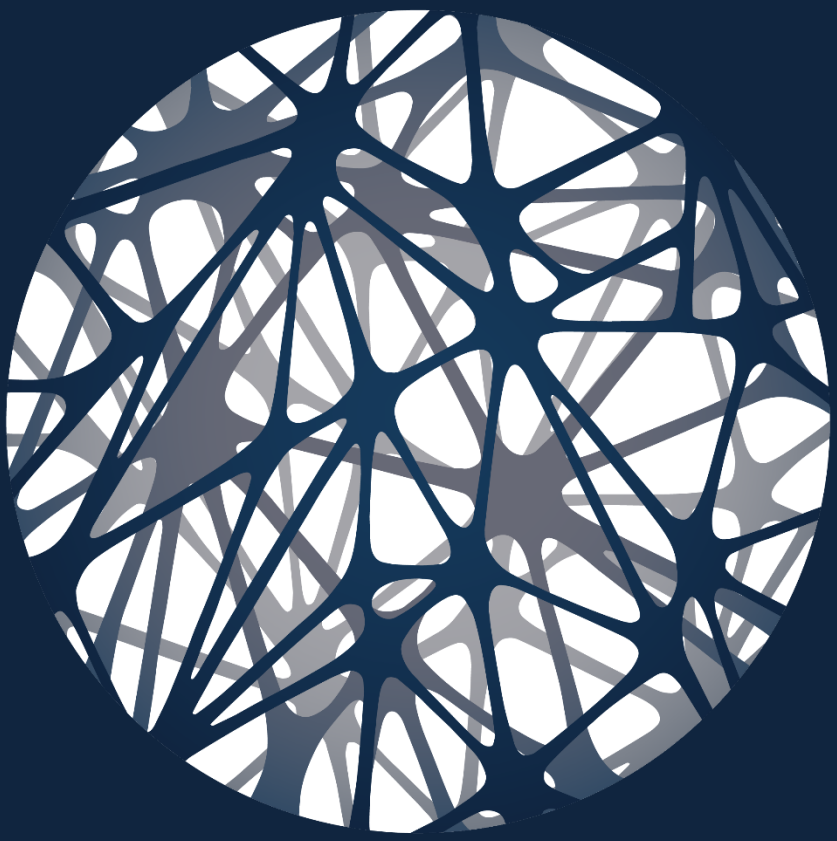
Novel circuits involved in *Drosophila melanogaster* virgin female sexual behaviours

Eliane Ochôa Arez de Carvalho



Dissertation presented to obtain the Ph.D degree in Molecular Biosciences

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

Oeiras,
September, 2021

Novel circuits involved in *Drosophila melanogaster* virgin female sexual behaviours

Eliane Ochôa Arez de Carvalho

Dissertation presented to obtain the Ph.D degree in Molecular Biosciences

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

Research work coordinated by:

PGCD
PROGRAMA DE PÓS-GRADUAÇÃO
CIÊNCIA PARA O DESENVOLVIMENTO

Fundação Champalimaud

FCT
Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

Oeiras, September 2021

UNIVERSIDADE NOVA DE LISBOA

Novel circuits involved in *Drosophila melanogaster* virgin female sexual behaviours

Eliane Ochôa Arez de Carvalho

A Dissertation

Presented to the Faculty

of Universidade Nova de Lisboa

in Candidacy for the Degree

of Doctor of Philosophy

Maria Luísa Vasconcelos

2021

The work presented in this dissertation was carried out under the Graduate Program Science for Development (PGCD, Programa de Pós-Graduação Ciência para o Desenvolvimento), at the Champalimaud Centre for the Unknown (Lisbon, Portugal) and under the supervision of Dr Maria Luísa Vasconcelos. Financial support was given by a doctoral fellowship from the Portuguese Science and Technology Foundation (FCT, Fundação para a Ciência a Tecnologia), with the reference SFRH/BD/113753/2015 attributed to Eliane Ochôa Arez de Carvalho and by the Champalimaud Foundation.

To Marco and Gabriela

Acknowledgements

I would like to express here my gratitude for all people who accompanied me in this journey and who, in most varied ways, supported me, nurtured me, and always motivated me to continue.

To my life partner and our daughter, to whom I dedicate this thesis, for never letting go of my hand and ensuring that my feet are firmly on the ground. I am grateful to them for always making me a better person, for all understanding, motivation, companionship and above all, for their love. I am also grateful to my parents, Sílvia and Tozé, and my aunt and uncle, Diná and Zé, for guiding my life and for always supporting me unconditionally. My brothers, Marcus, Kevin and Luis, and my cousin Claudio, for being my best cheerleaders, and to the rest of the family who have supported me throughout my journey.

To my supervisor, Luísa, for believing in me and giving me the opportunity to be part of an extremely enriching environment; because she has an unwavering patience and calm, the perfect antagonists for my "hyperactivity". But above all, for her humanity and for having taught me so much.

To my thesis committee, Carlos and Marta, for all support throughout these years and for the wise advice that helped me make some important choices for my professional future.

To my lab mates, with whom I shared defeats and conquests, for the exchange of ideas and thoughts outside the box. In particular, a huge thanks to Cecilia for the final push. I cannot fail to express my gratitude to two former lab members, Márcia and Sophie, for patiently introducing me to the wonderful world of neuroscience and the fruit fly.

To the squad, Anabel, Susana, and Tânia, for being part of my life and allowing me to be part of theirs.

To my friends, for every moment of peace and harmony and for being a breath of fresh air in my life.

Table of contents

Summary

Courtship is a set of species-specific behaviours that provide a way by which male and female communicate, allowing the display of fitness of one sex (male) to the other (female) and leading to mating. This innate behaviour present in all animals, is crucial for reproduction and species survival. In *Drosophila melanogaster*, courtship consists of a series of stereotyped actions performed by the male towards the female, while she evaluates him by the sensory cues presented to her. At the end, the male may decide to attempt copulation, but it is the female who will decide whether or not to mate.

Our goal was to contribute to the understanding of the neural circuit that mediates female behaviours during courtship, not only her receptivity but also the behaviours she displays to accomplish her decision of accepting or rejecting the male.

In chapter I we used a single pair receptivity assay and a temperature-inducible neuronal inhibitor to screen eight GAL4 lines for the effect of silenced brain neurons on receptivity. We found that silencing *70A09*-GAL4 brain neurons drastically reduces female receptivity and increases walking speed. The increase in speed is courtship song-specific, as escape from a looming threat or from a courtship-impaired male is not intensified. Activation of 70A09 neurons leads to pausing, confirming the role of these neurons in escape modulation. We suggest that *70A09*-GAL4 neurons are necessary to supress female escape in a courtship context.

In chapter II we used a split-GAL4 intersection of the 70A09 line with a *doublesex*-expressing line, to investigate the role of the *70A09-dsx* positive neurons in female receptivity. We observed the expression of subsets of brain *dsx* neurons (aDN, pC1a, pC1b and vpoDN), whose silencing reduces virgin female receptivity and increases the display of immature-like behaviours, albeit receptive virgin behaviours were not affected. These neurons are not the ones modulating the speed phenotype observed in chapter I, as silencing them does not affect female walking speed during courtship. We employed another intersectional approach to restrict 70A09 neuronal expression to pC1a, in order to access the individual role of these neurons in female receptivity. Although this intersection did not show the expression of the pC1a alone, we present

evidence that these neurons are necessary to trigger female receptivity, but do not affect any of the premating behaviours.

This work provides important insights into female premating behaviours and how these behaviours and receptivity are encoded in the brain.

Resumo

A corte é um conjunto de comportamentos específicos da espécie, que constituem a comunicação entre o macho e a fêmea, permitindo a exibição da aptidão de um interveniente (macho) ao outro (fêmea) e levando ao acasalamento. Este comportamento inato, presente em todos os animais, é crucial para a reprodução e sobrevivência das espécies. Na *Drosophila melanogaster*, a corte é constituída por uma série de ações estereotipadas que o macho direciona à fêmea, enquanto ela o avalia através das pistas sensoriais que lhe são apresentadas. No final, o macho pode tentar a cópula, mas é a fêmea quem decidirá se acasala ou não.

Este estudo teve como objetivo contribuir para o conhecimento do circuito neuronal que modula os comportamentos da fêmea durante a corte, não só a sua recetividade, mas também os comportamentos que ela exibe até decidir se aceita ou rejeita o macho.

No capítulo I, utilizámos um teste de recetividade de um único par e um inibidor neuronal induzível por temperatura para rastrear oito linhas GAL4 quanto ao efeito da inibição dos neurónios cerebrais na recetividade da fêmea. Verificámos que o silenciamento dos neurónios *70A09*-GAL4 reduz drasticamente a recetividade da fêmea e aumenta a sua velocidade de marcha. O aumento da velocidade é uma resposta específica à canção de corte, uma vez que a fuga de uma ameaça eminente ou de um macho com corte deficiente não é intensificada. A ativação dos neurónios 70A09 induz a que as fêmeas parem, confirmando o papel destes neurónios na modulação da fuga. Sugerimos que os neurónios *70A09*-GAL4 são necessários para suprimir a fuga da fêmea num contexto de corte.

No capítulo II, recorremos a uma interseção split-GAL4 da linha *70A09*-GAL4 com uma linha que expressa *doublesex*, para investigar o papel dos neurónios *70A09-dsx* na receptividade da fêmea. Observámos a expressão de subconjuntos de neurónios cerebrais *dsx* (aDN, pC1a, pC1b e vpoDN), cujo silenciamento reduz a recetividade da fêmea virgem e aumenta a exibição de comportamentos característicos de fêmeas imaturas, embora os comportamentos de virgens recetivas não tenham sido afetados. Estes neurónios não são os que modulam o fenótipo de

velocidade observado no capítulo I, uma vez que, quando silenciados, a velocidade de marcha da fêmea durante a corte não é afetada. Com o objetivo de perceber o papel dos neurónios pC1a na recetividade da fêmea, utilizámos outra interseção para restringir a expressão neuronal da 70A09 ao pC1a. Embora esta intersecção não tenha revelado a expressão apenas do pC1a, apresentamos evidências de que estes neurónios são necessários para desencadear a recetividade da fêmea, mas não afetam nenhum dos comportamentos manifestados pré-cópula.

Este trabalho fornece dados importantes sobre os comportamentos pré-cópula da fêmea e, como estes comportamentos e a receptividade são codificados no cérebro.

Thesis publications

Arez, E., Mezzera, C., Neto-Silva, R.M. et al. Male courtship song drives escape responses that are suppressed for successful mating. Sci Rep 11, 9227 (2021). https://doi.org/10.1038/s41598-021-88691-w

List of acronyms and abbreviations

5-HT	5-hydroxytryptamine or serotonin
5-HT7	serotonin receptor type 7
7,11-HD	heptacosadiene
7,11-ND	nonacosadiene
7-T	tricosene
AMMC	antennal mechanosensory and motor center
CHC	cuticular hydrocarbon
CNS	central nervous system
CsChrimson	light-gated cation channel
cVA	11-cis-vaccenyl acetate
DN	descending neuron
dsf	*dissatisfaction* gene
dsx	*doublesex* gene
DsxF	female-specific Doublesex protein
dsxF	female *doublesex* isoform
DsxM	male-specific Doublesex protein
e.g.	*exempli gratia*, Latin for “for exemple”
EN	excitatory neuron
fru	*fruitless* gene
GAL4	yeast transcription activator protein
GAL80	yeast negative regulatory protein
GRN	gustatory receptor neuron

i.e.	*id est*, Latin for “that is”
IN	inhibitory neuron
IPI	inter-pulse interval
JO	Johnston’s organ
JON	Johnston’s organ neuron
Kir2.1	inward rectifying potassium channel 2.1
LED	light-emitting diode
OE	ovipositor extrusion
ORN	olfactory receptor neuron
SEZ	suboesophageal zone
SPSN	sex-peptide sensory neuron
sxl	*sex-lethal* gene
tra	*transformer* gene
Tra	Transformer protein
Tra-2	Transformer-2 protein
UAS	upstream activating sequence
VNC	ventral nerve cord
VPO	vaginal plate opening

General Introduction

Animal behaviour studies the ways animals interact with members of their species, with organisms of other species and with the environment. Thus, studies on animal behaviour help us understand how animals find and defend resources, avoid predators, chose mates or reproduce and, ultimately, how animals' interactions influence the survival and reproduction of the individuals.

Behaviours can be defined as changes in the activity of an organism in response to a stimulus, whether external or internal. Innate behaviours, *i.e.*, behaviours that are triggered by some sensory stimuli without prior experience or learning, are very important for survival. The majority of animal species can be identified by certain behaviours, which have been shaped throughout evolution and are species-specific (Gahan, 2005). The inheritance of behavioural pattern is very useful for animals to react quickly and often means the difference between life and death. For example, when avoiding predators, searching for food or choosing a mating partner, animals display innate behaviours, although some aspects of these behaviours can be modifiable by experience (Baker, Taylor and Hall, 2001). The behaviour of an animal must require an amount of information about the individual's environment and its internal state (Scott, 2005). For this, all behaviours depend on the nervous system for initiation, coordination and execution. However, in most of the cases it is the endocrine system that determines when a particular behaviour is performed through its influence on the development and physiological state of the animal (Gahan, 2005). For example, the transition to sexuality in fruit flies is controlled by the juvenile hormone, a key regulator of many aspects of insect physiology (Wyatt and Davey, 1996). So, it is fair to say that animal behaviours need the cooperative actions of the endocrine, sensory and central nervous systems to occur (Scott, 2005).

Assigning behavioural functions to neural structures has been a central goal in neuroscience. Specifically, neuroscience aims to understand how neural circuits are built and function to allow individuals to perceive the environment and perform specific behaviours based on those perceptions. Because innate behaviours appear to be stereotyped action patterns and species-specific, it has been suggested that

the neuronal substrates necessary for their execution are genetically determined and programmed since animals' development (Baker, Taylor and Hall, 2001). Therefore, innate behaviours constitute a strong system to study as they allow us to grasp the link between behaviours, the genes that build them and their neural substrates. The fruit fly *Drosophila melanogaster* is a great model to pursuit this kind of studies. Its simplicity and genetic tractability help us understand the genetic, cellular and neuronal mechanisms underlying animal behaviour. Although we cannot directly translate fruit flies' basic principles to humans or other mammals, they can give us insights on how similar circuits are structured and function in other species.

This work focuses on *D. melanogaster* virgin female behaviour during courtship, a crucial innate behaviour for the survival of the species. It aims at identifying neurons involved in female receptivity behaviour and to contribute to the understanding of the neuronal processes that modulate the female's acceptance or rejection of a courting male.

Genetic basis of *Drosophila melanogaster* sexual development

As it happens in most of sexual-reproducing animals, female and male *D. melanogaster* differ in their anatomy, although gender differentiation goes beyond the anatomical differences. Both male and female exhibit distinct behaviours when it comes to reproduction, which does not need any prior experience or learning from their conspecifics. It has been shown that the ability to perform sex-specific behaviours is dependent on sexual dimorphism in the fly central nervous system (CNS). Early studies that aimed to define structures in the fly CNS responsible for sex-specific behaviours used gynandromorphs, sex mosaic flies that contain both male and female characteristics (Hotta and Benzer, 1976; Hall, 1977, 1979; von Schilcher and Hall, 1979; Szabad and Fajszi, 1982; Tompkins and Hall, 1983; Ferveur and Greenspan, 1998). These investigations demonstrated a correlation between defined regions of the brain and ventral nerve cord (VNC) and the sex-specific behaviours. For example, a study suggested that the posterior dorsal brain

near the mushroom body is necessary for the display of male-like behaviours (Hall, 1979), while the anterior dorsal brain is essential for female-like behaviours and receptivity (Tompkins and Hall, 1983). Thus, it was suggested that the nervous tissue must be masculine or feminine for the fly to perform appropriate sex-specific behaviours, which must be under the control of sex-determination mechanisms.

The hypothesis that sexual behaviours are under genetic control led to studies on the genes that regulate *Drosophila* sexual development and behaviour. In fact, sexually dimorphic features of fruit flies are under the control of the "sex-determination cascade" (Figure 1) (reviewed in (Cline and Meyer, 1996; Yamamoto *et al.*, 1998; Christiansen *et al.*, 2002). On the top of this cascade is the *sex-lethal* gene (*sxl*) which is activated when the ratio of X chromosomes to autosomes is greater than 1, *i.e.*, only in female flies (X/X). The *sxl* promotes the expression of the splicing factor *transformer* (*tra*) that leads to the production of the female-specific Tra protein which, in combination with the non-sex-specific Transformer-2 protein (Tra-2), regulates the splicing of the sex-determination genes *doublesex* (*dsx*) and *fruitless* (*fru*) (reviewed in (Greenspan and Ferveur, 2000)). The lack of Tra activity in males allows *dsx* and *fru* to be expressed into their male isoforms Dsx^M and Fru^M, respectively, whereas the presence of this protein in females leads to the alternate splicing of *dsx* into the Dsx^F isoform, but the absence of any isoforms of Fru (reviewed in (Baker, Taylor and Hall, 2001; Manoli, Meissner and Baker, 2006; Yamamoto, Sato and Koganezawa, 2014)).

Genetic dissection with mutations has shown the connection between the sex-determination hierarchy genes and the control of behaviour (reviewed in (Hall, 1994) (Billeter, Goodwin and O'Dell, 2002)). Females with mutations in the *sxl* and *tra* genes develop into males, anatomically and behavioural, whereas their expression in males leads them to attract other mature males and to display low courtship levels towards females. These mutated males also synthetise the female aphrodisiac pheromone, contrary to the mutated females. These findings indicate that *sxl* and *tra* control behavioural, morphological and biochemical aspects of female sexual differentiation. In males, the *fru* gene is responsible for the specification of the neuronal circuitry that expresses the courtship behaviour (reviewed in (Hall, 1994;

Greenspan and Ferveur, 2000; Manoli, Meissner and Baker, 2006; Villella and Hall, 2008; Yamamoto and Koganezawa, 2013); (Billeter, Goodwin and O'Dell, 2002; Demir and Dickson, 2005; Stockinger *et al.*, 2005)). Mutations in the male-specific *fru* isoform disrupt some or all behaviours of the courtship repertoire, while its expression in females lead them to court other females. The *dsx* also plays an important role in the sex specification by supporting the development of neuronal and non-neuronal cells in a sex-specific manner (reviewed in (Yamamoto, Jallon and Komatsu, 1997; Baker, Taylor and Hall, 2001; Yamamoto, Sato and Koganezawa, 2014); (Billeter, Goodwin and O'Dell, 2002)). For example, the female isoform Dsx^F regulates the female morphology and the production of female-specific aphrodisiac pheromones, whereas the male isoform Dsx^M activates genes required for male morphology and, together with *fru*, regulate aspects of the courtship repertoire (Villella and Hall, 1996; Billeter *et al.*, 2006; Rideout, Billeter and Goodwin, 2007). In fact, *dsx* and *fru* are co-expressed in the male CNS and their respective transcripts (Dsx^M and Fru^M) are mutually required for the specification of a complete male-specific CNS and, consequently, for the correct presentation of male sexual behaviours (Rideout, Billeter and Goodwin, 2007; Rideout *et al.*, 2010; Kimura *et al.*, 2008).

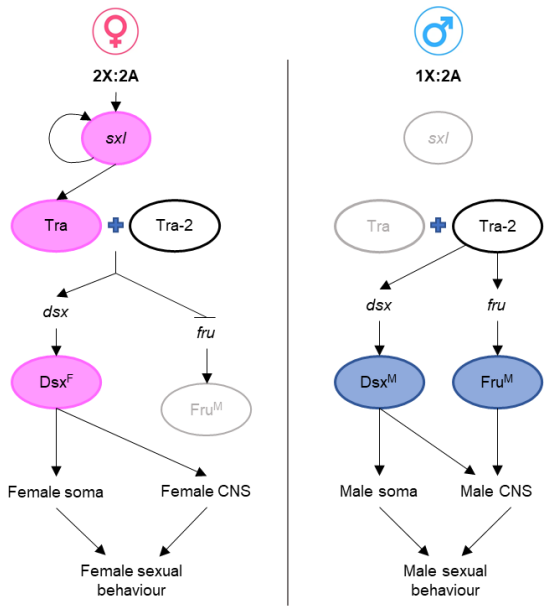


Figure 1. Schematic of the sex-determination hierarchy highlighting the functional activities of *dsx* and *fru*. Black lines or colours indicate active; grey indicates inactive or non-functional. Female-specific proteins are pink, male-specific proteins are blue and non-sex-specific proteins are white.

The direct role of *dsx*^{F} in the regulation of female-specific behaviours is poorly understood. Taylor and co-workers (Taylor *et al.*, 1994) demonstrated that females that lack the Dsx^{F} isoform and express the male-specific Dsx^{M} develop male-like external structures but behave as females. The female-specific *dsx* isoform was shown to be required to prevent male-specific neuronal formation in the female CNS, as the lack of Dsx^{F} expression in mutant females led them to express male-specific courtship neurons (Kimura *et al.*, 2008). These results indicate that the female anatomic and neuronal specification is directly dependent on the *dsx*. However, as was observed in regards to *fru* in males (Rideout, Billeter and Goodwin, 2007; Kimura *et al.*, 2008), *dsx* may act synergistically with other genes to generate female-specific behaviours. For example, the *dissatisfaction* (*dsf*) gene was shown to be necessary for flies to display appropriate sexual behaviour and to undergo sex-specific neural development, as mutations in this gene lead males to court both males and females and to present copulation defects, while females show low receptivity with egg laying defects. (Finley *et al.*, 1998). This gene is a by-product of the sex-determination cascade that acts downstream *tra* and, although it acts independently of *dsx* and *fru*, a possible cooperative action is not ruled out (Finley *et al.*, 1997, 1998).

Although there are other genes that contribute to the regulation of sex-specific behaviours, independently of the sex-determination cascade (reviewed in (Hall, 1994; Yamamoto, Jallon and Komatsu, 1997; Yamamoto *et al.*, 1998; Singh and Singh, 2016); (Billeter, Goodwin and O'Dell, 2002)), there is no doubt that genes within the sex-determination hierarchy are crucial for the specification of flies' masculinity and femininity.

Courtship in *Drosophila melanogaster*

Female-Male attraction is crucial for species to reproduce and survive. For them to be attracted to each other, a good communication must occur. A benefit of communication is the reduction of uncertainty about the status or intentions of the individuals involved, which allow them to make the most suitable decision in mate selection, therefore contributing for their survival and/or that of their species.

Male-specific sexual behaviours

Drosophila male courtship is composed by a sequence of stereotyped events, first described in wild type flies by Sturtevant (Sturtevant, 1915) and in mutant strains by Bastock and Manning (Bastock and Manning, 1955) (reviewed in (Yamamoto and Koganezawa, 2013)). Briefly, when a male encounter a female he will orient towards her, tap her abdomen with the forelegs, extend and vibrate one wing to produce the species-specific courtship song, lick female's genitalia and attempt to copulate (Figure 2) (Spieth, 1952; Bastock and Manning, 1955).

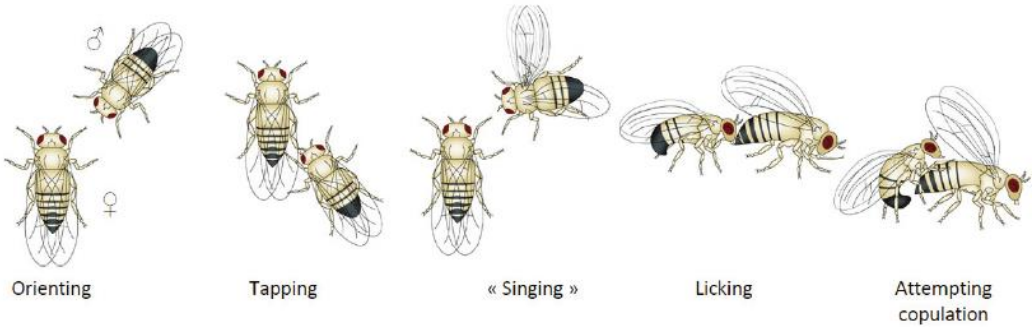


Figure 2. *Drosophila melanogaster* male courtship behaviours. The male fruit fly orients towards the female, then follows her, taps her abdomen and sings a species-specific courtship song by vibrating one wing. Finally, he licks the genitalia of the female and curls his abdomen in an attempt to copulate with her (Bontonou and Wicker-Thomas, 2014).

Besides the sensory inputs provided by a courting male, major factors that influence female receptivity is her age and mating status. If the female is too young or has previously mated, she will reject the male and copulation will not occur. But if she is mature, she will slow down, open her vaginal plates and allow copulation (Spieth, 1952; Markow and Hanson, 1981; Lasbleiz, Ferveur and Everaerts, 2006). It is thought that the subtle female behaviours inform the male about the quality of his displays and/or her receptivity state, which give him useful clues of how to proceed with the courtship.

Female-specific sexual behaviours

It is thought that until the female has obtained all information from the male, that allow her to make a confident judgement, she needs to prevent copulation without decreasing his interest. So, whether or not copulation occurs is dictated by the female, which will depend on her age and mating status, *i.e.*, immature virgin and mated females are unreceptive and mature virgin are receptive to mate.

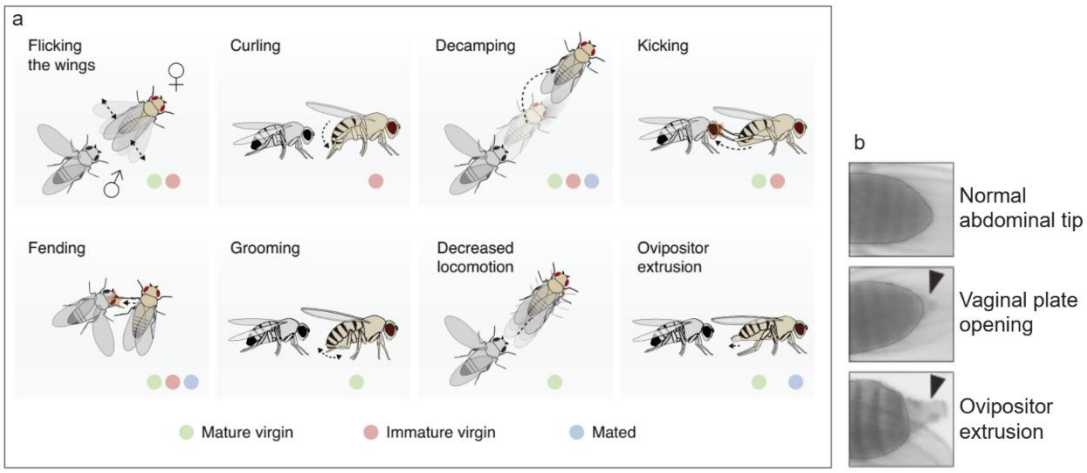


Figure 3. Behavioural components of courtship in the female *Drosophila melanogaster* at different receptivity states. (a) Illustration of the behavioural modules exhibited by immature virgin, mature virgin and mated females. Flicking the wings: wings undergo one or several rapid lateral flicks, curling: the female curls the tip of the abdomen downwards, decamping: the female runs, jumps, or flies away, kicking: the female kicks backwards with her hind legs, fending: the female extends her leg on the side such that she keeps an individual distance from the other flies, grooming: the female grooms her abdomen, decreased locomotion: the female slows down prior to copulation, ovipositor extrusion: the female pushes the vaginal plates posteriorly so that they project from the tip of the abdomen as a tube-like structure. **(b)** Dorsal view of female abdomens either not extruding or performing vaginal plate opening or ovipositor extrusion. Black arrowheads indicate the ovipositor. ((a) (Aranha and Vasconcelos, 2018) and (b) adapted from (Mezzera *et al.*, 2020)).

The best known *Drosophila* precopulatory behaviours are rejection behaviours and, although performed at different levels and possibly with different meanings, they are displayed by both receptive and unreceptive females (Spieth, 1952; Connolly and Cook, 1973; Spieth, 1974; Dukas and Scott, 2015; Lasbleiz, Ferveur and Everaerts, 2006; Mezzera *et al.*, 2020; F. Wang, Wang, Forknall, Parekh, *et al.*, 2020). During courtship females may display behaviours such as curling, wing flicking, ovipositor extrusion, fending, decamping and kicking (Figure 3) (Spieth, 1952; Connolly and Cook, 1973; Tompkins *et al.*, 1982; Villella and Hall, 2008). When courted, immature

flies present curling and wing flicking more often, while mature females tend to perform more kicking with her hindlegs and decamping (Bastock and Manning, 1955; Spieth, 1952; Connolly and Cook, 1973). Despite the initial rejection, a receptive female will eventually slow down and open the vaginal plates to allow copulation (Connolly and Cook, 1973; Tompkins *et al.*, 1982; K. Wang *et al.*, 2020; Mezzera *et al.*, 2020). Once mated, female will display mostly ovipositor extrusion (OE) to block copulation (Lasbleiz, Ferveur and Everaerts, 2006; F. Wang, Wang, Forknall, Parekh, *et al.*, 2020; Mezzera *et al.*, 2020).

Three behaviours have been suggested as good predictors of copulation success: pausing, grooming and vaginal plate opening (VPO, also referred as partial ovipositor extrusion). Usually, prior to copulation, females decrease their locomotor activity, either by pausing or slowing down, and groom the abdomen more often (Markow and Hanson, 1981; Tompkins *et al.*, 1982; Lasbleiz, Ferveur and Everaerts, 2006; Bussell *et al.*, 2014; Coen *et al.*, 2014; Aranha *et al.*, 2017). Although it is not clear, female's abdominal grooming may serve to actively spread cuticular compounds and possibly, to stimulate the male to attempt copulation (Lasbleiz, Ferveur and Everaerts, 2006). Vaginal plate opening is performed only by sexually mature females, either virgin or mated (Connolly and Cook, 1973; Lasbleiz, Ferveur and Everaerts, 2006; Mezzera *et al.*, 2020) and, when coupled with male licking, increases the probability of a male to attempt copulation (Mezzera *et al.*, 2020). So, it is not surprising that immature females elicit low levels of copulation attempts from males (Connolly and Cook, 1973; Mezzera *et al.*, 2020). The extrusion of the ovipositor, a characteristic rejection of mated females, also entices the male to attempt copulation and at similar levels as elicited by virgins-exhibiting VPO (F. Wang, Wang, Forknall, Parekh, *et al.*, 2020; Mezzera *et al.*, 2020). Again, the probability of a male attempt to copulate increases with the co-occurrence of OE and licking (Mezzera *et al.*, 2020). Interestingly, an unsuccessful copulation attempt upon virgin VPO exposure does not discourage the male to continue courting, whereas the opposite is observed when the OE is presented by a mated female (F. Wang, Wang, Forknall, Parekh, *et al.*, 2020). Thus, these two behaviours, with opposite meanings when it comes to female receptivity, may give the male important gustatory cues that may help him decide how much

effort he will apply for a given female. In fact, no other female's rejection behaviour is so effective in preventing copulation, and subsequent male courtship, as the ovipositor extrusion does (Connolly and Cook, 1973).

The VPO and the OE are courtship-specific behaviours, as they are only displayed in the context of courtship and elicited by the male song (Lasbleiz, Ferveur and Everaerts, 2006; F. Wang, Wang, Forknall, Parekh, *et al.*, 2020; K. Wang *et al.*, 2020; Mezzera *et al.*, 2020). Another curious correlation between female and male's behaviour was found for the male courtship song and female locomotor activity. Actually, these two behaviours appear to be mutually conditioned, *i.e.*, male song intensity seems to positively evolve with female's locomotor activity, while the latter decreases in receptive females with intense courtship song (von Schilcher, 1976; Crossley, Bennet-Clark and Evert, 1995; Trott *et al.*, 2012; Bussell *et al.*, 2014; Coen *et al.*, 2014; Vaughan *et al.*, 2014; Clemens *et al.*, 2015; Deutsch *et al.*, 2019).

The sensory modalities of courtship

The interaction in which mating partners exchange sensory information, is under the influence of the "specific mate recognition system" (Paterson, 1985). As the name implies, this system allows the effective recognition of conspecific sexual partners and leads to species reproductive isolation. Mate recognition is accomplished by the species-specific sensory signals or cues. Thus, during the sexual interplay, both partners exchange signals that belong to multiple sensory modalities that can be visual, auditory, olfactory and/or tactile. In *Drosophila melanogaster*, vision gives the male directional information about other fly's position. Additionally, olfaction and gustation will tell him if the pair is male or female and of the right species. Because female's investment into the next generation is much higher than that of the male, she needs to make sure that she will choose the best candidate. For that, female uses mainly olfaction to sense male pheromones and, highly important, audition to hear the courtship song that he plays.

Each sensory signal transferred between mating partners plays an important, but not determinant role in reproductive success. It is likely that they act together, or redundantly, in shaping female and male behaviour since disrupting only one sensory modality does not prevent courtship and copulation, albeit reducing their levels (Tompkins, Hall and Hall, 1980; Gailey, Lacaillade and Hall, 1986; Joiner MlA and Griffith, 1997).

Vision

For *D. melanogaster* male fly, vision is necessary for him to detect female's motion and to help him initiate and direct male courtship. In fact, motionless *Drosophila* females are subjected to less courtship than mobile females (Tompkins *et al.*, 1982). On the other hand, both female and female-like motion trigger courtship initiation and entice the male in chasing behaviours (Cook, 1979; Tompkins *et al.*, 1982; Agrawal, Safarik and Dickinson, 2014; Kohatsu and Yamamoto, 2015). Although the female movement is highly important for the male, the way she looks also gives him important visual cues. Larger females that harbour bigger abdomens were shown to present greater lifetime fecundity and to be more attractive to males (Long *et al.*, 2009). So, it is not surprising that vision-impaired males, or males courting in the dark, present abnormal courtship and take more time to copulate when compared to normal males (Spieth and Hsu, 1950; Markow, 1975; Tompkins *et al.*, 1982; Markow, 1987). For female flies, vision is not as important as for males, as female receptivity is similar in light and dark conditions (Sakai *et al.*, 2002). Interestingly, blind females copulate faster than females that see when paired with normal males (Tompkins *et al.*, 1982), which suggests that they rely on olfactory and auditory cues to make their decision.

