

Investigation of Sleep Homeostasis:

a Chronic Imaging Study

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I dedicate this thesis to my mentors

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“Start by doing what's necessary, then what's possible;
and suddenly you are doing the impossible.”

— Saint Francis of Assisi

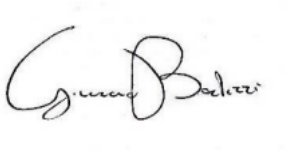
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Declaration

I, Giacomo Bertuzzi, declare that the work presented here is an original work. I was involved in all the stages of the work. External contributions have been acknowledged in the work. None of the results and ideas presented here have been submitted elsewhere.



14-May-2022

Signature *Date*

Abstract

Drosophila shows complex behaviours but performs these with relatively few neurons. Genetic tools for mapping brain circuits and perturbing neurons, together with the ability to record neural activity, offer the potential to link behaviours with brain circuits and dynamics. Neurobehavioural dynamics are complex and short experiments do not capture dynamics occurring over multiple days.

To overcome this limitation, I developed a chronic imaging protocol which allows the recording of neurobehavioural dynamics of a fly living for days on a spherical treadmill under a two-photon microscope. To validate this approach, I followed the Ca^{2+} dynamics in a genetic line (23E10-GAL4) labelling the switch neurons controlling sleep and wake. Sleep was measured as locomotor inactivity lasting for 5 or more minutes.

I explored the relationship between the dendritic Ca^{2+} dynamics in the switch neurons and sleep. I found that some of the features describing the dendritic Ca^{2+} dynamics correlate with “future sleep” and the time of day.

To overcome another limitation of current experiments investigating sleep homeostasis, I developed a protocol to sleep deprive flies during imaging experiments. I proved that the protocol is effective in causing sleep rebound after sleep deprivation and does not affect the lifespan of the experimental fly.

Our study paves the way for new experiments investigating sleep homeostasis and the detrimental effects of sleep deprivation over extended periods of time. It is also a validation of the virtues of Drosophila as a model for systems neuroscience.

Keywords— long-term imaging – sleep homeostasis – neurobehavioural dynamics

Table of Contents

Chapter 1 - Introduction…… 12

1.1 Sleep in Drosophila: a general overview……..... ……. 12

1.2 Neural correlates of sleep in Drosophila: the central role of the “sleep switch”…..... 17

1.3 Feeding and sleep: an intertwined relation…….…….... 22

1.4 Measuring and disrupting sleep in Drosophila ..... ……. 26

1.5 Neural representation of naturalistic behaviours: a technical challenge ..... ……. 29

1.6 Objectives of the current research ..... ……. 32

Chapter 2 – Setting up a chronic neurobehavioural experiment…….. 35

2.1 A setup for a chronic neurobehavioural experiment with head-fixed flies..... ……. 35

2.1.1 A feeding system for the fly under the microscope……..……..…. … 39

2.1.2 A chronic fly preparation for imaging using two-photon microscopy……..…. 52

2.2 A pipeline to record for days the neurobehavioural dynamics of Drosophila. ……. 56

Chapter 3 – Chronic imaging of the genetic line 23E10…….. 64

3.1 Ca^{2+} imaging of 23E10 neurons……..……. 64

3.1.1 Ca^{2+} dynamics in the dendrites……..……..……. 71

3.1.2 Ca^{2+} dynamics in the axons….. ..... …… …. 80

3.2 Correlation analysis of different parameters describing Ca^{2+} dynamics ..... …..…… 83

Chapter 4 – Correlating Ca2+ dynamics in 23E10 dendrites and sleep…… 89

4.1 Mean GCaMP6f fluorescence in the dendrites correlates with the time of the day..... 90

4.2 Ca^{2+} dynamics in the dendrites and sleep before and after imaging ..... …..…… 93

4.3 Ca^{2+} dynamics between the two hemispheres could be linked with sleep……..…. 110

Chapter 5 – Sleep deprivation under a two-photon microscope…… 114

5.1 A protocol for feeding and imaging before, during and after sleep deprivation…….. 115

5.2 Air puffs are effective in eliciting an active locomotor response…….…. 119

5.3 Random puffs of air for 12-h induce sleep rebound after sleep deprivation ……. 123

Chapter 6 – Discussion…… 129

6.1 Chronic monitoring of a fly under a two-photon microscope: a new chapter…… 150

Chapter 7 – Material and methods……... 156

7.1 Drosophila strains and culture ……..…..…… 156

7.1.1 Holidic medium……. 156

7.2 Behavioural tracking……..…….. 158

7.3 Two-photon calcium imaging……..…… 160

7.4 Sleep deprivation under the two-photon microscope……..…..……. 162

7.5 Generation of figures and statistics…… …… 163

References……... 164

Supplementary material…… 181

List of Figures

Figures chapter 1 - Introduction

1.1 Representation of the central complex and the "sleep switch"…… 18

Figures chapter 2 – Setting up a chronic neurobehavioural experiment

2.1 Adapting a two-photon setup for chronic behavioural recordings under the microscope….. 39

2.2 A liquid holidic medium…….…….. 42

2.3 Holidic food diet does not alter sleep pattern nor the amount of sleep..…….. 44

2.4 Head-fixed flies respond well to liquid holidic diet and automatic feeding…….…….. 47

2.5 Flies survive for days under a two-photon microscope and show sleep and wake……. 50

2.6 Sleep quantity and quality are in the physiological range in flies living under a 2-photon….. 52

2.7 A chronic imaging preparation does not affect the physiological sleep pattern of the flies… 56

2.8 A pipeline for chronic monitoring of flies under a two-photon microscope… ….……. 59

2.9 A chronic imaging experiment does not affect sleep quantity and quality……..……. 61

2.10 A less disruptive imaging protocol helps flies to have a more consolidated sleep……..... 64

Figures chapter 3 – Chronic imaging of the genetic line 23E10

3.1 Ca^{2+} imaging of 23E10 neurons. Ca^{2+} oscillations are visible to the naked eye……. 69

3.2 An analysis of Ca^{2+} dynamics……..……..…… 71

3.3 A qualitative analysis of Ca^{2+} dynamics in the 23E10 dendrites over multiple days…..... 74

3.4 Distinct Ca^{2+} dynamics in distinct dendritic regions of 23E10 neurons… ……..... 77

3.5 Ca^{2+} dynamics of the entire dendritic region well represent regional variation of activity… 80

3.6 A qualitative analysis of Ca^{2+} dynamics in the 23E10 axons over multiple days……..... 83

3.7 A correlation analysis of different parameters describing Ca^{2+} dynamics … …..…… 86

3.8 Dendritic Ca^{2+} dynamics in the two hemispheres are positively correlated…….. 89

Figures chapter 4 – Ca^{2+} dynamics in 23E10 dendrites and sleep

4.1 The time of the day influences dendritic Ca^{2+} dynamics in the 23E10 neurons……..... 93

4.2 Descriptive features of Ca^{2+} dynamics in the 23E10 dendrites correlate with the amount of sleep in the 30 minutes when the imaging session occurred……..... 96

4.3 Distinct features of the Ca^{2+} traces in the 23E10 dendrites correlate with the amount of sleep after the imaging session, but not with the sleep before (30min bin)…. 99

4.4 Distinct features of the Ca^{2+} traces in the 23E10 dendrites correlate with the amount

of sleep after the imaging session, but not with the sleep before (1h bin)……..……. 102

4.5 Distinct features of the Ca^{2+} traces in the 23E10 dendrites correlate with the amount of sleep after the imaging session, but not with the sleep before (2h bin)……..... 105

4.6 The strongest correlation is found when considering sleep 1h after the bin0…….. 108

4.7 Peaks' number in traces could be a predictor of sleep the imaging session…… 110

4.8 The goodness of the prediction of sleep from the number of peaks in an imaging trace depends on the time of the day and negatively correlate with the change in fluorescence (ΔR/R0)…….... 111

4.9 Ca^{2+} dynamics in the two hemispheres show negative correlation for a limited time…….. 114

4.10 Negative correlation of dendritic Ca^{2+} dynamics occurs after reduced sleep……... 116

Figures chapter 5 – Sleep deprivation under a two-photon microscope

5.1 Coherence between the feeding protocol and the circadian locomotor rhythmicity allows changing of the light-dark cycle without losing the biphasic sleep pattern..... 119

5.2 An imaging protocol to follow dendritic Ca^{2+} dynamics before, during and after sleep deprivation in a two-photon setup……..……..…… 121

5.3 Developing a protocol to sleep deprive flies for 12h under a two-photon microscope..…… 124

5.4 Random puffs of humidified air for 12h elicits a strong behavioural response in head-fixed flies freely walking on a spherical treadmill… ……..…… 127

5.5 Overnight sleep deprivation using random puffs of humidified air induces sleep rebound…… 130

Figures chapter 7 – Material and methods

7.1 An overview…… 154

Supplementary materials

8.1 Chronic imaging protocols with hm feeding at fixed timing influence timing and quantity of sleep, but not the circadian rhythmicity of the locomotor activity…… 185

8.2 Circular HeatMaps show higher Ca2+ activity in the axons compare to the dendrites…….. 187

8.3 Variation of Ca^{2+} imaging traces during 24h……. 189

1 | Introduction

1.1 Sleep in Drosophila: a general overview

The mystery of sleep has fascinated people since ancient times. The 2nd-century Roman physician Galen attempted the first neuroscientific definition of sleep, considering it both a rest response that allows our brain to escape from the rigor of our waking experience as well as a phase that is subject to the intensity of the waking experience (Oberhelman, 1983). Since Galen, extensive research on animal models has been conducted. Animals sleep in diverse manners, but there are several general features(Campbell and Tobler, 1984):

- The animal is immobile (in most cases), with a species-specific posture.
- The threshold to elicit behavioural responses to stimuli is higher than when the organism is awake.
- The timing and amount of sleep are regulated through circadian and homeostatic mechanisms.
- A sleeping organism must wake up upon sufficiently intense stimulation.

The beneficial effects of sleep, as well as the detrimental effects of sleep loss, have focused attention on the mechanism that regulates sleep. Knowing the dynamics behind this universal physiological process could help to control both positive and negative effects of sleep loss.

Examining sleep in animal models, where behavioural, genetic, and physiological analyses are easier than in humans, provides an opportunity to understand sleep at

the molecular level as well as at the level of the neuronal circuits. For these reasons Drosophila melanogaster has been a very useful model to study the correlates of sleep and wake.

The first systemic reports about the alternation of periods of activity and quiescence in Drosophila date back to 1956 (Roberts, 1956). Only in the year 2000, two independent laboratories showed that prolonged inactivity in Drosophila could be indeed a sleep-like status, satisfying the criteria defining sleep (Hendricks et al., 2000; Shaw et al., 2000).

Sleep in Drosophila has commonalities as well as differences with human, and more generally mammalian sleep.

Two characteristics of sleep that are shared with humans are a more consolidated sleep at night (Liu et al., 2015) and more reduced and fragmented sleep during later stages of life (Vienne et al., 2016). In both flies and humans, homeostatic and circadian factors make synergistic contributions to the regulation of sleep. Differently from humans, most flies are active in the early hours of the morning and at dusk and less active in between (Liang et al., 2019). Moreover, sharp-wave ripples and sleep spindles (elements of human sleep) have not been observed in flies, while slow-waves have been found both in humans and Drosophila sleep circuits (Raccuglia et al., 2019; Yap et al., 2017).

In both mammals and flies, the control of body movements is dependent on the arousal state (van Swinderen et al., 2004; Uchida et al., 2021) . When flies are asleep an uncoupling of body movement from brain activity is observed (Tainton-Heap et al., 2021). As it has been seen in mammals (Vyazovskiy and Harris, 2013), the neural activity in the fly brain changes, both at the level of single neurons and across the whole brain(van Alphen et al., 2013; Bushey et al., 2015; Tainton-Heap et al., 2021). The changes go in the direction of general neural depression, without

suppression of brain activity, and inhibition of sensory processing (Tainton-Heap et al., 2021). That said, a recent study demonstrates that flies may process—perhaps subconsciously—'salient' information requiring interpretation, such as smell, and this type of processing of information could happen during sleep and wake up a fly; similarly to what has been observed in mammals. Modulation of the arousal threshold is dependent on the internal states of the animal. For example, starved flies are woken up by certain food-related odours (French et al., 2021).

Flies appear to have distinct sleep stages(van Alphen et al., 2013; Tainton-Heap et al., 2021), although an open question is whether these stages perform analogous functions to the ones found in human sleep. It is not known whether specific sets of neurons regulate different stages of sleep, but recent work suggests this could be the case(Yap et al., 2017).

Drugs affect sleep both in flies and humans. For example, caffeine and amphetamines induce increased locomotor activity (Nall et al., 2016; Karam et al., 2022); anti-insomnia drug Gaboxadol and hydroxyzine induce rest (Shaw et al., 2000). Although, while the same drug could cause the same behavioural effect in both species the modality to reach similar effects is not always equivalent (Shafer and Keene, 2021).

Flies as well as humans sleep more after certain experiences, reflecting a need for more sleep and underling the role of sleep in achieving many different functions. For example, a socially enriched experience, that for a fly usually involves courtship and fighting behaviour, is linked with increased and deeper sleep (Ganguly-Fitzgerald et al., 2006; Lone et al., 2016).

Behind this observed behaviour there is most likely a need to process the plastic changes occurring in the brain during experiences that require learning and plasticity in the brain (Tononi and Cirelli, 2014; Vorster and Born, 2015).

As much as certain experiences induce and/or consolidate sleep in mammals and insects, some others suppress sleep. For example, sexual arousals could compensate for the lack of sleep (Beckwith et al., 2017) and starvation induce food-searching behaviour (e.g. increase in locomotion) (Keene et al., 2010). Food and sexual reproduction can represent a higher order of priority compared to sleep; however, suppression of sleep always comes with a price.

One of the main consequences of sleep deprivation is shared between flies and humans and involves learning and memory deficits. Flies in need of sleep perform badly in learning tasks (Seugnet et al., 2008). Moreover, lack of sleep reduces courtship behaviour and success in reproduction in flies and mice (Kayser et al., 2014).

The beneficial effect of sleep has been shown in *Drosophila*: enforced sleep in mutants that have learning and memory deficits is able to restore normal learning levels without fixing the underlying brain problems (Dissel et al., 2015). Interestingly, increased sleep improves memory consolidation in humans, mice, rats, cats and flies (Lei et al., 2022).

The continue research into why we sleep and what happens when we do it, led scientists exploring the link between metabolite clearance, fundamental to remove toxic proteins from the brain, and sleep. Recent research with mice and flies showed that a dramatic accumulation of ROS (reactive oxygen species) in the gut, following sleep deprivation, could lead to death (Vaccaro et al., 2020). Interestingly, sleep loss could cause death through the above mechanism, but it has been shown that flies could survive without sleep, at least in a controlled laboratory setting (Geissmann et al., 2019). From work using Drosophila it seems that the relationship between sleep and oxidative stress is bidirectional. On one side oxidative stress triggers sleep (Hill et al., 2018; Kempf et al., 2019), on the other side sleep influences the general health

of the animal and its ability to respond to environmental stress acting as an antioxidant for both the body and the brain (Neckameyer and Nieto-Romero, 2015; Hill et al., 2018).

Overall, sleep research in Drosophila keeps highlighting the conserved functions of sleep and drives the search for the molecular mechanisms underpinning the alternation between wake and sleep as well as the neuronal machinery at the circuit level.

1.2 Neural correlates of sleep in Drosophila: the central role of the “sleep switch”

Sleep is an actively regulated process and not just the result of reduced activity of the brain compared to waking. It is a complex behavioural phenomenon that involves the whole brain. During sleep, there is a reorganisation of neuronal activity(Tainton-Heap et al., 2021).

The transition between sleep and wakefulness ultimately depends upon a very limited number of neurons, which act as “sleep switch” (Saper et al., 2001; Pimentel et al., 2016). This idea is at the base of the *flip-flop model* and represents the predominant model in sleep research (Saper et al., 2010). The switch from wake to sleep or vice versa relies on the antagonism between two groups of neurons: the wake and sleep-promoting neurons. The model has been further developed following the discovery of neural oscillators and advanced knowledge of dynamics relative to the two brain hemispheres (Dunmyre et al., 2014; Tera A. Glaze,Sonya Bahar, 2021).

Integrating the flip-flop model with the two-process model, it has been proposed that the “sleep switch” responds to both the homeostatic and circadian control of sleep (Rempe et al., 2010). The homeostatic regulation has been considered sleep-inducing, while the circadian system has been associated with arousal (Cavanaugh et al., 2016).

The homeostatic control of sleep, which depends on the time the organism spends awake, is finely integrated with the circadian system of neurons, which sets the time the organism is supposed to sleep (Deboer, 2018). Sleep homeostasis and circadian timing of sleep operate as separate systems, even though they are finely integrated with each other (Deboer, 2018). For example, clock neurons have been found to

signal to circuits involved in sleep homeostasis in several species. Occasionally, the homeostatic sleep drive arises outside of the normal circadian rest period, to recover sleep lost after sleep deprivation(Schwarz et al., 2021).

Interestingly, many of the neurons involved in the regulation of sleep homeostasis have different functions, and most of the time are also directly involved in coordinating behaviour during wakefulness. Sensory stimuli are continuously selected, suppressed, and integrated to decide the action to take in a specific context.

In the fruit fly brain, the “sleep switch” consists of about 20 neurons in total (Donlea et al., 2018). While the dendrites of these neurons are evenly distributed between the two hemispheres in two distinct clusters, their axons extend into the dorsal fan-shaped body (dFB), a sub-structure of the central complex in the central brain of Drosophila, reason why the “sleep switch” neurons are also called dFB neurons (Figure1.1).

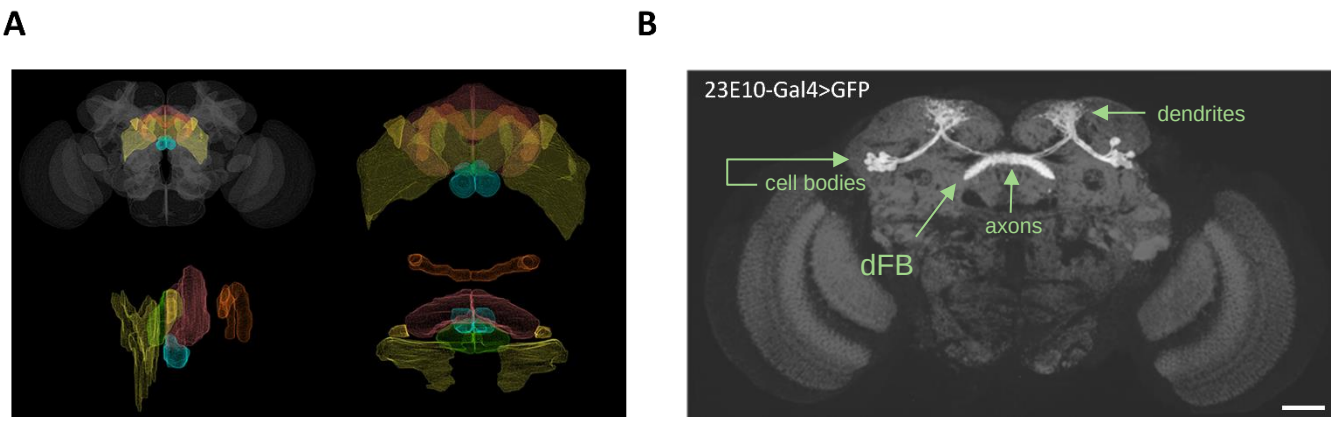


Figure 1.1 Representation of the central complex and the “sleep switch”.

Figure 2.1 Representation of the central complex and the "sleep switch".

(A) A representation of the central complex with a rendering using NeuroGFX (Givon et al., 2011). Clockwise from top left: whole brain, front view of the central complex, side view of the central complex, top view of the central complex.The central complex neuropils are in the following colours ■ the protocerebral bridge (PB), ■ fan-shaped body (FB), ■ ellipsoid body (EB), and ■ noduli (NO). Accessory neuropils are in the following colours ■ the bulb (BU) and the ■ lateral accessory lobe (LAL) **(B)** A representation of the neurons labelled by the genetic line R23E10. This image has been taken from www.janelia.org. Front view of a 3D rendering of a confocal stack showing the R23E10 expression pattern (white) along with immunohistochemical staining against nc82 (gray). The images were compiled with imageJ by compressing a z-stack of confocal sections taken at a distance of 1.5 µm between each individual confocal plane (scale bar = 50 µm).

The brain of Drosophila could be defined by a set of neuropils (around 50), which are then grouped in regions. The central complex is a region comprising four main neuropils and two accessory neuropils, showed in Figure 1.1. Each neuropil is then divided in sub-regions, accordingly to their orientation: this is the case of the dorsal fan-shaped body, sub-region where the sleep switch has the axons.

Interestingly, the central complex is a multisensory processing unit: the incoming sensory information is gated and processed and then a specific behavioural response is coordinated. It has been proposed that the central complex has a role in generating a very primitive form of self-awareness, integrating self-generated motion with external stimuli to create a neural simulation of an animal's movement through space (Tanimoto, 2019). It is therefore not surprising that the "sleep switch" output (the dFB axons) resides in fact in the central complex of Drosophila.

The induction of sleep is determined by a change in the electrical state of the dFB neurons. During wakefulness the input resistance and membrane time constants increase, the rise in the electrical excitability leads to a firing state. When dFB neurons are firing the fruit fly switches to a sleep state (Pimentel et al., 2016).

The change in the excitability of the dFB neurons is finely regulated by external signalling as well as intracellular dynamics and depends on the modulation of membrane ion channels and membrane ion currents. Specifically, it has been shown that potassium channels play a fundamental role, three in particular: Shaker, Shab and Sandman(Pimentel et al., 2016; Kempf et al., 2019).

Shaker and its β modulatory subunit Hyperkinetic are regulated by the levels of reactive oxygen species (ROS) and are the link between increased sleep and levels of ROS. Indeed, increased levels of ROS in the dFB neurons induce the oxidation of the cofactor nicotinamide dinucleotide phosphate (NADPH), which is bound to Hyperkinetic. The effect is a reduced inactivation of the A-type current, carried by

Shaker. Shaker and Shab are required for repetitive action potential generation (Pimentel et al., 2016; Kempf et al., 2019).

Sandman is a two-pore-domain potassium channel that is critical for the hyperpolarisation of the dFB neurons. When the dFB are entering the firing modality Sandman is internalised. When the fly is about to wake up, the firing of the dFB declines thanks to the upregulation of Sandman, and the consequent presence of voltage-independent leak currents. The upregulation of Sandman is mediated by dopamine, which also causes the downregulation of Shaker and Shab (Pimentel et al., 2016).

Interestingly, the dopamine signalling influencing the excitability of the dFB neurons recalls the mammalian regulation of sleep-promoting neurons (the ventrolateral preoptic nuclei of the hypothalamus) , inhibited by dopamine(Oishi and Lazarus, 2017).

The dynamics of the network regulating the sleep switch are quite complex and only a few circuits have been explored systematically. It is known that dFB neurons are regulated by the circadian clock neurons and some arousal-promoting neurons downregulates dFB activity through dopamine signalling (Liu et al., 2012; Ni et al., 2019). It is also known that dFB neurons are regulated by R5 neurons, which represent the homeostatic regulation of sleep (Liu et al., 2016). Other non-central complex neurons seem to influence dFB activity through different neuronal pathways, although much evidence is still lacking (Sitaraman et al., 2015; Cavanaugh et al., 2016; Qian et al., 2017). The inputs to the dFB are both sleep-promoting glutamatergic and arousal-promoting dopaminergic. Interestingly, inputs to the dFB seem to reach both the dendrites and the axons of the neurons(Ni et al., 2019).

Recently a lot of effort has been put in understanding how the dFB effectively put the fly to sleep.

dFB neurons form an inhibitory synapse with a group of neurons called “helicon cells”, which coordinate locomotor responses to visual input(Donlea et al., 2018; Tainton-Heap et al., 2021). When the sleep drive is high and the dFB are “on” the helicon cells are inhibited. These helicon cells make also synapses with ring neurons (R5) in the ellipsoid body of the central complex. R5 neurons not only are an important centre that keeps track of sleep pressure, but they are also responsible to select behavioural locomotor responses. When helicon cells are inhibited by the dFB, the activity of R5 neurons is drastically reduced (Donlea et al., 2018). Recently, it has been suggested that dFB neurons are key to synchronise the electrical patterns of R5 and arousal mediating neurons (helicon cells) (Raccuglia et al., 2022). Interestingly, it has been shown that the dFB also signal to a group of neurons named *hugin+.* These neurons are known to be the circadian output neurons regulating locomotor activity rhythms (Schwarz et al., 2021).

Less is known about the role of the dFB in gating mechanical stimuli and the mechanism of inhibition of sensory neurons not related to the visual system.

Another mystery is related to the quality of sleep. Sleep duration does not necessarily correlate with sleep depth. It is not known if the dFB neurons have a role in determining the quality of sleep. It is likely that a fine regulation of sleep quality depends on a number of different neurons as well as specific activity patterns. Recording of local field potentials (LFPs) in the fruit fly brain have registered 7-10Hz oscillation predominated immediately after a fly fell asleep and just before it woke up and was comparatively less evident in the middle of the sleep bout. Interestingly, 7-10Hz oscillations were seen upon genetic induction of sleep, with the activation of the dFB, but not with anti-insomnia drugs (Yap et al., 2017). A closer investigation

of differences in oscillator brain activity during sleep could shine a light on the regulation of sleep quality by the brain, assuming that sleep is regulated by the brain and for the brain.

1.3 Feeding and sleep: an intertwined relation

Sleep is a plastic behavioural state finely regulated by a combination of ecologically and physiologically relevant environmental factors. Temperature changes (Lamaze et al., 2017) as well as social interactions (Li et al., 2021) affect sleep quantity and quality. Moreover, the availability and the quality of food directly regulate sleep (Linford et al., 2012; Chouhan et al., 2021). Not surprisingly, the regulation is bidirectional: quantity and quality of sleep influenced by external agents and changes in the internal state could in turn affect feeding (Murphy et al., 2017) and social interaction (Kayser et al., 2015). Mutual correlations and causalities between sleep and nutrition are particularly important to understand since both feeding and sleep are vital for the surviving of each single animal during its lifespan.

In 1906 Sherrington formulated the *singleness of action principle*, stating animals could only select one action at a time (Sherrington, 1906). In 1943 Maslow formulated his *hierarchy of need theory*, postulating that the fulfilment of basic physiological needs has priority over higher needs(Maslow, 1943). In other words, needs such as food, water and sleep must be met before needs such as mating and cooperation. How and when flies prioritize sleep over feeding and vice versa?

Behavioural prioritization is plastic and context dependent (Cheriyamkunnel et al., 2021). *Drosophila melanogaster*, or the “lover of the dew” as the Greek name suggests, has a crepuscular activity, which coincides with the opportunity for feeding. It feeds on fruits and microorganisms, such as yeast, associated with fruit. Although flies are generalist feeders, they are sensitive to changes in their internal nutrient status (Mazzotta et al., 2021).

Feeding is such a critical behaviour for flies that they evolved the ability of discriminating nutritional and non-nutritional sugars (Fujita and Tanimura, 2011). It takes just 20 seconds for a fly to sense the nutritional value of a food source (Qi et al., 2015). The consequences of feeding decisions reflect on the quality of sleep (Chen et al., 2016). Ingestion of non-nutritional sugars induce fragmentation of sleep and lighter sleep (lower arousal threshold)(Hasegawa et al., 2017). Interestingly, like humans, flies ingesting a high quantity of sugar alone suppress their need for sleep(Hasegawa et al., 2017). Yeast in combination with sugar promotes wakefulness if the sugar levels are low and sleep if they are high (Catterson et al., 2010). Although we are lacking knowledge about the bidirectional regulation of sleep and feeding, emerging evidence point to a role of the gut microbiota(Silva et al., 2021). What is certain is the need of a careful design of a diet for experiments of sleep in flies.

Looking at the brain of fruit flies it is interesting how neurons relevant for the processing of information relative to feeding and sleep are in the same brain area. Sugars and amino acids as well as dangerous metabolites are sensed by distinct gustatory receptor neurons (GRNs) (Montell, 2009). These neurons are in the sensilla, which could be found on the proboscis, but also on the wings' margins. In the suboesophageal ganglion (SOG), GRNs synapse with projecting neurons, which in turn synapse with higher brain centres (Montell, 2009). Among these centres, there is the superior medial protocerebrum (SMP), where the arborisations of many sleep-related neurons are found (Cavanaugh et al., 2016; Donlea et al., 2018). In this area gustatory information is integrated into circuits governing sleep/wake behaviour (Beckwith and French, 2019).

The absence of gustatory inputs for around six hours leads to a food-searching behaviour (KOPEĆ, 1928). Starved flies suppress their sleep and increase their

locomotor activity. Their hyperactivity is rescued by the perception of gustatory stimuli, with an immediate effect in the presence of a sweet stimulus (Yu et al., 2016).

Refeeding following starvation induces sleep (Murphy et al., 2016). Intriguingly this sleep is deeper compared to pre-meal, and it is characterised by higher arousal thresholds. It positively correlates with the volume of food ingested and with the nutrients present in the food (e.g., protein and salt induce deeper post-feeding sleep) (Murphy et al., 2016). A possible key player in promoting sleep after feeding could be a subset of Allostatin A-positive neurons. Thermogenetic activation of these neurons promotes sleep, reduces locomotion, and suppresses feeding (Chen et al., 2016).

The ability of switching between mutually exclusive behaviours such as sleep and feeding relies on dynamics yet to be understood. Recently, it has been proposed that a specific group of neurons, called P1 neurons, could be the integrator of different behavioural states, such as feeding, sleep and courtship (Deutsch et al., 2020).

Flies are equipped with mechanisms to sense internal and external changes and to adapt to an ever-changing environment, it is likely that the system developed to be highly plastic and to embrace different regulatory mechanisms in different situations, both at the cellular and connectivity level.

In 1982 Borbely introduced the *two-process model* to explain sleep regulation (Borbély, 1982). Process-C represented the regulation of sleep by the circadian system and holds the information for sleep timing. Process-S represented the homeostatic regulation of sleep, encoding the need of sleep based purely on tiredness. The *two-process model* remains influential in the design of experiments and data evaluation (Borbély et al., 2016). Perhaps the model could be integrated

considering the large number of findings of the past three decades and could include Process B, which could integrate distinct behavioural drives. What is certain is the need for a “sleep switch”, integrating all the processes and putting the animal to sleep.

1.4 Measuring and disrupting sleep in Drosophila

The easiest way to measure sleep is to record the locomotor activity of the animal object of study, considering that the main feature of sleep is a sustained physical inactivity. Since the mutagenesis studies by Benzer in the 1970s that changed the field of the circadian rhythms (Konopka and Benzer, 1971), the tools to measure the locomotor activity of flies have not changed much.

Sleep in Drosophila is usually measured using the *Drosophila* activity monitor system (DAMS; Trikenetics Inc., Waltham, MA). DAMS allows to measure sleep in populations of flies and to do it for several days. Conceptually, the system works similarly to what has been used by Benzer. Each fly is housed in individual glass tubes, secured between food and cotton. The individual tubes are placed in monitors and an infrared beam crosses the centre of each tube. When the fly, walking in the glass tube, crosses the beam, the fly's activity is detected by a collector connected with a computer. Sleep is classified as a period of inactivity of 5 minutes or more. The clear advantage of DAMS is the possibility of screening large numbers of flies, this advantage has led to many discoveries in a short time (Pfeiffenberger et al., 2010). After genetic screening and monitoring of activity using DAMS, many sleep-regulating molecules and circuits have been found. As much as this approach has been successful, there are limitations. The spatial resolution and the accuracy of the sleep measurements are low (Zimmerman et al., 2008). On the two sides of the infrared beam, the fly in the glass tube could still be active. The flies considered inactive could be feeding, grooming, could be sick or just immobile and vigilant, instead of sleeping. Also, the system does not provide an accurate measure of sleep quality, providing only information about consolidated sleep or fragmented sleep, by

measuring the frequency of beam crossing. Another bias of the system could come if the fly sits right in the middle of the beam, when too much movement may be detected (Gilestro, 2012). Finally, the system is unable to record interacting flies.

An alternative way to assess sleep in flies is to use video-recording systems and computer vision analysis. Different tools have been developed in recent years, from the FlyPi project (Maia Chagas et al., 2017) to the Ethoscope platform (Geissmann et al., 2017), with many labs using their own customised video-recording system (Donelson et al., 2012). What these systems have in common is the use of cameras (usually more than one) to record videos with high resolution. The advantage is the possibility of processing these videos in real-time or offline. The development of these tools met the need of affordability and scalability.

Interestingly, with Ethoscope fly behaviour could trigger delivering of stimuli, in a way independent from the experimenter (Geissmann et al., 2017). This represents a useful tool for neuroscientists.

Software for automatic recognition of a subset of behaviours (e.g., grooming, movement of the proboscis and wings in flies) have revolutionised the field and have led to important discoveries in biology (Qiao et al., 2018). Most of the advantage of video-tracking systems comes from the software used to analyse the recordings. Sleep is a complex behaviour and there is more and more the need to combine the measure of inactivity with other behavioural measures for an accurate description of the process.

Finally, sleep researchers interested in understanding the neuronal dynamics of sleep have started assessing the locomotor activity of flies with a system composed of: 1) an air-suspended ball (Buchner, 1976) and 2) cameras to record the ball and fly's movement while the fly, walking on the ball, is head-fixed under a two-photon microscope (Seelig et al., 2010). The advantage of this system is the possibility of

measuring sleep together with neuronal activity since the air-suspended ball could fit both in a rig for electrophysiology and under a two-photon microscope. This type of tracking is limited to a single fly per ball per experiment, and it is not the best approach to screen many flies, e.g., for profound defects due to genetics and consequently isolate new genetic variants. Although the system of tracking a fly's movement using an air-suspended ball dates to the 1970s (Buchner, 1976), only recently it has been shown to key to connect neural activity and behaviour (Ribeiro et al., 2018).

A significant insight in the physiology of sleep could come from studies of sleep deprivation. A lot of effort has been put in to developing sleep-deprivation techniques that could be integrated with the tools already in use to measure locomotor activity, such as the SNAP (The Sleep Nullifying Apparatus) (Melnattur et al., 2020). In Drosophila mechanical sleep deprivation has been performed using an orbital shaker(Kayser et al., 2015), a rotating device (Geissmann et al., 2019), or a vibrating platform (Öztürk-Çolak et al., 2020).

The effect of sleep deprivation is assessed by measuring sleep rebound after the treatment; this is also the only way to differentiate the effect of stress from the effects of sleep deprivation (Cirelli and Bushey, 2008).

The advantage of the techniques in use is the flexibility they offer, since most of the time, flies loaded in monitor for (video)tracking remain in the same tubes before, during and after sleep deprivation.

However, except for the Ethoscope (Geissmann et al., 2017), the technique in use target flies not specifically and independently of their actual activity.

A method for sleep depriving flies for several hours during simultaneous physiological recordings is not currently available.

1.5 Neural representation of naturalistic behaviours over multiple timescales: a technical challenge

Observing the structure and physiology of cells in live animals over extended periods of time is essential to unravel the mechanisms that underlie experience-dependent plasticity, neural development, neurodegeneration, and aging (Adam and Mizrahi, 2011; Mostany et al., 2013; Nishiyama et al., 2014). Sleep is a plastic behaviour: aging, quality and quantity of feeding, stress, intense social experiences, learning, neurodegeneration, and changes of habits influence the neuronal machinery controlling sleep and affect sleep drive (Raven et al., 2018; Matenchuk et al., 2020; Radwan et al., 2021).

Sleep drive is built up during periods of wake and dissipated during periods of rest (Pimentel et al., 2016), however the temporal dynamics of this process are not yet clear. Does the dissipation of sleep drive occur with different dynamics throughout 24-hour cycles? Dissipation of sleep drive depends on the activity of the sleep homeostat, i.e., the dorsal fan-shaped body neurons (dFB) (Pimentel et al., 2016). How does the activity of the dFB change during 24-hour cycles?

While long-term in vivo imaging (>24h) of the intact nervous system has been possible for several animal model species in the past years (Asokan et al., 2020; Wu et al., 2022), the field of fruit fly research has not benefited from this approach. Time-lapse studies in either naturally translucent animals, such as C.Elegans (Gritti et al., 2016), or mice with implanted optical windows (Steinzeig et al., 2017) have uncovered longitudinal dynamics for cellular morphology and function, impossible to

capture otherwise. The tiny fruit fly has a fragile and opaque exoskeleton. Implanting an external optical window did not seem possible until recently.

First, researchers from University of California created a chronic transparent window on the fruit fly's head to monitor neural activity in freely walking flies (Grover et al., 2016). The developed technique, called Flyception, made possible the observation of neural representation of naturalistic behaviours, such as mating.

The Flyception system, while being an exceptional tool to investigate the fly brain, is limited by the type of imaging that can be done. The camera sensor and lensing are far away from the fly, the resolution of the brain imaging is limited, and fine neuronal dynamics in axons and dendrites remain elusive.

Only a couple of years after the publication of Flyception, a group of researchers from Stanford shared a procedure for long-term in vivo imaging in the fly brain for up to 50 days (Huang et al., 2018). For the first time a single fly brain is imaged repeatedly for days. The biggest drawback of the approach is the epifluorescence widefield imaging used for monitoring calcium dynamics. Increased fluorescence background makes this type of imaging less suitable for imaging in deeper structures, and visible excitation light interferes with behaviour. Also, the procedure presented does not allow continuous imaging of brain dynamics: each experimental fly is placed in a vial with fly food while not under the microscope for an imaging recording session.

