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Sleep regulation in Drosophila

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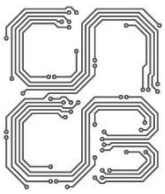

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Master Thesis

SLEEP REGULATION IN DROSOPHILA

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ENB MSc Neurosciences

Graduate School of Systemic Neurosciences

Ludwig-Maximilians-Universität München

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Oriol Pavón Arocas: *Sleep regulation in Drosophila*

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"As long as our brain is a mystery, the universe, the reflection of the structure of the brain will also be a mystery"

Santiago Ramón y Cajal (1852-1934)
1906 Nobel Prize Laureate

Sleep regulation in Drosophila

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Abstract

Sleep is a ubiquitous process preserved across all types of animals, but its functional implications and the underlying cellular and molecular mechanisms are far from being understood. In the fruit fly *Drosophila melanogaster*, a group of neurons projecting to the dorsal Fan-shaped Body (dFB) are implicated in homeostatic sleep regulation and can induce sleep on demand upon activation. Dopamine is an aminergic neurotransmitter with wake-promoting effects. Work in our lab revealed a strong inhibitory effect of dopamine on dFB neurons suggesting that dopamine achieves its arousal effects via directly shutting down sleep-promoting dFB neurons. Here, I have used a photoactivatable GFP-based tracing method to identify a small number of dopaminergic neurons residing in the PPL1 cluster that send projections to dFB neurons. In parallel, I have carried out an *in silico* screen of *Drosophila* transgenic lines to look for potential pre- and post-synaptic partners of the dFB to discover new structures with a possible role in the neural mechanisms of sleep regulation in *Drosophila*.

Keywords

Sleep, arousal, *Drosophila*, dopamine, dorsal Fan-shaped Body, photoactivatable GFP

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A tots vosaltres, gràcies.

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CHAPTER I

INTRODUCTION

Sleep

At first glance, sleep is a paradoxical process. Entering periodic states of immobility and quiescence has no obvious immediate gain; on the contrary, it would seem to place an organism at a clear survival disadvantage. Nonetheless, sleep is a ubiquitous process preserved across all types of animals including mammals, birds, reptiles, amphibians, fish and invertebrates (for a review see Campbell & Tobler 1984). Although the length and structure of sleep varies in different taxa, the common feature remains that most animals dedicate significant amounts of time to sleep every day.

In mammals, sleep can be defined according to the following behavioural criteria and their electrophysiological correlates: the adoption of a stereotypical posture, the maintenance of quiescence, an elevated arousal threshold and state reversibility upon stimulation (Campbell & Tobler 1984). Humans typically need between 7 and 9 hours of sleep per day, which corresponds to about a third of our lives (Hirshkowitz et al. 2015), whereas rhesus monkeys sleep around 12 hours a day (Crowley et al. 1972) and rats and mice around 13 hours (Twyver 1969).

Conserved over the course of evolution, the still unknown functions of sleep are believed to play an essential role for survival. The transition from sleep to wakefulness and vice versa is a critical step and is regulated by two different systems that interact with each other (Borbély 1982): the circadian system anticipates periodic environmental changes and matches sleep to specific periods of the day and night cycle, whereas the homeostatic system senses changes in internal states and sleep need and is in charge of use dependent increases in sleep time (sleep rebound).

Over the past several decades experiments in mammals have started to dissect out the role of specific wake-promoting nuclei (Saper et al. 2010). Such nuclei are formed by heterogeneous groups of high numbers of neurons, a characteristic that has hindered attempts to identify and characterise cellular circuits and mechanisms involved in the regulation of sleep. One way to overcome this complexity is to move to simpler model organisms that are still able to generate complex behaviours like sleep. The animal model I used in this project is the fruit fly *Drosophila melanogaster.*

Drosophila as a model organism for studying sleep

The fruit fly *Drosophila melanogaster* presents itself as an appealing animal model with a nervous system that contains a manageable number of neurons compared to that of mammals and yet is capable of generating complex behaviours. The fly's genome is known in detail (Adams et al. 2000), and there is an extensive and comprehensive genetic toolbox available that allows the neurogenetic dissection of many behaviours (Venken et al. 2011). Researchers are now able to direct gene expression to multiple specific subsets of neurons by means of binary systems such as GAL4-UAS and LexA-LexAop (Fischer et al. 1988; Brand & Perrimon 1993; Lai & Lee 2006) and interfere with specific neural circuits to dissect how the brain works (Zemelman et al. 2002; Lima & Miesenböck 2005; Claridge-Chang et al. 2009).

Perhaps one of the most important accomplishments of behavioural genetics is the discovery of *period,* a *Drosophila* mutant with alterations in the circadian clock that set the ground for the understanding of the molecular and cellular basis of circadian sleep control (Konopka & Benzer 1971). In the past few years, *Drosophila* has also emerged as a powerful model system to study the

cellular underpinnings of sleep. Two seminal papers published simultaneously by independent research teams revealed that episodes of prolonged rest in *Drosophila* correspond to a sleep-like state (Hendricks et al. 2000; Shaw et al. 2000) that meets the criteria of sleep (Campbell & Tobler 1984). First, periods of quiescence are associated with a preferred location and increased arousal thresholds. In addition, quiescent states (sleep) exhibit homeostatic regulation: a substantial increase in sleep is observed after sleep deprivation (sleep rebound). Sleep rebound is dissociated from circadian control. Finally, and similarly to mammals, rest in *Drosophila* is age-dependent and can be modulated by stimulants and hypnotics such as caffeine or antihistamines. These two studies provided the starting point for a genetic dissection of the neural mechanisms of sleep regulation in *Drosophila*.

The dorsal Fan-Shaped Body: output arm of the sleep homeostat

Although the importance of sleep is undisputed, the functional implications of this mysterious process are far from being understood. Two of the most revisited theories regarding the function of sleep are the synaptic homeostasis hypothesis (Tononi & Cirelli 2006) and the involvement of sleep in memory consolidation (Diekelmann & Born 2010). Recent studies in *Drosophila* have established direct connections between sleep and restoration of behavioural plasticity (Bushey et al. 2011; Dissel et al. 2015) and between sleep and memory consolidation (Donlea et al. 2011; Berry et al. 2015).

In order to be able to address more concrete questions about sleep regulation and its benefits to the nervous system it is crucial to understand the circuitry underlying sleep itself. A major breakthrough in this direction was accomplished in 2011 when a group of neurons projecting to the dorsal fan-shaped body (dFB) were found to induce sleep on demand upon activation (Donlea et al. 2011). These neurons strongly resemble the ExF/2 neurons and can be specifically targeted by means of C5-GAL4, 104y-GAL4 and C205-GAL4 lines (Young & Armstrong 2010) and also by the more recently discovered R23E10-GAL4 line (Jenett et al. 2012), which shows a particularly high specificity for dFB neurons with little or no transgene expression detectable elsewhere (Donlea et al. 2014) (Figures 1A and 1B).

Further studies have implicated the dFB neurons specifically in homeostatic sleep regulation (Donlea et al. 2014). Whole-cell recordings have revealed that individual dFB neurons can exist in different states of excitability (Figure 1C) despite being otherwise anatomically indistinguishable (Li et al. 2009; Donlea et al. 2014). In addition, the intrinsic properties of these neurons are modulated by sleep pressure, shifting their input-output function according to sleep need (Figure 1D). Mutants in which this modulation is disrupted present sleep deficits and lack homeostatic sleep regulation. To date, the neuronal signals that instruct these changes remain elusive.

The discovery of a structure in the fly brain with a central role in sleep homeostasis raises the possibility of untangling the neural mechanisms of sleep regulation and of tackling questions such as which structures in the brain provide inputs to dFB neurons or receive outputs from them, which neurotransmitters mediate these connections, and what the cellular and molecular components of the circuit are.