Chemosensation

The discrimination between males and females is in large part mediated by sex-specific pheromones (Ferveur, 2005). Pheromones with low volatility are recognised

mostly by gustatory receptors neurons (GRNs) present in the fly proboscis, legs, wings and in the female vaginal plates; while the high volatile pheromones are recognised mainly at the olfactory level, in olfactory receptors neurons (ORNs) present in the 3rd segment of the antenna and maxillary palps (reviewed in (Vosshall and Stocker, 2007; Kohl, Huoviala and Jefferis, 2015)). The most abundant female-specific cuticular hydrocarbon (CHC) is the 7,11-heptacosadiene (7,11-HD) followed by the 7,11-nonacosadiene (7,11-ND), while the male presents the 7-tricosene (7-T) (reviewed in (Greenspan and Ferveur, 2000; Laturney and Billeter, 2014)). The female 7,11-HD has a particular importance in her attractiveness, promoting male courtship and inducing wing vibration (Venard and Jallon, 1980; Antony *et al.*, 1985). Interestingly, this female pheromone is under the control of the sex-determination cascade with the *dsx* female protein (Dsx^F) being sufficient and necessary for the expression of 7,11-HD (Waterbury, Jackson and Schedl, 1999). On the other hand, the male 7-T has a dual role in stimulating *D. melanogaster* females and inhibiting intermale courtship (Antony *et al.*, 1985; Grillet, Dartevelle and Ferveur, 2006). Moreover, the female-specific 7,11-HD is not produced by the sibling *D. simulans*, acting as attractant to *D. melanogaster* males and repellent to *D. simulans* males (Savarit *et al.*, 1999; Billeter *et al.*, 2009; Clowney *et al.*, 2015), which suggests a role of 7,11-HD in species reproductive isolation (reviewed in (Sato and Yamamoto, 2020)).

It is thought that most of the chemical substances that act during courtship of mature flies are detected by contact, during the acts of tapping and probably licking. Contact-mediated chemosensation is processed by GRNs which send inputs to the suboesophageal zone (SEZ) of the fly brain (reviewed in (Vosshall and Stocker, 2007)). During tapping, males sense the females' or other males' CHCs through gustatory cells harboured in the forelegs. Three gustatory receptors (Gr) were identified as having a role in courtship (Gr32a, Gr33a and Gr68a) (reviewed in (Kohl, Huoviala and Jefferis, 2015)). Studies with mutants show that these receptors are involved in inhibiting male-male courtship, in the perception of inhibitory pheromones and in the control of correct courtship song (Miyamoto and Amrein, 2008; Moon *et al.*, 2009; Koganezawa *et al.*, 2010; Fan *et al.*, 2013). Furthermore, foreleg gustatory

neurons, that express the ion channel coding genes *ppk23*, *ppk25* and *ppk29*, have been demonstrated to promote male-female courtship and inhibit intermale courtship (Liu *et al.*, 2012; Starostina *et al.*, 2012; Thistle *et al.*, 2012; Toda, Zhao and Dickson, 2012). These genes belong to a class of ion channels from the *pickpocket* family thought to be involved in gustation and mechanoreception (Thistle *et al.*, 2012).

Pheromones involved in long distance communication are probably volatile and would be detected before the first physical contact. It is likely that the majority of insects express volatile compounds, although sex-specific odours that contribute to long range premating communication are not well known. An interesting example is the bombykol, produced by the female of silk moth *Bombyx mori*. These females release this compound to signal their availability and can attract males from meters away (reviewed in (Regnier and Law, 1968; Wicker-Thomas, 2007; Gomez-Diaz and Benton, 2013). The best-known *Drosophila melanogaster* volatile pheromone is the male-specific 11-cis-vaccenyl acetate (cVA). The cVA is a lipid produced in the male ejaculatory bulb, firstly described as an aggregation pheromone (Bartelt, Schaner and Jackson, 1985) and, more recently, as an intermale courtship inhibitor, a male-male aggression activator and a female receptivity enhancer (reviewed in (Kohl, Huoviala and Jefferis, 2015)). In fact, cVA is transferred to females during mating, which function as a volatile pheromone to inhibit courtship of the female by subsequent males (Butterworth, 1969; Guiraudie-Capraz, Pho and Jallon, 2007). This pheromone is detected through the olfactory receptor Or67d expressed in *fru*+ ORNs in the antenna, which activates a sexually dimorphic neuronal circuit that modulates aggression in males and sexual attraction in females (Ha and Smith, 2006; Kurtovic, Widmer and Dickson, 2007; Datta *et al.*, 2008; Ruta *et al.*, 2010; Kohl *et al.*, 2013). Interestingly, while cVA increases female receptivity through Or67d, long-term exposure to cVA activates the Or65a which decreases female receptivity for about a day after mating (Lebreton *et al.*, 2014). This mechanism may be the first triggering females unreceptivity to further mates, independently of the sex-peptide effect, the so called copulation effect (Manning, 1967; Chapman *et al.*, 2003). Recently, the female-specific (Z)-4-undecenal, a volatile pheromone whose precursor is the 7,11-

HD, was shown to elicit flight attraction in both sexes and courtship in males (Lebreton *et al.*, 2017; Borrero-Echeverry *et al.*, 2021).

The pheromonal profile of the flies may also give cues about flies' age since the full repertoire of sex-specific pheromones is established during the first two days after eclosion (Jallon and Hotta, 1979; Tompkins, Hall and Hall, 1980). This may be the reason why young males elicit courtship from mature males and court both male and female flies (McRobert and Tompkins, 1983). Pheromones also induce age-dependent copulation advantage in males, as sensitivity of the Or47b to palmitoleic acid, a pheromone that promotes male courtship, increases with age through the action of the juvenile hormone (Lin *et al.*, 2016). Thus, 7-days old males will court more vigorously than 2-days old males, being preferred by the females.

While male vision is important in guiding his position during courtship (Kimura, Sato, Yamamoto, *et al.*, 2015), females' chemosensory cues determine for how long the male pursues an object, *i.e.*, his persistence (Agrawal, Safarik and Dickinson, 2014).

Audition

The courtship song, also called "love song", may be the most important auditory cue for the female and, together with volatile pheromones, allows individuals' communication at a distance. This song is species-specific and plays a crucial role in species recognition, as well as providing to the female information about the male's fitness (Spieth, 1974; Kyriacou and Hall, 1982; Ritchie, Halsey and Gleason, 1999). The male song consists of sine song and pulse song with rhythmic elements, which contains information specific to each *Drosophila* species (Kyriacou and Hall, 1986; Kyriacou, van den Berg and Hall, 1990). Briefly, the sine song is a humming sound played at a frequency of 140-170 Hz, whereas the pulse song presents a frequency of 150-300 HZ and is interleaved with inter-pulse intervals (IPIs) (von Schilcher, 1976; Kyriacou and Hall, 1982). The species-specific pattern of the courtship song is

characterized by the succession of pulse songs and IPIs, which present a length of ≈35 ms in *D. melanogaster*.

The importance of the song in mating success was shown in a variety of studies using mutants (reviewed in (Hall, 1994; Yamamoto, Jallon and Komatsu, 1997; Singh and Singh, 2016)). From ion channels to factors involved in controlling gene expression, there are several genes that appear to modulate the courtship song repertoire. For example, the *cacophony* (*cac*) gene is important for a proper display of the pulse song and IPI, as well as mating success, as *cac* mutant males display abnormal courtship song and show decreased copulation rate when compared to wild type males (Kulkarni and Hall, 1987). Additionally, auditory mutant females, as well as females courted by wingless males, fail to be stimulated by the song (Bennet-Clark and Ewing, 1967; Schilcher, 1976; von Schilcher, 1976; Eberl, Duyk and Perrimon, 1997).

The female senses the song through the vibration of the arista on their antenna (Figure 4) (Cook, 1973). This feather-like structure, in the 3rd antennal segment, serves as sound receiver and vibrates in response to particle velocity. Song is perceived by specialized neurons in the Johnston's organ (JO), housed in the 2nd antennal segment, which detects sound, gravity and wind (Kamikouchi *et al.*, 2009; Yorozu *et al.*, 2009). Movement of the arista and the 3rd segment of the antenna causes the stretching or compression of the cilia of the JO neurons (JONs), activating or inactivating them. Depending on the type of stimuli received, JONs then transmit the information to one of the five zones of the antennal mechanosensory and motor center (AMMC) in the fly brain (Kamikouchi *et al.*, 2009; Yorozu *et al.*, 2009). The neurons that project to the zones A and B are sensitive to vibratory stimuli such as courtship song, while those that innervate zones C to E are more sensitive to static stimuli, such as wind and gravity. In fact, silencing AMMC-B neurons or removal of the arista, reduces female response to song and consequently her receptivity (Vaughan *et al.*, 2014; Yamada *et al.*, 2018).

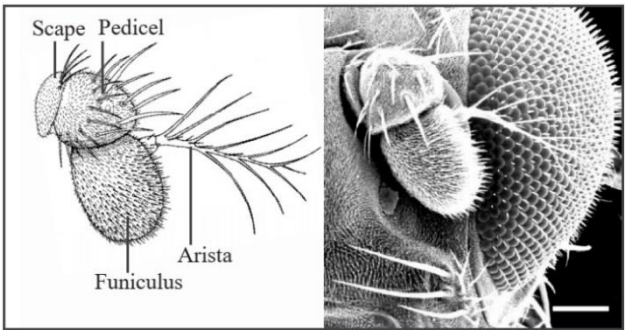


Figure 4. *Drosophila melanogaster* antenna. Schematic drawing (left) and scanning electronmicrograph (right, 0.1 mm). Each antenna is composed of three segments, the scape, the pedicel and the funiculus, the latter carrying the feather-like arista (Göpfert and Robert, 2002).

Although male auditory cues present a higher importance for mating success, female auditory cues displayed in both precopulatory and copulatory moments are likely to play an important role in the mating process. Female acoustic signals emitted by movement stimulates the male to initiate courtship and, more recently described, female song displayed during copulation seems to modulate male ejaculate allocation (Ejima and Griffith, 2008; Kerwin and Philipsborn, 2020; Kerwin, Yuan and von Philipsborn, 2020).

The importance of different sensory signals during a sexual encounter may change as courtship progresses, *i.e.*, visual cues are used in early stages of the mate detection and recognition, whereas chemical and acoustic stimuli are more potent during later phases of courtship (Spieth, 1974; Lasbleiz, Ferveur and Everaerts, 2006). The change in male and female behaviours during the course of courtship suggests that they are coordinated in a sex-specific manner that might increase mutual recognition and arousal.

Neuronal basis of female receptivity

The neuronal basis of male courtship behaviours has been intensively studied in the past years, possibly motivated by the stereotyped behavioural pattern and easily

observed in *Drosophila* males. Despite the recent efforts in unveiling the neuronal basis underlying females' premating behaviours, little is known about how the female brain processes internal and external cues to generate a specific response.

Most of what we know on the neuronal basis of female receptivity was obtained through the study of postmating neurons, *i.e.*, the study of female postmating behaviours that are triggered after copulation. After mating, female internal state undergoes a series of changes that lead female to become temporarily unreceptive, increase the egg laying and adapt her nutritional choices, as a result of the postmating switch (Manning, 1967; Kubli, 2003; Ribeiro and Dickson, 2010; Walker, Corrales-Carvajal and Ribeiro, 2015). These changes are triggered by the binding of the sex-peptide, transferred during ejaculation, to its receptor expressed in *dsx* and *fru* positive sex-peptide sensory neurons (SPSNs) located in the uterus (Yapici *et al.*, 2008; Häsemeyer *et al.*, 2009; Yang *et al.*, 2009; Rezával *et al.*, 2012). These neurons project to the abdominal ganglion of the VNC where they connect to the *dsx*-expressing SAG neurons (Feng *et al.*, 2014), which in turn send input to higher regions in the brain. Recent findings demonstrated that SAG neurons directly input onto female-specific *dsx*+-pC1 neurons in the central brain, to which they deliver information about the female mating status (F. Wang, Wang, Forknall, Patrick, *et al.*, 2020). Silencing any piece of this uterus-brain connection induces post-mating responses in virgin females.

The activity in the pC1 neurons promote female receptivity and is synergistically triggered by male cVA and courtship song (Zhou *et al.*, 2014), suggesting pC1 cluster as a site for multimodal integration in the brain. In virgin females, these neurons provide direct excitatory input to a pair of *dsx*+ descending neurons (vpoDNs), contributing for triggering female VPO (K. Wang *et al.*, 2020). They also play a role in preventing egg laying by providing indirect inhibitory input to *fru*+ oviposition descending neurons (oviDNs), through oviposition inhibitory neurons (oviINs) (F. Wang, Wang, Forknall, Patrick, *et al.*, 2020). While pC1 relay information about the mating status to these descending neurons (DNs), the display of a specific behaviour

requires information from other neurons. VPO excitatory (vpoENs) and inhibitory neurons (vpoINs) deliver song information to the vpoDNs and may ensure that these neurons respond specifically to the conspecific courtship song. On the other hand, the oviDNs receive substrate gustatory sensory cues mediated by oviposition excitatory neurons (oviENs), which help a mated female to choose a proper oviposition site. Besides the neuronal modulation that triggers the postmating responses in mated females, a pair of *dsx*-DNs in the brain were recently found to modulate the ovipositor extrusion, an important female' rejection behaviour (F. Wang, Wang, Forknall, Parekh, *et al.*, 2020; Mezzera *et al.*, 2020). The DNp13 neurons induce OE by mated females and respond to male song via synaptic input from pC2l auditory neurons (Deutsch *et al.*, 2019; F. Wang, Wang, Forknall, Parekh, *et al.*, 2020; Mezzera *et al.*, 2020), which were previously shown to induce ovipositor extrusion (Kimura, Sato, Koganezawa, *et al.*, 2015). The OE induced by DNp13 may be dependent on egg production or ovulation, since activation of DNp13 neurons do not induce OE in virgin as much as it was induced in mated females (F. Wang, Wang, Forknall, Parekh, *et al.*, 2020).

A significant role has been attributed to *fru* and, in more extent, to *dsx*-expressing neurons in female's reproductive behaviours. Although most of the studies focus on the neuronal modulation of postmating behaviours, other neurons were shown to modulate important aspects of female's premating behaviours. As mentioned before, receptive females decrease their locomotion before copulation, which is reflected by both increased pausing and decreased walking speed. *Abdominal-B* (*Abd-B*) -expressing neurons in the VNC and *apterous* -expressing neurons in the brain were shown to modulate these behaviours. The *Abd-B* neurons control female pausing during courtship (Bussell *et al.*, 2014), whereas *apterous*-expressing neurons are required for the appropriate reduction of the walking speed in the presence of a courting male (Aranha *et al.*, 2017). Activity in both neuronal groups is also required for female to copulate, as silencing them decreases virgin female receptivity. A more recent study described two groups of neurons within the ellipsoid body, R4d and R2/R4m, that are likely to control the behavioural switch from rejection to acceptance in virgin females (Ishimoto and Kamikouchi, 2020). Cholinergic R4d and GABAergic

R2/R4m neurons receive input from the PPM3 dopaminergic neurons, whose activity decreases female receptivity, as was observed for the activation of R4d neurons. Contrary, activity in R2/R4m neurons increases female receptivity and it was suggested that these neurons exert an inhibitory effect on R4d neurons through the release of GABA, attenuating premating rejection responses in virgin females.

Main goals

Understanding the functional architecture of neural circuits and how they mediate individuals' behaviours, is one of the main goals for those devoted to behavioural neuroscience. Despite the great evolution in the neuroscience field, little is known yet about how animals process internal and external cues to develop a mating-specific response. Neurons in the female brain are of great interest since they appear to be the site for higher-order processing that would integrate female mating status and male sensory cues to generate an appropriate response. To this end, *Drosophila melanogaster* courtship is an excellent behavioural paradigm.

Here, we focus on *Drosophila* female receptivity with the aim to (i) find neurons in the brain that mediate female receptivity to a courting male, (ii) anatomically characterise those neurons and (iii) study the female-specific premating behaviours that may be under their control.

We believe that this work will contribute to a better understanding of how sensory information elicits appropriate sexual behaviors and, maybe, how neurons are organized to modulate female receptivity.

CHAPTER I

70A09 neurons modulate female speed in the context of courtship

1.1. Summary

Persuasion is a crucial component of the courtship ritual needed to overcome contact aversion. In fruit flies, it is well established that the male courtship song prompts receptivity in female flies, in part by causing sexually mature females to slow down and pause, allowing copulation. Whether the above receptivity behaviours require the suppression of contact avoidance or escape remains unknown. Here we show, through genetic manipulation of neurons we identified as required for female receptivity, that male song induces avoidance/escape responses that are suppressed in wild type flies. First, we show that silencing 70A09 neurons leads to an increase in escape, as females increase their walking speed during courtship together with an increase in jumping and a reduction in pausing. The increase in escape response is specific to courtship, as escape to a looming threat is not intensified. Activation of 70A09 neurons leads to pausing, confirming the role of these neurons in escape modulation. Finally, we show that the escape displayed by the female results from the presence of a courting male and more specifically from the song produced by him. Our results suggest that courtship song has a dual role, promoting both escape and pause in females and that escape is suppressed by the activity of 70A09 neurons, allowing mating to occur.

1.2. Introduction

Mating rituals serve many different purposes, such as attracting potential mates, synchronizing reproduction, announcing the animal's species, sex and fitness, persuading the mate to overcome contact aversion (Tinbergen, 1964). A prospective mate that is unreceptive to the courtship advances will likely flee the scene (Lenschow and Lima, 2020).

In *Drosophila melanogaster* courtship, the male performs a series of distinct and stereotyped motor programs such as orienting towards the female, following her while extending and vibrating one wing producing a courtship song, quivering the abdomen, tapping and licking female's genitals and, finally, attempting copulation (Bastock and Manning, 1955; Hall, 1994; Fabre *et al.*, 2012). During male courtship the female exhibits behaviours that may be interpreted as rejection responses such as wing flicking, ovipositor extrusion, fending, decamping and kicking (Spieth, 1952; Connolly and Cook, 1973; Tompkins *et al.*, 1982; Villella and Hall, 2008). Although performed at different levels, rejection behaviours are displayed by both receptive and unreceptive females (Connolly and Cook, 1973; Lasbleiz, Ferveur and Everaerts, 2006; Dukas and Scott, 2015; F. Wang, Wang, Forknall, Parekh, *et al.*, 2020; Mezzera *et al.*, 2020) and constitute the means by which the female communicates with the male. Thus, receptive females are thought to temporarily reject the courting male to collect quantitative and qualitative information about him (Bastock and Manning, 1955; Lasbleiz, Ferveur and Everaerts, 2006; Villella and Hall, 2008; Ferveur, 2010). Despite mild rejections, a receptive female will eventually slow down and open the vaginal plates to induce the male to copulate (Connolly and Cook, 1973; Tompkins *et al.*, 1982; K. Wang *et al.*, 2020; Mezzera *et al.*, 2020). Female locomotor activity is tightly coupled with receptivity since unreceptive flies (either sexually immature, mated, or manipulated) do not slow down nor pause as much as receptive females (Connolly and Cook, 1973; von Schilcher, 1976; Tompkins *et al.*, 1982; Crossley, Bennet-Clark and Evert, 1995; Bussell *et al.*, 2014; Coen *et al.*, 2014; Aranha *et al.*, 2017; Ishimoto and Kamikouchi, 2020). More specifically, receptive females slow down in response to the male's courtship song (von Schilcher, 1976;

Crossley, Bennet-Clark and Evert, 1995; Bussell *et al.*, 2014; Coen *et al.*, 2014; Vaughan *et al.*, 2014; Clemens *et al.*, 2015; Deutsch *et al.*, 2019). The relationship between locomotor activity and song has been mechanistically explored in recent years. Besides auditory neurons (Kamikouchi *et al.*, 2009; Yorozu *et al.*, 2009; Vaughan *et al.*, 2014; Clemens *et al.*, 2015; Zhou *et al.*, 2015), the higher order pC2 neurons are involved in the regulation of locomotion upon song presentation (Deutsch *et al.*, 2019), as indicated by the negative correlation of speed and calcium responses of female pC2 neurons to a song stimulus. Genetic manipulation of pC2 activity indicates that other circuit elements must contribute to the locomotor tuning for the song, since activation of pC2 neurons leads to multiphasic speed responses and their silencing leads to a correlation between speed and the interpulse interval of the song which is uncorrelated in wild type females. pC1 neurons, which integrate multiple inputs such as internal sensing of the mating status (F. Wang, Wang, Forknall, Patrick, *et al.*, 2020) and the male pheromone cis-vaccenyl acetate (Zhou *et al.*, 2014), also respond to song (Zhou *et al.*, 2014), though how these contribute to a locomotor response has not been shown.

With the goal of understanding the behavioural and neuronal mechanisms of female receptivity, we combined detailed quantitative description of female behaviour during courtship with neuronal manipulations. These approaches inform each other. While detailed behavioural analysis constitutes a window into brain function as it allows the mapping of specific sets of neurons or circuits to specific behavioural outputs, the identification and manipulation of neurons involved in receptivity contribute to the dissection of the modular structure of receptivity. In a female receptivity screen, aimed at identifying brain neurons where higher order receptivity would take place, we identified a group of neurons (line 70A09) that, when silenced, render the female unreceptive. Specifically, when silencing 70A09 neurons, sexually mature flies in the presence of a courting male walk faster, pause less and jump more than control flies, behaviours that are hallmarks of an escape response, which could explain why they are unreceptive. However, even if escape is impeded, they still did not mate. Furthermore, the increased escape response was specific to the courtship context,

as these flies did not increase escape response triggered by general threats, such as a large overhead looming stimulus. Conversely, acutely activating 70A09 neurons lead to a halt in walking. We further confirmed the requirement of courtship to elicit the escape response by pairing *70A09*-silenced females with males that do not court. Finally, we showed that the courtship song is key to elicit escape. In summary, we identified a new role of the male courtship song in eliciting female escape and a set of neurons in the female brain that are involved in suppressing such courtship song-induced escape response. We propose that the male song has a dual role, first eliciting escape and providing the female with enough time to assess the male, until the decision to mate is made, upon which then the song prompts a decrease in locomotion and that activity in 70A09 neurons is necessary to suppress the initial song-induced escape.

Author contributions

Márcia M. Aranha together with Sophie Dias performed the initial fertility and receptivity screen. Ricardo M. Neto Silva and Marta A. Moita designed and performed the looming experiments. All other experiments were performed and analysed by Eliane Arez with the participation of Cecilia Mezzera in the activation, fruitless and wingless experiments.

1.3. Results

1.3.1. Silencing *70A09*-GAL4 brain neurons reduces female receptivity

In order to identify neurons involved in female receptivity, we performed a silencing screen of the Janelia GAL4 line collection (Jenett *et al.*, 2012). Silencing was achieved with the expression of an inward rectifier potassium channel, *Kir2.1* (Baines *et al.*, 2001), that reduces the probability for an action potential to occur by hyperpolarizing the neurons. To prevent developmental lethality, silencing was restricted to the adult stage using temperature sensitive GAL80 (McGuire, Mao and Davis, 2004) which inhibits the expression of *Kir2.1*. The control flies have the same genotype but a different temperature treatment, though all flies were tested at 25 ºC (see methods). We tested 1042 lines for fertility and identified 65 lines in which at least 25% of the silenced females did not produce progeny (n=20-25). Next, we tested these lines for receptivity. For this, we paired a single wild type naïve male and a silenced virgin female in an arena and quantified copulation within 30 minutes (Figure 1.2a). With this secondary screen we identified 20 lines that affected receptivity when silenced (Table 1). Finally, we selected eight lines based on the strength of the phenotype, absence of neurons known to affect receptivity, such as, sex-peptide sensing neurons (Yapici *et al.*, 2008; Häsemeyer *et al.*, 2009; Yang *et al.*, 2009), and confirmation that the phenotype results from neuronal disruption using *elav*-GAL80 (see below). We next retested these lines while restricting the neuronal manipulation to the brain using a flippase under the control of the orthodenticle promoter (*otd*) (Asahina *et al.*, 2014). The lines 70A09 and 57G02 showed a marked reduction in copulation when brain neurons were silenced in the adult female (Figure 1.1). The line 70A09 was selected for further analysis considering the more restricted expression pattern when compared to 57G02 (data not shown). The loss of receptivity when silencing neurons labelled by the line 70A09 was confirmed with constitutive silencing where no temperature treatment is applied (Figure 1.2b and Figure 1.1). In this case, the controls are the two parental lines (lines used in the cross to obtain test flies) crossed with the line w^{1118} which was the basis for the generation of all transgenic lines in this work, therefore providing a neutral genetic

Table 1. *Drosophila* female receptivity obtained from a genetic screening of a collection of GAL4 lines. Copulation rate upon silencing of GAL4 lines under the control of *TubGal80^{TS}*, with corresponding *p*-values calculated from Fisher's exact statistical test.

Stock #	Janelia ID	Associated gene	N	Copulation index (%)		statistics
				18 °C	30 °C	
48068	26H08	Fur1	21	87.5	64.6	p=0.0157 (*)
38693	49E12	5-HT2	24	85.4	85.4	p=1.2265
46027	52D08	GABA-B-R2	25	90.3	94.3	p=0.6595
45362	72B02	DopR	28	95.8	41.7	p<0.0001 (****)
47069	80F08	Dat	20	77.8	78.3	p=1.0000
46831	87B08	RunxB	27	88	82	p=0.5766
47720	70A09	5-HT7	21	72.9	12.5	p<0.0001 (****)
39510	70A08	5-HT7	21	84.1	60	p=0.0176 (*)
46676	72D03	D2R	23	95.8	81.3	p=0.5050
47902	22A08	nAcRalpha-96Aa	23	89.6	91.7	p=1.0000
39462	68B11	Octbeta2R	25	89.4	85.4	p=0.7589
46642	70H01	DopR2	23	87.5	95.8	p=0.2678
38843	52G04	5-HT1A	25	91.7	70.8	p=0.0169 (*)
45490	23E09	GRHRII	31	79.2	89.6	p=0.1631
39464	68C01	Octbeta2R	23	81.3	56.3	p=0.0147 (*)
47673	57G02	nAcRbeta-64B	23	95.8	43.8	p<0.0001 (****)
39544	70F08	Rdl	20	88.9	84.4	p=0.7578
39419	27A07	Fur1	25	95.8	89.6	p=0.4353
39511	70B01	5-HT7	23	100	93.8	p=0.2421
45471	22B06	fru	22	97.9	97.9	p=1.0000
46197	33H05	ct	22	85.4	85.4	p=1.0000
38744	50D04	5-HT2	17	85.1	89.6	p=0.5523
49631	94D02	en	24	68.8	25	p<0.0001 (****)
48043	22H11	fru	24	91.7	95.8	p=0.6773
48977	22C11	fru	24	91.7	2.1	p<0.0001 (****)
49193	26F06	Fur1	20	82.5	82.9	p=1.0000
48979	22D01	nAcRalpha-96Aa	23	91.7	81.3	p=0.2321
49017	23B08	nAcRalpha-30D	25	93.8	97.9	p=0.6170
49019	23B11	fru	22	92.3	92.3	p=1.0000
48992	22F07	nAcRalpha-96Aa	24	93.5	97.8	p=0.6166
49012	23A11	nAcRalpha-96Aa	25	88.4	55.8	p=0.0015 (**)
49021	23C03	fru	25	93.8	83.3	p=0.1986
49143	25H03	lz	29	93.8	50	p<0.0001 (****)
49006	23A03	AlstR	24	91.7	45.8	p<0.0001 (****)
49015	23B02	fru	22	100	93.6	p=0.2419
49175	26D04	Fur1	21	92.3	89.7	p=1.0000
49008	23A05	nAcRalpha-96Aa	25	93.8	85.4	p=0.3167
49602	64D03	Takr99D	24	93.2	97.7	p=0.6162
49926	36A05	ct	24	82.5	93.8	p=0.1753
49559	44H01	lilli	23	89.6	89.6	p=1.0000
49441	27H08	mam	23	91.7	81.3	p=0.2321
49457	28E01	Lmpt	23	91.7	85.1	p=0.3553
49299	22B04	Adar	23	88.9	62.9	p= 0.0130 (*)
39514	70B07	5-HT7	22	83.3	83.3	p=1.0000
49301	22C05	fru	23	92.1	76.3	p=0.1132
49342	29E06	Pkc53E	21	89.1	0	p<0.0001 (****)
49608	64H06	amn	21	87.8	82.9	p=0.7560
46643	70H08	DopR2	24	83.3	76.7	p=0.1505
49494	29F10	fdl	24	79.2	4.2	p<0.0001 (****)
49338	29B09	Pkc53E	14	83.7	82.9	p=1.0000
49944	37A02	nAcRalpha-96Aa	20	92.1	84.2	p=0.4799
49208	27A05	Fur1	25	88.6	86.1	p=1.0000
49459	28E05	Dscam	24	74.4	84.2	p=0.4009
49424	88E07	ems	27	93.8	2.1	p<0.0001 (****)
49428	94D06	en	24	97.2	97.1	p=1.0000
49907	35D04	ct	25	88.6	91.1	p=0.7391
48027	9F02	retn	23	91.5	95.8	p=0.4353
49320	25E10	Adf1	23	94.4	91.2	p=0.6690
48138	40A05	dsx	25	91.7	27.8	p<0.0001 (****)
49494	29F10	fdl	24	79.2	4.2	p<0.0001 (****)
49350	30G04	Nrg	14	81.3	27.1	p<0.0001 (****)
49523	30B11	ple	25	89.6	81.3	p=0.3864
46963	76B10	GABA-B-R3	25	85.4	85.4	p=1.0000
49384	39F10	SoxN	23	83.3	72.9	p=0.3235
46385	57F02	nAcRalpha-30D	24	81.3	50	p=0.0021 (**)

background. Constitutive silencing was used in the subsequent experiments of this work because it is not lethal and it involves simpler and faster husbandry compared to conditional silencing. To confirm that the observed phenotype was a consequence of neuronal disruption we used *elav*-GAL80 (Yang *et al.*, 2009) to prevent *Kir2.1* expression in neurons. We did not observe abolishment of receptivity in these females (Figure 1.2b), indicating that the reduced receptivity is a result of neuronal silencing.

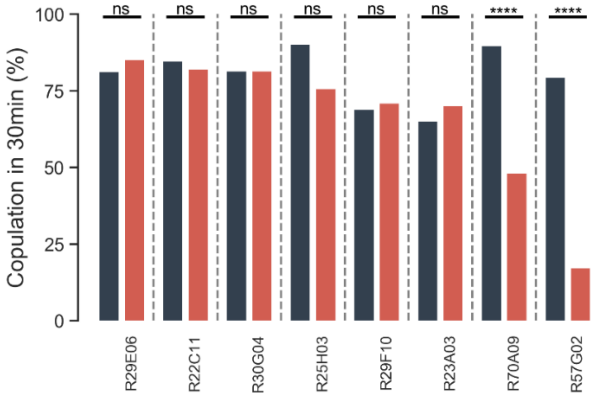


Figure 1.1. Screening of GAL4 lines for receptivity upon silencing of brain neurons. Receptivity of virgin females carrying the indicated GAL4 lines and *UAS-Kir2.1*, *TubGal80^{TS}*. Statistical analysis was performed with Fisher's exact test: ns = not significant, **** $p < 0.0001$. n=40-48.

Immunostaining of the *70A09*-GAL4 brain neurons revealed many neuronal groups that could play a role in female receptivity (Figure 1.2c). To identify the neurons responsible for the receptivity phenotype observed, we used two approaches that involved intersections with 70A09. In both approaches, in-house generated splitGAL4 and a LexA version of 70A09 were used to allow for more flexibility in the intersections (Figure 1.3). One approach was to generate intersections that separately label each of the groups of neurons that can be identified in the immunostaining. Using this approach, we labelled and tested i) the auditory sensory neurons (Figure 1.4a), ii) the local GABAergic antennal lobe neurons (Figure 1.4b), iii) neurons that express the insulin-like peptides in the pars intercerebralis (Figure 1.4c), iv) the lobula columnar neurons (LC17) (Figure 1.4d) and v) the protocerebral posterior lateral cluster (PPL3)

(Figure 1.4e). None of the separate groups recapitulated the *70A09*-GAL4 (from here on referred to as 70A09) silencing phenotype.

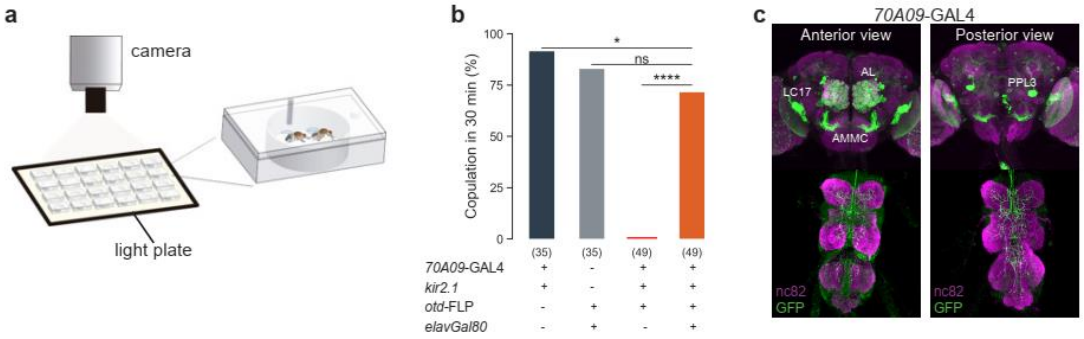


Figure 1.2. Silencing 70A09 brain neurons reduces female receptivity. (a) Schematic representation of the behavioural setup to test female receptivity. Mating arena containing mating pairs is highlighted. **(b)** Copulation rate of silenced and control females with n values shown in parentheses. Statistical analysis was performed with Fisher's exact test: ns = not significant; * $p<0.05$, **** $p<0.0001$. **(c)** Anterior and posterior views of female brain and VNC showing the expression pattern of *70A09*-GAL4/*otd-nls*:FLPo intersecting neurons. Neurons were visualised with anti-GFP (green) and the tissue counterstained with the synaptic marker nc82 (magenta).

