The role of small GTPases in regulating sleep in *Drosophila melanogaster*



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Abstract

Sleep is highly conserved across the animal kingdom, yet why and how we sleep remains largely unknown. Uncovering the molecular pathway underlying homeostatic sleep regulation is integral to understanding the origin and function of sleep, as well as for the development of spatiotemporally precise medication to treat sleep disorders.

In the model organism, *Drosophila melanogaster,* neurons that project to the dorsal fan shaped body (dFB neurons) form a critical part of the sleep homeostat. By switching between their electrically-silent (OFF) state and electrically-active (ON) state, dFB neurons respond to increasing sleep pressure by inducing sleep. Previous work identified that mutating *crossveinless-c (cv-c),* a gene encoding a Rho-GTPase activating protein (Rho GAP), locks dFB neurons in the OFF state and results in an insomniac phenotype. Since GAPs are essential for the hydrolysis of small GTPases from their active (GTP-bound) to their inactive (GDP-bound) state, this thesis aimed to uncover the role of small GTPases in sleep regulation. A combination of genetic interaction, behaviour and imaging experiments were used.

The work presented here demonstrates that active Rho1 is upregulated in dFB neurons of *cv-c* mutants, and that manipulating Rho1 in dFB neurons of *cv-c* mutants rescues the insomniac phenotype. In addition, knocking down Rho1 in *cv-c* mutants restores dFB neuron excitability. This suggests that Rho1 is an important part of the molecular pathway underlying sleep regulation. However, the nature of how Rho1 regulates dFB membrane excitability, and to what extent its interacting partner Rac1 is involved, remains unclear. The findings of this thesis suggest a vexingly complex role of small GTPases in regulating sleep and highlights the importance of finely-tuned regulation of these signalling proteins.

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Statement of Authorship

This thesis is the product of my own work. Some experiments were conducted in collaboration with Anissa Kempf, a post-doctoral fellow in the Miesenböck lab. Any experiments that were the product of collaboration will be stated in the figure legend.

Abbreviations

BDSC	Bloomington Drosophila Stock Centre
CA	Constitutively active
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
Cv-c	Crossveinless-c
DAM	Drosophila Activity Monitor
dFB	Dorsal Fan-shaped Body
DIA	Diaphanous
DLC	Deleted Liver Cancer
DN	Dominant Negative
EEG	Electroencephalogram
FRET	Fluorescence Resonance Energy Transfer
GAP	GTPase Activating Protein
GDI	Guanine Nucleotide Dissociation Inhibitor
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GTP	Guanosine-5'-triphosphate
LARG	Leukemia-Associated RhoGEF
LFP	Local Field Potential
LOV	Light-Oxygen-Voltage
MCT	Multiple Comparisons Test
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NREM	Non Rapid Eye Movement
OE	Overexpression
PA	Photo-activable
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline/Tween
PKN	Protein Kinase N
PR	Photo-recruitable
REM	Rapid Eye Movement
RFP	Red Fluorescent Protein
ROCK	Rho-associated coiled-coil forming kinase

ROI	Region of Interest
ROS	Reactive Oxygen Species
SAM	Sterile α Motif
SCN	Suprachiasmatic Nucleus
SD	Sleep deprived / Sleep deprivation
SEM	Standard Error of the Mean
sLNV	Small Ventral Lateral Neurons
START	Steroidogenic Acute Regulatory Protein-related Lipid-transfer
TRAP	Translating Ribosome Affinity Purification
UAS	Upstream Activating Sequence
VLPO	Ventrolateral Preoptic Nucleus
WASP	Wiskott-Aldrich Syndrome protein
WT	Wild-type
ZT	Zeitgeber

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1 Introduction

1.1 An introduction to sleep

Defining sleep

Sleep can be defined as a period of quiescence in which an animal disconnects itself from its external environment. This period of quiescence can be characterised using behavioural, physiological and pharmacological criteria. There are four key behavioural hallmarks of sleep (Campbell & Tobler, 1984):

1. Reversible immobility - the reversibility of sleep distinguishes it from hibernation, anaesthesia and coma

2. Reduced responsiveness to sensory stimuli - this presents itself as a higher arousal threshold

3. "Relaxed" species-specific posture

4. Homeostatic rebound - sleep deprivation is typically followed by a compensatory increase in sleep duration or in sleep depth

In addition to behavioural criteria, sleep has been associated with physiological changes. Electroencephalogram (EEG) recordings have revealed two distinct sleep stages present in mammals, birds and some invertebrates: Rapid Eye Movement (REM) sleep, which is associated with a low amplitude EEG signal and a high frequency power spectral density, and Non-Rapid Eye Movement (NREM) sleep, which is associated with a high amplitude EEG signal and a low frequency power spectral density in the 0.5–4.5 Hz range (Allada & Siegel, 2008). Physiologically distinct stages in sleep have also been identified in

Drosophila melanogaster. Using local field potential (LFP) recordings Alphen et al. (2013) identified distinct sleep stages and Raccuglia et al. (2019) found characteristic slow wave oscillations (0.5–4 Hz) in the sleep-regulating R5 network. Thus, physiological characteristics can be used to identify sleep and distinct sleep stages across the animal kingdom.

Sleep can also be defined based on molecular and pharmacological properties. Sleep across animal phyla is regulated by many of the same neurotransmitters and signalling pathways. For example, dopamine is a key wake-promoting neurotransmitter, which has been shown to suppress sleep in mice, *Drosophila*, and *Caenorhabditis Elegans* (Anafi et al., 2019). In addition, melatonin, antihistamines and GABAergic agonists have been shown to promote sleep in mammals, zebrafish, and *Drosophila* (Joiner, 2016). Melatonin was even found to increase daily sleep amount in *Hydra Vulgaris,* which lack a centralized nervous system (Kanaya et al., 2020). Thus, sleep across the animal kingdom shares not only similar behavioural and physiological characteristics, but is also regulated by many of the same neurochemical pathways. This points towards a single common evolutionary origin of sleep.

The ubiquity of sleep

Sleep has been observed in almost every animal including mammals, birds, reptiles, arthropods, molluscs and even in Cnidaria such as *Cassiopea* and *Hydra* (Campbell & Tobler, 1984; Kanaya et al., 2020; Nath et al., 2017). The ubiquity of sleep, and the remarkable similarity in its behavioural, physiological and pharmacological properties, strongly suggest that sleep evolved from a common ancestral origin. The probability that sleep evolved independently, several times, using the same biochemical regulatory

pathways in phylogenetically distant phyla is highly unlikely. Thus, with the identification of sleep in Cnidaria, it has been suggested that sleep evolved in the last common ancestor of Cnidaria and Bilateria, approximately 600 million years ago, as shown in Figure 1-1 (Anafi et al., 2019). REM and NREM sleep has been identified in birds, mammals and reptiles suggesting that REM evolved in early amniotes, approximately 300 million years ago (Shein-Idelson et al., 2016).

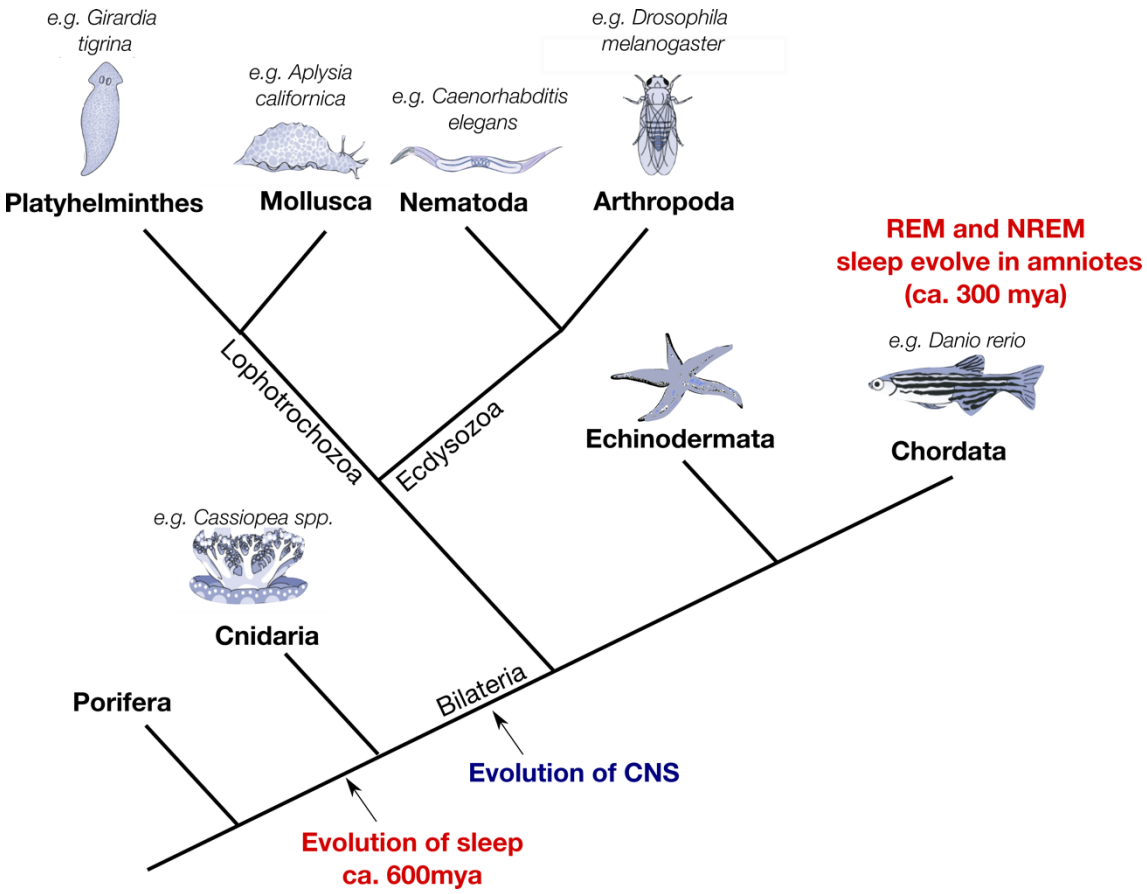


Figure 1-1: A pruned phylogenetic tree of the animal kingdom. Sleep has been described in Cnidaria and in Bilateria suggesting that sleep likely evolved 600 million years ago (mya), before the evolution of a centralised nervous system (CNS). Sleep has been identified in all species named. Sleep has yet to be studied in Echinodermata. Figure adapted from Anafi et al. (2019).

Despite the ubiquity of sleep, and its likely conserved nature, there is ample variability in sleep architecture and sleep duration across the animal kingdom. Elephants sleep on average 4 hours a day, whereas armadillos sleep around 20 hours a day (Joiner, 2016). In addition, whereas some animals consistently sleep the same amount every day, some animals exhibit great plasticity in their sleep duration. For example, the white-crowned sparrow (*Zonotrichia leucophrys gambelii*) sleeps on average two-thirds less when migrating, than when in a nonmigratory state (Rattenborg et al., 2004). Similarly, maternal killer whales have been observed to show very little resting behaviour for several months after giving birth (Lyamin et al., 2005). These variations are likely explained by different ecological pressures (Joiner, 2016). The adaptability in sleep behaviour across species might explain how sleep, a seemingly maladaptive behaviour, has been evolutionarily maintained and conserved for over 600 million years.

Functions of sleep

When considering ecological factors, sleep is largely a maladaptive state. During sleep, animals are less aware of their surroundings and thus, at greater predation risk. In addition, sleep is associated with various opportunity costs. Sleeping animals are not able to forage, feed, find mates or mate. As such, the majority of ecological factors are likely to select against the evolutionary conservation of sleep (Capellini et al., 2010). However, for sleep to have been maintained for over 600 million years it must provide some biological advantages that outweigh its ecological costs, or as Rechtschaffen said: *"If sleep doesn't serve an absolutely vital function, it is the greatest mistake evolution ever made"* (Rechtschaffen, 1971). Thus, intrinsic factors of sleep must give rise to positive selective pressures.

The intrinsic factors favouring sleep have been highlighted through the consequences of sleep deprivation. Animals that are sleep deprived suffer from cognitive deficits, emotional instability, reduced immune function and in extreme cases even death (Joiner, 2016). As such, some of the main functions of sleep include memory and learning, metabolic clearing, and immune system functioning (Joiner, 2016).

The importance of sleep for cognitive performance has been supported by the fact that short sleeping mutants consistently perform worse on memory dependent tasks (Bushey et al., 2007; Donlea et al., 2014). In addition, inducing sleep by activating sleep-promoting neurons, was shown to enhance memory consolidation (Donlea et al., 2011).. One hypothesis of why sleep is important for learning and memory consolidation is the Synaptic Homeostasis Hypothesis (Tononi & Cirelli, 2003). This hypothesis posits that memory consolidation is dependent on the downscaling of synaptic connections and that synaptic downscaling can only occur during sleep, when sensory inputs are reduced (Tononi & Cirelli, 2014).

Another hypothesis for the function of sleep is that it is important for metabolic clearance. Xie et al. (2013) proposed that sleep's function is to clear toxins and metabolites that accumulate during waking. The group found that during sleep there is a 60% increase in interstitial space, enabling an increased flow of cerebrospinal fluid and allowing for the clearance of toxic waste products during sleep (Xie et al. 2013). In addition, Van Alphen et al. (2021) found that proboscis extensions, which are predominant during deep sleep of *Drosophila*, facilitated waste clearance from the haemolymph and also helped reduce mortality after injury. Sleep also functions to clear reactive oxygen species (ROS), a theory first proposed by Reimund in 1994. ROS are produced in the mitochondria as a by-product of cellular metabolism. ROS were shown to be higher after sleep deprivation,

and manipulations increasing and decreasing ROS, increased and decreased daily sleep, respectively (Hill et al., 2018; Kempf et al., 2019). These lines of evidence strongly suggest that sleep has a restorative role and is important for metabolic clearance.

Sleep has also been tightly linked with immune system functioning. A forward genetic screen identified that genes involved in the immune response make up a large proportion of genes affected by sleep deprivation (Williams et al., 2007). In addition, Kuo and Williams (2014) found that increasing sleep in *Drosophila* increased resistance to a bacterial infection and strongly promoted survival, suggesting a clear role of sleep in maintaining a robust immune system.

Sleep is clearly important for memory consolidation and learning, metabolic clearance and immune functioning. These intrinsic roles for sleep, among others not discussed, likely act as positive selective pressures, conferring sleeping animals with an evolutionary advantage, and enabling the conservation of sleep across the animal kingdom. Sleep may also have been co-opted for these functions over time, and it is highly likely that the primordial function of sleep remains to be discovered. Uncovering the molecular mechanisms that regulate sleep will give us insight to both how and why sleep evolved in the first place. This could also uncover a universal function of sleep shared across the animal kingdom, that to this day remains elusive (Krueger et al., 2016).

The importance of studying sleep

Understanding the cellular and molecular mechanisms behind the wake-to-sleep switch can also pave the way for new methods of treating sleep disorders. Sleep disorders pose a significant burden on global human health. At least one third of adults in western societies report habitual sleep of 6 hours or less (Grandner, 2017), and at least 10%

experience chronic insomnia (Bhaskar et al., 2016). Inadequate sleep has been associated with diabetes and obesity (Nedeltcheva & Scheer, 2014), heart disease (Cappuccio et al., 2011) and often precedes the development of neurodegenerative diseases such as Alzheimer's and Parkinson's (Peter-Derex et al., 2015). Insomnia is also a key predictor of depression (Baglioni et al., 2011). Clearly, sleep and human wellbeing are inseparable. Elucidating the molecular mechanisms of sleep regulation will allow us to target drugs for sleep disorders with higher spatiotemporal precision, and will help us understand how sleep can be used to treat illnesses such depression. This will be invaluable for improving life quality and health across the globe.

1.2 Sleep in *Drosophila*

Drosophila melanogaster as a model organism

The model organism *Drosophila melanogaster* provides a powerful tool to study the molecular regulation of sleep. *Drosophila* are cheap, easy to breed and have a short generation time. In addition, the availability of its fully sequenced and well characterised genome, the simplicity of its chromosome arrangement (4 chromosomes), and the low degree of genetic redundancy, makes the fruit fly an incredible genetic toolkit (Ugur et al., 2016). Balancer chromosomes (which prevent recombination) and phenotypic markers make genetic work in the fly easier than in other model organisms. Stock centres such as the Bloomington Drosophila Stock Centre (BDSC) hold a vast array of different fly lines, with the BDSC holding more than 80, 000 different stocks. The availability of these fly lines allows for quick and easy screening of genes in specific cell populations and make it possible to gain mechanistic insights of many different behaviours.

D. melanogaster fulfil the behavioural criteria of sleep and sleep on average 15-18 hours per day. They exhibit a midday siesta but mainly sleep at night (Shaw et al., 2000). Sleep in *Drosophila* is defined as quiescent periods lasting at least 5 minutes, as flies that are inactive for this period of time show behavioural hallmarks of sleep such as an increased arousal threshold and a relaxed posture (Hendricks *et al*, 2000). In addition, LFP recordings suggest that after 5 minutes of inactivity, *Drosophila* brain activity is decreased (Alphen et al., 2013; Nitz et al., 2002). Therefore, flies that are inactive for longer than 5 minutes meet both behavioural and physiological criteria of sleep.

Drosophila also fulfil the molecular criteria of sleep: the molecular pathways underlying sleep in flies, mammals and humans are highly conserved. Neurotransmitters such as

dopamine, GABA and acetylcholine affect sleep in *Drosophila* as they do in mammals (Joiner, 2016). In addition, several signal transduction pathways are conserved. For example, overexpressing ligands of the epidermal growth factor receptor (EGFR) pathway resulted in an increase in sleep in flies, but also in mammals and *C. elegans* (Allada & Siegel, 2008). Genetically, 60% of the *Drosophila* genome is homologous to that of humans, and many genes identified in forward screens of the fruit fly have been found to be genes responsible for human diseases (Ugur et al., 2016). For example, mutations in the human homolog of *Period*, one of the genes forming part of the circadian clock in *Drosophila,* was found to be responsible for advanced sleep phase syndrome, a disorder which makes humans wake up and go to sleep approximately 4 hours earlier than normal (Toh et al., 2001). This highlights that uncovering molecular pathways in *Drosophila* can have a profound relevance for human health and suggests that *D. melanogaster* is an appropriate model to study sleep.

The two process model of sleep regulation

Sleep in flies and mammals is regulated by two processes: the circadian system and the sleep homeostat, as shown in Figure 1-2 (Borbély, 1982). The circadian system regulates the timing of sleep (and other behaviours) through endogenous oscillations that span 24 hours. Light, temperature and food act as "zeitgebers" which help synchronise the 24 hour rhythm of the clock. Nonetheless, circadian rhythms are self-sustained: mice and flies reared in constant darkness continue to exhibit typical wake/ rest rhythms across a 24 hour period (Allada & Chung, 2010). The endogenous circadian rhythms are driven by a transcriptional-translational feedback loop which involves the genes *Period* and *Clock* (among others). In brief, CLOCK, a DNA-binding transcription factor, together with Cyc, binds to the promoter of *Period*. *Period* encodes the protein PER, which forms a

heterodimeric complex with TIM (encoded by *Timeless*) and together they enter the nucleus and negatively regulate the activity of CLOCK, thus forming a negative feedback loop (Luo, 2021). Many genes are regulated in a similar fashion, giving rise to daily 24 hour rhythms in an animal's biochemistry, physiology and behaviour. The transcriptional cycles of individual cells are synchronised by pacemaker neurons in the brain. In *Drosophila,* lateral neurons expressing the pigment dispersing factor (PDF) regulate the clocks of downstream cells, whereas in mammals, the suprachiasmatic nucleus (SCN) is the central pacemaker and is essential for circadian rhythmicity (Luo, 2021).

The sleep homeostat is responsible for inducing sleep once a threshold of sleep deficit has been surpassed. It is hypothesised that internal markers, signalling sleep need, accumulate throughout the waking day (Borbély, 1982). These signal to the sleep homeostat (a set of sleep promoting neurons) to induce sleep. Sleep, in turn, reverses these internal changes back to a baseline level. The internal markers of sleep pressure are still ill-defined yet could hold the key to understanding why animals sleep (Donlea et al., 2014).

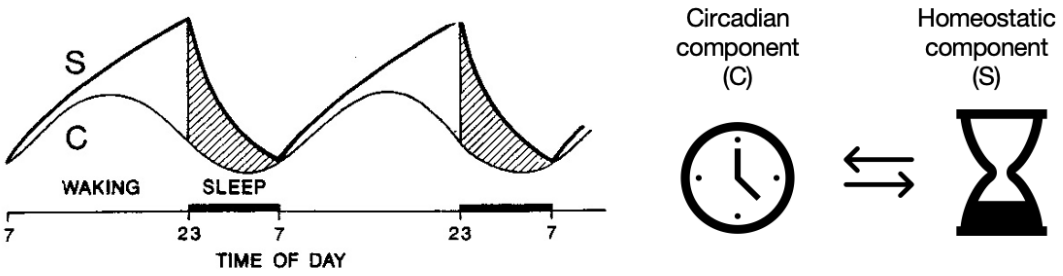


Figure 1-2: The two process model of sleep (Borbély, 1982). C represents the propensity of an animal to sleep, which is determined by the circadian clock. S, represents sleep debt which accumulates throughout the waking day and rapidly declines upon the onset of sleep. S reflects the homeostatic component of sleep and is analogous to an hourglass, where sleep onset is induced once a threshold of sleep deficit is surpassed.

Various studies suggest that the circadian and homeostatic systems regulate sleep independently. For example, arrhythmic animals with lesions in the SCN exhibited the same sleep duration and homeostatic rebound as rats with normal circadian rhythms (Mistlberger et al., 1983; Tobler et al., 1983). Furthermore, mutations affecting homeostatic sleep regulation had no effect on circadian rhythms, with mutants exhibiting normal circadian rhythmicity even in constant darkness (Donlea et al., 2014). However, contradictory evidence suggests that the circadian system and the sleep homeostat do interact. SCN lesions in the squirrel-monkey (*Saimiri sciureus*) resulted in a four hour increase in daily sleep (Edgar et al., 1993). In addition, mutations in circadian clock genes such as *clock, period and timeless* were shown to affect sleep duration and/or sleep rebound in both mice (Naylor et al., 2000) and *Drosophila* (Shaw et al., 2002). Most recently, it was found that the sleep homeostat suppresses circadian outputs through *Hugin+* neurons in *Drosophila* which function downstream to circadian clock neurons (Schwarz et al., 2021). These studies suggest that although some underlying pathways may act independently, generally the circadian and homeostatic systems interact. This interaction might be crucial to ensure that an animal stays awake, when sleep might be dangerous, despite high sleep pressure. Thus, although the circadian system and the homeostat are often studied in isolation it is important to consider that pathways underlying one might also affect the other.

dFB neurons: a sleep promoting centre

In the *Drosophila* brain, various "sleep centres" have been implicated in the homeostatic regulation of sleep. This includes the mushroom body which comprises of 2500 Kenyon cells (Pitman et al., 2006; Sitaraman et al., 2015), the pars intercerebralis (Foltenyi et al., 2007), the ellipsoid body (Liu et al., 2016) and the fan shaped body (Donlea et al., 2011). It is likely that these different sleep centres perform different roles of the sleep homeostat.

A set of neurons, 12 in each hemisphere, which project to the dorsal fan shaped body (dFB), likely make up the output arm of the homeostat. Activation of these dFB neurons induces sleep (Donlea et al., 2011), and silencing them through dopaminergic activation reduces sleep (Liu et al., 2012). In addition, flies in which the excitability of dFB neurons has been dampened, exhibit an insomniac phenotype and do not show a sleep rebound after sleep deprivation (Donlea et al., 2014). Together, this suggests that dFB neurons are crucial for homeostatic sleep regulation in the fly.

dFB neurons are located in the central complex and receive known input from two populations: R5 neurons in the ellipsoid body, and dopaminergic neurons in the paired posterior lateral 1 (PPL1) and the paired posterior medial 3 (PPM3) (Q. Liu et al., 2012; S. Liu et al., 2016). R5 neurons (previously called R2 neurons) encode sleep drive and act upstream to dFB neurons to regulate sleep homeostasis. Activating R5 neurons generates sleep pressure, however, this is only the case when dFB neurons are intact. Interestingly, the excitability of R5 neurons is modulated by sleep pressure: their spontaneous action potential firing rate is upregulated with waking time and after sleep deprivation, and this is accompanied with structural plastic changes (Liu et al., 2016). In addition to R5 neurons, dFB neurons receive inhibitory input from dopaminergic neurons.

Dopamine released from the PPL1 acts on dFB neurons via DopR, a G-protein coupled dopamine receptor and reduces sleep (Q. Liu et al., 2012). Pimentel et al. (2016) showed that dopamine application to dFB neurons locked them in an electrically silent, hyperpolarised state. Locking dFB neurons in this OFF state, triggers wakefulness. As such, dFB neurons, which receive excitatory and inhibitory inputs from R5 neurons and dopaminergic neurons, respectively, are in a prime location to sample sleep pressure and act as the sleep homeostat, inducing sleep once a threshold of sleep deficit has surpassed.

dFB neurons alternate between an electrically active (ON) and an electrically silent (OFF) state and this correlates with the behavioural state of the fly. When a fly is in a state of low sleep drive (for example after dopamine application), dFB neurons are electrically silent (OFF). In contrast, when flies are in a state of high sleep drive, for example at the end of the day or after sleep deprivation, dFB neurons are electrically active (ON). This was shown by current clamp experiments which found that the input-output function, input resistance and membrane time constant, all measures of intrinsic neuronal excitability, increase after sleep deprivation in dFB neurons of wild-type flies, and decrease after dopamine application (Donlea et al., 2014; Pimentel et al., 2016).

The switch between the OFF and ON state of dFB neurons is modulated by two potassium channels:

1. Shaker: a voltage-gated potassium channel which mediates voltage-gated A-type currents (Cirelli et al., 2005)

2. Sandman: a two-pore potassium leak channel that is internalised in a vesicle during sleep, but fuses with the plasma membrane during waking (Pimentel et al., 2016). Non-A-type currents flow through Sandman.

The two channels work antagonistically to regulate sleep drive. When sleep drive is high, dFB neurons are characterised by a high level of A-type currents (which flow through Shaker) and a low level of leak currents (which flow through Sandman). When sleep drive is low (e.g after dopamine application), A-type currents in dFB neurons are reduced and non-A type currents (leak currents) are quadrupled. The A-type currents and non-A type currents were attributed to the potassium channels Shaker and Sandman respectively, because knocking down *Shaker* reduced A-type currents, and knocking down *Sandman* reduced non-A type currents. In addition, RNAi-mediated knockdown of *Shaker* reduced sleep, whereas knockdown of Sandman increased sleep relative to parental controls (Pimentel et al., 2016). Thus, both Sandman and Shaker are critical in mediating the wake-sleep switch.

The reduced level of leak currents in the ON state, and the observation that blocking exocytosis with botulinum neurotoxin C locked neurons in an electrically active state, suggests that Sandman is localised at the plasma membrane during waking, but is internalised in a vesicle at sleep onset (Pimentel et al., 2016). This suggests that the endocytosis of Sandman is critical for dFB neurons to switch from OFF (state of low sleep drive) to ON (state of high sleep drive), and thus for sleep induction. The role of the different potassium channels and the switch between ON and OFF is depicted in Figure 1-3.

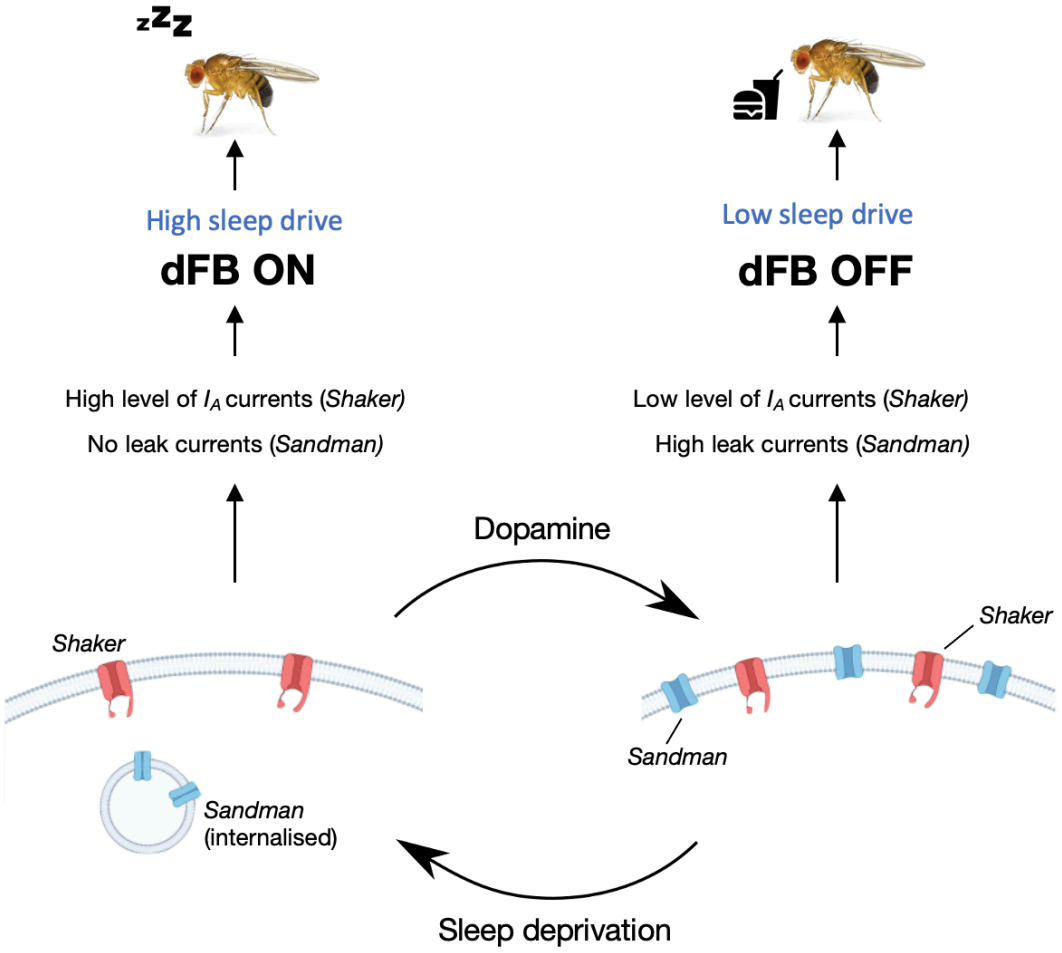


Figure 1-3: A schematic representation of how *Shaker* and *Sandman* work antagonistically to drive the ON-OFF switch of dFB sleep promoting neurons. (*Adapted from Donlea, 2017)*

Understanding what signals or processes switch dFB neurons ON would reveal what drives sleep onset in *Drosophila* and even in mammals, if the sleep mechanism is conserved. dFB neurons show a remarkable similarity to a set of sleep-promoting neurons in the ventrolateral preoptic area (VLPO) of the hypothalamus in mammals (Sherin et al., 1996). Like dFB neurons, VLPO neurons modulate their activity with increasing sleep pressure (Alam et al., 2014). In addition, both groups of cells are the target of general anaesthetics (Kottler et al., 2013; Moore et al., 2012) and both use inhibitory transmitters and homologous peptidergic signalling pathways (Sherin et al., 1998; Szymusiak et al., 1998). Thus, uncovering the molecular pathways that underlie the OFF-ON switch of dFB neurons is likely to shed light on the mechanisms that control sleep in mammals.

Recently, ROS were identified as a key signal regulating the wake-to-sleep switch (Hill et al., 2018; Kempf et al., 2019). ROS, which accumulate with increasing sleep pressure, regulate the activity of dFB neurons through the NADPH cofactor of Hyperkinetic, a Kvβ subunit of Shaker. The oxidation of NADPH, which is likely caused by the accumulation of ROS during waking, was shown to slow the inactivation of A-type currents (flowing through Shaker) and boost dFB action potentials, concomitantly inducing sleep (Kempf et al., 2019). However, ROS and Shaker in itself are insufficient to switch dFB neurons from OFF to ON. The generation of ROS, through the optogenetic activation of miniSOG, was not able to switch a dFB neuron from an OFF to an ON state (unpublished results). This suggests that another mechanism must be in place to enable the wake-to-sleep switch. It is parsimonious to suggest that this mechanism is reliant on Sandman, and that for the OFF-ON switch to occur, Sandman must be endocytosed. This has led to the hypothesis that an unknown signal regulating the exo-endocyting cycling of Sandman is key for sleep induction in animals.

1.3 The sleep regulatory role of *crossveinless-c (cv-c)*

The notion that small GTPases may be involved in regulating the wake-to-sleep switch comes from previous work in the Miesenböck lab which found that mutating *crossveinless-c (cv-c)*, a gene encoding a Rho-GTPase activating protein (RhoGAP) (Denholm et al., 2005), led to an insomniac phenotype (Donlea et al., 2014).

Flies carrying transheterozygous mutations for *cv-c* slept significantly less during the day and night than heterozygous and parental controls (Figure 1-4A). In addition, *cv-c* mutants did not exhibit a sleep rebound after sleep deprivation, whereas heterozygous controls did (Figure 1-4E). *cv-c* mutants did not exhibit any defects in circadian rhythms suggesting that Cv-c exclusively modulates the homeostatic regulation of sleep (Figure 1-4F). Cv-c was found to act specifically in dFB neurons to regulate sleep: expressing wild-type *cv-c (UAS-cv-c)*, specifically in dFB neurons, in an otherwise *cv-c* mutant fly rescued the insomniac phenotype, as shown in Figure 1-4B. Furthermore, knocking down *cv-c* exclusively in dFB neurons, using a RNAi line driven by the *R23e10-GAL4* driver (which labels dFB neurons), resulted in an insomniac phenotype (Figure 1-4C). Interestingly, *cv-c* mutants showed similar memory deficits to sleep-deprived wildtype flies (Figure 1-4D), suggesting they are in a state of high sleep pressure. Together, these experiments suggest that *cv-c* plays a critical role in dFB neurons, and is essential for the proper homeostatic regulation of sleep.