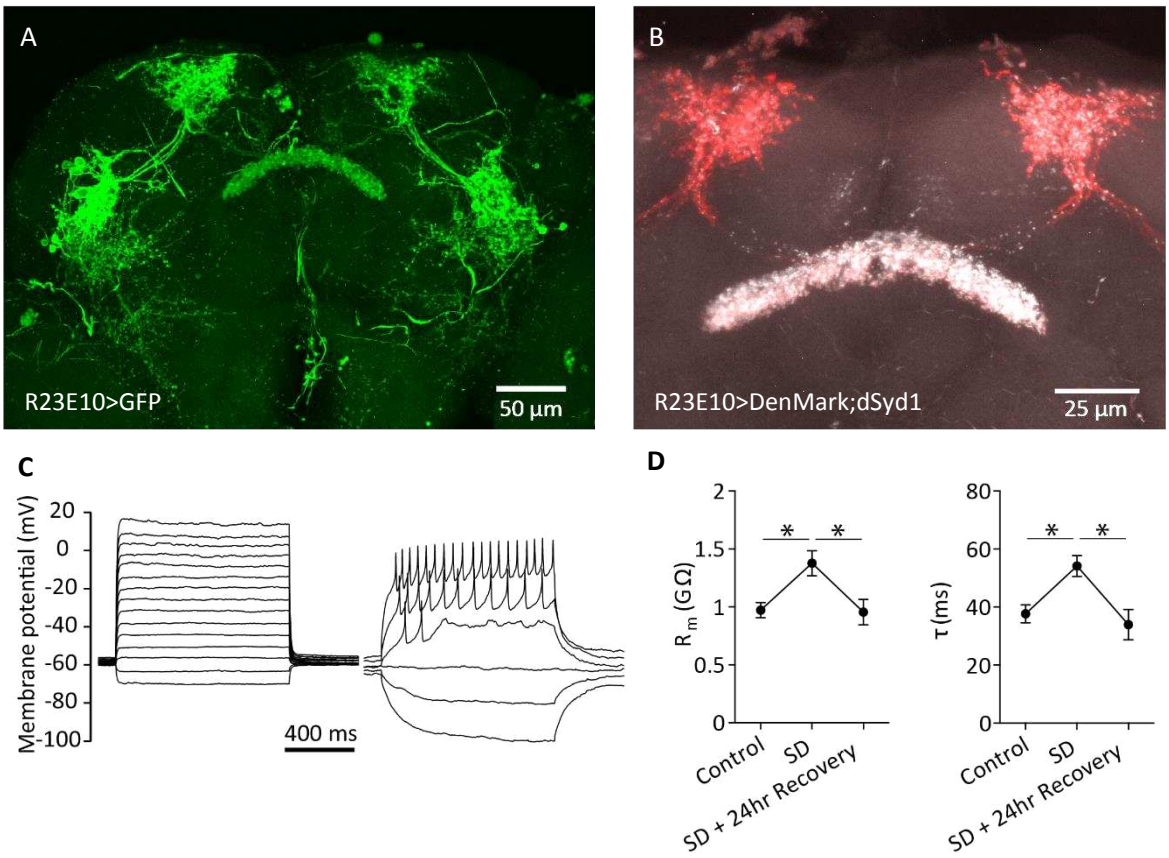


Figure 1. Morphology and electrophysiological properties of sleep-control neurons | (A) Maximum intensity projections of confocal sections show that R23E10-LexA drives expression on sleep-promoting dFB projecting neurons. (B) DenMark and dSyd1 reveal the polarity of the dFB neurons. DenMark (red) is a dendritic marker, dSyd1 (white) is a pre-synaptic marker. Dendrites are located dorsally and axons project to the dorsal layer of the fan shaped body (image courtesy of Jeffrey Donlea). (C) Examples of membrane potential changes evoked in dFB neurons by injection of current steps (modified with permission from Donlea et al. 2014). (D) Sleep history alters the input resistance (R_m) and the membrane time constant (τ_m) of dorsal FB neurons. SD stands for sleep deprivation. Asterisks denote significant differences in pairwise post hoc comparisons (modified with permission from Donlea et al. 2014).

Dopaminergic regulation of sleep and arousal

In mammals, evidence suggests that several aminergic neurotransmitters, including dopamine, serotonin, noradrenaline and histamine, play a role in the regulation of the sleep/wake cycle (Boutrel & Koob 2004; Saper et al. 2005). Psychostimulant drugs that enhance dopaminergic transmission have wake-promoting effects and arousing properties both in rodents (Isaac et al. 2003) and in *Drosophila* (Andretic et al. 2005). In addition, mutations of the *Drosophila* dopamine transporter DAT/fumin that increase synaptic levels of dopamine translate into decreased sleep times and increased arousal (Kume et al. 2005). Further to playing an important role in different types of arousal (Van Swinderen & Andretic 2011), dopamine also mediates several other behaviours, including aversive reinforcement and appetitive motivation (Claridge-Chang et al. 2009; Waddell 2010).

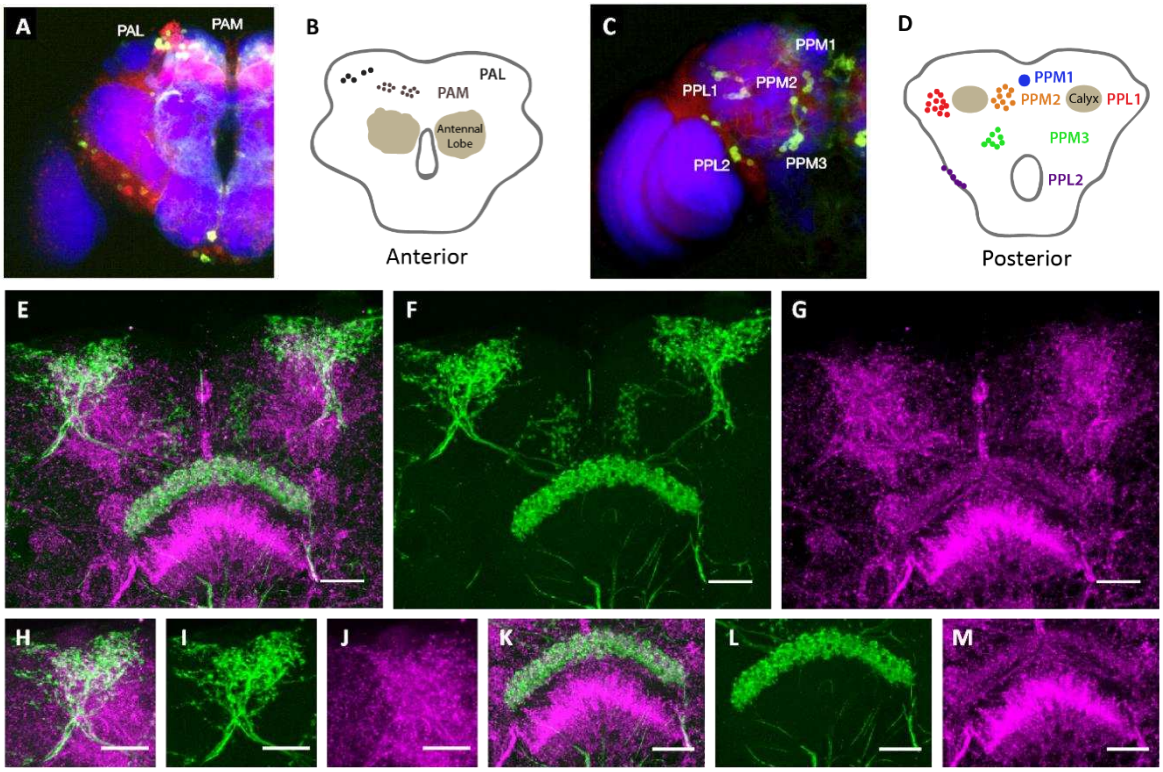


Figure 2. Dopaminergic clusters of the TH-GAL4 line relative to the projections of dFB neurons from R23E10-LexA | (A and C) Anterior and posterior view of the different dopaminergic clusters in the *Drosophila* TH-GAL4 line. Adapted with permission from Claridge-Chang et al. 2009. (B and D) Scheme of the location of the dopaminergic cell bodies captured by the TH-GAL4 line. (E-G) Maximum intensity projections of confocal sections of the posterior half of whole-mount adult brains of flies expressing TH-GAL4>mCherry (magenta) and R23E10-LexA>GFP (green). (H-M) Detailed view of the dendritic arbour (H-J) of R23E10-LexA>GFP overlapped with TH-GAL4>mCherry and the Fan-shaped Body (K-M). All scale bars represent 25 µm. PAM: protocerebral anterior medial. PAL: protocerebral anterior lateral. PPL: protocerebral posterior lateral. PPM: protocerebral posterior medial.

Multiple lines of evidence thus suggest that dopamine promotes wakefulness in *Drosophila*, but the underlying dopaminergic circuit remains to be determined. Dopamine is secreted by 200-300 neurons in the central fly brain (Budnik & White 1988), which are organised into different clusters according to their location and projection patterns (Claridge-Chang et al. 2009; Mao & Davis 2009) (Figures 2A to 2D). It has been previously described that specific subsets of dopaminergic neurons are involved in concrete, differentiated functions (Claridge-Chang et al. 2009; Waddell 2013; Lin et al. 2014). Could this also be the case in the sleep/wake circuit? If so, which of the dopaminergic clusters play a role in regulating sleep and arousal and which are their synaptic targets?

Recent studies addressing these questions revealed that dopaminergic neurons interact closely with the dFB sleep-promoting neurons to modulate sleep (Liu et al. 2012; Ueno et al. 2012) and sensitivity to anaesthetics (Kottler et al. 2013). These studies showed that dopaminergic neurons addressable by the TH-GAL4 line (Friggi-Grelin et al. 2003) make synaptic connections with dFB neurons and that DA1/DopR receptors in dFB neurons mediate the wake promoting effects of dopamine in *Drosophila* (Liu et al. 2012; Ueno et al. 2012). Furthermore, the same groups tried to pin down the specific

neurons responsible for dopamine signalling to the dFB, reaching conflicting conclusions. Using Mosaic Analysis of a Repressible Cell Marker (MARCM) (Lee & Luo 1999), Ueno and colleagues found a single dopaminergic neuron within the protocerebral posterior medial 3 (PPM3) cluster projecting to the FB (although not to the dorsal layer). Transient activation of this neuron (or group of cells containing this neuron) reduced sleep (Ueno et al. 2012). Interestingly, using an independent approach, Liu and colleagues generated restricted dopaminergic GAL4 drivers based on the TH-GAL4 line and linked the wake-promoting effects of dopamine to a pair of protocerebral posterior lateral 1 (PPL1) neurons projecting to the dFB (Liu et al. 2012). It remains unclear which are the direct effects of dopamine on the intrinsic properties of dFB neurons and which of the dopaminergic neurons in the fly brain produce such effects.

