Investigation of the decision-making and time-keeping abilities of SIFamide signalling in *Drosophila melanogaster*

by
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Abstract

Drosophila melanogaster is an invaluable model organism for the study of basic neuroscience. Using two previously characterized mating behaviours (Longer- and Shorter-Mating Duration), this research aims to further our knowledge of the neural circuit involved in each, and shed light on the mechanism by which four SIFamide producing neurons are involved in both. We also seek to investigate the involvement of core circadian clock genes in interval timing mechanisms. To do so, we investigated the populations of SIFamide receptor expressing neurons necessary for each behaviour and studied the contribution of circadian clock genes within the SIFamide signalling pathway. Our main experimental approach consisted of population specific knock-downs of the SIFamide receptor, the impact of which was assessed using a simple behavioural assay. This approach was complemented by rescue experiments and feminization of neurons. Finally, our investigation of the circadian clock was mediated by circadian gene knock-downs in SIFamide expressing neurons. Our results show that SIFamide signalling for each mating behaviour is mediated by segregated signalling to different, non male-specific SIFamide receptor expressing neuronal populations. We further demonstrate that SIFamide expressing neurons are not involved in the interval timing mechanism of these mating behaviours via core circadian gene contribution. This work presents preliminary results towards the investigation of a novel model of decision-making via neuronal signalling.

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List of Abbreviations

aa – amino acid

AstC – Allatostatin C

burs – Bursicon

CCAP – Crustacean cardioactive peptide

Cha – Choline acetyltransferase

CLK/*clk* – clock GENE and *protein*

CNS – Central nervous system

COMT – Catechol-O-methyltransferase

CREB – cAMP response element-binding protein

cry – cryptochrome protein

Crz – Corazonin

CYC/*cyc* – cycle GENE and *protein*

DARPP-32 – Dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa

dbt – double-time protein

Df – fly line lacking the sex peptide receptor removed from genome by deletion

DH44 – Diuretic hormone 44

Drosophila – *Drosophila melanogaster*

dsRNA – double stranded RNA

dsx^{F} – doublesexF protein

dsx^{M} – doublesexM protein

EH – Eclosion hormone

$elav^{c155}$ – embryonic lethal abnormal vision promoter

FLP – flip recombinase

FRT – FLP recombination target

fru – fruitless protein

GABA – gamma-Aminobutyric acid

GAD – Glutamic acid decarboxylase – synthetic enzyme for GABA

$GAL80^{ts}$ – Temperature sensitive GAL80 denatured above 29°C

GPCR – G-protein coupled receptor

IR – inverse repeat

KD – knock-down

Kir2.1 – potassium channel

LK – Leucokinin

LMD – Longer-Mating-Duration

mip – Myoinhibitory peptide

ms – Myosuppressin

NachBach – bacterial sodium (Na^+) channel

NP – Neuropeptide

NPF – Neuropeptide F

NT – neurotransmitter

One-way ANOVA – one-way analysis of variance statistical test

PDF – Pigment Dispersing Factor

PER/*per* – period GENE and *protein*

proc – Proctolin

RNAi – interference RNA

SIFa – SIFamide

SIFaR – SIFa receptor

SIFaR^{B322} – loss of function SIFaR allele

SMD – Shorter-Mating-Duration

sNPF – short Neuropeptide F

Sxl – Sex-lethal protein

Tdc2 – Tyrosine decarboxylase 2

TH – Tyrosine hydroxylase – rate limiting enzyme in synthesis of dopamine

TIM/*tim* – timeless GENE and *protein*

TK – Tachykinin

TNT.G – tetanus toxin light chain

tra/tra2 – transformer/transformer2 proteins

Trh – Tryptophan hydroxylase – rate limiting enzyme in synthesis of serotonin

tsh – teashirt protein

tubP – tubulin promoter

UAS – Upstream Activation Sequence

VGlut – Vesicular glutamate transporter

VNC – ventral nerve cord

2R – right arm of the second chromosome

3R – right arm of the third chromosome

5-HT – serotonin

Acknowledgements

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Introduction

Behaviours make up an organism's everyday life. The more complex examples of behaviours, such as courtship and mating, are made up of highly stereotyped sequences of events (movements, sounds, etc.). These behaviours are instructed by the brain and rely on intricate neural circuits. Deciphering these networks and their role in modulating or driving specific behaviours is essential to our understanding of the brain as a whole.

Behaviours occur sequentially and many are highly time-dependant. For instance, courtship cannot occur at the same time as mating and one must follow the other. In addition, to each of these behaviours is attributed a specific duration. Such behaviours are ideal models of interval-timing. interval-timing is involved in many behaviours and represents a key function of the neural networks that underlie them.

Some neural circuits contain a single or small subset of neurons whose activation is sufficient to induce a specific behaviour. These cells are called command neurons and constitute a crucial element of the neural circuit they belong to. Finally, certain behaviours are sex dependant. In most species, courtship is only performed by males. Similarly, males and females display different mating behaviours with males often mounting the stationary females. Such behaviours are called sexually dimorphic. However, sexual dimorphism is not exclusively behavioural, it is also present at the cellular and molecular levels. Certain proteins are sex specific and their expression in cells can induce a 'masculinization' or 'feminization' of their structure. This is particularly important

in neural circuits as it can lead to sex-specific arborisation of neurons and synapses.

The concepts of interval-timing and command neurons are therefore central to our understanding of the brain as a whole, as is our knowledge of the importance of sexual dimorphism. The model organism *Drosophila melanogaster* (Drosophila) is ideal to study the neural mechanisms underlying interval-timing and command neurons.

Neural Circuits

Neurons represent the brain's unit of computation. They send and receive inputs from one another, and are capable of complex computational processing. However, individual neurons cannot work on their own (Augustine et al., 2001). They must make functional connections with each other to create intricate networks called neural circuits (Augustine et al., 2001). Neural circuits underlie many major functions of an organism. They are involved in endocrine control, behavioural control and reflexes alike. Their complexity varies on the task they control, from a direct reflex arc to a complex behaviour or endocrine signalling. They can all, however, be reduced to three major components: afferent inputs, an interneuronal system and an efferent output system (motor, endocrine, etc.) (Augustine et al., 2001). These must all work in tandem to insure normal behaviour. They must encode signals from multiple sources and information such as time and place.

To properly understand the brain's role in directing behaviour, fundamental concepts governing neural circuits and their information processing must be elucidated.

Interval timing

Many behaviours are highly time-dependant. They rely on absolute or relative time-keeping in the minutes' range. Such behaviours are models of interval timing.

Interval timing is the ability to count time on the seconds to minutes time scale (Agostino et al., 2011b). It is the least understood of two time-keeping clock mechanism currently known. The other clock, which has been studied more extensively and is better understood, is the circadian clock. In the recent past, both clock mechanisms have recently been shown to overlap at the molecular level.

Circadian timing is the ability to count time on a 24-hour time scale. The genetic basis of the clock mechanism necessary to generate circadian rhythm was first introduced by Ronald Konopka and Seymour Benzer in 1971, with the discovery of the first circadian clock mutants and the period gene (Konopka and Benzer, 1971). Since then, many studies have further elucidated the mechanisms underlying circadian rhythmicity and their importance in many behaviours. In its simplest form, the circadian system is defined by three interacting components: (i) an input pathway integrating external cues that influence (ii) the central oscillator which generates the rhythm, and (iii) output pathways which feed into

the neural circuits of many biological processes dependent on circadian rhythmicity (Agostino et al., 2011b). The central oscillator is conserved between Drosophila and mammals and consists of an intricate feedback loop with a 24-hour period. In Drosophila, the complex formed by proteins *clock (clk)* and *cycle (cyc)* in the nucleus causes transcription of the genes PERIOD (PER) and TIMELESS (TIM), which are then transcribed into the *period (per)* and *timeless (tim)* proteins. These proteins bind together to form a *per-tim* complex which is translocated to the nucleus where it inhibits transcription of CLOCK (CLK) and CYCLE (CYC) in a negative feedback loop. Several mechanisms, including *double-time (dbt)* phosphorylation of *per,* and *cryptochrome (cry)*-mediated degradation of *tim*, lead to the degradation of the *per-tim* complex and the subsequent reformation of the *clk-cyc* complex. This continuous cycle entrains the 24-hour period at the basis of the circadian rhythm.

Much less is currently known about the basis of interval timing. The mechanism driving interval timing is still unknown in both vertebrates and invertebrates. Several models of interval timing have been proposed, both at the transcriptional level and from a molecular perspective. The case has been made that glutamatergic activation of the striatum and dopamine signalling could both be involved in molecular regulation of interval timing by activating CREB and DARPP-32 (Agostino et al., 2011b). In addition, polymorphisms in the *Catechol-O-methyltransferase* (COMT) and the dopamine D2 receptor have been shown to influence the speed and accuracy of the internal interval-timing clock (Reuter et al., 2005).

In spite of this relative gap in knowledge, a clear link has been found between the core circadian clock genes and interval timing. In 1980, Kyriacou and Hall found that the interval between tone pulses of the Drosophila male courtship was significantly altered in three different *per* mutants, suggesting a partially overlapping molecular basis for interval and circadian timing (Kyriacou and Hall, 1980). In addition, Agostino *et al.* recently reported that time of day influences the estimation of short intervals in mice, while several groups have revealed that the timing of auditory and visual signals varies with time-of-day in humans (Agostino et al., 2011c; Chandrashekaran et al., 1991; Kuriyama et al., 2005).

Because the underlying mechanisms and the neuronal signalling required to regulate interval timing represent a large gap in the scientific knowledge, their elucidation is paramount to the study of neuroscience. This is also the case for other fundamental principles of neural circuits such as the 'command neurons'.

'Command neurons' and Decision-Making Neurons

The notion of single neurons playing a central role in eliciting a behaviour was introduced as early as 1938 by Wiersma in the crayfish, and 1956 when Hagiwara and Watanabe experimentally reproduced the typical sound reflex in the cicada with a single stimulation of the motor nerve fiber innervating the sound muscle (Hagiwara and Watanabe, 1956; Wiersma, 1938). In 1964, Wiersma and Ikeda characterized a group of five interneurons responsible for the 'bilateral rhythmic swimmeret movements' in the crayfish which they referred to as 'command neurons'(Wiersma and Ikeda, 1964). The term referred to a cell or

group of cells capable of eliciting a specific, defined behaviour following experimental activation (Kupfermann and Weiss, 1978). However, this pioneering paper did not offer a formal definition of the concept. In 1971, Kupfermann and Weiss published a review of 'the command neuron concept' (Kupfermann and Weiss, 1978). The early inferences drawn from Wiersma's work defined an ON switch type of neuron which would induce a given behaviour when activated beyond threshold. However, most neural circuits involve redundant circuitry and feedback loops, complicating the task of designating a single neuron as 'commanding' (Kupfermann and Weiss, 1978). In addition, 'command neurons' are not themselves the cause of a given behaviour and must be activated by specific inputs to elicit a behavioural response.

A key controversy of the 'command neuron' concept resides in the fact that putative command neurons were not defined by their natural ability to induce behaviours but rather on the basis that their experimental activation did so (Kupfermann and Weiss, 1978). Furthermore, the term has been applied for interneurons and motor neurons, individual neurons and small groups of neurons without distinction.

In their review, Kupfermann and Weiss attempted to provide a functional definition of neurons previously referred to as 'command neurons'. They characterized 'command neurons' as neurons both necessary and sufficient to induce a naturally occurring behaviour and extended this definition to small subsets of neurons with the same properties which they referred to as command systems. Finding and characterizing new command systems or neurons is

important to further our knowledge of these highly specialized network components.

This notion of command neurons is computationally crucial within neural circuits because it attributes a higher weight factor to a specific ‘behavioural switch’ which becomes sufficient to induce the given behaviour it commands. The same concept can be applied to a subset of neurons located at the intersection of two neural circuits. If the neural networks underlying two related but mutually exclusive behaviours, such as varying flight pattern behaviours, intersect at a given neuronal cluster, the shared neurons may act as a toggle switch between the two behaviours. In this model, the system would no longer be uniquely ‘commanding’ but would need to compute complex signals in order to ‘decide’ which behaviour may be more appropriate. In the context of this research, we define such neurons which make up the intersection between two distinct neural circuits as decision-making neurons, and propose that they may be crucial to the proper discrimination between related behaviours.

Interval timing and decision-making neurons are not present in all neural circuits. However, they are likely to be found in complex, time dependent behaviours which may be driven by similarly complex neural circuits. A simple animal model is therefore required to study these concepts in an accessible and controlled behavioural paradigm which can be easily manipulated and reproduced. With its smaller nervous system, highly characterized and stereotyped behaviours, and the numerous tools available for its study, Drosophila is an ideal model in which to do so.

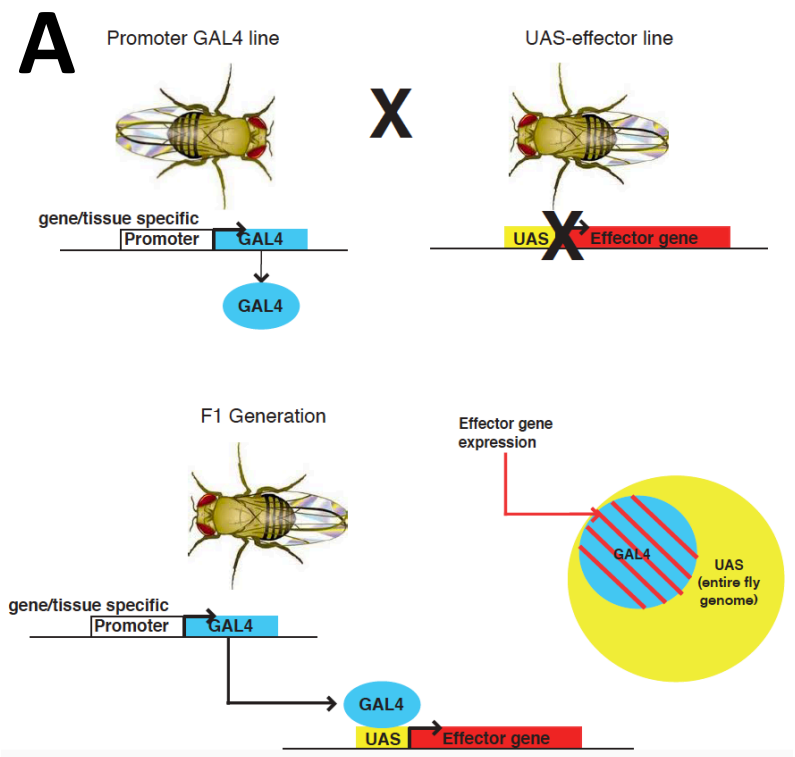
Drosophila melanogaster

Drosophila, also known as the fruit fly, is an invertebrate model organism. It has been extensively used in behavioural research, specifically in the context of courtship, mating, feeding, circadian rhythmicity and aggression (Alekseyenko et al., 2013; Cavanaugh et al., 2014; Choi et al., 2015; Crickmore and Vosshall, 2013; Hoopfer et al., 2015; Schlichting et al., 2016; Shirangi et al., 2016). Drosophila is also used in the study of several diseases (Lu and Vogel, 2009). Many major scientific discoveries stem from its study: the first potassium channel (*shaker*), the circadian clock genes and the immune system *toll* pathway to name only a few, were all cloned or discovered in Drosophila (Konopka and Benzer, 1971; Papazian et al., 1987).

The fruit fly presents many key advantages for the in-depth study of neural circuits and interval timing. The Drosophila central nervous system (CNS) is composed of ~100 000 neurons split between the brain and the ventral nerve cord (VNC). This makes the fruit fly a simple and approachable system. Despite this relatively minuscule number of neurons, the Drosophila brain shares many similarities with that of mammals. The main neurotransmitters are all conserved between species (GABA, 5-HT, Dopamine, Glutamate, Acetylcholine) while the complexity provided by a multitude of neuronal cell types is similar to that of the human brain (Fischbach and Dittrich, 1989; Venken et al., 2011). In addition, fruit flies exhibit simple, reproducible and quantifiable behaviours which can be studied using reliable and reproducible assays. Furthermore, powerful genetic tools are available for the dissection of neural circuits. The sequencing of the fruit

fly's entire genome in 2000, and the development of a genome wide RNAi library in 2007 are important resources for the study of Drosophila (Adams et al., 2000; Dietzl et al., 2007). These tools are combined to a set of powerful binary systems, the most common of which is the UAS-GAL4 system, which allow for any transgene to be expressed in any chosen subset of neurons expressing a specific, common promoter (Venken et al., 2011).

