universität wien

DISSERTATION

Titel der Dissertation

Neural control of the female mating decision in *Drosophila melanogaster*

angestrebter akademischer Grad

Doktor/in der Naturwissenschaften (Dr. rer.nat.)

Verfasserin / Verfasser:	Kai Feng
Matrikel-Nummer:	0701378
Dissertationsgebiet (lt. Studienblatt):	Molekulare Biologie
Betreuerin / Betreuer:	Dr. Barry Dickson

Wien, im 20.05.2010

Table of Contents

Contributions and acknowledgements

This work could not have been done without the help and support of the following:

Barry Dickson provided constant support and guidance throughout my PhD;

Martin Häsemeyer performed the GRASP experiments in this thesis, shared a lot of useful thoughts and ideas throughout the project and provided valuable comments on this thesis;
Christian Schusterreiter developed the original video tracking system from which our current system is derived;
Guangnan Tian helped me with the GAL4 silencing screen;
Christopher Masser shared the fly work and information regarding the lethality in the GAL4 silencing screen

Martin Colombini and colleagues in the workshop helped to build our experimental apparatus;
Thomas Micheler, Christian Machacek and Wolfgang Lugmayr provided technical support on the web interface and software compiling of our video tracking system;
Christopher Masser and Bidaye Salil generated the enhancer tiling GAL4 lines;
Alex Keene recorded the courtship song, shared a lot of useful discussions in the early phase of my PhD and provided helpful comments and suggestions on some chapters of this thesis;
Jai Yu introduced me into fruit flies and helped me with the staining and image registration procedures together with Tianxiao Liu;
Ruth Fuchs, Attila Gyorgy, Katarina Bartalska and Stephie Wandl provided technical support on fly husbandry and molecular biology;
Pawel Pasierbek provided technical support on confocal microscopy;
Simon Rumpel and Carrie Cowan monitored my progress through the PhD and provided helpful suggestions as my PhD committee members;
Kristin Scott and Eric Kubli generously shared fly stocks;
The service departments at the IMP/IMBA provided indispensible research support;
I am grateful to all current and past members of Dickson lab for very nice atmosphere in the lab and a lot of useful discussions and ideas.

Summary

Neural control of the female mating decision in *Drosophila melanogaster*

Animals make behavioural decisions based on their internal physiological states and the sensory information they receive from outside world. For most species, whether and with whom to mate are amongst the most critical decisions they have to make in their life time. In *Drosophila*, although males usually initiate courtship and display an elaborate courtship ritual, it is the female who makes the final decision whether or not mating occurs. Both her own internal physiology and the external cues from the courting male are critical inputs to the female's mating decision. Courtship song is the primary sensory cue provided by the male to stimulate the female's receptivity. In order to specifically study the effect of courtship song on females, in the absence of any confounding cues from the male, we have established a system which allows us to precisely monitor a single fly's locomotion while providing defined acoustic stimuli to it. Female flies change their locomotion in response to courtship song, even when there is no male present, but the pattern of change is dependent on the female's mating status and sexual maturity. Mature virgin females slow down upon hearing the song, which suggests acceptance of a fictive courting male. In contrast, immature virgins and mated females speed up in response to courtship song. The mating switch in song response is mediated by the sex peptide receptor. Despite the differential responses, females in different states are all tuned to the conspecific interpulse interval, suggesting that song recognition itself is not dependent on either sexual maturity or mating status. In addition, we could show that fru^{M}, the male-specific isoform of fruitless, blocks female song responses as well as other female sexual behaviours. To identify the neural substrates of female mating behaviour, we also performed a neuronal silencing screen for receptivity with a collection of GAL4 lines. Out of this screen, lines which show post-mating-like behaviour in virgins have been identified. The arborization pattern of the neurons labeled by these lines and GRASP signals with the sex peptide sensing neurons in the reproductive tract suggest they are likely to relay information of female mating status to the central brain.

Zusammenfassung

Neuronale Kontrolle des weiblichen Paarungsverhaltens in Drosophila melanogaster

Tiere fällen in ihrem Verhalten Entscheidungen, die sowohl von ihrem internen physiologischen Zustand als auch von den von der Aussenwelt kommenden sensorischen Informationen abhängen. Für die meisten Arten ist die Entscheidung, ob und mit wem die Paarung vollzogen werden soll eine der wichtigsten Entscheidungen im Leben. In Drosophila beginnen die Männchen gewöhnlich mit der Balz und stellen ein aufwändiges Balzverhalten zur Schau. Es ist jedoch das Weibchen, das die endgültige Entscheidung darüber fällt, ob es zur Paarung kommt. Diese Paarungsentscheidung des Weibchens wird von ihrem physiologischen Zustand und den Signalen, die vom balzenden Männchen ausgehen, beeinflusst. Der Balzgesang ist das vorrangige sensorische Signal, das vom Männchen gesendet wird, um die Empfänglichkeit des Weibchens zu erhöhen.

In der vorliegenden Arbeit wurde experimenteller Aufbau entwickelt, der es ermöglicht, präzise die Lokomotion einer einzelnen Fliege zu messen, während ein definierter auditorischer Stimulus gegeben wird. Auf diese Weise kann spezifisch die Auswirkung des Balzgesangs auf das Weibchen in Abwesenheit anderer störender, vom Männchen ausgehender Signale untersucht werden. Weibliche Fliegen verändern ihr Lokomotionsmuster in Reaktion auf den Balzgesang auch in Abwesenheit eines Männchens. Die Art und Weise der Veränderung hängt dabei vom Verpaarungszustand und der sexuellen Reife des Weibchens ab. Reife, unverpaarte Weibchen verlangsamen ihre Lokomotion, wenn sie den Balzgesang hören, was die Akzeptanz eines fiktiven, balzenden Männchens nahelegt. Im Gegensatz dazu beschleunigen nicht ausgereifte unverpaarte Weibchen und bereits verpaarte Weibchen ihre Lokomotion in Reaktion auf den Balzgesang. Diese Verhaltensänderung in der Reaktion auf den Balzgesang ist vom Sex Peptide Rezeptor abhängig. Trotz der unterschiedlichen Reaktionen sind Weibchen in den verschiedenen Zuständen alle auf das konspezifische Interpulsintervall des Balzgesangs eingestimmt, was darauf schliessen lässt, dass das Erkennen des Balzgesangs als solchen weder von der sexuellen

Reife noch dem Verpaarungszustand abhängt. Des weiteren konnten wir zeigen, dass fru^M, die spezifisch im Männchen auftretende Isoform von fruitless, die Reaktion des Weibchens auf den Balzgesang sowie andere, spezifisch weibliche Verhaltensmuster aufhebt. Um die neuronale Basis des weiblichen Paarungsverhaltens zu identifizieren, wurde ein neuronaler Silencing Screen mit einer Sammlung von GAL4 Linien durchgeführt, in dem die Empfänglichkeit des Weibchens getestet wurde. In diesem Screen wurden GAL4 Linien gefunden, für die unverpaarte Weibchen das Verhalten verpaarter Weibchen zeigen. Die Arborisierungen der Neurone, die in diesen Linien markiert werden sowie die GRASP Signale mit den Sex Peptide sensitiven Neuronen in den Reproduktionsorganen weisen darauf hin, dass diese Neurone vermutlich Informationen über den Verpaarungsstatus des Weibchens in zentrale Hirnstrukturen senden.

Introduction

Overview

All animals, even those with relatively simple brains, need to constantly choose appropriate actions based on sensory stimuli and internal physiological states. In some cases, sensory inputs convey a reflexive behavioural output. For example, one withdraws the arm immediately after touching a hot surface. In other cases, multiple sensory cues and internal states are processed and integrated to initiate higher order decision making. For example, a dog chooses whether to eat or not when food is provided and this decision is made based on quality of the food and satiety of the dog itself. It is a fascinating question to ask how neural circuits execute such action selection by processing and integrating information representing both outside world and internal states. With relative simplicity of its nervous system, robustness of its behaviours, accessibility in laboratory and state-of-art genetic tool box to dissect its neural circuits, the fruit fly *Drosophila melanogaster* provides a great opportunity to address this question.

Sexual behaviour in Drosophila

The brains of *Drosophila* are relatively small containing ~100,000 neurons compared to hundreds of billions in the human brain, yet these animals perform complex and motivated behaviours (Sokolowski 2001; Krashes, DasGupta et al. 2009). One of the most well-studied behaviours is the male courtship behaviour (Sturtevant 1915; Spieth 1974; Hall 1994). Male flies are attracted by visual or acoustic cues caused by a female's movement (Tompkins, Gross et al. 1982; Ejima and Griffith 2008) and pheromonal cues from a female's cuticle (Amrein 2004). Males initiate courtship by following and orienting towards his target female. If the female is stationary, he taps her with his forelegs. He usually proceeds by extending one of his wings to produce a species-specific song while following or circling around the female. An unreceptive female will decamp or reject the courting male by kicking, flicking wings or extruding her

ovipositor. If the female is receptive, she will slow down her locomotion to allow the male to lick her genitalia with his proboscis and attempt to copulate by bending his abdomen (Figure 1). If the attempted copulation fails, the male will cease for a moment and start courting again with orienting or singing. After several rounds of these sequential steps, eventually the female will stop and assume an appropriate posture to allow the copulation to happen or keep rejecting the male until he gives up (Greenspan and Ferveur 2000). This elaborate ritual performed by male flies is innate, which means a male fly in isolation after birth can perform all the steps without any learning from other males.

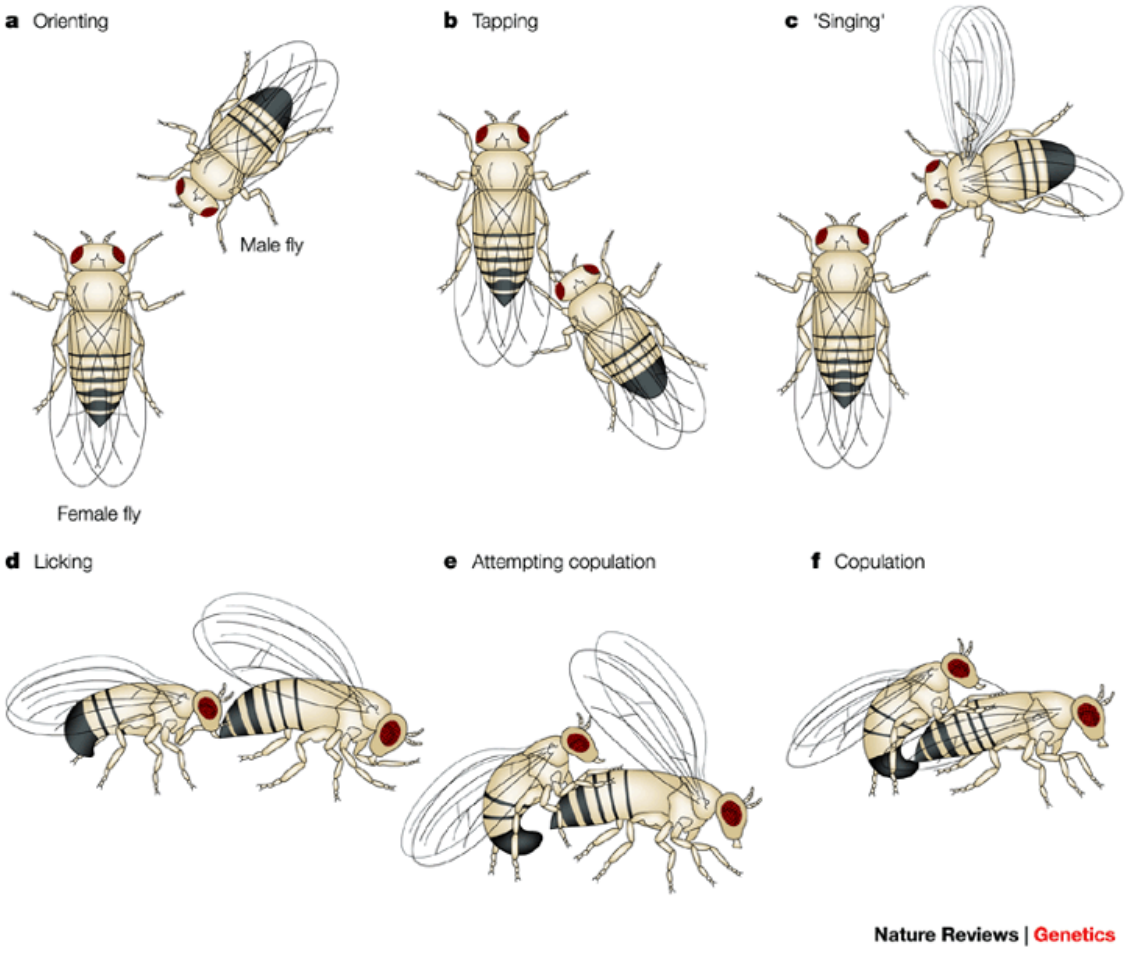


Figure 1. Courtship ritual of *Drosophila melanogaster*

(A-F) Courtship steps sequentially performed by a male fly. When a male fly indentifies a female, (A) he starts the courtship by orienting towards her, (B) then taps her with his forelegs, (C) and sings a species-specific courtship song by vibrating one of his wings. (D) He proceeds by licking her genitalia with his proboscis and finally, (E) curls

his abdomen in an attempt to copulate with her. (F) If the female is receptive, the copulation happens. Adapted from (Sokolowski 2001).

After decades of investigation by many labs, numerous mutants affecting different aspects of male courtship have been isolated (Gill 1963; Castrillon, Gonczy et al. 1993; Hall 1994; Yamamoto, Jallon et al. 1997). Among those, most have been found to be defective in general functions like vision or rhythm, etc. Therefore the observed courtship defects are likely the results of a more general defect in neural functions. Some which "specifically" affect courtship behaviour were mapped to a locus named as "*fruitless*" (Gailey and Hall 1989). The gene was independently cloned by two groups and it was revealed to be very complex with four promoters and eight exons (Ito, Fujitani et al. 1996; Ryner, Goodwin et al. 1996). Of particular importance, the S exon driven by P1 promoter has a binding site of the sex-determination factor Transformer (Tra) and its cofactor Transformer-2 (Tra-2), which leads to sex-specific splicing of the S exon containing transcripts (Heinrichs, Ryner et al. 1998; Lam, Bakshi et al. 2003). In males, these transcripts splice at the default donor site resulting in fusion with common exons shared by both sexes and can be translated into proteins called Fru^M, whereas in females the corresponding transcripts splice alternatively due to the binding of Tra, resulting in a premature stop codon that leads to no detectable protein (Usui-Aoki, Ito et al. 2000).

By mutating the Tra binding site or the splicing donor site in the S exon, flies constitutively splicing in default male or female way were made by homologous recombinations. The behavioural phenotypes of these flies suggest that Fru^M is required in males for courtship behaviour and is sufficient to drive females to court when ectopically expressed in females (Demir and Dickson 2005). Fru^M is expressed in about 1500 neurons in a male fly, which represents about 2% of the nervous system. *GAL4* knocked into the S exon recapitulates the expression pattern of endogenous Fru^M. Blocking neurotransmission of these neurons selectively during adulthood leads to loss of courtship behaviour in male flies (Manoli, Foss et al. 2005; Stockinger, Kvitsiani et al. 2005), which suggests Fru^M expressing neurons form the key "circuits" for the male courtship behaviour. Recently a lot of effort was focused on further dissecting functions of subsets of *fru* circuits and some progresses have been made (Koganezawa, Haba et al. ; Manoli and Baker 2004; Kurtovic, Widmer et al. 2007; Datta, Vasconcelos et al.

2008; Kimura, Hachiya et al. 2008), although it is still early days to solving the problem how *fru* circuits give rise to such complex yet well controlled behaviours.

To date, the majority of studies on mating behaviour in *Drosophila* have focused on males; the female behaviour has often been viewed as relatively passive and subtle. Although males seem to play a more active role in mating, it is the female who makes the final decision whether mating occurs or not. Numerous factors contribute to the female's mating decision. External factors include environmental factors like temperature and humidity, and more importantly, the signals emitted from the courting male which are essential for females to assess the male quality. Internal factors include sexual maturity and mating status of females. It has also been suggested that previous experience of local male quality can modulate female mating decision (Dukas 2008). How does the brain of a female fly sense these external and internal signals and use them to guide the mating decision? It is an interesting biological question by itself and also an attractive paradigm for understanding the neural mechanisms of information integration and decision making (Dickson 2008).

Female mating decision

The mating costs for males and females are asymmetric. Unlike other species in which males contribute to their offspring by parental care or nutrition in the ejaculate (Markow and Ankney 1984), in *Drosophila melanogaster*, the major investment of males is the effort of an elaborate courtship display. It is usually of a male's interest to mate frequently using inexpensive sperm to maximize his reproductive success. For the females, egg production competes for nutrition with somatic growth and maintenance and mating itself has been suggested to be costly (Fowler and Partridge 1989; Chapman, Liddle et al. 1995). So it is important for the females to carefully choose males with high reproductive fitness. This is achieved by evaluating the signals emitted from the courting male during the courtship ritual. To maximize her reproductive success and reduce the cost, females also need to coordinate mating with their reproductive physiology. This needs tight regulation of mating behaviour in pace with oogenesis, ovulation and sperm storage. Therefore, every time a female fly encounters a courting male, the decision of whether to mate

with him is crucial to her reproductive success and this decision has to be made based on both her internal physiological states and the external cues from the courting male (Figure 2).

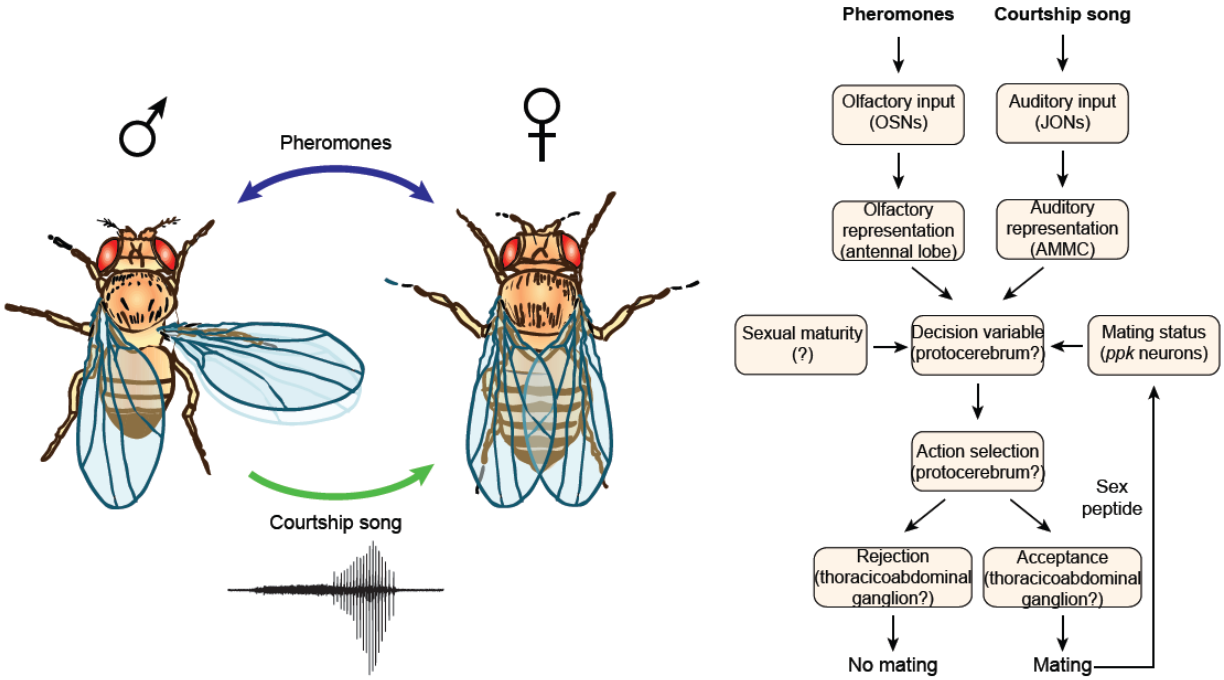


Figure 2. Female mating decision: the elements and potential neural substrates.