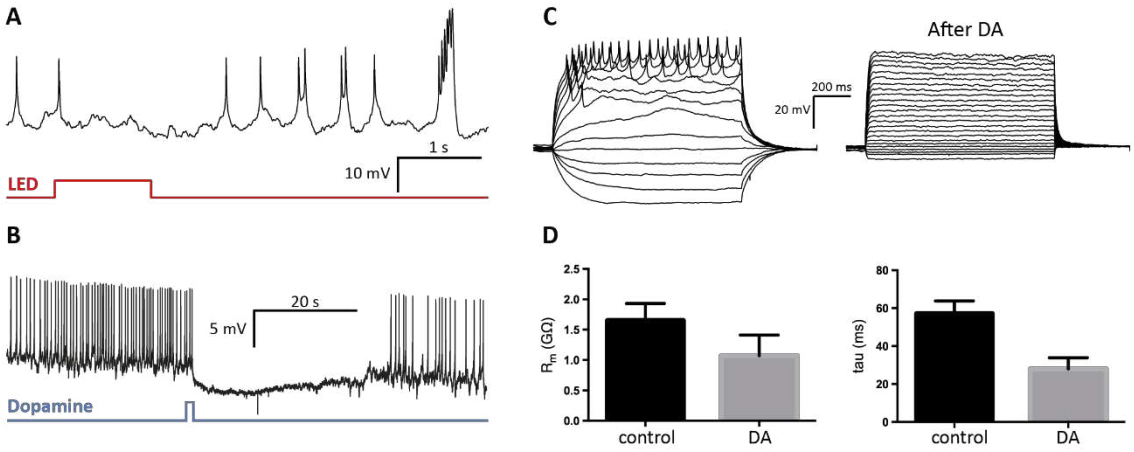


Figure 3. Dopamine suppresses activity in the dFB | (A) Transient photoactivation of the TH-GAL4 neurons by expressing Chrimson suppresses the activity of dFB neurons. (B) Direct application of dopamine (DA) on the dendritic tuft of dFB neurons mimics the effect of activating the full subset of TH-GAL4 neurons. (C) Long term application of dopamine in the dendritic arbour switches dFB neurons to an unexcitable state. (D) Long term application of dopamine in the dendritic arbour decreases the input resistance (R_m) and the time constant (τ_m) of sleep-promoting dFB neurons. Error bars represent SEM.

Experiments in our lab revealed that optogenetic activation of TH-GAL4 neurons while recording from dFB neurons has a strong inhibitory effect capable of suppressing tonic spiking in these neurons (Figure 3A) (Pimentel et al. in prep). Interestingly, direct application of brief pulses of dopamine to the dendritic arbour of dFB neurons mimics the effects of activation of the full subset of neurons comprised by the TH-GAL4 line (Figure 3B), suggesting that the dendritic arbour is the main locus of action of dopamine in dFB neurons. In addition, long term application of dopamine on the dendritic tuft induces a drastic shift in the intrinsic properties of dFB neurons (decreasing the R_m and τ_m) and suppresses the generation of spikes (Figures 3C and 3D). These changes resemble the different excitability states reported previously. This line of evidence suggests that dopamine achieves its wake-promoting effects via directly shutting down the sleep-promoting dFB neurons, which constitute the output arm of the sleep homeostat. The main aim of my project is to identify the dopaminergic neurons within the TH-GAL4 line that project to the dendritic tuft of the dFB neurons and regulate sleep and arousal.

Mapping neural circuits via photoactivatable GFP tracing

Identifying how neural networks are organised and interact with one another has been a key challenge since the beginnings of neuroscience research. Notable efforts date as far back as Santiago Ramón y Cajal (Ramon y Cajal 1889), whose drawings of stained subsets of structures in the nervous system inspired the neuron doctrine. Progress in our understanding of neural circuits has gone hand in hand with the development of more sophisticated techniques. Better microscopy and labelling techniques have been key to establishing the detailed anatomical structure of the nervous system, whereas the ability to label and photoactivate defined subsets of neurons allow us to search for functional connections. Tracing techniques have continued to evolve in parallel with the increasing depth of our understanding of the brain (for a review see Lanciego & Wouterlood 2011), and now range from anterograde and retrograde tracings, for example by means of biocytin (Horikawa & Armstrong 1988; King et al. 1989) or horseradish peroxidase (HRP) (Kristensson & Olsson 1971), to monosynaptic circuit tracing with *Rabies virus* (Deshpande et al. 2013; Callaway & Luo 2015). A constant flow of discoveries of new and interesting molecules drives novel applications and techniques that help us navigate the intricate complexity of neural circuits.

One example of such molecules is a novel variant of the *Aequorea victoria* green fluorescent protein (GFP) (Shimomura et al. 1962; Chalfie et al. 1994; Tsien et al. 1997), named photoactivatable GFP (PA-GFP) (Patterson & Lippincott-Schwartz 2002). Photoactivation consists of the rapid conversion of a molecule to its fluorescent state by intense irradiation at a specific wavelength. Upon photoactivation, PA-GFP shifts from a barely fluorescent form to an intensely fluorescent form and diffuses throughout the cell to achieve a uniformly distributed equilibrium (Patterson & Lippincott-Schwartz 2002). Since its development, PA-GFP has been readily combined with the powerful genetic toolbox available in *Drosophila* and the three-dimensional, spatially resolved capabilities of two-photon laser scanning microscopy (Denk et al. 1990) to develop a combined genetic and optical neural tracing method to study neuronal processes of individual neurons in the fly brain (Datta et al. 2008; Claridge-Chang et al. 2009). More recently, researchers have managed to generate enhanced versions of PA-GFP, namely C3PA-GFP and SPA-GFP (Ruta et al. 2010), that have increased the sensitivity and usability of these tools (Aso et al. 2014).

PA-GFP tracing permits non-random, optically guided circuit mapping by labelling anatomically or genetically targeted subsets of neurons (Datta et al. 2008). In this project, I have used two independent binary systems to express 10xUAS-SPA-GFP in dopaminergic neurons and mCherry in sleep-promoting dFB neurons. This has allowed me to target discrete regions of the dFB neurons via mCherry guided two-photon visualization and specifically photoactivate the dopaminergic projections present in that region of interest. Once the photoactivated PA-GFP has diffused and filled the cells, it is possible to identify which dopaminergic neurons target the dFB and are part of the sleep/wake circuit. In parallel, I have carried out an *in silico* screen of *Drosophila* transgenic lines to look for potential pre- and post-synaptic partners of the dFB to discover new structures with a possible role in the neural mechanisms of sleep regulation in *Drosophila*.

CHAPTER II

METHODS

Fly strains

Fly stocks were grown on standard cornmeal agar medium and maintained on a 12-hour light / 12-hour dark cycle at 25ºC and 60% humidity. The following *Drosophila melanogaster* strains were obtained from in-house databases existing at the Centre for Neural Circuits and Behaviour: UAS-mCD8::GFP, LexAop-rCD2::GFP, UAS-mCD8::mCherry, LexAop-mCherry and LexAop-rCD2::GFP;UAS-mCD8::mCherry. R23E10-LexA (Pfeiffer et al. 2010) and R84C10-GAL4 (Jenett et al. 2012) were gifts from Gerald Rubin. 10xUAS-SPA/CyO;MKRS/TM2 was a gift from Richard Axel.

For photoactivatable GFP tracing experiments, balanced TH-GAL4 flies (Friggi-Grelin et al. 2003) were crossed with 10xUAS-SPA/CyO;MKRS/TM2 flies (Aso et al. 2014) to yield 10xUAS-SPA/CyO;TH-GAL4/MKRS. In parallel, balanced R23E10-LexA flies were crossed with a balanced LexAop-mCherry line to yield R23E10-LexA/CyO;LexAop-mCherry/Ser. Finally, 10xUAS-SPA/CyO;TH-GAL4/MKRS flies were paired with R23E10-LexA/CyO;LexAop-mCherry/Ser virgins to obtain the experimental flies R23E10-LexA/10xUAS-SPA;TH-GAL4/LexAop-mCherry.

Expression patterns were visualised by crossing balanced GAL4 driver lines to balanced responder strains carrying UAS-mCD8::GFP or UAS-mCD8::mCherry and balanced LexA driver lines to balanced responder strains carrying LexAop-rCD2::GFP or LexAop-mCherry to yield the following experimental flies: R23E10-LexA/CyO;LexAop-rCD2::GFP/Ser, R23E10-LexA/CyO;LexAop-mCherry/Ser, R23E10-LexA/LexAop-rCD2::GFP;R84C10-Gal4/UAS-mCD8::mCherry, UAS-mCD8::mCherry/CyO;TH-GAL4/Sb and R23E10-LexA/UAS-mCD8::mCherry;TH-GAL4/LexAop-rCD2::GFP.

Photoactivatable GFP tracing

Brains of 3- to 7-day old flies were dissected in a Sylgard-coated Petri dish filled with extracellular solution containing 103 mM NaCl, 3 mM KCl, 5 mM TES, 8 mM trehalose, 10 mM glucose, 7 mM sucrose, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$ (pH 7.3) continuously equilibrated with a mixture of 95% O_2/5% CO_2. Once dissected, the brains were mounted posterior up (antennal lobes facing down) in a microscope slide previously coated with Poly-L-lysine (Sigma-Aldrich) to prevent them from moving. Brains were under constant perfusion with carbogenated extracellular solution for the full duration of the experiments.

Photoactivation experiments were performed using a two-photon laser scanning microscope (Chameleon Ultra II, Coherent) modulated by Pockels Cells (Conoptics 302RM) and coupled to the scan engine of a Movable Objective Microscope (Sutter Instruments) equipped with a 20x/1.0NA W-Plan-Apochromat objective (Zeiss). Emitted photons were separated from excitation light by a series of dichromatic mirrors and dielectric and coloured glass filters and detected by GaAsP photomultiplier tubes (Hamamatsu Photonics H10770PA-40 SEL) for green and red fluorescence. Photomultiplier currents were amplified (Laser Components HCA-4M-500K-C) and passed through a custom-designed integrator circuit to maximize the signal-to-noise ratio. The microscope was controlled through ScanImage r3.8 run in MATLAB 2012a (MathWorks) via a PCI-6110 DAQ interface board (National Instruments).

The dFB of experimental flies was labelled with LexAop-mCherry by driving expression under the R23E10-LexA promoter. In addition, ten copies of a UAS-SPA transgene (Aso et al. 2014) were expressed in dopaminergic neurons under the control of the TH-GAL4 promoter. By exciting the samples with light centred at 1000nm with an intensity of 3 to 6 mW (as measured at the specimen), the mCherry expressed by dFB neurons could be visualised and a 3D volume was selected as a target for photoactivation. The Pockels Cells bias voltage was calibrated for each wavelength used (710 nm, 925 nm and 1000 nm) and adjusted to obtain maximum signal-to-noise ratio in each phase of the experiment.

