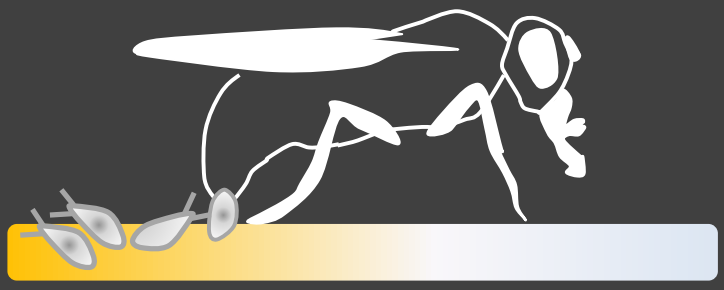
Neuronal control of suppression, initiation and completion of egg deposition in *Drosophila melanogaster*

Cristina Oliveira Ferreira



Dissertation presented to obtain the Ph.D degree in Neuroscience

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
December, 2021

UNIVERSIDADE NOVA DE LISBOA

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Cristina Oliveira Ferreira

A Dissertation
Presented to the Faculty
of Universidade Nova de Lisboa
in Candidacy for the Degree
of Doctor of Philosophy

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To the future

Acknowledgments

This 7-year journey was definitely not a smooth one with a lot of difficult moments in the course and, if I reach the end of this path, that was only made possible because I am fortunate to be surrounded by people that are good listeners, patient and caring.

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There are three people which, without their unconditional love and support, I would have never finished this PhD. *This thesis is specially dedicated to: my parents, Emilia and Carlos, and to my companion, Ricardo Neto.*

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Título

Controlo neuronal da supressão, iniciação e terminação da deposição do ovo em *Drosophila melanogaster*

Resumo

O comportamento de oviposição é um aspeto central na biologia reprodutiva dos insetos tendo um impacto profundo na sobrevivência e fitness das espécies. Em semelhança à maioria das espécies de insetos, as fêmeas *Drosophila* evoluíram para investir a sua energia na produção de muita progenia em detrimento de cuidado maternal. A relação mãe-progenia termina com a seleção de um local apropriado para a deposição dos ovos que deverá alimentar e proteger a progenia de eventos ambientais extremos e predadores. Enquanto grande progresso tem sido feito na compreensão das bases neuronais da seleção de substrato, existe pouco conhecimento sobre a organização e função de circuitos motores que controlem a deposição do ovo. Esta tese, através da combinação de análise comportamental com a caracterização de circuitos neuronais, fornece conhecimento sobre a arquitetura de circuitos motores inferiores que executam e regulam a deposição do ovo.

No segundo capítulo, descrevemos as fases e os elementos comportamentais associados com oviposição em fêmeas *D. melanogaster* originárias de uma estirpe selvagem. Caracterizamos três fases distintas deste comportamento complexo: deposição do ovo, contorções abdominais e exploração. Estabelecemos que a fase de deposição do ovo − quando o ovo é posto − é sempre seguida pela fase de contorções abdominais, enquanto que a fase de exploração − onde a mosca procura por um local ideal de oviposição − é opcional. Cada fase tem um programa comportamental motor específico que pode progredir numa sequência de comportamentos ou não. Todos estes elementos comportamentais são executados para promover a degustação do substrato, a deposição do ovo e a ovulação. Esta descrição detalhada do comportamento de oviposição em moscas selvagens oferece uma estrutura para questionar os substratos neuronais adjacentes.

No terceiro capítulo, fornecemos nova evidência sobre um circuito motor no gânglio abdominal − os neurónios OvAbg − que são suficientes e necessários para o comportamento de oviposição. Usando uma abordagem interseccional baseada no perfil de neurotransmissores para obter subgrupos funcionais, de-

scobrimos que os neurónios OvAbg têm três populações neuronais com contribuições diferentes para a oviposição. Experiências de ativação optogenética mostram que os subgrupos glutamatérgicos (OvAbg/VGlut) e colinérgicos (OvAbg/Cha) estão envolvidos na iniciação e terminação da fase da deposição do ovo. Além disso, mostramos que o silenciamento genético de um subgrupo distinto de neurónios GABAérgicos (OvAbg/Gad1) não tem efeito no número de ovos depositados, no entanto, a sua ativação optogenética inibe oviposição. Estes resultados fortemente sugerem que, durante oviposição, os neurónios OvAbg/Gad1 estão silenciados e, quando ativos, podem bloquear a execução de oviposição.

Este trabalho fornece informações importantes sobre a identidade e arquitetura de substratos motores inferiores que controlam um comportamento complexo através da descoberta do papel importante do circuito OvAbg no controlo de oviposição. Este trabalho serve de base para a descoberta de como diferentes circuitos estão conectados para coordenar comportamento.

Abstract

Egg-laying behaviour is a central aspect of insect´s reproductive biology with a profound impact on species fitness and survival. Like most insect´s species, *Drosophila* females evolved to invest their energy on the production of many offspring over providing maternal care. The mother-offspring interaction ends with the selection of an appropriate site to deposit the eggs, which should allow proper feeding and protection of the larvae from life-threatening environmental elements and predators. While great progress has been made to understand the neuronal basis of egg-laying site selection, very little is known about how, once a substrate is selected, the appropriate motor circuits are organized and activated to deposit an egg. In this thesis, by combining behavioural analysis and neuronal circuit characterization, we provide insights into the architecture of lower motor circuits executing and regulating egg deposition.

In the second chapter, we describe the phases and the behavioural elements associated with egg-laying in wild-type *D. melanogaster* females. We characterize three distinct phases of this complex behaviour: egg deposition, abdominal contortions and exploration. We show that the egg deposition phase − when the egg is laid − is always followed by the abdominal contortions phase, whereas the exploration phase - when the fly searches for appropriate egg-laying sites - is optional. Each phase has a specific behavioural motor programme which can progress in a behavioural sequence or not. All these behavioural elements are executed to promote substrate probing, egg deposition and ovulation. This detailed description of egg-laying behaviour in wild-type flies offers a framework to interrogate the underlying neuronal substrates.

In the third chapter, we provide novel evidence on an abdominal ganglion motor circuit − OvAbg neurons − which is sufficient and necessary for egg-laying behaviour. Using an intersectional approach based on neurotransmitter profile to obtain functional sub-groups, we find that OvAbg neurons harbor three neuronal populations with different contributions to egg laying. Optogenetic activation experiments show that glutamatergic (OvAbg/VGlut) and cholinergic (OvAbg/Cha) subsets are involved in the initiation and completion of egg deposition, respectively. Furthermore, we show that genetic silencing of a distinct subset of GABAergic (OvAbg/Gad1) neurons has no effect on the number of eggs laid, yet, optogenetic activation abolishes egg-laying. These results

strongly suggest that, during egg-laying, OvAbg/Gad1 neurons are silent and, when active, may gate egg-laying execution.

The work presented here provides unprecedented insights into the identity and architecture of lower motor substrates controlling a complex behaviour by finding the OvAbg circuitry as a key player in the control of egg-laying. This work sets the stage for unravelling how different circuits are connected to coordinate behaviour.

Author Contributions

Cristina Ferreira (CF) and Maria Luisa Vasconcelos (MLV) conceived and designed the project. All experiments were performed and analysed by CF, with the exception of Figures 2.1, 2.2, 3.4, 3.5, 3.6, 3.12 and 3.13 where Miguel Gaspar (MG) contributed with python analysis. CF wrote the thesis.

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List of Abbreviations

AA: Acetic Acid

Abg: Abdominal ganglion

aDNs: anterior Dorsal Neurons

AL: Antennal Lobe

Cha: Choline acetyltransferase

CHC: Cuticular Hydrocarbons

CNS: Central Nervous System

CS: Canton S

cVA: 11-cis-Vaccenyl Acetate

DNs: Descending Neurons

DsRed: red fluorescent protein

dsx: doublesex

dTdc2: Tyrosine decarboxylase 2

EM: Electron Microscopy

fru: fruitless

Gad1: Glutamic acid decarboxylase 1

GFP: Green Fluorescent Protein

Gr: Gustatory receptor

GRASP: GFP Reconstitution Across Synaptic Partners

GSCs: Germline Stem Cells

GSNs: Gustatory Sensory Neurons

JAABA: Janelia Automatic Animal Behaviour Annotator

LH: Lateral Horn

MB: Mushroom Body

nc82: Bruchpilot

nsyb: neuronal synaptobrevin

OA: Octopamine

OAMB: Octopamine receptor in mushroom bodies

Octβ2R: Octopamine β2 receptor

Or: Olfactory receptor

OSNs: Olfactory sensory Neurons

oviDNs: oviposition Descending Neurons

oviENs: oviposition excitatory Neurons

oviINs: oviposition inhibitory Neurons

PBS: Phosphate Buffered Saline

PFA: Paraformaldehyde

PMR: Post-Mating Response

PNs: Projection Neurons

PU: Posterior Uterine

s.d.: standard deviation

SAG: Sex Peptide Abdominal Ganglion

SEM: Standard Error of the Mean

SEZ: Subesophageal Zone

SP: Sex Peptide

SPR: Sex Peptide Receptor

SPSNs: Sex Peptide Sensory Neurons

VGlut: Vesicular Glutamate transporter

VNC: Ventral Nerve Cord

wt: wild-type

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Chapter 1

General Introduction

How the Central Nervous System (CNS) generates a sequence of motor actions to produce behaviour, once a decision is signalled by the brain, is a long-standing question in behavioural neuroscience. It is a complex task as this motor output needs to be: (1) tightly coordinated in time so that the appropriate sequence of behavioural elements is accomplished; (2) synchronised with the animal internal state (e.g. mating, feeding status); and (3) flexible enough to cope with unexpected changes in the environment.

The fruit fly has a relatively simple nervous system (∼200,000 neurons) that manages to perform complex behaviours. As such, the fly has been a prime model in behavioural neurobiology research for many years. The current available genetic toolkit, combined with minimal culturing requirements and a short life cycle allow flexible and diverse approaches to scientific questions. The study presented here explores the fruit fly's potential, along with the study of egg-laying behaviour, in order to strengthen our knowledge about the general principles governing animal motor behaviour.

1.1 Egg-Laying: Innate and Reproductive Behaviour

Animals are born with a set of pre-defined behavioural programmes − innate behaviours − that are reliably executed in response to specific stimuli (Tinbergen, 1991). These behaviours are supported by genetically hardwired neural circuits and do not rely on learning (Kim et al., 2017). Reproductive behaviours are included in the repertoire of innate behaviours. Fly sexual behaviours have been highly significant for the study of relevant questions such as: differences in the genetic architecture of gender-specific behaviours, molecular and neuronal basis of social interactions, multisensory integration, neuromodulation and the motor execution of behavioural programmes (Aranha and Vasconcelos, 2018; Kim et al., 2017; Laturney and Billeter, 2014).

Egg-Laying behaviour is a sexually dimorphic behaviour essential for the survival and fitness of oviparous species, displayed by females in the post-copulatory phase. Its correct implementation requires the coordination of physiological and behavioural changes that occur to meet the metabolic and nutritional demands of egg production and maturation. Given that *Drosophila* females do not display maternal behaviours, it is crucial that the behavioural changes include decisions regarding the sites where to lay the eggs to ensure

the proper larvae nutrition, and protection from environmental threatening factors. Thus, egg-laying is a complex behaviour and its regulation implies the coordination between the peripheric system and the CNS in order to produce the correct output in space and time. Specifically, as postulated by Cury et al. (2019), three variables should be considered:

1. **When** (mating, circadian regulation and the value of the egg-laying site);
2. **Where** (egg-laying site selection);
3. **How to lay an egg** (behavioural motor programmes and underlying neuronal substrates).

In the next sections of this introductory chapter, I will review the current knowledge on the neuroethology of egg-laying, following the three aforementioned points.

1.2 When to lay an egg

1.2.1 Female Post-Mating Response

After copulation, female flies initiate a series of changes in different components of their behaviour known as the female Post-Mating Response (**PMR**). The genetic, physiological and neuronal basis of this response in *Drosophila melanogaster* have been thoroughly studied over the past twenty years (Carvalho et al., 2006; Chen et al., 1988; Heifetz et al., 2014, 2000; Hussain et al., 2016b; Liu and Kubli, 2003; Mack et al., 2006; McGraw et al., 2008; Peng et al., 2005; Rezával et al., 2014; Soller et al., 1999). The PMR is initiated soon after mating (Liu and Kubli, 2003; McGraw et al., 2008; Shao et al., 2019) and it can persist for several days (Garbe et al., 2016; Peng et al., 2005; Ravi Ram and Wolfner, 2005). The three behavioural hallmarks of the PMR are: a decrease in female sexual receptivity to new courting males (Chen et al., 1988; Connolly and Cook, 1973), an increase in the rate of egg laying (Chen et al., 1988; Heifetz et al., 2000) and a change in the feeding habits that correlates with the nutritional demand of egg production, which is characterized by increased preference for yeast, salt and polyamines-enriched food (Barnes et al., 2008; Carvalho et al., 2006; Hussain et al., 2016b; Ribeiro and Dickson, 2010; Walker et al., 2015).

1.2.2 Activating the Post-Mating Response

The activation of the PMR was shown to be mostly dependent on the action of a male-specific peptide – **Sex Peptide (SP)** (Garbe et al., 2016; Peng et al., 2005; Ribeiro and Dickson, 2010; Walker et al., 2015). During copulation, SP is transferred together with the male sperm into the female reproductive tract (Chen et al., 1988; Liu and Kubli, 2003; Peng et al., 2005). The neuronal basis of SP-dependent modulation in the female fly is well dissected (Feng et al., 2014; Häsemeyer et al., 2009; Jang et al., 2017; Rezával et al., 2014, 2012; Yang et al., 2009; Yapici et al., 2008). SP binds to **Sex Peptide Receptor (SPR)**, which is expressed in a group of internal **SP Sensory Neurons** (**SPSNs**) located in the uterus (Häsemeyer et al., 2009; Yang et al., 2009). SPR activation reduces SPSNs' excitability, which in turn dampens the activation of their postsynaptic partners located in the fly Ventral Nerve Cord (VNC) – the **SP Abdominal Ganglion** (**SAG**) Neurons. SAG neurons project to the brain where they relay the information to initiate the PMR (Feng et al., 2014). The SAG downstream targets in the central brain were recently identified, thereby unveiling more details about the PMR implementation by the CNS (Wang et al., 2020, 2021). Although SP plays a key role in inducing the PMR, evidence is growing on SP-independent PMR mechanisms. In particular, chronic exposure to the sex pheromone 11-cis-Vaccenyl Acetate (cVA) (Lebreton et al., 2015) and the physical act of copulation (Shao et al., 2019) contribute to the female PMR.

Most of the post-mating behaviours are the output of neuronal circuits developmentally controlled by two transcription factors, ***fruitless (fru)*** and ***doublesex (dsx)*** (Demir and Dickson, 2005; Feng et al., 2014; Häsemeyer et al., 2009; Rezával et al., 2014, 2012; Rideout et al., 2010; Walker et al., 2015; Wang et al., 2021; Yang et al., 2009).

1.2.3 Circadian Modulation

The timing of laying an egg is also regulated by the circadian rhythm. Monitoring the egg-laying activity of *D. melanogaster* in a 12h Light:Dark (L:D) cycle shows a 24h periodicity that is characterized by a peak in egg deposition at the onset of the dark phase (Allemand, 1976; Asburner et al., 2005; Sheeba et al., 2001). The egg deposition rhythm is maintained when flies are subjected to different photoperiod and temperature conditions, as well as after changes in the protein diet quality (Howlader et al., 2006; Sheeba et al., 2001). The persis-

tence of the egg-laying rhythm under atypical conditions suggests the existence of a circadian regulator of egg-laying. After copulation, the PMR needs to be integrated with the circadian clock in order to precisely control the timing of egg-laying. How the CNS orchestrates this integration is still unknown.

1.2.4 Egg-laying Site Value

The 'when' to lay an egg is not only dependent on the post-copulatory responses and the circadian rhythm. The control over the site where the eggs are laid is of major importance for species perpetuation. *D. melanogaster* does not display maternal behaviours and the larvae show low mobility, thereby it is crucial that females find a good location to lay their eggs. That location should provide protection from predators and pathogenic microorganisms, environmental stressors, and should also be able to sustain larvae feeding. Therefore, in spite of the drive to lay eggs after mating, if the substrate conditions are not ideal for egg-laying, pregnant flies will pause egg deposition and search for a better egg-laying site. Indeed, there is evidence that females withhold eggs in the reproductive system until circumstances favour egg deposition (Allemand, 1976; Asburner et al., 2005; Drummond-Barbosa and Spradling, 2001; Kacsoh et al., 2015; Kurz et al., 2017; Lefèvre et al., 2012; Yang et al., 2008, 2015). Different environmental cues can trigger a pause in egg-laying at distinct stages of the process, which can manifest as: 1) a decrease in the rate of egg production; 2) a delay in the ovulation of mature eggs (passing an egg from the ovaries to the uterus); and 3) apoptosis of egg precursor cells, which can be induced in extreme environmental conditions, thereby halting egg production. Depriving flies of an appropriate substrate for egg-laying (Asburner et al., 2005; Drummond-Barbosa and Spradling, 2001; Yang et al., 2008, 2015), as well as extreme temperatures (Asburner et al., 2005) are sufficient to reduce egg deposition in mated females. Drummond-Barbosa et al. (2001) observed that mated females maintained in a protein-poor diet reduce the egg production rate. Exposure to predators or to pathogenic bacteria have also been shown to decrease egg deposition and induce egg retention in the ovaries (Kacsoh et al., 2015; Kurz et al., 2017; Lefèvre et al., 2012). A study conducted by Kacsoh et al. (2015) found that apoptosis of the egg precursor cells is observed when mated *Drosophila* are exposed to female parasitoid wasps. Interestingly though, a different strategy is observed when flies are exposed to pathogenic bacteria (ex. *Escherichia coli*) characterized by

the accumulation of mature eggs in the ovaries (delay in ovulation) (Kurz et al., 2017).

1.3 Where to lay an egg

1.3.1 How to Sense the Environment

As already mentioned, fruit flies are very selective about the site where they lay the eggs and, consequently, they may spend a variable amount of time searching for a proper egg-laying spot (Cury and Axel, 2021; Vijayan et al., 2021; Yang et al., 2008, 2015). It is widely accepted that *D. melanogaster* has a strong preference for laying eggs in rotting fruits, which provide a soft substrate. Flies use multiple sensory modalities to search for possible substrates: olfaction, gustation, vision and mechanosensation are all important to provide accurate information about the substrate qualities. The sensory cues that stimulate or inhibit egg-laying in *D. melanogaster* are well-known (reviewed in (Cury et al., 2019) and (Aranha and Vasconcelos, 2018) (Fig. 1.1).