The latest contributions to the field show how quickly the community of fruit fly researchers is moving towards a different kind of imaging experiments. Andres Flores and colleagues presented their setup and workflow to monitor continuously the neurobehavioural dynamics of a fly living under a 2-photon microscope for up to 7 days (Flores-Valle et al., 2022). Robots are used to assist surgery and to feed the fly during the experiments, which also experience a system of virtual reality

integrated in the setup. An air-suspended ball is used to track locomotor behaviour, using the approach previously described. While Andres Flores and colleagues offer the invaluable opportunity of exploring neuronal and behavioural dynamics over days with high-temporal-resolution monitoring, the system presented is expensive and technically complex. Max Jameson Aragon and colleagues reported a way to record neural structures and activity in behaving Drosophila for up to 12 hours, without removing the cuticle (Aragon et al., 2022). To achieve this, they compressed the air sacs underneath the brain cuticle. They used multiphoton imaging, and they showed how their approach could be used with a two-photon microscope. While the presented approach is promising and extremely exciting for the field, it does not yet offer the possibility of imaging deeper structures, such as the central complex, using two-photon imaging.

1.6 Objectives of the current research

Observing multiple epochs of extended naturally occurring sleep while simultaneously recording the neuronal dynamics of the “sleep switch” of Drosophila over days has been the main drive of this work. Linking neural activity to complex behaviour represents one of the main interests in neuroscience.

Scientists can observe cell activities of the nervous system non-invasively using genetically encoded calcium indicators, i.e., GECIs, and calcium ion imaging technology (Oh et al., 2019). When cells are electrically active the intracellular concentration of calcium ions increases and GECI, sensitive to free calcium ions, undergo a change in conformation. The change in conformation of GECI, following the binding of calcium ions, stimulates the emission of fluorescence, which can be collected and recorded by means of two-photon fluorescence microscope or optical fibre recording (Lohr et al., 2021). Thanks to GECIs like GCaMP It is possible to detect changes in calcium ions caused by a single action potential, with high temporal precision (Zhang et al., 2021). Genetically it is possible to express GECIs either throughout the life of a cell or only at certain stage of development. This offers extreme flexibility to the experimenter and the advantage of following the neural activity of specific cells up to several months (Zhang et al., 2021).

Two-photon imaging with GECIs allows for high spatial resolution. It is possible to track the fluorescence signals of either cell bodies or cell substructural units, such as axons, dendrites, or local synapses, even in small neurons deep inside the brain. Importantly, two-photon imaging allows recording from populations of neurons and multiple dendritic arbors, fundamental to understanding neural circuit function. For these reasons and considering that most of the processing in insect neurons is

localised in arbors that are distant from the soma (Seelig et al., 2010), the two-photon microscope is preferable to other recording techniques to investigate brain dynamics at different levels. Whole-cell patch-clamp, for example, only reveals the physiology of the cell body and recordings from multiple neurons are difficult and impractical in small animals like Drosophila. Although, recording techniques different from two-photon microscopy are irreplaceable for answering certain classes of questions: patch-clamp electrophysiology, for example, offer a unique window into synaptic and other transmembrane currents.

To explore for long periods of time the properties of a brain circuit of Drosophila in the context of the behaviour we decided to use two-photon microscopy. .

Chronic (>24h) monitoring of freely walking Drosophila under a 2-photon microscope requires:

1. Adapting a 2-photon setup for chronic behavioural recordings under the microscope
2. Selecting a diet and a feeding protocol for chronic behavioural recordings
3. Defining a workflow to chronically image the fruit fly's brain while recording fly's behaviour

The first part of this work will describe the development of the procedure for the long-term recordings. The effort culminated in chronic neurobehavioural experiments lasting for up to 7 days. Our goal was to explore the Ca^{2+} dynamics recorded in the dFB at various times over consecutive days and investigate the relationship between those dynamics and sleep. This work contributes to the knowledge of the physiology of the Drosophila sleep switch and it is relevant for future chronic neurobehavioural studies.

Sleep shows dynamics at multiple timescales. Behaviourally, sleep duration and timing are known to change during 24 hours in Drosophila; sleep regularity is defined

by the comparison of the amount of sleep-wake during consecutive days. Studying sleep physiology over multiple days allows a better understanding of how biological systems adapt to experience-dependent changes.

Despite the importance of observing the physiology of living organisms, the study of a disrupted physiology could reveal important biological properties. Consequently, in the last part of this work, I will present how I designed a way to deprive of sleep Drosophila while living under a 2-photon microscope. Our work shows for the first time that is possible to perturb the physiology of a fly during a chronic imaging experiment.

The road is still long to answer the numerous questions about sleep and the relative brain circuits. This work is a small step in giving a more accurate view of what could occur in a close to natural setting to the wider research field.

2 | Setting up a chronic neurobehavioural experiment

Chronic monitoring of neurobehavioural dynamics of individual flies under a two-photon microscope requires the following: (1) the fly must be kept alive and well for at least 24 hours under the two-photon microscope; it must therefore be fed regularly. (2) An experimental preparation must be devised that allows for access to the brain for several days. Studying neurons involved in the regulation of sleep requires also to think about preserving the circadian rhythmicity of the fly.

In this chapter I will describe how I developed an experimental procedure which allows chronic imaging during sleep-wake cycles of head-fixed flies.

2.1 A setup for a chronic neurobehavioural experiment with head-fixed flies

As shown in Figure 2.1, a two-photon set-up was adapted to perform chronic neurobehavioural recordings.

Sleep is a fundamental behaviour which could be monitored continuously over multiple days. Sleep, considered as extended epochs of immobility lasting for >5 minutes, was monitored using a ball-tracking system. A 6 mm polystyrene ball suspended on a constant humidified air stream was placed close underneath the fly (mounted on a 3-D printed chamber under a 2-photon microscope, with its head and thorax fixed to the chamber and the legs free to move) such that rotation of the ball by the fly's legs simulated walking. The rotation of the air-supported ball was recorded by a GuppyPro camera (equipped with a magnification lens and bandpass

filter to reject visible light and infrared light form the 2-photon laser), at a mean acquisition rate of 22.80Hz. The trackball was illuminated using an infrared LED (Thorlabs) at low intensity. Forward and turning velocities were computed by custom-written software using MATLAB (developed and written by Clifford Talbot). Inactivity was defined as duration of periods where the movement trace did not cross the activity threshold .

The humidity and temperature were carefully controlled to maintain 25C and 60% humidity in the cage, to mimic the conditions of flies used for sleep experiments in the laboratory. A 12h-light:dark-cycle was imposed by using a Thorlabs LED * with a neutral white light (light on 08:00 – light off 20:00; turned off during imaging sessions). The position of the LED was carefully chosen to stimulate the cryptochrome, the main photoreceptor of fruit flies, known to regulate locomotor circadian rhythms and located in the eyes of Drosophila (Jiang et al., 2018).

To minimize external interference, flies were fed without removing them from the experimental setup, while they remain head fixed to the 3-D printed chamber under the objective. I designed a system to deliver liquid food to the fly, in an automatic and reliable way. Using a computer-controlled micromanipulator (XYZ Zaber Technologies): first, the air-supported ball was removed; subsequently, droplets of holidic food medium (presented in the following section) were delivered close to the fly. This process was controlled using custom-written software in LabView and was monitored using a GuppyPro camera controlled by AVT SmarView software.

For each fly tested in the long-term imaging experiments a video capturing a lateral view of the fly and the track ball was recorded. The videos lasted for the entire duration of the experiment and were recorded at a mean acquisition rate of 22.8 Hz. This translates into a (maximum) yeld of some 10^7 images per fly.

* LED Output Power 740mW/880mW - Irradiance 7.7 µW/mm2 – Viewing Angle 150°

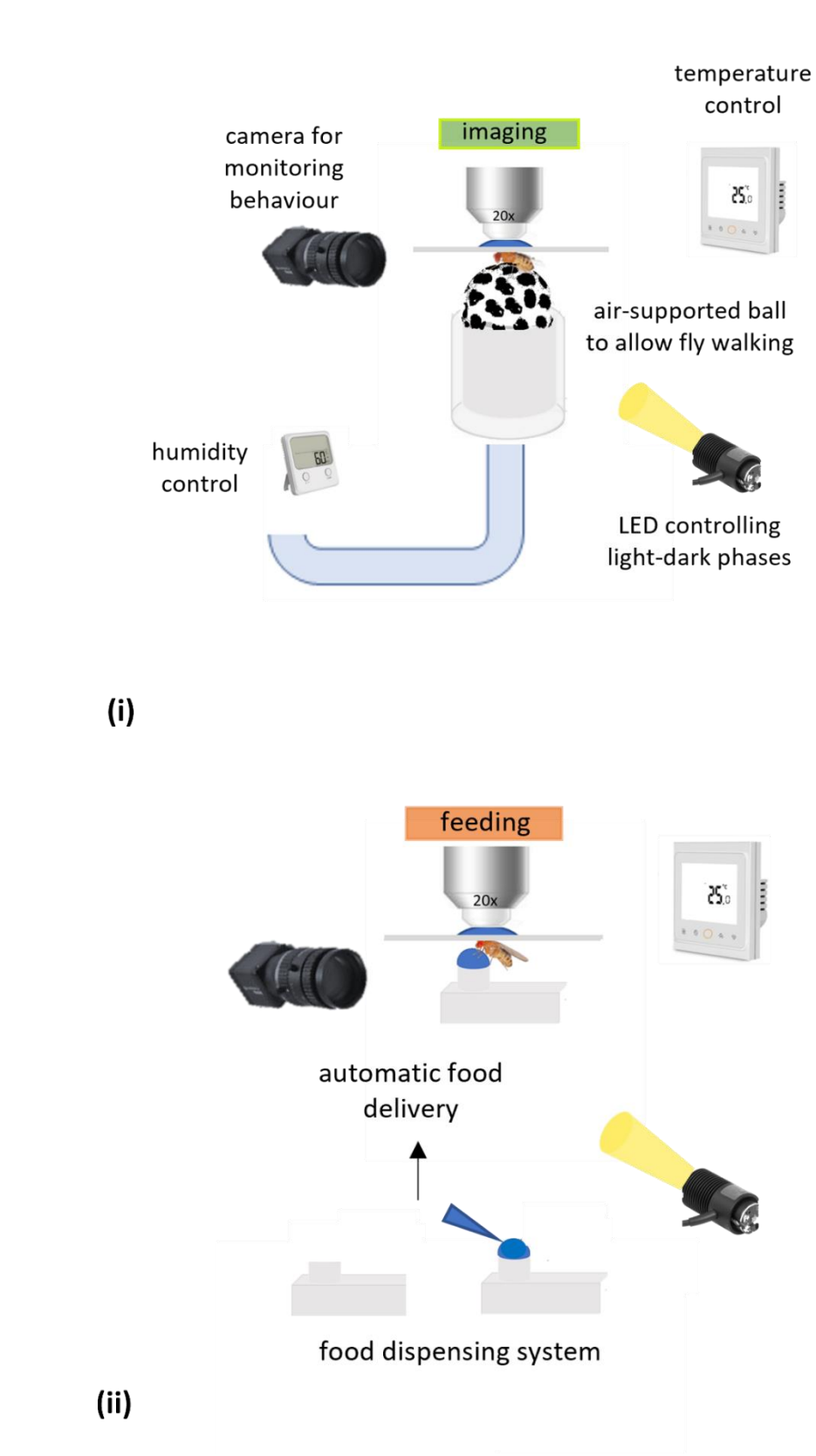


Figure 3.1 Adapting a two-photon setup for chronic behavioural recordings under the microscope

2.1 Adapting a two-photon setup for chronic behavioural recordings under the microscope.

(A) Schematic of the apparatus to monitor the locomotor behaviour and feeding the fly. **(i)** In the two-photon microscope we placed the following: an LED controlling light-dark phases (Thorlabs, 400-700nm wavelength), a temperature and humidity controller (ThermoFisherScientific), a camera (GuppyPro). The fly is head-fixed, and the 3D-printed chamber (custom-made) allows the exposed fly brain to be separated from intact legs and eyes, allowing stimulation, feeding and walking on the ball. The small air-supported ball floats on a stream of humidified air. The house air is filtered (La-Man 520b and Wilkerson M03-02-MX0) and regulates by two pressure regulators (RS 703-6104). The air passes the valve, passes through a regulator flowmeter (Key Instruments, MR3A14BVBN) and then a bottle containing water. The ball holder is 3D-printed and custom-made, it has a hemispherical bowl for the ball. The schematic shows the 20X, 1.0 NA multiphoton water immersion objective (W-Plan-Apochromat, Zeiss). **(ii)** We used a 3D-printed holder (custom-made) for the automatic delivery of the food. The holder has a bowl to collect a drop (~10µL) of liquid. Adult flies eat approx. between 1.3 and 2.3 microliter/day and the rate of ingestion varies during the 24h, with 2/3 of the daily ingestion occurring during the light period (Ja et al., 2007). We used a camera (GuppyPro) to watch the fly and position the food in front of the proboscis. The fly could reach the holder with its legs and easily reach the food extending its proboscis. The holder for the food and the holder for the ball are moved thanks to a XYZstage (Zaber), fine movements of adjustment are made manually using the controls of a micromanipulator.Both holders are robust and reusable.

2.1.1 A feeding system for a fly in the setup

One of the main challenges of chronic imaging experiments is feeding the experimental fly while it lives under the two-photon microscope. During the entire length of the experiment, the fly's head and thorax are fixed. While the fly can move its proboscis towards a source of food, it cannot move around looking for food, nor decide freely when it is time to eat. Therefore, the fly food should meet certain criteria: it should be easy to access for the fly with natural proboscis extensions; the food should be easily delivered in the experimental set-up without disturbing the fly; the protocol should allow for maintaining the fly healthy between two feeding sessions; the protocol should offer the possibility to monitor food ingestion in the fly, in an easy and straight-forward way. "Standard" medium used to maintain *Drosophila* in laboratories is a combination of potato starch, cornmeal, corn syrup, mal, molasses, banana, rolled oats, beet syrup, treacle and one of a variety of dead or fresh live yeasts (Piper et al., 2014). Even though these food recipes contain the components that flies need to survive and reproduce, they do not represent a solution for chronic imaging experiments. For chronic experiments under the microscope, I modified a recipe of Matthew D W Piper and colleagues (Figure 2.2) for an "holidic" medium (hm) that supports adult lifespan and egg laying to the same extent as oligidic food and increases the bioavailability of drugs (Piper et al., 2014).

A

	Ingredient	Stock	Amount per litre	note
Base	Buffer	10x: 30ml/l glacial acetic acid 30g/l KH_2PO_4 10g/l $NaHCO_3$	100ml	Filter solution In sterile conditions
Sugar	Sucrose		17.2g	
Amino Acids	L-isoleucine L-leucine L-tyrosine		1.82g 1.21g 0.42g	
Metal ions	$CaCl_2$ $6H_2O$ $CuSO_45H_2O$ $FeSO_47H_2O$ $MgSO_4$ $MnCl_2$ $4H_2O$ $ZnSO_4$ $7H_2O$	1,000x:250 g/l 1,000x:2,5 g/l 1,000x:25 g/l 1,000x:250 g/l 1,000x:1 g/l 1,000x:25 g/l	1ml 1ml 1ml 1ml 1ml 1ml	
Cholesterol	Cholesterol	20mg/ml in EtOH	5 ml	
	Water (milliQ)		1 l –V1	V1 is the sum of all the following volumes
Autoclave 15 min/120°C	**For the passages below**:	**use sterile conditions**	**All steps:**	**constant stirring**
Amino acids	Essential amino acid stock solution	8g/l L-arginine 10g/l L-histidine 19g/l L-lysine (HCl) 8g/l L-methionine 13g/l L-phenylamine 20g/l L-threonine 5g/l L-tryptophan 28g/l L-valine	60.51ml	
	Nonessential amino acid stock solution	35g/l L-alanine 17g/l L-asparagine 17g/l L-aspartic acid 1g/l L-cysteine HCl 25g/l L-glutamine 32g/l glycine 15g/l L-proline 19g/l L-serine	60.51ml	Add one at the time with just ¾ of the total volume. Adjust pH with 90%of volume and then add liquid to reach the total volume.
	Sodium glutamate Stock solution	100g/l sodium glutamate	15.13ml	
Vitamins	Vitamin solution	125x: 0.1g/l thiamine (aneurin) 0.05g/l riboflavin 0.6g/l nicotinic acid 0.775g/l Ca pantothenate 0.125g/l pyridoxine (HCl) 0.01g/l biotin	14ml	
	Sodium folate	1,000x:0.5 g/l	1ml	
Other nutrients		125x: 6.25g/l choline chloride 0.63g/l myo-inositol 8.13g/l inosine 7.5g/l uridine	8ml	
Preservatives	Propionic acid Nipagin	110g/l methyl 4-hydroxybenzoate in 95%EtOH	6ml 15ml	
Storage:	**4°C /dark room**	**Best to aliquot**		

Figure 2.2 A holidic medium

2.2 A liquid holidic medium

(A) Table with the ingredients for the liquid holidic medium (*hm*). Notes are important for a correct preparation of the food and for ensuring the fly has all the nutrients it needs. Doses are for 1L *hm*.

With experiments of video-tracking, I tested flies placed in tubes with either hm or “standard” brown food to measure any difference in sleep pattern. As shown in Figure 2.3, there is neither a difference in the sleep pattern during a 24-hour day(Figure 2.3,A) nor is there a change in the total sleep of the two groups of flies (Figure 2.3,B). Lifespan was also not affected (data not shown).

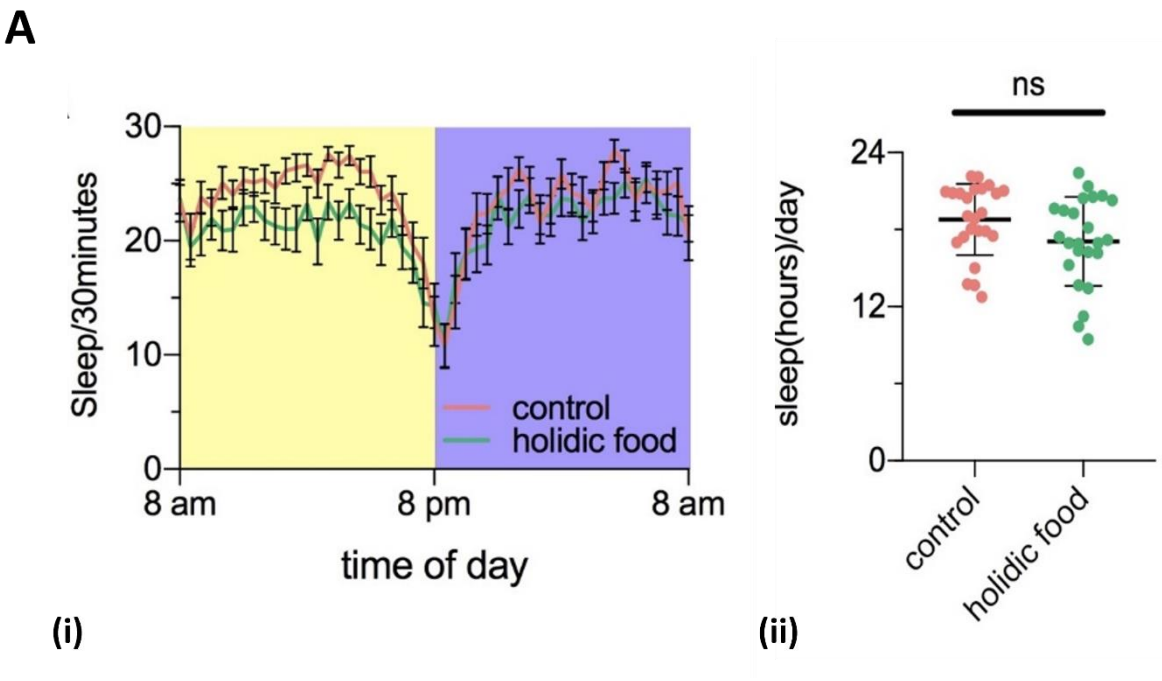


Figure 2.3 Holidic food diet does not alter sleep pattern nor the amount of sleep

(A) Sleep during a 24-hour day in flies fed with holidic medium (green, *n*=26 flies) or with brown food as a control (salmon, *n*=24 flies). Data are means ± s.e.m. Two-way repeated-measures ANOVA of the hour-by-hour sleep time course detected no significant difference between flies fed with standard food and flies fed with holidic medium (P=0.9336). **(B)** Total sleep/day in control and experimental flies (circles denote individual flies; horizontal lines indicate group means). Mann-Whitney test detected no significant effect in the total sleep/day (P=0.720).

Holidic medium can be kept solid for normal fly maintenance and liquid when needed for experiments, by changing the concentration of agar in the medium. Liquid food gives the advantage to monitor food ingestion easily. As previously mentioned, a video camera (GuppyPro) was positioned in the set-up to monitor proboscis movements and the rate of success in delivering the source of fly food. As shown in Figure 2.4,A (i-iv), flies interact with the liquid food presented with the automatic system approach, extending the proboscis, and eventually ingesting liquid hm, with consequent enlargement of the abdomen. Figure 2.4,A (iv) shows the abdomen with blue food dye colouring after feeding. Flies that respond well to the automatic feeding, with a constant positive interaction with hm, have a better chance of being healthy throughout the chronic imaging experiment and are more reliable for studies of homeostatic processes, such as sleep homeostasis (data not shown). A schematic protocol for feeding the fly during the chronic imaging is shown in Figure 2.4,B. Food availability controls the animal's "circadian behaviour"(Dreyer et al., 2019) and the circadian system fails to control normal rhythmicity when animals are stressed due to absence of food (Dreyer et al., 2019). Accordingly, I observed hyperactive behaviour when flies were kept without food for more than 4 hours during the day and 6 hours during the night (not shown). I decided to feed flies at regular intervals of 3 hours during the day, to let the fly undisturbed as much as possible, and leave the fly without food during night for 6 hours. The time points where the fly is fed are decided according to the known sleep pattern and the 12 hours of light: darkness, considering that when the fly is normally more active consumes and requires more energy.

I tested flies for several positive interactions with the food, activity and grooming before and after each feeding session. I determined a proboscis extension index

(P.I.) to quantify the response of flies to the holidic diet and the feeding protocol (Figure 2.4,C).

The interaction of the flies with the liquid food when presented with the automatic feeding is not always constant. Some flies need multiple presentations of the stimulus to naturally extend their proboscis. Flies that extend their proboscis for long time during a feeding interval do not always respond similarly during the following feeding interval (Figure 2.4,C).

While overall flies respond well to the holidic diet and the automatic feeding over the duration of the protocol, the differences in flies' food behaviour suggested the need for a more selective procedure prior the chronic neurobehavioural experiment. Only healthy flies that respond consistently to the automatic feeding, for at least three feeding sessions, should be chosen for chronic recordings.

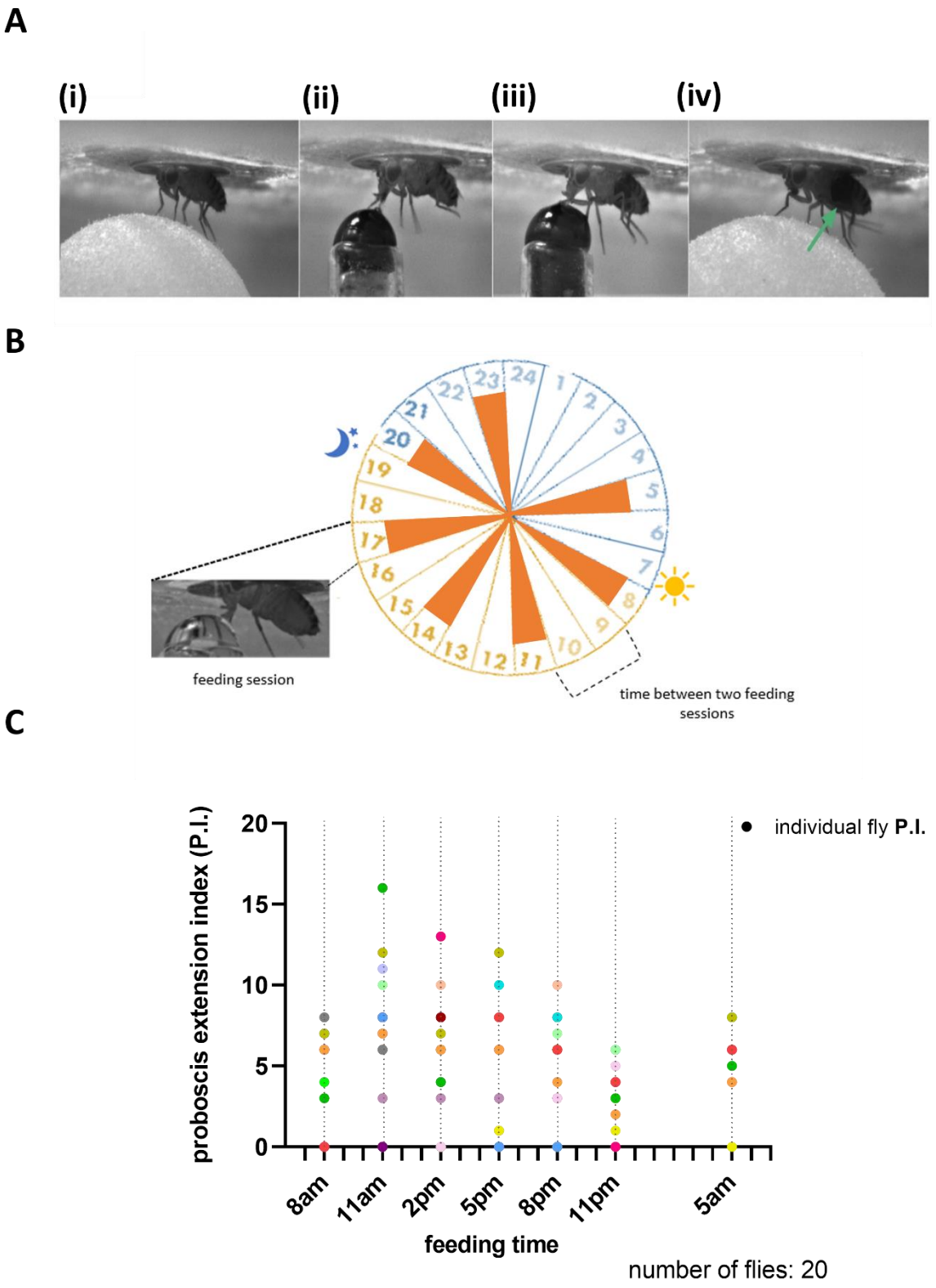


Figure 2.4 Over 24h, head-fixed flies respond well to liquid holidic diet under a 2-P microscope.

2.4 Over 24h, head-fixed flies respond well to liquid holidic diet under a 2-P

(A) Head-fixed fly under the two-photon microscope while successfully feeding. Holidic food is coloured with Brilliant Blue dye. **(i)** before food presentation, **(iv)** after ingestion of food: (iv) the dark spot on the abdomen of the fly, caused by food ingestion. **(B)** Feeding protocol used to test head-fixed flies under the two-photon microscope. Seven feeding sessions, marked with the orange triangle. Interval between feeding sessions is 3h, except for the interval between the last feeding session of the day at midnight and the first session of the day at 6am, which is of 6h. **(C)** Flies respond differently to food presentation: some with long proboscis extensions, others with short and repetitive or a combination of long and short extensions and some with no extensions. These different responses are represented by the “proboscis extension index”. For each session of feeding, the score of the proboscis extension index was measured taking in consideration the following values: 3 for each long extension, 1 for each short extension. For each timepoint, each dot refers to the score of one individual fly. Different scores for different trials for the same fly could be tracked looking at the colours. Each fly has a specific colour.

As shown in Figure 2.5, head-fixed flies survive for days under the two-photon microscope, showing bouts of inactivity alternated with activity. Figure 2.5,A shows a movement trace of an example-fly that lived under the microscope for days. To quantify the extent of these bouts, the trace was binarized, as shown in Figure 2.5,A (binarized trace in blue). The trace was smoothed by computing the moving average of each bin using a sliding-window of 10 time-bins. This helped remove small deflections that could be attributed to noise and not to fly activity . An empirically determined threshold of 2.5 (arbitrary units) was used to binarize this trace. This decision was necessary since when the fly is not walking the software still records a signal that could be interpreted as movement. Only when the fly is walking the acceleration of the ball cause a significant increase in the intensity of the signal. The threshold of 2.5 represents the minimal acceleration that defines fly's movement. A signal above 2.5 identifies the bouts of activity. Following convention, sleep was defined as inactivity of at least five minutes.

When looking at the sleep profile in flies that are not head-fixed scientists have relied on averages of many flies. While these averages show a consistent pattern of circadian rhythmicity, this does not necessarily hold true for individual flies and considering individual days. I compared the sleep profile of head-fixed flies with the one of flies placed in tubes for video tracking. Figure 2.5,B presents the sleep profile of a single head-fixed fly over days, while Figure 2.5,C shows the sleep profile of control flies (monitored using the DAM system, see chapter 6). Note the variability between days both in control flies and head-fixed flies. Despite this variability, all flies show an increased locomotor activity when the light phase ends (8pm).

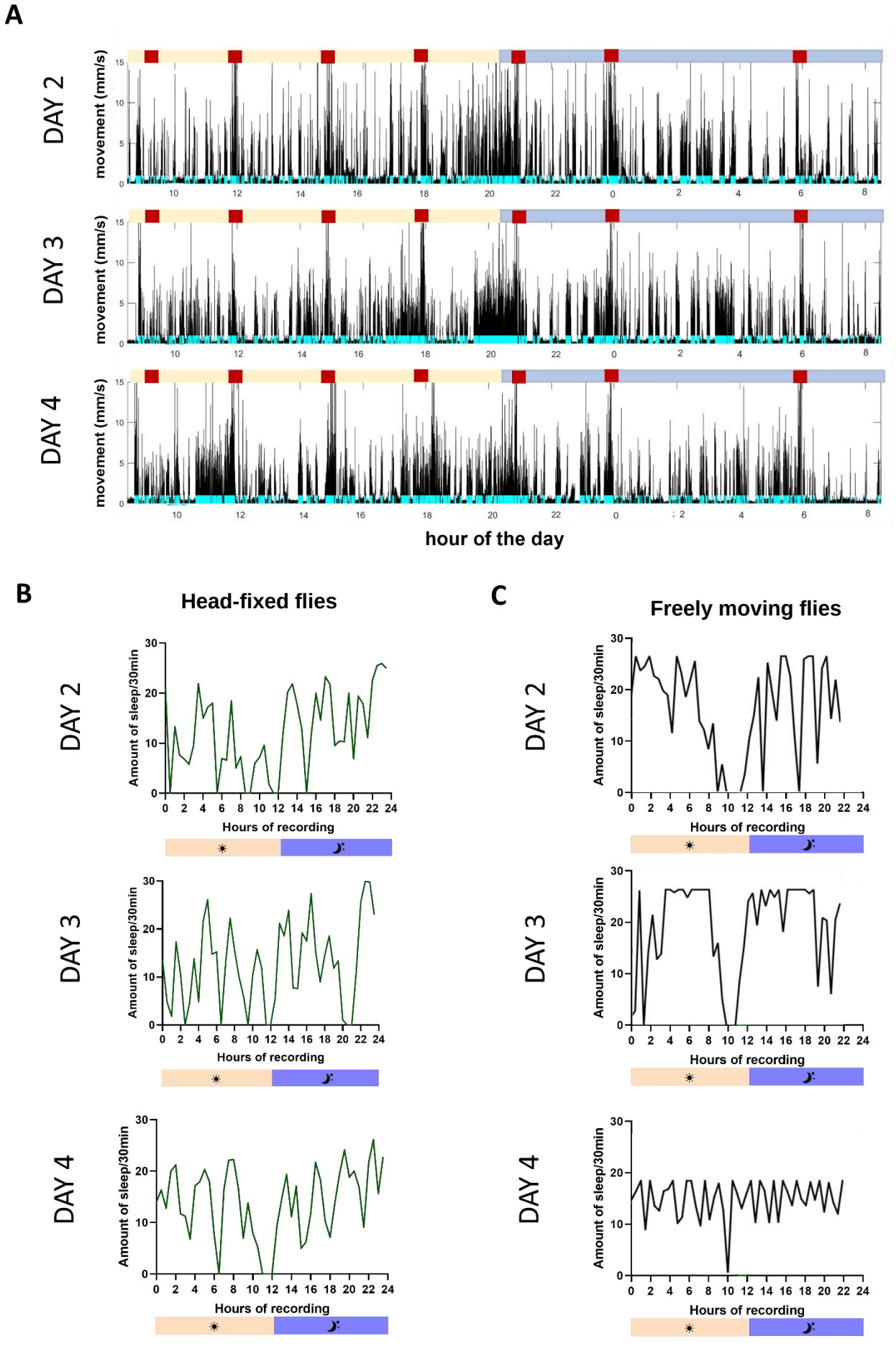


Figure 2.5 Head-fixed flies respond well to liquid holidic diet and automatic feeding

2.5 Head-fixed flies respond well to liquid holidic diet and automatic feeding

(A) Locomotor trace from ball-tracking in consecutive days. On the *y* axes of the three graphics, the speed measured as millimetres per second. On the *x* axes, the hours of the day (from 8am, when lights are switched ON, to 8am, 24h later). The yellow bar on the top of each graph indicates the daytime, the blue bar the night-time. Lights switch OFF at 8pm. The red squares along the bars represent the feeding intervals. Here are represented only the days in-between the first and last day. One single fly is considered. In each of the three graphics you see the raw trace in black, which reflects the rotation of the ball, and a blue trace, after we applied a threshold to the raw trace. The threshold is standardized for all the experiments. The bouts of activity are in blue. The trace in blue is then used to distinguish the individual sleep bouts (inactivity>5min). **(B)** Sleep profile extracted from the locomotor trace of three consecutive days. On the *y* axes of the three graphics, the amount of sleep every 30minutes. On the *x* axes, the hours of the day (day starts at 8am and lasts 24h). The bars below each graphics indicate respectively the day and the night (lights switch off at 8pm). The trend of the green line indicates the trend of the amount of sleep during 24h. The three days are relative to one single experiment with one fly. **(C)** Video-tracking of a freely moving fly in a single glass tube (5mm diameter) over consecutive days. Video-tracking is explained in the last chapter of this work. Here three sleep profiles from three consecutive days are used as an example for comparing them with the sleep profiles of head-fixed flies living for days under a two-photon microscope. To appreciate the increase of activity at the end of the light phase and the variability in the amount of sleep between days. On the *y* axes of the three graphics, the amount of sleep every 30minutes. On the *x* axes, the hours of the day (day starts at 8am and lasts 24h). The bars below each graphics indicate respectively the day and the night (lights switch off at 8pm).

As shown in figure 2.6, A and B, sleep quantity in head-fixed flies living for days under a 2-photon microscope does not change from control flies. Sleep during the night is higher than sleep during the day. Sleep quality is defined by the variation in number and length of sleep bouts. Figure 2.6, C and D, shows that the duration of every sleep-bout change during the day as well as the number of sleep-bouts. In both groups, sleep bouts are longer when lights are off, and the number of sleep bouts decreases during the day.

The developed feeding system allows the survival of head-fixed flies for days and preserves the circadian rhythmicity of their locomotor activity.

