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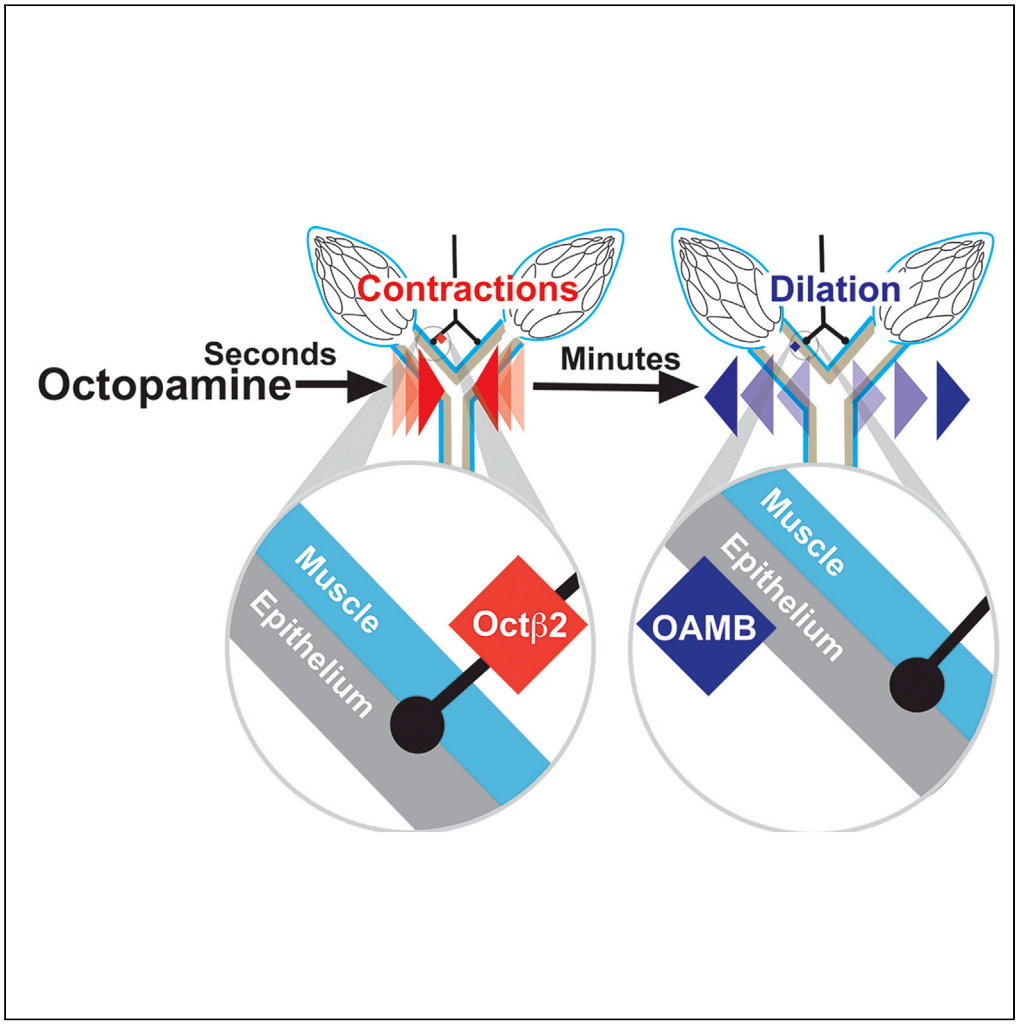

Sonali A. Deshpande, Ethan W. Rohrbach, James D. Asuncion, ..., Felix E. Schweizer, Hugo J. Bellen, David E. Krantz

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Highlights

The receptors Octβ2 and OAMB mediate oviduct muscle contraction and relaxation

The receptors are detectably expressed in neurons and epithelia but not muscle cells

The control of visceral muscles in flies and mammals may share common features

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Regulation of *Drosophila* oviduct muscle contractility by octopamine

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SUMMARY

Octopamine is essential for egg-laying in *Drosophila melanogaster*, but the neuronal pathways and receptors by which it regulates visceral muscles in the reproductive tract are not known. We find that the two octopamine receptors that have been previously implicated in egg-laying–*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* respectively. Interactions with glutamatergic pathways modify the effects of octopamine. Octopaminergic activation of Octβ2R on glutamatergic processes provides a possible mechanism by which octopamine initiates lateral oviduct contractions. We speculate that aminergic 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* and larger insects such as locusts (Lim et al., 2014; Lange, 2009; Hana and Lange, 2017; White et al., 2021; Yang et al., 2009; Zhou et al., 2012; Avila et al., 2012; Rubinstein and Wolfner, 2013; Rezaval et al., 2014; Gou et al., 2014; Yoshinari et al., 2020; Andreatta et al., 2018; Dustin Rubinstein et al., 2014; Middleton et al., 2006; Rodriguez-Valentin et al., 2006; Lee et al., 2003, 2009, 2016; El-Kholy et al., 2015; Li et al., 2015; Donini and Lange, 2004).

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., 2014; Hana and Lange, 2017; Zhou et al., 2012; Avila et al., 2012; Rubinstein and Wolfner, 2013; Rezaval et al., 2014; Gou et al., 2014; Yoshinari et al., 2020; Andreatta et al., 2018; Lange, 2009; Dustin Rubinstein et al., 2014; Middleton et al., 2006; Rodriguez-Valentin et al., 2006; Lee et al., 2003, 2009, 2016; El-Kholy et al., 2015; Li et al., 2015; Yang et al., 2009; Cossio-Bayugar 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; Schneider et al., 2019; Spencer and Hu, 2020; Sanders et al., 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; Schneider et al., 2019; Spencer and Hu, 2020; Sanders et al., 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 (Lee et al., 2003, 2009, 2016; Dustin Rubinstein et al., 2014; Lim et al., 2014; Meiselman et al., 2018; 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*

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OAMB— are essential for fertility (Lee et al., 2003, 2009; McKinney et al., 2020; Li et al., 2015; Lim et al., 2014; Rezaval et al., 2014; Dustin Rubinstein et al., 2014; Yoshinari et al., 2020; Schneider et al., 2012; Pauls et al., 2018; Monastirioti et al., 1995; 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; Rodriguez-Valentin et al., 2006; Tamashiro and Yoshino, 2014a, 2014b). 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; McGaw and Curtis, 2013; Wu and Cooper, 2012; Audsley and Weaver, 2009; Clark et al., 2008). 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; Schneider et al., 2019; Spencer and Hu, 2020; Sanders et al., 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; Schneider et al., 2019; Spencer and Hu, 2020; Sanders et al., 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 (Lee et al., 2003, 2009; Lim et al., 2014); 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) reveal 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 (Lee et al., 2003, 2009; Lim et al., 2014) and also interact with glutamatergic pathways previously shown to govern contractility (Rodriguez-Valentin et al., 2006; Yang et al., 2009; Gou et al., 2014; Castellanos et al., 2013; Hasemeyer et al., 2009; Middleton et al., 2006).