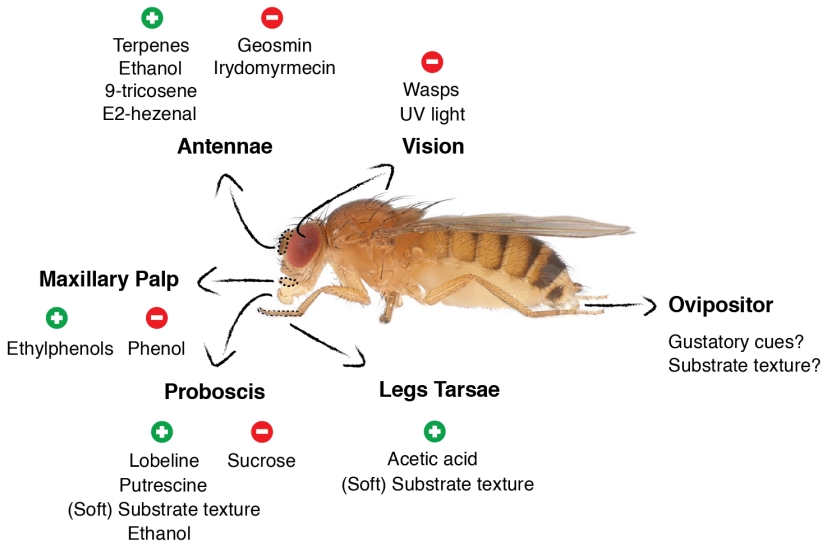


Figure 1.1: Summary of the sensory systems and some of the attractive (⊕) and aversive (⊖) egg-laying cues involved in egg-laying site selection.
Female *D. melanogaster* figure adapted from the collection of *Drosophila* species images available in Nicolas Gompel´s laboratory website (`http://gompel.org/images-2`).

For most of the sensory cues, the first-order sensory neurons are identified. However, much less is known about how these peripheric sensory circuits com-

municate with higher-brain centers to accomplish egg-laying site selection. In the next sections, I will review some of the sensory cues identified to positively (attractive) or negatively (aversive) modulate egg-laying.

Olfactory cues

Flies smell odours from a distance or in site by using the antennae and the maxillary palps. The inventory of olfactory cues evaluated by *D. melanogaster* during the choice of an egg-laying site is vast.

Egg-laying is stimulated by volatile **limonene** and **valencene** terpenes present in *Citrus* fruits, both detected by Olfactory Sensory Neurons (OSNs) expressing the receptor Or19a located in the antennae. The attraction to *Citrus* fruits protects the larvae from parasitoid wasps, which are repelled by the smell of valencene (Dweck et al., 2013). **Ethanol**, one of the main metabolites of fermentation present in rotting fruits, is simultaneously an attractive odour for egg-laying (Azanchi et al., 2013) and a potential toxic compound for the larvae (Schumann et al., 2021). Consequently, ethanol preference is dose and context-dependent. Female flies prefer to deposit eggs in food substrates containing the most ecologically beneficial concentrations of ethanol (3-5%) (Azanchi et al., 2013). Yet, they lay eggs in high-ethanol concentration substrates (10-15%) when facing predators, as a way to protect the larvae from infection (Kacsoh et al., 2013; Milan et al., 2012). Evidence suggests that both the olfactory and gustatory systems can contribute for ethanol discrimination (Azanchi et al., 2013). **Ethylphenols** derived from fruit antioxidants promote egg-laying, and its detection in adult flies is done by maxillary palp neurons expressing the receptor Or71a (Dweck et al., 2015). Egg-laying site selection is also modulated socially through olfactory cues released by other females and by males. The pheromone **9-tricosene** deposited by males in food sites was shown to work as an aggregation and an egg-laying attractive cue for females. It is detected by Or7a-expressing neurons in the antennae, the same olfactory sensory neurons that also detect the leaf volatile **E2-hexenal**, another egg-laying enticing compound (Lin et al., 2015). Mated females also provide information about high-quality egg-laying sites to other females by marking the food spots with a combination of female and male chemicals, including species-specific **cuticular hydrocarbons (CHC)** and the pheromone **cVA** (present in the male sperm) (Duménil et al., 2016).

In contrast, female flies avoid laying eggs in substrates with the volatile compounds **geosmin** and **phenol** produced by toxic microbes (e.g. bacterias and mold) (Mansourian et al., 2016; Stensmyr et al., 2012). The egg-laying avoidance of geosmin is mediated by the activation of sensory neurons expressing the olfactory receptor Or56a in the antennae (Chin et al., 2018; Stensmyr et al., 2012), while phenol is detected by Or46a-expressing neurons housed in the maxillary palps (Mansourian et al., 2016). Flies also avoid laying their eggs in spots enriched in odorants derived from parasitoid wasps (e.g.wasp sex pheromone - **iridomyrmecin**), an aversion mediated by olfactory receptors Or49a and Or85f expressed in the antennae (Ebrahim et al., 2015).

Gustatory cues

Flies use the proboscis and the legs, which house a wide pallet of Gustatory Sensory Neurons (GSNs) that can detect non-volatile cues (Ling et al., 2014; Montell, 2009). There is also strong evidence, from looking at the behaviour of mated flies and gene expression analysis, that the ovipositor (a tube-like structure protruding from the tip of the female abdomen) may also contribute to gustatory substrate discrimination, even though the sensory mechanisms of ovipositor recognition remain unknown (Cury and Axel, 2021; Montell, 2009; Yang et al., 2008).

Drosophila females show preference to lay eggs in substrates containing: the bitter-tasting compound **lobeline**, detected by Gr66a-expressing neurons located in the pharynx of the proboscis (Joseph and Heberlein, 2012); **Acetic Acid** (AA, a product of fruit fermentation), a preference mediated by sour gustatory neurons located in the legs tarsae and expressing the ionotropic receptors IR25a and IR76b (Chen and Amrein, 2017; Gou et al., 2014; Joseph et al., 2009); and polyamine-rich food (e.g. **putrescine**), a preference mediated by IR76b-expressing neurons housed in the proboscis labellum (Hussain et al., 2016a). **Sucrose** is not by default an attractive substrate for egg-laying. Yet, flies lay eggs on sucrose-rich substrates when it is the sole option in the environment (Yang et al., 2008, 2015). Sucrose preference, alike ethanol, is a great example of how egg-laying site selection is often based on relative-value decisions that are modulated by the environmental context during egg-laying.

In the course of egg-laying site selection, the substrate preferences may contrast with an innate positional avoidance for the same substrates. Indeed, these

competing behavioural drives are observed for AA and lobeline (Joseph et al., 2009; Joseph and Heberlein, 2012). By analysing the fly position in a 2-choice egg-laying assay, Joseph et al. (2009) showed that mated females spend less time in the AA-containing food substrate in comparison to food lacking AA, even though they ultimately move towards to the AA-containing substrate to deposit the eggs. The AA positional aversion is mediated by the olfactory system (Chen and Amrein, 2017; Joseph et al., 2009).

Mechanosensory cues

While the olfactory and gustatory inputs for egg-laying site selection have been extensively explored, much less is known about the contribution of mechanosensation. An important physical cue for egg-laying is the **substrate texture**. *D. melanogaster* females prefer to lay eggs in softer substrates, a preference that contrasts with other *Drosophila* species (Crava et al., 2019a; Karageorgi et al., 2017; Wu et al., 2019; Zhang et al., 2020). Indeed, flies must be equipped with the proper machinery to sense substrate textures, since they are capable to distinguish subtle hardness differences as small as 0.05% (Zhang et al., 2020). Recently, two studies established a role for the proboscis labellum and the legs tarsae in discriminating a spectrum of substrate textures (Wu et al., 2019; Zhang et al., 2020).

Besides the contribution of the legs and the proboscis, flies may also discriminate substrate texture by using the ovipositor. There are two types of ovipositor morphology observed across *Drosophila* species: a blunt ovipositor (e.g. *D. melanogaster*) or a serrated and enlarged ovipositor (e.g. *D. suzukii*) (Crava et al., 2019b). The ovipositor design is correlated with the preference to lay eggs in intact *vs.* rotting fruits, which relates to high *vs.* low hardness substrates, respectively. Divergent to *D. melanogaster*, the pest *D. suzukii* uses intact fruits as an egg-laying site and a serrated ovipositor facilitates egg deposition by cutting through the fruit skin. An RNA sequencing analysis showed that the ovipositor harbours mechanosensory and chemosensory channels, which could function on the detection of substrate qualities (Crava et al., 2019a). Further studies are required to elucidate the role of the ovipositor during egg-laying site selection.

Visual cues

Vision acts on egg-laying site choice by detecting **light**, **substrate colour** and the presence of **predators** (Guntur et al., 2017; Kacsoh et al., 2015; Solar et al., 1974; Zhu et al., 2014). Females avoid depositing eggs on sites exposed to UV-light, which is sensed by the R7-photoreceptor neurons (Zhu et al., 2014). Exposure to parasitoid wasps triggers an alert signal transmitted from exposed to naïve females through the display of wing movements. The reception of this signal by the naïve females is dependent on vision (Kacsoh et al., 2015).

1.4 How to lay an egg

1.4.1 Female Reproductive Anatomy

The *Drosophila* reproductive system occupies a large part of the abdomen cavity. It comprises two large ovaries connected to the uterus through the oviducts (lateral and common oviducts). There are three sperm storage organs (two spermathecae and the seminal receptacle) and one accessory gland on each side (parovaria) that release secretions important for ovulation (Sun and Spradling, 2013) and egg deposition (Cury et al., 2019) (Fig. 1.2). Each ovary contains 16-20 ovarioles, which house the egg chambers at 14 different developmental stages, including the Germline Stem Cells (GSCs) that proliferate and differentiate into a mature egg in a process known as oogenesis (Cury et al., 2019; Drummond-Barbosa, 2019; Klowden, 2009). Octopamine (OA), neuropeptide F and ecdysone have been implicated in GSCs proliferation and their production is stimulated after mating via SP-dependent modulation (reviewed in (Drummond-Barbosa, 2019)). Beyond the egg production process, two additional physiological events need to occur before the egg is deposited in the environment: ovulation and fertilization. Ovulation − passing an egg from an ovary to the uterus − is dependent on the degradation of a layer of follicle cells encapsuling the mature oocyte, which are important during egg maturation (Deady and Sun, 2015; Klowden, 2009). A single egg is then propelled through the oviduct to reach the uterus where the sperm stored in the storage organs fertilizes the egg (Cury et al., 2019; Klowden, 2009). The last step for egg-laying is the expulsion of the egg into the environment. This physical act is ensured by the extrusion of the ovipositor carrying the fertilized egg.

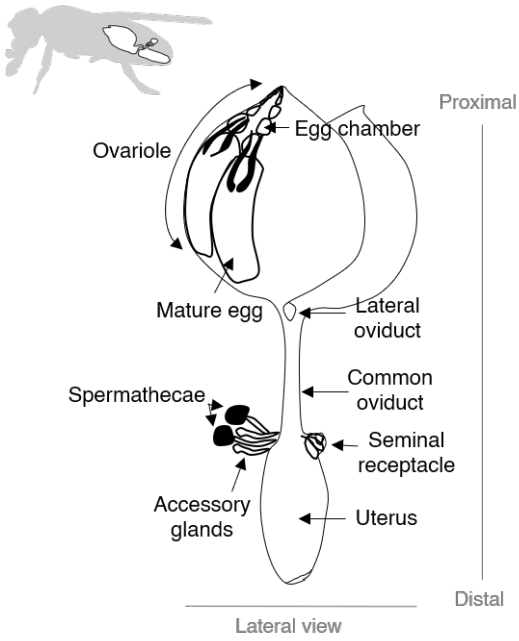


Figure 1.2: Anatomy of *Drosophila* female reproductive system.

1.4.2 Behavioural Motor Programmes

Unlike sexual behaviours that have been studied for many decades, the only existing description of egg-laying behaviour in *D. melanogaster* at the moment my thesis work started consisted in the study by Yang et al. published in 2008. The analysis of egg expulsion events in wild-type (wt) flies revealed the existence of a cycle that is repeated for each egg laid comprising three behavioural motor programmes: a search programme, an ovipositor motor programme, and a clean and rest programme (Fig. 1.3). Prior to egg deposition, females initiate the **search programme** by spending a variable amount of time foraging for an egg-laying site. Females explore the environment by probing the substrate with the legs, proboscis and the ovipositor (Yang et al., 2008; Zhang et al., 2020). Interestingly, a search-like behaviour is displayed for every egg laid, even if the environment has not changed since the last search (Gou et al., 2014; Yang et al., 2008, 2015). This egg expulsion-search behaviour likely guides more accurate decisions through the continuous accumulation of sensory evidence, which ultimately culminates in a substrate choice and the initiation of the ovipositor motor programme.

The **ovipositor motor programme** represents the consummatory phase of egg-laying behaviour, and is marked by the egg deposition in the substrate. It involves downward abdomen bending to contact the substrate, followed by the extrusion and insertion of the ovipositor into the substrate to expel and burrow the egg (Yang et al., 2008).

Egg expulsion is followed by the **clean and rest programme**, during which the females groom the ovipositor with the hindlegs, while remaining immobile (Yang et al., 2008). Egg expulsion does not occur if eggs are not ovulated, even when optogenetically activating neurons that trigger egg expulsion (Vijayan et al., 2021). The presence of an ovulated egg in the reproductive tract was shown to activate the attraction to AA-containing substrates (Gou et al., 2014), thereby initiating a new cycle of search leading to egg deposition.

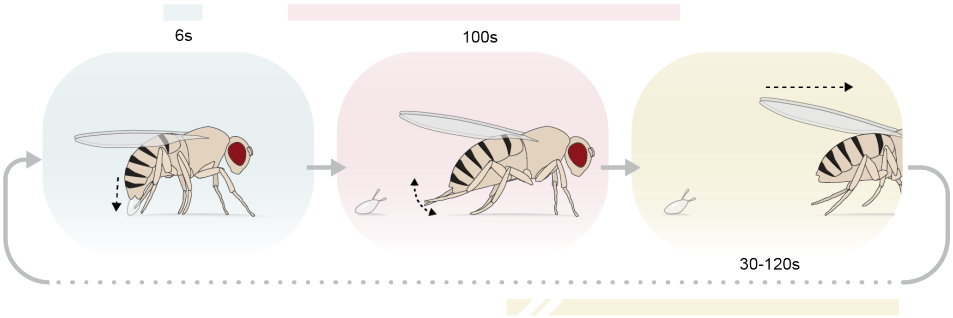


Figure 1.3: Egg-laying motor programmes.
Egg expulsion occuring during the ovipositor motor programme (left) is followed by cleaning of the abdomen and resting (center). Next, the fly explores the environment to select a site to deposit a new egg (right). Adapted from (Aranha and Vasconcelos, 2018).

1.5 Egg-laying Circuits

1.5.1 When and Where: From mating to the decision of laying an egg

From the previous sections, it is evident that the decision for an egg-laying site is not a simple process, as environments can be complex and the female's preference is modulated by a variety of external and internal factors. As mentioned above, different sensory modalities are involved in sensing the substrate and the environment. However, how the sensory systems communicate with higher-order brain centers to produce a representation of substrate suitability

is poorly understood. Regarding the internal factors, on the other hand, the circuits involved in the female PMR (referred in Section 1.2) need to first relay the mating signal to the brain and, secondly, integrate this signal with the circuits dedicated to the perception of egg-laying cues involved in the deliberation of substrate suitability. I will next introduce three recent works which provided insights into the brain circuits processing egg-laying cues, and coordinating mating and egg-laying (Fig. 1.4).

Firstly, an olfactory circuit underlying the integration of the aversive cue geosmin was unveiled. In the brain, odours are first processed in dedicated glomeruli of the Antennal Lobe (AL), where the signal is relayed to Projection Neurons (PNs). The PNs send their axons to the Mushroom Body (MB) and the Lateral Horn (LH), two centers crucial for associative learning and innate odour responses, respectively (reviewed in (Masse et al., 2009)). There is evidence that PNs encoding aversive egg-laying odours project to a common region in the LH, opening the possibility for a domain dedicated to integrate egg-laying aversive olfactory signals and, thus, eliciting similar behavioural responses (Chin et al., 2018; Ebrahim et al., 2015; Huoviala et al., 2018). Mapping the LH targets of the geosmin PNs (**DA2 PNs**) using the *Drosophila* brain electron microscopy (EM) dataset uncovered a large array of postsynaptic partners, the majority innervating brain regions involved in multimodal sensory integration. Among the downstream partners of DA2 PNs, the **LHAV1a1** neurons were found to be necessary for the geosmin avoidance behaviour during egg-laying (Huoviala et al., 2018).

While the previous work strengthened the knowledge of how an aversive egg-laying cue is integrated in the brain, the next two studies deciphered a central circuit integrating mating status and egg-laying cues. Wang et al. (2020) found that, in virgin females, the VNC SAG neurons (discussed in Section 1.2) activate the female-specific *dsx+* **pC1** cluster in the brain which, in turn, activates the **oviposition inhibitory neurons** (**oviINs**). In mated females, the inhibition of SPSN-SAG-pC1-oviINs pathway by the SP is necessary for egg-laying (Fig. 1.4). The oviINs are also integrated in a circuit constituted by the **oviposition excitatory neurons** (**oviENs**). In contrast to oviINs' role, oviENs promote egg-laying in mated females and respond to attractive egg-laying cues. Furthermore, a pair of *dsx+* **anterior dorsal neurons** (**aDNs**) are also integrated in the oviENs-oviINs circuit (Fig. 1.4) (Nojima et al., 2021). The aDNs process multiple sensory modalities, though olfaction accounts for most of the synaptic

input. Silencing aDNs abolishes the preference of mated females to lay eggs in substrates enriched in male-pheromones without affecting the number of eggs laid, suggesting a role for aDNs in egg-laying site selection rather than on the motor execution of this behaviour (Nojima et al., 2021). These findings established a circuit model of how the brain coordinates mating and egg-laying.

In the next section, I will expand these circuits to their downstream partners relaying motor commands to the VNC.

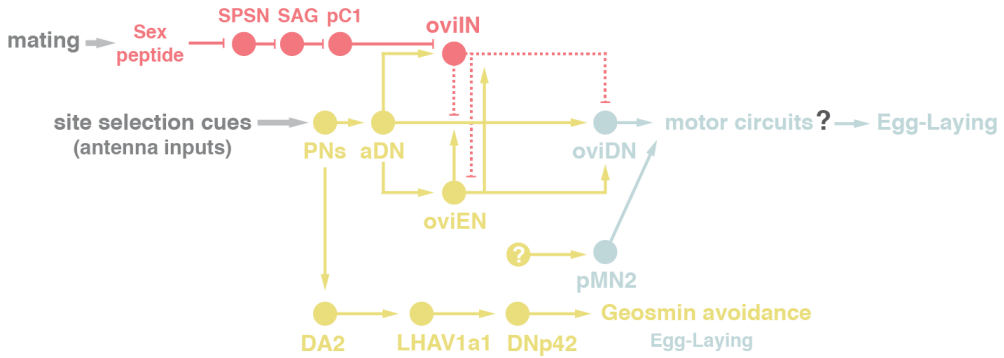


Figure 1.4: Diagram of neuronal circuits involved in egg-laying and their modulation by mating and site selection cues.
Interactions between different neurons involved in the PMR (red), egg-laying site selection (yellow) and execution (light green). In the brain, mating status is processed by pC1-oviIN neurons and substrate cues are integrated by oviEN and aDN neurons. In mated females, pC1-oviIN pathway is silent and, thus, the activity of oviENs and aDNs promotes egg-laying when a suitable substrate is encountered. oviIN connections depicted by dashed lines represent the absence of inhibitory input onto oviEN, oviDN and aDN neurons in mated females. Colour scheme in accordance with Fig. 1.3. Adapted from (Nojima et al., 2021) and (Wang et al., 2020).

