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Synopsis

To generate an appropriate behavioral output the nervous system has to integrate information about the outside world as well as the internal state of the animal. *Drosophila melanogaster* females show a striking switch in their reproductive behavior after mating. When presented with the exact same sensory cues virgin females will accept males for copulation whereas the same female will reject male advances after she was mated. This switch in reproductive behavior is triggered by a small peptide, the Sex peptide (SP), transferred from the male to the female during mating. SP activates a specific receptor, the Sex-peptide-receptor (SPR), which is broadly expressed in the female's nervous system and reproductive tract. Here, we pinpoint the action of SPR to a small subset of internal sensory neurons that innervate the female uterus and oviduct and that project to the central nervous system. These neurons express both *fruitless*, a marker of neurons implicated in sex-specific behaviors, and *pickpocket*, a marker for proprioceptive neurons. We show that SPR expression in these neurons is both required and sufficient to orchestrate the switch in reproductive behavior. These neurons therefore offer an entry point into a neuronal circuit that integrates information about the outside world with the internal state of the animal to produce appropriate behavioral actions.

Synopse

Um angemessenes Verhalten zu generieren muss das Nervensystem Informationen über die Umgebung mit Informationen über den internen Zustand des Tieres verrechnen. Drosophila melanogaster Weibchen ändern ihr Verhalten nach der Paarung grundlegend. Während sich Jungfrauen mit Männchen paaren weisen Weibchen diese nach der Paarung zurück. Diese Verhaltensänderung wird duch ein Polypeptid, das sogenannte Sex-peptid (SP) ausgelöst, welches während der Paarung vom Männchen zum Weibchen übertragen wird. SP aktiviert einen spezifischen Rezeptor, den sogenannten Sex-peptid Rezeptor (SPR), welcher in vielen Zellen des Nervensystems und der weiblichen Sexualorgane exprimiert wird. Hier zeigen wir, dass SPR in einer kleinen Anzahl interner sensorischer Nervenzellen, die den Uterus und Ovidukt innervieren und ins zentrale Nervensystem projezieren, gebraucht wird, um die Verhaltensänderung auszulösen. Diese Nervenzellen exprimieren sowohl fruitless, welches in Nervenzellen, die für Paarungsverhalten wichtig sind, exprimiert wird, als auch pickpocket, welches in proprioceptiven Nerven exprimiert wird. Wir zeigen, dass Expression von SPR in diesen Neuronen sowohl nötig als auch ausreichend ist, um die Verhaltensänderung nach der Paarung auszulösen. Diese Neurone stellen daher einen Zugang zu einem Neuronalen Schaltkreis dar, der Informationen über die Umgebung mit internen Informationen über den Zustand des Tieres verrechnet, um angemessene Verhaltensweisen auszulösen.

Introduction

A major goal in neuroscience is to investigate how behaviors are encoded in the nervous system and how neuronal circuits compute information to create appropriate behavioral outputs.

To find and define underlying principles of how the nervous system accomplishes different tasks, behaviors are often viewed as being wired in a dedicated neuronal circuit. These behavioral circuits should contain a dedicated set of sensory neurons, gathering information about the outside world and the internal state of the animal relevant to the behavior, neurons integrating this information and generating commands as well as central pattern generators that execute motor programs associated with the behavior. Neurons in these circuits can of course be involved in more than one behavior, e.g. sensory neurons gathering information relevant to a variety of behavioral tasks. To characterize neuronal circuits and to identify how they can integrate sensory information leading to an appropriate behavioral output it is important to identify the individual components. Knowing key neuronal players on the sensory, integration and output level will aid in the understanding of the structure of the circuit as a whole and through physiological analysis to identify the underlying principles of how nervous systems compute information and encode behaviors.

The study of innate behaviors is especially suited to understand building blocks of neuronal circuits. Innate behaviors are behaviors that do not need to be learned but can be performed by naïve animals without any prior experience. This suggests that the behavior is both under genetic control and that it is more or less hard-wired into the nervous system (Baker et al., 2001). Because of this, it is very likely that a dedicated neuronal circuit exists which encodes the behavior. Since innate behaviors should be under tight genetic control, it is furthermore likely that genes can be used to identify neuronal circuits and circuit elements.

An exemplary innate behavior is the courtship ritual performed by *Drosophila melanogaster* males towards females. When *D. melanogaster* males are paired with

virgin females they perform a courtship ritual that consists of a series of steps ultimately leading to copulation (Hall, 1994; Spieth, 1974). Naïve males that have been isolated post-eclosion can display the full courtship sequence, indicating that this behavior is indeed innate (Baker et al., 2001; Hall, 1994). The neuronal circuit controlling the courtship behavior is set up by the *fruitless* gene (Hall, 1978; Ito et al., 1996; Ryner et al., 1996). *Fruitless* is a putative transcription factor that is sex-specifically spliced leading to a functional product in males but the absence of Fruitless protein in females (Heinrichs et al., 1998; Ito et al., 1996; Lam et al., 2003; Ryner et al., 1996). Remarkably the male isoforms of *fruitless* are both necessary and sufficient for courtship behavior (Demir and Dickson, 2005; Manoli et al., 2005). Males in which the protein is spliced in the female manner are unable to perform courtship towards females (Demir and Dickson, 2005). If on the other hand male splicing is forced in females these will now behave like males and court other females (Demir and Dickson, 2005). *Fruitless* is expressed almost exclusively in the nervous system in about 2000 neurons encompassing sensory neurons, central neurons and motor neurons (Manoli et al., 2005; Stockinger et al., 2005). Acutely silencing all *fruitless* expressing neurons completely abolishes courtship behavior in males while other behaviors are fully intact (Manoli et al., 2005; Stockinger et al., 2005). Remarkably activation of *fruitless* neurons in beheaded flies can induce the production of courtship song, a very important step in courtship (Clyne and Miesenbock, 2008). This shows that the innate courtship behavior is indeed under tight genetic control and that the master control gene *fruitless* can be used to get access to the neuronal circuit controlling this behavior.

A very interesting question is how nervous systems select behaviors that are appropriate in a given context. Animals are often faced with the choice of conflicting actions, e.g. whether to fight or flee when faced with a threat or whether to accept or reject a prospective mate. To accomplish this task, behavioral circuits presumably need to integrate information about the external world with information about the internal state of the animal. Ultimately the behavioral decision should depend on information relayed by sensory neurons as well as neurons detecting internal states of the animal relevant to the given behavior. To understand how the nervous system

integrates this information and selects the appropriate action it is important to identify the key neuronal players.

A good model system to study action selection is the mating-induced behavioral switch in female *Drosophila melanogaster*. Virgin *D. melanogaster* females readily accept courting males for copulation (Manning, 1967). Like many other insects, *D. melanogaster* females store sperm for a prolonged time after mating (Bloch Qazi et al., 2003; Lefevre and Jonsson, 1962). They therefore do not critically depend on mating frequently to fertilize a large proportion of their eggs. Accordingly *D. melanogaster* females dramatically change their behavior after mating as long as they have sperm in storage (Gillott, 2003; Manning, 1967). Mated females start to search for suitable oviposition sites and lay their eggs (Bloch Qazi et al., 2003; Gillott, 2003; Manning, 1967; Yang et al., 2008). At the same time they will actively reject courting males (Gillott, 2003; Manning, 1967). This means that the female nervous system, faced with the very same sensory cues from a courting male, will produce two opposing behavioral outputs depending on the internal mating state. Since reproductive behaviors are critical for the survival of a species it is very likely that this behavioral switch is under tight genetic and neuronal control much like courtship behavior itself. This makes it an ideal and tractable entry point into how internal physiological states modulate action selection.

The mating-switch is induced by a small peptide, the sex-peptide (SP), synthesized in the male accessory glands and transferred to the female during mating (Chapman et al., 2003; Chen et al., 1988; Liu and Kubli, 2003). SP is both necessary and sufficient to induce the post-mating switch, since females mating to males lacking SP behave like virgins after mating (Chapman et al., 2003; Liu and Kubli, 2003) whereas injection of synthetic SP into virgin females induces post-mating behaviors (Chen et al., 1988). The molecular target of SP, called Sex-peptide-receptor (SPR) has recently been identified (Yapici et al., 2008). Females lacking SPR behave like virgins after mating (Yapici et al., 2008). They still accept courting males for copulation and do not induce egg-laying (Yapici et al., 2008). Furthermore, contrary to wildtype virgins they are insensitive to injection of sex-peptide (Yapici et al., 2008). SPR is expressed in a large number of neurons as well as in the female reproductive tract (Yapici et al., 2008). Its

function in mediating the post-mating switch is confined to the *fruitless* expressing neurons however, since expression of SPR in *fruitless* neurons is both necessary and sufficient to mediate the SP induced post-mating switch (Yapici et al., 2008). This means that sex-peptide via SPR acts on the *fruitless* circuit in females to induce the behavioral switch in mating behaviors.

In order to be able to address the question how the nervous system integrates external sensory information with information about the internal mating state to decide whether to accept or reject a courting male, it is crucial to know the exact cellular target of sex-peptide.

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Review: How the *Drosophila* male influences female mating decisions

Introduction

Mating is a key social behavior. In insects as in many other species the actual act of mating is preceded by a more or less stereotyped courtship ritual. Often males perform elaborate courtship sequences while females choose based on these courtship rituals which male they will accept for copulation.

Recently a lot of progress has been made in *Drosophila melanogaster* in identifying mating signals, their cognate receptors as well as neurons sensing these signals. At the same time organizational structures of higher order circuits are beginning to emerge. This opens exciting possibilities in the research of the neurobiology of decision making. Together with the availability of genome sequences of other related *Drosophilids* as well as more distantly related insect species the study of the molecular evolution of mating signals comes well within reach especially since strategies for transgenesis in other insect species are under active development. Behavioral and neuroethological studies of mating behaviors in other insect species should also give new insight into how mating signals are interpreted differently in species employing differing mating strategies. The identification of mating signals and their cognate receptors at the same time promises to offer novel, specific and effective strategies for insect population control. Considering the large economic and medical impact of insect agricultural pests and disease vectors this is a very important area of applied research.

The goal of this review is to highlight different aspects and recent advances in identifying how *Drosophila* males manipulate female mating decisions and to attempt to show possible intriguing directions for future research. The review consists of three major parts. First we will highlight how sexual cooperation and sexual conflict can arise in insect mating systems. In the second part we will give a review of male courtship signals and how they are detected and processed in the

female nervous system. The last part will be dedicated to how the male employs post-mating signals to control female behavior even after mating took place.

Cooperation and conflict in *Drosophila* mating

Decisions about when and with whom to mate have huge evolutionary consequences. To achieve the maximum reproductive success, strategies for males and females generally differ and the optimal strategy is species dependent as well.

It is usually in the male's interest to mate frequently (Lessells, 2006). This will maximize his reproductive success by guaranteeing a large share of parenthood in the next generation. At the same time female polyandry will potentially diminish his reproductive success. In insects females usually store sperm after mating that is subsequently used to fertilize a large amount of eggs. Therefore depending on the mode of sperm utilization this led to the evolution of differing male mating strategies. In insect species in which sperm of males mating first is rarely displaced from storage by subsequent mates, males will usually guard females until they reach sexual maturity to ensure that they are the first to mate with the female (Wigby and Chapman, 2004). However in species like *Drosophila melanogaster* with second male sperm precedence, i.e. in which the sperm of the male mating second will displace the sperm of the first mate from storage, it is generally in the male's interest to prevent female remating. The male will therefore not only try to employ pre-mating signals to gain courtship success but also post-mating signals to reduce female polyandry (Wigby and Chapman, 2004).

A female's reproductive success will be influenced by the fitness of her mate (Lessells, 2006). It is therefore in the female's interest to only allow males for copulation that have a high reproductive fitness. Both pre- and post-copulatory mechanisms allow *Drosophila* females to choose high-quality males. During courtship rituals females can assess their suitor's quality by evaluating the quality of mating signals presented by the males (Dickson, 2008). These mating signals are relayed by different sensory modalities such as olfaction, taste and audition which presumably allows a more thorough sampling of the quality of the presumptive mate (Spieth, 1974). The female will ultimately evaluate these mating signals to decide

whether to accept the given male for copulation or not (Dickson, 2008). After copulation sperm-competition for the fertilization of the ova can ensue if sperm from multiple males is present in the reproductive tract (Wigby and Chapman, 2004). This can serve as another means of male reproductive quality control. In frequently remating species post-copulatory mechanisms are presumably more important for mate quality assessment whereas pre-copulatory choice should be of greater importance in species that remate at lower frequency.

To maximize her reproductive success it is in the female's interest to fertilize as many of her eggs as possible. This is especially true in insects where maternal care usually ceases at the time of egg-laying. In species in which females are able to store sperm and therefore fertilize their eggs for a prolonged time after mating it is generally advantageous to females to have enough sperm in storage at any given time to fertilize all their eggs. Because of the ability to store sperm a female's reproductive success does not directly depend on maximizing the number of mates especially since it has been suggested that mating itself is associated with a survival cost (Jennions and Petrie, 2000; Polak and Markow, 1995). The female however has to guard herself against bad mate choice and male sterility. Remating should be of advantage to the female if she encounters a male of higher fitness than her previous mate. The optimal remating strategy also depends on other factors. In some *Drosophila* species for example the parental investment into egg-production is shared between males and females since females are able to use the male ejaculate for nutritional purposes (Markow and Ankney, 1984). These species in general show a higher remating frequency (Markow and Ankney, 1984). Female remating can also be of advantage in rapidly changing environments since it increases the genetic diversity of the offspring. For these reasons the female optimum for remating usually lies at intermediate levels (Lessells, 2006).

In *Drosophila melanogaster* there is little cooperation in mating. Males do not offer nuptial gifts and their main energy investment into the offspring is via the presentation of an elaborate courtship ritual to convince females of their quality. There is clearly sexual conflict over remating as well. While it is in the female's interest not to remate too frequently she will still gain reproductive fitness by

remating at intermediate levels. A male's reproductive fitness however will always decrease if a female remates. Males will therefore evolve strategies to suppress remating below the female's optimum while females will evolve strategies to resist this manipulation (Lessells, 2006). This sexual conflict over mating strategies should shape the sexual behavior displayed by *Drosophila melanogaster*.

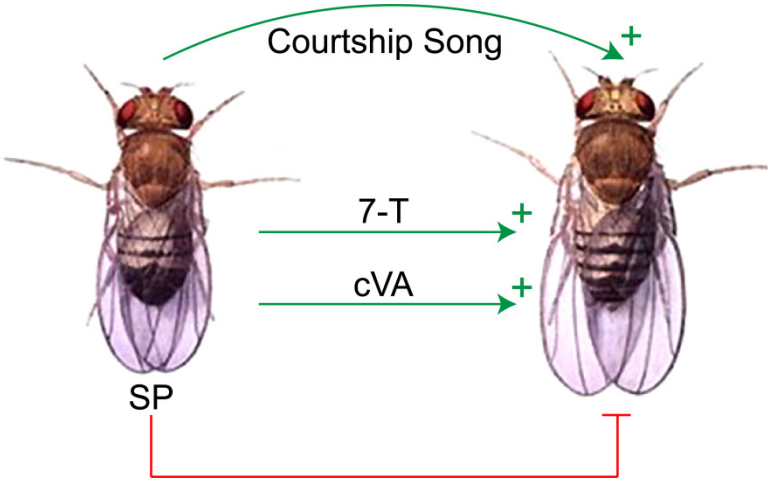

Figure 1: Pre- and post-mating signals influencing female receptivity
Schematic of pre- and post-mating signals relayed from males to females that enhance (green) or inhibit (red) female receptivity. 7-T: 7-Tricosene; cVA: cis-vaccenyl-acetate

Male signals that stimulate females to mate

Drosophila male courtship

Drosophila melanogaster males display an elaborate courtship sequence towards conspecific females. Courtship is an innate behaviour since naïve males that have never encountered a female are able to perform successful courtship (Baker et al., 2001; Hall, 1994). Courtship in *Drosophila melanogaster* proceeds as a loose sequence of steps. The male first orients towards the female fly, follows it, extends its wing to produce courtship song and taps and licks the female abdomen (Hall, 1994). Finally the male will attempt copulation, and if he managed to convince the female copulation will ensue. The elaborate courtship ritual presumably serves the purpose to give the female ample cues on which she can base her mating decision. That male courtship performance is indeed coupled to female choosiness is nicely illustrated by a set of experiments undertaken by B. Holland and WR. Rice. They

could show that males raised in an artificially monogamous environment significantly reduce their courtship investment towards females (Holland and Rice, 1999). This is likely the consequence of decoupling courtship investment from courtship success as well as reducing courtship competition. This experiment exemplifies how male mating strategies evolve in response to the structure of the local population. In this context it would also be interesting to see how female mating strategies can influence male courtship investment on an evolutionary scale. One could predict that males coevolving with females displaying an artificially high remating rate would also reduce their courtship investment since the value of courtship success would decrease.

The main identified cues presented to the female by the male in *D. melanogaster* during courtship are pheromones and the male's courtship song (Figure 1). Contrary to crickets or moths were the courtship song or pheromones respectively play a role in attracting mates (de Bruyne and Baker, 2008; Hedwig, 2006) for *D. melanogaster* these cues seem to have more exclusive roles in mate selection rather than mate attraction since males and females usually meet and breed on food sources (Markow and O'Grady, 2005).

Courtship song

Drosophila melanogaster males produce song during courtship by unilaterally vibrating a wing (Hall, 1994; Spieth, 1952). This courtship song is a key mating signal (Markow, 1987; Rybak et al., 2002). Males lacking wings and therefore unable to sing have very low courtship success (Bennet-Clark and Ewing, 1967; Rybak et al., 2002; Schilcher, 1976) and deaf females display very low receptivity towards courting males (Markow, 1987). Playback of recorded song can rescue the courtship success of wingless males to wildtype levels (Bennet-Clark and Ewing, 1967; Schilcher, 1976) clearly demonstrating that it is indeed the lack of courtship song that reduces female receptivity towards wingless males.