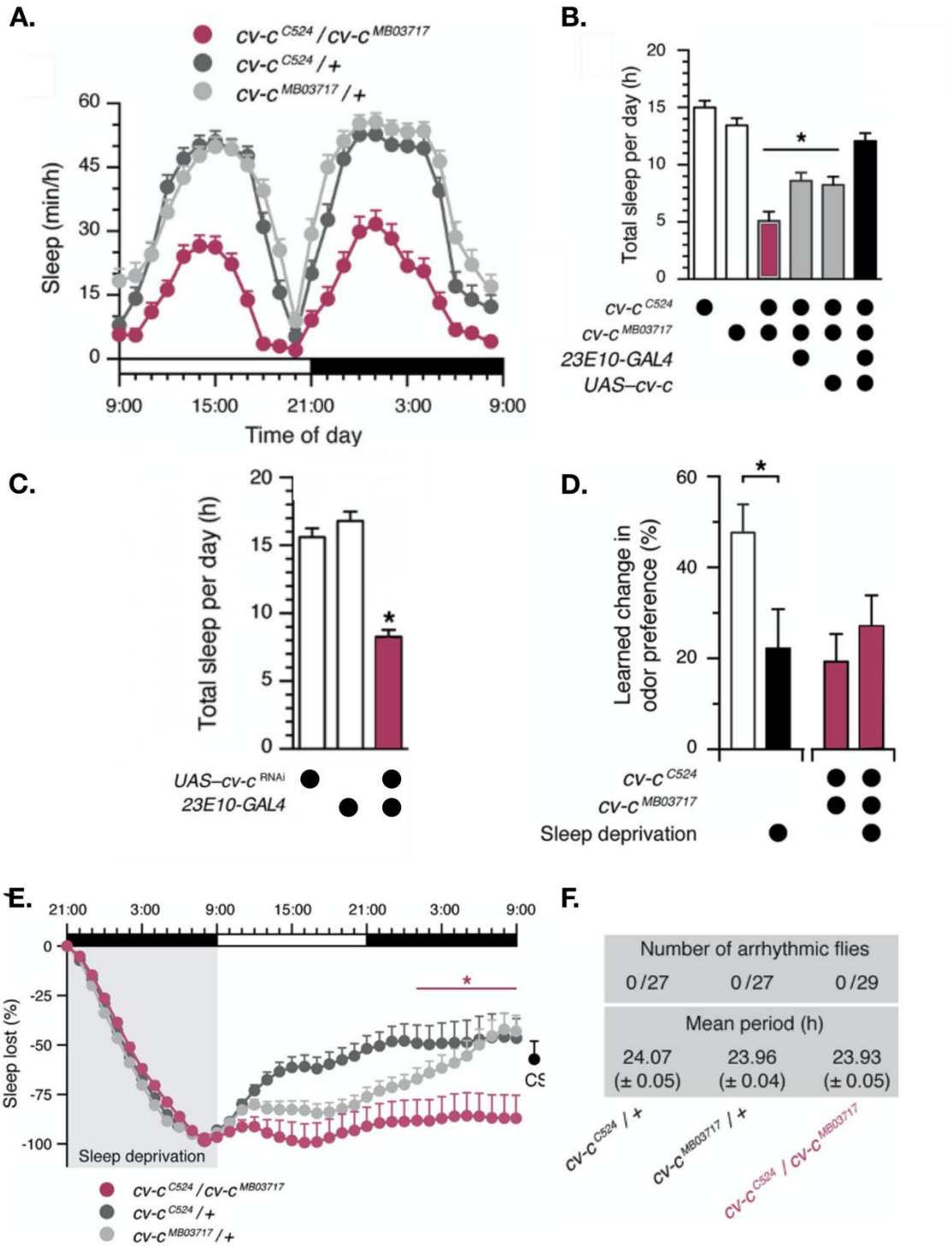


Figure 1-4: Key figures from Donlea et al (2014). A) 24 hour sleep profile of *cv-c^{c524}/cv-c^{MB03717}* transheterozygous mutants and heterozygous controls. **B)** Expressing *UAS-cv-c* in dFB neurons (using *R23e10-GAL4*) rescues the sleep insomniac phenotype of *cv-c^{c524}/cv-c^{MB03717}* mutants. **C)** Expressing *UAS-cv-c^{RNAi}* in dFB neurons decreases sleep by 50% (Compared to controls). **D)** *cv-c^{c524}/cv-c^{MB03717}* mutants have impaired short term memory, similar to sleep-deprived wildtype flies. **E)** *cv-c^{c524}/cv-c^{MB03717}* mutants show less sleep rebound after 12 hour night sleep than heterozygous controls. **F)** *cv-c^{c524}/cv-c^{MB03717}* mutants show no change in circadian rhythmicity during 7 days in constant darkness. *n* of flies for plots and statistical tests can be found in Donlea et al (2014). Data is presented as means ± SEM. *R23e10-GAL4* is the driver used to target dFB neurons.

In addition, current clamp experiments showed that the dFB neurons of *cv-c* mutants were locked in an electrically silent state (Donlea et al., 2014). Whereas wildtype dFB neurons increased in excitability after sleep deprivation, *cv-c* mutant dFB neurons did not. This is shown in the current clamp data in Figure 1-5A and B, where input resistance (R_m) and membrane time constant (T_m) are used as measures of excitability. R_m reflects the voltage response to an injected current, whereas T_m reflects the temporal window in which inputs can summate to excite a cell. The higher the R_m and T_m values, the more excitable the neuron (Donlea et al., 2014). In addition, recent voltage clamp experiments showed that leak currents (non-A-type) are higher in *cv-c* mutants compared to wildtype, as shown in Figure 1-5C. This posits that Sandman channels are localised (or possibly locked) at the plasma membrane in *cv-c* mutants. Together, these results suggest that Cv-c is required for dFB neurons to compute sleep pressure, turn ON, and induce sleep.

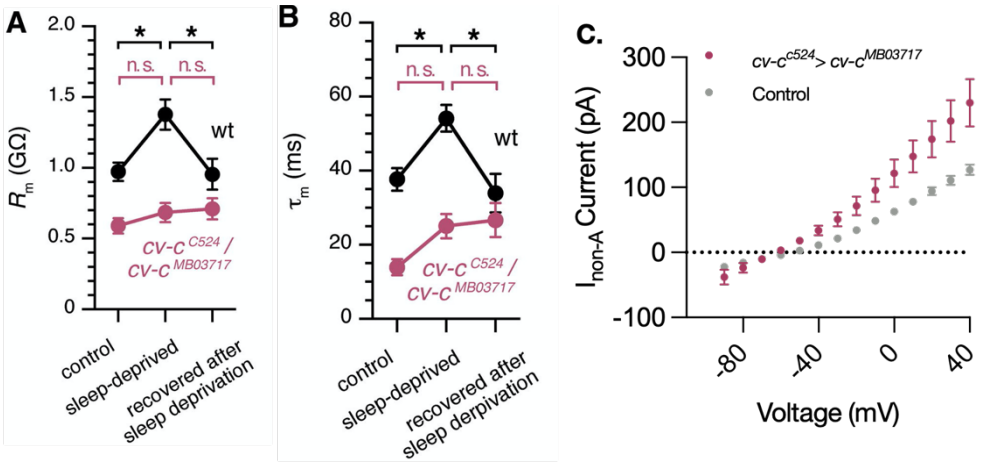


Figure 1-5: Current clamp and voltage clamp of *cv-c* mutant vs. control dFB neurons. A) R_m (Input resistance) is higher in control than in *cv-c* dFB neurons, and changes with sleep pressure in control but not *cv-c* mutant dFB neurons. **B)** T_m (Time constant) is higher in control than *cv-c* dFB neurons and changes with sleep pressure in control but not *cv-c* mutants. Two-way ANOVA detected significant effects of sleep history ($F[2,231] = 6.856$, $p = 0.0013$) and genotype ($F[1,231] = 38.96$, $p < 0.0001$). Asterisks denote significant differences in pairwise post hoc comparisons. **C)** Non A-type current measured for different voltage steps. Two-way ANOVA detected a significant interaction between voltage and genotype ($P = 0.0187$). A and B are figures from Donlea et al., (2014). Figure C is unpublished data from Anissa Kempf.

Understanding why Cv-c is important for the wake-to-sleep switch will reveal the molecular mechanisms underlying sleep homeostasis in dFB neurons. *cv-c* encodes a protein that is 1017 amino acids in length and is homologous to Deleted Liver Cancer 1 (DLC1), DLC2 and DLC3 in mammals (Denholm et al., 2005; Sotillos et al., 2018). DLC1 has a known role in controlling cell migration and tumour metastasis. Knockdown of DLC1 causes an increase in tumorigenesis, whereas its overexpression inhibits the growth of tumour cells (Xue et al., 2008). Like DLC1, the Cv-c protein contains three evolutionarily conserved domains: a N-terminal sterile α motif (SAM), a steroidogenic acute regulatory protein-related lipid-transfer (START) domain, and a GTPase activating domain (GAP) (Sotillos et al., 2018). Although the SAM and START domain play some importance in DLC1's role in suppressing tumours (Barras & Widmann, 2013; Healy et al., 2008; Joshi et al., 2020), the main functional capacity of Cv-c is dependent on its GAP activity (Sotillos et al., 2018).

GAPs play an important role in negatively regulating small GTPases. Small GTPases are a family of monomeric proteins that act as molecular switches by cycling between an active (GTP-bound) and an inactive (GDP-bound) state. While GAPs negatively regulate small GTPases by hydrolysing the GTP molecule to GDP, Guanine Exchange Factors (GEFs), activate small GTPases, by stimulating the release of GDP. When in its active GTP-bound state, small GTPases localise at the plasma membrane and bind to downstream effectors to elicit a myriad of biological processes. When in its inactive GDP-bound state, small GTPases bind to Guanine Dissociation Inhibitors (GDIs) which remove them from the cytoplasm and protect them from degradation. Thus, GEFs, GAPs and GDIs tightly regulate the cycling of small GTPases between their active and inactive states, as shown in Figure 1-6.

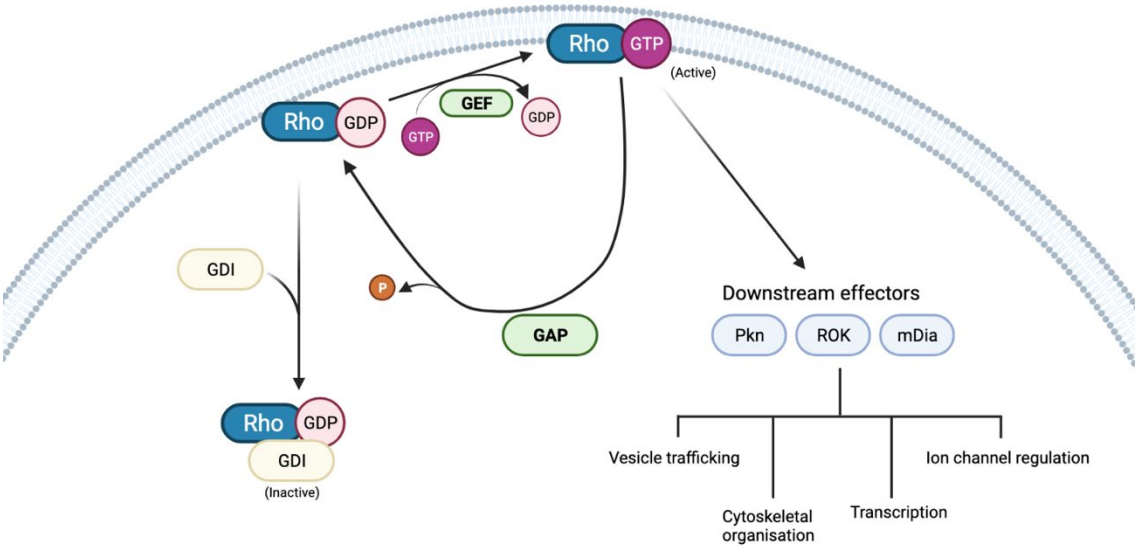


Figure 1-6: The molecular switch of Rho small GTPases. Rho small GTPases cycle between their inactive (GDP-bound) state and their active (GTP-bound) state through antagonistic regulation of GEFs and GAPs. When in its active state, Rho-GTP binds to downstream effectors such as PKN, ROK and mDia, which are involved in various cellular processes. In its inactive state, Rho GTPases bind to GDIs. *Figure made with Biorender.com*

The Ras superfamily of small GTPases, which is the largest and best studied superfamily, is composed of five main families: Ras, Rho, Ran, Rab and Arf GTPases (Bishop & Hall, 2000). *cv-c* is known to specifically contain a Rho-GTPase activating domain, suggesting that it plays an important role in regulating at least one of the eight types of Rho GTPases in *Drosophila.* Rho1, Rac1 and Cdc42 are the best studied Rho GTPases in *Drosophila* (Luo, 2000). Preliminary evidence from TRAP (Translating Ribosome Affinity Purification) RNA isolation, followed by sequencing, confirmed that all eight Rho family GTPases present in the *Drosophila* genome are indeed expressed in dFB neurons as shown in Figure 1-7. Among these, Rho1 was most abundant. This suggests that Rho1 is a likely substrate of Cv-c in dFB neurons.

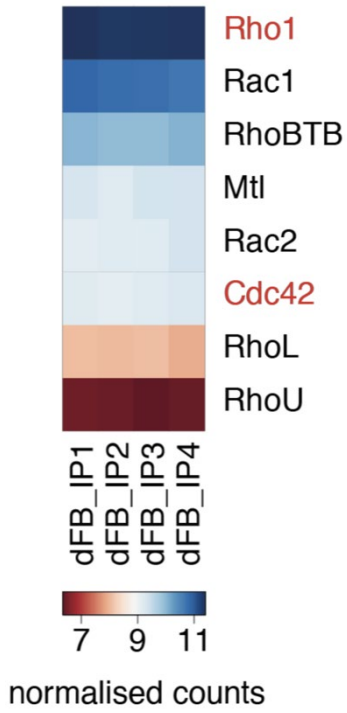


Figure 1-7: Relative expression of Rho family GTPases in dFB neurons. RNA was isolated from dFB neurons using the TRAP method. Each column represents a different replicate. Blue denotes strong expression, red denotes weak expression. Unpublished data from Anissa Kempf.

Indeed, in vitro and in vivo experiments have shown that both DLC1 and Cv-c preferentially show GAP activity toward Rho1. Sato et al. (2010) conducted in vitro cell free assays and found that Cv-c exhibited GAP activity towards Rho1 and Cdc42, but not towards Rac1. In vivo experiments further demonstrated that Rho1 was the main substrate of Cv-c regulating the dendritic arborization of neurons (Sato et al., 2010). In addition, Denholm et al. (2005), found that morphogenic defects in the Malpighian tubules of *cv-c* mutants were rescued by knocking down Rho1 but not by knocking down Cdc42 or Rac1. Similarly, in mouse cells, knocking down Rho1 in DLC1 mutants helped reduce the associated tumour growth phenotype back to wildtype levels (Xue et al., 2008). These studies suggest that Rho1-GTP is typically upregulated in *cv-c* mutants. Thus, Rho1 is the main substrate for Cv-c and its mammalian homolog DLC1. However, we cannot fully exclude the possible role of other Rho-GTPases because Cv-c substrate specificity is likely to be regulated in a tissue-dependent manner (Denholm et al., 2005).

Together, both previous literature and preliminary experiments suggest that Cv-c plays a key role in regulating Rho1-GTP levels, by hydrolysing it to Rho1-GDP. Therefore, it is parsimonious to hypothesise that in *cv-c* mutants, Rho1-GTP is upregulated, and that Rho1, or possibly other Rho family GTPases, are causing the deficits in sleep

homeostasis. Since the membrane excitability of dFB neurons is modulated by potassium channels, it is likely that Rho GTPases have a sleep regulatory effect by either:

1) regulating the endo-exocytosis of Sandman, whose leak currents are key in mediating the OFF-ON switch.

2) directly modulating ion channels (Sandman or Shaker) which affect the membrane excitability of dFB neurons.

3) modulating the structural plasticity of dFB neurons which in turn affects membrane excitability.

Indeed, Rho GTPases are known to be involved in vesicular trafficking (Symons & Rusk, 2003), ion channel regulation (Jin, 2009) and neural structural plasticity (Luo, 2000).

1.4 Small GTPases

The role of small GTPases in vesicular trafficking

Rho GTPases play an important role in vesicle trafficking through their ability to regulate the actin cytoskeleton (Chi et al., 2013). When active, Rho GTPases bind to various downstream effectors that are involved in actin polymerisation. For example, Rho activates ROCK (Rho-associated coiled coil-containing protein kinases) which is known to rearrange the actin cytoskeleton, and Rac1 and Cdc42 are known to interact with actin nucleators from the WASP (Wiskott-Aldrich Syndrome Protein) family (Bishop & Hall, 2000). Actin polymerisation or depolymerisation affects the formation of vesicles, vesicle transport and vesicle targeting (Croisé et al., 2014). Thus, Rho GTPases, through their downstream effectors are important for both endocytosis and exocytosis. For example, Lamaze et al. (1996) found that expressing constitutively active forms of Rac and Rho reduced transferrin-receptor mediated endocytosis by inhibiting the formation of clathrin-coated vesicles. In addition, Rac and Rho have been implicated in exocytosis (reviewed in Ory & Gasman, 2011): constitutively active Rho and Rac stimulated exocytosis while inhibiting Rho and Rac reduced exocytosis in mast cells (Norman et al., 1996). Interestingly, the mammalian Cv-c homolog DLC3 has been shown to coordinate endocytic membrane trafficking, suggesting a potential role for Cv-c in endocytosis (Braun et al., 2015). Rho GTPases are also known to act as scaffolding proteins for the recruitment of proteins important in vesicle transport and formation. For example, RhoGTPases are known to interact with Rab GTPases, which are integral to endocytosis (Hutagalung & Novick, 2011). Thus, it is plausible that Rho GTPases regulate endocytosis through intermediate small GTPases, as well as via the actin cytoskeleton.

The role of small GTPases in ion channel regulation

Rho family GTPases have also been implicated in the direct regulation of ion channels (Jin, 2009). Overexpressing RhoA (the mammalian homolog of Rho1) was shown to reduce the activity of Kv1.2, a voltage dependent potassium channel of the Shaker family (Cachero et al., 1998). Further studies found that RhoA regulates the ion channel by modulating channel endocytosis via the downstream effector ROCK (Stirling et al., 2009). It is thus likely that in *Drosophila* a similar mechanism exists, whereby Rho GTPases regulate membrane excitability, either by directly modulating potassium ion channels or by regulating the endocytosis or exocytosis of ion channels.

The role of Rho family GTPases in regulating structural plasticity

Another mechanism by which Rho GTPases may affect membrane excitability is through changes in structural plasticity. With their known role in regulating the actin cytoskeleton, Rho GTPases have been implicated in regulating structural plasticity of axons and dendrites. For example, live imaging experiments revealed that Rac and Rho play antagonistic roles in dendrite remodelling: Rac promotes dendritic branching, whereas Rho decreased dendritic extensions (Li et al., 2000). In addition, Cv-c was shown to regulate dendritic elongation in *Drosophila* by negatively regulating Rho1 (Sato et al., 2010). This suggests that Rho GTPases play a role in regulating dendritic structure.

Rho1 has also been found to regulate axonal plasticity in small ventral lateral neurons (s-LNVs), a set of clock neurons in *Drosophila* (Petsakou et al., 2015). The axonal projections of sLNVs are maximally spread in the morning, and are contracted in the evening. This axonal plasticity was found to be mediated by Rho1. Rho1-GTP was found to be highest in the evening, when sLNV projections were contracted, and overexpressing

Rho1 locked sLNv projections in a contracted state. Interestingly, these changes in axonal structure also correlated with the excitability of clock neurons: sLNV neurons are most excitable at dawn when axonal termini are maximally spread (Cao & Nitabach, 2008). This suggests that Rho1 may influence membrane excitability by affecting the structural plasticity of neurons. Indeed, Liu et al. (2016) found that structural changes in ellipsoid body neurons were critical in generating sleep drive. Therefore, it is parsimonious to suggest that small GTPases may regulate the excitability of dFB neurons, and thus sleep, by mediating axonal and dendritic structural plasticity.

Interactions between Rho, Rac and Cdc42

There is a significant amount of crosstalk between small GTPases, as well as between their effectors, which contribute to the complexity of Rho GTPase signalling. In many biological processes, Rho and Rac have been found to act antagonistically. For example, in neuritogenesis small GTPases act antagonistically. While Rac and Cdc42 are required for neurite outgrowth, Rho inhibits neurite initiation and induces neurite retraction (Govek et al., 2005; Leeuwen et al., 1997). Similar antagonistic interactions were observed in migrating cells: Rho, which is predominantly active at the back of the cell, regulates stress fibre and focal adhesion assembly and promotes cell contraction, whereas Rac, which is localised predominantly at the leading edge of migrating cells, stimulates membrane protrusion and ruffling (Hall, 2005; Sander et al., 1999). In addition, Rho and Rac play antagonistic roles in the organisation of the germ cell environment in *Drosophila* (Sarkar et al., 2007). Therefore, both across species and across cell types, Rho and Rac often act antagonistically. However, it is important to consider that this is not consistently a *bona fide* rule. Some studies show that Rho and Rac act in parallel pathways, or in some cases even activate each other (Guilluy et al., 2011; Ridley et al., 1992).

The potential sleep regulatory role of small GTPases

It is clear that small GTPases are involved in a myriad of biological processes. Many of these, discussed above, suggest a role for small GTPases indirectly modulating membrane excitability. This, together with the finding that flies lacking the RhoGAP Cv-c are insomniac, reinforces the rationale for investigating the role of small GTPases in regulating sleep in the dFB neurons of *Drosophila melanogaster.*

1.5 Hypotheses

This thesis aims to investigate whether small GTPases play a role in regulating sleep via dFB neurons, and if so, how. Three main hypotheses were posited:

Hypothesis 1: Rho1 is upregulated in *cv-c* mutants, and this upregulation of Rho1 is what is causing the deficits in sleep homeostasis. This hypothesis is based on previous literature suggesting that Cv-c acts as a GAP of Rho1.

Hypothesis 2: Rac1 acts antagonistically to Rho1 in dFB neurons, and so has an opposing role in regulating sleep. This hypothesis is based on previous literature which suggests that Rho1 and Rac1 often act in an antagonistic manner.

Hypothesis 3: Small GTPases regulate the endocytosis of Sandman, thereby affecting the membrane excitability of dFB neurons. This hypothesis is based on the known role of small GTPases in vesicular trafficking and ion channel regulation.

2 Methods

To investigate the role of small GTPases in the wake-to-sleep switch I used a behavioural and an imaging approach. The behavioural approach involved genetically manipulating small GTPases, by introducing overexpression (OE), constitutively active (CA), dominant negative (DN) or RNAi constructs, and investigating their effect on sleep behaviour. These experiments involved a myriad of fly strains, genetic crosses and sleep measurements, and are outlined in Section 2.1 and 2.2. Optogenetics (Section 2.3) was also used to correlate genetic manipulation and sleep behaviour. The imaging experiments aimed to quantify the activity of small GTPases by using a fluorescence-based sensor. This involved dissections, immunohistochemistry and confocal imaging and the techniques are outlined in Section 2.4. Finally, statistical methods are explained and justified in Section 2.5.

2.1 Fly husbandry and genetics

2.1.1 Fly strains

All flies used in this study were from the species *Drosophila melanogaster.* Flies were reared in a 12-hour light, 12-hour dark cycle at 25°C at 60% relative humidity on standard corn agar food, made in-house (Recipe in Appendix). For temperature sensitive experiments (GAL80ts), flies were raised at 18°C. Stocks were flipped every 20-25 days and kept at room temperature. A list of all fly strains used in the study can be found in the Appendix.

For behaviour and imaging experiments, mated females were selected one day post-eclosion and housed in vials with 15-20 flies. The flies were anaesthetized with CO_2 during collection. Only female mated flies were collected and used in this study because mating status and sex influences sleep behaviour (Garbe et al., 2016; Ly et al., 2018).

To specifically manipulate genes in dFB neurons I made use of the GAL4-UAS system. This binary expression strategy involves the yeast transcription factor GAL4 and the responder transgene UAS (Upstream activating sequence) (Duffy, 2002). The UAS controls the expression of the coding sequence of a particular gene of interest. Transcription of the UAS responder relies on the presence of the GAL4 transgene, so only when both transgenes are present, will the gene of interest be expressed. The GAL4 transcriptional activator can be placed under control of endogenous regulatory elements, and thus can be expressed in specific tissues or cells of interest (Brand & Perrimon, 1993). This enables targeted expression of a gene of interest.

To target dFB neurons I used the *R23e10-GAL4* driver (Figure 2-1). This was previously shown to drive expression specifically in the dorsal fan shaped body circuit (Jenett et al.,

2012), and has previously been used to target the specific sleep-promoting population of interest (Pimentel et al., 2016).

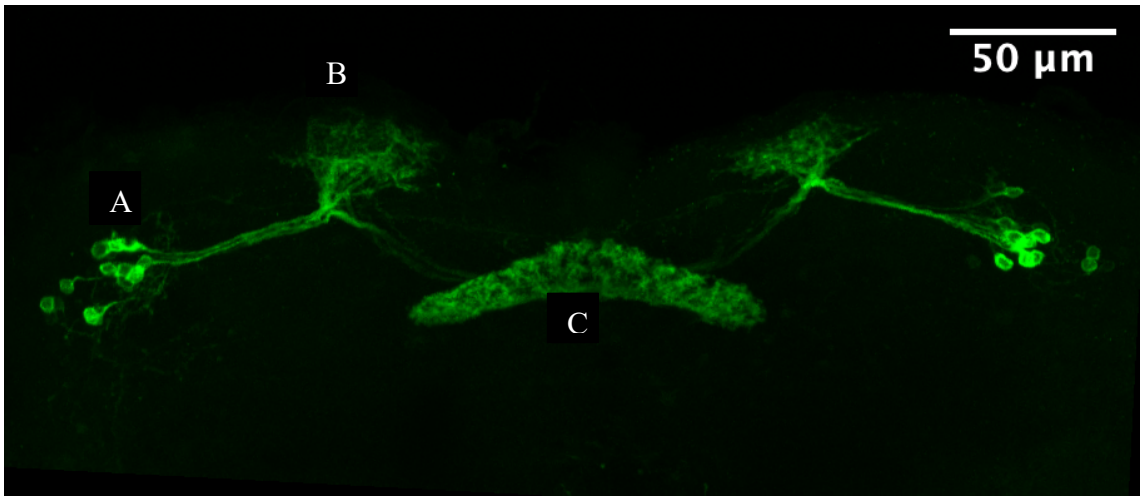


Figure 2-1: dFB neurons labelled using *R23e10-GAL4*-driven expression of *UAS-mCD8::GFP*. A: cell bodies. B: Dendritic projections. C: Axonal projections (dorsal fan shaped body)

2.1.2 *cv-c* mutants

Homozygous *cv-c* mutants are lethal so transheterozygous mutants were used to study the role of Cv-c. The following two allelic combinations were used:

- $cv\text{-}c^{c524}$ and $cv\text{-}c^{MB03717}$
- $cv\text{-}c^{1}$ and $cv\text{-}c^{MB03717}$

$cv\text{-}c^{c524}$ is a point mutation generated by Denholm et al. (2005), $cv\text{-}c^{1}$ is a spontaneous mutation identified by Stern (1934) and $cv\text{-}c^{MB03717}$ is a transposable element insertion generated by Bellen et al. (2011). Both allelic combinations were used interchangeably because the sleep profiles were very similar, and certain UAS transgenes were easier to combine with certain *cv-c* alleles. Most behavioural experiments were verified in both backgrounds.

2.1.3 Making recombinants

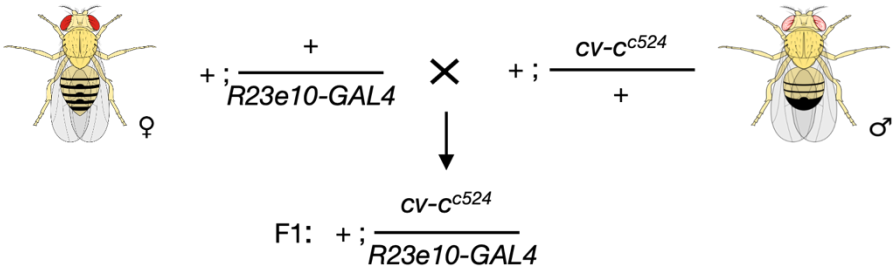
Since the *cv-c* alleles, many UAS transgenes of interest, as well as the *R23e10-GAL4* driver were all located on the third chromosome, I performed sequential recombination in order to combine transgenes onto the same chromosome. Recombinants were generated through a series of crosses, as outlined in Figure 2-2. Flies were genotyped to verify the presence of both transgenes and primers were designed in order to target the transgenes of interest. Primers were ordered as DNA Oligos in tubes from Sigma-Aldrich. The primer sequences used are shown in Table 1. Genotyping was conducted using a standard PCR protocol. It is important to note that several recombinants that were used in this study were generated by previous members of the lab.

Table 1: Primers used for genotyping. The melting temperature for all primers used was between 61-65°C and the GC% was between 45-55%.

Primer Name	Sequence (5' – 3')
GAL4 Forward	CTCCCAAAACCAAAAGGTCTCCG
GAL4 Reverse	CGTCGCCAAAGAACCCATTATTG
UAST Forward	AGCAAATAAACAAGCGCAGC
UAST Reverse	TGCTCCCATTCATCAGTTCC
eGFP Forward	CGATGCCACCTACGGCAA
eGFP Reverse	GCTTGTGCCCCAGGATGTT
PKN-RBD Reverse	ATGGACATACAGCTCTCCGG
Rac1 Reverse	GTCGAACACGGTGGGTATGT
Rho1 Reverse	GTTGGGATCATTTCGCAAAT
Cdc42 Reverse	AATGGTGTGTAATCTCGGGC
Rab5 Reverse	CCTGGTACTCGTGGAACTGT

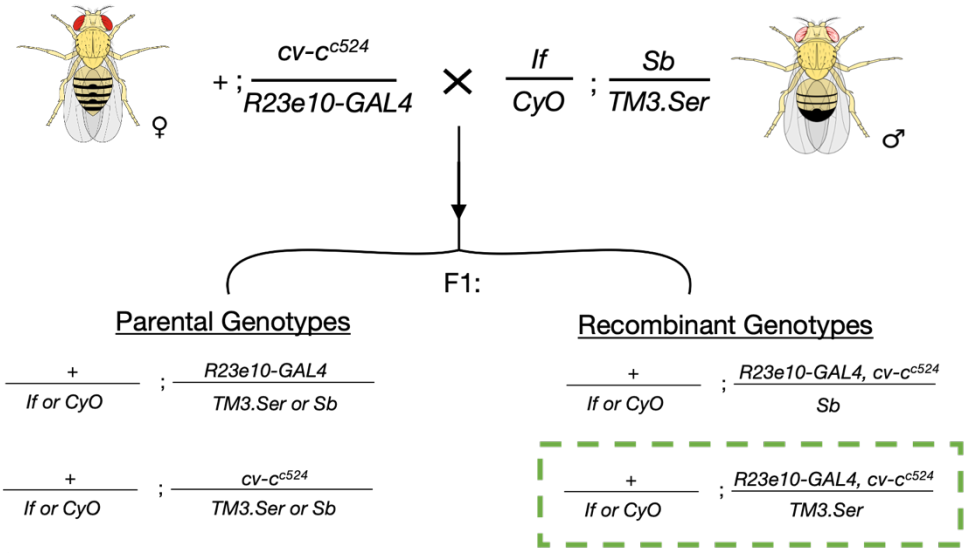
Cross 1:

To recombine the *R23e10-GAL4* driver with a *cv-c* mutant allele (both on the 3rd chromosome) females and males carrying the respective transgenes were crossed.



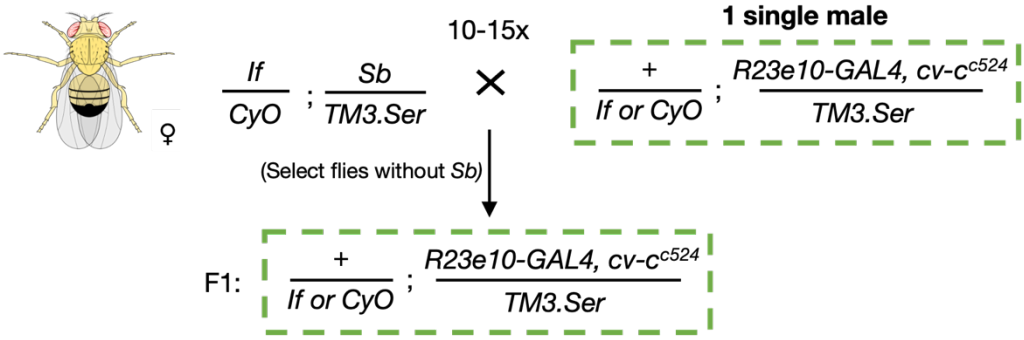
Cross 2:

Recombination only occurs in females, so virgin females from F1 were selected and crossed with double balancer males



Cross 3:

Potential recombinant individual males (highlighted in green) were crossed with several double balancer virgins in order to generate a series of stable stocks. The lower the frequency of recombination, the more crosses should be set up at this stage.



F1 flies from cross 3 were genotyped for the presence of both transgenes (i.e *R23e10-GAL4* and *cv-c^{c524}*) . If both transgenes are present, a stable stock can be generated.

Figure 2-2: Diagram illustrating step by step crosses used to generate an example recombinant line. *TM3. Ser* and *CyO* were used as balancers. *If* and *Sb* are markers.

2.1.4 Constitutively active and dominant negative constructs

In order to manipulate small GTPase activity I made use of CA and DN constructs which lock GTPases in their GTP- or GDP-bound state. CA constructs harbour an amino acid substitution of Val for Gly at residue 12 which prevents GTP hydrolysis (Bishop & Hall, 2000). As such, *UAS-Rho.V12* and *UAS-Rac.V12* generate CA forms of Rho and Rac respectively, under the control of UAS. DN constructs work by competing with endogenous Rho GTPases for binding to GEFs (Bishop & Hall, 2000). DN constructs have a higher affinity to GEFs and harbour a mutation which prevent it from interacting to downstream target proteins. This prevents endogenous GTPases from binding to GEFs, and thus prevents downstream responses (Feig, 1999). The constructs used in the study were all obtained from the BDSC and are listed in the fly strains table in the Appendix.

2.1.5 GAL80ts for conditional expression

Since small GTPases play key roles in development I wanted to limit the expression of transgenes to adult flies. To do this I used a version of GAL80 which is temperature sensitive (GAL80ts) developed by Matsumoto et al. (1978). GAL80 is a protein which antagonises GAL4 and inhibits its ability to bind to UAS (Lue et al., 1987). When flies are kept at 18°C, GAL80ts represses the activity of GAL4 and thus prevents GAL4 from inducing expression of the transgene of interest. At 30°C, GAL80ts becomes inactive allowing GAL4 to bind to UAS. To use this technology, previous members of the lab recombined GAL80ts with the *R23e10-GAL4* driver line. This enabled me to drive the expression of genes of interest specifically in dFB neurons and only in adults, by raising flies at 18°C (where GAL4 activity is repressed) and shifting them to 31°C during behavioural experiments (where GAL4 activity is enabled).

2.2 Sleep measurements

2.2.1 DAM system

Mated female flies aged 3-5 days were individually loaded into 60mm glass tubes using an aspirator. Approximately 5-10mm of standard fly food was added to one end of the glass tube and sealed with wax, and a cotton plug was inserted at the other end of the tube. The tubes were then loaded into a Drosophila Activity Monitor (DAM 2, TriKinetics). The DAM system measures the locomotor activity of 32 individual flies by recording each time a fly crosses an infrared beam spanning the middle of the tube, as shown in Figure 2-3.

