DISSERTATION

Molecular and genetic analysis of
Drosophila melanogaster female reproductive behaviours

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To my family and friends for their faith in science and me.

Preface: Genes, neurons and behaviour

Animals are born with defined sets of innate behaviours. For example, almost all living species of ants form social colonies where individual members have roles like catching and transporting the pray, looking after the eggs and larval ants in the nest and defending the colony from external predators. Or consider a salmon species (Atlantic salmon *Salmo salar*) that is typically born in a river tributary. After spending couple of months in the river they are born the young salmons migrate to the sea for a year or two till they come back to breed in the same river they are born. One other example is the common garden spider *Araneus diadematu,* building orb webs with defined structures in less than half an hour (Ridley 1995). Why do ants have different roles in the colony? What makes the salmon return home? How does the garden spider know the rules of building the orb web? Such examples can be increased and combined in one fascinating question: How and why do animals behave? Studies done in the last century have increased our knowledge about animal behaviour. Now we know from Charles Darwin about the evolution of species through a sexual selection of traits that fits to their environment best, from Carl Wernicke and Paul Broca, that a functional nervous system is necessary for certain behaviours, from Camillo Golgi and S. Ramon Cajal, about neurons, the building blocks of the nervous system, and from Thomas Hunt Morgan, about how behavioural traits are transmitted from one generation to the other via genes. These scientists together with many others contributed from different directions to answer the fundamental questions of neuroscience, starting from the function of single neurons to how to build functional neural circuits that combine information from internal and external environment of an animal to produce certain types of behaviours. In the last century, by the help of model organisms and the emerging molecular and genetic tools, many studies are done to reveal the general principles of animal behaviour. *Drosophila melanogaster*, because of its simple nervous system, its variety of innate behaviours and the availability of genetic and molecular tools has become an important player in this era of seeking the secrets about genes, neurons and behaviour.

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SUMMARY

Innate behaviours are essential for many aspects of animal lifespan; therefore they are robust and regulated at multiple levels. These behaviours are hard wired in the nervous system and are regulated by internal and external factors. Moreover, analogous behaviours exist in many species from higher organisms to genetically tractable animal models. Thus, innate behaviours are good systems to study how genes regulate neurons to produce different behavioural responses.

In many species, mating is an essential innate behaviour that is necessary for the survival and continuity of the species. It requires multiple levels of control on animal's behaviour. For example in many species, mating induces a dramatic switch in female reproductive behaviour and physiology. In most insects, this switch is triggered by factors present in the male's seminal fluid. How these factors exert such profound effects in females is poorly understood. In order to understand the molecular mechanisms underlying this phenomenon in female *Drosophila melanogaster*, we established a high throughput egg laying assay which allows us to check the mating status of the females together with the switch from virgin to mated stage in the nervous system. Based on this assay we performed a genome wide neuronal screen using an inducible RNAi library. The screen uncovered genes involved in female receptivity, egg laying and the neuronal switch mediating the transition in female behaviour after mating. The first gene we characterized from this screen is the receptor for the sex peptide (SP), the primary trigger of the post-mating response in this species. The sex peptide receptor (SPR) is a G-protein coupled receptor that is specifically activated by low nanomolar concentrations of SP. It is expressed in the female's reproductive tract, and in the brain and ventral nerve cord of both sexes. Females that lack *SPR* function, either entirely or only in the nervous system, fail to respond to SP and continue to show virgin behaviours even after mating. We also identified *Tβh* and *VMAT*, genes regulating octopamine biosynthesis and transport respectively, with post-mating defects similar to SPR mutants. Therefore, we performed the initial behavioural analysis to check their possible relation with SPR signalling. We found, even though Tβh mutant females failed to show post mating responses, they responded to high amounts of injected SP.

ZUSAMMENFASSUNG

Angeborene Verhalten sind essentiell für viele Aspekte im Leben eines Tieres, aus diesem Grund sind sie robust und werden auf vielen Ebenen durch interne und externe Faktoren reguliert. Die Netzwerke, die diesen Verhalten unterliegen bestehen aus vorgeformten Verbindungen zwischen Neuronen im Nervensystem. Wir finden ähnliche Verhaltensweisen in verschiedenen Arten - von hoch entwickelten bis einfachen, jedoch genetisch zugänglichen, Organismen. Daher bieten angeborene Verhaltensweisen ein gutes Modell um zu untersuchen wie Gene Neuronaktivität regulieren und damit verschieden Verhaltensmuster erzeugen.

Bei vielen Arten ist das Paarungsverhalten ein sehr wichtiges angeborenes Verhalten, dass notwendig ist für das Überleben und den Erhalt der Art. Paarungsverhalten sind auf vielen Ebenen reguliert. So durchlaufen zum Beispiel Weibchen in vielen Arten nach der Paarung eine dramatische Veränderung hinsichtlich ihres reproduktiven Verhaltens und ihrer internen Physiologie. In vielen Insekten wird dieser Wandel durch Faktoren hervorgerufen, die in der männlichen Samenflüssigkeit vorkommen. Wie diese Faktoren derartige tiefgreifende Effekte hervorrufen ist erst wenig verstanden. Um die molekularen Mechanismen zu untersuchen, die diesem Phänomen in *Drosophila melanogaster* unterliegen, haben wir eine Assay etabliert, das das Eierlegverhalten adressiert. Mit Hilfe dieses Assays ist es möglich den Paarungsstatus zusammen mit der Verhaltensänderung -vom jungfräulichen zum gepaarten Zustand-in hohem Durchsatz zu analysieren. Basierend auf diesem Assay haben wir, unter Nutzung einer induzierbaren RNAi -Bibliothek, einen genomweiten neuronalen Screen durchgeführt. Dieser Screen enthüllte Gene, die involviert sind in die Rezeptivität, das Eierlegeverhalten sowie die Post-Paarungsverhaltensänderung von *Drosophila melanogaster* Weibchen. Das erste Gen, das wir untersucht haben ist der Rezeptor für das sogenannte Sex Peptid (SP), dem Hauptfaktor für die Post-Paarungsverhaltensänderung bei Weibchen dieser Art. Der Sex Peptid Rezeptor (SPR) ist ein G-Protein gekoppelter Rezeptor, der das Sex Peptid im nanomolar Bereich spezfisch bindet. Er wird im reproductiven Trakt in Weibchen und dem

Zentralnervensystem beider Geschlechter exprimiert. Weibchen, denen die Rezeptoraktivität entweder völlig oder nur im Zentralnervensystem fehlt können nicht auf die Präsents des Sex Peptides reagieren und behalten ihr jungfräuliches Verhalten selbst nach der Paarung bei. Wir haben außerdem die Gene *Tβh* und *VMAT*, die die Biosynthese und den Transport des Neurotranmitters Oktopamin regulieren, identifiziert. Da ihre Unterdrückung Post-Paarungsdefekte ähnlich denen der SPR Mutanten hervorruft, haben wir erste Verhaltensanalysen druchgeführt. Diese haben ergeben, dass obwohl Tβh mutante Weibchen keine Post-Paarungsverhaltensänderungen zeigen, sie dennoch auf hohe Dosen injezierten Sex Peptids reagieren.

INTRODUCTION

Mating behaviour and *Drosophila melanogaster*: a genetic model to study innate behaviours

Mating is one of the fundamental processes in animal behaviour that involves selection of the best partner for reproduction and survival of the species. Thus, mechanisms that control this highly important process are complex and tightly controlled by several internal and external factors. External factors mostly consist of different sensory stimuli for the recognition of an appropriate mate and vary from species to species. For example in frogs (Watson and Kelley 1992; Holmes, Chan et al. 2008), crickets (Libersat, Murray et al. 1994; Wagner and Reiser 2000) and song birds (Bentley, Wingfield et al. 2000; Nowicki and Searcy 2004), auditory information produced by a male specific song is vital for the acceptance of the female, while in rodents olfactory cues specifies the sex specific responses (Johnston and Rasmussen 1984; O'Connell and Meredith 1984; White, Fischer et al. 1984). Moreover internal factors are also critical for the regulation of mating behaviours. In many species, copulation with a male induces changes in female behaviour that are controlled by either factors from the male seminal fluid (Swanson 2003; Wigby and Chapman 2005) and/or the changes of female hormones due to the presence of a fertilized egg (Fuyama 1995; Fuyama and Ueyama 1997) or an embryo in the uterus (Groothuis, Dassen et al. 2007; Khan, Bellefontaine et al. 2008). Because mating is a robust behaviour with tightly regulated sequential events, it represents a good model to study how genes and neurons regulate innate behaviours.

Drosophila melanogaster has a complex mating behaviour in which male and female flies have particular roles to achieve successful copulation (Hall 1994; O'Dell and Kaiser 1997) (figure1). The male fruit fly initiates the courtship ritual by tapping and following the female fly and singing a species specific song. In response, the mature virgin female fly slows down and allows the male to lick her genitalia with his proboscis. This is followed by male's first attempt to copulation by bending his abdomen. If the first attempt fails, the male fly continues courting till the female accepts him by opening up her vaginal plate for copulation (Hall 1994; Wasserman

2000). Copulation duration is species specific, in *Drosophila melanogaster* it is approximately 20 minutes (Hall 1994; O'Dell and Kaiser 1997). When females are immature (1-2 two days old) or mated, they reject the male by stereotypic behaviours such as decamping, kicking and flicking or extruding their ovipositor (Hall 1994). Mutations effecting different steps of male courtship behaviour have been identified through many genetic screens (Yamamoto and Nakano 1999). Most studied ones include putative zinc-finger transcription factors *fruitless* (*fru*) (Taylor, Villella et al. 1994; Ito, Fujitani et al. 1996; Ryner, Goodwin et al. 1996; Demir and Dickson 2005) and *doublesex* (*dsx*) (Baker and Wolfner 1988; Villella and Hall 1996), RNA splicing factor *transformer* (*tra*) (McRobert and Tompkins 1985; Taylor, Villella et al. 1994), and tailles-like nuclear receptor *dissatisfaction* (*dsf*) (Finley, Taylor et al. 1997). Recently *fru*-expressing neurons have been shown to be necessary for courtship behaviour in males, as well as the male isoform of *fru* to be sufficient to induce male courtship behaviour in females (Demir and Dickson 2005; Stockinger, Kvitsiani et al. 2005).

So far most of the studies done on *Drosophila melanogaster* mating focus on the components of male courtship behaviours and less attention has been paid to female reproductive behaviours. Female flies have a less active role during courtship then males, mostly eliciting the male sexual drive with multiple sensory cues (Marcillac and Ferveur 2004; Wedell 2005; Legendre, Miao et al. 2008). The major role of the female fly during courtship is the decision to accept or reject the male and is strictly regulated with several factors. After mating females undergo a series of physiological changes allowing fertilization and deposition of eggs as well as sperm storage (Fuyama and Ueyama 1997; Heifetz and Wolfner 2004; Peng, Zipperlen et al. 2005; Wigby and Chapman 2005; Ram and Wolfner 2007; Wolfner 2007). The dramatic switch that happens in female behaviour due to mating is an excellent example for how innate behaviours are regulated with multiple internal and external factors. Thus, it is an attractive system to identify the mechanisms that control such changes on the cellular and molecular levels.

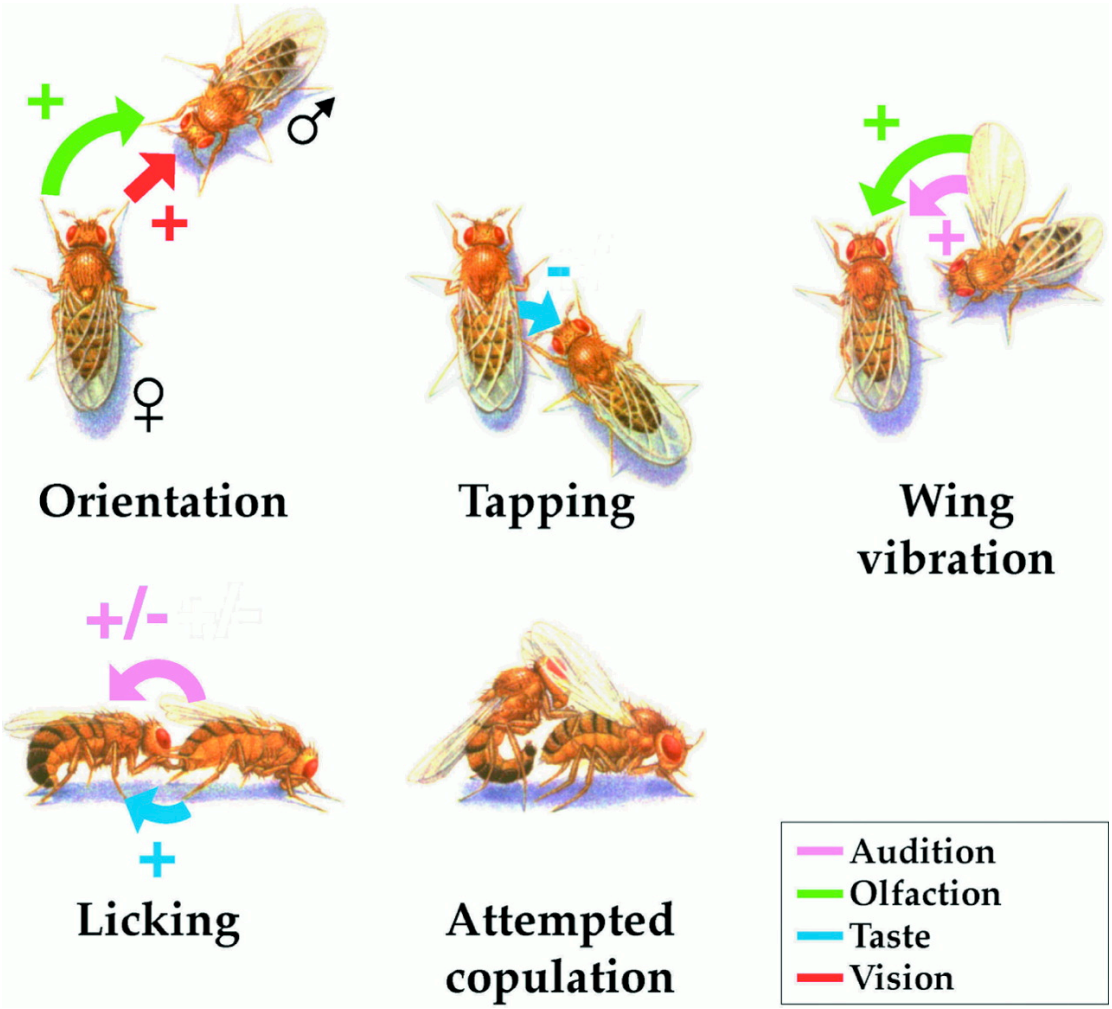

Figure 1 *Drosophila melanogaster* mating ritual

Mating starts, when male flies recognize and orient themselves towards the females by using visual and olfactory sensory cues. It continues with tapping, where the male senses the gustatory cues on the female fly and starts singing a species specific song. The courtship song is recognized by the female, leading to a slowing down response in locomotion. In the following steps of courtship, the male licks the female genital and attempts copulation by bending his abdomen. These sequential events continue until female accepts copulation or rejects the male. The image is adapted from (Greenspan and Ferveur 2000)

Drosophila female reproductive system

To understand the regulation of female mating behaviour, it is important to know the basic anatomy of the system. The *Drosophila melanogaster* reproductive system consists of two ovaries, sperm storage organs, uterus and vulva (figure2). Each ovary is composed of 10-20 ovarioles that are held together with a peritoneal sheath of muscle fibres (Soller, Bownes et al. 1999). The proximal ends of each ovariole form a pedicel that is interconnected to the lateral oviduct through the calyx. The common oviduct forms from multiple lateral oviducts and enlarges at the posterior side to form the uterus. The uterus is composed of multiple layers of muscle tissue that is heavily innervated by the neurons coming from the abdominal ganglia. These neurons regulate the uteral muscle contractions which allows the egg movement inside the oviduct and the sperm movement from the uterus to both of the sperm storage organs (Bloch Qazi, Heifetz et al. 2003).

Drosophila melanogaster have two distinct types of sperm storage organs that are located at the anterior end of the uterus (figure2). The seminal receptacle is the primary sperm storage organ. It is a thin, blind-ended tubule from which sperm is initially released for the fertilization of an egg in the uterus (Adams and Wolfner 2007). When the sperm is depleted from the seminal receptacle, the spermathecal stores start releasing the sperm (Lefevre and Jonsson 1962). A pair of spermathecea is located dorsally to the seminal receptacle and is composed of a capsule surrounded with epithelial tissue. Upon sperm entry to the uterus, these epithelial cells release large amounts of fluid to the spermathecal capsule, which is proposed to be important for the sperm maintenance (Heifetz and Wolfner 2004). The size of the sperm storage organs is directly related with the length of the sperm tail that is species specific. In extreme cases such as *D. bifurca,* it can reach to a size about 81mm, approximately 20 times longer than length of the female carrying them (Miller and Pitnick 2003). So far, it is not clear why flies need to have two sperm storage organs, but it might be explained by the distinct roles of spermethecea and seminal vesicle that has not been identified yet.

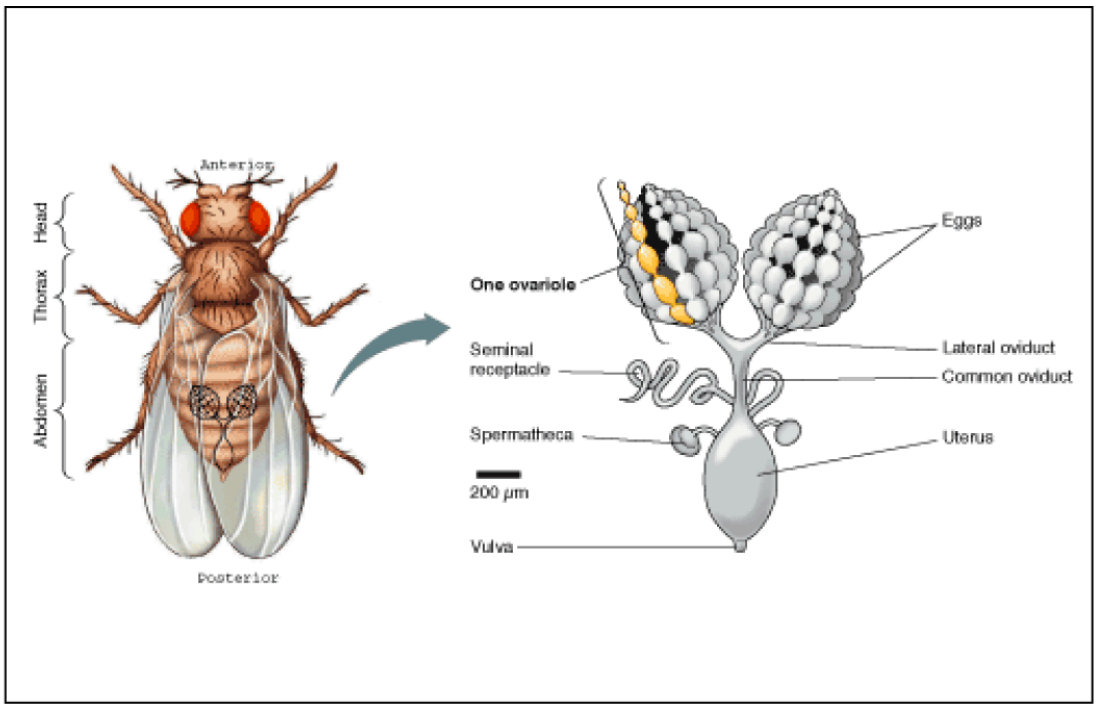

Figure 2

Drosophila melanogaster **female reproductive system**. The image is adapted from Principles of Developmental Biology Fred Wilt, University of California at Berkeley Sarah Hake, University of California at Berkeley ISBN 0-393-97430-8 (2003)

Drosophila female reproductive behaviours

Drosophila melanogaster female reproductive behaviours can be divided into two stages separated by mating. Before mating, female flies are receptive and retain their eggs but after mating they become refractory, increase their ovulation rate, initiate sperm storage and start laying eggs. These sequential events and factors regulating female behaviours before and after mating have been widely studied in insects and will be summarized here.