The male courtship song in *D. melanogaster* consists of two components. Short sound pulses (Bennet-Clark and Ewing, 1967), also termed pulse-song, and sinusoidal hums, the so-called sine song (Schilcher, 1976). It has been shown that the main role

of sine-song is a priming effect of the females. Playing sine-song to females before pairing them with males will subsequently increase their receptivity (Schilcher, 1976). The main important component during courtship however is the pulse song. Playback of pulse song alone during courtship of wingless males can rescue their copulation success almost to wildtype levels (Bennet-Clark and Ewing, 1967; Schilcher, 1976). The most critical parameter evaluated by the female is the length of the interval between the pulses. *D. melanogaster* females clearly prefer song with an inter-pulse-interval (IPI) of 34ms (Bennet-Clark and Ewing, 1969; Schilcher, 1976). Since different closely related *Drosophila* species have different inter-pulse-intervals this has been suggested to be important for species recognition. To what extent song quality is involved in sexual selection has not been addressed in *D. melanogaster*. However in *D. montana* field and laboratory studies have shown a clear link between female song preference and gain of female reproductive fitness. In this species females can exercise sexual selection based on courtship song characteristics (Ritchie et al., 1998) and mating to males with preferred song characteristics is correlated with higher off-spring survival (Aspi and Hoikkala, 1995; Hoikkala et al., 1998). This illustrates that male courtship song can indeed relay information about his fitness to the female. Very likely selective pressure led to similar mate evaluation strategies in *D. melanogaster* for which it has been shown that allowing mate choice increases female reproductive success (Partridge, 1980). It would be interesting to see to what extent song quality relates to male courtship success and reproductive fitness and if song quality would deteriorate in an environment with reduced courtship competition, such as an artificially monogamous environment (Holland and Rice, 1999).

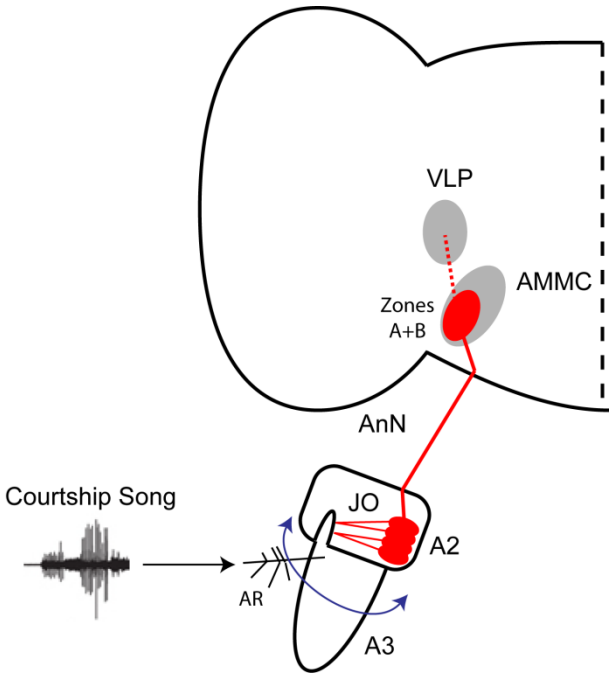

Figure 2: Neuronal pathways of *Drosophila* audition
Schematic of identified neurons in *Drosophila* audition. Suggested connectivity is indicated by a dashed line.
AR: Arista; JO: Johnston's organ; A2/3: Second and third antennal segment; AnN: Antennal Nerve; AMMC: Antennal mechanosensory and motor centre; VLP: Ventrolateral protocerebrum

The courtship song is sensed via the female's antenna (Figure 2) (Gopfert and Robert, 2001, 2002; Kernan, 2007; Todi et al., 2004). The arista on the base of the third antennal segment serves as an air-speed sensor and gets deflected by sound (Gopfert and Robert, 2001, 2002; Kernan, 2007; Todi et al., 2004). This leads to a rotation of the third antennal segment relative to the second segment (Caldwell and Eberl, 2002; Gopfert and Robert, 2001, 2002; Tauber and Eberl, 2003). The second antennal segment houses the Johnston's organ (JO) which consists of stretch receptors that sense the antennal rotation (Figure 2) (Kernan, 2007; Kim et al., 2003). This neuronal signal is subsequently relayed to a specific brain structure, the so called antennal mechanosensory and motor center (AMMC) (Figure 2) (Kamikouchi et al., 2006). In contrast to olfaction where the detection of certain stimuli has been mapped to defined regions in the antennal lobe a separation of stimulus detection in the auditory pathway is just beginning to emerge. Recently it has been shown that JO neurons not only detect sound but also sense gravity

(Kamikouchi et al., 2009) and wind (Yorozu et al., 2009). These stimuli are detected by distinct neuronal populations in Johnston's organ that also project to different zones in the AAMC (Kamikouchi et al., 2009; Yorozu et al., 2009). Candidate second-order neurons in the sound-sensing pathway have been identified as well (Kamikouchi et al., 2009) with a region in the ventrolateral protocerebrum appearing as the main target of putative sound-sensitive interneurons (Figure 2) (Kamikouchi et al., 2009).

A major question in *Drosophila* audition is where the tuning to a specific courtship song arises. The antennal system is likely anatomically tuned to only respond well to certain frequencies. Also the peripheral difference in response to gravity or wind versus sound shows that peripheral tuning exists (Kamikouchi et al., 2009; Yorozu et al., 2009). These different stimuli however lead to very distinct deflection patterns in the antenna as is not necessarily expected for different sounds. It is therefore likely that the task of identifying a sound-mixture as the "courtship song" is accomplished by higher-order circuits. Especially the tuning to a certain inter-pulse-interval of the courtship song as observed in *Drosophila melanogaster* is unlikely to occur in the periphery. The identification of higher order circuits that accomplish this timing task should yield valuable information about how tuning and gating of sensory stimuli can be accomplished in the nervous system. It will also be interesting to investigate how plastic female preferences for certain male courtship song characteristics are. In crickets it has been shown that female song preference can shift based on general song quality of the local male population (Wagner et al., 2001).

Pheromonal signals

Pheromonal signals play an important role during courtship. Males that lack cuticular hydrocarbons, one major source of insect pheromones, have considerably reduced courtship success (Billeter et al., 2009; Rybak et al., 2002) and females that are smellblind have significantly reduced receptivity towards courting males (Markow, 1987).

To date two male pheromones that stimulate female receptivity have been identified. 7-Tricosene (7-T) is a male specific cuticular hydrocarbon that can

stimulate female receptivity since females show a lower mating latency towards males producing higher levels of 7-T or males that are perfumed with high levels of the pheromone (Grillet et al., 2006). Another male specific pheromone is cis-vaccenyl acetate (cVA). This pheromone is synthesized in the ejaculatory bulb (Butterworth, 1969). Females that are unable to detect cVA are considerably less receptive towards courting males (Kurtovic et al., 2007) whereas females engineered to constitutively sense cVA show increased receptivity (Ronderos and Smith, 2010). This indicates that cVA is an important mating signal stimulating female receptivity.

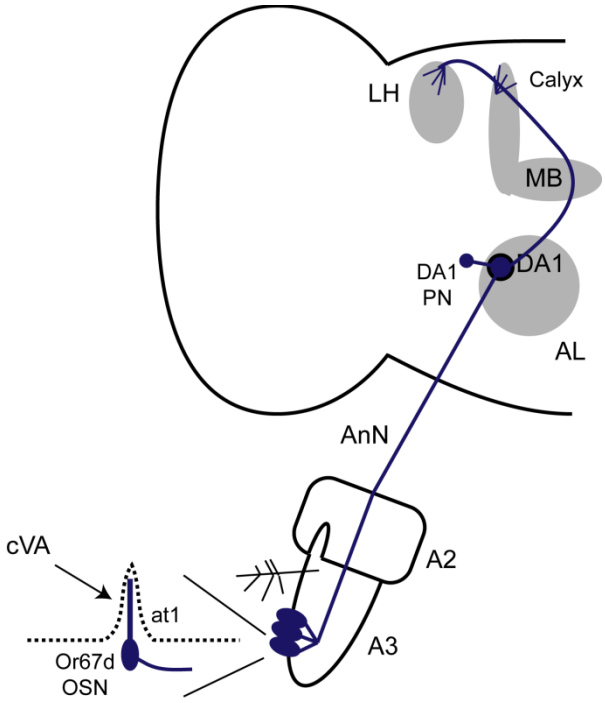

Figure 3: Neuronal pathways of cVA detection
Schematic of neurons involved in the detection of cVA and the relay of the sensory information.
cVA: cis-vaccenyl-acetate; OSN: Olfactory sensory neurons; at1: Sensillum housing Or67d OSN's; A2/3: Second and third antennal segment; AnN: Antennal Nerve; AL: Antennal lobe; DA1: Glomerulus DA1; PN: Projection neuron; MB: Mushroom body; LH: Lateral horn

Cis-vaccenyl acetate is sensed via the dedicated olfactory receptor Or67d (Figure 3) (Kurtovic et al., 2007) which is expressed on trichoid sensilla in both males and females (Couto et al., 2005; Fishilevich and Vosshall, 2005; Kurtovic et al., 2007). Females lacking Or67d are significantly less receptive towards courting males

(Kurtovic et al., 2007) indicating that evaluation of this pheromonal signal plays an important role in female mating decisions. Or67d does not seem to be the receptor for cVA itself however but rather the receptor for the odorant binding protein LUSH that changes conformation upon binding of cVA (Laughlin et al., 2008). Activated LUSH is bound by Or67d and its essential co-receptor SNMP a CD36 homologue implicated in pheromone detection in a variety of insect species (Benton et al., 2007). SNMP is expressed in other olfactory receptor neurons as well making them potential candidates for the detection of yet unidentified pheromones (Benton et al., 2007). The Or67d neurons project to a single glomerulus in the antennal lobe of the fly brain, the DA1 glomerulus (Figure 3) (Couto et al., 2005; Kurtovic et al., 2007) where they connect to a specific class of lateral projection neurons (Figure 3) (Datta et al., 2008). Both the Ord67d neurons and their cognate projection neurons express the *fruitless* gene (Datta et al., 2008; Kurtovic et al., 2007) which is a master regulator of sexual behaviors in flies (Anand et al., 2001; Baker et al., 2001; Demir and Dickson, 2005; Ito et al., 1996; Ryner et al., 1996). This shows an interesting wiring principle of neurons implicated in sensing mating signals that could be exploited to identify other neurons detecting stimuli relevant to courtship and mating decisions. The DA1 projection neurons arborize in both the mushroom body and the lateral horn in the fly brain (Figure 3) (Jefferis et al., 2007). Strikingly the DA1 projection in the lateral horn is sexually dimorphic indicating that detection of cVA is potentially differentially processed in male and female flies (Datta et al., 2008). This makes sense since cVA has disparate effects in the sexes. While stimulating receptivity in females it reduces courtship in males towards other males as well as females (Kurtovic et al., 2007). Recently it has also been shown that cVA stimulates male-male aggression via Or67d (Wang and Anderson, 2010). These differing effects suggest that information relayed via Or67d feeds into multiple circuits controlling different behaviors.

While the information of one pheromonal signal diverges onto different downstream circuits, information from different pheromones such as cVA and 7-T has to converge in the brain. Contrary to cVA the receptor for 7-T is not yet identified. Its

identification together with the cognate receptor and projection neurons could provide insight into where in the brain these pheromonal signals are integrated.

Comparisons between pheromone-less and wingless males have shown a synergistic effect between pheromonal and auditory cues on female receptivity. While wingless males are still able to win females over males neither presenting auditory nor olfactory cues are unsuccessful in courtship (Rybak et al., 2002). This argues that auditory and olfactory cues are integrated in the female central nervous system to evaluate her suitor's quality and based on that information to decide whether to mate or not. The identification of neurons in which this conversion of information relevant to a given behavior occurs and how these neurons integrate differing signals such as multiple pheromones or olfactory and auditory information should be an important goal of future research.

Male signals that modulate female behavior post-mating

The molecular mechanism of behavioral control

In *Drosophila melanogaster*, females become refractory to further mating attempts for about one week after mating to a wildtype male under laboratory conditions (Manning, 1962). At the same time they increase their egg-laying rate dramatically (Bloch Qazi et al., 2003; Kubli, 2003). This dramatic and long-lasting behavioral change in response to the mating status is correlated with the presence of sperm in storage (Manning, 1962). In some insects it has indeed been suggested that the presence of sperm in the storage organs is sensed via stretch receptors and directly responsible for the reduction in receptivity (Sugawara, 1979).

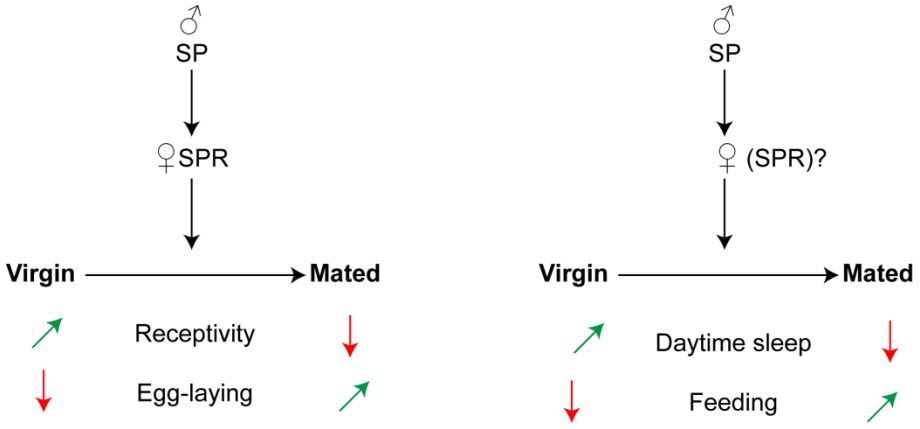

Figure 4: Post-mating responses induced by the Sex-peptide in females
Green arrows indicate an increase in the behavior, red arrows a suppression.
SP: Sex-peptide; SPR: Sex-peptide-receptor

In *Drosophila melanogaster* however a mechanism has evolved by which the male directly influences the female behavioral state after mating (Figures 1 and 4). In *Drosophila melanogaster*, like in many other insects, males transfer various peptides to the female during mating together with sperm. Among these peptides is a 36 amino acid peptide, called the sex peptide (SP). It is synthesized exclusively in the male accessory gland (Chen et al., 1988) and transferred to the female during mating (Lung and Wolfner, 1999; Pilpel et al., 2008). If wildtype females mate with males lacking this peptide they subsequently behave like virgins within one day after mating (Chapman et al., 2003; Liu and Kubli, 2003). This indicates that the sex-peptide is required to induce the long-term behavioral changes in the female after mating. SP is also sufficient to induce these changes since the injection of synthetic SP into the hemolymph renders females unreceptive and induces egg-laying just like mating does (Chen et al., 1988). Male derived SP is therefore the main component informing the female central nervous system about the mating state. The physiological response to SP can be divided into two branches (Figure 5). Via its C-terminus SP induces female refractoriness and oviposition (Schmidt et al., 1993) whereas the N-terminal half stimulates the release of juvenile hormone (JH) from the *corpora allata* (Figure 5) (Fan et al., 2000; Partridge, 1980). JH in turn stimulates oocyte progression thereby coordinating ovulation with oviposition (Bownes, 1989).

Administration of SP itself however only has a short-term effect on female behavior. For a long-term switch of female mating behavior sperm storage is required as is also evident from experiments with spermless males which only induce short-term refractoriness in females while still transferring SP (Kalb et al., 1993). This sperm-induced long term effect is termed sperm effect. SP is known to bind to sperm tales from which it is slowly released by cleavage in the reproductive tract (Kubli, 2003; Peng et al., 2005). It is therefore very likely that SP mediates the sperm effect (Chapman et al., 2003; Kubli, 2003; Liu and Kubli, 2003; Peng et al., 2005).

The molecular target of SP in the female has long been elusive. The receptor for SP has recently been identified through the course of a pan-neuronal genome-wide RNAi screen (Yapici et al., 2008). The Sex-peptide-receptor (SPR) is a G-protein coupled receptor related to other peptide receptors. SPR mutant virgins are as receptive as wildtype virgins. They are completely insensitive to SP however. Females lacking SPR therefore do not show the mating induced behavioral switch. They remain receptive after mating and don't induce egg-laying (Yapici et al., 2008). They are furthermore completely insensitive to injection of synthetic SP which renders wildtype virgins unreceptive (Chen et al., 1988; Yapici et al., 2008) and using a cell-based assay it has been demonstrated that SPR is indeed a molecular target of SP (Yapici et al., 2008). These results clearly demonstrate that SPR is the master regulator of the mating induced behavioral switch in females.

SPR signals through pertussis-toxin sensitive G-Proteins in the target cells upon activation (Yang et al., 2009). Due to a mutation in the Gαi protein in *Drosophila* that presumably renders it Pertussis-toxin insensitive (Katanaev et al., 2005) these results argue that SPR signals via $G_{\alpha o}$ rather than $G_{\alpha i}$. The targets downstream of $G_{\alpha o}$ in these neurons are less clear, experiments manipulating signaling activity of protein kinase A suggest however that signaling via PKA can influence the post-mating switch (Yang et al., 2009).

The transfer of SP is giving the male ultimate and far-reaching control over female reproductive behaviors (Figure 4). Not only do females become refractory to further mating attempts and initiate egg-laying behaviors but their sleep-cycle and feeding

behavior also changes upon mating in a sex-peptide dependent manner (Figure 4) (Carvalho et al., 2006; Isaac et al., 2010). This signaling system presumably has advantages to the female as well. It relays part of the burden of coordinating reproductive and other behaviors with the presence of sperm to the male. That the production and transfer of SP is associated both with a relevant energy cost and a clear reproductive benefit was recently demonstrated. Males can change the amount of transferred SP based on how competitive the environment is (Wigby et al., 2009). In the presence of competitor males they transfer significantly more sex-peptide during mating than in an environment without male competition (Wigby et al., 2009). At the same time in a competitive environment a male's reproductive success is higher if he transfers more SP (Wigby et al., 2009). This suggests that males strategically allocate this key post-mating signal to reach a maximum reproductive benefit while minimizing their energy investment.