To photoconvert PA-GFP from its inactive to its intensely fluorescent form (Patterson & Lippincott-Schwartz 2002; Datta et al. 2008; Claridge-Chang et al. 2009; Ruta et al. 2010; Aso et al. 2014) the laser was tuned to 710 nm and the selected volume of tissue containing the dendritic arbour of neurons projecting to the dFB (or other target areas) was illuminated with an intensity between 2 and 4 mW. The photoactivation scan was performed at 512×512 pixels resolution with a pixel size of 0.03-0.05 µm and a pixel dwell time of 3.2 µs. The target volume comprised 4 to 15 µm of the dendritic arbour or axonal projections from neurons projecting to the dFB and it was divided into z-slices with 2 to 4 µm steps, depending on the orientation of the sample. Before moving the focal plane to the following z-slice, each pixel was scanned 4 times successively using the frame-averaging function of the microscope software (ScanImage r3.8). The number of repetitions of the photoactivation scan varied between 90 and 150 repetitions, depending on the target volume, the expression levels of PA-GFP and the depth of the photoactivation target. Each repetition was separated by a 15 to 30 seconds period to allow diffusion of the photoactivated GFP and minimize photobleaching.

In initial experiments, the right or left half of the brain was photoactivated at a pixel size of 0.25 µm. The target volume comprised 60 µm and was divided in z-steps of 5 µm. This successfully revealed the main dopaminergic clusters present in the TH-GAL4 line.

Brains were set for a 30 min period to allow diffusion of the photoactivated PA-GFP and then prepared for confocal microscopy as follows: fixed for 20 min under vacuum with 4% paraformaldehyde in phosphate buffered saline (PBS, 1.86 mM NaH_2PO_4, 8.41 mM Na_2HPO_4, and 175 mM NaCl), washed multiple times in PBS, embedded in Vectashield (Vector Labs), sealed with nail polish and kept at 4ºC in the dark until confocal image acquisition.

Confocal microscopy

For imaging of native GFP or mCherry fluorescence, brains were dissected in the same extracellular solution used in the photoactivatable GFP tracing experiments, fixed for 20 min under vacuum with 4% paraformaldehyde in PBS and washed multiple times in PBS. Brains were kept on ice during the whole procedure until mounting. All brains were embedded in Vectashield (Vector Labs), sealed with nail polish and kept at 4ºC in the dark.

All confocal images, including the ones from photoactivated brains, were collected on a Leica TCS SP5 X confocal microscope with a 25x/0.95NA W or a 40x/1.30NA Oil objective (Leica) and processed with Fiji and Adobe Illustrator.

Image processing

Confocal stacks from photoactivatable GFP experiments were processed in Fiji as follows: a maximum projection image was generated from the green channel (PA-GFP) and ROIs were drawn around putatively photoactivated cell bodies. These ROIs were used as a mask to obtain the fluorescence z-profiles from the confocal brain stacks using a custom-written macro. Extracted data were imported to Igor Pro v6.37 and analysed with a custom-written macro in order to perform background subtraction and obtain the following parameters from each ROI: the maximum fluorescence intensity and the depth of the putative somata. A photoactivation profile for each brain was established from the obtained values.

Representative maximum projections of photoactivated brains were generated in Fiji and processed in Adobe Illustrator.

In silico screen

In an attempt to identify GAL4 lines driving expression to structures that could potentially be pre- or post-synaptic partners of neurons projecting to the dFB, a screen across a library of 3496 registered Janelia Farm GAL4 *Drosophila* brain stacks (Jenett et al. 2012) was run in MATLAB R2014b (MathWorks) using a customised version of a script written and kindly shared by Oliver Barnstedt (Centre for Neural Circuits and Behaviour, University of Oxford, Oxford, UK).

Given that the R23E10 line was unavailable in the Janelia Farm database at the time this screen was performed, an anatomical comparison was carried out to demonstrate clear structural overlapping of R23E10-LexA with its closest resembling line, the R84C10-GAL4 (Jenett et al. 2012) (Figure 7). R84C10 was then chosen as the template line for the screen.

The Segmentation Editor Plugin for Fiji was used to draw three different ROIs in the registered R84C10 z-stack: the dendritic tuft of the neurons projecting to the dFB, the gap generated by the structure of the dendritic arbour, and the dFB region (Figure 8). Each ROI was converted into a 3D template file and the script was used to calculate the absolute and relative overlap between the three ROIs and each of the 3496 registered Janelia Farm GAL4 *Drosophila* brain stacks and to compile a list where the lines with strongest overlap ranked on top. The first 100 hits for each of the six conditions (absolute and relative overlap for the three ROIs) were individually assessed and scored from 0 to 10 (being 10 the best possible score).

Brain stacks with a score superior to 8 were merged with a R84C10 stack in Fiji and analysed further. Features such as structural connectivity between both lines, number of cells labelled by the driver (taken as an indicative of how clean the line was) and labelling of structures different from the dFB were taken into account before compiling a final list of potentially interesting GAL4 driver lines with a possible role in regulating or interacting with dFB neurons. The GAL4 driver lines present in the final list were ordered from Bloomington Stock Centre and kept at the Centre for Neural Circuits and Behaviour for future behavioural testing.

CHAPTER III

RESULTS

Photoactivation of PA-GFP successfully reveals the main dopaminergic clusters captured by the TH-GAL4 line

In a set of proof of principle experiments, I drove expression of ten copies of PA-GFP in flies under the control of the TH-GAL4 driver, which captures most dopaminergic neurons. I targeted the photoactivation scan to half of the brain with a two-photon laser scanning microscope (Figure 4A). After photoactivated PA-GFP diffused and filled the cells, I could identify the main dopaminergic clusters addressed by the TH-GAL4 line (Figures 4B to 4D) present in the posterior part of the *Drosophila* brain. The results of these experiments support the accuracy and suitability of the method for the aims of my project.

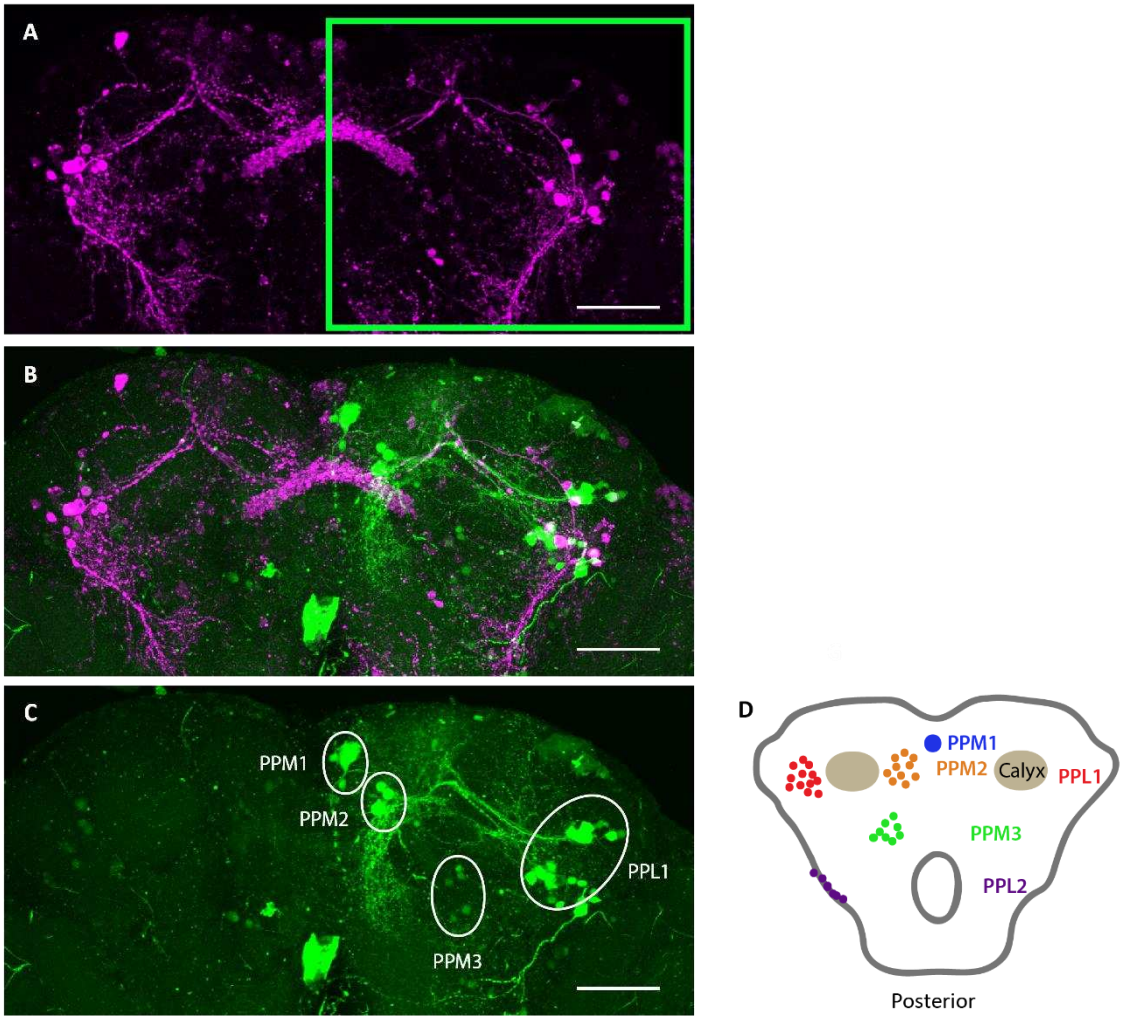


Figure 4. Clusters of dopaminergic neurons as revealed by PA-GFP | (A) Maximum intensity projection of mCherry expression controlled by R23E10-LexA shows the structure of the sleep homeostat of the fruit fly (magenta). The green square signals the area selected for photoactivation. (B-C) Maximum intensity projections of 10xUAS-SPA labelled dopaminergic neurons captured by TH-GAL4 (green). White circles indicate distinct sets of dopaminergic neurons revealed after photoactivation. (D) Scheme of the distinct subsets of dopaminergic neurons captured by TH-GAL4. Scale bars indicate 50 µm.