1.5.2 How: from the decision to the execution of laying an egg

Descending Outputs

The decision of laying an egg is executed due to the activity of **Descending Neurons (DNs)** that relay information from the brain to the VNC. The VNC, the functional analogous of the vertebrate´s spinal cord, is connected with the body thoracic appendages (legs and wings) and abdominal segments/organs to regulate physiology and execute motor programmes. It receives descending signals from the brain to initiate motor actions, but also sends ascending signals carrying motor and sensory feedback (Fig. 1.5b) (Court et al., 2020; Hsu and Bhandawat, 2016). The VNC is located posteriorly to the brain and comprises

by three pairs of thoracic units (T1, T2 and T3) fused with a region called the Abdominal ganglion (Abg) (Fig. 1.5a) (Court et al., 2020).

Up to now, three DNs − **pMN2**, **DNp42** and **oviDNs** − were found to be involved in egg-laying behaviour, even though, only DNp42 and oviDNs were characterized in a more integrative way within egg-laying circuits (Huoviala et al., 2018; Kimura et al., 2015; Vijayan et al., 2021; Wang et al., 2020). Huoviala et al. identified that the geosmin avoidance LHAV1a1 neurons output to the descending neuron DNp42 (Fig. 1.4). Silencing DNp42 abolishes geosmin avoidance during egg-laying, as observed upon LHAV1a1 inactivation. The female-specific *dsx+* pMN2 was the first DN described to elicit the motor elements linked with egg deposition (Fig. 1.4) (Kimura et al., 2015). More recently, a set of dimorphic *fru+* oviposition descending neurons (oviDNs) were characterized as necessary and sufficient for egg-laying. Silencing oviDNs abolishes egg-laying in mated females, whereas their activation triggers the motor elements for egg deposition: abdomen bending to contact the substrate, ovipositor extrusion and egg expulsion. Imaging oviDNs activity during spontaneous egg-laying revealed that its signal rises during the search period and peaks during egg deposition. Interestingly, the rate of rise in oviDNs activity during exploration is modulated by substrate quality, which strongly affects how quickly oviDNs hit the threshold to initiate the motor programme for egg expulsion (Vijayan et al., 2021). The activity of oviDNs is not affected by mating, as abdomen bending and ovipositor extrusion are similarly triggered in virgin and mated females, which suggests a mating-dependent modulation in brain circuits upstream of OviDNs (Wang et al., 2020). Importantly, oviDNs are integrated in the egg-laying circuit composed by oviIN, oviEN and aDN neurons (Fig. 1.4) (Wang et al., 2020). Further studies are required to characterize the downstream partners of oviDN neurons in order to draw a more complete picture of a sensory-motor pathway dedicated to egg-laying.

Motor Executors

How is egg-laying execution, downstream of DNs, implemented? The motor circuits involved in the execution of the egg-laying behavioural programme (search, egg deposition and clean and rest) are poorly explored.

The neural substrates that integrate commands from the brain to generate motor outputs are located in the VNC and are referred to as **lower motor**

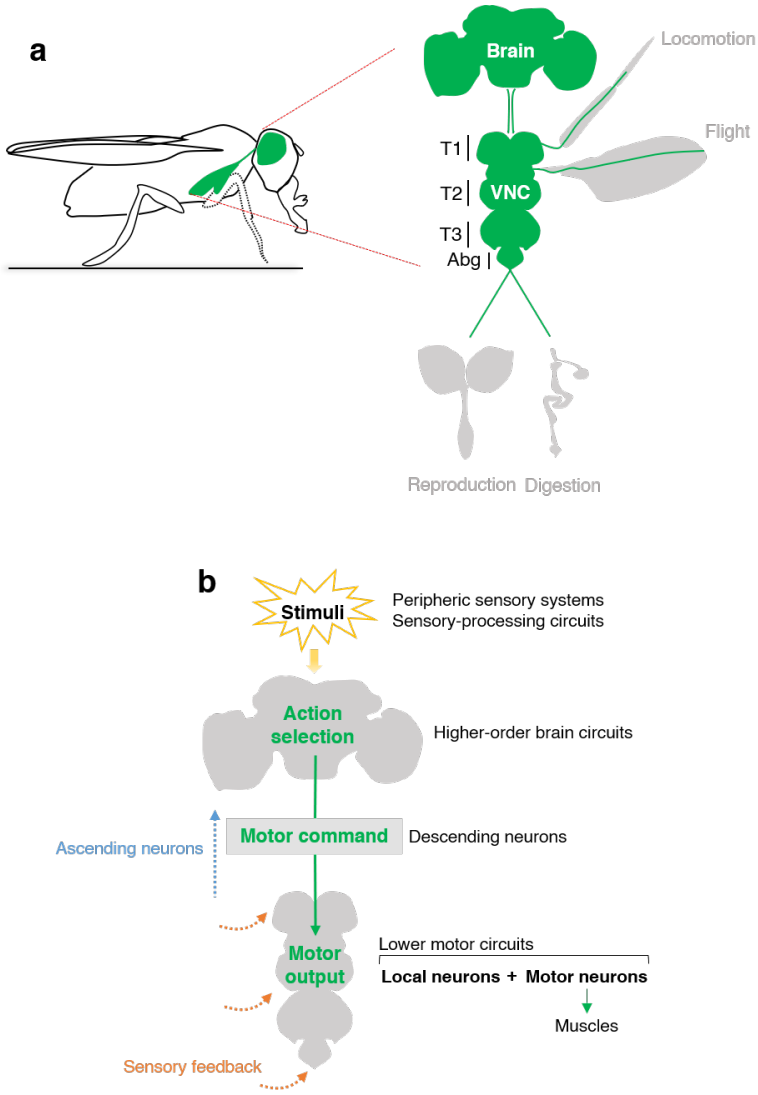


Figure 1.5: Logic of motor control by the *Drosophila* CNS.
(a) Structural and functional organization of the VNC.
(b) In order to generate motor behaviour, the nervous system integrates a variety of stimuli; those are first detected by the peripheric sensory systems (e.g. probosics, antennae, legs, ovipositor) and associated sensory circuits, which relay the sensory information to the brain. In the brain, the information is integrated in higher brain circuits involved in motivation, learning, choice and internal state, which select a behavioural outcome. The behaviour comes to action *via* brain descending neurons that activate lower motor circuits in the VNC involved in the execution and coordination of motor behaviour. Mechanosensory, proprioceptive and chemical feedback from the periphery into the VNC, as well as ascending inputs into the brain, are an important source of sensory feedback to the system; such feedback allows to fine tune behaviour accordingly with the external conditions and, also, ensures a coordinated sequence of motor events.

circuits. These circuits comprise local neurons and motor neurons. While motor neurons generate motor output by directly innervating muscle fibers, local neurons act on downstream motor neurons to control behaviour (Fig. 1.5b) (Purves, 2001; Venkatasubramanian and Mann, 2019).

The motor execution of egg-laying implies the coordination among behaviours that promote ovulation, fertilization, search and, ultimately, egg deposition into the substrate. There is supporting evidence that *dsx*-expressing neurons are key in the control of egg-laying behaviour. Silencing either the activity of all ***dsx+* neurons**, or a restricted subset of **∼9 VNC *dsx+*/octopaminergic neurons**, is sufficient to reduce, or even abolish egg-laying in mated females; in turn, their activation triggers an increase in the number of eggs laid by virgin females (Rezával et al., 2014; Rideout et al., 2010). Yet, how and which sub-populations of the *dsx+* VNC network control the egg-laying motor programme is still to be uncovered.

The mechanisms by which the VNC controls the oviduct muscles to promote ovulation are fairly understood. The female reproductive tract is a muscular system which distends when the egg passes through, being innervated by motor and sensory processes. There is mounting evidence that ovulation in *Drosophila* is modulated by **glutamate** and **OA** (Lee et al., 2003; Lim et al., 2014; Rodríguez-Valentín et al., 2006). While OA promotes oviduct relaxation by binding to OAMB and Octβ2R receptors in the oviduct wall, glutamate induces oviduct contractions (Lee et al., 2003; Lim et al., 2014; Rodríguez-Valentín et al., 2006). Impairing octopaminergic signalling results in sterile females with enlarged ovaries full of mature eggs that are not ovulated and, therefore, not laid (Lee et al., 2003; Li et al., 2015; Lim et al., 2014; Monastirioti, 2003; Monastirioti and Linn, 1996). Interestingly, the re-establishment of OA production in a population of Abg neurons innervating the ovaries and oviducts is sufficient to rescue the sterile phenotype (Monastirioti, 2003). Moreover, stimulating a cluster of Abg glutamatergic neurons innervating the oviduct triggers reproductive tract contractions (Gou et al., 2014) while its silencing causes eggs jammed in the oviducts (Castellanos et al., 2013; Gou et al., 2014; Yang et al., 2008).

1.6 Analogous Lower Motor Circuits

1.6.1 *Drosophila* Male Sexual and Locust Egg-laying Behaviours

Insects are prime models to study the neuronal basis of motor behaviours. Works on *D. melanogaster* and locust species (*Locusta migratoria migratorioides* and *Schistocerca americana*) have been fundamental to our comprehension of the identity and function of lower motor circuits in a wide range of behaviours, from locomotion to reproduction (Ayali and Lange, 2010; Feng et al., 2020; Howard et al., 2019). Here, I will briefly review two studies, which provide general principles of how motor circuits are organized to coordinate reproduction in insects. I will first introduce a circuit in *Drosophila* males involved in the successful execution of genital coupling during courtship (Pavlou et al., 2016). Next, I will review work on circuits controlling different behaviours during egg deposition in locust females (Ayali and Lange, 2010; Lange, 2009a,b).

The study undertaken by Pavlou et al. showed that the initiation and termination of genital coupling in males is coordinated by an Abg circuit composed by two *dsx+* populations: a glutamatergic subset (***dsx+*/VGlut+**) that innervates the male genitalia, and a local GABAergic subset (***dsx+*/Gad1+**). The *dsx+*/VGlut+ neurons initiate copulation by allowing genital coupling with the female, whereas *dsx+*/Gad1+ neurons mediate genital uncoupling to terminate copulation, likely by gating the activity of the *dsx+*/VGlut+ population. This circuit is modulated by sensory feedback from ***dsx+* mechanosensory neurons** located in the genitalia, and is poised to connect with local dopaminergic neurons to modulate copulation persistence (Fig. 1.6a).

The work developed over the past years in locust has been instrumental to understand how VNC circuits coordinate a wide range of behaviours. Here, I will focus on the motor control of egg-laying behaviour. The knowledge presented here is based on three exhaustive reviews on the field (Ayali and Lange, 2010; Lange, 2009a,b). The locust reproductive system has identical anatomical structure to the one in *Drosophila*: two ovaries linked by lateral oviducts converging on a central oviduct connected with the genital chamber or uterus. There is one spermatheca to store sperm and a pair of accessory glands. During egg-laying, females dig a deep hole in the substrate with the ovipositor valves to burrow the eggs. While digging the substrate, females retain the ovulated

eggs in the lateral oviducts and, only once the digging process is completed, the eggs descend to the uterus for fertilization and expulsion. Therefore, the coordination between digging, egg retention and fertilization behaviours is crucial for successful egg-laying. How is this coordination accomplished to produce an orderly sequence of behaviours? Three motor circuits located in the abdominal ganglion were shown to control digging, egg retention and fertilization (Fig. 1.6b). The **digging circuit** innervates the ovipositor valves to induce contractions for substrate digging. An **egg-retention** circuit whose activity stimulates oviduct contractions ensures that eggs are not expelled during the digging process. The coordinated activity of digging and egg-retention neurons ensures that eggs are only expelled once the hole is completed. The digging circuit receives sensory feedback from mechanosensory neurons in the ovipositor valves to maintain or stop digging behaviour. The egg then descends to the uterus where fertilization must occur before expulsion. A **fertilization circuit** promotes contractions of the spermatheca for sperm release and fertilization. The fertilization module likely receives feedback from sensory neurons sensing the egg in the uterus to ensure fertilization at the appropriate timing (Ayali and Lange, 2010; Lange, 2009a,b).

Taken together, these studies allow us to speculate that the same circuit logic may be applied in the control of egg-laying behaviour in *D. melanogaster*.

In my thesis work, I drew inspiration from the work outlined in the previous sections, and used *D. melanogaster* egg-laying behaviour as a paradigm to further our understanding of the mechanisms underlying motor behaviour.

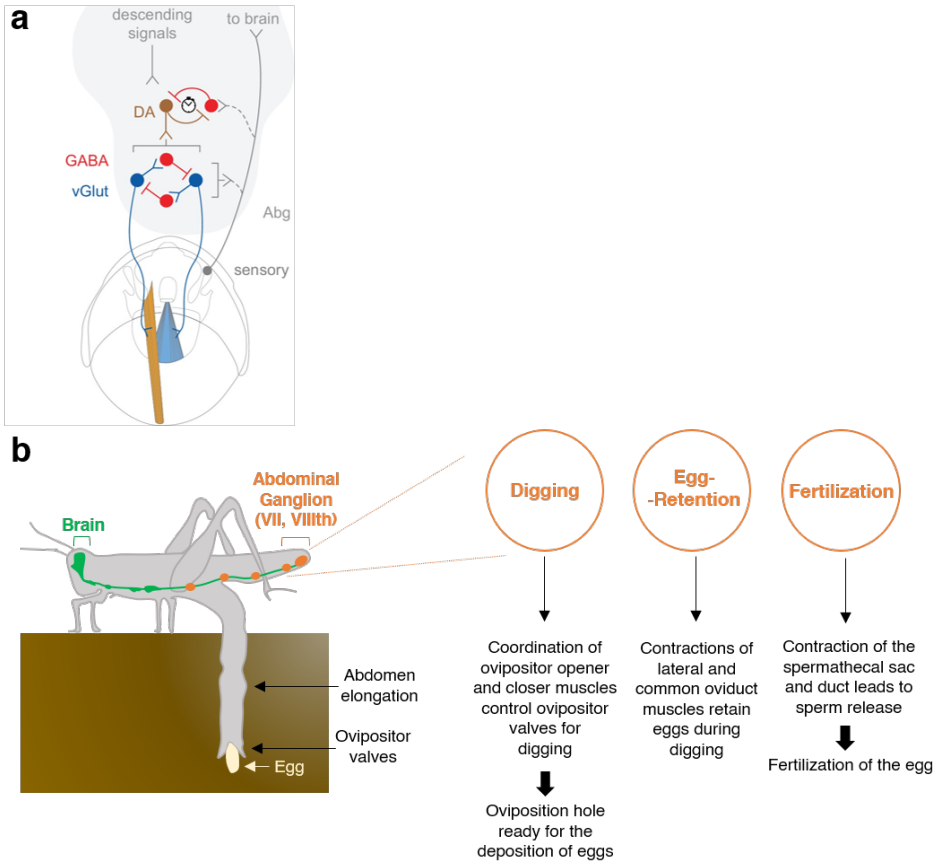


Figure 1.6: VNC motor circuits controlling reproductive behaviours in insects.
(a) Model of the tripartite circuit controlling genital coupling in *Drosophila* male. Adapted from (Pavlou et al., 2016).
(b) The three motor circuits located in the Abg that control egg-retention, digging and fertilization behaviours in locust. These circuits are located in the VII and VIIIth segments of the Abg. Egg-laying is accomplished by the elongation of the abdomen into the substrate, while digging a hole by using the ovipositor valves. Adapted from (Lange, 2009a).

Chapter 2

Egg-laying behaviour in wild type flies

2.1 Characterization of egg-laying behaviour

In order to understand how the execution of the egg-laying motor programme is coordinated, we analysed wild-type egg-laying behaviour in detail. Fig. 2.1a shows a schematic representation of the behavioural setup. Single mated females were placed in an arena lined with 1% agarose on three walls. Videos were recorded for 45 minutes. We first analysed the temporal structure of egg expulsion (Fig. 2.1b). For the duration of the video, each female laid from 4 to 17 eggs, with a median of 11.5 (Fig. 2.1c). All eggs were laid on the agarose walls and 94% of eggs were buried in the agarose. The median inter-egg expulsion interval calculated for each female ranges from 2 to 3 minutes with the exception of one fly (fly#7) that showed a median of 5 minutes (Fig. 2.1d). These data indicate a low inter-individual variability when rearing conditions and environment are controlled. To obtain a detailed description of egg-laying behaviour, we analysed egg-laying motor elements which we associated with different egg-laying phases. Egg-laying behaviour involves: 1) an exploration phase where the fly presumably searches for appropriate egg-laying sites, 2) the egg deposition phase in which the ovipositor motor programme is activated to lay an egg, and 3) the rest phase. We chose to call the rest phase, 'abdominal contortions', because we and others (Bräcker et al., 2019; Cury and Axel, 2021) observed that this phase is accompanied by strong and prolonged abdominal contortions, as described below. Fig. 2.1e shows videoframes capturing each of the different egg-laying motor elements. Some behavioural denominations were based on other descriptions of egg-laying (Bräcker et al., 2019; Cury and Axel, 2021). Egg deposition motor elements all progress in a sequence culminating in egg expulsion and ending with grooming of the terminalia. We show more than one videoframe for egg pushing as it has two different postures. Abdominal contortions, as mentioned earlier, are the single motor element of its phase. We defined exploration motor elements as elements where females probe the substrate either with the ovipositor or the proboscis without progressing in continuous sequence to egg expulsion.

