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Daniel Rodrigues Machado **Integration of Sleep and Social Behavior in *Drosophila melanogaster***
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Universidade do Minho
Escola de Medicina

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Integration of Sleep and Social Behavior in *Drosophila melanogaster*

Tese de Doutoramento em Medicina

Trabalho efetuado sob a orientação da
Professora Kyunghee Koh
e do
Professor Tiago Gil Rodrigues Oliveira

janeiro de 2019

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, 15 / 01/ 2019

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The adventure of scientific exploration is certainly a thrilling and absorbing journey. However, along the exciting results, disconcerting questions, intriguing puzzles and small and big wonders one may encounter, come long periods of hard work, spotted here and there with a tempting degree of frustration. In the face of such difficulties dedication, persistency and a clear mind are absolute must-haves. These qualities, justifiably attributed to a certain "scientific charisma" of the researcher, are mostly achieved out of the lab, with family, friends and colleagues who, through their always-ready provocative questions and curiosity and company and support, give all the obstacles one might face a smaller meaning in comparison with the awe one encounters in Science and in Nature.

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Resumo

É ainda pouco claro como o cérebro decide entre necessidades incompatíveis. Uma possível abordagem a esta questão, considerando que o sono é incompatível com virtualmente qualquer outro comportamento, passa por explorar a tomada de decisão que leva um animal a dormir ou a responder a outras necessidades críticas. Neste trabalho investigamos como é que dois comportamentos fundamentais, o sono e o cortejamento, são integrados numa resposta coordenada. Demonstramos que os machos *Drosophila* suprimem o sono em favor do cortejamento quando o seu *drive* sexual é elevado e uma fêmea está presente, indicando que o balanço entre o *drive* do sono e o *drive* sexual determina o comportamento do macho. Descrevemos os neurónios MS1 (Male Specific 1), que estão envolvidos especificamente na regulação do sono do macho: a ativação dos neurónios MS1 induz uma redução significativa do sono especificamente nos machos, enquanto que o seu silenciamento bloqueia a supressão do sono induzida pela fêmea. Demonstramos ainda que a supressão do sono induzida pelos neurónios MS1 é mediada pela octopamina, um ortólogo da noradrenalina dos mamíferos. Interessantemente, apesar da sua regulação dimórfica do sono, descobrimos que os neurónios MS1 não expressam o fator de transcrição Fruitless^{M} (FRU^{M}), específico dos machos e que é necessário ao seu comportamento de cortejamento. Em vez disso, os neurónios MS1 formam um circuito sexualmente dimórfico com os neurónios FRU. Os nossos resultados sugerem que os neurónios MS1 e FRU estabelecem um circuito bidirecional em que os neurónios MS1 recebem *inputs* de neurónios FRU e, por sua vez, estimulam a atividade de vários grupos FRU de forma a promoverem um estado de alerta que permite ao macho suprimir o sono para cortejar a fêmea. Recentemente, descobrimos um segundo *driver*, MS2-Gal4, também associado com a regulação do sono do macho. A ativação dos neurónios MS2 suprime o sono nos machos, enquanto que a sua inibição impede a supressão do sono induzida pela fêmea. Descobrimos que um pequeno grupo de neurónios MS2 expressa o gene *fru* e se localiza na região mAL, uma região do cérebro envolvida na regulação dos neurónios P1. A ativação de uma subpopulação dos neurónios mAL suprime o sono em machos, enquanto que o seu bloqueio previne a redução do sono observada na presença de fêmeas. A investigação sobre o papel dos neurónios MS2 no sono dos machos e sobre a sua interação com os neurónios MS1 e P1 seria relevante para detalhar o nosso conhecimento sobre os mecanismos neuronais que coordenam o conflito entre sono e cortejamento.

Abstract

The question of how the brain decides between conflicting needs is still poorly understood. A possible approach to the problem, considering that sleep is incompatible with virtually all other behaviors, is to explore the decision-making process that leads an animal either to sleep or to engage in other pressing behaviors. In this work, we investigate how two essential behaviors, sleep and courtship, are integrated in a coordinated manner. We show that *Drosophila* males suppress sleep to engage in courtship when females are present and sex drive is high, indicating that the balance between sleep drive and sex drive determine the behavior of the male. We describe the MS1 (Male Specific 1) neurons involved in male-specific regulation of sleep; activation of MS1 neurons leads to markedly reduced sleep specifically in males and silencing of MS1 neurons block female-induced male sleep suppression. We also show that male sleep suppression induced by MS1 requires octopamine, an ortholog of mammalian norepinephrine. Interestingly, despite their sexually dimorphic role in sleep regulation, we found that MS1 neurons do not express the male-specific transcription factor Fruitless^{M} (FRU^{M}), which is required for male courtship behavior. Instead MS1 neurons form a sexually dimorphic circuit with the FRU-expressing neurons. Our data suggest that the MS1 and FRU neurons establish a bidirectional circuit, in which MS1 receive input from certain FRU neurons and in turn induce the activity of several FRU neuronal clusters to promote a state of heightened sexual arousal that allows the male to stay awake and court females. Recently we found a second driver, MS2-Gal4 associated with male-specific sleep regulation; activation of MS2 neurons suppresses sleep in males and inactivation of MS2 neurons block female-induced sleep suppression. We found that a small number of MS2 neurons are *fru*-positive and are located in the mAL, a region previously described to modulate the activity of P1 neurons. We found that activation of a broader set of mAL neurons suppresses sleep in males, and blocking them prevents sleep reduction in male-female pairs. Further investigation of the role of MS2 neurons in male sleep regulation and their interaction wtih MS1 and P1 neurons will advance our understanding of the neural mechanisms underlying the coordination between sleep and courtship.

Contents

Abbreviations

3IY – 3-Iodo Tyrosine

AD – activating domain

AD – Activation Domain of split Gal4

AHL – Adult Hemolymph Like medium

BRP - Bruchpilot

CLK – CLOCK

CNS – Central Nervous System

CRY – CRYPTOCHROME

CYC – CYCLE

DAM – *Drosophila* Activity Monitoring system

DBD – DNA binding domain

DBD – DNA Binding Domain of split Gal4

dFSB – dorsal Fan-Shaped Body

DN1 – Dorsal clock Nerouns 1

dsx – doublesex

EB R2 – R2 ring of the Ellipsoid Body

F – female

FLP – Flippase enzyme

FRT – Flippase Recognition Target

fru – fruitless

GECI – Genetically Encoded Calcium Indicator

GFP – Green Fluorescent Protein

GRASP – GFP Reconstitution Across Synaptic Partners

LFP – Long Field Potentials

M – male

mAL interneurons – medial Anterior-Lateral interneurons

MF – male-female pair of flies

MM – male-male pair of flies

MS1 neurons – Male Specific 1 neurons

MS2 neurons – Male Specific 2 neurons

NREM – Non-Rapid Eye Movement sleep

PAL – protocerebral anterior lateral node

PER – PERIOD

PI – Pars Intercerebralis

PKA – Protein Kinase A

ppk23 – pickpocket 23

Rdl – Resistant to dieldrin

REM – Rapid Eye Movement sleep

SHY – Synaptic Homeostasis Hypothesis

SMPa – superior medial, anterior protocerebrum

SOG – subesophageal ganglion

Tdc – Tyrosine decarboxylase

TH – Tyrosine Hydroxilase

TIM – TIMELESS

TNT – tetanus toxin

TRIC – Transcriptional Reporter of Intracellular Calcium

TβH – Tyramine β-hydroxilase

VTA – Ventral Tegmental Area

WT – wildtype

Chapter 1

Introduction

1.1. Sleep as an essential behavior

It is well known that even a small reduction in the necessary daily amount of sleep can have a large impact on human cognition and performance (Wennberg et al. 2017; Killgore 2010; Dongen et al. 2003; Krause et al. 2017; Banks et al. 2005; Zhao et al. 2017). However, despite its importance for optimal performance and survival, the physiological functions of sleep are still a mystery. Several theories have been proposed to explain what happens in the brain during sleep that is essential for the proper function of animals.

A number of studies suggest that sleep is important for learning and memory consolidation. After being exposed to new experiences humans sleep more deeply, with higher slow-wave activity, a hallmark of sleep need (Tononi & Cirelli 2014). Additionally, while the encoding of new memories occurs during wakefulness, the consolidation of such memories occurs preferentially during sleep (Rasch & Born 2008; Marshall & Born 2007; Wagner & Born 2008).

The Synaptic Homeostasis Hypothesis (SHY), proposed by Tononi & Cirelli, proposes that sleep is important for the stability and consolidation of synapses(Tononi & Cirelli 2014). According to SHY, during wakeful periods synapses undergo net potentiation, which is associated with the acquisition of new information. During sleep, downscaling of synapses occurs, allowing for the stability of the neuronal circuits activated during wakeful periods (Diering et al. 2017; Vivo et al. 2017; Huber et al. 2004).

Sleep may also be important to reduce the amount of energy consumed by the brain (Tononi & Cirelli 2014). This is likely an important task, considering the high metabolic rates exhibited by the brain: about 25% of the total glucose consumed (Tononi & Cirelli 2014).

Recently, Nedergaard and colleagues proposed that the reduced activity of the central nervous system (CNS) during sleep permits the glymphatic system to clear toxic metabolic byproducts accumulated during wake. Therefore, sleep could be important for the "detoxification" of the brain(Xie et al. 2013).

Even though these theories provide interesting hypotheses about the physiological functions of sleep, the question of why we sleep remains unanswered. In addition, the molecular and neuronal substrates of sleep remain largely elusive. Thus, understanding how sleep is regulated is necessary for

the development of new treatments not only for sleep disorders, but also for pathologies that often present sleep disturbances, such as depression, anxiety disorders, and most neurological disorders (Wennberg et al. 2017; Krause et al. 2017; Dongen et al. 2003; Banks et al. 2005; Kawada 2017).

1.2. *Drosophila* as a model for sleep studies

Sleep is conserved across several species, from humans to fruit flies, to fish, birds and even nematodes such as *Caenorhabditis elegans* (Hendricks et al. 2000; Rattenborg et al. 2016; Oikonomou & Prober 2017; Siegel 2009; Raizen et al. 2008). In the early 2000's, Hendricks *et al.* and Shaw *et al.* described sleep in *Drosophila* for the first time, and observed that four main behavioral features of sleep are present in both humans and *Drosophila* (Hendricks et al. 2000; Shaw et al. 2000). First, like humans, flies are mostly immobile during sleep. However, this state is reversible by strong stimuli, distinguishing sleep from a coma. Second, while asleep, flies exhibit an increased arousal threshold, meaning their responsiveness to external stimuli is significantly reduced. Third, the sleep-wake cycle of flies is under circadian control. Flies sleep mostly at night and take short naps during the day; in addition, flies display peaks of activity at dawn and dusk. Fourth, and importantly, after prolonged wakefulness, flies show rebound sleep, characterized by longer sleep episodes. This homeostatic regulation of sleep is also observed in humans.

Evidence from pharmacological studies further demonstrates that the physiological mechanisms of sleep are conserved. Drugs that modulate sleep in humans have similar effects in flies. For example, hypnotic drugs, like antihistamines, induce sleep in humans as well as in flies (Shaw et al. 2000). On the other hand, somnolytic agents, such as caffeine, modafinil and amphetamines, suppress sleep in both humans and flies (Shaw et al. 2000; Andretic et al. 2005; Hendricks et al. 2003).

Sleep is also associated with specific alterations of the electrical activity of the brain and, in humans, electrophysiological studies are used to assess sleep. During sleep, the asynchronous, high-frequency electrical waves associated with wakefulness are replaced by deep, low-frequency, highly-synchronous waves of Non-Rapid Eye Movement (NREM) sleep, a hallmark of sleep need (Donlea et al. 2017; Vyazovskiy & Harris 2013). NREM sleep then alternates with Rapid Eye Movement (REM)

sleep, which has shorter episodes and is characterized by arrhythmic waves and absence of muscle tonus (Donlea et al. 2017). In *Drosophila*, sleep is also associated with an overall alteration of the brain's activity, as revealed by electrophysiological and calcium (using GCaMP) measurements conducted in the central brain of live flies (Alphen et al. 2013; Bushey et al. 2015; Nitz et al. 2002). The analysis of field potentials (LFP) revealed an overall reduction of the electrical activity during sleep (Alphen et al. 2013). Moreover, during prolonged sleep episodes the electrical activity of the brain varies across time and is correlated with oscillations in the arousal threshold of the fly (Alphen et al. 2013).

Due to technical challenges of electrophysiological measurements, sleep in flies is usually assessed by analyzing their behavior. Because flies exhibit a significantly higher arousal threshold after 5 minutes of inactivity, sleep in flies is generally defined as immobility for at least 5 minutes (Huber et al. 2004). This can be assessed, for example, using video-tracking software or the *Drosophila* Activity Monitoring (DAM) system, a high throughput automated system that measures activity in flies to infer their sleep profile (Pfeiffenberger et al. 2010; Gilestro 2012).

The exploration of sleep neurochemistry revealed that the same neurotransmitters modulate sleep in mammals and flies (Sehgal & Mignot 2011; Cirelli 2009). In *Drosophila*, octopamine is a wake-promoting neurotransmitter (Seidner et al. 2015; Crocker & Sehgal 2008; Crocker et al. 2010). It is synthetized in a two-step pathway: (1) tyrosine is converted to tyramine by the enzyme tyrosine decarboxylase (Tdc); (2) tyramine is converted to octopamine by the enzyme tyramine β-hydroxilase (TβH). Octopamine has 4 known receptors – Octβ1R, Octβ2R, Octβ3R and OAMB. Of these, OAMB is known to be involved in sleep regulation (Crocker et al. 2010). It is analogous to the mammalian β adrenergic receptor and is expressed in several neurons, including the *pars intercerebralis* (PI), a structure in the fly's brain thought to be analogous to the mammalian hypothalamus (Crocker et al. 2010; Toivonen & Partridge 2009). Octopamine acts on the OAMB receptor to promote wakefulness, a similar effect to the one exerted in the mouse hypothalamus by noradrenaline from the *locus coeruleus* (Rolls et al. 2017; Crocker et al. 2010). Manipulation of the octopaminergic system further supports the role of octopamine as a wake-promoting agent. For example, the artificial activation of *tdc2*-positive neurons (*tdc2* is neuron-specific) suppresses sleep in flies, while inhibition causes an

increase of sleep (Crocker & Sehgal 2008; Seidner et al. 2015). Similarly, mutations in the *tdc2* and *tβh* genes that cause octopamine depletion increase sleep (Crocker & Sehgal 2008).

Along with octopamine, dopamine is a major wake-promoting signal (Kume et al. 2005; Andretic et al. 2005). In humans, for example, psychostimulants that promote dopamine accumulation in the synaptic cleft (e.g.: cocaine, amphetamines, methamphetamines) have a potent sleep-suppressing effect (Herrmann et al. 2017; Urbano et al. 2017). Likewise, flies fed with methamphetamines sleep significantly less than flies fed with vehicle food. Additionally, loss-of-function mutations of the *Drosophila* dopamine transporter *fumin* mimic the effect of methamphetamines (Kume et al. 2005). On the other hand, blocking the synthesis of dopamine by either inhibiting tyrosine hydroxylase (TH), which produces dopamine, or by feeding flies with 3-iodo tyrosine (3IY), which blocks synthesis of dopamine – has a sleep-promoting effect (Ueno et al. 2012).

GABA has sleep-promoting effects in both mammals and fruit flies (Lancel 1999; Agosto et al. 2008). In humans, drugs that promote GABA signaling are used as sleep-inducing agents, while in *Drosophila* mutations of the GABA receptor *Resistant to dieldrin* (*Rdl*) cause a reduction in sleep latency (Chung et al. 2009).

Drosophila has also helped to uncover genes underlying sleep regulation. Genes first discovered to modulate sleep in fruit flies were later shown to also have an effect in mammals. *shaker*, for example, encodes a voltage-gated K^+ channel and its mutation causes sleep reduction in *Drosophila* (Cirelli et al. 2005). *Kcna2*, the mammalian homolog of *shaker*, encodes a Kv1.2 voltage-dependent channel that reduces sleep in mice (Douglas et al. 2007). Studies in fruit flies also revealed that the PKA/CREB signaling pathway, which is involved in learning, memory recording and cognition, also modulates sleep (Levin et al. 2017; Hellman et al. 2010; Crocker & Sehgal 2008; Wu et al. 2009; Vecsey et al. 2009). Decreases of cAMP promote sleep, while increases of cAMP promote wakefulness. The role of the PKA/CREB pathway as a sleep modulator was confirmed in mammals, as alterations of the levels of cAMP affect sleep in rats and mutations of CREB impair sleep in mice (Vecsey et al. 2009; Hellman et al. 2010). These findings highlight the contributions of *Drosophila* to unveil the regulatory mechanisms of sleep in mammals.

Fruit flies constitute a powerful model for sleep studies and allow for a detailed analysis of the genetic and neural basis of sleep regulation. With a short lifespan, easy handling and a vast array of

genetic tools, *Drosophila* allows a systematic, unbiased and high throughput study of complex behaviors. The relatively small and simple *Drosophila* genome and nervous system also makes it possible to trace the biological connections from genes to neuronal circuits, to behavioral output more readily than in mammals.

Among some of the most versatile genetic tools employed in *Drosophila* research are the binary expression systems, such as Gal4/UAS and LexA/LexAop, derived from yeast and bacteria, respectively (Brand & Perrimon 1993; Yagi et al. 2010). These systems employ a transcription factor – Gal4 or LexA, that binds to a specific DNA sequence – UAS or LexAop, respectively, to activate the transcription of downstream sequences. These two systems, Gal4-UAS and LexA-LexAop, are independent of each other, and can be used to restrict transgene expression to a small number of neurons defined by the intersection of the two systems. Thus, using the binary systems we can manipulate the expression of any gene with temporal and spatial precision, a feature essential for the exploration of complex biological phenomena.

In addition to the binary expression systems, other methods are useful for the exploration of neuronal circuits. For instance, the GFP Reconstitution Across Synaptic Partners (GRASP) technique uses a GFP split into two non-functional components, one component expressed in the pre-synaptic cell and the other in the post-synaptic cell (Fan et al. 2013; Feinberg et al. 2008). If both cells are close enough to permit contact, the GFP protein is reconstituted to its functional form and a fluorescent signal is visible. Another useful reagent is GCaMP, a genetically encoded calcium indicator (GECI) that uses a GFP fused with the calcium-sensitive protein calmodulin to detect changes in the intracellular levels of calcium (Grienberger & Konnerth 2012). In neurons, increased calcium levels are associated with increased neuronal activity. GCaMP detects such increases in calcium and thus works as an indirect reporter of neuronal activity (Berlin et al. 2015; Tolö et al. 2013).

In summary, the presence of sleep in fruit flies and the existence of powerful genetic tools make *Drosophila* an excellent model for sleep studies.

1.3. Modulation of sleep by different behavioral drives

In both flies and mammals, sleep is regulated by two main systems: the circadian system and the homeostatic system. The circadian system controls the timing for sleep using endogenous molecular oscillators, and the homeostatic system regulates the need for sleep based on recent sleep history (Borbély & Achermann 1999).

The molecular mechanisms underlying the circadian clock are conserved in flies and humans (Donelson & Sanyal 2015; Allada et al. 2001). The *Drosophila* molecular clock consists of a transcriptional feedback loop in which CLOCK (CLK) and CYCLE (CYC) (a homolog of mammalian BMAL1) promote the transcription of their own repressors, PERIOD (PER) and TIMELESS (TIM) (the CRYPTOCHROME, CRY, plays the role of TIM in mammals) (Borbély & Achermann 1999; Allada & Chung 2010). The daily oscillation of CLK/CYC and PER/TIM renders a circadian cycle that takes approximately 24 hours. In flies, the neuronal substrate of the clock is a network of about 150 neurons in the brain that can be entrained by external clues, such as light and temperature (Cavanaugh et al. 2016; Light et al. 2016). This entrainment is of major importance because it permits the individual to anticipate and adapt to daily changes in the surrounding environment.

The homeostatic system is still not well understood. However, advances in *Drosophila* studies have helped to shed some light onto the subject, and some sleep centers, such as the dorsal fan-shaped body (dFSB) and the R2 ring of the ellipsoid body (EB R2) were shown to be involved in sleep homeostasis (Liu et al. 2016; Donlea et al. 2014) .The dFSB is a sleep-promoting structure localized in the medial dorsal brain and receives projections from the ExFI2 neurons. These neurons show increased electrical excitability in response to higher sleep pressure caused by sleep deprivation, and induce sleep when artificially stimulated, even in well-rested flies (Donlea et al. 2014; Donlea et al. 2011). A nearby structure, the EB R2 may encode sleep drive (Liu et al. 2016). In sleep-deprived flies, the R2 neurons exhibit an increase in synaptic terminals. However, if the R2 neurons are silenced, sleep rebound is attenuated, suggesting R2 neurons are involved in the modulation of sleep homeostasis.

