Identification and characterisation of a novel aldo-keto reductase regulating sleep in *Drosophila melanogaster*

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Department of Life Sciences
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A thesis submitted for the degree of Doctor of Philosophy

2017

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Herewith I confirm that the work enclosed herein is my own original written work.

I am the sole and independent author of this written work and I report scientific results that I have collected myself, and with the assistance of students co-supervised by me in the course of my PhD studies as specifically acknowledged.

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To my family,
the one I made and the one I was given

Acknowledgements

Personal

I would like to thank my wonderful lab for their patience and support.

Institutional

Anne Petzold's PhD scholarship was funded by the Edmond Safra Foundation.

Some antibodies were received as kind gifts from other laboratories such as anti-GadI (Rob Jackson, Tufts University), anti-DDC (Jay Hirsh, University of Virginia), and anti-sNPFR (Kweon Yu, KRIBB).

We would also like to thank Dr. Waddell (CNCB, Oxford) for generously sharing fly stocks and Dr. Jongkyeong Chung (Seoul National University) for sharing the MIP-Gal4 line.

We would also like to thank the Facility for Imaging by Light Microscopy (FILM) of Imperial College London and its most helpful staff, particularly Stephen Rothery, for their expert assistance. FILM is part supported by funding from the Wellcome Trust (grant 104931/Z/14/Z) and BBSRC (grant BB/L015129/1).

We would also like to thank PhD candiate Jess Sharrock for her help with thin layer chromatography, and Dr. Katrin Kierdorf for her most helpful comments on earlier versions of this thesis.

Fruitful discussions on the project by Drs. Tony Southall and Nick Franks (Imperial College London) acting as Anne Petzold's Progress Review Panel were also very much appreciated.

Student projects contributing to this study

Copulation data was collected by Solweig Schlumberger as part of her B.Sc. project co-supervised by Anne Petzold and entitled "A novel Aldo-Keto Reductase in Drosophila melanogaster linking sleep and sex" (2017).

The homeostatic sleep response was characterised by Ho Yan Grace Wu as part of her M.Res. project co-supervised by Anne Petzold and entitled "A novel regulator of sleep homeostasis in Drosophila melanogaster" (2017).

Abstract

Sleep is an important behaviour that has been observed in all animals studied so far, including *Drosophila melanogaster*. While a range of studies demonstrates that sleep is crucial to preserve an animal's cognitive and physical health, relatively little is known about its regulation. Specifically, the molecular mechanisms that regulate sleep are still unclear, although a strong heritable component prevails. While the gene network governing sleep timing – the circadian system – is comparatively well described, the network calibrating the sleep homeostat is still vague. The study of sleep-regulatory mechanisms was greatly advanced by the adoption of the relatively simple model organism *Drosophila melanogaster*. Following seminal studies that demonstrated the suitability of *Drosophila* for the study of sleep, several sleep mutants were identified. Among those, *hyperkinetic* – a member of the aldo-keto reductase (AKR) family of enzymes – was one of the first *Drosophila* sleep mutants ever described. The abundance and importance of AKRs for human health and disease in combination with their conservation in *Drosophila* provides an excellent basis to examine whether AKRs systematically act as molecular regulators of sleep behaviour.

Thus, we produced the first *in silico* study of potential *Drosophila* AKRs. We experimentally tested potential AKRs for neural sleep-regulatory roles through a nervous system-wide and Mushroom body-specific screen. We identified and further characterised the neural populations expressing particular candidates and the role these candidate AKRs fulfil to modulate sleep behaviour.

We identified a novel AKR, CG10638, which specifically acts through a pair of wake-promoting peptidergic projection neurons termed "inferior contralateral interneurons" (ICLI) whose inhibition is necessary to relieve sleep pressure built up during the day, or specifically induced through dedicated sleep deprivation. We also demonstrate that ICLI neurons receive inputs from the major 'clock' neurons of the fly brain, and show that CG10638 negatively regulates ICLI neurons to acutely gauge the sleep homeostat and to integrate sleep pressure with the circadian network.

We also identified a Mushroom body-specific sleep-regulatory AKR, CG3397, that may be close to Hyperkinetic in structure and function.

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List of Abbreviations

ab . . . . . **antibody**

ACER . . . . . *angiotensin-converting enzyme-related*

ACh . . . . . Acetylcholine

AKR . . . . . Aldo-keto reductase

ARY . . . . . *aldehyde reductase Y*

BDSC . . . . . Bloomington Drosophila Stock Centre

BRP . . . . . *bruchpilot*

cDNA . . . . . clonal DNA

CLK . . . . . *clock*

CRY . . . . . *cryptochrome*

CT . . . . . Circadian time

CYC . . . . . *cycle*

DAM . . . . . Drosophila Activity Monitor

DD . . . . . Dark:Dark schedule

dFSB . . . . . dorsal fan-shaped body

DGDP . . . . . Drosophila Genome Disruption Project

DILP . . . . . *Drosophila* insulin-like peptide

DN$1-3$ . . . . dorsal neurons, subpopulations 1-3

DSHB . . . . . Drosophila Hybridoma Bank

dsRNA . . . . dorsal-stranded RNA

DOW . . . . . Disc-over-water method

dPC . . . . . **dorsal protocerebrum**

EEG . . . . . Electroencephalogram

EB . . . . . Ellipsoid body

ELAV . . . . . *Embryonic Lethal, Abnormal Vision*

FABP . . . . . *fatty acid binding protein*

FAD . . . . . flavin adenine dinucleotide

gDNA . . . . . genomic DNA

GFP . . . . . Green Fluorescent Protein

GRASP . . . . GFP Reconstitution Across Synaptic Partners

GWA . . . . . Genome-wide association study

HK . . . . . *hyperkinetic*

Hrct . . . . . Hypocretin

IPC . . . . . Insulin-producing cell

IR . . . . . Inverted repeat

KC . . . . . Kenyon cell (of the MB)

K_v . . . . . voltage-gated K^+ channel

LD . . . . . Light:Dark schedule

l-LN_v . . . . . large ventral lateral neurons

s-LN_v . . . . . small ventral lateral neurons

MB . . . . . Mushroom body

MIP . . . . . Myoinhibitory peptide/Allostatin B

MSA . . . . . Multiple sequence alignment

NMJ . . . . . Neuro-muscular junction

SH . . . . . *shaker*

PDF . . . . . Pigment-dispersing factor

PDFR . . . . . PDF receptor

PER . . . . . *period*

PI . . . . . Pars intercerebralis

PPI . . . . . Protein-protein interaction network

qRT-PCR . . . Quantitative real-time polymerase chain reaction

QVR . . . . . *quiver*

RDL . . . . . Resistant-to-dieldrin

RFP . . . . . Red Fluorescent Protein

RNAi . . . . . RNA interference

ROS . . . . . Reactive oxygen species

SCN . . . . . Suprachiasmatic nucleus

SD . . . . . **Sleep deprivation**

shRNA . . . . short-hairpin RNA

SHY . . . . . Synaptic homeostasis hypothesis

sNPF . . . . . small Neuropeptide F

sNPFR . . . . sNPF receptor

SOG . . . . . Subesophageal ganglion

SP . . . . . Sex Peptide

SPR . . . . . SP receptor

SSS . . . . . *sleepless*

SWA . . . . . Slow-wave activity

SWS . . . . . Slow-wave sleep

TIM . . . . . *timeless*

TRPA1 . . . . Transient receptor potential channel, subtype A1

TRiP . . . . . Transgenic RNAi Resource Project

TTFL . . . . . Transcription-translation feedback loop

UAS . . . . . Upstream-activating sequence

VDRC . . . . . Vienna Drosophila Resource Centre

WAKE . . . . *wide awake*

ZT . . . . . Zeitgeber

1 Introduction

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1.1 Essential considerations regarding sleep behaviour

1.1.1 What is sleep and is it important?

What is sleep?

Sleep is a reversible condition characterized by physical quiescence, e.g., immobility in humans and many other animals, and an elevated arousal threshold (Campbell et al. 1984). Often, sleep is associated with a species-specific posture, e.g., lying down in humans and many other animals (Campbell et al. 1984). Crucially, sleep is under circadian and homeostatic control: the timing of sleep in a species is generally regular within the nychthemeron (Beersma et al. 2007; Fisher et al. 2013; Hendricks et al. 2004) and animals temporarily deprived of sleep generally respond with an increase in sleep duration and/or intensity (Allada et al. 2017).

Despite being associated with overt quiescence, sleep is a heterogenous state. Instead, it is organized in different stages that have traditionally been defined according to their specific signatures in electroencephalographic (EEG) recordings (Siegel 2002, part I). In mammals and birds, brain activity as measured by EEG switches between fast frequencies of low amplitude during wake and rapid eye movement (REM) sleep and slow frequencies of high amplitude during slow-wave activity (SWA), with cycles of REM sleep and different stages of SWA systematically alternating throughout the night (Siegel 2002, part I). In humans, different stages of sleep as defined by their EEG signature are associated with different sleep depth as indicated by the intensity of stimuli sufficient for arousal (Hirshkowitz 2004).

Sleep characteristics differ among members of a species, in part due to natural genetic variation. For instance, sleep-related human EEG profiles differ strongly between individuals (Lewandowski et al. 2013) and the heritability of such EEG profiles is relatively high (Ambrosius et al. 2008; De Gennaro et al. 2008). Even on an individual level, sleep is not a uniform behaviour, but differs throughout life depending on gender, age, food availability, environmental conditions, reproductive state, and other factors (Siegel 2005).

Is sleep important?

A set of seminal studies demonstrated the deleterious effects of sleep deprivation and suggest that sleep is absolutely necessary to maintain an organism's normal functions.

Sleep-deprived rats on a disk over water are on the brink of death The debilitating effects of chronic sleep deprivation were demonstrated by seminal studies in the rodent model by Allan Rechtschaffen using the disk-over-water (DOW) method which he developed. In the DOW approach, the experimental animal is placed on a disk suspended over water which starts to rotate once the animals shows signs of sleep as indicated by its EEG profile. The rat would slide into the water – which rats typically avoid – unless it starts walking. An otherwise unperturbed control animal is placed on the other side of the disk (Rechtschaffen et al. 1995).

Using the DOW method, Rechtschaffen showed that sleep deprivation elicited a strong rebound sleep response (Rechtschaffen et al. 1983). During prolonged sleep deprivation, rats failed to maintain their regular temperature rhythms, appeared increasingly unhealthy, developed skin lesions, and lost weight (Rechtschaffen et al. 1983). Eventually, prolonged sleep deprivation culminated in death (Rechtschaffen et al. 1983).

Prolonged sleep deprivation impairs a range of bodily and cognitive functions This initial study by Rechtschaffen did not detect major immune deficiencies in the sleep-deprived group (Benca et al. 1989), but subsequent studies

and studies from other groups showed that prolonged sleep deprivation breaks down host defense mechanisms in rodents (Bergmann et al. 1996; Everson 1993).

In humans, lack of sleep severely affects cognitive performance (Durmer et al. 2005; Van Dongen et al. 2003) and decision making (see Pilcher et al. 1996 for a meta-analysis), and also impacts on the immune system (Moldofsky et al. 1989). Chronic sleep deprivation – such as shift work – poses a medical risk factor. It increases, among other diseases, cancer risk (Haus et al. 2013). In fact, the debilitating effects of shift work are now a recognised syndrome termed shift work disorder (Wright et al. 2013).

1.1.2 How is sleep controlled?

Initial studies of sleep regulation in mammals

Lessons from encephalitis lethargica A century ago, the neurologist Constantin von Economo first noted the symptoms of a 'sleeping sickness' that would soon bear his name: 'von Economo disease' or encephalitis lethargica (Economo 1930), an atypical form of encephalitis. He noted that patients show "a sopor [an abnormally deep sleep or stupor] of different degree varying from simple somnolence to the deepest sopor in which the patients may sleep for weeks and months but from which in the majority of cases, it is possible to arouse them" (Economo 1930).

The reversibility of the state distinguished the condition from coma; the brevity of this reversal marked the condition as a dangerous disease. From extensive *post mortem* studies in patients, von Economo and others concluded that the condition was associated with brain lesions specifically located in the midbrain and the brainstem (Dale et al. 2003).

The rising dominance of EEG sleep signatures In 1937, Frédéric Bremer introduced his "encephale isolé" preparation, a transection approach where the cerebrum of a cat is disconnected from the rest of the brain at different levels which allows the investigation of the contribution of midbrain and brainstem structures on the overt sleep behaviour and the electroencephalogram (EEG) profile in detail

(Kerkhofs et al. 2000). With the advent of EEG, sleep research quickly moved to EEG as the tool of choice to study sleep in a non-invasive way in humans and laboratory mammals (Borbely et al. 1981; Moruzzi et al. 1949; Sallanon et al. 1989; Swett et al. 1968).

Initial insights into sleep regulation in mammals In brief, the timing of sleep in mammals relies on a functional 'master clock', the suprachiasmatic nucleus (SCN, Mistlberger 2005), a structure that is also essential for temperature regulation and its close association with sleep (Eastman et al. 1984). This circadian sleep drive is an output of the 'molecular clock' – a system of clock proteins that rhythmically control their own expression and thereby maintain rhythmic levels – that has initially been identified in fruit flies (section 1.4, Fig. 1.7). Sleep behaviour is further shaped by other, interacting brain regions, especially the thalamus, hypothalamus, and the reticular formation (Eban-Rothschild et al. 2017; Saper et al. 2005). While the regulation of sleep *timing* is under circadian control, sleep can occur in the absence of a circadian clock. For instance, sleep – as well as sleep-temperature dynamics – still occur in rats with lesions of the SCN (Baker 2005), and mice carrying mutations of clock genes retain the ability to sleep (DeBruyne et al. 2006; Shiromani et al. 2004). Interestingly, the EEG profile during slow wave sleep (SWS) – SWA – is largely independent of circadian drives and explained by sleep pressure: SWA is enhanced when sleep pressure is increased due to dedicated sleep deprivation, and, if subjects are given opportunities to nap, its prevalence increases throughout the waking day (Dijk 2009).

Two-process model of sleep regulation

Generally, sleep is thought to be governed by two antagonistic processes: circadian drive (process C) and homeostatic sleep drive (process S, Borbély 1982).

On the one hand, sleep/wake rhythms follow a 24 h pattern with diurnal animals such as humans and fruit flies sleeping mainly during the night. On the other hand, the longer an animal stays awake, the stronger the drive to sleep becomes, while sleep drive dissipates when the animal is asleep. The circadian and homeostatic

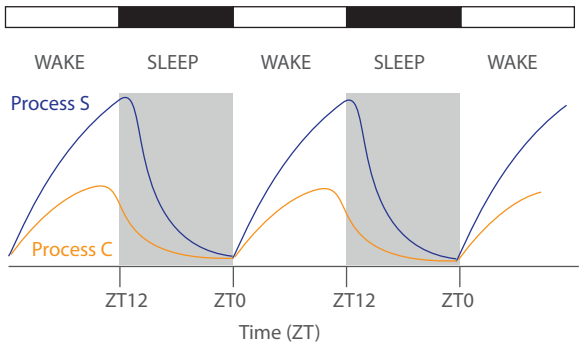


Figure 1.1: Two-process model of sleep regulation. This model proposes two partly antagonistic mechanisms that interact to produce alternating sleep/wake rhythms. Here, circadian mechanisms prepare the organism to initiate sleep at times appropriate for the species, e.g., towards the night in diurnal species, and to terminate sleep at appropriate times leading up to the main activity period, e.g., towards the morning. A homeostatic drive traces sleep pressure accumulating during wake until sleep is initiated. Once both drives peak, e.g., in the evening for diurnal species, sleep is iniated, and once both drives dissipate, sleep is terminated. The model predicts that homeostatic sleep drive maintains sleep towards the end of the night to counteract the rousing influence of the circadian system. This illustration is adapted from Borbély 1982 who first proposed this model.

sleep drives work in opposition: the circadian process drives wakefulness during the main part of the day, until sleep pressure is high enough to counteract the circadian process and initiate sleep (Borbély 1982; Lockley et al. 2012, see Fig. 1.1).

In general terms, circadian processes determine *when* to sleep, while homeostatic processes impact on sleep quality - *how long* or *how deep* we sleep, for instance. In mammals, SWA is often used as a marker of homeostatic sleep pressure (Dijk 2009).

The two-process model of sleep regulation is quite broad. While it does make strong assumptions about the existence of independent, albeit interacting circadian and homeostatic processes, it does not preclude, e.g., the neural correlates or the molecular underpinnings that govern these regulatory processes, which may be vastly different across species, and may even differ considerably within the lifetime of individuals. It thereby provides a valuable unifying framework for the study of sleep across phylogeny (Borbély et al. 2016; Skeldon et al. 2014).

1.1.3 Conservation of sleep behaviour across species

Is sleep ubiquitous?

The dominance of the EEG discouraged sleep research of animals where this tool does not apply such as insects which lack a cortical structure (Vorster et al. 2015; Zimmerman et al. 2008). Thus, in order to investigate sleep throughout phylogeny, the wider field adapted a definition of sleep that is broad and consists of mainly behavioural criteria as introduced in section 1.1.

While some comprehensive reviews find evidence for the ubiquity of sleep throughout the animal kingdom (Campbell et al. 1984; Cirelli et al. 2008), other reviews caution that sleep behaviour is highly diverse among species depending on their size, ecological conditions, and many other factors and thus should not be interpreted as a single type of behaviour (Siegel 2005).

Sleep in non-mammalian model systems

Sleep in fishes The zebrafish *Danio rerio* exhibits regular rest:activity patterns that are under circadian control with the main portion of activity occurring during the day (Hurd et al. 1998). This is true of both the adult (Hurd et al. 1998) and the larval stage (Cahill et al. 1998). In zebrafish, sleep is regulated by the hypocretin (Hcrt) system (Prober et al. 2006; Yokogawa et al. 2007), a peptide crucially involved in narcolepsy and in healthy sleep regulation in mammals (Ebrahim et al. 2002; Siegel et al. 2001). An initial study by David Prober and collegues demonstrated that the circuitry of Hcrt+ neurons is similar to that of mammals in structure and function. For instance, overexpression of Hcrt promotes wakefulness in zebrafish as it does in mammals (Prober et al. 2006). Similarly, mutations of the Hcrt receptor heavily destabilise sleep, probably through a disruption of sleep-promoting GABA release (Yokogawa et al. 2007).

Sleep in round worms The round worm *Caenorhabditis elegans* also exhibits quiescence during lethargus – the period preceding each of the four moults at regular times of the day (Raizen et al. 2008). Consequently, it experiences four

episodes of potentially sleep-like behaviour in the course of its short life. Using an automated system to mechanically stimulate the dish that the worms inhabit, Raizen et al. show that the arousal threshold of worms is increased in the lethargus phase. Additionally, disturbance preceding the lethargus stage increase quiescence during this lethargus stage, indicating homeostatic rest regulation (Nagy et al. 2014; Raizen et al. 2008). Lethargus is timed by the *lin-42* gene – a homologue of the *Drosophila* and mammalian clock gene *period* – indicating that this behaviour is under circadian control (Jeon et al. 1999). A recent study demonstrated that rest can also be induced by modulation of O_2 levels, and that the induced rest state is accompanied by neural quiescence (Nichols et al. 2017).

Conservation of sleep behaviour in insects

Generally, rhythmic rest:activity cycles that are under circadian control have long been known to be a common feature among animals including insects (Brady 1974; Zimmerman et al. 2008). However, relatively little attention has been paid to the rest phase of these cycles. In their seminal review on animal sleep, Campbell and Tobler note that "[...] the presence of sleep among insects remains highly debatable" (Campbell et al. 1984). However, they go on to present a body of evidence suggesting that the insect rest state may be considered a sleep-like state.

A sleep-like state in honeybees Early evidence for sleep-like states in insects comes from studies of the diurnal forager bee *Apis mellifica carnea*: firstly, the activty pattern of this bee is regular and under circadian control, peaking around mid-day (Kaiser et al. 1983). Forager bees exhibit different arousal thresholds at different times of the night, indicating that their rest may be a dynamic state with stages of different depth (Sauer et al. 2003). Additionally, the firing activity recorded from an optomotor interneuron in response to a moving target exhibits circadian variation with little activity during the night, and an increase in responsivity when the animal was given an arousing air puff (Kaiser et al. 1983). Later studies detected evidence for a homeostatic rest reponse: forager bees respond to continuous disturbance with an increase in both the duration and the intensity of rest when the disturbance

occured during their main rest phase – at night – but not when it occured in their main activity phase (Sauer et al. 2004). The dynamic and duration of sleep-like behaviour changes drastically throughout life in the forager bee, although it is difficult so differentiate whether this is a pure age-dependent effect or due to the change in caste which is both age-dependent and associated with a change in tasks (Klein et al. 2008). Interestingly, forager bees exhibit a preference for the site of sleep, and their preffered site of sleep changes with a change in caste (Klein et al. 2014).

A sleep-like state in cockroaches The cockroach *Leucophaea maderae* exhibits regular rest periods that are under circadian control (Roberts 1960; Roberts 1962). *Leucophaea* spends around 60 % of time per day in rest and exhibits an activity increase in the beginning of the night (Tobler 1983). Evidence for circadian modulation of neural excitability in *Leucophaea* has been specifically reviewed by Terry Page (Page 2009). Manually depriving these nocturnal animals of rest for 3 h before lights-off decreases activity in the beginning of the night, pointing to the existence of a homeostatic rest response (Tobler 1983). Studies of the scorpion *Pandinus heterometrus* (Tobler et al. 1988) and another cockroach, *Blaberus giganteus* (Tobler et al. 1992), reported similar findings.

1.1.4 Conservation of sleep behaviour in fruit flies

Campbell and Tobler already noted that insects, although not well studied at that point for their rest behaviour, exhibit long periods of immobility during rest (Campbell et al. 1984).

Two pioneering studies have directly addressed the question whether rest behaviour in *Drosophila melanogaster* can reasonably be considered sleep. The first such study focussed on the question whether rest in fruit flies was actively regulated and found that, firstly, flies sought out a particular location – in the vicinity of food medium – to rest, and rest periods were regular in timing and duration (Hendricks et al. 2000). Furthermore, the baseline rest profile of clock mutants – per^0 and tim^0 – were severely dampened compared to wildtype control

flies (Hendricks et al. 2000). These initial findings strongly suggest that sleep in flies is under circadian regulation. Since sleep in other species is also under homeostatic control, Hendricks et al. subjected flies to mechanical stimuli of different intensity and detected an increased arousal threshold in resting flies compared to mobile siblings. Additionally, flies that have been sleep deprived during their main rest phase – at night – subsequently increased the amount of sleep, while the same disruption during their active phase – during the day – did not, although sleep deprivation was achieved by manual perturbation in this study. Conversely, flies sleep less when exposed to caffeine (Hendricks et al. 2000), a somnolytic agent that antagonises the adenosine A1 receptor in mice (Greenstone 1990) and humans (Biaggioni et al. 1991). These findings strongly suggest that rest in *Drosophila* is an essential behaviour that is precisely regulated by both circadian and homeostatic mechanisms and shares the main characteristics of sleep in other species.

Another study later that year by Shaw et al. confirmed the homeostatic response to sleep deprivation using an automated system for sleep disruption (Shaw et al. 2000). It also confirmed the dampening of the sleep profile in clock mutants and identified additional proteins known to correlate with sleep deprivation in rats to be upregulated in flies (Shaw et al. 2000).

1.1.5 Why do animals, including fruit flies, sleep?

The presence of specific EEG signatures during sleep reflects a systematic shift in neural activity which points to the functions fulfilled by sleep in relation to the nervous system: the functions most commonly associated with sleep are energy conservation, restorative functions on a cellular level such as metabolite clearance, and synaptic homeostasis which includes neural processes relating to memory consolidation and cognitive performance (Chakravarti et al. 2017; Harbison et al. 2009; Miyazaki et al. 2017; Siegel 2005). These putative functions are not mutually exclusive, but are likely dependent on each other and share some regulatory pathways (Zielinski et al. 2016).

Metabolic homeostasis Sleep has been proposed to be a state of "adaptive immobility" that serves to conserve energy similar to seasonal periods of hibernation in some animals (Berger et al. 1995; Schmidt 2014; Siegel 2009; Walker et al. 1980). In fact, sleep and metabolism are intimately linked. In a recent review, Maria Yurgel and collegues point out that insufficient sleep is associated with metabolic diseases such as diabetes and obesity, and that, conversely, metabolic state modulates sleep levels and architecture (Yurgel et al. 2015). For instance, in rats, chronic sleep deprivation leads to weight loss, despite an increase in energy consumption (Rechtschaffen et al. 1983).

In flies, there is also limited evidence for the benefit of sleep for energy homeostasis: generally, the metabolic state in respect to nutrition also impacts on sleep, and changes to sleep affects metabolic rate (the relationship between nutrition and sleep in flies is discussed in section 1.3.4). Challenges to energy homeostasis such as starvation or dietary restriction impact on sleep in fruit flies. For instance, sleep in flies is associated with a decrease in metabolic rate – measured in terms of CO_2 consumption – or energy expenditure during sleep (Stahl et al. 2017). While starved flies sleep less and are more active as they forage for food, they are also able to decrease their metabolic rate in response to starvation, indicating that metabolic rate and sleep are governed by separable mechanisms (Stahl et al. 2017). Additionally, the level of one of the main energy sources in *Drosophila* – glucagon stores – vary throughout the day and are highest during the night (Zimmerman et al. 2003). Flies resistant to starvation due to increased triglyceride stores show adaptive changes in sleep behaviour (Masek et al. 2014; Thimgan et al. 2015), while flies resistant to dessication through increased glucagon stores do not (Thimgan et al. 2015), indicating that sleep levels are not just a consequence of energy levels, but are differentially regulated depending on the specific nutritional environment.

Aside from bodily energy homeostasis, sleep may also aid the clearance of metabolites from the brain. In mammals, the brain claims a high metabolic load (Clarke et al. 1999) producing a high number of metabolic waste products which are potentially toxic. This is particularly apparent under conditions of disease such

as neurodegeneration that sees the accumulation of neurotoxic proteins such as tau and β-amyloid (Nedergaard 2013). Clearance of those substances from the brain is achieved by recirculating cerebrospinal fluid (CSF) through interstitial fluid (ISF) and exchanging it through the glymphatic system – a dedicated vascular system formed by glial cells (Jessen et al. 2015). Sleep may greatly benefit this process: a recent study in mice detected a large decrease in the volume of interstitial space during sleep measured in terms of the expansion of an injected tracer and direct measurements of the interstitial space volume (Xie et al. 2013). In humans, an association between the accumulation of neurotoxic proteins and insufficient sleep has also been observed (e.g., Nesse et al. 2017).

Memory consolidation and synaptic homeostasis Sleep and memory formation are strongly linked. In fact, the different stages of sleep discernible from the EEG profile of a sleeping mammal may differentially contribute to memory consolidation (Stickgold 2005; Vorster et al. 2015). The interaction between memory consolidation and sleep suggests that sleep supports the maintenance of neural processes. Early studies on molecular sleep regulation put forward the synaptic homeostasis hypothesis (SHY), suggesting that sleep serves to downscale synapses that underwent short-term growth during waking hours in order to decrease the metabolic load put on neurons (Tononi et al. 2003, 2006).

There is evidence supporting this hypothesis in mammals. For instance, markers of synaptic number and strength are increased after wake and decreased after sleep (Cirelli et al. 2000; Vyazovskiy et al. 2014) and sleep-related modulation of synaptic potentiation by receptor trafficking has also been observed (Vyazovskiy et al. 2008). Structurally, dendritic spines undergo synaptic remodeling during sleep (Maret et al. 2011; Vivo et al. 2017; Yang et al. 2014; Yang et al. 2012).

There is ample evidence that synaptic plasticity benefits from sleep also in flies, thereby benefiting cognitive performance. Similar to mammals, sleep contributes to learning and memory consolidation in flies: while experiences during wake can alter sleep need in *Drosophila* (Bushey et al. 2011; Donlea et al. 2009), it fails to

do so in known memory mutants (Ganguly-Fitzgerald et al. 2006). Furthermore, flies that sleep very little due to environmental or genetic manipulations are poor learners (Bushey et al. 2007; Glou et al. 2012; Seugnet et al. 2008). Conversely, some memory mutants are also short sleepers (Liu et al. 2008), and an increase in sleep can rescue those deficits in some mutants (Dissel et al. 2015, 2017).

There is further, both molecular and structural evidence for sleep-related synaptic homeostasis in flies: the level of synapse-specific proteins is increased after wake in comparison to sleep and as a consequence of sleep deprivation (Bushey et al. 2011; Gilestro et al. 2009). In flies, synaptic rescaling has also been observed and these changes in synaptic morphology are affected by prolonged sleep deprivation (Bushey et al. 2011; Donlea et al. 2009). Also, sleep can also modulate synaptic potentiation via receptor trafficking (Wu et al. 2014a). However, synaptic rescaling can also occur rapidly in the absence of sleep (Frank et al. 2006). There is also a long list of candidate genes that may be differentially expressed during sleep and wake, particularly genes relating to neural plasticity (Cirelli et al. 2000; Mackiewicz et al. 2009).

Thus, in both mammals and flies, sleep could provide a window of opportunity to prune synapses unspecifically activated during wake, thereby increasing the signal-to-noise ratio of neural firing patterns and decreasing energy expenditure (Tononi et al. 2014). While there is extensive evidence supporting this hypothesis, a *causal* link between synaptic upscaling and sleep induction is still elusive, or, as a critical review recently noted "[...] it is not clear why stronger synapses should make one sleepy" (Frank 2012). It is of note that synaptic homeostasis can occur rapidly and without sleep, at least in the neuro-muscular junction (NMJ, Frank et al. 2006) – a well-established model to study neural plasticity in *Drosophila melanogaster* – and in the rodent cortex (Ibata et al. 2008; Yang et al. 2012). Thus, while SHY provides a potential mechanism to explain the relationship between sleep and memory formation observed on a behavioural level, it does not provide a causal link and should be treated as an informative, but simplified hypothesis.

Drosophila provide an excellent model organism to investigate the molecular mechanism linking synaptic plasticity and sleep. For instance, a combination of studies in mammals and flies identified such a mechanism relating to receptor trafficking: in mammals, receptor recycling by the proteasome may negatively regulate synaptic homeostasis (Willeumier et al. 2006). In fruit flies, levels of *Notch* – a highly conserved transmembrane receptor involved in development and, possibly, maintenance of cell-cell adhesion and communication – are dynamically regulated by ubiquitination following endocytosis in flies (Jékely et al. 2003) and mammals (reviewed in Sorkin et al. 2009). Regulation of *Notch* is crucial to reach the advanced degree of complexity of certain axonal arborizations (Ables et al. 2011; Hassan et al. 2000), to achieve acute synaptic plasticity in response to activity and/or experience (Ables et al. 2011; Bivort et al. 2009), and to successfully form protein synthesis-dependent long-term memory (Ge et al. 2004; Presente et al. 2004, reviewed in Ables et al. 2011). Interestingly, synpatic plasticity through *Notch* signalling may be sleep-dependent as disruption of the *Notch* regulator *bun* affects sleep homeostasis (Seugnet et al. 2011).

1.2 Why and how to study sleep in fruit flies

1.2.1 Hereditability sleep studies from humans to fruit flies (and reverse)

Sleep: a partly heritable, complex trait

It is of importance to note that sleep is a complex trait. While chronic sleep deprivation in rats induced using the DOW method identified a large body of genes that exhibit changes in transcription in response to sleep loss (Cirelli et al. 2006), there are only few genes identified where, conversely, a single mutation strongly affects sleep behaviour in mammals (Tafti et al. 2002). Similarly, in fruit flies, two mutagenesis studies found that only 1 % of several thousand mutant lines showed sleep-related effects greater than two standard deviations from the population average, while more lines had smaller effects on sleep (Cirelli et al. 2005b; Wu et al. 2008). However, when taking into account several loci, the association of genes with sleep traits increases (Harbison et al. 2013).

Motivation for heritability studies of sleep

The search for gene products that affect sleep in mammals has centered around two main non-invasive strategies. Genome-wide association studies (GWA) identify variations such as single nucleotide polymorphisms (SNPs) in gene loci that are associated with a variability of particular sleep traits such as overall sleep duration and latency to sleep onset. Alternatively, gene expression studies search for gene targets whose expression changes in a sleep-dependent manner, often after some form of sleep deprivation (Crocker et al. 2010a; Tafti et al. 2002). Together with GWA studies and forward screens undertaken in *Drosophila*, some conserved genetic targets and pathways emerge.

Throughout more than a century of studying sleep disorders, researchers repeatedly noted that many sleep conditions run in families.

Familial sleep disorders For instance, the initial scientific reports of narcolepsy focussed on dogs, and noted the heritability of the condition deducible from their pedigree (Westphal 1877). Later studies isolated a particular disruption of the hypocretin system – a mutation in the *hypocretin receptor 2* – as the cause of the nacrolepsy syndrome in humans, dogs, and other mammals (Chemelli et al. 1999; Lin et al. 1999). Gene defects do not only contribute to the pathophysiology of narcolepsy (Mignot 1998), but also other devastating sleep disorders such as fatal familial insomnia (Montagna et al. 2003), advanced sleep phase (Jones et al. 1999) and delayed sleep phase syndrome (Ancoli-Israel et al. 2001) as well as restless leg syndrome (Lazzarini et al. 1999; see review of human sleep genetics by Dauvilliers et al. 2005).

Familial sleep traits Furthermore, self-reporting human twin studies imply that both qualitative aspects of sleep such as bed time/waking time and quantitative aspects such as sleep duration, and even susceptibility to sleep disturbance, are driven by a detectable genetic determination (Castro 2002; Gottlieb et al. 2007; Heath et al. 1990; Klei et al. 2005; Partinen et al. 1983). Similarly, not only wake-related EEG profiles exhibit strong heritability (Beijsterveldt et al. 1996), but also sleep-related EEG profiles (Ambrosius et al. 2008; De Gennaro et al. 2008).

Sleepiness-related genes The first sleep trait study that focussed on families – the Framingham Heart Study Offspring Cohort – identified a significant association of sleepiness with circadian components (*CSNK2A2*, *PROK2* and *CLOCK*), and a locus around the gene *PDE4D*, among others (Gottlieb et al. 2007). *PDE4D* encodes a phosphodiesterase that degrades cAMP, which acts as a signal transduction molecule. Interestingly, the fruit fly counterpart of *PDE4D* is the *dunce* gene, a locus already implicated in sleep regulation (Hendricks et al. 2001). A screen for biomarkers of sleepiness in fruit flies identified amylase, an enzyme that converts starch into sugars, as a gene that is upregulated in response to sleep deprivation (Thimgan et al. 2011). Interestingly, expression of this gene is also upregulated in

humans under similar conditions (Thimgan et al. 2011). However, the limited scope of that study cannot directly link amylase levels with sleepiness.

Sleep duration-related genes A recent extensive GWA study for gene variants associated with sleep duration across several European populations identified the *ABCC9* locus as a potential sleep regulatory gene (Allebrandt et al. 2013). *ABCC9* encodes an ATP-sensitive potassium channel subunit, SUR2. SUR2 is conserved in fruit flies, and mildly affects sleep as well (Allebrandt et al. 2013).

Sleep deprivation-response genes A recent study in humans found an overlapping range of genes affected by insufficient sleep, including genes associated with the immune system (e.g., *interleukin-6/IL6*), circadian rhythms (e.g., *PER*, *CYC*, and *CLOCK*) and sleep homeostasis (e.g., *KCNV2*) as well as oxidative stress (i.e., peroxidases) and metabolism (e.g., the glucose transporter *GLUT3*), and detected a general dampening of circadian expression profiles of otherwise cycling genes (Moller-Levet et al. 2013). Interestingly, the involvement of interleukins such as IL6 in sleep has been observed in an independent study in rats (Thimgan et al. 2013) and may be conserved in mice (Morrow et al. 2005), and an upregulation of immune-response genes after sleep deprivation has also been observed in fruit flies (Thimgan et al. 2013).

1.2.2 The power of genetic studies in fruit flies

Advantages of the fruit fly model *Drosophila melanogaster* is an attractive model organism that is easy and cheap to keep. Flies can be raised at high numbers as they are prolific – females can lay up to around 100 eggs a day for up to 20 days (Jennings 2011). Their short reproductive cycle of around 10 days at 25 °C means that genetic manipulations are easily tractable through the progeny (Jennings 2011). In fact, the availability of the whole genome sequence of *Drosophila melanogaster* from the year 2000 onwards (Adams et al. 2000) led to the observation that flies share a large number of genes with mammals (Yoshihara et al. 2001) and paved the way for in-depth genetic studies.

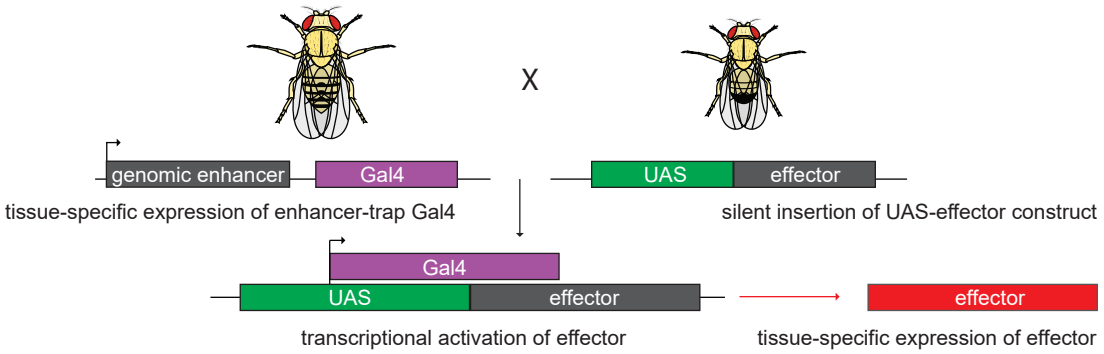


Figure 1.2: The UAS/Gal4 system. The yeast transcriptional activator *gal4* is expressed by genomic enhancers either due to random insertions of Gal4 into these regions, or by combining the Gal4 sequence with a dedicated promoter sequence. The exogenous Gal4 protein is expected to be non-functional in the fly unless it encounters the yeast upstream activation sequence (UAS) to which it binds to 'drive' transcription of UAS – along with gene sequences combined with UAS which are termed 'effectors'. This system was pioneered by Brand et al. 1993 and the schema was inspired by St Johnston 2002.

Effector	Exemplary purpose	Pioneered by
mCD8::GFP	Visualisation of Gal4 expression	Lee et al. 1999
siRNA	RNAi-mediated gene knock down	Dietzl et al. 2007
TRPA1	Heat-dependent depolarisation	Hamada et al. 2008 Parisky et al. 2008
Kir2.1	Membrane hyperpolarisation	Baines et al. 2001
Shibire	Synaptic block	Vicario et al. 2001
CsChrimson	Optogenetic neural activation	Klapoetke et al. 2014

Table 1.1: Examples of effectors of the UAS/Gal4 system

Their progress was greatly advanced by the genetic toolbox available for flies such as transgenic systems to manipulate cells' genetic makeup and/or activity levels in a spatially and temporally restricted manner. These tools include RNAi-mediated gene silencing to accompany comprehensive libraries of mutant lines that cover large parts of the genome (Hendricks et al. 2004). The basic principle of these transgenic systems – the UAS/Gal4 system – is depicted in Fig. 1.2. Similar alternative systems such as the lexA/lexAop system have also been developed (del Valle Rodríguez et al. 2012).

The large toolbox of effector lines allows the combination of genetic and circuit-based strategies such as combinations of electrical, thermogenetic, or optogenetic neural silencing/activation and RNAi-mediated knock down. Common examples

of effectors are listed in Table 1.1.

Pioneering studies in behavioural genetics in the fruit fly model: clock work *Drosophila melanogaster* have long been used to study the circadian system, ever since the first circadian gene was identified in fruit flies in 1971 by the work of Ronald Konopka (discussed in section 1.4). Although it took two decades and major advances in the field of genetics to identify further core components of the fly 'clock', the promising fact remains that the core clock is conserved in mammals in genetic components and in principle (Andreani et al. 2015; Dubowy et al. 2017).

1.2.3 Genetic trait analysis of sleep in fruit flies

The last decade has seen dedicated genome-wide screens for candidate sleep regulatory genes in fruit flies including transcriptome screens, as well as forward and reverse genetic screens, or a combination. Such GWA studies are correlative. In *Drosophila*, these can be combined with directed genetic manipulations that target the candidate gene(s) identified in a forward screen, often within specific target populations using aforementioned driver lines (see section 1.2.2, Hendricks et al. 2004).

Transcriptome screens One set of studies undertook extensive screens for genes whose expression was altered in response to sleep deprivation (e.g., Cirelli et al. 2005b and Williams et al. 2007). For instance, the first such study assessed the transcriptome after the normal waking day, after a period of dedicated sleep deprivation and after a night of undisturbed sleep, and detected a range of genes that responded to increasing sleep pressure, including a number of genes involved in metabolism and the stress response (Cirelli et al. 2005b). The second such study collected evidence for an interaction of sleep with immune response mechanisms (Williams et al. 2007).

Another type of study aims to detect genes whose transcriptional variation upholds phenotypic variation within the population in regards to sleep. The first such study in flies found a relatively high degree of association of sleep traits with genetic loci, as well as a relatively high degree of sexual dimorphism which coincides

with the enrichment of sleep-response genes in sexual organs (ovaries, testis), particularly of genes associated with day-specific sleep traits (Harbison et al. 2009).

Forward genetic screens Another set of studies screened for gene mutations affecting sleep behaviour. In fruit flies, random mutagenesis screens are based on screening the offspring of male flies with randomly mutated germ cells using X-rays or chemicals such as EMS (e.g., Cirelli 2003; Shi et al. 2014) and/or random insertions of transposable elements (e.g., Harbison et al. 2008; Harbison et al. 2013). These studies detected a number of potential sleep-regulatory genes (e.g., Shi et al. 2014), including potential regulators of metabolism (Harbison et al. 2008), and confirmed the relatively high degree of heritability of sleep traits in general, and the relatively high degree of sexual dimorphism in particular (Harbison et al. 2013).

1.3 Parameters shaping sleep behaviour in fruit flies

1.3.1 Main features

Constraints of fruit fly behaviour

Drosophila melanogaster are small insects of the order Diptera. It undergoes four life stages developing from an egg into a larva, before it pupates and emerges as an adult fly. The duration of their developmental, reproductive and life cycle is sensitive to temperature (Klein et al. 2015). While a sleep-like state has been demonstrated in the roundworm *C. elegans* which is similar to *Drosophila melanogaster* larvae in size and a range of behaviours (Raizen et al. 2008), the existence of sleep in *Drosophila melanogaster* at the larval stage is still elusive. Therefore, all behavioural sleep studies in *Drosophila melanogaster* have been conducted with adult flies.

Drosophila melanogaster are sexually dimorphic. The basic anatomy of an adult female and an adult male fly is illustrated in Fig. 1.3. Their nervous system undergoes major reconstruction during metamorphosis (Truman 1990). The central nervous system of the adult *Drosophila* is located in the head (Fig. 1.4A, B) and consists of the brain equivalent, the protocerebrum, and a spinal cord homologue, the ventral nerve cord (VNC, Fig. 1.4C).

As small, diurnal insects they are prone to dessication and react acutely to changes in temperature and light. For instance, as their genus name *Drosophila* – 'dew lover' – hints, they preferentially eclose from their pupal case in the early morning to emerge as an adult. During their adult life, fruit flies are also diurnal and exhibit their highest degree of activity in the fresher morning – followed by a second peak of activity in the cooling evening (Dubowy et al. 2017). The species name *melanogaster* refers to their dark abdomen.

Their common name 'fruit fly' or 'vinegar fly' refers to the observation that they live on fermenting fruit, particularly on the yeast growing on it, and spend a considerable amount of their time foraging (Becher et al. 2012). Foraging involves tasting potential food substrates by extending the proboscis (Fig. 1.4A) into the

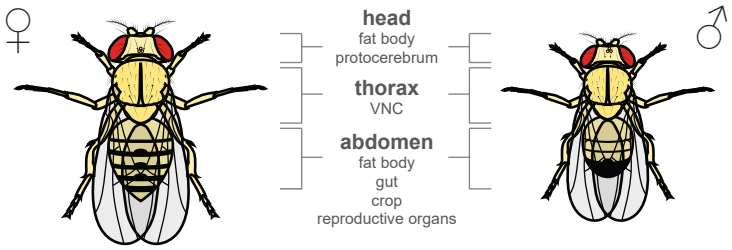


Figure 1.3: Illustration of a female and male *Drosophila melanogaster*. Relevant organs are annotated and the male and female fly schema is an adaptation sourced from `https://commons.wikimedia.org/wiki/` under the Creative Commons license.

food substrate to establish contact with taste neurons (Becher et al. 2012). The proboscis extension reflex is a useful behaviour that can be exploited to evaluate a fly's responsivity to a range of substances (Shiraiwa et al. 2007). Additionally, a major portion of activity is dedicated to reproductive behaviours such as searching for and courting of females in males, and egg laying-related behaviours in females (Klein et al. 2015). Preceding mating, *Drosophila melanogaster* performs a courtship "ritual" that was originally described by Bastock et al. 1955. Courtship is an innate behaviour of *Drosophila melanogaster* that involves a series of stereotyped behaviours on the part of both the male and the female (Ferveur 2010). The male courtship behaviours are more obvious to the observer and have been characterised in detail. Briefly, when a male encounters a female he (1) orients toward the female and chases her, (2) taps the female with his forelegs, (3) starts "singing" a "courtship song" with the help of wing vibrations, (4) licks her genitalia, and (5) attempts copulation. If the female is receptive and spreads her wings, the male is able to mount and (6) successfully copulates for 10-20 min (Ejima et al. 2007). Fruit flies also respond to social cues in order to defend territory or food (Nilsen et al. 2004).

Measuring sleep in fruit flies

Throughout the decades, tools to measure activity in fruit flies gained considerably in temporal resolution: early studies of activity rhythms in flies used eclosion assays (e.g., Konopka et al. 1971) which signify the time a fly emerges from the pupae and can thus only be used as a general measure of activity rhythms within a population.

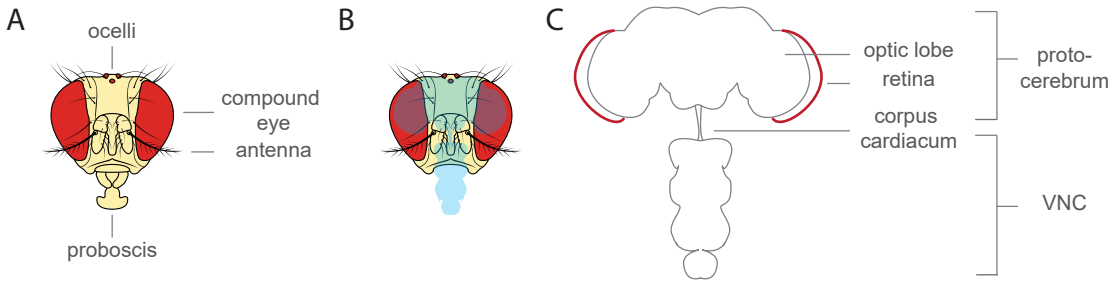


Figure 1.4: Illustration of the head and brain of *Drosophila melanogaster*. A, Head, B, Approximate location of the central nervous system, C, Central nervous system. A-B, The illustration of the head is an adaptation of a schema sourced from `https://commons.wikimedia.org/wiki/` under the Creative Commons license.

The next decade saw the introduction of ultrasound systems that provide an excellent resolution to detect even small movements (e.g., Shaw et al. 2000).

However, the most common assay to study behavioural rhythms and rest in flies are *Drosophila* Activity Monitors (DAMs) produced by Trikinetics Inc (Fig. 1.5). In principle, DAMs can only distinguish between two states: a fly can be "active" – pass the beam – or "inactive" – does not pass the beam. Additionally, the highest temporal resolution DAMs provide is 1 min.

These monitors were intially adopted to study activity rhythms of individual circadian mutant flies (Hamblen et al. 1986), and adopted by Shaw et al. to study rest:activity rhythms in regards to sleep (Shaw et al. 2000).

In experimental practice, sleep in fruit flies is traditionally defined in terms of periods of immobility of at least 5 min in duration (Hendricks et al. 2000; Shaw et al. 2000). This time window is arbitrary and was chosen as it correlates with other measures: for instance, Shaw et al. found that rest:activity profiles detected by an ultrasound system – which provides a temporal resolution of 2 s – were similar to those produced by DAMs (Shaw et al. 2000). Furthermore, rest bouts in *Drosophila* are typically at least 5 min in duration (Hendricks et al. 2000), and flies resting for at least 5 min exhibit the increase in arousal threshold indicative of sleep (Andretic et al. 2005; Huber et al. 2004; Shaw et al. 2000)[1].

[1]The "5 min-rule" received dedicated discussion in several reviews, see, e.g., Ho et al. 2005.



Figure 1.5: *Drosophila* Activity Monitors (DAMs). Monitors consist of an array of infra-red beams; flies are kept in "activity tubes" containing food medium which are orthagonally inserted into the monitor array.

Sleep architecture and timing

A regular sleep profile of a population of flies across several days is depicted in Fig. 1.6. In flies, rest:activity rhythms are under circadian control (Konopka et al. 1971; Zehring et al. 1984). In order to describe a circadian behaviour, the field of chronobiology has adopted a nomenclature that will be adhered to in this study. This nomenclature stems from initial work by Jürgen Aschoff (see, e.g., Aschoff 1966). Briefly, endogenous daily rhythms are synchronised to produce a regular, overt behaviour by exogenous cues termed "zeitgeber" ("ZT") or "time givers" such as light or temperature. Under the standard light:dark cycles of a laboratory, ZT0 usually coincides with lights-on and the onset of activity in diurnal organisms, while ZT12 usually coincides with lights-off. In the absence of exogenous cues under "free-running conditions", biological rhythms have to be synchronised based on endogenous cues of the circadian system alone and this "circadian time" is abbreviated to CT with CT0 coinciding with the onset of activity of diurnal organisms (see also the Dictionary of Circadian Rhythms collected by the Circadian Rhythm laboratory and hosted by Boise State University, `http://www.circadian.org/dictionary.html`).

Regarding fly sleep timing, their activity peaks in the morning – at dawn – and in the evening – at dusk, and another time in the afternoon under certain conditions (De et al. 2013). Sleep is almost absent at these times (Fig. 1.6, see also Andretic et al. 2005). Consequently, the largest portion of the overall day-specific sleep centers around ZT6 ("noon") and is often referred to as the fly 'siesta'

(Fig. 1.6, see also Andretic et al. 2005). Night sleep is relatively homogeneously distributed througout the dark phase (Andretic et al. 2005). While the onset of day sleep is gradual within a population and varies vastly between flies, the onset of night sleep is sharp (Andretic et al. 2005).

The level of sleep consolidation differs between day and night: flies is generally more consolidated during the night which manifests, e.g., in a lengthening of the average bout duration in the night compared to the day (Andretic et al. 2005). This observation is corroborated by the finding that flies resting during the night show higher arousal thresholds than flies resting in the day (Huber et al. 2004). Furthermore, arousal thresholds may even differ throughout the night (Faville et al. 2015). Flies exhibit the highest basal arousal threshold after around 30 min of inactivity in the night (Alphen et al. 2013) and an additional increase after sleep deprivation (Alphen et al. 2013). These findings support the hypothesis of varying sleep depth in fruit flies associated with a stage-like architecture reminiscent of that found in mammals, although with a considerable lower degree of robustness and stereotypy.

Sleep behaviour varies widely with extreme long sleepers sleeping 3 times as long as extreme short sleepers (Andretic et al. 2005). The variability – particularly of night sleep – is large also between different laboratory strains, and even within and between strains that were inbred for many generations (Schlichting et al. 2014; Zimmerman et al. 2012).

A few genes have so far been identified that specifically alter night sleep in *Drosophila melanogaster*. One such candidate is *angiotensin-converting enzyme-related* (ACER, Carhan et al. 2011; Svetec et al. 2015), a human Ace2 homologue which is a member of the angiotensin-converting enzyme (ACE) peptidase family that is involved in the metabolism of angiotensin peptides which in turn are involved in blood pressure regulation in mammals (Eriksson et al. 2002; Lambert et al. 2008). Another candidate is TfAP-2 which may be involved in the development of sleep-regulatory structures such as 'clock' neurons (Kucherenko et al. 2016).

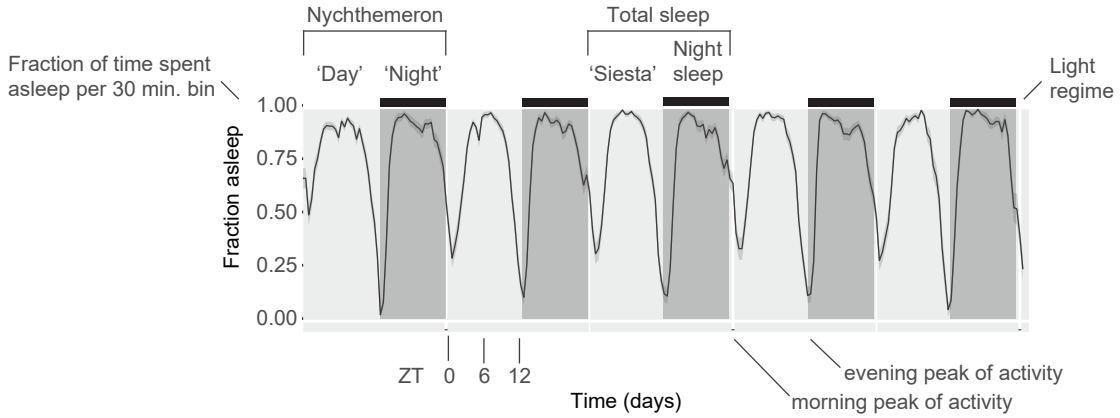


Figure 1.6: Exemplary typical sleep profile of a population of male fies as used in this study. 'Day' refers to the light portion of the nychthemeron and usually starts at ZT0; 'night' refers to the dark portion and usually starts at ZT12. Sleep levels are quantified in terms of the amount of time spent asleep per fly per 30 min bin. Sleep consists of a night-specific portion, and a day-specific portion peaking around ZT6 and often termed 'siesta' to distinguish it from daily, or 'total sleep'.

Taken together, both genes and environment interact to shape sleep behaviour, and the manipulation of one can help bring the contribution of the other into the light.

Homeostatic sleep response

Similar to mammals, fruit flies react to sleep loss with sleep rebound, i.e., they require more sleep after sleep deprivation (Hendricks et al. 2000; Huber et al. 2004; Shaw et al. 2000). Following sleep deprivation, sleep episodes are longer and more consolidated – less frequently interrupted by brief awakenings – than in control flies (Huber et al. 2004). Interestingly, prolonged automated sleep deprivation may be lethal to flies, especially when the molecular clock is additionally perturbed (Shaw et al. 2002).

Sleep deprivation in fruit flies is usually accomplished through mechanical disturbance (e.g., Shaw et al. 2002) but sleep can also be disrupted through other environmental stimuli such as strong light (Kayser et al. 2014) or social interaction (Ganguly-Fitzgerald et al. 2006; Gilestro et al. 2009). Interestingly, the magnitude of sleep rebound grows with the duration of preceding sleep deprivation (Huber et al. 2004)

Interestingly, neurochemical agents known to affect sleep in humans similarly affect fruit flies: caffeine (Hendricks et al. 2000; Shaw et al. 2000) and amphetamines (Andretic et al. 2005) reduce sleep in fruit flies, while histamines increase sleep (Shaw et al. 2000). Similarly, flies respond to the wake-promoting drug Modafinil with decreased sleep and decreased sleep rebound after withdrawal (Hendricks et al. 2003). At least certain volatile anaesthetic agents that are active in humans similarly affect fruit flies (Campbell et al. 1994; MacMillan et al. 2017).

Electrophysiological signature

To test whether neural excitability differs between rest and wake in flies, local field potentials (LFPs) were recorded in the brain when flies were either resting or awake: in this preparation, an electrode is placed in the medial protocerebrum to record activity near sleep regulatory centers, and a control electrode is placed in the optic lobe. The fly is tethered, but usually able to move to some degree (Nitz et al. 2002) or even placed on an air-suspended ball to allow for a full range of walking movements (Alphen et al. 2013). Similar to earlier studies of the forager bee *Apis mellifica carnea*, those initial recordings indicate that sleep does have an electrical signature that is different from the waking state: generally, electrical activity seems to be reduced during rest (Nitz et al. 2002) and this is not a consequence of immobility *per se* (Swinderen et al. 2004). A similar reduction of neural activity during rest has also been observed using *in vivo* Ca^{2+} imaging of tethered flies (Bushey et al. 2015).

1.3.2 Sexual dimorphism

***Fruitless* determines the sex-specific differentiation of the nervous system** *Drosophila melanogaster* is a sexually dimorphic insect (compare Fig. 1.3) with an elaborate courtship "ritual" whose presence and characteristics are associated with the *fruitless* gene (*fru*, Gailey et al. 1989) which is involved in the sex-specific differentiation of the nervous system underlying these behaviours (Stockinger et al. 2005). Interestingly, a GWA study of sleep factors in *Drosophila melanogaster* found

an association of sleep with the *fru* locus (Harbison et al. 2013), although direct evidence of an interaction of the *fru* locus and sleep behaviour is still elusive.

Sleep is generally similar between the sexes Similar to males, virgin and mated female fruit flies exhibit a morning and evening peak of activity. Consequently, sleep is almost absent around dawn and dusk in both sexes (Andretic et al. 2005). Also, sleep occurs mainly during the night portion of the nychthemeron (Isaac et al. 2010, but Zimmerman et al. 2012). Similarly, sleep is also more consolidated in the night than it is during the day (Andretic et al. 2005; Huber et al. 2004; Isaac et al. 2010). At night, both mated and virgin females sleep about as long as males do (Isaac et al. 2010).

Notable sex-related differences Generally, female flies sleep less than male flies, particularly post-mating (Helfrich-Förster et al. 2000; Isaac et al. 2010; Zimmerman et al. 2012). This is due to a strong decrease in day sleep after mating (Garbe et al. 2016; Isaac et al. 2010). This change in sleep in mated females is related to the deposition of male sex peptide (SP) present in the sperm at copulation since females mated with *SP*-deficient males do not exhibit a decrease in day sleep (Garbe et al. 2016; Isaac et al. 2010). Interestingly, females that are deficient of the *SP receptor* (*SPR*) lack other post-mating responses such as egg laying, but their sleep behaviour has not been evaluated (Yapici et al. 2008). At lights-off, mated females fall asleep very quickly – slightly earlier than males – indicating that their loss of day sleep results in sleep deprivation (Isaac et al. 2010). Conversely, virgin females – who exhibit a degree of day sleep similar to males – fall asleep even later than males (Isaac et al. 2010).

Similar observations on day sleep reduction in mated females were made regarding a closely related *Drosophila* species, *Drosophila suzukii* (Ferguson et al. 2015).

1.3.3 Environment

Natural populations of *Drosophila melanogaster* undergo local adapation resulting in genetic and phenotypic variation correlated with geographical location (Jong

et al. 2003). Many environmental factors that impact on fly activity levels such as light, temperature, and crowding differ vastly between latitudes, and it is therefore not surprising that local adaptation on the genetic and behavioural level has been detected for circadian rhythm (Hut et al. 2011) and sleep (Svetec et al. 2015). For instance, a recent study of North American *Drosophila melanogaster* populations found that flies from lower latitudes sleep more than flies from high latitutes; an effect which is mainly driven by variation in night sleep (Svetec et al. 2015).

Even within inbred laboratory strains, sleep behaviour varies considerably under baseline conditions (Zimmerman et al. 2012) and in response to environmental challenges such as changes in light and temperature, nutrition, the social environment, stress, and health status which are discussed below.

Light

Entrainment to ambient light through the visual system Akin to most insects, *Drosophila melanogaster* has an elaborate visual system comprised of a retina and four ganglia contained in the optic lobe (Sato et al. 2013). The retina is a compound eye that contains several hundred ommatidial units, and each in turn contains eight different types of photoreceptor neurons (R1-R8) to detect light. These photoreceptors detect light via wavelength-specific rhodopsins located in the photoreceptor rhabdomere and relay them to the visual ganglia of the optic lobe (Zhu 2013).

Ambient light-driven wakefulness by a subset of clock neurons Studying the role of light in fly sleep is easily confounded by the strong effect that light has on circadian rhythms (Dubowy et al. 2017). In fact, systematic changes in ambient light levels or pulses of strong light can be used to entrain flies or to shift their endogenous phase (Emery et al. 2000). This is true for temperature as well, albeit to a lesser degree (Dubowy et al. 2017). Light generally induces activity in *Drosophila* (Martin et al. 1999) and it modulates the neural excitability of core clock neurons, the large LN_v (l-LN_v): for instance, spontaneous activity of l-LN_v increases with light (Sheeba et al. 2008a) which usually coincides with increased activity during

waking hours in these diurnal flies. Conversely, when the neural activity of l-LN$_v$ is artificially increased and the l-LN$_v$ are very active during the dark period – at night – night sleep is suppressed (Shang et al. 2008; Sheeba et al. 2008a).

Sleep during continuous light Flies are photosensitive and generally prefer ambient light of low intensity (Rieger et al. 2007). Strong continuous light severely disrupts the molecular clock and may thereby mask sleep-specific effects. Thus, effects of continuous light on circadian and sleep behaviour in fruit flies is usually studied using low intensity light regimes ('moonlight', Schlichting et al. 2014). 'Moonlit' nights increase activity levels during the night and decreases them during the day (Schlichting et al. 2014). When 'moonlit' nights are combined with gradual light increases in the beginning of the day and a gradual decrease in the beginning of the night – mimicking dawn and dusk – the moonlight-induced night activity decreases (Schlichting et al. 2015), indicating that flies are able to regulate sleep in response to 'natural' ambient light levels.

Sleep during continuous dark Sleep of *Drosophila melanogaster* in continuous dark has received little attention. There is, however, a body of research on activity patterns in continuous darkness which can be used as a cautious reference for sleep behaviour. In this diurnal fly, darkness generally decreases activity (Martin et al. 1999). In an initial response to continuous darkness, fruit flies shift most of their sleep into the subjective night. Over time in continuous dark, the circadian clock eventually dampens and becomes desynchronised over time resulting in a dampening of the rest:activity profile (Helfrich-Förster et al. 2000). Particularly, the bimodal activity profile – featuring distinct morning and evening peaks – gives way to a unimodal activity profile with a single peak (Helfrich-Förster et al. 2000).

Temperature

The timing of certain biological processes such as the cyclic expression of clock genes need to be insensitive to physiological temperatures (Kidd et al. 2015). For small poikilotherms like *Drosophila melanogaster*, thermoregulation is essential for

survival (Klein et al. 2015). Consequently, their thermotaxis is acute and tightly controlled (Klein et al. 2015) and relies on a subset of a highly conserved family of ion channels – Transient Receptor Potential (TRP) cation channels (Fowler et al. 2013) – and on ionotrophic receptors (Chen et al. 2015).

High temperatures rising within a physiological range – from 25 °C to 29 °C – affect the sleep profile of fruit flies with opposing effects on day and night sleep: high temperatures increase day sleep, and decrease night sleep (Chen et al. 2015; Lamaze et al. 2017; Parisky et al. 2016). While day sleep becomes more consolidated into longer sleep bouts, night sleep becomes more fragmented with shorter sleep bouts (Parisky et al. 2016). High temperature-induced increases of night activity provoke a sleep rebound independent of the circadian clock (Parisky et al. 2016). Also, timing of the male 'siesta' is amenable to temperature: siesta sleep onset increases in response to temperature increases up to around 30 °C, when this response reverses and the onset of sleep is delayed (Lamaze et al. 2017). This response relies on the thermosensor TRPA1 (Lamaze et al. 2017). From an ethological perspective, this may be an essential response to avoid dessication especially at noon or 'siestatime', when it is usually both hottest and brightest (Parisky et al. 2016). These findings emphasise that day and night sleep fulfil different functions and are under different regulatory constraints.

Social environment

The social environment has a strong effect on a range of behaviours in *Drosophila melanogaster* and its effect on sleep has been tested both under crowded conditions and with paired interactions.

Paired interactions: male-male aggression Male flies are territorial and engage in aggressive behaviours when confined to a small space and competing for food or females. After prolonged exposure, males will usually disengage from active fighting, but the presence of a competitor still acts as a sleep deprivation stimulus and leads to sleep rebound (Gilestro et al. 2009). Males kept in male-only

pairs show a progressively higher amount of rebound sleep once isolated the longer they were kept in groups (Lone et al. 2016).

Paired interactions: male-female interactions and the male sex drive For fruit flies, mating and sleep are exclusive behaviours. Mating in *Drosophila*, particularly in females, follows the circadian clock and mainly occurs during the day (Sakai et al. 2001). The male sex drive is so strong, however, it can override the circadian influence, and can be harnessed as a sleep deprivation stimulus: when paired with a female, male flies tirelessly engage in courtship also during the night and sleep very little (Fujii et al. 2007). The only rest period occurs around dusk (Fujii et al. 2007); this increased rest could reflect a sleep rebound from the nocturnal courtship activities. Males impaired in their ability to detect olfactory cues do not engage in nocturnal courtship activities and consequently do not change their rest:activity pattern (Fujii et al. 2007); they also do not seem to engage in social interaction when kept with a male as indicated by a lack in rebound sleep when isolated (Lone et al. 2016).

Grouped conditions: when social housing competes with sleep The analysis of group behaviour is hampered by the fact that the most wide-spread activity monitoring system – Drosophila Activity Monitors (DAMs) produced by Trikinetic Inc. – rely on the detection of infrared beams disrupted by a fly passing through them. In principle, this approach cannot discriminate between a signal derived from a single or multiple flies. Although video-based tools able to handle multiple flies are available (Branson et al. 2009), most studies circumvent this problem by recording sleep behaviour individually *after* the social treatment. Once isolated, flies sleep more than individually housed flies, supposedly recovering sleep lost when in a group (Ganguly-Fitzgerald et al. 2006). This increase in sleep is specific to the day portion of the nychthemeron and rises with increasing group size (Ganguly-Fitzgerald et al. 2006). Sleep deprivation by mechanical means also elicits a day-specific homeostatic response (Ganguly-Fitzgerald et al. 2006). Interestingly, animals that are impaired in their ability to sense their social environment – blind

and smell-impaired flies – do not show an increase in sleep levels due to grouped housing (Ganguly-Fitzgerald et al. 2006). However, the day-specific increase in sleep persists across multiple days (Ganguly-Fitzgerald et al. 2006), and is thus unlikely to reflect an acute homeostatic sleep response. Other studies also report an increase in sleep levels due to grouped housing or 'social enrichment' (Bushey et al. 2011; Liu et al. 2015).

1.3.4 Nutritional state

Striking a balance between sleep and feeding Feeding patterns in *Drosophila melanogaster* are regular throughout the day, peaking in the morning (Xu et al. 2008). Its feeding behaviour follows a stereotypic pattern: flies forage for food, particularly when hungry (Lee et al. 2004). When the presence of food is detected during foraging, fruit flies cease to move and initiate a meal by extending the proboscis into the food source (Edgecomb et al. 1994). Ingestion-induced signalling processes terminate a meal (Pool et al. 2014). Feeding and sleeping are exclusive behaviours in *Drosophila* and are timed to occur mainly around noon and at night (sleep) or at dawn and dusk (feeding, compare, e.g., Murphy et al. 2016). This rhythm is driven by the circadian system: flies deficient in a functional molecular clock delay feeding even until the night, thereby decreasing the amount of sleep they obtain (Xu et al. 2008).

Food composition impacts on sleep Like mammals, fruit flies respond to sweet and bitter tastes via a range of gustatory receptors (GRs), but flies can also evaluate food based solely on its nutritional content (Dus et al. 2011). For instance, among ten sugars sampled, sucrose is the sugar with the highest palatability and the highest nutritional value, while L-fucose is very sweet and palatable, but low in caloric value (Stafford et al. 2012). Fruit flies learn to prefer the sugar higher in nutritional value, indicating that nutritional evaluation and taste are separable in *Drosophila* (Stafford et al. 2012).

Acutely elevated levels of dietary sugar – such as a diet high in sucrose for 12-48 h – increase activity levels in both sexes and affect sleep architecture (Catterson et al.

2010; Linford et al. 2012). Interestingly, it seems to be the gustatory value that drives sugar-induced changes of sleep as flies deficient in sugar-sensing gustatory receptors sleep normally (Linford et al. 2012).

Effects of dietary protein (yeast) levels are mild and sexually dimorphic: in males, high protein decreases sleep duration during both day and night, but increases sleep duration in females during the day (Catterson et al. 2010). This may be unsurprising considering the differences in energy and nutritional needs of both sexes particularly after mating (Gorter et al. 2016).

Depletion of lipid stores also affects the homeostatic sleep response (Thimgan et al. 2015). In fact, the liver and white adipose tissue homologue – the fat body (Fig. 1.3) – may be one of the main structures that buffers sleep against starvation and other disruptive stressors: for instance, flies deficient in *brummer* – an adipose triglyceride lipase – have increased triglyceride stores and an increased homeostatic sleep response (Grönke et al. 2005; Thimgan et al. 2010), and flies deficient in *lsd2 – lipid storage droplet 2* – have decreased triglyceride levels and a decreased homeostatic sleep response (Thimgan et al. 2010).

Starvation-induced sleep suppression: when sleep competes with foraging Similar to mammals (Danguir et al. 1979; Rechtschaffen et al. 1983), sleep deprivation in fruit flies is associated with changes in feeding. In flies, a starvation protocol usually involves a baseline recording of flies on either normal food medium or a combination of sucrose and agar, and their subsequent transferral to pure agar. The agar medium ensures access to water to prevent dessication, while holding no caloric value (see, e.g., Keene et al. 2010). Flies survive this kind of total starvation only for a limited amount of time; depending on environmental conditions, flies die in the course of two days (Keene et al. 2010). Such starvation induces hyperactivity in the day, probably reflecting intensified foraging (Lee et al. 2004). In fact, flies with disrupted foraging regulation due to a mutation in the *foraging* gene refrain from suppressing sleep in response to starvation (Keene et al. 2010). Also, starvation reduces sleep in a sexually dimporphic manner: in females, it reduces sleep quickly,

both during the day and night of acute starvation; in males, starvation takes up to 12 hours to impact on sleep (Keene et al. 2010). Flies with a disrupted molecular clock are particularly prone to starvation-induced sleep suppression (Keene et al. 2010).

Flies with reduced levels of *translin* – an RNA/DNA binding protein – do not reduce sleep during periods of starvation (Murakami et al. 2016). *Translin*-deficient flies do not only sleep more than flies with normal *translin* levels when fed and when starved, they also exhibit reduced levels of metabolic rate in terms of CO_2 consumption (Stahl et al. 2017). A similar mechanism might apply to another mutation – *Drosophila activity-regulated cytoskeleton-associated* (*dARC*) – that renders flies resistant to starvation through the suppression of starvation-induced hyperactivity (Mattaliano et al. 2007).

1.3.5 Age

A mutual relationship between aging and sleep In the laboratory, flies live for around 50-80 days, whereby their exact lifespan depends on many factors such as diet and temperature (Grotewiel et al. 2005). During that time, flies age across many dimensions (Grotewiel et al. 2005). Age-related sleep deterioration, namely sleep loss and sleep fragmentation, are present in both mammals (Hasan et al. 2012; Pandi-Perumal et al. 2002) and flies (Kayser et al. 2014; Koh et al. 2006; Shaw et al. 2000). The relationship between sleep quality and lifespan is mutual: many short sleeping fly mutants are also short-lived (e.g., Bushey et al. 2007, 2010; Robertson et al. 2013; Stavropoulos et al. 2011) and a mutant fly line outbred for a very short sleeping phenotype – *insomnia-like* (*ins-l*) – shows signs of early aging (Seugnet et al. 2009).

The life span of *Drosophila* is closely linked to feeding, as dietary restriction extents their life span (Bass et al. 2007; Grandison et al. 2009; Libert et al. 2007) and high calorie diets shorten it (Yamazaki et al. 2012). A life-shortening high calorie diet also leads to a deterioration of sleep quality (Yamazaki et al. 2012). Conversely, inhibition of insulin signaling improves sleep quality even in older flies (Metaxakis et al. 2014). Since sleep timing is under clock control, some deterioration

of sleep quality may be related to the age-related weakening of the circadian clock (Koh et al. 2006; Luo et al. 2012; Rakshit et al. 2012). Similar effects on sleep have been found for life-shortening temperature increases (Koh et al. 2006). Taken together, these findings indicate that age-related deterioration of sleep quantity and quality is a function of physiological rather than chronological age.

Sleep parameters change with age Aging affects sleep in fruit flies in ways similar to mammals. For instance, young flies sleep more than old flies (Shaw et al. 2000; Zimmerman et al. 2012). In fact, sleep is longest in very young flies immediately after eclosion, i.e., in the first light period of the first adult day (Kayser et al. 2014; Shaw et al. 2000), which may be crucial for post-eclosion brain development (Kayser et al. 2014) and cognitive performance (Seugnet et al. 2011). In the very young fly, remnants of cycling ecdysone – a steroid necessary for post-embryonic development – could partly account for this day-specific increase in sleep (Ishimoto et al. 2010). Sleep becomes not only shorter but also more fragmented with age (Koh et al. 2006; Koudounas et al. 2012; Shaw et al. 2000) and more susceptible to disturbance and sleep deprivation (Kayser et al. 2014). Age-related sleep fragmentation stresses flies: induction of sleep fragmentation through light stimulation leads to sleep rebound implying that it produces sleep deprivation, and it also recruits oxidative stress and insulin pathways (Williams et al. 2016).

Accumulation of sleep-disrupting stressors with age Interestingly, studies of flies deficient in *forkhead box* (*foxo*) – a key regulator of the insulin pathway – exhibit hypersensitivity to paraquat-induced oxidative stress (Zheng et al. 2007). The stability of the circadian clock is dampened by high levels of oxidative stress, and by age in both flies and humans (Wilking et al. 2013). The expression of a range of genes is associated with the coincident increase of oxidative stress levels with age (Zou et al. 2000) and the susceptibility to additional, paraquat-induced oxidative stress is augmented in *foxo* mutants (Zheng et al. 2007). Given that both aging and sleep deprivation induce cellular stress and are accompanied by

profound changes in sleep (Koh et al. 2006; Shaw et al. 2002), oxidative stress may be one of the factors that destabilises sleep with age.

1.4 What drives fruit fly sleep? Part I: circadian sleep regulation

Similar to mammals, sleep in fruit flies is governed by circadian entrainment and homeostatic drives and thus reflects the two-process model (Dubowy et al. 2017; Sehgal et al. 2007). Importantly, circadian and homeostatic mechanisms of sleep regulation are not only separable conceptually but also genetically as sleep can occur without a functional clock. For instance, flies deficient in clock-regulating genes still sleep, although their sleep differs from sleep of normal flies (Hendricks et al. 2000; Shaw et al. 2000, 2003). The two following sections address the current knowledge about how these mechanisms work to regulate sleep in *Drosophila*.

1.4.1 The fly 'clock'

The 'molecular clock'

The nature of the 'molecular clock' and its constituent proteins have been first identified in *Drosophila melanogaster* (e.g., Konopka et al. 1971, see Stanewsky 2014 for a recent review). In fact, it was a circadian mutant that set off the field of behavioural genetics: screening for mutants with altered eclosion time – at that time the key parameter to evaluate the circadian rhythm within a population – Ronald Konopka was the first to identify fly lines with altered rhythms and to map these traits to a single gene: *period* (Konopka et al. 1971). A succession of genes that form the core circadian clock network were identified in the next decades: *timeless* (*tim*, Sehgal et al. 1994), *clock* (*clk*, Allada et al. 1998), and *cycle* (*cyc*, Rutila et al. 1998), along with the photoreceptor *cryptochrome* (*cry*, Emery et al. 1998; Stanewsky et al. 1998). An extremely simplified schema illustrating the interaction of only the core clock components is depicted in Fig. 1.7.

A system of clock neurons

While the molecular clock is cell-autonomous, it orchestrates overt circadian behaviour mainly through a particular set of neurons. Clusters of clock neurons –

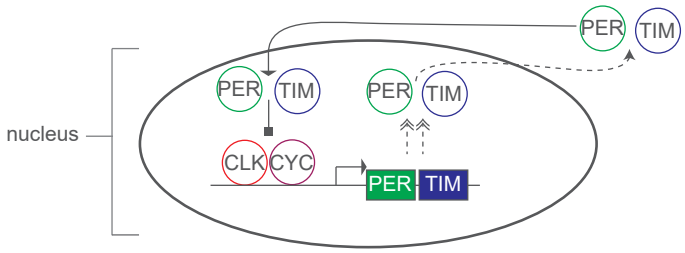


Figure 1.7: Illustration of the circadian transcription-translation feedback loop (TTFL). The 'clock' consists of a TTFL of a particular set of genes, *clk* and *cyc*, that activate the transcription of a second set of genes, *per* and *tim*, whose proteins in turn down-regulate CLOCK and CYCLE activity, and a posttranslational loop that maintains rhythmic phosphorylation of PER and TIM to regulate their stability and expression (Hardin 2005). Environmental entrainment of the 'clock' is ensured by the light-dependent degradation of TIM that relies on CRY (Hardin 2005), but can be achieved by other factors as well, such as temperature or social cues (Kaushik et al. 2007; Levine 2002). The schema is inspired by Dubowy et al. 2017, but omits many proteins known to regulate the circadian clock in order to provide an illustration of the principle of the TTFL only.

identified through high expression levels of the core clock gene *per* – are comprised of about 150 neurons in total (Dubowy et al. 2017; Johard et al. 2009).

A schema outlining the location and projection pattern of clock neurons as far as they are directly implicated in sleep is depicted in Fig. 1.8. Briefly, an exclusive set of neurons that expresses pigment dispersing factor (PDF) are the master pacemaker neurons of the fly brain: for instance, mutations in *pdf* severely disrupt the circadian rhythm (Renn et al. 1999). PDF+ neurons were first detected with an antibody for PDH, a crustacean pigment-dispersing hormone, that specifically labels two small groups of lateral neurons, a group consisting of relatively large cell bodies – the large LN_vs (l-LN_vs) – and a group consisting of relatively small cell bodies – the small LN_vs (s-LN_vs, Helfrich-Forster et al. 1993). The extended clock neuron network includes PDF- clusters of dorsal neurons (DN_{1-3}) and lateral dorsal neurons (LN_d, Kaneko et al. 2000).

Neural transmission in the clock network

PDF: the main transmitter PDF is expressed throughout the arborisations of PDF+ neurons (Helfrich-Forster et al. 1993) including the projection of the s-LN_v into the dPC through the dorsal projection (Fig. 1.8). The molecular clock

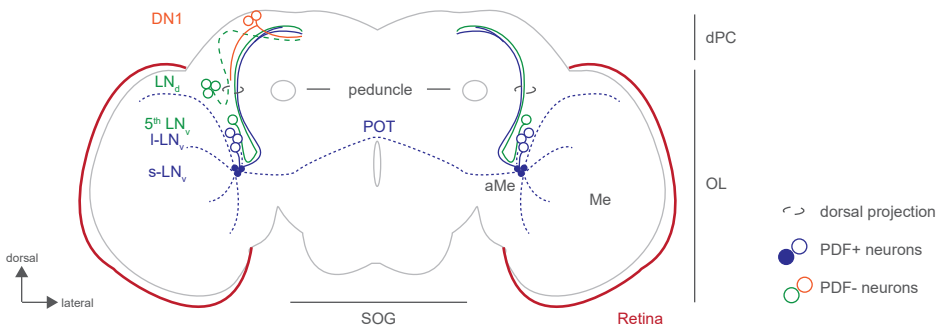


Figure 1.8: Neuroanatomical substrates involved in circadian sleep regulation. The core clock network consists of the PDF+ large LN_v (l-LN_v) and small LN_v (s-LN_vs, and the PDF- dorsal neurons (DN_{1-3}) and lateral dorsal neruons (LN_d). While the l-LN_v extend from the accessory medulla (aMe) to mainly arborise throughout the medulla (Me) of the optic lobe (OL), touching on the s-LN_v, and cross talk through the posterior optical tract (POT), the s-LN_v form a fiber tract that runs into the dorsal protocerebum (dPCR) and recruits other clock neurons including the DN_1, DN_3 and LN_d (Helfrich-Forster et al. 2007). Interestingly, these latter neurons all terminate in the dPC (Kaneko et al. 2000). The DN_1 cluster extends into the pars intercerebralis (PI) of the dPC and connects to the s-LN_v in this region (Blanchardon et al. 2001). The schema is inspired by Helfrich-Förster 1997 and Kaneko et al. 2000, but omits clusters of clock neurons that have not yet been directly implicated in sleep regulation. Peduncle, MB peduncle, SOG, subesophageal ganglion.

drives the activity dynamics in PDF+ neurons (Depetris-Chauvin et al. 2011; Kaneko et al. 2000; Klose et al. 2016) including secretion of PDF from the dorsal projection (Park et al. 2000). Studies directly applying PDF to downstream target neurons or acutely activating PDF+ neurons detected pronounced excitation by PDF binding to its receptor, PDFR (Depetris-Chauvin et al. 2011; Seluzicki et al. 2014; Vecsey et al. 2014; Wu et al. 2008).

PDFR: receptor of the clock network PDF is homologous to mammalian vasoactive intestinal peptide (VIP), a peptide released from the SCN, and the PDF receptor (PDFR) is homologous to the mammalian VIP receptor VPAC2 and the Calcitonin receptor. A triad of studies found the PDFR to be a classical G protein-coupled hormone receptor, a receptor class that regulates cyclic AMP (cAMP, Hyun et al. 2005; Lear et al. 2005; Mertens et al. 2005). The PDFR responds to the VIP homologue PDF, but also to the Calcitonin homologue DH31 (Shafer et al. 2008). PDFR activation increases cAMP and PKA which stabilises

the molecular clock (Sabado et al. 2017) and synchronises neural activity across clock neurons (Li et al. 2014). For instance, the s-LN$_v$ react strongly to PDF (Klose et al. 2016). Here, the PDFR acts as an autoreceptor to stabilise clock gene expression (Lin 2004; Mezan et al. 2016). The particular components of the PKA pathway employed in clock neurons may be heterogenous (Duvall et al. 2013) and may involve miRNAs (Chen et al. 2017; Zhang et al. 2013).

Hierarchy and synchronisation of the clock network

While the molecular clock drives rhythmic activity in clock neurons, it does so in interaction with the environment. For instance, clock neurons of the dorsal cluster preferentially entrain to temperature (Miyasako et al. 2007; Parisky et al. 2016), while PDF+ neurons are most readily entrained by light, mainly through a CRY-dependent mechanism: CRY responds to the light spectrum indicative of morning, i.e., blue light, coinciding with flies' highest activity levels (Cao et al. 2008). In order to adapt to variations in day length across seasons, the circadian regulation has to be flexible to allow for a lengthening and a shortening of the period, leading to the proposal of a 'two-oscillator model' (Daan et al. 1976; Pittendrigh et al. 1976). This has been difficult to address experimentally: under constant light conditions, CRY constantly degrades *tim* and the molecular clock breaks down; leading to arrhythmicity (Busza 2004; Ceriani et al. 1999; Emery et al. 2000; Rosato et al. 2001). In *cry*-deficient flies, however, this does not happen and a desynchronization developing into two different components, a short period and a long period, can be observed (Yoshii et al. 2004).

A pair of studies was the first to show the neuroanatomical separation of morning and evening oscillators: they identified the PDF+ LN$_v$ to be crucial for morning anticipation and the additional recruitment of the PDF- LN$_d$ to be involved in evening anticipation (Grima et al. 2004; Stoleru et al. 2004). While there is conflicting evidence on the exact hierarchy, it seems that the s-LN$_v$ drive predominantly the morning peak of activity in response to CRY activation and

strongly influence the evening peak of activity as well (Picot et al. 2007; Rieger et al. 2006; Stoleru et al. 2005).

1.4.2 Process C: sleep regulation through the clock network

Modulation of sleep drive by wake-promoting s-LN$_v$

The s-LN$_v$ are most responsive to PDF and most active at dawn (Klose et al. 2016). Activation of the LN$_v$ and the dorsal clusters by expressing a depolarising ion channel, *shaw*, leads to an overexcretion of PDF in the dorsal protocerebrum (dPC) and increases locomotor activity at night (Hodge et al. 2008), suggesting that this activation may suppress sleep. PDF levels in the dorsal projection are positively correlated with short periods, indicating that LN$_v$ favor activity over rest (Wülbeck et al. 2008). Conversely, *PDF*-deficient flies sleep more (Chung et al. 2009).

Fruit flies exhibit sleep both during the day and the night and the associated regulatory mechanisms may differ (section 1.3.1). Conversely, the main activity peaks in *Drosophila* occur in the morning and in the evening (Yoshii et al. 2012). There is mounting evidence that s-LN$_v$ drive the morning peak of activity, e.g., the complexity of their dorsal arborisation, its PDF load and the morning-specific peak in excitability (Depetris-Chauvin et al. 2014), thereby producing a nearly complete absence of sleep at this time (section 1.3.1). Similarly, overstabilisation of the PDFR-dependent cAMP cascade decreases sleep: for instance, the reduction of a miRNA, GW182, which reduces expression of the cAMP phosphodiesterase *dunce* that normally accompanies PDFR activation, also suppresses day sleep (Zhang et al. 2013). The morning peak and coincident absence of sleep may in part be due to temperature regulation: flies delay 'siesta' sleep at high temperatures (Lamaze et al. 2017). This adaptive behaviour depends on the connection of DN$_1$ to thermosensitive circuits. In fact, DN$_1$ neurons project downwards to inhibit the 'evening pacemakers', LN$_d$ and the 5th LN$_v$, as well as the 'morning pacemaker', the s-LN$_v$, to create a time window for siesta sleep to occur, especially when morning temperatures are

high (Guo et al. 2016), and, similarly, to promote sleep late at night when the activating influence of wake-promoting s-LN$_v$ is rising (Kunst et al. 2014).

The LN$_v$ are less active during the night (Buhl et al. 2016) coinciding with decreased arousal and the main sleep phase; activation of the LN$_v$ increases arousal and decreases sleep (Shang et al. 2008; Sheeba et al. 2008a). Similarly, GABA from as yet unidentified sources exerts inhibiting influence on l-LN$_v$ and s-LN$_v$ – which express the GABA$_A$ receptor *resistant-to-dieldrin* (*rdl*) and the GABA$_B$ receptor GABA$_B$R2 – to promote sleep, particularly at night (Agosto et al. 2008; Chung et al. 2009; Gmeiner et al. 2013; Parisky et al. 2008). Interestingly, while a reduction of the GABA$_B$ receptor in l-LN$_v$ deceases late night sleep – consistent with a dampening influence on the morning pacemaker by GABA – it does not affect circadian rhythms (Gmeiner et al. 2013). Furthermore, the levels of the RDL regulator *wide awake* (*wake*) trace increasing sleep pressure and are highest in the evening (Liu et al. 2014). WAKE is regulated by the molecular clock and aids RDL trafficking to the cell membrane which is crucial to decrease membrane excitability and to initiate sleep: reduction of RDL in l-LN$_v$ strongly delays night sleep (Liu et al. 2014).