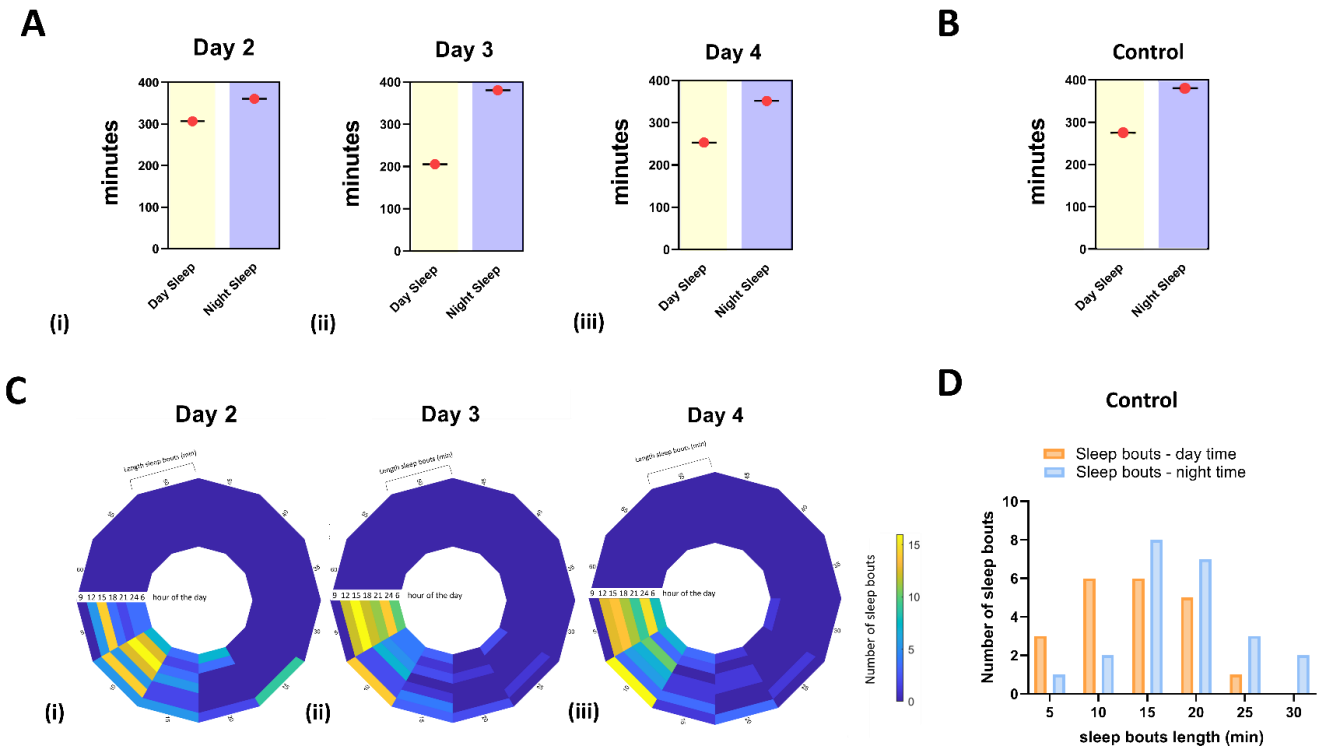


Figure 2.6 Flies survive for days under a two-photon microscope and show alternation of sleep and wake

2.6 Flies survive for days under a two-photon microscope and show alternation of sleep and wake

(A) Sleep quantity during the day and the night in consecutive days. These data come from tracking the ball movements under the two-photon microscope. For one single fly from **(i)** to **(iii)** to appreciate the consistency in the different amount of sleep during daytime and night-time. On the y axes, the total amount of sleep measured in minutes. **(B)** Average sleep quantity during the day and the night for a control fly. The data come from a video-tracking experiment (more about the video-tracking experiment in the last chapter of this work). **(C)** Sleep quality measured as length of sleep bouts in consecutive days. The three histograms show the number of sleep bouts of different length in different time intervals during a day (24h). The numbers in the external ring of each histogram indicate the minutes a sleep bout would last. The colours indicate the number of sleep bouts of that specific length. From blue, 0 sleep bouts of that length, to yellow, 15 sleep bouts of that length. The time intervals correspond to the time in-between feedings. For example, the outer ring refers to the time that goes from 6am to 9am. The following ring, the time that goes from 9am till 12. To appreciate the variation of sleep bouts length during a single day and the different length of sleep bouts at the beginning of the day and at the endo of the day. The data are from one single fly, living under a two-photon microscope. **(D)** Sleep quality measured as length of sleep bouts in consecutive days. The data refer to a single fly during a video-tracking experiment. The data are the result of averaging between three days. On the y axis the number of sleep bouts. On the x axis the length of sleep bouts in minutes. To appreciate the difference in sleep bouts length between the first part of the day and the last part of the day.

2.1.2 A chronic fly preparation

The challenge for chronic imaging *in vivo* is creating a chronic fly preparation for repeated imaging of neural dynamics. The opaque exoskeleton of the fruit fly demands the use of microsurgery to gain access to the central nervous system (Huang et al., 2018). *Drosophila* would not stay healthy during a two-day imaging-session with an open brain window. Long-term exposure (over several days) of the exposed brain extracellular solution used for imaging experiments may affect the flies' health. Another difference between conventional imaging approaches and long-term imaging experiments is the need to preserve the movement of the proboscis for feeding over multiple days. In conventional imaging experiments, to reduce movement artifacts the proboscis is fixed. Moreover, a recent study suggested that a deep sleep stage in Drosophila requires proboscis extensions (van Alphen et al., 2013), that should be allowed if interested in the neural correlates of sleep homeostasis.

To overcome these difficulties and enable longitudinal imaging studies, I sealed the brain window with a transparent optical adhesive. To identify the adhesive that matches the needs of the experiment in terms of refractive index, viscosity and hardness, different glues were tested. The criteria for deciding between the adhesive included the quality of the sealed brain window, recovery rate of the fly after surgery, and quality of imaging (signal-to-noise ratio, movement of the brain underneath the sealed glue window). I tested the low toxicity silicone adhesive Kwik-Sil (World Precision Instrument), the UV-curable glue NOA68, NOA61, NOA63, NOA83 and NOA1385 (Norland Optics). Kwik-Sil does not solidify properly and consistently, with the result that a lot of flies do not survive the surgery. Moreover, the quality of

the imaging window was never good enough for chronic imaging a deep brain structure (*e.g*., dFB neurons). I selected the optical adhesive NOA13825 (Norland Optics). NOA68, NOA61 and NOA63 failed to provide a brain window clear enough to identify the neuronal structures expressing GCaMP6f and tdTomato. In addition, the curing process using these glues was extremely slow and time-consuming. NOA13825 has a refractive index close to that of water, cures quickly, is solid and stabilizes the brain, which improved some limitations of previous approaches using other Norland adhesives. NOA13825 let me image the dFB axons, located deep into the brain. However, because the chosen adhesive displays oxygen inhibition, it had to be cured in an inert atmosphere. It was therefore cured in a custom-made enclosed chamber that was perfused with CO2 (the overall curing procedure and, thus, the flies' exposure to the CO2 lasted no longer than 45 seconds). The curing process occurred using a UV gun (Opticure 2000 UV lamp, Norland). Adhesive, UV gun and CO2 chamber are shown in Figure 2.7,A. Figure 2.7,B shows an example of the chronic preparation: in Figure 2.7,B (i) the fly before the application of the glue, in Figure 2.7,B (ii) the fly brain window after the application of the glue. Figure 2.7,B (iii) and (iv) show the position of the fly in the chamber on the day of the chronic imaging experiment, the sealed brain window has maintained its integrity.

The procedure has no noticeable effect on sleep-wake profiles of flies. Flies with chronic preparation and control flies placed in tubes for video tracking of locomotor activity show similar sleep pattern (Figure 2.7,C) and average sleep/day (Figure 2.7,D).

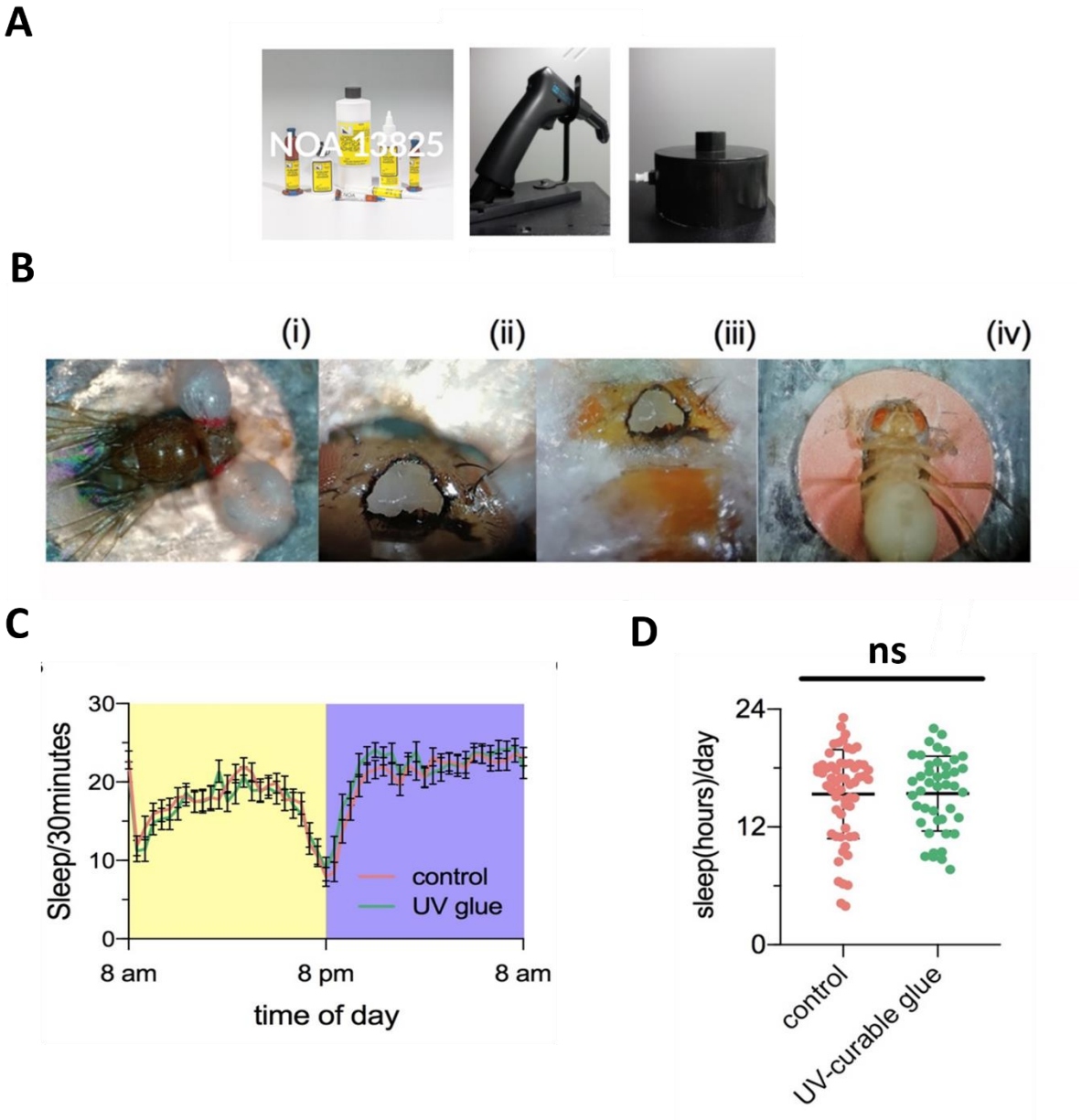


Figure 2.7 A chronic imaging preparation does not affect the physiological sleep pattern of the flies

2.7 A chronic imaging preparation does not affect the physiological sleep pattern of the flies

(A) Chronic imaging preparations require: a UV-curable glue, a UV gun, an isolated chamber for CO_2 diffusion. The glue is stored in a dark place at room temperature. Once opened, each bottle has a shelf life of three months. The UV gun needs a battery, and to be charged before its use. The chamber allows to have a space with limited oxygen, thanks to the constant flow of CO_2 into the chamber. The fly is placed in the chamber for the curing process. The gun is introduced from the hole on the top of the chamber, while the CO_2 flows from the small hole on the side (on the left in the picture ,in white) **(B)** Flies are prepared for chronic imaging: **(i)** creation of a brain window (~250 × 200 μm^2) **(ii)** sealing of brain window with UV-curable glue **(iii)** fixing of the experimental fly on a chamber after two-three days of recovery from surgery **(iv)** the experimental fly is free to move legs and antennae, proboscis and eyes are free from vax. **(C)** Sleep during a day (24h) in flies with brain window sealed with UV-curable glue (green, *n*=43 flies) or without (salmon, *n*=59 flies). Data are means ± s.e.m. Two-way repeated-measures ANOVA of the hour-by-hour sleep time course detected no significant difference between flies with and without brain window (P=0.9333). **(D)** Total sleep/day in control and experimental flies (circles denote individual flies, horizontal lines indicate group means). Mann-Whitney test test detected no significant effect in the total sleep/day (P=0.8956).

2.2 A timeline to record for days the neurobehavioural dynamics of Drosophila

Figure 2.8 shows the timeline to record for days the neurobehavioural dynamics of *Drosophila*. 2-3 days prior to the experiment, 3-4 day-old cold-anaesthetized female flies were placed into a custom mount and head-fixed using eicosane (Sigma). Cuticle, adipose tissue, and trachea above the brain were manually removed, and the resulting window is sealed using a thin layer of translucent UV-curable glue. Flies were allowed to recover from the procedure for approximately 20-40 minutes before they were removed from the mount and placed individually into vials for 2-3 days until the start of the experiments. On the recording day, flies were first pre-screened visually for gross health deficits. Healthy flies were cold-anaesthetized for at least 30 minutes prior to the following selection process: the fly displaying the highest imaging quality (negligible brain movement, high baseline fluorescence intensity in red and green channel – see chapter 3) and health (size and activity of the fly) is selected. Moreover, as mentioned in the previous section, the ability of the fly to feed under the microscope was tested before the initiation of the chronic experiment (i.e., its propensity to extend its proboscis to reach food droplet). While this selection is clearly not blind, it is unlikely that a selection bias was introduced, because the selection criteria pertained exclusively the general health of the fly and stability of the imaging preparation, not to behavioural or neural activity. I am not aware of any behavioural phenotype that could have been implicitly selected for by choosing flies that extended their proboscis more readily.

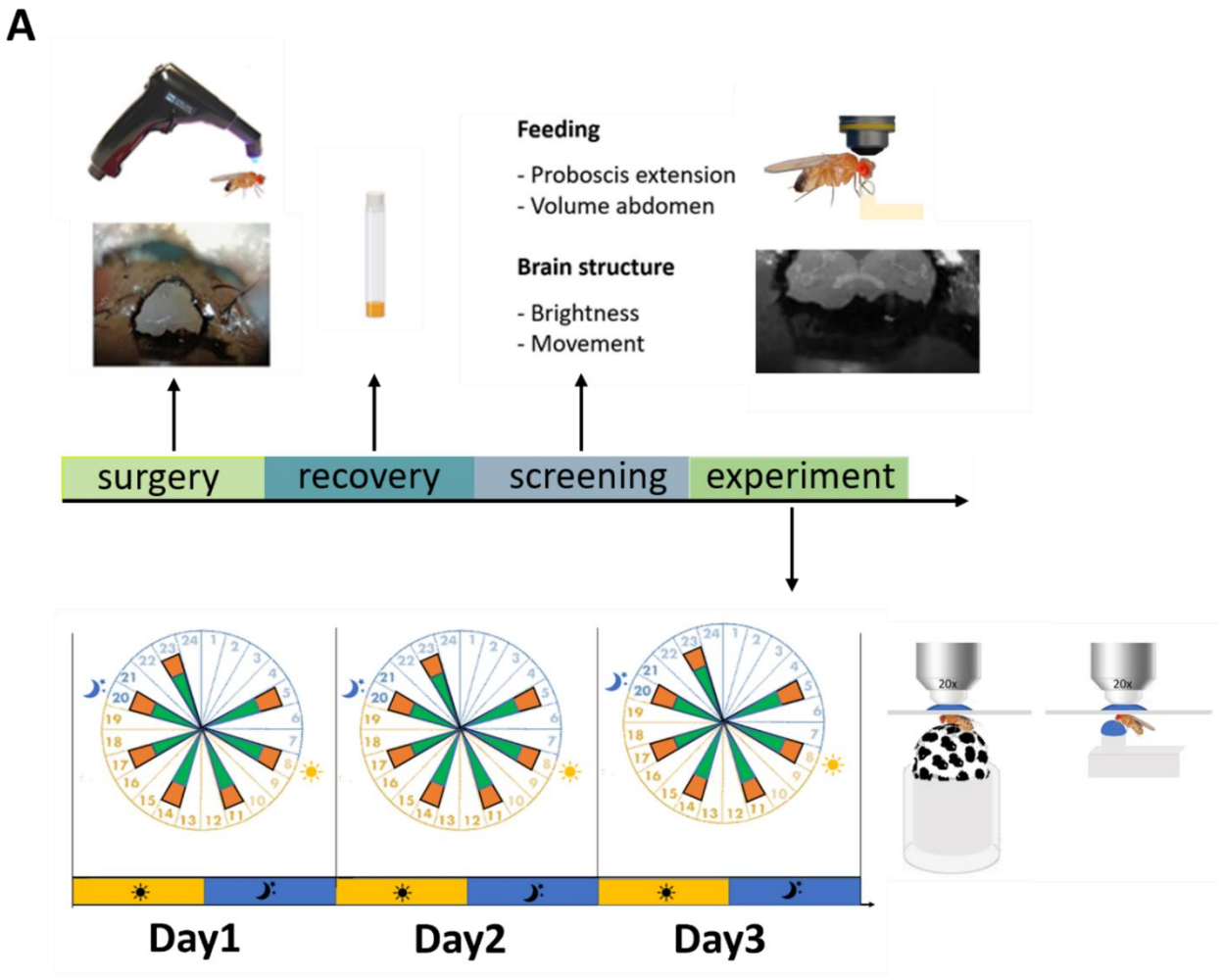


Figure 2.8 A timeline for chronic monitoring of flies under a two-photon microscope

(A) The timeline in four stages: 1) the surgery 2) the recovery 3) the testing 4) the experiment. The surgery starts with 3 days old flies. Flies are placed in vials alone for recovery, for approximately two days. Testing usually occurs on the third day after the surgery. The fly which passes the examination goes into the experimental stage. In the schematic, the protocol for imaging (green segments) and for feeding (orange segments).

This procedure allowed chronic neurobehavioural monitoring of individual flies for up to 7 days. As shown in Figure 2.9, sleep quantity and quality were not altered during the entire length of the experiment (a recording of one single fly over days). The average sleep profile of a head-fixed fly with a sealed brain window (Figure 2.9,A (i)) shows how the procedure does not affect the circadian rhythmicity of the flies, with increased locomotor activity closer to the time when lights go off. Control flies, head-fixed and without brain window, show a similar sleep profile (Figure 2.9,B (i)). Experimental flies sleep more at night (Figure 2.9, A (ii) and B (ii)) and have fewer and shorter sleep bouts during the day (Figure2.9,A (iii) and B (iii)) , like control flies.

Considering different days of the chronic neurobehavioural experiment, sleep quantity and quality show a physiological range of variation (Figure 2.9, E).

That said, as shown in Supplementary Figure1, if flies are fed only at certain times of the day and the food is not available ad libitum the feeding interval become a zeitgeber. While the variability between daytime sleep and nighttime remains constant, the total sleep is generally lower in flies fed in specific intervals under the two-photon microscope than control flies fed ad libitum in glass tubes. Moreover, sleep seems to occur more frequently after the each feeding interval.

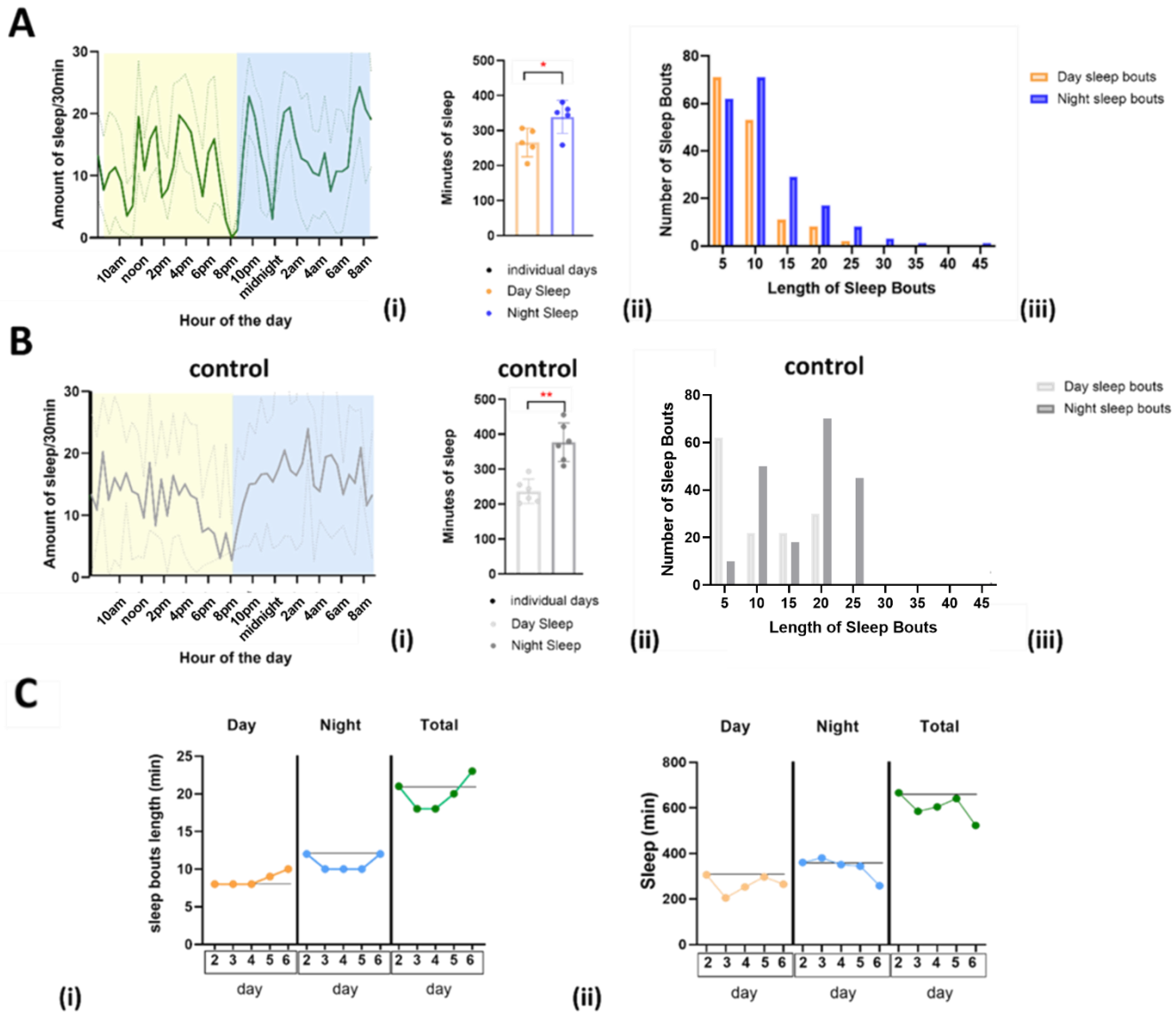


Figure 2.9 A chronic imaging experiment does not affect sleep quantity and quality

2.9 A chronic imaging experiment does not affect sleep quantity and quality

(A) Sleep of a fly living for seven days under a two-photon microscope. First and last day has been excluded **(i)** The thick line represents the average sleep profile over 7 days. Dotted lines represent the variation in the sleep profile among different days. On the y axis, the total amount of sleep every 30 minutes. On the x axis, the hours of the day, starting from 8am (lights ON at 8am and OFF at 8pm). **(ii)** Quantity of sleep during the day and during the night. Each dot represents individual days. Total sleep is measured in minutes. To appreciate the difference between day and night. Paired *t*-test detected a significant difference (P<0.05*). Error bars are ± s.e.m. **(iii)** Quality of sleep measured as length of sleep bouts in consecutive days. To appreciate the difference between day and night. Longer sleep bouts occur at night. **(B)** Sleep of a fly living for seven days in a glass tube for a video-tracking experiment. The first day has been excluded. (i) Sleep profile. On the y axis, the total amount of sleep every 30 minutes. On the x axis, the hours of the day, starting from 8am (lights ON at 8am and OFF at 8pm). Thick line represents the average sleep profile over 6 days. Dotted lines represent the variation in the sleep profile over days. **(ii)** Quantity of sleep during the day and during the night. Each dot represents individual days. Total sleep is measured in minutes. To appreciate the difference between day and night. paired *t*-test detected a significant difference (P<0.01**). Error bars are ± s.e.m. **(iii)** Quality of sleep measured as length of sleep bouts in consecutive days. To appreciate the difference between day and night. Longer sleep bouts occur at night. **(E)** Variation over consecutive days of sleep quality and quantity. Data from a single fly living for days under a two-photon microscope. Each dot represents the average value for that single day. **(i)** On the y axis, the sleep bouts length measured in minutes. On the x axis the consecutive days. Day 1 has been excluded. Total sleep represents the average sleep bout length in 24h. To appreciate the consolidation of sleep quality over the days. **(ii)** On the y axis, the amount of sleep measured in minutes. On the x axis the consecutive days. Total sleep represents the average sleep in 24h.

Overall, an invasive imaging protocol influences the behaviour during these long-term recordings. I tested two different imaging protocols. Protocol A has 9 imaging sessions in every 24h-period, which in many cases happen one hour before or after feeding sessions (Figure 2.10, A (i)). Protocol B has 7 imaging sessions in every 24h-period, which precede the feeding sessions (Figure 2.10, B (i)). While total sleep does not change using either Protocol A or Protocol B (Figure 2.10,C) and the sleep profile maintains its circadian rhythmicity (Figure 2.10, A (ii) and B (ii)), sleep seems more consolidated when using Protocol B (longer sleep bouts). The data presented in the following chapters were collected using both Protocol A and Protocol B.

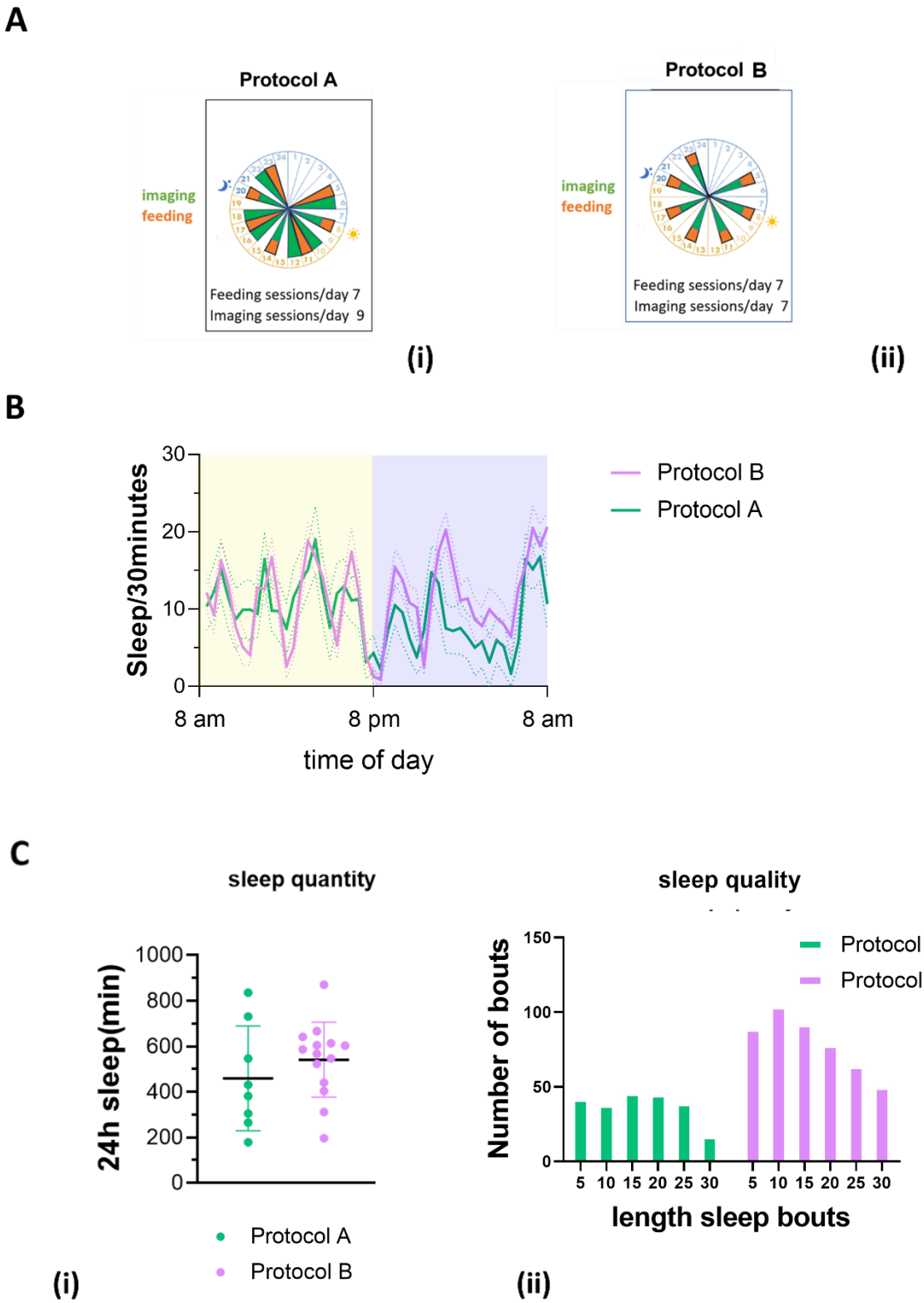


Figure 2.10 A less disruptive imaging protocol helps flies to have a more consolidated sleep

2.10 A less disruptive imaging protocol helps flies to have a more consolidated sleep

(A) Schematic of *Protocol A* in **(i)**. There are 7 feeding sessions and 9 imaging sessions. The imaging sessions are every other hour. The first imaging session is at 6am and the last one at 10pm. Schematic of *Protocol B* in **(ii)**. There are 7 feeding sessions and 7 imaging sessions. The imaging sessions are every 3 hours. The first imaging session is at 5am and the last one at 11pm. **(B)** The sleep profile of flies tested with *Protocol A* (*n*= 6, where *n* represents the number of days. Flies=4) and with *Protocol B* (*n*=13, where *n* represents the number of days. Flies = 4).On the y axis, the total amount of sleep every 30 minutes. On the x axis, the hours of the day, starting from 8am (lights ON at 8am and OFF at 8pm). Tick line represents the average sleep. Dotted lines represent error bars. **(C)** In **(i)** the total quantity of sleep for flies tested with *Protocol A* and *Protocol B*. (circles denote individual flies, horizontal lines indicate group means). Kolmogorov-Smirnov test detected no significant effect in the total sleep/day (P=0.970). In **(ii)** sleep quality of flies tested with *Protocol A* and *Protocol B*. On the y axis, the total number of bouts of a specific length. On the x axis, the length of the sleep bouts in minutes (bins are chosen to have a difference of 5 minutes, max and minimum length are also arbitrary).

3 | Chronic imaging of the genetic line 23E10

3.1 Ca^{2+} imaging of 23E10 neurons

Having established an experimental setting that allows for chronic imaging in *Drosophila*, I tested different Ca^{2+} indicators as reporters of neural activity in the dorsal fan-shaped body neurons (dFB). The choice of the probe to measure Ca^{2+} dynamics is important to capture biological meaningful Ca^{2+} events. Different probes can capture different Ca^{2+} transients, which differ in size and temporal course. Using the GAL4/UAS system, I expressed in dFB-neurons: GCaMP6f, GCaMP6s and GCaMP7b, GCaMP7c and GCaMP7f, high -performance GFP-based calcium indicators for imaging activity in neuronal populations (Dana et al., 2019). GCaMP indicators have the general advantage of a favorable dynamic range, partially because of their lower basal fluorescence compared to FRET-based indicators, for example. Single fluorescent protein indicators are also more suitable for long-term assays, due to their reduced potential for photobleaching, the absence of substantial cellular toxicity and negligible perturbation of cellular functions (Whitaker, 2010). I expressed the different probes in different genetic lines, all known to target the dFB region and used to manipulate sleep in *Drosophila* (Donlea et al., 2011; Ueno et al., 2012; Matheson et al., 2021): C5-GAL4, 104y-GAL4, C205-GAL4, 84C10-GAL4, 23E10-GAL4.

My decision of using GCaMP6f in the genetic line 23E10 for chronic imaging was based on the following observations. First, the line is more restricted than the other lines targeting the dFB region, and under the two-photon microscope I could clearly observe the expression of the brighter probes in anatomical defined regions, the dendritic regions (one for each hemisphere), the axons and cell bodies. This was

not the case with the other lines, where the expression of the reporter wasn't clearly defined to distinct sub-regions, except for the line 84C10-GAL4. Moreover, only with the line 23E10-GAL4 and 84C10-GAL4 I could observe rapid fluctuation in the fluorescence, with a pattern of activity clear to the naked eye, when expressing GCaMP6f. I can't exclude that this observation was influenced by the difficulty I had in visualizing clearly the clusters of dendrites and axons of the neurons in all the other lines. Second, the line 23E10 is the preferred line used to record and manipulate the activity of dFB neurons in many laboratories, as well as being the main line used in my laboratory. The decision of using GCaMP6f was instead based on the presence of a Ca^{2+} dynamics observed with the naked eye. GCaMP6f has fast kinetics compared to GCaMP6s, GCaMP7b, GCaMP7c. The fast variants of the GCaMP indicators could report changes in fluorescence within ~50ms. The high sensitivity in detecting Ca^{2+} transients come with lower brightness(Zhang et al., 2021).

In chronic two-photon imaging, photobleaching of the fluorophore can become problematic. Hence, a bright sensor that requires less laser-power is desirable. GCaMP7-indicators are brighter than the previous generation; however, the increased brightness comes with higher Ca^{2+}-affinity, which could result in a lower dynamic range to detect Ca^{2+} fluctuations (Pérez Koldenkova and Nagai, 2013). Using GCaMP7f I failed to detect Ca^{2+} -oscillations, which instead are very evident using GCaMP6f. One explanation could be that using GCaMP7f there is a saturation of Ca^{2+}- binding sites that are already close to saturation.

Imaging artifacts represent one problem occurring when imaging Ca^{2+}-dynamics in neurons. Even slight movements of the cells could lead to a misleading interpretation of Ca^{2+}-traces. To overcome this problem, I decided to express a structural marker in the line 23E10, together with a Ca^{2+}-sensor. Looking for the best combination of

brightness and stability, I tested the red fluorescent proteins mCherry and tdTomato. I chose the membrane-bound tdTomato reporter (R9D11-CD4::tdTomato), for its high levels of brightness and a level of expression in the line 23E10 that could let me observe fine dendrites and axons in the subcellular compartments.

Fluorescence intensity is influenced by sensor expression. Since both UAS-GCaMP6f and UAS-tdTomato are located on the 2nd chromosome, I created and used a recombinant line UAS-GCaMP6f-tdTomato for the chronic imaging experiments. This approach let us use homozygous flies for UAS-GCaMP6f-tdTomato on the 2nd chromosome and homozygous flies for R23E10-GAL4 on the 3rd chromosome.

I chose dendrites of dFB neurons as starting point for our chronic imaging experiments because they are more superficial and much brighter than the axons. That said, collection of axonal data is on-going. When attempting the imaging of the cell bodies of dFB neurons I had difficulties to distinguish the individual cells. Cell bodies of dFB neurons tend to overlap. While a heterogeneity in their activity looked promising, I decided to focus our attention on the input (dendrites) and output (axons) branches of the dFB neurons.

In Figure 3.1,A a schematic of the dFB neurons highlight the different substructures within the 23E10 line. Figure 3.1,B shows the expression of tdTomato and GCaMP6f in dendrites and axons. Figure 3.1,C show a time series of snapshots of activity in one single recording session: rapid Ca^{2+} oscillations, visible to the naked eye.

Durations of individual imaging sessions were limited to 3-minute long “snapshots” of dFB activity, to avoid photo-bleaching and adverse thermal effects due to repeated long recordings. Acquisition rate was 16.68Hz (256 pixels/frame, approx.. 15images per second) and the laser power was set to levels below approx.14 mW (when measured after the objective with a ThorLabs power-meter).

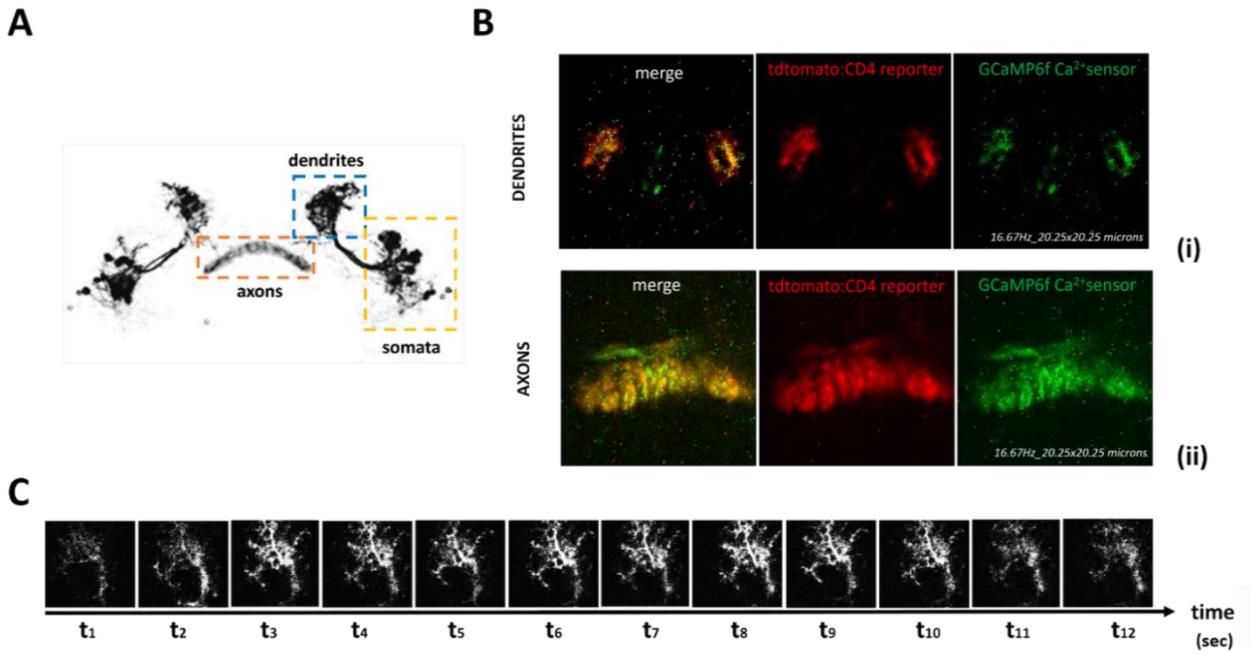


Figure 3.1 Ca^{2+} imaging of 23E10 neurons. Ca^{2+} oscillations are visible to the naked eye

(A) A schematic of the dorsal fan-shaped body (dFB) neurons labelled by the 23E10 genetic line. The orange square indicates the output branch of the neurons (the axons). The blue square indicates the input branch of the neurons (the dendrites). There are two dendritic arbors, one for each hemisphere. The yellow square indicates the area of the cell bodies. Cell bodies are in both hemispheres. **(B)** Fluorescence images of 23E10-GAL4>GCaMP6f-UAS fly brain. Expression of tdTomato:CD4 (the reporter) and GCaMP6f (the sensor) in **(i)** the dendrites and in **(ii)** the axons. Images are 'stacked' to provide a maximal intensity projection. Maximum intensity projection from imaging acquisition using a two-photon microscope (window sealed with UV-curable glue). Resolution at 16.67Hz. 20.25x20.25 microns. **(C)** Example of time-lapse images, time series of images from one recording session of 3 minutes. Time is measured in seconds. Each image is representative of the images taken in one second. Recording at 16.67Hz using a two-photon microscope (256 pixels/frame, approx.15images per second). Region of interest: dendritic arbor of one hemisphere. In white the GCaMP6f signal and in black is the background.

