashi et al. 2013; Deady and Sun 2015). Uncovering conserved mechanisms of egg development and release in tractable models has the potential to aid the development of new treatments for patients with infertility.

Ovulation, or rupture of a mature oocyte from the ovary, is a requisite step preceding successful fertilization. Canonically, ovulation is initiated when a surge of luteinizing hormone (LH) is released from the anterior pituitary. LH acts as a ligand for the LH receptor (LHR) on mural granulosa cells of a developing follicle in the ovary. LHR then stimulates ovulation via both $G\alpha_s$ and $G\alpha_q$ G-protein coupled receptor (GPCR)

signaling pathways (Breen et al. 2013). In mammals, LH binding to LHR is required for at least three different behaviors involved in ovulation—oocyte meiosis resumption, cumulus expansion, and follicular rupture (Curry and Smith 2006). LHR activity increases prostaglandin and progesterone release, which in turn induces expression of enzymes such as ADAMTS, plasminogens, and matrix metalloproteinases (MMPs) that facilitate the degradation of specific follicle cells. The spatially and temporally precise coordination of these enzymatic and transcriptional changes is required for successful follicle rupture. Cox-2 null mice lack prostaglandin synthesis and do not ovulate (Matsumoto et al. 2001). Mice that are null for the progesterone receptor also fail to ovulate (Lydon et al. 1996). And altering prostaglandin signaling without ablating it can produce mislocated follicle cell rupture where oocytes are deposited into the ovary instead of into the peritoneal space (Curry and Smith 2006). Similarly, follicle rupture almost completely ceases when progesterone signaling is reduced. There remains a need to understand which follicle cells and intracellular pathways facilitate prostaglandin and progesterone roles. LH, prostaglandin, and progesterone signaling is also complex and affects multiple other processes in female fertility such as preparation of the uterus for implantation and corpora lutea maintenance during pregnancy (Kumar and Sait 2011), actions which must be considered by therapies targeting its pathways.

Furthermore, LH signaling pathways represent only a fraction of the total number of required signals involved in ovulation, and little is known about the interaction between different neuromodulatory and hormonal systems. Anovulation in PCOS is caused by an imbalance of LH and follicle stimulating hormone (FSH) (Kalro, Loucks, and Berga 2001), and this condition has been shown to associate with increased

plasma levels of noradrenaline (Lansdown and Rees 2012; Greiner et al. 2005). The neuromodulation of mammalian fertility either directly or via regulation upstream of LH, however, remains only vaguely defined. Determination of specific neural mechanisms relevant to female fertility will continue to add valuable options to the list of potential targets for treating anovulation.

**Need for a model to study neuromodulation of fertility**

Considering the prevalence of female infertility and the cost of this condition to society, defining the cellular mechanisms that promote each required process from follicle development to egg implantation is essential. Disorders such as PCOS underscore the complexity of signal regulation and the unresolved roles of CNS and PNS neurotransmission contributing to infertility. A model system is needed to elucidate such mechanisms and identify novel therapeutic strategies. Authors of studies in mouse models acknowledge that expansive cellular interactions and genetic redundancies in rodent models make identifying key components of fertility similarly difficult (Liu et al. 2013). *Drosophila melanogaster* on the other hand represent a genetically tractable model with rapid sexual maturation and a relatively simple nervous system ideal for studying both fertility and aminergic neuromodulation.

The Drosophila model offers the benefits of a simpler genome with fewer redundancies while still maintaining complex physiological processes. The numbers of nervous system cells, neural connections, and neural signaling molecules is also greatly reduced in comparison to mammalian models. Due to the fruit fly's short lifespan, much of the female's nutritional intake is directed toward a constant process of oogenesis and

ovulation from within the first day post eclosion. This system of high throughput egg laying is being increasingly well documented, and the vast genetic toolkit available in Drosophila has established it an ideal model system in which to study translational concepts in ovulation and fertility (Jiang et al. 2021). Still, most previous studies have focused exclusively on mechanisms downstream of neural signals. The specific neurons regulating distinct processes involved in egg laying remain disconnected from their target reproductive tissues in most signaling models. It is now time to more accurately characterize such circuitry. Doing so will not only improve efforts to understand human infertility but also offer valuable insights into how large groups of neurons coordinate behavior between the central nervous system and the periphery. One key question in this field is whether neurons that produce the same signaling molecule signal redundantly or whether individual neurons have unique roles in communicating with distant organs. In this introduction chapter, I will describe the physiological processes involved in Drosophila egg laying, a central role for aminergic neurotransmission, and existing methods that can be used to investigate this model.

**Female Drosophila reproductive tract anatomy and ovulation**

Female reproductive anatomy is relatively conserved throughout most animals; females have two bilaterally symmetric ovaries, wherein development of oocytes occurs. The ovaries release the mature oocyte into their respective oviduct (which is the receptacle and transporter for the oocyte, similar to the role of the fallopian tube). The oocyte is transported along the oviduct toward the uterus. This general anatomy and physiology is descriptive of animals from humans to Drosophila. Furthermore, similar to

human females, Drosophila store sperm internally and undergo internal fertilization. Whether an oocyte is fertilized or not, it will be oviposited. In contrast to humans, embryogenesis occurs completely externally in Drosophila.

One aspect that is dramatically different between Drosophila and humans is the capacity for egg production; Drosophila are able to fully develop and lay more than 70 eggs per day at their peak fertility. This enormous reproductive capacity is largely due to their process of oogenesis. Each Drosophila ovary contains approximately 15-20 ovarioles wherein oocyte development occurs. Approximately seven developing follicles (egg chambers) are found within one ovariole and are connected to each other via stalk cells. Similar to a developing mammalian “follicle”, Drosophila also have a layer of somatic cells surrounding the developing oocyte. These somatic cells in humans are granulosa and theca cells, wherein in Drosophila they are termed “follicle cells”.

Each ovariole is isolated from other ovarioles by an encapsulating ovariole muscle sheath. The group of 15-20 ovarioles per ovary are contained within the peritoneal sheath, which envelops the entire ovary. At the anterior-most region of the ovariole is a region called the germarium which is composed of germline stem cells and somatic stem cells which will ultimately give rise to the egg chamber. The germline stem cell divides to produce a 16-cell germline cyst, wherein one of the germ cells is specified as the oocyte and the remaining 15 become the nurse cells, which support the development of the oocyte. A stage one follicle is completely enveloped by a follicle-cell layer and this will bud off, retaining connections via stalk cells to egg chambers posterior- and anterior- to itself. Only after the cyst has developed to 16 cells, the follicle-cell layer migrates to completely enclose the 16-cell cyst. This highly specific cell

movement is the first of many these dynamic and complex follicle cells will undergo. Throughout the first six stages of oogenesis, the follicle cells will divide around nine times, to around 800 cells, to maintain coverage of the growing germ cells (King 1970). During stages six-nine, the follicle cells cease mitosis and undergo endoreplication. During stage nine, the majority of follicle cells move posteriorly to cover the continuously growing oocyte, leaving only ~50 cells (termed “stretch cells”) covering the 15 nurse cells. Amazingly, between stage nine and 10, ~6-10 follicle cells at the anterior region detach from the follicle-cell layer and move through the 15 nurse cells to reach the anterior border of the oocyte. These cells will ultimately produce the micropyle (region of the egg chamber that is permeable to sperm). The main-body follicle cell layer continues to cover the growing oocyte between stages 11-14 and undergo gene amplification of genes regulating chorion synthesis. Little is known regarding the fate of the follicle cells after the stage 14 oocyte is ovulated (Verheyen and Cooley 1994) (Spradling, 1993).

Many checkpoints exist to coordinate external cues with this high-energy demanding process (Peterson et al. 2015). Upon successful completion of the requisite checkpoints, an egg chamber will advance to a preovulatory, stage 14 follicle marked by increased expression of receptors for the fly orthologue of noradrenaline, octopamine (Oa). Oocytes less mature than stage 14 are not ovulated; the only oocytes to be found outside the ovary have completed egg maturation (Verheyen and Cooley 1994) and have been ovulated, suggesting mechanisms to prevent premature follicles from being ovulated. Similar feedback mechanisms may be in place to prevent the follicle-cell layer

from entering the oviduct after expulsion of the oocyte, but this has not been investigated.

Successful development of a mature oocyte is the first compulsory phase in female fertility. Oocytes are then ovulated, transported to the uterus, oviposited, and, if fertilized, undergo embryogenesis.

**Egg passage through the reproductive tract**

The environment into which an oocyte will be ovulated influences whether it will be ovulated or not. The egg is first accepted from the ovary into the lateral oviduct, then is promptly shuttled to the common oviduct, before pausing in the uterus prior to oviposition. The lateral and common oviduct muscles are typical insect visceral muscles that contain skeletal and smooth muscle characteristics: there are striations, and they have slow, rhythmic contractions (Middleton et al. 2006). The oviduct muscles circularly envelop the oviduct lumen. The oviduct epithelium, which surrounds the lumen, has microvilli that likely assist in oocyte transport through secretions (Middleton et al., 2006). Uterine musculature is much more intensive than the oviduct but structurally similar (Middleton et al. 2006). Approximately 70 times per day, the oviduct must correctly coordinate the previously described events for transporting large oocytes all the way from the ovary to the uterus, positioning them correctly, and preparing them for fertilization.

Proper tonus of the oviduct musculature is essential for an egg to be able to pass through; if the circular oviduct is tightly contracted, the egg will not be able to be ovulated. The role that neuromodulators such as Oa have on the muscle tonus on the

oviduct has been intensively studied. Middleton and colleagues (2006) characterized the innervation of the female reproductive tract: nerves branch from the abdominal median nerve trunk to intensively innervate the female reproductive tract, all of which express the octopamine-synthesizing enzyme tyrosine decarboxylase 2 (Tdc2). Post-mating increases in Oa have been demonstrated to be essential in relaxing the oviduct musculature (Rubinstein and Wolfner 2013), a process that is dependent on ovulin. Data in support of this claim come from measuring sarcomere lengths after mating in response to increased Oa after mating.

Instead of Oa acting directly upon oviduct muscle, there is a body of evidence suggesting that Oa receptors on the oviduct epithelium, which lies between the oviduct muscle and the oviduct lumen, regulate oviduct muscle. From these studies, Oamb and Oct2βR on the oviduct epithelium activate Ca2+ and cAMP pathways, respectively. This model describes intracellular pathways that could increase nitric oxide signaling from the epithelium to signal to the oviduct muscle to stimulate relaxation (H.-G. Lee, Rohila, and Han 2009; Lim et al. 2014b). The common oviduct is intensively innervated by Tdc2(+) neurons and some glutamatergic neurons. Using immunohistochemistry to identify neurotransmitter accumulation at synaptic boutons, one group was able to associate how different neuromodulators were released over time in response to mating (Heifetz et al., 2014). They found the major neurotransmitter within the oviduct is octopamine; however they also found there is also a considerable amount of serotonin activity (Heifetz et al. 2014). Currently, it is unclear what the role of serotonin is in regulating the oviduct. Nevertheless, the relatively narrow oviduct undergoes many morphological changes to allow for ovulation to occur and for the oocyte to be

transported to the uterus. In Chapter 2 of this dissertation, novel pathways for regulating oviduct muscle contraction and dilation are described in which Oa receptor-expressing neurons (OaRNs) play an intermediate role between Oa neurons and reproductive tissues. There is overlap in the populations of OaRNs and glutamatergic reproductive tract neurons, and the indirect neuromodulation of egg laying by Oa via OaRNs represents a vitally understudied aspect of egg laying regulation.

Although most research involving the oviduct-ovulation relationship has been focused upon the oviduct muscles, the epithelium also maintains important roles for regulating egg transport. Secretions from the secretory glands of the spermathecae and parovaria into the oviduct lumen are also essential for proper ovulation. Using a lozenge knockout fly to eliminate reproductive glands, a significant impact of secretory cells on ovulation can be observed. Knockdown of secretory cells specifically within the reproductive tract reveals a correlation between the efficiency of ovulation to the number of properly formed secretory cells (J. Sun and Spradling 2013). This new evidence directly implicates communication between the products that are secreted within the oviduct and the ovary to coordinate ovulation. In Chapter 4 of this dissertation, evidence is presented showing Oamb-dependent regulation of spermathecal secretory cells by Oa.

**Ovipostion**

After an oocyte has been matured, ovulated, and transported, it can finally be laid. Females can retain eggs in their uterus for extended periods of time until they find an appropriate environment/substrate where to lay their egg. The time an egg spends in

the uterus can be estimated by using the following formula: [time to lay one egg (time given/total eggs laid) = time in uterus (time to lay one egg * percent of eggs found within the uterus) + time to ovulate (time to lay one egg – time in uterus)] (J. Sun and Spradling 2013).This method is an estimate and relies on an experiment wherein females are dissected and the location of an oocyte within the reproductive tract is recorded. Using this estimation, transport through the oviduct is almost instantaneous, whereas females can hold an egg in their uterus for ~10 minutes. This conclusion has been verified by time-lapse image series of fly abdomen cross-section during the egg laying process (Mattei et al. 2015).

If the egg has been successfully fertilized, embryogenesis will occur externally. For a species to propagate, it is essential its embryos are viable. An egg must therefore be laid in an optimal environment, a process that represents a model system for studying simple decision-making processes (Yang et al. 2008). Drosophila females go through a very stereotyped behavior that precedes oviposition term the “Ovipositor motor program”: 1. immediately prior to oviposition, a female will extend the posterior-most part of her abdomen so her ovipositor is contacting the egg-laying substrate, then she will lay the egg; 2. Cleaning and Resting – the female will touch her ovipositor with her hind legs as if to clean it, then remain immobile for a few seconds; 3. Searching – after the female has rested, she will search for another appropriate site to lay an egg, to repeat these stereotyped steps (Yang et al. 2008). Studies have identified a subset of ILP8 neurons (similar to mammalian relaxin, responsible for widening the cervix for birth) that are expressed in the reproductive tract near the uterus and may regulate this behavior. Hyperpolarizing these neurons resulted in abolition of the ovipositor motor

program, and thus no egg laying. Furthermore, silencing of ILP8-neurons resulted in an “egg jamming” phenotype; eggs were found in the lateral oviduct and the common oviduct, a highly uncommon phenotype (Yang et al. 2008). Command neurons found in the brain (oviposition descending neurons, OviDNs) stimulate the ovipositor motor program following mating, but the circuitry linking these neurons to the ovipositor tissue and mechanisms coordinating each step of the process remain unknown (F. Wang et al. 2020; Feng et al. 2014).

The preferred substrates that a female will choose to lay her eggs are well characterized. Drosophila have been found to be attracted to a substrate with acetic acid (AA) to lay their eggs (Gou et al. 2014; Yang et al. 2008; Joseph et al. 2009). AA is a natural byproduct of fermenting fruit, a preferred food choice of fruit flies. Females prefer to oviposit their eggs on a substrate containing AA, a behavior mediated by the gustatory system (Joseph et al., 2009). Using pox-neuro mutants to transform taste bristles into mechanosensory bristles without gustatory receptors, a neural pathway has been shown to be required for exerting preference for AA-containing substrates. Intriguingly, despite having AA as a preferred substrate to oviposit, females naturally exhibit positional avoidance toward AA mediated by primary olfactory organs (Joseph et al. 2009). Surgical removal of the 3rd antennal segments in olfactory organs reversed positional avoidance of AA-containing substrates (Joseph et al. 2009). Further studies into the neuronal circuitry of the preferences to oviposit on AA-containing substrates uncovered a set of reproductive tract neurons. that drives female behavior to go towards the AA-containing substrate when an egg is ready to be deposited from the uterus (Gou et al. 2014). It is hypothesized that the mechanical stretching of the reproductive tract by

an egg is sufficient to produces signals that affect behavior to guide a female toward the AA-containing substrate, and ppk+ neurons within the reproductive tract may facilitate such mechanosensation. After reducing activity of ppk+ neurons, females present with multiple eggs stuck within their reproductive tract, however they do not exhibit the AA preference that was typical of an “egg-jam” phenotype (Gou et al. 2014). The circuits linking ppk+ neurons within the reproductive tract that sense the presence of an egg to other processing centers within the VNC or CNS, however, remain unclear. In Chapter 4 of this dissertation, a subset of ppk(+) neurons in the uterus are shown to express Oa receptors, suggesting their involvement in the octopaminergic regulation of egg laying.

Further studies into Drosophila oviposition preference determined that a female will prefer to lay an egg on softer substrate rather than a hard substrate and a plain substrate to a bitter (lobeline) substrate (Yang et al. 2008). Females will feed from a sucrose-containing substrate but avoid laying their eggs there in a dose-dependent manner (Yang et al., 2008). This preference is prescribed to a subset of Gr5a sweet taste receptor neurons. When Gr5a neuronal activity was reduced by overexpressing hyperpolarizing Kir2.1, females reduced their avoidance to the sucrose-containing substrate (Yang et al. 2008). This avoidance is also reduced when the sucrose-containing substrate becomes too far away from other options, suggesting there is a decision based upon effort – is it worth it to travel further to lay eggs for that substrate? It is possible that the females are exhibiting a behavior to prevent the first instar larvae from hatching from the embryo and having to travel too far for a sucrose-containing food course. A follow-up study determined that another gustatory receptor (Gr66a) is

necessary for both the situational avoidance to lobeline and also the attraction to egg laying on such substrates (Joseph and Heberlein 2012).

Another preferred oviposition substrate for female fruit flies is ethanol, another byproduct of fermenting, rotten fruit. Unlike the AA-containing substrate preference, females do not actively avoid ethanol-containing substrates (Azanchi, Kaun, and Heberlein 2013; van Delden and Kamping 1990; Richmond and Gerking 1979; Siegal and Hartl 1999). Using a similar approach to the AA-preference study, researchers removed the third antennal segment and found that the preference for ethanol-containing media was not eliminated, suggesting that ovipositing females are attracted through different sensory input than their antennae (Azanchi, Kaun, and Heberlein 2013). They also used the pox neuro mutants to render their gustatory spines as mechanosensory spines, and this manipulation made females avoid ethanol-containing oviposition sites, directly implicating the gustatory system in ethanol sensing. They further determined the circuitry regulating the preference for ethanol-containing substrates as an oviposition site, and they found dopaminergic neurons as a likely candidate. They silenced a subset of dopaminergic neurons (PAM neurons) by using tetanus toxin (UAS-TeTx) and showed a decreased response to ethanol, suggesting these are the subset of neurons regulating ethanol preference (Azanchi, Kaun, and Heberlein 2013).

Oviposition and ovulation are tightly related. An egg needs to be ovulated before it can be oviposited. Further, because a female will hold onto an egg until it finds a preferred substrate, it would be wasteful for a female to ovulate again before the previous egg has been laid. A feedback mechanism must occur between the uterus

having an egg in it and the ovary to prevent another from being ovulated. Data from Yang et al 2008 suggest the possibility that ILP8-neurons are not only responsible for the substrate selection for egg laying but also show a potential role in relaying an inhibitory signal to the ovaries to prevent ovulation. It seems unlikely that the feedback mechanism would come from the ppk+ mechanosensitive neurons, because the phenotype of silencing those neurons involves only one egg being present within the oviduct (Gou et al. 2014). Deciphering this phenotype becomes tricky experimentally. Depending on the method of anesthesia, one could artificially induce ovulation and therefore increase the presence of eggs within the reproductive tract. For example, anesthesia with CO2 increases the presence of eggs within the lateral oviducts (Kaneuchi et al. 2015). Nevertheless, the potential feedback mechanism is currently unknown but would be very interesting to uncover. In Chapter 4 of this dissertation, OaRNs are shown to compose new subsets of neurons that may help untangle distinct circuit pathways regulating oviposition.

**Roles for octopamine in fertility**

Many years of work have demonstrated that one imperative signal required for ovulation and subsequent egg laying is octopamine (Oa), with the first example characterizing octopamine-null female flies in 1996 (Monastirioti, Charles E. Linn, and White 1996). Oa is synthesized from tyramine through tyramine β-hydroxylase (TβH) and tyramine is synthesized from tyrosine through tyrosine decarboxylase (Tdc). Using a null mutant for TβH (Monastirioti et al., 1996), females are unable to synthesize Oa, are unable to ovulate, and experience a dramatic mature follicle-retention phenotype.

Females unable to produce their own tyramine (Tdc-null) also are sterile with a severe egg-retention phenotype.

To further investigate this strong phenotype, efforts have focused on identifying involved Oa receptors and their mode-of-action to promote egg laying. Females that are defective for the Oamb receptor are unable to ovulate (Lee et al. 2003; Lee, Rohila, and Han 2009). Lee et al 2003 developed several Oamb mutants, wherein they characterized the sterility and egg-retention phenotypes. They demonstrated the oviduct had the highest expression level of Oamb, followed by mature follicle cells in the ovary. To assay ovulation, they used 3-4 day old virgin females mated 1:1 with wild type males for one hour, then examined the reproductive tract on ice at various time points. The most striking phenotype observed was 12 hours after mating; their mutant alleles were "ovulated" 0-10% compared to their controls at 60%. The role of Oamb in mature follicle cells in promoting follicle rupture and ovulation has since been extensively documented (Deady and Sun 2015).

Follow-up experiments to finding this receptor being responsible for ovulation came from the same group when they sought to determine the tissue in which Oamb was responsible for regulating ovulation. In their 2009 study, they developed a "reproductive system GAL4" (RS-GAL4), by using a fragment of the Oamb gene that was specifically expressed in the oviduct epithelium. To determine if the oviduct epithelium was the site of Oamb's action to regulate ovulation, they used an Oamb mutant ($Oamb^{286}$) and their RS-GAL4 driver to ectopically express Oamb specifically in the oviduct epithelium. Control females "ovulated" around 60% and the mutants "ovulated" around 35%. When they performed a rescue experiment, they successfully

reduced the ovulation defect. Females with overexpression of either isoform of Oamb (AS or K3) in the oviduct epithelium in the $Oamb^{286}$ mutant ovulated at levels similar to control (70-60%). These data suggest an essential role for Oamb in the oviduct epithelium to regulate ovulation (Lee, Rohila, and Han 2009). However, these conclusions have been challenged by upstream roles for Oamb shown in follicle cell rupture, and the role of the oviduct epithelium in egg laying as well as the roles of Oa receptor expression there remain poorly defined. Chapter 2 of this dissertation suggests roles for Oamb and Octβ2R in regulating oviduct muscle contractility.

The same group also investigated a possible role for another OA receptor in the oviduct epithelium – Octβ2R (Lim et al. 2014). They determined Octβ2R mutant flies are also sterile, a phenotype attributable to a lack of ovulation. To assay ovulation, they used 4-5 day old virgins mated 1:3 with wild type males (10 virgins, 30 males / vial) for 18 hours then dissected on ice. If there was an egg within the oviduct (lateral or common) or the uterus, she was recorded as “ovulated”. Wild type females “ovulated” at around 85% whereas *Oct2βR* mutant females “ovulated” at ~25%, despite showing normal copulation, sperm storage, and post-mating rejection behavior. To determine the site of Octβ2R’s action upon ovulation, they attempted a rescue experiment, wherein they used their previously generated “reproductive-system” RS-GAL4 driver to drive UAS-Octβ2R specifically in the oviduct epithelium– in this fly, the female does not express Octβ2R anywhere except her oviduct epithelium. When they used these females to assay for ovulation, they “ovulated” at levels comparable to control (85-95%), suggesting the site for Octβ2R’s influence in ovulation was also the oviduct epithelium, similarly to their findings with Oamb. When they expressed Oamb (either isoform) or

UAS-Octβ3R in the oviduct epithelium to attempt to rescue the Octβ2R mutant phenotype, it significantly increases ovulation rates (~35-50%) from Octβ2R mutant rates (~20%) but does not reach control levels of ~85%, suggesting potential non-overlapping roles of the different OA receptors in ovulation. The current model is that 1. Octβ2R increases cAMP in the oviduct epithelium to activate PKA and that may induce secretions from the oviduct into the lumen; and 2. Oamb increases [Ca2+] in the oviduct epithelium, increasing CaMKII activity which could induce nitric oxide signaling to relax the oviduct musculature or to also contribute to luminal secretions.

As mentioned previously, developing egg chambers in the Drosophila ovary exist beneath two layers of musculature, which have also been hypothesized to be another site of action of octopamine in regulation of ovulation. The layer closest to the egg chamber is the ovariole muscle sheath. Each ovariole has its own muscle sheath enveloping its contents. This layer contains circular bands of muscle fibers that surround the egg chambers, and do not run anteriorly-posteriorly (Middleton et al. 2006). One layer more distal from the ovariole muscle sheath is the ovarian sheath, or peritoneal sheath, which surrounds the entire ovary. The peritoneal sheath is a large mesh-like network with many gaps that surrounds each ovary (Hudson et al. 2008; Middleton et al. 2006). Both of these muscle networks contain irregular patterns of the thick and thin filaments that are characteristic of “supercontractile” insect visceral muscles, suggesting the ability of these muscles to contract to less than 50% of their resting length. The physiology of these ovarian muscles has been described with a method where ovaries can be live imaged to generate ovariograms based on their movements (indicating a contraction). It has been determined that the musculature of

the female reproductive system (ovaries, peritoneal sheath, oviduct, spermathecae) contract rhythmically but independently from each other. Peritoneal sheath contractions at the base of the ovary can be observed in ex-vivo preparations, and the amplitude of these contractions is augmented with OA application (Middleton et al. 2006).

Recent work has shown that Oa signals also increase germ line stem cell proliferation in anterior regions of the ovary via Oamb receptor activity in escort cells (Yoshinari et al. 2020). Such signaling is part of a broader post-mating response in which numerous fertility behaviors become disinhibited following female reproductive organ exposure to signals in male seminal fluid. Precisely how Oa from CNS neurons is delivered to different regions of the reproductive tract, remains an unresolved question. As described here, Oa signaling pathways have been described that target every reproductive organ (ovaries, oviducts, accessory glands, and uterus). The actions of Oa at each organ, however, differ and must be strictly coordinated. Distinguishing neural pathways responsible for the regulation of each distinct stage of egg laying is a primary goal of ongoing projects. In Chapter 5 of this dissertation, it is shown that individual octopaminergic neurons may each have unique reproductive targets and roles. OaRNs expressing different subtypes of Oa receptors may also divide the post-mating circuit into functional subgroups. Such data helps establish the octopaminergic neuromodulation of egg laying as a potentially powerful model in which to study fundamental principles of system neuromodulation. Previously, such models have been restricted to very select circuits such as the crab somatogastric ganglion due to the difficulty in assaying neurotransmitter/neuromodulator effects in complex circuits with larger numbers of neurons (Marder and Bucher 2007).

**Need for a complete model of octopaminergic pathways in egg laying**

Follicle cell rupture assays used to investigate ovulation in Drosophila have recently been incorporated into a workflow for evaluating nonhormonal contraceptive treatments for humans (Jiang et al. 2021). Such work highlights the translational nature of the female fly reproductive system. Still, such assays are performed ex-vivo in the absence of neural inputs and depend on time-consuming dissections of each ovariole. If the neural systems that influence the process of egg laying can be better defined, translational fertility assays could be expanded to include fully in vivo scenarios. Strict and clear definitions of ovulation, oviduct passage, and oviposition must also be standardized in order to properly evaluate the locations of fertility effects.

To measure “ovulation” in Drosophila, it is important to remember a few key components of the ovulatory process. 1) For ovulation to occur, mature eggs must be present in the ovary. If there is a mutation that causes a reduction in oogenesis, there will be decreased ovulation. Waiting a few days post-eclosion and feeding females with wet yeast prior to an experiment should increase the number of available eggs within the ovary. A characteristic phenotype of anovulation is egg retention – when there is a build-up of mature stage 14 egg chambers in the ovary. In this condition, females have many mature follicles but are unable to ovulate. Due to these phenomena, quantification of mature follicles present within the ovary is necessary for a read-out in egg laying experiments to attribute an “ovulation” defect. 2) Mating induces many behavioral and physiological changes. Therefore it must be considered before performing an ovulation assay. For example, mating increases octopamine release through ovulin-induced

mechanisms, and increases the rate of ovulation (Rubinstein and Wolfner, 2014). The status of mating for each female tested must be uniform throughout the experimental conditions. 3) If females are expected to mate prior to an ovulation assay, it is important to confirm that they have normal copulation behavior. If a specific genetic alteration impairs copulation behavior, it could also impact the post-mating response. If that is the case, then what would be measured would be a measurement of successful or unsuccessful copulation. 4) The transportation of an egg throughout the reproductive tract could influence the rate of ovulation, as demonstrated through experiments investigating oviduct secretions, contractions, and post-mating physiological and morphological changes. Egg transport is a complex process and therefore shouldn't be a read-out for "ovulation".

An egg is first ovulated and received in the lateral oviduct, and then it is transported to the uterus and eventually oviposited. These steps are separate from each other, and each can indicate its own set of phenotypes. If the oviduct lacks secretions or is deformed or uncoordinated in its contractions, for example, the oocyte will be transported at a slower rate or not at all (J. Sun and Spradling 2013). Considering effects in each of these systems separately is imperative for combining the currently disparate models of follicle development, ovulation, oviduct passage, and oviposition. As shown by each chapter of this dissertation, octopaminergic mechanisms offer a powerful means for uniting models of fertility behavior in the fly. The model circuits described here establish a novel system relevant to fertility assays (OaRN networks) (Chapter 4) and define previously unrecognized heterogeneity among egg

laying circuit neurons that can guide understandings of neuromodulatory circuit functions throughout biology (Chapters 4 and 5).

## CHAPTER 2: REGULATION OF DROSOPHILA OVIDUCT MUSCLE CONTRACTILITY BY OCTOPAMINE

### SUMMARY

Octopamine is essential for oviposition (egg laying) in Drosophila melanogaster, but the neuronal pathways and receptors in the oviposition circuit by which octopamine regulates visceral muscle contractility in the reproductive tract are not known. We find that the two octopamine receptors that have been previously implicated in oviposition–OAMB and Octβ2R are expressed in octopaminergic and glutamatergic neurons that project to the reproductive tract, peripheral ppk(+) neurons within the reproductive tract and epithelial cells that line the lumen of the oviducts. Further optogenetic and mutational analyses indicate that octopamine regulates both oviduct contraction and relaxation via Octβ2 and OAMB receptors respectively. Interactions with glutamatergic pathways modify the effects of octopamine on both the epithelium and muscle. Octopaminergic activation of Octβ2R on glutamatergic processes provides a possible mechanism by which octopamine initiates lateral oviduct contractions. We speculate that the aminergic, neuromodulatory pathways in the oviposition circuit may be comparable to some of the mechanisms that regulate visceral muscle contractility in mammals.

## INTRODUCTION

Mammals and invertebrates such as Drosophila express multiple receptor subtypes for the same neurotransmitter and deciphering their respective roles will be essential to understand circuit activity and behavior. We are using the fly oviposition circuit as a model to investigate this issue, building on pioneering work in Drosophila as well as larger insects such as locusts (Lim et al. 2014b; Orchard and Lange 1985; 1986; Wong and Lange 2014; White, Chen, and Wolfner 2021; Yang et al. 2009; Zhou et al. 2012; Avila et al. 2012; Middleton et al. 2006; Rodríguez-Valentín et al. 2006; Rezával et al. 2012; 2014; Gou et al. 2014; Yoshinari et al. 2020; Andreatta et al. 2018; H.-G. Lee et al. 2003; H.-G. Lee, Rohila, and Han 2009; H. Lee et al. 2016; El-Kholy et al. 2015; Li et al. 2015; Rubinstein and Wolfner 2013).

Both glutamate and the aminergic neuromodulator octopamine have been implicated in the regulation of the oviposition circuit and post-mating behavior in Drosophila and other insects (Lim et al. 2014b; Hana and Lange 2020; Zhou et al. 2012; Avila et al. 2012; Rubinstein and Wolfner 2013; Rezával et al. 2014; Gou et al. 2014; Yoshinari et al. 2020; Andreatta et al. 2018; Lange 2009; Middleton et al. 2006; Rodríguez-Valentín et al. 2006; H.-G. Lee et al. 2003; H.-G. Lee, Rohila, and Han 2009; H. Lee et al. 2016; El-Kholy et al. 2015; Li et al. 2015; Yang et al. 2009; Cossío-Bayúgar et al. 2012). One crucial function of the oviposition circuit is to regulate the contraction and relaxation of the visceral muscles that line the oviducts, thus allowing passage of the egg through the reproductive tract (Dustin Rubinstein et al., 2014). Similar rhythmic contractions occur within most if not all mammalian viscera including the gut and genitourinary tract (McHale et al. 2006; S. Schneider, Wright, and Heuckeroth 2019;

Spencer and Hu 2020; Sanders, Ward, and Koh 2014). The neuromodulatory regulation of these contractile processes requires a complex interplay between cells in the central nervous system and the periphery which remains incompletely understood (McHale et al. 2006; S. Schneider, Wright, and Heuckeroth 2019; Spencer and Hu 2020; Sanders, Ward, and Koh 2014). Now classic studies in the locust reproductive tract have uncovered some of the neuronal elements governing visceral muscle contractility in insects (Lange 2009). The tools available in Drosophila allow additional detailed probes of the underlying molecular-genetic and cellular pathways (H.-G. Lee, Rohila, and Han 2009; H.-G. Lee et al. 2003; H. Lee et al. 2016; Lim et al. 2014b; Meiselman, Kingan, and Adams 2018; F. Wang et al. 2020).

In Drosophila, a cluster of eight to ten octopaminergic neurons in the abdominal ganglion (AbG) innervate the reproductive tract, and genetic studies suggest that at least two octopamine receptors-- Octβ2R and OAMB -- are essential for fertility (H.-G. Lee, Rohila, and Han 2009; H.-G. Lee et al. 2003; McKinney et al. 2020; Li et al. 2015; Lim et al. 2014b; Rezával et al. 2014; Rubinstein and Wolfner 2013; Yoshinari et al. 2020; A. Schneider et al. 2012; Pauls et al. 2018; Monastirioti, Charles E. Linn, and White 1996; Monastirioti 2003). The role of each receptor in regulating contractility in the oviducts and the insect reproductive tract as a whole remains unclear (Middleton et al. 2006; Rodríguez-Valentín et al. 2006; Tamashiro and Yoshino 2014). Establishing the function of specific octopaminergic receptors and the mechanisms by which they regulate contractility represents a critical step toward deciphering the principles that govern the oviposition circuit. Studies of the fly oviposition circuit could also identify general principles governing the neuromodulation of visceral muscle in classical

invertebrate models such as larger insects and crustaceans (Lange 2009; McGraw, Clark, and Wolfner 2008; Wu and Cooper 2012; Audsley and Weaver 2009; McGaw and Curtis 2013). Although the regulation of central pattern generators for locomotion in the CNS and in ganglia have been studied extensively in these species, the molecular mechanisms by which invertebrate visceral muscles are regulated by neuromodulatory inputs to the muscle tissue remain poorly described.

In mammals, many neuromodulatory inputs are indirectly routed to visceral muscle cells via receptors expressed in nearby neurons or interstitial cells (McHale et al. 2006; S. Schneider, Wright, and Heuckeroth 2019; Spencer and Hu 2020; Sanders, Ward, and Koh 2014). These include the modulation of gut contraction by receptors expressed on interstitial cells and by neurons within the mesenteric ganglia (McHale et al. 2006; S. Schneider, Wright, and Heuckeroth 2019; Spencer and Hu 2020; Sanders, Ward, and Koh 2014). The possibility that similarly indirect pathways may regulate the Drosophila reproductive tract has been suggested previously, based on the genetic rescue of an octopamine receptor mutant and expression in the epithelium (H.-G. Lee, Rohila, and Han 2009; H.-G. Lee et al. 2003; Lim et al. 2014b); however, the notion that in invertebrates, visceral muscles might be indirectly regulated via receptors expressed on other cell types has otherwise received little attention.

We find that the lateral and common oviducts (LO and CO respectively) show distinct patterns of Octβ2R and OAMB expression, that the LO and CO are differentially regulated, and that Octβ2R and OAMB perform distinct roles in the regulation of oviduct muscle contractility. Our data also suggest that some of the effects of octopamine on muscle may be mediated indirectly via receptors on non-muscle cells as previously

suggested (H.-G. Lee et al. 2003; H.-G. Lee, Rohila, and Han 2009; Lim et al. 2014) and also interact with glutamatergic pathways previously shown to govern contractility (Rodríguez-Valentín et al. 2006; Yang et al. 2009; Gou et al. 2014; Castellanos, Tang, and Allan 2013; Middleton et al. 2006; Häsemeyer et al. 2009).

## RESULTS

### Octβ2R and OAMB are differentially expressed in the lateral and common oviducts

Drosophila express six subtypes of octopamine receptors including OAMB, Octβ1R, Octβ2R, Octβ3R, Oct-TyrR, also known as Tyr1R, (El-Kholy et al. 2015; Han, Millar, and Davis 1998; Evans and Maqueira 2005; Ohhara et al. 2012; Kutsukake et al. 2000; Bayliss, Roselli, and Evans 2013) and the more recently discovered Octα2R (Qi et al. 2017). Both RT-PCR and a series of GAL4 drivers show expression of OAMB, Octβ2R, Oct-TyrR in the female reproductive tract (El-Kholy et al. 2015; Li et al. 2015; H.-G. Lee, Rohila, and Han 2009; Lim et al. 2014b; H.-G. Lee et al. 2003). Here we focus on OAMB and Octβ2R, since these have been previously suggested to be required for oviposition (Lim et al. 2014b; Li et al. 2015; H.-G. Lee et al. 2003; H.-G. Lee, Rohila, and Han 2009).

To more precisely determine the location and cell types that express each receptor, we generated a new set of MiMIC lines in which GAL4 was inserted within the endogenous locus of each receptor (P.-T. Lee et al. 2018) (Supplemental Fig. 1). Some of these lines have been described previously (P.-T. Lee et al. 2018). Since expression of GAL4 in the MiMIC locus is controlled by the endogenous regulatory regions within

each gene, the pattern “mimics” that of endogenous protein expression more accurately than most standard GAL4 transgenes (P.-T. Lee et al. 2018) (Note that these lines label the cells that express the endogenous protein, but do not provide information on its subcellular localization).

We observe robust expression of both OAMB and Octβ2R in the epithelial cells that line the lumen of the lateral and common oviducts (Fig. 1, A-i, A-iii, A-iv, B-i, B-iii) consistent with the expression pattern driven by a GAL4 line containing a portion of the OAMB gene (Lim et al. 2014b; Li et al. 2015; H.-G. Lee et al. 2003; H.-G. Lee, Rohila, and Han 2009). However, Octβ2R is exclusively expressed in the epithelia within the lateral oviduct (Fig. 1, B-i iii). While we also detect an immunofluorescent signal in the common oviduct, it arises from thin processes that are morphologically distinct from the epithelium (Fig. 1, B-iv). In contrast to Octβ2R, OAMB is expressed in the epithelium of both the lateral and common oviducts (Fig. 1, A-iii, A-iv).

In addition to the epithelium, we detect OAMB expression in several subtypes of nonneuronal cells within the reproductive system. These include the follicle cells surrounding the egg as previously reported (Deady and Sun 2015), phalloidin(-) cells in the caps of the parovaria glands (data not shown) which are required for ovulation (J. Sun and Spradling 2013), and consistent with previous functional experiments, phalloidin (-) cells both in the caps of the spermathecae and in the seminal receptacle (Avila et al. 2012). We focus here on the function of OAMB and Octβ2R in the oviducts.

Both OAMB and Octβ2R are expressed in an extensive network of thin processes throughout the oviducts (Fig. 1, A-vii, B-iv, B-vii, Supplemental Fig. 2). We confirmed the neuronal identity of both the OAMB(+) and Octβ2R(+) processes via co-

labeling with the neuronspecific glycoprotein nervana, recognized by the “anti-HRP” antibody (B. Sun and Salvaterra 1995) (Fig. 1. A-vii, B-vii and data not shown). Subsets of processes expressing OAMB and Octβ2R were detected on the lumenal and external faces of the oviducts (Supplemental Fig. 2).

Octopaminergic cell bodies in the AbG that express OAMB and Octβ2R have been previously identified in the AbG (McKinney et al. 2020) and both OAMB and Octβ2R axons project through the Median Abdominal Nerve (MAN) and into the reproductive tract. (Fig. 1, A-i, A-vi, B-i). The reproductive tract is extensively innervated by octopaminergic projections (Supplemental Fig. 3) (Pauls et al. 2018) and others have shown that octopaminergic nerve terminals at the larval NMJ in body wall muscle express at least two types of autoreceptors (Koon et al. 2011). To determine whether octopaminergic cells that project to the reproductive tract also express OAMB and/or Octβ2R as potential autoreceptors (McKinney et al. 2020) we performed a series of co-localization experiments. Using a Tdc2-LexA driver to express membrane bound RFP in Tdc2(+) cells and the MiMIC-GAL4 lines for each receptor, we confirmed the expression of Octβ2R in the midline Tdc2(+) somata within the AbG (Fig. 2) (McKinney et al. 2020). However, in contrast to an earlier report (McKinney et al. 2020), we detect Octβ2R expression in most, if not all, of the midline Tdc2(+) cells in the AbG (Fig. 2). We do not detect OAMB in any of the midline Tdc2(+) neurons in the AbG (Supplemental Fig. 4). Similarly, none of the OAMB(+) processes in the reproductive tract appear to co-express Tdc2 (data not shown). We conclude that a subset of the Octβ2(+) processes present in the oviducts, but none of the OAMB(+) processes, represent projections from octopaminergic neurons in the AbG.

In addition to octopamine, glutamate released from ILP7(+) neurons regulates the oviposition circuit, and ILP7(+) processes project to the oviducts (Yang et al. 2009; Rodríguez-Valentín et al. 2006; Gou et al. 2014; Castellanos, Tang, and Allan 2013). To determine if ILP7 neurons might also express Octβ2R, we performed additional co-localization experiments using the ILP7- LexA driver and the Octβ2R-GAL4 MiMIC line. We detect expression of Octβ2R expression in at least four ILP7(+) cells in the AbG (Fig. 3). These data raise the possibility that octopamine could regulate the function of ILP7(+) neurons, either at the level of cell bodies and processes in the AbG or the nerve terminals that innervate the reproductive tract.

We detect expression of both OAMB and Octβ2R in multiple, peripheral, neuronal cell bodies within the reproductive tract (Fig. 1A-vii, 1B-vii, Supplemental Fig. 5). The labeled somata are embedded within the muscle cells of the uterus and project anteriorly into the oviducts (Fig. 1, A-vii, B-vii). All peripheral Octβ2(+) neurons that we detect also express ppk1 (Supplemental Fig. 5B) a channel involved in mechanosensation in the reproductive tract as well as the larval body wall and also implicated in post-mating behavioral changes (Yang et al. 2009; Gou et al. 2014; Rezával et al. 2014; Gorczyca et al. 2014; Zelle et al. 2013; Mauthner et al. 2014). By contrast, some peripheral OAMB(+) cell bodies do not express ppk1 (Supplemental Fig. 5A). The location of the cell bodies that express ppk1 and Octβ2R or OAMB in the uterus is consistent with the location of a subset of ppk1(+) neurons that have been shown to regulate glutamatergic neurons in the oviposition circuit and the post-mating response (Yang et al. 2009; Gou et al. 2014; H. Lee et al. 2016; Häsemeyer et al. 2009). By contrast, we do not detect expression of Octβ2R or OAMB in a second subset

of ppk1(+) neurons which also regulate the post-mating response and localize within peripheral nerves near the lateral oviducts (Yang et al. 2009; Gou et al. 2014; H. Lee et al. 2016; Häsemeyer et al. 2009). The expression of Octβ2R and OAMB in ppk1(+) cells raise the possibility that octopaminergic activation of cells in the periphery could modulate the post-mating response, but we have not yet tested this hypothesis.

We did not detect expression of OAMB or Octβ2R in muscle cells labeled with phalloidin in the oviduct or elsewhere in the reproductive tract (Fig. 1 and data not shown). We cannot exclude the possibility that the MiMIC lines we have used failed to detect expression in muscle, either because the levels are too low, or because introduction of the trojan exon for the MiMIC selectively disrupted expression in muscle. Moreover, RNAi knockdown of Octβ2R RNA using a muscle driver was reported to cause female infertility (Li et al. 2015). We find that some drivers used for expression in muscle are also expressed at other sites within the AbG and the oviducts (Supplemental Fig. 6). Therefore, ectopic expression of the Octβ2R RNAi in cells other than muscle, e.g. neurons, may have contributed to the previously observed infertility (Li et al. 2015). With these caveats in mind, our data suggest that octopaminergic regulation of muscles in the fly reproductive tract may be mediated indirectly via receptors expressed in nonmuscle cells, as previously suggested in Drosophila (H.-G. Lee et al. 2003; H.-G. Lee, Rohila, and Han 2009; Lim et al. 2014) and reminiscent of some neuromodulatory inputs to mammalian visceral muscles (McHale et al. 2006; A. Schneider et al. 2012; Spencer and Hu 2020; Sanders, Ward, and Koh 2014). It has been previously suggested that glutamate and octopamine may be released from the same cells to regulate the oviposition circuit (Rodríguez-Valentín et al. 2006). To address this

possibility, we performed additional co-localization experiments. Since the glutamatergic regulation of the reproductive tract is mediated by ILP7(+) neurons (Gou et al. 2014; Castellanos, Tang, and Allan 2013), we compared the localization of markers expressed using ILP7-LexA to labeling using an antibody to Tdc2 (αTdc2, COVALAB 00013519). We do not detect any overlap (Fig. 4), suggesting that glutamate and octopamine are released by different cells in the oviposition circuit. These data are also consistent with the observation that expression of UAS-VGluTdsRNAi in octopaminergic neurons (TDC2-GAL4) did not disrupt egg-laying (Castellanos, Tang, and Allan 2013).

It has been suggested that proctolin may contribute to oviduct contractions in both flies and larger insects (Orchard and Lange 1986; Lange 2009; Rodríguez-Valentín et al. 2006), and at least some ILP7 cells in the Drosophila nerve cord express mRNA encoding proctolin (A. M. Allen et al. 2020). To determine whether the Tdc2(+) or ILP7(+) cells in the AbG could store and release proctolin, we co-labeled Tdc2(+) or ILP7(+) cells with a commercially available antibody to proctolin. We do not detect expression of proctolin in Tdc2(+) cells (Fig. 5). By contrast, at least three ILP7(+) cells in the distal AbG were co-labeled for proctolin (Fig. 5) raising the possibility that glutamate and proctolin might be co-released.

**Octopaminergic neurons in the AbG stimulate lateral but not common oviduct activity**

To determine the function(s) of Octβ2R and OAMB expressing neurons and the effects of octopamine on the oviducts we optogenetically stimulated the octopaminergic (Tdc2(+)) neurons in the AbG. Our initial experiments using a standard version of ChR2

yielded inconsistent results (data not shown). We therefore used modified versions of ChR2 with increased light sensitivity--ChR2-XXM and ChR2-XXL (Scholz et al., 2017, Dawydow et al., 2014)--for all the experiments shown here. We did not detect any differences between the response of ChR2-XXM and ChR2-XXL and they were used interchangeably. To express ChR2 and ChR2-XXM/L in octopaminergic neurons, we used the driver Tdc2-GAL4 (See Supplemental Fig. 3 for overview of expression) (Cole et al. 2005). To allow stimulation of both the soma and more peripheral processes, we employed an “Intact Preparation” in which minimal dissection techniques were used to expose the AbG and reproductive system while preserving the entire CNS and peripheral neuronal connections (Fig. 6A, B).

Several previous reports have indicated that octopamine can induce muscle relaxation in locusts and flies (Dustin Rubinstein and Wolfner 2014; Lange 2009; Rodríguez-Valentín et al. 2006; Cook and Wagner 1992). By contrast, bath-applied octopamine drives contractions in crickets (Tamashiro and Yoshino 2014; Mizunami and Matsumoto 2017), and movements previously observed at the base of the reproductive tract in Drosophila may in fact represent oviduct contractions (Middleton et al. 2006; Meiselman, Kingan, and Adams 2018). We find that optogenetic activation of octopaminergic neurons (30 s) results in rhythmic contractions 18.11 ± 2.09 (n=9) of the lateral oviducts during the period of stimulation (Fig. 6C, D). Contractions occurred with a latency of 5.3±1.3 s and ceased within 2 seconds of ending the light stimulus. To further explore this response, we repeated the stimulus twice more with intervening rest periods and observed a similar number of contractions and latency to contraction following stimulation (Fig. 6D). Together, these data show that activation of

octopaminergic neurons can induce acute, repetitive contractions of muscle in the lateral oviduct. Interestingly, using the same stimulation protocol in the same preparations, we did not detect any contractions in the CO following optogenetic stimulation of Tdc2(+) (data not shown). These data suggested that the LO and CO may be differentially regulated by octopaminergic inputs. We hypothesize that these effects are mediated by octopamine, but we cannot rule out a contribution from co-released tyramine.

To verify these results, we performed additional experiments using calcium probes to better visualize muscle cells. Representative traces and the average of all traces are shown in Fig. 6 (E-F), and Supplemental Fig 7 (A-B) respectively (see also Video 1). To visualize cytosolic calcium in muscle, we expressed the red shifted calcium indicator RCaMP1b in muscle using the driver 24B-GAL4 and expressed UAS-ChR2-XXL in neurons using Tdc2-LexA. As shown in Video 1, calcium transients and contractions occurred simultaneously. Therefore, the regions of interest that we quantified show changes in fluorescence (ΔF) that result from both the intrinsic fluorescence of RCaMP as well as movement of the tissue, and the traces in Fig. 6 represents the aggregate change caused by both movement and changes in cytosolic calcium.