While the circadian and the homeostatic systems explain how daily sleep-wake rhythms are regulated and how sleep need is modulated by sleep history, they are insufficient to explain how sleep

is integrated with other critical behaviors. Although it is essential for optimal performance, sleep is incompatible with virtually all other behaviors: while asleep, animals lose the chance to forage for food, fight for territory or find a sexual partner (Zhao et al. 2017; Griffith 2013). Besides, sleep usually occupies several hours of the day and constitutes a period of higher vulnerability to predators. Therefore, a contextualized suppression of sleep might be evolutionarily advantageous, as it offers the individual the opportunity to engage in other critical behaviors (Allada & Siegel 2008). Thus, there must be mechanisms that allow the brain to decide whether to sleep or to engage in other pressing behaviors.

Several recent studies highlight the coordinated regulation of sleep and other behaviors. For example, studies in rodents revealed that dopaminergic neurons from the dorsal raphe and from the ventral tegmental area promote wakefulness when the animal is exposed to salient stimuli, such as an attractive sexual partner, a novel object, savory food or a predator's odor (Cho et al. 2017; Eban-rothschild et al. 2016). Importantly, these studies offer insight into how the mammalian brain suppresses sleep in the presence of relevant motivational factors, such as sex drive, curiosity, hunger and fear. Findings in *Drosophila* also highlight the conflict between sleep and other critical needs. For instance, starved flies sleep less than fed ones, perhaps to forage for food (Mcdonald et al. 2010), and mated female flies display a reduction in daytime sleep, possibly to lay eggs (Isaac et al. 2010).

Specific social contexts, such as the ones associated with mating rituals, also modulate sleep. Male arctic sandpiper birds, for example, suppress sleep for 3-week periods – with no apparent impairment of performance – to compete with other males for the opportunity to mate with females, providing further evidence that sleep can be suppressed in favor of other important behaviors (Lesku et al. 2012). Sexual context also modulates sleep in *Drosophila*. Groups of flies with males and females sleep less than groups of flies with only one sex, presumably because flies in groups with both sexes engage in mating (Liu et al. 2015). Furthermore, video analysis of MF pairs revealed that the proximity between animals follows a daily cycle distinct from cycling of locomotor behavior of individual flies (Fujii et al. 2007). These studies suggest that male flies suppress sleep in order to engage in sexual activity, a behavior analogous to the one described for male sandpipers and rodents.

However, although several neuronal populations have been described to regulate sleep or courtship in *Drosophila*, the neuronal circuitry necessary to integrate both behaviors in a concerted

and contextualized manner has not yet been described. Determining this circuitry would be a significant advancement into our understanding of how the brain balances sleep with other behaviors.

1.4. The courtship behavior in *Drosophila*

In *Drosophila*, male-specific behavior, such as courtship, is determined by two main genes: *doublesex* (*dsx*) and *fruitless* (*fru*) (Ito et al. 1996; Ryner et al. 1996; Baker et al. 2001; Stockinger et al. 2005; Rideout et al. 2010; Yamamotot & Koganezawa 2013). The main function of *dsx* is to determine the soma of the fly. In females, DsxF specifies female genitalia, and in males, DsxM determines male genitalia (Rideout et al. 2010). DsxM also plays a role in learned social behaviors (Pan & Baker 2014). On the other hand, *fru* determines the behavior of the fly, and has a sexually dimorphic expression in the nervous system, encoding a functional protein FRU^M in males, and a non-functional protein FRU^F in females (Ryner et al. 1996; Stockinger et al. 2005; Von Philipsborn et al. 2014). *fru* also encodes a third isoform – FRU^C – common to both males and females, which is not involved in the determination of the sex of the nervous system (Song et al. 2002).

FRU^M is both necessary and sufficient to determine male-specific courtship behavior (Stockinger et al. 2005; Kohatsu et al. 2011). In fact, males that were manipulated to express fru^F, instead of fru^M, exhibit courtship deficits and orient their sexual activity towards other males, forming courtship chains, in which each fru^F male courts while being simultaneously courted by other fru^F males (Stockinger et al. 2005). These abnormal behaviors are also observed when *fru*-expressing neurons are silenced. Interestingly, females engineered to express fru^M or in which *fru* neurons are activated by heat induced cation channel, *dTrpA1*, mimic the behavior of fru^F males (Stockinger et al. 2005).

Male *Drosophila* courtship follows a complex ritual of multiple stereotypical steps: orienting, chasing, singing, tapping, licking, attempted copulation, and eventually, copulation (Greenspan & Ferveur 2000; Bastock & Manning 1955). First, the male starts by orienting his body toward the female – orienting – and then proceeds to chase her – chasing. If the female allows some proximity, the male extends and vibrates one of his wings to produce the courtship song – singing. The unilateral extension of the wing is an easily recognizable element of the courtship ritual and is often used to assess the courtship performance of the male. Then, through the forelegs, the male samples the gustatory

pheromones of the female by tapping her in the abdomen – tapping. Eventually, the male may lick the female's genitalia – licking – and may also try to copulate – attempted copulation. If the female is receptive, she will open her wings and vaginal plate and allow the male to successfully copulate – copulation. In the dark, it is possible to note an extra step – "scanning", in which the male extends both wings while attempting to find the female (Krstic et al. 2009).

In males, the expression of *fru* determines the development of a male-specific network of approximately 2000 neurons, organized in several clusters that process different aspects of male behavior, from the sensory input to the central processing of external and internal information, to the activation of output motor programs [84,89,92–](Koganezawa et al. 2010). For example, the pheromonal information from other flies may be detected by *fru*-positive *pickpocket 23* (*ppk23*) neurons of the foreleg and then transmitted through specialized ascending pathways to processing centers in the brain, such as the P1 neurons (Toda et al. 2012; Kallman et al. 2015; Clowney et al. 2015). These neurons then integrate information from the sensory inputs and from the internal state of the animal to compute how the male should interact with other flies (Koganezawa et al. 2016; Pattnaik et al. 2016; Kallman et al. 2015; Kohatsu et al. 2011; Kimura et al. 2008).

The P1 neurons are essential to coordinate male-specific behavior. They form a male-specific cluster of about 20 to 30 fru^{+} dsx^{+} double-positive cells in the dorsal, lateral posterior protocerebrum and are a central node in the regulation of male sexual activity (Pattnaik et al. 2016; Kimura et al. 2008). The P1 neurons are intensely excited by female sensory cues, as indicated by the increase of GCaMP signal in P1 neurons of males exposed to virgin females (Kohatsu et al. 2011). P1 neurons thus integrate sensory inputs induced by females with inputs that translate the internal state of the male – for example, mating drive – to promote oriented courtship in a contextualized fashion.

As a key processing center of sexual behavior, P1 neurons are both necessary and sufficient to induce sustained courtship, even if excited just by a momentary stimulus and in the absence of a target (Kohatsu & Yamamotot 2015; Philipsborn et al. 2011). Indeed, the transient stimulation of P1 neurons, using the *TrpA1* channel or the red-light sensitive channelrhodopsin, is sufficient to evoke persistent courtship. Therefore, regulatory systems may be in place to restrain the activity of P1 neurons to the appropriate social, sexual and environmental contexts. Among such regulatory systems, it is important to note the role of the medial anterior-lateral (mAL) interneurons as direct inhibitors of

the P1 cluster (Clowney et al. 2015). The mAL neurons are *fru*-positive sexually dimorphic GABAergic cells localized just above the antennal lobe of the male brain. They constitute the inhibitory branch of a sensory pathway that conveys pheromone signals from the *ppk23* neurons in the forelegs to processing centers in the brain, such as the P1 neurons (Clowney et al. 2015). Interestingly, although they respond to pheromones from both sexes, mAL neurons respond more intensely to female pheromones. Because the P1 neurons are immediately downstream of the mAL, contact with a female elicits mAL neurons to inhibit the P1 neurons. Therefore, the mAL neurons may help to suppress the persistent activity of the P1 cluster, thus helping to limit the mating activity of the male to the appropriate social context (Clowney et al. 2015).

The activity of P1 neurons and, consequently, the engagement of males in courtship, takes into consideration not only the sensory inputs triggered by females, but also the male mating drive, which is the internal state that translates the motivation of the male to engage in courtship. The mating drive is higher in sex-deprived males, and lower after multiple sexual encounters (Anderson 2016; Zhang et al. 2016).

Recent data indicate that dopaminergic circuits monitor the recent sexual history of the male to modulate the mating drive (Zhang et al. 2016). The *fru*-positive aSP4 neurons, integrated in the protocerebral anterior lateral (PAL) node, are an example of cells that are part of such dopaminergic circuits. aSP4 neurons exhibit a progressive reduction of activity as sexual satiety increases, and their exogenous activation can reverse the reduced courtship in sexually satiated males (Zhang et al. 2016). Information regarding sexual satiety may be transmitted by aSP4 neurons directly to P1 neurons through contacts in the superior medial, anterior protocerebrum (SMPa). Hence, the P1 neurons integrate sensory stimuli triggered by females and the internal state of the male, to promote a concerted behavioral response.

In this work, we describe how *Drosophila* males decide between conflicting behavioral drives under specific social contexts. Specifically, we explore the decision between sleep and courtship, two incompatible but essential behaviors. In the presence of a female, a male may choose to suppress sleep in order to engage in courtship. The suppression of sleep does not occur when males are paired with other males or when males are sexually satiated, indicating that the male sexual arousal may act as a modulator of sleep. The dynamic interaction between sleep and sex drive is further supported by

the observation that the male sexual performance is affected by an increased sleep drive. Moreover, we describe the octopaminergic MS1 (Male Specific 1) neurons as mediators of the decision between sleep and courtship. The MS1 neurons suppress sleep exclusively in males and mediate sleep suppression secondary to sexual arousal induced by females, as males with inhibited MS1 neurons do not lose sleep in the presence of females and display courtship deficits. Finally, we show that, in males, the MS1 neurons, which do not express FRU interact with the FRU neuronal network to suppress sleep and to promote a sustained state of sexual arousal.

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Aims

- Understand how sex drive and sleep drive are integrated in the brain, allowing the male to decide between sleep and courtship.

- Describe the role of MS1, MS2 and P1 neurons in the regulation of male sleep.

Chapter 2

Experimental work

Chapter 2.1.

Identification of octopaminergic neurons that modulate sleep suppression by male sex drive.

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Identification of octopaminergic neurons that modulate sleep suppression by male sex drive

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Abstract

Molecular and circuit mechanisms for balancing competing drives are not well understood. While circadian and homeostatic mechanisms generally ensure sufficient sleep at night, other pressing needs can overcome sleep drive. Here, we demonstrate that the balance between sleep and sex drives determines whether male flies sleep or court, and identify a subset of octopaminergic neurons (MS1) that regulate sleep specifically in males. When MS1 neurons are activated, isolated males sleep less, and when MS1 neurons are silenced, the normal male sleep suppression in female presence is attenuated and mating behavior is impaired. MS1 neurons do not express the sexually dimorphic FRUITLESS (FRU) transcription factor, but form male-specific contacts with FRU-expressing neurons; calcium imaging experiments reveal bidirectional functional connectivity between MS1 and FRU neurons. We propose octopaminergic MS1 neurons interact with the FRU network to mediate sleep suppression by male sex drive.

Introduction

Sleep is mainly regulated by two processes: the circadian process, which controls the timing of sleep, and the homeostatic process, which modulates sleep drive based on sleep-wake history (Borbely and Achermann, 1999). However, because sleep is incompatible with virtually all other behaviors, sometimes it may be advantageous to forgo sleep in order to engage in other critical behaviors (Siegel, 2012). For example, male arctic sandpipers that sleep the least during 3-week mating periods produce the most offspring (Lesku et al., 2012). Elucidating the neural mechanisms underlying the choice between sleep and sex, two behaviors critical for the fitness of individuals and species, would provide valuable insights into the general problem of balancing conflicting needs.

Sleep in *Drosophila* shares many features with sleep in humans. Like humans, flies adjust their sleep behavior depending on other needs (Griffith, 2013). Starved flies sleep less than well-fed flies, presumably to forage for food (Keene et al., 2010); female flies sleep less after mating, presumably to lay eggs (Isaac et al., 2010); and mixed-sex groups of flies sleep less than single-sex groups, presumably to engage in sexual activities (Liu et al., 2015). Although several neuronal populations that regulate sleep or courtship in the fly nervous system have been identified (Auer and Benton, 2016; Chakravarti, 2016; Griffith, 2013; Yamamoto and Koganezawa, 2013), neural substrates underlying coordinated regulation of sleep and sexual behavior remain elusive.

Here we demonstrate that the balance between sleep and sex drives determine whether male flies sleep or court, and describe a newly identified neuronal group mediating sleep suppression by male sexual arousal. Earlier studies have shown that norepinephrine and its *Drosophila* counterpart octopamine act as wake-promoting signals (Aston-Jones and Bloom, 1981; Carter et al., 2010; Crocker and Sehgal, 2008). We found that a small number of octopaminergic neurons, which we named MS1 (Male Specific 1), regulate the decision between sleep and courtship in males. Activating MS1 neurons reduced sleep specifically in males, and silencing MS1 neurons led to decreased female-induced sleep loss and impaired mating behavior. The male-specific isoform of the FRU transcription factor FRU^M, which we will refer to as FRU for simplicity, is expressed in ~1500 neurons that range from peripheral sensory neurons to motor neurons, forming a circuit that controls courtship behavior (Auer and Benton, 2016; Kimura et al., 2005; Manoli et al., 2005; Stockinger et al., 2005; Yamamoto and Koganezawa,

2013). We found that MS1 neurons do not express FRU, but instead interact with the FRU neural circuit; calcium imaging experiments revealed that MS1 neurons act both upstream and downstream of FRU neurons. We propose that octopaminergic MS1 neurons communicate with the FRU courtship circuit bidirectionally to promote sexual arousal and establish a state of enhanced readiness for sustained courtship.

Materials and Methods

Fly stocks

Flies were raised on standard food containing molasses, cornmeal, and yeast under a 12h:12h light:dark cycle. MS1-Gal4 (BDSC#12837) (Bellen et al., 2004), *Tdc2*-LexA (BDSC#52242) (Shearin et al., 2013), UAS-*TrpA1* (BDSC#26263) (Hamada et al., 2008), UAS–m*CD8::GFP* (BDSC#5137) (Lee and Luo, 1999), lexAop2-*mCD8::GFP*, UAS-IVS-*mCD8:: RFP* (BDSC#32229), UAS-*NaChBac::eGFP* (BDSC# 9466) (Luan et al., 2006), UAS-*TNT* (BDSC#28838) (Sweeney et al., 1995), LexAop2-*FLPL* (BDSC#55820), GMR23E10-Gal4 (BDSC#49032), GMR71G01-lexA (BDSC#54733), UAS-*GCaMP6m* (BDSC#42750), and iso31 (w^{1118}) (BDSC#5905) were obtained from the Bloomington Stock Center. UAS-FRT-stop-FRT-*mCD8::GFP*, UAS-FRT-stop-FRT-*Dscam::GFP* and UAS-FRT-stop-FRT-*nSyb::GFP* and *fru-FLP* (Yu et al., 2010), LexAop-*GCaMP6m* and UAS-*P2X2* (Lima and Miesenbock, 2005), LexAop-*P2X2* (Yao et al., 2012), and $fru^{4\text{-}40}$ (Demir and Dickson, 2005) lines were obtained from Barry Dickson; $dsx^{683\text{-}7058}$ and $dsx^{1649\text{-}9625}$ mutants (Chatterjee et al., 2011), and *fru*-LexA (Mellert et al., 2010) from Bruce Baker; LexAop-Gal80 (Thistle et al., 2012) and LexAop-*spGFP11::CD4* (Gordon and Scott, 2009) from Kristin Scott; UAS- *spGFP1-10::NRX* (Fan et al., 2013) from Nirao Shah; P1-split Gal4 (Inagaki et al., 2014) from David Anderson; Tbh^{nm18} mutants (Monastirioti et al., 1996) from Maria Monastirioti; and $Oamb^{286}$ mutants (Lee et al., 2003) from Kyung-An Han. Fly lines used in behavioral experiments were outcrossed to an isogenic background (iso31) for at least five generations, except for the *Tbh* and *Oamb* lines.

Sleep analysis

For sleep analysis, 4- to 7-day-old flies entrained to a 12h:12h LD cycle were placed in glass tubes containing 5% sucrose and 2% agar. Flies were raised and monitored at 25°C except where noted. Males and females were housed together in groups of ~30 flies until they were loaded into tubes. For experiments involving TrpA1, flies were raised in LD at 22°C and monitored for 1 day at

22°C to determine baseline levels, 1 day at 28-29°C to activate the TrpA1 channel, and 1 day at 22°C to examine recovery. For TNT experiments comparing MM and MF pairs, flies were raised and assayed at 22°C because initial data suggested that UAS-TNT/+ controls behaved differently from other controls at 25°C, perhaps due to leaky TNT expression. Activity data were collected in 1-min bins using *Drosophila* Activity Monitoring (DAM) System (Trikinetics). Beam breaks from single-beam (SB) monitors with infrared (IR) detectors at a single location or inter-beam movements from multi-beam (MB) monitors with IR detectors at 17 locations (Garbe et al., 2015) were used to measure sleep as a period of inactivity lasting at least 5 min (Huber et al., 2004). SB monitors were used for all experiments involving isolated flies, while both SB and MB monitors were used in the MF interaction experiments. We found that although the absolute sleep levels were somewhat higher with SB monitors, the same pattern of reduced sleep in MF compared to MM pairs was seen using SB or MB monitors. For video recording, flies were loaded into 7 mm x 16 mm x 3 mm recording arenas. For nighttime recording, a USB webcam (Logitech Webcam Pro 9000) and infrared LEDs were used as previously described (Faville et al., 2015), and for daytime recording, a digital camera (Sony DCR-SX63) and white LEDs were used. For sleep deprivation experiments, flies placed in SB monitors or recording arenas were deprived of sleep using mechanical stimulation. A multi-tube vortexer fitted with a mounting plate (Trikinetics) was used to apply mechanical stimulation for 3 sec every min. Satiety manipulation was essentially as described (Zhang et al., 2016), except that single virgin males were grouped with 10-15 virgin females for 4.5-5.5 hr to induce satiety. Mating behavior (courtship and copulation) was scored at the beginning and end of the satiety assay, which confirmed that males were satiated by the end of the assay. Immediately following the satiety manipulation, shortly before ZT12, individual male flies were aspirated into monitor tubes or recording arenas that contained control females for sleep assay in the MF condition. For DAM data, sleep parameters were analyzed using a MatLab-based software, SleepLab (William Joiner). For video data, sleep amount of individual flies was manually scored for the first 5 min of each nighttime hour, except for the sleep deprivation experiment, where the first 5 min of each 30-min interval during 6 hours after deprivation was scored. Scoring was done blind to the experimental condition and genotype. In cases where only one fly in a MF pair was active, we used male courtship behavior to determine its sex.

Analysis of mating behavior

For simultaneous analysis of courtship and sleep during the night, videos recorded under infrared light were manually scored for courtship and sleep during 5 min periods as indicated. For analysis of courtship during the day, virgin male flies were collected and housed in groups of ~10 on standard fly food for 4-8 days. Iso31 virgin females (3-7 days post-eclosion) were used in non-competitive assays, which were performed during the day phase (ZT1-6). For non-competitive courtship assays, a male and female were gently aspirated into a plastic mating chamber (15 mm diameter and 3 mm depth) covered with a clear plastic plate, and were kept separated until a divider was removed after ~10 min. For the light condition, dim light (~25 lux) was used because bright light has been shown to interfere with courtship performance of *white* males (Krstic et al., 2013). For the dark condition, infrared light was used. Flies were recorded for 90 min using a USB webcam (Logitech Webcam Pro 9000) and scored blind to experimental condition. Courtship index was determined as the fraction of total time a male was engaged in courtship activity during a period of 5 min or until successful copulation after courtship initiation. Courtship activity included orienting, chasing, singing, attempted copulation, and "scanning", a behavior specific to the dark condition, where the male extends both wings in search of a female (Krstic et al., 2009). Only males that took at least 1 min to copulate after courtship initiation were included in the computation of courtship index. Competitive copulation assays were performed under dim red light using Canton-S virgin females. Males of different genotypes were marked with a small dot of acrylic paint on their thorax. Two males and one female were aspirated into each well of a 12-well plate, and the first male to successfully copulate within 90 min was determined the winner. Trials in which neither male succeeded in copulating were not included in the analysis.