Pre-mating behaviours

Receptivity

Like in many insects, *Drosophila melanogaster* females are not active in the courtship ritual, but their decision to mate or reject the male is critical for the continuity of the species. In addition mating costs are different for males and females. Unlike males that mate multiple times and produce huge numbers of sperm, female flies mate few times and produce less number of protein rich eggs. Mating also decreases the life span of females. (Barnes, Wigby et al. 2008). Therefore, female receptivity is tightly regulated with internal and external factors.

Internal factors regulating female receptivity include the endogenous reproductive state and circadian rhythm of virgin females (Howlader and Sharma 2006; Krupp, Kent et al. 2008). Endogenous reproductive state is controlled by sexual maturity and seminal fluid components. Immature virgin females are not receptive to courting males and they reject them by stereotypic behaviours such as decamping, kicking and flicking. Until now, how sexual maturaty regulates female receptivity is poorly understood (Fuyama 1995; Soller, Bownes et al. 1999; Wasserman 2000; Wedell 2005). One candidate molecule is juvenile hormone (JH) that has been shown to regulate several processes during drosophila development and metamorphosis (Dubrovsky, Dubrovskaya et al. 2002; Gruntenko, Karpova et al. 2003; Raushenbakh, Adon'eva et al. 2004; Tu, Yin et al. 2005; Liu, Li et al. 2008). JH is produced in corpus allatum (CA) that is localized at the posterior region of the brain (Moshitzky,

Fleischmann et al. 1996). Its production is mainly regulated by insulin like peptides that are secreted from pars inter-cerabralis (Tu, Yin et al. 2005; Rauschenbakh, Karpova et al. 2007). Acp70a or sex peptide, one of the accessory gland molecules, (SP) also stimulates the production of JH (Fan, Rafaeli et al. 1999). In the reproductive system, JH controls oocyte maturation and vitolegenes and JH deficiency caused by mutations in the *apterous* gene reduces receptivity and causes sterility (Shtorch, Werczberger et al. 1995). Further, when immature virgins are implanted with CA taken from mature virgins, they become sexually active 24 hours earlier (Shtorch, Werczberger et al. 1995). However, the molecular mechanisms of JH action in controlling female behaviour are not yet identified.

The other factor controlling endogenous reproductive stage is mating and seminal fluid components from the male. These mechanisms will be explained in the later sections.

Second internal factor regulating the receptivity is circadian rhythms. Like most animals *Drosophila melanogaster*, have a daily rhythmic activity that is controlled by an endogenous clock (Howlader and Sharma 2006; Krupp, Kent et al. 2008). The female mating behaviour is under restricted control of this clock that is governed by the oscillations of circadian clock genes; *period* (per), timeless (*tim*) and disconnected *(disco)* (Fuyama 1995; Soller, Bownes et al. 1999; Wasserman 2000; Wedell 2005). How circadian rhythm regulates female receptivity is poorly understood but one suggested mechanism is by changing the female attractiveness. Because male flies mainly use olfactory cues to initiate courtship, the amount of female attractive pheromones is important for the male sexual drive. Thus, circadian regulation on pheromone production might affect the intensity of male courtship and indirectly change the female receptivity. Recently, supporting this hypothesis, the main pheromone production enzyme, desaturase1 (*desat1*), in females was shown to be transcriptionally regulated by clock genes. (Dubrovsky, Dubrovskaya et al. 2002; Gruntenko, Karpova et al. 2003; Raushenbakh, Adon'eva et al. 2004; Tu, Yin et al. 2005; Liu, Li et al. 2008).

The external factors controlling female mating are mainly related to the fitness of the courting male but also involve temperature and humidity (Gilbert and Richmond

1982; Ritchie, Halsey et al. 1999; Gruntenko, Karpova et al. 2003). Different chemical and acoustic signals allow females to estimate the male fitness and allow them to discriminate the species (Ejima and Griffith 2008). Chemical signals consist of male specific sex pheromones acting as aphrodisiacs to stimulate copulation in females. These pheromones are mainly long-chain hydrocarbon molecules and are produced in specific cells called oenocytes. So far, in *Drosophila melanogaster* two male specific pheromones, 7-tricosene (7-T) and cis-vaccenyl acetate (cVA) has been shown to decrease receptivity by reducing the female attractiveness (Grillet, Dartevelle et al. 2006; Ha and Smith 2006; Kurtovic, Widmer et al. 2007).

The acoustic signals mainly consist of the male courtship song. It is critical for females to choose the right mate (Ritchie, Halsey et al. 1999). In response to courtship song, mature females slow down allowing males to attempt copulation. The copulation latency of wingless males or aristaless (hearing segment of antenna in flies) females is elevated compared to wild type pairs. The characteristics of courtship song are determined by the inter-pulse intervals and vary between species. For example the mean interpulse interval in *D. melanogaster*, *D. simulans* and *D. marituana* is 30-35 milliseconds (msec), 50-55 msec and 35-50 msec, respectively (Hoikkala, Aspi et al. 1998; Ritchie, Halsey et al. 1999).

By combining the information from external and internal factors female flies accept or reject courting males. Current proposed models on mating decision of females suggests interconnected relations of these factors on the molecular and circuitry levels but little experimental evidence is present.

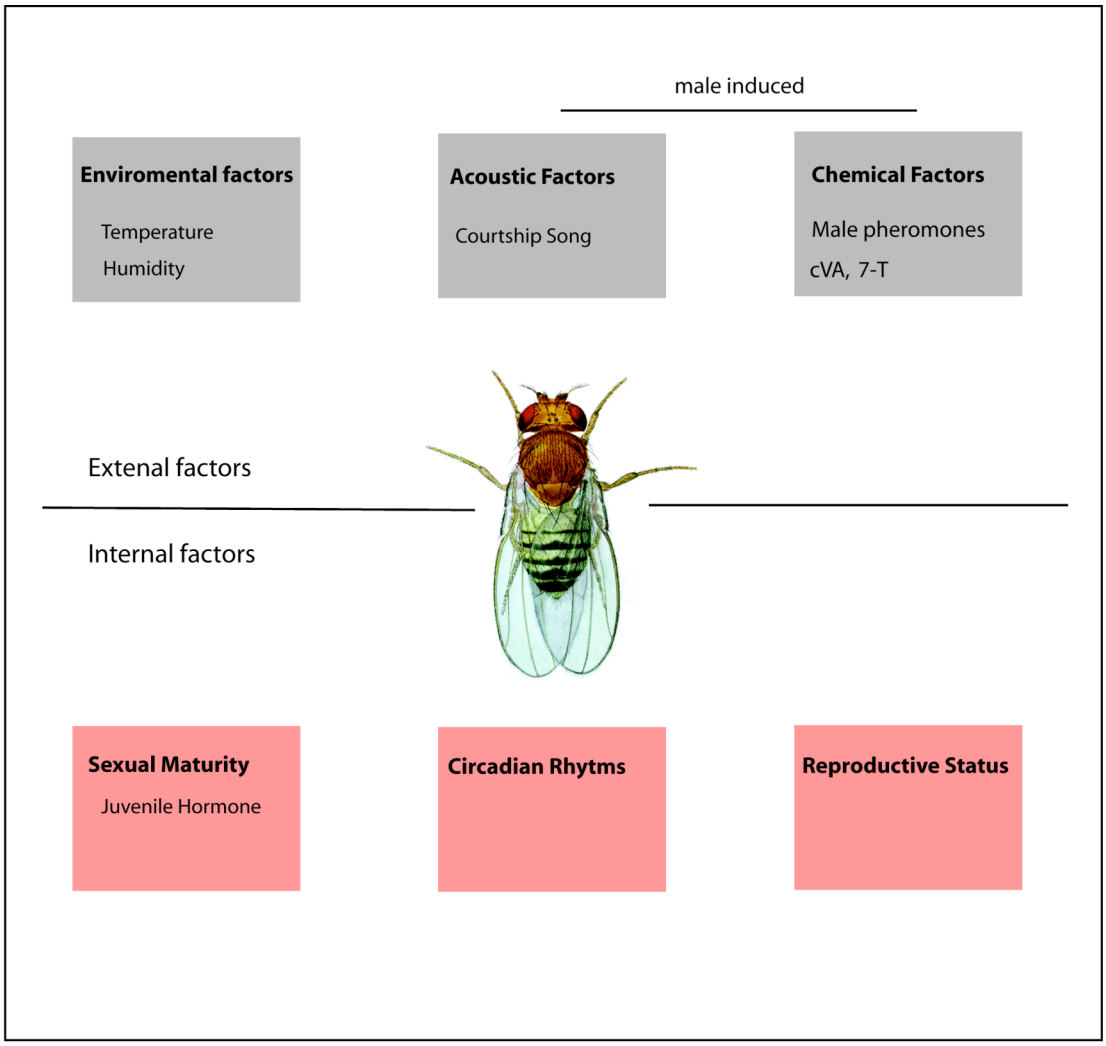

Figure 3 **Female Receptivity is regulated with multiple factors.**

These factors can be classified in to two groups: Internal and external. Internal factors include sexual maturity, circadian rhythm and reproductive status. External factors consist of environmental regulations and male induced sensory stimulations.

Post-mating behaviours

Ovulation and egg laying

Ovulation is an essential process for egg production (Heifetz, Yu et al. 2001). Many insect species ovulate only after mating but in *Drosophila melanogaster*, it happens also in adult virgin females at lower rates (Fuyama and Ueyama 1997). Mating increases the levels of ovulation within 1.5 hours in parallel with sperm storage (Fuyama and Ueyama 1997)). During ovulation, mature oocytes are transferred from the ovaries to the uterus through the lateral and common oviducts where they are fertilized (Bloch Qazi, Heifetz et al. 2003).

Seminal fluid components, accessory gland proteins and sperm increase the ovulation rate by possibly acting on multiple targets in the reproductive tract (Bloch Qazi, Heifetz et al. 2003; Heifetz and Wolfner 2004). Many of these target molecules and the neuro-modulators they regulate in females are unknown in *Drosophila melanogaster* but evidence from other insect species such as locust (*Locusta migratoria*), suggests the possible roles of octopamine, glutame, proctolin and SchistoFLRFamide (Lazarovici and Pener 1978; Newland and Yates 2008). Recent studies done on fly mutants lacking either of the two enzymes of the octopamine production cascade, tyrosine beta hydroxylase (*tbh*) and thyramine decarboxylase (*tdc*) or octapamine receptor subtype, octopamine in mushroom bodies (*oamb*) supports this hypothesis. Mutants of these genes show defects in egg laying and/or ovulation suggesting the role of octopamine in *Drosophila melanogaster* ovulation and egg deposition behaviours (Monastirioti, Linn et al. 1996; Lee, Seong et al. 2003; Monastirioti 2003).

Egg laying is a result of different physiological processes including oogenesis, ovulation and egg-fertilization. Mating elevates egg laying rate by inducing changes in the female reproductive tract and the nervous system through seminal fluid components that are transferred during copulation (Chapman, Herndon et al. 2001; Kubli 2003). Circadian rhythm is also proposed to contribute to egg laying regulation but there are some controversial results regarding its control by clock genes. The expression of the main circadian clock genes *tim* and *per* are constant in ovaries and

don't oscillate with dark and light cycles (Howlader, Paranjpe et al. 2006; Howlader and Sharma 2006). Nutritional state and temperature also affect egg laying rate (Lee, Simpson et al. 2008). Therefore, it is not clear if egg laying is purely regulated by the endogenous clock or it is a cyclical process where cycles depend on environmental factors.

Neural control on egg laying is also present. For example silencing of *fru* neurons (Kvitsiani and Dickson 2006) or ablation of mushroom bodies (MB) (Fleischmann, Cotton et al. 2001) increases egg laying rate in virgin females. In addition, insulin like peptide 7 (ilp-7) expressing neurons are recently reported to regulate the egg laying site selection in *Drosophila melanogaster* (Yang, Belawat et al. 2008). These findings suggest that egg laying is regulated on different levels through possibly different molecular mechanisms.

Sperm storage

Sperm storage is an important process for reproductive success. It prolongs the amount of time that sperm can be used, separates insemination and fertilization, increases sperm competition in cases of multiple mating and extends duration of egg laying and refractory period of females (Lefevre and Jonsson 1962). In some species, it also allows females to choose the best quality sperm from multiple-mating experiences. During copulation, *D. melanogaster* males transfer approximately 4000 sperm to females of which 1000 get stored in sperm storage organs, mostly in the seminal receptacle and partially in the spermathecea, for a period of two weeks (Lefevre and Jonsson 1962; Neubaum and Wolfner 1999; Bloch Qazi, Heifetz et al. 2003). Sperm accumulation starts just before the end of mating and reaches its peak approximately 1 hour after copulation (Bloch Qazi, Heifetz et al. 2003). Male and female flies play different roles in sperm storage: The female reproductive tract is responsible for the contractions for the movement of sperm and releases fluids for sperm absorption and protections (Heifetz and Wolfner 2004; Middleton, Nongthomba et al. 2006). Male-based mechanisms involve sperm motility and seminal proteins. Sperm storage regulates fertilization; the release of sperm from sperm storage organs is in parallel with ovulation rate to avoid egg and sperm waste and to decrease the rate of polyspermy (Kubli 2003; Swanson 2003; Chapman and

Davies 2004). It also affects receptivity; females that mate with spermless males show decreased post mating responses and remate frequently. This is also known as the sperm effect (Swanson 2003).

So far few genes have been identified effecting sperm storage in *D. melanogaster*. One example is a reactive oxygen species-producing enzyme, glucose dehydrogenase (*gld*) that is released from the spermathecae and vaginal plate (Schiff, Feng et al. 1992). *Gld* mutants store fewer sperm and stored sperm are distributed unevenly between two spermathecae (Iida and Cavener 2004). One other example is lozenge (*lz*) mutants that are defected in spermathecal development. *Lz* encodes for a putative transcription factor and several alleles have been reported to cause different spermathecal phenotypes (Green and Green 1956). *Lz* mutant females are also reluctant to mate due to increased rates of spontaneous ovulation (Fuyama 1995).

Sperm storage also allows sperm competition. Sperm competition is defined as the competition between the sperm from two or more males within the female reproductive tract. In *D. melanogaster* it occurs in multiple ways. Some studies also suggest that males produce short and unfertile sperm as a cheap filler to delay female remating. These short sperm are proposed to protect the long and fertile sperm from spermacite that females produce or from the sperm of other males (Holman, Freckleton et al. 2008). Another sperm competition mechanism is sperm displacement. Female remating causes the release of stored sperm due to the presence of new male ejaculate. In studies where GFP-labeled sperm is used to track the sperm's path, it was shown that sperm displacement occurs after second male transfers sperm to female and only from one of her sperm storage organs (Price, Dyer et al. 1999). These processes are good examples of male induced mechanisms to control female behaviour and to gain dominance on female's progeny.

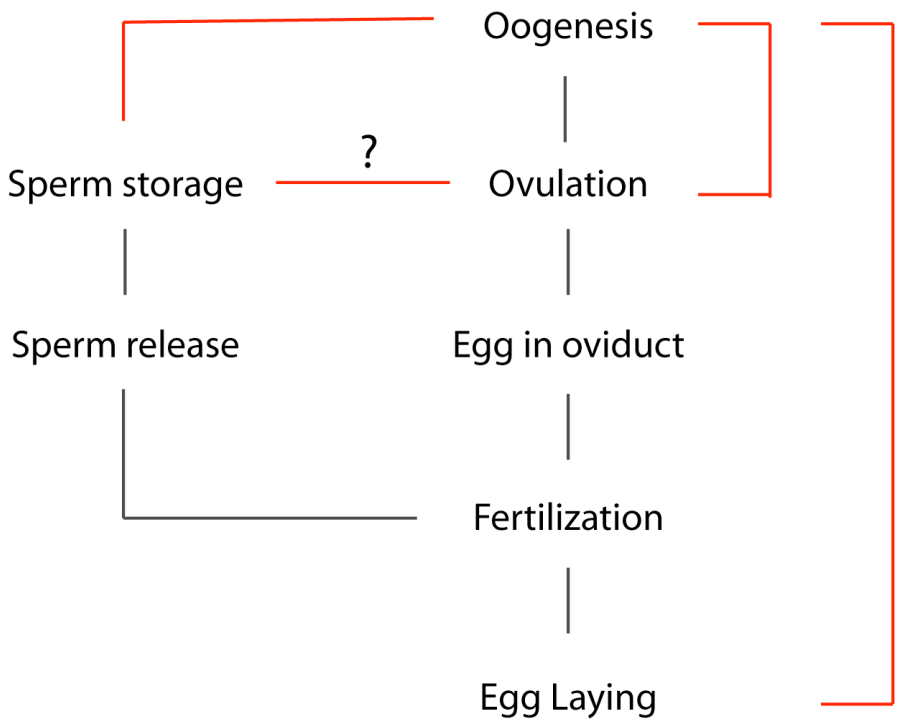

Figure 4 Sequence of events for female reproductive behaviours.
Female reproduction starts with the maturation of eggs in oogenesis. Mature oocytes are released from the ovaries to the oviducts where they are fertilized with sperm after mating. Mating facilitates ovulation and initiates egg laying and sperm storage. These events are tightly linked to each other and controlled by parallel mechanisms. The red lines represent possible regulatory connections between different reproductive behaviours. Black lines represent the sequence of events.

Remating

Remating is an evolutionary mechanism for females to increase the heterogeneity of the progeny and to protect themselves against male sub-fertility and sterility. Many insect, fish, reptile and mammal species reported to engage in remating at different frequencies (Owens 2002; Singh, Singh et al. 2002; Sprenger, Faber et al. 2008; Yamane, Kimura et al. 2008). It has been also widely studied among natural and laboratory strains of genus *Drosophila* (Singh, Singh et al. 2002). For example *D. pachea* females remate multiple types within one day, while in *D. subobscura* remating occurs rarely. In *Drosophila melanogaster,* females don't remate before 5 to 7 days if they are mated with a wild type male. The frequency of remating depends on several factors including the amount of sperm stored, seminal fluid components, quantity of eggs laid and levels of nutrition (Singh, Singh et al. 2002; Ram and Wolfner 2007). Some reports also suggest, remating is influenced by density of the population due to high incidence rates of courtship in crowded conditions (Crudgington, Beckerman et al. 2005). Genetic analyses done on strains artificially selected according to their remating speeds, show involvement of the second and X chromosomes but no particular gene have been identified regulating remating frequencies. One possible candidate is a cAMP-specific phosphodiesterase encoding gene *dunce (dnc)* that is located on the 3D4 of X chromosome. Mutations in *dnc* cause sexual hyperactivity in female flies. However several defects including associative and nonassociative learning phenotypes seen in *dnc* mutants argue against its specific role for female remating regulation (Bellen and Kiger 1987)

The female remating phenomenon has become an interesting topic for evolutionary biologist because of its association with sexual selection by means of regulating sperm usage patterns and sperm competition (Singh, Singh et al. 2002). Moreover, remating is also shown to be controlled by neural activity (Yamamoto and Nakano 1999; Fleischmann, Cotton et al. 2001), therefore it is also an attractive model for understanding the basis of neural modulation on behaviour.