The second approach was to intersect the 70A09 line with lines of genes involved in generating sexually differentiated circuits, *fruitless* (*fru*) and *doublesex* (*dsx*) (Villella and Hall, 2008). Immunostaining of the intersection of 70A09 with *fru* shows labelling of local antennal neurons and auditory sensory neurons, corresponding to GABAergic neurons of the line 70A09 (Figure 1.4f and 1.4b). Some additional labelling is observed in the protocerebrum corresponding to neurons located in the ventral nerve cord (VNC) that project to the brain since the intersection in this case is not restricted to the brain. The *fru* intersection line was not tested further since *fru*-positive brain neurons were shown in the first approach to not be involved in the receptivity phenotype (Figure 1.4a, b and f) and the *fru*-positive ascending neurons are out of the scope of this work. Silencing *dsx*-positive 70A09 (*70A09*∩*dsx*) neurons does lead to a reduction of receptivity (Figure 1.4g). Immunostaining of this intersection (Figure 1.4g) showed labelling of pC1 neurons which had been shown to modulate receptivity (Zhou *et al.*, 2014; K. Wang *et al.*, 2020). In fact, the degree of reduction in receptivity resembled that observed by Zhou et al. (Zhou *et al.*, 2014). However, this reduction in receptivity is partial and does not explain the complete

abolishment of receptivity observed in the *70A09*-silenced females, hence other neurons must be involved. The candidates are smaller cells with diffuse innervation which remain untested.

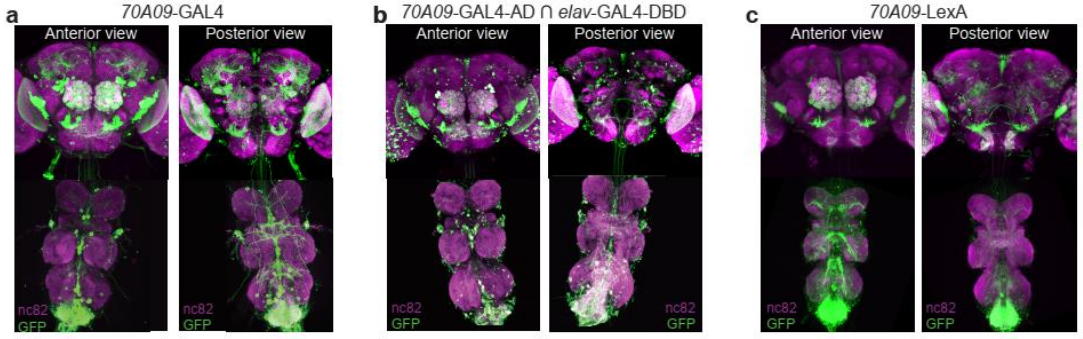


Figure 1.3. Anatomical characterisation of three driver lines under the control of 70A09 enhancer. Confocal images of brains and VNCs from female flies carrying **(a)** *70A09*-GAL4 and *UAS-CD8::GFP*, **(b)** *70A09*-AD∩*elav*DBD and *UAS-CD8::GFP*, **(c)** *70A09*-LexA and *LexAop-CD2-GFP*. GAL4, split-GAL4 and LexA-driven expression is shown in green while the synaptic marker nc82 is shown in magenta.

In summary, 70A09 labels brain neurons involved in female receptivity which include but are not restricted to pC1 neurons.

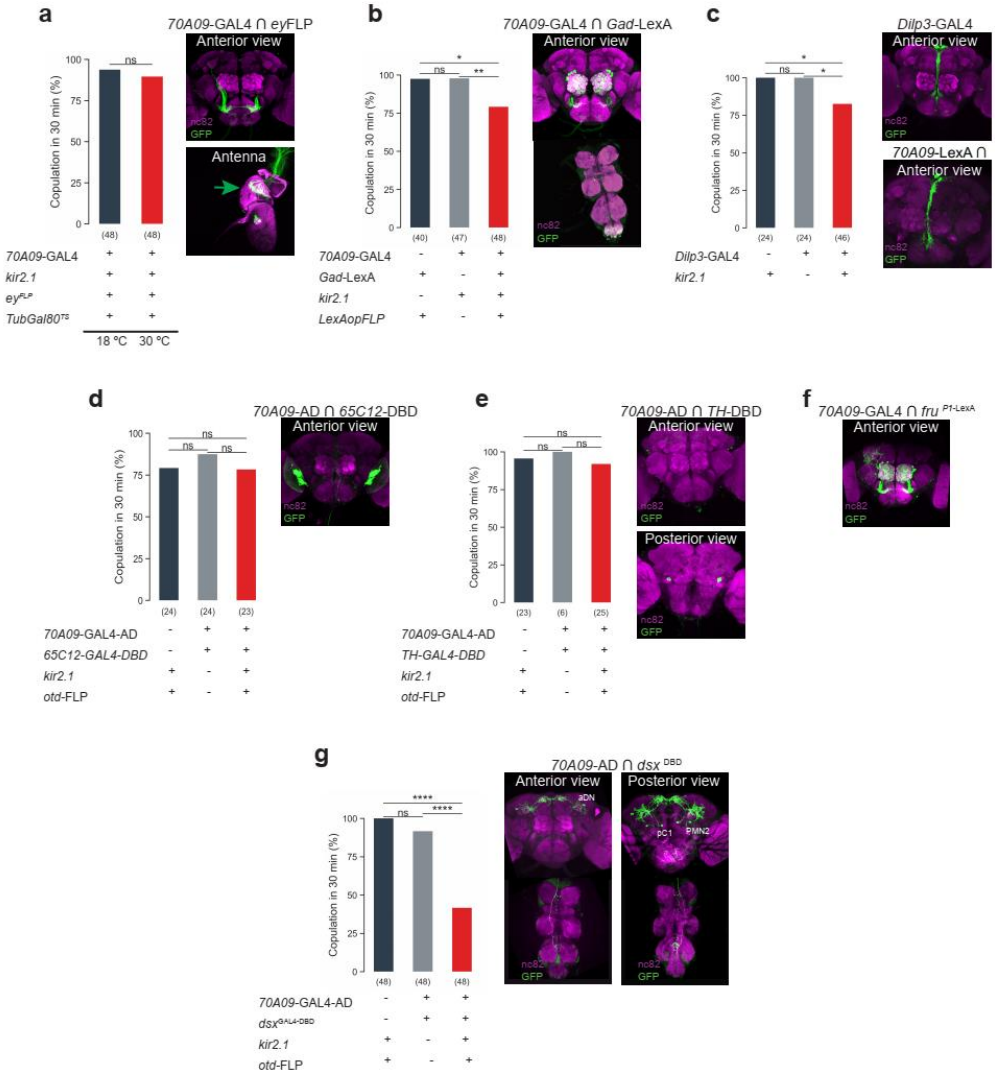


Figure 1.4. Female receptivity phenotype upon silencing different subsets of 70A09 neurons. (a–e, g) Copulation rate of silenced and control females (left) when silencing different sets of 70A09-positive neurons shown in confocal images (right). For all the mating analysis statistical analysis were performed with Fisher's exact test: ns = not significant, **p*<0.05, ***p*<0.01, *****p*<0.0001. n values are shown in parentheses. **(a)** Anterior view of female brain (top) and view of the Johnston's organ in the antenna (arrow, bottom) showing sensory neurons obtained from the intersection of *70A09*-GAL4 with *ey*-FLP. **(b)** Anterior view of female brain and VNC showing the expression pattern of *70A09*-GAL4 intersected with *gad*-LexA. **(c)** Anterior view of female brain showing *Dilp3*-GAL4 expression (top) and *70A09*-LexA intersected with *Dilp3*-GAL4 (bottom). For mating experiment only *Dilp3*-GAL4 was used to drive *kir2.1* expression in *ilp3*-expressing neurons. **(d)** Anterior view of female brain showing the LC17 neurons obtained from the intersection of *70A09*-GAL4-AD with 65C12-GAL4-DBD. **(e)** Anterior and posterior views of female brain showing the expression pattern of *70A09*-GAL4-AD intersected with TH-GAL4-DBD. **(f)** Anterior view of female brain showing 70A09-*fruitless* positive neurons obtained from the intersection of *70A09*-GAL4 with *fru*^{P1}-LexA. **(g)** Anterior and posterior views of female brain showing 70A09-*doublesex* positive neurons obtained from the intersection of *70A09*-GAL4-AD with *dsx*$^{GAL4\text{-}DBD}$. For all these confocal images, neurons were visualised with anti-GFP (green) and the tissue counterstained with the synaptic marker nc82 (magenta).

1.3.2. *70A09*-silenced females escape in response to male courtship

To characterise the behaviour of *70A09*-silenced females during courtship, we now used a setup that allows tracking the flies (Figure 1.5a). We analysed flies' behaviours from the start of courtship up to 10 minutes or until copulation (in those cases where copulation occurred in less than 10 minutes). We recorded single pairs for 20 minutes or until copulation to account for variability in latency to court (Figure 1.5b). First, we tested the female receptivity phenotype to validate the use of the setup. We observed that receptivity is also abolished in the arena with a different size, shape and lighting (Figure 1.5c). To confirm that the reduced copulation rate is due to reduced receptivity rather than reduced attractiveness of the female, we measured the courtship elicited by these females. We observed that males take about the same time to initiate courtship and court at the same levels silenced and control females (Figure 1.5b and 1.5d).

Female locomotor activity is one of the most reliable indicators of the female's willingness to copulate (Connolly and Cook, 1973; von Schilcher, 1976; Tompkins *et al.*, 1982; Crossley, Bennet-Clark and Evert, 1995; Bussell *et al.*, 2014; Coen *et al.*, 2014; Aranha *et al.*, 2017; Ishimoto and Kamikouchi, 2020), therefore we measured walking speed and pausing levels. Given that courtship happens in bouts, we quantified walking speed in three distinct moments of courtship dynamics, represented in Figure 1.5e: before courtship starts, during courtship ('courtship ON'), and during intervals between courtship bouts ('courtship OFF'). Quantification of walking speed during courtship ON revealed that *70A09*-silenced females walk at a substantially higher speed than control females (Figure 1.5f). It is known that unmanipulated females slow down during courtship (Connolly and Cook, 1973; von Schilcher, 1976; Tompkins *et al.*, 1982; Crossley, Bennet-Clark and Evert, 1995; Bussell *et al.*, 2014; Coen *et al.*, 2014; Aranha *et al.*, 2017; Ishimoto and Kamikouchi, 2020). Thus, the difference in walking speed during courtship could result from silenced females not responding to male courtship, *i.e.*, not slowing down like control females. To address this, we compared walking speed during courtship ON with other moments. We observed that rather than sustaining the speed, *70A09*-silenced

females increase walking speed during courtship ON compared to before courtship (Figure 1.5g). The increase in speed is acute since, during courtship OFF, *70A09*-silenced females return to the walking speed exhibited before courtship. This observation is in sharp contrast with control females that reduce the walking speed during courtship ON and sustain this reduced speed during courtship OFF.

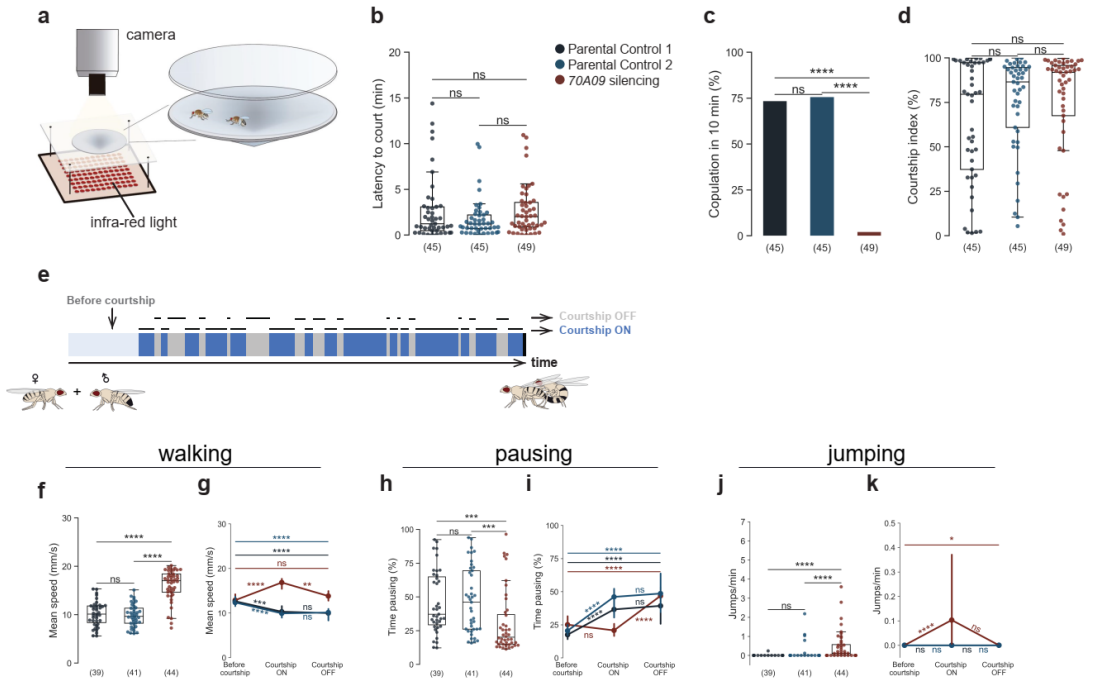


Figure 1.5. *70A09*-silenced females escape in response to male courtship. (a) Schematic representation of the behavioural setup to quantify and characterise receptivity behaviour (Aranha *et al.*, 2017). **(b)** Male latency to court. Genotypes: *w-/UAS>STOP>kir2.1*; *otd-nls*:FLPo/+; + (Parental Control 1), w-; +; *70A09*-GAL4/+ (Parental Control 2) and w-/*UAS>STOP>kir2.1*; *otd-nls*:FLPo/+; *70A09*-GAL4/+ (*70A09* silencing). **(c)** Copulation rate of silenced and control females. **(d)** Courtship index toward silenced and control females. **(e)** Schematic representation of the male courtship dynamic: before courtship (period from the start of recording to the start of courtship), courtship ON (bouts of courtship) and courtship OFF (bouts of non-courtship). **(f–k)** Behavioural effects of silencing 70A09 brain neurons on female mean walking speed (4 – 50 mm/s) **(f, g)**, female pausing **(h, i)** and number of jumps per minute **(j, k)**, during courtship ON periods (**f, h, j**) or in different moments of courtship dynamics **(g, i, k)**. Statistical analysis was performed with Fisher's exact test **(c)**, Kruskal-Wallis test **(b, d, f, h, j)** and Friedman's test **(g:** parental control 1 and 70A09 silencing**, i, k)** followed by post hoc pairwise Dunn's test with Bonferroni correction, repeated measures ANOVA followed by post hoc multiple pairwise paired t-test with Bonferroni correction **(g:** parental control 2**)**: ns = not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. n values are shown in parentheses.

Next, we analysed female pausing as it has been reported to increase during courtship (Tompkins *et al.*, 1982; Bussell *et al.*, 2014). We found that *70A09*-silenced

females pause less during courtship compared to control females (Figure 1.5h). Comparing across different courtship moments we found that, contrary to courtship ON moments, pausing increases in courtship OFF (Figure 1.5i).

The increase in walking speed and reduced rest are means for the female to escape the male. A third way to escape the male is to take off in flight, which in an enclosed arena results in a jump. For this reason, we investigated whether jumping was affected in manipulated flies. Indeed, during courtship ON *70A09*-silenced females jump more than control flies (Figure 1.5j). Jumping in *70A09*-silenced females is strongly increased during courtship ON compared to before courtship (Figure 1.5k). During courtship OFF jumping decreases though not significantly, suggesting that the females remain aroused.

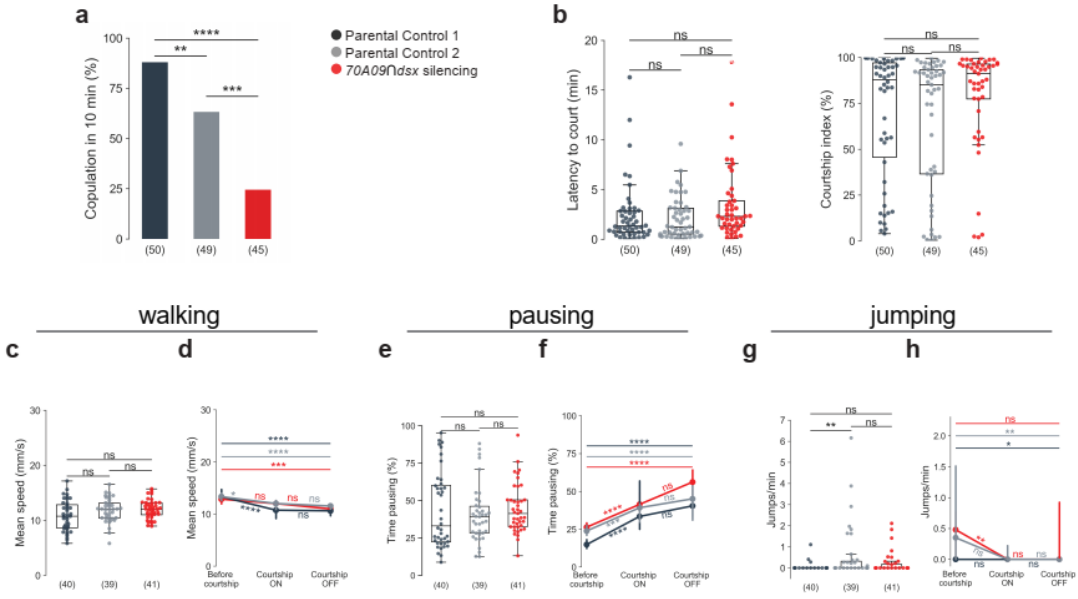


Figure 1.6. Copulation, courtship and locomotion behaviour of *70A09∩dsx* silenced females. (a) Copulation rate of silenced and control females. Genotypes: w-/*UAS>STOP>kir2.1*; *otd-nls:*FLPo/+; + (Parental Control 1), w-; *70A09*-GAL4-AD/+; *dsx*$^{GAL4\text{-}DBD}$/+ (Parental Control 2) and w-/*UAS>STOP>kir2.1*; *70A09*-GAL4-AD/*otd-nls:*FLPo; *dsxGAL4-DBD*/+ (*70A09∩dsx* silencing). **(b)** Male latency to court (left) and courtship index toward silenced and control females (right). **(c-h)** Behavioural effects of silencing *70A09∩dsx* neurons on female mean walking speed (4 – 50 mm/s) **(c, d)**, **(e)** female pausing **(e, f)** and number of jumps per minute **(g, h)**, during courtship ON periods **(c, e, g)** or in different moments of courtship dynamics **(d, f, h)**. Statistical analysis was performed with Fisher's exact test **(a)**, Kruskal-Wallis **(b, e, g)** and Friedman's test **(d:** *70A09∩dsx* silencing**, f and h)** followed by post hoc Dunn's test with Bonferroni correction, one-way ANOVA followed by post hoc Tukey's test **(c)**, repeated measures ANOVA followed by post hoc multiple pairwise paired t-test with Bonferroni correction **(d:** parental controls**)**: ns = not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. n values are shown in parentheses.

In the previous section we have shown that the receptivity phenotype was partially due to *70A09∩dsx* neurons. To test whether this subset of 70A09 neurons is also involved in the escape phenotype, we tested the *70A09∩dsx* silencing in the tracking setup. Analysis of walking speed, pausing and jumping shows that *70A09∩dsx*-silenced females do not escape a courting male (Figure 1.6). In other words, *dsx* neurons within the 70A09 line are not involved in the courtship-induced escape phenotype.

Altogether, our findings suggest that activity in 70A09 neurons is required for females to suppress escape responses during courtship.

1.3.3. Silencing 70A09 neurons does not increase escape responses upon threat

To determine if 70A09 neurons are involved in general escape responses, we tested the response of *70A09*-silenced females to looming stimuli. When exposed to looming in an enclosed arena, fruit flies have been shown to display different defensive responses, namely freezing, running and jumping. To analyse escape responses, (Card, 2012; von Reyn *et al.*, 2014; Gibson *et al.*, 2015; Zacarias *et al.*, 2018) *i.e.*, running and jumping, of *70A09*-silenced females, we adapted a previously established behavioural paradigm (Figure 1.7a) (Zacarias *et al.*, 2018). Single flies were transferred to a covered arena and allowed 2 minutes to explore. This baseline period was followed by 5 minutes during which the flies were exposed to 7 repetitions of a looming stimulus, displayed on a computer monitor angled above the arenas (Figure 1.7a). To examine the profile of escape responses, we plotted the average speed of the flies aligned to looming onset (Figure 1.7b). We found that the speed was constant and similar between unmanipulated and silenced flies before stimulus onset. Upon looming onset, flies showed a sharp decrease in their speed, which was followed by a rapid increase in locomotion that was less pronounced for silenced flies. The elevation in speed relative to that observed before looming onset was more noticeable for control flies than for *70A09*-silenced females.

To better characterise this disparity in escape responses, we quantified the difference in speed (delta speed) between a defined time window (0.5 sec) after looming offset and before looming onset (Figure 1.7c). We found that the increase in speed in response to looming stimuli was significantly lower for *70A09*-silenced females compared to controls. The less vigorous escape responses observed for silenced females in response to threat differ from what was observed in the context of courtship.

In response to a courting male, besides increased walking speed and reduced pausing, silenced flies also show an increase in jumps, that likely correspond to take-off attempts. Therefore, we also investigated jumping responses upon visual threat. We quantified the number of escape jumps per fly for the different genotypes during

the baseline and stimulation periods (Figure 1.7d). During the stimulation period, silenced females jumped significantly more than both controls. However, we found that during baseline silenced females also jumped significantly more than unmanipulated females. Given this result, we asked if the increase in jumps observed during stimulation relative to those observed in baseline was significantly higher for silenced females. For each genotype, we calculated the difference between the number of jumps observed during stimulation and baseline (delta jumps) (Figure 1.7e), and we found a significant difference between the silenced condition and only one of the controls.

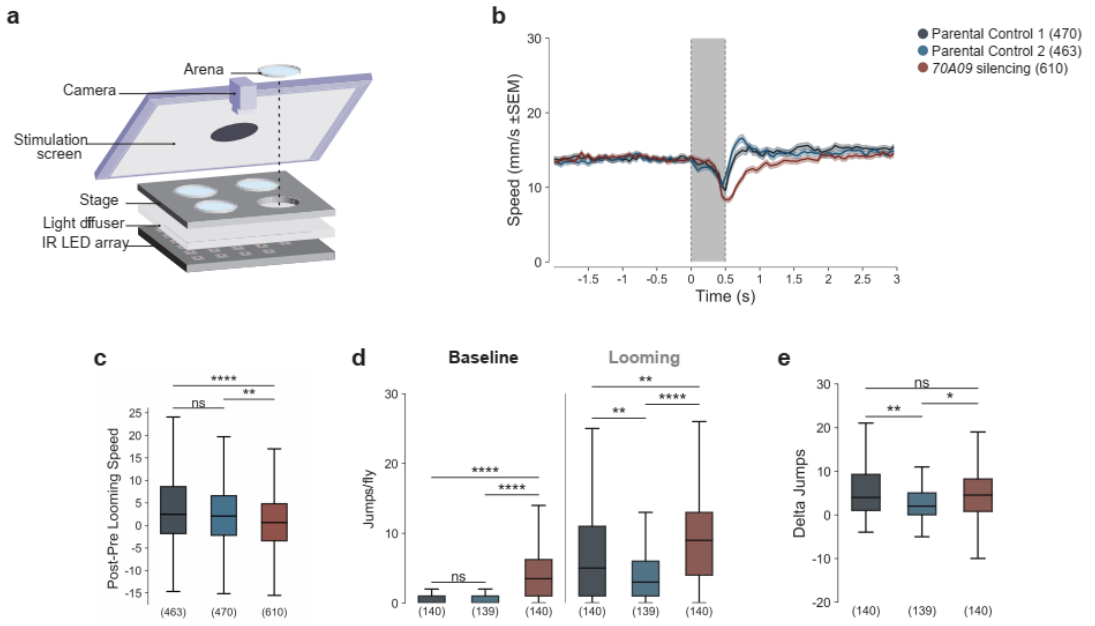


Figure 1.7. Silenced 70A09 females show less vigorous escape responses when exposed to a threat. (a) Schematic of the experimental setup used to characterise escape behaviours in response to looming stimuli. Genotypes: *+/w-, UAS>STOP>kir2.1; otd-nls*:FLPo/+; + (Parental Control 1), *+/w-; +; 70A09*-GAL4/+ (Parental Control 2) and *+/w, UAS>STOP>kir2.1; otd-nls*:FLPo/+; *70A09*-GAL4/+ (*70A09* silencing). In **(b)** and **(c)** only looming events where flies were walking before and after the stimulus were included. **(b)** Looming-triggered speed profile. Average (±SEM) speed in a time window around looming. Dashed lines indicate looming onset and offset. Shaded grey indicates looming duration **(c)** Change in speed caused by stimulus presentation (pre-looming period subtracted from post-looming period). **(d)** Number of jumps per fly during the baseline and stimulation period. **(e)** Increase in the number of jumps per fly during stimulation relative to baseline (jumps per fly during stimulation subtracted from jumps per fly during baseline). Center line, median; box limits, upper (75) and lower (25) quartiles; whiskers, 1.5x interquartile range. Statistical analysis was performed with Kruskal-Wallis test, followed by post hoc pairwise Dunn's test with Bonferroni correction: ns = not significant, **p*<0.05, ***p*<0.01, *****p*<0.0001. n values are shown in parentheses and indicate the number of looming events in **(b)** and **(c)**, and the number of flies in **(d)** and **(e)**.

Together, these results indicate that the increased escape displayed by *70A09*-silenced females in the context of courtship is a specific response to the courting male, not observable in a general threat context.

1.3.4. *70A09*-silenced females are unreceptive independently of escape availability

The increased walking speed of *70A09*-silenced females during courtship raises the question of whether the absence of mating is merely a consequence of the inability to slow down. To address this question, we restricted the walking space of the arenas used for screening with the introduction of an adapter (restricted arenas, Figure 1.8a). In this new version the space is 6 mm x 5 mm x 4,5 mm, which allows movement but not running (consider for reference that a fly is around 2 mm long). We paired single flies for 20 minutes and analysed for up to 10 minutes after courtship initiation.

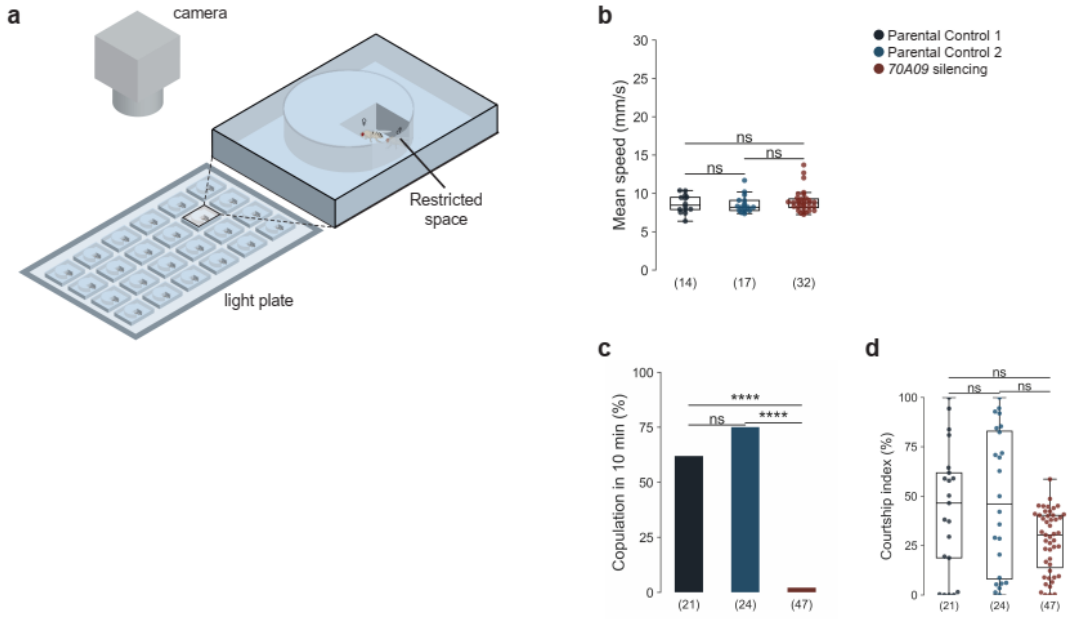


Figure 1.8. Silenced 70A09 females remain unreceptive when escape is impeded. (a) Schematic representation of the behavioural setup to test female receptivity when the female is not allowed to walk away from the male. **(b)** Female mean walking speed (4 – 50 mm/s) during courtship ON periods. Genotypes: w */UAS>STOP>kir2.1*; *otd-nls*:FLPo/+; + (Parental Control 1), w-; +; *70A09*-GAL4/+ (Parental Control 2) and w-/*UAS>STOP>kir2.1*; *otd-nls*:FLPo/+; *70A09*-GAL4/+ (*70A09* silencing). **(c)** Copulation rate of silenced and control females. **(d)** Male courtship index toward silenced and control females. Statistical analysis was performed with Fisher's exact test **(c)** and, Kruskal-Wallis test followed by post hoc pairwise Dunn's test with Bonferroni correction **(b, d)**: ns = not significant, ****p<0.0001. n values are shown in parentheses.

We confirmed that indeed in these arenas *70A09*-silenced females do not speed up but rather walk at similar speed of control females (Figure 1.8b). We found that, in

this context, silenced females still did not mate (Figure 1.8c). The male courtship index is similar in all conditions showing that the difference in copulation rate does not result from low male drive (Figure 1.8d). In sum, our results show that 70A09 females are unreceptive independently of their ability to escape.

1.3.5. Courtship, and specifically courtship song, is required for 70A09-dependent escape suppression

To confirm that increase in walking speed, reduction in pausing and increase in jumps in *70A09*-silenced females is a response to courtship, we paired female flies with *fru* mutant males that do not court (Demir and Dickson, 2005). Since courtship was absent, we used the distance between the flies as a proxy for courtship as we have previously shown that below 5.5 mm there is a 95,5% likelihood of courtship ('courtship distance') (Aranha *et al.*, 2017).

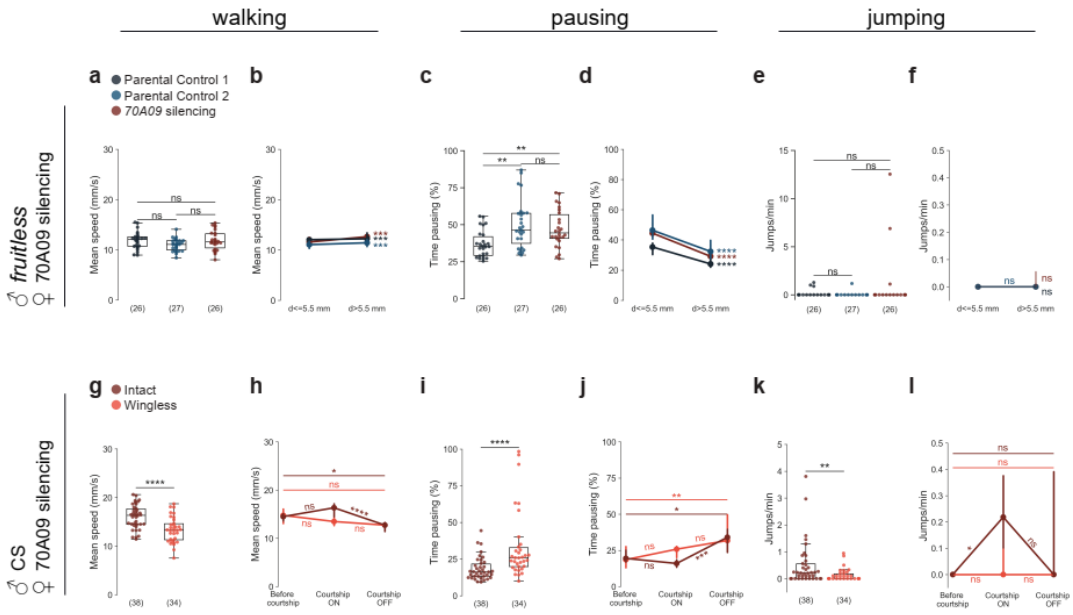


Figure 1.9. Silenced 70A09 females do not escape when coupled with courtship-impaired males. **(a–f)** Non courting *fruitless* mutant males paired with females of each of the genotypes: w */UAS>STOP>kir2.1*; *otd-nls*:FLPo/+; + (Parental Control 1), w-; +; *70A09*-GAL4/+ (Parental Control 2) and w-/*UAS>STOP>kir2.1*; *otd-nls*:FLPo/+; *70A09*-GAL4/+ (*70A09* silencing).). **(a, b)** Female mean walking speed (4 – 50 mm/s), **(c, d)** female pausing and **(e, f)** number of jumps per minute, at courtship distance **(a, c, e)** or within and outside courtship distance **(b, d, f)**. **(g–l)** Canton-S males intact and with wings removed (Wingless) paired with silenced 70A09 females (*70A09* silencing). **(g, h)** Female mean walking speed (4 – 50 mm/s), **(i, j)** female pausing and **(k, l)** number of jumps per minute, during courtship ON periods **(g, i, k)** or in different moments of courtship dynamics **(h, j, l)**. Statistical analysis was performed with one-way ANOVA followed by post -hoc Tukey's test **(a)**, paired t-test **(b)**, Kruskal-Wallis test **(c, e)** and Friedman's test **(h, j, l)** followed by post hoc pairwise Dunn's test with Bonferroni correction, Wilcoxon signed rank test **(d, f)**, unpaired t-test **(g)** and Mann-Whitney U test **(i, k)**: ns = not significant, *p<0.05, **p<0.01, *** p<0.001, ****p<0.0001. n values are shown in parentheses.

We observed no difference between the walking speed of *70A09*-silenced females and control females at courtship distance (Figure 1.9a), as well as, no difference in walking speed between courtship distance and not courtship distance for all conditions (Figure 1.9b). These results indicate that the changes in female walking speed require courtship from a male, the mere presence of a male not being sufficient to trigger them. Pausing levels were also very different from those observed in females paired with a courting male. At courtship distance, silenced flies pause either as much or more when compared to the parental controls (Figure 1.9c). In all conditions there is more pausing at courtship distance (Figure 1.9d). Finally, jumps were nearly absent in all conditions (Figure 1.9e and 1.9f). From our results, we conclude that a courting male and not the mere presence of a male triggers escape in *70A09*-silenced females.