To understand the temporal sequence of the different behavioural elements, we plotted the probability of each motor element around the moment of egg expulsion (Fig. 2.2a-d). During egg deposition phase, the motor elements leading to egg expulsion are performed every time an egg is deposited (Fig. 2.2a). After an egg is expelled, the female curls the abdomen while walking away, which can

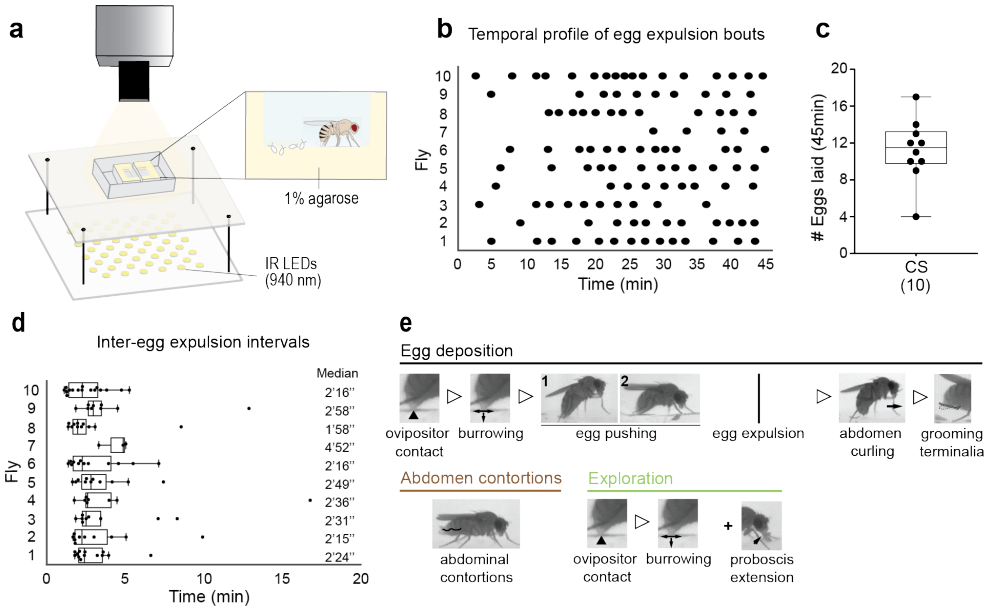


Figure 2.1: Egg-laying behaviour in wild type flies.
(a) Schematic of setup and arenas used to record egg-laying behaviour. Each arena is composed by two chambers allowing to record two flies simultaneously. The egg-laying substrate used in this study was 1% agarose. Infrared (IR) LEDs were used for illumination and IR cameras were used to record fly behaviour. **(b)** Raster plot showing the temporal profile of egg expulsion bouts. Each black dot marks the moment of egg expulsion. n = 10 flies. **(c)** Number of eggs laid by mated Canton S (CS) flies. Median = 11.5; total number of flies = 10; total number of eggs = 112. **(d)** Time intervals between egg expulsion bouts per fly. Each dot represents the interval between consecutive egg deposition bouts. Median values of the distribution for each fly are shown on the right side. n = 10 flies. **(e)** Video snapshots illustrating the different egg-laying motor patterns analysed within each egg-laying phase. Open triangles between snapshots denote sequential behaviours. *Ovipositor contact* (marked by the black arrowhead) is defined by abdomen bending accompanied by extrusion of the ovipositor and contact with underlying substrate. It can culminate in egg expulsion or not. *Burrowing* is characterized by scratching the surface eventually leading to digging the substrate with the ovipositor. *Egg pushing* is characterized by a rigid posture that initiates at the end of burrowing behaviour and accompanies egg expulsion. Note that this behaviour can be displayed in two different postures: 1. erect body posture (fly head is elevated in relation to the abdomen, or 2. leaning body posture (fly leans towards the substrate). *Abdomen curling* is characterized by curling the abdomen followed by walking forward, either lifting the curled abdomen, or by dragging it through the substrate. *Grooming terminalia* is self-explanatory. *Abdominal contortions* are undulated abdominal movements (represented by the shape of the black line) accompanied by extrusion of the ovipositor. *Proboscis extension* is characterized by the proboscis being extended to contact the substrate (marked by the black arrow).

be followed by grooming terminalia (Fig. 2.2b). While ovipositor contact, burrowing, egg pushing and abdomen curling are fixed elements of egg deposition, grooming terminalia is optional. A few seconds after the egg is expelled, the probability of abdominal contortions rises (Fig. 2.2c). The timing of abdominal contortions is very variable though abdominal contortions generally increase a few seconds after egg expulsion and decrease in the minute leading up to egg expulsion. It is likely that abdominal contortions correspond to the moment of ovulation since the next phase, exploration, is activated by ovulation (Gou et al., 2014). As abdominal contortions subside, the exploration motor programme initiates (Fig. 2.2d). Females contact the substrate by extending the proboscis and by bending the abdomen to reach the surface with the extruded ovipositor. A small peak of proboscis extension is also observed after egg expulsion indicating there may also be an association of this behaviour with the post-egg expulsion motor sequence. Part of these ovipositor contacts are accompanied by burrowing. These two behavioural elements are the same as those observed in the egg deposition programme except that they are not followed by egg pushing and expulsion. An overview of exploration and egg deposition phases together with the respective motor elements is provided in Supplementary Video 1. Abdominal contortions phase is represented in Supplementary Video 2.

We next analysed the transition probabilities between the different phases (Fig. 2.2e). This analysis revealed that egg deposition is always followed by abdominal contortions. Abdominal contortions are either followed by exploration or transitions directly to egg deposition with similar likelihood. Once the female initiates exploration she does not return to abdominal contortions without depositing an egg first. Similarly, once the female deposits an egg, she does not explore without going through abdominal contortions first. In our quantification of phase transitions, we did not consider proboscis extension, as it is not exclusive to the exploration phase nor to egg-laying behaviour. Still, the results suggest that exploration is optional in this setup.

Here we described seven behavioural elements and associated the elements with each phase, which sets the stage to analyse the neuronal circuits of egg-laying.

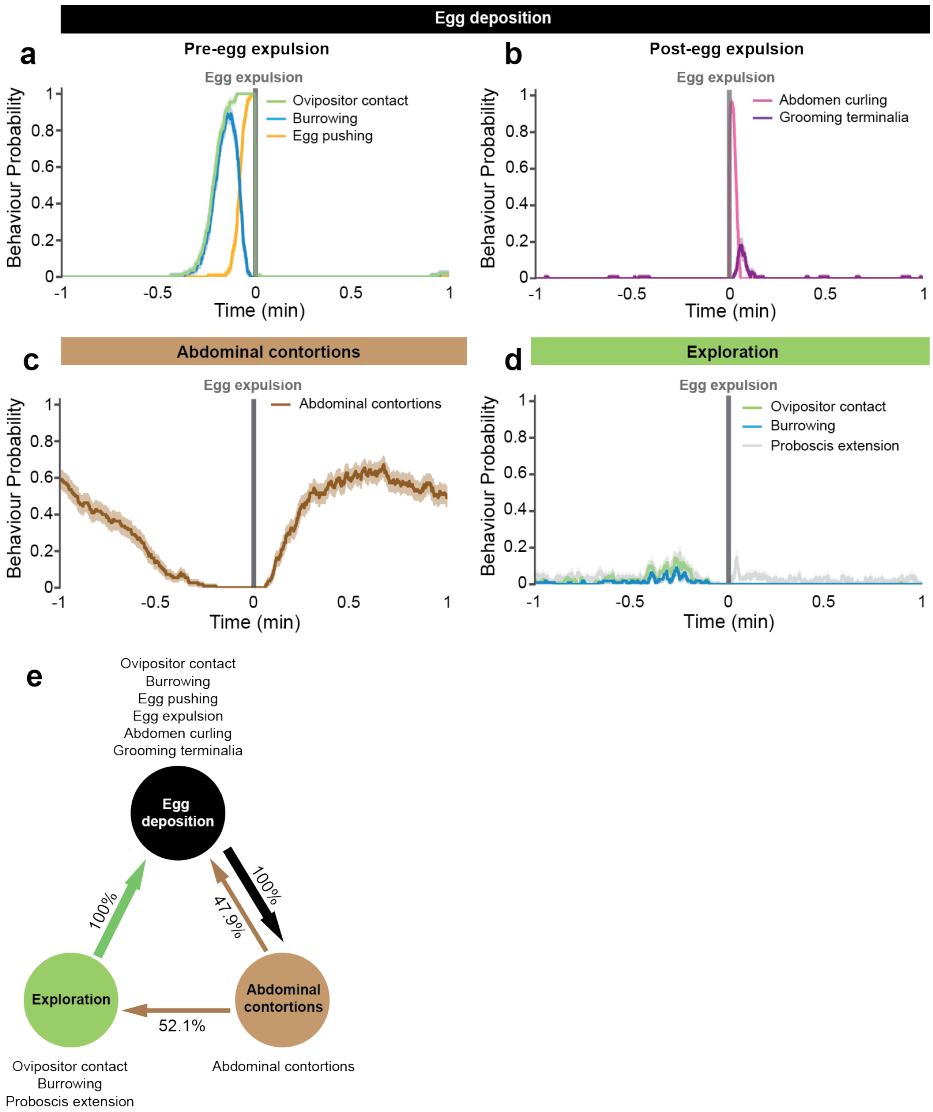


Figure 2.2: Egg-laying behaviour in wild type flies *(continued)*.
(a), **(b)**, **(c)** and **(d)** Probabilities of indicated female egg-laying behaviours during a 1-min time window around egg expulsion (time = 0, represented by the grey vertical line). Shaded area represents the Standard Error of the Mean (SEM). n = 112 egg expulsions. **(e)** Transition probabilities of the egg-laying phases. Phases: egg deposition (black), exploration (green) and abdominal contortions (brown). Arrow thicknesses represent the degrees of transition likelihood. n = 10 flies.

Chapter 3

Motor control of egg-laying behaviour

3.1 Activity of OvAbg neurons promotes egg deposition

Organs in the abdominal region of the flies connect to the central nervous system at Abg of the VNC. Since the reproductive organs are located in the abdomen and many egg-laying behavioural elements involve abdominal movement, this region is candidate to identify neurons involved in the execution of egg-laying behaviours. Therefore, we performed an activation screen of split-Gal4 lines (Jenett et al., 2012; Pavlou et al., 2016; Tirian and Dickson, 2017) selected based on Abg expression and, from this screen, we selected a line − we will call OvAbg − for further investigation. Interestingly, the OvAbg line encompasses regulatory fragments of the sex determination gene, *dsx*, widely known to control sex specific reproductive behaviours in flies (Mezzera et al., 2020; Rideout et al., 2010; Villella and Hall, 2008; Zhou et al., 2014). The OvAbg line anatomy reveals a group of neurons exclusively localized in the Abg (Fig. 3.1a). OvAbg neurons innervate other ganglia of the VNC, the Subesophageal Zone (SEZ) (Fig. 3.1b), and the reproductive system, specifically in the lateral oviducts and the distal uterus (Fig. 3.1c).

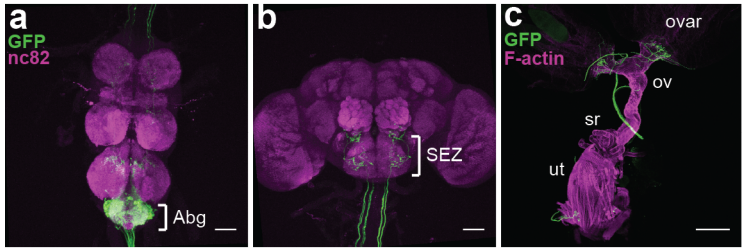


Figure 3.1: OvAbg anatomy.
(a), **(b)** and **(c)** Confocal images of female VNC (a), brain (b) and reproductive system (c) of OvAbg neurons and corresponding innervations stained with anti-GFP (green) to reveal the OvAbg anatomy and nc82 for neuropil. Anti-F-actin was used in (c) to visualize the muscle fibers. Abg: abdominal ganglion; SEZ: subesophageal zone; ovar: ovary; ov: oviducts; sr: seminal receptacle; ut: uterus. Anti-GFP is targeting the fluorescent protein Venus from OvAbg > CsChrimson-mVenus expressing flies. Scale bars a), b) 50 µm and c) 200 µm.

Additionally, we found one OvAbg projection in the seventh abdominal segment of the body wall muscle (Supplementary Fig. 4.1a and b). To identify the polarity of OvAbg neurons, we used synaptotagmin and DenMark labelling,

which revealed presynaptic terminals in the SEZ and mixed labelling of processes in the Abg and lateral oviducts (Supplementary Fig. 4.1c-k).

To investigate the role of OvAbg neurons in egg-laying, we optogenetically activated them using CsChrimson (Klapoetke et al., 2014) with 6 stimuli of 10 seconds with a 20 second interval between stimuli (Fig. 3.2a). The neuronal activation always, and only, leads the mated female to assume an egg pushing posture (Fig. 3.2b, Supplementary Video 3). In contrast, the control females, which have the same genetic background as OvAbg but no regulatory element for splitAD (Hampel et al., 2015), never assume the egg pushing posture during light stimulation. Fig. 3.2c depicts the egg pushing posture of an OvAbg activated female and a wild type female during unmanipulated egg-laying for comparison. To address whether mating status affects the output of OvAbg neurons, we activated virgin OvAbg females. We observed that virgin females, like mated females, assume an egg pushing posture in all light ON periods (Fig. 3.2b). To test the contribution of the OvAbg projections in the brain to the activation phenotype, we activated headless females. We observed that similarly to intact OvAbg females, headless OvAbg females assume an egg pushing posture at every light stimulation (Fig. 3.2d-e), indicating that OvAbg brain projections do not contribute to this activation phenotype. Next, we quantified egg expulsion during the activation protocol. We observed that nearly half of the test flies expel an egg during the activation protocol whereas control females do not expel eggs (Fig. 3.2f). Most of the eggs are expelled during the first stimulation period (Fig. 3.2g). These results show that activity of OvAbg consistently leads to an egg pushing posture but does not always lead to egg expulsion.

Given that OvAbg neurons control a female specific behaviour, we asked whether these neurons are present in the male. Indeed, there are male OvAbg neurons albeit fewer and with dimorphic projections (Supplementary Fig. 4.1l-n). Activation of OvAbg neurons in males always triggers two motor elements associated with copulation, abdomen curling and aedeagus extrusion, suggesting an analogous role of OvAbg neurons in male reproduction (Supplementary Fig. 4.1o and p).

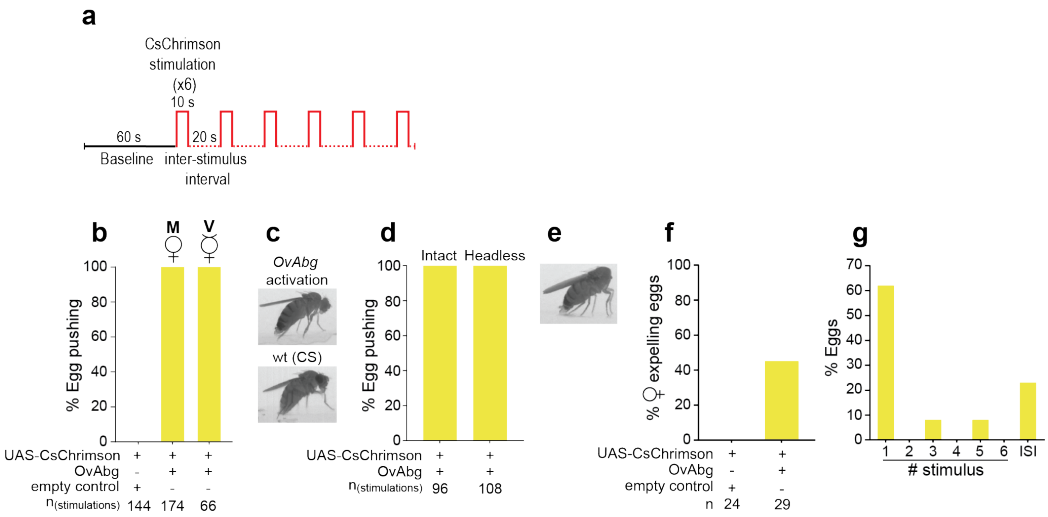


Figure 3.2: Activity of OvAbg neurons promotes egg deposition.
(a) Neuronal CsChrimson activation protocol. **(b)** Percentage of stimulation events in which Mated (M) and Virgin (V) OvAbg flies displayed an egg pushing-like posture during photoactivation with CsChrimson. n = 144 (control), 174 (M, OvAbg) and 66 (V, OvAbg) stimulations. **(c)** Video snapshots (lateral view) of an OvAbg female (top) displaying an egg pushing-like posture in response to the stimulation with CsChrimson. A snapshot of a CS fly is also shown (bottom) for comparison of the egg pushing posture performed by wt flies during natural egg-laying behaviour. **(d)** Percentage of stimulation events in which headless OvAbg flies displayed an egg pushing posture during photoactivation with CsChrimson. n = 96 (control) and 108 (OvAbg) stimulations. **(e)** Video snapshot (lateral view) of a headless OvAbg female performing egg pushing-like posture in response to the stimulation with CsChrimson. **(f)** Percentage of OvAbg females laying eggs during photoactivation with CsChrimson. n = 24 (control) and 29 (OvAbg) females. **(g)** Percentage of eggs laid by OvAbg females during stimulation periods and Inter-Stimulus Intervals (ISI). n = 13 eggs.

To further investigate the role of OvAbg activity in egg-laying, we performed silencing experiments using the inwardly rectifier potassium channel Kir2.1 (Baines et al., 2001). For egg-laying experiments, females were paired with males on apple juice agar plates and monitored for two hours for copulation. The number of eggs laid by mated females over 24 hours was nearly abolished by OvAbg silencing (Fig. 3.3a). Notably, we observed that 88.7% test and 75% control flies mated, showing that OvAbg neurons are not involved in female receptivity (Supplementary Fig. 4.1q).

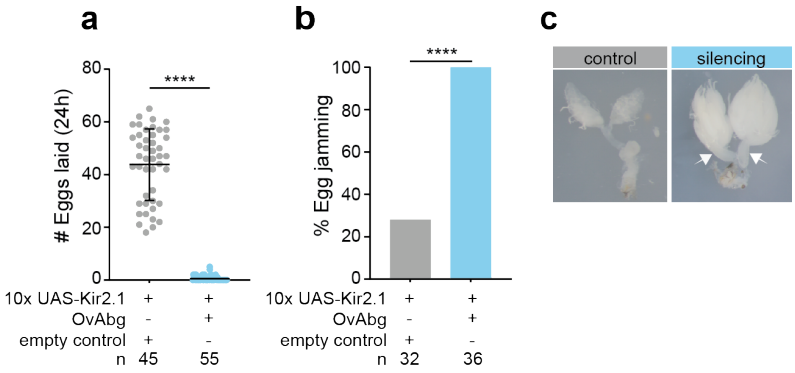


Figure 3.3: Chronic silencing of OvAbg neurons blocks egg-laying and promotes egg-jamming.
(a) Number of eggs laid per female in the 24h after mating during inhibition of OvAbg neurons. n = 45 (control) and 55 (OvAbg) females. Mean ± s.d. is shown on top the scatter plot. Mann-Whitney test, ****$p < 0.0001$. **(b)** Percentage of females with eggs jammed in the lateral oviducts during inhibition of OvAbg neurons. n = 32 (control) and 36 (OvAbg) females. Fisher´s exact test, ****$p < 0.0001$. **(c)** Image representing an OvAbg female reproductive system (right) with two eggs jammed (white arrows) in the lateral oviducts and a control (left) reproductive system with no eggs in the oviducts. Note that, besides the egg arresting, OvAbg silenced females also have enlarged ovaries containing more mature eggs when compared with control ovaries.

This result together with the observation that flies survive and appear healthy with constitutive silencing of OvAbg neurons indicates that they are specifically involved in egg-laying. To ascertain that egg-laying defect does not result from a defect in egg production, we dissected the reproductive system. We observed eggs jammed in the lateral oviducts in all test flies together with an excess of mature eggs in the ovaries (Fig. 3.3b-c), indicating that egg production is not affected.

In this series of experiments, we found a group of neurons that are part of the egg-laying motor circuits as they are directly involved in the execution of egg pushing and egg expulsion, and that are necessary for egg-laying. These Abg neurons provide a great entry point to address how different circuits coordinate to execute egg deposition.

3.2 Silencing OvAbg neurons disrupts all motor elements associated with egg-laying behaviour

We have shown that upon activation of OvAbg neurons a single egg deposition motor element - egg pushing - is induced and that OvAbg silenced females do not lay eggs. How do OvAbg silenced females behave? Do they perform all the behaviour elements with the exception of egg pushing, or are other motor elements are affected?

To answer these questions we used the anion channelrhodopsin GtACR1 (Mohammad et al., 2017) for acute optogenetic silencing of OvAbg neurons. We analysed 15 minutes of light stimulation as well as 10 minutes pre- and 5 minutes post-stimulation (Fig. 3.4a). Acute silencing of OvAbg neurons blocked egg-laying; the number of eggs laid during the stimulation was severely reduced and partially recovered in the post-stimulation period (Fig. 3.4a and b).