Circadian modulation of sleep drive through synaptic homeostasis of s-LN$_v$ arborisation

The synaptic terminals of the dorsal projection of s-LN$_v$ undergo rhythmic remodelling that follows a circadian, sleep-reliant pattern: their complexity is lowest in the night (Fernández et al. 2008; Gorostiza et al. 2014), and this dynamic is lost in circadian mutants (Fernández et al. 2008), and it depends on sleep for maintenance (Donlea et al. 2009). Conversely, there is mounting evidence that axonal remodeling of the dorsal projection impacts on sleep: generally, in PDF+ LN$_v$, transcription is largely confined to two main time windows: midday and midnight (Huang et al. 2013), which coincide with main sleep phases. Fittingly, the expression of most genes associated with axon guidance peak in the night (Huang et al. 2013), and rhythmic expression patterns were also observed for ion channels (Huang et al. 2013). More specifically, disruption of the axonal aborisation through manipulation of the histone acetyltransferase (HAT) enzyme Tip60 (Pirooznia et al. 2012), the retrograde bone

morphogenetic protein (Gorostiza et al. 2014), Mmp1 (Depetris-Chauvin et al. 2014), and Aβ42 (Song et al. 2016) disrupts circadian rhythm and sleep.

It has not yet been specifically tested why a disruption of the dorsal projection of s-LN$_v$ also disrupts sleep. However, the wake-promoting role of the s-LN$_v$ together with their morphological plasticity points to the hypothesis that the wake-promoting s-LN$_v$ generally antagonise sleep-regulatory downstream neurons and the influence over their downstream partners needs to be limited, at least in part, by a retraction of synaptic connections in order to allow sleep to occur.

PDF & sNPF: antagonistic sleep regulators of s-LN$_v$

A dedicated study on varicosities of s-LN$_v$ using electron microscopy detected both dense core vesicles (DCV) indicative of a peptidergic, paracrine transmitter and clear vesicles (CV) indicative of a fast, synaptic neurotransmitter (Yasuyama et al. 2010). Since virtually all DCV were PDF+, PDF may be the main peptidergic transmitter of the s-LN$_v$ (Yasuyama et al. 2010). The search for the fast-acting transmitter has recently revealed a candidate, glycine (Frenkel et al. 2017). However, PDF+ neurons co-release other peptides including ion transport peptide (ITP, Hermann-Luibl et al. 2014), the neuropeptide-Y (NPY) homologue neuropeptide-F (NPF, Lee et al. 2017), and small NPF (sNPF, Johard et al. 2009; Lee et al. 2009; Shang et al. 2013).

Among those, sNPF is expressed in s-LN$_v$ (Johard et al. 2009; Kula-Eversole et al. 2010). In general, sNPF is sleep-promoting: for instance, activation of sNPF+ neurons promotes sleep especially during the day, and the *sNPF*^{00448} mutant is short-sleeping (Shang et al. 2013)[2]. Furthermore, activation of the sNPF receptor (sNPFR) has effects that antagonise those of the PDFR: in a larval model, PDF release activates motor neurons and muscles expressing the PDFR, whereas sNPF release inhibits motor neurons expressing the sNPFR (Shang et al. 2013; Vecsey et al. 2014). Furthermore, sNPF application also inhibits neurons that naturally express the sNPFR, i.e., DILP neurons (Vecsey et al. 2014, section 1.5).

[2]It is of note that Chen et al. 2013 describe some effects of *sNPF* deficiency on sleep levels that are incongruent with the previous study by Shang et al. 2013. This could, in part, be due to the different sNPF+ populations targeted.

Similar to the PDFR, the sNPFR also acts through a G-protein cAMP cascade (Vecsey et al. 2014). It is homologous to members of the NPY receptor family and NPY receptors act in the mammalian SCN to modulate circadian rhythms (see discussion in Johard et al. 2009).

1.5 What drives fruit fly sleep? Part II: homeostatic sleep regulation

After decades of circadian research in fruit flies, two seminal studies by the groups of Amita Sehgal and Giulio Tononi demonstrated that rest in fruit flies resembles sleep and can thus be effectively used to study the complex factors that influence homeostatic sleep drive (Hendricks et al. 2000; Shaw et al. 2000, discussed in section 1.3.1). The homeostatic drive is particularly apparent when fruit flies are deprived of sleep: upon sleep deprivation, flies prolong their rest periods and need more intense stimuli to be aroused (Hendricks et al. 2000). Sleep rebound is a critical experimental component to distinguish factors that influence circadian processes from those driving homeostatic processes.

1.5.1 Neural regulators and neurotransmitter systems of process S

Several neurotransmitter systems and neuroanatomical structures have been implicated in homeostatic sleep regulation in fruit flies. Major sleep regulatory centres of the fly brain are depicted in Fig. 1.9.

Mushroom bodies (MB)

Mushroom bodies (MB, Fig. 1.9, blue) are a bilateral neuropil structure mainly comprising around 2000 intrinsic Kenyon cells (KCs) per hemisphere (Aso et al. 2009; Strausfeld et al. 1998). KCs employ acetylcholine (ACh) as their major fast transmitter (Barnstedt et al. 2016). KCs are third-order olfactory neurons that are clustered into three main subpopulations: γ KCs that form part of the horizontal lobe as well as $\alpha\beta$ and $\alpha'\beta'$ that each make up parts of the horizontal and vertical lobes (Crittenden et al. 1998). The MB forms an association matrix in which each of its 15 compartments contains the dendrites of one mushroom body output neuron (MBON) and receives specific dopaminergic reinforcement signals by dopaminergic neurons (DANs, Aso et al. 2014b). The tripartite synapse between KC, MBON, and

DAN allows the positive/negative reinforcement of a given odour to bias behaviour towards/away from it (Aso et al. 2014a; Owald et al. 2015).

Two early studies implicated MB in sleep regulation: one study found pronounced sleep suppression when inhibiting the MB through expression of *shibire* – a dynamin mutation that arrests synaptic endocytosis (Chen et al. 2002; Vicario et al. 2001) – and also when ablating the MB through hydroxyurea injection (Pitman et al. 2006). The second study exploited a previous finding regarding the role of PKA signalling in sleep regulation: this earlier study had already identified mutations that disrupted the PKA pathway and led to memory deficits and changes in sleep, whereby mutations inhibiting PKA signalling such as *rutabaga* promoted sleep and mutations enhancing PKA signalling such as $dunce^{ML}$ suppressed sleep (Hendricks et al. 2001). The second MB study exploited these findings and enhanced PKA activity through expression of an additional catalytic PKA subunit in different regions of the brain and found a sleep-suppressing effect of PKA activity in the MB (Joiner et al. 2006). In light of later studies that relate increases in cAMP-PKA signalling to increased Ca^{2+} influx in MB (Pavot et al. 2015), these findings point to a generally wake-promoting role of the MB.

These studies did not specifically address whether MB are involved in the homeostatic regulation of sleep. Other studies point to the *potential* of the MB to take part in the circadian modulation of sleep: for instance, activation of MB does not only promote arousal, but can also deconsolidate circadian rhythm (Gorostiza et al. 2014). Similarly, modulation of serotonin levels in the MB affects circadian rhythms and sleep (Yuan et al. 2006), but the specific molecular mechanisms and downstream pathways involved are still elusive. Importantly, even ablation of MB reduces but does not annihilate sleep (Joiner et al. 2006), indicating that brain regions other than the mushroom bodies partake in sleep initiation and regulation.

Internal MB microcircuits differentially regulate sleep. Firstly, the role of KCs in sleep regulation is heterogeneous: KCs that predominantly project into the $\alpha'\beta'$ lobes generally suppress sleep and activate glutamatergic MBONs, while KCs that predominantly project into the γ lobe generally promote sleep and project to

cholinergic MBONs (Aso et al. 2014a). Activation of a combination of sleep and wake-promoting KCs does not affect sleep, pointing to competitive mechanisms (Sitaraman et al. 2015a). Secondly, the effects of MBON activation are similarly heterogeneous: specifically, the 'wake-promoting' MBONs reside in the γ and β' lobes ($\gamma4$, $\gamma5$, $\beta'2$), and the 'sleep-promoting' MBONs reside in the γ and α' lobes ($\gamma3$, $\gamma2$, $\alpha'1$) and some MBONs share downstream targets (Aso et al. 2014a). Thirdly, activation of the third part of the tripartite MB synapse, DANs, excites wake-promoting MBONs and consequently suppresses sleep (Sitaraman et al. 2015b). Conversely, excitability of DANs increases with sleep pressure and decreases with sleep in a manner that facilitates memory retention, a major function of the MB (Berry et al. 2015). Additionally, GABAergic input to wake-promoting $\alpha'\beta'$ neurons via a set of dorsal paired medial (DPM) neurons increases sleep (Haynes et al. 2015).

The central complex and the dorsal fan-shaped body (dFSB)

The first dedicated screen for brain structures that support sleep homeostasis identified the dorsal fan-shaped body (dFSB, Fig. 1.9, purple) as a major sleep regulatory centre: thermogenetic activation of the dorsal fan-shaped body (dFSB) promotes both day and night sleep (Donlea et al. 2011). The dFSB is exceptionally active in young flies which exhibit the highest amount of sleep (Kayser et al. 2014). Importantly, sleep induction via the dFSB is reversible: "sleep-induced" flies in which dFSB neurons were acutely depolarised are just as easily aroused as parental control flies and respond to sleep deprivation normally – distinguishing dFSB-dependent sleep induction from paralysis or passive immobility (Donlea et al. 2011). Sleep induction through activation of the dFSB benefits memory (Donlea et al. 2011).

The fly brain accommodates hundreds of dopaminergic neurons that form several subgroups based on location and expression pattern (Mao et al. 2009). Human psychostimulants that act via dopamine pathways, such as caffeine and amphetamines, similarly reduce sleep in fruit flies, while pharmacological depletion of dopamine using 3-iodo-tyrosine increases sleep (Andretic et al. 2005; Shaw et al. 2000). Similarly, mutation of *fumin* – which encodes the dopamine transporter

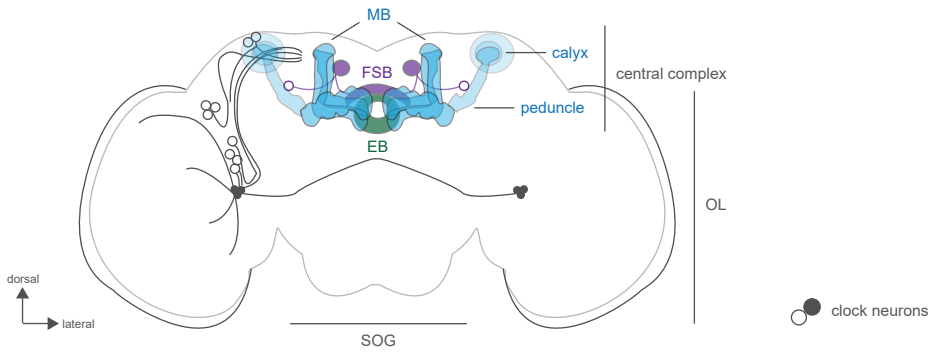


Figure 1.9: Neuroanatomical substrates involved in homeostatic sleep regulation. Two main sleep-regulatory centres have been identified in the fly: the mushroom bodies (MB, blue) and the dorsal fan-shaped body (dFSB, purple). The latter is part of the the central complex which also includes the ellipsoid body (EB, green) and two structures that have so far not been directly implicated in sleep, namely the protocerebral bridge and noduli (not shown).

(DAT) – produces hyperactivity and suppresses sleep (Kume 2005). Follow-up studies of the *fumin* mutant identified a single dopaminergic input neuron to the dFSB that acted through the dopamine receptor, DopR or DAT1 (Liu et al. 2012; Ueno et al. 2012). Excitability of dFSB neurons is closely related to arousal from sleep in fruit flies and dopaminergic input inhibits sleep-promoting dFSB neurons to enhance arousal (Pimentel et al. 2016). The arousing effects of metamphetamine decreases with increasing sleep debt, pointing towards other homeostatic systems that can override arousal-promoting dopamine signaling (Andretic et al. 2005).

Similarly, the ellipsoid body (EB, Fig. 1.9, green), a central circular structure innervating the dFSB, among others, also acts as a 'homeostat': neural excitability and structural plasticity as well as NMDA receptor expression in subsets of EB neurons positively correlate with levels of sleep pressure modulated by acute sleep deprivation (Liu et al. 2016). Conversely, changes in neural excitability of these neurons affects both synpatic plasticity and sleep levels, indicating a causal link between EB neuron activity and sleep drive (Liu et al. 2016) which may then be used to drive activity in the dFSB, the potential 'effector' arm of the fly sleep homeostat (discussed in Liu et al. 2016).

1.5.2 Neural regulators and neuropeptide systems of process S

Peptides involved especially in the regulation of the interaction between metabolism and sleep in *Drosophila melanogaster* are secreted by a cluster of cells in the pars intercerebralis (PI) that shares functional homology with the mammalian hypothalamus.

Insulin-dependent integration of sleep and metabolism

Drosophila insulin-like peptides (DILPs) are functionally homologous to mammalian insulin on the protein level (Teleman et al. 2010). Fruit flies have 8 DILPs and a subset is expressed by DILP producing cells (IPCs, Fig. 1.10) which are located in the PI, and potentially a few other neurons (Cong et al. 2015; Kannan et al. 2013). IPCs secrete DILPs into the haemolymph to reach a wide range of tissues (Teleman et al. 2010). Only one *Drosophila* insulin receptor, DInR, has so far been described (Kannan et al. 2013). It seems to be expressed in different tissues in the fly body, including the protocerebrum (Teleman et al. 2010) and the ventral nerve cord (VNC[3]). Functional DILP secretion by IPCs is important for energy homeostasis: it is involved in the response to hunger (Zhan et al. 2016) and starvation (Kannan et al. 2013) and regulated by the fat body in part through release of the leptin homologue Unpaired2 (Upd2) in response to nutritional state (Rajan et al. 2012). Interestingly, IPCs receive inputs from the circadian system (Barber et al. 2016; Cavanaugh et al. 2014; He et al. 2017) to tune, for instance, the timing of feeding (Barber et al. 2016) and the timing of sleep in the context of the current nutritional state (below).

IPCs are wake-promoting: for instance, DILP and DInR levels are positively associated with sleep duration and stabilise circadian rhythms, at least in male flies (Cong et al. 2015). This study also detected expression of at least one DILP, $DILP_2$, in a few other neurons of the fly brain aside from IPCs, including neurons implicated in circadian behaviour (LN_v and LN_d, Cong et al. 2015). A separate study also found that the activity levels of DILP+ neurons is positively associated with sleep,

[3]For expression of DInR in VNC compare the FlyLight Gal4 lines generated for CG18402 (InR) on `http://flweb.janelia.org/cgi-bin/flew.cgi`.

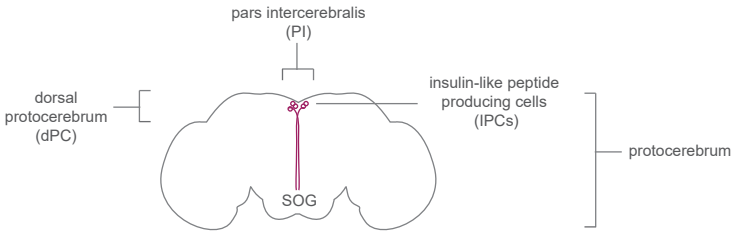


Figure 1.10: Insulin-like peptide producing cells (IPCs). Location of IPCs in the pars intercerebralis (PI) region of the dPC.

particularly night sleep (Crocker et al. 2010b). However, while total ablation of IPCs in females seems to mildly increase sleep in the day in combination with a low caloric diet (Broughton et al. 2010), another study in female flies failed to detect a change in sleep following upregulation of DILPs in PI neurons (Erion et al. 2012).

Taken together, while DILP signalling through IPCs is involved in the stabilisation of both sleep and metabolism, these results do not conclusively show that IPCs are the substrate that *integrates* sleep and metabolic regulation.

Insulin-associated integration of sleep and metabolism

The fat body is a sleep-promoting antagonist to IPCs Insulin and glucagon signalling are antagonistically coordinated in mammels and flies (Kim et al. 2004): IPCs project to the neuroendocrine gland corpus cardiacum which secretes the functional homologue of human glucagon – adipokinetic hormone (AKH) – into the fat body to mobilize energy reserves under conditions of starvation (Kim et al. 2004). AKH-dependent signalling impacts directly on behaviour: for instance, starvation-induced sleep-suppression may be mediated by AKH (Lee et al. 2004; Yu et al. 2016). In the same vein, disruption of ACER in the fat body suppresses sleep (section 1.3.1, Carhan et al. 2011).

Allostatin A acts through IPCs to modulate sleep and feeding Activation of Allostatin A (AstA) in a subset of AstA+ neurons – PLP neurons – suppresses feeding and increases sleep by decreasing food-related arousal (Chen et al. 2016). This increase in sleep may in turn decrease energy requirements, since AstA+ neuron activation is not followed by a feeding rebound, but allows increased feeding

when these flies are starved (Chen et al. 2016), and flies deficient in *AstA* or the AstA receptor, *Dar-2*, seem to have normal or even increased energy stores (Hentze et al. 2015; Hergarden et al. 2012). PLP neurons seem to receive inputs from clock neurons (Chen et al. 2016), and PLP neurons in turn terminate near the PI where IPCs express *Dar-2* (Hentze et al. 2015). Considering the site of arborization of PLP neurons in the input region of the dFSB around the PI, it is also possible that PLP neurons might impact on sleep levels through a modulation of the dFSB which would be more consistent with the sleep-promoting role of the dFSB rather than the wake-promoting role of IPCs.

Allostatin B balances sleep and arousal Allostatin B/Myoinhibitory peptide (MIP) is a ligand of the sex peptide receptor (SPR, Kim et al. 2010) and is expressed by a cluster of around 40 neurons located, partly, in the PI (Min et al. 2016). Silencing particularly of cholinergic MIP+ neurons in the PI seems to send a satiety signal as it decreases arousal to food odours and feeding, while activation of MIP+ neurons increases body weight and triglyceride levels (Min et al. 2016). Fittingly, reduction of MIP promotes sleep, potentially due to the lack of inhibition of wake-promoting clock neurons of the fly brain by MIP through SPR (Oh et al. 2014). A subset of MIP+ neurons – two pairs of lateral neurons (ICLI, ADLI) and sPI neurons in the PI – co-express the Natalisin (NTL) peptide (Jiang et al. 2013), and NTL+ ICLI neurons as well as NTL- PLP neurons[4] co-express a functional Tyramine receptor (TyrR), Huang et al. 2016). Disruption of NTL+ neuron activity affects sexual arousal (Jiang et al. 2013) in a manner dependent on the TyrR: reduction of TyrR levels in NTL+ neurons increases sexual arousal, and so does activation of NTL+ neurons while TyrR levels are reduced (Huang et al. 2016). Whether NTL+ TyrR+ neurons directly *integrate* sleep and sexual arousal remains elusive. However, specifically the reduction of MIP in NTL+ neurons does not alter sleep (Oh et al. 2014), but NTL signaling itself could have an impact on sleep: the

[4]It is unclear whether the 'PLP neurons' described by Chen et al. 2016 are identical to the AllostatinB+ PLP neurons described by Huang et al. 2016.

NTL domain is strikingly similar to the RFamide receptor DMSR-1 which regulates stress-induced sleep in *C. elegans* (Iannacone et al. 2017).

A link of IPCs and sickness sleep Drosophila sleep more when subjected to cellular stress through, for instance, bacterial infection (Kuo et al. 2010), tissue stress (Kuo et al. 2010), and painful heat (Lenz et al. 2015) and such 'sickness sleep' may be beneficial for survival (Kuo et al. 2014a,b). One route that might initiate sickness sleep could be the immune pathway involving EGF and NFkB: up- and downregulation of ligands of the Epidermal Growth Factor Receptor (EGFR) is positively associated with sleep levels during day and night (Foltenyi et al. 2007) and the expression of one ligand, *rhomboid-1* (*rho*) could be pinpointed to the PI region (Foltenyi et al. 2007). Sickness sleep in invertebrates has recently been reviewed by Kristen Davis & David Raizen (Davis et al. 2017).

Other neuropeptides involved in the integration of sleep and metabolism

Leucokinin induces food coma Leucokinin is a peptide released from a small number of neurons in the protocerebrum and the VNC to modulate feeding, particularly meal size (Al-Anzi et al. 2010). A pair of Leucokinin+ neurons has been found to receive inputs from clock neurons and project to locomotion and sleep centers of the fly – EB and FSB – to relay the circadian dynamic of rest:activity rhythms (Cavey et al. 2016).

Fruit flies often rest after a meal. A dedictated study by Keith Murphy et al. detected an increased arousal threshold to vibration stimuli in flies immediately following a meal indicating that these flies were not only resting, but actually sleeping after a meal (Murphy et al. 2016). Postprandial sleep requires inhibition of Leucokinin receptor-expressing neurons such as those of the FSB (Murphy et al. 2016). Since both feeding (Xu et al. 2008) and sleep (Hendricks et al. 2000; Shaw et al. 2000) are under circadian control, the occurrence of postprandial sleep does not necessarily mean that Leucokin+ neurons *integrate* sleep and feeding. However, *translin* also acts through Leucokinin+ neurons to modulate starvation-induced

sleep suppression (Murakami et al. 2016), and big meals after a period of prolonged starvation induce a particularly stong increase in sleep (Regalado et al. 2017).

NPF and sNPF There are many other neuropeptides that are involved in the regulation of both sleep and feeding. Briefly, NPF has been found to modulate feeding (Wu et al. 2003) and sleep in a sex-specific manner (He et al. 2013).

Similarly, sNPF regulates feeding (Hong et al. 2012; Lee et al. 2004) and sleep (Chen et al. 2013; Shang et al. 2013). Importantly, sNPF levels correlate with sleep pressure (Chen et al. 2013). Furthermore, sNPF+ neurons may be particularly involved in tuning the homeostatic sleep response (see also section 1.4.2, Shang et al. 2013).

1.5.3 Molecular regulators

Genes that have so far been identified to affect sleep behaviour center around four clusters: circadian regulators (section 1.4), ion channels, modifiers of ion channels/neurotransmission, and other signalling pathways, e.g., insulin signalling (section 1.3.4) and redox signalling.

Homeostatic control of synaptic transmission through Shaker and associated regulators

Changes in neural excitability drives circadian rhythm dynamics (e.g., Hodge et al. 2008) and is crucial for homeostatic sleep regulation.

Shaker: a regulator of membrane excitability The first homeostatic sleep phenotype identified by random screens is *minisleep* and results from a mutation in the α subunit of a voltage-gated K^+ channel (Cirelli et al. 2005b). This channel is termed 'Shaker' channel since mutant flies shake their legs under ether anaesthesia (Kaplan et al. 1969). Shaker is the founding member of the voltage-gated potassium channel (K_v) family 1 (Frolov et al. 2012): specifically, it is a fast delayed rectifier K_v channel involved in membrane repolarisation (Frolov et al. 2012). Fittingly, mutations of the *shaker* locus are associated with neural hyperexcitability as

measured at the neuro-muscular junction (NMJ, e.g., Lee et al. 2008; Wang et al. 2000) and in heterologous systems (Lichtinghagen et al. 1990).

Certain *shaker* (*sh*) mutations reduce total daily sleep duration by about 50 % – but do not affect circadian rhythm (Cirelli et al. 2005b) – as well as reducing lifespan and cognitive performance (Cowan et al. 1986), and sensitivity to certain anaesthetics (Walcourt et al. 2001). The expression of *shaker* seems to be confined to the head (Cirelli et al. 2005b), and might be strongest in the mushroom bodies (Rogero et al. 1997; Wu et al. 2010). As discussed in section 1.5.1, the role of the *Drosophila* MB in sleep regulation is complex: different MB subcircuits promote or suppress sleep (Sitaraman et al. 2015a). Earlier studies that demonstrated Shaker expression in the MB did not address whether the Shaker protein is expressed at different levels in different parts of the MB (Rogero et al. 1997; Wu et al. 2010). Thus, it is still a hypothesis to be tested whether the hyperexcitability observed in Shaker mutants is present in wake-promoting MB subcircuits and explains the strong sleep suppression observed in the *sh* mutant.

Interestingly, *shaker* is also expressed in the dFSB and – in conjunction with another potassium channel, *sandman* – modulates dFSB neuron excitability and arousal levels (Pimentel et al. 2016). As discussed in section 1.5.1, the dFSB is a sleep-promoting structure whose activation promotes sleep. The RNAi-mediated reduction of *shaker* expression in the dFSB *decreases* neural excitability in the dFSB and *suppresses* sleep; presumably because slow-activating rectifier mechanisms dominate membrane repolarisation in the absence of the fast-activating rectifier Shaker (Pimentel et al. 2016).

The *shaker* locus is conserved from mice to humans (*KCNA1*, Albrecht et al. 1995), where it is associated with distinct channelopathies (D'Adamo et al. 2015; Syrbe et al. 2015). The *shaker* homologue in mice, *Kcna2*, is also associated with a sleep phenotype and abnormal EEG profiles, including seizures (Douglas et al. 2007; Espinosa et al. 2004; Vyazovskiy et al. 2002).

The most prominent group of potentially sleep regulatory channel modifiers center around the Shaker channel. Foremost, the Shaker channel is associated

with a β subunit encoded in the *hyperkinetic* (*hk*) locus (Chouinard et al. 1995; Gulbis 2000) which gates the Shaker channel according to intracellular levels of its co-factors NADPH and NADP+ (Bähring et al. 2001; Kilfoil et al. 2013; Weng et al. 2006). Crystallographic analysis of Hk identified this protein to belong to the family of aldo-keto reductases (AKR, Gulbis 2000). *Hk* mutations replicate the effects of *sh* mutations in respect to leg shaking under ether anaesthesia (Kaplan et al. 1969; Wang et al. 2000), sleep reduction by up to 50 %, shortened life span, and defects in cognitive performance (Bushey et al. 2007; Joiner et al. 2007). In mice, mutations of this locus (*Kcnab1*) are associated with a similar phenotype (Giese et al. 1998; Rettig et al. 1994). The *KCNAB1* locus is conserved in humans and implicated in epileptic pathology (Busolin et al. 2011).

The expression and membrane localisation of Sh relies on an extracellular, GPI-anchored protein termed QUIVER (QVR) or SLEEPLESS (SSS, Koh et al. 2008; Wu et al. 2008). QVR might also bind NADPH (Humphreys et al. 1996; Wang et al. 2000) and affects Shaker channel activation and thereby membrane excitability in a manner similar to Hk (Dean et al. 2011; Wu et al. 2014b). Mutations in *qvr* replicate the *hyperkinetic/minisleep* phenotype in respect to reduced sleep and lifespan, hyperactivity and leg shaking (Koh et al. 2008).

Furthermore, mutations in *crossveinless-c*, a locus that encodes the Rho-GTPase-activating protein (Rho-GAP), also severely reduce sleep duration by 50 % or more (Donlea et al. 2014). Rho-GAP may specifically activate neurons of the dFSB in response to sleep deprivation to increase homeostatic sleep drive until the flies recovered sleep lost (Donlea et al. 2014). The molecular targets of Rho-GAP are still elusive, but are likely to include small GTPases of the Rho family (e.g., RhoA, Rho1, Rac1, Rac2; Denholm et al. 2005). For instance, the small GTPase RhoA is known to regulate the α subunit of a channel of the Shaker family, the delayed rectifier K+ channel $K_v1.2$, which is encoded by the *KCNA2* locus in humans (Cachero et al. 1998).

Homeostatic control of synaptic transmission through receptor modifiers

Receptor expression and trafficking impact on membrane excitability and are under sleep-dependent regulation. For instance, *sss* might provide a direct link between sleep regulation and membrane excitability: SSS modulates membrane excitability through inhibition of nicotinic Acetylcholine receptors (nAChR, Shi et al. 2014; Wu et al. 2009; Wu et al. 2014b), in a manner that benefits sleep (Wu et al. 2014b). SSS also interacts with another AChR subunit, *redeye*, which is sleep-promoting (Shi et al. 2014). SSS may be mainly expressed in the MB (Wu et al. 2010).

Similarly, levels of the NMDAR in input neurons to the dFSB closely trace sleep pressure and are crucial to regulate excitability to correctly tune sleep drive (Liu et al. 2016, see also Tomita et al. 2015).

Redox homeostasis

Recurrent hypotheses of the elusive function of sleep propose a protective role from oxidative stress (section 1.1.5). The metabolic load of neurons is immense and these cells are prone to damage by oxidative stress, one of the leading factors of neurological diseases (see, e.g., Higgins et al. 2010). Generally, redox homeostasis is tightly coupled to the circadian rhythm and driven by circadian clock proteins (Musiek et al. 2013). However, regulation of redox homeostasis is, to some degree, autonomous from the molecular clock: a recent study of redox oscillations that focussed on peroxiredoxin (PRX) proteins – a major sink for cellular reactive oxygen species (ROS) – found that PRXs are uncoupled from the TTFL and act independently of the molecular clock. As such, they could represent a redox oscillator and a promising path to tease apart cause and effect of redox and circadian oscillations (Milev et al. 2015). Interestingly, even sleep deprivation can induce oxidative stress (Gopalakrishnan et al. 2004; Villafuerte et al. 2015).

1.6 A potential role of aldo-keto reductases in sleep regulation

One of the first *Drosophila* sleep mutants was *hyperkinetic* (*hk*), a regulatory β subunit of the K_v channel Shaker (see above). Interestingly, Hk is also an aldo-keto reductase (AKR) (Gulbis 2000; Gulbis et al. 1999). While there is mounting evidence for the implication of AKRs in human health and disease, *Drosophila* AKRs have not received dedicated study. An overview of our current knowledge of AKRs will be given in this section; *Drosophila* AKRs will be discussed in a dedicated chapter (chapter 3).

1.6.1 The AKR superfamily

Aldo-keto reductases (AKRs) are a group of structurally-related monomeric NADPH-dependent oxidoreductases (Bohren et al. 1989). They catalyse the conversion of carbonyl substrates such as aldoses and ketoses to alcohols using NADH or NADPH as cofactors (Penning 2015). Most plants and animals express several *akr* genes with the highest activity detected for aldehyde reductase in the kidney and liver of mammals, birds, reptiles, amphibians, and fishes (O'Connor et al. 1999).

Most AKRs preferentially bind NADPH instead of NADH (Barski et al. 2008). However, NADP+ is more abundant in cells than NADPH (Pollak et al. 2007), thus favouring reduction over oxidation. Importantly, the NADH/NAD+ and NADPH/NADP+ ratios are dissociated in active cells: while biosynthesis of NADH is bound to cellular metabolism such as glycolysis and respiration, the NADPH/NADP+ ratio is kept at a constant high (Pollak et al. 2007). With constant co-factor supply, AKR function in metabolism and detoxification can be maintained under a wide range of energetic states.

The AKR superfamily is a rapidly growing protein family now comprised of 16 families with well over 190 members (Penning 2015), compared to 150 members of 15 families a few years ago (Mindnich et al. 2009) and an initial number of 42 members in 6 families 20 years ago (Jez et al. 1997b). The Perelman School of Medicine at the University of Pennsylvania hosts a database of AKR superfamily

members (Hyndman et al. 2003). This resource has recently been updated (Penning 2015) and the current organisation of AKR families is listed below (Table 1.2). AKR families 2-5 and 8-13 are species-specific. Within the AKR superfamily, families exhibit less than 40 % sequence identity with other families, and AKRs with more than 60 % sequence identity form a sub-family (Jez et al. 1997b).

1.6.2 Structural properties, molecular and biological functions

Structural properties

AKRs are comprised of a characteristic $\beta - \alpha - \beta$ motif or TIM (triosephosphate isomerase) barrel which is compact, but adaptable, to bind a wide range of carbonyl substrates (Jez et al. 1997a). The active site residues are highly conserved across AKRs, particularly the spatial arrangement of Asp-50, Tyr-55, Lys-84, and His-117 (Jez et al. 1997a, rat Akr1c9 numbering). The cofactor binding pocket is highly affine for pyridine nucleotides which AKRs use as cofactors particularly for the reduction of aldoses and ketoses (Wilson et al. 1998) and shows a particularly high degree of conservation with Asp-50, Asn-167, Gln-190, and Ser-271 (Jez et al. 1997a, rat Akr1c9 numbering). Variation in the loops towards the C-terminal end of the barrel convey substrate specificity, although the substrate binding pocket itself seems to be quite conserved, particularly at residues Ala-52, Tyr-55, Trp-86, and His-117 (Jez et al. 1997a, rat Akr1c9 numbering). The degree of conservation among AKRs can be used to organise its members down to the level of subfamilies (compare Jez et al. 1997a; Penning 2015).

The majority of human genes undergoes alternative splicing which gives rise to different protein isoforms derived from the same gene locus (Stamm et al. 2005). While no dedicated study of alternative splicing events throughout the AKR superfamily has been undertaken, a thorough literature research presented by Oleg Barski and collegues collects ample evidence for alternative splicing among AKR genes, albeit limited mostly to mammalian AKRs (Barski et al. 2013). Alternative splicing is particularly evident for members of the AKR6 family which comprises

Family	Description
AKR1	aldehyde and aldose reductases, hydroxysteroid dehydrogenases, steroid 5β reductases
AKR2	mannose and xylose reductases
AKR3	yeast AKRs
AKR4	chalcone and codeinone reductases
AKR5	gluconic acid reductases
AKR6	β subunits of voltage-gated K^+ channels
AKR7	aflatoxin dialdehyde and succinic semialdehyde reductases
AKR8	pyridoxal reductases
AKR9	aryl alcohol dehydrogenases
AKR10	Streptomyces AKRs
AKR11	Bacillus AKRs
AKR12	Streptomyces sugar aldehyde reductases
AKR13	hyperthermophilic bacteria reductases
AKR14	E.coli reductases
AKR15	Mycobacterium reductases
AKR16	V. cholera reductases

Table 1.2: List of AKR families within the superfamily according to a recent review by Penning 2015.

$K_v\beta$ subunits; this is also the only AKR family where the existence of (mammalian) isoforms has been experimentally validated and differential functions demonstrated (e.g., England et al. 1995; Rettig et al. 1994).

General molecular and biological functions

AKRs play an important role in a wide range of metabolic pathways where they interact with other carbonyl-metabolizing enzymes, e.g., aldehyde and alcohol dehydrogenases, cytochrome P450s (CYPs) and glutathione S-transferases (GSTs). Other AKRs are involved in redox homeostasis, and also gene transcription through nuclear receptor signalling (Penning et al. 2007). Another focal function of AKRs is the transformation and detoxification of potentially harmful xenobiotic or endogenous aldehydes and ketones derived from the environment as nutrient or toxin, or from metabolism (Bachur 1976; Barski et al. 2008; Penning et al. 2007). Many AKRs are associated with human diseases and may serve as therapeutic targets (Chen et al. 2012; Penning et al. 2007). For instance, the conversion of

glucose into sorbitol by a mammalian aldose reductase is greatly enhanced when glucose levels are high due to diabetes mellitus and the harmful build-up of sorbitol might be circumvented by inhibition of this enzyme (Tomlinson et al. 1994).

The largest AKR families are family 1 (33 members) – mainly comprising aldehyde and aldose reductases, and hydroxysteroid dehydrogenases – and family 6 (14 members), which are $K_v\beta$ subunits.

AKR1 family The exemplary AKR1 member is AKR1A1. This protein is mainly expressed in the liver and kidney, but also in the cerebrum, where it preferentially catalyses the NADPH-dependent reduction of aromatic and aliphatic aldehydes, and biogenic amines (O'Connor et al. 1999). An involvement in the oxidative stress response has also recently been demonstrated (Li et al. 2013). In humans, mutations of the *AKR1A1* locus is associated with non-Hodgkin's lymphoma (Lan et al. 2007) and increased cardiotoxicity (Bains et al. 2008).

AKR6 family Less is known about AKRs of family 6 and only three human members are described so far (Barski et al. 2008). Structurally, AKR6 members form $K_v\beta$ subunits that assemble with α subunits early in channel biogenesis and aid in channel trafficking (Campomanes et al. 2002; Fink et al. 1996; Manganas et al. 2000; Shi et al. 1996).

A comparatively well-studied mechanism is the regulation of the rat Shaker family potassium channel $K_v1.4$ by the $K_v\beta2$ protein. Generally, $K_v\beta2$ associates with $K_v1.4$ to aid channel trafficking (Shi et al. 1996) and to regulate channel activity (Accili et al. 1997; Rettig et al. 1994). Jun Weng and collegues identified two potential substrates of $K_v\beta2$ – 4-carboxybenzaldehyde (4-CB) and 4-cyanobenzaldehyde (4-CY) – and confirmed that substrate turnover by $K_v\beta2$ requires the co-factor NADPH (Weng et al. 2006). Furthermore, this study demonstrated that the availability of the co-factor NADPH is also crucial for the ability of $K_v\beta2$ to modulate $K_v1.4$ activity: specifically, binding of $NADP^+$ by $K_v\beta2$ increases the open probability of $K_v1.4$, while binding of NADPH decreases the open probability (see Fig. 1.11 for an illustration of this relationship, Weng et al. 2006). Another study confirmed the

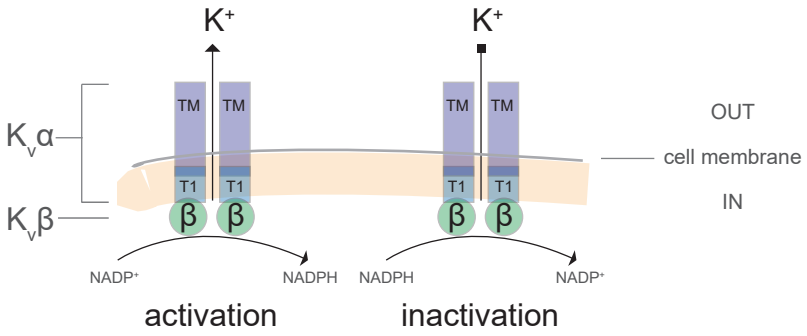


Figure 1.11: Schema of the Shaker complex. The Shaker complex is a voltage-gated K^+ (K_v) channel that consists of an α subunit forming a transmembrane (TM) and a linker domain (T1) and a β subunit such as HK that binds the co-factor $NADP^+$ or NADPH which induces conformational changes that opens ($NADP^+$) or closes (NADPH) the TM domain (inspired by Kilfoil et al. 2013).

specificity of $K_v\beta 2$ for benzaldehydes and identified several other substrates, further demonstrating that $K_v\beta 2$ subunits are indeed catalytically active AKRs (Tipparaju et al. 2008). However, these studies did not investigate whether substrate binding itself was relevant for channel gating functions.

The substrate binding domains of AKR6 proteins are relatively different from other AKRs, their catalytic activity is considerably weaker, and the relationship between co-factor binding and substrate binding for channel gating is still unclear; these differences between AKR6 members and members of other AKR families imply that the oxidoreductase-related functions of AKR6 proteins is still elusive (Barski et al. 2008).

AKR6 proteins are implicated in health and disease: for instance, deregulation or mutation of $K_v\beta$ subunits is associated with channelopathies in humans (Busolin et al. 2011; Chen et al. 2012; Heilstedt et al. 2001).

Sleep-related functions A direct involvement of mammalian AKRs in sleep or circadian rhythm regulation has so far not been demonstrated. However, the expression of at least some AKRs follow a circadian pattern (*Euphausia superba*, head, Teschke et al. 2011), particularly in peripheral tissues (*Mus musculus*, kidney, Zuber et al. 2009), and the transcription of at least one AKR, *AKR1B7*, responds to

increasing intracellular cAMP (*Mus musculus*, kidney, Lin et al. 2015; adrenal gland, Aigueperse et al. 1999). Interestingly, AKRs are involved in the prostaglandin pathway (Díez-Dacal et al. 2011; Kabututu et al. 2009) – prostaglandin is a key sleep-inducing molecule (Urade et al. 2011). Also, Shaker and sleep

1.6.3 Expression and function of insect AKRs

As discussed in section 1.6, aldo-keto reductases (AKRs) are NADPH-dependent oxidoreductases (Bohren et al. 1989) that are organised into a superfamily comprising different families of mainly mammalian, bacterial, and yeast AKRs (Penning 2015). Our knowledge of insect AKRs is greatly limited and the small number of dedicated studies is briefly addressed below. Generally, insect AKRs are functionally diverse and involved in developmental processes such as ecdysis, but also digestion and sensation, as well as neurotransmission.

Regulation of ecdysis and development

Spodoptera littoralis In the cotton leafworm *Spodoptera littoralis*, the AKR 3-dehydroecdysone 3α-reductase (3DE) is related to development due to its steroid activity (Takeuchi et al. 2000). The enzyme 3DE is expressed in the Malphigian tubules – a homologue of the mammalian kidneys and adrenal gland – at early stages of development. This enzyme is crucial for the inhibition of the molting hormones, ecdysteroids: 3DE irreversibly converts ecdysteroids into 3-dehydroecdysone which is then converted into 3-epiecdysone; ecdysteroids are crucial regulators of ecdysis, the molting of the larval cuticle (Takeuchi et al. 2000).

Bombyx mori An AKR of the AKR2E family and two integument esterases have been detected in the molting fluid of the silkworm *Bombyx mori* (Zhang et al. 2014). RNAi-mediated knock down experiments of these targets indicate an involvement in development (Zhang et al. 2014). Specifically, the silkworm expresses an AKR, 3-dehydroecdysone 3β-reductase (3DE-3β-reductase) which catalyses the reversible conversion of 3-dehydroecdysone into ecdysone – mainly in the genitalia, fat body, head, epidermis and hemocytes – to maintain ecdysone

at levels appropriate for development (Yang et al. 2011). The structure of this enzyme has been resolved: it reduces carbonyl compounds including isatin, but also progesterone, in an NADPH-dependent manner and has been suggested as the candidate AKR member AKR2E4 (Yamamoto et al. 2014). Similarly, the structure of a related AKR of this organism, AKR2E5, has been resolved: this enzyme also reduces carbonyl compounds such as glyceraldehyde, xylose, and progesterone in an NADPH-dependent manner (Yamamoto et al. 2016).

Aedis aegypti The ovarian ecdysteroid production in the females of the mosquito *Aedes aegypti* may also in part be regulated by an AKR (Sieglaff et al. 2005). In this organism, Aldehyde dehydrogenase 3 (Adh3), a potential AKR, is involved in the juvenile hormone pathway – a critical regulator of morphogenesis and reproduction – where it catalyses the conversion of farnesal to farnesoic acid which can then be converted into juvenile hormone (Rivera-Perez et al. 2013).

Antennae-related functions

The moth *Helicoverpa armigera* expresses two functional AKR1 members – specifically NADPH-dependent aldehyde reductases – in their antennae (Guo et al. 2014). The authors of this study exclude an involvement of this activity in pheromone pathways, but do not offer an alternative hypothesis regarding its function.

Also, the beetle *Holotrichia parallela* expresses, for instance, *Aldo-keto reductase-like* in its antennae in a sexually dimorphic fashion although the functional relevance of this enzyme, as well as its full expression pattern, is still elusive (Zhang 2016).

Considering the main role of the insect antennae as sensory organs of smell and touch (Chapman 1998) and the range of functions AKRs fulfil (Penning 2015), the expression of AKRs in these species could aid in the detection and elimination of xenobiotic substances.

Digestion

In the termite *Coptotermes gestroi*, the aldo–keto reductase CgAKR-1 is produced in the salivary gland, proventriculus, and foregut and helps to degrade a particular type of cellulose (Tramontina et al. 2017).

1.6.4 Expression and function of fruit fly AKRs

Only two *Drosophila melanogaster* genes have yet been specifically identified as AKRs in previous studies: Hk and CG10863.

Hk The first identification of the expression of a functional AKR in insects has in fact been Hk of *Drosophila melanogaster*: in 1995, Scott Chouinard and collegues not only demonstrated that Hk regulates membrane excitability through the Shaker channel, but also identified Hk as a $K_v\beta$ subunit due to its sequence similarity with mammalian $K_v\beta$ subunit which had already been identified as AKR members (Chouinard et al. 1995). This prediction from sequence similarity has later been confirmed through structural studies (Gulbis 2000; Gulbis et al. 1999). Research relating to Hk will be specifically discussed in section 1.6.5.

CG10638 A study dedicated to the development of a signed protein-protein interaction network for *Drosophila melanogaster* identified the gene CG10638 as a potential *Drosophila*AKR and experimentally validated the involvement of the protein in proteasome regulation (Vinayagam et al. 2014). Other screens suggest a role of CG10638 in transcriptional regulation and metabolism (Gruenewald et al. 2009; O'Keefe et al. 2011). Research relating to CG10638 will be specifically discussed in section 1.6.6.

CG10863 A study by Eric Luccio identified further members of the AKR superfamily by searching for candidates that might show NADH/NADPH dual specificity as indicated by sequence comparison with the dual-specificity enzyme xylose reductase (AKR2B5) of the yeast *Candida tenuis* (Di Luccio et al. 2006). This study included the *Drosophila melanogaster* AKR CG10863; through *in silico*

modelling CG10863 is placed in the AKR2E3 family, and steady-state assays identified its co-substrate as glyceraldehyde which CG10863 likely metabolises in an NADPH-dependent manner (Di Luccio et al. 2006).

1.6.5 Hk – a sleep-regulatory fruit fly AKR

Interaction with the Shaker channel

The voltage-gated K^+ (K_v) channel Shaker is associated with a β subunit encoded by the *hyperkinetic* (*hk*) locus (Chouinard et al. 1995; Gulbis 2000). Binding of either co-factor NADP+ or NADPH has differential effects on channel gating: while binding of NADPH inactivates the channel, binding of NADP+ prevents inactivation (Fig. 1.11, Bähring et al. 2001; Kilfoil et al. 2013; Weng et al. 2006).

Mutations of Shaker regulators such as *sss*/*qvr* and *hk* render flies hypersensitive to increased levels of reactive oxygen species (ROS) induced by the pesticide paraquat, probably due to their inability to downregulate neural excitability in compensation (Wang et al. 2000). *Hk*-deficient flies also accumulate high levels of ROS in presynaptic terminals (Ueda et al. 2008) and are prone to neurodegeneration (Fergestad et al. 2006).

Aside from their direct role in channel gating and membrane excitability, $K_v\beta$ subunits can also act as chaperones and aid in the membrane trafficking and localisation of α subunits (Accili et al. 1997; Shi et al. 1996) in part in cooperation with cyclin-dependent kinases (cdk, Vacher et al. 2011). They may also be crucial for linking α subunits to the intracellular scaffold and to promote channel clustering (Honoré et al. 1992; Levin et al. 1996; Nakahira et al. 1998; Tiffany et al. 2000). Some of those chaperone functions may rather depend on co-factor than on substrate binding (Campomanes et al. 2002). Such potential chaperone functions of Hk have not yet been directly investigated.

Sleep-regulatory functions of HK

Bushey et al. carried out an initial sleep study of flies carrying mutations of the *hk* locus: these flies sleep around 30-54 % less than normal flies (Bushey et al.

2007). The *hk* mutation affects both day and night sleep; this observation is most pronounced in males, due to already low day sleep levels in females (Bushey et al. 2007). Interestingly, *hk* mutations do not impair the homeostatic sleep response in this study (Bushey et al. 2007). The introduction of an additional *sh* mutation does not provoke a further reduction in sleep confirming that Hk and Sh act through the same mechanism to affect sleep (Bushey et al. 2007). Additionally, *hk* mutant flies show an ether anaesthesia-induced shaking phenotype similar to *sh* mutant flies (Bushey et al. 2007; Kaplan et al. 1969) and a similar abnormal light-induced jumping response (Ikeda et al. 1970b) as well as similar learning impairments (Bushey et al. 2007).

An exact list of Hk+ neurons is still elusive as no antibody exists to far, but several substrates have been proposed. For instance, the light-induced jump response depends on motoneurons in the ventral thoracic ganglion (Ikeda et al. 1970b), but *hk* mutations – along with *sh*, *qvr*, and *eag* mutations – also produce aberrant neural excitability at the neuromuscular junction (NMJ, Wang et al. 2000). Regarding the central nervous system, Hk is assumed to exert its effects through the MB, although there is only indirect evidence for this hypothesis: it mainly derives from the observation that Hk forms a complex with Sh, and Sh, in turn, is expressed mainly in the MB (Rogero et al. 1997; Wu et al. 2010). Sh also suppresses sleep when reduced in the dFSB (Pimentel et al. 2016), although no direct association study with Hk in this structure has yet been undertaken.

Another line of studies relates Hk function to clock neurons: clock neurons such as the l-LN$_v$ express the blue light receptor CRY which resets the molecular clock in the morning (section 1.4). CRY is likely to be descended from DNA photolyases – light-activated enzymes crucial for DNA repair in response to UV-A light and light of similar wavelength (Lin et al. 2005) and it contains two co-factors that absorb light (chromophores), one of which is flavin adenine dinucleotide (FAD, Lin et al. 2005). FAD is a redox co-factor like NAD+ and NADP+ and might modulate the transcription of circadian genes in the context of metabolic load: for instance, in mammals, FAD acts to stabilise CRY in the nucleus (Hirano et al. 2017). In flies,

CRY does not only sense blue light, but also enhances neural activity of the l-LN$_v$ in response (Fogle et al. 2011). This CRY action relies on FAD's redox sensing properties as application of the flavin-specific redox inhibitor diphenyleneiodonium chloride (DPI) and increasing oxidative stress levels by overexpression of *superoxide dismutase* (*SOD*) or H_2O_2 application diminish the light-induced neural activation (Fogle et al. 2011, 2015). Interestingly, both the light-induced behavioural startle and the neural activation also diminish in *hk*-deficient flies in the l-LN$_v$ (Fogle et al. 2015), and this observation holds not only for blue light (Fogle et al. 2015), but also for UV light (Baik et al. 2017), opening up the possibility that HK interacts with FAD to mediate neural activity in response to CRY activation.

Taken together, Hk may function as a neuroprotective redox sensor to modulate membrane excitability according to cellular stress levels and thereby initiate sleep and associated neural maintenance (discussed in Gulbis et al. 1999; Weng et al. 2006). In mice, the *hk* homologue *Kcnab1* is expressed in the brain (Gulbis et al. 1999) and cognitive impairments of *Kcnab1* mutant mice point to a similar function (Giese et al. 1998).

1.6.6 CG10638 – a regulator of the fruit fly proteasome?

Putative substrates and functions

This gene has not yet received detailed study, but it did surface in a range of screens, however, providing clues to its function which center around metabolism and the regulation of protein levels through the proteasome (section 3.1).

Metabolism and redox state Oxidative insult in the form of high oxygen levels and paraquat exposure increases expression levels of CG10638 (Girardot et al. 2004; Landis et al. 2004). This is also true when hyperoxia-induced expression levels are measured specifically in the head (Gruenewald et al. 2009), and a parallel upregulation is seen for chaperones which act to prevent oxidative stress-induced neural damage (Gruenewald et al. 2009). Furthermore, in cell lines carrying a mutation of *SIN3* – the main compound of the histone deacetylation complex which

represses transcription – CG10638 expression levels are increased along with genes involved in mitochondrial respiration and cell cycle regulation (Pile et al. 2003). Similarly, CG10638 levels are also affected by SWI/SNF chromatin remodeling factors which act in conjunction with the ATPase *Brahma* (*Brm*, Waldholm et al. 2011). Together, the Brm-SWI/SNF complex regulates transcription levels and alternative pre-mRNA processing in a manner that ties alternative splicing to the energy and oxidative stress state of the cell (Fontana et al. 2017).

Development The Notch and the RTK-Ras pathways play a major role in development. An investigation of potential integrators of Notch and RTK cross-talk found that upregulation of *ras* levels, but not of *notch* or their combination suppresses CG10638 expression (among others, Hurlbut et al. 2009).

Transcriptional regulation RNAi-mediated knock down of *WWOX* – an atypical tumour suppressor – also reduces CG10638 expression levels, and *WWOX* overexpression increases CG10638 levels (O'Keefe et al. 2011), marking CG10638 as a potential interactor of WWOX. In the context of other WWOX interactors such as superoxide dismutase (SOD) and the NAD^+-dependent isocitrate dehydrogenase (Idh), WWOX could be part of a mechanism to prevent DNA damage due to oxidative stress (O'Keefe et al. 2011). Despite the close tracing of *WWOX* levels, CG10638 was not further assessed as part of that study. The authors hypothesise, however, that CG10638 may be involved in sorbitol production as part of the glucose metabolism (O'Keefe et al. 2011). Another oncology study focussed on *p53* – a central tumour suppressor that regulates gene transcription – in order to detect *p53* downstream, and potentially protective, targets. Here, CG10638 was downregulated in response to radiation stress in normal flies, and upregulated in response to radiation stress in *p53* mutant flies, although the response was not robust across replicates (Akdemir et al. 2007).

Proteasome regulation The proteasome is a crucial component of proteostasis through protein degradation marked by ubiquitination (Tanaka 2009). Dysregulation of proteasome activity is associated with aging (Löw 2011) and diseases such as neurodegeneration and cancer (Dahlmann 2007).

Recent work to develop a signed protein-protein interaction network screened for potential positive and negative regulators of the proteasome and identified two unexpected candidates, the positive regulator enolase and the negative regulator CG10638 (Vinayagam et al. 2014). In order to validate these – and other – candidates, the authors employed two experimental strategies: firstly, the expression of candidate genes was reduced in cultured muscle cells through RNAi-mediated knock down under the control of the muscle-specific *Dmef2*-Gal4 driver, and the accumulation of ubiquitinated proteins assessed through histological examination of ubiquitin protein levels. Secondly, the expression of candidate genes was similarly reduced in S2+ cells and the enzymatic activity of the proteasome was assessed through a luminescent assay. These experiments confirmed CG10638 to increase proteasome activity and to consequently decrease ubiquitinated protein levels *in vitro* and *in vivo* (Vinayagam et al. 2014). Taken together, these findings mark CG10638 as a negative regulator of the proteasome (Vinayagam et al. 2014).

1.7 Summary and study aims

Rest behaviour in fruit flies is a sleep-like state

A vast body of research on the molecular and physiological mechanisms of sleep regulation accumulated over the last decades. Due to the complexity of sleep behaviour and the nature of the experiments, this body of knowledge is mostly derived from studies in vertebrate and invertebrate model organisms, particularly in fruit flies as they provide a less complex system with a broad genetic toolbox.

Progress has been accelerated by the acceptance of a behavioural instead of an EEG-based definition of sleep.

Rest is part of the daily behavioural dynamics of *Drosophila melanogaster*. The rest state in fruit flies qualifies as sleep as it is associated with periods of quiescence that are accompanied by decreased responsiveness to sensory stimuli. Importantly, this state is reversible by stimulation at appropriate intensities, and it is not only under circadian, but also under homeostatic control.

Robustness and variability of sleep behaviour

Fruit flies are diurnal and their sleep timing is under circadian control: they exhibit two main sleep phases, a shorter day-specific sleep episode around noon ('siesta') and a longer, more consolidated night-specific phase terminated by morning anticipation at dawn. Fruit flies are also sexually dimorphic. While sleep is largely similar between the sexes and independent of mating state, there are some notable differences: female flies sleep generally less than males and daytime sleep is particularly low in mated females.

Flies respond to somnolytic and arousing pharmacological agents known to affect sleep in mammals. There may be neural signatures associated with the rest state in flies in general and different stages of sleep in particular.

Individual factors such as age, mating state, hunger and experience, and environmental factors such as temperature and light, but also social interactions, shape sleep quantity and quality. Social experience affects sleep in flies as far as the – largely sensory – ability of the fly to engage in it. Competing behaviours

such as courtship and aggression can interfere with sleep structure and demand. Sleep requirements also vary according to accumulated sleep debt. The variability of sleep behaviour along many dimensions both between individuals and between different laboratory strains indicates that environment and experience play a major part in determining the particular sleep characteristics of a fly.

When wake antagonises sleep

Flies have a molecular circadian system that is homologous to the mammalian system. Here, the TTFL runs in a set of core clock neurons to module their activity. These core clock neurons express the neuropeptide transmitter PDF to synchronise the activity of downstream targets. While some downstream targets such as the DN_1 cluster are involved in modulating day sleep in response to ambient temperature, the core clock neurons, particularly the s-LN_v are wake-promoting: the neural activity of s-LN_v is under circadian control and drives behavioural arousal and activity, particularly the activity peak around dawn. Thus, sleep must be achieved by inhibition of s-LN_v, e.g., through GABAergic neural inhibition, as well as plastic changes of s-LN_v arborisations and their PDF load. The PDFR is a G-protein coupled receptor that acts through the PKA-cAMP pathway to stabilise the molecular clock and neural activity in the clock network in a manner that promotes activity. However, s-LN_v also express sNPF, an inhibitory peptide transmitter that could antagonise PDF signalling to modulate sleep at times of high PDF activity, such as the daytime.

This hypothesis is complemented by mounting evidence: for instance, PDF release from s-LN_v promotes waking; release of sNPF rather promotes sleep. Additionally, GABA has long been associated with the induction of sleep. For instance, in humans, extreme cases of insomnia are treated with $GABA_A$ antagonists (Hobson et al. 2002). In flies, an ionotropic $GABA_A$ receptor acts through clock neurons to aid sleep, together with metabotropic $GABA_B$ receptors in the large and small LN_vs.

Converging mechanisms of circadian and homeostatic sleep regulation

The l-LN$_v$s and s-LN$_v$s in concert with the MB and IPCs of the pars cerebralis PI, antagonised by the fat body and modulated by further peptidergic inputs, promotes wakefulness. Conversely, the dFSB, antagonised by subgroups of dopaminergic neurons, together with the EB, promotes sleep.

In light of previously identified potential sleep-regulatory genes and pathways, mechanisms of sleep regulation so far proposed center around SHY and the associated modulation of membrane excitability. According to SHY, sleep supports the maintenance of neural processes by, e.g., downscaling synapses that underwent short-term growth during waking hours, which is generally under circadian control. For instance, research on *Drosophila* sleep mutants identified the Shaker channel (Sh) and its regulators (Hk, Qvr, SSS, Rye) that modulate its expression, localisation, and response properties and consequently membrane excitability. In this case, the neural population that expresses the Shaker system has been narrowed down to the mushroom bodies (MB).

Additionally, redox cycling impacts on membrane excitability in the main mammalian clock, the SCN (Wang et al. 2012). While similar experiments have not been conducted in fruit flies yet, induction of low, but continuous oxidative stress affects sleep in flies in a manner similar to aging by rendering it more fragmented and loosening the tight coupling to circadian rhythm (Koh et al. 2006).

Study aims

Interestingly, a crystallographic characterisation of Hk identified this protein as belonging to the enzymatic group of NADPH-dependent oxidoreductases or aldo-keto reductases (Gulbis 2000). With the exception of Hk, aldo-keto reductases (AKRs) of fruit flies have so far not been further characterised, but studies in their human counterparts suggest that they are involved in a multitude of functions regarding health and disease including circadian rhythmicity, neural transmission, and redox homeostasis. *Drosophila melanogaster* AKRs, including Hk, receive detailed functional analysis in chapter 3.

This project aims to characterise the role of AKRs in sleep regulation inspired by the promising involvement of Hk in the neural regulation of sleep behaviour.

First, we take advantage of the availability of the recently updated *Drosophila melanogaster* genome and the wide range of genome mining tools to perform an *in silico* study of potential fruit fly AKRs. In a second step, we experimentally test the involvement of *Drosophila* AKRs in neural sleep regulation. To this end, we ascertain which AKRs are expressed in the nervous system. Then, we perform an RNAi-mediated knock down screen restricted to the nervous system to search for sleep-regulatory candidates. In parallel, we also perform a similar screen restricted to the MB as it is a major structure of the fly brain that has long been implicated in sleep regulation. Potential sleep-regulatory candidates are validated through alternative approaches: these include the examination of mutations associated with the candidate loci, and the sensitivity of candidate expression to sleep challenges such as sleep deprivation. We further characterise potential candidates in regards to the particular neural populations that express them, the role of these neurons in sleep regulation, and the particular role of the candidate in modulating circadian and/or homeostatic mechanisms of sleep regulation through these neurons.

2 Material and Methods

2.1 *In silico* tools

2.1.1 Collection of candidate AKR genes

Although a systematic nomenclature, the AKR superfamily, has been created by Drs. Hyndman and Penning and is hosted by the Perelman School of Medicine of the University of Pennsylvania (`http://www.med.upenn.edu/akr/`, Jez et al. 2001), this compilation is largely limited to human, mouse and rat AKRs and *Drosophila melanogaster* AKRs are missing except for Hyperkinetic (Hk).

We collected genomic loci predicted to encode an AKR domain from the *Drosophila melanogaster* genome release 6 by the Berkeley Drosophila Genome Project (BDGP) as of July 2014 (FB2014-04, `http://flybase.org`).

Their domain annotation is sourced from the InterPro annotation of *Drosophila melanogaster* genes (`http://www.ebi.ac.uk/interpro/`) which integrates predictive models of a range of member databases to produce a unifying protein classification tool (Jones et al. 2014; Mitchell et al. 2015). Specifically, annotations of AKRs in *Drosophila melanogaster* (NCBI taxonomic ID # 7227) within InterPro are derived from the PANTHER database ("Protein ANalysis THrough Evolutionary Relationships", `http://pantherdb.org`). PANTHER is a tool of the Gene Ontology Reference Genome Project developed to classify proteins through a combination of

manual and algorithmic curation to enable the accurate prediction of the functions of unknown proteins from their evolutionary relationship with known proteins (Mi et al. 2013).

2.1.2 Functional analysis and conservation of protein sequences

Conservation of *Drosophila melanogaster* AKRs

Multiple sequence alignment Protein sequences of candidate fly AKRs were compared with each other and a common reference AKR, rat AKR1c9, through multiple sequence alignments (MSA) as implemented in the MUSCLE webtool ("MUltiple Sequence Comparison by Log- Expectation", `http://www.ebi.ac.uk/Tools/msa/muscle/`) due to its high accuracy (Edgar 2004). Conserved residues were manually annotated according to rat AKR1c9 numbering following a similar approach by Jez et al. 1997b.

Pairwise sequence alignment Pair-wise sequence comparisons were generated using EMBOSS Needle to evaluate the sequence identity of pairs of *Drosophila melanogaster* AKRs (Rice et al. 2000). This tool is available from `http://www.ebi.ac.uk/Tools/psa/emboss_needle/` and was used with the default parameters, i.e., a gap open penalty of 10, and a gap extend penalty of 0.5, an end gap open penalty of 10 and an end gap extend penalty of 0.5.

Orthologue search In order to connect *Drosophila melanogaster* AKRs with the large body of mammalian AKR studies, we searched for the most likely orthologues, i.e., those proteins with the highest levels of sequence identity, in the well-characterised laboratory model organism *Mus musculus* using BLAST ("Basic Local Alignment Search Tool"). Specifically, we used the NCBI BLASTP webtool (protein-protein blast, `https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins`) at default settings.

Functional prediction

Gene Ontology analysis FlyBase was also used as an initial resource for the gene ontology analysis. The annotation of AKR protein domains follows PROSITE signatures and was generated using the scanprosite tool at default settings (`http://prosite.expasy.org/`, Castro et al. 2006; Sigrist et al. 2002).

Prediction of protein-protein interactions Protein-protein interactions (PPI) were collected from two sources, the 'SignedPPI' and the 'STRING' databases. The SignedPPI network (`http://www.flyrnai.org/SignedPPI/`) contains a protein-protein interaction (PPI) network of *Drosophila melanogaster* proteins that is 'signed' in the sense that it incorporates activation/inhibition relationships derived from both experimental data from cell-based RNAi screens in turn stored in dedicated databases (SignaLink, KEGG, STKE) and on prediction (Vinayagam et al. 2014). Similarly, the STRING database ("Search Tool for the Retrieval of Interacting Genes/Proteins", `https://string-db.org/`, Mering et al. 2005) provides a PPI network across organisms that incorporates both experimental data and predictions. Main STRING parameters used are a minimum interaction score of 0.4 and up to 20 interactors.

FlyAtlas 2.0 FlyAtlas is an online resource (`http://flyatlas.gla.ac.uk/flyatlas/index.html`) that provides a comprehensive overview of gene expression across *Drosophila melanogaster* tissues. It contains microarray-based gene expression profiles across the main adult tissues covering a large portion of the *Drosophila melanogaster* transcriptome and provides information on the abundance of a particular mRNA and its tissue enrichment in comparison to the whole fly (Chintapalli et al. 2007). We listed the expression of *Drosophila melanogaster* AKRs according to FlyAtlas database in respect to the tissues they are most abundantly expressed in ("enriched") and to indicate whether they are expressed in the nervous system ("NS expressed").

Ingredient	Concentration (%)	Source
Brewer's yeast	10	Whole Foods Market
Polenta	2	Whole Foods Market
Fructose	8	Whole Foods Market
Agar	0.8	Whole Foods Market
15:100 Nipagin:EtOH	0.5	Sigma Aldrich
Proprionic acid	0.5	Sigma Aldrich

Table 2.1: Fly medium: contents and their concentration relative to solvent (tap water). Main suppliers include Whole Foods Market Inc., Austin, TX, and Sigma Aldrich Co. LLC., Munich, Germany.

2.2 Animals

2.2.1 Husbandry

Sources Stocks were obtained from the Bloomington Drosophila Stock Center (BDSC, `http://flystocks.bio.indiana.edu/`, NIH P40OD018537) unless otherwise stated. The majority of RNAi lines was obtained from the Vienna Drosophila Research Center (VDRC). The wildtype strain used to generate parental controls, backcross mutant lines, and to calibrate experimental conditions was w^{1118} and similarly obtained from the VDRC.

Some lines were provided as kind gifts by other laboratories as referenced.

Maintenance Unless otherwise stated, flies were kept on standard medium (Table 2.1) under a regular light:dark (LD) schedule with 12 hours of light. Fly stocks were kept in vials (vials: VWR, # 734-2255, plugs: VWR, # 734-2875) at room temperature of around 22 °C and expanded in bottles (bottles: VWR, # 734-2673, plugs: VWR, # 734-2673). Experimental lines were raised at 25 °C and 60 % humidity in dedicated incubators (OvaEasy 380 Advance Ex Series II, Brinsea Products Ltd., Weston Super Mare, UK).

Temperature-sensitive genotypes – such as those including UAS-TRPA1 – were raised at 18 °C and 60 % humidity.

2.2.2 Stocks derived from insertional mutagenesis

To evaluate the involvement of *Drosophila melanogaster* AKRs in sleep regulation, we tested several lines carrying mutations that disrupted AKR loci. These mutants have been generated through insertional mutagenesis of transposable DNA elements as part of the Drosophila gene disruption project (GDP, `http://fruitfly.org/p_disrupt/index.html`).

Insertional mutagenesis by transposable elements

Transposable elements Transposons are parasitic DNA sequences that can insert and spread across the hosts' genome. A group of transposons, P-elements, occur naturally in *Drosophila melanogaster* and might have been acquired through parasitic mites (Engels 1992). In order to "jump" the genome, P-elements contain an enzyme, a transposase, that facilitates the excision and insertion of P-elements from and into the host DNA, thereby creating random mutations, as well as a recognition site for the transposase (Hummel et al. 2008). The ability of transposons to "jump" throughout the genome can be harnessed to generate mutations, usually by injecting a "P plasmid" that contains recognition sites, an insertion marker, and, if desired, other genes of choice, into a fly embryo that expresses the transposase (Hummel et al. 2008). Other transposable elements are also used in *Drosophila*, employing similar mechanisms to achieve mutagenesis (e.g., Bellen et al. 2004, 2011; Metaxakis et al. 2005). This principle is also termed insertional mutagenesis.

Transposon insertion strategies Fly lines derived from three different transposable elements were used for this project, namely P-element, piggyBac and Minos transposon lines. While integration of Minos transposons is essentially random, P elements preferentially insert into promoter regions, and piggyBac elements insert into hotspots and coldspots (Bellen et al. 2011). These transposable plasmids include different features:

- P-element lines incorporate visible markers (*mini-white*, w[+mC], and *mini-yellow*, y[+mDint2]), and a GAL4 site (Bellen et al. 2004).

Gene affected	Insertion site	Transformant	Transposon	Source	Order #
CG10638	69C4	EY21723	EP	BDSC	23110
	69C3	e01020	PBac	BDSC	17916
	69C3-69C4	f00334	PBac	BDSC	18315
	69C3	MB06512	Minos	BDSC	25511
CG3397	87A4	EY04742	EPg	BDSC	15768

Table 2.2: List of mutant lines. "Transposon" refers to the particular type of transposable elment used to generate the mutant, and different ensueing types are abbreviated "EP", P-element, "Minos", Minos element, and "PBac", PiggyBac.

- Minos lines carry a fluorescent marker (GFP) and a Gal4 site (Metaxakis et al. 2005).
- PiggyBac lines carry a visible marker (*mini-white*, w[+mC]) and a FRT site that allows FLP-mediated recombination between other FRT-containing elements to enable further mutagenesis (Bellen et al. 2011).

Mutant stocks

The GDP has accumulated an extensive collection of mutant lines carrying single transposon insertions in different genes covering up to 95 % of the genome. Mutant stocks used in this study are listed in Table 2.2.

2.2.3 Actuator lines: RNAi-mediated knock down

The UAS/Gal4 system and similar approaches allow the conditional temporally and spatially restricted expression of genes of choice (section 1.2). For the purpose of this chapter, lines "driving" gene expression, e.g., Gal4 lines, will be termed "driver lines". Lines containing the effector transgene, e.g., UAS lines, will be termed "actuator lines" if they were used to modulate gene expression levels or cellular activity, or "reporter lines" if they were used to mark cellular compartments or passively monitor cellular activity.

We screened *Drosophila melanogaster* AKRs for a sleep phenotype under conditions of RNAi-mediated reduction of AKR expression using dedicated RNAi lines as described below.

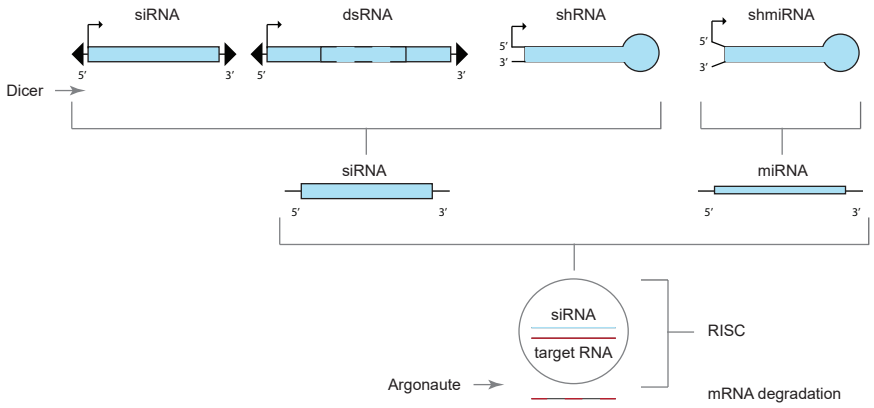


Figure 2.1: Schema of genetically encoded *in vivo* RNAi strategies. Four types of RNAi constructs are commonly used: short interference or siRNA, long double-stranded or dsRNA, short hairpin or shRNA, and short hairpin micro or shmiRNA. While siRNA are small RNA duplexes of around 20 nucleotides in length that are directly injected into the embryo to achieve a transient reduction of the gene of interest, the other RNA constructs can be integrated into the genome to be endogenously expressed. Both shRNA and shmiRNA consist of a small single-stranded RNA of around 20-30 nucleotides that folds in on itself to create a loop structure or "hairpin". Long dsRNA are typically around 350 nucleotides in length. In the RNAi pathway, the "seed" RNA sequence is processed by the RNase II enzymes Dicer and Dosha into short, interfering siRNA or miRNA, which are then loaded into the RNA-induced silencing complex (RISC) where the single-stranded siRNA hypridises with the target mRNA. The hybridised target mRNA is then degraded by the RNase Argonaute. Schema inspired by Perrimon et al. 2010.

RNAi-mediated knock down of gene expression

RNAi interference (RNAi) is a naturally occurring protective mechanisms of post-transcriptional gene silencing to degrade target mRNA that is complementary to certain "trigger" sequences such as viral RNA or transposons (Kavi et al. 2008, chapter I).

The RNAi machinery can be harnessed to reduce the production of proteins without the introduction of mutations: here, "seed" RNA sequences complementary to the gene of interest – also termed "inverted repeats" or "IR" – are either directly injected into the embryo or transfected into cells, or endogenously expressed as transgenes (Fig. 2.1, Perrimon et al. 2010). Since the RNAi machinery relies on the RNA-processing enzyme Dicer, the expression of an additional Dicer transgene can increase RNAi efficiency in some cases (Dietzl et al. 2007; Perrimon et al. 2010).

Insertion of the RNA transgene into the genome can be achieved through

various strategies, mainly through transposon-mediated random insertions, or targeted insertion into dedicated sites recognised by enzymes that facilitate their integration (Perrimon et al. 2010).

An RNAi-based approach is susceptible to nonspecific effects (Wang et al. 2015). These unintended effects can be due to (1) a partial sequence complementarity of the intended target transcript with other, unintended transcripts, (2) an immune response triggered by siRNAs, (3) general interference with the endogenous RNAi machinery through high levels of exogenous siRNAs (Jackson et al. 2010). Unindented effects can be augmented by co-expression of *Dicer*, as increased Dicer levels enhance the RNAi machinery in general, not just regarding the target gene (Cullen 2006), and Dicer fulfils complex, RNAi-independent functions (Wang et al. 2015).

RNAi libraries derived from different strategies

Three libraries of RNAi lines were used in this study: these consist of two first generation libraries, KK and GD lines, sourced directly from the VDRC, and the newer generation TRiP lines which were generated by the Harvard Medical School and sourced from BDSC.

First generation RNAi lines Two sets of RNAi lines used in this study employ hpRNAs.

GD library This collection has been generated by P-element mediated transformation of inverted repeats of short target gene fragments producing hpRNAs ("hairpin RNAs") together with 10 copies of UAS sites into the fly genome (Dietzl et al. 2007). The GD library is powerful due to its large scale: it covers up to 88 % of the genome (Dietzl et al. 2007). However, insertion of P-elements into the genome is prone to create mutations: P-elements may insert into coding regions of other loci, thus producing false positives. Additionally, P-elements may also insert into transcriptionally silent regions, thus producing false negatives (Bellen et al. 2011).

KK library This library was generated by ϕC31-integrase-mediated transformation of hpRNAs into a specific (attP) landing site recognised by ϕC31-integrase. The tools used to design the hpRNAs is described in Arziman et al. 2005 and Horn et al. 2010. The KK strategy avoids insertional mutagenesis or silent insertions, but – in this particular case – may also produce errors: the host line used for transformation of the hpRNA featured a previously undetected second attP site on the same chromosome (chr2L) which may show higher transformation rates than the originally annotated attP site and that may interrupt the developmentally relevant *tiptop* locus, thus affecting morphological development and pupal lethality (Green et al. 2014).

Next generation RNAi lines TRiP lines were also generated by ϕC31-integrase-mediated transformation of RNA constructs into a specific attP landing site. Two types of RNA constructs were used in several next generation vectors: long dsRNAs in vectors VALIUM1 and VALIUM10 (Ni et al. 2007), and shRNAs in vectors VALIUM20 and VALIUM22 (Ni et al. 2009). Importantly, this second generation of vectors contains insulator sequences which increase the level of conditional expression (Ni et al. 2009), but which may also increase the level of basal expression (Markstein et al. 2008). Thus, lines generated with vectors other than VALIUM10 should exhibit full knock down without additional expression of Dicer (`http://www.flyrnai.org`).

RNAi stocks

Lines RNAi lines used in this study are listed in Table 2.3 together with the particular library they derive from.

Validation All RNAi lines were validated for sequence identity with their respective target gene in silico using dsCheck (`http://dscheck.rnai.jp/`). DsCheck provides an *in silico* tool to chop the RNA sequences into siRNAs of 19 nucleotides each and to then blast the potential siRNAs against the *Drosophila melanogaster* genome to find hits other than the target mRNA, or "off-target" hits (Naito et al. 2005).

Target gene	Source	Order #	Transformant ID	Library
CG10638	VDRC	31306	7046	GD
	VDRC	41787	10458	GD
CG2767	VDRC	104191	104174	KK
CG6083	VDRC	25749	11809	GD
CG6084	VDRC	27551	11810	GD
CG9436	VDRC	24026	14215	GD
	VDRC	102521	111881	KK
CG10863	VDRC	48619	16330	GD
CG12766	VDRC	50403	16667	GD
CG18547	VDRC	100490	104487	KK
CG12224	VDRC	31700	7535	GD
hk	VDRC	47805	15937	GD
CG3397	VDRC	34604	10955	GD
	BDSC	25818	JF01836	TRiP

Table 2.3: List of RNAi lines.

Only RNA constructs with a perfect match to the target RNA were used. If more than one RNAi line was available for a particular AKR, the construct with the lowest number of predicted off-targets was used.

RNAi and mutant lines associated with the main phenotype were validated for knock down efficiency using qRT-PCR and quantitative immunohistochemistry.

2.2.4 Actuator lines: regulators of neural excitability

We successfully identified neurons that express the candidate CG10638. In order to confirm that this substrate had the potential to modulate sleep, we conditionally activated these neurons by expressing a temperature-sensitive actuator – UAS-TRPA1 – while recording the locomotion profile of flies, preceded by several days of baseline recording.

We also tried to silence these neurons by expressing a protein that blocks synaptic signalling – UAS-TNT.

Pre-synaptic temperature-sensitive actuators: TRPA1 The *Drosophila* TRPA1 channel is an endogenous temperature- and voltage-gated cation channel that is transiently activated by temperatures above the preferred level. The common

activation threshold for dTRPA1 is around 30 °C (Laursen et al. 2015). It is naturally expressed in dedicated warmth-activated anterior cell (AC) neurons which control thermotaxis (Hamada et al. 2008). Ectopic expression of TRPA1, together with carefully timed windows of temperature rises, can be used to depolarise neurons in a temporally restricted manner (Hamada et al. 2008; Parisky et al. 2008; Pulver et al. 2009; Rosenzweig et al. 2008). For instance, expression of TRPA1 has been used to detect brain structures involved in the homeostatic sleep response (Donlea et al. 2011) and to investigate the role of PDF+ l-LN$_v$ in sleep regulation (Parisky et al. 2008).

While neural thermoactivation is widely used to induce neural activity, no dedicated study investigated the potential of such forced activation to lead to neurodegeneration.

The stock used in this study is a UAS-dTRPA1 insertion generated by Paul Garrity (Rosenzweig et al. 2008, BDSC # 26263).

Post-synaptic regulators of excitability: TNT Expression of the tetanus neurotoxin light chain (TeTxLC or TNT) protein blocks synaptogenesis in larvae (Baines et al. 1999) and adult flies (Heisenberg et al. 2002) due to cleavage of synaptobrevin (n-syb) by TNT (Sweeney et al. 1995). TNT has been successfully expressed in PDF+ neurons to test the possibility of a fast-acting, synaptic neurotransmitter in addition to the known peptidergic transmitter PDF (Umezaki et al. 2011).

The stock used in this study is a UAS-TeTxLC insertion generated by Sean Sweeney and Cahir O'Kane (Sweeney et al. 1995, BDSC # 28838).

2.2.5 Reporter lines: fluorescent markers of cellular compartments, activity, and connectivity

We also aimed to describe the neural substrate of the candidate CG10638 anatomically and functionally through expression of reporter lines.

Fluorescent markers of cell anatomy

Identification of PDF+ neurons through expression of a conjugated fluorescent marker In order to identify PDF+ neurons without anti-PDF

staining – which is incompatible with other crucial antibodies used in this study – we used a PDF-RFP reporter line: here, a part of the regulatory *pdf* sequence is fused to the mRFP1 sequence, a mouse red fluorescent protein, leading to strong expression of RFP in nucleus, soma, and projections (Blanchard et al. 2010). The PDF-RFP reporter line was generated by the Blau laboratory (Ruben et al. 2012).

Characterisation of driver lines through expression of fluorescent markers In order to validate and characterise driver lines, we expressed the UAS-mCD8::GFP construct under their control, where "mCD8" refers to the mouse lymphocyte marker CD8, a transmembrane protein. This construct is particularly valuable to visualise neural projections as the GFP signal is distributed along their membrane and the surface/membrane ratio is favourable in neurites (Lee et al. 1999). The stock used in this study is the UAS-mCD8::GFP line generated by Liqun Luo (Lee et al. 1999, BDSC # 5137).

Additionally, we used a lexAop-rCD2-RFP construct to express RFP under the control of the 247-lexA driver (below) in order to examine the relative position of CG10638-expressing cells and the MB. This construct combines the rat transmembrane protein CD2 with the fluorescent reporter RFP and was generated by Paul Bosch and Iswar Hariharan (Bosch et al. 2015, BDSC # 58755).

Identification of neural polarity using markers DenMark and syt::GFP To define neural polarity, we expressed transgenic dendritic and axonal markers under the control of an appropriate driver. The dendritic transgene is DenMark, a construct based on the mouse ICAM5 protein which locates to dendritic compartments including the postsynapse. In order to visualise dendritic membranes, the DenMark construct is a fusion of ICAM5 and the fluorescent marker m-Cherry and faithfully reports dendritic morphology (Nicolaï et al. 2010). The axonal marker is a sytI::GFP construct: here, the vesicular protein synaptotagmin (syt) is conjugated to the fluorescent reporter GFP to recruit GFP to the presynaptic membrane (Zhang et al. 2001).

The DenMark stock used in this study was generated by Bassem Hassan (Nicolaï et al. 2010, BDSC # 33062) and the syt::GFP stock was generated by Kendal Broadie (Zhang et al. 2001, BDSC # 6925).

Reporters of synaptic connection: GRASP

To further confirm potential synaptic partners of CG10638's neural substrate with PDF+ neurons, we employed the "GFP Reconstitution Across Synaptic Partners" (GRASP) approach developed by Feinberg et al. 2008 for *C. elegans* and implemented in *Drosophila* by Gordon et al. 2009.

In brief, the GFP β barrel consists of 11 strands. To generate GRASP components, two essential and complementary GFP components are separated ("split") into two transgenes, namely strand 1-10 and strand 11 (Feinberg et al. 2008). For fly GRASP, split-GFP$^{1-10}$ is associated with a UAS and split-GFP^{11} with a lexAop promoter. In principle, only when the expression of the Gal4 and the lexA driver is directed to two neurons that establish synaptic contact should the two split-GFP components come together to form a functional GFP protein that emits a fluorescent signal (Gordon et al. 2009). Importantly, the split-GFP components are conjugated to the transmembrane protein CD4 to ensure localisation to the synaptic membrane (Feinberg et al. 2008).

For instance, this technique has been successfully employed to investigate the circadian plasticity of synaptic connections of s-LN$_v$ (Gorostiza et al. 2014).

The split-GFP lines used in this study were generated by Gordon et al. 2009 and the components are available from BDSC (# 58755).

Reporters of neural activity: CaLexA

To monitor the cumulative neural activity under conditions of sleep deprivation, we expressed an endogenous Ca^{2+} reporter, CaLexA. The full genotype is $\frac{LexAop-CD8::GFP-2A-CD8::GFP}{LexAop-CD8::GFP-2A-CD8::GFP}; \frac{UAS-mLexA::VP16-NFAT,lexAop-rCD2::GFP}{TM6B,Tb}$.

In brief, this construct employs the Nuclear factor of activated T cells (NFAT), a Ca^{2+} responsive transcription factor. In the CaLexA construct, NFAT is conjugated to a driver sequence, lexA. When lexA-NFAT is recruited to the nucleus in response

to Ca^{2+} influx, it activates the expression of the LexAop-GFP reporter line which leads to the production of membrane-bound GFP. Spatial precision of the CaLexA construct is provided by joining it with a UAS sequence that restricts CaLexA expression to Gal4-expressing driver lines (Masuyama et al. 2012).

CaLexA has been successfully used to monitor neural activity in sleep deprivation experiments (Kayser et al. 2014; Liu et al. 2016).

We expressed the CaLexA construct under the control of the driver targeting the neural substrate of CG10638. The CaLexA line used in this study was generated by Jing Wang (Masuyama et al. 2012, BDSC # 66542).

2.2.6 Driver lines

P element insertion stocks

Driver lines used for the RNAi sleep screen include the pan-neuronal ELAV and the pan-MB ok107 Gal4 lines.

The ELAV-Gal4 driver is a commonly used pan-neuronal driver that exploits the expression of ELAV (*Embryonic lethal, Abnormal Vision*) in virtually all neurons, although transient expression of *ELAV* in glial cells and neural progenitor cells during larval development has been detected (Berger et al. 2007). The original ELAV-Gal4 line is a P-element insertion ($elav^{C155}$) generated by Corey Goodman (Lin et al. 1995), but the line used in this study is a transgene that is expressed at moderate levels (Casas-Tintó et al. 2017) and was added to the BDSC collection by Peter Kolodziej (BDSC # 8765).

The ok107 driver line is a strong pan-MB driver (Aso et al. 2009). It is also a P element insertion (ok^{107}) generated by Cahir O'Kane (BDSC # 854) and olfactory acuity may be affected in these flies (Connolly et al. 1996).

Fly Light stocks

One set of MB drivers used in this study (Table 2.4) was derived from the Janelia Fly Light collection which now encompasses 7000 Gal4 lines (Jenett et al. 2012).

The enhancer fragments were selected as described by Pfeiffer et al. 2008 and inserted into a dedicated site (attP2, Jenett et al. 2012).

The expression pattern has been identified in adult brains and VNC through expression of an mCD8::GFP construct and the data is available through the Janelia Fly Light website (`https://www.janelia.org/project-team/flylight`).

Another set of driver lines used in this study, 'split-Gal4' lines, were also derived from the Janelia collection. Here, two enhancer fragments are combined to create the construct fused to the Gal4 sequence in order to produce an expression pattern that is more specific than those based on individual enhancer fragments (Luan et al. 2006).

Vienna Tiles stocks

Another set of driver lines was derived from the Vienna Tiles (VT) collection hosted by the VDRC. The VT driver lines consist of short genomic DNA fragments of 2 kB that encompass parts of the upstream and downstream regulatory sequences as described by Pfeiffer et al. 2008. These fragments are integrated into the genome at a defined position (attP2) as described by Kvon et al. 2014.

The VT collection now contains more than 8000 Gal4 lines. The expression of enhancer fragments has been validated in *Drosophila* larvae (Kvon et al. 2014). Data of adult expression is also available through the VDRC website (`http://brainbase.imp.ac.at/bbweb`).