Male seminal fluid molecules and sex peptides

The seminal fluid of *Drosophila melanogaster* consists of more than 80 proteins and peptides that are transferred to females together with sperm during copulation (Chapman and Davies 2004; Walker, Rylett et al. 2006). These peptides and proteins have action sites on the female reproductive tract and nervous system inducing specific responses such as increase in oogenesis and ovulation, decrease in receptivity, increase in feeding rate and stimulation of immune responses (Neubaum and Wolfner 1999; Tram and Wolfner 1999; Chapman, Herndon et al. 2001; Ravi Ram, Ji et al. 2005; Ram and Wolfner 2007). The variety of roles that seminal fluid molecules have, suggests their important functions in regulating reproductive behaviours of insects.

The main synthesis site of seminal fluid molecules is the secretory cells that are present in the paired accessory glands. The secondary sites are the ejaculatory ducts and the ejaculatory bulb (Chapman and Davies 2004). In situ hybridization experiments from accessory gland extracts together with the EST tag screens have so far identified 80-100 Acps in the *Drosophila melanogaster* genome, which are named according to their cytological locations (eg. Acp70a, Acp26Aa) (Swanson, Clark et al. 2001). Other seminal fluid molecules are synthesized in the secondary sites and include the anti-aphrodisiac pheromone cis-Vaccenyl acetate (cVA), the anti fungal peptide Drosomycin, the anti bacterial peptide Andropin and polymorphic carboxylesterase Esterase-6. Even though many seminal fluid molecules are identified, function of a few is known (Chapman and Davies 2004). Table 1 summarizes the synthesis sites of seminal fluid molecules with the nature of the substances.

Site of synthesis	Nature of secreted substances
Accessory gland main cells	An estimated 83 accessory gland proteins, many with unknown functions. Acps include peptides, prohormones, glycoproteins, enzymes (putative proteases, protease inhibitors, lipases) and antibacterial peptides
Accessory gland secondary cells	Filaments of unknown constituents
Ejaculatory duct	Dup 99B (peptide) Esterase-6 (enzyme) Glucose dehydrogenase (enzyme) Andropin (peptide) Drosomycin (peptide)
Ejaculatory bulb	PEB-me (protein) cis-Vaccenyl acetate (lipid) Esterase-6 (enzyme) Drosomycin (peptide)

Table 1 Summary of site and nature of seminal fluid molecules (Chapman and Davies 2004)

The best-characterized seminal fluid molecule is Acp70a or also known as 'the sex peptide '(SP). SP is responsible for the dramatic changes in female behaviour after mating such as decrease in receptivity (about 5-6 days) and increase in egg laying rate (Chapman, Bangham et al. 2003; Swanson 2003). Genetic and behavioural studies done through 1960s to 1980s first revealed the presence of such a substance in the seminal fluid. It is followed by the experiment in which HPLC separated fragments of accessory gland extracts were injected to virgin females. The fractions inducing the post mating responses were then analyzed by peptide sequencing, leading to the identification of a 36 amino acid peptide (Chen and Buhler 1970). Further analysis showed this peptide is encoded by the Acp70a gene (Chen and Buhler 1970; Chen, Stumm-Zollinger et al. 1988). Analysis on the Acp70a locus showed, SP is synthesized as a 55- amino acid precursor containing a 19 amino acid long signal peptide that is cleaved off during secretion from the accessory glands (Cirera and Aguade 1997). It then binds to the sperm tail and is transferred to the female flies during copulation. In the female reproductive tract SP has to be cleaved off from to sperm tail to reach its target molecules (Peng, Chen et al. 2005). The current model proposes SP to cross over vaginal wall to enter hemolymph where it is transferred to its targets (Chen, Stumm-Zollinger et al. 1988; Pilpel, Nezer et al. 2008).

So far, molecular and structural analysis identified many futures of SP and its roles on female behaviours. The C- terminus of SP is highly conserved, containing two cysteines that form a disulfide bridge and responsible for the decrease in receptivity and the increase in egg laying rate (Liu and Kubli 2003; Rexhepaj, Liu et al. 2003). The tryptophan-rich N –terminal binds to sperm and have been suggested to up-regulate juvenile hormone synthesis in corpus allatum leading to elevated vitellogenesis, subsequent oogenesis and oviposition of mated females (Moshitzky, Fleischmann et al. 1996; Peng, Chen et al. 2005). Recently, the region close to N-terminus of SP has also been shown to trigger immune response by elevating the anti-microbial peptide synthesis in the female reproductive tract (Peng, Zipperlen et al. 2005; Domanitskaya, Liu et al. 2007). Multiple roles of SP in regulating female post mating behaviours makes its target molecules appealing for further understanding of the female reproductive behaviours on the cellular and molecular levels.

Unfortunately, despite the effort spent to identify the SP targets in the last decade, no target molecule has been identified.

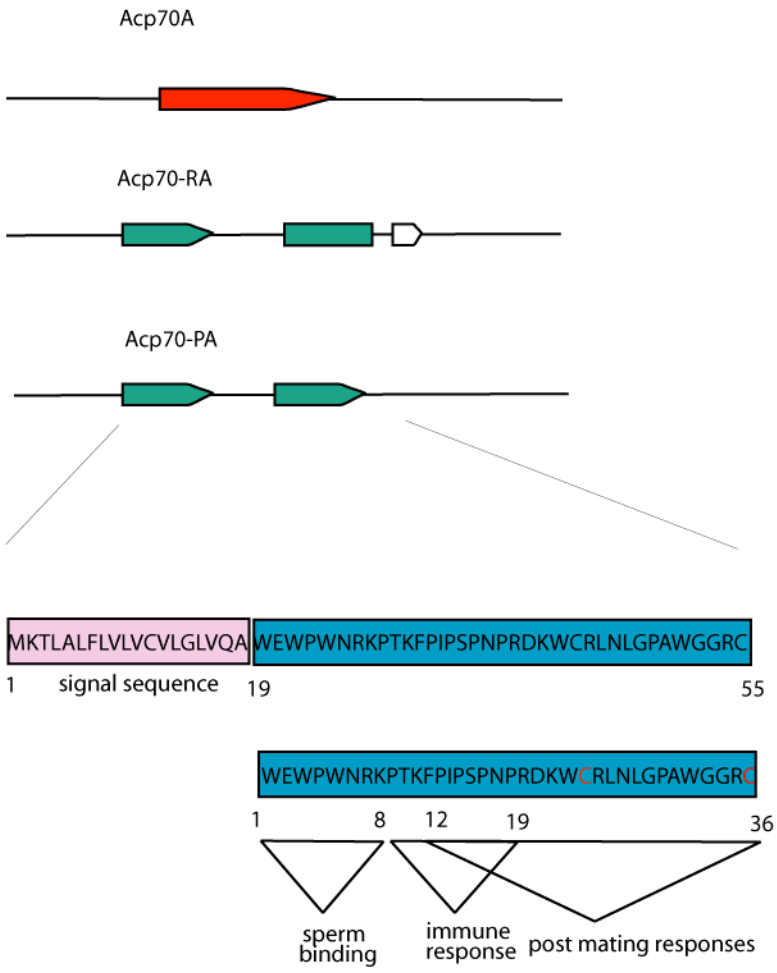

Figure 5 Acp70a locus and sex peptide.

Acp70a gene region is 266bp consisting two exons and a small intron. The primary transcript encodes for a 55-aminoacid precursor containing a 19-aminoacid long signal peptide. The signal sequence is cleaved off during secretion from the accessory glands. The mature peptide is a 36-aminoacid containing a disuphide bridge on the C terminus making this region cyclical. Different regions have been shown to regulate several responses in female behaviour.

Aim of the thesis

In this project we mainly focus on the link between genes and behaviour in *Drosophila melanogaster*, and tried to answer how female fruit flies regulate their behaviours before and after mating, which genes are involved in these process and what are the possible mechanisms that control acceptance vs. rejection of a courting male. The screen uncovered candidate genes regulating different steps of female behaviours. Further characterization of these genes might lead to better understanding of the female reproductive behaviours on the cellular and molecular levels. By studying female reproductive behaviours, our long-term goal was to understand the basis of an innate behaviour in a simple organism and to identify the general principles of how genes regulate neurons to produce behavioural responses.

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Chapter I:
Identification and characterization of the sex peptide receptor

The *Drosophila* sex peptide receptor mediates the post-mating switch in female reproductive behaviour

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Summary

Mating in many species induces a dramatic switch in female reproductive behaviour and physiology (Gillott 2003). In most insects, this switch is triggered by factors present in the male's seminal fluid. How these factors exert such profound effects in females is unknown. Here, we identify the receptor for the *Drosophila melanogaster* sex peptide (SP), the primary trigger of the post-mating response in this species (Chen, Stumm-Zollinger et al. 1988; Liu and Kubli 2003). The sex peptide receptor (SPR) is a G-protein coupled receptor that is specifically activated by low nanomolar concentrations of SP. It is expressed in the female's reproductive tract, and in the brain and ventral nerve cord of both sexes. Females that lack *SPR* function, either entirely or only in the nervous system, fail to respond to SP. Such females continue to show virgin behaviours even after mating. SPR is highly conserved structurally and functionally across the insect order, opening up the prospect of novel strategies to control the reproductive and host-seeking behaviours of important agricultural pests and human disease vectors.

Introduction

At various stages in their lifespan, animals can undergo dramatic switches in their (potential) patterns of innate behaviour. These behavioural switches provide an attractive model to explore the genetic and neural control of innate behaviours in general. Some of the most strikingly dimorphic behavioural patterns relate to mating and reproduction. For example, males and females of the same species typically perform distinct mating behaviours that are programmed genetically during development (Arthur, Jallon et al. 1998; Morris, Jordan et al. 2004), and in some species can also be switched in the adult in response to social cues (Munday, Buston et al. 2006). In *Drosophila melanogaster*, the behavioural switch that determines male or female mating behaviour is evidently set during development (Arthur, Jallon et al. 1998) by the sex-specific transcripts of the *fruitless* (*fru*) gene (Demir and Dickson 2005).

A second example of such a behavioural switch occurs in the adult females of many species as a result of mating. For example, in most insect species, virgin females are receptive to courting males and retain their eggs; whereas those that have mated are unreceptive and lay eggs. These changes in female behaviour and physiology are induced by factors produced in the male and transferred along with sperm during mating (Gillott 2003). In *Drosophila*, the primary trigger of this behavioural switch is the sex peptide (SP), a 36 amino acid peptide produced in the male accessory gland (Chen, Stumm-Zollinger et al. 1988; Liu and Kubli 2003). How SP exerts its effects on female behaviour and physiology is unknown, although it has been suggested the SP might act in part by modulating the activity of neurons that express *fru* (Dietzl, Chen et al. 2007). An essential first step in unravelling the effects of SP on female behaviour is to identify and localize the SP receptor(s) in the female. Here, we take this first step.

Results

CG16752 is required for post-mating responses induced by SP

We identified the gene *CG16752*, predicted to encode a G-protein coupled receptor (GPCR), in an ongoing genome-wide transgenic RNAi screen for genes required in the female nervous system for the post-mating switch in reproductive behaviour. Specifically, we found that expression of a *CG16752* RNAi transgene (Dietzl, Chen et al. 2007) (*UAS-CG16752-IR1*) with the pan-neuronal driver *elav-GAL4* led to a dramatic reduction in egg laying. To more carefully examine this egg laying phenotype, and to additionally assess mating receptivity of virgin and mated females, we used a protocol in which individual virgin females were first tested for receptivity to naïve males. Those females that mated were then allowed to lay eggs for 48 hours before being retested for receptivity to a second naïve male (Fig. 1a). In these assays, we used wild-type females as controls that do switch, as well as females carrying either *elav-GAL4* or *UAS-CG16752-IR1* alone. As controls that do not show post-mating behaviours, we used wild-type females mated to *SP* null mutant males (Liu and Kubli 2003), as well as virgin females. In the initial mating assays with virgin females, all genotypes were equally receptive (Fig. 1b), indicating that *CG16752* knock-down does not affect the mating receptivity of virgin females. In contrast, mated *CG16752* RNAi females laid dramatically fewer eggs than the negative controls (Fig. 1c), and unlike these controls, they remated at high frequency (Fig. 1d). In both aspects, mated *CG16752* RNAi females were indistinguishable both from wild-type virgins and from wild-type females previously mated to *SP* null males (Figs. 1c, d).

To control for potential off-targeting effects of the initial RNAi transgene, we generated a second independent line, *UAS-CG16752-IR2*, that targets a different region of the gene (Fig. 1e). In all three assays, this new RNAi line gave results indistinguishable from those obtained with the original line from the genome-wide library (Figs. 1b-d). We also identified a molecularly-defined deficiency (Parks, Cook et al. 2004), *Df(1)Exel6234*, that removes 88 kb from the chromosomal region 4F10-5A2, including *CG16752* and 4 other annotated genes (Fig. 1e). We verified the

molecular breakpoints of this deficiency, confirmed that it deletes the *CG16752* gene, and found that females homozygous for this deficiency were fully viable and had no obvious defects in the gross anatomy of their nervous system or reproductive organs. When tested in parallel in the same series of receptivity and egg laying assays, *Df(1)Exel6234* homozygous females showed post-mating defects indistinguishable from those obtained by RNAi knock-down of *CG16752* (Figs. 1b-d).

By mating *CG16752* RNAi or deficiency females to a *dj-GFP* to visualize sperm, we confirmed that sperm were transferred and stored normally in these animals. We thus postulated that the failure of these females to switch to post-mating behaviours could be due to a lack of sensitivity to SP. To test this directly, we injected SP into the haemolymph of *Df(1)Exel6234* homozygous virgins and wild-type controls, and then paired these virgins 5 hr later with naïve wild-type males. As expected, wild-type virgins injected with SP were unreceptive to these males, whereas those injected with buffer alone were as receptive as uninjected virgins (Fig. 1f). In contrast, *Df(1)Exel6234* virgins remained receptive even following injection with SP (Fig. 1f). Taken together, these genetic data demonstrate that the GPCR encoded by *CG16752* is required for the post-mating switch in female reproductive behaviour triggered by SP.

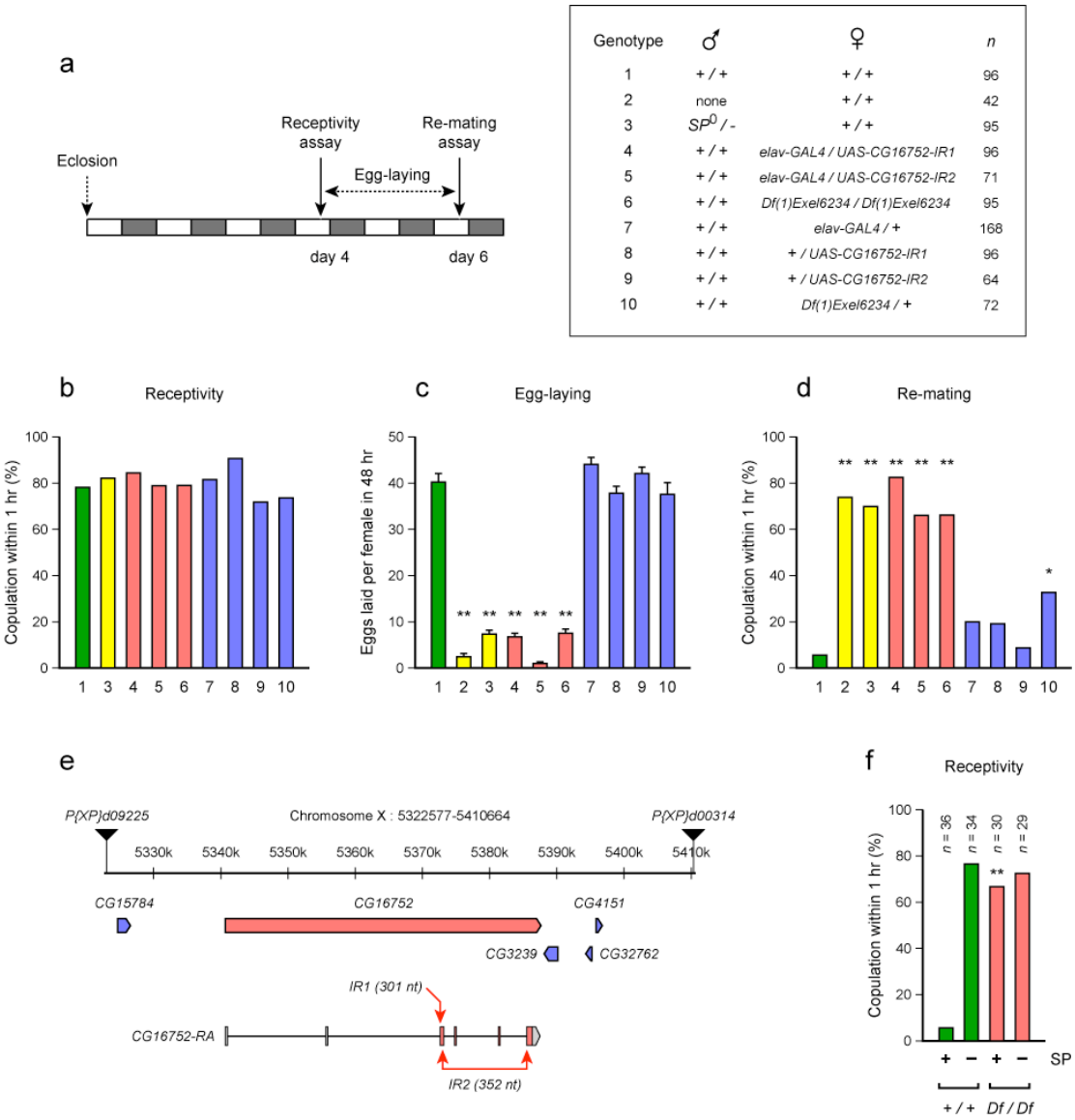
a
Eclosion
Receptivity assay
Re-mating assay
Egg-laying
day 4
day 6
Genotype ♂ ♀ n
1 + / + + / + 96
2 none + / + 42
3 SP0 / - + / + 95
4 + / + elav-GAL4 / UAS-CG16752-IR1 96
5 + / + elav-GAL4 / UAS-CG16752-IR2 71
6 + / + Df(1)Exel6234 / Df(1)Exel6234 95
7 + / + elav-GAL4 / + 168
8 + / + + / UAS-CG16752-IR1 96
9 + / + + / UAS-CG16752-IR2 64
10 + / + Df(1)Exel6234 / + 72
b
Receptivity
Copulation within 1 hr (%)
c
Egg-laying
Eggs laid per female in 48 hr
d
Re-mating
Copulation within 1 hr (%)
e
P{XP}d09225
Chromosome X : 5322577-5410664
P{XP}d00314
5330k 5340k 5350k 5360k 5370k 5380k 5390k 5400k 5410k
CG15784
CG16752
CG4151
CG3239
CG32762
IR1 (301 nt)
CG16752-RA
IR2 (352 nt)
f
Receptivity
Copulation within 1 hr (%)
n = 36
n = 34
n = 30
n = 29
SP
+ / +
Df / Df

Fig. 1 | *CG16752* is required for post-mating responses induced by SP

(**a**) Protocol for behavioural experiments. The *elav-GAL4* driver line additionally carried *UAS-Dcr-2* to enhance RNAi potency[9] (genotypes 4, 5 and 7).