We observed rhythmic fluctuations in fluorescence in the visceral muscle cells of the lateral oviduct following optogenetic stimulation of octopaminergic neurons (Fig. 6E and Supplemental Fig 7). We did not observe any response in the CO (Fig 6E'). These results confirm our observations using movement alone that optogenetic stimulation of

octopaminergic neurons activates rhythmic activity in visceral muscles within the LO but does not cause detectable effects on the muscles of the CO.

**ILP7 neurons can induce contractions in both the lateral and common oviducts**

For comparison, we next tested the effects of optogenetically stimulating glutamatergic neurons in the AbG. We used the drivers ILP7-LexA and ILP7-GAL4 to express channel rhodopsin in the glutamatergic neurons previously shown to innervate the reproductive tract (Castellanos, Tang, and Allan 2013; Gou et al. 2014). We monitored the response of the oviducts both in the absence of a calcium reporter (not shown) and using RCaMP1b as described above for octopaminergic neurons (Fig. 6F, F', Video 2). Similar to our results using Tdc2(+) neurons, the ΔF/F for ILP7(+) cells in the RCaMP experiments represents the aggregate effects of both muscle contractions and changes in cytosolic calcium. In contrast to octopaminergic neurons, expression of ChR2 in the ILP7/glutamate cells was sufficient to consistently induce calcium transients and contractions, and ChR2-XXM/L was not required to detect a response (not shown). However, for consistency, ILP7-LexA>LexAop-ChR2-XXL was used for direct comparison to Tdc2-LexA-LexAop-ChR2-XXL (Fig. 6E, E', F, F').

Optogenetic stimulation of ILP7(+) neurons activated muscles in both the lateral and the common oviduct (Fig. 6F, F') although the response of the lateral oviduct following optogenetic stimulation of ILP7(+) neurons was less consistent (7/10 preparations) compared to common oviduct contractions (11/12 preparations) and occurred with a slightly longer latency following stimulation (Supplemental Fig. 8). While optogenetic stimulation of both Tdc2(+) and ILP7(+) neurons led to calcium transients

and contractions of the LO, the average number of events was higher for Tdc2 stimulation: 21.2 ± 5.3 (mean + SEM, n=9) over 30 sec for Tdc2 versus vs 11± 3.7 over 30 sec (n=7) for ILP7. In addition, for most ILP7 preparations (6 of 7) the calcium transients and contractions stopped while stimulation was ongoing, as compared to Tdc2 experiments in which calcium transients and contractions continued through the entire stimulation period in 9 of 9 preparations. These differences notwithstanding, the response of both the LO and CO to ILP7 stimulation underscores the previously established and central role for glutamate in the regulation of muscle contractions. By contrast, the more restricted effect of octopaminergic neurons on activity in the LO but not the CO suggests a more specialized role in modulating more discrete aspects of the oviposition circuit.

**Peripheral octopamine and glutamate receptors induce oviduct contractions**

Optogenetic stimulation of neurons in the AbG or bath application of octopamine could potentially result in activation of octopamine receptors either within the central nervous system or the periphery. It also remained possible that co-released tyramine could contribute to the effects we observed. To determine whether octopamine and octopamine receptors in the periphery and within the reproductive tract were sufficient to generate lateral oviduct  contractions, we tested the effects of bath applied octopamine on reduced preparations in which the CNS had been removed: (1) an “Abdominal Fillet” preparation in which the MAN was cut and inputs from the AbG were thereby eliminated (Fig. 7A, A’) and (2) an “Isolated Preparation” in which the MAN was

cut and the reproductive tract was completely dissected out of the abdomen (Fig. 7B, B'). In addition, the nerves that connect the lateral oviducts and the uterus were severed in the "Isolated Preparation" (Fig. 7B, B', Supplemental Fig. 9). Initial dose-response experiments in the isolated preparation showed LO contractions at OA concentrations of >100 micromolar (Supplemental Fig. 10) and 1 mM was used for all further experiments to maximize the observed effects.

Application of 1 mM octopamine but not saline (HL3.1) alone to an Abdominal Fillet was followed by the initiation of contractions in the lateral oviduct in 6/6 preparations. The average number of contractions observed was 15.6 ± 2.05 (mean +/- SEM, n=6) over the initial oneminute observation period (Fig. 7C') (see Video 4). Application of octopamine to an Isolated Preparation was followed by lateral oviduct contractions in 5/5 flies with an average of 22.2 ± 6.5 contractions over 1 minute (Fig 7D'). In both the Isolated and Abdominal Fillet preparations, we observed a period of quiescence of ~30-60 sec after the contractions, followed by additional bouts of rhythmic contractions (data not shown). We did not detect contractions of the common oviduct following application of octopamine using either the Isolated Preparation or Abdominal Fillet (data not shown) consistent with the effects of optogenetically stimulating Tdc2(+) neurons.

Previous studies have suggested that both the ovaries and the calyx region at the base of the ovaries contract following bath application of octopamine (Middleton et al., 2006, Meiselman et al., 2018). To confirm the difference between contractions within the LO versus other sites within the reproductive tract, we performed additional bath application experiments using calcium sensors, similar to our experiments using

optogenetics. Expression of GCaMP (Fig. 7E) and RCaMP (Video 3, 4) via the muscle driver 24B-GAL4 helped to localize specific regions where contractions occur and to differentiate the muscles of the LO from the peritoneal sheath of the ovary (Chen et al., 2013, Vajente et al., 2020). As shown in Video 3, calcium transients and contractions of both ovaries and LO can sometimes be detected following bath application of octopamine. However, contractions of the ovaries versus LO can be readily distinguished and can occur independently. Changes in fluorescence and contractions in the ovaries but not the LO are seen at the end of Video 3, and Video 4 shows a preparation in which the LO but not the ovaries contracted in response to octopamine. In this paper we have focused on oviduct contractions. We will investigate the pathways responsible for ovary contractions in future experiments.

We used preparations expressing GCaMP6 and RCaMP1 to further quantify the effects of OA (Fig. 7, Supplementary Fig. 11). Using an Isolated Preparation, we observed rhythmic GCaMP6 activity in the lateral oviduct muscle following bath application of octopamine but not saline alone (Fig. 7E). As observed for optogenetic stimulation, the peaks of fluorescence represent both an increase in cytosolic calcium and muscle movement. The average number of peaks in traces of the lateral oviduct following bath application of octopamine was 19.0 ± 3.9 (n=5, mean +/-SEM), similar to the number of contractions scored in Fig. 7C, D in the absence of a calcium reporter. We observed a longer latency between the application of octopamine and initiation of calcium transients in the Abdominal Fillet (16.5±3.4 sec) versus application to an Isolated preparation (6.4±2.3 sec) (Supplemental Fig. 11) possibly due to the time required for diffusion of octopamine to its site of action within the abdomen.

In contrast to the lateral oviduct (Fig. 7E'), we observed minimal changes in fluorescence in the common oviduct in response to octopamine (Fig. 7E''), consistent with the lack of detectable movement in the common oviduct in response to optogenetic stimulation (Fig. 6E'). Together, these data show that the effects of octopamine differ between the lateral and common oviducts and suggest that the lateral and common oviducts may represent distinct functional units within the same circuit. We note that contraction of the lateral oviduct in response to OA occurs in the absence of the AbG or the peripheral nerves that connect the uterus to the lateral oviduct (in the Isolated Preparation). Therefore, the subset of peripheral nerves that connect the anterior and posterior regions of the reproductive tract and are cut in the Isolated Prep (see Fig. 1 and Supplemental Fig. 9) are not required for OA-induced LO contractions.

Although it remains possible that we failed to detect octopamine receptors in muscle cells, their apparent absence using the MiMIC lines suggest that the oviduct muscles are activated through receptors expressed on non-muscle cells, i.e. via an indirect mechanism. Possible indirect pathways include activation of octopamine receptors that are expressed on either the epithelial cells as suggested previously (Lee et al., 2003, Lee et al., 2009, Lim et al., 2014) or on one or more of the neuronal processes in the reproductive tract that express Octβ2R and/or OAMB.

To similarly explore the site of action of glutamate's affects, we bath applied glutamate (10 mM) to the Abdominal Fillet or Isolated Preparations (Fig. 7F, F') expressing GCaMP. Using the Abdominal Fillet, we observed a single sustained contraction in the common oviduct (Video 5, Fig 7F'). We also observed two to ten calcium transients in the lateral oviduct in the Abdominal Fillet in response to glutamate

(5 ± 1.2, mean + SEM, n=5) (Video 5, Fig. 7F''). In the Isolated Prep we observe activity in the common oviduct similar to the Abdominal Fillet but no more than one brief contraction in the lateral oviduct in response to glutamate (Video 6 and data not shown).

The response of the LO and CO to glutamate in the Abdominal Fillet Preparation, indicates that, similar to the effects of OA on the LO, the AbG is not required and that the relevant glutamate receptors reside within the reproductive tract. However, the difference between the response of the Isolated Preparation and the Abdominal Fillet to glutamate suggest that peripheral nerves that connect the uterus and lateral may contribute to glutamate induced contractions of the LO, in contrast to the effects of OA (see above).

In addition to glutamate and octopamine, the peptide neurotransmitter proctolin has been shown to regulate oviduct contractions in both flies and larger insects (Adams and O'Shea, 1983, Holman and Cook, 1985, Orchard and Lange, 1986, Lange et al., 1986, Rodriguez-Valentin et al., 2006). Since we find that proctolin is stored in a subset of ILP7 cells in the AbG, we tested the effects of bath applied proctolin (data not shown). Bath applied proctolin (10-10 to 10-4 M) had no effect on the lateral oviduct in either the Isolated Preparation or Abdominal Fillet (data not shown). Also, proctolin applied in combination with glutamate failed to alter the effects of glutamate on the LO (data not shown). By contrast, we observed contractions of the CO in response to bath applied proctolin as previously reported (Rodriguez- Valentin et al., 2006) (data not shown).

It has been suggested that the effects of OA on the reproductive tract may be mediated indirectly via receptors in the epithelium (Lee et al., 2003, Lee et al., 2009,

Lim et al., 2014) and we observe expression of both Octβ2R and OAMB in epithelial cells (Fig. 1). It has been previously shown that octopamine can increase calcium levels in the oviduct epithelium (Meiselman et al., 2018). To confirm these data and also test the effects of glutamate, we expressed the calcium indicator GCaMP in the epithelium using the driver RS-GAL4, which represents a fragment of the OAMB receptor gene (Lee et al., 2009). We used an Isolated Preparation to maximize visibility of the epithelium. In response to octopamine, we observe an increase in fluorescence in the epithelium within both the LO (Fig. 8B) and CO (Fig. 8C). Conversely, we observe a decrease in fluorescence in response to glutamate (Supplemental Fig. 12). The increase in fluorescence in the epithelium in response to OA appeared to be sustained throughout the 1-minute observation period (Fig. 8B, C). By contrast, the response of muscle cells to OA and glutamate was either transient or rhythmic (Fig 7). In addition, while only the LO muscle responded to either bath applied OA (Fig. 7E, E'') or optogenetic stimulation of Tdc2 neurons (Fig 6E, E'), the epithelium of both the LO and CO showed an increase in calcium in response to octopamine. Together, these data suggest that the epithelium may contribute to the regulation of processes that are common to both the LO and CO.

**Octopamine induces slow relaxation of the oviducts**

Based on the results of previous studies in both flies and other insects, we hypothesized that octopamine might cause a delayed dilation of the oviducts in addition to the more acute octopamine-induced contractions that we observed (Rodriguez-Valentin et al., 2006, Dustin Rubinstein et al., 2014, Lange, 2009). However, we found it

difficult to visualize dilation in the Intact Preparation and the Abdominal Fillet, limiting our ability to test whether optogenetic stimulation of OA neurons could induce oviduct dilation. We therefore relied on the use of the Isolated Preparation and bath-applied octopamine for all dilation experiments. After 1 minute of recorded baseline activity, octopamine or vehicle was added to the preparation and images were taken for an additional 9 min. Images were analyzed by measuring the apparent two-dimensional width at three sites in the common oviduct: anterior, mid and posterior (Fig. 9A). A fourth measurement was made at the approximate juncture between the calyx and the lateral oviduct as a proxy for both regions (indicated as “Calyx” in Fig. 9A). Over the course of 10 minutes, we observed a significant increase in the width of the calyx/lateral oviduct and the anterior common oviduct (Fig. 8B, B’, C). A recording of the entire 10 min period sped up 50x is shown in Video 7. These data indicate that octopamine can cause relaxation as well as contraction in the oviducts. However, contraction appears to represent a more acute response to octopamine, while relaxation occurs over a slower time course.

**Interactions between octopamine and glutamate modify their effects**

It has been previously suggested that octopamine and glutamate have opposing effects on oviduct contractions in both Drosophila and locusts (Rodriguez-Valentin et al., 2006, Lange, 2009, Dustin Rubinstein et al., 2014) and our data support this as one way in which glutamatergic an octopaminergic pathways might interact. To test whether octopamine and glutamate might interact in other ways, we sequentially bath applied octopamine and glutamate and recorded the response of both the lateral and common

oviduct. In previous studies testing the interactions between glutamate and OA in Drosophila (Rodriguez-Valentin et al., 2006), the MAN was electrically stimulated during bath application, potentially effecting other signaling pathways in addition to those mediated by OA and glutamate. Moreover, both the AbG and reproductive tract were present in these studies (Rodriguez-Valentin et al., 2006), allowing activation of receptors in the CNS. To more directly test the effects of glutamate and octopamine within the reproductive tract only, we used an Isolated Prep and did not employ electrical stimulation.

We expressed UAS-RCaMP1b using the driver 24B-GAL4 to observe the combined effects of glutamate and octopamine on muscle cells. For these experiments, the first agonist was applied and, after an additional 2 minutes, the second agonist was added. The responses to the first agonists were similar to those depicted in Fig. 7. Both Fig. 10 and Supplemental Fig. 12 shows the response to the second agonist in continued presence of the first.

Application of octopamine alone to an Isolated Preparation induced rhythmic calcium events in the lateral oviduct (Fig. 10B, see also Fig. 7). Preincubation for 2 minutes with glutamate on average blunted the octopamine-induced rhythmic fluctuations in fluorescence (due to both movement and changes in cytosolic calcium) within the lateral oviduct muscle (Fig. 10B'). In the CO, application of octopamine in the absence of glutamate caused minimal changes in the fluorescent signal in the common oviduct. Addition of octopamine following preincubation with glutamate unexpectedly induced rhythmic calcium transients in the common oviduct (Fig. 10C') that were not seen in the presence of octopamine alone (Fig. 10C and 7E''). The full set of

experiments showing all combinations of glutamate before and after octopamine in both the LO and CO are shown in Supplemental Fig. 12. These data support previous studies indicating that glutamatergic and octopaminergic pathways interact to regulate the oviposition circuit (Rodriguez-Valentin et al., 2006, Dustin Rubinstein et al., 2014, Lange, 2009) but suggest that under some conditions the interactions may be complex and perhaps synergistic. We have not yet tested the mechanism underlying these interactions and they will be the subject of future experiments.

**OAMB and Octβ2R regulate distinct effects of octopamine**

Although both OAMB or Octβ2R are required for female fertility (Lee et al., 2003, Lim et al., 2014, Li et al., 2015) their potential roles in either oviduct contraction or dilation are not known. To address this question, we tested the effects of mutations in both OAMB and Octβ2R (Lee et al., 2009, Lee et al., 2003, Lim et al., 2014). In an Isolated Preparation exposed to octopamine (Fig. 11A), Octβ2R mutants rarely displayed any lateral oviduct contractions following bath application of octopamine as compared to controls from the same genetic background (w1118) (Fig. 11A'). By contrast, we did not detect a decrease in the number of octopamine-induced lateral oviduct contractions (Fig. 11A'') or the latency to contractions (Supplemental Fig. 13) in OAMB mutants compared to the matched genetic background rosy (ry). Although the OAMB mutant appeared marginally more responsive than the control, this difference was not statistically significant (Fig. 11A''). These data strongly suggest that Octβ2R is required for octopamine-induced lateral oviduct contractions. Moreover, since the AbG and peripheral nerves were removed for these experiments, the Octβ2R receptors

responsible for these effects must be in the periphery and intrinsic to the reproductive tract. These might include Octβ2R receptors expressed in epithelial cells, peripheral neurons that localize to the lateral oviduct, or distal processes from the AbG neurons that project to the lateral oviduct. It remains possible that an occult group of Octβ2R receptors expressed in muscle cells stimulate oviduct contractions; however, as shown above, we are unable to detect Octβ2R expression in muscle using the MiMIC lines, and due to a lack of an available antibody to Octβ2, immunocytochemical detection is not feasible.

Similar to the Isolated Preparation, we did not detect any contractions in Octβ2R mutants using the Abdominal Fillet prep (Fig. 11B, B', n=10 animals). However, in contrast to the Isolated Preparation, OAMB mutants showed a significantly lower number of contractions than controls in the Abdominal Fillets (Fig. 11B"). Together these data suggest that although Octβ2R is required for contractions, OAMB may play an additional regulatory role. In addition, differences in the effects of OA on oviduct contractions in the Isolated Prep versus the Abdominal Fillet suggest that the contribution of OAMB receptors may depend in part on the peripheral nerves that connect the uterus and LO.

We next determined the effects of OAMB and Octβ2R mutants on octopamine-induced oviduct dilation (Fig. 11C). We again used an Isolated Preparation and quantified dilation as described for wild type flies (see Fig. 9). In many of the mutant flies, the lateral oviduct contained an egg during the observation period making it difficult to perform measurements of the calyx or lateral oviduct. We therefore focused on the anterior common oviduct for quantitating the effects of the mutants. Dilation of

the anterior common oviduct in the Oct□2R mutant was comparable to both wild type flies (data not shown) and a matched genetic background control (w1118) (Fig. 11C') indicating that Octβ2R is not required for oviduct dilation. Conversely, we did not detect dilation of the oviducts following application of octopamine to OAMB mutants, indicating that OAMB is required for oviduct dilation (Fig 11C''). Together, our data show that OAMB and Octβ2R receptors are required for different aspects of visceral muscle activity and primarily mediate oviduct relaxation and contraction, respectively. It remains possible that oviduct dilation could vary depending on the presence or absence of the peripheral nerves. However, we were unable to test this possibility due to the difficulty of observing dilation in the Abdominal Fillet preparation.

Bath application of octopamine to an Abdominal Fillet resulted in robust LO contractions, and our mutational analysis showed that Octβ2R is required for LO contractions. We reasoned that direct optogenetic activation of cells/tissue within the Abdominal Fillet might help to identify which tissue(s) within the reproductive tract may contribute to the OA dependent LO contractions. Following our experiments that showed a strong effect of the Octβ2R mutation on LO contractions (Fig. 11), we expressed Ch2-XXM using the driver Octβ2- GAL4 as a positive control. We observed LO contractions in 6 of 7 preparations (Fig. 12A, B). The latency to contraction and duration are shown in Supplemental Figure 14. We also observed contraction of the CO following optogenetic stimulation of Octβ2(+) tissues (Supplemental Figure 14).

We performed similar experiments using drivers for each of the tissue/cell types in the reproductive tract in which we detected Octβ2 expression (Fig. 12C). In contrast to our results using an intact preparation, optogenetic activation of the distal processes

of Tdc2(+) neurons failed to drive rhythmic contractions of the LO (Fig. 12D). Similarly, we failed to detect repetitive contractions using either one of two drivers for epithelial cells, or ppk1-GAL4 to express ChR2-XXM (Fig. 12D). By contrast, using the ILP7 driver to express ChR2-XXM in the Abdominal Fillet we observed repetitive LO contractions in 3 of 5 preparations and a single CO  contraction in 5 of 5 preparations (Fig. 12D, Video 8). These results suggest that activation of Oct□2 receptors on ILP7 nerve terminals might contribute to the LO contractions we observe in response to OA.

## DISCUSSION

The aminergic regulation of both central and peripheral circuits is conserved from flies to mammals and the fly oviposition circuit represents a powerful genetic model to explore the underlying mechanisms (White et al., 2021, Lim et al., 2014, Meiselman et al., 2018, Rodriguez-Valentin et al., 2006, Hasemeyer et al., 2009, Rezaval et al., 2014, Castellanos et al., 2013). We have used optogenetics and receptor mutants to explore the roles of octopamine on oviduct contractility. We find that the regulation of oviduct contractility is complex and that OA contributes to both contraction and dilation. The two OA receptors previously shown to be required for female fertility – Octβ2 and OAMB-- show distinct expression patterns and primarily regulate contraction and dilation respectively. We have confirmed the central function of glutamate in governing contractions (Lange, 2009, Rodriguez- Valentin et al., 2006, Castellanos et al., 2013, Gou et al., 2014), but present additional data suggesting a more complex role for glutamate and unexpected interactions with octopaminergic pathways.

Previous studies have consistently suggested that glutamate drives contractions in the reproductive tract, but the reported effects of octopamine have varied depending on both the species and the specific sites within the reproductive tract (Dustin Rubinstein et al., 2014, Lange, 2009, Kalogianni and Theophilidis, 1995, Lange and Orchard, 1986, Cook and Wagner, 1992, Hana and Lange, 2017, Tamashiro and Yoshino, 2014b, Rodriguez-Valentin et al., 2006, Middleton et al., 2006, Rubinstein and Wolfner, 2013). In particular, several previous reports have indicated that octopamine can induce muscle relaxation in locusts and flies (Dustin Rubinstein et al., 2014, Lange, 2009, Rodriguez-Valentin et al., 2006, Cook and Wagner, 1992). By contrast, bath applied octopamine has been reported to drive contractions in crickets (Mizunami and Matsumoto, 2017, Tamashiro and Yoshino, 2014b), and movements observed at the base of the reproductive tract may represent oviduct contractions in flies (Middleton et al., 2006, Meiselman et al., 2018). We find that optogenetic activation of octopaminergic neurons and bath applied octopamine results in rhythmic contractions and calcium transients in the LO but in the absence of glutamate, but neither have a detectable effect on the CO. Bath applied OA also causes dilation of the oviducts but with a longer time course than contractions.

We speculate that methodological differences may account for some of the differences between our findings and others including perhaps the simultaneous electrical stimulation of the MAN (Rodriguez-Valentin et al., 2006) and variations in the concentrations of OA (Middleton et al., 2006, Rodriguez-Valentin et al., 2006). In addition, the effects of OA on the LO versus the CO are different and can be difficult to distinguish based on movement alone. By expressing a calcium sensor in muscle, the

contribution of the ovaries, LO and CO to movement within the reproductive tract as whole are easier to discern. Finally, it is possible that some of our observations could have been confounded by tyramine co-released from octopaminergic neurons and perhaps activation of tyramine receptors by bath-applied octopamine. Tyramine has been shown to regulate the reproductive tract in Drosophila (Avila et al., 2012), other insects (Hana and Lange, 2017, Donini and Lange, 2004) and related species such as ticks (Cossio-Bayugar et al., 2012), and at least one tyramine receptor is expressed in the Drosophila reproductive tract (El-Kholy et al., 2015). Further experiments will be needed to explore the potential effects of tyramine on oviduct relaxation and contraction in Drosophila.

Differences between the responses of the LO and the CO to OA and glutamate may be important for the function of the oviposition circuit. Bath application of glutamate or optogenetic stimulation of ILP7 neurons drives contractions in both the common and lateral oviducts. By contrast, the response to bath applied OA in the absence of glutamate and optogenetic stimulation of Tdc2 neurons is restricted to the LO. In addition, the response of the CO is primarily confined to a single contraction, while the LO undergoes a series of rhythmic contractions. Further experiments will be needed to determine the function of each of these effects. It is possible that both are required for forward movement of the egg through the oviducts. However, lateral oviduct contractions have other functions. For example, some contractions of the LO may, in addition to contractions in the ovary, help to elicit mechanical activation of the egg (York-Andersen et al. 2015; Horner and Wolfner 2008; Kaneuchi et al. 2015). While retrograde movement of eggs has not been described in Drosophila, contractions to

promote egg-retention is well described in digging insects such as locust (reviewed in (Lange, 2009)). It is therefore conceivable that the function of some contractions in flies could be to retard forward movement of the egg, perhaps during selection of an oviposition site. Parsing the contribution of each anatomical and neuronal element within the oviposition circuit will be critical to understand the complex interplay between multiple neuromodulatory pathways within this circuit. Moreover, we speculate that the logic underlying the function of each element and their interactions may be applicable to other circuits in both the periphery and the CNS.

Comparing the responses and receptor expression patterns within the oviduct provides important clues to the mechanism by which OA regulates its function. We find that bath application of OA induces a sustained increase in cytosolic calcium in the epithelium of both the LO and the CO. While OAMB is expressed at both sites, Octβ2 is only expressed in the epithelium of the LO, suggesting that cytosolic calcium in epithelial cells may be regulated primarily by OAMB. Genetic rescue experiments indicate that OAMB expression in the epithelium is required for egg laying (Lee et al., 2003, Lee et al., 2009, Lim et al., 2014) and we show that OAMB mutants are unable to dilate the oviduct in response to bath applied OA. These data are consistent with the idea that OAMB signaling in the epithelium may indirectly regulate muscle relaxation as previously suggested based on genetic rescue of fertility (Lee et al., 2003, Lee et al., 2009, Lim et al., 2014).

In contrast to OAMB, Octβ2 appears to be primarily responsible for contractions rather than dilation. In addition to the epithelium, Octβ2 is expressed in at least three subtypes of neurons in the reproductive tract. These include ppk1(+) cells whose

somata reside in the periphery and both glutamatergic/ILP7(+) and Tdc2(+) processes that project from their cell bodies in the AbG. We used optogenetics to test whether one of these cell types might contribute to OA-induced LO contractions. Importantly, these optogenetic experiments were performed using a reduced Abdominal Fillet preparation to eliminate any contribution from cell bodies in the AbG. Optogenetic activation of ppk1(+) neurons, the epithelium and Tdc2(+) neurons had minimal effects on LO contractions in the Abdominal Fillet. By contrast, activation of ILP7(+) neurons induced repetitive contractions in a subset of preparations. Together with our additional observations that glutamate or octopamine can induce LO contractions in an Abdominal Fillet, we speculate that the mechanism by which OA initiates LO contractions may be via activation of Octβ2 on ILP7 terminals and the release of glutamate. The relevant signaling pathways might be similar to those at the larval NMJ in which both Octβ1R and Octβ2R regulate the function of glutamatergic nerve terminals (Koon et al., 2011, Koon and Budnik, 2012).

Our experiments interrogating the contribution of processes in the periphery depended on their differential sensitivity to optogenetic stimulation. Removing the somata of Tdc2 neurons in an Abdominal fillet ablated their response of the remaining distal processes to optogenetic stimulation. By contrast, the response of distal ILP7 processes was preserved in the absence of cell bodies. The response of distal ILP7(+) but not Tdc2(+) processes to optogenetic stimulation could reflect differences in their sensitivity to depolarization or downstream processes such as the ability of depolarization to induce calcium influx (Xing and Wu, 2018a, Xing and Wu, 2018b,

Harrigan et al., 2020). These differences may also explain the relative insensitivity of Tdc2(+) neurons to stimulation using a standard ChR2 variant rather than ChR2-XXM/L.

To more definitively test the hypothesis that activation of ILP7 neurons is involved in OA-mediated LO contractions, we expressed two RNAi transgenes targeting Octβ2R in lLP7(+) neurons. We similarly used RNAi to test the more general idea that activation of Octβ2 on neurons rather than those on epithelial cells is responsible for LO contraction. Thus far, the results have been inconclusive, and we anticipate that genetic rescue experiments will be needed to evaluate each of these possibilities. The function of octopamine receptors on both ppk1(+) neurons and Tdc2(+) neurons also remains unclear. Octβ2R could potentially act as an autoreceptor on Tdc2(+) nerve terminals as described at the larval NMJ (Koon et al., 2011, Koon and Budnik, 2012). In ppk1(+) cells, it is possible that Octβ2R and/or OAMB could modify mechanosensory activity or perhaps regulate signaling to neurons in the AbG (Gou et al., 2014, Yang et al., 2009, Hasemeyer et al., 2009, Lee et al., 2016), but further experiments will be needed to test these hypotheses.

We did not detect expression of Octβ2R or OAMB in muscle cells, suggesting that most octopaminergic effects on oviduct dilation or contractions are mediated via receptors expressed in either neurons or the epithelium (Lee et al., 2009, Lee et al., 2003, Lim et al., 2014). However, we cannot completely rule out the possibility that we failed to detect expression using the MiMIC lines and that OA receptor expression in muscle contributes to contractility as suggested for the effects of Octβ2R on fertility (Li et al., 2015) (but see Supplemental Fig. 6). Indeed, we believe that the effects of both OA and glutamate are complex and that multiple signaling pathways are active within

the oviposition circuit. We also cannot rule out the possibility that developmental effects of the Octβ2R and OAMB mutants influenced our results. Future experiments using genetic rescue during development versus adulthood will be important to assess this.

Whether the effect of OA on the oviduct musculature is causal for its effect on fertility remains to be tested. OAMB is active at multiple sites within the reproductive tract including the sperm storage organs and follicle cells (Deady and Sun, 2015, Avila et al., 2012). It is possible that the loss of fertility caused by octopaminergic pathways is independent of those that regulate either oviduct contractions or dilation.

In sum, we find that octopamine regulate the oviduct contractions via two distinct receptors and that interactions with glutamate may further modify the activity of these pathways. This complex network of receptors and the mechanisms by which they interact will clearly require further experiments to fully understand. Studies of the crab stomatogastric ganglion has yielded fundamental insights into rhythm generation. It appears that the rhythm of visceral muscles could follow a different logic and we propose that these and other experiments using the fly oviposition circuit will enhance our understanding of the evolutionarily conserved logic by which octopamine and other biogenic amines regulate circuit function and behavior.

**Limitations of Study**

One limitation of this study is that we still do not know the precise mechanisms by which octopaminergic activation of Octβ2 and OAMB induce muscle contractions and relaxation. A second limitation is that we cannot rule out a contribution to these activities from tyramine.

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**Contributions**

SD, DEK, EWR conceptualized the work and wrote the manuscript. SD, EWR with assistance from DJS and ES performed the live imaging experiments with the exception of those measuring dilation. JLA and AE conducted the dilation experiments. EWR, JLA and JH performed the labelings and confocal microscopy. FES wrote the custom software and edited the manuscript. P-T,L generated the GAL4 conversion and performed preliminary characterizations of their expression patterns in HJB labs. HLB leads the Genome Disruption Project (in collaboration with Norbert Perrimon and Allan

Spradling). SD performed all other experiments and analyses with assistance from DJS and AE. All co-authors read and approved the final version of the paper.

## FIGURES AND LEGENDS

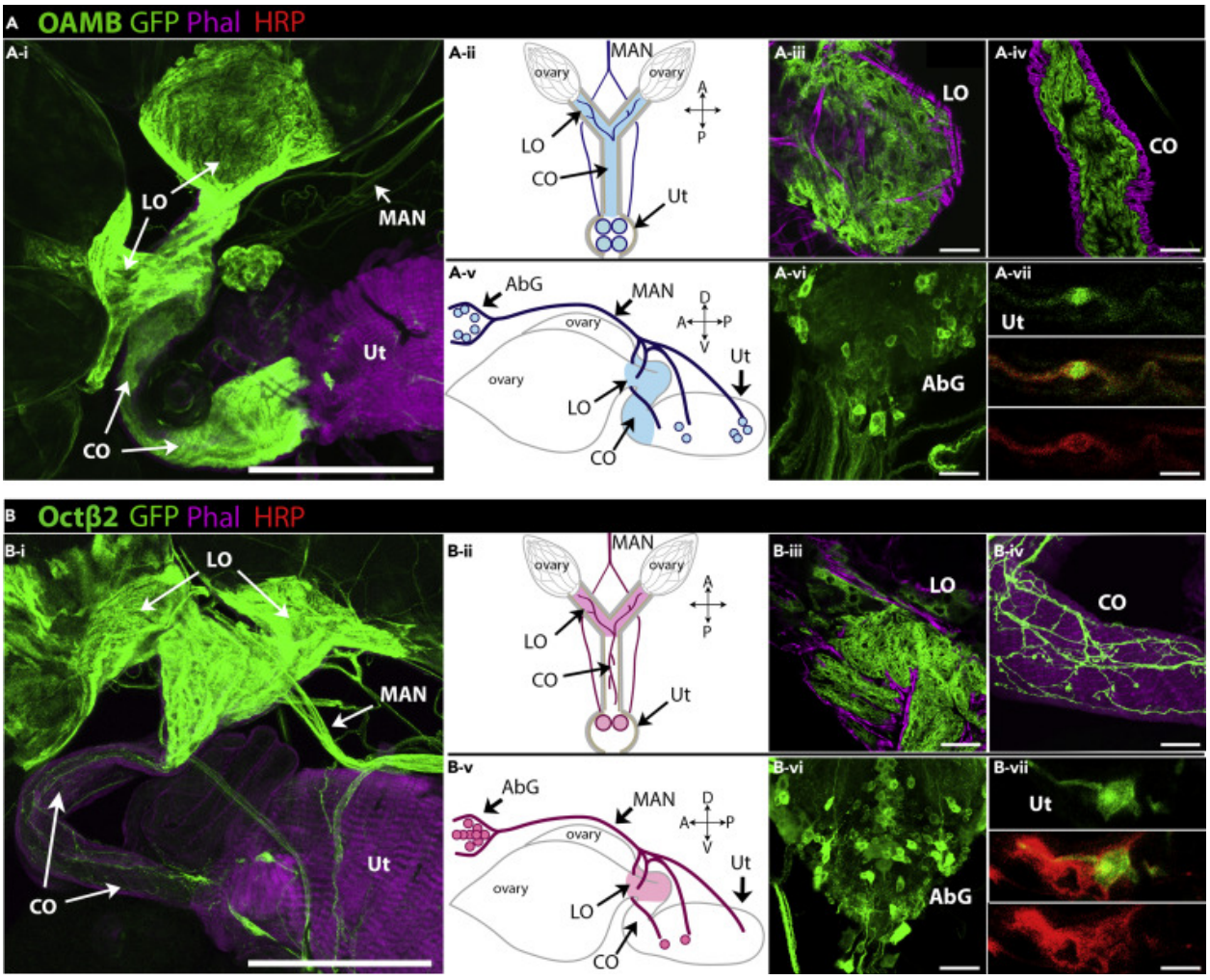


**Figure 1. OAMB and Octβ2 receptor expression in the oviducts.**

A-i. Overview of OAMB expression in the female reproductive system using OAMB-T2A-GAL4 to express UAS-mCD8-GFP (anti GFP-488, green) co-labeled with phalloidin coupled to Alexa Fluor 555 ("Phal", magenta). A-ii. Cartoon depicting OAMB expression in the epithelium of both the common (CO) and lateral (LO) oviducts (blue shaded areas) and in processes (blue lines) of OAMB(+) neurons (blue circles) innervating the

LOs from the MAN and local neurons. A-iii, A-iv. Higher magnification views of A-i, showing epithelial expression of OAMB in both the LO and CO. A-v. Cartoon depicting a sagittal view of the reproductive system in its natural in vivo conformation. A-vi. Higher magnification view of the OAMB expressing cells in the abdominal ganglion (AbG) from the preparation shown in A-i. A-vii. OAMB(+) cell body embedded in the uterus (Ut) muscle visualized via OAMB-T2A-GAL4 >> UAS-GFP.nls (anti GFP- 488, green) and co-labeled with the neuronal marker anti-HRP (anti HRP-568, red) B-i. Overview of Octβ2 expression in the female reproductive system using Octβ2-T2A-GAL4 to express UAS-mCD8-GFP (anti GFP-488, green) and co-labeled with phalloidin (“Phal”, magenta). B-ii. Cartoon depicting Octβ2 expression in the LO epithelium (pink shaded area) and neuronal processes (pink lines). B-iii, B-iv. Higher magnification of B-i showing expression of Octβ2 in the LO and CO. B-v. Cartoon depicting a sagittal view of the reproductive system in its in-vivo conformation. B-vi. Higher magnification view of the Octβ2 expressing cells in the abdominal ganglion (AbG) in B-i. B-vii Octβ2(+) neuron embedded in the uterus muscle visualized using Octβ2-T2A-GAL4 >>UAS-GFP.nls (anti GFP-488, green) and co-labeled with the neural marker anti-HRP (anti HRP-568, red). A-i, A-vi, B-i, B-iv, and B-vi are projections and Aiii, A-iv, A-vii, B-ii, and B-vii are single confocal slices. Scale bars: A-i, B-i: 200 μm; A-iii, A-iv, A-vi, B-iii, B-iv, B-vi: 20 μm; A-vii, B-vii: 10μm.

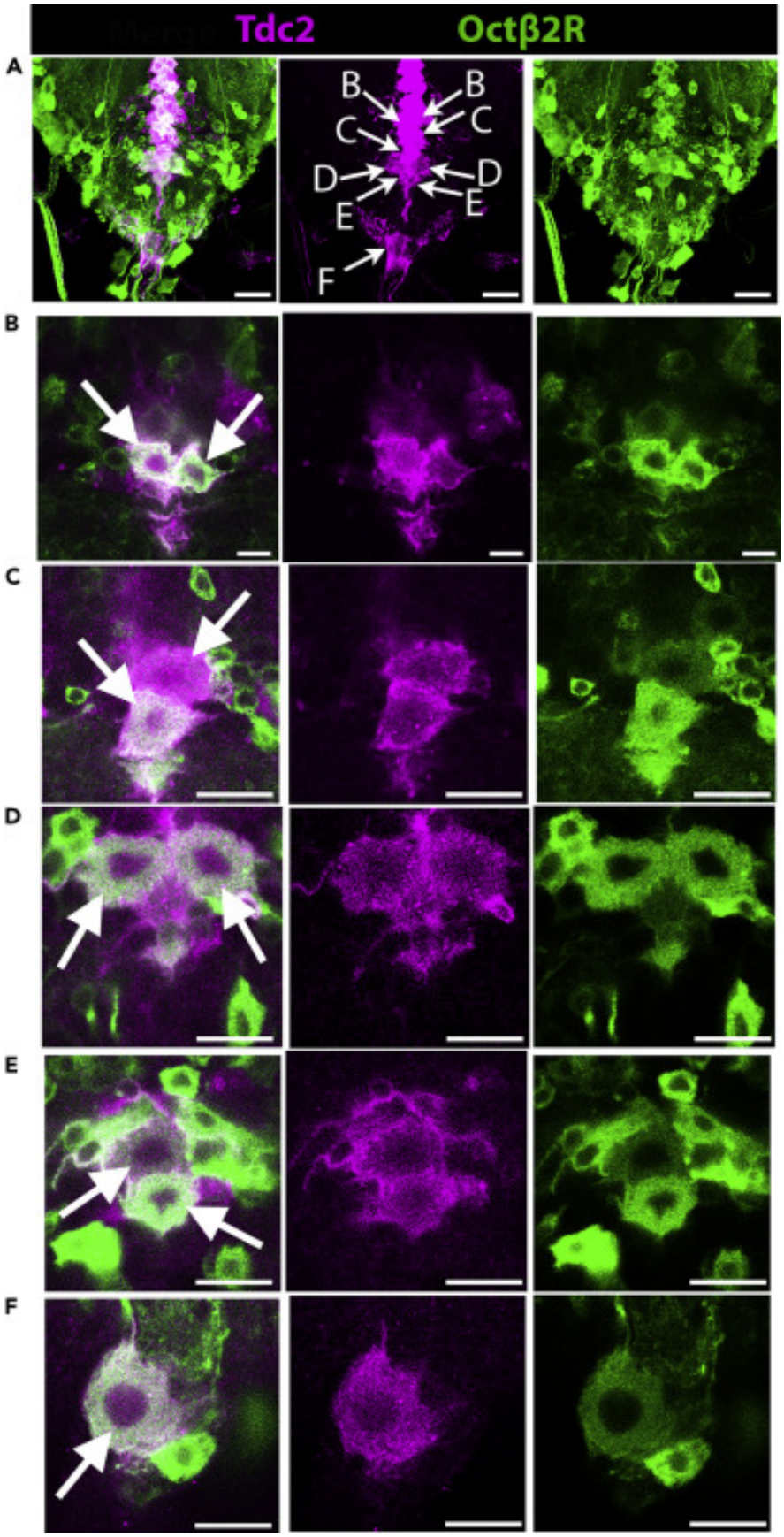
Tdc2
Octβ2R
A
B
C
D
E
F
B
B
C
C
D
D
E
E
F

**Figure 2. Expression of Octβ2 in Tdc2(+) neurons within the AbG.**

Cells were labeled with the drivers Tdc2-LexA and Octβ2-T2A-GAL4 as indicated and labeled with antibodies to RFP (magenta) and GFP (green) respectively. A confocal stack (A) and individual optical sections (B-F) are shown. Arrows in A indicate the cells shown in B-F. Scale bars in A: 20 microns; B-F: 10 microns.

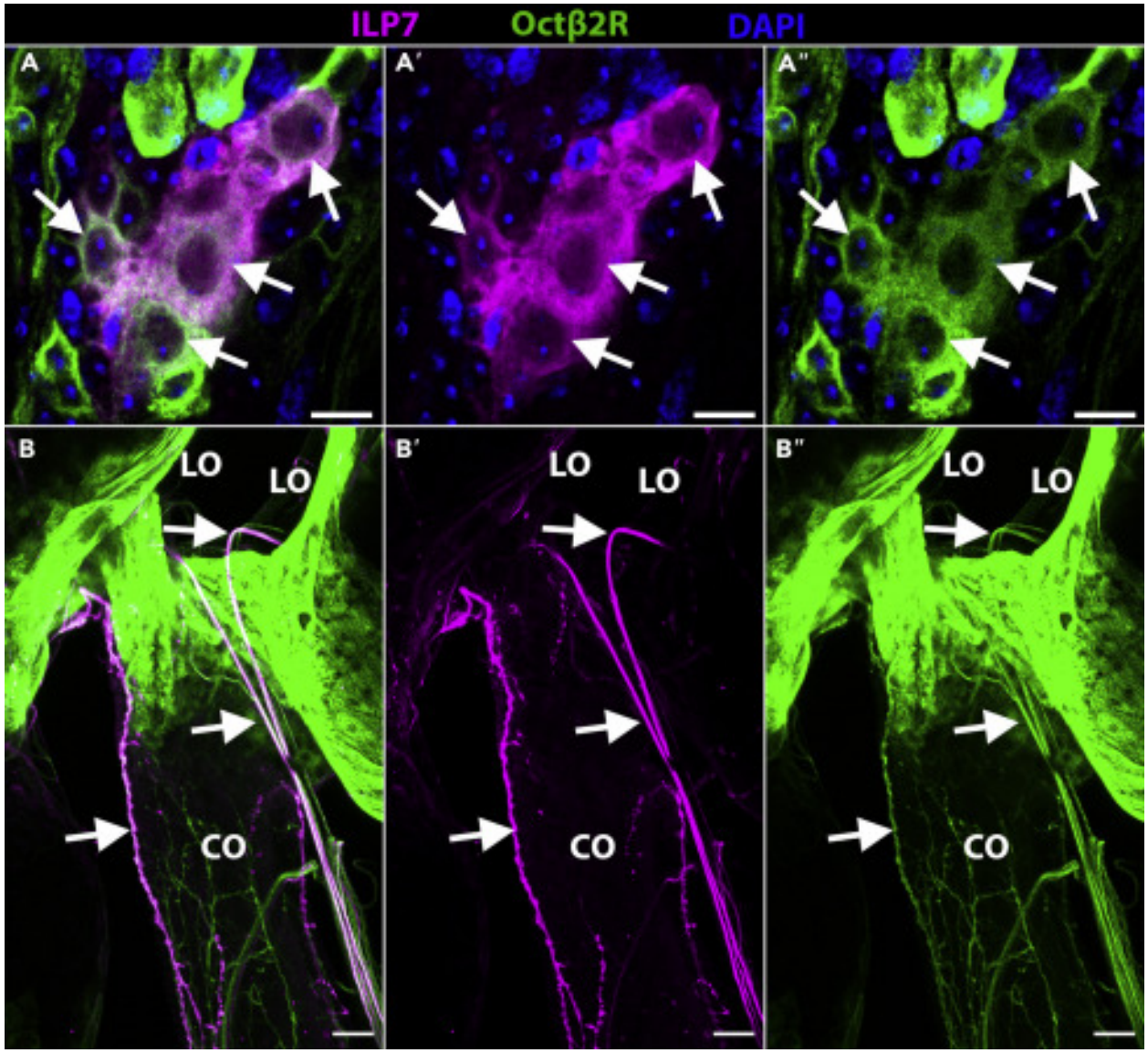

**Figure 3. Octβ2R and ILP7 are co-expressed in AbG neurons that innervate the oviducts.**

A-A''. Neural somata in the AbG. B-B'' shows processes in the LO and CO from the same preparation as in A. White arrows indicate co-expression of Octβ2R-T2A-GAl4>UAS-mCD8:GFP (anti-GFP, green) and ILP7-LexA>LexAop-CD2-RFP (anti-RFP, magenta). Scale bars = A: 5μm; B: 30 μm.

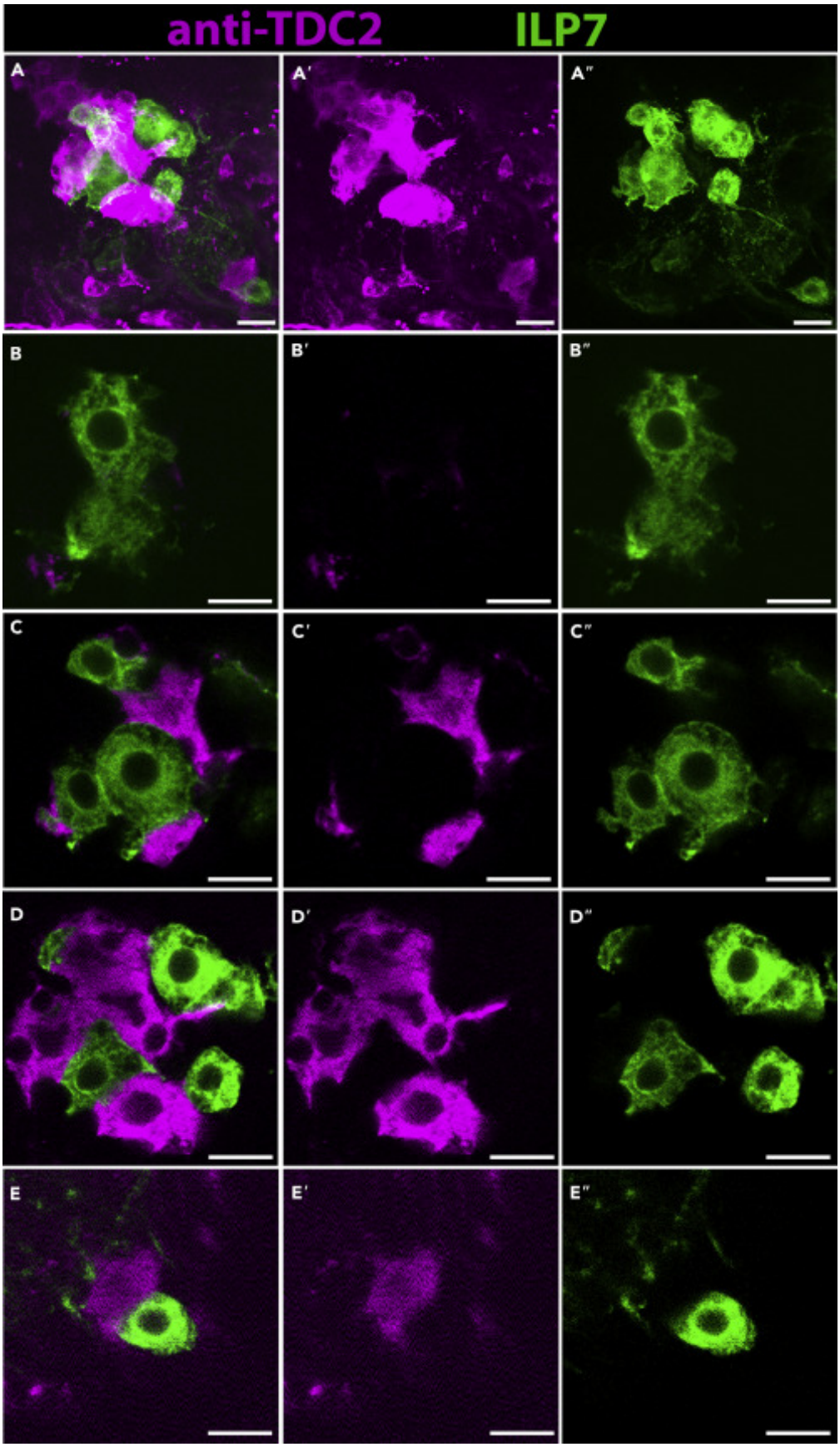
anti-TDC2
ILP7
A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''
E
E'
E''

**Figure 4. ILP7(+) and Tdc2(+) cells within the AbG are distinct.**

Flies expressing ILP7-GAL4 and UAS-mCD8-GFP were co-labeled with antibodies to GFP (A''-E'', green) and Tdc2 protein (A'-E', magenta) and the channels merged (A-E). A confocal stack (A-A'') and individual optical slices are shown (B-E''). We do not detect co-localization in any of the optical slices. Scale bars: 10 μm.