A courting male produces different stimuli that may lead the female to escape. They could be the visual stimulus of an approaching animal, the scent of male pheromone or the song that the male produces with wing vibration. Given that song has been shown to modulate the speed of the female during courtship, albeit to reduce it (von Schilcher, 1976; Crossley, Bennet-Clark and Evert, 1995; Bussell *et al.*, 2014; Vaughan *et al.*, 2014; Clemens *et al.*, 2015; Deutsch *et al.*, 2019), we decided to test the role of song in the response of *70A09*-silenced females. For this we paired *70A09*-silenced females with wild type males that were either intact or with the wings removed ('wingless'). We first confirmed that courtship index is not affected by wing removal (Figure 1.10). We then analysed the female walking speed in the two different conditions. We found that, during courtship, the walking speed of *70A09*-silenced females was lower for females paired with wingless males (Figure 1.9g). During the different moments of courtship, the walking speed of females paired with wingless males never changed whereas control silenced females with intact males, as previously found (Figure 1.5f), increased their walking speed during courtship ON moments compared to courtship OFF moments (Figure 1.9h). In this experiment, however, the walking speed of *70A09*-silenced females with intact males is not significantly different between baseline and courtship ON moments (Figure 1.9h), unlike what was previously found (Figure 1.5f), which may be a reflection of the higher

baseline walking speed observed in this experiment. Analysis of pausing during courtship ON revealed that *70A09*-silenced females paired with wingless males pause more than those paired with intact males (Figure 1.9i). Across the different moments of courtship *70A09*-silenced females paired with wingless males have similar pausing levels with a small increase of pausing in courtship OFF compared to before courtship (Figure 1.9j). Finally, *70A09*-silenced females paired with wingless males jump very little during courtship ON (Figure 1.9k) or any other moment of the video (Figure 1.9l) whereas *70A09*-silenced females paired with intact males significantly increase jumps during courtship ON compared to before courtship with no significant difference in courtship OFF moments. These results clearly show that a courting male that is unable to produce song does not elicit any type of escape in *70A09*-silenced females, *i.e.*, that song is a trigger for escape in *70A09*-silenced females.

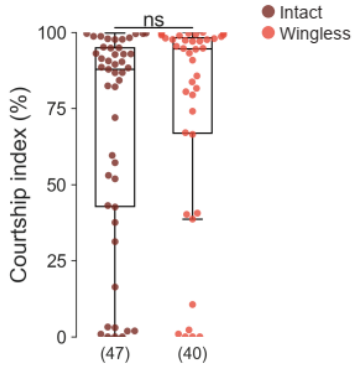


Figure 1.10. Courtship index of wingless and intact males. Male courtship index of intact males and males without wings towards 70A09 silenced females. Genotype: *w-/UAS>STOP>kir2.1; otd-nls*:FLPo/+; *70A09*-GAL4/+. Statistical analysis was performed with Kruskal-Wallis test, followed by pairwise comparisons using Mann–Whitney U test with Bonferroni correction: ns = not significant. n values shown in parentheses.

1.3.6. Activation of 70A09 neurons leads to female pausing but not mating

Silencing 70A09 neurons leads to decreased receptivity, which is accompanied by increased escape (higher walking speed, less pausing and more jumping) during courtship. We sought to explore the effect of activating these neurons during courtship. To this end, we expressed the red shifted channelrhodopsin, csChrimson (Klapoetke *et al.*, 2014) in 70A09 neurons. We recorded single pairs of courting flies for 9 minutes. The red light was off during the first 3 minutes, it was turned on from minute 3 to 6 and was again off for the last 3 minutes in order to allow within-video comparisons.

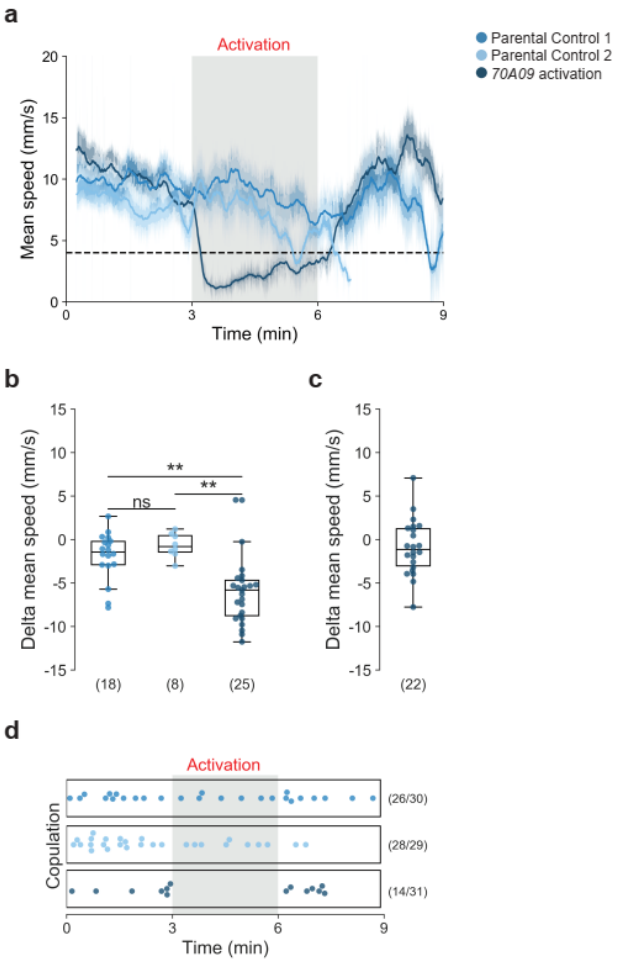


Figure 1.11. Activation of *70A09*-GAL4 brain neurons drastically reduces female speed. (a) Female mean speed calculated by rolling average for 15 seconds with standard error of the mean (SEM) represented. Genotypes: w-; *otd-nls*:FLPo/+; *UAS>STOP>Chrimson.mVenus* (Parental Control 1), w-; +; *70A09*-GAL4/+ (Parental Control 2) and w-; *otd-nls*:FLPo/+; *70A09*-GAL4/*UAS>STOP>Chrimson.mVenus* (70A09 activation). **(b)** Difference in flies mean speed between **(b)** activation-baseline periods for all genotypes and **(c)** lightsOFF-baseline periods for 70A09 activation. **(d)** Copulation of activated and control females with the distribution according to latency to copulation. Number of receptive females out of the total number of females are shown in parenthesis. Statistical analysis was performed with Kruskal-Wallis test **(b)**, followed by post hoc pairwise Dunn's test with Bonferroni correction and, Wilcoxon rank-sum test **(c)**: ns = not significant, **p<0.01. n values are shown in parentheses.

In this experiment, we quantified speed which includes pausing and jumping (as opposed to walking speed which does not). We observed that upon light activation the test flies drastically reduced their speed while the speed of control flies was unchanged (Figure 1.11a and 1.11b). In fact, activated females paused during light on, only performing lateral displacement prompted by the courting male. Once the light was off, activated females recovered their speed to values similar to those prior to activation as shown by comparing the delta of the speed during lights off and baseline to a database with a random group of values with a similar range varying around zero (Figure 1.11c, *p*=0.3359). Given that silencing 70A09 neurons reduces receptivity, we wondered what would be the effect of activation of these neurons on receptivity. Analysis of the latency to copulate shows that activated flies did not mate during light on and resume mating once the light turns off whereas control females mate throughout the whole video, indicating that activation of 70A09 neurons leads to a reduction of receptivity (Figure 1.11d). Courtship remains high throughout the experiment (Figure 1.12). It is unclear whether it is activation and silencing of the same or a different set of neurons within the 70A09 expression that leads to loss of receptivity.

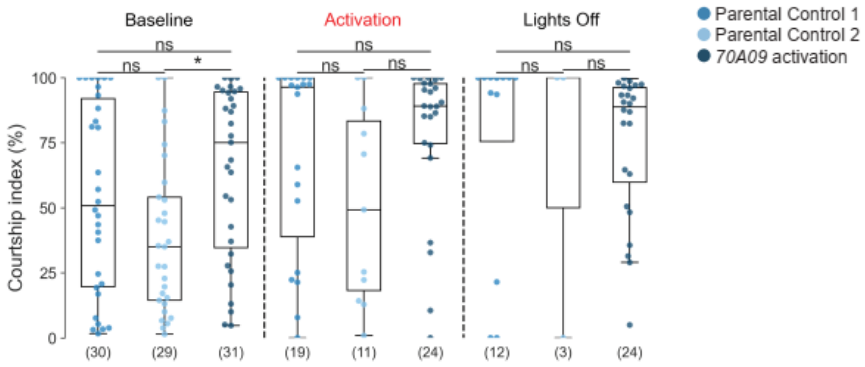


Figure 1.12. Male courtship in each period of the activation experiment. Male courtship index toward activated and control females, for each moment of the activation experiment. Genotypes: w-; *otd-nls*:FLPo/+; *UAS>STOP>Chrimson.mVenus* (Parental Control 1), w-; +; *70A09*-GAL4/+ (Parental Control 2) and w-; *otd-nls*:FLPo/+; *70A09*-GAL4/*UAS>STOP>Chrimson.mVenus* (70A09 activation). Statistical analysis was performed with Kruskal-Wallis test, followed by post hoc Dunn's test with Bonferroni correction: ns = not significant, *p<0.05. n values are shown in parentheses.

With these experiments we found that, in terms of speed, activation of 70A09 neurons leads to the opposite phenotype of silencing them during courtship ON. We speculate that in wild type receptive females these neurons are gradually activated during courtship ultimately leading to female pausing.

1.4. Discussion

Courtship allows animals to display and evaluate their qualities before they choose a mate. In most species, males initiate courtship and females decide whether or not to mate (Pycraft, 1914). Reproductive decisions have a powerful impact in the survival of the species and thus the communication between courtship partners is vital. To understand courtship behaviours, we must focus on how the partners communicate which sometimes involves subtle cues.

Here we reveal a novel layer of regulation of female speed in the context of courtship. Specifically, we found that escape suppression is a fundamental and hitherto unknown step of the female's response to courtship. When modulation of the 70A09 is absent, females escape the courting male continuously and vigorously. One could assume that these females are not able to perceive courtship from the male, perceiving instead an approaching animal, which would lead them to escape. But in fact, these females are recognizing the courtship song and this stimulus is inducing escape. Our work suggests that part of the female brain is interpreting song as aversive while another part processes song as a signal to slow down. We propose that activity in 70A09 brain neurons tips the scale to slowing down. Escape is occasionally observed in wild type receptive virgins, usually early in courtship. We speculate that courtship is initially aversive to the female which with continued courtship is adjusted to an opportunity to mate leading to reducing the speed and eventually accepting the male.

While our work highlights the impact of acoustic stimuli produced by the male during courtship, it is well established that chemical stimuli such as cis-vaccenyl acetate (cVA) and cuticular hydrocarbons are a major component of communication between flies and play a role in the female's decision to mate (Rybak, Sureau and Aubin, 2002; Grillet, Dartevelle and Ferveur, 2006; Kurtovic, Widmer and Dickson, 2007). This work opens the way to investigate how chemical stimuli contribute, in combination with courtship song, to the modulation of female speed during courtship.

Wild type unreceptive flies, *i.e.*, immature virgins and mated females, respond to courtship differently from receptive virgins. Immature virgins do not slow down and

pause less than mature virgins (Connolly and Cook, 1973; von Schilcher, 1976; Bussell *et al.*, 2014). Mated females do not display high walking speed, as immature females do, but show a positive correlation between song amount and their speed (Connolly and Cook, 1973; Coen *et al.*, 2014). In sum, some features of the natural unreceptive states are common to 70A09 silencing phenotype indicating that 70A09 neurons may be differently active in receptive and unreceptive females.

Besides a role in escape modulation, we have also uncovered a role of 70A09 neurons in receptivity that is separable from the ability to escape. Although it is clear from wild type behaviour the close link between speed modulation during courtship and receptivity, it remains to be elucidated which 70A09 neuron(s) are involved in receptivity and escape phenotype.

A recent study characterised neurons in the central brain, pC2l, that are tuned to courtship song and modulate the locomotor response in a sex-specific manner (Deutsch *et al.*, 2019). Though the exact identity of 70A09 neurons which are involved in the observed escape phenotypes is unknown, it is clear that they do not overlap with *dsx*-positive pC2l since we have shown that the *dsx* subset of 70A09 neurons do not show an escape phenotype. Moving forward it would be interesting to investigate how pC2l and 70A09 neurons interact to produce a locomotor response to song.

In conclusion our work shed a light on the interactions between mating partners, by revealing a new role of the male courtship song and identifying a set of brain neurons responsible for the song-induced female slowing down. The male song is a courtship cue with a dual role and opposite effects on the female: it first induces escape, providing the female with enough time to assess the male, until the decision to mate is made, and then it prompts a decrease in locomotion, which in turns will allow the male to get closer to the female and eventually copulate. The activity in 70A09 neurons is necessary for suppressing the song-induced escape by prompting a decrease in locomotion and allowing to advance the courtship plot. Our findings highlight the complexity of male-female interactions during courtship, revealing a dual response of the female to courtship song.

1.5. Material and methods

Drosophila stocks

Fly strains and sources are as follows: Canton-S (CS), w^{1118} (Morata and Garcia-Bellido, 1973), GMR70A09-GAL4 and all lines in receptivity screen (Jenett *et al.*, 2012), *65C12*-GAL4-DBD (Wu *et al.*, 2016), UAS-*Kir2.1* (Baines *et al.*, 2001), Tub-GAL80TS (McGuire, Mao and Davis, 2004); *otd-nls*:FLPo (Asahina *et al.*, 2014), UAS>STOP>*Kir2.1* (Yang *et al.*, 2009), UAS>STOP>*CD8-GFP* (Hong *et al.*, 2009), *8xLexAop2-FLP*$_L$ (Pan, Meissner and Baker, 2012), Gad-LexA (Diao *et al.*, 2015), $elav^{GAL4\text{-}DBD}$ (Luan *et al.*, 2006), ey-FLP (Therrien, Wong and Rubin, 1998), *elav*-GAL80 (Yang *et al.*, 2009), *Dilp3*-GAL4 (Buch *et al.*, 2008), TH-GAL4-DBD (Aso *et al.*, 2014) provided by Gerald Rubin (Janelia Research Campus, HHMI), *UAS>STOP>csChrimson.mVenus* (Klapoetke *et al.*, 2014) flp-out version provided by Vivek Jayaraman, fru^{LexA} (Mellert *et al.*, 2010), fru^{GAL4} (Demir and Dickson, 2005) and $dsx^{GAL4\text{-}DBD}$ (Pavlou *et al.*, 2016).

Construction of transgenic lines

The *70A09*-LexA and *70A09*-GAL4-AD DNA constructs were generated by Gateway™ cloning technology (Invitrogen). The entry clone (pCR8TM/GW/TOPO®; Invitrogen™) carrying the 70A09 enhancer fragment(Pfeiffer *et al.*, 2008), generously provided by Gerald Rubin (Janelia Research Campus, HHMI), was cloned into pBPLexA::p65Uw (Addgene plasmid #26230) and pBPp65ADZpUw (Addgene plasmid #26234). DNA constructs were verified by restriction enzymatic digestion with *XbaI* (New England Biolabs #R0145) for 2 hours at 37ºC and purified using QIAGEN® Plasmid Midi Kit (Cat Nº. 12145), prior to injection into flies. Plasmid was injected into y^1 w^{67c23}; P{CaryP}attP40 flies (Markstein *et al.*, 2008) by adapting a protocol from Kiehart et al. (Kiehart, Crawford and Montague, 2007).

Immunostaining and microscopy

Adult brains and VNCs were dissected in cold phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (PFA) in PBL (PBS and 0.12M Lysine) for 30 minutes at room-temperature (RT), washed three times for 5 minutes in PBT (PBS and 0.5% Triton X-100) and blocked for 15 minutes at RT in 10% Normal Goat Serum (NGS, Sigma) in PBT. Tissues were incubated with the primary antibodies in blocking solution for 72 hours at 4ºC. The following primary antibodies were used: rabbit anti-GFP (1:2000, Molecular probes, cat# A11122), and mouse anti-nc82 (1:10, Developmental Studies Hybridoma Bank). Samples were washed three times for 5 minutes in PBT and incubated in Alexa Fluor secondary antibodies (1:500, Invitrogen) for 72 hours at 4ºC. The following secondary antibodies were used: anti-rabbit IgG conjugated to Alexa 488 and anti-mouse IgG conjugated to Alexa 594. Samples were washed three times for 5 minutes in PBT and mounted in VectaShield medium (Vector Laboratories, Cat# H1000). Images were acquired on a Zeiss LSM 710 confocal microscope using 20x objective or 25x Immersion objective (Zeiss). After acquisition, colour levels were adjusted using Fiji (Schindelin *et al.*, 2012).

Behavioural experiments

Fly husbandry

Flies were raised in standard cornmeal-agar medium at 25 ºC and 70% relative humidity in a 12h:12h dark:light cycle, unless otherwise indicated. For all experiments both female and male flies were collected under CO_2 anaesthesia, soon after eclosion, and raised in regular food vials. Flies were raised in isolation for fertility and receptivity experiments. Females were raised in groups of up to 25 per vial for looming experiments. For acute neuronal silencing experiments, female flies and males were raised at 18 ºC from 6 to 14 days. Manipulated flies were incubated at 30 ºC for 24h, whereas control flies were maintained at 18ºC. Both controls and manipulated flies, as well as males, were shifted to 25 ºC 24 hours before the behavioural assay to prevent the effect of temperature treatment on the behaviour.

For chronic neuronal silencing, female flies and males were raised at 25 ºC from 4 to 8 days.

Unless specified, the flies used in behavioural experiments were 4-8 days old virgin females and males, and were tested in the same conditions as rearing (25 °C and 70% humidity).

Fertility screen

To allow mating, a male and a female were paired in a food vial for 30 minutes after which the male was removed. One week later the vial was checked for progeny. For each line 20-25 females were tested. The lines for which at least 25% of the females did not produce progeny were selected for further testing. In this initial large-scale screen, controls were not used.

Female receptivity

To test female receptivity, a single female was gently aspirated and transferred into circular acrylic chambers (small arenas: 16 mm in diameter x 4.5 mm height) and paired with a male. Individual pairs were recorded for 30 minutes using SONY HDR-CX570E, HDR-SR10E, HDR-XR520VE or HDR-PJ620 video cameras (1440 x 1080 pixels; 25 frames per second). A white LED was used as backlight source (Edmund optics, cat# 83-875).

Receptivity with female tracking

To allow the detailed behaviour analysis, a single female was gently aspirated and transferred to a custom-made circular arena with a conical-shaped bottom that avoid flies walking on the walls (Simon and Dickinson, 2010) (detailed arenas: 40 mm in diameter), allowing to track them as described in Aranha et al. (Aranha *et al.*, 2017). Each female was allowed to habituate to the new environment for about 10 minutes and then paired with a male. Movies were acquired in dim light using an infrared 940

nm LED strip (SOLAROX) mounted on an electric board developed by the Scientific Hardware Platform. Flies were recorded in grayscale (1024 x 1024 pixels, 60 frames per second), with a camera mounted above the arena (PointGrey FL3-U3-32S2M-CS with a 5 mm fixed focal length lens (Edmund Optics)) with a Hoya 49 mm R72 infrared filter, for 20 minutes or until copulation occurred. Female flies paired with fruitless mutant males were recorded for 10 minutes. Bonsai(Lopes *et al.*, 2015) was used for movie acquisition. To generate wingless males, individual CS male flies were anesthetized with CO_2 approximately 15-20 hours before the experiment. Wings were bilaterally cut at their base with microscissors or microforceps (World Precision Instruments) under a scope. Flies were allowed to recover at 25ºC until the experiment.

Receptivity in a restricted space

To test receptivity in a restricted space, the small arenas were modified by inserting an acrylic adaptor, thus reducing the walking surface (restricted arenas: 6 mm length x 5 mm width x 4.5 height). Single females were gently aspirated and transferred into the restricted arenas. Female flies were allowed to habituate to the new environment for about 10 minutes before being paired with the male. Movies were acquired in dim light using an infrared 940 nm LED strip (SOLAROX) mounted on an electric board developed by the Scientific Hardware Platform. Flies were recorded for 20 minutes in grayscale (1024 x 1024 pixels, 60 frames per second), with a camera mounted above the arena (PointGrey FL3-U3-32S2M-CS with a 16 mm fixed focal length lens (Edmund Optics)) with a Hoya 49 mm R72 infrared filter. This setup allowed us to record two pairs of flies at the same time. Bonsai (Lopes *et al.*, 2015) was used for movie acquisition.

Looming experiment

Behavioural apparatus and paradigm: Visual stimulation was delivered on a monitor (Asus ROG Strix XG258Q, 24.5") tilted at 45 degrees over the stage where the

arenas were placed. This stage was backlit by an infrared (940nm) LED array developed by the Scientific Hardware Platform. A 3mm (TBC) white opalino was placed between the LED array and the arenas to ensure homogeneous illumination. We recorded behaviour at 60Hz using a USB3 camera (FLIR Blackfly S, Mono, 1.3MP) with a 730nm long pass filter (Lee Filters, Polyester 87 Infrared). Behavioural arenas were 30 mm in diameter and 4 mm in height, and were built from opaque white and transparent acrylic sheets. Single flies were transferred to each behavioural chamber using a mouth aspirator. After being transferred, flies were allowed to habituate to the new environment for a period of 2 minutes. The duration of this baseline period was set based on the median duration that a male takes to start courting the female (latency to court). This baseline period was followed by a stimulation period that lasted 5 minutes, and during which 7 looming stimuli were presented with an ISI that ranged between 10 and 20 seconds. Videos were acquired using Bonsai (Lopes *et al.*, 2015) at 60 Hz and width 1104 x height 1040 resolution.

Looming stimulus: Looming stimuli were presented on the above-mentioned monitor running at 240Hz refresh rate; stimuli were generated by a custom Bonsai workflow (Lopes *et al.*, 2015). The looming effect was generated by a black circle that increased in size over a white background. The visual angle of the expanding circle can be determined by the equation: $\theta(t) = 2\tan{-1}\,(l / vt)$, where l is half of the length of the object and v the speed of the object towards the fly. Virtual object length was 1 cm and speed 25 cm s−1 (l / v value of 40 ms). Each looming presentation lasted for 500 ms. Object expanded during 450 ms until it reached a maximum size of 78° where it remained for 50 ms before disappearing.

Activation experiment

For the activation experiment, the female flies were individually collected and allowed to age in cornmeal-agar food containing 0.2 mM all trans-Retinal (Sigma, R2500) and reared in dim light until the experiment.

The same setup described in the *Receptivity with female tracking* section was used. For the light stimulation a high-powered 610 nm LEDs arrays interspersed between

the infrared LEDs on the blacklight board was used. The arena was irradiated with a power in the 4-4.7 mW/cm^2. A female and a male were gently aspirated and transferred in the arenas. They were allowed to habituate and only when the male started courting the video recording was started. Videos were recorded for 9 minutes or until copulation. The activation protocol included a baseline that lasts 3 minutes, followed by light stimulation during 3 minutes and a post-activation period of 3 minutes.

Data processing

In order to quantify female receptivity, a custom-made software was developed to track the flies and compute the time to copulation, when it occurred. To quantify flies' behaviours, FlyTracker (Eyjolfsdottir *et al.*, 2014) was used to track the two flies and output information concerning their position, velocity, distance to the other fly, among others. A Courtship Classifier developed in the lab using the machine learning-based system JAABA (Kabra *et al.*, 2013) was run to automatically identify courtship bouts. Subsequently, in-house developed software PythonVideoAnnotator (https://biodata.pt/python_video_annotator) was used to visualize courtship events generated by JAABA and manually correct them if necessary. Annotations were done from the beginning of courtship and during 10 min or until copulation. PythonVideoAnnotator was also used to manually annotate the copulation time, considering the whole duration of the video.

For the looming experiment, two main features were extracted from the videos using a custom-built Bonsai workflow: centroid position and pixel change in a 72 x 72px ROI around the fly.

Quantification and statistical analysis

Data analysis was performed using Python 3 scripts for all experiments, except for the copulation rate for small arenas receptivity experiments, for which GraphPad

Prism Software version 7.0 was used. All data, except those from flies excluded due to tracking errors, were analysed.

Female receptivity and male behaviour parameters

The latency to copulation was calculated from the beginning of male courtship. With exception of latency to copulation, all quantifications were performed for the first 10 minutes of courtship or until copulation, whichever happened first. Male courtship index was calculated as the ratio between courtship frames and the total number of frames.

Female locomotor parameters during male courtship

For the characterisation of female locomotor activity, mean speed, pausing and jumping were quantified. Since courtship is a prerequisite, we selected only videos with courtship index equal or above 20%. The three behaviours were separately quantified in three different moments: i) before courtship starts (# frames before courtship initiation), ii) courtship ON (# frames of courtship since courtship initiation) and iii) courtship OFF (# frames of not courtship since courtship initiation). For the experiment with *fruitless* mutant males, since courtship was absent, the three behaviours were quantified when the distance between the two animals was below 5.5 mm, which is a proxy for courtship, and compared to the same behaviours when the distance was above 5.5 mm. The distance information was extracted from the FlyTracker output (see *Data processing* section).

Walking frames were defined as the frames in which female speed was within the range of 4-50 mm/s and the mean walking speed for each fly was calculated by the sum of speed values divided by the number of walking frames. Pausing frames were defined as the frames in which the fly speed was below 4 mm/s, as reported previously (Bussell *et al.*, 2014). The pausing percentage was obtained normalizing the number of pausing frames over the total number of frames for each courtship moment. Jumps were defined as instantaneous female speed above 70 mm/s. We

set this value based on the discontinuity in the speed distribution and on the presence of peaks in the raw, un-binned speed data. Since a high number of peaks were observed for speed values above 50 mm/s (upper limit for walking speed), manual observation of random peaks was performed. Below 70 mm/s most of the peaks corresponded to fly transitions from the lid to the bottom of the arena and/or decamping. Therefore, we set the threshold for jumps at 70 mm/s. For the activation experiment, no speed filter was applied. To observe females' speed during the whole video recording, rolling average and standard error of the mean (SEM) applied to 15 seconds were calculated.

Female locomotor parameters during looming stimulus

Using the centroid position, a fly was considered to be walking if its speed was higher than 4 mm/s and lower than 75 mm/s. We identified jumping events by detecting peaks in the raw data. A fly was classified as having jumped if its instantaneous speed exceeded 75 mm/s, a threshold identified by a discontinuity in the speed distribution. The speed plots represent all the moments in which the speed was below the jump threshold for those looming events in which the flies were walking in the 0.5 sec bin preceding looming onset, and in the 0.5 sec bin from 2.0 to 2.5 sec after loom offset.

For statistical analysis of all experiments, Fisher's exact test was performed to compare the copulation rate between two different groups. Prior to statistical testing, Levene's test was used to assess variance homogeneity and Shapiro-Wilk test were used to assess normality across all individual experiments. Independent groups were subjected to unpaired t-test (n=2) or one-way ANOVA followed by post hoc pairwise Tukey's test (n≥3) if parametric assumptions were satisfied. If not, Mann-Whitney U test (n=2) or Kruskal-Wallis test followed by post hoc Dunn's test (n≥3) was used. For dependent groups, paired t-test (n=2) or repeated measures ANOVA followed by post hoc multiple pairwise paired t-test (n≥3) were applied if parametric assumptions were satisfied. If not, Wilcoxon signed-rank test (n=2) or Friedman's test followed by post hoc Dunn's test (n≥3) was used. Bonferroni correction to *p*-values was applied when

multiple comparisons were performed. Wilcoxon rank-sum test was used to compare one data group with a dataset of random values with median around zero and variance equivalent to the experimental group. The sample size for each condition is indicated in each plot. All the statistical details related to the figures are included in Tables A1-A6 (see *Appendix A* in page 76). The difference in sample size for the same condition in different analysis is due to the different thresholds applied.

1.6. Appendix A

Table A1. Statistical details related to Figures 1.1, 1.2 and 1.4 (Section *1.3.1*)

Figure	groups	n	normally distributed	statistical test	p value	dfs	
1.1	R29E06	40	NA	Fisher's exact test	18 C vs 30 C = 0,1957	1	
	R22C11	44	NA	Fisher's exact test	18 C vs 30 C = 0,2140	1	
	R30G04	48	NA	Fisher's exact test	18 C vs 30 C = 0,2056	1	
	R25H03	40	NA	Fisher's exact test	18 C vs 30 C = 0,0741	1	
	R29F10	48	NA	Fisher's exact test	18 C vs 30 C = 0,1715	1	
	R23A03	40	NA	Fisher's exact test	18 C vs 30 C = 0,1687	1	
	R70A09	48	NA	Fisher's exact test	18 C vs 30 C = 7,72E-06	1	
	R57G02	48	NA	Fisher's exact test	18 C vs 30 C = 3,77E-08	1	
1.2b	a) UAS>STOP>Kir2.1; otd-FLP; 70A09GAL4/elavGAL80	49	NA	Fisher's exact test	a vs b = 0,0291; a vs c = 0,3011; a vs d = 0,0000	3	
	b) UAS>STOP>Kir2.1; ; 70A09GAL4	35	NA				
	c) ; otd-FLP; elavGal80	35	NA				
	d) UAS>STOP>Kir2.1; otd-FLP; 70A09GAL4	49	NA				
1.4a	UAS>STOP>kir2.1/eyFLP;+;70A09GAL4/TubGal80TS	48	NA	Fisher's exact test	18 C vs 30 C = 0,7145	1	
1.4b	a) w-;8xLexAop2FLP;Gad-LexA	40	NA	Fisher's exact test	a vs b = 1,0000; a vs c = 0,0102; b vs c = 0,0076	2	
	b) w-; UAS>STOP>Kir2.1; 70A09-GAL4	47	NA				
	c) 70A09GAL4ÇGad-LexA > UAS-Kir2.1	48	NA				
1.4c	a) w-; UASKir2.1; +	24	NA	Fisher's exact test	a vs b = 1,0000; a vs c = 0,0442; b vs c = 0,0442	2	
	b) w-; Dilp3 GAL4; +	24	NA				
	c) Dilp3 GAL4>Kir2.1	48	NA				
1.4d	a) UAS>STOP>Kir2.1;otd-FLP;	24	NA	Fisher's exact test	a vs b = 0,7008; a vs c = 1,0000; b vs c = 0,4614	2	
	b) ;70A09-AD;65C12-DBD	24	NA				
	c) otd-FLP ∩ 70A09-AD ∩ 65C12-DBD > Kir2.1	23	NA				
1.4e	a) UAS>STOP>Kir2.1;otd-FLP;	23	NA	Fisher's exact test	a vs b = 1,0000; a vs c = 1,0000; b vs c = 1,0000	2	
	b) ;70A09-AD;TH-DBD	6	NA				
	c) otd-FLP ∩ 70A09-AD ∩ 65C12-DBD > Kir2.1	25	NA				
1.4g	a) UAS>STOP>Kir2.1;otd-FLP;	48	NA	Fisher's exact test	a vs b = 0,1171; a vs c = 0,0000; b vs c = 0,0000	2	
	b) ;70A09-AD;Dsx^{DBD}	48	NA				
	c) otd-FLP ∩ 70A09-AD ∩ Dsx^{DBD} > Kir2.1	48	NA				

NA: not applicable

Table A2. Statistical details related to Figures 1.5 and 1.6 (Section *1.3.2*)

Figure	groups	n	normally distributed	statistical test	p value	dfs	
1.5b	a) parental control 1	45	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 1,0000; b vs c = 0,2765. H = 2,85	2	a vs b: 88
	b) parental control 2	45	no				a vs c: 92
	c) 70A09 silencing	49	no				b vs c: 92
1.5c	a) parental control 1	45	NA	Fisher's exact test	a vs b = 1,0000; a vs c = 0,0000; b vs c = 0,0000	2	
	b) parental control 2	45	NA				
	c) 70A09 silencing	49	NA				
1.5d	a) parental control 1	45	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 0,9978; b vs c = 1,0000. H = 0,95	2	a vs b: 88
	b) parental control 2	45	no				a vs c: 92
	c) 70A09 silencing	49	no				b vs c: 92
1.5f	a) parental control 1	39	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 1,14E-09; b vs c = 2,13E-11. H = 58,62	2	a vs b: 78
	b) parental control 2	41	no				a vs c: 81
	c) 70A09 silencing	44	no				b vs c: 83
1.5g	a) parental control 1	35	no	Friedman test with post hoc Dunn's test	before vs ON = 0,0005; before vs OFF = 4,00E-05; ON vs OFF = 1,0000. Q = 36,40	2	68
	b) parental control 2	39	yes	rmANOVA with post hoc mpPaired t-test	before vs ON = 3,00E-06; before vs OFF = 3,32E-07; ON vs OFF = 1,0000. F = 45,03	2	76
	c) 70A09 silencing	43	no	Friedman test with post hoc Dunn's test	before vs ON = 5,60E-05; before vs OFF = 0,9674; ON vs OFF = 0,0030. Q = 21,91	2	84
1.5h	a) parental control 1	39	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 0,0008; b vs c = 0,0003. H = 19,32	2	a vs b: 78
	b) parental control 2	41	no				a vs c: 81
	c) 70A09 silencing	44	no				b vs c: 83
1.5i	a) parental control 1	38	no	Friedman test with post hoc Dunn's test	before vs ON = 5,00E-06; before vs OFF = 3,50E-05; ON vs OFF =1,0000. Q = 33,21	2	74
	b) parental control 2	41	no		before vs ON = 1,00E-06; before vs OFF = 2,16E-08; ON vs OFF = 1,0000. Q = 48,20	2	80
	c) 70A09 silencing	43	no		before vs ON = 1,0000; before vs OFF = 3,00E-05; ON vs OFF = 7.70E--05. Q = 34,09	2	84
1.5j	a) parental control 1	39	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,8379; a vs c = 7,66E-08; b vs c = 1,78E-05. H = 35,57	2	a vs b: 78
	b) parental control 2	41	no				a vs c: 81
	c) 70A09 silencing	44	no				b vs c: 83
1.5k	a) parental control 1	39	no	Friedman test with post hoc Dunn's test	before vs ON = 0,6325; before vs OFF = 0,1860; ON vs OFF = 1,0000. Q = 3,50	2	76
	b) parental control 2	41	no		before vs ON = 1,0000; before vs OFF = 1,0000; ON vs OFF = 1,0000. Q = 0,40	2	80
	c) 70A09 silencing	44	no		before vs ON = 3,00E-06; before vs OFF = 0,0123; ON vs OFF = 0,1328. Q = 24,00	2	86