Regions of interest (ROIs) from green and red imaging channels were manually selected, and the mean fluorescence intensities for each frame were extracted using ImageJ. The resulting time-series were then analysed further using custom-written code in MATLAB 2017b. The green-to-red ratiometric signal (dR_t/R_0) was computed as $dR_t/R_0=(R_t-R_0)/R_0$, where $R_t=F^g_t/F^r_t$, and R_0 is the 10^{th} percentile of R_t. Representative imaging traces of normalized (e.g., $dF_t/F_0=(F_t-F_0)/F_0$ with F_0 the 10^{th} percentile of F_t) GCaMP6f, tdTomato and dR/R are shown in Figure 3.2,A. Thanks to the chronic preparation, the fly brain does not move significantly and the tdTomato signal looks stable, although this was not always the case. In Figure 3.2,B (i) it is possible to appreciate how the ratiometric signal is a good representation of the Ca^{2+}-dynamics in the ROI. For further analysis of comparison between different traces I used the mean fluorescence of the ratiometric traces (mean dR/R). To validate the use of the mean dR/R, I calculated the standard deviation (SD) and the root mean square (RMS) for each trace and compare them with the mean dR/R (Figure 3.2,B (ii)).

As is evident from the representative traces in Figure 3.2, I detected transient changes in GCaMP fluorescence (GCaMP-transients), this was true both in dendrites and axons. The mean fluorescence of a trace is just one of the features that could be extracted from the traces. Using the *findpeaks* functions in MATLAB, adapted to the set of data collected, I programmatically identified: 1) the local maxima, or peaks in each trace (corresponding to each transient), 2) the peaks width, 3) the interval between peaks (Figure 3.2,C).

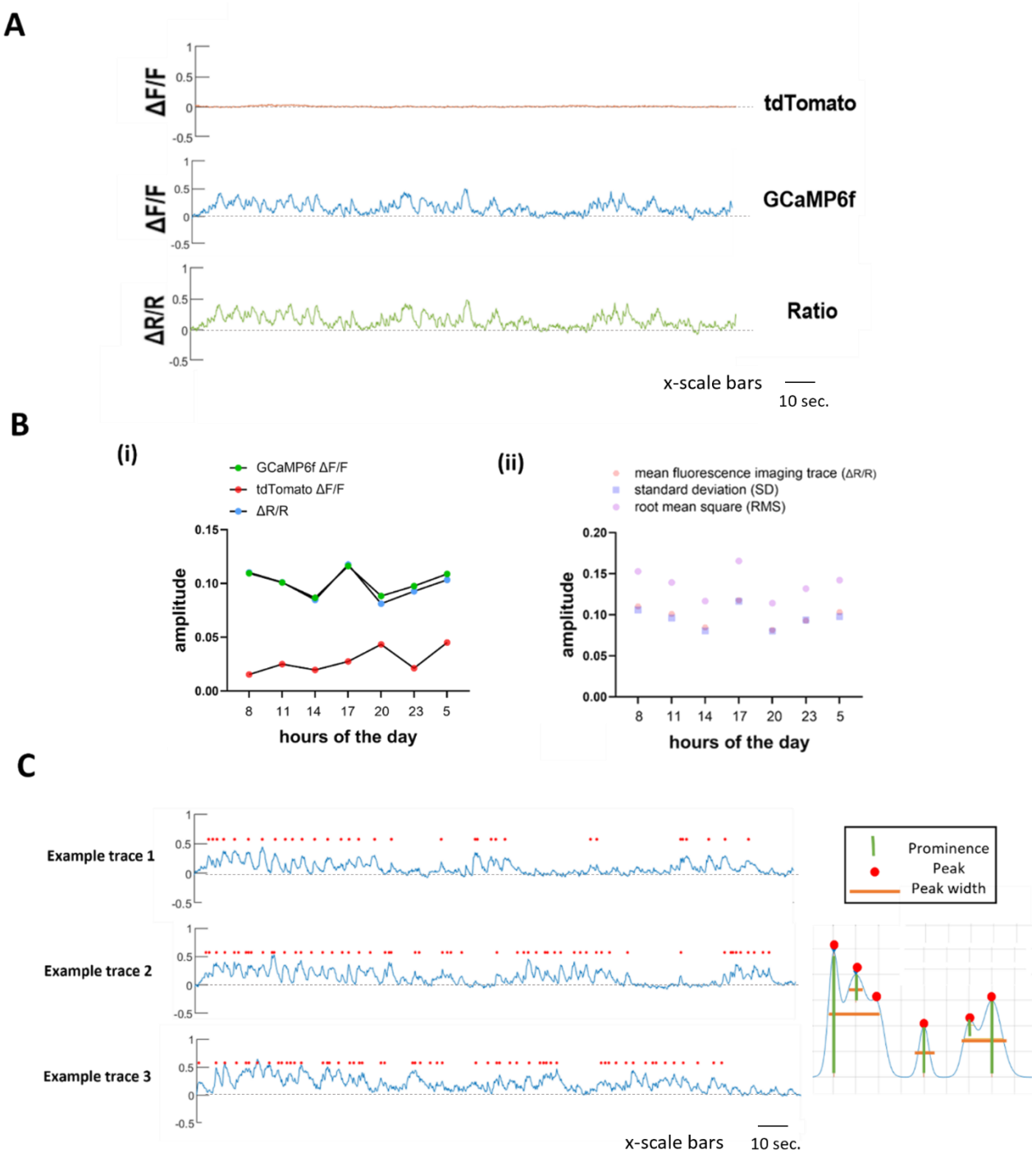


Figure 3.2 An analysis of Ca^{2+} dynamics

3.2 An analysis of Ca^{2+} dynamics

(A) Three representatives Ca^{2+} traces from one single imaging session of one dendritic arbor (one hemisphere). Each imaging session last for 3minutes and was recorded at 16.67Hz (256 pixels/frame). The green is the ratiometric trace and is the trace used for further analysis. Consider checking the last chapter of this work for more detailed info. On the y axes the amplitude of the change in fluorescence. On the x axes (not shown) the time. Each recording lasted ~ 3 minutes. In red trace the fluorescence changes in the tdTomato signal and in blue the one in the GCaMP6f signal. Both signals are normalized (see last chapter of this work for more details). **(B)** The validity of using the ratiometric trace. Example from a day of a chronic imaging experiment. On the y axes the amplitude of the change in fluorescence. On the x axes the time of the day (first recording at 8am, last recording at 5am). **(C)** The ratiometric trace is used for processing of the signal using a built-in Matlab function *(findpeaks)*. Illustration of three example traces and the identification of the peaks. Peaks are highlighted with red dots. The amplitude of peaks in each trace is defined as the mean value of ΔR/R0 of individual peak. All traces are recorded at 16.67Hz (256 pixel/frame). Objective, 20X.

3.1.1 Ca^{2+} dynamics in the dendrites

Changes in Ca^{2+} as a proxy for neural activity in the dendrites of the dFB (Figure 3.3,A) were recorded at specific times during the day (either 9 times in 24h, using Protocol A, or 7 times using Protocol B). While I could find two dendritic arbors, one for each hemisphere, I focused our attention on the dendritic arbor in just one hemisphere.

With the goal of quantifying dFB activity patterns when measured by Ca^{2+} -imaging I detected transient GCaMP excursions and measured different parameters relative to these transients (e.g., peaks intensity, peak's location, peaks width, peaks interval). I was interested in quantifying the frequency, regularity, and intensity with which these transients occur, aiming to find a pattern of activity that I could link with either the time of the day or sleep homeostasis.

As shown in Figure3.3,B, I organized the traces per day and time of the day , so that I could compare Ca^{2+}-dynamics between different time of the day and different days. I created circular heatmaps (using the MATLAB function *CirHeatmap*) where I could analyse the distribution and intensity of the GCaMP transients. (Figure 3.3,B (i-iii)).

GCaMP-transients have a repetitive occurrence and their amplitude and prevalence vary among days and time of the day. Most of the transients occurs at the begging of each recording session (Supplementary Figure2 (i)). The intensity of the peaks tends to be higher in the middle of the day as well as the interval between peaks tends to be lower. I could observe a progressive shift in the time interval between peaks from a time of the day to another. In the example in Figure 3.3,B (ii), from 11am to 5pm the peaks progressively become closer one with another, and the same could be observed from 8pm to 8am.

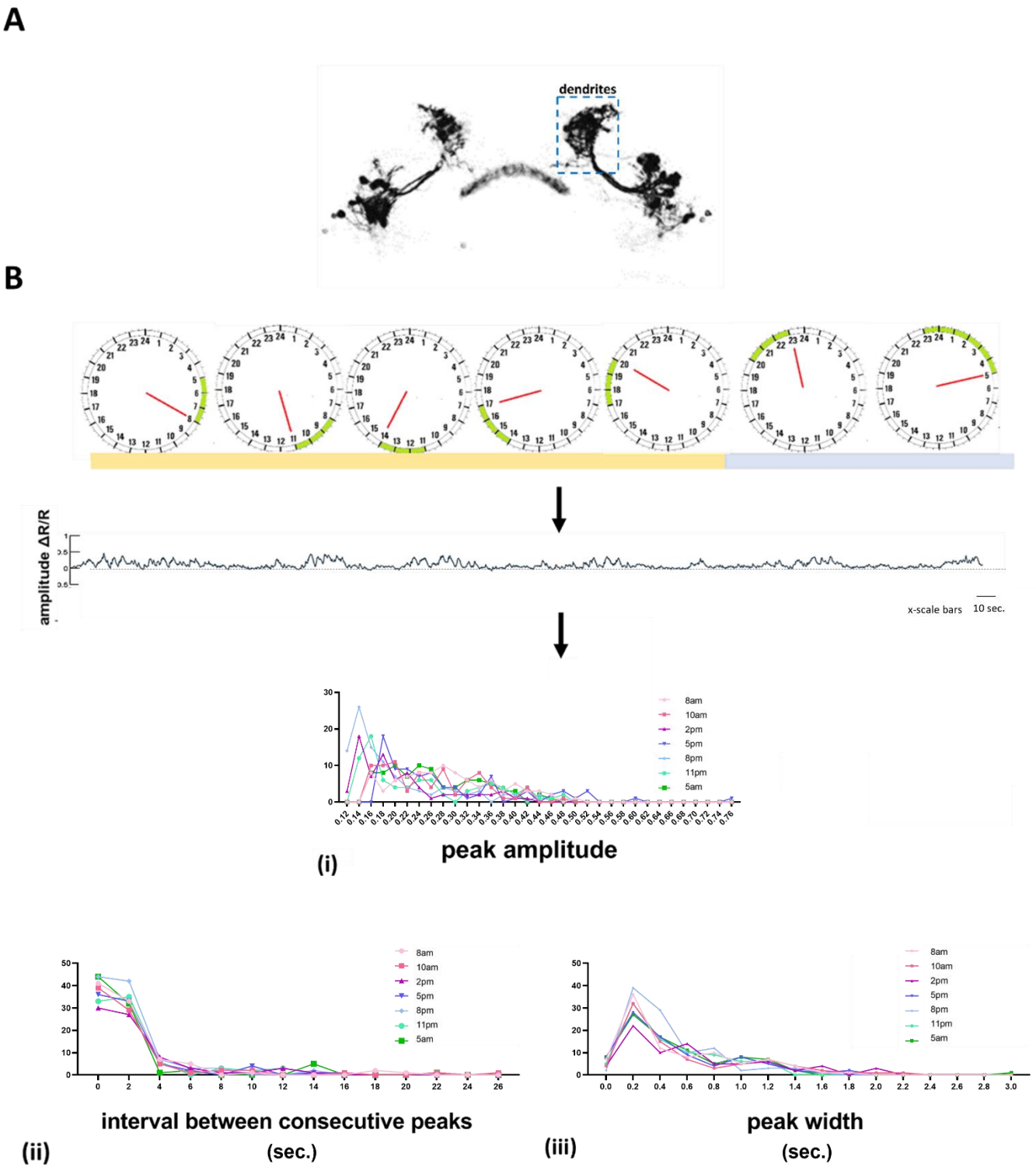


Figure 3.3 A qualitative analysis of Ca2+ dynamics in the 23E10 dendrites over multiple days

3.3 Qualitative analysis of Ca^{2+} dynamics in the 23E10 dendrites over days

(A) A schematic of the area recorded. In the blue square the dendritic arbor of one hemisphere. This image is a rendering of the dFB neurons, created using a confocal stack of 23E10 neurons and Adobe Illustrator. **(B)** An illustration showing the different hours of the day when we recorded the Ca^{2+} activity in the dFB dendrites. In green the time interval between the preceding imaging session and the imaging session at the time indicated by clock hand in red. Below, one dendritic Ca^{2+} trace used for illustrative purposes. For each of these traces we extracted descriptive features. The data collected in each day were then organized in histograms. **(i)** An example of a histogram describing the distribution of peaks in the trace. On the x axis the amplitude the peaks in ascending order. On the y axis, the number of peaks of a specific amplitude. Traces recorded at different times have different colours.**(ii)** An example of a histogram describing the distribution of peaks intervals. On the x axis the distance between peaks in ascending order. On the y axis, the number of intervals of a specific amplitude. Traces recorded at different times have different colours. **(iii)** An example of a histogram describing the distribution of peak width in each trace. On the x axis the peak width in ascending order. On the y axis, the number of peaks with a specific peak width. Traces recorded at different times have different colours.

When recording from the dendric arborization of dFB neurons I noticed distinct activity pattern in two regions. Evidence from previous work in the laboratory has suggested the possibility that the population of dFB neurons is heterogeneous. Different Ca^{2+}-dynamics in the dendrites could be due to the different activation of individual cells in the population of neurons. I wanted to investigate the distribution, intensity, and shape of the Ca^{2+}-transient in distinct regions of the dendrites. I identify an upper and a lower region (Figure 3.4, A).

Looking at the mean fluorescence (Figure 3.4,B), the upper ROI contributes to most of the activity recorded when looking at the entire dendritic arbour. Figure 3.4,C shows example traces from the UP,DOWN ROIs, simultaneously recorded, and from the entire dendritic region.

Ca^{2+} - transients in the upper region are increased in number and frequency, and this could be seen not only within a recording session, but between different times of the day (Figure 3.4,C).

While distinct temporal and spatial activity in dendritic regions could be due to the functional diversity of the neurons in the population of 23E10 neurons, it is important to notice the anatomical differences of the two regions. Dendritic length and number, spine number and dendritic spine density should be taken in consideration when considering Ca^{2+}-dynamics differences within the dendritic arbor.

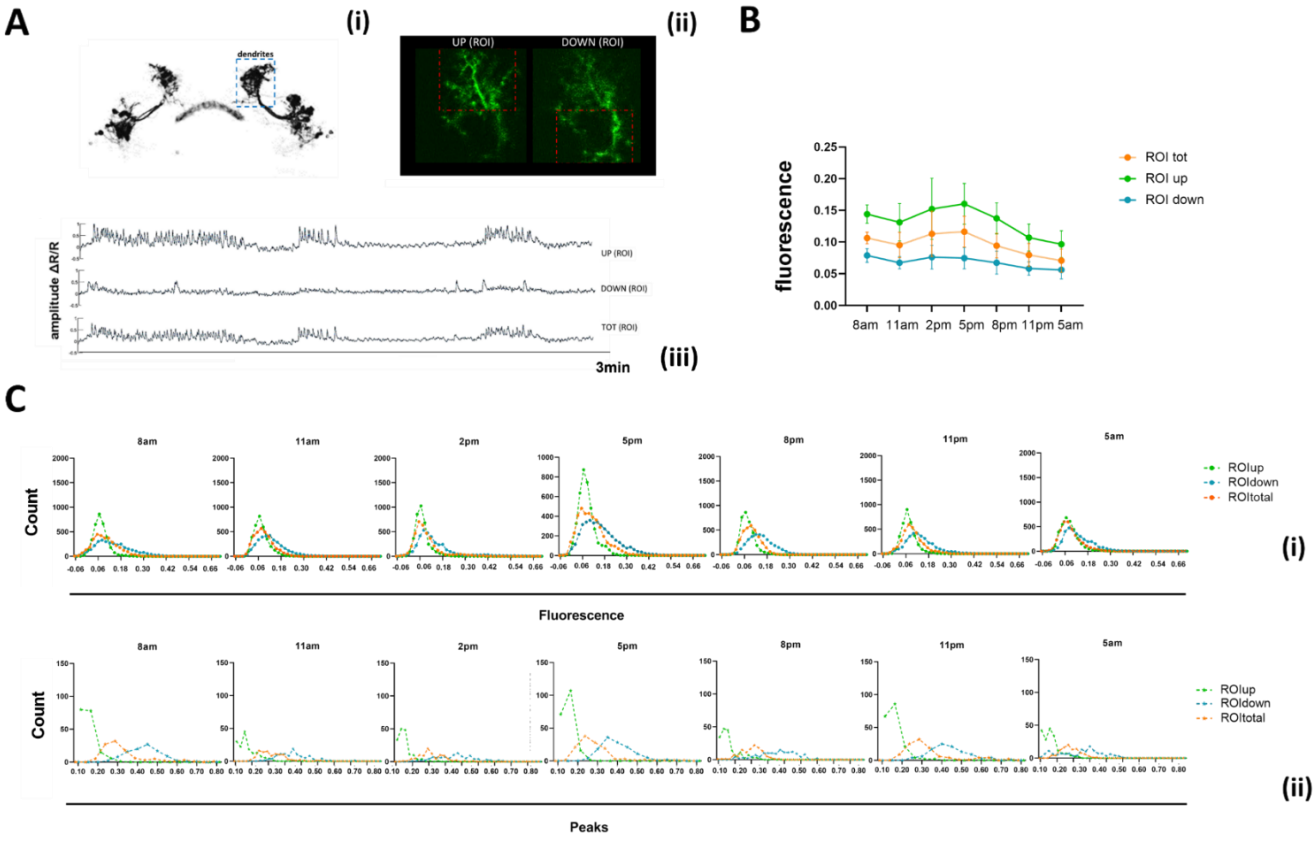


Figure 3.4 Distinct Ca^{2+} dynamics in distinct dendritic regions of 23E10 neurons

3.4 Distinct Ca^{2+} dynamics in distinct dendritic regions of 23E10 neurons

(A) In **(i)** schematic of the area recorded. In the blue square the dendritic arbor of one hemisphere. In **(ii)** images are 'stacked' to provide a maximal intensity projection of the dendrites. In green the signal from GCaMP6f. Two squares with red dotted lines indicate the upper (left square) and lower (right square) region of interest (ROI). Images taken using a 20X, acquisition at 16.67Hz. In **(iii)** 3 examples of Ca^{2+} traces extracted in the three regions of interests (ROI up, ROI down and total ROI (ROIup+ROIdown). Traces are $\Delta R/R_0$. Each trace lasts ~3minutes. **(B)** An example showing the variation of the fluorescence for one fly during one day of recordings (*Protocol B*). Data are means ± s.e.m. On the x axis the seven consecutive imaging sessions. On the y axis the amplitude of the mean fluorescence for each specific trace. Green line refers to the trace extracted from the upper region. The blue trace refers to the trace extracted from the lower region. The orange refers to the trace extracted considering the entire dendritic region. **(C)** Example of histograms obtained from the analysis of the Ca^{2+} dynamics in the three regions of interest. The top line of histograms in **(i)** shows the change in the fluorescence distribution along the consecutive recording traces. For each graph, on the x axis the amplitude of the transients in ascending order. On the y axis, the number of transients of a specific amplitude. These data refer to all the recordings for one single fly (4days/7 recordings per day). The bottom line in **(ii)** shows the distributions of peaks amplitude along the consecutive recording traces. For each graph, on the x axis the amplitude the peaks in ascending order. On the y axis, the number of peaks of a specific amplitude. These data refer to all the recordings for one single fly (4days/7 recordings per day).

Investigating the correlation between different regions in the dendritic area, we found a significant correlation between the upper and lower ROI (Figure 3.5,A) and between the entire ROI and the distinct ROIs (Figure3.5,B and C). The elevated R squared of these correlations and the distribution of the individual data strongly suggests the functional integration of the upper and lower region of interest.

For further analysis, presented in chapter 3, I will consider the entire dendritic ROI to relate Ca^{2+}-dynamics in 23E10 dendrites with behaviour.

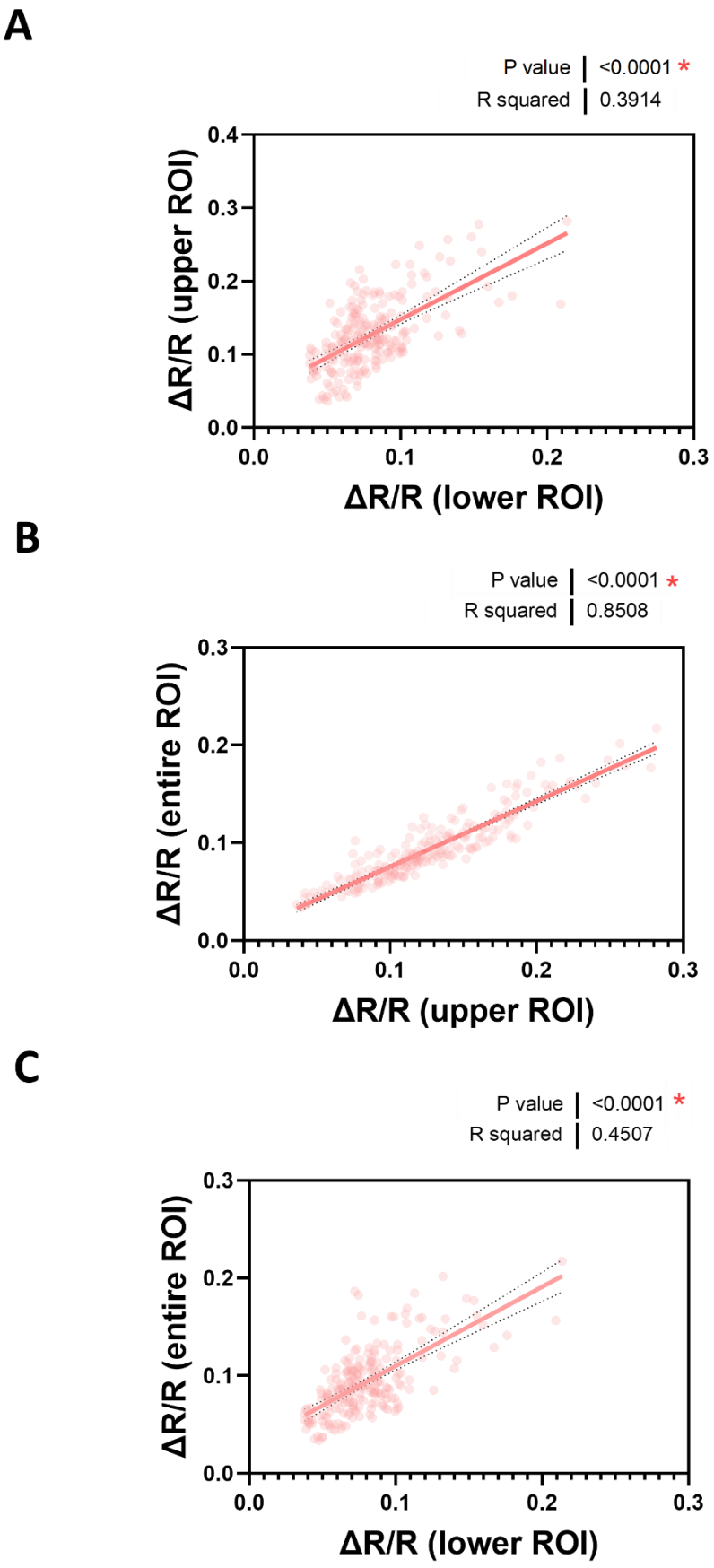


Figure 3.5 Ca^{2+} dynamics of the entire dendritic region well represent regional variation of activity

3.5 Ca^{2+} dynamics of the entire dendritic region well represent regional variation of activity

(A) A simple linear regression between the upper and lower ROI (region of interest) of the dendrites. P value< 0.0001* R squared=0.3914 R= 0.6256 **(B)** A simple linear regression between the entire ROI (upperROI+lowerROI) and the upper ROI. Considering the dendritic arbor of one hemisphere. P value< 0.0001* R squared=0.8508 R= 0.9223 **(C)** A simple linear regression between the entire ROI (upperROI+lowerROI) and the lower ROI. Considering the dendritic arbor of one hemisphere. P value< 0.0001* R squared=0.4507 R= 0.6713.

3.1.2 Ca^{2+} dynamics in the axons

I decided to repeat a qualitative analysis of imaging traces over days for the data collected imaging the axons (Figure3.6). I am currently in the process of collecting and analysing more data relative to the Ca^{2+}-dynamics in 23E10 axons.

Analogously to the dendrites, Ca^{2+}-dynamics in the axons changes between days and time of the day (Figure3.6,B (i-iii) and Supplementary Figure2 (ii)). Differently to the dendrites, Ca^{2+}-transients are more intense when lights are off (8pm-8am), around 5am I registered a high number of high intensity peaks (Figure3.6,B(i) and Supplementary Figure2 (ii)). Comparing axons and dendrites, the number of peaks is higher and the distance between peaks lower in the axons. Also, the distance between peaks is less variable in the axons. Peaks are least close one with another when lights are on, during the first part of the day, and become progressively closer one with another later during the last part of the day (Fiigure3.6,B(i) and Supplementary Figure2 (ii)). When looking at peak's width, Ca^{2+}-transient are larger in the axons than they are in the dendrites. Moreover, I could observe a progressive increase in the number of larger Ca^{2+}-transients from lights on to lights off (Figure 3.6,B (iii)).

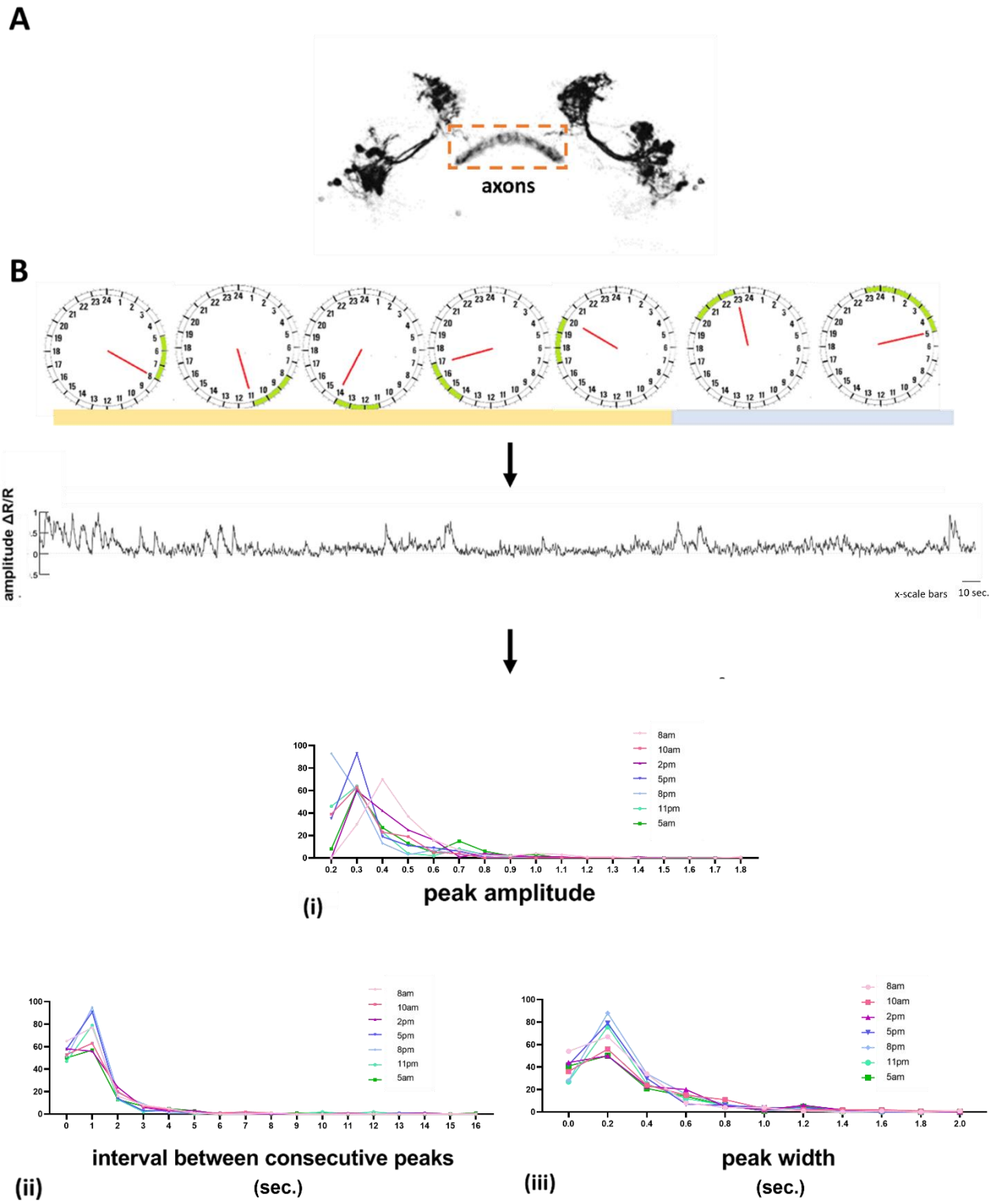


Figure 3.6 A qualitative analysis of Ca^{2+} dynamics in the 23E10 axons over multiple days

3.6 A qualitative analysis of Ca^{2+} dynamics in the 23E10 axons over days

(A) A schematic of the area recorded in an imaging session. In the red square the axonal region. **(B) (i)** An illustration showing the different hours of the day when we recorded the Ca^{2+} activity in the dFB axons. Below, one dendritic Ca^{2+} trace used for illustrative purposes. For each of these traces we extracted descriptive features. The data collected in each day were then organized in histograms. **(i)** An example of a histogram describing the distribution of peaks in the trace. On the x axis the amplitude the peaks in ascending order. On the y axis, the number of peaks of a specific amplitude. Traces recorded at different times have different colours.**(ii)** An example of a histogram describing the distribution of peaks intervals. On the x axis the distance between peaks in ascending order. On the y axis, the number of intervals of a specific amplitude. Traces recorded at different times have different colours. **(iii)** An example of a histogram describing the distribution of peak width in each trace. On the x axis the peak width in ascending order. On the y axis, the number of peaks with a specific peak width. Traces recorded at different times have different colours.

3.2 Correlation analysis of different parameters describing Ca^{2+} dynamics

In this exploratory analysis I wanted to also understand how different features of the trace were relating with each other. One of the aims was to better describe the change of the Ca^{2+}-transients during the experiment. In addition, with the goal of correlating the Ca^{2+}-transients with behaviour, I wanted to use this analysis to validate the correlation between the behaviour and different features of the trace.

As shown in Figure 3.7, all features of the trace are correlated, considering both data collected imaging dendrites and axons. Individual flies show similar trends of correlation with some exception, for example in Figure 3.7,A (ii) and (iii). Although in Figure 3.7,C I show data from a single fly (data collection in progress) , I could appreciate that the correlation trend for the different features of the trace hold true for both axons and dendrites.

Peaks width, peaks interval and number of peaks are features of the trace more interconnected, instead the fluorescence of the trace mildly correlate with the above-mentioned features. Overall, the higher the average peaks' interval, peaks width and fluorescence, the lower is the number of peaks in a trace.

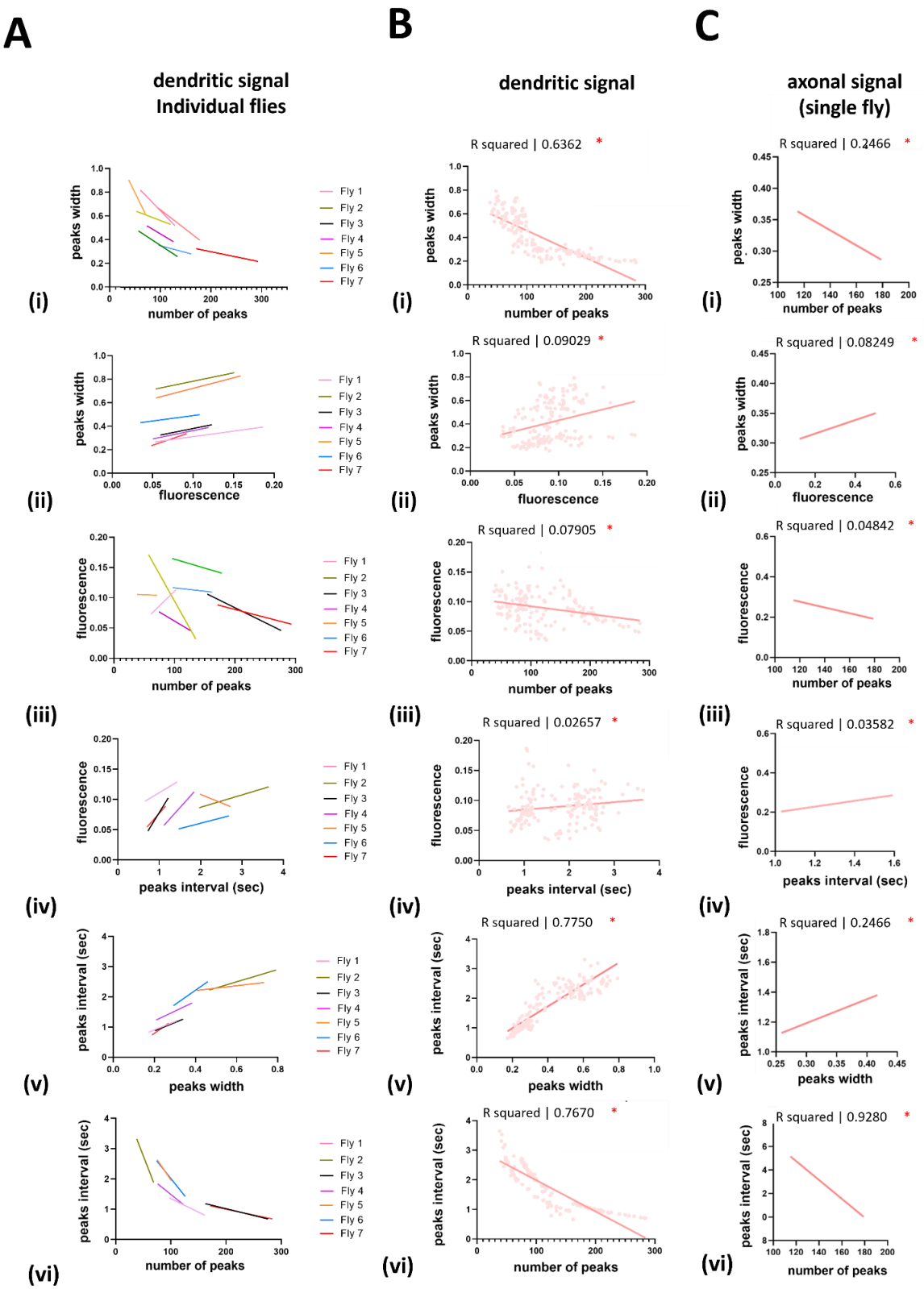


Figure 3.7 A correlation analysis of different parameters describing Ca^{2+} dynamics

3.7 A correlation analysis of different parameters describing Ca^{2+} dynamics

(A) From **(i)** to **(vi)** graphs showing the result of a series of simple linear regressions. Each graph shows a series of coloured lines. Each line refers to one single linear regression analysis for one fly. Lines of the same colour belong to the same fly. To appreciate the general trend of the slopes. Number of flies analysed=7. **(B)** From **(i)** to **(vi)** each graph shows the result of a simple linear regression analysis. We pooled all data from all flies. (Number of flies analysed=7). In each graph each dot represents one single data point. **(C)** From **(i)** to **(vi)** each graph shows the result of a simple linear regression analysis. Data come from one single fly.

In view of the fact that 23E10 neurons have one dendritic arbor for each hemisphere (Figure 3.8,A) , I asked if the two hemispheres were correlated and if the relation between different parameters describing Ca^{2+} dynamics was the same in both hemispheres.