The UAS-GAL4 system involves a transactivator, the yeast transcription activator protein GAL4, and its binding sequence, the Upstream Activation Sequence (UAS) (Brand and Perrimon, 1993). This system allows for any transgene located downstream of the UAS to be expressed in cells where the promoter driving GAL4 expression is active (Figure 1A). Since the UAS-GAL4 system was developed in 1993, several similar systems and intersectional tools have been designed (Brand and Perrimon, 1993). Other binary systems include the LexAop-LexA and QUAS-QF systems (Potter et al., 2010; Szüts and Bienz, 2000; Venken et al., 2011). In addition, a specific repressor exists for the GAL4 and QF transactivators, GAL80 (Figure 1B) and QS respectively (Venken et al., 2011). Promoter-driven flip recombinases (FLP) can also be used to further restrict transgene expression patterns in a mechanism similar to that of the cre-LoxP system in mice. The recombinase acts on FLP recombination targets (FRTs) to excise via recombination the genomic portion located between them (Golic and Lindquist, 1989). The FLP-FRT system can be used either to express the transgene in the FLP expressing cells by removing an FRT-flanked stop-cassette from between the UAS site and the transgene (flip-in) or to prevent


A
Promoter GAL4 line
X
UAS-effector line
gene/tissue specific
Promoter
GAL4
GAL4
UAS
Effector gene
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UAS
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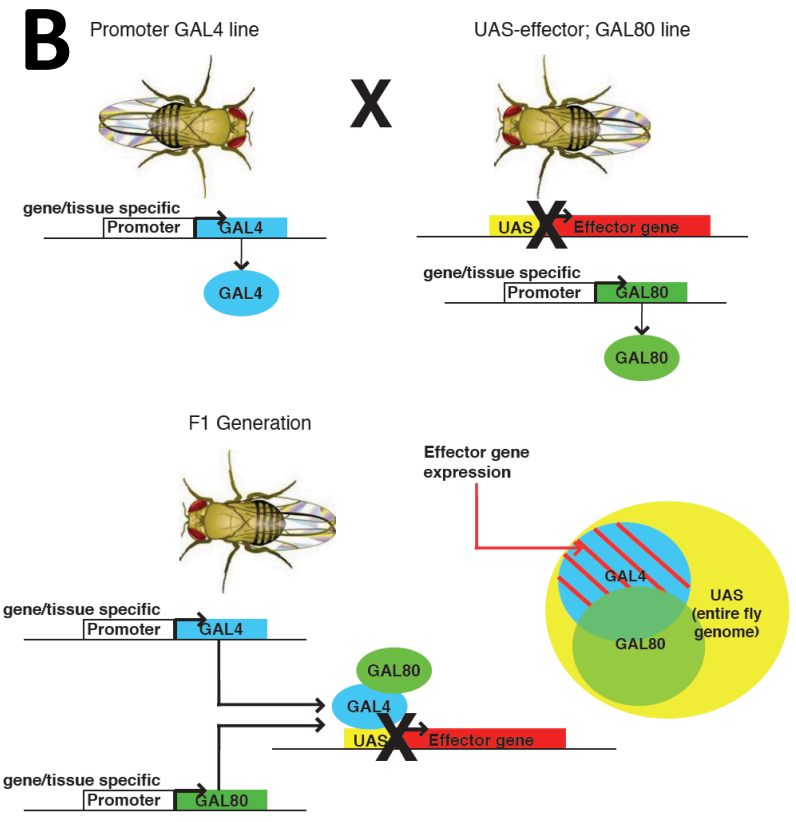
B
Promoter GAL4 line
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Promoter
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UAS
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GAL80
F1 Generation
Effector gene
expression
GAL4
UAS
(entire fly
genome)
GAL80
gene/tissue specific
Promoter
GAL4
GAL80
GAL4
UAS
Effector gene
gene/tissue specific
Promoter
GAL80

Figure 1. Mode of action of UAS/GAL4 binary system

Crossing scheme and mode of action of the (A) UAS/GAL4 binary system and (B) UAS/GAL4/GAL80 system.

expression of the transgene in these cells when the FRT sites flank the transgene sequence itself (flip-out).

This vast array of genetic tools, combined to a full genome RNAi library and UAS-transgene combinations allowing for labelling, activation, hyperpolarization and silencing of neurons as well as knock-down or knock-in of proteins makes Drosophila an ideal model in which to study neural circuits and their fundamental principles. However, strong behavioural paradigms are required in order to do so.

Shorter and Longer-Mating Duration

Among the behaviours studied in Drosophila from a neural circuit perspective, two provide a model of interval-timing and decision-making neurons at once. ‘Shorter-Mating-Duration’ (SMD) and ‘Longer-Mating-Duration’ (LMD) are male specific behaviours dependant on social context (Figure 2).

SMD is the shortening of mating duration that occurs when group-reared flies are exposed to females. SMD relies on gustatory sensory inputs. The neural network underlying this behaviour involves short Neuropeptide F (sNPF) signalling and the mushroom body, as well as the *clock* and *cycle* components of the circadian clock (Kim et al., 2016).

LMD is a rival-mediated response exhibited by group-reared males in the form of a lengthened mating duration (Kim et al., 2012). The sensory input necessary to drive LMD is visual (Kim et al., 2012). Its neural network includes a feedback loop between Pigment Dispersing Factor (PDF) and Neuropeptide F

(NPF) producing neurons as well as the *period* and *timeless* clock components. The ellipsoid body is also required for learning and memory consolidation (Kim et al., 2012, 2013).

These pieces of the neural networks necessary for LMD and SMD are specific to each behaviour. LMD and SMD therefore rely on different neural networks. However, certain behaviours may share parts of the same neural circuitry. The intersecting neurons involved can have important decision-making roles, determining which behaviour will occur. Because LMD and SMD are highly related behaviours, we wondered if their neural circuits might intersect. Previous unpublished work showed that both behaviours require SIFamide (SIFa) signalling (Figure 3C and F). As the only currently known point of intersection between the neural circuits of LMD and SMD, SIFa producing neurons could be capable of discriminating between inputs to direct males towards either behaviour depending on social context. They are therefore ideal decision-making candidates.

As models of interval timing, LMD and SMD seem to corroborate the argument for circadian modulation of the molecular interval clock. Previous results have shown that LMD is disrupted in *per*, *tim*, *dbt* and *cry* mutants, while *clk* and *cyc* directly influence SMD (Kim et al., 2012, 2016). As the molecular mechanism underlying interval timing is still unknown, it is difficult to predict how many components are required for its function. Since it is a clock, we can predict that it may require similar basic components to the circadian clock. Though interval timing has also been associated with an exponential decay mechanism,

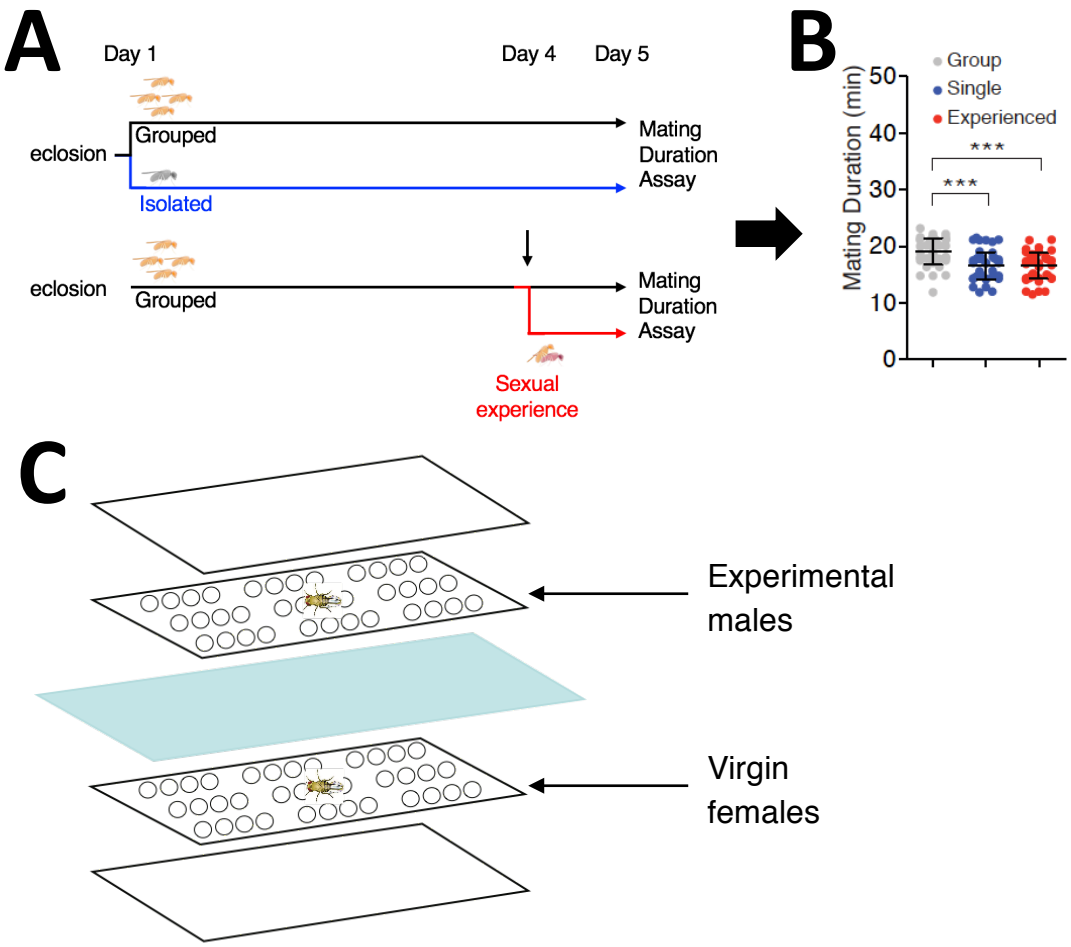


Figure 2. Behavioural chamber, paradigms and analysis of LMD and SMD
(A) Schematic representation of the timeline of the LMD and SMD behavioural paradigms and (B) graphic representation of normal LMD (grey v. blue) and SMD (grey v. red) behaviours. Represented in (C) is a schematic of a mating chamber.

this type of clock would be exposed by changes in temperature. As both LMD and SMD are visible at temperatures of 20-29°C, this timing mechanism was discarded as a possible explanation for interval timing of these behaviours. We therefore presumed that the mechanism regulating interval timing of LMD and SMD has the same basic structure as the circadian clock and specifically, requires a central oscillator. The role of SIFa signalling in regulating timing of LMD and SMD is still unknown. However, the oscillators required for LMD and SMD can either be located before, after, or within the SIFa neurons. If interval timing relies on a mechanism that differs from the circadian oscillator, locating its molecular clock at the intersection of these neural circuits would allow for a single mechanism to regulate two behaviours.

SIFamide and SIFamide Receptor

SIFa is a neuropeptide produced by four neurons of the *pars intercerebralis*, the Drosophila equivalent of the hypothalamus. The *SIFa* gene coding for this 72 amino acid (aa) peptide is located on the right arm of the second chromosome (2R) of the fly's genome. SIFa specifically binds to its receptor, the SIFa receptor (SIFaR). SIFaR is a 758 aa protein encoded by the *SIFaR* gene, located on 3R of the genome. SIFaR is a class A GPCR, first characterized in 2006 by Jørgensen *et al.* (Jørgensen et al., 2006). In Drosophila, the SIFa-SIFaR signalling network is involved in promoting sleep, the modulation of sexual behaviour and courtship, and circadian outputs for rest:activity rhythms (Cavanaugh et al., 2014; Park et al., 2014; Sellami and Veenstra, 2015; Terhzaz et al., 2007).

SIFamide is a neuropeptide. As such, it can act as a neurotransmitter secreted from axon terminals following an action potential, or as a neurohormone secreted from the cell into extracellular space (or the blood stream). This property complicates the study of SIFa as it can be involved in signalling independently of cell activation via different pathways.

Based on the preliminary results already reported, SIFa neurons and SIFa signalling present a highly interesting model for the study of neural networks and their fundamental properties. A pan-neuronal knock-down of SIFa induces a loss of both LMD and SMD, suggesting that the neuropeptide is required for both behaviours (Figure 3C). However, constitutively hyperpolarizing SIFa neurons induces a loss of LMD but not SMD (Figure 3D) while a constitutive depolarization of the same neurons reverses this phenotype by affecting SMD but not LMD (Figure 3E). These results indicate that neurons must be depolarized for the induction of LMD and hyperpolarized in order to induce SMD. Furthermore, preventing exocytosis in SIFa neurons through the use of the tetanus toxin light chain (*UAS-TNT.G*) induces a loss of both mating duration behaviours (Figure 3F). This result suggests that SIFa neurons are capable of secreting SIFa whether they are depolarized or hyperpolarized.

Taken together, these results indicate that SIFa neurons may act to determine which behaviour will take place by integrating received inputs, inhibitory in the context of SMD and excitatory for LMD, and directing downstream signalling in accordance.

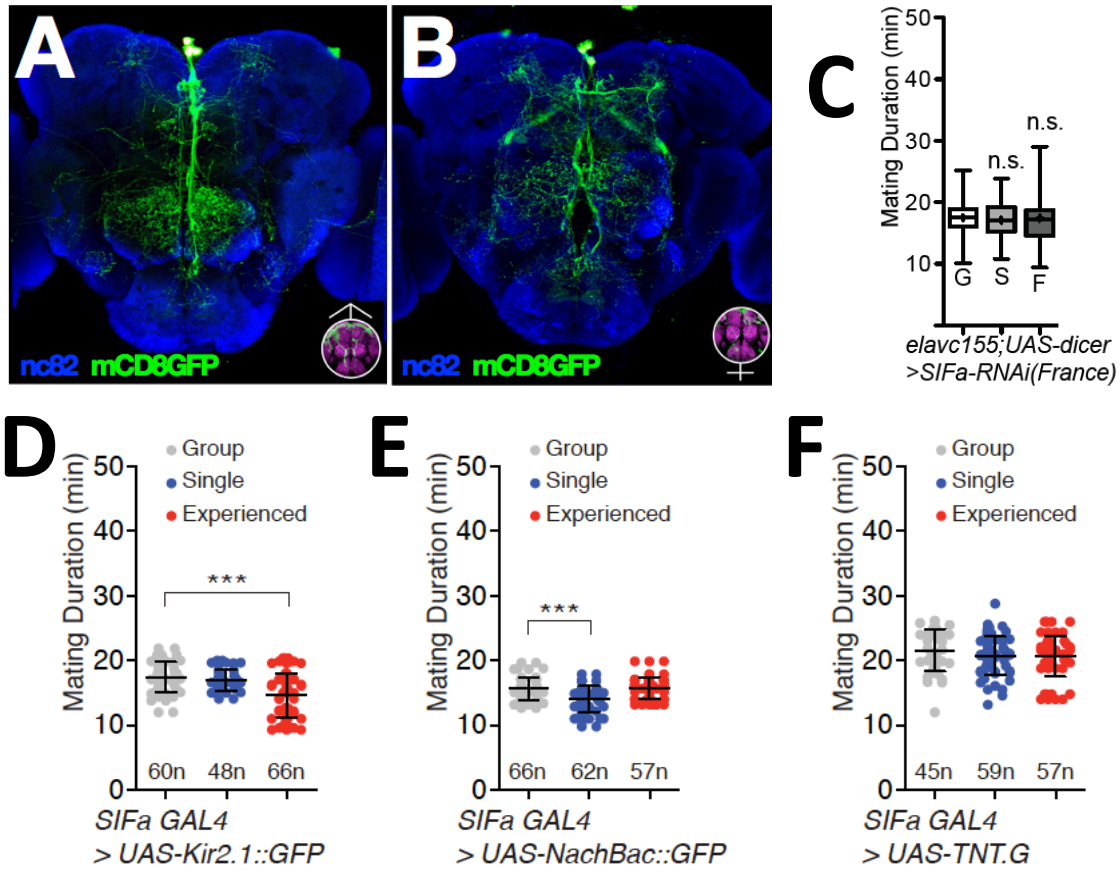


Figure 3. SIFa signalling is necessary for both LMD and SMD.

SIFa producing neurons of the *pars intercerebralis* labelled in the (A) male (left) and (B) female (right) brain with *CD4tdGFP* and *RedStinger*, presynaptic active zones labelled with *nc82* (C) Mating duration assay of elav^{c155};*UAS-dicer*;*UAS-SIFa*-RNAi males (D) Mating duration assay of *SIFa* hyperpolarization experiment (E) Mating duration assay of *SIFa* activation experiment (F) Mating duration assay for inhibition of exocytosis in *SIFa* neurons. G, group-reared; S, single-reared: F, sexually experienced. Asterisks represent significant differences (*** $p<0.0001$) obtained by One Way Anova Kruskall-Wallis test with a Dunn's column comparison post-test. (Woo Jae Kim unpublished data)

SIFa neurons and their signalling therefore define an intersection between the LMD and SMD neural circuits. These neurons stray from the classical definition of command neurons provided by Kupfermann and Weiss as they are involved in not one but two, mutually exclusive behaviours. SIFa neurons represent a hub functioning to integrate and differentiate converging signals in order to redistribute outputs driving different behaviours. They represent a more complex level of computation than command neurons. Based on these preliminary observations, we defined SIFa neurons in the context of LMD and SMD as 'putative decision-making neurons', due to their potential ability to differentiate two behaviours and direct downstream signalling consequently (Figure 5A). How they do so remains to be seen.