The female mating decision relies on multiple variables that include sensory inputs from the courting male (pheromones and courtship song) and her own physiological states (sexual maturity and mating status). Parentheses indicate relevant neurons or regions. OSNs, olfactory receptor neurons; GRNs, gustatory receptor neurons; JONs, Johnston's organ neurons; AMMC, antennal mechanosensory and motor center. Adapted from (Dickson 2008) and minor modifications were made.

Manning suggested there are two separate processes controlling female sexual behaviour. The first is "switch on", which determines whether a female is "accessible" to the courtship of males. The second is "courtship summation", which summates the various stimuli from a courting male until a critical level is reached, when the female allows him to mount (Manning 1967a). The "switch on" is largely controlled by the internal physiology of females including sexual maturity and mating status. The "courtship summation" depends mainly on acoustic and pheromonal cues from courting males (Figure 2). Although the neuroethology of how these factors contribute to

the female mating decision has been explored extensive in 60s and 70s, the genetic and neuronal mechanisms of these processes only started to emerge in recent years.

Internal factors

Sexual maturity

It has been known for a long time that female flies do not mate until several hours after eclosion. By two days of age, females become fully receptive, which means if they are paired individually with males, mating happens in short period (normally below 30 minutes). Manning found that the receptivity of female flies increased gradually from almost 0 to 100 percent between 24 and 40 hours after eclosion (Manning 1967a). Interestingly, the courtship time, which is the time from courtship initiation to copulation, showed a bimodal distribution with one group copulating within 15 minutes and another group having no copulation in 1 hour. This suggests a female fly is either fully receptive or unreceptive, which is independent of the amount of courtship from the male suitor (Manning 1967a). Therefore, for each individual female, it seems that a sudden transition in receptivity happens between 24 and 40 hours after eclosion (Manning 1967a).

The mechanism of this change is still largely unknown. However, juvenile hormone has been implicated in this process (Ringo 1996). By inhibiting the development of adult characters, juvenile hormone plays an important role in insect metamorphosis (Wyatt and Davey 1996). Juvenile hormone becomes active again early in adulthood where it is secreted from the corpora allata and functions to stimulate oocyte development (Wyatt and Davey 1996). There are multiple lines of evidence that suggest juvenile hormone is involved in the development of female sexual receptivity. First, the development of sexual receptivity, ovarian maturation, and corpus allatum activity in early adulthood are temporally correlated (Ringo 1996). Second, topical application of juvenile hormone accelerated the onset of receptivity as did implanting active corpora allata in pupae (Manning 1966). Third, mutant flies of *apterous* gene, which is defective in juvenile hormone production, had very low receptivity (Ringo, Werczberger et al. 1991). However, juvenile hormone seems not to control receptivity through stimulating the ovary

growth because females on a diet lacking protein matured normally in sexual receptivity while their ovary growth was arrested (Manning 1967a).

Mating status

Drosophila melanogaster females become refractory to further mating for 8-10 days after mating with wild-type males (Manning 1962). This process is accompanied by a dramatic increase in egg laying rate and known as the "post-mating switch" (Kubli 2003). The regain of receptivity is associated with depletion of sperm by fertilization and egg-laying (Manning 1967a).

Two distinct mechanisms were proposed to cause the post-mating switch: the first is "copulation effect", which lasts for 1-2 days when females are mated with spermless males; the second is "sperm effect", which lasts for 8-10 days until sperm is exhausted in wild-type flies (Manning 1962; Manning 1967a). During copulation, there are about 80 proteins and peptides in the seminal fluid transferred to females together with sperm (Chapman and Davies 2004; Walker, Rylett et al. 2006). Injection of either of two peptides from the male seminal fluid, sex peptide (SP, also known as Acp70A) or DUP99B, into the female hemolymph induced a switch in receptivity and egg laying rate for 1-2 days (Chen, Stumm-Zollinger et al. 1988; Saudan, Hauck et al. 2002). Ectopic expression of SP in female fat bodies or under control of a heatshock promoter also leads to unreceptivity and high egg laying rate (Aigaki, Fleischmann et al. 1991). Another protein ovulin (also known as Acp26Aa) was shown to stimulate egg laying rate on day 1 after mating (Herndon and Wolfner 1995; Chapman, Herndon et al. 2001). However, only females mated with males lacking SP showed impaired post-mating switch from 12 hours on after mating, which suggests SP is required for the long-lasting "sperm effect" (Chapman, Bangham et al. 2003; Liu and Kubli 2003).

SP is synthesized initially as a 55-amino acid precursor consisting of a 19-amino acid signal peptide that is cleaved off when it is secreted from the accessory glands (Cirera and Aguade 1997). SP binds to sperm tail with the N-terminal end and is transferred to the female reproductive tract during copulation. The C-terminus of the peptide gets cleaved off from sperm tail in the female reproductive tract and released gradually to induce the long-lasting post-mating

switch (Schmidt, Choffat et al. 1993; Peng, Chen et al. 2005a). Some unbound full length SP can also be detected in the hemolymph of mated females and is cleaved there (Pilpel, Nezer et al. 2008). The binding of SP to sperm explains the discrepancy concerning time frames between injection experiments and results of SP null males. Thus, SP is the molecular basis of "sperm effect" whereas sperm is merely the carrier. When mated with a spermless male, the female still receives SP from the male, although the storage cannot last for a long time and this explains most of the "copulation effect". Therefore, SP is the key molecule responsible for the post-mating switch, whereas DUP99B and ovulin only play minor role on the first day after mating (Liu and Kubli 2003). However, it is not entirely clear at this moment if other mechanical or chemical factors contribute to the refractoriness to remating in the first couple of hours after mating.

Besides regulating receptivity and egg laying, SP was shown to upregulate juvenile hormone production in corpus allatum with its N-terminus, which in turn elevates oogenesis and ovulation (Moshitzky, Fleischmann et al. 1996). SP also stimulates the immune response in mated females by elevating the anti-microbial peptide synthesis (Peng, Zipperlen et al. 2005b). In addition, food intake behaviour (Carvalho, Kapahi et al. 2006) and sleep cycle (Isaac, Li et al. 2009) in females were recently shown to change upon mating in a sex-peptide-dependent manner.

The receptor for SP has been identified recently in a pan-neuronal genome-wide transgenic RNAi screen for egg laying (Yapici, Kim et al. 2008). When *sex peptide receptor (SPR)* was knocked down in all the neurons, mated females didn't show the post-mating switch but rather behaved like virgins. They were also completely insensitive to the injection of synthetic SP into hemolymph (Yapici, Kim et al. 2008). The *SPR* gene encodes a G-protein coupled receptor. The binding of SP was examined with a cell culture assay in Chinese hamster ovary cells. In the assay, ligand-mediated GPCR activation triggers a luminescent flash through $G\alpha_q$-dependent Ca^{2+} pathway. When *SPR* was expressed in the cultured cells together with the Ca^{2+} reporter aequorin and one of the chimaeric G proteins $G\alpha_{qi}$ or $G\alpha_{qo}$, but not $G\alpha_{qs}$ or $G\alpha_q$ alone, treatment of SP or DUP99B in higher concentration could induce robust luminescence (Yapici, Kim et al. 2008). These results indicate SPR signals through $G\alpha_i$ or $G\alpha_o$. Therefore, cAMP but not Ca^{2+} might be involved in the downstream signaling of SPR and this is consistent with previous

results that the mutant of *dunce*, which encodes a cAMP phosphodiesterase, is insensitive to SP either from males or injections (Chapman 1996).

Orthologues of *SPR* are detected in most sequenced insect genomes including many species of *Drosophila, Aedes and Bombyx*. However, genes encoding SP-like peptides were only discerned in a few *Drosophila* species closely related to *D. melanogaster* (Yapici, Kim et al. 2008). It is also puzzling that SPR is expressed broadly in the central nervous system of males as well as females (Yapici, Kim et al. 2008), yet it is only required in a few sensory neurons in the female reproductive tract for the post-mating switch (see later sections). Recently, Kim and colleagues identified a highly conserved family of peptides across a wide range of invertebrate species, myoinhibitory peptides (MIPs), as SPR ligands (Kim, Bartalska et al. 2010; Yamanaka, Hua et al. 2010). However, MIPs clearly doesn't induce the post-mating switch, no matter whether injected into the hemolymph of females or transferred from males during copulation (Kim, Bartalska et al. 2010).

External factors

Pheromonal signals

While the internal physiology of a female sets a permissive or restrictive condition, it is the sensory cues from the courting male that trigger the female mating decision. Information regarding the species and fitness of the male is presumably encoded in these signals (Ritchie, Townhill et al. 1998; Rybak, Sureau et al. 2002). Among them, chemical signals play an important role. Males lacking cuticular hydrocarbons have significantly reduced mating success (Rybak, Sureau et al. 2002; Billeter, Atallah et al. 2009) while smellblind females are much less receptive to courting males (Markow 1987).

So far two male pheromones which stimulate female receptivity have been identified. Cis-vaccenyl acetate (cVA) is a volatile pheromone synthesized in the male ejaculatory bulb (Butterworth 1969). The olfactory receptor Or67d is involved in sensing cVA. *Or67d* knock-out

females showed significantly reduced receptivity to courting males and the mutant males showed higher male-male courtship and reduced male-male aggression (Kurtovic, Widmer et al. 2007; Wang and Anderson 2009). This indicates cVA is an important signal for both sexes to evaluate their partners for the mating decisions. Or67d is expressed in trichoid sensilla in both sexes (Couto, Alenius et al. 2005; Fishilevich and Vosshall 2005). An essential co-receptor SNMP is also required for the detection of cVA in the olfactory sensory neurons expressing *Or67d* (Benton, Vannice et al. 2007). Or67d has recently been shown to be the receptor of odorant binding protein LUSH in a changed conformation upon binding cVA, rather than the receptor of cVA itself (Laughlin, Meehan et al. 2008).

The non-volatile pheromone 7-Tricosene (7-T) is a male specific cuticular hydrocarbon that stimulates female receptivity. Males producing higher levels of 7-T or males perfumed with high levels of this pheromone need less courtship time to persuade the females to mate with them (Grillet, Dartevelle et al. 2006). The receptor and downstream signaling of 7-T are still unknown.

Courtship song

During courtship, the most conspicuous behaviour is the male circling around the female while extending and vibrating one of his wings. The importance of this action had been demonstrated by Sturtevant almost one century ago when he cut off the males' wings and observed little success in their mating (Sturtevant 1915). One could imagine visual stimuli are provided by the wing extension and it should also cause air flow which ventilates pheromones. Shorey found acoustic signals were produced by the wing vibration and recorded the "courtship song" from the males (Shorey 1962). However, whether the courtship song is able to stimulate female receptivity wasn't clear until Bennet-Clark and Ewing found that playback of a simulated song could rescue a wingless male's mating speed (Bennet-Clark and Ewing 1967).

Acoustic communication is widely used by animals to coordinate their behaviours (Gerhardt and Huber 2002). In *Drosophila*, each species produces a unique pattern of sounds (Waldron 1964; Ewing and Bennet-Clark 1968; Bennet-Clark and Ewing 1970) and it is thought to be involved in species recognition, sexual selection and sexual stimulation. Report on other acoustic stimuli

than courtship song for *Drosophila* is sparse (Paillette, Ikeda et al. 1991). In *Drosophila melanogaster*, the courtship song consists of pulse and sinusoidal components (Figure 3A). The trains of monocyclic pulses arranged in bursts are usually referred as "pulse song" and the "sine song", which is a ~160Hz sinusoidal hum, precedes and/or follows some but not all pulse bursts (von Schilcher 1976a).

Playback experiments clearly demonstrated that the pulse song can increase the mating speed of wingless males when played during courtship (Bennet-Clark and Ewing 1967; von Schilcher 1976b; Kyriacou and Hall 1982; Ritchie, Halsey et al. 1999). However, whether stimulation of pulse song before courtship can be summated by females to enhance their receptivity is a matter of debate (Bennet-Clark, Ewing et al. 1973; von Schilcher 1976b; Kyriacou and Hall 1984). Playback of sine song during courtship doesn't seem to increase the mating speed of wingless males (Kyriacou and Hall 1982). But pre-stimulation with sine song before males were introduced was reported to increase the female receptivity (von Schilcher 1976b).

The pulse song was also shown by von Schilcher to stimulate males to increase their activity and courtship. Even when there are no females present, males can be aroused to court each other and form a "courtship chain" when artificial playback of the pulse song is provided (von Schilcher 1976a; Eberl, Duyk et al. 1997). It may also be advantageous for a male to get instantly aroused when it hears other males singing, perhaps because this is a good indication there are virgin females nearby (von Schilcher 1976a).

The power for both sine song and pulse song is concentrated below 250 Hz and the patterns are rather simple and stereotyped (Figure 3A). The limited acoustic stimuli space allows each parameter tested individually or in combination during behavioural assays or neural recordings (Murthy 2010). So far, the interpulse interval (IPI) draws most attention as a critical parameter to stimulate the abovementioned behavioural responses (Bennet-Clark and Ewing 1969; Tomaru and Oguma 1994a; Tomaru and Oguma 1994b). The IPI, measured as time from start of one pulse to start of the next pulse, seems to be species-specific. In *Drosophila melanogaster*, the average IPI is about 34 msec at 25˚C. Its sibling species *Drososophila simulans* sings a song with similar pulses, but the average IPI is about 48 msec (Ewing and Bennet-Clark 1968). Therefore, the average IPI could potentially serve as a species recognition cue. Playback

experiments confirmed that artificial songs can best stimulate the female receptivity when it is of the species-specific IPI (Bennet-Clark and Ewing 1969). The IPI of both species was also reported to fluctuate rhythmically with periods of 55 sec in *D. melanogaster* and 35 sec in *D. simulans* (Kyriacou and Hall 1980; Alt, Ringo et al. 1998). This species-specific cycle is controlled by the circadian gene *period* (Kyriacou and Hall 1980; Kyriacou and Hall 1986; Wheeler, Kyriacou et al. 1991). Although some results indicate the rhythm is the most important parameter to stimulate female receptivity (Kyriacou and Hall 1982; Greenacre, Ritchie et al. 1993; Ritchie, Townhill et al. 1998), it became controversial when others reported failure to detect these cycles and suggested that incorrect statistics had been used to show the IPI is rhythmic (Crossley 1988; Ewing 1988; Logan and Rosenberg 1989; von Schilcher 1989).

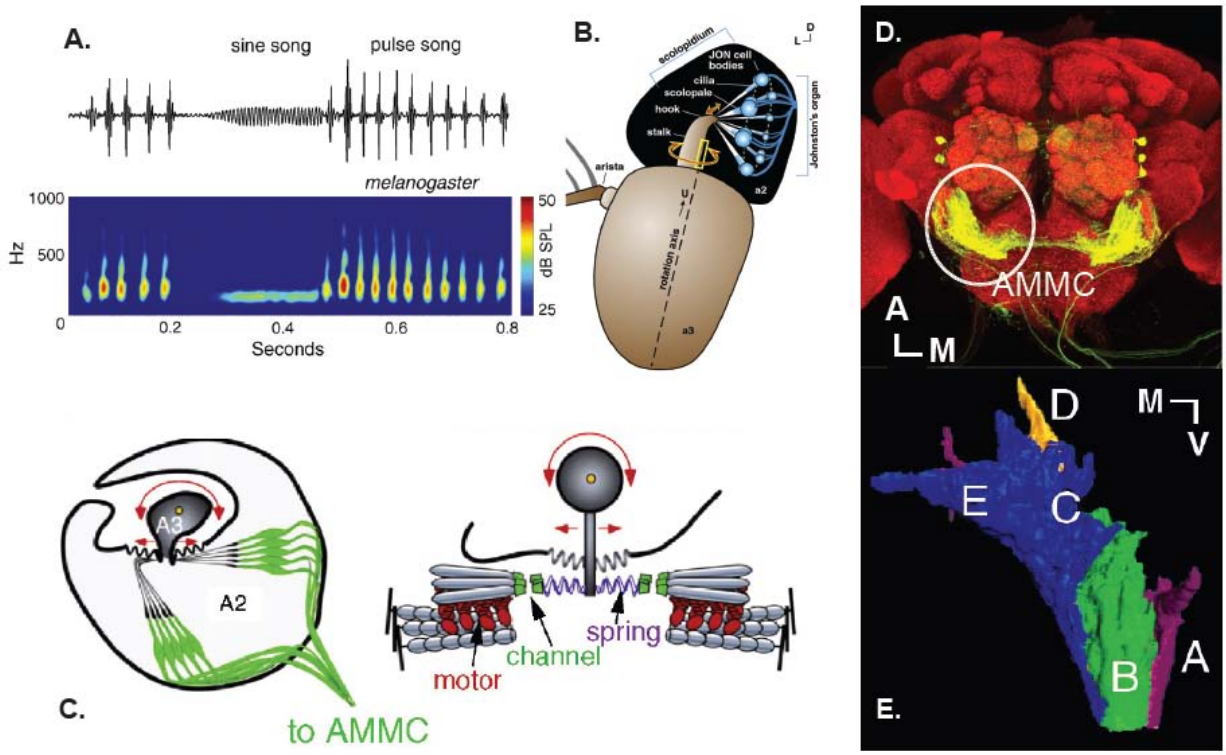


Figure 3. The courtship song and audition in *Drosophila*

(A) Top, an example trace of courtship song of *Drosophila melanogaster*. Bottom, the spectrogram (20 msec sliding fft window) for this clip. Adapted from (Murthy 2010). (B) Schematic of the antennal ear of *Drosophila*. The feather-like arista and the third segment of antenna (a3) form a rigid rod which is connected to the second segment

of antenna (a2) through a hook. Displacement of the arista rotates the a3 relative to a2 and this twist stretches the primary auditory neurons in a2. Adapted from (Kamikouchi, Shimada et al. 2006). (C) Cross-section of the a2/a3 joint and gating-spring model of the transduction module. The proposed transduction module consists of gating springs, force-gated channels and adaptation motors. Adapted from (Murthy 2010). (D) The antennal mechanosensory and motor center (AMMC) in a *Drosophila* brain (white circle). Green channel is staining of mCD8GFP driven by the *nan-GAL4*, which labels all the JO neurons. Red channel is staining of NC82, which is a synaptic marker to highlight the neuropil regions. Maximum projection of confocal stacks is shown. (E) The AMMC can be subdivided to five zones, A-E. Adapted from (Kamikouchi, Inagaki et al. 2009).

Audition in Drosophila

In parallel with behavioural experiments on the functions of courtship song, studies on the audition of *Drosophila* also began in the 1960s. Manning found that the mobility of aristae of a female is very important for her receptivity, indicating arista is the receiver of courtship song (Manning 1967b). Direct electrophysiological recordings from antennal nerve in response of sounds confirmed the "ear" of flies is located in the antenna (Ewing 1978).

Different from human ear as a sensor of sound pressure, the antenna of *Drosophila* responds to particle velocity, which is suitable for near field sound from a small source like a male fly's wing (Bennet-Clark 1971). The mechanical transduction of acoustic signals in the antenna started to unravel in the last decade with the help of laser Doppler vibrometry (Gopfert and Robert 2002). By measuring the displacement of different parts of antenna in response to sound, Gopfert and Robert found the arista is rigidly attached to the third segment of antenna and they together connect to the second segment via a small hook (Gopfert and Robert 2001; Figure 3B). Particle movement of the air induced by sound causes the feather-like arista and the third segment of antenna to rotate around the hook and this twist stretches the primary auditory neurons in the second segment of antenna to convert the mechanical energy to electrical signals in neurons (Gopfert and Robert 2001). Despite the overt difference in the design between fly ear and human ear, striking similarities shared by them in the actuating functions were revealed by analysis of dead flies and genetic mutants that affects different parts of the auditory machinery (Gopfert and Robert 2003; Gopfert, Humphris et al. 2005). They both nonlinearly alter their tuning dependent

on the intensity of the stimulus and show spontaneous twitches in the absence of sound (Gopfert and Robert 2003). They also both have power gain by actively amplifying the mechanical input (Gopfert, Humphris et al. 2005). Recent studies have provided evidence that these active processes and the transduction from mechanical vibration to electrical signals could be mediated by mechanisms similar to vertebrate hair cells. By using an electrode to apply stepped forces to a charged fly while simultaneously measuring the displacement of antenna and recording from the antennal nerve, Gopfert and colleagues found evidence supporting direct mechanotransducer gating in the fly's ear (Albert, Nadrowski et al. 2007). They demonstrated in another study that a gating spring model containing a transducer channel and a motor (Figure 3C) can explain all the active mechanics and nonlinearity of a fly ear (Nadrowski, Albert et al. 2008). Such a quantitative understanding will facilitate the identification of transducer proteins, which are still unknown in vertebrates, by genetic screens in flies (Murthy 2010).