PPL1 neurons project to the dendritic arbour of sleep-promoting dFB neurons

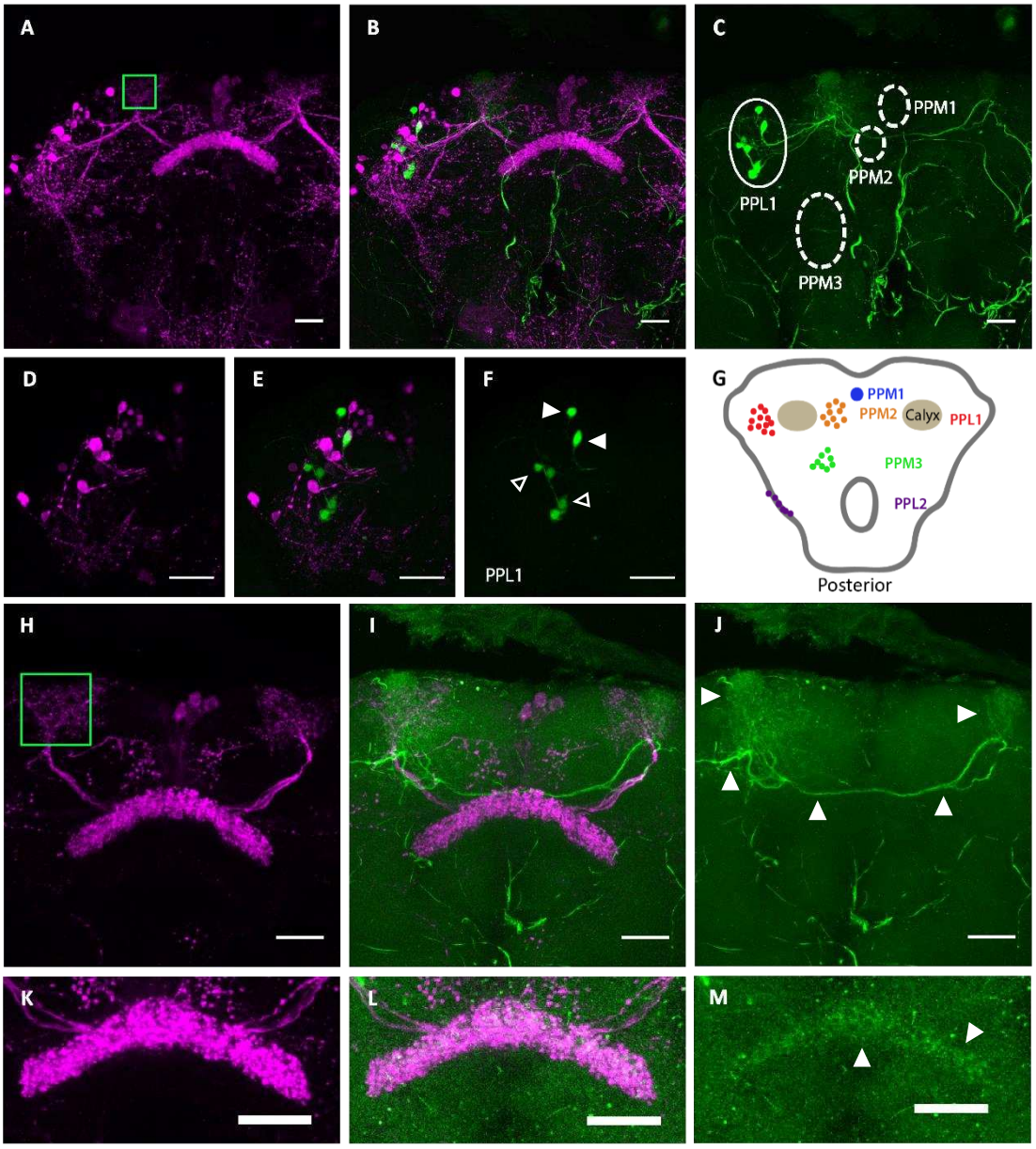
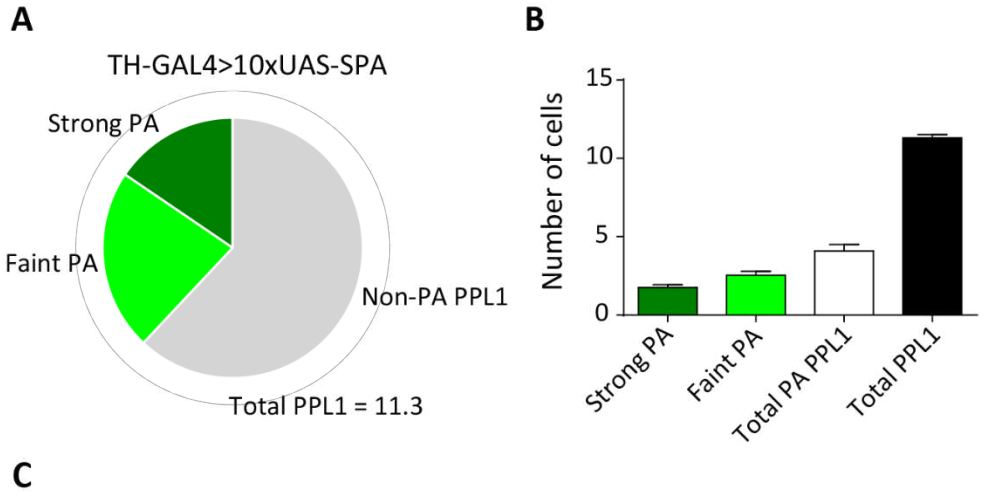


Figure 5. A small number of dopaminergic PPL1 neurons project to the dendritic arbour of sleep-promoting dFB neurons | (A-C) Photoactivation of the dendritic tuft of dFB neurons (green square) in flies expressing mCherry under the control of R23E10-LexA (magenta) and 10xUAS-SPA under the control of TH-GAL4 (green) reveals a small number of dopaminergic neurons exclusively from the PPL1 cluster (solid circle), but non from the other clusters captured by TH-GAL4 (dashed circles). Images are maximum intensity projections of confocal stacks. (D-F) Zoomed maximum intensity projections of the PPL1 cluster reveal strongly labelled (solid arrowheads) and weakly labelled (empty arrowheads) dopaminergic neurons (green). (G) Scheme of the distinct subsets of dopaminergic neurons captured by TH-GAL4. (H-M) Maximum intensity projections of the neurites (solid arrowheads) from photoactivated PPL1 neurons. All scale bars represent 25 µm.

Once the parameters for the photoactivation scans were established I set out to determine which dopaminergic neurons target sleep-promoting dFB neurons and are part of the sleep/wake circuit. As before, I expressed 10xUAS-SPA in the dopaminergic neurons captured by the TH-GAL4 driver. In addition, in order to restrict the photoactivation scans to regions relevant to the sleep/wake circuit, I took advantage of the complementary LexA system to express mCherry in the sleep-promoting dFB neurons addressed by the R23E10-LexA driver. This allowed me to visualise dFB neurons and their projections and exclusively target the dendritic tuft (or any other region of interest) of sleep-promoting dFB neurons with a two-photon laser scanning microscope (Figure 5A and 5H). Diffusion of the photoactivated PA-GFP to the somata revealed that only dopaminergic neurons belonging exclusively to the PPL1 cluster were activated (Figures 5A to 5C). A detailed analysis indicated that some neurons are more strongly labelled than others (Figures 5D to 5F). In addition, activated PA-GFP filled the neurites of such neurons revealing that at least one of the labelled neurons has contralateral projections (Figures 5H to 5J) and another one has projections to the dFB (Figures 5K to 5M).

Quantification of all the photoactivated brains consistently revealed two strongly labelled PPL1 neurons and two to three not as strongly labelled PPL1 neurons (Figure 6). Altogether, around four PPL1 neurons per hemisphere project to the dendritic tuft of sleep-promoting dFB neurons.



C

	Mean	SEM	N
Strong PA	1,75	± 0,18	12
Faint PA	2,55	± 0,25	11
Total PA PPL1	4,08	± 0,42	12
Total PPL1	11,3	± 0,21	10

Figure 6. A subset of PPL1 neurons project to the dendritic tuft of dFB neurons | (A-B) Number of PPL1 neurons identified after photoactivation of the dendritic tuft region of sleep-promoting dFB neurons in flies expressing 10xUAS-SPA under control of TH-GAL4. Error bars represent SEM. (C) Summary table of mean, SEM and number of photoactivated brains.

To summarise, these data indicate that a subset of dopaminergic neurons residing in the PPL1 cluster are responsible for the wake-promoting effects of dopamine on the dendritic tuft of dFB neurons forming the output arm of the sleep homeostat in the fruit fly. Furthermore, the data also suggest that between two and four PPL1 neurons in each hemisphere mediate such effects.

Identification of putative pre- and post-synaptic partners of the dFB

In an attempt to identify GAL4 lines driving expression to structures that could potentially be pre- or post-synaptic partners of neurons projecting to the dFB, I first compared the expression patterns of R23E10-LexA, the line that specifically targets sleep-promoting dFB neurons, with R84C10-GAL4, a line that addresses a subset of dFB neurons that resemble those captured by R23E10-LexA (Figure 7) but that are unable to induce sleep upon transient activation. This comparison revealed an almost identical structural expression pattern: both lines drive expression to a subset of dFB neurons with different functions but with the same anatomical structure. This, in addition to the fact that R84C10 was registered in the Janelia Farm GAL4 library whereas R23E10 was not, allowed me to use R84C10 as the template for my GAL4 screen across 3496 lines.