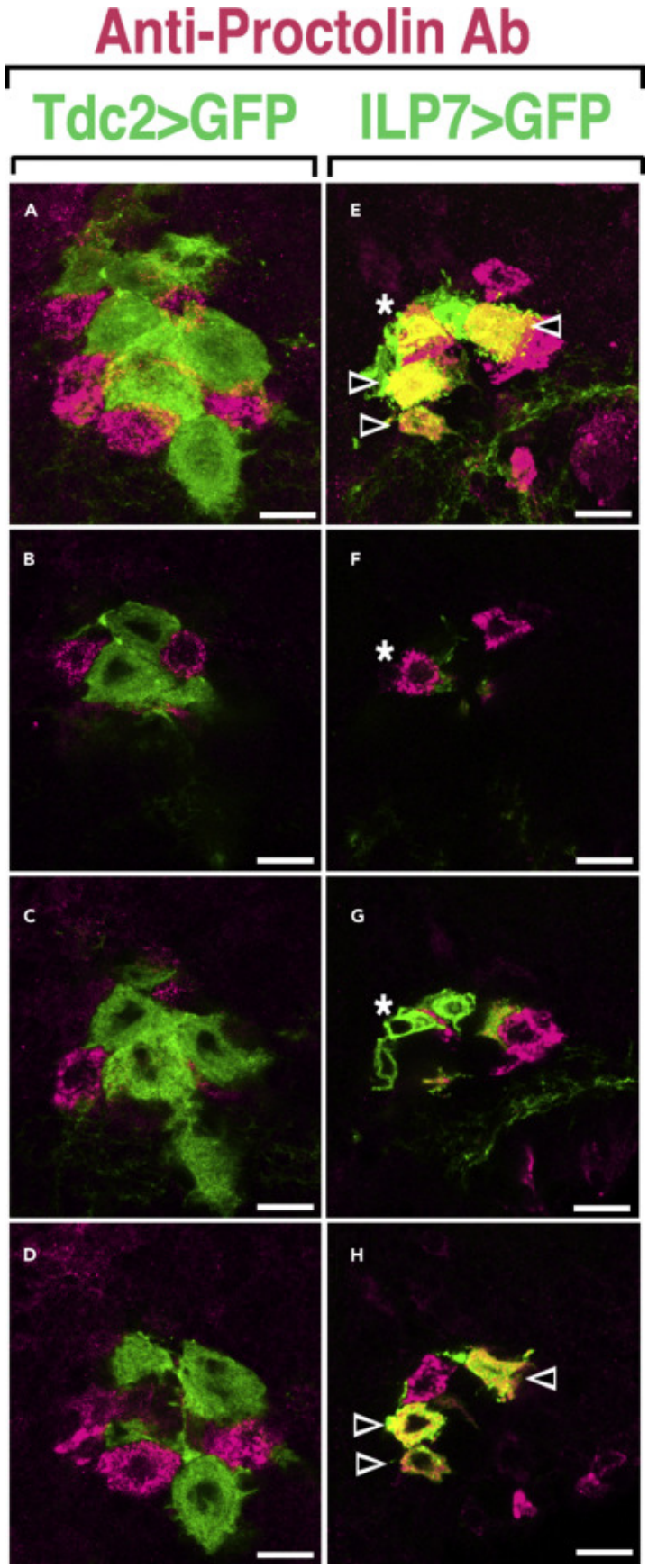
Anti-Proctolin Ab
Tdc2>GFP
ILP7>GFP
A
B
C
D
E
F
G
H

**Figure 5. Expression of proctolin in a subset ILP7(+) but not Tdc2(+) neurons.** Somata in the AbG expressing UAS-mCD8-GFP using either Tdc2-GAL4 (A-D) or ILP7-GAL4 (E-F) were co-labeled with anti-GFP (green) and anti-proctolin (magenta). A maximal intensity projection (A, E) and individual optical slices from the same stack (B-D, E-H) are shown. Arrowheads indicate co-localization. The asterisk indicates a region in which the labels overlap but do not co-localize thus indicating two distinct cells. Scale bars: 10μm.

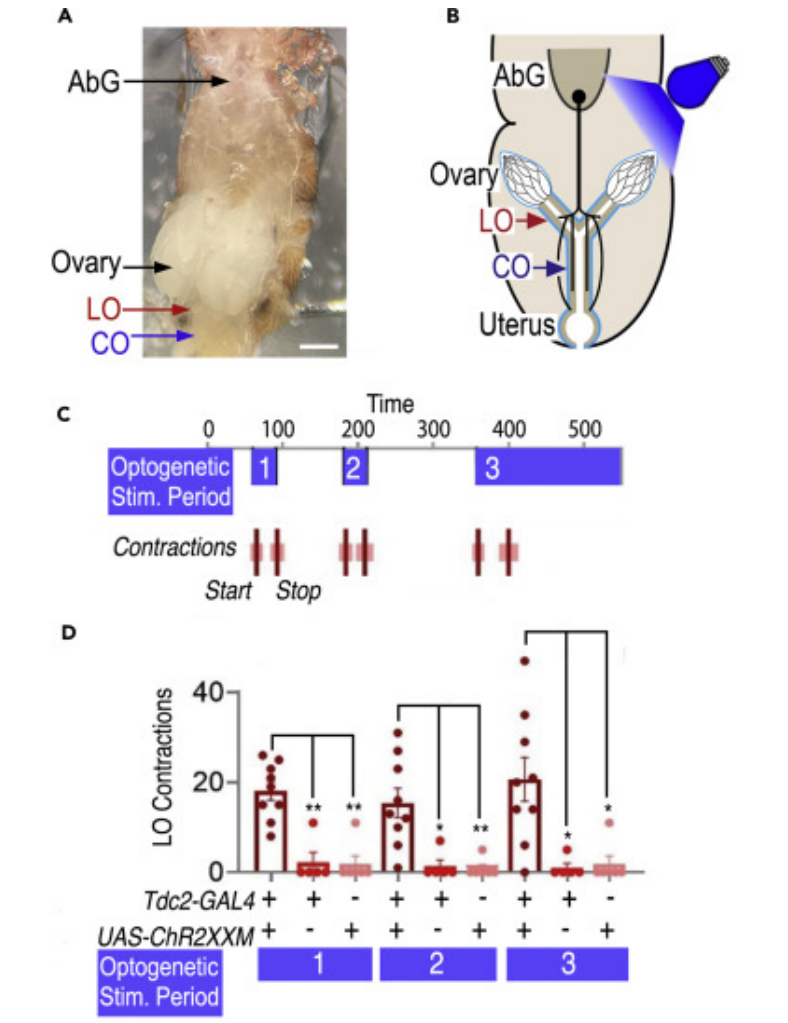
A
AbG
Ovary
LO
CO
B
AbG
Ovary
LO
CO
Uterus
C
Time
0
100
200
300
400
500
Optogenetic Stim. Period
1
2
3
Contractions
Start
Stop
D
LO Contractions
40
20
0
Tdc2-GAL4 + + - + + - + + -
UAS-ChR2XXM + - + + - + + - +
Optogenetic Stim. Period
1
2
3
E
Tdc2 >> ChR2XXL
LO
Δ F/F (n)
200
100
0
-20
0
20
40
E'
Tdc2 >> ChR2XXL
CO
Δ F/F (n)
200
100
0
-20
0
20
40
F
ILP7 >> ChR2XXL
LO
Δ F/F (n)
400
200
0
-20
0
20
40
Time (sec)
F'
ILP7 >> ChR2XXL
CO
Δ F/F (n)
400
200
0
-20
0
20
40
Time (sec)

**Figure 6. Optogenetic stimulation of Tdc2 and ILP7 neurons induces oviduct contractions.**

A. Micrograph of the “Intact Preparation” used for optogenetic stimulation. The Abdomnal Ganglion (AbG), Ovary, Lateral Oviduct (LO) and Common Oviduct (CO) are indicated. Scale bar: 200 microns. The anterior portion of the fly and the posterior end of the abdomen are not visible. B. Schematic of the Intact Preparation used for optogenetic stimulation. The black circle and lines extending from the AbG represent neurons and neuronal projections to the reproductive tract. C. Time course of optogenetic stimulation. Following an initial baseline recording, the preparation was optogenetically stimulated three times. The mean time between lights-on and the first contraction and between lights-off and the last contraction are indicated by vertical red lines (n=9, pink rectangles = SEM) D. Optogenetic stimulation of Tdc2>>UAS-ChR2-XXM expressing neurons (n=9), but neither Tdc2-GAL4 (n=5) or UAS-ChR2-XXM (n=5) alone induced lateral oviduct contractions (Kruskal Wallis test, $p<0.0001$, with Dunn’s multiple comparisons post-hoc test, * < 0.05, **< 0.01) during the three successive stimulation periods. E-F. Sample traces showing the effects of optogenetic stimulation using the oviduct muscle driver 24B-GAL4 to express RCaMP1b. $\Delta F/F(n)$ represents the aggregate change in signal caused by both movement and an increase in RCaMP fluorescence with “n” indicating normalization (see Methods) in muscles of either the lateral oviduct (red traces) or common oviduct (blue traces). The flies used for each experiment expressed either Tdc2-LexA>>LexAopChRXXL, 24B-GAL4>>UAS-RCaMP (E and E’) or ILP7-LexA>>LexAop-ChXXL, 24B-GAL4>>UAS- RCaMP (F and F’) as

indciated. Grey traces in each panel represent controls expresing 24B-GAL4>>UAS-RCaMP alone.

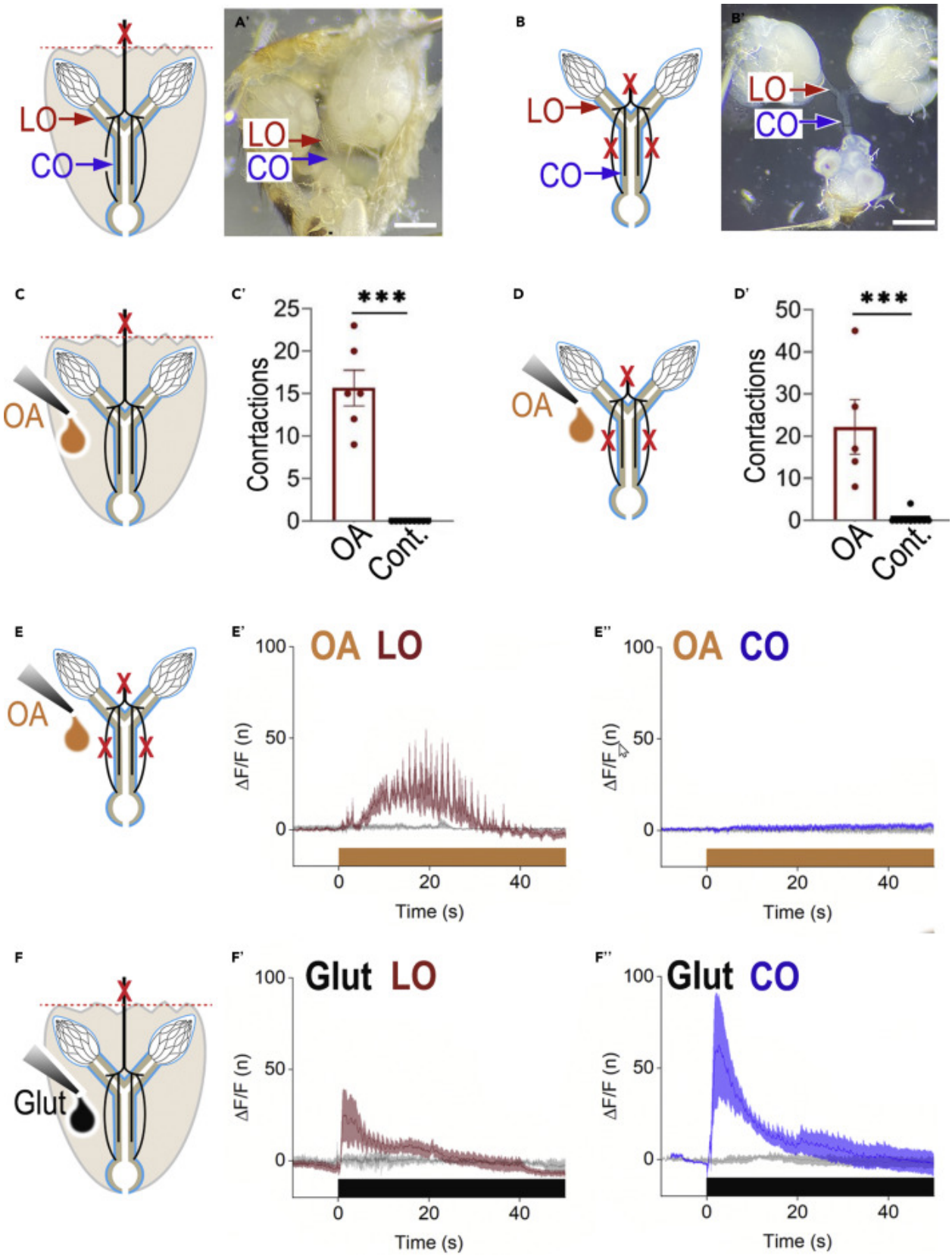
A'
LO
CO
B
B'
LO
CO
C
OA
C'
***
Conrtactions
OA
Cont.
D
OA
D'
***
Conrtactions
OA
Cont.
E
OA
E'
OA LO
ΔF/F (n)
Time (s)
E''
OA CO
ΔF/F (n)
Time (s)
F
Glut
F'
Glut LO
ΔF/F (n)
Time (s)
F''
Glut CO
ΔF/F (n)
Time (s)

**Figure 7. Octopamine and glutamate regulate lateral oviduct contractions.**

A. Schematic of an Abdominal Fillet. The dotted red line indicates the cut made at the anterior end of the abdomen. The red "X" indicates the severed MAN. A'. Micrograph of an Abdominal Fillet prep. BB'. Schematic (B) and micrograph (B') of an Isolated Prep. Red X's indicates the severed peripheral nerves and MAN. Scale bars: 200 microns. C-C'. Addition of OA to an Abdominal Prep (C) and the number of lateral oviduct contractions (C') seen after addition of octopamine (OA) or saline control (n=6, Mann-Whitney test, *** $p<0.001$). D-D'. Addition of OA to an Isolated Preparation (D) and the number of lateral oviduct contractions (D') seen after addition of octopamine or saline control (n=5, Mann-Whitney test, ***$p<0.001$). E-E''. Addition of OA to an Isolated Prep (E) and the observed $\Delta F/F$ in muscles of the lateral oviduct (E', red trace, n=6) and common oviduct (E'', blue trace, n=6) after addition of octopamine or saline (grey traces, n=6 in E, n=7 in E''). F-F''. In an Abdominal Fillet (F), glutamate stimulates an increase in rhythmic fluorescent activity in muscles of both the lateral oviduct (F', red trace, n=6) and common oviduct (F'', blue trace, n=5). Gray traces represent saline controls (F' n=3, F'' n=4). To allow comparison across preparations, time 0 on the x axis has been normalized to the initiation of the first event in each recording.

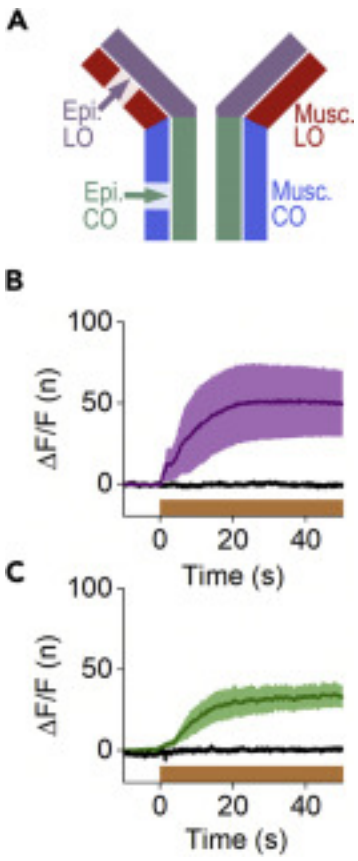


**Figure 8. Octopamine increases cytosolic calcium in the epithelium.**

A. Schematic showing the relationship between the epithelium (Epi.) and the muscle (Musc.) in the lateral oviduct (LO) and common oviduct (CO). B. Average response of epithelium in the LO following bath application of octopamine (purple trace, n=4,) or saline (black trace n=4). C. Average response of epithelium in the CO following bath application of octopamine (green trace n=6,) or saline (black trace n=3). Orange bar indicates application of OA or saline control.

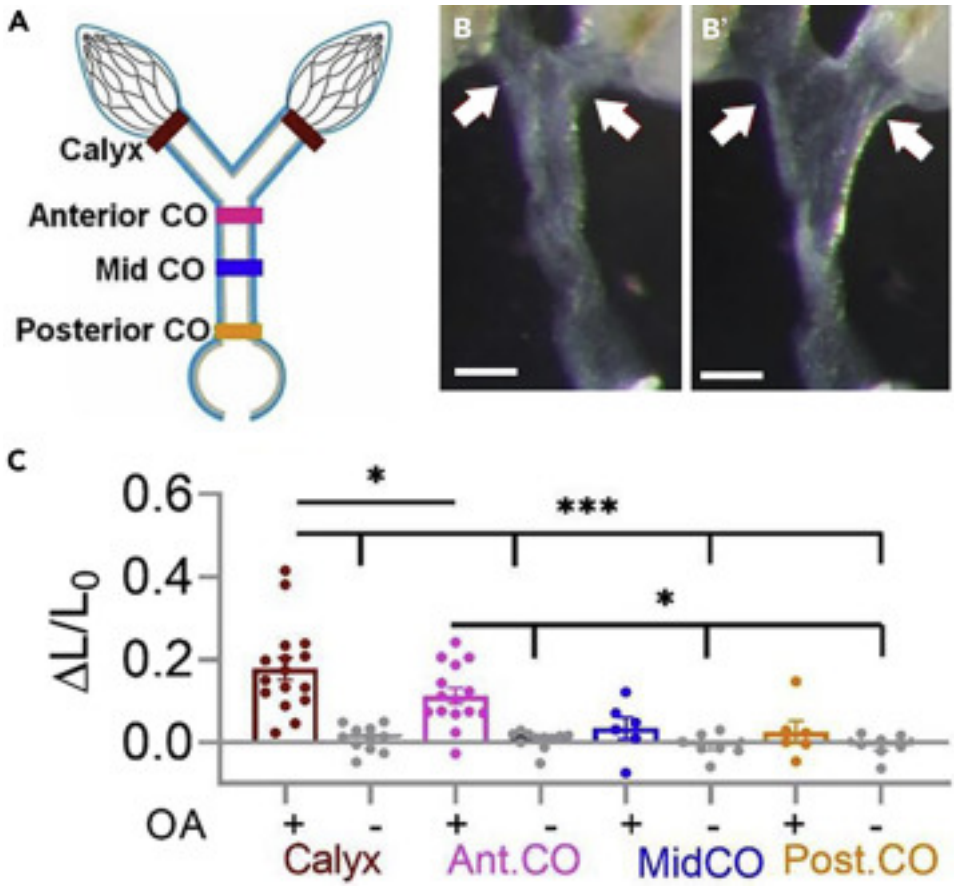


**Figure 9. Octopamine causes oviduct dilation as well as contraction.**

A. Regions of fly oviduct measured in dilation assays. B-B'. Light micrographs showing the reproductive tracts before (B) and 9 min after (B') addition of octopamine. Arrows indicate dilation of calyx/lateral oviduct and anterior common oviduct. Scale bars: 50 microns. C. Those who blade never fade (ΔL = Lfinal – L0) normalized to the initial width (L0) (Kruskal-Wallis test, $p<0.0001$, with Dunn's multiple comparisons post-hoc test, ***0.001, ** 0.01, *0.05, n=6-14 as indicated in by data points in panel C. Folds or other disruptions in the tissue prevented measurement and decreased the n for some sites).

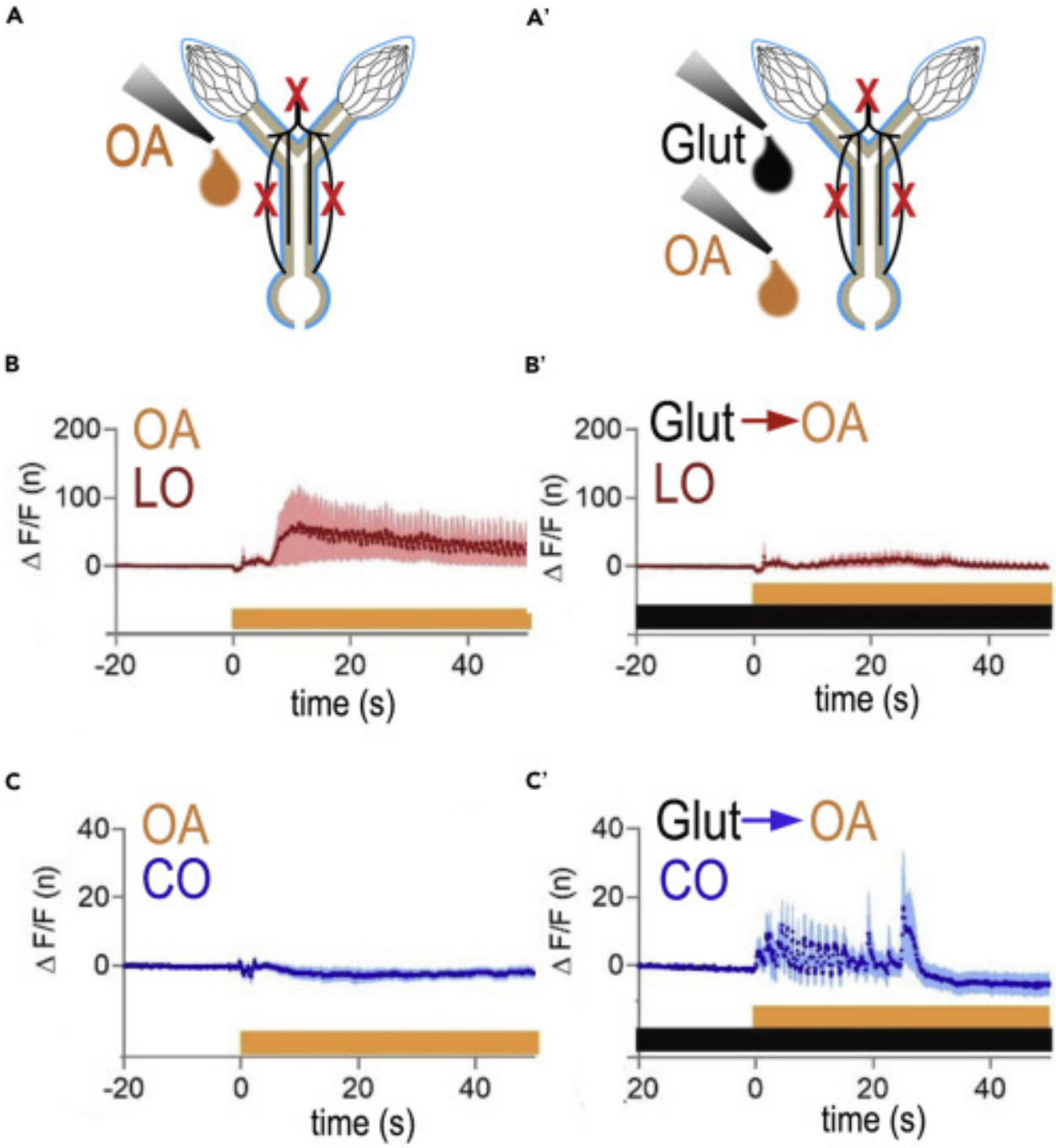


**Figure 10. Potential interactions between octopamine and glutamate.**

Octopamine alone added to an Isolated Prep (A) initiates a rhythmic response in lateral oviduct muscle cells (B, n=11) but not the CO (C, (n=10). Following preincubation in glutamate (A'), the effects of octopamine on the LO are blunted (B', n=11). Application

of glutamate followed by octopamine drives rhythmic events in the common oviduct muscle (C', n=11) that are not seen following octopamine alone (C, n=10).

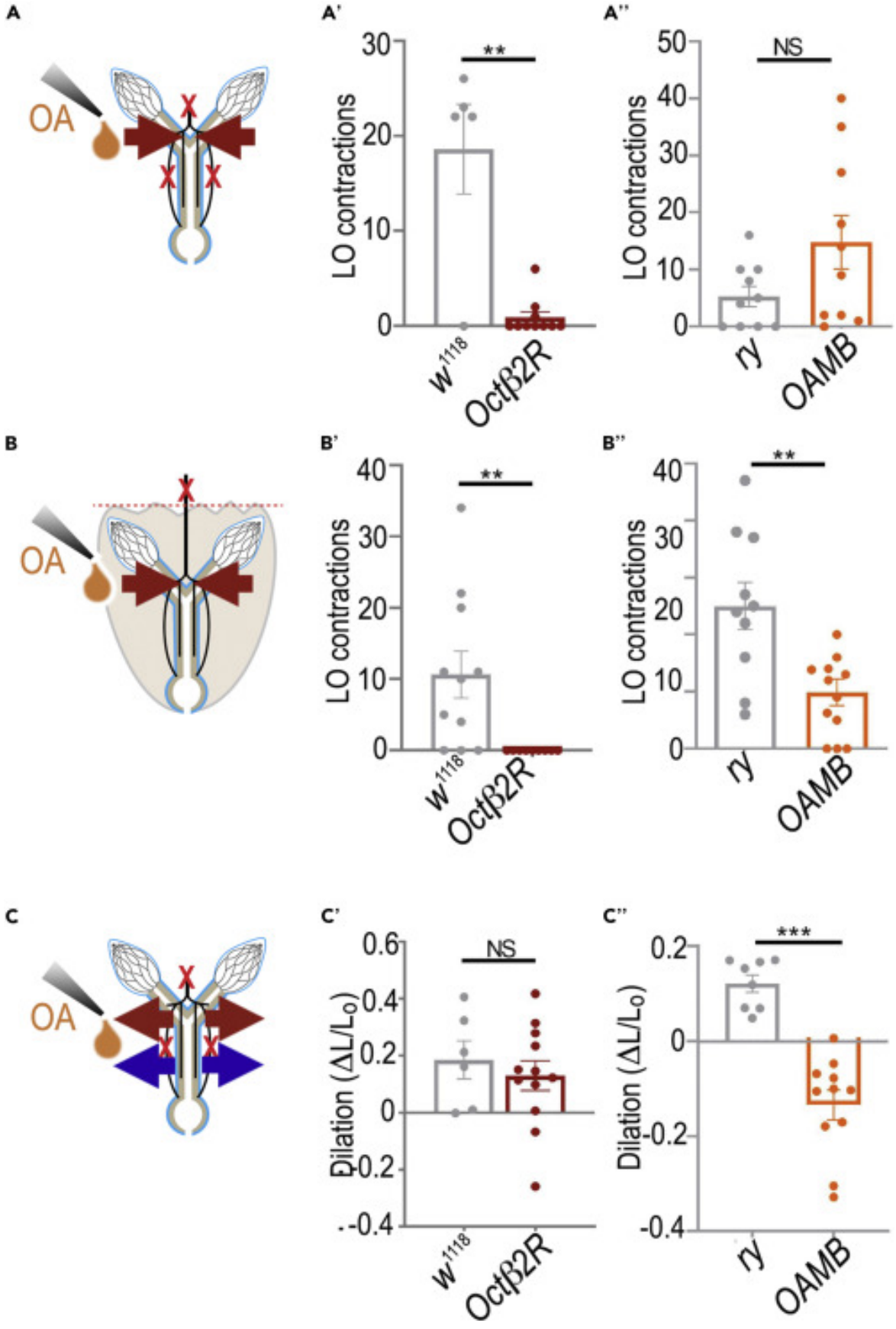
A
OA
A'
LO contractions
30
20
10
0
**
w1118
OctβR2R
A''
LO contractions
50
40
30
20
10
0
NS
ry
OAMB
B
OA
B'
LO contractions
40
30
20
10
0
**
w1118
OctβR2R
B''
LO contractions
40
30
20
10
0
**
ry
OAMB
C
OA
C'
Dilation (ΔL/Lo)
0.6
0.4
0.2
0
-0.2
-0.4
NS
w1118
OctβR2R
C''
Dilation (ΔL/Lo)
0.2
0
-0.2
-0.4
***
ry
OAMB

**Figure 11. Octβ2 and OAMB are required for contraction and dilation respectively.** A. Isolated Preparation used to quantify lateral oviduct contractions (red arrows) in response to bathapplied octopamine (OA). A'. Contractions in the Octβ2 mutant and genetically matched controls (w1118). A". Contractions in the OAMB mutant and genetically matched controls (rosy indicated as ry), B. Abdominal Fillet used for measuring lateral oviduct contractions. B'. Contractions in the Octβ2 mutant and control. B". Contractions in OAMB mutant and controls. C. Isolated Preparations used to quantify dilation in Octβ2, OAMB mutants and controls. C'. Dilation of anterior CO in Octβ2 mutants and controls. C". Dilation of anterior CO in OAMB mutants and controls (n=5-10 per condition as indicated by data points, Mann-Whitney test, **0.01, *** 0.001).

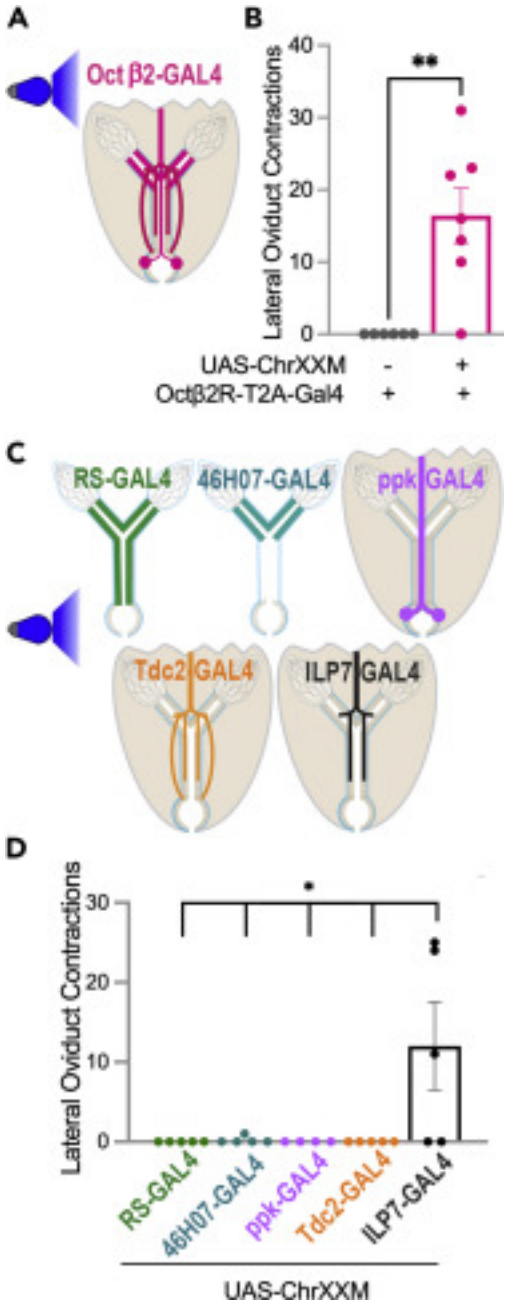


**Figure 12. Optogenetic stimulation of peripheral Octβ2 and ILP7 expressing processes induces lateral oviduct contractions.**

A. Optogenetic activation of Octβ2(+) tissue induces lateral oviduct contractions. A. Tissue detected to express Octβ2 including the epithelium, descending Tdc2(+) processes from the AbG, descending ILP7(+) processes from the AbG and peripheral

ppk1(+) neurons intrinsic to the reproductive tract. B. Number of lateral oviduct contractions in 30 seconds (n=7) in flies expressing Octβ2R-MiMIC-T2A-GAL4>UAS-ChR2-XXM versus UAS-ChR2-XXM alone (n=6, Students t-test, $p<0.01$). C. Specific drivers used to express ChR2-XXM in the epithelium (GMR46H07-GAL4 indicated as "46H07-GAL4" and OAMB-RS-GAL4/"RS-GAL4"), Tdc2(+) processes ("Tdc2-GAL4"), ILP7(+) processes ("ILP7-GAL4") and peripheral ppk1(+) neurons (ppk1-GAL4/"ppk-GAL4"). An Abdominal Fillet was used for experiments with the neuronal drivers Tdc2-GAL4, ILP7-GAL4 and ppk1- GAL4. To reduce the potential contribution of local neuronal processes, an Isolated Preparation was used for the epithelial drivers 46H07-GAL4 and RS-GAL4. D. Number of lateral oviduct contractions in 30 seconds using the indicated drivers (n=4-5, as indicated by data points, One way ANOVA, * $p<0.05$).

## STAR MATERIALS AND METHODS

### Resource availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, David E. Krantz (dkrantz@ucla.edu).

### Materials availability

Fly lines generated in this study have been deposited to Bloomington Drosophila Stock Center (BDSC, Bloomington, IN): Octβ2-MiMIC-T2A-GAL4 (M13416-TG4.2); BDSC#67511 OAMB-MiMIC-T2A-GAL4 (M12417-TG4.1); BDSC #67506

### Data and code availability

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request. All data reported in this paper will be

shared by the lead contact upon request. Code: Original Labview code for the normalization program we used was written by Felix Schweizer and available at https://ucla.box.com/s/u8372zvb7awvikaovgjqo6tdefat21ub.

**Construction of MiMIC-T2A-GAL4 lines**

Receptor-MiMIC-T2A-GAL4 flies were generated as described (Lee et al., 2018) and deposited in the Bloomington Drosophila Stock Center (BDSC, Bloomington, IN): OAMB: stock #67506; Octβ2: #67511 (see Supplemental Fig. 1).

**Fly husbandry and stocks**

Flies were raised in mixed sex vials on cornmeal/sucrose/yeast/sucrose/dextrose/agar medium at 25ºC and 50-70% humidity under a 12:12 light: dark cycle. Tdc2-GAL4, Tdc2-LexA, 46H07- GAL4, 24B-GAL4, UAS-GCaMP6m, LexAop-CD2-RFP, UAS-GFP.nls, LexA-OP-ChR2UASRCaMP1b, the Octβ2 mutant, ppk1-GAL4, also known as ppk-GAL4 as described in (Grueber et al., 2007), and GMR46H07-GAL4, were obtained from the BDSC. We thank the following people for generously supplying the following additional lines: Dr. Kyung-An Han (University of Texas, El Paso) for OAMB286 and OAMB-RS-GAL4; Dr. Bing Ye (University of Michigan) for ppk1-LexA (Gou et al., 2014), Dr. Rebecca Yang (Duke) for ILP7-LexA, and Dr. Robert Kittel (University of Würzberg) for UAS-ChR2-XXM and -XXL.

**Dissections**

Female flies 5-7 days post-eclosion were anesthetized on ice, then immobilized on Sylgard disc glued to a standard microscope slide. All dissections were performed in ice cold HL3.1 (Feng et al., 2004). To anatomically isolate pre- and post-synaptic elements of the circuit, we developed a series of increasingly reduced dissections: 1) “Intact

Preparation” (see Fig. 6A): For the optogenetic experiments shown in Fig. 6 we performed a minimal dissection to generate an “Intact Preparation” which preserved all processes that connect the AbG to the reproductive tract and well as the peripheral nerves that connect the lateral oviduct to the uterus. The legs and wings were removed, and the fly was immobilized with ventral side facing up using insect pins bent into a staple-shape and inserted through the tip of the abdomen and over the cervical connective. Using sharp forceps, the terminal sternites of the thorax and last 4 abdominal plates were removed to expose the abdominal ganglion and reproductive tract. The anterior sternites of the abdomen were left in place. 2) “Abdominal Fillet” preparation (see Fig. 7A): To disrupt inputs from the AbG but preserve the peripheral nerves and the endogenous conformation of the reproductive tract, we developed an “Abdominal Fillet” preparation, also performed on a Sylgard disc. The abdomen was first separated from rest of the fly body using microscissors. The sternal plates were then removed to expose the reproductive organs. The dorsal tergites and internal organs were left intact. The tissue was stabilized by pinning the ovaries and the uterus to the Sylgard substrate. The Abdominal Fillet was used for both optogenetic and bath-application experiments. 2) “Isolated Preparation” (see cartoon Fig. 7B): To remove inputs from the AbG and also disrupt the communication through a subset of peripheral nerves within the reproductive tract we used an “Isolated Preparation”. After immobilizing the flies on ice, the abdomen was separated from rest of the fly body using microscissors. The abdominal cuticle was removed, and the reproductive tract isolated from the gut and fat bodies. The tissue was stabilized by pinning the anterior tip of the ovaries and the distal end of the uterus to the Sylgard substrate with insect pins.

Peripheral neurons connecting the lateral oviduct to the uterus were cut using either a microscissors or sharp forceps.

**Immunohistochemistry**

All samples were dissected in phosphate saline buffer and labeled as described (Greer et al., 2005). Briefly, samples were fixed in 4% paraformaldehyde for 30min and blocked in 5% normal goat serum for 30 minutes, washed 3x with PBST (0.3% Triton-X 100 in PBS), and incubated in primary antibodies overnight at 4ºC. After incubation in secondary antibodies for 2hr at ambient temperature, the samples were cleared using 25% glycerol and mounted on a bridged slide using Diamond Prolong mounting media (Thermofisher 36966), Fluoromount-G or (SouthernBiotech #0100-01) Fluoromount-G with DAPI (SouthernBiotech #0100-20). Confocal images were obtained using a Zeiss LSM 880 confocal microscope with Zen software. Images were processed using Fiji/ImageJ software (Schindelin et al., 2012). All antibodies, their sources, and concentrations are listed in Supplementary Table 1.

**Live imaging of muscle and epithelium**

Live imaging experiments were performed in HL3.1 solution (Feng et al., 2004). After recording a baseline in HL3.1 alone, HL3.1 containing octopamine or glutamate (or HL3.1 alone as a control) was added manually to obtain the indicated final concentrations (1mM or 10 mM respectively) and mixed using gentle trituration. Mechanical disturbance of the tissue occasionally caused contractions of the lateral oviduct (data not shown). Therefore, in all experiments, care was taken to avoid touching or disturbing the reproductive tract during buffer exchange. The objective was carefully cleaned after each experiment. Calcium activity was visualized by expressing

GCaMP6m or RCaMP1b in the tissue of interest. Images were captured under a Zeiss Achroplan water immersion 10x objective on a Zeiss Axio Examiner Z1 microscope with a CCD camera (Andor iXon 897, Oxford Instruments, Oxfordshire, England) at a capture rate of 12 frames/sec using Andor IQ2 software and a Lambda DG-4 Xenon light source (Sutter). Imaging of GCaMP and RCaMP without optogenetic stimulation was performed using standard Chroma filter sets 41001 and 41007a respectively. Images were analyzed using Fiji/ImageJ software (Schindelin et al., 2012). For all Regions of interest (ROIs) an off-target area was selected as background. Changes in fluorescence are reported as the background-subtracted difference in the change in fluorescence divided by baseline (ΔF/F= [(F peak - F baseline)/ F baseline]). ΔF/F values were normalized to the variance over 5-10 seconds before light or drug application and normalized traces indicated as ΔF/F(n). Traces were also de-trended using a second order polynomial. The signal was normalized and detrended using a custom script written in LabView https://ucla.box.com/s/u8372zvb7awvikaovgjqo6tdefat21ub. Preparations in which eggs were observed to be moving into the lateral oviduct or showed ovaries without eggs were discarded from the data set.

**Quantitation of oviduct contractions**

To quantitate oviduct contractions in optogenetic experiments, the number of events observed in video recordings of each experiment were manually counted for the duration of the stimulation period: 30 sec for all experiments except for stimulation period number 3 in Fig 6, C D which was 4 minutes. Oviduct contractions were counted for 1 min following the addition of octopamine or glutamate. A longer period of

observation was used for bath application experiments because of the relatively long and variable latency to contractions following addition of octopamine. Contractions of the LO were defined by decrease in the distance between ovaries and a characteristic contraction of the oviduct tissue. These movements can be distinguished from random movements of the prep in either the x-y plane or the z axis or contractions of the ovaries (see e.g. Video 3).

**Quantitation of oviduct relaxation/dilation**

Mated female flies 5-7 days post eclosion were used for relaxation/dilation experiments. The reproductive system was dissected from the abdomen in HL3.1 and both the MAN and peripheral nerves were severed to generate an “Isolated Preparation” (see above). The preparation was transferred to fresh HL3.1 and observed for 10 minutes, with digital images captured at either 1 frame per second or 1 frame per 5 seconds. After 1min of recorded baseline activity, octopamine was added to the preparation for a final concentration of 1mM. To quantitate relaxation/dilation and changes in luminal volume in two-dimensional images, the width/diameter of the reproductive tract at the indicated positions was measured. The calyx and lateral oviduct were treated as a single unit. Measurements were made using Fiji/Image J in one-minute intervals before and after application of octopamine. The data is expressed as □L/L0, with □L representing the difference between baseline (L0) and the width at the indicated time. For most experiments, the reproductive tract was visualized on a Zeiss STEMI SV11 trinocular stereo microscope using either a Canon EOS DSLR still camera or a Dinolite USB videocamera AM7023CT inserted into the trinocular port (see Video 7). For a subset of experiments, images were acquired using a Zeiss Axio Examiner Z1 microscope fitted

with a 10x objective with a Andor iXon 897 camera, as described above for observing oviduct contractions.

**Optogenetic stimulation**

Mated female flies were raised in standard food containing 80 □M all-trans retinal from 1day post eclosion until tested (5-7 days). Stimulation was performed using a Lambda DG-4 light source (Sutter) and the standard light path of an AxioExaminer microscope to illuminate the entire field of view at 1mW/mm2 power (measured using a Thorlabs digital handheld optical power meter). For experiments quantitating contractions without simultaneous calcium imaging, optogenetic stimulation was performed using a standard filter set for GCaMP (see Live imaging above). To improve visualization of the tissue, the preparation was illuminated from the side using an external LED mounted on a ringstand. For simultaneous RCaMP imaging and optogenetic stimulation, a custom filter set that included a dual band excitation filter with peaks at 484 and 561 (FF01-484/561), a 593 nm high pass dichroic (FF593-Di03), and the single band emission filter (FF01-620/52) was used. To start and stop stimulation, a single band excitation filter (FF01-562/40) inserted into a custom holder within body of the microscope, was manually moved in and out of the light path respectively for baseline imaging of RCaMP (exposure either 542-582 nm light) or simultaneous imaging of RCaMP and excitation of ChR2 (exposure to both 473 – 495 nm and 546 – 576 nm light). Optogenetic stimulation of lLP7(+) glutamatergic neurons was performed using a standard variant of ChR2. Our initial experiments using a standard variant of ChR2 in octopaminergic cells yielded inconsistent results (data not shown) and we therefore tested the effects of octopaminergic cells using the more sensitive, red-shifted variant Chrimson (Klapoetke

et al., 2014). The high sensitivity of Chrimson led to muscle movement under ambient light, making it difficult to control its effects (not shown). We therefore turned to two recently developed variants of ChR2 (ChR2-XXM and ChR2-XXL) that are more sensitive to light than standard ChR2 variants, but less sensitive than Chrimson (Dawydow et al., 2014, Scholz et al., 2017). The kinetic properties ChR2-XXM and ChR2-XXL differ (Dawydow et al., 2014, Scholz et al., 2017), but both responded similarly to stimulation under the conditions used for our experiments (data not shown).

**Quantification and Statistical Analysis**

Data were analyzed in Prism 9 (GraphPad; San Diego CA, USA). Group means were compared using two-tailed t tests or one- or two-way ANOVAs, with repeated-measures where appropriate, followed by pairwise comparisons with multiple comparisons tests as indicated. Sample sizes (n) for each experiment are depicted in each figure panel or in the appropriate figure legend. All group averages shown in data panels depict mean ± SEM unless otherwise indicated.

**Supplemental Videos**

1) Optogenetic stimulation of Tdc2 neurons in an Intact Preparation

2) Optogenetic stimulation of ILP7 neurons in an Intact Preparation

3) Octopamine induced contractions of both the ovaries and lateral oviducts in an Isolated Preparation

4) Octopamine induced contractions of only the lateral oviducts in an Isolated Preparation

5) Glutamate induced contractions in an Abdominal Fillet Prep

6) Glutamate induced contractions in an Isolated Prep

7) Octopamine induced dilation of the oviducts (50x speed)

8) Optogenetic stimulation of ILP7 neurons in an Abdominal Fillet

## CHAPTER 3: PRECISE CRISPR-CAS9-MEDIATED MUTATION OF A MEMBRANE TRAFFICKING DOMAIN IN THE DROSOPHILA VESICULAR MONOAMINE TRANSPORTER GENE

### SUMMARY

Monoamine neurotransmitters such as noradrenalin are released from both synaptic vesicles (SVs) and large dense-core vesicles (LDCVs), the latter mediating extrasynaptic signaling. The contribution of synaptic versus extrasynaptic signaling to circuit function and behavior remains poorly understood. To address this question, we have previously used transgenes encoding a mutation in the *Drosophila* Vesicular Monoamine Transporter (*dVMAT*) that shifts amine release from SVs to LDCVs. To circumvent the use of transgenes with non-endogenous patterns of expression, we have now used CRISPR-Cas9 to generate a trafficking mutant in the endogenous *dVMAT* gene. To minimize disruption of the *dVMAT* coding sequence and a nearby RNA splice site, we precisely introduced a point mutation using single-stranded oligonucleotide repair. A predicted decrease in fertility was used as a phenotypic screen to identify founders in lieu of a visible marker. Phenotypic analysis revealed a defect in the ovulation of mature follicles and egg retention in the ovaries. We did not detect defects in the contraction of lateral oviducts following optogenetic stimulation of octopaminergic neurons. Our findings suggest that release of mature eggs from the ovary is disrupted

by changing the balance of VMAT trafficking between SVs and LDCVs. Further experiments using this model will help determine the mechanisms that sensitize specific circuits to changes in synaptic versus extrasynaptic signaling.

## INTRODUCTION

Biogenic amines such as serotonin and dopamine regulate circuit function and behavior in both mammals and model invertebrates such as *Drosophila melanogaster*. While some amine receptors localize to classical synapses, many others localize to extrasynaptic sites (Fuxe et al., 2010). Amine release within classical synaptic structures is mediated by synaptic vesicles (SVs) which cluster at defined presynaptic release sites (Dittman and Ryan, 2019). Extrasynaptic release is thought to be mediated in part by large dense-core vesicles (LDCVs) which, unlike SVs, do not cluster at presynaptic active zones (Trueta et al., 2012). The requirement for each type of vesicle release for most aminergic circuits is not known.

Vesicular monoamine transporters (VMATs) are required for the storage of amines in both SVs and LDCVs (Blakely and Edwards, 2012). Rodents and humans contain two VMAT genes while *C. elegans* and *Drosophila melanogaster* contain only one (Liu and Edwards, 1997, Greer et al., 2005, Duerr et al., 1999). The *Drosophila* VMAT gene (*dVMAT*) encodes two RNA splice variants, dVMAT-A and dVMAT-B, which are expressed in neurons and a subset of glia, respectively (Greer et al., 2005, Chang et al., 2006). We have used dVMAT-A as a model for VMAT trafficking since its C-terminal, trafficking domain appears similar to mammalian VMATs (Greer et al., 2005). Mutation of a tyrosine-based endocytosis motif or deletion of the distal C-terminus blocks rapid internalization of dVMAT-A from the cell surface in cultured cells and nerve terminals in vivo (Grygoruk et al., 2010, Grygoruk et al., 2014). Mutation of the tyrosine motif or deletion of the distal C-terminus (the “D3” truncation mutant) also reduces the localization of dVMAT-A to SVs in vivo (Grygoruk et al., 2010, Grygoruk et

al., 2014). Both mutations also lead to a corresponding increase in the localization of dVMAT-A to LDCVs, although the trafficking events that cause the shift to LDCVs remains unclear (Grygoruk et al., 2010, Grygoruk et al., 2014).

To study the behavioral effects of dVMAT-A trafficking in vivo, we have previously used mutant *dVMAT* transgenes encoding the tyrosine mutant or the C-terminal deletion to "rescue" the phenotype of a *dVMAT* null mutant (Grygoruk et al., 2014). Interestingly, some behaviors appeared to be severely compromised by shifting the relative amounts of dVMAT-A that localize SVs versus LDCVs, while others appeared to be minimally affected (Grygoruk et al., 2014, Wasserman et al., 2015). These data suggest that for some circuits, a precise balance of SV and LDCV release is essential, while for others, it is dispensable. However, we were concerned that by using transgenic expression constructs, some aspects of the mutant phenotype might be caused by non-physiological patterns of expression, or a mismatch with the endogenous developmental profile of *dVMAT*. The introduction of mutations into the endogenous *dVMAT* gene using CRISPR-Cas9 has the potential to circumvent these problems. In addition, avoiding the use of transgenes for dVMAT expression facilitates the introduction of other transgenic probes for optogenetics or live imaging, both of which will be useful for analyzing circuit activities potentially disrupted by changes in dVMAT trafficking.