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; Arakawa et al., 1990; Han et al., 1998; Evans and Maqueira, 2005; Ohtani et al., 2006; Ohhara et al., 2012; Bayliss et al., 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; Lee et al., 2003, 2009; Lim et al., 2014). Here we focus on *OAMB* and *Octβ2R* because these have been previously suggested to be required for oviposition (Lim et al., 2014; Li et al., 2015; Lee et al., 2003, 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 (Lee et al., 2018) (Figure S1). Some of these lines have been described previously (Lee et al., 2018). Because 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 (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 (Figures 1A-i, 1A-iii, 1A-iv, 1B-i, and 1B-iii) consistent with the expression pattern driven by a GAL4 line containing a portion of the OAMB gene (Lim et al., 2014; Li et al., 2015; Lee et al., 2003, 2009). However, *Octβ2R* is exclusively expressed in the epithelia within the lateral oviduct (Figures 1B-i and 1B-iii). Although we also detect an immunofluorescent signal in the common oviduct, it arises from thin processes that are morphologically distinct from the epithelium (Figure 1B-iv). In contrast to *Octβ2R*, *OAMB* is expressed in the epithelium of both the lateral and common oviducts (Figures 1A-iii and 1A-iv).

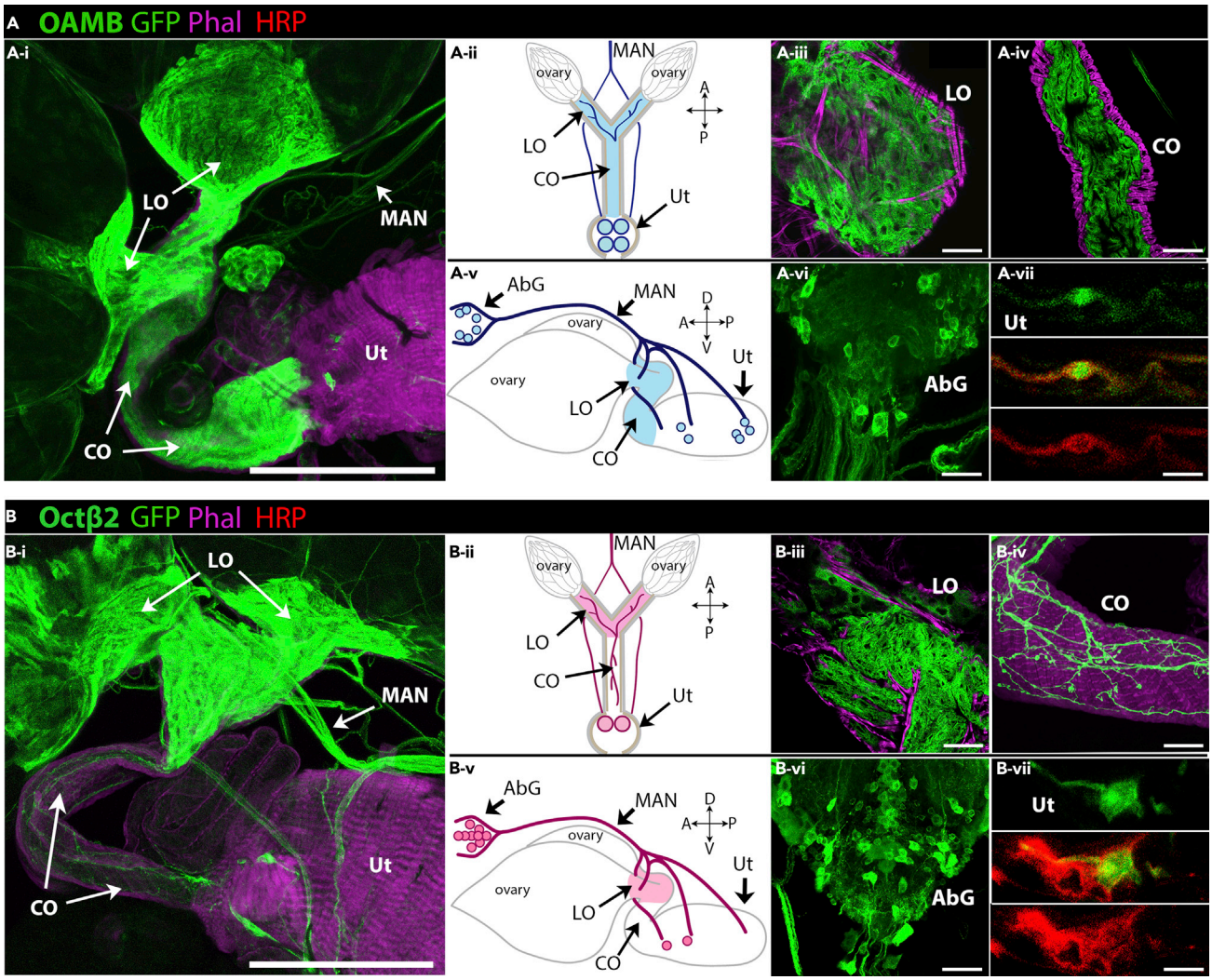


Figure 1. *OAMB* and *Octβ2* receptor expression in the oviducts

(A) (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 and 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) (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 and 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 A-iii, 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.

In addition to the epithelium, we detect *OAMB* expression in several subtypes of non-neuronal 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 (Sun and Spradling, 2013), and consistent with previous functional experiments, phalloidin (−) cells both in the caps of the spermathecae and in the seminal receptacle

(Clark and Lange, 2003; 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 (Figures 1A-vii, 1B-iv, 1B-vii, and S2). We confirmed the neuronal identity of both the OAMB(+) and Octβ2R(+) processes via co-labeling with the neuron-specific glycoprotein *nervana*, recognized by the "anti-HRP" antibody (Sun and Salvaterra, 1995) (Figures 1A-vii, 1B-vii and data not shown). Subsets of processes expressing *OAMB* and *Octβ2R* were detected on the lumenal and external faces of the oviducts (Figure S2).

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 (Figures 1A-i, 1A-vi, and 1B-i). The reproductive tract is extensively innervated by octopaminergic projections (Figure S3) (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; Koon and Budnik, 2012). 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 (Figure 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 (Figure 2). We do not detect OAMB in any of the midline Tdc2(+) neurons in the AbG (Figure S4). 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; Rodriguez-Valentin et al., 2006; Gou et al., 2014; Castellanos et al., 2013). To determine if ILP7 neurons might also express *Octβ2*, 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 (Figure 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 (Figures 1A-vii, 1B-vii, and S5). The labeled somata are embedded within the muscle cells of the uterus and project anteriorly into the oviducts (Figures 1A-vii and 1B-vii). All peripheral *Octβ2*(+) neurons that we detect also express *ppk1* (Figure S5B) 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; Adams et al., 1998; Gou et al., 2014; Zelle et al., 2013; Rezaval et al., 2012; Mauthner et al., 2014; Gorczyca et al., 2014). By contrast, some peripheral OAMB(+) cell bodies do not express *ppk1* (Figure S5A). 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; Lee et al., 2016; Hasemeyer 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; Lee et al., 2016; Hasemeyer 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 (Figure 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 *Octβ2* 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 (Figure S6).