Other driver lines

Other driver lines used in this study are listed in Table 2.4 together with the driver lines discussed above. Some of these lines are P-element insertions, e.g., repo^{Gal4}, ok107^{Gal4}, TyrR^{Gal4}, and produce phenotypic effects, e.g., ok107^{Gal4} (Connolly et al. 1996) and TyrR^{Gal4} (Huang et al. 2016).

Driver	System	Source	Expression	Reference
ELAV	Gal4	BDSC	pan-neuronal	Lin et al. 1995
ok107	Gal4	BDSC	MB	Connolly et al. 1996
247	lexA	BDSC	MB	Pitman et al. 2011
R34B09	Gal4	BDSC	MB, $\alpha\beta$ lobes	Jenett et al. 2012
R16A06	Gal4	BDSC	MB, γ lobe	Jenett et al. 2012
R35B12	Gal4	BDSC	MB, $\alpha'\beta'$ lobes	Jenett et al. 2012
MB607B	Gal4	JF	MB, γm sublobes	Luan et al. 2006
MB009B	Gal4	JF	MB, γm	(Luan et al. 2006)
MB418B	Gal4	JF	MB, $\alpha'\beta'$ sublobes	Luan et al. 2006
MB463B	Gal4	JF	MB, $\alpha'\beta'$ sublobes	Luan et al. 2006
MB005B	Gal4	JF	MB, $\alpha'\beta'$ sublobes	Luan et al. 2006
MB185B	Gal4	JF	MB, $\alpha\beta$ comp.	Luan et al. 2006
VT026326	Gal4	BDSC	ICLI neurons	Kvon et al. 2014
Repo	Gal4	BDSC	pan-glial	Sepp et al. 2001
PDF	Gal4	BDSC	PDF+ neurons	Park et al. 2000
PDF	lexA	R. Stanewsky	PDF+ neurons	Shang et al. 2008
MIP	Gal4	J. Chung	MIP+ neurons	Min et al. 2016
R61A01	Gal4	BDSC	MIP+ TyrR+ neurons	Jenett et al. 2012
TyrR	Gal4	BDSC	MIP+ TyrR+ neurons	Huang et al. 2016

Table 2.4: List of driver lines.

2.3 Experimental crosses

Backcrossing of mutant lines

Transposon insertion lines were backcrossed to the wildtype strain common to this study (w^{1118}, VDCR # 60100) for at least five generations to reduce genetic variability due to differences in the background strains (Hospital 2005).

W^{1118} flies of the same stock were also used as wildtype controls.

UAS/Gal4 system

Generally, to combine actuator or reporter lines with the appropriate driver lines, we kept a common crossing protocol. Here, Gal4 or lexA stocks were kept in bottles at 25 °C and virgins were collected within 4 h after eclosion according to common phenotypic criteria of a female virgin fly and kept in vials under standard conditions. Young male flies at 2-7 days of age were collected from UAS or lexAop stocks.

For each cross, 3-4 female virgins were crossed to 2 male flies of the appropriate genotype.

We also performed crosses to generate parental controls: we crossed female virgins of the Gal4 or lexA stocks to a "wildtype" stain, w^{1118} (VDCR # 60100), which we used throughout this study. Similarly, we crossed female virgins of the UAS or lexAop stocks with male w^{1118} flies. These parental controls were used alongside the experimental crosses and treated in the same manner according to the same protocols. Thus, we can compare the results obtained from experimental lines with flies that carry the UAS and Gal4 alleles separately; this approach allows us to estimate potential genetic background variation and/or unspecific expression of the actuator which could generate a false positive result or mask an experimental effect.

Crossing schemes

Crosses for the nervous system-wide RNAi-mediated sleep screen were obtained as given in the exemplary cross 2.1. Here, the driver line ELAV-Gal4 is crossed to the homozygous IR line on chromosome 2.

$$w^*; \frac{ELAV - Gal4}{CyO}; + \times yw^{1118}; \frac{UAS - IR}{UAS - IR} \tag{2.1}$$

Crosses for the MB-specific RNAi-mediated sleep screen were obtained as given in the exemplary cross 2.2.

$$w^*; \frac{ok107 - Gal4}{CyO}; + \times yw^{1118}; \frac{UAS - IR}{UAS - IR} \tag{2.2}$$

All RNAi constructs of the KK library as well as TRiP constructs were inserted in chromosome 2; RNAi lines derived from the GD library are inserted either in chromosome 1, 2, or 3.

Crosses to obtain the expression of reporters or other actuators under the control of the relevant driver line were performed similiarly.

2.4 Behaviour

2.4.1 Baseline sleep recording

Preparation Flies were briefly anaesthesized with CO_2 and inserted into tubes (5 × 65 mm) containing fly medium (henceforth termed "activity tubes"). Flies were given at least 24 h to recover from anaesthesia and the social environment before recording. Unless otherwise stated, flies were young – 1 to 4 days old on the first day of recording – and male. Habituation and recording was performed in dedicated incubators (Ova Easy 380 Advance ExSeries II, Brinsea Products Ltd., Weston-Super-Mare, UK) running a regular light regime (LD) at 25 °C and 60 % humidity. The regular light:dark schedule ("LD") was equivalent to 12 h of light followed by 12 h of darkness.

Sleep recording and acquisition The activity of individual flies was recorded using Drosophila Activity Monitors (model DAM2, TriKinetics, Waltham, MA, USA): here, activity tubes with single flies are inserted into a monitor array. The mid-line of the array is marked with an infra-red beam which is blocked by a passing fly. The temporal resolution is moderate: specifically, the activity of individual flies is recorded in terms of the number of beam crossings per min. Data is acquired by the DAMSystem3 Data Acquisition Software supplied by the manufacturer.

For regular baseline recordings, at least 6 days were recorded, whereby the first two days were regarded as the habituation period and discarded for analysis (Fig. 2.2A).

According to the conventional definition of sleep in *Drosophila melanogaster* (Shaw et al. 2000), a fly that has been inactive, – did not cross the midline – for more than 5 min is considered to have been asleep until the next beam crossing occurs.

2.4.2 Free running conditions

Light regimes To tease apart homeostatic and circadian sleep drives, flies were subjected to a range of light regimes. For free running conditions, several days of LD were followed by constant darkness ("DD", Fig. 2.2B). "DD" was achieved

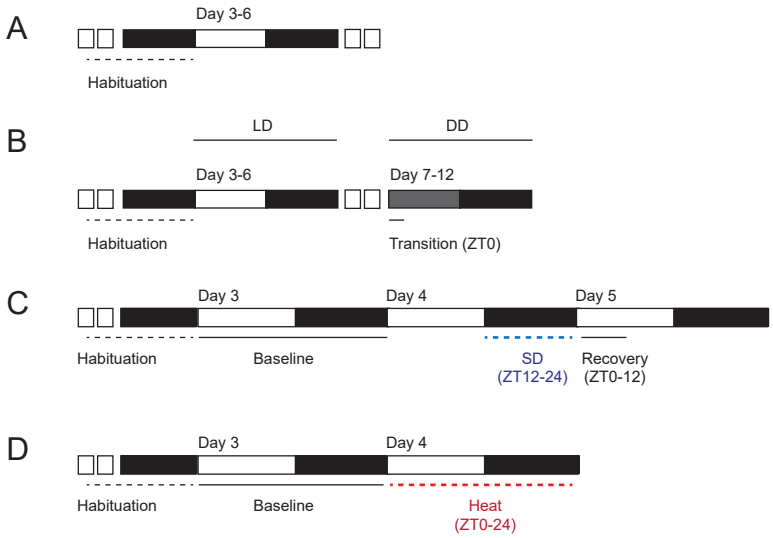


Figure 2.2: Behavioural protocols. A, Baseline recording. B, Free running regime. C, Sleep deprivation regime. D, Heat activation. Indicated days signify days that were included in the analysis.

by turning off the lights in the incubator and enclosing DAM monitors in a film changing bag to block off any light. Due to the physical manipulation of the flies during the transition period from LD to DD, the first three hours following the manipulation were excluded from analysis.

Transition period In order to compare sleep characteristics under different light conditions, we subjected flies to several days of LD followed by constant darkness and compared the last two LD days with the first two DD days (Fig. 2.2B).

Free running behaviour Flies were subjected to a prolonged period of darkness in which the "molecular clock" lacked resetting light input ("free running conditions") as depicted in Fig. 2.2B. At least 5 days of entrainment (LD) were followed by at least 7 days under free running conditions (DD). Only flies with recordings of 5 full days or more were considered for further analysis.

2.4.3 Sleep deprivation

Baseline sleep of male flies 1-4 days of age was recorded as described.

For the purpose of sleep deprivation (SD), DAM2s were mounted on an orbital flatbed shaker (VWR, PSU-10i, # 444-0933). The shaker was programmed to move according to a pseudo-randomized protocol consisting of bouts of shaking for the duration of 5 to 30 s every 1 to 7 min. This protocol has been found to be an efficient strategy to achieve SD in the lab and is consistent with conventional automated SD strategies targeting *Drosophila melanogaster* (e.g., Shaw et al. 2000).

Unless otherwise stated, flies were sleep deprived for 12 h overnight, from ZT (Zeitgeber) 12 to ZT 0 on the next day. Control flies were equally anaesthetised, inserted into activity tubes and DAM2s, and kept in the same incubator at the same time. The SD regime is depicted in Fig. 2.2C.

2.4.4 Heat activation

In order to acutely activate our neurons of interest, we expressed a TRPA1 transgene under the control of an appropriate specific driver as indicated in the figure legends.

Here, experimental and control flies were raised under appropriate conditions as listed above to avoid premature activation. Similarly, baseline sleep of male experimental and parental control flies at 1-4 days of age was recorded essentially as described, but at a lower temperature of 22 °C.

For the purpose of heat activation, the ambient temperature in the incubator was raised to 29 °C. This temperature lies at the lower range of the activation threshold of TRPA1 to balance the specific heat activation and generally temperature-related effects, as the latter could mask experimental effects due to the strong impact of temperature on sleep in flies (section 1.3). Temperature were elevated for 24 h and subsequently brought down to the previous level. The heat-activation regime is depicted in Fig. 2.2D.

2.4.5 Quantification

Sleep behaviour

Quantification Behavioural data was first processed using the rethomics package, a dedicated R package for the analysis of animal sleep behaviour. The rethomics

Parameter	Description
Total sleep	Fraction of time spent immobile per 24 h
Night sleep	Fraction of time spent immbolie during subjective night
Day sleep	Fraction of time spent immobile during subjective day
Sleep bout #	Number of continuous periods of immobility
Sleep bout length	Length of continuous periods of immobility
Sleep latency	Time to first sleep bout after light transition
Activity level	Number of beam breaks per min

Table 2.5: Sleep parameters.

package is currently developed in the lab (Geissmann et al. *in prep.*) and available from `https://github.com/gilestrolab/rethomics`. Data was further analysed in the R environment running in RStudio.

It is important to point out that within the technical DAM framework, an animal can fulfil only one of two states: it is either "active", i.e., passes the beam within a defined period of time, or "inactive", i.e., does not pass the beam within a defined period of time. Data sets were curated such that dead animals were discarded, whereby an animal that has been continuously immobile – did not pass the beam – for a minimum of 12 h is considered dead.

Sleep parameters Sleep was defined by continuous periods of immobility of a minimum of 5 min in duration. This is a common proxy parameter for sleep and correlates with a decreased arousal threshold in flies and other defining criteria of sleep (e.g., Shaw et al. 2002 and Huber et al. 2004).

A typical sleep profile is illustrated in Fig. 1.6; it depicts the average fraction of time spent asleep per 30 min bin. The envelope reflects the standard error of the mean ("sem").

The main sleep/activity parameters extracted from the raw data are given in Table 2.5.

Within the constraints of the temporal resolution, flies can exhibit an activity bout of a minimum of 1 min in duration. According to the "5 min rule", a sleep bout is of a minimum duration of 5 min.

Category		Criteria
R	Strongly rhythmic	peak(s) above significance level with power > 100
WR	Weakly rhythmic	peak(s) above significance level with power < 100
AR	Arrhythmic	peak(s) below significance level

Table 2.6: Criteria for circadian rhythm categories, significance level was set to $p<0.05$.

Circadian behaviour

The temporal distribution of activity bouts was analysed for circadian rhythmicity using ActogramJ. This tool is a FiJi plugin (Schindelin et al. 2012, `https://fiji.sc/`) and was developed by Johannes Schindelin with the group of Charlotte Helfrich-Förster (Schmid et al. 2011, `http://132.187.25.13/actogramj/manual.html`). The use of ActogramJ for this study was inspired by Zantke et al. 2013. Briefly, the Lomb-Scargle algorithm – a spectral analysis of time series derived from Fourier analysis – was used to determine periodicities, i.e., the free running period length and rhythm power. For the purpose of this study, the free running period length is equivalent to the period with the highest associated power value that meets the significance requirement of $p<0.05$ and is expressed in hours. Also, the highest power value per animal is considered its maximum rhythm strength and is also reported. The power reflects the height of the period peak in the Lomb-Scargle periodogram above the significance threshold which is conventionally, but arbitrarily chosen at α=0.05, and power is thus expressed in "arbitrary units" ("AU").

Furthermore, rhythm behaviour of individual flies was categorised as rhythmic, weakly rhythmic, or arrythmic, according to the criteria given in Table 2.6.

ActogramJ was used to visually inspect actograms of individual flies, to select representative actograms of individual flies, or – for populations with strong rhythmicity – to generate smoothed average actograms per genetic population.

2.5 Quantitative real-time reverse-transcription PCR

2.5.1 Primary considerations

Quantitative real-time reverse-transcription PCR (qRT-PCR) is a standard molecular biology technique that provides the most sensitive detection of sparsely expressed mRNA or subtle differences in mRNA expression (Bustin et al. 2004). Thus, we opted for qRT-PCR to measure mRNA expression levels of different *Drosophila melanogaster* AKRs. Experimental procedures and conditions, together with particular quality considerations, are discussed below.

Polymerase chain reaction (PCR) Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) is a technical variant of PCR in which fragments of DNA are amplified from very low levels. The technique was developed by Kary Mullins (Valasek et al. 2007) and involves the annealing of two DNA primers complementary to the 3' of the sense and anti-sense target DNA to the target strand which allows the DNA polymerase to synthesise a new strand. These steps – DNA denaturation into single strands, primer annealing, DNA elongation – are temperature sensitive; thermal cycling (systematic alteration of heating and cooling) is a common way to control and repeat this reaction for maximum yield. Since the DNA amplified from the original DNA template is in turn used as DNA template in each new cycle, a chain reaction is triggered that produces target DNA exponentially (Valasek et al. 2007).

Reverse transcription PCR and qRT-PCR are fundamentally different in purpose. While PCR allows the exponential amplification of template DNA, qRT-PCR targets the transcriptome: in qRT-PCR, the target DNA is usually derived from mRNA through reverse transcription. The reverse transcriptase synthesises a single stranded complementary DNA from the mRNA strand. To distinguish genomic DNA from complementary DNA, the latter is often referred to as "cDNA" in contrast to genomic or "gDNA". The amplification of cDNA is

predictably exponential; it is limited only by the availability of starting reagents. Thus, the initial mRNA levels can be concluded from the real-time increase from one cycle to the other (real-time RT-PCR) or from the amount of final product (end-point RT-PCR, Smith et al. 2009).

Real-time (RT) quantification In real-time approaches, the amount of cDNA is monitored throughout the amplification process through the detection of fluorescent dyes or probes. "Probes" are conjugated to primers and emit a fluorescent signal when bound to double-stranded DNA (Smith et al. 2009). For the present study, we employed an intercalating fluorescent dye, SYBR Green, that emits a fluorescent signal upon excitation when bound to double-stranded DNA (Smith et al. 2009). Real-time detection of cDNA amplification using SYBR Green provides the greatest dynamic range and sensitivity of common real-time strategies (Schmittgen et al. 2000). If the amplification from one cycle to the next were completely efficient, then each cycle should produce double the amount of cDNA as the last one, until the reagents supplied to each reaction run out and amplification plateaus (Valasek et al. 2007).

The "threshold cycle" (C_T) reflects the initial amount of mRNA present in the sample: the lower the C_T value, and the earlier amplification reached plateau, the higher was the initial amount of mRNA (Smith et al. 2009).

Constraints and caveats The main caveats challenging a successful qRT-PCR include the inherent biological variability within samples and the variability of the real-time reporter, as well as considerations of template and primer quality and the suitability of the reference genes (Bustin et al. 2004). While the genome is essentially identical in all cells of a particular organism, the levels of gene *transcription* can vary greatly due to developmental, physiological and environmental factors, introducing high levels of inherent variability (Bustin et al. 2004). The capture of transcriptional variation due to experimental manipulations is usually the goal of qRT-PCR and requires robust and reliable results distinguishable from inherent noise levels. Also,

RNA degrades easily, and RNA templates can easily be contaminated with much more stable DNA (Bustin et al. 2004).

2.5.2 Sample collection, preparation, and reverse transcription

Experimental conditions

Generally, flies were kept under standard conditions, i.e., 25 °C and 60 % humidity, until samples were collected. Unless otherwise stated, samples were collected between ZT0 and ZT4.

RNAi-mediated knock down We used qRT-PCR to evaluate the efficiency and specificity of the RNAi-mediated knock down using two different RNA transgenes targeting CG10638. Here, we drove IR expression in the nervous system under the control of ELAV and collected head samples from male experimental and parental control flies.

Baseline expression We measured baseline mRNA levels of fly AKRs in young male w^{1118} flies in different tissues, namely the brain, head, and body, and compared their enrichment in the context of fly AKRs as a group.

Timecourse To measure mRNA expression throughout 24 h, we separated young male flies into groups of 12 animals and entrained them to LD for several days before taking samples every 3 h starting at ZT0 to obtain 8 timepoints in total.

Homeostatic response We assessed the expression response to sleep deprivation by subjecting groups of young male w^{1118} flies to the sleep deprivation regime as described above.

Sample collection

Brains of young male w^{1118} flies were dissected in ice-cold PBS. Heads and bodies were separated on cold ice. A sample consisted of 40 dissected brains, or 12 heads, or 5 bodies.

Samples were immediately transferred to ice-cold Trizol (Thermo Scientific # 15596026) and subsequently homogenised through bead-beating in a tissue homogeniser.

Unless RNA extraction proceeded immediately, samples were stored at -80 °C until RNA could be extracted.

Sample preparation

RNA extraction RNA was extracted essentially as described by **Emery2007** Briefly, samples are transferred to 800 µl Trizol (Thermo Scientific # 15596026) immediately after dissection. After the addition of 160 µl chloroform (VWR # 22711.260), samples are homogenised and centrifuged for 15 min at 12000 g. RNA is transferred to a new tube and precipitated with 400 µl of isopropanol (VWR # 20842.323). After 10 min at room temperature, RNA is centrifuged for 15 min at 12000 g at 4 °C. The supernatant is discarded and the RNA pellet washed several times with 75 % Ethanol (VWR # 20821.296). After the RNA is briefly air-dried to allow the Ethanol to evaporate, it is re-suspended in RNAse-free H_2O and incubated at 50 °C to ensure that RNA is completely dissolved. This protocol yielded RNA of 60-130 ng µl^{-1} for brains, 150-350 ng µl^{-1} for heads, and 300-1000 ng µl^{-1} for bodies.

In order to ensure a high template quality, only samples with optical density (OD) ratios (A_{260}/A_{280}) of 1.9-2.1 of a calibrated spectrophotometer (NanoDrop 2000c, Thermo Scientific, Washington, DA) were considered for reverse transcription.

DNAse treatment DNase I treatment of all samples was performed as follows: 1 µl DNase I (NEB # M0303S) in DNase I reaction buffer (NEB # B0303S) was added to 10 µl of sample and incubated at 37 °C for 15 min. After addition of 1 µl 25 mM EDTA, samples were incubated for another 15 min at 65 °C. RNA was briefly centrifuged and kept on ice until further processing or immediately stored at -80 °C.

Reverse transcription

For reverse transcription, we opted for the ProtoScript MuLV first strand DNA synthesis kit (NEB # E6300S) performed essentially according to the manufacturer's instructions. Briefly, 0.5 µg µl^{-1} DNase I treated RNA is mixed with dT_{23} primers at 0.2 μM and dNTPs at 200 μM and incubated at 70 °C for 5 min, then chilled on ice. The RNA-primer mix is added to the RT buffer containing the Murine RNase inhibitor and M-MuLV reverse transcriptase, then incubated at 42 °C for 60 min. The enzyme is inactivated at 80 °C for 5 min, followed by a brief centrifugation and an addition of H_2O to bring the cDNA to the appropriate concentration. Since our samples were generally of high quality, we opted for oligo-dT primers (dT_{23}) for the initiation of reverse transcription as they are specific to mRNA and allow us to measure the levels of more than one transcript within the same sample.

2.5.3 Primer design

Primer design Primers of target sequences of the *Drosophila melanogaster* genome (release 5, `flybase.org`) were designed with the Primer3 algorithm implemented in Ugene software (Ugene 1.12, UniPro, Novosibirsk, Russia). Preference was given to primers spanning at least one intronic region to allow us to detect contamination by gDNA through agarose gel electophoresis. Where possible, primers were limited to a cDNA amplicon product size of 200-350 bp. Primers were evaluated *in silico* for target sequence complementarity using Primer-BLAST (NCBI, `https://www.ncbi.nlm.nih.gov/tools/primer-blast/`) and for specificity using agarose gel electrophoresis of amplicons produced by PCR.

Reference genes We aimed to assess differences in expression in response to experimental conditions and therefore opted for their quantification relative to reference genes. The choice of reference genes is crucial and complex: reference genes should be genes whose expression is stable across experimental conditions and that are expressed in a range that matches the target genes. Generally, using more than one reference gene is preferable to improve consistency and robustness. We

chose RpL32 and RpII215 as reference genes which were selected based on published evaluations of qRT-qPCR reference genes for *Drosophila melanogaster* Ling et al. 2011; Ponton et al. 2011. We independently evaluated these references for target complementarity using Primer-BLAST and tested them for specificity using Taq PCR followed by aagarose gel electrophoresis. To address their stability across experimental conditions, we diluted the inital mRNA concentration to 40 mM based on the concentration measured in a 0.5 µl aliquot per sample using the NanoDrop.

The main drawback of the relative quantification method is the the reference genes are to be used throughout to provide a unifying internal standard.

2.5.4 PCR

For PCR, we opted for the Taq DNA polymerase PCR as available from the supplier (New England Biolabs # M0273) and proceeded according to manufacturer's instructions. Reactions were run in small volumes of 12 µl with a DNA template concentration of $0.02\,\mu g\,\mu l^{-1}$ and a $MgCl_2$ concentration of 25 mM.

Thermal cycling conditions are listed in Table 2.8.

2.5.5 Amplification and real-time data collection

We used the intercalating dye SYBR Green to monitor cDNA amplification in real-time. Here, a fluorescent signal is emitted when SYBR Green is bound to the double-stranded DNA. Samples at a concentration of $40\,ng\,\mu l^{-1}$ were added to Fast Sybr Green Master Mix (Life technologies # 4385612) in Fast Optical 96 well plates (ABI # 4346906) and run in a StepOne Real-Time PCR Instrument (Applied Biosystems # 4376357). ROX dye – which is pre-calibrated on StepOne systems – was used as a passive reference in all reactions to control for subtle loading differences between wells.

Thermal cycling conditions are listed in Table 2.9.

All reactions that included template were run in triplicate, with one No template control (NTC) for each primer pair to evaluate primer self-amplification and primer

Target	Sequence	cDNA (bp)	gDNA (bp)
CG10638-A	GCCATGGAAAAGCTGGTAAA	335	396
	GGAATGACACCCAGACCAAC		
CG10638-B	CAGAAACTGAAACGCGAACA	109	183
	CGCCCAGTAGCTTTAATTGC		
CG10863	AAATGGAGAAGCTCGTGGAA	200	256
	GGGACAGTAGGCGGTCACTA		
CG12766	GAGTATGCCTGCCGAAAGAC	285	404
	TTGATTGTGGATTGGCTTGA		
CG6083	GAAGTGGTGACCCAAGCAGT	289	353
	TGTCACTGCCCGACTTGTAG		
CG18547	CGCGAACTACGGTTTTGATT	253	253
	CAAGCTCTTCTCCACGCTTT		
CG3397	GCCAGCTACTTTCGTCAAGG	318	318
	TCCAACTCGTAACGTGCAAC		
CG9436	CTTAGCAACCTGGGTTTGGA	328	328
	AGTAGGCGCAGATGACCAGT		
G2767	AGCTGGGTATCGCCACATAG	260	627
	AGCTTCCGTCCTCGTTGATA		
CG6084	GATGGTGTTGAGGCCAAGAT	296	362
	TTGACCAGACCCTCTTCCAC		
CG12224	GAAGGTGCGTCGTATGGAGT	320	320
	TTCACACTCTCCCGAGCTTT		
Hk	AGAGTTCGCACTTTTCGCG	593	864
	GCTACCATTGCGCCATGGATCC		
ARY	TAATGATCCCAGCGGACTTT	657	882
	AGCGGCAATAATCAACCAAG		
Period	CGCCCATTCCCTCCGTGATG	94	94
	CCATTGCTGTCCGTGTATTTCTTGTT		
RpL32	ATGCTAAGCTGTCGCACAAATG	107	107
	GTTCGATCCGTAACCGATGT		
RpII215	GTGAAGCGCGTACAGTTCG	218	817
	AGGTCGATGTGTCCAAAGTGA		

Table 2.7: Primer list, forward primer listed first. The *period* primer was taken from Sanchez et al. 2010.

Step	Temperature (°C)	Duration
Initial denaturation	95	90 s
Cycle (35×)	95	15 s
	58	30 s
	72	30 s
Final extension	72	5 min
Hold	8	Forever

Table 2.8: Cycling conditions (PCR).

Step	Temperature (°C)	Duration
Cycle (40×)	95	20 s
	95	3 s
	60	30 s

Table 2.9: Cycling conditions (qRT-PCR).

contamination. We also included Melt curves in all of our qRT-PCR assays in order to examine amplicon specificity.

2.5.6 Quantification

Data acquisition Fluorescence of the intercalating agent, SYBR Green, was measured against the passive reference, ROX dye. Data was initially processed using the StepOne qPCR Analysis software v2.0 (Thermo Scientific, Waltham, MA), to acquire Melt curves and several quantitative parameters for each reaction.

Parameters Initially, "R_n" values are calculated which indicate the fluorescence measured for the reporter SYBR Green normalised to the passive reference, ROX dye. The ΔR_n reflects the magnitude of the normalised fluorescence signal generated by the reporter SYBR Green for each cycle during amplification relative to the baseline derived from the initial PCR stages where the rate of amplification is low. Then, C_T values are calculated for each reaction. "C_T" reflects the threshold cycle, i.e., the cycle in which the amplification passes a threshold that is above the baseline and derived from the rate of amplification measured during the exponential phase.

Quantification Data was further processed and analysed in R running in RStudio. We employed the relative quantification approach to assess amplification levels relative to a set of common reference genes in order to detect differences between experimental treatments. Here, C_T values of the target transcripts were normalized to the geometric mean of the two reference genes (ΔC_T, Eq. 2.3).

$$\Delta C_T = C_{Ttarget} - C_{Treference} \tag{2.3}$$

The difference between target and reference genes is expressed in terms of as fold change ($FC_{\Delta C_T}$, Eq. 2.4) and the data variance is combined ($\sigma_{\Delta C_T}$, Eq. 2.5).

$$FC_{\Delta C_T} = 2^{-\Delta C_T} \tag{2.4}$$

$$\sigma_{\Delta C_T} = \sqrt{\sigma^2_{target} + \sigma^2_{reference}} \tag{2.5}$$

Due to the wide range of expression of *Drosophila melanogaster* AKRs in different tissues, the abundance of all targets is expressed on the log-scale of base 10. Differences between experimental conditions ($FC_{\Delta\Delta C_T}$) were expressed as percentage difference to the average $FC_{\Delta C_T}$ of the control group (Eq. 2.6).

$$FC_{\Delta\Delta C_T} = \frac{FC_{\Delta C_{TExp}}}{FC_{\Delta C_{TControl}}} \times 100 \tag{2.6}$$

For timecourse measurements, one set of samples covering all timepoints was run per plate. Due to the variation between plates, samples per plate were normalised to the ZT0 sample of that plate. The $FC_{\Delta C_T}$ values of samples was normally distributed and normalised to the average of $FC_{\Delta C_T}$ values of ZT0 samples. Variation in the ZT0 sample was expressed as the difference of ZT0 values across plates to the average $FC_{\Delta C_T}$ of ZT0.

2.6 Immunohistochemistry

2.6.1 Antibodies

The immunohistochemistry toolbox: antibodies

Antibodies, or immunoglobulins (Ig), are naturally occurring proteins that recognise unique molecules of harmful proteins or organisms called antigens to target them for neutralisation by the immune system (Janeway 2001). Immunohistochemistry exploits the high affinity antibodies have for antigens: target proteins can be cloned and themselves used as antigens to raise antibodies that can then be used to trace the expression pattern of the target protein throughout the organism and to evaluate effects of mutations or environmental manipulations on the pattern or level of the target protein (Patel 1994).

Initial preparations for *Drosophila melanogaster* immunohistochemistry stem from the work of Zalokar & Erk (eggs, Zalokar et al. 1977) and Mitchison and Sedat (embryos, Mitchison et al. 1983). Generally, immunohistochemistry protocols combine the incubation of a tissue with a primary antibody raised against the target protein, together with or followed by the incubation with a secondary antibody that in turn recognises the primary antibody and that expresses a marker that can be used to detect the location of the secondary antibody in the tissue. We chose the most common type of secondary antibody – antibodies conjugated to fluorescent probes.

Primary antibodies

Polyclonal and monoclonal antibodies Antibodies are produced by a certain type of immune cells: B cells. There are polyclonal and monoclonal antibodies, whereby a polyclonal antibody is in fact a collection of antibodies produced by different cells, while monoclonal antibodies are antibodies that are produced by a single B cell (Lipman et al. 2005). Polyclonal antibodies tend to be less specific than monoclonal antibodies as they often bind to more than one epitope per antigen. Therefore, polyclonal antibodies can exhibit higher affinity and greater sensitivity to protein expressed at low levels than monoclonal antibodies, but they may be less accurate and are prone to cross-reactivity (Lipman et al. 2005). Single B cells

can be harvested and fused to cell lines to generate large volumes of monoclonal antibodies. This principle has been exploited to a large scale by the Drosophila Hybridoma Bank (DSHB, `http://dshb.biology.uiowa.edu/`) which manages a large collection of antibodies.

Primary antibodies generated against CG10638 We used custom-made antibodies against CG10638. These were raised against synthetic peptides generated in the lab by Giorgio Gilestro based on protein sequences suggested by Ab-Designer (Pisitkun et al. 2012, `https://hpcwebapps.cit.nih.gov/AbDesigner/index.jsp`). Two peptides were generated for each isoform, corresponding to exposed loop regions, i.e., DDNTLLPKNEDDVLQ (ab# 1), YTPLGKPKPDIQKPD (ab# 2), EDLMPHENGQLRTND (ab# 3), GRPKACNPLPDYYTD (ab# 4). Peptide antibodies were raised and purified by Eurogentec (anti-peptide programme AS-DOUB-LXPGUI).

A list of primary antibodies used in this study is provided in Table 2.10.

Secondary antibodies

Secondary antibodies aid in the detection of primary antibodies generated in a particular host. They are generated by immunising a host species with primary antibodies from the target species. Additionally, the kind of secondary antibodies used in this study are conjugated to a fluorescent probe to increase staining specificity and to enable ready, albeit indirect detection of the primary antibody through confocal microscopy (Lipman et al. 2005).

The primary drawback of secondary antibodies that rely on fluorescent probes is that within one preparation (1) primary antibodies need to be raised in different species that are not prone to cross-species reactivity, and (2) the secondary antibodies must carry fluorescent probes clearly distinguishable from each other.

A list of secondary antibodies used in this study is provided in Table 2.11.

Antigene	Raised in	Conc.	Source	Cat. #
CG10638-A (1)	guinea pig	1:200	custom	-
CG10638-A (2)	guinea pig	1:200	custom	-
CG10638-B (3)	guinea pig	1:200	custom	-
CG10638-B (4)	guinea pig	1:200	custom	-
VGluT	rabbit	1:500	gift from H. Aberle	-
GABA	rabbit	1:200	Sigma Aldrich	A2052
GadI	rabbit	1:1000	gift from R. Jackson	-
ChAT*	mouse	1:200	DSHB	ChAT4B1
DDC	rat	1:200	gift from J. Hirsh	-
TH	rabbit	1:1000	Sigma	T8700
5HT	rabbit	1:200	abcam	ab66047
PDF*	mouse	1:200	DSHB	PDF C7
Repo*	mouse	1:200	DSHB	8D12
sNPFR	rat	1:200	gift from K. Yu	-
N Cadherin*	mouse	1:200	DSHB	DN-Ex 8
nc82*	mouse	1:200	DSHB	nc82
mCD8*	rat	1:400	Thermo Scientific	MCD0800
GFP	rabbit	1:400	abcam	ab290
RFP	rabbit	1:400	abcam	ab62341

Table 2.10: List of primary antibodies, monoclonal antibodies are marked with an asteriks.

Antigene	Fluorophore	Conc.	Source	Cat. #
rabbit	Alexa 488	1:400	abcam	ab150077
rabbit	Alexa 568	1:400	abcam	ab175471
mouse	Alexa 488	1:400	abcam	ab150113
mouse	Alexa 568	1:400	abcam	ab175473
rat	Alexa 488	1:400	abcam	ab150157
rat	Alexa 647	1:400	abcam	ab150167
chicken	Alexa 488	1:400	abcam	ab150169
guinea pig	Alexa 488	1:400	abcam	ab150185
guinea pig	Alexa 568	1:400	abcam	ab175714
guinea pig	Cy5	1:200	abcam	ab102372

Table 2.11: List of secondary antibodies.

2.6.2 Sample preparation and staining

Samples Unless otherwise stated, immunohistochemistry was performed in young male flies at 2-5 days of age and entrained to LD.

Brain dissection and fixation Fly brains were dissected essentially as described in Helfrich-Forster et al. 2007. Briefly, flies were immobilized on ice immediately before dissection. Brains and/or the VNC were dissected in ice-cold 0.01 % PBS (Sigma # P4417) and fixed in 4 % PFA for 20 min (Triton-X: Sigma # X100-5ML, PFA: Sigma # 158127) on a nutator at 4 °C.

Staining and mounting Washing and staining was performed essentially as described by Wu et al. 2006. Briefly, brains were washed thoroughly with 0.03 % PBST and blocked with 5 % goat serum in 0.03 % PBST (abcam # ab156046) for at least 30 min. Brains were incubated with primary antibody in 0.03 % PBST for two days, washed thoroughly, and incubated with secondary antibody in 0.03 % PBST for two days. Brains were again thoroughly washed and left to rest in mounting medium (Vectashield, Vector laboratories # H-1000) overnight, before being mounted on standard glass microscope slides (slide: VWR # 631-1553, coverslip: VWR # 631-0146) under a regular light microscope. Respective concentrations of primary and secondary antibodies are listed in Table 2.10 and 2.11.

Modification for GRASP experiments For GRASP stainings, only a background staining using the ubiquitous neural marker Bruchpilot was performed. Here, dissection and processing was performed under light levels as low as possible. Brains were briefly fixed as described above, washed three times in 0.03 % PBST, and blocked with 5 % goat serum for 30 min. Samples were incubated with the primary antibody (anti-nc82, Table 2.10) for 2 h, washed three times with 0.03 % PBST and incubated in the secondary antibody (anti-mouse Alexa 568, 1:200, Table 2.11) overnight before being washed and mounted as described above.

2.6.3 Quantification

Data acquisition Images of whole-mount stained fly brains were acquired on a Zeiss 510 confocal microscope running the Zeiss ZEN software for light microscopy (Zeiss, Oberkochen, Germany). Images were taken at a resolution of 1024×1024 p or 512×512. Confocal stacks of whole-mount brains were taken at 20× magnification and 0.5 µm sections, and somata were imaged at 40× magnification and 0.5 µm sections.

Qualitative analysis All images were processed and analysed in ImageJ (FiJi distribution, Schindelin et al. 2012, `https://fiji.sc/`). Some images were only examined qualitatively. Those include stainings to determine co-expression of two proteins within a cell, e.g., neurotransmitter determination, and the evaluation of neural projection patterns. Qualitative descriptions are based on at least three images or image stacks taken from different brains. For co-expression experiments, channels were recorded separately and validated for bleed through.

Quantitative analysis Images or image stacks in which the fluorescence intensity was to be quantified were not further processed in any way. Sections were acquired at 0.5 µm steps and the section in which the nucleus reached the maximum diameter was selected for quantification. Average pixel intensity was measured in the respective ROI drawn around the cell body, and in a region of the same ROI shape outside of the cell body to use as a measure of background intensity for normalisation (Eq. 2.7). ROIs were drawn using the freehand tool.

$$\Delta F = \frac{F_{Soma}}{F_{Background}} \tag{2.7}$$

Representative images are depicted with the false color scale “fire” ranging from low intensity (blue) to high intensity (red).

Average soma diameter was measured using the scale tool.

2.7 Pharmacological and fitness assays

2.7.1 Paraquat exposure

Paraquat is an insecticide that induces high levels of oxidative stress in flies (Krůček et al. 2015; Rzezniczak et al. 2011). Paraquat has been used, for instance, to test the oxidative stress susceptibility of *hk* mutant flies (0-10 mM, Wang et al. 2000).

In order to measure survival upon paraquat exposure, young male flies of the appropriate genotype were sorted into vials in groups of 10 and were left to recover from anaesthesia for at least 24 h. At ZT1, flies were transferred to new vials containing 1% Agar (Sigma # 05040) to provide water and starved for 3 h. At ZT4, flies were transferred to new vials containing 1 % Agar and filter paper soaked with 400 µl of paraquat (0-20 mM, Sigma # 36541) dissolved in 5 % sucrose. Flies kept in paraquat-treated vials and transferred back to standard conditions, i.e., 25^{o}C and 60% humidity, until all flies had died. Death was recorded hourly or daily, depending on the experimental condition.

2.7.2 Shaking leg assay

Certain *hk* mutations are associated with a shaking phenotype (Kaplan et al. 1969). There is no quantitative protocol to assess shaking in fruit flies. Thus, we developed a simple protocol to quantify the degree of shaking in flies in the context of positive and negative references. Here, young adult flies of the appropriate genotype were transferred into empty vials and briefly left to recover from anaesthesia. A plug was drenched in ether and each vial was closed with the ether plug for a short time until a majority of the flies ceased moving.

Shaking legs under anaesthesia and recovery were qualitatively scored under a standard light microscope. A positive reference – the hk^1 mutant – and a negative reference – the genetic background control w^{1118} – was introduced to all experimenters to demonstrate the range of expected shaking from 0 (no shaking) to 5 (heavy shaking). Experimenters were blind to the genotype of the animals when scoring. Each genotype was scored by at least four experimenters and

each experimenter scored each genotype at least twice to provide a measure of consistency. Positive and negative references were interspersed with experimental flies to provide a measure of accuracy.

2.7.3 Courtship assay

To test the association of the sleep phenotype with arousal in the form of sexual arousal, we subjected flies to a courtship assay. We tested both male-male and male-female interactions. The fundamental steps of the naturally occurring courtship behaviour of *Drosophila melanogaster* has been briefly described in section 1.3.1.

Paradigm A courtship assay was implemented essentially as described in Nichols et al. 2012. Virgin males were aged for 3-5 days, and virgin females were aged for 2-3 days in small groups of 10-15 animals. Male flies to be tested were inserted into glass tubes that are similar to "activity tubes" but longer (11 mm), and left to recover from anaesthesia overnight. Importantly, we used activity tubes instead of the typical 1×1 cm chambers. The latter commonly result in successful participation of up to 100 % of flies (e.g., Huang et al. 2016). We opted for tubes to achieve moderate levels of participation of around 50 %. This was crucial in our case to leave a dynamic range for both impairments and improvements in courtship initiation and copulation success.

Paired interactions For male-male interactions, flies were paired for 1 h and subsequently separated. Males were then left to recover overnight before being subjected to male-female interactions. For male-female interactions, flies were paired and directly observed for 2 h, then separated.

Scoring Courtship and copulation behaviours were scored based on consistent parameters inspired by Ejima et al. 2007 and listed in Table 2.12. For male-male interactions, only parameters relating to courtship could be observed as copulation is anatomically not possible between male *Drosophila*; fighting was not assessed.

Parameter	Description
Courtship initiation	Proportion of flies wing singing
Courtship latency	Time from t_0 to wing singing
Courtship duration	Time from courtship initiation to copulation initiation
Copulation success	Proportion of flies per group copulating
Copulation latency	Time to copulation from t_0

Table 2.12: Copulation and courtship parameters, "t_0" refers to the introduction of the male or female target fly. Wing singing was used as the behavioural proxy to define courtship initiation as it is a clearly observable behaviour that rarely occurs spontaneously.

Typically, 5 male flies per genotype and 15 flies in total were tested per replicate. Target flies were of the w^{1118} genotype.

2.7.4 Nutritional status

Body weight

Wet bodyweight was measured in young male flies at 4 to 6 days of age with *ad libitum* access to food throughout development and adult life and kept under standard conditions, i.e., 25 °C and 60 % humidity, and a regular LD schedule.

Flies were collected between ZT2 and ZT4 and immediately weighed. Wet body weight was measured on a precision balance (Mettler-Toledo International Inc.).

Thin layer chromatography

Thin layer chromatography (TLC) was used to determine the triglyceride content in experimental and control groups.

TLC was performed essentially as described in (Bligh et al. 1959). Briefly, groups of 10 male flies per genotype aged 5-7 days that were LD entrained, able to feed *ad libitum* and kept under standard conditions were collected and homogenised in 100 µL 3:1 Chloroform:Methanol. A dilution series of standard samples was prepared using lard dissolved in 3:1 Chloroform:Methanol. 2.5 µL of experimental samples and standards were applied to the TLC plate and run in 4:1 Hexane:Ethyl Ether. Once the mobile phase reached the cut-off line, the plate was sprayed with the oxidising agent Ceric Ammonium Heptamolybdate and baked at 80 °C for 20 min.

2.7.5 Quantification

Data of pharmacological and nutritional assays was collected in Microsoft Excel (Microsoft Office Suite v2016, Microsoft, Redmond, WA) and analyses in R running in RStudio.

The two shaking scores per genotype per experimenter were averaged.

For body weight measurements, 3-5 flies per tube were weighed per replicate and the average weight per fly was calculated per tube.

Triglyceride levels were calibrated according to the relative standard curve derived from the reference samples.

2.8 Statistical analysis

Normality tests Data sets were tested for normal distribution using Shapiro-Wilk normality test at an α level of 0.05.

Parametric data sets For normally distributed data sets, parametric tests were used to test for differences between conditions. We used the Welsh two sample t-test to test paired independent samples, and the test statistic ("t"), degrees of freedom and p-value is reported. We employed ANOVA to compare across multiple groups and for co-variance analysis, and the test statistic ("F"), degrees of freedom and p-value is reported.

To visualise normally distributed, parametric data sets, results were represented in bar plots. Throughout, bar height reflects the average and error bars reflect the standard error of the mean ("sem").

Non-parametric data sets For non-normally distributed data sets, non-parametric tests were used to test for differences between conditions. We used Wilcoxon rank sum test to test paired independent samples, and the test statistic ("W") and p-value is reported. To compare across multiple groups and for co-variance analysis we employed Kruskal-Wallis test followed by Dunn's post hoc test, and the test statistic ("χ^2"[1]) and p-value is reported. Pearson's χ^2 test was used to compare copulation and courtship frequency, and the test statistic ("χ^2"), degrees of freedom and p-value is reported.

To visualise non-normally distributed, non-parametric data sets, results were represented in box plots. Throughout, the top and the bottom of the box represent the 25^{th} and 75^{th} percentile and the bar within the box represents the 50^{th} percentile, i.e., the median. The whiskers extend to within 1.5 of the inter-quartile range of the upper and lower quartile, respectively.

[1]Since the Kruskall Wallis test statistic approximates a χ^2 distribution, the value of the test statistic is typically indicated by "χ^2".

Code	Description
*	p<0.05
**	p<0.01
***	p<0.001
n.s.	not significant (p>0.05)

Table 2.13: Significance code.

Significance code The significance code used throughout all figures is given in Table 2.13. Importantly, p<0.05 was set for level of significance throughout.

3

Results: candidate sleep-regulatory AKRs in fruit flies

3.1 *In silico*: collection of potential fruit fly AKRs

The expression patterns and functions of all fly AKRs except Hk are elusive. Thus, in a first step, we aim to collect candidate AKRs of *Drosophila melanogaster* by identifying genomic loci that encode an AKR domain and subject these candidates to extensive *in silico* research, together with a literature review of genetic and phenotypic screens that could indicate the molecular processes that these proteins are involved in. We further evaluate aspects of conservation between mammalian and fly AKRs. Eventually, we evaluate these candidates for expression in the nervous system to select those potentially involved in neural sleep control.

3.1.1 Further candidate AKRs

Collection of AKRs The defining feature of AKRs is their NADPH-dependent oxidoreductase domain. We detected a total of 12 distinct genomic loci that encode such AKR motifs in fruit flies (Table 3.1).

Gene symbol	Flybase symbol	UniProt symbol
CG10638	FBgn0036290	Q9VTY2
hk	FBgn0263220	M9PE66
CG3397	FBgn0037975	Q9VGF1
CG12224	FBgn0037974	Q9VGF2
CG18547	FBgn0037973	Q9VGF3
CG9436	FBgn0033101	Q7JVH6
CG6083	FBgn0036183	Q9VTL0
CG6084	FBgn0086254	M9PF61
CG10863	FBgn0027552	Q9Y112
CG12766	FBgn0035476	Q9VZK8
CG2767	FBgn0037537	Q9VHX4
ary	FBgn0058064	D2A6K3

Table 3.1: Loci encoding AKR motifs in fruit flies according to the BGDP release 6 of the *Drosophila melanogaster* genome. The annotation of UniProt identifiers follows NCBI gene cards (`http://www.ncbi.nlm.nih.gov/gene`). Some AKRs have alternative gene symbols: *Hk* is also known as CG32688, along with a number of little used alternative symbols, i.e., CG17779, CG2287, CG32688, CG34319, CG43388, and CG43388. CG40064 has been previously published under the more distinct name of aldehyde reductase Y (ARY, Koerich et al. 2008). A little used alternative symbol for CG10638 is CG32101.

3.1.2 Genomic location, potential expression and function

Loci The *Drosophila melanogaster* AKRs we detected span eight loci, mostly clustering on chromosome 3, with three groups of AKRs being immediate "neighbours", i.e., CG12766/CG10863, CG6083/CG6084, and CG18547/CG12224/CG3397. As these "neighbours" also have high sequence identity and are phylogenetically closely related (Supplementary Fig. 7.2), these groups might reflect gene duplication events. One AKR, CG9436, is located on chromosome 2. Two AKRs are located on sex-specific chromosomes: CG32688 (*hk*) on the X and CG40064 (*ary*) on the Y chromosome (Table 3.2).

Isoforms In order to evaluate the specificity of RNAi lines available to target *Drosophila* AKRs, we assessed the potential presence of protein isoforms. Generally, we found that AKR genes are small with a median of 5 exons (range: 1-10) and a median length of around 1,700 bp (range: 1303-30139). Several AKRs (40 %) may encode multiple transcripts: CG6084 (4), CG10638 (3), CG32688 (9), and CG12224

Gene symbol	Location	Length	Exons	Transcripts
CG10638	3L:12,498,106	3206	8	3
Hk	X:10,233,347	30139	10	9
CG3397	3R:11,983,098	1444	3	1
CG12224	3R:11,980,704	1919	3	2
CG18547	3R:11,976,574	1817	4	1
CG9436	2R:6,755,601	1303	1	1
CG6083	3L:11,622,528	1598	4	1
CG6084	3L:11,620,535	3847	8	4
CG10863	3L:3,941,008	1458	6	1
CG12766	3L:3,938,624	1502	6	1
CG2767	3R:8,005,924	2187	5	1
ARY	Y:702,430	1619	5	2

Table 3.2: Genomic loci and predicted isoforms encoding AKR domains in *Drosophila melanogaster*.

(2), ARY (2) (see Table 3.2, median of 1 transcript, range: 1-9). Interestingly, alternative transcripts of the largest locus, *hk*, fall into two main groups: a group of four short isoforms with up to 7 exons (PK, PE, PL, PM) and a group of five long isoforms with up to 10 exons (RG, RH, RF, RD, RI), although most of these transcripts are completely or near identical on the protein level (73.5-99.7 % identity) indicating that only few distinct proteins derive from this locus (Supplementary Table 7.1). An experimental study by Scott Chouinard also detected only one unique isoform derived from the *hk* locus (Chouinard et al. 1995). Several other transcripts are redundant on the protein level: both transcripts of CG12224 are identical and two transcripts of CG10638 (A, D) are identical, while transcript B is distinct (Supplementary Table 7.1). All of the four transcripts of CG6084 are extremely similar with a sequence identity between 92.7-97.1 % suggesting that they may not all encode distinct transcripts (Supplementary Table 7.1).

3.1.3 Conservation

In order to evaluate the level of conservation among *Drosophila* AKRs within and across species, we aligned *Drosophila* protein sequences with a reference rat AKR and annotated commonly conserved AKR residues.

Conserved residues

Reference: rat Akr1c9 The conventional exemplary AKR member is rat Akr1c9 which also serves as a unifying numbering reference; it contains those residues regarded as highly conserved and essential for AKR functionality (Jez et al. 1997b; Penning 2015). These residues have been briefly listed in section 1.6.

"Typical" and "atypical" AKRs In respect to conserved residues, fly AKRs can be separated into two groups: one larger group – CG10638, CG10863, CG12766, CG9436, ARY, CG6083, CG6084, and CG2767 – exhibits a relatively high degree of conservation to mammalian AKRs such as rat Akr1c9 (Table 3.3). Conservation is particularly strict in respect to the active site and the substrate binding pocket, although two AKRs – CG10638-PB and both isoforms of ARY – substitute the serine residue at position 271 for Alanine (Table 3.3). The degree of conservation is notably lower in the other, smaller group – Hk, CG3397, CG12224 and CG18547 – which will be termed the "atypical" group in contrast to the larger, "typical" group.

Position in AKR superfamily

We aimed to tentatively place the other *Drosophila melanogaster* AKRs in the AKR superfamily tree by searching for potential ortholgues among mammalian AKRs, specifically in the common model organism *Mus musculus*.

Hk Hk is also the only *Drosophila melanogaster* AKR that has already been incorporated into the AKR superfamily tree (# AAC46631, `http://www.med.upenn.edu/akr/`). Along with the human KCNABs, it is assigned to family 6, and forms the only member of the insect-specific subgroup B.

Mammalian orthologues In respect to their sequence identity to the common mammalian laboratory animal, *Mus musculus*, fly ARKs fall into three groups: AKRs of the "atypical" group – Hk, CG3397, CG12224, and CG18547 – show relatively high structural identity to the mouse *Kcnab1* and *Kcnab2* genes of the AKR6 family (Table 3.4). Another group of AKRs is relatively similar to members

	AS			CB				SB			
Protein	50	55	84	50	167	190	271	52	55	86	117
Rat Akr1c9	D	Y	K	D	N	Q	S	A	Y	W	H
CG10638-PA	D	Y	K	D	N	Q	S	A	Y	W	H
CG10638-PB	D	Y	K	D	N	Q	A	A	Y	W	H
CG10863	D	Y	K	D	N	Q	S	A	Y	W	H
CG12766	D	Y	K	D	N	Q	S	A	Y	W	H
CG9436	D	Y	K	D	N	Q	S	A	Y	G	H
ARY-PC	D	Y	K	D	N	Q	A	A	Y	W	H
ARY-PD	D	Y	K	D	N	Q	A	A	Y	W	H
CG6083	D	Y	K	D	N	Q	S	A	Y	W	H
CG6084-PA	D	Y	K	D	N	Q	S	A	Y	W	H
CG6084-PB	D	Y	K	D	N	Q	S	A	Y	W	H
CG2767	D	Y	K	D	N	Q	S	A	Y	P	H
Hk-PG	D	H	K	D	R	Q	A	S	H	Y	H
Hk-PH	D	H	K	D	R	Q	A	S	H	Y	H
Hk-PI	D	H	K	D	R	Q	A	S	H	Y	H
Hk-PF	D	H	K	D	R	Q	A	S	H	Y	H
Hk-PD	D	H	K	D	R	Q	A	S	H	Y	H
Hk-PK	D	H	K	D	R	Q	A	S	H	Y	H
Hk-PE	D	H	K	D	R	Q	A	S	H	Y	H
Hk-PM	D	H	K	D	R	Q	A	S	H	Y	H
Hk-PL	D	H	K	D	R	Q	A	S	H	Y	H
CG3397	D	Y	K	D	A	V	I	A	Y	A	H
CG12224-PB	-	-	-	-	A	V	I	-	-	-	H
CG12224-PC	-	-	-	-	A	V	I	-	-	-	H
CG18547	D	Y	K	D	A	V	M	A	Y	A	H

Table 3.3: Conserved residues of AKR proteins in *Drosophila melanogaster* (rat Akr1c9 numbering) for active site (AS), co-factor binding pocket (CB), and substrate-binding pocket (SB).

of the subfamily Akr1c, e.g., CG10638, CG10863, and CG12766, and another group is relatively similar to members of the subfamily Akr1b, e.g., CG6083, CG6084, CG602767, and ARY (Table 3.4).

3.1.4 Putative biological functions

Gene ontology analysis

The Gene Ontology Consortium ventures predictions of molecular processes and biological functions that gene products may be involved in based on either experimental evidence, where available, or based on sequence homology to already

Gene symbol	Hit	Identity [%]
CG10638	AKR1c19	46
Hk	Kcnab1	50
CG3397	Kcnab2	56
CG12224	Kcnab2	25
CG18547	Kcnab2	59
CG9436	Akr1a1	44
CG6083	Akr1b3	44
CG6084	Abr1b10	62
CG10863	Akr1c19	48
CG12766	Akr1c1	43
CG2767	Akr1b3	43
ARY	Akr1b3	40

Table 3.4: Potential orthologues of *Drosophila melanogaster* AKRs in *Mus musculus*.

characterised proteins (Consortium 2013, see also `http://geneontology.org/`). Molecular processes and biological functions are categorised and given unique identifiers ("GO" numbers). For all fly AKRs except Hk, their function is largely hypothetical and even their identification as AKRs is mostly based on prediction from sequence homology and structural similarity with AKRs of other species rather than experimental evidence.

We performed a gene ontology analysis of *Drosophila* AKRs as given in Table 3.5. Generally, *Drosophila melanogaster* AKRs are comprised of unspecified oxidoreductases, aldehyde reductases, alcohol dehydrogenases, and $K_v\beta$ subunits (Table 3.5). Half of *Drosophila melanogaster* AKRs, e.g., CG10638, CG9436, CG6083, CG6084, CG10863, and CG12766, may encode aldehyde reductases and would be expected to perform simple reduction processes. Another group – Hk, CG3397, CG12224, ARY, and CG18547 – are either $K_v\beta$ subunits or do not have a function specified apart from their general involvement in oxidoreduction. One AKR, CG2767, may encode an alcohol dehydrogenase (Table 3.5).

Experimental evidence from previous studies

Although AKR genes of *Drosophila melanogaster* have not received dedicated study, several genetic and phenotypic screens as well as transcriptomic studies imply AKRs

Gene symbol	Predicted biological processes/molecular function	GO
CG10638	cellular aldehyde metabolism	GO:0004032
	oxidation-reduction process	GO:0055114
Hk	circadian sleep/wake cycle, sleep	GO:0005249
	protein oligomerization	GO:0051259
	flight behaviour	GO:0007629
	potassium ion transport	GO:0006813
	Voltage-gated K+ channel activity	GO:0005249
CG3397	oxidation-reduction	GO:0055114
	potassium ion transport	GO:0006813
CG12224	oxidation-reduction	GO:0055114
CG18547	oxidation-reduction	GO:0055114
CG9436	oxidation-reduction	GO:0055114
	aldehyde reductase	GO:0004032
CG6083	oxidation-reduction	GO:0055114
	aldehyde reductase	GO:0004032
	mushroom body development	GO:0016319
CG6084	oxidation-reduction	GO:0055114
	aldehyde reductase	GO:0004032
CG10863	oxidation-reduction	GO:0055114
	aldehyde reductase	GO:0004032
CG12766	oxidation-reduction	GO:0055114
	aldehyde reductase	GO:0004032
CG2767	oxidation-reduction	GO:0055114
	alcohol dehydrogenase (NADP+)	GO:0008106
	chaeta development [Ssdp]	GO:0022416
	wing disc development [Bx]	GO:0035220
ARY	oxidation-reduction	GO:0055114

Table 3.5: Predicted molecular functions and biological processes of *Drosophila melanogaster* AKRs.

in a range of functions and the main findings are listed in Supplementary Table 7.1. Common denominators of AKR function include responses to metabolic stress such as low temperature and starvation, neural and muscle development and motor control, as well as neural maintenance (Supplementary Table 7.1).

Adult nervous system and circadian regulation Two AKRs, HK and CG3397, may be involved in neurotransmission (Table 3.5). The role of Hk as a β subunit of the Shaker channel has been described elsewhere (section 1.5). The functional prediction for CG3397 is based on sequence homology with Hk and the mouse β

subunit, *Kcnab2*, and is tentatively supported by a gene expression screen of muscle dystrophia (Montana et al. 2006). Additionally, expression of CG3397 is up-regulated upon release from circadian drive, e.g., in circadian clock mutants (*per*, *clock*, *cycle*, Lin et al. 2002). There are two other AKRs, i.e., CG9436 and CG6084, that have a connection to the circadian system: both genes are expressed in PDF+ neurons, whereby CG6084 expression undergoes circadian cycling (Kula-Eversole et al. 2010).

Gene regulation Interestingly, several AKRs may be implicated in the regulation of gene expression and protein degradation, e.g., CG10638 (Vinayagam et al. 2014; Waldholm et al. 2011), CG18547 (Crona et al. 2013; Nelson et al. 2005; Ren et al. 2011), CG6084 (Lim et al. 2011), and CG2767 (Bozoky et al. 2009; Bronstein et al. 2010). Some AKRs are implicated in development: CG18547 may be involved in developmental gene regulation (Crona et al. 2013) and CG2767 plays a role in body development (Bozoky et al. 2009; Bronstein et al. 2010). Furthermore, CG6083 may play a role in MB development, as knock down of CG6083 expression results in fused γ lobes (Kobayashi et al. 2006).

Stress response Several AKRs are differentially expressed in response to stress: for instance, CG18547, CG9436, and CG6084 respond to starvation (Fujikawa et al. 2009). While CG10863 and CG12766 respond to hypoxia (Vermeulen et al. 2013), CG3397 and CG10638 respond to high oxygen-induced stress (Gruenewald et al. 2009). CG9436 expression responds to immune challenge (De Gregorio et al. 2001; Irving et al. 2001). Generally, hypoxia-associated genes may mainly be expressed in the digestive system such as the fat body, e.g., CG10638, CG3397, CG10863, and CG12766, as were starvation-associated genes, e.g., CG18547, CG96436, and CG6084 (Supplementary Tables 7.1 and 7.2).

Sex-related behaviours Two AKRs may mainly be involved in sex-related behaviours: *ary* is located on the Y-chromosome (Koerich et al. 2008, Table 3.2) and is probably exclusively expressed in the testis (Supplementary Table 7.2). CG12224 might play a role in female post-mating behaviour (McGraw et al. 2008).

3.2 Initial experimental evidence

3.2.1 Expression in the nervous system

We assessed the expression level of AKR genes in the head to test whether the Y-linked target, *ary*, was indeed absent from the head. Then, expression of AKRs except for *ary* is measured in the brain relative to the body in order to test which AKRs were expressed and potentially enriched in the brain.

All *Drosophila melanogaster* AKRs could be successfully amplified (Fig. 3.1A). CG10863 is among the most abundant both in the head (Fig. 3.1A) and in the rest of the body (Supplementary Fig. 7.11A).

The isoforms of CG10638 are expressed differently in the head with isoform A being more abundant ($W=9$, $p<0.05$, Fig. 3.1C). CG10638, isoform B ("isoB"), is consistently expressed at low levels in the head, brain, and body on par with the supposedly MB-related CG6083 (see Kobayashi et al. 2006). Isoform A is expressed at moderate levels among *Drosophila* AKRs (Fig. 3.1A, Fig. 7.11A, B). Among AKRs expressed in the head, both isoforms of CG10638 are the only targets that are enriched in the brain (brain/body ratio>1, Fig. 3.1D).

Ary is expressed at negligible levels in the head, but at higher levels in the body (Fig. 3.1, $W=0$, $p<0.05$, Fig. 3.1B), consistent with the notion that it is testis-specific.

3.2.2 Nervous system-wide RNAi sleep screen

Initial experiments

As a first step to investigate the relevance of AKRs for fly sleep, we conducted a small RNAi screen among *Drosophila melanogaster* AKRs with the exception of ARY, a Y-linked AKR that is likely to be exclusive to the testis (see above). Thereby, we drove expression of RNAi-lines with ELAV-Gal4, a driver that is expressed at intermediate levels throughout the nervous system, in order to target virtually all neurons. As a negative control, we also knocked down the exogenous

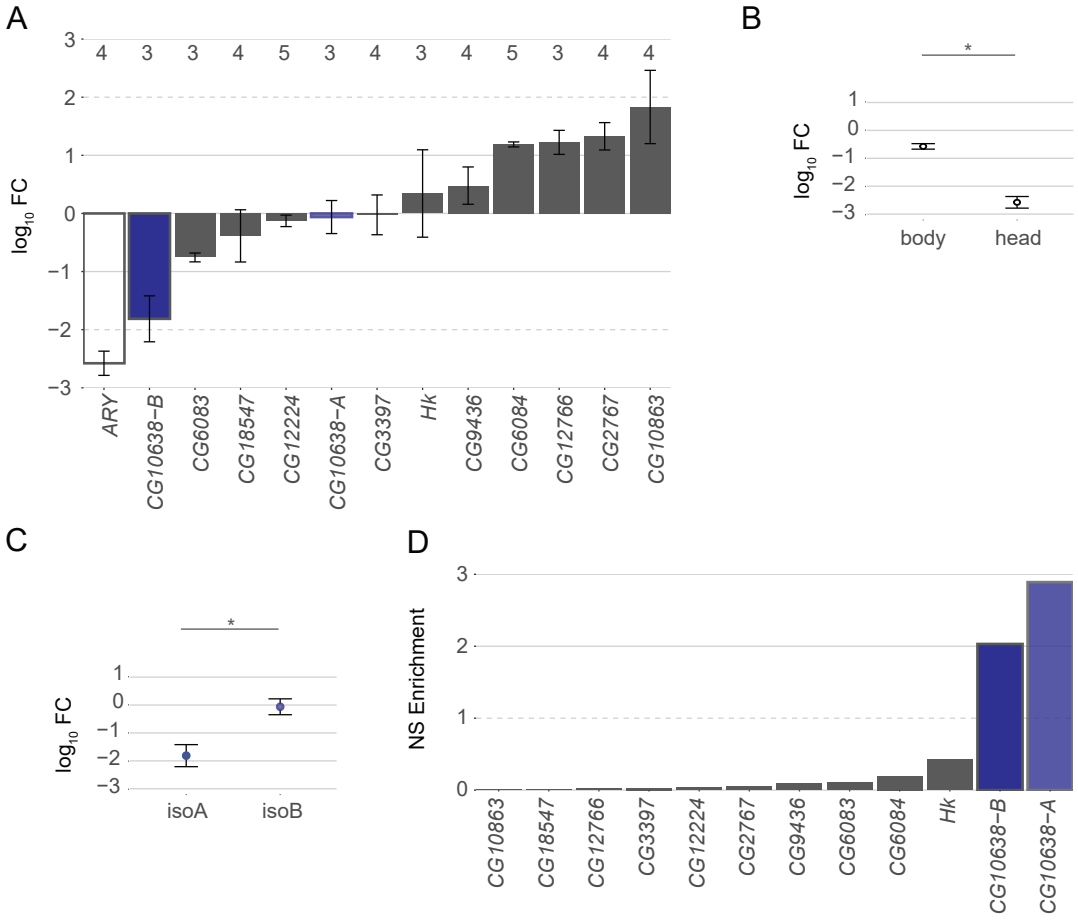


Figure 3.1: Expression of *Drosophila melanogaster* AKRs in the fly head. A, Expression of AKRs in the head (sample size indicated above respective bar). B, Expression of *ary* in the head and the rest of the body (n=4/tissue). C, Expression of both predicted isoforms of CG10638 (amplification from largest exclusive exon, n=3/target). D, Adult enrichment of AKRs in protocerebrum relative to the rest of the body. A, D, Bar height reflects mean, error bars in A reflect sem. B, C, Dot reflects median, whiskers reflect 1.5 of inter-quartile range. B, C, Wilcoxon rank sum test, "*" signifies $p<0.05$.

target GFP, and we used knock down of *hk*, the only known AKR to affect sleep, as the most suitable positive control.

Initially, we combined the ELAV driver with a UAS-Dicer2 construct in order to increase knock down efficiency. However, this led to high levels of lethality when targeting CG2767 (KK104174) – only a few unhealthy survivors could be tested and showed low activity and high sleep levels (not shown). They also had underdeveloped wings and were smaller than the parental controls (not shown). Also, complete lethality beyond the second day of eclosion was observed when targeting

CG18547 (KK104487). The few newly eclosed flies that could be examined also had underdeveloped wings and were smaller than the parental controls (not shown). While we observed similar levels of lethality when using a stronger driver, nsyb-Gal4 (not shown), we cannot differentiate whether lethality in these lines was due to high knock down efficiency or the over-expression of *Dicer per se*, or a combination of both.

General effects on sleep demand and architecture

Using the ELAV driver without the addition of Dicer2 was a viable option and produced flies that hatched normally and appeared grossly normal.

Upon knock down of AKRs in the nervous system (ELAV>IR), flies spend around 65 % of the time asleep (Fig. 3.2C, population median, red line). Generally, knock down of AKRs does not affect sleep demand as the average sleep level of experimental lines is not different from parental control flies except for a limited number of candidates (Fig. 3.2D). Also, knock down of AKRs does not affect sleep architecture as the average sleep bout length is largely similiar between experimental and parental control flies except for a limited number of AKRs (Fig. 3.2F). The average bout length upon knock down of AKRs in the nervous system is around 44.5 min (Fig. 3.2E, population median, red line).

To test for unspecific effects due to the artificial activation of the RNAi machinery, we expressed an RNAi transgene complementary to GFP. GFP-IR would not be expected to have an effect: it does not have a target in the *Drosophila melanogaster* genome as GFP is a protein exogenous to the fly. Indeed, expression of GFP-IR in the whole nervous system did not affect sleep duration (Fig. 3.2D, $\chi^2(2)=18.5$, $p<0.001$; *post hoc* ELAV>IR-GFP:ELAV/+, $p=0.481$, ELAV>IR-GFP:IR-GFP/+, $p<0.05$). GFP-IR flies sleep around 76 % of the time, compared to 75 % and 82 %, respectively, in parental controls (Fig. 3.2D). Expression of GFP-IR in the nervous system does also not affect sleep architecture; sleep bouts in ELAV>GFP-IR flies last around 21 min, which is on par with parental control flies which sleep on average around 19 min and 27 min per bout (Fig. 3.2F, $\chi^2(2)=5.8$, $p>0.05$).

However, expression of Hk-IR in the nervous system does substantially reduce sleep levels (Fig. 3.2C, green line). As indicated in the sleep quantification in Fig. 3.2D , reduction of *hk* significantly suppresses sleep ($\chi^2(2)$=59.5, p<0.001; *post hoc* as annotated) and RNAi flies sleep approximately 3.5 h per day – or around 15 % – less than parental controls. Interestingly, the expression of Hk-IR does not suppress sleep levels homogeneously: instead, it leads to a reduction of mostly night-specific sleep and here affects particularly sleep in the middle of the night producing a biphasic sleep pattern (Fig. 3.2B). Initial studies of fruit fly sleep had already noted that, within a population, sleep is particularly concentrated in the beginning of the night, between ZT14 and ZT16, after which the propensity for sleep decreases, together with sleep pressure (Hendricks et al. 2000). Also, the neural activation of the l-LN_v specifically suppresses night sleep (Sheeba et al. 2008b). Incidentally, Hk is expressed in the l-LN_v and has been demonstrated to modulate their excitability (Fogle et al. 2015). The sleep suppression achieved through expression of Hk-RNAi fits into the two-process model of sleep regulation which states that sleep pressure dissipates throughout the night and predicts that it should be easier for the rising influence of the circadian system throughout the night to overcome homeostatic processes to limit sleep, particularly when the homeostatic system is perturbed through, e.g., reduced levels of rest-promoting Hk in clock neurons.

Expression of Hk-IR in the nervous system also substantially reduces sleep bout length (Fig. 3.2F, $\chi^2(2)$=35.02, p<0.001; *post hoc* as annotated). Sleep bouts of ELAV>Hk-IR flies are on average 6.5 min – or around 30 % – shorter than those of parental controls (Fig. 3.2F). While both day and night sleep are affected, night sleep is suppressed more strongly in ELAV>Hk-IR flies (Fig. 3.2B).

Identification of another candidate: CG10638

Expression of CG10638-IRs in the nervous system reduces sleep below the levels of ELAV>Hk-IR (Fig. 3.2C, blue line reflects sleep levels achieved by expression of GD10458/“IR# 2”). The expression of GD7046 (“IR# 1”) suppresses sleep by 3.5 h per day – or around 15 % – compared to parental controls (Fig. 3.2D,

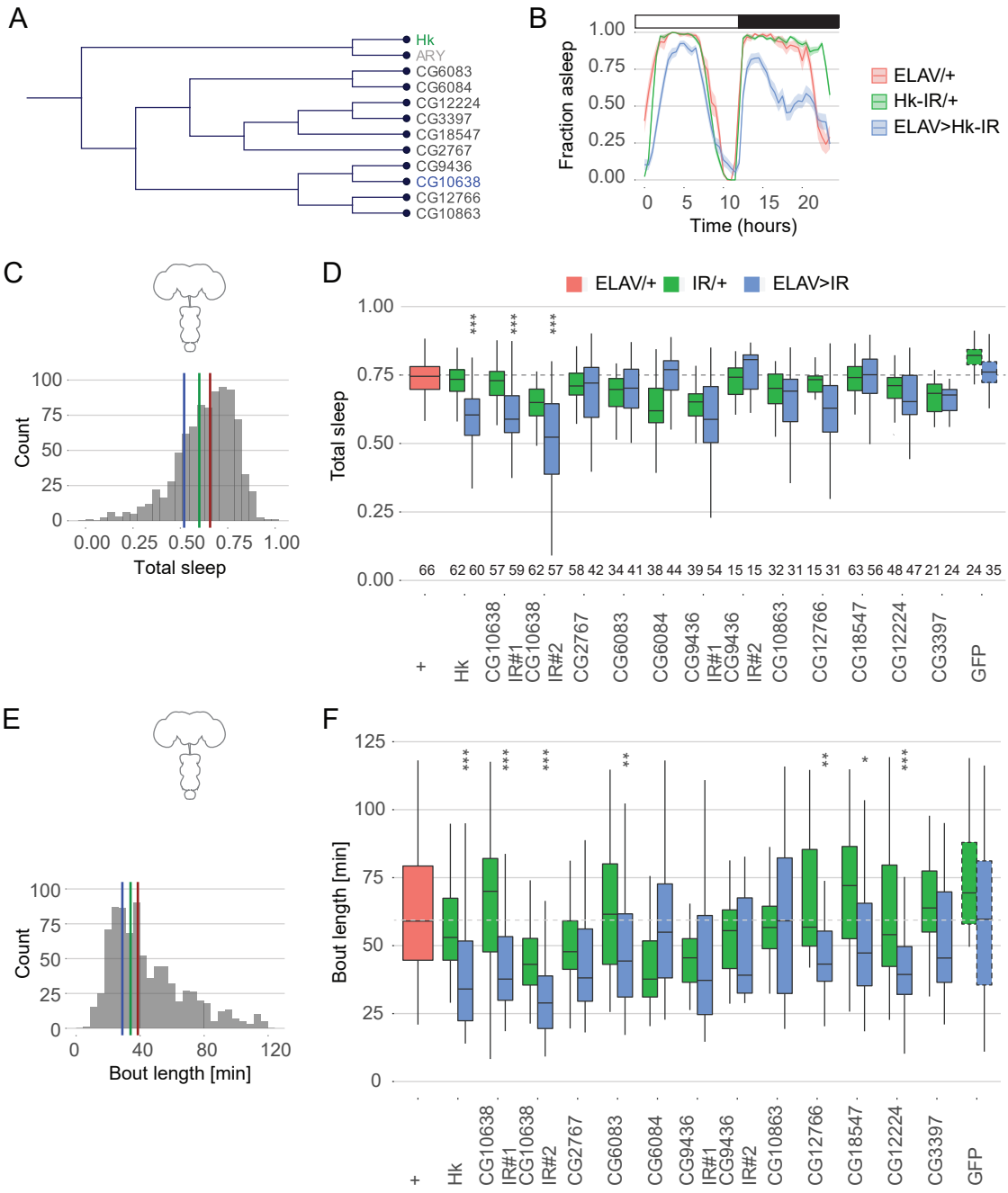


Figure 3.2: RNAi-based sleep screen of AKRs (ELAV>IR). A, Simplified cladogram of *Drosophila melanogaster* AKR loci generated using CLC Sequence Viewer 7.8.1. B, Sleep profile produced by expression of Hk-IR with ELAV-Gal4, wrap of three days of baseline recording, sample size as in D. C, Histogram of sleep levels per experimental line (ELAV>IR) with binwidth of 0.04, vertical lines reflect ELAV>CG10638-IR# 2 (blue), ELAV>Hk-IR (green), and the population median (red). D, Total sleep per day per genotype, sample size as annotated. E, Histogram of sleep bout length with binwidth of 5, lines reflect genotypes as in C. F, Sleep bout length, sample size as in C. D, F, Boxes reflect 25^{th} (top) and 75^{th} (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. D, F, Kruskal Wallis test to compare sleep levels/bout length between experimental group (ELAV-IR) and parental controls (ELAV/+, IR/+) and the lowest significance level common to both parental control lines in comparison to the experimental line is indicated above the experimental group; "*" reflects $p<0.05$, "**" reflects $p<0.01$, "***" reflects $p<0.001$, "n.s." signifies "not significant". Dashed line reflects median sleep levels of parental control ELAV-Gal4/+.

$\chi^2(2)$=49.1, p<0.001; *post hoc* as annotated). The expression of the other RNA transgene, GD10458 ("IR# 2"), suppresses sleep more severely by 4.3 h per day – or around 18 % – compared to parental controls (Fig. 3.2D, $\chi^2(2)$=79.8, p<0.001; *post hoc* as annotated).

Expression of CG10638-IRs in the nervous system also severely reduces sleep bout length below the level of ELAV>Hk-IR (Fig. 3.2E, blue line reflects genotypes as in C). The expression of IR# 1 in the nervous system shortens sleep bouts by 5.5 min or around 26 % compared to parental controls (Fig. 3.2E, $\chi^2(2)$=35.3, p<0.001; *post hoc* as annotated). Expression of IR# 2 shortens sleep bouts more severely by 6 min or around 30 % (Fig. 3.2E, $\chi^2(2)$=50.9, p<0.001; *post hoc* as annotated).

Effects of other AKRs on sleep architecture

While the nervous system-wide knock down of AKRs other than Hk and CG10638 did not affect sleep levels (Fig. 3.2D), the knock down of other AKRs destabilises sleep, i.e., reduces the average sleep bout duration (Fig. 3.2F).

The nervous system-wide expression of CG12224-IR shortens sleep bouts by 5.5 min or around 23 % compared to parental controls ($\chi^2(2)$=21.7, p<0.01; *post hoc* as annotated) and the expression of CG6083-IR shortens sleep bouts by 5 min or around 26 % compared to parental controls ($\chi^2(2)$=11.1, p<0.01; *post hoc* as annotated).

Two other AKRs have only very mild effects on sleep architecture: the expression of CG12766-IR shortens sleep bouts by 2 min or around 10 % compared to parental controls ($\chi^2(2)$=12.9, p<0.01; *post hoc* as annotated) and the expression of CG18547-IR shortens sleep bouts by 1 min or around 5 % compared to parental controls ($\chi^2(2)$=17.3, p<0.01; *post hoc* as annotated).

3.2.3 Mushroom body-specific RNAi sleep screen

We restricted the reduction of AKRs to the MB in order to test their sleep-related effects for this main sleep-regulatory structure.

General effects on sleep demand

We used ok107-Gal4 to drive expression of RNAi transgenes in the MB and this manipulation generally does not affect sleep levels except for a limited number of candidates (Fig. 3.3C). Upon knock down of AKRs in the MB, flies sleep around 73 % of the time (Fig. 3.3A, red line reflects population median).

The expression of GFP-IR in the MB does not affect sleep levels: ok107>GF-IR flies sleep around 82 % of the time, on par with parentol controls which sleep around 83 % and 78 % of the time, respectively (Fig. 3.3C, $\chi^2(2)=21.0$, $p<0.001$; *post hoc* ok107>GFP-IR:ok107/+, $p=0.669$, ok107>GFP-IR:IR-GFP/+, $p<0.05$).

However, expression of Hk-IR in the MB does mildly suppress sleep: ok107>Hk-IR flies sleep around 5 % less per day than parental controls (Fig. 3.3C, $\chi^2(2)=11.0$, $p<0.05$; *post hoc* as annotated). Sleep suppression is mainly restricted to the latter part of the night: Hk-IR flies sleep approximately 1.2 h – or 5 % – less per night than parental control flies, but only approximately 0.5 h – or 2 % – less per day (Fig. 3.3B).

The MB-specific knock down of CG10638 reduces sleep below the population average (Fig. 3.3A, blue line reflects the sleep level achieved by expression of GD7046). However, this was true only for one trangene, IR# 1, which reduces sleep by 2.8 h – or 11.5 % – per day compared to parental controls (Fig. 3.3C, $\chi^2(2)=25.0$, $p<0.001$; *post hoc* as annotated). Expression of IR# 2 does not significantly affect sleep: RNAi flies sleep around 68 % of the time per day, on par with parental control flies which sleep around 64 % or 76 % of the time per day, respectively (Fig. 3.3C, $\chi^2(2)=33.6$, $p<0.001$; *post hoc* ok107>IR# 2:ok107/+, $p<0.05$, ok107>IR# 2:IR# 2/+, $p=0.1102$). It is of note, however, that the parental control lines, UAS-IR# 2 and ok107-Gal4, substantially differ from each other (ok107/+:IR# 2/+, $p<0.001$). Low sleep levels in the parental control UAS-IR# 2 could mask potential effects of the experimental line, ok107>IR# 2.

Identification of another candidate: CG3397

Knock down of another AKR, CG3397, in the MB suppresses sleep to a lower level than CG10638-IR (Fig. 3.3A, green line reflect sleep levels achieved by expression

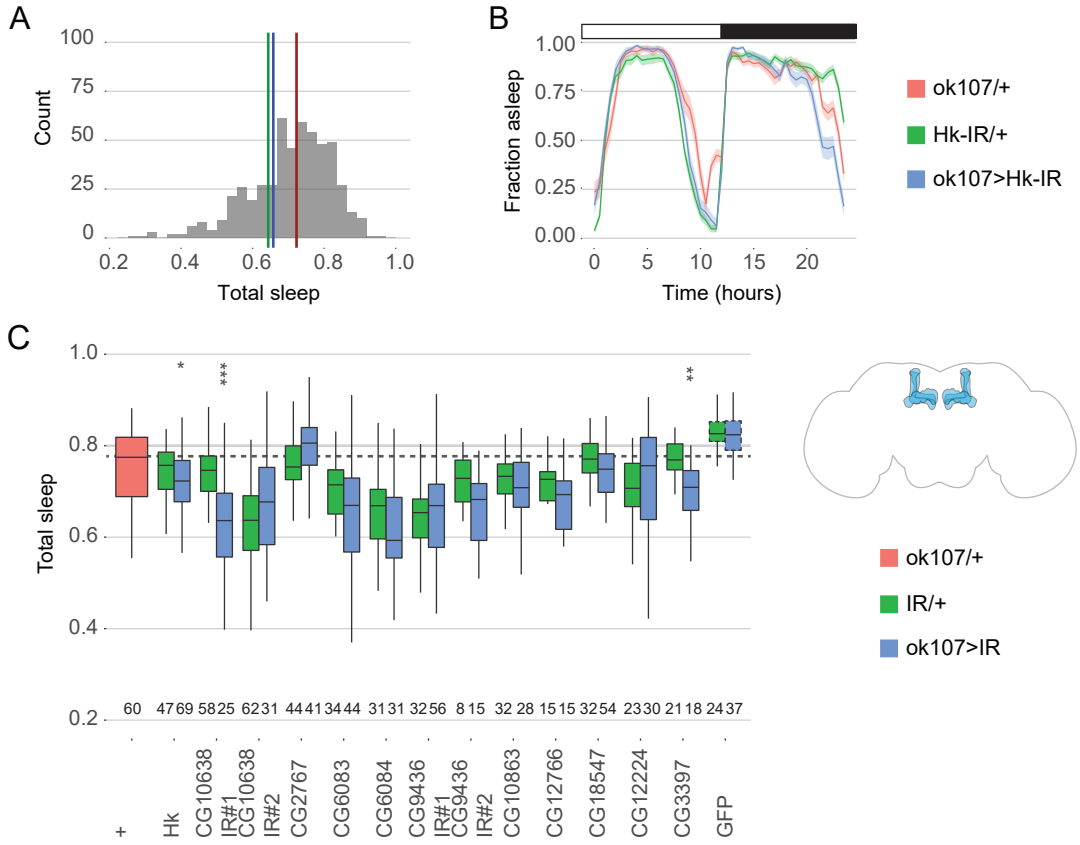


Figure 3.3: RNAi-based sleep screen of AKRs (ok107>IR). A, Histogram of sleep levels per experimental line (ok107>IR) with binwidth of 0.04, lines reflect ok107>CG10638-IR# 1 (blue), ok107>CG3397-IR (green), and the population median (red); sample size as in C. B, Sleep profile produced by expression of Hk-IR under control of ok107, wrap of three days of baseline recording, sample size as in C. C, Total sleep per day per genotype, sample size as annotated. C, Boxes reflect 25$^{\text{th}}$ (top) and 75$^{\text{th}}$ (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. C, Kruskal Wallis test to compare sleep levels/bout length between experimental group (ok107-IR) and parental controls (ok107/+, IR/+) and the lowest significance level common to both parental control lines in comparison to the experimental line is indicated above the experimental group; "*" reflects $p<0.05$, "**" reflects $p<0.01$, "***" reflects $p<0.001$, "n.s." signifies "not significant". Dashed line reflects median sleep levels of parental control ok107-Gal4/+.

of GD10955). In fact, ok107>CG3397-IR flies exhibit a reduction of sleep levels by 2.9 h – or 12 % – per day compared to parental controls which both sleep around 75 % of the time (Fig. 3.3C, $\chi^2(2)=11.4$, $p<0.001$; *post hoc* as annotated).

3.3 Summary

We detected 12 genomic loci that are predicted to encode at least one AKR domain. We have characterised *Drosophila melanogaster* AKR proteins in respect to potential isoforms and their potential functions using *in silico* tools to complement our literature research. To tentatively put *Drosophila melanogaster* AKRs in the context of the AKR superfamily, we also discerned the level of conservation of *Drosophila* AKRs within and across species, particularly regarding potential orthologues in mammals.

Regarding their rate of conservation, we identified two groups of AKRs: one group with a relatively high degree of conservation of canonical residues that comprises potential AKR1 proteins, mainly aldehyde reductases, and another group with a low degree of conservation that comprises potential AKR6 proteins, i.e., β subunits of ion channels. Among the latter group, two AKRs have been validated by earlier studies (Hk) or are predicted through our *in silico* analysis (CG3397) to be involved in neural transmission due to their similarity with the mammalian *Kcnab1* and *Kcnab2* genes. Previous studies have identified several AKRs as potential regulators of transcription and protein levels; this includes a potential negative regulator of the proteasome, CG10638.

In a next step, we experimentally assessed the expression of *Drosophila melanogaster* AKRs in different tissues to identify potential neural regulators. We identified an AKR that is enriched in the brain, CG10638, and one AKR that is likely absent from the brain, ARY. We then tested the effect of nervous system-wide reductions of AKRs on sleep behaviour. As the MB are a major structure in the fly brain that has long been implicated in sleep regulation, we also tested sleep-related effects of AKR reduction in this structure in particular.

We found that the strong nervous system-wide knock down of two AKRs, CG18547 and CG2767, is associated with adult lethality, suggesting that these AKRs may play a role in development. Using a nervous system-wide driver that is expressed at moderate levels, we we able to test all experimental genotypes and identified two AKRs, Hk and CG10638, whose downregulation suppresses sleep.

Regarding CG10638, both RNAi transgenes we tested – which are predicted to each target only one of the two isoforms – suppresses sleep when driven in the nervous system. Interestingly, when we restricted our screen to the MB by using a strong MB-specific driver, ok107, we achieved a mild sleep suppression through the knock down of Hk, and the knock down of one of the transgenes targeting CG10638, as well as the knock down of CG3397. These findings indicate that Hk has indeed the potential to modulate sleep through the MB. However, the finding that the expression of CG3397-RNAi in the nervous system does not affect sleep, while the MB-specific expression of this RNAi does, is puzzling. These seemingly discrepant findings are likely due to the different strengths of both drivers: the pan-neural driver ELAV, which we used for the nervous system-specific screen, is expressed at moderate levels, while the ok107 driver, which we used for the MB-specific screen, is expressed at very high levels and has thus a higher potential to drive strong expression of CG3397 in the MB than ELAV. Fittingly, the sleep suppression achieved through expression of CG3397-RNAi is relatively mild. Secondly, the expression of only one of our CG10638-RNAi lines suppresses sleep when expressed in the MB, while the nervous system-wide expression of both transgenes suppresses sleep. This finding has several possible explanations, all of which are, at least in part, addressed experimentally in the next chapter: (1) CG10638 could have a role outside of the MB and it would therefore be informative to identify the specific neuronal populations that CG10638 is expressed in. (2) The RNAi transgenes may not be isoform-specific and/or may compensate for each other, thereby masking potential isoform-specific effects. Here, the assessment of CG10638 expression levels in RNAi and mutant flies could help to unmask such a mechanism. (3) The parental control of one of the RNAi transgenes, IR# 2/+, exhibits lower levels of sleep than other parental controls, e.g., IR# 1/+ and ELAV-Gal4/+, indicating that there may be unintended background expression of the RNAi transgene outside Gal4-driven populations. This latter possibility gives further emphasis to the importance of the identification of potentially isoform-specific neural populations.