A strong argument can also be made for the involvement of SIFa-SIFaR signalling in driving the interval clock of LMD and SMD. If interval timing relies on a central oscillator, the ones required for LMD and SMD may be located before, after or within SIFa neurons. If the respective oscillators of SMD and LMD are located upstream of SIFa neurons, these must differentiate between the two distinct inputs in order to direct signalling to the correct downstream neurons (Figure 4A). Another possibility is that SIFa neurons themselves, encode time-keeping for these behaviours. In this case, inputs received from LMD or SMD neural circuits would be integrated directly to produce the correct chronology (Figure 4B). Finally, it is possible that time-keeping for each behaviour is encoded downstream of the SIFa producing neurons (Figure 4C). SIFa receptor-expressing neurons could then be responsible for such computations. Finally, it is

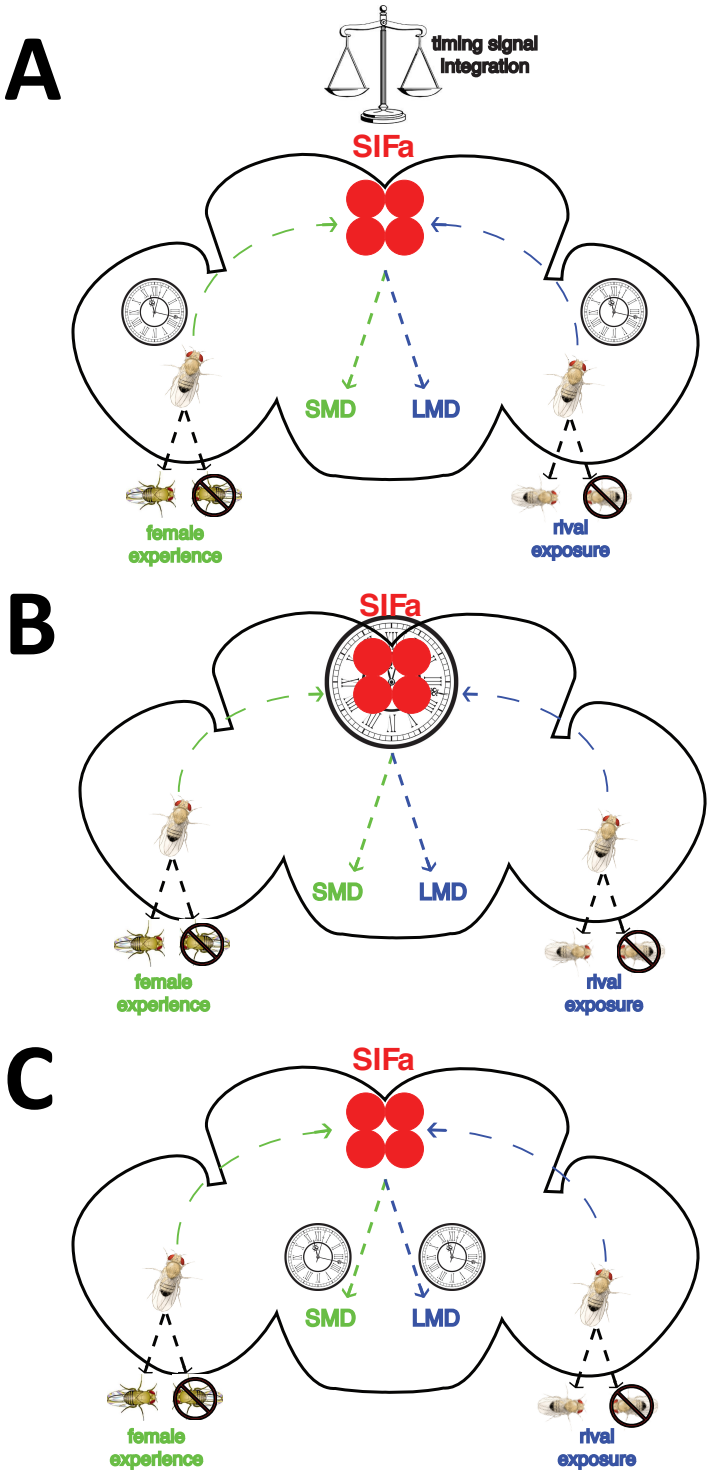


Figure 4. Schematic representation of the core molecular interval timing clock mechanism in LMD and SMD
It may be located (A) upstream of SIFa neurons, (B) within these neurons or (C) downstream of SIFa neurons.

possible that interval timing relies on a different mechanism from the circadian clock. SIFa neurons still constitute ideal candidates to play a major role in the computation of time. As they are located at the intersection of the LMD and SMD neural circuits, they represent a hub of information processing. LMD and SMD neural networks would be more computationally efficient if they relied on a single molecular clock to generate both of their rhythms. Regardless of the scenario, there is a strong possibility that SIFa signalling is involved in the time-keeping of both behaviours. While the role and expression of circadian clock genes in SIFa neurons have not been elucidated, these neurons have already been implicated in the regulation of sleep time and rest: activity rhythms (Cavanaugh et al., 2014). Identifying output pathways required for each behaviour should shed further light on the neural circuits required for both LMD and SMD.

Sexual Dimorphism

Sexual dimorphism is characterized by differences in appearance between males and females of the same species. These differences can be seen at the level of proteins, cells, organisms and behaviours among other things and result from the inheritance of sex-specific patterns in the genetic material. Such differences may be particularly important in the context of sex-specific behaviours. Because LMD and SMD are male specific behaviours, sexual dimorphism could play an integral role in determining the SIFaR+ neuronal populations responsible for each. In addition, SIFa neurons themselves display a high level of sexual dimorphism which may be required for accurate signalling to

SIFaR+ neurons as they could be the result of sex-specific signalling pathways (Figure 3A and B).

The sex-determining pathway of *Drosophila melanogaster* involves the expression of *Sex-lethal* (*Sxl*) and *transformer/transformer2* (*tra/tra2*), which leads to the repression of *fruitless* (*fru*) and the expression of *doublesexF* (dsx^F) in females. In males however, *Sxl* is not expressed, which leads to the alternative splicing of *tra* into a non-protein forming mRNA and the expression of *fru* and dsx^M (Salz and Erickson, 2010). These sex-specific differences in protein expression can result in the 'masculinization' or 'feminization' of cells leading to the emergence of sex-specific functions or signalling networks (Pavlou et al., 2016). Neurons expressing the male-specific genes *fru* and dsx^M may therefore be specifically required for LMD and SMD.

Hypothesis and Objectives

Based on the preliminary data highlighted above and previous literature, we hypothesize that SIFa signalling is involved in the LMD and SMD neural networks via two different output pathways which independently signal to discrete groups of neurons (one for each behaviour) (Figure 5A and B). In addition, we hypothesize that SIFa-SIFaR signalling is involved in the computation of interval timing required for both of these behaviours through the involvement of the core circadian clock genes (Figure 5C). The objective of this thesis is to identify the localization and identity of SIFaR-expressing neurons required for both LMD and SMD and to study the involvement of the core clock components in this signalling pathway, in relation to these behaviours. To study our hypotheses, we will focus on the following objectives:

I. Identify the localization of SIFaR-neurons involved in either SMD or LMD within the CNS. We will focus on knocking down the SIFaR in specific regions of the CNS and behaviourally infer the relevance of these regions for LMD and SMD in the context of SIFa signalling.
II. Determine the identity of the neurons found in aim I. A combination of behaviour studies will be used to determine whether the previously discovered neurons are sexually dimorphic and which neurotransmitter or neuropeptide they produce.
III. Study the involvement of SIFa neurons in the time-keeping required for LMD and SMD. Core circadian clock genes will be knocked down

specifically in the SIFa+ neurons. The behavioural effect on LMD and SMD will be assayed experimentally.

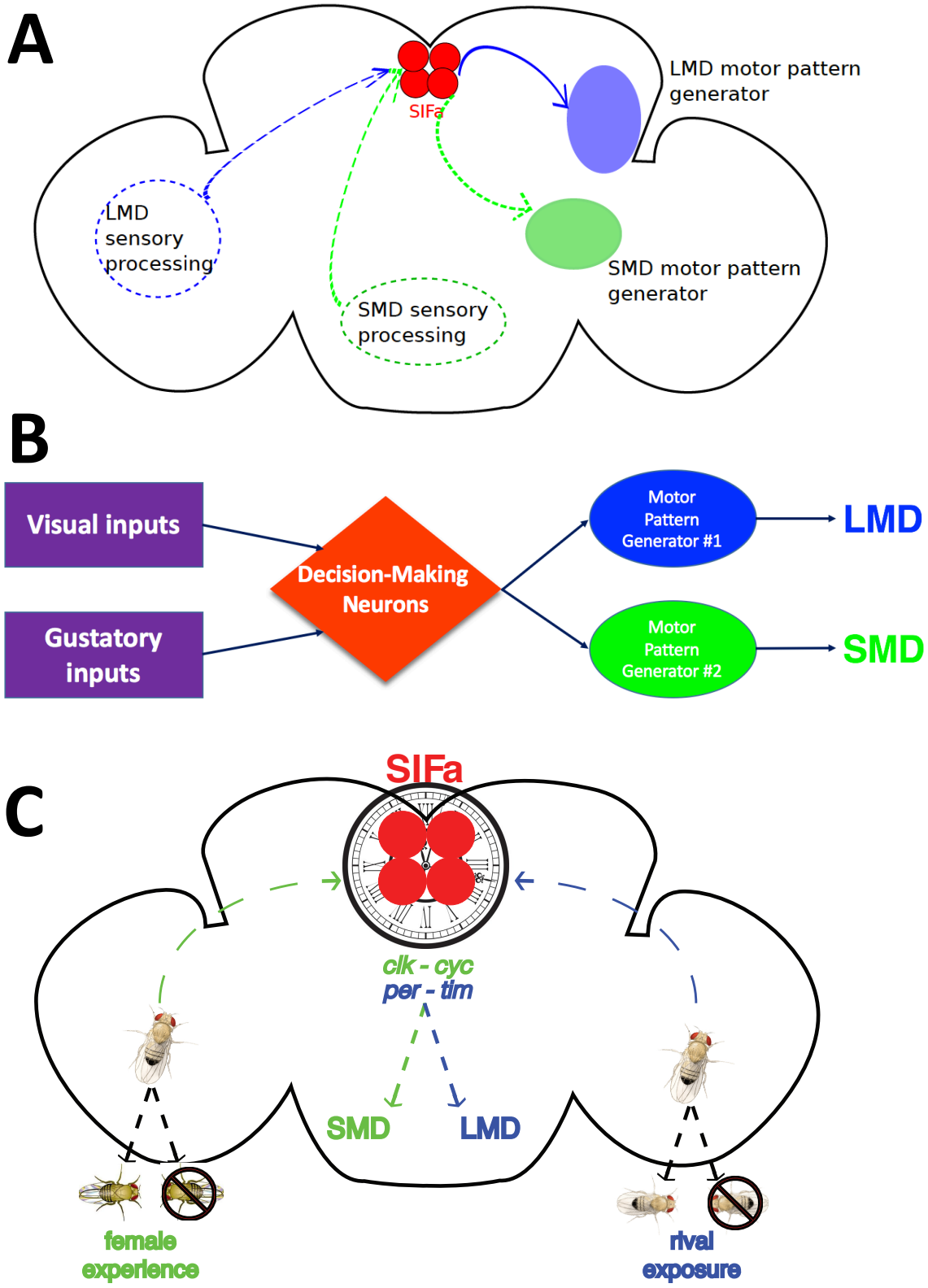


Figure 5. Hypothetical working models of SIFa signalling and interval timing control within LMD and SMD neural circuits.

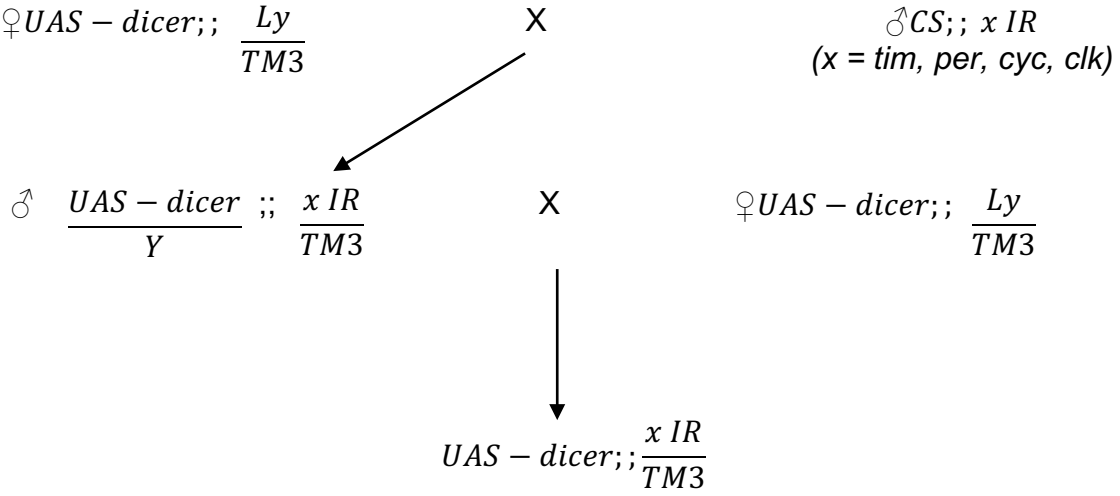
Materials and Methods

Fly Lines

Most GAL4 and RNAi lines were obtained from the Bloomington Stock Center (Indiana), except circadian clock inverse repeat (IR) lines, which were obtained from the Vienna Stock Center. *SIFa GAL4* was provided to our laboratory by the Veenstra group. Their overall type and function are summarized in Table 1.

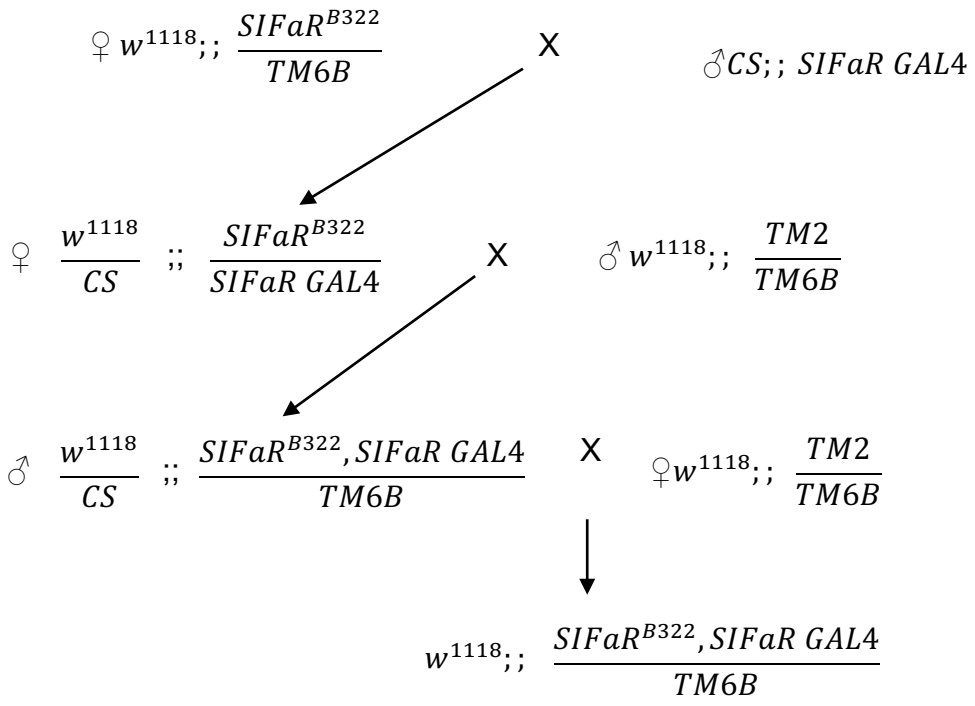
UAS-Dicer containing lines

Lines containing *UAS-dicer* on the X chromosome were generated by back-crossing IR lines to female virgin *UAS-dicer;; Ly/TM3* twice:



Recombinant rescue lines

Lines used for rescue experiments were obtained by crossing recombinant *CS;;SIFaR GAL4,SIFaR^{B322}* males (from each *SIFaR GAL4* line) with *CS; UAS-SIFaR; SIFaR^{B322}* virgin females. The recombinant lines were generated using the following crossing scheme:



Rescue Experiments

Rescue experiments were performed by reintroducing wild type SIFaR in *SIFaR* mutants. The mutant line used contained the *SIFaR*^{B322} allele, a loss of function allele generated using a p-element insertion within the *SIFaR* coding sequence. Because this allele of the *SIFaR* gene is homozygous lethal, rescue experiments consisted of two parts, with lethality rescue preceding the behaviour rescue which excluded all GAL4 drivers that did not rescue lethality. All rescue experiments were performed by driving wild type *SIFaR* expression with one of four *SIFaR GAL4s* at our disposal, in the mutant line (Table 1). Lethality rescue was assessed by comparing the number of homozygous and heterozygous progeny. Behaviour rescue was assessed using the standard experimental paradigms of LMD and SMD (Figure 2).