(Continue next page)

(Table A2 continue)

Figure	groups	n	normally distributed	statistical test	p value	dfs	
1.6a	a) parental control 1	50	NA	Fisher's exact test	a vs b = 0,0050; a vs c = 0,0000; b vs c = 0,0002	2	
	b) parental control 2	49	NA				
	c) 70A09 ∩ Dsx silencing	45	NA				
1.6b	a) Courtship latency, parental control 1	50	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 0,1231; b vs c = 0,0761. H = 6,05	2	a vs b: 97
	b) Courtship latency, parental control 2	49	no				a vs c: 93
	c) Courtship latency, 70A09 ∩ Dsx silencing	45	no				b vs c: 92
	a) Courtship index, parental control 1	50	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,6554; a vs c = 1,0000; b vs c = 0,2101. H = 3,43	2	a vs b: 97
	b) Courtship index, parental control 2	49	no				a vs c: 93
	c) Courtship index, 70A09 ∩ Dsx silencing	45	no				b vs c: 92
1.6c	a) parental control 1	40	yes	one-way Anova with post hoc Tukey's HSD test	a vs b = 0,1140; a vs c = 0,0533; b vs c = 0,9000. F = 3,24	2	a vs b: 77
	b) parental control 2	39	yes				a vs c: 79
	c) 70A09 ∩ Dsx silencing	41	yes				b vs c: 78
1.6d	a) parental control 1	35	yes	rmANOVA with post hoc mpPaired t-test	before vs ON = 3,00E-05; before vs OFF =1,00E-06; ON vs OFF =1,0000. F = 36,60	2	68
	b) parental control 2	39	yes		before vs ON =0,0131; before vs OFF = 5,10E-05; ON vs OFF = 0,3609. F = 21,19	2	76
	c) 70A09 ∩ Dsx silencing	41	no	Friedman test with post hoc Dunn's test	before vs ON = 0,0625; before vs OFF = 0,0002; ON vs OFF = 0,2741. Q = 32,63	2	80
1.6e	a) parental control 1	40	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 0,7043; b vs c = 0,6954. H = 1,91	2	a vs b: 77
	b) parental control 2	39	no				a vs c: 79
	c) 70A09 ∩ Dsx silencing	41	no				b vs c: 78
1.6f	a) parental control 1	38	no	Friedman test with post hoc Dunn's test	before vs ON =7,79E-08; before vs OFF =5,48E-10; ON vs OFF =1,0000. Q = 48,21	2	74
	b) parental control 2	39	no		before vs ON =0,0001; before vs OFF = 1,40E-05; ON vs OFF = 1,0000. Q = 25,08	2	76
	c) 70A09 ∩ Dsx silencing	41	no		before vs ON = 7,00E-06; before vs OFF = 1,64E-10; ON vs OFF = 0,2000. Q = 46,24	2	80
1.6g	a) parental control 1	40	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,0033; a vs c = 0,1574; b vs c = 0,5279. H = 10,76	2	a vs b: 77
	b) parental control 2	39	no				a vs c: 79
	c) 70A09 ∩ Dsx silencing	41	no				b vs c: 78
1.6h	a) parental control 1	39	no	Friedman test with post hoc Dunn's test	before vs ON = 0,1870; before vs OFF =0,0324; ON vs OFF =1,0000. Q = 7,00	2	76
	b) parental control 2	39	no		before vs ON =0,0512; before vs OFF = 0,0057; ON vs OFF = 1,0000. Q = 11,94	2	76
	c) 70A09 ∩ Dsx silencing	41	no		before vs ON = 0,0027; before vs OFF = 0,4346; ON vs OFF = 0,1882. Q = 11,38	2	80

NA: not applicable

rmANOVA: repeated measures ANOVA

mpPaired t-test: multiple pairwise paired t-test

Table A3. Statistical details related to Figure 1.7 (Section *1.3.3*)

Figure	groups	n	normally distributed	statistical test	p value	dfs	
1.7c	a) parental control 1	463	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,1893; a vs c = 2,78E-07; b vs c = 0,0021. H = 31,09	2	a vs b: 931
	b) parental control 2	470	no				a vs c: 1071
	c) 70A09 silencing	610	no				b vs c: 1078
1.7d	a) baseline, parental control 1	140	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 4,99E-17; b vs c = 3,36E-16. H = 94,78	2	a vs b: 277
	b) baseline, parental control 2	139	no				a vs c: 278
	c) baseline, 70A09 silencing	140	no				b vs c: 277
	a) looming, parental control 1	140	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,0020; a vs c = 0,0036; b vs c = 9,93E-11. H = 44,20	2	a vs b: 277
	b) looming, parental control 2	139	no				a vs c: 278
	c) looming, 70A09 silencing	140	no				b vs c: 277
1.7e	a) parental control 1	14	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,0019; a vs c = 1,0000; b vs c = 0,0253. H = 12,83	2	a vs b: 29
	b) parental control 2	17	no				a vs c: 44
	c) 70A09 silencing	32	no				b vs c: 47

Table A4. Statistical details related to Figure 1.8 (Section *1.3.4*)

Figure	groups	n	normally distributed	statistical test	p value	dfs	
1.8b	a) parental control 1	14	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 1,0000; b vs c = 0,9758. H = 0,98	2	a vs b: 29
	b) parental control 2	17	no				a vs c: 44
	c) 70A09 silencing	32	no				b vs c: 47
1.8c	a) parental control 1	21	NA	Fisher's exact test	a vs b = 0,5192; a vs c = 0,0000; b vs c = 0,0000	2	
	b) parental control 2	24	NA				
	c) 70A09 silencing	47	NA				
1.8d	a) parental control 1	21	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 0,2886; b vs c = 0,1010. H = 5,58	2	a vs b: 43
	b) parental control 2	24	no				a vs c: 66
	c) 70A09 silencing	47	no				b vs c: 69

NA: not applicable

Table A5. Statistical details related to Figures 1.9 and 1.10 (Section *1.3.5*)

Figure	groups	n	normally distributed	statistical test	p value	dfs	
1.9a	a) parental control 1	26	yes	one-way Anova with post hoc Tukey's HSD test	a vs b = 0,1304; a vs c = 0,9000; b vs c = 0,1722. F = 2.39	2	a vs b: 51
	b) parental control 2	27	yes				a vs c: 50
	c) 70A09 silencing	26	yes				b vs c: 51
1.9b	a) parental control 1	26	yes	paired t-test	≤ 0.5mm vs 1 > 0.5mm = 0,0003; t= -4,16	50	
	b) parental control 2	27	yes		≤ 0.5mm vs 1 > 0.5mm = 0,0006; t= -3,93	52	
	c) 70A09 silencing	26	yes		≤ 0.5mm vs 1 > 0.5mm = 0,0004, t= -4,07	50	
1.9c	a) parental control 1	26	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,0032; a vs c = 0,0094; b vs c = 1,0000. H = 12,96	2	a vs b: 51
	b) parental control 2	27	no				a vs c: 50
	c) 70A09 silencing	26	no				b vs c: 51
1.9d	a) parental control 1	26	no	Wilcoxon signed rank test	≤ 0.5mm vs 1 > 0.5mm = 1,33E-05; w = 4,0	50	
	b) parental control 2	27	no		≤ 0.5mm vs 1 > 0.5mm = 1,70E-05; w = 10,0	52	
	c) 70A09 silencing	26	no		≤ 0.5mm vs 1 > 0.5mm = 3,29E-05, w = 12,0	50	
1.9e	a) parental control 1	26	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 1,0000; b vs c = 0,8879. H = 1,34	2	a vs b: 51
	b) parental control 2	27	no				a vs c: 50
	c) 70A09 silencing	26	no				b vs c: 51
1.9f	a) parental control 1	26	no	Wilcoxon signed rank test	≤ 0.5mm vs 1 > 0.5mm = 0,4990; w = 10,0	50	
	b) parental control 2	27	no		≤ 0.5mm vs 1 > 0.5mm = 0,1614; w = 8,0	52	
	c) 70A09 silencing	26	no		≤ 0.5mm vs 1 > 0.5mm = 0,8590; w = 21,0	50	
1.9g	a) intact	38	yes	t- test	a vs b = 3,60E-05; t= -4,41	70	
	b) wingless	34	yes				
1.9h	a) intact	36	no	Friedman test with post hoc Dunn's test	before vs ON = 0,1198; before vs OFF = 0,0202; ON vs OFF = 6,00E-06. Q = 27,72	2	70
	b) wingless	28	no		before vs ON = 1,0000; before vs OFF = 0,0720; ON vs OFF = 0,5228. Q = 10,50	2	54
1.9i	a) intact	38	no	Mann-Whitney U test	a vs b = 2,39E-05; U = 285,00	70	
	b) wingless	34	no				
1.9j	a) intact	34	no	Friedman test with post hoc Dunn's test	before vs ON = 0,2958; before vs OFF = 0,0428; ON vs OFF = 0,0001. Q = 21,94	2	66
	b) wingless	29	no		before vs ON = 0,5396; before vs OFF = 0,0064; ON vs OFF = 0,2503. Q = 10,83	2	56
1.9k	a) intact	38	no	Mann-Whitney U test	a vs b = 0,0022; U = 402,50	70	
	b) wingless	34	no				
1.9l	a) intact	32	no	Friedman test with post hoc Dunn's test	before vs ON = 0,0271; before vs OFF = 0,6068; ON vs OFF = 0,5444. Q = 4,61	2	62
	b) wingless	36	no		before vs ON = 1,0000; before vs OFF = 1,0000; ON vs OFF = 1,0000. Q = 1,46	2	70
1.10	a) intact	47	no	Mann- Whitney U test	a vs b = 0,0680; U = 764,50	85	
	b) wingless	40	no				

Table A6. Statistical details related to Figures 1.11 and 1.12 (Section *1.3.6*)

Figure	groups	n	normally distributed	statistical test	p value	dfs	
1.11b	a) parental control 1	18	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 0,0024; b vs c = 0,0014. H = 17,84	2	a vs b: 24
	b) parental control 2	8	no				a vs c: 41
	c) 70A09 activation	25	no				b vs c: 31
1.11c	a) 70A09 activation	22	no	Wilcoxon rank-sum test	a vs b = 0,3359; w = -0,96	42	
	b) generated dataset*	22	no				
1.11d	a) parental control 1	30	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,1726; a vs c = 1,0000; b vs c = 0,0571. H = 6,60	2	a vs b: 57
	b) parental control 2	29	no				a vs c: 59
	c) 70A09 activation	31	no				b vs c: 58
1.12	a) baseline, parental control 1	30	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,2853; a vs c = 0,9287; b vs c = 0,0215. H = 7,35	2	a vs b: 57
	b) baseline, parental control 2	29	no				a vs c: 59
	c) baseline, 70A09 activation	31	no				b vs c: 58
	a) activation, parental control 1	19	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,3490; a vs c = 1,0000; b vs c = 0,3119. H = 3,08	2	a vs b: 28
	b) activation, parental control 2	11	no				a vs c: 41
	c) activation, 70A09 activation	24	no				b vs c: 33
	a) light OFF, parental control 1	12	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 0,1566; b vs c = 0,8825. H = 4,24	2	a vs b: 13
	b) light OFF, parental control 2	3	no				a vs c: 34
	c) light OFF, 70A09 activation	24	no				b vs c: 25

* The generated dataset is a dataset of random values with median around zero and variance equivalent to the experimental dataset

CHAPTER II

70A09-*doublesex* neurons modulate receptivity and immature virgin behaviours in response to courtship

2.1. Summary

Communication between mating partners is decisive for a proper mate's choice. In *Drosophila melanogaster*, male cues contribute to the female's arousal and trigger neurons that modulate female acceptance behaviours, such as the vaginal plate opening, which are required for a successful copulation to occur. *Doublesex* neurons expressed in the female brain play an important role in the modulation of female receptivity, although the mechanisms by which they do so are poorly understood. Here we show that a subset of brain *dsx* neurons, within 70A09 expression, are required for females to display high levels of receptivity and reduced levels of rejection behaviours. First, we observed that silencing *70A09∩dsx* neurons, namely pC1a, pC1b and vpoDN, induces females to display unreceptive-like behaviours characteristic of very young virgins and, that activation of these neurons induces female flies to open the vaginal plates. Second, we show that silencing pC1a alone seems to be sufficient to drastically decrease female receptivity, although it has no effect on female sexual behaviours displayed in response to a courting male. Our results suggest that distinct pC1 neurons regulate female behaviours and receptivity differently.

2.2. Introduction

Mating is very important for species survival. For that, individuals display a set of innate behaviours which allow animals to interact and assess their qualities before commit to reproduction. In *Drosophila melanogaster*, as in many animal species, the male courts and the female decides whether or not to mate (Pycraft, 1914). Therefore, female's decision represents a key factor for sexual selection and species evolution.

Male courtship is composed by a series of distinct and stereotyped motor programs including orienting towards the female, chasing, vibrating the wings and attempting copulation (Bastock and Manning, 1955; Hall, 1994). When courted, a mature receptive virgin female will walk away until she slows down and opens the vaginal plates to allow copulation (Connolly and Cook, 1973; Tompkins *et al.*, 1982; Mezzera *et al.*, 2020; K. Wang *et al.*, 2020). She also exhibits behaviours that may be interpreted as rejection responses such as wing flicking, ovipositor extrusion, fending, decamping and kicking (Spieth, 1952; Connolly and Cook, 1973; Tompkins *et al.*, 1982; Villella and Hall, 2008). Although performed at different levels, rejection behaviours are displayed by both receptive and unreceptive females (Connolly and Cook, 1973; Lasbleiz, Ferveur and Everaerts, 2006; Dukas and Scott, 2015) and constitute the means by which the female communicates with the male. Thus, receptive females are thought to temporarily reject the courting male to collect quantitative and qualitative information about him (Bastock and Manning, 1955; Villella and Hall, 2008; Ferveur, 2010).

Once copulation has occurred, female internal state undergoes a series of changes that lead female to become temporarily unreceptive, increase the egg laying and adapt her nutritional choices (Manning, 1967; Kubli, 2003; Ribeiro and Dickson, 2010; Walker, Corrales-Carvajal and Ribeiro, 2015). These changes are triggered by the male sex-peptide, which is transferred to the female during copulation (Aigaki *et al.*, 1991; Chapman *et al.*, 2003; Liu and Kubli, 2003), and detected by sex-peptide sensory neurons (SPSNs) in the female reproductive tract (Yapici *et al.*, 2008; Häsemeyer *et al.*, 2009; Yang *et al.*, 2009). The postsynaptic partners of SPSNs have

been identified in a set of abdominal ganglion ascending neurons (SAG) that expresses the sex-determination gene doublesex (*dsx*). Together, SPSNs and SAGs define a reproductive organ-brain connection in female sexual receptivity (Feng *et al.*, 2014). Recent works from Dickson's lab went further in the characterisation of this circuit (F. Wang, Wang, Forknall, Patrick, *et al.*, 2020; K. Wang *et al.*, 2020). They have shown that SAG neurons send input to female-specific pC1 brain neurons and that pC1 activity supresses female egg laying (F. Wang, Wang, Forknall, Patrick, *et al.*, 2020). Additionally, pC1 directly inputs onto a pair of female-specific descending neurons (vpoDN) that triggers female's vaginal plates opening (K. Wang *et al.*, 2020), a behaviour indispensable for the flies to accept a courting male. Moreover, Zhou *et al.* (Zhou *et al.*, 2014) demonstrated that pC1 neurons respond to cVA and courtship song, promoting female receptivity. The female *dsx*-expressing pC1 cluster (Lee, Hall and Park, 2002; Rideout *et al.*, 2010) is composed by five morphologically distinct pC1 cells, named as pC1a-pC1e (F. Wang, Wang, Forknall, Patrick, *et al.*, 2020). Besides the role in female sexual behaviours, some studies revealed that the activation of female pC1 subsets drives male-specific behaviours (Rezával *et al.*, 2016; Wu, Bidaye and Mahringer, 2019) and female-female aggression (Palavicino-Maggio *et al.*, 2019; Schretter *et al.*, 2020). Taken together, the studies suggest that pC1 cluster could be not only an integration site for multiple courtship stimuli, but also a central piece in the network that controls both mated and virgin female behaviours. Still, the neural basis of virgin female (*i.e.*, premating) sexual behaviours remains poorly characterised.

We have already shown that *70A09-dsx+* neurons are involved in female receptivity and that they do not modulate any aspect of female locomotion during courtship. Here we performed a detail analysis and characterisation of female sexual behaviours displayed in response to a courting male. Specifically, when silencing the *70A09-dsx+* neurons, sexually mature flies display immature-like rejection behaviours when courted, although mature virgin rejection behaviours are not affected. Neuronal activation of *70A09-dsx+* females induces vaginal plate opening. However, silencing these neurons does not reduce the display of this behaviour by mature virgin females.

We sought to restrict 70A09 expression to the pC1 cells expressed by the intersection with *dsx*. We observed the expression of the pC1a and a drastic reduction in female receptivity upon silencing, showing that the activity in pC1a alone is necessary for receptivity. We also observed that silenced females behave similar to controls, both for rejection and acceptance behaviours, suggesting that pC1b and/or vpoDN are the ones modulating immature virgin female's behaviours displayed by *70A09-dsx+* silenced females during male courtship.

This work raises new hypothesis for further investigation on pC1 neurons. It will possibly help to identify new roles attributed to these female-specific brain neurons and to understand the mechanisms by which they modulate female receptivity.

Author contributions

All experiments were performed and analysed by Eliane Arez.

2.3. Results

2.3.1. *70A09-dsx+* brain neurons comprise aDN, pC1a, pC1b and vpoDN

In order to find neurons, within 70A09 expression (*Chapter I*), involved in female receptivity behaviour, we conducted several intersectional approaches (Figure 1.4). By intersecting *70A09*-expressing line with a *dsx*-expressing line, we found that silencing *70A09-dsx+* (*70A09∩dsx*) neurons reduced female receptivity (Figure 1.4g). Immunostaining of the *70A09∩dsx* brain neurons (Figure 2.1) showed the labelling of the aDN neurons in the anterior part of the brain. In the posterior part, we observed a subset of the female pC1 cluster (Lee, Hall and Park, 2002) and the female-specific PMN2 neurons (Kimura, Sato, Koganezawa, *et al.*, 2015).

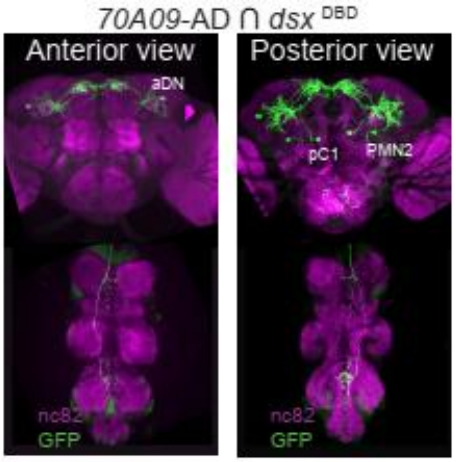


Figure 2.1. *70A09-dsx+* brain neurons expression. Anterior and posterior views of female brain showing 70A09-*doublesex* positive neurons obtained from the intersection of *70A09*-GAL4-AD with *dsx*$^{GAL4\text{-}DBD}$. Neurons were visualised with anti-GFP (green) and the tissue counterstained with the synaptic marker nc82 (magenta).

The variable expression observed in *70A09∩dsx* female brains led us to perform a quantitative anatomical analysis (Table 2). We noted that the aDN neurons appear in both hemispheres of all brain samples, whereas PMN2 neurons appear in five brains whose presence varies between one or two hemispheres. The pC1 cells appear in all brain samples varying the number of pC1 cells being labelled and the hemispheres in which they appear. All six brains presented at least one pC1 cell labelled in both hemispheres, while two pC1 cells appear at low frequencies and rarely in both brain hemispheres.

Table 2. *70A09-dsx+* neurons quantification and localization. Number of brain samples and number of hemispheres in which each cell is expressed.

Cell type	One hemisphere	Both hemispheres
aDN	-	6/6
One pC1 cell	-	6/6
Two pC1 cells	3/6	1/6
PMN2	3/6	2/6

To verify the identity of each *70A09∩dsx* neuron and to address, specifically, which pC1 cell types are labelled in this intersection, we performed the *MultiColor FlpOut* technique (MCFO) (Nern, Pfeiffer and Rubin, 2015). This technique allowed us to stochastically label a small number of cells in different colours, in order to analyse the neurons individually. For this analysis, a heat-shock of 25 minutes was applied prior to flies' dissection. MCFO analysis (Figure 2.2) allowed us to validate the identification of brain aDN and PMN2 neurons. By comparing individual *70A09*-pC1 cells anatomy with each of the female pC1 cells (F. Wang, Wang, Forknall, Patrick, *et al.*, 2020), we concluded that 70A09-*dsx* intersection labels both pC1a and pC1b neurons.

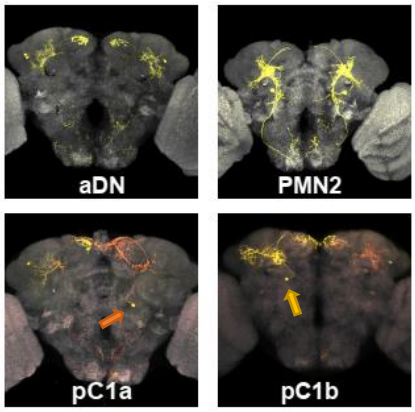


Figure 2.2. *70A09∩dsx* cell types. Cell types obtained by applying the MultiColor Flp-Out (MCFO) technique on females expressing *70A09*-AD and *dsx*^{DBD} transgenes. Neurons were visualised with anti-FLAG (yellow), anti-V5 (orange) and the tissue counterstained with the synaptic marker nc82 (grey).

The aDN neurons are present in both male and female *Drosophila* (Rideout *et al.*, 2010), being dimorphic in neurite morphology and regulating different behavioural outputs (Nojima *et al.*, 2021). In females, aDN neurons connect to olfactory and oviposition-related neurons modulating oviposition site selection, but not female's copulation rates (Nojima *et al.*, 2021). Using a line (*VT042851*-AD) (Tirian and Dickson, 2017) that labels only the aDN neurons in the brain when intersected with a *dsx*-expressing line (Figure 2.3), we confirmed that silencing these neurons does not affect virgin female receptivity (19 silenced females out of 21 copulated: 90.5%).

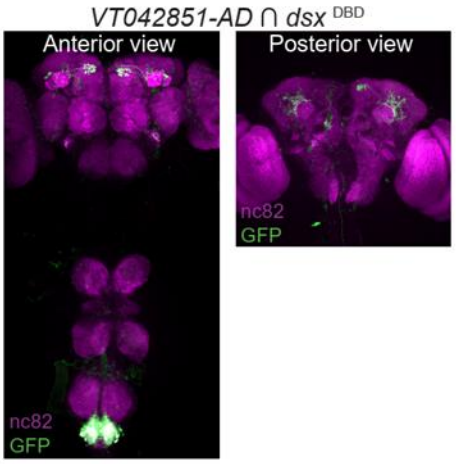


Figure 2.3. aDN neurons expression. Anterior and posterior views of female brain showing aDN neurons obtained from the intersection of *VT042851*-GAL4-AD with *dsx*$^{GAL4\text{-}DBD}$. Neurons were visualised with anti-GFP (green) and the tissue counterstained with the synaptic marker nc82 (magenta).

PMN2 neuron was shown to modulate ovipositor extension in the context of oviposition (Kimura, Sato, Koganezawa, *et al.*, 2015). However, a recent work described a brain descending neuron (vpoDN), anatomically similar to PMN2, whose activity is necessary for virgin females to open the vaginal plates and accept the male (K. Wang *et al.*, 2020). If the PMN2 was in fact the vpoDN, we would expect that female flies performed vaginal plate opening upon activation. So, to check if the neuron labelled in the 70A09-*dsx* intersection is the PMN2 or the vpoDN, we performed an optogenetic activation of *70A09∩dsx* virgin females. For this, we used a different setup (Figure 2.4a) in which we recorded two flies at the same time during 4 minutes. One minute baseline was applied for the flies to adapt to the arena, followed by six optogenetic stimuli separated by an interval of 20 seconds (Figure 2.4b). We observed that upon neuronal activation, manipulated females exhibited

vaginal plate opening (arrow in Figure2.4c left and 2.4d) while this was not observed for virgin control females (Figure 2.4c right and 2.4d), which confirms that *70A09∩dsx* labels the vpoDN. Considering this, and that the silencing of vpoDN abolishes female copulation (K. Wang *et al.*, 2020), we would expect a higher reduction in copulation rate of silenced *70A09∩dsx* virgin females (Figure 1.4g, ≈50%). Thus, we presume that vpoDN activity in one of the hemispheres is sufficient to induce females to open the vaginal plates.

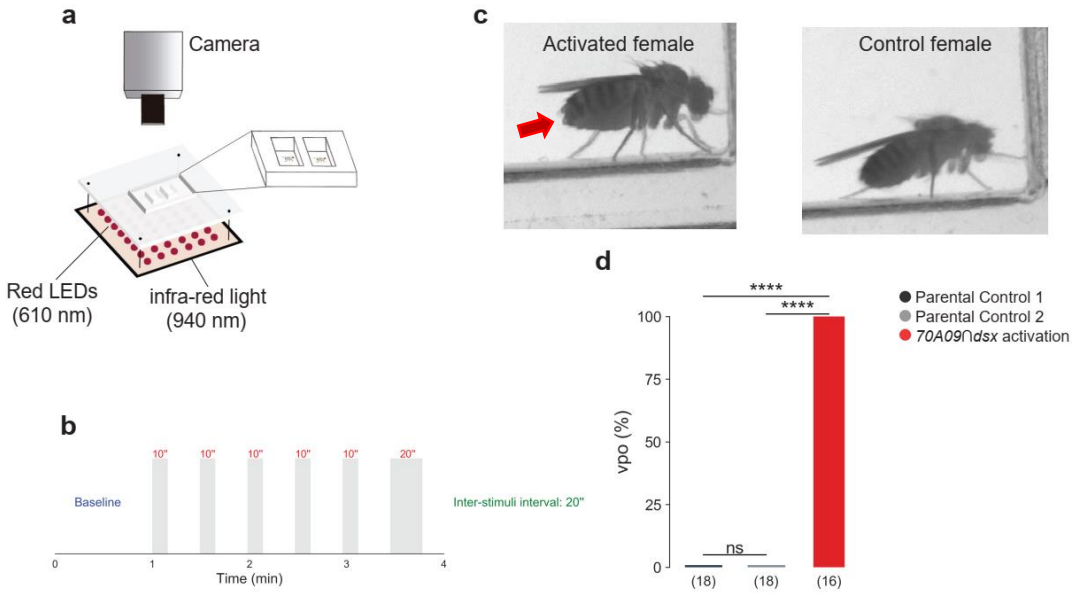


Figure 2.4. *70A09∩dsx* activation leads females to open the vaginal plates. (a) Schematic representation of the behavioural setup to test for female vaginal plate opening. **(b)** Activation protocol applied: 1 minute baseline and 6 optogenetic activation stimuli separated by a 20 seconds' interval. **(c)** 70A09-*dsx*+ female performing vaginal plate opening (arrow, left) and control female (right) upon neuronal optogenetic activation. **(d)** Percentage of females that open vaginal plates while activated. Genotypes: *w-/UAS>STOP>kir2.1*; *otd-nls*:FLPo/+; + (Parental Control 1), *w-; 70A09*-GAL4-AD/+; $dsx^{GAL4\text{-}DBD}$/+ (Parental Control 2) and *w-/UAS>STOP>kir2.1*; *70A09*-GAL4-AD/*otd-nls*:FLPo; $dsx^{GAL4\text{-}DBD}$/+ (*70A09∩dsx* silencing). Statistical analysis was performed with Fisher's exact test: ns = not significant, ****p<0.0001. n values are shown in parentheses.

Altogether, these observations corroborate that the aDN neurons are not involved in female receptivity and suggest that *70A09-dsx+* pC1 and/or vpoDN may be the ones modulating female receptivity.

2.3.2. Silenced *70A09-dsx+* females display immature-like behaviours

We have already shown that silenced *70A09∩dsx* virgin females do not increase their speed in response to a courting male (Figure 1.6c-h of *Chapter I*). Thus, we asked whether silencing *70A09∩dsx* brain neurons lead females to display other rejection behaviours, as an expression of the decreased receptivity. To answer this, we characterise the behaviour of *70A09-dsx+*-silenced females during courtship, using a setup that allows flies' tracking (Figure 1.5a). We analysed flies' behaviours from the start of courtship up to 5 minutes or until copulation. Again, we tested the female receptivity to validate the use of the setup. We observed a decrease in receptivity (Figure 2.5a) at the same degree as was observed when a different setup was used (Figure 1.4g). The reduction in copulation is due to females' reduced receptivity since both silenced and control females elicited similar male courtship levels (Figure 2.5b).

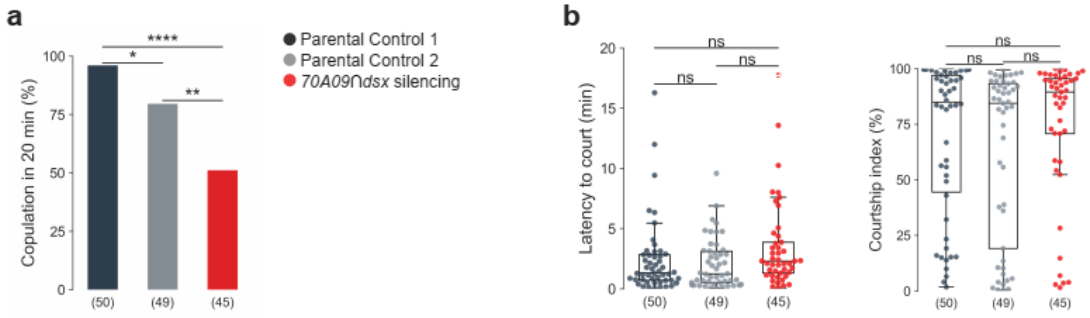


Figure 2.5. *70A09∩dsx* silenced females present reduced copulation but elicited similar courtship levels when compared to controls. (a) Copulation rate of silenced and control females. Genotypes: *w-/UAS>STOP>kir2.1*; *otd-nls*:FLPo/+; + (Parental Control 1), *w-; 70A09*-GAL4-AD/+; *dsx*$^{GAL4\text{-}DBD}$/+ (Parental Control 2) and *w-/UAS>STOP>kir2.1*; *70A09*-GAL4-AD/*otd-nls*:FLPo; *dsx*$^{GAL4\text{-}DBD}$/+ (*70A09∩dsx* silencing). **(b)** Male latency to court (left) and courtship index (right). Statistical analysis was performed with Fisher's exact test **(a)** and Kruskal-Wallis test, followed by post hoc pairwise Dunn's test with Bonferroni correction **(b)**: ns = not significant, **p*<0.05, ***p*<0.01, *****p*<0.0001. n values are shown in parentheses.

To check if these neurons modulate females' sexual behaviours, we observed and quantified different rejection behaviours displayed during male courtship. We chose to analyse the curling usually displayed only by unreceptive immature female flies, wing flicking and kicking is performed by both immature and mature virgin females (Spieth, 1952; Connolly and Cook, 1973), and ovipositor extrusion displayed mainly

by mated flies to block copulation (F. Wang, Wang, Forknall, Parekh, *et al.*, 2020; Mezzera *et al.*, 2020). Since *70A09∩dsx* neurons comprise the vpoDN, we also quantified the vaginal plate opening, displayed by mature virgin females as a mean to allow copulation (Connolly and Cook, 1973; Tompkins *et al.*, 1982; K. Wang *et al.*, 2020).

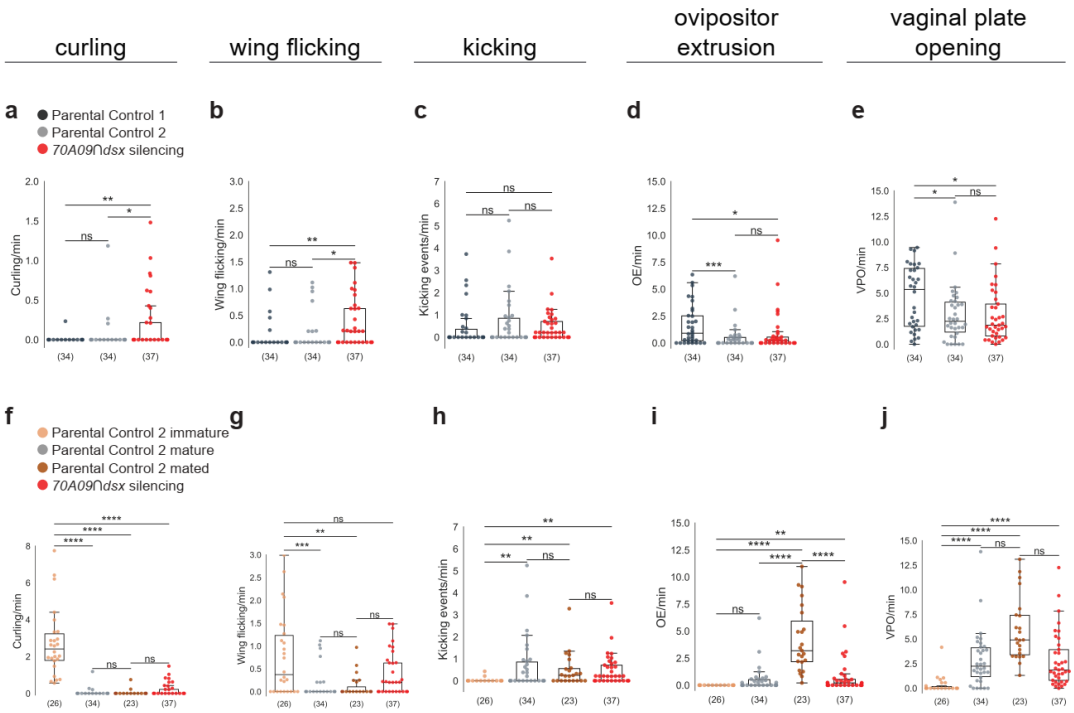


Figure 2.6. Silencing *70A09∩dsx* neurons leads females to display immature-like behaviours. Curling, wing flicking, kicking and ovipositor extrusion bouts per minute of courtship, displayed by **(a-e)** virgin silenced and parental controls females and, **(f-j)** virgin silenced females and parental control 2 females at different stages of fly maturity and mating status. Genotypes: *w-/UAS>STOP>kir2.1*; *otd-nls*:FLPo/+; + (Parental Control 1), *w-; 70A09*-GAL4-AD/+; $dsx^{GAL4\text{-}DBD}$/+ (Parental Control 2) and *w-/UAS>STOP>kir2.1*; *70A09*-GAL4-AD/*otd-nls*:FLPo; $dsx^{GAL4\text{-}DBD}$/+ (*70A09∩dsx* silencing). Statistical analysis was performed with Kruskal-Wallis test, followed by post hoc pairwise Dunn's test with Bonferroni correction: ns = not significant, **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. n values are shown in parentheses.