Through our AKR-wide sleep screen, we have (1) confirmed the sleep-related role of Hk in the nervous system in general and the MB in particular, (2) identified a candidate AKR that severely affects sleep when reduced in the nervous system (CG10638), and (3) identified a second candidate AKR that affects sleep when reduced in MB in particular (CG3397). The next two chapters are dedicated to our validation and characterisation of the candidate CG10638 (chapter 4) and CG3397 (chapter 5).

4

Results: candidate CG10638

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4.1 Approach

Our initial experiments presented in chapter 3 identified the candidate AKR CG10638 as a potential sleep-regulatory gene. First, it is not only expressed in, but enriched in the brain to the highest degree among potential *Drosophila melanogaster* AKRs (Fig. 3.1). It also lists in the FlyAtlas database as a gene that is expressed in the brain (Supplementary Table 7.2), but no specific substrate or target cell population is known. Secondly, reduction of CG10638 mRNA levels in the brain suppresses sleep, indicating a sleep-promoting role (Fig. 3.2).

Next, we aimed at further elucidating the role of this candidate gene in sleep regulation regarding (1) the robustness and generality of this phenotype, (2) the neural substrate, and (3) potential mechanisms. These investigations were preceded by a characterisation of the gene locus *in silico*.

Certain lines of experiments presented in this chapter were conducted as part of student projects under the day-to-day supervision of Anne Petzold. Specifically, the copulation data presented in Fig. 4.30 and Fig. 4.31, as well as the related Fig. 7.8 in Appendix, section 7.2.3, was collected by Solweig Schlumberger as part of her B.Sc. project entitled "A novel Aldo-Keto Reductase in Drosophila melanogaster linking sleep and sex" (2017). Anne Petzold developed the project, defined the methodology and specific experiments to be conducted and cultured the flies of the relevant genotypes. Solweig Schlumberger conducted the copulation assays under the direct supervision of Anne Petzold who also analysed the results.

Also, Ho Yan Grace Wu collected parts of the datasets relating to the homeostatic sleep response (Fig. 4.29, Fig. 4.26, Fig. 4.23) as part of her M.Res. project entitled "A novel regulator of sleep homeostasis in Drosophila melanogaster" (2017) under the day-to-day supervision of Anne Petzold. Anne Petzold developed the project, defined the methodolody and experiments, cultured the flies of the relevant genotypes, and

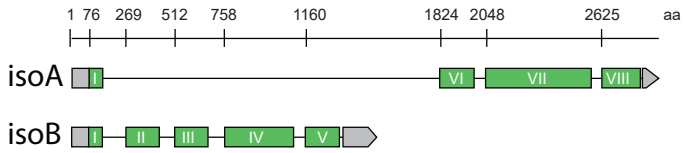


Figure 4.1: Annotated gene locus CG10638.

conducted the initial experiments. Mrs. Wu conducted additional experiments under direct supervision by Anne Petzold who also analysed the results.

4.2 *In silico:* the candidate AKR CG10638

4.2.1 Protein isoforms and conservation

Initially, we characterised this locus in respect to potential alternative isoforms and their predicted enzymatic activities and potential functions.

Gene locus and protein isoforms The candidate AKR CG10638 (alternatively CG32101, FlyBase symbol FBgn0036290, UniProt symnol Q9VTY2) is a small AKR of around 35.8 kDa (ExPasy, `http://web.expasy.org`) encoded by 8 exons (chapter 3, Table 3.2). Of the three predicted transcripts, two are identical on the protein level (CG10638-A and CG10638-D, Supplementary Table 7.1) leaving two predicted isoforms, "isoA" and "isoB", which are encoded by almost mutually exclusive exons (Fig. 4.1).

The isoforms are relatively different from each other: aligning the protein sequence of isoA and isoB results in a sequence identity of 53 % and a sequence similarity of 71 % (Table 4.1). This is a smaller degree of identity than that shared by isoA with its closest match, CG10863 (57 %), but the closest match for isoB (Supplementary Table 7.1).

Conservation CG10638 belongs to the "typical" AKR group with a relatively high number of conserved residues that make up the active site, substrate binding pocket, and co-factor binding pocket. However, while isoA exhibits perfect conservation of essential AKR residues compared to the reference AKR, rat Akr1c9, isoB substitutes

	Residues	Percent
Length	317	
Identity	168/317	53
Similarity	225/317	71
Gaps	7/317	2.2

Table 4.1: Pairwise sequence alignment of both isoforms.

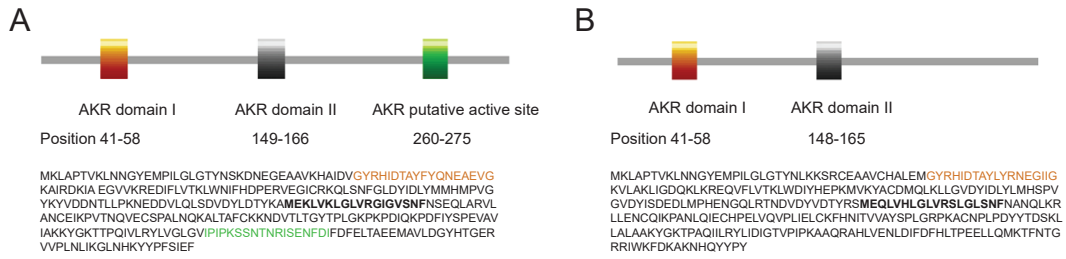


Figure 4.2: Identification and localisation of protein domains adapted from graphical output of protein domain search. A, isoform A. B, isoform B.

Serine-271 with an Alanine as do AKRs of the "atypical" group (chapter 3, Table 3.3). In this context, it is of note that, according to prediction, both isoforms contain an AKR domain, but isoB may not contain a functional active site domain due to this substitution (Fig. 4.2).

4.2.2 Putative biological functions

Gene ontology analysis Specifically, CG10638 might belong to the aldehyde reductases and would be expected to perform simple redox reactions (chapter 3, Tables 3.5). It shows the highest degree of homology with mouse AKR1c19 (chapter 3, Table 3.4) which is a reductase for isatin and other xenobiotic alcohols in the liver and gut (Ishikura et al. 2005). Interestingly, isatin may be an endogenous compound that modulates levels of stress and anxiety (Bhattacharya et al. 1991).

Protein-protein interactions However, consistent with previously discussed transcriptomic screens, predicted protein-protein interactions place CG10638 at the heart of a range of essential processes centering around protein production and metabolism (Fig. 4.3). These putative processes include: proteasome regulation

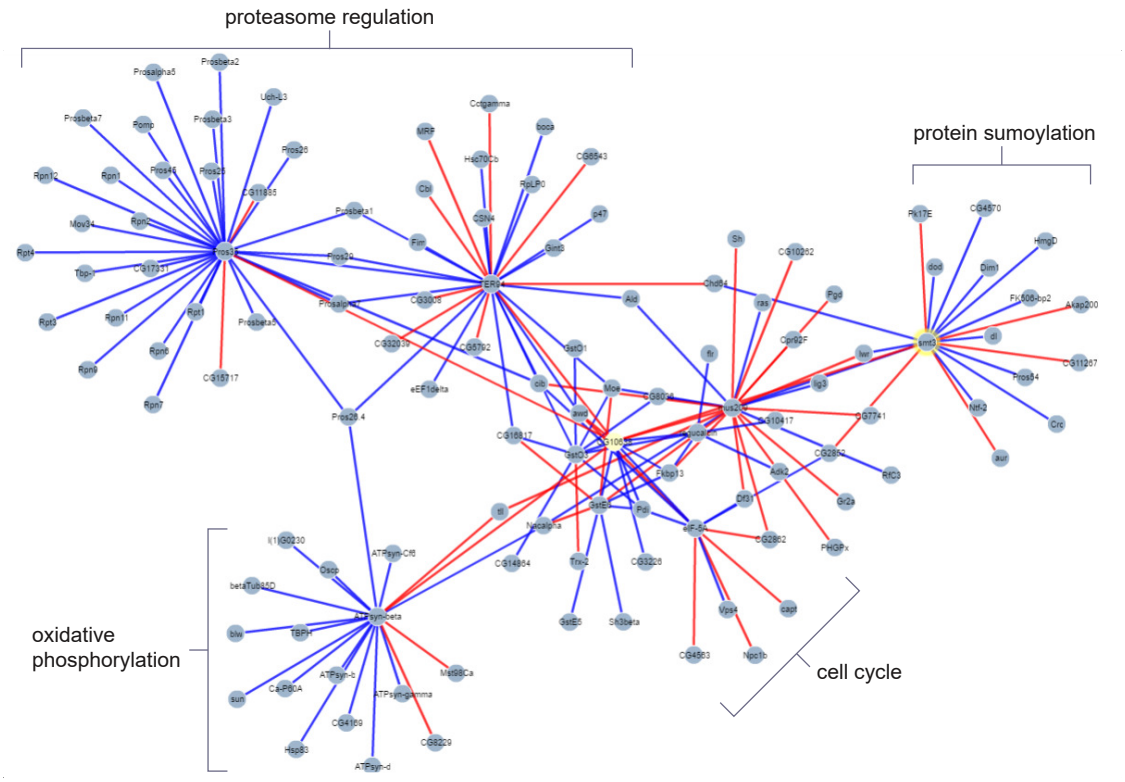


Figure 4.3: Annotated potential signed protein-protein interactions based on SignedPPI.

based on its potential interaction with the core proteasome protein Pros-35, protein sumoylation based on its potential interaction with the *Drosophila* SUMO protein Smt3, oxidative phosphorylation based on its potential interaction with the mitochondrial ATP synthase β, and cell cycle regulation based on its potential interaction with the eukaryotic translation inhibition factor eIF5A (Fig. 4.3).

Furthermore, predictions from cross-species similarities suggest an involvement of CG10638 in the sorbitol pathway through the interaction with the Sorbitol dehydrogenases Sodh-1 and Sodh-2 (Fig. 4.4). CG10638 may also interact with the Leucine-rich repeat kinase (Lrrk). A particularly high association score of 0.92, however, is associated with the potential interaction of CG10638 with fabp (fatty acid binding protein) inferred from the interaction of human AKR1B1 with FABP5/7 derived from the BioGRID interaction database (Fig. 4.4).

Fabp expression oscillates in a circadian manner in the rodent brain (Gerstner et al. 2006; Gerstner et al. 2008) and in *Drosophila melanogaster* (Gerstner et al. 2011). In *Drosophila*, it is a dendritic protein that benefits dendritic morphology

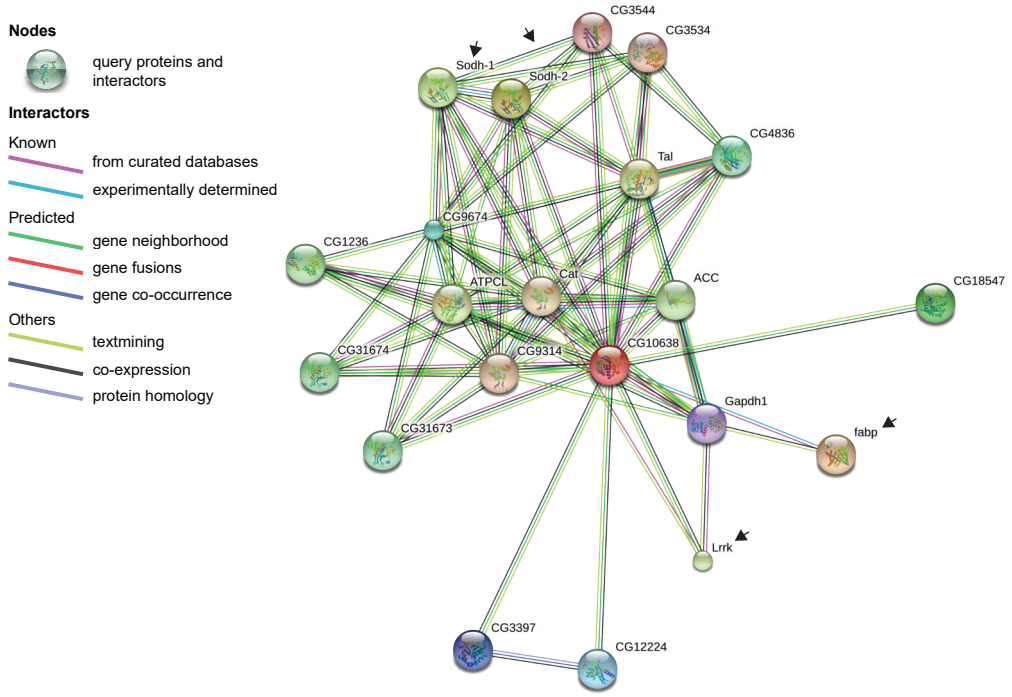


Figure 4.4: Protein-protein interactions predicted by STRING. Potential interaction partners discussed in the text are marked with an arrow.

(Misra et al. 2016) and its constitutive overexpression reduces sleep levels (Gerstner et al. 2011). Acute overexpression of *fabp*, however, increases day sleep due to an enhanced consolidation of sleep bouts and benefits long-term memory formation (LTM, Gerstner et al. 2011). Acute overexpression of *fabp* has been successfully used to improve memory performance in known *Drosophila* memory mutants supposedly due to increased sleep levels (Dissel et al. 2015). Intriguingly, *fabp* levels may be regulated by ubiquitination (Ramirez et al. 2015).

4.3 Robustness and scope of sleep phenotype

4.3.1 Nervous system-wide RNAi-mediated knock down

In a second important step, we aimed to ascertain the robustness of the phenotype through different routes: first, we tested the effects of RNAi-mediated nervous system-wide reduction of CG10638 on sleep in combination with *Dicer* over-expression. Secondly, we tested whether CG10638 hypomorphs exhibit detectable sleep suppression. For both approaches, we confirmed the efficacy of CG10638 reduction using qRT-PCR.

Confirmation of lines

Sequence specificity Both IR lines targeting CG10638 are expected to be transcript-specific: both exhibit a perfect match with only one isoform, and exhibit no comparable match with other genes (Table 4.2).

qRT-PCR In order to experimentally test the specificity and efficacy of RNAi transgenes, we measured mRNA levels of both transcripts in ELAV>IR# 1 and ELAV>IR# 2 flies, as well as parental controls. IR expression in the whole nervous system reduces expression of CG10638: with either RNAi transgene, isoA is equally reduced. Conversely, IR# 2 reduces expression of isoB more severely than IR# 1 (Fig. 4.5).

In respect to knock down efficiency, isoA is reduced by expression of both IR lines by around 45 % (Fig. 4.5, $F(2, 12)=793.5$, $p<0.001$, *post hoc* as annotated). IsoB is also reduced through the expression of both IR lines, but at different levels of efficiency: IR# 1 reduces isoB by around 20 %, while IR# 2 reduces isoB by around 40 % (Fig. 4.5, $F(2, 11)=566.3$, $p<0.001$, *post hoc* as annotated).

In respect to knock down specificity, IR# 1 reduces both transcripts equally (IR# 1: isoA:isoB, $p=0.292$), while IR# 2 exhibits higher specificity for isoB (IR# 2: isoA:isoB, $p<0.001$).

hpRNA	mis=0	mis=1	mis=2	target
IR# 1	318	0	0	CG10638-A
	0	3	3	CG17492
	0	2	11	CG12766
	0	0	4	CG10638-B
IR# 2	287	0	0	CG10638-B
	0	2	5	CG4320
	0	2	3	CG14861
	0	0	4	CG10638-A

Table 4.2: On- and off-target search for CG10638-IRs using dsCheck, "mis=0" number of complete matches, "mis=1" number of matches with one mismatch, "mis=2" number of matches with two mismatches. Only the first three hits and the other respective isoform are listed.

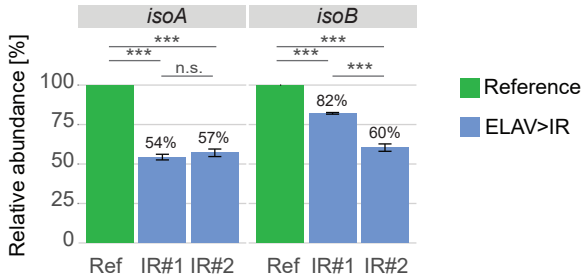


Figure 4.5: Efficiency of RNAi-mediated knock down in the nervous system of young male flies in respect to expression levels of CG10638. ELAV-IR flies were compared with parental control flies (ELAV/+) raised under the same conditions. The average expression level of CG10638 relative to reference genes in parental control flies served as reference group, n=3/group, and expression levels of targets relative to the reference group are annotated above the respective bar in percent. Bar height reflects mean, error bars reflect sem. Results of ANOVA were annotated as follows: "*" reflects $p<0.05$, "**" reflects $p<0.01$, "***" reflects $p<0.001$, , "n.s." signifies "not significant".

Sleep phenotype

Nervous system-wide RNAi-mediated knock down (ELAV>IR) As introduced in the previous chapter 3, expression of CG10638-IR in the nervous system suppresses sleep (Fig.3.2D). As this chapter is specifically concerned with the candidate CG10638, the initial phenotype detected using the ELAV driver will be examined in more detail here.

Expression of both CG10638-IRs reduces sleep levels (Fig. 4.6C, $\chi^2(4)=112.91$, $p<0.001$; *post hoc* as annotated). As described in chapter 3, the expression of

CG10638-IR in the nervous system achieves a reduction of sleep levels by 3.5-4.3 h compared to parental controls.

The sleep profiles produced by expression of CG10638-IRs in the nervous system are depicted in Fig. 4.6A and B. Expression of IR# 1 in the nervous system suppresses sleep particularly in the night (Fig. 4.6A). Expression of IR# 2 also suppresses sleep (Fig. 4.6B). The differential timing of RNAi-mediated effects between the two IR lines leads to a similar reduction of night sleep (Fig. 4.6G, $\chi^2(4)$=112.8, p<0.001; *post hoc* ELAV>IR# 1:ELAV:IR# 2, p=0.919, otherwise as annotated). However, the IR lines produce significantly different effects on day sleep: the nervous system-wide expression of IR# 2 suppresses day-specific sleep below the level of the parental control ELAV/+ and below the level of the other IR line, ELAV>IR# 1 (Fig. 4.6E). However, day-specific sleep in ELAV>IR#1 flies is not significantly different from the other parental control, IR# 2/+ as these flies also exhibit a low level of day sleep (Fig. 4.6E, $\chi^2(4)$=78.1, p<0.001; *post hoc* ELAV>IR# 1:ELAV:IR# 2, p<0.01, otherwise as annotated).

Regarding the sleep architecture, we observed that the expression of both CG10638-IRs reduces the average duration of sleep episodes (Fig. 3.2F). Specifically, the expression of IR# 1 in the nervous system – which suppresses night sleep – also decreases the duration of night sleep bouts, while the expression of IR# 2 does not (Fig. 4.6H, $\chi^2(4)$=95.5, p<0.001; *post hoc* as annotated). Conversely, the expression of IR# 2 in the nervous system – which suppresses day sleep – decreases the duration of day sleep bouts, while the expression of IR# 1 does not (Fig. 4.6F, $\chi^2(4)$=35.5, p<0.001; *post hoc* as annotated)

In order to evaluate whether a reduction of CG10638 reduces sleep levels or merely induces hyperactivity, we analysed daily activity levels in RNAi flies and parental controls in terms of the average number of activity bouts per min. Expression of IR# 1 in the nervous system does produce an increase in activity levels, but expression of IR# 2 does not, suggesting that the sleep phenotype associated with CG10638 knock down is not due to hyperactivity (Fig. 4.6D, $\chi^2(4)$=107.56, p<0.001; *post hoc*

ELAV>IR# 1:ELAV/+, p<0.001, ELAV>IR# 1:IR# 1/+, p<0.01, ELAV>IR# 2:ELAV/+, p<0.001, ELAV>IR# 2:IR# 2/+, p=0.135).

Nervous system-wide RNAi-mediated knock down with *Dcr2* co-expression (ELAV>Dcr2;IR) We also tested whether another ELAV-Gal4 line and co-expression of *Dcr2* would suppress sleep.

Sleep profiles produced by expression of IR# 1 and IR# 2 in the nervous system together with *Dcr2* are depicted in Fig. 4.7A and B. Indeed, expression of both IRs suppresses sleep (Fig. 4.7C, $\chi^2(4)=27.05$, p<0.001, *post hoc* as annotated). Similarly, we assessed activity levels in RNAi flies and parental controls that co-expressed *Dcr2* and found that activity levels were not affected in these genotypes (Fig. 4.7D, $\chi^2(4)=13.44$, p<0.05; *post hoc* ELAV>Dcr2;IR# 1:ELAV>Dcr2, p=0.998, ELAV>Dcr2;IR# 1:IR# 1/+, p=0.894, ELAV>Dcr2;IR# 2:ELAV>Dcr2, p=0.101, ELAV>IR# 2:IR# 2/+, p=0.983).

While this set of experiments generally corroborated the sleep phenotype we initially observed, co-expression of *Dcr2* did affect the time window in which the RNAi-mediated phenotype was particularly pronounced: our initial experiments showed that particularly expression of IR# 2 suppressed both day and night sleep, and the reduction of night sleep was relatively stable throughout the night (compare sleep profile in Fig. 4.6B). Co-expression of *Dcr2*, however, shifted the entire sleep reduction into the night and produced a pronounced through in the middle of the night (compare sleep profile in Fig. 4.7B). These findings do not allow specific conclusions about the potential involvement of Dcr2 in the production of this phenotype. It is of note, however, that co-expression of Dcr2 can affect the RNAi machinery and lead to unspecific or even toxic effects. Additionally, Dcr2 has a range of complex, RNAi-unrelated functions which may be perturbed by over-expression of this protein. Such technical caveats of the use of Dcr in RNAi studies are discussed in more detain=l later (see section 6.5). In order to avoid the sleep phenotype associated with CG10638 to be obscured by interference of Dcr2, we conducted remaining experiments without the co-expression of *Dcr2*.

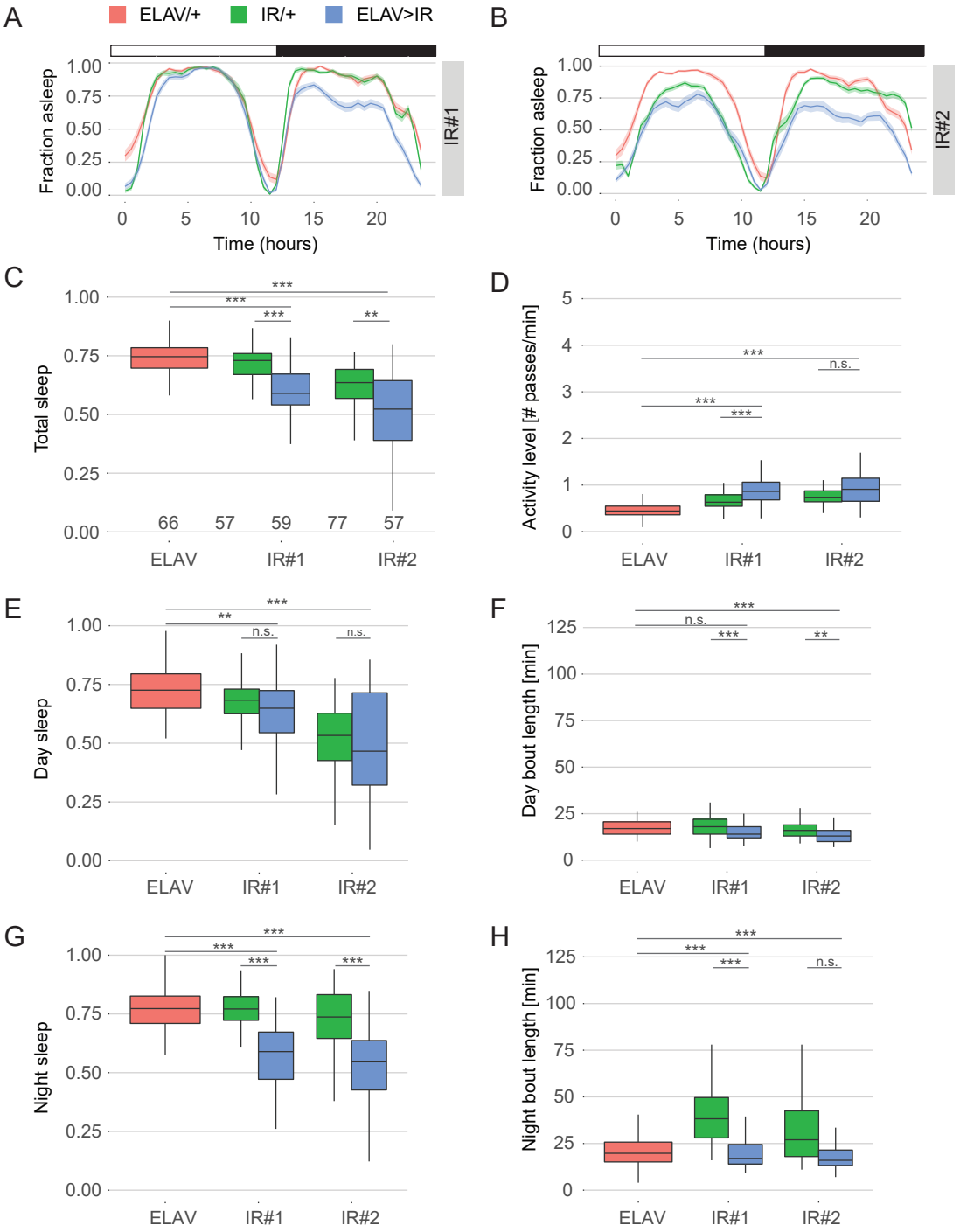


Figure 4.6: Sleep upon knock down of CG10638 in the nervous system (ELAV>IR). A, Sleep profile of ELAV>IR# 1 and parental controls, wrap of three days of baseline recording. B, Sleep profile of ELAV>IR# 2 as in A. C, Daily sleep levels. D, Activity levels. E, Sleep levels during the day. F, Sleep bout length during the day. G, Sleep levels during the night. H, Sleep bout length during the night. A-H, Sample size as annotated in C. C-H, Boxes reflect 25th (top) and 75th (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. C-H, Kruskal Wallis test to compare sleep/activity parameters between experimental group (ELAV-IR) and parental controls (ELAV/+, IR/+); "*" reflects $p<0.05$, "**" reflects $p<0.01$, "***" reflects $p<0.001$, "n.s." signifies "not significant".

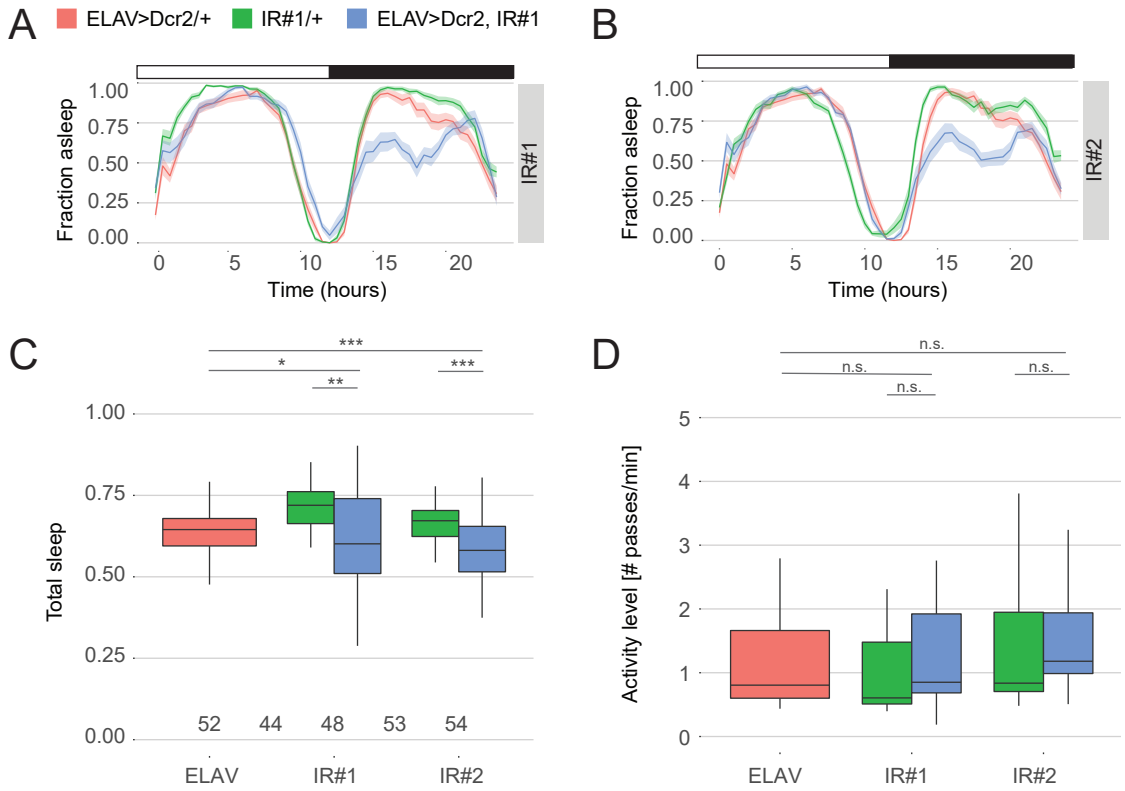


Figure 4.7: Sleep upon knock down of CG10638 in the nervous system (ELAV>Dcr;IR). A, Sleep profile of ELAV>Dcr;IR# 1 and parental controls, wrap of three days of baseline recording. B, Sleep profile of ELAV>Dcr;IR# 2 as in A. C, Daily sleep levels. D, Activity levels. A-D, Sample size as annotated in C. C-D, Boxes reflect 25^{th} (top) and 75^{th} (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. C-D, Kruskal Wallis test to compare sleep/activity parameters between experimental group (ELAV-IR) and parental controls (ELAV/+, IR/+); "*" reflects $p<0.05$, "**" reflects $p<0.01$, "***" reflects $p<0.001$, "n.s." signifies "not significant".

Circadian rhythm

In light of our *Dcr2* co-expression experiments, where we observed a shift of the sleep time window amenable to RNAi-mediated reduction of CG10638 levels, we tested whether the sleep suppression we observed was merely due to the difficulty of the organism to *schedule* sleep, i.e., an impairment in sleep *timing*, which would indicate that CG10638 modulates sleep mainly through the circadian sleep drive. Thus, we tested whether knock down of CG10638 in the nervous system affects the circadian clock by subjecting RNAi flies and parental controls to free-running conditions.

Representative actograms of ELAV>CG10638-IR flies and parental controls are

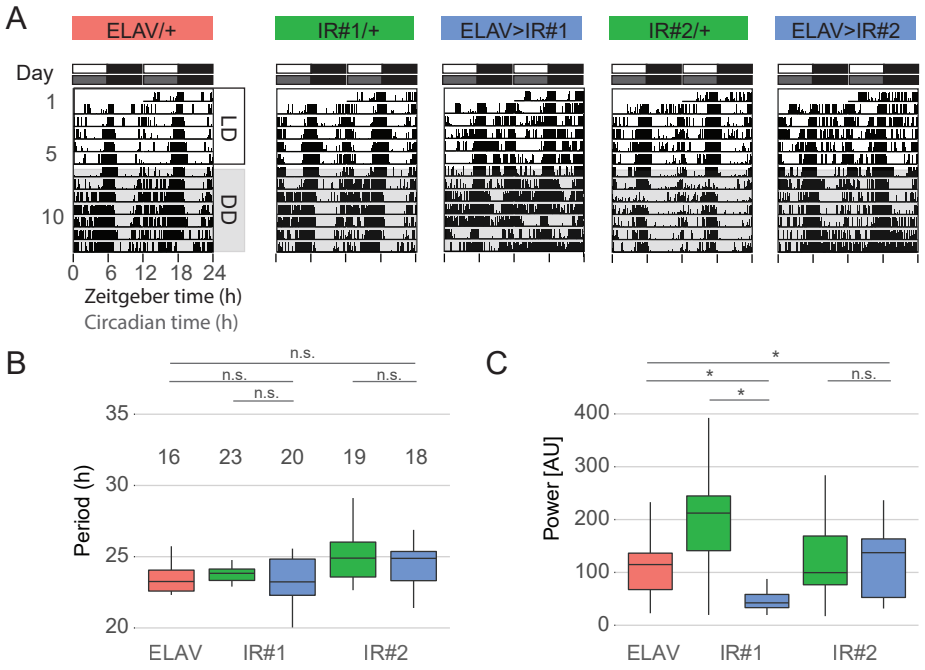


Figure 4.8: Circadian phenotype upon knock down of CG10638 in the nervous system. A, Free running period. B, Rhythm power. C, Representative actograms of experimental genotypes and parental controls. B-C, Sample size as annotated in B. Boxes reflect 25th (top) and 75th (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. B-C, Kruskal Wallis test to compare circadian parameters between experimental group (ELAV-IR) and parental controls (ELAV/+, IR/+); "*" reflects p<0.05, "n.s." signifies "not significant".

shown in Fig. 4.8A and the rhythm analysis of RNAi flies and parental controls is given in Table 4.3.

The length of the free-running period is unaltered in flies expressing CG10638-IRs in the nervous system: the period length of ELAV>IR# 1 flies is 23.3 h, on par with parental controls which exhibit a period length between 23.8 h and 23.3 h (Table 4.3, Fig. 4.8B), although the large variation in period length in ELAV>IR# 1 flies could indicate a destabilisation of the circadian regulation (Table 4.3). The period length of ELAV>IR# 2 flies is 24.9 h, which is also similar to those of parental controls of between 24.9 h and 23.8 h (Table 4.3, Fig. 4.8B, $\chi^2(4)=11.24$, p<0.05; *post hoc* ELAV>Dcr2;IR# 1:ELAV>Dcr2, p=0.474, ELAV>Dcr2;IR# 1:IR# 1/+, p=0.105, ELAV>Dcr2;IR# 2:ELAV>Dcr2, p=0.723, ELAV>Dcr2;IR# 2:IR# 2/+, p=0.76).

However, while rhythm power is unaffected by expression of IR# 2, it is mildly weakened by expression of IR# 1 (Fig. 4.8C, $\chi^2(4)=34.35$, p<0.001; *post hoc*

Genotype	N	Period Mdn	Range	Power Mdn	Range	SR	WR	AR
ELAV/+	16	23.26	22.32-25.73	114.86	22.74-232.97	63	38	0
IR# 1/+	23	23.83	22.90-30.35	212.27	19.52-392.42	87	13	0
ELAV>IR# 1	20	23.33	16.75-30.82	43.05	19.41-264.91	15	80	5
IR# 2/+	19	24.90	17.17-29.12	99.59	17.26-283.94	47	53	0
ELAV>IR# 2	18	24.92	21.40-26.88	139.56	31.69-452.28	56	44	0

Table 4.3: Circadian rhythm upon RNAi-mediated knock down of CG10638 in the nervous system (ELAV>IR). Categories of rhythm strength are as follows: strongly rhythmic ("SR"), weakly rhythmic ("WR"), and arrhythmic ("AR").

ELAV>Dcr2;IR# 1:ELAV>Dcr2, $p<0.05$, ELAV>Dcr2;IR# 1:IR# 1/+, $p<0.05$, ELAV>Dcr2;IR# 2:ELAV>Dcr2, $p<0.05$, ELAV>Dcr2;IR# 2:IR# 2/+, $p=0.979$). This mild weakening of rhythmicity due to the expression of IR# 1 in the nervous system leads to an increased incidence of weakly rhythmic flies ("WR") in this genotype compared to parental controls (Table 4.3).

4.3.2 Constitutive hypomorphs for CG10638

In order to evaluate the robustness of the sleep phenotype associated with the CG10638 locus in an RNAi-independent manner, we tested an insertion line that disrupts this locus (Fig. 4.9A).

Confirmation of mutant lines

Before testing the mutant line for altered sleep behaviour, we validated the reduction of CG10638 expression due to the disruption of the locus by the EY21723 insertion.

Generally, the insertion EY21723 considerably decreases the expression levels of both isoforms by around 80 % for isoA and by around 45 % for isoB in the homozygous mutant (Fig. 4.9B). Interestingly, when the mutation was kept heterozygous, expression of isoA was moderately reduced by around 25 %, while expression of isoB was upregulated by around 50 % (Fig. 4.9B, isoA: $F(2, 6)=450.6$, $p<0.001$, *post hoc* $CG10638^{EY21723/+}$:WT, $p<0.001$, $CG10638^{EY21723}$:WT, $p<0.001$; isoB: $F(2, 6)=57.92$, $p<0.001$, *post hoc* $CG10638^{EY21723/+}$:WT, $p<0.01$, $CG10638^{EY21723}$:WT, $p<0.01$).

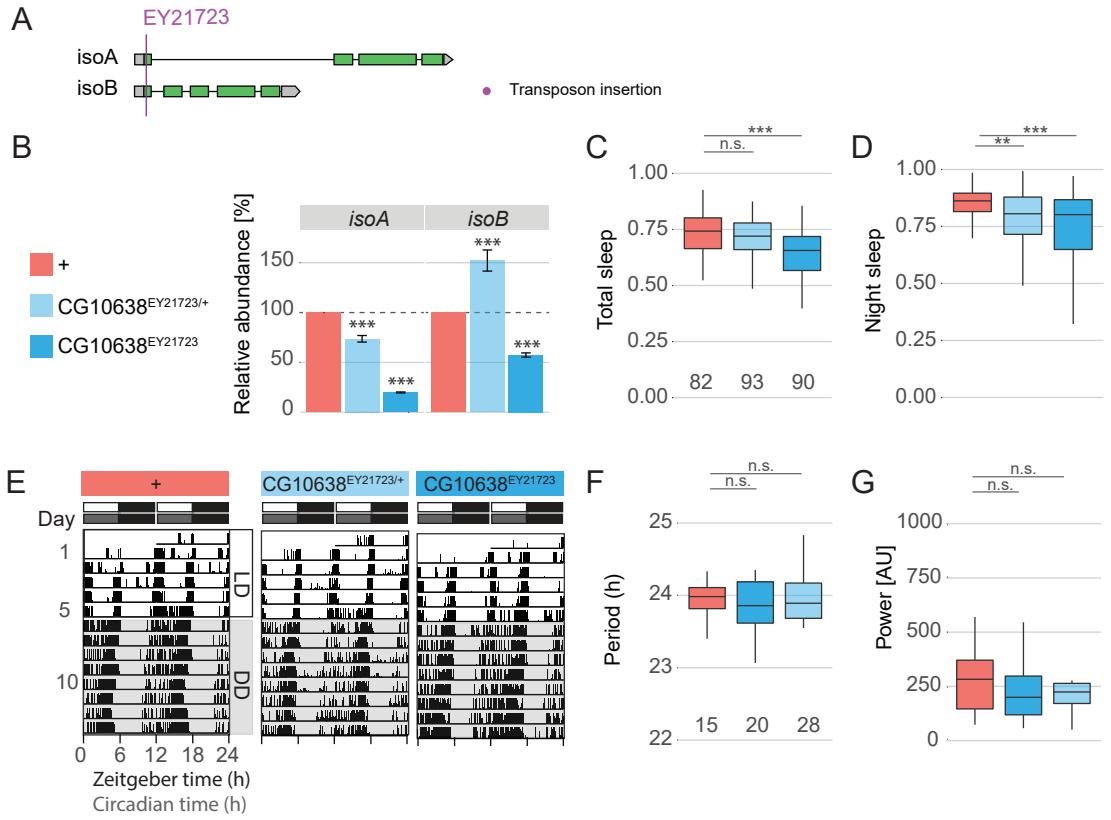


Figure 4.9: Hypomorph CG10638^{EY21723}. A, Schema of the locus including annotation of the CG10638^{EY21723} allele. B, Relative abundance of CG10638 expression in heads of young male flies, "w^{1118} serves as genetic background control (n=3/genotype). Bar height reflects mean, error bars reflect sem. Results of ANOVA indicated above respective bar. C, Daily sleep levels of young male CG10638^{EY21723} flies and genetic background control, sample size as annotated. D, Sleep levels during the night, sample size as in C. E, Representative actograms of individual CG10638^{EY21723} flies throughout the course of the experiment, respective genotypes indicated above the actogram. F, Free-running period length, sample size as annotated. G, Rhythm power, sample size as in F. C-G, Colorcode of genotypes as in B. C-D, F-G, Boxes reflect 25th (top) and 75th (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. C-D, F-G, Kruskal Wallis test to compare sleep/circadian parameters between mutant flies and wildtype controls. Throughout, "*" reflects p<0.05, "**" reflects p<0.01, "***" reflects p<0.001, "n.s." signifies "not significant".

Genotype	N	Period (h)		Power		R (%)	
		Mdn	Range	Mdn	Range	SR	WR
+	15	23.91	23.40-25.19	282.76	55.81-569.73	87	13
EY21723/+	20	23.71	21.55-25.37	197.26	46.91-607.35	95	5
EY21723	28	23.86	23.07-27.45	190.08	57.33-545.47	89	11

Table 4.4: Circadian rhythm of CG10638^{EY21723} mutant. The "arrhythmic" column ("AR") was omitted as no arrythmic animals were detected.

Sleep behaviour

The hypomorph mutant line CG10638^{EY21723} exhibits mildly reduced sleep levels: homozygous mutants sleep around 2.9 h – or 12 % – less than WT controls, and heterozygous mutants sleep around 3.8 h – or 16 % – less (Fig. 4.9C, $\chi^2(2)$=25.54, p<0.001, *post hoc* CG10638$^{EY21723/+}$:WT, p=0.212, CG10638^{EY21723}:WT, p<0.001). Sleep suppression in CG10638^{EY21723} mutants is particularly apparent during the night (Fig. 4.9D, $\chi^2(2)$=18.63, p<0.001, *post hoc* as annotated).

Circadian rhythm

In order to discern whether the sleep disruption we detected in CG10638^{EY21723} mutants was mainly due to a destabilisation of the circadian sleep drive, we subjected mutant flies to free-running conditions and assessed their rest:activity rhythms.

In CG10638^{EY21723} mutants, the circadian rhythm is not affected (Fig. 4.9E). The free-running period length of 23.7 h in the heterozygous mutant, and of 23.9 h is on par with the period length of WT controls of 23.9 h (Table 4.4, Fig. 4.9F, $\chi^2(2)$=0.9959, p=0.61). Rhythm power is also normal in mutants compared to WT controls, both in the heterozygous and homozygous combination (Table 4.4, Fig. 4.9G, $\chi^2(2)$=1.58, p=0.45).

4.4 Associated phenotypes

The only *Drosophila melanogaster* AKR that has so far been been investigated in detail is Hk (see section 1.6.5). Before Hk received dedicated study due to its involvement in sleep, it had already been investigated for two other phenotypes: hypersensitivity to the oxidative stress-inducing agent paraquat, and leg shaking under ether anaesthesia (see section 1.6.5).

A shaking leg phenotype has long been associated with mutations of the Shaker channel which Hk regulates (see section 1.5.3, Kaplan et al. 1969) and the association of both both loci with this phenotype points to a shared mechanism: in brief, ether exposure leads to an activation of motoneurons of the thoracic ganglion which manifests in rhythmic bursts of neural activity in hk mutants that, presumably, cannot be down-regulated in compensation in the absence of Shaker channel inactivation usually provided by Hk (Ikeda et al. 1970a).

Secondly, the *hk* locus has also been associated with a hypersensitivity to exposure with the oxidative stress-inducing agent paraquat Wang et al. 2000. In brief, exposure to paraquat induces the production of superoxide radicals through oxidation of NADPH, leading to both the built-up of ROS and a depletion of NADPH levels (**Smith1985**). Depletion of NADPH, in turn, thwarts the inactivation of the excitatory Shaker channel by Hk, also leading to neural hyperexcitability and, presumably, neurodegeneration and early death (see section 1.6, Fig. 1.11, and also the discussion in Wang et al. 2000).

Thus, to test whether CG10638 was also involved in the regulation of neural excitability, for instance through a Shaker-related mechanism, we tested both flies with an RNAi-mediated knock down of CG10638 levels in the nervous system and $CG10638^{EY21723}$ mutant flies for both a hypersensitivity to paraquat and an ether-induced shaking leg phenotype.

4.4.1 Sensitivity to oxidative stress

In order to test whether oxidative stress susceptibility is associated with the CG10638 locus in the manner that is described for *hk*, we treated both ELAV>CG10638-IR

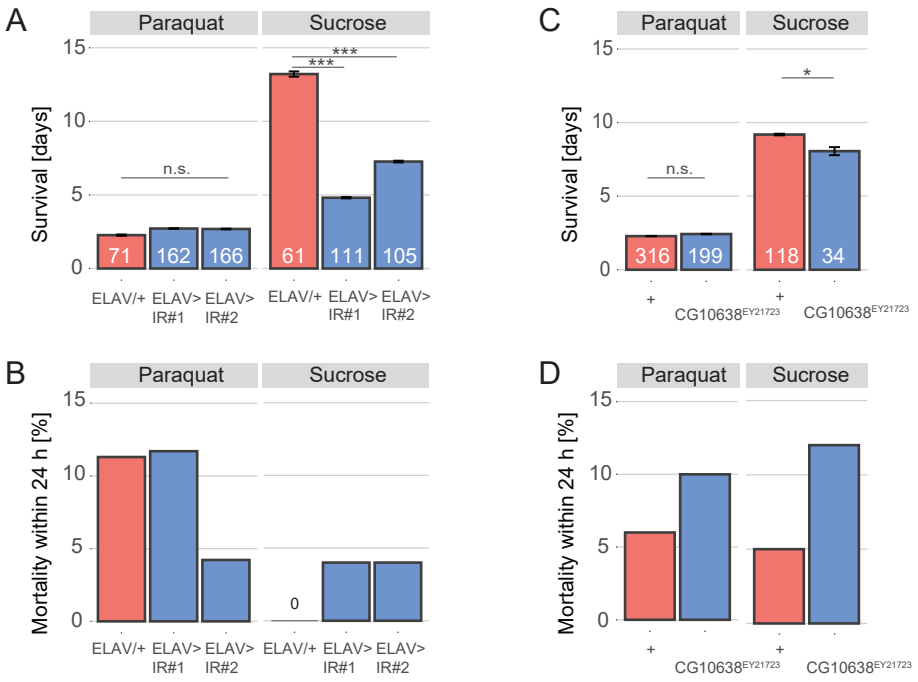


Figure 4.10: Sensitivity to chronic paraquat (PQ) treatment of young (1-3 days old) RNAi and mutant flies. A, Survival of RNAi flies of chronic treatment with 20 mM PQ dissolved in 5 % sucrose or sucrose alone. Sample size as annotated. B, Mortality within 24 h of flies as in A. C, Same as in A for CG10638^{EY21723} mutants. Sample size as annotated. D, Same as in B for CG10638^{EY21723} mutants. A, C, Bar height represents mean, error bars represent sem; in some cases, error bars do not rise above bar. A, C, ANOVA, "*" reflects p<0.05, "**" reflects p<0.01, "***" reflects p<0.001, "n.s." signifies "not significant". B, D, Bar height reflects percentage of flies dead within 24 h.

and mutant flies from a young age onwards – flies were 1-3 days old at the start of the experiment – chronically with high concentrations of paraquat of 20 mM dissolved in 5 % sucrose preceded by a short period of starvation, and assessed survival rates until all flies had died. Control flies were subjected to sucrose alone.

Short-term mortality under chronic paraquat treatment is comparable between RNAi flies and parental controls: 12 % of flies expressing IR# 1 and 4 % of flies expressing IR# 2 in the nervous system died within 24 h compared to 11 % of WT control flies (Fig. 4.10B). Long-term mortality under paraquat treatment is also similar: RNAi flies died after around 2.7 days compared to 2.3 days in WT control flies (Fig. 4.10A). Curiously, the control treatment consisting of sucrose alone did reduce the average lifespan of RNAi flies of both transgene lines: ELAV>IR# 1 flies died after around 4.8 days, and ELAV>IR# 2 flies died

after around 7.3 days, while WT flies survived this treatment for around 13.2 days (Fig. 4.10A, treatment: $F(2,670)=511.7$, $p<0.001$, genotype: $F(1,670)=84.8$,$p<0.001$, treatment × genotype: $F(1,670)=114.7$, $p<0.01$, *post hoc* ELAV/+ S:PQ, $p<0.001$, ELAV>IR# 1 S:PQ, $p<0.001$, ELAV>IR# 2, $p<0.001$, ELAV/+:ELAV>IR# 1 S, $p<0.001$, ELAV/+:ELAV>IR# 2 S, $p<0.001$, ELAV/+:ELAV>IR# 1 PQ, $p=0.86$, ELAV/+:ELAV>IR# 2 PQ, $p=0.899$).

In CG10638^{EY21723} mutants, both their short-term mortality and long-term mortality during chronic paraquat treatment is comparable to WT: after 24 h, 10 % of mutant flies and 6 % of WT control flies have died (Fig. 4.10D). Under continuous treatment, mutant flies die after around 2.5 days compared to 2.3 days for WT (Fig. 4.10C). Similar to RNAi flies, sucrose treatment itself had discernible effects on lifespan: when treated with sucrose alone, 12 % of mutant flies died within 24 h compared to 5 % of WT control flies (Fig. 4.10D). Similarly, mutant flies chronically treated with sucrose alone died after around 8.1 days, while WT control flies died after around 9.2 days (Fig. 4.10C, treatment: $F(1,663)=1187.74$, $p<0.001$, genotype: $F(1,663)=0.24$,$p=0.624$, treatment × genotype: $F(1,663)=8.15$, $p<0.01$, *post hoc* WT S:PQ, $p<0.001$, CG10638^{EY21713} S:PQ, $p<0.001$, WT:CG10638^{EY21713} S, $p<0.05$, WT:CG10638^{EY21713} PQ, $p=0.873$).

4.4.2 Shaking

We tested both RNAi and mutant flies for leg shaking under ether anaesthesia using the hk^1 mutation as a positive reference together with ELAV>Hk-IR flies.

ELAV>CG10638-IR flies do shake more than the parental control flies of the ELAV-Gal4/+ genotype, but they shake less than the main positive control, Hk: the shaking score of RNAi flies is around 1.7, the score of the parental control line reaches 0.6, and the *hk* mutant hk^1 consistently received the maximum score of 5 (Fig. 4.11A). It is important to note, however, that expression of Hk-IR in the nervous system also produces only a low degree of shaking of 1.8 which is on par with ELAV>CG10638-IR flies (Fig. 4.11A, $\chi^2(4)=17.48$,

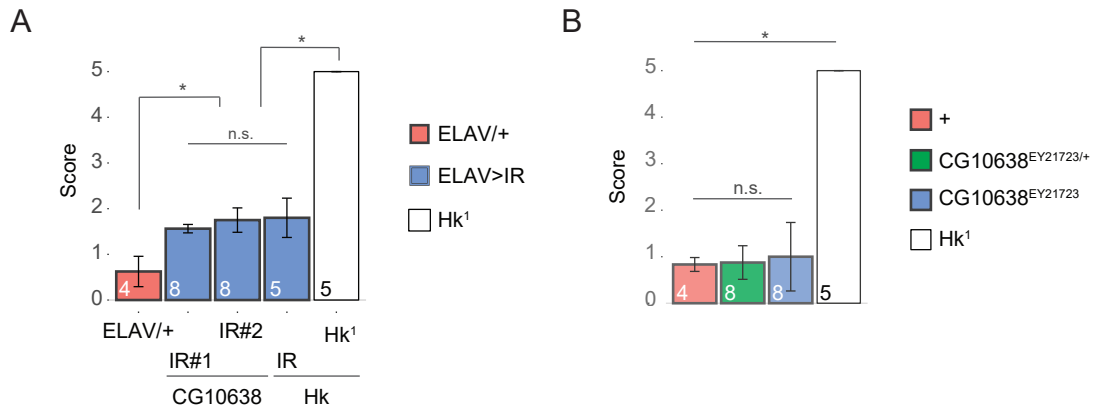


Figure 4.11: Shaking leg phenotype of ELAV>CG10638-IR flies and CG10638^{EY21723} mutants in response to acute exposure to ether. A, Magnitude of shaking under ether anaesthesia in RNAi flies, sample size as annotated. B, Same for CG10638^{EY21723} mutants, sample size as annotated. A, B, Bar height represents mean, error bars represent sem; in some cases. A, B, ANOVA, "*" reflects p<0.05, "n.s." signifies "not significant".

p<0.001, *posthoc* Hk^1:ELAV>CG10638-IR# 1, p<0.01, Hk^1:ELAV>CG10638-IR# 2, p<0.01, Hk^1:ELAV>Hk-IR# 1, p<0.05, ELAV/+:ELAV>CG10638-IR# 1, p<0.05, ELAV/+:ELAV>CG10638-IR# 2, p<0.05, ELAV/+:ELAV>Hk-IR# 1, p<0.05, ELAV>Hk-IR:ELAV>G10638-IR# 1, p=0.378, ELAV>Hk-IR:ELAV>G10638-IR# 2, p=0.463).

CG10638^{EY21713} mutants do not shake: the degree of shaking associated with CG10638 mutants of 1 is on par with that of the WT control w^{1118}/"+" of 0.8 (Fig. 4.11B), and the shaking score of CG10638^{EY21713} mutants is considerably lower than that of the positive control, hk^1, which consistently received the maximum score of 5 (Fig. 4.11B, $\chi^2(3)$=11.07, p<0.05, *posthoc* Hk^1:CG10638^{EY21713}, p<0.01, Hk^1:CG10638$^{EY21713/+}$, p<0.01, Hk^1:WT, p<0.001, CG10638^{EY21713}:CG10638$^{EY21713/+}$, p=0.47).

4.5 Histological identification of neural substrates

We identified CG10638+ neurons by help of dedicated antibodies. Since the antibody stained very specific peptidergic neurons, we were successful in selecting a restricted driver line, VT026326, that targets CG10638+ neurons, and employed it to confirm the behavioural phenotype via RNAi-mediated reduction of CG10638 in these neurons.

4.5.1 CG10638 insertion line MB06512

Expression levels of CG10638 isoforms are vastly different with isoA being expressed at much higher levels than isoB (Fig. 3.1C). The stark difference in expression makes it unlikely that they are expressed in the same neural population. Next, we aimed at ascertaining the neural substrates that express both or either isoform.

In a first step, we used one of the transposon insertion lines targeting the CG10638 locus, MB06512. This line was generated by Metaxakis et al. 2005 as part of a study into the possibility of employing the transposable Minos element from *Drosophila hydei* to generate random mutations throughout the *Drosophila melanogaster* genome. This insertion carries a GFP reporter (Fig. 4.12A) and inserted into exon IV which forms part of the isoA transcript (Fig. 4.12B). This GFP reporter sequence would be expected to be expressed in cells that express isoA if the insertion of the GFP coding sequence in the isoA locus actually gives rise to a fusion protein. To test whether such a fusion occurs, one can attempt to amplify a transcript predicted to cover both coding sequences of isoA and GFP through qRT-PCR. The successful amplification of such a product indicates the transcription of mRNA that could be employed by the cell to generate a GFP:isoA fusion protein. We did not, however, validate that the 3xP3-EGFP reporter sequence was actually fused to isoA upon transcription. Thus, we interpret the GFP signal we detected with caution and conducted further experiments to investigate the expression of CG10638 transcripts.

GFP is expressed in several cells of the fly brain (Fig. 4.12C, E): specifically, GFP is expressed in a subset of cells that stain positive for the glial cell marker

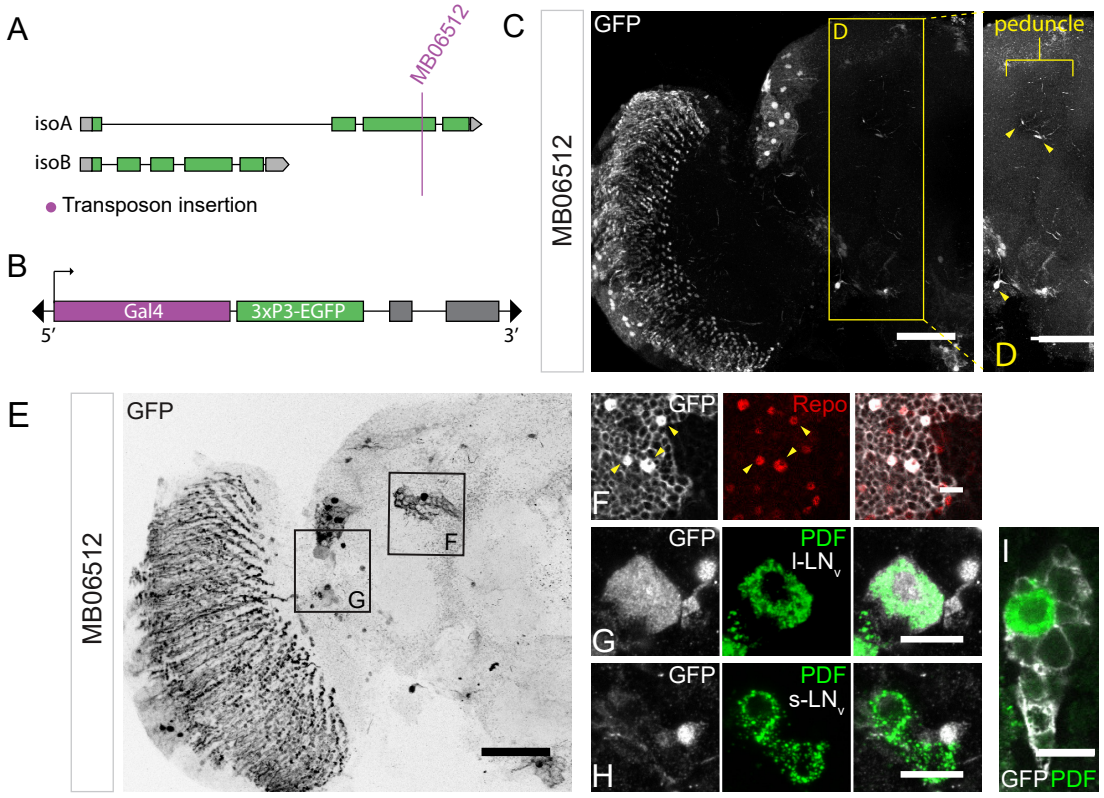


Figure 4.12: GFP-expression driven by insertion line MB06512. A, Insertion of MB06512 in the CG10638 locus, adapted from Metaxakis et al. 2005. B, Plasmid MB06512. C, Confocal projection of inner protocerebrum of whole-mount adult brain of CG10638^{EY21713} mutant. GFP signal (white) reflects cells that produce GFP from the transgene inserted into the CG10638 locus. D, Magnified section of GFP+ soma and projections as in C. E, Confocal projection of posterior protocerebrum of whole-mount adult brain of CG10638^{EY21713} mutant, genotype as in C, GFP signal in black to increase visibility. F, Magnified section of GFP+ cells stained with anti-repo. G, Magnified section of GFP+ cells stained with anti-PDF. H, Magnified section of GFP+ cells stained with anti-PDF. I, Magnified section of GFP+ cells 'wrapped' around a l-LN$_v$ soma. Scale bars: C, E, 50 µm, D, F-H, 10 µm.

Repo (Fig. 4.12F), and in PDF+ cells, namely the l-LN$_v$ (Fig. 4.12G), but not in the s-LN$_v$ (Fig. 4.12H). Some GFP+ glial cells form small networks and surround PDF+ neurons (Fig. 4.12I). Additional weak expression was found in the interior protocerebrum, namely a weakly stained projection around the peduncle and a ventrolateral cell body (Fig. 4.12C-D).

4.5.2 Expression of isoA in PDF+ neurons

In a next step, two peptides per transcript A and B were prepared in the lab to use as antigens to produce antibodies against either isoform; four antibodies were generated in total. One antibody (anti-isoB, # 3) will be discussed in detail in section 4.5.3 due to its specific staining pattern.

Representative confocal projections of stainings using the other antibodies are given in Fig. 4.13I-K with anti-nc82 (bruchpilot) used as a background staining to visualise the whole brain.

Representative confocal sections of adult flies of the PDF::RFP to indicate the location of the PDF+ neurons, l-LN$_v$ and s-LN$_v$, counter-stained with antibodies against the isoforms of CG10638. One of the antibodies against isoA (ab# 2) labels the large lateral neurons which express RFP under PDF control (PDF-Gal4>mCD2::RFP, Fig. 4.13, C). This staining indicates that isoA may be expressed in the l-LN$_v$. The other antibodies did not stain the l-LN$_v$ neurons above background (compare Fig. 4.13A, E, G with Fig. 4.13C). No antibody stained above background in the s-LN$_v$ (Fig. 4.13, B, D, F, H).

4.5.3 Expression of isoB in ICLI neurons

Expression of isoB in a pair of lateral neurons One of the antibodies against isoB (ab# 3) exclusively labels a pair of lateral neurons with extended projections into the dorsal protocerebrum and a midline projection where both neurons cross over to the other side of the brain (Fig. 4.14A). This arrangement is depicted in Fig. 4.14C. There is no discernible expression in the VNC (Fig. 4.14B). The somata of these lateral neurons are relatively large spanning around 12 µm in diameter (Fig. 4.14D), which is reminiscent of peptidergic neurons.

Lateral neurons are part of the MIP+ cluster Peptidergic neurons involved in sleep regulation – aside from clock neurons which are located mediolaterally – include, for instance, MIP+ neurons which were described in detail by Min et al. 2016. To test whether CG10638-isoB+ neurons are part of the MIP+ set, we

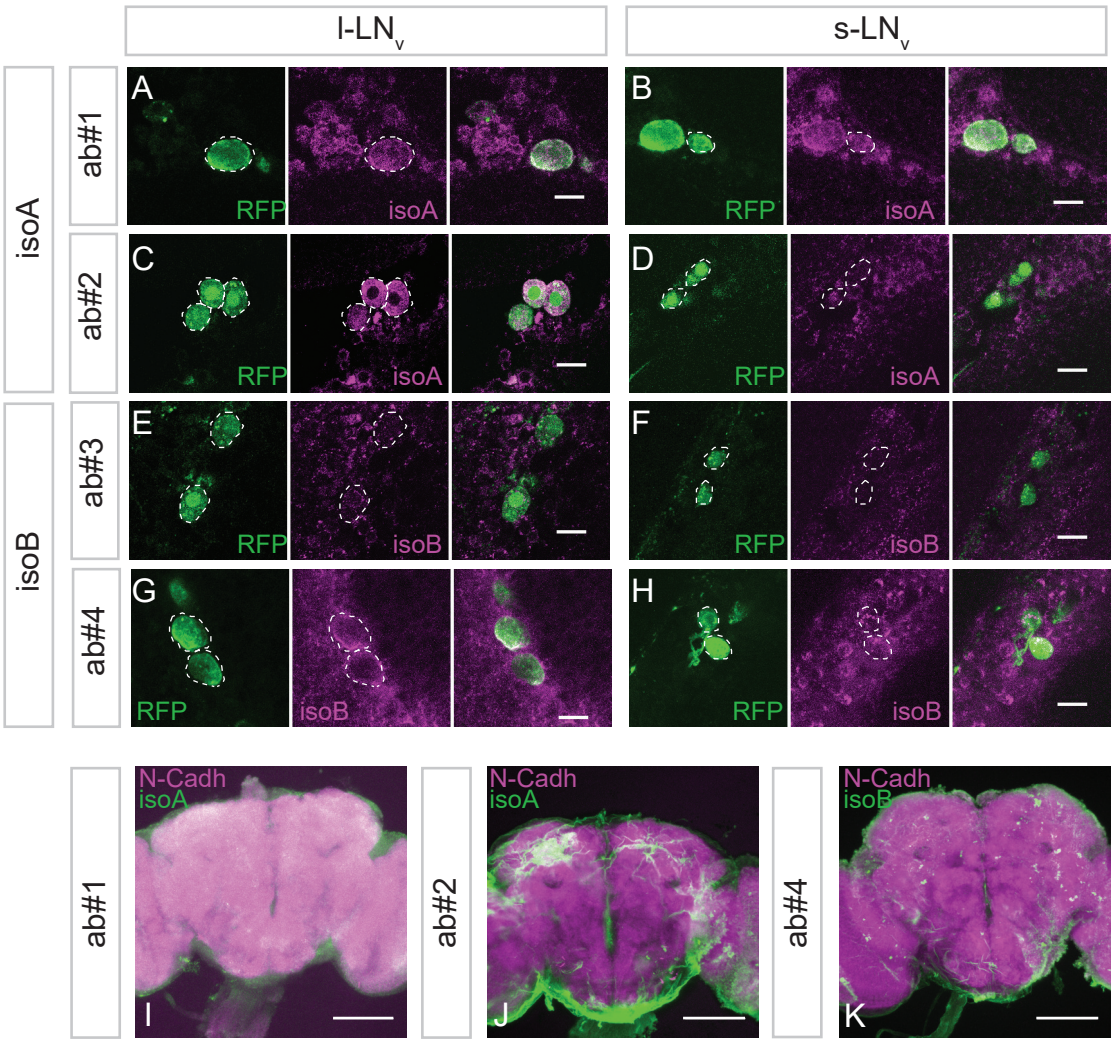


Figure 4.13: Expression of CG10638 in the lateral protocerebrum. A-H, Expression of CG10638 in adult PDF::RFP reporter flies detected by antibodies (ab) # 1-2 against isoA and ab# 3-4 against isoB in l-LN$_v$ (left) and s-LN$_v$ (right), scale bars: 10 µm. PDF::RFP signal was enhanced through staining with an anti-RFP antibody, CG10638 isoforms were stained with custom-made antibodies against isoA (ab# 1-2) and isoB (ab# 3-4) peptide sequences. I-K, Confocal projection of adult whole-mount brains stained with ab # 1-2, 4 (green, ab # 3 is shown in the detail in the following figure). Anti-nc82/bruchpilot (magenta) was used for background staining. Scale bars: 100 µm.

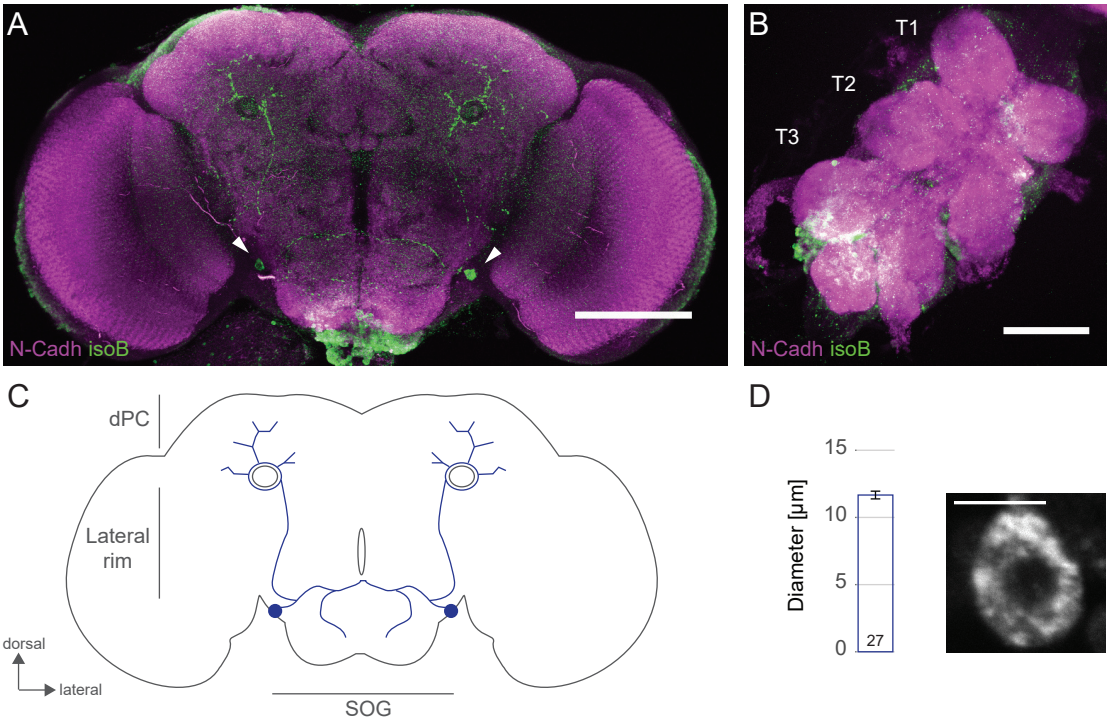


Figure 4.14: Expression of CG10638 (isoB) in the dPC. A, Confocal projection of adult whole-mount brain stained with ab # 3 against isoB (green) and anti-nc82/bruchpilot (magenta) for background staining. B, Same as A of VNC. C, Schema of isoB+ projections. D, Diameter of isoB+ somata (n=27 cells) accompanied by a representative confocal image. Bar height reflects mean, error bars reflect sem. Scale bars: A-B, 100 µm, D, 10 µm.

visualised MIP+ neurons by expressing an mCD8::GFP construct under the control of the MIP driver described in Min et al. 2016.

There are several MIP+ neurons in the brain that are captured by the MIP driver line and annotated in Fig. 4.15A. These include a pair of ventrolateral neurons termed "ICLI" by Min et al. 2016 ("inferior contralateral interneurons", Fig. 4.15A, inset). Anti-isoB stains this pair of ventrolateral neurons both in the somata (Fig. 4.15C) and varicosities (Fig. 4.15D), but does not stain, for instance, another set of ventromedial neurons (Fig. 4.15E, see Fig. 4.15B for an overview of this region).

The peptidergic ICLI neurons do not seem to co-express any of the common neurotransmitters we tested, i.e., acetylcholine, serotonin, dopamine, GABA, or glutamate (Supplementary Fig. 7.10).

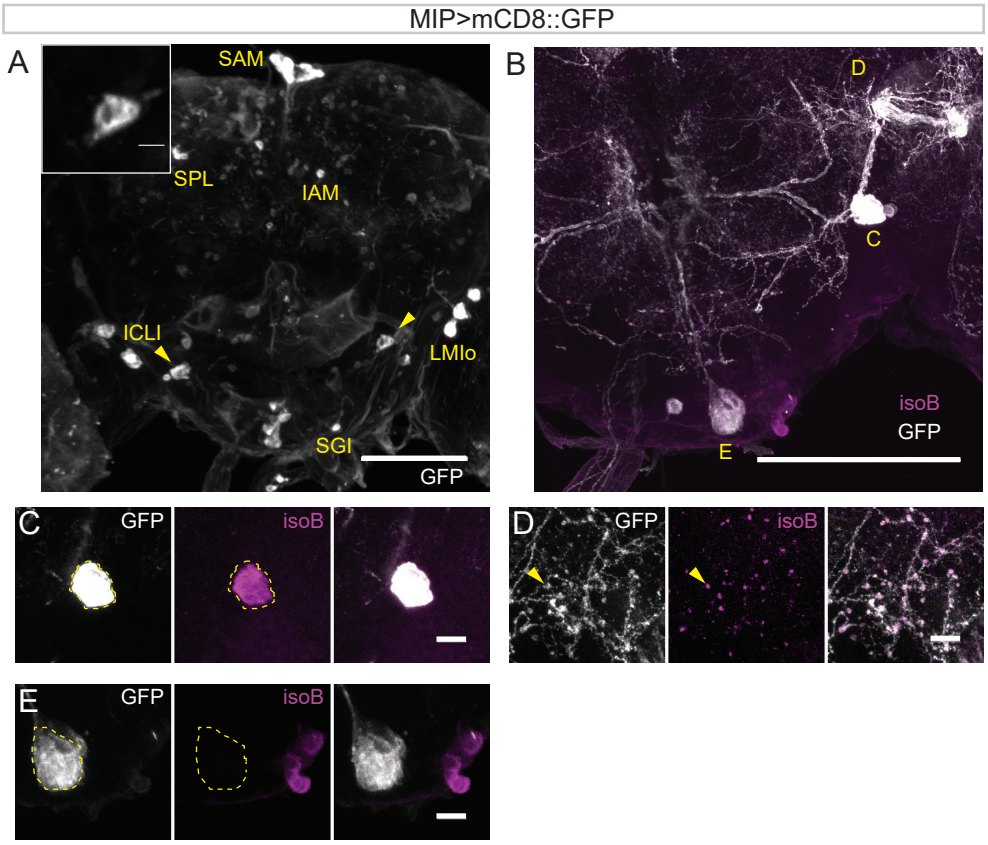


Figure 4.15: Expression of CG10638 in MIP+ neurons. A, Confocal projection of adult whole-mount brain of MIP>mCD::GFP reporter flies in the protocerebrum to visualise expression pattern of MIP-Gal4. GFP signal is shown in white. Inset shows a magnification of the soma of a ventrolateral neuron. B, Confocal projection of ventrolateral protocerebrum of MIP>mCD::GFP (white) counter-stained with anti-isoB (ab# 3, magenta). C, Magnified subsection of B, the isoB+ soma. D, Magnified subsection of B, isoB+ varicosities. E, Magnified subsection of B, an isoB- soma. Scale bars: A-B, 50 µm, C-E, 10 µm.

4.5.4 Driver line for isoB+ ICLI neurons

Confirmation of driver line

The driver line VT026326 has projections reminiscent of those of ICLI neurons. We visualised these projections by expressing mCD8::GFP under the control of VT026326 (Fig. 4.16A).

Counter-staining with anti-isoB in the soma ((Fig. 4.16D) and in central (Fig. 4.16E) and dorsal projections (Fig. 4.16F, see Fig. 4.16C for an overview of this region) confirms that this driver line indeed labels ICLI neurons; it also exhibits

very limited expression in the VNC (Fig. 4.16B).

Anti-isoB also labels varicosities of ICLI projections. To visualise specifically synapses, we drove expression of the syt::GFP construct with VT026326 and detected robust overlap of isoB and GFP (Fig. 4.16G), although isoB+ varicosities are also located outside albeit nearby GFP+ synaptic regions, indicating that CG10638 may be a perisynaptic protein.

Confirmation of antibody

Reliability In order to ascertain whether anti-isoB reliably correlates with expression levels of isoB, we induced RNAi-mediated reduction of CG10638 expression in ICLI neurons through expression of CG10638-IR# 2 under the control of the VT026326 driver. Indeed, the fluorescence intensity of the isoB staining is reduced by around 70 % in this genotype compared to parental controls (Fig. 4.17B, $F(2, 20) = 17.08$, $p<0.001$, *post hoc* VT026326>IR# 2:VT026326/+, $p<0.001$, VT026326>IR# 2:IR# 2/+, $p<0.001$, IR# 2:VT026326/+, $p=0.999$, see Fig. 4.17A for representative sections).

Specificity Anti-isoB stains a pair of lateral neurons (Fig. 4.14A, B) that were identified as MIP+ ICLI neurons (Fig. 4.15). Other antibodies raised against CG10638 do not stain ICLI neurons above background levels (Fig. 4.18, A-B and D, staining of anti-isoB, ab# 3, included in C for comparison).

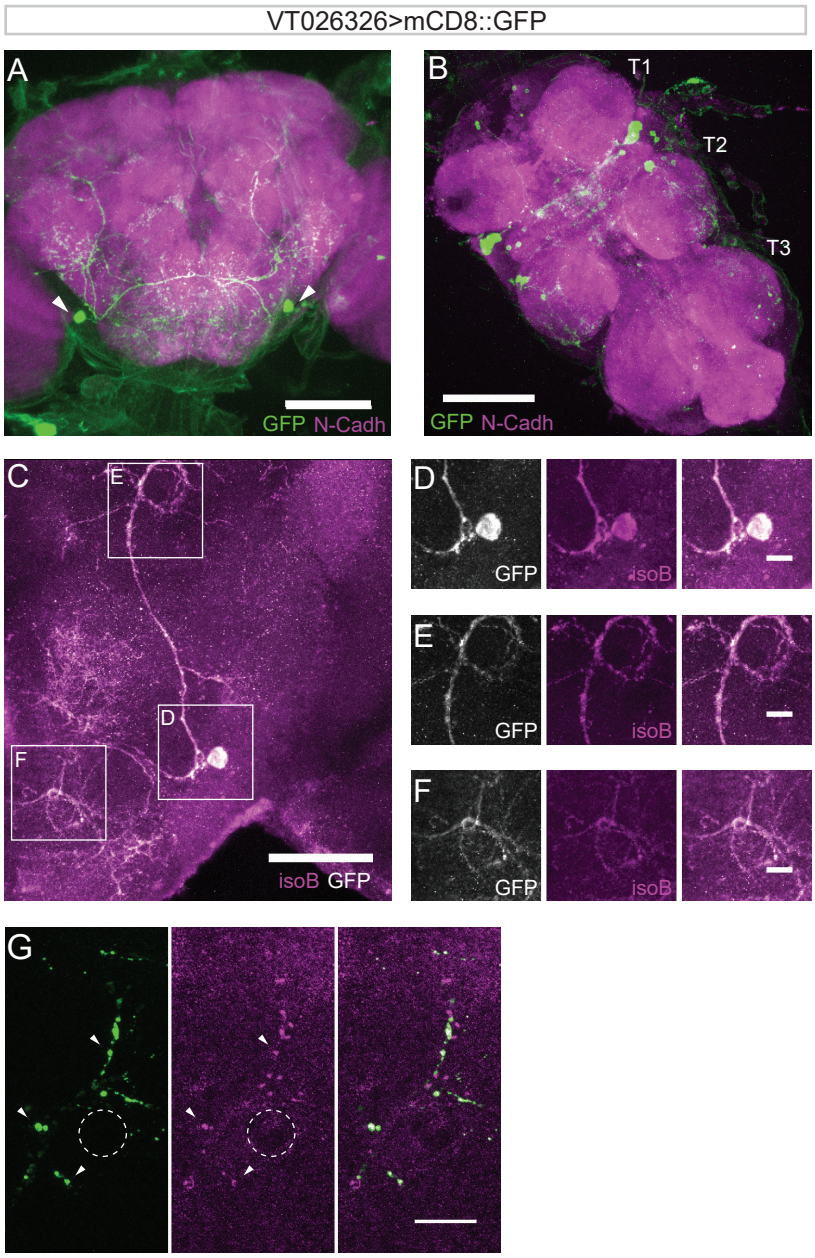


Figure 4.16: Driver line for CG10638-isoB+ neurons (VT026326-Gal4). A, Confocal projection of adult whole-mount brains of VT026326>mCD8::GFP reporter flies. GFP signal is shown in green and background staining of anti-nc82/bruchpilot is shown in magenta. B, Same as A for VNC. C, Confocal projection of ventrolateral protocerebrum of VT026326>mCD8::GFP reporter flies showing the location of ICLI neuron soma and projections (white) and a background staining of anti-nc82/bruchpilot (magenta). D, Magnified section of C, the isoB+ soma. E, Same as D, isoB+ dorsal projection. F, Same as D, isoB+ midline projection. G, Confocal projection of VT026326>syt:GFP reporter flies showing syt-driven GFP signal along isoB+ varicosities of the dorsal projection of ICLI neurons, dashed line indicates approximate location of MB peduncle. Scale bars: A-B, 100 µm, C, 50 µm, D-F, 10 µm, G, 20 µm.

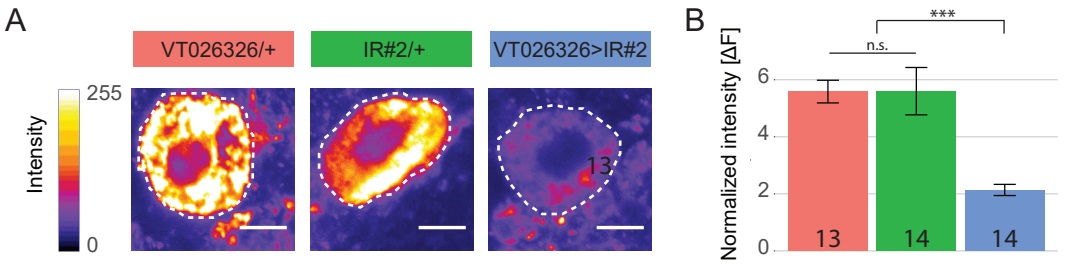


Figure 4.17: Reliability of isoB antibody staining in the dorsal protocerebrum. A, Representative sections through the soma, genotype indicated above image, scale bar 5 µm. B, Fluorescence intensity relative to background, sample size as annotated. Bar height reflects mean, error bars reflect sem. B, ANOVA; "***" reflects p<0.001, "n.s." signifies "not significant".

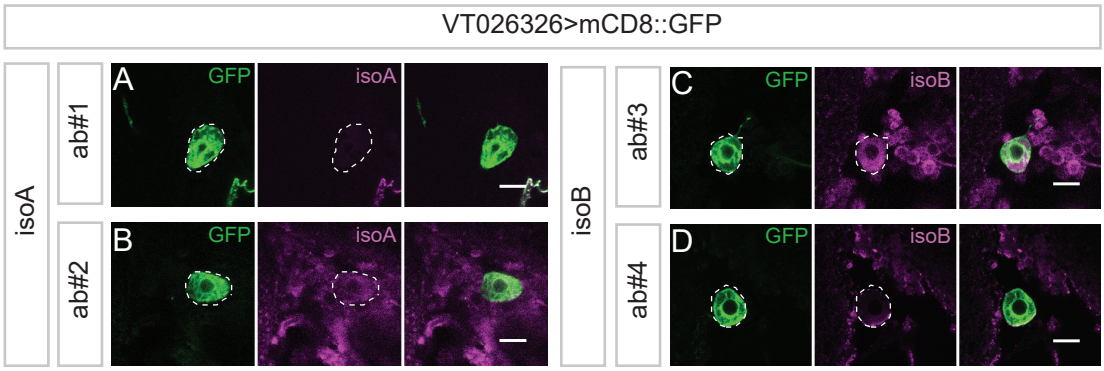


Figure 4.18: Specificity of isoB expression in the dorsal protocerebrum. A-D, Staining of CG10638 ab# 1-4 (magenta) in ICLI neurons which were labelled by VT026326>mCD8::GFP (green). Scale bars: A-D, 10 µm.

4.6 Behavioural confirmation of neural substrates

4.6.1 PDF+ neuron-specific RNAi-mediated knock down

RNAi-mediated knock down of CG10638

One route to test whether isoA is actually functional in PDF+ neurons in light of the sleep phenotype it produces when reduced in the whole nervous system is to modulate the expression levels of this gene in PDF+ neurons while recording flies' baseline locomotion levels.

To this end, we expressed CG10638-IRs under the control of the PDF+ neuron-specific driver PDF-Gal4. Indeed, expression of both IR# 1 (Fig. 4.19A) and IR# 2 (Fig. 4.19B) suppresses sleep. Interestingly, reduction of CG10638 levels in PDF+

neurons mainly affects night sleep and produces a biphasic nightly sleep profile which is particularly apparent when expressing IR# 2 (Fig. 4.19B) in PDF+ neurons, which is similar to the sleep profile produced when expressing Hk-IR in the nervous system using ELAV-Gal4 (see section 3.2.2). Specifically, expression of IR# 1 in PDF+ neurons reduces sleep levels by 2.4 h or around 10 % compared to parental controls, and expression of IR# 2 reduces sleep levels by 3.8 h or around 16 % compared to parental controls (Fig. 4.19C, $\chi^2(4)=85.80$, $p<0.001$; *post hoc* as annotated).

The sleep suppression is specific to the night portion of sleep (Fig. 4.6E, $\chi^2(4)=123.1$, $p<0.001$; *post hoc* as annotated). Interestingly, the sleep profile of both PDF>IR line shows a distinct 'dip' in sleep levels in the middle of the night around ZT18; this effect is particularly strong when IR# 2 is expressed under the control of PDF (Fig. 4.19A, B). There is no significant effect of CG10638-IR expression in PDF+ neurons on day sleep (Fig. 4.19D, $\chi^2(4)=22.1$, $p<0.001$; *post hoc* PDF>IR# 1:PDF/+, $p=0.849$, PDF>IR# 1:IR# 1/+, $p=0.107$, PDF>IR# 2:PDF/+, $p<0.01$, PDF>IR# 2:IR# 2/+, $p=0.089$).

Sleep timing is also affected: in particular, both day and night sleep is delayed. A delay in siesta sleep is particularly apparent: expression of IR# 1 in PDF+ neurons delays siesta sleep by 24.5 min, and expression of IR# 2 delays siesta sleep by 45 min compared to control flies (Fig. 4.19F, $\chi^2(4)=76.38$, $p<0.001$; *post hoc* as annotated). The sleep delay is also present for night sleep: expression of IR# 1 in PDF+ neurons delays night sleep by 23.5 min, and expression of IR# 2 delays night sleep by 36.5 min compared to parental controls (Fig. 4.19E, $\chi^2(4)=153.51$, $p<0.001$; *post hoc* as annotated).

RNAi-mediated knock down of Hk

The *Drosophila melanogaster* AKR Hk acts through the l-LN$_v$ to modulate neural excitability according to blue light stimulation of the blue light photoreceptor CRY (see section 1.6.5). In order to compare the potential role of CG10638 (isoA) in l-LN$_v$ with the activity of Hk, we also reduced Hk expression through the expression of an Hk-specific RNAi transgene under the control of the PDF driver.