Immunohistochemistry and GRASP

For whole mount immunostaining, fly brains were fixed in 4% paraformaldehyde (PFA) for 30 min, dissected, and blocked in 5% normal goat serum for 1 h at RT. The following primary antibodies

were used: rabbit anti-GFP (Molecular Probes Cat# A-21312, RRID:AB_221478) at 1:500, mouse anti-RFP (Rockland Cat# 600-401-379, RRID:AB_2209751) at 1:500, BRP (DSHB Cat# nc82, RRID:AB_528108) at 1:150; anti-HA (Covance Research Products Inc Cat# MMS-101R-500, RRID:AB_10063630) at 1:1000; and anti-DSX (kind gift from Bruce Baker) at 1:300. The secondary antibodies, Alexa Fluor 488 goat anti-rabbit (Thermo Fisher Scientific Cat# A11008, RRID:AB_143165) and Cy5 goat anti-mouse (Thermo Fisher Scientific Cat# A10524, RRID:AB_2534033) were used at 1:1000. Primary and secondary antibodies were incubated at 4°C overnight. For GRASP experiments, fly brains were fixed in PFA for 30 min at RT, and imaged immediately without immunostaining. Images were obtained on a Leica SP8 confocal microscope.

Calcium Imaging

4- to 7-day-old flies that were housed individually and entrained to LD cycles were anesthetized on ice and dissected in adult hemolymph-like saline (AHL (Wang et al., 2003)), and brains were mounted on a glass-bottom chamber containing AHL. A custom-built gravity-dependent perfusion system was used to control perfusion flow. Leica SP8 confocal microscope was used to acquire 20 to 25 slices (~2.5 μm/slice) of the antero-dorsal, ventral, or posterior brain every 2.5 or 5 sec for 3 to 5 min. 2.5 mM ATP in AHL was delivered for 1 min after 1 min of baseline measurements. FIJI was used to compute projections of relevant confocal slices, and regions of interest (ROIs) were selected using images taken at high laser intensity. The average intensity of the ROIs during the 30-sec period before the start of ATP perfusion was used as the baseline measurement, F^0. For each time point, normalized ΔF, $(F-F^0)/F^0$, was computed.

Statistical Analysis

To compare multiple groups, one-way ANOVAs were performed followed by Tukey or Dunnett post-hoc tests. Two-way ANOVAs were performed to test for the interaction in experiments involving

two factors. Student's *t* tests were used to compare pairs of groups. To compare sleep bout durations, which are not normally distributed, Kruskal-Wallis tests were performed, followed by Dunn's post hoc tests. Log-rank tests were used for cumulative courtship initiation rate and cumulative copulation success rate in non-competitive mating assays. For competitive copulation data, binomial tests were used to assess whether the observed percentage was different from 50%. Bonferroni corrections were applied to correct for multiple tests performed on data from the same flies (e.g., daytime and nighttime). All experiments were repeated on at least two separate occasions using flies from independent genetic crosses, and pooled data are presented.

Results

Balance between sex and sleep drives determines courtship vs sleep behavior

To determine the effects of sexual stimuli on male sleep, we measured sleep in wild-type flies in different social settings: isolated male (M) or female (F) flies, and male-male (MM) or male-female (MF) pairs using multi-beam or single-beam Drosophila Activity Monitors (DAMs) (see Materials and methods). Sleep amount was markedly reduced in MF pairs relative to MM pairs (Figure 1A,B and Figure 1 – figure supplement 1). As expected, isolated females exhibited reduced daytime sleep relative to isolated males, and the reduction was comparable to the daytime sleep reduction in MF relative to MM pairs (Figure 1B and Figure 1 – figure supplement 1B), consistent with the possibility that the difference in daytime sleep between MF and MM pairs is largely due to female wakefulness. In contrast, nighttime sleep loss in MF relative to MM pairs is considerably greater than the difference in sleep amount between isolated males and females (Figure 1B and Figure 1 – figure supplement 1B), which suggests that the nighttime sleep loss in MF pairs is not simply due to the presence of another fly or sex differences in sleep amount between males and females in isolation.

To assess nighttime behavior of individual flies in MM or MF pairs, we made video recordings under infrared light, which revealed that males paired with females spent much of the night engaged in courtship (Video 1). To quantify this observation, we manually scored courtship and sleep-wake behavior of individual flies for the first 5 minutes every hour during the 12-hr dark period. Male behavior was categorized as sleep, courtship, or wake without courtship, whereas female behavior was categorized as sleep or wake. Individual male flies slept more in MM pairs than in MF pairs (Figure 1C), with awake males spending most of their time courting in MF pairs (Figure 1D), a behavior not exhibited by either male in MM pairs. Pairs of flies tended to be awake or asleep together, and only ~2% of the time was a female awake while its male partner was asleep. As a result, sleep in a pair of flies (defined as when both flies are asleep) is a good measure of sleep in a single male fly in the pair (Figure 1C). These results demonstrate that males spend much of the night courting instead of sleeping when paired with females, and validate the use of DAMs to measure sleep in pairs of flies. Our data, together with previous work that employed video tracking to conclude that daily rhythms in the proximity

between flies in MF pairs are driven by male sex drive (Fujii et al., 2007), led to the idea that male flies possess mechanisms for suppressing sleep in the presence of female flies.

If sex drive underlies female-induced male sleep loss, sexually satiated males would not exhibit sleep loss in the presence of females. To test this prediction, we employed a recently developed satiety assay (Zhang et al., 2016). As previously shown, male flies housed with a number of virgin females exhibited reduced courtship and copulation behaviors over a 4.5-hr period (Figure 2 - figure supplement 1). When paired with a female in a DAM, a satiated male that had been grouped with virgin females slept more than a naive male that had been grouped with other males (Figure 2A). Video analysis confirmed that satiated males exhibited increased sleep accompanied by decreased courtship index (Figure 2B,C). These data suggest that when sex drive is satisfied, the normal level of nighttime sleep drive is sufficient to allow males to sleep in the presence of females.

In a complementary experiment, we tested whether excessive sleep drive can overcome sex drive in non-satiated males by depriving them of sleep by mechanical stimulation. Whereas MF pairs slept less than MM pairs under non-deprived conditions, MF pairs slept as much as MM pairs following 6 hours of sleep deprivation (Figure 2D). Video analysis confirmed that sleep-deprived males slept more and courted less than non-deprived males (Figure 2E,F). These results demonstrate that excessive sleep drive can overcome sex drive.

We next examined how activation of the sleep-promoting dorsal fan-shaped body (dFSB) affects sleep in MF pairs. The dFSB is thought to function in the output arm of the sleep homeostat (Donlea et al., 2014; Donlea et al., 2011). We induced sleep by activating dFSB using the R23E10-Gal4 driver (Donlea et al., 2014) to express the bacterial sodium channel NaChBac. As expected, activation of dFSB induced sleep in isolated males (Figure 2 – figure supplement 2). Notably, males with activated dFSB slept almost as much when paired with control females (MF^c) as when paired with control males (MM^c) at night (Figure 2G). In contrast, parental controls, i.e., males carrying either R23E10-Gal4 or UAS-*NaChBac* alone, exhibited the normal pattern of reduced nighttime sleep in MF^c relative to MM^c pairs (Figure 2G). These data provide further evidence that elevated sleep drive suppresses sexual behavior. Together, our data demonstrate that the relative strength of sleep drive and sex drive determines whether a male engages in sleep or courtship.

MS1 neuronal activity regulates male sleep and courtship

What are the neural mechanisms underlying the decision between sleep and courtship? In an ongoing screen for neuronal populations regulating sleep in *Drosophila*, we isolated MS1-Gal4, an enhancer trap line that is associated with sexually dimorphic regulation of sleep. The Gal4 insertion (BG02822) is in an intron of the gene encoding Multidrug-Resistance like Protein 1 (MRP1). It is not known whether MRP1 plays a role in sleep or courtship. We employed the Gal4/UAS binary expression system to express the warmth-sensitive TrpA1 channel in MS1 neurons, and activated MS1 neurons by shifting the temperature from 22°C to 29°C. We found that activation of MS1 neurons led to decreased sleep in isolated males, but not in isolated females (Figure 3A). Males with activated MS1 neurons did not exhibit male-specific behaviors, such as courtship and aggression, but instead exhibited locomotor behavior typically observed in awake flies, i.e., pacing the perimeter of the recording arena (Video 2). Although males with activated MS1 neurons lost about 2/3 of nighttime sleep relative to controls at 29°C, they slept no more than the control males when the temperature was returned to 22°C (Figure 3A), suggesting that sleep loss due to activated MS1 neurons does not lead to recovery sleep. Since the TrpA1 channels were activated for only one day during the adult stage, these results indicate that MS1 neurons function in adult male flies to promote wakefulness. Constitutive activation of MS1 neurons by NaChBac expression also resulted in male-specific sleep reduction (Figure 3B), demonstrating that both chronic and acute activation of MS1 neurons affect male sleep.

When we expressed tetanus toxin (TNT) in MS1 neurons to block neurotransmission, sleep in isolated males was not altered (Figure 3C). We hypothesized this may be because MS1 neurons become activated only under specific social contexts. Indeed, whereas silencing of MS1 neurons via TNT expression did not affect sleep in MM^c pairs (experimental males paired with control males), it led to a significant occlusion of the nighttime sleep loss in MF^c pairs (Figure 3C). For further analysis of male sleep and courtship behavior in MF^c pairs, we examined videos recorded under infrared light. We quantified sleep amount and courtship index for the first 5 minutes every nighttime hour. Males with silenced MS1 neurons slept more and courted less than parental control males (Figure 3D,E). These

data suggest that activation of MS1 neurons is dependent on female cues and is required to keep males awake in the presence of females, presumably so that they can engage in sustained courtship.

The above experiment was conducted at night when sleep drive is high and MF pairs had been together for over a day. To further examine the role of MS1 neurons in male mating behavior, we performed courtship and copulation assays during the day immediately after a virgin female was introduced to a virgin male. Inhibition of MS1 activity by TNT expression had little effect on courtship index and copulation latency when assayed under white light conditions (Figure 3F,G). Since the sleep-suppressing effects of MS1 activity manipulation were stronger in the dark, we repeated the assays in infrared light during the subjective day. We found that whereas inhibition of MS1 activity by TNT expression had little effect on courtship index, it had a significant effect on copulation latency (Figure 3F,G). Since males with silenced MS1 neurons can successfully copulate in the light condition, it is unlikely that MS1 neuronal activity is directly required for copulation. Instead it may be required for timely progression through stages of courtship in the dark. Consistent with delayed copulation in these assays, MS1>TNT males were also less successful in mating when competing against control males (Figure 3H). Together, our findings suggest that MS1 neuronal activity is important for optimal mating performance in the dark, especially during the night when sleep drive is high.

Octopamine mediates male sleep regulation by MS1 neurons

We next examined the expression pattern of MS1-Gal4 using membrane-bound GFP, and found a restricted pattern of expression in 3 to 7 neurons in the subesophageal ganglion (SOG) near the midline and ~4 pairs of neurons in the dorsal brain (Figure 4A). No cell bodies were detectable in the ventral nerve cord, although there were descending projections from the central brain (Figure 4B). Differences between male and female expression patterns were not apparent. We noticed that some of the projection patterns of the MS1 neurons in the SOG resemble those of octopaminergic neurons (Busch et al., 2009; Busch and Tanimoto, 2010). In addition, previous findings showed that sleep loss through activation of octopaminergic neurons does not result in rebound sleep (Seidner et al., 2015), and light inhibits the wake-promoting effects of octopamine (Shang et al., 2011). These observations

led us to hypothesize that some of the MS1 neurons are octopaminergic. To test this idea, we expressed RFP in MS1 neurons and GFP in TDC2-expressing octopaminergic neurons (TDC2 is an enzyme required for octopamine synthesis in the nervous system (Cole et al., 2005)). We found that all MS1 neurons in the SOG are octopaminergic (Figure 4C). Consistent with this finding, co-expression of Gal80, an inhibitor of Gal4, in TDC2 neurons removed all MS1-Gal4 activity in the SOG (Figure 4D).

To test whether the octopaminergic subset of MS1 neurons underlie male-specific sleep suppression, we restricted NaChBac expression to the non-octopaminergic subset of MS1 neurons using Gal80 in TDC2 neurons. Indeed, we found that activation of non-TDC2 MS1 neurons did not alter sleep in males (Figure 4E). Furthermore, mutations in Tyramine β-hydroxylase (Tbh), another protein involved in octopamine synthesis (Monastirioti et al., 1996), and OAMB, an octopamine receptor implicated in sleep regulation (Crocker et al., 2010), prevented the male sleep loss induced by activation of MS1 neurons (Figure 4F). These data show that a small number of octopaminergic neurons in the SOG mediate male-specific arousal through the OAMB receptor.

MS1 and FRU neurons appear to be connected anatomically

Given the male-specific role of MS1 neurons in sleep regulation and the central role of the FRU circuit in the regulation of male behaviors, we employed two approaches to test whether MS1 neurons express FRU. First, we used the FLP-FRT system to restrict GFP expression to the intersection of MS1-Gal4 and *fru*-LexA (Mellert et al., 2010), and second, we used *fru*-LexA to express GFP in FRU neurons while simultaneously expressing RFP under the control of MS1-Gal4. We did not detect any overlap between the MS1 and FRU populations (Figure 5A,B and Figure 5 – figure supplement 1A), nor did we detect DOUBLESEX (DSX), another transcription factor important for male sexual behavior, in MS1 neurons (Figure 5C).

Three sets of octopaminergic neurons in the SOG have been characterized previously, but MS1 neurons appear to be distinct based on 3 lines of evidence. First, 3 FRU-expressing octopaminergic neurons in the SOG underlie the decision between courtship and aggression (Certel et al., 2010), but MS1 neurons do not express FRU. Second, a subset of octopaminergic neurons in the SOG defined by

Tdc2-Gal4 and *Cha*-Gal80 mediate aggression (Zhou and Rao, 2008), but the combination of MS1-Gal4 and *Cha*-Gal80 removes all expression from the SOG. Third, VPM5 is implicated in memory formation (Burke et al., 2012), but cell body locations and projection patterns suggest MS1 neurons do not include VPM5. Thus, MS1 neurons are distinct from previously characterized octopamingeric neurons.

Interestingly, we observed a group of FRU neurons in close proximity to the MS1 neurons in the SOG (Figure 5B), suggesting a possible interaction between the two neuronal groups. We thus examined whether MS1 and FRU neurons can form synaptic contacts by employing a version of GRASP (GFP Reconstitution Across Synaptic Partners) in which one of the GFP fragments is fused to NEUREXIN (NRX) for synaptic targeting (Fan et al., 2013; Feinberg et al., 2008; Gordon and Scott, 2009). We observed that punctate reconstituted GFP (i.e., GRASP) signals in the region surrounding the esophagus were more pronounced in male brains compared with female brains (Figure 5D,E). GRASP signals were not observed in control flies, which demonstrates that the observed GRASP signals are not due to leaky transgene expression (Figure 5 – figure supplement 1B). These data demonstrate sexual dimorphism in the potential contacts between MS1 and FRU neurons, which may account for the male-specific effects on sleep with activation of MS1 neurons.

To determine anatomical connectivity between MS1 and FRU neurons, we examined patterns of pre- and post-synaptic markers in MS1 neurons. When the NSYB pre-synaptic marker was targeted to the octopamingeric subset of MS1 neurons, we observed expression in multiple brain areas known to be involved in male reproductive behavior (Yamamoto and Koganezawa, 2013; Zhang et al., 2016), including the antennal lobe, lateral horn, lateral protocerebrum, anterior superior medial protocerebrum, and mushroom body regions (Figure 5F). Since FRU neurons also send projections to these regions, it is possible that MS1 neurons make synaptic contacts with several clusters of FRU neurons that were not detected by the GRASP technique. In contrast to the pre-synaptic marker, the DSCAM post-synaptic marker was enriched around the esophagus (Figure 5G), where we observed strong GRASP signals, suggesting that MS1 neurons receive input from the FRU circuit through this region. However, the NSYB pre-synaptic marker was also observed in the region surrounding the esophagus (Fig 5F), indicating octopaminergic MS1 neurons are positioned to receive and send information through this region of the brain.

MS1 and FRU neurons are connected functionally

The potential anatomical connection between MS1 and FRU neurons suggest that the two populations may be functionally connected. We first tested whether MS1 neurons act upstream of FRU neurons by expressing ATP-dependent channel P2X2 (which is not normally expressed in the fly) in MS1 neurons and using live imaging of GCaMP6m in FRU neurons to measure calcium fluctuations in dissected brains. Upon application of ATP, a number of FRU neurons exhibited a marked increase in the GCaMP6m signal (Figure 6A,B), indicating that MS1 neurons act upstream of several FRU clusters. Based on the position of their cell bodies, one of these clusters appeared to be P1 interneurons in the posterior brain. Using the more restricted R71G01-LexA driver, we confirmed an excitatory input from MS1 to P1 neurons (Figure 6 – figure supplement 1).

To test how broadly MS1 neurons influence activity of the FRU circuitry, we examined connectivity with multiple other FRU subpopulations. One of these bilateral FRU cluster located above the antennal lobes is likely to be mAL, which provides inhibitory input to P1 neurons (Clowney et al., 2015; Kallman et al., 2015). In addition, although we could not confirm their identity, a neuronal pair in the superior protocerebrum may be FRU-expressing dopaminergic aSP4 neurons (Figure 6A), whose activity has recently been shown to reflect mating drive (Zhang et al., 2016). Quantification of the GCaMP6m signal in the cell bodies of mAL neurons as well as the arch neuropil region heavily innervated by FRU neurons showed that stimulation of MS1 neurons led to strong calcium responses in males but not in females (Figure 6B). Since P1 neurons are not present in females, excitatory input from MS1 to P1 is also male specific. Additional unidentified FRU clusters responded strongly to activation of MS1 neurons, indicating that MS1 neurons provide broad excitatory input to the FRU circuit.

Consistent with the idea that MS1 neurons act upstream of FRU neurons, we found that activation of MS1 neurons in a *fru* mutant background had little effect on male sleep (Figure 7A). In addition, MS1 activation in a *dsx* mutant background produced little effect on male sleep (Figure 7A), suggesting that MS1 neurons also act upstream of *dsx*-expressing neurons. P1 neurons express both FRU and DSX (Kimura et al., 2008), and our data show that they exhibit calcium responses to MS1 stimulation, and thus are a prime candidate for a FRU cluster acting downstream of MS1 neurons to

promote wakefulness. Indeed, activation of P1 neurons using a highly restricted P1-split Gal4 driver (Inagaki et al., 2014) resulted in a strong reduction in male sleep, but had no effect on female sleep as expected from the sexually dimorphic nature of P1 (Figure 7B).

Our data suggest P1 neurons act downstream of MS1 neurons to mediate sleep regulation. Artificial activation of P1 has been shown to trigger courtship behaviors in isolated males (Kohatsu et al., 2011; von Philipsborn et al., 2011), and similarly we observed wing extension in males with activated P1 neurons (Video 3). However, wing extension did not last beyond the first hour of daytime thermogenetic activation of P1 neurons, and continued activation led to behavior typical of awake flies such as pacing, feeding, and grooming (Video 4). In contrast, MS1 activation did not produce wing extension at any time (Videos 2-4), perhaps because activation of P1 via MS1 is not strong enough to trigger courtship behavior. Our data suggest MS1 neurons affect mating success indirectly by providing octopamingeric arousal input to the courtship circuit.

Based on the role of MS1 neurons in female-induced male sleep suppression and the role of FRU neurons in sensory processing of female presence, we hypothesized that MS1 neurons also act downstream of FRU neurons. Indeed, we found that when we used P2X2 and ATP to active the FRU circuit, MS1 neurons in the SOG exhibited an increased GCaMP6m signal in a male-specific fashion (Figure 7C,D). We did not detect calcium responses in MS1 neurons when we stimulated P1 neurons using R71G01-LexA (max $\Delta F/F^0 = 0.01 \pm 0.01$), which suggests that MS1 and P1 neurons do not form a positive feedback loop and FRU neurons other than the P1 cluster act upstream of MS1 neurons. Collectively, our data show that MS1 neurons receive input from FRU neurons and, in turn, send excitatory input to several FRU clusters to balance sleep and sexual behaviors specifically in male flies.

Discussion

Behavioral choice is a continual challenge facing organisms with multiple goals. Here we have investigated the choice between two essential behaviors: sleep and sex. We found that male flies suppressed sleep in the presence of females, and that sexual satiety or elevated levels of sleep drive attenuated female-induced male sleep suppression. These findings demonstrate that sleep and sex drives compete to control behavior, highlighting the importance of motivational factors such as sex drive in sleep regulation.