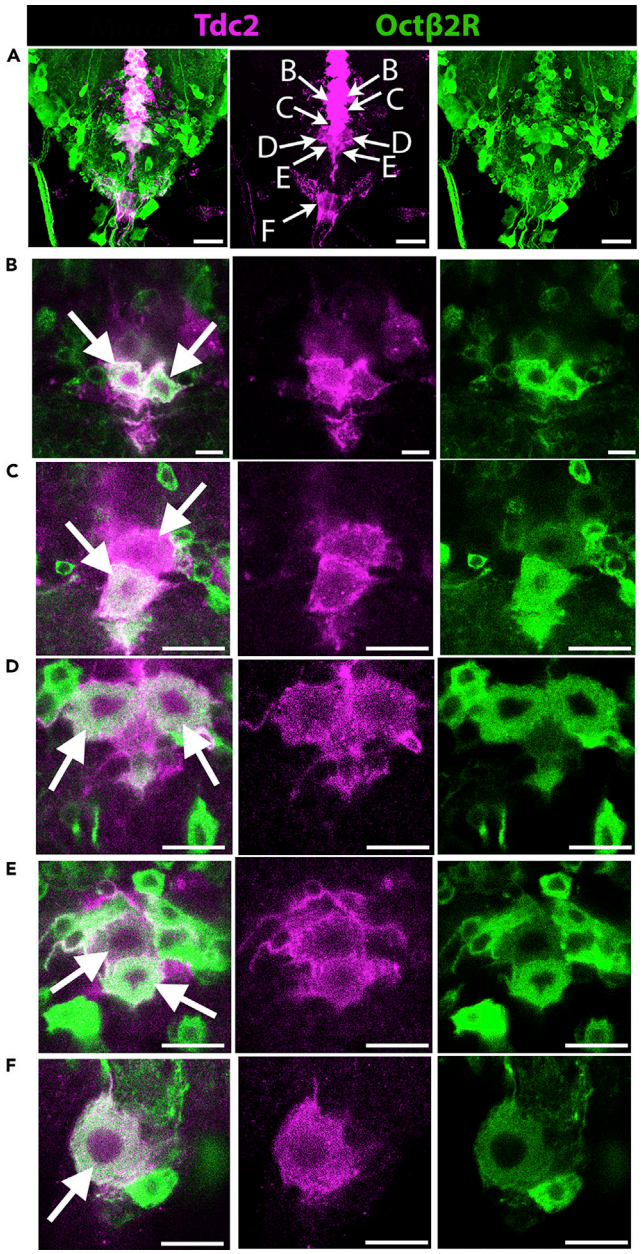


Figure 2. Expression of *Octβ2* in Tdc2(+) neurons within the AbG

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.

Therefore, ectopic expression of the *Octβ2R* RNAi in cells other than muscle, e.g. neurons, may have contributed to the previously observed infertility phenotype (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 non-muscle cells, as previously suggested in *Drosophila* (Lee et al., 2003, 2009; Lim et al., 2014) and reminiscent of some neuromodulatory inputs to mammalian visceral muscles (McHale et al., 2006; Schneider et al., 2019; Spencer and Hu, 2020; Sanders et al., 2014).

It has been previously suggested that glutamate and octopamine may be released from the same cells to regulate the oviposition circuit (Rodriguez-Valentin et al., 2006). To address this possibility, we performed

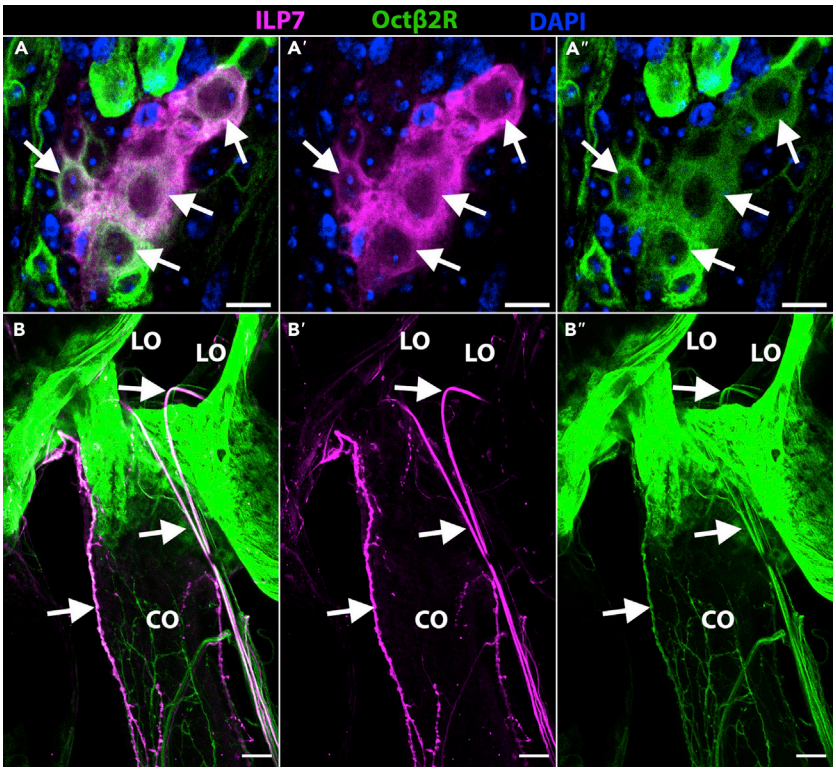


Figure 3. *Octβ2R* and *ILP7* are co-expressed in AbG neurons that innervate the oviducts

(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.

additional co-localization experiments. Because the glutamatergic regulation of the reproductive tract is mediated by ILP7(+) neurons (Gou et al., 2014; Castellanos et al., 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 (Figure 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-DVGLUT-dsRNAi* in octopaminergic neurons (*Tdc2-GAL4*) did not disrupt egg-laying (Castellanos et al., 2013).

It has been suggested that proctolin may contribute to 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), and at least some ILP7 cells in the *Drosophila* nerve cord express mRNA encoding proctolin (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 (Figure 5). By contrast, at least three ILP7(+) cells in the distal AbG were co-labeled for proctolin (Figure 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 Figure S3 for overview