The understanding of neural representation of auditory stimuli only started recently. Kamikouchi and colleagues carried out a detailed anatomical characterization of the primary auditory neurons in the Johnston's Organ (JO). The JO is a chordotonal organ that comprises ~480 monodendritic, ciliated mechanosensory neurons in the second segment of antenna (Kamikouchi, Shimada et al. 2006). These neurons project along the antennal nerve to the antennal mechanosensory and motor center (AMMC) in the brain (Kamikouchi, Shimada et al. 2006; Figure 3D). With GAL4 lines expressing in subsets of JO neurons and stochastic single cell labeling, Kamikouchi et al. found each subgroup of JO neurons innervate a stereotyped region in the AMMC (Figure 3E), although it doesn't reveal a clear spatial organization like glomeruli for olfactory neurons (Kamikouchi, Shimada et al. 2006). Two recent studies examined the responses of subsets of JO neurons to different types of mechanical stimuli using calcium imaging. It seems JO neurons can be classified by their sensitivity to static deflection of the arista and sound. The JO neurons innervating the C and E region in AMMC are responsive to gravity and wind and the response remains as long as the arista is displaced. The JO neurons innervating the A and B regions respond to sound or transiently to the onset and offset of a displacement of arista (Kamikouchi, Inagaki et al. 2009; Yorozu, Wong et al. 2009). Since all the JO neurons attach to the same antennal receiver, different sensitivity to vibration and deflection should reflect distinct transduction machinery of different cell types (Kamikouchi, Inagaki et al. 2009).

Numerous genes and proteins important for the development and function of the fly's ear have been identified with the power of fly genetics (Eberl, Duyk et al. 1997; Walker, Willingham et al. 2000; Caldwell and Eberl 2002; Kim, Chung et al. 2003; Gong, Son et al. 2004; Eberl and Boekhoff-Falk 2007; Sun, Liu et al. 2009). Among them, a group of TRP Channels have been suggested to play different roles and function in distinct subsets of auditory receptor neurons. Two TRPV channels *nanchung* and *inactive* which are expressed in all JO neurons were first indentified as hearing mutants (Kim, Chung et al. 2003; Gong, Son et al. 2004), but later experiments suggested they are also important for gravity sensing (Sun, Liu et al. 2009). A TRPN channel, *nompC*, expressed specifically in the A and B subgroups of JO neurons, is required for hearing but not gravity sensing (Walker, Willingham et al. 2000; Kamikouchi, Inagaki et al. 2009; Sun, Liu et al. 2009). Two TRPA channels, *painless* and *pyrexia*, which are expressed in a distinct subset of JO neurons and cap cells respectively, are required for gravity sensing only (Sun, Liu et al. 2009). How these channels are gated by the mechanical inputs to the antenna remains to be uncovered.

Neural circuits controlling female mating decision

To date our understanding of the neural circuits underlying female mating decision is largely restricted to the early processing of each sensory modality (Figure 2). The male specific volatile pheromone cVA is known to be sensed by the olfactory sensory neurons expressing *Or67d* (Kurtovic, Widmer et al. 2007). The *Or67d* positive neurons project to the DA1 glomerulus in the antennal lobe of the fly brain (Couto, Alenius et al. 2005; Fishilevich and Vosshall 2005). It seems that the activity of Or67d neurons is sufficient to mediate the behavioural responses to cVA. When BmOR1, the receptor for a silkmoth pheromone bombykol, is expressed in the Or67d neurons in the mutant background, artificial activation of these neurons by applying bombykol elicits behavioural responses that mimic the responses to cVA in wild-type flies (Kurtovic, Widmer et al. 2007). A specific class of olfactory projection neurons were identified with photoactivatable GFP to innervate the DA1 glomerulus and project to the higher brain centers mushroom body and lateral horn (Datta, Vasconcelos et al. 2008). Both *Or67d* neurons and their cognate projection neurons are *fru* positive and fru^M is required for these neurons to

generate sexual dimorphic projection patterns (Stockinger, Kvitsiani et al. 2005; Datta, Vasconcelos et al. 2008). The sexual dimorphic arborization of the DA1 neurons in the lateral horn indicates that the same sensory input is potentially fed into different downstream circuits in males and females to generate disparate behavioural responses.

Our knowledge about auditory processing circuits currently lags behind that of the olfactory system. As discussed above, we already know a subset of JO neurons are responsible for hearing and they project to the A and B regions of AMMC. It remains to be uncovered which neurons are specifically involved in detecting the species-specific courtship song and how this information is integrated with other sensory inputs to guide the mating decisions. Several types of neurons have been suggested to innervate distinct regions of AMMC (Kamikouchi, Inagaki et al. 2009). Behavioural and physiological experiments are needed to answer whether and how they are involved downstream in processing the auditory information.

The identification of SPR greatly facilitates the understanding of neuronal mechanisms underlying the post-mating switch. By knocking down *SPR* by RNAi or expressing a membrane-bound SP in a collection of GAL4 drivers, recently two groups found *ppk-GAL4* labels neurons where *SPR* expression is necessary and sufficient for the post-mating switch (Hasemeyer, Yapici et al. 2009; Yang, Rumpf et al. 2009). Based on the previous results that expression of *SPR* in *fru* neurons is also necessary and sufficient for the post-mating switch (Yapici, Kim et al. 2008), SPR should function in the intersection of fru^{GAL4} and *ppk-GAL4* to control the behavioural switch after mating. The cell bodies of these internal sensory neurons are located in the female reproductive tract and they send their projections to the abdominal ganglion in the ventral nerve cord. Silencing of these neurons with a temperature sensitive dynamin mutant ($shibire^{ts}$) leads to mated-female-like behaviours in virgins including increased egg laying and reduced receptivity and it seems to account for all the effects of silencing fru^{GAL4} neurons (Kvitsiani and Dickson 2006; Haesemeyer, Yapici et al. 2009; Yang, Rumpf et al. 2009). These results suggest that activation of SPR and subsequent signaling via $G\alpha_o$ and cAMP pathway lead to a reduction of neuronal activity or synaptic release, which is responsible for the switch of post-mating behaviours (Haesemeyer, Yapici et al. 2009; Yang, Rumpf et al. 2009; Hasemeyer 2010).

The advances on the neural pathways sensing cVA and SP should be mostly attributed to the identification of receptors. To search neural circuits responsible for the downstream processing of sensory information, integration of multimodality and action selection seems to be more difficult partially because we don't have a gene or molecule as a handle. The number of classical mutants that affect female receptivity is very limited (Yamamoto, Jallon et al. 1997; Juni and Yamamoto 2009) and they are usually accompanied with other nonspecific defects (Nakano, Fujitani et al. 2001). One exception is the neuropeptide SIFamide (Terhzaz, Rosay et al. 2007). Knock down of SIFamide by RNAi leads to hyperreceptivity in females and high male-male courtship, and the mutant flies are seemingly normal otherwise. It seems that SIFamide is only expressed in four neurons in the pars intercerebralis in adult flies and ablation of these neurons by overexpressing pro-apoptotic genes under control of *SIFamide-GAL4* phenocopies the RNAi knock down (Terhzaz, Rosay et al. 2007). Since the arborization of the *SIFamide* neurons is broad and there are no *in vivo* experiments done so far on the putative SIFamide receptor (Roller, Yamanaka et al. 2008), it is still not clear how *SIFamide* functions to control female receptivity. Since the pars intercerebralis houses a lot of neurosecretory neurons and destruction of this structure inhibits both oviposition and receptivity in a lot of insects including *Drosophila melanogaster* (Ringo 1996), it is possible that there are other neurosecretory neurons regulating female sexual behaviours directly or indirectly yet to be identified.

Another piece of evidence about neurons important for female mating decision came from classical analysis of gynandromorphs to ask which neurons must be female for the female receptivity. A group of neurons in the dorsal anterior brain have been suggested to be necessary and sufficient for female receptivity when they are bilaterally female (Tompkins and Hall 1983). However, the resolution of this study is far from single-cell level and we still have no clue about how these neurons might execute the control on female mating decisions.

Tools for dissecting neural circuits in Drosophila

Individual neurons are the functional units of the nervous system. To study how neural circuits give rise to animal behaviours, we need to first identify individual neurons or group of neurons

that share some similarity in their morphology and functions; then we have to measure activities of these neurons in order to understand their physiology and perturb them in order to understand their functions by establishing causality between their activities and behaviours. In model systems like *Drosophila,* recent progress in the development of tools has provided great opportunity for genetic dissection of neural circuits (Luo, Callaway et al. 2008).

To repeatedly access certain neurons or cell types, we take advantage of enhancers in the genome. If we already know a gene is specifically expressed in the target neurons, the best way is to use the cis-regulatory elements of this gene to mimic its endogenous expression. However, in most of the cases, there are no such specific genetic markers ready to use. An alternative strategy is to integrate an effector together with a minimal promoter randomly into the genome and employ the enhancers close to the integration site to drive the expression of the effector. With these "enhancer trap" methods (Bellen, O'Kane et al. 1989), we can generate a lot of transgenic lines and screen for interesting expression pattern or functional relevance. A limitation of these methods is that the expression is often too broad to be useful because usually multiple enhancers are "trapped". Recent development of the site-specific integration based on the phage ΦC31 integrase (Groth, Fish et al. 2004) and the genome-wide "enhancer bashing" method is promising to cope with this limitation. By taking relatively short fragments of genomic regions containing cis-regulatory elements and carefully choosing a specific site in the genome to minimize the interference of local environment, "enhancer bashing" offers more restricted expression patterns and flexibility in further subdividing these patterns (Pfeiffer, Jenett et al. 2008).

Binary expression strategies based on transcription factors and its DNA binding sites, like the GAL4/UAS system (Brand and Perrimon 1993), have been proven to be extremely useful in increasing the expression level of transgenes, and more importantly, providing the combinatorial power of reusing the *GAL4* lines and *UAS-transgenes*. Recent development of other binary systems, including the lexA (Lai and Lee 2006) and Q systems (Potter, Tasic et al. 2010), provides the possibility of expressing different effector transgenes in distinct populations of neurons simultaneously in the same animal.

Another binary strategy is based on DNA recombinase and its recognition site. The yeast Flipase/FLP recognition target has been introduced into *Drosophila* (Golic and Lindquist 1989).

Flp/FRT system together with the transcription factor based binary systems, their suppressors (Lee and Luo 1999; Potter, Tasic et al. 2010) and the split halves of the transcription factors (Luan, Peabody et al. 2006) provide tremendous possibilities of intersectional analysis of neurons labeled by two or more cis-regulatory elements.

Neuroanatomy in flies has benefited greatly from the abovementioned methods. But sometimes sparse labeling of neurons is difficult to achieve even with intersectional methods. A new approach based on photoactivatable GFP (PA-GFP) has been used for anatomical tracing of neurons labeled by a relatively broad driver (Datta, Vasconcelos et al. 2008). Photoactivation of a small region of neuropil can label all the PA-GFP expressing neurons intersecting this region. The converted GFP that fluoresces at high level can diffuse out of the photoactivated region to light up the somata and other arborizations of the candidate neurons. This method can be used to identify potential postsynaptic partners of a given neuron by photoactivating the region where the axon of this neuron terminates. Another GFP-based method, GFP reconstitution across synaptic partners (GRASP), has been developed and introduced to flies recently to study the connectivity between neurons (Feinberg, Vanhoven et al. 2008; Gordon and Scott 2009). By expressing N-terminal and C-terminal parts of GFP under control of membrane-bound signals in two different cell types, one can see the reconstituted GFP only when both cell types have close contacts. Other powerful tools for neuroanatomy like Brainbow (Livet, Weissman et al. 2007) and transsynaptic labeling (Ugolini 1995) have been available in other organisms and extensive efforts are being made to develop these techniques in flies.

Genetic methods for neurophysiology have been emerging in recent years. Compared with electrophysiology, optical imaging with genetically encoded indicators of neuronal activities is less invasive, more tolerant to the small size of neurons in *Drosophila* and able to record from multiple cells in the same time. A wide range of genetically encoded indicators sensitive to Ca^{2+} (Miyawaki, Llopis et al. 1997; Baird, Zacharias et al. 1999; Griesbeck, Baird et al. 2001; Nakai, Ohkura et al. 2001), cyclic nucleotide (DiPilato, Cheng et al. 2004) or pH (Miesenbock, De Angelis et al. 1998) have been developed and applied in *Drosophila*. Among those, the Ca^{2+} indicator G-CaMP has been the most widely used (Wong, Wang et al. 2002; Marella, Fischler et al. 2006). GCaMP3, an improved version with higher signal-noise ratio, faster kinetics and better

photostability (Tian, Hires et al. 2009), will be very useful for physiologically dissecting neural circuits in the future.

Loss- and gain-of-function experiments are essential for assigning gene functions to biological processes. Likewise, the causal relationships between the functions of neural circuits and animal behaviours can only be established when we manipulate the activity of these neurons and observe changes in behaviours. Besides knocking down important genes in relevant neurons with tissue specific RNAi (Dietzl, Chen et al. 2007) and overexpressing pro-apoptotic genes (White, Tahaoglu et al. 1996) or neural toxins (Han, Stein et al. 2000) to simply kill the cells, we can perturb the neural activity by directly blocking the synaptic release or altering the excitability of the neurons. The commonly used effectors to silence neurons include a temperature-sensitive dominant-negative mutant of dynamin, Shibire^{ts1}, which blocks the synaptic transmission at restrictive temperatures (Kitamoto 2001); the tetanus toxin light chain (TNT) which cleaves synaptobrevin to prevent vesicle fusion (Sweeney, Broadie et al. 1995); and an inward rectifying potassium channel, Kir2.1, which hyperpolarizes neurons to prevent action potential generation (Baines, Uhler et al. 2001). The options of activating neurons with genetically encoded effectors include a sodium channel NaChBac whose overexpression boosts the neural activity (Ren, Navarro et al. 2001; Nitabach, Wu et al. 2006); a light-gated cation channel Channelrhodopsin-2, which is inducible by blue light with kinetics in milliseconds (Nagel, Szellas et al. 2003; Zhang, Ge et al. 2007); a temperature-sensitive TRP channel TRPA1 which starts to be activated when the temperature is above 25˚C (Hamada, Rosenzweig et al. 2008; Pulver, Pashkovski et al. 2009); a ligand-gated ion channel the ionotropic purinoceptor $P2X_2$ which can be activated by light-induced release of caged ATP (Lima and Miesenbock 2005); and a photoactivated adenylyl cyclase (PAC) which allows light-induced manipulation of cellular cAMP levels (Schroder-Lang, Schwarzel et al. 2007). With these tools available, we can perform loss- and gain-of-function experiments in specific neurons to map the neural circuits important for animal behaviours.

Aim of the thesis

With the increasingly powerful genetic tool box, the stage has been set for exploring the function of neural circuits in *Drosophila* (Olsen and Wilson 2008). Audition is the primary sensory modality for a female to make the mating decision (Markow 1987; Tomaru and Oguma 2000; Rybak, Sureau et al. 2002), yet the least understood sensory modality in terms of processing in higher order neural circuits. To map the downstream auditory circuits and study how these circuits are involved in female mating decision making, we urgently need a high-throughput behavioural assay that allows us to specifically assess the auditory responses in females, in the absence of any confounding cues from the male. In the first part of this project, we aim to establish such a behavioural assay to quantitatively measure the responses of females alone to auditory stimuli under the restrain of their internal physiological states. We hope to explore the higher order auditory processing circuits with this assay later on.

In parallel, an unbiased screen of female receptivity with a collection of *GAL4* lines is performed to identify the neural substrates for each component of female mating decision making (Figure 2). We hope some of the downstream circuits of each sensory modality could emerge in the screen and converge at some point. Our long-term goal is to reconstitute the neural circuits responsible for all components for female mating decision making and use it as an example to understand the basic principles of information processing and how animal behaviours are generated in neural circuits.

Results

Part I Quantitative analysis of female behavioural responses to courtship song

Automated quantitative analysis of song responses

Courtship song stimulates the female's receptivity, which is typically measured as the latency to copulation. In order to specifically study the female's response to courtship song, in the absence of any other confounding cues from the male, we require an assay to measure the response of a single female to song alone. Playback of the male's courtship song has been reported to induce virgin females to slow down their locomotion (von Schilcher 1976a; Crossley, Bennetclark et al. 1995). We therefore sought to develop a more rigorous quantitative assay system that would allow us to explore this behavioural response to song in more detail. Our system (Figure 1A) consists of a simple perspex rack with 28 individual chambers covered by mesh on both sides, each 10 × 45 mm in area and 3 mm in height. Flies can be easily loaded into these chambers through a sliding cover. The rack is then placed horizontally inside a sound-proof box, where it is evenly illuminated from above. A digitally recorded courtship song is played through a set of three speakers, while a video camera records the flies' movement. We also developed customized video tracking software to quantify the movement of single flies in each of the 28 chambers. The position of each fly in every frame of the video is automatically detected and the locomotion speed is then derived (Figure 1B).

In our standard assay, the fly's movement is recorded for a total of 6 minutes. The first 3 minutes are in silence to allow the flies to settle down and serve as an internal control. Then, the song (or any control sound) is played in six 5-second bursts, each followed by 25 seconds of silence. An example of the locomotion of a single wild-type virgin female in response to a burst of pulse song is shown in Figure 1C (top). Consistent with previous observations, the flies walk in small bouts during the time of silence (Martin 2004; Valente, Golani et al. 2007). Therefore the locomotion speed for each fly is quite stochastic at a given time point. This is evident in the

noisy trace of locomotion speed of a single fly over time. By averaging the speed across the 28 flies and 6 song repetitions in one assay, we obtained much smoother curves during the time of silence and we observe pronounced changes after the song starts (Figure 1C, bottom). Therefore, we use the maximal change of the average speed after the song is played normalized against the basal activity before song starts (Δv / v) to quantify the responses of flies to a song in later analysis (see methods for details). So a single data point in all of the subsequent analyses represents 28 flies, each exposed to 6 song repetitions.