We observed that silenced *70A09∩dsx* females perform curling and wing flicking (Figure 2.4a and 2.4b, respectively) at higher levels when compared to virgin background controls. On the other hand, kicking and ovipositor extrusion (Figure 2.6c and 2.6d, respectively), were displayed at similar levels as virgin control females. Interestingly, silenced females perform vaginal plate opening at the same level as

controls (Figure 2.6e). This may be caused by the fact that vpoDN is not consistently expressed in both brain hemispheres (PMN2 in Table 2), which suggests that neuronal activity in a single vpoDN is sufficient to induce the opening of the vaginal plates by silenced *70A09∩dsx* females. Additionally, both vpoDNs must be silenced to reduce the levels of VPO by virgin females The results show that silencing *70A09∩dsx* brain neurons leads the female to display an immature-specific rejection behaviour, while mature behaviours are not affected. We thought it was important to quantify the behaviours displayed by females at different stages of sexual maturity and mating status, in the same setup and with a control genetic background, to directly compare their behaviour to silenced *70A09∩dsx* females. Considering that both parental control virgin females behave similarly, we chose to test only parental control 2 females. Immature females were 6 to 8 hours-old, mature females were 4 to 7 days-old and mated females were tested 24-hours post mating. We confirmed that immature virgin females perform more curling and wing flicking when compared to both mature virgin and mated females (Figure 2.6f and 2.6g, respectively), while kicking, OE and VPO were hardly observed in these females (Figure 2.6h and 2.6i, respectively). When *70A09∩dsx* silenced females were compared with immature virgin females, we observed that the curling is displayed at lower levels than immature (Figure 2.6f) whereas wing flicking is displayed at levels similar to immature females (Figure 2.6g). As predicted, mated females performed higher levels of OE when compared to any of the other conditions (Figure 2.6i). Surprisingly, we found no differences between mated and both control mature and silenced virgin females when the VPO was compared (Figure 2.6j), contrary to what was observed by Wang and her colleagues (F. Wang, Wang, Forknall, Parekh, *et al.*, 2020). In mated females VPO is observed at the edges of the OE, suggesting that it is a transition between absence and presence of OE, as had been reported previously (Mezzera *et al.*, 2020). Other behaviours are also similar to mature virgins.

These results suggest that *70A09∩dsx* brain neurons' activity suppresses immature-like rejections by mature virgin females.

2.3.3. Silencing *70A09-VT2064+* neurons drastically reduces female receptivity

In order to check if an individual pC1 cell activity modulates female receptivity differently than the activity of two or more pC1 cells and to separate the effect of the pC1 from the vpoDN effect, we attempted to restrict 70A09 neuronal expression to pC1a and/or pC1b. To do that, we intersected the *70A09*-AD line with a pC1-expressing line, the *VT002064*-DBD line (Tirian and Dickson, 2017), hereby called as *70A09∩VT2064*. Despite only one pC1 cell is expressed (Figure 2.7a, yellow arrows in 2.7b), this intersectional approach did not restrict *70A09* expression as we expected, with innervations into the AMMC (green arrow, Figure 2.7a), a region involved in the auditory processing (Kamikouchi, Shimada and Ito, 2006; Kamikouchi *et al.*, 2009), together with other unspecific neurons.

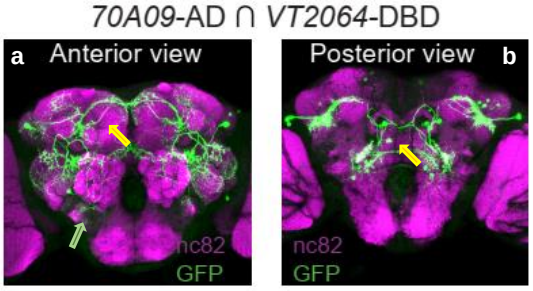


Figure 2.7. *70A09-VT2064+* brain neurons expression. Anterior and posterior views of female brain showing 70A09-*VT2064* positive neurons obtained from the intersection of *70A09*-GAL4-AD with *VT002064*-GAL4-DBD. **(a)** yellow arrow: pC1 projection; green arrow: AMMC innervation. **(b)** yellow arrow: pC1 cell. Neurons were visualised with anti-GFP (green) and the tissue counterstained with the synaptic marker nc82 (magenta).

In order to analyse in detail the anatomy of *70A09-VT2064* intersection, we applied the MCFO technique (Nern et al. 2015) to individually analyse each neuron. We observed the expression of pC1a (Figure 2.8a, yellow arrows in Figure 2.8b) and a projection that seems downstream the AMMC (green arrows, Figure 2.8b). Although this intersection shows expression in brain regions known to be connected with *Drosophila* auditory circuit (Clemens *et al.*, 2015; Matsuo *et al.*, 2016), none of the neurons labelled seem to be part of the sexual circuitry in *Drosophila* (Auer and Benton, 2016), with exception of the pC1a. Additionally, these neurons do not express either *fruitless* (Figure 1.4f in *Chapter I*) or *doublesex* (Figure 2.1).

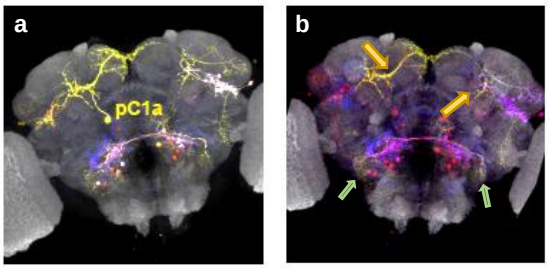


Figure 2.8. *70A09∩VT2064* cell types. Cell types obtained by applying the MultiColor Flp-Out (MCFO) technique on females expressing *70A09*-AD and *VT002064*-DBD transgenes. **(b)** yellow arrows: pC1a projections; green arrows: AMMC innervation. Neurons were visualised with anti-HA (magenta), anti-FLAG (yellow), anti-V5 (red) and the tissue counterstained with the synaptic marker nc82 (grey

Next, we checked if the silencing of *70A09∩VT2064* affects virgin female receptivity. We observed that *70A09∩VT2064* silenced females drastically decrease their receptivity (Figure 2.9a) and that this decrease is not due to low female attractiveness, since males court silenced females at the same levels as controls (Figure 2.9b).

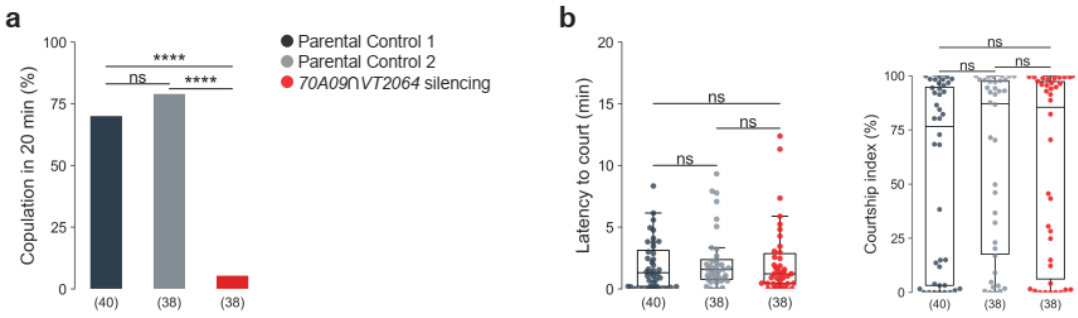


Figure 2.9. Silencing *70A09∩VT2064* reduces virgin female receptivity. (a) Copulation rate of silenced and control females. Genotypes: *w-/UAS>STOP>kir2.1*; *otd-nls*:FLPo/+; + (Parental Control 1), *w-; 70A09*-GAL4-AD/+; *VT002064*-GAL4-DBD/+ (Parental Control 2) and *w-/UAS>STOP>kir2.1*; *70A09*-GAL4-AD/*otd-nls*:FLPo; *VT002064*-GAL4-DBD/+ (*70A09∩VT2064* silencing). **(b)** Male latency to court (left) and courtship index (right). Statistical analysis was performed with Fisher's exact test **(a)** and Kruskal-Wallis test, followed by post hoc pairwise Dunn's test with Bonferroni correction **(b)**: ns = not significant, ****p<0.0001. n values are shown in parentheses.

Taken together, our results suggest that the pC1a neuron is the more likely to modulate the female receptivity phenotype observed upon silencing of *70A09∩VT2064* neurons and, that its activity is necessary for females to display high levels of receptivity.

2.3.4. *70A09-VT2064+* neurons activity does not modulate female's behaviours in response to male courtship

We asked whether *70A09-VT2064* neurons modulate female-specific behaviours to male courtship, in addition to receptivity. To answer this question, we manually annotated females' behaviours similarly to what was done for *70A09∩dsx* silenced females (*Section 2.3.2*).

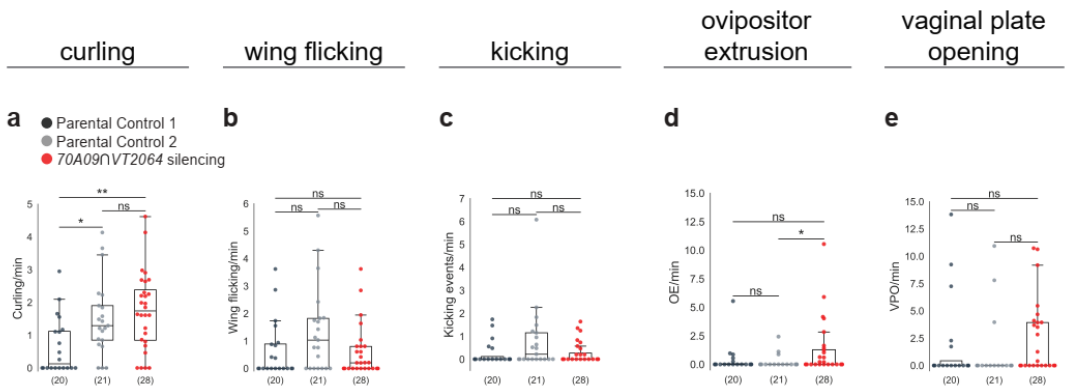


Figure 2.10. *70A09∩VT2064* silenced females do not behave differently than controls. (a) Curling, **(b)** wing flicking, **(c)** kicking, **(d)** ovipositor extrusion and **(e)** vaginal plate opening bouts per minute of courtship, displayed by virgin silenced and parental controls females. Genotypes: *w-/UAS>STOP>kir2.1*; *otd-nls*:FLPo/+; + (Parental Control 1), *w-; 70A09*-GAL4-AD/+; *VT002064*-GAL4-DBD/+ (Parental Control 2) and *w-/UAS>STOP>kir2.1*; *70A09*-GAL4-AD/*otd-nls*:FLPo; *VT002064*-GAL4-DBD/+ (*70A09∩VT2064* silencing). Statistical analysis was performed with Kruskal-Wallis test, followed by post hoc pairwise Dunn's test with Bonferroni correction: ns = not significant, **p*<0.05, ***p*<0.01. n values are shown in parentheses.

When we analysed the female's rejection behaviours (*e.g.,* curling, wing flicking, kicking or ovipositor extrusion), we observed that *70A09∩VT2064* silenced females perform wing flicking and kicking (Figure 2.10b and 2.10c, respectively) at similar levels as controls, whereas curling and ovipositor extrusion (Figure 2.10a and 2.10d, respectively) are performed at higher levels by silenced females when compared to parental control 1 and 2, respectively. In regard to vaginal plate opening (Figure 2.10e), an acceptance behaviour that allow male copulation, we found no difference between silenced and control females. So, similar levels of rejection and acceptance behaviours were observed when silenced and control females were compared. Taking these observations into account, we would expect that silenced females

copulate at similar levels as controls (Figure 2.9a). Thus, we wondered how silenced females avoid male copulation. One hypothesis is that silenced females are subjected to less copulation attempts by the male. To check this, we quantified male's copulation attempts per minute of courtship.

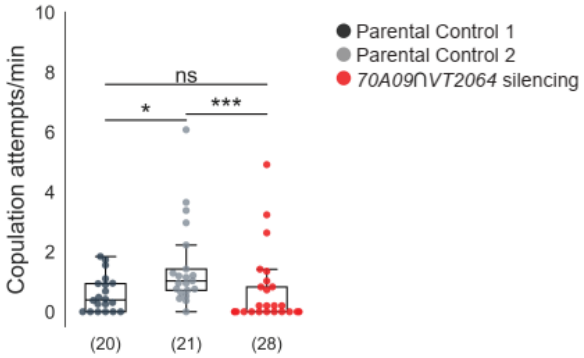


Figure 2.11. Male copulation attempts towards *70A09-VT2064*+ silenced and control females. Copulation attempts per courtship minute. Genotypes: *w-/UAS>STOP>kir2.1*; *otd-nls*:FLPo/+; + (Parental Control 1), *w-; 70A09*-GAL4-AD/+; *VT002064*-GAL4-DBD/+ (Parental Control 2) and *w-/UAS>STOP>kir2.1*; *70A09*-GAL4-AD/*otd-nls*:FLPo; *VT002064*-GAL4-DBD/+ (*70A09∩VT2064* silencing). Statistical analysis was performed with Kruskal-Wallis test, followed by post hoc pairwise Dunn's test with Bonferroni correction: ns = not significant, *p<0.05, ***p<0.001. n values are shown in parentheses.

We observed that silenced females are subjected to copulation attempts at similar levels as parental control 1 and at lower levels when compared to parental control 2 (Figure 2.11). These findings may explain the differences in copulation rates observed between silenced females and parental control 2 (Figure 2.9a), but do not explain these same differences when silenced females are compared with parental control 1, *i.e.*, similar copulation attempt levels but different copulation rates. Because these results (Figures 2.10 and 2.11) do not fully explain the receptivity phenotype observed for *70A09∩VT2064* silenced females, we decided to check the female's locomotor activity in response to a courting male. This aspect is a good indicator of the female's willingness to copulate since unreceptive females do not slow down nor pause as much as receptive females (Connolly and Cook, 1973; von Schilcher, 1976; Tompkins *et al.*, 1982; Crossley, Bennet-Clark and Evert, 1995; Bussell *et al.*, 2014; Coen *et al.*, 2014; Aranha *et al.*, 2017; Ishimoto and Kamikouchi, 2020). Thus, we

checked if the silencing of *70A09∩VT2064* neurons leads females to increase the speed, pause less and/or jump more in response to male courtship. According to our analysis (Figure 2.12), silencing *70A09∩VT2064* neurons do not induce females to behave as unreceptive. Although silenced females jump more than controls during courtship ON moments (Figure 2.12e and f), they walk at similar speed (Figure 2.12a and b) and pause at same levels (Figure 2.12c and d) as virgin control females.

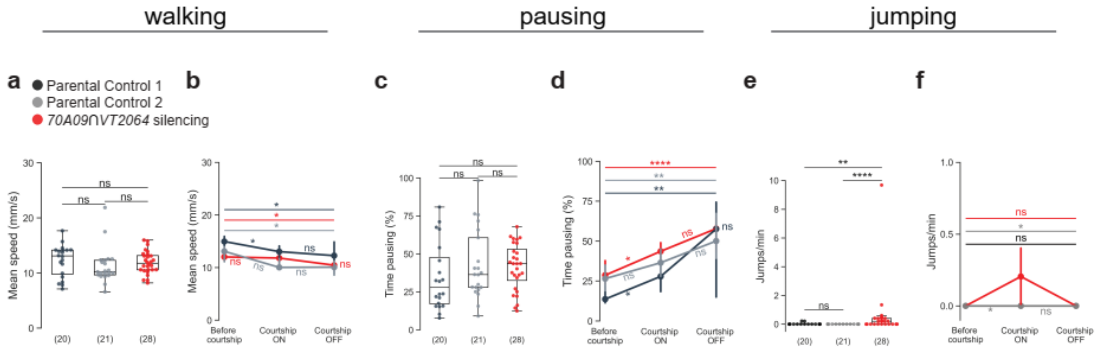


Figure 2.12. Silencing *70A09∩VT2064* neurons do not induce significative changes in virgin females' locomotor activity. (a) Females mean walking speed (4 – 50 mm/s), **(c)** percentage of time females spend pausing and **(e)** number of jumps per minute, during courtship ON periods. **(b)** females mean walking speed (4 – 50 mm/s), **(d)** percentage of time females spend pausing and **(f)** number of jumps per minute, in different moments of courtship dynamics. Genotypes: *w-/UAS>STOP>kir2.1*; *otd-nls*:FLPo/+; + (Parental Control 1), *w-; 70A09*-GAL4-AD/+; *VT002064*-GAL4-DBD/+ (Parental Control 2) and *w-/UAS>STOP>kir2.1*; *70A09*-GAL4-AD/*otd-nls*:FLPo; *VT002064*-GAL4-DBD/+ (*70A09∩VT2064* silencing). Statistical analysis was performed with **(a, c, e)** Kruskal-Wallis test, followed by post hoc pairwise Dunn's test with Bonferroni correction and, **(b, d, f)** repeated measures ANOVA followed by post hoc multiple pairwise paired t-test or Friedman's test followed by post hoc pairwise Dunn's test with Bonferroni correction: ns = not significant, **p*<0.05, ***p*<0.01, *****p*<0.0001. n values are shown in parentheses.

Taken together, these results led us to conclude that *70A09∩VT2064* neurons do not modulate any of the female's sexual behaviours. If we follow our assumption that the pC1a neuron is the more likely to modulate the female receptivity, we can speculate that pC1a activity alone is not necessary for females to display sexual behaviours in response to a courting male, though they may be necessary for females to display high levels of receptivity.

2.4. Discussion

Reproductive decisions have a huge impact on species survival. Thus, accepting a proper mate is one of the most important decisions a female will make during her existence. The communication between courtship partners is crucial for this process. The female assesses the quality of the male from the courtship behaviours that he displays while she gives him information about her receptivity through female-specific sexual behaviours. This type of female-male interaction was shown in a recent study that establishes a correlation between female vaginal plate opening or ovipositor extrusion and male licking (Mezzera *et al.*, 2020). This work also demonstrates that the coexistence of these two behaviours stimulates the male to attempt copulate.

Here we sought to investigate if the brain d*oublesex* neurons modulate female sexual behaviours, in specific, pC1a, pC1b and/or vpoDN. Inactivation of this group of neurons leads female flies to present behavioural hallmarks characteristic of immature virgin females which are not yet receptive. Still, silenced females manifest other rejection and acceptance behaviours similarly to mature receptive females. *70A09∩dsx* manipulated females present levels of vaginal plate opening similar to virgin controls. The unilateral expression of the vpoDN in some of the brains observed suggests that both vpoDNs must be silenced to reduce the levels of vaginal plate opening by virgin females. On the other hand, vpoDN activity in both brain hemispheres, together with the activity of pC1a and pC1b, may be necessary to suppress immature-like behaviours by virgin females. However, Zhou *et al.* showed that in immature virgins pC1 neurons respond to male cues (*e.g.*, cVA and courtship song), similarly to mature virgins, and that pC1 activation does not render immature females receptive (Zhou *et al.*, 2014), which indicates that immature responses to a courting male may be modulated by factors that regulate flies' sexual maturity such as hormones.

The adult *Drosophila* central brain connectome (Clements *et al.*, 2020; Xu *et al.*, 2020) revealed an interconnection between female-specific pC1 neurons. This tool allowed us to search the inputs and outputs within pC1 cluster, we observed that pC1a provides synaptic inputs to all the other pC1s. Curiously, pC1 cluster receives

numerous synaptic inputs from SAG neurons and pC1a is the one receiving the majority of them, followed by pC1c and pC1b (F. Wang, Wang, Forknall, Patrick, *et al.*, 2020). Thus, we speculate that pC1a alone is necessary for females to display high levels of receptivity, although it does not modulate female sexual behaviours, and that pC1b and vpoDN may be the ones modulating immature flies' behaviours. Additionally, activation of subsets of the female pC1 cluster drives male-specific behaviours (Rezával *et al.*, 2016; Wu, Bidaye and Mahringer, 2019) and female-female aggression (Palavicino-Maggio *et al.*, 2019; Schretter *et al.*, 2020), suggesting that pC1 neurons drive distinct behaviours through distinct downstream circuits. This may explain why silencing pC1a and pC1b, or the whole pC1 cluster (Zhou *et al.*, 2014), does not reduce completely female receptivity and even manipulated flies, that show to be receptive to a courting male, display a kind of aggression behaviour upon copulation (see Figure B1 in *Appendix B*, page 112). Recurrent connectivity between pC1 subsets and the vpoDN were also shown (K. Wang *et al.*, 2020), indicating that downstream neurons to pC1 may also contribute to modulate different aspects of female behaviour.

In conclusion, our work highlights the importance of the pC1a in female receptivity, by revealing that activity of these neurons alone is necessary for high copulation rates by virgin females. However, neuronal activity of pC1a is not necessary for females to display appropriate behaviours in response to a courting male, indicating that other neurons, probably downstream, modulate female sexual behaviours. for this specific case, we propose that the activity of pC1b and/or vpoDN is necessary to suppress immature-like behaviours in mature virgin females.

Future work is necessary to clarify (i) how each pC1 cell modulates female receptivity, (ii) what kind of behaviours each one regulates and (iii) how they communicate with each other to generate a proper behavioural output.

2.5. Material and methods

Drosophila stocks

Fly strains and sources are as follows: Canton-S (CS), w^{1118} (Morata and Garcia-Bellido, 1973), *otd-nls*:FLPo (Asahina *et al.*, 2014), UAS>STOP>*Kir2.1* (Yang *et al.*, 2009), UAS>STOP>*CD8-GFP* (Hong *et al.*, 2009), 20xUAS-*CD8::GFP* (Pfeiffer et al. 2010), UAS>STOP>*csChrimson.mVenus* (Klapoetke *et al.*, 2014) (flp-out version provided by Vivek Jayaraman), $dsx^{GAL4\text{-}DBD}$ (Pavlou *et al.*, 2016), *VT002064*-GAL4-DBD and *VT042851*-GAL4-AD (Tirian and Dickson, 2017) and *70A09*-GAL4-AD (generated in the lab as described previously at section 1.5 in *Construction of transgenic lines*). The following fly stock was used for MCFO: pBPhsFlp2::PEST;; pJFRC201-10XUAS>STOP>myr::smGFP-HA,pJFRC240-10XUAS>STOP>myr::smGFP-V5-THS-10XUAS>STOP>myr::smGFP-FLAG (Nern, Pfeiffer and Rubin, 2015).

Immunostaining and microscopy

For standard immunostainings, adult brains and VNCs were dissected in cold phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (PFA) in PBL (PBS and 0.12M Lysine) for 30 minutes at room-temperature (RT), washed three times for 5 minutes in PBT (PBS and 0.5% Triton X-100) and blocked for 15 minutes at RT in 10% Normal Goat Serum (NGS, Sigma) in PBT. Tissues were incubated with the primary antibodies in blocking solution for 72 hours at 4ºC. The following primary antibodies were used: rabbit anti-GFP (1:2000, Molecular probes, cat# A11122), and mouse anti-nc82 (1:10, Developmental Studies Hybridoma Bank). Samples were washed three times for 5 minutes in PBT and incubated in Alexa Fluor secondary antibodies (1:500, Invitrogen) for 72 hours at 4ºC. The following secondary antibodies were used: anti-rabbit IgG conjugated to Alexa 488 and anti-mouse IgG conjugated to Alexa 594. Samples were washed three times for 5 minutes in PBT and mounted in VectaShield medium (Vector Laboratories, Cat# H1000).

To perform the MCFO-technique staining, adult brains were dissected in cold phosphate-buffered saline (PBS), fixed in 2% paraformaldehyde (PFA) in PBL (PBS and 0.12M Lysine) for 55 minutes at room-temperature (RT), washed four times for

10 minutes in PBT (PBS and 0.5% Triton X-100) and blocked for 90 minutes at RT in 10% Normal Goat Serum (NGS, Sigma) in PBT. Tissues were incubated with the primary antibodies in blocking solution for 4 hours at RT and then for 48 hours at 4ºC. The following primary antibodies were used: rat anti-FLAG Tag (1:200, Novus Biologicals, cat# NBP1-06712), rabbit anti-HA Tag (1:300, Werfen, cat# C29F4) and mouse anti-nc82 (1:10, Developmental Studies Hybridoma Bank). Samples were washed five times for 15 minutes in PBT and incubated in Alexa Fluor secondary antibodies (1:500, Invitrogen) in blocking solution for 4 hours at RT and then for 72 hours at 4ºC. The following secondary antibodies were used: anti-rabbit IgG conjugated to Alexa 594, anti-rat IgG conjugated to Alexa 488 and anti-mouse IgG conjugated to Alexa 647. Samples were washed 5 times for 15 minutes in PBT and blocked for 90 minutes at RT in 5% Normal Mouse Serum (NMS, Sigma) in PBT. After NMS removal, samples were incubated with DL550 mouse anti-V5 antibody in 5% NMS in PBT for 4 hours at RT and then for 48 hours at 4ºC. Samples were washed five times for 15 minutes in PBT and mounted in VectaShield medium (Vector Laboratories, Cat# H1000).

Images were acquired on a Zeiss LSM 710 confocal microscope using 20x objective or 25x Immersion objective (Zeiss). After acquisition, colour levels were adjusted using Fiji (Schindelin *et al.*, 2012).

Behavioural experiments

Fly husbandry

Flies were raised in standard cornmeal-agar medium at 25ºC and 70% relative humidity in a 12h:12h dark:light cycle, unless otherwise indicated. For all experiments both female and male flies were collected under CO_2 anaesthesia, soon after eclosion, and raised in isolation in regular food vials. For neuronal silencing, female flies and males were raised at 25ºC from 4 to 8 days.

Unless specified, the flies used in behavioural experiments were 4-8 days old virgin females and males, and were tested in the same conditions as rearing (25°C and 70% humidity).

Female receptivity

To test female receptivity, a single female was gently aspirated and transferred into circular acrylic chambers (small arenas: 16 mm in diameter x 4.5 mm height) and paired with a male. Individual pairs were recorded for 30 minutes using SONY HDR-CX570E, HDR-SR10E, HDR-XR520VE or HDR-PJ620 video cameras (1440 x 1080 pixels; 25 frames per second). A white LED was used as backlight source (Edmund optics, cat# 83-875).

Receptivity with female tracking

To allow the detailed behaviour analysis, a single female was gently aspirated and transferred to a custom-made circular arena with a conical-shaped bottom that avoid flies walking on the walls (detailed arenas: 40 mm in diameter; Simon and Dickinson 2010), allowing to track them as described in Aranha et al. (Aranha *et al.*, 2017). Each female was allowed to habituate to the new environment for about 10 minutes and then paired with a male. Movies were acquired in dim light using an infrared 940 nm LED strip (SOLAROX) mounted on an electric board developed by the Scientific Hardware Platform. Flies were recorded in grayscale (1024 x 1024 pixels, 60 frames per second), with a camera mounted above the arena (PointGrey FL3-U3-32S2M-CS with a 5 mm fixed focal length lens (Edmund Optics)) with a Hoya 49 mm R72 infrared filter, for 20 minutes or until copulation occurred. Bonsai (Lopes *et al.*, 2015) was used for movie acquisition.

Activation experiment

For the activation experiment, female flies were individually collected and allowed to age in cornmeal-agar food containing 0.2 mM all trans-Retinal (Sigma, R2500) and reared in dim light until the experiment.

To allow the analysis of the behaviour, a custom-made rectangular arena (15 x 6 x 3 mm), that allows the recording of two single flies at the same time, was used. Movies were acquired in dim light using an infrared 940 nm LED strip (SOLAROX) mounted

on an electric board developed by the Scientific Hardware Platform. Flies were recorded in grayscale (1328 x 1048 pixels, 60 frames per second), with a camera mounted above the arena (PointGrey FL3-U3-13S2M-CS with a 5 mm fixed focal length lens (Edmund Optics)) with a Hoya 49 mm R72 infrared filter. For light stimulation a high-powered 610 nm LEDs arrays interspersed between the infrared LEDs on the blacklight board was used. The arena was irradiated with a power that varied from 3.63 to 10.65 mW/cm2. Bonsai (Lopes *et al.*, 2015) was used to acquire the movies and trigger that activation stimulus protocol. Females were gently aspirated and transferred to the arenas. They were allowed to habituate for four minutes before start recording and videos were recorded for four minutes. The activation protocol included a baseline of 1 minute, followed by five light stimuli of 10 seconds each and a sixth light stimulus of 20 seconds, interspaced by a 20-second interval.

Data processing

In order to quantify female receptivity, a custom-made software was developed to track the flies and compute the time to copulation, when it occurred. To quantify flies' behaviours, FlyTracker (Caltech) (Eyjolfsdottir *et al.*, 2014) was used to track the two flies and output information concerning their position, velocity, distance to the other fly, among others. A Courtship Classifier developed in the lab using the machine learning-based system JAABA (Kabra *et al.*, 2013) was run to automatically identify courtship bouts. Subsequently, in-house developed software PythonVideoAnnotator (https://biodata.pt/python_video_annotator) was used to visualize courtship events generated by JAABA and manually correct them if necessary. Annotations were done from the beginning of courtship and during 5 minutes or until copulation. PythonVideoAnnotator was also used to manually annotate copulation time, considering the whole duration of the video, and female's behaviours displayed during courtship.

Quantification and statistical analysis

Data analysis was performed using Python 3 scripts for all experiments, except for the copulation rate for small arenas receptivity experiments, for which GraphPad Prism Software version 7.0 was used. All data, except those from flies excluded due to tracking errors, were analysed.

Female receptivity and male behaviour parameters

All quantifications were performed for the first 5 minutes of courtship or until copulation, whichever happened first. Male courtship index was calculated as the ratio between courtship frames and the total number of frames.

Female sexual behaviours and locomotor parameters during male courtship

For the characterisation of female behaviours, sexual behaviours such as curling, wing flicking, kicking, ovipositor extrusion and vaginal plates opening were quantified. The curling is defined by the abdominal downward bending and the ovipositor extrusion consists in pushing the vaginal plates, from the tip of the abdomen, as a tube-like structure. For each behaviour the number of bouts were normalized by the total courtship time in minutes.

For the characterisation of female locomotor activity, mean speed, pausing and jumping were quantified. The three behaviours were separately quantified in three different moments: i) before courtship starts (# frames before courtship initiation), ii) courtship ON (# frames of courtship since courtship initiation) and iii) courtship OFF (# frames of not courtship since courtship initiation). Walking frames were defined as the frames in which female speed was within the range of 4-50 mm/s and the mean walking speed for each fly was calculated by the sum of speed values divided by the number of walking frames. Pausing frames were defined as the frames in which the fly speed was below 4 mm/s, as reported previously17. The pausing percentage was obtained normalizing the number of pausing frames over the total number of frames for each courtship moment. Jumps were defined as instantaneous female speed

above 70 mm/s. We set this value based on the discontinuity in the speed distribution and on the presence of peaks in the raw, un-binned speed data.

Since courtship is a prerequisite, we selected only videos with courtship amount equal or above 30 seconds.

For statistical analysis of all experiments, Fisher's exact test was performed to compare the copulation rate between two different groups. Prior to statistical testing, Levene's test was used to assess variance homogeneity and Shapiro-Wilk test was used to assess normality across all individual experiments. Independent groups were subjected to Kruskal-Wallis test followed by post hoc Dunn's test, since they do not satisfy parametric assumptions. For dependent groups, repeated measures ANOVA followed by post hoc multiple pairwise paired t-test was applied if parametric assumptions were satisfied. If not, Friedman's test followed by post hoc Dunn's test was used. Bonferroni correction to *p*-values was applied when multiple comparisons were performed. The sample size for each condition is indicated in each plot. All the statistical details related to the figures are included in Tables B1-B4 (see *Appendix B* in pages 113-116). The difference in sample size for the same condition in different analysis is due to the different thresholds applied.

2.6. Appendix B

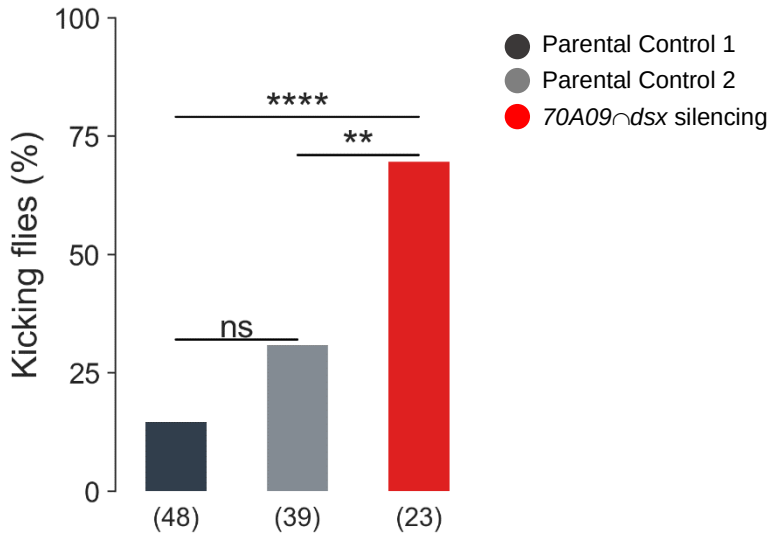


Figure B1. Percentage of flies that present kicking upon copulation. Genotypes: *w-/UAS>STOP>kir2.1*; *otd-nls*:FLPo/+; + (Parental Control 1), *w-; 70A09*-GAL4-AD/+; $dsx^{GAL4\text{-}DBD}$/+ (Parental Control 2) and *w-/UAS>STOP>kir2.1*; *70A09*-GAL4-AD/*otd-nls*:FLPo; $dsx^{GAL4\text{-}DBD}$/+ (*70A09*∩*dsx* silencing). Statistical analysis was performed with Fisher's exact test: ns = not significant (p=0.1160), **p=0.0040, ****p=0.0000. n values are shown in parentheses.