Table 1. Summary of lines used

Type	Lines Used		Effect
UAS	NachBac	Bacterial Sodium Channel	Induce cell depolarization/activation
	TNT.G	Tetanus toxin light chain	Prevent exocytosis
	Kir 2.1	Potassium Channel	Induce cell hyperpolarization
	RNAi/IR lines	Interference RNA lines	Induce gene-specific knock down
Driver	$elav^{c155}$	Pan-neuronal driver	Drives UAS-transgene expression in neurons
Mutant	$SIFaR^{B322}$	SIFaR mutant generated by Piggybac insertion	Homozygous lethal SIFaR mutant used for rescue
GAL4	various	GAL4 construct under promoter control	Drive expression of UAS-transgene in labelled cells
GAL80	tsh GAL80	GAL80 driven by VNC specific promoter	Inhibit UAS activation by GAL4 in VNC

Fly Food

Males undergoing the behavioural paradigm were reared in vials containing a different food recipe from that used for crosses, and stable lines. Genetic crosses, behavioural crosses and stable lines used for male and virgin collections were kept in vials and bottles containing our standard food recipe:

- 84.31% Water
- 0.95% Agar
- 1.3% Inactive yeast
- 4.72% Corn flour
- 0.61% Soy flour
- 7.1% Molasses
- 0.71% Tegosept solution
- 0.25% Propionic acid
- 0.05% Phosphoric acid

Males undergoing the rearing portion of the behavioural experiments were placed in the 25°C incubator, in vials containing food enriched in soy flour:

- 84.31% Water
- 0.95% Agar
- 1.3% Inactive yeast
- 5.33% Soy flour
- 7.1% Molasses
- 0.71% Tegosept solution
- 0.25% Propionic acid
- 0.05% Phosphoric acid

This soy flour enriched food proved to be more adequate for assays as it remained solid even after five days at 25°C and allowed males to feed for the duration of the behavioural paradigm without the risk of drowning or sticking to the food. All flies were kept in an environment with a 12h:12h light: dark cycle and 55% humidity.

Behavioural assay

Male flies obtained from crosses were collected immediately following eclosion and separated in two conditions and incubated at 25°C: (i) single-reared males where 100 individual males were isolated in each vial and (ii) group-reared males where 200 males were placed by groups of five in each vial (Figure 2A). Four days later, group-reared males (ii) were again separated into two groups of 100 individuals: one group remained unchanged (ii) while the other (iii) was transferred into new vials containing 3X female virgins lacking the sex peptide receptor (Df). All flies were returned to 25°C incubation (Figure 2A). The next day (5), males of each condition were anaesthetized and placed in the behavioural chambers above new female virgins Df but physically separated from them by a plastic film (Figure 2C). Each chamber contained thirty-six (36) pairs of flies (male-female) and two chambers were filled for each condition for a total of 72 pairs each (i-iii). Filled chambers were labelled according to the condition of males contained inside and incubated for 90 minutes at 25°C to recover from anaesthesia. Following recovery, chambers were taken out of the incubator and the plastic film was removed to allow physical contact between the male and female of each pair. The start and end time of mating was then recorded for each pair visually with the aid of a standard timer. When a trend was noticed but no statistically significant differences recorded, the experiment was repeated and results were combined from the two experiments to ensure statistical accuracy of the results.

Statistical analysis

The mating start and end times from each male-female pair was entered into Microsoft Excel following each assay to generate the mating duration by subtracting the start from the end time. The lists of mating durations generated for each rearing condition of the males were then transcribed to Prism where the data was analysed using a one-way analysis of variance (One-way ANOVA). As we did not analyse the data with an assumption of normality, the non-parametric Kruskall-Wallis test was used. The Dunn's multiple comparison post hoc test was used to obtain p values comparing each pair of data sets because each one contained a different n number (Figure 2B)**.** Figures were made in Adobe Illustrator using the graphs generated in Prism and saved as .pdf files for subsequent use.

Results

Elucidating the basic principles regulating neural network activity and characteristic neuronal signalling mechanisms is an essential task of neuroscience. Our behavioural models of LMD and SMD in *Drosophila melanogaster* present a unique opportunity to study a potentially novel neuronal signalling mechanism. Preliminary results have suggested that SIFa signalling participates in both behaviours in a discrete capacity. The goal of this thesis is to determine which populations of SIFaR-expressing neurons are involved in each behaviour downstream of SIFa neurons and to propose a mechanism by which these two distinct populations of neurons could be receiving SIFa inputs.

1. SIFaR+ neurons are necessary and sufficient for LMD and SMD

To validate that SIFa signalling required for LMD/SMD was directed at SIFaR-expressing neurons, pan-neuronal and SIFaR drivers were used to mediate an RNAi driven knock-down (KD).

Identification of a functional SIFaR RNAi line

To begin analysing the role of SIFaR+ neurons in LMD and SMD, we screened all available Bloomington SIFaR RNAi lines (BL44068, BL34947 and BL25831) using the pan-neuronal driver $elav^{c155}$. Each of these lines codes for a double stranded RNA (dsRNA) molecule of varying lengths and covers a different region of the SIFaR gene (Figure 6). In order to enhance the RNAi (interference RNA) efficiency, a copy of the *Dicer* gene was also placed under GAL4 control (*UAS-Dicer*). Results indicate that only pan-neuronal KDs with two of the three RNAi

lines successfully recapitulate the lack of LMD and SMD seen in SIFa knock-downs (Figure 7A and C).

Analysis of the data shows that knock-downs induced by both BL34947 (Figure 7A) and BL25831 (Figure 7C) caused flies of all social conditions (single-reared, group-reared and sexually experienced) to mate for statistically similar times. This indicates a disruption of LMD and SMD induced by SIFaR KD. Analysis of the data obtained from the BL44068 mediated KD (Figure 7B) showed that group-reared, naïve flies mated significantly longer than single-reared flies, while sexually experienced, group-reared flies mated for a significantly shorter amount of time than their naïve counterparts. These results show normal LMD and SMD (Figure 7B). We concluded that BL25831 and BL34947 *SIFaR RNAi* are sufficient to induce a KD of SIFaR recapitulating the results of the SIFa knock-down, while BL44068 *SIFaR RNAi* is not.

Further studies of the two remaining RNAi lines of interest revealed that BL25831 flies seemed less healthy than the flies from the BL34947 line. In addition, we were unable to make a stable homozygous line of flies carrying this RNAi because the flies were either too sick to reproduce properly or sterile. As a result, we focused our attention on BL34947 for future SIFaR knock-down experiments.

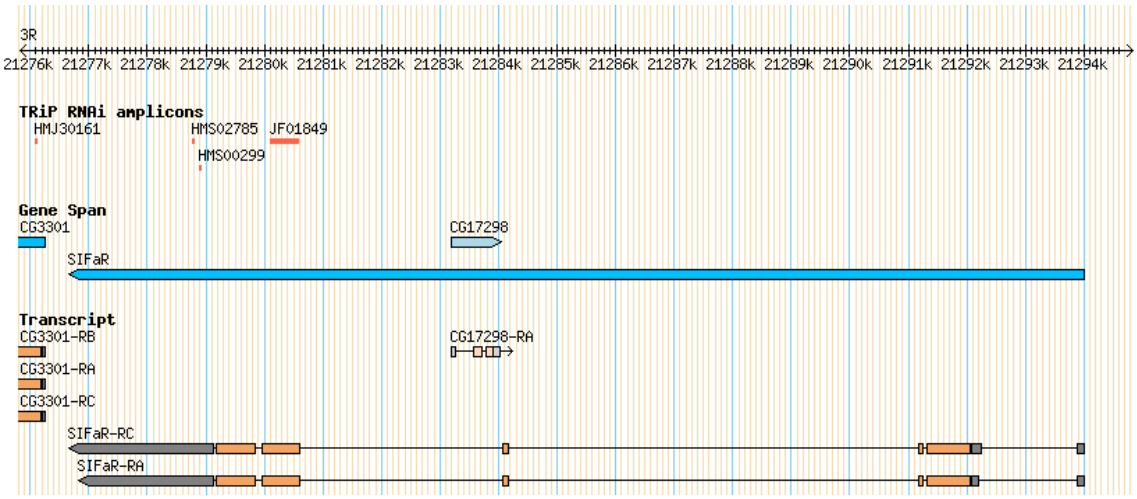


Figure 6. SIFaR RNAi alignment.
The figure illustrates the GBrowse (http://flybase.org) alignment of the three RNAi used with the complete region of the *SIFaR* genomic sequence and the SIFaR transcripts. dsRNA-HMS02785 is the RNA used to generate fly line BL44068, dsRNA-HMS00299 was used to generate line BL34947 and dsRNA-JS01849, BL25831.

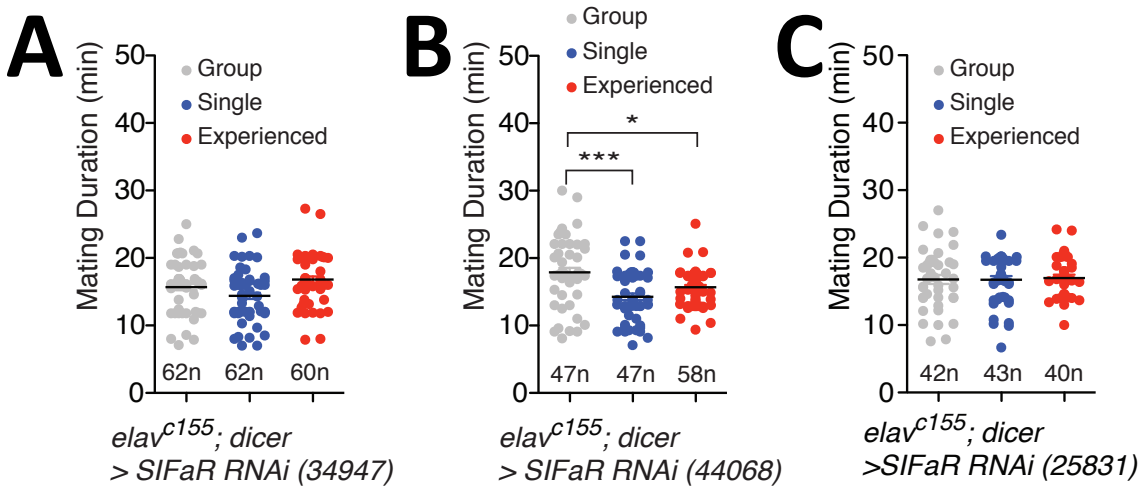


Figure 7. Analysis of various SIFaR RNAi efficiency.
Mating duration assay results obtained from a pan-neuronal knock-down of SIFaR through the use of different available Bloomington RNAi lines (A) UAS-SIFaR BL34947 (B) UAS-SIFaR BL44068 (C) UAS-SIFaR BL25831. G, group-reared; S, single-reared: F, sexually experienced. Data was analyzed with a 1-Way Anova Kruskall-Wallis test with a Dunn's column comparison post-test. * indicates statistical significance at $p<0.01$ and *** indicates statistical significance at $p<0.0001$.

Identification of SIFaR+ neurons necessary and sufficient for LMD and SMD

To narrow down the population of SIFaR+ neurons necessary for mating duration behaviours, available *SIFaR-GAL4* lines were screened via RNAi mediated SIFaR knock-down. Because each *GAL4* line is created using a different 200bp portion of the *SIFaR* promoter, each of the four lines screened labels different, partially overlapping populations of SIFaR+ neurons (Supplemental figure 1 A-D). Results of the KDs showed that each of the four *GAL4* lines screened contained neurons necessary for both LMD and SMD. In the case of *SIFaR GAL4* lines BL49041, BL49061 and BL46391, group-reared, single-reared and sexually experienced males all mated for statistically similar times (Figure 8A, B and D and Supplemental figure 1A, B and D). A SIFaR KD in the neurons labelled by *SIFaR GAL4* BL49087 showed a complete reversal of the mating duration lengths observed during normal LMD and SMD (Figure 8C and Supplemental figure 1D). Single-reared naïve males mated significantly longer than the group-reared naïve males who also mated for a significantly shorter amount of time than their sexually experienced counterparts (Figure 8C).

Taken together, these results show that SIFaR+ neurons are necessary for both LMD and SMD.

We further studied the SIFaR+ population of neurons to find the cells sufficient for LMD and SMD. We performed rescue experiments by reintroducing wild type SIFaR in *SIFaR* mutants. The mutant line used contained the loss of function allele $SIFaR^{B322}$. We first attempted to rescue lethality before moving on to rescue behaviour excluding *GAL4* drivers that did not rescue lethality (Table 2

and Figure 9). Results showed that rescue attempts using *GAL4* drivers *49087* and *49061* produced viable homozygous progeny while drivers *49041* and *46391* did not (Table 1). This data indicates that neuronal populations labelled by *SIFaR GAL4s 49061* and *49087* contain the SIFaR expressing cells sufficient to rescue lethality. Neurons labelled by *SIFaR GAL4s 49041* and *46391* are not.

Behavioural rescue experiments were then performed to identify the population of SIFaR+ neurons sufficient for LMD and SMD. Results showed that group-reared, naïve males mated for a significantly longer period of time than single-reared and sexually experienced group-reared males when endogenous SIFaR was reintroduced in the neuronal population labelled by *SIFaR GAL4 49087* (Figure 9B). No mating was observed when endogenous SIFaR was reintroduced in the cells labelled by *SIFaR GAL4 49061* (Figure 9A). This data indicates that the neuronal population labelled by *SIFaR GAL4 49087* contains cells sufficient for viability as well as LMD and SMD, while *SIFaR GAL4 49061* labelled cells only include neurons sufficient for viability.

These results show that SIFaR expressing neurons labelled specifically by *SIFaR GAL4 49087* are both necessary and sufficient for LMD and SMD.

Conclusion

We therefore identified a functional *SIFaR RNAi* line and demonstrated that SIFaR neurons labelled by *SIFaR GAL4 49087* are necessary and sufficient for both LMD and SMD.

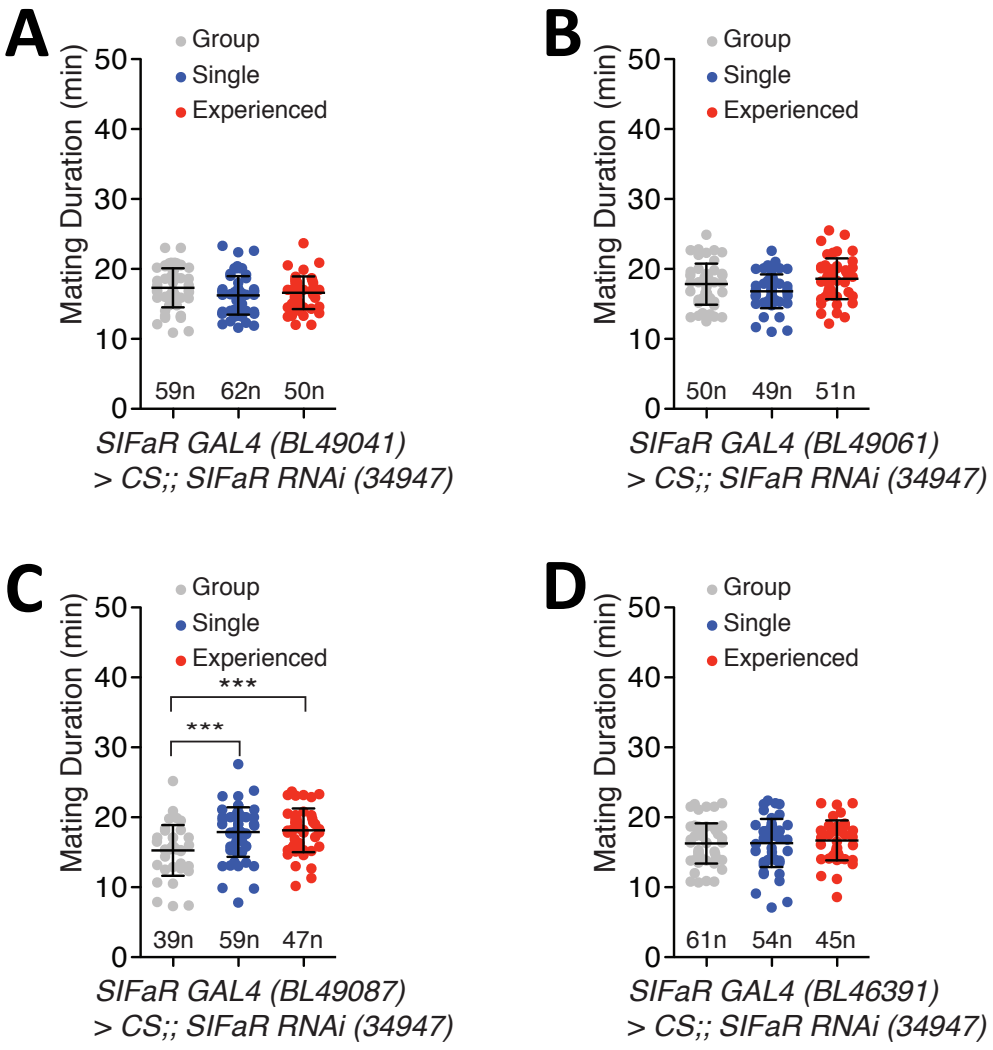


Figure 8. SIFaR expressing neurons are necessary for LMD and SMD.
Mating duration assay results obtained from a knock-down of SIFaR in neuron patterns labelled by various *SIFaR GAL4* lines (A) *SIFaR GAL4* BL49041 (B) *SIFaR GAL4* BL49061 (C) *SIFaR GAL4* BL49087 (D) *SIFaR GAL4* BL46391. G, group-reared; S, single-reared: F, sexually experienced. Data was analyzed with a 1-Way Anova Kruskall-Wallis test with a Dunn's column comparison post-test. *** symbol indicates statistical significance at $p<0.0001$.