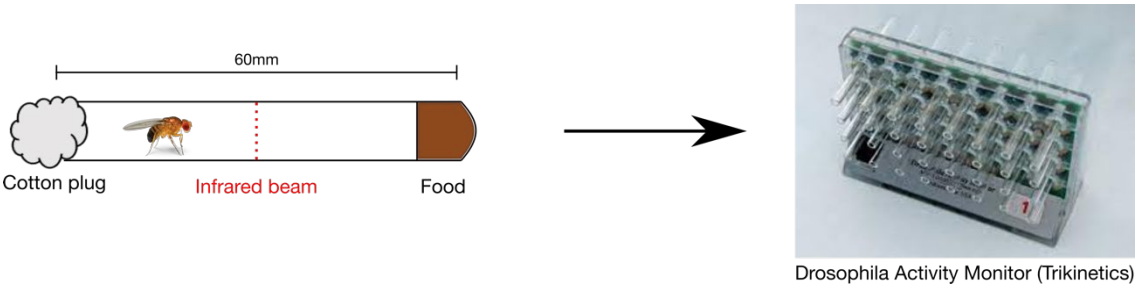


Figure 2-3: Drosophila Activity Monitor: Flies are individually inserted into 60mm glass tubes. 32 tubes can be loaded into one Drosophila Activity Monitor which records the activity of the flies using an infrared beam.

The monitors were placed in a temperature controlled incubator (PHC, MIR-254-PE) in a 12-hour light and 12-hour dark cycle at 25°C. Sleep was defined as any period in which a fly did not cross the infrared beam for 5 minutes or more. This is based on the conventional definition of *Drosophila* sleep (Hendricks et al., 2000; Shaw et al., 2000). Deceased flies were excluded from the analysis. Tubes which contained larvae were also excluded from the analysis.

2.2.2 Sleep deprivation

Flies were sleep deprived using a Sleep Nullifying Apparatus. The Sleep Nullifying Apparatus (SNAP) mechanically sleep deprives flies by snapping back and forth from −60° to +60° every 10 seconds (Melnattur et al., 2020). DAMs with 32 flies each were loaded into the SNAP and the activity of the flies was monitored while sleep deprivation occurred. This method of sleep deprivation was shown to prevent >95% of night time sleep in flies and was shown to induce a similar homeostatic response as when flies were sleep deprived by hand (Melnattur et al., 2020). Importantly, the SNAP machine was tested on stress-sensitive flies, which were found to behave similarly after sleep deprivation as wild type flies (Shaw et al., 2002). For all my experiments I sleep deprived flies during night-time for 12 hours (from 8pm to 8am). Flies that were deceased or got stuck to food were excluded from the analysis.

2.2.3 Sleep analysis

The data collated from the DAM system was analysed using a Sleep and Circadian Analysis MATLAB Program (S.C.A.M.P) script written by Christopher Vecsey (Donelson et al., 2012). Using the script the following parameters were quantified:

- Sleep duration: This is the total number of hours slept by a fly in a 24 hour period. The fly is considered to be sleeping when it is inactive for 5 minutes or more.

- P(Doze): The probability that an active fly will stop moving (Wiggin et al., 2020).

- Activity per waking minute: This is the number of beam brakes per waking minute. This measure (intensity of waking activity) can be used to see whether any manipulations inhibit locomotion. A low value denotes reduced locomotor activity, whereas a high value denotes hyperactivity.

- Average number of sleep bouts: This is the number of sleep episodes exhibited by an individual fly in a 24 hour period. A sleep episode is considered a period in which the fly is inactive for 5 minutes or more.

- Average bout duration: This is the mean duration of a sleep episode. This can be used as a measure of how fragmented the sleep is.

Unless otherwise stated, the values of day 2 and day 3 after loading were averaged for parameter quantification. Data from at least 3 independent experiments, from 3 independent crosses and loaded on different days were pooled.

2.3 Optogenetics

2.3.1 Set up

The optogenetics set up was developed by Clifford Talbot (Kempf et al., 2019). 2-3 day old mated female flies were inserted into 60mm glass tubes and loaded into a set of 20 custom built light-tight chambers, each with a high-power blue LED (Osram Opto Semiconductors LB W5SM-FZHX-35-0, 467 nm). A liquid cooling system, with a centrifugal pump (RS 702-6891), liquid heat exchangers (Thermo Electric Devices LI102), and a CPU cooler (Corsair CW-9060007-WW), was used to remove excess heat from the LEDs. The apparatus was set up in a temperature controlled incubator (Sanyo MIR-154) and behaviour experiments were conducted at 25°C.

To track the activity of the flies in the optogenetic set up, a high resolution camera (Thorlabs DCC1545M) with a 8 mm lens (Thorlabs MVL8M23) was used. A long-pass filter (Thorlabs, FEL800nm) was used to reject photostimulation light. A script written by Clifford Talbot in LabView (National Instruments) extracted position data from video images. Using a MATLAB script (also written by Clifford Talbot) this data was translated to activity and sleep histograms for each individual fly, where a period of inactivity lasting at least 5 minutes was classified as sleep.

2.3.2 Photorecruitable Rho-GEF (LARG)

To optogenetically activate endogenous Rho1 with high temporal precision I made use of a LOV-based photorecruitable Rho-GEF (PR-Rho-GEF) developed by Rich et al. (2020). The PR-Rho-GEF consists of two components: a Light-Oxygen-Voltage (LOV) domain fused to a membrane-localised SsRA peptide, and a cytoplasmic SspB peptide fused to the catalytic domain (DH) of leukemia-associated RhoGEF (LARG). LARG is a Rho-GEF that was found to be a potent Rho activator in mammalian systems (Jaiswal et al.,

2013). The LOV domain family of proteins are sensitive to blue light with an absorption peak at 450nm (Pudasaini et al., 2015). Upon blue light exposure, the LOV domain undergoes a conformational change which exposes the SsrA peptide, and enables the recruitment of SspB. Thus, as shown in Figure 2-4, blue light results in the recruitment of the LARG-DH to the plasma membrane, where it functions to activate Rho1. This optogenetic probe was found to be a robust method to acutely activate Rho1 in several *Drosophila* tissues, including the larval central nervous system (Rich et al., 2020). In addition, this type of optogenetic activation is reversible: the SspB-bound Rho-GEF construct returns to its inactive cytoplasmic location within minutes.

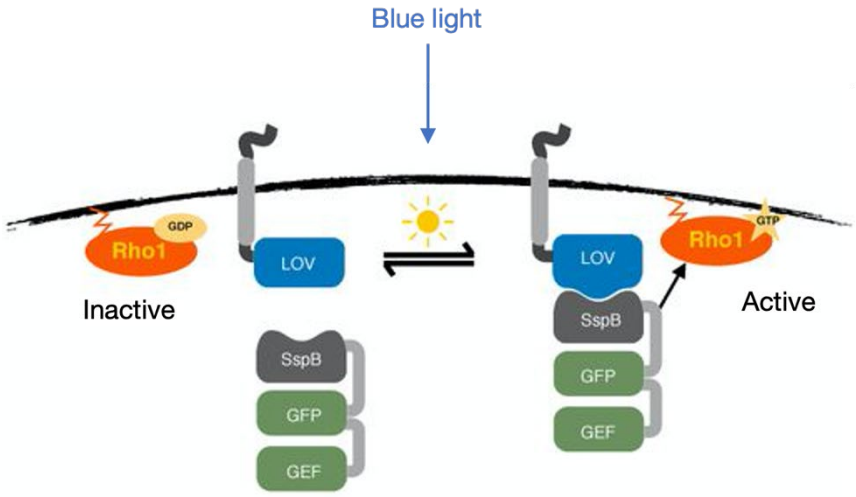


Figure 2-4: Illustration adapted from Rich et al. (2020) showing how LOV-based photoactivable LARG(DH) works to activate Rho1. Upon blue light exposure, the LOV-SsRA domain undergoes a conformational change which recruits SspB to the plasma membrane. SspB is fused to a GFP tag and a Rho-GEF catalytic domain (LARG-DH). Translocation of LARG-DH to the plasma membrane acutely activates Rho1.

To use this optogenetic probe in dFB neurons I combined the SsRA-bound LOV construct with the *R23e10-GAL4* line. SspB-LARG was constitutively expressed using a ubiquitin driver. The transgenes were then combined in a final cross as shown in Figure 2-5. To

control for any effects of the LOV domain SspB-mScarlet, instead of SspB-LARG(DH), was expressed. mScarlet does not interact with Rho1. All crosses were raised in darkness in order to prevent any LOV domain activation during development.

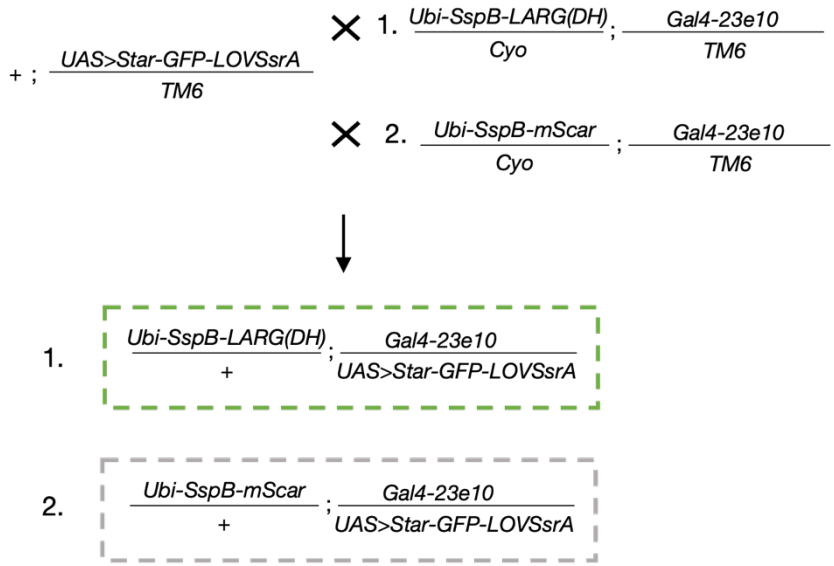


Figure 2-5: Crosses to express photoactivable Rho-GEF constructs in dFB neurons. Genotype 1 contains the catalytic domain of LARG constitutively expressed by the Ubi promoter and the UAS-LOV domain, driven specifically in dFB neurons using the *R23e10-GAL4* line. Genotype 2 is the control which replaces the LARG domain with mScarlet.

2.3.3 Photoactivatable Rac1

To activate Rac1 with high temporal precision I made use of a LOV-based photoactivable Rac (PA-Rac) construct developed by Y. I. Wu et al., (2009) and Wang et al. (2010). Similar to the photoactivable LARG construct, this construct takes advantage of the blue light sensitivity of the LOV domain. Wu et al. (2009) fused constitutively active Rac1 to a LOV2- Jα sequence. In the dark, the LOV domain blocks the binding of effectors to Rac1, but upon blue light exposure the Jα helix (an extension of the LOV domain) unwinds. This frees up the effector binding sites and leads to constitutive activation of Rac1, as shown in Figure 2-6. The group also fused the LOV domain to a dominant negative mutation of Rac1, enabling inhibition of Rac1 activity with high spatial and

temporal precision. It is important to note that this construct works by activating/inhibiting exogenous Rac1, whereas the photorecruitable Rho-GEF construct works by activating endogenous small GTPases.

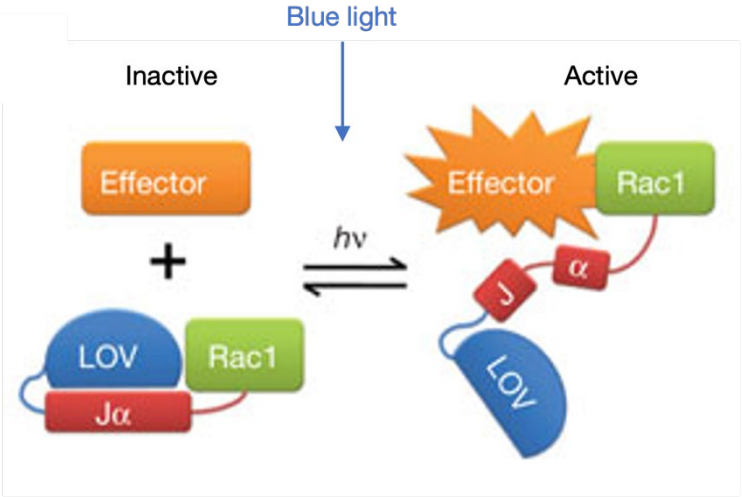


Figure 2-6: Diagram of how LOV-bound photoactivable Rac becomes activated upon blue light exposure, adapted from (Wang et al., 2010). In the dark, LOV-Jα complex prevents Rac1 from binding to downstream effectors. Upon blue light exposure, LOV-Jα undergoes a conformational change and Rac1 is constitutively active and binds to downstream effectors.

Flies carrying *UAS-PA-RacQ61L* (constitutively active PA-Rac1) and *UAS-PA-RacT17N* (dominant negative PA Rac1), were crossed with flies carrying the *R23e10-GAL4* construct, to drive the constructs specifically in dFB neurons. As a light insensitive control, *UAS-C450M-PA-RacQ61L* was expressed. This construct contains the constitutively active form of PA-Rac1, but it is light insensitive because it harbours a mutation in the LOV2 domain.

All crosses were raised in darkness in aluminium covered vials.

2.3.4 Optogenetic protocol

Female flies expressing PR-Rho-GEF or PA-Rac1 constructs, or their respective controls were collected upon eclosion and placed into aluminium covered vials in groups of 10-15. 2-3 days after collection, flies were loaded into the optogenetic set-up in a temperature-controlled incubator (Sanyo MIR-154). Experimental and control genotypes were loaded side by side (e.g. 10 light-sensitive flies, 10 light-insensitive flies). Positioning of genotypes was alternated in every replicate. The incubators were set to a dark-dark cycle.

Flies were kept in darkness for one full 24 hour period (Baseline day). The light protocol was then initiated at 8am (ZT 0) on the following day for 24 hours. This was followed by a recovery day in darkness. During the 24 hour light stimulation period, the blue light LEDs (467nm) delivered a continuous train of 3-ms light pulses (~28 mW/cm^2) at 20 Hz. This light intensity was based on a previous protocol (Donlea et al., 2018) and was tested on wildtype flies to ensure it did not significantly affect sleep. The percentage difference in sleep duration between the baseline and light stimulation days was calculated to determine the effect of light activation on sleep. In wildtype flies the percentage decrease in sleep upon blue light activation was around 10-20%.

Deceased flies and tubes that had larvae crawling were excluded from the analysis. The *n* numbers for these experiments are low because only 20 flies could be loaded per experiment and larvae often disrupted sleep measurements which meant that a lot of flies had to be excluded.

2.4 Confocal Imaging

2.4.1 Rho1 Biosensor

In order to determine the level of Rho1-GTP activity I made use of a GFP-based Rho1 activity probe developed by Simões et al. (2006). This probe (PKNG58AeGFP) consists an eGFP tag fused to three Rho1-GTP binding domains from the Rho1 downstream effector Protein Kinase N (PKN). When Rho1 is in its active, GTP-bound state, it binds to the probe and recruits it to the plasma membrane. The probe fluorescence is weak when localised in the cytoplasm, but upon binding of activated Rho1, the probe is concentrated at the membrane to give intense fluorescence (Petsakou et al., 2015). The construct also includes a point mutation at amino acid 58, which reduces the probe's affinity to Rac1. In vitro binding assays showed that the PKN-eGFP probe binds specifically to Rho-GTPγS at ratios of 5:1 to 10:1 (Simões et al., 2006). This sensor was used by Petsakou et al. (2015) to monitor how Rho1-GTP levels change throughout the day in lateral ventral neurons, suggesting that it is a reliable approach to study Rho1 activity in neurons.

2.4.2 Immunohistochemistry

The staining protocol used was adapted from Wu & Luo (2006). Flies aged 5-8 days old were immobilized at specific time points on ice and dissected in Phosphate Buffer Solution (PBS) using forceps (Dumont N°5). Brains were dissected clean and all trachea was removed in order to maximise antibody penetrance. The brains were fixed for 30 minutes at room temperature in 4% Paraformaldehyde in PBS and then washed twice briefly in 0.5% PBST (2.5ml Triton X-100, 497.5ml PBS), followed by three 20 minute washes. Brains were blocked overnight at 4°C in PBST with 10% Normal Goat Serum (Sigma G6767) on a shaker (RPM 35). Brains were then incubated with primary antibodies, followed by secondary antibodies for 2 days each, at 4°C on a shaker (RPM

35). Primary and secondary antibodies were diluted in 5% NGS and 0.5 % PBST. Before and after secondary antibody incubation, brains were washed in 0.5% PBST, twice briefly, followed by three 20 minute washes. Before mounting, brains were briefly soaked in chilled 50% glycerol (Sigma). Brains were mounted with VectaShield (Vector Labs H1000) on polysine coated microscope slides (VWR, 631-0107), covered with 1.5mm cover slips (VWR, 631-0121) and sealed with nail polish.

Primary antibodies used were chicken anti-GFP (1:1000, ABCAM 13970) and rabbit anti-dsRed (1:500, TaKaRa 632496). Secondary antibodies used were Alexa Fluor 488 goat anti-chicken (1:1000, Invitrogen A11039) and Alexa Fluor Plus 555 goat anti-rabbit (1:1000, Invitrogen A32732).

2.4.3 Image acquisition

Mounted brains were imaged using a Leica TCS SP5 confocal microscope at 25 magnification with a water immersion objective (HCX IRAPO L 25x0.95, Leica). Image stacks were collected at 1024 x1024 at 2.0 digital zoom and optimised step size. Depending on the experiment, a step size of 0.29µm or 0.5µm was used.

For quantifying Rho1 biosensor activity, all imaging settings were kept the same in order to allow for fair comparison between groups. A 10% laser intensity at 100% gain was selected for the 488 laser, whereas a 3% laser intensity at 50% was selected for the 555 laser. Images were acquired with a pin hole of 55.88, with a speed of 400Hz, and using bidirectional and line by line sequential scanning. These settings were selected to ensure no bleed through between the channels and no oversaturation in the brightest conditions (Jonkman et al., 2020).

2.4.4 Image analysis

Images were analysed and quantified using Fiji. A z stack containing the region of interest (ROI) was selected for each image and a sum of slices projection was generated. A ROI around the dFB axons was traced in the tdTomato channel (as shown in Figure 2-7) and mean fluorescence intensity was quantified for both GFP and tdTomato signal. Background levels were determined by the mean fluorescence intensity of a randomly selected region in the surrounding area of the ROI. Background fluorescence was then subtracted from the ROI fluorescence. In order to normalise for variability between stainings and dissections, the mean fluorescence of the GFP signal after background subtraction was divided by the mean fluorescence of the tdTomato signal after background subtraction. This normalised GFP/tdTomato ratio was used to determine Rho1-GTP activity. Images were quantified blindly and any images in which the morphology of the dFB projections was deformed were excluded from quantification.

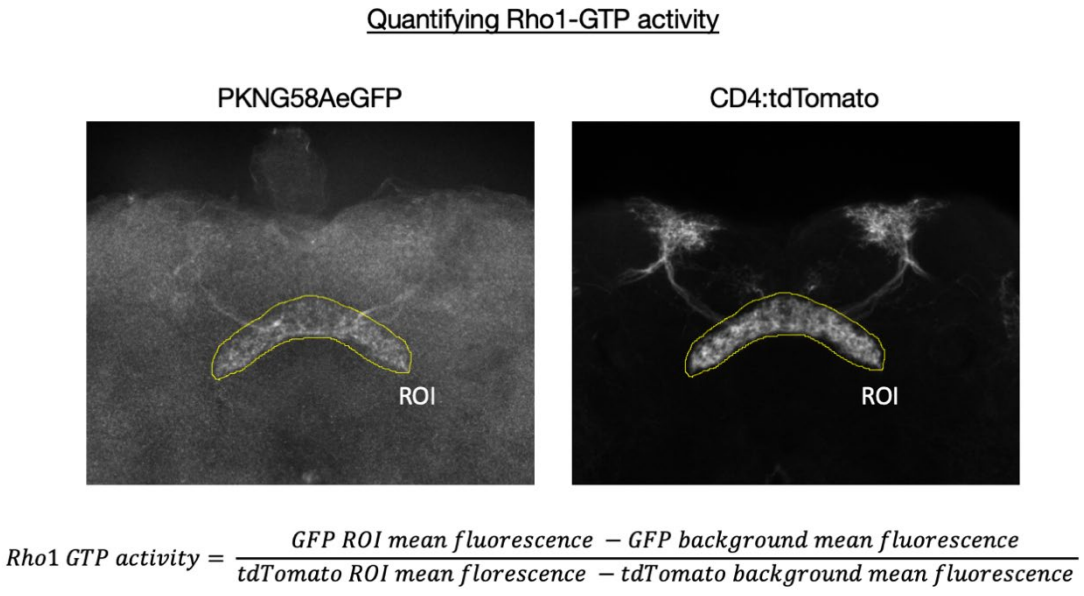


Figure 2-7: Quantifying Rho1-GTP activity using the PKNG58eGFP biosensor. GFP signal was normalised to tdTomato signal, after background subtraction. These images were acquired from the same brain, in two different channels using sequential scanning. The genotype of the brain shown is: *UAS-CD4:tdTomato; R23e10-GAL4 > UAS-PKNG58AeGFP.*

2.4.5 Calculating axonal plasticity

To calculate axonal plasticity, I used a script kindly gifted by Justin Blau (Petsakou et al., 2015). Using Fiji, a JPEG image sequence was generated from each z stack. The image sequence with 310.3 x 310.3 μm images with 1024 x 1024 pixel resolution was then imported into MATLAB. The axonal projections of dFB neurons were selected as the ROI. The tdTomato channel (555) was used for calculating axonal plasticity. The MATLAB script quantified the z-spread, y-spread, x-spread of the region of interest. This is based on pixels with >70% of the maximum fluorescent intensity, thus filtering out any background noise. The 3D spread was then calculated by multiplying the z, y and x spread values. The axonal volume was quantified using the script which calculates the total amount of pixels. More detail on how the script quantifies the spread values is described in the supplementary methods of Petsakou et al. (2015).

2.5 Statistical Analysis

For each experiment, data from at least 3 independent replicates were pooled. Data was handled in Microsoft Excel and statistics were performed using GraphPad Prism 9. For each data set I assessed the assumptions of normality and constant variance by looking at the Q-Q residuals plot, histograms, the D'Agostino-Pearson test and Shapiro-Wilk test of normality. This information was used to determine whether a parametric (one-way ANOVA or T-test) or a non-parametric test (Kruskal-Wallis) was most appropriate. This combinatorial approach was used because simply testing for normality is flawed: in a large data set, mild deviations will result in a significant result, even though mild deviations of normality are not problematic for parametric tests (Krzywinski & Altman, 2014). To compare means of different groups, the one-way ANOVA or Kruskal-Wallis test was followed by a Tukey or Dunnett test (parametric), or a Dunn's multiple comparisons test (non-parametric). P values < 0.05 were considered significant, and the respective levels of significance were denoted with the following asterisks: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Error bars represent the standard error of the mean (SEM), unless otherwise stated. Figures were put together using Inkscape and Biorender.

For behaviour experiments, GAL4 and UAS lines were each crossed with Canton S flies (wildtype) to serve as parental controls. Each experimental group was compared statistically to both parental controls with a post-hoc multiple comparisons test. Multiple comparisons test will be abbreviated to MCT throughout.

3 Results

3.1 The role of Rho1 in regulating sleep

cv-c was shown to play a key role in regulating the membrane excitability of dFB neurons: flies carrying transheterozygous mutations for *cv-c* are insomniac and their dFB neurons are locked in an electrically silent state. Since *cv-c* encodes a RhoGAP, it is likely that its small GTPase substrate is involved in the molecular pathway underlying the dFB ON-OFF switch.

The first step of this project was to identify the substrate of Cv-c. Previous literature found that Cv-c mainly exhibits GAP activity towards Rho1, but also exhibits partial GAP activity towards Rac1 and Cdc42 (Denholm et al., 2005; Sato et al., 2010). In addition, preliminary work (mentioned in Section 1.3) identified that Rho1 is the small GTPase that is most abundantly expressed in dFB neurons. Thus, I first sought out to test whether the substrate of Cv-c in dFB neurons is Rho1.

The main method in which substrate specificity of Rho-GAPs has been elucidated is through rescue experiments. Since Cv-c is known to negatively regulate its small GTPase substrate, it is likely that phenotypes observed in *cv-c* mutants are caused by high levels of the active small GTPase substrate. Thus, knocking down or reducing the substrate should rescue the *cv-c* mutant phenotype back to wildtype levels.

Dominant negative (DN) constructs, as introduced in 2.1.4, lock small GTPases in their GDP-bound (inactive) form, thereby reducing their activity. I introduced the DN constructs of Rho1, Rac1 and Cdc42 into the transheterozygous *cv-c* mutant background and measured sleep as described in Section 2.2. The data is shown in Figure 3-1.

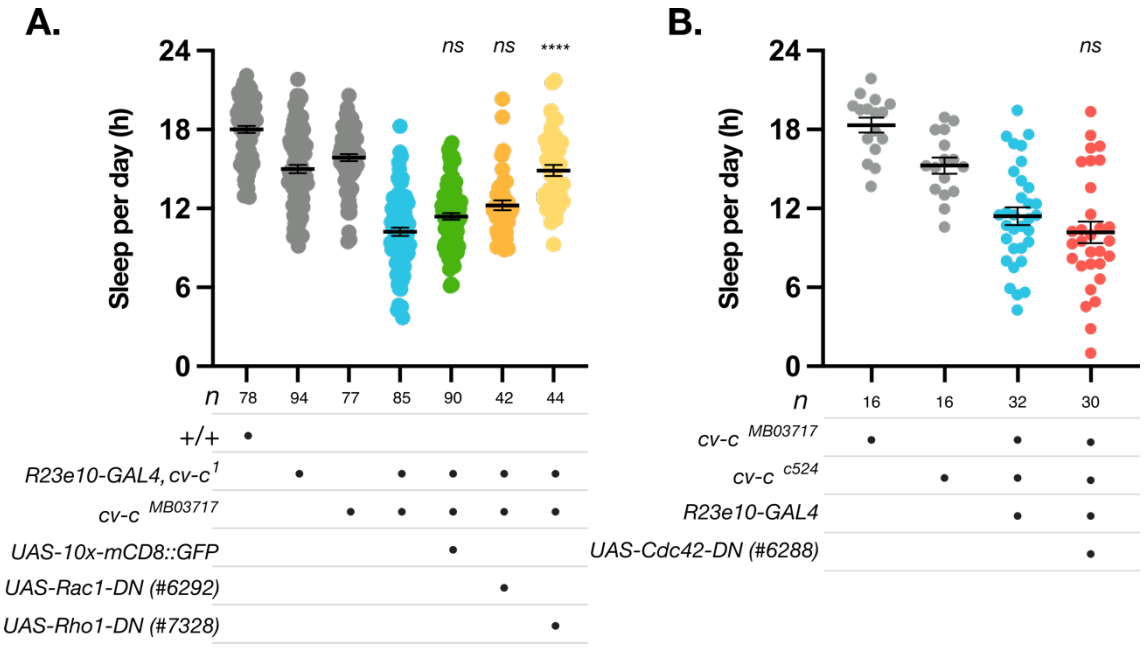


Figure 3-1: Dominant negative Rho1, but not dominant negative Rac1 or Cdc42 rescues the *cv-c* insomniac phenotype. Data is presented as means ± SEM; each circle represent an individual fly. *n* represents the number of flies for each genotype. **A).** Average sleep duration of *cv-c*^{1}/*cv-c*^{MB03717} + dominant negative Rac1 and Rho1, with controls. Data was pooled from 6 independent experiments conducted on separate days. Values were averaged across day 2 and 3. Kruskal Wallis test was significant (X^2= 314.7, p<0.0001). Asterisks denote significant differences in pairwise Dunn's post-hoc comparisons test compared to transheterozygous *cv-c* group (blue). **B)** Average sleep duration of *cv-c*^{c524}/*cv-c*^{MB03717} + dominant negative Cdc42 and controls. ANOVA was significant (F=18.7, p<0.0001) Asterisks denote significant differences in pairwise Tukey's post-hoc comparisons test compared to transheterozygous *cv-c* group (blue). Data shown in B was collected by a previous member of the lab (Michael Song), and thus the controls and *cv-c* background are different. However, the behaviour also holds true in the *cv-c*^{1}/*cv-c*^{MB03717} background. Numbers next to the genotypes are the BDSC fly number.

Experimental groups were statistically compared to *cv-c* transheterozygous mutants (blue) using a post-hoc test, and differences are denoted by the asterisks. *cv*-c transheterozygous mutants slept on average 10 hours a day, whereas controls slept between 15-18 hours. When DN Rho1 was introduced in *cv-c* mutants, specifically in dFB neurons (using the *R23e10-GAL4* driver), sleep returned to the level of heterozygous parental controls (15 hours). This rescue in sleep was not observed when DN Rac1 or DN Cdc42 were introduced. Expression of DN Rac1 in *cv-c* mutants did increase sleep slightly (by 2 hours) but this was not statistically significant (P > 0.99, Dunn's MCT). To control for effects of introducing a UAS transgene, *UAS-10x-mCD8::GFP* (membrane-

bound GFP) was introduced in *cv-c* mutants. This resulted in a partial increase in sleep (1.2 hours) but this was not statistically significant ($P > 0.99$, Dunn's MCT). Therefore, these experiments, together with the RNA sequencing data previously collected from the lab, strongly suggest that Rho1 is the sleep-relevant substrate of Cv-c in dFB neurons. Furthermore, it suggests that Rho1 is upregulated in *cv-c* mutants, and this upregulation is causing the defects in sleep homeostasis. This led me to investigate the role of Rho1 in regulating sleep.

3.1.1 Manipulating Rho1 in *cv-c* mutants

With Rho1 identified as the main sleep-relevant substrate of Cv-c in dFB neurons, I sought to test how different genetic manipulations of Rho1 would influence sleep behaviour in *cv-c* mutants. If Rho1 is upregulated in *cv-c* mutants, as suggested by preliminary experiments, then I would expect Rho1 to have a sleep-reducing effect. Thus, I hypothesised that genetic manipulations that increase Rho1 levels, such as constitutively active (CA) constructs or overexpression (OE) of Rho1, should further reduce sleep in *cv-c* mutants (i.e. exacerbate the insomniac phenotype), and genetic manipulations that reduce Rho1 levels, such as DN or RNAi constructs should increase sleep in *cv-c* mutants (i.e. rescue sleep to baseline levels).

I introduced four different genetic constructs (Rho1 DN, Rho1 RNAi, Rho1 OE, Rho1 CA) into the $cv\text{-}c^{1}/cv\text{-}c^{MB03717}$ background and drove the constructs specifically in dFB neurons using the *R23e10-GAL4* line. Constructs that work to decrease Rho1-GTP levels are shown in yellow/orange, whereas constructs that increase Rho1-GTP levels are shown in pink/purple in Figure 3-2. Parental controls are shown in grey. *UAS-mCD8::GFP* (green) was added as an additional control. Asterisks in all figures represent the post-hoc analysis of experimental groups compared to the transheterozygous *cv-c* group (blue), unless otherwise denoted.

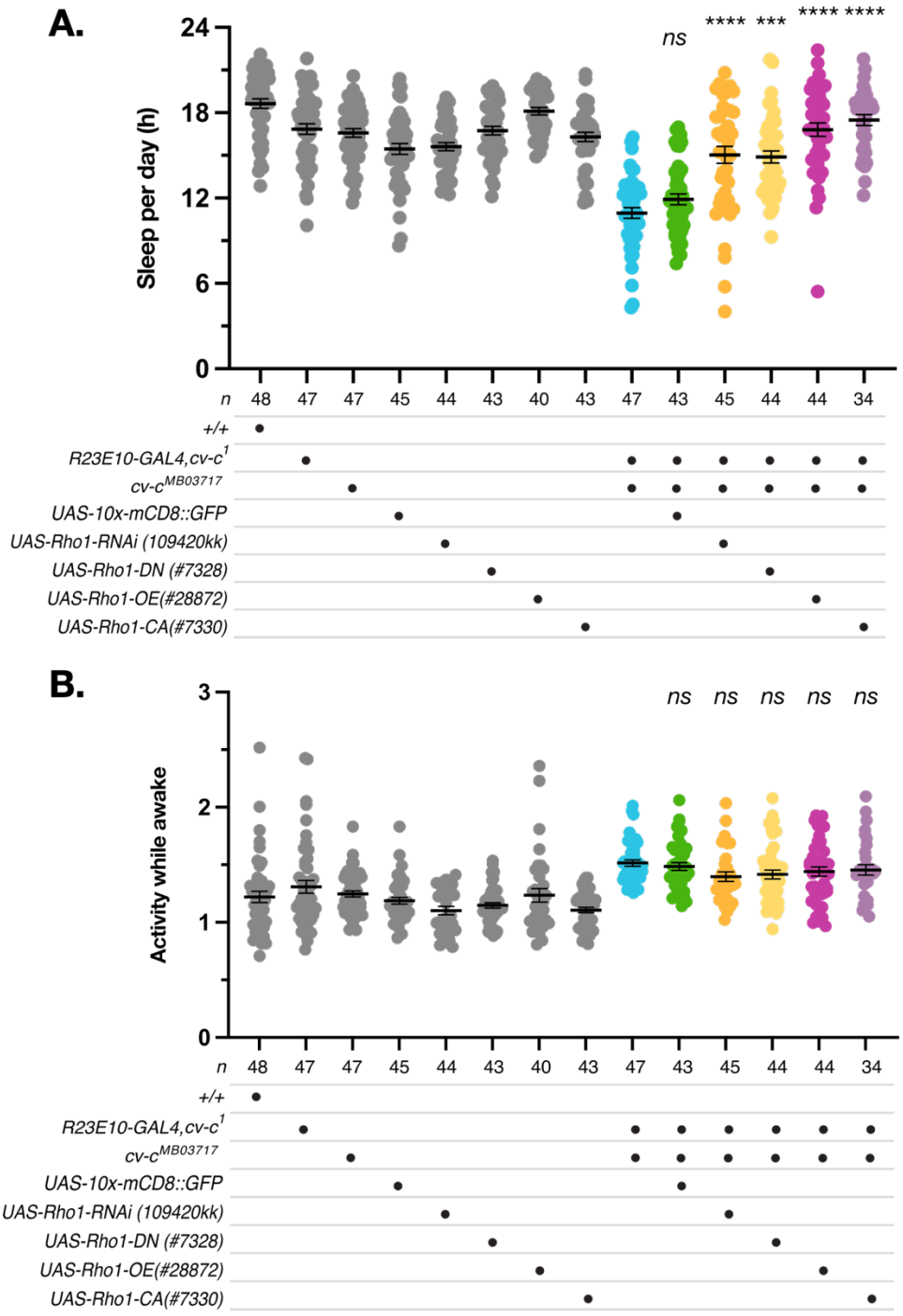


Figure 3-2: The effect of Rho1 manipulations on sleep duration and activity while awake. A) Average sleep duration in hours plotted for different genotypes. **B)** Activity while awake: average number of beam brakes per minute awake. Data is presented as means ± SEM; each circle represent an individual fly. *n* represents the number of flies for each genotype. Data was pooled from 3 independent experiments conducted on separate days. Values were averaged across day 2 and 3. Kruskal Wallis test was significant for A ($X^2 = 239.5$, $p<0.0001$) and B ($X^2 = 210.7$, $p<0.0001$). Asterisks denote significant differences in pairwise Dunn's post-hoc comparisons test compared to transheterozygous *cv-c* group (blue). Dominant negative data set and controls is the same as shown in Figure 3-1. Numbers next to the genotypes are the BDSC fly number.

