Mapping the neural circuits that modulates the molecular switch between alternative interval timing behaviours in *Drosophila melanogaster*

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Abstract

Neuropeptides are central modulators of many functions including male-specific mating behaviours. Understanding how these chemical messengers modulate the neural substrates are still not well understood but remains important for biological research. In *Drosophila melanogaster*, two well-defined microcircuits (Longer-Mating-Duration (LMD) and Shorter-Mating-Duration (SMD)), are used to understand the underlying mechanisms of how neuropeptide interactions modulate temporal information in mating behaviours. In our study, we investigated the influence of SIFamide receptor-mediated signaling and its association to both LMD and SMD. We performed several RNAi-based screens where we identified and mapped out seven different types of neuropeptidergic neurons which were found to be important to either LMD and/or SMD. Following this analysis, we highlight three independent signaling pathways which are necessary to describe the cellular mechanics of the neuropeptides involved. Firstly, we infer that synaptic contacts between proctolin and SIFamide neurons in the subesophageal ganglion mediate inhibition in SMD whereas proctoclin as a neuropeptide modulates both LMD and SMD in a non-synaptic manner. Secondly, we describe an existing insulin-related microcircuit that is modulated by the inputs of Dimmed (DIMM), a transcription factor, through adipokinetic hormone, allatostatin A, and leucokinin to exhibit SMD. Thirdly and lastly, we discuss our interpretations of how capability neurons in the central brain resolves a potential disinhibition microcircuit in LMD via olfactory based signaling in the antennae lobe. In summary, our results contribute to establishing a model system to study neuropeptidergic microcircuits in complex mating behaviours

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List of Abbreviations

AstA – Allatostatin A

AstC – Allatostatin C

Burs – Bursicon

CCAP – Crustacean cardioactive peptide

CLK/*clk* – Clock protein and gene

CPPB – CAPA precursor peptide B

CRZ – Corazonin

DAG - Diacylglycerol

Dar-2 – Allatostatin A receptor

DSK – Drosulfakinin

DIMM- - Dimmed-negative

DIMM+ - Dimmed-positive

DH31 – Diuretic hormone 31

DH44 – Diuretic hormone 44

DILP - Drosophila insulin-like-peptide

EH – Eclosion hormone

ETH – Ecdysis triggering hormone

elav^{c1555} – Embryonic lethal abnormal vision promoter

FMRFa - FMRFamide

$GAL80^{ts}$ – Inhibitor of *GAL4* that is temperature sensitive to denature at 29°C

GPCR – G-protein coupled receptor

GRASP – GFP Reconstitution Across Synaptic Partners

Hs-hid – Heat shock-head involution defective

KCNJ2 - Inward-rectifying K+ channel

LMD – Longer-Mating-Duration

LK – Leukokinin

LKR – Leukokinin receptor

MIP – Myoinhibitory peptide

MS – Myosupressin

NaChBach – Bacterial sodium channel

NDS – Normal donkey serum

NPF – Neuropeptide F

nSyb – Neuronal synaptobrevin

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

Pburs – Partner of Bursicon

PDF – Pigment dispering factor

PKC – Protein kinase C

Proc – Proctolin

RNAi – RNA interference

SOG – Subesophageal ganglion

SNARE – SNAP Receptor

sNPF – Small Neuropepide F

SIFa – SIFamide

SIFaR – SIFamide receptor

STD – Standard Deviation

TNT.G – Tetanus toxin light chain

UAS – Upstream Activation Sequence

VDF – Virginizer deficiency

Acknowledgements

First and foremost, I give my gratitude to my supervisor, Dr. Woo Jae Kim. Thank you for being such an excellent mentor, your wealth of knowledge, and giving me the opportunity to conduct research under your supervision. I want give credit to my co-supervisor, Dr. Jean-Claude Beïque, and my TAC members, Dr. Antonio Colovita and Dr. Leonard Maler. You all gave me overwhelming support and guidance which allowed me to complete my studies. I would also like to thank Dr. Seung Gee Lee for teaching me everything when I first arrived at the lab, including the recombination crosses. I cannot forget my partner in crime at the lab, Ehteshamul Islam (Sam). You and I spent many days and nights together, but we enjoyed every moment of it. I still won't never forget that you would go to Tim Horton's for the potato wedges and not the coffee. I give many thanks to our technicians, Oksu Shin and Jasdeep Kaur, both were the glue to our lab since they were always available whenever I needed anything. I Especially give a shout out to Elsie Lebedev for completing an illustration for my thesis and those countless nights going for coffee and green tea during our time at the lab. You also taught me how to identify TM2 balancer, very helpful indeed! Next, I have to recognize Khoi-Nguyen Ha Nguyen (Joseph) and Shatha Atieh for helping me with my experiments. I don't think I would have completed all those assays without your help. Thank you Hadi for your presence and teaching me your culture, I hope to see you again soon. Lastly, I thank Ana Baraa, Ash, Ciara, Mahdi, Brittany, Dorthy, Addy, Tiffany, Hanan, Yan, Tulsi, Catherine, Lina, Ammar, Hertek, Sneha, Ayoub, Kendyll, Yasmine, Katelyne, Hanan, Mohcene, Grace, Ji Yeun, my family and friends, and everyone else for the memorable experience I had at the University of Ottawa. Thank you so much everyone!

Introduction

The human brain is complex organ that modulates various physiological and behavioural functions. It is composed of approximately 86 billion neurons which are systemically organized into complex neural circuits (Boto & Tomchik, 2019). Each neuron forms a synapse, a site where electrical and chemical information is transferred from one neuron to another. The full mechanisms by which the brain encodes and translates information for many different biological functions remains elusive. The use of methodologies that will manipulate neural constructs through behavioural genetics is an ideal model to examine this phenomenon.

All organic life contains DNA for the use of storage and transfer of information (Connell & Hofmann, 2011). From single cell organisms to humans, this data can be used to further understand how biological processes interact with the nervous system. Our approach is to use simple organisms and their neurobiology as it will yield important insights in understanding how genetic information is translate into brain-behaviour interactions.

Drosophila Melanogaster

The fruit fly, *Drosophila melanogaster*, is an invertebrate organism that has been extensively used for biological research. Its first documentation was during the early 1900s where it was used by Thomas Hunt Morgan to study the role of chromosomes in the theory of inheritance. Decades later, Dr. Morgan was awarded the 1933 Nobel Prize in Physiology or Medicine for his research in heredity (Jennings, 2011). Now, more than

1800 labs world-wide are currently the *Drosophila* for nearly all aspects of biological research (Hales, Korey, Larracuente, & Roberts, 2015).

Fruit flies are ideal candidates to investigate various cellular and molecular mechanisms. One of its valuable features is that they contain approximately 100,000 neurons and about 14,000 genes (Pandey & Nichols, 2011). Fewer neurons and genes allows greater insights to understand highly complex biological processes by narrowing down mechanistic focus. Fruit flies are also suitable for scientific experiments due to their low maintenance and ability to generate several offspring. These flies are commonly raised in a 25°C environment which allows them to reach adulthood in 10 days (Venken, Simpson, & Bellen, 2011b). Therefore, both of these factors allow fruit flies to be appropriate genetic models for many aspects of research.

The genome of *D. melanogaster* is engineered in such a way where modern techniques and designs can be used to identify novel neural circuits. For example, the fruit fly contains a genetic toolkit which incorporates the *GAL4*/*UAS* binary system. *GAL4*, originally derived from yeast, is designed to be expressed in a tissue-specific manner and acts as a transcriptional activator (Venken et al., 2011b). *GAL4* binds to an enhancer region, the Upstream Activation Sequence (*UAS*), and facilitates the activation of a selected transgene (Figure 1). Further adjustments can be made to spatially restrict the expression of *GAL4* to smaller subsets or certain types of cells by introducing *GAL80*, a *GAL4*-inhibitor (Figure 2). This binary system is also supported by a vast collection of *GAL4/UAS* lines provided by large-scale *Drosophila* stock centers to allow diverse biological experiments to be performed (Jenett et al., 2012). Therefore, it is evident that *D. melanogaster* is an appropriate organism to investigate lesser known mechanisms as it

provides a versatile approach to manipulate cells or genes and uncover its association to complex behaviours.

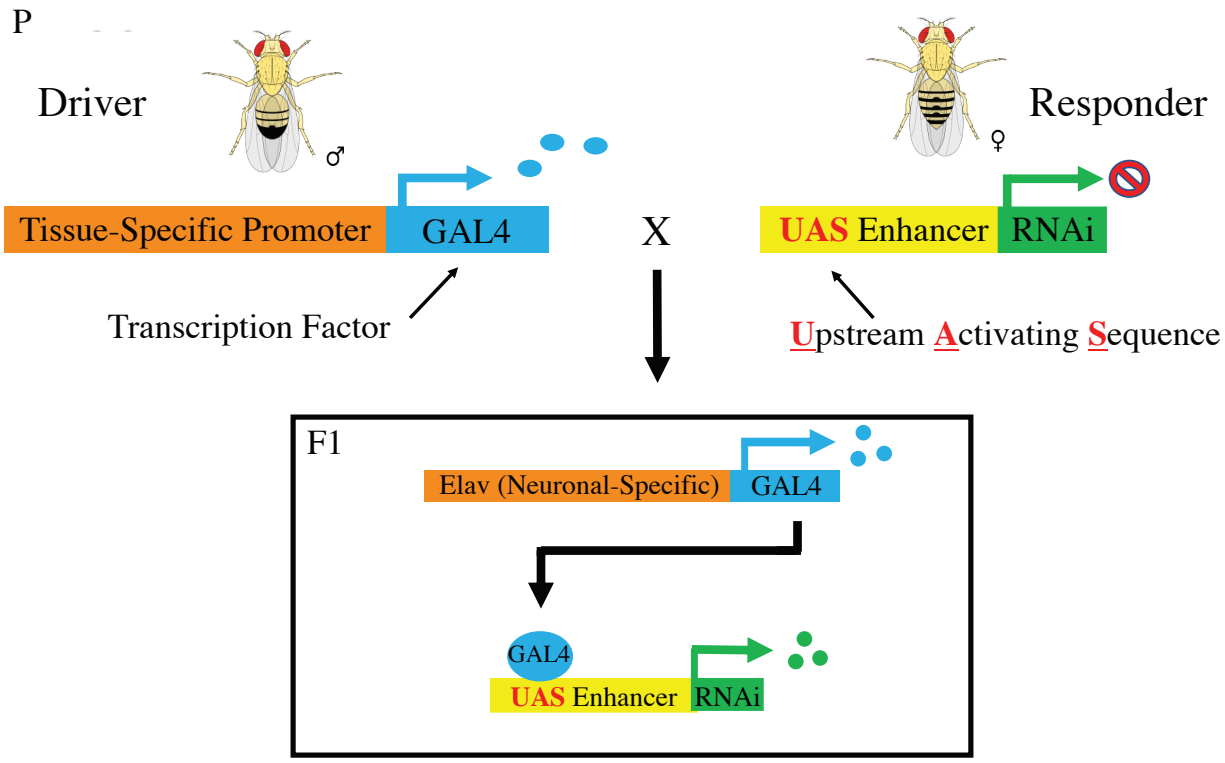


Figure 1. *GAL4-UAS* System. A male fly which contains *GAL4*, a transcription factor, is crossed with a *UAS* (Upstream Activation Sequence) transgenic female fly that occupies a gene of interest. The F1 generation is used as it contains both binary constructs. As *GAL4* is tissue-specific, expression and effects of the desired gene(s) will be limited to selected tissue. For example, our F1 generation displays a phenotype where knockdown of a selected transgene is applied to elav labeled cells (neuronal-specific marker).

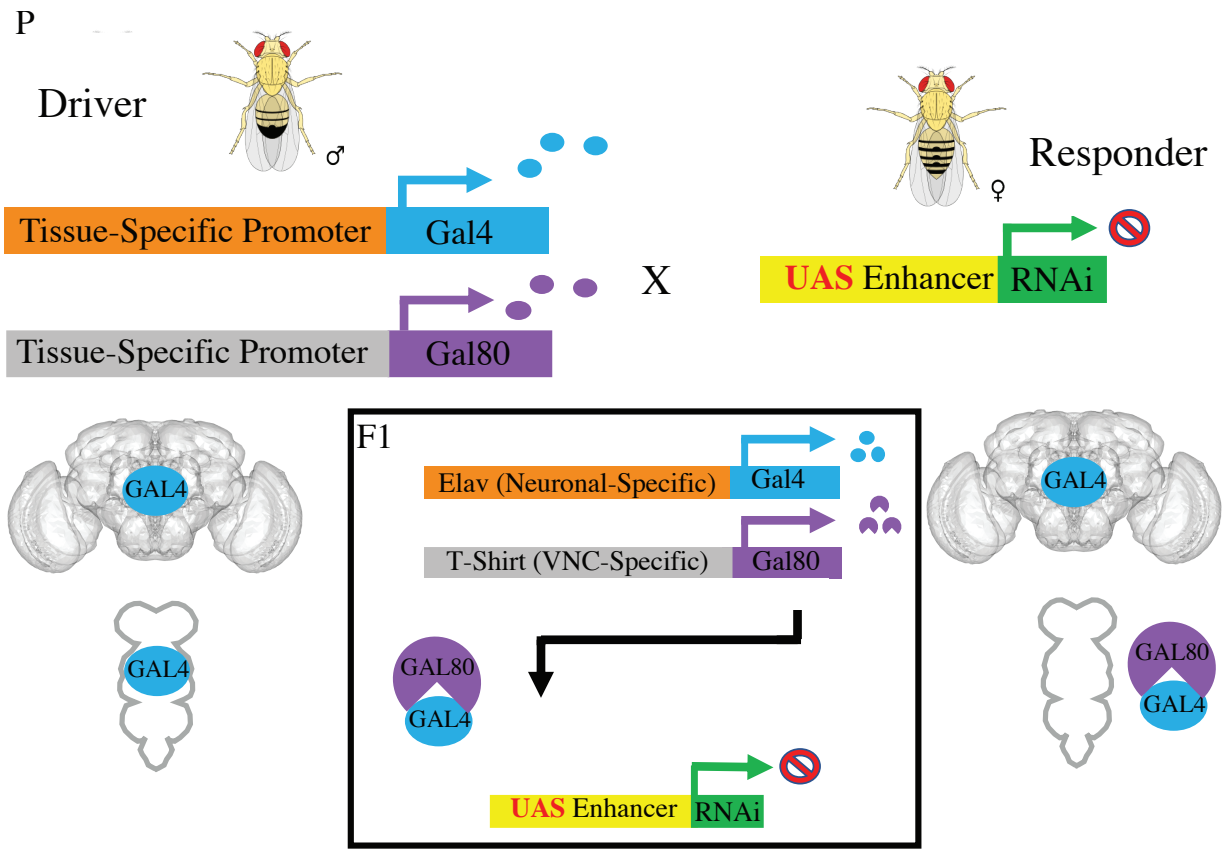


Figure 2. *GAL4-GAL80-UAS* System. A male fly that contains both *GAL4* and *GAL80* (inhibitor of GAL4) construct is crossed with a *UAS* transgenic female fly that contains gene of interest to be expressed. The F1 generation is used for experimental purposes as it contains *GAL4, GAL80*, and *UAS* construct. In contrast to our first example in Figure 1, knockdown of a specific gene is restricted to only the central brain neurons and not the ventral nerve cord (VNC) neurons.