(**b**) Receptivity of virgin females of the indicated genotypes, scored as the percentage of females that copulated within 1 hr. $P > 0.01$ for all comparisons against +/+ (genotype 1), χ^2-test with Bonferroni correction.

(**c**) Number of eggs laid per female during the 48 hr immediately after copulation. Data are mean ± s.e.m. ** $P < 0.001$, Tukey's multiple comparison test.

(**d**) Re-mating frequency for females tested 48 hr after the initial mating. * $P < 0.01$, ** $P < 0.001$ for all comparisons against +/+ (genotype 1), χ^2-test with Bonferroni correction.

(**e**) Organization of the *CG16752* genomic region. The region deleted in *Df(1)Exel6234* is shown. This deficiency derives from a precise deletion of interval between P-element insertions *P{XP}d09225* and *P{XP}d00314* (ref. 10), and includes the 4 annotated genes indicated. *UAS-CG16752-IR1* targets nucleotides 552-582 of the *CG16752-RA* transcript, and *UAS-CG16752-IR2* targets nucleotides 869-1220 (spanning 4 exons).

(**f**) Receptivity of wild-type or *Df(1)Exel6234* homozygous virgin females assayed 5 hr after injection with either 12pmol SP (+) or Ringer's solution alone (-).

***CG16752* encodes a specific sex peptide receptor**

To test whether *CG16752* might encode the SP receptor itself, we expressed a *CG16752* cDNA in mammalian CHO cells together with the Ca^{2+} reporter aequorin. In this assay, ligand-mediated GPCR activation triggers a luminescent flash via the $G\alpha_{q/11}$-dependent Ca^{2+} pathway (Le Poul, Hisada et al. 2002). We detected only a very weak response to SP in these cells, even at concentrations as high as 10μM (Fig. 2a). It has been suggested that SP responses might involve the cAMP rather than the Ca^{2+} pathway (Harshman, Loeb et al. 1999), and so we suspected that our initial failure to detect a strong SP response might be because CG16752 couples to G proteins other than $G\alpha_{q/11}$. Accordingly, we cotransfected these cells with constructs encoding one of three different chimeric G-proteins ($G\alpha_{qs}$, $G\alpha_{qi}$ or $G\alpha_{qo}$) designed to divert $G\alpha_s$-, $G\alpha_i$- or $G\alpha_o$-dependent signals, respectively, from the cAMP pathway into the Ca^{2+} pathway (Conklin, Farfel et al. 1993). Expression of $G\alpha_{qi}$ or $G\alpha_{qo}$, but not $G\alpha_{qs}$, resulted in robust Ca^{2+} responses to SP (Fig. 2a).

The response to SP is highly specific, as we did not detect comparable levels of activation to any of 8 other *Drosophila* peptides, even at 10μM (Fig. 2b; see Methods). Amongst the closest relatives of *CG16752* in *Drosophila* are *CG2114* and *CG8784*, which encode receptors for FMRFamides and hugin-γ, respectively (Meeusen, Mertens et al. 2002; Park, Filippov et al. 2002). Neither of these peptides activated CG16752, and conversely, expression of CG2114 or CG8784 in CHO cells conferred sensitivity to their respective ligands, but not to SP (Fig. 2b). In a dose-response assay, we determined that SP activates CG16752 with an EC_{50} of 1.3nM (Fig. 2c). The closely related peptide, DUP99B, which can induce the same post-mating responses as SP (Saudan, Hauck et al. 2002) activates CG16752 with an EC_{50} of 7.3nM. Thus, both SP and DUP99B specifically activate CG16752 at physiological concentrations, and in the low nanomolar range typical for such peptide-GPCR interactions (Saudan, Hauck et al. 2002). We thus conclude that *CG16752* encodes a functional receptor for SP that couples to $G\alpha_{qi}$ and/or $G\alpha_{qo}$ to regulate cAMP levels. We henceforth refer to this receptor as the sex peptide receptor, SPR.

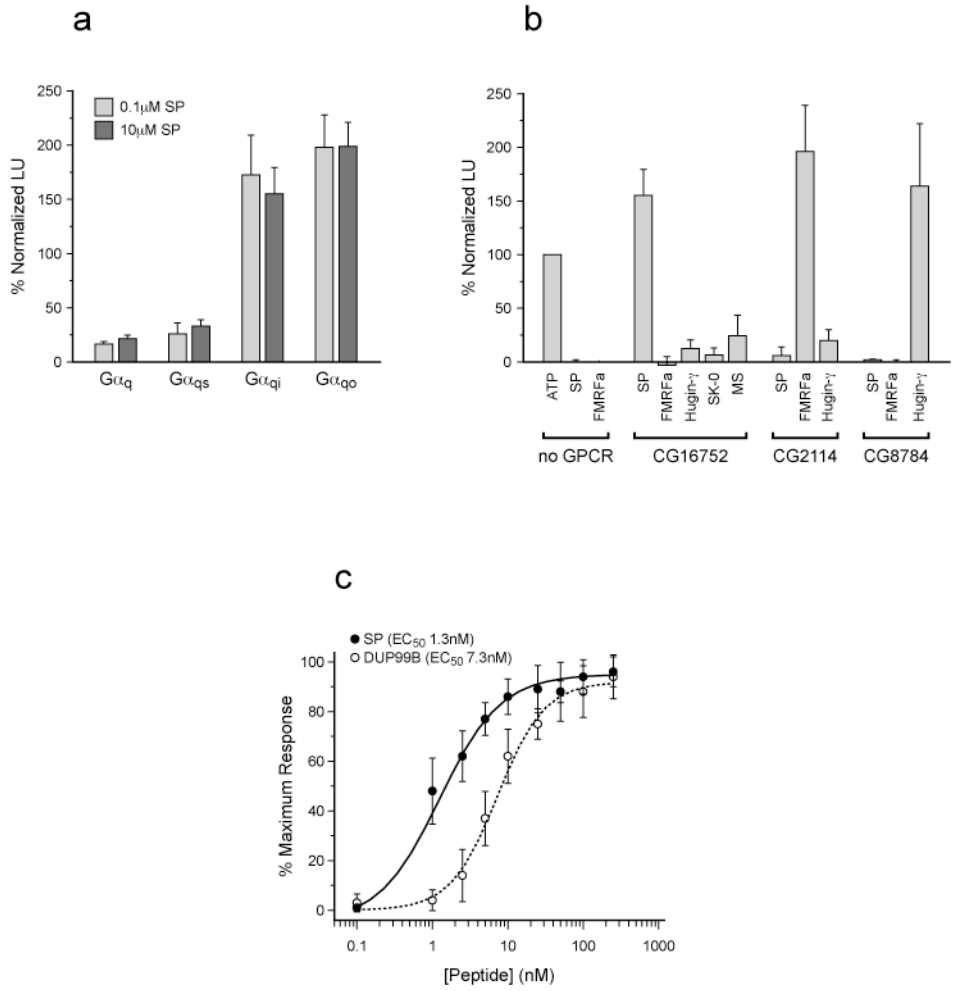

Fig. 2 | *CG16752* encodes a specific sex peptide receptor

(**a**) Luminescence responses of CHO cells expressing CG16752, aequorin and either one of the three chimeric G-proteins ($G\alpha_{qs}$, $G\alpha_{qi}$, or $G\alpha_{qo}$) or no additional G protein (endogenous $G\alpha_q$). Cells were treated with either 0.1μM or 10μM SP, and responses normalized against the response to 25μM ATP, which activates Ca^{2+} signalling via the endogenous P_2Y_2 receptor (100%). (**b**) Luminescene responses of CHO cells expressing the indicated GPCR and aequorin upon exposure to various peptide ligands (10μM), normalized against responses to 25μM ATP (100%). Cells expressing CG16752 or no additional GPCR were co-transfected with $G\alpha_{qi}$. Data are mean ± s.d. (n = 5–8). (**c**) Dose-response curves of CHO cells expressing CG16752, aequorin and $G\alpha_{qi}$ treated with SP or DUP99B. Each data point is mean ± s.d. (n = 8).

SPR is expressed in the nervous system and female reproductive tract

To define the primary cellular targets of SP, we generated antisera against an N-terminal region of SPR. These antisera revealed high levels of SPR expression in the female reproductive organs, in particular in the spermathecae, the primary sites for long-term sperm storage (Bloch Qazi, Heifetz et al. 2003) and the lower oviduct (Fig. 3a,c,d). Staining with the anti-SPR antisera was restricted to the cell membrane (Fig. 3d) and was absent in *Df(1)Exel6234* homozygous females (Fig. 3b), confirming the specificity of these antisera. SPR could not be detected in the male reproductive organs.

SP is also thought to pass into the haemolymph and ultimately act directly on targets in the central nervous system (CNS) (Ottiger, Soller et al. 2000). Indeed, staining the adult female CNS with anti-SPR revealed broad expression on the surface regions of both the brain (Figs 3e-g) and ventral nerve cord (VNC, Fig. 3h). Expression was most prominent in ventral regions of the suboesophageal ganglion (SOG), the cervical connective (cc), and many nerve roots in the brain and VNC. The restricted staining on the surface of the CNS was not an artefact due to poor antibody penetration, as we could reliably detect SPR in central brain regions upon ectopic expression of a *UAS-SPR* transgene in selected brain regions. CNS staining was completely absent in *SPR* null mutants, and greatly reduced in the *elav-GAL4 UAS-SPR-IR1* females (Fig. S1). In contrast to receptors for neuropeptides that are released within the CNS, the superficial localization of SPR is consistent with its role in detecting a ligand that circulates in the haemolymph and reaches central targets by crossing the blood-brain barrier. We observed a very similar CNS staining in males (Fig. S1), suggesting that SPR may have additional functions unrelated to its role in female reproductive behaviour. We have not been able to detect any abnormalities in the mating behaviour of *SPR* null males. SPR could not be detected in embryos or larvae, nor in any other adult tissues. Overall, the distribution of SPR concords remarkably well with the reported binding sites of radiolabelled SP applied to whole-female tissue sections *in vitro* (Ottiger, Soller et al. 2000).

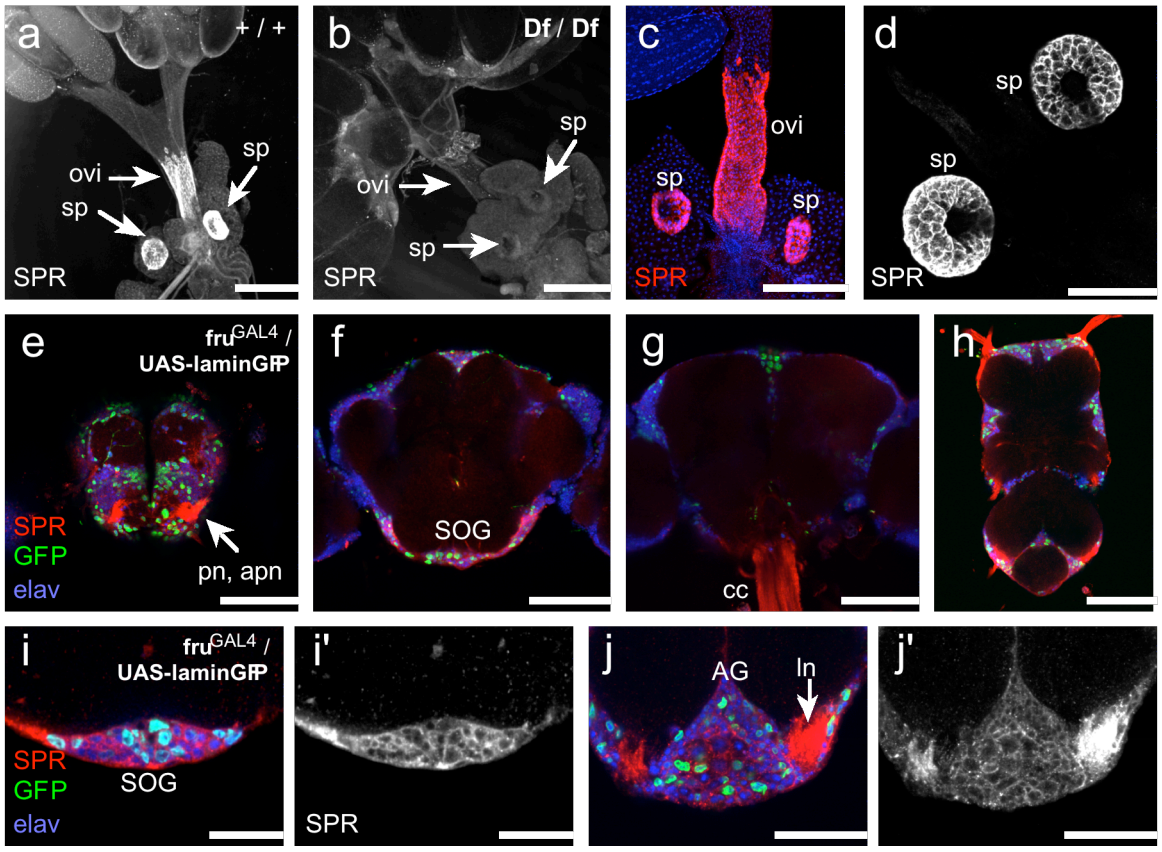

Fig. 3 | SPR is expressed in the nervous system and female reproductive tract

(**a, b**) Reproductive organs of wild-type (**a**) and *Df(1)Exel6234* homozygous (**b**) females stained with anti-SPR. ovi, oviduct; sp, spermathecae. Scale bar: 200μm. (**c, d**) Higher magnification views of wild-type oviduct and spermathecae stained with anti-SPR (red in **c**). The sample in (**c**) is counterstained with DAPI (blue). Scale bars: 200μm in **c**, 100μm in **d**. (**e-h**) Confocal sections of the brain (**e-g**) and ventral nerve cord (**h**) of *fru*^{GAL4} / *UAS-laminGFP* female stained with anti-SPR (red), anti-GFP (green) and anti-Elav (blue). (**e-g**) are sections from the anterior, middle, and posterior of the brain. pn, pharyngeal nerve, apn, accessory pharyngeal nerve; SOG, suboesophageal ganglion; cc, cervical connective. **e-g** are oriented with dorsal up; **h** with anterior up. Scale bars: 100μm. (**i, i'**) and (**j, j'**) Higher magnification views of the suboesophageal ganglion (SOG, **i, i'**) and abdominal ganglion (AG, **j**, **j'**), oriented as in **e-h**. ln, leg nerve. Scale bars: 25μm in **i, i'**, 50μm in **j, j'**.

SPR function is required in *fru* neurons

Post-mating responses can be induced in virgin females by blocking synaptic transmission of neurons that express the sex-specific P1 transcripts of the *fru* gene, leading to the speculation that SP might exert its effects in part by modulating the activity of these *fru* neurons (Dietzl, Chen et al. 2007). Consistent with this hypothesis, we found that some of the central neurons that express SPR are also positive for *fru*, as reported by the fru^{GAL4} driver (Fig. 3e-j). In particular, SPR appeared to be expressed in many fru^{GAL4}-positive neurons in the SOG and throughout the VNC. To test whether SPR function is required in *fru* neurons to trigger a post-mating response, we used the fru^{GAL4} driver and *UAS-SPR-IR1* to specifically knock-down SPR in these cells. These females showed normal receptivity as virgins, but after mating they laid only few eggs and re-mated at high frequency (Fig. 4a-c).

To test whether expression in *fru* neurons is also sufficient for the post-mating switch, we introduced fruGAL4 and UAS-SPR into SPR deficient females. In these females, SPR is only expressed in the *fru* neurons, yet we observed complete rescue of the re-mating phenotype (Fig. 4c) and partial but significant rescue of the egg laying phenotype (Fig. 4b). Together, these RNAi and rescue experiments strongly support the notion that SP triggers the post-mating behavioural switch primarily by modulating the activity of a subset of the fru neurons.

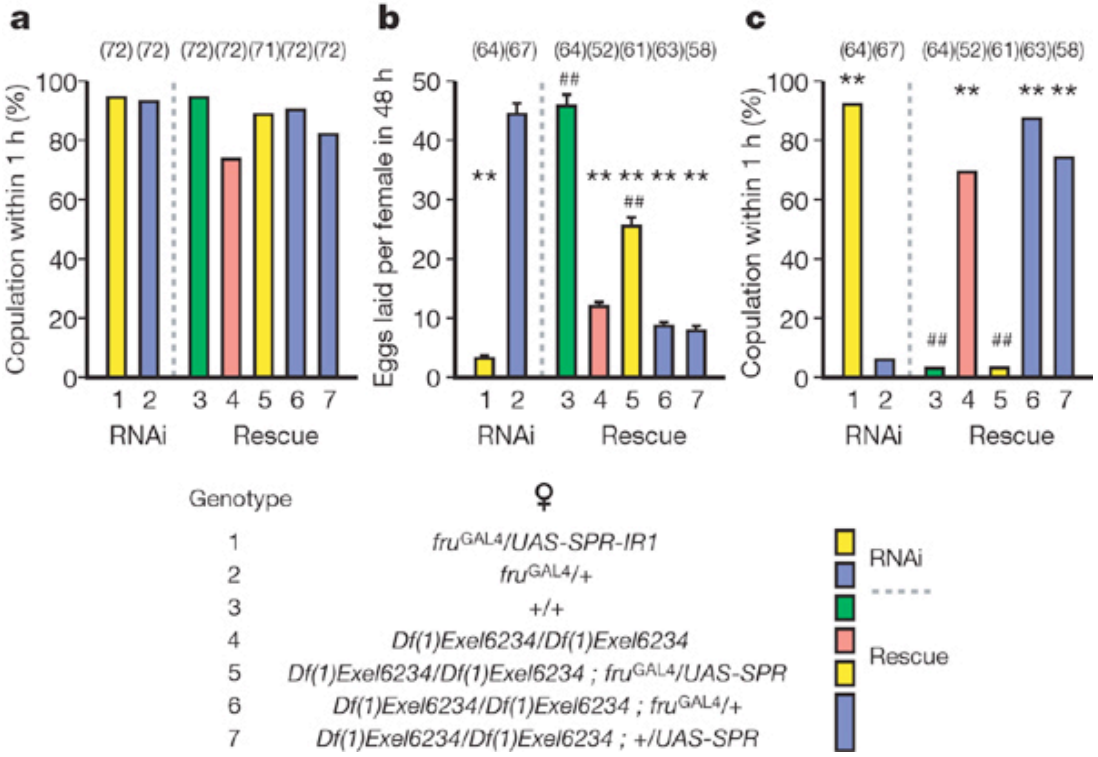

Fig. 4 | SPR function is required in *fru* neurons

Receptivity (a), egg laying (b) and re-mating (c) assays for females of the indicated genotype, mated with wild-type males and assayed according to the protocol of Fig. 1a. For the RNAi experiments, the fruGAL4 line additionally carried UAS-Dcr-2 (genotypes 1 and 2). The RNAi (genotypes 1 and 2) and rescue (genotypes 3–7) data are from distinct experimental cohorts. Data in b are shown as mean s.e.m. Double asterisk, $P < 0.001$ compared to wild-type females (genotypes 2 or 3); # #, $P < 0.001$ compared to deficiency females (genotype 4); Student's t-test (b) and 2 test (c).