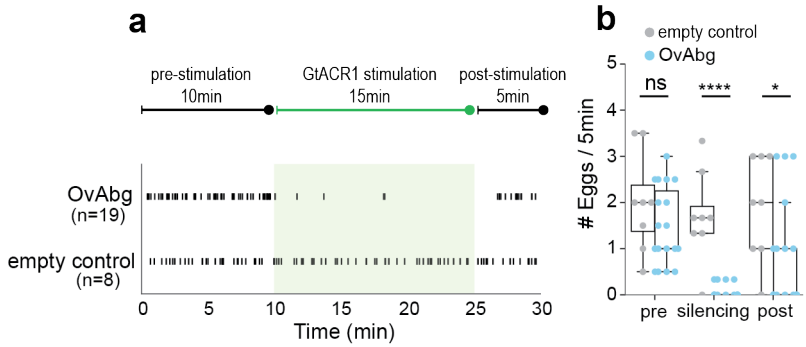


Figure 3.4: Acute silencing of OvAbg neurons blocks egg-laying
(a) (top) Neuronal GtACR1 silencing protocol scheme. Green shaded area represents the stimulation period. (bottom) Raster plot shows the egg expulsion events. n = 19 (OvAbg) and 8 (control) flies. **(b)** Quantification of the number of eggs laid per fly by OvAbg and control females during 5 min periods. n = 19 (OvAbg) and 8 (control) flies. Pre: Mann-Whitney test, ns $p \geq 0.05$; Silencing: Mann-Whitney test, ****$p < 0.0001$; Post: Mann-Whitney test, *$p < 0.05$.

Analysis of the behavioural elements showed that, with the exception of grooming, all egg deposition motor elements are abolished during stimulation and partially recovered post-stimulation (Fig. 3.5a-e). The expulsion of four eggs during the silencing period was done without using most of the egg deposition motor programme (Supplementary Video 4). The number of terminalia grooming bouts does not differ from control during silencing (Fig. 3.5e).

However, the time the female spent grooming the terminalia is much larger in the test condition during silencing (Fig. 3.5f). Interestingly, both measures of grooming are reduced compared to control in the post-stimulation period, suggesting a rebound effect on circuits modulating grooming behaviour. The data, so far, shows a wide effect of silencing OvAbg neurons in all the egg deposition elements.

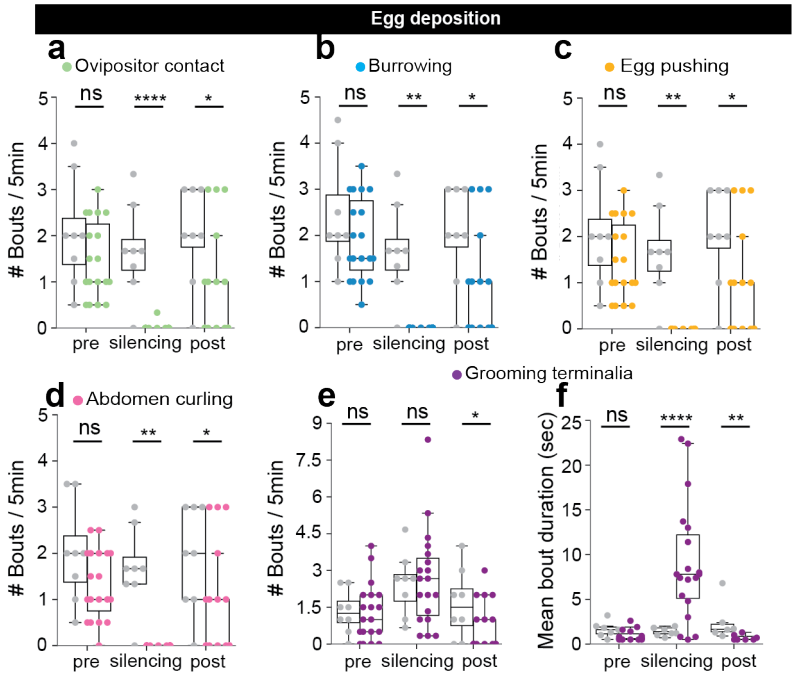


Figure 3.5: Silencing OvAbg neurons disrupts all motor elements associated with egg deposition phase.
Quantification of the number of behaviour bouts during 5 min periods. n = 19 (OvAbg) and 8 (control) flies. **Pre**: Mann-Whitney test in **(a)**, **(c)** and t-test in **(b)**, **(d)**, **(e)** ns ≥ 0.05; **Silencing**: Mann-Whitney test in **(a)**, **(e)**, ****p < 0.0001 and ns ≥ 0.05 and t-test in **(b)**, **(c)**, **(d)**, **p < 0.01; **Post**: Mann-Whitney test in **(a)**, **(b)**, **(c)**, **(d)**, **(e)**, *p < 0.05. **(f)** Quantification of the mean duration of grooming terminalia bouts. n = 19 (OvAbg) and 8 (control) flies. **Pre**: Mann-Whitney test, ns p≥ 0.05; **Silencing**: t-test, ****p < 0.0001; **Post**: Mann-Whitney test, **p < 0.01.

We next analysed the other egg-laying phases. Abdominal contortions are reduced compared to control, both in number of bouts (Fig. 3.6a) and behaviour duration (Fig. 3.6b). Additionally, the intensity of the contortions and the extent of the ovipositor extrusion are reduced in test flies compared to controls, as exemplified in Fig. 3.6c. The behaviour elements of the exploration motor programme are reduced during silencing (Fig. 3.6d-f). Interestingly, both the

exploration and abdominal motor programmes during the post-stimulation period are not different from the respective controls (Fig. 3.6a-b, d-f), indicating that the regulation of these phases is simpler than that of the egg deposition programme where the inhibitory effects of GtACR1 stimulation persist.

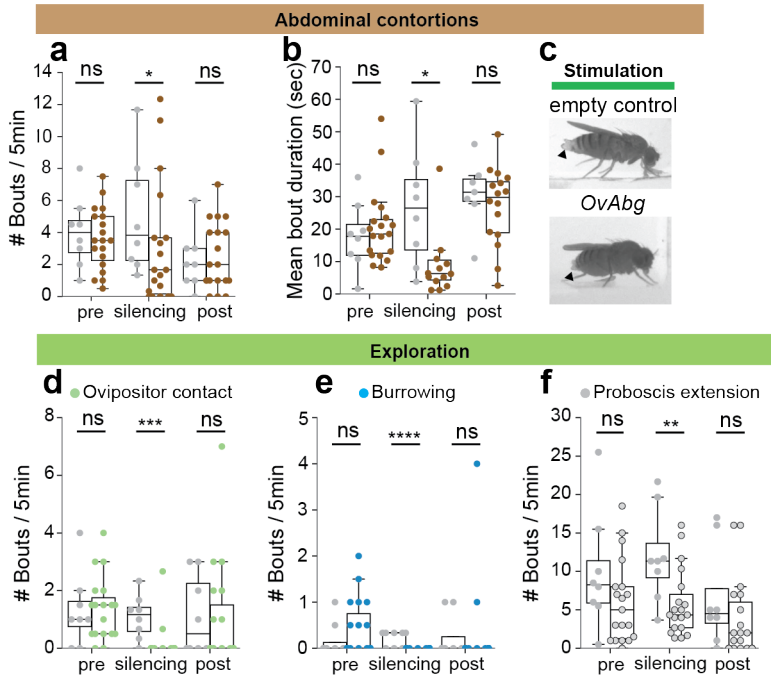


Figure 3.6: Silencing OvAbg neurons disrupts all motor elements associated with abdominal contortions and exploration phases
(a) and **(b)** Quantification of the number of abdominal contortions bouts during 5 min periods and the corresponding bout mean duration. n = 19 (OvAbg) and 8 (control) flies. **Pre**: t-test in **(a)** and Mann-Whitney test in **(b)**, ns $p \geq 0.05$; **Silencing**: Mann-Whitney test in **(a)** and **(b)** *$p < 0.05$; **Post**: Mann-Whitney test in **(a)** and t-test in **(b)**, ns $p \geq 0.05$. **(c)** Video snapshots of test (bottom) and control (top) flies displaying abdominal contortions during the silencing period. Silenced flies also display less extended ovipositor extrusions during abdominal contortions (arrowheads). **(d)**, **(e)** and **(f)** Exploration phase-associated motor elements and corresponding quantification of the number of behaviour bouts during 5 min periods. n = 19 (OvAbg) and 8 (control) flies. **Pre**: t-test in **(d)** and Mann-Whitney test in **(e)** and **(f)**, ns $p \geq 0.05$; **Silencing**: Mann-Whitney test in **(d)**, **(e)**, and **(f)**, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; **Post**: Mann-Whitney test in **(d)**, **(e)**, and **(f)**, ns $p \geq 0.05$.

Our findings show that silencing OvAbg neurons affects all phases of egg-laying behaviour. This dramatic result could reflect a direct involvement of OvAbg neurons in all egg-laying phases. Alternatively, they could reflect an

arrest on the egg-laying cycle (Fig. 2.2e) induced by the loss of egg pushing behaviour and inability to complete egg deposition.

3.3 Activity of GABAergic OvAbg neurons blocks egg-laying

To address how different neurons within the OvAbg population contribute to the execution of egg-laying, we used an intersectional approach to obtain functional subgroups (Diao et al., 2015). The GABAergic OvAbg (OvAbg/Gad1) neurons will be discussed here while cholinergic and glutamatergic OvAbg neurons will be discussed in the ensuing sections. OvAbg/Gad1 neurons (∼96 neurons, n=7 flies) are local interneurons with sparse and faint projections to other VNC ganglia (Fig. 3.7a). No projections of OvAbg/Gad1 neurons were observed in the brain (Fig. 3.7b) or the reproductive system (Fig. 3.7c).

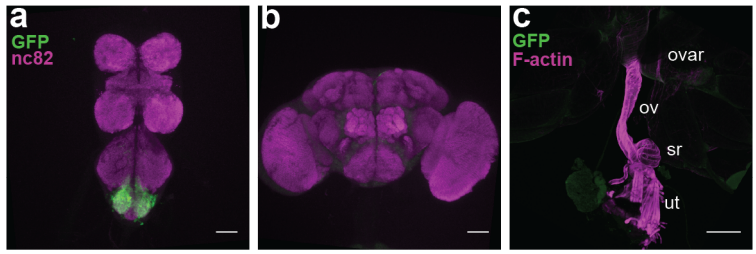


Figure 3.7: GABAergic OvAbg anatomy.
(a), **(b)**, and **(c)** Confocal images of female VNC (a), brain (b) and reproductive system (c) of OvAbg/Gad1 neurons and corresponding innervations stained with anti-GFP (green) to reveal the anatomy and nc82 for neuropil. Anti-F-actin was used in (c) to visualize the muscle fibers. ovar: ovary; ov: oviducts; sr: seminal receptacle; ut: uterus. Anti-GFP is targeting the fluorescent protein Venus from OvAbg/Gad1-LexA > CsChrimson-mVenus expressing flies. Scale bars a), b) 50 µm and c) 200 µm.

Silencing OvAbg/Gad1 had no effect on the number of eggs laid in 24h (Fig. 3.8a) and its optogenetic activation did not elicit any behaviour associated with the egg-laying motor programme (data not shown). Therefore, if OvAbg/Gad1 neurons contribute to egg-laying, they may do so by inhibiting egg-laying. To test this, we activated OvAbg/Gad1 neurons overnight (16h activation) and measured the number of eggs laid. We observed that activation of OvAbg/Gad1 abolishes egg-laying (Fig. 3.8b) and, the dissection of the ovaries at the end of the experiment, revealed that the eggs are jammed at the lateral oviduct (Fig. 3.8c-d).

In summary, the results show that, during egg-laying, OvAbg/Gad1 neurons are silent, and that activity in OvAbg/Gad1 neurons prevents egg-laying. This subset of OvAbg neurons contributes to opposing outcomes compared to the

general line and, thus, have the potential to gate egg-laying execution by other neurons in the OvAbg population.

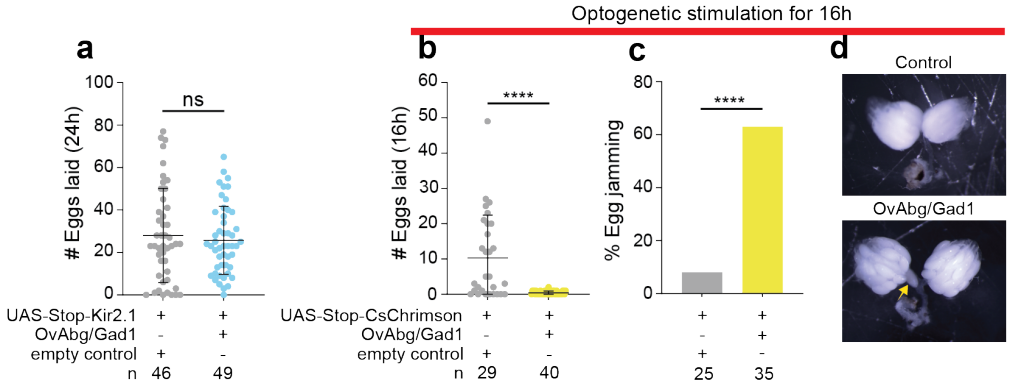


Figure 3.8: Activation of OvAbg GABAergic neurons is sufficient to block egg-laying.
(a) Number of eggs laid per female in the 24h after mating during inhibition of OvAbg/Gad1 neurons. n = 46 (control) and 49 (OvAbg/Gad1) females. Mean ± s.d. is shown on top of the scatter plot. Mann-Whitney test, ns $p \geq 0.05$. **(b)** Number of eggs laid per female during the 16h photoactivation with CsChrimson of OvAbg/Gad1 neurons. n = 29 (control) and 40 (OvAbg/Gad1) females. Mean ± s.d. is shown on top of the scatter plot. Mann-Whitney test, ****$p < 0.0001$. **(c)** Percentage of females with eggs jammed in the lateral oviducts after 16h photoactivation of OvAbg/Gad1 neurons. n = 25 (control) and 35 (OvAbg/Gad1) females. Fisher´s exact test, ****$p < 0.0001$. **(d)** (bottom) Image representing a reproductive system of an OvAbg/Gad1 female after 16h photoactivation with one egg jammed (yellow arrow) in the lateral oviduct and a control (top) reproductive system with clear oviducts. Note that OvAbg/Gad1 activated females also have enlarged ovaries containing more mature eggs when compared with control ovaries (similar to OvAbg silencing egg jamming phenotype, see figure 3.3c).

3.4 Cholinergic OvAbg neurons are necessary and sufficient for egg deposition

Cholinergic neurons (OvAbg/Cha) are a large fraction of OvAbg neurons (Fig. 3.9a) that include projections to the brain (Fig. 3.9b) and the reproductive system (Fig. 3.9c). Silencing OvAbg/Cha neurons leads to a severe reduction in the number of eggs laid (Fig. 3.9d) and a large fraction of the females display egg jamming in the lateral oviduct (Fig. 3.9e). These results show a very similar phenotype to that observed when silencing all OvAbg neurons (Fig. 3.3a-b).

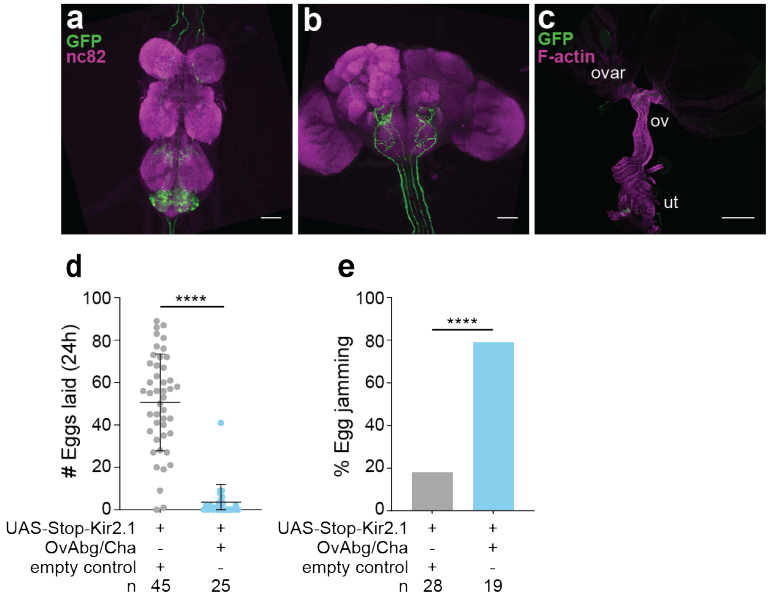


Figure 3.9: Silencing Cholinergic OvAbg neurons blocks egg-laying. **(a)**, **(b)**, and **(c)** Confocal images of female VNC (a), brain (b) and reproductive system (c) showing OvAbg/Cha neurons and corresponding innervations stained with anti-GFP (green) to reveal the membranes and nc82 for synapses. Anti-F-actin was used in (c) to visualize the muscle fibers. ovar: ovary; ov: oviduct; ut: uterus. Anti-GFP is targeting the fluorescent protein Venus from OvAbg/Cha-LexA > CsChrimson-mVenus expressing flies. Scale bars a), b) 50 µm and c) 200 µm. **(d)** Number of eggs laid per female in the 24h after mating during inhibition of OvAbg/Cha neurons. n = 45 (control) and 25 (OvAbg/Cha) females. Mean ± s.d. is shown on top of the scatter plot. Mann-Whitney test, ****$p < 0.0001$. **(e)** Percentage of females with eggs jammed in the lateral oviducts during inhibition of OvAbg/Cha neurons. n = 28 (control) and 19 (OvAbg/Cha) females. Fisher´s exact test, ****$p < 0.0001$.

Likewise, activation of OvAbg/Cha neurons using the protocol shown in Fig. 3.2a leads to both virgin and mated females assuming an egg pushing

posture each time the light is ON (Fig. 3.10a-b, Supplementary Video 5), as observed when all OvAbg neurons are activated (Fig. 3.2b-c). Interestingly, quantification of the number of females laying eggs during the stimulation protocol revealed that all OvAbg/Cha females laid one egg (Fig. 3.10c), in contrast to less than half of OvAbg females (Fig. 3.2f).