Most *Drosophila* mutations generated via CRISPR-Cas9 have used double stranded (ds) homologous repair and vectors that include a screening marker, such as dsRED (Gatz et al., 2013, Sebo et al., 2013, Yu et al., 2013). Although the subsequent removal of dsRED or other markers can leave a small DNA scar, this is irrelevant for

large deletions and “knock outs”. By contrast, even a small scar can be disruptive if introduced within a coding sequence. The splice acceptor site for the last coding exon of dVMAT-A is adjacent to the trafficking domain, further increasing the risk for disrupting trafficking via mutations in this region. “Scarless”/“seamless” dsDNA repair represents one method to precisely introduce point mutants without introducing any other changes in the genomic DNA sequence (Xie et al., 2014, Ye et al., 2014, Gotze et al., 2022, Paquet et al., 2016). Single stranded (ss) DNA homologous repair represents an alternative approach and does not require the specific recognition sequence used for some ds scarless methods (Xie et al., 2014, Ye et al., 2014, Soldner et al., 2011, Yang et al., 2013). In addition, ss repair has been suggested to be less prone to random integration events and may be more efficient than ds methods (Boel et al., 2018).

Most ssDNA repair methods employ a relatively short (~100 bp) oligonucleotide, prohibiting inclusion of a cDNA encoding a visible marker to assist in screening transformants. Co-conversion techniques in which a second marker gene is mutagenized may be used to circumvent this problem (Levi et al., 2020), but necessitate the use of multiple sgRNAs and increase the possibility of off-target effects. Molecular screening techniques for point mutants also have been proposed (Touroutine and Tanis, 2020) and include the relatively simple addition of novel restriction sites into the repair vector (Cong et al., 2013).

We have taken an alternative strategy to precisely modify the *dVMAT* gene without a visible marker to identify founders, and used a primary phenotypic screen based on our prior experiments using *dVMAT* transgenes (Grygoruk et al., 2014). Since *dVMAT* transgenes that disrupt trafficking to SVs show reduced female fertility

(Grygoruk et al., 2014), we reasoned that fertility could be used to screen for generation of the same mutation in the endogenous gene via CRISPR-Cas9. We report here the generation of the new allele *dVMAT*$^{D3}$. We have used the *dVMAT*$^{D3}$ mutation to analyze which sites within the oviposition circuit may be disrupted by changes in dVMAT trafficking.

## RESULTS

### Generation of a *dVMAT* allele and phenotypic screening

dVMAT-A, the neuronal splice variant of dVMAT, contains dileucine and tyrosine-based trafficking motifs within the C-terminus (Fig 1A) (Grygoruk et al., 2010, Grygoruk et al., 2014). A *dVMAT-A* transgene lacking this region ("D3") showed reduced trafficking to SVs and increased trafficking to LDCVs (Fig 1B) (Grygoruk et al., 2010, Grygoruk et al., 2014). The splice acceptor site for the last coding exon of dVMAT-A is adjacent to a dileucine motif and the site used to introduce a stop codon in the D3 mutant (Fig 1C, D) (Grygoruk et al., 2014). To introduce a stop codon at the same site within the endogenous *dVMAT* gene without disrupting the nearby DNA sequence, we designed a single stranded (ss) DNA repair construct. Four bases in total were changed (Fig 1E, magenta) to introduce the stop codon, mutate the guide PAM, and introduce a silent BglII site to facilitate genotyping. Note that the premature stop codon is contained within the BglII site.

Our initial attempt using oligos with a total length of 200 bp failed (not shown), and others have suggested that, counterintuitively, shorter ssDNA oligos may be more effective (Yang et al., 2013). We therefore repeated the same procedure using an oligo

with a total length of 90 bp (for sequence see Methods). One hundred embryos were injected with the guide RNA and ssDNA repair template to produce germline mosaics. Five progeny from each germline mosaic were mated to a balancer and backcrossed to generate 500 lines of which 450 survived. Homozygous females from each line were selected by the absence of the *Cy* marker and crossed to wild type males to assay for potential defects in female fertility. Of the 450 lines tested, 57 lines from 17 different germline mosaics exhibited a decrease in the number of eggs per vial by visual inspection.

As a secondary screen, we used PCR to amplify a 1-kb region bracketing the site of the mutation and the new BglII site. The presence of two bands at 460 and 580-bp rather than an uncleaved 1-kb PCR product indicated the presence of the BglII site and the premature stop codon (Fig 1F). To confirm the presence of the mutated stop codon, genomic DNA was extracted from 57 candidates of the original 450 lines. Of the first 16 that were analyzed by PCR, seven were confirmed to contain the BglII site. The PCR products were then sequenced to determine whether the PAM site was also mutated and to rule out additional spurious mutations. The PCR products of the remaining 41 fly lines were subjected to Sanger sequencing to simultaneously interrogate all of the mutated sites. Of the 57 lines we sequenced, 23 contained the intended four base pair mutations without any additional changes. An additional 30 of the 57 lines (30/450 of total fly lines from 12 germline mosaics) contained missense mutations, insertions or deletions other than the intended changes. Four lines were false positives and later determined to be both genotypically and phenotypically wild type. We selected five of the sequenced lines for further analysis: four that contained the intended mutations

without any additional spurious changes (F8A, F32A, M23A, M30A) and one that was genotypically and phenotypically wild type (F7B). The F7B line was used as a genetically-matched control for later molecular and behavioral assays.

We probed the selected lines for dVMAT protein expression using a previously described antibody to the N-terminus of dVMAT-A (1G, H) (Romero-Calderón et al., 2008)] and an anti-CSP loading control (1H). We did not detect a significant difference between the four *dVMAT*$^{D3}$ mutant lines, the F7B control and an additional wild type (Canton S) control (Fig. 1G, H). These data are consistent with our previous observations using transgenic *dVMAT* mutants whichindicate that disruption of fertility in response to altered trafficking can occur independent of any changes in expression levels (Grygoruk et al., 2014).

**Phenotypic analysis**

To more precisely quantitate the apparent defect in oviposition used for screening, we performed fertility (Fig 2A) and fecundity (Fig 2B) assays as previously described [26]. Both fertility and fecundity were markedly reduced in lines containing the *dVMAT*$^{D3}$ mutant compared to control, but slightly higher than the null *dVMAT* mutant, which we have previously shown to lay few if any eggs [27] (Fig 2A, B). By contrast, larval locomotion of the *dVMAT*$^{D3}$ mutant lines did not significantly differ from the genetically matched F7B control or CS larvae (Fig 2C).

Since the four *dVMAT*$^{D3}$ mutant lines behave similarly and showed similar levels of protein expression, we chose one (F8A) for further analysis. The survival of the F8A mutant line was reduced compared to the F7B control (Fig 2D) and showed a slight

increase in feeding (Fig 2E). These data are consistent with the results obtained using *UAS-DVMAT-D3* transgene to genetically rescue the *dVMAT* null and validate our previous observation that *dVMAT* trafficking mutants disrupt a subset of amine-dependent circuits, but have less severe effects on others (Grygoruk et al., 2014). Our current results also demonstrate the use of ssDNA repair and a phenotypic screen to introduce point mutations into a gene with previously identified phenotype.

**Site of the fertility defect**

Some mutations that disrupt octopaminergic signaling cause defects in ovulation with retention of eggs within the ovary (Simon et al., 2009, Deady et al., 2015); by contrast, other mutants have been reported to primarily manifest defects downstream of ovulation and retain eggs within the calyx and oviducts (Cole et al., 2005). To specifically determine if *dVMAT*$^{D3}$ would show a defect in ovulation, we quantified the number of mature follicles and the distribution of eggs within the reproductive tract. We find that the ovaries in *dVMAT*$^{D3}$ mutant contain an elevated number of mature follicles relative to controls (Fig 3A). In addition, most eggs were found in the ovaries in the mutant (Fig 3B), in contrast to the controls and WT flies in general, in which eggs rapidly pass through the reproductive tract (Sun and Spradling, 2013). These data suggest that *dVMAT*$^{D3}$ causes a defect in ovulation (Deady et al., 2015).

The entire female reproductive tract in *Drosophila* is innervated by octopaminergic projections from the ventral nerve cord, and multiple events in the reproductive tract in addition to follicle cell rupture are thought to be regulated by octopamine (White et al., 2021, Rezaval et al., 2014). We have shown that octopamine

regulates contraction of the lateral oviducts in *Drosophila* (Deshpande et al., 2022), raising the possibility that *dVMAT*$^{D3}$ might disrupt oviduct contractility. To test this, we introduced a transgenic probe for optogenetic stimulation into the *dVMAT*$^{D3}$ background, and into a control (Canton S) background. We used *Tdc2-LexA* to express the channelrhodopsin variant ChR2-XXL in octopaminergic neurons and optogenetically stimulated Tdc(+) neurons for 30 seconds. To help quantify contractions, we expressed a second probe, RCaMP1b, in the oviduct muscles using the driver *24B-GAL4*. To determine if the response would be depleted by multiple periods of stimulation, the stimulus was repeated a second time after a 30 second rest period. We did not detect a difference in the number of contractions between mutant and control during either of the stimulation periods (Fig 3C).

## DISCUSSION

In vitro studies have revealed fundamental information about the molecular machinery responsible for transporter trafficking (Li et al., 2022, Wu et al., 2015, Moron et al., 2003, Xu et al., 2022). Additional in vivo models are required to determine the effects of transport on behavior and the contribution of the endogenous milieu to transport activity (Sun et al., 2020, Ingram et al., 2021, Bu et al., 2021, Fagan et al., 2020, Gowrishankar et al., 2018, Bowton et al., 2014, Meinke et al., 2022, Fischer et al., 2022, Kasture et al., 2019, Haase et al., 2021, Janickova et al., 2017). The expression of mutations as exogenous transgenes represents a powerful approach to determine how alterations in trafficking may disrupt behavior; however, transgenic expression systems may not fully capture the regulatory patterns of the endogenous gene. The use

of transgenes for gene expression can also complicate the use of additional transgenic probes for neuronal stimulation and imaging. Here we report the use of CRISPR-Cas9 to disrupt trafficking signals in the C-terminus of the endogenous *dVMAT* locus, and initial phenotypic analysis of the new allele.

We screened a relatively large number of lines (450 total from 100 injected founders) to ensure that we would obtain a candidate. Although it is difficult to predict the efficiency of a given homologous repair construct, the number of candidates we obtained suggest that smaller phenotypic screens for other mutations may be sufficient, assuming that the oligo repair construct is optimized to $\leq$ 90 bp (Yang et al., 2009). We suggest that a phenotypic screen may be used to identify CRISPR-Cas9 mutants in other genes in cases in which standard marker-based screens using a dsDNA construct are not convenient, e.g., because a site for a scarless insertion is not apparent. In addition, the presence of DNA repeats can complicate the generation of the relatively large homology arms used for some ds repair constructs. Indeed, parallel attempts to generate a dsDNA construct for introduction of a premature stop codon in *dVMAT* were hampered by repeats in the 3' UTR (data not shown).

Consistent with our previous findings using a *dVMAT-D3* transgene, we find that the endogenous CRISPR-Cas9 mediated *dVMAT*$^{D3}$ mutation impairs female fertility and fecundity, but has no detectable effect on baseline, larval locomotion (Grygoruk et al., 2014). We also show retention of mature follicles in the ovaries, suggesting that loss of synaptic signaling or an increase in extrasynaptic signaling may disrupt follicle rupture. Follicle rupture is mediated by OAMB (Deady and Sun, 2015) and future experiments

will more specifically explore the relationship between octopamine release by *dVMAT*$^{D3}$ and the response of OAMB receptors in follicle cells.

It is possible that *dVMAT*$^{D3}$ directly disrupts follicle cell rupture by changing octopamine release within the ovaries. Alternatively, it is possible that octopaminergic synapses in the CNS *upstream* of the reproductive tract are indirectly responsible for this phenotype. The methods we have established to express optogenetic and imaging transgenes in the *dVMAT*$^{D3}$ mutant background will be useful to explore upstream elements of the oviposition circuit in future experiments. Here, we have used optogenetics coupled with live imaging to analyze the effects of *dVMAT*$^{D3}$ on oviduct contractility *downstream* of ovulation. We do not detect a difference between *dVMAT*$^{D3}$ and wild type controls for the contraction of lateral oviduct muscle in response to optogenetic stimulation of octopaminergic neurons. These data indicate that at least one element of the oviposition circuit downstream of ovulation is not disrupted by *dVMAT*$^{D3}$. However, octopamine also regulates dilation of the oviducts, another process downstream of ovulation that may influence fertility (Rodríguez-Valentín et al. 2006; Deshpande et al. 2022). Dilation can be easily visualized when the reproductive tract is dissected out of the abdomen and octopamine is bath-applied to the reproductive tract (Rodríguez-Valentín et al. 2006; Deshpande et al. 2022). Unfortunately, visualization of dilation is difficult with the intact preparations that we have used here and are required for optogenetic stimulation of octopamine neurons within the abdominal ganglion ( Deshpande et al. 2022). Since we cannot test the effects of *dVMAT*$^{D3}$ using bath-applied octopamine, we cannot rule out the possibility that *dVMAT*$^{D3}$ causes a defect in oviduct dilation.

In sum, while other aspects of the oviposition circuit may be disrupted by *dVMAT*$^{D3}$, our current data suggest that retention of mature eggs in the ovaries is likely to be responsible for the decrease in fertility. Moreover, at least some downstream processes such as oviduct contractions appear to be unaffected by a shift in octopamine release from SVs to LDCVs.

In future experiments, we will use additional drivers to express RCaMP in subsets of other neurons to analyze the effects of *dVMAT*$^{D3}$ on aminergic circuits unrelated to oviposition. These include an octopaminergic circuit in the visual system that regulates the response of flies to odor plumes during flight (Wasserman et al., 2015). Ongoing experiments will determine whether *the dVMAT*$^{D3}$ line replicates the phenotype that we have previously seen using a *UAS-DVMAT-D3* transgene Wasserman et al., 2015). If so, expression of RCaMP in visual system neurons combined with *dVMAT*$^{D3}$ may be used to investigate the underlying mechanisms.

Our previous data indicates that the *dVMAT-D3* transgene traffics less to SVs, and we speculate that some aspects of the *dVMAT*$^{D3}$ phenotype is due to a decrease in amine release from SVs. However, in addition to a decrease in sorting to SVs, we have previously shown that the *dVMAT-D3* transgene localizes more to LDCVs than the wild type transporter (Grygoruk et al., 2014). Therefore, it is possible that increase in amine release from LDCVs may also contribute to the *dVMAT*$^{D3}$ phenotype. Further experiments using mutations that more specifically disrupt sorting to SVs, may resolve this issue. Alternatively, it may not be possible to reduce dVMAT sorting to SVs without increasing its localization to LDCVs; another mutation (Y600A) also led to an increase in the localization of dVMAT to LDCVs (Grygoruk et al., 2014). We speculate that, at least

in flies, there may be communication between the pathways for biogenesis and/or recycling of SVs and LDCVs, and at present, we can only conclude that the *dVMAT*$^{D3}$ phenotype results from a change in the balance of amine release between SVs and LDCVs.

The variety of octopamine-dependent processes within the oviposition circuit and the CNS provide a model to further probe the effects of dVMAT trafficking on circuit function. We speculate that further experiments using *dVMAT*$^{D3}$ coupled with optogenetics and imaging will help to elucidate the circuit properties that govern synaptic versus extrasynaptic signaling.

## MATERIALS AND METHODS

### Husbandry

Mixed populations of male and female flies were raised on cornmeal-molasses agar media at 25°C and 20-40% humidity with a 12-hour light/dark cycle.

### Mutagenesis and screening

Potential sites for CRISPS-Cas9 based mutagenesis in the *dVMAT* gene were selected using the online program CRISPR Optimal Target Finder (http://targetfinder.flycrispr.neuro.brown.edu/) and the guide RNA sequence gattgcagTCTTTGATCTA. The guide RNA vector was constructed by self-annealing the oligonucleotide containing the guide sequence plus 5' and 3' recognition sites for the expression vector (CTTC- gattgcagTCTTTGATCTA) and its reverse complement (TAGATCAAAGActgcaatc-CAAA), followed by ligation into a unique BbsI site in the

vector pU6-2-BbsI-chiRNA (Gratz et al., 2013). The single guide RNA plasmid was co-injected (Best Gene, Inc.) with the 90-nt single-stranded repair oligonucleotide acccataccctttttccatttcatttctgtgattgcagTCTTAGATCTGCAGACGCGATCGGGCTCAGGTA CGTTATGTAACCTATCAAAA (mutated bases underlined) into *vas-Cas9* expressing embryos (BDSC #51323). Of the 120 injected embryos,100 survived to adulthood and were mated to the balancer line *Tft/CyO* for two generations to obtain 450 stable lines expressing the mutagenized chromosome over *CyO*. To phenotypically screen for the presence of a *dVMAT* mutant, individual females from each line homozygous for the mutagenized chromosome were mated to wild type (Canton S) males and vials were visually inspected for evidence of reduced fertility and fecundity. To verify the presence of the mutation and the absence of spurious changes, genomic DNA from each one of the 57 lines was isolated and subjected to PCR using the primers TATGTATGCTCCATTGCTGACG and CCAAAGAAGGACTCTCACAAGC. PCR products were digested with BglII (NEB) followed by gel electrophoresis and/or DNA sequencing using the oligo TATGTATGCTCCATTGCTGACG (Genewiz).

**Western blots**

For western blots, 10 heads per genotype (5 males, 5 females) from flies aged 3-7 days were homogenized in 140mM NaCl, 10mM Tris-Cl pH 8.0, 0.5mM EGTA, 1% Tx-100, 0.1% sodium deoxycholate, 0.1% SDS, with a protease inhibitor cocktail (Roche cOmplete Mini). Following centrifugation to remove debris (13000g, 5 min, ambient temperature) the supernatant was mixed with 1/5 volume of 5X SDS-PAGE sample buffer. One head-equivalent per lane was loaded on a 10% polyacrylamide gel and

transferred to nitrocellulose. The top and bottom portions of the blot were probed with a rabbit primary antibody to the dVMAT amino terminus (1:1000, [9]) or mouse anti-CSP (1:1000, Developmental Studies Hybridoma Bank) respectively (2 hrs, RT) followed by the appropriate HRP-conjugated secondary antibodies (anti-mouse, 1:2000, MEMD Millipore Corp, anti-rabbit, 1:2000, Invitrogen). The blot was washed and exposed to the HRP substrate SuperSignal West Pico PLUS (Thermo Scientific) and imaged on an Azure Biosystems C400 scanner. The area under the peak of bands representing dVMAT and CSP were quantified (ImageJ/Fiji) and dVMAT signal normalized to the CSP loading control for comparison across samples.

**Larval locomotion**

Two to three larvae were placed on the food for 30 sec to acclimate and locomotion was scored as the number of 0.4 cm grids crossed over 2 minutes. Their speed was calculated as centimeters per second (cm/s) using Multi-Worm Tracker software (Sweirczek et al., 2011).

**Survival**

Flies were housed in vials (5 males, 5 females per vial) and scored for survival each day until all flies were dead. Two hundred flies per genotype were tested.

**Fertility**

Flies were collected under $CO_2$ anesthesia 0–5 days before mating. One virgin female was mated with four $w^{1118}$ males in vials at room temperature. Twelve days after mating,

parents were removed. Candidates were scored as fertile if the vial contained at least one larva, pupa, or adult over the subsequent twelve days. A population of 70 flies were tested five times in replicate experiments. Fertility is expressed as the proportion of the population capable of producing at least one viable progeny.

**Fecundity**

Five females of the indicated genotype were mated to five $w^{1118}$ control males in a vial for 3 days. Mated flies were passed into a new vial each day for 12 days. The number of eggs laid per vial on each day was scored manually. Twenty flies per genotype were tested and the number of eggs were counted each day for 12 days. Fecundity is expressed as number of eggs laid per female per day over 12 days.

**Mature Follicle Quantification**

Five-day-old females fed with wet yeast for one day and mated to 10 wild type Canton S males (5 females: 10 males) in bottles. After two days, ovaries were dissected from twenty flies per genotype, stained with DAPI, and mounted, with stage 14 mature follicles within each ovary quantified as described (Deady and Sun, 2015).

**Egg Distribution within the reproductive Tract**

To determine the location of the egg in the reproductive tract, single-pair matings were set up between one virgin female and one Canton S male. Flies were allowed to mate for 6 hours, an interval sufficient for all females to reach a steady state level of ovulation and egg laying (Deady and Sun, 2015, Sun and Spradling, 2013). Females were then

frozen (-80˚C, 5 min) and reproductive tracts were dissected to examine the location of the eggs.

**Optogenetics and live-imaging**

All flies used for optogenetic experiments contained two copies of *Tdc2-LexA* and one copy each of*, LexAop-CHR-XXL, 24B-Gal4, and UAS-RCaMP1b* and were homozygous for either *dVMAT*$^{D3}$ or wild type *dVMAT*. Mixed cultures of males and female flies were reared in low-light conditions (to avoid uncontrolled stimulation of ChR) on standard cornmeal-molasses agar media containing 80μM retinol for 2 days, from 4 to 6 days post-eclosion. At 6 days post-eclosion female flies were pinned, ventral side up to a Sylgard platform and immersed in HL3.1 as described (Deshpande et al., 2022). The ventral cuticle at the junction between the thorax and abdomen and a portion of the abdomen was carefully removed to expose the abdominal ganglion and the oviducts respectively. The preparations were imaged using an upright Zeiss AxioExaminer microscope fitted with a 10x immersion objective and a custom filter set that included a dual band excitation filter with peaks at 484 nm and 561 nm (FF01-484/561), a 593 nm high pass dichroic (FF593-Di03), and the single band emission filter (FF01-620/52). Two collimated LED light sources (565 nm and 470 nm, Thorlabs) were coupled using a “custom multi-LED source for microscope illumination” and passed through the epifluorescence light path of the microscope for visualization of the oviducts or optogenetic stimulation respectively. Following 1 min of baseline recording, optogenetic stimulation using the 470 nm LED was initiated manually via the driver (DC2200,

Thorlabs) for 30 sec followed by a 30 sec rest period and a second 30 sec stimulation period. Contractions were manually counted as described (Deshpande et al., 2022).

**Feeding Assay**

Groups of ten flies 3-5-days old were starved for 24 hours on 1% agar medium as a water source. After starvation, flies were transferred to blue food (10% sucrose, 5% active yeast, 1% agar, and 4% blue food dye (McCormick) and allowed to feed for 15 mins. The amount of blue food dye ingested was measured using spectrophotometric measurement of the blue dye as previously described [60]. In brief, 10 flies were homogenized in 400 µL PBS and centrifuged (14,000 rpm for 3 min at ambient temperature). 250 µL of the supernatant was aspirated into a fresh tube, avoiding the pelleted debris and re-centrifuged (14,000 rpm, 3 min, at ambient temperature). 200 µL of the supernatant was loaded into a 96 well microplate for absorbance readings (Biotek Synergy 2 Multi-Mode Plate Reader). The corrected absorbance of the dye was calculated by subtracting the absorbance at 750 nm (outside of blue dye profile) from the absorbance at 630 nm (peak of blue dye) as previously described (Albin et al., 2003).

## FIGURES AND LEGENDS

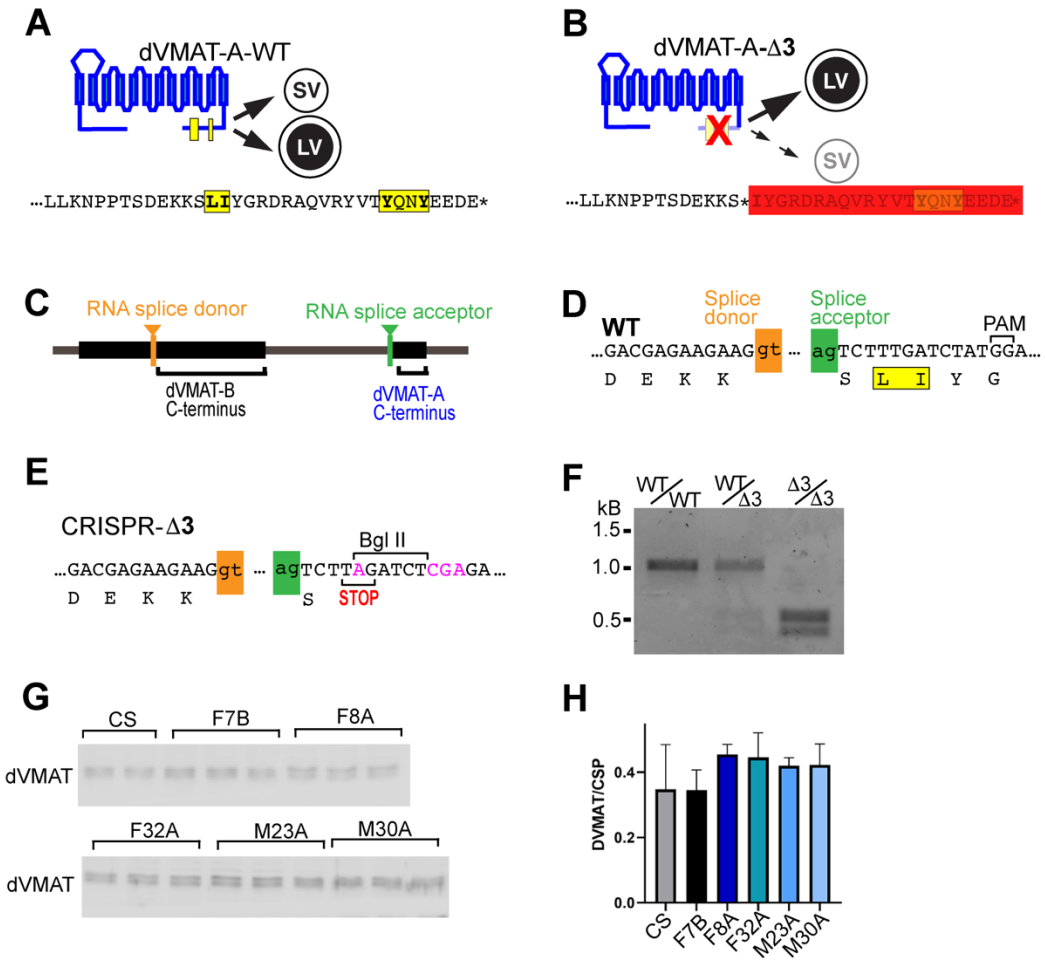


**Figure 1. CRISPR-Cas9 based mutagenesis and phenotypic screen for mutants.**

A. The neuronal isoform of dVMAT (dVMAT-A) contains at least two trafficking motifs (yellow rectangles) within the C-terminal cytoplastic domain, which allow sorting to both synaptic vesicles (SVs) and large dense-core vesicles (LDCVs). B. Ablation of this domain disrupts dVMAT-A trafficking to SVs and increases trafficking to LDCVs. C. *dVMAT* gene expresses two splice variants. D. The splice acceptor site for dVMAT-A is proximal to a dileucine motif. E. The ssDNA repair construct mutated four base pairs (magenta). F. The BglII site allows cleavage of a PCR product in *dVMAT*$^{D3}$ that is uncut

in WT. F. A western probed for DVMAT. G. Quantitation of the DVMAT bands normalized to a CSP loading control.

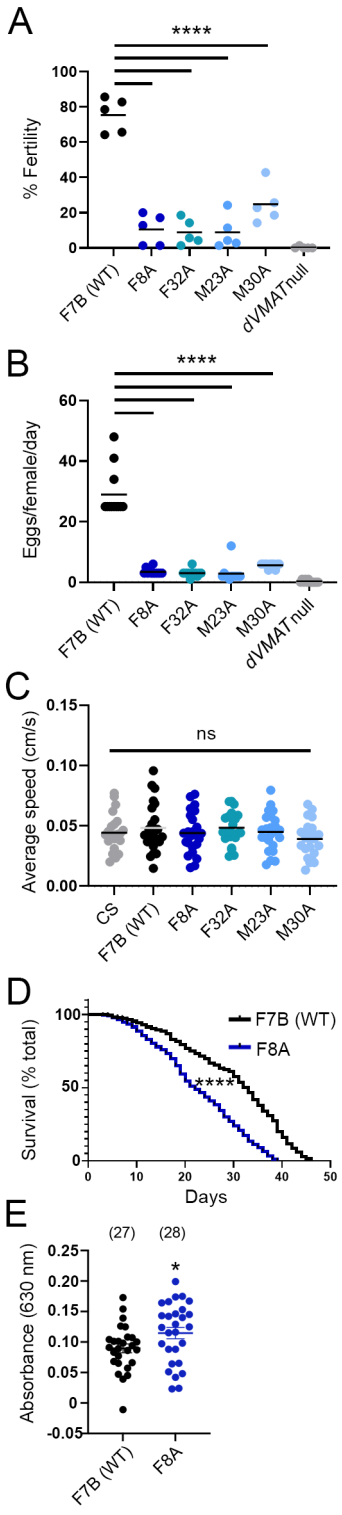


**Figure 2. Phenotypic analysis.**

Fertility (A), fecundity (B) and larval locomotion (C) were assayed for four genotypically verified *dVMAT*$^{D3}$ mutants, a control line genotypically wild type at the *dVMAT* locus (F7B) and wild type Canton S flies (CS). One-way ANOVA with Bonferroni's multiple comparisons test, p≤0.0001****, mean indicated as horizontal lines. No significant differences in locomotion (C) were detected between mutant and wildtype larva (One way ANOVA). D. Adult survival on standard food was quantified for one of *dVMAT*$^{D3}$ alleles (F8A) and a control (F7B) with Kaplan Meier analysis by Mantel-Cox Test, p≤0.0001****, N=200 per group, median survival F7B = 32.5 days, F8A = 22.0 days. E) Quantification via spectrophotometry of ingested blue dye (n = 27-28 groups of 10 flies), two-tailed unpaired t test (p≤0.0332*).

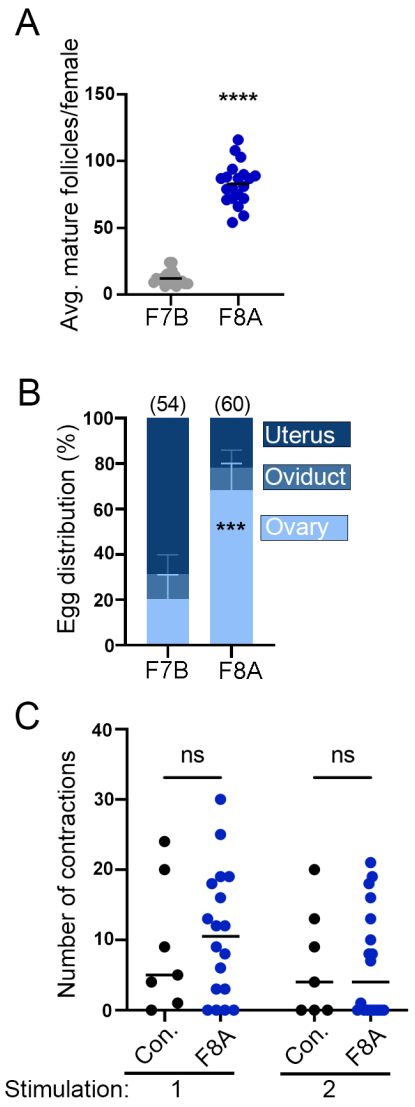


**Figure 3. *dVMAT$^{D3}$* mutants retain mature eggs in their ovaries.**

A. Quantification of mature follicles in female ovaries post egg-laying in F7B controls (grey) and the F8A *dVMAT$^{D3}$* mutant (blue). Graphs show individual datapoints and group means, (F7B n=19 flies and F8A n=20 flies, two-tailed unpaired t test, p≤0.0001****). B. Location of the egg within the reproductive tract (RT) after 6 hours of mating. Graphs show mean ± 95% confidence interval, (n=54 flies for F7B and 60 flies for F8A), Fisher' exact test (p≤0.001***). C. Optogenetic stimulation of Tdc2-LexA>>LexAop-CHR-XXL in

either a control ("Con.") fly harboring WT *dVMAT* in a $w^{1118}$ background (black) or $dVMAT^{D3}$ (F8A) mutant (blue) induces similar numbers of lateral oviduct contractions.

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## CHAPTER 4: DROSOPHILA CELLS THAT EXPRESS OCTOPAMINE RECEPTORS CAN EITEHR INHIBIT OR PROMOTE OVIPOSITION

### SUMMARY

Adrenergic signaling is known to play a critical role in regulating female reproductive processes in both mammals and insects. In *Drosophila*, the ortholog of noradrenaline, octopamine (Oa), is required for ovulation as well as several other female reproductive processes. Loss of function studies using mutant alleles of receptors, transporters, and biosynthetic enzymes for Oa have led to a model in which disruption of octopaminergic pathways reduces egg laying. However, neither the complete expression pattern in the reproductive tract nor the role of most octopamine receptors in oviposition is known. We show that all six known Oa receptors are expressed in peripheral neurons at multiple sites within in the female fly reproductive tract as well as in non-neuronal cells within the

sperm storage organs. The complex pattern of Oa receptor expression suggests the potential for differential regulation of specific subtypes of peripheral neurons in the reproductive tract that are responsible for the inhibition of egg-laying in unmated flies. Indeed, activation of some neurons that express Oa receptors inhibits oviposition, and neurons that express different subtypes of Oa receptor can affect different stages of egg laying. Stimulation of some Oa receptor expressing neurons (OaRNs) also induces contractions in lateral oviduct muscle and activation of non-neuronal cells in the sperm storage organs by Oa generates OAMB-dependent intracellular calcium release. Our results are consistent with a model in which adrenergic pathways play a variety of complex roles in the fly reproductive tract that includes both the stimulation and inhibition of the oviposition.

## INTRODUCTION

Oocyte development, fertilization, and ovulation are regulated by multiple steroid and peptide hormones as well as aminergic neuromodulators in both mammals and invertebrates. The regulation of some processes is surprisingly conserved, allowing the use of relative simple systems to explore the underlying mechanisms (White, Chen, and Wolfner 2021; Kamhi et al. 2017; E. M. Knapp et al. 2020; E. Knapp and Sun 2017). These include the adrenergic regulation of oocyte development and ovulation mediated by noradrenalin in mammals and its structural ortholog octopamine (Oa) in *Drosophila melanogaster* (Deady and Sun 2015; J. Kim et al. 2021; Hoshino and Niwa 2021; Yoshinari et al. 2020; Andreatta et al. 2018; J. Kim et al. 2021; L. Wang et al. 2022; J.

Kim and You 2022; Čikoš et al. 2007; Schmidt et al. 1985; Kannisto, Owman, and Walles 1985; Kobayashi et al. 1983; Blum et al. 2004).

In mammals, noradrenaline is known to directly modulate reproductive tract function (Lawrence and Burden 1980; M. T. Itoh et al. 2000; Masanori T. Itoh and Ishizuka 2005; Ricu et al. 2008) with proposed roles in ovarian steroid synthesis (Garrido et al. 2018; Adashi and Hseuh 1981) and muscle contractions (Virutamasen, Wright, and Wallach 1973; Kannisto, Owman, and Walles 1985; Kobayashi et al. 1983). Polycystic ovarian syndrome (PCOS) is a common anovulatory disease affecting millions of women in every region of the world (Wolf et al. 2018), and it has been shown to present with increased sympathetic signal release to the ovaries and elevated plasma noradrenaline levels (Lansdown and Rees 2012; Greiner et al. 2005). Determining conserved mechanisms by which adrenergic signaling regulates female fertility may aid in the development of novel therapeutic strategies for PCOS and other anovulatory conditions.

In *Drosophila* and other insects, the steps required for egg-laying (oviposition) include follicle development, follicle rupture, ovulation, passage through the oviducts and egg deposition (White, Chen, and Wolfner 2021; J. Sun and Spradling 2013; Häsemeyer et al. 2009; Feng et al. 2014; F. Wang et al. 2020; Deady and Sun 2015; Mattei et al. 2015; Avila et al. 2012; Orchard and Lange 1985; Lange 2009). Oa contributes to the regulation of most, if not all these processes in *Drosophila* and other insect species (Kamhi et al. 2017; Andreatta et al. 2018; Orchard and Lange 1985; Lange 2009; Hana and Lange 2020; Wong and Lange 2014; Rezával et al. 2012; Yoshinari et al. 2020; White, Chen, and Wolfner 2021).

*Drosophila melanogaster* expresses six Oa receptors (Qi et al. 2017; Balfanz et al. 2005; Maqueira, Chatwin, and Evans 2005; Evans and Maqueira 2005; Farooqui 2007; El-Kholy et al. 2015). Two of these, Oamb and Octβ2R, have been established as critical for fertility and linked to several physiological processes involved in oviposition (H.-G. Lee et al. 2003; H.-G. Lee, Rohila, and Han 2009; Lim et al. 2014a; Deshpande et al. 2022; Deady and Sun 2015; Avila et al. 2012; Li et al. 2015). Expression of both receptors in the epithelium of the oviduct regulates egg laying (H.-G. Lee, Rohila, and Han 2009; Lim et al. 2014a). Oamb's expression in ovarian follicle cells is required for follicle rupture (Deady and Sun 2015). Oamb expression in parovarian organs as well as the seminal receptacle and spermathecae may play a role in sperm storage dynamics (Avila et al. 2012; 2010). We have recently shown that Oamb and Octβ2R are required for contraction and dilation of the lateral oviducts respectively (Deshpande et al. 2022). The effects of Octβ2R may be mediated via expression in either neurons (Deshpande et al. 2022) or muscle (Li et al. 2015). It is unclear whether Oamb or Octβ2R may regulate additional processes involved in oviposition, and they are widely expressed at a number of additional sites throughout the reproductive tract and CNS, The potential role for Oamb and Octβ2R expressing neurons in oviposition-linked circuits is particularly unclear, since most studies have focused on their function in non-neuronal tissues such as the epithelium and follicle cells (H.-G. Lee, Rohila, and Han 2009; Lim et al. 2014a; H.-G. Lee et al. 2003; Li et al. 2015; Deady and Sun 2015; White, Chen, and Wolfner 2021). With regard to the other four Oa receptors expressed in *Drosophila* -- Octα2R, Octβ1R, Octβ3R Oct-TyrR—it is not known whether they play any role in oviposition, or in which type(s) of cells in the reproductive tract they may be

expressed (El-Kholy et al. 2015). Here and in a previous report, we have used a panel of molecular tools for high-fidelity expression of each receptor to determine where they are expressed in the reproductive tract and how cells expressing each one may contribute to oviposition (Deshpande et al. 2022).

Although Oa regulates multiple processes within the reproductive tract, the most commonly reported phenotype for mutations that reduce octopaminergic signaling is a decrease in the release of eggs from the ovary (ovulation) with a resultant decrease in egg laying (White, Chen, and Wolfner 2021; Pang et al. 2022). This phenotype has been observed with loss of function mutants or RNAi for the receptors Oamb (H.-G. Lee et al. 2003; H.-G. Lee, Rohila, and Han 2009; Deady and Sun 2015) and Octβ2R (Lim et al. 2014a; Li et al. 2015), an enzyme required for Oa synthesis, tyrosine-β-hydroxylase (TbH) (Monastirioti, Charles E. Linn, and White 1996; Cole et al. 2005; Monastirioti 2003) and the transporter responsible for its storage and release from secretory vesicles (Simon et al. 2009). Oa also has the potential to influence fertility via actions in the oviducts or uterus (Deshpande et al. 2022; Lim et al. 2014b; Li et al. 2015; H.-G. Lee, Rohila, and Han 2009; H.-G. Lee et al. 2003); however, effects that occur downstream of follicle rupture can be epistatically occluded by retention in the ovaries.

Both gain of function and loss of function mutations can be useful for genetic analyses, e.g., for epistatic experiments to determine the order of genetic or biochemical events. With perhaps one notable exception (Hoff et al. 2011), gain of function transgenes for *Drosophila* Oa receptors are not available. Moreover, over-expressing a receptor is unlikely to increase the activity of post-synaptic neurons if the presynaptic input is not amplified. Because the Oa receptors are $G_{\alpha(s,q)}$ type GPCR's,

directly activating neurons in which the Oa receptors are expressed represents an alternative approach to probe their potential functions. The molecular tools available in *Drosophila* are well suited to drive expression of probes that activate or inhibit specific subsets of neurons. These include the temperature-sensitive alleles for the dTrpA1 $Ca^{2+}$ channel and Kir2.1 $K^{+}$ channel that can be combined with lines such as “MiMICS” in which a GAL4 or LexA transcription factor is embedded within the endogenous receptor gene to precisely replicate its temporal and spatial patterns of expression (Diao et al. 2015; Venken et al. 2011; McKinney et al. 2020; Deshpande et al. 2022; P.-T. Lee et al. 2018).

We have used a panel of six MiMIC lines to map the expression in the reproductive tract and the potential function(s) in oviposition of the six known *Drosophila* Oa receptors (McKinney et al. 2020; Deshpande et al. 2022). We demonstrate co-expression of Oa receptor subtypes with the mechanosensitive marker ppk1.0 and acetylcholine marker ChAT in uterine cells previously shown to regulate oviposition (Yoshinari et al. 2020; Yang et al. 2009; Gou et al. 2014; F. Wang et al. 2020; Deshpande et al. 2022; Rezával et al. 2014; 2012). Activation of neurons that express at least three of the receptors stimulates oviduct contraction, and we show that Oamb regulates the acute response of cells in the sperm storage organs to Oa. Behavioral assays indicate that activation or inhibition of neurons that express each receptor impacts oviposition in a different way, but in contrast to most previous studies in which Oa has been suggested to promote oviposition (Deady and Sun 2015; Cole et al. 2005; Li et al. 2015; H.-G. Lee, Rohila, and Han 2009; Lim et al. 2014a; Simon et al. 2009; Monastirioti, Charles E. Linn, and White 1996; J. Kim et al. 2021; Hoshino and Niwa

2021; White, Chen, and Wolfner 2021), our data suggest a complementary role for octopamine receptor-expressing neurons (OaRNs) in the inhibition of egg-laying. This includes a potentially novel role for Oamb-expressing neurons to promote egg retention in the uterus.

## MATERIALS AND METHODS

### Experimental model and subject details.

Flies were raised in mixed sex vials on cornmeal/sucrose/yeast/sucrose/dextrose/agar medium at 25°C and 50–70% humidity under a 12:12 light: dark cycle unless otherwise noted. Mated or virgin female flies 5–7 days post eclosion were used for all experiments. Fly lines used in this study are listed in Supplementary Table S1.

### Fly Husbandry and stocks.

Publicly available fly lines with noted identifiers were obtained from the BDSC (Listed in Supplementary Table 1). We thank the following people for generously supplying the following additional lines: Dr. Kyung-An Han (University of Texas, El Paso) for *OAMB-RS-GAL4*; Dr. Bing Ye (University of Michigan) for *ppk1.0-LexA* and *ppk-Gal4* (Gou et al., 2014), Soo Hong Min (Harvard) for *elav-Gal80* (Yang et al. 2009), Dr. Robert Kittel (University of Würzberg) for *UAS-ChR2-XXM*, and Dr. Mark Frye (UCLA) for *UAS-Kir2.1* and *tub-Gal80$^{ts}$*.

**Immunofluorescence staining.**

To visualize the expression patterns of the Oa receptor genes, complete reproductive systems from flies harboring *UAS-mCD8-GFP* and either Oa receptor *MiMIC-T2A-Gal4*'s or *ChAT-Gal4* were dissected in phosphate buffered saline (PBS) at 25 °C with each innervating VNC and MAN connection left intact. VNC + Reproductive Tract preparations were then fixed in 4% paraformaldehyde (PFA) for 15 min and washed in PBS containing 0.3% (vol/vol) Triton X-100 (PBT) for 30 minutes. Following fixation, preparations were washed for 1 hour in blocking buffer containing 5% (vol/vol) normal goat serum (NGS) (Cat# G9023, Sigma-Aldrich) in PBT. This blocking buffer was then replaced for new blocking buffer containing the primary antibody mouse anti-GFP (1:500, Ref# A11120, Invitrogen), and samples were incubated for 24–48 hr at 4 °C. The sample was then washed in PBT for 3 hr at 25 °C before being incubated in blocking buffer containing the secondary antibody AF488-conjugated goat-anti-mouse (1:500, Ref# A21202, Invitrogen) at 4 °C for 24 hr. After being washed again in PBT for 1 hr at 25 °C, the preparations were optically cleared in 25% glycerol for 1 hr at 25 °C and then mounted on Superfrost slides (Cat# 12-550-143, Fisherbrand) with bridged Glass Cover Slips #0 (Cat# 72198-10, Electron Microscopy Sciences) in Fluoromount-G mounting media (Cat# 0100-01, SouthernBiotech). For co-staining of muscle cells, preparations were dissected and processed as described above, except AF555-conjugated Phalloidin stain (1:500, Ref# A34055, Invitrogen) was included in the secondary antibody solution. For co-labeling of *Ppk1.0-LexA*, preparations were dissected and processed as described above, except rabbit anti-dsRed (1:500, Cat# 632496, Takara Bio) was added to the primary antibody solution and AF568-conjugated

goat-anti-rabbit (1:500, Ref#A31572, Invitrogen) was added to the secondary antibody solution. For co-staining of neural cells, preparations were dissected and processed as described above, except Rhodamine (TRITC) conjugated rabbit anti-horseradish peroxidase (HRP) stain (1:500, Code# 323-025-021, Jackson Immunoresearch) was included in the secondary antibody solution.

**Optogenetic stimulation of lateral oviduct contractions.**

To study the effect of OaRN stimulation on oviduct behavior, optogenetic stimulations of OARNs were performed on abdominal filet preparations as previously described (Deshpande et al. 2022). Flies harboring one copy of *Octβ1R*, *Octβ3R*, or *Oct-TyrR MiMIC-T2A-Gal4* and one copy of *UAS-ChR2-XXM::tdTomato* were compared to control flies with one copy of the *MiMIC-T2A-Gal4* that had been crossed with *Canton-S* (CS) flies. Live imaging of stimulation responses focused on recording lateral oviduct behavior, and lateral oviduct contractions were identified as previously described (Deshpande et al. 2022).

**Accessory glands live imaging.**

To determine Oa's effects on the accessory gland cellular activity, we prepared fly reproductive tracts for live imaging using the previously described "isolated preparation" method (Deshpande et al. 2022). Instead of focusing on oviduct regions, however, we recorded live imaging from the accessory glands, using Zeiss Achroplan water immersion 10x objective on a Zeiss Axio Examiner Z1 microscope with a CCD camera (Andor iXon 897, Oxford Instruments, Oxfordshire, England) at a

capture rate of 12 frames/sec, Andor IQ2 software, and a 555 nm LED light source (Thorlabs). RCaMP1b signal emission was captured using the standard Chroma filter set 41007a. Images were analyzed using Fiji/ImageJ software (Schindelin et al. 2012). For all Regions of interest (ROIs), an off-target area of equal size was selected as background. Changes in fluorescence are reported as the background-subtracted difference in the change in fluorescence divided by baseline (dF/F = [(F peak - F baseline)/F baseline], where F baseline = average RCaMP signal during the 1 min before Oa bath application). In all experiments, 1 min of baseline activity was recorded before Oa was bath applied to the preparation at the indicated concentration. Recording was conducted for 4 min following Oa addition. All flies tested were 4 days post eclosion and carried either one copy of *24b-Gal4* or *40B09-Gal4* and one copy of *UAS-RCaMP1b*. RNAi experiments used flies that additionally included one copy each of *UAS-dicer2* and *UAS-OAMB-RNAi*. Mated flies were cohoused with *CS* males after sorting 1 day post eclosion. Virgin flies were sorted and housed without males.

**Fertility Assays.**

Egg laying assays were conducted as previously described (E. Knapp and Sun 2017). In brief, virgin flies harboring indicated *Gal4* alleles and either *UAS-TrpA1*, *UAS-Shibire$^{ts}$*, or *UAS-Kir2.1* and *TubGal80$^{ts}$* were collected and housed at 22 °C. At 5 days post eclosion, these virgins were mated with *CS* males and either kept at 22 °C for control experiments or shifted to 29 °C to facilitate hyperactivation or suppression of neural activity via transgene expression. The number of eggs laid on molasses plates were then counted every day for 2 days and averaged. Egg laying times were

calculated by dividing 1440 min by the total number of eggs laid per female per day. Following 2 day egg laying experiments, females' ovaries were dissected for quantification of mature follicles as previously described (E. Knapp and Sun 2017). Ovaries were fixed in 4% PFA for 15 min, stained with DAPI (1:1000, Prod#62248, Thermo Scientific), and mounted on Superfrost slides with 24x30-1 Cover Glass (Fisherbrand). Mature follicles in each set of ovaries were quantified via fluorescent imaging using a Zeiss LSM 880 confocal microscope. To partition egg laying times into ovulation time, oviduct time, and the uterus time, partitioning ratios were determined by dissecting reproductive tracts after separate 6 hr mating experiments and determining the percentage of females with eggs in the oviduct or uterus as previously described (E. Knapp and Sun 2017).

## RESULTS

### Multiple Octopamine receptors are expressed by central and peripheral neurons that innervate the reproductive tract

We visualized the expression of each one of the six *Drosophila* Oa receptors using a panel of *MiMIC-T2A-Gal4* lines (McKinney et al. 2020; Diao et al. 2015; Venken et al. 2011). Consistent with previous reports (H.-G. Lee et al. 2003), we detect expression of Oamb in follicle cells that surround the mature oocyte (Fig. 1, B.i, filled arrow) and expression of both Oamb and Octβ2R in epithelial cells that line the lumen of the oviducts (Fig. 1, B.i, B.v, open arrows). We did not detect the expression of Oa receptors other than Oamb in follicle cells (Fig. 1 and data not shown). Functional data

also support a role for Oamb in somatic escort cells in regulating the germline stem cell lineage, exclusive of its role in ovulation and follicle cell rupture (Yoshinari et al. 2020). Determining the expression of other Oa receptors in intra-ovariole cells will require further studies.