How widespread this male induced female switch is among the animal kingdom is not clear. SPR itself is highly conserved at least among insect species (Yapici et al., 2008) and the SPR homologs of species as distant as *Aedes aegypti* and *Bombyx mori* can be activated by *Drosophila melanogaster* SP in a cell culture assay (Yapici et al., 2008). The sex-peptide itself however cannot be detected in distantly related species and even only in some of the *Drosophilidae*. Within the *Drosophilidae* the presence of the peptide seems to correlate well with a low remating frequency whereas it is generally absent from highly remating species (BJD, unpublished). This suggests that in these species the male induced female behavioral switch is probably absent. It would be interesting to see however if the basic circuitry is still present and female refractoriness could be induced by ectopic application of *D. melanogaster* SP. The fact that SPR is still present in these species is not surprising. Its widespread expression in the nervous system (Yapici et al., 2008) suggests that it probably has more functions than controlling the female mating switch. This idea is supported by the fact that SPR is not only the receptor for sex-peptide but also for prothoracicostatic peptides in *Bombyx morii* as well as for other myo-inhibitory-peptides in a variety of insect species (Kim et al., 2010; Yamanaka et al., 2010). In general however the absence of sex-peptide homologs does not rule out that female

reproductive behavior is controlled by male derived peptides. The SP gene is very small and genes subject to sexual selection like most Acps generally evolve very fast (Wolfner, 2002) which makes the search for homologs very difficult. This becomes apparent in *Helicoverpa zea* where a male derived peptide has been identified (HezPSP) which has pheromonostatic effects in females (Eliyahu et al., 2003; Kingan et al., 1995). This peptide has no sequence homology to *D. melanogaster* SP but it has a disulphide bridge in the exact same position (Eliyahu et al., 2003; Kingan et al., 1995). Whether this peptide can activate SPR has not been demonstrated. It also has pheromonostatic activity in the closely related *Helicoverpa armigera* however (Eliyahu et al., 2003) in which injection of *D. melanogaster* SP strongly represses PBAN dependent sex-pheromone biosynthesis as well (Fan et al., 2000). This argues that a male-derived SP female SPR signaling axis is relatively ancient and that control of female mating decisions via male derived peptides is widespread among insects.

Neuronal mechanisms of behavioral control

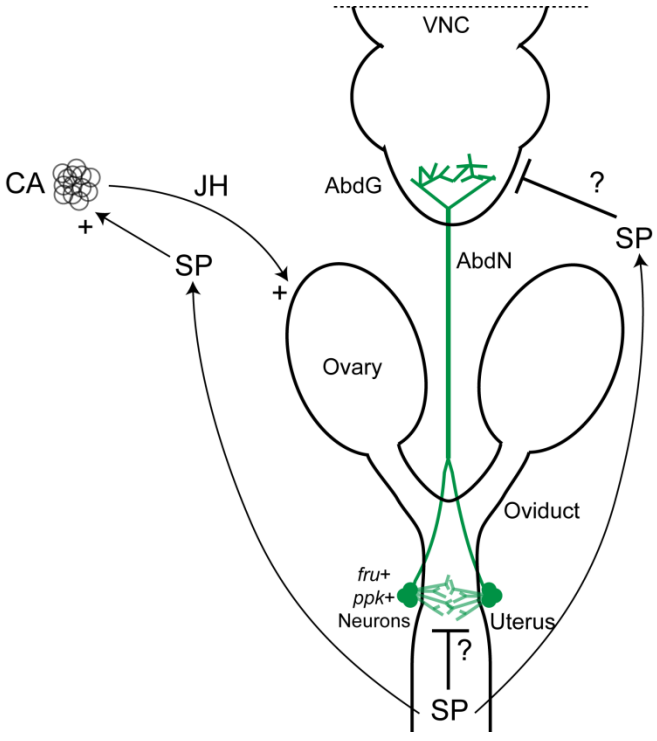

Figure 5: Neuronal and physiological basis of the SP effect
Schematic depicting the neurons sensing SP via SPR as well as the pathway of the humoral response to SP.
SP: Sex-peptide; CA: *Corpora allata*; JH: Juvenile hormone; AbdN: Abdominal nerve; AbdG: Abdominal ganglion; VNC: Ventral nerve cord.

Sex-peptide-receptor is widely expressed in the nervous system of both males and females (Yapici et al., 2008). For the mating induced switch however SPR expression is both required and sufficient in a small set of internal sensory neurons located on the uterus in the female genital tract (Hasemeyer et al., 2009; Yang et al., 2009). These neurons express both *fru*-Gal4, a marker for neurons important in sexual behaviors (Demir and Dickson, 2005; Kvitsiani and Dickson, 2006; Manoli et al., 2005; Stockinger et al., 2005) as well as *ppk*-Gal4 a marker of proprioceptive neurons (Adams et al., 1998; Grueber et al., 2003; Zhong et al., 2010). The neurons send arborizations both into the uterus as well as along the inner epithelium of the reproductive tract (Hasemeyer et al., 2009) and might therefore be involved in coordinating ovulation and fertilization. Even in the absence of SP *Drosophila melanogaster* females are unreceptive for about 4 hours after mating (Liu and Kubli, 2003). The uterine PPK neurons sensing the sex-peptide have morphological similarity to the mechanosensory neurons sensing the presence of a spermatophore in the *bursa* in some species of moth leading to suppression of receptivity (Adams et al., 1998; Hasemeyer et al., 2009; Sugawara, 1979). It is interesting to speculate that the uterine *ppk+* neurons might sense the conformational change of the uterus that occurs upon mating (Adams and Wolfner, 2007; Avila and Wolfner, 2009) and thereby mediate the short-term post-mating response in a female-intrinsic manner. The extrinsic control of remating via a male-derived peptide might therefore have evolved to hijack and stabilize an intrinsic system responsible to coordinate egg-laying and receptivity with the presence of sperm.

Like silencing synaptic transmission of all *fru*-Gal4 neurons (Kvitsiani and Dickson, 2006), the silencing of the *ppk*-Gal4 neurons induces post-mating behaviors in virgin female flies (Hasemeyer et al., 2009; Yang et al., 2009) and the behavioral phenotype of silencing all *fru*-Gal4 neurons can indeed be mainly attributed to the *ppk+/fru+* neurons (Yang et al., 2009). This suggests that activation of SPR and subsequent signaling via $G_{\alpha o}$ leads to a reduction in neuronal activity or synaptic release which is responsible for the switch to female post-mating behaviors (Hasemeyer, 2010, Chapters 1 and 2; Hasemeyer et al., 2009; Yang et al., 2009). The internal sensory neurons on the uterus send processes to the abdominal ganglion of the ventral

nerve cord where they terminate in a ventral region (Hasemeyer, 2010, Chapters 1 and 2; Hasemeyer et al., 2009; Yang et al., 2009). The abdominal ganglion has been implicated in controlling female reproductive behaviors since it houses octopaminergic neurons controlling oviposition (Monastirioti, 2003). Since SP controls disparate behaviors such as egg-laying, receptivity, sleep and feeding (Carvalho et al., 2006; Chapman et al., 2003; Chen et al., 1988; Isaac et al., 2010; Liu and Kubli, 2003) it is likely that the mating signal gets relayed to higher order centers in the brain by second-order projection neurons. A likely target site for these would be the suboesophageal ganglion (SOG). Neurosecretory cells in the SOG have been implicated in moth to control pheromone biosynthesis via the release of pheromonotropic factors such as PBAN (Sato et al., 1994). These neurosecretory cells normally display rythmic activity but have been shown to be long-term inhibited in *Bombyx mori* after mating with a fertile male (Ichikawa, 1998). The role of the *ppk+ fru+* internal sensory neurons is likely to relay information about the female mating state to relevant circuits in the CNS and thereby allow the female to adjust the behavioral output based on the presence or absence of sperm in her reproductive tract.

During courtship the *Drosophila* female nervous system integrates information of various mating signals such as courtship song and pheromones with the information about the internal mating state to ultimately form the decision whether to accept the male for copulation or not. How premating signals are integrated with the information about the mating state relayed by the *ppk+ fru+* neurons can serve as an important paradigm of how the nervous system adjusts behavioral decisions based on the internal physiological state of the animal. To identify potential neurons that perform this integration task it is crucial to identify the neurons downstream of the SP sensing neurons. By tracing how external and internal sensory information flow through the brain it should be possible to identify key neuronal players on which this information converges.

Plasticity of female mating decisions

To maximize her reproductive success a female should try to mate with a high quality male. The decision to mate could be based upon a fixed threshold in which every male that passes the test is allowed to copulate. However this would have a number of disadvantages for the female. If the local male population is of general low quality the fixed threshold might prevent her from mating altogether or simply not allow the female to rank individual male quality. In the former case her reproductive success would be zero in the latter the benefit of mate choice would be severely compromised. The decision could however be based upon an adaptive threshold that allows the female to first sample the local male population and subsequently make her mating decision based on the sample quality. *D. melanogaster* females reach sexual maturity only about 40 hours after eclosion giving them ample time to gauge the quality of the local male population (Manning, 1967). And indeed it has been shown that *D. melanogaster* females can shift their mating preferences according to their experience. *D. melanogaster* females prefer large males over small males and accordingly display a higher mating latency towards small males. If however the females experience courtship exclusively by small males post eclosion their mating latency towards small males decreases (Dukas, 2005). This suggests that females can indeed adjust their mating threshold based on the average quality of the male population.

To what extent female remating decisions are based on an adaptive threshold is far less clear. Remating should be mainly beneficial for a female when she encounters a male of higher reproductive quality than her previous mate. Using male strains that differed in their reproductive fitness it was shown that remating frequency in *Drosophila melanogaster* indeed strongly correlates with male genetic background but not with male reproductive quality (Byrne and Rice, 2005). This argues against a mechanism that couples perceived male quality to an adaptation of remating intervals.

Conclusions and perspectives

Mating behaviors are intimately linked to the survival of a species. The evaluation of mating signals should give males important information about whom to court and females about whom to accept for copulation. Mating strategies are shaped by conflict and cooperation between the sexes. During *Drosophila* courtship males present mating signals to females that ultimately influence her decision to mate or not. Two important components are the volatile pheromone cis-vaccenyl acetate and the courtship song. Both cues have an attractive role increasing female receptivity towards courting males. After mating *Drosophila* males have a high interest in keeping a female from remating since this would diminish their reproductive success. *Drosophila melanogaster* males transfer a small peptide in their seminal fluids which is necessary and sufficient to suppress female receptivity and at the same time to coordinate oviposition and ovulation with the availability of sperm. Information about these pre- and post-mating signals should be integrated in the female nervous system, presumably modulated by prior experience, and ultimately guide the decision whether to mate or not.

How do neuronal circuits evolve? *Drosophila* species differ to a great extent in their remating intervals as well as their courtship behaviors (Markow and O'Grady, 2005). Where defined enhancer elements labeling central circuit elements are known these could potentially be used to label corresponding neurons across species and thereby open the door to comparative circuit evolution. Together with the study of molecular evolution of mating signals this could lead to important insights into how differing mating strategies are encoded in the nervous system.

While a few key mating signals and their cognate receptor neurons are known the next big step will be to identify neurons integrating information relevant to courtship and receptivity. Tracing how information flows through the brain and where it converges will be very interesting from a circuit perspective since it can serve as a paradigm of how complex behavioral circuits are organized. Tackling the physiology of integration centers processing information from various channels will give important insights into how decisions are formed in the nervous system. To start addressing these questions it will be important to identify relevant higher order

neurons. This means an anatomical understanding of the circuit involved in mating behaviors is crucial. The identification and characterization of higher order neurons will require a combination of behavioral measurements after silencing/activation of candidate neurons, anatomical data about connectivity as well as physiological data to establish how individual input neurons influence responses in an integration neuron. Defining higher order circuit elements will be a daunting task but can yield insight into general principles of sensory integration and decision making that might well be applicable to circuit elements in more complex nervous systems such as our own.

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Chapter 1

Sensory neurons in the *Drosophila* genital tract regulate female reproductive behavior

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Summary

Females of many animal species behave very differently before and after mating. In *Drosophila melanogaster*, changes in female behavior upon mating are triggered by the sex peptide (SP), a small peptide present in the male's seminal fluid. SP activates a specific receptor, the sex peptide receptor (SPR), which is broadly expressed in the female reproductive tract and nervous system. Here, we pinpoint the action of SPR to a small subset of internal sensory neurons that innervate the female uterus and oviduct. These neurons express both *fruitless* (*fru*), a marker for neurons likely to have sex-specific functions, and *pickpocket* (*ppk*), a marker for proprioceptive neurons. We show that SPR expression in these *fru*+ *ppk*+ neurons is both necessary and sufficient for behavioral changes induced by mating. These neurons project to central targets in the abdominal ganglion and/or suboesophageal ganglion - regions of the central nervous system that have been implicated in the control of reproductive behaviors in *Drosophila* and other insects. These studies reveal how mating status is sensed in *Drosophila* females and begin to delineate the neural circuitry that controls female reproductive behavior.

Introduction

An animal's behavioral choices depend not only on sensory input from the external environment, but are also guided by internal states that must be sensed and conveyed to the relevant neural circuits in the CNS. The reproductive behaviors of *Drosophila melanogaster* females provide an ideal model system to explore the molecular and neural mechanisms that sense internal states and modulate behavioral choices (Dickson, 2008). Virgin females are sexually receptive and lay only very few eggs. In contrast, females that have already mated are refractory to further mating attempts and begin to lay eggs (Bloch Qazi et al., 2003; Kubli, 2003).

Accordingly, the central circuits that select specific behavioral actions in response to a courting male or a suitable egg-laying substrate must be informed of the female's mating status.

The switch from virgin to mated female behavior does not occur in females that have mated to males lacking SP, a 36-amino acid peptide present in the male seminal fluid (Chapman et al., 2003; Chen et al., 1988; Liu and Kubli, 2003). Conversely, injection of synthetic SP or expression of transgenic SP induces virgin females to behave as though they had mated (Aigaki et al., 1991; Chen et al., 1988). SP binds to the surface of sperm, but is gradually released once the sperm enters the female reproductive tract (Peng et al., 2005a). These data suggest that detection of SP is the signal that indicates the presence of sperm in the reproductive tract. If sperm are available, the female fertilizes and lays her eggs and is reluctant to mate again. If not, the female suppresses egg-laying and is ready to mate. How is the SP signal conveyed to the CNS, and how does it modulate CNS circuits that control female mating behaviors?

The prevailing view is that SP is transported across the epithelium of the genital tract, enters the haemolymph, and acts directly on CNS targets (Kubli, 2003). Indeed, SP, like many other male seminal fluid proteins (Lung and Wolfner, 1999; Monsma et al., 1990; Ravi Ram et al., 2005), can be detected in the haemolymph of mated but not virgin females (Pilpel et al., 2008). Synthetic SP also triggers a post-mating response when injected directly into the haemolymph of virgin females (Chen et al., 1988). However, an alternative possibility is that the SP signal is conveyed to the CNS by a direct neural pathway from the reproductive tract. There is no evidence to date for such a pathway in *Drosophila*, but this model has been proposed for some species of moth in which unidentified male substances elicit an analogous post-mating response (Foster, 1993; Giebultowicz et al., 1990; Jurenka et al., 1993). An important first step towards resolving these possibilities, and ultimately understanding how SP acts to modulate behavioral circuits in the CNS, is to identify the cellular targets of SP.

We recently identified a molecular receptor for SP, called SPR, a member of the Gprotein coupled receptor family (Yapici et al., 2008). Females that lack SPR fail to respond to SP, both the natural SP transferred during mating as well as synthetic SP injected directly into the haemolymph. SPR is broadly expressed in the female reproductive tract and nervous system. SPR expressed in the epithelial tissues of the reproductive tract might contribute to immune and/or tissue remodeling responses that are also induced by mating (Adams and Wolfner, 2007; Domanitskaya et al., 2007; Peng et al., 2005b). The behavioral responses to mating can be entirely attributed to SPR function in the nervous system (Yapici et al., 2008). Moreover, expression of SPR in neurons that express the sex-specific transcripts of the *fruitless* (*fru*) gene is both necessary and sufficient for these behavioral responses (Yapici et al., 2008), supporting the notion that SP might act on some subset of the *fru* neurons (Kvitsiani and Dickson, 2006).

The *fru* gene labels ~2000 different neurons, including both sensory and central neurons (Billeter and Goodwin, 2004; Lee et al., 2000; Manoli et al., 2005; Stockinger et al., 2005). The specific neuronal targets for SP, and the route by which SP reaches these targets, therefore remain unknown. Here, we show that post-mating behavioral responses are mediated by SPR function in a set of just 2–3 internal sensory neurons located on either side of the uterus. These sensory neurons have rich arborizations within the lumen of the reproductive tract and project to central targets in the abdominal and/or suboesophageal ganglia. We propose that SP modulates the signals that these neurons convey to the CNS, thereby regulating the central circuits that govern female reproductive behavior.

Results

A GAL4 screen identifies neurons that require SPR function

We initially identified SPR in a genome-wide pan-neuronal RNAi screen (Yapici et al., 2008). In this screen, we crossed the pan-neuronal *elav-GAL4* driver to a genome-wide collection of RNAi transgenes (Dietzl et al., 2007) and scored female progeny for egg-laying defects. The *elav-GAL4 UAS-SPR-IR* females not only laid very few eggs after mating, but also remained sexually receptive. Thus, despite mating, *elav- GAL4*

UAS-SPR-IR females, like *SPR* null mutants, continue to behave as though they were virgins (Yapici et al., 2008).

To define the cellular requirement for SPR function, we now inverted the logic of this screen. We crossed the *UAS-SPR-IR* transgene to a large collection of GAL4 lines, scoring the female progeny for egg-laying defects in the same fashion. In each of these *GAL4* lines, the GAL4 transcriptional activator is expressed in a random but stereotyped subset of cells, reflecting the action of known or unknown cis-regulatory sequences. In these cells, any *SPR* function should now be inhibited by the *UAS-SPR-IR* transgene. From an initial survey of 998 GAL4 lines, we identified a set of 59 lines that resulted in a strong and reproducible egg-laying defect (Figure 1A). Many of these lines were found to be broadly expressed, as revealed with a *UAS-mCD8-GFP* transgene. We therefore focused our attention on a final set of 7 lines with restricted and distinct patterns of neuronal expression.

For each of these lines we performed a series of secondary assays to confirm the egg-laying defect, and also to examine the sexual receptivity of both virgin and mated females (Figure 1B). For all 7 *GAL4* lines, SPR knock-down resulted in reduced egg-laying and increased re-mating of females that had already mated, but little if any change in the receptivity of virgin females (Figures 1C-E). These defects were indistinguishable from those observed upon pan-neuronal SPR knock down with the *elav-GAL4* driver (Figures 1C-E), or in *SPR* null mutant females (Yapici et al., 2008).