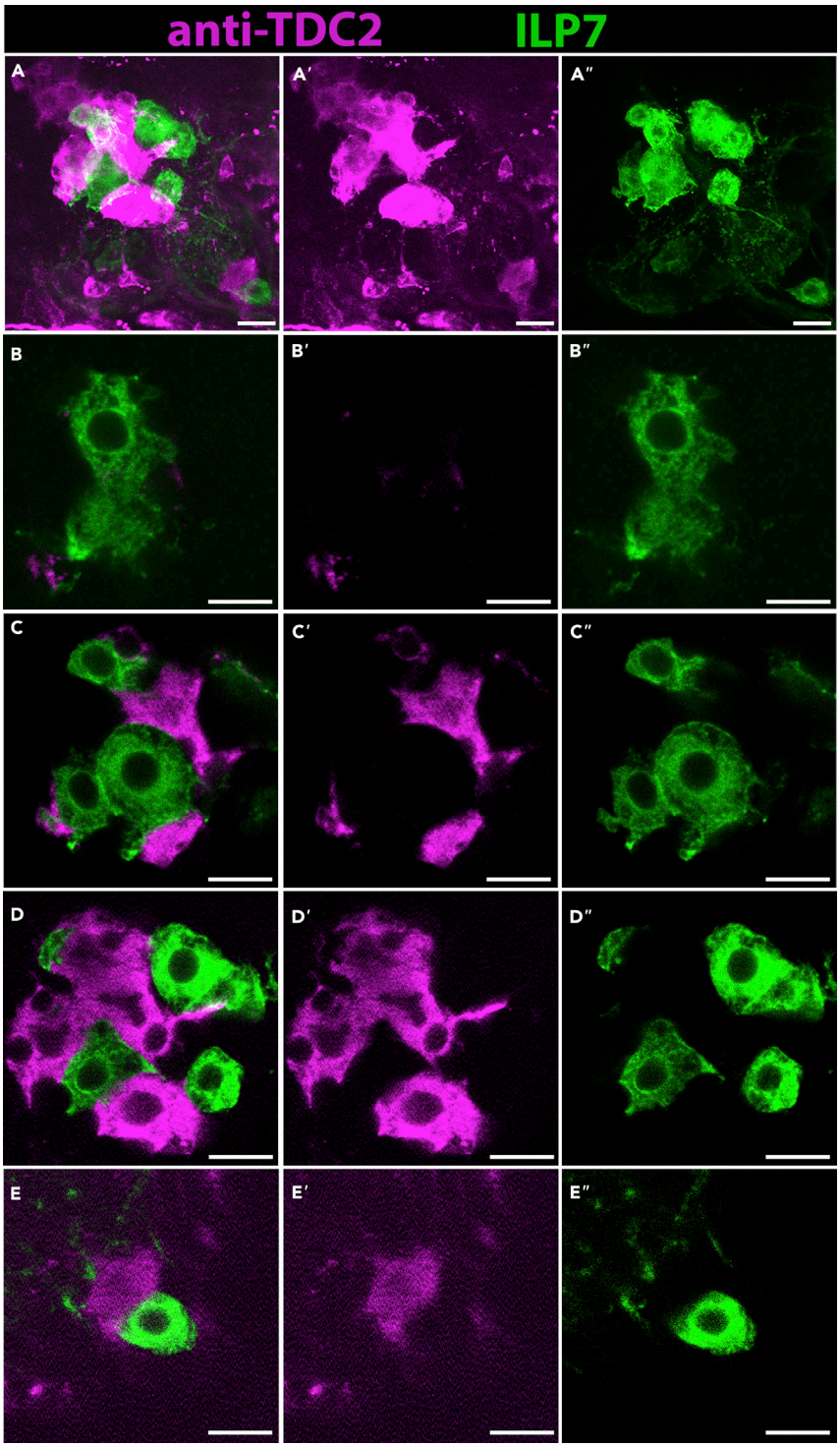


Figure 4. ILP7(+) and Tdc2(+) cells within the AbG are distinct

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.

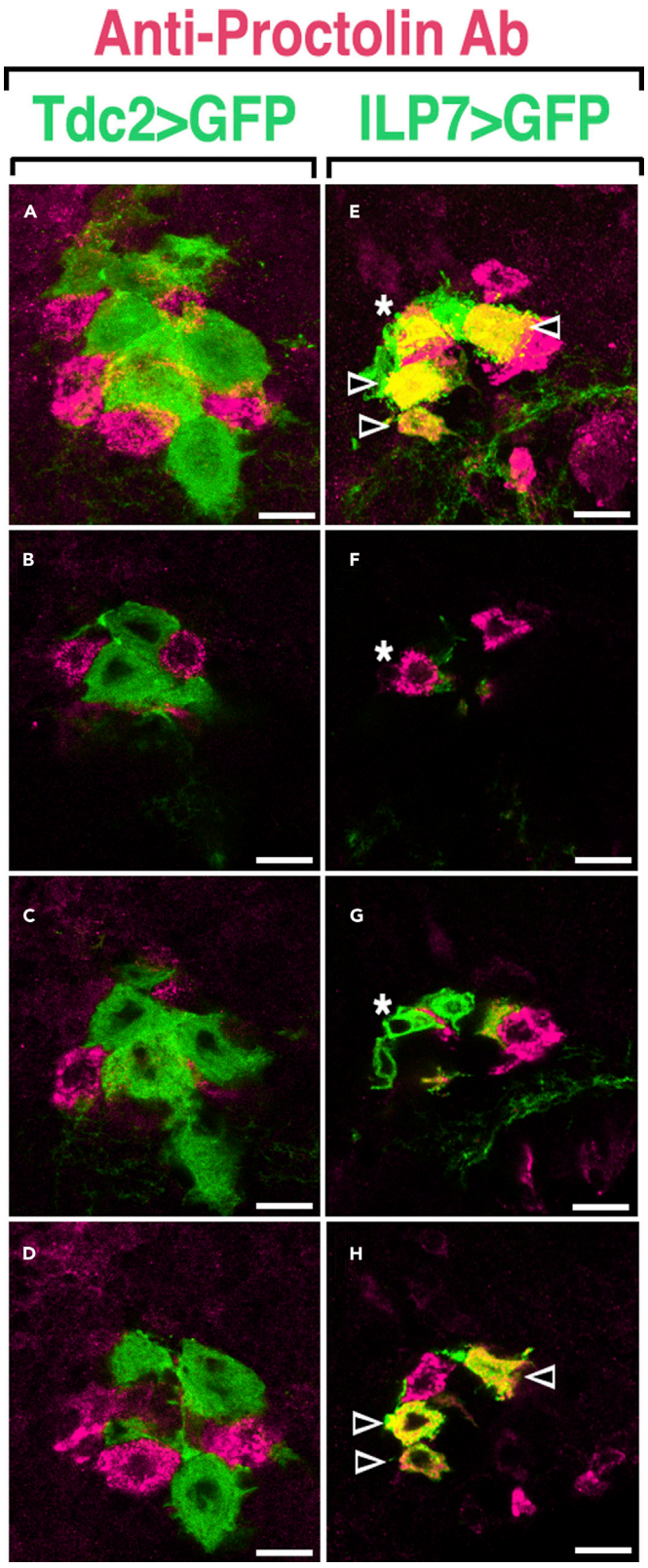

Anti-Proctolin Ab
Tdc2>GFP
ILP7>GFP
A
B
C
D
E
F
G
H

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 and F) were co-labeled with anti-GFP (green) and anti-proctolin (magenta). A maximal intensity projection (A and E) and individual optical slices from the same stack (B–D and 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.

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 (Figures 6A and 6B).

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 drives contractions in crickets (Mizunami and Matsumoto, 2017; Tamashiro and Yoshino, 2014b), and movements previously observed at the base of the reproductive tract in *Drosophila* may in fact represent oviduct contractions (Middleton et al., 2006; Meiselman et al., 2018). We find that optogenetic activation of octopaminergic neurons (30 s) results in rhythmic contractions 18.11 $\pm$ 2.09 (n = 9) of the lateral oviducts during the period of stimulation (Figures 6C and 6D). Contractions occurred with a latency of 5.3 $\pm$ 1.3 s and ceased within 2 s 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 (Figure 6D). Together, these data show that activation of octopaminergic neurons can induce acute, repetitive contractions of muscle in the lateral oviduct. Of interest, 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.