Structural and functional conservation of insect SPRs

The *SPR* gene has been highly conserved during the course of insect evolution, as we can readily identify putative orthologues in most sequenced insect genomes, including *D. pseudoobscura*, the mosquitos *Aedes aegypti* and *Anopheles gambiae*, the moth *Bombyx mori*, and the beetle *Tribolium castaneum* (Figs. 5). Putative vertebrate orthologues are less apparent (Fig. 5a). To test for functional conservation of the insect SPR family, we isolated *SPR* cDNAs from each of these 5 insect species and tested them for responses to *D. melanogaster* SP in the CHO cell assay. SP was a potent activator of the *D. pseudoobscura*, *A. aegypti*, and *B. mori* receptors, with EC_{50}s of 4.3nM, 167nM and 63nM respectively (Figs. 6b-d). These receptors also responded to DUP99B with lower sensitivity (Figs. 6b-d), but not to any of the other 8 control peptides, including FMRFamides and hugin-γ. The receptors from *A. gambiae* and *T. castaneum* were not activated by either SP or DUP99B, even at 10μM (Fig. 6a). However, we do not have any other means to confirm that these receptors are functionally expressed in the CHO cells. Nonetheless, the functional conservation of *SPR* genes from *Drosophila*, *Aedes*, and *Bombyx* (Fig. 5b), together with the observation that *D. melanogaster* SP can induce post-mating responses in the moth *Helicoverpa armigera* (Fan, Rafaeli et al. 1999), strongly suggests that the family of receptors we have identified are likely to mediate post-mating changes in female reproductive behaviour and physiology across much of the insect order.

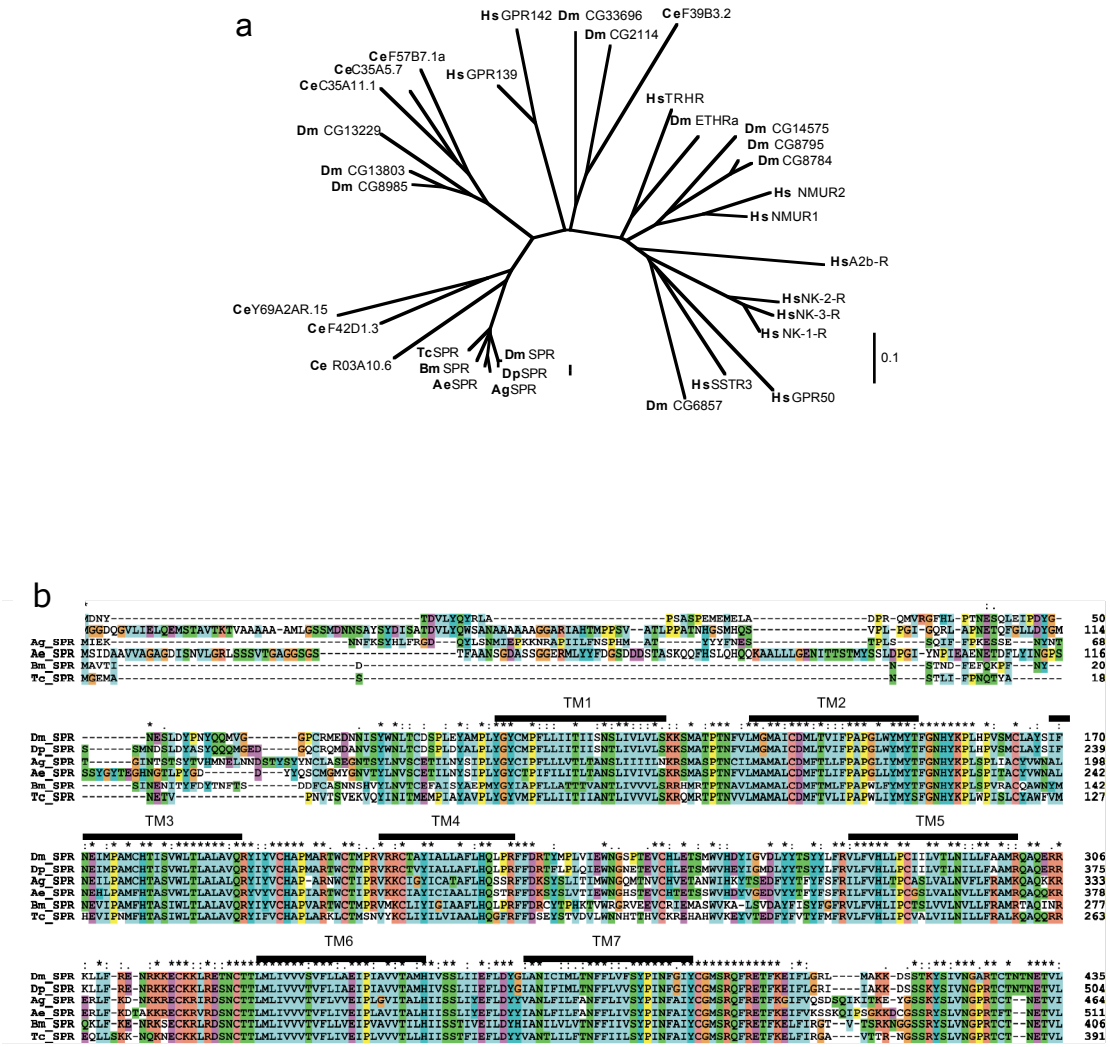

Fig. 5 | Structural and functional conservation of insect SPRs

(**a**) Phylogenetic tree of predicted insect SPRs and related *Drosophila*, *C. elegans* and human GPCRs. Scale bar: 0.1 amino acid replacements per site. (**b**) Multiple alignment of insect SPRs, prepared using Clustal X (Jeanmougin, Thompson et al. 1998).

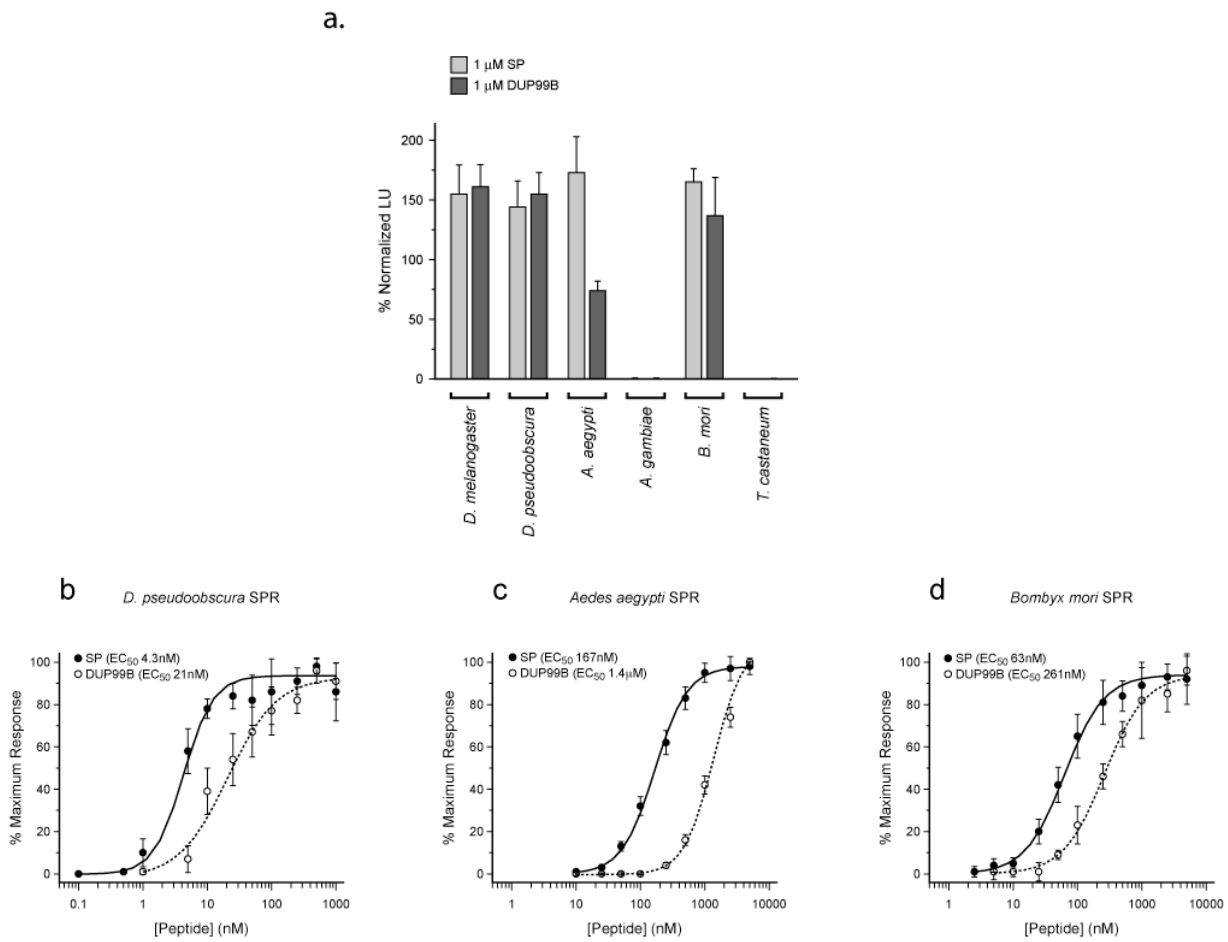

Fig. 6 | Structural and functional conservation of insect SPRs

(a) Luminescene responses of CHO cells expressing the SPR orthologue of the indicated species, together with aequorin and $G\alpha_{qi}$, and treated with *D. melanogaster* SP or DUP99B (1μM). Cells expressing either *A. gambiae* or *T. castaneum* SPR showed no responses at 10μM (not shown). Data are normalized against responses to 25μM ATP (100%). Data are mean ± s.d. (n = 6). (**b-d**) Dose-response curves of CHO cells expressing various insect SPRs, aequorin, and $G\alpha_{qi}$ treated with *D. melanogaster* SP or DUP99B. Each data point is mean ± s.d. (n = 6). (**e**) Luminescene responses of CHO cells expressing the SPR orthologue of the indicated species, together with aequorin and $G\alpha_{qi}$, and treated with *D. melanogaster* SP or DUP99B (1μM). Cells expressing either *A. gambiae* or *T. castaneum* SPR also showed no responses at 10μM (not shown). Data are normalized against responses to 25μM ATP (100%). Data are mean ± s.d. (n = 6).

Conclusion

In conclusion, the data presented here provide strong evidence that SPR is the receptor for SP, and that activation of SPR initiates the chain of events that ultimately lead to dramatic changes in female reproductive behaviour and physiology. Our identification of SPR now paves the way for defining these events at the molecular, cellular, and circuit levels. Furthermore, because SPR is so highly conserved across insect species, it can now provide the basis for cellular assays to identify SP-like activities in other species, and to develop novel approaches for controlling the reproductive and host-seeking behaviours of several important agricultural pests and human disease vectors.

Methods

Fly stocks. *UAS-SPR-IR1* (*UAS-CG16752-IR1*) was obtained from the genome-wide transgenic RNAi library (Dietzl, Chen et al. 2007) maintained at the Vienna *Drosophila* RNAi Center. *UAS-SPR-IR2* was generated by cloning a 352 bp PCR product from the RE15519 cDNA (Drosophila Genomics Resource Center) as an inverted repeat into a custom-designed *UAS* vector, and then inserting this transgene into a specific 2nd chromosome site (VIE-28b) using the ϕC31 system (Groth, Fish et al. 2004). *UAS-SPR* was generated by cloning the entire *SPR* coding region from RE15519 into a similar custom-designed *UAS* vector, followed by integration at a different site on the 2nd chromosome (VIE-72a). The *Df(1)Exel6234* stock (Parks, Cook et al. 2004) was obtained from the Bloomington *Drosophila* Stock Center and verified by a series of PCRs on genomic DNA extracted from homozygous and control flies. The original line was then recombined with $white^{+}$, and crossed for 3 generations into a Canton S background. Canton S was used as wild-type in all experiments. Other stocks used were *elav-GAL4* (Luo, Liao et al. 1994), fru^{GAL4} (Stockinger, Kvitsiani et al. 2005), SP^{0} / TM3, Sb (Liu and Kubli 2003), Δ^{130} / TM3, Sb (Liu and Kubli 2003), *UAS-laminGFP* (Aza-Blanc, Lin et al. 2000), and *dj-GFP* (Santel, Blumer et al. 1998). *SP* null males were SP^{0} / Δ^{130} (Liu and Kubli 2003). Both the *elav-GAL4* and fru^{GAL4} stocks additionally carried a *UAS-Dcr-2* insertion on the X chromosome (Dietzl, Chen et al. 2007).

Behavioural assays. All flies were raised on semidefined medium (Backhaus 1984) at 25°C in a 12 hr:12 hr dark:light cycle. Virgin males and females were collected at eclosion. Males were aged individually for 5 days; females were aged for 4 days in groups of 10–15. All assays were performed at circadian time 6:00–10:00, and on at least 3 independent occasions. For assays performed according to the protocol in Fig. 1a, single female and male virgins were paired in 10 mm diameter chambers and videotaped for 1 hr. The time to copulation was recorded for each female. Those females that copulated were then transferred to single food vials for 48 hr, and the number of eggs laid by each female was counted manually. Females were then re-tested for receptivity in the same manner in pairings with naïve Canton S males. The data set for the *elav-GAL4* / + controls is pooled data from two separate series of

experiments in which the *elav-GAL4* driver was crossed to each of the respective parental strains for the two *UAS-SPR-IR* transgenes. These two sets of *elav-GAL4* / + controls were not significant different in any of the assays. SP injections into the abdomen of virgin females were performed as described previously (Schmidt, Choffat et al. 1993). Following injection, females were transferred to individual food vials and tested after 5 h for receptivity with a naïve Canton S male.

CHO cell assays. CHO-K1 cells were transiently transfected essentially as described previously[25]. The relevant GPCRs were expressed from constructs prepared by cloning the entire open reading frame in a pcDNA3.1 (+) vector (Invitrogen). Expression constructs for CG2114[30], CG8784[15], the chimeric G proteins ($G\alpha_{qs}$/qs5-HA, $G\alpha_{qi}$/qi5-HA, and $G\alpha_{qo}$/qo5-HA)[13] and codon-optimized aequorin (hucytaeqpcDNA3)[31] have been described previously. Luminescent signals were measured with a Synergy2 photometer (BioTek). The *Drosophila* peptides used in this study are as follows ('a', amidated C termini; pQ, pyro-glutamic acid; P, hyroxyproline; **C**, cysteine residues linked by disulphide bridge): FMRFamide-2 (DPKQDFMRFa), FMRFamide-3 (TPAEDFMRFa), sulfakinin (SK)-0 (NQKTMSFa), SK-1 (FDDYGHMRFa), SK-2 (GGDDQFDDYGHMRFa), myosuppressin (MS; TDVDHVFLRFa), hugin-γ (pQLQSNGEPAYRVRTPRLa), pyrokinin (PK)-2 (SVPFKPRLa), synthetic sex peptide (SP; WEWPWNRKPTKFPIPSPNPRDKW**C**RLNLGPAWGGR**C**), and synthetic DUP99B (DUP99B; pQDRNDTEWIQSQKDREKW**C**RLNLGPYLGGR**C**). These peptides were synthesized using the Fmoc-strategy and solid-phase method on an ABI 433A Peptide Synthesizer and purified with HPLC. For SP and DUP99B, purified peptides were folded prior to a second HPLC purification by incubating them in 0.01 M ammonium bicarbonate (pH 8) containing 3% DMSO for 36 h.

Immunohistochemistry. A synthetic peptide corresponding to the predicted N-terminal 21 amino acids of the mature SPR (PTNESQLEIPDYGNESLDYPNC-OH) was conjugated to KLH and used to generate rabbit antisera (Gramsch Laboratories). SPR antisera were cleaned by incubating with equal volume of *Df(1)Exel6234* embryos overnight at 4 ^{o}C. Wandering 3rd instar larva and 8–10 d virgin females and males were dissected under PBS (pH7.4). Tissues were fixed for overnight at 4^{o}C in

4% paraformaldehyde in PBS (or in some cases at room temperature for 2 h). The tissues were incubated in primary antibody (1:500) for 48 hr at 4°C, and in secondary antibody for 24 hr at 4°C. Other antibodies used were: rat anti-elav (1:500; ref. 32), mouse anti-GFP (1:1000; Chemicon), Alexa 488-conjugated goat anti-rabbit, Alexa 568-conjugated goat anti-mouse and Alex 633-conjugated goat anti-rat (all 1:1000; Molecular Probes). Images were acquired with a Zeiss LSM 510/Axiovert 200M and processed in Adobe Photoshop.

Cloning of other insect *SPR* genes. *SPR* orthologues were identified by TBLASTN searches on the relevant genome assemblies, and gene structures predicted using Genscan (http://genes.mit.edu/GENSCAN.html). The complete ORF of each *SPR* orthologue was amplified by RT-PCR using the following primers: *D. psudoobscura*, forward 5'-atgggcggcgatcaaggggt, reverse 5'-ggcaccaacatcaccaatta; *A. aegypti* forward 5'-atgtcaattgatgctgcggta, reverse 5'- cgttggttctgtgtgacaaa; *A. gambiae* forward 5'-atgattgaaaaaaataatttcaag, 5'-cctgctatctaaccacagt; *B. mori* forward 5'-atggcggtcaccatagacaa, reverse 5'-ggcttaaagcacagtttcgt; *T. castaneum* forward 5'-atgggcgagatggcgtcgaac, reverse 5'-tcaacattgagtttgtcctaa. *D. pseudoobscura* was obtained from the Tucson *Drosophila* Stock Center (stock number, 14011-0121). Frozen stocks of *Aedes aeqypti* (MRA-735B) and *Anopheles gambiae* (MRA-132B) were obtained from the MR4 Resource Center (VA). *Tribolium castaneum* and *Bombyx mori* were gifts from Drs Gregor Bucher (Johann-Friedrich-Blumenbach-Institute, Germany) and Dušan Zitnan (Slovak Academy of Science, Slovakia), respectively. The predicted protein sequences were analyzed with TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) to confirm the presence of seven transmembrane domains. The nucleotide sequences and translations of SPR reported in this paper have been deposited in the GenBank database and have the following accession numbers: *D. pseudoobscura*, EU106873; *Aedes aegypti*, EU106874; *Anopheles gambiae*, EU106875; *Bombyx mori*, EU106876; and *Tribolium castaneum*, EU106877.