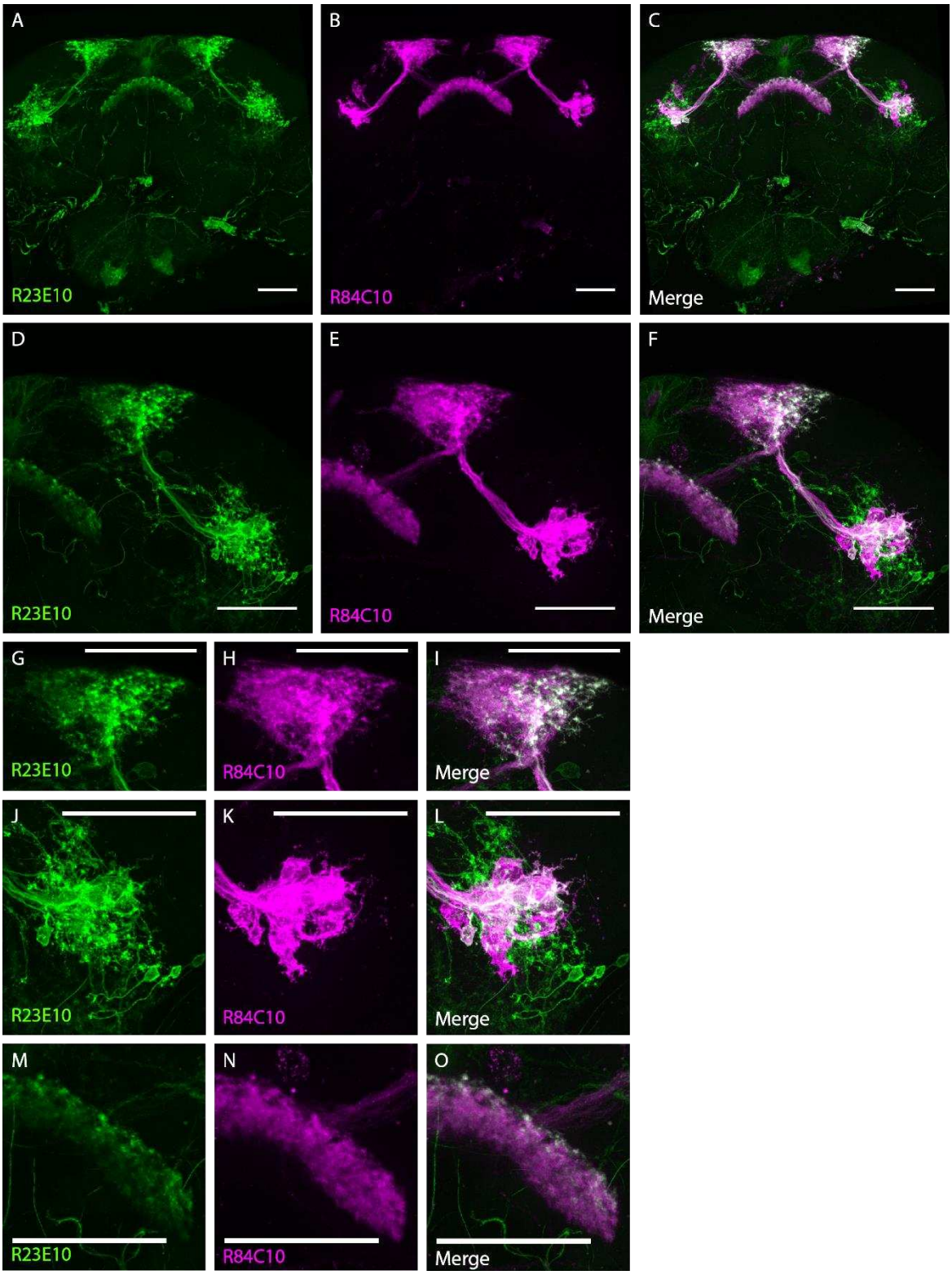


Figure 7. R84C10 and R23E10 have overlapping expression patterns in dFB neurons | (A-F) Maximum intensity projections of confocal sections of GFP expression under control of R23E10-LexA (green) and mCherry under control of R84C10-GAL4 (magenta). Zoomed projections reveal clear overlap in the dendritic tuft (G-I), somata (J-L) and axons (M-O) of both drivers. All scale bars correspond to 50 µm.

To find putative pre-synaptic partners I selected the area of the dendritic tuft of dFB neurons and the canal generated by the structure of the dendritic arbour as regions of interest (ROI) (Figure 8). In addition, I selected the dorsal layer of the Fan-shaped Body as a third ROI to identify lines that might contact the axons of dFB neurons (Figure 8), therefore being possible post-synaptic partners of dFB neurons. I then obtained a list of GAL4 lines sorted by decreasing intensity of overlap with the respective ROIs and individually selected the potentially interesting ones. Based on the anatomical interaction between the lines found in the screen and the R84C10 line I compiled a list with a total of 42 GAL4 *Drosophila* lines that could be functionally linked to the dFB (Table 1 and Figure 9). Finally, I searched the reference stock numbers and the insertion genes of these lines in Bloomington Stock Centre and FlyBase and ordered them for future behavioural testing (Table 2).

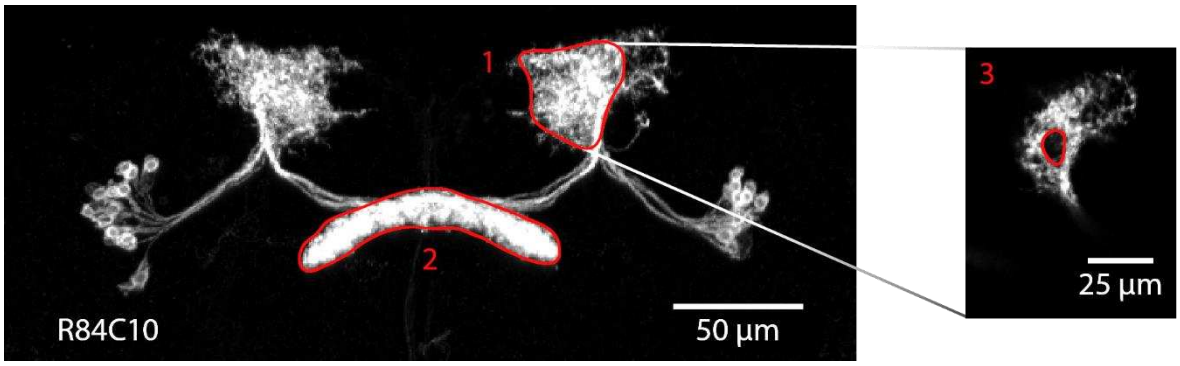


Figure 8. Schematic representation of the ROIs selected for the *in silico* screen of GAL4 driver lines | Maximum intensity projection of the registered brain stack for the *Drosophila* R84C10-GAL4 line. (1) ROI delineating the dendritic tuft of neurons projecting to the dFB. (2) ROI containing the dorsal layer of the Fan-shaped Body, where the axons of dFB neurons reside. (3) ROI surrounding the canal that crosses the dendritic arbour of dFB neurons.

Dendritic Tuft		**Gap in Dendritic Tuft**		**dFB region**	
n = 10		n = 16		n = 16	
Total Overlap	**Relative Overlap**	**Total Overlap**	**Relative Overlap**	**Total Overlap**	**Relative Overlap**
n = 3	n = 7	n = 8	n = 8	n = 8	n = 8

Table 1. Summary table of the total number of lines selected from the results of the screen | 10 lines revealed expression patterns with substantial anatomical overlap in the dendritic arbour of dFB neurons, 16 lines with the canal within the dendritic tuft and 16 with the dorsal layer of the FB.