We and others have previously shown that Octβ2R is expressed in multiple neurons within the reproductive system as well as neurons in the CNS that descend from the abdominal ganglion (AbG) (Deshpande et al. 2022; McKinney et al. 2020). We find that Octa2R, Octβ1R, Octβ3R, and Oct-TyrR expressing cells also extend processes throughout the reproductive system (Fig. 1, A, B, chevrons, C, D, yellow circles, Sup. Fig. 1). Based on labeling with a neuron-specific antibody to a neuron specific epitope ("anti-HRP"), most of these processes appear to be neuronal (Sup. Fig. 2 and data not shown).

As others have reported, we detect neuronal cell bodies that express Oamb, Octa2R, Octβ1R, Octβ2R, Octβ3R and Oct-TyrR in the Abdominal Ganglion (AbG) (Fig. 1, A, chevrons) (McKinney et al. 2020). We further confirm that projections from these neurons extend into the median abdominal nerve (MAN) that connects the AbG to the reproductive system (Fig. 1, A, B, stars).

We also observe multiple peripheral neurons that express Oamb, Octa2R, Octβ1R, Octβ2R, Octβ3R and Oct-TyrR and are embedded within the MAN and/or the reproductive tract itself (Fig. 1, B, chevrons). Cells embedded within uterine muscle layers appear to project ascending processes through the MAN into the CNS, whereas cells embedded within the MAN are difficult to distinguish as either afferent or efferent (Fig. 1, chevrons). Single cell labeling methods will be necessary to definitively

determine the projection patterns and connectivity of each neuron. In contrast to Oamb and Octb2R, we do not detect expression of Octa2R, Octβ1R, Octβ3R or Oct-TyrR in any of the epithelial cells that line the lumen of the oviducts (Fig. 1 and data not shown).

**Cholinergic ppk1.0(+) neurons express multiple subtypes of octopamine receptors**

Sex peptide (SP) released by males during copulation regulates the post-mating response of females via disinhibition of oviposition (P. S. Chen et al. 1988; Aigaki et al. 1991; Soller, Bownes, and Kubli 1997; Chapman et al. 2003; Häsemeyer et al. 2009; Yang et al. 2009; Avila et al. 2010). The set of neurons that expresses the SP receptor (SPR) is also known to include peripheral neurons that express a form of acid-sensing sodium channel ppk, ppk1. A “ppk1.0” driver has been made to express specifically in these reproductive-tract ppk1(+) neurons. (Gou et al. 2014; Häsemeyer et al. 2009; Greenspan 1980; Yoshinari et al. 2020). We previously demonstrated co-expression of *ppk1.0-LexA* with the *MiMIC-T2A-Gal4* drivers representing Octβ2R and Oamb (Deshpande et al. 2022). Here we again used the *ppk1.0-LexA* driver to determine if other Oa receptors are expressed in ppk1.0(+) cells within the reproductive tract, focusing on a specific cluster of three stereotypically localized ppk1.0(+) neurons in the anterior uterus. Since some ppk1 cells are known to be a subset of the cholinergic SPR(+) neurons, we also co-labeled ppk1.0-expressing cells with ChAT-Gal4 (Fig. 3, A) (Yoshinari et al. 2020; Grueber et al. 2007; 2003).

We find that Octα2R, Octβ1R, and Octβ3R are expressed in overlapping sets of neurons that co-express both ppk1.0-LexA and ChaT-Gal4 (Fig. 2). These include at

least three neurons embedded within the muscle cells that envelop the anterior uterus (Fig. 1, Bii, Biii, Biv, chevrons). Of these, Octβ3R is expressed in all three cells (Fig. 2, D) while Octβ1R and Octα2R are expressed in two each (Fig. 2, B, C). We also observe ChAT expression in several adjacent cells that do not express ppk1.0-LexA(+) (Fig. 2, A, open white arrows). It is possible that these cells express ppk1 (and SPR) but are not included in the more restricted subset of cells labeled by the ppk1.0 driver. At least one Octα2R(+) cell is ppk1.0(-) but ChAT(+). At least two Octα2R(+) cells and three Octβ3R(+) cells (Fig. 2, Bi, Di) are ppk1.0(-) but ChAT(+). We did not detect any neural cell bodies in the uterus that labeled with one of the Oa receptor drivers but did not express ChAT (data not shown). In sum, we observe a complex pattern of Oa receptor expression in the peripheral neurons within the reproductive tract that have been previously associated with the post-mating response. By extension, activation of these cells by Oa would have the potential to retard egg-laying in unmated flies.

### *Oamb* is expressed in non-neural secretory gland and uterine cells

In a previous report, we focused on Oamb expression in relatively anterior regions within the reproductive tract and the role of Oamb in lateral oviduct contractility (Deshpande et al. 2022). Oamb also regulates the function of more posterior regions of the reproductive tract, including the function of the parovarian glands and the spermathecae, one of two sperm storage organs in the fly (Avila et al. 2012). The second sperm storage organ is the seminal receptacle, a compact, muscular tube attached to the uterus, also regulated by Oa (Avila et al. 2012). The parovarian glands are bilaterally symmetric structures posterior to spermatheca thought to also play a role

in hormone secretion, however, their function remains poorly understood (Ito and Tomioka 2016; Wilson et al. 2017; Peng et al. 2005; Avila et al. 2012; Findlay et al. 2014; Ram and Wolfner 2009; Claudia Fricke et al. 2013; C. Fricke et al. 2009). Using the *Oamb* MiMIC line, we show for the first time robust expression of *Oamb* in the parovarian glands (Fig. 3, C) and several sites within the sperm storage organs including the spermathecal secretory cells (Fig. 3, B), cells in the seminal receptacle that appear to represent an epithelial layer luminal to the muscle (Fig. 3, A), and neuronal processes that innervate each organ (Fig. 3, A, B, C, stars). We also detect a cluster of small cells expressing *Oamb* embedded between the muscle cells of the posterior uterus (Fig. 3, D). The cells in the posterior uterus extend processes from their somata and thus appear morphologically neuronal; however, a subset of at least three such Oamb(+) cells is not detectably labeled with the commonly used neuronal marker anti-HRP/nirvana, and could potentially represent another cell type (Fig. 3, E, F, G).

**Optogenetic stimulation of OaRNs drives lateral oviduct muscle contractions**

We have previously shown that optogenetic stimulation of neurons that express Octb2R induces lateral oviduct contractions (Deshpande et al. 2022). To determine whether the other OaRNs might also promote oviduct contractility, we expressed the channelrhodopsin variant *ChR2-XXM* using each of the Oa receptor MiMIC Gal4 lines. We employed a previously described assay in which optogenetic stimulation of octopaminergic neurons was paired with quantitation of lateral oviduct contractions (Fig. 4) (Deshpande et al. 2022). We used a preparation in which the AbG had been removed and the reproductive tract remained in situ within the abdomen (Fig. 4, A)

(Deshpande et al. 2022). In addition to Octb2R(+) neurons, we find that optogenetic activation of Octβ1R(+) and Oct-TyrR(+) neurons can reliably induce lateral oviduct contractions. Stimulation of only two of five preparations using the Octb3R MiMIC driver was followed by contractions (Fig. 4, B). In addition to lateral oviduct contractions, stimulation of *Octb3R* expressing neurons was followed by contraction of the common oviduct (Supplementary Video 1). Importantly, although the oviduct epithelium expresses both Octb2R and Oamb, we have previously shown that optogenetic stimulation of epithelial cells has no detectable effects on oviduct muscle contractions (Deshpande et al. 2022). In addition, we do not detect expression of any Oa receptors in the muscles of the reproductive tract (Fig. 1 and (Deshpande et al. 2022)). We therefore conclude that cells expressing Octβ1R, Octb3R and Oct-TyrR that respond to optogenetic stimulation are likely to be neurons, although it is not possible to rule out another, novel type of electrically excitable cell.

**Bath applied octopamine elicits Oamb-dependent global calcium changes in sperm storage organs**

In addition to optogenetic stimulation of *Tdc2*(+) and OaRNs, bath applied Oa induces muscle contractions and calcium transients within the muscle cells of both the ovaries and the lateral oviducts (Middleton et al. 2006; Deshpande et al. 2022). It has been hypothesized that epithelial expression of *Oamb* or *Octβ2R* in the oviduct may facilitate Oa-dependent regulation of muscle behavior (Lim et al. 2014). Muscle cells that can be labeled with the *24B-Gal4* driver (Martínez-Azorín et al. 2013) also surround the lumen of the seminal receptacle, where we find expression of Oamb in an epithelial-

like layer (Figure 3A). To determine if OA effects the muscle of the seminal receptacle, we used *24B- Gal4* to express *UAS-RCaMP1b* and quantitated changes in fluorescence (DF/F) at this site as a measure of cytosolic calcium at baseline and in response to bath applied Oa. We observed frequent spontaneous calcium transients at baseline in the muscle cells of the seminal receptacle. We also observed an increase in global calcium levels in the seminal receptacle following application of Oa (Fig. 5, A, B). Interestingly, calcium transients in the seminal receptacle appeared as waves, both at baseline and in the presence of Oa (Supplementary Video 2). The rate of these calcium transients was unaffected by Oa bath application (data not shown). In similar experiments, we were unable to detect any changes in fluorescence or contractions within the muscle layers of the uterus (data not shown), but we cannot rule out a subtle effect on muscle activity at this site.

**Oamb elicits calcium transients in secretory cells within the spermatheca**

The secretory cells in the spermathecae gland have been suggested to be regulated by Oamb (Avila et al. 2012) and, as shown above (Fig. 3, B), we find that *Oamb* is robustly expressed in the spermathecae in both neural and non-neural cell types. We did not detect the expression of any Oa receptors other than Oamb in the non-neural cells within these organs (Fig. 1, B, and data not shown). The *40B09-Gal4* line is expressed specifically in the secretory cells of the spermathecae as demonstrated by baseline RCaMP signal (Fig. 5, D). We sought to determine the acute effects of Oa on these secretory cells, which have been previously shown to be involved in spermathecal sperm storage (Schnakenberg, Matias, and Siegal 2011; A. K. Allen

and Spradling 2008; Filosi and Perotti 1975). We expressed *UAS-RCaMP1b* using the driver *40B09-Gal4* and bath applied Oa (or vehicle) to “isolated” preparations of the reproductive tract that had been dissected out of the abdomen. We observe a dose-dependent increase in cytosolic calcium within *40B09-Gal4* expressing cells in response to Oa at concentrations as low as 100nM (Fig. 5, C, D).

To directly test the contribution of the Oamb receptor to calcium transients in spermathecal cells, we used the driver *40B09-Gal4* to co-express *UAS-RCaMP1b* with a previously tested RNAi transgene directed against *Oamb* mRNA (Perkins et al. 2015). A large number of changes also occur in both the brain and female reproductive tract in response to SP contained within seminal fluid held by the spermatheca (Ito and Tomioka 2016; Wilson et al. 2017; Peng et al. 2005; Avila et al. 2010; Findlay et al. 2014; Ram and Wolfner 2009; Claudia Fricke et al. 2013; C. Fricke et al. 2009; Chapman et al. 2003; Rezával et al. 2012; Yang et al. 2009). Therefore, we also tested whether mating would have any effect the response to Oa. Knockdown of *Oamb* significantly blunted the calcium response to Oa in the spermathecae of mated flies but not those of virgin flies (Fig. 5, E, F). Intriguingly, Expression of *Oamb-RNAi* via *40B09-Gal4* did not affect baseline spermathecal calcium levels of mated flies but significantly reduced that of virgin flies (Fig. 5, G), although it is difficult to accurately compare absolute fluorescence levels across different preparations. These data indicate that Oamb may regulate the baseline calcium level of spermatheca secretory cells in virgins and the response of these cells to Oa in mated flies. Based on the results obtained from secretory cell knockdown of *Oamb*, these effects appear to occur via cell autonomous mechanisms perhaps distinct from the nonautonomous, indirect mechanisms by which

by which we have previously proposed Oa regulates reproductive muscles (Deshpande et al. 2022).

**Hyperactivating Oa receptor expressing neurons induces defects in egg laying behavior**

We next determined how neuons expressing each Oa receptor might affect reproductive processes required for female fertility. We expressed the temperature-sensitive cation channel, *UAS-dTrpA1*, with our panel of Oa receptor MiMIC Gal4 lines and first tested egg laying behavior at 29ºC, a temperature at which dTrpA1 activates neurons (Rosenzweig et al. 2005; Hamada et al. 2008). We hypothesized that activation of some if not all of the OaRNs might promote oviposition, similar to previously shown roles for Oa signaling in promoting ovulation (Deady and Sun 2015; White, Chen, and Wolfner 2021; Pang et al. 2022; Cole et al. 2005; Yoshinari et al. 2020). However, we found that activation of OaRNs in female flies led to a significant decrease in egg laying rate compared to controls (Fig. 6, A). We did not detect a change in egg laying rate in control flies expressing any of the Gal4 drivers +/ - *UAS-dTrpA1* and maintained at the permissive 22ºC (Sup. Fig. 3).

Egg laying consists of ovulation (release of eggs from ovary), egg transport through the oviduct, and oviposition (releasing eggs from the uterus to the external substrate). To determine which steps of the egg-laying process might be affected by activating each cell type via *UAS-dTrpA1*, we assayed the distribution of eggs in the female reproductive tract and calculated the average time of each egg spent in ovulation, the oviduct, or in the uterus.

When Oamb(+) cells were activated, the time required for both ovulation and oviposition was significantly longer than controls (Figure 6B), indicating that these females exhibit a retention of eggs in both the ovaries and uterus. Activating either Octα2R(+)-or Octβ1R(+) cells impairs ovulation but not oviduct passage (Fig. 6, C, E). Conversely, activating Octβ2R(+) cells disrupts oviduct passage but does not detectably alter ovulation (Fig. 6, F). Activation of Octβ3R(+) neurons increases the time required for both ovulation and oviduct transport (Fig. 6, G). For Oct-TyrR(+) cells, we observe a slightly longer average time for the overall egg-laying process with a trend toward increased time spent in the uterus but do not detect a significant change in the time spent in any stage of egg passage (Fig. 6, D). Overall, these findings support the idea that octopaminergic circuits regulate multiple aspects of egg-laying. However, our data now suggest that in addition to Oamb and Octβ2R, Octβ1R, Octβ3R, Octα2R and Oct-TyrR expressing cells may also contribute to the regulation of oviposition. They also suggest that Oa signaling may inhibit as well as promote egg laying via some pathways.

Due to Oamb's expression in multiple non-neural cell types in the reproductive tract (E. M. Knapp, Deady, and Sun 2018; Lim et al. 2014b; H.-G. Lee, Rohila, and Han 2009; H.-G. Lee et al. 2003; Li et al. 2015), we aimed to determine whether expression of *UAS-dTrpA1* in neurons is required for the decrease in egg laying we observed following activation of the all Oamb(+) cells. To test this, we utilized the temperature sensitive *elav-Gal80* transgene to inhibit Gal4-induced expression of the hyperactivating channel in neurons, and assayed egg laying. Our results show that *elav-Gal80* suppression of *Oamb-T2A-Gal4* in *UAS-dTrpA1* hyperactivation experiments produces near-control egg laying rates (Fig. 6, H). (Differences between these elav-Gla80 flies

and control flies may be due to either incomplete inhibition of *dTrpA1* expression in Oamb(+) neurons or a small contribution of hyperactivity in elav (-), Oamb(+) cells to a decrease in egg laying.) We also confirm that expression of *dTrpA1* by the oviduct epithelium driver OAMB-RS-Gal4 has no effect on egg laying (Fig. 6, I), consistent with the idea that, unlike neurons, non-excitable cells are not responsive to depolarization.

**Ovarian egg retention following hyperactivation of octopamine receptor expressing cells**

An increase in the time required for ovulation (time within the ovary) might be caused by defects either in follicle cell rupture and/or oocyte development. To differentiate between these possibilities we examined the ovaries of the females post egg laying experiments. We observed a significant retention of stage 14 mature follicles (Fig. 7, A) following activation of cells expressing Oamb, Octa2R, Octb1R, Octb2R and Octb3R, but not Oct-TyrR. Thus, although Oamb activation in follicle cells promotes ovulation, activation of other octopamine receptor-expressing cells has the opposite effect and appears to impede ovulation. Since *dTrpA1* acts to depolarize cells, the primary candidates for these effects are most likely neurons in the post-mating circuit that provides octopaminergic signals to the ovary. These include processes shown to directly innervate the ovary (Fig. 1) as well as upstream octopaminergic signaling event in the CNS that have the potential to indirectly regulate ovulation. Previous studies using *dTrpA1* suggest that both the direct and indirect effects we observe are due to changes in neuronal signaling; however, we cannot rule out the possibility that other electrically active cells could play a role.

In sum, our data suggest that some processes regulated by octopamine receptor expressing cells may retard rather than induce ovulation. These observations stand in contrast to previous studies in which Oamb activation promoted ovulation via follicle cell rupture and increased the number of germ cells (Deady and Sun 2015; Yoshinari et al. 2020).

**Activation of Octα2R or Oct-TyrR expressing cells induces follicular atresia**

In contrast to the other groups tested, we found that activation of Oct-TyrR expressing cells did not lead to a severe retention in mature follicles (Fig. 7, A). We also observed that activation of Octα2R expressing cells elicited mature follicle retention in only about half of the females. Considering both genotypes displayed a decrease in egg laying, we decided to further examine the ovaries to probe for any defects in oogenesis. DAPI staining revealed signs of cell death during mid-oogenesis with both genotypes displaying nurse cell nuclei fragmentation in stages ~6-9 (Fig. 7, B). Germline cell death in mid-oogenesis can be a result of developmental abnormalities, environmental stress, or drug treatment (Jenkins, Timmons, and McCall 2013). Nutritional deprivation and more specifically protein starvation is the most prominent cause of mid-oogenesis induced cell death (Barth et al. 2011). This starvation induced response slows oogenesis to save costly nutritional resources and is known to be partially regulated by insulin and ecdysone signaling pathway (Terashima et al. 2005; Burn et al. 2015; Pritchett and McCall 2012). Together with these studies, our results suggest that some egg laying effects caused by hyperactivating Oct-TyrR or Octα2R expressing cells may be due to CNS changes in nutrition or hormonal regulation.

**Hyperactivating glutamatergic/cholinergic but not octopaminergic neurons inhibits egg laying**

To explore the mechanism by which Oa receptor expressing cells may regulate egg development and oviposition, we used additional drivers to express *UAS-dTrpA1* in subtypes of neurons that express Oa receptors. As shown in Fig. 3 and (Yoshinari et al. 2020), ppk(+) cells and ChAT(+) cells in the reproductive tract express multiple subtypes of Oa receptors. We have shown that a subset of glutamatergic neurons that localize to the AbG and project to the reproductive tract also express Oa receptors (Deshpande et al. 2022). To test the possibility that Oa receptors could mediate a reduction in egg-laying via activation of glutamatergic or cholinergic pathways, we expressed *UAS-dTrpA1* in ppk1.0(+), VGluT(+) or ChAT(+) cells. Following activation of cholinergic neurons or ppk1.0(+) neurons, we observe a decrease in egg laying rate (Fig 8, yellow, pink) and an increase in ovulation & oviduct passage time (Fig. 8, D) similar to the activation of cells expressing the receptors Octa2R and Octb1R. Activation of VGluT(+) glutamatergic neurons resulted in a significant decrease in egg laying rate and an increase in oocyte retention time in the ovary and oviducts similar to the effects of cells expressing the receptors Octb2R and Octb3R (Fig 8A, green, C). By contrast, we failed to detect any decrease or increase in egg-laying when we expressed *UAS-dTrpA1* in octopaminergic neurons using the driver *Tdc2-Gal4* (Cole et al. 2005). Similarly, others have shown that *UAS-dTrpA1* expressed in Tdc2 and Tbh neurons does not elevate egg-laying beyond WT levels, although it rescues the reduction in oviposition caused by exposure to parasitoid wasps, (Pang et al. 2022) .

**Silencing OaRNs promotes egg laying behavior in both mated and virgin flies**

If one of the functions of OaRNs is to inhibit oviposition, then blocking the function of these cells could potentially promote oviposition. To test this hypothesis, we used the Oa receptor MiMIC lines to express the inward rectifying Kir2.1 channel (*UAS-Kir2.1*) and thereby dampen neuronal excitability (Johns et al. 1999). We utilized *tubGal80$^{ts}$* to specifically express *Kir2.1* during egg-laying experiments and avoid effects of silencing OaRNs during development. When flies are shifted to 29ºC to facilitate *Kir2.1* expression, we observed an increase in egg-laying for flies expressing Kir2.1 in Octα2R, Octβ2R(+), or Octβ3R(+) cells (Fig. 9, A, orange, green, blue) but not Oamb(+) or Octβ1R(+) cells (Fig. 10, A, red, teal). To further test whether inhibiting Oa receptor-expressing cells could promote oviposition, we utilized the transgene *UAS-shibire$^{ts}$* (*UAS-Shi$^{ts}$*), a temperature sensitive mutant form of *dynamin,* that inhibits neurotransmission via blockade of the exocytotic cycle (Brand and Perrimon 1993; Kitamoto 2001). We again observed a slight increase in egg laying rate when cells expressing Octα2R or Octβ2R were silenced (Fig. 9, B, orange, green respectively), but did not detect any increase following silencing of cells expressing Octβ1R or Octβ3R (Fig 9, B, teal, blue respectively). When *Oamb-T2A-Gal4/UAS-shibire$^{ts}$* flies were shifted to the restrictive temperature, most females died during the first 24 hrs (data not shown) precluding further analysis. We did not test Oct-TyrR in these experiments because hyperactivating Oct-TyrR expressing cells had little detectable effect on egg laying and Oct-TyrR does not appear to be expressed express in SPR(+), ppk1.0(+) neurons in the uterus (Fig. 6 and data not shown).

One of the most prominent behavioral changes that occurs after mating is an increase in egg laying (Yang et al. 2009; Rezával et al. 2012; 2014; Yoshinari et al. 2020; Chapman et al. 2003; Ram and Wolfner 2009; Avila et al. 2012; White, Chen, and Wolfner 2021). If one of the functions of OaRNs is to inhibit oviposition, this activity might be expected to be particularly important in virgin females. To test this hypothesis, we repeated our experiment expressing *UAS-Shi$^{ts}$* in OaRNs using virgin rather than mated females. We observe a dramatic increase in egg-laying in all the cell types we tested including those expressing Octa2R, Octb1R, Octb2R, and Octb3R (Fig 9, C). We recognize that daily egg deposition counts can become increasingly variable as egg laying increases (Sup Fig. 3, A). This caveat aside, these data further suggest that in addition to activating processes in the reproductive tract that promote oviposition such as follicle cell rupture, Oa may play an important role in inhibiting oviposition, especially under conditions in which egg-laying would not be productive such as in virgin flies.

## DISCUSSION

It has been known for decades that Oa regulates female fertility and the oviposition circuit in *Drosophila* and other insects (Lange 2009; Tamashiro and Yoshino 2014; White, Chen, and Wolfner 2021; Monastirioti, Charles E. Linn, and White 1996; Zheng et al. 2021). In *Drosophila*, some loss of function mutants that disrupt Oa synthesis and/or release share a common phenotype marked by retention of mature oocytes in the ovaries (Deady and Sun 2015; Cole et al. 2005; Monastirioti, Charles E. Linn, and White 1996; Monastirioti 2003; Andreatta et al. 2018). Similarly, studies utilizing mutations in *Oamb* and *Octb2R* have demonstrated a decrease in ovulation and

retention of mature follicles within the ovaries (H.-G. Lee et al. 2003; H.-G. Lee, Rohila, and Han 2009; Lim et al. 2014b; Li et al. 2015). Oamb also regulates sperm storage, secretory cell activity, oviduct dilation (Avila et al. 2012; Middleton et al. 2006; D. S. Chen, Clark, and Wolfner 2022) and germline stem cell proliferation (Yoshinari et al. 2020; Hoshino and Niwa 2021), while Octb2R is required for lateral oviduct contractions (Deshpande et al. 2022). *Drosophila* express four other Oa receptors in addition to Oamb and Octb2R (Balfanz et al. 2005; Maqueira, Chatwin, and Evans 2005; Qi et al. 2017; McKinney et al. 2020) but their expression patterns and function in the reproductive system have remained unknown.

To gain further insight into the mechanisms by which Oa may regulate oviposition, we have used a panel of high-fidelity Gal4 “MiMIC” lines to map expression in the reproductive tract of all the known *Drosophila* Oa receptors. We have previously shown that *Oamb* and *Octb2R* are expressed in peripheral neurons proximal to the oviducts and uterus as well as central neurons that project from the AbG to the reproductive tract (Deshpande et al. 2022). Here we show that multiple peripheral neurons that localize to the reproductive tract also express *Octa2R*, *Octβ1R, Octβ3R* and *Oct-TyrR*. These include cell bodies proximal to the oviducts in the MAN and embedded in the musculature of the uterus. Most of these co-express *ChaT* and label with the neuronal marker HRP. Exceptions include a small subset of cells in the posterior uterus that do not appear to label with anti-HRP.

Using a restricted *ppk1.0-LexA* driver known to express in a subset of afferent SPR(+) neurons, we confirm that one example anterior uterine cluster of at least three peripheral neurons in the post-mating circuit also co-expresses the cholinergic marker

*ChaT* (Yoshinari et al. 2020). We further show that these post-mating circuit neurons all express octopamine receptors. Two neurons express *Octα2R*, *Octβ1R*, and *Octβ3R*, while one neuron expresses only Octβ3R. Other neurons that appear to localize to the same cluster but are ppk1.0(-) express *ChaT* and *Octα2R* but no other Oa receptor. Such partially overlapping co-expression suggests that the post-mating circuit might be divided into functional units that differ by their expression of different Oa receptor subtypes.

Other cell types proposed to express OA receptors include follicle cells that surround the developing oocyte and epithelial cells that line the lumen of the oviducts (Deady and Sun 2015; Sun and Spradling 2013; Lim et al. 2014; Lee, Rohila, and Han 2009; Lee et al. 2003; Li et al. 2015; White, Chen, and Wolfner 2021). We also show that cells which line the lumen of the seminal receptacle express *Oamb*, similar to its expression in the epithelium of the oviducts (Deshpande et al. 2022; Lim et al. 2014; Li et al. 2015; Lee, Rohila, and Han 2009; Lee et al. 2003) and that $Ca^{2+}$ levels in the muscle of the seminal receptacle are sensitive to Oa. These results suggest that the epithelial cells of the seminal vesicle may control the surrounding muscle similar to the mechanism previously proposed for the oviducts (H.-G. Lee et al. 2003; H.-G. Lee, Rohila, and Han 2009; Lim et al. 2014b). We are intrigued by the appearance of wave-like patterns in the seminal receptacle $Ca^{2+}$ activity and speculate that this may play a role in sperm movement within the lumen of the organ, similar to function of muscle contractions in the movement of eggs within the oviducts.

**Acute effects in the oviducts and sperm storage organs**

Previous results indicate that loss of *Octb2R* blocks contraction of the lateral oviducts and optogenetic activation of *Octb2R* expressing neurons can induce lateral oviduct contractions (Deshpande et al. 2022). We find that optogenetic activation of *Octb1R* and *Oct-TyrR* expressing neurons can also induce lateral oviduct contractions. Since mutation of *Octb2R* essentially blocks contractions caused by bath applied Oa, the possibility that Octb1R, Octb2R, and Oct-TyrR represent three equally important, parallel pathways within the reproductive tract that mediate oviduct contraction seems unlikely. Rather, we speculate that Octb1R and Oct-TyrR are more likely to be active in neurons within the CNS and upstream of Octb2R. Alternatively, it remains possible that some of the cells that express *Octb2R* also express *Octb1R* and *Oct-TyrR*, but that only the function of Octb2R is required for contractions. Further co-labeling studies and the development of mutations in *Octb1R* and *Oct-TyrR* will help to distinguish between these possibilities.

While previous studies have demonstrated a requirement for Oa in the regulation of sperm storage, the more acute effects of octopaminergic signaling in sperm storage organs have been less clear. We show that Oa induces calcium transients in secretory cells of the spermathecae and that this effect is blocked by knockdown of *Oamb* within these cells. These data are consistent with a previously assigned role for Oamb in sperm storage (D. S. Chen, Clark, and Wolfner 2022; Avila et al. 2012). The relatively high sensitivity of the spermatheca cells to Oa may reflect differences in the relative affinity of Octb2R versus Oamb, or perhaps differential access of the receptors to bath applied Oa. Concentration-dependent effects have also been observed in the

reproductive tracts of other insect species exposed to Oa (Abdoun et al. 1995; Wong and Lange 2014; Lange 2009; Xu et al. 2017).

**Systemic effects in oviposition uncovered using gain of function transgenes**

Our functional data using dTrpA1 indicate that activating OaRNs can impede ovulation and egg laying. We confirmed these effects using Kir2.1 and $Shi^{ts}$ to inhibit cells that express Oa receptors and observe an increase in egg-laying. We were initially surprised by these data since previous studies have focused on octopaminergic processes that appear to facilitate ovulation and oviposition (Pang et al. 2022; D. S. Chen, Clark, and Wolfner 2022; White, Chen, and Wolfner 2021; Lim et al. 2014b; Li et al. 2015; H.-G. Lee et al. 2003; Middleton et al. 2006; Monastirioti 2003; Monastirioti, Charles E. Linn, and White 1996; Deady and Sun 2015). We suggest that the use of gain of function transgenes to activate neurons can uncover effects that are less obvious using loss of function receptor mutants and RNAi transgenes. We also suggest that the use of both gain of function and loss of function strategies are important for probing oviposition, since the same gene products may be active in multiple, sequential processes and subject to epistatic effects. In particular, the epistatic relationship between follicle rupture and other processes involved in oviposition may require the use of gain of function methods. For Oa signaling mutants in which oocytes never leave the ovary, *downstream* effects in the uterus may be difficult or impossible to detect. Therefore, we speculate that the unusual uterine retention phenotype that we report may be absent in loss of function *Oamb* mutants because the oocytes are trapped at an upstream site in the ovary (Deady and Sun 2015; H.-G. Lee et al. 2003; H.-G. Lee,

Rohila, and Han 2009; Yoshinari et al. 2020). Similarly, oviduct retention might be occluded by upstream retention of mature follicles in the ovaries. If so, retention of eggs in the ovary seen with *Octb2R* knock-down or mutants might have occluded downstream effects in the oviducts (Lim et al. 2014b; Li et al. 2015).

Although the epistatic relationship is less obvious, we suggest that some of the effects we observe may reflect disruption of processes *upstream* of follicle rupture. Instances of follicular atresia observed in Octα2R and Oct-TyrR hyperactivation assays suggest that circuits expressing these receptors can produce defects in follicle development, possibly by disrupting fly nutritional intake or homeostatic systems. Though this phenotype seems to be only partially penetrant, such effects may occlude effects on any downstream egg laying processes. The lack of any obvious impairment to follicular development in hyperactivation assays involving the other Oa receptors, however, suggests that the effects we observe for most Oa receptor expressing cells are likely due to direct disruption of reproductive tract behavior rather than broader, metabolic mechanisms able to affect multiple organ systems.

CNS circuits that regulate oviposition include pCL1 neurons in the brain that innervate oviposition descending neurons (oviDNs) (F. Wang et al. 2020; Feng et al. 2014). It is tempting to speculate that OaRNs might regulate pCL1 or oviDN, or additional excitatory or inhibitory neurons within the same circuit (F. Wang et al. 2020). We are particularly drawn to the observation that hyperactivation of Oamb(+) neurons results in retention of eggs in the uterus just prior to deposition (Fig. 6, B). Following follicle rupture, eggs ovulate and pass through the oviduct in a very short amount of time in WT flies and without significant delay under baseline conditions (Mattei et al.

2015). By contrast, flies can retain fertilized eggs in their uterus until sensory inputs indicate that egg laying can occur in a predator/toxin free environment (Pang et al. 2022). We speculate that Oamb(+) neurons in the CNS may represent a behavioral choice point and regulate the decision to deposit eggs. Further studies of the CNS connectome combined with single cell sequencing, and optogenetics will be needed to test this hypothesis and identify the underlying circuits.

**Possible cell and molecular mechanisms**

It remains unclear why hyperactivation of presynaptic Tdc2 neurons with dTrpA1 does not appear to increase egg-laying. Similarly, *dTrpA1* expression in Tdc2 and Tbh neurons rescued a reduction in oviposition caused by exposure to parasitoid wasps, but did not elevate egg-laying beyond WT levels (Pang et al. 2022). We speculate that, if the effects of Oa are as complex as we suggest and it acts to both promote and retard ovulation and oviposition, simultaneous activation of all octopaminergic pathways might not appear to have any effects under some conditions. It is also possible that, under some circumstances, octopamine and tyramine have opposing effects in oviposition as they do for larval locomotion (Saraswati et al. 2004). Further experiments using intersectional drivers that are specific for subsets of octopaminergic neurons may be needed to detect a net loss or gain in fertility in the absence of exogenous stimuli such as threats from parasitoid wasps (Pang et al. 2022). A previously described intersectional approach using *doublesex* is useful for expression in the multicellular cluster of octopaminergic neurons that innervates the reproductive tract, but cannot be

used to stimulate individual octopaminergic neurons within the cluster (Rezával et al. 2014).

Further experiments will also be needed to determine which post-synaptic neurons that express specific Oa receptors are responsible for the effects we observe. We recognize that the effects of hyperactivating or silencing OaRNs on egg laying are similar to those seen in experiments involving the SPR(+) neurons of the post-mating circuit, where neural activity is correlated with inhibition of egg laying processes (Yoshinari et al. 2020; Yang et al. 2009). Based on our findings of co-expression between Oa receptors, *ChAT*, and *ppk1.0* in peripheral neurons, it is possible that some of our experiments using *Oa-receptor* divers produce expression of dTrpA1 or Kir2.1 in the same cells as in similar experiments using *SPR* drivers that achieve the same effect. We therefore speculate that neurons in the peripheral SPR(+) post mating circuit that express Oa receptors represent a specific group of neurons that may be responsible for some of the egg laying phenotypes we observe. Intriguingly, individual cells in this group express differing profiles of Oa receptor subtypes, and different Oa receptor subtype drivers affect different stages of the egg laying process as seen in egg laying time percent assays. These results may suggest that distinct post-mating circuit neurons inhibit different stages of egg laying until SP silences their activity. Further intersectional studies using SPR and OaRN drivers could prove useful to uncover single-cell heterogeneity in the post-mating circuit.

Importantly, all the transgenes we have used here act by directly activating or inhibiting neuronal activity rather that activating or inactivating Oa receptors. Oa receptors, like most other GPCRs can have net “inhibitory” or “excitatory” effects which

can vary across cell types, downstream effectors and the subcellular location of the receptors (Robb et al. 1994; M. Wang et al. 2007). The coupling of octopamine receptors to excitatory G proteins and downstream effectors has been extensively examined *in vitro* and in the epithelial cells within the reproductive tract (Y.-C. Kim et al. 2013; H.-G. Lee, Rohila, and Han 2009; Debnath, Williams, and Bamber 2022; Xu et al. 2017). We therefore speculate that effects seen in neuron hyperactivation experiments using Oa-receptor drivers may be similar to the effects of increased Oa signaling to such cells. Further experiments will be needed to more precisely determine the *in vivo* effects of Oa receptors in neurons within the oviposition circuit and CNS as well as how each may influence egg-laying.

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**Data Availability Statement**

Fly strains are available upon request and/or from the Bloomington Stock Center as indicated. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

## FIGURES AND LEGENDS

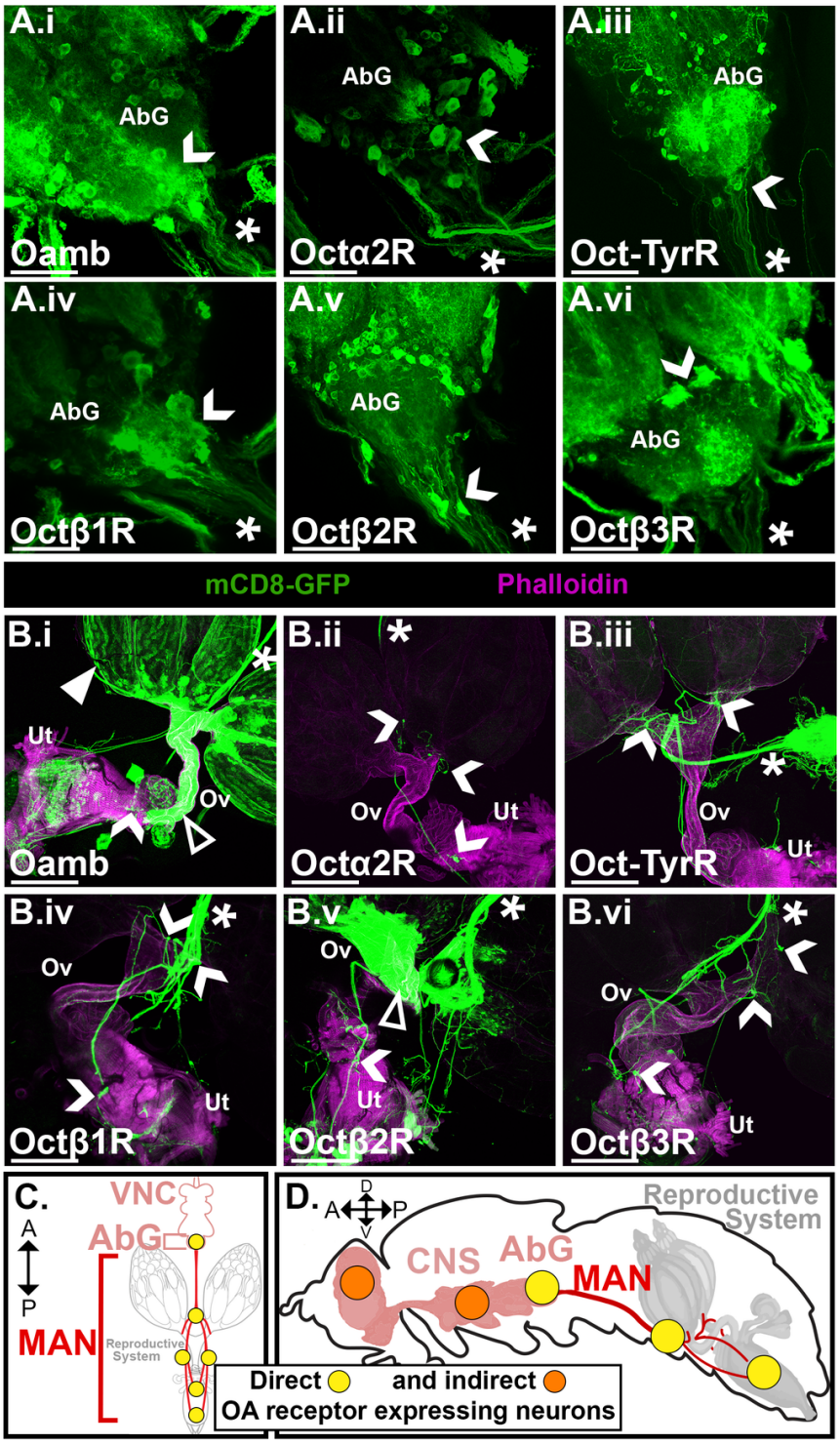



**Figure 1. Octopamine receptor expression in cells associated with the reproductive system.** A panel of octopamine receptor drivers (*MiMIC-T2A-Gal4* lines

(BDSC)) was used to drive *UAS-mCD8-GFP*. For each genotype, the Abdominal ganglion (AbG) (**A.**) and the reproductive system (**B.** Ov=oviduct, Ut=uterus) were dissected then labeled with anti-GFP ALEXAfluor-488 (green) and Phalloidin-555 (magenta) to visualize OA receptor expressing cells and muscles respectively. Labeled nerves in the medial abdominal nerve (MAN, stars) project from neurons in the CNS to reproductive system (**A.** chevrons) and from neurons in reproductive system back to the CNS (**B.** chevrons). OAMB is also expressed in follicle cells (filled arrow). Both OAMB and Octβ2R are expressed in epithelial cells (open arrows). **C.** Ventral-view cartoon depicting the approximate locations of neural clusters expressing octopamine receptor directly associated with the reproductive system. **D.** Sagittal-view cartoon showing the approximate locations of groups of octopamine receptor expressing neurons in the full CNS and reproductive system. Orange circles indicate clusters of cells known to express octopamine receptors that are not directly associated with the reproductive system but could potentially influence oviposition.

Scale bars = 20μm (**A.**) and 200μm (**B.**)

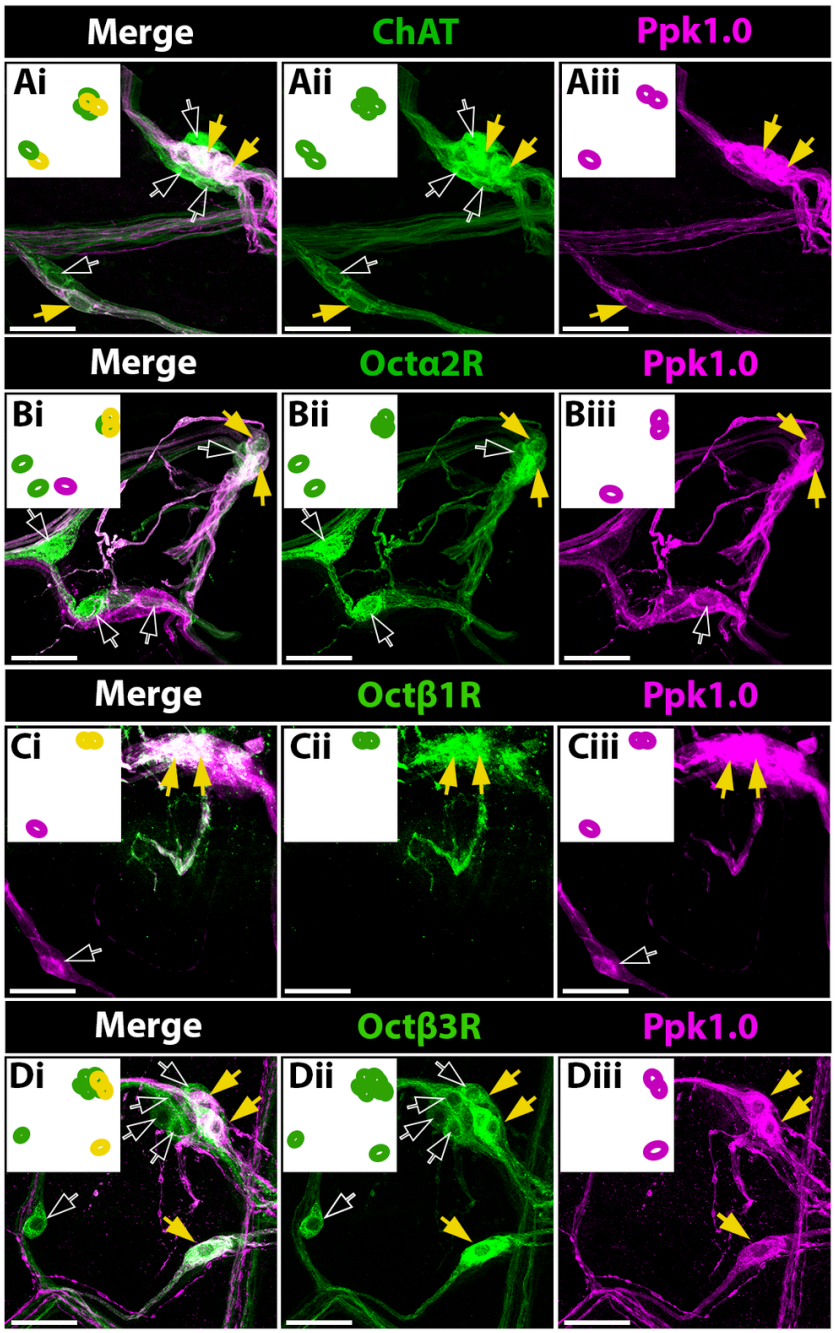



**Figure 2. Peripheral ppk1.0(+) neurons express OA receptors and are cholinergic.** Octopamine receptor MiMIC Gal4's (Receptor MiMIC insertion -t2a-Gal4) or *ChAT-Gal4* were used to drive *UAS-mCD8-GFP* and *Ppk1.0-LexA* was used to drive LexAop-CD2-RFP. Octα2R (**A)**, Octβ1R (**B**), Octβ3R (**C**), and ChAT (**D**) expression was assessed among three stereotyped ppk1.0(+) cell bodies embedded within the anterior uterus.

Images are maximum signal projections through 20μm. Insets show cell body locations expressing each of the compared drivers with yellow ovals in “merge” insets indicating coexpression.

Scale bars = 10μm

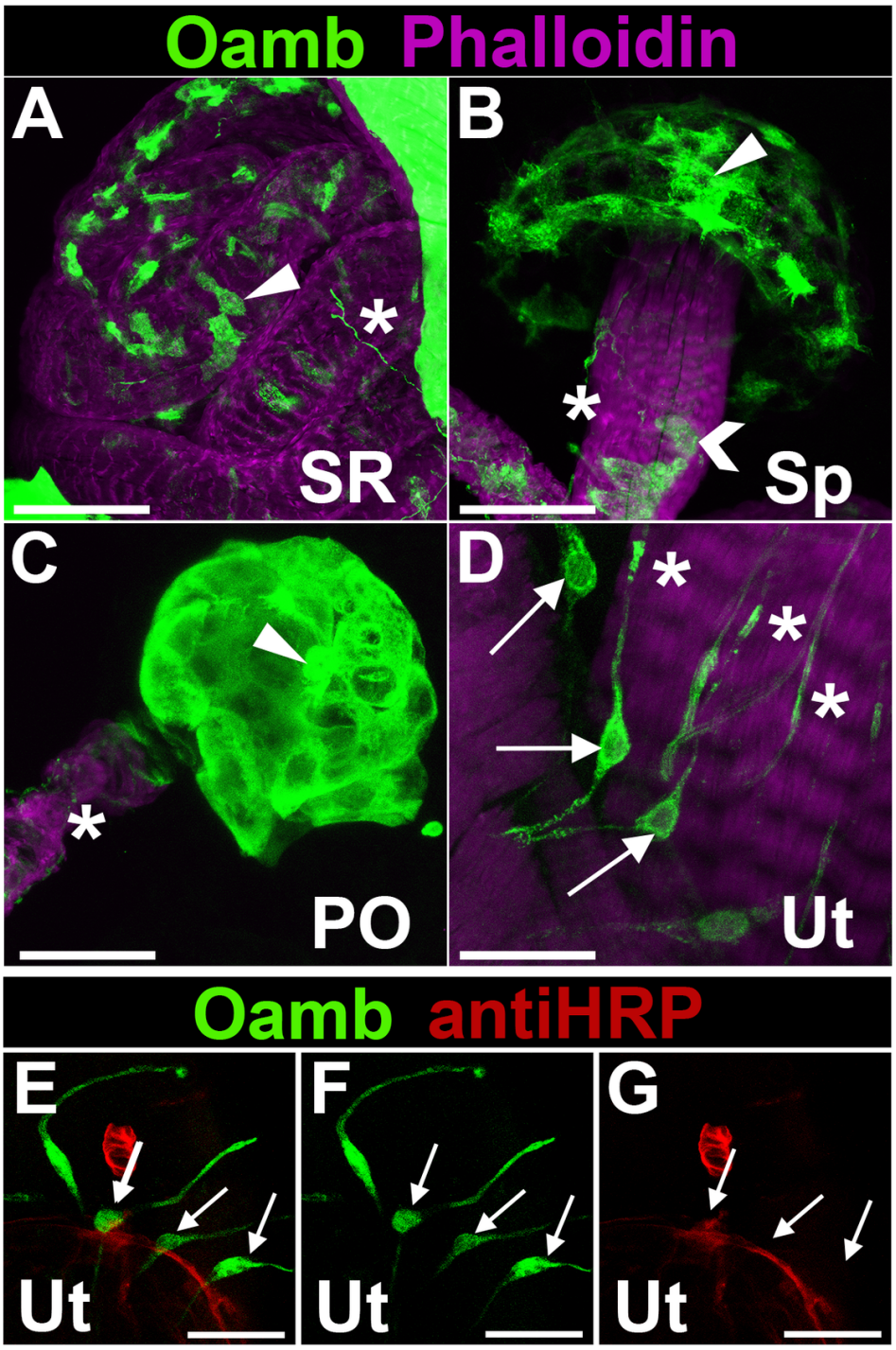


**Figure 3. OAMB is expressed in the seminal receptacle and spermathecae.** OAMB-t2a-Gal4 was used to drive *UAS-mCD8-GFP*. For each genotype, the reproductive system was dissected then labeled with anti-GFP ALEXAfluor-488 (green) and Phalloidin-555 (magenta) to visualize OAMB expressing cells and muscles respectively. We find OAMB expression in non-muscle cells in the seminal receptacle (SR) (**A.**), spermathecae (Sp) (**B.**), paraovia (PO) (**C.**), and posterior uterus (Ut) (**D.**). Despite neuron-like morphology, the posterior uterus cells do not label with anti-HRP (**E.-G.**) Scale bars = 10μm

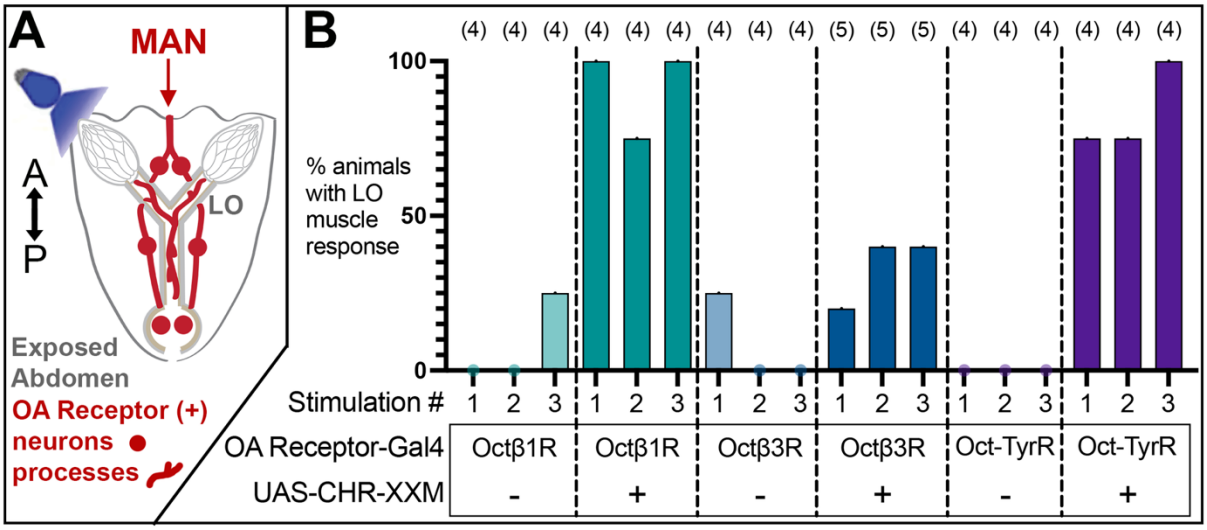



**Figure 4. Optogenetic stimulation of OA receptor expressing neurons drives lateral oviduct (LO) contraction. A.** Fly abdomens were dissected to expose the reproductive organs, and neurons with cell bodies or processes in the periphery were stimulated via channelrhodopsin. **B.** The probability of a LO contraction response to each of 3 successive stimulations per fly was then quantified. Stimulation of *Octβ1R-t2a-Gal4* and *Oct/Tyr-t2a-Gal4* expressing cells reliably induces LO contraction. Stimulation of Octβ3R-t2a-Gal4 expressing cells sometimes produces LO contraction, with a higher probability of response after the first stimulation. Control flies had two

instances of spontaneous contractions but otherwise did not respond to light stimulations.