Phylogenetic analysis. Using the insect SPRs, we performed NCBI-BLASTP searches[33] against the NCBI non-redundant protein database and collected all *H.sapiens*, *Drosophila melanogaster* and *C.elegans* entries that were below a highly significant e-value of 1e-5. In an alternative approach, we built a profile hidden

Markov model (HMM) (Eddy 1998) out of the insect SPR conserved region and collected additional proteins with a significant e-value below 0.001. A 90 per cent redundant protein set (without recent duplications, sequencing errors and splice variants) was aligned using MUSCLE (Tomkiewicz, Muzeau et al. 2004) and graphically processed with Clustal X (Jeanmougin, Thompson et al. 1998). The phylogenetic tree was calculated with PHYLIP (Felsenstein 2005) using the Jones-Taylor-Thornton matrix as distance algorithm and the neighbour-joining method for tree calculation. The image was generated with the help of Phylodendron (© 1997 by D.G. Gilbert). Sequences and NCBI accession numbers: *Drosophila melanogaster*: CG13229 (gb|AAM28948.1|), CG13803 (gb|AAF47633.2|), CG8985 (gb|AAF47635.2|), CG2114 (tpg|DAA00378.1|), CG33696 (ref|NP_001027122.1|), ETHRa (gb|AAO20966.1|), CG8795 (ref|NP_731788.1|), CG8784 (ref|NP_731790.1|), CG14575 (ref|NP_996140.1|), CG6857 (ref|NP_523404.2|); *Caenorhabditis elegans*: R03A10.6 (emb|CAA93674.2|), Y69A2AR.15 (gb|AAK68559.2|), F42D1.3 (emb|CAB03091.2|), F57B7.1a (emb|CAA98492.1|), C35A5.7 (emb|CAA94909.2|), C35A11.1 (gb|AAB66039.3|), F39B3.2 (gb|AAB07577.2|); *Homo sapiens*: GPR142 (ref|NP_861455.1|), GPR139 (sp|Q6DWJ6|), TRHR (ref|NP_003292.1|), NMUR2 (ref|NP_064552.2|), NMUR1 (gb|AAH36543.1|), A2b_R (ref|NP_000667.1|), NK-1_R (gb|AAA59936.1|), NK-2_R (gb|AAB05897.1|), NK-3_R (gb|AAB21706.1|), GPR50_Hs (gb|AAI03697.1|), SSTR3_Hs (ref|NP_001042.1|).

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Author contributions

N.Y. and C.R. identified *D. melanogaster SPR* in the RNAi screen, N.Y. performed the initial molecular analysis and all behavioural assays, and Y-J.K. performed the cellular assays and immunohistochemistry and cloned *SPR* orthologues from other insects. B.J.D. supervised the project and wrote the manuscript together with N.Y. and Y-J.K.

Author information

The authors declare no competing interests. Correspondence and requests for materials should be addressed to B.J.D. (dickson@imp.ac.at).

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Chapter II:

Genetic programming of female mating behaviours in *Drosophila melanogaster* and octopaminergic regulation on female receptivity and remating

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Summary

In many species, innate behaviours are regulated by multiple external and internal signals. One classic example of such behaviours is female reproductive behaviours in *Drosophila melanogaster*. Upon mating, female fruit flies go through major behavioural changes. They reduce their receptivity to courting males and start to lay eggs. These behavioural switches are mainly induced by male seminal fluid components, which act on the female nervous system. Here we used this switch in female behaviour to identify genes regulating different steps of female mating. We carried out a genome-wide neuronal RNAi screen for reduced egg laying and identified genes with various mating phenotypes. Furthermore we classified the screen hits into three phenotypical classes and focused on the post-mating defective genes for further understanding of post-mating switch in females. We focused on two post-mating defective genes that are responsible for octopamine bio-synthesis and transport and postulated a novel role for octopamine in regulating female receptivity and post-mating switch.

Introduction

Animals are born with defined sets of innate behaviours. These behaviours are hardwired in the nervous system and not only essential for the fitness of the animals but necessary for the survival of the species (Manoli, Meissner et al. 2006; Menzel, Leboulle et al. 2006). The molecular mechanisms that regulate the wiring and function of the nervous system to produce these behaviours are subject to multiple divisions of neuroscience but they are still poorly understood on the molecular level. Innate behaviours are also excellent models to study the connection between genes and behaviour, due to following reasons. First, neural circuits that regulate genetically encoded behaviours are less complicated than circuits controlling higher cognitive functions (Zucker 1972; Carew and Kandel 1977; Zeigler 1989). Second, they exist in almost all biological organisms including genetically tractable animal models (Aston-Jones, Chen et al. 2001; Komiyama and Luo 2006). Finally, fundamental principles of these behaviours might be conserved among species. Thus, identification of molecules in genetically tractable systems might lead to understanding of analogous behaviours in other organisms.

Mating is an innate behaviour, which consists of multiple steps (White, Fischer et al. 1984; Hall 1994; Sprenger, Faber et al. 2008). Although those behavioural steps vary among species, the basic principles of mating behaviour are shared in many unrelated organisms. In most species, mating decisions are made by females (Ziegler, Kentenich et al. 2005; Moore 2007; Gow 2008). Females accept or reject courting males in response to the combination of internal and external sensory stimuli. Male courtship is also influenced by the sensory stimuli from females, such as female sex pheromones that initiate sexual arousal in males (Marcillac and Ferveur 2004). Thus, mating behaviours can be seen as an interconnected feedback loop, where two sexes exchange information through sensory cues and modulate their behavioural responses according to this information flow (Reid and Stamps 1997; White 2004; Phelps, Rand et al. 2006).

Drosophila melanogaster has a well-defined mating ritual in which male and female flies perform multiple behavioural steps in order to make a successful copulation (Hall 1994). Male flies execute courtship behaviour by producing multiple sensory stimuli such as courtship song, licking and tapping. Female flies respond to these sensory stimuli by either allowing males to copulate by decreasing their locomotion or rejecting males by extruding their ovipositor (Hall 1994). So far, male courtship behaviour has been widely studied to understand the basic principles of mating behaviours. However, female behaviours have received relatively little attention. Female mating behaviour is an excellent model to study the basis of innate behaviours and the molecular mechanisms that regulate them. Mating changes female behaviour mainly through male seminal fluid components that are transferred during copulation (Fuyama and Ueyama 1997; Gillott 2003; Liu and Kubli 2003; Chapman and Davies 2004). Analysis on female post-mating switch allows the identification of molecules inducing functional changes in the nervous system leading to different behavioural responses. One key molecule for female mating switch is an accessory gland molecule, Acp70a, also known as the sex peptide (SP). SP is the main regulator of two post-mating responses; decrease in receptivity and increase in egg laying rate (Chapman, Bangham et al. 2003; Liu and Kubli 2003). Recently SP receptor (SPR) has been identified by our group (Yapici, Kim et al. 2008). However how SPR regulates the behavioural switch in females is poorly understood.

Egg laying is regulated with multiple factors. Therefore, there might be several reasons for a decrease in egg laying activity. (Heifetz, Yu et al. 2001; Heifetz and Wolfner 2004; Horner, Czank et al. 2006). Thus, assays for egg laying allow identification of defects in multiple steps of female mating behaviours. In this study, we have performed a genome wide RNAi screen, using a semi quantitative egg laying assay and identified 28 genes controlling female mating behaviours on various behavioural steps. We focused on genes responsible for the post mating switch defects, which includes SPR and two additional genes implicated in octopaminergic signalling, for further understanding of post mating switch behaviour in females.

Results

A genome wide transgenic RNAi screen identifies genes with egg laying defects

Screen system

To identify genes regulating female reproductive behaviours, we have performed a transgenic RNAi screen by using a semi quantitative egg laying assay. Since we focused on the neuronal control of female behaviour, we targeted gene knock down specifically to the nervous system. This allowed us to exclude most of the phenotypes caused by genes required for the structural development of the female reproductive tract as well as genes controlling vital functions in different stages of development.

Neuronal RNAi was achieved using an *elav*-GAL4 driver (Luo, Liao et al. 1994) in combination with UAS-IRs obtained from the Vienna Drosophila RNAi Center (VDRC) (Dietzl, Chen et al. 2007). *Elav*-GAL4 is expressed in the entire nervous system, from early development throughout adult stages. This long-term expression allows accumulation of short hairpins in the neurons for efficient knock down (figure 1b-d). UAS-IR transgenes contain 300-400 base pair (bp) short gene fragments that are cloned as inverted repeats under an UAS promoter. The expression of UAS-IR transgenes by GAL4-UAS system produces small interfering RNAs that interact with mRNA transcripts, eventually leading to their degradation via RNAi pathway (figure1a) (Tabara, Grishok et al. 1998). Previous studies from several organisms including *C.elegans* show, that RNAi is less efficient in neurons than in other cell types (Kennedy, Wang et al. 2004). Therefore, we introduced a UAS-*dcr2* transgene to enhance the RNAi potency and to increase the efficiency of gene knock down (Dietzl, Chen et al. 2007).

To test egg laying, UAS-IR males were crossed to the driver line virgins. From this cross, 20-30 females were collected during first three days after eclosion (figure 2a). During these three days of period, females were kept together with their male siblings and allowed to mate with them. Egg laying assays were performed in the following three consecutive days and the number of eggs were scored semi quantitatively on a scale from 1 to 5 at the end of each day. According to our screening criteria, an

average 3-day score of 3 or above was considered as a putative positive (figure 2b). Putative positives were retested blindly in the same manner in order to decrease the false positive discovery rates.

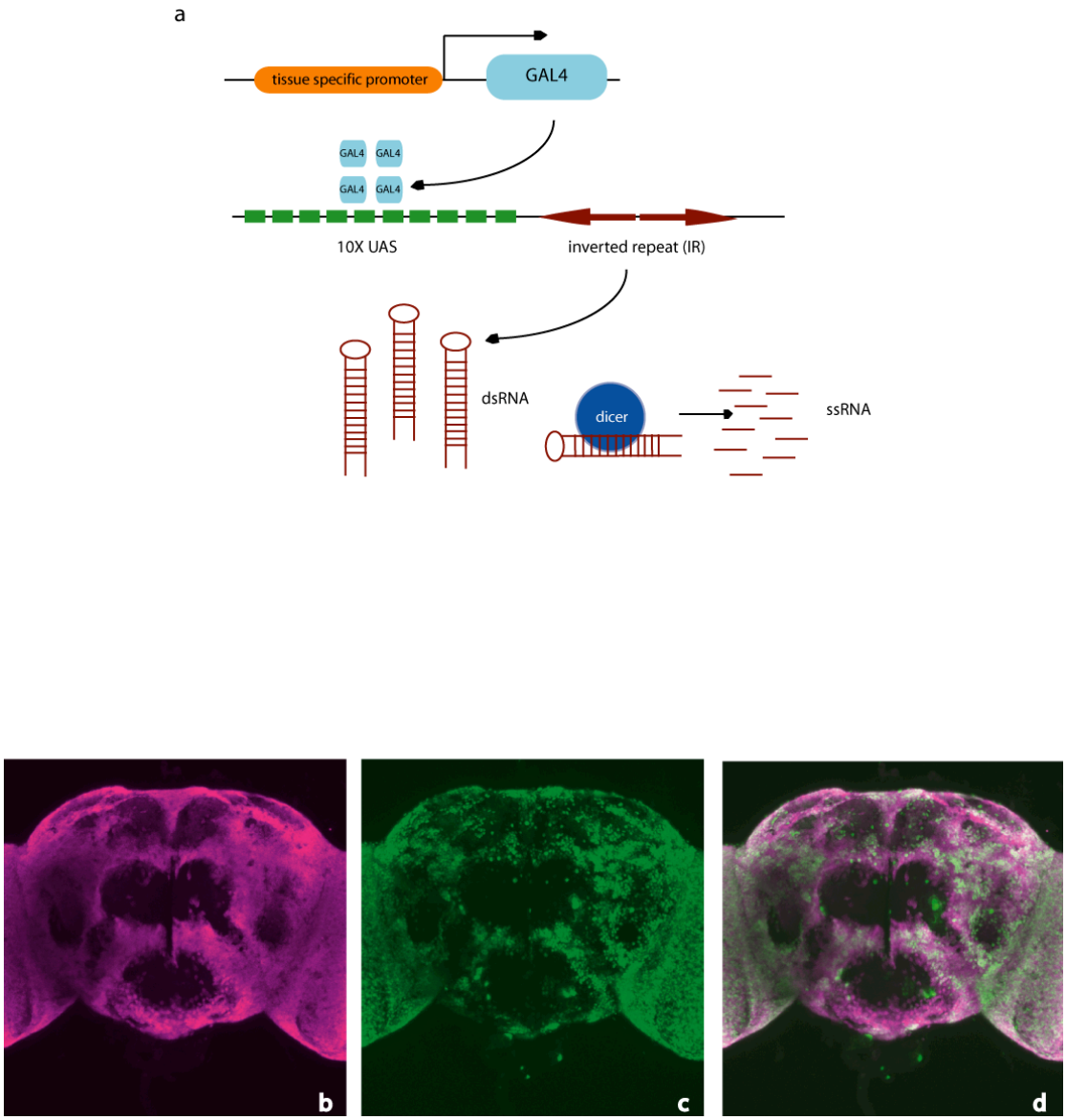
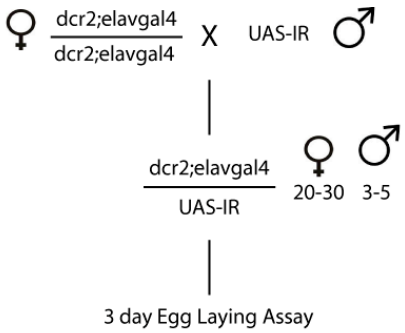


Figure 1 Transgenic RNAi system and *elav*-GAL4 expression.

(a) GAL4 /UAS system for inducible RNAi knock down. GAL4 is expressed in the target tissue and by binding to the upstream activation sequence (UAS), initiates synthesis of hairpins in consequence leading to degradation of target RNA. (b-d) Elav-GAL4 driver line is ubiquitously expressed in the nervous sytem. As an example, staining of adult fly brain *elav*-GAL4 driving UAS-*nLacZ* with *elav* (b) and (c) β-gal antibodies, show colocalization (d).

a.



b.

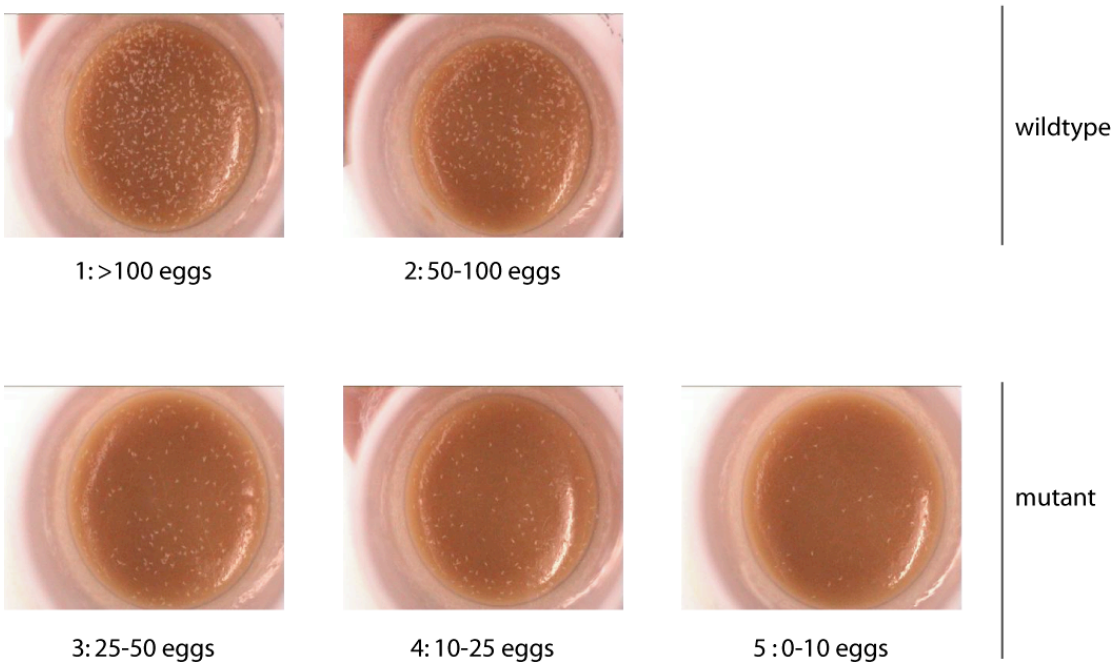


Figure 2 Egg laying screen outline

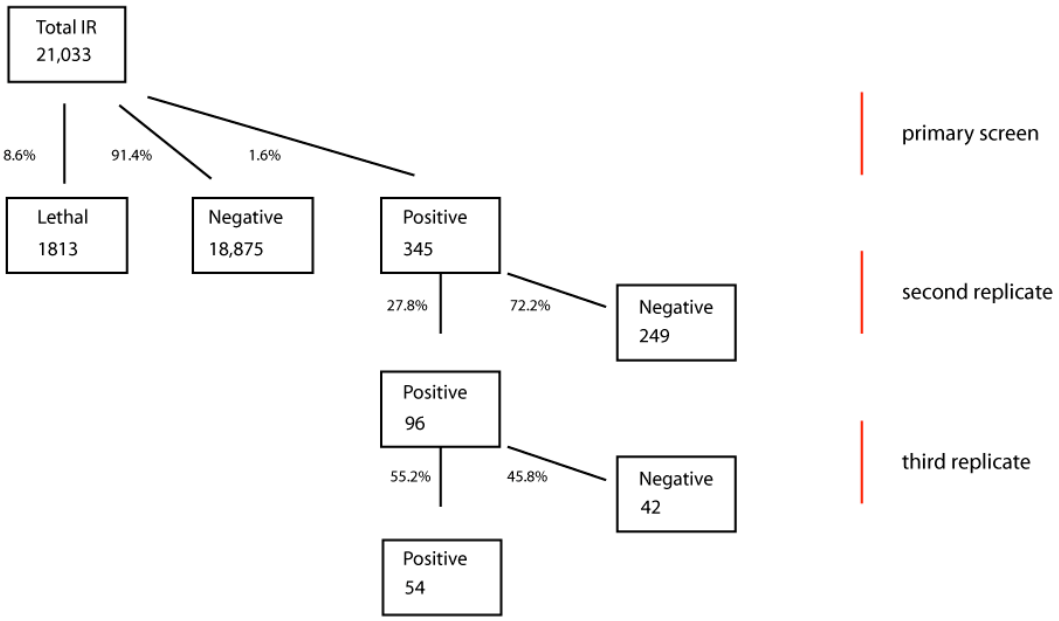
(a). Crossing scheme for the screen. UAS-*dcr2*; *elav*-GAL4 homozygous virgins were crossed to UAS-IRs males from the VDRC stock centre. From the resulting progenies 20-30 females were collected into fresh food vials and number of eggs laid was scored every day for three consecutive days. (b) Scoring criteria for the semi quantitative egg laying assay. Scores 3-5 were considered as egg laying defective (mutant). A line was defined as a putative positive if the phenotype was average 3 or above during the 3 day assay period.

Screen results

By using the semi quantitative assay, we screened 21,092 UAS-IR lines, covering 12,214 genes (figure 3a). 1,796 lines (8.5%) representing 1282 genes were lethal or severely weak. These genes are likely ones associated with essential functions for development and/or function of the nervous system. Lethality phenotype was distributed to different developmental stages, possibly due to knock-down effects on different steps of development and/or depending on the potency of the hairpin (figure 3b). In some cases we couldn't define the lethal phase precisely. These lines fell into the undefined lethal category (figure3b).

In the primary screen, 345 lines (1.6 %) representing 336 genes were positive in the 3-day-egg laying assay. These lines were retested twice, by repeating the same assay in a blind manner alongside the primary screen. At the end of these assays, 54 lines representing 53 genes were confirmed as positives (figure 3a). We analyzed these genes in higher resolution to investigate causes of such reduced egg laying phenotype.

a.



b.