Previously, we demonstrated that these defects can indeed be attributed to a failure to respond to SP, as injecting synthetic SP into the haemolymph renders control virgins unreceptive, but has little if any effect on *elav-GAL4 UAS-SPR-IR* virgins (Yapici et al., 2008). Similarly, we confirmed that expressing *UAS-SPR-IR* with the most restricted of our positive *GAL4* lines, *ppk-GAL4*, also significantly suppresses the response of virgin females to synthetic SP (Figure 1F).

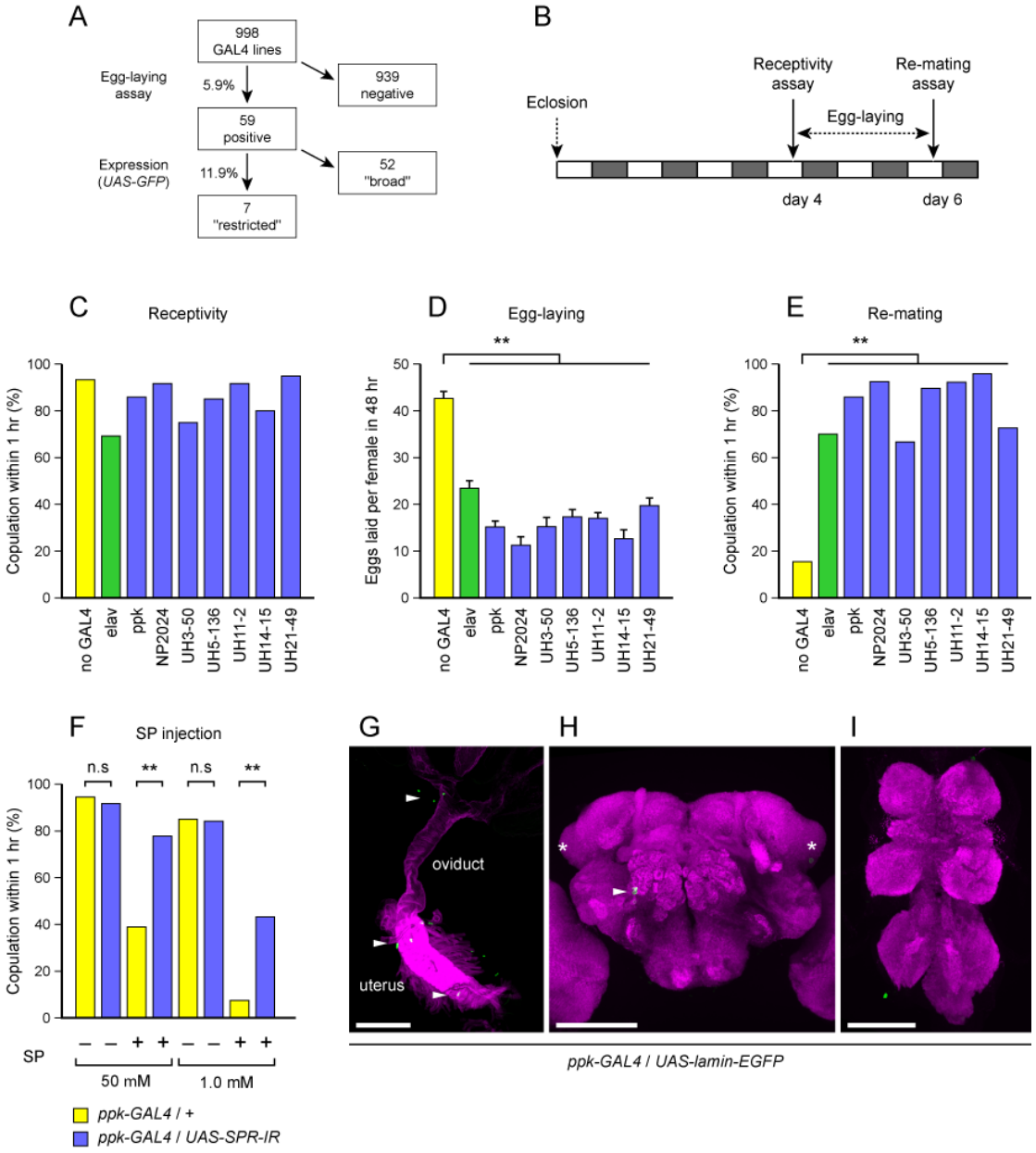


Figure 1: Identification of GAL4 lines in an SPR RNAi screen
(A) Overview of the primary screen.
(B) Protocol for the secondary assays in (C-E).
(C) Receptivity of virgin females carrying the indicated GAL4 line and *UAS-SPR-IR*, scored as the percentage of females that copulated within 1 hr. *n* = 59–120.
(D) Number of eggs laid per female during the 48 hr period after mating, *n* = 43–112. Data are shown as mean ± s.e.m. ** *P* < 0.0001, Student's *t*-test.
(E) Re-mating frequencies, *n* = 42–110. ** *P* < 0.0001, exact binomial test.
(F) Receptivity of virgin females of the indicated genotype upon injection with either 50 µM SP, 1.0 mM SP, or buffer alone (–). *n* = 36–40. n. s., *P* > 0.05; ** *P* < 0.0001; exact binomial test.
(G) Reproductive tract of *ppk-GAL4 UAS-lamin-EGFP* female stained with anti-GFP (green) and phalloidin (magenta). Arrowheads indicate locations of *ppk*+ neurons. Scale bar, 200 µm.
(H and I) Brain (H) and ventral nerve cord (I) of *ppk-GAL4 UAS-lamin-EGFP* female stained with anti-GFP (green) and the synaptic marker mAb nc82 (magenta). Arrowhead indicates a *ppk*+ neuron near the antennal nerve, asterisks indicate the positions of weakly stained cells lateral to the protocerebrum. Scale bar, 100 µm.

SPR is required in *ppk*+ sensory neurons in the female reproductive tract

Increased re-mating is not an obligatory consequence of reduced egg-laying (Barnes et al., 2007), yet we observed high re-mating frequencies with all 7 *GAL4* lines identified on the basis of their egg-laying defects. This suggests that the two postmating responses might be mediated by SPR function in a common set of cells, rather than the direct action of SP on distinct neuronal circuits for egg-laying and receptivity. Accordingly, we sought to determine the sites of expression that are common to all 7 *GAL4* lines.

Preliminary analyses identified *ppk-GAL4* as the line with the most restricted expression pattern. Using a nuclear targeted *UAS-lamin-EGFP* reporter, we found that *ppk-GAL4* drives expression almost exclusively in peripheral sensory neurons in the legs, wings, and body wall, as well as a small number of neurons associated with the female reproductive tract (Figure 1G). No cells are consistently labeled within the central nervous system (Figures 1H and 1I). Only occasionally could we detect one or two *ppk*+ cell bodies near the base of the antennal nerve, and sometimes also weak expression in one or two cells located lateral to the dorsal protocerebrum (Figure 1H). No *ppk*+ cells could be detected within the ventral nerve cord (Figure 1I).

Like *ppk-GAL4*, all 6 of the other positive *GAL4* lines also label cells along the reproductive tract (Supplemental Figure 1). In particular, all of these lines also label 2–3 sensory neurons located on either side of the uterus. In contrast, the *ppk*+ sensory neurons in the legs, wings, and body wall were not labeled by all other positive *GAL4* lines, and are also unlikely sites for the detection of a male seminal fluid protein. Accordingly, we conclude that the behavioral changes induced by SP are dependent upon *SPR* function in the *ppk*+ reproductive tract sensory neurons.

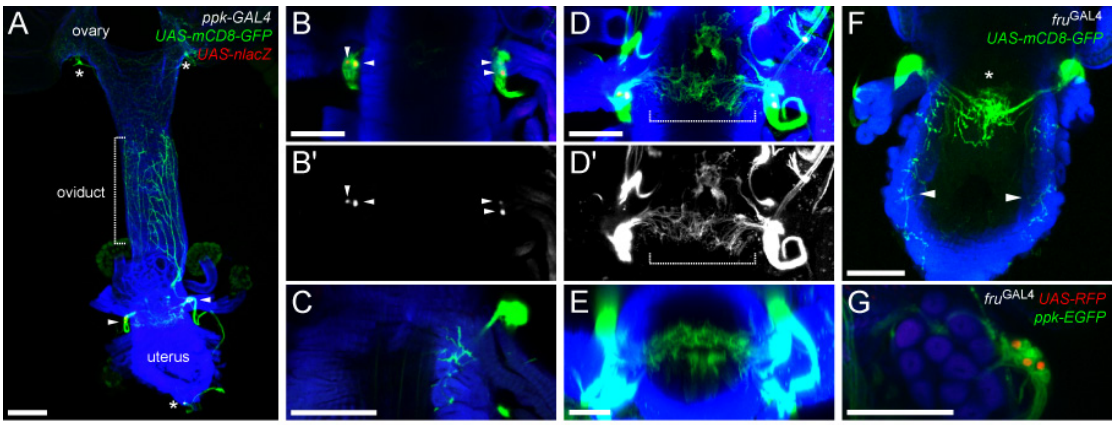


Figure 2: *ppk*$^{+}$ *fru*$^{+}$ sensory neurons innervate the female reproductive tract
(A-E) Reproductive tract of *ppk-GAL4 UAS-mCD8-GFP UAS-nlacZ* females, stained with anti-GFP (green), anti-β-galactosidase (red), and phalloidin (blue).
(A) Oviduct and uterus. Arrowheads indicate *ppk*+ cell bodies flanking the uterus, dashed line indicates projections along the oviduct, and asterisks indicate additional *ppk*+ neurons near the base of the ovaries and the tip of the uterus.
(B and B') Higher magnification views showing 2 *ppk*+ neurons on each side of the uterus (arrowheads). (B') shows the anti-β-galactosidase staining alone.
(C) Confocal section showing processes of *ppk*+ neurons that penetrate between the muscle and epithelial cells to enter the lumen of the uterus.
(D and D') Processes of *ppk*+ neurons in the lumen of the uterus (dashed line).
(E) View along the central axis of the uterus, which is surrounded by a ring of muscle fibres (blue). Processes of *ppk*+ neurons in the uterus lumen (green) presumably lie on or near the inner surface of epithelial cells.
(F) Reproductive tract of *fru*GAL4 *UAS-mCD8-GFP* female stained with phalloidin (blue), with GFP fluorescence in green. Asterisk indicates arborizations in the uterus, which includes fine processes extending to the lower uterus that are less obvious with *ppk-GAL4* (arrowhead).
(G) High magnification confocal image of the uterus of a *ppk-EGFP fru*GAL4 *UAS-hist-RFP* female, stained with phalloidin (blue) and showing GFP fluorescence in green and RFP fluorescence in red.
Scale bars: (A), 100 μm; (B-G), 50 μm.

ppk+ *fru*+ sensory neurons innervate the reproductive tract

As visualized with a membrane-tethered *UAS-mCD8-GFP* reporter, the *ppk*+ reproductive tract neurons project fine processes between the muscle and epithelial cell layers to enter and arborize within the lumen of the uterus (Figures 2A-E). An additional branch bifurcates close to the soma and arborizes extensively in the lower regions of the common oviduct (Figure 2A). As judged by confocal microscopy, the arborizations in both the uterus and lower oviduct run along the inner surface of the epithelial cell layer. We had previously mapped the requirement for SPR function to the set of ~2000 neurons that express the sex-specific P1 transcripts of the *fru* gene (Yapici et al., 2008). These neurons are distributed in clusters throughout the

peripheral and central nervous systems (Billeter and Goodwin, 2004; Lee et al., 2000; Manoli et al., 2005; Stockinger et al., 2005), and contribute functionally to both male (Manoli et al., 2005; Stockinger et al., 2005) and female (Kvitsiani and Dickson, 2006) mating behaviors.

We therefore suspected that some or all of the *ppk*+ neurons might also be *fru*+. Indeed, in *fru*-GAL4 *UAS-mCD8-GFP* females we observed GFP+ neurons near the uterus that appeared identical to the *ppk*+ neurons, with similar arborizations in the upper uterus and the oviduct (Figure 2F). The *fru*-GAL4 driver is stronger than *ppk-GAL4*, revealing additional fine arborizations extending into the lower uterus (Figure 2F). *fru*-GAL4, like several of the other positive *GAL4* lines from our initial screen, did not label the *ppk*+ cells near the base of the ovary and the tip of the uterus.

To test whether the *ppk*+ and the *fru*+ uterus sensory neurons are the same, we combined a *ppk-EGFP* reporter (Grueber et al., 2003) with *fru*-GAL4 *UAS-hist-RFP*, allowing us to visualize both *ppk*+ and *fru*+ cells in the same animal. Indeed, the cells that expressed the cytoplasmic *ppk-EGFP* reporter were also positive for the nuclear *UAS-hist-RFP* reporter driven by *fru*-GAL4 (Figure 2G).

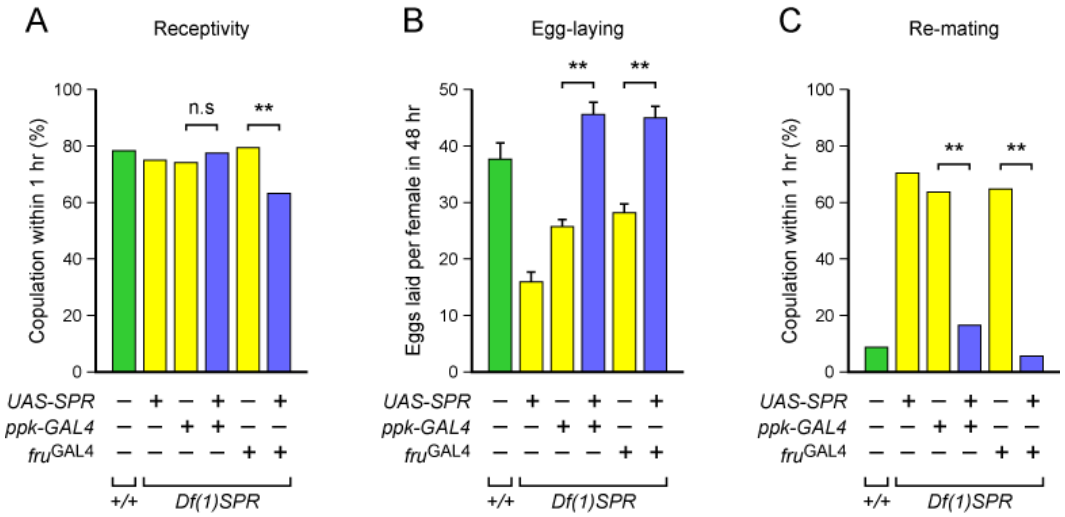


Figure 3: Rescue of SPR function in *ppk*$^{+}$ neurons
Females of the indicated genotypes were assayed according to the protocol in Figure 1B. *n* = 60 for the first two genotypes, and 112–120 for all others. *Df(1)SPR* is the cantonized *Df(1)Exel6234* strain (Yapici et al., 2008). n.s., $P > 0.05$; ** $P < 0.0001$; exact binomial tests in (A) and (C), Student's t-test in (B). Data in (B) are mean ± s.e.m. Note that, in slight contrast to (Yapici et al., 2008), we observe a small but significant reduction in virgin receptivity upon expression of *UAS-SPR* with *fru*GAL4 (A), possibly as a result of *SPR* overexpression. We also obtain a full restoration of egg-laying with *fru*GAL4, whereas previously we obtained only partial rescue (Yapici et al., 2008). However, our *SPR* deficiency females also now lay slightly more eggs than previously observed, possibly due to the recent accumulation of modifier mutations.

SPR expression in *ppk+ fru+* neurons is sufficient for the mating switch

The results of our RNAi knock-down experiments establish that *SPR* function is required in the *ppk+ fru+* uterus sensory neurons, but they do not preclude an additional requirement for *SPR* function in other cells. To test this possibility, we used *ppk-GAL4* to drive a *UAS-SPR* transgene in *SPR* null mutant females. In these females *SPR* function is present only in *ppk+* cells. In assays for virgin receptivity, egg-laying, and re-mating frequency, these females behaved indistinguishably from the wild-type control females (Figure 3). In contrast, *SPR* mutants carrying either but not both of the two transgenes laid significantly fewer eggs after mating and re-mated at high frequency (Figure 3). We also confirmed our previous finding (Yapici et al., 2008) that expression of *SPR* in *fru+* neurons alone is also sufficient to restore the post-mating switch in *SPR* null mutant females (Figure 3). The simplest interpretation of these data is that the behavioral changes induced by mating are due to SP acting exclusively on the *ppk+ fru+* uterus sensory neurons.

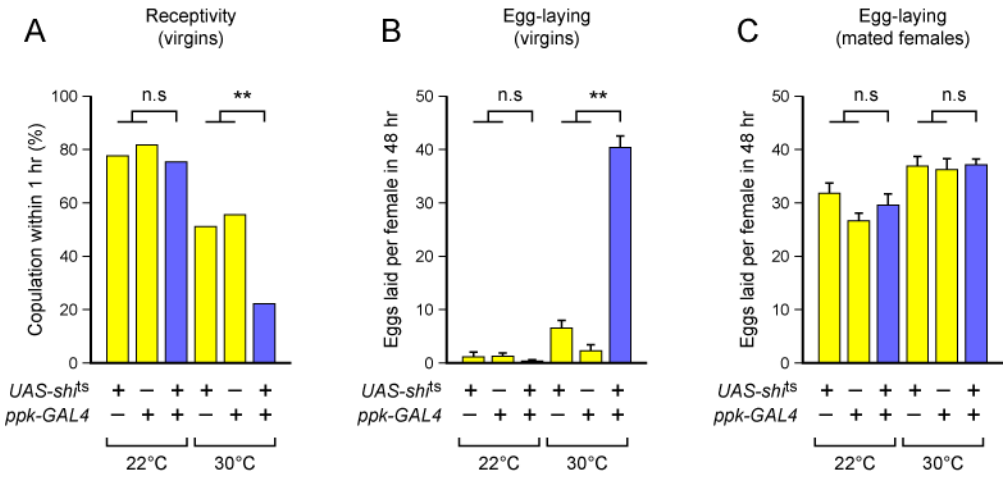


Figure 4: Silencing the *ppk*$^{+}$ neurons
(A) Receptivity of virgin females raised at 22°C and kept at the indicated temperature for 90 min before and 60 min during the mating assay. *n* = 137–190 for assays at 22°C, *n* = 90 for all genotypes at 30°C. n.s., *P* > 0.05; ** *P* < 0.0001; exact binomial test.
(B) Number of eggs laid by virgin females raised at 22°C and then kept at the indicated temperature for 2 days. *n* = 50–55. Data are shown as mean ± s.e.m. n.s., *P* > 0.05; ** *P* < 0.0001; Student's *t*-test.
(C) Number of eggs laid by females raised at 22°C, mated to wild-type males, and then kept at the indicated temperature for 2 days. *n* = 39–66. Data are shown as mean ± s.e.m. n.s., *P* > 0.05, Student's *t*-test.