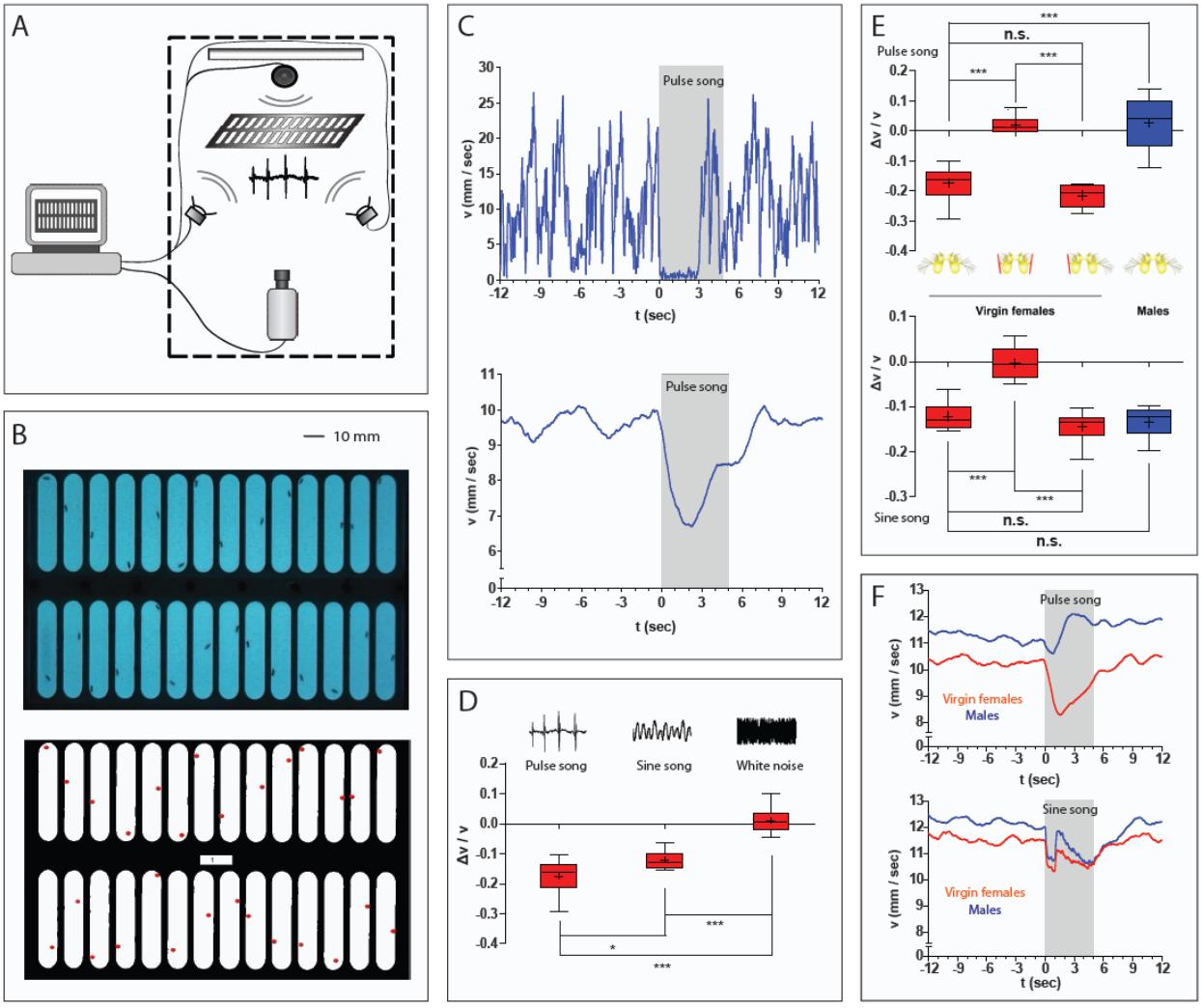


Figure 1. Quantitative analysis of song responses

(A) Schematic of the experimental setup. (B) We developed tracking software to analyze the videos. An example frame clipped from a video is shown on top. Our tracking software detects the position of each fly (bottom). Further analysis is done automatically based on the tracking results. (C) Top, an example trace of locomotion speed of a

single wild-type virgin female. This fly completely stopped its locomotion when a 5-second burst of pulse song is played (gray bar). Bottom, locomotion speed averaged across the 28 flies and 6 song repetitions in this assay. (D) The quantification of virgin female's responses to pulse song, sine song and white noise. (E) The quantification of wild-type fly's responses to pulse song (top) and sine song (bottom). Intact virgin females, virgin females with their arista bilaterally and unilaterally removed and intact males were tested. (F) Time courses of the responses of virgin females and males to pulse song (top) and sine song (bottom). Each trace was averaged across all the assays shown in E. In D and E, each box plotting consists of n = 8 to 9 data points; each data point represents an assay of 28 flies tested with 6 song repetitions. In each plot, the box represents the interquartile range; the whiskers represent the min and max, the line in the box represents the median and the "+" represents the mean. Single asterisk, $P < 0.05$; triple asterisk, $P < 0.001$; n.s., $P > 0.05$; Student's t-test.

In our initial experiments, we used our setup to assess the responses of mature wild-type virgins to a natural pulse song, natural sine song, and white noise. Consistent with previous findings (von Schilcher 1976a), virgin females showed a pronounced slowing response to pulse song, a more modest slowing in response to sine song, and no response to white noise (Figure 1D). The responses to both pulse and sine song were eliminated by removing both arista, confirming that they are mediated by audition. Females with one arista removed responded normally, confirming that these results were not due to the effects of the surgery. Males showed a similar slowing response to sine song, but did not slow down in response to pulse song (Figure 1E). If anything, males rather sped up in response to pulse song, although this effect was somewhat variable and statistically not significant (Figure 1E). Precise time courses reveal that the responses of virgin females and males to sine song were quite similar (Figure 1F bottom), whereas the responses to pulse song were dramatically different (Figure 1F top). Except for the brief and small drop in speed initially, the males indeed sped up in response to pulse song (Figure 1F top), which is consistent with previous results (von Schilcher 1976a; Crossley, Bennetclark et al. 1995; Kowalski, Aubin et al. 2004). Because the male's basal activity declines fast in the 12-second window before the song starts (Figure 1F top), we tend to overestimate the baseline (v) by taking the average. When we calculate the Δv for the males, the overestimated v leads to further underestimation of the speeding up effect and overestimation of the effect of the initial brief slowing down. This explains why the net effect Δv was only slightly above 0 for males.

Maturation of song responses and mating receptivity

Virgin females are not sexually receptive on the first day after eclosion, becoming fully receptive only at 2 days of age (Manning 1967a). To test whether changes in receptivity correspond to locomotion in response to song, we examined the responses of virgin females at varying ages, from 6 hours to 24 days after eclosion (Figure 2A). The baseline locomotion of these females, as well as their slowing response to sine song, remained fairly constant with age, although very young and very old females were slightly less active. In contrast, responses to pulse song varied markedly. At 6 and 24 hours after eclosion, virgins sped up significantly in response to song. Between 2–4 days, however, they showed their maximal slowing response, which was only mildly attenuated as they aged further. Therefore, the response to courtship song switches dramatically on the second day after eclosion, from an apparently aversive to a submissive response.

This switch in song responses is mirrored in the receptivity of virgins to courting males. If intact mature males were provided in the same chambers, with or without additional song, 6-hour old females were completely unreceptive, 3-day old females maximally receptive, and older virgins slightly less receptive (Figure 2B). A similar pattern was seen when wingless (mute) males were added and the pulse song alone played in 5-second bursts at 25-second intervals, whereas females at all ages were largely unreceptive to wingless males without additional song playback (Figure 2B). For very young or older females, receptivity was indistinguishable when intact males or wingless males plus song were provided. However, playback was less effective around the time of maturation (1 day, $P = 0.0564$ and 0.0026, wingless + song versus winged – song and winged + song; 3 day, $P < 0.0001$, wingless + song versus winged ± song; Fisher's exact test). We speculate one of the possible reasons could be that the newly matured virgins are more sensitive to the song quality or context.

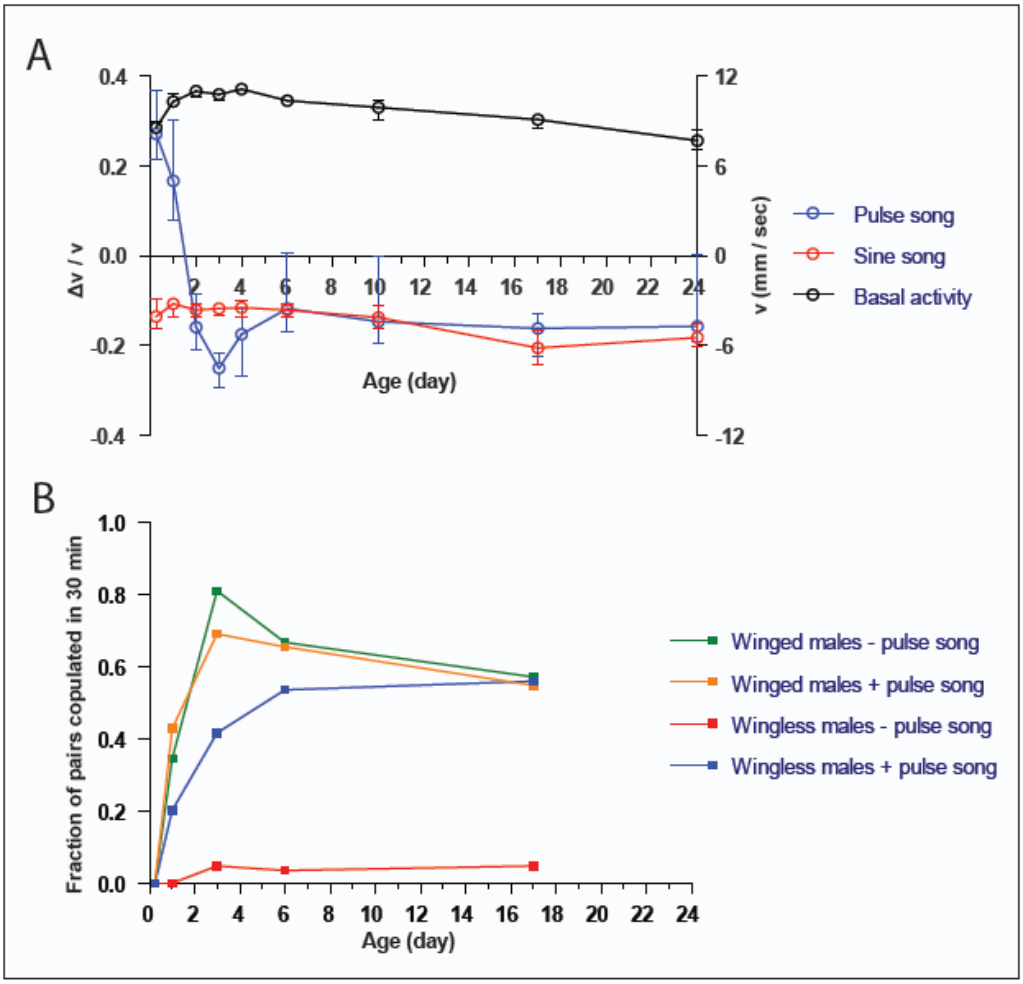


Figure 2. Maturation of song responses and mating receptivity

(A) Responses of virgin females at indicated ages to pulse song and sine song (left y axis). Basal activity of flies in these experiments is represented by the average speed during the period of silence (right y axis). Depicted are the median (circle) and the interquartile range (whiskers); *n* = 10 for each group. (B) Mating receptivity of wild-type virgin females at indicated ages to wingless or intact males with or without pulse song playback. These experiments were done in the same environment as the song response assay. When the song was played, it started 3 minutes after the females and males were mixed and 25-second silence followed each 5-second burst of song playback. 84 pairs were tested for each data point.

Sex Peptide Receptor signaling modulates song responses

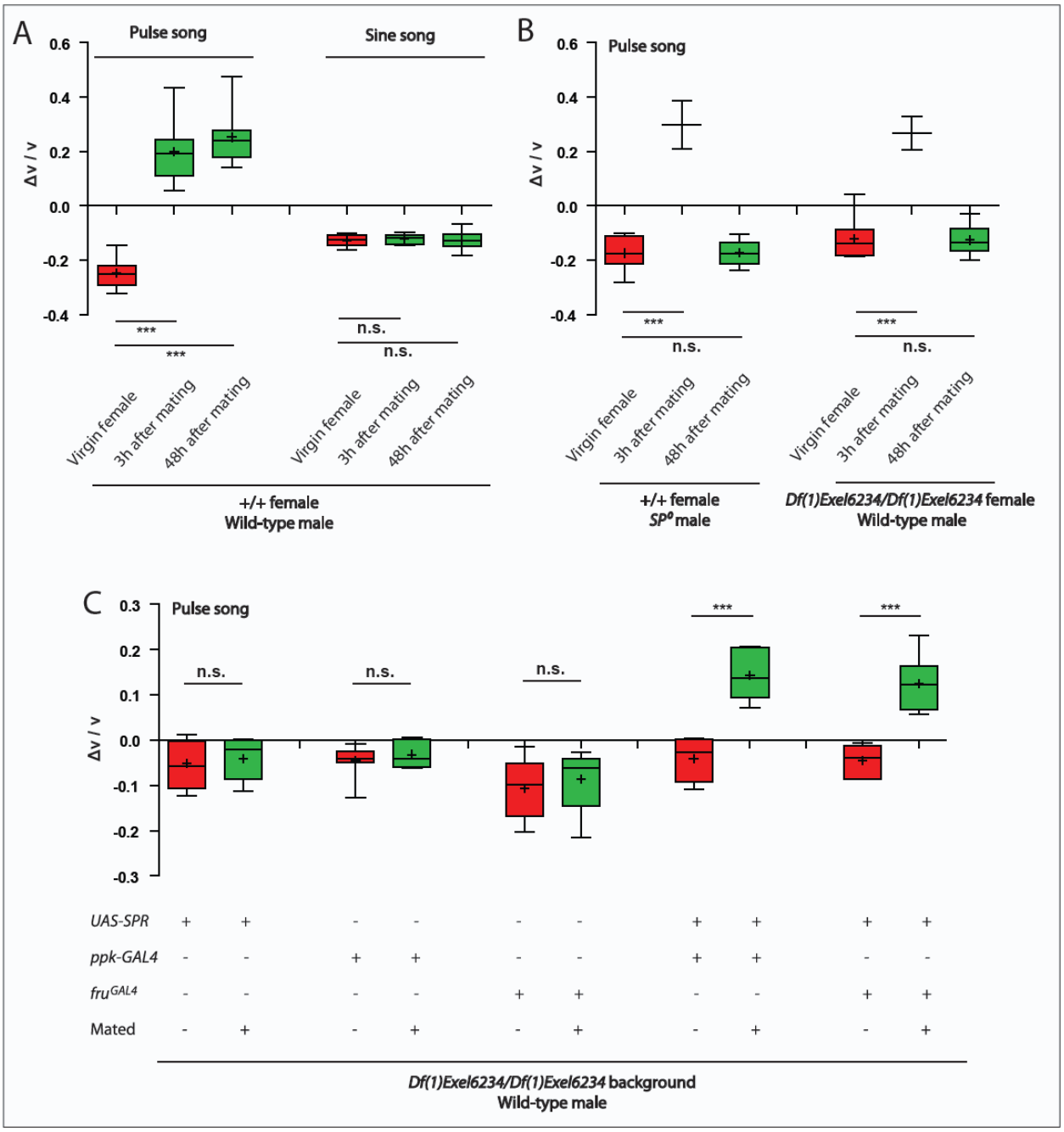
Females that have recently mated are not receptive to courting males (Manning 1962). We found that mated females also do not slow down in response to pulse song. Rather, like immature virgins, mated females show a seemingly aversive response, increasing their locomotion upon

detecting the pulse song. For wild-type females, this switch happens within 3 hours after mating and persists when examined 2 days later (Figure 3A). In contrast, the responses to sine song are not affected by mating (Figure 3A). Mating receptivity is regulated by the sex peptide (SP) a male seminal fluid protein that activates a specific receptor (SPR) in sensory neurons of the female reproductive tract (Chen, Stumm-Zollinger et al. 1988; Yapici, Kim et al. 2008; Hasemeyer, Yapici et al. 2009; Yang, Rumpf et al. 2009). Wild-type females that had mated with SP^0 males (Liu and Kubli 2003) and females homozygous for the deficiency *Df(1)Exel6234* that covers the *SPR* gene with wild-type males (Yapici, Kim et al. 2008) became fully receptive like virgins 48 hours after mating. However, under these two conditions, females didn't start to remate for at least 4 hours after mating (Liu and Kubli 2003; Yapici, unpublished observations). Similarly, we found that wild-type females having mated with SP^0 males and deficiency females lacking *SPR* with wild-type males, like virgins, also slow down in responses to pulse song 48 hours after mating. But during the short refractory period after mating, they do switch to speeding up (Figure 3B).

Recently, it has been shown that the *ppk-GAL4* and fru^{GAL4} double positive neurons in the female reproductive tract are responsible for the *SPR* mediated post-mating switch of receptivity and egg laying (Hasemeyer, Yapici et al. 2009; Yang, Rumpf et al. 2009). By overexpression of SPR in *ppk-GAL4* or fru^{GAL4} neurons in the deficiency females lacking *SPR*, we found that the speeding up responses to pulse song were rescued in these females 48 hours after mating. Therefore, it is likely that the responses to pulse song in females are regulated by the same neuronal pathway that controls the switch of receptivity and egg laying after mating.

Figure 3. Sex peptide signaling modulates song responses

(A) Responses to pulse song and sine song of wild-type virgin females, mated females 3 hours and 48 hours after mating with wild-type males; n = 8 for each group. (B) Responses to pulse song of wild-type females mated with SP^0 males and *SPR* deficiency females; n = 10, 2, 6, 7, 2, 7 for each group from left to right. *Df(1)Exel6234* is a deficiency covering *SPR*. (C) Rescue of song responses of *SPR* deficiency females with *ppk-GAL4 and* fru^{GAL4} and controls; all mated females are tested 48 hours after mating; n = 5 to 9 for each group. In each plot, the box represents the interquartile range; the whiskers represent the min and max, the line in the box represents the median and the "+" represents the mean. Triple asterisk, $P < 0.001$; n.s., $P > 0.05$; Student's t-test.



Tuning of pulse song responses

The *Drosophila melanogaster* pulse song has a typical mean interpulse interval (IPI) of approximately 34 msec (Ewing and Bennet-Clark 1968). The IPI is believed to be a critical component of species discrimination, as other species have distinct IPIs (e.g., a closely related species *Drosophila simulans* has a typical mean IPI of approximately 48 msec; (Ewing and

Bennet-Clark 1968), and mating is best stimulated in playback experiments by the courtship song containing the correct species-specific IPI (Bennet-Clark and Ewing 1969; Kyriacou and Hall 1982). To assess whether our assays of single females reflect the same preference, and also to ask whether immature virgins, mature virgins, and mated females all have the same tuning curve, we systematically varied the IPI across a range of 10–68 msec. All three types of females responded maximally to songs with an IPI in the range of 25–43 msec, with greatly attenuated responses below 20 msec or above 50 msec (Figure 4A). These data suggest that frequency tuning and song recognition is likely to occur upstream of the circuits that mediate the distinct behavioural response to song.

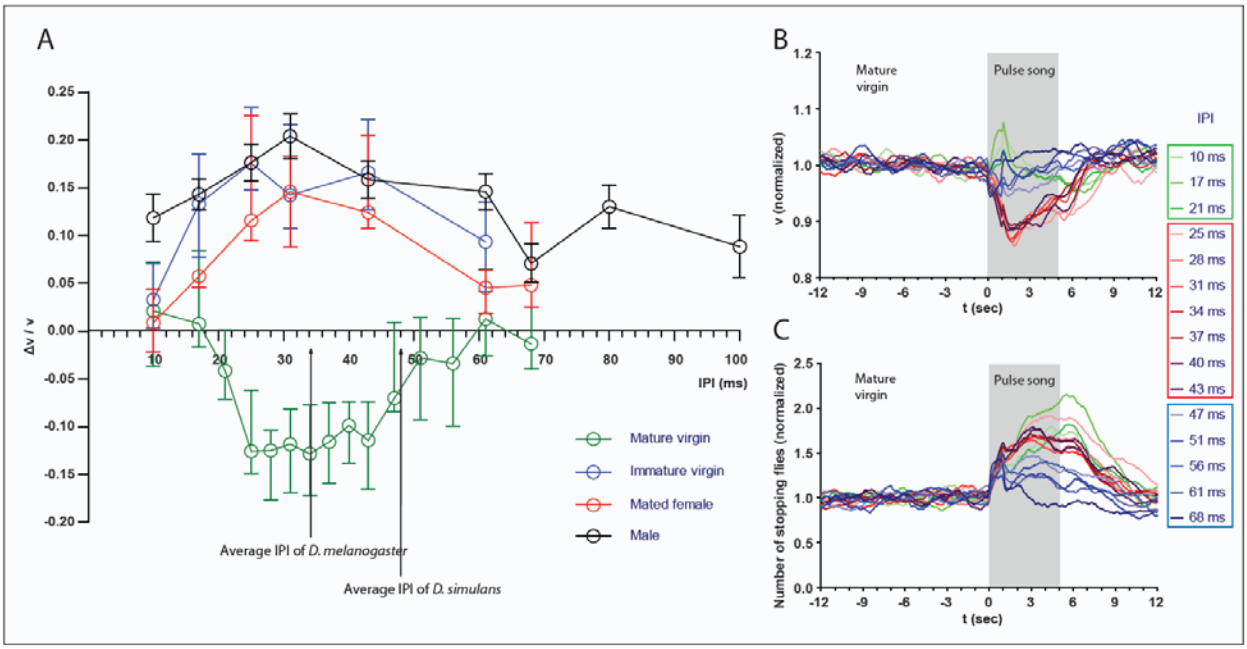


Figure 4. Tuning of pulse song responses

(A) Pulse songs with different interpulse intervals (IPIs) were artificially generated by digitally placing silence of various durations after a pulse clipped from a natural song and repeating these patterns. Tuning curves to this series of songs were plotted for mature virgin females, immature virgin females, mated females and males. In each plot, the circle represents the median and the whiskers represent the interquartile range; n = 5 to 11 for each group. The average IPIs of the courtship songs of *D. melanogaster* and *D. simulans* males were indicated as references. (B) Time courses of the average locomotion speed of mature virgin females in response to pulse songs with different IPIs normalized against the basal level. (C) Time courses of the number of stopping flies (see methods) of mature virgin females in response to pulse songs with different IPIs normalized against the basal level.