Surprisingly, all Rho1 manipulations resulted in a statistically significant increase in sleep in comparison to *cv-c* mutants, as shown in Figure 3-2A. While Rho1-RNAi and Rho1-DN increased sleep by almost 40% compared to *cv-c* mutants, introducing Rho1-OE and Rho1-CA in dFB neurons of *cv-c* mutants increased sleep by almost 60%. All Rho1 manipulation groups slept significantly more than the mCD8::GFP control in *cv-c* mutants, suggesting that the observed increase in sleep is not due to unspecific UAS transgene or overexpression effects. In addition, the sleep duration of the four Rho1 manipulations were not statistically different from the sleep duration of the parental UAS controls (*UAS-x* > +), or from the GAL4 control (*R23e10-GAL4* > +) suggesting that all manipulations resulted in a full rescue of the *cv-c* sleep phenotype. However, while Rho1-RNAi and Rho1-DN groups still slept significantly less that the wildtype strain (+/+) ($P<0.0001$ for both, Dunn's MCT), total sleep duration in *cv-c* + Rho-OE and *cv-c* + Rho1-CA was indistinguishable from total sleep duration observed in wildtype flies ($P > 0.99$ and $P = 0.4$, respectively, Dunn's MCT). This suggests that both overexpressing and knocking down Rho1 in dFB neurons of *cv-c* mutants increases sleep duration, but overexpressing Rho1 has a stronger sleep promoting effect.

To ensure that these effects are really due to sleep and not due to locomotor defects, the number of beam brakes per waking minute, which reflects the average level of activity while awake, was plotted (Figure 3-2B). All experimental groups (carrying the transheterozygous *cv-c* mutations) showed a slight hyperactive phenotype, and were significantly more active than parental controls. However, between the experimental groups there were no significant differences. This suggests that introducing Rho1 manipulations into the *cv-c* mutant background does not affect locomotor activity while awake. Further analysis into the sleep architecture is shown in Figure 3-3A.

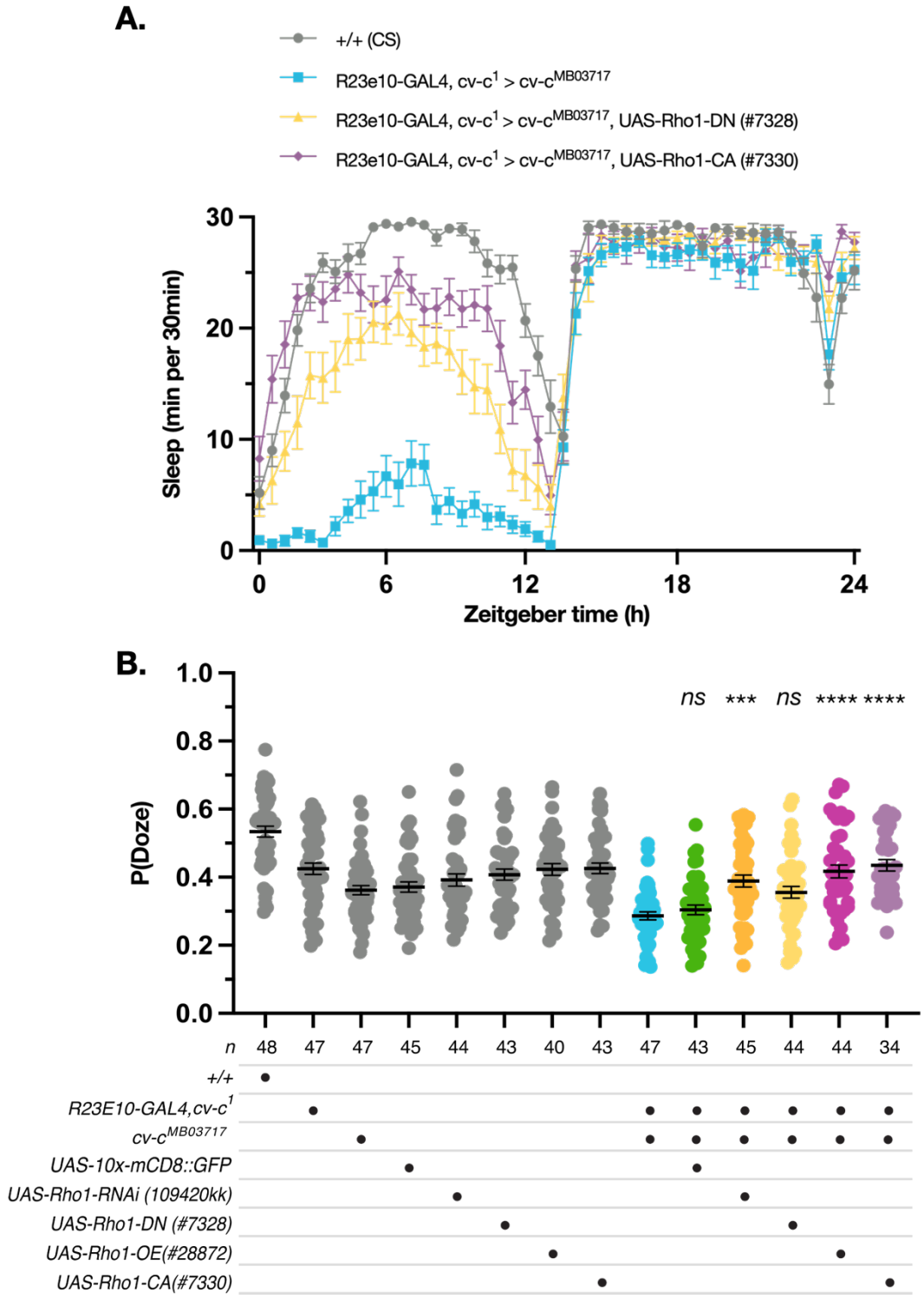


Figure 3-3: The effect of Rho1 manipulations on A) sleep architecture and B) P(Doze). A) Minutes of sleep per 30 minute bin plotted across 24 hours. Average of day 2 and day 3 of a representative experiment plotted. B) Data is presented as means ± SEM; each circle represent an individual fly. n represents the number of flies for each genotype Data was pooled from 3 independent experiments conducted on separate days. Values were averaged from day 2 and 3. ANOVA test was significant ($F=14.8$, $p<0.0001$) and asterisks denote significant differences in pairwise Tukey's multiple comparisons test compared to transheterozygous *cv-c* group (blue).

Figure 3-3A depicts that *cv-c* mutants (in this combination of $cv\text{-}c^{1}/cv\text{-}c^{MB03717}$) mainly sleep less during the day, and thus the rescue in sleep caused by introducing Rho-OE and Rho-DN is mainly observed in day sleep. Importantly, Rho1 manipulations did not affect circadian rhythmicity; all genotypes exhibited a normal wake-sleep cycle. The differences in sleep between groups were mainly attributable to the number of sleep episodes, rather than the duration of the sleep episodes (data not shown). This suggests that *cv-c* mutants that carry Rho1 manipulations are more likely to fall asleep when awake. To test this idea, the P(Doze) parameter was plotted. As shown in Figure 3-3B, the probability to "Doze" is significantly reduced in the *cv-c* mutants and the mCD8::GFP control, but is statistically rescued to the level of parental controls when Rho1 is either overexpressed, knocked down (RNAi), or mutated (CA). DN is not statistically different from *cv-c* mutants but leans towards the other Rho1 manipulations (P =0.1102, Tukey's MCT).

To validate these findings I conducted a series of further experiments. First, I tested the DN, OE and CA construct in a second *cv-c* mutant background ($cv\text{-}c^{c524}/\ cv\text{-}c^{MB03717}$). The $cv\text{-}c^{c524}/\ cv\text{-}c^{MB03717}$ background was used in initial studies by Donlea et al., (2014). The sleep profile of these *cv-c* mutants closely resembles that of $cv\text{-}c^{1}/\ cv\text{-}c^{MB0371}$, but the insomniac phenotype is slightly more pronounced. The RNAi line had already been verified in this background previously by other lab members so I did not include it. As shown in Figure 3-4, all groups (DN, OE and CA) significantly increased sleep in comparison to the transheterozygous *cv-c* group. Thus, these results are consistent across the different *cv-c* mutant backgrounds.

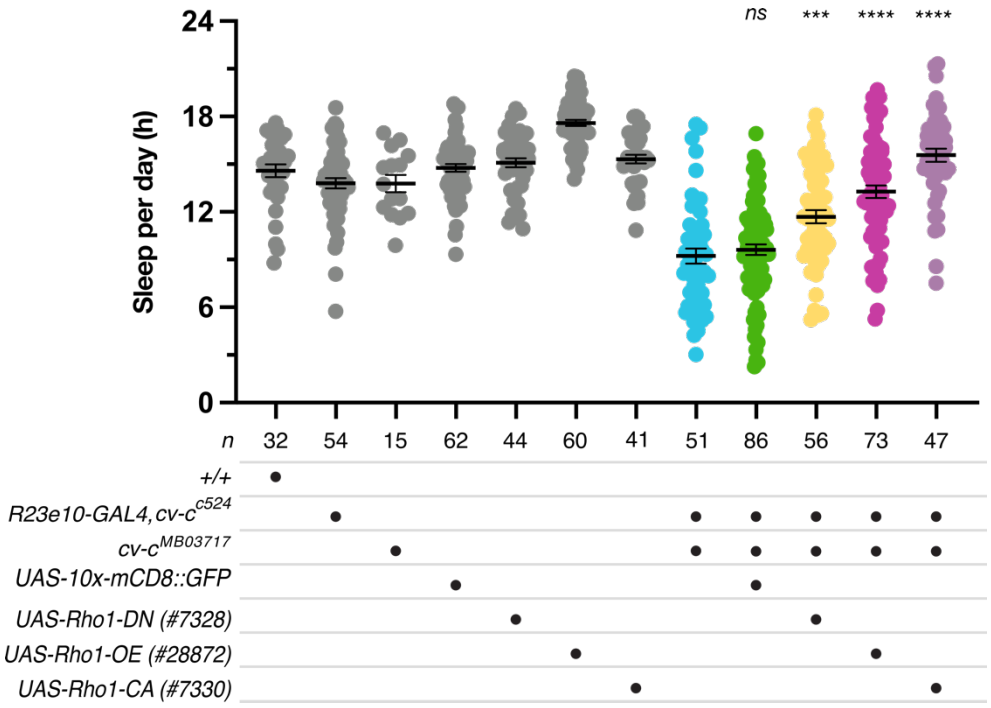


Figure 3-4: The effect of Rho1 manipulations on sleep duration in the *cv-c^{c524}/cv-c^{MB03717}* *background.* Average sleep duration in hours plotted for different genotypes. Data is presented as means ± SEM; each circle represent an individual fly. *n* represents the number of flies for each genotype. Data was pooled from 4 independent experiments conducted on separate days. Not all controls were included in all replicates, hence different *n* numbers. Values were averaged across day 2 and 3. ANOVA test detected significant differences between genotypes ($F = 49.64$, $p<0.0001$) Asterisks denote significant differences in pairwise Tukey's post-hoc comparisons test compared to transheterozygous *cv-c* group (blue).

Secondly, I looked at whether overexpressing Rho1 (which resulted in a counterintuitive finding) would also rescue the insomniac phenotype observed in flies expressing *UAS-cv-c-RNAi* in dFB neurons. This experiment was particularly necessary since the *cv-c^{MB03717}* allele carries a GAL4 insertion which can drive the expression of UAS transgenes in other brain structures, e.g. the optic lobe and the mushroom body (see the appendix for images). I wanted to be sure that the "surprising" effect I was seeing was really due to the role of Rho1 in dFB, rather than due to off target effects in other brain structures. Indeed, overexpressing Rho1 in the dFB of flies co-expressing UAS-*cv-c-RNAi* rescued the insomniac phenotype, as shown in Figure 3-5.

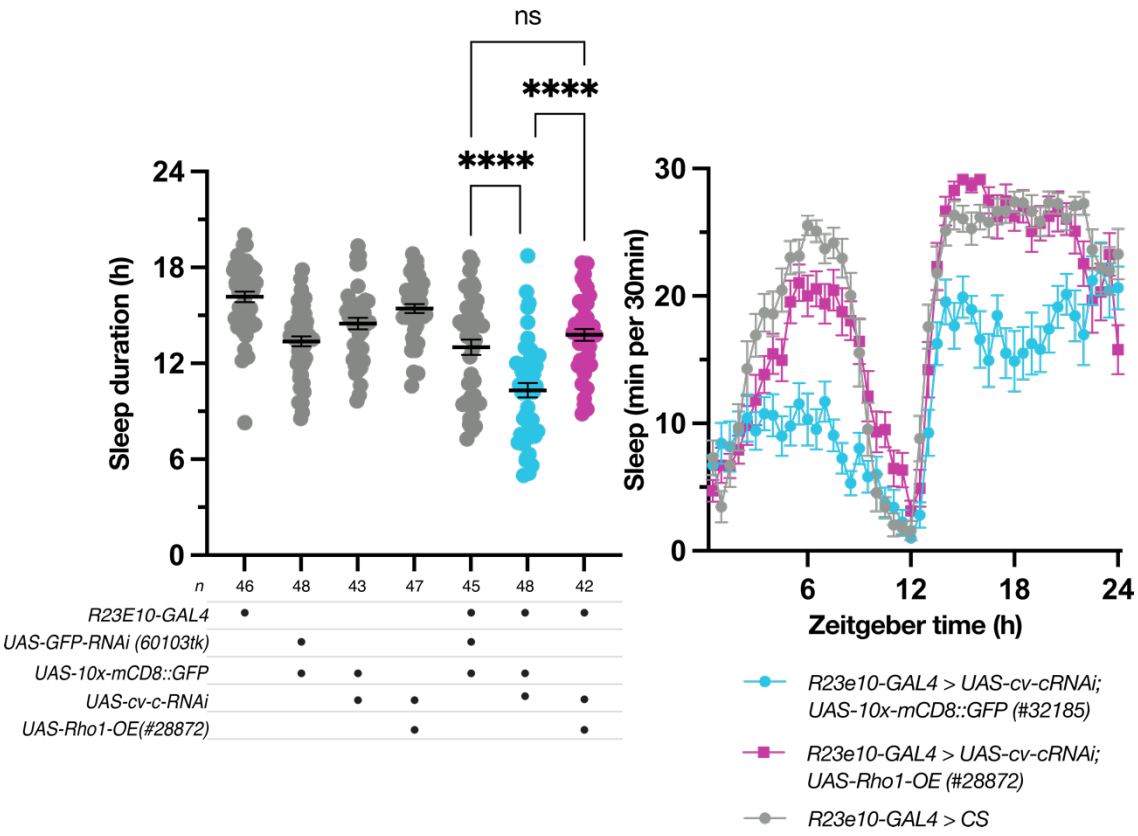


Figure 3-5: Rho1-OE rescues the insomniac phenotype observed in flies expressing *cv-c*-RNAi in dFB neurons. A) Sleep per day plotted (h). Data is presented as means ± SEM; each circle represent an individual fly. *n* represents the number of flies for each genotype. Values were averaged from day 2 and 3. ANOVA test was significant ($F=28.09$, $p<0.0001$) and asterisks denote significant differences in pairwise Tukey's multiple comparisons test. **B)** Minutes of sleep per 30 minute bin plotted across 24 hours for key groups. These experiments were loaded with the help of Anissa Kempf.

These experiments suggest the conflicting finding that increasing Rho1-GTP activity (by expressing Rho1-OE or Rho1-CA) in dFB neurons of *cv-c* mutants results in the same sleep behaviour phenotype as knocking down Rho1 (with a RNAi or DN construct). Both types of manipulations rescue the insomniac phenotype observed in *cv-c* mutants. This result is puzzling and prompts two main questions.

1. If high levels of Rho1 are responsible for the insomniac phenotype in *cv-c* mutants, then why does overexpressing Rho1 result in a sleep promoting effect?

2. How can overexpressing and knocking down Rho1 result in the same behavioural phenotype?

To answer these questions I decided to switch to an imaging approach. My aim here was to first to validate the model: Is Rho1 really upregulated in *cv-c* mutants? And secondly, to verify if Rho1 is really being upregulated and downregulated in the OE and DN groups, respectively.

3.1.2 Imaging for Rho1-GTP activity

To test whether Rho1 is upregulated in *cv-c* mutants, I used a GFP-based biosensor (PKNG58AeGFP) which binds to GTP-bound (active) Rho1. This allowed me to quantify Rho1-GTP activity in *cv-c* mutants, wildtype (WT) flies, and the DN and OE groups. I expressed the sensor in dFB neurons using the *R23e10-GAL4* line, and immobilised and dissected brains at Zeitgeber (ZT) 0-2, ZT 6-8 and ZT 10-12, as shown in Figure 3-6. I decided to quantify Rho1-GTP activity at different times of day because it was previously found that Rho1 levels change throughout the waking day in sLNV neurons (Petsakou et al., 2015).

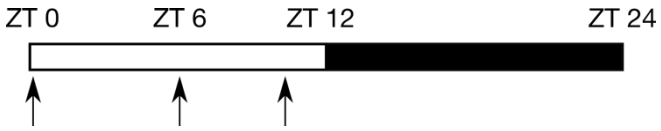


Figure 3-6: Timeline indicating timing of dissections. ZT denotes Zeitgeber time. Flies were maintained in a 12 hour light and dark cycle. Flies were immobilised on ice at ZT 0-2, ZT 6-8 and ZT 10-12. These experiments were conducted in two batches: ZT6-8 dissections were conducted on separate days from the other groups and on flies from different crosses. For each condition, at least 3 independent replicates were conducted.

I dissected the following genotypes:

$$\textbf{WT} = \frac{\textit{UAS-CD4:Tdtomato}}{\textit{UAS-PknG58A-eGFP}} \; ; \; \frac{\textit{R23e10-GAL4}}{+}$$

$$\textit{cv-c}^{\textit{MUT}} = \frac{\textit{UAS-CD4:Tdtomato}}{\textit{UAS-PknG58A-eGFP}} \; ; \; \frac{\textit{R23e10-GAL4, cv-c}^{c524}}{\textit{cv-c}^{MB03717}}$$

$$\textit{cv-c}^{\textit{MUT}} + \textbf{Rho OE} = \frac{\textit{UAS-CD4:Tdtomato}}{\textit{UAS-PknG58A-eGFP}} \; ; \; \frac{\textit{R23e10-GAL4, cv-c}^{c524}}{\textit{cv-c}^{MB03717}\textit{, UAS-Rho1-OE (\#28872)}}$$

$$\textit{cv-c}^{\textit{MUT}} + \textbf{Rho DN} = \frac{\textit{UAS-CD4:Tdtomato}}{\textit{UAS-PknG58A-eGFP}} \; ; \; \frac{\textit{R23e10-GAL4, cv-c}^{c524}}{\textit{cv-c}^{MB03717}\textit{, UAS-Rho1-DN (\#7328)}}$$

The normalised Rho1-GTP fluorescence was calculated based on the PKNG58AeGFP/CD4:tdTomato ratio as outlined in Section 2.4.4 in order to control for any variability between stainings and dissections. The axonal branch was selected as the region of interest for quantification because the PKNG58AeGFP signal was the strongest there. The DN group was only included for the ZT 6-8 dissections due to time constraints. The results are shown in Figure 3-7.

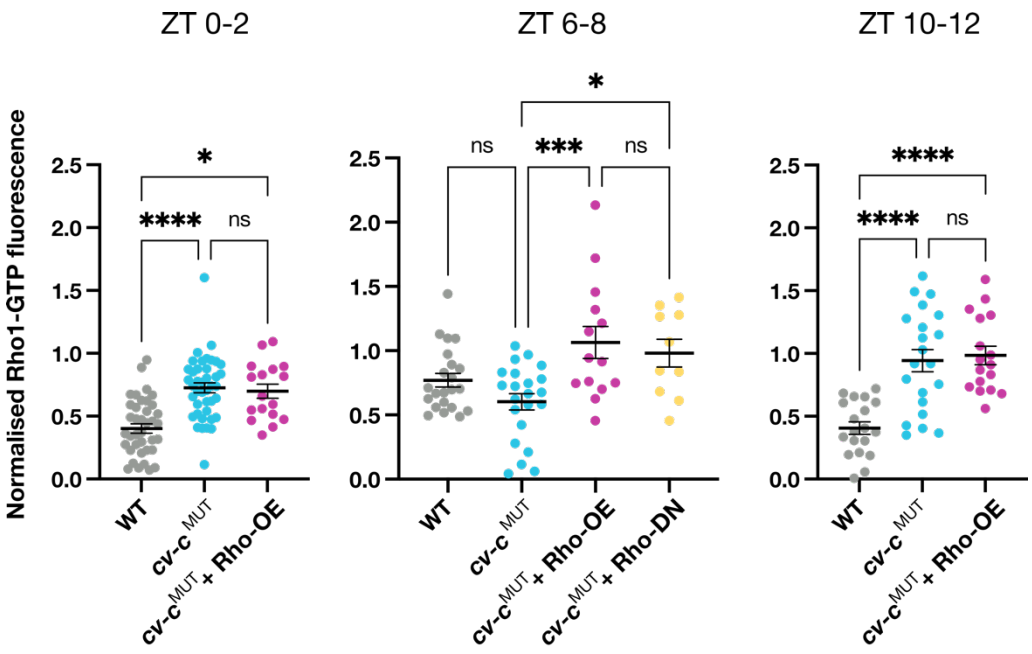


Figure 3-7: Rho1-GTP fluorescence (PKNG58AeGFP/CD4:tdTomato) in dFB axons of WT, *cv-c* mutants and *cv-c* mutants + Rho OE flies at ZT0-2, ZT6-8 and ZT10-12. Data is presented as means ± SEM; each circle represent an individual fly brain. One way ANOVA for all groups was significant ($F = 12.8$, $p<0.0001$). Asterisks denote statistical differences in Tukey's multiple comparisons test. Data is pooled from at least 3 independent replicates for each time point. ZT 6-8 dissections were conducted on separate days to ZT 0-2 and ZT 0-12 dissections and on flies from different crosses. *n* in order of appearance: ZT0-2 (39, 41, 17), ZT6-8 (22, 22, 14, 10), ZT 10-12 (19, 21, 17)

The results suggest that Rho1-GTP activity is significantly higher in *cv-c* mutants than in WT flies at ZT 0-2 and ZT 10-12 ($P<0.0001$, Tukey's MCT) but not at ZT 6-8 ($P=0.76$, Tukey's MCT). Notably, the Rho1-GTP activity in the WT group is much higher at ZT 6-8 than at other timepoints. Considering that the flies dissected at ZT 6-8 were from different crosses, and dissected on different days to the other groups, this could be due to an experimental artefact. However, it is also plausible that there is an upregulation of Rho1-GTP activity in WT dFB neurons, which peaks at midday and drops before sleep onset.

Rho1-GTP activity in the *cv-c*^{MUT} + Rho-OE group is not statistically different to Rho1-GTP activity in *cv-c* mutants at ZT 0-2 and ZT 10-12. This suggests that overexpressing Rho1 in dFB neurons of *cv-c* mutants does not further increase Rho1 levels at these time points. Intriguingly, Rho1-GTP activity in *cv-c*^{MUT} + Rho-DN at ZT6-8 is the same as *cv-c*^{MUT} + Rho-OE. This suggests that introducing dominant negative Rho1 leads to internal compensation that upregulates Rho1-GTP levels. This may explain the conflicting behavioural results, in which both Rho1-OE and Rho1-DN result in an increase in sleep. However, only a few flies of the Rho1-DN genotype were dissected, and only at ZT 6-8, so these findings should be considered with caution.

While GFP levels differed significantly between genotypes and time points ($P< 0.0001$, One way ANOVA), the tdTomato fluorescence did not change significantly between any genotypes or time points (All groups were NS in Tukey's MCT, One way ANOVA was $P= 0.0326$). This confirms that the differences we see are really due to changes in Rho1 activity. This is further highlighted in the representative images shown in Figure 3-8.

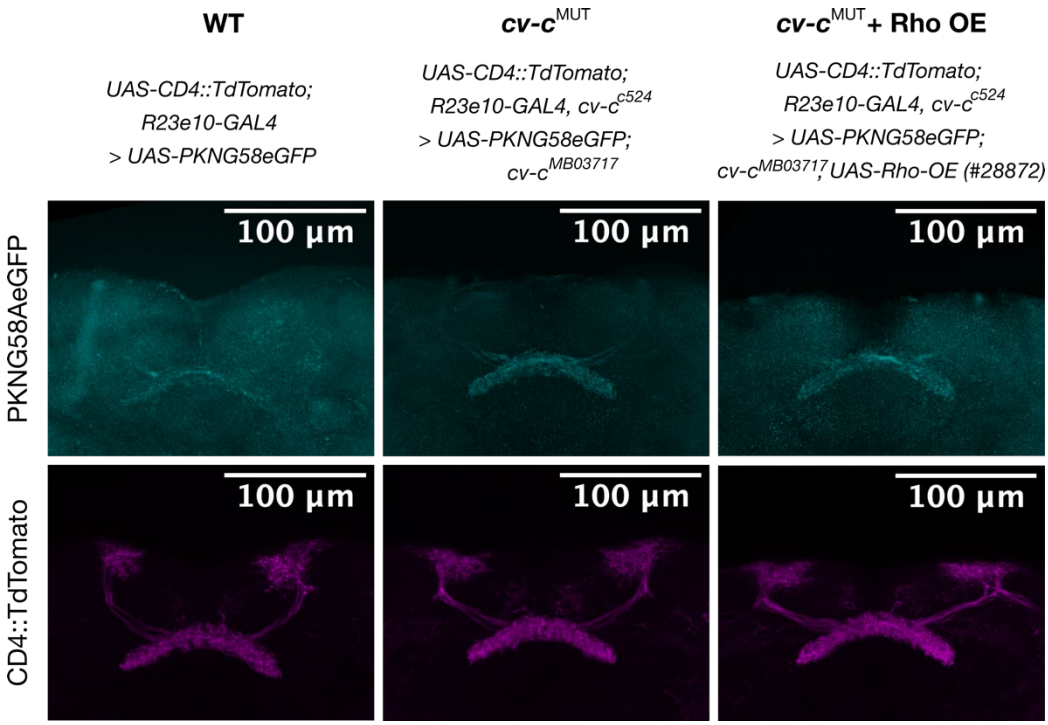


Figure 3-8: Maximum projection z stack of representative dFB projections of WT, *cv*-c^{MUT} and *cv*-*c*^{MUT} + Rho OE groups at ZT 0. All images are from the same replicate.

To ensure that introducing the *UAS-PKNG58AeGFP* and *UAS-CD4:tdTomato* transgenes do not alter the sleep phenotypes, I analysed the sleep of all genotypes prior to dissection. The data is shown in Figure 3-9.

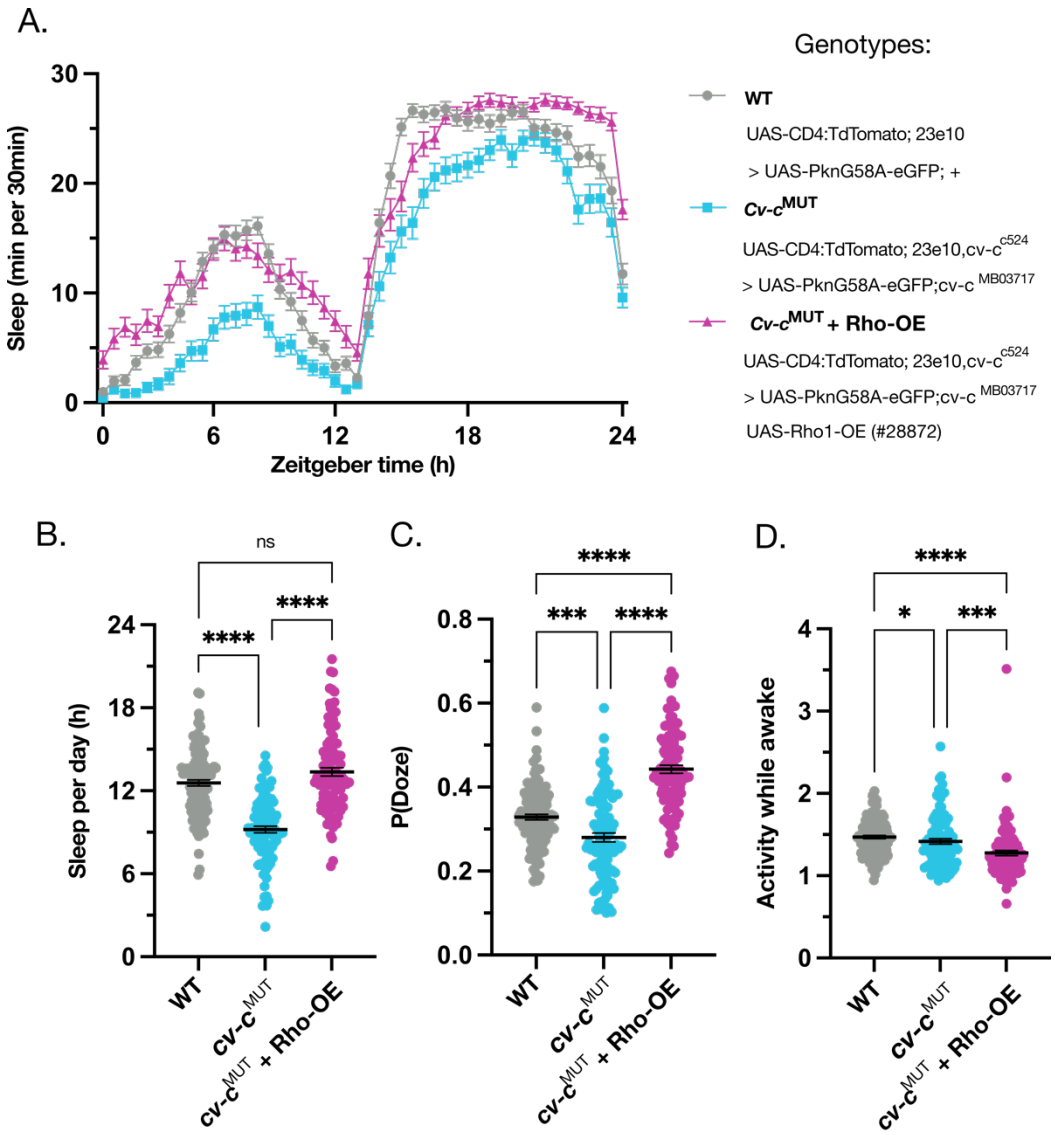


Figure 3-9: Behaviour of flies used for imaging experiments. Genotypes for all graphs are denoted on top right. Data for all plots was pooled from the same 3 experiments, conducted on separate days. One way ANOVA test was used in B ($F = 72.9$,$p<0.0001$) and C ($F = 84.5$, $p<0.0001$) and a Kruskal-Wallis test was used in D ($X^2 = 39.6$, $p<0.0001$). Asterisks denote significant differences in pairwise Tukey's post-hoc comparisons test. Number of flies for each genotype: WT = 116, *cv-c*^{MUT} = 102, *cv-c*^{MUT} + Rho OE = 102. **A)** Sleep profile: Minutes of sleep per 30 minute bin plotted across 24 hours. **B)** Hours of sleep per day (mean ± SEM; each circle represent an individual fly). **C)** Probability that fly will stop moving (mean ± SEM; each circle represent an individual fly). **D)** Beam brakes per waking minute (mean ± SEM; each circle represent an individual fly).

In the presence of *UAS-PKNG58AeGFP* and *UAS-CD4:tdTomato*, *cv-c* mutants still sleep significantly less than both WT and *cv-c*^{MUT} + Rho-OE groups. However, it is important to note that the behavioural phenotypes are slightly attenuated: *cv-c* mutants sleep around 3 hours less than WT rather than 5-8 hours less. In addition, there was significant variability between experiments: the insomniac phenotype in some experiments was significantly more pronounced than in others. Nonetheless, the pooled percentage reduction in sleep in *cv-c* mutants compared to WT is around 30%, which is consistent with previous behaviour experiments. The probability to doze was similar to previous behavioural experiments, with *cv-c* mutants exhibiting a lower probability to stop moving than both WT and *cv-c*^{MUT} + Rho-OE. Activity while awake was slightly lower in the *cv-c* mutants, and more significantly lower in *cv-c*^{MUT} + Rho-OE. This suggests that the additional transgenes lead to slight defects in locomotor activity. Despite this, the behaviour of dissected flies closely resembles the behaviour of my initial experiments.

Is Rho1 activity associated with changes in sleep pressure?

To better understand the role of Rho1 in homeostatic sleep regulation I decided to investigate how Rho1 is associated with sleep pressure. *cv-c* mutants are known to be in a state of high sleep pressure (they show cognitive deficits) however, they cannot discharge this sleep pressure into changes in membrane excitability of dFB neurons (and therefore sleep), as suggested by their electrically silent dFB neurons (Donlea et al., 2014). Therefore, high Rho1 levels (as seen in *cv-c* mutants) might reflect sleep need.

To investigate if Rho1-GTP levels are associated with an increase in sleep drive, I quantified Rho1 levels (using the PKNG58AeGFP sensor) of flies that had been mechanically sleep deprived for 12 hours during the night vs rested flies, as shown in Figure 3-10 and Figure 3-11. I did this for three genotypes: WT, *cv-c*^{MUT} and *cv-c*^{MUT} + Rho-OE. The DN group was not included due to time constraints.

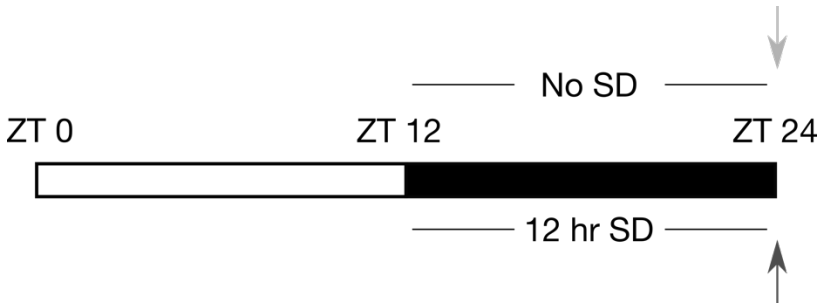


Figure 3-10: Timeline of dissections in sleep deprived (SD) vs rested flies. Flies were loaded on the same day, but one group was exposed to 12 hours of sleep deprivation in SNAP, while the other group was allowed to rest. Both groups were dissected at 8am (ZT 0-2) the following morning.