I found that between the two hemispheres there is a significant positive correlation (Figure3.8,A (i-ii)). I then observed that different features of the traces recorded in the two hemispheres relate one with each other in a similar way.

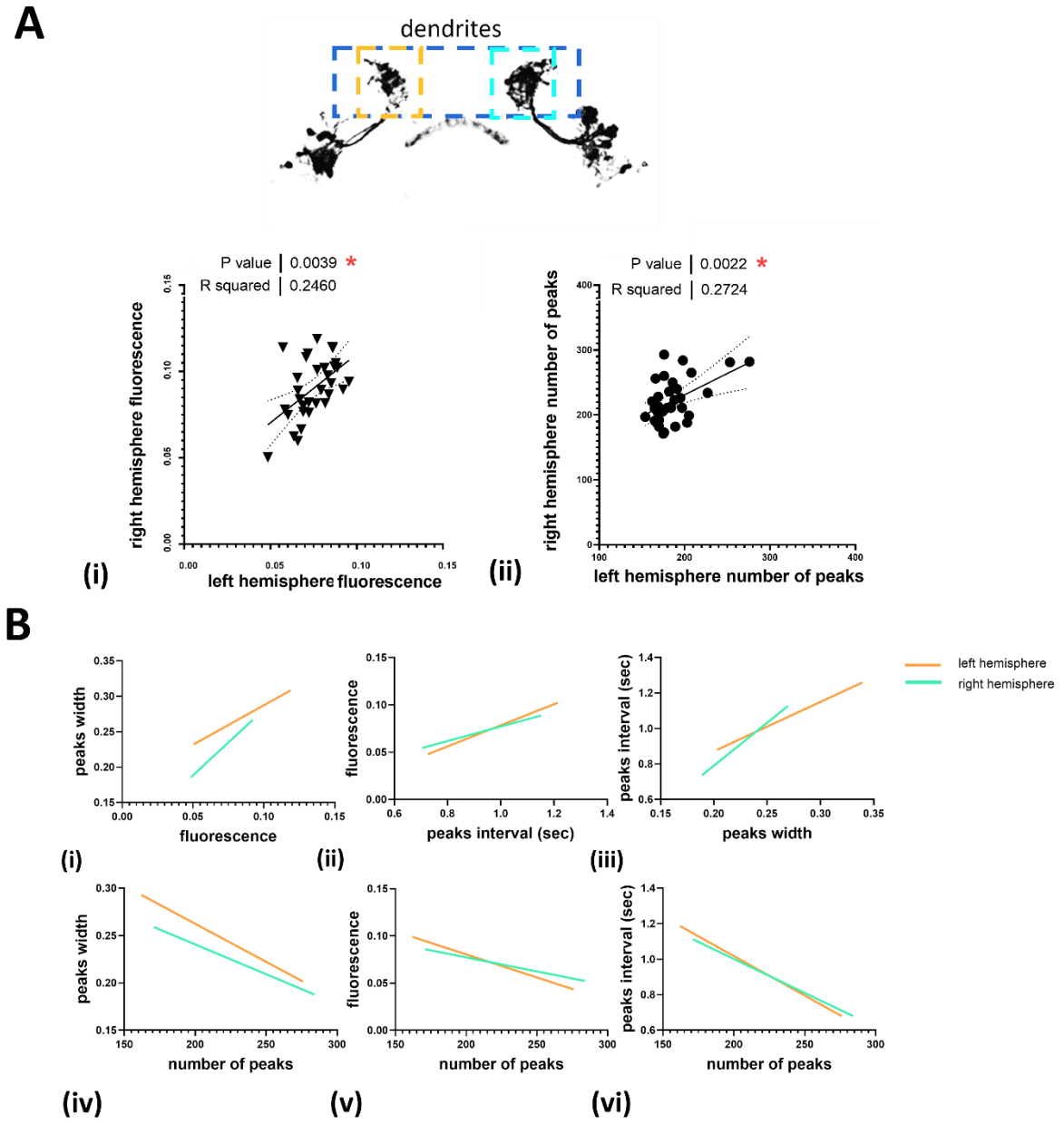


Figure 3.8 Dendritic Ca^{2+} dynamics in the two hemispheres are positively correlated

3.8 In the 23E10 line dendritic Ca^{2+} dynamics in the two hemispheres are positively correlated

(A) On the top, schematic of the area recorded. In the blue square the dendritic arbors of the two hemispheres. Within the orange and light blue squares the individual dendritic arbors. On the bottom **(i)** shows the result of a simple linear regression. On the y axis the mean fluorescence in the right hemisphere. On the x axis the mean fluorescence of the left hemisphere. P value= 0.0039* R squared=0.2460 R= 0.4959 **(ii)** shows the result of a simple linear regression. On the y axis the number of peaks in the traces in the right hemisphere. On the x axis the number of peaks in the traces in the left hemisphere. P value= 0.0022** R squared=0.2724 R= 0.5219 The data shown come from simultaneous, consecutive recordings of the two-hemispheres in one fly. **(B)** From **(i)** to **(vi)** each line shows the result of a simple linear regression analysis. To appreciate the same trend for both hemispheres.

4 | Correlating dendritic Ca^{2+} dynamics and sleep

The genetic line 23E10 labels the dorsal fan-shaped body neurons (dFB). The dFB are embedded in a recurrent circuitry that controls homeostatic sleep. Over the past years, an elaborate framework has been developed, which describes how this *somnostat* – and the dFB as its output unit – accumulates sleep pressure during waking and discharges it during sleep (Pimentel et al., 2016). The evolution of this framework is fueled by the rapid progress in discovering components on the circuit, cellular, and molecular levels (Kempf et al., 2019; Raccuglia et al., 2022). Yet, it is not known if the neural representation of sleep pressure changes within hours, minutes or whether the dFB merely represents the moment-by-moment (in)activity of the fly. Recent unpublished work from our laboratory suggests that dFB neuronal activity neither represents the moment-by-moment activity nor the initiation or termination of movement episodes. In this chapter I will present our investigation of the relation between Ca^{2+}-dynamics in the 23E10 dendrites and sleep, considering a window of time between 30minutes and several hours. The analysis presented here will be limited to the data collected recording the dendritic arbor of 23E10 neurons, because of the larger pool of data.

4.1 Mean GCaMP6f fluorescence in the dendrites correlates with the time of the day

In the first place, I asked myself if the time of the day had any influence on the neural activity at the level of the 23E10 dendrites. Homeostatic and circadian processes control sleep, its timing and duration, even though very little is known about the mechanisms regulating these two processes. The current hypothesis in the field is that the circadian system influences sleep acting directly and indirectly on sleep circuits to induce wake (Cavanaugh et al., 2016). It is also known that the circadian system is thought to increase its wakefulness drive toward the end of the light phase, to prevent sleep when the homeostatic drive is high (Allada and Chung, 2010; Guo et al., 2018).

I found that the mean fluorescence of Ca^{2+}-transients recorded in the dendrites correlates with the time of the day (Figure 4.1). Considering multiple recordings in specific time of the day and the average fluorescence of Ca^{2+}-transients I found: 1) a positive correlation of the mean fluorescence with the time of the day during daytime ; 2) a negative correlation of the mean fluorescence with the time of the day during nighttime (Figure 4.1, B). When considering individual flies, I could also appreciate the same trend of correlation, as shown in Figure 4.1,C. Over multiple flies and multiple days I could observe an increase of the fluorescence of Ca^{2+}-transients during the day, till the time of higher locomotion during 24h, after which I observed a decrease in the fluorescence of the transients (Figure 4.1,A). Interestingly, an analogous trend is observed when considering the root-mean-square (RMS) of the traces, with the exception that the highest values of RMS are at 8pm (Supplementary Figure3).

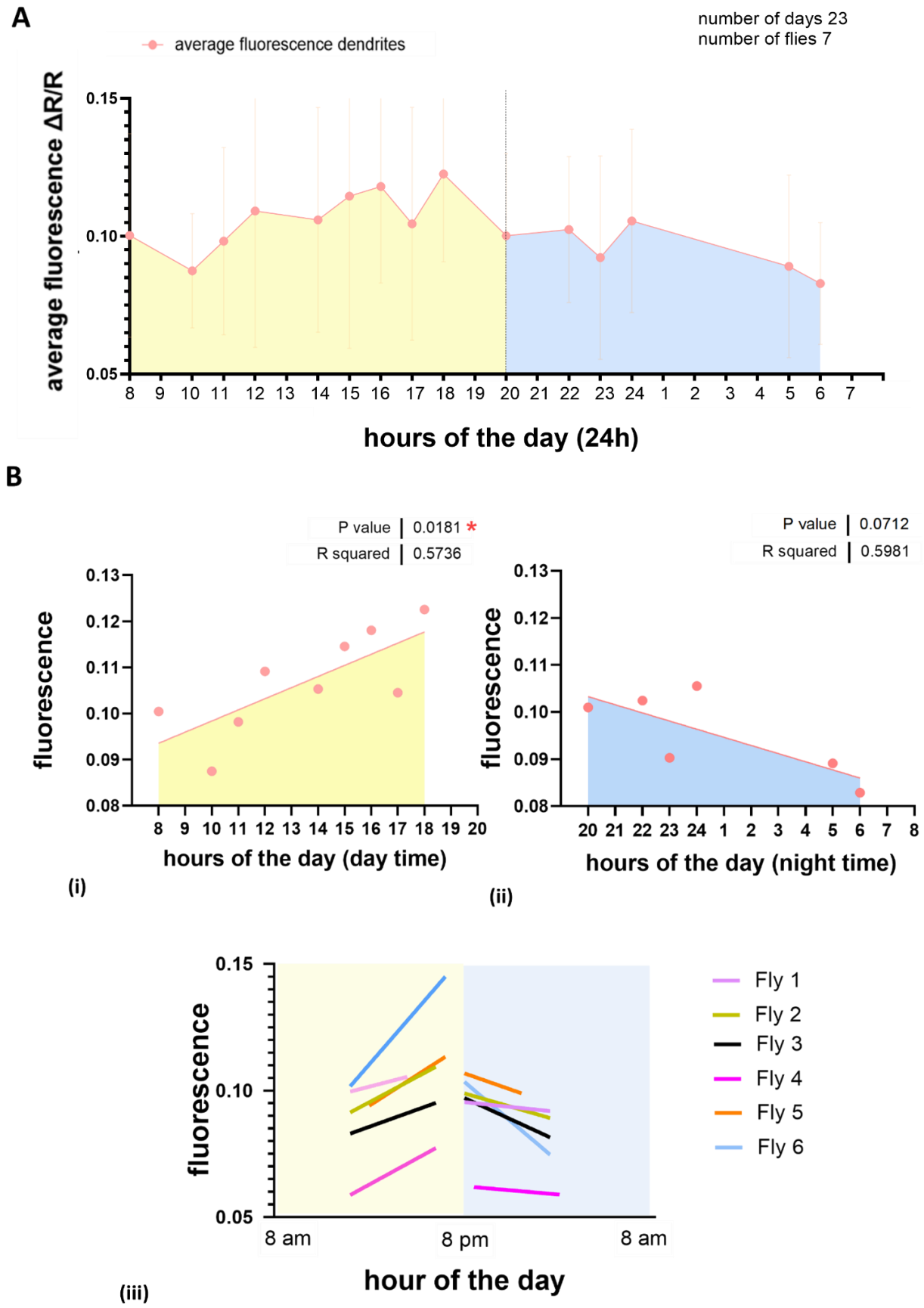


Figure 4.1 The time of the day influences dendritic Ca^{2+} dynamics in the 23E10 neurons

4.1 The time of the day influences dendritic Ca^{2+} dynamics in 23E10 neurons

(A) Average fluorescence change in the dendritic signal throughout 24h. On the x axis the time of the day (from 8am to 8am, 24h). On the y axis the amount of fluorescence. All the values are extracted from ratiometric traces. Salmon dots are averages ± s.e.m. Number of flies=6. Number of days=23. To appreciate the difference in trend during the periods with lights ON and lights OFF. **(B)** Graphs showing a simple linear regression analysis. Each point is the average of all the data recorded at that specific time of the day. Number of flies=6. Number of days=21. For the daytime P value=0.0181 R squared=0.5736. For the night-time P value=0.0712 R squared=0.5981. **(C)** Each line in the graph shows the result of a simple linear regression for one fly (over consecutive days of the experiment). Lines of the same colour belong to the same fly. For daytime and for night-time data we have used two separate linear regression analysis.

4.2 Ca^{2+} dynamics in the dendrites of 23E10 neurons and sleep before and after imaging

Sleep homeostasis is by definition the process regulating the need of sleep and dictates how much the organism will sleep in the near future. Our challenge was to investigate the representation of behaviour in the dFB over minutes and hours using short “snapshots” (3-minute long) of dFB neural activity over several days. To relate these “snapshots” to behaviour I used the features of recorded Ca^{2+} traces (mean peaks width, peaks number, mean peaks’ interval, mean fluorescence) and the measures of sleep extracted from the locomotor trace (amount of sleep, number of sleep bouts, length of sleep bouts). Here I will present a correlation analysis between the features of the Ca^{2+} traces and the amount of sleep before, during and after the imaging sessions.

When analysing sleep using the DAM system (see methods section) the approach is to consider 30minutes bin and quantify the amount of sleep every 30 minutes. Similarly, I decided to quantify sleep/30minutes when analysing the locomotor trace from ball-tracking in our chronic neurobehavioural experiments. When considering the 30 minutes bin when I recorded the imaging session, I found a mild but significant correlation between the amount of sleep and the four features I picked to describe the Ca^{2+} dynamics in the dendrites. Interestingly, the fluorescence has the lowest correlation with the amount of sleep in the 30minutes when the imaging session occurred (Figure 4.2,B (iv)). It is worth mentioning that looking at individual flies, not all flies show a similar correlation trend, indeed some show an opposite trend (Figure 4.2,C). This could mitigate the correlations found when considering all set of data from multiple flies. I will discuss this variability later in the text.

When correlating the 30minutes bin when imaging occurred and the imaging sessions, I need to take in consideration that in those 30minutes include partially sleep before and after the imaging session. To investigate the correlation sleep before and after the imaging session and the features of the Ca^{2+} traces, I proceeded to consider the 30 minutes bin before and after the 30 minutes bin when the imaging session occurred.

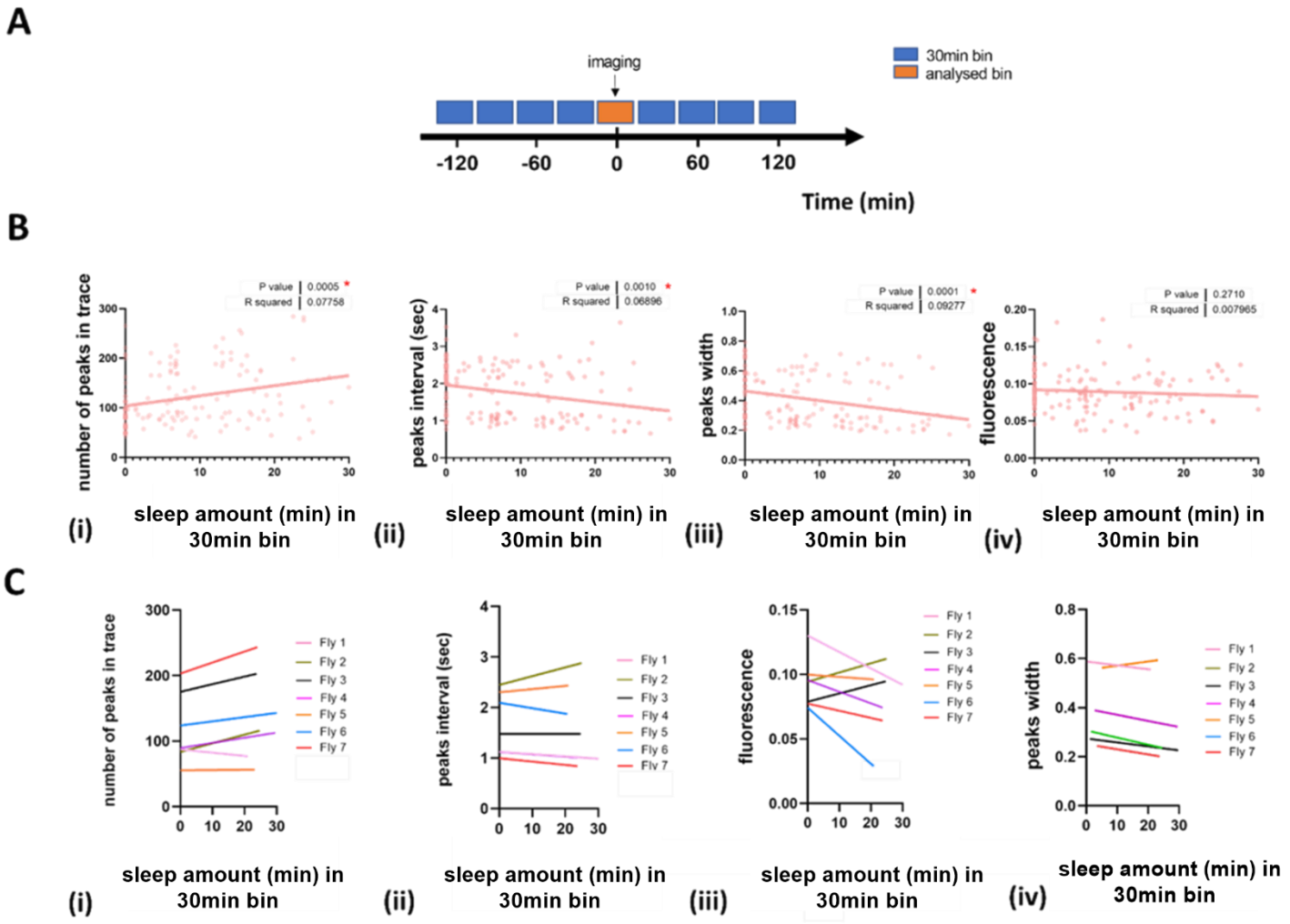


Figure 4.2 Descriptive features of Ca^{2+} dynamics in the 23E10 dendrites correlate with the amount of sleep in the 30 minutes when the imaging session occurred

4.2 Descriptive features of Ca^{2+} dynamics in the 23E10 dendrites correlate with the amount of sleep in the 30 minutes when the imaging session occurred.

(A) A schematic of the approach used for the analysis. Each square represents a bin of 30minutes. Imaging recording occurs at time 0. The bin 0 represent the 30minutes when the imaging session occurred. The colour orange represents the bin analysed in this figure. We looked at the amount of sleep in the bin 0 **(B)** From **(i)** to **(iv)** the result of simple linear regression analysis. Each point in each graph represents the data from one recording. All recordings from different flies have been considered. In **(i)** P value= 0.0005*** R squared=0.07758 **(ii)** P value= 0.0010** R squared=0.06896 **(iii)** P value= 0.0001*** R squared=0.09277 **(iv)** P value= 0.007965 R squared=0.2460. On the y axis the feature of the traces taken in consideration for one specific analysis. On the x axes the amount of sleep in the bin 0. **(C)** From **(i)** to **(iv)** each graph shows the trend of regression for individual flies. Line of different colours represent different flies. The same colour indicates one the same fly in all the graphs. On the y axis the feature of the traces taken in consideration for one specific analysis. On the x axes the amount of sleep in the bin 0. Number of flies=7.

As shown in Figure 4.3,C the sleep in the 30 min after the 30min bin when the imaging occurred significantly correlates with the number of peaks, the mean peaks' interval, the peak width, and the fluorescence of the recorded trace. While this is not true for the sleep happening in the 30 minutes before the 30 minutes when I imaged (Figure 4.3,B). Of all the features of the trace, the number of peaks in the traces better correlates with the sleep in the analysed bin after the imaging (Figure 4.3,D).

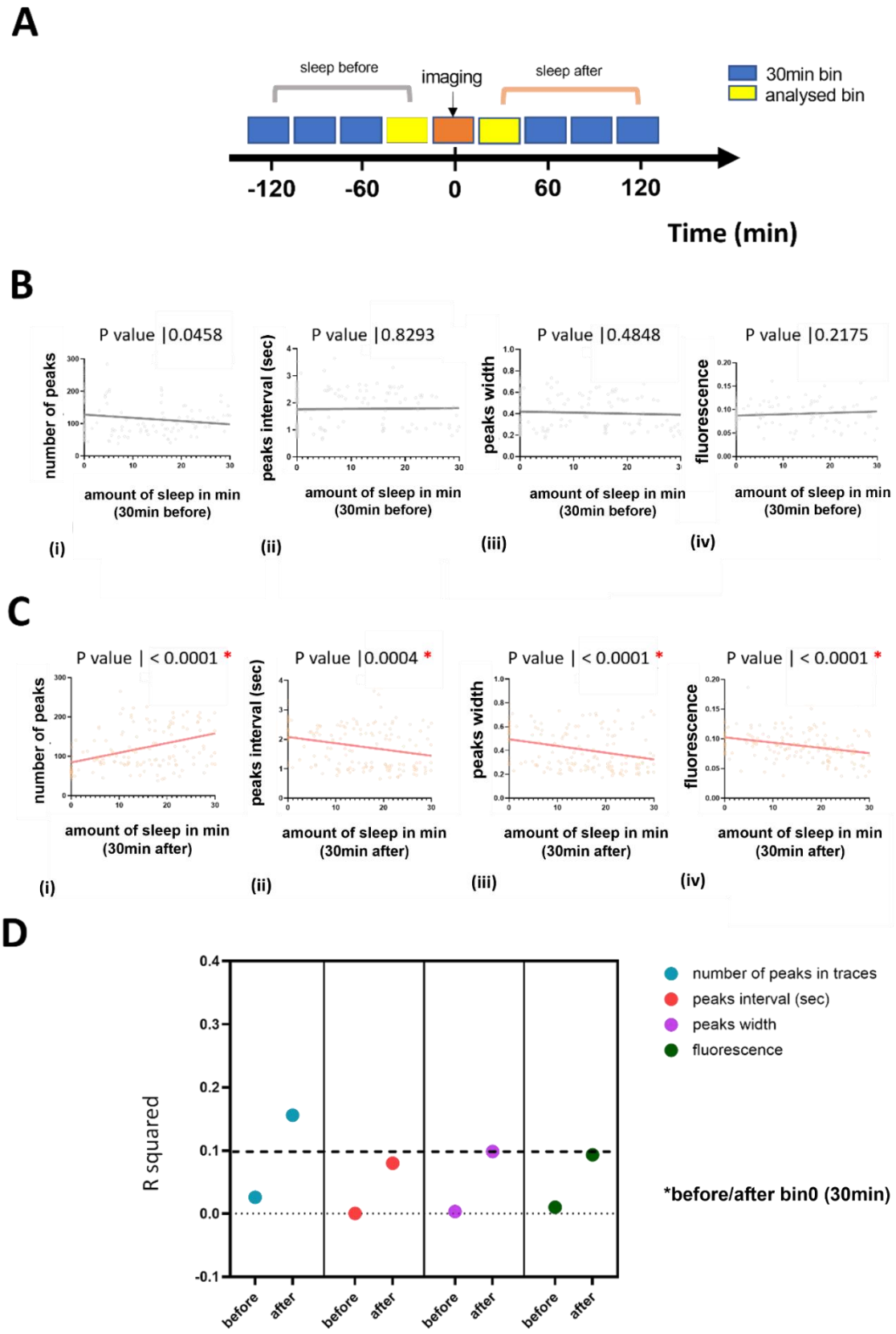


Figure 4.3 Distinct features of the Ca^{2+} traces in the 23E10 dendrites correlate with the amount of sleep after the imaging session, but not with the sleep before (30min bin)

4.3 Distinct features of the Ca^{2+} traces in the 23E10 dendrites correlate with the amount of sleep after the imaging session, but not with the sleep before (30min bin)

(A) A schematic of the approach used for the analysis. Each square represents a bin of 30minutes. Imaging recording occurs at time 0. Time bin 0 is in orange. The analysed bins are in yellow. We looked at the 30minutes before and after the bin 0. We considered two bins and the amount of sleep occurred in those bins. **(B)** From **(i)** to **(iv)** simple linear regressions between the amount of sleep in the 30min before bin0 and the features of the trace. Each point in each graph represents the data from one recording. Number of flies=7. **(C)** From **(i)** to **(iv)** simple linear regressions between the amount of sleep in the 30min after bin0 and the features of the trace. Each point in each graph represents the data from one recording. Number of flies=7. **(D)** Summary of the variation of R squared values. Dots of the same colour refers to the same feature of the trace. On the x axis “before” and “after” referring to the bin analysed in a specific linear regression analysis.

When I looked at the sleep one hour before and after the bin when the imaging occurred (Figure 4.4,A), I found that all the features of the Ca^{2+} traces strongly correlate with the “sleep after” (Figure 4.4,C). Again, the number of peaks in trace better correlate with the “sleep after”(Figure 4.4,D). The “sleep before” does not show any correlation with the recorded traces (Figure 4.4,B).

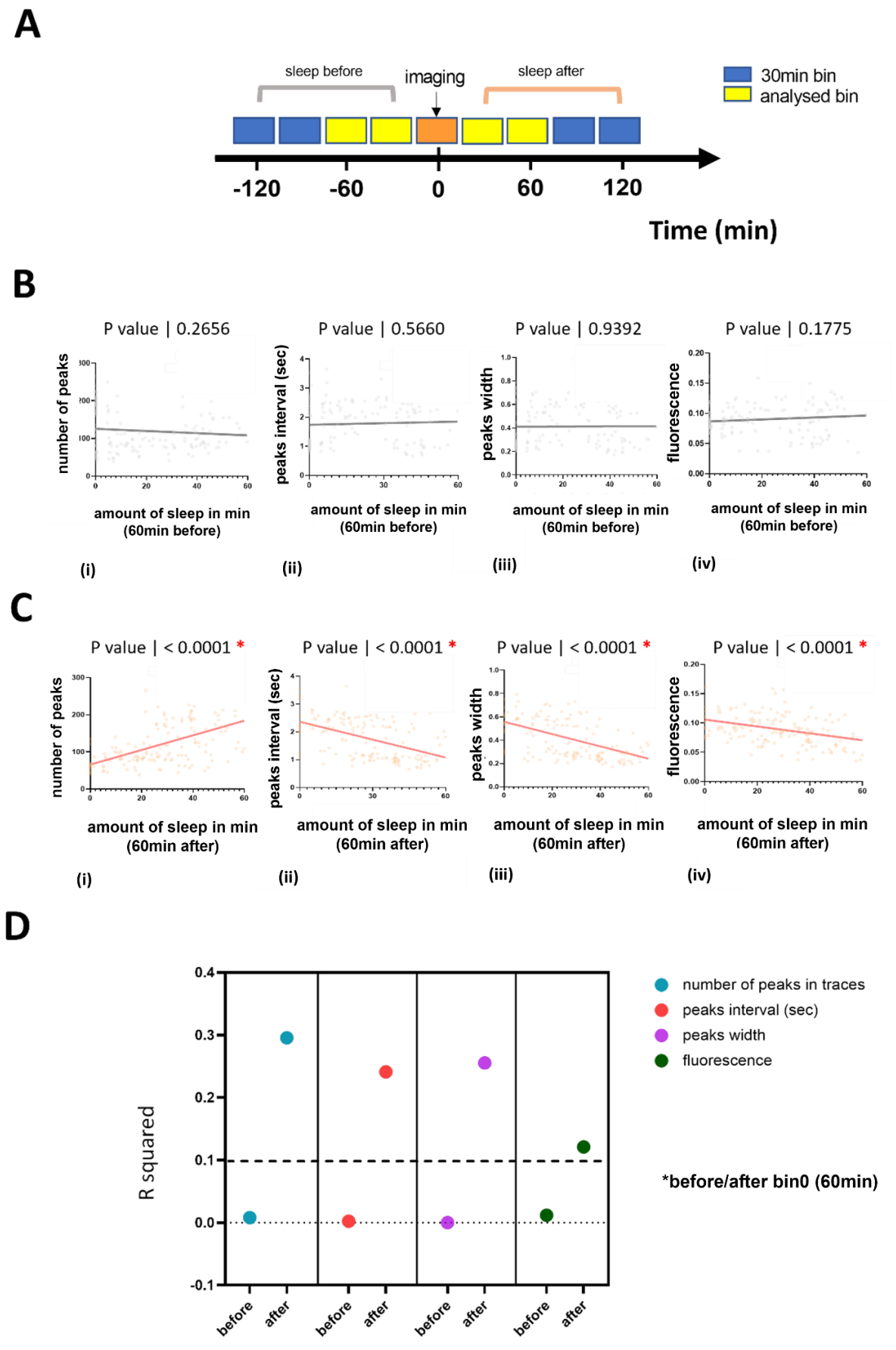


Figure 4.4 Distinct features of the Ca^{2+} traces in the 23E10 dendrites correlate with the amount of sleep after the imaging session, but not with the sleep before (60min bin)

4.4 Distinct features of the Ca^{2+} traces in the 23E10 dendrites correlate with the amount of sleep after the imaging session, but not with the sleep before (1h bin)

(A) A schematic of the approach used for the analysis. Each square represents a bin of 30minutes. Imaging recording occurs at time 0. Time bin 0 is in orange. The analysed bins are in yellow. We looked at the 1h before and after the bin 0. We considered two bins and the amount of sleep occurred in those bins. **(B)** From **(i)** to **(iv)** simple linear regressions between the amount of sleep in 1h before bin0 and the features of the trace. Each point in each graph represents the data from one recording. Number of flies=7. **(C)** From **(i)** to **(iv)** simple linear regressions between the amount of sleep in 1h after bin0 and the features of the trace. Each point in each graph represents the data from one recording. Number of flies=7. **(D)** Summary of the variation of R squared values. Dots of the same colour refers to the same feature of the trace. On the x axis “before” and “after” referring to the bin analysed in a specific linear regression analysis.

Surprisingly, the sleep in the two hours after imaging shows a weaker but significant correlation with the recorded Ca^{2+} traces (Figure 4.5,C), while no significant correlation could be observed considering the sleep in the two hours before imaging (Figure 4.5,B).

Once again, the peak numbers are better correlated with “sleep after” (Figure 4.5,D).

I decided to focus the analysis within a time window that would not exceed the max time between two consecutive imaging sessions (3hours). This is relevant especially because the recording session in itself would break any sleep bout, which in flies could last even 60-120minutes. The effect of disrupted sleep would have jeopardized the results. On top of this, the higher the period of movement considered, the higher the probability to identify a large amount of sleep in that period and eventually find a significant correlation with.

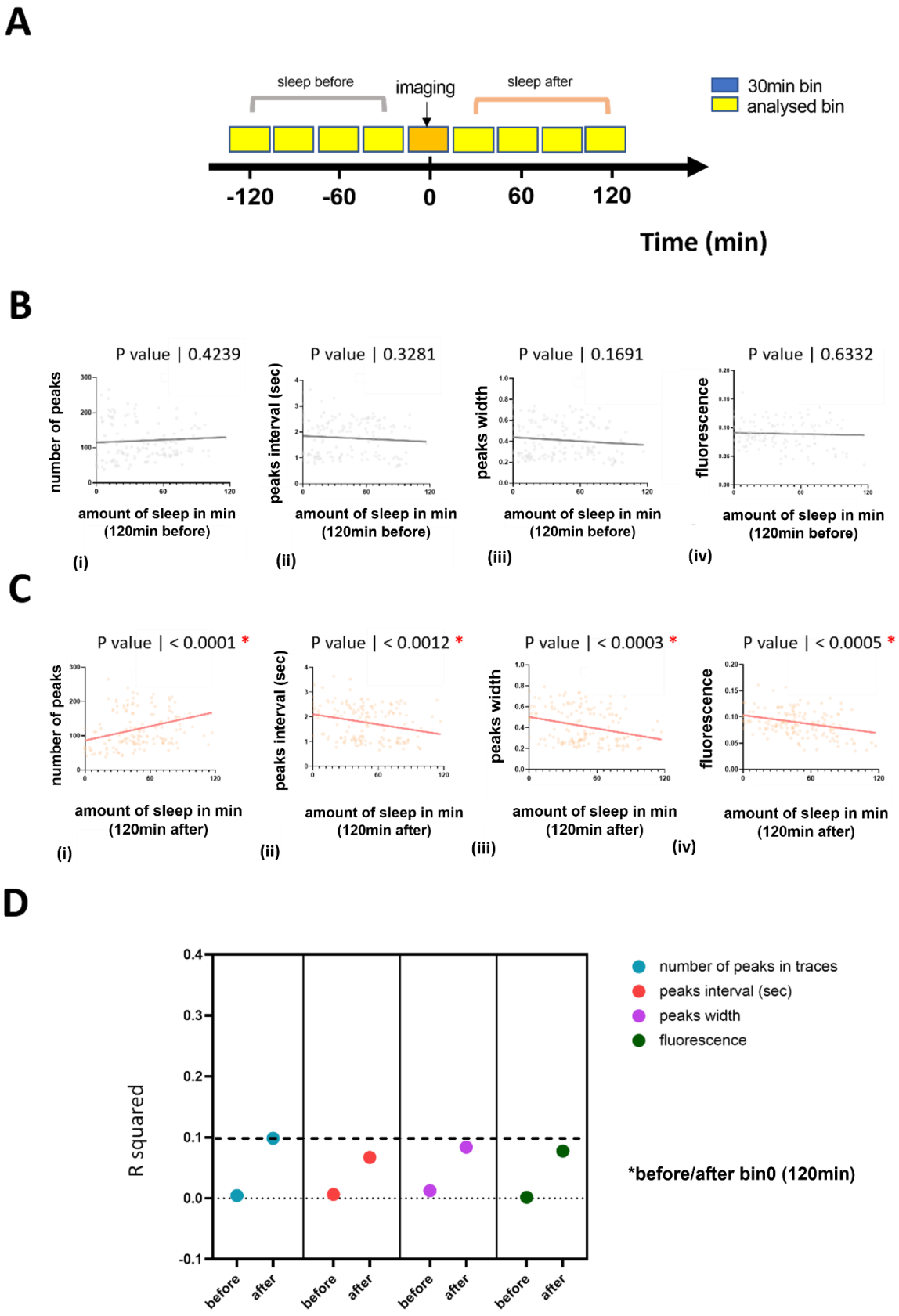


Figure 4.5 Distinct features of the Ca^{2+} traces in the 23E10 dendrites correlate with the amount of sleep after the imaging session, but not with the sleep before (120min bin)

4.5 Distinct features of the Ca^{2+} traces in the 23E10 dendrites correlate with the amount of sleep after the imaging session, but not with the sleep before (2h bin)

(A) A schematic of the approach used for the analysis. Each square represents a bin of 30minutes. Imaging recording occurs at time 0. Time bin 0 is in orange. The analysed bins are in yellow. We looked at the 2h before and after the bin 0. We considered two bins and the amount of sleep occurred in those bins. **(B)** From **(i)** to **(iv)** simple linear regressions between the amount of sleep in the 2h before bin0 and the features of the trace. Each point in each graph represents the data from one recording. Number of flies=7. **(C)** From **(i)** to **(iv)** simple linear regressions between the amount of sleep in the 2h after bin0 and the features of the trace. Each point in each graph represents the data from one recording. Number of flies=7. **(D)** Summary of the variation of R squared values. Dots of the same colour refers to the same feature of the trace. On the x axis “before” and “after” referring to the bin analysed in a specific linear regression analysis.

In every window of time considered in this analysis: 1) the higher is the number of peaks in a trace, the more is the sleep after the recording session; 2) the higher is the mean interval between peaks, the little is the “sleep after”; 3) the higher is the mean peak width, the little is the “sleep after”; 4) the higher is the fluorescence, the little is the “sleep after”. In other words, a trace with a high number of peaks, a short interval between peaks, little peaks’ width it is likely to predict that the fly is going to sleep soon.

How good is this prediction? I observe the best correlation between the Ca^{2+}-traces and the “sleep after” when I consider the window of time of 1 hour after the 30 minutes when I recorded the imaging session (Figure 4.6,A). Of all the features of the trace, the peaks number could be considered the best predictor of the locomotor behaviour of the fly in the following hour, while the fluorescence is the worst predictor (Figure 4.6,B).