For virgin female tuning that we mapped in higher resolution, we found a plateau of maximal responses to IPIs between 25 msec to 43 msec. This is also evident in the time courses of the responses, where responses to IPIs between 25 msec to 43 msec form a cluster which is clearly separated from shorter or longer IPIs (Figure 4B). This exactly reflects the IPI range of male courtship song in *D. melanogaster*, which centers at about 34 msec and has a standard deviation of about 6 msec (Popov, Sitnik et al. 2003). It seems that sudden transitions happen between 21 msec and 25 msec and between 43 msec and 47 msec.

The slowing down effect is contributed significantly by flies that completely cease their locomotion upon hearing the song. Therefore we also examined the proportion of flies that stopped (see methods) in responses to songs with varying IPIs (Figure 4C). We found that the low IPIs were actually as pro-stopping, if not more, as the intermediate IPIs that elicited the maximal slowing down in average speed. The contribution of the stopping flies to the average speed must be compensated by increased speed of other flies when songs with low IPIs were played. In contrast, the longer IPIs showed an attenuated effect of making flies stop. Therefore, we suggest that the low and high boundaries of the tuning curve are mediated by different mechanisms.

We also examined the tuning of males (Figure 4A). Like females, they also showed maximal responses to the intermediate IPIs that are within the natural range of courtship song. But they still responded markedly to the IPIs out of this range, which is consistent with previous results (von Schilcher 1976a).

Fru^M blocks female responses to pulse song

fruitless (*fru*) is one of the two pivotal genes downstream of *transformer* (*tra*) in the sex-determination hierarchy in *Drosophila* (Heinrichs, Ryner et al. 1998). Together with *doublesex*, alternative splicing of *fru* in the two sexes specifies sex-specific neural circuitry and behaviour (Demir and Dickson 2005; Kimura, Ote et al. 2005; Manoli, Foss et al. 2005; Stockinger, Kvitsiani et al. 2005; Vrontou, Nilsen et al. 2006; Rideout, Billeter et al. 2007; Kimura, Hachiya et al. 2008; Rideout, Dornan et al. 2010). The male specific isoform fru^M was shown to be

necessary for male courtship behaviour and when ectopically expressed in females, renders the females some aspects of male courtship behaviour but abnormality in sexual behaviours typical for females like receptivity and egg laying (Demir and Dickson 2005). Since the song responses we have reported are clearly sex-dimorphic, we sought to ask what roles fru^M plays to specify these sex-dimorphic behaviours. Females bearing one copy of fru^M, which is forced to splice in the male form to produce functional fru^M proteins, lost the responses to pulse song typical for both immature virgins and mature virgins (Figure 5A). They however didn't gain the speeding up responses of males. Their responses to sine song remain normal, which suggests the general auditory functions of these flies are not affected (Figure 5B). Therefore, these data suggest the responses to pulse song in females might be controlled by neural circuits that are amenable to the regulation of fru^M.

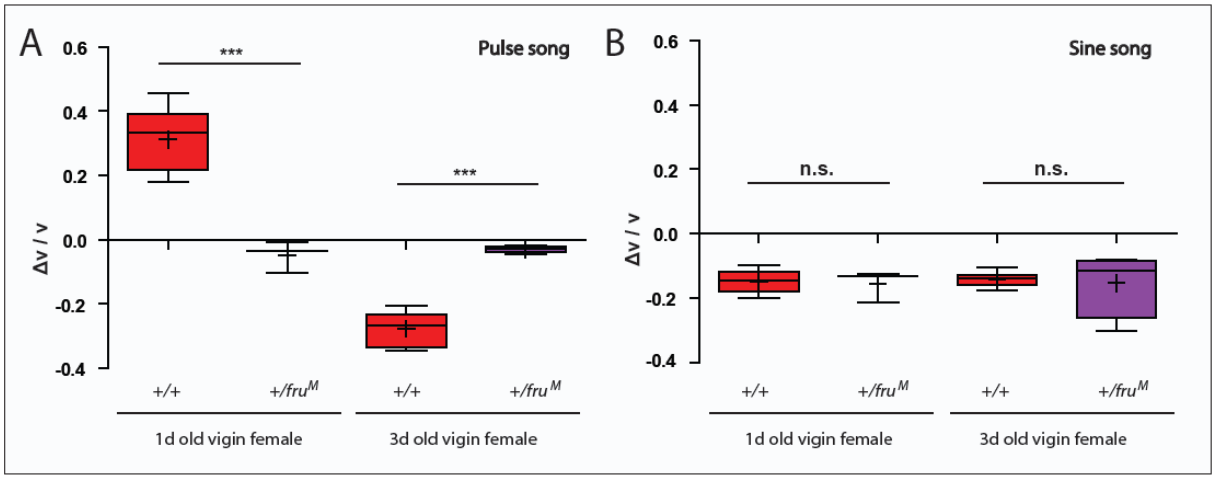


Figure 5. Fru^M blocks female responses to pulse song

(A) Responses 1d and 3d old fru^M virgin females and wild-type controls to pulse song. (B) Responses 1d and 3d old fru^M virgin females and wild-type controls to sine song. In both A and B, n = 10, 3, 10, 4 from left to right. In each plot, the box represents the interquartile range; the whiskers represent the min and max, the line in the box represents the median and the "+" represents the mean. Triple asterisk, $P < 0.0001$; n.s., $P > 0.05$; Student's t-test.

Part II A neuronal silencing screen identifies neural substrates for female sexual behaviours in Drosophila

A neuronal silencing screen for female sexual behaviours

Neural circuits underlying female sexual behaviours in *Drosophila* are poorly understood (Dickson 2008). Therefore, we carried out an unbiased neuronal silencing screen to search for the neural substrates of female sexual behaviours. We chose to screen an enhancer tiling *GAL4* collection generated in the lab (Masser and Bidaye, unpublished results) because it tends to label smaller population of neurons and provides more flexibility for further dissection with the known enhancer elements (Pfeiffer, Jenett et al. 2008). By crossing the *GAL4* drivers to a transgenic line expressing *UAS-Kir2.1*, an inward rectifying potassium channel (Baines, Uhler et al. 2001), we selectively silenced the neurons labeled by the driver lines in the F1 generation (Figure 6A). We tested the receptivity of virgin females by pairing them with wild-type males and recording videos for 30 minutes. We also qualitatively scored egg laying of both virgins and mated females (Figure 6B). In total we have screened 1680 lines. About half of the lines were lethal at various stages after crossing to *UAS-Kir2.1*. About 42% of the lines that were viable and didn't show any obvious general defects went through the behavioural tests (Figure 6C). Out of these lines, we found 35 lines showing various behavioural phenotypes, which are summarized in Figure 6D. Among those, 17 lines had receptivity below 30% but showed no phenotype in egg laying; 9 lines showed elevated egg laying in virgins but normal receptivity; 5 lines showed elevated egg laying in virgins and extremely low receptivity; 3 lines didn't lay a single egg but the receptivity was only slightly subnormal; and 1 line had very low receptivity while not laying any eggs. We haven't found lines that are normally receptive and retain eggs (but still lay a few) after they are mated, which was seen in flies with *SPR* knocked down in the *ppk* and *fru* double positive neurons (Hasemeyer, Yapici et al. 2009; Yang, Rumpf et al. 2009). This might be due to the fact that our egg laying assay is not sensitive enough, or alternatively, it reflects how the relevant circuits operate (silencing of the *ppk* and *fru* double positive neurons lead to the opposite phenotypes (Hasemeyer, Yapici et al. 2009; Yang, Rumpf et al. 2009)).

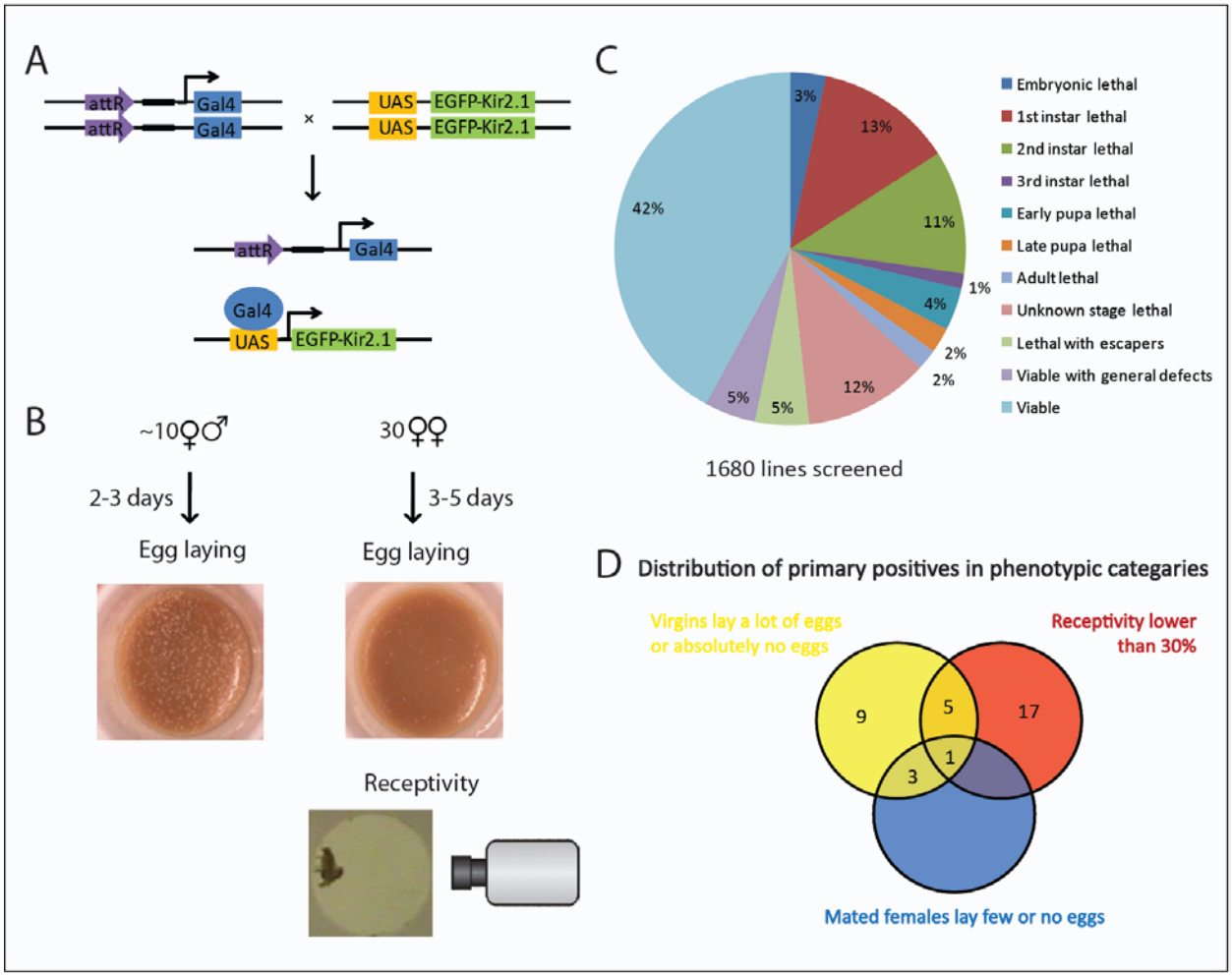


Figure 6. A neuronal silencing screen for female sexual behaviours

(A) In our screen, enhancer tiling *GAL4* lines were crossed to a transgenic line expressing an inward rectifying potassium channel *Kir2.1*. (B) Schematic of the behavioural assays for egg laying and receptivity. In our assay, we collect about 10 females together with several males and check their egg laying two to three days later while presuming they are mated. Wild-type females should have laid a lot of eggs (picture on the left; Yapici 2008). We also collect 30 virgins and age them for 3-5 days before we check their egg laying and receptivity. The wild-type virgins usually lay only a few eggs at this age (picture on the right; Yapici 2008). In the receptivity assay, we pair the virgins with single wild-type males and videotape for 30 minutes. The number of pairs copulated during this time are scored manually after the assay. (C) Classification of the phenotypes in the 1680 lines we have screened. (D) Distribution of primary positives in phenotypic categories.

GAL4 Lines are identified that label neurons important for receptivity and egg laying

In those primary positive lines that were retested, some were confirmed and showed rather strong phenotypes. The virgins of 3 lines after crossing to *UAS-Kir2.1*: *454-GAL4*, *45154-GAL4* and *3280-GAL4*, were completely unreceptive in 30 minutes after pairing with wild-type males (Figure 7A) and showed elevated egg laying rate that is typically seen in mated females (Figure 7B). Another line *7068-GAL4* showed extremely low receptivity yet laid normal amount of eggs as virgins (Figure 7A and 7B). To exclude the possibility that developmental defects due to constant silencing led to the behavioural phenotypes, we used the TARGET system to restrict the expression of *Kir2.1* to adulthood (McGuire, Mao et al. 2004). By expressing a temperature-sensitive GAL80 under control of a ubiquitous promoter, we suppressed *Kir2.1* expression at permissive temperature (22 ˚C) and only released it by shifting the flies to the restrictive temperature (30 ˚C) two days before the assay. While controls that were constantly in restrictive temperature showed normal receptivity, acute silencing of neurons labeled by 3 lines *454-GAL4*, *7068-GAL4* and *45154-GAL4* all led to reduced receptivity (Figure 7C). These results suggest that activity of the neurons labeled by these lines is important for female receptivity and egg laying.

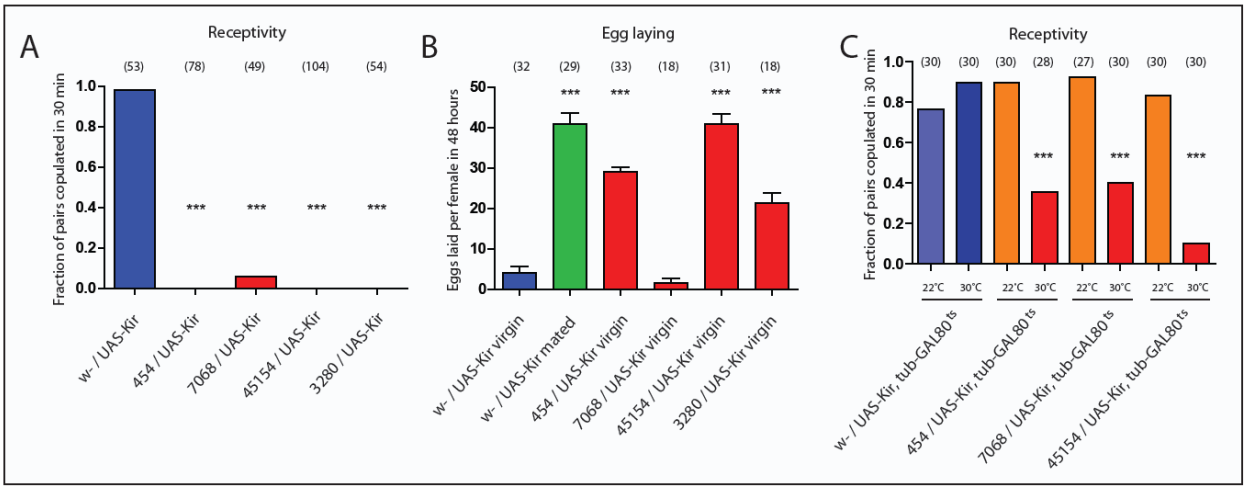


Figure 7. Identification of lines with impaired receptivity and egg laying in virgins

(A) Receptivity was severely impaired in 4 *GAL4* lines driving the expression of *Kir2.1*. (B) Egg laying rate in virgins was elevated in 3 lines except for 7068. (C) Acute silencing of neurons labeled by 3 *GAL4* lines only in adulthood leads to reduced receptivity. Parentheses denote the *n* for each column. Triple asterisk, $P < 0.0001$. In A,

Fisher's exact test, compared with the first column; in B, Student's *t*-test, compared with the first column; in C, Fisher's exact test, compared with the 22˚C control for each genotype.

Expression pattern and GRASP with *ppk* neurons

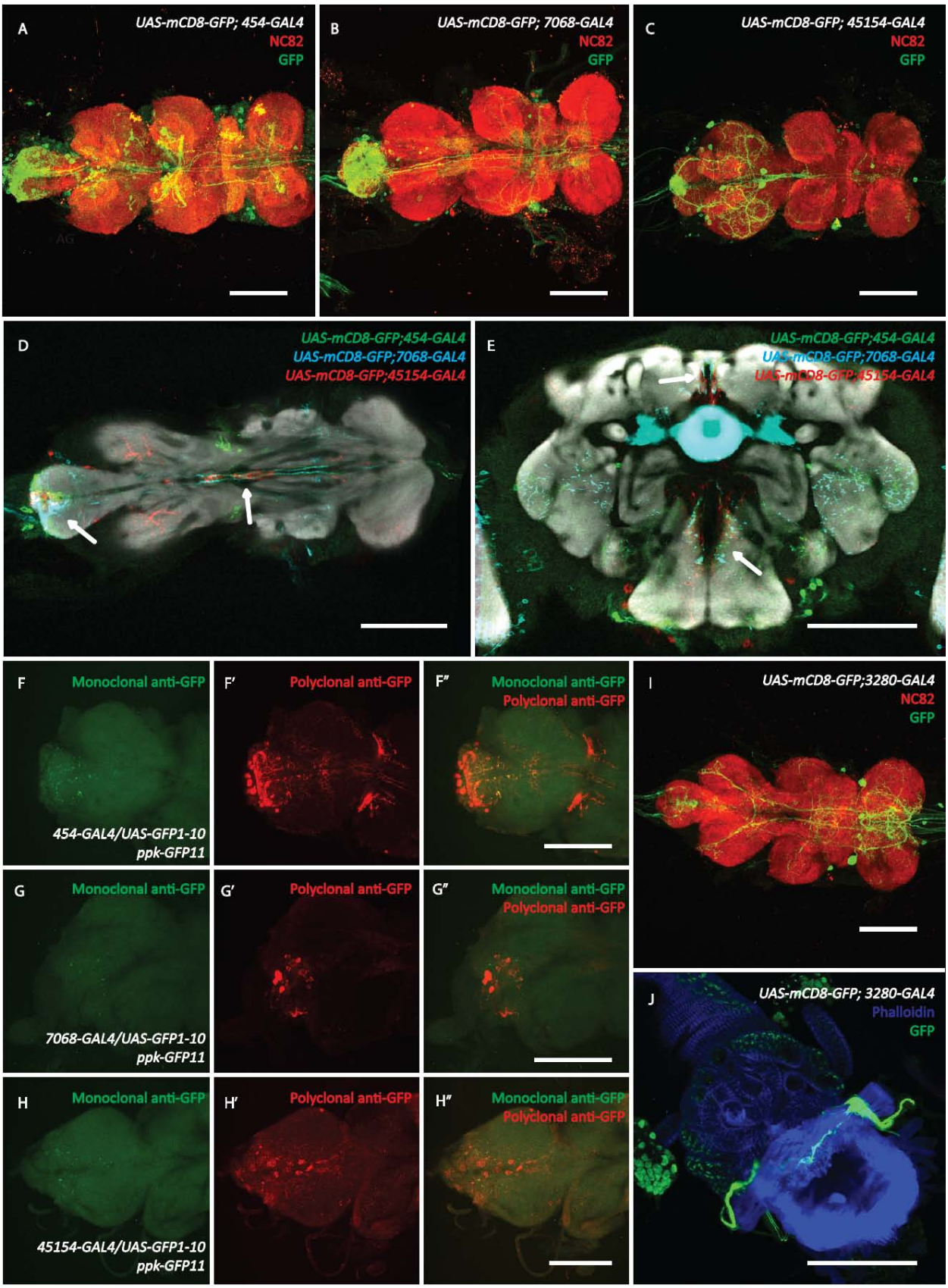
In order to assess the expression pattern of the *GAL4* lines in adult females, we expressed a membrane-tethered green fluorescent protein, mCD8GFP, under control of the *GAL4* lines. A few clusters of neurons and neuronal processes were labeled in the brain and ventral nerve cord (VNC) of each line. A region highlighted in all of the lines that showed impaired receptivity is the abdominal ganglion (Abg) in the VNC (Figure 8A-8C, 8I). To accurately compare neurons labeled by different *GAL4* lines in different tissue samples, we applied a non-rigid image registration algorithm to the samples against standard templates (Rohlfing and Maurer 2003; Jefferis, Potter et al. 2007; Yu 2009). In the overlay of the registered images of *454-GAL4*, *7068-GAL4*, *45154-GAL4*, we found an anchor-like structure of neuronal processes in the dorsal posterior Abg were labeled by all three lines (Figure 8D). Overlaps can also be seen in the median bundle and arborizations surrounding the oesophagus in the brain (Figure 8E). These regions are interconnected by two nerve tracts running along the dorsal midline of the VNC and the suboesophageal ganglion (SOG) in the brain (Figure 8D). However, we couldn't resolve the origin of these neurites or identify overlapping cell bodies because the expression of these lines are not restricted enough.