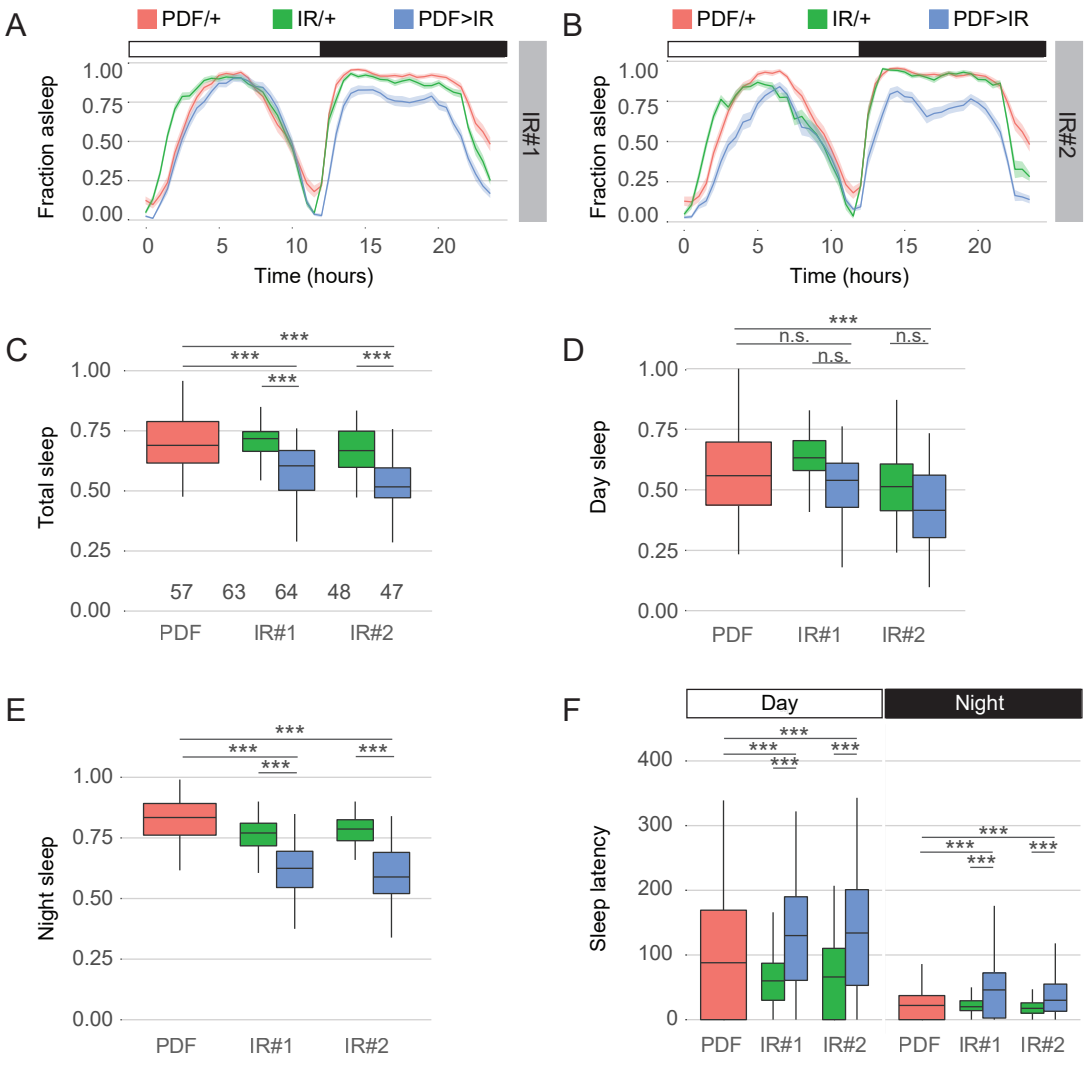


Figure 4.19: Sleep upon knock down of CG10638 in PDF+ neurons. A, Sleep profile of PDF>IR# 1 and parental controls, wrap of three days of baseline recording. B, Sleep profile of PDF>IR# 2 as in A. C, Daily sleep levels. D, Sleep levels during the day. E, Sleep levels during the night. F, Latency to day and night sleep. A-F, Sample size as annotated in C. C-F, Boxes reflect 25^{th} (top) and 75^{th} (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. C-F, Kruskal Wallis test to compare sleep parameters between experimental flies (PDF>IR) and parental controls (PDF/+, IR/+); "*" reflects $p<0.05$, "**" reflects $p<0.01$, "***" reflects $p<0.001$, "n.s." signifies "not significant".

The *hk*$^{\text{‘}}$ mutant has been named due to its characteristically abberrant activity patterns (Kaplan et al. 1969). Since the effects of RNAi-mediated reduction of Hk have not been tested in detail, we first evaluated the activity levels of PDF>Hk-IR flies. The RNAi-mediated reduction of Hk in PDF+ neurons does not produce hyperactivity (Fig. 4.20A, $\chi^2(2)=15.02$, $p<0.001$, *post hoc* PDF>Hk-IR:PDF/+, $p<0.001$, PDF>Hk-IR:Hk-IR/+, $p=0.6$).

The constitutive *hk*^{1} mutation suppresses both day and night sleep (Bushey et al. 2007). We thus tested the effects of RNAi-mediated reduction of Hk specifically in PDF+ neurons which comprise the s-LN$_v$ and l-LN$_v$, and the ensuing sleep profile of experimental and parental control flies is given in Fig. 4.20B. Similar to the *hk*^{1} mutant, reduction of Hk in PDF+ neurons tends to suppress sleep (Fig. 4.20A, $\chi^2(2)=14.18$, $p<0.001$, *post hoc* as annotated). This sleep suppression is mainly concentrated on night sleep, which is suppressed by around 2.4 h or 13 % compared to parental controls (Fig. 4.20E, $\chi^2(2)=20.5$, $p<0.001$, *post hoc* as annotated). Day sleep is not affected by the reduction of Hk expression levels in PDF+ neurons (Fig. 4.20D, $\chi^2(2)=17$, $p<0.001$, *post hoc* PDF>Hk-IR:PDF/+, $p=0.25$, PDF>Hk-IR:Hk-IR/+, $p<0.05$).

4.6.2 ICLI neuron-specific RNAi-mediated knock down

To test whether ICLI neurons are a substrate of CG10638 activity, we tested whether reduction of CG10638 in ICLI neurons would impact on sleep levels and/or architecture.

Indeed, daily sleep levels are lowered due to the expression of either IR# 1 or IR# 2 under the control of the ICLI neuron-specific driver VT026326: VT026326>IR# 1 flies sleep 2.64 h – or around 11 % – less than parental controls (Fig. 4.21C). Even more dramatically, VT026326>IR# 2 flies sleep 3.7 h – or around 15.5 % – less than parental controls (Fig. 4.21C, $\chi^2(4)=163.39$, $p<0.001$; *post hoc* as annotated).

Both siesta and night sleep are affected (Fig. 4.6A, B), but the effects on the siesta are more severe: expression of IR# 1 in ICLI neurons reduces siesta sleep by 1.8 h or 15 %, while expression of IR# 2 reduces siesta sleep by 2.8 h or

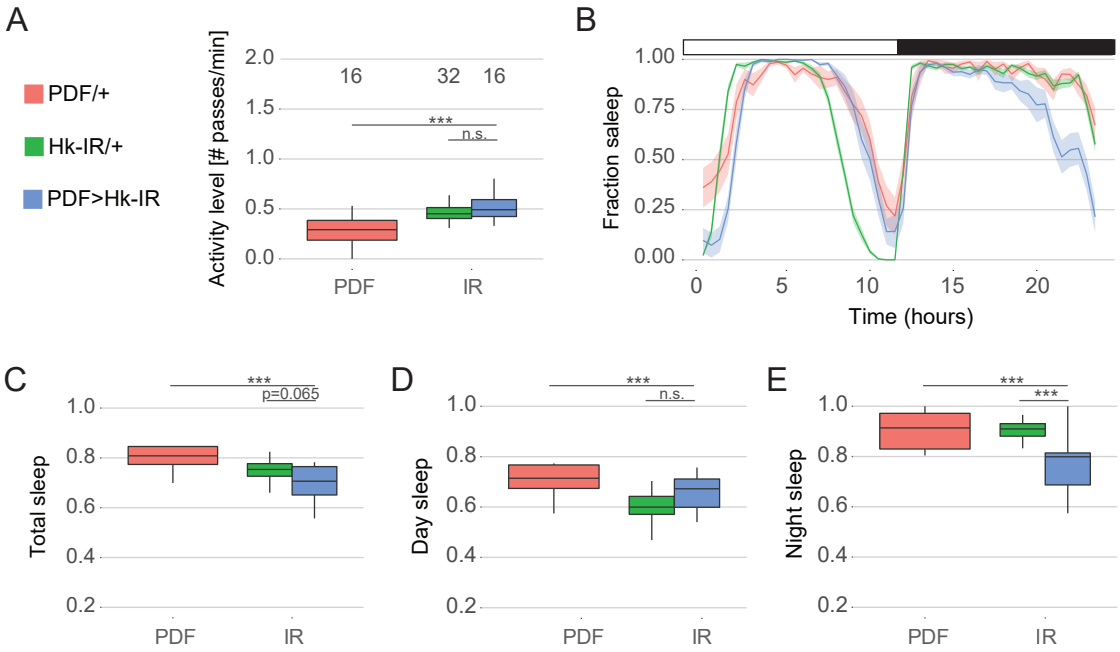


Figure 4.20: Sleep upon knock down of Hk in PDF+ neurons. A, Activity level of PDF>Hk-IR and parental control flies, wrap of three days of baseline recording. B, Sleep profile of PDF>Hk-IR and parental controls. C, Daily sleep levels. D, Sleep levels during the day. E, Sleep levels during the night. A-E, Sample size as annotated in A. A, B-E, Boxes reflect 25^{th} (top) and 75^{th} (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. A, B-E, Kruskal Wallis test to compare sleep parameters between experimental flies (PDF>Hk-IR) and parental controls (PDF/+, Hk-IR/+); "*" reflects p<0.05, "**" reflects p<0.01, "***" reflects p<0.001, "n.s." signifies "not significant".

23 % (Fig. 4.21D, $\chi^2(4)$=181.68, p<0.001; *post hoc* as annotated). Siesta sleep is also destabilised through CG10638-IR expression manifest in shortened sleep bouts of RNAi flies: VT026326>IR# 1 flies sleep on average 6.5 min or 33 % less long per bout than parental controls, and VT026326>IR# 2 sleep around 7 min or 40 % less (Fig. 4.21E, $\chi^2(4)$=104.56, p<0.001; *post hoc* VT026326>IR# 1:VT026326/+, p<0.01, VT026326>IR# 1:IR# 1/+, p<0.001, VT026326>IR# 2:VT026326/+, p<0.001, VT026326>IR# 2:IR# 2/+, p<0.001). Siesta sleep is not fragmented, however: the number of sleep bouts is normal in RNAi flies (Supplementary Fig. 7.7A).

Regarding night sleep, the expression of IR# 1 in ICLI neurons does not affect night sleep levels as RNAi flies sleep around 9.4 h compared to around 9.8 h in parental controls, but the the expression of IR# 2 in ICLI neurons mildly

suppresses sleep with RNAi flies sleeping around 9.0 h and parental controls sleeping around 9.7 h per day (Fig. 4.21F, $\chi^2(4)=69.68$, $p<0.001$; *post hoc* VT026326>IR# 1:VT026326/+, $p<0.001$, VT026326>IR# 1:IR# 1/+, $p=0.698$, VT026326>IR# 2:VT026326/+, $p<0.001$, VT026326>IR# 2:IR# 2/+, $p<0.01$). The expression of either IR in ICLI neurons has mild effects on night sleep architecture and leads to subtle sleep fragmentation (Supplementary Fig. 7.7B-C).

The expression of either RNAi transgene in ICLI neurons also delays sleep: VT026326>IR# 1 flies initiate siesta sleep around 47.5 min or 40 % later than parental controls, and night sleep around 7.5 min or 24 % later (Fig. 4.21G). Even more dramatically, VT026326>IR# 2 flies initiate siesta sleep around 79.5 min or 47 % later than parental controls, and night sleep around 4.5 min or 16 % later (Fig. 4.21G, Latency to siesta: $\chi^2(4)=92.22$, $p<0.001$; *post hoc* as annotated, latency to night sleep: $\chi^2(4)=160.81$, $p<0.001$; *post hoc* as annotated).

The RNAi-mediated sleep phenotype associated with the CG10638 locus is not generally due to hyperactivity: while the expression of IR# 1 in ICLI neurons increases activity, expression of IR# 2 does not (Fig. 4.21H, $\chi^2(4)=123.88$, $p<0.001$; *post hoc* VT026326>IR# 1:VT026326/+, $p<0.001$, VT026326>IR# 1:IR# 1/+, $p<0.001$, VT026326>IR# 2:VT026326/+, $p<0.001$, VT026326>IR# 2:IR# 2/+, $p=0.298$).

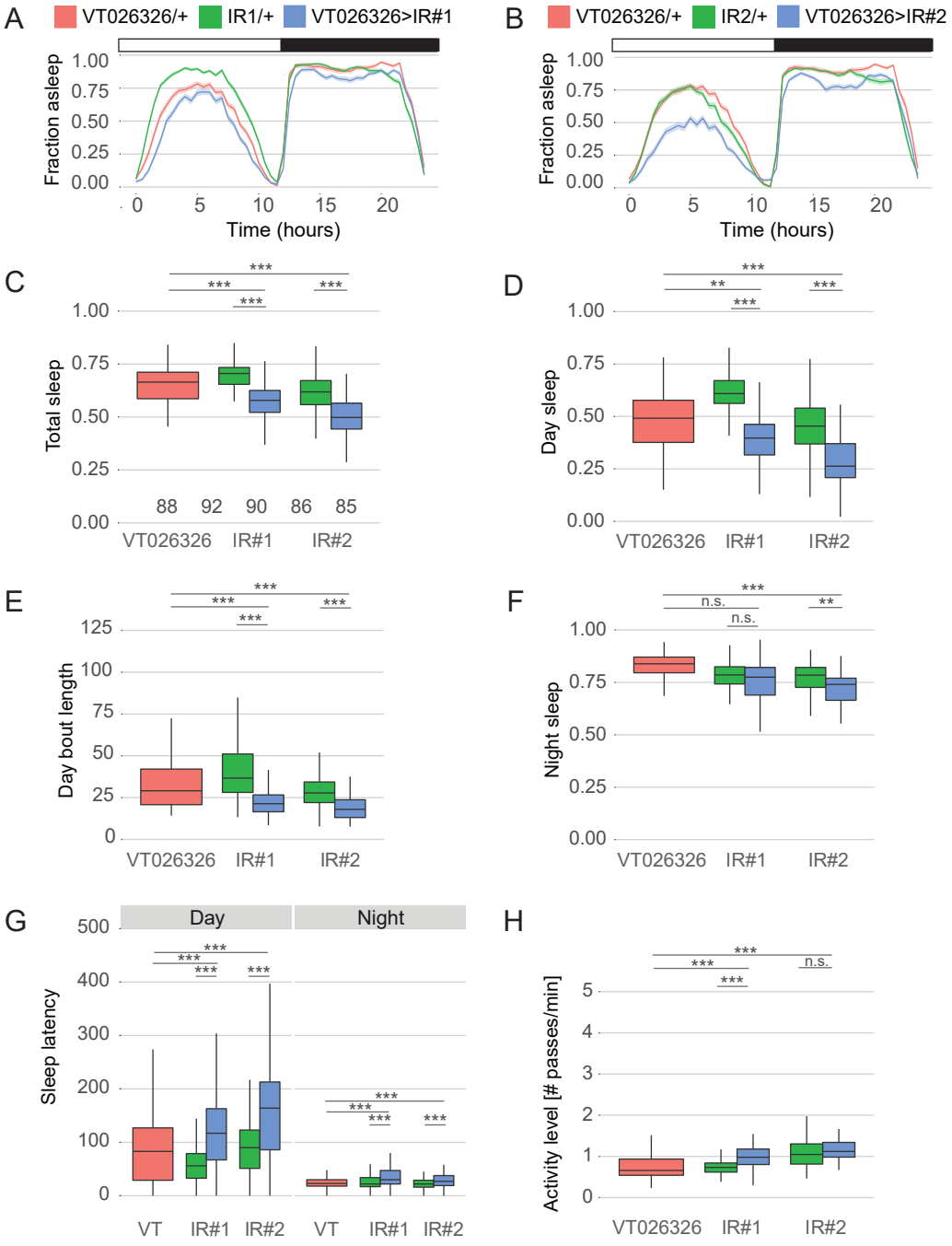


Figure 4.21: Sleep phenotype of flies with knock down of CG10638 in ICLI neurons under regular light conditions. A, Sleep profile of VT026326>IR# 1 flies and parental controls, wrap of three days of baseline recording. B, Sleep profile of VT026326>IR# 2 as in A. C, Daily sleep levels. D, Sleep levels during the day. E, Length of sleep bouts during the day. F, Sleep levels during the night. G, Latency to siesta and night sleep. H, Activity levels. A-H, Sample size as annotated in C. C-H, Boxes reflect 25^{th} (top) and 75^{th} (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. C-H, Kruskal Wallis test to compare sleep parameters between experimental flies (VT026326>IR) and parental controls (VT026326/+, IR/+); "*" reflects $p<0.05$, "**" reflects $p<0.01$, "***" reflects $p<0.001$, "n.s." signifies "not significant".

4.7 Potential sleep regulatory mechanisms through ICLI neurons

We tested whether CG10638 act through circadian or homeostatic processes: first, we tested the effect of CG10638 reduction in isoB+ ICLI neurons under free-running conditions and sleep deprivation. Secondly, we assessed CG10638 mRNA and protein levels under conditions of increasing sleep pressure, as well as the physiological response to sleep deprivation of ICLI neurons to correlate it with the expression response.

4.7.1 General involvement of ICLI neurons in sleep regulation

Sleep effects of ICLI neuron activation

In order to ascertain independently of the RNAi-mediated approach whether ICLI neurons were capable of modulating sleep, we activated these neurons using the thermosensitive TRPA1 construct under the control of the VT026326 driver for the course of one day (24 h) preceded by a baseline recording of several days. Due to the strong, wake-promoting effect of light, we tested ICLI neuron activation both under regular light conditions and in acute darkness spanning the duration of the heat activation.

Sleep does not differ between genotypes at the restrictive temperature of 22 °C: sleep levels are similar under a regular light regime (Fig. 4.22C, $\chi^2(2)=2.15$, $p=0.34$). Sleep levels are also similar in acute darkness (Fig. 4.22D, $\chi^2(2)=18.98$, $p<0.001$, *post hoc* VT026326>TRPA1:VT026326/+, $p<0.001$, VT026326>TRPA1:TRPA1/+ $p=0.15$).

Heat-activation of ICLI neurons at the permissive temperature of 29 °C reduces sleep in a precise manner (Fig. 4.22A, B). Activation of ICLI neurons under regular LD conditions reduces specifically night sleep (Fig. 4.22F, $\chi^2(2)=82.24$, $p<0.001$, *post hoc* as annotated), but has no effect on day sleep (Fig. 4.22E, $\chi^2(2)=19.37$, $p<0.001$, *post hoc* VT026326>TRPA1:VT026326/+, $p<0.001$, VT026326>TRPA1:TRPA1/+ $p=0.814$). Heat-activation of ICLI neurons in darkness reduces both day sleep (Fig.

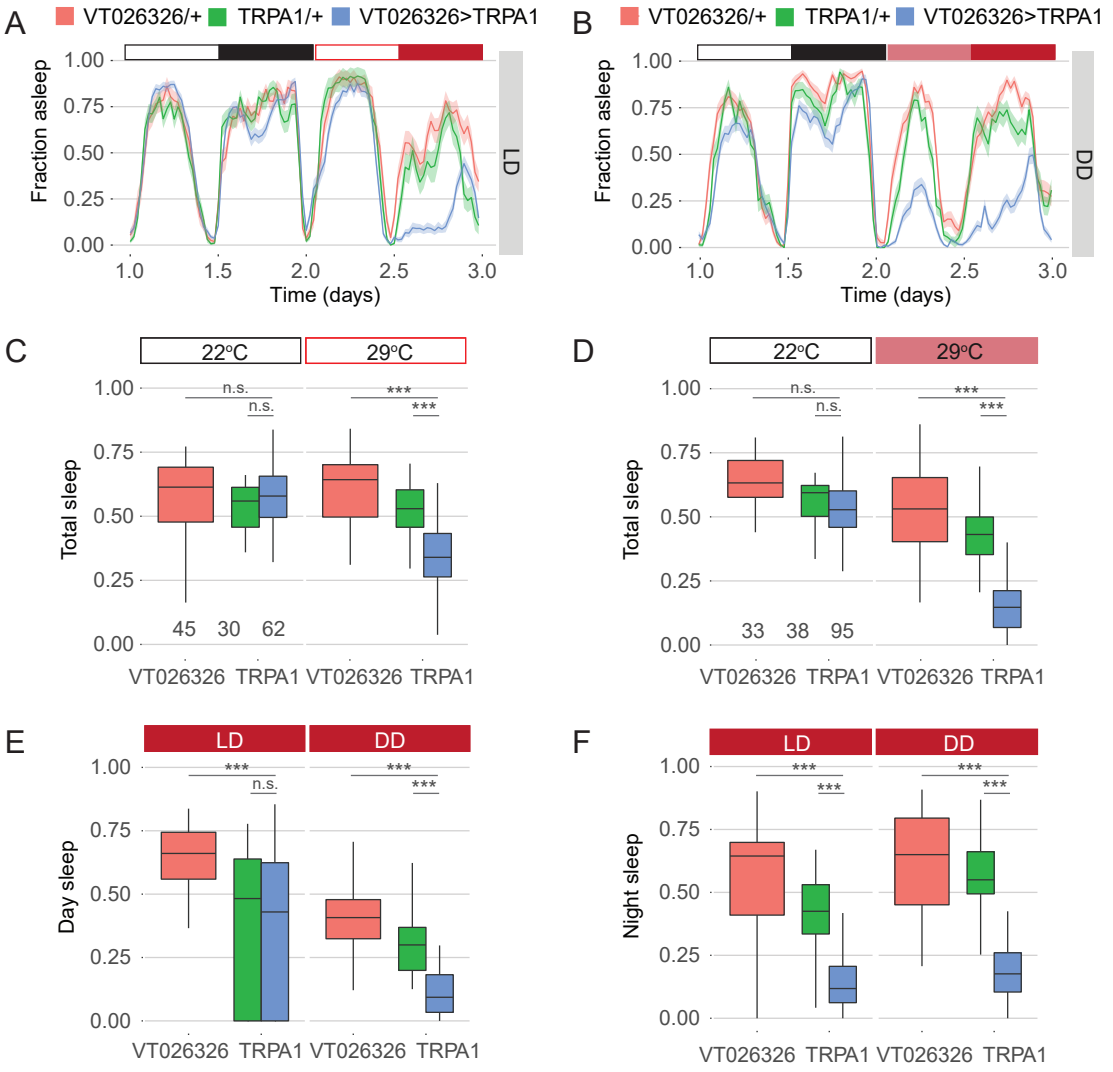


Figure 4.22: Sleep upon activation of ICLI neurons under different light regimes. A, Sleep profile of VT026326>TRPA1 in LD. B, Sleep profile of VT026326>TRPA1 in darkness. C, Daily sleep levels in LD at restrictive and permissive temperature, sample size as annotated. D, Same as in C in darkness, sample size as annotated. E, Daily sleep levels during (subjective) day in both conditions at permissive temperature. F, Same as in E for (subjective) night. C-F, Boxes reflect 25^{th} (top) and 75^{th} (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. C-F, Kruskal Wallis test to compare sleep parameters between experimental flies (VT026326>TRPA1) and parental controls (VT026326/+, TRPA1/+) under different light and temperature conditions, whereby "*" reflects $p<0.05$, "**" reflects $p<0.01$, "***" reflects $p<0.001$, "n.s." signifies "not significant".

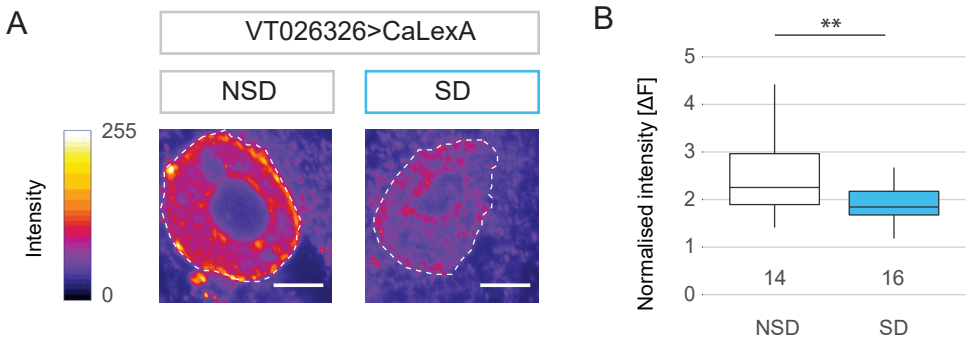


Figure 4.23: Effects of sleep deprivation on ICLI neuron activity. A, Representative confocal sections through the ICLI soma for SD and NSD treatment, scale bars: 5 µm. B, Fluorescence intensity in ICLI somata for both treatments, sample size as annotated. Boxes reflect 25th (top) and 75th (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. B, Wilcoxon rank sum test to compare staining intensity between conditions; "**" reflects p<0.01.

4.22E, $\chi^2(2)$=80.09, p<0.001, *post hoc* as annotated) and night sleep (Fig. 4.22F, $\chi^2(2)$=82.24, p<0.001, *post hoc* as annotated).

Effects of sleep deprivation on ICLI neuron activation

Given that ICLI neurons have the potential to modulate sleep, we next aimed to investigate whether this relationship is mutual, i.e., whether sleep also modulates ICLI neuron activity. To this end, we expressed the Ca^{2+} indicator CaLexA in ICLI neurons and subjected flies of this genotype to overnight SD.

Representative sections of ICLI neuron somata of each treatment are depicted in Fig. 4.23A. Sleep deprivation reduces the GFP signal in ICLI neurons (Fig. 4.23 B, W=179, p<0.01). Background staining levels do not differ between treatments (data not shown, W=17.47, p=0.765).

4.7.2 Sleep-related expression of CG10638

In order to test whether sleep-related effects of CG10638 were related to circadian or homeostatic sleep regulation, we tested whether (1) expression of CG10638 traces circadian time or sleep pressure, (2) expression of CG10638 responds to homeostatic challenge, and (3) changes in mRNA levels translate to changes in the protein level.

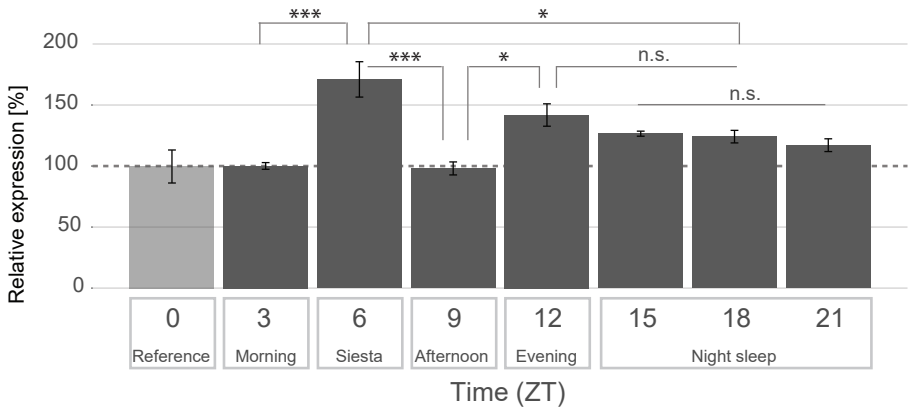


Figure 4.24: Expression profile of CG10638 (isoB) in ICLI neurons throughout the day, n=3/ZT. Bar height reflects mean, error bars reflect sem. Results of ANOVA: $F(6, 14)=11.45$, $p<0.001$, *post hoc* ZT3:ZT6, $p<0.001$, ZT3:ZT9, $p=0.999$, ZT3:ZT12, $p<0.05$, ZT3:ZT15, $p=0.227$, ZT3:ZT18, $p=0.317$, ZT3:ZT21, $p=0.676$, ZT6:ZT9, $p<0.001$, ZT6:ZT12, $p=0.151$, ZT6:ZT15, $p<0.05$, ZT6:ZT18, $p<0.01$, ZT6:ZT21, $p<0.01$, ZT9:ZT12, $p<0.05$, ZT9:ZT15, $p=0.168$, ZT9:ZT18, $p=0.239$, ZT9:ZT21, $p=0.564$, ZT12:ZT15, $p=0.767$, ZT12:ZT18, $p=0.637$, ZT12:ZT21, $p=0.288$, ZT15:ZT18, $p=0.999$, ZT15:ZT21, $p=0.967$, ZT18:ZT21, $p=0.993$).

Expression of CG10638 throughout the day

First, we ensured that we were able to capture the expression dynamics of transcripts known to systematically change in the course of the day. These include *bruchpilot* (*brp*, Supplementary Fig. 7.12A) and *period* (*per*, Supplementary Fig. 7.12B).

Expression of isoB changes throughout the day (Fig. 4.24). IsoB expression is low directly after sleep, both after night sleep (ZT0) and after siesta sleep (ZT9), and elevated at times coinciding with or immediately preceding regular sleep episodes: isoB levels are upregulated by around 70 % at ZT6 compared to ZT0, and by around 42 % at ZT12 compared to ZT0 (Fig. 4.24). Elevated expression levels of ZT6 sharply decrease back to those of the early morning (ZT0-3) by ZT9 (Fig. 4.24). Elevated expression levels of ZT12 gradually decrease with dissipating sleep pressure in the course of the night from ZT15 (27 %) through ZT18 (24 %) to ZT21 (21 % compared to ZT0) back to early morning levels of ZT0-3 (Fig. 4.24, see legend for statistical analysis).

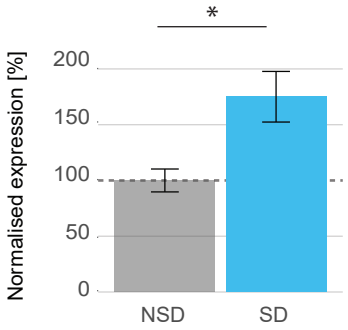


Figure 4.25: Expression of CG10638 (isoB) in ICLI neurons in response to SD in young male WT flies, n=7/treatment. Bar height reflects mean, error bars reflect sem. Asteriks reflects result of t-test to compare normalised expression levels between treatments, whereby "*" reflects $p<0.05$.

Expression of CG10638 in response to sleep deprivation

Transcript level Expression of isoB mRNA was upregulated in response to sleep deprivation (Fig. 4.25, $t(6)=-3.551$, $p<0.05$). Expression of the clock gene *per* and the other isoform of CG10638, isoA, was unaffected by sleep deprivation (Supplementary Fig. 7.13A, B).

Protein level In order to test whether isoB protein levels also trace sleep pressure, we measured isoB protein levels in ICLI neurons immediately after sleep (ZT0, "S") and after the waking day (ZT12, "W") and detected an upregulation of isoB after the waking day (Fig. 4.26B, $W=0$, $p<0.001$).

Similarly, an increase in sleep pressure due to dedicated sleep deprivation is associated with accumulation of isoB protein in ICLI neurons (Fig. 4.26D, $W=33$, $p<0.01$).

4.7.3 CG10638 – a regulator of sleep homeostasis

In order to test whether sleep-related effects of ICLI-specific RNAi flies were due to a circadian misregulation of sleep timing or an insensitivity to sleep pressure, we tested whether (1) sleep effects persisted in constant darkness, (2) circadian rhythms were destabilised, and (3) the homeostatic sleep response was perturbed in these flies.

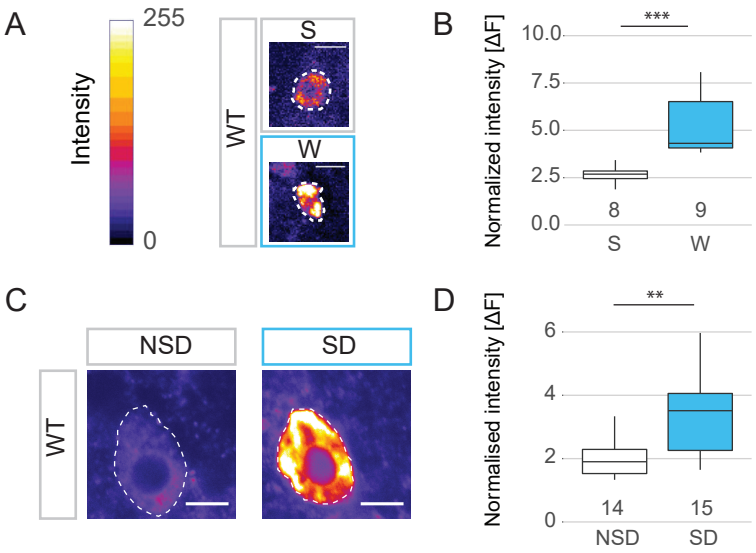


Figure 4.26: Expression of isoB in response to sleep pressure in male WT flies. A, Representative sections though ICLI somata. B, Fluorescence intensity of isoB staining at two different timepoints; ZT0 is associated with low sleep pressure (slept/"S"), ZT12 is associated with high sleep pressure (wake/"W"), sample size as annotated. C, Same as A for SD treatment. D, Fluorescence intensity of isoB staining under two conditions, sleep deprived ("SD") and slept ("NSD"), sample size as annotated. A, C, Scale bars: 10 µm. B, D, Boxes reflect 25th (top) and 75th (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. Asteriks reflect result of Wilcoxon rank sum test to compare staining intensity between conditions, whereby "**" reflects $p<0.01$, "***" reflects $p<0.001$.

Sleep in constant darkness

We assessed the persistence of the sleep phenotype of LD-entrained VT026326>CG10638-IR flies by comparing two days in a regular LD schedule with two following days of constant darkness (Fig. 4.27A).

Animals generally react to constant darkness with sleep suppression during the subjective day: siesta sleep is lower across all genotypes in DD compared to LD by around 3.6 h or 34 % for VT026326>IR# 2 flies, by around 3.8 h or 45 % for IR# 2/+ flies, and by around 6.48 h or 67 % for VT026326/+ flies (Fig. 4.27B, treatment: $F(1, 170)=99.45$, $p<0.001$, genotype: $F(2, 170)=62.1$, $p<0.001$, treatment × genotype: $F(2, 170)=3$, $p=0.052$). Parental control flies do not exhibit a significant change in night sleep in DD compared to LD: night sleep of IR# 2/+ flies is around 6 % lower in DD compared to LD, and around 2 % lower in VT026326/+ flies

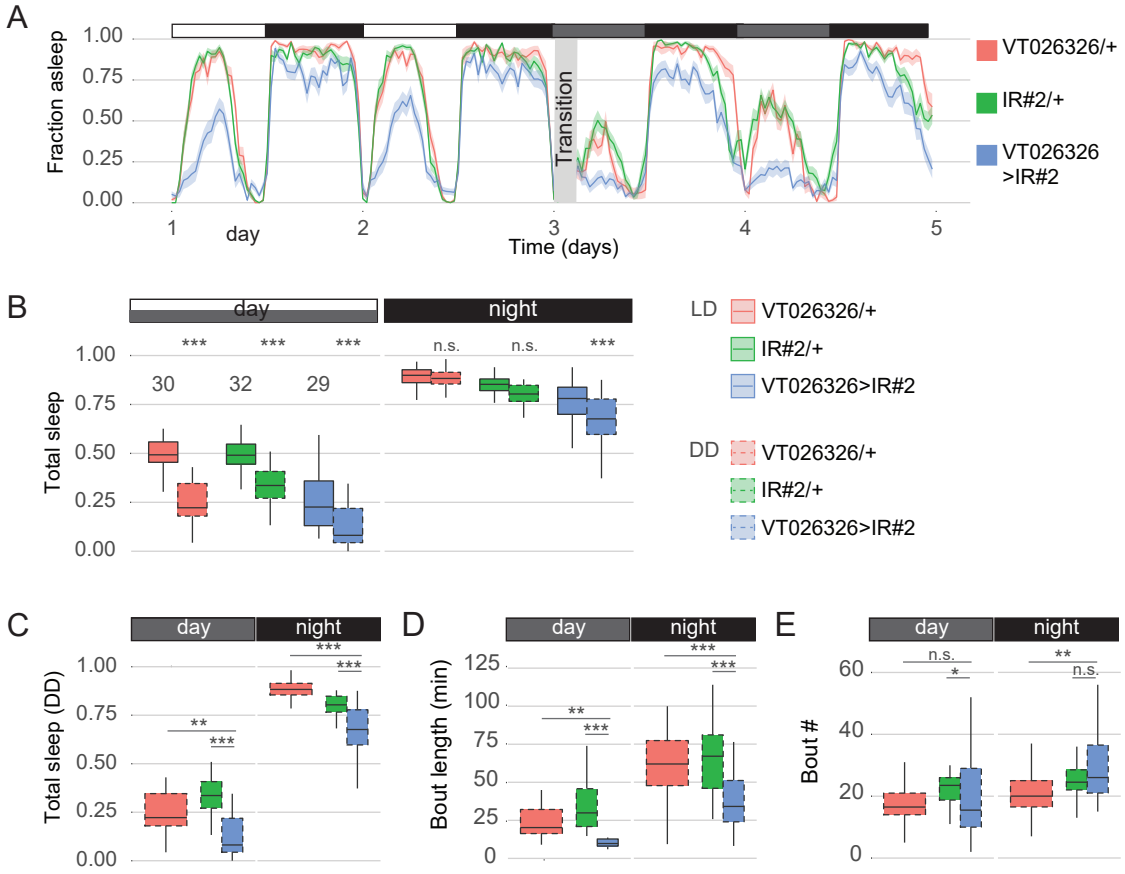


Figure 4.27: Sleep upon knock down of CG10638 in ICLI neurons under constant darkness. A, Sleep profile of VT026326>IR# 2 and parental controls. B, Daily sleep levels in LD and DD. C, Sleep in DD: daily sleep levels. D, As in C: sleep bout length. E, As in C: sleep bout number. A-E, Boxes reflect 25th (top) and 75th (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. B-E, Kruskal Wallis test to compare sleep parameters between flies of the experimental genotype (VT026326>IR# 2) and parental controls (VT026326/+, IR# 2/+) and between treatment conditions; "*' reflects p<0.05, "**" reflects p<0.01, "***" reflects p<0.001, "n.s." signifies "not significant".

(Fg=ig. 4.27B). However, in VT026326>IR# 2 flies, night sleep is mildly lowered in DD in comparison to LD by around 2.4 h or 14 % (Fig. 4.27B, treatment: $F(1, 170)=15.19$, $p<0.001$, genotype: $F(2, 170)=36.7$, $p<0.001$, treatment × genotype: $F(2, 170)=3.18$, $p<0.05$, *post hoc* VT026326/+ LD:DD, $p=0.972$, IR# 2/+ LD:DD, $p=0.61$, VT026326>IR# 2 LD:DD, $p<0.001$).

Thus, sleep suppression in response to ICLI neuron-specific knock down of CG10638 persists in darkness: sleep is suppressed in VT026326>IR# 2 flies during the subjective day by around 4.7 h or 29 % compared to parental controls (Fig. 4.27C,

$\chi^2(2)$=34.71, p<0.001; *post hoc* as annotated). Under acute darkness, night sleep is also significantly suppressed by expression of IR# 2 in ICLI neurons: RNAi flies sleep around 3.8 h or 23 % less than parental control flies during the subjective night (Fig. 4.27C, $\chi^2(2)$=45.41, p<0.001; *post hoc* as annotated).

The destabilisation of sleep manifest in a shortened duration, but a normal frequency of sleep bouts (Fig. 4.21E), also persists in darkness: in VT026326>IR# 2 flies, an average day sleep bout lasts 8 min, compared to 14.3 min and 19 min, respectively, for parental control flies, and an average night sleep bout lasts 19 min, compared to 34 min and 43 min for parental controls (Fig. 4.27D, Day bout length: $\chi^2(2)$=28.31, p<0.001; *post hoc* as annotated, Night bout length: $\chi^2(2)$=19.6, p<0.001; *post hoc* as annotated).

The frequency of sleep bouts is normal: VG026326>IR# 2 flies sleep in around 15.5 bouts per subjective day, and in around 26 bouts per subjective night, compared to 16.5 and 23.5 day bouts, and 20 and 24.5 night bouts, respectively for parental control flies (Fig. 4.27E, Day sleep bout # : $\chi^2(2)$=7.92, p<0.05, *post hoc* VT026326>IR# 2:VT026326/+, p=0.718, VT026326>IR# 2:IR# 2/+, p<0.05; Night sleep bout # : $\chi^2(2)$=11.03, p<0.001, *post hoc* VT026326>IR# 2:VT026326/+, p=0.446, VT026326>IR# 2:IR# 2/+, p<0.01).

Circadian rhythm

To test the stability of the circadian system under conditions of CG10638 in ICLI neurons, VT026326>IR# 2 flies were subjected to several days of light entrainment before being transferred to constant darkness. Actograms showing average the activity profiles of each genotype are included in Fig. 4.28A.

Generally, animals of all genotypes are rhythmic: the vast majority of flies across all genotypes tested falls into the "rhythmic" ("R") category (Table 4.5). VT026326>IR# 1 flies do not differ from parental controls in respect to free running period length which is 23.5 h in RNAi flies, and 23.8 h and 23.7 h, respectively, in control flies (Table 4.5). Similarly, expression of IR# 2 in ICLI neurons is associated with a period length of 24 h which is comparable with the period length of parental

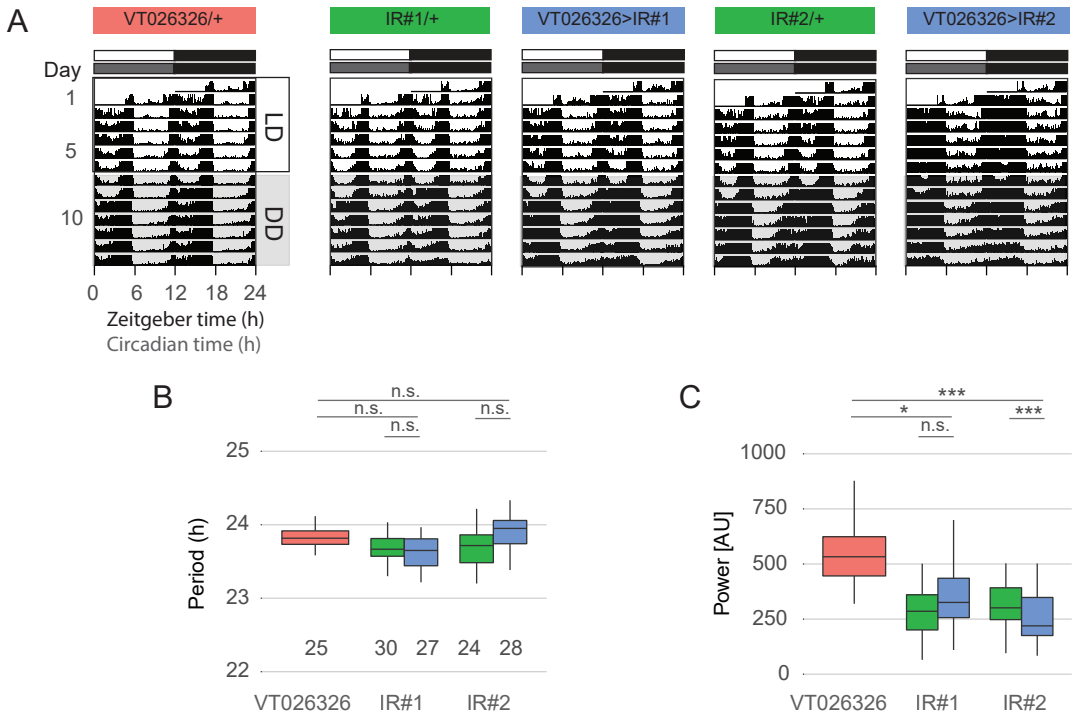


Figure 4.28: Circadian rhythm upon CG10638 knock down in ICLI neurons. A, Actograms showing the average activity pattern per genotype under free running conditions for RNAi flies and parental controls throughout the course of the experiment. Genotype as annotated above actogram. B, Period length under free running conditions. C, Rhythm power as in B. A-C, Sample size as annotated in B. Boxes reflect 25th (top) and 75th (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. Asteriks reflect result of Kruskal Wallis test to compare circadian parameters between flies of the experimental genotype (VT026326>IR) and parental controls (VT026326/+, IR/+) and between treatment conditions, whereby "*' reflects p<0.05, "**" reflects p<0.01, "***" reflects p<0.001, "n.s." signifies "not significant".

control flies of 23.7 h and 23.8 h, respectively (Table 4.5, Fig. 4.28B, $\chi^2(4)$=19.25, p<0.001, *post hoc* VT026326>IR# 1:VT026326/+, p=0.991, VT026326>IR# 1:IR# 1/+, p=0.644, VT026326>IR# 2:VT026326/+, p=0.292, VT026326>IR# 2:IR# 2/+, p=0.871).

However, rhythms are mildly weakened by expression of IR# 2 in ICLI neurons: in VT026326>IR# 2 flies, rhythm power is reduced compared to parental control flies, while this is not the case for expression of IR# 1 (Table 4.5, Fig. 4.28C, $\chi^2(4)$=37.79, p<0.001, *post hoc* VT026326>IR# 1:VT026326/+, p=0.846, VT026326>IR# 1:IR# 1/+, p=0.91, VT026326>IR# 2:VT026326/+, p<0.001, VT026326>IR# 2:IR# 2/+, p<0.001).

Genotype	N	Period (h) Mdn	Range	Power Mdn	Range	R (%)
VT026326/+	25	23.83	18.98-24.12	524.56	40.97-877.81	100
IR# 1/+	30	23.67	20.90-24.56	227.10	30.67-501.57	97
VT026326 IR# 1	27	23.52	20.80-28.45	309.54	52.33-1003.65	96
IR# 2/+	24	23.73	23.2-24.46	301.01	95.31-688.54	100
VT026326 IR# 2	28	23.95	20.02-28.23	215.60	33.73-683.04	100

Table 4.5: Circadian rhythm upon knock down of CG10638 in ICLI neurons. Further rhythm categories were omitted as they occurred very rarely.

Homeostatic sleep response

In order to assess whether sleep-related effects of ICLI-specific RNAi flies were due to insensitivity to sleep pressure, we sleep deprived flies overnight and measured their ability to rebound (Fig. 4.29A). We measured rebound sleep for different time windows: the immediate sleep deprivation response from ZT0-ZT3 (Fig. 4.29B) and a delayed sleep deprivation response from ZT3-ZT6 (Fig. 4.29C).

Sleep profiles of experimental and parental control flies throughout the course of the experiment are depicted in Fig. 4.29A.

Our sleep deprivation regime successfully disrupted sleep across all genotypes: sleep deprivation reduced sleep levels by around 80 % across genotypes (Fig. 4.29D, treatment: $F(1, 285)=559.42$, $p<0.001$, genotype: $F(2, 285)=10.4$, $p<0.001$, treatment×genotype: $F(2, 316)=5.24$, $p<0.01$, *post hoc* as annotated). During the time immediately following sleep deprivation (ZT0-ZT3), parental controls were able to compensate for lost sleep through an increase in sleep levels by around 60 %, while flies of the experimental line VT026326>IR# 2 were not (Fig. 4.29E, treatment: $F(1, 277)=44.89$, $p<0.001$, genotype: $F(2, 277)=11.24$, $p<0.001$, treatment×genotype: $F(2, 316)=3.23$, $p<0.05$, *post hoc* VT026326/+ SD:NSD, $p<0.05$, IR# 2/+ SD:NSD, $p<0.001$, VT026326>IR# 2 SD:NSD, $p=0.18$).

Conversely, in the next time step (ZT3-ZT6), sleep in parental control flies decreases back to NSD levels, while sleep duration in experimental flies rises above NSD levels, indicating a delayed homeostatic sleep response (Fig. 4.29F, treatment: $F(1, 277)=20.47$, $p<0.001$, genotype: $F(2, 277)=19.54$, $p<0.001$, treatment×genotype:

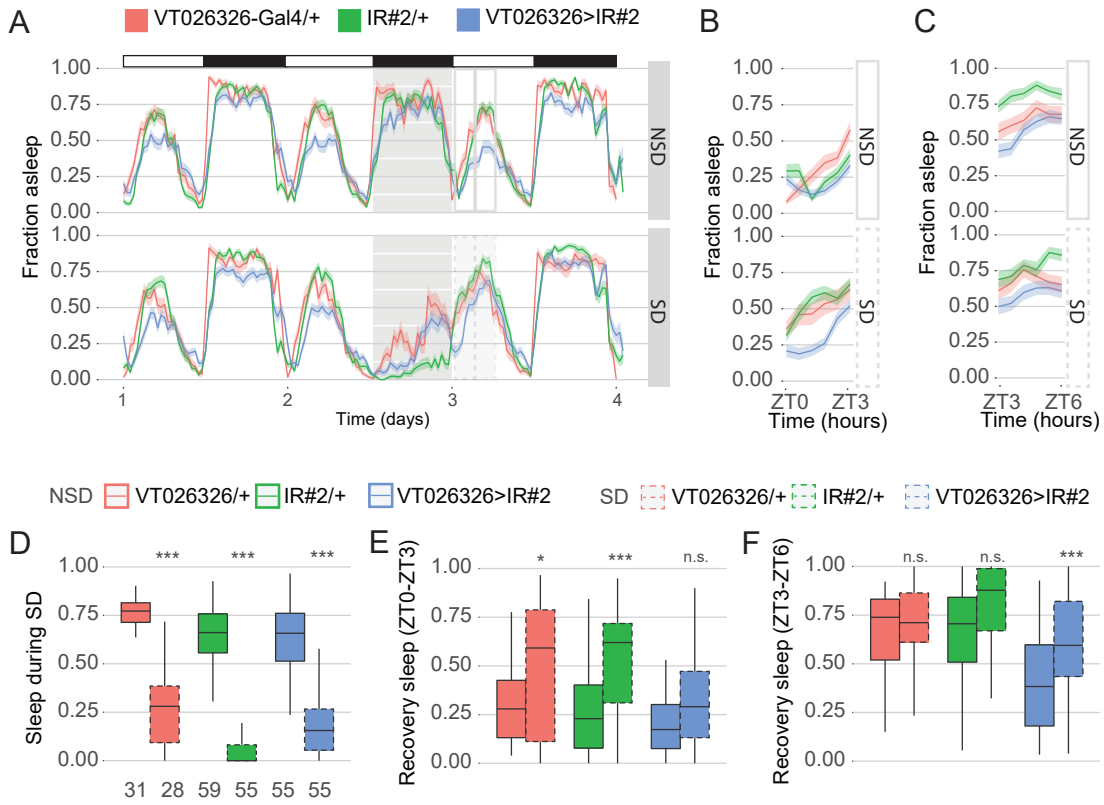


Figure 4.29: Homeostatic sleep response upon knock down of CG10638 in ICLI neurons. A, Sleep profile of VT026326>IR# 2 and parental controls throughout the experiment. B, Magnification of recovery sleep following SD (ZT0-ZT3). C, Same as B for ZT3-ZT6. D, Sleep during SD from ZT12-ZT0. E, Recovery sleep from ZT0-ZT3. F, Recovery sleep from ZT3-ZT6. A-F, Sample size as annotated in D. Boxes reflect 25th (top) and 75th (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. Asteriks reflect result of Kruskal Wallis test to compare circadian parameters between flies of the experimental genotype (VT026326>IR) and parental controls (VT026326/+, IR) and between treatment conditions, whereby "*' reflects p<0.05, "**" reflects p<0.01, "***" reflects p<0.001, "n.s." signifies "not significant".

F(2, 316)=2.74, p=0.066, *post hoc* VT026326/+ SD:NSD, p=0.997, IR# 2/+ SD:NSD, p=0.154, VT026326>IR# 2 SD:NSD, p<0.001).

4.7.4 CG10638 – a regulator of arousal

We tested arousal-modulating effects of CG10638 through a sleep-independent route and tested ICLI-specific RNAi flies for sexual arousal. We chose sexual arousal as it is known to be modulated by neurons of the MIP+ cluster (Jiang et al. 2013). Thus, we tested sexual arousal and mating success in flies of the same genotype as

presented by Jiang et al. 2013 to serve as a positive control; these consist of flies in which MIP+ neurons were silenced through the expression of UAS-TNT under the control of an MIP-specific driver (section 7.2.3). We confirmed the copulation phenotype of MIP>TNT flies described by Jiang et al. 2013, but did not detect an impairment in sexual arousal as measured in terms of frequency of and latency to courtship initiation (Supplementary Fig. 7.8).

In respect to VT026326>IR# 2 flies, when paired with a mature, virgin female, male RNAi flies were more likely to copulate within an hour than parental controls: specifically, while 76 % of RNAi flies copulated within 1 h, only 41 % of IR# 2/+ and 31 % of VT026326/+ parental control flies did so (Fig. 4.30A, $\chi^2(2)=14.61$, $p<0.001$). This effect persists even after 2 h of exposure to a female, when 81 % of RNAi flies – but only 59 % of IR# 2/+ and only 46 % of VT026326/+ parental control flies – had copulated (Fig. 4.30B, $\chi^2(2)=9.56$, $p<0.01$). Within this 2 h window, among flies that achieved copulation success, VT026326>IR# 2 flies were quicker to copulate: on average, RNAi flies copulated after 37 min, instead of around 60 min as observed for parental control flies (Fig. 4.30C, $\chi^2(2)=10.23$, $p<0.05$, *post hoc* as annotated). The shorter latency to copulation of VT026326>IR# 2 flies is aided by their tendency to initiate courtship faster: RNAi flies initiated courtship after around 14 min, while parental controls took 4-5 min longer to do so (Fig. 4.30D, $\chi^2(2)=9.27$, $p<0.05$, VT026326>IR# 2:IR# 2/+, $p=0.152$, VT026326>IR# 2:VT026326/+, $p<0.01$).

In a next step, we aimed to test whether reduction of CG10638 in ICLI neurons generally augments sexual arousal, or whether it merely increases the sensitivity to the presence of a female. Thus, we subjected flies with an ICLI-specific knock down of CG10638 to male-male interactions and scored the prevalence of male-male courtship. This behaviour is rare in flies, and was absent, for instance, in genotypes tested as a positive control for male-female interactions (Supplementary Fig. 7.8D). Interestingly, sexual arousal of VT026326>IR# 2 flies was pronounced to such a degree that they initiate male-male courtship: while the incidence of male-male courtship was very low in IR# 2/+ (24 %) and in VT026326/+ parental control flies (17 %), 67 % of VT026326>IR# 2 flies initiated male-male courtship (Fig. 4.31B,

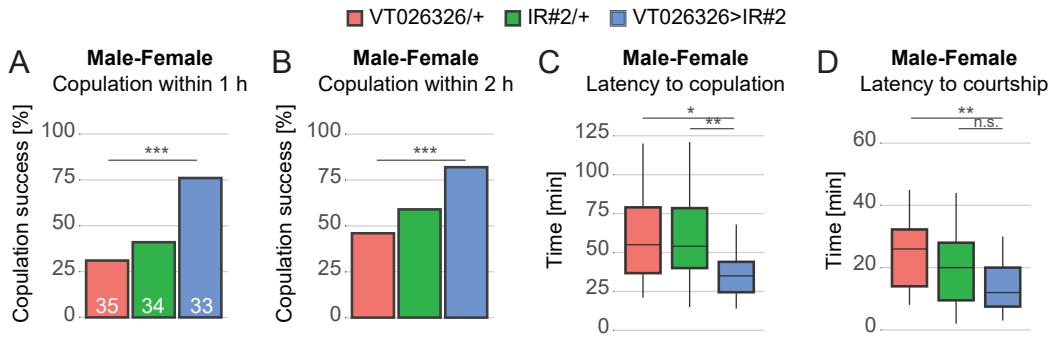


Figure 4.30: Sexual arousal of male flies upon CG10638 knock down in ICLI neurons presented with a virgin female for up to 2h between ZT2 and ZT4. A, Copulation success in male-female interactions of young male RNAi flies and parental controls within 1 h. B, Copulation success within 2 h. C, Latency to copulation within 2 h. D, Latency to courtship initiation. A-D, Sample size annotation in A reflects number of male-female pairs/genotype. A-B, Bar height reflects percentage of flies that successfully copulated. Asteriks reflect results of Pearson's χ^2 test. C-D, Boxes reflect 25$^{\text{th}}$ (top) and 75$^{\text{th}}$ (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. Asteriks reflect result of Kruskal Wallis test to compare behavioural parameters between flies of the experimental genotype (VT026326>IR) and parental controls (VT026326/+, IR). A-D, Throughout, "*' reflects p<0.05, "**" reflects p<0.01, "***" reflects p<0.001, "n.s." signifies "not significant".

$\chi^2(2)=17.86$, $p<0.001$). The distribution of male-male courtship initiation events throughout the course of 40 min (after which no courtship initiation occurred) per genotype is depicted in Fig. 4.31A.

Another phenotype associated with MIP+ neurons, increased feeding and bodyweight, was not detectable in VT026326>IR# 2 flies (Supplementary Fig. 7.9).

4.7.5 A s-LN$_v$-to-ICLI neuron-dependent arousal pathway

In order to put the CG10638-dependent, arousal-promoting role of ICLI neurons into context, we aimed to elucidate the network context of these neurons. Thus, we further analysed their projection pattern and connection partners.

Projection pattern

Polarity ICLI neurons have projections into the dorsal protocerebrum in addition to a midline projection (Fig. 4.14A). In order to determine the polarity of ICLI

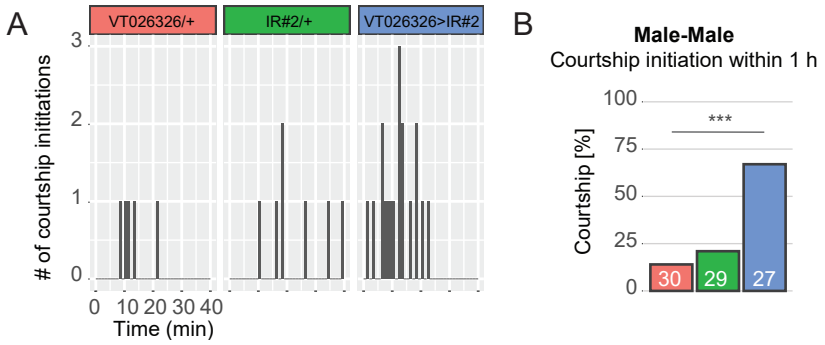


Figure 4.31: Sexual arousal of male flies upon CG10638 knock down in ICLI neurons presented with a another male for up to 1h between ZT2 and ZT4. A, Distribution of courtship initiation events in male-male interactions of young male RNAi flies and parental controls within 1 h. B, Percentage of courtship initiation events per genotype. Sample size annotation reflects number of male-male pairs/genotype. Bar height reflects percentage of flies that initated courtship. Asteriks reflect results of Pearson's χ^2 test, whereby "***" reflects p<0.001.

projections, we expressed the dendritic marker Denmark and the axonal marker sytI under the control of VT026326.

Dendritic or input regions were detected along the dorsal projection (Fig. 4.32A) including the ring around the MB peduncle (Fig. 4.32A'). Synaptic or output regions were not as readily detectable. However, we detected clusters of sytI+ varicosities in the SOG region (Fig. 4.32B). The midline projection expresses both dendritic and synaptic markers, indicating cross-talk between ICLI neurons as they project into the contralateral side of the protocerebrum (Fig. 4.32C). The basic anatomy of ICLI neurons is depicted in a schema in Fig. 4.32D.

MB peduncle Strikingly, ICLI neurons curve upwards in the direction of the MB peduncle before proceeding to project further into the dorsal protocerebrum (Fig. 4.14A). The dorsal projections of ICLI neurons wrap around the peduncle of the mushroom bodies (Fig. 4.33A), but do not seem to enter the fibre tract (Fig. 4.33B).

Neural partners

ICLI input region in s-LN$_v$ output region The dorsal projection of ICLI neurons enters the output region of the dorsal projection of s-LN$_v$ into the dorsal protocerebrum (dPC). In order to test whether the s-LN$_v$ and ICLI neurons are

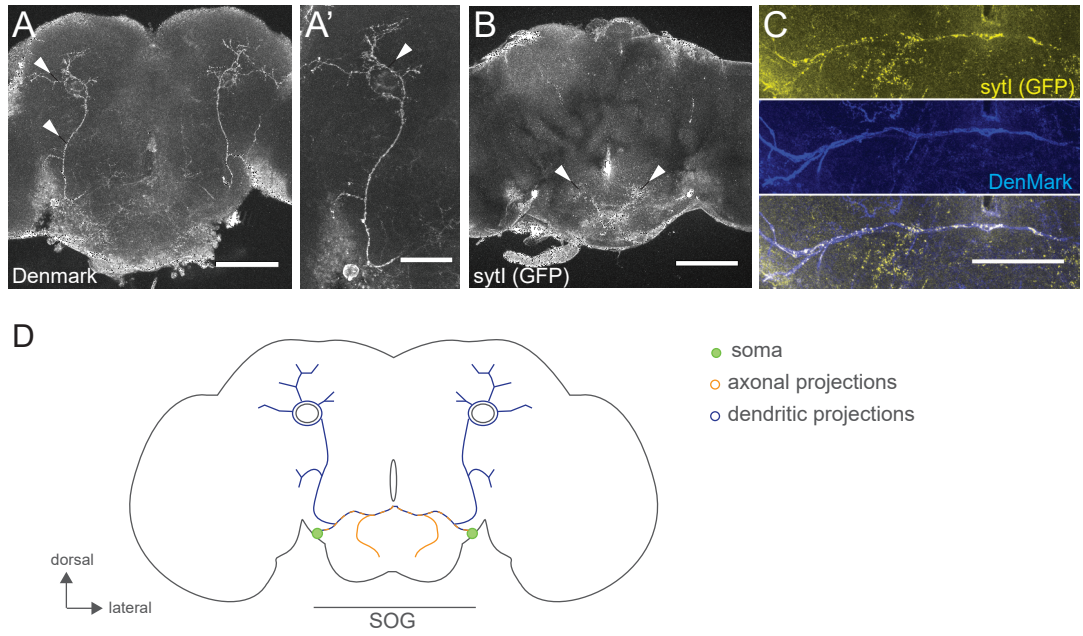


Figure 4.32: Polarity of ICLI neurons. A-A', Confocal projection of whole-mount adult brains of VT026326>DenMark reporter flies. B, Same as A for VT026326>sytI:GFP reporter flies. C, Co-expression of VT026326>DenMark;SytI. D, Illustration indicating ICLI neuron location, polarity, and localisation of input and output regions. Scale bars: A, B, 50 µm, A', C, 20 µm

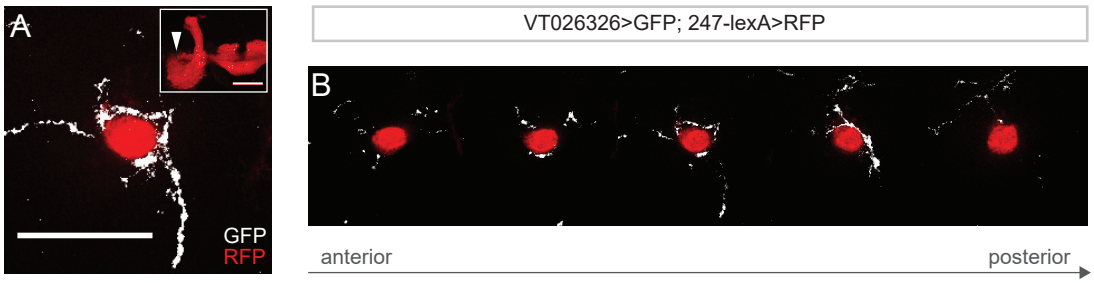


Figure 4.33: ICLI neurons bypass the mushroom body peduncle. A, Confocal projection of VT026326>mCD8::GFP visualised in 247-lexA::RFP background, inset reflects the region of the MB peduncle investigated, scale bars: 20 µm. B, Representative saggital sections, 5 µm step size.

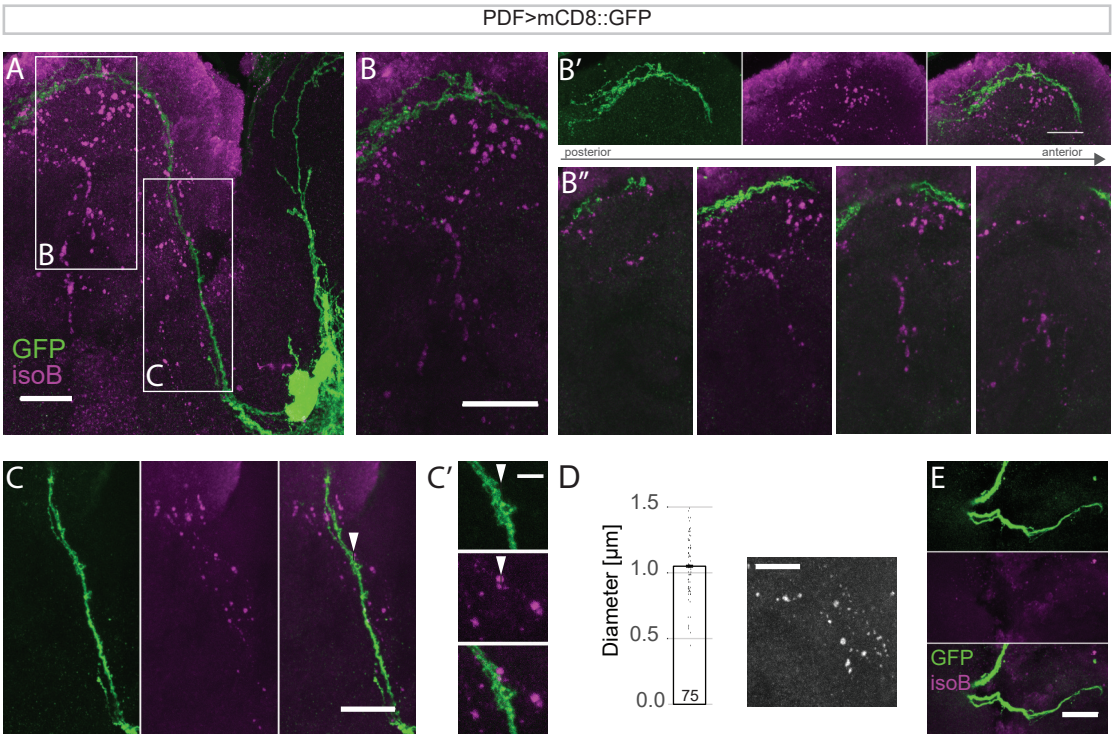


Figure 4.34: Projection of ICLI neurons into s-LN$_v$ output region in dPC - the expression pattern of PDF+ neurons is visualised by driving the reporter mCD8::GFP using PDF-Gal4. A-E, Confocal projection of isoB staining in PDF>mCD8::GFP genetic background: anti-isoB is depicted in magenta, anti-GFP staining is depicted in green. A, Confocal projection of isoB staining in dPC in PDF>mCD8::GFP genetic background. B-B", Magnified section of A, dPC. B, Connection of isoB+ and PDF+ arbours. B', Magnified section of B. B", Saggital sections through the dPC covering the ICLI input region. C-C', Magnified section of A, lateral PC. C, Initial segment of PDF+ dorsal projection. C', Magnified section of C, details of isoB+ varicosities intermingled with PDF+ terminals. D, Diameter of isoB+ varicosities in dorsal PC, n=75 varicosities. Size of varicosities are depicted along the y-axis to illustrate their variability, bar height reflects mean. E, Magnified section of isoB staining along PDF+ midline projection. Scale bars: A-C, 20 µm, C'-E, 10 µm.

indeed connected, we expressed the mCD8::GFP construct under the control of the (1) PDF driver and to counter-stain for isoB, and (2) VT026326 driver to counter-stain for PDF.

ICLI neurons enter the dorsal output region of s-LN$_v$: intermingling of CG10638-isoB+ terminals with PDF+ terminals is discernible in the PDF>GPF background (Fig. 4.34A). In the PDF>GFP background, CG10638-isoB+ terminals enter the PDF+ dorsal projection, but also cover a large area ventral to the PDF+ projection (Fig. 4.34B-B"). CG10638-isoB+ terminals are also present around the initial

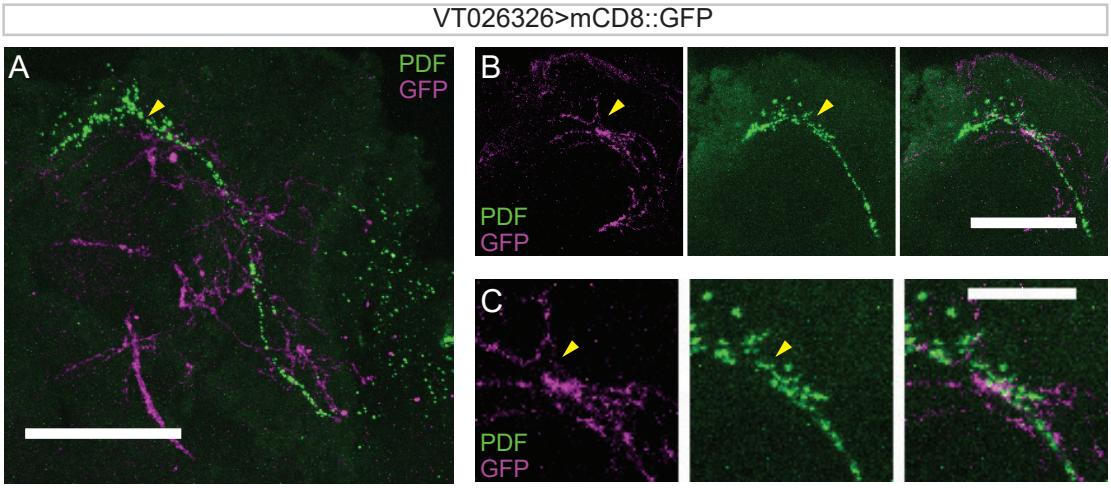


Figure 4.35: Projection of ICLI neurons into s-LN$_v$ output region in dPC - the expression pattern of ICLI neurons is visualised by driving the reporter mCD8::GFP using VT026326-Gal4. A, Confocal projection of anti-PDF staining in dPC in VT026326>mCD8::GFP genetic background. B, Magnified subsection of A. C, Magnified subsection of B. Scale bars: A-C, 20 µm.

segment of the PDF+ dorsal projection (Fig. 4.34C), at times in very close proximity (Fig. 4.34C'). We consistently detected such CG10638-isoB+ varicosities of variable size in the dorsal protocerebrum and lateral rim, with varicosities in the dorsal protocerebrum being relatively large with a diameter of around 1 µm (Fig. 4.34D). No CG10638-isoB+ terminals are detectable along the PDF+ midline projection formed by the l-LN$_v$ (Fig. 4.34E).

In the VT026326>GFP background, intermingling with of CG10638-isoB+ varicosities with PDF+ terminals is also discernible (Fig. 4.35A, G, see also the magnification in Fig. 4.35B). In this combination, the GFP+ projections from ICLI neurons curving around to follow the PDF+ dorsal projection are particularly clear to observe (Fig. 4.35C).

Synaptic connection between ICLI and s-LN$_v$ In order to ascertain whether ICLI neurons actually form synaptic connections with s-LN$_v$, we expressed one split-GFP component (spGFP$^{1-10}$) under the control of the PDF-lexA driver, and the other split-GFP component (spGFP^{11}) under the control of the VT026326 driver. Indeed, the combined expression of spGFP components in PDF+ and

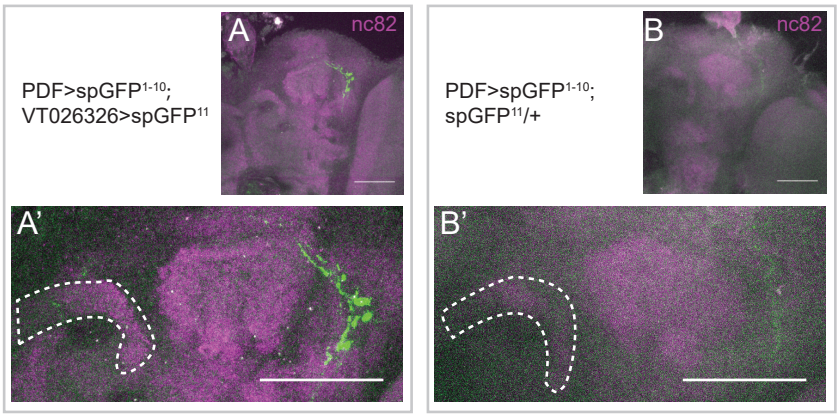


Figure 4.36: Synaptic connections of ICLI neurons. A, Confocal projection of whole-mount adult brain of PDF>spGFP$^{1-10}$;VT026326>spGFP^{11} genotype. GRASP signal (green) is shown together with anti-bruchpilot background staining (magenta). A', Magnified subsection of A. B, Confocal projection of whole-mount adult brain of PDF>spGFP$^{1-10}$;spGFP^{11}/+ control genotype. GRASP signal is absent, anti-bruchpilot background staining is present (magenta). B', Magnified section of B. Antibodies: A-B', nc82 (magenta), Scale bars: A-B', 50 µm. A', B', Dashed lines indicate the shape of the protocerebral bridge for positional reference.

ICLI neurons produces a functional, detectable GFP signal (Fig. 4.36A), while the omission of the VT026326 driver does not (Fig. 4.36B).

Input signals

sNPF receptor The s-LN$_v$ are known to express PDF and sNPF. In light of the reduction of ICLI neuron activity we observed under SD, we tested whether ICLI neurons could receive sNPF input as the sNPF receptor (sNPFR) has been found to exert inhibitory effects (section 1.4.2). Thus, we tested whether ICLI neurons could receive inhibitory inputs from the s-LN$_v$ via sNPF by staining ICLI neurons with an anti-sNPFR antibody obtained from Kweon Yu.

We visualised ICLI neurons by driving expression of mCD8::GFP under the control of the VT026326 driver. Indeed, ICLI neurons stain positive for the sNPFR (Fig. 4.37A), both in the dorsal projections (Fig. 4.37B) and the soma (Fig. 4.37C). Similarly, sNPFR and CG10638-isoB stainings overlap in ICLI somata of wildtype flies (Fig. 4.37D).

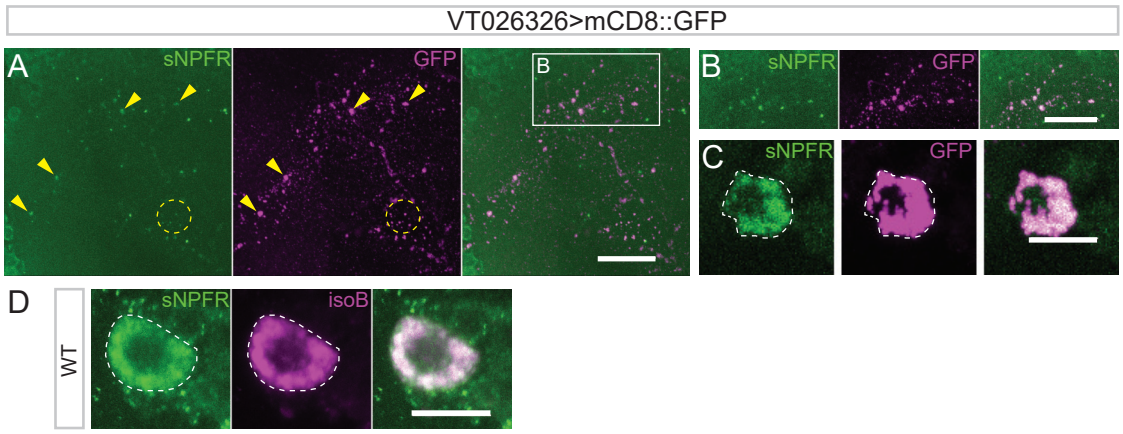


Figure 4.37: Expression of sNPFR in ICLI neurons. A, Confocal projection of dPC in VT026326>GFP reporter flies stained with anti-sNPFR. B, Magnified section of A. C, Confocal section through sNPFR+ soma in the VT026326>GFP background. D, Confocal section through soma stained for sNPFR and CG10638-isoB in WT background. Scale bars: A-B, 20 µm, C-D, 10 µm.

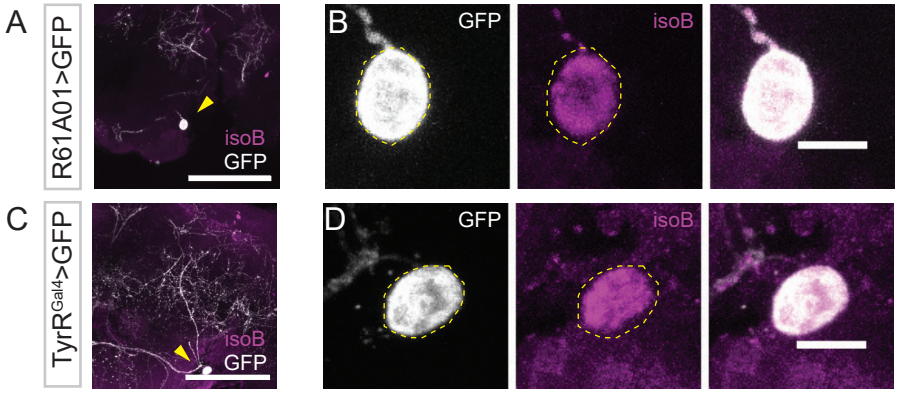


Figure 4.38: Expression of Tyramine receptor (TyrR) in ICLI neurons. A, Expression pattern of TyrR driver, projection of the ventrolateral protocerebrum. B, Magnification of A, representative section of soma. C, Expression pattern of the TyrR^{Gal4} driver, projection as in A. D, Magnification of C, representative section of soma. Scale bars: A, C, 50 µm; B, D, 10 µm.

Tyramine receptor A study by Huang et al. 2016 describes the expression of the Tyramine receptor (TyrR) in neurons strongly reminiscent of ICLI neurons. To adress this possibility, we visualised TyrR+ neurons by expressing mCD8::GFP under the control of two TyrR drivers (Fig. 4.38A, C). One driver line, TyR^{Gal4} (Fig. 4.38C), was generated by Huang et al. 2016. Indeed, anti-isoB stains the somata of TyrR+ neurons (Fig. 4.38B, D).

4.8 Summary

Our initial results pointed to the AKR CG10638 as a potential sleep regulator of *Drosophila melanogaster*. In this chapter, we aimed to validate and further characterise this candidate.

We confirmed the sleep phenotype associated with the CG10638 locus through another set of RNAi-mediated knock down experiments in the nervous system and through the examination of a hypomorph mutant line. The sleep phenotype comprises a pronounced deficit in sleep maintenance manifest in a severe suppression of sleep levels due to shortened sleep episodes. We also detected limited evidence that CG10638 knock down in the nervous system mildly weakens rest:activity rhythms, but did not detect an impairment in maintaining endogenous circadian rhythms *per se*. We also tested both RNAi and mutant flies for two other phenotypes commonly associated with the exemplary *Drosophila melanogaster* AKR, Hk, namely leg shaking and hypersensitivity to paraquat-induced oxidative stress. We did not detect an association of the CG10638 locus with either of these phenotypes. Thus, we conclude that it is unlikely that CG10638 acts predominantly through the Shaker complex. However, we found that both RNAi and mutant flies are hypersensitive to a sucrose-only diet which could indicate an involvement of CG10638 in sugar metabolism.

Through initial immunohistochemical experiments, we identified two potential neural populations that express CG10638. These include the PDF+ l-LN$_v$ – which predominantly express isoA – and a pair of MIP+ ventrolateral neurons termed ICLI neurons – which predominantly express isoB. In a first step, we validated these neural populations as functional substrates of CG10638 by restricting the RNAi-mediated knock down of CG10638 to PDF+ and to ICLI neurons, respectively. Of the two IR lines we employed for this study, both lines lead to a similar reduction of isoA, but only one line, IR# 2, leads to a pronounced reduction of isoB. Thus, while the CG10638-related RNAi transgenes we employed for this study were theoretically isoform-specific, our expression data demonstrate that they have differential effects on isoform levels, but are not isoform-specific.

Similarly, our assessment of the expression of CG10638 isoforms in the heterozygous $CG10638^{EY21723/+}$ mutant revealed that the down-regulation of one isoform can be associated with the up-regulation of another isoform, potentially to support compensatory mechanisms. Thus, we caution that behavioural results obtained through RNAi-mediated knock down of CG10638 need to be interpreted in the context of further experiments. Particularly, isoform-specific conclusions cannot be drawn from our RNAi experiments alone.

In a second step, we confirmed that reduction of CG10638 levels in PDF+ neurons using both IR lines suppresses sleep, particularly night sleep, and produces a biphasic night sleep profile. Using the hk^1 mutant, previous studies implicated the AKR Hk in the neural regulation of l-LN_v in response to blue light, but did not further assess a relation to sleep regulation (Fogle et al. 2015). We found that the effects of a PDF+ neuron-specific RNAi-mediated reduction of Hk levels are milder than the effects we detected for a nervous system-wide knock down of Hk (compare sleep profiles in Fig. 4.20B and Fig. 3.2B) and milder than the effects described for the hk^1 mutant by Daniel Bushey and collegues (Bushey et al. 2007). These findings suggest that Hk may act in other brain structures in a similar vein, i.e., to benefit sleep. For instance, our initial results demonstrate a sleep-supporting role for Hk in the MB which has been suggested due to the role that the Hk-associated α subunit, Shaker, plays in the MB (Bushey et al. 2007). Additionally, both the nervous system-wide knock down of Hk and the nervous system-wide knock down of CG10638 produces a biphasic night sleep profile with a through – a particularly pronounced sleep suppression – in the middle of the night. This is also true of PDF+ neuron-restricted knock down of CG10638, but not of PDF+ neuron-restricted knock down of Hk; the latter rather produces a mild and gradual sleep suppression in the second half of the night. As discussed in section 3.2.2, the two-process model predicts a dissipation of sleep pressure with sleep, i.e., throughout the night. Thus, a disruption of sleep-promoting regulators such as CG10638 and Hk could affect night sleep increasingly with the dissipation of other sleep-supporting mechanisms and the rising influence of the wake-promoting circadian system. Additionally, previous

studies that systematically probed arousal throughout the day and night in fruit flies detected a higher propensity for flies to wake up in the middle of the night compared to the beginning and end of the night: specifically, in the middle of the night, flies needed weaker stimuli to be aroused from sleep than in the beginning and end of the night (Alphen et al. 2013). Thus, sleep in the middle and the second half of the night seems to be more amenable to sleep disruption than the beginning of the night, and it may not be surprising that the disruption of molecular regulators of sleep had pronounced effects particularly at these times of the night.

Importantly, we also confirmed that the reduction of CG10638 levels in ICLI neurons also generally suppresses sleep, but we observed that we achieved a greater degree of sleep suppression with the IR line that reduces isoB levels more severely than isoA levels. Importantly, the reduction of isoB levels in ICLI neurons mainly suppresses day sleep which coincides with isoB expression levels. Through qRT-PCR experiments, we assessed the expression of isoB throughout the day and observed that isoB is expressed at high levels during the day, particularly during times of high sleep pressure. Specifically, isoB expression is particularly high at the time preceding the siesta, and at the time preceding night sleep; isoB levels are also upregulated in response to dedicated sleep deprivation. We confirmed these expression dynamics through immunohistochemisty: here, we detected high isoB protein levels at times of high sleep pressure, i.e., after the waking day in comparison to the morning, and after dedicated sleep deprivation.

We found that ICLI neurons are wake-promoting and that their activation strongly suppresses sleep. A natural route of ICLI neuron activation could be provided by the s-LN$_v$ as we demonstrate in this study that ICLI neurons innervate the output region of and form synaptic connections with s-LN$_v$. We found that ICLI neurons express the sNPFR which opens up the possibility that ICLI neurons receive input from s-LN$_v$ through sNPF release. An overview of the ICLI neuron anatomy, polarity, and connection partners is depicted in Fig. 4.39.

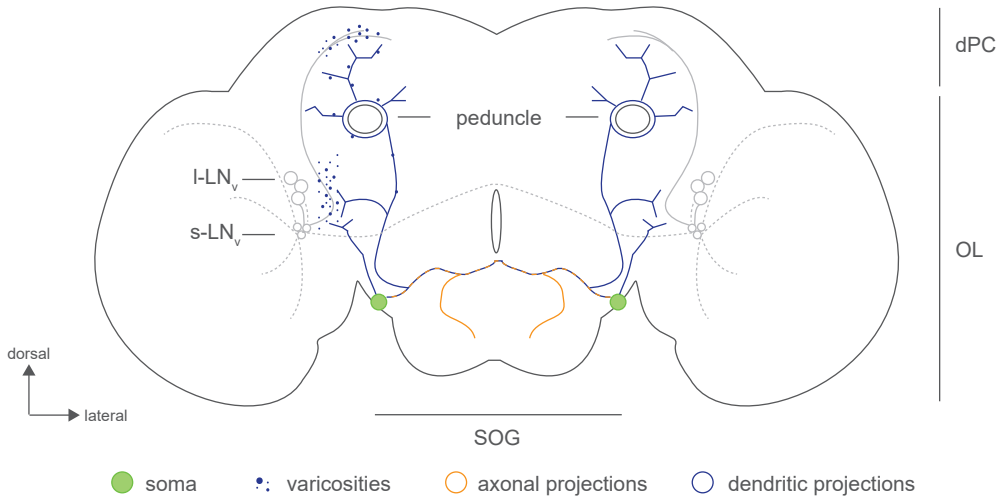


Figure 4.39: Schema of ICLI neurons polarity and inputs/outputs.

Furthermore, we confirmed that ICLI neurons are arousal-promoting and strongly respond to sexual cues. Reduction of CG10638 levels in ICLI neurons does not only severely suppress sleep, but also increases sexual arousal.

Taken together, we have validated that the CG10638 locus is associated with a sleep phenotype in a manner that is distinct for both isoforms. Generally, isoA inhibits activity of l-LN$_v$ during the night to benefit sleep, and isoB modulates ICLI neuron activity during the day to enable siesta sleep. Specifically, CG10638 protein and mRNA levels trace sleep pressure. ICLI neurons are wake-promoting MIP+ and TyrR+ neurons that express the sNPFR. Our experiments show that CG10638 negatively regulates ICLI neuron activity at times of high sleep pressure to counteract the arousing role of ICLI neurons which may be associated with wake-promoting input from the s-LN$_v$.

5

Results: candidate CG3397

5.1 Literature and *in silico* research relating to CG3397

Initial experiments presented in chapter 3 identified CG3397 as a potential sleep modulator of the MB since reduction of CG3397 in this brain structure suppresses sleep. Little is known about this gene so far.

Gene locus and protein isoforms

CG3397 (FlyBase symbol FBgn0037975, UniProt symnol Q9VGF1) is a candidate *Drosophila melanogaster* AKR of around 38 kDa (ExPasy, `http://web.expasy.org`). A schema of the gene locus is depicted in Fig. 5.1. It contains 3 exons (chapter 3, Table 3.2) that are predicted to encode only one isoform (chapter 3, Table 3.2).

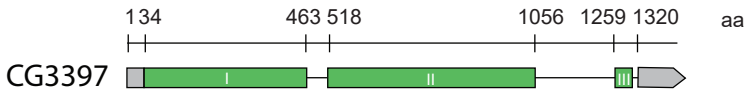


Figure 5.1: Annotated gene locus CG3397.