Table 2. Rescue of *SIFaR*^{B322} mutant lethality with multiple *SIFaR GAL4* lines.

GAL4 Line Tested	# of males	# of Females	# of 6B	# of non-6B**
49041 #1*	18	17	35	0
49041 #2	20	15	35	0
49061 #2	18	17	19	16
49061 #3	13	22	14	21
49087 #2	16	17	16	19
49087 #3	12	23	17	18
46391 #1	148	186	334	0

* Multiple recombinant *SIFaR GAL4, SIFaR*^{B322} lines were tested for *49041 GAL4*, *49061 GAL4* and *49087 GAL4*.

** Presence of non-6B flies indicates rescue of the lethality phenotype.
Data produced by Alexander Kwan (UROP student)

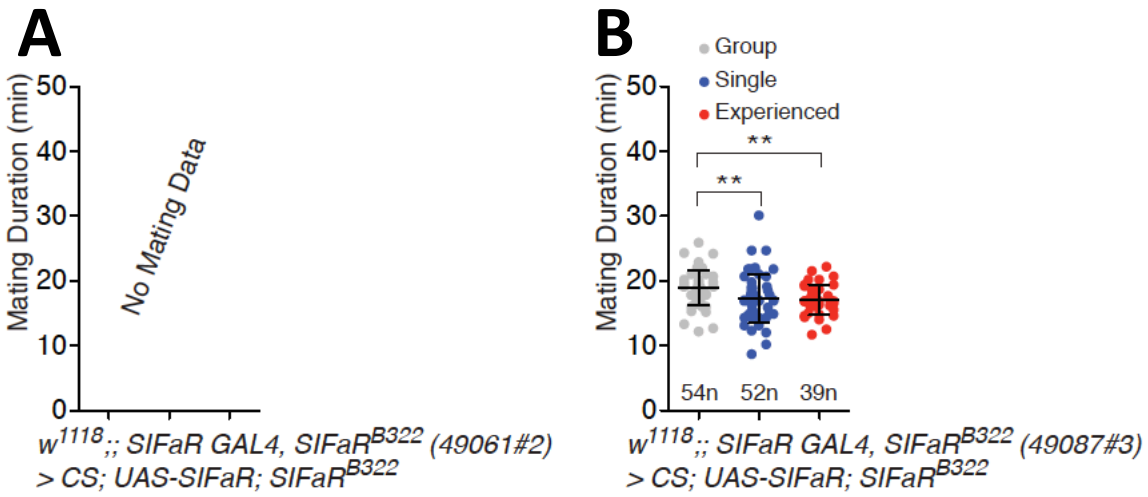


Figure 9. A single SIFaR driver line contains the neurons sufficient for LMD and SMD.

Mating behaviour rescue assay performed with *SIFaR GAL4* lines capable of rescuing lethality (A) *SIFaR GAL4* (49061), $SIFaR^{B322}$ (B) *SIFaR GAL4* (49087), $SIFaR^{B322}$. Data was analyzed with a 1-Way Anova Kruskall-Wallis test with a Dunn's column comparison post-test. ** indicates statistical significance at $p<0.004$. Data produced by Alexander Kwan (UROP student)

2. Localization of SIFaR+ neurons involved in LMD and SMD

Once the neuronal population sufficient for LMD and SMD was narrowed to a single *GAL4* expression pattern, intersectional experiments were performed to identify their anatomical localization. The *Drosophila* CNS is comprised of the brain and the VNC. To determine in which of these regions SIFaR+ target neurons were located, a partial neuronal SIFaR KD was performed to exclude neurons of the VNC. We used the VNC specific promoter *teashirt* (*tsh*) to drive *GAL80* expression and restrict *RNAi* activity to the brain (Figure 10B).

Results showed that these knock down mutants exhibited normal LMD and SMD (Figure 10B). Mating duration was shorter for single-reared and sexually experienced males than it was for group-reared males (Figure 10B). In contrast a pan-neuronal, $elav^{c155}$ mediated KD of SIFaR resulted in similar mating durations for all social conditions (Figure 10A).

This data indicates that Drosophila neurons outside of the VNC are not necessary for LMD and SMD.

Conclusion

We therefore show with this data that SIFaR expressing neurons responsible for both behaviours are located not in the brain, but in the VNC.

3. Characterization of SIFaR+ neurons involved in LMD and SMD

Neural circuits are composed of organized clusters of neurons which communicate with one another through dendritic and axonal projections using

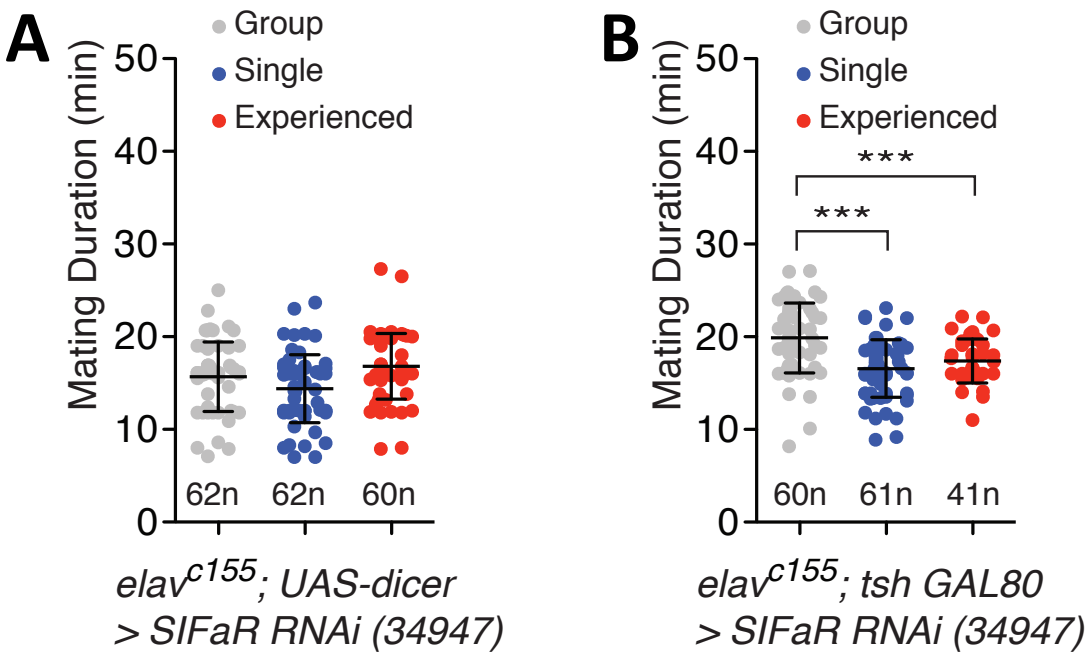


Figure 10. SIFaR expressing neurons necessary for LMD and SMD are located in the VNC.

Mating duration assay results obtained from (A) a pan-neuronal SIFaR knock-down using the driver *elav*^{c155} and (B) a pan-neuronal SIFaR knock-down excluding the VNC, with the combination of the driver *elav*^{c155} and the repressor *tsh GAL80*. Data was analyzed with a 1-Way Anova Kruskall-Wallis test with a Dunn's column comparison post-test. *** indicates statistical significance at $p<0.0001$.

signalling molecules and their specific receptor. In the case of LMD and SMD, these circuits involve sensory neurons, a memory center and several interneurons which transmit information from the primary sensory organs to the genitalia. The behavioural output resulting from the activation of these neural circuits is a highly stereotyped comportment which requires precise and well timed muscle activation. The previous results have already shown that SIFa neurons communicate with downstream neurons and therefore do not directly induce muscle contraction or relaxation (Figure 10). Because the target SIFaR-expressing cells necessary for LMD and SMD are neurons and not muscles, they can be characterized by their own signalling molecule.

Broad neurotransmitter screening of SIFaR+ neurons

The first screen performed in the scope of this investigation concerned neurotransmitters (NTs). NTs are expressed by large, discrete populations of neurons which offer an ideal target for a primary screen. Several NTs are expressed in the *Drosophila* nervous system, most of which are conserved with mammals. The main NTs found in the *Drosophila* CNS are dopamine, octopamine, glutamate, serotonin, GABA and acetylcholine. NT system specific SIFaR knock-down mutants were generated, and mating duration was assayed for LMD and SMD. Results showed that SIFaR+ neurons expressing GABA, dopamine, serotonin, octopamine and acetylcholine were all necessary to induce SMD (Figure 11A, C-F). All showed a disruption of SMD highlighted by the similar mating times of group-reared and sexually experienced males. However, none of the mutant males tested showed any disruption in LMD as single-reared males

mated for significantly shorter intervals than their group-reared counterparts (Figure 11A-F). Unlike other NTs, glutamate showed no involvement for either behaviour as mating duration of group-reared males was significantly longer than that of single-reared and sexually experienced males (Figure 11F).

These results demonstrate that neurons expressing NTs GABA, dopamine, serotonin, octopamine and acetylcholine are necessary for SMD while no NT-expressing neurons are involved in LMD. While we can conclude that SIFaR expressing neurons necessary for LMD do not express NTs, this data does not indicate which type of signalling molecule is required downstream of SIFa neurons for SMD. We can only determine that NTs are expressed by SIFaR+ neurons required for this behaviour, not that these NTs are themselves used by these neurons for SMD signalling.

NTs are expressed by large, discrete populations of neurons. In addition, NT systems do not account for all neurons of the *Drosophila* CNS. While these results show that none of the NT systems are involved in LMD, they do little to narrow down the neurons involved in SMD.

Narrow neuropeptide screening of SIFaR+ neurons

Following the NT screen, we hypothesized that SIFaR+ neurons necessary for LMD were peptidergic and did not express any of the *Drosophila* NTs, since NPs are the other main category of neuronal signalling molecules. Neuropeptides (NPs) are short chains of amino acids secreted by neurons which act as signalling molecules. They can be co-secreted with NTs or other NPs and are expressed in *Drosophila* by small groups of neurons within the CNS.

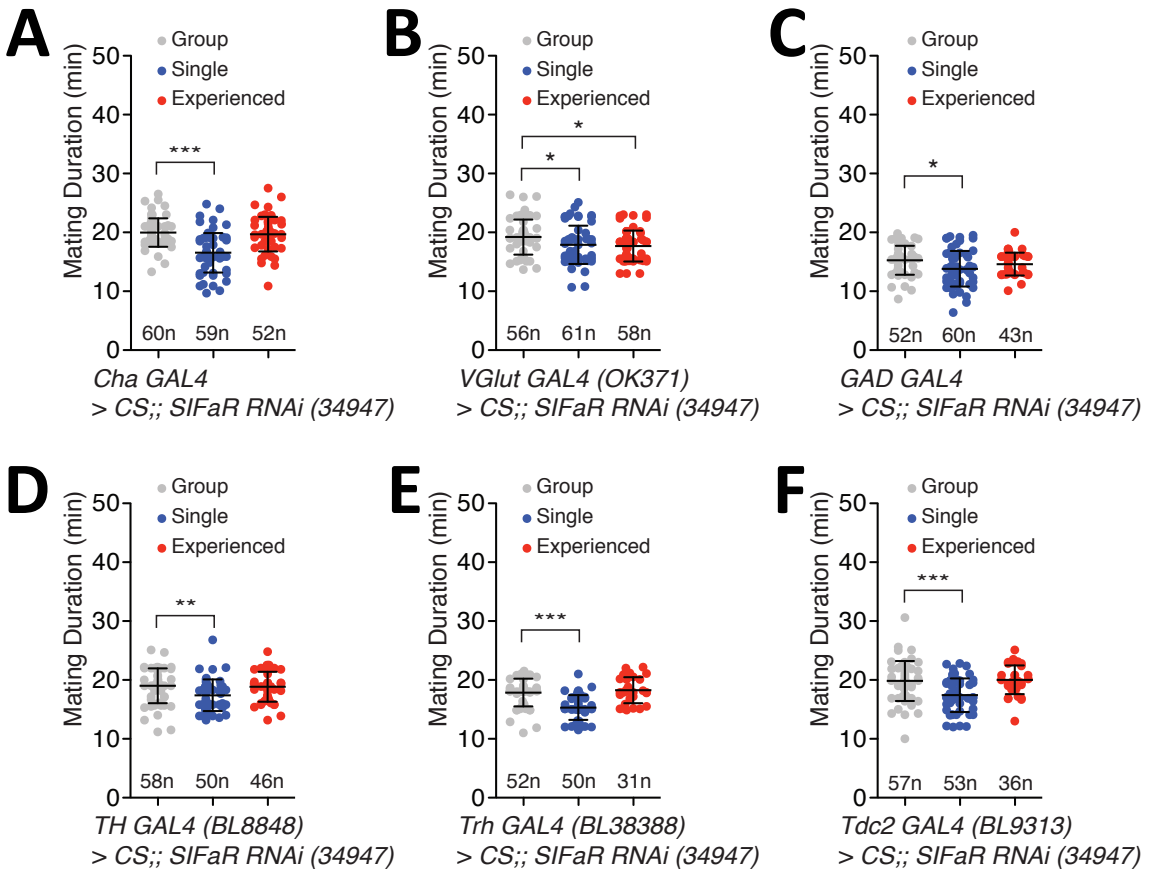


Figure 11. Neurotransmitter systems contain neurons necessary for SMD but not LMD.

SIFaR knock down with SIFaR RNAi (34947) in (A) Cholinergic neurons (Cha GAL4), (B) Glutamatergic neurons (VGlut GAL4), (C) GABAergic neurons (Gad GAL4), (D) Dopaminergic (TH GAL4) and (E) serotonergic (Trh GAL4) neurons, and (F) octopaminergic neurons (Tdc2 GAL4). Data was analyzed with a 1-Way Anova Kruskall-Wallis test with a Dunn's column comparison post-test. * indicates statistical significance at $p<0.01$, ** indicates statistical significance at $p<0.001$ and *** indicates statistical significance at $p<0.0001$.

A second screen was therefore performed to test *NP-GAL4* specific SIFaR KD mutants. Results showed that a SIFaR knock-down in corazonin, eclosion hormone and tachykinin neurons did not affect either behaviour (Figure 12A-C). A knock-down in AstC, leucokinin, DH44 and myoinhibitory peptide expressing neurons abolished SMD but not LMD (Figure 12D-G). Finally, knocking down the SIFaR in bursicon and proctolin expressing neurons caused a loss of both behaviours while a SIFaR KD in myosuppressin expressing neurons was responsible for the loss of LMD but not SMD (Figure 12H, I and K). The transcription factor DIMMED was also tested (Figure 12J). DIMMED is a transcription factor expressed almost exclusively by most Drosophila peptidergic cells (Park et al., 2008). Results showed that knocking down the SIFaR in *DIMM* labelled neurons caused a loss of SMD but not LMD (Figure 12J).

Taken together, these results indicate that SIFaR target neurons necessary for SMD include neurons expressing every NT except glutamate, as well as most NP and *DIMM+* neurons. On the contrary, target neurons necessary for LMD only include neurons expressing proctolin, bursicon and myosuppressin which are *DIMM-*.

Conclusion

We showed that SIFa signalling required for SMD is mediated by non-glutamatergic NT expressing neurons and most neuropeptidergic neurons while LMD relies on SIFa signalling to bursicon, proctolin and myosuppressin expressing neurons only. The SIFaR expressing neurons receiving inputs for both behaviours are therefore separated in two distinct populations.