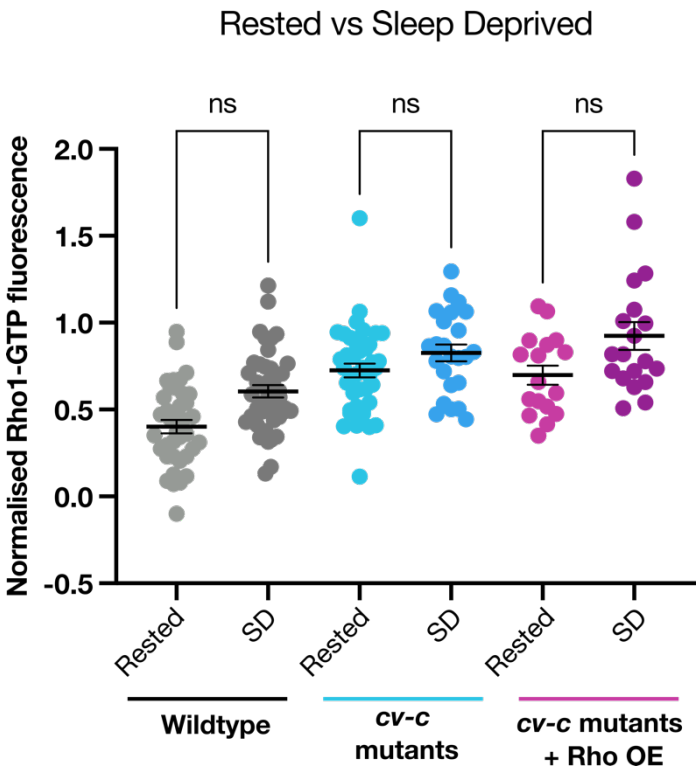


Figure 3-11: Rho1-GTP fluorescence (PKNG58AeGFP/CD4:tdTomato) in dFB axons of rested and sleep deprived (SD) WT, *cv-c* mutant and *cv-c* mutant + Rho OE flies. Data is pooled from at least 3 independent replicates and is presented as means ± SEM; each circle represent an individual fly brain. One way ANOVA for all groups was significant ($F = 12.8$, $p<0.0001$). NS denotes no statistical differences in Tukey's multiple comparisons test. *n* = WT R (39), WT SD (43), Cv-c R (41), Cv-c SD (24), *cv-c*^{MUT} + Rho OE R (17), *cv-c*^{MUT} + Rho OE SD (19).

Figure 3-11 depicts that there were no significant differences in Rho1-GTP fluorescence between the rested and sleep deprived groups of any of the genotypes (Tukey's MCT). However, there is a tendency for Rho1-GTP activity to be elevated after sleep deprivation in all groups. This is especially pronounced in the WT group. When the normalised Rho1-GTP fluorescence values of rested WT (mean = 0.4) and sleep deprived WT (mean = 0.6) were compared using an unpaired t-test, a statistically significant difference was detected ($t(80) = 3.934$, $P = 0.0002$). Looking at individual replicates, I observed that this upregulation in Rho1-GTP activity after sleep deprivation was significant in 3 out of 6 replicates, but not significant in the other 3 replicates. The efficiency of sleep deprivation was the same across all 6 replicates. It is therefore difficult to conclude to what extent the

changes in Rho1 activity are really due to changes in sleep pressure. It is also worth noting that the difference in the mean Rho1-GTP activity between rested and sleep deprived flies is more pronounced in WT and *cv-c*^{MUT} + Rho OE groups (a difference of 0.2 and 0.22 respectively), than in *cv-c* mutants (a difference of 0.1). This may suggest that the upregulation of Rho1 after sleep deprivation is attenuated in *cv-c* mutants, and this attenuation may lead to a lack of sleep rebound found in *cv-c* mutants.

In addition, I analysed the sleep rebound of these three genotypes after sleep deprivation (Figure 3-12). The sleep profile in Figure 3-12B suggests that *cv-c* mutants rebound less after sleep deprivation than WT and *cv-c*^{MUT} + Rho OE flies. However, the differences in rebound sleep during recovery day are not statistically significant (Tukey's MCT), as suggested in Figure 3-12A. Rebound was only measured in a small proportion of flies, and it is likely that differences in rebound would become significant with an increase in *n* numbers.

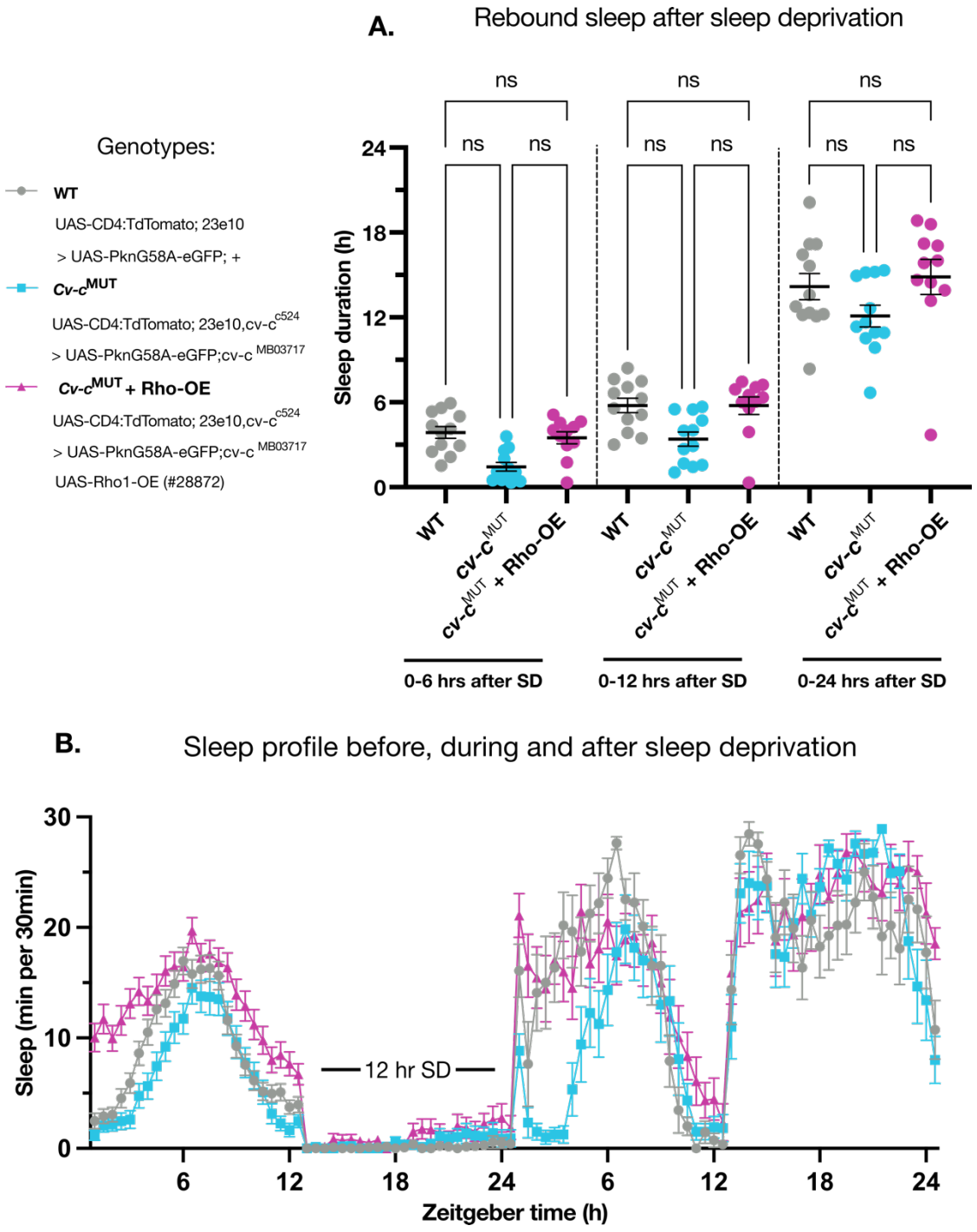


Figure 3-12: Sleep behaviour after sleep deprivation in flies. A) Sleep duration quantified for the first 6 hours, first 12 hours and 24 hours after sleep deprivation in all genotypes. *n* = WT (12), *cv-c* ^{MUT}(12), *cv-c*^{MUT} + Rho OE (11). One way ANOVA for all groups was significant (F = 72.6, p<0.0001,). NS denotes no statistical differences in Tukey's multiple comparisons test. **B)** Sleep profile of flies before, during and after sleep deprivation. Minutes of sleep per 30 minute bin plotted. First 24 hours pooled from two experiments (*n* = 96 for all groups). Second 24 hours is the sleep profile of leftover flies from both experiments (same *n* as Figure A).

Overall, the imaging results provide answers to the previous two questions posited in Section 3.1.1:

1. Rho1 is higher in *cv-c* mutants than in WT at most time points of the day. This evidence supports our initial hypothesis that Rho1 is the substrate of Cv-c.

2. Rho-OE and Rho-DN both lead to high levels of Rho1 activity in *cv-c* mutants at ZT 6-8. This might explain why introducing Rho-OE and Rho-DN in *cv-c* mutants result in the same behavioural phenotype.

In addition, I found that Rho1-GTP activity has a tendency to increase after sleep deprivation in WT flies, which suggests that Rho1-GTP activity might be correlated with high sleep pressure.

3.1.3 The effect of Rho1 on dFB axonal plasticity

Rho1 is known to play an important role in regulating the actin cytoskeleton. This has implications for the development of dendrites and axons. Rho1 has been found to promote axonal retraction (Gonzalez-Billault et al., 2012) and to decrease dendritic extensions (Li et al., 2000). In addition, disrupting the cycling of Rho1 between its active (GTP) and inactive (GDP-bound) state by expressing either CA or DN mutations led to failed axon outgrowth (Luo, 2000). Therefore, it is plausible that overexpressing or knocking down Rho1 in *cv-c* mutants leads to changes in the structure of axons and this in turn influences dFB excitability or affects downstream signalling. In addition, although mutating *cv-c* was found to have no effect on dFB morphology (Donlea et al., 2014), the axonal volume was never quantified and statistically compared to WT. Therefore, I decided to quantify the 3D spread and volume of dFB axonal projections of the different genotypes. I decided to focus on axonal structure, rather than dendritic structure, because dendritic branches, by eye, looked consistent across genotypes, whereas there was quite some variability in axonal spread. In addition, the Rho1 biosensor was expressed most strongly in the axons, suggesting that Rho1 is most active there. To quantify the 3D spread and volume of dFB axons a script developed by Justin Blau was used.

Figure 3-13 depicts the 3D spread and volume of CD4:tdTomato labelled axonal projections of dFB neurons of WT, *cv-c*^{MUT} and *cv-c*^{MUT} + Rho OE at ZT 0-2.

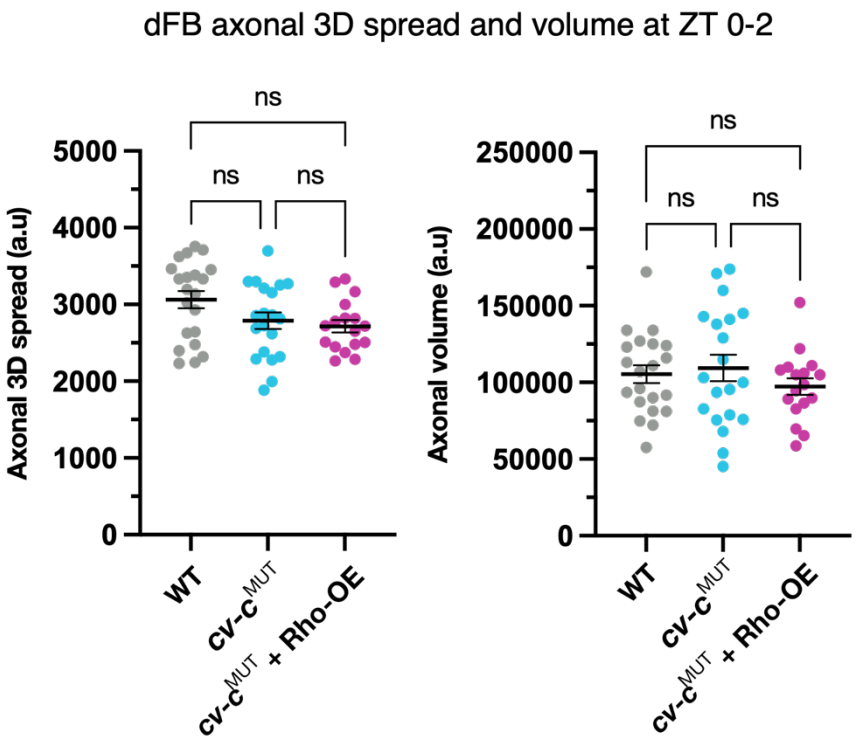


Figure 3-13: Quantification of dFB axonal structure. A) 3D spread: x* y* z spread of pixel values. One-way ANOVA was not significant (F=3.114, P = 0.052). **B)** Axonal volume: total number of pixel values. One-way ANOVA was not significant (P=0.479, F= 0.745). *n* for both plots: WT (21), *cv-c*^{MUT} (20), *cv-c*^{MUT} + Rho OE (17). Data is presented as means ± SEM; each circle represent an individual fly brain. Values were pooled from 3 experiments.

One-way ANOVA analysis revealed that there were no statistically significant differences between the three genotypes in 3D spread (P = 0.052) or axonal volume (P = 0.479) of dFB neurons. However, it is important to note that the P value of the 3D spread is close to the cut-off point of 0.05, and it appears that WT dFB neurons have a slightly larger 3D spread than the other groups. Nonetheless, it seems that there are no obvious differences in axonal morphology between the different genotypes.

A second point of investigation was to look at whether dFB neurons change their axonal spread or volume throughout the waking day. This is of significant interest because Petsakou et al. (2015) found that axonal projections of sLNV neurons were spread out in the morning, and contracted in the evening, and that these changes in axonal plasticity were mediated by Rho1, with low Rho1 levels detected in the morning and high Rho1 levels detected in the evening. Thus, I decided to compare the 3D spread and volume of WT dFB neuron axonal projections at ZT 0-2 to ZT 10-12.

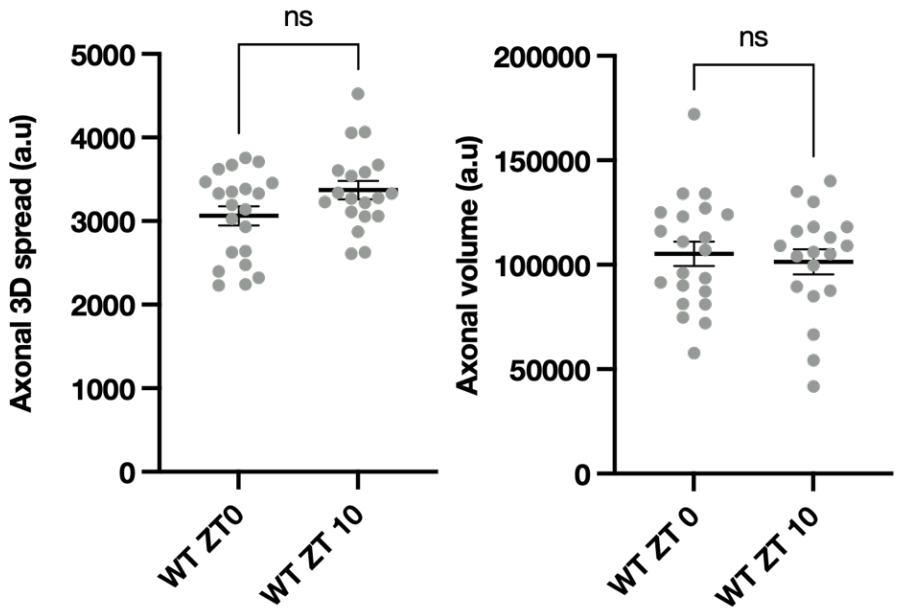


Figure 3-14: Axonal 3D spread and volume of wildtype dFB neurons at ZT0 and ZT10. Unpaired T-test was conducted to analyse the different time points. 3D spread t-test detected no significant differences ($t(38) = 1.950$, $P = 0.059$). Axonal volume t-test detected no significant differences ($t(38) = 0.464$, $P = 0.645$). Ns for both plots: WT ZT 0 (21), WT ZT 10 (19). Data is presented as means ± SEM; each circle represent an individual fly brain. Values were pooled from 3 experiments.

The dFB axons are 10% more spread out (three-dimensionally) at ZT 10 than at ZT 0 however no statistically significant differences were detected ($t(38) = 1.950$, $P = 0.059$, T-test). No statistical differences were detected in axonal volume ($t(38) = 0.464$, $P = 0.645$) of dFB neurons. This suggests that dFB axons do not spread out or contract throughout the waking day.

Since structural plastic changes were found to correlate with sleep drive in ellipsoid neurons (Liu et al., 2016), I asked whether there are structural plastic changes in the axonal projections of dFB neurons that correlate with sleep pressure. I therefore quantified the axonal volume and 3D spread of dFB neurons of sleep deprived vs rested flies of all genotypes as shown in Figure 3-15. The MCT revealed no significant difference in the 3D spread of axonal volume of rested and sleep deprived flies of all genotypes. This suggests that the axonal structure in dFB neurons is not affected by sleep pressure.

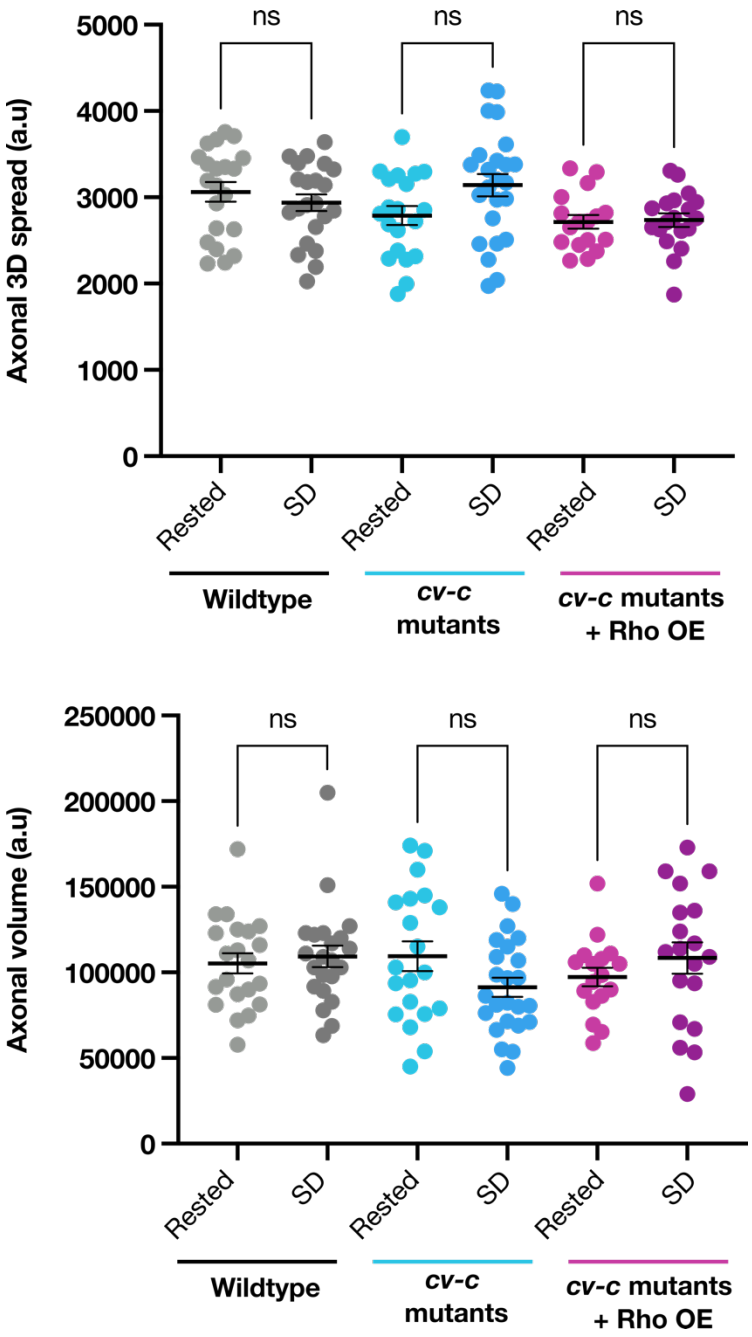


Figure 3-15: Axonal 3D spread and volume in rested vs sleep deprived (SD) WT, *cv-c* mutant and *cv-c* mutant + Rho OE flies. One way ANOVA for 3D spread was significant (F = 2.834, P = 0.019) but no significant differences were detected in Tukey's Multiple Comparison Test. One way ANOVA for axonal volume was not significant (F = 1.256, P = 0.288,). Data is presented as means ± SEM; each circle represent an individual fly brain. Values were pooled from 3 experiments. N for all groups was between 17-24.

Overall, it seems like Rho1 in dFB neurons does not play a critical role in regulating axonal structure. To confirm this I plotted the fluorescence values and their respective 3D spread and axonal volume values for all groups in order to determine if there is a correlation between Rho1-GTP activity and axonal spread or volume. This is shown in Figure 3-16.

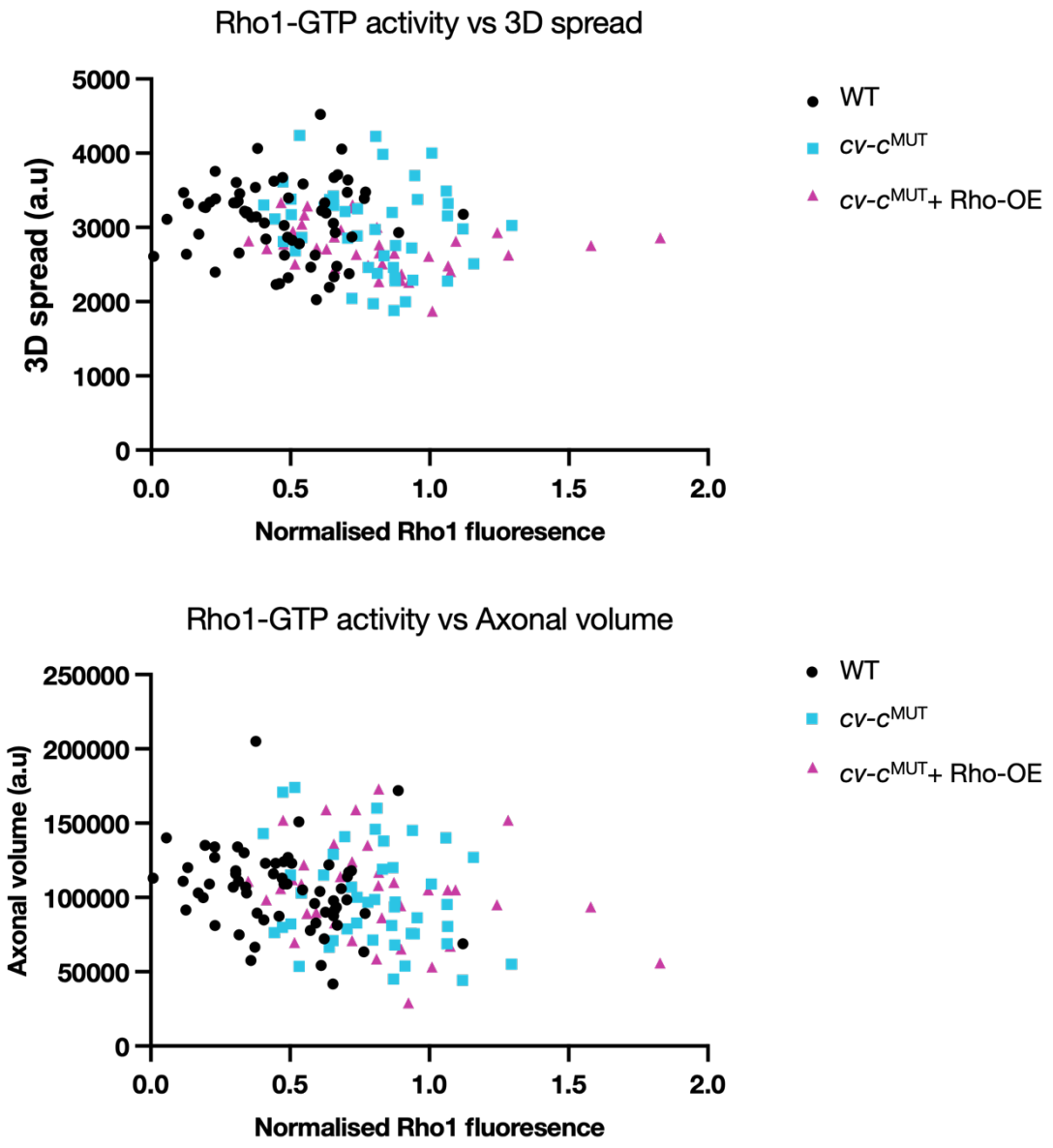


Figure 3-16: Rho1-GTP activity (PKNG58AeGFP/CD4:tdTomato ratio) does not correlate with 3D spread or with axonal volume in any genotypes. Correlation coefficient for 3D spread R = -0.157. Correlation coefficient for Axonal volume R = -0.251. All genotypes were pooled for R coefficient calculation. Values were pooled from 3 independent experiments.

The respective correlation coefficients of 3D spread (R = -0.157) and axonal volume (R = -0.251) compared to normalised Rho1 fluorescence (of all genotypes) suggest that Rho1-GTP activity is not significantly correlated with any changes in axonal structure. In addition, when the correlation coefficients of the different genotypes were calculated independently, the coefficients were very similar, suggesting that no manipulations affect the relationship between Rho1 and its role in regulating the axonal structure of dFB neurons.

Overall, I can conclude that mutating *cv-c* or overexpressing Rho1 in *cv-c* mutants has no obvious effect on axonal structure. The effect of knocking down Rho1 in *cv-c* mutants on dFB axonal structure was not quantified, but based on the correlation coefficient calculated in Figure 3-16, and observation by eye, it is unlikely that *cv-c*^{MUT} + Rho-DN would have any differences in axonal structure. In addition, unlike in sLNV neurons, dFB neurons do not contract and spread out their axonal projections throughout the waking day. Furthermore, no plastic changes in dFB axons were observed after sleep deprivation. This suggests that any changes in dFB membrane excitability are not mediated by changes in axonal structure or plasticity. It is important to consider that this does not rule out the possibility that Rho1 might affect dFB dendrites or soma. However, by eye, there were no obvious differences in dFB dendritic or somatic morphology.

3.1.4 Linking Rho1 activity to dFB excitability

To understand how changes in Rho1-GTP results in differences in sleep behaviour I performed electrophysiology experiments in collaboration with Anissa Kempf. This enabled us to identify how Rho1-GTP levels correlate with dFB membrane excitability.

Current clamp experiments from Donlea et al (2014) found that the membrane time constant and input resistance, two measures of membrane excitability, of *cv-c* mutant dFB neurons were significantly lower than those of control dFB neurons. In addition, voltage clamp experiments (*unpublished data)* found that *cv-c* mutant dFB neurons have higher levels of leak (non-A-type) currents than controls. This suggests that the dFB neurons of *cv-c* mutants are locked in an electrically-silent, high conductance state.

Since *cv-c* mutants have higher levels of Rho1-GTP than WT, I asked whether Rho1-GTP directly affects dFB excitability. I hypothesised that if high Rho1 in *cv-c* mutants is causing the insomniac phenotype, then knocking down Rho1 should rescue dFB excitability back to WT levels. Anissa Kempf conducted a series of current and voltage clamp experiments on dFB neurons of control flies (grey), *cv-c* mutant flies (blue) and *cv-c* mutant + Rho1 RNAi flies (orange). The experimental setup of these electrophysiology experiments is the same as in Kempf et al., (2019) and Pimentel et al., (2016). The membrane time constant, input resistance and the non-A-type currents in response to voltage clamp steps are shown in Figure 3-17.

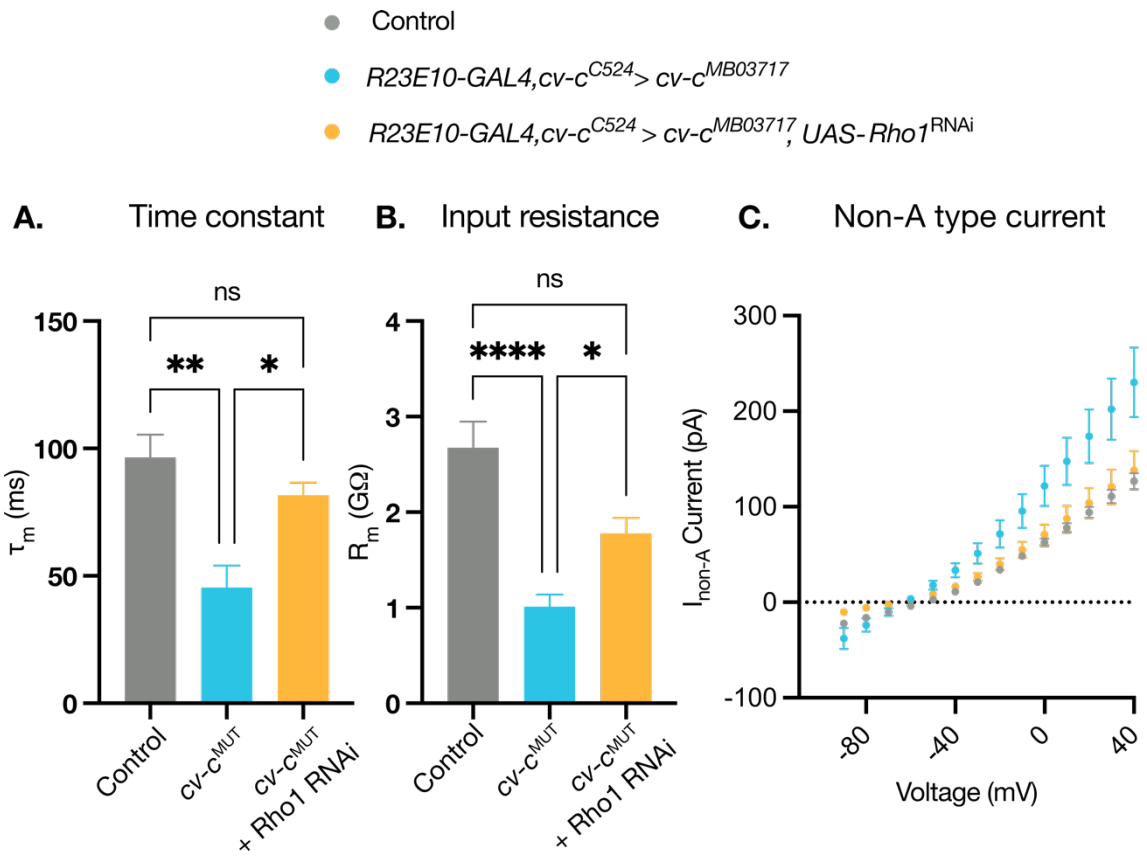


Figure 3-17: Membrane Time Constant (T_m), Input resistance (R_m) and level of non-A type currents of *cv-c* mutant dFB neurons is rescued back to control levels when Rho1 RNAi is expressed in dFB neurons. Mean value plotted with SEM as error bars. Kruskal Wallis test for A ($X^2 = 12.74$, $P = 0.0017$) and B ($X^2 = 18.07$, $P < 0.0001$) were both significant. Asterisks denote significant differences in pairwise Dunn's multiple comparisons test. For C, Two way ANOVA found a significant interaction between genotype and voltage ($P<0.0047$). Flies were prepared by Celina Hartmann. Experiments and analysis was perfomed by Anissa Kempf.

The current clamp experiments (A and B) show that the T_m and R_m values of *cv-c* mutant dFB neurons are rescued back to control levels when Rho1 RNAi is expressed in dFB neurons. No significant difference was detected between the control and the *cv-c*^{MUT} + Rho1 RNAi group in both excitability parameters (Dunn's MCT). Since a high time constant and high input resistance reflect a more excitable neuron (Donlea et al., 2014), these results indicate that knocking down Rho1 in *cv-c* mutants is critical for dFB neurons to become excitable.

The voltage clamp experiment (Figure 3-17C) found that the level of non-A-type currents in the *cv-c*^{MUT} + Rho1 RNAi group closely resembles the control. Therefore, knocking down Rho1 in dFB neurons of *cv-c* mutants lowers the amount of currents flowing through Sandman. This prompts the idea that Rho1 levels dictate the amount of Sandman currents in dFB neurons and that Rho1 might be regulating the endocytosis of Sandman. Overall, these experiments highlight a clear link between the level of Rho1-GTP and dFB excitability. This strongly suggests that Rho1 plays an important role in regulating the dFB OFF- ON switch, and thus the wake- sleep switch.

3.1.5 Optogenetic activation of Rho1

With differences in the level of Rho1-GTP in *cv-c* mutants directly correlating with changes in dFB excitability, I wanted to investigate whether manipulating Rho1 in the dFB neurons of wildtype flies has an effect on sleep. Although mutating *cv-c* or overexpressing Rho1 in *cv-c* mutants had no effect on axonal structure, it does not rule out the possibility that developmental effects are contributing to the sleep phenotypes. I therefore restricted Rho1 manipulation to adulthood.

Using a LOV-based optogenetic probe developed by Rich et al. (2020), which activates Rho1 through the blue light dependent recruitment of the PR-Rho-GEF LARG, I investigated how the acute activation of Rho1 in adulthood affects sleep duration in otherwise WT flies. The PR-RhoGEF was acutely activated with blue light for 24 hours, as shown in the protocol (Figure 3-18). Sleep during baseline, light activation and recovery day is plotted in Figure 3-19. The sleep profile during light activation, and the percentage difference of sleep during light activation vs baseline is plotted in Figure 3-20.