A number of wake-promoting neuronal populations in flies and mammals have previously been identified (Afonso et al., 2015; Brown et al., 2012; Crocker et al., 2010; Joiner et al., 2006; Liu et al., 2012; Parisky et al., 2008; Pitman et al., 2006; Saper et al., 2010; Sitaraman et al., 2015; Ueno et al., 2012; Weber, 2016), but why activation of these neurons leads to wakefulness is largely unclear. Since sleep is incompatible with many other behaviors, it is plausible that the role of some arousal centers is to keep animals awake so that they can address other pressing needs. For instance, dopaminergic ventral tegmental area (VTA) neurons in mice are required for maintaining wakefulness in the presence of motivating stimuli such as food and sexual partners (Eban-Rothschild et al., 2016), and LEUCOKININ-expressing neurons in *Drosophila* promote wakefulness under starvation conditions (Murakami et al., 2016). Our research identified a small number of octopaminergic neurons in the SOG that regulate male sleep specifically in a sexual context. MS1 neurons act in concert with the FRU circuit to promote wakefulness and courtship, suggesting that activation of MS1 neurons tips the balance in favor of courtship over sleep. The selective advantage of being able to inhibit sleep drive in a sexual context is demonstrated by our finding that inhibition of MS1 neurons places male flies at a disadvantage when they must compete for sexual partners.

Whereas total sleep deprivation by external stimulation led to suppression of courtship and wakefulness, partial sleep loss due to activation of MS1 neurons did not lead to rebound sleep. This may be because activation of MS1 neurons mimics self-motivated sleep loss in a sexual context, which allows flies to sleep when sleep drive is sufficiently high, and thus prevents accumulation of excessive sleep drive that leads to rebound sleep. Octopamine signaling may inhibit accumulation of sleep drive (Seidner et al., 2015), and thus may be especially well suited for adaptive, self-motivated sleep loss

under conditions where wakefulness is required for something important such as sex and food (Siegel, 2012). Consistent with this view, octopamine mediates starvation-induced foraging behavior (Yang et al., 2015). The noradrenergic system in humans, which is similar to the *Drosophila* octopaminergic system, may function in an analogous manner to allow important motivational factors such as sex drive, fear, and hunger to overcome sleep drive.

In addition to promoting wakefulness, octopamine regulates other behaviors such as aggression (Hoyer et al., 2008; Zhou and Rao, 2008), choice between courtship and aggression (Certel et al., 2007), memory formation (Burke et al., 2012), egg laying (Monastirioti et al., 1996), and foraging (Yang et al., 2015). MS1 neurons in the SOG are distinct from previously characterized octopaminergic neurons, and play a novel role as a link between sleep and courtship circuits. Each of the behaviors modulated by octopamine may be mediated by distinct subsets of octopaminergic neurons.

Earlier research documented sex differences in sleep (Isaac et al., 2010; Krishnan and Collop, 2006), yet little has been known about the neural mechanisms underlying sexually-dimorphic regulation of sleep. Enhanced neuronal activity in a subset of dorsal clock neurons (DN1s) was proposed to underlie elevated *siesta* in males relative to females (Guo et al., 2016), but DN1 activation has similar effects on sleep in males and females. The MS1 neurons are unusual in regulating sleep only in males. The sexual dimorphism does not appear to stem from differences in MS1 neurons themselves, but rather from sexually dimorphic connectivity between MS1 and FRU neurons at both anatomical and functional levels. We found that MS1 stimulation elicits calcium responses in several FRU clusters specifically in males. Two of the clusters, P1 and mAL, play important roles in courtship (Clowney et al., 2015; Kallman et al., 2015; Kimura et al., 2008; Koganezawa et al., 2016; Kohatsu et al., 2011; Kohatsu and Yamamoto, 2015). An additional pair in the superior protocerebrum, which may be aSP4 neurons, has been shown to signal mating drive (Zhang et al., 2016), raising the possibility that MS1 neurons modulate mating drive depending on social context. A widespread increase in the excitability of the FRU circuit may keep males in a sexually aroused state and provide enhanced sensitivity to cues from females.

In addition to providing excitatory input to several FRU clusters, MS1 neurons receive excitatory input from the FRU circuit. Since MS1 neurons are important for male sleep regulation in the presence of a female especially in the dark, and many FRU neurons respond to female pheromones directly or

indirectly, it is plausible that the message conveyed to MS1 neurons from FRU neurons concerns female pheromones. The specific neuronal groups that communicate directly with MS1 neurons have yet to be identified. Nevertheless, MS1 neurons are well positioned to translate the detection of female cues into an arousal signal for sustained courtship. A heightened state of arousal may be especially important for successful mating when sleep drive is high and vision is limited, conditions under which MS1 activity strongly impacts sleep and courtship.

Our work demonstrates that sex drive and sleep drive are integrated in a circuit that contains optopaminergic neurons and FRU neurons, and provides a valuable entry point for investigating the neural circuitry underlying the coordination of sleep and courtship, and more generally the choice between competing behaviors.

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Figure legends

Figure 1. Balance between sleep drive and sex drive determines male sleep levels. **(A)** Sleep profile in 30-min intervals for wild-type (iso31) flies in isolation (M for male, F for female) or in pairs (MM for male-male, MF for male-female) using multi-beam monitors. Corresponding data using single-beam monitors are shown in Figure 1 – figure supplement 1. In all figures, the white and black bars below the x-axis indicate light and dark periods, respectively. N = 29-44. **(B)** Daytime and nighttime sleep amount of flies shown in (A). **(C)** Nighttime sleep profile in 5-min bins each hour for wild-type (iso31) flies from video analysis. Sleep amount was manually scored for the first 5 min of each hour for individual males (M w/ F) or females (F w/ M) in MF pairs or individual males in MM pairs (M w/ M). Collective sleep amount of MM and MF pairs, i.e., when both flies were asleep, is presented on the right. N=15-24. **(D)** Percentage of three types of male behavior, i.e., sleep, courtship, and wake but not courtship, during the 5-min bins shown in (C). In all figures, error bars represent SEM; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; ns = not significant. Two-way ANOVA (B) or one-way ANOVA (C) followed by Tukey post hoc test. Significance of the interaction between the two factors (single vs pair and presence vs absence of a female) is indicated in (B). Sleep amounts obtained for 5-min intervals were summed for ANOVA in (C) and significant differences between conditions are indicated above the brackets. Sleep amount was not significantly different between individual males and females in MF pairs (M w/ F vs F w/ M).

Figure 1 – figure supplement 1. Single-beam DAM data demonstrating reduced male sleep in the presence of females. **(A)** Sleep profile in 30-min intervals for wild-type (iso31) flies in isolation (M, F) or in pairs (MM, MF). N = 45-52. **(B)** Daytime and nighttime sleep amount for flies shown in (A). Two-way ANOVA followed by Tukey post hoc test (B). Significance of the interaction between the two factors (single vs pair and presence vs absence of a female) is indicated.

Figure 2. Sexual satiety and sleep deprivation attenuate female-induced sleep loss in males. **(A)** Sleep profile of wild-type (iso31) MF pairs including satiated or non-satiated (naive) males assayed using single-beam monitors. N=31-33. **(B-C)** Sleep amount (B) and courtship index (C) of males in MF

pairs from video analysis of the first 5-min every nighttime hour. N=38-40. **(D)** Sleep profile of wild-type (iso31) MM or MF pairs without sleep deprivation or 6-h sleep deprivation in the early night measured using single-beam monitors. Dotted rectangles indicate the period of sleep deprivation by mechanical stimulation. N = 38-42. **(E-F)** Sleep amount (E) and courtship index (F) of males in MF pairs without sleep deprivation or 6-h sleep deprivation in the early night. Sleep during the first 5-min every 30 min during 6 h after sleep deprivation (ZT18 – ZT24) was scored from videos. N=40-48. **(G)** Sleep profile of "experimental" (R23E10>NaChBac, R23E10/+, or NaChBac/+) males, paired with iso31 control females (MF^c) or control males (MM^c) measured using multi-beam monitors. N = 28-30. Student's *t* test (A-G) with Bonferroni correction (A, D, G). For statistical analysis, sleep amounts were summed and courtship index averaged over the periods indicated by the brackets above the sleep profiles or as noted on the y-axis of the bar graphs. For simplicity, in all figures involving comparison of a genotype against two parental controls, significant differences are indicated only if the experimental group differed significantly from both control groups in the same direction.

Figure 2 – figure supplement 1. Successful manipulation of sexual satiety in male flies. **(A)** Percent of satiated males shown in Figure 2A exhibiting courtship or copulation behavior. Mating behavior was examined twice during the first hour (initial) and twice during the last hour (end) in 4.5-h or 5.5-h assays. N = 5 groups of 6-7 males. **(B)** Percent of satiated males shown in Figure 2B exhibiting courtship or copulation behavior. N=7 groups of 6 males. Student's paired *t* test (A, B).

Figure 2 – figure supplement 2. Activation of dFSB induces sleep in isolated males. Sleep profile of R23E10>NaChBac and parental control (R23E10/+ or NaChBac/+) males in isolation. N = 40-57. Daytime and nighttime sleep amounts were summed for one-way ANOVA followed by Dunnett post hoc test relative to both parental controls.

Figure 3. MS1 neuronal activity regulates sleep and mating behavior in males. **(A)** Sleep profile of male (left) and female (right) flies expressing the warmth-activated TrpA1 channel in MS1 neurons (MS1>TrpA1) and control flies (MS1/+ and TrpA1/+). TrpA1 was activated by temperature shift from 22°C to 29°C. N = 32-40. **(B)** Sleep profile of flies expressing the NaChBac bacterial sodium channel

in MS1 neurons and parental controls. N = 30-36. **(C)** Sleep profile of males expressing tetanus toxin in MS1 neurons (MS1>TNT) and parental controls (MS1/+ and TNT/+) in isolation (M) or paired with iso31 control males (MM^c) or females (MF^c) in multi-beam monitors. N = 37-49. **(D-E)** Sleep amount (D) and courtship index (E) of males with silenced MS1 neurons (MS1>TNT) and parental controls paired with control females. Videos were manually scored for sleep and courtship index during the first 5-min bins every hour at night and values summed (D) or averaged (E) for statistical analysis. N=26-27. **(F-G)** courtship index (F), and cumulative copulation success (G) over 90-min mating assays. Males with silenced MS1 neurons (MS1>TNT) and parental controls were assayed in dim white light (left) or infrared light (right). N=23-26. **(H)** Percentage of successful copulation by males with silenced MS1 neurons (MS1>TNT) against MS1/+ or TNT/+ controls in 90-min competitive copulation assays under dim red light. N = 29-34. One-way ANOVA followed by Dunnett post hoc test relative to both parental controls (A-F); log rank test (G); binomial test relative to 50% (H).

Figure 4. MS1 neurons in the SOG are octopaminergic. **(A)** Confocal projection of a whole-mounted MS1> CD8::GFP male (left) or female (right) adult central brain. Antibodies against GFP (green) and Bruchpilot (BRP, magenta) were used for immunostaining. Scale bar: 100 μm. **(B)** Confocal projection of the ventral nerve cord of a male or female expressing CD8::GFP under the control of MS1-Gal4. Scale bar: 50 μm. **(C)** Top: confocal projection of a male central brain expressing RFP (magenta) in MS1 neurons (using Gal4-UAS) and GFP (green) in Tdc2 neurons (using LexA-LexAOp). Scale bar: 100μm. Bottom: Magnified view of the SOG region indicated by the rectangle in the corresponding image in the top row. Arrows point to neurons co-expressing MS1>RFP and *Tdc2*>GFP. Scale bar: 50μm. **(D)** Expression of NaChBac::GFP in all MS1 neurons (left, MS1-Gal4, *Tdc2*-LexA/UAS-*NaChBac*) and in the non-octopaminergic subset (right, MS1-Gal4, *Tdc2*-LexA/UAS-*NaChBac*; LexAop-Gal80). Expression of Gal80, a suppressor of Gal4, in TDC2 neurons removed NaChBac::GFP expression specifically in the SOG. Scale bar: 100 μm. **(E)** Sleep profile of male flies of the indicated genotypes. N = 75-78. **(F)** Sleep profile of males in which MS1 neurons were activated with TrpA1 expression at 28°C in an iso31 control (left), Tbh^{nm18} mutant (middle), or $Oamb^{286}$ mutant (right) background. N = 20-165. One-way ANOVA followed by Dunnett post hoc test relative to MS1>NaChBac, Tdc2-LexA/+ flies (E) or both parental controls (F).

Figure 5. MS1 neurons appear to form synaptic contacts with FRU neurons. **(A)** Confocal projection of a male brain expressing CD8::GFP in the intersection between MS1-Gal4 and *fru*-FLP (MS1-Gal4/UAS-FRT-stop-FRT-CD8::GFP; FRU-FLP/+). Scale bar: 100 μm. **(B)** Confocal projection of the SOG region of a male brain expressing RFP (magenta) in MS1 neurons (MS1-Gal4 > UAS-mCD8::RFP) and GFP (green) in FRU neurons (*fru*-LexA > LexAOp-CD8::GFP). *The single MS1 neuron and two FRU neurons are at different focal planes. Scale bar: 25 μm. **(C)** Confocal projection of the SOG region of a male brain expressing CD8::GFP under the control of MS1-Gal4. The brain was co-stained using antibodies against GFP and DSX. Arrows point to DSX positive neurons. Scale bar: 25 μm. **(D)** Confocal projection of a male (left) or female (right) brain expressing spGFP1-10::NRX driven by MS1-Gal4 and spGFP11::CD4 driven by *fru*-LexA. Reconstituted GFP fluorescence was detected without antibody staining. Scale bar: 100 μm. A negative control image is presented in Figure 5 – figure supplement 1B. **(E)** Magnified view of the region indicated by the rectangle in (D). Scale bar: 25 μm. **(F)** Confocal projections of male brains expressing the pre-synaptic marker NSYB in the octopaminergic subset of MS1 neurons (MS1-Gal4 > UAS-FRT-stop-FRT-*nSyb*, *Tdc2*-LexA > LexAop-FLP). Axon terminals are visible in antennal lobes (AL), lateral protocerebrum (LP), lateral horn (LH), anterior superior medial protocerebrum (SMPa), mushroom body calyx (Calyx), and the region surrounding the esophagus (indicated by the rectangle). Scale bar: 100 μm. **(G)** Posterior view of a male brain expressing the post-synaptic marker DSCAM in the octopaminergic subset of MS1 neurons (MS1-Gal4 > UAS-FRT-stop-FRT-*Dscam*, *Tdc2*-LexA > LexAop-FLP). DSCAM expression is highly enriched around the esophagus (indicated by the rectangle). Scale bar: 100 μm.

Figure 5 – figure supplement 1. MS1 neurons do not express FRU but likely form synaptic contacts with FRU neurons. **(A)** Confocal projection of the lateral protocerebrum of a male brain expressing RFP (magenta) in MS1 neurons (MS1-Gal4 > UAS-CD8::RFP) and GFP (green) in FRU neurons (*fru*-LexA > LexAOp-CD8::GFP). Scale bar: 50 μm. **(B)** Confocal projections of a male brain expressing split GFP fragments in MS1 and FRU neurons (left, MS1-Gal4/UAS- spGFP1-10::NRX; *fru*-LexA /LexAop-spGFP11) and a negative control male brain (right, MS1-Gal4/UAS-spGFP1-10::NRX/+; LexAop-spGFP11/+). Reconstituted GFP fluorescence was detected without antibody staining. Scale bar: 100 μm.

Figure 6. Experimental activation of MS1 neurons elicits calcium responses in FRU neurons. **(A)** GCaMP6m increase (ΔF) in FRU neurons of a dissected male brain in which MS1 neurons are activated by P2X2 expression and ATP perfusion. Antero-dorsal (left) and posterior (right) views are presented using the "fire" look-up table. AL: antennal lobes. Arrows point to a pair of neurons that may be aSP4. Scale bar: 100 μm. **(B)** Normalized GCaMP6m response ($\Delta F/F^0$) in the cell bodies of mAL and P1 neurons, and the arch region in male (M), female (F), or negative control (C) brains. Female brains do not exhibit calcium responses in P1 neurons because these neurons are male specific. Flies carrying UAS-P2X2, *fru*-LexA, and LexAop-GCaMP6m, but not MS1-Gal4 served as negative controls. Fluorescence traces (top) and peak responses (bottom) are presented. Gray rectangles indicate 2.5 mM ATP perfusion. N = 4-9. Student's *t* test (B).

Figure 6 – figure supplement 1. MS1 stimulation induces calcium responses in P1. P2X2 was expressed under the control of MS1-Gal4, while GCaMP6m was expressed under the control of R71G01-LexA. A posterior view is presented using the "fire" look-up table. Scale bar: 100 μm.

Figure 7. Activation of P1 neurons suppresses male sleep and experimental activation of FRU neurons elicits calcium responses in MS1 neurons. **(A)** Sleep profile of males expressing NaChBac in MS1 neurons (MS1>NaChBac) and control males (MS1/+ and NaChBac/+) in an iso31 control (left), $fru^{LexA/4\text{-}40}$ mutant (middle) and $dsx^{683/1649}$ mutant (right) background. N = 11-83. **(B)** Sleep profile of male (M) and female (F) flies in which P1-split Gal4 (Inagaki et al., 2014) was used to drive UAS-*TrpA1* (P1 > *TrpA1*) and parental control flies (P1/+ and *TrpA1*/+). TrpA1 was activated on the 2nd day by raising the temperature from 22°C to 29°C. N = 82-91. **(C)** GCaMP6m response to ATP (ΔF) in MS1 neurons of a male brain in which FRU neurons are activated by P2X2 expression and ATP perfusion. **(D)** Normalized GCaMP6m response ($\Delta F/F^0$) in the cell bodies of MS1 neurons in males (M) and females (F). N = 5-7. One-way ANOVA followed by Dunnett post hoc tests (A, B); Student's *t* test (C, D).

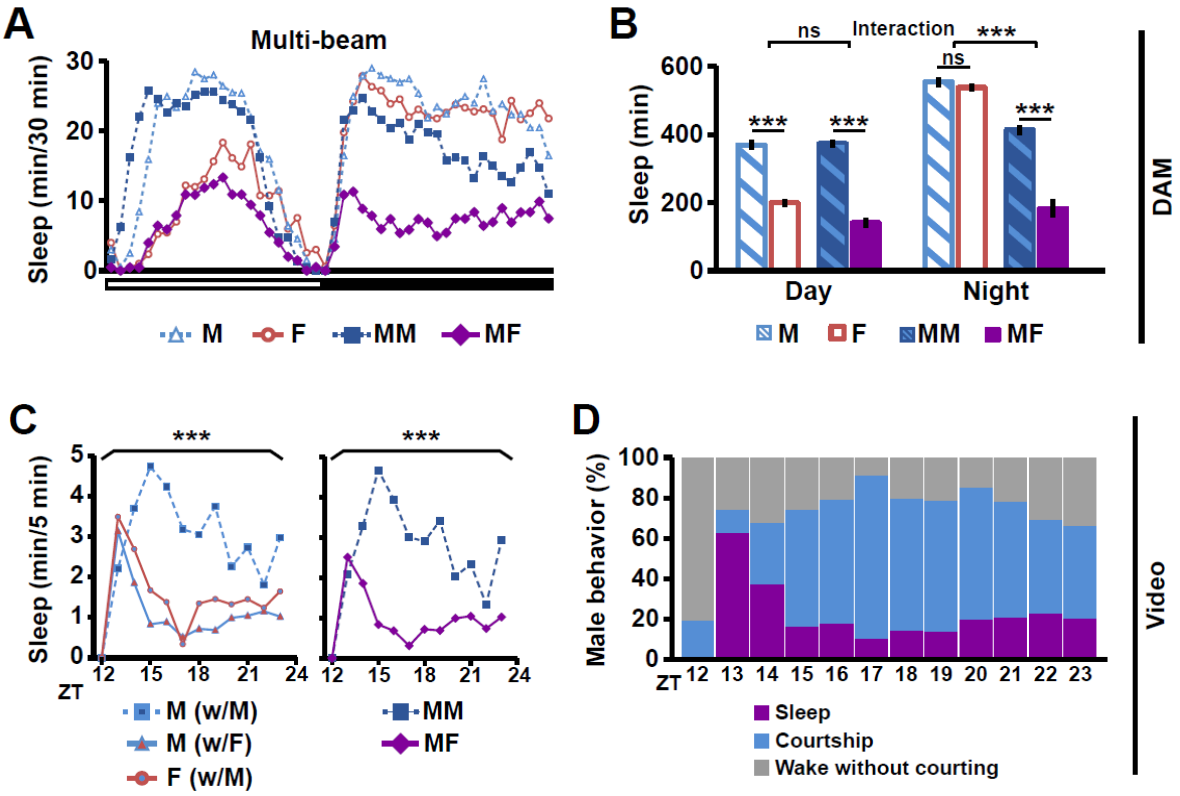
Video legends

Video 1. Wild-type male-male (MM) and male-female (MF) pairs at ~ZT18 under infrared light. While most MM pairs slept, many males paired with females engaged in courtship behaviors including chasing and wing extension.