Silencing *ppk*+ *fru*+ neurons induces post-mating behaviors in virgin females

We used *ppk-GAL4* and a *UAS-shi*^{ts} transgene (Kitamoto, 2001) to acutely block synaptic transmission from *ppk*+ neurons. At the restrictive temperature of 30°C, the dominant-negative *shi*^{ts} dynamin mutant blocks synaptic vesicle recycling. Normal synaptic transmission is rapidly restored once flies are returned to the permissive temperature of 22°C (Kitamoto, 2001). When we silenced *ppk*+ neurons by culturing flies for 90 min at 30°C, virgin females were significantly less receptive to mating than control females lacking either *ppk-GAL4* or *UAS-shi*ts, as well as virgins that carried both but were maintained at 22°C (Figure 4A). Indeed, the receptivity of virgin females with silenced *ppk*+ neurons was reduced to levels typically observed in wild-type mated females (Figures 1E and 3B).

Similarly, virgin *ppk-GAL4 UAS-shi*^{ts} females also laid many eggs when they were maintained for 2 days at 30°C (Figure 4B), at a rate comparable to wild-type mated females (Figures 1D and 3C). Indeed, for females with silenced *ppk*+ neurons, egglaying rates were equally high regardless of whether or not they were allowed to

mate before being transferred to 30°C (Figures 4B and 4C). In contrast, and as expected, control females laid very few eggs as virgins (Figure 4B) but large numbers of eggs after mating (Figure 4C).

Silencing the *ppk*+ neurons thus induces post-mating behaviors in virgin females. The same effect has previously been observed upon silencing of all *fru*+ neurons (Kvitsiani and Dickson, 2006), which of course also includes the *ppk*+ *fru*+ uterus sensory neurons. Silencing these neurons evidently mimics exposure to SP.

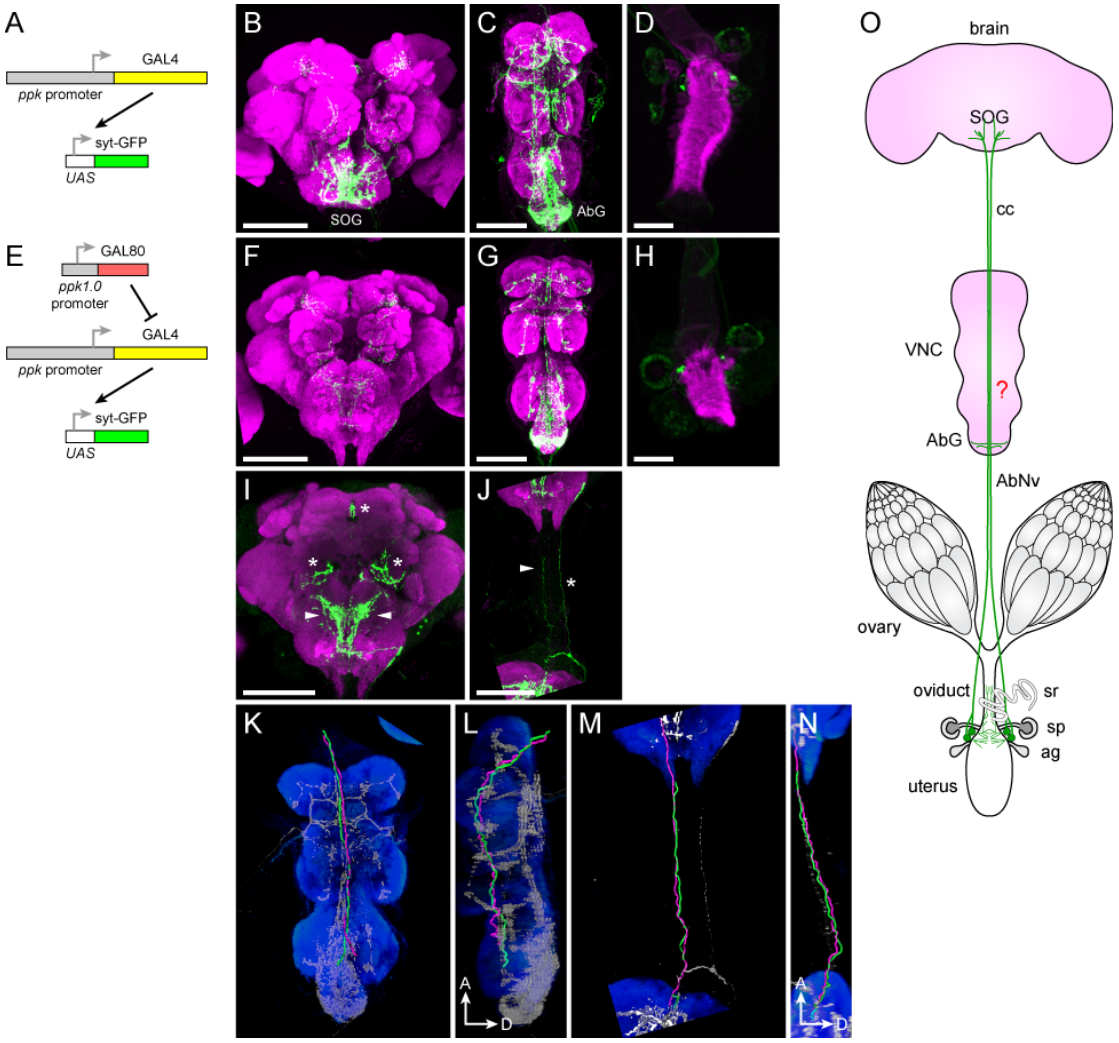


Figure 5: Central projections of *ppk*+ neurons
(A-D) GFP expression in *ppk-GAL4 UAS-syt-GFP* (A) female. Brain (B) and ventral nerve cord (C) stained with anti-GFP (green) and mAb nc82 (magenta). Reproductive tract (D) stained with anti-GFP (green) and phalloidin (magenta). SOG, suboesophageal ganglion; AbG, abdominal ganglion.
(E-H) GFP expression in *ppk-GAL4 ppk1.0-GAL80 UAS-syt-GFP* (E) female, showing brain (F), ventral nerve cord (G) and reproductive tract (H). Samples were stained and imaged under identical conditions to those shown in (B-D).
(I and J) Single confocal section of the posterior brain of a *ppk-GAL4 ppk1.0-GAL80 UAS-syt-GFP* female (I), imaged at higher gain than in (F). Note the extensive terminal arborizations of *ppk*+ fibres in the medial posterior SOG (arrowheads), which can be traced to a medial ascending pair of medial *ppk*+ fibres visible in the maximum intensity projection of cervical connective (J, arrowhead) Asterisk in (J) indicates an additional lateral fibre that originates from a cell loosely associated with the prothoracic ganglion and terminates in the lateral SOG. This lateral fibre is frequently lost during dissection. Termini in other brain regions (asterisks in I) are derived from *ppk*+ fibres that appear to enter through the antennal nerve (Figure 1H and Supplemental Figure 2B)
(K–N) Tracings of *ppk*+ fibres along the ventral aspect of the nerve cord (K and L) and the cervical connective (M and N) of a *ppk-GAL4 ppk1.0-GAL80 UAS-syt-GFP* female, stained with mAb nc82 (blue) and anti-GFP (grey). The two medial GFPpositive pathways are traced in green and magenta. (K) and (M) show ventral views, (L) and (N) lateral views.
(O) Schematic, indicating uterus *ppk*+ neurons (green), with central projections along the abdominal nerve (AbNv) to the AbG, and most likely also SOG. cc, cervical connective; sr, seminal receptacle; sp, spermathecae; ag, accessory gland (parovaria).
Scale bars: 100 µm.

Central projections of *ppk+ fru+* sensory neurons

We sought to determine the central projections of the *ppk*+ neurons by combining *ppk-GAL4* with either the membrane marker *UAS-mCD8-GFP* (Supplemental Figures 2AC) or the pre-synaptic marker *UAS-syt-GFP* (Figures 5A-D). Because very few if any CNS cells are *ppk+* (Figures 1H and 1I), any GFP+ processes in the CNS presumably derive from peripheral *ppk*+ neurons. This includes, but unfortunately is not limited to, the *ppk*+ *fru*+ sensory neurons on the uterus. We observed that these neurons contribute to the GFP+ fibers to the abdominal trunk nerve, the processes of which arborize extensively within the abdominal ganglion. At least some of the GFP+ fibers from the abdominal trunk nerve extend further anteriorly, but are difficult to trace through the thoracic ganglia due to the many additional GFP+ processes that enter through the leg and wing nerves.

Fortunately, we found that a proximal 1.0 kb promoter fragment from the *ppk* gene drives expression in most of the *ppk*+ leg neurons, but not in the uterus neurons (Supplemental Figures 2D-G). We therefore used this *ppk1.0* promoter to drive expression of GAL80, a repressor of GAL4, and combined this *ppk1.0-GAL80* transgene with *ppk-GAL4 UAS-syt-GFP* (Figure 5E-N). In these animals, the uterus neurons were still strongly labeled (Figure 5H), but reporter expression within the ventral nerve cord was now largely restricted to two sets of readily distinguished fibers that enter through the abdominal trunk and the mesothoracic nerves. (Figure 5G). The dense set of *ppk*+ termini in the abdominal ganglion was still present, whereas those in the thoracic ganglia were greatly reduced. Those that remain appeared to arise predominantly, if not exclusively, from processes that enter through the mesothoracic nerve.

In 3D confocal images from these animals, we could now trace a medial pair of bilateral GFP+ fibers that emerge from the dense network in the abdominal ganglion, traverse the entire length of the nerve cord along its ventral side (Figures 5K and 5L), and extend through the cervical connective (Figures 5J, 5M, and 5N) into the brain. These ascending fibers terminate with dense arborizations in the posterior region of the suboesphageal ganglion (SOG; Figure 5I). *ppk1.0-GAL80* suppresses marker expression in almost all other *ppk*+ inputs to the brain, with the exception of

processes that enter near the antennal nerve (asterisks in Figure 5I) and cells near the prothoracic ganglion that extend an ascending lateral fiber that terminates in the lateral SOG (asterisk in Figure 5J). As none of these processes come into proximity of the medial posterior SOG, we conclude that the GFP+ termini in this region arise exclusively from the ascending fibers from the abdominal nerve.

Our various attempts to perform single-cell labeling of the *ppk*+ uterus neurons have thus far been unsuccessful. We therefore cannot state with certainty that this ascending *ppk*+ pathway originates from the uterus neurons, and not some other unidentified *ppk*+ sensory neurons that also contribute to the abdominal nerve. Nonetheless, our data indicate that the neural signal generated or modulated by SP is conveyed to targets in the abdominal ganglion of the nerve cord, and most likely also to targets in the posterior suboesophageal ganglion in the brain (Figure 5O).

Discussion

SPR acts in *ppk*+ *fru*+ sensory neurons innervating the uterus

Here we have described a set of internal *ppk*+ *fru*+ sensory neurons in the female reproductive tract, and provided evidence that SPR functions in these neurons to trigger the behavioral changes induced by SP upon mating. The identification of SPR and its function in these neurons was made possible by transgenic RNAi technology – in particular a genome-wide library of RNAi transgenes and a large collection of GAL4 lines to target gene interference to specific cells. First, we identified SPR by screening the RNAi library with a pan-neuronal GAL4 line (Yapici et al., 2008). Now, we have screened a GAL4 library with this *SPR* RNAi line in order to identify the specific neurons in which *SPR* function is required. This iterative approach provides a powerful means to successively and systematically narrow down the genes and neurons that contribute to specific behaviors – an important first step in defining the underlying molecular and circuit mechanisms.

We have not been able to unambiguously detect SPR protein in these *ppk*+ *fru*+ sensory neurons by light microscopy. We presume it would be located on the arborizations of these neurons within the lumen of the uterus, but these processes lie on the surface of epithelial cells that themselves express high levels of SPR.

Nonetheless, we are confident that these neurons do indeed express SPR, and that activation of SPR in these neurons induces post-mating behaviors. This conclusion rests on two complementary sets of observations. First, SPR is required in both *ppk*+ and *fru*+ cells, as evidenced by the lack of a post-mating response upon knock-down of SPR in either cell population. Second, we can restore the post-mating response in *SPR* null mutant females by expressing SPR in either *ppk*+ or *fru*+ cells alone. This precludes the possibility that SPR is required in both a *ppk*+ *fru*- and a *ppk*- *fru*+ cell population, forcing the conclusion that SPR acts exclusively in cells that are both *ppk*+ and *fru*+. The sensory neurons innervating the uterus are the only cells we have been able to identify that express both of these markers. There are typically 4–6 such cells, and we do not yet know if they are functionally equivalent, or if egg-laying and receptivity are regulated by two distinct cell subtypes.

Models for SP action

Silencing synaptic transmission of *ppk*+ *fru*+ neurons mimics the activity of SP, in that both cause virgin females to become unreceptive and initiate egg-laying. Thus, an attractive hypothesis is that activation of SPR by SP, directly or indirectly, reduces the synaptic output of these neurons. The *ppk*+ *fru*+ neurons may be the only sensory neurons in the uterus (M. H., unpublished), and, like other *ppk*+ neurons (Adams et al., 1998; Grueber et al., 2003), they are probably mechanosensory. They may therefore have an important function as uterus stretch receptors in the coordination of sperm transfer, fertilization, and egg release. As such, they may have two distinct functional states, depending on the presence or absence of sperm. SP may switch these neurons between these two states. Because receptivity can be genetically uncoupled from egg production and egg-laying (Barnes et al., 2007), we infer that SP can also act independently of any stretch signal in the uterus. Modulation of receptivity and egg-laying might be mediated through either distinct *ppk*+ *fru*+ subtypes or distinct central synapses.

How might SP regulate these sensory neurons? We can envision two possibilities. First, the *ppk*+ *fru*+ neurons may detect SP in the reproductive tract and alter their firing rate accordingly. In this model, passage of SP into the haemolymph would not be required to induce the post-mating response. A second possibility is that SP

enters the circulatory system and acts presynaptically to modulate the release of these neurons at their central targets. The fact that SP can indeed be detected in the haemolymph of mated females (Pilpel et al., 2008) does not in itself exclude the former possibility. At least some effects of SP, such as stimulating juvenile hormone synthesis in the corpus allatum (Moshitzky et al., 1996), probably do require SP to enter the haemolmyph, but not all of SP's effects are mediated by this route (Domanitskaya et al., 2007). Similarly, the fact that SP triggers a post-mating response even when injected directly into the haemolymph (Chen et al., 1988) is also consistent with either model. The somata and some processes of the *ppk*+ *fru*+ neurons lie outside the uterus and presumably are readily accessible to factors in the haemolymph. We also note that a neural rather than a circulatory route has been proposed to mediate postmating responses in several species of moths (Foster, 1993; Giebultowicz et al., 1990; Jurenka et al., 1993). However, this conclusion is based upon the loss of this response upon nerve cord transection, a result predicted by both of these models. Thus, both models appear to be consistent with available evidence from studies in *Drosophila* and other species, and distinguishing between them will require detailed studies of the physiological properties of the *ppk*+ *fru*+ neurons and their response to SP.

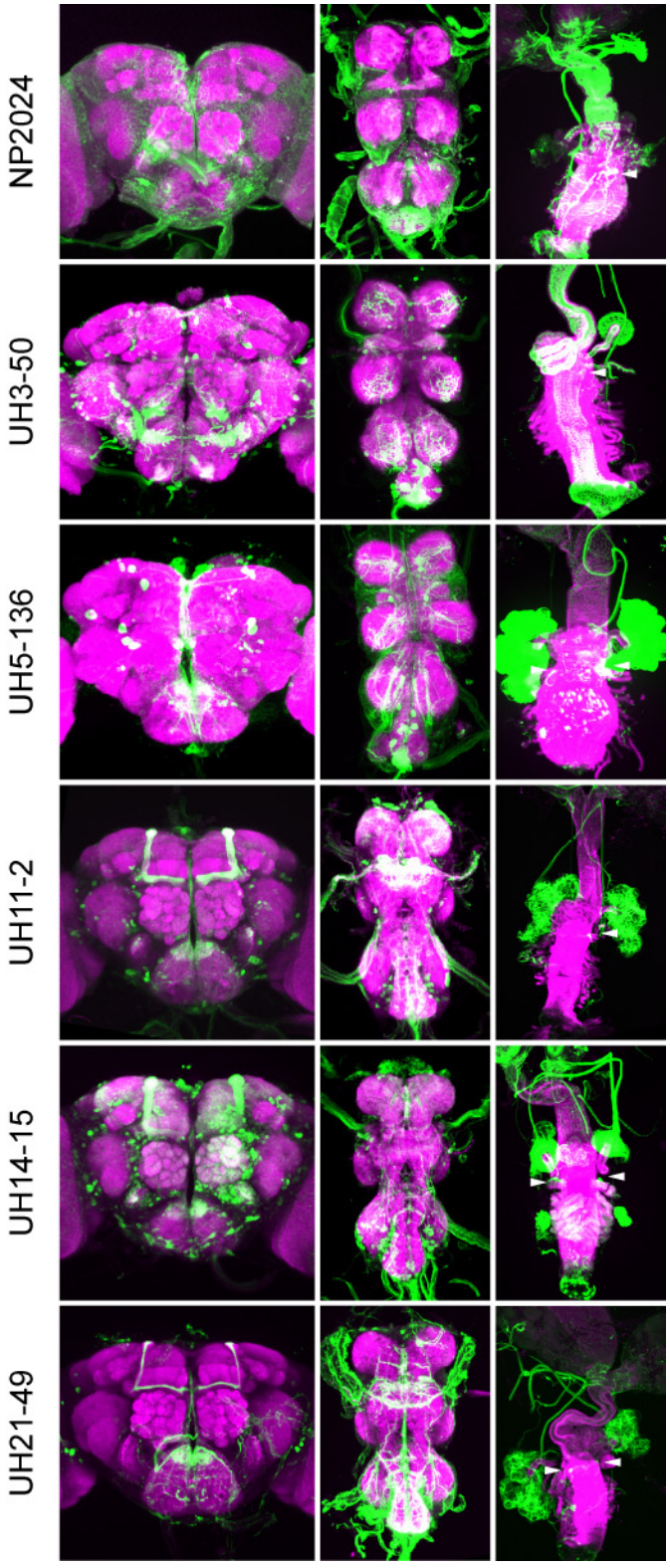
Central targets of *ppk*+ *fru*+ sensory neurons

The central targets of the *ppk*+ *fru*+ sensory neurons include the abdominal and/or suboesophageal ganglia – regions of the CNS likely to contain circuits that mediate behavioral responses to mating. The abdominal ganglion also houses the octopaminergic neurons that innervate the muscles of the ovary, oviduct, and uterus, and are believed to regulate the release and passage of mature eggs from the ovary to the uterus (Cole et al., 2005; Middleton et al., 2006; Monastirioti, 2003; Monastirioti et al., 1996; Rodriguez-Valentin et al., 2006). We suspect that these octopaminergic neurons are direct or indirect targets of the *ppk*+ *fru*+ sensory neurons of the reproductive tract, and that these circuits serve to coordinate ovulation and oviposition with the detection and utilization of sperm.