We think the expression in the abdominal ganglion is particularly interesting because the afferent projections from the SP sensing neurons in the female reproductive tract terminate in this region (Hasemeyer 2010). Silencing of the SP sensing neurons with *ppk-GAL4* led to unreceptive virgins laying elevated amount of eggs, which resembles mated females (Hasemeyer, Yapici et al. 2009; Yang, Rumpf et al. 2009). This raises the possibility that the similar phenotypes we saw by silencing the neurons labelled by the abovementioned lines could come from a common neural pathway. We found the expression pattern of *3280-GAL4* resembled the SP sensing neurons in the female reproductive tract and their projections in the Abg (Figure 8I-8J; Hasemeyer, unpublished results). Females remated at high rate when *SPR* is knocked down in *3280-GAL4* expressing neurons with RNAi (data not shown), which suggests this line indeed labels the SP sensing neurons. However, we didn't find the SP sensing neurons labeled by *454-*

GAL4, *7068-GAL4* and *45154-GAL4* in the female reproductive tract (data not shown). To assess the possibility that these lines label downstream neurons of the SP sensing neurons, we used GFP reconstitution across synaptic partners (GRASP) to check the connectivity between the neurons labeled by these lines and by *ppk-GAL4* (Feinberg, Vanhoven et al. 2008; Gordon and Scott 2009). We fused *ppk* promoter with a membrane-tethered *CD4::spGFP11* and used the GAL4 lines to drive the expression of *UAS-CD4::spGFP1-10*. If these two populations of neurons have processes in close proximity, full GFP can be reconstituted and recognized by a mouse monoclonal antibody specifically. We used a rabbit polyclonal GFP antibody that also recognizes the spGFP1-10 to orient ourselves to the neurons labeled by the GAL4 lines. As shown in Figure 8F-8H", strong GRASP signals can be detected in *454-GAL4*, and weaker ones in *7068-GAL4* and *45154-GAL4*. The localization of these signals is similar to the axonal terminals of the SP sensing neurons (Hasemeyer 2010)also see Figure 8I). These results suggest the GAL4 lines we identified could potentially label downstream neurons of the SP sensing neurons in the female reproductive tract, although further experiments are required to pinpoint the neurons responsible for the behavioural phenotypes and confirm the connectivity physiologically.

Figure 8. Expression pattern and GRASP with *ppk* neurons

(A-C) The expression patterns in the ventral nerve cord (VNC) of *454-GAL4*, *7068-GAL4* and *45154-GAL4*. Maximal projection of confocal stacks. (D-E) Overlay of the stainings registered against standard VNC and brain templates (gray) using a non-rigid warping algorithm. Confocal section for both VNC and brain. Arrows, colocalization of the three lines detected in the abdominal ganglion, two nerve tracks in the dorsal midline of the VNC, regions surrounding the esophagus and median bundle in the brain. (F-H") GRASP with *ppk-CD4::spGFP11*, stained with a mouse monoclonal antibody specific for the reconstituted full GFP (green) and a rabbit polyclonal GFP antibody that also recognizes the spGFP1-10 (red). (I-J) The expression pattern of *3280-GAL4* in the VNC and female reproductive tract. Maximal projection of confocal stacks. Scale bar, 100 μm.


A
UAS-mCD8-GFP; 454-GAL4
NC82
GFP
B
UAS-mCD8-GFP; 7068-GAL4
NC82
GFP
C
UAS-mCD8-GFP; 45154-GAL4
NC82
GFP
D
UAS-mCD8-GFP;454-GAL4
UAS-mCD8-GFP;7068-GAL4
UAS-mCD8-GFP;45154-GAL4
E
UAS-mCD8-GFP;454-GAL4
UAS-mCD8-GFP;7068-GAL4
UAS-mCD8-GFP;45154-GAL4
F
Monoclonal anti-GFP
454-GAL4/UAS-GFP1-10
ppk-GFP11
F′
Polyclonal anti-GFP
F″
Monoclonal anti-GFP
Polyclonal anti-GFP
G
Monoclonal anti-GFP
7068-GAL4/UAS-GFP1-10
ppk-GFP11
G′
Polyclonal anti-GFP
G″
Monoclonal anti-GFP
Polyclonal anti-GFP
H
Monoclonal anti-GFP
45154-GAL4/UAS-GFP1-10
ppk-GFP11
H′
Polyclonal anti-GFP
H″
Monoclonal anti-GFP
Polyclonal anti-GFP
I
UAS-mCD8-GFP;3280-GAL4
NC82
GFP
J
UAS-mCD8-GFP; 3280-GAL4
Phalloidin
GFP

Discussion

A quantitative assay for auditory responses in locomotion

To study the neuronal mechanisms of auditory processing and how auditory information is used to guide mating decisions in females, we require a reliable readout of the response to song when perturbing the female's genetics or physiological states. We also need to assess the female's preference for different songs when manipulating the song patterns. The female's response to song was often assessed by confining a group of females with mute males supplemented by playback of songs and measuring the mating speed (Bennet-Clark and Ewing 1969). There are two drawbacks of this method: first, the males emit other confounding cues that bring in unnecessary variables; second, males can also be stimulated by courtship song (von Schilcher 1976a; Crossley, Bennetclark et al. 1995) and therefore it is difficult to distinguish between the effect of song on females and males. To directly assess the female's response to song is difficult in *Drosophila* (Ritchie, Townhill et al. 1998). In two previous studies, locomotion of single-sex groups was examined while courtship song was played and females were observed to slow down in response to pulse song (von Schilcher 1976a; Crossley, Bennetclark et al. 1995). These results were thrown into confusion when another computer-tracking-based study failed to observe single female's response to song alone (Kowalski, Aubin et al. 2004).

Normal females become stationary before copulation occurs (Cook 1973; Tompkins, Gross et al. 1982) and in almost all cases a bout of pulse song was seen directly before copulation (von Schilcher 1976b), which suggests pulse song might be able to trigger females to stop. Although Kowalski et al. suggested females only slow down in response to pulse song in the context of males, the temporal resolution in their study (1 frame / sec) might be insufficient for them to observe brief stopping of single females (Kowalski, Aubin et al. 2004).

Although individual female's responses to a bout of song are highly variable, we observed robust reduction of the average speed in virgin females in response to pulse song. Indeed, we found the

slowing down effect is rather short-lasting and the dynamics are fast (Figure 1C and 1F). We also observed that most of the copulations happened immediately after a burst of pulse song started in the mating experiments with wingless males shown in Figure 2B, which confirmed the trigger-like function of pulse song (von Schilcher 1976b). Therefore, the choice of temporal structure of song playback and the time windows to compare the speed change is crucial for observing female response to song in locomotion.

Song responses as a proxy for mating decisions

Immature virgin females were shown to have no response to pulse song, which was different from the mature virgins that slowed down (von Schilcher 1976a). In contrast, we found that immature virgins speed up robustly in response to pulse song. The discrepancy can be explained by the difference in methods between these studies. von Schilcher quantified the number of moving flies instead of the average speed we used in our assay. This could have prevented him from observing the acceleration of immature virgins in response to pulse song.

We found song response is also regulated by mating status and mated females speed up in response to pulse song. Given the observation that locomotor activity of females is negatively correlated with their receptivity (Cook 1973), it is interesting to note that mated females as well as immature virgins show apparently “aversive” responses to pulse song in the absence of courting males compared to the “submissive” responses of mature virgins. We suggest pulse song alone might be sufficient to trigger a female to make a mating decision to a fictive male based on her own physiological states. Therefore, we could use female’s response to pulse song in locomotion as a proxy for female’s mating decision to study the underlying neural circuits.

We have noted that the responses in the controls of the *SPR* rescue experiments are diminished (Figure 3C). This is surprising to us because these lines were backcrossed for five generations to our wild-type standard line and therefore they should share the same genetic background. But given the fact that our standard line is not an isogenic stock, selection for certain trait could happen as a bottleneck effect in the single female crosses. Since the difference between virgin

and mated female is clear in the rescued flies but absent in the controls, we think our conclusion that *SPR* acts in *ppk* and *fru* neurons to modulate the female responses to pulse song is still valid.

The *SPR* deficiency females switch to speeding up in a brief period after mating, of which the mechanism is still not understood. One explanation is the *ppk* and *fru* double positive neurons might also be gated by mechanical stimuli. The uterus where these neurons locate undergoes conformational change after mating and Acp36DE, a male accessory gland protein, is required for this change (Avila and Wolfner 2009). A recent study found *ppk* actually encodes a DEG/ENaC protein that is involved in mechanical nociception (Zhong, Hwang et al. 2010). It would be interesting to test the *ppk* mutant females or wild-type females that mated with males lacking Acp36DE to ask if they remate and how they respond to the pulse song shortly after mating.

Female preference for conspecific IPI

Tuning for conspecific sounds is a conserved feature of auditory systems from insects to primates (Machens, Gollisch et al. 2005; Petkov, Kayser et al. 2008). Systematically mapping the female preference for certain song parameters will shed light on the roles of courtship song on speciation and sexual selection. It will also benefit our understanding of the neuronal mechanisms underlying such tuning. In *Drosophila melanogaster*, the main target for female tuning is thought to be the interpulse interval (IPI) rather than the carrier frequency (Bennet-Clark and Ewing 1969; Rybak, Aubin et al. 2002). Our results confirmed the female tuning to conspecific IPI and suggested that this tuning is not modulated by sexual maturity or mating status (Figure 4A).

Our quantitative analysis further revealed some interesting features of the IPI tuning. First, the tuning curve is a relatively broad plateau with steep slopes on both sides rather than a bell-shaped curve. Since we were looking at a population response, it is possible that individual females have slightly different preferences and the plateau reflects this variation. Alternatively, the tuning curve might be entirely a result of the auditory processing in females. Second, the

females do show behavioural responses to lower or higher IPIs out of the tuning range and these responses are different, which is evident in the precise time courses of the average speed and the number of stopping flies (Figure 4B and 4C). These results suggest the IPI tuning is unlikely a consequence of filtering during the early processing in the auditory system. The "wrong song" is actually "perceived" by the flies and generates less obvious behavioural responses, although it doesn't stimulate receptivity. This notion is corroborated by the fact that we could even detect behavioural responses of females to a single pulse (data not shown). A more obvious example is, a song with heterospecific IPI could elicit rejection in *Drosophila biauraria* females (Tomaru, Matsubayashi et al. 1995).

We used a constant IPI in these experiments for simplicity. It is unlikely that the average IPI is the only target for female preference. When we played the natural pulse song, the females showed greater responses than when any of the songs with a constant IPI was played (compare Figure 1E and Figure 4A). In addition, the rhythm in IPI was suggested to affect female preference in mating (Kyriacou and Hall 1982; Ritchie, Townhill et al. 1998). Nevertheless, our behavioural tuning curves should provide a starting point for mapping the neuronal tuning to auditory stimuli with physiological methods, which is essential for studying the neuronal mechanisms underlying auditory processing and song pattern recognition.

Sexual dimorphic responses to pulse song

Males and females respond differently to pulse song (von Schilcher 1976a; Crossley, Bennetclark et al. 1995; Kowalski, Aubin et al. 2004; Figure 1E and 1F). We have shown that the female responses could be blocked by ectopic expression of fru^M, yet these females didn't gain the male responses (Figure 5A). This is consistent with the observations that grouped fru^M females cannot be stimulated by pulse song to court each other more vigorously while fru^F males that lack fru^M protein show normal pulse-song-induced courtship (data not shown). These data suggest that the male responses to pulse song are *doublesex (dsx)* dependent. It is generally believed that these two transcription factors in the sexual determination pathway specify the sexual dimorphic neural circuits which in turn control the sexual specific behaviours (Demir and

Dickson 2005; Manoli, Foss et al. 2005; Rideout, Billeter et al. 2007; Clyne and Miesenbock 2008; Kimura, Hachiya et al. 2008). With the neuronal populations expressing these two transcription factors well characterized and genetic tools to access these neurons available (Manoli, Foss et al. 2005; Stockinger, Kvitsiani et al. 2005; Kimura, Hachiya et al. 2008; Yu 2009; Rideout, Dornan et al. 2010), we are at a good starting point to narrow down which neurons are responsible for the sexual dimorphic responses to pulse song.

Besides the overt differences, we noticed two sexual dimorphic features in the responses. First, compared with females that only show "phasic" responses, males respond "tonically" to pulse song. The female responses decay fast and this process starts while the song is still playing (Figure 1F). In contrast, the male responses sustain for longer time even after the song ends (Figure 1F), which is consistent with previous results (von Schilcher 1976a). Second, the tuning curve of males to IPI seems to be broader than females, at least in the long-IPI end. Female responses show a pronounced reduction to IPIs below 17 msec or above 68 msec in both average locomotion speed (Figure 4A) and mating speed with wingless males (Bennet-Clark and Ewing 1969). In contrast, males respond robustly even to IPIs above 80ms (von Schilcher 1976a; Figure 4A). To IPIs shorter than 17 ms, von Schilcher found drastically diminished responses (von Schilcher 1976a), which is different from our results (Figure 4A). However, we noticed that the male responses to IPIs below 17 msec are much less sustainable (data not shown). These results suggest that the sexual dimorphic control of song responses may occur in song pattern recognition as well as action selection and motor output.

Conclusion I

We have established a quantitative assay to assess song responses of single flies. The precise quantification and high temporal resolution in our system allow us to reveal rather subtle and brief behavioural responses that are difficult to uncover with traditional methods. The automated video tracking system provides a reliable solution for handling rather large number of animals in genetic screens. By characterizing the responses of wild-type flies under different physiological states to various song types, we have established a baseline for genetic manipulations. The next

step is to explore the neural circuits underlying auditory processing and female mating decision by probing altered behavioural responses in animals with specific neuronal activity perturbed genetically.

A neuronal silencing screen for female sexual behaviours

Traditionally, the genetic approach to understand the neural mechanisms underlying behaviours is rather indirect. It involves isolating genes essential for the behaviours and mapping the neurons in which the genes function. The development of new tools in recent years has made it possible to make specific perturbations in genetically labeled neurons and directly assess the neuronal functions in certain behaviours (Luo, Callaway et al. 2008). As an analogue to forward genetics, we could screen random GAL4 lines unbiasedly in certain behavioural paradigms. Alternatively, as an analogue to reverse genetics, we could preselect GAL4 lines based on the expression patterns and specifically perturb neurons in some candidate regions in the nervous system. We decided to use an unbiased approach to screen for neural substrates underlying female sexual behaviours because little knowledge is available so far about the relevant circuits. We expect to identify neural circuits responsible for different aspects of female receptivity and egg laying, including sensory processing, action selection and motor control, although our screen is still in the early stages. With the knowledge of the specific behavioural phenotypes and expression patterns of the positive lines, we should be able to do more targeted secondary assays to tease out the components and finally assemble a whole picture for the neural control of female mating decision.

Candidate second-order neurons for the SP sensors

We found very low receptivity and elevated egg laying in virgin females when neurons labeled by several *GAL4* lines are silenced (Figure 7). We also included *7068-GAL4* in the analysis, which showed normally low egg laying rates in virgins (Figure 7). *7068-GAL4* labels neurons morphologically similar to the octopaminergic neurons that control ovulation (Monastirioti 2003)

and neuronal arborizations were found in the ovary and oviduct. Therefore, we speculate that there might be a separate population of neurons directly suppressing egg laying in this line.

Low receptivity and elevated egg laying resemble the behaviours of wild-type mated females and virgin females in which the neurons sensing SP are silenced (Hasemeyer, Yapici et al. 2009; Yang, Rumpf et al. 2009). The preliminary GRASP results suggest that neurons labeled by these lines might form synaptic connections with the *ppk* expressing neurons that sense SP. Although it is tantalizing to hypothesize that the behavioural phenotypes we saw when silencing these *GAL4* lines are mediated by the downstream neurons of the *SP* sensors, we still have several steps to go before we can make any conclusions.

Each of these *GAL4* lines labels several clusters of neurons in the brain and VNC. We haven't been able to identify the neurons specifically responsible for the behavioural phenotypes. Several methods can be used to circumvent this problem. First, we could stochastically label subsets of the neurons and correlate the behavioural phenotypes and neurons labeled in each animal. This method has been used to successfully identify a cluster of motor neurons that control the proboscis extension reflex (Gordon and Scott 2009). Second, if multiple lines label the same cluster of neurons, we could use the split-GAL4 system (Luan, Lemon et al. 2006) or other combinatory methods (Potter, Tasic et al. 2010) to specifically manipulate the overlapping neurons in behavioural experiments. If any of these methods work, we should also be able to resolve the projection patterns of the candidate neurons because the labeling would be much sparser.

To establish the connectivity between the candidate neurons and the SP sensors, we need to circumvent some caveats in the GRASP experiments. First, we used the *ppk* promoter to drive the expression of one of split-GFP halves. Since a lot of peripheral sensory neurons other than the SP sensors are labeled by this promoter and project to the abdominal ganglion (Abg), we need to show these GRASP signals come from the right neurons. *3280-GAL4*, in which the projections of the SP sensors in the Abg are clearly resolvable (Figure 8I), should be able to help us to circumvent this problem. Second, the GRASP system was not tested rigorously in *Drosophila* to show that signals can only be detected when synapses are formed. Therefore, we need either anatomical evidence from electron microscopy to show the synaptic connections or

physiological evidence that these neurons are functionally coupled. One method to achieve this is to activate the SP sensors with *TrpA1* (Pulver, Pashkovski et al. 2009) while monitoring the activity of candidate downstream neurons with Ca^{2+} imaging (Tian, Hires et al. 2009). Third, while the GRASP signals were very strong with *454-GAL4* and clearly colocalized with some neurites labeled by the *GAL4* line, the signals were much weaker with *7068-GAL4* and *45154-GAL4* and the colocalization was less clear. Possible reasons include that these lines are much weaker and the polyclonal anti-GFP antibody doesn't stain GFP1-10 in the neurites labeled by the *GAL4* lines very well. We need to repeat the experiments and increase the signal-noise ratio to exclude the possibility that these signals are non-specific.

The similarity of phenotypes and expression patterns in these lines suggest that they might label some neurons in common. However, we cannot prove this until we examine the expression patterns driven by these enhancer tiles in the same animal. By fusing the enhancer tiles with a fluorescent protein indicator or using other binary expression systems (Lai and Lee 2006; Potter, Tasic et al. 2010), we should be able to achieve such double labeling. Alternatively, these lines may label different parts of the neural circuits that control female sexual behaviours. In this case, it would be interesting to examine whether they are interconnected and how information flows in these circuits.