Table B1. Statistical details related to Figure 2.4 (Section *2.3.1*)

Figure	groups	n	normally distributed	statistical test	p value	dfs	
2.4d	a) parental control 1 b) parental control 2 c) 70A09∩dsx activation	18 18 16	NA NA NA	Fisher's exact test	a vs b = 1,0000; a vs c = 0,0000; b vs c = 0,0000	2	

NA: not applicable

Table B2. Statistical details related to Figures 2.5 and 2.6 (Section *2.3.2*)

Figure	groups	n	normally distributed	statistical test	p value	dfs	
2.5a	a) parental control 1 b) parental control 2 c) 70A09∩dsx silencing	50 49 45	NA NA NA	Fisher's exact test	a vs b = 0,0147; a vs c = 0,0000; b vs c = 0,0047	2	
2.5b	a) Courtship latency, parental control 1 b) Courtship latency, parental control 2 c) Courtship latency, 70A09∩dsx silencing	50 49 45	no no no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 0,1231; b vs c = 0,0761. H = 6,05	2	
	a) Courtship index, parental control 1 b) Courtship index, parental control 2 c) Courtship index, 70A09∩dsx silencing	50 49 45	no no no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,4213; a vs c = 1,0000; b vs c = 0,3330. H = 3,17	2	
2.6a	a) parental control 1 b) parental control 2 c) 70A09∩dsx silencing	34 34 37	no no no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 0,0032; b vs c = 0.0306. H = 12,00	2	
2.6b	a) parental control 1 b) parental control 2 c) 70A09∩dsx silencing	34 34 37	no no no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 0,0036; b vs c = 0.0168. H = 12,42	2	
2.6c	a) parental control 1 b) parental control 2 c) 70A09∩dsx silencing	34 34 37	no no no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,9342; a vs c = 0,3336; b vs c = 1,0000. H = 2,59	2	

(Continue next page)

(Table B2 continue)

Figure	groups	n	normally distributed	statistical test	p value	dfs	
2.6d	a) parental control 1 b) parental control 2 c) 70A09∩dsx silencing	34 34 37	no no no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,0026; a vs c = 0,0192; b vs c = 1,0000. H = 12,51	2	
2.6e	a) parental control 1 b) parental control 2 c) 70A09∩dsx silencing	34 34 37	no no no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,0582; a vs c = 0,0381; b vs c =1,0000. H = 7,74	2	
2.6f	a) parental control 2 immature b) parental control 2 mature c) parental control 2 mated d) 70A09∩dsx silencing	26 34 23 37	no no no no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 2,65E-15; a vs c = 8,19E-13; a vs d = 3,57E-11; b vs c = 1,0000; c vs d = 1,0000. H = 82,47	3	
2.6g	a) parental control 2 immature b) parental control 2 mature c) parental control 2 mated d) 70A09∩dsx silencing	26 34 23 37	no no no no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,0003; a vs c = 0,0037; a vs d =0,3893; b vs c = 1,0000; c vs d = 0,3327. H = 20,03	3	
2.6h	a) parental control 2 immature b) parental control 2 mature c) parental control 2 mated d) 70A09∩dsx silencing	26 34 23 37	no no no no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,0095; a vs c = 0,0027; a vs d = 0,0011; b vs c = 1,0000; c vs d = 1,0000. H = 17,82	3	
2.6i	a) parental control 2 immature b) parental control 2 mature c) parental control 2 mated d) 70A09∩dsx silencing	26 34 23 37	yes no no no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,0374; a vs c = 5,61E-14; a vs d = 0,0036; b vs c = 1,48E-07; c vs d = 2,74E-06. H = 62,21	3	
2.6j	a) parental control 2 immature b) parental control 2 mature c) parental control 2 mated d) 70A09∩dsx silencing	26 34 23 37	no no no no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,82E-05; a vs c = 1,36E-12; a vs d =9,14E-06; b vs c = 0,0065; c vs d = 0,0065. H = 55,41	3	

NA: not applicable

Table B3. Statistical details related to Figure 2.8 (Section *2.3.3*)

Figure	groups	n	normally distributed	statistical test	p value	dfs	
2.8a	a) parental control 1	40	NA	Fisher's exact test	a vs b = 0,4412; a vs c = 0,0000; b vs c = 0,0000	2	
	b) parental control 2	38	Na				
	c) 70A09∩VT2064 silencing	38	NA				
2.8b	a) Courtship latency, parental control 1	40	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 1,0000; b vs c = 1,0000. H = 0,80	2	
	b) Courtship latency, parental control 2	38	no				
	c) Courtship latency, 70A09∩VT2064 silencing	38	no				
	a) Courtship index, parental control 1	40	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,7935; a vs c = 1,0000; b vs c = 1,0000. H = 1,29	2	
	b) Courtship index, parental control 2	38	no				
	c) Courtship index, 70A09∩VT2064 silencing	38	no				

NA: not applicable

Table B4. Statistical details related to Figures 2.10, 2.11 and 2.12 (Section *2.3.4*)

Figure	groups	n	normally distributed	statistical test	p value	dfs	
2.10a	a) parental control 1	20	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,0442; a vs c = 0,0021; b vs c = 1,0000. H = 12,01	2	
	b) parental control 2	21	no				
	c) 70A09∩VT2064 silencing	28	no				
2.10b	a) parental control 1	20	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,2179; a vs c = 1,0000; b vs c = 0,1702. H = 4,49	2	
	b) parental control 2	21	no				
	c) 70A09∩VT2064 silencing	28	no				
2.10c	a) parental control 1	20	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,1746; a vs c = 1,0000; b vs c = 0,3395. H = 4,06	2	
	b) parental control 2	21	no				
	c) 70A09∩VT2064 silencing	28	no				
2.10d	a) parental control 1	20	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 0,2298; b vs c = 0,0451. H = 6,58	2	
	b) parental control 2	21	no				
	c) 70A09∩VT2064 silencing	28	no				
2.10e	a) parental control 1	20	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 0,4990; b vs c =0,1050. H = 4,74	2	
	b) parental control 2	21	no				
	c) 70A09∩VT2064 silencing	28	no				
2.11a	a) parental control 1	20	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,0388; a vs c = 0,9401; b vs c = 0,0006. H = 14,16	2	
	b) parental control 2	21	no				
	c) 70A09∩VT2064 silencing	28	no				

(Continue next page)

(Table B4 continue)

Figure	groups	n	normally distributed	statistical test	p value	dfs	
2.12a	a) parental control 1	20	yes	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,1531; a vs c = 1,0000; b vs c = 0,3078. H =4,31	2	
	b) parental control 2	21	no				
	c) 70A09∩VT2064 silencing	28	yes				
2.12b	a) parental control 1	19	yes	rmANOVA with post hoc mpPaired t-test	before vs ON = 0,0356; before vs OFF = 0,0191; ON vs OFF = 1,0000. F = 14,96	2	36
	b) parental control 2	19	no	Friedman test with post hoc Dunn's test	before vs ON = 0,0776; before vs OFF = 0,0192; ON vs OFF = 1,0000. Q = 13,37	2	
	c) 70A09∩VT2064 silencing	28	no		before vs ON = 1,0000; before vs OFF =0,0182; ON vs OFF = 0,1830. Q = 12,07	2	
2.12c	a) parental control 1	20	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 0,5188; a vs c = 0,3326; b vs c = 1,0000. H = 2,89	2	
	b) parental control 2	21	no				
	c) 70A09∩VT2064 silencing	28	yes				
2.12d	a) parental control 1	20	no	Friedman test with post hoc Dunn's test	before vs ON = 0,0168; before vs OFF = 0,0012; ON vs OFF = 1,0000. Q =24,40	2	
	b) parental control 2	21	no		before vs ON = 0,2271; before vs OFF = 0,0045; ON vs OFF = 4869. Q = 10,29	2	
	c) 70A09∩VT2064 silencing	28	yes	rmANOVA with post hoc mpPaired t-test	before vs ON = 0,0137; before vs OFF = 3,20E-05; ON vs OFF = 0,0821. F = 25,01	2	54
2.12e	a) parental control 1	20	no	Kruskal-Wallis test with post hoc Dunn's test	a vs b = 1,0000; a vs c = 0,0010; b vs c = 5,10E-05. H = 22,27	2	
	b) parental control 2	21	yes				
	c) 70A09∩VT2064 silencing	28	no				
2.12f	a) parental control 1	20	no	Friedman test with post hoc Dunn's test	before vs ON = 1,0000; before vs OFF = 1,0000; ON vs OFF = 0,4718. Q = 2,00	2	
	b) parental control 2	21	no		before vs ON = 0,0363; before vs OFF = 0,0363; ON vs OFF = 1,0000. Q = 8,00	2	
	c) 70A09∩VT2064 silencing	28	no		before vs ON = 0,1941; before vs OFF = 1,0000; ON vs OFF = 0,6096. Q = 1,48	2	

rmANOVA: repeated measures ANOVA

mpPaired t-test: multiple pairwise paired t-test

GENERAL DISCUSSION

In this work we presented data that identify *70A09*-GAL4 brain neurons as critically involved in female receptivity and whose activity is necessary to suppress female escape from a courting male. *70A09*-expressing brain neurons that modulate female speed receive information from auditory-processing neurons, since we have shown that silenced females increased their walking speed in a courtship song-dependent manner. Next, we analysed the intersection of 70A09 line with *dsx* and revealed that *70A09∩dsx* neurons modulate immature behaviours. These neurons had been shown to modulate receptivity (pC1a/b and vpoDN) and egg laying (aDN) (Zhou *et al.*, 2014; K. Wang *et al.*, 2020; Nojima *et al.*, 2021). Silencing these neurons decreases female receptivity at similar levels as observed when the whole pC1 cluster was silenced (Zhou *et al.*, 2014). We also revealed that the activity of pC1a alone is necessary for females to display high levels of receptivity and that, pC1b and vpoDN activity is likely to be required to suppress immature-like rejection behaviours.

Here we provide a discussion of the main findings described in the previous chapters, put them into context and highlight some suggestions for future investigation on the circuits here described.

Serotonergic system as a modulator of female locomotor activity in a courtship context

The 70A09 promotor expresses a fragment of the serotonin-receptor gene 5-HT_7, whose expression is detected in the CNS and VNC of both larvae and adult *Drosophila*. The neurotransmitter serotonin (5-HT) regulates a variety of functions in animals such as sleep and circadian rhythms, mood, emotional behaviour, sensory processing and motor activity, among others. It is also known to interact with other neurotransmitter systems through the activation of serotonin receptors located on cholinergic, dopaminergic, GABAergic or glutamatergic neurons (reviewed in (Crispino, Volpicelli and Perrone-Capano, 2020)). Both $5HT_7$-expressing neurons and 5-HT-releasing neurons have been implicated in *Drosophila* sexual motivation (Becnel *et al.*, 2011; Pooryasin and Fiala, 2015) and motor activity in both larvae and adult flies (Silva *et al.*, 2014; Pooryasin and Fiala, 2015; Majeed *et al.*, 2016; Aimon

et al., 2019; Howard *et al.*, 2019). These studies demonstrated that blocking the active site of serotonin cognate receptor 5-HT_7, knockdown serotonin receptors or, inactivating either 5-HT_7-expressing or 5-HT-releasing neurons increases flies' locomotor activity. On the other hand, inhibiting the reuptake of serotonin or activating neurons expressing 5-HT_7 or 5-HT reduces the locomotor activity. Pooryasin and Fiala (Pooryasin and Fiala, 2015) also shown that activation of 5-HT neurons reduces both female and male mating success. Another study showed that inactivation of the 5-HT_7 reduces male sexual motivation and female receptivity (Becnel *et al.*, 2011), as blocking the active site of this receptor decreases the frequency of courtship displays by males and reduces copulation levels by both males and females. Curiously, these 5-HT_7-inactive females did not exhibit obvious rejection behaviours, but they generally avoided contact with the male. These findings evidence a negative correlation between serotonin and locomotor activity, *i.e.*, higher levels of serotonin lead to low locomotor activity and *vice versa*, although its correlation with female receptivity is not clear. Additionally, the serotonergic effect on flies' locomotion was observed in different behavioural contexts, which suggests that the serotonergic neurons may act in combination with behavioural modulatory neurons, contributing to orchestrate selective and context-dependent behaviours. Though studies characterizing 5-HT_7 neurons did not show any anatomic similarity with *70A09*-expressing brain neurons (Becnel *et al.*, 2011; Howard *et al.*, 2019), evidence points 70A09 neurons as part of the serotonergic system.

Speed-modulatory neurons as downstream neurons to female sexual circuitry

It is not clear which *70A09*-GAL4 neurons modulate female speed during courtship and whether these neurons modulate both locomotion and female receptivity, or, if these neurons act downstream the female sexual circuitry to generate courtship-dependent locomotor behaviours.

The fact that the activation of *70A09*-GAL4 brain neurons generate such an unequivocal motor output led us to speculate that these neurons are descending

neurons or connect with descending neurons, since descending neurons integrate information in the brain and communicate it to the VNC, modulating motor behaviours (Hsu and Bhandawat, 2016; Cande *et al.*, 2018; Namiki *et al.*, 2018). In fact, activation of some descending neurons led to flies' quiescence (Cande *et al.*, 2018) and, some of them, present projections to VNC similar to the ones observed in *70A09*-GAL4 anatomic analysis. To what receptivity concerns, we were also not able to determine if neurons that modulate speed are the same modulating receptivity. Similarly to our work, other studies showed that both activation or silencing of 5-HT or 5-HT_7-expressing neurons reduces female receptivity (Becnel *et al.*, 2011; Pooryasin and Fiala, 2015). We presume that, if *70A09*-GAL4 comprises neurons that independently modulate speed and receptivity, speed-modulatory neurons may act downstream the female sexual circuitry. As a matter of fact, 70A09 expresses neurons in the brain that modulate *Drosophila* female receptivity with no role in the regulation of flies' speed (pC1 and vpoDN). In receptive females, activation of these neurons may trigger the serotonergic system, increasing the sexual motivation state of the fly and leading to copulation-facilitating behaviours. Nevertheless, the genetic tools available were not sufficient for us to confirm this hypothesis.

Doublesex **pC1a as a mating status sensor that influences distinct behavioural outputs**

The female pC1 cluster is composed by five distinct cells (from pC1a to pC1e) and it is necessary for virgin females to display high levels of receptivity (Zhou *et al.*, 2014; Rezával *et al.*, 2016). In this work we showed that silencing pC1a, pC1b and vpoDN decreases virgin female receptivity and, curiously, at the same level as observed when the whole pC1 cluster was silenced (Zhou *et al.*, 2014). Silenced females display normal levels of vaginal plate opening suggesting that, in this particular case, pC1a and pC1b are the ones modulating female receptivity.

Female pC1 cluster integrates the internal state in the *Drosophila* brain and modulates multiple behaviours such as responses to male song and receptivity, as well as aggressive and male-like courtship behaviours (Rezával *et al.*, 2016; Wu,

Bidaye and Mahringer, 2019; Deutsch *et al.*, 2020; Schretter *et al.*, 2020). In fact, different stimulation levels of the pC1 lead to distinct behavioural outputs as was shown by the work of Rezával and her colleagues (Rezával *et al.*, 2016). Low stimulation increases female receptivity, but when a strong stimulation was applied, a latent courtship circuitry is activated in the female brain inducing male-like behaviours. A specific pC1 cell, the pC1d, was reported to induce female aggression and male-typical behaviours in female flies (Wu, Bidaye and Mahringer, 2019; Deutsch *et al.*, 2020; Schretter *et al.*, 2020). The differences in behavioural outputs found between these works may be related with the tools used for neuronal stimulation and with the differences in the activation protocols applied, that led to different neuronal stimulation levels. Another interesting finding was the apparent involvement of the pC1d in female receptivity. Although activation did not affect female receptivity, silencing of pC1d decreased it, suggesting that baseline activity is necessary for normal female receptivity (Wu, Bidaye and Mahringer, 2019; Deutsch *et al.*, 2020). However, and contrary to pC1d-induced aggression, reduced female copulation promoted by neuronal inactivation does not occur via acetylcholine, suggesting that pC1d may release multiple neurotransmitters (Wu, Bidaye and Mahringer, 2019), each one modulating distinct behaviours. This kind of hierarchical control of *Drosophila* behaviours was also reported for the male-specific P1, a subset of the *dsx*+-pC1 cluster (Zhang *et al.*, 2018). Similar to the female-pC1, P1 neurons serve as a higher center that integrates both internal and external cues, driving both male aggression and mating behaviours, which is also dependent on the activation level (von Philipsborn *et al.*, 2011; Hoopfer *et al.*, 2015; Koganezawa, Kimura and Yamamoto, 2016).

How does silencing only two pC1 cells reproduce the same phenotype as the whole pC1 cluster? Does each pC1 cell modulate different behaviours and have distinct effects on female receptivity? Do they interact with each other to generate context-dependent social behaviours?

Recently, female pC1 cluster was suggested as a mating status integrator, as it receives information from the postmating-inducing SAG neurons, with the pC1a subtype receiving the majority of those inputs (F. Wang, Wang, Forknall, Patrick, *et al.*, 2020). The now available *Drosophila* brain connectome (Clements *et al.*, 2020; Xu *et al.*, 2020) allowed us to observe reciprocal connections within the pC1 cluster, but the pC1a appears to be the only one that communicates with all the other pC1 cells. Thus, we suggest that pC1a is the key integrator of the mating status and its individual activity is sufficient to induce female receptivity. According to its activation level or status, the pC1a may exert different effects on the downstream pC1 cells modulating distinct behaviours that will depend not only on the mating status but also on the sensory external cues. For example, mated females are known to be more aggressive than virgin females, in the context of competition for egg laying sites (Bath *et al.*, 2017). So, the activity of the pC1a may (i) exert an inhibitory effect onto aggression-modulatory pC1d neurons in virgin females or, (ii) modulate the release of distinct neurotransmitters by the pC1d that will target distinct downstream neurons, inducing distinct context-dependent behaviours. If any of these hypotheses is true, we believe that pC1a will have similar modulatory effects on other female pC1 cells that may regulate specific social behaviours.

Taken all the results together, we hypothesise that the receptivity phenotype observed upon silencing of *70A09*-GAL4 brain neurons (Chapter I) was due to the combination of receptivity modulatory pC1 neurons and walking speed modulatory 5-HT_7-expressing neurons. This hypothesis was also raised by the fact that silencing pC1 neurons do not induce higher levels of typical female rejection behaviours (Chapter II), which suggest that the pC1s need the coordinated action of other neurons to reproduce an appropriate female sexual behaviour. We also hypothesise that distinct pC1 neurons modulate female receptivity in opposite directions, as the silencing of only two pC1s led to a reduction of ≈50% in female receptivity, whereas the silencing of pC1a alone drastically reduced female receptivity.

Future work

Chapter I

Considering that *70A09*-GAL4 expresses descending neurons, besides the vpoDN (described in Chapter II), we propose that intersecting this line with DNs-expressing lines may help us to restrict the expression specifically to descending neurons, which will allow us to verify if these are the speed-modulatory neurons. The DNb01 and DNd02-expressing lines are good candidates for this intersection since activation of this neurons lead to flies quiescence (Cande *et al.*, 2018). Restricting the expression to the desired neurons will also help us to determine the role that they may have in female receptivity.

To find if 70A09 neurons that modulate female speed belong to the serotonergic system, we propose the knockdown of 5-HT_7 in *70A09*-GAL4 brain neurons through RNAi strategies. If the speed-modulatory neurons belong to the serotonergic neurons, we expect to observe an increase in speed of manipulated female flies. On the other hand, if the activity of 5-HT_7 on 70A09 neurons is necessary for females to be receptive, we expect to see also a decrease in female receptivity.

Chapter II

To understand the role of pC1a alone in female receptivity, it would be important to identify other lines that label only pC1a, within the pC1 cluster, to allow confirmation of the phenotype observed in this chapter with the *70A09∩VT2064* line.

Being able to restrict neuronal expression to 70A09 speed-modulatory neurons and to pC1a neurons, will allow us not only to clarify our hypotheses but also facilitate further characterization in terms of neurotransmitters release and functional connectivity.

REFERENCES

Agrawal, S., Safarik, S. and Dickinson, M. (2014) 'The relative roles of vision and chemosensation in mate recognition of Drosophila melanogaster', *Journal of Experimental Biology*, 217(15), pp. 2796–2805. doi: 10.1242/jeb.105817.

Aigaki, T. *et al.* (1991) 'Ectopic expression of sex peptide alters reproductive behavior of female D. melanogaster', *Neuron*, 7(4), pp. 557–563. doi: 10.1016/0896-6273(91)90368-A.

Aimon, S. *et al.* (2019) 'Fast near-whole–brain imaging in adult Drosophila during responses to stimuli and behavior', *PLOS Biology*. Edited by J. Dubnau, 17(2), p. e2006732. doi: 10.1371/journal.pbio.2006732.

Antony, C. *et al.* (1985) 'Compared behavioral responses of maleDrosophila melanogaster (Canton S) to natural and synthetic aphrodisiacs', *Journal of Chemical Ecology*, 11(12), pp. 1617–1629. doi: 10.1007/BF01012116.

Aranha, M. M. *et al.* (2017) 'apterous Brain Neurons Control Receptivity to Male Courtship in Drosophila Melanogaster Females', *Scientific Reports*, 7(1), p. 46242. doi: 10.1038/srep46242.

Aranha, M. M. and Vasconcelos, M. L. (2018) 'Deciphering Drosophila female innate behaviors', *Current Opinion in Neurobiology*, 52, pp. 139–148. doi: 10.1016/j.conb.2018.06.005.

Asahina, K. *et al.* (2014) 'Tachykinin-Expressing Neurons Control Male-Specific Aggressive Arousal in Drosophila', *Cell*, 156(1–2), pp. 221–235. doi: 10.1016/j.cell.2013.11.045.

Aso, Y. *et al.* (2014) 'The neuronal architecture of the mushroom body provides a logic for associative learning', *eLife*, 3, p. e04577. doi: 10.7554/eLife.04577.

Auer, T. O. and Benton, R. (2016) 'Sexual circuitry in Drosophila', *Current Opinion in Neurobiology*, 38, pp. 18–26. doi: 10.1016/j.conb.2016.01.004.

Baines, R. A. *et al.* (2001) 'Altered Electrical Properties in *Drosophila* Neurons Developing without Synaptic Transmission', *The Journal of Neuroscience*, 21(5), pp. 1523–1531. doi: 10.1523/JNEUROSCI.21-05-01523.2001.

Baker, B. S., Taylor, B. J. and Hall, J. C. (2001) 'Are complex behaviors specified by dedicated regulatory genes? Reasoning from Drosophila.', *Cell*, 105(1), pp. 13–24. doi: 10.1016/s0092-8674(01)00293-8.

Bartelt, R. J., Schaner, A. M. and Jackson, L. L. (1985) 'cis-Vaccenyl acetate as an aggregation pheromone inDrosophila melanogaster', *Journal of Chemical Ecology*, 11(12), pp. 1747–1756. doi: 10.1007/BF01012124.

Bastock, M. and Manning, A. (1955) 'The Courtship of Drosophila melanogaster', *Behaviour*, 8(2/3), pp. 85–111.

Bath, E. *et al.* (2017) 'Sperm and sex peptide stimulate aggression in female Drosophila', *Nature Ecology & Evolution*, 1(6), p. 0154. doi: 10.1038/s41559-017-0154.

Becnel, J. *et al.* (2011) 'The Serotonin 5-HT7Dro Receptor Is Expressed in the Brain of Drosophila, and Is Essential for Normal Courtship and Mating', *PLoS ONE*. Edited by T. Zars, 6(6), p. e20800. doi: 10.1371/journal.pone.0020800.

Bennet-Clark, H. C. and Ewing, A. W. (1967) 'Stimuli provided by Courtship of Male Drosophila melanogaster', *Nature*, 215(5101), pp. 669–671. doi: 10.1038/215669a0.

Billeter, J.-C. *et al.* (2006) 'Isoform-specific control of male neuronal differentiation and behavior in Drosophila by the fruitless gene', *Current biology: CB*, 16(11), pp. 1063–1076. doi: 10.1016/j.cub.2006.04.039.

Billeter, J.-C. *et al.* (2009) 'Specialized cells tag sexual and species identity in Drosophila melanogaster', *Nature*, 461(7266), pp. 987–991. doi: 10.1038/nature08495.

Billeter, J.-C., Goodwin, S. F. and O'Dell, K. M. C. (2002) 'Genes mediating sex-specific behaviors in Drosophila', *Advances in Genetics*, 47, pp. 87–116. doi: 10.1016/s0065-2660(02)47003-4.

Bontonou, G. and Wicker-Thomas, C. (2014) 'Sexual Communication in the Drosophila Genus', *Insects*, 5(2), pp. 439–458. doi: 10.3390/insects5020439.

Borrero-Echeverry, F. *et al.* (2021) 'The female pheromone (Z)-4-undecenal mediates flight attraction and courtship in Drosophila melanogaster', *bioRxiv*, p. 2021.01.06.425638. doi: 10.1101/2021.01.06.425638.

Buch, S. *et al.* (2008) 'Opposing Effects of Dietary Protein and Sugar Regulate a Transcriptional Target of Drosophila Insulin-like Peptide Signaling', *Cell Metabolism*, 7(4), pp. 321–332. doi: 10.1016/j.cmet.2008.02.012.

Bussell, J. J. *et al.* (2014) 'Abdominal-B Neurons Control Drosophila Virgin Female Receptivity', *Current Biology*, 24(14), pp. 1584–1595. doi: 10.1016/j.cub.2014.06.011.

Butterworth, F. M. (1969) 'Lipids of Drosophila: A Newly Detected Lipid in the Male', *Science*, 163(3873), pp. 1356–1357. doi: 10.1126/science.163.3873.1356.

Cande, J. *et al.* (2018) 'Optogenetic dissection of descending behavioral control in Drosophila', *eLife*. Edited by K. Scott, 7, p. e34275. doi: 10.7554/eLife.34275.

Card, G. M. (2012) 'Escape behaviors in insects', *Current Opinion in Neurobiology*, 22(2), pp. 180–186. doi: 10.1016/j.conb.2011.12.009.

Chapman, T. *et al.* (2003) 'The sex peptide of Drosophila melanogaster: Female post-mating responses analyzed by using RNA interference', *Proceedings of the National Academy of Sciences*, 100(17), p. 9923. doi: 10.1073/pnas.1631635100.

Christiansen, A. E. *et al.* (2002) 'Sex comes in from the cold: the integration of sex and pattern', *Trends in genetics: TIG*, 18(10), pp. 510–516. doi: 10.1016/s0168-9525(02)02769-5.

Clemens, J. *et al.* (2015) 'Connecting Neural Codes with Behavior in the Auditory System of Drosophila', *Neuron*, 87(6), pp. 1332–1343. doi: 10.1016/j.neuron.2015.08.014.

Clements, J. *et al.* (2020) neu *Print: Analysis Tools for EM Connectomics*. preprint. Neuroscience. doi: 10.1101/2020.01.16.909465.

Cline, T. W. and Meyer, B. J. (1996) 'Vive la différence: males vs females in flies vs worms', *Annual Review of Genetics*, 30, pp. 637–702. doi: 10.1146/annurev.genet.30.1.637.

Clowney, E. J. *et al.* (2015) 'Multimodal Chemosensory Circuits Controlling Male Courtship in Drosophila', *Neuron*, 87(5), pp. 1036–1049. doi: 10.1016/j.neuron.2015.07.025.

Coen, P. *et al.* (2014) 'Dynamic sensory cues shape song structure in Drosophila', *Nature*, 507(7491), pp. 233–237. doi: 10.1038/nature13131.

Connolly, K. and Cook, R. (1973) 'Rejection Responses by Female Drosophila melanogaster: Their Ontogeny, Causality and Effects upon the Behaviour of the Courting Male', *Behaviour*, 44(1/2), pp. 142–166.

Cook, R. (1979) 'The courtship tracking of Drosophila melanogaster', *Biological Cybernetics*, 34(2), pp. 91–106. doi: 10.1007/BF00365473.

Cook, R. M. (1973) 'Courtship processing in Drosophila melanogaster. I. Selection for receptivity to wingless males', *Animal Behaviour*, 21(2), pp. 338–348. doi: 10.1016/S0003-3472(73)80076-4.

Crispino, M., Volpicelli, F. and Perrone-Capano, C. (2020) 'Role of the Serotonin Receptor 7 in Brain Plasticity: From Development to Disease', *International Journal of Molecular Sciences*, 21(2). doi: 10.3390/ijms21020505.

Crossley, S. A., Bennet-Clark, H. C. and Evert, H. T. (1995) 'Courtship song components affect male and female Drosophila differently', *Animal Behaviour*, 50(3), pp. 827–839. doi: 10.1016/0003-3472(95)80142-1.

Datta, S. R. *et al.* (2008) 'The Drosophila pheromone cVA activates a sexually dimorphic neural circuit', *Nature*, 452(7186), pp. 473–477. doi: 10.1038/nature06808.

Demir, E. and Dickson, B. J. (2005) 'fruitless Splicing Specifies Male Courtship Behavior in Drosophila', *Cell*, 121(5), pp. 785–794. doi: 10.1016/j.cell.2005.04.027.

Deutsch, D. *et al.* (2019) 'Shared Song Detector Neurons in Drosophila Male and Female Brains Drive Sex-Specific Behaviors', *Current Biology*, 29(19), pp. 3200-3215.e5. doi: 10.1016/j.cub.2019.08.008.

Deutsch, D. *et al.* (2020) 'The neural basis for a persistent internal state in Drosophila females', *eLife*. Edited by M. R. Carey et al., 9, p. e59502. doi: 10.7554/eLife.59502.

Diao, Fengqiu *et al.* (2015) 'Plug-and-Play Genetic Access to Drosophila Cell Types using Exchangeable Exon Cassettes', *Cell Reports*, 10(8), pp. 1410–1421. doi: 10.1016/j.celrep.2015.01.059.

Dukas, R. and Scott, A. (2015) 'Fruit fly courtship: The female perspective', *Current Zoology*, 61(6), pp. 1008–1014. doi: 10.1093/czoolo/61.6.1008.

Eberl, D. F., Duyk, G. M. and Perrimon, N. (1997) 'A genetic screen for mutations that disrupt an auditory response in Drosophila melanogaster', *Proceedings of the National Academy of Sciences of the United States of America*, 94(26), pp. 14837–14842. doi: 10.1073/pnas.94.26.14837.

Ejima, A. and Griffith, L. C. (2008) 'Courtship Initiation Is Stimulated by Acoustic Signals in Drosophila melanogaster', *PLoS ONE*. Edited by B. Brembs, 3(9), p. e3246. doi: 10.1371/journal.pone.0003246.

Eyjolfsdottir, E. *et al.* (2014) 'Detecting Social Actions of Fruit Flies', in Fleet, D. et al. (eds) *Computer Vision – ECCV 2014*. Cham: Springer International Publishing, pp. 772–787.

Fabre, C. C. G. *et al.* (2012) 'Substrate-Borne Vibratory Communication during Courtship in Drosophila melanogaster', *Current Biology*, 22(22), pp. 2180–2185. doi: 10.1016/j.cub.2012.09.042.

Fan, P. *et al.* (2013) 'Genetic and Neural Mechanisms that Inhibit Drosophila from Mating with Other Species', *Cell*, 154(1), pp. 89–102. doi: 10.1016/j.cell.2013.06.008.

Feng, K. *et al.* (2014) 'Ascending SAG Neurons Control Sexual Receptivity of Drosophila Females', *Neuron*, 83(1), pp. 135–148. doi: 10.1016/j.neuron.2014.05.017.

Ferveur, J. F. and Greenspan, R. J. (1998) 'Courtship behavior of brain mosaics in Drosophila.', *Journal of neurogenetics*, 12(4), pp. 205–226. doi: 10.3109/01677069809108559.

Ferveur, J.-F. (2005) 'Cuticular Hydrocarbons: Their Evolution and Roles in Drosophila Pheromonal Communication', *Behavior Genetics*, 35(3), pp. 279–295. doi: 10.1007/s10519-005-3220-5.

Ferveur, J.-F. (2010) 'Drosophila female courtship and mating behaviors: sensory signals, genes, neural structures and evolution', *Current Opinion in Neurobiology*, 20(6), pp. 764–769. doi: 10.1016/j.conb.2010.09.007.

Finley, K. D. *et al.* (1997) 'dissatisfaction, a gene involved in sex-specific behavior and neural development of Drosophila melanogaster', *Proceedings of the National Academy of Sciences of the United States of America*, 94(3), pp. 913–918. doi: 10.1073/pnas.94.3.913.

Finley, K. D. *et al.* (1998) 'dissatisfaction Encodes a Tailless-like Nuclear Receptor Expressed in a Subset of CNS Neurons Controlling Drosophila Sexual Behavior', *Neuron*, 21(6), pp. 1363–1374. doi: 10.1016/S0896-6273(00)80655-8.

Gahan, P. B. (2005) 'Life: the science of biology (7th edn) W. K. Purves, D. Sadava, G. H. Orians and H. C. Heller, W. H. Freeman & Co, 1121 pp., ISBN 0-7167-9856-5 (2004)', *Cell Biochemistry and Function*, 23(3), pp. 221–221. doi: https://doi.org/10.1002/cbf.1179.

Gailey, D. A., Lacaillade, R. C. and Hall, J. C. (1986) 'Chemosensory elements of courtship in normal and mutant, olfaction-deficient Drosophila melanogaster', *Behavior Genetics*, 16(3), pp. 375–405. doi: 10.1007/BF01071319.

Gibson, W. T. *et al.* (2015) 'Behavioral Responses to a Repetitive Visual Threat Stimulus Express a Persistent State of Defensive Arousal in Drosophila', *Current Biology*, 25(11), pp. 1401–1415. doi: 10.1016/j.cub.2015.03.058.