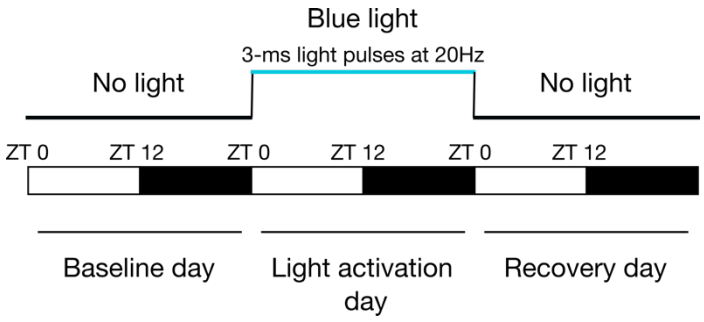


Figure 3-18: Optogenetic protocol

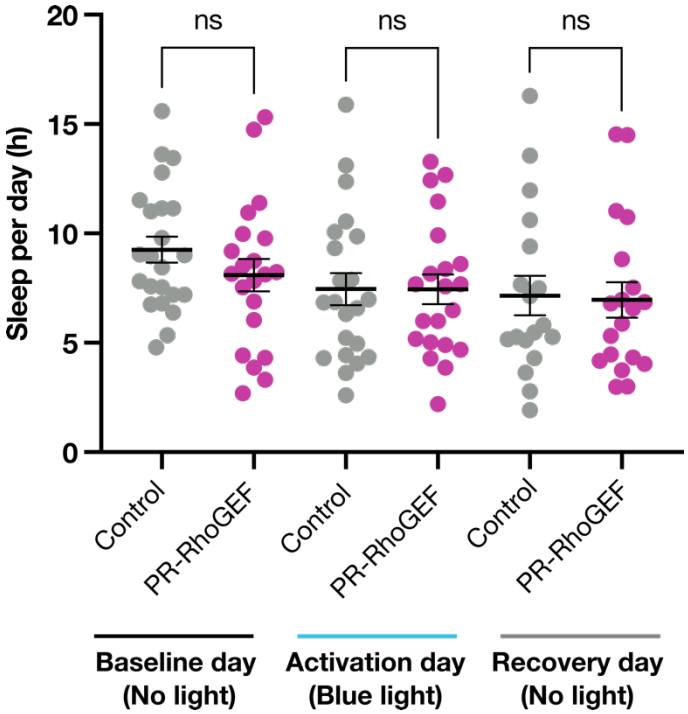


Figure 3-19: Sleep duration (h) during baseline, light activation and recovery day in control (*Ubi-SspB-mScar; R23e10-GAL4 > UAS-LOV-SsrA*) and PR-Rho-GEF (*Ubi-SspB-LARG(DH); R23e10-GAL4 > UAS-LOV-SsrA*) flies. ANOVA was not significant (P = 0.241, F =1.369). Pairwise comparisons in Tukey's multiple comparisons test were not significant, as shown in the graph. *n* = 23, 21, 22, 21, 18, 19 in order of appearance (left to right). Data is presented as means ± SEM; each circle represent an individual fly. Values were pooled from 5 independent experiments.

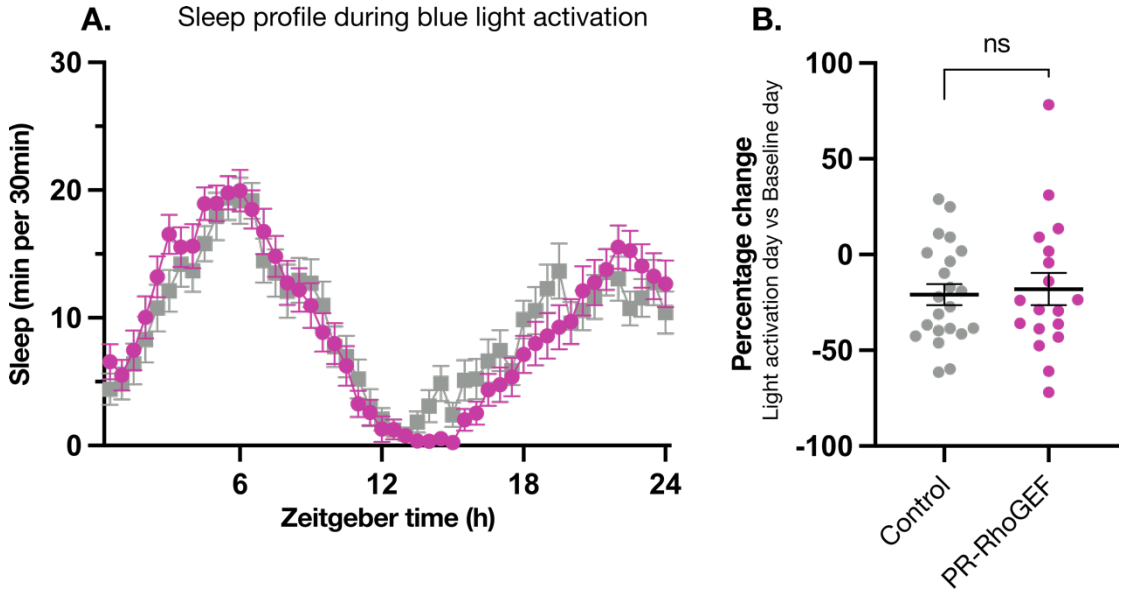


Figure 3-20: A) Sleep profile during blue light activation. Minutes of sleep per 30 minute bin plotted. **B) Percentage difference: (sleep during blue light activation – sleep during baseline day) / baseline day.** Unpaired t-test between control and PR-RhoGEF group was not significant (t(38) = 0.2893, P = 0.774). N = Control (22), PR-RhoGEF (18). Values were pooled from 5 independent experiments.

No significant differences between the control and PR-Rho-GEF group were detected during baseline, activation or recovery day ($F = 1.369$, $P = 0.241$, One way ANOVA). In addition, the percentage difference in sleep (baseline vs light activation day) was not significantly different between the control and PR-RhoGEF ($t(38) = 0.289$, $P = 0.774$). Thus, photoactivating Rho1 using a PR-RhoGEF does not result in any changes in sleep duration. In addition, no changes in circadian rhythmicity were observed (Figure 3-20). It is important to note that for these experiments, only one copy of the GEF domain was present, whereas in Rich et al. (2020) homozygotes were used. Thus, it may be worthwhile to repeat these experiments using homozygotes.

3.1.6 Summary

This section investigated the role of Rho1 in regulating sleep. This involved manipulating Rho1 in *cv-c* mutants, imaging for Rho1-GTP activity, current and voltage clamp experiments (conducted by Anissa Kempf), quantification of dFB axonal structure and optogenetic activation of Rho1 in otherwise wildtype flies.

The key findings that emerged were:

1. Overexpressing and knocking down Rho1 in *cv-c* mutants rescues the insomniac phenotype
2. In dFB axons, Rho1-GTP is higher in *cv-c* mutants than in wildtype flies
3. Rho1-GTP is upregulated after sleep deprivation and at ZT 6-8 in wildtype flies
4. Rho1-GTP activity does not correlate with 3D spread or volume of dFB neuron axonal projections
5. Knocking down Rho1 in *cv-c* mutants increases the excitability of dFB neurons back to wildtype levels
6. Optogenetic activation of Rho1 using PR-Rho-GEF (LARG) has no significant effect on sleep

These results suggest a vexingly complex role of Rho1-GTP in dFB neurons. One reason why deciphering the role of Rho1 in regulating sleep might produce conflicting results is that small GTPases do not act in isolation: there is a significant amount of crosstalk between small GTPases. Therefore, I sought to investigate if Rho1 interacts with Rac1 and whether Rac1 itself has a sleep regulatory role.

3.2 The role of Rac1 in regulating sleep

A well-studied small GTPase interaction is the antagonistic relationship between Rho1 and Rac1. Rho1 and Rac1 are known to act antagonistically in axon pathfinding, neurogenesis, cell migration and more, as outlined in Section 1.4. With insomniac *cv-c* mutants implicating a role for Rho1 in regulating sleep, I asked whether Rac1 plays an antagonistic role in dFB neurons.

I approached this question using a three-pronged approach. Firstly (3.2.1), I investigated whether manipulations of Rac1 in *cv-c* mutants would rescue the sleep phenotype, thus counteracting the high levels of Rho1. Secondly (3.2.2), I investigated what happens when we simultaneously overexpress and knockdown Rho1 and Rac1 in WT flies and vice versa. Thirdly (3.2.3), I used an optogenetic probe to activate and inhibit Rac1 with high spatio-temporal precision in dFB neurons of WT flies.

3.2.1 Manipulating Rac1 in *cv-c* mutants

If Rho1 and Rac1 antagonistically regulate each other in dFB neurons then we would expect Rac1 to be downregulated when Rho1 is upregulated. Thus, in *cv-c* mutants, where Rho1 is upregulated, we would expect Rac1 to be downregulated. This suggests that overexpressing Rac1 in *cv-c* mutants should rescue the insomniac phenotype, either indirectly (by downregulating Rho1), or directly (by regulating ion channels involved in modulating the membrane excitability of dFB neurons).

To test this hypothesis I introduced four UAS constructs into the $cv\text{-}c^{1}/cv\text{-}c^{MB03717}$ background: one DN construct (BDSC #6292), two WT overexpression constructs (BDSC #6293 and #28874) and one CA construct of Rac1 (BDSC #6291), and expressed them in dFB neurons using the *R23e10-GAL4* driver. The two overexpression constructs both express WT Rac1 under UAS control but the constructs were generated by different labs and the mutations are located on different chromosomes. *UAS-mCD8::GFP* was used as an additional UAS control. Average sleep duration in hours and the average activity levels while awake were quantified and plotted in Figure 3-21. Parental controls are shown in grey. Asterisks in all graphs represent statistical post-hoc comparison of experimental vs. the transheterozygous *cv-c* group (blue), unless otherwise denoted.

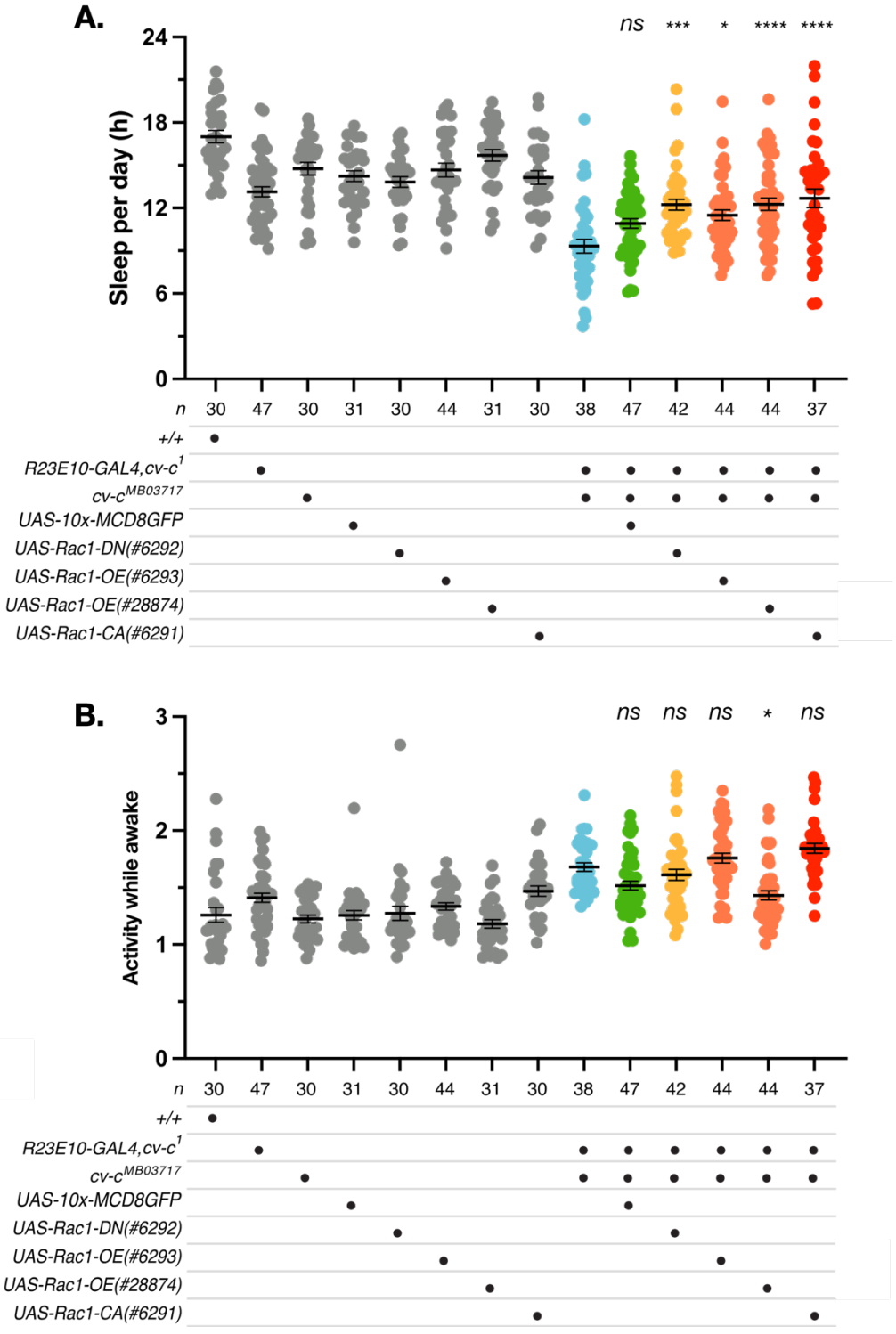


Figure 3-21: The effect of Rac1 manipulations on total sleep duration and activity while awake in cv-c1/cv-cMB03717 background. A) Average sleep duration in hours plotted for different genotypes. **B)** Activity while awake: average number of beam brakes per minute awake. Data is presented as means ± SEM; each circle represent an individual fly. n represents the number of flies for each genotype. Data was pooled from 3 independent experiments conducted on separate days. Values were averaged across 2 days (day 2 and 3). ANOVA ($F = 19.81$, $p < 0.0001$) and Kruskal-Wallis test was significant ($X2 = 245.3$, $p < 0.0001$) for A and B, respectively. Asterisks denote significant differences in pairwise post-hoc comparisons test compared to transheterozygous cv-c group (blue).

When compared to *cv-c* mutants, all Rac1 manipulations in *cv-c* mutants led to a significant increase in sleep as shown in Figure 3-21A (Tukey's MCT). However, introducing *UAS-mCD8::GFP* (membrane-bound GFP) into the dFB of *cv-c* mutants also led to a 17% increase in sleep. When this group was compared to the Rac1 manipulation groups then no statistical significant differences were detected as shown in Table 2. Thus, we cannot exclude that the increase in sleep we observe when overexpressing Rac1 in *cv-c* mutants is due to nonspecific UAS transgene effects or off-target overexpression effects.

Table 2: Post-hoc Tukey's multiple comparison test comparing Rac1 manipulation groups to *cv-c* mutant + *UAS-mCD8::GFP* control

Group being compared to *R23e10-GAL4, cv-c^{1} > cv-c^{MB03717}, MCD8:GFP*	Mean Diff.	95.00% CI of diff.	Significance	P value
R23e10-GAL4, cv-c^{1} > cv-c^{MB03717}, UAS-Rac-DN (#6292)	-1.322	-3.280 to 0.6348	ns	0.6574
R23e10-GAL4, cv-c^{1} > cv-c^{MB03717}, UAS-Rac-OE (#6293)	-0.5857	-2.519 to 1.348	ns	>0.9999
R23e10-GAL4, cv-c^{1} > cv-c^{MB03717}, UAS-Rac-OE (#28874)	-1.35	-3.284 to 0.5832	ns	0.5953
R23e10-GAL4, cv-c^{1} > cv-c^{MB03717}, UAS-Rac-CA (#6291)	-1.763	-3.788 to 0.2632	ns	0.1865

Interestingly, the DN Rac1 construct resulted in a similar increase in sleep as the OE and CA Rac1 constructs. This closely resembles the observations found when manipulating Rho1 in *cv-c* mutants.

As observed in 3.1.1, the activity while awake of *cv-c* mutants is slightly elevated in comparison to parental controls. Introducing Rac1 manipulations into the *cv-c* mutant background results in some variability in activity, however none of the groups, apart from *UAS-Rac1-OE (#28874)* are significantly different from one another (Tukey's MCT). The variability in activity while awake does not correlate with sleep behaviour suggesting that the sleep phenotypes we see are not due to any locomotor deficits.

To verify the results I repeated the experiment for select candidates (BDSC #28874 and 6291) in the *cv-c*^{c524}/*cv-c*^{MB03717} background. As shown in Figure 3-22, expressing Rac1-OE (#28874) and Rac1-CA (#6291) in dFB neurons of *cv-c*^{c524}/*cv-c*^{MB03717} mutant flies did not affect sleep duration significantly.

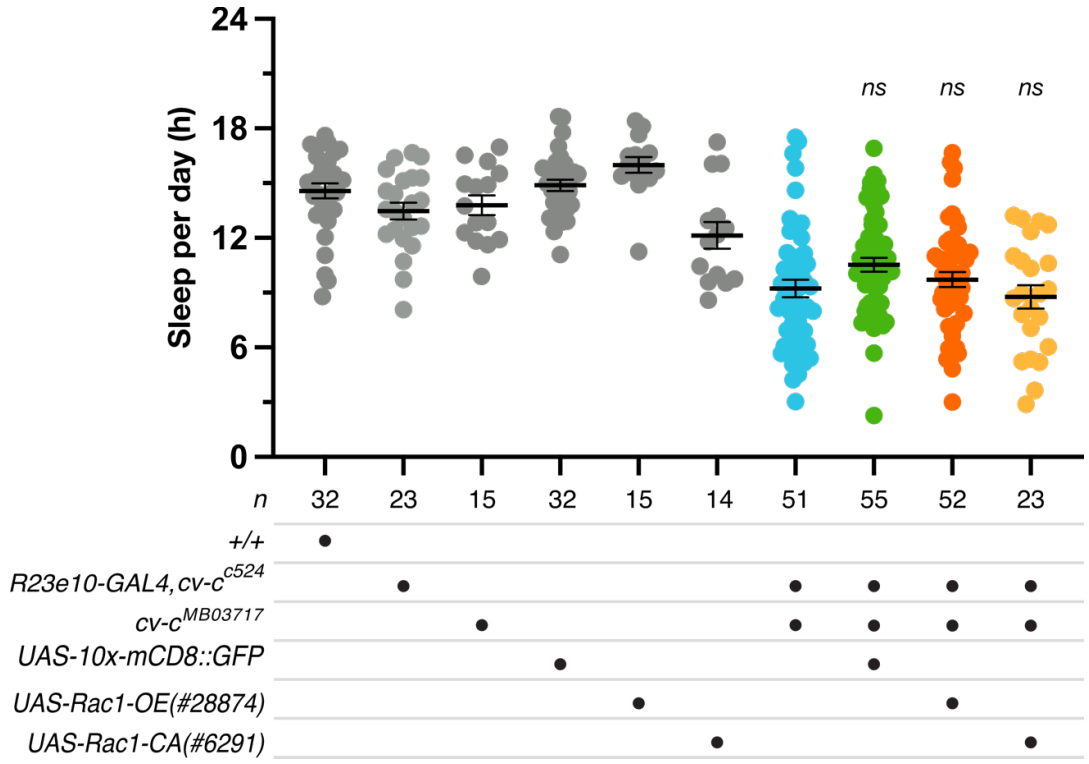


Figure 3-22: The effect of Rac1 manipulations in the *cv-c*^{c524}/*cv-c*^{MB03717} background on sleep per day (h). Data is presented as means ± SEM; each circle represent an individual fly. n represents the number of flies for each genotype. Data was pooled from 3 independent experiments conducted on separate days. UAS controls from 28874 and 6291 were only included in 1 out of 3 experiments. Values were averaged across 2 days (day 2 and 3). ANOVA was significant ($F = 27.10$, $p < 0.0001$). Asterisks denote significant differences in pairwise Tukey's Multiple Comparison test compared to transheterozygous *cv-c* group (blue).

In addition, overexpressing Rac1 in flies co-expressing *UAS-cv-c-RNAi* in dFB neurons had no effect on the insomniac phenotype as shown in Figure 3-23. No significant difference in total day sleep was detected between the *UAS-cv-c-RNAi* + *UAS-MCD8::GFP* (control) group and the *UAS-cv-c-RNAi* + *UAS-Rac1-OE* group (Tukey's MCT). Looking at the sleep profile, it looks like Rac1-OE results in a slight increase in night sleep, in comparison to the *cv-c* group, however a t-test found no statistical difference in night sleep between the two groups (t(29) = 1.98, P=0.057).

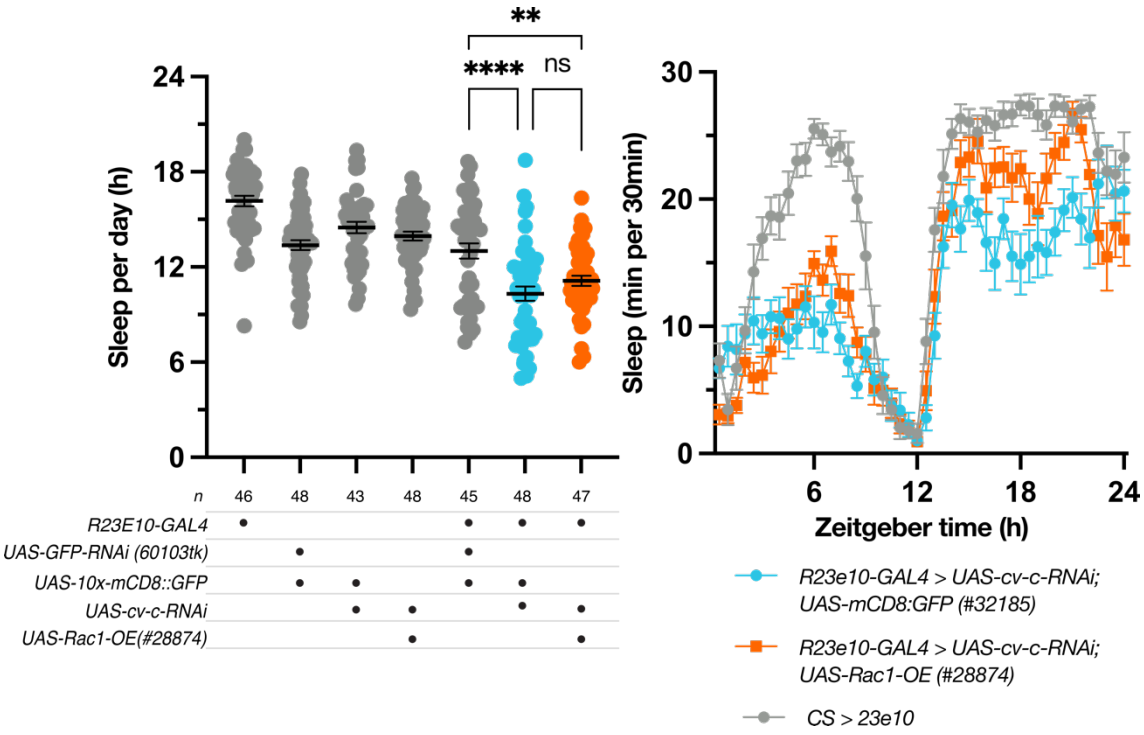


Figure 3-23: Overexpressing Rac1 in dFB neurons does not rescue the insomniac phenotype of UAS-cv-c-RNAi expressing flies. A) Sleep per day plotted (h). Data is presented as means ± SEM; each circle represent an individual fly. n represents the number of flies for each genotype. Values were averaged from day 2 and 3. ANOVA test was significant (F=28.09, p<0.0001) and asterisks denote significant differences in pairwise Tukey's multiple comparisons test. **B)** Minutes of sleep per 30 minute bin plotted across 24 hours for key groups.

Together, these experiments suggest that Rac1 does not rescue the insomniac phenotype observed in *cv-c* mutants or *cv-c* RNAi knockdown. However, we cannot exclude the possibility that internal compensation in *cv-c* mutants, and the upregulation of Rho1, is itself counteracting the role of Rac1 in regulating sleep. Thus, I decided to further investigate whether Rho1 and Rac1 act antagonistically in WT flies.

3.2.2 Rho1 and Rac1 antagonism in dFB neurons

If the hypothesis that Rho1 and Rac1 act antagonistically in dFB neurons is true then we would expect Rho1 to be upregulated and Rac1 to be downregulated in *cv-c* mutants. To see whether the relative balance of Rho1 and Rac1 in *cv-c* mutants is causing the insomniac phenotype, I tried to mimic this shift in WT flies. I hypothesised that overexpressing Rho1 and knocking down Rac1 in dFB neurons of otherwise WT flies would lead to an insomniac phenotype. Since a correct Rho1/ Rac1 balance is integral for many developmental processes I restricted transgene expression to adulthood by making use of the GAL80ts system, as described in 2.1.5. At 18°C, GAL80 represses the activity of GAL4 and the UAS-transgenes are not expressed. At 31°C, GAL80 is degraded and GAL4 can bind to UAS to induce transgene expression. I measured sleep in flies at 18°C for 2 days and then shifted them to 31°C and measured their sleep for 2 days, as shown in Figure 3-24. I then quantified sleep at day 2 (18°C) and day 4 (31°C) and calculated the percentage difference in sleep, as shown in Figure 3-25. To control for any UAS or RNAi effects I expressed *UAS-mCD8::GFP* and *UAS-RNAi-GFP* in dFB neurons using the *R23e10-GAL4* line.

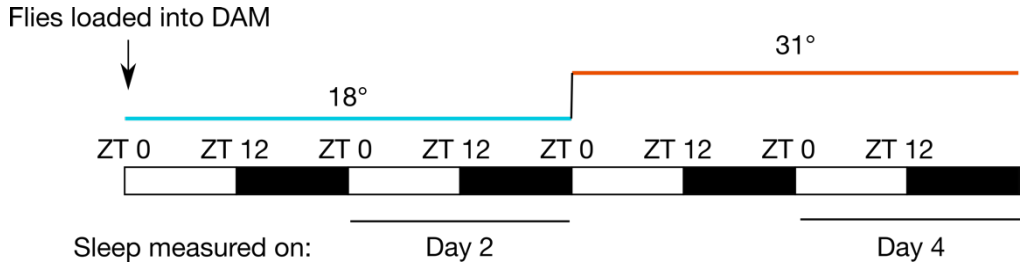


Figure 3-24: Timeline of experiments using GAL80ts

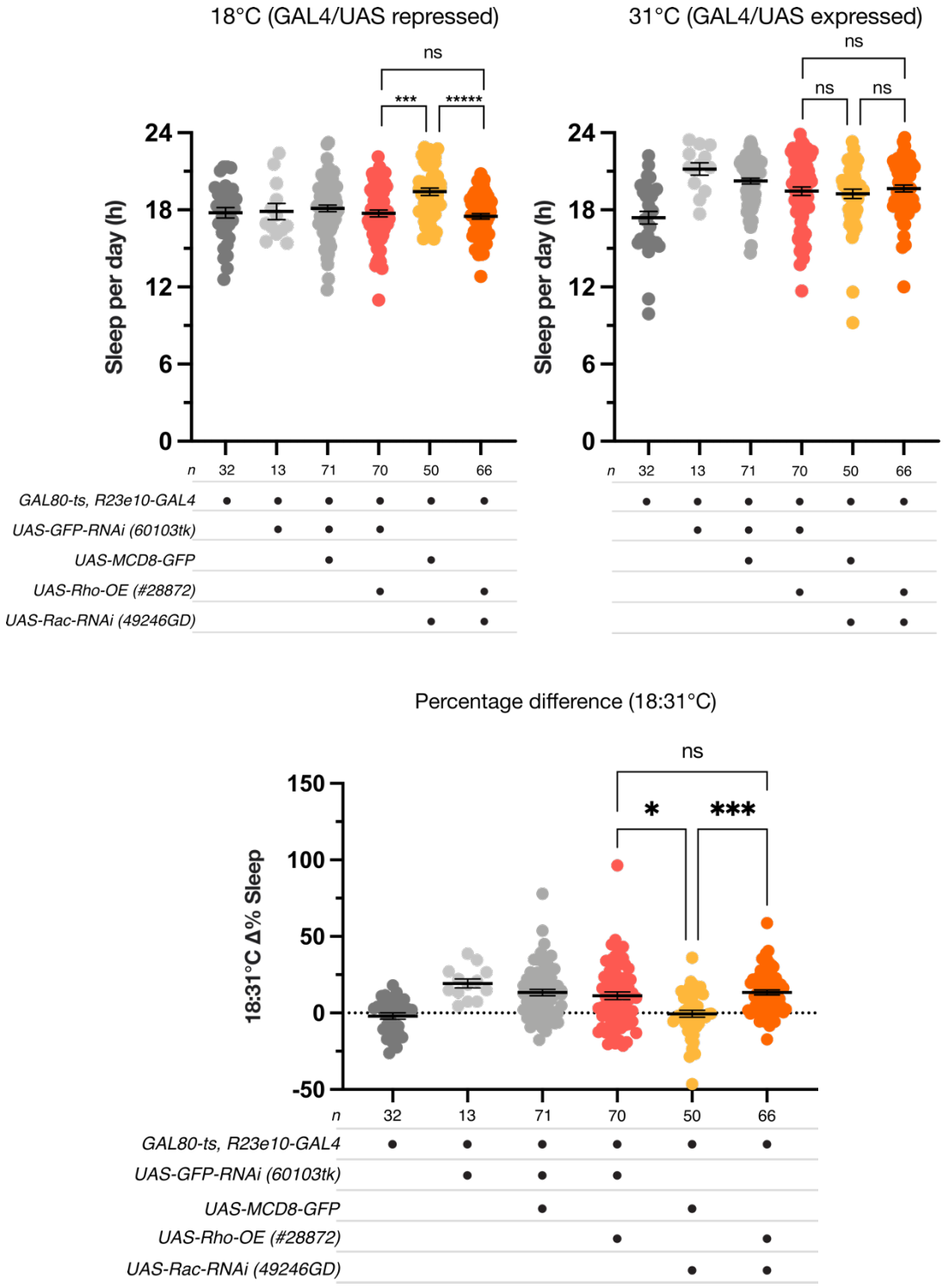


Figure 3-25: The effect of simultaneously overexpressing Rho1 and knocking down Rac1 in dFB neurons on sleep. A) Sleep per day (h) plotted for day 2 (18°C) and day 4 (31°C). **B)** Percentage difference in sleep at 18°C to 31°C. Data is presented as means ± SEM; each circle represent an individual fly. n represents the number of flies for each genotype. Values were pooled from 5 independent experiments. *GAL80ts- R23e10-GAL4* and *60103tk* control genotypes were only included in one replicate, hence lower n numbers. Kruskal Wallis test at 18° ($X^2 = 5.55$, $P<0.0001$) and 31° ($X^2 = 31.38$, $P<0.0001$) were significant. Kruskal Wallis test of percentage difference was also significant ($X^2 = 42.64$, $P<0.0001$). Asterisks denote post-hoc pairwise comparisons test.

At 18°C all genotypes sleep a similar amount (around 18 hours) apart from flies expressing *GAL80ts, R23e10-GAL4 > UAS-Rac1-RNAi*, which exhibit elevated levels of sleep. At 31°C, where GAL80ts is degraded and UAS transgenes are expressed, all groups expressing an RNAi line (either the *UAS-GFP-RNAi* control, or *UAS-Rac1-RNAi*) sleep around 19 hours a day. Thus, overexpressing Rho1, knocking down Rac1 or simultaneously overexpressing Rho1 and knocking down Rac1 in dFB neurons resulted in no significant differences in sleep at 31°C (Dunn's MCT). When plotting the percentage difference in sleep between 18°C and 31°C, significant differences between the three groups are detected, as denoted by the pairwise comparisons plotted in Figure 3-25. Knocking down Rac1 thus appears to reduce the temperature induced increase in sleep. This observation might however be attributable to leaky expression at 18°C. Overall, simultaneously increasing Rho1 levels and reducing Rac1 levels in dFB neurons using GAL80ts has no clear effect on sleep.

I next looked at whether shifting the Rho1-Rac1 balance in the opposite direction would have a sleep promoting effect. Rac1 was overexpressed in dFB neurons using the *UAS-Rac1-OE (#28874)* line and Rho1 was knocked down in dFB neurons using the *UAS-Rho1-RNAi (109420kk)* line. The protocol and the controls used in this experiment were the same as in Figure 3-25 (both experiments were conducted at the same time). Sleep per day at 18°C, 31°C and the percentage difference is plotted in Figure 3-26.

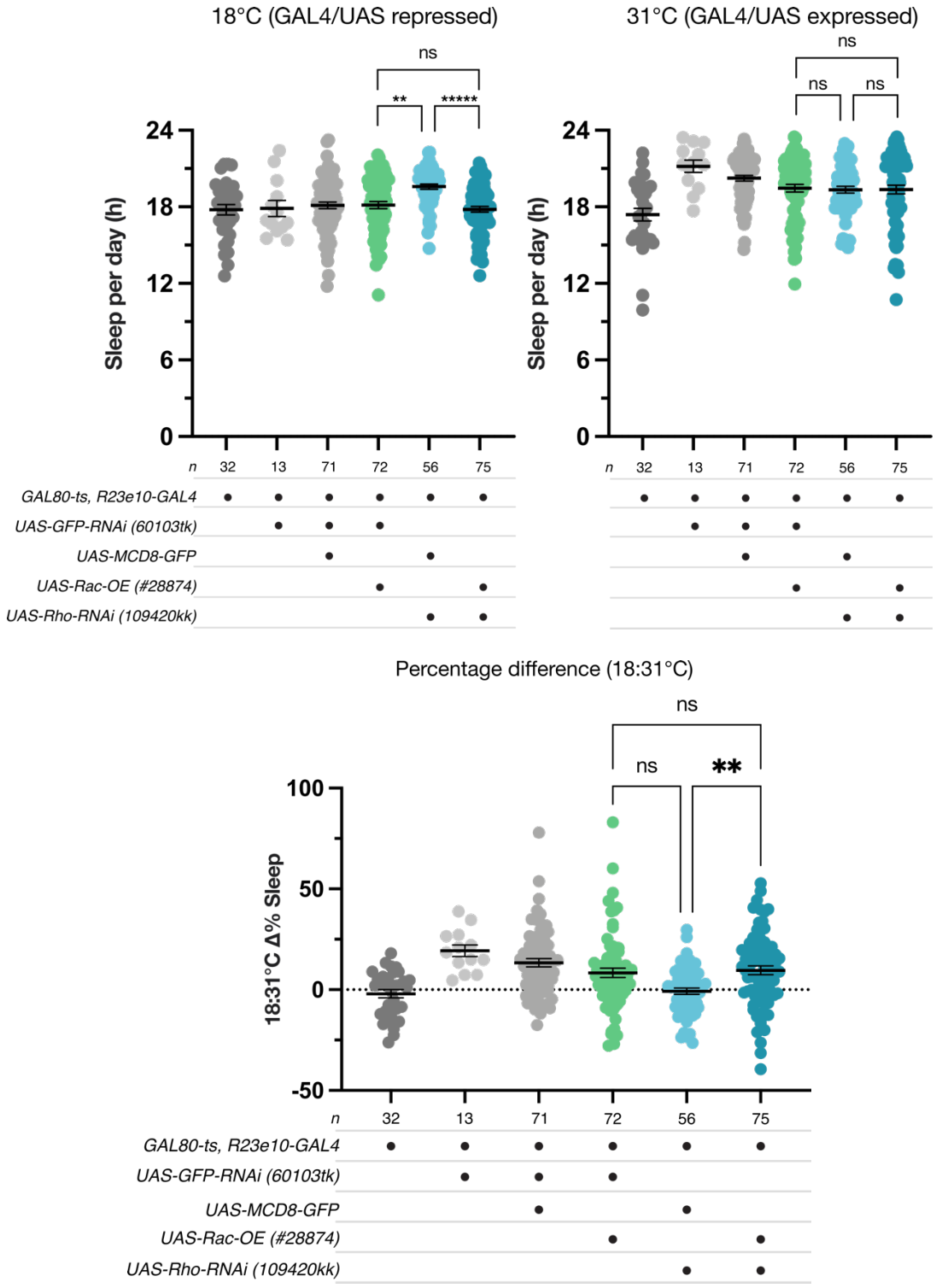


Figure 3-26: : The effect of simultaneously overexpressing Rac1 and knocking down Rho1 in dFB neurons on sleep. A) Sleep per day (h) plotted for day 2 (18°C) and day 4 (31°C). **B)** Percentage difference in sleep at 18°C to 31°C. Data is presented as means ± SEM; each circle represent an individual fly. n represents the number of flies for each genotype. Values were pooled from 5 independent experiments. *GAL80ts- R23e10-GAL4* and *60103tk* control genotypes were only included in one replicate, hence lower n numbers. Kruskal Wallis test at 18° ($X^2 = 30.1$, $P<0.0001$) and 31° ($X^2 = 30.66$, $P<0.0001$) were significant. Kruskal Wallis test in B was also significant ($X^2 = 43.66$, $P<0.0001$). Asterisks denote pairwise comparisons in Tukey's Multiple Comparison Test.