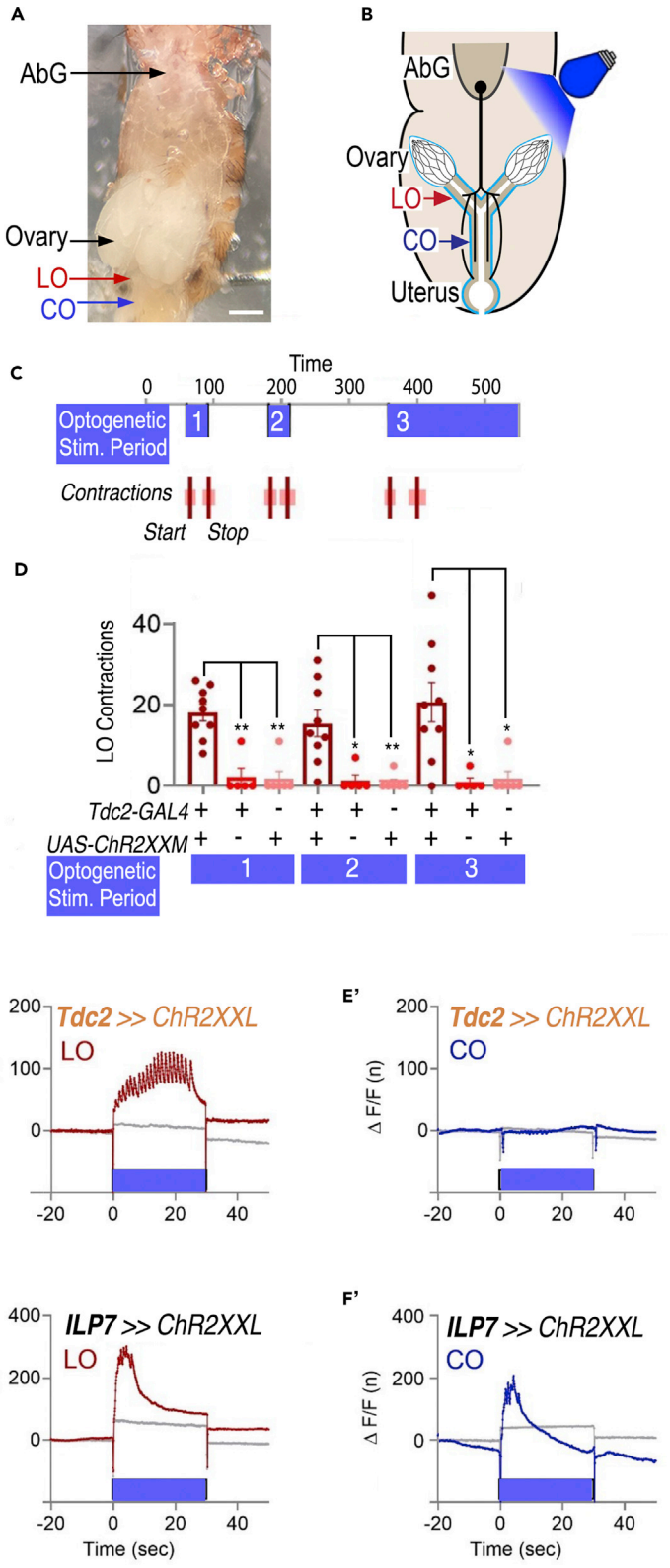
To verify these results, we performed additional experiments using calcium probes to better visualize muscle cells. Representative traces and the average of all traces are shown in Figures 6 (E and F), and S7 (A and B) respectively (see also Video S1). To visualize cytosolic calcium in muscle, we expressed 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 S1, 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 Figure 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 (Figures 6E and S7). We did not observe any response in the CO (Figure 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 channelrhodopsin in the glutamatergic neurons previously shown to innervate the reproductive tract (Castellanos et al., 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 (Figures 6F, 6F', and Video S2). 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* (Figures 6E, 6E', 6F, and 6F').

Optogenetic stimulation of ILP7(+) neurons activated muscles in both the lateral and the common oviduct (Figures 6F and 6F') although the response of the lateral oviduct following optogenetic stimulation of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Tdc2 >> ChR2XXL
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ILP7 >> ChR2XXL
LO
400
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Time (sec)
F'
ILP7 >> ChR2XXL
CO

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 and F) Sample traces showing the effects of optogenetic stimulation using the oviduct muscle driver *24B-GAL4* to express RCaMP1b.ΔF/F(n) represents the aggregate change in signal caused by both movement and an increase in RCaMP fluorescence with "n" indicating normalization (see STAR 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. Gray traces in each panel represent controls expresing *24B-GAL4>>UAS-RCaMP* alone.

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 (Figure S8). Although 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 s for Tdc2 versus vs 11 ± 3.7 over 30 s (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 (Figures 7A and 7A') and (2) an "Isolated Preparation" in which the MAN was cut and the reproductive tract was completely dissected out of the abdomen (Figures 7B and 7B'). In addition, the nerves that connect the lateral oviducts and the uterus were severed in the "Isolated Preparation" (Figures 7B, 7B', and S9). Initial dose-response experiments in the isolated preparation showed LO contractions at OA concentrations of ≥100 μM (Figure S10) 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 1-min observation period (Figure 7C') (see Video S4). 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 min (Figure 7D'). In both the Isolated and Abdominal Fillet preparations, we observed a period of quiescence of ~30–60 s 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