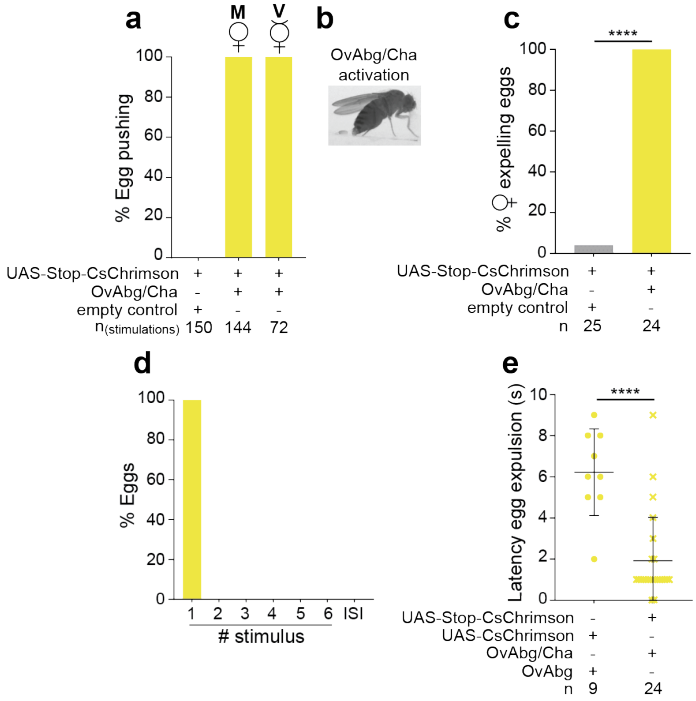


Figure 3.10: OvAbg Cholinergic neurons are involved in egg pushing and expulsion.
(a) Percentage of stimulation events in which OvAbg/Cha flies displayed an egg pushing-like posture during photoactivation with CsChrimson. n = 150 (control), 144 (M, OvAbg/Cha) and 72 (V, OvAbg/Cha) stimulations. **(b)** Video snapshot (lateral view) of an OvAbg/Cha female displaying an egg pushing-like posture in response to the stimulation with CsChrimson. **(c)** Percentage of OvAbg/Cha females that lay eggs during photoactivation with CsChrimson. n = 25 (control) and 24 (OvAbg/Cha) females. Fisher´s exact test, ****$p < 0.0001$. **(d)** Percentage of eggs laid by OvAbg/Cha females during stimulations and ISI. n = 24 eggs. **(e)** Latency (seconds) to egg expulsion (period of time to egg expulsion during stimulation) of OvAbg and OvAbg/Cha photoactivated females. n = 9 (OvAbg) and 24 (OvAbg/Cha) females. Mean ± s.d. is shown on top of the scatter plot. Mann-Whitney test, ****$p < 0.0001$.

Additionally, all eggs laid during the stimulation protocol by OvAbg/Cha females were laid during the first stimulus (Fig. 3.10d), whereas egg-laying timing by OvAbg females during the stimulation protocol was variable, with females

laying eggs in the third and fifth stimulus as well as in the interstimulus intervals (Fig. 3.2g). The results show that, upon activation, if and when an egg is laid is variable for OvAbg, but not for OvAbg/Cha females. We also quantified, within the 10 second stimulation bout, when the females expelled the egg. We found a striking difference between OvAbg and OvAbg/Cha females (Fig. 3.10e), with OvAbg females taking a longer time to expel the egg. The increased variability regarding when the egg is expelled during the stimulation protocol, and the increased latency to lay an egg upon light ON of the OvAbg females compared to the OvAbg/Cha females, likely results from inhibition by the GABAergic neurons in the OvAbg population. Activation of OvAbg neurons encompasses simultaneous activation of inhibitory OvAbg/Gad together with egg-laying promoting OvAbg/Cha, which results in conflicting information leading to variability and delay of the behavioural execution.

Overall, the results indicate that OvAbg/Cha neurons underlie the execution of egg pushing leading to egg expulsion. Unlike egg-laying behaviour of wild-type flies, eggs expelled by optogenetically activated OvAbg/Cha females were never buried and were equally distributed between the acrylic and the agarose surfaces, highlighting the importance of other behavioural components for egg-laying site selection and egg burial.

3.5 Glutamatergic OvAbg neurons contribute to egg deposition initiation

We found that glutamatergic OvAbg (OvAbg/VGlut neurons) is the smallest functional group composed by ∼10-18 neurons (n=4 flies). We observed projections in the VNC, some unilateral and some bilateral and, occasionally, we observed projections in the brain (Fig. 3.11a-b). This group of neurons also projects to the lateral oviducts and distal uterus (Fig. 3.11c).

Optogenetic activation of OvAbg/VGlut neurons using the protocol depicted in Fig. 3.2a in mated females triggered ovipositor contact-related behaviours (Supplementary Video 6). We found that 55% of the stimulations lead to ovipositor contact behaviour (Fig. 3.11d-e, bright yellow box), while incomplete ovipositor contacts (ovipositor not touching the substrate) (Fig. 3.11e, pale yellow box) were identified in 21% of the stimulation periods (Fig. 3.11d). Additionally, in a small fraction of the stimulations (7%), flies displayed burrowing behaviour accompanying ovipositor contacts (Fig. 3.11d). Finally, we observed in 14% of stimulations that flies either extruded the ovipositor with straight abdomen or bent the abdomen without extruding the ovipositor. Control flies did not display any of these behaviours during light ON (Fig. 3.11d). Interestingly, we found that virgin and mated OvAbg/VGlut females display different behavioural phenotypes upon activation. In virgins, 66% of the stimulations do not evoke any behaviour (Fig. 3.11d). Ovipositor extrusion or abdomen bending was observed in 18% of the stimulations. Ovipositor contact was identified in only 15% of the stimulations and burrowing behaviour display was residual (1% stimulations) (Fig. 3.11d). This result suggests that the behavioural output of OvAbg/VGlut neurons is modulated by the mating status.

In the first section of this study, we characterized ovipositor contact and burrowing as motor elements displayed by pregnant females in the exploration and egg deposition phases. The phenotype of OvAbg/VGlut activation provides evidence for a role of OvAbg/VGlut in controlling these behaviours, which could be specific to one of those phases, or be implicated in both. To test this, we investigated in detail the egg-laying behaviour in OvAbg/VGlut females silenced with the potassium channel Kir2.1, since a GtACR1 tool that allows this type of genetic intersection is not available. We video-recorded silenced flies for 15 minutes and annotated their behaviour, as well as that of the control flies.

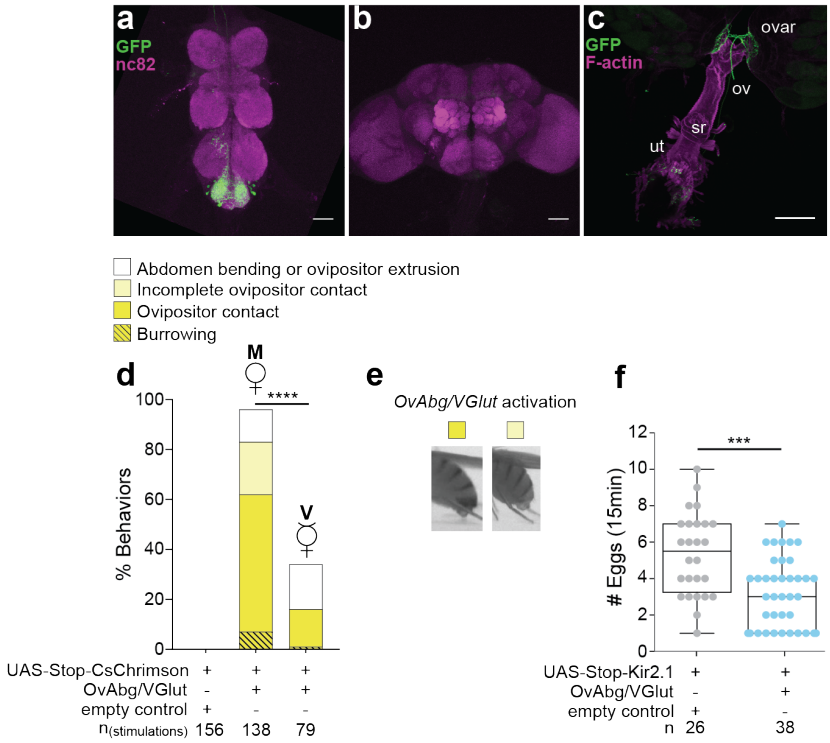


Figure 3.11: OvAbg Glutamatergic neurons are involved in ovipositor contact and burrowing behaviours.
(a), **(b)** and **(c)** Confocal images of female VNC (a), brain (b) and reproductive system (c) showing OvAbg/VGlut neurons and corresponding innervations stained with anti-GFP (green) to reveal the anatomy and nc82 for synapses. Anti-F-actin was used in c) to visualize the muscle fibers. ovar: ovary; ov: oviducts; sr: seminal receptacle; ut: uterus. Anti-GFP is targeting the fluorescent protein Venus from OvAbg/VGlut-LexA > CsChrimson-mVenus expressing flies. Scale bars a), b) 50 µm and c) 200 µm. **(d)** Percentage of stimulation periods in which OvAbg/VGlut Mated (M) and Virgin (V) flies displayed ovipositor contact, incomplete ovipositor contact, burrowing, abdomen bending or ovipositor extrusion behaviours during photoactivation with CsChrimson. n = 156 (control), 138 (M, OvAbg/VGlut) and 79 (V, OvAbg/VGlut) stimulations analysed. Fisher´s exact test, ****$p < 0.0001$. **(e)** Video snapshots (lateral view) of OvAbg/VGlut females displaying ovipositor contact and incomplete ovipositor contact behaviours in response to the stimulation with CsChrimson. **(f)** Number of eggs laid per female while silencing OvAbg/VGlut neurons. n = 26 (control) and 38 (OvAbg/VGlut) females. Mann-Whitney test, ***$p < 0.001$.

Chronic silencing of OvAbg/VGlut neurons reduced, but did not abolish, the number of eggs laid (Fig. 3.11f), suggesting that this neuronal subset is not necessary for egg-laying in contrast to OvAbg/Cha group.

Analysis of the behavioural elements shows that OvAbg/VGlut silencing affects egg deposition behaviours up to egg expulsion: ovipositor contact, bur-

rowing and egg pushing (Fig. 3.12a, b and c). Silenced OvAbg/VGlut females displayed fewer bouts of each behaviour per egg, although we still observed manipulated flies that perform these behaviours at levels comparable to control. It should be noted that the behavioural variability observed in OvAbg/VGlut neuronal manipulations (both the optogenetic activation and chronic silencing) could reflect the variability in the number of neurons and innervations labelled obtained from the splitGal4/LexA intersectional method.

The reduction in the expression of motor elements that precede egg expulsion results in abnormal initiation of the egg deposition bout (Supplementary Video 7). Surprisingly, the post-egg expulsion behaviours - abdomen curling and grooming - were not reduced (Fig. 3.12d and e). In fact, grooming was slightly increased, which may be associated with the deficient egg deposition (Fig. 3.12e). Indeed, the increased grooming in test females is specifically associated with the egg deposition events, rather than a generalised increase in grooming terminalia throughout the video (Supplementary Fig. 4.2). The contortions phase was not affected (Fig. 3.12f). Glutamate has been shown to be involved in oviduct contractions (Rodríguez-Valentín et al., 2006), which presumably occur during the contortions phase. Our results indicate that there is a distinct group of glutamatergic neurons, not labelled by OvAbg, involved in oviduct contractions. The exploration phase, which shares two motor elements with the egg deposition phase (ovipositor contact and burrowing) and also includes proboscis extension was also not affected (Fig. 3.13a-c).

Egg-laying in *Drosophila* culminates in egg expulsion subterraneously in the substrate. Since silencing OvAbg/VGlut neurons reduces pre-egg expulsion behaviours, we investigated if silenced females had less eggs buried. Indeed, by comparing the number of eggs dropped (not buried) on the agarose surface in relation to the total number of eggs laid, we observed that OvAbg/VGlut silenced females had less eggs buried in the agarose substrate (Fig. 3.13d) in comparison to control flies.

In summary, the activation and silencing results strongly suggest that glutamatergic OvAbg neurons are involved in the initiation of the egg deposition motor sequence. Although OvAbg/VGlut neurons are not necessary for egg-laying, egg-laying is less efficient in OvAbg/VGlut silenced flies due to deficient egg deposition initiation. Furthermore, OvAbg/VGlut neurons play a role in progeny survival as silencing OvAbg/VGlut leads to fewer eggs buried in the substrate and, therefore, exposed to predation and adverse climate conditions.

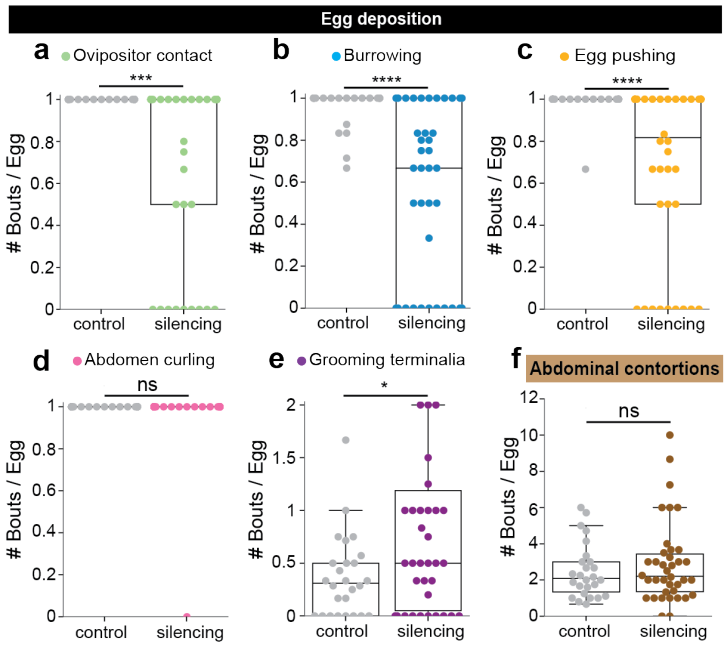


Figure 3.12: OvAbg Glutamatergic neurons contribute to egg deposition initiation.
(a), **(b)**, **(c)**, **(d)** and **(e)** Egg deposition-associated motor elements and corresponding quantification of the number of behaviour bouts normalized for the number of eggs laid per female. n = 26 (control) and 38 (OvAbg/VGlut) flies. Mann-Whitney test in a, b, c, d and e, ****p $<$ 0.0001, ***p $<$ 0.001, *p $<$ 0.05, ns p$\geq$ 0.05. **(f)** Quantification of the number of abdominal contortions bouts normalized for the number of eggs laid per female. n = 26 (control) and 38 (OvAbg/VGlut) flies. Mann-Whitney test, ns p $\geq$ 0.05.

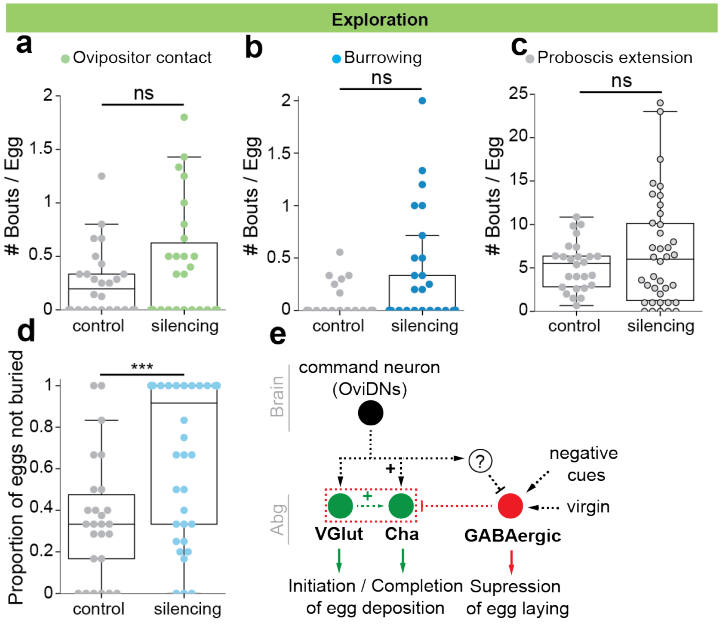


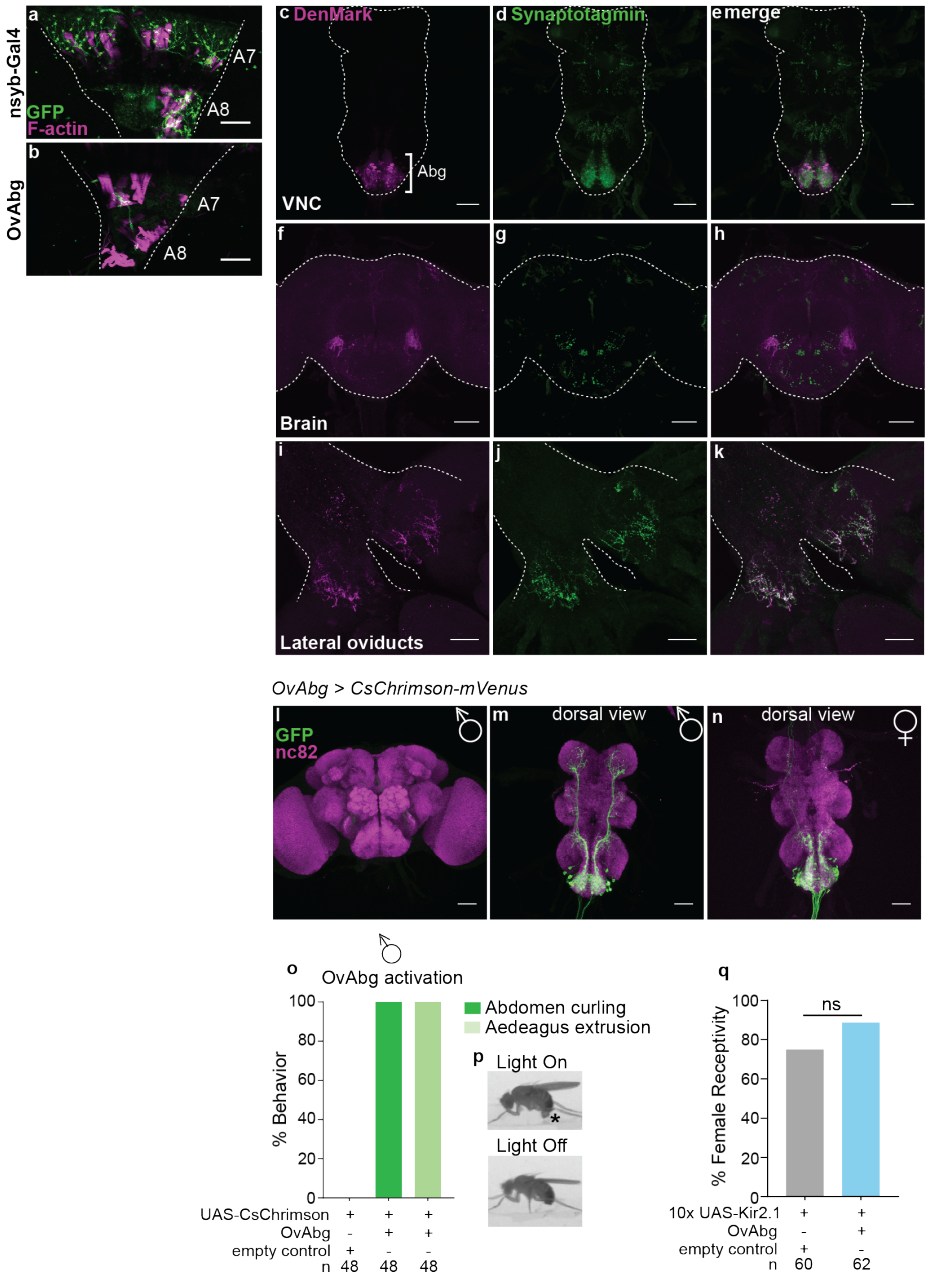
Figure 3.13: OvAbg Glutamatergic neurons contribute to egg deposition initiation *(continued).*

(a), **(b)** and **(c)** Exploration phase-associated motor elements and corresponding quantification of the number of behaviour bouts normalized for the number of eggs laid per female. n = 26 (control) and 38 (OvAbg/VGlut) flies. Mann-Whitney test in a, b and c, ns $p \geq 0.05$. **(d)** Proportion of eggs not buried per fly. n = 26 (control) and 38 (OvAbg/VGlut) flies. Mann-Whitney test, ***$p < 0.001$. **(e)** Proposed model of how glutamatergic, cholinergic and GABAergic OvAbg neurons may function in a local circuit to control and execute egg deposition behaviour. Solid arrows are based on our data and dashed arrows represent hypothetical connections.