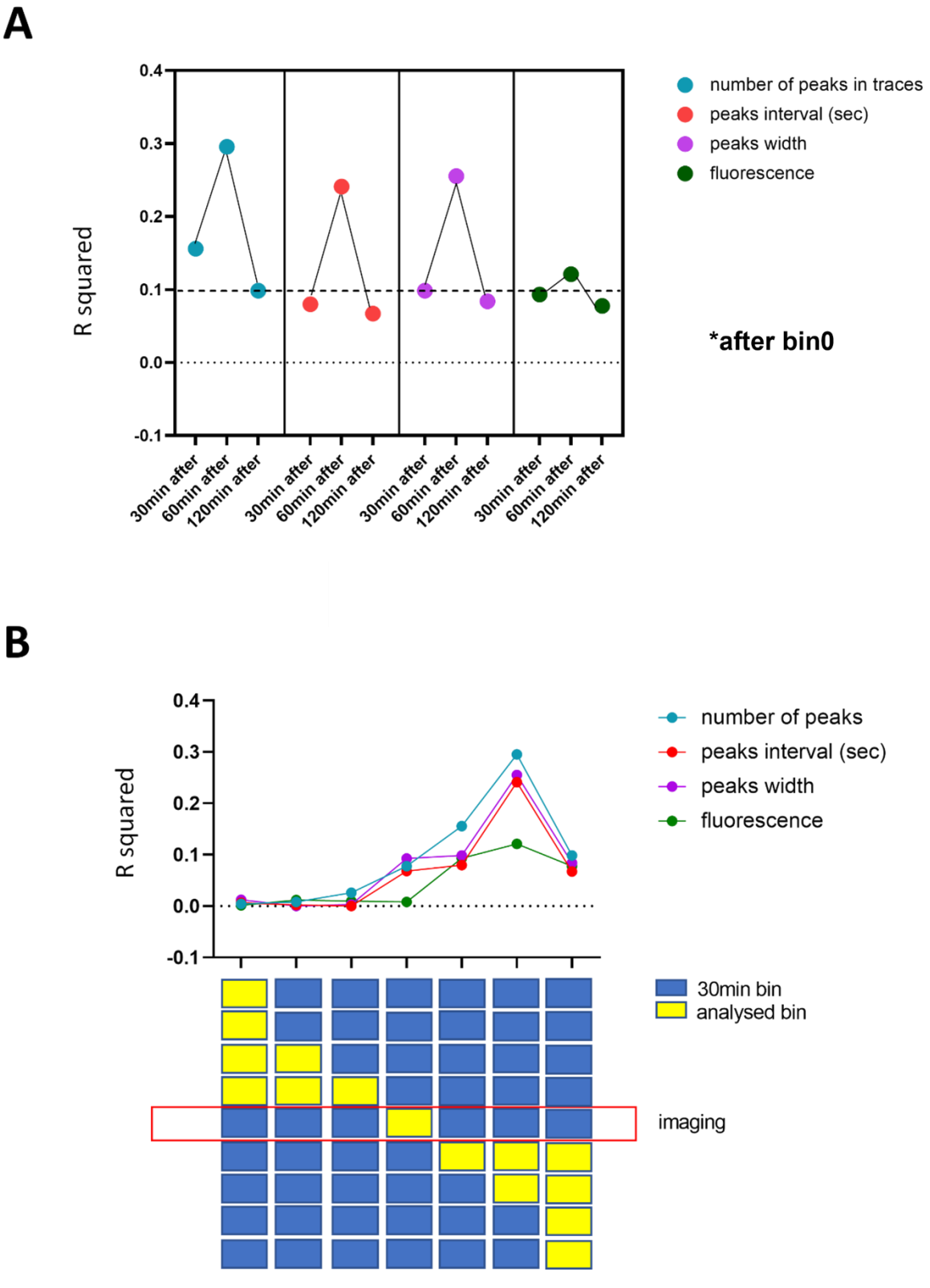


Figure 4.6 The strongest correlation is found when considering sleep 1h after the bin0

4.6 The strongest correlation is found when considering the amount of sleep 1h after the bin0

(A) and (B) Summary of the variation of R squared values, which reflect simple linear regressions considering the Ca^{2+} traces and sleep after bin0. Dots of the same colour refers to the same feature of the trace. On the x axis each label refers to the bin analysed in a specific linear regression analysis.

These findings are supported by the observation of the trend of the average number of peaks in traces and the average amount of sleep/30minutes in 24h. As shown in Figure 4.7, just before the amount of sleep increases, I observe an increase in the average number of peaks in traces.

A

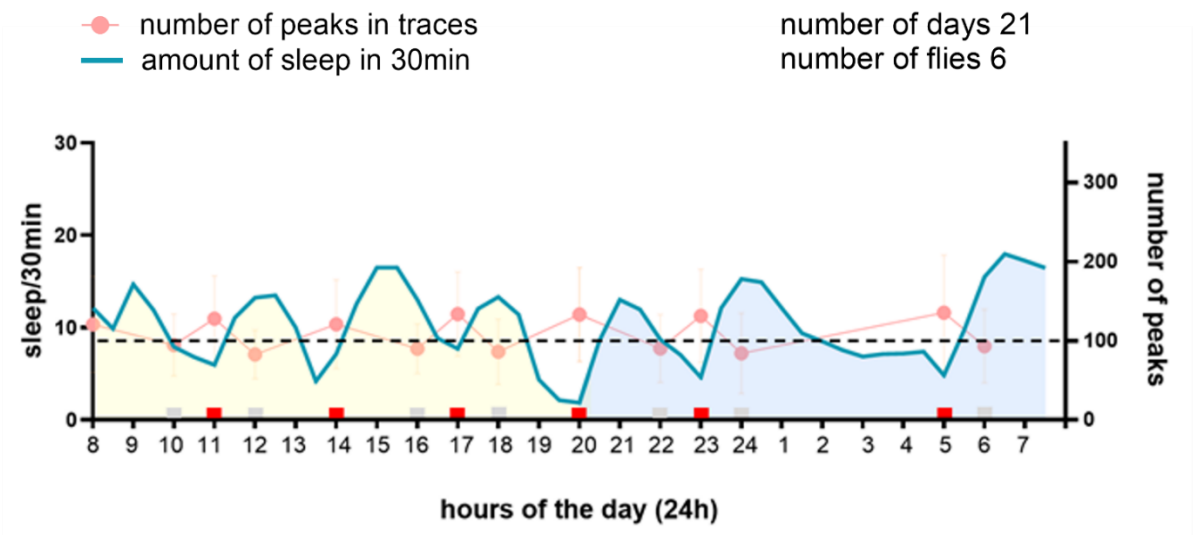


Figure 4.7 The number of peaks in traces could be a predictor of sleep after the imaging session

(A) On the x axis the time of the day in hours (24h). On the right y axis, the amount of sleep every 30 minutes. On the left y axis, the number of peaks in the trace. Each salmon dot refers to the average number of peaks (±sem) found in the Ca^{2+} traces recorded at that specific time of the day. Corresponding to each dot a small square indicating if the number of peaks is either above (red square) or below (grey square) a set threshold. The blue line follows the variation in the amount of sleep in an entire day. It represents an average of the amount of sleep of 6 flies over 21 days.

Interestingly, I found that the goodness of the prediction of sleep from the number of peaks in an imaging trace depends on the time of the day and negatively correlates with the change in fluorescence ($\Delta R/R_0$). As shown in Figure 4.8, the R squared values decreases when fruit flies would normally have their peak of locomotor activity, dictated by the circadian clock.

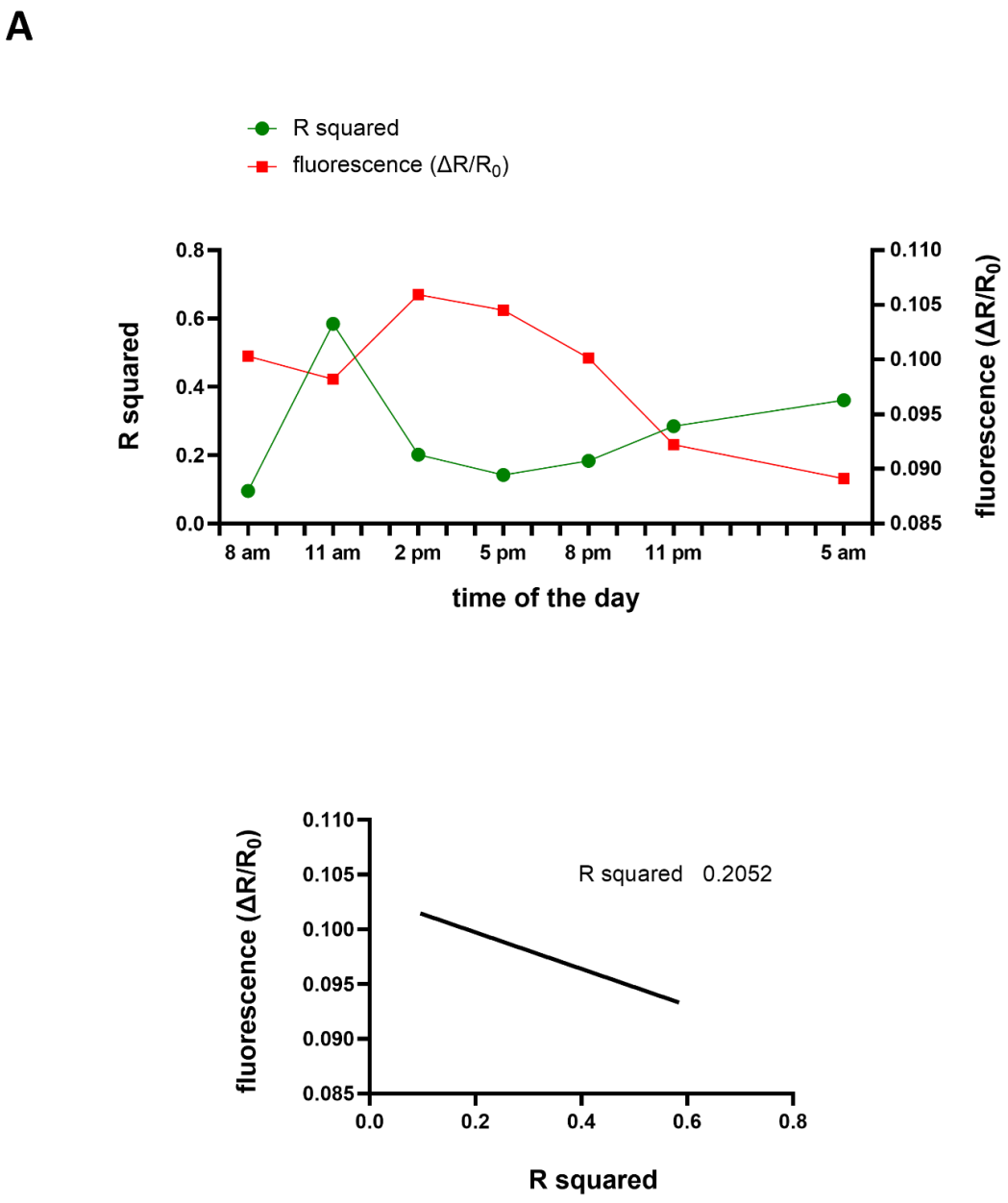


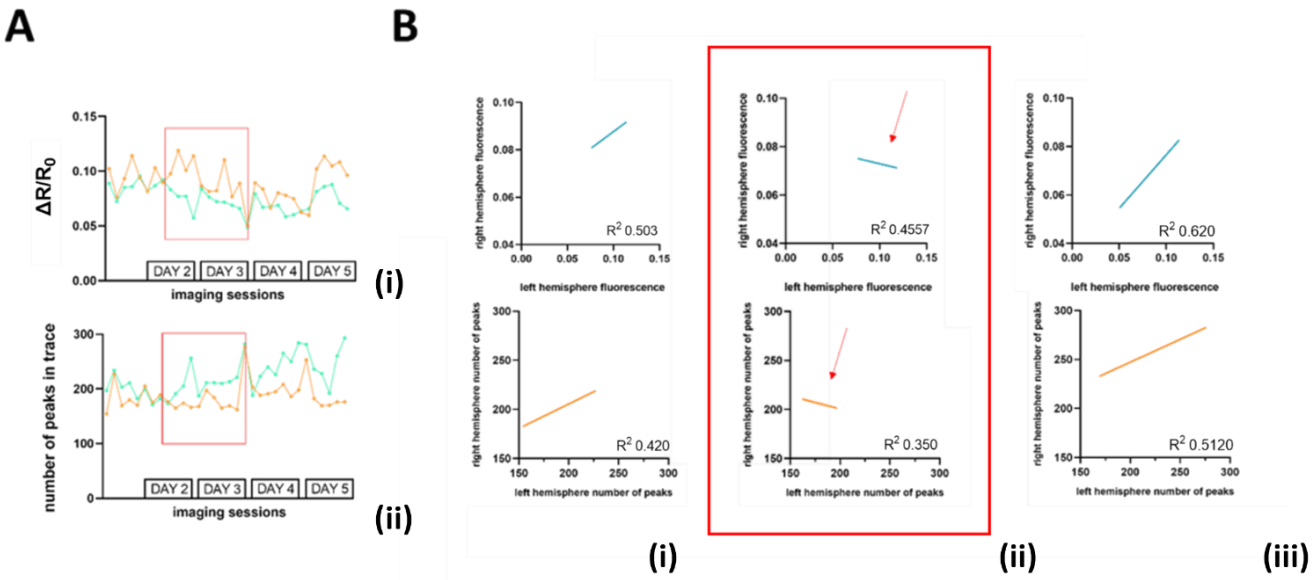
Figure 4.8 The goodness of the prediction of sleep from the number of peaks in an imaging trace depends on the time of the day and negatively correlate with the change in fluorescence ($\Delta R/R_0$)

4.8 The goodness of the prediction of sleep from the number of peaks in an imaging trace depends on the time of the day and negatively correlate with the change in fluorescence ($\Delta R/R_0$)

(A) Example from one single fly. The change in the R squared over the course of 24h is plotted together with the change in the mean fluorescence ($\Delta R/R_0$) of traces recorded in different times of the day. R squared results from simple linear regression between the number of peaks in a recorded trace and the amount of sleep in 1h after the recorded trace. The mean fluorescence and the R squared negatively correlate during 24h.

4.3 Ca^{2+} dynamics between the two hemispheres could be linked with sleep

In the previous chapter I showed the presence of a positive correlation between the dendritic arbors of the two hemispheres. Interestingly, when I looked at the features of the Ca^{2+} imaging traces over time I noticed that the traces recorded from the two hemispheres were not always correlating in the same way (Figure 4.8,A). Indeed, for a limited time I found a negative correlation between the two traces instead of a positive correlation. Curiously, this negative correlation was not just limited in time, but also limited to certain features of the trace, such as the peaks number (Figure 4.8,B).



4.9 Dendritic Ca^{2+} dynamics in the two hemispheres show negative correlation for a limited amount of time

(A) Variation of the values relative to the mean fluorescence **(i)** and the number of peaks **(ii)** in a Ca^{2+} imaging trace. Each dot is representative of one recording. On the x axis the consecutive imaging sessions and the day when this imaging sessions were recorded. On the y axis the variation of distinct features. The red square indicates the time interval considered for our analysis. **(B)** Variation of the correlation between the values of one hemisphere and the other. Considered features of the trace: fluorescence and number of peaks. In **(i)** the result of a simple linear regression analysis considering the values recorded before the time marked by the red square. R^2=0.503 correlation between the fluorescence recorded in one hemisphere vs the one recorded in the other hemisphere. R^2=0.420 correlation between the number of peaks recorded in one hemisphere vs the one recorded in the other hemisphere. In **(ii)** the result of a simple linear regression analysis considering the values recorded within the time marked by the red square. R^2=0.4557 correlation between the fluorescence recorded in one hemisphere vs the one recorded in the other hemisphere. R^2=0.350 correlation between the number of peaks recorded in one hemisphere vs the one recorded in the other hemisphere. In **(iii)** the result of a simple linear regression analysis considering the values recorded after the time marked by the red square. R^2=0.620 correlation between the fluorescence recorded in one hemisphere vs the one recorded in the other hemisphere. R^2=0.5120 correlation between the number of peaks recorded in one hemisphere vs the one recorded in the other hemisphere.

I asked myself if this phenomenon could be linked with sleep. To my surprise I found that the sleep before the time window when the two hemispheres have a negative correlation is less than the average sleep for the analysed fly (Figure 4.9). I observed a change in the traces from around 5pm of the 2nd day till 5am of the 4th day, considering 24h from lights on (8am). During the 3rd day, when for an entire 24h the two hemispheres were negative correlated, the total sleep was higher than the rest of the other days (Figure 4.9,A (iii)). Night and day sleep during the 3rd day was also higher than the other days (Figure 4.9,A (iv-v)). When I considered a cycle of 24h from 5pm of the 2nd day, when I observed the change in Ca^{2+} dynamics between the two hemispheres, I found that the total sleep was much higher than the sleep in the 24h before and slightly higher of the total sleep in the 24h after (Figure 4.9, (ii)). Looking at the sleep profile of the analysed fly (Figure 4.9,A (i)) I could appreciate how sleep during day 4 is higher comparing it with the sleep during day 1 and partially day 2, suggesting a high average baseline sleep.

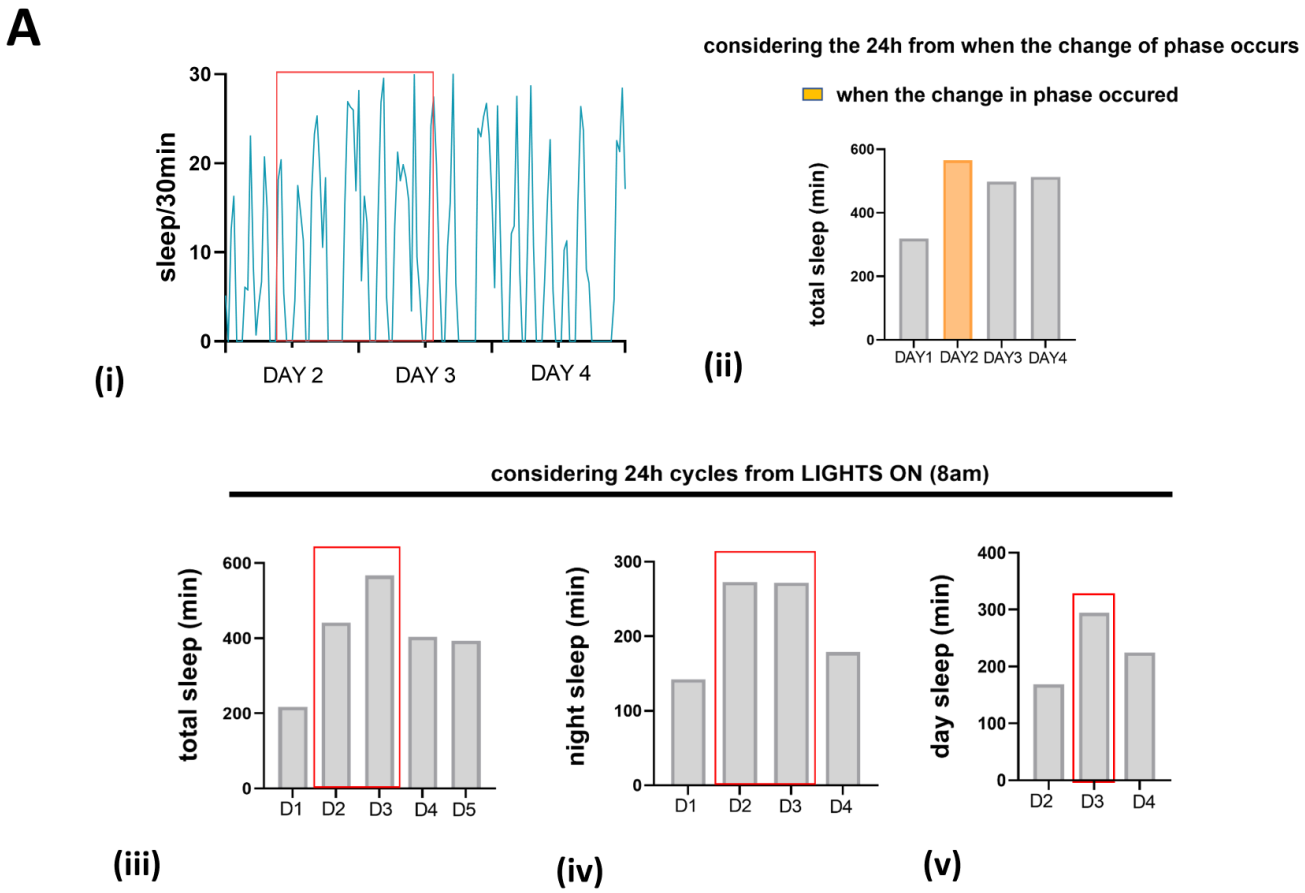


Figure 4.10 Negative correlation of dendritic Ca^{2+} dynamics in the two hemispheres occurs after reduced sleep

(A) The variation in the amount of sleep before, during and after the time-window marked by the red square. In **(i)** the sleep profile of the analysed fly. On the y axis the amount of sleep for every 30 minutes. On the x axis the consecutive days. Each single day is 24h starting at 8am. In **(ii)** we define a day 24h starting at the time point when we saw a change in the trend of correlation between the left and right hemisphere. From **(iii)** to **(v)** we define a day 24h starting when lights are ON at 8am. The red squares indicate the time when we observed the change in correlation between the hemispheres.

5 | Sleep deprivation under a two-photon microscope

As much as the experimental approach developed and presented in the previous chapters could be informative and relevant to provide evidence of the physiology of sleep in *Drosophila*, integrating to this approach a system to control the behaviour of the fly during chronic neurobehavioural experiments would be extremely useful. I decided to develop a protocol to disrupt rest in *Drosophila* while performing long recordings of the neuro behavioural dynamics of a fly living under a two-photon microscope.

Sleep-deprivation has been extensively studied in *Drosophila*. Insufficient sleep disrupts learning (Li et al., 2009), suppresses aggressive behaviour (Kayser et al., 2015), and impairs a wide range of essential processes such as alertness, metabolic activity (Stahl et al., 2017), and immune function (Kuo and Williams, 2014). Sleep-loss in *Drosophila* is linked with a change in the electrical activity of the dFB neurons and in their metabolic state, with increased firing rate and mitochondrial reactive oxygen species after a night of sleep deprivation (Kempf et al., 2019). In this chapter I present the work that led to development of a system to sleep deprive flies under a two-photon microscope.

5.1 Adapting a protocol for feeding and imaging before, during and after sleep deprivation

Studies on sleep deprivation in flies have shown that sleep deprivation is more effective when it occurs during the relative night of the fly (Potdar et al., 2017; Kempf et al., 2019). A recent work to study the accumulation of ROS in dFB neurons shows that mitochondrial ROS production rises after a night of sleep deprivation, but not if the same protocol of sleep deprivation is used during the day or if it is prolonged on the following day after the night sleep deprivation (Kempf et al., 2019).

With these premises I decided to design a protocol to allow simultaneously sleep deprivation of the fly walking on the ball and multiple imaging sessions overnight.

I first changed the relative light and dark phase to have lights off at 10am of the experimenter time instead of 8pm, with the aim of facilitating the collection of data. I then tested a feeding protocol to fit with the new light-dark cycle. As shown in Figure 5.1,A the new protocol had 7 feeding sessions, all very close to the time when usually the fly is more active. I found that this protocol does not alter the rhythmicity of the fly (Figure 5.1,B (i)). Flies are more active at the end of the light phase, and they would sleep more at night than during the light phase (Figure 5.1,B (ii)).

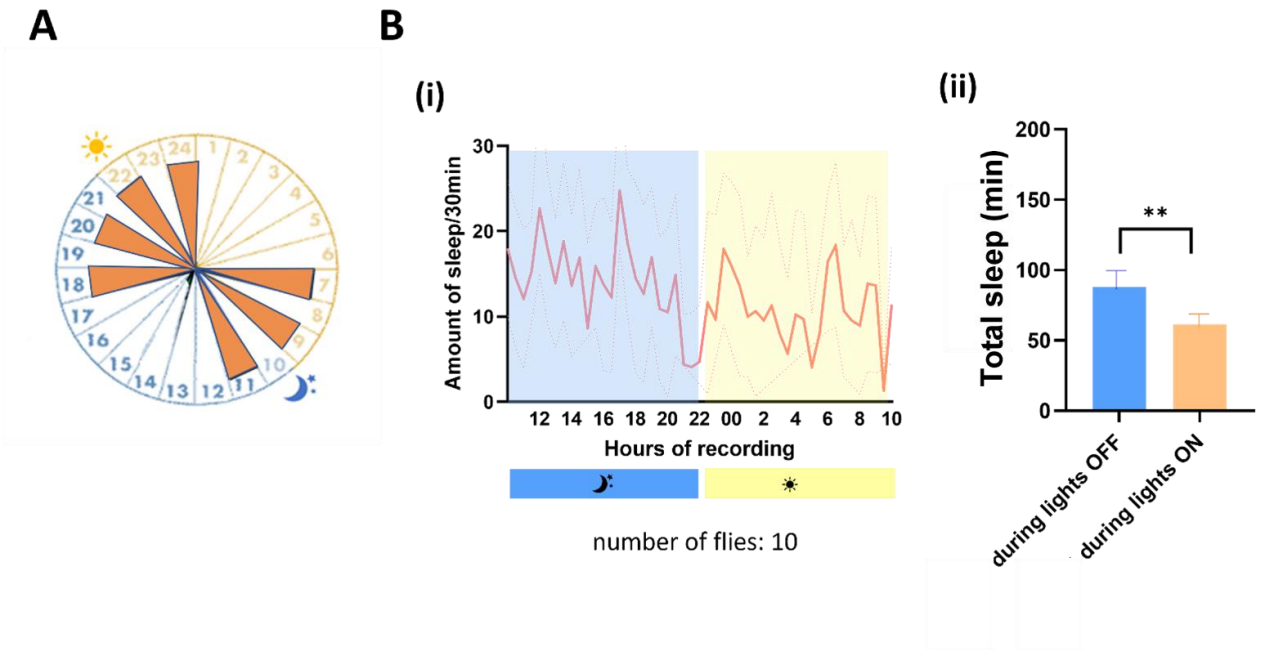


Figure 5.1 Coherence between the feeding protocol and the circadian locomotor rhythmicity allows changing of the light-dark cycle without losing the biphasic sleep pattern

(A) An illustration of the feeding protocol with flies entrained in a light cycle with lights ON at 10pm (experimenter time) and lights OFF at 10am (experimenter time). **(B)** Sleep of flies tested with the feeding protocol in (A). In **(i)** the sleep profile of 10 flies. Dotted lines represent the error bars (± sem). In **(ii)** the amount of sleep during the relative night and day. A day: 24h from 10pm (experimenter time). Kolmogorov-Smirnov test detected significant effect in the total sleep/day (P=0.0054). Data are means ± s.e.m.

Next, I suggested two imaging protocols, which I am currently testing (Figure 5.2). A more invasive imaging protocol, shown in Figure 5.2,A, would give us the possibility to potentially observe a progressive increase in the neural activity of dFB neurons with the increase of sleep pressure caused by the sleep deprivation protocol. A total of six imaging sessions every other hour during the sleep deprivation overnight. The following night is used as a control, with one imaging session after light are off, one in the middle of the night and one before lights are on.

In Figure 5.2,B is shown the second protocol I am proposing. This protocol is less invasive during the already stressful sleep deprivation. The number of imaging sessions during sleep deprivation would be the same as any other night: one at the begging of the night, one in the middle and one at the end of the night. The advantage of this protocol is that it offers a window for imaging the neural dynamics of the dFB during the sleep rebound, while the first protocol presented leaves the fly undisturbed during the sleep rebound period after sleep deprivation. With the second protocol, during daytime the imaging sessions would be: two imaging sessions 1 hour apart at the beginning of the light phase and two imaging sessions 1 hour apart at the end of the light phase. One aim would be to compare both the behaviour and the neural dynamics in the dFB before sleep deprivation and during the period of sleep rebound. Moreover, it is not known for how long sleep deprivation affects the Ca^{2+} dynamics of the dFB neurons. Understanding the neural representation of sleep deprivation and of its consequences over multiple days is part of the current effort.

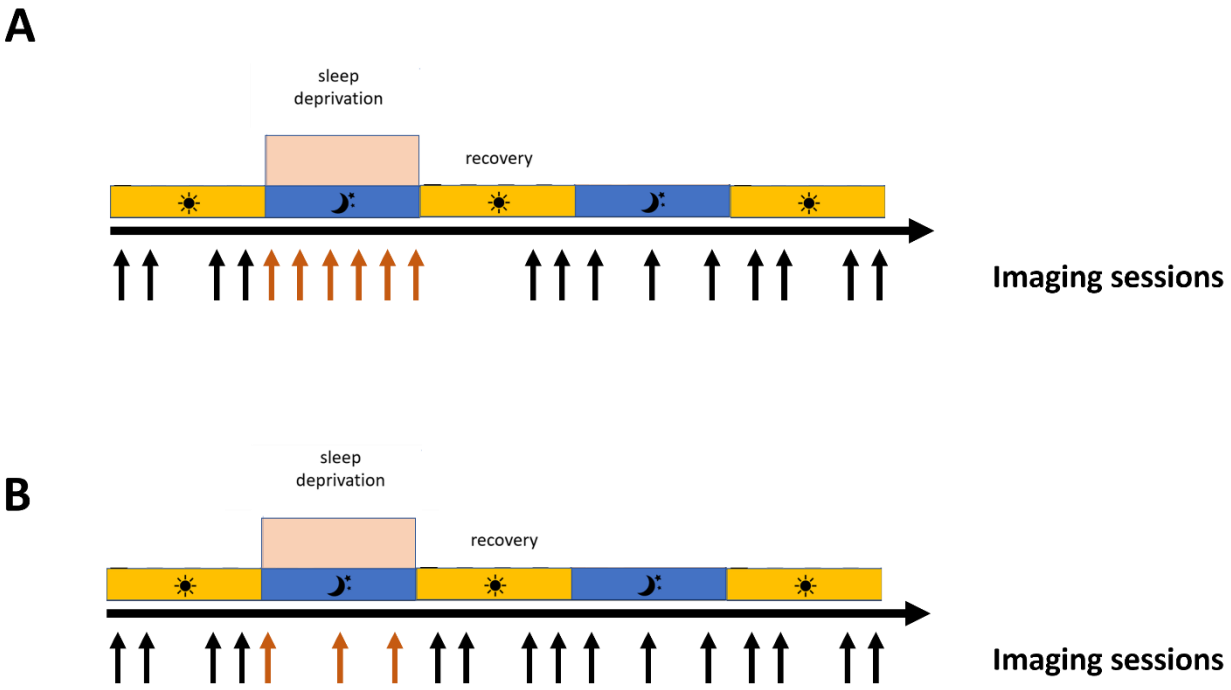


Figure 5.2 An imaging protocol to follow dendritic Ca^{2+} dynamics before, during and after sleep deprivation in a two-photon setup

(A) An illustration of the first of the two the imaging protocols used for sleep deprivation under the two-photon microscope. Each arrow represents an imaging session. Imaging sessions occur every other hour during sleep deprivation. During the first phase of sleep rebound there are no imaging sessions. Total number of imaging session during each day (excluding day with sleep rebound): 4. **(B)** An illustration of the second of the two the imaging protocols used for sleep deprivation under the two-photon microscope. During sleep deprivation there are only three imaging sessions, without any difference from the control condition (night without sleep deprivation). During sleep rebound there are imaging sessions at the beginning and end of the relative day.

5.2 Air puffs are effective in eliciting an active locomotor response in head-fixed flies

Among the different possible ways to sleep deprive *Drosophila*, I decided to first test a protocol consisting of repetitive air puffs directed at the experimental fly, causing air puff stress (APS). Air puffs have been used already to test the locomotor response of flies (Wolf et al., 2002; Lebestky et al., 2009) and thanks to a custom-written code and an Arduino platform, air puffs could be delivered in a computer-controlled way to a fly living under a two-photon set up (John-Paul Moszynski built the software for delivering controlled air puffs).

I tested flies 4-7 days old, to match the age of flies during chronic neurobehavioural recordings. Repetitive air puffs were presented in different ways to head-fixed flies freely walking on a spherical treadmill. All puffs were delivered frontally, with a pressure of 25Psi/3LPM. All the protocols were executed during the relative night for the experimental fly.

In a first protocol (Figure 5.3,A) I delivered puffs of air every 2 minutes. To mark the start of the puffs I turned lights on for 20 seconds (light are off during the relative night of the experimental fly), after 3 seconds I delivered a series of air puffs: 10 air puffs every 3 seconds, each puff lasting 200ms (Figure 5.3,A (ii)). This protocol was effective in inducing a locomotor response in head-fixed flies (Figure 5.3,a (i)): a progressive acceleration Is observed in flies receiving the train of air puffs, following which flies return to their pattern of activity. This was in line with what was observed in the literature (Lebestky et al., 2009). Although this effect was observed repeatedly, it was not consistent during the entire length of the relative night (white marks in Figure 5.3,A (ii) represent movement). Since I wanted to sleep deprive the head-

fixed fly for the entire length of the night, I tested a second protocol. In the second protocol (Figure 5.3,B) I delivered 200ms puffs of air every 3 seconds for the entire night. While flies responded well to the first hours of the protocol, the intensity of the train of puffs was too high and flies did not survive the night.

I finally tested the last protocol presented here: puffs of air of random intensity were delivered randomly for 20 seconds, every 20 seconds (Figure 5.4).

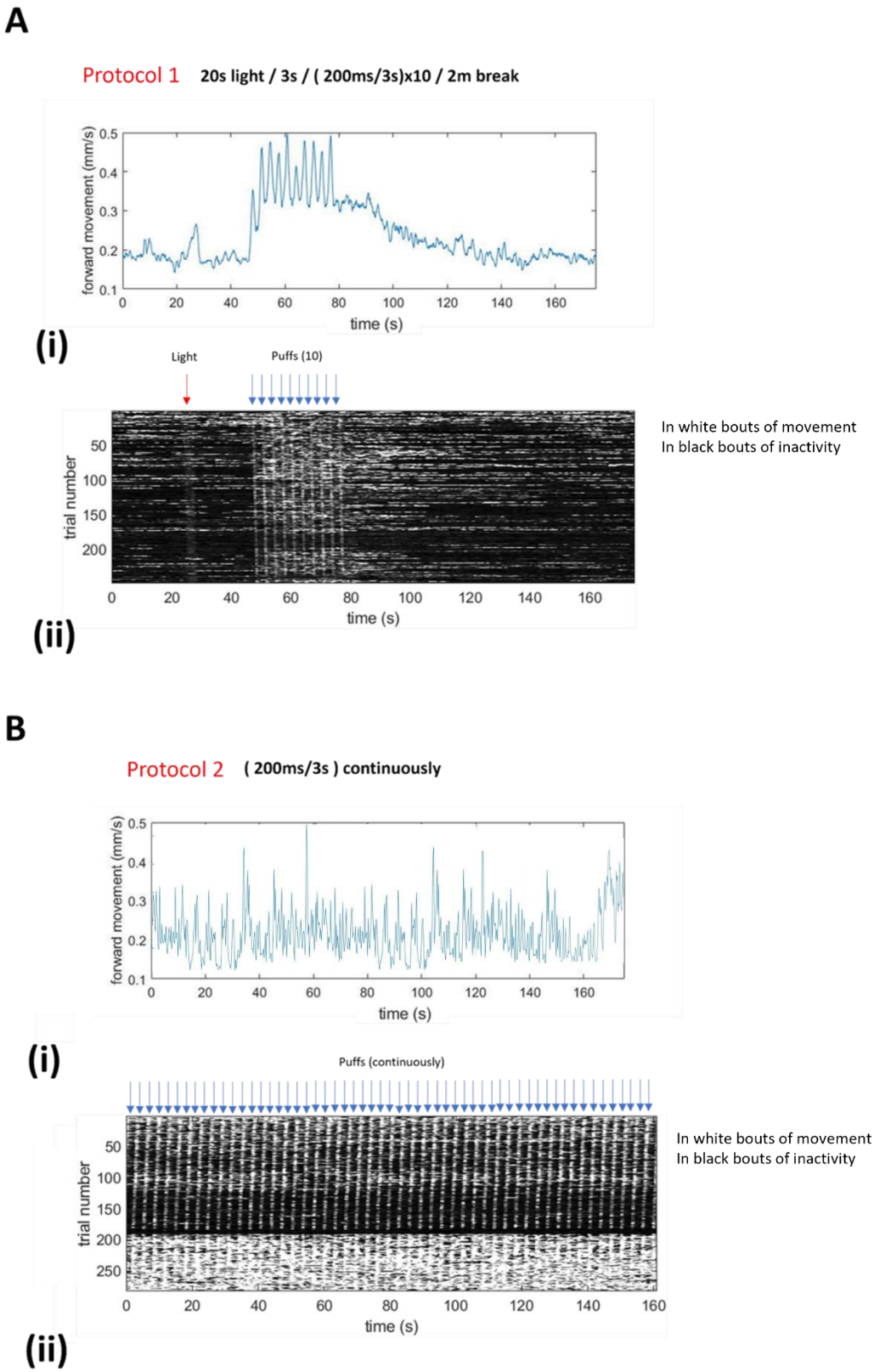


Figure 5.3 Developing a protocol to sleep deprive flies for 12h under a two-photon microscope

5.3 Developing a protocol to sleep deprive flies for 12h under a two-photon microscope

(A) The speed of one single fly using *Protocol 1.* **(i)** The speed of the fly in one single trial. On the y axis the speed, measured in mm/s. On the x axis the length of one single trial in seconds. In each trial 10 puffs of air lasting for 200ms. After 40s to appreciate the four peaks corresponding to the puffs of air. **(ii)** The speed of the fly during different consecutive trials. On the y axis the number of trials starting from the top. On the x axis the length of a trial in seconds. In white are the bouts of movement and in black the bouts of inactivity. The arrows indicate the timing of the light lasting for 20s, 3s before the 10 puffs, and of the 10 air puffs. **(B)** The speed of one single fly using *Protocol 2.* **(i)** The speed of the fly in one snapshot of 3minutes, during the length of the protocol. On the y axis the speed, measured in mm/s. **(ii)** Continuous puffs of air lasting for 200ms. In white are the bouts of movement and in black the bouts of inactivity.