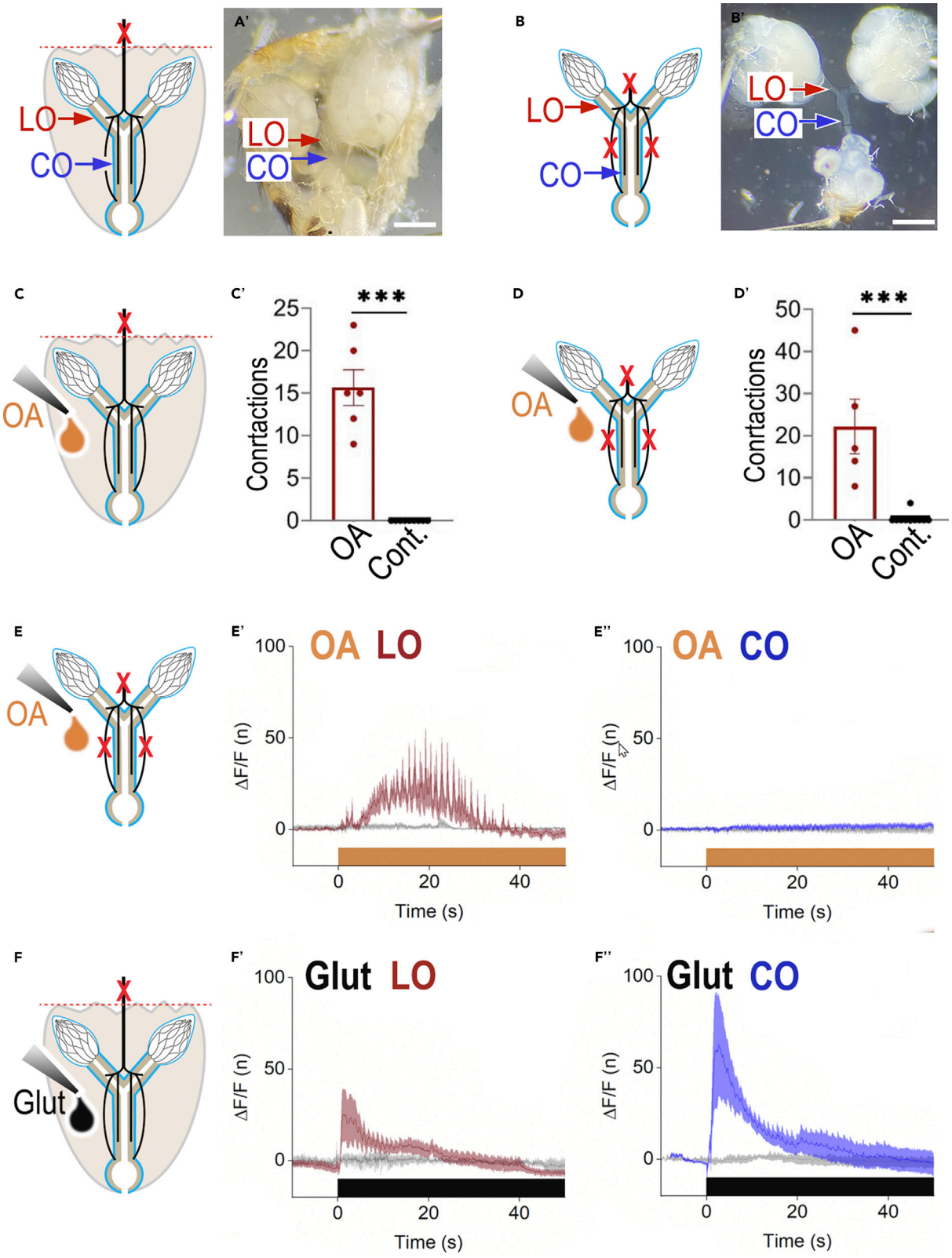
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Figure 7. Octopamine and glutamate regulate lateral oviduct contractions

(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.
(B and B') Schematic (B) and micrograph (B') of an Isolated Prep. Red X's indicates the severed peripheral nerves and MAN. Scale bars: 200 microns.
(C and C') Addition of OA to an Abdominal Fillet 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 and 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 (gray 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.

optogenetics. Expression of GCaMP (Figure 7E) and RCaMP (Videos S3 and S4) 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 S3, 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 Videos S3 and S4 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 (Figures 7 and S11). Using an Isolated Preparation, we observed rhythmic GCaMP6 activity in the lateral oviduct muscle following bath application of octopamine but not saline alone (Figure 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 $\pm$ 3.9 (n = 5, mean +/−SEM), similar to the number of contractions scored in Figures 7C and 7D 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 $\pm$ 3.4 s) versus application to an Isolated preparation (6.4 $\pm$ 2.3 s) (Figure S11) possibly because of the time required for diffusion of octopamine to its site of action within the abdomen.

In contrast to the lateral oviduct (Figure 7E'), we observed minimal changes in fluorescence in the common oviduct in response to octopamine (Figure 7E''), consistent with the lack of detectable movement in the common oviduct in response to optogenetic stimulation (Figure 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 Figures 1 and S9) 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, 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 (Figures 7F and 7F') expressing GCaMP. Using the Abdominal Fillet, we observed a single sustained contraction in the common oviduct (Video S5 and Figure 7F'). We also observed two to ten calcium transients in the lateral oviduct in the Abdominal Fillet in response to glutamate (5 $\pm$ 1.2, mean $\pm$ SEM, n = 5) (Video S5 and Figure 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 S6and data not shown).

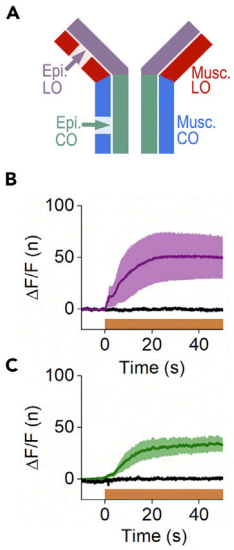

Figure 8. Octopamine increases cytosolic calcium in the epithelium

(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.

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). Because 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, 2009; Lim et al., 2014) and we observe expression of both *Octβ2R* and *OAMB* in epithelial cells (Figure 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 (Figure 8B) and CO (Figure 8C). Conversely, we observe a decrease in fluorescence in response to glutamate (Figure S12). The increase in fluorescence in the epithelium in response to OA appeared to be sustained throughout the 1-min observation period (Figures 8B and 8C). By contrast, the response of muscle cells to OA and glutamate was either transient or rhythmic (Figure 7). In addition, while only the LO muscle responded to either bath applied OA (Figures 7E and 7E'') or optogenetic stimulation of Tdc2 neurons (Figures 6E and 6E'), 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.

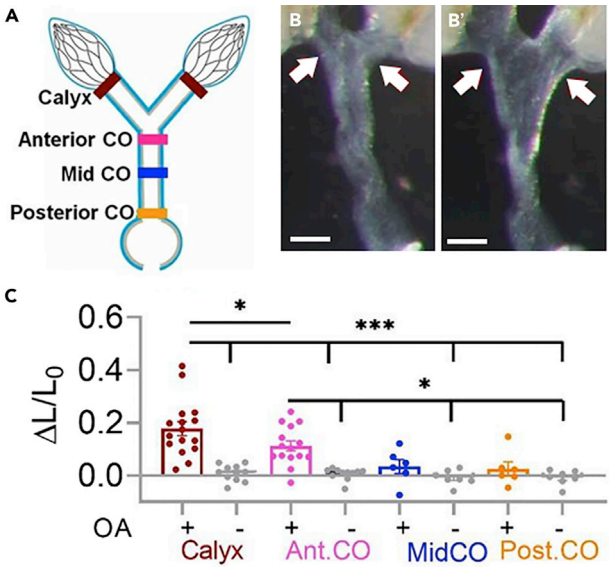

Figure 9. Octopamine causes oviduct dilation and contraction

(A) Regions of fly oviduct measured in dilation assays.

(B and 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) Change in the width of each region after octopamine application ($\Delta L = L_{final} - L_0$) normalized to the initial width (L_0) (*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 by data points in panel C. Folds or other disruptions in the tissue prevented measurement and decreased the n for some sites).