Chapter 4

Supplementary Information

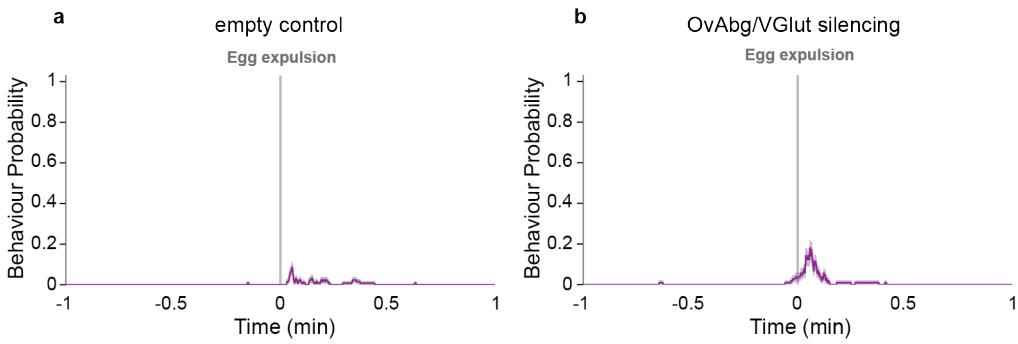
4.1 Supplementary Figure 4.1



Supplementary Figure 4.1

(a) and **(b)** Confocal images of female abdominal muscles in the nsyb-Gal4 (a) and OvAbg line (b). Neuronal innervations are stained with anti-GFP (green) and muscle fibers with anti-F-actin (magenta). A7 and A8 indicate the position of the abdominal segments. nsyb-Gal4 line expression is shown for comparison with the OvAbg line expression. Anti-GFP is targeting the fluorescent protein GFP and Venus from nsyb-Gal4 > mCD8::GFP and OvAbg > CsChrimson-mVenus flies. Scale bars a) and b) 50 µm. **(c-k)** Confocal images of OvAbg neuronal polarity in the female VNC (c-e), brain (f-h) and reproductive system (i-k). Dendrites (inputs) are labelled using the somatodendritic marker, DenMark, and axons (outputs) are labelled using the synaptic vesicle marker, Synaptotagmin. Anti-GFP is targeting EGFP-tagged Synaptotagmin and anti-DsRed is targeting mCherry-tagged DenMark. Scale bars c-k) 50 µm. **(l-n)** Confocal images of male brain (l), as well as male (m) and female (n) dorsal view of VNC showing OvAbg neurons and corresponding innervations stained with anti-GFP (green) to reveal the anatomy and nc82 for synapses. Anti-GFP is targeting the fluorescent protein Venus from OvAbg > CsChrimson-mVenus expressing flies. Scale bars l), m) and n) 50 µm. **(o)** Percentage of stimulation events in which male OvAbg flies displayed abdomen curling and aedeagus extrusion behaviours during photoactivation with CsChrimson. n = 48 (control) and 48 (OvAbg) stimulations. **(p)** Video snapshot (lateral view) of OvAbg male displaying abdomen curling and aedeagus extrusion (asterisk) behaviours in response to the stimulation with CsChrimson (top). A snapshot of the same male during a light off period (below) is also shown for comparison. **(q)** Percentage of receptive females during inhibition of OvAbg neurons. n = 60 (control) and 62 (OvAbg) females. Fisher´s exact test, ns $p \geq 0.05$.

4.2 Supplementary Figure 4.2



a and **b** Probabilities of grooming terminalia behaviour during a 1-min time window around egg expulsion for (a) control and (b) OvAbg/VGlut silenced flies. Time = 0 minutes marks the moment of egg expulsion (represented by the grey vertical line). n = 126 (control) and n = 105 (OvAbg/VGlut) egg expulsions.

4.3 Supplementary Videos

Videos can be accessed trough this link: `www.biorxiv.org/content/10.1101/2021.08.23.457359v1.supplementary-material`

4.3.1 Supplementary Video 1

Motor elements displayed by Canton S mated females during the exploration and egg deposition phases.

4.3.2 Supplementary Video 2

Abominal contortions displayed by Canton S mated females during the abdominal contortions phase.

4.3.3 Supplementary Video 3

CsChrimson optogenetic stimnulation of OvAbg neurons in mated females. The first part of the video shows egg pushing accompained by egg expulsion. The second part of the video shows egg pushing without egg expulsion. The red dot on the top right side of the video marks the stimulation period.

4.3.4 Supplementary Video 4

Egg deposition bout during GtACR1 inhibition of OvAbg neurons in mated females. The green dot on the top right side of the video marks the stimulation period.

4.3.5 Supplementary Video 5

CsChrimson optogenetic stimnulation of OvAbg/Cha neurons in mated females. The first part of the video shows egg pushing accompained by egg expulsion. The second part of the video shows egg pushing without egg expulsion. The red dot on the top right side of the video marks the stimulation period.

4.3.6 Supplementary Video 6

CsChrimson optogenetic stimnulation of OvAbg/VGlut neurons in mated females eliciting ovipositor contact behaviour. The red dot on the top right side of the video marks the stimulation period.

4.3.7 Supplementary Video 7

Egg deposition bout during Kir2.1 inhibition of OvAbg/VGlut neurons in mated females.

4.4 Supplementary Table 1. Fly stocks

Stocks	Source	Reference
Canton S (CS)	Lab stock	-
w-; empty-p65-AD(attp40); +	Bloomington Drosophila Stock Center (BDSC), 71210	Hampel et al. (2015)
w-; VT038154-p65.AD(attp40); +	BDSC, 74056	Tirian et al. (2017)
w-;+; dsx^{DBD}	-	Pavlou et al. (2016)
w-; +; R57C10-GAL4(attp2)	BDSC, 39171	Jenett et al. (2012)
y^1w-; Gad1(MI09277)-LexA:QFAD; +	BDSC, 60324	Diao et al. (2015)
y^1w-; ChaT(MI04508)-LexA:QFAD; +	BDSC, 60319	Diao et al. (2015)
y^1w-; VGlut(MI04979)-LexA:QFAD; +	BDSC, 60314	Diao et al. (2015)
w-; +; 20xUAS-CsChrimson-mVenus(attp2)	BDSC, 55136	Klapoetke et al. (2014)
w-, 20xUAS-CsChrimson-mVenus(attP18);+;+	BDSC, 55134	Markstein et al. (2008)
w-;; 20xUAS>Stop>CsChrimson-mVenus(attp2)	FLP-out version providedby Vivek Jayaraman	Klapoetke et al. (2014)
DL; +; 10xUAS-Kir2.1-EGFP	Provided by Moita laboratory	-
w-;UAS>Stop>Kir2.1-EGFP(VIE-19A);+	-	Asahina et al. (2014)
w-; +; 20xUAS-GtACR1(attp2)	-	Mohammad et al. (2017)
w-; 8xLexAop2-FLP(attp40); +	BDSC, 55820	Pan et al. (2012)
w-; +; 8xLexAop2-FLP(attp2)	BDSC, 55819	Pan et al. (2012)
w-; +; 20xUAS-mCD8::GFP(attp2)	BDSC, 32194	Pfeiffer at al. (2010)
w-; UAS-DenMark2, UAS-syt.EGFP	BDSC, 33064	Nicolai et al. (2010)

4.5 Supplementary Table 2. Full genotypes used in experiments

Figures	Genotypes
Figure 2.1 and 2.2	Canton S (CS)
Figure 3.1 and 3.2	Control: *w-; empty-p65-AD(attp40) / +; dsx^{DBD} / 20xUAS-CsChrimson-mVenus(attp2)* Test: *w-; VT038154-p65-AD(attp40) / +; dsx^{DBD} / 20xUAS-CsChrimson-mVenus(attp2)*
Figure 3.3	Control: *w- / DL; empty-p65-AD(attp40) / +; dsx^{DBD} / 10xUAS-Kir2.1-EGFP* Test: *w- / DL; VT038154-p65-AD(attp40) / +; dsx^{DBD} / 10xUAS-Kir2.1-EGFP*
Figure 3.4-3.6	Control: *w-; empty-p65-AD(attp40) / +; dsx^{DBD} / 20xUAS-GtACR1(attp2)* Test: *w-; VT038154-p65-AD(attp40) / +; dsx^{DBD} / 20xUAS-GtACR1(attp2)*
Figure 3.7 and 3.8b-d	Control: *w-; empty-p65-AD(attp40) / 8xLexaop2-FLP; dsx^{DBD}, UAS>Stop>CsChrimson-mVenus(attp2) / Gad1-LexA:QFAD (MI09277)* Test: *w-; VT038154-p65-AD(attp40) / 8xLexaop2-FLP; dsx^{DBD}, UAS>Stop>CsChrimson-mVenus(attp2) / Gad1-LexA:QFAD (MI09277)*
Figure 3.8a	Control: *w-; empty-p65-AD(attp40), UAS>Stop>Kir2.1-EGFP(VIE-19A) / 8xLexaop2-FLP(attp40); dsx^{DBD} / Gad1-LexA:QFAD (MI09277)* Test: *w-; VT038154-p65-AD(attp40), UAS>Stop>Kir2.1-EGFP(VIE-19A) / 8xLexaop2-FLP(attp40); dsx^{DBD} / Gad1- LexA:QFAD (MI09277)*
Figure 3.9a-c and 3.10a-d	Control: *w-; empty-p65-AD(attp40) / 8xLexaop2-FLP(attp40); dsx^{DBD}, UAS>Stop>CsChrimson-mVenus(attp2) / ChaT-LexA:QFAD (MI04508)* Test: *w-; VT038154-p65-AD(attp40) / 8xLexaop2-FLP(attp40); dsx^{DBD}, UAS>Stop>CsChrimson-mVenus(attp2) / ChaT-LexA:QFAD (MI04508)*
Figure 3.9d-e	Control: *w-; empty-p65-AD(attp40), UAS>Stop>Kir2.1-EGFP(VIE-19A) / 8xLexaop2-FLP(attp40); dsx^{DBD} / ChaT-LexA:QFAD (MI04508)* Test: *w-; VT038154-p65-AD(attp40), UAS>Stop>Kir2.1-EGFP(VIE-19A) / 8xLexaop2-FLP(attp40); dsx^{DBD} / ChaT-LexA:QFAD (MI04508)*
Figure 3.10e	1. *w-; VT038154-p65-AD(attp40) / +; dsx^{DBD} / 20xUAS-CsChrimson-mVenus(attp2)* 2. *w-; VT038154-p65-AD(attp40) / 8xLexaop2-FLP(attp40); dsx^{DBD}, UAS>Stop>CsChrimson-mVenus(attp2) / ChaT-LexA:QFAD (MI04508)*
Figure 3.11a-e	Control: *w-; empty-p65-AD(attp40) / VGlut-LexA:QFAD (MI04979); dsx^{DBD}, UAS>Stop>CsChrimson-mVenus(attp2) / 8xLexaop2-FLP(attp2)* Test: *w-; VT038154-p65-AD(attp40) / VGlut-LexA:QFAD (MI04979); dsx^{DBD}, UAS>Stop>CsChrimson-mVenus(attp2) / 8xLexaop2-FLP(attp2)*
Figure 3.11f; 3.12 and 3.13	Control: *w-; empty-p65-AD(attp40), UAS>Stop>Kir2.1-EGFP(VIE-19A) / VGlut-LexA:QFAD (MI04979); dsx^{DBD} / 8xLexaop2-FLP(attp2)* Test: *w-; VT038154-p65-AD(attp40), UAS>Stop>Kir2.1-EGFP(VIE-19A) / VGlut-LexA:QFAD (MI04979); dsx^{DBD} / 8xLexaop2-FLP(attp2)*
Supplementary Figure 4.1a-b	a. *w-; +; GMR57C10-GAL4(attp2) / 20xUAS-mCD8::GFP(attp2) (nsyb)* b. *w-; VT038154-p65-AD(attp40) / +; dsx^{DBD} / 20xUAS-CsChrimson-mVenus(attp2)*
Supplementary Figure 4.1c-k	*w-; VT038154-p65-AD(attp40) / UAS-Synaptotagmin-EGFP, UAS-DenMark2-mCherry; dsx^{DBD} / +*
Supplementary Figure 4.1l-n	*w-, 20xUAS-CsChrimson-mVenus(attP18); VT038154-p65-AD(attp40) / +; dsx^{DBD} / +*
Supplementary Figure 4.1o-p	Control: *w-; empty-p65-AD(attp40) / +; dsx^{DBD} / 20xUAS-CsChrimson-mVenus(attp2)* Test: *w-; VT038154-p65-AD(attp40) / +; dsx^{DBD} / 20xUAS-CsChrimson-mVenus(attp2)*
Supplementary Figure 4.1q	Control: *w- / DL; empty-p65-AD(attp40) / +; dsx^{DBD} / 10xUAS-Kir2.1-EGFP* Test: *w- / DL; VT038154-p65-AD(attp40) / +; dsx^{DBD} / 10xUAS-Kir2.1-EGFP*
Supplementary Figure 4.2a-b	Control: *w-; empty-p65-AD(attp40), UAS>Stop>Kir2.1-EGFP(VIE-19A) / VGlut-LexA:QFAD (MI04979); dsx^{DBD} / 8xLexaop2-FLP(attp2)* Test: *w-; VT038154-p65-AD(attp40), UAS>Stop>Kir2.1-EGFP(VIE-19A) / VGlut-LexA:QFAD (MI04979); dsx^{DBD} / 8xLexaop2-FLP(attp2)*

Chapter 5

Experimental Procedures

5.1 Fly stocks and husbandry

See Supplementary Table 1 and 2 for genotypes of *Drosophila* used in this study. Fruit flies *D. melanogaster* were raised in standard cornmeal-agar medium, using Vienna food recipe (in 1 Liter of water: 80 g molasses-barley malt, 22 g beet syrup, 80 g corn flour, 18 g granulated yeast, 10 g soy flour, 8 g agar-agar, 8 mL propionic acid, 12 mL 15% nipagin, 35 mL Bavistin), at 25ºC and 70% relative humidity in a 12h dark:12h light cycle. Detailed information on fly housing and age for each experiment are indicated in the relevant section.

5.2 Immunohistochemistry

Adult brains, VNCs, reproductive systems and abdomen cuticles were dissected in cold Phosphate-Buffered Saline (PBS) and immediately transferred to cold Paraformaldehyde (PFA) 4% in PBL (PBS with 0.12 M Lysine) and fixed for 30 min at Room Temperature (RT), washed three times for 5 min in PBT (PBS with 0.5% Triton X-100) and blocked for 30 min at RT in 10% normal goat serum in PBT (Sigma, cat# G9023). Samples were incubated with the primary antibodies in blocking solution, for 72h at 4°C. The following primary antibodies were used: rabbit anti-GFP 1:1000 (Molecular Probes, cat#A11122), chicken anti-GFP 1:1000 (abcam, ab13970), mouse anti-nc82 1:10 (Developmental Studies Hybridoma Bank, cat# AB2314866), rabbit anti-DsRed 1:1000 (Takara, cat# 632496). Samples were washed three times for 5 min in PBT and incubated in Alexa Fluor 488 or 594 secondary antibodies 1:500 (Invitrogen) for 72h at 4ºC. To counterstain the female reproductive system, Alexa 594-conjugated phalloidin (Molecular Probes, cat# A12381) was used. Samples were washed three times for 5 min in PBT and mounted in VectaShield medium (Vector Laboratories, cat#H-1000). Images were acquired on a Zeiss LSM 710 confocal microscope using a 25X immersion objective (Zeiss) for the brains/VNCs and a 10X objective (Zeiss) for the reproductive systems and abdomen cuticles. After acquisition, colour levels were adjusted using Fiji (Schindelin et al., 2012) for optimal display.

5.3 Preparation of flies to be assayed

Low fly density crosses (10-15 virgin females x 5 males per bottle) were used in all experiments for rearing flies with the appropriate genotype. In order to maximize the occurrence of egg deposition events during behavioural experiments, we followed the egg-laying deprivation protocol described by Yang et al. (2015) in all experiments, except in the 24h egg-laying assays. Briefly, groups of 5-7 virgin females per vial of the appropriate genotypes and 2-3 Canton S males (for mating) were collected into normal food vials with the exception of optogenetic experiments in which flies were housed in normal food containing all-trans-Retinal (Sigma, R2500) (all-trans-Retinal concentrations used: 0.2 mM for CsChrimson activation and 0.4 mM for GtACR1 silencing). In contrast with the original protocol (Yang et al., 2015), wet yeast paste was not supplied to the food. Flies were left in the vials for 4 to 7 days at 25ºC and 70% relative humidity. Behavioural assays were performed within that 4–7 days' time window.

5.4 Behavioural assays

5.4.1 24h egg-laying assay

Single virgin females were gently aspirated and transferred to 35 mm Petri dishes of 10 mm of height (Thermo Fisher Scientific) coated with apple agar (750 mL water, 250 mL apple juice, 19,5 g agar, 20 g sugar, 10 mL 10% nipagin) and incubated with a naive CS male for 2h under constant observation to check for mating occurrence. Plates where mating did not happen were discarded. Flies were kept in the plate for 24h before eggs were counted. After egg counting, the female´s reproductive system was dissected to measure egg jamming.

5.4.2 Egg-laying arena and substrate

Custom made small rectangular-shaped arenas with 2 chambers were designed to allow recording of 2 flies simultaneously. Each chamber measures 1.8 (H) x 1.2 (L) x 0.3 (D) cm. During behavioural assays, the chambers were partially filled with the egg-laying substrate, which in this study was always 1% agarose (SeaKam® LE Agarose, cat# 50004) diluted in distilled water (Milli-Q® Water Purification Systems Merk). Flies had a free walking space of 1.5 x

0.7 x 0.3 cm in the chamber for egg-laying. The base and the lid of the arena were made of white opaque and transparent acrylic, respectively.

5.4.3 Detailed behaviour

To analyse the motor elements associated with egg-laying behaviour, females were collected soon after eclosion and housed in groups following the egg-laying deprivation protocol described above. Aged 4-7 days females were tested. Flies were gently aspirated into the egg-laying arena and behaviour was recorded at 20 frames per second during 45 min for CS (Fig. 2.1 and 2.2) and during 15 min for OvAbg/VGlut silenced females (Fig. 3.11f, 3.12, 3.13). The same fly handling procedure was performed for optogenetic experiments in which egg-laying motor elements were analysed (for more detailed information, see the optogenetic stimulation section).