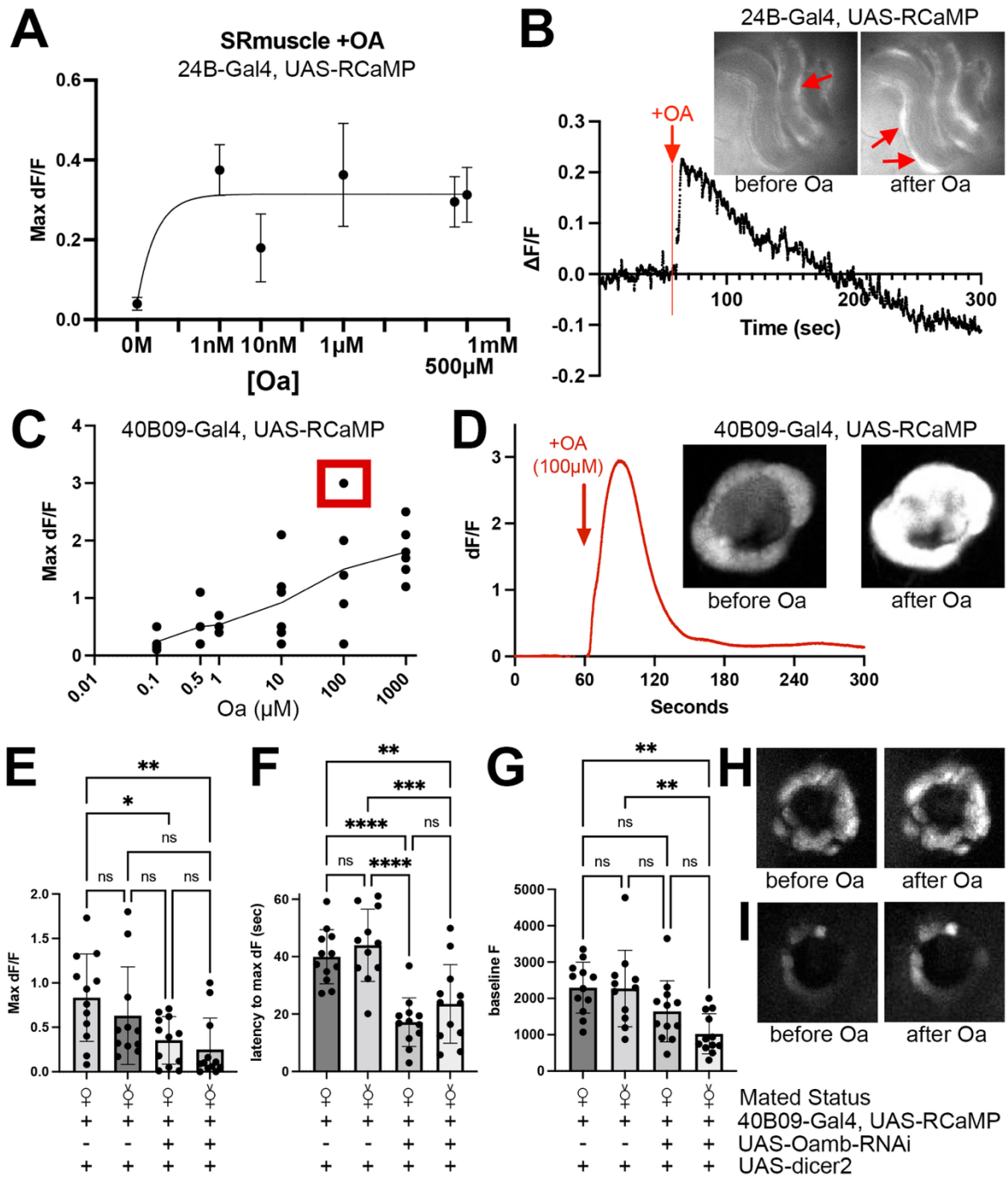



**Figure 5. OAMB mediates sperm storage gland responses to octopamine. A-B.** UAS-RCaMP1b expression was driven by the muscle driver 24B-Gal4, and octopamine

was applied to reproductive systems dissected in HL3.1. **A.** Seminal receptacle muscle cells increase free intracellular calcium in response to octopamine doses ranging from 1nM to 1mM. **B.** Sample trace and images from a recording where 1μM OA was delivered. **C-G.** *UAS-RCaMP1b* expression was also driven by the spermatheca secretory cell driver 40B09-Gal4, and octopamine was again applied to reproductive systems dissected in HL3.1. **C.** The secretory cells of the spermathecae robustly increase free intracellular calcium levels in response to octopamine doses ranging from 100nM to 1mM. **D.** Example trace from **C.** (Red box) including before and after images. **E.** Knocking down OAMB in the spermathecae secretory cells reduces their RCaMP response to OA. **F.** Knocking down OAMB in the spermathecae secretory cells reduces the duration of their response to OA. **G.** Knocking down OAMB in the spermathecae secretory cells affects the baseline Ca2+ signal in virgins. Baseline and post-Oa RCaMP signal from mated (**H.**) and virgin (**I.**) spermathecae experiencing Oamb knockdown revealed disturbed arrangements of cells with fluorescent signal compared to controls.

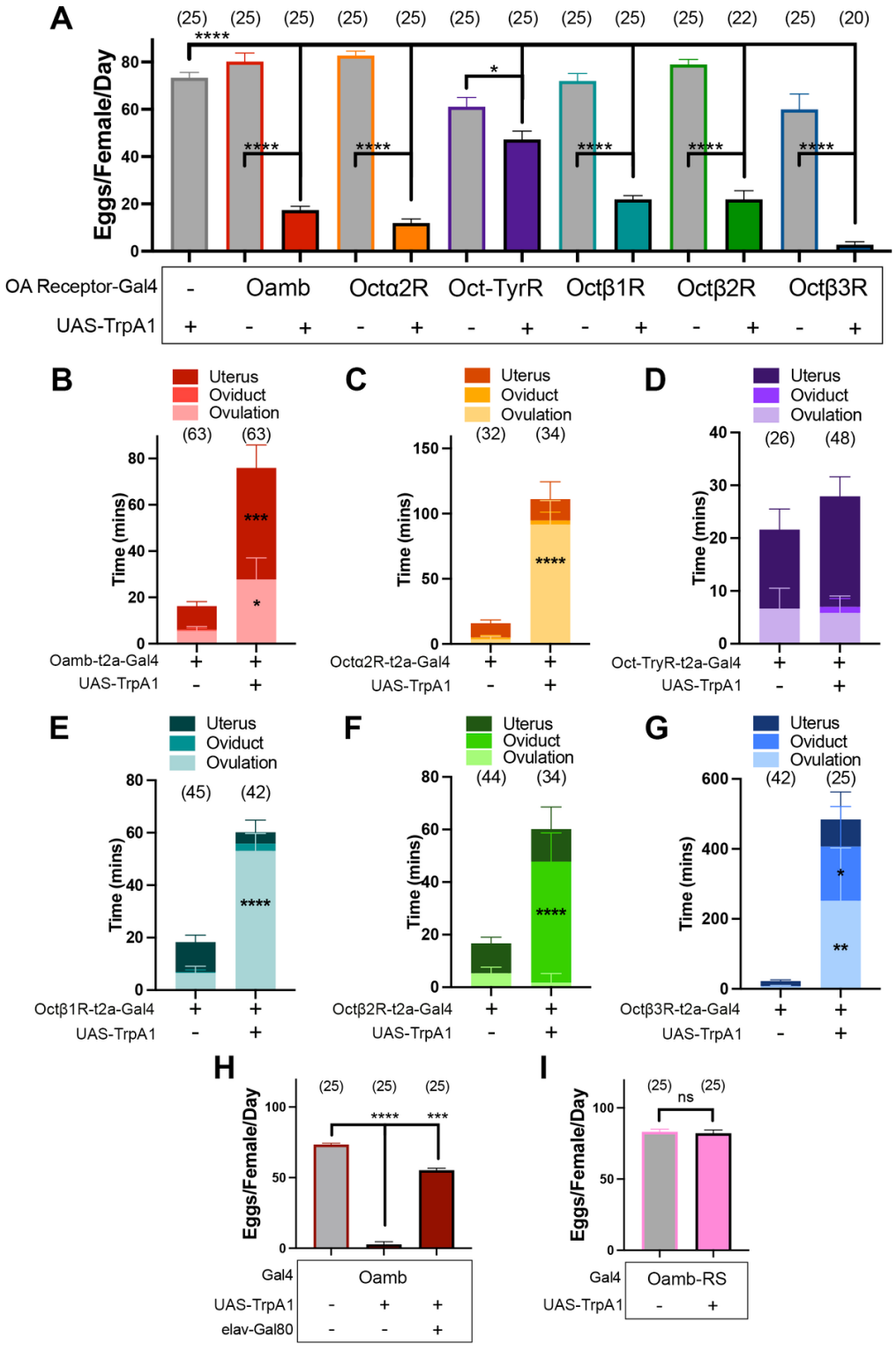



**Figure 6. Activating circuits expressing each different octopamine receptor impairs distinct aspects of egg laying. A.** Expression of *UAS-TRPA1$^{ts}$* via all

octopamine receptor MiMIC Gal4 drivers during a 48 hour egg laying period significantly decreases counts of eggs laid per female per day **B-G.** Activating neurons expressing each receptor had distinct effects on ovulation and/or passage through the reproductive tract, including retardation of both ovulation and passage through the uterus (A, OAMB), ovulation alone (C, Octα2R and E, Octβ1R), oviduct passage alone (F, Octβ2R), and ovulation and oviduct passage (G. Octβ3R). Activation of Oct/Tyr cells (D) did not show statistically significant changes to any particular step of egg laying despite trending toward retardation of ovulation and oviduct passage. **H.** *Elav-Gal80* expression changes the effect of OAMB cell hyperactivation on egg laying. **I.** Hyperactivation of oviduct epithelial cells using *OAMB-RS-Gal4* to drive *UAS-TRPA1$^{ts}$* has no effect on egg laying.

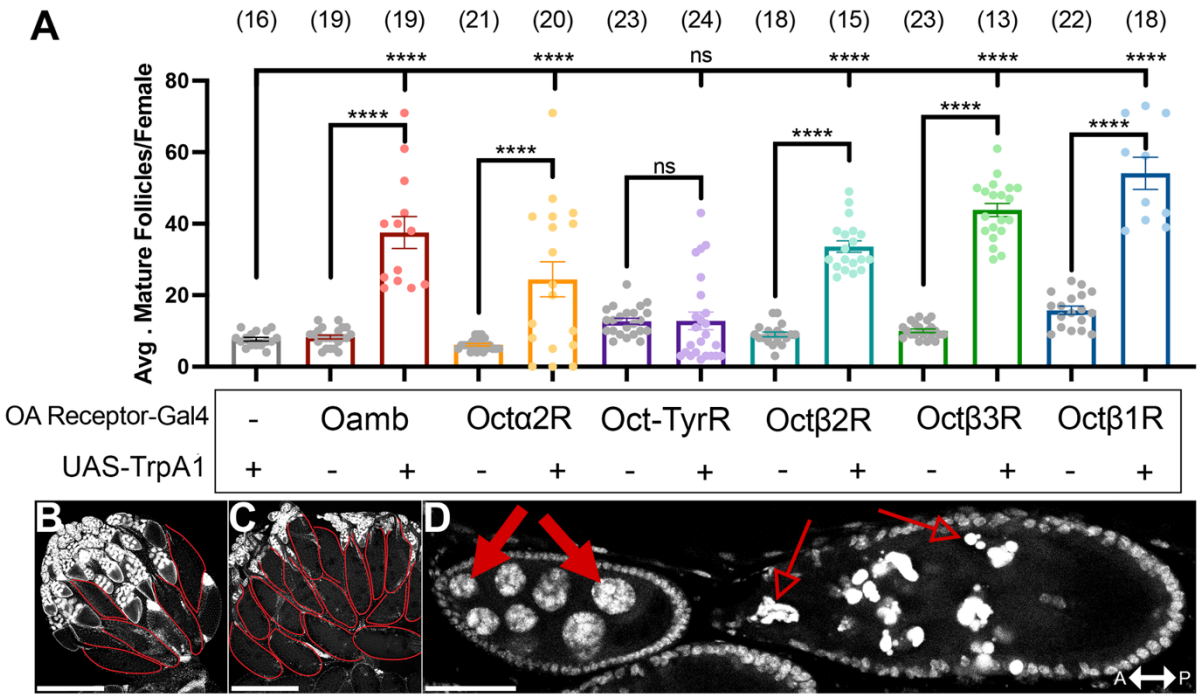



**Figure 7. Mature follicles develop but fail to ovulate when octopamine receptor expressing neurons are hyperactivated. A.** Mature follicle counts per female at time of dissection following a 48hr mating period. **B.** Example ovary from control dataset with

5 mature follicles (red ovals). **C.** Example ovary from TrpA1-expressing dataset with 16 mature follicles (red ovals). **D.** In some Octα2R and Oct-TyrR hyperactivation flies, DAPI stains reveal that mid-stage follicles appear to experience nuclear fragmentation in nurse cells. Solid red arrows indicate normal nuclei in an example ovariole. Empty red arrows indicate fragmented nuclei in the same ovariole.

Scale bar = 200 μm (B,C), 50 μm (D).

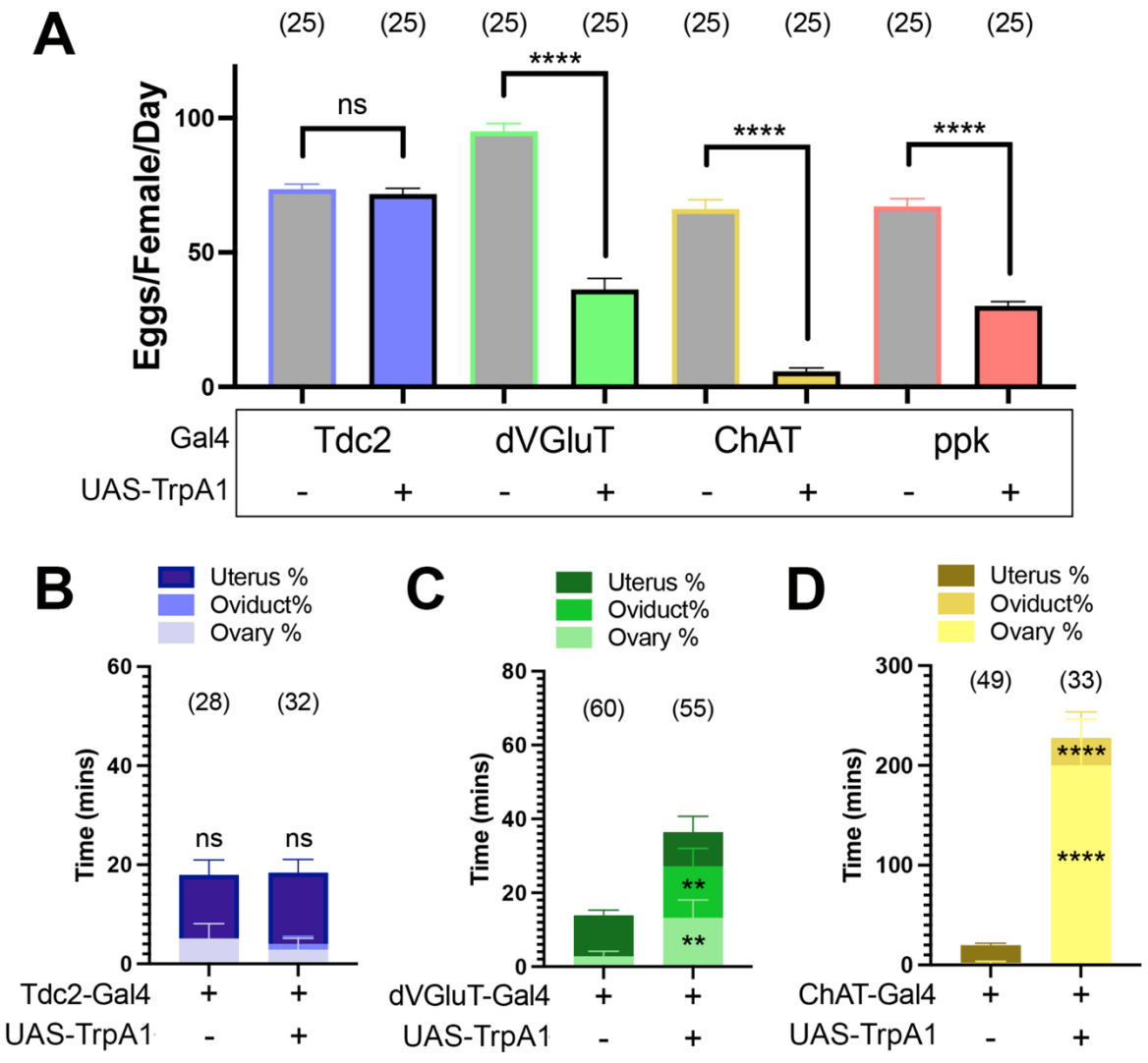



**Figure 8. Hyperactivating other cell populations known to partially overlap with octopamine receptor expression produces varied effects on egg laying. A.**

Averages of eggs laid per day over a 48-hr egg laying assay using *UAS-TRPA1$^{ts}$* to hyperactivate cells expressing each indicated Gal4 suggest that hyperactivating Tdc2(+) cells (blue) or RS-Gal4(+) cells (pink) has no effect on egg laying and while hyperactivating dVGluT(+) (green), ChAT(+) (yellow), or ppk(+) (red) cells produces egg laying deficits. **B.** Tdc2(+) cell hyperactivation does not significantly alter the amount of time it takes eggs to pass any egg laying process. **C.** dVGluT(+) cell hyperactivation induces defects in both ovulation and oviduct passage. **D.** ChAT(+) cell hyperactivation produces more pronounced ovulation and oviduct passage defects.

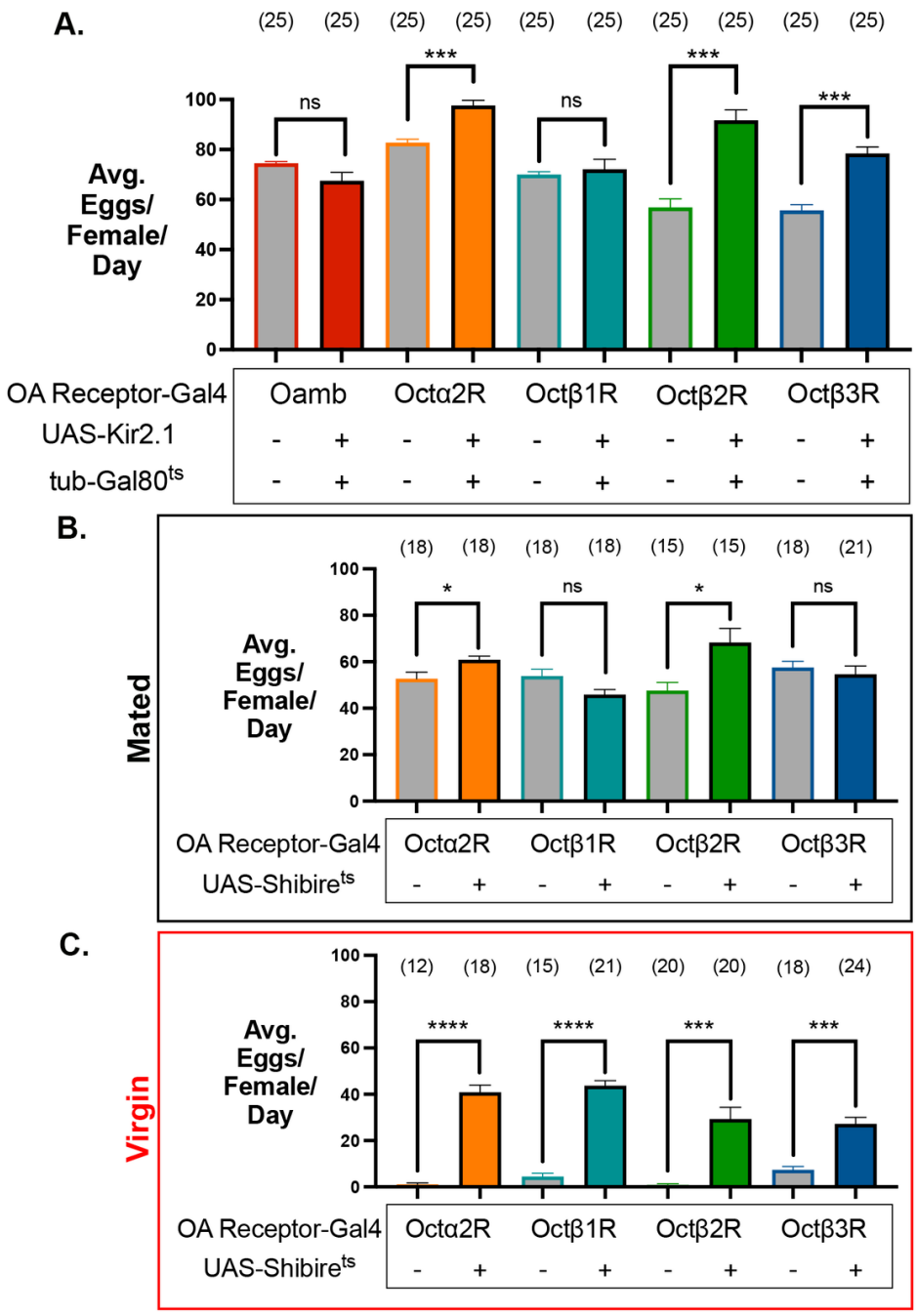
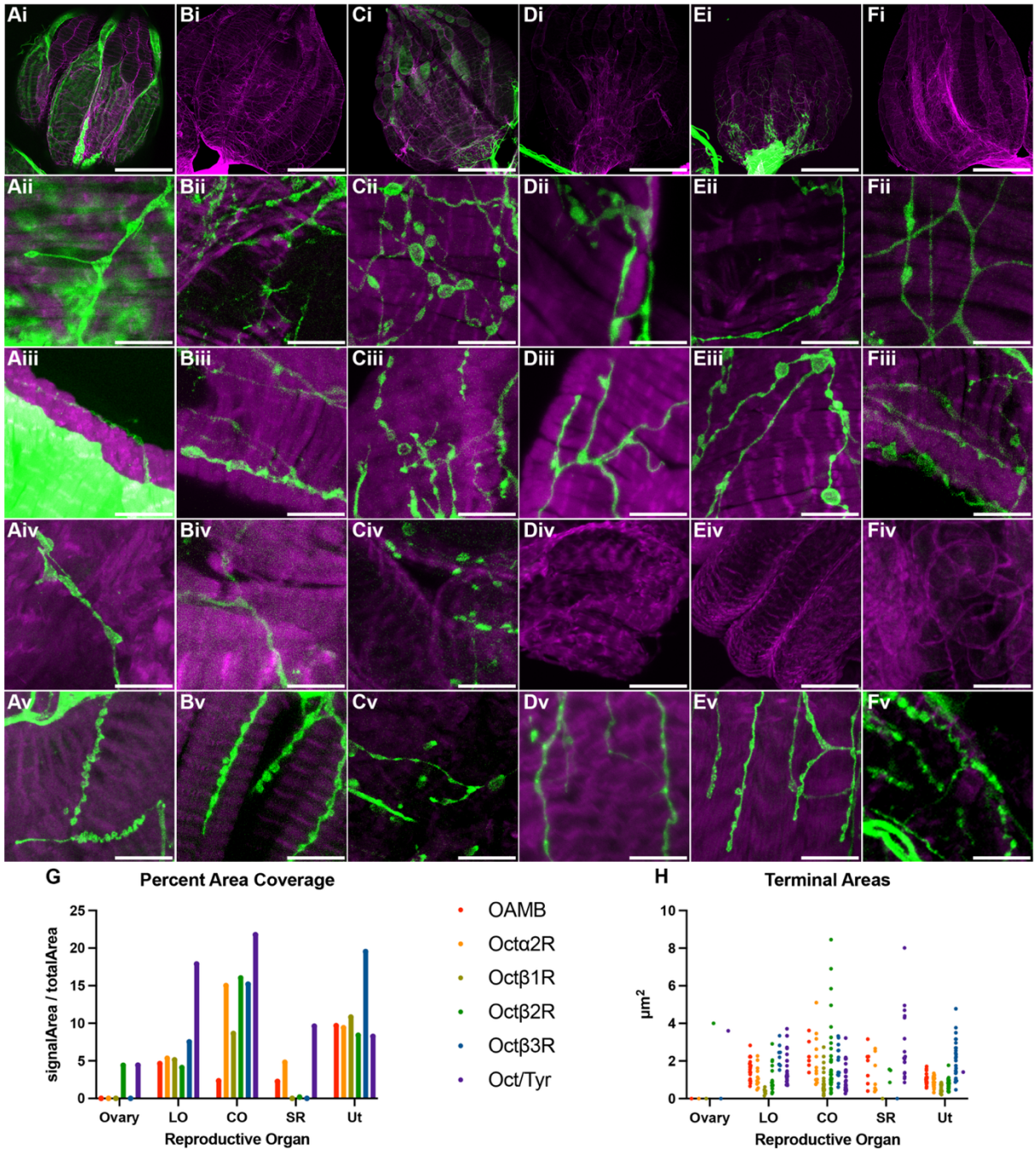



**Figure 9. Silencing circuits expressing OA receptors increases egg laying in both virgin and mated flies.** Octopamine receptor MiMIC Gal4's (Receptor MiMIC insertionr-t2a-Gal4) were used to drive UAS-*Kir2.1* (**A.**) or UAS-*Shibire*$^{ts}$ (**B., C.**). At the

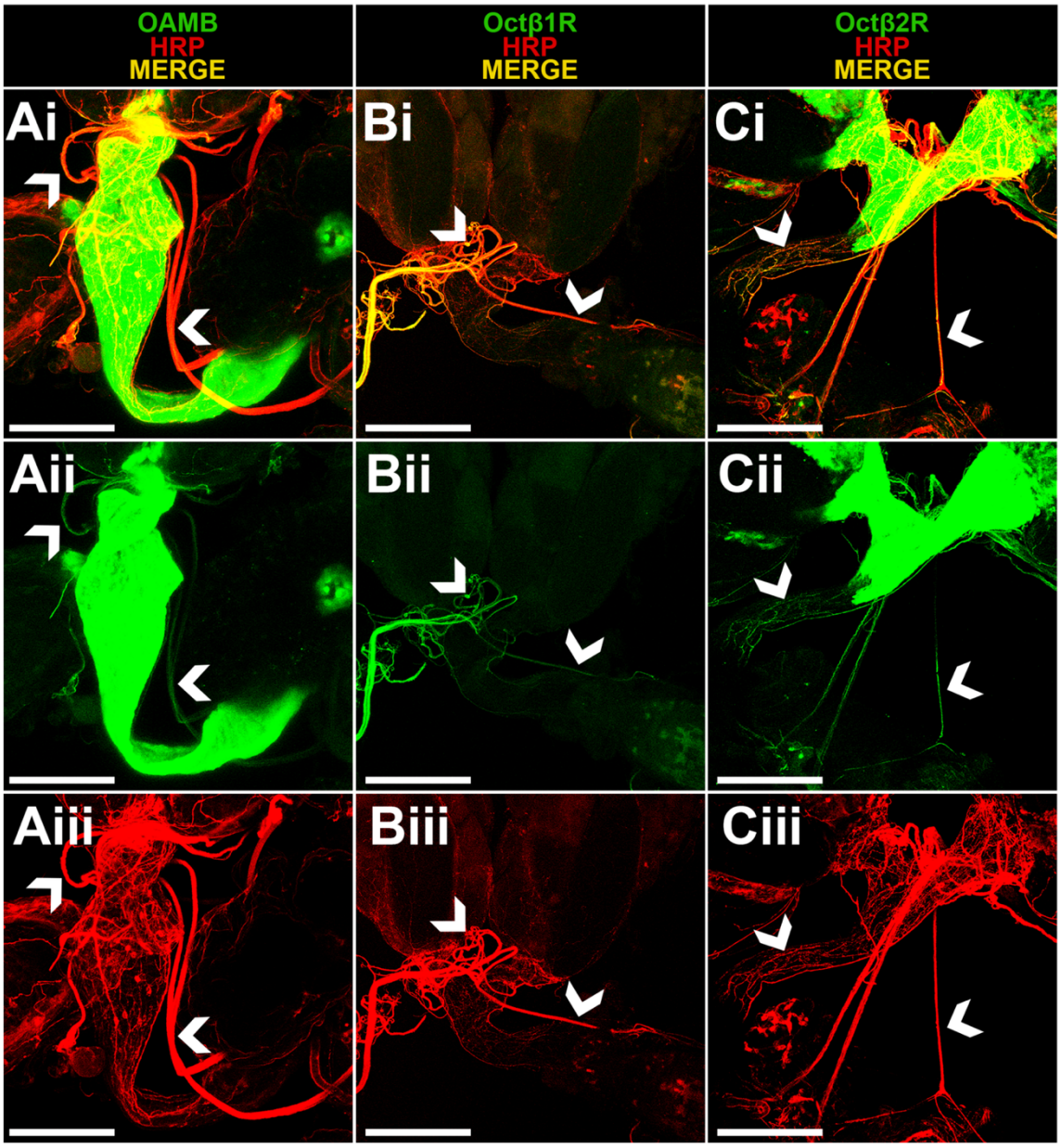
permissive temperature, egg laying increased in some mated lines (**A.**, **B.**) and all virgin lines (**C.**). Egg laying did not significantly decrease in any condition.



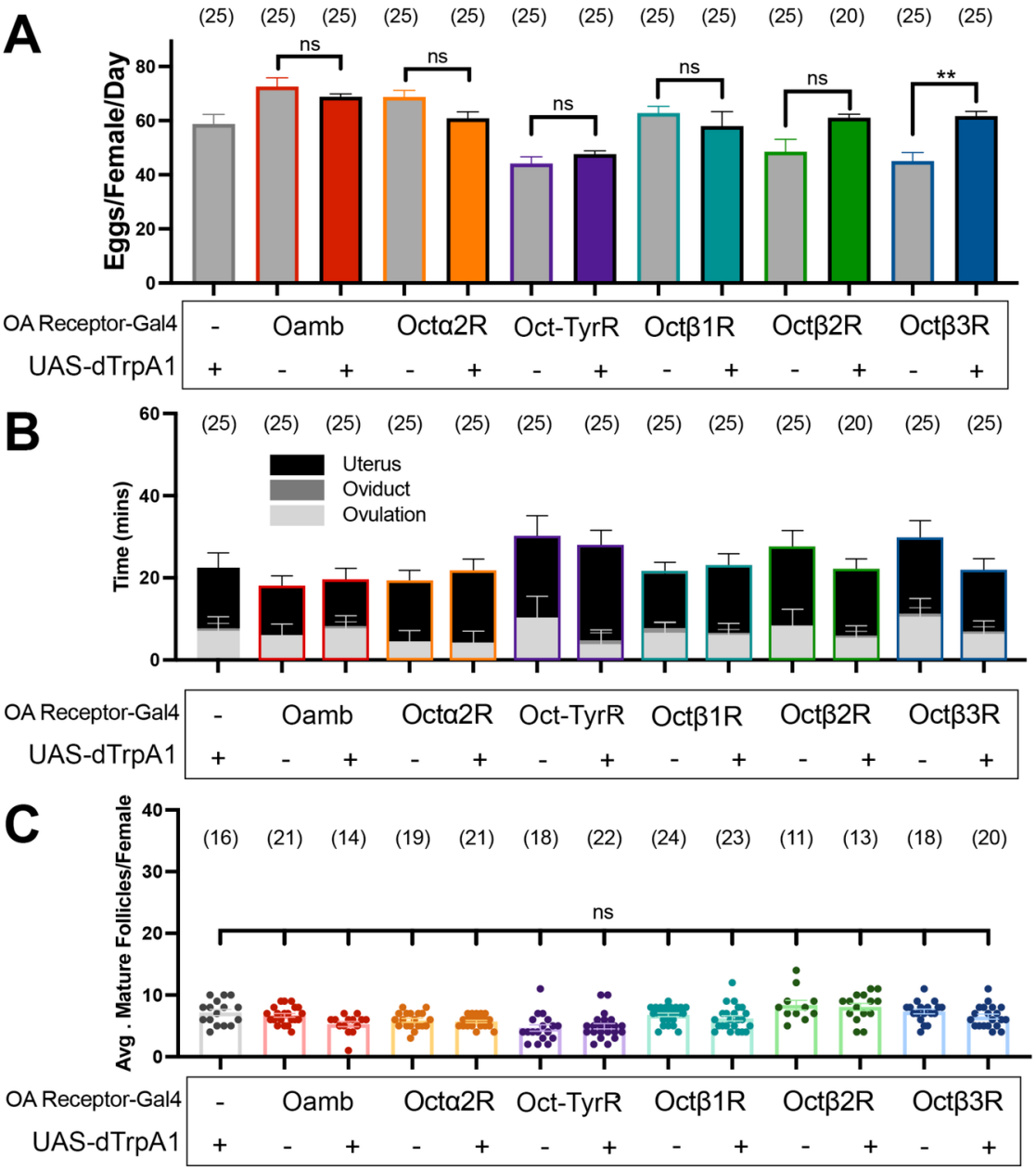

**Supplemental Figure 1. Octopamine receptors are expressed by neurons with terminals in reproductive organs.** *Oamb-t2a-gal4* (**A**), *Octα2-t2a-Gal4* (**B**), *Oct-TyrR-*

*t2a-Gal4* (**C**), *Octβ1R-t2a-Gal4* (**D**), *Octβ2R-t2a-Gal4* (**E**), and *Octβ3R-t2a-Gal4* (**F**) were used to drive *UAS-mCD8-GFP* expression. Reproductive systems were dissected and stained with anti-GFP antibody (green) and phalloidin f-actin stain (magenta). Representative nerve terminals were captured in the ovaries (**A-F, i**), calyx regions (**A-F, ii**), common oviducts (**A-F, iii**), seminal receptacles (**A-F, iv**), and uteruses (**A-F, v**). The approximate density of innervation in each region by neurons expressing each different receptor type was then quantified by calculating the area innervating nerves took up as a percent of equivalent areas of tissue across different preparations (**G**). From the same images, the approximate size of representative terminals was also calculated (**F**).

Scale bars = 100 μm (A-F, i) and 10 μm (A-F, ii-v)




**Supplemental Figure 2. Octopamine receptors are expressed by cells in the reproductive system that can be labelled with a neuron-specific antibody.** MiMIC Oa Receptor Gal4's were used to express *UAS-mCD8-GFP*. Signal from GFP (**Aii**, **Bii**, **Cii**) and HRP (**Aiii**, **Biii**, **Ciii**) antibody labels were compared (**Ai**, **Bi**, **Ci**) in reproductive systems and attached MANs. Representative regions of co-labeling are indicated by chevrons. Scale Bars = 200 μM.




**Supplemental Figure 3. Oa-Receptor-t2a-Gal4, UAS-TRPA1ts flies kept at a restrictive temperature of 22°C did not show defects in fertility compared to controls.** Neither egg laying (**A**), egg passage through any specific reproductive organ (**B**), nor mature follicle development (**C**) was significantly reduced in flies harboring the same alleles as in Figure 3 but kept at a temperature restricting TRPA1$^{ts}$ expression.

Flies harboring *Octβ3R-t2a-Gal4* and *UAS-TRPA1$^{ts}$* displayed a slight increase in egg laying (A, blue). However, their egg laying rate was not significantly different from the Gal4 (-) control (A, grey).

**Supplementary Video 1. Stimulation of Octβ3R neurons drives lateral and common oviduct contraction.** Stimulation of CHR2-XXM from *UAS-CHR2-XXM* driven by *Octβ3R-T2A-Gal4* in isolated reproductive tract preparations (Deshpande et al. 2022) drives lateral and common oviduct contractions that sometimes force eggs backwards in the oviduct. Optogenetic stimulation was initiated at 6 sec and persists for 14 sec.

**Supplementary Video 2. Transient calcium wave activity in the muscle of the seminal receptacle.** Spontaneous RCaMP1b fluorescence in the muscle of the seminal receptacle presents as waves of increasing signal both before and after addition of Oa. The clip has been sped up 3x (1 min at 12fps reduced to 20 sec at 36fps). 1μM Oa was added 10 sec into the 20 sec video. A Global Ca2+ response to Oa, but no detectable change in the rate of Ca2+ transients can be observed.

# CHAPTER 5: HETEROGENEITY IN ROLES AND EXCITABILITY AMONG A CENTRAL CLUSTER OF OCTOPAMINERGIC NEURONS THAT REGULATES DROSOPHILA EGG LAYING

## INTRODUCTION

Many aminergic nuclei such as locus coeruleus and the raphe contain multiple neurons that release the same aminergic neurotransmitter and project to similar targets (Poe et al. 2020). In some cases, anatomical, molecular or functional differences can distinguish similar subsets of cells within these nuclei (Andrade and Haj-Dahmane 2013; Huang and Paul 2019; Soiza-Reilly and Commons 2014; Borodovitsyna et al. 2020; Chandler et al. 2019). Markers for many other subtypes of aminergic neurons are lacking, making it difficult to identify them or determine their post-synaptic targets. In addition, since many aminergic neurons use volume rather than synaptic transmission, standard molecular tools that use synaptic markers to map connectivity are often unapplicable. As a result, the functional heterogeneities between many aminergic neurons remain poorly understood.

Aminergic nuclei in mammals contain thousands or millions of neurons depending on the species, thereby complicating the identification of specific subtypes (Poe et al. 2020). In addition, neuron location within nuclei is not precisely fixed. By contrast, the relatively small number of neurons and their stereotyped location in invertebrates has greatly facilitated the analysis of their function, as evidenced by classical studies in C. elegans crab, lobster and locust (Basu and Kravitz 2003; Lange 2009; Bargmann and Marder 2013; Harris-Warrick and Marder 1991). We are similarly

using *Drosophila melanogaster* to analyze the projections and functional properties of an aminergic cluster in the ventral nerve cord of the female fly. These studies take advantage of the many genetic tools available in this system as well as the relatively small number of neurons that comprise most aminergic clusters or “nuclei” in the fly.

A cluster of ~10 octopaminergic neurons in the abdominal ganglion of the ventral nerve cord innervates the female reproductive tract and is part of the oviposition (egg-laying) circuit (Monastirioti et al. 1995; Rezával et al. 2014; Pauls et al. 2018). The output of the cluster can be quantified by a variety of distinct assays because octopamine release is required for sperm storage, egg maturation, and contractility of the oviducts in flies as well as other insects (White, Chen, and Wolfner 2021; Rezával et al. 2014; Rodríguez-Valentín et al. 2006; Hana and Lange 2017; Yoshinari et al. 2020; Middleton et al. 2006; Meiselman, Kingan, and Adams 2018; Avila et al. 2012). The specific targets innervated by each cell and the potential differences between their physiology and function are not known. Identifying the targets of these cells and their physiological properties would be a key step toward understanding the oviposition circuit and would enhance this circuit’s utility as a model to study the way in which individual cells within aminergic nuclei regulate circuit function and behavior.

Using the single cell labeling technique Multi Color Flip Out (MCFO) (Nern, Pfeiffer, and Rubin 2015), we show that individual neurons within this cluster have discrete targets. Optogenetic stimulation experiments comparing restricted and inclusive octopaminergic Gal4 drivers show that different subset of octopaminergic neurons regulate muscle behavior to different degrees. The most effective subset in driving lateral oviduct contractions includes specific octopaminergic neurons that innervate the

calyx region of the reproductive tract, between the lateral oviduct and the ovary. Patch clamp recordings of two adjacent neurons from this model cluster also show differences in electrophysiological excitability but similar inhibitory regulation. These data establish a framework for studying the function of individual aminergic neurons in and how they regulate behavior.

## RESULTS

### Individual octopaminergic neurons innervating the reproductive tract have distinct targets.

The cluster of octopaminergic neurons that projects to the reproductive tract includes ~10 midline cell bodies near the ventral surface of the abdominal ganglion (Rezával et al. 2014). Similar to midline, unpaired neurons in other species such as locust, each one of these cells extends a single, large process that branches into two bilaterally symmetric extensions. Each of the two branches includes an extensive arborization with the ganglion and a long process that projects through the median abdominal nerve (MAN) into the reproductive tract (Pauls et al. 2018). To map the projections of each cell, we used the single-cell labeling technique MCFO (Nern, Pfeiffer, and Rubin 2015). In brief, expression of three transgenes with different molecular tags allows labeling of individual cells with three different secondary antibodies. Expression is limited to one neuronal subtype using the GAL4/UAS system and stochastic recombination of the tagged transgenes restricts labeling to a relatively small number of cells. Although a driver specific for octopaminergic neurons in the brain is available it does not label the octopaminergic neurons in the AbG (Schneider et al.

2012). We therefore used Tdc2-GAL4 to express MCFO in both octopaminergic and tyraminergic cells but confined our analysis to the most posterior, midline octopaminergic neurons. To map the projections of these cells, we dissected the reproductive tract together with the attached ventral nerve cord of preparations labeled using MCFO. We first determined whether any processes in the reproductive tract (RT) were labeled and then assessed whether one two or three of the three potential MCFO tags were visible in specific processes, representing each tag in RBG images by red, green, and/or blue channels. The combinations of blue+green, red +green, and red+blue are seen as turquoise, yellow, and pink respectively. Expression of all three channels appears as various shades of orange to pink depending on the relative levels of expression of each tag. We next determined which cells in the OA cluster near the tip of AbG were labeled and which one(s) corresponded to the labeling of processes that we observed in the RT. In some cases, an individual cell could be unambiguously assigned to projections in only one region of the reproductive tract. In other cases, more than one set of processes and/or more than one cell were identically labeled. The identity of these cells was therefore ambiguous within the confines of a single experiment. However, by comparing the data from several experiments, it was possible to deduce the identity of some of these cells.

Fig 1 (2 27 19i) shows an overview of the labeled reproductive system (Fig 1A) and ventral nerve cord (Fig 1B) as well as magnified views of the indicated areas (Fig1C, D, E). Yellow labeling in the stalks of the two spermathecae is visible (Fig 1C, arrows) consistent with the co-expression of green and blue MCFO tags in these processes. Additional processes and boutons labeled in red (but neither green nor blue)

are also present (Fig 1C, asterisks), indicating that the Sp receive input from an additional neuron other than the yellow cell in Fig 1B. We also observe red labeling of the uterus (and Ut, see also 1D) as well as the posterior common oviduct (data not shown). A blue-labeled process branches at the base of the ovary and fine extensions of these processes extensively innervate the body of the ovary (Fig 1A, E).

In the VNC, we detect several labeled cell bodies near the posterior tip the AbG that extend dorsally into the neuropil. For convenience, we designate this as the Poster Octopaminergic Cluster (POC) and distinguish it from additional, more anterior neuropil (Fig 1B, Ant Npl) and cell bodies. Within the POC, a single TdC2(+) cell is labeled yellow (2 27 19i, Fig 1B, arrow) and can be visualized in both the red and green channels (not shown). Since the only region of the reproductive tract that is similarly labeled are the stalks of the spermathecae (Fig 1C, arrows), we can unambiguously identify this cell as innervating the spermathecae (Sp). Its position near the posterior tip of the AbG provides useful a reference point for other, more anterior cells within the cluster. We have identified 8 other preps in which cell at the tip of the VNC projects to the Sp. Together with Fig 1, these data indicate that at least one cell in the POC projects exclusively to the spermathecae. Bilateral labeling of both spermathecae is consistent with the bilaterally symmetric processes of midline octopaminergic neurons that have been described in the fly, locusts and other insects (Rezával et al. 2014; Pauls et al. 2018; Lange 2009).

Immediately anterior to the yellow cell is a red cell body (Fig 1B, arrowhead). Another, small red cell can be seen at a more anterior and ventral position (Fig 1B, asterisk). Further anteriorly, a single blue cell body and dorsally extending process can

be visualized (Fig 1B, double arrowhead). Since processes in both ovaries are labeled blue, we conclude that this cell innervates the ovaries. We cannot specifically assign a cell to the red labeled processes in either the Sp or the Uterus using this preparation alone since at least two red cells are present in the POC.

The preparation shown in Fig 2 indicates that at least three distinct cells in the POC innervate the calyx at the base of ovary. They also confirm that at least one cell is specific for the ovaries+calyx, identify a second cell that innervates the spermathecae, and identify a cell that innervates the calyx+LO. In Fig 2A (3 18 19i), turquoise (green+blue) processes can be visualized in the spermathecae. Other processes visible in either the blue or green channel--but not both-- are visible in the calyx at the base the ovary (Fig 1A, C). The appearance of turquoise in in the calyx Fig 1A is caused by overlap between distinct green and blue processes that can be distinguished in a high-resolution image (Fig 1C). The blue processes present in the calyx (Fig 1A) extend into the LO but the green processes do not. Conversely, at least one green process innervates the body of each ovary while the blue processes do not (Fig 1A, asterisks). In contrast to the green processes, red processes arborize extensively through a large area of the ovary beyond the calyx (Fig 2A) similar to the blue processes in Fig 1 (2 27 19 i). Faint green labeling of the Uterus and posterior oviduct are also present but difficult to visualize in here (Fig 2A and data not shown).

To identify specific cell bodies that innervate each labeled region of the RT, we imaged the VNC as in Fig 1. A small turquoise cell body in the VNC can be visualized here (Fig 2A inset, arrow). Its anterior-posterior position is similar to that of the small red cell body in Fig 1 (2 27 19 i). More anteriorly, turquoise labeling can be seen in a

confocal stack of the VNC, but single confocal slices indicate that the turquoise labeling results from overlap of adjacent blue and green cell bodies (Fig 2, D,C, insets). Therefore, these data indicate that the turquoise cell is specific for the spermathecae. However, unlike the cell that innervates the spermathecae in Fig 1, the turquoise cell in Fig 2 is not at the tip of the POC and may correspond to the red labeling of the spermathecae in Fig 1. Based on its size and position, it is likely to correspond to the red cell labeled with an asterisk in Fig 1.

The labeling of the VNC shows a that a single blue cell (Fig 2A VNC inset) and two red cells; however, the more anterior red cell projects into the anterior neuropil (not shown) and only one red cell body is present in the POC. We therefore conclude that this cell innervates the ovaries. Similarly, since we observe a single blue cell in the POC and detect blue processes in the LO but not elsewhere in the RT, we conclude that the blue cell shown in Fig 2 innervates the calyx+LO. The distinct green and blue labeling of the calyx indicates that in addition to the blue cell, at least one green cell innervates the calyx and sends a few processes into the ovary. At least three cells are labeled green in the RT this preparation and we therefore cannot determine which one is responsible for innervating the calyx versus other regions of the based on this preparation alone; however, we have paired the green cell labeled with an asterisk with the calyx based on additional data discussed below. Together, these data support those from figure 1 indicating that the POC contains at least one cell that resides just anterior to the center of the POC is specific to the ovaries(+calyx) and does not innervate other regions of the RT. The data in Fig 2 also shows that, in addition to the cell that innervates the

calyx+ovaries, at least two additional cells project to the calyx. At least one of these can be assigned to the blue cell near the anterior extent of the POC.