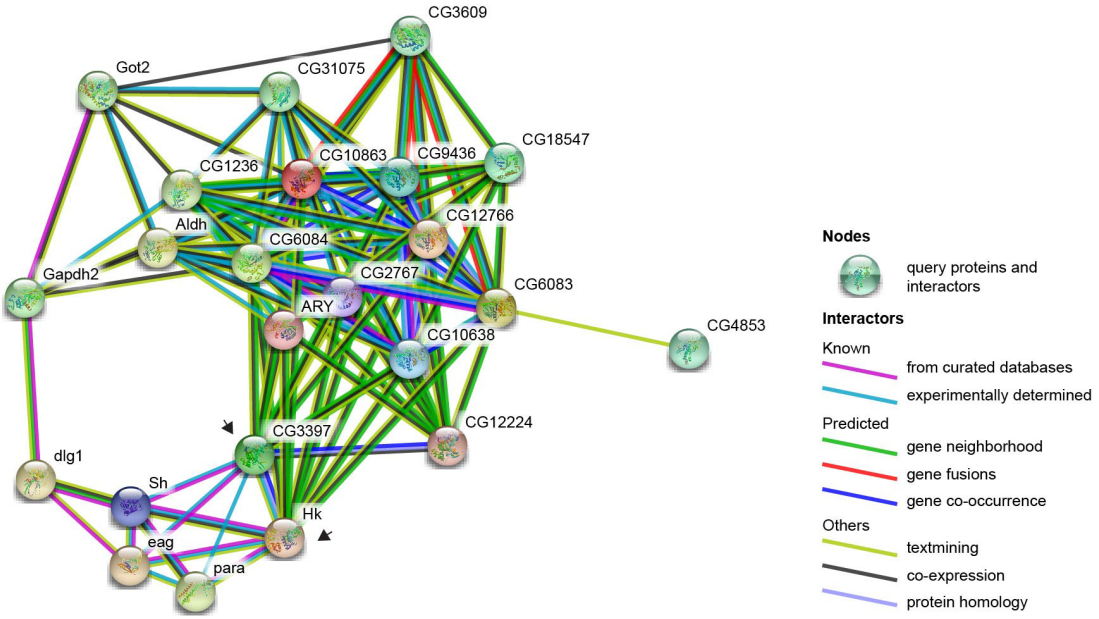


Figure 5.2: Predicted protein-protein interactions of AKRs (STRING PPI). Arrows indicate interaction partners discussed in the text.

Putative function

CG3397 belongs to the "atypical" group of *Drosophila melanogaster* AKRs with a relatively low rate of conservation of core AKR residues in a manner similar to Hk (chapter 3, Table 3.3) and shares high sequence identity with other AKRs of this group (Supplementary table 7.1). Due to its similarity to Hk, CG3397 is predicted to be involved in K^+ ion transport and associated neural transmission (Table 3.5). Protein-protein interaction networks also place CG3397 close to Hk (Fig. 5.2).

Similar to Hk, CG3397 may be an oxidative stress sensor: the expression of this gene is upregulated in response to high oxygen-induced oxidative stress (Gruenewald et al. 2009).

The neural substrates of Hk have not been comprehensively identified as no antibody exists. However, functional studies suggest that Hk nodulates neural activity in PDF+ neurons (section 1.6.5). Interestingly, CG3397 has also been indirectly related to the circadian clock (Lin et al. 2002), and the STRING database also predicts an association with Reg-5 – rhythmically expressed gene 5 – and Reg-2 (Fig. 5.3).

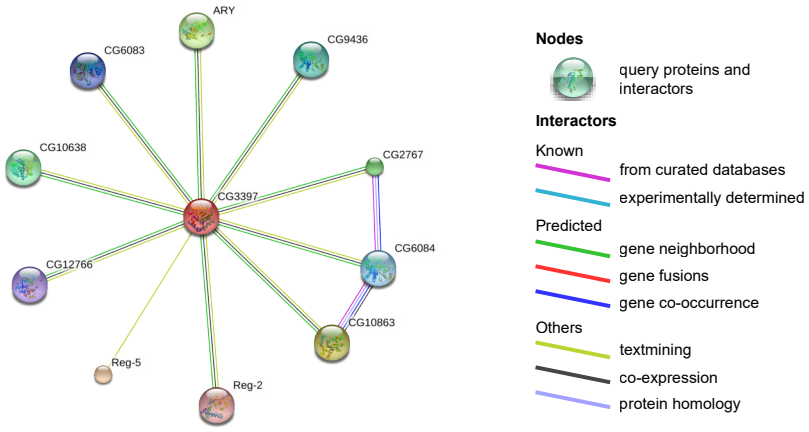


Figure 5.3: Predicted protein-protein interactions of CG3397 (STRING PPI).

A study focussed on *Drosophila* genes that respond to mutations in the *Myosin Heavy Chain* (*Mhc*) locus and ensueing muscle dystrophy detected an upregulation of CG3397 – among many others – in *Mhc* mutants which suggests that CG3397 may be involved in compensatory mechanisms (Montana et al. 2006).

5.2 Robustness and scope of sleep phenotype

Initial experiments identified CG3397 as a potential sleep modulator, and suggested the MB as its potential neural substrate since reduction of CG3397 in this brain structure suppresses sleep. We aimed to further assess robustness and scope of the phenotype and its association with the MB.

We evaluated the robustness of the phenotype using two independent strategies: we tested (1) another RNAi line targeting the CG3397 locus, and (2) a CG3397 mutant line.

5.2.1 RNAi-mediated knock down in whole MB

After an initial *in silico* search confirmed that CG3397 is predicted to encode only one isoform for which at least two RNAi lines were available, we sought to confirm the MB-specific sleep phenotype of CG3397 using independent RNAi and MB driver lines. We were also able to confirm the phenotype in an RNAi-independent manner by testing a CG3397 mutant insertion, EY04742.

Sequence specificity Both IR lines targeting CG3397 might have off-targets. A potential off-target with the highest match is the *Drosophila melanogaster* AKR CG12224 (Table 5.1).

MB-specific knock down Expression of the RNAi transgene GD10955 suppresses sleep as has already been described in section 3.2 (Fig. 3.3C; data included in Fig. 5.4 for comparison). A separate TRiP RNAi line that targets the CG3397 transcript also suppresses sleep: flies of the ok107>JF01835 genotype sleep around 2 h – or 12 % – less than parental control flies (Fig. 5.4, JF01836: $\chi^2(2)=23.56$, $p<0.001$, *post hoc* as annotated).

Since sleep was reduced to lower levels by expression of JF01836, we focussed on this line for further RNAi experiments. The line JF01836 will be denoted "IR" in this chapter.

hpRNA	mis=0	mis=1	mis=2	target
GD10955	325	0	0	CG3397
	51	74	76	CG12224-A
	0	4	12	CG18547
JF01836	412	0	0	CG3397
	127	74	80	CG12224-A
	0	4	12	CG18547

Table 5.1: On and off-target search for CG3397-IRs; "mis=0" number of complete matches, "mis=1" number of matches with one mismatch, "mis=2" number of matches with two mismatches. Only the first three hits are listed.

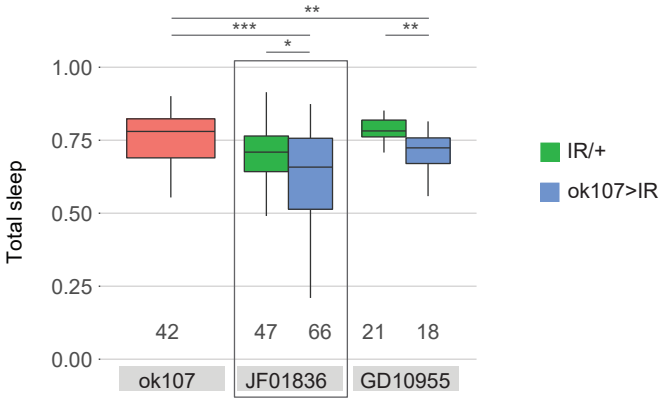


Figure 5.4: Daily sleep levels in two MB-specific RNAi lines targeting CG3397, sample size as annotated. Boxes reflect 25th (top) and 75th (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. Asteriks reflect result of Kruskal Wallis test to compare sleep levels between flies of the experimental genotype (ok107>IR) and parental controls (ok107/+, IR), whereby "*' reflects p<0.05, "**" reflects p<0.01, "***" reflects p<0.001, "n.s." signifies "not significant".

The sleep profile of flies expressing CG3397-IR under the control of the MB driver ok107 is depicted in Fig. 5.5A. IR expression in the MB reduces night sleep by around 1.9 h or 10 % compared to parental controls (Fig. 5.5B, $\chi^2(2)$=17.15, p<0.01, *post hoc* as annotated). The expression of CG3397-IR in the MB does not affect day sleep: RNAi flies sleep around the same amount of time as parental controls do, i.e., around 8 h during the day (Fig. 5.5C, $\chi^2(2)$=46.17, p<0.001, ok107>IR:ok107/+, p<0.001, ok107>IR:IR/+, p=0.723).

Flies expressing CG3397-IR in the MB also have mildly destabilised sleep: night sleep bout duration is shorter for RNAi flies than for parental controls by around 6.8 min or 22 % (Fig. 5.5D, $\chi^2(2)$=8.26, p<0.05, *post hoc* as annotated).

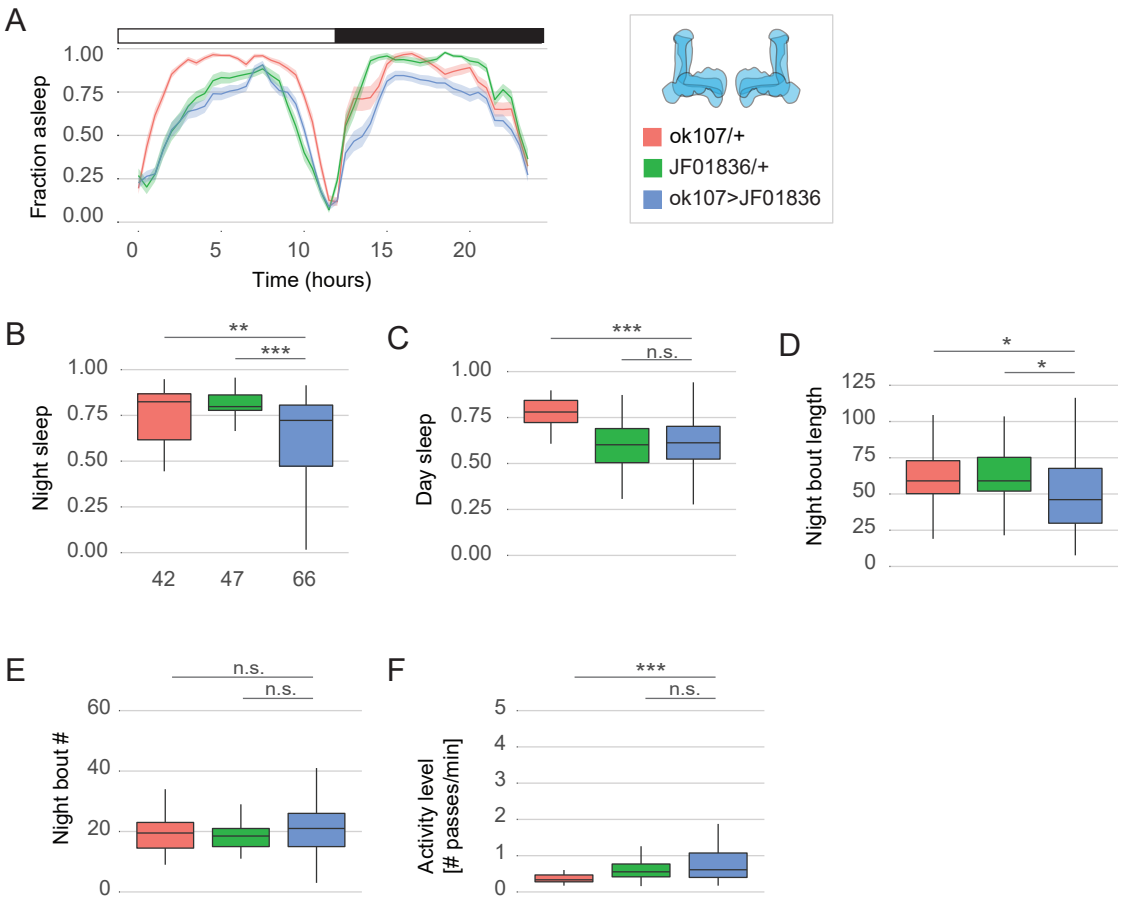


Figure 5.5: Sleep upon MB-specific knock down of CG3397. A, Sleep profile. B, Sleep levels during the night. C, Sleep levels during the day. D, Length of sleep bouts during the night. E, Sleep bout # during the night. F, Activity level. A-F, Sample size as annotated in B. Boxes reflect 25^{th} (top) and 75^{th} (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. Asteriks reflect result of Kruskal Wallis test to compare sleep parameters between flies of the experimental genotype (ok107>IR) and parental controls (ok107/+, IR), whereby "*' reflects $p<0.05$, "**" reflects $p<0.01$, "***" reflects $p<0.001$, "n.s." signifies "not significant".

The number of sleep bouts, however, is similar to parental controls, i.e., 19.7 min (Fig. 5.5E, $\chi^2(2)=1.16$, $p=0.56$).

The RNAi-mediated sleep phenotype associated with the CG3397 locus cannot be explained by hyperactivity since the activity level of ok107>CG3397-IR flies is similar to that of control flies (Fig. 5.5F, $\chi^2(2)=20.16$, $p<0.001$, ok107>IR:ok107/+, $p<0.001$, ok107>IR:IR/+, $p=0.547$).

5.2.2 RNAi-mediated knock down in MB subcircuits

There is a large collection of MB-specific driver lines available (Aso et al. 2009, 2014b) allowing us to dissect particular MB subcircuits that CG3397 might modulate. Thus, we expressed CG3397-IR under the control of drivers that target particular MB lobes and sublobes.

MB lobes

As described in section 1.5, the *Drosophila* MB have three main lobes: the $\alpha\beta$, $\alpha'\beta'$, and γ lobe. The driver lines we used were "R34B09" which expresses primarily in the $\alpha\beta$ lobes, "R35B12" which expresses primarily in the $\alpha'\beta'$ lobes, and "R10A06" which expresses primarily in the γ lobe.

RNAi-mediated reduction of CG3397 in particular MB lobes has differential effects: CG3397-IR expression in $\alpha\beta$ and γ lobes suppresses sleep (Fig. 5.6A). Expression of CG3397-IR in the $\alpha'\beta'$ lobes does not affect sleep levels (Fig. 5.6A, $\chi^2(6)=82.33$, $p<0.001$, *post hoc* R35B12>IR:IR/+, $p=0.42$, otherwise as annotated).

MB sublobes

The *Drosophila* MB lobes are again divisible into compartments, or sublobes (section 1.5) which are targeted specifically by a new generation of Gal4 lines termed "split-Gal4" lines (section 2.3). A number of split-Gal4 lines have been used in a dedicated study by Divya Sitamaran and collgues to specifically target MB sublobes in order to narrow down the sleep-regulatory role of MB to individual compartments: here, the split-Gal4 driver lines were used to drive the expression of a heat-activated actuator – UAS-TRPA1 – which induces neural activation to then measure ensueing changes in sleep levels (Sitaraman et al. 2015b). From this study, we selected 'wake-promoting' and 'sleep-promoting', as well as 'neutral' driver lines to test the effect of CG3397 knock down on the sleep-related activity levels of MB compartments. Particularly, sublobes of the γ lobe associated with an *increase* in sleep is γm (γ medial) targeted by MB607B and a 'neutral' γm line not associated with a sleep phenotype when TRP-activated is MB009B; sublobes of the $\alpha'\beta'$ lobe associated with a *decrease* in

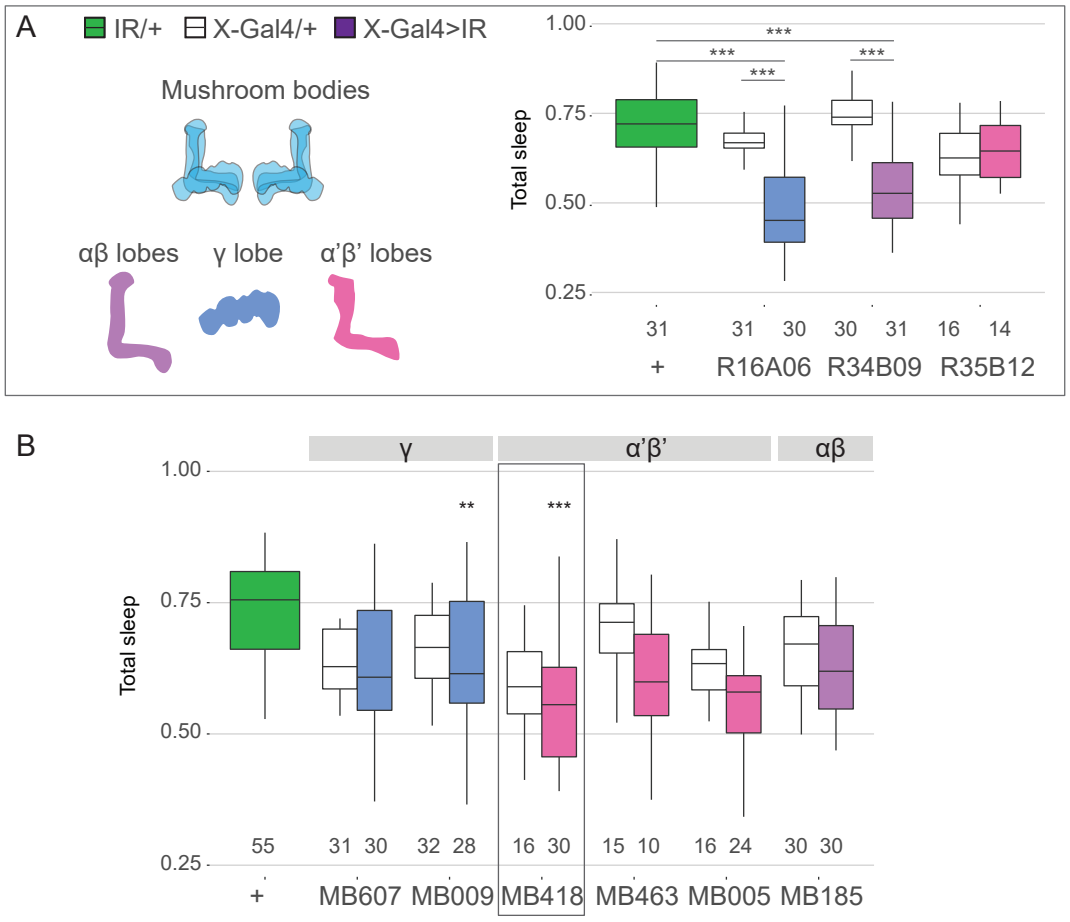


Figure 5.6: Sleep levels upon RNAi-mediated knock down of CG3397 in MB lobes and sublobes. A, Sleep levels upon knock down in MB lobes, sample size as annotated. B, Sleep levels upon knock down in MB sublobes, sample size as annotated. Boxes reflect 25th (top) and 75th (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. Asteriks reflect result of Kruskal Wallis test to compare sleep parameters between flies of the experimental genotype (ok107>IR) and parental controls (ok107/+, IR), whereby "*' reflects p<0.05, "**" reflects p<0.01, "***" reflects p<0.001, "n.s." signifies "not significant".

sleep are targeted by MB463B, MB418B and MB005[1]; and sublobes of the $\alpha\beta$ lobe were generally not associated with a change in sleep levels (Sitaraman et al. 2015b).

Throughout the three lobes, RNAi-mediated reduction of CG3397 in sublobes suppresses sleep: expression of CG3397-IR in the γm sublobe driven by MB009B mildly suppresses sleep, as well as the expression of CG3397-IR driven by MB185B in $\alpha\beta$ sublobes. We observed the strongest effect by CG3397 knock down in $\alpha'\beta'$

[1]All $\alpha'\beta'$ lobe-specific split-Gal4 lines produced a sleep phenotype when driving TRPA1 in this study, see Fig. 1B in Sitaraman et al. 2015b

sublobes using MB418B (Fig. 5.6B, $\chi^2(12)=57.2$, $p<0.001$, *post hoc* as annotated).

5.2.3 Mutant CG3397^{EY04742}

Sleep levels are reduced both in the homozygous and the heterozygous CG3397^{EY04742} mutant in comparison to WT controls (Fig. 5.7A). Specifically, daily sleep levels are reduced in the CG3397^{EY04742} mutant by around 2.9 h or 17 % in the homozygous mutant, and by around 3.8 h or 22 % in the heterozygous mutant compared to WT flies (Fig. 5.7B, $\chi^2(2)=14.5$, $p<0.001$, *post hoc* CG3397^{EY04742}:CG3397$^{EY04742/+}$, $p=0.356$, otherwise as annotated).

Night sleep is more dramatically affected by the expression of CG3397-IR in the MB: homozygous mutants sleep around 1 h or 9 % less than WT flies, while heterozygous mutants sleep around 3.6 h or 34 % less (Fig. 5.7C, $\chi^2(2)=20.45$, $p<0.001$, *post hoc* CG3397^{EY04742}:CG3397$^{EY04742/+}$, $p<0.01$, otherwise as annotated).

Effects on day sleep are milder than night-specific effects: homozygous mutants sleep around 1.7 h or 21 % less than WT flies, while heterozygous mutants sleep around 0.5 h or 7 % less (Fig. 5.7D, $\chi^2(2)=10.35$, $p<0.05$, *post hoc* CG3397^{EY04742}:CG3397$^{EY04742/+}$, $p<0.05$, otherwise as annotated).

Sleep architecture is only mildly affected in CG3397^{EY04742} mutants, particularly the heterozygous mutant. Night sleep is destabilised: in heterozygous mutants, night sleep bouts are shortened to 16 min, while the bout length of homozygous mutants is similar to that of WT flies, i.e., around 34.5 min (Fig. 5.7F, $\chi^2(2)=12.69$, $p<0.001$, *post hoc* WT:CG3397$^{EY04742/+}$, $p<0.01$, WT:CG3397^{EY04742}, $p=0.388$, CG3397^{EY04742}:CG3397$^{EY04742/+}$, $p<0.01$). The night bout number, however, is similar between CG3397^{EY04742} and WT controls, i.e., around an average of 25 bouts per night (Fig. 5.7E, $\chi^2(2)=0.5$, $p=0.78$).

Conversely, during the day, heterozygous mutants exhibit less sleep episodes of around 19 bouts, while homozygous mutants sleep in the same number of bouts as WT flies do, i.e., 34 bouts (Fig. 5.7G, $\chi^2(2)=14.79$, $p<0.001$, *post hoc* WT:CG3397$^{EY04742/+}$, $p<0.001$, WT:CG3397^{EY04742}, $p=0.235$, CG3397^{EY04742}:CG3397$^{EY04742/+}$, $p<0.01$). The day bout length, however, is similar

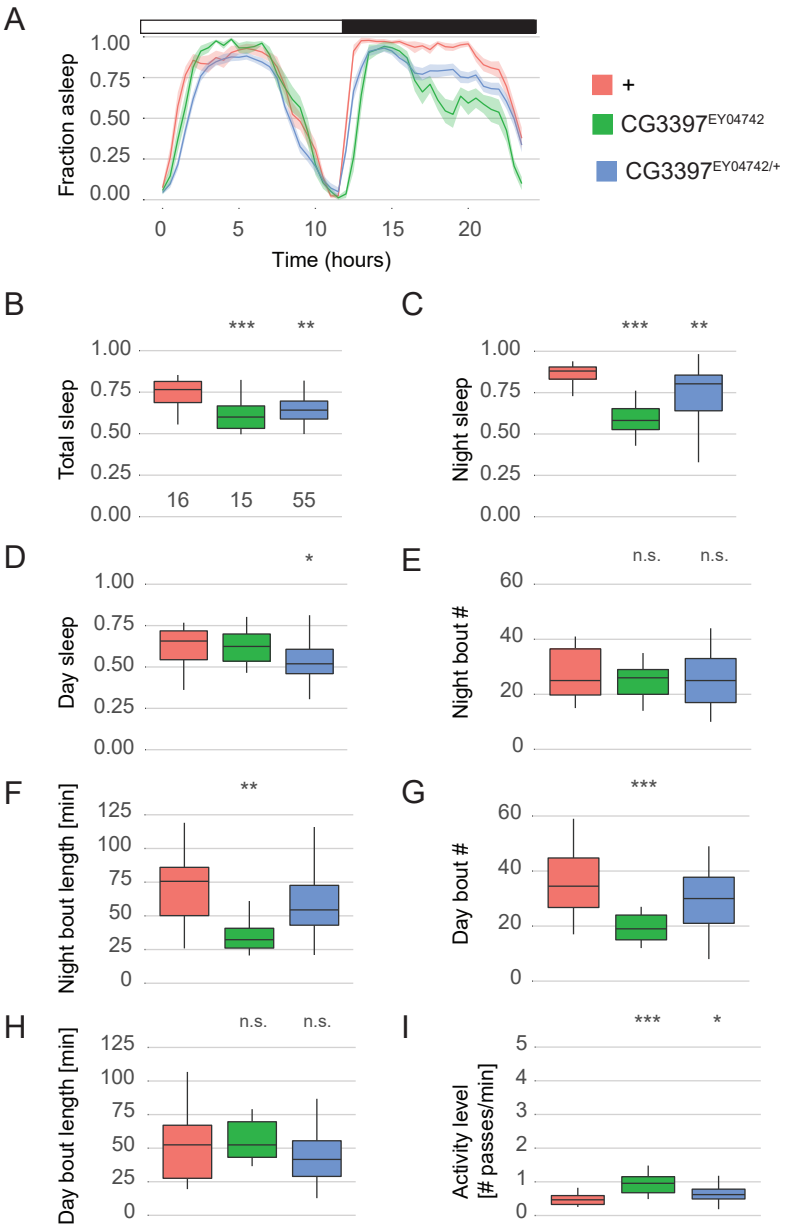


Figure 5.7: Sleep of CG3397^{EY04742} mutants. A, Sleep profile of homozygous and heterozygous mutants, wrap of three days of baseline recording. B, Daily sleep levels. C, Sleep levels during the night. D, Sleep levels during the day. E, Sleep bout # during the night. F, Sleep bout length during the night. G, Sleep bout # during the day. H, Sleep bout length during the day. I, Average daily activity level. A-I, Sample size as annotated in B. Boxes reflect 25th (top) and 75th (bottom) percentile, bar within the box reflects median, whiskers reflect 1.5 of inter-quartile range. Asteriks reflect result of Kruskal Wallis test to compare sleep parameters between mutant flies and wildtype controls, whereby "*' reflects p<0.05, "**" reflects p<0.01, "***" reflects p<0.001, "n.s." signifies "not significant".

in the CG3397^{EY04742} mutant to WT flies, i.e., 19 min (Fig. 5.7H, $\chi^2(2)$=3.78, p=0.15).

The activity levels of CG3397^{EY04742} mutants are increased compared to WT control flies (Fig. 5.7I, $\chi^2(2)$=17.16, p<0.01, *post hoc* CG3397^{EY04742}:CG3397$^{EY04742/+}$, p<0.05, otherwise as annotated).

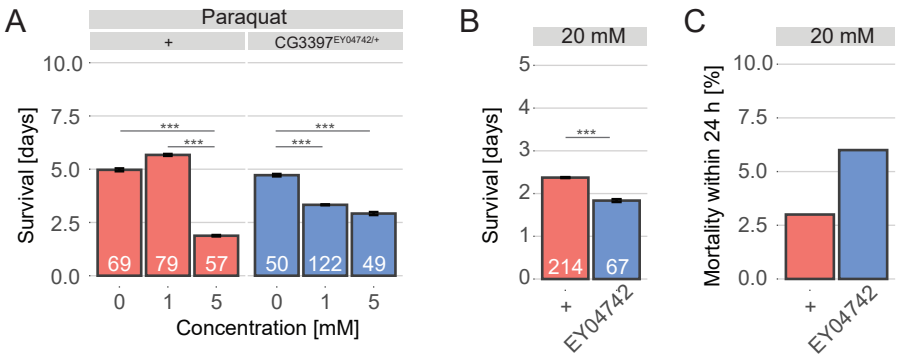


Figure 5.8: Oxidative stress susceptibility of CG3397$^{EY04742/+}$ mutants. A, Survival of WT and mutant flies chronically treated with increasing concentrations of PQ, sample size as annotated. B, Same as A for 20 mM PQ, sample size as annotated. C, Mortality within 24 h. A-C, Bar height reflects mean. A-B, Error bars reflect sem, although error bars do not rise above bar in most cases. Asteriks reflect results of ANOVA, whereby "*' reflects p<0.05, "**" reflects p<0.01, "***" reflects p<0.001, "n.s." signifies "not significant". Treatment: F(1,422)=152.13, p<0.001, genotype: F(1,422)=32.94, p<0.001, treatment×genotype: F(1,422)=33.94, p<0.001, *post hoc* WT 0 mM:1 mM, p=0.054, WT 0 mM:5 mM: p<0.001, CG3397$^{EY04742/+}$ 0 mM:1 mM, p<0.001, CG3397$^{EY04742/+}$ 0 mM:5 mM, p<0.001, 0 mM WT:CG3397$^{EY04742/+}$, p=0.946, 1 mM WT:CG3397$^{EY04742/+}$, p<0.001, 5 mM WT:CG3397$^{EY04742/+}$, p<0.001. Only heterozygous mutants were used in this experiment due to the difficulty in raising homozygous mutants at sufficient numbers.

5.3 Associated phenotypes

We also investigated the similarity of CG3397 with Hk by asssessing common phenotypes associated with the *hk* mutation – such as shaking and oxidative stress susceptibility – for an association with the CG3397 locus.

5.3.1 Sensitivity to oxidative stress

Paraquat (PQ) is an insecticide that is lethal to flies. At lower concentrations between 0 mM and 5 mM, both WT flies and CG3397$^{EY04742/+}$ mutant flies die of paraquat at a largely similar rate: at the control treatment consisting of 5 % sucrose (0 mM PQ), both mutant and WT flies die after around 5 days (Fig. 5.8A). At 1 mM PQ, mutant die slightly earlier than WT flies, i.e., 2.5 days earlier (Fig. 5.8A), and at 5 mM PQ, WT flies die slightly earlier than mutant flies, i.e., 1 day earlier (Fig. 5.8A, see legend for statistical analysis).

At high concentrations of paraquat (20 mM) CG3397$^{EY04742/+}$ mutants are

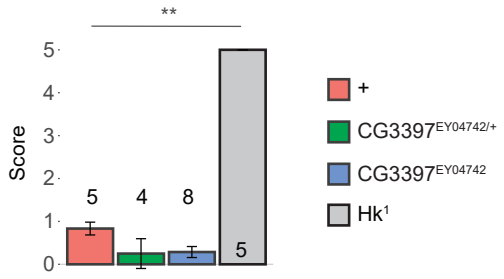


Figure 5.9: Shaking phenotype of RNAi (ok107>IR) and mutant flies, sample size as annotated above bar. Bar height reflects mean, error bars reflect sem. Asteriks reflect results of ANOVA, whereby "**" reflects p<0.01.

especially susceptible to paraquat-induced oxidative stress: mutant flies die earlier than WT flies both in the short term and in the long term. At high concentrations, 6.3 % of mutant flies died within 24 h compared to 3 % of WT flies (Fig. 5.8C). In the long term, mutant flies died around 1 day earlier than WT flies from high concentrations of PQ (Fig. 5.8B, $F(1,279)=13.68$, $p<0.001$).

5.3.2 Shaking

CG3397 is not associated with a leg shaking phenotype: heterozygous and homozygous CG3397^{EY04742} mutants have a low shaking score of 0.25 and 0.3, respectively, which is similar to the shaking intensity observed in WT flies (0.8). Therefore, CG3397^{EY04742} mutants do not shake at the pronounced levels detectable in Hk^{1} mutant flies which consistently reaches the maximum score of 5 (Fig. 5.9, $\chi^2(3)=14.92$, $p<0.001$, *post hoc* Hk^{1}:WT, $p<0.01$, Hk^{1}:CG3397$^{EY04742/+}$, $p<0.001$, Hk^{1}:CG3397^{EY04742}, $p<0.001$, CG3397^{EY04742}:CG3397$^{EY04742/+}$, $p=0.38$).

5.3.3 Homeostatic sleep response

Hk has so far not been associated with a homeostatic phenotype (Bushey et al. 2007). In order to test whether CG3397 expression levels respond to sleep pressure, we sleep deprived adult male flies overnight to measure mRNA levels of both CG3397 and Hk. While previous studies showed the absence of homeostatic sleep response on the behavioural level (Bushey et al. 2007), we now also demonstrate that *hk*

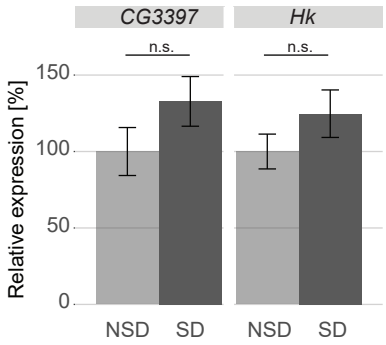


Figure 5.10: Expression of CG3397 and Hk in response to SD, n=4/treatment. Bar height reflects mean, error bars reflects sem, although error bars do not rise above bar in most cases. Asteriks reflect results of t-test to compare expression levels in response to treatment for each transcript, whereby "*' reflects p<0.05, "**" reflects p<0.01, "***" reflects p<0.001, "n.s." signifies "not significant".

mRNA does not respond to SD (Fig. 5.10, W=4, p=0.343). Similarly, the expression levels of CG3397 are also stable under SD (Fig. 5.10, W=3, p=0.2).

5.4 Summary

In our initial RNAi-mediated sleep screen directed at the MB, we detected a candidate AKR that suppressed sleep when knocked down in the MB. In this chapter, we aimed to validate and to further characterise this candidate.

In a first step, we tested another IR construct targeting the CG3397 locus and confirmed that this second line produces similar sleep-related effects. We also examined a CG3397 mutant line for a possible sleep phenotype. Both the MB-specific expression of the second RNAi construct, and the mutant line are associated with a sleep phenotype similar to the one we initially detected.

We also tested the second RNAi construct under the control of drivers that target one of the three main lobes of the MB as well as sublobes and found an association of CG3397 activity across MB compartments, particularly in the $\alpha'\beta'$ lobes.

Our *in silico* research revealed that CG3397 is relatively similar to Hk in structure and is predicted to perform similar functions. Thus, we tested other phenotypes associated with the *hk* mutation to examine their similarity functionally. Indeed, the CG3397 locus does share some functional similarities with Hk, but not all of the aspects we tested. Similar to Hk, CG3397 expression levels do not respond to SD. In a further similarity to Hk, CG3397 mutants are hypersensitive to paraquat-induced oxidative stress. However, other than Hk, CG3397 mutants are not susceptible to ether-induced shaking.

6 Discussion

6.1 Motivation and aim of this study

Sleep is a ubiquitous, or near-ubiquitous behaviour conserved across phylogeny even down to insect species (Campbell et al. 1984). As discussed in chapter 1, a range of functions has been attributed to sleep. While their hierarchy is still elusive, their essentiality for normal physical and cognitive functions is well established: ethological circumstances that chronically curtail sleep – such as shift work in humans and social housing in flies – impact negatively on health and cognitive performance. Even an acute lack of sleep is associated with a decline in cognitive and physical fitness in humans, rodents, and flies.

The first sleep mutation ever detected affect a fly K_v channel, *shaker* (*sh*), in the previously identified sleep center of the fruit fly brain, the MB (Cirelli et al. 2005a). The Shaker complex plays a major role in gauging neural excitability in a manner benefiting sleep (Pimentel et al. 2016; Wu et al. 2014b). The regulatory subunit of this complex in flies, Hyperkinetic (Hk) – which is associated with a sleep phenotype similar to *shaker* mutants (Bushey et al. 2007) – ties Shaker channel activity to cellular stress levels due to its sensitivity to ROS levels in the form of free NADP+ (Fogle et al. 2015). Its NADP/NADPH-dependent oxidoreductase

activity marks HK as an aldo-keto reductase (AKR), a conserved group of enzymes that play a major role in human health and disease (Gulbis 2000).

The abundance and importance of this enzymatic group in humans in combination with its conservation in fruit flies provides an excellent basis to examine whether AKRs systematically act as molecular regulators of sleep behaviour. In this study, we aimed to identify *Drosophila melanogaster* AKR genes aside from *hk* to examine their potential involvement in sleep regulation. To this end, we performed a thorough *in silico* study of potential AKR loci of the *Drosophila melanogaster* genome, and employed a range of genome mining tools to find their predicted biological functions. We then experimentally tested these potential AKRs for a sleep-regulatory role through a nervous system-wide and a MB-specific RNAi-mediated forward screen. We also identified and further characterised the neural populations expressing particular candidates and the role the candidate AKRs play in these cells to modulate sleep behaviour.

6.2 Screen for potential AKR sleep regulators

6.2.1 Identification of candidate AKRs

Approach

As described in chapter 1, a major sleep regulator in *Drosophila* is the AKR Hk. Therefore, we evaluated whether other AKRs fulfilled a similar, sleep-regulatory function. To this end, we searched the *Drosophila melanogaster* genome for AKR domains and characterised candidate loci. We further investigated the translated proteins from potential AKR genes and their predicted expression and function. Following *in silico* data collection, we moved on to discern which AKRs are actually expressed in *Drosophila* and which candidates were expressed primarily in the brain, in order to gauge the probability for a neural sleep regulator such as Hk. All AKRs except the Y-linked, likely testis-specific ARY were tested for sleep-related functions in an RNAi screen that either encompassed the whole nervous system or was specific to the MB.

Drosophila melanogaster AKRs

Our *in silico* research presented in chapter 3 identified a total of 12 genomic loci that might encode AKR domains, i.e., NADPH-dependent oxidoreductase domains. These potential AKRs are mostly clustered on chromosome 3, except for CG9436 – which is located on chromosome 2 – and the two sex chromosome-linked AKRs, *hk* (X-linked), and *ary* (Y-linked).

Potential AKRs fall into two main groups: one group exhibits high similarity with mammalian *Kcnab1* and *Kcnab2* genes which encode regulatory β subunits grouped into the AKR6 family. These include the already identified Hk, as well as CG3397, CG12224, and CG18547. Of these, the AKR CG3397 is the most likely to be involved in neurotransmission in a manner similar to Hk due to their high degree of sequence identity. Another, larger group exhibits high similarity with the AKR1 family of aldehyde and aldose reductases. These include CG10638, CG10863, CG12766, CG9436, CG6083, CG6084, CG2767, and ARY. The X-linked Hk and the Y-linked ARY have already been described (Bushey et al.

2007; Vibranovski et al. 2008), but all other *Drosophila melanogaster* AKRs have not received dedicated functional study, although they did surface as positive hits in a number of genetic screens, mainly concerned with stress such as hypoxia, hyperoxia, or starvation, or neural or muscle development, or neural maintenance (chapter 3 and Supplementary Table 7.1).

Drosophila melanogaster AKRs encode 5 exons on average which give rise to only one transcript in the majority of cases; for those AKRs with multiple predicted transcripts, the potential isoforms are often nearly identical. A notable exception is CG10638 which is predicted to encode two distinct isoforms, termed "isoA" and "isoB" for the purposes of this study (section 3.1). We find that isoA and isoB exhibit a relatively low degree of sequence identity of around 50 %. While isoA exhibits perfect conservation of canonical AKR residues, isoB substitutes the Ser-271 residue for an Alanine. The Ser-271 residue is part of the co-factor binding pocket and highly conserved among AKRs, although a mutagenesis study of this particular residue to discern its specific effects on co-factor binding has not been undertaken (Jez et al. 1997a). However, studies of dual NADH/NADPH specificity in the related superfamily of short-chain dehydrogenase/reductases (SDR) demonstrate a shift in co-factor specificity due to the mutation of a Serine residue of the co-factor binding pocket (Cho et al. 2003; Nakanishi et al. 1997). Another study detected an involvement of the Serine residue in co-factor specificity also in AKRs (Di Luccio et al. 2006). These findings open up the possibility that isoB may not directly respond to the $NADP^+$/NADPH ratio of the cell; alternatively, CG10638 may rather respond to NAD^+ and/or NADH, or may not act according to cellular oxidative stress levels at all. These alternatives would have to be addressed by a dedicated study of the enzymatic kinetics of CG10638 at different $NADP^+$:NADPH concentrations, and of the differences between the isoforms in this regard. Such molecular studies have been carried out to investigate the substrate and co-factor binding properties of the rat $K_v\beta$ AKR (Weng et al. 2006), and to discern the potential dual NADH/NADPH specificity of the *Drosophila melanogaster* AKR CG10863 (Di Luccio et al. 2006). Such molecular experiments for CG10638 require

knowledge of its potential substrates. Our *in silico* research revealed that the closest potential mouse orthologue of this AKR is AKR1c19. A detailed study of the substrate specificity of the mouse AKR1c19 by Shuhei Ishikura and collegues provides a starting point for the investigation of substrate and co-factor properties of CG10638: for instance, this study suggests that AKR1c19 may exhibit NADH-linked activity *in vivo*, and also identified several potential substrates *in vitro*, including isatin (Ishikura et al. 2005), a molecule involved in the modulation of stress and anxiety levels (Bhattacharya et al. 1991).

Although it was not within the scope of this study, the identification of potential biological functions of *Drosophila* AKRs would be greatly enhanced by a dedicated *in silico* investigation into their *structural* properties. The protein structure of Hk has been resolved (Gulbis et al. 1999), including its adaptation to interactions with its α subunit, Shaker (Gulbis 2000), providing an initial model template for the structural characterisation of other potential $K_v\beta$ subunits. Similarly, the structure of several AKR1 members in other species have also been resolved (reviewed in Jez et al. 1997a), providing model templates for the structural characterisation of other potential *Drosophila* AKRs as well. In this vein, such a study has been undertaken by Eric Luccio to identify novel AKRs: this study included the potential *Drosophila* CG10863 and characterised its enzymatic properties in the context of a structural model (Di Luccio et al. 2006).

6.2.2 Candidate sleep regulators

Several candidate AKRs are abundantly expressed throughout the body and brain

Through initial work presented in chapter 3, we successfully detected all predicted *Drosophila melanogaster* AKRs in the body. Also, most AKRs are present in the head with the exception of *ary*. This Y-linked gene is essentially absent from the brain and likely specific to the testis (section 3.2.1). This expression pattern is not unprecedented among AKRs: several mammalian AKRs are expressed predominantly in the testis (Pratt-Hyatt et al. 2013), and mutations of one AKR1,

AKR1C2/AKR1C4, are associated with the development of ambiguous genitalia in humans due to the involvement of this protein in the testosterone pathway (Flück et al. 2011; Penning 2015). Due to its sex-specific expression and absence from the nervous system, ARY was not included in further functional sleep studies.

Similar to AKRs of other species, *Drosophila melanogaster* AKRs are expressed primarily in the body (see for expression of mammalian AKRs, e.g., O'Connor et al. 1999; Pratt-Hyatt et al. 2013; Tu et al. 2009). We detected high expression levels of several AKRs in the body, particularly of the oxidative stress-responsive potential aldehyde reductases CG6083, CG10863, and CG12766. In light of the relevance of liver and white adipose tissue homologue – the fat body – in the organisation of daily, regular behaviours such as sleep and feeding (Grönke et al. 2005; Thimgan et al. 2010, 2015, section 1.5), these candidates pose a promising starting point for an investigation into the involvement of *Drosophila melanogaster* AKRs in behavioural organisation by the fat body. The Malpighian tubules of *Drosophila* could serve as a secondary target as they are the renal equivalent of the human kidneys and adrenal gland, express a range of human disease genes, and express, for instance, *sh* and *hk* (Wang et al. 2004).

A notable exception among mammalian AKRs that do show expression in the nervous system are members of family 1 (Pratt-Hyatt et al. 2013), particularly the aldehyde reductase AKR1A1 which has been detected in the cerebrum (O'Connor et al. 1999). Interestingly, one fly AKR is enriched in the brain – CG10638 – to an extent that exceeds Hk; this AKR is relatively similar to other AKRs of the AKR1 family and also predicted to be an aldehyde reductase (section 4.2). This particular AKR encodes two isoforms – isoA and isoB – that are expressed at vastly different levels in all tissues tested with higher expression levels of isoA, although both isoforms are enriched in the brain relative to the rest of the body.

As this study provides an initial characterisation of *Drosophila melanogaster* AKRs in general and their putative sleep-regulatory function in particular, we limited our search to *neural* regulators of sleep in order to achieve specific, robust, and interpretable results: the two-process model of sleep regulation provides a

framework to dissect mechanisms that drive sleep (section 1.1.2), the mechanisms that drive sleep that have so far been described in detail are mainly related to the nervous system (sections 1.5 and 1.4), and the effects of neural populations on sleep are largely based on their identifiable position within a network. This level of specificity is very difficult to achieve when perturbing peripheral tissues due to the abundance of potential secondary effects. However, follow-up studies should address the possibility of a more general involvement of *Drosophila melanogaster* AKRs in fly health and fitness, including sleep behaviour.

A limited number of AKRs specifically modulate sleep

Our RNAi-based sleep screen among *Drosophila melanogaster* AKRs revealed evidence for the involvement of a limited number of candidate AKRs in neural sleep regulation.

Given the detectable expression of *Drosophila melanogaster* AKRs in the head in general, and the nervous system-specific enrichment of one AKR – CG10638 – in particular, we tested fly AKRs for their role in neural sleep regulation through RNAi-mediated knock down in the nervous system. We used a moderate driver line to express RNAi transgenes targeting AKRs as we observed lethality issues using a stronger approach for CG18547 and CG2767 when RNAi flies failed to develop past the larval stage and/or past the hardening of the cuticle during early adulthood (section 3.2.2).

We find that both CG18547 and CG2767 are expressed at low levels in the adult nervous system. Given the role that insect AKRs fulfil in ecdysis (section 1.6.3), it is possible that these two *Drosophila melanogaster* AKRs benefit specifically development through the ecdysteroid pathway which would generalise the involvement of AKRs in insect ecdysis to *Drosophila*. A systematic measurement of CG18547 and CG2767 in the molting fluid and the body of *Drosophila melanogaster* larvae could ascertain whether these AKRs were expressed during these developmental stages.

Regarding sleep, the reduction of AKRs in the nervous system generally does not affect sleep, although there are two exceptions: we were successful in confirming

Hk as a neural sleep regulator and in identifying another AKR – CG10638 – as a candidate whose reduction suppresses and destabilises sleep in a manner distinct from Hk. Upon pan-neural knock down of CG10638 using two independent RNAi transgenes, flies strongly suppress sleep.

Regarding the mounting evidence for the sleep-regulatory role of the MB, we also tested the effects of MB-specific RNAi-mediated knock down. Here, we confirmed the sleep-regulatory role of Hk specific to the MB, although the degree of sleep suppression due to RNAi-mediated reduction of Hk in the MB is smaller than the pan-neuronal reduction, suggesting that Hk acts through neural populations other than the MB to modulate sleep. We also identified another AKR, CG3397, that might promote sleep through MB-dependent activity. We did not detect sleep suppression due to the RNAi-mediated reduction of CG3397 in the whole nervous system, however. This discrepancy could be due to the different strengths of the pan-neural ELAV and the pan-MB ok107 driver: ELAV drives moderate levels of actuator expression which proved to be important for our study in order to avoid lethality associated with some *Drosophila* AKRs (see above). The MB-specific driver, ok107, however, is one of the strongest MB drivers (Aso et al. 2009). Also, the MB-specific knock down of CG10638 using one RNAi transgene suppresses sleep, although this is not the case for the other RNAi transgene. The absence of lethality issues, as well as the limited amount of candidates we isolated from the MB-specific screen – i.e., Hk, CG3397, and, possibly, CG10638 (isoA) – suggests that *Drosophila* AKRs generally do not fulfil sleep regulatory functions in the MB. However, the MB-specific sleep suppression of CG3397 knock down – compared with the mild effect of Hk knock down in the MB and the pronounced effect of Hk knock down in the whole nervous system – suggests that, within the nervous system, CG3397 could fulfil a sleep-regulatory role specific to the MB in contrast to Hk. Thus, we confirmed, for the first time, that Hk has indeed the potential to modulate sleep through MB-specific activity. Furthermore, we identified another candidate AKR – CG3397 – that might promote sleep. Our *in silico* research presented in chapter

3 identified CG3397 as an AKR that is relatively similar to Hk in structure and (predicted) function, including the potential involvement in K^+ ion transport.

6.3 Characterisation of a novel sleep regulator: CG10638

6.3.1 Involvement of both isoforms in sleep behaviour

The sleep phenotype associated with both isoforms is robust

The coincident sleep-related effects of a nervous system-specific knock down and the natural brain enrichment of CG10638 marks this AKR as a promising neural sleep regulator.

Our *in silico* research presented in chapter 3 revealed that CG10638 has two isoforms, "isoA" and "isoB". As briefly discussed in section 1.6, the existence of different isoforms derived from alternative splicing is not uncommon among AKRs: some mammalian isoforms of the AKR6 family have been investigated *in vitro* (Barski et al. 2013) and potential splice variants of farnesal dehydrogenases of *Aedes aegypti* have been characterised *in vitro* and *in vivo* (Rivera-Perez et al. 2013).

Thus, in experiments presented in chapter 4, we used two RNAi constructs to target the CG10638 locus which are predicted to be specific to one isoform only. However, when we tested the knock down efficiency and specificity achieved through expression of the RNAi transgenes in the nervous system, we found that, while both RNAi transgenes indeed reduce CG10638 expression, this reduction is not entirely isoform-specific: both RNAi lines reduce isoA levels by around 50 %, but only one RNAi line, GD10458, also strongly reduces isoB. When we used these RNAi constructs to reduce CG10638 expression in the nervous system, we detected pronounced sleep suppression for both constructs in two separate sets of experiments. While the expression of both constructs similarly suppresses night-specific sleep, the expression of the transgene GD10458 also suppresses day sleep. In light of the reduction of CG10638 isoforms we actually achieve with this approach, we hypothesise that both isoforms may be involved in sleep regulation, and that the reduction of isoA suppresses night sleep, and the reduction of isoB suppresses day sleep, and, possibly, night sleep as well.

We corroborated the sleep phenotype associated with the CG10638 locus using CG10638^{EY21723} mutants which carry a plasmid insertion in the exon common to both isoforms. The homozygous mutant exhibits a strong reduction of expression levels of both isoforms by around 55-75 %. Interestingly, in the heterozygous mutant, expression levels of isoA are mildly decreased, but expression levels of isoB 'overshoot' to around 150 % of wildtype levels.

While both our RNAi-based and our mutant-based approach are valid to study the general involvement of CG10638 in sleep regulation, we cannot use these gentic tools alone to differentiate the role of the two isoforms. We encounter two main drawbacks with two RNAi transgenes that target the CG10638 locus: while expression of the RNAi transgenes has some differential effects on isoform expression, it is not isoform-specific. Thus, the sleep behaviour we observe when expressing RNAi transgenes could be due to a combined effect of both isoA and isoB reduction. Additionally, we cannot exclude that CG10638 isoforms may compensate for each other. This consideration is corroborated by our observation that, in the case of the heterozygous CG10638^{EY21723} mutant, reduced isoA levels are associated with an increase of isoB expression.

Thus, we combined our behavioural investigations using RNAi and mutant lines with other isoform-specific approaches to distinguish both isoforms as distinctly as possible: for instance, we quantified isoform-specific expression levels under different experimental conditions using qRT-PCR, and characterised the neurons expressing either or both isoforms through immunohistochemistry.

The sleep phenotype associated with both isoforms is likely not directly linked to oxidative stress

Our *in silico* analysis discussed above revealed that at least one isoform, isoA, contains the canonical AKR residues essential for NADPH-dependent oxidoreduction.

Since at least one of the isoforms *may* respond to oxidative stress, we tested an association of the CG10638 locus with the paraquat-induced oxidative stress response. We chose this particular method of oxidative stress induction as it has been demonstrated to provoke oxidative stress hypersensitivity in the mutant of another

Drosophila melanogaster AKR, hk^1 (Wang et al. 2000). We detected hypersensitivity to paraquat-induced oxidative stress neither in flies with nervous system-wide RNAi-mediated knock down of CG10638 nor in CG10638^{EY21723} mutants. These findings do not preclude the possible involvement of one or both CG10638 isoforms in oxidative stress regulation: in both RNAi flies and mutants, we detected low residual expression of CG10638 which may be sufficient to support compensatory mechanisms. Similarly, the hk^1 mutant exhibits high mortality of around 50 % within 48 h to high concentration of paraquat (10 mM), while wildtype flies exhibit a high survival rate of around 97 % (Wang et al. 2000). Since we observed a mortality rate of around 10 % already within 24 h for wildtype flies, our high concentration of paraquat (20 mM) may have masked a lethality effect in RNAi and mutant flies of CG10638. Thus, another set of experiments should be conducted with lower concentrations of paraquat, e.g., 10 mM, to increase the survival time of wildtype flies and leave more room for the detection of a potential decrease in survival time for RNAi and mutant flies of CG10638.

The sleep phenotype associated with both isoforms is not directly linked to circadian sleep regulation

In the framework of the two-process model of sleep regulation, the circadian and the homeostatic sleep drive interact to shape sleep (section 1.1.2). The circadian system comprising sets of "clock neurons" such as the small and large LN_v (s-LN_v, l-LN_v) and aided by other neurohormonal and neuropeptidergic populations, is crucial for sleep *timing* such as its organisation into distinct portions of siesta and night sleep (section 1.4). Other brain structures such as the MB, the dFSB and other aminergic and neuropeptidergic populations, together with peripheral tissues, modulate sleep *quality* particularly in response to acute sleep pressure (section 1.5).

We rule out that isoB modulates the circadian sleep drive: flies with RNAi-mediated knock down of CG10638 in the nervous system, as well as CG10638^{EY21723} mutants, exhibit largely normal circadian rhythms. We did not detect evidence for a disruption of the circadian rhythm in CG10638^{EY21723} mutants, neither in the heterozygous, nor in the homozygous combination. Similarly, the RNAi transgene

that produces particularly strong reductions of isoB (GD10458) does not alter circadian rhythms. However, we did detect a mild weakening of the circadian rhythm when we expressed the RNAi transgene that mainly targets isoA (GD7046) in the nervous system. This is consistent with the destabilisation of sleep we initially observed when we reduced CG10638 levels using GD7046 in the nervous system in the RNAi-based sleep screen (section 4.3.1).

Thus, we conclude that CG10638 does not mainly modulate sleep through the circadian sleep drive and that the main difference between the sleep profile of flies with reduced levels of CG10638 and control flies is sleep suppression, instead of a shift of the sleep time window. We cannot draw isoform-specific conclusions regarding the involvement of CG10638 isoforms in circadian and/or homeostatic sleep regulation as our genetic tools are not entirely isoform-specific. However, our findings could point to a role for isoB that is independent of the circadian system, while isoA may modulate the circadian sleep drive without perturbing the circadian system in general. To explore this possibility, we aimed to identify the sleep-regulatory neural populations that express isoA and/or isoB.

Both isoforms act through distinct neural populations

The vastly different expression levels of CG10638 isoforms suggest that they are expressed in different tissues (section 3.2.1). In a set of immunohistochemical experiments presented in chapter 4, we identified the neural populations that predominantly express one isoform in two steps: firstly, in the CG10638^{MB06512} mutant line – which carries a GFP insertion in the CG10638 locus – we detected GFP expression in the l-LN$_v$ and in a ventrolateral cell body, as well as fine projections in the vicinity of the MB peduncle. Secondly, we designed peptides to generate isoform-specific antibodies which we used to detect CG10638+ cells in the central nervous system. Through these stainings, we detected predominant expression of isoA in l-LN$_v$ and predominant expression of isoB in a pair of MIP+ ventrolateral "ICLI" neurons that project towards the MB peduncle and beyond.

We confirmed these expression patterns in two steps: firstly, we directed the RNAi-mediated knock down of CG10638 to ICLI neurons using a driver line which we validated expresses specifically in ICLI neurons, and detected a reduction of isoB protein levels in RNAi flies. Secondly, we tested behaviourally whether the RNAi-mediated knock down of CG10638 in l-LN$_v$ and ICLI neurons, respectively, would suppress sleep as we had observed in similar experiments with a nervous system-wide driver. Indeed, expression of either RNAi transgene targeting CG10638 – which are both expected to reduce isoA levels – in l-LN$_v$ suppresses, destabilises, and delays sleep, particularly in the night. Similarly, expression of either RNAi transgene in ICLI neurons suppresses and destabilises sleep, particularly during the day, albeit more severely for the RNAi transgene that efficiently reduces isoB expression levels.

Thus, we conclude that CG10638 isoforms are expressed in particular neural populations: while isoA is predominantly expressed in l-LN$_v$, isoB is predominantly expressed in ICLI neurons.

The action of isoA in l-LN$_v$ could mainly account for the strong suppression of night sleep we observed in the initial nervous system-wide RNAi-based experiment. As discussed in section 1.4.2, the l-LN$_v$ are wake-promoting; they drive high activity levels particularly in the morning through an increase in neural activity particularly in the second half of the night which needs to be inhibited through GABA receptor signalling to maintain sleep until the morning (Gmeiner et al. 2013; Liu et al. 2014).

The action of isoB in ICLI neurons mainly benefits day sleep. The day-specific effect is considerably stronger when we reduce isoB specifically in ICLI neurons in comparison to a reduction driven by ELAV in the whole nervous system. This could be due to relatively moderate expression levels of ELAV in comparison to the ICLI-specific driver line, VT026326, and/or the combined effect of reducing isoA in l-LN$_v$ and isoB in ICLI neurons. As discussed in section 1.5.2 and confirmed through our stainings described in section 4.5, MIP+ neurons are a relatively large population of around 40 cells that include neurons of the PI and SOG, as well as several other neurons distributed throughout the brain including ICLI neurons (Min et al. 2016). Cholinergic subsets of MIP+ neurons such as a pair of inferior anterior medial (IAM)

neurons are associated with food-related arousal and the regulation of bodyweight (Min et al. 2016), and a more specific subset of MIP+ neurons consisting of ICLI and very few other neurons is associated with sexual arousal (Jiang et al. 2013, see also Huang et al. 2016). Feeding and mating occur preferentially during the day in the diurnal insect *Drosophila melanogaster* (e.g., Xu et al. 2008, section 1.3.4, and Sakai et al. 2001, section 1.3.3) and it would thus not be surprising that changes in arousal levels regarding food and potential mates may impact on day sleep levels.

6.3.2 Isoform A: a sleep timer that runs on sugar?

IsoA supports night sleep through l-LN$_v$

As described in section 4.3, we found that the expression of both RNAi transgenes that target CG10638 in the nervous system similarly reduce the expression of isoform A and also lead to pronounced suppression of night sleep. Our immunohistochemical experiments point to an expression of CG10638, primarily of isoA, by the l-LN$_v$. The expression of both RNAi transgenes specifically in PDF+ neurons comprising s-LN$_v$ and l-LN$_v$ replicates the night-specific sleep suppression and strongly affects night sleep dynamics: reduction of CG10638 in PDF+ neurons suppresses and destabilises night sleep, and strongly delays both day and night sleep (section 4.6).

This finding is consistent with mounting evidence that implicates the l-LN$_v$ in sleep regulation: GABAergic inhibition of l-LN$_v$ is crucial for the suppression of activity and the maintenance of sleep particularly during the night, as well as for the timely initiation of night sleep, but is not necessary for the maintenance of circadian rhythms (Gmeiner et al. 2013; Liu et al. 2014, section 1.4.2). Thus, we hypothesise that isoA aids in the inhibition of the wake-promoting l-LN$_v$ during the night to support sleep.

IsoA activity is likely distinct from Hk activity

Another AKR, Hk, also acts through the l-LN$_v$: a dedicated study by Keri Fogle and colleagues investigated the l-LN$_v$-specific activity of Hk in response to light simulation (Fogle et al. 2015). Here, a pulse of blue light is given around ZT18 and

activates the l-LN$_v$; this neural activation stems from the excitation of CRY by blue light, which, in turn, inhibits Hk and releases its channel inactivation of K$_v$ channels such as ether-a-go-go (Fogle et al. 2015, section 1.6.5). This relationship of Hk with membrane excitability also manifests in an ether-induced shaking phenotype (see section 1.6.5). We did not detect evidence for a similar shaking phenotype associated with the CG10638 locus. This does not preclude the possibility that CG10638 is involved in neurotransmission: both the CG10638^{EY21723} mutant and the ELAV>CG10638-IR flies exhibit low residual expression of CG10638 which may enable sufficient protein production to compensate ether-induced effects. Furthermore, studies focussed on the ether-induced shaking phenotype of *hk* mutants did not identify a neural population responsible for this behaviour (Wang et al. 2000). It is possible that CG10638 is not naturally expressed in cells that mediate shaking behaviour or not at sufficient levels.

However, our experiments suggest that Hk and CG10638 do not act through the same substrates, e.g., the Shaker complex, in a similar manner to modulate sleep: in our RNAi-based approach, both the nervous system-wide and the PDF+ neuron-specific reduction of Hk has different sleep-related effects in comparison to the reduction of CG10638. For instance, the nervous system-wide reduction of Hk suppresses both day and night sleep as has been reported for the *hk*^{1} mutant (Bushey et al. 2007), while the PDF+ neuron-specific reduction of Hk suppresses sleep only in the latter half of the night which is consistent with the sleep-promoting role of Hk in the regulation of l-LN$_v$ activity which increase their level of activity in the second half of the night, unless they are inhibited (see section 1.6.5). Conversely, the nervous system-wide reduction of CG10638 suppresses mainly night sleep, as does the PDF+ neuron-specific reduction of CG10638, where the night sleep suppression is particularly strong in the middle of the night. Furthermore, our *in silico* analysis revealed that, among *Drosophila melanogaster* AKRs, CG10638 – particularly isoform A – is relatively distant to Hk and more closely related to other aldehyde reductases such as CG10863 (section 4.2). Experimental evidence collected by Eric Di Luccio demonstrates that CG10863 is an NADPH-dependent aldehyde reductase

that metabolises glyceraldehyde, but also isatin, among other substrates (Di Luccio et al. 2006), which partly overlaps with the substrate specificity of the putative CG10638 orthologue, the mouse AKR1c19 (Ishikura et al. 2005).

Putative involvement of isoA in glycolysis

Importantly, both flies with RNAi-mediated knock down of CG10638 in the nervous system – particularly flies with predominant knock down of isoA – and $CG10638^{EY21713}$ mutant flies are hypersensitive to a diet that consists exclusively of 5 % sucrose, a disaccharide combining glucose and fructose. In comparison to our standard fly medium which contains 8 % fructose, this a relatively low sucrose concentration. Sucrose-only diets are generally unhealthy for flies and can induce sugar toxicity even at relatively low concentrations: for instance, a previous study found an increased susceptibility to paraquat treatment in wildtype flies when paraquat was combined with a sucrose-only diet of 1 % sucrose instead of a standard diet comprising cornflour, sucrose, and yeast (Rzezniczak et al. 2011). *Drosophila* encounter high levels of sugar in their natural diet (Chng et al. 2016, see sections 1.3.1 and 1.3.4) and evolved specific adaptive mechanisms to maintain carbohydrate homeostasis in the short term, although chronic exposure to a diet very high in sugar leads to metabolic diseases such as obesity and diabetes even in flies (Chng et al. 2016). High dietary sugar levels recruit molecular regulators of the fat body that have been implicated in sleep regulation such as *Unpaired2* and *lipid storage droplet 2*, as well as the neural insulin-pathway centred around the sleep-regulatory IPCs (see sections 1.3.4 and 1.5.2).

The increased sensitivity to a sucrose-only diet we observed in flies with decreased levels of CG10638 implies this AKR in carbohydrate metabolism. In fact, CG10638 could participate in carbohydrate homeostasis mechanisms such as glycolysis that break down carbohydrates to prevent sugar toxicity (Chng et al. 2016). This hypothesis is supported by the predicted interaction of CG10638 with Sodh-1 and Sodh-2 (Sorbitol dehydrogenase 1 and 2) which derives from the interaction of Sord (Sorbitol dehydrogenase) and AKR1d1 in mouse (section 4.2), and it

is not unprecedented even among human AKRs: the human aldose reductase (AKR1B1) has a core function in glycolysis where it metabolises glucose to sorbitol with the help of NADPH before sorbitol is converted to fructose by sorbitol dehydrogenase in the sorbitol (polyol) pathway (Yabe-Nishimura 1998). This AKR1B1-dependent mechanism is important to regulate potentially toxic sorbitol levels (Tomlinson et al. 1994).

An isoA-dependent modulation of glucose levels could be a possible mechanism to aid the sleep-promoting inhibition of the l-LN$_v$ at night. The modulation of membrane excitability through glucose metabolism by AKRs is not unprecedented. For instance, in pancreatic β cells, Shaker-type K$_v$ channels can be modulated by glucose: high glucose levels increase the K$_v$ current, leading to membrane hyperexcitability (Yoshida et al. 2009).

Taken together, CG10638 may negatively regulate the activity of the l-LN$_v$ to support sleep maintenance at times of rising l-LN$_v$ activity. Regulation of l-LN$_v$ activity by CG10638 may be achieved through the lowering of glucose levels by CG10638-dependent glycolysis and a concomitant decrease of membrane excitability of l-LN$_v$ modulated by K$_v$ channels. Electrophysiological experiments need to be undertaken to test this hypothesis: a first set of experiments should test whether the reduction of CG10638 levels – through an RNAi-mediated strategy or mutation of the gene locus – modulates the membrane excitability of l-LN$_v$. In a second step, epistasis experiments of CG10638 and Sodh-1 and Sodh-2 could discern whether these enzymes have similar sleep-related effects, and whether they act through the same pathway. A third step should elucidate whether the manipulation of this pathway specifically impacts on sleep through l-LN$_v$.

6.3.3 ICLI neurons: a wake-promoting output of s-LN$_v$

ICLI neurons receive input from s-LN$_v$

Our immunohistochemical experiments presented in chapter 4 revealed that isoB is exclusively expressed in ICLI neurons. ICLI neurons possess a striking anatomy: they consist of a pair of ventrolateral somata that connect when crossing to the

contralateral side of the brain to project to the contralateral dorsal protocerebrum, thereby by-passing the MB peduncle. Through the expression of a dendritic marker in ICLI neurons, we show that ICLI neurons receive input in the dorsal protocerebrum. In fact, through the expression of a GFP probe bridging synaptic partners, we demonstrate that ICLI neurons receive input from a set of clock neurons, the s-LN$_v$, in the dorsal protocerebum (section 4.7.5). We also consistently found large accumulations of isoB+ varicosities that intermingle with the PDF+ dorsal projection of the s-LN$_v$, both in the dorsal protocerebrum and the lateral rim (section 4.7.5). Our finding that ICLI neurons connect to the s-LN$_v$ in the dorsal protocerebum is supported by previous work that identified ICLI neurons as outputs of PDF+ neurons: a study conducted by Christiane Helfrich-Forster and collegues ectopically over-expressed PDF in order to detect neurons that had the enzymatic capacity to synthesise PDF, i.e., were likely peptidergic, and that were able to respond to PDF, i.e., were likely to express the PDFR. This study detected both of these abilities in ICLI neurons ("neuron 8", Helfrich-Förster et al. 2000).

We also detect expression of the sNPFR in the projections and somata of ICLI neurons, opening up the possibility that ICLI neurons receive input from the s-LN$_v$ through their release of the inhibitory neuropeptide transmitter sNPF (section 4.7.5).

Inspired by another study that investigated TyrR+ neurons (Huang et al. 2016), we demonstrate that ICLI neurons are identical to TyrR+ neurons described in that study, although ICLI neurons receive Tyramine from as yet unidentified sources. Interestingly, one of the first transcriptome studies directed at clock neurons found that *Tyrosine decarboxylase 2* (*Tdc2*), encoding an enzyme crucial for the synthesis of Tyramine, is expressed and cycles in the s-LN$_v$ and that reduction of *Tdc2* in s-LN$_v$ dampens activity and the circadian rhythm (Huang et al. 2013).

Thus, we conclude that ICLI neurons receive input from s-LN$_v$ in the dorsal protocerebrum via PDF and sNPF. A possible release of Tyramine from s-LN$_v$ to ICLI neurons is speculative at this point due to the lack of functional evidence.

ICLI neurons are arousal- and wake-promoting

ICLI neurons are wake-promoting As an RNAi-independent strategy to discern whether ICLI neurons have the potential to modulate sleep and wake, we thermogenetically activated ICLI neurons and found that the acute activation of these neurons promotes wake (section 4.7.1). Specifically, under a regular light schedule, this wake-promoting role of ICLI neurons is restricted to the night; in acute darkness, the activation of ICLI neurons additionally suppresses day sleep. This striking light-dependent modulation points to the existence of different mechanisms regulating day and night sleep under natural conditions. At this point, the mechanisms governing the light-related modulation of sleep behaviour through ICLI neurons are speculative. These findings demonstrate, however, that changes to ICLI neuron activity have the potential to modulate both day and night sleep.

If ICLI neurons were truly wake-promoting, they would be expected to be inhibited at times of high sleep pressure in order to allow sleep to occur. To test this hypothesis, we sleep deprived flies that expressed a Ca^{2+} reporter in ICLI neurons to trace neural activity in response to sleep deprivation. Indeed, we found that ICLI neurons are inhibited during periods of acute sleep deprivation (section 4.7.1).

IsoB-mediated inhibition of ICLI neurons promotes sleep to the expense of sex Through immunohistochemical experiments, we confirmed the expression of MIP by ICLI neurons which was proposed by an earlier study by Min et al. 2016. Here, cholinergic subsets of MIP+ neurons were identified as particular substrates of food-related arousal associated with MIP+ neurons (Min et al. 2016). We also tested ICLI neurons for a bodyweight and feeding phenotype, but found no evidence for a role of ICLI neurons in this behaviour (section 4.7.3). This finding is consistent with our observation that ICLI neurons do not express the common marker of cholinergic neurons, choline acetyltransferase (ChAT), and are thus unlikely to be cholinergic (section 7.3).

Another study by the same research group focussed on a more restricted subset of MIP+ neurons, NTL+ neurons, that includes the ICLI neurons, and found that

silencing of NTL+ neurons using TNT led to a *decrease* in male-female courtship and copulation success (Jiang et al. 2013). Another study also described a copulation phenotype associated with ICLI neurons – in that study identified as TyrR+ neurons and confirmed by us to be ICLI neurons – with silencing of these neurons using TNT leading to an *increase* in sexual arousal that manifests in increased courtship in male-female but also in male-male interactions (Huang et al. 2016). We could not provide further direct evidence to resolve these discrepant results since we found the expression of TNT in ICLI neurons using our driver to be lethal. However, our expression of RNAi transgenes targeting CG10638 – which might affect ICLI neuron activity more mildly – produces viable offspring that demonstrated a clear *increase* in sexual arousal, both in male-female and in male-male interactions, corroborating previous findings of an involvement of ICLI neurons in sexual arousal as demonstated by Huang et al. 2016.

Our observation of an increased sexual arousal in flies with reduced levels of isoB in ICLI neurons are not only consistent with earlier studies (compare Huang et al. 2016), but also with our finding that isoB likely acts to inhibit ICLI neurons to allow sleep to occur. Under normal circumstances, the initiation and maintenance particularly of day sleep requires the withdrawal from arousing stimuli such as sexual cues. For instance, recent fruit fly studies have shown that sleep deprivation – and the ensueing need for rebound sleep – suppress sexual arousal that manifests in a decrease of male courtship (Chen et al. 2017). Conversely, our study points to the hypothesis that decreased sleep levels provide a window of opportunity for sex-related behaviours to occur, suggesting that the regulation of sleep and sex is intimately linked.

Our findings suggest that ICLI neurons are arousal- and wake-promoting output neurons of the s-LN$_v$.

6.3.4 Isoform B: a molecular sleep homeostat of ICLI neurons

A negative regulator of ICLI neurons

We observed a sleep suppression due to the reduction of CG10638, particularly isoB, in ICLI neurons. IsoB modulates sleep in a distinct manner through ICLI neurons: isoB mainly consolidates siesta sleep; it also supports night sleep, particularly under conditions of sleep-destabilising prolonged darkness (sections 4.6 and 4.7.3). Consistent with earlier studies described in section 1.3.3, we observed that under prolonged darkness, control flies suppress sleep during the subjective day and shift most of their sleep into the subjective night. With reduced isoB levels in ICLI neurons, flies do not fully accomplish this night shift of sleep (section 4.7.1). Similarly, isoB knock down in ICLI neurons delays the initiation of siesta sleep by almost 50 % (section 4.6). These findings suggest that isoB fulfils an inhibitory role in ICLI neurons to promote the initiation and maintenance of sleep at times of intense wake-promoting input from s-LN$_v$, in the morning and in the evening.

No involvement of isoB in circadian sleep regulation through ICLI neurons

In light of the impact of isoB on sleep initiation and the connection of ICLI neurons to s-LN$_v$, we examined whether isoB modulates ICLI neuron activity mainly to support the circadian drive, i.e., whether isoB in ICLI neurons acts predominantly to stabilise rest:activity rhythms and sleep timing. When we reduced isoB expression levels in ICLI neurons and subjected flies to free running conditions of constant darkness, we did not detect a disruption of the circadian rhythm in RNAi flies. This corroborated our previous experiments using a nervous system-wide driver for RNAi-based experiments and the CG10638^{EY21713} mutant in which we also did not find evidence for a disruption of the circadian rhythm (sections 4.3).

Thus, we rule out that isoB acts through ICLI neurons mainly to modulate the circadian sleep drive.

IsoB expression traces sleep pressure

We found that expression levels of isoB mRNA systematically change during the day where it exhibits two peaks: isoB mRNA levels are particularly high around siesta sleep and in the period leading up to night sleep (section 4.7.2). Similarly, on the protein level, we found that isoB accumulates in the course the waking day compared to the morning. In short, while isoB is differentially expressed at different times of the day, it is not expressed in a classically cycling pattern reminiscent of, e.g., the canonical 'clock gene' *period* whose expression levels gradually increase in the beginning of the day and peak in the beginning of the night before gradually decreasing throughout the night (Hardin et al. 1990).

Our molecular observations of the isoB expression dynamics coincide with the behavioural effects of RNAi-mediated isoB reduction in ICLI neurons which leads to the delay and suppression of siesta sleep, and the delay of night sleep. There is mounting evidence that day sleep provides the main time window for homeostatic sleep rebound to occur (e.g., Ganguly-Fitzgerald et al. 2006; Huber et al. 2004). Also, the parallel upregulation of isoB levels in response to acute sleep deprivation and at times after prolonged wake, i.e., noon and evening, support the notion that day sleep is largely driven by mounting sleep pressure. Our molecular and behavioural findings suggest that isoB expression could correlate with acute oxidative stress levels that rise with prolonged wakefulness (Villafuerte et al. 2015). As discussed in section 1.2, sleep deprivation and oxidative stress are closely linked: for instance, oxidative stress response genes are consistently upregulated in transcriptome studies of sleep deprived animals (Moller-Levet et al. 2013). In *Drosophila*, oxidative damage with age may contribute to a deterioration of sleep quality (Koh et al. 2006). Similarly, as discussed in section 1.1.5, one of the main functions of sleep may be the alleviation of metabolic load put on neurons through unspecific synaptic growth during wake. In fact, prolonged sleep deprivation leads to a build up of damaging oxidative stress levels in neurons (Villafuerte et al. 2015). It is not unprecedented among AKRs to be expressed according to acute oxidative stress levels: the transcription of AKR1 genes responds to oxidative stress through the action of the oxidative/electrophilic

stress-responsive Nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor of the leucine zipper family (Penning 2017; Wu et al. 2012). The transcription factor Nrf2 is conserved in *Drosophila* in sequence and in function: for instance, flies express a homologue of *Nrf2*, *Cnc-C*, which is also involved in proteasome regulation in this organism (Grimberg et al. 2011). Also, flies over-expressing the Nrf2 repressor *Keap1* (*Kelch-like ECH-Associated Protein 1*) exhibit increased resistance to paraquat-induced oxidative stress (Sykiotis et al. 2008).

To test the hypothesis of oxidative stress-dependent transcription of isoB, the previously discussed replication of the paraquat-induced oxidative stress assay at differing concentrations would provide crucial insights: if levels of isoB were indeed regulated by oxidative stress levels to modulate sleep, then isoB could be upregulated in flies subjected to oxidative stress induced, e.g., by paraquat, at least locally in its neural substrate, ICLI neurons. In fact, previous studies detected an upregulation of CG10638 in response to high oxygen-induced oxidative stress along with neuroprotective chaperones (Girardot et al. 2004; Gruenewald et al. 2009; Landis et al. 2004). Also, studies relating CG10638 to the Brm-SWI/SNF complex (Waldholm et al. 2011) – a major regulator of transcription modulated my oxidative stress levels (Fontana et al. 2017) – support the notion of an oxidative stress-related expression of CG10638 (section 1.6.6). In light of these tissue-wide transcriptomic studies, the neural substrate and tools we validated in this study enable the assessment of isoB protein levels in a restricted neural population and provide a local target to measure the effects of oxidative stress levels on isoB transcription. Taken together, such experiments would provide a promising route to investigate the relevance of oxidative stress-dependent transcription regulation by Nrf-2 in a specific cellular population not only *in vivo*, but in regards to behavioural function.

IsoB supports sleep homeostasis through ICLI neurons

If isoB would be a true homeostatic regulator in ICLI neurons, it would be expected to accumulate with increasing sleep pressure. When we acutely sleep-deprived flies and measured isoB expression levels immediately in the morning, we found an

increase both of isoB mRNA in the head, and an increase of isoB protein levels specifically in ICLI neurons (section 4.7.2). Furthermore, if isoB would play an active role in shaping ICLI neuron activity according to acute levels of sleep pressure, we hypothesised that a disruption of isoB expression should affect the homeostatic sleep response. Indeed, when we acutely sleep deprived flies with RNAi-mediated knock down of CG10638 in ICLI neurons for the course of one night, RNAi flies showed a delayed homeostatic sleep response compared to parental control flies (section 4.7.3). This observation points to a weakening of the homeostatic drive in the face of reduced CG10638 levels in ICLI neurons such that the wake-promoting circadian drive cannot be overcome to allow rebound sleep to occur.

Our findings support a role of isoB in homeostatic sleep regulation that aids in sleep initiation and maintenance according to acute levels of sleep pressure as indicated, for instance, by rising oxidative stress levels.

Our molecular and behavioural evidence demonstrates that isoB in ICLI neurons does not only initiate and consolidate siesta sleep, but also initiates and maintains the homeostatic sleep response during the day (see above). Thus, the flexibility in adapting the sleep schedule partly against the circadian clock and the environment seems to depend, in part, on the role isoB plays in ICLI neurons. Previous studies demonstrate a pronounced and distinct increase in day sleep – as well as a milder increase in night sleep – in the pdf^{01} mutant (Parisky et al. 2008) and the *pdfr* mutant (han^{5304}, Chung et al. 2009) compared to wildtype controls. These findings indicate that mechanisms antagonising PDF signalling may be particularly important to support day sleep. Our results point to a wake-promoting role of ICLI neurons, likely through excitatory PDF signalling by s-LN_v that is antagonised by isoB to allow sleep to occur at times when the circadian sleep drive most antagonises the sleep drive, at nap time. Interestingly, when we examined the cellular and subcellular location of isoB in ICLI neurons in more detail, we detected isoB not only in the soma and projections, but also in and/or around synaptic terminals of ICLI neurons, where we also observed expression of the sNPFR (section 4.7.5).

6.3.5 Future routes to explore neural inhibition through isoB activity

As part of this study, we focussed on the functional implications of isoform B of CG10638 for sleep regulation. We show that isoB is expressed by MIP+ ICLI neurons which we demonstrate to make connections with the s-LN$_v$ and to be also TyrR+, sNPFR+, and likely PDFR+. The expression of isoB correlates with sleep pressure, and it acts in ICLI neurons to promote sleep during the day, particularly when sleep pressure is high, antagonising the wake-promoting input from s-LN$_v$.

The molecular functions of CG10638 have not been specifically assessed as part of any study. However, consistent with our *in silico* search, protein-protein interaction networks and experimental evidence identify CG10638 generally as a negative regulator of the proteasome (Vinayagam et al. 2014). Furthermore, our search of another protein-protein interaction database, STRING, predicted an interaction of CG10638 with fabp (fatty acid binding protein). As discussed in section 4.2, *fabp* levels are linked to sleep: specifically, constitutive overexpression of *fabp* reduces sleep levels (Gerstner et al. 2011). Fabp is also a dendritic protein involved in dendritic morphology (Misra et al. 2016) and, importantly, levels of *fabp* may be regulated by ubiquitination (Ramirez et al. 2015). The putative interaction of CG10638 and fabp suggests that CG10638 may regulate FABP protein levels in order to gauge the connectivity of ICLI neurons to the wake-promoting s-LN$_v$ in a manner that benefits sleep. This mechanism is not unprecedented: the dorsal projection of s-LN$_v$ exhibits a circadian rhythm of plastic growth and retraction (Fernández et al. 2008). This rhythmic remodelling is supported by sleep (Donlea et al. 2009) and underlies the circadian dynamic of the establishment of connections with its synaptic partners (Gorostiza et al. 2014). The putative interaction of CG10638 and fabp should be examined further, e.g., by testing whether acute changes in *fabp* expression levels impact on ICLI neuron morphology in the dorsal protocerebrum in a manner that impacts on its connectivity with the s-LN$_v$, and whether such changes replicate the sleep phenotype associated with the CG10638 locus. For instance, if the s-LN$_v$ indeed exert a wake-promoting influence over ICLI

neurons, the dendritic arbor of ICLI neurons in the dorsal protocerebrum could be retracted at regular sleep times when s-LN$_v$ activity is still high, e.g., during siesta, or at times of acutely high sleep pressure.

An alternative, not necessarily incommensurate role of isoB in ICLI neurons could be related to receptor homeostasis: ICLI neurons express the inhibitory sNPFR (section 4.7.5) and, probably, the excitatory PDFR (Helfrich-Förster et al. 2000). A possible route for the modulation of wake-promoting ICLI neurons lies in the differential release of the antagonising neuropeptides PDF and sNPF by the s-LN$_v$ and/or the modification of PDF and sNPF receptor levels by ICLI neurons. This hypothesis is supported by several recent studies: a decrease in the excitability of s-LN$_v$ during the night generally benefits sleep (Depetris-Chauvin et al. 2014; Shang et al. 2008; Sheeba et al. 2008a). This night-specific inhibition is, at least in part, due to GABAergic input (Agosto et al. 2008; Chung et al. 2009; Gmeiner et al. 2013; Parisky et al. 2008). Less is known about the day-specific regulation of s-LN$_v$ that permits siesta sleep. The high activity levels of s-LN$_v$ generally antagonise day sleep (Depetris-Chauvin et al. 2014; Shang et al. 2008; Sheeba et al. 2008a). However, s-LN$_v$ express not only the excitatory neuropeptide PDF, but also the inhibitory neuropeptide sNPF (Johard et al. 2009; Lee et al. 2009; Shang et al. 2013). Thus, times of high excitability of s-LN$_v$ do not necessarily have to promote the activation of its target neurons, but could also lead to inhibition due to sNPF release (see Vecsey et al. 2014). Interestingly, the $sNPF^{00448}$ mutant suppresses sleep predominantly during the day, but also during the night (Shang et al. 2013), while the pdf^{01} mutant enhances sleep, predominantly during the day, but also during the night (Chung et al. 2009). Furthermore, the thermogenetic activation of sNPF+ neurons suppresses day and night sleep under both regular light schedules and in constant darkness (Shang et al. 2013). The authors of this study hypothesise that the sleep-promoting effect of sNPF+ neuron activation is due to the activation of the s-LN$_v$ and support this finding by demonstrating sleep suppression due to the RNAi-mediated reduction of *sNPF* in s-LN$_v$ (Shang et al. 2013). Conversely, another study demonstrated that the overexpression of *PDF* in ICLI neurons increases activity

in constant darkness (Helfrich-Förster et al. 2000, see also discussion in Choi et al. 2010). That study focussed on circadian rhythm and detected a mild destabilisation of circadian rhythm through *PDF*-overexpressing ICLI neurons (Helfrich-Förster et al. 2000), but the observed increase in activity levels could be indicative of sleep suppression due to increased PDF signalling. In light of the negative regulation that isoB exerts over ICLI neurons to promote sleep, a promising route lies in an investigation of CG10638 as a receptor chaperone: CG10638 may be involved in receptor trafficking in a manner that, e.g., benefits the sNPFR and hinders the PDFR. At times when isoB levels are high – around siesta time and at times preceding night sleep – and sleep pressure is high, this increased isoB expression could aid in the downregulation of the PDFR. Such a downregulation of the PDFR would be associated by a decreased control of s-LN_v over ICLI neuron activity through PDF, and a relatively increased sensitivity to the inhibitory neuropeptide sNPF. In this context, the potential interaction of CG10638 with leucine-rich repeat kinase (Lrrk), which we detected through our *in silico* analysis (section 4.2), is particularly notable as *lrrk2* is a Parkinson-related disease gene that has a crucial role in the trafficking of dopamine receptors (Rassu et al. 2017). Importantly, it is not unprecedented for AKRs to act as receptor chaperones: several $K_v\beta$ subunits direct the localisation of $K_v\alpha$ subunits to the membrane *in vitro*, and the role of $K_v\beta$ subunits in directing $K_v\alpha$ subunits to the dendritic membrane has been demonstrated *in vivo* (Campomanes et al. 2002). Future studies should address the possibility of a receptor chaperone role of CG10638. For instance, if isoB were indeed involved in sleep-promoting receptor maintenance in ICLI neurons, the ICLI neurons with reduced levels of CG10638 should be more excitable by PDF, but less responsive to sNFP application, particularly during the day, in comparison to parental control flies. Similarly, immunohistochemical experiments should reveal whether CG10638 levels are indeed associated with PDFR and/or sNPFR levels.

Evidence of a *causal* relationship between CG10638 levels and ICLI neuron inhibition would be particularly valuable in the identification of an exemplary

molecular mechanism in a specific neural subset that provides an interface to connect the circadian and the homeostatic sleep drive.

6.4 Characterisation of a novel sleep regulator: CG3397

6.4.1 A novel sleep-regulator of MB microcircuits

Approach

We directed a set of our initial RNAi-based screen specifically to the MB as this is a major structure of the fly brain that has long been implicated in sleep regulation (section 1.5). Initial results from our MB-specific screen point to a potential *Drosophila* AKR that might be involved in sleep regulation in the MB, CG3397. We confirmed the initial phenotype using two independent RNAi transgenes directed against the CG3397 locus, as well as the $CG3397^{EY04742}$ mutant. Since our *in silico* research revealed considerable sequence identity of CG3397 and Hk, we specifically investigated this AKR for functional similarities with Hk.

Association of CG3397 and Hk with the MB

CG3397 could be neural regulator: we detected moderate expression levels in the head and brain similar to *hk* (section 3.2.1). We tested both CG3397 and *hk* for an association with sleep regulation in general by inducing a nervous system-wide RNAi-mediated knock down of these genes (section 3.2.2). We also tested a potential MB-specific role by directing the knock down specifically to the MB (section 3.2.3).