5.4.4 Optogenetics

For all experiments using CsChrimson, except in the 16h egg-laying assay (Fig. 3.8b-d), the stimulation protocol included 1 min baseline period followed by 6 repetitions of 10 s red-light stimuli with a power of 4.40 mW/cm^2 and 20 s interval between stimuli. Fly behaviour was recorded at 20 frames per second, except in the OvAbg line activation experiments (Fig. 3.2b-g) in which we used 15 frames per second. In the OvAbg headless females' photoactivation (Fig. 3.2d), the head was gently cut using dissection forceps (Dumont #55 Forceps, 11295-51) under CO2 anaesthesia. Flies were transferred to the egg-laying arena and allowed to recover from this procedure for 5-10 min before photoactivation. In the 16h photoactivation egg-laying assay (Fig. 3.8b-d), mated females were transferred to the apple agar plates (described in the 24h egg-laying assay section). The stimulation protocol included constant red-light with a power ranging 4.19-4.85 mW/cm^2 during 16h. At the end of this period, the eggs were counted and the reproductive system was dissected to measure egg jamming. For the silencing experiment using GtACR1 (Fig. 3.4-3.6), the stimulation protocol included a pre-stimulation period that lasted for 10 min, followed by constant green-light stimulation with a power of 5-6.23 mW/cm^2 during 15 min and a post-stimulation period of 5 min. Videos were recorded at 20 frames per second.

5.4.5 Image capture

Flies were filmed using a camera mounted above the arena (Teledyne Flir Flea3 FL3-U3-13S2M) equipped with a 16mm fixed focal length lens (Edmund Optics) and with a resolution of 1328 x 1048 pixels. For illumination, an infrared light using a 940 nm LED strip (SOLAROX) and a Hoya 49 nm R72 infrared filter (to reduce interference of visible light) were used. An electronic HARP LED array interface v1.3 and an HARP LED array v2.0, developed by the Champalimaud Foundation Hardware Platform, were used to evoke a high-powered 610nm (CsChrimson activation) and 527nm (GtACR1 silencing) light. Bonsai software (Lopes et al., 2015) was used to trigger the optogenetics stimuli and to acquire the videos as avi files.

5.5 Quantification and statistical analysis

5.5.1 Data processing

After movies were acquired, the in-house developed software Python VideoAnnotator was used to manually annotate the time and duration of all egg-laying motor elements under analysis. Annotations were done for the total duration of the video in all experiments.

5.5.2 Quantification of behaviours

Data and statistical analysis were performed using custom Python scripts in Fig. 2.1b-d; Fig. 2.2a-e; Fig. 3.4-3.6; Fig. 3.11f; Fig. 3.12 and 3.13a-d. All the other analysis were performed using Prism 9 (GraphPad Software, La Jolla, CA).

The inter-egg expulsion intervals (Fig. 2.1d) were calculated as:

Inter-egg expulsion intervals = first frame of egg expulsion bout - last frame of previous egg expulsion bout (per fly)

The product was converted to minutes.

The number of eggs per 5 minutes (Fig. 3.4b) was calculated as:

 # Eggs / 5 min = (sum # egg expulsions) / 5 minutes (per fly)

The number of behaviour bouts per 5 minutes (Fig. 3.5a-e; Fig. 3.6a, d-f) was calculated as:

 # Bouts / 5 min = (sum # behaviour frames) / 5 minutes (per fly)

The number of behaviour bouts per egg (Fig. 3.12a-f; Fig. 3.13a-c) was calculated as:

 # Bouts / Egg = (sum # behaviour frames) / total # egg expulsions (per fly)

The mean bout duration (Fig. 3.5f and Fig. 3.6b) was calculated as:

mean bout duration = # behaviour frame duration (s) / total # behaviour bouts (per fly)

Where the behaviour frame duration is given by the last frame subtracted to the first frame of each behaviour bout converted to seconds.

The proportion of eggs not buried (Fig. 3.13d) was calculated as:

Proportion of eggs not buried = (sum # eggs not buried) / total # eggs (per fly)

Egg expulsion bouts in which it was not possible to determine whether the egg is buried or not were excluded from this analysis.

The percentage of behaviour displayed by flies during photoactivation (Fig. 3.2b,d; Fig. 3.10a and Fig. 3.11d) was calculated as:

% Behaviour = (sum # stimuli with behaviour) / total # stimuli

The percentage of females laying eggs (Fig. 3.2f and Fig 3.10c) was calculated as:

% Females laying eggs = (sum # females that laid eggs) / total # of females

The percentage of eggs laid during the photoactivation protocol (Fig. 3.2g and Fig. 3.10d) was calculated as:

% Eggs = (sum # eggs laid on each stimulus or ISI) / total # eggs

The latency for egg expulsion (Fig. 3.10e) was calculated as:

Latency egg expulsion = first frame corresponding to egg expulsion event – first frame corresponding to the stimulus initiation (per fly)

The product was converted to seconds.

The percentage of eggs jammed (Fig. 3.3b; Fig. 3.8c and Fig. 3.9e) was calculated as:

% Eggs jammed = (sum # reproductive systems with eggs in the oviducts) / total # reproductive systems

To investigate the egg-laying phases transition probability (Fig. 2.2e), we first chose an egg-laying motor element to represent each phase: egg expulsion represented the egg deposition phase, abdominal contortions represented the abdominal contortions phase and ovipositor contacts that do not progress to egg expulsion represented the exploration phase. Proboscis extension and burrowing behaviours were not considered for the exploration phase transition analysis because: 1) proboscis extension displays a continuous occurrence during egg-laying behaviour (see Fig. 2.2d); 2) proboscis extension behaviour is not exclusive to egg-laying, being also displayed in other behavioural contexts (ex: feeding); 3) burrowing behaviour is displayed along with ovipositor contact but with lower frequency (see Fig. 2.2d). We calculated for the total video duration (45 min), the likelihood of all transitions between phases by using a first order Markov Chain analysis (Slater, 1973). Phases transitions were identified as changes in the selected behavioural patterns for each phase.

To calculate the probability of the egg-laying behaviours around egg expulsion (Fig. 2.2a-d and Supplementary Fig. 4.2a-b) we aligned all the egg expulsion events of all flies and we counted how many behaviours were occurring in each of the 1200 frames (1 minute) preceding and following the end of egg expulsion. We then normalized the counts over the total number of egg expulsions. To represent the egg expulsion event, the last frame for each event was selected. We excluded from the analysis egg expulsion events that are less than 1200 frames from the start or end of the video.

5.5.3 Statistical analysis

Boxplot in Fig. 2.1c indicates the median flanked by the 25th and 75th percentiles (box) and whiskers showing the maximum and minimum values. In all the other boxplots the whiskers represent the 5th and 95th percentiles. Error bars when shown are mean ± s.d.. Prior to statistical testing, normality across all individual experiments was verified using the Shapiro's Test, D'Agostino's Test, Anderson-Darling test and Kolmogorov-Smirnov in Prism 9 analysis. Only Shapiro´s and D'Agostino´s tests were used in Python analysis. To assess the data variance homogeneity, Levene´s and Bartlett´s tests were used for non-normally and normally distributed data, respectively. In all experiments, we performed pairwise comparisons. Mann-Whitney test was used in non-normally

distributed data. t-test was used in normally distributed data and the t-test with Welch´s correction was applied when data had unequal variances. Fisher´s exact test was used in contingency tables. The P value is provided in comparison with the control and indicated as * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, **** for $p < 0.0001$, and 'ns' for non-significant ($p \geq 0.05$).

Chapter 6

General Discussion and Conclusion

6.1 Discussion

Egg-laying is an excellent model for exploring the architecture of lower motor control, as it is a complex behaviour that comprises different phases, each with a specific behavioural repertoire. The results presented in this doctoral thesis contributed to elucidate how the VNC controls motor behaviours of the egg-laying process. We first performed a detailed analysis of the behavioural elements and the phases occurring during egg-laying, which provided a fundamental framework to then address the neuronal basis of egg-laying behaviour. We next identified and investigated the role of three distinct neuronal populations with differential roles for the execution and regulation of egg-laying behaviour. A general discussion of these fundamental results and their implications to our knowledge of how the CNS works is presented below.

6.2 Egg-laying behaviour in wild-type flies

Complementary to Yang et al. (2008), we showed that egg-laying behaviour is structured in three phases - egg deposition, abdominal contortions and exploration. Each phase includes a specific and stereotyped repertoire of behavioural elements conserved across *Drosophila* species (Bräcker et al., 2019) and executed to promote substrate probing, egg deposition and ovulation. Different groups simultaneously working on this topic reached complementary results that were recently published (Cury and Axel, 2021; Vijayan et al., 2021), highlighting the need in the field for a more comprehensive characterization of this complex behaviour.

We show that the egg deposition phase follows a reliable behavioural sequence in which grooming terminalia is the only optional behaviour, as also shown by Cury and Axel (2021). We established that egg deposition always transits to abdominal contortions, the sole behaviour of this phase, which is in line with Vijayan et al. showing that abdominal contortions represent the ovulation period and, thus, are critical for the positioning of a new egg to be laid (Vijayan et al., 2021). The exploratory phase shares common behavioural elements with the egg-deposition phase but, in this case, they are used by flies to sample the substrate and do not culminate in egg expulsion. The exploratory behaviours are not performed in a behavioural sequence and can be displayed with different timings, in contrast with the egg deposition motor programme. The variability in the sequence and timing of exploratory behaviours, which was also reported by Cury and Axel (2021), may offer to the organism a behavioural flexibility to cope with changes in the environment during egg-laying site selection. On this analysis, we did not distinguish proboscis extension bouts performed for feeding or exploration in the context of egg-laying. However, since the substrate we used lacks nutritive cues and the flies tested are well fed, we hypothesize that most of the proboscis extension bouts observed in our experiments are specific for egg-laying behaviour. Interestingly, females show reduced exploration in our behavioural assays and this phase is optional. Two factors may explain these observations: 1) there may be exploration that does not include the motor elements we considered and, instead, flies use mechanosensory and chemosensory information from the legs and antennae; 2) we use very small arenas with a restricted space for exploration and without complex sensory cues. This feature may allow a quick spatial and sensory recognition of the environment making

exploration less frequent. Future work on the features of exploration and site selection should use more complex arenas and environments.

In this work, moments of distinct behaviours were annotated manually, which represents an important investment of time and limits the use of this behavioural assay in high-throughput experiments. The implementation of automated annotation systems (e.g. DeepLabCut, JAABA) to track fly kinematics and classify egg-laying motor elements will certainly offer an advantage for future studies (Kabra et al., 2013; Mathis et al., 2018).

Overall, this detailed description of egg-laying behaviour provided a framework to interrogate the underlying neuronal substrates.

6.3 OvAbg neurons: lower motor control of egg-laying behaviour

This study offers unprecedented insights into the lower motor control of egg-laying through the characterization of a novel population located in the Abg − the OvAbg neurons. The analysis of their anatomy and function showed that OvAbg neurons are critical for egg-laying behaviour and belong to the egg-laying motor circuits. We show that OvAbg is a dimorphic population involved in the control of reproductive behaviours, as shown by the distinct CNS anatomy and function between females and males. The differential regulation of *dsx* expression supports sex-specific behaviours (Kohl et al., 2013; Nojima et al., 2021; Pavlou et al., 2016; Rideout et al., 2010). We assume that we are targeting part of the *dsx+* population at the Abg based on our intersectional approach using splitGal4 lines controlled by *dsx* regulatory regions, and given the anatomy reported for *dsx+* neurons in the VNC (Pavlou et al., 2016; Rideout et al., 2010).

The Abg egg-laying circuit is poised to receive commands from the brain for the execution of egg deposition. Which are the upstream targets of OvAbg neurons? OviDNs innervations at the VNC are anatomically poised to connect with OvAbg neurons (Vijayan et al., 2021; Wang et al., 2020). Tools to study connectivity between neurons, such as GRASP (Feinberg et al., 2008) and optogenetic stimulation integrated with calcium imaging, await the development of new genetic drivers labelling OviDNs and OvAbg populations, respectively.

The OvAbg neurons provided a great entry point to address how different circuits coordinate egg deposition. A detailed discussion of the three abdominal ganglion populations characterized in this study follows below.

6.4 Execution of Egg Deposition: Glutamatergic and Cholinergic OvAbg circuits

We characterized two sub-populations within the OvAbg circuit − a glutamatergic and a cholinergic − that implement the egg deposition motor programme.

The OvAbg/VGlut circuit represents a restricted fraction of the *dsx+* glutamatergic-expressing neurons in the Abg of *Drosophila* females. Given the total number of *dsx+* neurons expressing glutamate at the female Abg (∼100 neurons) reported by Pavlou et al (2006), we reasoned that OvAbg/VGlut population represents only 10-18% of all Abg glutamatergic *dsx+* neurons. The neuronal manipulations of OvAbg/VGlut population uncovered a specific role in the initiation of the egg deposition motor sequence through the execution of ovipositor contact and burrowing behaviours. Interestingly, these motor elements of egg deposition, that are also displayed during the exploration phase, were not affected in the exploration phase when these neurons were silenced, suggesting a different control for the same motor elements during different phases.

Curiously, the fact that OvAbg/VGlut neurons are involved in the initiation of the egg deposition sequence led us to speculate that they could represent the analogous neurons on the female Abg to the male-specific glutamatergic neurons controlling the initiation of genital coupling during male courtship (Pavlou et al., 2016). Additionally, the OvAbg/VGlut population could be the equivalent circuit in the fly to the digging motor circuit found in locust females (see introduction section 1.6). Burrowing behaviour is one of the motor elements elicited by OvAbg/VGlut activation and, complementary to our work, this behaviour was recently associated with digging the substrate to allow subterraneous egg deposition (Cury and Axel, 2021). Based on the architecture of the locust egg deposition circuits, we hypothesized that the OvAbg/VGlut population could locally interact with an egg-retention circuit to block egg expulsion while digging is still occurring.

Egg-laying is mostly performed by mated females, although virgin females may deposit unfertilized eggs residually. Activation of OvAbg/VGlut neurons leads to fewer events of egg deposition initiation in virgin females when compared to mated females, thus suggesting that mating status modulation of egg-laying is occurring locally at the Abg, in addition to the modulation in the brain (Feng et al., 2014; Shao et al., 2019; Wang et al., 2020). Local modulation may result from direct octopaminergic modulation or in downstream targets.

The OvAbg/Cha circuit represents a broad population that is necessary and sufficient for egg deposition. Optogenetic activation of OvAbg/Cha neurons triggers the completion of egg deposition through the execution of egg pushing and egg expulsion, while their silencing abolishes egg-laying and promotes egg-jamming. The egg-jamming phenotype suggests a role for OvAbg/Cha neurons in oviduct contractions. Such modulation is most likely indirect, because we did not find any projections between this population and the lateral oviducts. As discussed in the introductory section, a role for OA in the oviduct activity during egg-laying has been intensely studied in insects. Do OvAbg/Chat neurons belong to the octopaminergic network involved in this process? We propose that the OvAbg population is unlikely to be octopaminergic, given the differences in the Abg cell body anatomy between OvAbg and the octopaminergic representative dTdc2-GAL4 line (data not shown). Nonetheless, cholinergic OvAbg neurons may still modulate reproductive function by responding to octopaminergic signalling (e.g. through the expression OAMB and/or Octβ2R receptors). An RNAi screening targeted to octopaminergic receptors and functional connectivity experiments should help clarify at the role of OA on the OvAbg circuit function.

Our findings hint at a possible interaction between the OvAbg glutamatergic and cholinergic populations in the execution of egg deposition. How is this interaction set so that flies perform a sequential order of behavioural elements leading to egg expulsion? Are OvAbg/VGlut presynaptic to OvAbg/Cha neurons? If glutamatergic OvAbg were upstream partners of cholinergic OvAbg neurons in a linear sequential motor circuit, we would expect that activation of OvAbg/VGlut population elicited egg pushing and egg expulsion, which are never observed upon this manipulation. However, we cannot rule out the possibility for such a sequential pathway because OvAbg/VGlut neurons may work

together with other excitatory descending input to activate OvAbg/Cha population (see model schematic, Fig. 3.13e).

It is also interesting to speculate that both populations may receive sensory feedback from the reproductive system to shape the egg deposition motor programme. For example, Posterior Uterine (PU) sensory neurons located in the uterus (Cury and Axel, 2021) could relay feedback to OvAbg/VGlut neurons about the presence of the egg in the ovipositor during burrowing behaviour and, therefore, modulate the activity of this motor circuit to fine tune the progression along the behavioural sequence to complete egg deposition.

6.5 Regulation of Egg-laying: GABAergic OvAbg circuit

The GABAergic OvAbg circuit represents about half (∼46%) of all *dsx+* GABAergic-expressing neurons in the Abg according with Pavlou et al (2006). While the glutamatergic and cholinergic OvAbg populations represent motor circuits involved in the implementation of the egg deposition motor programme, the OvAbg/Gad1 population seems to play a more regulatory role on egg-laying. This evidence comes from the fact that (1) the number of eggs laid is not affected when OvAbg/Gad1 neurons are silenced and (2) optogenetic activation does not elicit any behavioural element associated with the egg-laying motor programme (data not shown) but, instead, is sufficient to block egg-laying. Taken together, our data indicate that OvAbg/Gad1 activity negatively regulates egg-laying whereby they need to be silent during egg-laying, likely through inhibitory descending input. Local suppression of egg-laying may be required when negative egg-laying cues arise or in virgin females (mating status modulation), which could be mediated by an increase in activity in the GABAergic population. OvAbg/Gad1 neurons could suppress egg-laying by acting on the glutamatergic and cholinergic populations (see model schematic, Fig. 3.13e). We may hypothesize that OvAbg/VGlut and OvAbg/Gad1 populations form a circuit to coordinate egg deposition initiation and suppression with analogous architecture to the one proposed by Pavlou et al. (2016). If OvAbg/Gad1 neurons indeed supress egg-laying in unfavourable environmental conditions, to which negative cues do they respond? Who is relaying the information about the environment to OvAbg/Gad1? We envision

that it could be either transmitted from sensory systems (e.g. legs, reproductive system) or from descending input coding negative cues (e.g. DNp42 pathway for geosmin avoidance).

As a conclusion, we identified different populations of OvAbg neurons involved in the control of egg-laying motor output. The fundamental questions on the connectivity between these OvAbg populations require further investigation, which is currently limited to the genetic drivers available to label different OvAbg neurons. Future screenings on additional enhancer lines (e.g. enhancer bashing) and different expression systems (e.g. LexA driver lines) would allow to independently label and manipulate each of the relevant clusters. This will open new possibilities of dissecting the circuit. More recently, the EM-based connectivity for the T1, T2 and T3 VNC segments was recently published highlighting the sensory and motor networks controlling limb movement (Phelps et al. 2021). Mapping the structural connectivity of the Abg awaits future work and would certainly contribute for our understanding on how the OvAbg circuit coordinates and regulates egg-laying behaviour.

6.6 Conclusion

1. Our findings provide a detailed description of egg-laying. We described the different motor elements, their participation in different egg-laying phases and how flies transition from one phase to the next. This description facilitates the goal of linking a complex behaviour – egg-laying – with its neuronal underpinnings.

2. We present insights into the logic of egg deposition motor circuits. This work serves as a stepping stone to dissect ascending and descending communication with the brain, to extend neuronal dissection and connectivity of egg-laying populations and address mechanisms of local mating status modulation of egg-laying.

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