The preparation shown in Fig 3 (3 18 19 iii) is notable for the relatively large number of cells in the posterior OC that are labeled with a single color and can be assigned to identically projections in the RT. 3 18 19 iii. Processes in the RT are labeled in yellow in the LO and calyx and in red in both the LO and the anterior region of the CO. We do not detect any labeling of processes in the ovaries. More posteriorly, we observe very extensive pink/orange labeling in posterior region of the common oviduct and the uterus (Fig 3 A, 3 18 19 iii). Additional green processes in the posterior common oviduct and descending toward the Uterus are present but are more difficult to see in this image (data not shown). On the stalks of the spermathecae, we detect two sets of processes; one set that appears pink/orange (white arrows) and is labeled in all three RBG channels (Fig 3D). A second set is blue (white arrowheads) (Fig 3D). These data confirm that at least two different cells innervate the spermathecae.

In the VNC from this preparation (Fig 3B) (3 18 19 iii) we detect one yellow, one red, two green, two blue, and two pink/orange cells. Another area in the VNC that appears to be labeled yellow can be seen at the intersection of a blue cell (white arrowhead) and a green cell (asterisk). This can be more easily resolved into distinct red, green and blue cells in the inset of Fig 3E and therefore and does not represent a second yellow cell body in the cluster.

The most anterior red cell projects into the anterior neuropil rather than to the RT (not shown). Therefore, the one other red cell in Fig 3B, 3 18 19 iii can be unambiguously assigned to the processes that innervate both the lateral oviducts and

common oviduct LO+CO. Similarly, the yellow cell in the VNC cluster in 3 18 19 iii can be assigned to a region at the base of the ovary that includes the calx and lateral oviduct. It's position near the anterior neuropil indicates that this cell is at or near the anterior end of the cluster. In Fig 2, the blue cell which labels the lateral oviduct (3 18 19 i) is also located at or near the anterior end of the cluster.

Further comparison of the VNC and the reproductive tract (3 18 19 iii) indicates that the two pink/orange cells innervate the Sp and the PCO+Ut (Fig 3D, F). Comparison to Fig 1 (2 27 19 i) suggests that the smaller, more posterior pink cell innervates the spematecae, similar to the yellow cell in Fig 1D. We therefore assign the other more anterior pink cell in Fig 3F to pink processes in posterior common oviduct and the uterus (Fig3, F). At least two green cells are also present in the VNC (Fig 3B, black arrow and asterisk, see inset) and we observe green processes as well as pink processes in posterior common oviduct and the uterus, although the green processes are less prominent Fig 3A (here in 3 18 19 iii) These data suggest that at least two distinct cells may innervate the posterior common oviduct and uterus.

Labeling of the posterior common oviduct (Fig 4A, B) (2 27 19 iv) and uterus (Fig 4A, C) by two distinctly colored sets of processes is more easily visualized in Fig 4(A-C). In the VNC, one blue cell and one green cell can be identified within octopaminergic cluster (Fig 4D (2 27 19 iv)). We do not detect any labeled cells more posterior to the blue and we do not detect blue or green labeling of the spermathecae here. Further comparison to Fig 3 (3 18 19 iii) indicates that blue cell body that innervates the PCO+Ut in Fig 4 (2 27 19 iv) is at a similar if not equivalent position as the large pink cell body that innervates the PCO+Ut in Fig 3 (3 18 19 iii). We designate this cell as

Posterior CO+Uterus (PCOU) or PCOU-P. The second, slightly more anterior cell innervates the CO+Ut and is labeled green Fig 4 (2 27 19 iv). We designate this cell as PCOU Anterior or PCOU-A. It appears to be equivalent to large pink cell that innervates the PCO and Ut in Fig 3 (3 18 19 iii).

Red processes in Fig 4A (2 27 19 iv) can be seen both in the stalks of the spermathecae and the ovary. At least one large and one nearby smaller red cell body (asterisk) can be seen in the VNC (Fig 4D) (2 27 19 iv). We cannot clearly distinguish which one innervates each target based on these images alone. However, comparison to the VNC shown in Fig 1 (2 27 19 i) suggests that the small cell bodies labeled red and indicated with asterisks in both Fig 4 (2 27 19 iv) and Fig 1 (2 27 19 i) may innervate the Sp. Further comparison to Fig 1 indicates that at least two distinct cells innervate the spermathecae, one at the posterior tip of the cluster and another more anteriorly. *Additional Comparison to 3 18 19 iii allows us to confirm that the blue cell in 3 18 19 iii clustered with the red and green cells is also likely to innervate the Sp. By extension, the more anterior blue cell in 3 18 19 iii is likely to project to the anterior neuropil.*

The preparation in Fig 5 illustrates the complex innervation in the calyx. Fig 5A and 5B (3 18 19 ii) show blue, red, green and yellow processes in the calyx. The blue processes appear to be completely restricted to the calyx (Fig 5A) while some yellow processes extend into the ovary (Fig 5 A add label to 5A). The red processes in the calyx do not extend into the ovary but rather the lateral oviduct. Two sets of green processes are present: one broadly innervates the body of the ovaries and can also be seen traversing the calyx. Additional green processes (data not shown) project to the

uterus and the CO like those shown in Fig 4 (2 27 19 iv). Together these data indicate that at least three distinct processes (blue, red, yellow) innervate the calyx in addition to green processes that pass through the calyx to innervate the ovary more broadly.

Comparison of the labeling in the VNC and RT allows us to map the origin of the blue, yellow and red processes. Labeling of the POC includes a single blue cell at or near the anterior end of the cluster (Fig 5C, E) (3 18 19 ii). Since we only observe one set of blue processes in the RT, we conclude that this cell innervates the calyx. As noted above, it has few if any projections into the rest of the ovary or the lateral oviduct and the arborization appears to be confined to the calyx. By contrast, a single yellow cell (Fig 5C, F) can be assigned to the yellow processes that innervate the calyx, but also send a few processes into the bottom third of the ovary (Fig 5A). Only a single red cell is present in the POC and we detect a single site with red processes in the RT, a region encompassing the calyx and a portion of the LO (Fig 5 (3 18 19 ii). The cell appears to represent the anterior end of the PO cluster similar to the yellow cell in Fig 3 (3 18 19 iii) which also projects to the calyx and LO. We designate this cell as “Cal+LO”. The presence of multiple green cells makes it difficult to definitively identify their targets in this preparation shown in Fig 5 but at least one of them must innervate the similarly labeled ovary.

The preparation shown in Fig 6 uncovered a cell that may be exclusive to the uterus. Labeling of the RT revealed red processes in the uterus and in the posterior region of the CO (Fig 6 A, C). By contrast, we detect additional green processes in the uterus, but not the adjacent posterior region of the CO. Another set of green processes are present at the base of the ovary/calyx and extend for a short distance into the LO.

The stalks of the Sp are partially obscured in this image, yet are decorated with red and/or green processes (data not shown). Blue processes in 2 27 19 iii are present at the base of the ovary and extend into the LO.

A prominent red cell near the posterior end of the POC can be seen in in both Fig 6A and the Fig 6C inset (2 27 19 iii). A green cell and a blue cell of similar size lie at a more anterior and ventral positions within the VNC. A smaller green cell is immediately posterior and ventral to the blue cell (Fig 5B inset).

The size and position of the red cell body in Fig 6A inset and 6C (2 27 19 iii) is consistent with that of the more posterior cell which innervates the posterior oviduct and uterus in Fig 1 (2 27 19 i) (the larger red cell), Fig 4 (2 27 19 iv) (blue) and Fig 3 (3 18 19 iii) (larger pink cell). It does not appear that there is a smaller red cell body more anteriorly as is found in 2 27 19 i, and a small red cell near the posterior green cell (not visible in max projection) may innervate the Sp (data not shown). We detect only one blue cell body and one set of blue processes thus allowing us to assign this cell to the Cal+LO. Note that it is at or near the anterior extent of the cluster, similar to the yellow Cal+LO cell in Fig 3 (3 18 19iii). The partially obscured green cell body is immediately posterior and dorsal to the blue cell the calyx is labeled green in 2 27 19 iii. Since the cell that innervates the calyx and projects partially into the ovary is in a similar, posterior position in Fig 5 (3 18 19 ii), we believe that the posterior green cell is also Cal+Ov here in Fig 6 (2 27 19 iii). The green processes in the Ut are therefore derived from the large green cell midway between the anterior and posterior ends of the cluster. Importantly, the more posterior cells seen here Fig 6(red cell), Fig 1(2 17 19i, red cell) Fig 3 (3 18 19iii, pink cell), and Fig 4 (2 27 19 iv, blue cell and green cell) that innervate the Ut also

innervate the Post CO. By contrast we see green labeling here (Fig 6) in the Ut but not the Post CO. We conclude that there is a distinct cell within the cluster which exclusively innervates the Ut and that it lies anterior to the two PO+Ut cells.

**Stimulating subsets of POC neurons can induce lateral oviduct muscle contractions.**

To test the functional output of different POC neurons, we used a series of TβH-GAL4 lines that appear to express in subsets of POC neurons (Jenett et al. 2012) and assayed lateral oviduct muscle behavior, which has been shown to be affected by OA signaling (Fig. 7A) (Deshpande et al. 2022). Intriguingly, using the channelrhodopsin allele *UAS-CHR2-XXM-tdTomato*, we find that multiple different sets of *TβH-GAL4* expressing cells each seem capable of affecting repetitive oviduct muscle contraction (Fig. 7B). These experiments are still a work in progress. More preparations need to be tested so that minor differences in lateral oviduct contraction latency, rate, and duration may be assessed, and more *TβH-GAL4* lines need to be tried. However, at least one *TβH-GAL4* ("TβH2") seems to express in cells innervating the lateral oviduct region and stimulation of these cells via CHR2 reliably induces repetitive lateral oviduct contractions (Fig. 7, B, D). Interestingly, other *TβH-GAL4* lines such as "TβH1" that do not appear to include expression in cells innervating the lateral oviduct regions also seem capable of inducing lateral oviduct contractions (data analysis underway).

**TβH2 expresses in 4 specific POC neurons.**

To further validate our MCFO mapping and assess which specific POC cells may contribute to lateral oviduct contractions, we next compared the expression of TβH-GAL4 drivers to the expression of *Tdc2-LexA*, which mimics the expression pattern of *Tdc2-GAL4* (Burke et al. 2012). Figure 8 details an example experiment representing this comparison using the TβH-GAL4 allele that most reliably induced lateral oviduct contractions ("TβH2") and a *LexAop::CD2-RFP, UAS::mCD8-GFP* reporter sequence. For each preparation, confocal images were taken through the VNC and RT at 500nm and 1μm intervals, respectively. Both maximum signal projections (Fig. 8, A,F) and single-slice image analyses (Fig. 8, B-E) were used to assay coexpression in 11 midline Tdc2 (+) neurons at the posterior end of the abdominal ganglion (Fig. 8, A-E) and projections throughout the RT (Fig. 8F). For the *TβH-GAL4* line that most robustly induces lateral oviduct contractions when stimulated via channelrhodopsin, we find coexpression with *Tdc2-LexA* in 4 of the 11 assayed Tdc2 (+) cells. Of these coexpressing cells, at least one must be included in the set of POC cells due to coexpression in the RT (Fig. 8F). Comparing these results and repeated experiments (n=8, data not shown) to the mapped neurons in figures 1-6, we conclude that either cell "8" or "9" (Fig. 8D) likely represents a calyx-innervating cell depicted in Figures 2, 3, or 5. Another two coexpressing cells, "10" and "11" (Fig. 8E) may be octopaminergic neurons that contribute to oviduct behavior, but due to their more-anterior localization we conclude that these cells are less likely to be POC neurons innervating the reproductive system. Experiments to determine the overlap between other *TβH-GAL4* allleles and *Tdc2-LexA* are currently underway.

**Neurons in the POC have different excitabilities.**

To determine the electrophysiological properties of the cells in the OA cluster innervating the RT, we performed whole cell, patch clamp recordings from somata in the AbG. To distinguish Tdc (+) cells from other neurons in the AbG, we expressed the marker mCD8-GFP using Tdc2-GAL4, the same driver we used for MCFO experiments. The marker was easily visualized in live images while patching (Fig. 9, C-D) and after fixation and (Fig. 9, A, B). We chose to focus on two large cell bodies at the posterior tip of the cluster ("VNC2" and "VNC3") because they could be easily visualized and consistently distinguished from each other and the rest of the cluster due to spacing between their localizations and that of other cells. Our anatomical data suggest that both cells project to the Posterior CO and the Uterus. Access to the VNC for electrophysiological recording required disruption of the glial sheath that surrounds it, which led to slight changes in the absolute position of the OA neurons (Fig. 9 A, B compared to C). VNC2 and 3 could nonetheless be consistently identified as the first and second large, midline cells at the posterior tip of the cluster (Fig. 9, A-E). To confirm that we were recording from same cells that we had imaged using confocal microscopy, we injected biocytin into cells during recordings and visualized the cluster using confocal microscopy after each experiment (Fig. 9, A, B). Digital rotation (Fig. 9B) of a horizontally oriented VNC (Fig. 9A) following a recording from VNC2 shows that the VNC3 cell body is slightly ventral to VNC2 in addition to being more anterior.

Using whole cell path clamp, we detected relatively few spontaneous action recordings in either VNC2 or 3 in our initial, baseline recordings (data not shown, also

see traces at 0 current injection (Fig. 9, F-H)). To determine if a baseline inhibitory potential was responsible for the apparent quiescence of the cells, current was progressively injected and the number of action potentials after each injection was recorded (Fig. 9, F, G). The number of action potentials elicited by each current step was significantly lower for VNC3 compared to VNC2 (Fig. 9H). These data suggest that VNC3 may be inherently less excitable than VNC2 or receive stronger inhibitory inputs, despite innervating a similar target.

**Neurons in the POC have different responses to picrotoxin and ivermectin.**

To explore the baseline inhibitory inputs to VNC2 and VNC3, we bath applied the inhibitory channel blocker picrotoxin (Ffrench-Constant and Roush 1991; ffrench-Constant et al. 1993; Stilwell et al. 2006). We again injected current in a stepwise fashion and quantified the number of action potentials that were elicited at each step, both before and after treatment with picrotoxin (Fig. 10, A, B). Application of picrotoxin resulted in a dramatic leftward shift of the current-response curve in both VN2 and VNC3 (Fig. 10, C-F). However, the maximal excitability of VNC3 remained lower than VNC2, consistent with the recordings shown in Figure 9.

In addition to GABA gated inhibitory channels, Drosophila express a glutamate gated chloride channel (GluCl) that is also responsive to picrotoxin (Etter et al. 1999; Cully et al. 1996). We are not aware of a specific GluCl antagonist. Therefore, to determine whether GluCl might contribute to the inhibitory control of VNC2 and/or VNC3, we tested the effects of the GluCl agonist ivermectin (data not shown). In addition, since both VNC2 and VNC3 were relatively quiescent at baseline, we also

tested the effects of ivermectin after first applying picrotoxin. We detect a rightward shift in the current-response curves of both VNC2 and VNC3 in response to ivermectin following picrotoxin (Fig. 10, C-F); however, the response of VNC2 was significantly more robust than VNC3. In VNC2, activation of GluCl appeared to restore the level of inhibition seen prior to the initial application of picrotoxin. By contrast, application of ivermectin to VNC3 returned the cell to an intermediate level of excitability midway between baseline and the more excitable state seen after application of picrotoxin. These data are consistent with lower level of excitability observed for VNC3 compared VNC2 and further underscore the subtle differences between these two similar octopaminergic cells.

**POC neurons express the Glu-Cl receptor**

To further explain the physiological differences between VNC2 ("POC2") and VNC3 ("POC3"), we tested for GluCl expression in POC neurons. Because POC3 behavior changed in response to ivermectin treatment whereas POC2 behavior did not (Fig. 10, E, F), we hypothesized that perhaps POC3 expressed GluCl, the target of ivermectin agonism, while POC2 might not. Comparing the expression of *Tdc2-LexA* to that of *GluCl-Gal4* with multiple angles of signal analysis, however, suggests no expression of GluCl in POC2 or POC3 (Fig. 11, A,B). Rather, *GluCl* appears to express in cells with non-neural morphology that envelop the entire VNC. This expression pattern implies that the processes of POC2, which extend along the ventro-posterior edge of the AbG, pass between GluCl (+) cells( Fig. 11, B, bottom row, asterisk) while

the processes of POC3, which extend dorsally though the neuropil of the AbG, do not (Fig. 11, B, middle row, asterisk).

## DISCUSSION

We have identified 10 cells in the AbG that innervate specific regions of the RT. These include two cells each that innervate the Sp and posterior common oviduct, three for the Calyx +/- portions of the ovaries or LO, and one each for the the ovaries, the lateral oviducts, and the common oviducts. The positions within the OA cluster for some cells were unambiguously identified in individual MCFO preparations. Others could be deduced by comparing labeled cells across multiple preparations. Electrophysiology of two cells shows that they share a common baseline inhibition yet are differentially excitable. These data form the basis for further functional studies of each cell and how they may interact to regulate the oviposition circuit.

We have named the cells in two ways: one as a descriptor of the organs they innervate and a second representing their approximate order along the midline of the AbG from posterior to anterior. The most posterior cell that we have unambiguously identified innervates the Sp and we designate it VNC1 or Sp1. It can be seen in Fig 1 as a single yellow cell in the cluster and in Fig 3 as the more posterior pink cell. We have observed it in an additional 12 preparations (not shown). The two innervation patterns within the stalks of the Sp are very similar, and boutons from each cell are adjacent to one another at multiple points. The functional relevance of each cell remains to be determined.

Both VNC2 and VNC3 innervate the uterus and the posterior portion of the common oviduct. However, VNC3 extends processes in more posterior uterine regions than VNC2. This overlapping-yet-partially-distinct pattern of innervation is similar to the nature of the multiple Sp and calyx-innervating cells. We detect innervation of both the Posterior oviduct and Uterus representing either VNC2 or VNC3 in 10 preps including those shown in Fig 1, 2 (albeit faint), 3, 4 (5) and 6. In contrast to VNC2 and VNC3, VNC4 innervates the uterus but not the common oviduct. In addition, innervation of the Ut appears to be more posterior than VNC2 or 3. VNC5 innervates both the LO and CO. It's location can be seen in Figure 5 posterior and ventral to VNC3 and we designate this VNC4 or LOCO.

Eight large ventral unpaired medial (VUM) cells have been previously suggested to innervate the RT (Rezával et al. 2014). In addition to these, we have identified a smaller, midline cell at the tip of the AbG that innervates the Sp (Sp1) and a second, small and more anterior cell that also innervates the Sp (Sp2). Thus, in total we have identified innervation patterns and positions within the VNC of 10 midline cells than innervate the RT, suggesting that two of these cells may be Tdc2 (+), dsx (-) despite their innervation of female-specific organs. Based roughly on their position from posterior to anterior, we designate the posterior cell that innervates the Sp (Sp1) as VNC1. The next two cells innervate the Ut as well as the Post CO and we designate these VNC2 and 3. We designate the second more anterior Sp cell (Sp2) as VNC4. The LO cell is at the anterior end of the cluster just anterior to the calyx cells. We designate LO VNC10, and the two calyx cells as VNC8 and 9. The Ut, ovary, LO+CO represent the remaining cells in the center of the cluster: VNC5, 6 and 7. The Ovary cell call be

assigned to VNC6 based on its position immediately posterior to the calyx cells in Figures 1, 2, and 5. The LO+CO cell is adjacent to the VNC3 (PCOU-P) and VNC4 (Sp2) and we designate it as VNC5. We suggest that the Post Ut cell is also likely to be adjacent to VNC3, 4 and 5 and we designate it VNC6. Comparison of (2 27 19 iii  3 18 19 iii suggests that the smaller green cell in 3 18 19 iii (asterisk) innervates the posterior uterus.

The fly connectome has been mapped in the brain using EM and ongoing efforts are likely to map the VNC (Scheffer et al. 2020). However, these experiments require serial tracing of neuronal processes in adjacent sections. The length of the processes that project from the VNC to the RT would render a similar EM reconstruction prohibitively difficult. The use of non-synaptic modes of neuronal communication by many aminergic neurons also reduces the power of techniques that require close synaptic contacts.

Single cell labeling techniques that map the projections of presynaptic neurons can help to overcome these difficulties. Molecular methods to label individual neurons can also facilitate electrophysiological studies. The stereotyped position of invertebrate neurons enhances to power of this technique and was pioneered in the STG of the crab. By contrast, both the electrophysiological properties and the projection patterns of most aminergic neuromodulatory neurons in the fly remain unclear.

Some of aminergic circuits in the fly consist of a single aminergic ell, e.g. CSD or DPM, similar to the aminergic circuits in C. elegans. However, many other aminergic cells are part of larger clusters, e.g. the DA clusters in the adult brain.  The number of aminergic neurons in mammalian nuclei are orders of magnitude higher than flies.

Nonetheless, the logic underlying the regulation of multiple downstream targets by clusters of aminergic cells may be similar. We speculate that small clusters of aminergic neurons observed in flies might be used to model the function aminergic nuclei such as the LC and Raphe.

To determine how individual neurons within an aminergic cluster or nucleus may act in concert to control a common target, we have focused on clusters in the fly that contain more than one or two cells. At the same time, to allow the precise identification of each cell in a cluster and its respective target a relatively small number of cells is useful. In the central brain, these criteria are met by the DA clusters and the serotonergic cells that innervate the optic lobe (Sampson et al. 2019). The OA cluster in the AbG that innervates the reproductive tract, abbreviated here as the POC, provides another useful model. It has been studied extensively because of its proposed role in oviposition, and the number of cells in the cluster as well as the organs in the RT that are innervated has been previously determined. However, the projection patterns of each individual cell are not known. Without this information, it is difficult to distinguish difference between the functions of each cell. On the one hand, all of the cells in the cluster may project in a similar pattern throughout the RT, thus regulating downstream targets en masse. Conversely, each cell may innervate a completely different target.

While genetic reagents such as split GAL4 lines to label specific neuromodulatory cells have been developed for other circuits, similar tools to comprehensively map OA cells in either the brain or nerve cord have not yet been developed. We have therefore taken advantage of a stochastic method to overcome this problem. We find that despite similar neurochemical profiles, many OA cells in the AbG

project to distinct targets in the reproductive tract. By contrast, several also appear to have similar projection patterns.

Ongoing work will determine the relationship between POC neuron innervation targets and POC neuron roles in behavior. Currently available data suggests that multiple POC neurons may contribute to lateral oviduct contraction behaviors but that these contributions may vary by individual neuron. The fact that indirect circuits may facilitate such oviduct behaviors (Deshpande et al., 2022) suggests that different POC neurons may have different roles in information processing involving both downstream neurons and the RT. Some POC neurons may affect oviduct contractions directly while others may affect the same behavior via alternate, indirect pathways.

The differences in electrophysiological properties between POC2 and POC3 establish further heterogeneity in the function of POC neurons. Reponses to picrotoxin that suggest chronic inhibition of POCs by GABA receptor channel activity implicate a possible mechanism by which the POC neurons are kept dormant by GABAergic signals until mating occurs. The reconstitution of inhibition following ivermectin application to POC2 but not POC3 also shows that individual POC neurons can be differentially modulated once activated. This effect may represent a means for coordinating the activation of different stages of the egg laying process (ovulation, oviduct passage, oviposition).

Together, these data help establish the *Drosophila* POC as a model aminergic cluster useful for studying heterogeneity among aminergic neurons with shared characteristics such as localization. This cluster of aminergic neurons governs a wide range of reproductive behaviors, yet mechanisms underlying the coordination required

for successful fertility remain unknown. Recent work such as that in Chapter 4 of this dissertation has shown that different types of OA receptors and different types of postsynaptic cells may contribute to different effects of octopamine signaling in different regions of the RT. The ongoing work in this chapter for the first time suggests mechanisms by which the presynaptic octopaminergic neurons themselves may also contribute to octopamines diverse roles in fly fertility.

## MATERIALS AND METHODS

### MCFO Immunohistochemistry

To map the projection targets of individual POC neurons, multi-Color Flip Out (MCFO) experiments were carried out using *Tdc2-Gal4* (Cole et al., 2005) and *UAS-MCFO7* (Nern et al., 2015). Flies were aged to 7-10 DPE, and reproductive systems with adjoined central nervous systems were dissected in PBS. Preparations were then fixed in 4% PFA, washed 3x in PBS + 0.3% tritonX100 (PBT), and blocked in 5% normal goat serum (NGS) in PBT for 2 hours at room temperature. Primary antibodies against V5, HA, and FLAG epitopes (MsαV5, RbαHA, RtαFLAG) were then applied in blocking solution overnight at 4C. Preparations were washed 8x in PBT at room temperature before secondary antibodies (α-Ms-488, α-Rb-555, α-Rt-633) were applied for 3 hours in blocking solution at room temperature. Following secondary antibody application, preparations were washed another 8x, cleared in 25% glycerol in PBS overnight at 4C, and mounted on slides with bridged cover slips so that tissue was not compressed.

Mounted preparations were then imaged using a Zeiss LSM 880 confocal microscope and images were analyzed using publicly available ImageJ software.

**Optogenetics and Lateral Oviduct Contraction Assay**

To study the effect of stimulating different subsets of POC neurons, optogenetic stimulations of POC neurons were performed on intact preparations as previously described (Deshpande et al. 2022) using a series of *TβH-Gal4* alleles ("TβH1-5") known to express in subsets of octopaminergic neurons. Flies harboring one copy of TβH1, TβH2, TβH3, TβH4, or TβH5 and one copy of *UAS-ChR2-XXM::tdTomato* were compared to control flies with one copy of *Tdc2-Gal4* and *UAS-ChR2-XXM::tdTomato* (positive control) or one copy of *UAS-ChR2-XXM::tdTomato* alone (negative control). Live imaging of stimulation responses using a Zeiss Axio Examiner system with ThorLabs LED illumination and an ANDOR iXon X3 camera focused on recording lateral oviduct behavior, and lateral oviduct contractions were identified as previously described (Deshpande et al. 2022).

**Coexpression Immunohistochemistry**

Coexpression between *TβH-Gal4* alleles, *GluCl-Gal4*, and *Tdc2-LexA* were performed using the dual reporter construct *UAS::mCD8-GFP, LexAop::CD2-RFP*. Flies were aged to 4-6 DPE, and reproductive systems with adjoined central nervous systems were dissected in PBS. Preparations were then fixed in 4% PFA, washed 3x in PBS + 0.3% tritonX100 (PBT), and blocked in 5% normal goat serum (NGS) in PBT for 2 hours at room temperature. Primary antibodies against GFP and RFP epitopes (MsαGFP, RbαdsRED) were then applied in blocking solution overnight at 4C. Preparations were

washed 8x in PBT at room temperature before secondary antibodies (α-Ms-488, α-Rb-555) were applied for 3 hours in blocking solution at room temperature with DAPI stain (1:1000). Following secondary antibody application, preparations were washed another 8x, cleared in 25% glycerol in PBS overnight at 4C, and mounted on slides with bridged cover slips so that tissue was not compressed. Mounted preparations were then imaged using a Zeiss LSM 880 confocal microscope and images were analyzed using publicly available ImageJ software.

**Electrophysiology**

Electrophysiological recordings from POC neurons were performed in flies harboring one copy each of *Tdc2-Gal4* and *UAS::mCD8-GFP*. Flies aged 4 DPE were anesthetized on ice before being dorsally glued to a recording chamber with UV-activated glue. HL3.1 solution was then used to fill the recording chamber, and a small window was dissected in the ventral cuticle over the AbG. GFP fluorescence was visualized so that POC neurons could be identified, and whole-cell patch-clamp recordings from POC2 or POC3 were performed with either continuous HL3.1 perfusion or perfusion of HL3.1 containing the indicated concentration of picrotoxin or ivermectin. During recordings, patched cells were filled with biocytin dye. Following recordings, preparations were unglued from the recording chamber via mechanical separation, and VNCs were dissected in cold PBS. Preparations were then fixed in 4% PFA, washed 3x in PBS + 0.3% tritonX100 (PBT), and blocked in 5% normal goat serum (NGS) in PBT for 2 hours at room temperature. Primary antibody against GFP (MsαGFP) was then applied in blocking solution overnight at 4C. Preparations were washed 8x in PBT at

room temperature before secondary antibodies (α-Ms-488, STREPTAVIDIN-555) were applied for 3 hours in blocking solution at room temperature. Following secondary antibody application, preparations were washed another 8x, cleared in 25% glycerol in PBS overnight at 4C, and mounted on slides with bridged cover slips so that tissue was not compressed. Mounted preparations were then imaged using a Zeiss LSM 880 confocal microscope and images were analyzed using publicly available ImageJ software.

## FIGURES AND LEGENDS

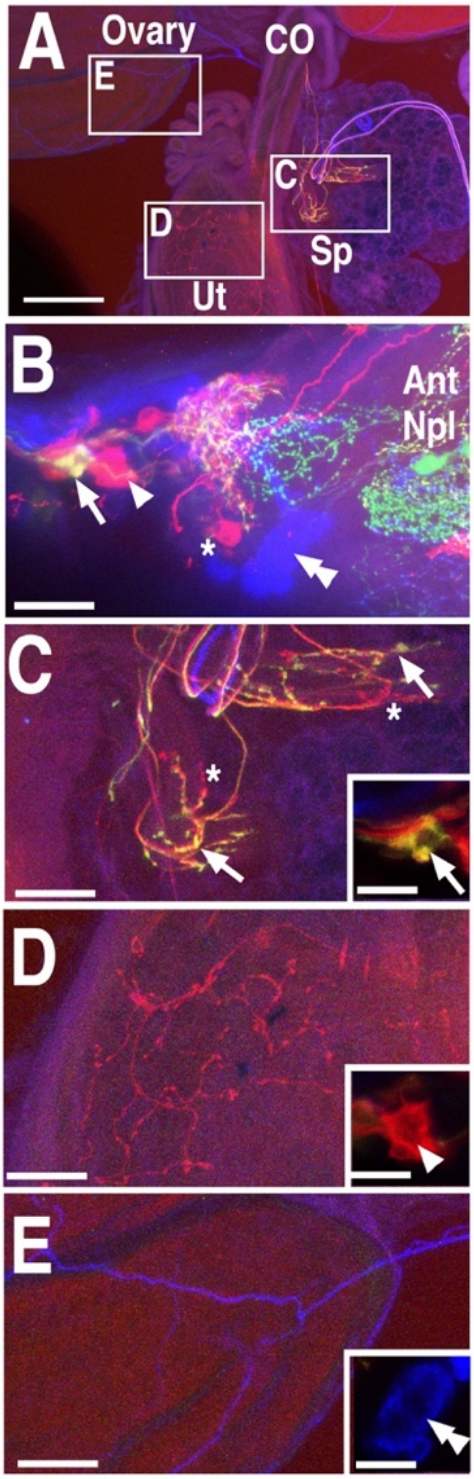



**Figure 1. (2 27 19i) A neuron at the tip of the AbG projects to the spermathecae while other cells innervate the ovary and uterus.** A. Overview of the labeled

reproductive tract with the Common Oviduct (CO), Spermathecae (Sp) Ovary, and Uterus (Ut) B. Labeling of the AbG shown as confocal stack. Arrow indicates a yellow cell label the Spermatheca (see insets in C, D, E for single optical plane. The Anterior Neuropil (Ant Npl) is indicated.  C, D, E. Panels correspond to boxed areas in A. Insets correspond to indicated in B. Scale bars: A, C, D, E. 50 microns. B, and insets in A, C.

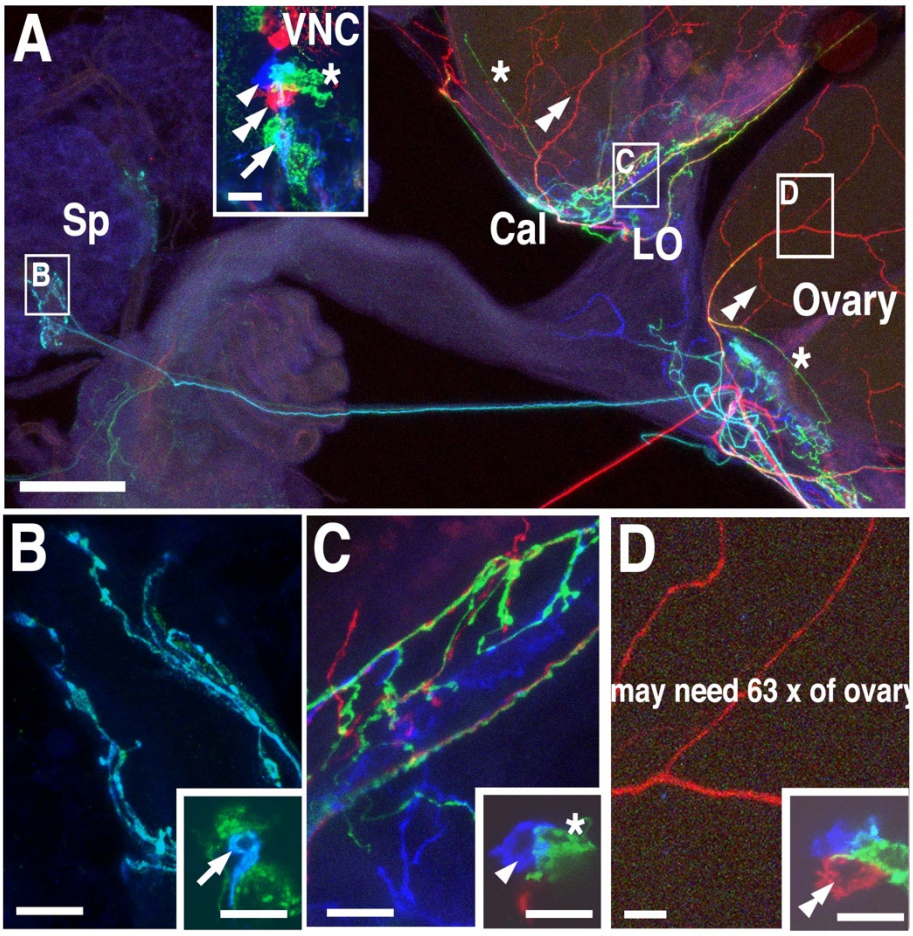



**Figure 2. (2 27 19 i) Neurons innervating the calyx, lateral oviducts, ovaries and spermathecae.** A. Overview of the labeled reproductive tract with the Spermathecae (Sp), Calyx (Cal), Lateral Oviduct (LO) and Ovary, indicated. The inset shows the labeled somata within the ventral nerve cord (VNC). Asterisks indicate a few, scant

green processes.  B, C, D. Panels correspond to boxed areas in A. Insets correspond to the cells indicated in B. The red cell (double arrowhead) can be unambiguously assigned to the calyx+ovaries based on this . Scale bars: A, 50 microns. B,C,D and insets in B,C,D: 10 microns.

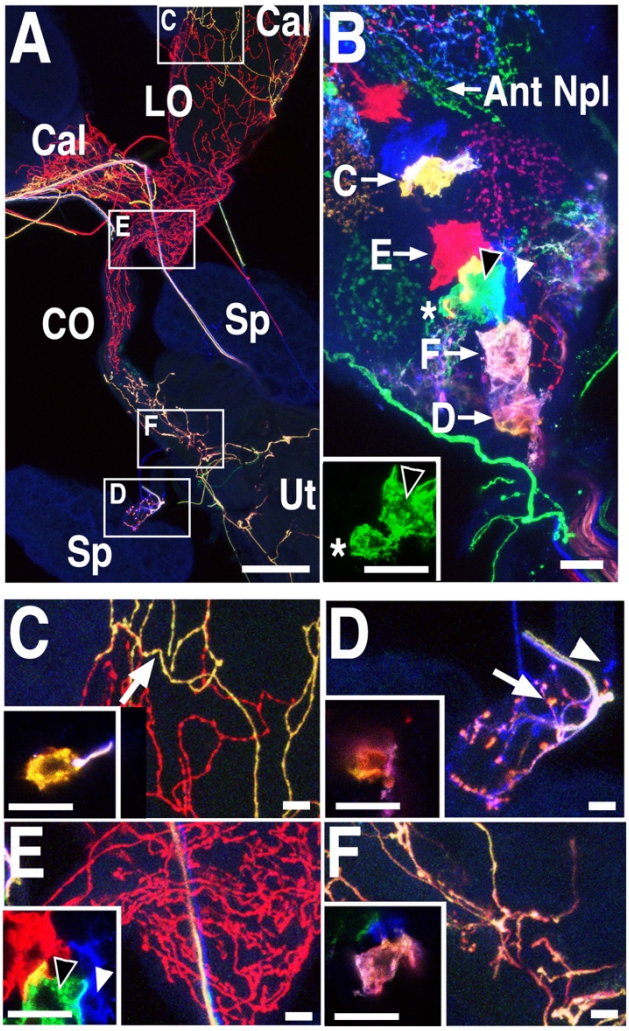



**Figure 3. (3 18 19 iii) Neurons innervating the Calyx, Lateral Oviduct, Anterior Common Oviduct, Posterior Common Oviduct, Ovary and Spermathecae.** A. Overview of the labeled reproductive tract with the Spermathecae (Sp), Calyx (Cal), Lateral Oviduct (LO), Common Oviduct (CO) and Uterus (Ut) indicated. B. A confocal

stack shows labeling of cell bodies in the POC and the more anterior neuropil (Ant Npl). Arrows point to cells C, D. Panels correspond to boxed areas in. Insets correspond to the cells indicated in B with a single arrowhead, double arrowhead, arrow or asterisk. The red cell (double arrowhead) can be unambiguously assigned to the calyx+ovaries based on this . Scale bars: A, 50 microns. B,C,D and insets in B,C,D: 10 microns.

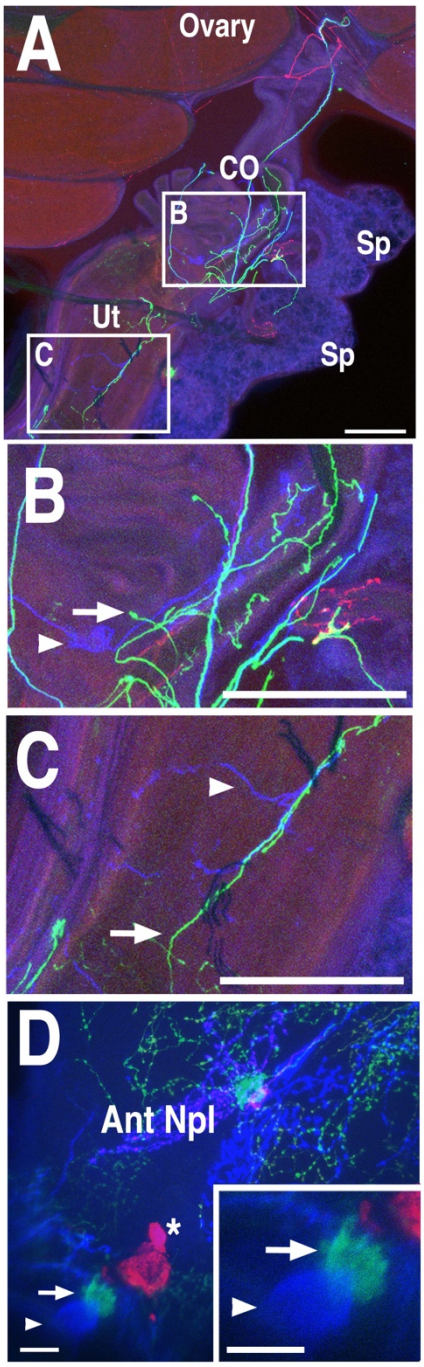



**Figure 4. Two neurons project to the posterior common oviduct and uterus.** A. Overview of the labeled reproductive tract with the Ovary, Common Oviduct, (CO), Spermathecae (Sp), and Uterus (Ut) indicated. The boxed regions shown at higher magnification (B, C) include the posterior CO (B) and the Uterus (C). D. Labeling cells in

the VNC includes one blue (arrowhead) and one green (arrow) cells. Two red cells are visible with the smaller one marked (*). Scale bars: A, 50 microns. B,C,D 5 microns. Inset in D: 10 microns.

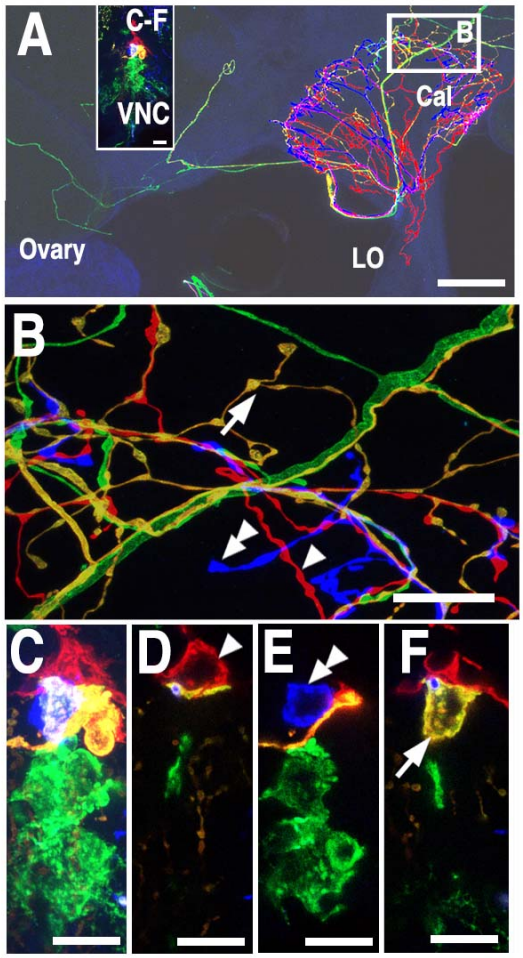



**Figure 5. Complex innervation of the calyx.** A. Overview of the labeled reproductive tract with the Ovary, Calyx (Cal) and Lateral Oviduct (LO) indicated. The boxed region is shown at higher magnification in B. The inset shows labeling of the VNC from the same prep and is shown at higher magnification in C-F. The arrow, single arrowhead and arrow indicate processes (B) and somata (C-F) of the red, , blue and yellow cell shown

in C-F. D,E, F are single optical sections of the confocal stack shown in C. Scale bars: A, 50 microns. B xxxx microns. Inset in A, and C-F: 10 microns.

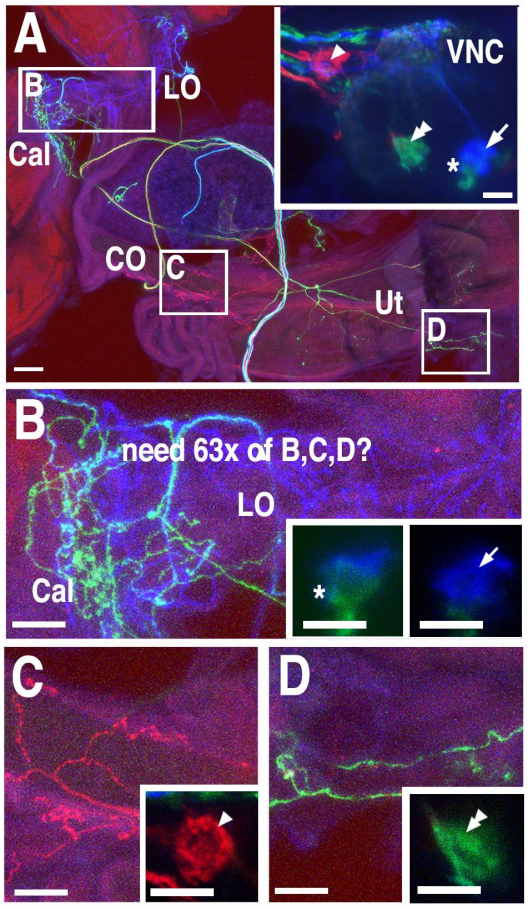



**Figure 6. Legend to follow.** This figure is a work in progress.

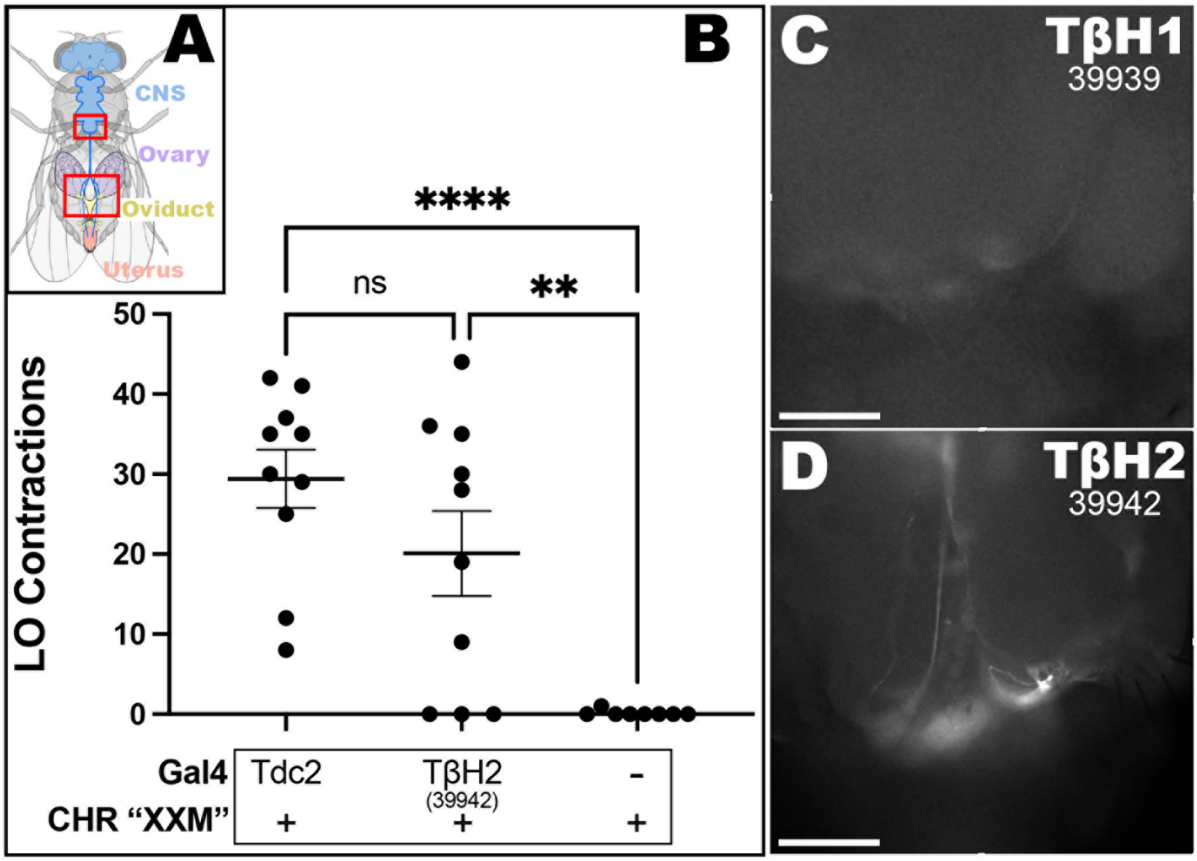



**Figure 7. Stimulating subsets of POC neurons that innervate the calyx can stimulate lateral oviduct muscle behavior.** An intact preparation (A.) exposing the AbG and oviduct via two small windows cut in the ventral cuticle/bodywall (red rectangles) was used to compare the effects of stimulating cells that express two distinct TβH-Gal4 alleles ("TβH1" and "TβH2"). B. UAS-CHR2(XXM)-tdTomato expression in TβH2 (+) cells reliably facilitated lateral oviduct contractions upon exposure to stimulating light, similar to the effects of stimulating Tdc2 (+) cells, whereas stimulation of TβH1 (+) cells or preparations lacking a Gal4 did not. C. Visualization of the tdTomato tag in TβH1 (+) cells did not show signal in lateral oviduct regions. D. Visualization of the tdTomato tag in TβH2 (+) cells showed signal in lateral oviduct regions.