Interval Timing

Timing and goal-oriented behaviours are necessary and interconnected variables for survival. To ensure one's safety, the ability to endure and prosper in a given environment will rely on the organism's capacity to perceive and gauge time. This phenomenon is associated with neuronal circuits being manipulated by specific behaviours through temporal information (Buhusi & Meck, 2005). For this reason, it is

important to investigate the neural basis of interval timing.

Interval timing is a specific timescale that is measured precisely within the seconds-to-minutes range (Buhusi & Meck, 2005). It is a timescale that may arguably be one of the most significant influences on a wide array of behaviours. Humans and other animals use interval timing for foraging (Kacelnik & Brunner, 2002), multi-step arithmetic (Sohn & Carlson, 2008), and decision-making (Buhusi & Meck, 2005). The latter serves our scientific interest as we intend to understand how the brain encodes temporal information and processes it into specific outputs or decisions. The utility of interval timing is directly correlated with everyday decisions, so it is important that we understand the theoretical and clinical basis of time perception. Though many studies have shown that humans utilize an assortment of interval timing behaviours (Buhusi & Meck, 2005), understanding how temporal information alters specific behaviours is important for biological sciences.

We are now beginning to understand how the neural circuits of time perception influence time-based decisions. Invertebrates have made several contributions in understanding fundamental processes in neuroscience. Though most invertebrates live relatively simple lives, they are engaged in a dynamic social setting where they must adapt appropriately to survive their environment. Thus, understanding how interval timing manipulates neural circuits in the *Drosophila* will allow us to broaden our genetic model and understand how these processes are modulated in the human brain.

Longer-Mating-Duration and Shorter-Mating-Duration

Competition among males for female copulation presents a significant influence over mating behaviours (Parker, Ball, Stockley, & Gage, 1997). Other factors which pose equal or greater influence is the socio-sexual environment by which a male fly is housed in. The term socio-sexual environment refers to a specific environmental situation where social influence modulates the mating behaviour of a male fly and as a consequence, the neural circuits that governs it are altered. Many species have developed highly sophisticated approaches to maximize their reproductive success due to socio-sexual environments. For example, male fruit flies will alter their behaviour in presence of other rivals by accurately controlling the length of their mating (i.e. mating duration). This phenomenon is important since mating duration is embedded into complex social constructs which directly influences neuronal circuits (W. J. Kim, Lee, Schweizer, et al., 2016; W. J. Kim, Jan, & Jan, 2012, 2013a). Though different mating behaviours are known to exist (Shuster, SM, Wade, 2001), two well-established paradigms in *Drosophila*, Longer-Mating-Duration (LMD) and Shorter-Mating-Duration (SMD), have defined microcircuits which will be important to understand the circuits underlying temporal information (W. J. Kim, Lee, Schweizer, et al., 2016; W. J. Kim, Jan, & Jan, 2012, 2013a).

LMD and SMD are context-based behaviours mediated by socio-sexual environments. LMD is a rival-specific mating behaviour that is induced when a male fly is placed with other rival males prior to copulation. This male fly (naïve condition or male flies raised with other male flies) will increase or extend their mating in comparison

to a non-rivaled male fly (singly-reared condition). Previous reports have described that visual cues, circadian clock genes *period* and *timeless*, and signaling between Pigment Dispersing Factor (PDF) and Neuropeptide F (NPF) are essential to mediate LMD (W. J. Kim et al., 2012, 2013a). Conversely, SMD is a sexual experience-specific mating behaviour that is exhibited when a male fly is presented in an enriched female environment. This fly (sexual-experience condition) will decrease or shorten their mating in comparison to the naïve condition. This paradigm relies on contact-based chemoreception through gustatory and mechanosensory cues, utilizes circadian clock genes *clock* and *cycle*, and neuropeptidergic signaling from Short Neuropeptide F (sNPF) to drive SMD (W. J. Kim, Lee, Auge, Jan, & Nung, 2016). It remains to be seen how socio-sexual conditions modulates the neuronal circuits in LMD and SMD as these factors are poorly understood. Since PDF, NPF, and sNPF are involved in our behaviours, we hypothesize that other neuropeptides are involved as well.

Neuropeptides

Neuropeptides are chemical messengers that modulates various functions in behaviour and physiology. Their molecule compositions are generally larger than neurotransmitters but smaller than hormones. In *D. melanogaster*, more than 50 neuropeptides have been identified and characterized to modulate various neural circuits and neural dynamics (Hewes & Taghert, 2001; Pauls et al., 2014; Vanden Broeck, 2001). From invertebrates to humans, neuropeptides have increasingly become more apparent to mediate cellular communication and thus have emerged as important players in brain-behaviour interactions.

Neuropeptides are widely diverse in their ability to alter the neural substrates for a given behaviour. They modulate their effects either through the soma and/or neurites to synaptic sites, as well as initiate long-range signaling to produce hormonal-mediate effects via dense core vesicles (Pol, 2012). Neuropeptides act by binding and activating their corresponding receptors to reconfigure a network by changing neuronal excitability and synaptic plasticity for one or many neurons (Nässel, 2002). This consequently leads to the activation of various cascades that includes feedback signaling, coordination, and sensory integration (Komuniecki, Hapiak, Harris, & Bamber, 2014). These pathways are generally initiated by several neuropeptides working in conjunction with each other, but it is also possible for a one type of neuropeptide to mediate these effects as well (Jékely et al., 2018). Besides knowing that neuropeptides and G-protein coupled receptors (GPCRs) are flexible and dynamic (Monastirioti, 2003), we have not yet elucidated their mechanistic interactions that results in circuit-level consequences.

In recent years, *Drosophila* research has shifted its attention to neural circuits that involves neuropeptidergic pathways. Many studies have described neuropeptides and its receptors in mammals to be closely related to previous ancestral species (Mirabeau & Joly, 2013) and are conserved evolutionarily (Nässel & Williams, 2014). This leads us to believe that studying mating duration will lead us to greater insights to understanding the fundamental mechanisms in neuropeptide-receptor signaling. While most studies have primarily focused on neuropeptide expression in *D. melanogaster*, few studies have characterized receptor expression. Targeting these circuits will be ideal because they are known to have restricted access to synaptic connections (Jékely et al., 2018), thereby providing an excellent model to understand neuropeptide interactions between mating

duration and socio-sexual environments.

Preliminary Research

SIFamide

SIFamide (SIFa) is a conserved neuropeptide among many insect, crustacean, and arachnid species (Veenstra, Rombauts, & Grbić, 2012; Verleyen, Huybrechts, & Schoofs, 2009). In *D. melanogaster*, this neuropeptide is expressed within the pars intercerebralis and has extensive dendritic and axonal terminal distributions throughout the nervous system (Verleyen et al., 2004). Though many studies have found SIFa to be involved in multiple distinct behaviours including appetite (Martelli et al., 2017), courtship (Terhzaz, Rosay, Goodwin, & Veenstra, 2007), and sleep (S. Park, Sonn, Oh, Lim, & Choe, 2014), there are no studies to describe its role in mating duration.

A previous investigation conducted by the Kim lab has reported that SIFa holds characteristics that constitutes a cellular switch between the activation of male-specific behaviours (Schweizer, 2018). In this study, SIFa was downregulated by combining *UAS*-SIFa-RNA interference (RNAi) with pan-neuronal elav-*GAL4* driver (neuronal-specific) and found that both LMD and SMD was disrupted (Figure 3.1), suggesting that SIFa is involved in both behaviours. Next, SIFa neurons were also found to regulate the neuronal activity of both LMD and SMD. This was shown by blocking synaptic transmission by cleaving neuronal synaptobrevin (nSyb), a SNAP Receptor (SNARE) protein involved in vesicle fusion (Sweeney, Broadie, Keane, Niemann, & Kane, 1995). The results showed that both LMD and SMD were disrupted when using SIFa-*GAL4* driver with tetanus

toxin light chain (TNT.G) (Figure 3.2) (Sweeney et al., 1995). Finally, preliminary results also reported that SIFa neurons were constitutively hyperpolarized using Kir2.1 (KCNJ2), an inward-rectifying K+ channel that prevents depolarization (Baines, Uhler, Thompson, Sweeney, & Bate, 2001). This showed that SMD displayed normal behaviour whereas LMD was disrupted (Figure 3.3). In contrast, SIFa neurons were also constitutively depolarized by NaChBac::GFP, a bacterial sodium channel that increases sodium conductance (Sheeba et al., 2008). This result showed that LMD exhibited normal behaviour whereas SMD was disrupted (Figure 3.4). Altogether, both results indicate that activating SIFa neurons disrupts SMD whereas deactivating SIFa neurons disrupts LMD.

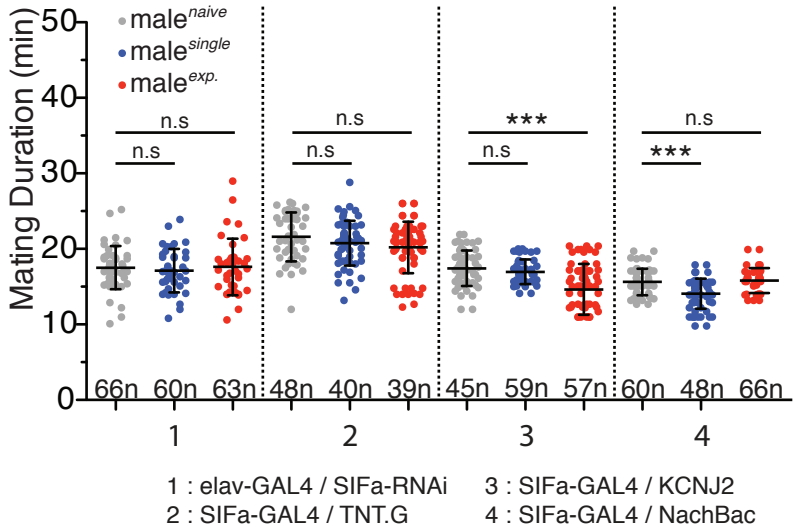


Figure 3. SIFa-mediated signaling controls the activation of both LMD and SMD. (1) Pan-neuronal (elav-*GAL4*) knockdown of SIFa (*UAS*-SIFa) disrupted both LMD and SMD. (2) Induction of synaptic blocker, *UAS*-TNT.G, into SIFa-*GAL4* cells disrupted both LMD and SMD (3) Constitutively hyperpolarizing SIFa-*GAL4* cells with *UAS*-KCNJ2 disrupted LMD but not SMD. (4) Constitutively depolarizing SIFa-*GAL4* cells with *UAS*-BaChBac disrupted SMD but not LMD. (1-4) Every dot in each functional analysis represents a single pair of flies (male and female). The numbers above the x-axis indicates n number whereas numbers below x-axis signifies different experiment. The bars in box and whisker plot indicate means ±Standard Deviations (STDs). Kruskal-

Wallis one-way analysis of variance (ANOVA) test was used for statistical analysis. NS indicates non-significance, * indicates significance ($p<0.05$), ** indicates significance ($p<0.01$) and *** indicates significance ($p< 0.001$) for comparisons to naive group. Experiments performed by Dr. Woo Jae Kim.

SiFamide Receptor

Neuropeptide-receptor pathways generally consist of G protein-coupled receptors (GPCRs), one of the largest class of receptors. GPCRs, including SIFamide Receptor (SIFaR) share common structural features (Jørgensen, Hauser, Cazzamali, Williamson, & Grimmelikhuijzen, 2006), but have different mechanics and ligands to enable their activation for various physiological and behavioural functions. In *D. melanogaster*, SIFaR-mediated signaling is involved in sleep, courtship, feeding, and sexual behaviour (Martelli et al., 2017; Sellami & Veenstra, 2015; Terhzaz et al., 2007). Though SIFaR is involved in many different behavioural paradigms, its functional significance to male-specific mating behaviours remains poorly understood.

A recent investigation conducted by the Kim lab identified that both LMD and SMD were modulated by SIFaR cells (Schweizer, 2018). This was shown by first using three available *UAS*-SIFaR-RNAi lines and combining with pan-neuronal elav-*GAL4* driver. *Dicer* (an enzyme that processes mRNA to siRNA and miRNA) was included to enhance the knockdown effect (Bernstein, Caudy, Hammond, & Hannon, 2001). The results showed that both LMD and SMD was disrupted in two of the three *GAL4* lines (Figure 4.1 and 4.2) whereas one of the three *GAL4* lines was not disrupted (Figure 4.3). There was also an attempt to create homozygous lines for the two effector lines, but this

was only successful with SIFaR-RNAi 34947. Therefore, the investigators concluded that all future SIFaR-RNAi-based screens will be performed using this line (Schweizer, 2018).

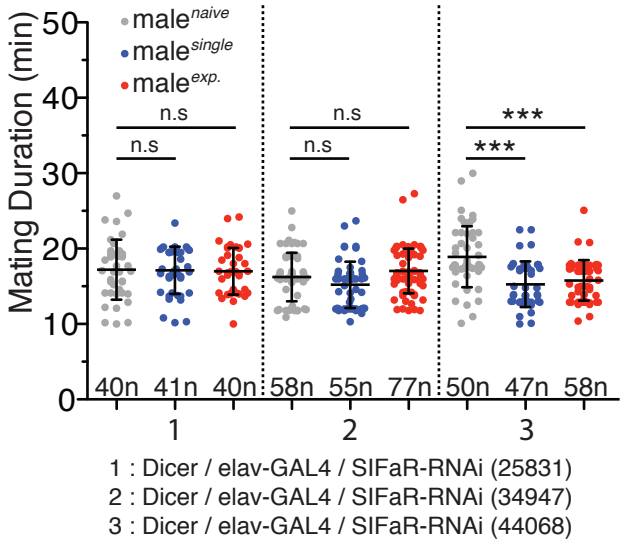


Figure 4. Investigating the functional impact of various SIFaR-RNAi lines. SIFaR was knockdown using various SIFaR-RNAi lines with pan-neuronal driver elav-*GAL4*. *Dicer* was incorporated to enhance RNAi effect. (1) LMD and SMD was disrupted using *UAS*-SIFaR-RNAi 25831 (2) LMD and SMD was disrupted using *UAS*-SIFaR-RNAi 34947. (3) LMD and SMD was not disrupted using *UAS*-SIFaR-RNAi 44068. Every dot in each functional analysis represents a single pair of flies (male and female). The numbers above the x-axis indicates n number whereas numbers below x-axis signifies different experiment. The bars in box and whisker plot indicate means ±Standard Deviations (STDs). Kruskal-Wallis one-way analysis of variance (ANOVA) test was used for statistical analysis. NS indicates non-significance, * indicates significance ($p<0.05$), ** indicates significance ($p<0.01$) and *** indicates significance ($p< 0.001$) for comparisons to naive group. Experiments performed by Justine Schweizer.

Next, it was important to determine which SIFaR-*GAL4* driver(s) elicits both LMD and SMD. This was completed by using four available SIFaR-*GAL4* drivers which expressed distinct fragments of the SIFaR promoter region. The difference in targeting distinct fragments of the promoter regions will yield diverse subset(s) of SIFaR cells (Jenett et al., 2012). When all four SIFaR-*GAL4* drivers were knockdown using *UAS*-

SIFaR-RNAi 34947, both LMD and SMD were disrupted in all SIFaR-*GAL4* drivers (Figure 5.1-5.4). Moreover, it was important to highlight that SIFaR-*GAL4* 49087 labeled cells showed that singly-reared and experienced-reared flies had an increased in mating duration when compared to naïve-reared flies (Figure 5.3). The researchers determined that cells within this driver were important to resolving a potential disinhibition circuit for both LMD and SMD.