The behavioural outcome of this experiment closely resembles Figure 3-25. At 18°C, all groups, apart from the *GAL80ts, R23e10-GAL4 > UAS-Rho RNAi* sleep the same amount. At 31°C all groups apart from the *GAL80ts, R23e10-GAL4* control sleep around 19 hours. When looking at the percentage difference in sleep between 18°C and 31°C it seems that knocking down Rho1 prevents the temperature induced increase in sleep observed in the other groups, including the GFP control group. It is difficult to conclude whether the differences observed in the percentage difference plots are really attributable to the act of knocking down Rho or Rac, or whether they are due to leaky expression at 18 degrees.

Circadian rhythmicity and activity while awake was not affected by any of the manipulations. In addition, other sleep parameters, such as the number of sleep episodes or the average sleep episode duration, were not obviously different between genotypes at 31°C (Day 4) (*Data not shown).* Overall, from these experiments we can deduce that shifting the Rho1-Rac1 balance in wildtype flies, using the GAL80ts system, has no significant effect on sleep. This suggests that Rho1 and Rac1 do not act antagonistically in dFB neurons. In addition, these experiments do not provide conclusive evidence that either GTPase plays any role in the molecular regulation of sleep. However, there are several issues with these experiments which may hinder our understanding of how small GTPases regulate sleep. These will be discussed in Section 4.

3.2.3 Optogenetic activation of Rac1

Since temperature itself has an effect on sleep I decided to use an additional approach to study the role of Rac1 in regulating sleep. I expressed photoactivable constitutively active Rac1 (PA-Rac1) and photoactivable dominant-negative Rac1 (PA-DN-Rac1) in dFB neurons, of otherwise wildtype flies, and investigated the effect of blue light on sleep in these genotypes. A light insensitive version of PA-Rac1 was used as a control. Blue light slightly reduced sleep in controls (by 11%) but this effect was not significant ($P = 0.385$, Tukey's MCT). The protocol and approach used was the same as for the PR-Rho GEF experiments and is outlined in Section 2.3. Sleep during baseline, blue light activation and recovery day is plotted in Figure 3-27. The sleep profile during activation day and the percentage difference in sleep between baseline and activation day is plotted in Figure 3-28.

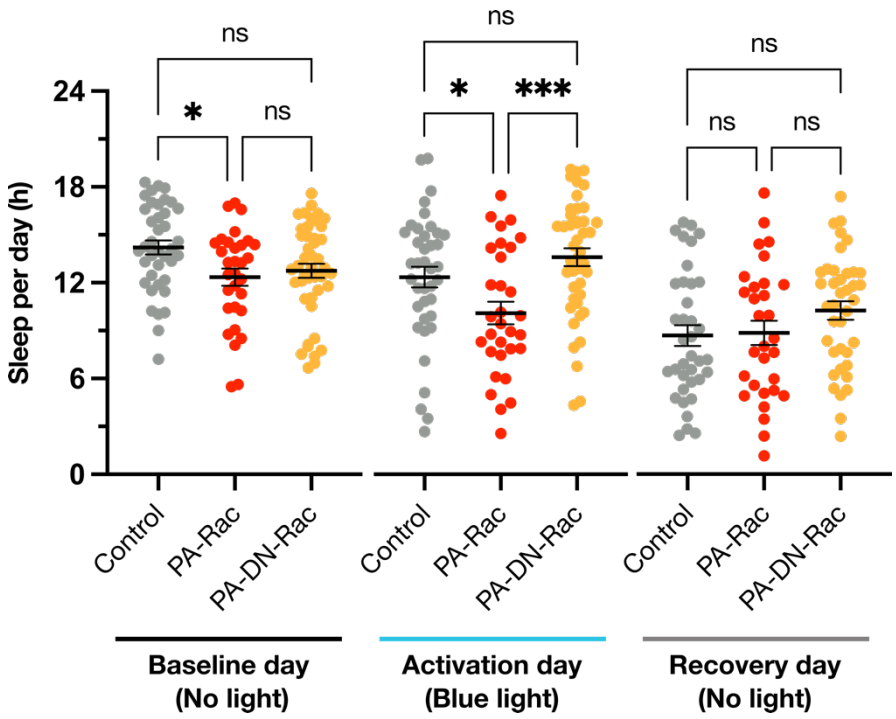


Figure 3-27: Sleep duration (h) during baseline, light activation and recovery day in Control (*R23e10-GAL4 > #32047*), PA-Rac (*R23e10-GAL4 > #32049*) and PA-DN-Rac (*R23e10-GAL4 > #31429*). Data is presented as means ± SEM; each circle represent an individual fly. Values were pooled from 10 independent experiments. One-way ANOVA was conducted on each day separately: Baseline day ($F = 4.236$, $P = 0.017$), Activation day ($F = 7.337$, $P=0.001$), Recovery day ($F = 1.817$, $P = 0.167$). Asterisks denote differences in Tukey's Multiple Comparison Test. N = 39, 31, 45, 39, 31, 44, 37, 30, 39 in order of appearance (left to right).

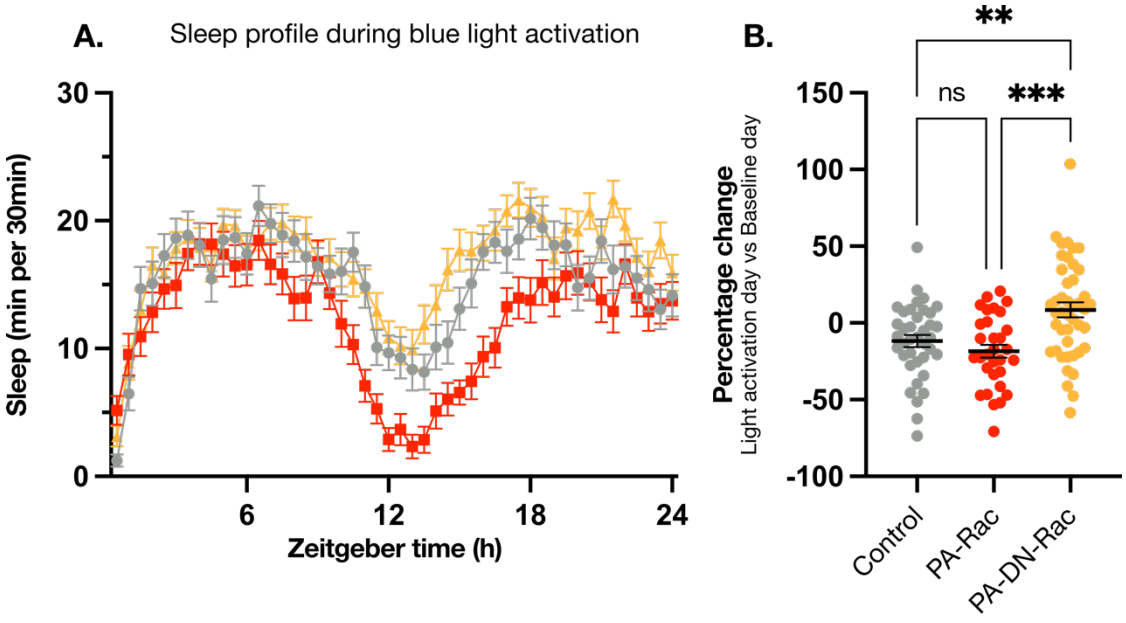


Figure 3-28: Sleep profile during blue light activation. Minutes of sleep per 30 minute bin plotted. **B) Percentage difference: (sleep during blue-light activation – baseline sleep) / baseline sleep**. One way ANOVA was significant ($F=10.18$, $P<0.0001$) and asterisks denote differences in Tukey's MCT. Values were pooled from 10 independent experiments. N = Control (38), PA-Rac (31), PA-DN-Rac (44).

In darkness, the presence of the PA-Rac and PA-DN-Rac transgenes results in slightly reduced sleep in comparison to the control group. (P =0.023 and P= 0.059, respectively, Tukey's MCT). During 24 hour blue light activation, sleep in both the control and the PA-Rac group is reduced, by 11% and 18% respectively, compared to baseline levels. In contrast, flies expressing PA-DN-Rac in dFB neurons actually sleep 8% more during blue light activation compared to baseline levels. This difference in response to blue light is highlighted in the Figure 3-28B. This suggests that photoactivating DN Rac1 in dFB neurons of otherwise wildtype flies has a significant sleep-promoting effect. In contrast, photoactivating CA Rac1 in dFB neurons has a slight sleep reducing effect. However, this effect is not significant in comparison to the control group when considering the percentage difference between baseline and activation sleep.

These results, in contrast to the GAL80ts data, suggest that Rac1 might after all play a role in regulating sleep via dFB neurons.

3.2.4 Summary

This section investigated the role of Rac1 in regulating sleep.

The key findings that emerged were:

1. Overexpressing or knocking down Rac1 in dFB neurons of *cv-c* mutants does not rescue the *cv-c* insomniac phenotype

2. Simultaneously overexpressing and knocking down Rho1 and Rac1 in an antagonistic fashion in adult flies (using GAL80ts) does not affect sleep duration

3. Optogenetic activation of a DN Rac1 construct in dFB neurons of otherwise WT flies results in a significant increase in sleep.

To what extent these observations truly reflect the role of Rac1 in regulating sleep in dFB neurons is unclear. It is likely that the behavioral approach to study the role of small GTPases in dFB neurons is not appropriate. Unfortunately, Rac1 activity could not be imaged as this would have helped elucidate how Rho1 and Rac1 interact in dFB neurons. Thus, further experiments will be needed to uncover the role of Rac1 in regulating sleep.

3.3 The role of small GTPases in the endocytosis of Sandman

Sleep in *Drosophila* is governed by the membrane excitability of dFB neurons which is regulated by the potassium channels Sandman and Shaker. Using voltage clamp experiments, Pimentel et al. (2016) found that Sandman is localised in the plasma membrane when dFB neurons are OFF (low sleep drive) and internalised when dFB neurons are ON (high sleep drive). Thus, the endocytosis of Sandman is critical for the OFF-to-ON / wake-to-sleep switch. Given the known role of small GTPases in vesicular trafficking I wanted to investigate whether they play a role in regulating the endocytosis of Sandman, thereby affecting dFB excitability.

3.3.1 Manipulating Rab5 in dFB neurons has an effect on sleep

One avenue to investigate whether the endocytosis of Sandman is important for regulating sleep is to study Rab small GTPases. Rab GTPases are known to be key regulators of endocytosis (Hutagalung & Novick, 2011). Rab5 which is involved in the early endocytic stages was found to interact with Sandman: Weiss et al. (2019) found that seizure-like phenotypes, observed in flies that have a higher retention of Sandman in early endosomes of cortex glial cells, were partially rescued by knocking down Rab5. This suggests that overexpressing Rab5 increases endocytosis, and thus promotes the retention of Sandman in early endosomes. In contrast, knocking down Rab5 (or expressing a DN construct) should reduce endocytosis, and so should promote the retention of Sandman at the plasma membrane. Indeed, various studies have found that CA Rab5 promotes endocytosis, whereas DN Rab5 reduces endocytosis (Gulappa et al., 2011; Somsel Rodman & Wandinger-Ness, 2000). Thus, manipulating Rab5 might affect the homeostatic regulation of sleep by modulating the endocytosis of Sandman. To test this, I expressed

Rab5-OE, Rab5-CA and Rab5-DN in dFB neurons of otherwise wildtype flies. Sleep per day and activity while awake is plotted in Figure 3-29.

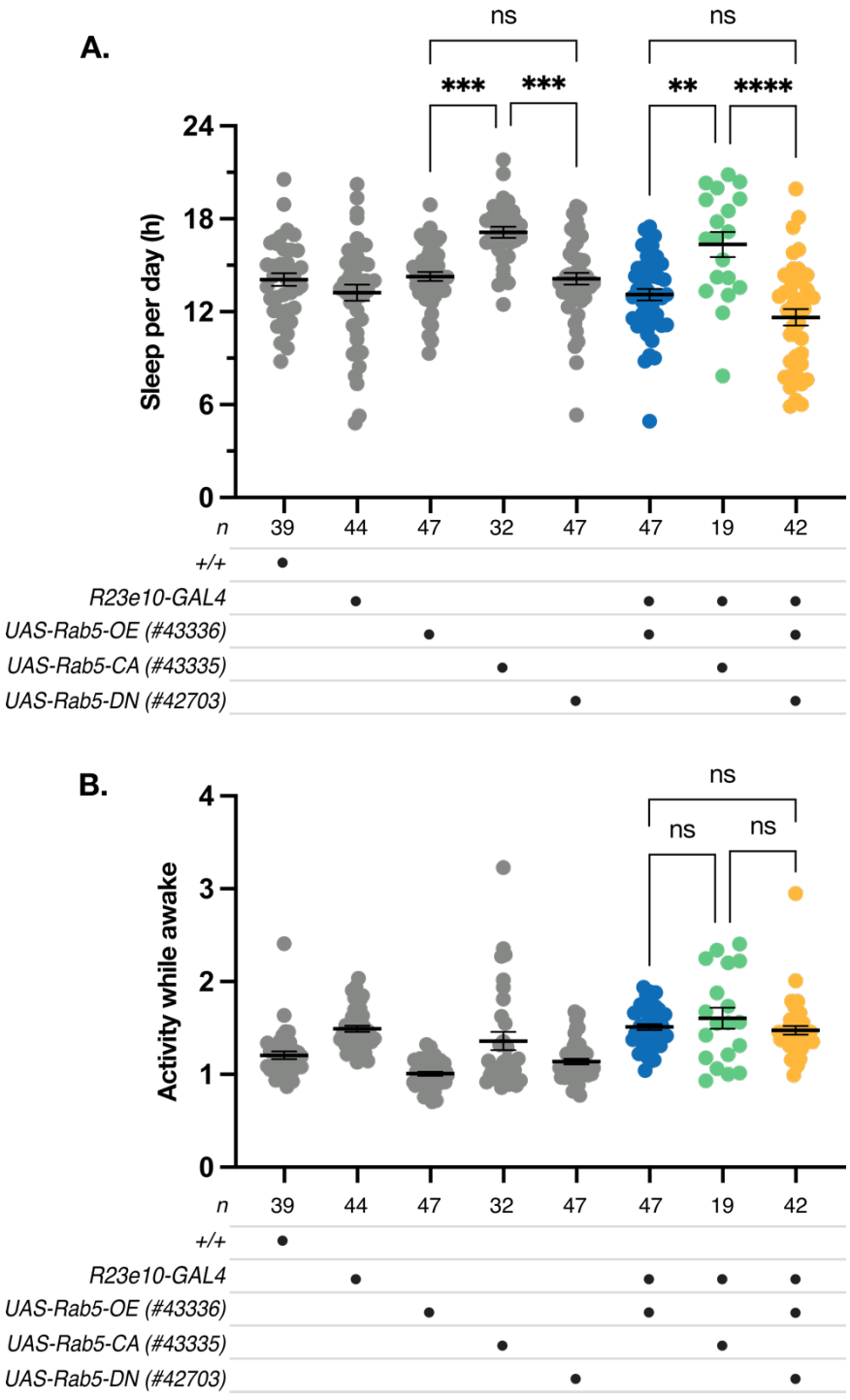


Figure 3-29: : The effect of Rab5 manipulations on sleep duration and activity while awake. A) Average sleep duration in hours plotted for different genotypes. One-way ANOVA was significant ($F = 16.70$, $P < 0.0001$) **B)** Activity while awake: average number of beam brakes per minute awake. Kruskal Wallis test was significant ($X^2 = 117$, $P < 0.0001$). Data is presented as means ± SEM; each circle represent an individual fly. n represents the number of flies for each genotype Data was pooled from 3 independent experiments conducted on separate days. Values were averaged across 2 days (day 2 and 3). Asterisks denote pairwise post-hoc comparisons between key groups.

Expressing CA Rab5 in dFB neurons results in a significant increase in sleep (+3 hours) in comparison to the *R23e10-GAL4* control (P=0.0031, Tukey's MCT). However, the undriven UAS control *(+ > UAS-Rab5-CA #43335*) also sleeps significantly more than the *R23e10-GAL4* control (P < 0.0001). This might be due to leaky expression or due to differences in genetic background. Thus, although it seems like increasing Rab5 activity in dFB neurons results in a sleep promoting effect, this observation needs to be considered with caution. Expressing DN Rab5 in dFB results in a significant decrease in sleep (-2.5 hours, P= 0.0018) in comparison to the undriven UAS control *(+ > UAS-Rab5-DN #42703*) and in a slight decrease in sleep (-1.5 hours) when compared to the *R23e10-GAL4* control. However, the latter is not statistically significant (P= 0.222, Tukey's MCT). Overexpressing WT Rab5 in dFB neurons did not significantly affect sleep duration.

Activity while awake, as plotted in Figure 3-29B, was not statistically different between the *R23e10-GAL4* driven Rab5 manipulations, however there is some variability between controls. Nonetheless, activity while awake variations do not reflect variations in sleep suggesting that the sleep phenotypes we observe are not due to locomotor deficits.

Overall, it seems that locking Rab5 in its constitutively active GTP-bound state promotes sleep, and reducing Rab5 activity by locking it in its GDP-bound state reduces sleep. However, these observations need to be taken with caution due to variability in controls. If these observations are true, they closely fit with the hypothesis that promoting endocytosis (thus internalizing Sandman) leads to a sleep promoting phenotype, whereas reducing endocytosis (thus retaining Sandman in the plasma membrane) leads to a wake promoting phenotype.

In *cv-c* mutants we expect that Sandman is retained at the plasma membrane (based on voltage clamp experiments mentioned earlier). This suggests that promoting endocytosis, by expressing CA Rab5, should rescue the sleep insomniac observed in *cv-c* mutants. In contrast, inhibiting endocytosis in *cv-c* mutants by expressing DN Rab5, should either have no effect on sleep, or should exacerbate the phenotype. To test this hypothesis, CA and DN Rab5 were recombined with *cv-c* alleles and expressed in dFB neurons. *UAS-mCD8::GFP* was also introduced in *cv-c* mutants to control for effects of introducing a UAS transgene into the mutant background. Sleep per day and activity while awake is plotted Figure 3-30.

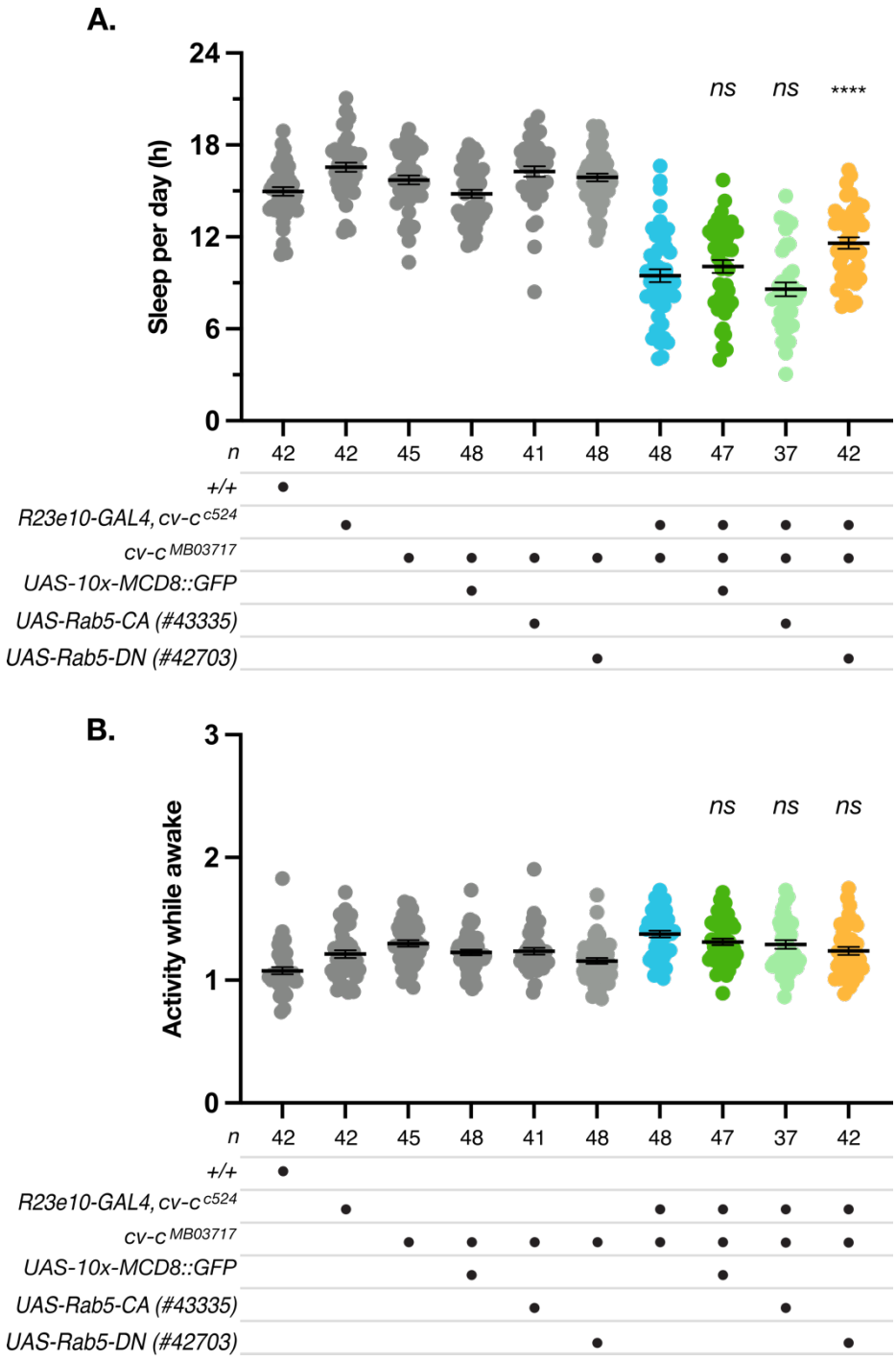


Figure 3-30: The effect of Rab5 manipulations in the *cv-c^{c524}/cv-c^{MB03717}* on sleep duration and activity while awake. A) Average sleep duration in hours. One-way ANOVA was significant ($F = 80.41$, $P < 0.0001$) **B)** Activity while awake: average number of beam brakes per minute awake. Kruskal Wallis test was significant ($X^2 = 72.91$, $P < 0.0001$). Data is presented as means ± SEM; each circle represent an individual fly. n represents the number of flies for each genotype Data was pooled from 3 independent experiments conducted on separate days. Values were averaged across 2 days (day 2 and 3). Asterisks denote pairwise post-hoc comparisons between respective groups and transheterozygous *cv-c* group (blue). These experiments were loaded with the help of Anissa Kempf.

Introducing Rab5-CA and Rab5-DN into the *cv-c*^{c524}/*cv-c*^{MB03717} background resulted in unexpected sleep phenotypes. Contrary to what was hypothesised, DN Rab5 led to a significant increase in sleep (+2.1 hours), when compared to the transheterozygous *cv-c* mutants (P = 0.0005, Tukey's MCT). CA Rab5 led to a slight decrease in sleep (-0.9 hours) although this is not statistically significant (P = 0.75, Tukey's MCT). When compared to the *UAS-mCD8::GFP* control, neither group is statistically different (P=0.053 and P=0.088, and respectively). Activity while awake was not significantly different between any experimentally manipulated groups.

This finding suggests that manipulating Rab GTPases in the *cv-c* background results in significantly different sleep phenotypes to the WT background. It is difficult to decipher what exactly is going on from these behavioural experiments, and therefore I, again, switched to an imaging approach to better understand how small GTPases are involved in regulating dFB excitability.

3.3.2 Staining for Sandman

Being able to visualise Sandman in dFB neurons would enable us to investigate whether overexpressing or knocking down small GTPases increases or reduces the endocytosis of Sandman. The aim of these experiments was to stain for Sandman using a CRISPR knock-in of RFP (Red Fluorescent Protein) -tagged Sandman. Flies homozygous for the Sandman-RFP constructs were combined with *UAS-MCD8::GFP*; *R23e10-GAL4* in order to see whether the RFP signal colocalises with the GFP membrane anchored signal in the dFB neurons. In addition, flies homozygous for the Sandman-RFP constructs were combined with *UAS-Rab5-GFP* driven by *R23e10-GAL4* to see if it colocalised with early endosomes. The rationale of these experiments was to see whether the distribution of Sandman between the plasma membrane and early endosome changes in flies with differing sleep pressure, and in WT flies vs. *cv-c* mutants.

Unfortunately, attempts to visualise Sandman failed. The RFP signal was sparse and closely resembled unspecific background staining of negative controls (in which Sandman-RFP was not expressed). This was further verified by merging the GFP and RFP channels, as shown in Figure 3-31. The RFP signal did not colocalise with either the plasma membrane or Rab5-tagged early endosomes in the soma, dendrites or axonal projections of dFB neurons. These results suggest that either the staining protocol did not work effectively (e.g. poor antibody penetrance), or that Sandman in dFB neurons is present at too low concentrations to be visualised. As a result the direct interaction between small GTPases and Sandman was not further investigated as part of this Masters thesis.

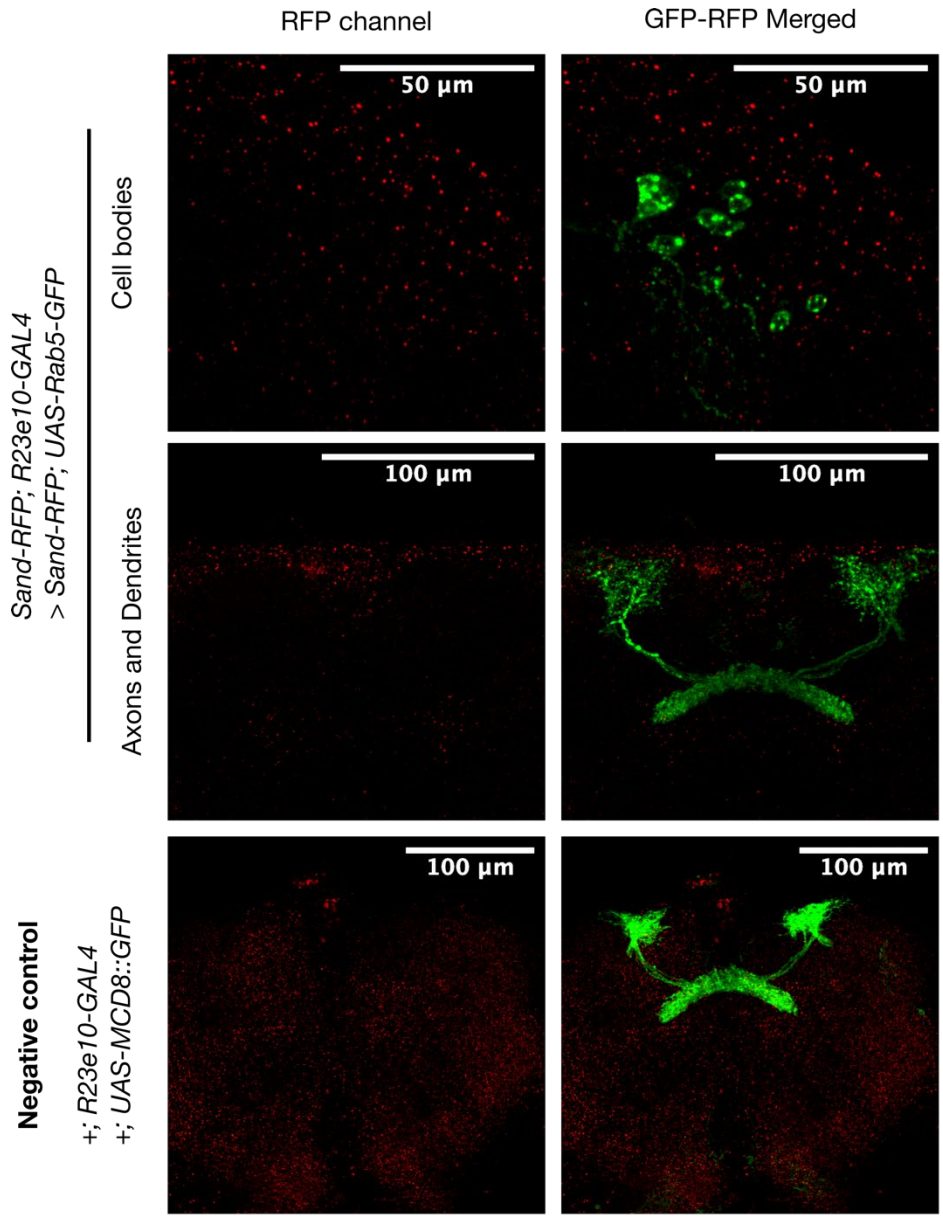


Figure 3-31: Staining for CRISPR Sandman-RFP knock-in in flies co-expressing UAS-Rab5-GFP in dFB neurons. Negative control shows similar levels of background RFP signal suggesting that RFP signal is likely the result of unspecific staining.

3.3.3 Summary

In conclusion, no direct link between the endocytosis of Sandman and the role of small GTPases could be established. This is mainly the result of methodological issues. Future work will have to look at troubleshooting these experiments in order to elucidate how small GTPases, Sandman and homeostatic sleep regulation interact.

4 Discussion

This thesis aimed to elucidate the molecular pathway in dFB neurons underlying wake-sleep regulation of *Drosophila melanogaster*. Previous work from the Miesenböck lab found that mutating Cv-c*,* a Rho-GAP, locked dFB neurons in an electrically silent state and led to defects in sleep homeostasis. This led to the idea that small GTPases, which are downregulated by Rho-GAPs, form an important part of the molecular pathway underlying homeostatic sleep regulation.

Using a combination of thermogenetic, optogenetic, behavioural and imaging approaches I investigated the role of Rho1, Rac1 and Rab5 in regulating sleep. The results suggest a vexingly complex role of small GTPases in regulating sleep and highlight the importance of finely-tuned spatiotemporal regulation of these regulatory proteins.

4.1 Does Rho1 regulate sleep?

With Rho1 identified as a candidate substrate for Cv-c, we hypothesised that active Rho1 is upregulated in *cv-c* mutants and that this upregulation in Rho1 is what is causing the deficits in sleep homeostasis. Indeed, using a Rho1-GTP biosensor (PKNG58AeGFP) I found that Rho1-GTP is higher in *cv-c* mutants compared to WT flies at most time points. However, what remains unclear is whether high Rho1 levels are responsible for the insomniac phenotype observed in *cv-c* mutants.

The high levels of Rho1-GTP in *cv-c* mutants point towards a sleep-reducing effect of Rho1 in dFB neurons. This is further supported by the finding that knocking down Rho1 in *cv-c* mutants using RNAi or a DN construct rescued the insomniac phenotype. However, overexpressing WT Rho1 or CA Rho1 in *cv-c* mutants also led to a rescue in

homeostatic sleep deficits: *cv-c*^{MUT} + Rho OE flies exhibited normal sleep duration (15 hours) and showed a homeostatic sleep rebound after sleep deprivation. Imaging experiments confirmed that Rho1 levels in *cv-c*^{MUT} + Rho OE flies were equal to that in *cv-c* mutants. If high Rho1 levels in *cv-c* mutants are causing the sleep insomniac phenotype, then why do *cv-c*^{MUT} + Rho OE flies, which have similarly high Rho1 levels, exhibit normal sleep behaviour?

Two possible scenarios are:

a. High Rho1-GTP levels have a sleep-reducing effect and the overexpression results are an artefact of overwhelming the finely-tuned endogenous system

b. High Rho1-GTP levels have a sleep-promoting effect and overcompensation in *cv-c* mutants results in an insomniac phenotype

To investigate if Rho1-GTP has a sleep-reducing or sleep-promoting effect (without internal compensation) I conditionally manipulated Rho1-GTP in adult WT flies using the PR-Rho-GEF LARG. These experiments found that acutely activating Rho1 had no significant effect on sleep. This could be due to the fact that only heterozygotes were used. Rich et al. (2020), who developed the PR-Rho-GEF, found that the copy number of the transgene was important and thus, homozygotes may be needed for acute activation of Rho1 *(personal communication).* Alternatively, the sleep regulation pathway may be dependent on the activation of Rho1 via specific GEFs (not LARG), or compensation in the WT system might cover any sleep effects. Either way, no clear effect of Rho1 on sleep in WT flies could be elucidated.

An interesting observation of the imaging experiments is that there is a tendency for Rho1-GTP to increase after sleep deprivation and at ZT 6-8 in WT flies. This suggests that Rho1-GTP might act as a molecular correlate for sleep pressure. However, the fact that Rho1-GTP levels are low at ZT 10-12, shortly before sleep onset, contradicts this idea. In addition, while *cv-c* mutants have electrically silent dFB neurons, sleep deprived flies have highly excitable dFB neurons (sleep deprivation increases the excitability of dFB neurons), but Rho1 levels are high in both groups. Therefore, these experiments suggest a vexingly complex role for how Rho1-GTP activity, sleep pressure and dFB excitability are linked. It is likely that these confusing findings are a result of an experimental artefact. Indeed, the upregulation of Rho1 after sleep deprivation was only found in three out of six replicates. Therefore, further experiments using a more sensitive Rho1 biosensor would be needed to verify these results. In addition, live imaging of Rho1, in combination with activity tracking of the imaged fly, could further verify how Rho1, its localisation, and sleep pressure are linked.