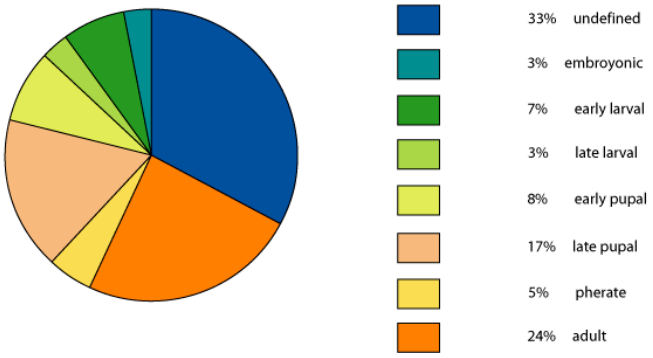


Figure 3 General overview of the egg laying screen.

(a) 21,033 UAS-IR lines representing 12,234 genes were screened. 8.6 % of the IR lines gave lethality phenotype at different stages of development. 1.6 % of the IR lines scored as primary positives. These lines were re-screened blindly twice resulting with 53 positive IR lines. (b) Distribution of lethality phenotype according to different stages of development. 33% of the IR lines considered as undefined lethal due to the absence of homozygous flies in the progeny.

Phenotype Classification

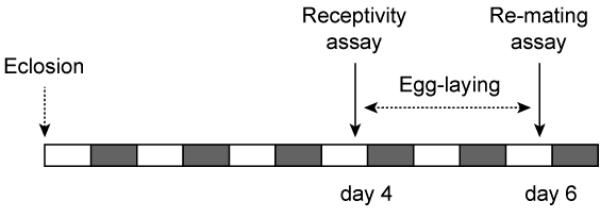
Multiple factors can cause reduced egg laying. The first possible cause is defects that reduce female receptivity. In this case, RNAi virgins are not receptive to courting males and refuse to copulate. This class could include genes regulating hearing, pheromone bio-synthesis and release, and locomotion (Wedell 2005; Grillet, Dartevelle et al. 2006). Second class of defects leading to reduced egg laying is associated with post-mating responsiveness. Mating induces fundamental changes in female behaviour. These changes, so far called post-mating responses are induced mainly by male seminal fluid components. SP, an accessory gland molecule, controls two of the main post-mating responses, elevated egg laying and female rejection. Therefore one reason why RNAi females fail to lay eggs is a lack of response to SP or to other seminal fluid components. This class of genes should include the SP receptor, as well as genes regulating SPR signalling. The third class of phenotype is defects in egg laying itself. Flies with this phenotype are expected to mate normally and exhibit all of the post mating responses, but fail to lay eggs. Finally, because in our primary assay we allowed RNAi females to mate with their own male siblings due to practical reasons, it is also possible that defects in male mating may cause in reduced female egg laying.

In order to distinguish among these possible phenotypes, we designed secondary behavioural assays in which we checked the general posture of the flies as well as defects in different steps of female reproductive behaviour in detail (figure 4a). For this purpose, we collected virgin RNAi females immediately after eclosion and aged them in groups of 10-15 in fresh food vials. At day 4, we placed virgin females with wild type males in courtship chambers and video taped the courtship behaviour for 1 hour. From these courtship videos, we calculated the percentage of copulation for each female in 1 hour. We also pay attention for the general defects in locomotion and posture of the flies to identify the possible unspecific receptivity defects mainly caused by general problems in the nervous system. All of the females from the receptive lines were transferred individually to fresh food vials for quantitative egg laying assay and allowed to lay eggs over the 48 hours. After the egg laying assay, we checked if females show post-mating rejection behaviour. In these assays, we placed the same females with naive wild type males in courtship chambers and scored for

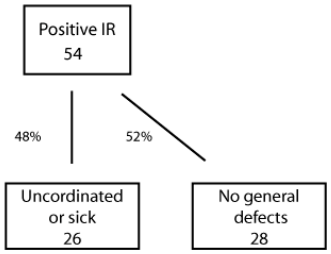
remating. We were looking for RNAi females, which fail to lay eggs and remate in high frequencies.

After the secondary assays, we found 26 lines with general posture and locomotion defects (unspecific-phenotype; figure 4b). The rest 28 lines did not show any abnormality in general locomotion. Among those, 10 lines were found unreceptive, 10 lines showed defects only in egg laying and only 3 lines had post-mating switch defect, whereby females fail to lay eggs and remate in high frequency. These lines include the SPR, tyrosine beta hydroxylase (*Tβh*) and vesicular monoamine transporter (*VMAT*). Remaining 5 lines did not show any obvious phenotype. These lines belong to either false positives or possible male mating defective lines (table1 and figure 4c).

a.



b.



c.

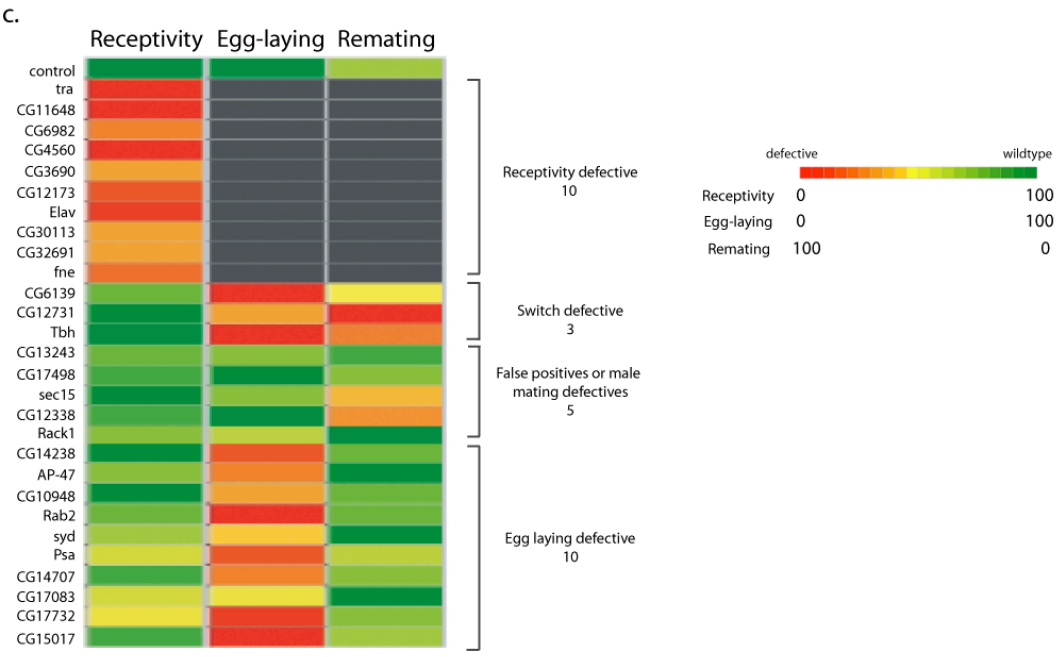


Figure 4 Secondary analysis of egg laying screen positives

(a) Protocol for secondary behavioural analysis. (b) Classification of positive lines according to defects in posture and locomotion. 28 lines showed no defects. (c) Classification of primary egg laying phenotypes into three categories. Receptivity defective lines weren't tested for egg laying or remating (shown by gray bars). In the heat map, red encodes for a defect in behaviour.

TransID	GeneName	GeneSymbol
853	CG8224	babo
4689	CG4676	CG4676
8875	CG3139	syt
10443	CG18009	Trf2
14174	CG9311	CG9311
22315	CG6937	CG6937
26019	CG7873	Src42A
26039	CG8025	CG8025
27341	CG10955	CG10955
28488	CG14359	CG14359
29646	CG32084	CG32084
30767	CG10186	CG10186
32255	CG13645	CG13645
33197	CG4088	lat
38610	CG11943	CG11943
39769	CG13176	CG13176
39937	CG17083	CG17083
40884	CG14199	CG14199
41029	CG12223	Dsp1
42279	CG15345	CG15345
42549	CG14181	CG14181
43120	CG4195	l(3)73Ah
45281	CG3689	CG3689
45339	CG4982	CG4982
47188	CG7007	VhaPPA1-1
50505	CG33500	unknown

Table 2 Lines with general posture and locomotion defects

Gene symbol	Gene name	Receptivity (n)	mated (n)	%	Egg-laying (n)	mean	stddev	Remating (n)	n mated	%	Class
control	**control**	300	278	92,7	274	37,7	12,1	272	75	27,6	Control
CG16724	tra	60	8	13,3							Rec
CG11648	CG11648	60	6	10,0							Rec
CG6982	CG6982	53	16	30,2							Rec
CG4560	CG4560	30	3	10,0							Rec
CG3690	EG:BACR7A4.13	60	24	40,0							Rec
CG12173	CG12173	60	12	20,0							Rec
CG4262	elav	60	11	18,3							Rec
CG30113	CG30113	48	18	37,5							Rec
CG32691	unknown	30	11	36,7							Rec
CG4396	fne	60	14	23,3							Rec
CG6139	CG6139	60	44	73,3	44	0,1	0,6	44	18	40,9	Post
CG12731	SPR	300	265	88,3	259	13,6	8,3	251	208	82,9	Post
CG1543	Tbh	60	55	91,7	55	0,0	0,0	55	37	67,3	Post
CG13243	BG:DS02252.1	60	46	76,7	45	28,0	12,4	44	7	15,9	False
CG17498	CG17498	57	46	80,7	46	34,7	11,7	46	11	23,9	False
CG7034	sec15	60	50	83,3	50	28,3	11,6	50	27	54,0	False
CG7111	Rack1	60	42	70,0	42	23,1	10,5	42	0	0,0	False
CG12338	CG12338	33	26	78,8	26	37,5	9,8	26	16	61,5	False
CG14238	CG14238	60	52	86,7	51	5,1	7,0	51	9	17,6	Egg
CG9388	AP-47	90	63	70,0	62	8,0	7,8	62	4	6,5	Egg
CG10948	CG10948	47	39	83,0	39	12,1	15,3	38	7	18,4	Egg
CG3269	Rab2	60	44	73,3	43	0,0	0,0	42	8	19,0	Egg
CG8110	syd	60	40	66,7	37	16,9	15,0	38	4	10,5	Egg
CG1009	Psa	60	35	58,3	35	4,3	8,1	35	11	31,4	Egg
CG14707	CG14707	60	47	78,3	46	9,0	14,7	46	12	26,1	Egg
CG17083	CG17083	60	36	60,0	35	20,6	11,3	35	4	11,4	Egg
CG17732	CG17732	60	33	55,0	20	2,5	5,5	20	5	25,0	Egg
CG15017	CG15017	49	40	81,6	40	0,8	2,8	40	11	27,5	Egg

Table1: Detailed behavioural analysis of 25 positive lines. All of the assays are performed according to the protocol in figure 4a.

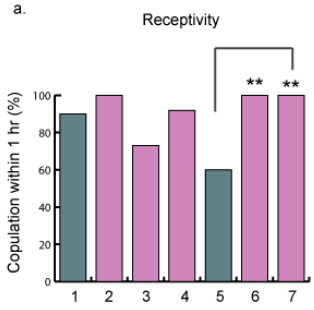
Role of Octopaminergic Signalling in the female post-mating

The *Tβh* and *VMAT* post-mating phenotype that we observed in our screen suggested a novel role for octopamine in regulating post-mating switch in females. Octopamine is one of the major neurotransmitters in the invertebrate nervous system. It regulates many behavioural processes including aggression (Stevenson, Dyakonova et al. 2005; Certel, Savella et al. 2007), learning (Farooqui 2007) and locomotion (Fox, Soll et al. 2006; Fussnecker, Smith et al. 2006; Ormshaw and Elliott 2006). Octopamine also regulates egg- laying behaviour. Mutants lacking two enzymes of the octopamine bio-sythesis cascade, *Tβh* and *tdc2* (tyrosine hydrolxase 2) are shown to fail in ovulation and egg deposition (Lee, Seong et al. 2003; Monastirioti 2003). To further characterize the observed post-mating switch defect, we performed a series of behavioural assays. We first confirmed the previous phenotypes detected in the secondary analysis of the screen hits by simply repeating the assays in the same manner (figure 5a-c). This time we also included SPR-IR as a positive control, which allowed us to compare the post-mating phenotypes caused by knockdown of different classes of molecules. In the initial mating assays, all of the genotypes were equally receptive (figure 5a). In egg laying assays, both *Tβh* and *VMAT* RNAi females failed to lay any eggs. This result was consistent with the previously reported phenotype of *Tβh* mutants (Monastirioti 2003). In addition to reduced egg laying, *Tβh* and *VMAT* RNAi females also remated at higher frequencies. The frequency of remating was higher in *Tβh* knock down than *VMAT*. This might be due the hairpin potency or to the existence of other monoamine transporters that will compensate the *VMAT* function. To eliminate the potential RNAi off-targeting effect, we tested a null allele of the *Tβh* gene, $T\beta h^{M18}$ (Monastirioti, Linn et al. 1996) using the same assays. Compared to the control line (CS), $T\beta h^{M18}$ mutants had elevated receptivity (figure 5a). In egg laying and remating assays, $T\beta h^{M18}$ mutants behaved similarly to RNAi lines, showing high remating and reduced egg laying. This phenotype was also similar to SPR mutants, *Df(1)Exel6234* (figure 5b-c). These results show that flies lacking octopamine in the nervous system fail to produce post-mating responses in egg laying and receptivity.

Tβh converts tyramine to octopamine (figure 5e). Therefore, Tβh mutants have elevated tyramine levels (Monastirioti 2003). To rule out the possibility that defects in post-mating responses are due to excessive tyramine, we tested tdc (tyramine decarboxylase) mutants. Tdc synthesizes tyramine from tyrosine. In *D. melanogaster* there are two tdc genes, *tdc1* and *tdc2*. *Tdc2* is specifically expressed in neurons and $Tdc2^{RO54}$ mutants lack both tyramine and octopamine in the nervous system (Cole, Carney et al. 2005). When we tested, $Tdc2^{RO54}$ flies phenocopied $T\beta h^{M18}$ in all of the assays; they laid no eggs and remate at higher frequencies even after mating. These results showed egg laying and remating defects seen in Tβh mutant females are indeed due to lack of octopamine in the nervous system (figure 5b-c).

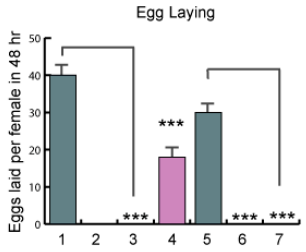
Because SPR and Tβh mutants show similar phenotypes in post-mating responses, we postulated that the failure of these females to switch to post-mating behaviours could be caused by a failure in SP response. To test this directly, we injected SP to the abdomen of $T\beta h^{M18}$ and $Tdc2^{RO54}$ virgin females. We also used *Df(1)Exel6234* and CS virgins as controls. 5 hours after injections, we paired these flies with wild type males. As expected, CS virgins were unreceptive to males, whereas *Df(1)Exel6234* virgins were still receptive after SP injections. In contrast to *Df(1)Exel6234*, both $T\beta h^{M18}$ and $Tdc2^{RO54}$ virgins responded to SP and became unreceptive as much as CS virgins did (figure 5d). These genetic data suggested octopamine is not likely one of the direct downstream components of SP-signalling.

a.

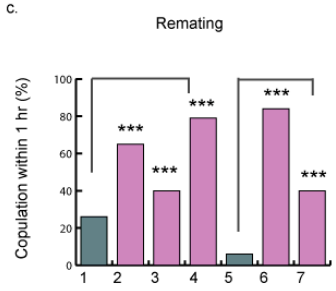


Genotype	♂	♀	*n*
1	+ / +	*elav-GAL4 / +*	60
2	+ / +	*elav-GAL4 / tbh-IR*	60
3	+ / +	*elav-GAL4 / VMAT-IR*	60
4	+ / +	*elav-GAL4 / SPR-IR*	55
5	+ / +	+ / +	81
6	+ / +	TßhM18	55
7	+ / +	*tdc2RO54*	21
8	+ / +	+ / +	39
9	+ / +	TßhM18	35
10	+ / +	*tdc2RO54*	21
11	+ / +	*Df(1)Exel6234 / Df(1)Exel6234*	17

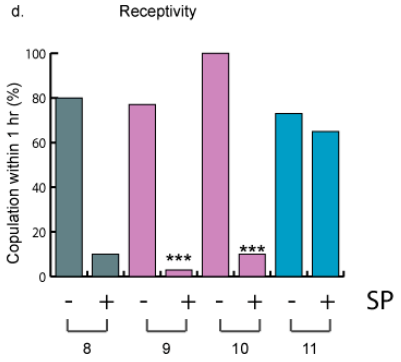
b.



c.



d.



e.

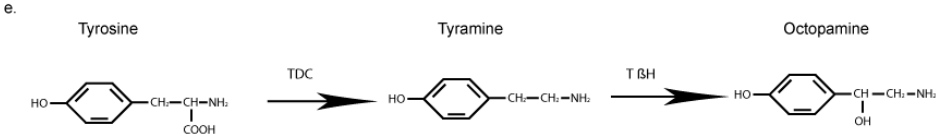


Figure 5

(**a**) Receptivity of virgin females of the indicated genotypes, scored as the percentage of females that copulated within 1 hr. $P > 0.01$ for RNAi all comparisons against *elav*-GAL4/+ (genotype 1), for the rest all comparisons against CS (genotype 5) χ^2-test.

(**b**) Number of eggs laid per female during the 48 hr immediately after copulation. Data are mean ± s.e.m. *** $P < 0.001$, Dunnet's multiple comparison test.

(**c**) Re-mating frequency for females tested 48 hr after the initial mating. *** $P < 0.001$ for RNAi all comparisons against *elav*-GAL4/+ (genotype 1), for the rest all comparisons against CS (genotype 5) χ^2-test.

(**d**) Receptivity of indicated genotype virgin females assayed 5 hr after injection with either 1mM SP (+) or Ringer's solution alone (-).

(e) The octopamine biosynthesis cascade.

Discussion

Here we present the results of a genome-wide transgenic RNAi screen for female egg laying behaviour and a potential new role for octopamine in regulating female post-mating responses. We used *D. melanogaster* female mating behaviour as a model to understand how genes regulate neuronal function to produce specific types of behaviours. By using this approach, we were able to identify potential candidate genes regulating specific steps of female mating behaviours in the fly nervous system. We focused on the post-mating defective class of genes to understand the female mating switch. By understanding post-mating switch, our aim was to establish a basic model for the neural modulation that control changes in behavioural responses.

The inducible RNAi technique we used is a powerful tool to study tissue specific functions of genes. Although classical mutagenesis screens lead to the discovery of many genes in *Drosophila melanogaster,* the tissue specificity is a problem especially to study genes with multiple functions. In particular for neural circuitry and behaviour research, it is important to check if behavioural phenotypes are due to defects in development. In this perspective inducible RNAi allows spatial and temporal control of gene knockdown particularly in the nervous system for behavioural analysis. Inducible RNAi is also useful to overcome the lethality phenotypes of genes with essential functions during development and allows studying the function of these genes in the adult animals. The RNAi knock down is also dependent on the driver line. In our screen, we chose an early pan-neuronal driver *elav*-GAL4 that allowed an efficient knockdown while at the same time having a low rate of lethality. Only 8,6% of the IR tested were lethal (figure3b). This rate is much lower than the lethality rate seen by a ubiquitous driver *A5C*-GAL4 (Dietzl, Chen et al. 2007). The lethality varied from embryonic stages to adults (figure3b). We haven't further characterized the lethality phenotypes but we believe this list contains potentially interesting genes regulating basic functions and/or wiring patterns of vital neural circuitries.