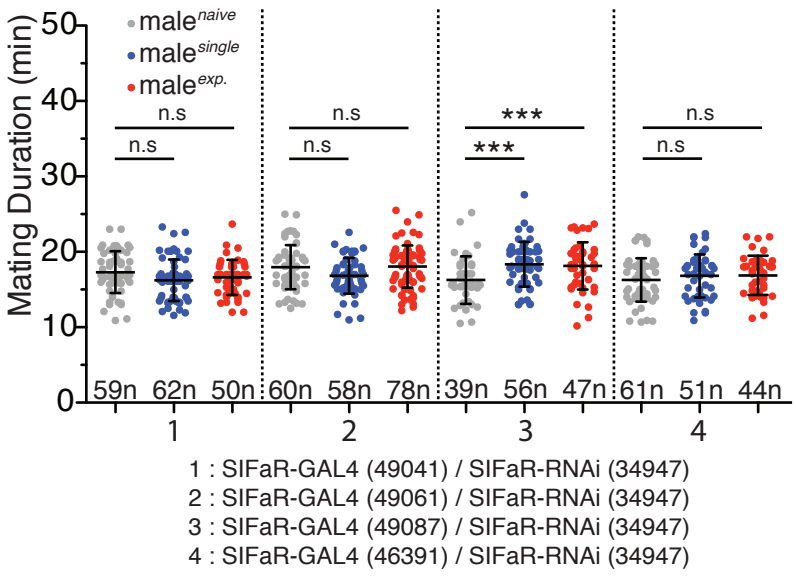


Figure 5. Identifying important SIFaR-*GAL4* drivers that modulates LMD and SMD. (1-2, 4) LMD and SMD were disrupted in SIFaR-*GAL4* drivers 49041, 49061, and 46391 were downregulated by using *UAS*-SIFaR-RNAi 34947. (3) Singly-reared and experience-reared flies had increased mating duration when compared naïve condition in SIFaR-*GAL4* drivers 49087 using *UAS*-SIFaR-RNAi 34947. Every dot in each functional analysis represents a single pair of flies (male and female). The numbers above the x-axis indicates n number whereas numbers below x-axis signifies different experiment. The bars in box and whisker plot indicate means ±Standard Deviations (STDs). Kruskal-Wallis one-way analysis of variance (ANOVA) test was used for statistical analysis. NS indicates non-significance, * indicates significance ($p<0.05$), ** indicates significance ($p<0.01$) and *** indicates significance ($p< 0.001$) for comparisons to naive group. Experiments performed by Justine Schweizer.

Hypothesis and Objectives

SIFaR-mediated signaling is essential to drive LMD and SMD. We hypothesize that two distinct signaling pathways must exist to elicit these mating paradigms through neurons which are neuropeptidergic and expresses SIFaR. The main objectives in this thesis will be to identify these neurons and create an anatomical map of their distribution throughout the nervous system. Additionally, we will evaluate the electrophysiological functions of the identified neurons to determine their functional significance on either LMD and/or SMD. Our goal is subdivided into several objectives which is as follows:

Objective A: Conduct RNAi-based genetic screening to identify neurons which are neuropeptidergic and expresses SIFaR that modulate either LMD and/or SMD.

A1. Perform *UAS*-SIFaR-RNAi (primary screen) in candidate neuropeptide-*GAL4*s to identify neuropeptidergic neurons that may be involved in LMD and/or SMD.

A2. Confirm candidate neuropeptide(s) involvement with *UAS*-neuropeptide-RNAi (secondary screen) in SIFaR-*GAL4* cells to verify its association to LMD and/or SMD.

Rationale: It is plausible that distinct subsets of neurons which are neuropeptidergic and expresses SIFaR are necessary to elicit these behaviours. The primary screen is intended to target neuropeptidergic neurons via *GAL4* drivers by knockdown of SIFaR using *UAS*-SIFaR-RNAi. In contrast, the secondary screen is designed to confirm whether targeted neuropeptide is involved by selectively downregulating its expression through RNAi in SIFaR-*GAL4* drivers. The combination of both screens will functionally suggest that one or more cells that modulates either LMD and/or SMD will be positive for SIFaR and a

selected neuropeptide of interest.

Objective B: Create a functional map of the SIFa/SIFaR microcircuit that regulates LMD and SMD.

B1. Perform double membrane labeling to SIFa and SIFaR cells by introducing SIFa-*LexA*/*lexAop*-mCD8GFP and SIFaR-*GAL4*/*UAS*-mCD8RFP to observe the morphological distribution between both cell-types.

Rationale: Assembling the morphological distribution of both SIFa and SIFaR neurons will provide clues to where the critical cells that mediate LMD and/or SMD are located.

Objective C: Construct an anatomical map of candidate neuropeptidergic neurons (objective A) and SIFaR cells which are involved in either LMD and/or SMD

All candidate neuropeptide-*GAL4* drivers will be combined with:

C1. *UAS*-mCD8RFP to visualize morphological distribution of selected neuron(s)

C2. *UAS*-Denmark to visualize dendritic arborization of selected neuron(s)

C3. *UAS*-sytGFP to visualize presynaptic terminals of selected neuron(s)

Rationale: Establishing a functional map between neuropeptidergic neurons and SIFaR cell bodies will provide insights of the cellular interactions which facilitates our male-specific mating behaviours.

Objective D: Determine the functional relevance of candidate neuropeptidergic neurons by altering its electrophysiological properties

Selected candidate neuropeptide-*GAL4* drivers will be combined with:

D1. *UAS*-KCNJ2 to prevent membrane depolarization in selected neurons

D2. *UAS*-NaChBac to induce sodium conductance in selected neurons

Rationale: Altering the electrophysiological functions of candidate neuropepitidergic neurons will describe how neuronal activity either activates or deactivates our mating paradigms.

METHODS

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Table 1. Overview of resources/fly Lines used to perform current study.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primary Antibodies		
Chicken polyclonal α-GFP	abcam	ab13970
Rabbit polyclonal α-dsRed	Living Colors	
Mouse monoclonal α-nc82	DSHB	AB_2314866
Secondary Antibodies		
TRITC	Jackson ImmunoResearch	111-025-003
Alexa Fluor 647	Jackson ImmunoResearch	115-605-003
Alexa Fluor 488	Jackson ImmunoResearch	112-545-003
Chemicals, Peptides, and Recombinant Proteins		
Ethyl Alcohol Anhydrous	Commercial Alcohols	P016EAAN
Fluoroshield Mounting Medium	abcam	ab104135
Normal Donkey Serum	abcam	ab7475
Paraformaldehyde (20%)	EMS	MR3482
Schneider's Drosophila Medium (1X)	Gibco	21720-024
Experimental Models: Organisms/Strains		
AKH-GAL4	Jan A. Veenstra	N/A
UAS-AKH-RNAi	Blooming Stock Center	34960
UAS-AKH-RNAi	Vienna Stock Center	11352
AstA-GAL4	Blooming Stock Center	51979
UAS-AstA-RNAi	Blooming Stock Center	25866
Burs-GAL4	Blooming Stock Center	40972
UAS-Burs-RNAi	Blooming Stock Center	26719
UAS-Burs-RNAi	Vienna Stock Center	13520
CAPA-GAL4	Blooming Stock Center	51969
UAS-CAPA-RNAi	Blooming Stock Center	28345
UAS-CAPA-RNAi	Vienna Stock Center	41124
Crz-GAL4	Blooming Stock Center	51976
UAS-Crz-RNAi	Vienna Stock Center	30670
UAS-Denmark, UAS-sytGFP	N/A	N/A
DH31-GAL4	Blooming Stock Center	51988
DH44-GAL4	Blooming Stock Center	51987
UAS-DH44-RNAi	Blooming Stock Center	25804
UAS-DH44-RNAi	Vienna Stock Center	108473
UAS-DH44-RNAi	Blooming Stock Center	45054
DSK-GAL4	Blooming Stock Center	51981
UAS-DSK-RNAi	Blooming Stock Center	25869
EH-GAL4	Blooming Stock Center	51974
ETH-GAL4	Blooming Stock Center	51982

UAS-ETH-RNAi	Blooming Stock Center	26242
FMRFa-GAL4	Blooming Stock Center	51990
UAS-FMRFa-RNAi	Blooming Stock Center	58197
UAS-KCNJ2-eGFP	Jan Lab	N/A
LK-GAL4	Blooming Stock Center	51993
UAS-LK-RNAi	Blooming Stock Center	14091
UAS-mCD8RFP, LexAop-mCD8GFP	Blooming Stock Center	32229
MIP-GAL4	Blooming Stock Center	51984
UAS-MIP-RNAi	Blooming Stock Center	41680
UAS-MIP-RNAi	Blooming Stock Center	5294
MS-GAL4	Blooming Stock Center	51986
UAS-MS-RNAi	Blooming Stock Center	26245
UAS-NaChBac-eGFP	Jan Lab	N/A
Pburs-GAL4	Blooming Stock Center	27142
SIFa-GAL4	Jan A. Veenstra	N/A
SIFaR-GAL4	Blooming Stock Center	49087
sNPF-GAL4	Blooming Stock Center	46382
Tsh-GAL80	N/A	N/A
TK-GAL4	Blooming Stock Center	51974
UAS-TK-RNAi	Blooming Stock Center	25800
UAS-TK-RNAi	Vienna Stock Center	103662
Software and Algorithms		
Adobe Illustrator CS3	Adobe	N/A
Prism 5 for Mac OS X	GraphPad Software	N/A
Other		
LSM800 AxioObserverZ1	Zeiss	N/A

Mating Duration Assay

Transgenic flies in this study were all raised and maintained in similar environments except when assigned to a specific experimental condition. Each fly was raised in 12-hour light:12-hour night cycles at 25°C in 55% relative humidity until mating assay. For each mating duration assay, the age of each male fly was between five to eight days old whereas the female fly was less than ten days old. The female fly or virginizer deficiency (VDF) flies are created by inserting hs-hid transgene into Y chromosome which are placed in 37°C water bath for 90 min. Hs-hid is temperature sensitive transgene that

activates caspases to induce programmed apoptosis.

Our LMD paradigm is measured when comparing the group-reared vs. single-reared treatment whereas SMD behaviour is calculated when you compare the group-reared vs. experienced-reared treatment (Figure 6). Male flies which are set in either group-reared or sexual-experienced-reared conditions are raised in a vial with ten males total whereas the singly-reared treatment contains only one male fly per vial. For SMD behaviour and one day prior to assay, a 2:1 ratio of female flies (20 flies or more) is added to the experienced-reared treatment to induce a female enriched environment.

Five days after the initial setup for assay, both male and female flies were anaesthetized using CO_2 and placed into their respective treatment groups in our mating chamber. VDF females were placed into the bottom compartment whereas the males placed in the upper compartment. A thin transparent film separates the male and female fly. After placing the flies into each of their compartments, the chamber is placed in 25°C incubator for 1.5 hours to allow flies to recover from CO_2. After designated time has passed, transparent film was removed and timer of one hour to record mating duration was initiated. Only the pair of flies that complete their mating duration within the one-hour cycle is recorded for further statistical analysis. All assays were performed between 10AM to 4PM.

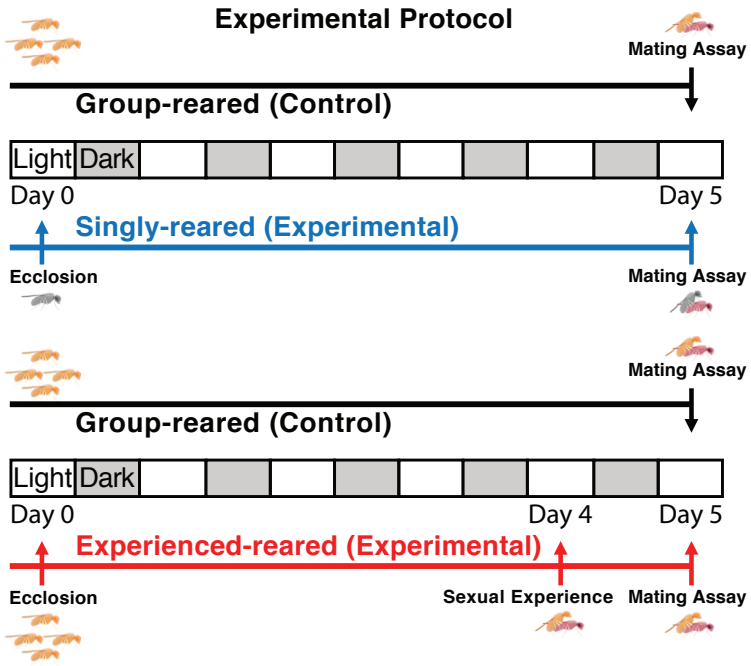


Figure 6. Experimental timeline to setup LMD and SMD paradigm. For LMD, male flies are assigned to either group-reared or singly-reared condition. Mating assay is performed five days after initial setup. For SMD, male flies are assigned to either group-reared or experience-reared condition. One day prior to assay, a 2:1 ratio or greater of VDF female flies are added to the experience-reared condition to include a female enriched socio-sexual environment. SMD mating assay is performed five days after initial setup.

RNAi-based Screen Analysis

Our RNAi-based strategy is intended to identify neuropeptides involved in our mating duration behaviours. This will be achieved by designing two types of screens that will determine which neuroepeptidergic neurons that express SIFaR are relevant for either LMD and/or SMD. Our *UAS*-SIFaR-RNAi screening (primary screen) will knockdown a known pathway important for our paradigm in candidate neuropeptidergic neurons (left side of Figure 7). After this screening, all candidate neuropeptides will be downregulated by implementing *UAS*-neuropeptide-RNAi (secondary screen) in SIFaR-*GAL4* labeled

cells (right side of Figure 7). Altogether, our screening approach will complement each other and the identity of novel neuropeptide function in male-specific mating behaviours will be achieved.

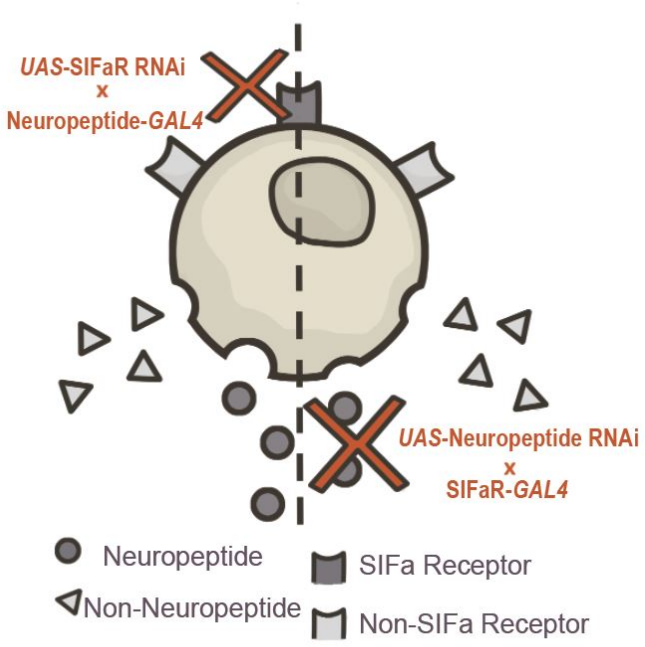


Figure 7. Experimental protocol for RNAi-based screens. Two distinct RNAi screens are designed to identify novel neuropeptide function in either LMD and/or SMD. The primary screen (left side) will knockdown SIFaR, an essential receptor, in candidate neuropeptides. Once candidate neuropeptides are recognized, a secondary screen (right side) will knockdown its expression in SIFaR-*GAL4* labeled cells. Illustration completed by Elizabeth Lebedev.