In our approach, the RNAi-mediated reduction of *hk* both in the nervous system in general and the MB in particular suppresses both day and night sleep and replicates the sleep phenotype described for the *hk* mutant hk^1 (Bushey et al. 2007). Hk is a β subunit of the voltage-gated K^+ channel Shaker and regulates the channel opening probability in response to the acute $NADP^+$/NADPH ratio: specifically, binding of NADPH activates HK to close the Shaker channel, while binding of $NADP^+$ prevents channel closing (Kilfoil et al. 2013, see also section 1.6.5). Mutations in the catalytic residues of the β subunit prevents successful inactivation and increase neural activity (Bähring et al. 2001). An RNAi-mediated reduced availability of Hk would likely mimic this effect, i.e., increase the open probability of the Shaker channel, and leave the cell hyperactive. This hypothesis

is corroborated by electrophysiological studies of the hk^1 mutant in which rhythmic spontaneous activity is increased in a cultured preparation (Yao et al. 1999) and in l-LN$_v$ of the intact brain (Fogle et al. 2015). Since the Shaker channel is expressed in the MB (Rogero et al. 1997; Wu et al. 2010), this structure seems likely to be one of the substrates of Hk activity as well. Our results show that Hk has indeed the potential to act through MB neurons. In light of the studies that demonstrate a wake-promoting role of the MB (Pitman et al. 2006 and Joiner et al. 2006; section 1.5.1), Hk may be one of the molecular regulators that is able to negatively modulate the neural excitability of the KCs in order to allow sleep to occur.

Similar experiments for the candidate CG3397 presented in chapter 3 revealed that its reduction in the whole nervous system did not affect overall sleep levels. However, a MB-specific reduction of CG3397 expression levels did substantially suppress sleep below the level of Hk, probably due to the high expression levels driven by the pan-MB ok107 driver in comparison to the pan-neuronal ELAV driver (discussed above).

As described in section 1.5, the MB lobes comprise three sublobes that are divided into sublobes and again into compartments. Based on a previous study of the role of particular MB sublobes in sleep regulation by Sitaraman et al. 2015b, we selected several driver lines that target sleep-regulatory MB sublobes. We detected a change in sleep levels due to the RNAi-mediated reduction of CG3397 in both the γ and $\alpha\beta$ lobes, but not in the $\alpha'\beta'$ lobe. Using drivers specific to particular compartments of sublobes, we detected a sleep-promoting role of CG3397 in compartments across lobes, particularly in the $\alpha'\beta'$m ($\alpha'\beta'$medial) compartment (MB418B).

The seemingly incongruent sleep effects between lobes and sublobes have also been observed by Divya Sitamaran and collegues in their study of sleep-regulatory MB sublobes: here, the activation of whole lobes did not produce changes in sleep levels, while the activation of particular compartments specific to a sublobe did (Sitaraman et al. 2015b). The authors hypothesise that the lack of sleep effects during the activation of whole lobes was due to the combined activation of sleep-promoting and wake-promoting compartments (Sitaraman et al. 2015b). Generally,

we detected an effect of CG3397 reduction in compartments that Divya Sitamaran and collegues describe as 'wake-promoting' due to the sleep suppressing effect of their activation, and we do not detect an effect of CG3397 reduction in the 'sleep-promoting' compartment, γdorsal (γd), described by Sitaraman et al. 2015b.

Taken together, we found a generally inhibitory role of CG3397 across wake-promoting MB subcircuits. In a next step, we experimentally assessed in further detail whether CG3397 and Hk share common functions.

6.4.2 A sleep regulator that mimicks Hk?

Our *in silico* research placed CG3397 in the vicinity of Hk: both are relatively similar in sequence, and both lack the high degree of conservation of essential AKR residues commonly found among AKRs, including *Drosophila melanogaster* AKRs. Also, Hk and CG3397 share the most similar mouse orthologue, *Kcnab2*, and protein-protein interaction networks predict a functional interaction of Hk and CG3397 (chapter 3).

Thus, we tested the CG3397 locus for an association with other phenotypes commonly associated with the *hk* mutation and found considerable overlap: as the phenotype *hyperkinetic* suggests, *hk* mutants exhibit abberrant activity patterns (Wang et al. 2000), and we detected an association of the CG3397 locus with hyperactivity, both in the CG3397^{EY047742} mutant and in flies with MB-specific knock down of CG3397, although the intensity of hyperactivity was mild, probably owing to a mild reduction of CG3397 expression levels in experimental flies.

Furthermore, the NADPH-dependent modulation of Hk activity implies that Hk may be a "redox sensor" that alters neural excitability in response to oxidative stress levels (Chouinard et al. 1995; Gulbis 2000; Gulbis et al. 1999). Indeed, Hk may modulate the neural excitability of clock neurons in response to blue light; light stimulation recruits the redox co-factor FAD which may act to suppress Hk-dependent Shaker inactivation (Fogle et al. 2015). This excitability phenotype manifests in two associated phenotypes observable in the hk^1 mutant: firstly, hk^1 mutants are susceptible to ether-induced neural hyperexcitability that leads to violent "shaking" of the limbs and abdomen (Bushey et al. 2007; Kaplan et al.

1969). Secondly, the redox sensing property of Hk manifests in a hypersensitivity to oxidative stress induced by, e.g., the insecticide paraquat (Weng et al. 2006). Consequently, we tested CG3397^{EY047742} mutants for both phenotypes. While we did not observe a shaking phenotype in these mutants, we did detect a susceptibility to oxidative stress, pointing to a potential role of CG3397 in pathways that compensate for rising oxidative stress levels. This hypothesis is supported by a transcriptome study that detected an upregulation of CG3397 in response to high oxygen-induced oxidative stress (Gruenewald et al. 2009).

A study focussed on the involvement of Hk in sleep did not detect an impairment of Hk mutants in homeostatic sleep regulation (Bushey et al. 2007). Our findings corroborate these results through a different route: we detected only a negligible upregulation of both *hk* and CG3397 mRNA levels in response to SD (see section 5.3).

Future perspectives

Taken together, CG3397 exhibits compelling similarity to Hk: the expression levels in brain and body and in response to sleep deprivation are similar. Both CG3397 and Hk play an inhibitory role in the generally wake-promoting MB, and CG3397^{EY04742} mutants are susceptible to oxidative stress, as are hk^{1} mutants. Their levels of sequence identity places CG3397 into the same AKR family as Hk, the insect Kvβ subunits, AKR6B.

We were able to pin-point the activity of CG3397 to particular MB compartments. In light of extensive libraries of MB drivers, these findings provide an excellent basis to test whether CG3397 could be one of the substrates that inhibit the recently identified wake-promoting MB subcircuits in order to allow night sleep initiation. Furthermore, as recent studies involve Hk in the regulation of l-LN$_v$ activity (Fogle et al. 2015), an additional role of CG3397 in these neurons cannot be ruled out. Another study detected CG3397 expression in PDF+ neurons; here, the expression of CG3397 was unaffected by a disruption of the molecular clock, indicating that CG3397 does not have a major role in circadian rhythm (Lin et al. 2002). However, an initial follow-up study should discern whether CG3397 also acts through the

l-LN$_v$ neurons to support. For instance, the preservation of the circadian rhythm in CG3397^{EY04742} mutants should be assessed.

It is still unclear at this stage whether CG3397 interacts with Hk and/or with the Shaker complex to exert its effects, and further epistasis experiments need to be undertaken to explore this possibility. Alternatively, in light of the receptor chaperone function of another molecular sleep regulator of the MB, SSS, and of KCNAB2 proteins of other species (section 1.5), further epistasis experiments should be able to discern whether CG3397 might aid in receptor trafficking and/or stabilisation.

6.5 Caveats and limitations of this study

6.5.1 Conceptual caveats

Validity of candidate AKR selection

Our collection of *Drosophila melanogaster* AKRs is based on the genome annotation available from the main fly genome database, FlyBase. Its protein domain annotation, including of AKR domains, is derived mainly from the PANTHER database, which provides a functional prediction based on similarity with proteins in other species (Mi et al. 2013).

The incorporation of a candidate AKR into the AKR superfamily is entirely based on its sequence identity with other, core members, as long as a functional expression has been demonstrated (Jez et al. 1997b). We confirmed the domain annotation of candidate AKRs through a comparison of conserved and essential AKR residues of *Drosophila melanogaster* AKRs with a commonly used reference rat AKR, AKR1c9. However, the presented list of *Drosophila melanogaster* AKRs should be considered a 'candidate list' only and not as a comprehensive and ultimate collection. As such a limited number of insect AKRs has been incorporated into the AKR superfamily which could serve as model proteins, it is possible that updates to existing protein annotation tools might produce further candidates.

Selection of target tissues

We intentionally biased our approach towards *neural* sleep regulators and conducted a nervous system-wide RNAi-mediated knock down screen. This focus on the nervous system was based on mounting evidence of the involvement of molecular regulators involved in sleep modulation through the nervous system, particularly the MB (e.g., Dubowy et al. 2017; Miyazaki et al. 2017; Tomita et al. 2017; section 1.5). This includes the particularly relevant work on the fly AKR Hk which may act primarily through two neural populations, the KCs of the MB, and the l-LN$_v$ (Fogle et al. 2015, see section 1.5). Thus, we conducted a parallel MB-specific screen to validate the putative involvement of Hk in MB-dependent

sleep regulation and to test whether other *Drosophila* AKRs could fulfil similar sleep-related functions in this brain structure.

However, brain structures other than the MB modulate sleep, especially the dFSB of the central complex. The involvement of the dFSB in sleep regulation became increasingly clear through the work of Jeff Donlea in recent years (e.g., Donlea et al. 2014, section 1.5).

Furthermore, peripheral tissues are involved in sleep regulation in *Drosophila*, particularly energy stores such as the fat body (e.g., Thimgan et al. 2010 and Thimgan et al. 2015, section 1.5). Similarly, as far as mammalian AKRs have been described, most are expressed at high levels throughout the body, particularly the liver and kidney, and are rarely expressed mainly, let alone specifically, in the nervous system (Barski et al. 2008).

Validity of the working definition of sleep

The widening acceptance in the sleep field of a behavioural definition of sleep has greatly advanced sleep research in model organisms where the gold standard of EEG recordings does not apply, such as insects. The behavioural definition of sleep requires that the sleeping animal seeks out a preferred position or location to engage in a period of quiescence or immobility that is reversible and under homeostatic regulation (Campbell et al. 1984, section 1.1). *Drosophila melanogaster* meet the defining criteria of sleep behaviour, and their sleep is similar to other species, including humans, in various respects (section 1.1.4).

Now, uninterrupted periods of immobility of 5 min or more are considered a sufficient criterion for sleep. This "5 min rule" has been discussed in section 1.3.1. It adds consistency to the analysis of fly sleep throughout the field as it provides a concrete, quantifiable criterion. Nevertheless, it is an arbitrary time window, although several studies have performed validation experiments: for instance, Joan Hendricks et al. found that the majority of resting episodes in flies last longer than 5 min (Hendricks et al. 2000). Also, this time window coincides with an increase

in the arousal threshold which poses an independent criterion for sleep (Andretic et al. 2005; Huber et al. 2004; Shaw et al. 2000).

A common tool to investigate sleep in flies are *Drosophila* Activity Monitors (DAMs) which provide a resolution of 1 min in the form of midline passes through the arena (Hamblen et al. 1986, section 1.3). The initial studies demonstrating that "sleep in Drosophila is a rest-like state" (Hendricks et al. 2000) used a combination of video and/or ultrasound-based activity detection systems with DAMs and found that these detection systems provide similar activity profiles (Ho et al. 2005).

Aside from the robustness of activity profiles deducible from DAM data, these monitors are not suitable for the detection of local movements such as feeding or fine movements such as grooming. Thus, we have not used DAM-based data beyond the analysis of general rest:activity patterns.

However, progress in the study of *Drosophila* rest:activity rhythms, first in the circadian framework as initiated by the Hamblen et al. 1986 study, and then in respect to sleep (Hendricks et al. 2000; Shaw et al. 2000), has been greatly advanced through the use of DAM devices. These monitors are suitable to exploit the considerable advantages of the fruit fly model: flies can be grown in large numbers, their whole genome is sequenced and annotated in detail, and specific and/or random mutations can be generated and evaluated easily; in DAM devices, the rest:activity profile of hundreds of flies can be tested at any one time allowing the analysis of their circadian rhythm and sleep patterns.

6.5.2 Technical limitations

Every study has technical limitations. In this section, we address those we were particularly concerned with and gave the greatest consideration when developing and optimising experimental protocols.

Environmental influences

As discussed in section 1.3, fly sleep is heavily influenced by environmental factors, particularly by light, temperature, the nutritional state and food composition, and

the social environment during development and adulthood (section 1.3.3). While we cannot completely rule out the interference of such factors, we took steps to keep the environmental conditions as stable as possible throughout and between experiments.

While a modulation of sleep through humidity has not yet been demonstrated, light and temperature strongly affect both sleep levels and sleep timing (section 1.3.3). Thus, most importantly, we performed experiments in dedicated incubators with stable light and temperature levels, and uniform humidity. Incubators were left undisturbed throughout the course of an experiment. Similar conditions were ensured when flies were raised and prepared for experiments.

Considering the impact of social enrichment on sleep levels, we isolated flies during the habituation phase prior to sleep experiments. As the effects of the social environment can persist for several days after isolation (Ganguly-Fitzgerald et al. 2006) and crowding conditions during development can affect sleep (Chi et al. 2014), we ensured that experimental flies were raised under the same conditions, i.e., at similar and low levels of crowding. This protocol also ensured unobstructed and *ad libitum* access to food since starvation has been demonstrated to affect sleep in *Drosophila* (section 1.3.4).

In the same vein, we used a standardised fly medium to rule out unintended variation of food components such as sugar and lipid levels which can modulate sleep levels and architecture (section 1.3.4).

Limitations of RNAi-mediated knock down

A particular caveat of RNAi screens is their propensity to produce false positives. This can be due to the inadvertent creation of mutations when the RNAi transgene inserts into a random location as, for instance, in RNAi lines of the GD library, due to the inadvertent insertion of the RNAi transgene into a second insertion site as, for instance, in RNAi lines of the KK library, or the design of RNAi transgene sequences that are complementary to other genes apart from the target gene (Jackson et al. 2010, see also section 2.2.3).

To address the latter possibility, we validated RNA transgene sequences *in silico* and generally excluded sequences with a high number of potential off-targets. In the case of CG3397, we employed both available hpRNA lines as both are predicted to have possible off-target effects regarding CG12224. RNAi-based results regarding CG3397 should thus be interpreted with caution. While we cannot rule out possible off-target effects through various channels, we confirmed our on-target effects through different routes: most importantly, we included RNAi transgenes that provided a negative and a positive control, i.e., RNAi lines against the exogenous GFP and the described sleep-regulatory AKR Hk. Additionally, we tested independent mutant lines, and measured the efficiency and the specificity of the reduction achieved for our main phenotypes.

However, a rescue experiment of AKR mutants would be a crucial test for specificity: in such an experiment, a transgene comprising the candidate locus could be expressed in the mutant background to compare sleep levels between transgenic "rescue" flies and mutant flies to evaluate whether the re-introduction of the candidate gene alleviates the sleep phenotype of the mutant flies. Such an approach, however, requires a line carrying a complete knock out of the candidate gene. Unfortunately, we detected low residual expression of candidate genes in the relevant mutant line, prohibiting this approach.

Another consideration is the presence of silent transgene insertions which can produce false negative results. Thus, our initial RNAi-based sleep screen should only be seen as an initial guidance as to which AKRs may strongly modulate sleep.

Furthermore, dosage is another factor that can affect the outcome achieved through RNAi-based knock down. A common recommendation to enhance RNAi efficiency is to co-express the RNAse Dicer, although this can increase the incidence of unspecific effects (see section 2.2.3, Cullen 2006; Wang et al. 2015), and be toxic to cells and trigger an immune response (Olejniczak et al. 2009). In the same vein, achieving reductions that are too high can have detrimental, even lethal effects, depending on the function and relevance of the gene(s) involved. For instance, we found that the reduction of certain AKRs is associated with lethality, particularly

two AKRs involved in gene transcription and regulation, CG18547 and CG2767. Both RNAi lines are part of the KK library which is prone to produce high siRNA levels due to an additional insertion of the hpRNA transgene, which is also associated with a potential disruption of the *tiptoe* locus (Green et al. 2014). As expression of the same transgenes at moderate levels avoided lethality, the AKRs CG18547 and CG2767 may in fact play a crucial role in embryonic development.

A route to avoid developmental lethality derived from the expression of particular transgenes is to combine the Gal4 driver line with a Gal80 construct under the control of an appropriate driver such as *tubulin*: Gal80 is a yeast repressor of Gal4 that can be transgenetically expressed in *Drosophila* to inhibit the expression of Gal4 *in vivo* (Duffy 2002). The spatial control of Gal80 can be achieved in ways similar to Gal4, by fusing the Gal80 sequence to an appropriate promoter sequence (del Valle Rodríguez et al. 2012). For instance, a *tubulin*-Gal80 transgene would be expressed pan-cellularly. The temporal control of Gal80 expression can be achieved through expression of the Gal80^{ts} mutant which renders Gal80 active at low temperatures of around 18 °C, but inactive at higher temperatures of 29 °C or above (del Valle Rodríguez et al. 2012). Alternatively, the GeneSwitch system employs a Gal4 construct that is activated by a steroid (RU486) supplemented in a fly's diet. The GeneSwitch construct is a chimeric protein combining a Gal4 with a mutated progesterone receptor–ligand binding domain which confers activation of Gal4 upon steroid binding (Nicholson et al. 2008). While being very valuable tools for the temporal restriction of gene expression, both Gal80^{ts} and GeneSwitch have drawbacks: the release of Gal80^{ts}-mediated inhibition requires high temperatures which profoundly disrupt the sleep profile as discussed in section 1.3.3. The GeneSwitch system, on the other hand, requires the supplementation of a fly's diet with a steroid, a progesterone (Nicholson et al. 2008). The steroid activity that many members of the AKR superfamily exhibit (Penning 2015) precludes the systematic use of the GeneSwitch system for our target genes.

An approach to limit lethality without the use of potentially disruptive systems is to culture RNAi transgene-expressing flies at lower temperatures of around 18 °C.

We generally avoided this strategy in order to provide the same developmental conditions for all crosses. We did test this approach for the nervous system-wide expression of RNAi transgenes directed against CG18547 and CG2767, but did not observe an improvement in survival of experimental flies to adulthood.

Limitations of driver lines

The design of driver lines relies on the ability of enhancer regions of particular genes to additionally drive the expression of transgenes conjugated to enhancer fragments or inserted directly into enhancer regions (section 1.2).

The temporal and spatial expression pattern of many driver lines has not been exclusively characterised. Studies that attempted to do so often found surprising effects such as expression of the 'pan-neuronal' driver ELAV in glial cells within a certain developmental time window (Berger et al. 2007). Additionally, many driver lines were generated through insertional mutagenesis and create themselves phenotypic mutations (section 2.3).

To address the latter possibility, we always tested driver lines – as well as actuator lines in most cases – together with experimental lines for the possible presence of phenotypes. We also confirmed the expression of all driver lines through immunohistochemistry in the adult brain. This approach still leaves open the possibility that (1) the driver expresses in cells or tissues outside of the protocerebrum, (2) the driver is expressed transiently before the animal is adult, and (3) a combination of both. To address the first possibility, we examined expression of driver lines in the VNC. However, we did not test for the presence of the driver in tissues outside of the nervous system nor in larvae.

Limitations of qRT-PCR

Since we were mainly interested in relative differences between experimental conditions, genotypes, or transcripts, we used the relative quantification method. We used the same reference genes throughout the study to provide a unifying standard. This method implies that we cannot make an assumption about absolute mRNA levels.

As levels of gene transcription have a high degree of inherent variability, we ensured a high template quality. Main modifications of the technical protocol to achieve high quality and consistency in our qRT-PCR assays are described in detail in section 2.5. Throughout, we tested baseline expression levels in at least two independent experiments, and increased this number for experimental conditions. We also included positive and negative controls when informative. For instance, when testing the expression of targets in the course of the day, we also measured the expression of a known cycling gene, i.e., *period*.

Generally, mRNA levels do not necessarily reflect protein levels. Thus, where possible, we tested differences between experimental groups not only on the level of mRNA expression, but also on the protein level using immunohistochemistry.

Limitations of immunohistochemistry

The main source of unreliability of immunohistochemistry relates to the specificity and robustness of antibodies, both primary and secondary (section 2.6).

Importantly, primary antibodies do not necessarily reflect the expression of the actual antigen, or, in other words, do not necessarily faithfully report the localisation of the target protein. This is particularly true of polyclonal antibodies, which are prone to nonspecific binding. Also, the affinity to the antigen may be low, at times below the detection level, which would produce a false negative result. This is particularly true of monoclonal antibodies.

Secondary antibodies are raised against defining antigens of the host species of the primary antibody. Thus, one of the main concerns with secondary antibodies is cross-species reactivity. This is a particular concern for co-expression studies. We addressed this concern by combining endogenously expressed reporters with antibody stainings, where possible.

Where possible, we tested whether our antibody stainings reflect actual protein levels by comparing stainings of animals with reduced protein levels through RNAi-mediated knock down of the respective transcript with parental controls. However, we were not able to use this approach throughout. Where we did employ this

approach, we indeed detected a coincident relative alteration of both mRNA and protein levels in experimental animals in comparison to parental controls (see section 4.5.4).

6.6 Conclusion and outlook

Our study provides the first candidate collection of *Drosophila melanogaster* AKRs alongside an *in silico* and literature-based analysis of their potential function in general, and an experimental investigation of their potential sleep-regulatory roles in neurons in particular. We have corroborated earlier studies that involve the *Drosophila* AKR Hk in sleep regulation, and we provide further evidence for a specific sleep-modulatory role of Hk in the l-LN$_v$ and the MB. Furthermore, we have identified another candidate sleep-regulatory AKR, CG3397, that acts within particular MB microcircuits to support night sleep, potentially by modulating membrane excitability in an NADPH-dependent manner similar to Hk. We have also identified another *Drosophila* sleep-regulatory AKR, CG10638, which encodes two isoforms, isoA and isoB. The putative sleep-regulatory molecular function of isoB is likely distinct from the molecular function of isoA: while isoA acts primarily through the l-LN$_v$ and could enhance their GABAergic inhibition that benefits night sleep, isoB acts primarily through output neurons of the s-LN$_v$, ICLI neurons, and could modulate the balance of PDFR-dependent wake-promoting excitation and sNPFR-dependent sleep-promoting inhibition of these neurons to increase sleep at times of high sleep pressure.

An electrophysiological study should investigate whether those potential fly AKRs belonging to family 6 actually impact on membrane excitability through the regulation of ion channels. Those studies have so far only been conducted for the fly AKR Hk (section 1.6.5), but a broader investigation of AKR6 family members in the powerful model organism of the fruit fly could greatly benefit our knowledge of mechanisms that involve *KCNAB1* and *KCNAB2* genes in human channelopathies (Busolin et al. 2011; Heilstedt et al. 2001).

Deregulation or mutation of mammalian AKRs are associated with metabolic syndromes and carcinogenesis (among other diseases, Chen et al. 2012; Penning et al. 2007) and greater insight into the potential use of *Drosophila melanogaster* as a model organism for AKR-related pathologies might benefit therapeutic research. Thus, a combination of biochemical and molecular studies should discern whether

potential fly AKRs belonging to family 1 fulfil canonical functions such as the reduction of particular types of aldehydes and amines. For instance, the potential involvement of one AKR, CG10638 (isoA) in both sorbitol production and sleep regulation poses a promising substrate to study the interaction between glucose metabolism and sleep in a simple model organism.

Sleep-related effects upon knock down of two AKRs, CG3397 and CG10638, other than Hk point towards the exciting possibility of a general role of this enzymatic group in sleep regulation. Particularly the potential function of the CG10638 isoform B as a molecular regulator of neural excitability in response to sleep pressure through synaptic homeostasis in general and/or receptor homeostasis in particular, could provide a missing link to balance the circadian sleep drive with sleep pressure.

AKRs could form a regulatory link between intracellular states and cellular excitability by modulating membrane excitability in response to, for instance, sleep pressure to provide a window of opportunity for synaptic downscaling and metabolic maintenance.

7 Supplemental results

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7.1 *In silico*

7.1.1 Hits in genetic and phenotypic screens

Genetic and phenotypic screens as well as transcriptomic studies imply AKRs in a range of functions and the main findings are listed in Table 7.1.

Annotation	References
CG10638	(S) glucose metabolism (O'Keefe et al. 2011)
	(S) Notch/RTK pathway (Hurlbut et al. 2009)
	(S) p53-dependent oncogenesis (Akdemir et al. 2007)
	(S) alternative pre-mRNA processing (Waldholm et al. 2011)
	(S) proteasome inhibition (Vinayagam et al. 2014)
	(S) hyperoxia (Gruenewald et al. 2009)
Hk	(P) shaking & Hyperexcitabilty (Kaplan et al. 1969)
	(P) sleep & lifespan (Bushey et al. 2007)
	(P) eag interaction (Wilson et al. 1998)
	(P) flight behaviour (Homyk et al. 1977)
CG3397	(S) hyperoxia (Gruenewald et al. 2009)
	(S) muscle structure/function, K_v β subunit? (Montana et al. 2006)
	(S) circadian clock (Lin et al. 2002)
CG12224	(S) post-mating gene response (McGraw et al. 2008)
CG18547	(S) starvation (Fujikawa et al. 2009)
	(S) KDM4A-dep. gene regulation/development (Crona et al. 2013)
	(S) polyO-dep. neurodegeneration (Nelson et al. 2005; Ren et al. 2011)
CG9436	(S) retrogene (Bai et al. 2008; Díaz-Castillo et al. 2012)
	(S) starvation (Fujikawa et al. 2009))
	(S) immune-induced (De Gregorio et al. 2001; Irving et al. 2001)
	(S) circadian system (Kula-Eversole et al. 2010)
CG6083	(S) fused medial γ lobe of MB (Kobayashi et al. 2006)
CG6084	(S) starvation (Fujikawa et al. 2009)
	(S) Dcr2 pathway (Lim et al. 2011)
	(S) circadian system (Kula-Eversole et al. 2010)
CG10863	(P) NADPH/NADH dual specificity (Di Luccio et al. 2006)
	(S) hypoxia (Vermeulen et al. 2013)
CG12766	(S) hypoxia (Vermeulen et al. 2013)
CG2767	(S) transcriptional regulation (Bozoky et al. 2009; Bronstein et al. 2010)
ARY	(S) Y-linked (Vibranovski et al. 2008)
	(P) unnecessary for spermogenesis/male fertility?
	(Vibranovski et al. 2008; Wasbrough et al. 2010)

Table 7.1: References relating to *Drosophila melanogaster* AKRs with (S) signifying gene-association screens and (P) signifying a phenotypic reverse genetics screens that detected a positive phenotype for the target.

Gene symbol	Enriched	NS expressed
CG10638	SG	YES
Hk	NS (brain, eye, VNC)	YES
CG3397	FB, sperm	YES
CG12224	MG	YES
CG18547	MG	YES
CG9436	Tubule	YES
CG6083	Testis	YES
CG6084	HG	YES
CG10863	eye, FB	YES
CG12766	MG	NO
CG2767	eye, crop	YES
ARY	Testis	NO

Table 7.2: Expression according to the FlyAtlas database (Chintapalli et al. 2007). FB, fat body, MG, midgut, HG, hindgut, SG, salivary gland, NS, nervous system, VNC, ventral nerve cord.

7.1.2 Expression and conservation

Expression according to FlyAtlas

Expression data derived from the FlyAtlas database is listed in Table 7.2. "Tissue-specific" is derived from the "adult enrichment" parameter in FlyAtlas and implies that the candidate is expressed at above average levels in a particular tissue. For reasons of simplicity, only those tissue with highest enrichment values are listed. "NS expressed" implies that the candidate is expressed in the nervous system, i.e., in brain, eye or VNC. Levels of enrichment are indicated by text colour as follows: black, enriched up to 5-fold, yellow, enriched up to 10-fold, orange, enriched up to 20-fold, red, enriched more than 20-fold.

Conservation of fruit fly AKRs within the species

This subsection and the next contain results of multiple sequence alignments (MSA) of *Drosophila melanogaster* AKRs in the context of rat Akr1c9 in order to conform to conventional AKR numbering suggested by Jez et al. 1997b and Penning 2015.

A matrix of the sequence identity of different AKR transcripts relative to each other is depicted in Table 7.1. The MSA was generated using MUSCLE Clustal 2.1 with default settings available from `http://www.ebi.ac.uk/Tools/msa/muscle`.

	Hk-PH	Hk-PK	Hk-PE	Hk-PL	Hk-PM	Hk-PI	Hk-PD	Hk-PG	Hk-PF	CG18547	CG12224-PB	CG12224-PC	CG3397
Hk-PH	100	98.4	98.4	98.4	98.4	98.4	98.4	98.4	98.4	22.4	19.16	19.16	21.7
Hk-PK	98.4	100	100	100	100	99.74	99.74	99.74	73.54	22.4	19.16	19.16	21.7
Hk-PE	98.4	100	100	100	100	99.74	99.74	99.74	73.49	22.4	19.16	19.16	21.7
Hk-PL	98.4	100	100	100	100	99.74	99.74	99.74	73.49	22.09	18.81	18.81	21.43
Hk-PM	98.4	100	100	100	100	99.74	99.74	99.74	73.49	22.09	18.81	18.81	21.43
Hk-PI	98.4	99.74	99.74	99.74	99.74	100	100	100	100	22.33	19.07	19.07	21.63
Hk-PD	98.4	99.74	99.74	99.74	99.74	100	100	100	100	22.4	19.16	19.16	21.7
Hk-PG	98.4	99.74	99.74	99.74	99.74	100	100	100	100	22.4	19.16	19.16	21.7
Hk-PF	98.4	73.54	73.49	73.49	73.49	100	100	100	100	22.4	19.16	19.16	21.7
CG18547	22.4	22.4	22.4	22.09	22.09	22.33	22.4	22.4	22.4	100	53.98	53.98	61.54
CG12224-PB	19.16	19.16	19.16	18.81	18.81	19.07	19.16	19.16	19.16	53.98	100	100	89.04
CG12224-PC	19.16	19.16	19.16	18.81	18.81	19.07	19.16	19.16	19.16	53.98	100	100	89.04
CG3397	21.7	21.7	21.7	21.43	21.43	21.63	21.7	21.7	21.7	61.54	89.04	89.04	100
CG2767	21.72	21.72	21.72	20.93	20.93	21.65	21.72	21.72	21.72	21.55	20.37	20.37	24.32
RAT_Akr1c9	21.95	21.95	21.95	21.36	21.36	21.88	21.95	21.95	21.95	20.27	18.31	18.31	20.82
ARY-PC	17.97	17.97	17.97	16.47	16.47	17.91	17.97	17.97	17.97	20.07	15.38	15.38	19
ARY-PD	19.21	19.23	19.29	17.7	17.7	19.43	19.49	19.49	19.49	19.67	15.87	15.87	18.06
CG10638-PB	21.51	21.51	21.51	21.13	21.13	21.43	21.51	21.51	21.51	21.6	15.79	15.79	18.95
CG9436	20	20	20	19.38	19.38	19.93	20	20	20	19.52	15.79	15.79	19.23
CG10638-PA	20.28	20.28	20.28	19.66	19.66	20.21	20.28	20.28	20.28	21.16	18.48	18.48	22.65
CG10638-PD	20.28	20.28	20.28	19.66	19.66	20.21	20.28	20.28	20.28	21.16	18.48	18.48	22.65
CG10863	22.86	22.86	22.86	22.15	22.15	22.78	22.86	22.86	22.86	18.84	17.22	17.22	20.28
CG12766	20.64	20.64	20.64	19.8	19.8	20.57	20.64	20.64	20.64	19.11	18.66	18.66	21.25
CG6083	18.15	18.15	18.15	17.35	17.35	18.09	18.15	18.15	18.15	18.43	16.43	16.43	18.06
CG6084-PA	20.64	20.64	20.64	19.93	19.93	20.57	20.64	20.64	20.64	21.16	16.51	16.51	20.21
CG6084-PD	21	21	21	20.34	20.34	20.92	21	21	21	21.16	17.37	17.37	20.49
CG6084-PB	18.69	18.47	18.53	18.01	18.01	18.67	18.73	18.73	18.73	19.42	17.37	17.37	19.41
CG6084-PC	18.36	18.15	18.21	17.65	17.65	18.35	18.41	18.41	18.41	19.42	16.51	16.51	19.14

CG2767	RAT_Akr1c9	ARY-PC	ARY-PD	CG10638-PB	G9436	CG10638-PA	CG10638-PD	CG10863	CG12766	CG6083	CG6084-PA	CG6084-PD	CG6084-PB	CG6084-PC
21.72	21.95	17.97	19.21	21.51	20	20.28	20.28	22.86	20.64	18.15	20.64	21	18.69	18.36
21.72	21.95	17.97	19.23	21.51	20	20.28	20.28	22.86	20.64	18.15	20.64	21	18.47	18.15
21.72	21.95	17.97	19.29	21.51	20	20.28	20.28	22.86	20.64	18.15	20.64	21	18.53	18.21
20.93	21.36	16.47	17.7	21.13	19.38	19.66	19.66	22.15	19.8	17.35	19.93	20.34	18.01	17.65
20.93	21.36	16.47	17.7	21.13	19.38	19.66	19.66	22.15	19.8	17.35	19.93	20.34	18.01	17.65
21.65	21.88	17.91	19.43	21.43	19.93	20.21	20.21	22.78	20.57	18.09	20.57	20.92	18.67	18.35
21.72	21.95	17.97	19.49	21.51	20	20.28	20.28	22.86	20.64	18.15	20.64	21	18.73	18.41
21.72	21.95	17.97	19.49	21.51	20	20.28	20.28	22.86	20.64	18.15	20.64	21	18.73	18.41
21.72	21.95	17.97	19.49	21.51	20	20.28	20.28	22.86	20.64	18.15	20.64	21	18.73	18.41
21.55	20.27	20.07	19.67	21.6	19.52	21.16	21.16	18.84	19.11	18.43	21.16	21.16	19.42	19.42
20.37	18.31	15.38	15.87	15.79	15.79	18.48	18.48	17.22	18.66	16.43	16.51	17.37	17.37	16.51
20.37	18.31	15.38	15.87	15.79	15.79	18.48	18.48	17.22	18.66	16.43	16.51	17.37	17.37	16.51
24.32	20.82	19	18.06	18.95	19.23	22.65	22.65	20.28	21.25	18.06	20.21	20.49	19.41	19.14
100	33.86	31.65	32.91	40.65	36.45	39.75	39.75	38.1	38.8	34.7	40.19	40.19	39.12	39.12
33.86	100	35.56	37.82	34.09	38.96	42.95	42.95	42.81	41.21	39.1	46.96	45.69	46.35	47.62
31.65	35.56	100	90.88	43.69	40	45.22	45.22	41.9	41.38	36.08	37.18	37.3	37.35	37.23
32.91	37.82	90.88	100	47.25	43.23	48.73	48.73	43.17	42.01	38.61	39.1	39.23	36.81	36.71
40.65	34.09	43.69	47.25	100	47.06	54.19	54.19	47.74	45.48	38.31	43	43.97	43.18	42.21
36.45	38.96	40	43.23	47.06	100	57.74	57.74	52.73	49.52	41.1	44.81	46.43	45.48	43.87
39.75	42.95	45.22	48.73	54.19	57.74	100	100	59.68	57.78	40.89	50.64	52.24	51.44	49.84
39.75	42.95	45.22	48.73	54.19	57.74	100	100	59.68	57.78	40.89	50.64	52.24	51.44	49.84
38.1	42.81	41.9	43.17	47.74	52.73	59.68	59.68	100	76.9	41.53	48.72	49.68	48.73	47.77
38.8	41.21	41.38	42.01	45.48	49.52	57.78	57.78	76.9	100	41.77	47.6	48.72	48.57	47.47
34.7	39.1	36.08	38.61	38.31	41.1	40.89	40.89	41.53	41.77	100	53.18	51.76	50.16	51.59
40.19	46.96	37.18	39.1	43	44.81	50.64	50.64	48.72	47.6	53.18	100	96.83	92.7	95.89
40.19	45.69	37.3	39.23	43.97	46.43	52.24	52.24	49.68	48.72	51.76	96.83	100	95.89	92.7
39.12	46.35	37.35	36.81	43.18	45.48	51.44	51.44	48.73	48.57	50.16	92.7	95.89	100	97.13
39.12	47.62	37.23	36.71	42.21	43.87	49.84	49.84	47.77	47.47	51.59	95.89	92.7	97.13	100

Figure 7.1: Sequence identity of AKR transcripts, annotation reflects self-identitity (grey box) and pairs with highest degree of identity per AKR (green box).

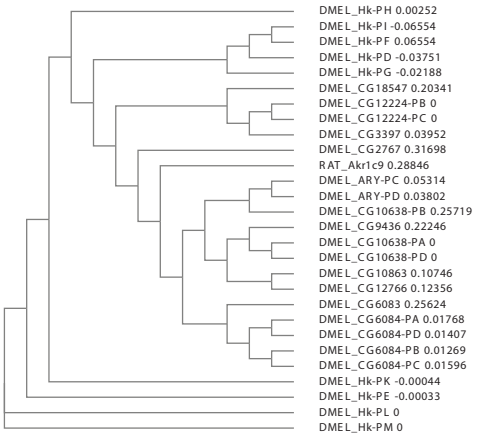


Figure 7.2: Simplified phylogenetic tree of *Drosophila melanogaster* AKRs.

A simplified phylogenetic tree based on the MSA of *Drosophila melanogaster* AKRs and rat Akr1c9 is depicted in Fig. 7.2. The MSA was generated using MUSCLE Clustal 2.1 with default settings available from `http://www.ebi.ac.uk` and passed onto the "Simple phylogeny" tool with default settings.

Conservation of fruit fly AKRs across species

Specifically, MSAs are annotated for conserved residues of the active site, the co-factor binding pocket, and the substrate binding pocket (see below).

Conserved residues of the active site

MSA of *Drosophila melanogaster* AKRs with annotation of conserved residues of the active site.

```
DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          ------------------------------------------------------------
DMEL_Hk-PM          ------------------------------------------------------------
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          MTPPRLSRYLMAVRGGRDGEQHSKNSQTDEEKQRDRQRNEREAKKEEQDAINYKRDKEQR
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------

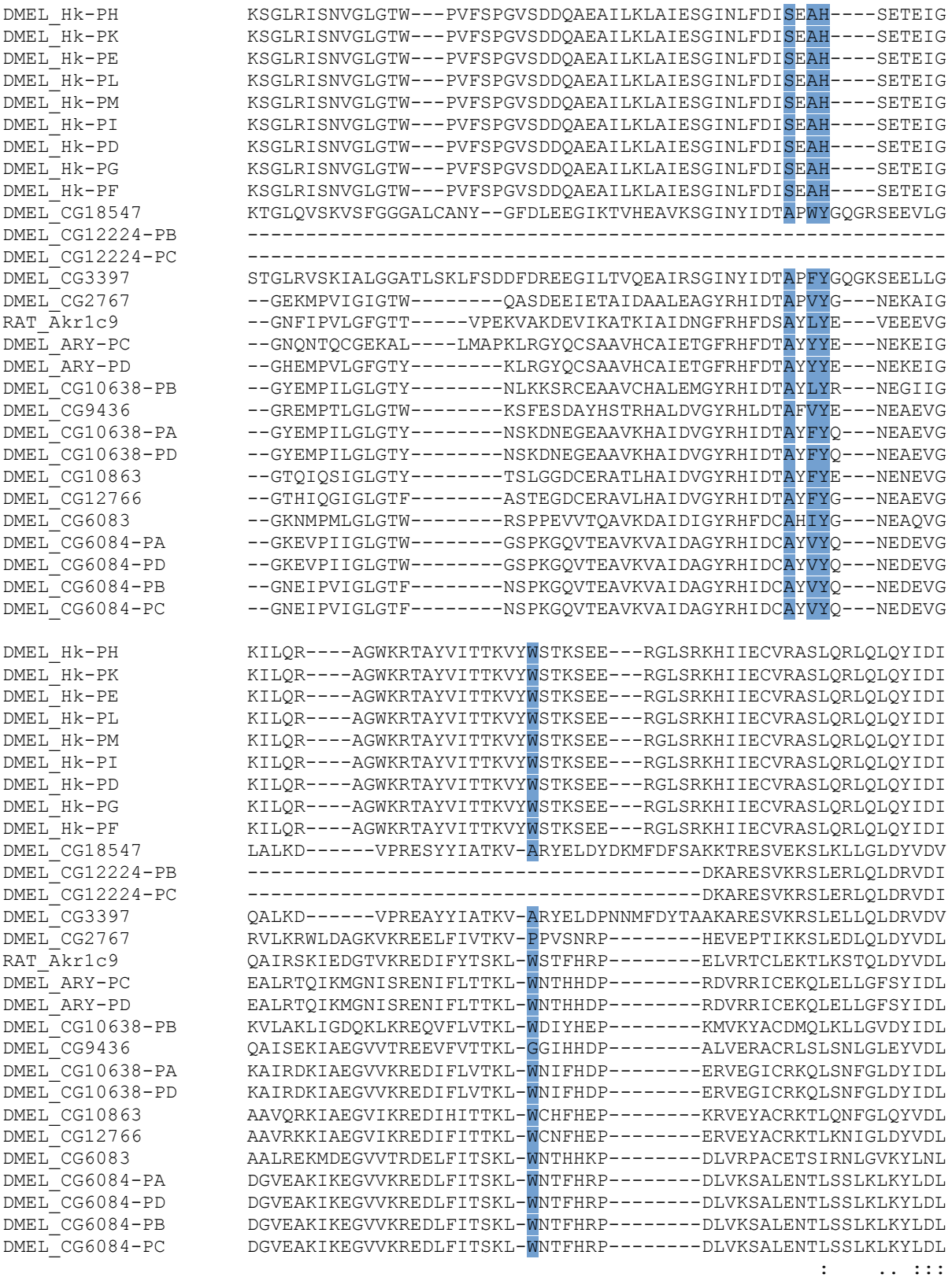
DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ----------------------MSMALCNLNGDGSAAQSTSQSQSQSPAATAAAAPLLP
DMEL_Hk-PE          ----------------------MSMALCNLNGDGSAAQSTSQSQSQSPAATAAAAPLLP
DMEL_Hk-PL          ----------------------MSMALCNLNGDGSAAQSTSQSQSQSPAATAAAAPLLP
DMEL_Hk-PM          ----------------------MSMALCNLNGDGSAAQSTSQSQSQSPAATAAAAPLLP
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          EARLLDFEIRRQQKRDEQQQQRQQQQQLQQHREHLQQQHRDQQQSSEKSPPTSSTAAAAH
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------
```

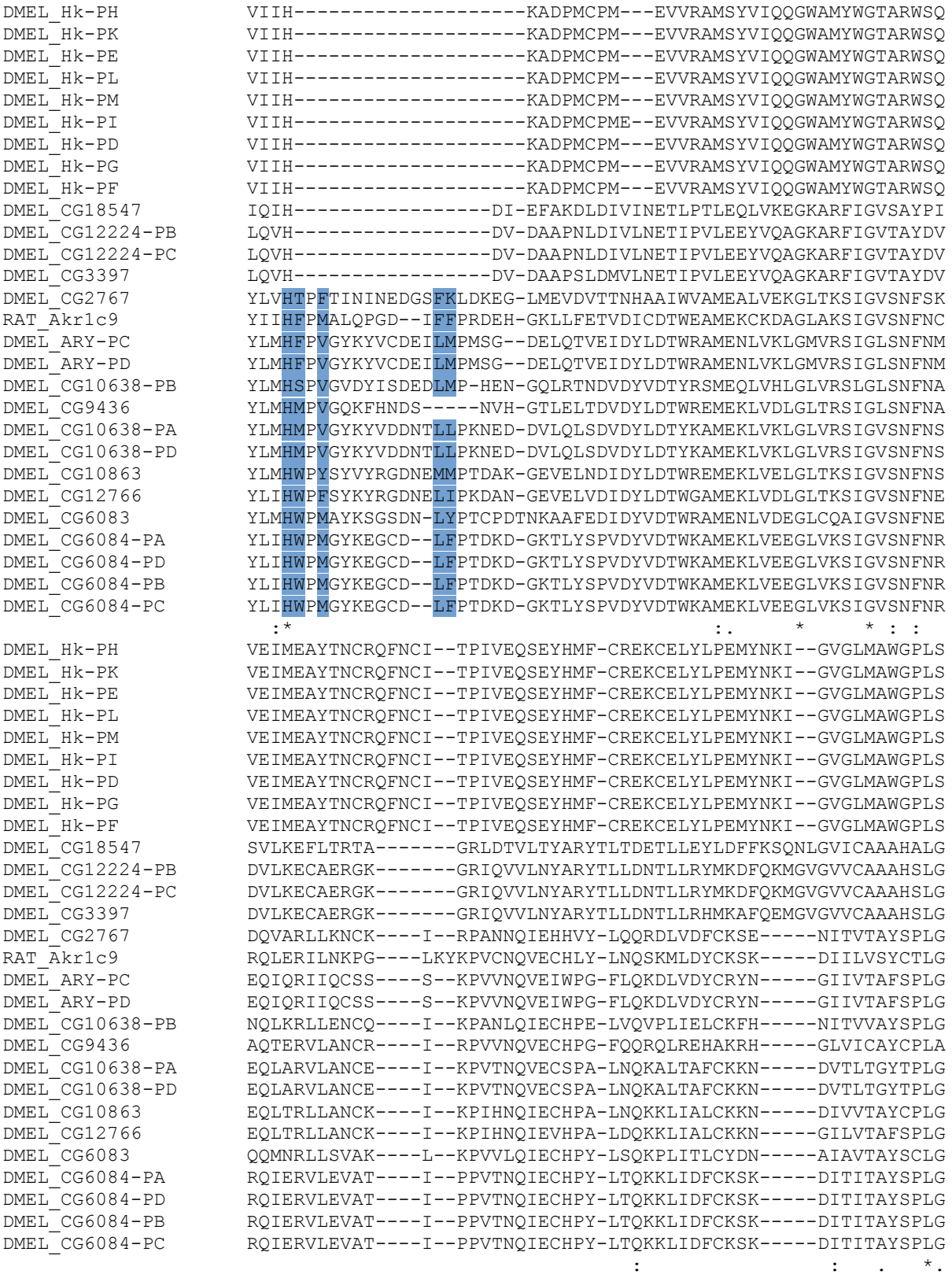
```
DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          HSHSHTLQPESTPLLLGHEQSGSAAPEGSGGGDVTDGAVTSIEMPTVVADDAGVPPLPLP
DMEL_Hk-PE          HSHSHTLQPESTPLLLGHEQSGSAAPEGSGGGDVTDGAVTSIEMPTVVADDAGVPPLPLP
DMEL_Hk-PL          HSHSHTLQPESTPLLLGHEQSGSAAPEGSGGGDVTDGAVTSIEMPTVVADDAGVPPLPLP
DMEL_Hk-PM          HSHSHTLQPESTPLLLGHEQSGSAAPEGSGGGDVTDGAVTSIEMPTVVADDAGVPPLPLP
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          QTHSHSQQQQQRQHLHLQQQHLQQQQQQQQQQQSTGSGSSNTPVTPKLQTPPPDRSLPPAF
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------

DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          LPQSTPQPLLMALPANLNFITGPTTQQLMIGNGAMGVIPSTNDSNNNNNNNNNVDNTSDE
DMEL_Hk-PE          LPQSTPQPLLMALPANLNFITGPTTQQLMIGNGAMGVIPSTNDSNNNNNNNNNVDNTSDE
DMEL_Hk-PL          LPQSTPQPLLMALPANLNFITGPTTQQLMIGNGAMGVIPSTNDSNNNNNNNNNVDNTSDE
DMEL_Hk-PM          LPQSTPQPLLMALPANLNFITGPTTQQLMIGNGAMGVIPSTNDSNNNNNNNNNVDNTSDE
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          RSRSIEREILYERKRFSQPETGEIKELDSTTSSSSTQQTFPGSAVGAGRGASAAVPGSGT
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------
```

```
DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          SNPVTIY-------------------------------------------------------
DMEL_Hk-PE          SNPVTIY-------------------------------------------------------
DMEL_Hk-PL          SNPVTIY-------------------------------------------------------
DMEL_Hk-PM          SNPVTIY-------------------------------------------------------
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          AIAVAVAGAGAGQSATLAGPPATAAAATAAIIPILPLGVATLRDDSTESEQSVTCAHNQL
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------

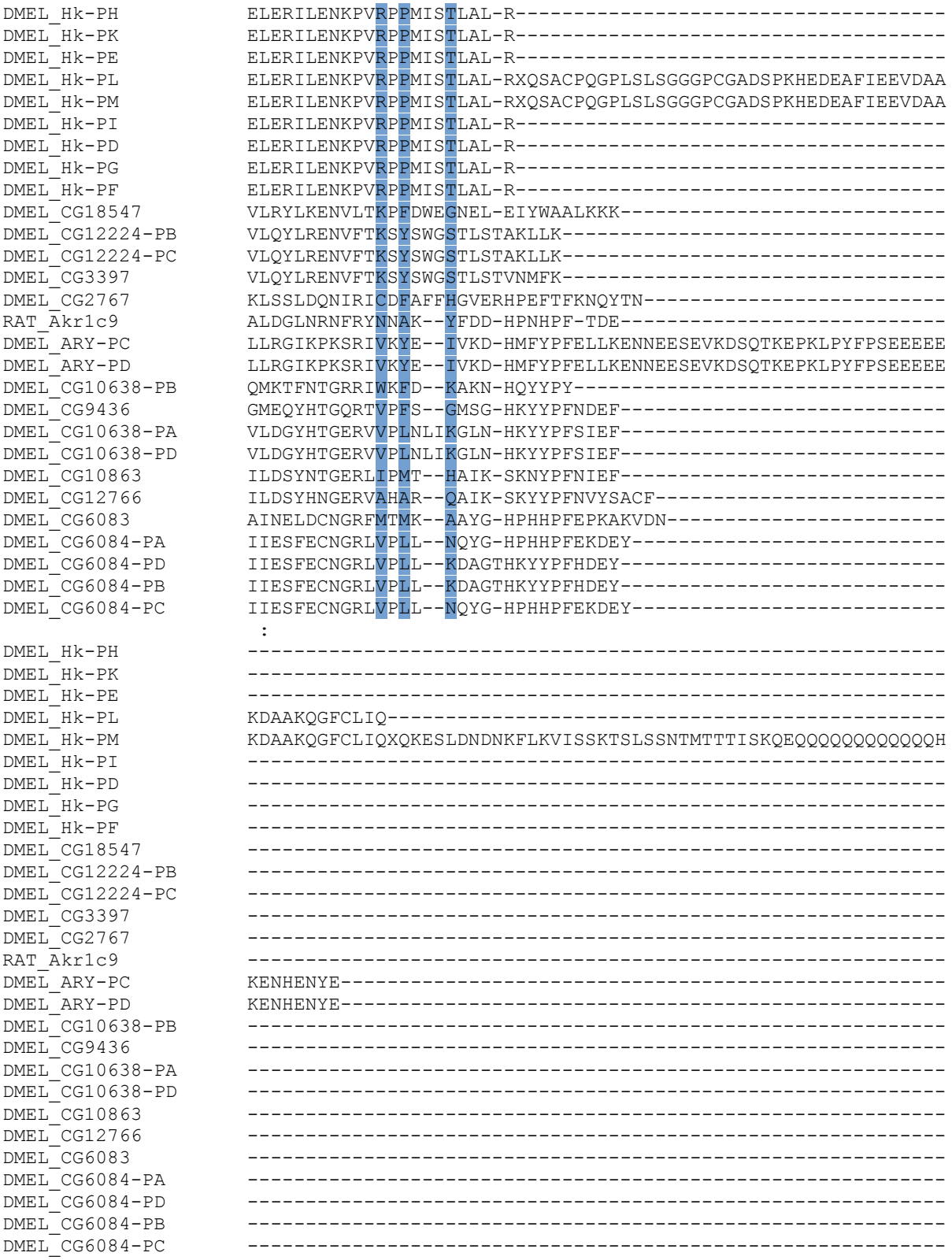
DMEL_Hk-PH          ----------MPTTASC--------------RSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PK          ------RCRAPIASLDCMEEFSG--------RSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PE          ------RCRAPIASLDCMEEFS---------RSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PL          ------RCRAPIASLDCMEEFS---------RSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PM          ------RCRAPIASLDCMEEFS---------RSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PI          ------MCRAPIASLDCMEEFSGTGGHLDFGRSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PD          ------MCRAPIASLDCMEEFSGTGGHLDFGRSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PG          ------MCRAPIASLDCMEEFSGTGGHLDFGRSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PF          DAYHRIMCRAPIASLDCMEEFSGTGGHLDFGRSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_CG18547        --------------------------------MSQTLPATYVKGFHDEAKVRRMEYRNLG
DMEL_CG12224-PB     ------------------------------------------------------MFDYSA--
DMEL_CG12224-PC     ------------------------------------------------------MFDYSA--
DMEL_CG3397         --------------------------------MSGSLPATFVKGFHDEEKVRRMEYRQLG
DMEL_CG2767         ------------------------------------------------MVNTKFLTFNN--
RAT_Akr1c9          ------------------------------------------------MDSISLRVALND--
DMEL_ARY-PC         ----------------------------------MIPADFLTSNIVEDNSQVAVILG--
DMEL_ARY-PD         ------MIPADFLTSNIVEDNSQV-------AVILGGNQNTQCGEKALLMAPKVRLSS--
DMEL_CG10638-PB     ------------------------------------------------MKLAPTVKLNN--
DMEL_CG9436         ------------------------------------------------MTNLAPTIRLNN--
DMEL_CG10638-PA     ------------------------------------------------MKLAPTVKLNN--
DMEL_CG10638-PD     ------------------------------------------------MKLAPTVKLNN--
DMEL_CG10863        ------------------------------------------------MSSKIPYVKHNN--
DMEL_CG12766        ----------------------------------------------MTGKVEYYFKHND--
DMEL_CG6083         ------------------------------------------------MSTPNFLLSN--
DMEL_CG6084-PA      ------------------------------------------------MAVPNVKFNN--
DMEL_CG6084-PD      ------------------------------------------------MAVPNVKFNN--
DMEL_CG6084-PB      -------MNTLMKIQLVRPVFRIT-------LRNYGKPERFMWCQKEYARAPKVVCLD--
DMEL_CG6084-PC      -------MNTLMKIQLVRPVFRIT-------LRNYGKPERFMWCQKEYARAPKVVCLD--
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DMEL_Hk-PH KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PK KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PE KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PL KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PM KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PI KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PD KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PG KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PF KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_CG18547 KTGLQVSKVSFGGGALCANY--GFDLEEGIKTVHEAVKSGINYIDTAPWYGQGRSEEVLG
DMEL_CG12224-PB --
DMEL_CG12224-PC --
DMEL_CG3397 STGLRVSKIALGGATLSKLFSDDFDREEGILTVQEAIRSGINYIDTAPFYGQGKSEELLG
DMEL_CG2767 --GEKMPVIGIGTW--------QASDEEIETAIDAALEAGYRHIDTAPVYG---NEKAIG
RAT_Akr1c9 --GNFIPVLGFGTT-----VPEKVAKDEVIKATKIAIDNGFRHFDSAYLYE---VEEEVG
DMEL_ARY-PC --GNQNTQCGEKAL----LMAPKLRGYQCSAAVHCAIETGFRHFDTAYYYE---NEKEIG
DMEL_ARY-PD --GHEMPVLGFGTY--------KLRGYQCSAAVHCAIETGFRHFDTAYYYE---NEKEIG
DMEL_CG10638-PB --GYEMPILGLGTY--------NLKKSRCEAAVCHALEMGYRHIDTAYLYR---NEGIIG
DMEL_CG9436 --GREMPTLGLGTW--------KSFESDAYHSTRHALDVGYRHLDTAFVYE---NEAEVG
DMEL_CG10638-PA --GYEMPILGLGTY--------NSKDNEGEAAVKHAIDVGYRHIDTAYFYQ---NEAEVG
DMEL_CG10638-PD --GYEMPILGLGTY--------NSKDNEGEAAVKHAIDVGYRHIDTAYFYQ---NEAEVG
DMEL_CG10863 --GTQIQSIGLGTY--------TSLGGDCERATLHAIDVGYRHIDTAYFYE---NENEVG
DMEL_CG12766 --GTHIQGIGLGTF--------ASTEGDCERAVLHAIDVGYRHIDTAYFYG---NEAEVG
DMEL_CG6083 --GKNMPMLGLGTW--------RSPPEVVTQAVKDAIDIGYRHFDCAHIYG---NEAQVG
DMEL_CG6084-PA --GKEVPIIGLGTW--------GSPKGQVTEAVKVAIDAGYRHIDCAYVYQ---NEDEVG
DMEL_CG6084-PD --GKEVPIIGLGTW--------GSPKGQVTEAVKVAIDAGYRHIDCAYVYQ---NEDEVG
DMEL_CG6084-PB --GNEIPVIGLGTF--------NSPKGQVTEAVKVAIDAGYRHIDCAYVYQ---NEDEVG
DMEL_CG6084-PC --GNEIPVIGLGTF--------NSPKGQVTEAVKVAIDAGYRHIDCAYVYQ---NEDEVG
DMEL_Hk-PH KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PK KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PE KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PL KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PM KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PI KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PD KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PG KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PF KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_CG18547 LALKD------VPRESYYIATKV-ARYELDYDKMFDFSAKKTRESVEKSLKLLGLDYVDV
DMEL_CG12224-PB ------------------------------------DKARESVKRSLERLQLDRVDI
DMEL_CG12224-PC ------------------------------------DKARESVKRSLERLQLDRVDI
DMEL_CG3397 QALKD------VPREAYYIATKV-ARYELDPNNMFDYTAAKARESVKRSLELLQLDRVDV
DMEL_CG2767 RVLKRWLDAGKVKREELFIVTKV-PPVSNRP--------HEVEPTIKKSLEDLQLDYVDL
RAT_Akr1c9 QAIRSKIEDGTVKREDIFYTSKL-WSTFHRP--------ELVRTCLEKTLKSTQLDYVDL
DMEL_ARY-PC EALRTQIKMGNISRENIFLTTKL-WNTHHDP--------RDVRRICEKQLELLGFSYIDL
DMEL_ARY-PD EALRTQIKMGNISRENIFLTTKL-WNTHHDP--------RDVRRICEKQLELLGFSYIDL
DMEL_CG10638-PB KVLAKLIGDQKLKREQVFLVTKL-WDIYHEP--------KMVKYACDMQLKLLGVDYIDL
DMEL_CG9436 QAISEKIAEGVVTREEVFVTTKL-GGIHHDP--------ALVERACRLSLSNLGLEYVDL
DMEL_CG10638-PA KAIRDKIAEGVVKREDIFLVTKL-WNIFHDP--------ERVEGICRKQLSNFGLDYIDL
DMEL_CG10638-PD KAIRDKIAEGVVKREDIFLVTKL-WNIFHDP--------ERVEGICRKQLSNFGLDYIDL
DMEL_CG10863 AAVQRKIAEGVIKREDIHITTKL-WCHFHEP--------KRVEYACRKTLQNFGLQYVDL
DMEL_CG12766 AAVRKKIAEGVIKREDIFITTKL-WCNFHEP--------ERVEYACRKTLKNIGLDYVDL
DMEL_CG6083 AALREKMDEGVVTRDELFITSKL-WNTHHKP--------DLVRPACETSIRNLGVKYLNL
DMEL_CG6084-PA DGVEAKIKEGVVKREDLFITSKL-WNTFHRP--------DLVKSALENTLSSLKLKYLDL
DMEL_CG6084-PD DGVEAKIKEGVVKREDLFITSKL-WNTFHRP--------DLVKSALENTLSSLKLKYLDL
DMEL_CG6084-PB DGVEAKIKEGVVKREDLFITSKL-WNTFHRP--------DLVKSALENTLSSLKLKYLDL
DMEL_CG6084-PC DGVEAKIKEGVVKREDLFITSKL-WNTFHRP--------DLVKSALENTLSSLKLKYLDL
: .. :::


DMEL_Hk-PH VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PK VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PE VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PL VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PM VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PI VIIH----------KADPMCPME--EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PD VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PG VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PF VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_CG18547 IQIH----------DI-EFAKDLDIVINETLPTLEQLVKEGKARFIGVSAYPI
DMEL_CG12224-PB LQVH----------DV-DAAPNLDIVLNETIPVLEEYVQAGKARFIGVTAYDV
DMEL_CG12224-PC LQVH----------DV-DAAPNLDIVLNETIPVLEEYVQAGKARFIGVTAYDV
DMEL_CG3397 LQVH----------DV-DAAPSLDMVLNETIPVLEEYVQAGKARFIGVTAYDV
DMEL_CG2767 YLVHTPFTININEDGSFKLDKEG-LMEVDVTTNHAAIWVAMEALVEKGLTKSIGVSNFSK
RAT_Akr1c9 YIIHFPMALQPGD--IFFPRDEH-GKLLFETVDICDTWEAMEKCKDAGLAKSIGVSNFNC
DMEL_ARY-PC YLMHFPVGYKYVCDEILMPMSG--DELQTVEIDYLDTWRAMENLVKLGMVRSIGLSNFNM
DMEL_ARY-PD YLMHFPVGYKYVCDEILMPMSG--DELQTVEIDYLDTWRAMENLVKLGMVRSIGLSNFNM
DMEL_CG10638-PB YLMHSPVGVDYISDEDLMP-HEN-GQLRTNDVDYVDTYRSMEQLVHLGLVRSLGLSNFNA
DMEL_CG9436 YLMHMPVGQKFHNDS-----NVH-GTLELTDVDYLDTWREMEKLVDLGLTRSIGLSNFNA
DMEL_CG10638-PA YLMHMPVGYKYVDDNTLLPKNED-DVLQLSDVDYLDTYKAMEKLVKLGLVRSIGVSNFNS
DMEL_CG10638-PD YLMHMPVGYKYVDDNTLLPKNED-DVLQLSDVDYLDTYKAMEKLVKLGLVRSIGVSNFNS
DMEL_CG10863 YLMHWPYSYVYRGDNEMMPTDAK-GEVELNDIDYLDTWREMEKLVELGLTKSIGVSNFNS
DMEL_CG12766 YLIHWPFSYKYRGDNELIPKDAN-GEVELVDIDYLDTWGAMEKLVDLGLTKSIGVSNFNE
DMEL_CG6083 YLMHWPMAYKSGSDN-LYPTCPDTNKAAFEDIDYVDTWRAMENLVDEGLCQAIGVSNFNE
DMEL_CG6084-PA YLIHWPMGYKEGCD--LFPTDKD-GKTLYSPVDYVDTWKAMEKLVEEGLVKSIGVSNFNR
DMEL_CG6084-PD YLIHWPMGYKEGCD--LFPTDKD-GKTLYSPVDYVDTWKAMEKLVEEGLVKSIGVSNFNR
DMEL_CG6084-PB YLIHWPMGYKEGCD--LFPTDKD-GKTLYSPVDYVDTWKAMEKLVEEGLVKSIGVSNFNR
DMEL_CG6084-PC YLIHWPMGYKEGCD--LFPTDKD-GKTLYSPVDYVDTWKAMEKLVEEGLVKSIGVSNFNR
DMEL_Hk-PH VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PK VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PE VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PL VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PM VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PI VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PD VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PG VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PF VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_CG18547 SVLKEFLTRTA-------GRLDTVLTYARYTLTDETLLEYLDFFKSQNLGVICAAAHALG
DMEL_CG12224-PB DVLKECAERGK-------GRIQVVLNYARYTLLDNTLLRYMKDFQKMGVGVVCAAAHSLG
DMEL_CG12224-PC DVLKECAERGK-------GRIQVVLNYARYTLLDNTLLRYMKDFQKMGVGVVCAAAHSLG
DMEL_CG3397 DVLKECAERGK-------GRIQVVLNYARYTLLDNTLLRHMKAFQEMGVGVVCAAAHSLG
DMEL_CG2767 DQVARLLKNCK----I--RPANNQIEHHVY-LQQRDLVDFCKSE-----NITVTAYSPLG
RAT_Akr1c9 RQLERILNKPG----LKYKPVCNQVECHLY-LNQSKMLDYCKSK-----DIILVSYCTLG
DMEL_ARY-PC EQIQRIIQCSS----S--KPVVNQVEIWPG-FLQKDLVDYCRYN-----GIIVTAFSPLG
DMEL_ARY-PD EQIQRIIQCSS----S--KPVVNQVEIWPG-FLQKDLVDYCRYN-----GIIVTAFSPLG
DMEL_CG10638-PB NQLKRLLENCQ----I--KPANLQIECHPE-LVQVPLIELCKFH-----NITVVAYSPLG
DMEL_CG9436 AQTERVLANCR----I--RPVVNQVECHPG-FQQRQLREHAKRH-----GLVICAYCPLA
DMEL_CG10638-PA EQLARVLANCE----I--KPVTNQVECSPA-LNQKALTAFCKKN-----DVTLTGYTPLG
DMEL_CG10638-PD EQLARVLANCE----I--KPVTNQVECSPA-LNQKALTAFCKKN-----DVTLTGYTPLG
DMEL_CG10863 EQLTRLLANCK----I--KPIHNQIECHPA-LNQKKLIALCKKN-----DIVVTAYCPLG
DMEL_CG12766 EQLTRLLANCK----I--KPIHNQIEVHPA-LDQKKLIALCKKN-----GILVTAFSPLG
DMEL_CG6083 QQMNRLLSVAK----L--KPVVLQIECHPY-LSQKPLITLCYDN-----AIAVTAYSCLG
DMEL_CG6084-PA RQIERVLEVAT----I--PPVTNQIECHPY-LTQKKLIDFCKSK-----DITITAYSPLG
DMEL_CG6084-PD RQIERVLEVAT----I--PPVTNQIECHPY-LTQKKLIDFCKSK-----DITITAYSPLG
DMEL_CG6084-PB RQIERVLEVAT----I--PPVTNQIECHPY-LTQKKLIDFCKSK-----DITITAYSPLG
DMEL_CG6084-PC RQIERVLEVAT----I--PPVTNQIECHPY-LTQKKLIDFCKSK-----DITITAYSPLG

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DMEL_Hk-PH          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PK          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PE          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PL          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PM          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PI          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PD          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PG          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PF          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_CG18547        LL---TNAGPQPWHP-----ASDEQKAIA----------------------------RK
DMEL_CG12224-PB     LL---RNAGPHASHP-----GSQEILAVA----------------------------KR
DMEL_CG12224-PC     LL---RNAGPHASHP-----GSQEILAVA----------------------------KR
DMEL_CG3397         LL---SNAGPQSWHP-----GSPELLAVG----------------------------KR
DMEL_CG2767         SK------GIAKFNAGAGIVRDLPDLMDI----------------------------PE
RAT_Akr1c9          SS------RDKTWVD-----QKSPVLLDD----------------------------PV
DMEL_ARY-PC         ----------QPNRK-----NHCPVYFFS----------------------------EG
DMEL_ARY-PD         ----------QPNRK-----NHCPVYFFS----------------------------EG
DMEL_CG10638-PB     ----------RPKAC-----NPLPDYYTD----------------------------SK
DMEL_CG9436         ----------RPQPA-----RQWPPFLYD----------------------------EH
DMEL_CG10638-PA     ----------KPKPD-----IQKPDFIYS----------------------------PE
DMEL_CG10638-PD     ----------KPKPD-----IQKPDFIYS----------------------------PE
DMEL_CG10863        ----------RPNPA-----EKTPNYIYD----------------------------AK
DMEL_CG12766        ----------RHNAE-----LRTPTFMYD----------------------------GK
DMEL_CG6083         -------SGHTPYEK-----PGAYPLLQH----------------------------PT
DMEL_CG6084-PA      SP-------NRPWAK-----AGDPVILEE----------------------------AK
DMEL_CG6084-PD      SP-------NRPWAK-----AGDPVILEE----------------------------AK
DMEL_CG6084-PB      SP-------NRPWAK-----AGDPVILEE----------------------------AK
DMEL_CG6084-PC      SP-------NRPWAK-----AGDPVILEE----------------------------AK

DMEL_Hk-PH          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PK          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PE          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PL          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PM          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PI          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PD          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PG          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PF          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_CG18547        ASEVCKERGVELGKLAMYYTMSGLPEVSTFLTGMQTRQLLRINLD-ANEV--GLSDKEQE
DMEL_CG12224-PB     GAEICQQRNVELGKLAMYYTMQLDG-AATFLIGIPNRKLLRINLDAIFD---GLTSHEQE
DMEL_CG12224-PC     GAEICQQRNVELGKLAMYYTMQLDG-AATFLIGIPNRKLLRINLDAIFD---GLTSHEQE
DMEL_CG3397         GAEICQKRNVELGKLAMYYTMQLDG-AATFLIGIPNRKLLRINLDAIFD---GLTSHEQE
DMEL_CG2767         VKEIAASHGKTPAQVLLRWIIDT---GVSAIPKSTNPARLKQNLD-VFDF--ELTAEEVA
RAT_Akr1c9          LCAIAKKYKQTPALVALRYQLQR---GVVPLIRSFNAKRIKELTQ-VFEF--QLASEDMK
DMEL_ARY-PC         MKRLVKKYKRSASQIVLRYLIDY---GVVPIPKAANPIHIKENLN-IFDF--KLDEADTR
DMEL_ARY-PD         MKRLVKKYKRSASQIVLRYLIDY---GVVPIPKAANPIHIKENLN-IFDF--KLDEADTR
DMEL_CG10638-PB     LLALAAKYGKTPAQIILRYLIDI---GTVPIPKAAQRAHLVENLD-IFDF--HLTPEELL
DMEL_CG9436         AQNLAKKYGRTTAQICLRYLVQL---GVVPLPKSSNKARIEENFR-VFDF--ELSPDDVA
DMEL_CG10638-PA     VAVIAKKYGKTTPQIVLRYLVGL---GVIPIPKSSNTNRISENFD-IFDF--ELTAEEMA
DMEL_CG10638-PD     VAVIAKKYGKTTPQIVLRYLVGL---GVIPIPKSSNTNRISENFD-IFDF--ELTAEEMA
DMEL_CG10863        VQAIGDKYKKSTAQVVLRYLIEI---GTIPLPKSSNPKRIEENFQ-IFDF--QLDAEDHA
DMEL_CG12766        VQAIADKYNKSIAQVVIRYVIEL---GTIPLPKSSNPKRIEENFN-VFDF--KLDAEDHA
DMEL_CG6083         ILAIAEKYERTAAQVLLRFQTQS---GIIVIPRSVSKQHMLDNFKRIWDF--ELAVDDIQ
DMEL_CG6084-PA      IKEIAAKKKKTPGQILIRYQVQR---ANIVIPKSVTKDRIESNFQ-VFDF--ELTPEEIE
DMEL_CG6084-PD      IKEIAAKKKKTPGQILIRYQVQR---ANIVIPKSVTKDRIESNFQ-VFDF--ELTPEEIE
DMEL_CG6084-PB      IKEIAAKKKKTPGQILIRYQVQR---ANIVIPKSVTKDRIESNFQ-VFDF--ELTPEEIE
DMEL_CG6084-PC      IKEIAAKKKKTPGQILIRYQVQR---ANIVIPKSVTKDRIESNFQ-VFDF--ELTPEEIE
                       :  .       : : :           :        :        :    *
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DMEL_Hk-PH ELERILENKPVRPPMISTLAL-R
DMEL_Hk-PK ELERILENKPVRPPMISTLAL-R
DMEL_Hk-PE ELERILENKPVRPPMISTLAL-R
DMEL_Hk-PL ELERILENKPVRPPMISTLAL-RXQSACPQGPLSLSGGGPCGADSPKHEDEAFIEEVDAA
DMEL_Hk-PM ELERILENKPVRPPMISTLAL-RXQSACPQGPLSLSGGGPCGADSPKHEDEAFIEEVDAA
DMEL_Hk-PI ELERILENKPVRPPMISTLAL-R
DMEL_Hk-PD ELERILENKPVRPPMISTLAL-R
DMEL_Hk-PG ELERILENKPVRPPMISTLAL-R
DMEL_Hk-PF ELERILENKPVRPPMISTLAL-R
DMEL_CG18547 VLRYLKENVLTKPFDWEGNEL-EIYWAALKKK
DMEL_CG12224-PB VLQYLRENVFTKSYSWGSTLSTAKLLK
DMEL_CG12224-PC VLQYLRENVFTKSYSWGSTLSTAKLLK
DMEL_CG3397 VLQYLRENVFTKSYSWGSTLSTVNMFK
DMEL_CG2767 KLSSLDQNIRICDFAFFHGVERHPEFTFKNQYTN
RAT_Akr1c9 ALDGLNRNFRYNNAK--YFDD-HPNHPF-TDE
DMEL_ARY-PC LLRGIKPKSRIVKYE--IVKD-HMFYPFELLKENNEESEVKDSQTKEPKLPYFPSEEEEE
DMEL_ARY-PD LLRGIKPKSRIVKYE--IVKD-HMFYPFELLKENNEESEVKDSQTKEPKLPYFPSEEEEE
DMEL_CG10638-PB QMKTFNTGRRIWKFD--KAKN-HQYYPY
DMEL_CG9436 GMEQYHTGQRTVPFS--GMSG-HKYYPFNDEF
DMEL_CG10638-PA VLDGYHTGERVVPLNLIKGLN-HKYYPFSIEF
DMEL_CG10638-PD VLDGYHTGERVVPLNLIKGLN-HKYYPFSIEF
DMEL_CG10863 ILDSYNTGERLIPMT--HAIK-SKNYPFNIEF
DMEL_CG12766 ILDSYHNGERVAHAR--QAIK-SKYYPFNVYSACF
DMEL_CG6083 AINELDCNGRFMTMK--AAYG-HPHHPFEPKAKVDN
DMEL_CG6084-PA IIESFECNGRLVPLL--NQYG-HPHHPFEKDEY
DMEL_CG6084-PD IIESFECNGRLVPLL--KDAGTHKYYPFHDEY
DMEL_CG6084-PB IIESFECNGRLVPLL--KDAGTHKYYPFHDEY
DMEL_CG6084-PC IIESFECNGRLVPLL--NQYG-HPHHPFEKDEY
DMEL_Hk-PH
DMEL_Hk-PK
DMEL_Hk-PE
DMEL_Hk-PL KDAAKQGFCLIQ
DMEL_Hk-PM KDAAKQGFCLIQXQKESLDNDNKFLKVISSKTSLSSNTMTTTISKQEQQQQQQQQQQQQH
DMEL_Hk-PI
DMEL_Hk-PD
DMEL_Hk-PG
DMEL_Hk-PF
DMEL_CG18547
DMEL_CG12224-PB
DMEL_CG12224-PC
DMEL_CG3397
DMEL_CG2767
RAT_Akr1c9
DMEL_ARY-PC KENHENYE
DMEL_ARY-PD KENHENYE
DMEL_CG10638-PB
DMEL_CG9436
DMEL_CG10638-PA
DMEL_CG10638-PD
DMEL_CG10863
DMEL_CG12766
DMEL_CG6083
DMEL_CG6084-PA
DMEL_CG6084-PD
DMEL_CG6084-PB
DMEL_CG6084-PC

```
DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          ------------------------------------------------------------
DMEL_Hk-PM          LQPQQYVQLQQQHLMASGSTSDSAAAVPIAAELQFDSLNNNNNNNNNNNTNGSPEQALPTS
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          ------------------------------------------------------------
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------

DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          ------------------------------------------------------------
DMEL_Hk-PM          DERQKRRSLLNFKSLDFHLKSLYSGLRSNSGTTPPSQPNSSVPAVDQPQRRMPYLRVESV
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          ------------------------------------------------------------
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------
```

```
DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          ------------------------------------------------------------
DMEL_Hk-PM          DPEEPRGSVLPGHSPSFLSPYSGVGGGAGSLQLPSNDGGTIRRSSTSDIVNCRRGGGAGG
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          ------------------------------------------------------------
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------


DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          ------------------------------------------------------------
DMEL_Hk-PM          SGGGAASAQDSRRPSTSDLLRKARERKGSEARMGRSVSHSGLTRGGPGGGGGGLGGRRTS
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          ------------------------------------------------------------
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------
```

```
DMEL_Hk-PH           ---
DMEL_Hk-PK           ---
DMEL_Hk-PE           ---
DMEL_Hk-PL           ---
DMEL_Hk-PM           MAF
DMEL_Hk-PI           ---
DMEL_Hk-PD           ---
DMEL_Hk-PG           ---
DMEL_Hk-PF           ---
DMEL_CG18547         ---
DMEL_CG12224-PB      ---
DMEL_CG12224-PC      ---
DMEL_CG3397          ---
DMEL_CG2767          ---
RAT_Akr1c9           ---
DMEL_ARY-PC          ---
DMEL_ARY-PD          ---
DMEL_CG10638-PB      ---
DMEL_CG9436          ---
DMEL_CG10638-PA      ---
DMEL_CG10638-PD      ---
DMEL_CG10863         ---
DMEL_CG12766         ---
DMEL_CG6083          ---
DMEL_CG6084-PA       ---
DMEL_CG6084-PD       ---
DMEL_CG6084-PB       ---
DMEL_CG6084-PC       ---

CODE
invariant and highly conserved regions
co-factor binding pocket residues
substrate binding pocket
```

Conserved residues of the co-factor binding pocket

MSA of *Drosophila melanogaster* AKRs with annotation of conserved residues of the co-factor binding pocket.