Video 2. Male flies with activated MS1 neurons (MS1 > *TrpA1*) and control males (MS1/+ or *TrpA1*/+) at 29°C. The flies were recorded at ~ZT17 under infrared light. MS1 activation led to pacing behavior typically observed in awake flies (note one of the control flies exhibiting the same behavior).

Video 3. Male flies with activated P1 neurons and control males (left) as well as male flies with activated MS1 neurons and control males (right) at 29°C. The flies were recorded ~30 min after the switch from 22°C to 29°C (ZT0.5) under white light. Wing extension is seen in males with activated P1 neurons (top left), but not in males with activated MS1 neurons (top right).

Video 4. Flies shown in Video 3 at ~ZT5 at 29°C. Males with activated P1 neurons no longer exhibited wing extension, but engaged in behavior typical of awake flies such as pacing, feeding, and grooming.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***
***
Sleep (min/5 min)
5
4
3
2
1
0
12
15
18
21
24
ZT
M (w/M)
M (w/F)
F (w/M)
MM
MF
D
Male behavior (%)
100
80
60
40
20
0
ZT 12 13 14 15 16 17 18 19 20 21 22 23
Sleep
Courtship
Wake without courting
Video

Figure 1.

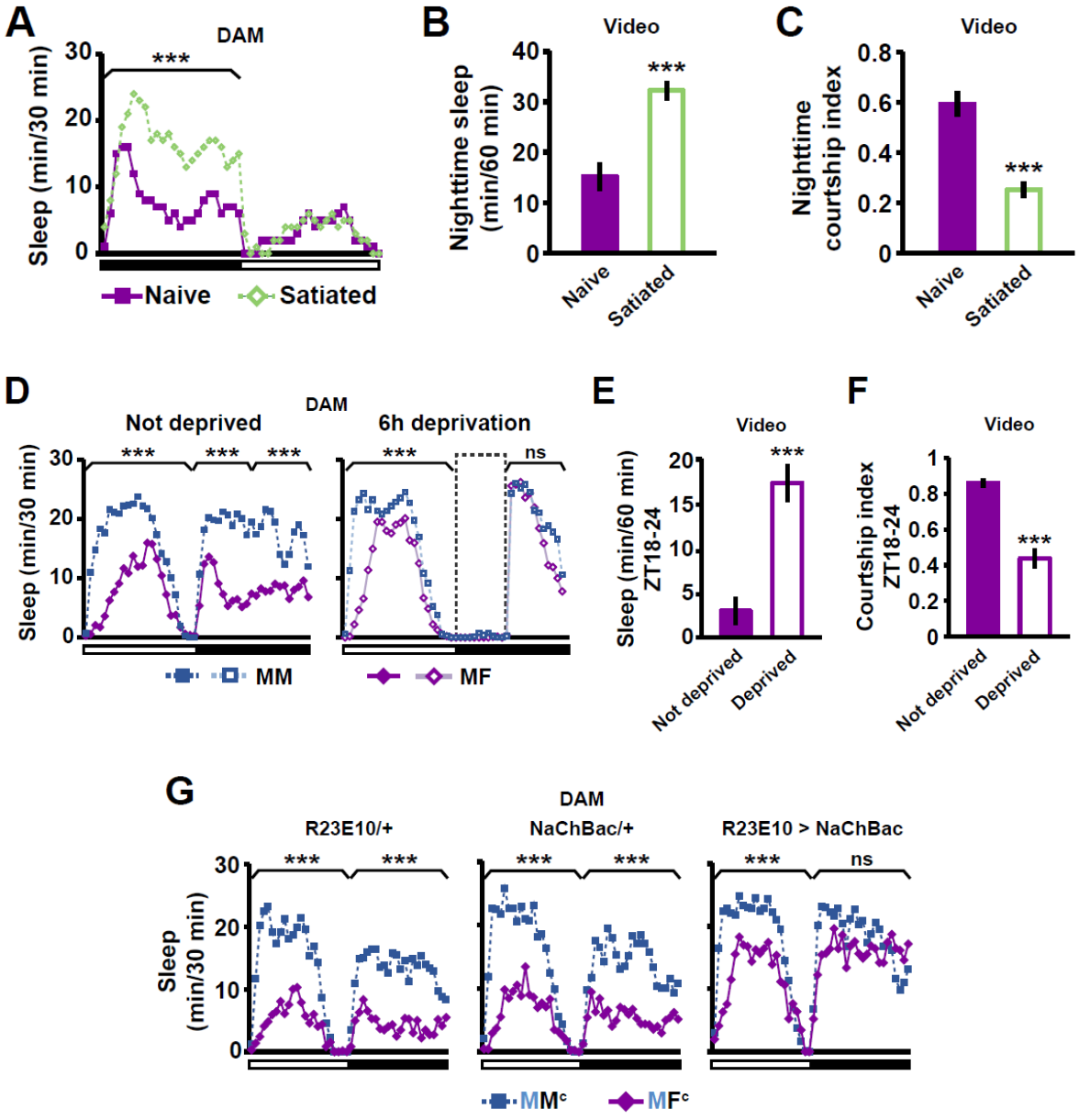
A
DAM
Sleep (min/30 min)
***
Naive
Satiated
B
Video
Nighttime sleep (min/60 min)
***
Naive
Satiated
C
Video
Nighttime courtship index
***
Naive
Satiated
D
DAM
Not deprived
6h deprivation
Sleep (min/30 min)
***
ns
MM
MF
E
Video
Sleep (min/60 min) ZT18-24
***
Not deprived
Deprived
F
Video
Courtship index ZT18-24
***
Not deprived
Deprived
G
DAM
R23E10/+
NaChBac/+
R23E10 > NaChBac
Sleep (min/30 min)
***
ns
MMc
MFc

Figure 2.

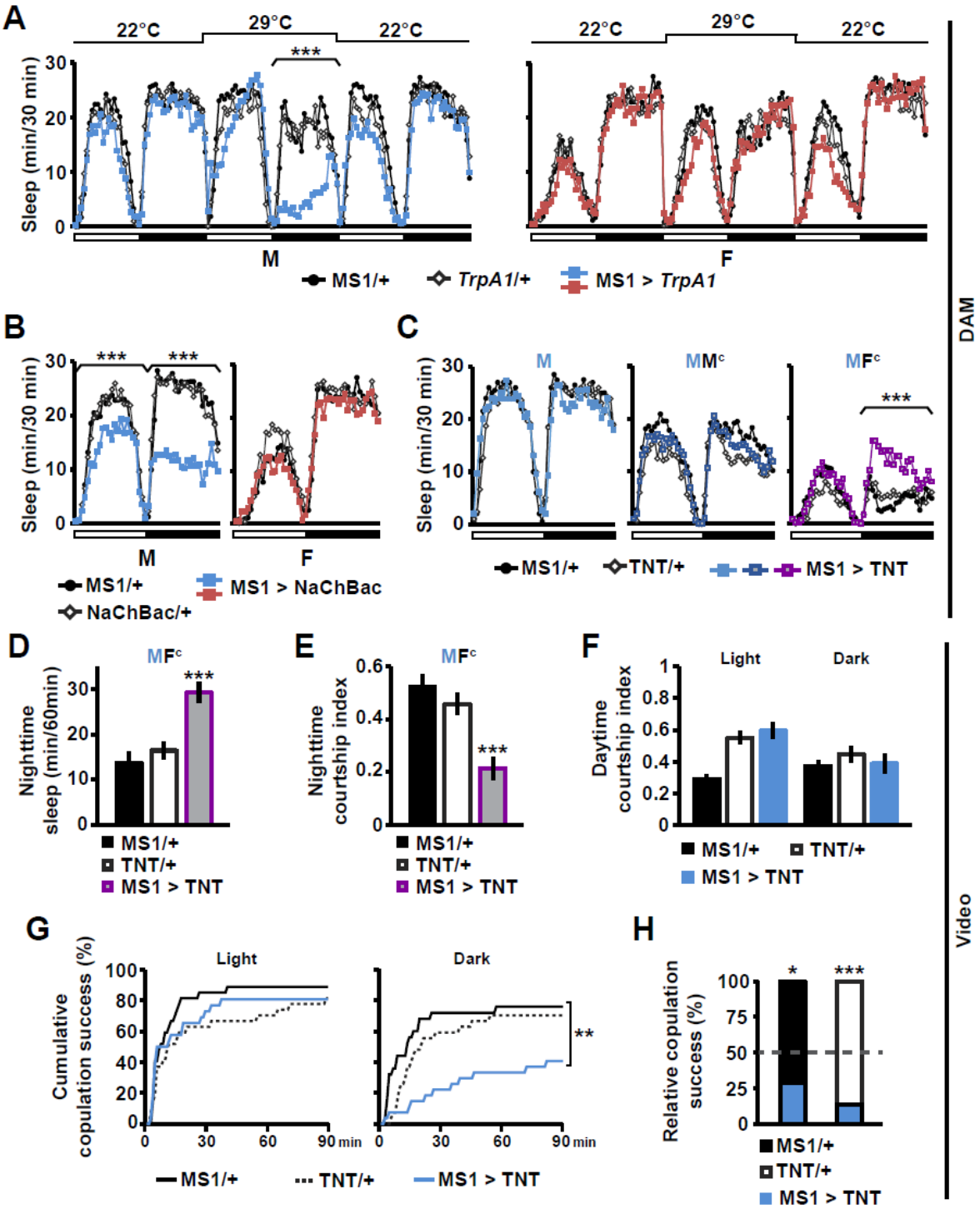
A
22°C
29°C
22°C
***
Sleep (min/30 min)
M
F
MS1/+
TrpA1/+
MS1 > TrpA1
B
C
MS1/+
NaChBac/+
MS1 > NaChBac
MS1/+
TNT/+
MS1 > TNT
DAM
D
E
F
Nighttime sleep (min/60min)
Nighttime courtship index
Daytime courtship index
Light
Dark
MS1/+
TNT/+
MS1 > TNT
G
H
Cumulative copulation success (%)
Relative copulation success (%)
90 min
Video

Figure 3.

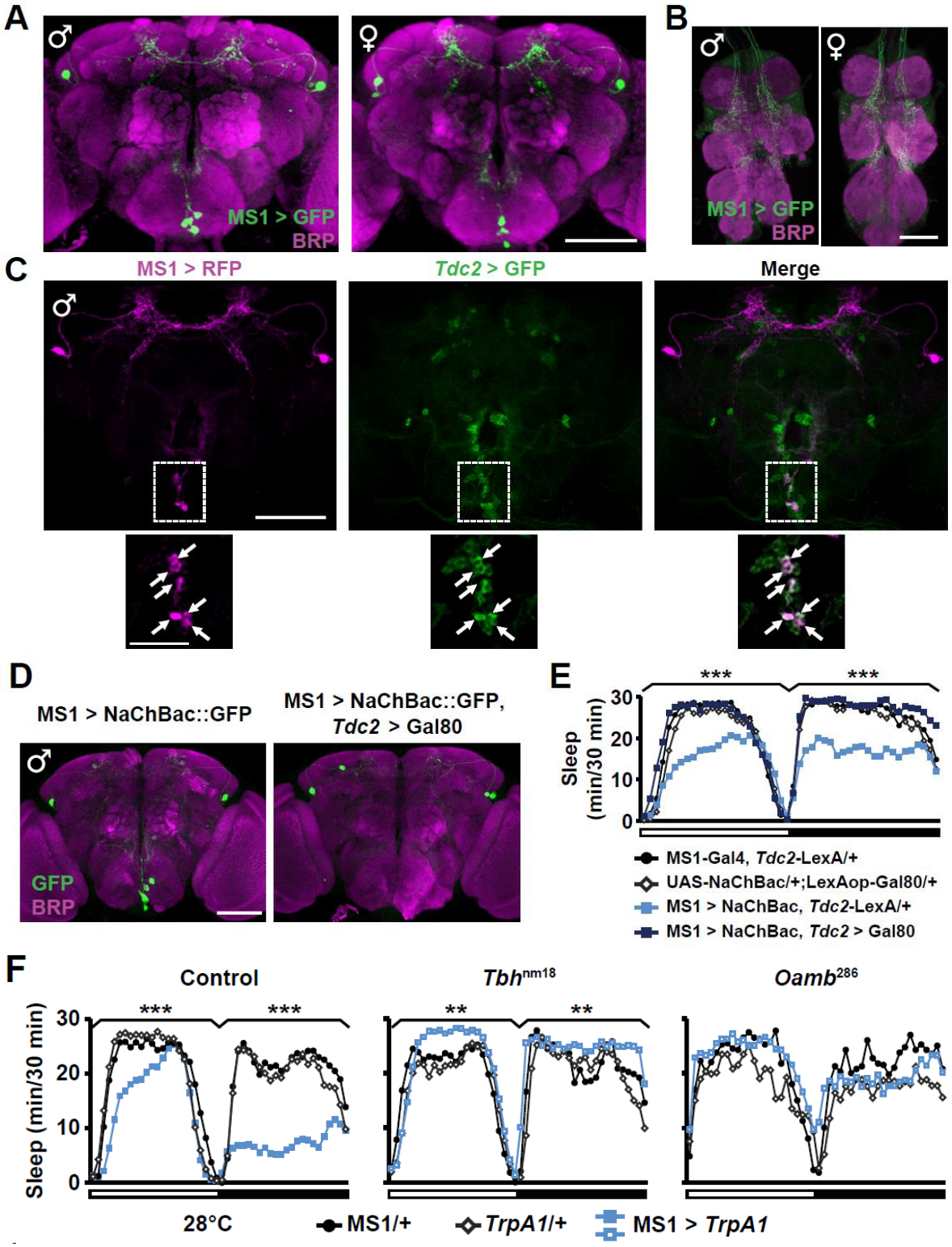
A
♂
♀
MS1 > GFP
BRP
B
♂
♀
MS1 > GFP
BRP
C
MS1 > RFP
Tdc2 > GFP
Merge
♂
D
MS1 > NaChBac::GFP
MS1 > NaChBac::GFP,
Tdc2 > Gal80
♂
GFP
BRP
E
***
***
Sleep
(min/30 min)
30
20
10
0
MS1-Gal4, Tdc2-LexA/+
UAS-NaChBac/+;LexAop-Gal80/+
MS1 > NaChBac, Tdc2-LexA/+
MS1 > NaChBac, Tdc2 > Gal80
F
Control
Tbh^nm18
Oamb^286
***
***
**
**
Sleep (min/30 min)
30
20
10
0
28°C
MS1/+
TrpA1/+
MS1 > TrpA1

Figure 4.

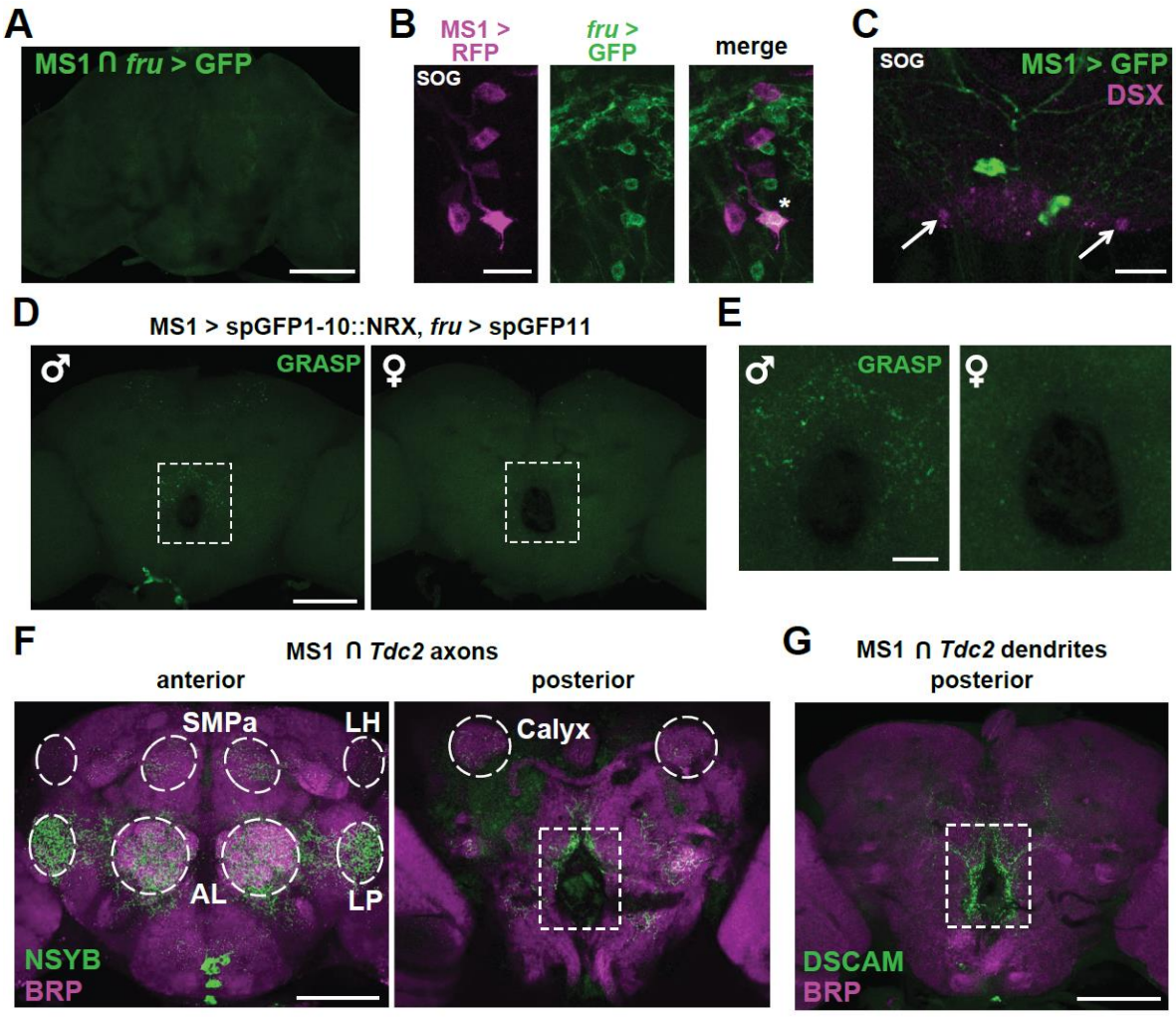
A
MS1 ∩ fru > GFP
B
MS1 > RFP
fru > GFP
merge
SOG
C
SOG
MS1 > GFP
DSX
D
MS1 > spGFP1-10::NRX, fru > spGFP11
GRASP
E
GRASP
F
MS1 ∩ Tdc2 axons
anterior
posterior
SMPa
LH
AL
LP
NSYB
BRP
Calyx
G
MS1 ∩ Tdc2 dendrites
posterior
DSCAM
BRP

Figure 5.

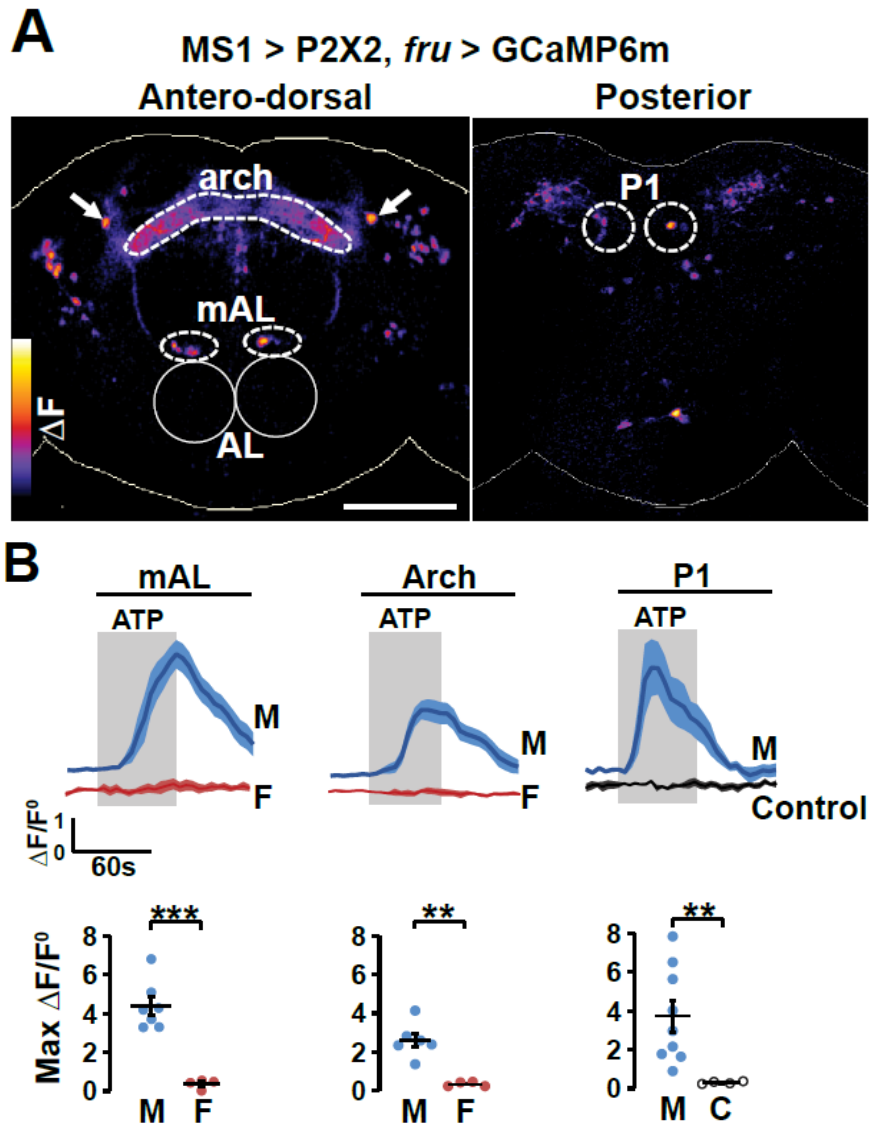
A
MS1 > P2X2, fru > GCaMP6m
Antero-dorsal
Posterior
arch
mAL
AL
P1
ΔF
B
mAL
Arch
P1
ATP
M
F
Control
ΔF/F0
60s
Max ΔF/F0
***
**
M
F
C

Figure 6.