When performing any genetic modification (i.e. RNAi) to either LMD and/or SMD, we are seeking a non-significant (n.s) which we describe the behaviour(s) to be disrupted. This indicates that knockdown of selected transgene is functionally relevant (Figure 8.2,

8.3, and 8.4). In contrast, when a result is significant (***) then this would signify that the behaviour(s) remains intact or normal and that the selected transgene is not functionally relevant (Figure 8.1, 8.2, and 8.3). It is also important to highlight that we only interpret our data if we observe a n.s result as we cannot conclude if a specific transgene is functionally involved if we detect *** result. This is because our wild-type phenotype displays a significant result (***) for both of our mating paradigms.

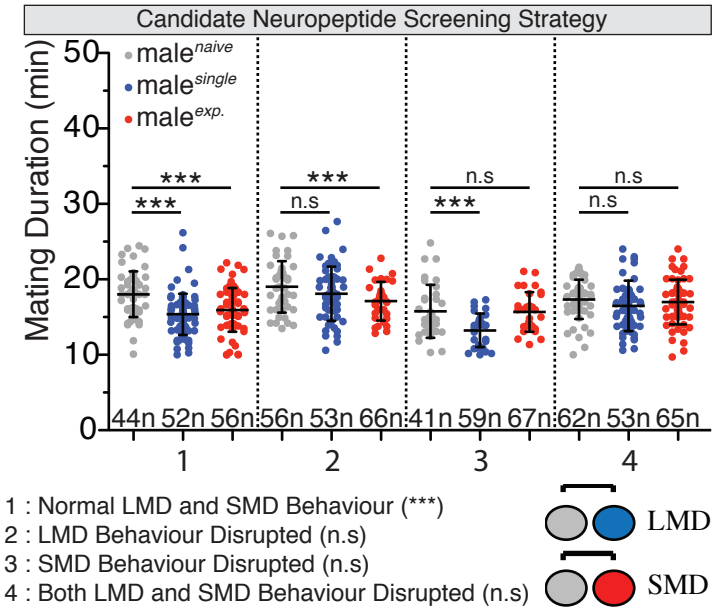


Figure 8. Hypothetical results for RNAi screening procedure. There are four possible interpretations for our results when using knockdown of selected transgene in specific type of tissue. (1) LMD and SMD remains intact for selected transgene in specific type of tissue. (2) Selected transgene disrupted LMD but not SMD in specific type of tissue. (3) Selected transgene disrupted SMD but not LMD in specific type of tissue. (4) Both LMD and SMD was disrupted in selected transgene in specific type of tissue. The numbers above the x-axis indicates n number whereas numbers below x-axis signifies different experiment. The bars in box and whisker plot indicate means ±Standard Deviations (STDs). Kruskal-Wallis one-way analysis of variance (ANOVA) test was used for statistical analysis. NS indicates non-significance, * indicates significance ($p<0.05$), ** indicates significance ($p<0.01$) and *** indicates significance ($p< 0.001$) for comparisons to naive group.

Image Analysis

All microscopic observations were conducted using Zeiss LSM800 AxioObserverZ1 mot Confocal Microscope (Carl Zeiss, Oberkochen, Germany) using x20 high aperture objective and scanned labeled specimens with a z-step size ranging from 0.6 to 1.0 µm. Microscopic images were further processed using ImageJ and Adobe Illustrator CS3.

Immunohistochemistry

For immunohistochemistry, adult *Drosophila* brains were dissected in ice-cold Schneider's medium (S2) and fixed in 1.2% paraformaldehyde for overnight period. Next, fly brains were rinsed eight times with 0.5% Triton X-100 (PBT) at room temperature with intervals of five minutes between each wash. The brains were then blocked in 5% normal donkey serum (NDS) for two hours then added primary antibody for overnight at 4°C. The sample is followed by fluorophore-conjugated secondary antibodies for one overnight at 4°C. Next, the brains were placed on antifade mounting solution (Invitrogen) on slides for imaging.

The following antibodies were used: chicken anti-GFP (abcam, 1:1000), rabbit anti-dsred (Clontech, Living colors dsRed polyconal AB, 1:250), mouse anti-nc82 (DSHB, monoclonal AB, 1:50).

Statistical Tests

Graphpad Prism 5 was used for statistical testing for all experimental conditions. An

independent t-test was conducted to compare two indepedent groups. Kruskal-Wallis test was used to compare group-reared with singly-reared and experienced-reared to determine significance. (*** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$), ns = non-significant).

Results

Unraveling the neural circuits that governs mating behaviours is important for modern neuroscience. One effective approach to achieve this goal is to conduct a RNAi-based screen to understand the relationship between mating duration and gene encoding neuropeptides. It is plausible that novel functions of several neuropeptides will be identified since some candidate genes are likely to be sensitive to gene silencing. Our next objective will be creating an anatomical map to visualize the morphological distribution of these neuropeptidergic circuits to further understand the mechanisms of LMD and SMD. In summary, our study is intended to segregate the integrative circuits between SIFa signaling and mating duration, thus creating a suitable model for studying neuropeptidergic circuit interactions.

Candidate Neuropeptide Screening via SIFaR-RNAi (Primary)

To begin our study, we performed a primary RNAi-based screen to determine which candidate neuropeptidergic neurons contains SIFaR and modulate either LMD and/or SMD. Neuropeptide-*GAL4* drivers and *UAS*-RNAi effector lines were obtained from various *Drosophila* stock centers and independent researchers (Table 1). Screening for candidate neuropeptides was performed using *D. melanogaster* by gene silencing

SIFaR via *UAS*-SIFaR-RNAi 34947 in candidate neuropeptide-*GAL4* labeled cells. In our primary screen, we assessed 19 different neuropeptides and found several significant and non-significant results that functionally modulated the mating duration of LMD and/or SMD (green column in Table 2). When downregulating SIFaR, we found that LMD was disrupted in Capability (CAPA)- (Figure 9.3), FMRFamide (FMRFa)- (Figure 9.4), and Myosuppressin (MS)-*GAL4* cells (Figure 9.6), SMD was disrupted in Adipokinetic Hormone (AKH)- (Figure 9.1), Allatostatin A (AstA)- (Figure 9.2), and Leucokinin (LK)-*GAL4* cells (Figure 9.5), and both LMD and SMD was disrupted in Proctolin (Proc)-*GAL4* cells (Figure 9.7). In summary, our findings suggest that LMD is mediated by CAPA, FMRFa and MS cells, SMD is mediated by AKH, AstA, and LK cells, and both LMD and SMD are mediated by Proc cells.

Table 2. Primary and Secondary RNAi-based Screens Used to Identify Candidate Neuropeptides

NP	ID	GAL4	RNAi	SIFaR-RNAi (34947)		SIFaR-GAL4 (49087)	
				LMD	SMD	LMD	SMD
AKH	Veenstra	GAL4		n.s	n.s		
	34960		RNAi			**	n.s
	v11352		RNAi			***	*
AstA	51979	GAL4		**	n.s		
	25866		RNAi			n.s	n.s
AstC	39448	GAL4		*	n.s		
	25868		RNAi			No Data	n.s
Burs	40972	GAL4		n.s	n.s		
	26719		RNAi			*	***
	v13520		RNAi			***	**
CAPA	51969	GAL4		n.s	***		
	28345		RNAi			***	No Data
	v41124		RNAi			n.s	No Data
Crz	51976	GAL4		***	**		
	v30670		RNAi			n.s	n.s
DH31	51988	GAL4		*	**		
DH44	51987	GAL4		***	n.s		
	25804		RNAi			***	*
	v108473		RNAi			No Data	***
	45054		RNAi			**	*
DSK	51981	GAL4		**	***		
	25869		RNAi			n.s	n.s
EH	51974	GAL4		***	**		
ETH	51982	GAL4		***	n.s		
	26242		RNAi	***	**		
FMRFa	51990	GAL4		n.s	*		
	58197		RNAi			n.s	No Data
LK	51993	GAL4		***	n.s		
	14091		RNAi			No Data	n.s
MIP	51984	GAL4		***	n.s		
	41680		RNAi			***	**
	5294		RNAi			***	**
MS	51986	GAL4		n.s	*		
	26245		RNAi			n.s	n.s
Pburs	v27142		RNAi			n.s	n.s
Proc	51972	GAL4		n.s	n.s		
	29570		RNAi			n.s	n.s
	v102488		RNAi			***	***
sNPF	46382	GAL4		**	***		
TK	51974	GAL4		**	*		
	25800		RNAi			n.s	n.s
	v103662		RNAi			n.s	***

(NP indicates neuropeptide; ID indicates identification from Bloomington Stock Centre, Vienna Stock Centre, or other researchers; GAL4 indicates selected neuropeptide crossed with SIFaR-RNAi; RNAi indicates selected neuropeptide crossed with SIFaR-GAL4)

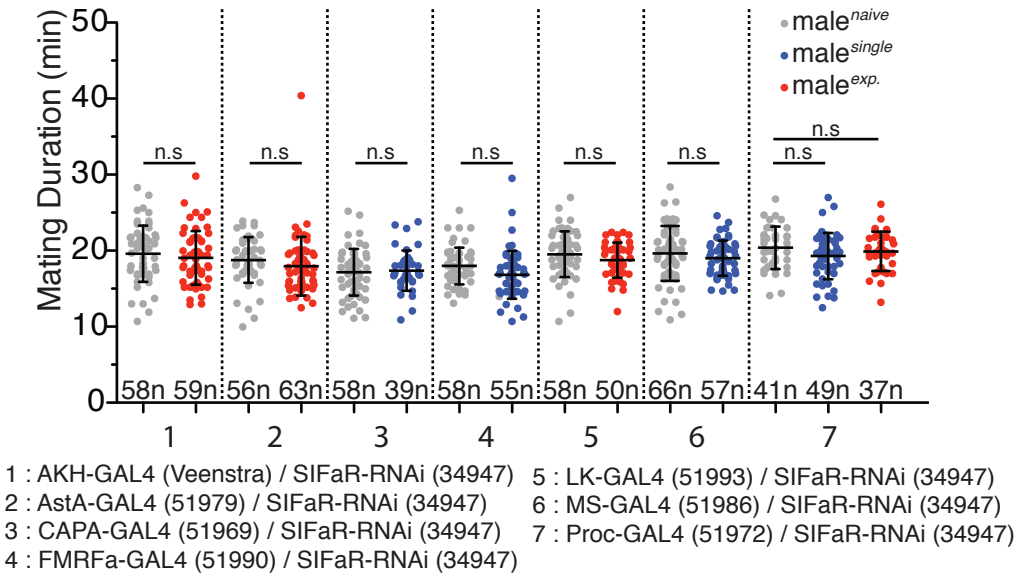


Figure 9. Seven candidate neuropeptides that may be involved in modulating LMD and/or SMD. (1-7) Knockdown of SIFaR via *UAS*-SIFaR-RNAi in various neuropeptide-*GAL4* drivers showed disruption in LMD and/or SMD. (1) SMD was disrupted in AKH-*GAL4* labeled cells. (2) SMD was disrupted in AstA-*GAL4* labeled cells. (3) LMD was disrupted in CAPA-*GAL4* labeled cells. (4) LMD was disrupted in FMRFa-*GAL4* labeled cells. (5) SMD was disrputed in LK-*GAL4* labeled cells. (6) LMD was disrupted in MS-*GAL4* labeled cells. (7) Both LMD and SMD was disrupted in Proc-*GAL4* labeled cells. The numbers above the x-axis indicates n number whereas numbers below x-axis signifies different experiment. The bars in box and whisker plot indicate means ±Standard Deviations (STDs). Kruskal-Wallis one-way analysis of variance (ANOVA) test was used for statistical analysis. NS indicates non-significance, * indicates significance ($p<0.05$), ** indicates significance ($p<0.01$) and *** indicates significance ($p< 0.001$) for comparisons to naive group.

Dimmed (DIMM) is a transcription factor that organizes differential neuronal functions and properties between one neuron to another (Park, Veenstra, Park, & Taghert, 2008). To determine if DIMM-positive (DIMM+) cells modulates LMD and/or SMD, we used *UAS*-SIFaR-RNAi 34947 with DIMM-*GAL4* driver and found that SMD but not LMD was disrupted when downregulating SIFaR (Figure 10). This suggests that SMD but not LMD relies on SIFaR in DIMM+ cells to elicit its behaviour (Schweizer, 2018).

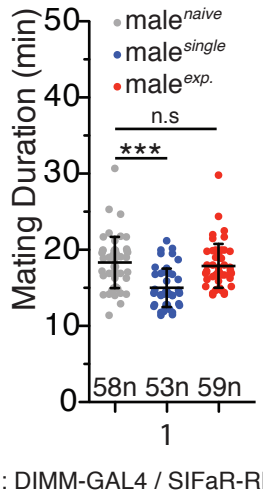


Figure 10. The transcription factor DIMM is important for SMD but not LMD. (1) Genetic disruption in SIFaR expression levels in DIMM cells disrupted SMD but not LMD behaviour. The numbers above the x-axis indicates n number whereas numbers below x-axis signifies different experiment. The bars in box and whisker plot indicate means ±Standard Deviations (STDs). Kruskal-Wallis one-way analysis of variance (ANOVA) test was used for statistical analysis. NS indicates non-significance, * indicates significance ($p<0.05$), ** indicates significance ($p<0.01$) and *** indicates significance ($p< 0.001$) for comparisons to naive group. Experiment performed by Justine Schweizer.

Confirmation Neuropeptide Screening via Neuropeptide-RNAi (Secondary)

Our primary screen identified seven candidate neuropeptides which were found to involved in either LMD and/or SMD. Verifying its functional involvement is achievable by designing a secondary neuropeptide RNAi-based screen via candidate neuropeptide-RNAi to knockdown its expression in SIFaR-*GAL4* labeled cells. This will confirm that the neuropeptide, as well as SIFaR is responsible for mediating our male-specific mating behaviours.

Our secondary neuropeptide-RNAi based screen used 28 different neuropeptide RNAi lines which targeted several of our candidate neuropeptides. Our results showed many significant and non-significant results among the RNAi lines (blue column in Table 2). For our selected results, we highlight that knockdown of candidate neuropeptides in SIFaR-*GAL4* drivers showed that CAPA (Figure 11.3), FMRFa (Figure 11.4), and MS disrupted LMD (Figure 11.6), AKH (Figure 11.1), AstA (Figure 11.2), and LK disrupted SMD (Figure 11.5), and Proc disrupted both LMD and SMD (Figure 11.7). In conclusion, our data indicates that CAPA, FMRFa, and MS are required for LMD, AKH, AstA, and LK are required for SMD, and Proc is required for both LMD and SMD.