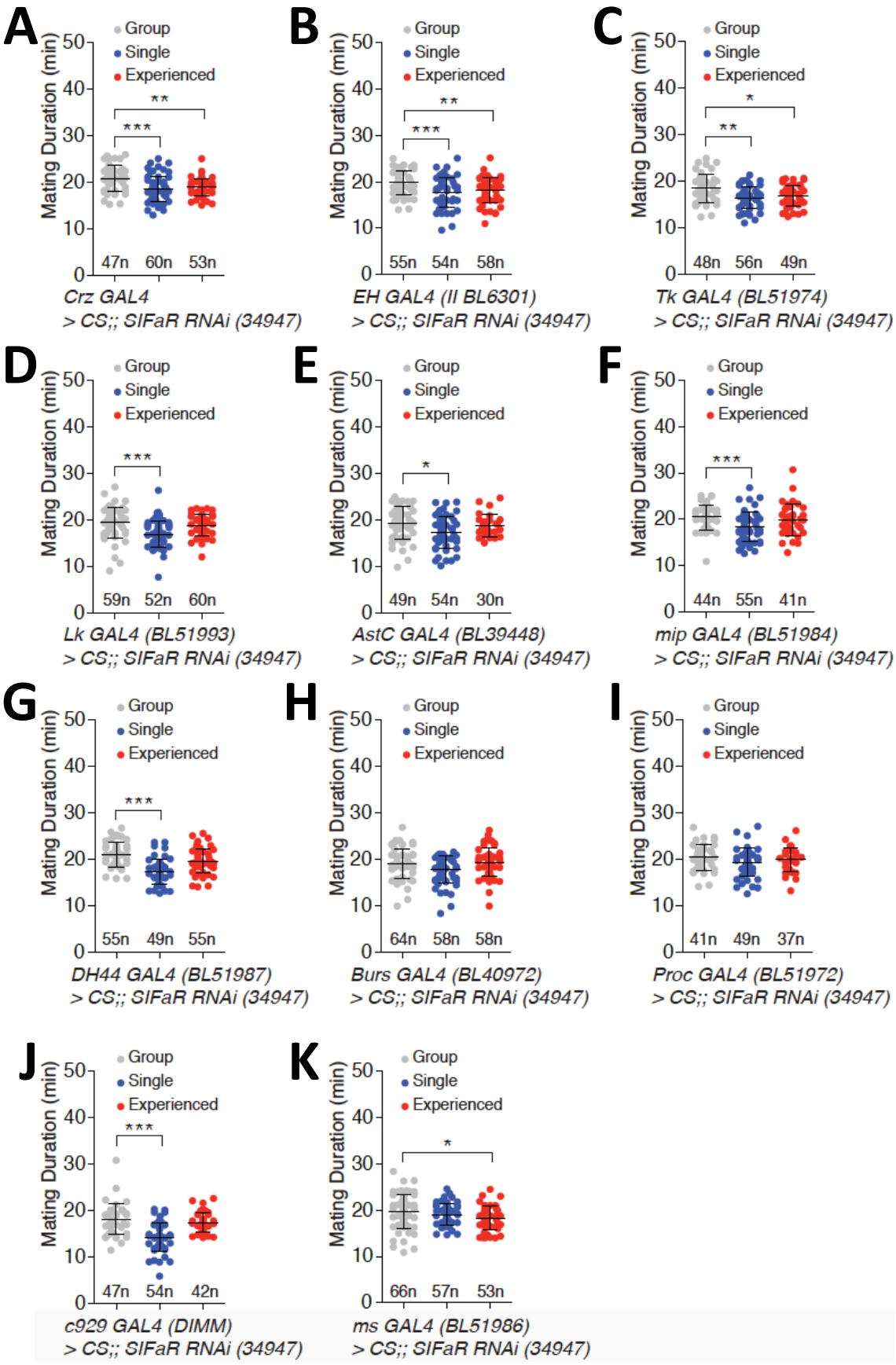

A
B
C
D
E
F
G
H
I
J
K
Group
Single
Experienced
Mating Duration (min)
47n 60n 53n
Crz GAL4
> CS;; SIFaR RNAi (34947)
55n 54n 58n
EH GAL4 (II BL6301)
> CS;; SIFaR RNAi (34947)
48n 56n 49n
Tk GAL4 (BL51974)
> CS;; SIFaR RNAi (34947)
59n 52n 60n
Lk GAL4 (BL51993)
> CS;; SIFaR RNAi (34947)
49n 54n 30n
AstC GAL4 (BL39448)
> CS;; SIFaR RNAi (34947)
44n 55n 41n
mip GAL4 (BL51984)
> CS;; SIFaR RNAi (34947)
55n 49n 55n
DH44 GAL4 (BL51987)
> CS;; SIFaR RNAi (34947)
64n 58n 58n
Burs GAL4 (BL40972)
> CS;; SIFaR RNAi (34947)
41n 49n 37n
Proc GAL4 (BL51972)
> CS;; SIFaR RNAi (34947)
47n 54n 42n
c929 GAL4 (DIMM)
> CS;; SIFaR RNAi (34947)
66n 57n 53n
ms GAL4 (BL51986)
> CS;; SIFaR RNAi (34947)

Figure 12. Behavioural impact of neuropeptide specific SIFaR knock-down

SIFaR knock down with SIFaR RNAi (34947) in (A) Corazonin+ neurons (*crz GAL4*), (B) Eclosion hormone+ neurons (*EH GAL4*), (C) Tachykinin+ neurons (*Tk GAL4*), (D) Leucokinin (*Lk GAL4*), (E) Allatostatin C+ (*AstC GAL4*) neurons, (F) myoinhibitory peptide+ neurons (*mip GAL4*), (G) Diuretic hormone 44+ (*DH44 GAL4*) neurons, (H) Bursicon+ (*burs GAL4*) neurons, (I) Proctolin+ (*proc GAL4*) neurons, (J) *Dimmed*+ (*c929 GAL4*) neurons and (K) Myosuppressin+ (*ms GAL4*) neurons. Data was analyzed with a 1-Way Anova Kruskall-Wallis test with a Dunn's column comparison post-test. * indicates statistical significance at $p<0.01$ ** indicates statistical significance at $p<0.001$ and *** indicates statistical significance at $p<0.0001$.

4. Investigation of *NP5270 GAL4*-labelled neurons in LMD and SMD

In addition to our study of signalling molecule based neuronal populations, we searched the literature for potentially relevant, mating duration related research. We found that Crickmore and Vosshall had previously described part of a neuronal circuit involved in the persistence of male copulation (Crickmore and Vosshall, 2013). Part of the neural circuit involved is labelled by *NP5270 GAL4*, which contains neurons that increase mating duration nine-fold. Based on their influence on mating duration, we hypothesized that these neurons may be critical for LMD and SMD. We reproduced the original lengthening of mating data induced by the silencing of *NP5270 GAL4* neurons (Figure 13A). Silencing these neurons by preventing exocytosis (*UAS-TNT.G*) quadrupled the mating duration in group-reared males. However, mating duration of single-reared and sexually experienced males was drastically shorter (20-30min) (Figure 13A). Therefore, even in these long-mating lines, LMD and SMD are unaffected. We then knocked-down SIFaR in the *NP5270 GAL4* expression pattern. Results showed that group-reared males mated longer than their single-reared counterparts but sexually experienced males mated for approximately the same duration indicating that SMD was affected but not LMD (Figure 13B). The same result was obtained when we constitutively activated *NP5270 GAL4* neurons with *NachBac* in an adult-specific manner (Figure 13C).

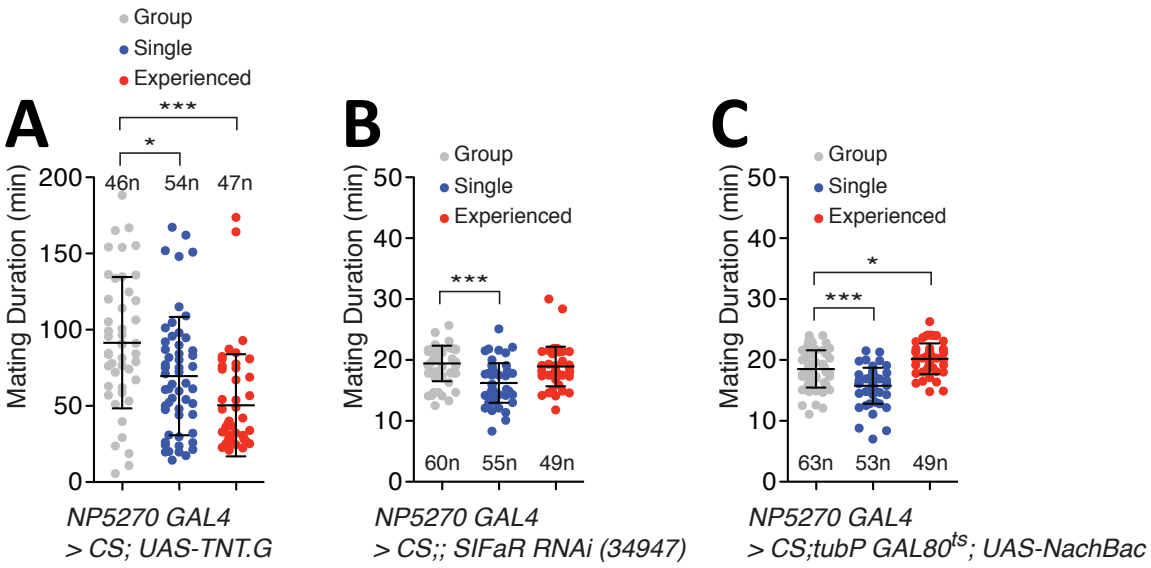


Figure 13. Involvement of *NP5270 GAL4* labelled neurons in LMD and SMD. Mating duration assay results from (A) the silencing of NP5270 labelled neurons using the light chain of tetanus toxin (*UAS-TNT.G*), (B) the knock down of SIFaR in NP5270 labelled neurons using *SIFaR RNAi* (*34947*) and (C) the constitutive activation of NP5270 labelled neurons during adulthood using *CS; tubP GAL80^{ts}; UAS-NachBac*. Data was analyzed with a 1-Way Anova, Kruskall-Wallis test with a Dunn's column comparison post-test. * indicates statistical significance at $p<0.01$ and *** indicates statistical significance at $p<0.0001$.

Conclusion

These results indicate that *NP5270 GAL4* labelled neurons are involved in SMD but not LMD and that there may be shared circuitry between the SMD neural network and that of persistence of copulation.

5. Investigation of male-specific SIFaR expressing neurons in mating duration

To further characterize SIFa signalling in LMD and SMD, we studied the importance of potential sexual dimorphism in this pathway. LMD and SMD are male-specific behaviours, it is therefore possible for the neural circuits involved in each to display a certain level of dimorphism indicating male-specific structures. SIFa neurons display a highly sexually dimorphic arborisation which may result in sex-specific signalling pathways (Figure 3A and B). Based on this rationale, we studied the impact of feminizing SIFa neurons on LMD and SMD (Figure 14C and D). However, feminizing these neurons either through the expression of female form *doublesexF (dsxF)* or the more efficient *sex lethal (sxl)* did not impact either mating behaviour as shown by the shortened mating durations of single-reared and sexually experienced males compared to group-reared males (Figure 14C and D).

We then studied the necessity of sexually dimorphic SIFaR neurons in LMD and SMD (Figure 14A and B). Results showed that SIFaR+ neurons with an active *dsx* promoter are not necessary for either behaviour (Figure 14B) while SIFaR+ neurons actively transcribing *fru* were necessary for SMD (Figure 14A). *Fruitless* is actively transcribed in both males and females. However, females do

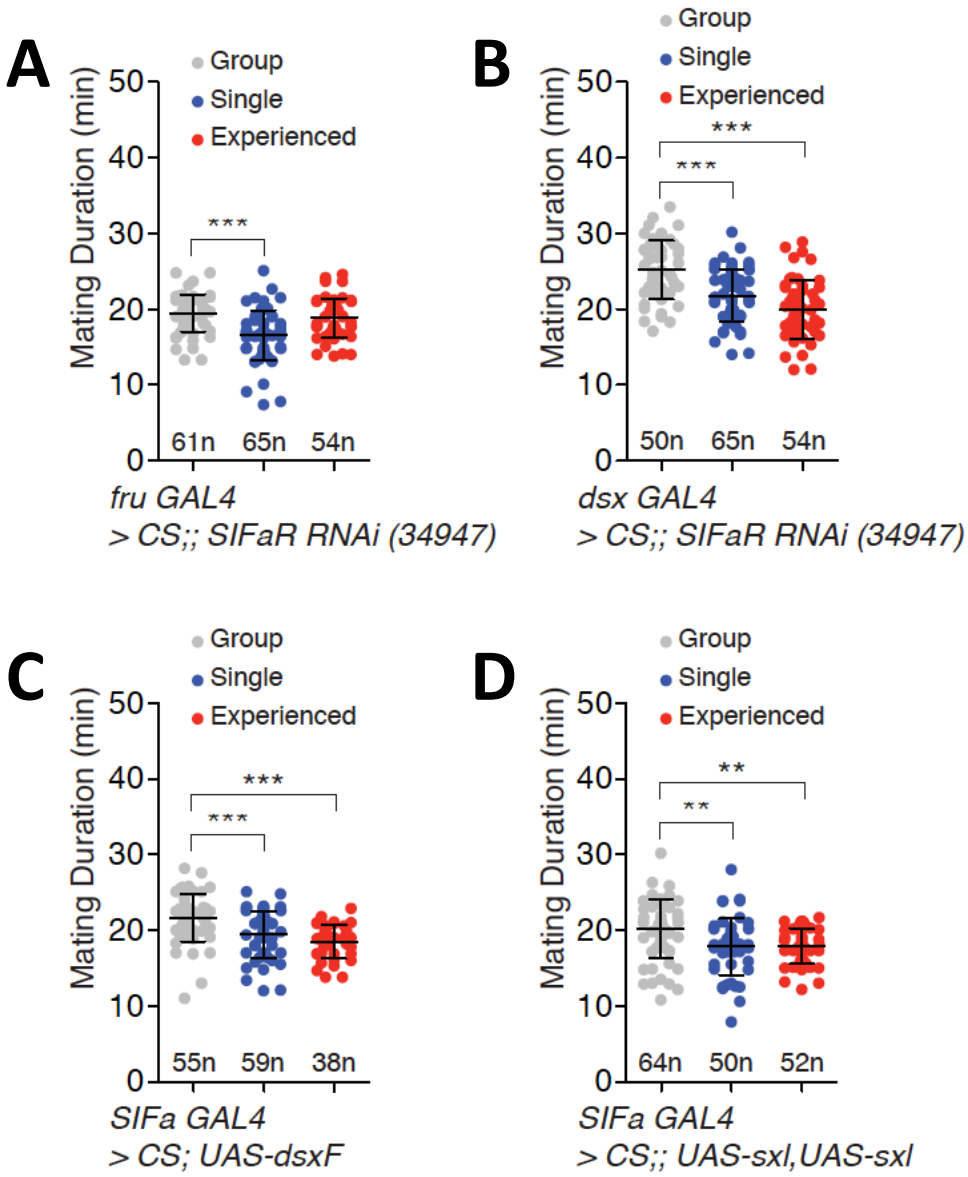


Figure 14. Involvement of SIFa and SIFaR neuron sexual dimorphism in SMD and LMD.

Feminization of SIFa neurons through knock-in expression of (A) doublesex^{F} (*UAS-dsx*^{F}) or (B) sex lethal (*UAS-sxl,UAS-sxl*). SIFaR knock-down in potentially dimorphic (C) doublesex expressing cells using the *dsx GAL4* driver or (D) fruitless expressing cells (*fru GAL4*). Data was analyzed with a 1-Way Anova, Kruskall-Wallis test with a Dunn's column comparison post-test. ** indicates statistical significance at $p<0.001$ and *** indicates statistical significance at $p<0.0001$.

not translate the RNAi and therefore lack the protein. Therefore, while this result does not prove that sexually dimorphic neurons are necessary for LMD, we conclude that neurons necessary for this behaviour are labelled by *fru GAL4*.

Conclusion

Taken together, these data indicate that sexual dimorphism is not essential in SIFa-SIFaR signalling in SMD and LMD. While *fru* mediated dimorphism of SIFaR neurons may play a role in SMD, other markers of sexual dimorphism are irrelevant in the context of LMD and SMD. Even so, since the *fru* promoter is active in both males and females, we cannot definitively conclude that *fru-GAL4* labelled SIFaR+ neurons are sexually dimorphic.

6. Investigation of core circadian clock genes in the SIFa-SIFaR signalling pathway

To conclude our investigation of SIFa-SIFaR signalling in the context of LMD and SMD, we investigated the impact of core circadian clock genes expression in SIFa+ neurons. We knocked-down *tim, per, clk* and *cyc* in SIFa+ neurons (Figure 15A-D). In each experiment, group-reared males mated significantly longer than single-reared and sexually experienced males (Figure 15A-D). Both LMD and SMD were therefore unaffected by these KDs.

Conclusion

We can therefore conclude that SIFa neurons are not involved in interval time-keeping of LMD and SMD through core circadian clock gene mediated

mechanisms. This does not exclude the possibility that these neurons may be involved in interval time-keeping through other mechanisms.

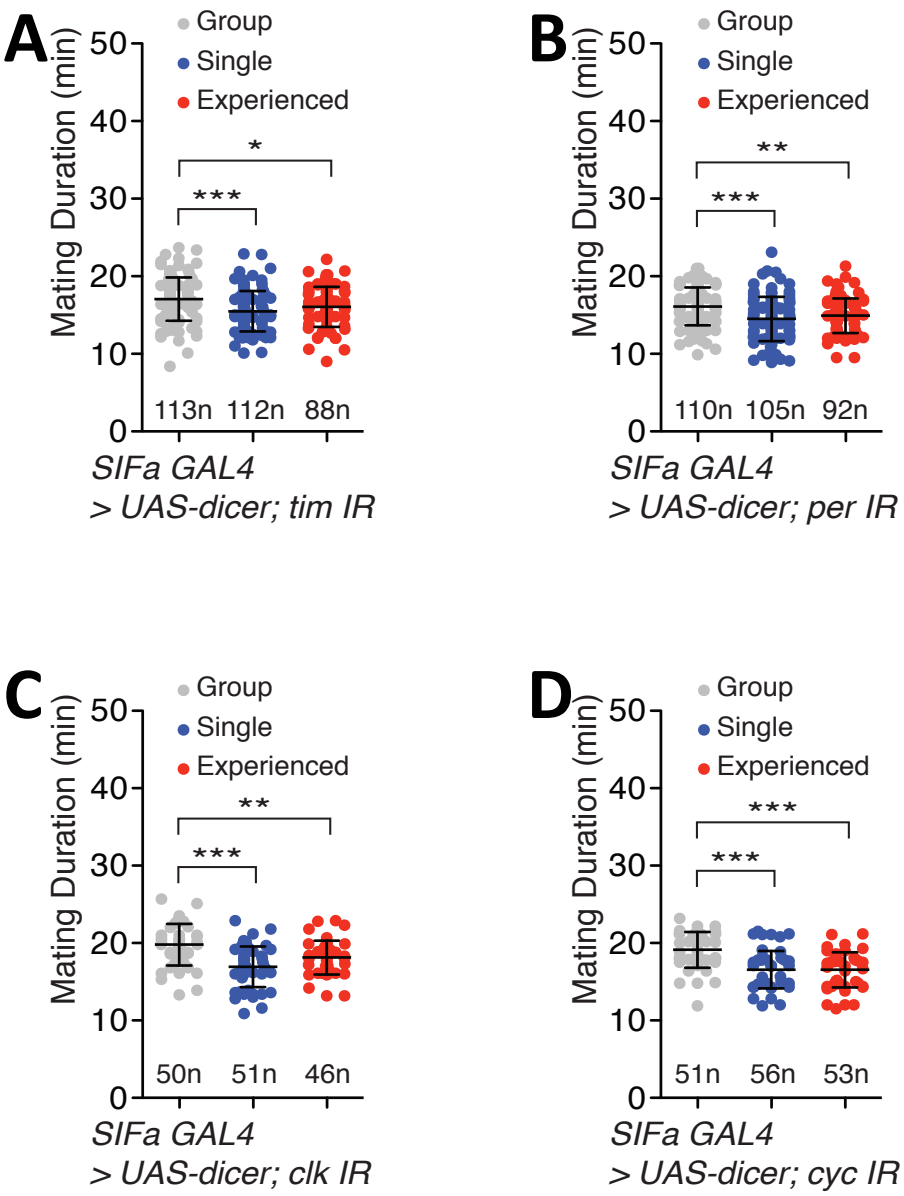


Figure 15. Expression of *timeless, period, clock* and *cycle* in SIFa neurons is not required for LMD and SMD.