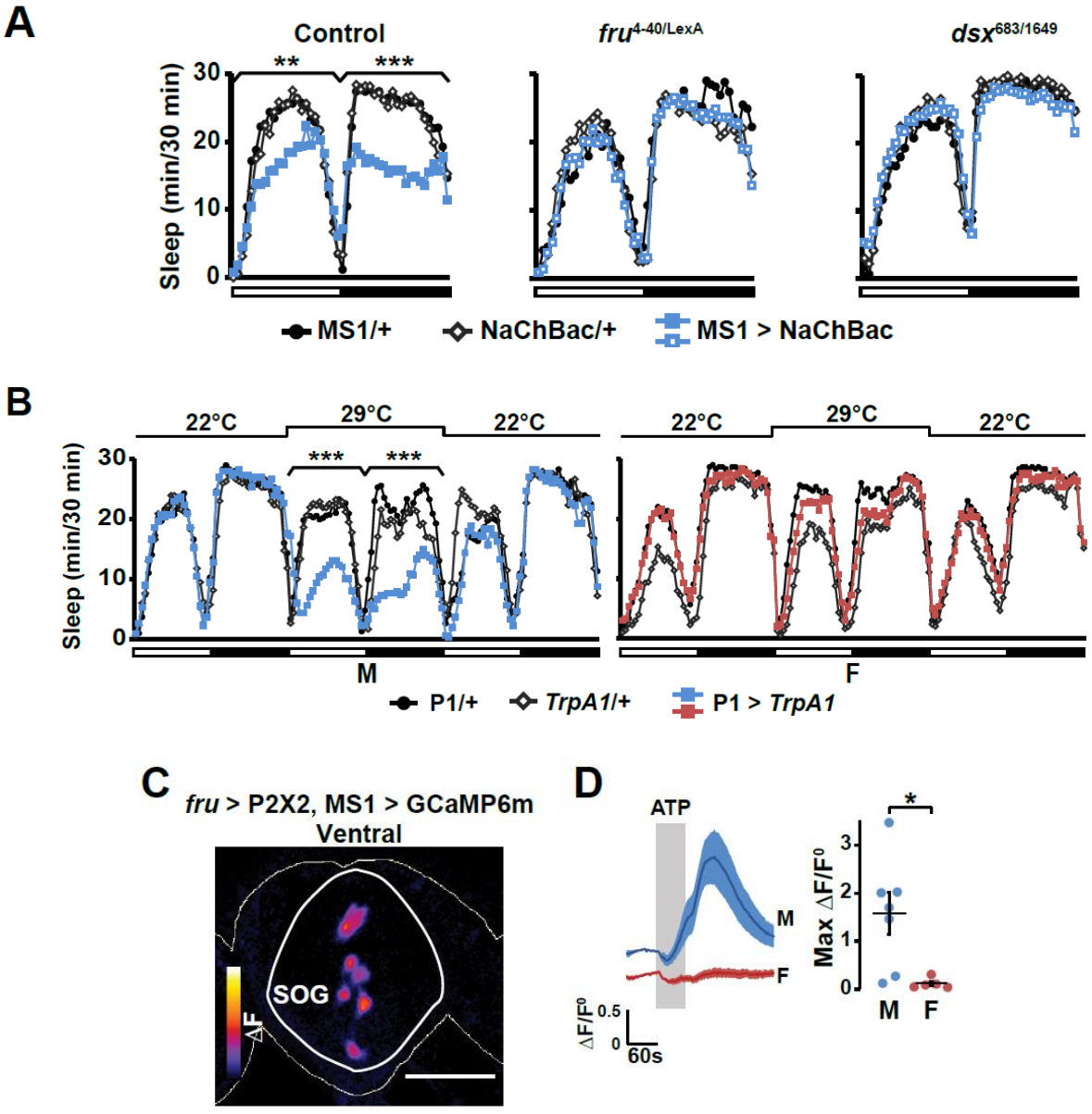
A
Control
fru4-40/LexA
dsx683/1649
Sleep (min/30 min)
30
20
10
0
**
***
MS1/+
NaChBac/+
MS1 > NaChBac
B
22°C
29°C
22°C
***
***
Sleep (min/30 min)
M
F
P1/+
TrpA1/+
P1 > TrpA1
C
fru > P2X2, MS1 > GCaMP6m
Ventral
SOG
ΔF
D
ATP
M
F
ΔF/F0
0.5
0
60s
Max ΔF/F0
3
2
1
0
*
M
F

Figure 7.

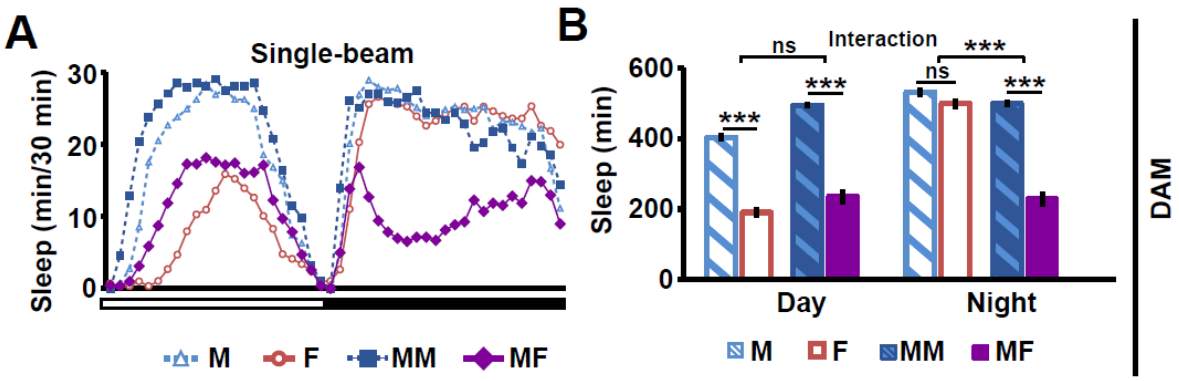

Figure 1. – figure supplement 1.

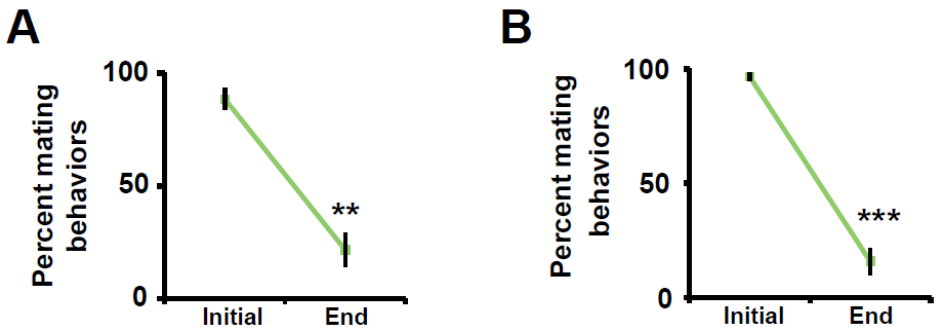

Figure 2. – figure supplement 1.

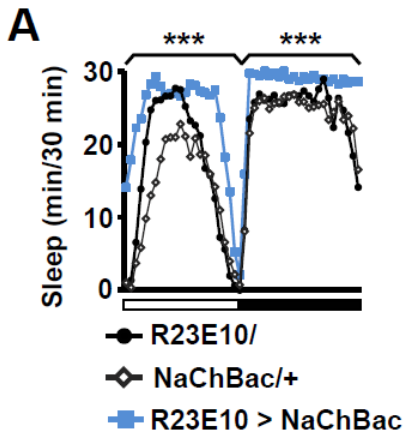

Figure 2. – figure supplement 2.

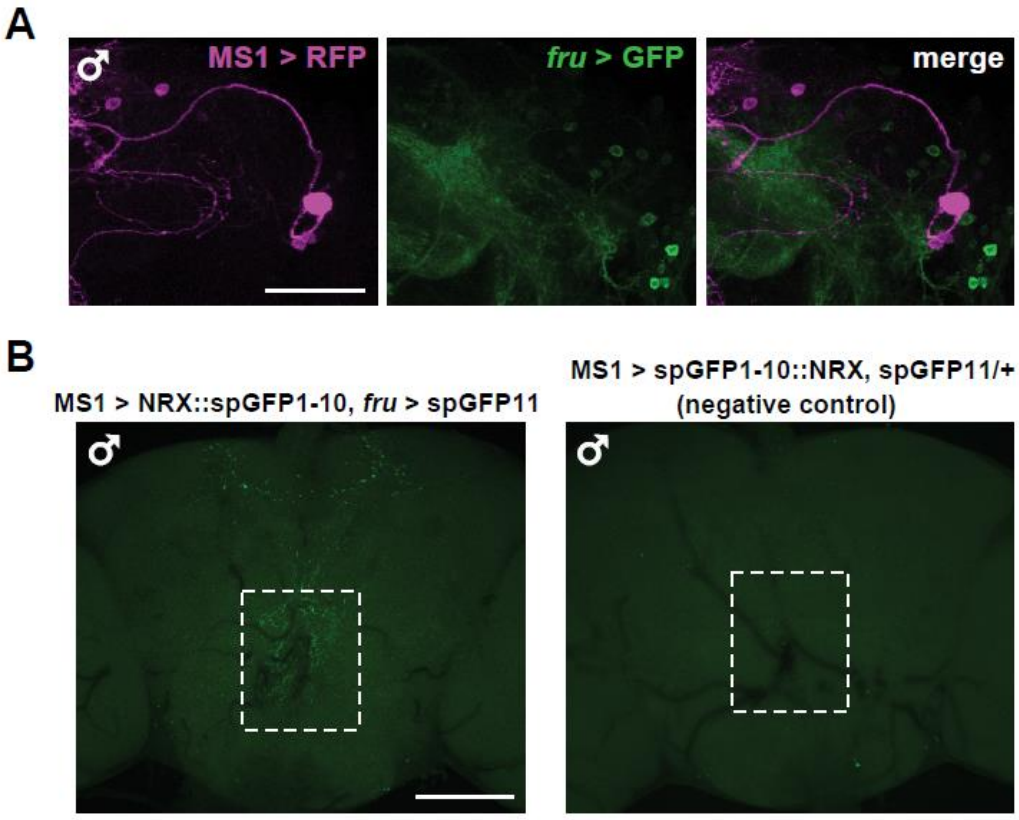

Figure 5. – figure supplement 1.

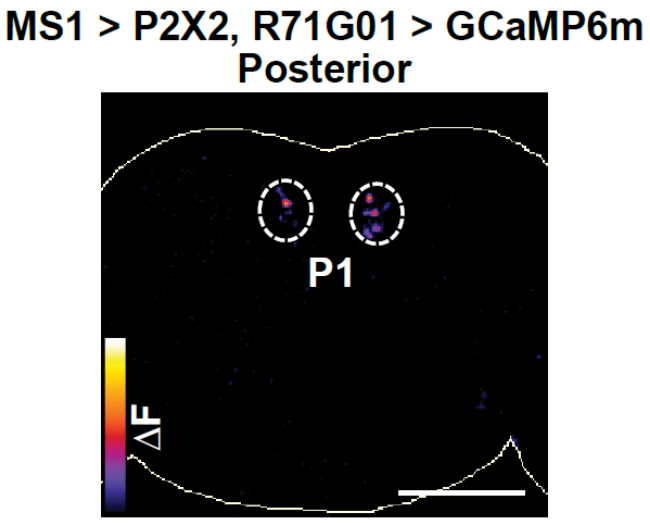

Figure 6. – figure supplement 1.

Chapter 2.2.

Identification of a novel neuronal population involved in the coordination of sleep and courtship.

Daniel R Machado, Dinis JS Afonso, Arzu Öztürk-Çolak, Kyunghee Koh.

(*Work in progress*)

(2017)

Identification of a novel neuronal population involved in the coordination of sleep and courtship.

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Abstract

Sleep is an essential behavior preserved among species. Like humans, the sleep of fruit fly *Drosophila melanogaster* follows a well-defined pattern regulated by two main systems: the circadian system and the homeostatic system, that control the timing and the need for sleep, respectively. However, there are many situations in which sleep is incompatible with other behaviors, such as when a male has to decide either to sleep or to court a female. Under these contexts, the two main systems of sleep regulation are insufficient to explain how the male decides which behavior to adopt. From previous studies, we learned that the male suppresses sleep to engage in courtship when paired with a female. We showed that the decision is mediated by octopaminergic MS1 neurons and *fru*-expressing P1 neurons. Here, we report a third group of neurons, MS2, whose activation suppresses sleep specifically in males. We found some MS2 neurons located in the medial, anterior lateral (mAL) region of the brain to be *fru*-positive. Using a different driver, we show that activation of a broad set of mAL neurons likely suppresses sleep exclusively in males, and their blocking prevents female-induced sleep reduction usually observed in males paired with females. These are surprising results considering mAL neurons were previously described as GABAergic neurons providing inhibitory input of P1 neurons, whose activation suppresses sleep in males. Therefore, we hypothesize that there are excitatory and inhibitory neuronal subpopulations within the mAL region and that the MS2 subset of mAL neurons mediate sleep suppression by male sex drive. Future work identifying and characterizing the precise subset of MS2 neurons responsible for male sleep regulation will help us advance our understanding of integration of sleep and sex.

Introduction

Sleep is an essential behavior preserved among species. The fundamental principles of sleep regulation are shared by humans and *Drosophila*. In both species the timing for sleep is regulated by a circadian system, while the need for sleep is monitored by the homeostatic system, which keeps track of recent sleep history to promote recovery sleep after prolonged periods of wakefulness (Allada et al. 2001; Allada & Chung 2010; Donelson & Sanyal, 2015; Light at al. 2016). However, the circadian and homeostatic systems are insufficient to explain how sleep is coordinated with other important behaviors, such as courtship. We previously showed that when a male is paired with a female, the male suppresses sleep in order to court the female. Sleep suppression is not observed when the male is paired with another male.

We also showed that the decision between sleep and courtship is mediated by MS1 neurons and FRU-expressing P1 neurons, a major processing center of courtship activity. We showed MS1 neurons use the neurotransmitter octopamine, an analog of noradrenaline, to suppress sleep exclusively in males, and activation of MS1 neurons can elicit calcium responses in several FRU-expressing neuronal clusters, including P1 and mAL clusters. In turn, MS1 neurons are stimulated by *fru*-expressing neurons (Machado et al. 2017). Furthermore, we demonstrated that activation of P1 neurons suppresses sleep in males, resembling the male-specific sleep suppression induced by the activation MS1 neurons. This finding was confirmed by two recent studies (Chen et al. 2017; Beckwith et al. 2017). However, the link between MS1 and P1 neurons appear to be indirect, and activation of MS1 neurons triggers calcium responses in multiple FRU clusters, suggesting that additional neurons besides MS1, P1 neurons are involved in the coordination between sleep and sex.

Here, we introduce a second group of neurons we named Male Specific 2 (MS2) that, like MS1 and P1 neurons, induces male-specific sleep reduction. We found that MS2 neurons in the subeosophageal ganglion (SOG) are octopaminergic but, unlike octopaminergic MS1 neurons in the SOG, do not regulate sleep. On the other hand, we found some MS2 neurons are FRU positive and are located in the mAL cluster. Therefore, we tested how the activity of mAL neurons affects sleep in males. Since mAL neurons were shown to inhibit P1 neurons, we were surprised to discover that artificial activation of mAL neurons suppresses sleep exclusively in males, which resembles the effects of P1

neuron activation. Additionally, blocking mAL neurons prevents female-induce sleep suppression usually observed in male-female pairs, further suggesting that at least some mAL neurons may provide excitatory input to P1 neurons. Given our previous finding that activation of MS1 neurons elicits calcium responses in mAL neurons (Machado et al. 2017), we hypothesize that FRU-expressing MS2 neurons in the mAL cluster and MS1 neurons may form a circuit that promotes the contextualized suppression of sleep in males paired with females. Exploring this circuit may offer an important advance in understanding how sleep and courtship are integrated.

Materials and Methods

Fly stocks

Flies were grown at 22°C on standard food containing molasses, cornmeal, and yeast under a 12 hr:12 hr light:dark cycle. MS2-Gal4 was obtained from Vienna Drosophila Research Center (VT012760). Tdc2-LexA (BDSC#52242), UAS-TrpA1 (BDSC#26263), UAS–mCD8::GFP (BDSC#5137), lexAop2-mCD8::GFP, UAS-IVS-mCD8::RFP (BDSC#32229), UAS-TNT (BDSC#28838), iso31 (w^{1118}) (BDSC#5905), R43D01-Gal4 (BDSC#64345) and Tdc2-Gal4-AD (BDSC#70862) were obtained from the Bloomington Stock Center. UAS-FRT-stop-FRT-mCD8::GFP and *fru*-FLP lines were provided by Barry Dickson.

Sleep assays

For the sleep assays we used the *Drosophila* Activity Monitoring (DAM) system (Trikinetics, Waltham, USA). Flies with three to four days were loaded into glass tubes with approximately 5 mm in diameter. The tubes were prepared with 2% agarose, 5% sucrose gel on one tip, and covered with yarn on the other tip, to avoid the flies from escaping. Sleep was inferred from the activity of the flies in the tubes, measured by a DAM monitor. Flies were considered to be asleep after periods of 5 or more minutes of immobility. Sleep assays with TrpA1 were done by exposing the flies to 22˚C for a first day, for adaption, and then exposing the flies for a second day at 22˚C, a third day at higher temperature (28˚C, 29˚C or 31˚C) and a final fourth day at 22˚C. Experiments with TNT were done at 22˚C to avoid leaky expression of UAS-*TNT*. All flies were tested under a circadian light cycle of 12 hr:12 hr. Sleep profiles and sleep parameters were obtained using SleepLab (William Joiner).

Immunohistochemistry

Whole mounted brains were dissected and fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature (RT), washed in 0.3% Triton-X phosphate solution (PBT 0,3%) for 2 hours and blocked in 5% normal goat serum for 1 hour at RT. We used the following primary antibodies: rabbit anti-GFP (Molecular Probes, Eugene, OR, Cat# A-21312, RRID:AB_221478) at 1:500, mouse anti-RFP (Rockland Cat, Limerick, PA, # 600-401-379, RRID:AB_2209751) at 1:500, and BRP (DSHB, Iowa

City, IA, Cat# nc82, RRID:AB_528108) at 1:150. The secondary antibodies, Alexa Fluor 488 goat anti-rabbit (Thermo Fisher Scientific, Waltham, MA, Cat# A11008, RRID:AB_143165) and Cy5 goat anti-mouse (Thermo Fisher Scientific, Waltham, MA, Cat# A10524, RRID:AB_2534033) were used at 1:1000. Primary and secondary antibodies were incubated at 4°C overnight, protected from light. Images were obtained on a Leica SP8 confocal microscope and analyzed with FIJI program.

Statistical analysis

Multiple group comparison was done through one-way ANOVAs followed by Tukey post-hoc tests.

Results

Activation of MS2 neurons induces male specific sleep suppression.

From an ongoing screen for sleep-regulatory neurons, we found another Gal4 line, VT012760, whose artificial activation using the TrpA1 ion channel suppresses nighttime sleep exclusively in males, resembling the MS1 activation phenotype (Figure 1A and 1B; (Machado et al. 2017). For simplicity, VT012760 will be referred to as MS2 (Male Specific 2). Interestingly, despite the severe sleep reduction during the night, the flies do not exhibit recovery sleep the day following the thermal stimulation, suggesting that the MS2 neurons are capable of suppressing sleep without inducing rebound.

MS2 neurons in the SOG are octopaminergic but do not induce male-specific sleep suppression.