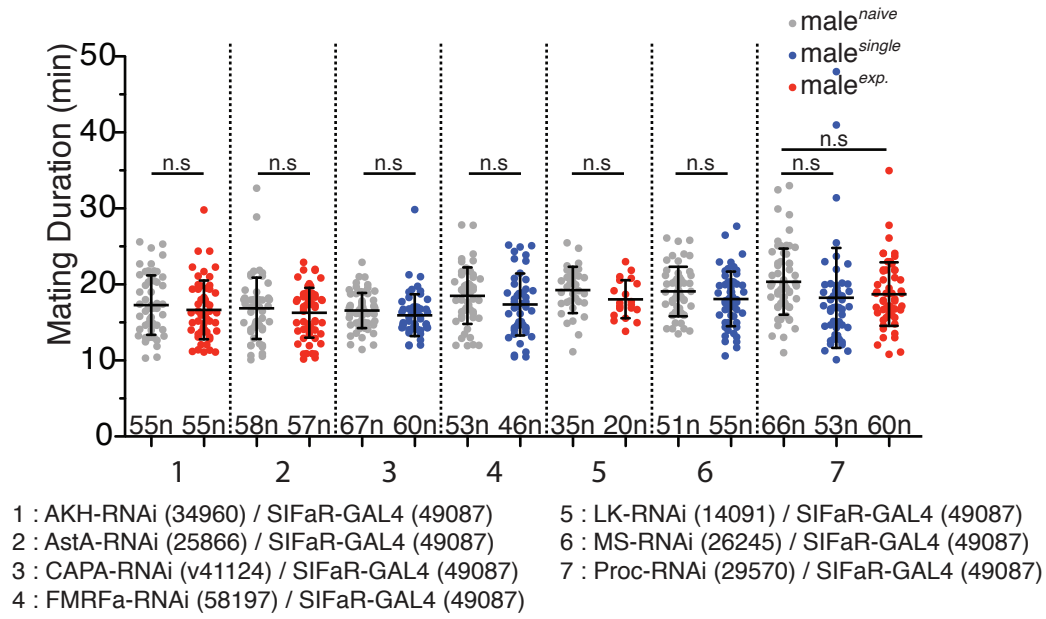


Figure 11. Seven candidate neuropeptides are functionally involved in modulating LMD and/or SMD. (1-7) Each candidate neuropeptide was knockdown with their respective RNAi line via *UAS*-Neuropeptide-RNAi in relevant SIFaR-*GAL4* labeled cells. (1) SMD was disrupted using *UAS*-AKH-RNAi. (2) SMD was disrupted using *UAS*-AstA-RNAi. (3) LMD was disrupted using *UAS*-CAPA-RNAi. (4) LMD was disrupted using *UAS*-FMRFa-RNAi. (5) SMD was disrupted using *UAS*-LK-RNAi. (6) LMD was disrupted using *UAS*-MS-RNAi. (7) Both LMD and SMD was disrupted using *UAS*-Proc-RNAi. The numbers above the x-axis indicates n number whereas numbers below x-axis signifies different experiment. The bars in box and whisker plot indicate means ±Standard Deviations (STDs). Kruskal-Wallis one-way analysis of variance (ANOVA)

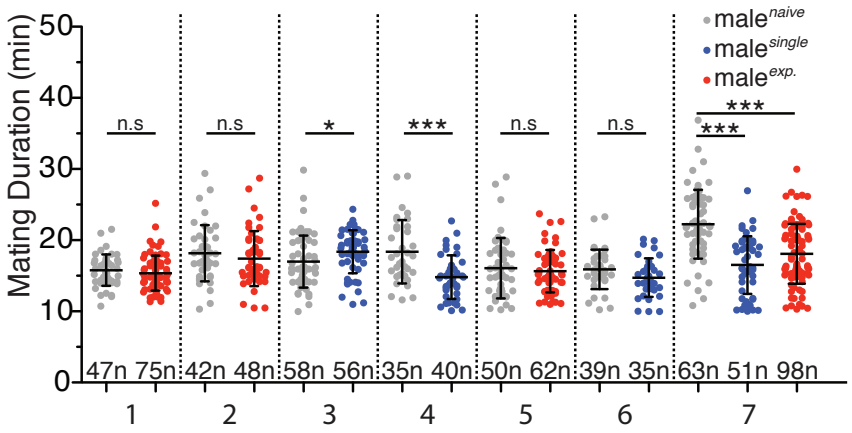
test was used for statistical analysis. NS indicates non significance, * indicates significance ($p<0.05$), ** indicates significance ($p<0.01$) and *** indicates significance ($p< 0.001$) for comparisons to naive group.

CAPA, FMRFa, and Proctolin in the Ventral Nerve Cord are required for LMD and/or SMD

The nervous system in *D. melanogaster* is subdivided into the central brain and ventral nerve cord (VNC). It is feasible to target a smaller neuronal subpopulation of target cells by performing intersectional strategies. To narrow down which cells are important for our mating duration paradigms, restricting *GAL4* expression to specific areas of the nervous system can achieved by incorporating a *GAL4*-inhibitor known as *GAL80*. T-shirt (Tsh) is a protein that is mostly expressed in the VNC region (Meissner, Manoli, Chavez, Knapp, & Lin, 2011) and can be combined with the inhibitor to create Tsh-*GAL80* (Venken, Simpson, & Bellen, 2011a).

To determine which region contains our important cells, we incorporated Tsh-*GAL80* to our secondary neuropeptide-RNAi based screen. This modified screen will reduce candidate neuropeptide expression via *UAS*-neuropeptide-RNAi in SIFaR-*GAL4* cells to the central brain only (Figure 2). The results of our modified secondary screen showed that MS disrupted LMD (Figure 12.6), knockdown of CAPA resulted in singly-reared flies to copulate longer than naïve condition (Figure 12.3), and FMRFa did not disrupt LMD (Figure 12.4). For SMD, we found that AKH (Figure 12.1), AstA (Figure 12.2), and LK all disrupted SMD when knockdown was applied to central brain only (Figure 12.5). Finally, both LMD and SMD were not disrupted when knockdown of Proc

was limited to central brain only (Figure 12.7). There are several different outcomes as a result of this screen. Firstly, MS neurons in the central brain modulate LMD, FMRFa neurons in the VNC mediate LMD, and CAPA neurons in both central brain and VNC differentially mediate LMD. Specifically, we observed a potential disinhibition circuit when knockdown of CAPA was restricted to central brain neurons only. Secondly, AKH, AstA, and LK neurons in the central brain are responsible for controlling SMD. Lastly, Proc neurons in the VNC regulate both LMD and SMD. In summary, we have found a multitude of neuropeptidergic circuits distributed across the nervous system which are involved in either LMD and/or SMD.



1 : AKH-RNAi (34960) / SIFaR-GAL4 (49087) / Tsh-GAL80
2 : AstA-RNAi (25866) / SIFaR-GAL4 (49087) / Tsh-GAL80
3 : CAPA-RNAi (v41124) / SIFaR-GAL4 (49087) / Tsh-GAL80
4 : FMRFa-RNAi (58197) / SIFaR-GAL4 (49087) / Tsh-GAL80
5 : LK-RNAi (14091) / SIFaR-GAL4 (49087) / Tsh-GAL80
6 : MS-RNAi (26245) / SIFaR-GAL4 (49087) / Tsh-GAL80
7 : Proc-RNAi (29570) / SIFaR-GAL4 (49087) / Tsh-GAL80

Figure 12. CAPA, FMRFa, and Proctolin modulates male-specific behaviours in VNC. (1-7) Each candidate neuropeptide was knockdown in central brain only using appropriate *UAS*-Neuropeptide-RNAi in SIFaR-*GAL4* labeled cells. (1) SMD was disrupted using *UAS*-AKH-RNAi with Tsh-*GAL80*. (2) SMD was disrupted using *UAS*-AstA-RNAi with Tsh-*GAL80*. (3) Singled-reared flies mated longer than naïve condition flies using *UAS*-CAPA-RNAi with Tsh-*GAL80*. (4) LMD was not disrupted using *UAS*-FMRFa-RNAi with Tsh-*GAL80*. (5) SMD was disrupted using *UAS*-LK-RNAi with Tsh-*GAL80*. (6) LMD was disrupted using *UAS*-MS-RNAi with Tsh-*GAL80*. (7) Both LMD and SMD was not disrupted using *UAS*-Proc-RNAi with Tsh-*GAL80*. The numbers above the x-axis indicates n number whereas numbers below x-axis signifies different

experiment. The bars in box and whisker plot indicate means ±Standard Deviations (STDs). Kruskal-Wallis one-way analysis of variance (ANOVA) test was used for statistical analysis. NS indicates non-significance, * indicates significance ($p>0.05$), ** indicates significance ($p<0.01$) and *** indicates significance ($p< 0.001$) for comparisons to naive group.

Critical LMD and SMD cells are expressed in both SIFaR-*GAL4* and SIFaR-*LexA* drivers

Our study has functionally confirmed seven neuropeptides to be involved in male-specific mating behaviours. The next task is to visualize the expression pattern for each neuropeptide as it will provide clues to determine their functional significance. To do this, two distinct binary systems, *GAL4/UAS* and *LexA/LexAop* (Venken et al., 2011a), will be used to target the expression of both SIFaR and the seven neuropeptides. Since our previous neuropeptide-*GAL4* screening was performed using the *GAL4/UAS* system, we decided to use *LexA/LexAop* system to visualize the expression pattern of SIFaR.

Before performing an anatomical-based screen for our selected neuropeptides, we must verify that SIFaR-*LexA* and SIFaR-*GAL4* cells contain the same or similar cells that modulate our male-specific mating behaviours. Our approach used fluorescent proteins to be overexpressed into the cytoplasmic compartment of the cells of interest, this will provide a morphological distribution of SIFaR cells (Venken et al., 2011a). To do this, we used *UAS*-mCD8RFP with SIFaR-*GAL4* and *LexAop*-mCD8GFP with SIFaR-*LexA* to drive membrane expression in both central brain and VNC. Our results revealed that both SIFaR-*GAL4* and SIFaR-*LexA* labeled cells show some variance in both optic lobes and VNC region. However, the expression pattern restricted to the medial subesophageal

ganglion (SOG) area was nearly identical (yellow arrowheads indicating overlapping SIFaR-*GAL4* and SIFaR-*LexA* cells in Figure 13). Our findings suggest that the SIFaR-*GAL4* and SIFaR-*LexA* cells contain the important cells that modulate LMD and SMD.

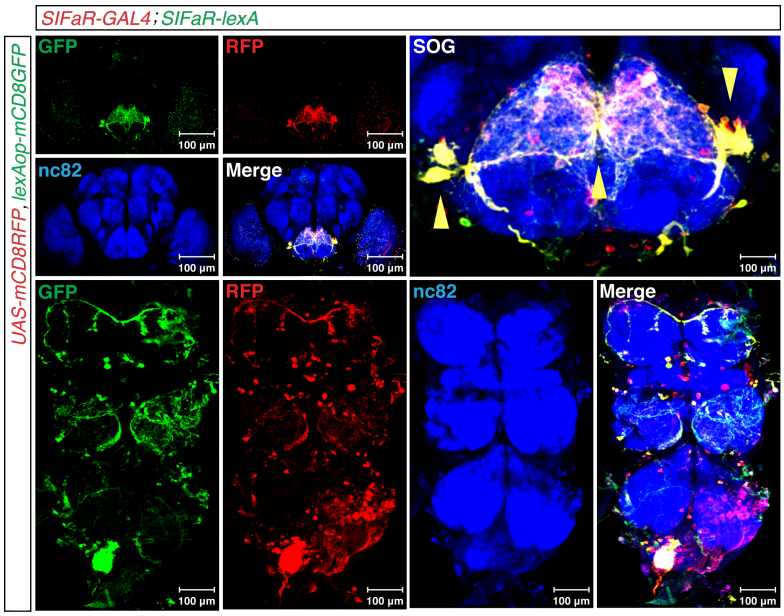


Figure 13. SIFaR-*GAL4* and SIFaR-*LexA* cells contain overlapping subpopulation cells which are critical to mediate LMD and SMD. Expression of *UAS*-mCD8RFP (red) shows the functional SIFaR-*GAL4* cells used in our RNAi-based screens whereas *LexA*-mCD8GFP (green) displays SIFaR-*LexA* cells to be used in our expression data. Yellow arrowheads indicate SIFaR-*GAL4* and SIFaR-*LexA* contain overlapping cell bodies in the SOG region. Brain neuropil (blue) is stained with nc82 that labels brunchpilot (brp), a cytoskeletal protein used for structural integrity (Smith & Taylor, 2011).

SIFa Forms Potential Synapses with SIFaR in the Medial SOG Region

Constructing a neuronal map between SIFa and SIFaR will provide a basis to understand their functional role in male-specific mating behaviours. To visualize the morphology between SIFa and SIFaR cell bodies, we used two distinct membrane

markers that merged SIFa-*GAL4* with *UAS*-mCD8RFP and SIFaR-*LexA* with *LexAop*-mCD8GFP. Our membrane expression data showed that SIFa has extensive distributions primarily towards the medial central brain and throughout the VNC region whereas SIFaR forms an expression pattern that is restricted to the SOG and superior region of the VNC. More importantly, our analysis displays potential overlapping cell bodies between SIFa and SIFaR in the medial SOG (yellow arrowheads in Figure 14), which suggests potential synapse formation.

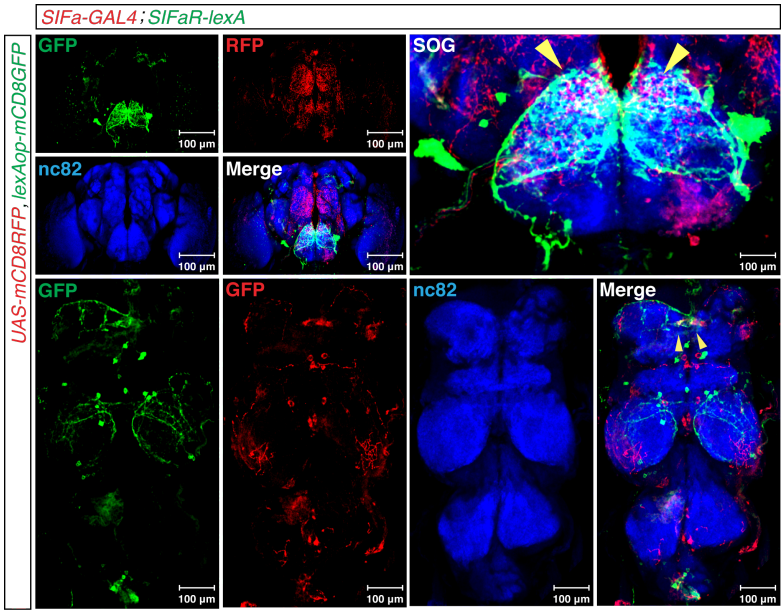


Figure 14. Morphological distribution between SIFa and SIFaR cells. SIFa-*GAL4* cells were labeled with *UAS*-mCD8RFP membrane marker (red) whereas SIFaR-*LexA* cells were labeled with *LexAop*-mCD8GFP (green) membrane marker. The yellow arrowheads indicate potential synapse formation in the SOG

To offer additional evidence whether synaptic formation between SIFa and SIFaR occurred in the SOG, we utilize a dendritic marker, DenMark, with a presynaptic marker, sytGFP (Venken et al., 2011a), into both SIFa-*GAL4* and SIFaR-*GAL4* drivers. Our results confirmed that SIFa axon terminals (green arrowheads in top half of Figure 15.1)

and SIFaR dendrites (red arrowheads in bottom half of Figure 15.2) were localized to the SOG region, indicating that synaptic contact is plausible.