5.2.1 Random air-puffs for 12h induce sleep rebound

As shown in Figure 5.4,A, puffs of humidified air of 25Psi/3LPM were delivered in a random fashion, each time for one of the following durations: 50ms(msec), 100ms, 150ms, 200ms. The train of random puffs was delivered every 20seconds in a 20seconds bin, for 12hours.

Random puffs of humidified air for 12h elicited a strong behavioural response in head-fixed flies freely walking on a spherical treadmill (Figure 5.4,B). Looking at the filtered movement trace in Figure 5.4,B, it is possible to follow the acceleration of sleep deprived flies versus the one of control flies (head-fixed without receiving air puffs) over the course of the relative night. The binarized trace (Figure 5.4,B) shows the increase in rest in the sleep deprived flies in the 12-h following sleep deprivation. This observation is confirmed by further analysis of the fly sleep.

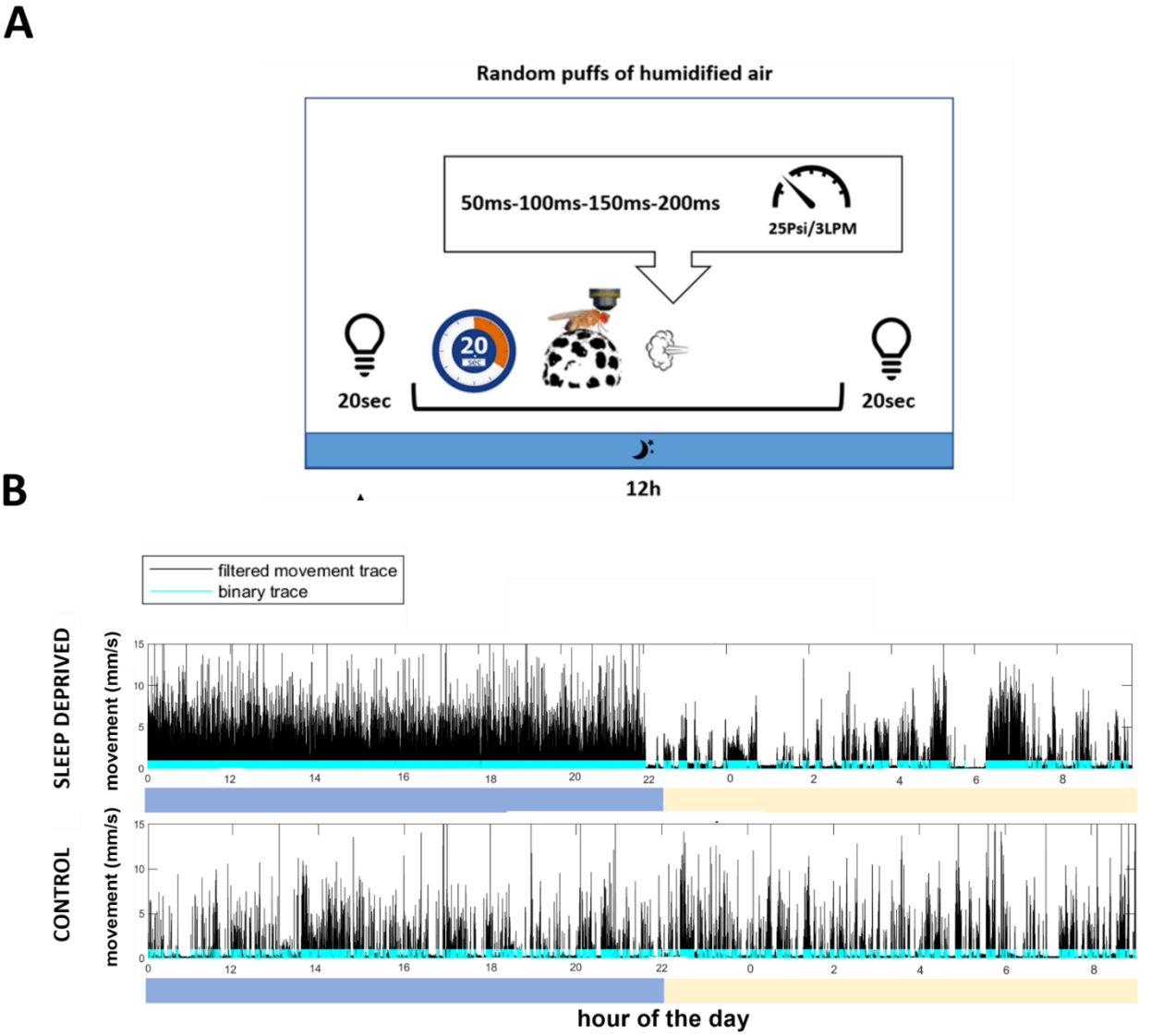


Figure 5.4 Random puffs of humidified air for 12h elicits a strong behavioural response in head-fixed flies freely walking on a spherical treadmill

5.4 Random puffs of humidified air for 12h elicits a strong behavioural response in head-fixed flies freely walking on a spherical treadmill

(A) An illustration of the *Protocol 3*. **(B)** Filtered and binarized traces from two flies during one single day (24h). On the x axis the hours of the day, from lights ON at 8am. On the y axis the speed, measured in mm/s. The top graph shows the speed of a fly during and after the sleep deprivation protocol. The bottom graph shows the speed of a fly used as a control (no sleep deprivation). Sleep deprivation occurs during the relative night.

The sleep profile in figure 5.5,A shows a significant increase in the sleep/30minutes during the light phase following sleep deprivation comparing it with control flies. Quantification of the amount of sleep (Figure 5.5,B) shows a significant increase in the sleep amount during the entire day after a night of sleep deprivation, interestingly the increase of sleep seems to be significantly higher during the first 6 hours of the light phase. One of the main features of sleep rebound is the timing when this happens: as soon as the sleep deprivation ends. Looking at the sleep bouts distribution of control and sleep deprived flies I also observe that sleep after sleep deprivation became more intense, with more and longer sleep bouts (Figure 5.5,C). Overall, random puffs of humidified air for 12 hours during the nighttime induced sleep rebound: they significantly increased the quantity and the quality of sleep during the light phase following air puffs stress.

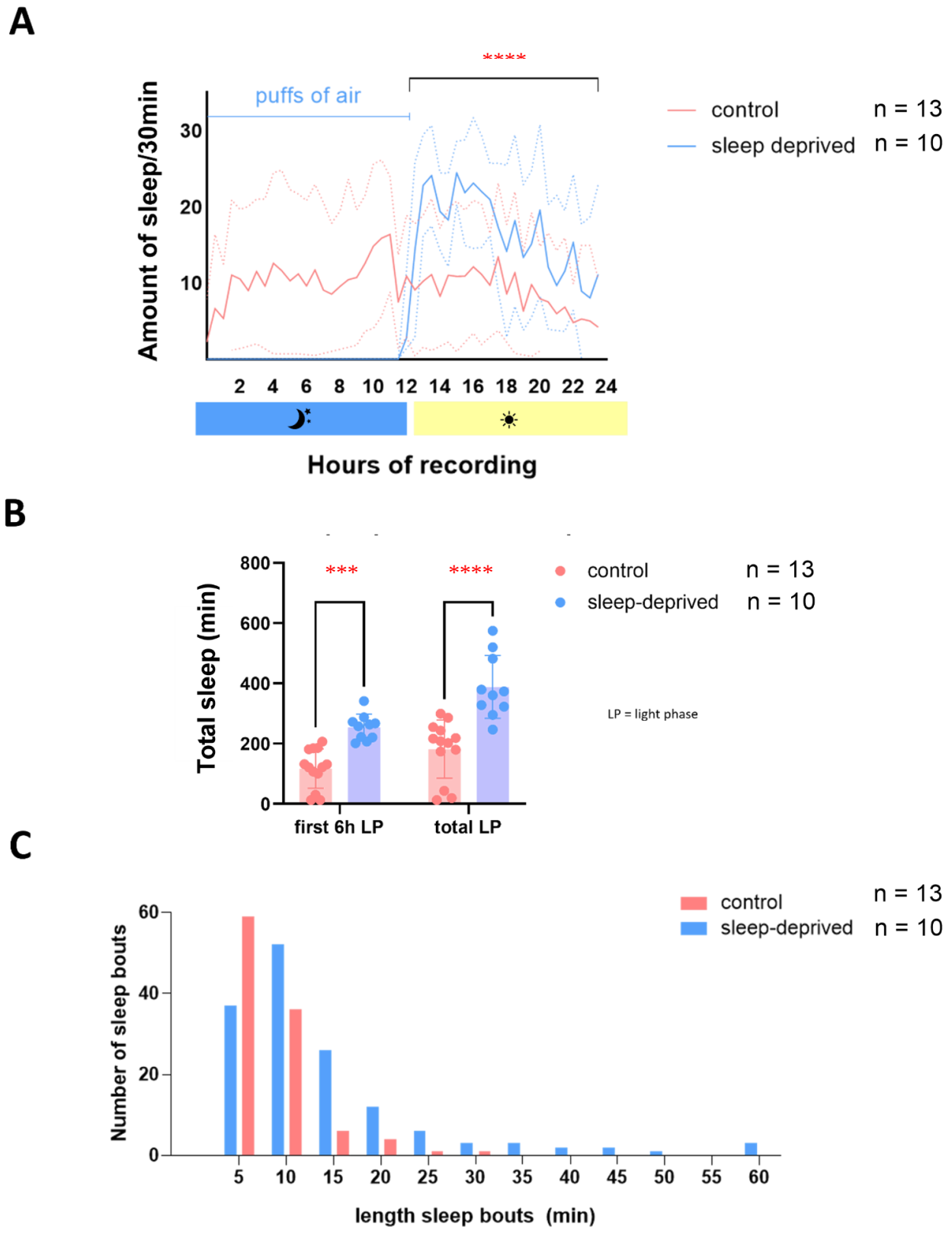


Figure 5.5 Overnight sleep deprivation using random puffs of humidified air induces sleep rebound

5.5 Overnight sleep deprivation using random puffs of humidified air induces sleep rebound

(A) The sleep profile of control and sleep-deprived flies. On the y axis the amount of sleep every 30minutes. On the x axis the hours of the day (24h). Data are means ± s.e.m. Two-way repeated-measures ANOVA detected a significant difference in sleep between sleep-deprived flies and controls during sleep rebound. (P=0.00007 number of control flies=13, number of sleep-deprived flies=10). **(B)** Comparison of the total amount of sleep measured in control and sleep-deprived flies. During the first 6h of the light phase and during the entire length of the light phase following the night of sleep deprivation. Two-way repeated-measures ANOVA detected a significant difference in sleep between sleep-deprived flies and controls during sleep rebound. (During the first 6h P=0.0008 During the entire length of the light phase P=0.000065 number of control flies=13, number of sleep-deprived flies=10). **(C)** Quality of sleep during the light phase following the night of sleep deprivation. Distribution of sleep bouts length. On the x axis the length of sleep bouts in bins of 5 minutes. On the y axis the number of bouts falling in a specific bin (number of control flies=13, number of sleep-deprived flies=10).

6 | Discussion

One fundamental question has fascinated scientists for generations: how does the brain process sensory information into motor responses and coordinate behaviour?

Different generations of scientists have tried to answer this question using the tools offered by their scientific community. Only at the beginning of the XX century, genetics contributed to an exponential growth of neurobehavioural experiments (Benzer, 1967). As powerful genetics is in verifying if specific neurons are necessary and sufficient for a behaviour (by either inactivating or stimulating neurons of interest) it lacks the ability to unveil how the information underlying a specific behaviour is represented by neural activity. In the last twenty years, the advance in the technology has helped scientists record the activity of the brain, at the circuit and single-cell level (Lewis et al., 2015).

Neurobehavioural experiments are challenging for two reasons: 1) only single individual animals could be studied for one recording session. Consistent results require animals tested in similar conditions, difficult to keep between different experiments. Confounding factors, such as stress, thirst, and hunger need to be taken into consideration together with individual differences. 2) defining neuronal activity and correlating distinctive features of the recorded signal with behaviour require an elaborate level of analysis. Neurons usually receive numerous stimuli, and their processing is influenced by the connection with other neurons and the internal state of the neuron itself.

The fruit fly *Drosophila melanogaster* has been extremely useful in understanding the connections between the brain and behaviour (Devineni and Scaplen, 2021). Thanks to the fly connectome the fly community has been presented with the

potential of study in dept the brain circuits of *Drosophila* (Scheffer et al., 2020). However, studying how the neural activity in the circuits results in computations and then in behaviour has been limited by the lack of validated experimental techniques, as I explained already in the first chapter of this work.

The work presented here was an exploratory study of neurobehaviour, which had the main goal of validating an experimental technique for chronic imaging the brain dynamics of a behaving fly using two-photon imaging. The advantage of using two-photon imaging is the possibility of monitoring the activity of a population of neurons and the neural dynamics in two structures of the neurons: the dendritic arbor and the axons.

In *Drosophila*, complex behaviours are generally performed using a relatively small number of neurons. I decided to investigate sleep and two dozen neurons projecting to the dorsal fan-shaped body (dFB) (out of 100,000 neurons of the fly brain), which could be identified using the genetic line R23E10 and represent the sleep switch of Drosophila (Donlea et al., 2014). Neurons comprising the dFB are embedded in a recurrent circuitry that controls homeostatic sleep (Donlea et al., 2018). In other words, these neurons are known to sense and correct sleep deficits. This system is integrated with the circadian clock, which controls sleep-wake cycles (Andreani et al., 2021; Raccuglia et al., 2022). Over the past years, an elaborate framework has been developed, describing how this *somnostat* – which has the dFB as its output unit – accumulates sleep pressure during waking and discharges it during sleep (Donlea et al., 2014; Liu et al., 2016; Raccuglia et al., 2019; Kempf et al., 2019). The evolution of this framework is fuelled by the rapid progress in discovering components on the circuit, cellular, and molecular levels. Yet, it is unknown how the information relative to the need of sleep is represented in the dFB neurons and how the variety of wake and sleep-promoting signals are processed and integrated into

the dFB dendrites, over the course of the day and over multiple days. Moreover, we are not aware of the time course over which the dFB neurons discharge sleep pressure to control sleep. Accumulation/decay of sleep pressure with a time constant of hours would point towards different cellular processes underlying sleep homeostasis than it would with a time constant of minutes. This is simply because the temporal dynamic range of the biochemical process in question must match the time course of the neuronal representation.

I used movement (the rotation of a spherical treadmill on which the fly rests or walk) as the behavioural measurement and the fluorescence emitted by the genetically encoded Ca^{2+}-sensor GCaMP6f as our neural activity measurement. When the fly was not moving for five or more minutes, rest was considered sleep (Hendricks et al., 2000; Shaw et al., 2000).

In the second chapter I presented the experimental set up and the protocol to monitor dFB activity and sleep-wake behaviour for up to seven days in individual flies. Measuring dFB neuronal activity with calcium imaging has the potential of capturing emergent functional properties of neuronal circuits, although calcium sensors are very sensitive to brain movement and photobleaching (Dana et al., 2019). I tested different ways to overcome these problems. Here I showed how I could successfully image for days the *Drosophila* brain through a chronic cranial window sealed with a thin layer of UV-curable glue. This glue has refractive index close to that of water, cures quickly, is solid and stabilises the brain, which improved some limitations of previous approaches. I showed the procedure to implant the cranial window, and the window itself. This allows chronic imaging without damaging the brain and reducing significantly photobleaching without influencing sleep-wake profiles of freely moving flies.

Our experimental set up implies head-fixation of the fly during the entire length of the experiment. Whereas this approach could be seen to limiting the behaviour of the animal, it is not proven that brain dynamics in head-fixed animals are substantially different from the ones in freely behaving animals. One of the main advantages of having head-fixed flies in the setup is the potential of controlling the sensory experience of the fly (Seelig et al., 2010). With this in mind, I designed a way to deliver food to the fruit fly during the experiment, without moving the fly from the microscope and minimizing any external interference. After testing different diets, I chose to deliver liquid holidic medium, adapting a recipe of Mathtew D W and colleagues (Piper et al., 2014). The evidence presented in the second chapter validate the use of liquid holidic medium for chronic monitoring of neurobehavioural dynamics under a two-photon microscope. The diet does not alter the sleep-wake cycle of flies and offers the possibility of investigating the effect of soluble drugs and alteration of the balance of nutrients, amino acids, vitamins on behaving animals during functional imaging. Moreover, the experimental set up presented here allows the study of feeding behaviour and brain dynamics over days.

I selected the feeding intervals and duration to maintain the walking behaviour of the fly over multiple days, as well as ensure sufficient walking activity after feeding (which could cause postprandial sleep (Murphy et al., 2016)). I showed that flies learn to feed under the microscope, despite the fact of showing inter individual variability in the response to the diet and the feeding intervals. As validated in chapter five, feeding is much needed closer to the peak of locomotor activity and undergoes a circadian regulation. When looking at the locomotor trace of individual flies extracted using video-tracking, it is possible to appreciate how freely moving flies have quite different pattern of activity one day from another and one fly from another. What most of them have in common is the increase locomotor activity

toward the end of the light phase.

In the third chapter of this work, I investigated the features of the signal recorded imaging for days the dFB neurons. Analysing Ca^{2+} signal patterns, and so extracting descriptors of the Ca^{2+} peaks is necessary for understanding kinetics of calcium signalling and downstream processing as well as the nature of the external signalling (Moein et al., 2018). Within a recorded imaging trace numerous information lie hidden. The relation between an external signal and a Ca^{2+} transient is not linear. The intensity of the stimulation and the number of stimuli influence the Ca^{2+} response (De Pittà et al., 2009). Moreover, several dynamical variables play a role in defining the response of a cell to stimuli: e.g., intracellular diffusion mechanisms, kinetics and/or level of expression of ion pumps and ion channels. Into this frame, I need to add the possible contribution of spontaneous Ca^{2+} events to the recorded signal (De Pittà et al., 2008).

Temporally, Ca^{2+} signals are evaluated as time-dependent changes in mean fluorescence, within a user-defined area, otherwise called region of interest (ROI). Fluorescence (defined by $\Delta R/R_0$) is not enough to characterize isolated transients, oscillation, and propagating waves (Francis et al., 2014). I focused on the following features of the traces: 1) the peak amplitude, 2) the peak width, 3) the interpeak interval, 4) the number of peaks. Information can be encoded in each of these features (Moein et al., 2018). In the past years has emerged that excitable cells could use either the frequency of peaks or their amplitude or both to decode informational input from multiple channels (Uhlén, 2004; De Pittà et al., 2009). The peak width, defined by the time difference between two points of the peak, reflects the duration of each peak and therefore the high activation of Ca^{2+} channels and increased intracellular Ca^{2+} concentration. Lastly, the distributions of interpeak interval (the time difference between two different peaks) could be meaningful for

downstream analyses since different cellular pathways may reflect different interpeak interval distributions (Moein et al., 2018). I showed that peaks width, peaks interval, number of peaks are all features of the trace correlated one with another. These features do not correlate with the $\Delta R/R_0$ (here in the text defined as “fluorescence”).

The rather regular appearing spiking behaviour has led to the misperception that Ca^{2+} signal could be treated like electrophysiological events, although the interpretation of Ca^{2+} dynamics is far more complex (De Pittà et al., 2009).

In our attempt to use the chronic imaging setup to follow the neural dynamics of the dFB neurons I decided to start our analysis looking at the dendritic compartments. The neurons project their dendrites in the two hemispheres, I showed there is a significant positive correlation between the activity in the dendrites in the two hemispheres. However, as I showed in chapter four, this positive correlation is not always present; I will discuss the implication of this phenomena later in the text.

While aware that calcium elevations from various compartments need to be considered altogether to infer the underlying spatiotemporal dynamics of electrical depolarization in the neuron, I aimed to report the dendritic signal over days for its potential in elucidating the dynamic changes of input to the dFB neurons throughout the length of the experiments. The dFB neurons receive competing wake and sleep-promoting stimuli, which vary during the day and among days and depend on the time the fly spent awake and the circadian clock (Ni et al., 2019; Raccuglia et al., 2022).

I found that Ca^{2+} transients have a repetitive occurrence and their amplitude and prevalence vary among days and time of the day. This reflects the ever-changing stimuli, the influence of the circadian clock and the subjective experience of the

external world, which is continuously changing. Interestingly most transients occur at the beginning of each recording session, this could be due to technical reasons rather than physiological. The laser switches on 30 seconds before each recording session, to adjust the focal plane, after finding the structure (around 10 second) the laser is switched off for then be switched on again for the recording session. This procedure could create an artificial excitation, therefore further experiments and analysis would be necessary to discriminate the nature of the initial transients recorded in most of the traces. For example, I could record different neuronal substructures, quantify the peaks only at the beginning of the trace (~20sec.) and compare different traces recorded at the same frequency and with a specific laser power.

When focusing on the variation of the features of the traces over the course of the day, I found that the intensity of the peaks tends to be higher in the middle of the day and the interval between peaks tends to be shorter. I also showed how the peaks progressively become closer one with another from 11am to 5pm, and the same could be observed from 8pm to 8am. Intriguingly, the locomotor peaks of activity in fruit flies are usually in the early morning and in the late afternoon.

I found that the mean fluorescence of Ca^{2+} transients recorded in the dendrites had changes dependent on the time of the day. Specifically, I saw a positive correlation of the mean fluorescence with the time of the day during daytime, and a negative correlation of the mean fluorescence with the time of the day during night-time. Over multiple flies and multiple days I could observe an increase of the fluorescence of Ca^{2+} transients during the day, till the peak time of locomotion during the 24h, after which I observed a decrease in the fluorescence of the transients.

The interpretation of the signal recorded in the dendrites of the dFB is complicated

for several reasons. A combination of signals from distinct populations of neurons converge to the dFB neurons (Ni et al., 2019). These signals reflect the circadian clock, arousal as well as sleep drive and influence the activity of the sleep switch. These upstream neurons regulate the activity of the dFB in opposite directions. There is a lack of understanding of the synaptic connections between neurons. It is not known if upstream neurons synapse onto the same or distinct dFB neurons. It is not known if the synapses are restricted to the dendrites or could be found in different locations in the neurons. Different neuronal pathways could elicit distinct effects on the sleep homeostat and the changes of activity in the neurons could be transient, dynamic, and highly state and context dependent. One way of dealing with this complexity is breaking the inputs in groups, for example considering sleep-promoting and activity-promoting neurons or classifying neurons for the nature of the neurotransmitter they preference (e.g., glutamatergic vs dopaminergic inputs).

The changes of Ca^{2+} dynamics with the time of the day could possibly be linked with the activity of the circadian clock. It is known that during different time of the day the circadian system modulate sleep behaviour (Allada and Chung, 2010; Cavanaugh et al., 2016; Guo et al., 2018). In particular, the circadian inhibition overpowers the homeostatic drive to sleep when there is a need for alertness at the end of the day (Liang et al., 2019),

It is not clear if the circadian clock uses a combination of direct and indirect modulation (e.g., through dopaminergic neurons (Liang et al., 2019)) of dFB neurons and which circadian clock neurons could be involved. In terms of Ca^{2+} imaging, it has been recently shown that five of the eight major pacemaker groups in the fly brain display fluctuations in the levels of intracellular Ca^{2+} during the 24-h, with a peak in the amount of Ca^{2+} in a specific time of the day. The peak of Ca^{2+} normally precedes by ~4 hours the behavioural output controlled by the clock cell (Liang et

al., 2016). Interestingly, the fluorescence change I observed in the dendrites for 24-h over multiple days resembles the change in Ca^{2+} levels in two groups of circadian cells: the dorsal lateral neurons (LNd) and the large ventral lateral neurons (lLNvs). Activation of the lLNv by selective channel expression disrupts sleep, whereas ablation increases sleep (Shang et al., 2008; Sheeba et al., 2008). A possible experiment would be to inactivate specific groups of clock neurons, such as LNd and lLNvs, and record the changes in Ca^{2+} fluorescence in the dFB dendrites, to verify the link between the two systems. However, it is also possible that the rise in Ca^{2+} fluorescence during the day and its decrease during the night is the result of different input stimulating the dFB neurons at a different time of the day

Another goal would be to understand if the changes in dendritic fluorescence during the day are hiding the inhibition of the dFB. In fact, an increase of Ca^{2+} does not define the nature of the stimuli, which could be either inhibitory or excitatory . Hyperpolarization-activated Ca^{2+}-permeable channels like Ih channels are an example of the channels that could be present at the level of the dendrites. This type of Ca^{2+} response is at the basis of cell-autonomous thalamic oscillations, crucial for promoting sleep in mammals, and more recently have been linked with the inhibition of helicon cells by the dFB (McCormick and Pape, 1990; Kanyshkova et al., 2009; Raccuglia et al., 2022). The idea that Ca^{2+} increases in the dFB dendrites are linked with inhibition would again relate the sleep homeostat to the circadian clock. It has been shown that the circadian clock directly inhibits sleep-promoting neurons (a non-MB subset of 201y-GAL4 cells), moreover, this inhibition occurs at specific times of the day (Cavanaugh et al., 2016). Interestingly, GRASP data show that these sleep-promoting neurons contact dendritic components of dFB neurons, so I could not exclude a possible indirect effect of the circadian system on the dFB (Cavanaugh et al., 2016).

The circadian system has been shown to have an indirect influence of another element of the sleep homeostat, the ellipsoid body. Distinct circadian oscillators through the agency of specific dopaminergic interneurons increase the probability of the ellipsoid body to favour activity over rest (Liang et al., 2019). Antagonistically the dFB suppresses sensory-triggered movements by inhibiting the ellipsoid body via helicon cells (Donlea et al., 2018).

The current model indicates that at night dFB neurons increase their activity to generate compound Ca^{2+} oscillations, which seem cell-autonomous, and the sleep homeostat circuit is in a synchronized state (Kempf et al., 2019; Raccuglia et al., 2022). The synchronized oscillations are believed to reduce neuronal activity, without suppressing it, as shown by local field potential measurement in *Drosophila* (Yap et al., 2017; Tainton-Heap et al., 2021).

It is not known how these oscillations control sleep and the whole brain electrical activity nor why inducing oscillations is facilitated at night. Recent findings show that synchronized states could be broken by salient sensory stimuli (French et al., 2021). Breaking synchronized states increases functional connectivity, which promotes the initiation of behavioural programs in other words awakes the fly (French et al., 2021). Interestingly, the slow waves oscillations of the synchronized state are reduced when hyperpolarizing dFB neurons (Raccuglia et al., 2022). The circadian clock reduces the inhibition of the homeostatic system over night (Collet et al., 2020). Furthermore, the circadian clock directly regulates also grooming behaviour, which occurs predominantly during short resting phases before the night-time sleep (Qiao et al., 2018).

I think that the change in the circadian inhibition of the sleep homeostat in the 24h could be observed looking at the dendritic Ca^{2+} dynamics. It is known that Ca^{2+}

oscillations in R5 neurons can be driven by TuBu neurons, which receive circadian input, and arises over the day to integrate circadian and homeostatic sleep drive (Guo et al., 2018; Lamaze et al., 2018; Raccuglia et al., 2019).

Sleep deprivation under the two-photon may reveal if the changes in fluorescence in the dendrites of the dFB and the activity-promoting clock neurons are related. If there is not an increase in the fluorescence during the day after the night of sleep deprivation than the circadian clock may be the one contributing to the observed changes in fluorescence. Increases in dendritic fluorescence after sleep deprivation would point to the influence of sleep homeostatic networks, above all the R5 neurons.

Analogously to the dendrites, Ca^{2+} dynamics in the axons changes between days and time of the day. Differently to the dendrites, Ca^{2+} transients are more intense when lights are off (8pm-8am), between 4am and 5am I registered a high number of high intensity peaks. I recorded a progressive increase in the number of larger Ca^{2+} transients from lights on to lights off. Comparing axons and dendrites, the Ca^{2+} transients are larger in the axons as well as the number of peaks is higher and the distance between peaks lower. Also, the distance between peaks is less variable in the axons. Generally, peaks are least close one with another when lights are on, during the first part of the day, and become progressively closer one with another later during the last part of the day. Although I reported the results from one single fly, I can speculate on the reported axonal Ca^{2+} dynamics.

Our observations are in line with the idea that the dFB axons are the output branch of the population of neurons. Processing the information (most likely already pre-processed) coming from the other neuronal compartments they induce sleep synapsing with a series of downstream targets. Little is known about the modulation

of the dFB axons by other neurons and the downstream effectors of the dFB. It is known that dopaminergic arousal neurons directly synapse on the dFB axons, reducing their activity (Pimentel et al., 2016). It is also known that dFB axons promote sleep releasing GABA and inhibiting octopamine arousal neurons as well as inhibiting helicon cells, which would otherwise excite R5 neurons (Donlea et al., 2018; Ni et al., 2019). Sleep induced by dFB seems to be particularly relevant at night (Kempf et al., 2019; Raccuglia et al., 2022). The increase of axonal Ca^{2+} transients when lights are off is in line with the idea that dFB activation has major implications during night-time. The higher axonal Ca^{2+} activity compared to the dendritic one has probably an easy explanation: in the axons we have the summation of the events occurring in the two hemispheres, when recording the Ca^{2+} dynamics in the dendrites I reported the data from a single hemisphere. Even if it is also possible a difference in the biophysical properties and we should not exclude the role of other neurons synapsing directly into the axonal region.

Of particular interest is the change of the features of the peaks. I have reported a change in peaks number and interval between peaks throughout the day and among different days. What do these features tell us? I observed the peaks in the axonal traces become more closer during lights off. I also saw the peaks becoming more closer in the dendrites when the fly is generally resting, during the peak of locomotor activity this trend does not appear to be present. I thought that the features related to the peaks in the traces could be linked with the amount of sleep, although I was not sure about the temporal dynamic of this relation. As shown in chapter four, I tested our hypothesis using the behavioural data, the locomotor trace simultaneously recorded throughout the chronic imaging experiment. Having more data relative to the dynamics of the dendrites, I focused our analysis on this substructure.

I found that the number of peaks is the best predictor of the locomotor behaviour of the fly instead the fluorescence showed to be the worst predictor. I also found that the features relative to the peaks best correlate with the amount of sleep occurring in the hour following the 30 minutes when I took the snapshot of the dendritic Ca^{2+} activity. A trace with a high number of peaks, a short interval between peaks, and little average width of the peaks is likely to predict that the fly is soon going to sleep. This observation is supported by another observation I made looking at the average number of peaks and the average locomotor trace considering all flies analysed: just before the amount of sleep increases, I saw an increase in the average number of peaks in the traces.

With our surprise, the R-squared relative to the relation between number of peaks in a trace and sleep in the hour after the recording, representing how good was the predictor, was changing throughout the 24 hours. Remarkably the R-squared did not only change with the time of the day, but also it negatively correlated with the fluorescence change in the imaging trace, which I mentioned above it also varied during the 24 hours.

The first consideration is the absence of correlation between the features of the peaks and the “sleep history”, defined as the sleep before (up to 2h) the 30min when I recorded the imaging trace. One reason could be that the dFB neurons are the effector of the sleep homeostat circuit, their activity is the result of a fine processing of all the stimuli: circadian and homeostatic, wake and sleep promoting. In the sleep homeostatic circuit there is a sensor (or possibly many sensors) which periodically samples the state of the fly (how much wake vs sleep), then there is an integrator which processes the information from the sensor(s) to determine the homeostatic sleep drive, and finally there is an effector that responds to this drive. The R5 neurons have been shown to be the integrator of the system (Liu et al., 2016).

Upstream of the dFB neurons they undergo “sleep-need”-dependent plastic changes. Sleep need elicits oscillatory signatures within the sleep homeostat circuit, although the dFB neurons seem to oscillate cell-autonomously and the oscillations induced by the sleep homeostat circuit seem to be time of the day dependent (Raccuglia et al., 2022). The current understanding is that sleep need is experienced by specific networks. When the dFB neurons are activated, the fly is put asleep(Pimentel et al., 2016). The Ca^{2+} dynamics in the dendrites of the dFB might unveil what is about to happen more than anything else.

The second consideration is about the time window when the amount of sleep shows a strong positive correlation with the number of peaks in the trace. The limitation to the analysis was the protocol for the imaging sessions. If the fly prior recording is asleep, the imaging session has the potential of waking up the fly. Interruption of sleep bouts and feeding just after the imaging session would jeopardize the results of our analysis. I decided to consider just the time in between imaging sessions, which could be up to 180minutes. From the 30min when I recorded the imaging trace to 1h after those 30min we have a progressive increase in the correlation between the amount of sleep and the number of peaks, which decreases just after this time window. One possible explanation is that flies are intermittent sleepers and privilege short sleep bouts (Martin et al., 1999; Wolf et al., 2002). Evolutionary it is safer to have an alternation of alertness and sleep whenever is possible, to decrease the chance that long uninterrupted sleep bouts would expose the fly to predators. This would be time of the day dependent, since in the evolution flies have developed the tendency of looking for food at dawn and dusk, and context dependent: availability of food in the area and presence of predators. The correlation found decreases after the hour probably because the arousal system pushes the fly to be awake. We could imagine that flies continuously assess the external world, once they evaluate that

the probability of finding food and predators is low, they attempt to sleep, this process, for the safety of the fly, needs to be periodic and regulated by the circadian, arousal system.

The above-mentioned regulation appears clear when I looked at the time-of-day variation of the R-squared relative to the relation between number of peaks in a trace and sleep in the hour after the recording. This observation together with the finding that this R-squared negatively correlated with the change in fluorescence during 24-h lead us to a third consideration. My current hypothesis is that the fluorescence could represent the influence of the circadian, arousal system on the dFB: actively decreasing the probability of falling asleep when the fly is supposed to look for food, mainly during the peak of locomotor activity at dusk. The sleep drive would be overcome by the arousal drive in a dynamic way during 24h. This hypothesis would need to be supported by further experiments.

A sleep deprivation experiment in the set-up would possibly show us a different trend of the R-squared values during the night of sleep deprivation.

It is worth to mention that in all our analysis I do not exclude the sleep that could be linked with food consumption, the reasons for this are the following. First, depending on the amount of food consumed and on the type of nutrients present in the food, the effect of the so-called “postprandial sleep” ranges from slightly arousing to strongly sleep inducing (Murphy et al., 2016). High amount of food ingested, protein, and salt induce postprandial sleep, which in average lasts 20min. This effect varies between individuals, and it is sensitive to the fruit fly’s internal clock (Murphy et al., 2016). Moreover, the data published so far refer to the effect of dietary components in isolation and we do not have information relative to the effect of a balanced diet. Lastly, from our experience flies do not eat at every feeding interval, and the amount

of food ingestion is highly variable. This is to take in consideration if we want to consider postprandial sleep as something completely different from other forms of sleep. I think that such distinction needs to have biological evidence, and therefore I considered to include in our analysis all kind of rest lasting >5min.

In the third and fourth chapters I took the chance of looking at the differences within the dendritic region of interest and at the dynamics between the two hemispheres.

In the dendritic arbor I reported the presence of two distinct regions. Their difference is expressed in distinct temporal and spatial Ca^{2+} activity. Transients in the upper region are more and more frequent, moreover the relative change in intensity of Ca^{2+}-transients between different time of the day is not consistent between the upper and lower region. The differences I described could be due to anatomical differences of the two regions: either the number and density of the dendrites or the angled position of the upper region respect to the lower one, physically up and down. Interestingly, the two regions are functionally integrated. Although this finding does not take in consideration that some dendrites could bridge between the upper and lower region and confound the analysis. It is likely that different inputs are received in distinct parts of the dendritic area, and a region of high integration (functional integration of the stimuli) coexist close to a region of low integration.

It has been recently shown that the line 23E10-GAL4 constitute a functionally heterogeneous group of cells (Jones et al., 2022). Curiously, the line labels also neurons that are not dFB, some involved in the regulation of sleep, but not part of the sleep homeostat circuit, and some not involved at all in sleep regulation. Nonetheless, the heterogeneity directly involves the dFB neurons projecting in the region I am taking in consideration. Currently, it is impossible to unequivocally assign a role in sleep promotion to all the dFB neurons (Jones et al., 2022). These

observations support the idea that the differences I saw at the level of the dendritic region, in terms of Ca^{2+} dynamics, could lie their foundations in the heterogeneity, functional and anatomical, of the dFB population. More experiments are needed to understand why the neurons in the population differ and how they operate together, although I could think that this diversity is important to regulate for example sleep quantity and quality.

The dFB neurons symmetrically project their dendrites into the two hemispheres. It is still not clear why this happens although it suggests the importance of looking at the dynamics of the two hemispheres. Interhemispheric interactions seem common throughout the animal kingdom (Güntürkün et al., 2020; Popov and Szyszka, 2020; Jablonka et al., 2021). Researchers have been looking at inhibition/excitation dynamics, there could synchronization between symmetric neural structures as well as contralateral inhibition to delay the other hemisphere. These interactions are temporary and often are relative to external stimuli (e.g., contralateral inhibition enhances the contrast of odor concentration gradient between both hemispheres in Drosophila (Mohamed et al., 2019)). The internal state of the animal could also induce changes in the interhemispheric interactions (Abeyratne et al., 2010). Hemispherical asymmetry distinctly changes with sleep state (NREM/REM) and within the different sleep stages of NREM sleep; it is also associated with sleep fragmentation and the presence of sleep apnea (Abeyratne et al., 2007; Swarnkar et al., 2007). Temporal differences in specific high-frequency oscillatory patterns between the left and right hippocampi have been found during slow-wave sleep (Villalobos et al., 2017). It is known that the brain operates at the edge of different dynamical regimes: the two simplest patterns are in-phase and anti –phase oscillations (Li and Zhou, 2011). Anti-phase pattern means that certain areas of the brain increase their activity, while others decrease it.