Next, we studied the expression pattern of MS2 neurons in both males and females. Using MS2-Gal4 to drive UAS-*CD8::GFP*, we found that MS2 neurons are organized in several clusters in the fly brain, namely a small group of approximately 2-3 cells in the SOG, a dense cluster in the antennal lobes, a few neurons in the dorsal brain, and two clusters in the lateral brain (Figure 2A). A comparison of MS2 neurons in males and females did not reveal obvious differences between the two sexes.

From previous studies we found that MS1 neurons in the SOG are octopaminergic and responsible for male-specific sleep suppression (Machado et al. 2017). Considering that stimulating MS2 neurons induces a sleep profile similar that of stimulating MS1 neurons, we examined whether MS2 neurons in the SOG are octopaminergic. To this end, we used Gal4/UAS to express RFP in MS2 neurons, and LexA/LexAop to express GFP in *tdc2*-positive neurons. We found that, indeed, the MS2 neurons of the SOG are *tdc2*-positive, octopaminergic neurons, as revealed by the overlapping expression of GFP and RFP in the SOG (Figure 2B).

We next sought to test whether the MS2 octopaminergic neurons contribute to suppress sleep exclusively in males. For this we used a split-Gal4 line. Split-Gal4 lines, in which the GAL4 is split in

two domains, activating domain (AD) and DNA binding domain (DBD), can be used to selectively target the intersection of two driver lines (Lin & Potter 2016). Only the cells that have both domains express functional GAL4, potentially restricting transgene expression to a very specific subset of cells. Combining Tdc2-Gal4-AD and MS2-Gal4-DBD, we made a MS2-Tdc2 split-Gal4 line and tested whether the activation of MS2-Tdc2^{+} affects sleep in males. This experiment revealed that activation of octopaminergic subset of MS2 neurons does not promote male-specific sleep reduction, indicating that sleep suppression is likely promoted by MS2 neurons that are not in the SOG (Figure 2C).

Some MS2 neurons are *fru*-positive and exhibit a sexually dimorphic expression pattern.

Given the involvement of the FRU circuit in male sleep regulation, we next asked whether some of the MS2 neurons are *fru*-positive. For this, we took advantage of the FLP-FRT system which allows targeting of transgenes to cells that express both the Flippase (FLP) enzyme and a Flippase Recognition Target (FRT) construct (Mellert et al. 2010). Thus, using MS2-Gal4 to express UAS-FRT-*mCD8::GFP* and *fru*-LexA to express FLP, we restricted the transcription of GFP specifically to potential MS2-Fru double-positive neurons. We found that the intersection between the MS2 and *fru* neuronal populations contains several mAL neurons present only in males (Figure 3), suggesting that these mAL neurons might be responsible for the male-specific sleep suppression by MS2 activation.

Manipulation of mAL neurons modulates male sleep

To examine whether mAL neurons influence male sleep in a sexual context, we employed R43D01-Gal4, a driver previously shown to include a broad set of mAL neurons (Clowney et al. 2015). Males carrying R43D01-Gal4 and UAS-TNT were paired with either a WT male (MM^{c}) or a WT female (MF^{c}) and the sleep of the pairs was measured. Surprisingly, we observed that when R43D01 neurons are silenced MF^{c} pairs do not exhibit a reduction of nighttime sleep and, in fact, show a sleep profile similar to the one of MM^{c} pairs (Figure 4A). One the other hand, MF^{c} control pairs (*R43D01*/+ and

TNT/+) show a reduction of nighttime sleep. To test whether activation of the R43D01 neurons has the opposite effect, i.e., suppression of sleep, we expressed the TrpA1 channel in the R43D01 neurons and tested individual males and females at 31°C. Indeed, males in which R43D01 neurons are activated exhibit a significant reduction of nighttime sleep (Figure 5B). These results are surprising because mAL neurons were previously described to inhibit P1 neurons in the presence of females (Clowney et al. 2015; Kallman et al. 2015), which would predict that manipulations of mAL neurons and P1 neurons would have opposite effects. Instead, it appears they cause similar effects on sleep. These results, together with the finding that some of the MS2 neurons are mAL neurons, suggest that the R43D01 neuronal population may include not only GABAergic, inhibitory neurons, as formerly described, but also excitatory neurons that might promote the activity of P1 neurons.

Discussion

We have shown that artificial activation of MS2 neurons promotes sleep suppression in males but not in females (Figure 2A and 2B). This phenotype resembles the one induced by activation of MS1 neurons, which modulate the conflict between sleep and sex by tilting the balance in favor of courtship. Intriguingly, the intersection between MS2 and *fru*-expressing neurons contains several mAL neurons present only in males. This is interesting in light of our previous finding that activation of MS1 neurons elicits enhanced calcium responses in mAL neurons (Machado et al. 2017). Moreover, activation of R43D01 neurons suppresses sleep in males and blocking of R43D01 neurons prevents the female-induced sleep reduction usually observed in MF pairs. Altogether, these are unexpected discoveries considering mAL neurons are thought to provide inhibitory input to the P1 cluster (Clowney et al. 2015). A possible explanation is that R43D01-Gal4 labels two antagonistic subpopulations: an inhibitory group and an excitatory group that includes Fru^+ MS2 neurons (Figure 5).

Previous studies have shown that some mAL neurons express GABA (Clowney et al. 2015), however it is not known whether there are non-GABAergic mAL neurons.

To clarify whether mAL includes two sub-populations, the FLP-FRT system could be used to restrict the expression of FRT-stop-FRT-*mCD8::GFP* specifically to Fru^+ MS2 neurons. In a complementary approach, we could use split-Gal4 lines, i.e., we could express the DNA binding domain of GAL4 in the MS2 population and the activation domain in the mAL neurons using the enhancer of R43D01-Gal4. The resulting split Gal4, MS2∩mAL-Gal4, could be used to direct GFP expression to the intersection of MS2 and mAL populations. Employing either system to restrict expression of GFP to a subset of MS2 neurons, we could co-immunostain male brains for GFP and GABA, using an anti-GABA antibody. If the MS2 subset of mAL neurons are non-GABAergic, they would be labeled only by GFP. Such an outcome would support the hypothesis that mAL neurons include two subpopulations: GABAergic and non-GABAergic. However, it might be that all MS2 neurons in the mAL are GABAergic. In that case, one might hypothesize that MS2 mAL neurons inhibit downstream neurons that are themselves inhibitory and block wake-promoting neurons, such as P1 neurons. Alternatively, it might

be that sleep suppression induced by activation of R43D01 neurons is caused by other non-mAL neurons.

The MS2∩mAL split Gal4 driver would also allow us to characterize the role of MS2∩mAL neurons in detail. If we learn that they do not express GABA, co-labeling experiments against multiple antibodies would help us determine which neurotransmitter system the neurons use. Expressing TrpA1 driven by the split Gal4 would permit us to test whether activation of MS2∩mAL neurons are sufficient to suppress sleep in males. Additionally, to assess the role of MS2∩mAL neurons in MF interactions, we could block the neurons using TNT and then pair the males with either WT females or WT males and measure sleep. If activating MS2∩mAL neurons suppresses sleep in isolated males, it would suggest such neurons may be part of the same sleep-modulatory circuits that include MS1 and P1 neurons. Moreover, if blocking MS2∩mAL neurons prevents sleep reduction of MF pairs, it would indicate that the activity of MS2∩mAL neurons is likely to mediate female-induced sleep suppression. Alternatively, if manipulating MS2∩mAL neurons does not affect sleep, it would suggest that neither MS2∩mAL neurons nor MS2 neurons in the SOG are the ones responsible for regulating sleep in males, and other MS2 neurons in other brain regions would have be investigated.

Since activation of MS1 neurons triggers calcium responses in some mAL neurons (Machado et al. 2017), we could also test whether activation of MS1 neurons triggers calcium responses in MS2∩mAL neurons. If the outcome is positive, we could determine whether the MS2∩mAL neurons are immediately downstream of MS1, by testing the response of MS2∩mAL neurons to octopamine using GCaMP. Additionally, we could use the P2X2-GCaMP system to examine whether the MS2∩mAL neurons send excitatory input to the P1 cluster.

Previous studies showed that female pheromones are processed in parallel excitatory and inhibitory circuits, and that mAL cluster acts in the inhibitory circuit (Clowney et al. 2015). They suggested that mAL-mediated inhibitory input may provide gain control or serve to limit the duration of courtship. Similarly, two antagonistic mAL sub-groups may contribute to the fine-tuning of male courtship. For example, upon the decision to court a female, the excitatory mAL neurons might act first, to suppress sleep and promote P1 activity and courtship. The inhibitory group might act afterwards, towards the conclusion of the mating ritual, helping to reduce the courtship promoted by P1 neurons. Alternatively, the two mAL subgroups may differ in their response to male and female

pheromones. For example, the excitatory group may respond to female pheromones whereas the inhibitory group responds to male pheromones. If we find that there are two subgroups of mAL neurons, further work could be performed to distinguish these possibilities.

Although more work is needed to identify and characterize the specific subset of MS2 neurons mediating male-specific sleep suppression, our results thus far provide a promising entry point for further investigation of neural mechanisms underlying sleep suppression by male sex drive.

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Figure legends

Figure 1. Activation of MS2 neurons suppresses sleep exclusively in males. **(A)** Sleep profile of male (left) and female (right) flies with MS2-Gal4 driving the temperature-sensitive channel TrpA1. To activate TrpA1 the flies were exposed to 28°C. N = 30-31. The sleep profiles represent minutes of sleep in intervals of 30 minutes. In all figures, the white bar bellow the x-axis represents the daytime/light period, and the black bar represents the nighttime/dark period. In all figures *p<0.05; **p<0.01; ***p<0.001; ns = not significant.

Figure 2. The MS2 neurons in the SOG are octopaminergic. **(A)** Confocal projection of the expression pattern of MS2 > CD8::GFP in the male brain (left) and in the female brain (right). The brains were immunostained for GFP (green) and Brunchpilot (BRP, magenta). Scale bar: 100 μm. **(B)** Top: confocal image of the male brain co-labeled with RFP in MS2 neurons (using Gal4-UAS; magenta) and with GFP in Tdc2 neurons (using LexA-LexAop; green). Scale bar: 100 μm. Bottom: magnified image of the SOG region outlined by the white rectangle in the images of the top row. The arrows point to neurons in which MS2 > RFP and *Tdc2* > GFP co-localize. **(C)** Sleep profile of males and females in which *MS2-Tdc2*split-Gal4 was used to express TrpA1 in MS2-Tcd2^{+} neurons. The flies were exposed to 29°C to activate TrpA1.

Figure 3. The MS2 neurons in the mAL region are *fru*-positive. On the left, confocal projection of a male brain expressing GFP in MS2-Fru double-positive neurons (green). The brain was also stained for BRP (magenta). On the right, magnified view of the MS2-Fru^{+} neurons of the mAL region of the brain, as outlined by the white rectangle on the left. Scale bar: 100 μm.

Figure 4. mAL neurons modulate sleep exclusively in males. **(A)** Sleep profile of males in which the R32D01 neurons are blocked by TNT (R43D01 > *TNT*), and respective parental controls (*R43D01*/+ and *TNT*/+), paired with either a WT male (MM^{c}; left) or a WT female (MF^{c}; right). N = 30-32. **(B)** Sleep profile of isolated males and females expressing the temperature-sensitive channel TrpA1 in R43D01 neurons. TrpA1 was activated by exposing the flies to 31°C. N = 25-32.

Figure 5. Hypothesis that proposes that the R43D01 neuronal population comprehends two sub-groups: an excitatory group and an inhibitory group. R43D01 neurons are represented by the orange box, which includes hypothetical excitatory mAL neurons (green circle) and inhibitory mAL neurons (red circle). According to the proposed hypothesis, the excitatory mAL group includes *fru*-expressing MS2 neurons. In this model, inhibitory mAL inhibit the P1 neurons, as described before. On other the hand, the excitatory group promotes the activity of P1 while inhibiting the activity of sleep-promoting centers. Sleep is regulated by inputs from both P1 and sleep centers.

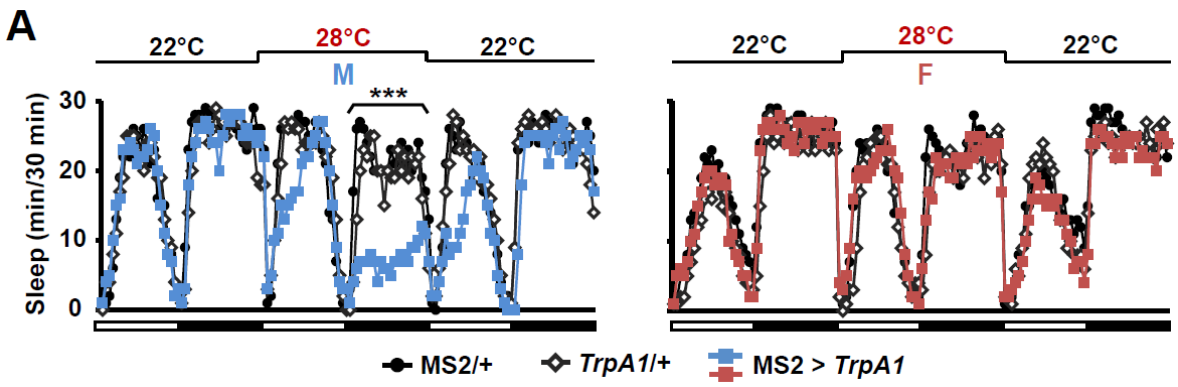
A
22°C
28°C
22°C
M
***
22°C
28°C
22°C
F
Sleep (min/30 min)
30
20
10
0
MS2/+
TrpA1/+
MS2 > TrpA1

Figure 1.

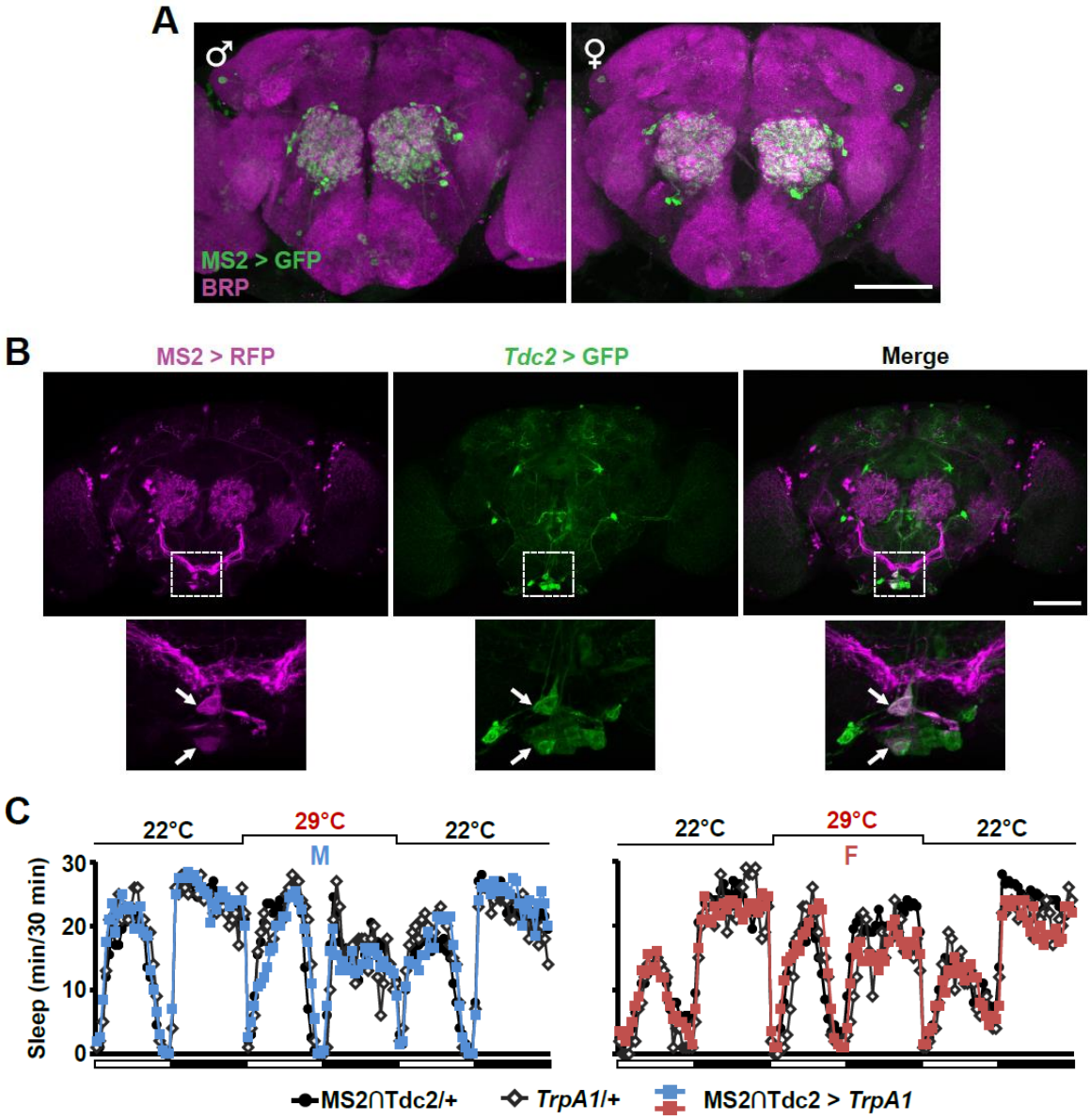
A
♂
♀
MS2 > GFP
BRP
B
MS2 > RFP
Tdc2 > GFP
Merge
C
22°C
29°C
22°C
M
F
Sleep (min/30 min)
30
20
10
0
MS2∩Tdc2/+
TrpA1/+
MS2∩Tdc2 > TrpA1

Figure 2.

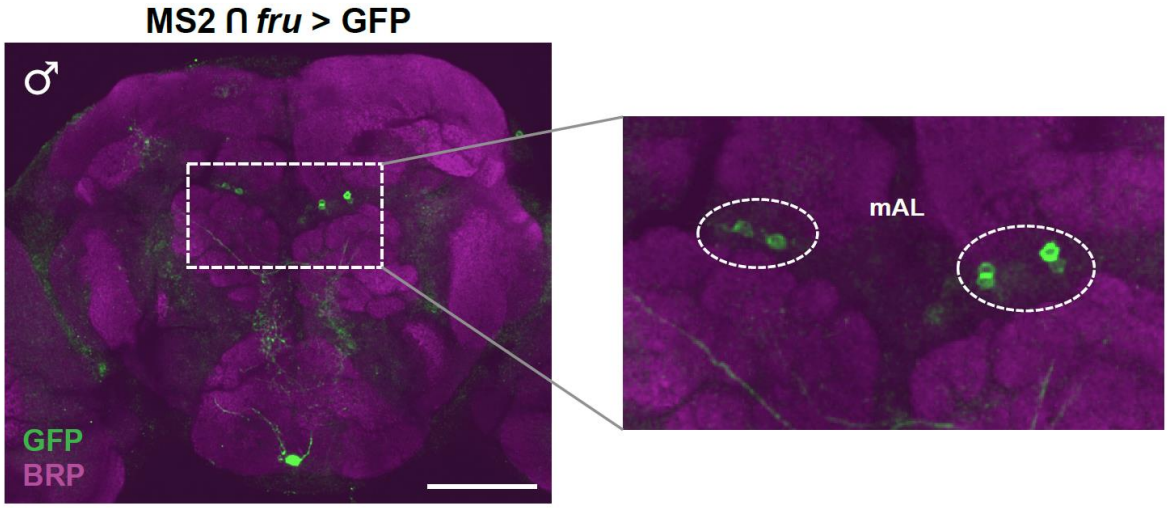

Figure 3.