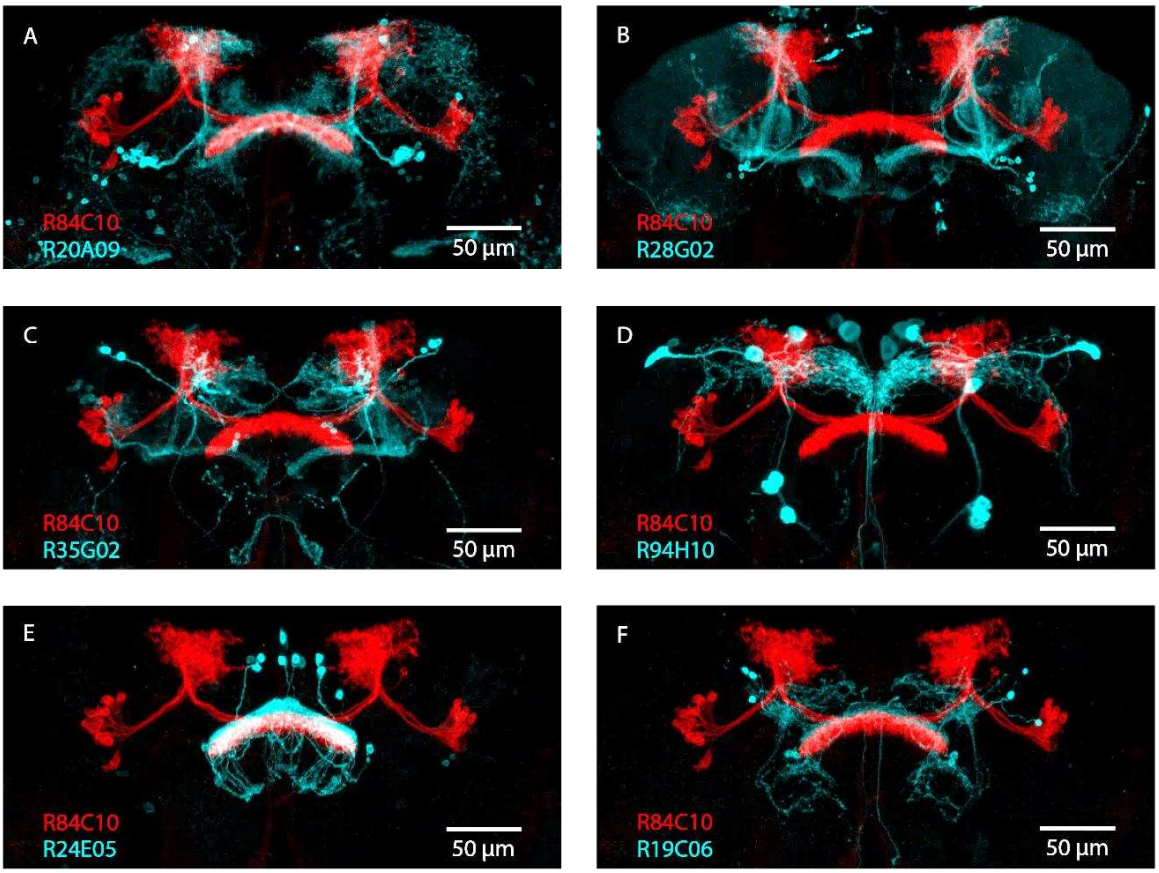


Figure 9. *In silico* screen results reveal putative pre- and post-synaptic partners of dFB neurons | (A-F) Examples of maximum intensity projections of registered Janelia Farm GAL4 brain stacks driving expression to neurons with projections to or from the dendritic tuft of dFB neurons (A-B), the gap generated in the dendritic arbour of dFB neurons (C-D) and the dorsal Fan-shaped Body (E-F).

Dendritic tuft of the neurons projecting to the dFB	GAL4 line	Bloomington stock number	Expresses GAL4 under the control of DNA sequences in or near	
Total overlap hits	R18E06*	48817	dlg1	MAGUK family – Guanylate kinase-like – Reproduction, gravitaxis
	R20A09*[a]	48875	trpm	LTrpC subfamily – Cation channel
	R92E10	40623	dpr15	Immunoglobulin-like – sensory perception of chemical stimulus
Relative overlap hits	R28G02*[a]	49466	mam	Mastermind family – neurogenic, development
	R16D06	48724	wb	Laminin – Cell migration, development

	R88F12	48398	inv	Engrailed homeobox family - Development
	R78G09	40015	SPR	G protein-coupled receptor, rhodopsin like – Mating, oviposition
	R44G12	47933	Rfx	DNA-binding – Nervous system development
	R19H11	49842	trpl	STrpC subfamily – Phototransduction, calcium transport
	R82C03	40135	CG9817	Zinc finger domain
Gap in dendritic tuft	**GAL4 line**	**Bloomington stock number**	**Expresses GAL4 under the control of DNA sequences in or near**	
Total overlap hits	R35G02*[a]	49918	slp1	Fork-head DNA-binding domain – Development, neuron projection development, morphogenesis
	R21D11	48945	Nplp1	Neuropeptide hormone activity, receptor agonist activity
	R24H02*	49097	CG32547	G protein-coupled receptor, rhodopsin-like – Neuropeptide receptor activity
	R15A04	48671	sc	bHLH domain - Development
	R32D11	49889	CadN	Cadherin – calcium ion binding, receptor activity, cell-cell adhesion
	R33D04	48113	ara	Homeobox DNA-binding domain – RNA pol II distal enhancer sequence-specific transcription factor
	R58G03	39193	CG6024	Low-density lipoprotein (LDL) receptor
	R82C06	40137	Octbeta3R	Octopamine receptor – G protein coupled, positive regulation of adenylate cyclase activity
Relative overlap hits	R45F03	50235	jar	Myosin-kinesin ATPase – motor activity

	R37G02*	49965	RhoGAPp190	Rho GTPase activating protein – mushroom body development, axogenesis, signal transduction
	R53G06	50448	Nos	Nitric oxide synthase – metabolism, development
	R17F09	48777	CG3371	DNA binding – positive regulation of Wnt signalling, development
	R51H05	41275	Vglut	L-glutamate transmembrane transporter, synaptic transmission
	R94H10[a]	47268	Atf-2	Activating transcription factor – Basic-leucine zipper domain
	R72D04	46677	D2R	Dopamine 2-like receptor – G-protein coupled via Gi/Go
	R14C11*	49256	so	Sine oculis homeobox family – DNA binding, circadian rhythm
dorsal Fan-shaped Body	**GAL4 Line**	**Bloomington stock number**	**Expresses GAL4 under the control of DNA sequences in or near**	
Total overlap hits	R24E05[a]	49081	Dl	EGF-like, notch binding, calcium binding – Cell cycle, migration, development
	R72G03	48309	CG31714	CUB domain, EGF-like, GPCR – G-protein coupled receptor activity
	R43D09	49553	pnt	DNA binding - Repressing transcription factor binding
	R84D12	40394	Eip93F	DNA binding HTH domain – development, programmed cell death
	R33E04	49752	mirr	Homeobox domain – gene expression regulation, development
	R56E07	39153	tna	Zinc finger - Zinc ion binding

	R91D10	40582	dpr12	Immunoglobulin – sensory perception of chemical stimulus, synaptic target recognition
	R65A06	39330	bib	Aquaporin-like – cation channel activity
Relative overlap hits	R19C06*[a]	48843	Dfd	unknown
	R38B06	49986	Nrt	Neurotactin – carboxylesterase, axon guidance, axon fasciculation
	R68B06	39458	TyrR	Octopamine-Tyramine receptor – G-protein coupled, phospholipase C-activating
	R24C07	49074	ana	Anachronism – TGF-beta, growth factor, development
	R48F11	50379	E2f	Transcription factor activity – development, growth
	R40E08	41238	dve	Homeobox domain – transcription factor, development
	R26B11*	49164	Fur1	Serine-type endopeptidase activity – glutamate receptor clustering, pre- and post-synaptic membrane organization
	R29A11	49881	fra	Netrin receptor activity – morphogenesis, development, axon guidance

Table 2. List of GAL4 driver lines, their Bloomington stock number and the gene were GAL4 is inserted | * indicates lines that appeared as a hit in two or more conditions of the screen (for example, R28G02 appeared in the dendritic arbour canal relative overlap, the dendritic tuft total overlap and the dendritic tuft relative overlap). [a] indicates lines whose expression patterns are depicted in Figure 9.

CHAPTER IV

DISCUSSION

Dopaminergic inputs target the effector arm of the sleep homeostat

In *Drosophila*, it has been established that the activity of a subset of neurons projecting to the dorsal layer of the Fan-shaped Body is sufficient for the induction and maintenance of sleep (Donlea et al. 2011). In addition, these neurons have been directly implicated in sleep homeostasis (Donlea et al. 2014) (Figure 1). Recent work has suggested that dopamine mediates sleep-regulation via specific connections to the dFB neurons (Liu et al. 2012; Ueno et al. 2012).

Work in our lab revealed a strong inhibitory effect of dopamine on dFB neurons, suggesting that dopamine achieves its arousal effects via directly shutting down sleep-promoting dFB neurons. Localised application of dopamine to the dendritic region of these neurons mimics the effects of optogenetic activation of the full subset of neurons comprised by the TH-GAL4 line, suggesting that the dendritic arbour is the main locus of action of dopamine in dFB neurons (Pimentel, et al. in prep) (Figure 3).

Previous studies have revealed that specific subsets of dopaminergic neurons are assigned to concrete, differentiated functions (Claridge-Chang et al. 2009; Waddell 2013; Lin et al. 2014). To test whether this is also true for dopaminergic modulation of the *Drosophila* sleep/wake circuit I used a PA-GFP-based tracing approach (Datta et al. 2008; Claridge-Chang et al. 2009; Ruta et al. 2010; Aso et al. 2014) to narrow down which dopaminergic neurons within the TH-GAL4 line project to the dendritic tuft of the dFB neurons. The data presented here indicates that a small subgroup of dopaminergic neurons residing in the PPL1 cluster project to the dendritic arbour of dFB neurons (Figures 5 and 6).

These results differ from data obtained by Ueno and colleagues, who identified a single unilaterally FB-projecting PPM3 neuron that has the ability to reduce sleep upon activation (Ueno et al. 2012). However, this PPM3 neuron does not project to the dorsal layer of the FB, and the magnitude of its effect on sleep reduction is substantially smaller than that seen after activating the full set of TH-GAL4 dopaminergic neurons, suggesting that this single neuron cannot fully account for the arousing effects of dopamine. In addition, the results described here suggest that there is little or no overlap between PPM3 neurons and the dFB neurons, suggesting that this particular neuron might exert its arousing influence indirectly via some other pathway.

In a parallel study, Liu and colleagues showed that, upon activation, restricted promoter lines based on the TH-GAL4 driver have wake-promoting influence (Liu et al. 2012). Following a combinatorial analysis they suggested that a pair of PPL1 neurons that project to the dFB are responsible for the arousing effects observed. However, Liu and colleagues did not directly nor exclusively activate single dopaminergic neurons to show that the PPL1-dFB neuron is sufficient to decrease sleep, and they propose that the pair of PPL1-dFB neurons may act in concert with other dopaminergic neurons to promote arousal.

Both lines of evidence suggest that more than one dopaminergic neuron may be required to shut down the output arm of the sleep homeostat of *Drosophila.* The data I present support this hypothesis and indicate that at least 4 PPL1 dopaminergic neurons from each hemisphere project to the dendritic tuft of dFB neurons and thus have the potential to regulate sleep. Furthermore, positive labelling of the dorsal layer of the FB after photoactivation indicates that one of these 4 PPL1 neurons is likely to be the PPL1-dFB neuron proposed by Liu and colleagues (Figures 2K to 2M).