Gomez-Diaz, C. and Benton, R. (2013) 'The joy of sex pheromones', *EMBO Reports*, 14(10), pp. 874–883. doi: 10.1038/embor.2013.140.

Göpfert, M. C. and Robert, D. (2002) 'The mechanical basis of Drosophila audition', *The Journal of Experimental Biology*, 205(Pt 9), pp. 1199–1208.

Greenspan, R. J. and Ferveur, J.-F. (2000) 'Courtship in Drosophila', p. 30.

Grillet, M., Dartevelle, L. and Ferveur, J.-F. (2006) 'A *Drosophila* male pheromone affects female sexual receptivity', *Proceedings of the Royal Society B: Biological Sciences*, 273(1584), pp. 315–323. doi: 10.1098/rspb.2005.3332.

Guiraudie-Capraz, G., Pho, D. B. and Jallon, J.-M. (2007) 'Role of the ejaculatory bulb in biosynthesis of the male pheromone cis-vaccenyl acetate in Drosophila melanogaster', *Integrative Zoology*, 2(2), pp. 89–99. doi: 10.1111/j.1749-4877.2007.00047.x.

Ha, T. S. and Smith, D. P. (2006) 'A pheromone receptor mediates 11-cis-vaccenyl acetate-induced responses in Drosophila', *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 26(34), pp. 8727–8733. doi: 10.1523/JNEUROSCI.0876-06.2006.

Hall, J. C. (1977) 'Portions of the central nervous system controlling reproductive behavior inDrosophila melanogaster', *Behavior Genetics*, 7(4), pp. 291–312. doi: 10.1007/BF01066800.

Hall, J. C. (1979) 'CONTROL OF MALE REPRODUCTIVE BEHAVIOR BY THE CENTRAL NERVOUS SYSTEM OF DROSOPHILA: DISSECTION OF A COURTSHIP PATHWAY BY GENETIC MOSAICS', p. 21.

Hall, J. C. (1994) 'The mating of a fly', *Science*, (264), pp. 1702–14.

Häsemeyer, M. *et al.* (2009) 'Sensory Neurons in the Drosophila Genital Tract Regulate Female Reproductive Behavior', *Neuron*, 61(4), pp. 511–518. doi: 10.1016/j.neuron.2009.01.009.

Hong, W. *et al.* (2009) 'Leucine-rich repeat transmembrane proteins instruct discrete dendrite targeting in an olfactory map.', *Nature neuroscience*, 12(12), pp. 1542–1550. doi: 10.1038/nn.2442.

Hoopfer, E. D. *et al.* (2015) 'P1 interneurons promote a persistent internal state that enhances inter-male aggression in Drosophila', *eLife*. Edited by M. Ramaswami, 4, p. e11346. doi: 10.7554/eLife.11346.

Hotta, Y. and Benzer, S. (1976) 'Courtship in Drosophila mosaics: sex-specific foci for sequential action patterns.', *Proceedings of the National Academy of Sciences*, 73(11), pp. 4154–4158. doi: 10.1073/pnas.73.11.4154.

Howard, C. E. *et al.* (2019) 'Serotonergic Modulation of Walking in Drosophila', *Current Biology*, 29(24), pp. 4218-4230.e8. doi: 10.1016/j.cub.2019.10.042.

Hsu, C. T. and Bhandawat, V. (2016) 'Organization of descending neurons in Drosophila melanogaster', *Scientific Reports*, 6(1), p. 20259. doi: 10.1038/srep20259.

Ishimoto, H. and Kamikouchi, A. (2020) 'A Feedforward Circuit Regulates Action Selection of Pre-mating Courtship Behavior in Female Drosophila', *Current Biology*, 30(3), pp. 396-407.e4. doi: 10.1016/j.cub.2019.11.065.

Jallon, J. M. and Hotta, Y. (1979) ‘Genetic and behavioral studies of female sex appeal in Drosophila’, *Behavior Genetics*, 9(4), pp. 257–275. doi: 10.1007/BF01068205.

Jenett, A. *et al.* (2012) ‘A GAL4-Driver Line Resource for Drosophila Neurobiology’, *Cell Reports*, 2(4), pp. 991–1001. doi: 10.1016/j.celrep.2012.09.011.

Joiner MIA, null and Griffith, L. C. (1997) ‘CaM kinase II and visual input modulate memory formation in the neuronal circuit controlling courtship conditioning’, *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 17(23), pp. 9384–9391.

Kabra, M. *et al.* (2013) ‘JAABA: interactive machine learning for automatic annotation of animal behavior’, *Nature Methods*, 10(1), pp. 64–67. doi: 10.1038/nmeth.2281.

Kamikouchi, A. *et al.* (2009) ‘The neural basis of Drosophila gravity-sensing and hearing’, *Nature*, 458(7235), pp. 165–171. doi: 10.1038/nature07810.

Kamikouchi, A., Shimada, T. and Ito, K. (2006) ‘Comprehensive classification of the auditory sensory projections in the brain of the fruit fly *Drosophila melanogaster*’, *The Journal of Comparative Neurology*, 499(3), pp. 317–356. doi: 10.1002/cne.21075.

Kerwin, P. and Philipsborn, A. C. von (2020) ‘Copulation Song in Drosophila: Do Females Sing to Change Male Ejaculate Allocation and Incite Postcopulatory Mate Choice?’, *BioEssays*, 42(11), p. 2000109. doi: https://doi.org/10.1002/bies.202000109.

Kerwin, P., Yuan, J. and von Philipsborn, A. C. (2020) ‘Female copulation song is modulated by seminal fluid’, *Nature Communications*, 11(1), p. 1430. doi: 10.1038/s41467-020-15260-6.

Kiehart, D. P., Crawford, J. M. and Montague, R. A. (2007) ‘Collection, dechorionation, and preparation of Drosophila embryos for quantitative microinjection.’, *CSH protocols*, 2007, p. pdb.prot4717. doi: 10.1101/pdb.prot4717.

Kimura, K. *et al.* (2008) ‘Fruitless and Doublesex Coordinate to Generate Male-Specific Neurons that Can Initiate Courtship’, *Neuron*, 59(5), pp. 759–769. doi: 10.1016/j.neuron.2008.06.007.

Kimura, K., Sato, C., Koganezawa, M., *et al.* (2015) ‘Drosophila Ovipositor Extension in Mating Behavior and Egg Deposition Involves Distinct Sets of Brain Interneurons’, *PLOS ONE*. Edited by E. M. C. Skoulakis, 10(5), p. e0126445. doi: 10.1371/journal.pone.0126445.

Kimura, K., Sato, C., Yamamoto, K., *et al.* (2015) ‘From the back or front: the courtship position is a matter of smell and sight in Drosophila melanogaster males’, *Journal of Neurogenetics*, 29(1), pp. 18–22. doi: 10.3109/01677063.2014.968278.

Klapoetke, N. C. *et al.* (2014) 'Independent optical excitation of distinct neural populations', *Nature Methods*, 11(3), pp. 338–346. doi: 10.1038/nmeth.2836.

Koganezawa, M. *et al.* (2010) 'The Shaping of Male Courtship Posture by Lateralized Gustatory Inputs to Male-Specific Interneurons', *Current Biology*, 20(1), pp. 1–8. doi: 10.1016/j.cub.2009.11.038.

Koganezawa, M., Kimura, K. and Yamamoto, D. (2016) 'The Neural Circuitry that Functions as a Switch for Courtship versus Aggression in Drosophila Males', *Current Biology*, 26(11), pp. 1395–1403. doi: 10.1016/j.cub.2016.04.017.

Kohatsu, S. and Yamamoto, D. (2015) 'Visually induced initiation of Drosophila innate courtship-like following pursuit is mediated by central excitatory state', *Nature Communications*, 6(1), p. 6457. doi: 10.1038/ncomms7457.

Kohl, J. *et al.* (2013) 'A Bidirectional Circuit Switch Reroutes Pheromone Signals in Male and Female Brains', *Cell*, 155(7), pp. 1610–1623. doi: 10.1016/j.cell.2013.11.025.

Kohl, J., Huoviala, P. and Jefferis, G. S. (2015) 'Pheromone processing in Drosophila', *Current Opinion in Neurobiology*, 34, pp. 149–157. doi: 10.1016/j.conb.2015.06.009.

Kubli, E. (2003) 'Sex-peptides: seminal peptides of the Drosophila male', *Cellular and Molecular Life Sciences (CMLS)*, 60(8), pp. 1689–1704. doi: 10.1007/s00018-003-3052.

Kulkarni, S. J. and Hall, J. C. (1987) 'Behavioral and cytogenetic analysis of the cacophony courtship song mutant and interacting genetic variants in Drosophila melanogaster', *Genetics*, 115(3), pp. 461–475.

Kurtovic, A., Widmer, A. and Dickson, B. J. (2007) 'A single class of olfactory neurons mediates behavioural responses to a Drosophila sex pheromone', *Nature*, 446(7135), pp. 542–546. doi: 10.1038/nature05672.

Kyriacou, C. P., van den Berg, M. J. and Hall, J. C. (1990) 'Drosophila courtship song cycles in normal and period mutant males revisited', *Behavior Genetics*, 20(5), pp. 617–644. doi: 10.1007/BF01065875.

Kyriacou, C. P. and Hall, J. C. (1982) 'The function of courtship song rhythms in Drosophila', *Animal Behaviour*, 30(3), pp. 794–801. doi: 10.1016/S0003-3472(82)80152-8.

Kyriacou, C. P. and Hall, J. C. (1986) 'Interspecific genetic control of courtship song production and reception in Drosophila', *Science (New York, N.Y.)*, 232(4749), pp. 494–497. doi: 10.1126/science.3083506.

Lasbleiz, C., Ferveur, J.-F. and Everaerts, C. (2006) 'Courtship behaviour of Drosophila melanogaster revisited', *Animal Behaviour*, 72(5), pp. 1001–1012. doi: 10.1016/j.anbehav.2006.01.027.

Laturney, M. and Billeter, J.-C. (2014) 'Neurogenetics of Female Reproductive Behaviors in Drosophila melanogaster', in *Advances in Genetics*. Elsevier, pp. 1–108. doi: 10.1016/B978-0-12-800271-1.00001-9.

Lebreton, S. *et al.* (2014) 'Love makes smell blind: mating suppresses pheromone attraction in Drosophila females via Or65a olfactory neurons', *Scientific Reports*, 4, p. 7119. doi: 10.1038/srep07119.

Lebreton, S. *et al.* (2017) 'A Drosophila female pheromone elicits species-specific long-range attraction via an olfactory channel with dual specificity for sex and food', *BMC Biology*, 15(1), p. 88. doi: 10.1186/s12915-017-0427-x.

Lee, G., Hall, J. C. and Park, J. H. (2002) 'DOUBLESEX GENE EXPRESSION IN THE CENTRAL NERVOUS SYSTEM OF DROSOPHILA MELANOGASTER', *Journal of Neurogenetics*, 16(4), pp. 229–248. doi: 10.1080/01677060216292.

Lenschow, C. and Lima, S. Q. (2020) 'In the mood for sex: neural circuits for reproduction', *Current Opinion in Neurobiology*, 60, pp. 155–168. doi: 10.1016/j.conb.2019.12.001.

Lin, H.-H. *et al.* (2016) 'Hormonal Modulation of Pheromone Detection Enhances Male Courtship Success', *Neuron*, 90(6), pp. 1272–1285. doi: 10.1016/j.neuron.2016.05.004.

Liu, H. and Kubli, E. (2003) 'Sex-peptide is the molecular basis of the sperm effect in Drosophila melanogaster', *Proceedings of the National Academy of Sciences*, 100(17), p. 9929. doi: 10.1073/pnas.1631700100.

Liu, T. *et al.* (2012) 'Two Drosophila DEG/ENaC channel subunits have distinct functions in gustatory neurons that activate male courtship', *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 32(34), pp. 11879–11889. doi: 10.1523/JNEUROSCI.1376-12.2012.

Long, T. A. F. *et al.* (2009) 'A Cost of Sexual Attractiveness to High-Fitness Females', *PLoS Biology*. Edited by R. Bonduriansky, 7(12), p. e1000254. doi: 10.1371/journal.pbio.1000254.

Lopes, G. *et al.* (2015) 'Bonsai: an event-based framework for processing and controlling data streams', *Frontiers in Neuroinformatics*, 9. doi: 10.3389/fninf.2015.00007.

Luan, H. *et al.* (2006) 'Refined Spatial Manipulation of Neuronal Function by Combinatorial Restriction of Transgene Expression', *Neuron*, 52(3), pp. 425–436. doi: 10.1016/j.neuron.2006.08.028.

Majeed, Z. R. *et al.* (2016) 'Modulatory Action by the Serotonergic System: Behavior and Neurophysiology in Drosophila melanogaster', *Neural Plasticity*, 2016, p. 7291438. doi: 10.1155/2016/7291438.

Manning, A. (1967) 'The control of sexual receptivity in female Drosophila', *Animal Behaviour*, (15), pp. 239–50.

Manoli, D. S., Meissner, G. W. and Baker, B. S. (2006) 'Blueprints for behavior: genetic specification of neural circuitry for innate behaviors', *Trends in Neurosciences*, 29(8), pp. 444–451. doi: 10.1016/j.tins.2006.06.006.

Markow, T. A. (1975) 'Effect of light on egg-laying rate and mating speed in phototactic strains of Drosophila', *Nature*, 258(5537), pp. 712–714. doi: 10.1038/258712a0.

Markow, T. A. (1987) 'Behavioral and sensory basis of courtship success in Drosophila melanogaster.', *Proceedings of the National Academy of Sciences*, 84(17), pp. 6200–6204. doi: 10.1073/pnas.84.17.6200.

Markow, T. A. and Hanson, S. J. (1981) 'Multivariate analysis of Drosophila courtship', *Proceedings of the National Academy of Sciences*, 78(1), pp. 430–434. doi: 10.1073/pnas.78.1.430.

Markstein, M. *et al.* (2008) 'Exploiting position effects and the gypsy retrovirus insulator to engineer precisely expressed transgenes', *Nature Genetics*, 40(4), pp. 476–483. doi: 10.1038/ng.101.

Matsuo, E. *et al.* (2016) 'Organization of projection neurons and local neurons of the primary auditory center in the fruit fly Drosophila melanogaster', *The Journal of Comparative Neurology*, 524(6), pp. 1099–1164. doi: 10.1002/cne.23955.

McGuire, S. E., Mao, Z. and Davis, R. L. (2004) 'Spatiotemporal Gene Expression Targeting with the TARGET and Gene-Switch Systems in Drosophila', *Science Signaling*, 2004(220), pp. pl6–pl6. doi: 10.1126/stke.2202004pl6.

McRobert, S. P. and Tompkins, L. (1983) 'Courtship of young males is ubiquitous inDrosophila melanogaster', *Behavior Genetics*, 13(5), pp. 517–523. doi: 10.1007/BF01065927.

Mellert, D. J. *et al.* (2010) 'Midline crossing by gustatory receptor neuron axons is regulated by *fruitless, doublesex* and the Roundabout receptors', *Development*, 137(2), pp. 323–332. doi: 10.1242/dev.045047.

Mezzera, C. *et al.* (2020) 'Ovipositor Extrusion Promotes the Transition from Courtship to Copulation and Signals Female Acceptance in Drosophila melanogaster', *Current Biology*, 30(19), pp. 3736-3748.e5. doi: 10.1016/j.cub.2020.06.071.

Miyamoto, T. and Amrein, H. (2008) 'Suppression of male courtship by a Drosophila pheromone receptor', *Nature Neuroscience*, 11(8), pp. 874–876. doi: 10.1038/nn.2161.

Moon, S. J. *et al.* (2009) 'A Drosophila Gustatory Receptor Essential for Aversive Taste and Inhibiting Male-to-Male Courtship', *Current Biology*, 19(19), pp. 1623–1627. doi: 10.1016/j.cub.2009.07.061.

Morata, G. and Garcia-Bellido, A. (1973) 'Behaviour in aggregates of irradiated imaginal disk cells ofDrosophila', *Wilhelm Roux' Archiv für Entwicklungsmechanik der Organismen*, 172(3), pp. 187–195. doi: 10.1007/BF00582074.

Namiki, S. *et al.* (2018) 'The functional organization of descending sensory-motor pathways in Drosophila', *eLife*, 7, p. e34272. doi: 10.7554/eLife.34272.

Nern, A., Pfeiffer, B. D. and Rubin, G. M. (2015) 'Optimized tools for multicolor stochastic labeling reveal diverse stereotyped cell arrangements in the fly visual system', *Proceedings of the National Academy of Sciences*, 112(22), pp. E2967–E2976. doi: 10.1073/pnas.1506763112.

Nojima, T. *et al.* (2021) 'A sex-specific switch between visual and olfactory inputs underlies adaptive sex differences in behavior', *Current Biology*. doi: 10.1016/j.cub.2020.12.047.

Palavicino-Maggio, C. B. *et al.* (2019) 'A small number of cholinergic neurons mediate hyperaggression in female *Drosophila*', *Proceedings of the National Academy of Sciences*, 116(34), pp. 17029–17038. doi: 10.1073/pnas.1907042116.

Pan, Y., Meissner, G. W. and Baker, B. S. (2012) 'Joint control of Drosophila male courtship behavior by motion cues and activation of male-specific P1 neurons', *Proceedings of the National Academy of Sciences*, 109(25), pp. 10065–10070. doi: 10.1073/pnas.1207107109.

Paterson, H. E. H. (1985) 'The Recognition Concept of Species', in Vrba, E. (ed.) *Species and Speciation*. Transvaal Museum Monograph No. 4. Pretoria.

Pavlou, H. J. *et al.* (2016) 'Neural circuitry coordinating male copulation', *eLife*, 5, p. e20713. doi: 10.7554/eLife.20713.

Pfeiffer, B. D. *et al.* (2008) 'Tools for neuroanatomy and neurogenetics in Drosophila', *Proceedings of the National Academy of Sciences*, 105(28), pp. 9715–9720. doi: 10.1073/pnas.0803697105.

von Philipsborn, A. C. *et al.* (2011) 'Neuronal Control of Drosophila Courtship Song', *Neuron*, 69(3), pp. 509–522. doi: 10.1016/j.neuron.2011.01.011.

Pooryasin, A. and Fiala, A. (2015) 'Identified Serotonin-Releasing Neurons Induce Behavioral Quiescence and Suppress Mating in Drosophila', *Journal of Neuroscience*, 35(37), pp. 12792–12812. doi: 10.1523/JNEUROSCI.1638-15.2015.

Pycraft, W. P. (1914) *The courtship of animals*. Hutchinson&Co.

Regnier, F. E. and Law, J. H. (1968) 'Insect pheromones', *Journal of Lipid Research*, 9(5), pp. 541–551. doi: 10.1016/S0022-2275(20)42699-9.

von Reyn, C. R. *et al.* (2014) 'A spike-timing mechanism for action selection', *Nature Neuroscience*, 17(7), pp. 962–970. doi: 10.1038/nn.3741.

Rezával, C. *et al.* (2012) 'Neural Circuitry Underlying Drosophila Female Postmating Behavioral Responses', *Current Biology*, 22(13), pp. 1155–1165. doi: 10.1016/j.cub.2012.04.062.

Rezával, C. *et al.* (2016) 'Activation of Latent Courtship Circuitry in the Brain of Drosophila Females Induces Male-like Behaviors', *Current Biology*, 26(18), pp. 2508–2515. doi: 10.1016/j.cub.2016.07.021.

Ribeiro, C. and Dickson, B. J. (2010) 'Sex peptide receptor and neuronal TOR/S6K signaling modulate nutrient balancing in Drosophila.', *Current biology : CB*, 20(11), pp. 1000–1005. doi: 10.1016/j.cub.2010.03.061.

Rideout, E. J. *et al.* (2010) 'Control of sexual differentiation and behavior by the doublesex gene in Drosophila melanogaster', *Nature Neuroscience*, 13(4), pp. 458–466. doi: 10.1038/nn.2515.

Rideout, E. J., Billeter, J.-C. and Goodwin, S. F. (2007) 'The sex-determination genes fruitless and doublesex specify a neural substrate required for courtship song', *Current biology: CB*, 17(17), pp. 1473–1478. doi: 10.1016/j.cub.2007.07.047.

Ritchie, M. G., Halsey, E. J. and Gleason, J. M. (1999) 'Drosophila song as a species-specific mating signal and the behavioural importance of Kyriacou & Hall cycles in D.melanogaster song', *Animal Behaviour*, 58(3), pp. 649–657. doi: 10.1006/anbe.1999.1167.

Ruta, V. *et al.* (2010) 'A dimorphic pheromone circuit in Drosophila from sensory input to descending output', *Nature*, 468(7324), pp. 686–690. doi: 10.1038/nature09554.

Rybak, F., Sureau, G. and Aubin, T. (2002) 'Functional coupling of acoustic and chemical signals in the courtship behaviour of the male *Drosophila melanogaster*', *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 269(1492), pp. 695–701. doi: 10.1098/rspb.2001.1919.

Sakai, T. *et al.* (2002) 'Light wavelength dependency of mating activity in the Drosophila melanogaster species subgroup', *Genes & Genetic Systems*, 77(3), pp. 187–195. doi: 10.1266/ggs.77.187.

Sato, K. and Yamamoto, D. (2020) 'Contact-Chemosensory Evolution Underlying Reproductive Isolation in Drosophila Species', *Frontiers in Behavioral Neuroscience*, 14. doi: 10.3389/fnbeh.2020.597428.

Savarit, F. *et al.* (1999) 'Genetic elimination of known pheromones reveals the fundamental chemical bases of mating and isolation in Drosophila', *Proceedings of the National Academy of Sciences*, 96(16), pp. 9015–9020. doi: 10.1073/pnas.96.16.9015.

Schilcher, F. von (1976) 'The function of pulse song and sine song in the courtship of Drosophila melanogaster', *Animal Behaviour*, 24(3), pp. 622–625. doi: 10.1016/S0003-3472(76)80076-0.

von Schilcher, F. (1976) 'The role of auditory stimuli in the courtship of Drosophila melanogaster', *Animal Behaviour*, 24(1), pp. 18–26. doi: 10.1016/S0003-3472(76)80095-4.

von Schilcher, F. and Hall, J. C. (1979) 'Neural topography of courtship song in sex mosaics ofDrosophila melanogaster', *Journal of comparative physiology*, 129(1), pp. 85–95. doi: 10.1007/BF00679915.

Schindelin, J. *et al.* (2012) 'Fiji: an open-source platform for biological-image analysis.', *Nature methods*, 9(7), pp. 676–682. doi: 10.1038/nmeth.2019.

Schretter, C. E. *et al.* (2020) *Neuronal circuitry underlying female aggression in* Drosophila. preprint. Neuroscience. doi: 10.1101/2020.05.27.118810.

Scott, G. (2005) *Essential Animal Behavior | Wiley*, *Wiley.com*. Available at: https://www.wiley.com/en-ar/Essential+Animal+Behavior-p-9780632057993 (Accessed: 27 April 2021).

Silva, B. *et al.* (2014) 'Serotonin Receptors Expressed in Drosophila Mushroom Bodies Differentially Modulate Larval Locomotion', *PLoS ONE*. Edited by B. A. Hassan, 9(2), p. e89641. doi: 10.1371/journal.pone.0089641.

Simon, J. C. and Dickinson, M. H. (2010) 'A New Chamber for Studying the Behavior of Drosophila', *PLoS ONE*. Edited by K. Hashimoto, 5(1), p. e8793. doi: 10.1371/journal.pone.0008793.

Singh, B. N. and Singh, A. (2016) 'The genetics of sexual behavior in Drosophila', *Advances in Genomics and Genetics*, 6, pp. 1–9. doi: 10.2147/AGG.S58525.

Spieth, H. T. (1952) ‘Mating behavior in the genus Drosophila (Diptera)’, *Bull AMNH*, (23), pp. 61–106.

Spieth, H. T. (1974) ‘Courtship Behavior in *Drosophila*’, *Annual Review of Entomology*, 19(1), pp. 385–405. doi: 10.1146/annurev.en.19.010174.002125.

Spieth, H. T. and Hsu, T. C. (1950) ‘The Influence of Light on the Mating Behavior of Seven Species of the Drosophila Melanogaster Species Group’, *Evolution*, 4(4), pp. 316–325. doi: https://doi.org/10.1111/j.1558-5646.1950.tb01401.x.

Starostina, E. *et al.* (2012) ‘A Drosophila DEG/ENaC Subunit Functions Specifically in Gustatory Neurons Required for Male Courtship Behavior’, *Journal of Neuroscience*, 32(13), pp. 4665–4674. doi: 10.1523/JNEUROSCI.6178-11.2012.

Stockinger, P. *et al.* (2005) ‘Neural Circuitry that Governs Drosophila Male Courtship Behavior’, *Cell*, 121(5), pp. 795–807. doi: 10.1016/j.cell.2005.04.026.

Sturtevant, A. H. (1915) ‘Experiments on sex recognition and the problem of sexual selection in Drosophila’, *j Anim Behav*, (5), pp. 351–66. doi: 10.1037/h0074109.

Szabad, J. and Fajszi, C. (1982) ‘CONTROL O F FEMALE REPRODUCTION IN DROSOPHILA: GENETIC DISSECTION USING GYNANDROMORPHS’, p. 18.

Taylor, B. J. *et al.* (1994) ‘Behavioral and neurobiological implications of sex-determining factors in Drosophila’, *Developmental Genetics*, 15(3), pp. 275–296. doi: 10.1002/dvg.1020150309.

Therrien, M., Wong, A. M. and Rubin, G. M. (1998) ‘CNK, a RAF-Binding Multidomain Protein Required for RAS Signaling’, *Cell*, 95(3), pp. 343–353. doi: 10.1016/S0092-8674(00)81766-3.

Thistle, R. *et al.* (2012) ‘Contact Chemoreceptors Mediate Male-Male Repulsion and Male-Female Attraction during Drosophila Courtship’, *Cell*, 149(5), pp. 1140–1151. doi: 10.1016/j.cell.2012.03.045.

Tinbergen, N. (1964) *Social Behaviour of Animals*. second. London: Champman and Hall.

Tirian, L. and Dickson, B. J. (2017) ‘The VT GAL4, LexA, and split-GAL4 driver line collections for targeted expression in the Drosophila nervous system’, *bioRxiv*, p. 198648. doi: 10.1101/198648.

Toda, H., Zhao, X. and Dickson, B. J. (2012) ‘The Drosophila Female Aphrodisiac Pheromone Activates ppk23+ Sensory Neurons to Elicit Male Courtship Behavior’, *Cell Reports*, 1(6), pp. 599–607. doi: 10.1016/j.celrep.2012.05.007.

Tompkins, L. *et al.* (1982) ‘The role of female movement in the sexual behavior ofDrosophila melanogaster’, *Behavior Genetics*, 12(3), pp. 295–307. doi: 10.1007/BF01067849.

Tompkins, L. and Hall, J. C. (1983) ‘IDENTIFICATION OF BRAIN SITES CONTROLLING FEMALE RECEPTIVITY IN MOSAICS OF DROSOPHILA MELANOGASTER’, p. 17.

Tompkins, L., Hall, J. C. and Hall, L. M. (1980) ‘Courtship-stimulating volatile compounds from normal and mutant Drosophila’, *Journal of Insect Physiology*, 26(10), pp. 689–697. doi: 10.1016/0022-1910(80)90042-6.

Trott, A. R. *et al.* (2012) ‘Song Choice Is Modulated by Female Movement in Drosophila Males’, *PLoS ONE*. Edited by M. Louis, 7(9), p. e46025. doi: 10.1371/journal.pone.0046025.

Vaughan, A. G. *et al.* (2014) ‘Neural Pathways for the Detection and Discrimination of Conspecific Song in D. melanogaster’, *Current Biology*, 24(10), pp. 1039–1049. doi: 10.1016/j.cub.2014.03.048.

Venard, R. and Jallon, J.-M. (1980) ‘Evidence for an aphrodisiac pheromone of femaleDrosophila’, *Experientia*, 36(2), pp. 211–213. doi: 10.1007/BF01953737.

Villella, A. and Hall, J. C. (1996) ‘Courtship Anomalies Caused by Doublesex Mutations in Drosophila Melanogaster’, *Genetics*, 143(1), pp. 331–344.

Villella, A. and Hall, J. C. (2008) ‘Chapter 3 Neurogenetics of Courtship and Mating in Drosophila’, in *Advances in Genetics*. Elsevier, pp. 67–184. doi: 10.1016/S0065-2660(08)00603-2.

Vosshall, L. B. and Stocker, R. F. (2007) ‘Molecular Architecture of Smell and Taste in *Drosophila*’, *Annual Review of Neuroscience*, 30(1), pp. 505–533. doi: 10.1146/annurev.neuro.30.051606.094306.

Walker, S. J., Corrales-Carvajal, V. M. and Ribeiro, C. (2015) ‘Postmating Circuitry Modulates Salt Taste Processing to Increase Reproductive Output in Drosophila’, *Current Biology*, 25(20), pp. 2621–2630. doi: 10.1016/j.cub.2015.08.043.

Wang, F., Wang, K., Forknall, N., Parekh, R., *et al.* (2020) ‘Circuit and Behavioral Mechanisms of Sexual Rejection by Drosophila Females’, *Current Biology*, 30(19), pp. 3749-3760.e3. doi: 10.1016/j.cub.2020.07.083.

Wang, F., Wang, K., Forknall, N., Patrick, C., *et al.* (2020) ‘Neural circuitry linking mating and egg laying in Drosophila females’, *Nature*, 579(7797), pp. 101–105. doi: 10.1038/s41586-020-2055-9.

Wang, K. *et al.* (2020) *Neural circuit mechanisms of sexual receptivity in* Drosophila *females*. preprint. Neuroscience. doi: 10.1101/2020.08.07.241919.

Waterbury, J. A., Jackson, L. L. and Schedl, P. (1999) 'Analysis of the doublesex female protein in Drosophila melanogaster: role on sexual differentiation and behavior and dependence on intersex.', *Genetics*, 152(4), pp. 1653–1667.

Wicker-Thomas, C. (2007) 'Pheromonal communication involved in courtship behavior in Diptera', *Journal of Insect Physiology*, 53(11), pp. 1089–1100. doi: 10.1016/j.jinsphys.2007.07.003.

Wu, M. *et al.* (2016) 'Visual projection neurons in the Drosophila lobula link feature detection to distinct behavioral programs', *eLife*, 5, p. e21022. doi: 10.7554/eLife.21022.

Wu, Y., Bidaye, S. S. and Mahringer, D. (2019) 'Drosophila female-specific brain neuron elicits persistent position- and direction-selective male-like social behaviors', *bioRxiv*, p. 594960. doi: 10.1101/594960.

Wyatt, G. R. and Davey, K. G. (1996) 'Cellular and Molecular Actions of Juvenile Hormone. II. Roles of Juvenile Hormone in Adult Insects', in Evans, P. D. (ed.) *Advances in Insect Physiology*. Academic Press, pp. 1–155. doi: 10.1016/S0065-2806(08)60030-2.

Xu, C. S. *et al.* (2020) *A Connectome of the Adult* Drosophila *Central Brain*. preprint. Neuroscience. doi: 10.1101/2020.01.21.911859.

Yamada, D. *et al.* (2018) 'GABAergic Local Interneurons Shape Female Fruit Fly Response to Mating Songs', *The Journal of Neuroscience*, 38(18), pp. 4329–4347. doi: 10.1523/JNEUROSCI.3644-17.2018.

Yamamoto, D. *et al.* (1998) 'From behavior to development: genes for sexual behavior define the neuronal sexual switch in Drosophila', *Mechanisms of Development*, 73(2), pp. 135–146. doi: 10.1016/S0925-4773(98)00042-2.

Yamamoto, D., Jallon, J. M. and Komatsu, A. (1997) 'Genetic dissection of sexual behavior in Drosophila melanogaster', *Annual Review of Entomology*, 42, pp. 551–585. doi: 10.1146/annurev.ento.42.1.551.

Yamamoto, D. and Koganezawa, M. (2013) 'Genes and circuits of courtship behaviour in Drosophila males', *Nature Reviews Neuroscience*, 14(10), pp. 681–692. doi: 10.1038/nrn3567.

Yamamoto, D., Sato, K. and Koganezawa, M. (2014) 'Neuroethology of male courtship in Drosophila: from the gene to behavior', *Journal of Comparative Physiology A*, 200(4), pp. 251–264. doi: 10.1007/s00359-014-0891-5.

Yang, C. *et al.* (2009) ‘Control of the Postmating Behavioral Switch in Drosophila Females by Internal Sensory Neurons’, *Neuron*, 61(4), pp. 519–526. doi: 10.1016/j.neuron.2008.12.021.

Yapici, N. *et al.* (2008) ‘A receptor that mediates the post-mating switch in Drosophila reproductive behaviour’, *Nature*, 451(7174), pp. 33–37. doi: 10.1038/nature06483.

Yorozu, S. *et al.* (2009) ‘Distinct sensory representations of wind and near-field sound in the Drosophila brain’, *Nature*, 458(7235), pp. 201–205. doi: 10.1038/nature07843.

Zacarias, R. *et al.* (2018) ‘Speed dependent descending control of freezing behavior in Drosophila melanogaster’, *Nature Communications*, 9(1), p. 3697. doi: 10.1038/s41467-018-05875-1.

Zhang, W. *et al.* (2018) ‘Hierarchical Control of Drosophila Sleep, Courtship, and Feeding Behaviors by Male-Specific P1 Neurons’, *Neuroscience Bulletin*, 34(6), pp. 1105–1110. doi: 10.1007/s12264-018-0281-z.

Zhou, C. *et al.* (2014) ‘Central Brain Neurons Expressing doublesex Regulate Female Receptivity in Drosophila’, *Neuron*, 83(1), pp. 149–163. doi: 10.1016/j.neuron.2014.05.038.

Zhou, C. *et al.* (2015) ‘Central neural circuitry mediating courtship song perception in male Drosophila’, *eLife*, 4, p. e08477. doi: 10.7554/eLife.08477.

ITQB-UNL | Av. da República, 2780-157 Oeiras, Portugal
Tel (+351) 214 469 100 | Fax (+351) 214 411 277

www.itqb.unl.pt