Our tracing experiments also suggest that some *ppk*+ fibres project from the abdominal trunk nerve right through to the SOG, potentially forming a direct neural

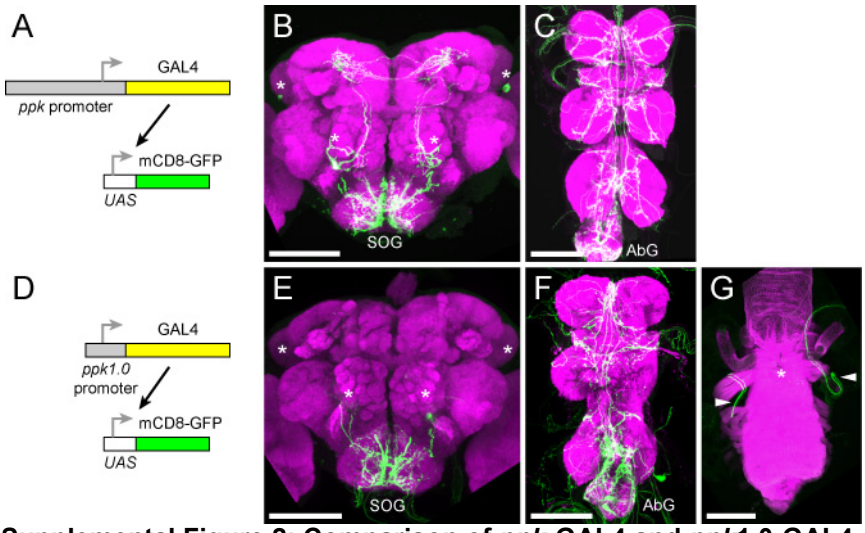
connection from the reproductive tract to the brain. We suspect that these projections may feed into neural circuits that regulate female mating receptivity and other postmating behaviors. Virgin females are enticed to mate by the male's courtship song. Most auditory sensory neurons project to the mechanosensory neuropil in the brain, adjacent to the SOG, but some also target the SOG itself (Kamikouchi et al., 2006). The proximity of the auditory processing centers and the ascending *ppk*+ projections raises the attractive possibility that mating modulates an early step in song processing. The SOG also contains processes of the Ilp7 neurons, which function in egg-laying site selection after mating (Yang et al., 2008). Direct evidence for mating-induced changes in SOG circuit function is lacking in flies, but has been obtained in other insects. In some species of moth, mating induces a long-term inhibition of neurosecretory cells in the SOG that regulate female pheromone biosynthesis, making mated females less attractive to other males (Ichikawa, 1998).

Having identified sensory neurons that detect SP in the reproductive tract, it will now be important to characterize the central pathways that process these signals to regulate female behavior. In the olfactory system, sensory neurons that detect pheromones are *fru*+ (Kurtovic et al., 2007; Root et al., 2008), as are their post-synaptic partners in the brain (Datta et al., 2008; Stockinger et al., 2005). Given that the sensory neurons that detect SP are also *fru*+, and many *fru*+ neurons are also located in both the abdominal and suboesophageal ganglia (Billeter and Goodwin, 2004; Manoli et al., 2005; Stockinger et al., 2005), it is enticing to think that a similar logic may apply in these pathways too. Elucidating the operation of these circuits should reveal how the female CNS integrates both external and internal information to switch between two very different behavioral patterns.



Supplemental Figure 1: Uterus sensory neurons labeled by additional positive GAL4 lines

Brain (left), ventral nerve cord (centre), and reproductive tract (right) of adult females carrying the indicated *GAL4* line and *UAS-mCD8-GFP*, stained with anti-GFP (green) and either mAb nc82 (magenta, brain and nerve cord) or phalloidin (magenta, reproductive tract). Arrowheads indicate GFP+ sensory neurons flanking the uterus.



Supplemental Figure 2: Comparison of *ppk*-GAL4 and *ppk*1.0-GAL4
(A–C) Brain (B) and ventral nerve cord (C) of *ppk-GAL4 UAS-mCD8-GFP* (A) female, stained with anti-GFP (green) and mAb nc82 (magenta). Asterisks in (B) indicate cell bodies in the brain that are not observed in other *GAL4* lines that were positive in *UAS-SPR-IR* assay. SOG, suboesophageal ganglion; AbG, abdominal ganglion.
(D–G) Brain (E), ventral nerve cord (F), and reproductive tract (G) of *ppk1.0-GAL4 UAS-mCD8-GFP* (D) female, stained with anti-GFP (green) and either mAb nc82 (magenta in E and F) or phalloidin (magenta in G). Compared to *ppk-GAL4*, *ppk1.0- GAL4* does not label neurons located near the antennal lobe and lateral protocerebrum (asterisks in E), and within the ventral nerve cord labels many of the fibres that enter through the thoracic and abdominal trunk nerves. *ppk1.0-GAL4* does not strongly label the uterus sensory neurons (asterisk in G), although other processes of the abdominal trunk nerve are labeled (arrowheads in G). Scale bars: 100μm.

Experimental Procedures

GAL4 screen

Virgin females homozygous for *UAS-SPR-IR1* on the 3rd chromosome (Yapici et al., 2008) and *UAS-Dcr2* on the first chromosome (Dietzl et al., 2007) were obtained from the appropriate Y, *hs-hid* stocks and crossed to males from the various GAL4 lines. 5–6 *UAS-SPR-IR* females were crossed to 3–5 *GAL4* males. Progeny were raised on semi-defined medium (Backhaus et al., 1984) at 25°C on a 12:12hr dark: light cycle. Parents were removed from the vial after 3 days, and adult progeny left in the vial for 3–4 days post-eclosion to allow mating. 20–30 adult females and 3–5 males were then removed and transferred to a fresh food vial, and again transferred to a fresh vial after 24 h and 48 h. After 72 h, the adult flies were discarded. The number of eggs in each of the three vials was estimated and scored on a 1-5 scale as follows: 1, ~100 or more eggs (normal); 2, ~50-100 eggs; 3, ~20-50 eggs; 4, ~5-20 eggs; 5, ~0-5 eggs. A three-day average score of 3 or more was regarded as positive. For a quick

assessment of GAL4 expression patterns, lines were crossed to *UAS-GFP* on the 2nd chromosome and the brains, ventral nerve cords, and reproductive tract were dissected from adult female progeny and examined live under wide field fluorescent microscopy.

Fly stocks

Most of the GAL4 lines were obtained from the Heberlein collection, supplemented with additional lines obtained from the NP consortium (Hayashi et al., 2002) and Douglas Armstrong (Armstrong et al., 1995), as well as numerous GAL4 lines generated or acquired in the Dickson lab over the past several years. *ppk-GAL4* was provided by Wesley Grueber (Grueber et al., 2003). The *ppk1.0-GAL4* and *ppk1.0-GAL80* lines were generated by cloning a 1.0 kb region immediately upstream of the *pickpocket* open reading frame into standard GAL4 and GAL80 expression vectors.

These transgenes were then integrated into a specific second chromosome site (VIE-72a) using the ϕc31 system (Groth et al., 2004). Other fly stocks used were *elav-Gal4* (Luo et al., 1994), *fru*GAL4 (Stockinger et al., 2005), *UAS-SPR*, *UAS-SPR-IR1* and *w+*; *Df(1)Exel6234* (Yapici et al., 2008), *UAS-lamin-EGFP* (provided by N. Stuurman), *UAS-hist-RFP* (Emery et al., 2005), *UAS-mCD8-GFP* (Lee and Luo, 1999), *UAS-syt-GFP* (Zhang et al., 2002), and *ppk-EGFP* (Grueber et al., 2003).

Behavioral assays

Quantitative assays for detailed phenotypic characterization were performed as described (Yapici et al., 2008). For the *UAS-shi*ts experiments, flies were raised, collected, and maintained at 22°C, and if appropriate shifted to 30°C 90 minutes before the assay. Assays for receptivity or egg-laying were then performed in parallel at 22°C and 30°C.

Immunohistochemistry and tracing of *ppk+* fibres

Staining of the CNS and reproductive tract were performed using rabbit anti-GFP (Torrey Pines Biolabs, 1:6000), mouse anti-GFP (Promega, 1:1000), mAb nc82 (DSHB, 1:20, (Wagh et al., 2006)) and/or rhodamine-phalloidin or Alexa647- phalloidin (both Molecular Probes/Invitrogen, 1:100). For axon tracing, the stained ventral nerve cord and brain was imaged at maximum optical resolution and high gain on a Zeiss LSM 510 confocal microscope. The image stack was deconvolved using Huygens Essential

(Scientific Volume Imaging) and a custom point spread function obtained from the confocal setup. Axons were traced in 3D using a custom module in Amira (Evers et al., 2005; Schmitt et al., 2004).

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Chapter 2

Signaling downstream of SPR and detailed analysis of the projections of the *ppk+ fru+* internal sensory neurons

Martin Haesemeyer and Barry J. Dickson

Summary

As in many species *Drosophila melanogaster* females behave very differently before and after mating. Before mating virgin females readily accept males for copulation and retain their eggs whereas after mating they will reject males and commence egg-laying. This dramatic behavioral switch is controlled by Sex-peptide (SP) a small male derived peptide found in the male seminal fluid. This peptide is synthesized in the male accessory gland and transferred to females during copulation. SP is both necessary and sufficient to elicit the behavioral switch. In the female SP is detected by a dedicated G-protein-coupled-receptor, the Sex-peptide-receptor (SPR). SPR is required in 4-6 *fruitless* (*fru+*) and *pickpocket* (*ppk+*) expressing internal sensory neurons on the uterus to elicit the female behavioral switch. Here we show that SPR in the *ppk+ fru+* neurons signals via $G_{\alpha o}$ and that these neurons project to a distinct region in the abdominal ganglion of the ventral nerve cord. Our findings contribute towards understanding of the physiology of these internal sensory neurons as well as aide the identification of downstream neurons that relay and potentially integrate information about the female mating state.

Introduction

Drosophila melanogaster females dramatically change their behavior according to their mating state. Virgin females accept courting males for copulation and retain their eggs, while the same females become refractory to courting males for approximately one week after mating and commence egg-laying (Bloch Qazi et al., 2003; Kubli, 2003). This behavioral switch is under the control of Sex-peptide (SP), a male derived peptide (Chapman et al., 2003; Chen et al., 1988; Liu and Kubli, 2003). SP is synthesized in the male accessory gland and transferred to the female during mating (Lung and Wolfner, 1999; Pilpel et al., 2008). In females SP activates a g-protein-coupled receptor, the sex-peptide-receptor (SPR), to elicit the post-mating

behavioral switch (Yapici et al., 2008). Despite its widespread expression, SPR is only required in a small set of internal sensory neurons on the female reproductive tract to orchestrate the mating switch (Hasemeyer et al., 2009; Yang et al., 2009). These neurons are located on the uterus and express both *pickpocket*-Gal4, a marker for proprioceptive neurons (Adams et al., 1998), and *fruitless*-Gal4, a marker for neurons involved in sex-specific behaviors (Manoli et al., 2005; Stockinger et al., 2005).

The molecular basis of signaling downstream of SPR in *ppk+ fru+* cells is not well understood. Cell-based assays indicated that SPR seems to signal via either $G_{\alpha i}$ or $G_{\alpha o}$ (Yapici et al., 2008). Behavioral data implicates protein-kinase-A (PKA) to be a downstream target of SPR (Yang et al., 2009). Expression of dominant-active PKA with *ppk*-Gal4 suppresses post-mating responses, while expression of a dominant-negative form induces egg-laying and partially suppresses receptivity (Yang et al., 2009). This led to the assumption that SPR signals via $G_{\alpha i}$ to reduce cAMP levels in the target cells (Yang et al., 2009). SPR signaling however was completely blocked by the expression of pertussis toxin (PTX) in *ppk* neurons (Yang et al., 2009). While PTX inhibits both $G_{\alpha i}$ and $G_{\alpha o}$ signaling in vertebrates (Moss and Vaughan, 1988), it is believed to only inhibit $G_{\alpha o}$ in *Drosophila* due to an amino acid substitution in the $G_{\alpha i}$ protein rendering it insensitive to the toxin (Katanaev et al., 2005). Here we show that SPR indeed signals via $G_{\alpha o}$ to elicit the SP dependent behavioral switch.

It is important to identify circuits downstream of the uterine *ppk+ fru+* neurons. This will further our understanding of how the mating signal is relayed to the central nervous system and ultimately how information about the mating state is integrated with external sensory information to create an appropriate behavioral output. SP has been shown to alter a range of behaviors in *Drosophila melanogaster*. It induces egg-laying and inhibits receptivity (Aigaki et al., 1991; Chen et al., 1988) and has been shown to inhibit siesta sleep and increase feeding in females (Carvalho et al., 2006; Isaac et al., 2010). Of these behavioral switches both egg-laying and receptivity have been shown to be mediated by the *ppk+ fru+* neurons (Hasemeyer et al., 2009; Yang et al., 2009) and it is likely that the change in sleep patterns is mediated by SPR in the same neurons. The fact that such different behaviors are all modulated by SP makes it likely that information about the mating state is relayed to and integrated

in higher brain centers. This means that the identification of neurons downstream of the *ppk+ fru+* neurons can lead to insights into how neural circuits for behaviors as different as mating and sleep are organized.

To identify putative downstream connecting partners it is important to know the exact location of the synaptic projections of the SP sensing neurons. These internal sensory neurons have been shown to project to the abdominal ganglion of the ventral nerve cord (Hasemeyer et al., 2009; Yang et al., 2009) and believed to potentially send processes to the suboesophageal ganglion in the brain (Hasemeyer et al., 2009). Using intersectional genetics here we show that the SP sensing neurons send processes to the ventral region in the abdominal ganglion of the ventral nerve cord. The identification and characterization of these processes should facilitate the identification of potential downstream neurons.

Results

SPR signals in *ppk+* neurons via $G_{\alpha o}$

In contrast to vertebrates, in *Drosophila* pertussis-toxin is believed to only inhibit signaling via the G-alpha protein $G_{\alpha o}$ (Katanaev et al., 2005). Expression of PTX with *ppk*-Gal4 abolishes post-mating responses in mated females while their receptivity as virgins remains unaffected (Figure1 and Yang et al., 2009). These females show reduced egg-laying after mating and remate at high levels (Figure 1C-D). Conversely driving expression of wildtype $G_{\alpha o}$ or a dominant active form of $G_{\alpha o}$ with *ppk*-Gal4 induces post-mating responses in virgin females (Figure 1B-C). These virgins are almost completely unreceptive towards courting males (Figure 1B) and lay eggs like mated females, whereas wildtype control virgins have low levels of egg-laying as expected (Figure 1C). However overexpression of $G_{\alpha i}$ in *ppk+* neurons does not affect female receptivity, egg-laying or remating (Figure 1B-D).

These data indicate that SPR signals via $G_{\alpha o}$ in the *ppk+ fru+* internal sensory neurons to induce the post-mating response.

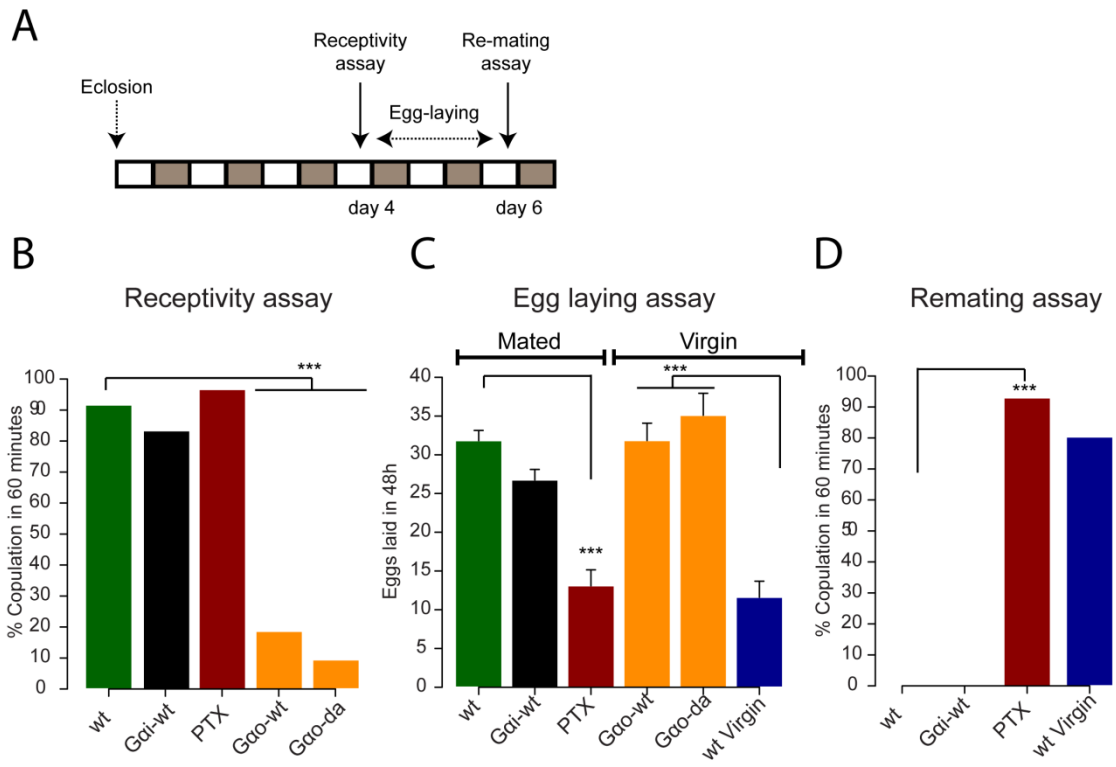

Figure1: SPR signals in *ppk*-Gal4 expressing neurons via $G_{\alpha o}$

(A) Protocol for the behavioral assays in C-E.