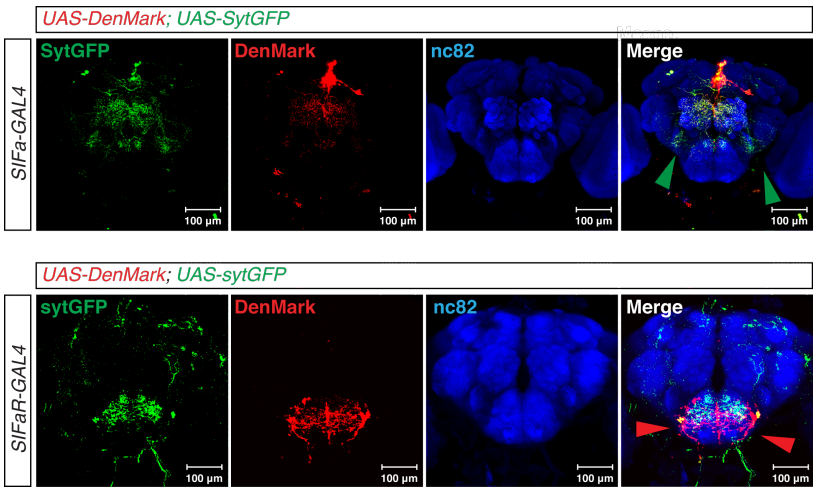


Figure 15. SIFa axon terminals and SIFaR dendrites are localized in SOG region. Anatomical data indicates potential synapses being formed between SIFa and SIFaR cells in the medial SOG region. Green arrowheads indicate presynaptic terminals of SIFa cells whereas red arrowheads indicate dendrites of SIFaR cells. Brain neuropil labeled with nc82. SIFa imaging data provided by Dr. Woo Jae Kim.

Functional Mapping of Candidate Neuropeptidergic Neurons

Visualizing the anatomical distribution of our selected neuropeptides will provide greater insights in understanding the association between neuropeptidergic signaling and our mating paradigms. To determine which candidate neuropeptidergic neurons express SIFaR, we used a double membrane marker to target both cell-types. We mapped the neuronal circuitry of each candidate neuropeptide by combining *UAS*-mCD8RFP with neuropeptide-*GAL4* drivers and *LexAop*-mCD8GFP with SIFaR-*LexA* drivers to generate membrane expression. Though the expression data of SIFaR-*LexA* cells may vary from each sample, we summarized the entirety of each sample into one generalized observation. Our expression data indicates that SIFaR contains between 20 to 50 cell

bodies in each optic lobe and has approximately five to nine pairs of cells formed in an intricate but symmetrical pattern in the SOG region. In the VNC, approximately eight to twelve cell bodies were found in the medial superior region whereas a large cluster of cells are found in the inferior region (Figure 14, 16, 17, 18, 19, 20, 21, and 22).

Our results for AKH found no neurons to be expressed in the central brain or VNC (Figure 16). Cell bodies for AstA were expressed in lateral optic lobes, potentially including one DIMM+ neuron in each lobe (blue arrowheads) and inferior VNC region (Figure 17). For CAPA neurons, we found four cell bodies in the superior SOG which includes one pair of DIMM+ (blue arrowheads) and one pair of DIMM-negative (DIMM-) (white arrowheads) and various cell bodies, including another pair of DIMM+ (blue arrowheads) found within the VNC region (Figure 18). Our expression data for FMRFa neurons reveal several cell bodies limited to the optic lobe region (Figure 19). We found one DIMM+ lateral horn leucokinin (LHLK) neuron (red arrowhead) and one subesophageal ganglion leucokinin (SELK) neuron that expresses SIFaR (yellow arrow) in the central brain (Figure 20). MS neurons were stained primarily in the optic lobe and medial SOG regions (Figure 21). The expression of Proc is distributed to both the central brain and VNC region. In the central brain, we found four Proc neurons that expresses SIFaR in the SOG (yellow arrowheads) and many cell bodies among the lateral VNC region. In summary, we have found many complex neuropeptidergic circuits which are interconnected with SIFaR-mediated signaling to elicit our mating paradigms.

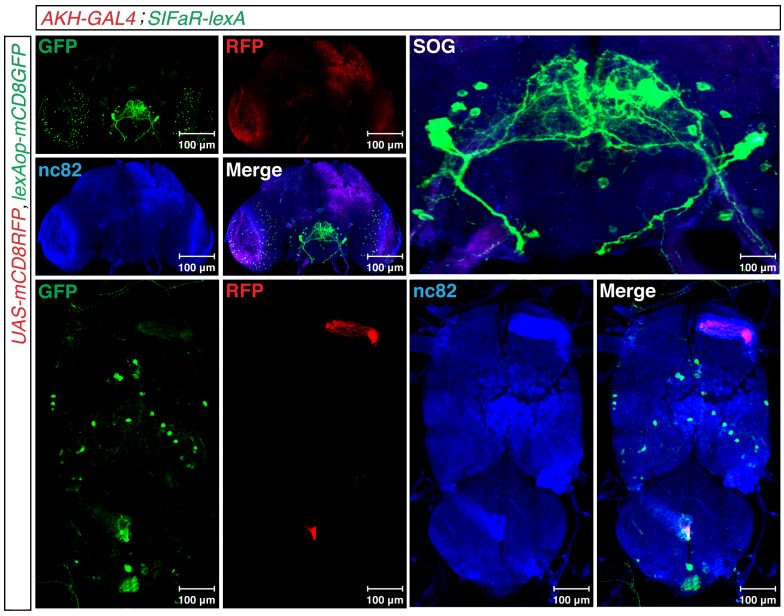


Figure 16. AKH cell bodies were not expressed in both the central brain and VNC. *UAS*-mCD8RFP was combined with AKH-*GAL4* (red) and *LexAop*-mCD8GFP was combined with SIFaR-*LexA* (green) to create membrane expression data. The *GAL4* driver used to target AKH showed no cells in the central brain or the VNC region. Many SIFaR cell bodies were found in optic lobes, five-six pairs in SOG, and approximately 15 cell bodies found among the VNC region. Adipokinetic Hormone (AKH).

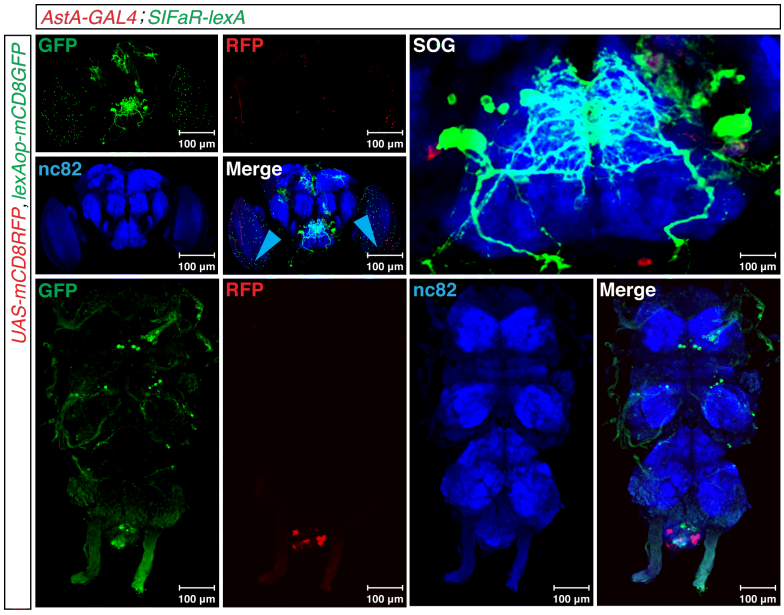


Figure 17. One pair of DIMM+ AstA cell bodies found in the optic lobes. *UAS*-mCD8RFP was combined with AstA-*GAL4* (red) and *LexAop*-mCD8GFP was combined with SIFaR-*LexA* (green) to create membrane expression data. AstA cells were detected in the distal optic lobes and two cell bodies in the SOG region. One pair of DIMM+ AstA cells was likely among the AstA cells in the optic lobes and another DIMM+ cluster in

the inferior VNC region. Many SIFaR cell bodies were found in optic lobes, five-six pairs in SOG, and about 15 cell bodies found among the VNC region. Blue arrowheads indicate DIMM+ neurons. Allatostatin A (AstA).

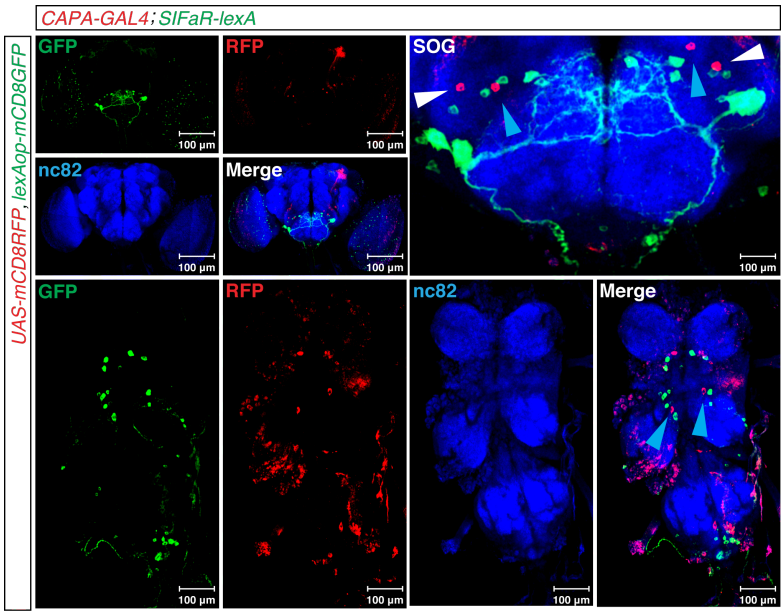


Figure 18. Two DIMM+ and two DIMM- CAPA neurons were detected in the SOG region. Double membrane labeling using SIFaR-*LexA*-labeled cells (green) and CAPA-*GAL4*-labeled cells (red). One pair of DIMM+ and one pair of DIMM- CAPA neurons were found in the SOG region and several cell bodies were found among the VNC region. Many SIFaR cell bodies were found in optic lobes, 4-5 pairs in SOG, and about 12 cell bodies found among the VNC region. Red arrowheads indicate DIMM+ neurons whereas white arrowheads indicate DIMM- neurons. Capability (CAPA).

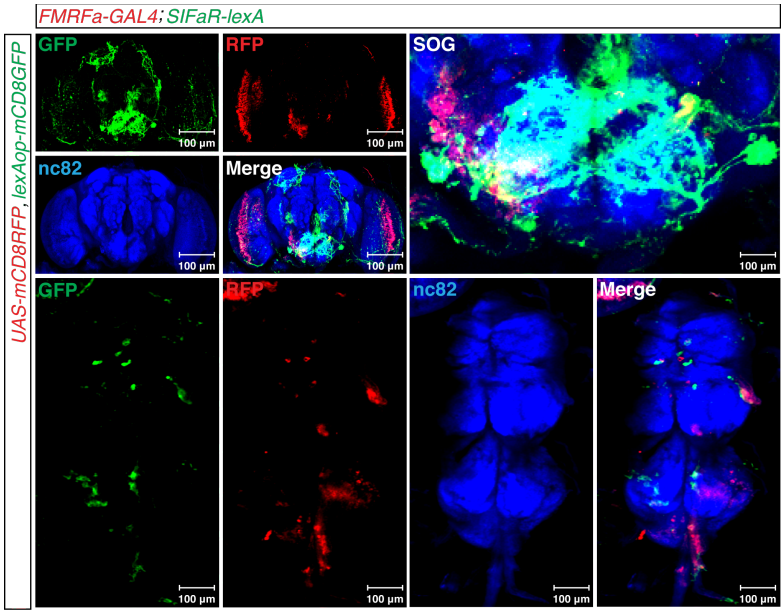


Figure 19. FMRFa-*GAL4* cell bodies are highly expressed in both optic lobes. Double immunolabeling with SIFaR-*LexA*-labeled cells (green) and FMRFa-*GAL4*-labeled cells (red). Hundreds of FMRFa cell bodies (red) are present in optic lobe region (Pyza & Meinertzhagen, 2003). Many SIFaR cell bodies were found in optic lobes and SOG, and 10 cell bodies found among the VNC region. FMRFamide (FMRFa).

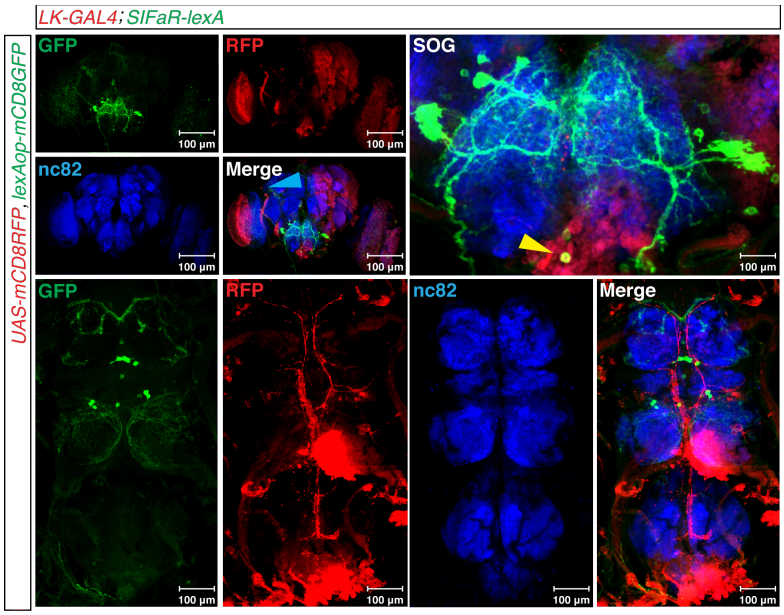


Figure 20. One DIMM+ SELK and one LHLK neuron identified to regulate SMD. Using *UAS*-mCD8RFP with LK-*GAL4*-labeled cells displayed one LHLK (red cell) and one DIMM+ SELK neuron (Yellow). Many SIFaR cell bodies were found in optic lobes, 5-6 pairs in SOG, and 6 cell bodies found among the VNC region. Blue arrowhead indicate DIMM+ neurons whereas yellow arrowhead indicates LK neuron expresses SIFaR. Leucokinin (LK).

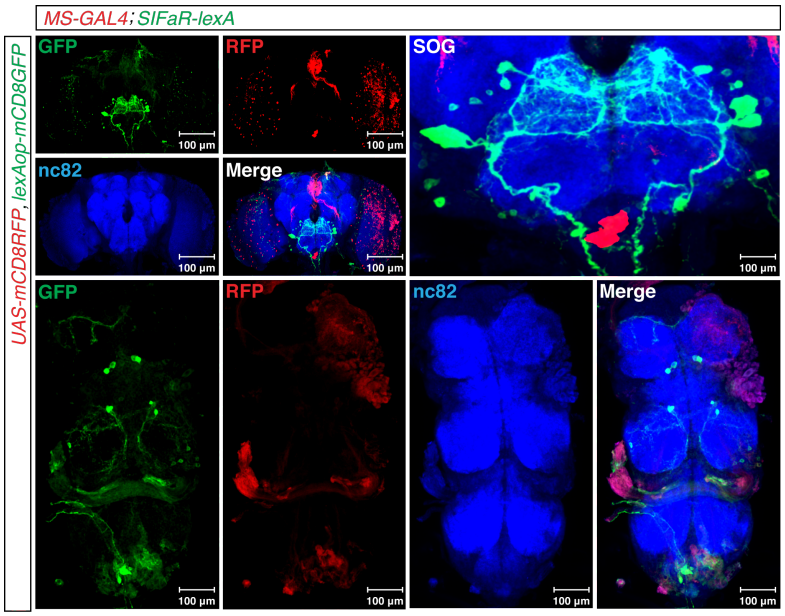


Figure 21. MS-*GAL4* cell expression is distributed primarily in the central brain . Double immunolabeling with SIFaR-*LexA*-labeled cells (green) and MS-*GAL4*-labeled cells (red). Several MS cell bodies were found in optic lobes and inferior medial SOG, and inferior VNC region.