One possibility for the confusing results is that overexpressing Rho1 in *cv-c* mutants disrupts the cytoskeletal structure of dFB neurons. However, quantification of axonal volume and 3D spread confirmed that dFB axonal projections are not affected by overexpressing Rho1.

Electrophysiology experiments conducted by Anissa Kempf, found that knocking down Rho1 in *cv-c* mutants rescued the electrophysiological properties of *cv-c* mutant dFB neurons back to that of WT dFB neurons. This strongly suggests that the high Rho1 levels in dFB neurons of *cv-c* mutants are what is locking them in an electrically silent state. In addition, voltage clamp experiments suggest non-A-type currents (flowing through Sandman) are high in *cv-c* mutants, but when Rho1 is knocked down, non-A-type currents

are reduced. This suggests that high Rho1 levels (found in *cv-c* mutants) might inhibit the endocytosis of Sandman.

An important consideration to take into account is that my findings are limited to the PKNG58AeGFP biosensor. The biosensor binds to Rho1 when it is in its active, GTP-bound state, and becomes recruited to the plasma membrane where its fluorescence becomes concentrated. When unbound, the biosensor is localised in the cytoplasm and the probe fluorescence is sparse and weak. Using this biosensor as a marker of Rho1-GTP activity relies on consistent image quality and consistency in the fluorophore expression. There was a significant amount of variability in how strongly the fluorophore was expressed across replicates. Therefore, a more sensitive and reliable biosensor, with lower background signal, should be used in the future. In addition, a higher resolution with an oil immersion objective, to reduce refraction, would be more appropriate for future experiments.

An additional source of concern is that the fluorescence of the unbound cytoplasmic biosensor might have been measured. The optical resolution used is likely too low to distinguish the membrane-bound GFP signal from the cytoplasmic GFP signal in the thin axonal branches of the dFB neurons. GFP fluorescence values might therefore reflect the general prevalence of the biosensor, rather than the membrane-localised Rho1-GTP-bound biosensor. This notion is further suggested by the lack of a negative control: introducing the DN Rho1 construct into *cv-c* mutants yielded a high Rho1-GTP fluorescence (probably due to internal compensation). A proper negative control would be needed to verify the validity of the biosensor: for example one could apply the Rho1 inhibitor exoenzyme C3 transferase. However, even if diffuse cytoplasmic fluorescence of the unbound biosensor was measured, differences in fluorescence between genotypes

are still likely attributable to the level of Rho1-GTP activity. Additionally, I conducted a few dissections (n = 7) using a different biosensor (Dia-GFP) and found that the dFB axonal fluorescence in WT brains was weaker than the fluorescence in *cv-c* mutant brains (data shown in the appendix). Thus, although the PKNG58AeGFP may not be completely representative of Rho1-GTP activity, it probably still accurately reflects differences in Rho1 activity between groups.

Alternatively, the puzzling results may be due to the complexity of small GTPase regulation. The *Drosophila* genome encodes 29 Rho-GEFs, 23 Rho-GAPs and 6-8 Rho-GTPases (Denk-Lobnig & Martin, 2019). This suggests that several GEFs and GAPs regulate the same Rho GTPases. Here we hypothesise that when *cv-c* is mutated, Rho1-GTP cannot be hydrolysed to Rho1-GDP and thus we have a higher level of active Rho1. However, with high GAP promiscuity, it is likely that several GAPs work to downregulate Rho1, and mutating *cv-c* may result in other GAPs compensating. Thus, the insomniac phenotype observed in *cv-c* mutants may not be down to the actual level of Rho1-GTP but due to the Rho-GEF to GAP ratio. For example, various studies, both experimental and computational, have found that the GEF:GAP ratio is key in determining the efficiency of GTPase activity cycling, and that the relative concentration of GEFs to GAPs is critical in determining the biochemical outcome (Goryachev & Pokhilko, 2006; Mason et al., 2016).

Rho GTPases are also finely spatially regulated. One small GTPase can perform distinct cellular functions when activated at different subcellular locations (Pertz, 2010). In my experiments I only measured Rho1-GTP activity in the axonal projections (which was due to the fact that the fluorescence of the biosensor was not strong enough in the dendrites or soma). Using a more sensitive biosensor one could quantify the relative ratio

of Rho1-GTP activity in axons, dendrites and soma. It is likely that the localisation rather than the concentration of Rho1-GTP is key in modulating dFB membrane excitability. If the function of Rho1 in regulating sleep is locally specific, then globally overexpressing or knocking down Rho1 in dFB neurons will interfere with the precise spatio-temporal signalling and localisation of the GTPase and result in unnatural, or difficult-to-interpret phenotypes.

Another possibility is that the sleep insomniac phenotype observed in *cv-c* mutants is due to GAP-independent functions of *cv-c.* In addition to the Rho-GAP domain, Cv-c has a SAM and START domain (Sotillos et al., 2018). These domains are known to be implicated in lipid membrane binding and lipid-transfer (Barras & Widmann, 2013). This suggests that Cv-c, like its mammalian homolog DLC1, might interact with phosphoinositides which are important lipid signalling molecules. Indeed the phosphoinositide 3-kinase gene has been linked to a sleep shortening phenotype (Allebrandt et al., 2017). In addition the lipid membrane domains of Cv-c likely play a critical role in localising it to the plasma membrane (Denholm et al., 2005). Therefore, it is possible that the insomniac phenotype observed in *cv-c* mutants is not due to the activity of Rho-GTPases, but actually due to GAP-independent functions of Cv-c.

In conclusion, high Rho1-GTP levels in *cv-c* mutants are likely the culprit of defects in sleep homeostasis, as suggested by knock-down genetic interaction experiments, imaging and electrophysiology experiments. However, why overexpressing Rho1 in *cv-c* mutants rescues the insomniac phenotype, and how Rho1-GTP levels affect sleep in WT flies remains unclear. The conflicting findings are likely the result of globally manipulating small GTPases that rely on finely tuned local regulation. More experiments, that take into account the importance of small GTPase localisation, will be needed in order to elucidate

the role of Rho1 in regulating sleep. In addition, more sensitive biosensors such as fluorescence resonance energy transfer (FRET) would enable more accurate visualisation and quantification of small GTPases, with higher specificity. Live imaging using two photon microscopy would furthermore enable one to verify whether Rho1-GTP activity is correlated with sleep pressure, a finding that would revolutionise our understanding of how sleep is regulated molecularly, if true.

4.2 Does Rac1 regulate sleep?

With *cv-c* mutants implicating a role of Rho1 in regulating sleep, I asked whether any interacting small GTPases are involved in the molecular pathway underlying wake-sleep regulation. Since Rho1 and Rac1 are known to act antagonistically in many cellular processes I hypothesised that Rac1 has an opposing role to Rho1 in dFB neurons and in the regulation of sleep. I approached this question using a three-pronged approach.

The first set of experiments looked at whether overexpressing or knocking down Rac1 in the dFB neurons of *cv-c* mutants (using OE, CA and DN constructs) would rescue the *cv-c* insomniac phenotype. I observed a slight upregulation in sleep when expressing Rac1 manipulating constructs in the $cv\text{-}c^{1}/cv\text{-}c^{MB03717}$ background but observed no rescue in sleep in the $cv\text{-}c^{c524}/cv\text{-}c^{MB03717}$ background. Differences in the behaviour are likely due to the fact that $cv\text{-}c^{1}$ is a hypomorphic mutant allele (gene is still expressed but at a reduced level), whereas $cv\text{-}c^{c524}$ is an amorphic mutation (gene is truncated and completely lacks the function of the WT gene). The $cv\text{-}c^{c524}/cv\text{-}c^{MB03717}$ background is therefore more appropriate to study the GAP functional role of Cv-c. Therefore, the experiments suggest that Rac1 does not antagonise Rho1 in the dFB neurons of *cv-c* mutants.

The second set of experiments investigated what happens when we simultaneously overexpress and knockdown Rho1 and Rac1 in the dFB neurons of WT flies using the GAL80ts system. No significant effect on sleep duration was observed when overexpressing Rho1 and knocking down Rac1 and vice versa. These results suggest that Rho1 and Rac1 do not play a critical role in regulating sleep in dFB neurons. However, there are various confounding factors that may affect our interpretation of these results. Firstly, temperature itself has an effect on sleep. Flies are ectotherms and temperature shifts can significantly affect their physiology and behaviour (Beckwith & French, 2019). Thus, the GAL80ts system may not be appropriate to study sleep due to confounding temperature x genotype x sleep interactions. Secondly, knocking down small GTPases in this experiment was achieved by expressing RNAi lines at day 3 (after 31°C induction degrades GAL80ts) and sleep was measured at day 4. It is plausible that the RNAi mediated knockdown of small GTPases takes longer than one day. Thirdly, I only measured sleep duration. It is possible that Rho1 and Rac1 interact to regulate sleep rebound, and so an effect may only be apparent when flies are sleep deprived.

A final set of experiments looked at the conditional, optogenetic activation of Rac1 OE and DN in dFB neurons using PA-Rac1. Here, expression of DN Rac1 had a sleep promoting effect, whereas expression of CA Rac1 had a slight sleep reducing effect (although not statistically significant). These experiments suggest that maybe Rac1 does have some sleep regulatory role after all. The reason why we see an effect of Rac1 on sleep here, but not in GAL80ts experiments might be due to differences in the way we measure sleep. Sleep in optogenetic experiments was measured using video-tracking rather than the traditional DAM system. While the DAM system only records the fly's activity when it crosses the laser beam spanning the middle of the tube, the video-tracking

system records the movement of the fly in the entire tube. Thus, the video-tracking system has a higher spatial resolution and may pick up on more subtle differences in sleep. Indeed, various studies have found that DAMs overestimate sleep amount and that video-tracking systems are more appropriate for detecting differences in sleep behaviour between genotypes or conditions (Faville et al., 2015; Geissmann et al., 2019). Thus, overexpressing and knocking down Rac1 might induce subtle differences in sleep that are too weak to detect using the DAM system. Alternatively, photoactivable Rac1 might result in more acute activation.

A key limitation to all these experiments is that overexpressing exogenous small GTPases can overwhelm the endogenous signalling system. Previous studies found that exogenous overexpression of one type of small GTPase leads to the depletion of other small GTPases (Boulter et al., 2010). This is due to GDI competition. When small GTPases are in their inactive, GDP-bound state, they bind to GDIs which keep them in the cytoplasm and prevent their degradation. Since the number of RhoGDIs is limited and shared between small GTPases, the exogenous overexpression of one small GTPase will lead to increased GDI competition and can lead to the displacement and degradation of other small GTPases. Indeed, Boulter et al. (2010) found that overexpression of Rho in HeLa cells reduced the level of endogenous Rac and Cdc42. Similarly the overexpression of Rac led to the degradation of endogenous Rho and Cdc42. Expressing CA and DN constructs also led to the degradation of endogenous small GTPases. This degradation was found to be specifically due to Rho-GDI competition, rather than antagonistic relationships between small GTPases, because the degradation of endogenous GTPases could be prevented by co-expressing RhoGDIs. Taking this into account, it is likely that overexpressing Rho1 in dFB neurons already leads to the depletion of Rac1 and vice versa. Thus, knocking

down one GTPase, while already overexpressing another, will not further affect the phenotype. This further highlights the complexity of small GTPase signalling and highlights that OE, CA and DN constructs may not be appropriate for elucidating their role in sleep.

While I hypothesised that Rho1 and Rac1 act antagonistically, it is likely that they act in the same pathway and promote each other rather than antagonise each other. Indeed, various studies have observed that Rho1 activates Rac1, or that Rac1 activates Rho1. The complexity of Rho1 and Rac1 crosstalk has been highlighted by the range of interactions discovered in different cellular systems across the literature, as shown in Figure 4-1.

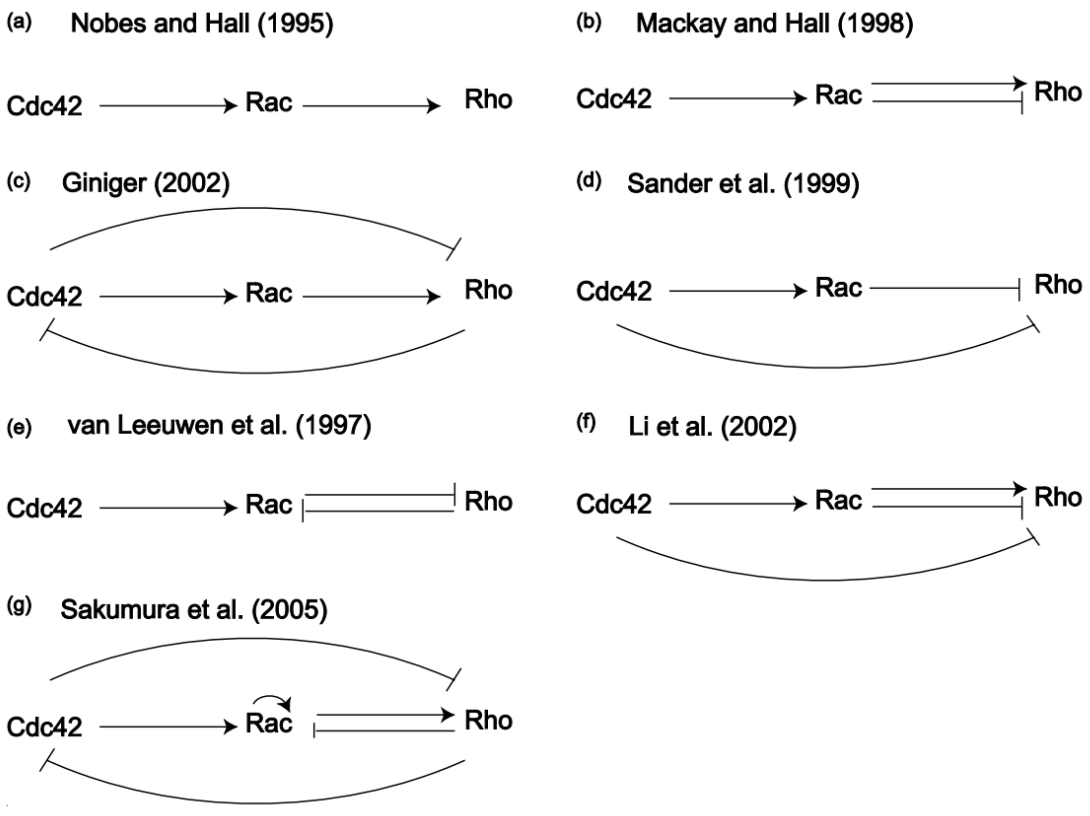


Figure 4-1: Figure from Jilkine et al., (2007) summarising the different types of interactions between Rho, Rac and Cdc42 that have been proposed in the literatuture.

In conclusion, the role of Rac1 in regulating sleep in dFB neurons, and how it interacts with Rho1 remains unclear. Experiments conducted here suggest that the interaction between Rac1 and Rho1 in dFB neurons is unlikely to be antagonistic. However, it is plausible that Rho1 and Rac1 are differentially localised in dFB neurons and that globally overexpressing and knocking down Rho1 and Rac1 in dFB neurons simply overwhelms the system and results in difficult-to-interpret results. Alternatively, Rho1 and Rac1 might regulate the same downstream effectors which affect sleep. Investigating potential downstream candidates of Rho1 and Rac1 that regulate sleep may help elucidate how they are involved in homeostatic sleep regulation. In addition the interaction of Rho1 and Rac1 could be better elucidated using FRET or other biosensors to simultaneously observe Rho1 and Rac1 levels. If Rho1 is upregulated, what is the endogenous activity of Rac1? Thus, experiments with higher spatial resolution are needed to better understand whether Rac1 plays a role in regulating sleep.

4.3 Do small GTPases regulate the endocytosis of Sandman?

Electrophysiology experiments found that knocking down Rho1 levels in *cv-c* mutants rescued the membrane excitability of dFB neurons back to control levels. This suggests that the level of Rho1-GTP directly affects dFB excitability. Since the membrane excitability of dFB neurons is modulated by potassium channels, I hypothesised that Rho GTPases affects membrane excitability by either:

1) regulating the endo-exocytosis of Sandman

2) by directly modulating ion channels (Sandman or Shaker)

3) by modulating the structural plasticity of the neurons, which in turn affect membrane excitability.

The finding that Rho1-GTP does not correlate with dFB axonal structural changes suggests that the third hypothesis is unlikely. Thus, I sought to investigate whether small GTPases regulate sleep by affecting the endocytosis of Sandman.

By manipulating Rab5, a small GTPase involved in early endocytic processes, I hoped to promote or reduce endocytosis in dFB neurons. I found that expressing Rab5-CA significantly increased sleep, and expressing Rab5-DN slightly decreased in sleep in comparison to the GAL4 and UAS control, respectively. This suggests that promoting the internalisation of Sandman, increases sleep, whereas inhibiting the internalisation of Sandman, decreases sleep. This supports our current model which suggests that the endocytosis of Sandman is critical for the OFF-to-ON switch of dFB neurons, and thus the wake-to-sleep switch (See Figure 1-3 for recap of model). However, controls also showed variability in sleep and so these observations need to be considered with caution.

Nevertheless, these findings suggest that manipulating early endocytic processes affects sleep.

Confusingly, expressing CA and DN Rab5 constructs in dFB neurons of transheterozygous *cv-c* mutants produced opposite behavioural phenotypes. Expressing DN Rab5 led to a significant increase in sleep and CA Rab5 had no significant effect on sleep in comparison to *cv-c* mutants. A possible explanation for these results is that endocytosis depends on various proteins working together in a finely-tuned system. Rab5-induced endocytosis might be dependent on the proper GTP-GDP cycling of Rho1. Indeed, Rab5 proteins have been found to interact with several Rho-GTPases (Margiotta & Bucci, 2019). Thus, mutating a Rho-GAP, and simultaneously overexpressing Rab5 might completely disrupt the endogenous regulation of endocytosis, thus producing difficult-to-interpret results.

To further explore how small GTPases regulate the endocytosis of Sandman I attempted to visualise the potassium channel using a CRISPR knock-in of RFP-fused Sandman. Unfortunately, attempts to visualise Sandman failed. A range of antibody dilutions and variations in the triton concentration should be tested in order to verify whether this is due to a staining issue. However, since the same antibodies and staining protocol were used for the Rho1 staining experiments, it is likely that RFP-fused Sandman is present at too low concentrations to be visualised. Future work will have to look at developing more sensitive tools to visualise Sandman, in order to elucidate how small GTPases, the endocytosis of Sandman and dFB membrane excitability are linked.

One suggestion on how to investigate whether Rho1 affects the endocytosis of Sandman, even without being able to visualise Sandman, is to quantify the number of Rab5 early

endosomes. By driving *UAS-Rab5-GFP* using *R23e10-GAL4* I was able to visualise early endocytic puncta in dFB neurons. An interesting future experiment would be to quantify the number of these Rab5 labelled puncta in dFB neuron cell bodies of *cv-c* mutants and WT flies. If high Rho1 levels in *cv-c* mutants are inhibiting endocytosis, then the number of Rab5 tagged endosomes in dFB neurons should be lower in *cv-c* mutants than in WT. Knocking down Rho1 in *cv-c* mutants should return the number of endosomes back to WT levels. This experiment would be an effective way to investigate whether Rho1 levels affect endocytosis in dFB neurons, and thus, whether this contributes to changes in membrane excitability. Unfortunately, the *UAS-Rab5-GFP* transgene is on the third chromosome and would have to be recombined with one of the *cv-c* alleles, a process which takes at least 4-6 weeks. As such, this experiment could not be included in this thesis.

It is important to consider that the role of small GTPases in vesicular trafficking is pleiotropic: Rho1 and Rac1 are involved in both endocytic and exocytic processes (Chi et al., 2013). This might further explain conflicting sleep phenotypes. Overexpressing or knocking down small GTPases might interfere with both endocytosis and exocytosis of Sandman. In addition, Rho GTPases can regulate vesicular trafficking through several different downstream pathways. Thus, a more informative approach to understand how the endocytosis of Sandman is regulated would be to study candidate downstream effectors that are critical to vesicular trafficking. Potential downstream candidates include ROCK and WASP which are involved in actin polymerisation and thus important for vesicular trafficking. How these downstream effectors regulate dFB membrane excitability could be determined by combining behavioural and electrophysiological experiments.

In conclusion, how small GTPases regulate dFB neuron membrane excitability remains unclear. It is likely that small GTPases affect homeostatic sleep regulation by regulating the endocytosis of Sandman, however this remains a hypothesis that needs to be further investigated. Experiments, such as quantifying Rab5 early endosomes, can help us understand how Rho1 regulates endocytosis. A direct link between dFB membrane excitability, the endocytosis of Sandman and small GTPases will depend on being able to visualise Sandman through imaging experiments, a feat that has not yet been possible.

4.4 Confounding factors

There are various additional confounding factors that may contribute to the conflicting results presented here. Firstly, to investigate the role of small GTPases I made use of CA and DN constructs. These constructs have been used since the early 1990s and have helped uncover the various functional roles and interacting partners of Rho GTPases (Ridley, 2012). However, DN and CA constructs interfere with the ability of small GTPases to cycle between their active and inactive state. This cycling is integral to their functional roles (Luo, 2000). Thus, phenotypes induced by expressing DN and CA constructs do not necessarily reflect the consequences of downregulating or upregulating small GTPases, respectively. Instead, the phenotypes may reflect the consequences of introducing a block at any stage in the cyclical reactions of small GTPases (Pertz, 2010). This could explain why I encountered the same behavioural phenotype when expressing CA and DN forms of the same small GTPase. This is not uncommon: several studies, especially in neurons, have reported that expressing the CA and DN constructs of the same small GTPase result in the same phenotype (Kuhn et al., 1998; Luo, 2000; Luo et al., 1994). Another significant limitation of DN constructs is that they act by sequestering

GEFs, not by directly inhibiting the endogenous Rho GTPase (Feig, 1999). This is an issue if the GEF acts not on one, but several Rho GTPases. Since Rho-GEF promiscuity is relatively common (Müller et al., 2020), it is likely that the expression of a DN construct of one Rho family member also reduces the activation of other Rho family members. Overall, although DN and CA constructs of small GTPases have been integral to discovering the various functional roles of small GTPases, using them to uncover the molecular pathway of sleep regulation may not be the most appropriate methodology.

It would also have been worthwhile to verify that the OE, CA and DN constructs used in this thesis were actually manipulating Rho1 or Rac1 levels in the correct manner. Although all transgenes were verified with genotyping, the constructs could have been further verified phenotypically using other GAL4 lines, or biochemically. Therefore, additional controls would be essential to determine whether the results observed in this thesis are a true reflection of small GTPase levels.

Another confounding factor of the experiments conducted in this thesis is that the *cv-c*^{MB03717} allele carries a GAL4 insertion which might drive the expression of UAS transgenes in other brain structures. Indeed, when imaging for Rho1 in *UAS-PKNG58AeGFP* expressing *cv-c* mutants, I found high levels of fluorescence in the optic lobes and particularly in the mushroom body (see Appendix). The mushroom body has been implicated in sleep regulation: broad activation of mushroom bodies promotes sleep (Pitman et al., 2006) and within the mushroom body, different neural subtypes have sleep-promoting and wake-promoting roles (Sitaraman et al., 2015). In addition, micro-RNAs that regulate sleep have been identified to function specifically within the mushroom body (Hobin et al., 2021; Wiggin et al., 2020). Therefore, the expression of UAS-transgenes within the mushroom body of *cv-c* mutants might have led to conflicting results.

Nevertheless, Rho1 and Rac1 were also manipulated in the *cv-c* RNAi background and the behavioural phenotypes were very similar to those observed in the *cv-c* mutant background. Thus, although it is important to consider that the UAS transgenes were partially expressed in brain structures other than the dFB, the sleep behavioural phenotypes observed and discussed are mainly due to the role of Rho1 and Rac1 in dFB neurons.

4.5 Conclusion

Overall, this thesis suggests either a vexingly complex role of small GTPases in regulating sleep in *Drosophila* or a paucity of reliable tools to define this role. The work presented here demonstrates that active Rho1 is upregulated in *cv-c* mutants and that manipulating Rho1 in dFB neurons of *cv-c* mutants rescues the insomniac phenotype. In addition, knocking down Rho1 in *cv-c* mutants rescues the membrane excitability of dFB neurons back to normal. This suggests that Rho1, and its regulation by the GAP Cv-c, is an important part of the molecular pathway underlying sleep regulation. However, to what extent Rho1's interacting partner Rac1 is involved, remains unclear. Furthermore, I was unable to come to firm conclusions regarding the role of small GTPases in regulating the endocytosis of Sandman. In addition to methodological limitations, the complexity of small GTPase regulation contributed to conflicting results. This complexity is likely the result of Rho-GEF and GAP promiscuity, cross-talk between small GTPases and the importance of tight spatiotemporal regulation of small GTPases. This complexity is not unique to small GTPases: the function of BMPs for example cannot be defined by their effects on phenotypes (Klumpe et al., 2020). This is because signalling molecules such as GTPases and BMPs can trigger different downstream effectors, and thus phenotypes, depending on where they are expressed, when they are expressed and at what

concentration. This highlights that studying keystone signalling molecules such as small GTPases requires much more finely-tuned manipulations. In conclusion, this thesis illustrates the complexity of small GTPase regulation and calls towards moving away from using DN and CA GTPase constructs to elucidate behavioural phenotypes.

5 Future Directions

Future experiments should further verify the role of small GTPases in homeostatic sleep regulation using alternative methods, such as FRET, as discussed earlier. In addition, the role of downstream candidates such as ROCK and WASP, as well as how they regulate the endocytosis of Sandman, will be critical to understanding how small GTPases regulate dFB neuron excitability and thus the wake-to-sleep switch.

Determining the signals upstream of small GTPases in dFB neurons may give us insight into why sleep evolved in the first place. Since mutating *cv-c* specifically disrupts homeostatic sleep regulation, it is likely that markers signalling sleep need feed into upstream pathways. Signals acting on small GTPases in dFB neurons likely originate from R5 neurons, since these have been identified as an upstream integrator of the sleep homeostat, signalling sleep need (S. Liu et al., 2016). It would also be interesting to investigate how the circadian system and the homeostat interact via small GTPases, since Rho1 has been found to cycle in sLNV clock neurons in *Drosophila.* Thus, uncovering the upstream signals acting on *cv-c* and small GTPases will help elucidate a complete molecular model of sleep regulation in *Drosophila,* and could help uncover the primordial function of sleep, that to this day remains elusive.

The close homology of small GTPases across the animal kingdom and the conserved nature of sleep regulatory pathways, strongly suggests that the role of small GTPases in regulating sleep is evolutionarily conserved. Notably, a Rho-GAP was found to be downregulated after sleep deprivation in the cnidarian *Hydra vulgaris* (Kanaya et al., 2020). This suggests that Rho GTPases have formed part of the molecular pathway underlying sleep since the origin of sleep. This further implies that understanding how

small GTPases regulate sleep in *Drosophila* will reveal a lot about the molecular nature of sleep regulation in mammals and humans. How these findings apply to mammalian systems will be of significant importance and could inaugurate a pipeline for the development of spatiotemporally precise sleep-disorder treating drugs. In a society where sleep is often not prioritised, and where sleep disorders have become a public health epidemic as declared by the CDC (Rodriguez, n.d.), uncovering the molecular nature of sleep will be invaluable for improving human wellbeing and health across the globe.

6 Appendix

Appendix A: Recipe for fly food

Table 3: Food was produced in-house by research technicians. This is the recipe for brown food which was used for all crosses, experiments and to maintain fly stocks.

Compound	Amount
Distilled Water	12L
Agar	81g
Yeast	300g
Cornmeal	750g
Molasses	450ml
Propionic acid	50.4ml
Tegosept	16.8g
Ethanol	84ml

Appendix B: Dia-GFP Rho1 activity biosensor confirms that Rho1-GTP is upregulated in dFB neurons of *cv-c* mutants

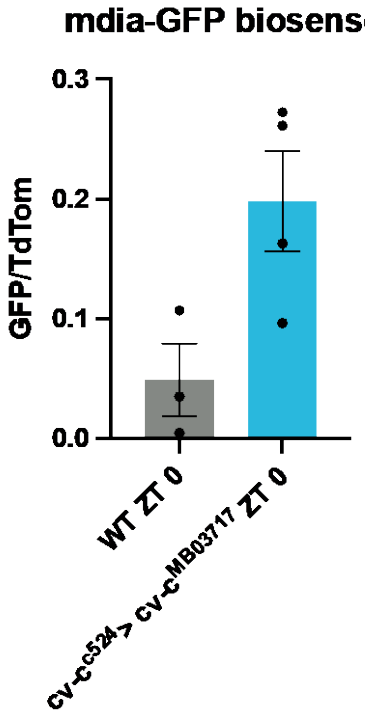


Figure 6-1: dia.RBD-GFP /CD4:tdTomato ratio quantified for WT and *cv-c* mutant flies immobilised at ZT 0. *n* = WT (3) and *cv-c* (4). The genotypes of the flies were: *UAS-P-dia.RBD-GFP (#52291); R23e10-GAL4 > UAS-CD4::tdTomato* and *UAS-P-dia.RBD-GFP (#52291); R23e10-GAL4, cv-c^{c524} > UAS-CD4::tdTomato; cv-c^{cMB03717}*

Appendix C: UAS-PKNG58AeGFP signal in the mushroom body of *cv-c* mutants

This is an example image in which Rho1-GTP fluorescence was very high in the mushroom body. This is not representative of all images (there was ample variability in the extent to which the GFP signal was visible in the mushroom body). Nonetheless, this suggests that *UAS-PKNG58AeGFP* was expressed in off-target brain structures due to the GAL4 insertion in *cv-c*^{MB02717}. Note that puzzlingly the *UAS-CD4:tdTomato* signal in the same fly could not be detected in the mushroom body.

The genotype of this fly was: *UAS-PKNG58AeGFP; R23e10-GAL4, cv-c*^{c524} *> UAS-CD4:tdTomato; cv-c*^{MB03717}*, UAS-Rho1-OE (#28872).*

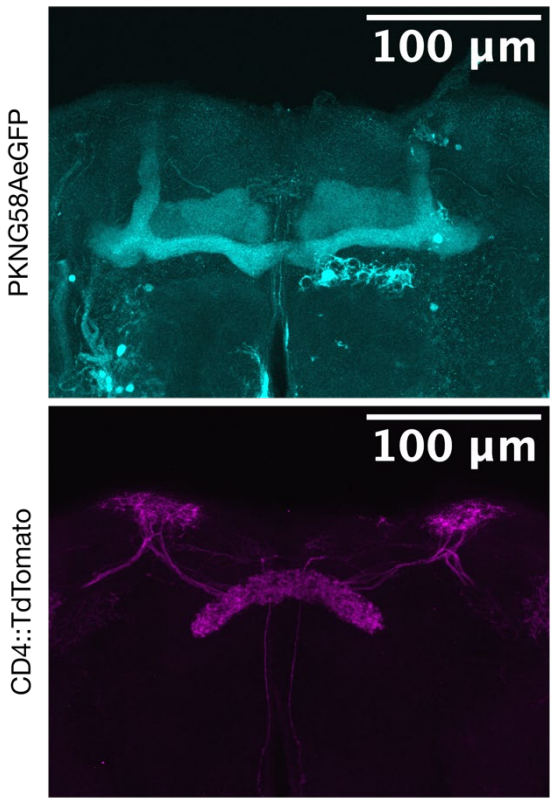


Figure 6-2: Example of PKN-GFP signal in mushroom body. Maximum intensity projection of a z stack that includes the mushroom body was generated.

D: Fly strains used in this study

Table 4: Fly stock name, source and type for all fly strains used in this study. BDSC refers to Bloomington Drosophila Stock Centre and CNCB refers to Centre for Neural Circuits and Behaviour

Fly stock	Source	Type
Canton-S (CS)	CNCB stock collection	Wild-type strain
cv-c^{c524}	CNCB stock collection	Mutant allele
cv-$c^{MB02717}$	CNCB stock collection	Mutant allele
cv-c^{1}	CNCB stock collection	Mutant allele
R23e10- GAL4	CNCB stock collection	Gal4-driver for dFB
GAL80ts, R23e10-GAL4	CNCB stock collection	Temperature sensitive Gal80 recombined with R23e10-Gal4 driver
UAS-mCherry-PA-RacQ61L	BDSC #32049	Photoactivable constitutively active Rac1
UAS-mCherrry-PA-RacT17N	BDSC #31429	Photoactivable dominant Rac1
UAS-Star-GFP-LOVSsrA	Michael Glotzer	LOV domain fused to SsrA under UAS control
Ubi>SspB-GFP-LARG	Michael Glotzer	LARG fused to SspB, driven by ubiquitin promoter
Ubi>SspB-mScar	Michael Glotzer	mScar fused to SspB, driven by ubiquitin promoter
UAS-CD4:tdTomato	CNCB stock collection	membrane anchored tdTomato
UAS-PKNG58AeGFP	James C. Hombria	Rho1-GTP biosensor. Expresses the Rho1 binding domain of PKN fused to GFP under UAS control.
UAS-P-dia.RBD-GFP	BDSC #52291	Rho1 GTP biosensor. Expresses the Rho1 binding domain of dia fused to GFP under UASp control.
Sandman-RFP (RFP-CG8713(20217))	WellGenetics	CRISPR/Cas9- mediated knock-in of a N-terminal RFP fusion protein of CG8713

Fly stock	Source	Type
UAS- cv-c RNAi	CNCB stock collection	UAS responder RNAi knockdown of *cv-c* under UAS control
UAS- mCD8::GFP	BDSC #32185	UAS responder mCD8 tagged GFP under the control of 10 UAS sequences
UAS- Rho1.V12	BDSC #7330	UAS responder: Constitutively active form of Rho1
UAS- Rho1.N19	BDSC #7328	UAS responder Dominant negative form of Rho1
UAS- Rho1.W	BDSC #28872	UAS responder Wild type Rho1
UAS- Rac1.V12	BDSC #6291	UAS responder Constitutively active form of Rac1
UAS- Rac1.N17	BDSC #6292	UAS responder Dominant negative form of Rac1
UAS- Rac1.L	BDSC #6293	UAS responder Wild type Rac1
UAS- Rac1. W	BDSC #28874	UAS responder Wild type Rac1
UAS- Cdc42.N17	BDSC #6288	UAS responder Dominant negative form of Cdc42
UAS-Rab5-OE	BDSC #43335	UAS responder: WT overexpression of Rab5
UAS-Rab5-CA	BDSC #43336	UAS responder: Constitutively active form of Rab5
UAS-Rab5-DN	BDSC #42703	UAS responder: Dominant negative form of Rab5
UAS-GFP-RNAi	CNCB stock collection (60103tk)	RNAi control
UAS-Rho1-RNAi (109420kk)	Vienna Drosophila Resource Centre	UAS responder RNAi knockdown of Rho1 under UAS control
UAS-Rac1 RNAi (49246GD)	Vienna Drosophila Resource Centre	UAS responder RNAi knockdown of Rac1 under UAS control

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