Besides receptivity and egg laying, multiple behaviours, including feeding (Carvalho, Kapahi et al. 2006), sleeping (Isaac, Li et al. 2009) and song responses (Figure 3B and 3C), are regulated by SP. At least for receptivity, egg laying and song responses, the *ppk* and *fru* double positive neurons in the female reproductive tract seem to act as a master switch (Hasemeyer, Yapici et al. 2009; Yang, Rumpf et al. 2009; Figure 3C). Where does the downstream neural pathway of the SP sensors segregate to control each of the behaviours? Identification of the second-order neurons of the SP sensors will provide a great opportunity to assess how neural circuits are organized to relay the same sensory information to control different behaviours.

Conclusion II

Female sexual behaviours in *Drosophila* are complex yet well controlled. Multiple sensory cues and internal states must be sensed and integrated in the brain to guide the mating decisions. We performed a neuronal silencing screen to search for the neural substrates underlying female sexual behaviours. In our initial results, *GAL4* lines labeling candidate second-order neurons of the SP sensors have been uncovered. Characterization of these lines might provide unique insights into how information is relayed from an internal sensor to the brain to regulate multiple behaviourals.

Materials and methods

Fly stocks

The wild-type standard line used in the song response assay is bearing a *hs-hid* transgene on the Y chromosome and derived from a laboratory *Canton S* stock. Another wild-type line from the same genetic background with a compound X chromosome bearing a *hs-hid* transgene was used to generate large quantity of males. SP^{0} is a gift from Eric Kubli (Liu and Kubli 2003); *UAS-EGFP-Kir2.1*, *UAS-EGFP-Kir2.1*, $tub\text{-}GAL80^{ts}$ and *UAS-CD4-spGFP1-10* are gifts from Kristin Scott (Fischler, Kong et al. 2007; Gordon and Scott 2009). *ppk-GAL4*, fru^{GAL}, *UAS-SPR* and *Df(1)Exel6234* (Stockinger, Kvitsiani et al. 2005; Yapici, Kim et al. 2008; Hasemeyer, Yapici et al. 2009) were backcrossed to the wild-type standard line for five generations. fru^{M} (Demir and Dickson 2005) was backcrossed to the wild-type standard line in 3 generations of single female crosses and 3 generations of single male crosses (since the fertility of fru^{M} females is extremely low, we have to set up many single female crosses and select the males bearing the fru^{M} allele in the progeny to set up single male crosses in order to get a lot of females for the next crosses). *ppk-CD4-GFP11* is generated by Martin Hasemeyer by subcloning the *CD4-spGFP11* DNA fragment into a construct containing the *ppk* promoter (Gordon and Scott 2009; Hasemeyer, Yapici et al. 2009). The enhancer tiling GAL4 lines are generated by Christopher Masser and Salil Bidaye in the lab.

All the flies used in this work were raised on standard cornmeal-yeast-agar medium at 25 ˚C and 60% humidity, except for the $tub\text{-}GAL80^{ts}$ flies that were raised in 22 ˚C and shifted to 25 ˚C two days before the behavioural assays. The flies bearing *hs-hid* transgene were heat-shocked for 1.5 hours during early pupa stage to generate large quantity of virgin females or males.

Behavioural assays

The song response assay was done as described in the main text. The videos were recorded with a SONY HDR-SR5CE camcorder at 25 fps with a resolution of 1440:1080. The original songs were recorded digitally from wild-type *Canton S* males in the lab by Alex Keene. The white noise was generated with a build-in function in the software Goldwave. A short clip of song was repeated until reaching the duration of 5 seconds to generate a song burst. The songs for the tuning experiments were generated by inserting various lengths of silence after a standard single pulse taken from the original pulse song and these patterns were repeated until the duration of 5 seconds was reached. The amplitude of the song playback was at about 100 dB SPL for the peak frequency in the position where the chambers are placed.

In the GAL4 screen, the virgin females from the crosses as well as wild-type males were collected shortly after eclosion and aged in groups of about 30 virgins or males for 3-5 days. Receptivity assays were performed in round Perspex chambers with a diameter of 10 mm and a height of 4 mm. Single tester virgin female and wild-type male were introduced into each chamber before the video recording started. The courtship was videotaped for 30 minutes with 30 pairs for each genotype. Number of pairs copulated within 5, 10 and 30 minutes were scored manually from the videos. The food tubes housing these virgins were examined for the virgin egg laying after the receptivity assay. Another 10 females and 10 males from the crosses were collected and aged together for 2-3 days before the food tubes were examined for the egg laying of mated females.

All the flies were reared in a 12h:12h light:dark cycle with the dawn at 8 am. All the behavioural assays were done between 1 pm to 5 pm.

Data analysis

The videos for the song response assay were tracked with customized MATLAB software. The coordinate of each fly in every frame was detected. Euclidean distance was calculated for each

fly between two consecutive frames to derive the speed (Figure 1C top shows an example of this original speed of a single fly). The speed for each fly throughout a video was averaged in a moving window of 25 frames before the average speed of the 28 flies was calculated for each frame. The sound track was used to detect the frame at which the song started (frame 0) for each video. The average speed of the 28 flies in the 600 frames centered at frame 0 was further averaged across the 6 repetitions of song bursts (Figure 1C bottom shows an example of this averaged trace across 28 flies and 6 song repetitions). Further computations were based on this average speed. The mean of the average speed in the 300 frames before frame 0 was calculated as the basal activity v. The maximum and minimum of the average speed in the 150 frames after frame 0 was denoted as v_{max} and v_{min}. The metric Δv we used to quantify the responses in one assay was calculated with the following formula: $\Delta v = (v_{max} - v) - (v - v_{min}) = v_{max} + v_{min} - 2v$.

In the time courses (Figure 1F and Figure 4B), the average speed across 28 flies and 6 song repetitions was further averaged and normalized against v across multiple assays for each experimental condition.

Euclidean distance between the frames T-2 and T+2 (time delay 160 msec) for each fly was calculated. If this distance was smaller than 2 pixels (0.2 mm), the fly was considered to stop at the frame T (slower than 1.25 mm/sec). The time courses (Figure 4C) were calculated with the same procedure of averaging and normalization as for the speed.

Immunohistochemistry and image registration

Immunohistochemistry and image registration were done by following the protocols described by Jai Yu (Yu 2009). Briefly, tissues were dissected in PBS and fixed with 4% PFA for 20 minutes at RT. After fixation, tissues were washed 3 times with PBST (PBS in 0.3% triton-X) and blocked in 10% goat serum blocking solution for 4 hours at RT before incubated in primary antibodies for 48 hours at 4 ˚C and in secondary antibodies for 72 hours at 4 ˚C. After both primary and secondary antibody incubation, tissues were washed 3 times for 15 min at RT and 1 time overnight at 4 ˚C. Confocal scanning was performed and the stacks were registered against

standard tissues (Yu 2009) with a non-rigid registration algorithm (Jefferis, Potter et al. 2007) in customized software Brainwarp.

Antibody concentrations:

Primary antibodies:	
Polyclonal rabbit anti-GFP (Torrey Pines)	1:3000
Monoclonal mouse anti-GFP (Sigma)	1:100
Mouse anti-nc82 (DSHB)	1:20
Secondary antibodies:	
Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen)	1:500
Alexa 488-conjugated goat anti-mouse IgG (Invitrogen)	1:500
Alexa 647-conjugated goat anti-mouse IgG (Invitrogen)	1:500
Alexa Fluor 647 phalloidin (Invitrogen)	1:100

References

Aigaki, T., I. Fleischmann, et al. (1991). "Ectopic expression of sex peptide alters reproductive behavior of female D. melanogaster." Neuron **7**(4): 557-63.

Albert, J. T., B. Nadrowski, et al. (2007). "Drosophila mechanotransduction--linking proteins and functions." Fly (Austin) **1**(4): 238-41.

Alt, S., J. Ringo, et al. (1998). "The period gene controls courtship song cycles in Drosophila melanogaster." Anim Behav **56**(1): 87-97.

Amrein, H. (2004). "Pheromone perception and behavior in Drosophila." Curr Opin Neurobiol **14**(4): 435-42.

Avila, F. W. and M. F. Wolfner (2009). "Acp36DE is required for uterine conformational changes in mated Drosophila females." Proc Natl Acad Sci U S A **106**(37): 15796-800.

Baines, R. A., J. P. Uhler, et al. (2001). "Altered electrical properties in Drosophila neurons developing without synaptic transmission." J Neurosci **21**(5): 1523-31.

Baird, G. S., D. A. Zacharias, et al. (1999). "Circular permutation and receptor insertion within green fluorescent proteins." Proc Natl Acad Sci U S A **96**(20): 11241-6.

Bellen, H. J., C. J. O'Kane, et al. (1989). "P-element-mediated enhancer detection: a versatile method to study development in Drosophila." Genes Dev **3**(9): 1288-300.

Bennet-Clark, H. (1971). "Acoustics of Insect Song." Nature **234**: 255-259.

Bennet-Clark, H. and A. W. Ewing (1969). "Pulse interval as a critical parameter in the courtship song of Drosophila melanogaster." Anim. Behav. **17**: 755-759.

Bennet-Clark, H. C. and A. W. Ewing (1967). "Stimuli providedby Courtship of Male Drosophila melanogaster." Nature **215**: 669-671.

Bennet-Clark, H. C. and A. W. Ewing (1970). "The love song of the fruit fly." Sci Am **223**(1): 85-90 passim.

Bennet-Clark, H. C., A. W. Ewing, et al. (1973). "The persistence of courtship stimulation in Drosophila melanogaster." Behav Biol **8**(6): 763-9.

Benton, R., K. S. Vannice, et al. (2007). "An essential role for a CD36-related receptor in pheromone detection in Drosophila." Nature **450**(7167): 289-93.

Billeter, J. C., J. Atallah, et al. (2009). "Specialized cells tag sexual and species identity in Drosophila melanogaster." Nature **461**(7266): 987-91.

Brand, A. H. and N. Perrimon (1993). "Targeted gene expression as a means of altering cell fates and generating dominant phenotypes." Development **118**(2): 401-15.

Butterworth, F. M. (1969). "Lipids of Drosophila: a newly detected lipid in the male." Science **163**(873): 1356-7.

Caldwell, J. C. and D. F. Eberl (2002). "Towards a molecular understanding of Drosophila hearing." J Neurobiol **53**(2): 172-89.

Carvalho, G. B., P. Kapahi, et al. (2006). "Allocrine modulation of feeding behavior by the Sex Peptide of Drosophila." Curr Biol **16**(7): 692-6.

Castrillon, D. H., P. Gonczy, et al. (1993). "Toward a molecular genetic analysis of spermatogenesis in Drosophila melanogaster: characterization of male-sterile mutants generated by single P element mutagenesis." Genetics **135**(2): 489-505.

Chapman, T. (1996). "Lack of response to sex-peptide results in increased cost of mating in dunce Drosophila melanogaster females " Journal of Insect Physiology **42**(11-12): 1007-1015.

Chapman, T., J. Bangham, et al. (2003). "The sex peptide of Drosophila melanogaster: female post-mating responses analyzed by using RNA interference." Proc Natl Acad Sci U S A **100**(17): 9923-8.

Chapman, T. and S. J. Davies (2004). "Functions and analysis of the seminal fluid proteins of male Drosophila melanogaster fruit flies." Peptides **25**(9): 1477-90.

Chapman, T., L. A. Herndon, et al. (2001). "The Acp26Aa seminal fluid protein is a modulator of early egg hatchability in Drosophila melanogaster." Proc Biol Sci **268**(1477): 1647-54.

Chapman, T., L. F. Liddle, et al. (1995). "Cost of mating in Drosophila melanogaster females is mediated by male accessory gland products." Nature **373**(6511): 241-4.

Chen, P. S., E. Stumm-Zollinger, et al. (1988). "A male accessory gland peptide that regulates reproductive behavior of female D. melanogaster." Cell **54**(3): 291-8.

Cirera, S. and M. Aguade (1997). "Evolutionary history of the sex-peptide (Acp70A) gene region in Drosophila melanogaster." Genetics **147**(1): 189-97.

Clyne, J. D. and G. Miesenbock (2008). "Sex-specific control and tuning of the pattern generator for courtship song in Drosophila." Cell **133**(2): 354-63.

Cook, R. M. (1973). "Courtship processing in Drosophila melanogaster. II. An adaptation to selection for receptivity to wingless males." Anim Behav **21**(2): 349-58.

Couto, A., M. Alenius, et al. (2005). "Molecular, anatomical, and functional organization of the Drosophila olfactory system." Curr Biol **15**(17): 1535-47.

Crossley, S. A. (1988). "Failure to Confirm Rhythms in Drosophila Courtship Song." Animal Behaviour **36**: 1098-1109.

Crossley, S. A., H. C. Bennetclark, et al. (1995). "Courtship Song Components Affect Male and Female Drosophila Differently." Animal Behaviour **50**: 827-839.

Datta, S. R., M. L. Vasconcelos, et al. (2008). "The Drosophila pheromone cVA activates a sexually dimorphic neural circuit." Nature **452**(7186): 473-7.

Demir, E. and B. J. Dickson (2005). "fruitless splicing specifies male courtship behavior in Drosophila." Cell **121**(5): 785-94.

Dickson, B. J. (2008). "Wired for sex: the neurobiology of Drosophila mating decisions." Science **322**(5903): 904-9.

Dietzl, G., D. Chen, et al. (2007). "A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila." Nature **448**(7150): 151-6.

DiPilato, L. M., X. Cheng, et al. (2004). "Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments." Proc Natl Acad Sci U S A **101**(47): 16513-8.

Dukas, R. (2008). "Learning affects mate choice in female fruit flies." Behavioral Ecology **16(4)**: 800-804.

Eberl, D. F. and G. Boekhoff-Falk (2007). "Development of Johnston's organ in Drosophila." Int J Dev Biol **51**(6-7): 679-87.

Eberl, D. F., G. M. Duyk, et al. (1997). "A genetic screen for mutations that disrupt an auditory response in Drosophila melanogaster." Proc Natl Acad Sci U S A **94**(26): 14837-42.

Ejima, A. and L. C. Griffith (2008). "Courtship initiation is stimulated by acoustic signals in Drosophila melanogaster." PLoS One **3**(9): e3246.

Ewing, A. W. (1978). "Antenna of Drosophila as a Love Song Receptor." Physiological Entomology **3**(1): 33-36.

Ewing, A. W. (1988). "Cycles in the Courtship Song of Male Drosophila-Melanogaster Have Not Been Detected." Animal Behaviour **36**: 1091-1097.

Ewing, A. W. and H. Bennet-Clark (1968). "The Courtship Songs of Drosophila " Behaviour **31**: 288-301.

Feinberg, E. H., M. K. Vanhoven, et al. (2008). "GFP Reconstitution Across Synaptic Partners (GRASP) defines cell contacts and synapses in living nervous systems." Neuron **57**(3): 353-63.

Fischler, W., P. Kong, et al. (2007). "The detection of carbonation by the Drosophila gustatory system." Nature **448**(7157): 1054-7.

Fishilevich, E. and L. B. Vosshall (2005). "Genetic and functional subdivision of the Drosophila antennal lobe." Curr Biol **15**(17): 1548-53.

Fowler, K. and L. Partridge (1989). "A cost of mating in female fruitflies." Nature **338**: 760 - 761.

Gailey, D. A. and J. C. Hall (1989). "Behavior and cytogenetics of fruitless in Drosophila melanogaster: different courtship defects caused by separate, closely linked lesions." Genetics **121**(4): 773-85.

Gerhardt, H. C. and F. Huber (2002). Acoustic communication in insects and anurans : common problems and diverse solutions. Chicago, University of Chicago Press.

Gill, K. S. (1963). "A mutation causing abnormal courtship and mating behavior in males of Drosophila melanogaster." Am. Zool. 3: s507.

Golic, K. G. and S. Lindquist (1989). "The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome." Cell **59**(3): 499-509.

Gong, Z., W. Son, et al. (2004). "Two interdependent TRPV channel subunits, inactive and Nanchung, mediate hearing in Drosophila." J Neurosci **24**(41): 9059-66.

Gopfert, M. C., A. D. Humphris, et al. (2005). "Power gain exhibited by motile mechanosensory neurons in Drosophila ears." Proc Natl Acad Sci U S A **102**(2): 325-30.

Gopfert, M. C. and D. Robert (2001). "Biomechanics. Turning the key on Drosophila audition." Nature **411**(6840): 908.

Gopfert, M. C. and D. Robert (2002). "The mechanical basis of Drosophila audition." J Exp Biol **205**(Pt 9): 1199-208.

Gopfert, M. C. and D. Robert (2003). "Motion generation by Drosophila mechanosensory neurons." Proc Natl Acad Sci U S A **100**(9): 5514-9.

Gordon, M. D. and K. Scott (2009). "Motor control in a Drosophila taste circuit." Neuron **61**(3): 373-84.

Greenacre, M. L., M. G. Ritchie, et al. (1993). "Female song preference and the period gene in Drosophila." Behav Genet **23**(1): 85-90.

Greenspan, R. J. and J. F. Ferveur (2000). "Courtship in Drosophila." Annu Rev Genet **34**: 205-232.

Griesbeck, O., G. S. Baird, et al. (2001). "Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications." J Biol Chem **276**(31): 29188-94.

Grillet, M., L. Dartevelle, et al. (2006). "A Drosophila male pheromone affects female sexual receptivity." Proc Biol Sci **273**(1584): 315-23.

Groth, A. C., M. Fish, et al. (2004). "Construction of transgenic Drosophila by using the site-specific integrase from phage phiC31." Genetics **166**(4): 1775-82.

Haesemeyer, M., N. Yapici, et al. (2009). "Sensory neurons in the Drosophila genital tract regulate female reproductive behavior." Neuron **61**(4): 511-8.

Hall, J. C. (1994). "The mating of a fly." Science **264**(5166): 1702-14.

Hamada, F. N., M. Rosenzweig, et al. (2008). "An internal thermal sensor controlling temperature preference in Drosophila." Nature **454**(7201): 217-20.

Han, D. D., D. Stein, et al. (2000). "Investigating the function of follicular subpopulations during Drosophila oogenesis through hormone-dependent enhancer-targeted cell ablation." Development **127**(3): 573-83.

Hasemeyer, M. (2010). Identification and characterization of the cellular targets of sex-peptide mediating the post mating switch in female *Drosophila melanogaster*, University of Vienna. **PhD thesis**.

Hasemeyer, M., N. Yapici, et al. (2009). "Sensory neurons in the Drosophila genital tract regulate female reproductive behavior." Neuron **61**(4): 511-8.

Heinrichs, V., L. C. Ryner, et al. (1998). "Regulation of sex-specific selection of fruitless 5' splice sites by transformer and transformer-2." Mol Cell Biol **18**(1): 450-8.

Herndon, L. A. and M. F. Wolfner (1995). "A Drosophila seminal fluid protein, Acp26Aa, stimulates egg laying in females for 1 day after mating." Proc Natl Acad Sci U S A **92**(22): 10114-8.

Isaac, R. E., C. Li, et al. (2009). "Drosophila male sex peptide inhibits siesta sleep and promotes locomotor activity in the post-mated female." Proc Biol Sci **277**(1678): 65-70.

Ito, H., K. Fujitani, et al. (1996). "Sexual orientation in Drosophila is altered by the satori mutation in the sex-determination gene fruitless that encodes a zinc finger protein with a BTB domain." Proc Natl Acad Sci U S A **93**(18): 9687-92.

Jefferis, G. S., C. J. Potter, et al. (2007). "Comprehensive maps of Drosophila higher olfactory centers: spatially segregated fruit and pheromone representation." Cell **128**(6): 1187-203.

Juni, N. and D. Yamamoto (2009). "Genetic analysis of chaste, a new mutation of Drosophila melanogaster characterized by extremely low female sexual receptivity." J Neurogenet **23**(3): 329-40.

Kamikouchi, A., H. K. Inagaki, et al. (2009). "The neural basis of Drosophila gravity-sensing and hearing." Nature **458**(7235): 165-71.