```
DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          ------------------------------------------------------------
DMEL_Hk-PM          ------------------------------------------------------------
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          MTPPRLSRYLMAVRGGRDGEQHSKNSQTDEEKQRDRQRNEREAKKEEQDAINYKRDKEQR
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------

DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ----------------------MSMALCNLNGDGSAAQSTSQSQSQSPAATAAAAPLLP
DMEL_Hk-PE          ----------------------MSMALCNLNGDGSAAQSTSQSQSQSPAATAAAAPLLP
DMEL_Hk-PL          ----------------------MSMALCNLNGDGSAAQSTSQSQSQSPAATAAAAPLLP
DMEL_Hk-PM          ----------------------MSMALCNLNGDGSAAQSTSQSQSQSPAATAAAAPLLP
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          EARLLDFEIRRQQKRDEQQQQRQQQQQLQQHREHLQQQHRDQQQSSEKSPPTSSTAAAAH
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------
```

```
DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          HSHSHTLQPESTPLLLGHEQSGSAAPEGSGGGDVTDGAVTSIEMPTVVADDAGVPPLPLP
DMEL_Hk-PE          HSHSHTLQPESTPLLLGHEQSGSAAPEGSGGGDVTDGAVTSIEMPTVVADDAGVPPLPLP
DMEL_Hk-PL          HSHSHTLQPESTPLLLGHEQSGSAAPEGSGGGDVTDGAVTSIEMPTVVADDAGVPPLPLP
DMEL_Hk-PM          HSHSHTLQPESTPLLLGHEQSGSAAPEGSGGGDVTDGAVTSIEMPTVVADDAGVPPLPLP
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          QTHSHSQQQQQRQHLHLQQQHLQQQQQQQQQQQSTGSGSSNTPVTPKLQTPPPDRSLPPAF
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------

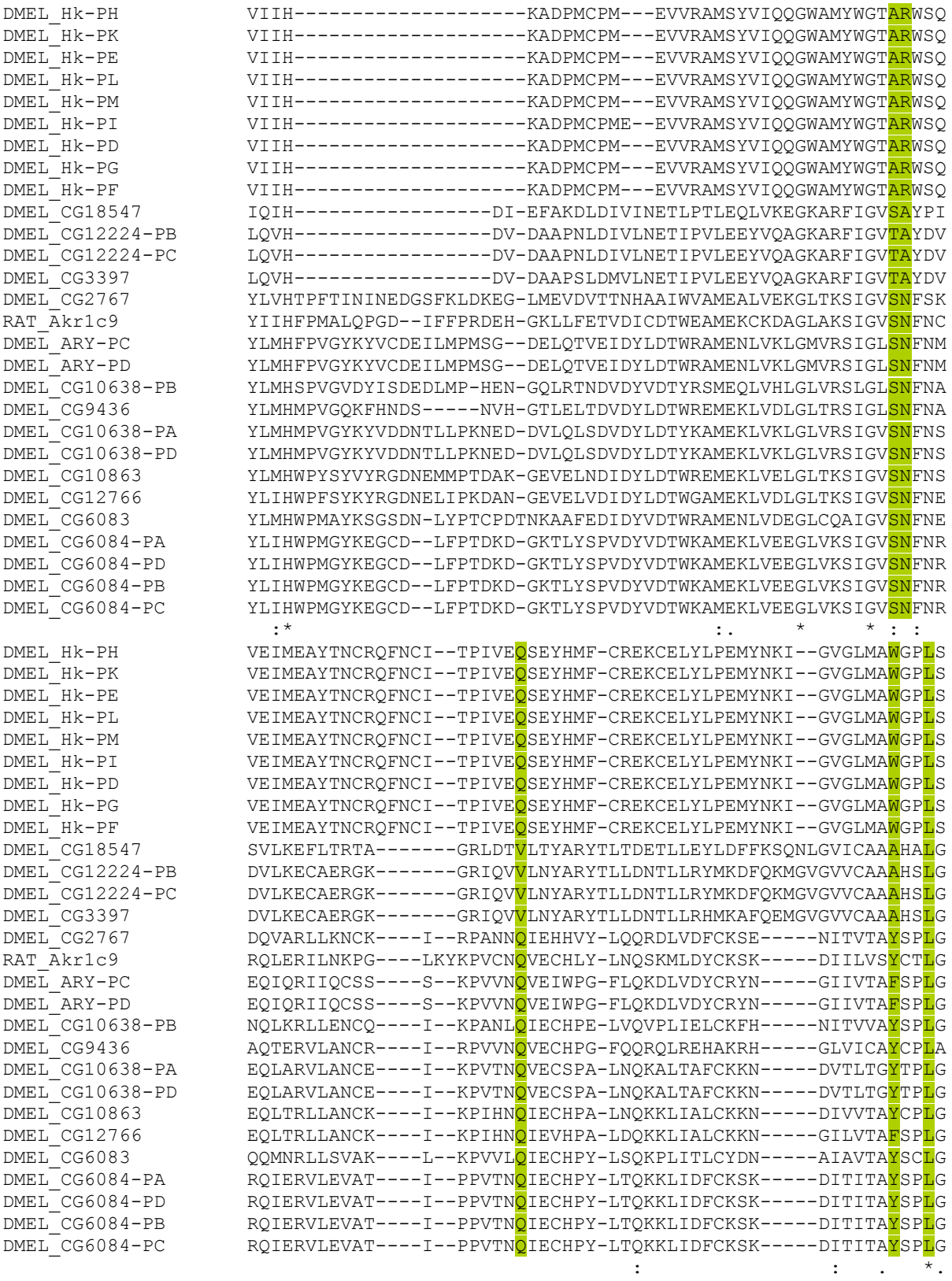

DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          LPQSTPQPLLMALPANLNFITGPTTQQLMIGNGAMGVIPSTNDSNNNNNNNNNVDNTSDE
DMEL_Hk-PE          LPQSTPQPLLMALPANLNFITGPTTQQLMIGNGAMGVIPSTNDSNNNNNNNNNVDNTSDE
DMEL_Hk-PL          LPQSTPQPLLMALPANLNFITGPTTQQLMIGNGAMGVIPSTNDSNNNNNNNNNVDNTSDE
DMEL_Hk-PM          LPQSTPQPLLMALPANLNFITGPTTQQLMIGNGAMGVIPSTNDSNNNNNNNNNVDNTSDE
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          RSRSIEREILYERKRFSQPETGEIKELDSTTSSSSTQQTFPGSAVGAGRGASAAVPGSGT
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------
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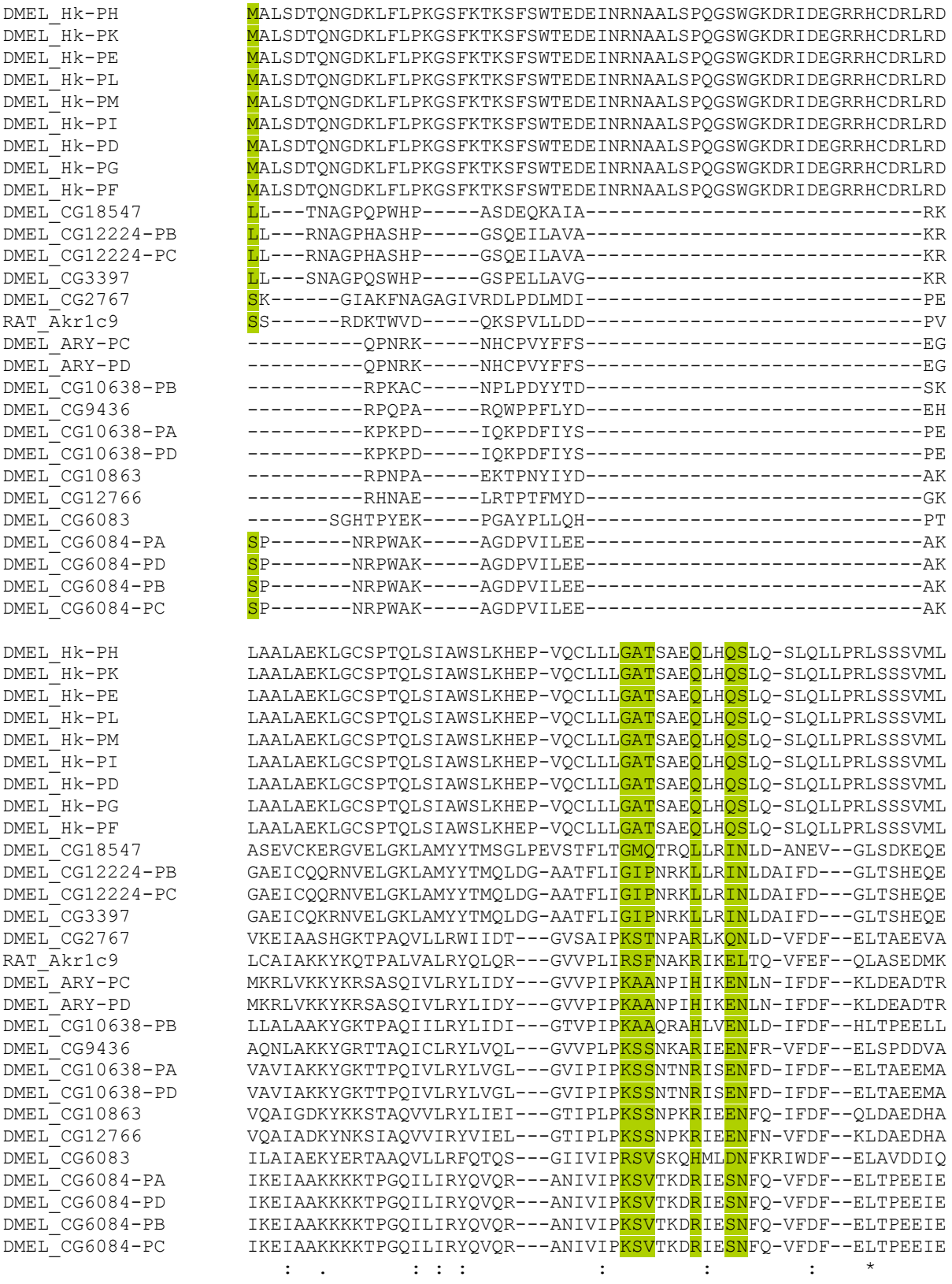
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DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          SNPVTIY-------------------------------------------------------
DMEL_Hk-PE          SNPVTIY-------------------------------------------------------
DMEL_Hk-PL          SNPVTIY-------------------------------------------------------
DMEL_Hk-PM          SNPVTIY-------------------------------------------------------
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          AIAVAVAGAGAGQSATLAGPPATAAAATAAIIPILPLGVATLRDDSTESEQSVTCAHNQL
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------

DMEL_Hk-PH          ----------MPTTASC--------------RSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PK          ------RCRAPIASLDCMEEFSG--------RSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PE          ------RCRAPIASLDCMEEFS---------RSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PL          ------RCRAPIASLDCMEEFS---------RSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PM          ------RCRAPIASLDCMEEFS---------RSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PI          ------MCRAPIASLDCMEEFSGTGGHLDFGRSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PD          ------MCRAPIASLDCMEEFSGTGGHLDFGRSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PG          ------MCRAPIASLDCMEEFSGTGGHLDFGRSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PF          DAYHRIMCRAPIASLDCMEEFSGTGGHLDFGRSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_CG18547        --------------------------------MSQTLPATYVKGFHDEAKVRRMEYRNLG
DMEL_CG12224-PB     ------------------------------------------------------MFDYSA--
DMEL_CG12224-PC     ------------------------------------------------------MFDYSA--
DMEL_CG3397         --------------------------------MSGSLPATFVKGFHDEEKVRRMEYRQLG
DMEL_CG2767         ------------------------------------------------MVNTKFLTFNN--
RAT_Akr1c9          ------------------------------------------------MDSISLRVALND--
DMEL_ARY-PC         ----------------------------------MIPADFLTSNIVEDNSQVAVILG--
DMEL_ARY-PD         ------MIPADFLTSNIVEDNSQV-------AVILGGNQNTQCGEKALLMAPKVRLSS--
DMEL_CG10638-PB     ------------------------------------------------MKLAPTVKLNN--
DMEL_CG9436         ------------------------------------------------MTNLAPTIRLNN--
DMEL_CG10638-PA     ------------------------------------------------MKLAPTVKLNN--
DMEL_CG10638-PD     ------------------------------------------------MKLAPTVKLNN--
DMEL_CG10863        ------------------------------------------------MSSKIPYVKHNN--
DMEL_CG12766        ----------------------------------------------MTGKVEYYFKHND--
DMEL_CG6083         ------------------------------------------------MSTPNFLLSN--
DMEL_CG6084-PA      ------------------------------------------------MAVPNVKFNN--
DMEL_CG6084-PD      ------------------------------------------------MAVPNVKFNN--
DMEL_CG6084-PB      -------MNTLMKIQLVRPVFRIT-------LRNYGKPERFMWCQKEYARAPKVVCLD--
DMEL_CG6084-PC      -------MNTLMKIQLVRPVFRIT-------LRNYGKPERFMWCQKEYARAPKVVCLD--
```

```
DMEL_Hk-PH          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PK          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PE          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PL          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PM          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PI          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PD          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PG          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PF          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_CG18547        KTGLQVSKVSFGGGALCANY--GFDLEEGIKTVHEAVKSGINYIDTAPWYGQGRSEEVLG
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         STGLRVSKIALGGATLSKLFSDDFDREEGILTVQEAIRSGINYIDTAPFYGQGKSEELLG
DMEL_CG2767         --GEKMPVIGIGTW--------QASDEEIETAIDAALEAGYRHIDTAPVYG---NEKAIG
RAT_Akr1c9          --GNFIPVLGFGTT-----VPEKVAKDEVIKATKIAIDNGFRHFDSAYLYE---VEEEVG
DMEL_ARY-PC         --GNQNTQCGEKAL----LMAPKLRGYQCSAAVHCAIETGFRHFDTAYYYE---NEKEIG
DMEL_ARY-PD         --GHEMPVLGFGTY--------KLRGYQCSAAVHCAIETGFRHFDTAYYYE---NEKEIG
DMEL_CG10638-PB     --GYEMPILGLGTY--------NLKKSRCEAAVCHALEMGYRHIDTAYLYR---NEGIIG
DMEL_CG9436         --GREMPTLGLGTW--------KSFESDAYHSTRHALDVGYRHLDTAFVYE---NEAEVG
DMEL_CG10638-PA     --GYEMPILGLGTY--------NSKDNEGEAAVKHAIDVGYRHIDTAYFYQ---NEAEVG
DMEL_CG10638-PD     --GYEMPILGLGTY--------NSKDNEGEAAVKHAIDVGYRHIDTAYFYQ---NEAEVG
DMEL_CG10863        --GTQIQSIGLGTY--------TSLGGDCERATLHAIDVGYRHIDTAYFYE---NENEVG
DMEL_CG12766        --GTHIQGIGLGTF--------ASTEGDCERAVLHAIDVGYRHIDTAYFYG---NEAEVG
DMEL_CG6083         --GKNMPMLGLGTW--------RSPPEVVTQAVKDAIDIGYRHFDCAHIYG---NEAQVG
DMEL_CG6084-PA      --GKEVPIIGLGTW--------GSPKGQVTEAVKVAIDAGYRHIDCAYVYQ---NEDEVG
DMEL_CG6084-PD      --GKEVPIIGLGTW--------GSPKGQVTEAVKVAIDAGYRHIDCAYVYQ---NEDEVG
DMEL_CG6084-PB      --GNEIPVIGLGTF--------NSPKGQVTEAVKVAIDAGYRHIDCAYVYQ---NEDEVG
DMEL_CG6084-PC      --GNEIPVIGLGTF--------NSPKGQVTEAVKVAIDAGYRHIDCAYVYQ---NEDEVG

DMEL_Hk-PH          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PK          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PE          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PL          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PM          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PI          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PD          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PG          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PF          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_CG18547        LALKD------VPRESYYIATKV-ARYELDYDKMFDFSAKKTRESVEKSLKLLGLDYVDV
DMEL_CG12224-PB     ---------------------------------------DKARESVKRSLERLQLDRVDI
DMEL_CG12224-PC     ---------------------------------------DKARESVKRSLERLQLDRVDI
DMEL_CG3397         QALKD------VPREAYYIATKV-ARYELDPNNMFDYTAAKARESVKRSLELLQLDRVDV
DMEL_CG2767         RVLKRWLDAGKVKREELFIVTKV-PPVSNRP--------HEVEPTIKKSLEDLQLDYVDL
RAT_Akr1c9          QAIRSKIEDGTVKREDIFYTSKL-WSTFHRP--------ELVRTCLEKTLKSTQLDYVDL
DMEL_ARY-PC         EALRTQIKMGNISRENIFLTTKL-WNTHHDP--------RDVRRICEKQLELLGFSYIDL
DMEL_ARY-PD         EALRTQIKMGNISRENIFLTTKL-WNTHHDP--------RDVRRICEKQLELLGFSYIDL
DMEL_CG10638-PB     KVLAKLIGDQKLKREQVFLVTKL-WDIYHEP--------KMVKYACDMQLKLLGVDYIDL
DMEL_CG9436         QAISEKIAEGVVTREEVFVTTKL-GGIHHDP--------ALVERACRLSLSNLGLEYVDL
DMEL_CG10638-PA     KAIRDKIAEGVVKREDIFLVTKL-WNIFHDP--------ERVEGICRKQLSNFGLDYIDL
DMEL_CG10638-PD     KAIRDKIAEGVVKREDIFLVTKL-WNIFHDP--------ERVEGICRKQLSNFGLDYIDL
DMEL_CG10863        AAVQRKIAEGVIKREDIHITTKL-WCHFHEP--------KRVEYACRKTLQNFGLQYVDL
DMEL_CG12766        AAVRKKIAEGVIKREDIFITTKL-WCNFHEP--------ERVEYACRKTLKNIGLDYVDL
DMEL_CG6083         AALREKMDEGVVTRDELFITSKL-WNTHHKP--------DLVRPACETSIRNLGVKYLNL
DMEL_CG6084-PA      DGVEAKIKEGVVKREDLFITSKL-WNTFHRP--------DLVKSALENTLSSLKLKYLDL
DMEL_CG6084-PD      DGVEAKIKEGVVKREDLFITSKL-WNTFHRP--------DLVKSALENTLSSLKLKYLDL
DMEL_CG6084-PB      DGVEAKIKEGVVKREDLFITSKL-WNTFHRP--------DLVKSALENTLSSLKLKYLDL
DMEL_CG6084-PC      DGVEAKIKEGVVKREDLFITSKL-WNTFHRP--------DLVKSALENTLSSLKLKYLDL
                                                                :    .. :::
```


DMEL_Hk-PH VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PK VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PE VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PL VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PM VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PI VIIH----------KADPMCPME--EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PD VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PG VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PF VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_CG18547 IQIH----------DI-EFAKDLDIVINETLPTLEQLVKEGKARFIGVSAYPI
DMEL_CG12224-PB LQVH----------DV-DAAPNLDIVLNETIPVLEEYVQAGKARFIGVTAYDV
DMEL_CG12224-PC LQVH----------DV-DAAPNLDIVLNETIPVLEEYVQAGKARFIGVTAYDV
DMEL_CG3397 LQVH----------DV-DAAPSLDMVLNETIPVLEEYVQAGKARFIGVTAYDV
DMEL_CG2767 YLVHTPFTININEDGSFKLDKEG-LMEVDVTTNHAAIWVAMEALVEKGLTKSIGVSNFSK
RAT_Akr1c9 YIIHFPMALQPGD--IFFPRDEH-GKLLFETVDICDTWEAMEKCKDAGLAKSIGVSNFNC
DMEL_ARY-PC YLMHFPVGYKYVCDEILMPMSG--DELQTVEIDYLDTWRAMENLVKLGMVRSIGLSNFNM
DMEL_ARY-PD YLMHFPVGYKYVCDEILMPMSG--DELQTVEIDYLDTWRAMENLVKLGMVRSIGLSNFNM
DMEL_CG10638-PB YLMHSPVGVDYISDEDLMP-HEN-GQLRTNDVDYVDTYRSMEQLVHLGLVRSLGLSNFNA
DMEL_CG9436 YLMHMPVGQKFHNDS-----NVH-GTLELTDVDYLDTWREMEKLVDLGLTRSIGLSNFNA
DMEL_CG10638-PA YLMHMPVGYKYVDDNTLLPKNED-DVLQLSDVDYLDTYKAMEKLVKLGLVRSIGVSNFNS
DMEL_CG10638-PD YLMHMPVGYKYVDDNTLLPKNED-DVLQLSDVDYLDTYKAMEKLVKLGLVRSIGVSNFNS
DMEL_CG10863 YLMHWPYSYVYRGDNEMMPTDAK-GEVELNDIDYLDTWREMEKLVELGLTKSIGVSNFNS
DMEL_CG12766 YLIHWPFSYKYRGDNELIPKDAN-GEVELVDIDYLDTWGAMEKLVDLGLTKSIGVSNFNE
DMEL_CG6083 YLMHWPMAYKSGSDN-LYPTCPDTNKAAFEDIDYVDTWRAMENLVDEGLCQAIGVSNFNE
DMEL_CG6084-PA YLIHWPMGYKEGCD--LFPTDKD-GKTLYSPVDYVDTWKAMEKLVEEGLVKSIGVSNFNR
DMEL_CG6084-PD YLIHWPMGYKEGCD--LFPTDKD-GKTLYSPVDYVDTWKAMEKLVEEGLVKSIGVSNFNR
DMEL_CG6084-PB YLIHWPMGYKEGCD--LFPTDKD-GKTLYSPVDYVDTWKAMEKLVEEGLVKSIGVSNFNR
DMEL_CG6084-PC YLIHWPMGYKEGCD--LFPTDKD-GKTLYSPVDYVDTWKAMEKLVEEGLVKSIGVSNFNR
DMEL_Hk-PH VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PK VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PE VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PL VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PM VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PI VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PD VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PG VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PF VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_CG18547 SVLKEFLTRTA-------GRLDTVLTYARYTLTDETLLEYLDFFKSQNLGVICAAAHALG
DMEL_CG12224-PB DVLKECAERGK-------GRIQVVLNYARYTLLDNTLLRYMKDFQKMGVGVVCAAAHSLG
DMEL_CG12224-PC DVLKECAERGK-------GRIQVVLNYARYTLLDNTLLRYMKDFQKMGVGVVCAAAHSLG
DMEL_CG3397 DVLKECAERGK-------GRIQVVLNYARYTLLDNTLLRHMKAFQEMGVGVVCAAAHSLG
DMEL_CG2767 DQVARLLKNCK----I--RPANNQIEHHVY-LQQRDLVDFCKSE-----NITVTAYSPLG
RAT_Akr1c9 RQLERILNKPG----LKYKPVCNQVECHLY-LNQSKMLDYCKSK-----DIILVSYCTLG
DMEL_ARY-PC EQIQRIIQCSS----S--KPVVNQVEIWPG-FLQKDLVDYCRYN-----GIIVTAFSPLG
DMEL_ARY-PD EQIQRIIQCSS----S--KPVVNQVEIWPG-FLQKDLVDYCRYN-----GIIVTAFSPLG
DMEL_CG10638-PB NQLKRLLENCQ----I--KPANLQIECHPE-LVQVPLIELCKFH-----NITVVAYSPLG
DMEL_CG9436 AQTERVLANCR----I--RPVVNQVECHPG-FQQRQLREHAKRH-----GLVICAYCPLA
DMEL_CG10638-PA EQLARVLANCE----I--KPVTNQVECSPA-LNQKALTAFCKKN-----DVTLTGYTPLG
DMEL_CG10638-PD EQLARVLANCE----I--KPVTNQVECSPA-LNQKALTAFCKKN-----DVTLTGYTPLG
DMEL_CG10863 EQLTRLLANCK----I--KPIHNQIECHPA-LNQKKLIALCKKN-----DIVVTAYCPLG
DMEL_CG12766 EQLTRLLANCK----I--KPIHNQIEVHPA-LDQKKLIALCKKN-----GILVTAFSPLG
DMEL_CG6083 QQMNRLLSVAK----L--KPVVLQIECHPY-LSQKPLITLCYDN-----AIAVTAYSCLG
DMEL_CG6084-PA RQIERVLEVAT----I--PPVTNQIECHPY-LTQKKLIDFCKSK-----DITITAYSPLG
DMEL_CG6084-PD RQIERVLEVAT----I--PPVTNQIECHPY-LTQKKLIDFCKSK-----DITITAYSPLG
DMEL_CG6084-PB RQIERVLEVAT----I--PPVTNQIECHPY-LTQKKLIDFCKSK-----DITITAYSPLG
DMEL_CG6084-PC RQIERVLEVAT----I--PPVTNQIECHPY-LTQKKLIDFCKSK-----DITITAYSPLG


DMEL_Hk-PH MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PK MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PE MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PL MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PM MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PI MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PD MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PG MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PF MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_CG18547 LL---TNAGPQPWHP-----ASDEQKAIA----------RK
DMEL_CG12224-PB LL---RNAGPHASHP-----GSQEILAVA----------KR
DMEL_CG12224-PC LL---RNAGPHASHP-----GSQEILAVA----------KR
DMEL_CG3397 LL---SNAGPQSWHP-----GSPELLAVG----------KR
DMEL_CG2767 SK------GIAKFNAGAGIVRDLPDLMDI----------PE
RAT_Akr1c9 SS------RDKTWVD-----QKSPVLLDD----------PV
DMEL_ARY-PC ----------QPNRK-----NHCPVYFFS----------EG
DMEL_ARY-PD ----------QPNRK-----NHCPVYFFS----------EG
DMEL_CG10638-PB ----------RPKAC-----NPLPDYYTD----------SK
DMEL_CG9436 ----------RPQPA-----RQWPPFLYD----------EH
DMEL_CG10638-PA ----------KPKPD-----IQKPDFIYS----------PE
DMEL_CG10638-PD ----------KPKPD-----IQKPDFIYS----------PE
DMEL_CG10863 ----------RPNPA-----EKTPNYIYD----------AK
DMEL_CG12766 ----------RHNAE-----LRTPTFMYD----------GK
DMEL_CG6083 -------SGHTPYEK-----PGAYPLLQH----------PT
DMEL_CG6084-PA SP-------NRPWAK-----AGDPVILEE----------AK
DMEL_CG6084-PD SP-------NRPWAK-----AGDPVILEE----------AK
DMEL_CG6084-PB SP-------NRPWAK-----AGDPVILEE----------AK
DMEL_CG6084-PC SP-------NRPWAK-----AGDPVILEE----------AK
DMEL_Hk-PH LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PK LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PE LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PL LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PM LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PI LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PD LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PG LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PF LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_CG18547 ASEVCKERGVELGKLAMYYTMSGLPEVSTFLTGMQTRQLLRINLD-ANEV--GLSDKEQE
DMEL_CG12224-PB GAEICQQRNVELGKLAMYYTMQLDG-AATFLIGIPNRKLLRINLDAIFD---GLTSHEQE
DMEL_CG12224-PC GAEICQQRNVELGKLAMYYTMQLDG-AATFLIGIPNRKLLRINLDAIFD---GLTSHEQE
DMEL_CG3397 GAEICQKRNVELGKLAMYYTMQLDG-AATFLIGIPNRKLLRINLDAIFD---GLTSHEQE
DMEL_CG2767 VKEIAASHGKTPAQVLLRWIIDT---GVSAIPKSTNPARLKQNLD-VFDF--ELTAEEVA
RAT_Akr1c9 LCAIAKKYKQTPALVALRYQLQR---GVVPLIRSFNAKRIKELTQ-VFEF--QLASEDMK
DMEL_ARY-PC MKRLVKKYKRSASQIVLRYLIDY---GVVPIPKAANPIHIKENLN-IFDF--KLDEADTR
DMEL_ARY-PD MKRLVKKYKRSASQIVLRYLIDY---GVVPIPKAANPIHIKENLN-IFDF--KLDEADTR
DMEL_CG10638-PB LLALAAKYGKTPAQIILRYLIDI---GTVPIPKAAQRAHLVENLD-IFDF--HLTPEELL
DMEL_CG9436 AQNLAKKYGRTTAQICLRYLVQL---GVVPLPKSSNKARIEENFR-VFDF--ELSPDDVA
DMEL_CG10638-PA VAVIAKKYGKTTPQIVLRYLVGL---GVIPIPKSSNTNRISENFD-IFDF--ELTAEEMA
DMEL_CG10638-PD VAVIAKKYGKTTPQIVLRYLVGL---GVIPIPKSSNTNRISENFD-IFDF--ELTAEEMA
DMEL_CG10863 VQAIGDKYKKSTAQVVLRYLIEI---GTIPLPKSSNPKRIEENFQ-IFDF--QLDAEDHA
DMEL_CG12766 VQAIADKYNKSIAQVVIRYVIEL---GTIPLPKSSNPKRIEENFN-VFDF--KLDAEDHA
DMEL_CG6083 ILAIAEKYERTAAQVLLRFQTQS---GIIVIPRSVSKQHMLDNFKRIWDF--ELAVDDIQ
DMEL_CG6084-PA IKEIAAKKKKTPGQILIRYQVQR---ANIVIPKSVTKDRIESNFQ-VFDF--ELTPEEIE
DMEL_CG6084-PD IKEIAAKKKKTPGQILIRYQVQR---ANIVIPKSVTKDRIESNFQ-VFDF--ELTPEEIE
DMEL_CG6084-PB IKEIAAKKKKTPGQILIRYQVQR---ANIVIPKSVTKDRIESNFQ-VFDF--ELTPEEIE
DMEL_CG6084-PC IKEIAAKKKKTPGQILIRYQVQR---ANIVIPKSVTKDRIESNFQ-VFDF--ELTPEEIE

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DMEL_Hk-PH          ELERILENKPVRPPMISTLAL-R-------------------------------------
DMEL_Hk-PK          ELERILENKPVRPPMISTLAL-R-------------------------------------
DMEL_Hk-PE          ELERILENKPVRPPMISTLAL-R-------------------------------------
DMEL_Hk-PL          ELERILENKPVRPPMISTLAL-RXQSACPQGPLSLSGGGPCGADSPKHEDEAFIEEVDAA
DMEL_Hk-PM          ELERILENKPVRPPMISTLAL-RXQSACPQGPLSLSGGGPCGADSPKHEDEAFIEEVDAA
DMEL_Hk-PI          ELERILENKPVRPPMISTLAL-R-------------------------------------
DMEL_Hk-PD          ELERILENKPVRPPMISTLAL-R-------------------------------------
DMEL_Hk-PG          ELERILENKPVRPPMISTLAL-R-------------------------------------
DMEL_Hk-PF          ELERILENKPVRPPMISTLAL-R-------------------------------------
DMEL_CG18547        VLRYLKENVLTKPFDWEGNEL-EIYWAALKKK----------------------------
DMEL_CG12224-PB     VLQYLRENVFTKSYSWGSTLSTAKLLK---------------------------------
DMEL_CG12224-PC     VLQYLRENVFTKSYSWGSTLSTAKLLK---------------------------------
DMEL_CG3397         VLQYLRENVFTKSYSWGSTLSTVNMFK---------------------------------
DMEL_CG2767         KLSSLDQNIRICDFAFFHGVERHPEFTFKNQYTN--------------------------
RAT_Akr1c9          ALDGLNRNFRYNNAK--YFDD-HPNHPF-TDE----------------------------
DMEL_ARY-PC         LLRGIKPKSRIVKYE--IVKD-HMFYPFELLKENNEESEVKDSQTKEPKLPYFPSEEEEE
DMEL_ARY-PD         LLRGIKPKSRIVKYE--IVKD-HMFYPFELLKENNEESEVKDSQTKEPKLPYFPSEEEEE
DMEL_CG10638-PB     QMKTFNTGRRIWKFD--KAKN-HQYYPY---------------------------------
DMEL_CG9436         GMEQYHTGQRTVPFS--GMSG-HKYYPFNDEF-----------------------------
DMEL_CG10638-PA     VLDGYHTGERVVPLNLIKGLN-HKYYPFSIEF-----------------------------
DMEL_CG10638-PD     VLDGYHTGERVVPLNLIKGLN-HKYYPFSIEF-----------------------------
DMEL_CG10863        ILDSYNTGERLIPMT--HAIK-SKNYPFNIEF-----------------------------
DMEL_CG12766        ILDSYHNGERVAHAR--QAIK-SKYYPFNVYSACF--------------------------
DMEL_CG6083         AINELDCNGRFMTMK--AAYG-HPHHPFEPKAKVDN-------------------------
DMEL_CG6084-PA      IIESFECNGRLVPLL--NQYG-HPHHPFEKDEY----------------------------
DMEL_CG6084-PD      IIESFECNGRLVPLL--KDAGTHKYYPFHDEY-----------------------------
DMEL_CG6084-PB      IIESFECNGRLVPLL--KDAGTHKYYPFHDEY-----------------------------
DMEL_CG6084-PC      IIESFECNGRLVPLL--NQYG-HPHHPFEKDEY----------------------------
                     :
DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          KDAAKQGFCLIQ--------------------------------------------------
DMEL_Hk-PM          KDAAKQGFCLIQXQKESLDNDNKFLKVISSKTSLSSNTMTTTISKQEQQQQQQQQQQQQH
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          ------------------------------------------------------------
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         KENHENYE------------------------------------------------------
DMEL_ARY-PD         KENHENYE------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------
```

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DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          ------------------------------------------------------------
DMEL_Hk-PM          LQPQQYVQLQQQHLMASGSTSDSAAAVPIAAELQFDSLNNNNNNNNNNTNGSPEQALPTS
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          ------------------------------------------------------------
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------

DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          ------------------------------------------------------------
DMEL_Hk-PM          DERQKRRSLLNFKSLDFHLKSLYSGLRSNSGTTPPSQPNSSVPAVDQPQRRMPYLRVESV
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          ------------------------------------------------------------
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------
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DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          ------------------------------------------------------------
DMEL_Hk-PM          DPEEPRGSVLPGHSPSFLSPYSGVGGGAGSLQLPSNDGGTIRRSSTSDIVNCRRGGGAGG
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          ------------------------------------------------------------
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------

DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          ------------------------------------------------------------
DMEL_Hk-PM          SGGGAASAQDSRRPSTSDLLRKARERKGSEARMGRSVSHSGLTRGGPGGGGGGLGGRRTS
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          ------------------------------------------------------------
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------
```

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DMEL_Hk-PH           ---
DMEL_Hk-PK           ---
DMEL_Hk-PE           ---
DMEL_Hk-PL           ---
DMEL_Hk-PM           MAF
DMEL_Hk-PI           ---
DMEL_Hk-PD           ---
DMEL_Hk-PG           ---
DMEL_Hk-PF           ---
DMEL_CG18547         ---
DMEL_CG12224-PB      ---
DMEL_CG12224-PC      ---
DMEL_CG3397          ---
DMEL_CG2767          ---
RAT_Akr1c9           ---
DMEL_ARY-PC          ---
DMEL_ARY-PD          ---
DMEL_CG10638-PB      ---
DMEL_CG9436          ---
DMEL_CG10638-PA      ---
DMEL_CG10638-PD      ---
DMEL_CG10863         ---
DMEL_CG12766         ---
DMEL_CG6083          ---
DMEL_CG6084-PA       ---
DMEL_CG6084-PD       ---
DMEL_CG6084-PB       ---
DMEL_CG6084-PC       ---
```

CODE

invariant and highly conserved regions

co-factor binding pocket residues

substrate binding pocket

Conserved residues of the substrate binding pocket

MSA of *Drosophila melanogaster* AKRs with annotation of conserved residues of the substrate binding pocket.

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DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          ------------------------------------------------------------
DMEL_Hk-PM          ------------------------------------------------------------
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          MTPPRLSRYLMAVRGGRDGEQHSKNSQTDEEKQRDRQRNEREAKKEEQDAINYKRDKEQR
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------

DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ----------------------MSMALCNLNGDGSAAQSTSQSQSQSPAATAAAAPLLP
DMEL_Hk-PE          ----------------------MSMALCNLNGDGSAAQSTSQSQSQSPAATAAAAPLLP
DMEL_Hk-PL          ----------------------MSMALCNLNGDGSAAQSTSQSQSQSPAATAAAAPLLP
DMEL_Hk-PM          ----------------------MSMALCNLNGDGSAAQSTSQSQSQSPAATAAAAPLLP
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          EARLLDFEIRRQQKRDEQQQQRQQQQQLQQHREHLQQQHRDQQQSSEKSPPTSSTAAAAH
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------
```

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DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          HSHSHTLQPESTPLLLGHEQSGSAAPEGSGGGDVTDGAVTSIEMPTVVADDAGVPPLPLP
DMEL_Hk-PE          HSHSHTLQPESTPLLLGHEQSGSAAPEGSGGGDVTDGAVTSIEMPTVVADDAGVPPLPLP
DMEL_Hk-PL          HSHSHTLQPESTPLLLGHEQSGSAAPEGSGGGDVTDGAVTSIEMPTVVADDAGVPPLPLP
DMEL_Hk-PM          HSHSHTLQPESTPLLLGHEQSGSAAPEGSGGGDVTDGAVTSIEMPTVVADDAGVPPLPLP
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          QTHSHSQQQQQRQHLHLQQQHLQQQQQQQQQQQSTGSGSSNTPVTPKLQTPPPDRSLPPAF
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------

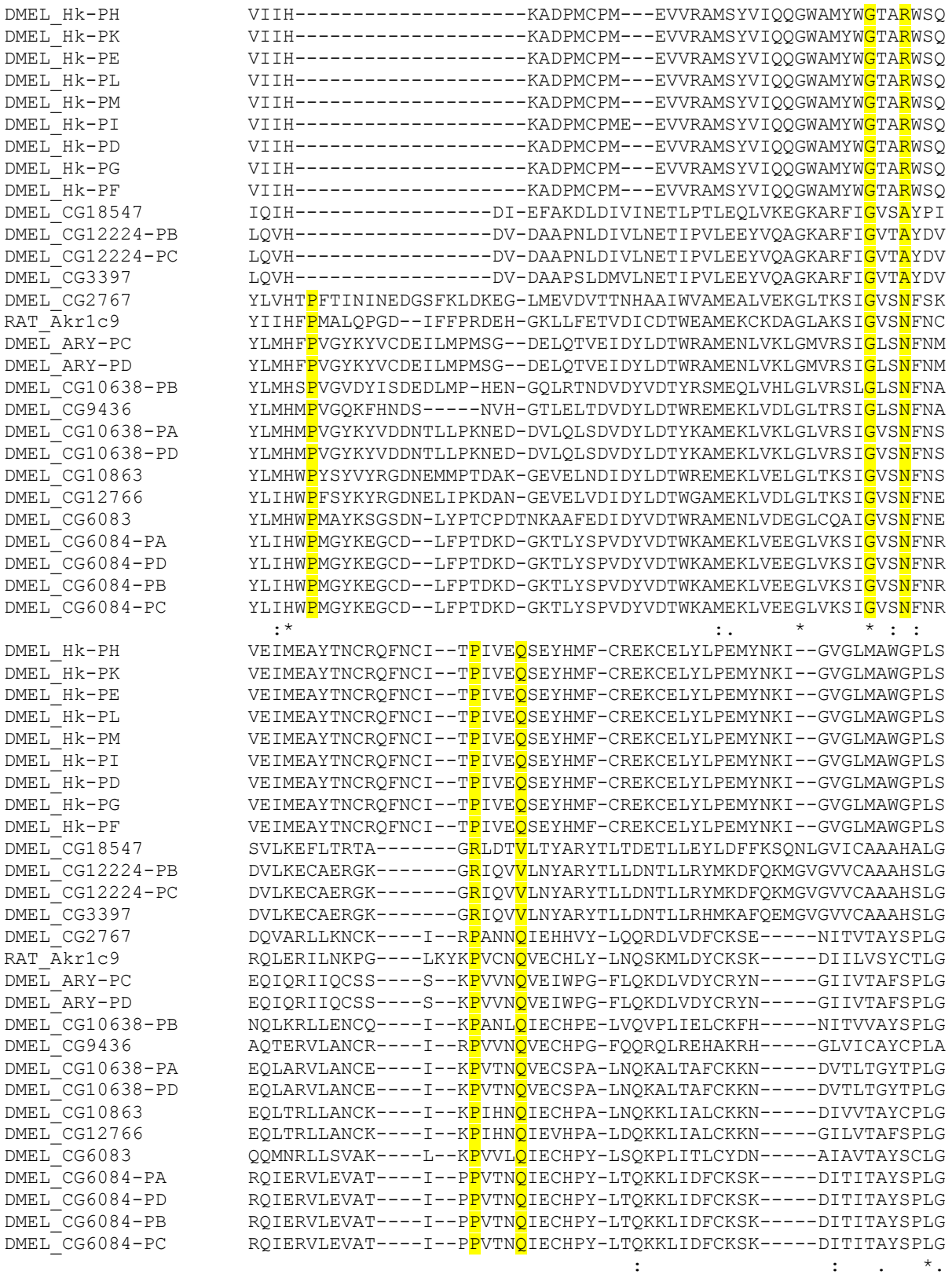
DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          LPQSTPQPLLMALPANLNFITGPTTQQLMIGNGAMGVIPSTNDSNNNNNNNNNVDNTSDE
DMEL_Hk-PE          LPQSTPQPLLMALPANLNFITGPTTQQLMIGNGAMGVIPSTNDSNNNNNNNNNVDNTSDE
DMEL_Hk-PL          LPQSTPQPLLMALPANLNFITGPTTQQLMIGNGAMGVIPSTNDSNNNNNNNNNVDNTSDE
DMEL_Hk-PM          LPQSTPQPLLMALPANLNFITGPTTQQLMIGNGAMGVIPSTNDSNNNNNNNNNVDNTSDE
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          RSRSIEREILYERKRFSQPETGEIKELDSTTSSSSTQQTFPGSAVGAGRGASAAVPGSGT
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------
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```
DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          SNPVTIY-------------------------------------------------------
DMEL_Hk-PE          SNPVTIY-------------------------------------------------------
DMEL_Hk-PL          SNPVTIY-------------------------------------------------------
DMEL_Hk-PM          SNPVTIY-------------------------------------------------------
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          AIAVAVAGAGAGQSATLAGPPATAAAATAAIIPILPLGVATLRDDSTESEQSVTCAHNQL
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------

DMEL_Hk-PH          ----------MPTTASC--------------RSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PK          ------RCRAPIASLDCMEEFSG--------RSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PE          ------RCRAPIASLDCMEEFS---------RSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PL          ------RCRAPIASLDCMEEFS---------RSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PM          ------RCRAPIASLDCMEEFS---------RSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PI          ------MCRAPIASLDCMEEFSGTGGHLDFGRSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PD          ------MCRAPIASLDCMEEFSGTGGHLDFGRSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PG          ------MCRAPIASLDCMEEFSGTGGHLDFGRSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_Hk-PF          DAYHRIMCRAPIASLDCMEEFSGTGGHLDFGRSISLGSNPALPLRHGSTPTPGLRYKNLG
DMEL_CG18547        --------------------------------MSQTLPATYVKGFHDEAKVRRMEYRNLG
DMEL_CG12224-PB     ------------------------------------------------------MFDYSA--
DMEL_CG12224-PC     ------------------------------------------------------MFDYSA--
DMEL_CG3397         --------------------------------MSGSLPATFVKGFHDEEKVRRMEYRQLG
DMEL_CG2767         ------------------------------------------------MVNTKFLTFNN--
RAT_Akr1c9          ------------------------------------------------MDSISLRVALND--
DMEL_ARY-PC         ----------------------------------MIPADFLTSNIVEDNSQVAVILG--
DMEL_ARY-PD         ------MIPADFLTSNIVEDNSQV-------AVILGGNQNTQCGEKALLMAPKVRLSS--
DMEL_CG10638-PB     ------------------------------------------------MKLAPTVKLNN--
DMEL_CG9436         ------------------------------------------------MTNLAPTIRLNN--
DMEL_CG10638-PA     ------------------------------------------------MKLAPTVKLNN--
DMEL_CG10638-PD     ------------------------------------------------MKLAPTVKLNN--
DMEL_CG10863        ------------------------------------------------MSSKIPYVKHNN--
DMEL_CG12766        ----------------------------------------------MTGKVEYYFKHND--
DMEL_CG6083         ------------------------------------------------MSTPNFLLSN--
DMEL_CG6084-PA      ------------------------------------------------MAVPNVKFNN--
DMEL_CG6084-PD      ------------------------------------------------MAVPNVKFNN--
DMEL_CG6084-PB      -------MNTLMKIQLVRPVFRIT-------LRNYGKPERFMWCQKEYARAPKVVCLD--
DMEL_CG6084-PC      -------MNTLMKIQLVRPVFRIT-------LRNYGKPERFMWCQKEYARAPKVVCLD--
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```
DMEL_Hk-PH          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PK          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PE          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PL          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PM          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PI          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PD          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PG          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_Hk-PF          KSGLRISNVGLGTW---PVFSPGVSDDQAEAILKLAIESGINLFDISEAH----SETEIG
DMEL_CG18547        KTGLQVSKVSFGGGALCANY--GFDLEEGIKTVHEAVKSGINYIDTAPWYGQGRSEEVLG
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         STGLRVSKIALGGATLSKLFSDDFDREEGILTVQEAIRSGINYIDTAPFYGQGKSEELLG
DMEL_CG2767         --GEKMPVIGIGTW--------QASDEEIETAIDAALEAGYRHIDTAPVYG---NEKAIG
RAT_Akr1c9          --GNFIPVLGFGTT-----VPEKVAKDEVIKATKIAIDNGFRHFDSAYLYE---VEEEVG
DMEL_ARY-PC         --GNQNTQCGEKAL----LMAPKLRGYQCSAAVHCAIETGFRHFDTAYYYE---NEKEIG
DMEL_ARY-PD         --GHEMPVLGFGTY--------KLRGYQCSAAVHCAIETGFRHFDTAYYYE---NEKEIG
DMEL_CG10638-PB     --GYEMPILGLGTY--------NLKKSRCEAAVCHALEMGYRHIDTAYLYR---NEGIIG
DMEL_CG9436         --GREMPTLGLGTW--------KSFESDAYHSTRHALDVGYRHLDTAFVYE---NEAEVG
DMEL_CG10638-PA     --GYEMPILGLGTY--------NSKDNEGEAAVKHAIDVGYRHIDTAYFYQ---NEAEVG
DMEL_CG10638-PD     --GYEMPILGLGTY--------NSKDNEGEAAVKHAIDVGYRHIDTAYFYQ---NEAEVG
DMEL_CG10863        --GTQIQSIGLGTY--------TSLGGDCERATLHAIDVGYRHIDTAYFYE---NENEVG
DMEL_CG12766        --GTHIQGIGLGTF--------ASTEGDCERAVLHAIDVGYRHIDTAYFYG---NEAEVG
DMEL_CG6083         --GKNMPMLGLGTW--------RSPPEVVTQAVKDAIDIGYRHFDCAHIYG---NEAQVG
DMEL_CG6084-PA      --GKEVPIIGLGTW--------GSPKGQVTEAVKVAIDAGYRHIDCAYVYQ---NEDEVG
DMEL_CG6084-PD      --GKEVPIIGLGTW--------GSPKGQVTEAVKVAIDAGYRHIDCAYVYQ---NEDEVG
DMEL_CG6084-PB      --GNEIPVIGLGTF--------NSPKGQVTEAVKVAIDAGYRHIDCAYVYQ---NEDEVG
DMEL_CG6084-PC      --GNEIPVIGLGTF--------NSPKGQVTEAVKVAIDAGYRHIDCAYVYQ---NEDEVG

DMEL_Hk-PH          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PK          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PE          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PL          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PM          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PI          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PD          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PG          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_Hk-PF          KILQR----AGWKRTAYVITTKVYWSTKSEE---RGLSRKHIIECVRASLQRLQLQYIDI
DMEL_CG18547        LALKD------VPRESYYIATKV-ARYELDYDKMFDFSAKKTRESVEKSLKLLGLDYVDV
DMEL_CG12224-PB     ------------------------------------DKARESVKRSLERLQLDRVDI
DMEL_CG12224-PC     ------------------------------------DKARESVKRSLERLQLDRVDI
DMEL_CG3397         QALKD------VPREAYYIATKV-ARYELDPNNMFDYTAAKARESVKRSLELLQLDRVDV
DMEL_CG2767         RVLKRWLDAGKVKREELFIVTKV-PPVSNRP--------HEVEPTIKKSLEDLQLDYVDL
RAT_Akr1c9          QAIRSKIEDGTVKREDIFYTSKL-WSTFHRP--------ELVRTCLEKTLKSTQLDYVDL
DMEL_ARY-PC         EALRTQIKMGNISRENIFLTTKL-WNTHHDP--------RDVRRICEKQLELLGFSYIDL
DMEL_ARY-PD         EALRTQIKMGNISRENIFLTTKL-WNTHHDP--------RDVRRICEKQLELLGFSYIDL
DMEL_CG10638-PB     KVLAKLIGDQKLKREQVFLVTKL-WDIYHEP--------KMVKYACDMQLKLLGVDYIDL
DMEL_CG9436         QAISEKIAEGVVTREEVFVTTKL-GGIHHDP--------ALVERACRLSLSNLGLEYVDL
DMEL_CG10638-PA     KAIRDKIAEGVVKREDIFLVTKL-WNIFHDP--------ERVEGICRKQLSNFGLDYIDL
DMEL_CG10638-PD     KAIRDKIAEGVVKREDIFLVTKL-WNIFHDP--------ERVEGICRKQLSNFGLDYIDL
DMEL_CG10863        AAVQRKIAEGVIKREDIHITTKL-WCHFHEP--------KRVEYACRKTLQNFGLQYVDL
DMEL_CG12766        AAVRKKIAEGVIKREDIFITTKL-WCNFHEP--------ERVEYACRKTLKNIGLDYVDL
DMEL_CG6083         AALREKMDEGVVTRDELFITSKL-WNTHHKP--------DLVRPACETSIRNLGVKYLNL
DMEL_CG6084-PA      DGVEAKIKEGVVKREDLFITSKL-WNTFHRP--------DLVKSALENTLSSLKLKYLDL
DMEL_CG6084-PD      DGVEAKIKEGVVKREDLFITSKL-WNTFHRP--------DLVKSALENTLSSLKLKYLDL
DMEL_CG6084-PB      DGVEAKIKEGVVKREDLFITSKL-WNTFHRP--------DLVKSALENTLSSLKLKYLDL
DMEL_CG6084-PC      DGVEAKIKEGVVKREDLFITSKL-WNTFHRP--------DLVKSALENTLSSLKLKYLDL
                                                                :    .. :::
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DMEL_Hk-PH VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PK VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PE VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PL VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PM VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PI VIIH----------KADPMCPME--EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PD VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PG VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_Hk-PF VIIH----------KADPMCPM---EVVRAMSYVIQQGWAMYWGTARWSQ
DMEL_CG18547 IQIH----------DI-EFAKDLDIVINETLPTLEQLVKEGKARFIGVSAYPI
DMEL_CG12224-PB LQVH----------DV-DAAPNLDIVLNETIPVLEEYVQAGKARFIGVTAYDV
DMEL_CG12224-PC LQVH----------DV-DAAPNLDIVLNETIPVLEEYVQAGKARFIGVTAYDV
DMEL_CG3397 LQVH----------DV-DAAPSLDMVLNETIPVLEEYVQAGKARFIGVTAYDV
DMEL_CG2767 YLVHTPFTININEDGSFKLDKEG-LMEVDVTTNHAAIWVAMEALVEKGLTKSIGVSNFSK
RAT_Akr1c9 YIIHFPMALQPGD--IFFPRDEH-GKLLFETVDICDTWEAMEKCKDAGLAKSIGVSNFNC
DMEL_ARY-PC YLMHFPVGYKYVCDEILMPMSG--DELQTVEIDYLDTWRAMENLVKLGMVRSIGLSNFNM
DMEL_ARY-PD YLMHFPVGYKYVCDEILMPMSG--DELQTVEIDYLDTWRAMENLVKLGMVRSIGLSNFNM
DMEL_CG10638-PB YLMHSPVGVDYISDEDLMP-HEN-GQLRTNDVDYVDTYRSMEQLVHLGLVRSLGLSNFNA
DMEL_CG9436 YLMHMPVGQKFHNDS-----NVH-GTLELTDVDYLDTWREMEKLVDLGLTRSIGLSNFNA
DMEL_CG10638-PA YLMHMPVGYKYVDDNTLLPKNED-DVLQLSDVDYLDTYKAMEKLVKLGLVRSIGVSNFNS
DMEL_CG10638-PD YLMHMPVGYKYVDDNTLLPKNED-DVLQLSDVDYLDTYKAMEKLVKLGLVRSIGVSNFNS
DMEL_CG10863 YLMHWPYSYVYRGDNEMMPTDAK-GEVELNDIDYLDTWREMEKLVELGLTKSIGVSNFNS
DMEL_CG12766 YLIHWPFSYKYRGDNELIPKDAN-GEVELVDIDYLDTWGAMEKLVDLGLTKSIGVSNFNE
DMEL_CG6083 YLMHWPMAYKSGSDN-LYPTCPDTNKAAFEDIDYVDTWRAMENLVDEGLCQAIGVSNFNE
DMEL_CG6084-PA YLIHWPMGYKEGCD--LFPTDKD-GKTLYSPVDYVDTWKAMEKLVEEGLVKSIGVSNFNR
DMEL_CG6084-PD YLIHWPMGYKEGCD--LFPTDKD-GKTLYSPVDYVDTWKAMEKLVEEGLVKSIGVSNFNR
DMEL_CG6084-PB YLIHWPMGYKEGCD--LFPTDKD-GKTLYSPVDYVDTWKAMEKLVEEGLVKSIGVSNFNR
DMEL_CG6084-PC YLIHWPMGYKEGCD--LFPTDKD-GKTLYSPVDYVDTWKAMEKLVEEGLVKSIGVSNFNR
DMEL_Hk-PH VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PK VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PE VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PL VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PM VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PI VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PD VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PG VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_Hk-PF VEIMEAYTNCRQFNCI--TPIVEQSEYHMF-CREKCELYLPEMYNKI--GVGLMAWGPLS
DMEL_CG18547 SVLKEFLTRTA-------GRLDTVLTYARYTLTDETLLEYLDFFKSQNLGVICAAAHALG
DMEL_CG12224-PB DVLKECAERGK-------GRIQVVLNYARYTLLDNTLLRYMKDFQKMGVGVVCAAAHSLG
DMEL_CG12224-PC DVLKECAERGK-------GRIQVVLNYARYTLLDNTLLRYMKDFQKMGVGVVCAAAHSLG
DMEL_CG3397 DVLKECAERGK-------GRIQVVLNYARYTLLDNTLLRHMKAFQEMGVGVVCAAAHSLG
DMEL_CG2767 DQVARLLKNCK----I--RPANNQIEHHVY-LQQRDLVDFCKSE-----NITVTAYSPLG
RAT_Akr1c9 RQLERILNKPG----LKYKPVCNQVECHLY-LNQSKMLDYCKSK-----DIILVSYCTLG
DMEL_ARY-PC EQIQRIIQCSS----S--KPVVNQVEIWPG-FLQKDLVDYCRYN-----GIIVTAFSPLG
DMEL_ARY-PD EQIQRIIQCSS----S--KPVVNQVEIWPG-FLQKDLVDYCRYN-----GIIVTAFSPLG
DMEL_CG10638-PB NQLKRLLENCQ----I--KPANLQIECHPE-LVQVPLIELCKFH-----NITVVAYSPLG
DMEL_CG9436 AQTERVLANCR----I--RPVVNQVECHPG-FQQRQLREHAKRH-----GLVICAYCPLA
DMEL_CG10638-PA EQLARVLANCE----I--KPVTNQVECSPA-LNQKALTAFCKKN-----DVTLTGYTPLG
DMEL_CG10638-PD EQLARVLANCE----I--KPVTNQVECSPA-LNQKALTAFCKKN-----DVTLTGYTPLG
DMEL_CG10863 EQLTRLLANCK----I--KPIHNQIECHPA-LNQKKLIALCKKN-----DIVVTAYCPLG
DMEL_CG12766 EQLTRLLANCK----I--KPIHNQIEVHPA-LDQKKLIALCKKN-----GILVTAFSPLG
DMEL_CG6083 QQMNRLLSVAK----L--KPVVLQIECHPY-LSQKPLITLCYDN-----AIAVTAYSCLG
DMEL_CG6084-PA RQIERVLEVAT----I--PPVTNQIECHPY-LTQKKLIDFCKSK-----DITITAYSPLG
DMEL_CG6084-PD RQIERVLEVAT----I--PPVTNQIECHPY-LTQKKLIDFCKSK-----DITITAYSPLG
DMEL_CG6084-PB RQIERVLEVAT----I--PPVTNQIECHPY-LTQKKLIDFCKSK-----DITITAYSPLG
DMEL_CG6084-PC RQIERVLEVAT----I--PPVTNQIECHPY-LTQKKLIDFCKSK-----DITITAYSPLG

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DMEL_Hk-PH          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PK          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PE          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PL          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PM          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PI          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PD          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PG          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_Hk-PF          MALSDTQNGDKLFLPKGSFKTKSFSWTEDEINRNAALSPQGSWGKDRIDEGRRHCDRLRD
DMEL_CG18547        LL---TNAGPQPWHP-----ASDEQKAIA----------------------------RK
DMEL_CG12224-PB     LL---RNAGPHASHP-----GSQEILAVA----------------------------KR
DMEL_CG12224-PC     LL---RNAGPHASHP-----GSQEILAVA----------------------------KR
DMEL_CG3397         LL---SNAGPQSWHP-----GSPELLAVG----------------------------KR
DMEL_CG2767         SK------GIAKFNAGAGIVRDLPDLMDI----------------------------PE
RAT_Akr1c9          SS------RDKTWVD-----QKSPVLLDD----------------------------PV
DMEL_ARY-PC         ----------QPNRK-----NHCPVYFFS----------------------------EG
DMEL_ARY-PD         ----------QPNRK-----NHCPVYFFS----------------------------EG
DMEL_CG10638-PB     ----------RPKAC-----NPLPDYYTD----------------------------SK
DMEL_CG9436         ----------RPQPA-----RQWPPFLYD----------------------------EH
DMEL_CG10638-PA     ----------KPKPD-----IQKPDFIYS----------------------------PE
DMEL_CG10638-PD     ----------KPKPD-----IQKPDFIYS----------------------------PE
DMEL_CG10863        ----------RPNPA-----EKTPNYIYD----------------------------AK
DMEL_CG12766        ----------RHNAE-----LRTPTFMYD----------------------------GK
DMEL_CG6083         -------SGHTPYEK-----PGAYPLLQH----------------------------PT
DMEL_CG6084-PA      SP-------NRPWAK-----AGDPVILEE----------------------------AK
DMEL_CG6084-PD      SP-------NRPWAK-----AGDPVILEE----------------------------AK
DMEL_CG6084-PB      SP-------NRPWAK-----AGDPVILEE----------------------------AK
DMEL_CG6084-PC      SP-------NRPWAK-----AGDPVILEE----------------------------AK

DMEL_Hk-PH          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PK          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PE          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PL          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PM          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PI          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PD          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PG          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_Hk-PF          LAALAEKLGCSPTQLSIAWSLKHEP-VQCLLLGATSAEQLHQSLQ-SLQLLPRLSSSVML
DMEL_CG18547        ASEVCKERGVELGKLAMYYTMSGLPEVSTFLTGMQTRQLLRINLD-ANEV--GLSDKEQE
DMEL_CG12224-PB     GAEICQQRNVELGKLAMYYTMQLDG-AATFLIGIPNRKLLRINLDAIFD---GLTSHEQE
DMEL_CG12224-PC     GAEICQQRNVELGKLAMYYTMQLDG-AATFLIGIPNRKLLRINLDAIFD---GLTSHEQE
DMEL_CG3397         GAEICQKRNVELGKLAMYYTMQLDG-AATFLIGIPNRKLLRINLDAIFD---GLTSHEQE
DMEL_CG2767         VKEIAASHGKTPAQVLLRWIIDT---GVSAIPKSTNPARLKQNLD-VFDF--ELTAEEVA
RAT_Akr1c9          LCAIAKKYKQTPALVALRYQLQR---GVVPLIRSFNAKRIKELTQ-VFEF--QLASEDMK
DMEL_ARY-PC         MKRLVKKYKRSASQIVLRYLIDY---GVVPIPKAANPIHIKENLN-IFDF--KLDEADTR
DMEL_ARY-PD         MKRLVKKYKRSASQIVLRYLIDY---GVVPIPKAANPIHIKENLN-IFDF--KLDEADTR
DMEL_CG10638-PB     LLALAAKYGKTPAQIILRYLIDI---GTVPIPKAAQRAHLVENLD-IFDF--HLTPEELL
DMEL_CG9436         AQNLAKKYGRTTAQICLRYLVQL---GVVPLPKSSNKARIEENFR-VFDF--ELSPDDVA
DMEL_CG10638-PA     VAVIAKKYGKTTPQIVLRYLVGL---GVIPIPKSSNTNRISENFD-IFDF--ELTAEEMA
DMEL_CG10638-PD     VAVIAKKYGKTTPQIVLRYLVGL---GVIPIPKSSNTNRISENFD-IFDF--ELTAEEMA
DMEL_CG10863        VQAIGDKYKKSTAQVVLRYLIEI---GTIPLPKSSNPKRIEENFQ-IFDF--QLDAEDHA
DMEL_CG12766        VQAIADKYNKSIAQVVIRYVIEL---GTIPLPKSSNPKRIEENFN-VFDF--KLDAEDHA
DMEL_CG6083         ILAIAEKYERTAAQVLLRFQTQS---GIIVIPRSVSKQHMLDNFKRIWDF--ELAVDDIQ
DMEL_CG6084-PA      IKEIAAKKKKTPGQILIRYQVQR---ANIVIPKSVTKDRIESNFQ-VFDF--ELTPEEIE
DMEL_CG6084-PD      IKEIAAKKKKTPGQILIRYQVQR---ANIVIPKSVTKDRIESNFQ-VFDF--ELTPEEIE
DMEL_CG6084-PB      IKEIAAKKKKTPGQILIRYQVQR---ANIVIPKSVTKDRIESNFQ-VFDF--ELTPEEIE
DMEL_CG6084-PC      IKEIAAKKKKTPGQILIRYQVQR---ANIVIPKSVTKDRIESNFQ-VFDF--ELTPEEIE
                       :  .       : : :           :        :        :    *
```

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DMEL_Hk-PH          ELERILENKPVRPPMISTLAL-R-------------------------------------
DMEL_Hk-PK          ELERILENKPVRPPMISTLAL-R-------------------------------------
DMEL_Hk-PE          ELERILENKPVRPPMISTLAL-R-------------------------------------
DMEL_Hk-PL          ELERILENKPVRPPMISTLAL-RXQSACPQGPLSLSGGGPCGADSPKHEDEAFIEEVDAA
DMEL_Hk-PM          ELERILENKPVRPPMISTLAL-RXQSACPQGPLSLSGGGPCGADSPKHEDEAFIEEVDAA
DMEL_Hk-PI          ELERILENKPVRPPMISTLAL-R-------------------------------------
DMEL_Hk-PD          ELERILENKPVRPPMISTLAL-R-------------------------------------
DMEL_Hk-PG          ELERILENKPVRPPMISTLAL-R-------------------------------------
DMEL_Hk-PF          ELERILENKPVRPPMISTLAL-R-------------------------------------
DMEL_CG18547        VLRYLKENVLTKPFDWEGNEL-EIYWAALKKK----------------------------
DMEL_CG12224-PB     VLQYLRENVFTKSYSWGSTLSTAKLLK---------------------------------
DMEL_CG12224-PC     VLQYLRENVFTKSYSWGSTLSTAKLLK---------------------------------
DMEL_CG3397         VLQYLRENVFTKSYSWGSTLSTVNMFK---------------------------------
DMEL_CG2767         KLSSLDQNIRICDFAFFHGVERHPEFTFKNQYTN--------------------------
RAT_Akr1c9          ALDGLNRNFRYNNAK--YFDD-HPNHPF-TDE----------------------------
DMEL_ARY-PC         LLRGIKPKSRIVKYE--IVKD-HMFYPFELLKENNEESEVKDSQTKEPKLPYFPSEEEEE
DMEL_ARY-PD         LLRGIKPKSRIVKYE--IVKD-HMFYPFELLKENNEESEVKDSQTKEPKLPYFPSEEEEE
DMEL_CG10638-PB     QMKTFNTGRRIWKFD--KAKN-HQYYPY---------------------------------
DMEL_CG9436         GMEQYHTGQRTVPFS--GMSG-HKYYPFNDEF-----------------------------
DMEL_CG10638-PA     VLDGYHTGERVVPLNLIKGLN-HKYYPFSIEF-----------------------------
DMEL_CG10638-PD     VLDGYHTGERVVPLNLIKGLN-HKYYPFSIEF-----------------------------
DMEL_CG10863        ILDSYNTGERLIPMT--HAIK-SKNYPFNIEF-----------------------------
DMEL_CG12766        ILDSYHNGERVAHAR--QAIK-SKYYPFNVYSACF--------------------------
DMEL_CG6083         AINELDCNGRFMTMK--AAYG-HPHHPFEPKAKVDN-------------------------
DMEL_CG6084-PA      IIESFECNGRLVPLL--NQYG-HPHHPFEKDEY----------------------------
DMEL_CG6084-PD      IIESFECNGRLVPLL--KDAGTHKYYPFHDEY-----------------------------
DMEL_CG6084-PB      IIESFECNGRLVPLL--KDAGTHKYYPFHDEY-----------------------------
DMEL_CG6084-PC      IIESFECNGRLVPLL--NQYG-HPHHPFEKDEY----------------------------
                     :
DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          KDAAKQGFCLIQ--------------------------------------------------
DMEL_Hk-PM          KDAAKQGFCLIQXQKESLDNDNKFLKVISSKTSLSSNTMTTTISKQEQQQQQQQQQQQQH
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          ------------------------------------------------------------
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         KENHENYE------------------------------------------------------
DMEL_ARY-PD         KENHENYE------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------
```

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DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          ------------------------------------------------------------
DMEL_Hk-PM          LQPQQYVQLQQQHLMASGSTSDSAAAVPIAAELQFDSLNNNNNNNNNNNTNGSPEQALPTS
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          ------------------------------------------------------------
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------

DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          ------------------------------------------------------------
DMEL_Hk-PM          DERQKRRSLLNFKSLDFHLKSLYSGLRSNSGTTPPSQPNSSVPAVDQPQRRMPYLRVESV
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          ------------------------------------------------------------
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
DMEL_ARY-PC         ------------------------------------------------------------
DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------
```

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DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          ------------------------------------------------------------
DMEL_Hk-PM          DPEEPRGSVLPGHSPSFLSPYSGVGGGAGSLQLPSNDGGTIRRSSTSDIVNCRRGGGAGG
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          ------------------------------------------------------------
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
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DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------

DMEL_Hk-PH          ------------------------------------------------------------
DMEL_Hk-PK          ------------------------------------------------------------
DMEL_Hk-PE          ------------------------------------------------------------
DMEL_Hk-PL          ------------------------------------------------------------
DMEL_Hk-PM          SGGGAASAQDSRRPSTSDLLRKARERKGSEARMGRSVSHSGLTRGGPGGGGGGLGGRRTS
DMEL_Hk-PI          ------------------------------------------------------------
DMEL_Hk-PD          ------------------------------------------------------------
DMEL_Hk-PG          ------------------------------------------------------------
DMEL_Hk-PF          ------------------------------------------------------------
DMEL_CG18547        ------------------------------------------------------------
DMEL_CG12224-PB     ------------------------------------------------------------
DMEL_CG12224-PC     ------------------------------------------------------------
DMEL_CG3397         ------------------------------------------------------------
DMEL_CG2767         ------------------------------------------------------------
RAT_Akr1c9          ------------------------------------------------------------
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DMEL_ARY-PD         ------------------------------------------------------------
DMEL_CG10638-PB     ------------------------------------------------------------
DMEL_CG9436         ------------------------------------------------------------
DMEL_CG10638-PA     ------------------------------------------------------------
DMEL_CG10638-PD     ------------------------------------------------------------
DMEL_CG10863        ------------------------------------------------------------
DMEL_CG12766        ------------------------------------------------------------
DMEL_CG6083         ------------------------------------------------------------
DMEL_CG6084-PA      ------------------------------------------------------------
DMEL_CG6084-PD      ------------------------------------------------------------
DMEL_CG6084-PB      ------------------------------------------------------------
DMEL_CG6084-PC      ------------------------------------------------------------
```

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DMEL_Hk-PH           ---
DMEL_Hk-PK           ---
DMEL_Hk-PE           ---
DMEL_Hk-PL           ---
DMEL_Hk-PM           MAF
DMEL_Hk-PI           ---
DMEL_Hk-PD           ---
DMEL_Hk-PG           ---
DMEL_Hk-PF           ---
DMEL_CG18547         ---
DMEL_CG12224-PB      ---
DMEL_CG12224-PC      ---
DMEL_CG3397          ---
DMEL_CG2767          ---
RAT_Akr1c9           ---
DMEL_ARY-PC          ---
DMEL_ARY-PD          ---
DMEL_CG10638-PB      ---
DMEL_CG9436          ---
DMEL_CG10638-PA      ---
DMEL_CG10638-PD      ---
DMEL_CG10863         ---
DMEL_CG12766         ---
DMEL_CG6083          ---
DMEL_CG6084-PA       ---
DMEL_CG6084-PD       ---
DMEL_CG6084-PB       ---
DMEL_CG6084-PC       ---

CODE
invariant and highly conserved regions
co-factor binding pocket residues
substrate binding pocket
```

Genotype	N	Period (h) Mdn	Range	Power Mdn	Range	R (%) SR	WR
+	15	23.91	23.40-25.19	282.76	55.81-569.73	87	13
e01020/+	23	23.98	22.55-30.67	224.93	31.87-864.47	78	22
e01020	27	24.13	19.25-28.97	116.63	6.17-802.96	56	44
f0334/+	18	23.97	22.25-26.75	154.58	24.28-1112.23	56	44
f0334	12	23.92	23.23-24.62	133.13	20.81-1033.36	67	33

Table 7.3: Circadian rhythm of CG10638 mutant lines.

7.2 Behaviour

7.2.1 Sleep and circadian rhythm of mutant lines

Sleep phenotypes Other alleles such as CG10638^{e01020}, CG10638^{f00334}, and CG10638^{EY21723} (Fig. 7.3A) only mildly affect sleep (Fig. 7.3B, C). The effects of CG10638^{EY21723} are covered in chapter 4. Additionally, among CG10638 mutant alleles, CG10638^{f00334} mildly reduces average daily sleep levels (Fig. 7.3B, $\chi^2(9)=89.12$, $p<0.001$, *post hoc* as annotated).

Transheterozygous combinations of CG10638^{f00334} and CG10638^{EY21723} affect sleep in the night portion of the day (Fig. 7.3C, $\chi^2(9)=73.79$, $p<0.001$, *post hoc* as annotated).

Circadian rhythm Circadian rhythm is generally not effected by CG10638 mutant alleles (Table 7.3).

Some alleles are associated with a mildly weakened rhythm reflected in lower rhythm power (Table 7.3): one allele, CG10638^{e01020}, does mildly weaken the circadian rhythm manifest in a reduction of rhythm power for that genotype when homozygous (Fig. 7.4C, $\chi^2(2)=7.69$, $p<0.05$, *post hoc* CG10638$^{e01020/+}$:WT, $p=0.823$, CG10638^{e010201}:WT, $p<0.05$), but does not affect average period length (Fig. 7.4B, $\chi^2(2)=2.09$, $p=0.35$), although an increase in the variability of period length in this population could indicate a destabilisation of period length (Fig. 7.4A, Table 7.3).

Another mutation, CG10638^{f0034}, does not affect circadian rhythm (Fig. 7.4A), neither in terms of period length (Fig. 7.4D, Kw, $\chi^2(2)=0.825$, $p=0.66$) nor rhythm

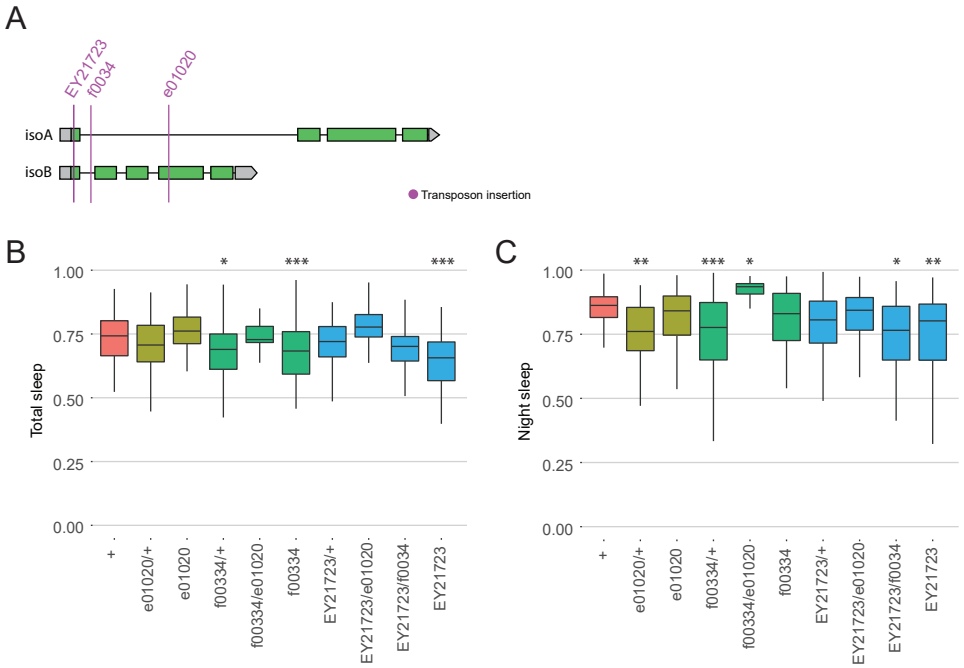


Figure 7.3: Sleep of other CG10638 mutant lines in homozygous, heterozygous, and transheterozygous combination. A, Schema of the CG10638 locus including annotation of insertion sites. B, Total daily sleep levels (ZT0-24). C, Total sleep during the night (ZT12-24). B-C, homozygotes: n=57-90, heterozygotes: n=51-97, transheterozygotes: n=31-57.

power (Fig. 7.4E, $\chi^2(2)$=4.65, p<0.05, but *pos hoc* CG10638$^{f00334/+}$:WT, p=0.168, CG10638^{f00334}:WT, p=0.056).

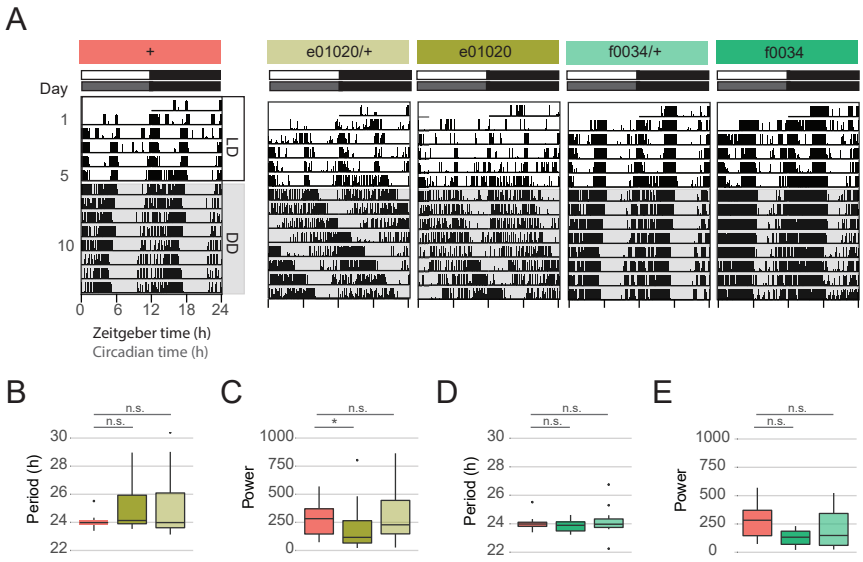


Figure 7.4: Circadian rhythm associated with CG10638 mutant alleles. A, Representative actograms of individual flies throughout the course of the experiment, respective genotypes indicated above the actogram. B, Period length for mutant CG10638^{e01020}. C, Power of mutant CG10638^{e01020}. D, Period length for mutant CG10638^{f0034}. E, Power of mutant CG10638^{f0034}. B-C, n=15-28. D-E, n=15-27.

7.2.2 Sleep behaviour of RNAi lines

MB06512-driven RNAi-mediated knock down

The transposable element MB06512 inserted into exon VII (Fig. 7.5A). The inserted sequence contains a GFP tag that exhibits limited expression in the brain, namely in glial cells (Fig. 4.12C); it also contains a Gal4 sequences which can, in principle, be used to drive expression of UAS constructs in these cells (Fig. 7.5B).

To test this potential function, we first tested whether the CG10638^{MB06512} allele itself is associated with a sleep phenotype. It is of note that this allele could only be tested in heterozygous combination due to the absence of an insertion marker. Under these conditions, this allele does not affect sleep (Fig. 7.5C, D, W=3.03, p=0.08).

Using the associated Gal4 insertion to drive CG10638-IR results in a moderate reduction of sleep for IR# 1 (Fig. 7.5D) and IR# 2 (Fig. 7.5E). The reduction in daily sleep (Fig. 7.5G, $\chi^2(4)$=111.58, p<0.001; *post hoc* as annotated) is driven by suppression of night time sleep (Fig. 7.5H, $\chi^2(4)$=87.48, p<0.001; *post hoc*

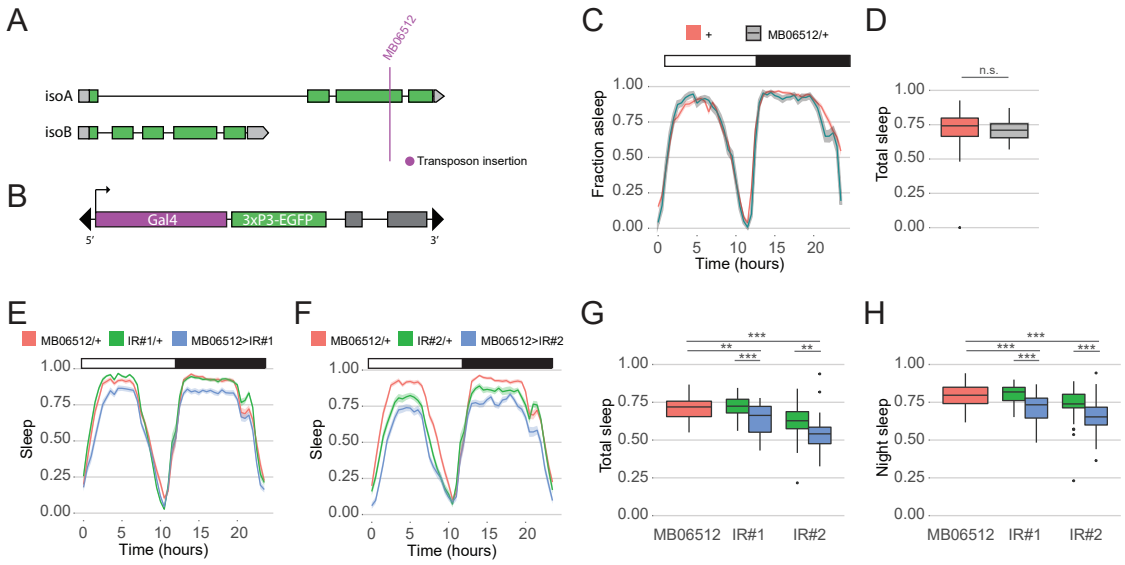


Figure 7.5: Sleep upon knock down of CG10638. A, Schema of CG10638 locus including annotation of MB06512 insertion site, inspired by **Metaxakis2015** B, Schema of MB06512 insertion. C, Sleep profile of $CG10638^{MB06512}$ line. D, Average fraction of time spent asleep per day by $CG10638^{MB06512}$. E, Sleep profile of MB06512>IR# 1. F, Sleep profile of MB06512>IR# 2. G, Average fraction of time spent asleep per day by MB06512>IR lines. H, Night sleep for RNAi lines. C-D, n=63-82. E-H, n=55-63.

as annotated) and, although more mildly, day sleep ($\chi^2(4)$=102.53, p<0.001; *post hoc* as annotated).

Glia-wide RNAi-mediated knock down

Since the absence of a phenotype could be due to the non-functionality of the Gal4 insertion and/or of glial cells in CG10638-mediated sleep regulation, we also tested sleep-related effects of RNAi-mediated knock down of G10638 in glial cells using the glial driver Repo-Gal4 (Awasaki et al. 2011; Stork et al. 2012).

Repo-Gal4 itself has a strong effect on the sleep profile (Fig. 7.6A). However, driving CG10638-IR using Repo-Gal4 does not alter sleep: it does not alter daily sleep levels overall (Fig. 7.6B, $\chi^2(2)$=14.42, p<0.001, *post hoc* Repo>IR# 1:Repo/+, p=0.694, Repo>IR# 1:IR# 1/+, p<0.001). Repo>CG10639-IR flies also exhibit normal sleep during the night (Fig. 7.6C, $\chi^2(2)$=32.34, p<0.001, *post hoc* Repo>IR# 1:Repo/+, p=0.662, Repo>IR# 1:IR# 1/+, p<0.001).

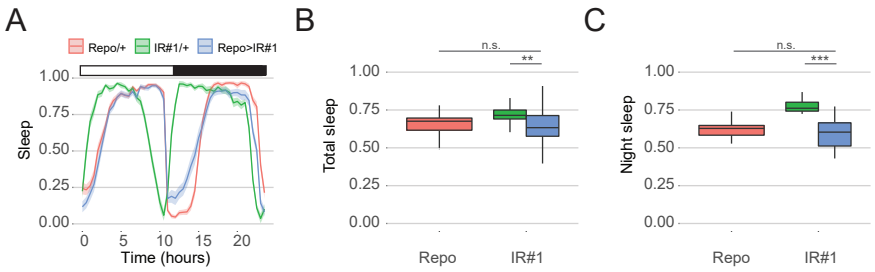


Figure 7.6: Sleep upon knock down of CG10638 in glial cells. A, Sleep profile of RNAi driven by Repo-Gal4. B, Total daily sleep levels. C, Sleep during the night. A-C, n=27-42.

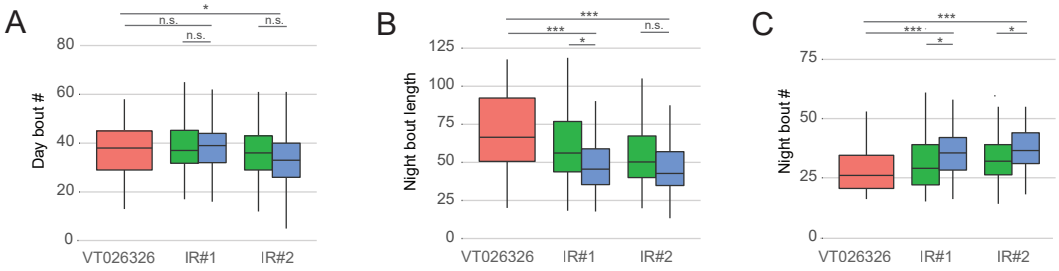


Figure 7.7: Additional sleep parameters upon knock down of CG10638 in ICLI neurons. A, Number of sleep bouts during the day. B, Length of sleep bouts during the night. C, Number of sleep bouts during the night. A-C, n=85-92.

ICLI-specific RNAi-mediated knock down

The main findings regarding the effect of CG10638-IR expression in ICLI neurons through the control of the CT026326 driver are presented in chapter 4. Here, additional aspects of sleep architecture will be discussed.

Knock down of CG10638 in ICLI neurons using either RNA transgene does not affect the number of siesta bouts (Fig. 7.7A, $\chi^2(4)=13.27$, $p<0.05$; *post hoc* VT026326>IR# 1:VT026326/+, $p=0.987$, VT026326>IR# 1:IR# 1/+, $p=0.996$, VT026326>IR# 2:VT026326/+, $p<0.05$, VT026326>IR# 2:IR# 2/+, $p=0.322$).

In respect to the night sleep architecture, expression of IR# 1 in ICLI neurons shortens the night bout length, but the expression of IR# 2 does not (Fig. 7.7B, $\chi^2(4)=44.02$, $p<0.001$; *post hoc* VT026326>IR# 1:VT026326/+, $p<0.001$, VT026326>IR# 1:IR# 1/+, $p<0.05$, VT026326>IR# 2:VT026326/+, $p<0.001$, VT026326>IR# 2:IR# 2/+, $p=0.106$).

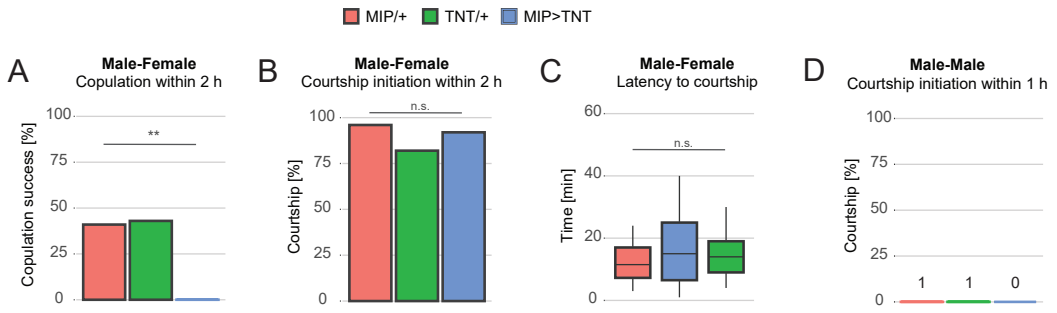


Figure 7.8: Mating behaviour upon MIP+ neuron silencing. A, Copulation success in male-female interactions of young male flies with MIP>TNT and parental controls within 2 h. B, Courtship initiation within 1 h. C, Latency to courtship initiation within 1 h. D, Courtship iniation in male-male interactions of young male flies with MIP>TNT and parental controls within 1 h. A-C, n=25-28 pairs/genotype, D, n=31-32 pairs/genotype.

The expression of either CG10638-IR mildly fragments night sleep by increasing the number of sleep bouts at this time (Fig. 7.7C, $\chi^2(4)=42.49$, $p<0.001$; *post hoc* as annotated).

7.2.3 MIP+ neuron-related phenotypes

Courtship and mating success

Silencing MIP+ neurons through the expression of UAS-TNT under the control of the MIP-specific driver MIP-Gal4 does not affect sexual arousal manifest in a normal courtship initiation response in males when subjected to a female on par with parental controls (Fig. 7.8B; $\chi^2(2)=0.385$, $p=0.825$).

However, courtship was not as fruitful for MIP>TNT flies which had only limited copulation success compared to parental controls (Fig. 7.8A; $\chi^2(2)=11.74$, $p<0.01$).

MIP>TNT flies do not show an inclination to initiate male-male courtship (Fig. 7.8D) and this disinterest is similar to parental controls (Fig. 7.8D; $\chi^2(2)=1.07$, $p=0.586$).

These findings generally replicate the phenotype described by Jiang et al. 2013 for Natalisin+ neurons, which are a subset of MIP+ neurons (Min et al. 2016).

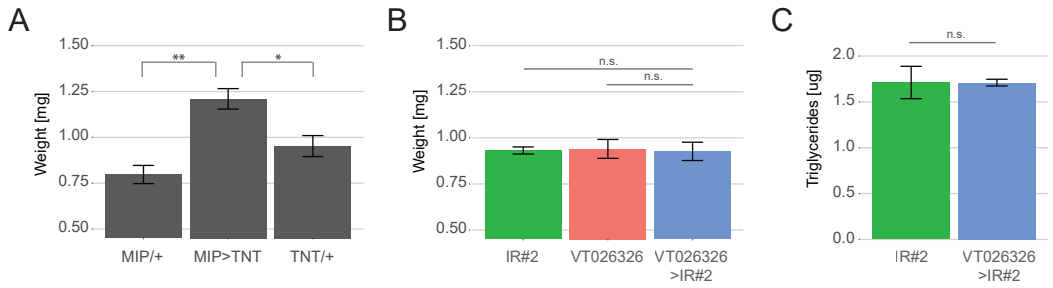


Figure 7.9: Body weight upon knock down of CG10638 in ICLI neurons. A, Body weight of males flies expressing MIP>TNT and parental controls (n=4/genotype). B, Body weight of male flies expressing VT026326>RRNAi# 2 and parental controls (n=7-12/genotype).

Bodyweight/Feeding

In a recent study, Min et al. 2016 described a bodyweight/feeding phenotype associated with MIP+ neurons. Since ICLI neurons are a subset of MIP+ neurons, we (1) used the experimental genotype described in Min et al. 2016 as a positive control to detect changes in bodyweight and (2) tested whether the ICLI subset specifically is also associated with such a phenotype.

Indeed, silencing of MIP+ neurons using TNT leads to an increase in wet body weight (Fig. 7.9A; $F(2, 9)=14.72$, $p<0.01$, *post hoc* MIP>TNT:MIP/+, $p<0.01$, MIP>TNT:TNT/+, $p<0.05$).

However, wet body weight is not affected by knock down of CG10638 in ICLI neurons in young male flies (Fig. 7.9B; $F(2, 25)=0.022$, $p=0.979$). Note that the effect of the expression of UAS-TNT driven by VT026326 could not be tested as this combination was lethal throughout.

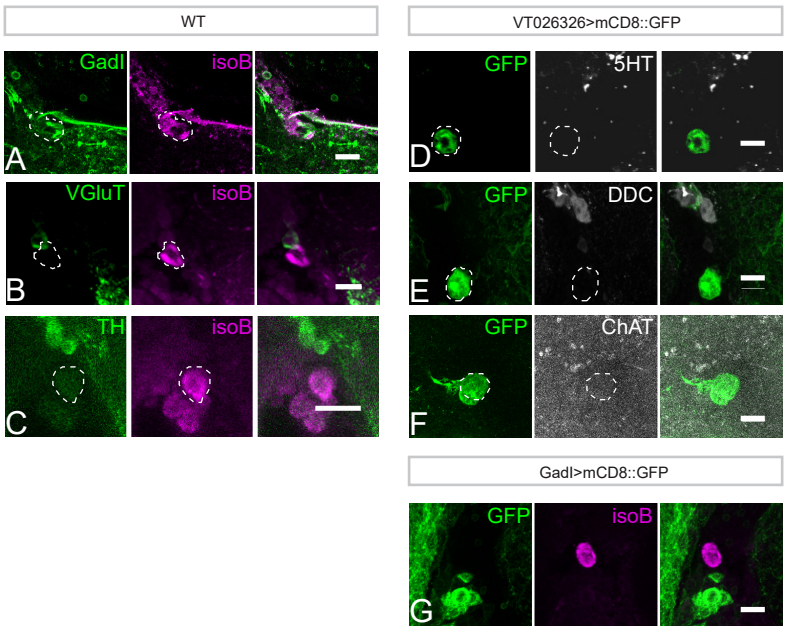


Figure 7.10: Neurotransmitter markers of ICLI neurons. A, GadI and CG10638-isoB co-staining. B, Same as A for VGluT. C, Same as B for TH. D, 5HT staining in VT026326>mCD8:GFP background. E, Same as D for DDC. F, Same as D for ChAT. G, CG10638-isoB staining in GadI>mCD8:GFP background. A-G, Scale bars: 10 µm.

7.3 Neurotransmitters of ICLI neurons

ICLI neurons stain positive for Glutamate decarboxylase (GadI, Fig. 7.10A), but are not part of the GadI-Gal4 expression pattern (Fig. 7.10G).

ICLI neurons are negative for the vesicular Glutamate Transporter (VGluT, Fig. 7.10B) and Choline Acetyltransferase (ChAT, Fig. 7.10F). They are also negative for 5-Hydroxytryptophan (5HT, Fig. 7.10D) and Tyrosine hydroxylase (TH, Fig. 7.10C), and for their common enzyme Dopa Decarboxylase (DDC, Fig. 7.10E).

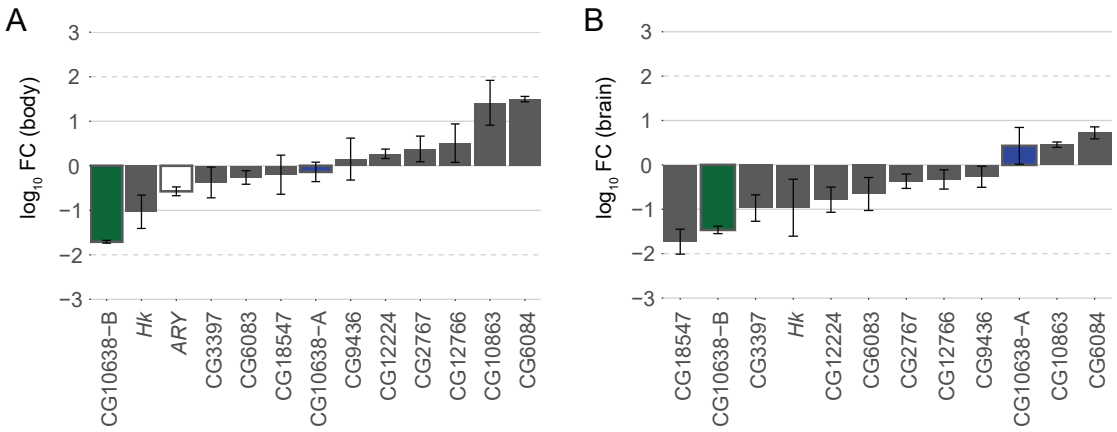


Figure 7.11: Expression of AKRs in body and brain. A, Expression in the whole body excluding the head (n=4-11/target). B, Expression in the brain (n=2-4/target).

7.4 Quantitative real-time PCR

Experimental evidence of AKR expression

AKRs are expressed at different levels, both in the body and brain (Fig. 7.11A, B). The targets CG6084 and CG10863 are most abundantly expressed in both tissues, and the targets CG10638-isoB, *hk*, and CG3397 are among the most sparsely expressed (Fig. 7.11A, B)

Additional data for the timecourse experiment

We investigated whether the candidate gene CG10638 (isoB) was expressed at systematically different levels in the course of the day in a manner reminiscent of classically cycling clock genes such as *per*. Indeed, we detected cycling expression of *per* in male fly head extracts, validating our sampling approach (Fig. 7.12A; $F(1, 22)=5.01$, $p<0.05$).

Levels of the synaptic protein *bruchpilot* (*brp*) have been shown to correlate with time awake (Gilestro et al. 2009). We detected a similar difference in *brp* mRNA levels in male head extracts from flies immediately after sleep (sacrificed at ZT0, “S”) in comparison to flies after the waking day (sacrificed at ZT12, “W”, Fig. 7.12A, $t(6)= -6.24$, $p<0.01$).

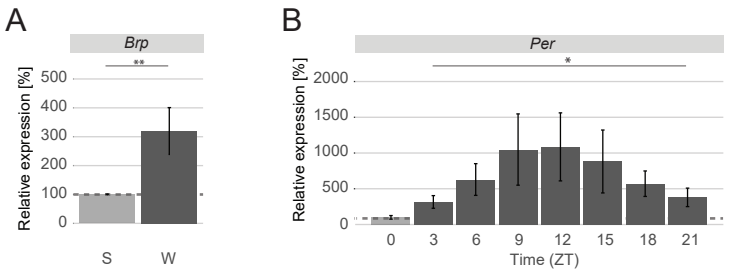


Figure 7.12: Expression of *brp* and *per*. A, Expression of *brp* in morning vs. evening. B, Timecourse of *per* expression. A-B, n=3/ZT.

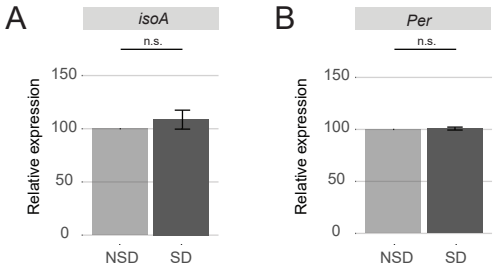


Figure 7.13: Expression in response to sleep deprivation. A, Expression levels of *isoA* in slept (NSD) and sleep-deprived flies (SD, n=7/treatment). B, Same as A for *per*.

Sleep deprivation

The expression levels of the cycling clock gene *per* do not respond to sleep deprivation (Fig. 7.13B, t(7)=-0.78, p-value=0.454).

Similarly, expression of isoform A of CG10638 does not respond to sleep deprivation (Fig. 7.13A, t(6)=-1.12, p-value=0.308).

"[...] if we wish to understand sleep in its most evolved form, i.e., in mammals, we must study its evolution by tracing sleep-like behaviours in invertebrates [...]"

— Scott S. Campell and Irene Tobler (Campbell et al. 1984)

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