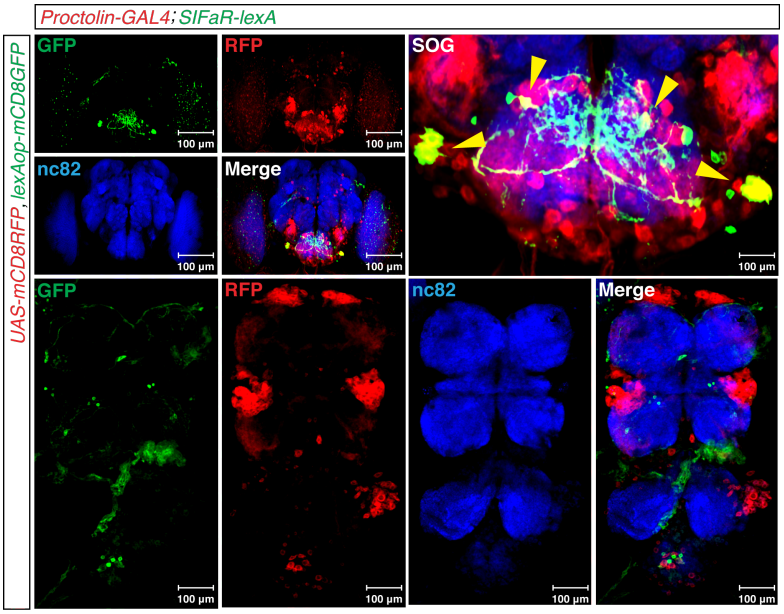


Figure 22. Four proctolinergic cells in the SOG have SIFaR expression. Double immunolabeling with SIFaR-*LexA*-labeled cells (green) and Proc-*GAL4*-labeled cells (red). Proc neurons were distributed throughout the nervous system but is concentrated in the medial SOG and lateral VNC region. In SOG, four Proc neurons are shown to express SIFaR (yellow arrowheads). Many SIFaR cell bodies were found in optic lobes, 5-6 pairs in SOG, and 6 cell bodies found among the VNC region. Proctolin (Proc).

Neuronal Activity of Proctolin Neurons Display LMD but not SMD

Our study identified seven neuropeptides to be functionally involved in either LMD and/or SMD. Due to the number of neuropeptides recognized, we decided to narrow our mechanistic focus to only Proc as it is the only neuropeptide required to generate both mating behaviours simultaneously.

To examine the functional role of Proc, we wanted to determine whether Proc neurons are within synaptic contact with SIFa neurons in the SOG region. To do this, we used *UAS*-Denmark and *UAS*-SytGFP with Proc-*GAL4* to drive expression of its axon terminals and dendrites. Our results confirmed that Proc neurons had dendritic expression in the SOG region whereas axon terminal projections were expressed towards the optic lobes, SOG, and lateral regions of the VNC (Figure 23). In summary, our expression results indicate that Proc neurons may potentially form synaptic contact with SIFa neurons in the SOG region.

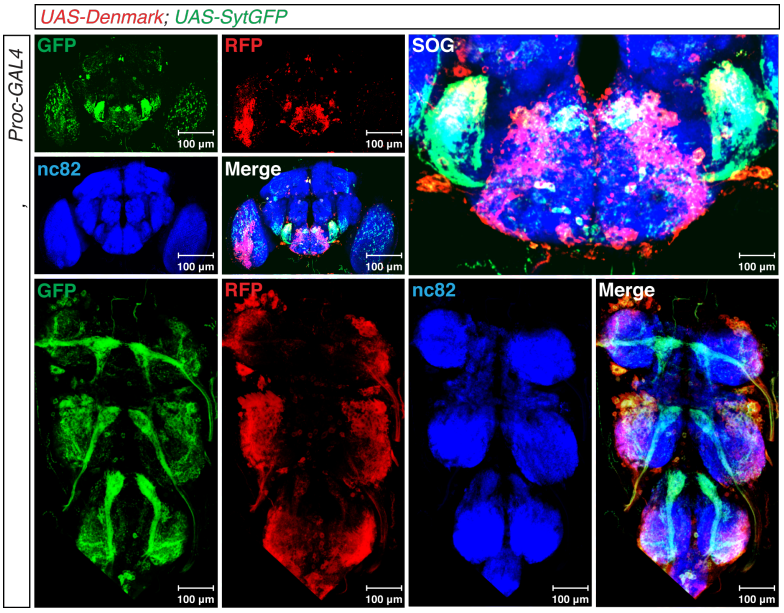


Figure 23. Axon terminals and dendritic expression pattern of Proc-*GAL4* in central brain and VNC. Anatomical data indicates potential Proc dendrites in the SOG that may interact with SIFa axonal terminals.

Our next objective was to determine the neuronal activity that underlies LMD and SMD by manipulating the electrophysiological properties of Proc-*GAL4* cells. To assess

how Proc neurons modulates LMD and SMD, we increased sodium conductance in Proc neurons by combining *UAS*-NaChBac with Proc-*GAL4* and found that LMD was mildly inhibited whereas SMD was disrupted (Figure 24.1). In contrast, we prevented membrane depolarization by expressing *UAS*-KCNJ2 with Proc-*GAL4* cells. Surprisingly, our results showed that LMD and SMD exhibited minimal disruption (Figure 24.2). Our findings suggest that the activation of Proc neurons induced an inhibitory effect to SMD via direct synaptic contact whereas LMD was mildly inhibited as adjacent circuits interconnected with LMD were disabled.

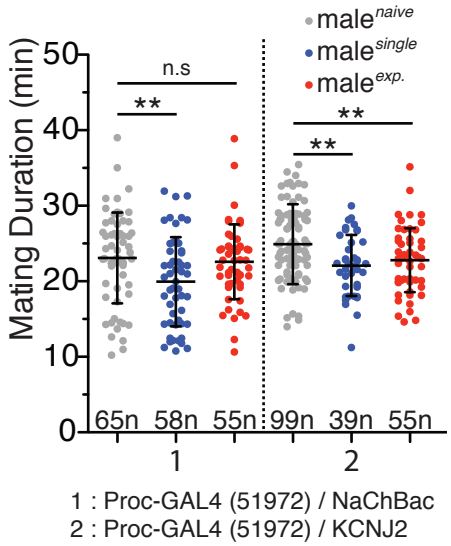


Figure 24. Activation Proc neurons displays LMD but not SMD. (1) LMD was mildly disrupted whereas SMD was disrupted by using inducing sodium conductance by NaChBac. (2) Both LMD and SMD was mildly disrupted introducing KCNJ, an inward-rectifier potassium ion channel. The numbers above the x-axis indicates n number whereas numbers below x-axis signifies different experiment. The bars in box and whisker plot indicate means ±Standard Deviations (STDs). Kruskal-Wallis one-way analysis of variance (ANOVA) test was used for statistical analysis. NS indicates non-significance, * indicates significance ($p<0.05$), ** indicates significance ($p<0.01$) and *** indicates significance ($p< 0.001$) for comparisons to naive group.

Discussion

D. melanogaster is an excellent model for identifying novel neuropeptide function in innate sexual behaviours. Along with previous studies indicating that neuropeptidergic circuits are associated with LMD and SMD, it has become increasingly more evident that other neuropeptides are involved as well.

Here, using behavioural genetics, we assessed 19 different neuropeptides and evaluated their functional significance on male-specific mating behaviours. Our RNAi-based screens identified seven neuropeptides and localized the important cell bodies for either LMD and/or SMD to specific regions of the nervous system. Additionally, we found that SIFa axonal terminals and SIFaR dendrites were primarily centralized towards the SOG region which potentially indicated synaptic contact between these cells. Subsequently, we examined the functional applications of Proc neurons by identifying potential synaptic contact with SIFa in the SOG region, as well as altering its electrophysiological functions which were found to be important for SMD but not LMD.

Our study presents and introduces three independent novel signaling pathways whereby SIFaR-mediated signaling recruits other neuropeptidergic circuits to elicit male-specific mating behaviours. The first pathway involves Proc neurons modulating both mating behaviours through synaptic and non-synaptic mechanisms. The second pathway describes DIMM to alter the cellular functions of AKH, AstA, and LK onto an existing hunger-driven circuit through insulin producing cells (IPCs) to accommodate SMD. In our last pathway, we discuss our interpretations where a plausible disinhibition circuit was resolved by CAPA neurons in the central brain through olfactory-mediated receptors.

Limitations in RNAi-based Genetic Screens

The utility of reverse genetics or RNAi-based screens is an excellent approach to identify novel functions in lesser known neuropeptides. As sequencing data and analysis of the *Drosophila* genome is widely available (Adams & O'Shea, 2014), this creates an opportunity to understand the relationship between mating duration and gene encoding neuropeptides. However, there are still several concerns in our screening strategy that must be addressed before we interpret and justify our results.

While using a systematic RNAi-based screen to identify candidate neuropeptides, it is conceivable that our methodology may not have accounted for all relevant neuropeptides involved in our mating paradigms. More than 50 neuropeptides have been characterized in *D. melanogaster* (Hewes & Taghert, 2001; Pauls et al., 2014; Vanden Broeck, 2001), but our study has only screened for 19 of them. We could have easily discounted other plausible modulators due to time and resource constraints. Additionally, we have also integrated *Dicer* gene into our screening process which is designed to enable the activation of RNA-induced silencing complex (RISC), a key multiprotein complex for RNAi (Sigoillot & King, 2012). Adding *Dicer* may have ineffectively downregulated a selected neuropeptide and thus producing a false-negative result. RNAi-based screens have been previously known to produce inconsistencies in gene silencing when observing behaviours (Dietzl et al., 2007).

Finally, we must also denote that our first genetic screen does not declare that the identified neuropeptides are required for their corresponding mating behaviour(s) since we are disrupting the SIFa-mediated pathway through SIFaR. Alternatively, it may be

that the neuropeptidergic neuron(s) that expresses SIFaR is utilized as relay signal to control the neuronal activities of LMD and SMD. In order to verify neuropeptide involvement, a second genetic screen must downregulate its function to confirm its relevance to its respective behaviour(s).

Proctolin Modulates LMD and SMD Through Two Different Mechanisms

Our study has identified two pairs of Proc neurons that expresses SIFaR in the SOG region to be functionally involved in male-specific mating behaviours. We showed that neuronally activating these neurons prompts LMD but not SMD (Figure 24.1) whereas deactivation results in both behaviours being mildly disrupted (Figure 24.2). We also showed that the role of Proc as a neuropeptide is required for both mating paradigms (Figure 9.7 and 11.7). While most studies have defined Proc to be associated with muscular function (Orchard, Lee, Silva, & Lange, 2011), there are no reports to show its relationship to mating duration. This implies that we found have a novel function where Proc modulates both mating behaviours through two different mechanisms. To rationalize our findings, Proc neurons may inhibit SMD through synaptic contact whereas LMD and SMD require the neuropeptide to elicit their respective behaviours (Figure 25).

To explain why LMD but not SMD is elicited through the activation of Proc neurons, our study focuses on potential synapses formed between SIFa (Figure 15.1) and Proc neurons in the SOG region (Figure 23). This reveals that cell-to-cell communication may be initiated in this area where activation of SIFa neurons (Figure 3.4) relays a signal to Proc neurons to deactivate SMD only (Figure 24.1). Since it is uncharacteristic for a neuropeptide to induce changes at the active zone (Nassel, 2009), we predict electrical

potential differences in Proc neurons caused SMD to be disrupted or inhibited. Conversely, we observed that the LMD circuit was minimally disrupted when Proc neurons were activated. Our consensus is that the presynaptic terminals of Proc neurons may be interconnected with other circuits associated with LMD to induce minimal disruption, but not enough to be considered a principle modulator. Confirmation of synaptic connectivity between SIFa and Proc neurons will need to be verified by using GFP Reconstitution Across Synaptic Partners (GRASP), a technique that uses a split GFP construct where one half of each complementary component are expressed in either the presynaptic or postsynaptic neuron (Feinberg et al., 2008). Our results also unexpectedly found that both LMD and SMD were slightly disrupted by the deactivation of Proc neurons (Figure 24.2). Our suggestion is that other compensatory circuits that modulates these behaviours must exist beyond the synaptic network that is controlled exclusively by Proc neurons. Since its axon terminals are distributed in the SOG and entire VNC region (Figure 23), we predict that these circuits are expressed outside of this area of the nervous system.

In our study, we also found that knockdown of Proc in SIFaR-*GAL4* cells and knockdown of SIFaR in Proc-*GAL4* cells disrupted both LMD and SMD (Figure 9.7 and 11.7), this indicates that Proc neurons receive inputs via SIFaR to modulate both mating paradigms. Neuropeptides can influence neural circuits through synaptic contact or non-synaptic mechanisms (Jékely et al., 2018). According to our results, the latter is likely the candidate. Smaller molecules such as neurotransmitters are commonly packaged into small clear vesicles which are found near the active zone of the presynaptic membrane whereas neuropeptides are stored and transported in large dense granular vesicles which

are found perisynaptically (Nässel & Winther, 2010). This implies that neuropeptide GPCRs are in the proximity of the perisynaptic area of the postsynaptic membrane which allows Proc to influence both LMD and SMD. Our previous studies in regard to temporal information suggest that Proc acts in a hormonal-mediated fashion to its peripheral targets to elicit our mating paradigms. For example, neuromodulation by a neuropeptide are transported over long distances and induced in a slow but sustained manner (Nässel & Winther, 2010). The slow activation is partially attributed to the mechanistic architecture of GPCRs which are activated by secondary messenger systems. Among many insect species, diacylglycerol (DAG) is a well-known secondary messenger that is used in the proctolinergic signaling (Baines, Lange, & Downer, 1990). DAG is a glyceride that acts as a secondary messenger in the phospholipase C pathway to activate protein kinase C (PKC), this will induce a specific intracellular cascading response (Vezenkov & Danalev, 2009). This activation via GPCRs corresponds with the timing of our mating behaviours as they both require several days in a specific socio-sexual environment to be displayed (W. J. Kim, Lee, Schweizer, et al., 2016; W. J. Kim et al., 2012, 2013a). To be specific, our LMD paradigm requires PDF-NDF interactions to modulate the activity of each other (W. J. Kim, Jan, & Jan, 2013b) whereas sNPF signaling is employed to elicit SMD (W. J. Kim, Lee, Schweizer, et al., 2016). Our presumption is that Proc may be integrated into a series of neuropeptidergic relays, that is, neuropeptide-to-neuropeptide communication with PDF, NDF, sNPF, and/or SIFa to which accounts for the time delay as seen in our behaviours. What is also not clear is whether one or both pairs of Proc neurons that expresses SIFaR in the SOG (Figure 22) are necessary to drive this phenomenon. Further investigations that utilizes intersectional approaches to segregate their functional

significance will be needed to determine their involvement in our mating behaviours.

DIMM Modulates Insulin-Related Microcircuit for SMD Behaviour

Our study identified AKH (Figure 9.1, 11.1 and 12.1), AstA (Figure 9.2, 11.2, and 12.2), and LK (Figure 9.5, 11.5, 12.5) in the central brain to be essential modulators of SMD behaviour. Moreover, we also found that DIMM, a transcription factor, is important for SMD only (Figure 10). Altogether, we describe a potential novel pathway whereby SMD utilizes an existing defined microcircuit to control the balance of insulin-sensitivity or *Drosophila* insulin-like-peptide (DILP)-sensitivity. Specifically, we propose that DIMM alters AKH, AstA, and LK neurons to modify its input to insulin producing cells (IPCs) to distribute its resources into either growth-related behaviours or sensory cues for reproduction (Figure 25).