(B) Receptivity of virgin females carrying *ppk*-Gal4 and the indicated UAS-Transgene. Wt control is *ppk*-Gal4 X w1118. Receptivity was scored as the percentage of females that copulated within 1 hr. n = 30-60. *** P < 0.001, Fisher's exact test, Holm's correction for multiple comparisons.

(C) Number of eggs laid per female during the 48hr period after the mating assay. Females were either mated or virgins as indicated. Data are shown as mean + sem. *** P < 0.001, Wilcoxon test, Holm's correction for multiple comparison.

(D) Re-mating frequencies. Wt Virgin controls are *ppk*-Gal4 X w1118 virgins to indicate wildtype initial receptivity levels. *** P < 0.001, Fisher's exact test, Holm's correction for multiple comparisons.

The *ppk+ fru+* internal sensory neurons send projections to the ventral side of the abdominal ganglion

Previously we used a combination of *ppk*-Gal4 and a *ppk*-promoter fragment Gal80 (ppk1.0-Gal80, Hasemeyer et al., 2009) to sparsely label nerve fibers from the abdominal nerve and trace the projections of the *ppk+ fru+* neurons of the uterus. While this approach considerably reduced the amount of projections originating from other *ppk+* cells it was not possible to unambiguously trace the projections of

the uterine neurons (Hasemeyer et al., 2009), because *ppk1.0*-Gal80 could not remove expression in all abdominal neurons labeled by *ppk*-Gal4 (Hasemeyer et al., 2009). Based on the projection pattern obtained using this Gal4/Gal80 combination we hypothesized that the *ppk+ fru+* neurons send projections to the abdominal ganglion of the ventral nerve cord as well as to the suboesophageal ganglion in the brain (Hasemeyer et al., 2009). To fully characterize the projections we sought a different method to unambiguously label the *ppk+ fru+* neurons on the uterus. Since the neurons on the uterus are the only neurons we identified to express both *ppk*-Gal4 and *fru*-Gal4 (Hasemeyer et al., 2009) the ideal strategy would be to use intersectional genetics to label the overlap between these markers.

Combining the flp/frt system (Golic and Lindquist, 1989) with the Gal4/UAS system (Brand and Perrimon, 1993) allows labeling the overlap between two expression domains. Flip-recombinase can be used to excise a transcriptional stop that is placed in between the UAS-sequence and a mCD8-GFP transgene, thereby allowing expression via Gal4 in the flip recombinase expression domain (Figure2A). A knock-in of flip-recombinase into the *fruitless* locus has been created in the lab and shown to be expressed in *fruitless*-Gal4 positive neurons (Yu, 2009). Unfortunately the intersection of *ppk*-Gal4 with *fruitless*-Flip-recombinase (fru^{Flp}) does not label any neurons (data not shown). This is most likely due to unfavorable timing between the peak of *fru*-Flp expression and that of *ppk*-Gal4 expression. The general sensory driver *pebbled*-Gal4 (peb-Gal4; Sweeney et al., 2007) however, shows intersectional expression with fru^{Flp} (Jai Yu, personal communication). *Peb*-Gal4 also labels the same number of neurons with the same morphology as *ppk*-Gal4 on the uterus in the female reproductive tract (Figure 2B; *PPK*-Gal4 and *Peb*-Gal4 around 6-7 neurons in cluster, *Fru*-Gal4 around 3-4 neurons) making it a good candidate to label the *ppk+ fru+* neurons on the uterus via intersection with fru^{Flp}.

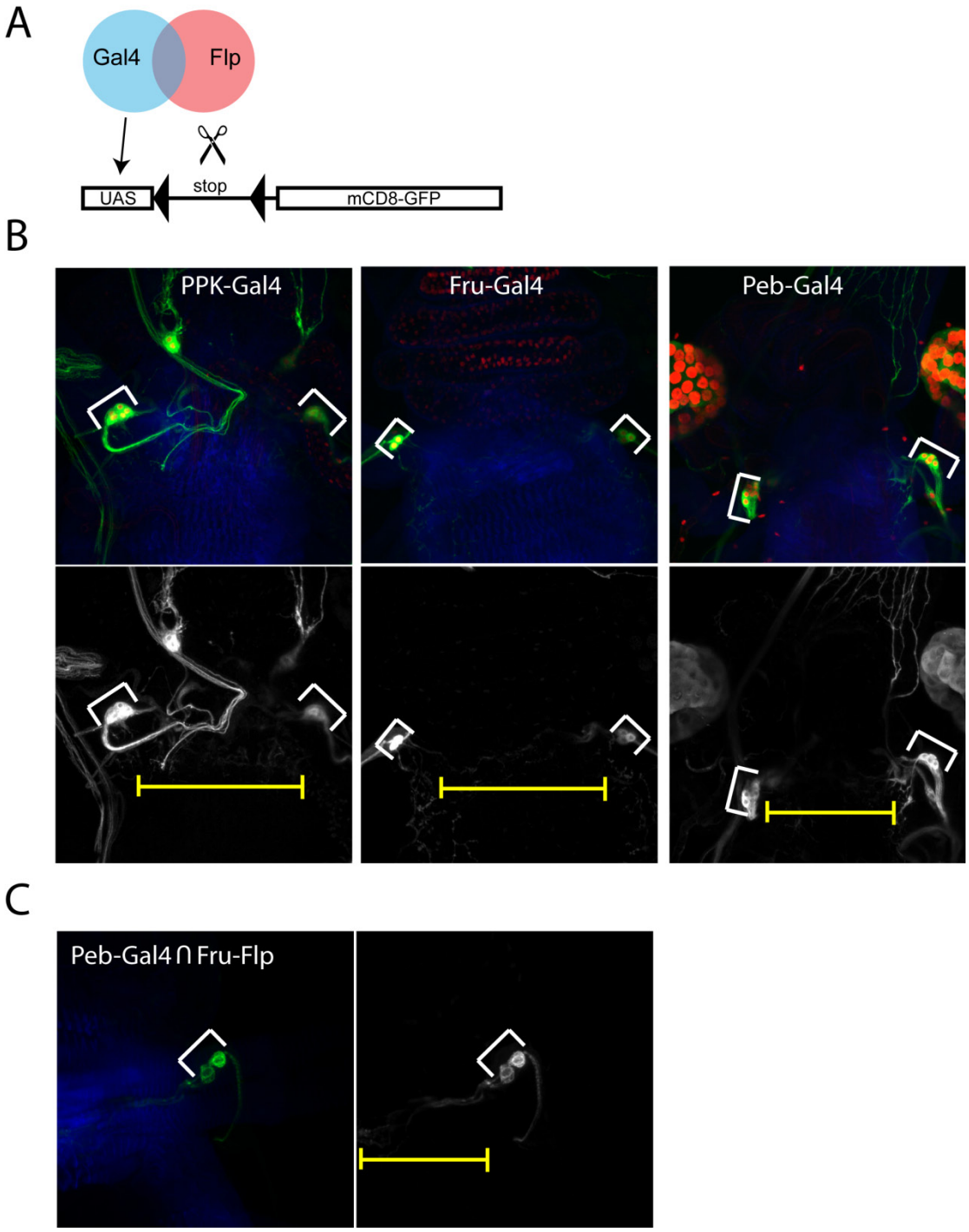

Figure2: Intersection of Peb-Gal4 and Fru-Flp labels the *ppk*+ *fru*+ internal sensory neurons.
(A) Schematic of the Gal4 Flp intersectional strategy.
(B) Expression of PPK-Gal4 (left), Fru-Gal4 (middle) and Peb-Gal4 (right) on the uterus, examined by crossing the indicated Gal4-line to a UAS-mCD8-GFP; UAS-hist-RFP reporter line, stained with anti-GFP (green), phalloidin (blue) and live RFP (red). Lower panel: GFP-channel only.
(C) Close-up of cell cluster labeled by intersection of Peb-Gal4 and Fru-Flp, stained with anti-GFP (green) and phalloidin (blue). Right panel: GFP-channel only.
(B-C) White brackets indicate location of cell-cluster present in all Gal4 lines which encompasses the *ppk*+ *fru*+ neurons. Yellow bar indicates the projections in the uterus.

The intersection of *peb*-Gal4 with *fru*^{Flp} indeed labeled the *ppk+ fru+* neurons on the uterus as judged by morphology of the labeled neurons (Figure 2C). Since the excision of the stop cassette by flip-recombinase is stochastic, the number of labeled cells on the uterus varies between animals (Arrows, Figure 3A). We therefore dissected the reproductive tract, ventral nerve cord and brain from each fly, keeping individual flies separated. In animals in which no cells on the uterus were labeled we did not observe any fibers in the abdominal nerve. This argues that all projections we observed entering the ventral nerve cord via the abdominal nerve originated from the internal sensory neurons on the uterus.

In rare cases we observed labeling of four neurons on one side of the uterus (Figure 3C, arrows) which should be the full set of *ppk+ fru+* internal sensory neurons (Hasemeyer et al., 2009). In these animals all projections entering the ventral nerve cord via the abdominal nerve (Figure 3D, arrows) terminated in the abdominal ganglion (Figure 3D, red bar). The other fibers observed in the ventral nerve cord did not arise from the abdominal nerve and were projections of other *fruitless* positive sensory neurons. This argues that the *ppk+ fru+* internal sensory neurons in fact do not send projections to the suboesophageal ganglion in the brain but that their axons rather terminate in the abdominal ganglion of the ventral nerve cord.

In most animals different subsets of the internal sensory neurons on the uterus were labeled (Figure 3A, arrows). We used these clones to analyze the projections in the abdominal ganglion in more detail. The analysis revealed that the neurons project to a distinct region on the ventral side of the abdominal ganglion (Figure 3B). Different neurons on the uterus seem to have slightly different patterns of projection. In general however the fibers enter via the abdominal nerve on the posterior dorsal side, project latero-ventral towards the anterior and then cross the midline to the contralateral side of the abdominal ganglion (Figure 3B). Some neurons have an additional medial branch, projecting towards the posterior along the ventral side (Figure 3B bottom panel, arrowhead).

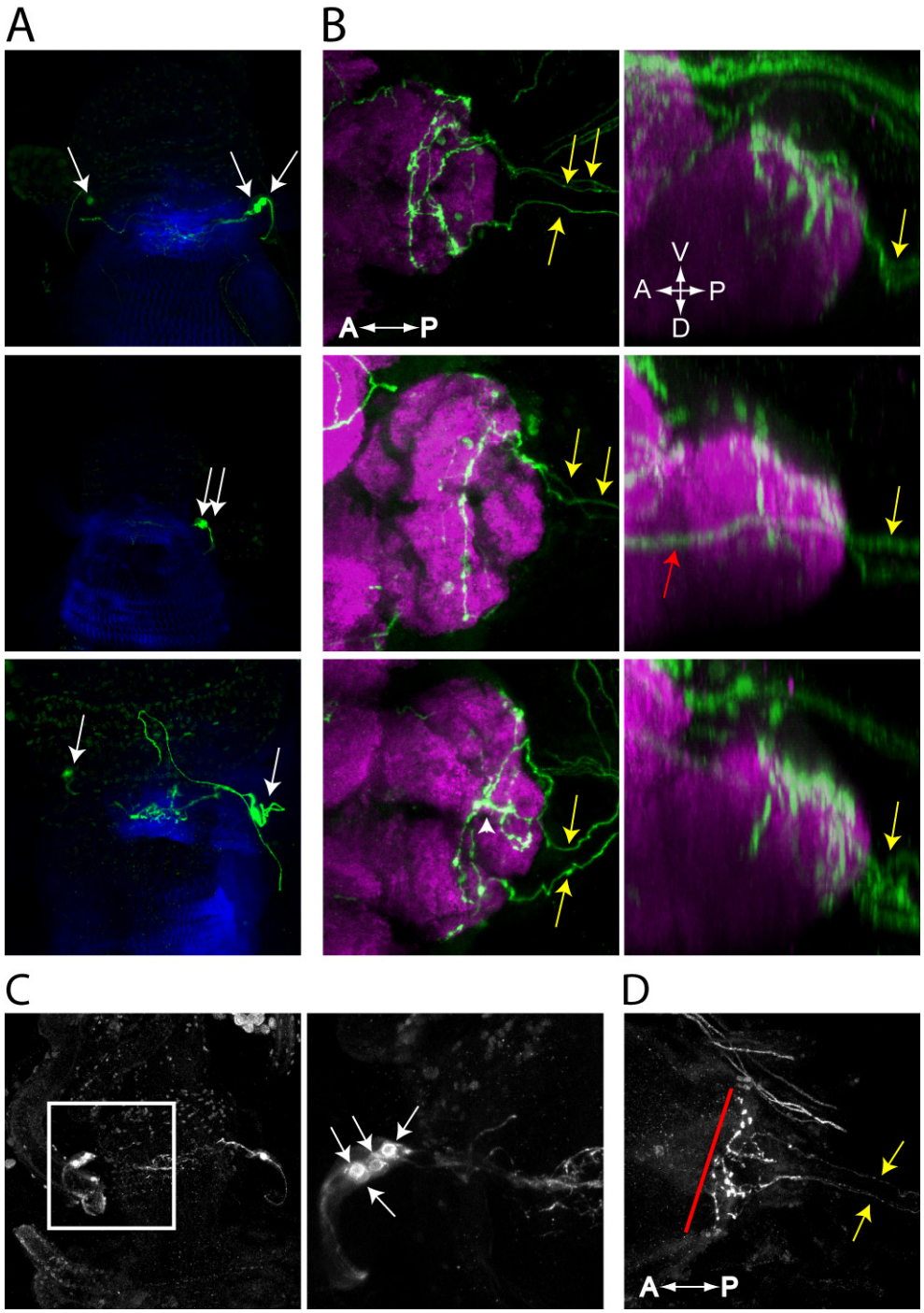

Figure3: The *ppk+ fru+* internal sensory neurons send projections to the ventral abdominal ganglion.
(A and B) Uterus (A) and abdominal ganglion (B) of 3 individual animals of Peb-Gal4; UAS-stop-mCD8-GFP; Fru-Flp, stained with anti-GFP (green), phalloidin (blue) and the neuropil marker Nc82 (magenta).
(B) Left panel is a maximum intensity Z-Projection, the right panel the corresponding Y-Projection. The anterior-posterior and dorso-ventral axes are indicated. White arrowhead indicates back-projection observed in some neurons.
(C) Uterus (left panel) and closeup of cell cluster (right panel) of a Peb-Gal4; UAS-stop-GFP1-10; FruFlp animal, stained with anti-GFP, labeling all *ppk+ fru+* cells in the cluster. White box indicates zoom region of right panel.

(D) Abdominal ganglion of the same animal shown in (C) stained with anti-GFP. No axons entering via the abdominal nerve project more anterior than the red bar.
(A-D) White arrows indicate individual cells labeled. Yellow arrows indicate individual axons in the abdominal nerve (B) or the boundaries of the abdominal nerve (D). Red arrows indicate, where necessary for disambiguation, projections that do not originate from the abdominal nerve.

Discussion

Our behavioral results suggest that SPR signals in *ppk*+ neurons via $G_{\alpha o}$ to elicit the behavioral switch. Specifically, the overexpression of either wildtype or dominant active forms of $G_{\alpha o}$ induces the mating switch in virgins (Figure 1) similar to blocking synaptic release of *ppk* expressing neurons via shi^{ts} (Hasemeyer et al., 2009; Yang et al., 2009), whereas blocking $G_{\alpha o}$ signaling via the expression of pertussis-toxin has the same effect as removing SPR from *ppk* neurons (Figure 1 and Hasemeyer et al., 2009; Yang et al., 2009). This suggests that activation of $G_{\alpha o}$ upon SP binding to SPR might reduce synaptic release from the *ppk+ fru+* sensory neurons and thereby activate the mating switch. So far physiological evidence of the SP effect on the neurons is missing and it is therefore not possible to say whether activation of SPR reduces activity in the *ppk+ fru+* neurons or if SPR acts in the presynaptic compartment to modulate transmitter release of the neurons. In the olfactory system it has been shown that GABA reduces synaptic release in olfactory sensory neurons (Olsen and Wilson, 2008). This GABA-ergic suppression of synaptic release has a fast component mediated by ionotropic GABA-A receptors and a slow component mediated by metabotropic GABA-B receptors (Olsen and Wilson, 2008). The GABA-B receptor mediated reduction in synaptic release is blocked by pertussis-toxin (Olsen and Wilson, 2008) and it is therefore intriguing to speculate that a similar mechanism might operate in the *ppk+ fru+* sensory neurons leading to a $G_{\alpha o}$ mediated reduction in synaptic release upon activation of SPR in the presynaptic compartment. Physiological characterization of the *ppk+ fru+* neurons is needed to further investigate this possibility.