Mating duration assay results from a SIFa+ neuron specific knock-down of (A) *tim* through *UAS-tim IR* (inverse repeat or RNAi), (B) *per* through *UAS-per IR,* (C) *clk* through *UAS-clk IR* and (D) *cyc* through *UAS-cyc IR. Dicer* was used to enhance the RNAi activity. Data was analyzed with a 1-Way Anova, Kruskall-Wallis test with a Dunn's column comparison post-test. * indicates statistical significance at $p<0.01$ ** indicates statistical significance at $p<0.001$ and *** indicates statistical significance at $p<0.0001$.

Discussion

Since the field of neuroscience was first established, many advances have been made to define the anatomy, function and chemistry of the nervous system and the cells it comprises. However, much remains to be discovered before we can claim to fully understand the brain and its extensions. In recent years, the aging of the population and the emergence of numerous neurological diseases and disorders has shifted the focus of the field toward more clinical applications. In spite of this, fundamental research is still necessary to further our knowledge of the brain and its cells.

This study aims to define a novel type of communication between neurons based on neuropeptide-mediated decision making. Using the model organism *Drosophila melanogaster* and the LMD/SMD behavioural paradigm previously described, we show that four neurons producing the neuropeptide SIFamide can provide inputs to two distinct target populations to differentially induce LMD or SMD. While the present research does not provide a mechanism for this communication, we present compelling evidence supporting a complex, bimodal signalling pathway. In addition, we study the importance of the identified SIFa+/SIFaR+ neurons for interval timing within the context of mating duration.

In this study, we demonstrate that SIFa signalling necessary and sufficient for LMD and SMD is mediated through SIFaR expressing neurons. Interestingly, we show that these receptor expressing neurons are located in the VNC. We therefore delineate a direct link between the brain and VNC, bridging an important gap between neural processing and muscle innervation/behavioural

output. Further study of these SIFaR+ neurons demonstrates a surprising lack of importance of sexual dimorphism in this signalling pathway. While SIFa neurons themselves are highly sexually dimorphic (Figure 3), we show that this dimorphism does not influence LMD and SMD (Figure 14). We were able to find a potential influence of sexual dimorphism on SIFaR expressing neurons, though this link does not provide definitive evidence for the involvement of male-specific structures and functions in LMD and SMD. Finally, we demonstrate that SIFa involvement in the encoding of interval timing is not mediated by core circadian clock genes though we do not exclude their role in time-keeping.

The research presented suggests that SIFa signalling plays a pivotal role in determining which mating behaviour, of LMD and SMD, will be exhibited. Based on the preliminary results shown in Figure 3, we hypothesized that SIFa signalling is directed at distinct populations of SIFaR+ neurons to induce either LMD or SMD. To define SIFa-SIFaR signalling in the context of mating duration, we used a targeted SIFaR knock-down approach.

SIFaR-expressing neurons are necessary and sufficient for both LMD and SMD

We identified two SIFaR RNAi lines which efficiently recapitulated the loss of LMD and SMD induced by SIFa KD when expressed pan-neuronally (Figure 7). This result not only verified that SIFa signalling is mediated by the SIFaR, it also showed that the SIFaR-expressing target population involved in both behaviours is entirely neuronal. However, we were unable to make a stable homozygous line from *SIFaR RNAi* BL25831, and lines combining neuronal GAL4 drivers with this

RNAi appeared to be developmentally delayed and showed signs of sickness (impaired mobility, smaller size). In addition, we were consistently unable to reproduce experimental results using this RNAi. For these reasons, we discarded this line from future studies and used *SIFaR RNAi* BL34947 for all subsequent KD experiments.

Once this line was identified, we used it to screen *SIFaR GAL4* lines to recapitulate the previous result (Supplemental figure 1). This screen demonstrated that SIFaR expressing neurons are specifically targeted by SIFa to induce LMD and SMD (Figure 8). We were therefore able to conclude that SIFaR expressing neurons are necessary for SIFa signalling in LMD and SMD. Interestingly, only one of the GAL4 lines tested was also sufficient to rescue both behaviours. *SIFaR GAL4* BL49087, which rescued lethality and behaviour in homozygous $SIFaR^{B322}$ mutants (Figure 9 and Table 2), was also the only GAL4 line to show a complete reversal of mating duration in the SIFaR KD experiment (Figure 8). This is significant because it suggests a potential disinhibition is involved in the LMD and SMD neural circuits downstream of SIFa neurons. It is however unlikely to be mediated by the SIFaR itself as only one of four SIFaR+ population of neurons was linked to this disinhibition. Although the SIFaR was characterized in 2006, very little is known about its function beyond the fact that it is a class A GPCR (Jørgensen et al., 2006). It is unknown whether the different SIFaR products are coupled to different G proteins and whether the SIFaR has an excitatory or inhibitory effect when activated by SIFa. If the reversal of mating duration observed in the *49087 GAL4* specific SIFaR KD is in fact due to

disinhibition, it is therefore more likely that the SIFaR+ neurons specific to this GAL4 are responsible for the activation of inhibitory downstream neurons though further research is required to verify this disinhibitory phenotype.

Disinhibition is particularly important in mammals for the initiation of movement in the striatum and constitutes an important area of research (Chevalier and Deniau, 1990). Several behavioural models of disinhibition have already been defined and studied in Drosophila (Jovanic et al., 2016; Lee et al., 2008; Olsen and Wilson, 2008). Sensory stimuli can often lead to several, competing behavioural outputs. Disinhibition is required between interneurons to select and maintain one of these behaviours over the others (Jovanic et al., 2016). In the context of LMD and SMD, the sensory inputs required to trigger each behaviour are distinct. However, the behavioural outputs are mutually exclusive and compete with one another. It is therefore possible that reciprocal inhibition of each circuit is involved in neuronal processing downstream of SIFaR-expressing neurons. Furthermore, the neural circuits involved in LMD and SMD regulate mating duration and it is highly probable that they modulate basic mating neural networks. In this context, SIFaR+ neurons may be involved in feed-forward inhibition of mating neurons, inducing a disinhibitory phenotype when this signalling pathway is silenced. Once specific cell populations of SIFaR+ neurons required for LMD or SMD are found, their specific response to SIFa signalling may be ellucidated by recording these neurons during optogenetic activation or inhibition of SIFa neurons.

Taken together, these results demonstrate that SIFa signalling involved in mating duration behaviours is mediated by SIFaR in SIFaR+ neurons. We further show that SIFaR expressing neurons labelled by *SIFaR (49087) GAL4* are necessary and sufficient to induce both LMD and SMD (Figure 9). Because the $SIFaR^{B322}$ mutant is homozygous lethal, our behavioural rescue experiments were preceded by lethality rescue experiments (Table 2). These showed that two of the four *SIFaR GAL4* lines were unable to rescue lethality (Table 2). We were therefore unable to use them in our behavioural rescue. Consequently, we cannot affirm that these GAL4 lines, *46391 GAL4* and *49041 GAL4*, do not label SIFaR+ neurons sufficient for behaviour as they are insufficient to rescue lethality. The use of a non-lethal *SIFaR* mutant would allow the verification of this fact, however, no such mutant currently exists.

SIFaR+ neurons necessary for mating duration behaviour are located exclusively in the VNC

We next studied the localization of SIFaR+ neurons involved in LMD and SMD. Interestingly, our results revealed that excluding VNC neurons from a pan-neuronal SIFaR KD did not cause a disruption of mating behaviour (Figure 10). We therefore show that SIFaR+ neurons necessary for LMD and SMD are located in the VNC. SIFa neurons possess projections extending as far as the abdominal ganglia in third instar larvae and research in adult Drosophila has recently shown these axonal projections to the VNC are conserved in adulthood (Martelli et al., 2017; Santos et al., 2007). These results do not therefore exclude synaptic transmission as a mode of SIFa signalling for LMD and SMD. The

localization of SIFaR+ neurons necessary for LMD and SMD in the VNC is particularly important. It provides a direct link between the sensory processing of these behaviours within the brain and the motor circuitry located within the VNC, most likely culminating in motoneuron signalling to the reproduction organ muscles.

We therefore define SIFa-SIFaR signalling as the first concrete neuronal link from brain sensory processing to VNC motor output for LMD and SMD.

SIFa signalling is directed to different SIFaR+ neuronal populations for LMD and SMD

Our NT screen results showed that while every NT system except glutamate contains SIFaR+ neurons necessary for SMD, none contained SIFaR+ neurons necessary for LMD (Figure 11). This suggested a wide network was responsible for the former behaviour, possibly including divergent signalling to neurons expressing various NTs. It also did not exclude the involvement of GABA mediated feed forward inhibition as part of this SIFaR+ downstream network of SMD, since SIFaR KD in GABAergic cells resulted in a loss of the behaviour (Figure 11). However, this KD did not result in a complete reversal of the mating duration (experienced males mating longer than the naïve ones) but only a return of mating duration to the control baseline. It is therefore unlikely that GABA signalling from SIFa activated neurons is responsible for the disinhibition-like phenotype observed and discussed previously.

On the other hand, the fact that none of the NT systems are involved in the LMD neural circuit immediately downstream of SIFa neurons is surprising. It

suggests a highly specialized signalling pathway limited to the involvement of a specific population of SIFaR+ neurons. Based on this results, we hypothesized that the signalling molecules used by these downstream neurons were NPs and not NTs. We therefore screened *NP GAL4* lines to KD the SIFaR and observe the contribution of each peptidergic neuronal population in SIFa-SIFaR signalling of the LMD neural circuit. While several KDs had no influence on either behaviour (corazonin, eclosion hormone, tachykinin), most *NP GAL4* driven KDs induced a loss of SMD (Figure 12A, B, C). This result was expected as we had already shown that the SIFaR+ neurons involved in this neural circuit formed a diverse population of cells expressing nearly every NT. Most NPs are co-expressed with NTs in neurons, which coincides with so many population specific KDs causing a loss of SMD. Only three *NP GAL4* mediated KDs caused a disruption of LMD. Due to the negative results of the *NT GAL4* mediated KDs, we already concluded that SIFaR+ neurons involved in this behaviour are not NT+. However, we can now establish that they express at least one NP of proctolin, bursicon and myosuppressin (Figure 12H, I, K). Interestingly, myosuppressin was the only population of neurons tested that influenced LMD without disrupting SMD. Proctolin and bursicon populations of neurons seemed to be involved in both behaviours. Furthermore, these SIFaR+ neurons are also *DIMM* negative (Figure 12J).

We therefore show that SIFaR+ neurons necessary for LMD and SMD are at the very least partly segregated. While it is possible that the same proc+ and burs+ neurons are involved in both behaviours, we show that some NT

expressing neurons are involved exclusively in SMD while ms-expressing neurons are necessary for LMD only. Based on these results, it is possible to hypothesize that proc+ and burs+ neurons are also divided in SMD and LMD circuits and are not individually involved in both behaviours. It is important to note that these results do not prove that proctolin, bursicon or myosuppressin are the signalling molecules these neurons use in the context of LMD and SMD neural signalling. Many Drosophila NPs are derived from a common precursors and co-expressed in neurons. The crustacean cardioactive peptide (CCAP) and bursicon (burs) for instance are derived from the same precursor and therefore co-expressed in most neurons (Dewey et al., 2004).

These results demonstrate that SIFa signalling required for LMD and SMD is directed to two distinct SIFaR+ neuronal populations. Although these two populations may contain a few overlapping neurons (proc+ or burs+), neural coding requires the integration of signals coming from an entire neuronal population and the fact that a subset of SIFaR+ neurons may be involved in both behaviours does not imply that the same SIFaR+ circuitry is shared between LMD and SMD. This result is particularly important because it provides a novel model of decision-making neurons. The original ‘command neuron’ or ‘command system’ as used by Wiersma and Ikeda, and later modeled by Kupfermann and Weiss was defined as a neuron or group of neurons integrating inputs to induce a behaviour (Kupfermann and Weiss, 1978; Wiersma and Ikeda, 1964). Here, we successfully demonstrate that SIFa signalling for LMD and SMD is mediated through distinct output pathways and therefore define a new ‘decision-making’

system in which SIFa neurons discriminate between LMD and SMD by integrating inputs to signal to two distinct neuronal populations, inducing two distinct behaviours. Based on previous research demonstrating that LMD relies on visual stimuli while SMD is dependent on gustatory inputs (Kim et al., 2012, 2016), we can further hypothesize that SIFa neurons receive distinct inputs from each neural circuit's sensory processing. This leads to the previously proposed model (Figure 5) according to which converging sensory inputs from LMD and SMD are integrated and analysed by SIFa neurons, leading to diverging signalling to the two SIFaR+ neuronal populations of the VNC described above.

While much work remains to prove that this model exists, it defines a novel mode of neuronal integration and signalling and offers new insights on the architecture of neural circuitry and neuronal computations.

Neurons involved in male persistence of mating are involved in SMD but not LMD

Based on previous literature showing the involvement of *NP5270 GAL4* labelled neurons in regulating persistence and duration of copulation, we tested our *SIFaR RNAi* KD line in this neuronal population (Crickmore and Vosshall, 2013). While Crickmore and Vosshall's work highlighted the persistence of male copulation in spite of exposure to stressful stimuli, the lines they tested displayed a considerable increase in mating duration (~900%) and we therefore hypothesized that the same neurons may be involved in LMD and SMD. We first reproduced the data presented by the authors to verify that silencing *NP5270 GAL4* neurons indeed results in a lengthening of mating duration (Figure 13A).

Our results show that, while mating duration is increased by approximately 400% in group-reared males, LMD and SMD are not affected and the mating duration of single-reared and sexually experienced males is far shorter (Figure 13A). Our subsequent experiments indicate that SMD but not LMD is affected by a SIFaR KD in the *NP5270 GAL4* labelled neuronal population (Figure 13B). These results coincide with data obtained from an adult-specific activation of the *NP5270 GAL4* neurons, suggesting that SIFa signalling to these neurons results in their depolarization (Figure 13C). In their paper, Crickmore and Vosshall demonstrated that GABAergic interneurons within the *NP5270 GAL4*+ neurons of the abdominal ganglion were necessary for the decrease of mating persistence and duration (Crickmore and Vosshall, 2013). The circuit they described also involved a parallel population of dopaminergic neurons working in opposition to the GABAergic neurons to lengthen copulation (Crickmore and Vosshall, 2013). These were not comprised within the *NP5270 GAL4* labelled population. However, the identified GABAergic neurons showed sexual dimorphism and expressed dsx^{M}. Our analysis of sexual dimorphism showed that SIFaR+, *dsx* expressing neurons are not involved in LMD and SMD (Figure 14B). We can therefore conclude that, though SIFaR+ neurons labelled by *NP5270 GAL4* in the abdominal ganglia are located in close proximity to the GABAergic neurons identified by Crickmore and Vosshall, they are not identical. The abdominal ganglia are rich in interneurons and it is possible that SIFaR+ neurons form relevant synaptic connections with those identified by Crickmore and Vosshall to regulate mating duration in a subtler manner.

Sexual dimorphism is not highly involved in SIFamide mediated signalling required for LMD and SMD

We further explored the importance of sexual dimorphism in SIFa-SIFaR signalling pathways involved in LMD and SMD. As previously mentioned, the duration of copulation is a male-dependent decision in *Drosophila melanogaster* (Beaver and Giebultowicz, 2004; Macbean and Parsons, 1967). As such, it is highly probable that any behaviour involving mating duration would rely on a sexually dimorphic neural circuit. The *fruitless* (*fru*) male specific gene has been shown to influence not only courtship, but also fertility and mating duration through the regulation of serotonin production in neurons of the abdominal ganglia innervating internal sex organs (Lee et al., 2001). In addition, the local circuitry coordinating male copulation identified by Pavlou *et al.* within the abdominal ganglia is entirely dimorphic and expresses *doublesex*^{M} (*dsx*^{M}) (Pavlou et al., 2016). We therefore investigated the influence of sexual dimorphism on SIFa-SIFaR signalling required for mating duration.