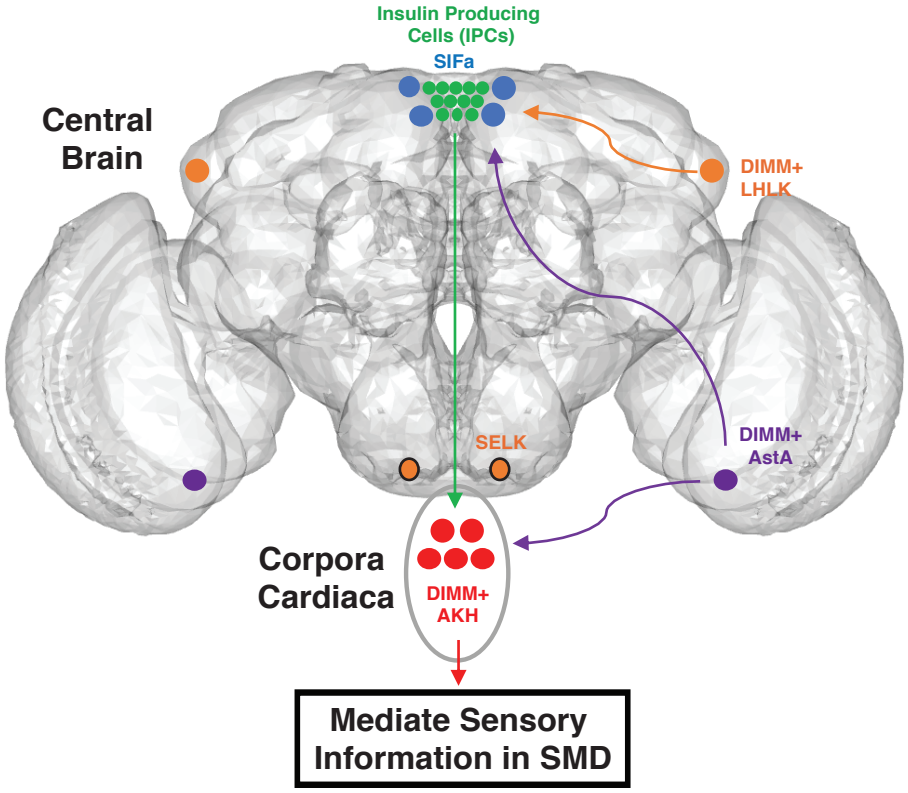


Figure 25. Hypothetical inhibition model for SMD sensory information. Our model highlights a theoretical mechanism that depicts a scenario where SMD behaviour in the adult brain of *D. melanogaster* is modulated by various neuropeptidergic inputs. We infer that DIMM+ (AKH, AstA, and LK) neuropeptidergic neurons mediates a response to IPCs to alter classical growth functions to recruit SMD salience. DIMM+ LHLKs and SELKs act on IPCs, DIMM+ AstA mediates feedback regulation between metabolic hormone release from IPCs and DIMM+ AKH.

DILP is a hormone that regulates energy homeostasis and glucose metabolism (Vogt & Bru, 2013) whereas AKH, an analogue of glucagon, induces the activation of stored energy (S. K. Kim & Rulifson, 2004). Together, both metabolic hormones act synchronously to modulate glucose regulation for feeding (Hong et al., 2012), foraging (Sun et al., 2017), and sexual attractiveness (Fedina, Arbuthnott, Rundle, Promislow, & Pletcher, 2017). We have compelling evidence to suggest that DIMM+ neuropeptides modulates this microcircuit to elicit SMD.

The first evidence for our hypothesis focuses on two pairs of LK neurons in the central brain, DIMM+ LHLKs and SELKs. Our knockdown approach (Figure 9.5, 11.5, 12.5) and expression data (Figure 20) indicates that both subsets of LK neurons might be involved. Each pair provides distinct functional roles where DIMM+ LHLKs are involved in locomotor activity, sleep and metabolism (Yurgel et al., 2019) and circadian pacemaker function (Cavey et al., 2016) whereas SELKs mediate food ingestion (Al-anzi et al., 2010) and gustatory behaviour (Yurgel et al., 2019). Since neither pair are found to be linked with mating duration, it would be interesting to determine whether one or both pairs are necessary. IPCs express LK receptors (LKR) (Yurgel et al., 2019) and have synaptic contacts with DIMM+ LHLKs (Zandawala et al., 2018) whereas SELKs have no apparent connections (Yurgel et al., 2019). We postulate that SELKs may be involved because they express SIFaR and likely induce their effects through long-range mechanics. However, LHLKs may have a greater role due to comparable LK- and DIMM-RNAi knockdown results in SIFaR-*GAL4* labeled cells (Figure 9.5, 10, and 11.5) and have direct synaptic contact with IPCs. A brief discussion of DIMM will reviewed at the end of this section.

The regulation of glucose between IPCs and DIMM+ AKH may also be a key modulator of SMD. There are 14 IPCs found in the pars intercerebralis which regulates metabolism, growth, and reproduction (Nässel, Liu, & Luo, 2015). These cells also have axonal projections towards DIMM+ AKH cell bodies in the corpora cardiaca (Kapan, Lushchak, & Luo, 2012; Meschi, Le, & Delanoue, 2019), a neurohemal region behind the central brain. Since both IPCs and AKH signaling regulates DILP-sensitivity (Nässel et al., 2015; D. Park et al., 2008), we propose that a dynamic balance of glucose levels in

sensory Gr5a neurons, critical cells that mediate SMD (W. J. Kim, Lee, Schweizer, et al., 2016), must be sustained to activate this pathway. We have supporting evidence from previous experiments that knockdown of octopamine or serotonin in SIFaR-*GAL4* labeled cells disrupted SMD (Schweizer, 2018). If SIFaR is expressed among IPCs then our observations are consistent with prior studies supporting that IPCs are modulated by octopamine (Crocker, Shahidullah, Levitan, & Sehgal, 2010) and serotonin (Nässel et al., 2015). Our anatomical data also indicates that AKH is not expressed in neither the central brain nor the VNC (Figure 16), but the neuropeptide is clearly essential (Figure 11.1). Several studies have reported that neuromodulation may occur beyond the nervous system in response to certain behaviours (Dickinson, Calkins, & Stevens, 2019). This could signify that DIMM+ AKH neurons (D. Park et al., 2008) in corpora cardiaca might be essential to regulate SMD behaviour.

How might IPCs and AKH mediate SMD? While it remains unclear whether IPCs directly influence mating duration, there are reports that both molecules mutually inhibit each other (Buch, Melcher, Bauer, Katzenberger, & Pankratz, 2008) and are known to regulate behaviours beyond their metabolic roles (Bharucha, Tarr, & Zipursky, 2008). For example, AKH Receptors (AKHRs) are expressed in several adipose and brain tissues, including Gr5a gustatory neurons (Bharucha et al., 2008). We disrupted SMD by producing a phenotype where male flies had become AKH-deficient in SIFaR-*GAL4* cells. Taken together, this outcome likely dampened the expression levels to maintain an appropriate balance of DILP which subsequently influenced the sensory Gr5a neurons. As memory processing has been shown to be influenced by insulin-mediated signaling by up to several days, (Lin, Senapati, & Tsao, 2019), our assumption is that our SMD

paradigm may utilize comparable mechanics. Thus, male flies with deficiencies in AKH have lost the ability to fine-tune this network or assign resources to mediate our SMD paradigm.

For our last neuropeptide, AstA, we have sufficient indication for its involvement in SMD behaviour. We found that knockdown of AstA (Figure 9.2) and DIMM (Figure 10) in SIFaR-*GAL4* cells disrupted SMD but not LMD and this effect was restricted to only the central brain (Figure 11.2). Given the data and literature, we have potentially identified one pair of DIMM+ AstA neurons in the optic lobes (Figure 17) to modulate feedback circuit mechanisms between AKH-IPC to elicit SMD. The role of AstA is involved in coordinating the balance and release of metabolic hormones from AKH and IPCs (Christina, Tayler, & Anderson, 2012). By silencing *Dar-2* (AstA receptor) in IPC and AKH cells, signaling by the AKH-IPC circuit was reduced and an accumulation of lipids was observed (Hentze, Carlsson, Kondo, Nässel, & Rewitz, 2015). The authors claimed that AstA-mediated signaling controls the output balance of AKH-IPC which is based on the importance of either growth or reproduction. This process is facilitated by AstA axonal projections in the protocerebrum, an area located superior to the SOG. Interestingly, IPCs also contains dense dendritic arborizations in this region (Hentze et al., 2015), this suggests that modulation might occur here. For our SMD paradigm, AstA neurons contributes to modify AKH-IPC circuits based on socio-sexual conditional inputs so that the correct ratio of metabolic cues is achieved. Our study downregulated AstA and disrupted DILP and AKH homeostasis in SMD circuits via AKH-IPCs which produced an abnormal function so that sensory information could not processed as described in our knockdown experiments.

In summary, we have provided evidence that indicates a potential novel signaling pathway where AKH, AstA, and LK are modified by DIMM to modulate SMD through IPCs. Much of the literature has primarily focused on these neuropeptides in the context of metabolic function in hunger-driven behaviour, presumably creating a one-sided viewpoint. Our findings have raised the possibility that insulin-specific circuits may play a parallel role in processing feeding, as well as mating behaviour. Our rationale for this model is simple, we propose DIMM modifies neuropeptidergic circuits to transmit information onto IPC circuits that is mating specific. DIMM cells are also referred to as large cells that release amidated peptides in an episodic manner (LEAPs) (D. Park et al., 2008). Their large somatic size may be an indicator of multiple functions implicated in several different behaviours than traditional neuropeptidergic cells. Though DIMM transcriptionally acts to affect cell growth and inhibit apoptosis in a time-dependent manner (Liu, Luo, & Nässel, 2016), its role in other cellular mechanisms are still not well characterized. Future studies will be conducted to determine the mechanisms of DIMM and how this transcription factor is relevant to male-specific mating behaviours.

Two DIMM- CAPA Neurons in the SOG Resolve Disinhibition in LMD

Our study has reported that CAPA neurons in both the central brain and VNC modulates LMD behaviour. We showed that knockdown of CAPA in SIFaR-*GAL4* cells disrupted LMD but not SMD (Figure 9.3 and 11.3). Interestingly, we found that the mating duration in singly-reared flies had increased in comparison to the naïve condition when CAPA was downregulated in the central brain only (Figure 12.3). Our study has found two DIMM-negative (DIMM-) and two DIMM+ CAPA neurons in the SOG

whereas several CAPA cell bodies, including DIMM+, were also localized to the medial VNC region (Figure 18). Our results suggest that CAPA-*GAL4* cells in the nervous system are required for LMD but only CAPA neurons in the central brain mediate the neuronal circuits to resolve disinhibition. Only one study has described a link between CAPA and mating behaviour (Diesner et al., 2018), but our study is the first to claim its association to disinhibition circuits. This section will focus on our interpretation where two DIMM- CAPA neurons in the SOG to resolve a disinhibition circuit to modulate LMD pathway.

CAPA is a neuropeptide expressed in various insect species (Koehler & Predel, 2010; Neupert, Russell, Russell, & Predel, 2010) and is most abundantly found in the neurohemal system (Predel & Wegener, 2006). Though many studies have examined CAPA to be involved in heart rate stimulation (Huesmann et al., 1995), rapid fluid secretion (Dow, Tublitz, Huesmann, Donnell, & Tublitz, 1995), and antidiuretic effects (Macmillan et al., 2018; Predel & Wegener, 2006), there is only one study to find its involvement in copulation behaviour (Diesner et al., 2018). This study claims that sexual behaviours induces an inhibitory response to female-specific pheromones when the ratio of CAPA concentrations are altered. (Diesner et al., 2018). However, the context of this study was investigated in *Agrotis ipsilin* male moths in the antennae lobe, a region that primarily processes and integrates olfactory sensory information (Schachtner, Schmidt, & Homberg, 2005). This evidence contradicts our LMD paradigm since we previously disregarded olfactory stimuli via Or83b mutant experiments (W. J. Kim et al., 2012) and that there is no CAPA expression in the antennae lobe of *D. melanogaster* (Nässel & Zandawala, 2018). As other olfactory receptors, Or47b and Or88a, are important for

mating and attraction in *D. melanogaster* (Dweck, Ebrahim, Thoma, Mohamed, & Keesey, 2015), it is conceivable that different olfactory-based receptors are salient to specific socio-sexual conditions as seen in our LMD paradigm. If olfactory signaling is involved in resolving our disinhibition circuit, then how might CAPA target the antenna lobe circuits despite having no CAPA cell bodies in the area? Neuropeptides are able to diffuse through the bloodstream by neurohemal organs to activate their corresponding GPCRs whether their spatial distance is near or far (Hewes & Taghert, 2001). In one study, CAPA precursor peptide B (CPPB) was found in the neurohemal organs in *D. melanogaster*. CPPB is an inactive pro-peptide that may be processed by prohormone convertase to transform it to the active form known as CAPA (Wegener, Reinl, Ja, & Predel, 2006). The physiological function of CPPB within the neurohemal organs is still not known (Predel & Wegener, 2006), but our deduction is that CPPB is carried through the bloodstream and synthesized as CAPA in the antennae lobe to resolve disinhibition circuits. Our study disrupted LMD through knockdown of CAPA in SIFaR-*GAL4* cells (Figure 11.3) which induced imbalances of CAPA concentrations, thus interrupting the inhibitory response via olfactory receptors to female-specific hormones. Since DIMM does not disrupt LMD (Figure 10), we narrowed our mechanistic focus to two DIMM-CAPA neurons in the SOG (Figure 12.3 and 18). To verify our hypothesis, CAPA receptor mapping in the antennae lobe should be a main target for future studies to elucidate mechanisms between temporal information and LMD.

Conclusion

D. melanogaster is an excellent model for investigating the relationship between different socio-sexual conditions and its influence to mating duration. Though it is clear that qualitative environmental stimuli contribute immensely to alter the neural substrates, how this influence results in neuropeptide-to-neuropeptide interactions remains largely unknown. Our study combined RNAi-based screening with behavioural analysis to identify novel neuropeptide function in male-specific mating behaviours. Seven distinct neuropeptides were identified and mapped to further understand how neuropeptide-receptor signaling modulates either LMD and/or SMD. Following our analysis, our study highlights three discrete signaling pathways where male-specific mating behaviours are elicited. Firstly, we describe that Proc neurons inhibit SMD synaptically whereas LMD and SMD are modulated non-synaptically via Proc neuropeptide. Secondly, we hypothesize a novel signaling pathway where DIMM+ alters cellular properties in AKH, AstA, and LK in a complex network that converges to IPCs which may act as a central regulator to control the activation and deactivation of SMD. Thirdly, we briefly discuss how CAPA neurons might resolve disinhibition circuits found in LMD through olfactory-mediated sensory information. We were unable to discuss the functional relevance of FMRFa and MS as there was no clear indication or sufficient evidence to infer any causal link to LMD.

In summary, our study highlights different neuropeptidergic systems which act synchronously in a multi-model network to elicit male-specific mating behaviours. We have attempted to rectify how each of selected neuropeptides are involved in either LMD

and/or SMD, but it is challenging to predict its full mechanisms as each have diverse functional influences (Nässel, 2018). We require additional neuropeptidergic evidence such as synaptic mapping as it would be informative to determine what interactions or constraints exists for these neuromodulators. As neuropeptide-receptor pathways are mostly functional conserved, our findings may be relevant towards processes that occurs in vertebrate species, including humans. In conclusion, our study contributes to further advance our understanding on the cellular mechanics that modulates male-specific mating behaviours in *D. melanogaster*.

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