Our previous hypothesis was that the *ppk+ fru+* sensory neurons on the uterus project directly to the brain (Hasemeyer et al., 2009). Since the activation of SPR seems to regulate disparate behaviors such as egg-laying, sexual receptivity, feeding

and sleep cycles in *Drosophila* females (Carvalho et al., 2006; Chapman et al., 2003; Chen et al., 1988; Isaac et al., 2010; Liu and Kubli, 2003) it is very likely that information about mating state is relayed to higher order brain centers and then fed into diverse behavioral circuits. Our results here however show that this relay could not be a direct one since the *ppk+ fru+* sensory neurons project exclusively to the abdominal ganglion of the ventral nerve cord (Figure 3). It is likely that they synapse with second order neurons in the abdominal ganglion that relay the information about the mating state to higher order centers. For the switch in egg-laying one can also envision a more direct route since the abdominal ganglion houses cell bodies of octopaminergic neurons that control oviposition (Monastirioti, 2003). In the olfactory system, specific *fru+* sensory neurons mediate sex-pheromone responses (Kurtovic et al., 2007) and synapse with second order neurons that are also *fru+* (Datta et al., 2008). It is intriguing to speculate that the same logic might apply for the *ppk+ fru+* sensory neurons detecting the mating state of the fly. Indeed there are *fru+* neurons in the abdominal ganglion of the ventral nerve cord that send processes to the suboesophageal ganglion in the brain (Yu, 2009). These neurons are plausible candidate downstream neurons, especially since in *Bombyx* the suboesophageal ganglion has been shown to house neurosecretory cells controlling receptivity behaviors (Ichikawa, 1998).

Unraveling the signaling downstream of SPR as well as analyzing the projection pattern of the *ppk+ fru+* sensory neurons are important first steps for a more detailed physiological analysis of the neurons as well as for investigating how they are feeding into downstream circuits.

Experimental Procedures

Fly stocks

UAS-Ptx, UAS-$G_{\alpha o}$ and UAS-$G_{\alpha o}^{GTP}$ were obtained from A. Tomlinson (Katanaev et al., 2005). UAS-Gαi was obtained from J. Knoblich (Schaefer et al., 2001). *Fru*^{Flp} was created by Ebru Demir and Jai Yi Yu by homologous recombination (Yu, 2009). Other fly stocks used were *Peb*-Gal4 (Sweeney et al., 2007), *PPK*-Gal4 (Grueber et al., 2003), *Fru*-Gal4 (Stockinger et al., 2005), UAS-hist-RFP (Emery et al., 2005) and UAS-FRT-stop-FRT-mCD8-GFP (Yu, 2009).

Behavioral assays

Quantitative assays for detailed phenotypic characterization were performed as described (Yapici et al., 2008).

Immunohistochemistry and tracing of *ppk+ fru+* neurons

Staining of the CNS and reproductive tract were performed using rabbit anti-GFP (Torrey Pines Biolabs, 1:6000), mouse anti-GFP (Promega, 1:1000), mAb nc82 (DSHB, 1:20, (Wagh et al., 2006)) and/or rhodamine-phalloidin or Alexa647- phalloidin (both Molecular Probes/Invitrogen, 1:100).

To trace the axons of the neurons labeled in *Peb-Gal4; UAS-FRT-stop-FRT-mCD8-GFP; Fru-Flp* animals the reproductive tract and ventral nerve cord of individual animals were dissected and stained. Tissues from different animals were kept separated to allow correlating projections with the neurons labeled in the individual animal. This precaution was necessary since the removal of the stop-cassette by Fru-Flp occurs in a stochastic manner.

All confocal stacks were acquired on a Leica TCS SP5. 3D projections of the stacks were performed using ImageJ (NIH).

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Discussion

To select behavioral actions that are relevant in a given context animals need to integrate both external sensory information as well as information about the internal and physiological state. How the nervous system integrates this information and forms a behavioral decision is not well understood. To address this question it is imperative to identify the neuronal circuit elements that convey external and internal sensory information as well as integration centers that process this information to form the behavioral decision.

We used the mating induced behavioral switch in *Drosophila melanogaster* as a paradigm to study decision making. Virgin females will readily accept courting males for copulation (Manning, 1967). After mating however when presented with the same sensory cues from a courting male those females will now reject the suitor's advances (Manning, 1967). This illustrates a strong influence of the internal physiological state of the female on the mating decision (Dickson, 2008).

While most external sensory neurons and their projections in *Drosophila* are fairly well characterized much less is known about neurons sensing internal physiological states. Here we characterized a set of internal sensory neurons that sense the female mating state. *Drosophila* females behave very differently before and after mating. This behavioral switch is induced by a small peptide, the sex-peptide (SP), which is synthesized in the male accessory gland and transferred to the female during mating (Chen et al., 1988; Pilpel et al., 2008). SP is detected in the female by a G-protein-coupled-receptor, the sex-peptide-receptor (SPR) (Yapici et al., 2008) which is required in the female nervous system to induce the sex-peptide mediated behavioral switch (Yapici et al., 2008). Here we could show that expression of SPR is both required and sufficient in a small set of internal sensory neurons on the female uterus with regard to its role in orchestrating the female behavioral change upon mating. These internal sensory neurons are characterized by the expression of both *fruitless*-Gal4 (*fru+*), a marker for neurons encoding sex-specific behaviors (Kvitsiani and Dickson, 2006; Manoli et al., 2005; Stockinger et al., 2005), as well as *pickpocket*-

Gal4 (*ppk*+) a marker for proprioceptive neurons (Adams et al., 1998). The neurons heavily innervate the uterus and send axonal projections to the ventral nerve cord (Hasemeyer, 2010: Chapter 2; Hasemeyer et al., 2009; Yang et al., 2009). Their dendritic arbors are similar in morphology to mechanosensory neurons innervating the bursa that control the post-mating switch in *P. rapae* (Sugawara, 1979). This, together with their expression of *pickpocket*-Gal4 suggests that the neurons have a mechanosensory role in addition to the detection of sex-peptide. They may therefore have a role in coordinating oviposition with ovulation and fertilization. In this context it is also interesting to note that not all *pickpocket*-Gal4 expressing neurons on the uterus are *fru+* (Hasemeyer, 2010: Chapter 2). It therefore might be that the *fruitless* negative neurons have a more exclusive mechanosensory role whereas the *fruitless* positive ones also acquired the ability to sense sex-peptide.

We could show that activation of $G_{\alpha o}$ signaling in *ppk*+ neurons mimics SP induced activation of SPR whereas inhibition of $G_{\alpha o}$ signaling abrogates post-mating responses (Hasemeyer, 2010: Chapter 2). This suggests that SPR signals in the *ppk+ fru+* internal sensory neurons via $G_{\alpha o}$ to induce the post-mating behavioral switch. Olfactory sensory neurons in *Drosophila* get inhibited presynaptically by GABA-ergic interneurons (Olsen and Wilson, 2008). The GABA-B receptor mediated part of this inhibition can be blocked by expression of pertussis-toxin in olfactory sensory neurons (Olsen and Wilson, 2008), suggesting that the inhibition is mediated by $G_{\alpha o}$ signaling. Together with the fact that conditional silencing of *ppk*-Gal4 expressing neurons can induce the post-mating response (Hasemeyer et al., 2009; Yang et al., 2009) this leads to the attractive hypothesis that SPR acts in the presynaptic compartment of the *ppk+ fru+* internal sensory neurons to reduce synaptic release of the neurons in mated females. This mode of action would require sex-peptide to leave the reproductive tract to act on the termini of the *ppk+ fru+* neurons in the ventral nerve cord, a route which is entirely plausible since SP can be detected in the haemolymph of mated females (Pilpel et al., 2008).

SPR expression is widespread in the nervous system (Yapici et al., 2008). Binding studies with labeled sex-peptide had also suggested a wide range of neuronal targets (Ding et al., 2003) which led to the hypothesis that SP globally acts on neuronal

circuits controlling mating behaviors (Kubli, 2003). The results presented here argue that the mating state is rather sensed by a group of internal sensory neurons and subsequently relayed to relevant circuits in the central nervous system. Since disparate behaviors such as receptivity, egg-laying, sleep and feeding undergo an SP dependent mating-induced switch (Carvalho et al., 2006; Chapman et al., 2003; Chen et al., 1988; Isaac et al., 2010; Liu and Kubli, 2003) this raises the question where the information relayed by these neurons diverges in the nervous system. While the SP signal has to diverge to modulate different neuronal circuits controlling various behaviors, it also has to converge and be integrated with external sensory information. Solving where and how this divergence and convergence of sensory information occurs in the nervous system promises to yield great insight into fundamental principles of neuronal circuit function.

To address how the information about the mating state is integrated in the nervous system it will be necessary to elucidate in which way SP modulates the physiology of the *ppk+ fru+* neurons. The fact that conditional as well as constitutive silencing of the neurons mimics SPR activation (Hasemeyer et al., 2009; Yang et al., 2009), putting virgins into a pseudo-mated state, suggests that the role of SPR might be to inhibit the internal sensory neurons, either by reducing their activity or by directly inhibiting synaptic release. There is indirect evidence suggesting that SP might activate SPR in the presynaptic compartment to inhibit synaptic release rather than modulating activity of the neurons themselves. *Ppk*-Gal4 expressing neurons are thought to be mechanosensory (Adams et al., 1998; Grueber et al., 2003) rather than chemosensory. Also here we have shown that SPR signals via $G_{\alpha o}$ which supports a presynaptic mode of action based on the analogy of GABA action via metabotropic receptors. It will however be important in the future to address this question directly using functional imaging or electrophysiology.

Sex-peptide has so far been shown to modulate different behaviors in females, namely receptivity, egg-laying, sleep and feeding (Carvalho et al., 2006; Chapman et al., 2003; Chen et al., 1988; Isaac et al., 2010; Liu and Kubli, 2003). For receptivity and egg-laying it is clear that the SP effect is mediated by the *ppk+ fru+* internal sensory neurons (Hasemeyer et al., 2009; Yang et al., 2009) which are likely to serve

as the sex-peptide sensor for feeding and sleep as well. This makes the neurons an interesting entry point for neuronal circuits controlling diverse behaviors. The identification of downstream neurons receiving input from the SP sensory neurons is therefore an important and exciting avenue of future research. Since SP controls a diverse set of behaviors that all depend on various sensory information it is likely that the information about the mating state is relayed to higher order centers in the fly's brain. We could show that the projections of the *ppk+ fru+* neurons on the uterus terminate in the abdominal ganglion of the ventral nerve cord. This raises two possibilities. The SP sensing neurons might connect to a dedicated set of projection neurons that relay the information about the mating state to neuronal circuits controlling the various behaviors or they might connect to different neurons that are already part of the circuits controlling egg-laying, receptivity, sleep and feeding. The former possibility is probably more plausible since it requires less signaling redundancy.

Different approaches can be used to identify putative second-order projection neurons relaying the information about the mating state. The *ppk+ fru+* positive neurons are likely to be excitatory (M.H., unpublished) which leads to the prediction that silencing downstream projection neurons would induce post-mating responses much like silencing of the SP sensory neurons does (Hasemeyer et al., 2009; Yang et al., 2009). A behavioral neuronal silencing screen assaying for the induction of post-mating responses can therefore lead to the identification of second-order projection neurons downstream of the *ppk+ fru+* internal sensory neurons. Silencing the second-order neurons can also address the question whether they are already specialized for a certain behavior or if they rather relay the information about the mating state to all relevant behavioral circuits. A complementary approach would be to anatomically identify potential downstream neurons based on the overlap of pre- and post-synaptic projections. This approach has the advantage that it is not biased towards a specific behavior assayed in a screen. It should therefore be especially useful for the identification of higher order neurons that receive input from second-order projection neurons since higher order centers are likely to be more specialized for a certain behavior. After anatomical identification and physiological verification

of connectivity the effect of perturbing the function of those higher order neurons could then be tested in a variety of behavioral paradigms.

A likely candidate set for second- as well as higher order neurons is the *fruitless* circuit. *Fru*-expressing neurons have been shown to be relevant both for male (Manoli et al., 2005; Stockinger et al., 2005) and female courtship behavior (Kvitsiani and Dickson, 2006). In the olfactory system both the neurons that detect the pheromone cVA, which is relevant for both male and female courtship, express *fruitless* (Kurtovic et al., 2007). These neurons connect to second order projection neurons that express *fruitless* as well and relay the sensory information to higher order centers such as the lateral horn and the mushroom body (Datta et al., 2008). Different classes of *fruitless*-expressing higher order neurons have been implicated in male courtship as well (Kimura et al., 2008; Kimura et al., 2005; Manoli and Baker, 2004) as have neurons forming the central pattern generator involved in generating male courtship song (Clyne and Miesenbock, 2008). This suggests that, at least for male behavior, neurons at all levels of information processing relevant to courtship express *fruitless*. It is therefore intriguing to speculate that a similar logic applies in females and that neurons downstream of the *ppk+ fru+* internal sensory neurons are *fru+* as well. A behavioral or anatomical screen of *fruitless* expressing neuronal subsets might therefore lead to the identification not only of second-order neurons relaying the mating signal but also of higher order neurons integrating external with internal sensory information.

In the context of receptivity it is likely that information about the male courtship song and the pheromone cVA, both of which stimulate females to mate (Kurtovic et al., 2007; Schilcher, 1976), converge and get integrated in some area of the brain to form the decision about whether to accept the male for copulation or not. At this point potential integration neurons are not known and, except for the olfactory pathway (Jefferis et al., 2007), even second order neurons are not well established (Figure 1).

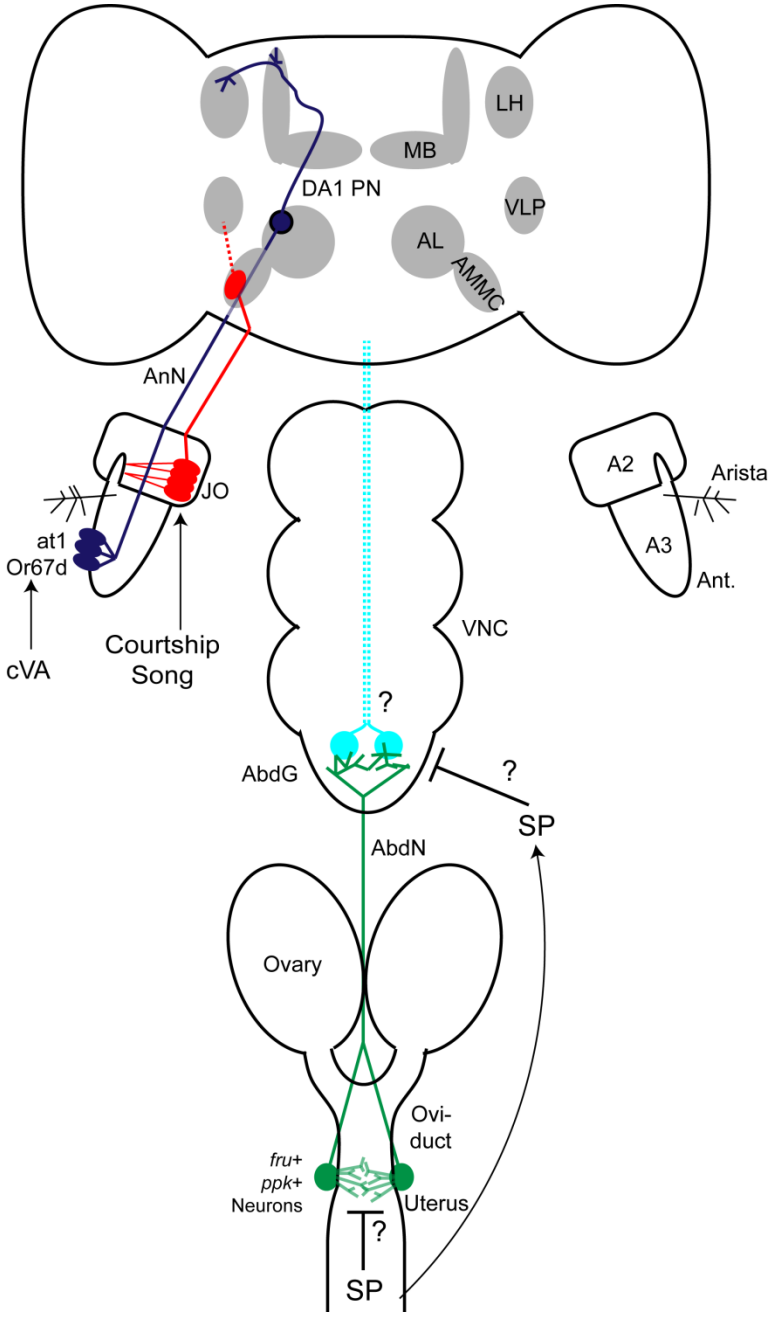

Figure 1: Neurons relaying information relevant to female receptivity
Schematic (not to scale) indicating stimuli known to be relevant for female receptivity behavior such as the SP-derived mating signal (SP), the pheromone cis-vaccenyl-acetate (cVA) and the male courtship song. Green indicates the SP-sensing *ppk+ fru+* internal sensory neurons for which the action of SP is unkown; blue the cVA-sensing Or67d neurons housed in at1 trichoid sensilla and their cognate projection neurons; red indicates Johnston's organs (JO) neurons sensitive to courtship song. The dashed red line indicates suggested second-order neurons for courtship song. The neurons depicted in cyan indicate hypothetical second order neurons relaying the SP response.
AbdN: Abdominal nerve; AbdG: Abdominal ganglion; VNC: Ventral nerve cord; Ant: Antenna; A2: Second antennal segment; A3: Third antennal segment; AnN: Antennal nerve; DA1 PN: Projection neurons relaying the cVA response from the DA1 glomerulus in the antennal lobe (AL); AMMC: Antennal mechanosensory and motor center; VLP: Ventro-lateral protocerebrum; MB: Mushroom body; LH: Lateral horn.

The identification of the SP-sensing neurons is only a first step in addressing how internal sensory information is processed in the nervous system since the neuronal integration centers are likely to be at least 2-3 synapses downstream of the *ppk+ fru+* neurons on the uterus. They nonetheless provide an important and valuable entry point into studying circuits controlling receptivity, egg-laying and potentially sleep and feeding. Especially the fact that they seem to orchestrate an all-or-none behavioral switch should make the identification of downstream circuits more straight-forward than for neurons relaying graded responses from external sensory systems. The identification of downstream circuits integrating external sensory information with information about the internal state of the animal will give insight into how nervous systems form behavioral decisions. The underlying principles of these processes are likely to be conserved even in nervous systems of higher complexity such as the mammalian brain. The *Drosophila* post-mating switch can therefore serve as a paradigm for how the internal state of an animal can modulate the value of external sensory information and therefore select behavioral actions that are appropriate in a given context.

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Poster: “Characterizing a potential role of toll receptors in olfactory targeting in *Drosophila melanogaster*”
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