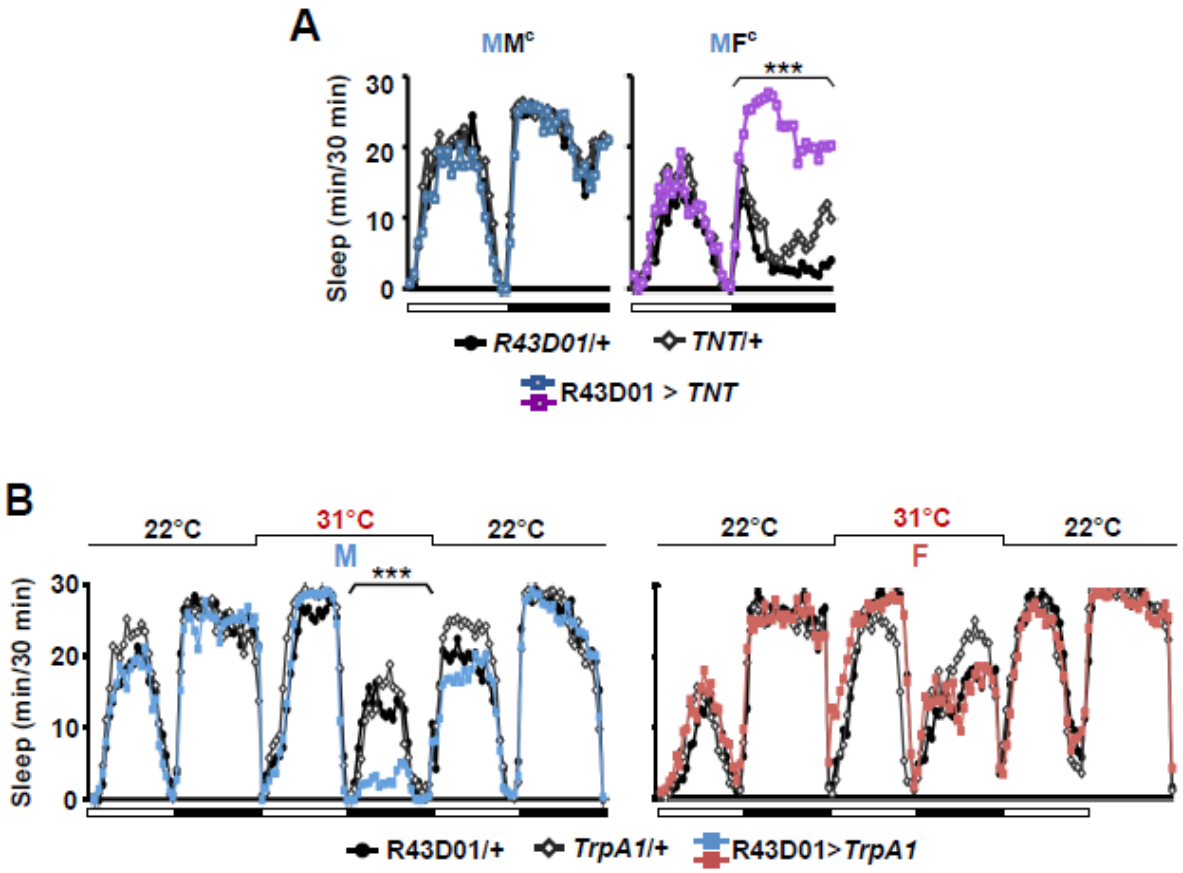
A
MM^c
MF^c
***
Sleep (min/30 min)
30
20
10
0
R43D01/+
TNT/+
R43D01 > TNT
B
22°C
31°C
22°C
M
***
F
Sleep (min/30 min)
R43D01/+
TrpA1/+
R43D01>TrpA1

Figure 4.

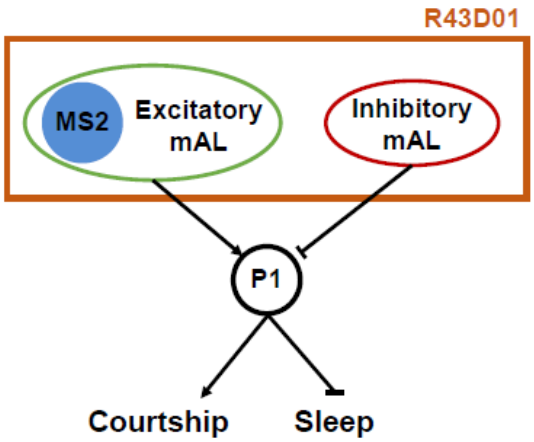
R43D01
MS2
Excitatory
mAL
Inhibitory
mAL
P1
Courtship
Sleep

Figure 5.

Chapter 3

Discussion, Conclusions and Future Directions

Discussion

Competition between sleep drive and sex drive modulates sleep in male flies

Animals face environmental challenges that constantly call for an adequate decision between essential behaviors. Because sleep is practically incompatible with any other behavior, under certain environmental and social conditions, it might be advantageous to suppress sleep and satisfy other critical needs (Allada & Siegel 2008; Isaac et al. 2010; Lesku et al. 2012; Griffith 2013). For example, mammalian studies revealed a dynamic conflict between sleep and feeding, as fasted animals sleep less than fed ones; on the other hand, sleep deprivation stimulates food intake (Rechtschaffen & Bergmann 2002; Taheri et al. 2004). Similarly, fruit flies sleep less when starved (Mcdonald et al. 2010).

In this work, we describe the conflict between sleep and courtship, two incompatible but important behaviors. We show that female presence induces a state of heightened sexual arousal in the male that leads to sleep suppression. Two other labs have recently confirmed some of our major findings (Beckwith et al. 2017; Chen et al 2017). The conflict between sex drive and sleep drive relies on a dynamic balance: increasing the sleep drive markedly affects the sexual performance of the males, making them sleep even when females are present and sex drive is high. On the other hand, males that are sexually satiated and thus experience reduced levels of sex drive, sleep significantly more than naïve, non-satiated males in the presence of females.

Behavioral outputs, in a given environmental and social context, depend on the integration of varied external cues and internal drives (Griffith 2013). Therefore, a certain behavior may be regulated not only by the specific internal drive that promotes that behavior, but by the outcome of the contextualized integration of different, even opposing, drives. Hence, sleep may not be modulated exclusively by sleep drive but, instead, may depend on the integration of multiple drives – for example, hunger, fear, sex drive, and sleep drive. Our findings highlight the importance of investigating how sleep is integrated with other behaviors.

The MS1 neurons mediate sleep suppression by male sex drive

How does the brain regulate the balance between sleep and courtship? In this work, we show that MS1 neurons play a central role in the integration of sleep and sex drives.

We found that the effects of manipulating the activity of the MS1 neurons are male-specific and context dependent. In isolated flies, artificial activation of MS1 neurons promotes wakefulness in males, but not in females. Importantly, silencing of MS1 neurons impairs courtship and impedes the loss of sleep elicited by females. Interestingly, silencing of MS1 neurons does not affect sleep in isolated males or males paired with other males, suggesting that MS1 neurons are normally active only when a potential sexual partner is present. The contextualized activation of the MS1 neurons only in the presence of females may, thus, be important to sustain an enhanced state of alertness that may confer reproductive advantage over other males.

An important feature of sleep behavior is the occurrence of rebound sleep after prolonged wakefulness (Shaw et al. 2000; Hendricks et al. 2000). Interestingly, the reduction of sleep induced by the MS1 neurons is not followed by recovery sleep. This supports the idea that, under certain conditions, it may be advantageous to reduce the amount of baseline sleep in order to satisfy other pressing behaviors. This is the case of the male sandpiper birds that suppress sleep for a 3-week period to compete with other males and court females (Lesku et al. 2012). Sleep loss does not appear to impair male performance as short-sleeping males are more successful at producing progeny than their peers that sleep more. Thus, in the presence of specific relevant stimuli, some sleep-suppressing neurons may act to promote a state of arousal that overcomes or inhibits the homeostatic accumulation of sleep drive. This may contribute to a reproductive advantage, as a male that is able to suppress sleep for long periods of time in order to engage in sexual activities is more likely to be successful than other males in competing for females.

The MS1 neurons use octopamine to suppress sleep in males

We have demonstrated that the MS1 neurons use octopamine to suppress sleep in males. MS1 neurons in the subesophageal ganglion (SOG) are octopaminergic, and mutations that interfere with octopamine synthesis block male sleep loss due to MS1 activation. In addition, and as mentioned before, the sleep loss caused by MS1 is not followed by a rebound response, a phenotype also observed with the activation of octopaminergic pathways (Seidner et al. 2015). A possible explanation for the absence of recovery could be that the octopaminergic circuits, including the MS1 octopamine-expressing neurons, interact with the sleep homeostat, either inhibiting or bypassing the neuronal pathways that monitor sleep accumulation. This raises the interesting idea that octopaminergic circuits may mediate a contextualized suppression of sleep in the presence of salient, motivational factors, a mechanism that allows the individual to suppress sleep and engage in relevant activities, such as sex or foraging for food. Octopamine is equivalent to the mammalian noradrenaline and, thus, the noradrenergic system may also mediate wakefulness promoted by motivational factors in mammals, including humans.

The octopaminergic system was shown to regulate many other behaviors besides sleep. In fact, octopamine is involved in the regulation of foraging, memory formation and some sexually dimorphic behaviors, such as egg laying, aggression and the conflict between aggression and courtship (Rezával et al. 2014; Yang et al. 2015; Iliadi et al. 2017; Kayser et al. 2015). Thus, different octopaminergic subpopulations may control different kinds of arousal and modulate the interaction between different behaviors and sleep. We propose that the MS1 octopamine-expressing neurons may constitute a particular group of octopaminergic neurons responsible for promoting wakefulness in males in the presence of sexual stimuli.

The MS1 neurons interact with the FRU circuitry to integrate sleep and sex drive

Although the expression pattern of the MS1 neurons shows no apparent differences between males and females, GRASP and GCaMP studies revealed sexually dimorphic interactions between MS1 and the FRU network. These data suggest that the male-specific effect of the MS1 circuit on sleep is not explained by the presence or absence of the MS1 neurons *per se*, but by differences in anatomical and functional connectivity to the FRU network. Sexually dimorphic connections between MS1 and FRU are supported by GRASP data, which suggests potential contacts between MS1 and FRU in the superior posterior slope, a region densely innervated by octopaminergic neurons. While GRASP suggests dimorphic anatomical connections between the MS1 and FRU populations, functional studies with GCaMP indicate that the MS1 neurons receive and transmit information to the FRU network.

Activation of the MS1 neurons elicits strong calcium responses in several Fru neuron clusters, including P1 and mAL, which are directly implicated in the regulation of the mating ritual (Kimura et al. 2008; Yamamotot & Koganezawa 2013; Clowney et al. 2015). Although additional work is necessary to confirm the identity of the neurons, MS1 activation may also elicit calcium responses in aSP4, which is thought to encode male sex drive (Zhang et al. 2016).

Although the P1 cluster acts downstream of the MS1 neurons, the MS1 input into the P1 neurons is not sufficiently strong to initiate courtship, suggesting that the MS1 neurons may be important to modulate, rather than trigger, courtship activity. In contrast, artificial excitation of the P1 neurons triggers courtship behaviors such as wing extension for the first 30 min or so, followed by increased locomotor activity and reduced sleep. This led us to propose a circuit in which the MS1 neurons modulate the activity of P1 and, then, the P1 neurons activate motor programs for courtship along with downstream circuits that regulate sleep. Recently, a second lab published data that supports this view. The P1 neurons have a pivotal role in coordinating sleep and courtship: although the activity of P1 is suppressed by increased sleep pressure, the activation of the P1 cluster promotes courtship through the stimulation of sex-dedicated circuits and suppresses sleep through the stimulation of wake-promoting DN1 neurons (Chen et al. 2017). Altogether, these findings suggest that the MS1 neurons promote the excitability of the FRU network, contributing to a state of heightened sexual arousal that keeps the male awake and more alert to the presence of females.

The communication between MS1 and the FRU network is bidirectional, in the sense that, besides exciting FRU neurons, the MS1 neurons also receive inputs from the FRU circuitry. As shown by GCaMP experiments, the MS1 neurons in the SOG show a significant calcium response to FRU neuron activation. Therefore, considering that several *fru*-expressing cells detect chemosensory signals from females and that the MS1 neurons promote wakefulness when a female is present, it is plausible that the MS1 neurons receive inputs from pheromone-sensing FRU neurons and promote sexual arousal that keeps the males awake for enhanced courtship.

The functional connection between MS1 neurons and the FRU network suggests that MS1 neurons may play a central role in the circuitry that integrates sleep and courtship. Therefore, we propose a model in which (1) MS1 neurons receive excitatory inputs from pheromone-sensing FRU neurons concerning the presence of females; (2) MS1 neurons then activate several FRU clusters such as P1 and mAL. (3) P1 neurons also receive inputs from the pheromone-sensing neurons and from other FRU neurons that control mating drive, such as aSP4, which may also receive inputs from MS1. (4) Under the appropriate social context and internal motivational state, the P1 neurons trigger courtship and suppress sleep (Figure 1). The MS1 neurons may, thus, amplify the chemosensory cues from females into a powerful arousing signal, promoting a sustained state of sexual arousal.

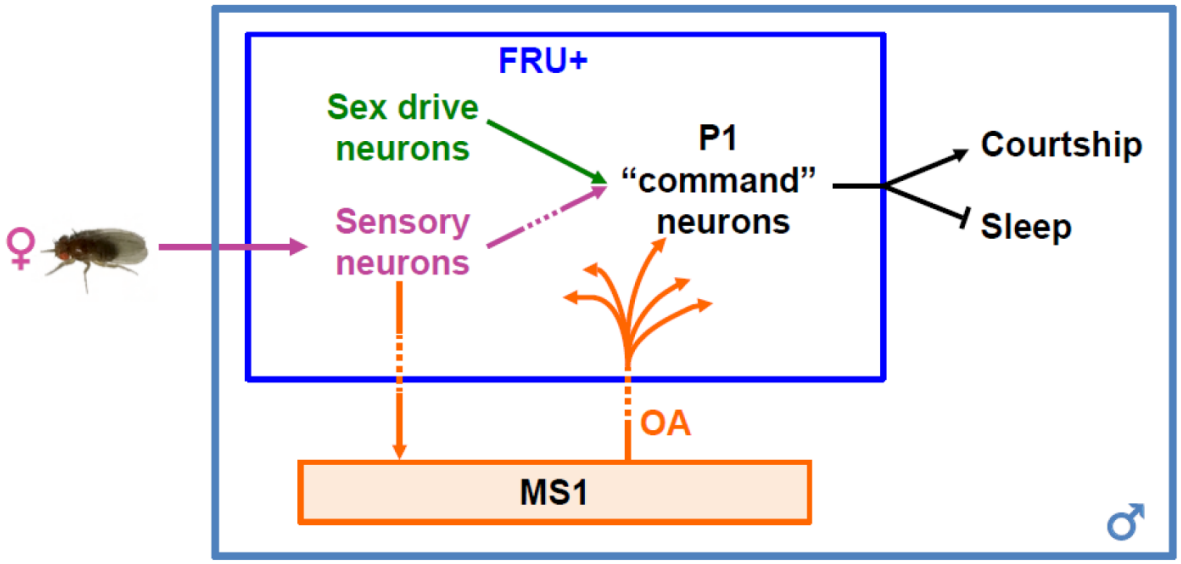

Figure 1. MS1-FRU circuitry underlying the integration of sleep and courtship. The female presence stimulates sensory FRU neurons that send excitatory inputs to mAL neurons and P1 neurons. Sex drive neurons and the mAL neurons also send information to the P1 cluster. The activation of the P1 cluster triggers courtship and suppresses sleep. MS1 neurons also receive inputs from sensory FRU neurons and, in turn, activate several FRU clusters, including the mAL and P1. The MS1 neurons may, thus, promote a sustained state of arousal by amplifying the sensory inputs from females processed by the FRU network. The bigger, dark blue box represents the male; the smaller, light blue box represents the FRU neuronal network; arrows with partially dashed lines represent neuronal connections that might de indirect.

The absence of sleep rebound following sleep suppression induced by activation of MS1, MS2 and P1 neurons might result from an adaptive homeostatic system.

An important property of sleep, present in both *Drosophila* and humans, is its homeostatic regulation, which promotes recovery sleep after prolonged periods of wakefulness (Donlea et al. 2017; Porkka-Heiskanen 2013; Huber et al. 2004). Therefore, the homeostatic system is essential to prevent sleep need form accumulating to deleterious levels (Shaw et al. 2002). Why and how, then, do MS1, MS2 and P1 neurons induce sleep suppression without promoting rebound sleep? A possible explanation might be a potential advantage for males that are able to suppress their sleep in order to pursuit a female, compared with males that are not able to surpass their sleep need to reproduce. This is the case of male arctic sandpiper birds; those who sleep the least produce the most progeny. This phenomenon provides further evidence that under specific sexual contexts sleep can be suppressed in favor of reproduction (Machado et al. 2017; Beckwith et al. 2017; Chen et al. 2017; Fujii et al. 2007).

According to our data, and data from other researchers, male flies exhibit vigorous sexual activity during the night (Fujii et al. 2007; Machado et al. 2017). This may be because the courtship ritual and subsequent copulation take about half an hour, during which both males and females are more exposed to threats from predators. Thus, it may be important for a male to suppress nighttime sleep in order to court a female. The beginning of daytime, the period in which sleep rebound is most evident, is an important time for flies: they start moving around and exploring their surroundings, foraging for food and, in the case of females, for a suitable place to lay eggs. Besides, daytime brings potential predators, in face of which flies must react fast. Thus, to suppress sleep during the night in order to reproduce, and to stay awake the following morning, might be an important strategy for flies to satisfy their biological needs without jeopardizing their capacity to react to a constantly changing environment.

It is important to note that, from our observations, it is not possible to infer whether the reduction of sleep during the night and the absence of recovery sleep in the early day have any deleterious consequences in the flies' performance, either in the short or long term. It is possible that, even if the flies are not affected after a night of short sleep, the effects of sleep deprivation for many

days might accumulate and eventually impair the performance of the individual later in life. To clarify this question, learning and memory tests may be administered to well-slept isolated males, sleep deprived isolated males, males paired with a male and paired with a female. The tests could be applied the day immediately after the night of pairing or deprivation, to test for short-term effects, and several days later to address potential long-term effects.

How could the homeostat allow for sleep reduction without sleep rebound the following day? We propose two hypotheses: (A) courtship-induced sleep reduction is not registered by the homeostat; (B) a sexual context changes the way flies sleep.

Hypothesis A proposes that, when a certain ecologically relevant behavior competes with sleep, the homeostatic circuits are modulated in such a way that potential deficits in sleep are not registered. As a consequence, recovery sleep is not promoted once the competing need is satisfied because the lack of sleep was not sensed by the homeostatic system in first place. Eventually, the circadian system guarantees that the animal is able to go back to its usual sleep schedule (Beckwith et al. 2017).

Hypothesis B proposes that the necessity to suppress sleep is compensated by changing the way a fly sleeps. According to this hypothesis, the sleep need of the male would not be significantly affected by the female presence because the male sleep adapts to that specific social condition. As observed in our sleep experiments with pairs of flies, the MF pairs exhibit increased sleep amount in the beginning of the night, the males starting to court the females only a few hours after lights turn off (Machado et al. 2017). It is possible that when the male is paired with a female, his sleep homeostatic system induces a deeper or more restorative sleep in the beginning of the night, to compensate for the sleep deprivation that will follow. This way, the male may get enough sleep to function properly without having to sleep more the next day. Because the MS1 and MS2 neurons are involved in the coordination between sleep and courtship, their activation may change the way the homeostat manages sleep.

A possible approach to test these hypotheses would be to assess the activity of neurons that encode sleep drive, such as EB R2 neurons (Liu et al. 2016). Indeed, a recent study used CalexA, a reporter of neuronal activity, to evaluate the activity of EB R2 neurons of isolated males, males exposed to females and males exposed to males. Surprisingly, significant increased levels of activity were

detected in the EB R2 neurons of males paired with females, suggesting the homeostatic system detects sleep deficits, even when caused by sexual interactions. Thus, these findings favor the hypothesis that, upon a relevant sexual encounter, sleep drive accumulates but instead of leading to rebound sleep, changes the way the male fly sleeps. Further investigation may help elucidate the neural mechanisms of the interaction between sexual arousal and sleep homeostasis.

Conclusions and Future directions

In conclusion, this work showed that the decision between sleep and sex, two incompatible, yet critical behaviors, relies on the balance between sleep drive and sex drive, which are integrated in a circuit composed of octopaminergic MS1 neurons, several FRU clusters, and a subset of MS2 neurons. The results raise many interesting questions including 1) whether MS1 neurons are activated by female cues; 2) which neurons mediate the connection between MS1 and P1 neurons; and 3) how P1 neurons regulate both sleep and courtship. The role of MS2 neurons in the decision between sleep and courtship is still unclear. Additional work is necessary to pinpoint the subset of MS2 neurons involved in male sleep regulation and to place them in the context of MS1 and P1 neurons. Regardless of the outcome of future studies, however, investigation of MS2 neurons is likely to produce novel insights into neural mechanisms underlying integration of sleep and courtship.

It is known that sleep is suppressed by hunger (Mcdonald et al. 2010; Leslie C. Griffith 2013), which raises the question of how hunger, sex drive, and sleep drive interact. Previous studies have shown that females exhibit marked behavioral changes after mating, such as reduced receptivity to courtship, increased egg laying, and reduced sleep (Rezával et al. 2014). However, the underlying neural mechanisms are not well understood, thus suggesting another interesting topic for future research.

By studying the conflict between sleep and courtship in fruit flies, the present work offers an entry point for investigating the problem of how the brain decides between conflicting needs. Exploring these questions in simpler animal models, such as *Drosophila melanogaster*, is a first step to understanding how more complex beings, such as humans, process conflicting needs in order to adapt their behavior to an ever-changing environment.

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