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 min 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 (Figure 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 Figure 9A). Over the course of 10 min, we observed a significant increase in the width of the calyx/lateral oviduct and the anterior common oviduct (Figures 9B, 9B', and 9C). A recording of the entire 10 min period sped up 50x is shown in Video S7. These data indicate that octopamine can cause relaxation and 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

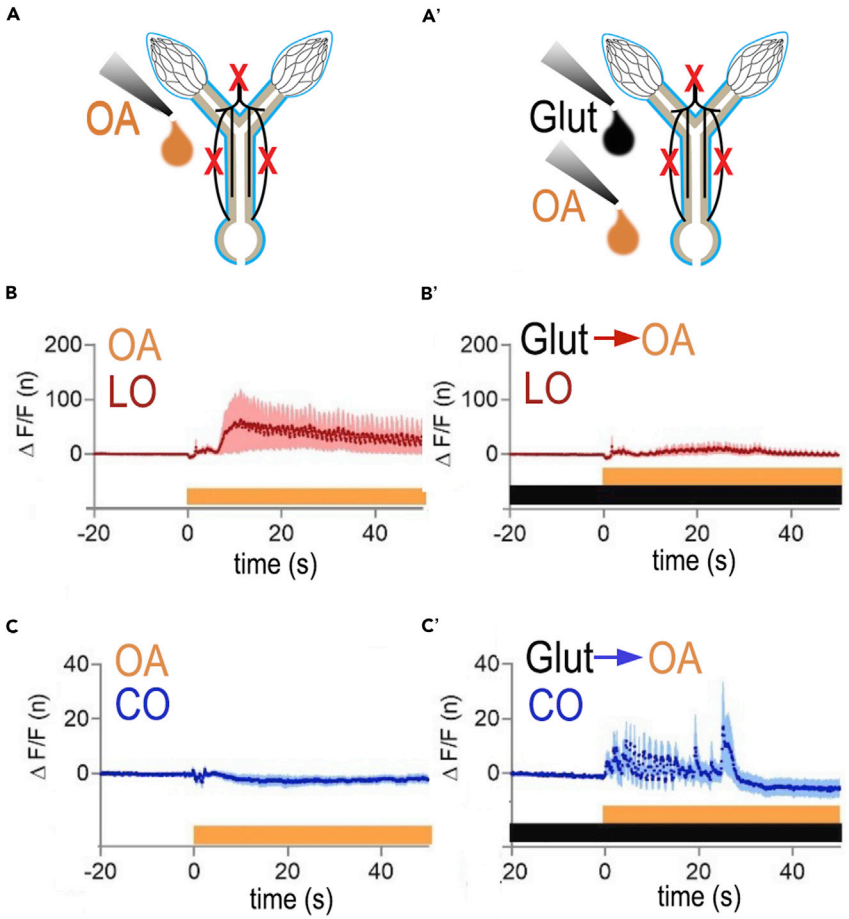

Figure 10. Potential interactions between octopamine and glutamate

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).

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 min, the second agonist was added. The responses to the first agonists were similar to those depicted in Figure 7. Both Figures 10 and S12 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 (Figure 10B, see also Figure 7). Preincubation for 2 min with glutamate on average blunted the octopamine-induced rhythmic fluctuations in fluorescence (because of both movement and changes in cytosolic calcium) within the lateral oviduct muscle (Figure 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 (Figure 10C') that were not seen in the presence of octopamine alone (Figures 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 Figure S12. These datasupport 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 *Octβ2* 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., 2003, 2009; Lim et al., 2014). In an Isolated Preparation exposed to octopamine (Figure 11A), *Octβ2R* mutants rarely displayed any lateral oviduct contractions following bath application of octopamine as compared to controls from the same genetic background (w^{1118}) (Figure 11A'). By contrast, we did not detect a decrease in the number of octopamine-induced lateral oviduct contractions (Figure 11A'') or the latency to contractions (Figure S13) 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 (Figure 11A''). These data strongly suggest that *Octβ2R* is required for octopamine-induced lateral oviduct contractions. Moreover, because 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 (Figures 11B and 11B', 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 (Figure 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 (Figure 11C). We again used an Isolated Preparation and quantified dilation as described for wild type flies (see Figure 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 *Octβ2R*mutant was comparable to both wild type flies (data not shown) and a matched genetic background control (w^{1118}) (Figure 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 (Figure 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 because of 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 (Figure 11), we expressed Ch2-XXM using the driver *Octβ2-GAL4* as a positive control. We observed LO contractions in 6 of 7 preparations (Figures 12A and 12B). The latency to contraction and duration are shown in Figure S14. We also observed contraction of the CO following optogenetic stimulation of Octβ2(+) tissues (Figure S14).

We performed similar experiments using drivers for each of the tissue/cell types in the reproductive tract in which we detected *Octβ2* expression (Figure 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 (Figure 12D). Similarly, we failed to detect repetitive contractions using either one of two drivers

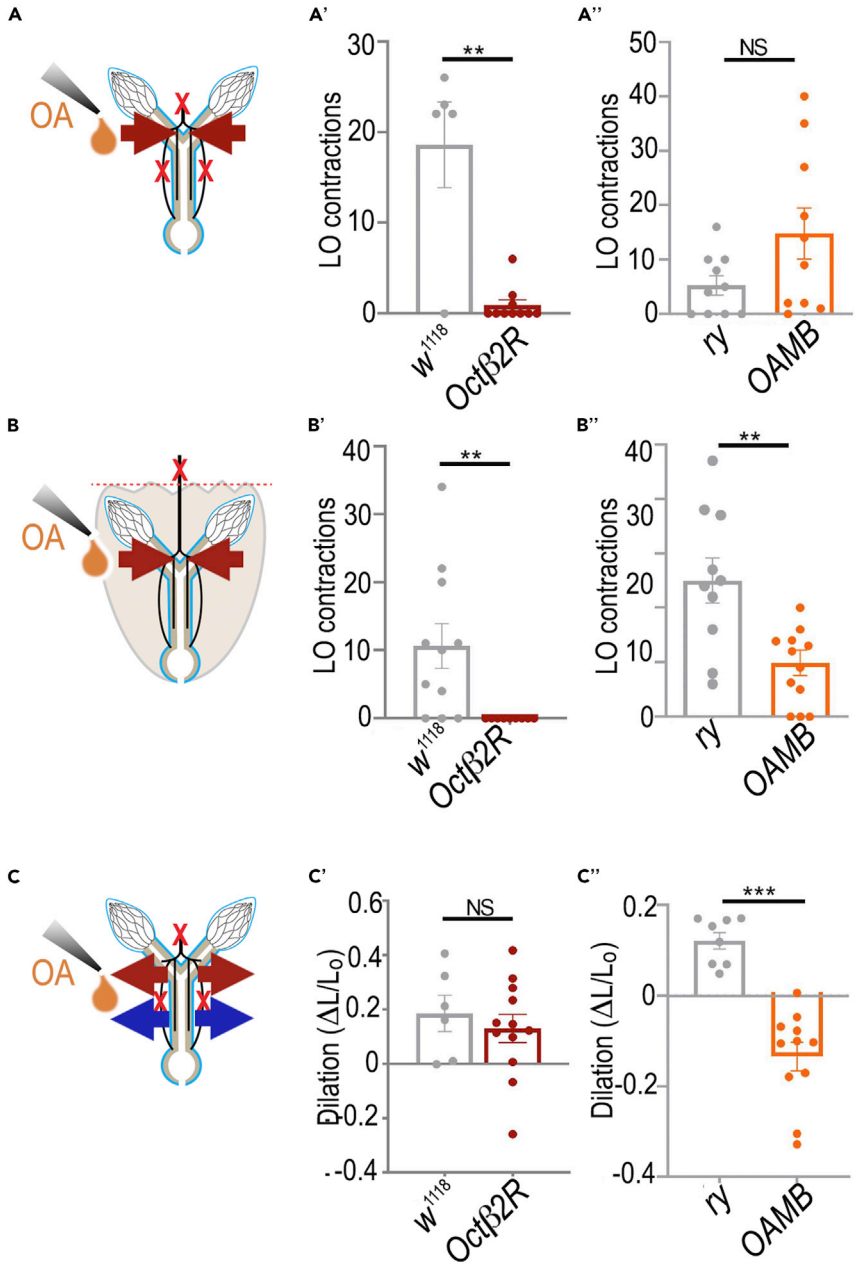

Figure 11. *Octβ2* and *OAMB* are required for contraction and dilation respectively

(A) Isolated Preparation used to quantify lateral oviduct contractions (red arrows) in response to bath-applied octopamine (OA). (A') Contractions in the *Octβ2*mutant and genetically matched controls (w^{1118}). (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).

for epithelial cells, or *ppk1-GAL4* to express ChR2-XXM (Figure 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 (Figure 12D and Video S8). These results suggest that activation of Octβ2 receptors on ILP7 nerve terminals might contribute to the LO contractions we observe in response to OA.