Scale Bars = 100 μm

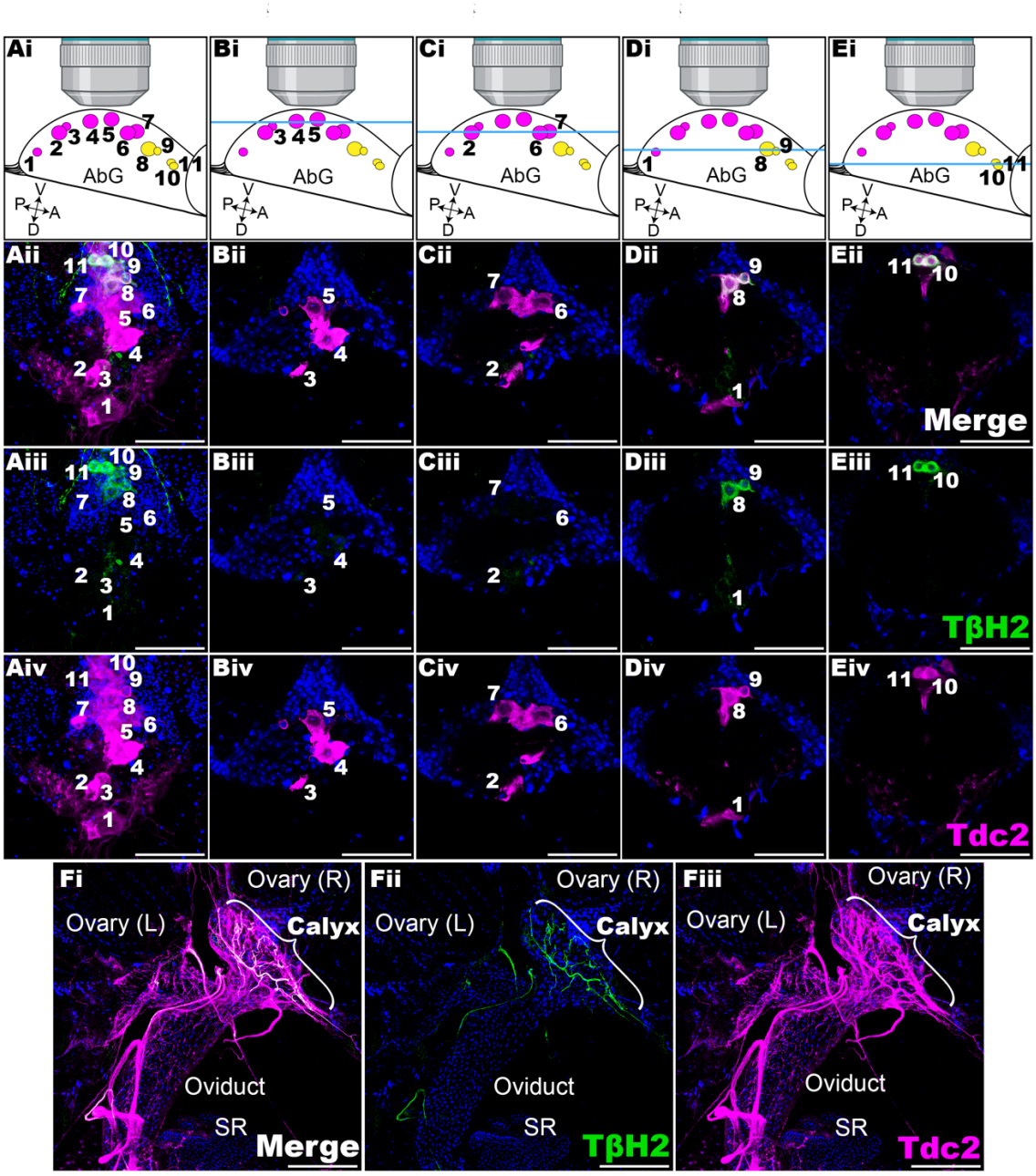



**Figure 8. The restricted TβH-Gal4 ("TβH2") expresses in POC neurons that innervate the calyx.** Ai. Side-view cartoon of Abdominal Ganglion (AbG) preparation and imaging angle. Aii-iv. Top-down maximum signal projection through the AbG (80

μm) overlaying signal from GFP (green), RFP (magenta), and DAPI (blue) labeling in a sample where TβH2-Gal4 drives UAS::mCD8-GFP and Tdc2-LexA drives LexAop::CD2-RFP. B-E. Images from 4 descending focal planes (Bi-Ei. blue lines) included in the maximum signal projection (A.). At least 4 cells are TβH2 (+), Tdc2 (+) (Ai-Ei, yellow circles). At least 7 cells are TβH2 (-), Tdc2 (+) (Ai-Ei, magenta circles). Fi-iii. In preparations of the reproductive tract, TβH2 (+), Tdc2 (+) projections innervate the calyx region between the ovaries and lateral oviducts.

Scale Bars = 10 μm (A-E) , 100 μm (F).

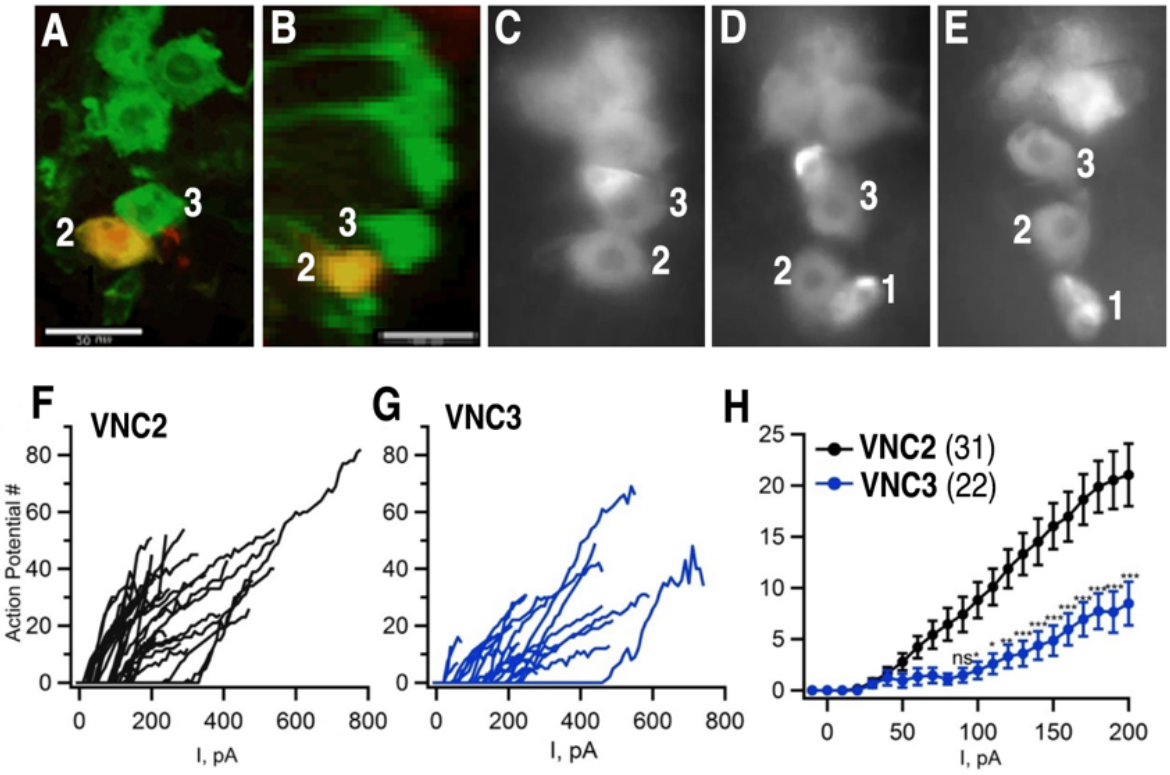



**Figure 9: VNC2 neurons are more excitable than VNC3 neurons.** Whole cell patch clamp was performed on VNC2 and 3. A,B. Confocal image of VNC2 and 3 in a horizontal position (A) and digitally rotated ~90 degrees (B). Tdc2 neurons are labeled

for GFP and VNC has been injected with biocytin. C-E. Three examples of preparations used for recording showing variations in distance between VNC2 and 3 and the variable presence of VNC1 in the field of view. The number of Action potentials vs current injection of VNC2 (F) and VNC3 (G) measured in Whole-cell current clamp mode measured in adult flies TDc2-GFP (4-8days old). H) Average of VNC2 (n=31) and VNC3 (n=22, mean + WEM) ns=0.1307, *p=0.0357 to 0.0122, ** p-0.0022, ***p=0.002 to <0.0001.

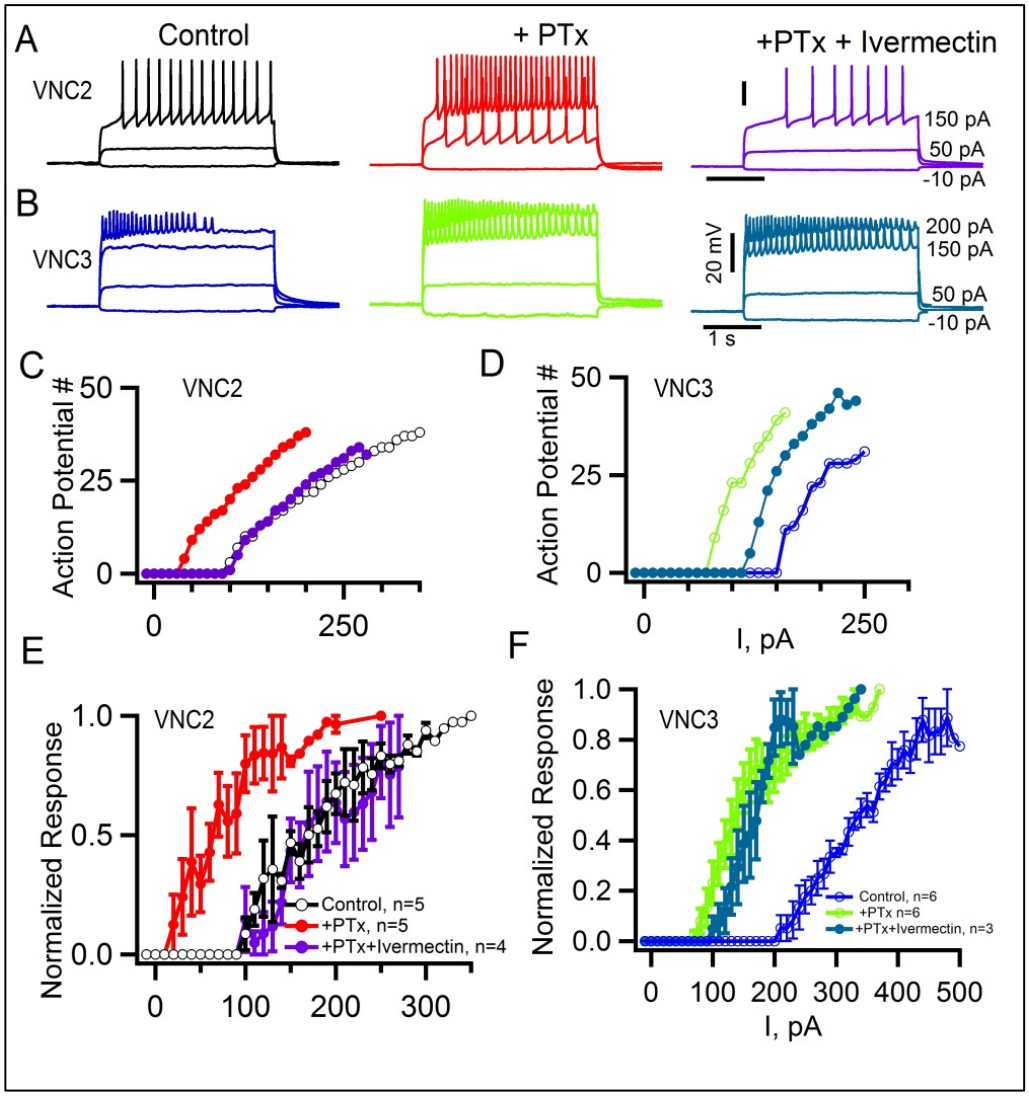


**Figure 10: Picrotoxin relieves tonic inhibition of VNC neurons and Ivermectin restores inhibition in VNC3.** Action potentials elicited at indicated current injections in control, after application of 100 µM Picrotoxin, (PTx) and in presence of 100 µM PTx +1 µM Ivermectin for VNC2 neuron **(A)** and VNC3 neuron **(B). (C)** The number of action potentials vs current injections of the VNC2 neuron (C) and VNC3 neruon (D). These are from the same neuron measured in (**A**) for VNC2 neuron and **(B)** for VNC3 neuron. The mean normalized response of VNC2 neurons in all the three conditions (control n=5, +PTx, n=5 and +PTx +Ivermectin, n=4) **(E)** and for VNC3 neuron **(F) (** control, n=6, +PTx= 6 and +PTx+Ivermectin , n=3).

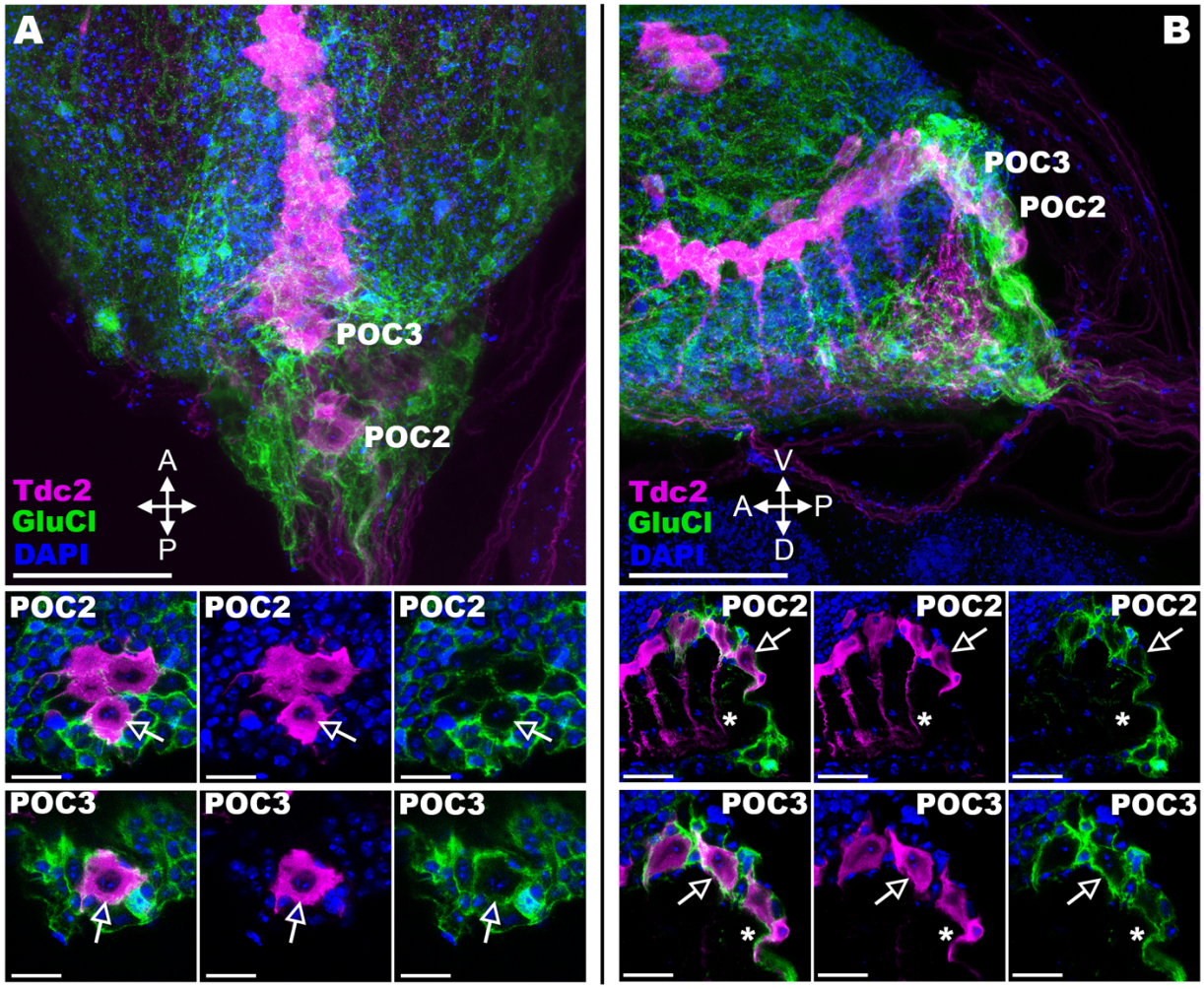



**Figure 11. POC neurons do not express the Glu-Cl receptor.** This figure is a work in progress. GluCl-Gal4 expression was compared to Tdc2-LexA expression using the reporters UAS::mCD8-GFP and LexAop::CD2-RFP. Membrane stains from top-down (A.) and side-angle (B.) views suggest no coexpression between Tdc2-LexA and GluCl-Gal4. However, POC2 and POC3 neuron projections in the AbG neuropil (B, high mag, asterisks) differ in their proximity to GluCl (+) cells.

## CHAPTER 6: SUMMARY OF MAJOR FINDINGS AND FUTURE DIRECTIONS

The aminergic regulation of both central and peripheral circuits is conserved from flies to mammals and the fly oviposition circuit represents a powerful genetic model to explore the underlying mechanisms (White et al., 2021, Lim et al., 2014, Meiselman et al., 2018, Rodriguez-Valentin et al., 2006, Hasemeyer et al., 2009, Rezaval et al., 2014, Castellanos et al., 2013). The work in Chapter 2 of this dissertation used optogenetics and receptor mutants to explore the roles of octopamine on oviduct contractility. The regulation of oviduct contractility is found to be complex, and OA contributes to both contraction and dilation. The two OA receptors previously shown to be required for female fertility – Octβ2 and OAMB-- show distinct expression patterns and primarily regulate contraction and dilation respectively. The central function of glutamate in governing contractions is confirmed (Lange, 2009, Rodriguez- Valentin et al., 2006, Castellanos et al., 2013, Gou et al., 2014), but additional data suggests a more complex role for glutamate and unexpected interactions with octopaminergic pathways.

Previous studies have consistently suggested that glutamate drives contractions in the reproductive tract, but the reported effects of octopamine have varied depending on both the species and the specific sites within the reproductive tract (Dustin Rubinstein et al., 2014, Lange, 2009, Kalogianni and Theophilidis, 1995, Lange and Orchard, 1986, Cook and Wagner, 1992, Hana and Lange, 2017, Tamashiro and Yoshino, 2014b, Rodriguez-Valentin et al., 2006, Middleton et al., 2006, Rubinstein and Wolfner, 2013). In particular, several previous reports have indicated that octopamine can induce muscle relaxation in locusts and flies (Dustin Rubinstein et al., 2014, Lange, 2009, Rodriguez-Valentin et al., 2006, Cook and Wagner, 1992). By contrast, bath applied octopamine has been reported to drive contractions in crickets (Mizunami and

Matsumoto, 2017, Tamashiro and Yoshino, 2014b), and movements observed at the base of the reproductive tract may represent oviduct contractions in flies (Middleton et al., 2006, Meiselman et al., 2018). In this work, it is determined that optogenetic activation of octopaminergic neurons and bath applied octopamine results in rhythmic contractions and calcium transients in the LO but in the absence of glutamate, but neither have a detectable effect on the CO. Bath applied OA also causes dilation of the oviducts but with a longer time course than contractions.

Methodological differences may account for some of the differences between these findings and others including perhaps the simultaneous electrical stimulation of the MAN (Rodriguez-Valentin et al., 2006) and variations in the concentrations of OA (Middleton et al., 2006, Rodriguez-Valentin et al., 2006). In addition, the effects of OA on the LO versus the CO are different and can be difficult to distinguish based on movement alone. By expressing a calcium sensor in muscle, the contribution of the ovaries, LO and CO to movement within the reproductive tract as whole are easier to discern. Finally, it is possible that some of the observations described here could have been confounded by tyramine co-released from octopaminergic neurons and perhaps activation of tyramine receptors by bath-applied octopamine. Tyramine has been shown to regulate the reproductive tract in Drosophila (Avila et al., 2012), other insects (Hana and Lange, 2017, Donini and Lange, 2004) and related species such as ticks (Cossio-Bayugar et al., 2012), and at least one tyramine receptor is expressed in the Drosophila reproductive tract (El-Kholy et al., 2015). Further experiments will be needed to explore the potential effects of tyramine on oviduct relaxation and contraction in Drosophila.

Differences between the responses of the LO and the CO to OA and glutamate may be important for the function of the oviposition circuit. Bath application of glutamate or optogenetic stimulation of ILP7 neurons drives contractions in both the common and lateral oviducts. By contrast, the response to bath applied OA in the absence of glutamate and optogenetic stimulation of Tdc2 neurons is restricted to the LO. In addition, the response of the CO is primarily confined to a single contraction, while the LO undergoes a series of rhythmic contractions. Further experiments will be needed to determine the function of each of these effects. It is possible that both are required for forward movement of the egg through the oviducts. However, lateral oviduct contractions have other functions. For example, some contractions of the LO may, in addition to contractions in the ovary, help to elicit mechanical activation of the egg (Heifetz et al., 2001, Horner and Wolfner, 2008, Kaneuchi et al., 2015). While retrograde movement of eggs has not been described in Drosophila, contractions to promote egg-retention are well described in digging insects such as locust (reviewed in (Lange, 2009)). It is therefore conceivable that the function of some contractions in flies could be to retard forward movement of the egg, perhaps during selection of an oviposition site. Parsing the contribution of each anatomical and neuronal element within the oviposition circuit will be critical to understand the complex interplay between multiple neuromodulatory pathways within this circuit. Moreover, the logic underlying the function of each element and their interactions may be applicable to other circuits in both the periphery and the CNS.

Comparing the responses and receptor expression patterns within the oviduct provides important clues to the mechanism by which OA regulates its function. Bath

application of OA induces a sustained increase in cytosolic calcium in the epithelium of both the LO and the CO. While OAMB is expressed at both sites, Octβ2R is only expressed in the epithelium of the LO, suggesting that cytosolic calcium in epithelial cells may be regulated primarily by OAMB. Genetic rescue experiments indicate that OAMB expression in the epithelium is required for egg laying (Lee et al., 2003, Lee et al., 2009, Lim et al., 2014) and OAMB mutants are unable to dilate the oviduct in response to bath applied OA. These data are consistent with the idea that OAMB signaling in the epithelium may indirectly regulate muscle relaxation as previously suggested based on genetic rescue of fertility (Lee et al., 2003, Lee et al., 2009, Lim et al., 2014).

In contrast to OAMB, Octβ2R appears to be primarily responsible for contractions rather than dilation. In addition to the epithelium, Octβ2R is expressed in at least three subtypes of neurons in the reproductive tract. These include ppk1(+) cells whose somata reside in the periphery and both glutamatergic/ILP7(+) and Tdc2(+) processes that project from their cell bodies in the AbG. Optogenetic activation of ppk1(+) neurons, the epithelium and Tdc2(+) neurons had minimal effects on LO contractions in the Abdominal Fillet. By contrast, activation of ILP7(+) neurons induced repetitive contractions in a subset of preparations. Together with additional observations that glutamate or octopamine can induce LO contractions in an Abdominal Fillet, it can be speculated that the mechanism by which OA initiates LO contractions may occur via activation of Octβ2R on ILP7 terminals and subsequent release of glutamate. The relevant signaling pathways might be similar to those at the larval NMJ in which both

Octβ1R and Octβ2R regulate the function of glutamatergic nerve terminals (Koon et al., 2011, Koon and Budnik, 2012).

Experiments interrogating the contribution of processes in the periphery depended on their differential sensitivity to optogenetic stimulation. Removing the somata of Tdc2 neurons in an Abdominal fillet ablated their response of the remaining distal processes to optogenetic stimulation. By contrast, the response of distal ILP7 processes was preserved in the absence of cell bodies. The response of distal ILP7(+) but not Tdc2(+) processes to optogenetic stimulation could reflect differences in their sensitivity to depolarization or downstream processes such as the ability of depolarization to induce calcium influx (Xing and Wu, 2018a, Xing and Wu, 2018b, Harrigan et al., 2020). These differences may also explain the relative insensitivity of Tdc2(+) neurons to stimulation using a standard ChR2 variant rather than ChR2-XXM/L.

To more definitively test the hypothesis that activation of ILP7 neurons is involved in OA-mediated LO contractions, two RNAi transgenes were expressed to target knockdown of Octβ2R in ILP7(+) neurons. RNAi was also used to test the more general idea that activation of Octβ2R on neurons rather than those on epithelial cells is responsible for LO contraction. Thus far, the results have been inconclusive, and further genetic rescue experiments will be needed to evaluate each of these possibilities. The function of octopamine receptors on both ppk1(+) neurons and Tdc2(+) neurons also remains unclear. Octβ2R could potentially act as an autoreceptor on Tdc2(+) nerve terminals as described at the larval NMJ (Koon et al., 2011, Koon and Budnik, 2012). In ppk1(+) cells, it is possible that Octβ2R and/or OAMB could modify mechanosensory activity or perhaps regulate signaling to neurons in the AbG (Gou et al., 2014, Yang et

al., 2009, Hasemeyer et al., 2009, Lee et al., 2016), but further experiments will be needed to test these hypotheses.

Expression of neither Octβ2R nor OAMB was detected in muscle cells, suggesting that most octopaminergic effects on oviduct dilation or contractions are mediated via receptors expressed in either neurons or the epithelium (Lee et al., 2009, Lee et al., 2003, Lim et al., 2014). However, a failure to detect expression using the MiMIC lines cannot be completely ruled out, and low levels of OA receptor expression in muscle may still contribute to contractility as suggested for the effects of Octβ2R on fertility (Li et al., 2015). Indeed it is likely that the effects of both OA and glutamate are complex and that multiple signaling pathways are active within the oviposition circuit. Developmental effects in Octβ2R or OAMB mutants also may have influenced the results presented here. Future experiments using genetic rescue during development versus adulthood will be important to assess this.

Whether the effect of OA on the oviduct musculature is causal for its effect on fertility remains to be tested. OAMB is active at multiple sites within the reproductive tract including the sperm storage organs and follicle cells (Deady and Sun, 2015, Avila et al., 2012). It is possible that the loss of fertility caused by octopaminergic pathways is independent of those that regulate either oviduct contractions or dilation.

In sum, Chapter 2 of this dissertation shows that octopamine regulates oviduct contraction via two distinct receptors, and  interactions with glutamate may further modify the activity of these pathways. This complex network of receptors and the mechanisms by which they interact will clearly require further experiments to fully understand. Studies of the crab stomatogastric ganglion have yielded fundamental

insights into rhythm generation. It appears that the rhythm of visceral muscles could follow a different logic, and this dissertation shows that these and other experiments using the fly oviposition circuit will enhance our understanding of the evolutionarily conserved logic by which octopamine and other biogenic amines regulate circuit function and behavior.

With regards to the VMAT variants described in Chapter 3 of this dissertaion, in vitro studies have revealed fundamental information about the molecular machinery responsible for transporter trafficking [43-48]. Additional in vivo models are required to determine the effects of transport on behavior and the contribution of the endogenous milieu to transport activity [49-58]. The expression of mutations as exogenous transgenes represents a powerful approach to determine how alterations in trafficking may disrupt behavior; however, transgenic expression systems may not fully capture the regulatory patterns of the endogenous gene. The use of transgenes for gene expression can also complicate the use of additional transgenic probes for neuronal stimulation and imaging. The use of CRISPR-Cas9 to disrupt trafficking signals in the C-terminus of the endogenous *dVMAT* locus is described here, and initial phenotypic analysis of the new allele is provided.

A relatively large number of lines (450 total from 100 injected founders) was screened to ensure that we would obtain a candidate. Although it is difficult to predict the efficiency of a given homologous repair construct, the number of candidates obtained suggested that smaller phenotypic screens for other mutations may be sufficient, assuming that the oligo repair construct is optimized to $\leq$ 90 bp [21]. A phenotypic screen may be similarly used to identify CRISPR-Cas9 mutants in other

genes in cases in which standard marker-based screens using a dsDNA construct are not convenient, e.g., because a site for a scarless insertion is not apparent. In addition, the presence of DNA repeats can complicate the generation of the relatively large homology arms used for some ds repair constructs. Indeed, parallel attempts to generate a dsDNA construct for introduction of a premature stop codon in *dVMAT* were hampered by repeats in the 3' UTR (data not shown).

Consistent with previous findings using a *dVMAT-D3* transgene, we find that the endogenous CRISPR-Cas9 mediated *dVMAT*$^{D3}$ mutation impairs female fertility and fecundity but has no detectable effect on baseline larval locomotion [11]. The mutation also presents with retention of mature follicles in the ovaries, suggesting that loss of synaptic signaling or an increase in extrasynaptic signaling may disrupt follicle rupture. Follicle rupture is mediated by OAMB [28] and future experiments will more specifically explore the relationship between octopamine release in *dVMAT*$^{D3}$ mutants and the activity of OAMB receptors in follicle cells.

It is possible that *dVMAT*$^{D3}$ directly disrupts follicle cell rupture by changing octopamine release within the ovaries. Alternatively, it is possible that octopaminergic synapses in the CNS *upstream* of the reproductive tract are indirectly responsible for this phenotype. The methods established here to express optogenetic and imaging transgenes in the *dVMAT*$^{D3}$ mutant background will be useful to explore upstream elements of the oviposition circuit in future experiments. Here, optogenetics is coupled with live imaging to analyze the effects of *dVMAT*$^{D3}$ on oviduct contractility *downstream* of ovulation. There exists no detectable difference between *dVMAT*$^{D3}$ and wild type controls for the contraction of lateral oviduct muscle in response to optogenetic

stimulation of octopaminergic neurons. These data indicate that at least one element of the oviposition circuit downstream of ovulation is not disrupted by *dVMAT*$^{D3}$. However, octopamine also regulates dilation of the oviducts, another process downstream of ovulation that may influence fertility [33, 42]. Dilation can be easily visualized when the reproductive tract is dissected out of the abdomen and octopamine is bath-applied to the reproductive tract [33, 42]. Unfortunately, visualization of dilation is difficult with the intact preparations that are required for optogenetic stimulation of octopamine neurons within the abdominal ganglion [42]. Since the effects of *dVMAT*$^{D3}$ cannot be tested using bath-applied octopamine, it cannot be ruled out that *dVMAT*$^{D3}$ causes a defect in oviduct dilation.

In sum, while other aspects of the oviposition circuit may be disrupted by *dVMAT*$^{D3}$, the data in Chapter 3 of this dissertation suggest that retention of mature eggs in the ovaries is likely to be responsible for the decrease in fertility. Moreover, at least some downstream processes such as oviduct contractions appear to be unaffected by a shift in octopamine release from SVs to LDCVs.

In future experiments, additional drivers could be used to express RCaMP in subsets of other neurons to analyze the effects of *dVMAT*$^{D3}$ on aminergic circuits unrelated to oviposition. These include an octopaminergic circuit in the visual system that regulates the response of flies to odor plumes during flight [12]. Ongoing experiments will determine whether *the dVMAT*$^{D3}$ line replicates other phenotypes previously seen using a *UAS-DVMAT-D3* transgene [12]. If so, expression of RCaMP in visual system neurons combined with *dVMAT*$^{D3}$ may be used to investigate the underlying mechanisms.

Previous data indicates that the *dVMAT-D3* transgene traffics less to SVs, and we speculate that some aspects of the *dVMAT*$^{D3}$ phenotype are due to a decrease in amine release from SVs. However, in addition to a decrease in sorting to SVs, we have previously shown that the *dVMAT-D3* transgene localizes more to LDCVs than the wild type transporter [11]. Therefore, it is possible that an increase in amine release from LDCVs may also contribute to *dVMAT*$^{D3}$ phenotypes. Further experiments using mutations that more specifically disrupt sorting to SVs, may resolve this issue. Alternatively, it may not be possible to reduce dVMAT sorting to SVs without increasing its localization to LDCVs; another mutation (Y600A) also led to an increase in the localization of dVMAT to LDCVs [11]. There may be communication between the pathways for biogenesis and/or recycling of SVs and LDCVs in flies, and at present, it is only clear that the *dVMAT*$^{D3}$ phenotype results from a change in the balance of amine release between SVs and LDCVs.

The variety of octopamine-dependent processes within the oviposition circuit and the CNS provide a model to further probe the effects of dVMAT trafficking on circuit function. We speculate that further experiments using *dVMAT*$^{D3}$ coupled with optogenetics and imaging will help to elucidate the circuit properties that govern synaptic versus extrasynaptic signaling.

It has been known for decades that Oa regulates female fertility and the oviposition circuit in *Drosophila* and other insects (Lange 2009; Tamashiro and Yoshino 2014; White, Chen, and Wolfner 2021; Monastirioti, Charles E. Linn, and White 1996; Zheng et al. 2021). In *Drosophila*, some loss of function mutants that disrupt Oa synthesis and/or release share a common phenotype marked by retention of mature

oocytes in the ovaries (Deady and Sun 2015; Cole et al. 2005; Monastirioti, Charles E. Linn, and White 1996; Monastirioti 2003; Andreatta et al. 2018). Similarly, studies utilizing mutations in *Oamb* and *Octb2R* have demonstrated a decrease in ovulation and retention of mature follicles within the ovaries (H.-G. Lee et al. 2003; H.-G. Lee, Rohila, and Han 2009; Lim et al. 2014b; Li et al. 2015). Oamb also regulates sperm storage, secretory cell activity, oviduct dilation (Avila et al. 2012; Middleton et al. 2006; D. S. Chen, Clark, and Wolfner 2022) and germline stem cell proliferation (Yoshinari et al. 2020; Hoshino and Niwa 2021), while Octb2R is required for lateral oviduct contractions (Deshpande et al. 2022). *Drosophila* express four other Oa receptors in addition to Oamb and Octb2R (Balfanz et al. 2005; Maqueira, Chatwin, and Evans 2005; Qi et al. 2017; McKinney et al. 2020) but their expression patterns and function in the reproductive system have remained unknown.

To gain further insight into the mechanisms by which Oa may regulate oviposition, the work presented in Chapter 4 of this dissertation used a panel of high-fidelity Gal4 “MiMIC” lines to map expression in the reproductive tract of all the known *Drosophila* Oa receptors. The work in Chapter 3 shows that *Oamb* and *Octb2R* are expressed in peripheral neurons proximal to the oviducts and uterus as well as central neurons that project from the AbG to the reproductive tract (Deshpande et al. 2022). Chapter 4 elaborates on this work to show that multiple peripheral neurons that localize to the reproductive tract also express *Octa2R*, *Octβ1R, Octβ3R* and *Oct-TyrR*. These include cell bodies proximal to the oviducts in the MAN and embedded in the musculature of the uterus. Most of these co-express *ChaT* and label with the neuronal

marker HRP. Exceptions include a small subset of cells in the posterior uterus that do not appear to label with anti-HRP.

Using a restricted *ppk1.0-LexA* driver known to express in a subset of afferent SPR(+) neurons, one example anterior uterine cluster of at least three peripheral neurons in the post-mating circuit is shown to co-expresses the cholinergic marker *ChaT* (Yoshinari et al. 2020). These post-mating circuit neurons all express octopamine receptors. Two neurons express *Octα2R*, *Octβ1R*, and *Octβ3R*, while one neuron expresses only Octβ3R. Other neurons that appear to localize to the same cluster but are ppk1.0(-) express *ChaT* and *Octα2R* but no other Oa receptor. Such partially overlapping co-expression suggests that the post-mating circuit might be divided into functional units that differ by their expression of different Oa receptor subtypes.

Other cell types proposed to express OA receptors include follicle cells that surround the developing oocyte and epithelial cells that line the lumen of the oviducts (Deady and Sun 2015; Sun and Spradling 2013; Lim et al. 2014; Lee, Rohila, and Han 2009; Lee et al. 2003; Li et al. 2015; White, Chen, and Wolfner 2021). Chapter 4 of this dissertation now shows that cells which line the lumen of the seminal receptacle also express *Oamb*, similar to its expression in the epithelium of the oviducts (Deshpande et al. 2022; Lim et al. 2014; Li et al. 2015; Lee, Rohila, and Han 2009; Lee et al. 2003) and that $Ca^{2+}$ levels in the muscle of the seminal receptacle are sensitive to Oa. These results suggest that the epithelial cells of the seminal vesicle may control the surrounding muscle similar to the mechanism previously proposed for the oviducts (H.-G. Lee et al. 2003; H.-G. Lee, Rohila, and Han 2009; Lim et al. 2014b). The appearance of wave-like patterns in the seminal receptacle muscle $Ca^{2+}$ activity is intriguing, and

such behavior may play a role in sperm movement within the lumen of the organ, similar to function of muscle contractions in the movement of eggs within the oviducts.

Previous results indicate that loss of *Octβ2R* blocks contraction of the lateral oviducts and optogenetic activation of *Octβ2R* expressing neurons can induce lateral oviduct contractions (Deshpande et al. 2022). Optogenetic activation of *Octβ1R* and *Oct-TyrR* expressing neurons can also induce lateral oviduct contraction. Since mutation of *Octβ2R* essentially blocks contractions caused by bath applied Oa, the possibility that Octβ1R, Octβ2R, and Oct-TyrR represent three equally important, parallel pathways within the reproductive tract that mediate oviduct contraction seems unlikely. It is more likely that Octβ1R and Oct-TyrR are active in neurons within the CNS and upstream of Octβ2R. Alternatively, it remains possible that some of the cells that express *Octβ2R* also express *Octβ1R* and *Oct-TyrR*, but that only the function of Octβ2R is required for contractions. Further co-labeling studies and the development of mutations in *Octβ1R* and *Oct-TyrR* will help to distinguish between these possibilities.

While previous studies have demonstrated a requirement for Oa in the regulation of sperm storage, the more acute effects of octopaminergic signaling in sperm storage organs have been less clear. This work shows that Oa induces calcium transients in secretory cells of the spermathecae and that this effect is blocked by knockdown of *Oamb* within these cells. These data are consistent with a previously assigned role for Oamb in sperm storage (D. S. Chen, Clark, and Wolfner 2022; Avila et al. 2012). The relatively high sensitivity of the spermatheca cells to Oa may reflect differences in the relative affinity of Octβ2R versus Oamb, or perhaps differential access of the receptors to bath applied Oa. Concentration-dependent effects have also been observed in the

reproductive tracts of other insect species exposed to Oa (Abdoun et al. 1995; Wong and Lange 2014; Lange 2009; Xu et al. 2017).

Functional data using dTrpA1 indicate that activating OaRNs can impede ovulation and egg laying. These effects were confirmed using Kir2.1 and $Shi^{ts}$ to inhibit cells that express Oa receptors, which produced an increase in egg-laying. Initially these data were surprising, since previous studies have focused on octopaminergic processes that appear to facilitate ovulation and oviposition (Pang et al. 2022; D. S. Chen, Clark, and Wolfner 2022; White, Chen, and Wolfner 2021; Lim et al. 2014b; Li et al. 2015; H.-G. Lee et al. 2003; Middleton et al. 2006; Monastirioti 2003; Monastirioti, Charles E. Linn, and White 1996; Deady and Sun 2015). The use of gain of function transgenes to activate neurons can uncover effects that are less obvious using loss of function receptor mutants and RNAi transgenes. The use of both gain of function and loss of function strategies are also important for probing oviposition, since the same gene products may be active in multiple, sequential processes and subject to epistatic effects. In particular, the epistatic relationship between follicle rupture and other processes involved in oviposition may require the use of gain of function methods. For Oa signaling mutants in which oocytes never leave the ovary, *downstream* effects in the uterus may be difficult or impossible to detect. Therefore, the unusual uterine retention phenotype reported here may be absent in loss of function *Oamb* mutants because oocytes are trapped at an upstream site in the ovary (Deady and Sun 2015; H.-G. Lee et al. 2003; H.-G. Lee, Rohila, and Han 2009; Yoshinari et al. 2020). Similarly, oviduct retention might be occluded by upstream retention of mature follicles in the ovaries. If

so, retention of eggs in the ovary seen with *Octb2R* knock-down or mutants might have occluded downstream effects in the oviducts (Lim et al. 2014b; Li et al. 2015).

Although the epistatic relationship is less obvious, some of the effects reported here via *in vivo* experiments may reflect disruption of processes *upstream* of follicle rupture. Instances of follicular atresia observed in Octα2R and Oct-TyrR hyperactivation assays suggest that circuits expressing these receptors can produce defects in follicle development, possibly by disrupting fly nutritional intake or homeostatic systems. Though this phenotype seems to be only partially penetrant, such effects may occlude effects on any downstream egg laying processes. The lack of any obvious impairment to follicular development in hyperactivation assays involving the other Oa receptors, however, suggests that the effects observed for most Oa receptor expressing cells are likely due to direct disruption of reproductive tract behavior rather than broader, metabolic mechanisms able to affect multiple organ systems.

CNS circuits that regulate oviposition include pCL1 neurons in the brain that innervate oviposition descending neurons (oviDNs) (F. Wang et al. 2020; Feng et al. 2014). It is tempting to speculate that OaRNs might regulate pCL1 or oviDN, or additional excitatory or inhibitory neurons within the same circuit (F. Wang et al. 2020). Particularly alluring is the observation that hyperactivation of Oamb(+) neurons results in retention of eggs in the uterus just prior to deposition (Fig. 6, B). Following follicle rupture, eggs ovulate and pass through the oviduct in a very short amount of time in WT flies and without significant delay under baseline conditions (Mattei et al. 2015). By contrast, flies can retain fertilized eggs in their uterus until sensory inputs indicate that egg laying can occur in a predator/toxin free environment (Pang et al. 2022). Oamb(+)

neurons in the CNS may therefore represent a behavioral choice point and regulate the decision to deposit eggs. Further studies of the CNS connectome combined with single cell sequencing, and optogenetics will be needed to test this hypothesis and identify the underlying circuits.

It remains unclear why hyperactivation of presynaptic Tdc2 neurons with dTrpA1 does not appear to increase egg-laying. Similarly, *dTrpA1* expression in Tdc2 and Tβh neurons rescued a reduction in oviposition caused by exposure to parasitoid wasps, but did not elevate egg-laying beyond WT levels (Pang et al. 2022). If the effects of Oa are as complex as this dissertation works suggests, where it it shown to both promote and retard ovulation and oviposition, then simultaneous activation of all octopaminergic pathways might not appear to have any effects under some conditions. It is also possible that, under some circumstances, octopamine and tyramine have opposing effects in oviposition as they do for larval locomotion (Saraswati et al. 2004). Further experiments using intersectional drivers that are specific for subsets of octopaminergic neurons may be needed to detect a net loss or gain in fertility in the absence of exogenous stimuli such as threats from parasitoid wasps (Pang et al. 2022). A previously described intersectional approach using *doublesex* is useful for restricting expression in the multicellular cluster of octopaminergic neurons that innervates the reproductive tract, but this tool cannot be used to help stimulate individual octopaminergic neurons within the cluster (Rezával et al. 2014).

Further experiments will also be needed to determine which post-synaptic neurons that express specific Oa receptors are responsible for the effects reported here. The effects of hyperactivating or silencing OaRNs on egg laying are recognizably

similar to those seen in experiments involving the SPR(+) neurons of the post-mating circuit, where neural activity is correlated with inhibition of egg laying processes (Yoshinari et al. 2020; Yang et al. 2009). Based on findings of co-expression between Oa receptors, *ChAT*, and *ppk1.0* in peripheral neurons shown here, it is possible that some experiments using *Oa-receptor* divers produce expression of dTrpA1 or Kir2.1 in the same cells as in similar experiments using *SPR* drivers that achieve the same effect. Neurons in the peripheral SPR(+) post mating circuit that express Oa receptors may thus represent a specific group of neurons responsible for some of the egg laying phenotypes we observe. Intriguingly, individual cells in this group express differing profiles of Oa receptor subtypes, and different Oa receptor subtype drivers affect different stages of the egg laying process as seen in egg laying time percent assays. These results may suggest that distinct post-mating circuit neurons inhibit different stages of egg laying until SP silences their activity. Further intersectional studies using SPR and OaRN drivers could prove useful to uncover single-cell heterogeneity in the post-mating circuit.

Importantly, all the transgenes used here act by directly activating or inhibiting neuronal activity rather that activating or inactivating Oa receptors. Oa receptors, like most other GPCRs can have net “inhibitory” or “excitatory” effects which can vary across cell types, downstream effectors and the subcellular location of the receptors (Robb et al. 1994; M. Wang et al. 2007). The coupling of octopamine receptors to excitatory G proteins and downstream effectors has been extensively examined *in vitro* and in the epithelial cells within the reproductive tract (Y.-C. Kim et al. 2013; H.-G. Lee, Rohila, and Han 2009; Debnath, Williams, and Bamber 2022; Xu et al. 2017). The

effects seen in neuron hyperactivation experiments using Oa-receptor drivers may be similar to the effects of increased Oa signaling to such cells. Further experiments will be needed to more precisely determine the *in vivo* effects of Oa receptors in neurons within the oviposition circuit and CNS as well as how each may influence egg-laying.

In Chapter 5 of this dissertation, 10 TDC2(+) cells in the AbG that innervate specific regions of the RT are identified and mapped. These include two cells that innervate the Sp, three for the Calyx +/- portions of the ovaries or LO, and one each for the Posterior oviduct, the ovaries, and the Lateral+Common Oviducts. The positions within the OA cluster for some cells were unambiguously identified in individual MCFO preparations. Others could be deduced by comparing labeled cells across multiple preparations. Ephys of two cells shows that they share a common baseline inhibition yet are differentially excitable. These data form the basis for further functional studies of each cell and how they may interact to regulate the oviposition circuit.

The cells have been named in two ways: one as a descriptor of the organs they innervate and a second representing their approximate order in the AbG from posterior to anterior. The most posterior cell that we have unambiguously identified innervates the Sp and we designate it VNC1 or Sp1. It can be seen in Fig 1 as a single yellow cell in the cluster and in Fig 3 as the more posterior pink cell. The two innervation patterns within the stalks of the Sp are very similar and boutons from each cell are adjacent to one another at multiple points. The functional relevance of each cell remains to be determined.

Both VNC2 and VNC3 innervate the uterus and the posterior portion of the common oviduct. Similar to the Sp, no differences have yet been detected between the

innervation patterns. We detect innervation of both the Posterior oviduct and Uterus representing either VNC2 or VNC3 in preps including those shown in Fig 1, 2 (albeit faint), 3, 4 (5) and 6. In contrast to VNC2 and 3, VNC4 innervates the uterus but not the common oviduct. In addition, innervation of the Ut appears to be more posterior than VNc2 or 3. VNC5 innervates both the LO and CO. It's location posterior to VNC1, 2 and can be seen in Fig 5. is posterior and ventral to VNC3 and we designate this VNC4 or LOCO.

Eight large VNC cells have been previously suggested to innervate the RT (Cole et al., 2005). This work identifies an additional smaller, midline cell at the tip of the AbG that innervates the Sp (Sp1) and a second, small and more anterior cell that also innervates the Sp (Sp2). The map included here in Chapter 5 thus identifies innervation patterns and positions within the VNC of 10 midline cells than innervate the RT. Based roughly on their position from posterior to anterior, the most posterior cell that innervates the Sp (Sp1) has been designated as VNC1. The next two cells innervate the Ut as well as the Post CO and these are designated VNC2 and 3 respectfully. The second, more anterior Sp cell (Sp2) is VNC4.

The LO cell is at the anterior end of the cluster just anterior to the calyx cells and is designated LO VNC10, while the two calyx-innervating cells are VNC8 and 9. The Ut, ovary, and LO+CO represent the remaining cells in the center of the cluster: VNC5, 6 and 7. The Ovary cell call be assigned to VNC6 based on its position immediately posterior to the calyx cells. The LO+CO cell is adjacent to the VNC3 (PCOU-P) and VNC4 (Sp2) and is designated as VNC5. The Post Ut cell is also suggested to likely localize adjacent to VNC3, 4 and 5, so it is designated VNC6.

The fly connectome has been mapped in the brain using EM, and ongoing efforts are likely to map the VNC. However, these experiments require serial tracing of neuronal processes in adjacent sections. The length of the processes that project from the VNC to the RT would render a similar EM reconstruction prohibitively difficult. However, other techniques may be used to map more distal connections, including GRASP and TANGO. EM to trace the processes from the VNC to the RT would be much more difficult, and the use of non-synaptic modes of neuronal communication by many aminergic neurons also reduces the power of techniques that require close synaptic contacts for mapping.

Single cell labeling techniques that map the projections of presynaptic neurons can help to overcome these difficulties. Molecular methods to label individual neurons can also facilitate electrophysiological studies. The stereotyped positions of invertebrate neurons enhances the power of this technique and has been pioneered in the STG of the crab. By contrast, both the electrophysiological properties and the projection patterns of most aminergic neuromodulatory neurons in the fly remain unclear.

Ongoing work will determine the relationship between POC neuron innervation targets and POC neuron roles in behavior. Currently available data suggests that multiple POC neurons may contribute to lateral oviduct contraction behaviors but that these contributions may vary by individual neuron. The fact that indirect circuits may facilitate such oviduct behaviors (Deshpande et al., 2022) suggests that different POC neurons may have different roles in information processing involving both downstream neurons and the RT. Some POC neurons may affect oviduct contractions directly while others may affect the same behavior via alternate, indirect pathways.

The differences in electrophysiological properties between POC2 and POC3 establish further heterogeneity in the function of POC neurons. Reponses to picrotoxin that suggest chronic inhibition of POCs by GABA receptor channel activity implicate a possible mechanism by which the POC neurons are kept dormant by GABAergic signals until mating occurs. The reconstitution of inhibition following ivermectin application to POC2 but not POC3 also shows that individual POC neurons can be differentially modulated once activated. This effect may represent a means for coordinating the activation of different stages of the egg laying process (ovulation, oviduct passage, oviposition).

Together, these data help establish the *Drosophila* POC as a model aminergic cluster useful for studying heterogeneity among aminergic neurons with shared characteristics such as localization. This cluster of aminergic neurons governs a wide range of reproductive behaviors, yet mechanisms underlying the coordination required for successful fertility remain unknown. Recent work such as that in Chapter 4 of this dissertation has shown that different types of OA receptors and different types of postsynaptic cells may contribute to different effects of octopamine signaling in different regions of the RT. The ongoing work in this chapter for the first time suggests mechanisms by which the presynaptic octopaminergic neurons themselves may also contribute to octopamines diverse roles in fly fertility.

Some of aminergic circuits in the fly consist of a single aminergic ell, e.g. CSD or DPM similar to the aminergic circuits in C. elegans. However, many other aminergic cells are part of larger clusters, e.g. the DA clusters in the adult brain. The number of aminergic neurons in mammalian nuclei are orders of magnitude higher than flies.

Nonetheless, the logic underlying the regulation of multiple downstream targets by clusters of aminergic cells may be similar. Small clusters of aminergic neurons observed in flies can be used to model the function of individual neurons among aminergic nuclei such as the LC and Raphe.

## CHAPTER 7: LITERATURE CITED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