Limitations of PA-GFP tracing

PA-GFP-based tracing methods permit non-random, optically guided circuit mapping by labelling anatomically or genetically targeted subsets of neurons (Datta et al. 2008). However, a known limitation is the difficulty to detect neurons in which the ratio of the total cytoplasmic *vs.* photoactivated volume is small (Aso et al. 2014). This could very well be the case in our scenario, in which the region of photoactivation is relatively small compared to the total volume of the cell. This is likely to be the reason underlying the different intensity of photoactivated neurons: neurons with fewer or thinner projections into the small volume selected for photoactivation will have a smaller number of PA-GFP molecules activated, which will translate into dimmer overall fluorescence. The larger the total volume of the neurons is compared to the photoactivated volume, the less successful the photoactivation will be in revealing the full extent of projections of the cell.

Such limitations might be the reason why attempts to photoactivate dopaminergic neurons innervating the dorsal layer of the FB, where 2 PPL1 neurons send axonal projections (FlyCircuit database# TH-F-200043), have proven unsuccessful (Figure S1). Inefficient labelling due to small photoactivated volumes will naturally constrain experiments that aim to perform detailed anatomical studies or experiments in which the somata are either distant or have thin projections connecting to the photoactivation site. Thus, we cannot rule out the possibility that additional dopaminergic neurons interact with dFB neurons to regulate sleep and arousal.

In addition, PA-GFP tracing currently lacks the ability to infer information about functional links between the identified PPL1 neurons and the sleep-promoting dFB neurons. For this reason it is also possible that not all 4 neurons identified establish functional connections to the dFB but rather have processes within the vicinity of the dendritic arbours of these neurons.

Because the effects of dopamine on dFB neurons are inhibitory (Figure 3), novel methods such as calcium-modulated photoactivatable ratiometric integrators (CaMPARI) (Fosque et al. 2015) cannot offer a solution. An alternative would be to establish paired recordings between individual PPL1 neurons and dFB neurons, a challenging venture given the lack of lines labelling exclusively and independently each one of the PPL1 neurons. Furthermore, it could also be the case that these unitary connections are not resolvable and that more than one dopaminergic neuron is required in order to achieve robust inhibition of dFB neurons. Unravelling the discrete components of the sleep/wake circuitry of flies will enable researchers to pose questions that address how external stimuli and homeostatic processes are integrated in identified neural networks.

Novel structures interacting with dFB projecting neurons

In an attempt to identify additional GAL4 lines labelling structures that are potentially pre- or post-synaptic partners of dFB neurons, I performed an *in silico screen* across a library of 3496 registered Janelia Farm GAL4 *Drosophila* brain stacks (Jenett et al. 2012). With the results of the screen I compiled a list comprising 42 GAL4 lines driving expression to structures overlapping with the dendritic tuft of dFB neurons, the canal generated by the structure of the dendritic arbour and the dorsal layer of the Fan-shaped Body (Figures 8 and 9, Table 1).

Some of the genes used to generate these lines are of particular interest. For instance, some genes contain the sequences of receptors to neurotransmitters with roles in the regulation of sleep/wake states such as dopamine (D2R) or octopamine (Octbeta3R and TyrR) (Crocker & Sehgal 2008). Other genes are involved in neurotransmitter transport (Vglut), receptor trafficking (Fur1), signal transduction (RhoGAPp190) or changes in membrane conductance (trpm, trpl), thus holding the potential of affecting or being affected by the structures within the ROIs selected for the screen. A combination of the genetic background of these lines, their structural expression patterns and their putative connectivity with dFB neurons hints towards potentially interesting lines. However, behavioural evidence is needed in order to demonstrate their role in sleep regulation.

I thus ordered the lines present in the final list (Table 2) from Bloomington Stock Centre. To establish if the structures captured by these lines are indeed pre- or post-synaptic partners of the sleep homeostat of *Drosophila*, the identified GAL4 drivers will be used to perform a behavioural screen. This can be achieved by expressing *dTrpA1*, a heat-inducible non-selective cation channel that can be used to depolarize neurons to trigger neurotransmitter release (Hamada et al. 2008), or *Shibire^{ts},* a temperature-sensitive dynamin mutant gene that permits spatial and temporal suppression of neurotransmission (van der Bliek & Meyerowitz 1991), while monitoring sleep/wake patterns. This approach will hopefully narrow down the number of candidates and help to identify novel structures that interact with the core of the sleep circuitry and will open new paths leading to uncharted territories of the neural machinery underlying sleep regulation in *Drosophila*.

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APPENDIX

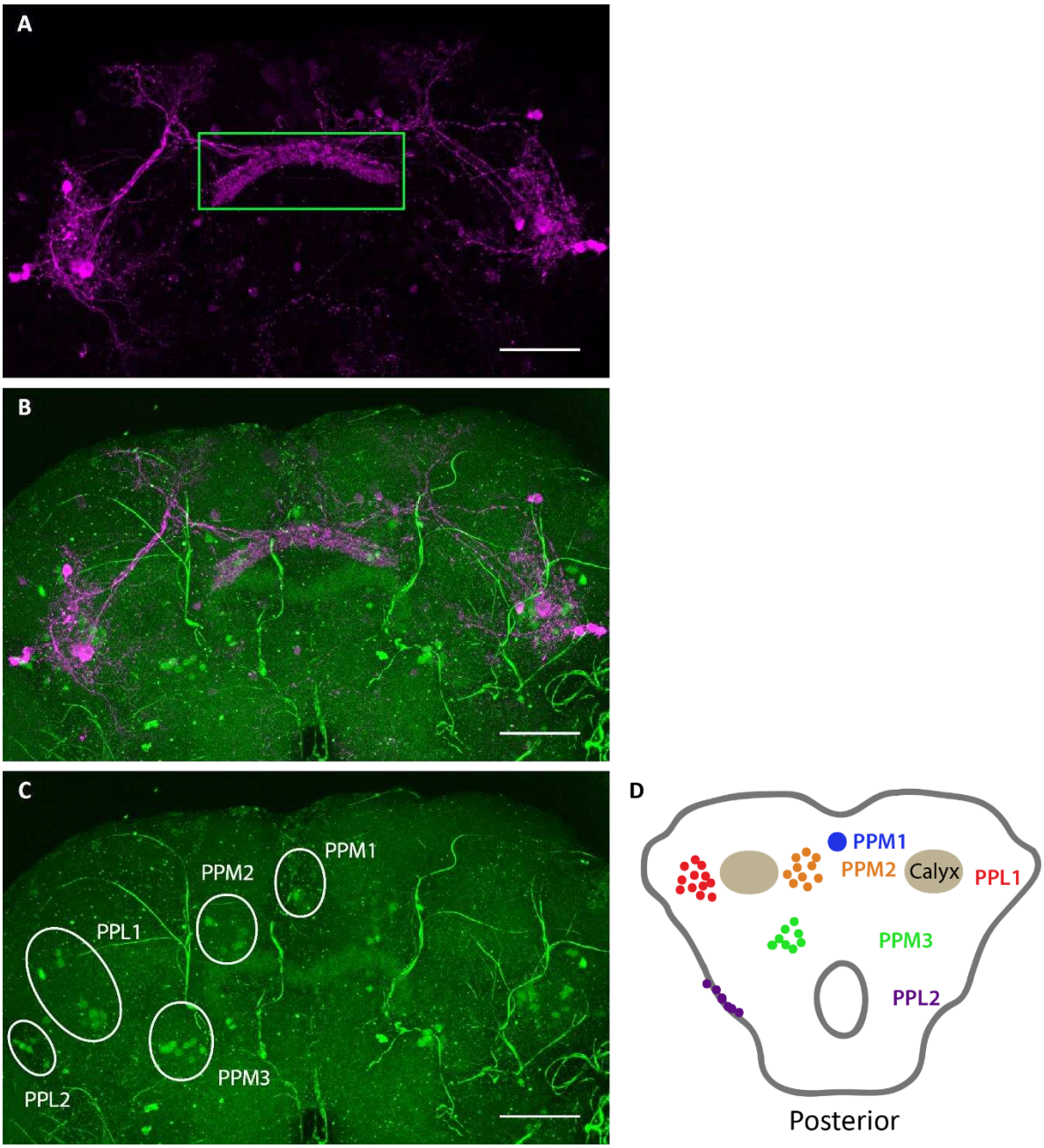

Figure S1. Photoactivation of the dorsal layer of the FB fails to reveal the two PPL1-dFB neurons| (A) Maximum intensity projection of mCherry expression controlled by R23E10-LexA shows the structure of the sleep homeostat of the fruit fly (magenta). The green square signals the area selected for photoactivation, corresponding to the dorsal layer of the FB. (B-C) Maximum intensity projections of 10xUAS-SPA labelled dopaminergic neurons captured by TH-GAL4 after photoactivation (green). White circles indicate distinct sets of dopaminergic neurons. Note that all clusters can be detected by the basal fluorescence of non-photoactivated PA-GFP when tuning the confocal settings to high powers. (D) Scheme of the distinct subsets of dopaminergic neurons captured by TH-GAL4. All scale bars indicate 50 µm.

Erklärung zur Bachelorarbeit/Masterarbeit

Hiermit versichere ich, dass die vorliegende Arbeit von mir selbstständig verfasst wurde und dass keine anderen als die angegebenen Quellen und Hilfsmittel benutzt wurden.
Diese Erklärung erstreckt sich auch auf in der Arbeit enthaltene Graphiken, Zeichnungen, Kartenskizzen und bildliche Darstellungen.

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I hereby confirm that I have written the accompanying thesis by myself, without contributions from any sources other than those cited in the text and acknowledgements.
This applies also to all graphics, drawings, maps and images included in the thesis.

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