In contrast to many advantages of inducible RNAi, there are also disadvantages that should be taken in to account when using the system. One major problem is off targeting effects of RNAi hairpin that can lead to wrong interpretation of the gene

function. Thus, phenotypes should be confirmed by multiple hairpins targeting different regions in the gene locus or by mutant alleles of the gene of interest. Another problem with transgenic RNAi is the efficiency of knockdown that is influenced by multiple factors such as the insertion site of the UAS-IR transgene, the specificity and the strength of the GAL4 driver and the processing of the hairpin in the target tissue. These problems may cause false negatives and false positives and should be considered when optimizing the screen assay. In our screen we neglected the false negatives and focused on decreasing the false positive rate. For this reason, we optimized our screen by repeating the semi quantitative egg laying assay for three consecutive days and by taking the average score as a final read-out. We also retested the putative positives by repeating the same procedure twice. By using this method, we were able to enrich the positive rate of 1,6% from the primary screen to 27,8% after the second replicate and to 55,2% after the third replicate. The confirmed 54 IR lines were tested in the behavioural analysis and most of them showed specific mating phenotypes. This indicated that our strategy to enrich the discovery rate of candidate genes regulating female mating was successful.

The first gene we have characterized from the screen was a receptor for SP, SPR (Yapici, Kim et al. 2008). To further understand how SP induces its effects through SPR on female mating circuitry and to analyze the post-mating switch behaviour in more detail, we focused on the post-mating defective genes that we have identified in the screen; *Tβh* and *VMAT*. These genes regulate octopamine biosynthesis and transport respectively. Therefore we postulated a possible octopaminergic regulation on female post-mating behaviours. To test this hypothesis, first we confirmed the primary RNAi phenotype with a null mutant, $T\beta h^{M18}$ and showed the post-mating phenotype that we have observed is indeed due to *Tβh* gene function. Because $T\beta h^{M18}$ flies have excessive tyramine, we tested Tdc2 mutants, $Tdc2^{RO54}$ that lack both octopamine and tyramine in the nervous system. These flies behaved similar to $T\beta h^{M18}$ therefore we attributed the $T\beta h^{M18}$ phenotype to lack of octopamine rather than to excessive amounts of tyramine in female nervous system. Next, we wondered if this defect in post-mating switch is due to sensitivity to SP. Therefore, we injected SP to $T\beta h^{M18}$ and $Tdc2^{RO54}$ virgins and tested if they show SP induced post-mating responses. Surprisingly, although these flies were insensitive to mating and

endogenous SP, they responded to injected SP and showed post-mating responses. This might be due to several reasons. One possibility is octopamine might be regulating the SP response in the SPR expressing neurons; therefore at the physiological conditions SP might fail to induce a functional response in SPR neurons when octopamine is missing. However injecting SP at high amounts might compensate the octopamine phenotype. Another possibility that might cause post-mating defects is problems with SP cleavage and transport. SP is transported to the female reproductive tract by binding to the sperm tail (Peng, Chen et al. 2005) and it has to be cleaved off to induce post-mating responses. Octopaminergic neurons are shown to regulate sperm storage (Monastirioti 2003; Middleton, Nongthomba et al. 2006) thus, one reason that octopamineless flies are insensitive to mating might be problems in sperm storage that will also effect the cleavage of SP from the sperm tail. We also detected elevated receptivity in *Tβh* mutants compared CS flies. Therefore octopamine can be a general regulator for receptivity in females. This might also explain the increased remating in these mutants. Further experiments are needed to explain the role of octopamine in regulating female receptivity and post-mating switch. These experiments should consists of behavioural assays to discriminate the effect of octopamine in receptivity and remating, imaging and/or electrophysiological analysis of neuronal activity in response to SP in the absence of octopamine and quantitative analysis of sperm storage and SP cleavage from the sperm tail in *Tβh* mutants.

In conclusion here we report our results from a genome wide RNAi screen for mating defects in the female nervous system of *Drosophila melanogaster*. We have identified 23 candidate genes with defects in either receptivity, egg laying or post-mating switch. From these genes, we focused on the post-mating defective class and with further behavioural analysis showed octopamine is not required for SP sensitivity but necessary for the induction of post-mating responses after mating.

Methods

Fly stocks.

All of the RNAi stocks were obtained from the genome-wide transgenic RNAi library (Dietzl, Chen et al. 2007) maintained at the Vienna *Drosophila* RNAi Center. Other stocks used were *elav-GAL4* (Luo, Liao et al. 1994), $T\beta h^{M18}$, $Tdc2^{RO54}$ (Hoyer, Eckart et al. 2008) and *Df(1)Exel6234* (Yapici, Kim et al. 2008). The *elav-GAL4* stock additionally carried a *UAS-Dcr-2* insertion on the X chromosome (Dietzl, Chen et al. 2007).

RNAi screen

Virgin females homozygous for both *UAS-Dcr2* on the X chromosome (Dietzl, Chen et al.) and *elav-GAL4* on the 3rd chromosome (Luo, Liao et al. 1994) were collected from a stock in which the Y chromosome carries a *hs-hid* transgene. Stock bottles containing 4-5 day-old larvae were transferred to 37°C waterbath for 60 minutes for two consequtitive days to kill the males, facilitating the large-scale collection of virgin females. 5-6 females were crossed to 3-5 males from the RNAi library (Dietzl, Chen et al.) maintained at the Vienna Drosophila RNAi Centre (VDRC). Parents were removed from the cross after three days and progeny were raised on semi-defined medium at 25°C and 70% humidity on a 12:12hr dark: light cycle. For semi-quatitative egg laying assay adult flies from the 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; 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. If no adults were obtained, or the majority died before the end of the 3rd day, the progeny were scored as lethal. These pan-neuronal lethal lines were not retested, and may include a small number of false positives.

Behavioural Assays:

All flies were raised on semi-defined medium[29] at 25°C in a 12 hr:12 hr dark:light cycle. Virgin males and females were collected at eclosion. Males were aged individually for 5 days; females were aged for 4 days in groups of 10–15. All assays were performed at circadian time 6:00–10:00, and on at least 3 independent occasions. For assays performed according to the protocol in Fig. 1, single female and male virgins were paired in 10 mm diameter chambers and videotaped for 1 hr. The time to copulation was recorded for each female. Those females that copulated were then transferred to single food vials for 48 hr, and the number of eggs laid by each female was counted manually. Females were then either re-tested for receptivity in the same manner in pairings with naïve Canton S males. The data set for the *elav-GAL4* / + controls is pooled data from two separate series of experiments in which the *elav-GAL4* driver was crossed to each of the respective parental strains for the two *UAS-IR* transgenes. SP injections into the abdomen of virgin females were performed as described previously (Schmidt, Choffat et al. 1993). Following injection, females were transferred to individual food vials and tested after 5 h for receptivity with a naïve Canton S male.

Immunohistochemistry

Brains were dissected in PBS and fixed with 4% PFA for 20 minutes at RT. After fixation, they were washed 3 times with PBS-T 0.1 % and incubated in primary antibodies for 24 hr at 4^{o}C, and in secondary antibodies for 3-5 hr at RT. Antibody concentrations were rat anti-elav (1:500), mouse anti-GFP (1:1000) and rabbit anti-β-gal (1:1000), Alexa 488-conjugated goat anti-rabbit, Alexa 568-conjugated goat anti-mouse and Alex 633-conjugated goat anti-rat (all 1:1000). Images were acquired with a Zeiss LSM 510/Axiovert 200M and processed in Adobe Photoshop.

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Author contributions

N.Y. performed the RNAi screen and all of the behavioural analysis.

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DISCUSSION

Genetic analysis of innate behaviours

One open question in biology is how animal behaviour is controlled in the nervous system. Starting from the late 19th century, scientists from different disciplines addressed this phenomenon from many perspectives. The early experiments of these neuroscientists mainly depended on either the anatomical studies or the correlative analysis of neural functions and behavioural responses. Although these techniques reveal many unknowns about the anatomy of the nervous system and its basic relations with behaviour, they fail to explain the molecular mechanisms underlying specific behavioural responses. These researchers were mainly lacking the tools to specifically manipulate the function of neurons for behavioural analysis. Today, the development of molecular and genetic tools in many model organisms gives us the chance to ask the same questions about neurons and behaviour in a more sophisticated way. For this reason, we choose to work on a genetically tractable organism, *Drosophila melanogaster.* In the last century, forward genetic screens using flies helped researchers to identify many key genes regulating developmental processes. Starting from late 1960s, Seymour Benzer from California Institute of Technology began to use forward genetic screens in *Drosophila melanogaster* to identify genes controlling behaviour. He and many others were successful to isolate several behavioural mutants by using this method. We therefore took a similar forward screening approach to identify key genes regulating female mating behaviours. We assume this approach will be a starting point to understand the molecular basis of these behaviours in more detail.

Drosophila melanogaster mating as a model for behavioural modulation

Drosophila melanogaster mating behaviour has been widely studied through molecular and genetic approaches (Hall 1994). It is an excellent model for genetic analysis of innate behaviours, especially to understand how sex specific behaviours are produced in the nervous system. For this reason, many forward genetic screens have been performed using male courtship behaviours. These screens identified

genes, when disturbed, causing defects in different steps of male mating behaviour. One of the good examples for this class of genes is a zinc finger transcription factor *fruitless* (*fru*) (Gailey and Hall 1989) that is necessary and sufficient for male courtship behaviour (Demir and Dickson 2005; Stockinger, Kvitsiani et al. 2005) 2005). In this study we focused on the female post-mating switch, which demonstrate a better model for behavioural changes than female to male behaviour transition seen in *fru* mutants. Female flies change their behaviour upon mating. Because these changes occur after the nervous system is established, they shouldn't be regulated by the wiring pattern of the system but by external or internal factors that female flies experience before and after mating. Compared to male courtship behaviour, female mating behaviours has so far received less attention. One reason is that female actions in courtship are not very apparent. The main role of females in courtship ritual is to accept or reject the male. Although female actions are not significant during courtship, female mating decisions are important. *Drosophila melanogaster* females don't remate frequently thus; choosing the right male for the production of healthy progeny is critical. For this reason, regulation of female receptivity is tightly controlled by internal and external factors. In addition, female post-mating switch is a good model to study the neural modulation on behaviour. Overall female mating behaviours represent a good system to identify genes regulating specific behavioural responses. For this reasons we performed a genome wide RNAi screen for female mating behaviours by using an egg laying assay. This assay allowed us to check multiple steps of female mating including receptivity, egg laying and post-mating switch. From the screen, we identified candidate genes regulating female receptivity, egg laying and post-mating switch. We were mainly interested in the post-mating defective class that presumably would include the receptor for SP (SPR), the key modulator of post-mating responses and other genes that would modulate the female mating switch. Identification of these genes was important to understand the molecular mechanisms of neuronal modulation on the female post-mating switch.

The Sex peptide receptor

Since the identification of the SP in 1988 (Chen, Stumm-Zollinger et al. 1988), one open question was how SP induces the behavioural switch in females. Many studies done by Eric Kubli and colleagues demonstrated how SP is transferred to the females

(Peng, Chen et al. 2005), where it binds to in the female nervous system and reproductive tract (Ottiger, Soller et al. 2000), which functional domains it carries (Aigaki, Fleischmann et al. 1991; Domanitskaya, Liu et al. 2007) and how it controls the temporal dynamics of the post-mating responses (Peng, Chen et al. 2005). These findings increased our knowledge about male originated regulation on females but fail to explain how female nervous system itself is regulated to produce the behavioural switch in mating. In order to understand the molecular mechanisms of the female post-mating switch, it was necessary to find target molecules of SP in the female nervous system. Unfortunately despite many efforts spent on classical genetic screens and/or candidate approaches, the SP receptor was not found.

In our egg laying screen, we identified a novel G-protein coupled receptor, CG16752 that showed post-mating switch defects when knocked down in the nervous system. In a series of behavioural and biochemical analysis, we showed CG16752 is a receptor for SP (SPR) and restricted SPR function to a subset of *fru* neurons (Yapici, Kim et al. 2008). These results supported our previous findings where we claimed the activity of *fru* neurons is required for female post-mating responses. Therefore we postulate a possible mechanism for SP action in which SPR is suggested to regulate the post-mating switch by silencing the neural activity of *fru* neurons. To test this hypothesis, further physiological experiments are necessary to observe neural activity in vivo where both *fru* and *SPR* is expressed.

Because SPR is widely expressed in the nervous system, we wondered if SPR is regulating egg laying and remating in different subsets of neurons. To test this assumption, we have recently screened a random set of GAL4 lines with SPR-IR in our semi-quantitative egg laying assay. Our aim was to identify GAL4 lines that would have the reduced egg laying phenotype but would not remate. Surprisingly, all of the lines we identified showed both of the post-mating responses (unpublished data). These data suggested that SPR is regulating egg laying and remating through the same set of neurons.

SPR is not only expressed in *fru* neurons where it regulates female post-mating behaviours but also in many other neurons in the brain and ventral nerve cord. We also found similar *SPR* expression pattern in males but so far, we couldn't identify

any obvious male courtship defects in *SPR* mutants. Therefore, one possibility is that there is an additional function of SPR, which is common for both male and female flies. This function might be distinct from regulating post-mating responses. Since in males SP is only found in the accessory glands, it is unlikely that this novel function of SPR is regulated with SP. This assumption suggests the presence of a novel ligand for SPR that should be present in the nervous system of both sexes. Consistent with this assumption, SPR has distinct relatives outside of the insect kingdom where SP itself is not present.

Apart from its contributions to the characterization of female post mating behaviours, SPR is also a potentially important target molecule for the reproductive control of insects. It is highly conserved in many insect species including disease vectors. We have shown in our study that SPRs from *Drosophila pseudoobscura*, *Adese aegypti*, and *Bombyx mori* responded to *Drosophila melanogaster* SP in the cell culture assay at different levels. This is strong evidence that the SPR homologs are functional in these species and might regulate analogous functions in post mating behaviours. In most of these insects, host-seeking behaviour is influenced by mating and egg laying. Therefore it can potentially be regulated with SPR function. Thus, regulating the SPR activity might be useful to control the reproductive rate of these pests to prevent the spread of infectious diseases that they carry.

Overall, identification of SPR is an important starting point to analyse the female post-mating behaviour from many perspectives. It might help us to understand the mechanisms how neuronal function regulates behavioural changes both on the cellular and molecular level.

Octopaminergic regulation on female receptivity and post mating switch

In the egg laying screen apart from SPR, we also identified *Tβh* and *VMAT*, genes regulating octopamine biosynthesis and transport respectively, with defects in post-mating behaviour when knocked down in the nervous system. We showed in genetic and behavioural analysis that this defect is due to the lack of octopamine but not due to SP insensitivity. Octopamine is one of the major neurotransmitters in the invertebrate nervous system (Roeder 1999). Lack of octopamine causes defects in

learning (Braun and Bicker 1992; Pribbenow and Erber 1996; Hammer and Menzel 1998), aggression (Stevenson, Dyakonova et al. 2005; Hoyer, Eckart et al. 2008) and locomotion (Fox, Soll et al. 2006; Fussnecker, Smith et al. 2006). It is also known to regulate egg laying (Monastirioti, Linn et al. 1996). Here we suggested a potential new role for octopamine in regulating female receptivity and post-mating switch. Our current data is insufficient to fully explain the octopaminergic modulation on female mating but one can speculate about different possibilities. Octopamine can be a general modulator for female receptivity. In our assays *Tβh* mutants showed elevated receptivity compared to CS flies. Thus, increased remating might be a consequence of hyper-receptivity phenotype. This possible explanation doesn't support the specific role of octopamine in regulating SP induced post-mating responses but doesn't eliminate the possibility that SPR function is modulated by octopamine. Octopamine has been previously shown in crickets to modulate neural excitation (Kinnamon, Klaassen et al. 1984; Walther and Zittlau 1998). Thus, one possibility how octopamine might regulate post-mating switch is by regulating the excitability of SPR neurons. In the absence of octopamine endogenous SP might not be able to induce a functional response in the SPR neurons but high levels of SP, as we used in our injection assays might compensate the octopamine role and induce the behavioural switch. To test this hypothesis, physiological experiments that will use imaging or electrophysiological techniques are necessary. In these experiments, response patterns of SPR neurons should be analysed upon SP induction in the presence or absence of octopamine. If our hypothesis is right then one will expect to see different SP responses in SPR neurons depending on the octopamine levels.

An alternative hypothesis is that octopamine might also be responsible for SP transport to SPR. SP is transferred to the female reproductive tract by binding to the sperm tail. To become functionally active, it has to be cleaved off (Peng, Chen et al. 2005). Octopaminergic neurons are shown to regulate sperm storage (Monastirioti 2003). Therefore, one reason that lack of octopamine causes post-mating defects is failure in sperm storage leading to cleavage defects of SP from the sperm tail. To test this possibility, we roughly checked the sperm storage ability of *Tβh* mutants. We found no apparent difference in sperm storage. These experiments were done in a very qualitative way, therefore should be repeated with quantitative analysis.

Another entrance point to elucidate the role of octopamine is the fact that it has multiple receptors with different expression profiles. Therefore, it is not surprising that the lack of octopamine causes multiple defects in behaviour. However, for a neurotransmitter that is critical for several biological processes, it is interesting that mutants are still viable without any obvious phenotypes. Most of the reported defects seen in *Tβh* mutants are found in detailed behavioural analysis. This suggests a general role for octopamine in neuro-modulation. To specifically analyse the multiple defects seen in *Tβh* mutants, it is necessary to identify the receptors for different behavioural phenotypes. For post-mating switch defect that we have found, one potential candidate is the octopamine receptor in mushroom bodies, *oamb.* Oamb was first identified with its specific expression in the mushroom bodies (Han, Millar et al. 1998). Afterwards, the expression was also found in the abdominal ganglia and in the reproductive tract (Lee, Seong et al. 2003). *Oamb* mutants have defects in egg laying very similar to *Tβh* mutants (Lee, Seong et al. 2003). Currently, we are in the process of testing mutant alleles of this receptor to check if they behave similar to *Tβh* mutants in post-mating assays. If we identify similar defects in post-mating switch, we can check the *oamb* neurons for SPR expression. The presence of an octopamine receptor subtype in SPR neurons with similar functions in post mating behaviour would support our hypothesis about octopaminergic regulation on SP response in these neurons.

Conclusion

For their survival, animals have to respond to the demands of their environment and change their behaviour by combining external and internal stimuli. These changes in behaviour are mainly regulated with the modulation of the nervous system in several ways. This project aimed to establish a genetic model to study the molecular mechanisms that regulate behavioural changes. For this purpose, we used *Drosophila melanogaster* as a genetic model to perform a genome wide RNAi screen for egg laying defects. The screen uncovered candidate genes with different molecular functions that caused defects in receptivity, egg laying and post-mating switch. We focused on the post-mating switch defective genes that caused defects in post-mating responses. Overall, our results set the first critical steps to the molecular analysis of a specific behavioural change. The female mating switch model we have established in

this project can now be used for further characterization of the female post-mating behaviours. In combinations with neuro-physiological approaches, these experiments might lead to the better understanding of how genes modulate neural functions to produce behavioural changes.

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- Sensory neurons in the *Drosophila* genital tract regulate female reproductive behaviour Martin Häsemeyer, Nilay Yapici and Barry J. Dickson Neuron (in revision)
- Genetic programming of female mating behaviours in *Drosophila melanogaster* and octopaminergic regulation on female receptivity and remating Nilay Yapici and Barry J. Dickson (manuscript).