In chapter four, I showed that I found a negative correlation between the dendrites in the left and right hemisphere that was limited in a defined window of time. The data are not enough to draw conclusions, although I could make some speculations. I observed that the sleep before the time window when the two dendritic arbors were in anti-phase was less than the baseline sleep for the analysed fly. Moreover, I found that in the 24h from when I started to see the anti-phase dynamic the total sleep was higher than the rest of the other days. Night and day sleep during the 3rd day was also higher than the other days.

In neuronal networks, such anti-phase pattern has been widely observed in experiments (Greicius et al., 2003; Mantini et al., 2007; Li and Zhou, 2011). Interestingly, anti-phase oscillation has been observed during the rest state and in anesthetized monkeys (Vincent et al., 2007). Although it is important to stress that the interpretation of an anti-phase pattern is context dependent. Transition of in-phase and anti-phase oscillations have also been reported within coupled neurons, for example in the coupled pre-Botzinger cells (Duan et al., 2017).

The reason for these patterns is still unclear and it could be either a direct or indirect consequence of the interaction between neural networks. Competition dynamics broadly exist in neuronal systems and could play a role in neuronal functions. Anti-phase synchronization of very small frequencies could result from the integration of low-frequency and high-frequency oscillations (Li and Zhou, 2011).

I think that there is a need for a much deeper investigation of the interhemispheric dynamics within the dFB population. Imaging the dFB neurons during sleep rebound after sleep deprivation could show if the anti-phase pattern observed is truly linked with sleep. In addition, analysis of the frequency, such as a Fourier transformation, could be insightful.

As presented in this section, experiments of sleep deprivation under the two-photon could be important to elucidate many aspects of the physiology of the dFB neurons. The fifth chapter of this work is dedicated to the development of the procedure to sleep deprive flies during chronic imaging experiments.

Sleep deprivation has been repeatedly used as a tool for investigating sleep regulation. In Drosophila, sleep deprivation has been achieved mainly using mechanical agitation, although gentle handling and thermogenetic have also been widely used (Shaw et al., 2002; Geissmann et al., 2019; Vaccaro et al., 2020). Sleep deprivation using vibrations showed high variability across individuals, and across days (Geissmann et al., 2019). Thermogenetic sleep deprivation while effective, it is always a genetic manipulation and does not consider confounding variables introduced with the change in temperature.

A novel behavioural paradigm for environmentally induced arousal was recently developed (Lebestky et al., 2009). They showed that delivering a succession of brief air puffs, closely spaced, could produce an extended period of hyperactivity, lasting up to 7-10minutes. Precisely, they reported a cumulative effect of the stimuli with a peak in the acceleration of the fly following a 6-puff exposure, following which flies were reducing their speed. The protocol increased both the frequency of the bouts of activity and the average speed during the bouts. Importantly, they reported that flies exposed to 6 puffs were significantly more reactive to following series of puffs, only if those were presented within the 30 min from the first 6 puffs. This study together with studies showing that mechanical startle could evoke a change in the internal state of the fly brain, inducing increases in brain activity (associated with arousal)(Cho et al., 2004; Wolf et al., 2002), convinced us to use air puffs for trying to sleep deprive flies under a two-photon microscope.

Our goal was to sleep deprive flies for as long as 12hours. It is difficult to keep animals awake for a long time because of the powerful homeostatic mechanism driving recovery of lost sleep. Our experiments showed that only random puffs of random length in 20second bouts for 12hours could sleep deprive flies. Continuous puffs would cause an excess of stress and lethality if prolonged for 12hours, while a repetitive series of 6 puffs over 12h would lead to habituation to the stimulus. The effect of sleep deprivation is usually measured by the presence and intensity of sleep rebound (Shaw et al., 2000). I proved that our protocol is not just effective in keeping the fly awake for 12h, but also it increases the amount of sleep /30minutes compared to controls once the protocol ends. I also found an increase in sleep depth (longer sleep bouts) during sleep rebound, as expected from the literature. Sleep rebound seems to be concentrated in the 6h following the protocol of sleep deprivation. It has been previously shown that flies recover ~30-40% of the sleep lost within 12h upon release from sleep deprivation (Hendricks et al., 2000).

It is important to note that our sleep deprivation occurs only during the relative night, I currently do not know if the protocol is effective also when is run during the relative day (lights on). Our decision of testing the protocol for 12h during the relative night is linked with previous findings(Potdar et al., 2017; Kempf et al., 2019). Kempf at all. showed that sleep deprivation for 12h overnight was effective in increasing ROS species in the dFB neurons, while sleep deprivation over the course of the day did not cause any significant increase. Furthermore, they showed that sleep deprivation prolonged for an entire day after a night of sleep deprivation was not significantly different from just 12h of sleep deprivation overnight. These findings are relevant since increase in the intracellular level of ROS has been proven to be responsible of the activation of the dFB neurons and induction of sleep. It would be interesting to look at the level of ROS after the night of sleep deprivation in the two-photon

setup. This could be done using a similar approach presented by Kempf at all., expressing MitoTimer in the 23E10-GAL4 line.

One relevant advantage of the protocol for sleep deprivation presented here is the possibility of video monitoring the fly behaviour, to ensure that flies are not damaged during the protocol. Protocols that use shaking for sleep deprivation could damage the fly, especially the legs, although this could not be normally assessed and can contribute to a misinterpretation of the data.

An important limitation of the results presented here is not considering the sexual dimorphism, which from recent findings seems substantial when considering sleep and sleep deprivation. For example, female flies sleep much less than males, and they are more sensitive to sleep deprivation, with an extended rebound upon long sleep restriction (Shaw et al., 2002). Recently it has been shown that depriving female flies of only the longest sleep episodes still led to a significant sleep rebound the subsequent morning, a phenomenon not seen in male flies (Geissmann et al., 2019). Therefore, it will be worth using male flies as well as female flies, to observe the above-mentioned behavioural differences and understand if those reflect on different neuronal dynamics.

In both male and female flies, the changes in sleep pressure seem to cycle in a circadian fashion (Borbély et al., 2016). Long sleep deprivation, lasting over days, does not eliminate the clock-regulated bouts of activity, suggesting the prioritization of the system for the circadian regulation of activity (Geissmann et al., 2019). It would be important to run experiments of sleep deprivation with clock mutants, in order to test the locomotor behaviour and validate previous observations. These experiments would also be important to understand if the activity of the dFB in these mutants is changed compared to the controls.

6.1 Chronic monitoring of a fly under a two-photon microscope: a new chapter

In the work presented here I have addressed three main questions: 1) Could we use a two-photon setup to monitor for days the brain and behavioural dynamics of Drosophila? 2) Could we study sleep homeostasis and the sleep homeostat using the developed experimental setup? 3) Could we manipulate the physiology of Drosophila in the setup and investigate the effect of sleep deprivation?

With this neurobehavioural study I have shown that it is possible. The approach is highly reproducible: 1) the costs are affordable for any size lab (~6.000,00£) with access to a two-photon microscope 2) the procedure requires training, although following the protocols this should be fast. This work opens new doors for experiments investigating not only sleep physiology, but also circadian regulation, feeding behaviour, stress response, learning, long-term memory, and even social behaviour. Indeed, it is feasible to modify the holder of the fly and add a chamber underneath where a second fly could be placed to interact with the experimental fly. For the very first time it is possible to simultaneously sleep deprive flies and measure neuronal activity.

I tried to answer the question “Which activity patterns in the dFB dendrites could be related to sleep?” Our hypothesis was that time-of-day modulation of sleep through the sleep homeostat could be reflected in the activity recorded in the dFB neurons. Our main findings are two: 1) there is a time-dependent modulation of Ca^{2+} fluorescence in dFB dendrites 2) the number of peaks in the recorded traces correlates with “sleep after”. Our findings led us to draw some conclusions, which are summarized in Figure 6.1.

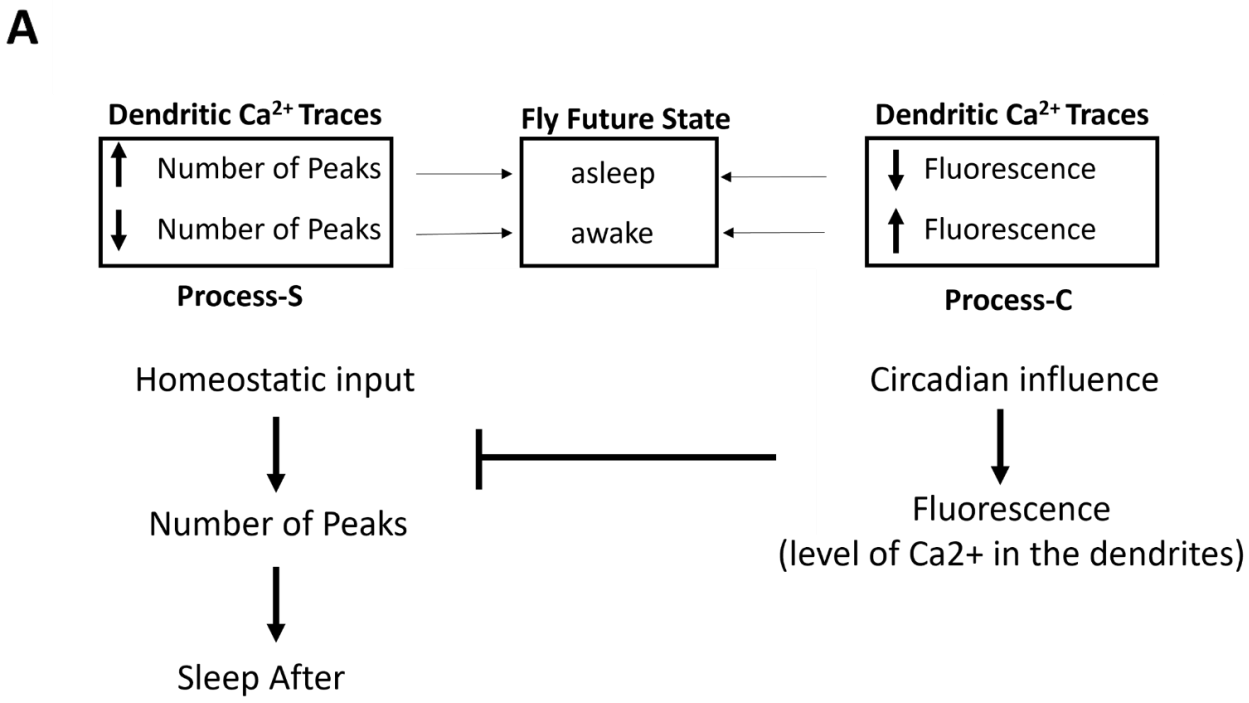


Figure 6.1 An overview

(A) The schematic shows a way to look at the results reported observing the dynamics of the dendrites of dFB neurons over days. The number of peaks could be linked with the influence of the sleep homeostat on the activity of the dFB (known to be exciatory).The change in the fluorescence could be linked with the influence of the circadian clock on the activity of the dFB (known to be inhibitory). When the fluorescence is high the ability of predicting the amount of sleep from the number of peaks is low. Overall, when the fluorescence is high the fly is most likely awake, when the number of peaks is high the fly is most likely asleep.

It is important to stress that these conclusions are a starting point for future experiments. The current set of data and experiments need to be integrated with a larger set. Nevertheless, it is worth sharing our insights.

The levels of homeostatic sleep pressure may depend upon the time of day that waking occurs, and not simply be a function of previous wake duration. The change in fluorescence in the dendrites together with the change in peaks number could predict the probability of sleep over wake. Process-S represent the homeostatic regulation and Process-C the circadian regulation,(Borbély et al., 2016). It is possible that the Process-C is directly involved in the modulation of excitability of the dFB, through the modulation of the Ca^{2+} levels in the dendrites. Flies are intermittent sleepers; it could be that flies try to release sleep pressure as soon as they can. Although, during the day, when the chance of finding food as well as finding predators is higher, the Process-C forces the fly to stay awake as much as possible. This could be achieved imposing a reduction in the excitability in the dFB neurons, known to put the fly asleep when they are in a firing state. The system is probably subject to more complex interactions and sleep quality, for example, could be regulated by a different mechanism. Another way of looking at the regulation of sleep is to look at the different known elements as oscillators. We have a main oscillator, which is the Process-C. This is also organized in a system of individual oscillators. We then have the Process-S, which has been recently proved to depend on oscillations for its functioning. And finally, we have the dFB, which seems to have an internal oscillator, probably dependent on the level of ROS. The dFB neurons, being the effector of the sleep regulatory mechanism, integrate the different oscillators to give a coherent response to the need of the fly.

The fundamental role of Process-C in shaping sleep has been shown in previous studies (Cavanaugh et al., 2016; Guo et al., 2018; Andreani et al., 2021; Raccuglia

et al., 2022). Previous findings have shown that clock mutants show a significant increase in sleep rebound after 12h of sleep deprivation, reclaiming ~100% of lost sleep (Shaw et al., 2002). Some clock mutants have shown an exaggerated response after 3h of sleep deprivation, for each minute of sleep deprivation flies were sleeping 3min more than the baseline sleep(Shaw et al., 2002). Moreover, the success of prediction of the level of Process-S using EEG data depends upon the time of the day (Borbély et al., 2016).

However, Process-C could also be influenced by Process-S, but very little work has been done to address this interaction. It is known that clock gene expression in the brain depends significantly upon prior sleep-wake history (Curie et al., 2015). Some studies have shown that the circadian amplitude of many neurobehavioural functions was function of sleep pressure, the higher the sleep pressure the lower the amplitude (Borbély et al., 2016). Interestingly, sleep deprivation attenuates phase shifts induced by light, suggesting a direct role of sleep pressure in attenuating circadian clock response to zeitgeber (Sahlem et al., 2014).

Process-C and Process-S interact continuously, as it has been proven by electrophysiological recordings in mammals (Deboer, 2018). The disruption of the synergy of Process-C and Process-S are probably responsible for the detrimental effects of sleep deprivation.

I am currently working on experiments of chronic imaging during sleep deprivation, and I am also aiming to look at the dynamics of the dFB during sleep rebound. Hopefully, my experiments will contribute to the understanding of the relation between Process-C and Process-S and how the dFB neurons modulate their activity to responds to the need of sleep of the fly.

I am also looking into analysing in different ways both the behavioural and imaging

data. I have days of video recording of flies during the chronic experiments. These videos contain relevant information. For example, I am interested in looking at the proboscis extensions of the fly. Researchers have observed a deep-sleep stage in Drosophila, during which fruit flies repeatedly extend and retract their proboscis. The number of proboscis extensions seem to increase at the end of the day (van Alphen et al., 2021). I observed the presence of proboscis extensions also in our experiments, although I have not performed a systematic analysis. It would be useful to understand: 1) if there is a relation between the proboscis extensions and the activity of the dFB 2) if this relation could mirror a distribution of deep-sleep bouts time-of-the-day dependent. Furthermore, I am interested in looking at the movement of the legs. Grooming is a behavioural state present in Drosophila (repeated execution of stereotyped legs 'movement), although it is not clear what is its function (Qiao et al., 2018). Studies suggested it confers survival and social benefit, sick flies do not groom (Mueller et al., 2022). Interestingly, grooming is regulated by the circadian clock, which decide when the fly grooms, and by another system, which regulates the duration of the grooming and depends on other behaviours. Flies in average spend ~13% of their waking time grooming (Qiao et al., 2018). Even if the time spent grooming in an average fly seems little, I want to understand if grooming influences our measure of sleep, since grooming flies do not walk.

There is a possibility that flies experience micro-intervals of sleep, during less than 5 minutes, and may contribute to sleep regulation. It would be revealing to find and characterize these micro-sleep bouts, which might be characterized by either micro-behaviours or a particular brain state or both.

Imaging data could be analysed again looking at the frequency domain, although this effort has already been started and it is currently carried through by Dr. Peter Hasenhuetl.

Sleep stages have not been addressed in this work, although the large amount of data collected has the potential of unveiling the presence of different sleep stages, in different time of the day. The biggest challenge of this chronic neurobehavioural studies is admittedly the handling of the data collected, which require a profound work of analysis and interpretation and it is time consuming. With the advance of automatic ways of analysis and with the power of collaborations these experiments will be more approachable and key to lead to important findings in neuroscience.

7 | Materials and methods

7.1 Drosophila strains and culture

D. melanogaster stocks were grown in standard medium containing yeast, sucrose, agar and molasses under a 12-light: dark-cycle at 25°C and nominal ambient humidity of approx. 65%. The genotype of flies used in this study were homozygous for *UAS-GCaMP6f* and *UAS-tdTomato* on the 2nd chromosome and homozygous for *23E10-GAL4* on their third chromosome. The driver line *23E10-GAL4* was used to target dFB neurons. The wild-type strain *Canton-S* was used as controls for behavioural experiments. Post eclosion, 4-5 female flies were placed into vials with 1-2 males. After 3-4 days chronic cranial windows were implanted. After 2-3 days of recovery flies were ready for experiments. During the chronic imaging experiments flies were fed with liquid *holidic medium*. Solid *holidic medium* was used to test the sleep pattern of flies, using video-tracking.

7.1.1 Holidic medium

A description of the components of the *holidic medium* is presented in chapter 2 (Figure 2.2). For the preparation of the medium there were two stages. In a first stage I prepared a solution with the following: buffer, metal ions, cholesterol, amino acids with low solubility (L-isoleucine, L-leucine and L-tyrosine), sugar and milliQ water. The volume of this solution equal 1L minus the volume of the second solution

added after autoclaving. The solution was autoclaved for 15min at 120°C and then cooled at room temperature, with continuous stirring to reach ~65°C. In a second stage amino acids, vitamins, nucleosides, choline, inositol, and preservatives were added to the first solution. The final solution was stored at 4°C in a dark place until used for the experiments. For the video-tracking experiments I used solid *holidic medium,* this was obtained adding agar (20g/L) to the first solution.

Note: the first and second solutions are prepared partially using aliquots from stock solutions. Except for the cholesterol stock solution, prepared in absolute ethanol, all the other solutions were prepared using milliQ water. Vitamin, FeSO4 and folic acid stocks are stored at -20°C, while amino acids stocks, buffer stock, cholesterol stock, and stock containing nucleosides, choline and inositol were stored at 4°C. These stocks were prepared using a sterile technique. I recommend checking the solubility of different components. I experienced that some amino acids solutions require adjusting of the pH to increase their solubility. Preparing all the solutions requires patience and in average around 8-9hours for all the procedure. I recommend to aliquot all the stock solutions and, if possible, also the final solution. From our experience food stock should not be maintained beyond one year. For each experiment I opened only one new falcon of *holidic medium*. I also recommend of using only the liquid component of the solution. I experienced that some components precipitate with time. Flies prefer liquid *holidic medium* at room temperature (rm). I normally leave the food in the fridge till few minutes before the feeding session, to leave time to the food to chill at room temperature. When using solid holidic medium I recommend testing different agar concentrations. I observed that flies do not like a stiff consistency, but a softer and stickier one.

For more information about the preparation of the liquid food I recommend checking the original paper by Piper at all.

7.2 **Behavioural tracking**

For activity and sleep monitoring using video-tracking experiments, I used a custom-made system. An adaptation of Trikinetics monitoring system by Dr Cliff Talbot and the Trikinetics software. Flies were placed individually in 60mm long and 5mm wide glass tubes. Each tube was closed by solid food from one side and from the other by cotton. For all the experiments the temperature was 25°C and the humidity 60%. Flies were reared on a 12hr day-night cycle, thanks to an LED controlled by a computer. Video were recorded using a GuppyPro camera. Periods of inactivity lasting at least 5 minutes were considered sleep. Immobile flies for the entire length of the experiment were excluded.

For activity and sleep monitoring under the two-photon microscope I used the rotation of a 6mm polystyrene ball suspended on a constant humidified air stream, as a proxy of fly's movement. The ball was carefully positioned underneath the fly, to allow the fly to freely walk on the ball. The fly should be positioned as if it was "climbing the ball", with the head in line with the centre of the ball (this facilitate fly's movements). The rotation of the air-supported ball was recorded by a GuppyPro camera. This camera was equipped with a magnification lens and a bandpass filter to reject visible light and infrared light from the 2-photon laser), at a mean acquisition rate of 22.80Hz. The trackball was illuminated using an infrared LED (Thorlabs) at low intensity. The ball was marked by a series of dots equally spaced to cover all the dimension of the ball. The marks were done using a marker visible with infrared light. Before each experiment, the ball was checked, to ensure the marks were still visible and the ball was clean. Ball's movements were computed by a custom-written software written by Dr Cliff Talbot. The analysis of the behaviour was then done

using MATLAB (R2019), using a custom-written code. For each fly I had one file containing the time-series of the fly's ball movement over the entire length of the experiment. For the analysis I considered the absolute values of the locomotion time-series. A binarized version of this movement time-series was used. The binarization was done empirically choosing an activity threshold. Inactivity was defined as the duration of periods where the movement trace did not cross the activity threshold. Periods of inactivity lasting at least 5 minutes were considered sleep.

Throughout the long-term experiments in the two-photon setup, I recorded a video of the flies. I used a GuppyPro camera and for the recording I used the same software used for tracking ball movements. Another camera (GuppyPro) was used to assess feeding behaviour, and the fly's response to liquid food. The "proboscis extension index" was used to represent the predisposition of the experimental fly to the diet based on liquid holidic medium. A value of 3 was given to each long proboscis extension, 1 for each short extension.

During all the behavioural recording in the long-term experiments, I used female flies head-fixed to a custom mount, using thermoplastic vax with a melting point of 52°C (Eicosan-Sigma).

7.3 **Two-photon calcium imaging**

I used a microscope with a movable objective (MOM, Sutter Instruments). To control the microscope, I used Scanimage (Vidrio Technologies) running on MATLAB R2016b. GCaMP6f and tdTomato fluorescence was excited by a Coherent Chamaeleon Ultra II two-photon laser (pulse duration: 140 fs; centre wavelength: 930 nm) and collected by a 20X, 1.0 NA multiphoton water immersion objective (W-Plan-Apochromat, Zeiss). Using a 302-m Pockels cell (Conoptics), the laser power was set to levels below approx. 10mW (using a ThorLabs power-meter). The emission paths for GCaMP6f and tdTomato fluorescence were separated by a 562nm edge (Brightline) dichroic beam splitter. Passed through a 520/60 single-band bandpass filter (GCaMP6f fluorescence) or a 641/75 single-band bandpass filter (tdTomato fluorescence) and detected by two separate photomultiplier tubes (Hamamatsu). The acquisition rate was 16.68Hz (256 pixels/frame).

Each recording session lasted for about 3 minutes. I processed the images for each recording session using ImagJ. I manually selected region of interests (ROIs) from green and red imaging channels and then extracted the mean fluorescence intensities for each frame. From ImageJ I imported the information relative to the change in fluorescence into MATLAB (R2017b or R2019). The resulting time series and that of a non-fluorescent brain area as background (for background subtraction – separately for green and red channels) were then analysed further using a custom-written code. The resulting traces were background subtracted, using the mean of the background time series. The time-resolved green-to-red ratiometric signal (dR_t/R_o) was computed as $dR_t/R_o = (R_t-R_o)/R_o$, where $R_t=F^g_t/F^r_t$, and R_o is the 10^{th} percentile of R_t. In some cases, photo-bleaching of the tdTomato signal was

observed, resulting in distortions of the dR/R signal. In these instances, the tdTomato signal was fit using a double-exponential function and the signal was corrected by a new baseline. For each trace I found the distinctive features of the trace: mean fluorescence, peaks amplitude, width, interval, and the number of peaks. For the identification of the peaks and their features I used the MATLAB function *findpeaks*.

7.4 Sleep deprivation under the two-photon

The sleep deprivation setup was built with the help of John-Paul Moszynski, who assembled the electric components. In all our experiments, a valve (The Lee Company) was controlled through an Arduino board by a computer. A TTL control input (5V=on, 0V=off) allowed the delivery of air to the fly. When the valve was on, the air flew through the tube (~5mm in diameter). The tube was positioned ~1cm from the fly's head. The air had a flow rate of 3 L/min (25Psi). The house air was filtered (La-Man 520b and Wilkerson M03-02-MX0) and regulated by two pressure regulators (RS 703-6104). The air passed the valve, passed through a regulator flowmeter (Key Instruments, MR3A14BVBN) and then a bottle containing water. The air delivered to the fly was humidified air, to increase survival of the fly throughout the experiment. Each air puff lasted 200ms in the first and second protocol used, while in the third it lasted randomly 50/100/150/200ms. The inter-puff interval was 3s in the first two protocols, while in the third protocol the interval was random in each 20s bin. In the first protocol every 2 minutes were delivered 10 puffs, in the second protocol puffs were delivered continuously and in the third one there was a random number of puffs every 20s.

7.5 Generation of figures and statistics

Figures were generated using GraphPad Prism 8 and MATLAB 2019b. For the histograms presented in this work I used the MATLAB function *CirHeatmap.* Statistical analyses for behavioural experiments were performed using GraphPad Prism 8. For comparison between multiple groups, ordinary one-way ANOVAs followed by Tukey's multiple comparison tests were used. All the video-tracking experiments were repeated at a different time with different batch of flies to ensure reliable conclusions. Individual tests and significance are detailed in figure legends. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

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A | Supplementary materials

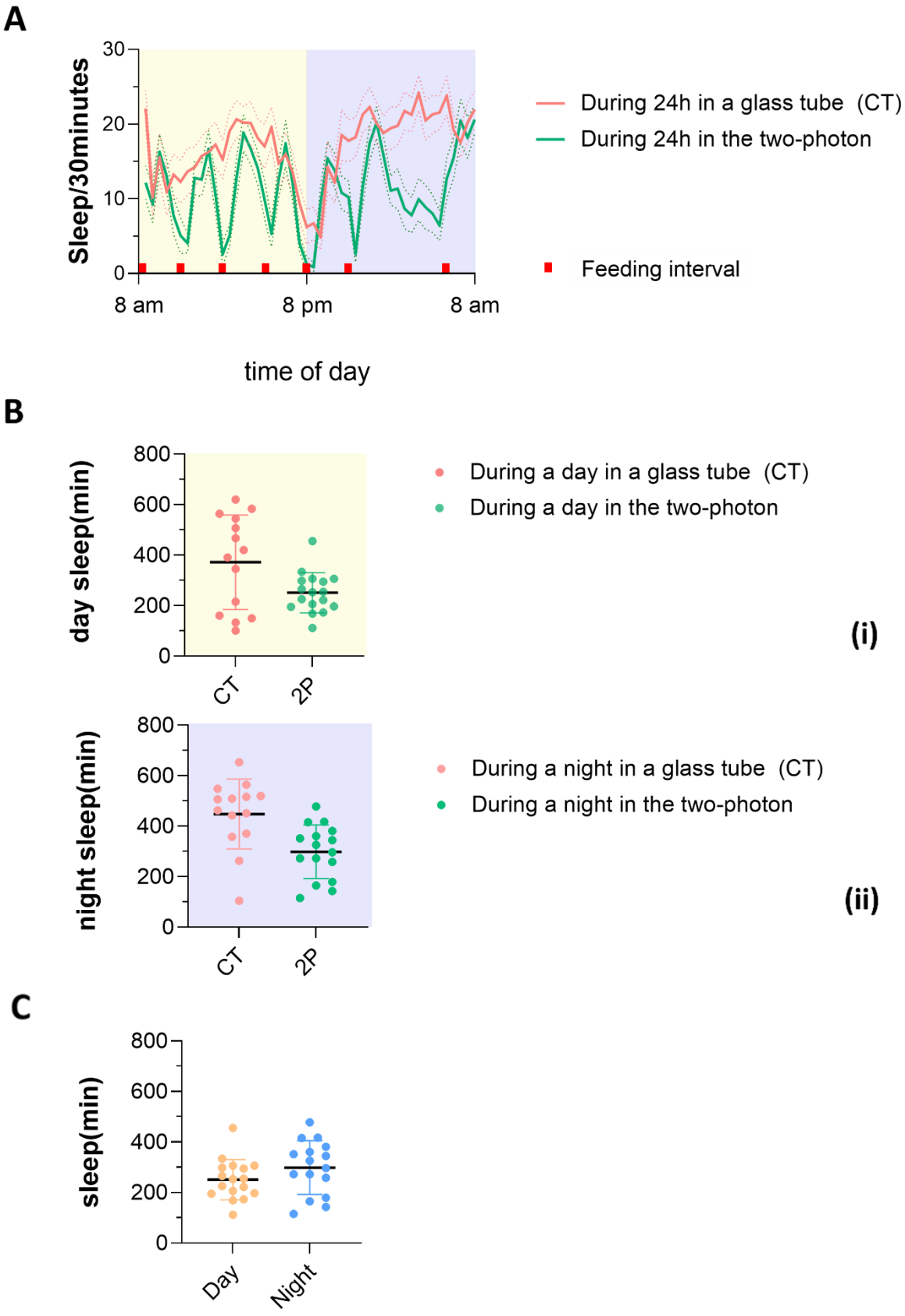


Figure1 Chronic imaging protocols with hm feeding at fixed timing influence timing and quantity of sleep, but not the circadian rhythmicity of the locomotor activity

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(A) Sleep profile (over 24h) of flies living in a glass tube with *ad libitum* food (salmon)(CT) and flies living under a two-photon microscope, fed at regular intervals (green)(2P). (Number of flies(n)=14 for CT and n=16 for 2P). Dotted lines represent deviation from the mean (bold line). **(B)** Total sleep during daytime (i) and nighttime (ii) in flies living in a glass tube with *ad libitum* food (salmon) and flies living under a two-photon microscope, fed at regular intervals (green). Error bars are ± s.e.m. Each dot represents one single fly. (Number of flies(n)=14 for CT and n=16 for 2P). **(C)** Total amount of sleep during daytime (n=16) and nighttime (n=16) in flies living under a two-photon microscope, fed at regular intervals (green).

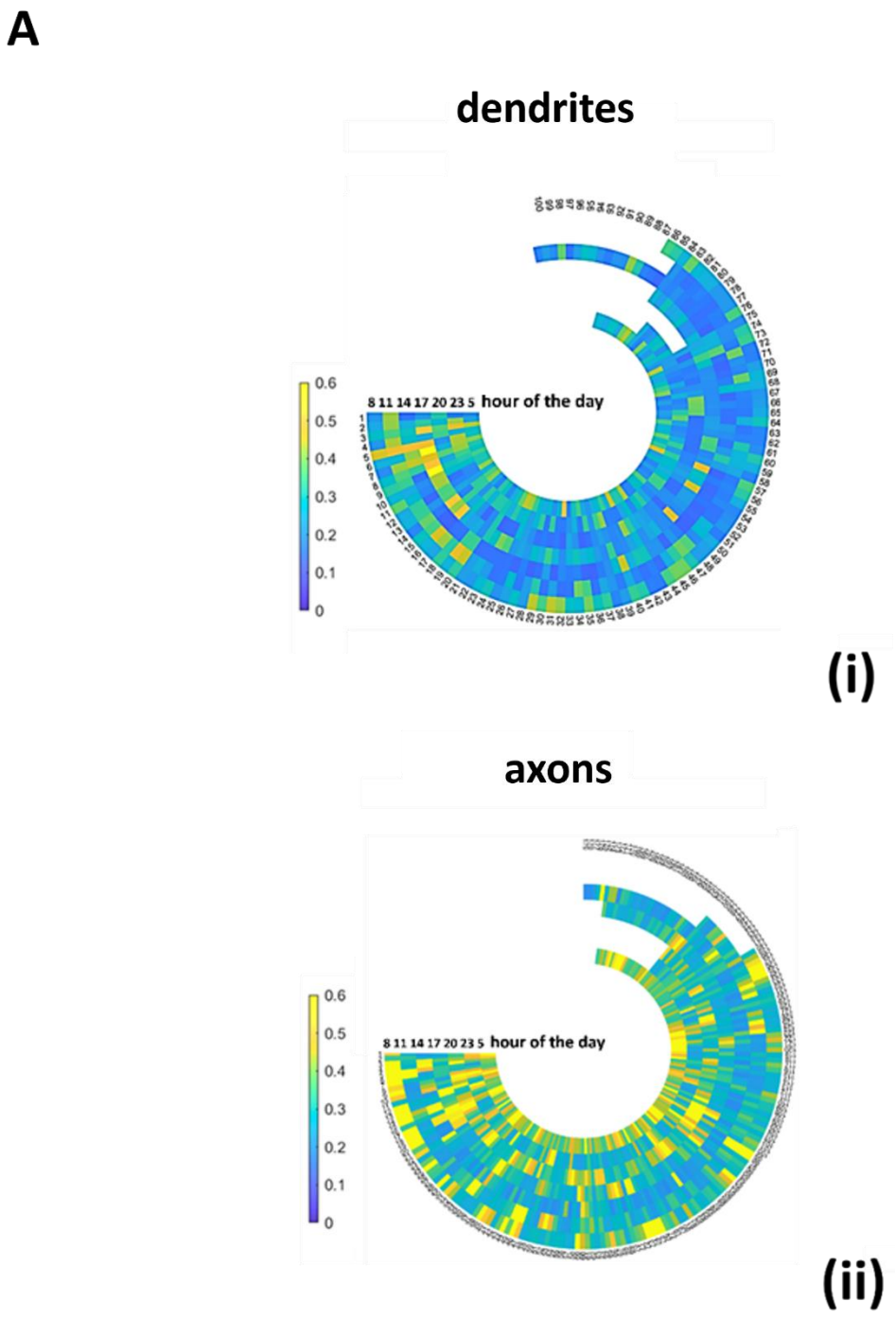


Figure2 Circular HeatMaps show higher Ca^{2+} activity in the axons compare to the dendrites

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(A) An example of a histogram describing the distribution of peaks in the trace. In the external ring the numbers indicate the peaks in the traces while the colour coding indicates the amplitude of the transients. From low amplitude in blue to high amplitude in yellow. Each ring refers to a single trace recorded at a specific time (timing of the recording at the beginning of each ring). **(i)** circular histogram for the recordings in the dendrites over 24h **(ii)** circular histogram for recordings in the axons over 24h.

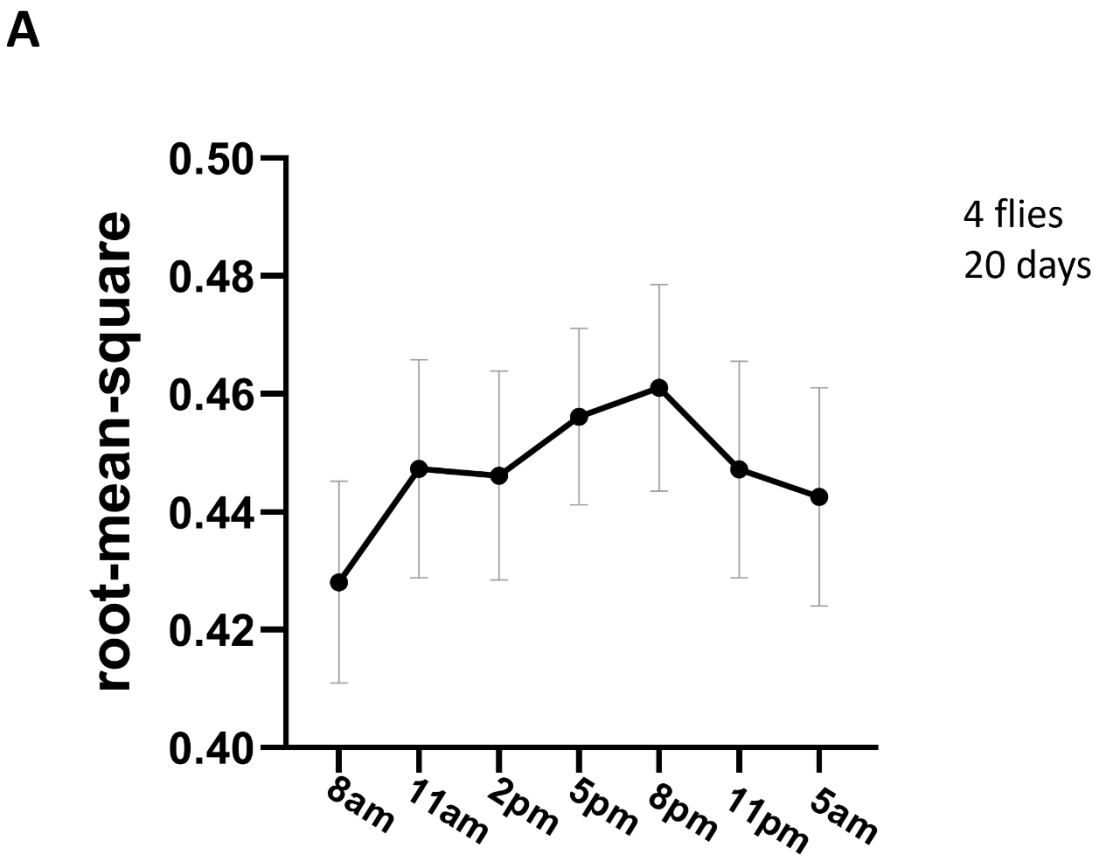


Figure3 Variation of Ca^{2+} imaging traces during 24h

(A) Root-mean-square (rms) calculation for traces recorded at different time of the day in a 24h period. Black dots are mean with error bars ±sem. Data are collected from 4 flies. Number of days 20.