SIFa neurons do not express *fru* (Sellami and Veenstra, 2015). However, they display a high level of sexual dimorphism in their arborisation which we hypothesized may be the result of *dsx*^{M} expression and lead to male specific functions (Figure 3A and B). We therefore studied the effects of feminizing these neurons on LMD and SMD. The sex-determining pathway of *Drosophila melanogaster* involves the expression of *Sex-lethal* (*Sxl*), *transformer/transformer2* (*tra/tra2*) and *dsx*^{F} in females. In males, *Sxl* is not expressed, which leads to the expression of *fru* and *dsx*^{M} (Salz and Erickson, 2010). We therefore feminized the SIFa neurons at different levels of this

pathway by inducing expression of *Sxl* or dsx^F. Our results revealed that LMD and SMD are unaffected by feminization of SIFa neurons (Figure 14C and D). Our next experiments focused on the involvement of sexually dimorphic SIFaR+ neurons in mating duration. As mentioned, SIFaR KD in *dsx*-active cells does not affect behaviour (Figure 14B) while the same experiment in *fru*-active cells affects SMD but not LMD (Figure 14A).

Therefore, it seems that sexual dimorphism does not play a vital role in SIFa-SIFaR signalling required for mating duration. The *fru GAL4* mediated KD of SIFaR does not convey conclusive evidence for the involvement of dimorphism in this signalling pathway since the *fru* promoter *fruP1* is active in females as well as males. The sex-specific difference in *fru* expression is the result of a *tra/tra2* mediated alternative splicing of the *fru P1* pre-mRNA in females which prevents translation (Goldman and Arbeitman, 2007). We can therefore not conclude that *fru* expression in SIFaR+ neurons is required for SMD, though it may have a role in this behaviour. In the case of LMD however, SIFa-SIFaR signalling does not involve sexual dimorphism.

These results are particularly surprising as recent research has demonstrated that the motor circuit coordinating male copulation is entirely sexually dimorphic and expresses dsx^M (Pavlou et al., 2016). However, SIFa-SIFaR signalling only constitutes one component of the neural circuits of SMD and LMD which contain other, sexually dimorphic, groups of neurons. LMD involves sexually dimorphic NPF expressing neurons and *cry*+ neurons (Kim et al., 2013) and SMD requires male-specific Gr5a neurons (Kim et al., 2016). It is

therefore possible that the particular SIFa-SIFaR signalling pathway required for both behaviours, receives and sends inputs to other sexually dimorphic neurons and is anchored within a generally male-specific circuit.

Core circadian clock genes are not involved in SIFa neurons' interval timing mechanism

Circadian clock genes have already been involved in interval timing mechanisms (Agostino et al., 2011b, 2011c; Kuczenski et al.). In addition, several studies have demonstrated that *clk, cyc, per* and *tim* can act independently of one another to regulate signalling cascades and behaviour (Agostino et al., 2011a; Goda et al., 2011; Seluzicki et al., 2014). Recently, these circadian clock genes have been linked to mating behaviour, and more importantly, LMD and SMD (Beaver and Giebultowicz, 2004; Kim et al., 2012, 2016; Kyriacou and Hall, 1980). Based on their role in modulating both LMD and SMD, we hypothesized that SIFa neurons held a key role in the regulation of interval timing involved in these behaviours. Previous work on LMD by Dr. Kim has already demonstrated that *tim* and *per* are involved in modulating LMD while *clk* and *cyc* participate in the modulation of SMD. In addition, this work also determined that *per* and *tim* gene expression was sufficient in PDF and NPF expressing neurons of the brain. Our KDs of core circadian clock genes in SIFa+ neurons show that these genes have no influence on mating duration behaviour in these cells. That is not to say however, that SIFa neurons are not involved in the mechanism of interval timing required for LMD and SMD. Since these neurons provide a direct link between the brain and the VNC, they must somehow encode timing information received from PDF and

NPF expressing neurons. One theory of interval timing relies on multiple biological oscillators located in series within neural circuits (Agostino et al., 2011b). If that is the case, then SIFa neurons could contain one of these oscillators, though not one that is dependent on circadian clock gene expression. In mammals, multiple NTs and their receptors have been linked to interval timing mechanisms, including dopamine and its receptors (Agostino et al., 2011b). It is possible that this mechanism is conserved in Drosophila. In this case, SIFa neurons would not be involved in this specific mechanism but SIFaR+ neurons could then become candidates. While part of the genetic interval timing mechanism of LMD has already been elucidated, the mechanism underlying interval timing of SMD is still unknown. SIFaR+ neurons located in the abdominal ganglia present interesting characteristics which make them ideal candidates to house portions of the interval timing mechanisms involved in mating duration behaviour. They are located in close proximity to several interneurons already involved in modulating duration and persistence of copulation and could also directly innervate the internal sexual organs of male flies. Their close proximity to the motor circuitry involved in the behavioural output of LMD and SMD means that they are primed to directly control the duration of muscle activity.

Overarching hypothesis of SIFa signalling within the LMD and SMD neural circuits

While the results discussed above define two distinct pathways of SIFa to SIFaR signalling for LMD and SMD, they do not explain the preliminary data which suggests that SIFa mediated signalling is required for LMD via SIFa neuron

activation, and SMD via SIFa neuron inhibition (Figure 3). Indeed, silencing the neurons with the TNT.G-mediated inhibition of exocytosis induces a loss of both LMD and SMD (Figure 3F). This result indicates that SIFa exocytosis is required for both behaviours. In addition, data obtained by constitutively activating SIFa neurons (NachBac) shows a loss of SMD but not LMD (Figure 3E). In contrast, constitutively inhibiting those neurons (Kir2.1) induces a loss of LMD but not SMD (Figure 3D). Taken together, these results demonstrate that the activation of SIFa neurons is necessary for LMD while their inhibition is required for SMD. We can therefore simultaneously conclude that SIFa is released by SIFa+ neurons during both their activation and inhibition. To explain these seemingly contradicting mechanisms, we propose a model according to which SIFaR+ neurons involved in each behaviour receive SIFa inputs from different signalling pathways. This model is possible due to the distinct populations of SIFaR+ neurons receiving SIFa inputs for each behaviour.

SIFa is a NP and can therefore be secreted locally at the synapse like a NT or more diffusely through paracrine signalling. We therefore hypothesize that SIFa is transmitted synaptically to one SIFaR+ population of neurons for LMD while it is transmitted through paracrine signalling to another for SMD (Figure 16). Such a model of neuronal signalling has not yet been described, which would make this a novel mechanism of bimodal signalling. The research presented previously takes the first step towards testing this model but future work is required to study the mode of SIFa signalling required for LMD and SMD. In addition, though several elements of the neural networks underlying LMD and

SMD have already been defined, they remain independent of one another. Further research will therefore be necessary to establish the neuronal pathways between sensory inputs and other established portion of LMD and SMD neural circuits, and SIFa neurons (Figure 16).

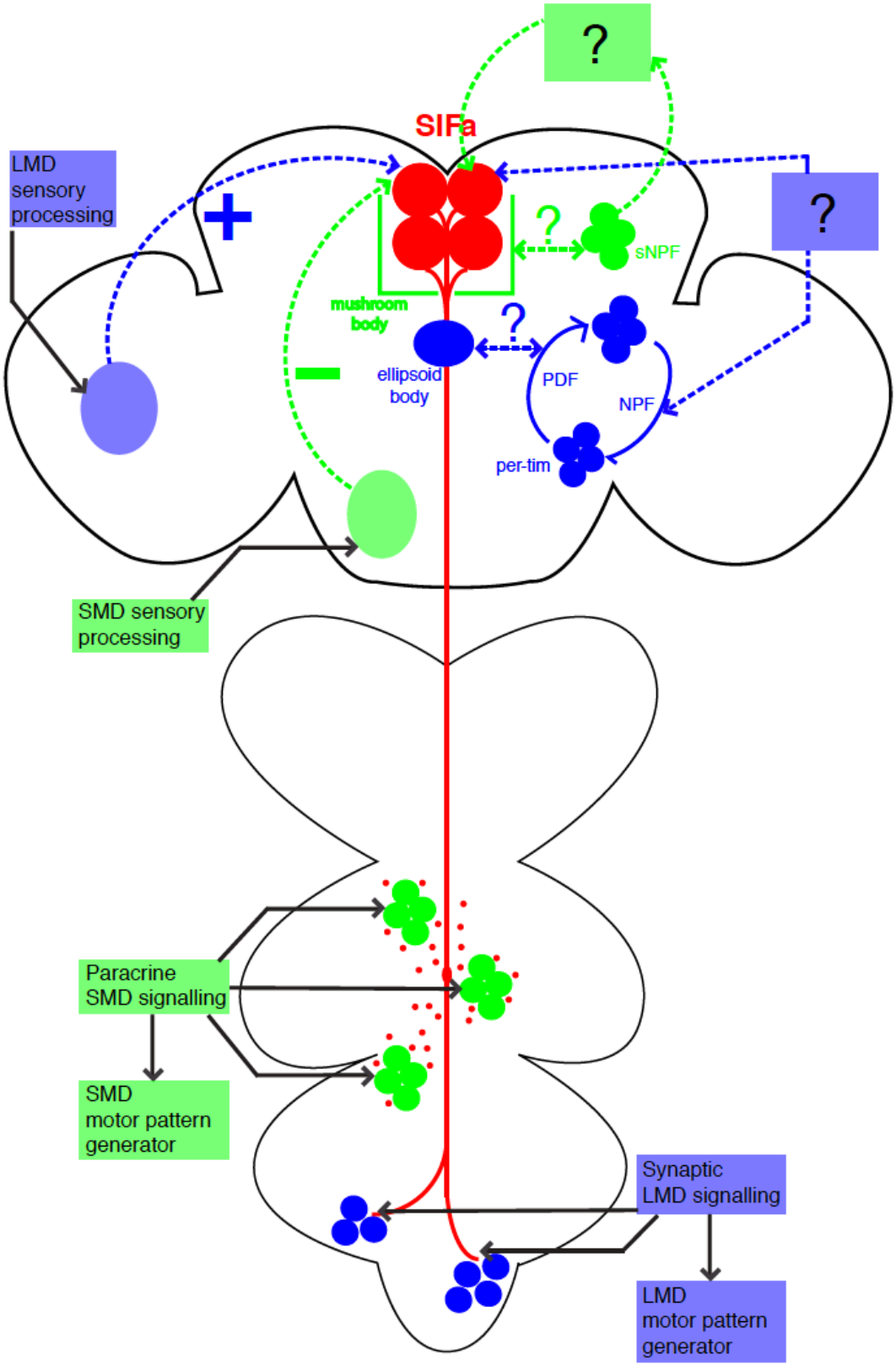


Figure 16. Overarching hypothetical model of SIFa signalling in LMD and SMD within already identified circuitry

Conclusion

In *Drosophila,* mating is the culmination of a highly stereotyped sequence of events called courtship. When males are presented with a female, they perform this chain of behaviours to woo the female, before attempting to mount her. This is the first crucial stage of mating, which is entirely dependent on the female. Once the female *Drosophila* has allowed the male to mount her, subsequent decisions are entirely male-dependent. The mating duration is one of these decisions. While courtship is a highly studied and well established sequence of events, much less is known about the mating behaviour itself.

This research sheds new light on the neural circuitry underlying two novel mating behaviours known as LMD and SMD. We demonstrate that the same group of four SIFa producing neurons is involved in neural pathways of both behaviours via signalling to partly independent SIFaR expressing neuronal populations. In addition, we begin to investigate the overarching question of how SIFa signalling induces LMD when these neurons are activated and SMD when they are inhibited. A hypothesis for this has already been formulated but further work is needed to investigate the behaviour of SIFa neurons in detail.

In addition, though behaviours are defined by an immediately visible phenotype, their persistence within species is determined by the evolutionary advantage they convey to the individuals. Successful mating is essential from an evolutionary standpoint as it ensures a high level of fitness and a high probability of passing genes on to the next generation. However, this advantage is offset by the energetic toll of sustained mating. Mating duration is therefore influenced by

its cost-benefit ratio which can be behaviourally illustrated by manipulating the social environment of *Drosophila* males prior to mating. While LMD illustrates the importance of fitness in a highly competitive environment, SMD most accurately exemplifies the energetic cost of repeated mating. The investigation of LMD and SMD in an evolutionary setting is therefore primordial to our understanding of behaviour.

Finally, though several key components of the neural circuits of each behaviour have been defined, neither one has been fully described. Establishing the full neuronal circuitry required for LMD and SMD should be a priority as it could shed light on basic principles governing all neural circuits.

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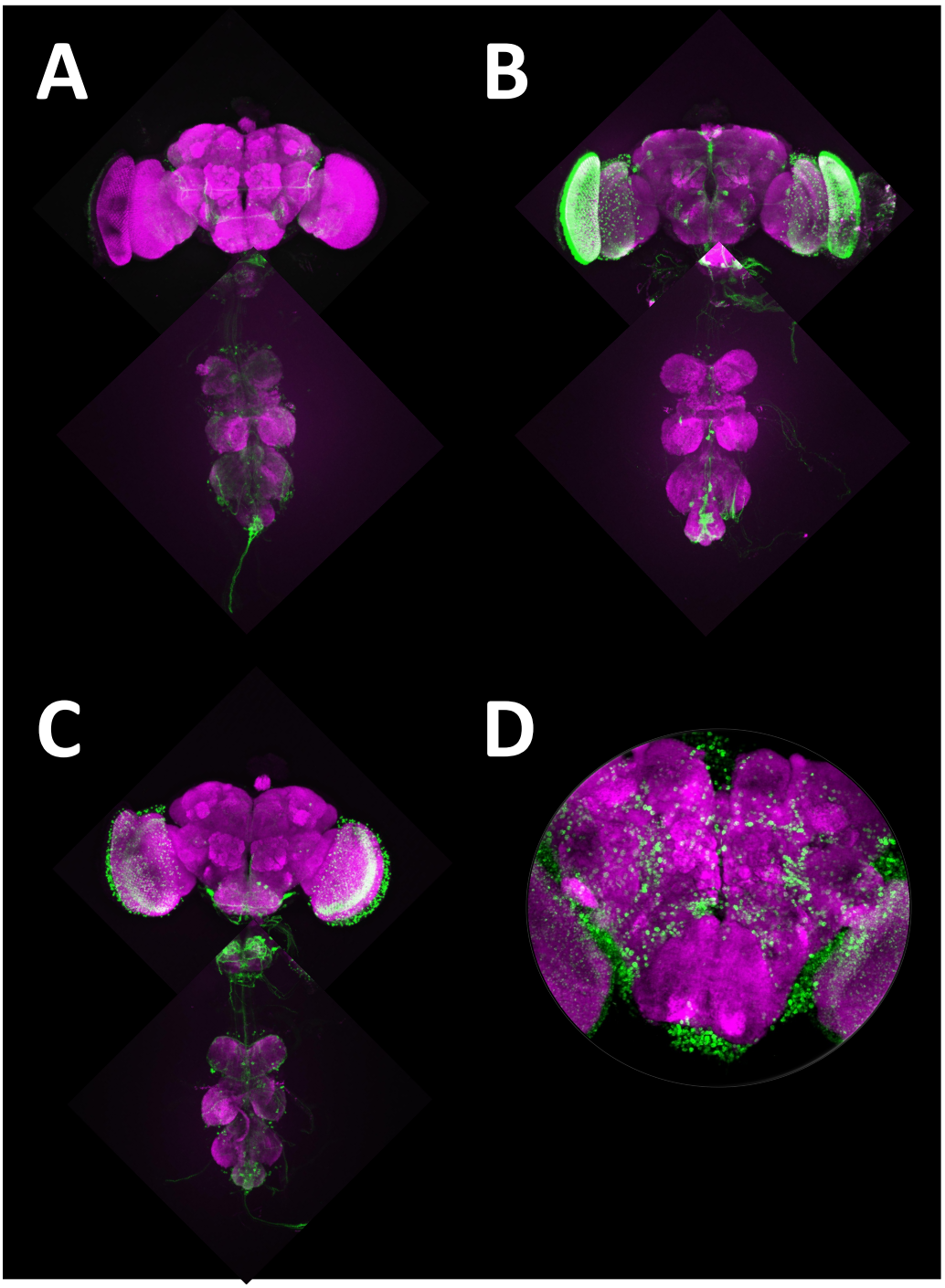
Appendice



Supplemental figure 1. Immunostaining data of *SIFaR GAL4* lines
(A-C). Data obtained from Bloomington Stock Center (http://flystocks.bio.indiana.edu) representing CNS neurons labelled by (A) *SIFaR GAL4* (49041), (B) *SIFaR GAL4* (49061) and (C) *SIFaR GAL4* (49087). (D) Data adapted from Dr. Kim seminar presenting neurons labelled by *SIFaR GAL4* (46391) within the brain lobes.