Kamikouchi, A., T. Shimada, et al. (2006). "Comprehensive classification of the auditory sensory projections in the brain of the fruit fly Drosophila melanogaster." J Comp Neurol **499**(3): 317-56.

Kim, J., Y. D. Chung, et al. (2003). "A TRPV family ion channel required for hearing in Drosophila." Nature **424**(6944): 81-4.

Kim, Y. J., K. Bartalska, et al. (2010). "MIPs are ancestral ligands for the sex peptide receptor." Proc Natl Acad Sci U S A **107**(14): 6520-5.

Kimura, K., T. Hachiya, et al. (2008). "Fruitless and doublesex coordinate to generate male-specific neurons that can initiate courtship." Neuron **59**(5): 759-69.

Kimura, K., M. Ote, et al. (2005). "Fruitless specifies sexually dimorphic neural circuitry in the Drosophila brain." Nature **438**(7065): 229-33.

Kitamoto, T. (2001). "Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons." J Neurobiol **47**(2): 81-92.

Koganezawa, M., D. Haba, et al. "The shaping of male courtship posture by lateralized gustatory inputs to male-specific interneurons." Curr Biol **20**(1): 1-8.

Kowalski, S., T. Aubin, et al. (2004). "Courtship song in Drosophila melanogaster: a differential effect on male–female locomotor activity." Canadian Journal of Zoology **82**: 1258-1266.

Krashes, M. J., S. DasGupta, et al. (2009). "A neural circuit mechanism integrating motivational state with memory expression in Drosophila." Cell **139**(2): 416-27.

Kubli, E. (2003). "Sex-peptides: seminal peptides of the Drosophila male." Cell Mol Life Sci **60**(8): 1689-704.

Kurtovic, A., A. Widmer, et al. (2007). "A single class of olfactory neurons mediates behavioural responses to a Drosophila sex pheromone." Nature **446**(7135): 542-6.

Kvitsiani, D. and B. J. Dickson (2006). "Shared neural circuitry for female and male sexual behaviours in Drosophila." Curr Biol **16**(10): R355-6.

Kyriacou, C. and J. Hall (1982). "The function of courtship song rhythms in Drosophila." Anim. Behav. **30**: 794-801.

Kyriacou, C. P. and J. C. Hall (1980). "Circadian rhythm mutations in Drosophila melanogaster affect short-term fluctuations in the male's courtship song." Proc Natl Acad Sci U S A **77**(11): 6729-33.

Kyriacou, C. P. and J. C. Hall (1984). "Learning and Memory Mutations Impair Acoustic Priming of Mating-Behavior in Drosophila." Nature **308**(5954): 62-65.

Kyriacou, C. P. and J. C. Hall (1986). "Interspecific genetic control of courtship song production and reception in Drosophila." Science **232**(4749): 494-7.

Lai, S. L. and T. Lee (2006). "Genetic mosaic with dual binary transcriptional systems in Drosophila." Nat Neurosci **9**(5): 703-9.

Lam, B. J., A. Bakshi, et al. (2003). "Enhancer-dependent 5'-splice site control of fruitless pre-mRNA splicing." J Biol Chem **278**(25): 22740-7.

Laughlin, S., T. Meehan, et al. (2008). "Prolactin levels in Western Lowland gorillas." Am J Primatol **70**(9): 897-900.

Lee, T. and L. Luo (1999). "Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis." Neuron **22**(3): 451-61.

Lima, S. Q. and G. Miesenbock (2005). "Remote control of behavior through genetically targeted photostimulation of neurons." Cell **121**(1): 141-52.

Liu, H. and E. Kubli (2003). "Sex-peptide is the molecular basis of the sperm effect in Drosophila melanogaster." Proc Natl Acad Sci U S A **100**(17): 9929-33.

Livet, J., T. A. Weissman, et al. (2007). "Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system." Nature **450**(7166): 56-62.

Logan, I. G. and J. Rosenberg (1989). "Identification of Cycles in the Courtship Song of Drosophila-Melanogaster - Comment." Animal Behaviour **37**: 860-860.

Luan, H., W. C. Lemon, et al. (2006). "Functional dissection of a neuronal network required for cuticle tanning and wing expansion in Drosophila." J Neurosci **26**(2): 573-84.

Luan, H., N. C. Peabody, et al. (2006). "Refined spatial manipulation of neuronal function by combinatorial restriction of transgene expression." Neuron **52**(3): 425-36.

Luo, L., E. M. Callaway, et al. (2008). "Genetic dissection of neural circuits." Neuron **57**(5): 634-60.

Machens, C. K., T. Gollisch, et al. (2005). "Testing the efficiency of sensory coding with optimal stimulus ensembles." Neuron **47**(3): 447-56.

Manning, A. (1962). "A sperm factor affecting the receptivity of *Drosophila melanogaster* females." Nature, Lond. **194**: 252-253.

Manning, A. (1966). "Corpus allatum and sexual receptivity in female Drosophila melanogaster." Nature **211**(5055): 1321-2.

Manning, A. (1967a). "The control of sexual receptivity in female Drosophila." Anim Behav **15**(2): 239-50.

Manning, A. (1967b). "Antennae and sexual receptivity in Drosophila melanogaster females." Science **158**(797): 136-7.

Manoli, D. S. and B. S. Baker (2004). "Median bundle neurons coordinate behaviours during Drosophila male courtship." Nature **430**(6999): 564-9.

Manoli, D. S., M. Foss, et al. (2005). "Male-specific fruitless specifies the neural substrates of Drosophila courtship behaviour." Nature **436**(7049): 395-400.

Marella, S., W. Fischler, et al. (2006). "Imaging taste responses in the fly brain reveals a functional map of taste category and behavior." Neuron **49**(2): 285-95.

Markow, T. A. (1987). "Behavioral and sensory basis of courtship success in Drosophila melanogaster." Proc Natl Acad Sci U S A **84**(17): 6200-4.

Markow, T. A. and P. F. Ankney (1984). "Drosophila Males Contribute to Oogenesis in a Multiple Mating Species." Science **224**(4646): 302-303.

Martin, J. R. (2004). "A portrait of locomotor behaviour in Drosophila determined by a video-tracking paradigm." Behav Processes **67**(2): 207-19.

McGuire, S. E., Z. Mao, et al. (2004). "Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila." Sci STKE **2004**(220): pl6.

Miesenbock, G., D. A. De Angelis, et al. (1998). "Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins." Nature **394**(6689): 192-5.

Miyawaki, A., J. Llopis, et al. (1997). "Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin." Nature **388**(6645): 882-7.

Monastirioti, M. (2003). "Distinct octopamine cell population residing in the CNS abdominal ganglion controls ovulation in Drosophila melanogaster." Dev Biol **264**(1): 38-49.

Moshitzky, P., I. Fleischmann, et al. (1996). "Sex-peptide activates juvenile hormone biosynthesis in the Drosophila melanogaster corpus allatum." Arch Insect Biochem Physiol **32**(3-4): 363-74.

Murthy, M. (2010). "Unraveling the auditory system of Drosophila." Curr Opin Neurobiol.

Nadrowski, B., J. T. Albert, et al. (2008). "Transducer-based force generation explains active process in Drosophila hearing." Curr Biol **18**(18): 1365-72.

Nagel, G., T. Szellas, et al. (2003). "Channelrhodopsin-2, a directly light-gated cation-selective membrane channel." Proc Natl Acad Sci U S A **100**(24): 13940-5.

Nakai, J., M. Ohkura, et al. (2001). "A high signal-to-noise Ca(2+) probe composed of a single green fluorescent protein." Nat Biotechnol **19**(2): 137-41.

Nakano, Y., K. Fujitani, et al. (2001). "Mutations in the novel membrane protein spinster interfere with programmed cell death and cause neural degeneration in Drosophila melanogaster." Mol Cell Biol **21**(11): 3775-88.

Nitabach, M. N., Y. Wu, et al. (2006). "Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods." J Neurosci **26**(2): 479-89.

Olsen, S. R. and R. I. Wilson (2008). "Cracking neural circuits in a tiny brain: new approaches for understanding the neural circuitry of Drosophila." Trends Neurosci **31**(10): 512-20.

Paillette, M., H. Ikeda, et al. (1991). "A new acoustic signal of the fruit-flies Drosophila simulans and D. melanogaster." Bioacoustics **3**: 247-254.

Peng, J., S. Chen, et al. (2005a). "Gradual release of sperm bound sex-peptide controls female postmating behavior in Drosophila." Curr Biol **15**(3): 207-13.

Peng, J., P. Zipperlen, et al. (2005b). "Drosophila sex-peptide stimulates female innate immune system after mating via the Toll and Imd pathways." Curr Biol **15**(18): 1690-4.

Petkov, C. I., C. Kayser, et al. (2008). "A voice region in the monkey brain." Nat Neurosci **11**(3): 367-74.

Pfeiffer, B. D., A. Jenett, et al. (2008). "Tools for neuroanatomy and neurogenetics in Drosophila." Proc Natl Acad Sci U S A **105**(28): 9715-20.

Pilpel, N., I. Nezer, et al. (2008). "Mating-increases trypsin in female Drosophila hemolymph." Insect Biochem Mol Biol **38**(3): 320-30.

Popov, A. V., N. A. Sitnik, et al. (2003). "The role of central parts of the brain in the control of sound production during courtship in Drosophila melanogaster." Neurosci Behav Physiol **33**(1): 53-65.

Potter, C. J., B. Tasic, et al. (2010). "The Q System: A Repressible Binary System for Transgene Expression, Lineage Tracing, and Mosaic Analysis." Cell(141): 536-548.

Pulver, S. R., S. L. Pashkovski, et al. (2009). "Temporal dynamics of neuronal activation by Channelrhodopsin-2 and TRPA1 determine behavioral output in Drosophila larvae." J Neurophysiol **101**(6): 3075-88.

Ren, D., B. Navarro, et al. (2001). "A prokaryotic voltage-gated sodium channel." Science **294**(5550): 2372-5.

Rideout, E. J., J. C. Billeter, et al. (2007). "The sex-determination genes fruitless and doublesex specify a neural substrate required for courtship song." Curr Biol **17**(17): 1473-8.

Rideout, E. J., A. J. Dornan, et al. (2010). "Control of sexual differentiation and behavior by the doublesex gene in Drosophila melanogaster." Nat Neurosci **13**(4): 458-66.

Ringo, J. (1996). "Sexual receptivity in insects." Annu Rev Entomol **41**: 473-94.

Ringo, J., R. Werczberger, et al. (1991). "Female sexual receptivity is defective in juvenile hormone-deficient mutants of the apterous gene of Drosophila melanogaster." Behav Genet **21**(5): 453-69.

Ritchie, M. G., E. J. Halsey, et al. (1999). "Drosophila song as a species-specific mating signal and the behavioural importance of Kyriacou & Hall cycles in D. melanogaster song." Anim Behav **58**(3): 649-657.

Ritchie, M. G., R. M. Townhill, et al. (1998). "Female preference for fly song: playback experiments confirm the targets of sexual selection." Anim Behav **56**(3): 713-717.

Rohlfing, T. and C. R. Maurer, Jr. (2003). "Nonrigid image registration in shared-memory multiprocessor environments with application to brains, breasts, and bees." IEEE Trans Inf Technol Biomed **7**(1): 16-25.

Roller, L., N. Yamanaka, et al. (2008). "The unique evolution of neuropeptide genes in the silkworm Bombyx mori." Insect Biochem Mol Biol **38**(12): 1147-57.

Rybak, F., T. Aubin, et al. (2002). "Acoustic communication in Drosophila melanogaster courtship: Are pulse- and sine-song frequencies important for courtship success?" Canadian Journal of Zoology-Revue Canadienne De Zoologie **80**(6): 987-996.

Rybak, F., G. Sureau, et al. (2002). "Functional coupling of acoustic and chemical signals in the courtship behaviour of the male Drosophila melanogaster." Proc Biol Sci **269**(1492): 695-701.

Ryner, L. C., S. F. Goodwin, et al. (1996). "Control of male sexual behavior and sexual orientation in Drosophila by the fruitless gene." Cell **87**(6): 1079-89.

Saudan, P., K. Hauck, et al. (2002). "Ductus ejaculatorius peptide 99B (DUP99B), a novel Drosophila melanogaster sex-peptide pheromone." Eur J Biochem **269**(3): 989-97.

Schmidt, T., Y. Choffat, et al. (1993). "The Drosophila-Melanogaster Sex-Peptide - a Molecular Analysis of Structure-Function-Relationships." Journal of Insect Physiology **39**(5): 361-368.

Schroder-Lang, S., M. Schwarzel, et al. (2007). "Fast manipulation of cellular cAMP level by light in vivo." Nat Methods **4**(1): 39-42.

Shorey, H. H. (1962). "Nature of the Sound Produced by Drosophila melanogaster during Courtship." Science **137**(3531): 677-678.

Sokolowski, M. B. (2001). "Drosophila: genetics meets behaviour." Nat Rev Genet **2**(11): 879-90.

Spieth, H. T. (1974). "Courtship behavior in Drosophila." Annu Rev Entomol **19**: 385-405.

Stockinger, P., D. Kvitsiani, et al. (2005). "Neural circuitry that governs Drosophila male courtship behavior." Cell **121**(5): 795-807.

Sturtevant, A. (1915). "Experiments on sex recognition and the problem of sexual selection in Drosophila." Anim Behav **5**: 351-366.

Sun, Y., L. Liu, et al. (2009). "TRPA channels distinguish gravity sensing from hearing in Johnston's organ." Proc Natl Acad Sci U S A **106**(32): 13606-11.

Sweeney, S. T., K. Broadie, et al. (1995). "Targeted expression of tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects." Neuron **14**(2): 341-51.

Terhzaz, S., P. Rosay, et al. (2007). "The neuropeptide SIFamide modulates sexual behavior in Drosophila." Biochem Biophys Res Commun **352**(2): 305-10.

Tian, L., S. A. Hires, et al. (2009). "Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators." Nat Methods **6**(12): 875-81.

Tomaru, M., H. Matsubayashi, et al. (1995). "Heterospecific inter-pulse intervals of courtship song elicit female rejection in Drosophila biauraria." Anim. Behav. **50**: 905-914.

Tomaru, M. and Y. Oguma (1994a). "Differences in Courtship Song in the Species of the Drosophila-Auraria Complex." Animal Behaviour **47**(1): 133-140.

Tomaru, M. and Y. Oguma (1994b). "Genetic basis and evolution of species-specific courtship song in the Drosophila auraria complex." Genet Res **63**(1): 11-7.

Tomaru, M. and Y. Oguma (2000). "Mate choice in Drosophila melanogaster and D. sechellia: criteria and their variation depending on courtship song." Anim Behav **60**(6): 797-804.

Tompkins, L., A. C. Gross, et al. (1982). "The role of female movement in the sexual behavior of Drosophila melanogaster." Behav Genet **12**(3): 295-307.

Tompkins, L. and J. C. Hall (1983). "Identification of Brain Sites Controlling Female Receptivity in Mosaics of DROSOPHILA MELANOGASTER." Genetics **103**(2): 179-195.

Ugolini, G. (1995). "Specificity of rabies virus as a transneuronal tracer of motor networks: transfer from hypoglossal motoneurons to connected second-order and higher order central nervous system cell groups." J Comp Neurol **356**(3): 457-80.

Usui-Aoki, K., H. Ito, et al. (2000). "Formation of the male-specific muscle in female Drosophila by ectopic fruitless expression." Nat Cell Biol **2**(8): 500-6.

Valente, D., I. Golani, et al. (2007). "Analysis of the trajectory of Drosophila melanogaster in a circular open field arena." PLoS One **2**(10): e1083.

von Schilcher, F. (1976a). "The role of auditory stimuli in the courtship of Drosophila melanogaster." Anim. Behav. **24**: 18-26.

von Schilcher, F. (1976b). "The function of pulse song and sine song in the courtship of Drosophila melanogaster." Anim. Behav. **24**: 622-625.

von Schilcher, F. (1989). "Have cycles in the courtship song of Drosophila been detected?" Trends Neurosci **12**(9): 311-3.

Vrontou, E., S. P. Nilsen, et al. (2006). "fruitless regulates aggression and dominance in Drosophila." Nat Neurosci **9**(12): 1469-71.

Waldron, I. (1964). "Courtship Sound Production in Two Sympatric Sibling Drosophila Species." Science **144**: 191-3.

Walker, M. J., C. M. Rylett, et al. (2006). "Proteomic identification of Drosophila melanogaster male accessory gland proteins, including a pro-cathepsin and a soluble gamma-glutamyl transpeptidase." Proteome Sci **4**: 9.

Walker, R. G., A. T. Willingham, et al. (2000). "A Drosophila mechanosensory transduction channel." Science **287**(5461): 2229-34.

Wang, L. and D. J. Anderson (2009). "Identification of an aggression-promoting pheromone and its receptor neurons in Drosophila." Nature **463**(7278): 227-31.

Wheeler, D. A., C. P. Kyriacou, et al. (1991). "Molecular transfer of a species-specific behavior from Drosophila simulans to Drosophila melanogaster." Science **251**(4997): 1082-5.

White, K., E. Tahaoglu, et al. (1996). "Cell killing by the Drosophila gene reaper." Science **271**(5250): 805-7.

Wong, A. M., J. W. Wang, et al. (2002). "Spatial representation of the glomerular map in the Drosophila protocerebrum." Cell **109**(2): 229-41.

Wyatt, G. R. and K. G. Davey (1996). "Cellular and molecular actions of juvenile hormone. 2. Roles of juvenile hormone in adult insects." Advances in Insect Physiology **26**: 1-155.

Yamamoto, D., J. M. Jallon, et al. (1997). "Genetic dissection of sexual behavior in Drosophila melanogaster." Annu Rev Entomol **42**: 551-85.

Yamanaka, N., Y. J. Hua, et al. (2010). "Bombyx prothoracicostatic peptides activate the sex peptide receptor to regulate ecdysteroid biosynthesis." Proc Natl Acad Sci U S A **107**(5): 2060-5.

Yang, C. H., S. Rumpf, et al. (2009). "Control of the postmating behavioral switch in Drosophila females by internal sensory neurons." Neuron **61**(4): 519-26.

Yapici, N. (2008). Molecular and genetic analysis of *Drosophila melanogaster* female reproductive behaviours, University of Vienna. **PhD thesis**.

Yapici, N., Y. J. Kim, et al. (2008). "A receptor that mediates the post-mating switch in Drosophila reproductive behaviour." Nature **451**(7174): 33-7.

Yorozu, S., A. Wong, et al. (2009). "Distinct sensory representations of wind and near-field sound in the Drosophila brain." Nature **458**(7235): 201-5.

Yu, J. Y. (2009). Mapping the courtship neuronal circuit of *Drosophila melanogaster*, University of Vienna. **PhD thesis**.

Zhang, W., W. Ge, et al. (2007). "A toolbox for light control of Drosophila behaviors through Channelrhodopsin 2-mediated photoactivation of targeted neurons." Eur J Neurosci **26**(9): 2405-16.

Zhong, L., R. Y. Hwang, et al. (2010). "Pickpocket is a DEG/ENaC protein required for mechanical nociception in Drosophila larvae." Curr Biol **20**(5): 429-34.

Curriculum Vitae

FENG, Kai
fengkaiking@gmail.com

Date of birth: JUL 07, 1983
Place of birth: Taiyuan, Shanxi, China
Nationality: People's Republic of China

Education:

2006- Dr. rer. Nat., the University of Vienna, Vienna, Austria
Mentor: Dr. Barry Dickson,
Research Institute of Molecular Pathology, Vienna, Austria

2002-2006 BSc, Shanghai Jiao Tong University, Shanghai, China

2005 Exchange student, Hong Kong University of Science and Technology, Hong Kong

1996-2002 Chengcheng High School, Taiyuan, China

1990-1996 Dananguan Primary School, Taiyuan, China

Oral and poster presentation in conferences:

2009 Neural Circuits Controlling Sexual Behavior, Janelia Farm Research Campus, VA, USA
Poster: Quantitative analysis of female responses to courtship song

2009 1st Vienna *Drosophila* Meeting, Vienna, Austria
Talk: Quantitative analysis of female responses to courtship song in *Drosophila*