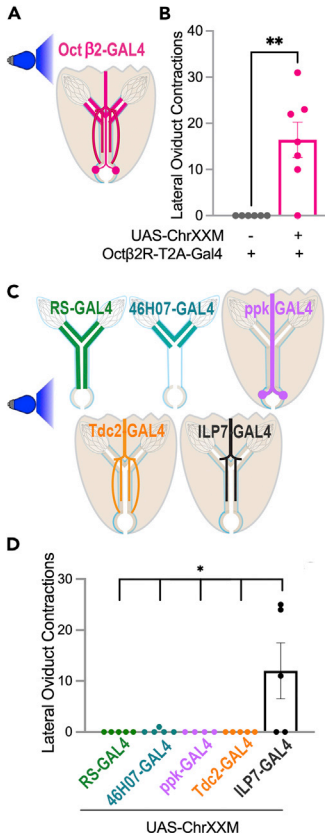

Figure 12. Optogenetic stimulation of peripheral *Octβ2* and *ILP7* expressing processes induces lateral oviduct contractions

(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 s (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 s using the indicated drivers (n = 4 and 5, as indicated by data points, *One way ANOVA*, *$p < 0.05$).

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 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 a 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 whereas 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 may 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). Although 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, 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. Although *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, 2009; Lim et al., 2014) and we show that *OAMB* mutants are unable to dilate the oviduct in response to bathe 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, 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, 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 ILP7(+) 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., 2003, 2009; 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 Figure S6). 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 have yielded fundamental insights into rhythm generation. We propose that further 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 the 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.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104697.

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AUTHOR CONTRIBUTIONS

SD, DEK, EWR conceptualized the work and wrote the manuscript. SD, EWR with assistance from DJS and EHS performed the live imaging experiments with the exception of those measuring dilation. JDA and AE conducted the dilation experiments. EWR, JDA 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.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti GFP-Alexa Fluor 488	Invitrogen	A-21311; AB_221477
Mouse "anti dsRED"	Takara	632393
Goat anti Mouse- Alexa Fluor 555	Invitrogen	A-21147; AB_2535783
Phalloidin-Alexa 555	Invitrogen	A34055
Rabbit Anti Tdc2	COVALABS	00013519
Rabbit Anti-proctolin	Jena Bioscience	ABD-032; AB_2892840
Chemicals, peptides, and recombinant proteins		
Octopamine	Sigma-Aldrich	O0250
Glutamate	Sigma- Aldrich	W328512
Proctolin	MedChem Express	HY-P0275 (/CS-7024)
Experimental models: Organisms/strains		
OAMB MiMIC-T2A-GAL4	Hugo Bellen; Bloomington *Drosophila* Stock Center (BDSC)	BDSC Stock #67506;
Octβ2β: MiMIC-T2A-GAL4	Hugo Bellen; BDSC	#67511
Tdc2-GAL4	BDSC	#9313
Tdc2-LexA	BDSC	#52242
GMR46H07-GAL4	BDSC	#50282
24B-GAL4	BDSC	#1767
UAS-GCaMP6m	BDSC	#91988
LexAop-CD2-RFP	BDSC	#58755
UAS-GFP.nls	BDSC	#4775; #4776
LexAop-ChR2-XXL	BDSC	#80161
UAS-RCaMP1b	BDSC	#63793
Octβ2 mutant PBac{w[+mC]= WH}Octbeta2R[f05679]	BDSC	#18896
ppk1-GAL4 (ppk-GAL4)	BDSC	#32079
$OAMB^{286}$	Kyung-An Han (UT, El Paso)	N/A
OAMB-RS-GAL4	Kyung-An Han (UT, El Paso)	N/A
ppk1-LexA	Bing Ye (U Michigan)	N/A
ILP7-LexA	Rebecca Yang (Duke)	N/A
UAS-ChR2-XXM	Dr. Robert Kittel (University of Würzberg)	N/A
UAS-ChR2-XXL	Dr. Robert Kittel (University of Würzberg)	BDSC #80161
Software and algorithms		
GraphPad Prism	GraphPad Software https://www.graphpad.com/	RRID:SCR_002798
Fiji/ImageJ software	PMID:22743772 https://imagej.net/software/fiji/downloads	RRID:SCR_002285
Normalization program in Labview	This paper	https://ucla.box.com/s/u8372zvb7awvikaovgjqo6tdefat21ub.

RESOURCE AVAILABILITY

Lead contact

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.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila lines: See key resources table. 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. Mated female flies 5–7 days post eclosion were used for all experiments.

METHOD DETAILS

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; *Octβ2β*: #67511 (see Figure S1).

Fly husbandry and stocks

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. *Tdc2-GAL4, Tdc2-LexA, 46H07-GAL4, 24B-GAL4, UAS-GCaMP6m, LexAop-CD2-RFP, UAS-GFP.nls, LexA-OP-ChR2UAS-RCaMP1b, 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 $OAMB^{286}$ 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 Figure 6A): For the optogenetic experiments shown in Figure 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 Figure 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 Figure 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 min, washed 3x with PBST (0.3% Triton-X 100 in PBS), and incubated in primary antibodies overnight at 4°C. After incubation in secondary antibodies for 2 h 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 key resources table.

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 ($\Delta F/F$ = [(F peak - F baseline)/F baseline]). $\Delta F/F$ values were normalized to the variance over 5–10 s before light or drug application and normalized traces indicated as $\Delta F/F(n)$. Traces were also de-trended using a second order polynomial. The signal was normalized and de-trended 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 dataset.

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 s for all experiments except for stimulation period number 3 in Figures 6C and 6D which was 4 min. 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 a 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 S3).

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 min, with digital images captured at either 1 frame per second or 1 frame per 5 s. 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/ImageJ in 1-min intervals before and after application of octopamine. The data is expressed as $\Delta L/L_0$, with ΔL representing the difference between baseline (L_0) 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 S7). 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 μM 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/mm^2 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 of muscle and epithelium 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 *ILP7*(+) 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). A Mann-Whitney test was used for the pairwise analysis shown in Figures S8 and S13. For all other experiments, group means were compared using either two-tailed t tests or one- or two-way ANOVAs, followed by pairwise comparisons as indicated. The 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. p values are shown as * <0.05, **< 0.01, ***<0.001.