IMPERIAL

Genome Dynamics and Sleep: Exploring the Connection Between Heterochromatin Modifications, DNA Damage Response, and Sleep Regulation

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Statement of originality

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Abstract

Sleep is a fundamental behavior characterized by a state of inactivity, which has been observed in a wide range of species. Despite its conservation in evolution, the precise purpose of sleep remains shrouded in mystery. The application of innovative tools has significantly contributed to the exploration of the regulating mechanisms and functions involved in sleep, although further investigations is still required. In this thesis, I have examined sleep in two different contexts, using *Drosophila melanogaster* as model system. In the first part of this work, I combined a range of transcriptomics and immunostaining experiments, to analyze how gene expression changes in the brain upon acute and chronic sleep deprivation (SD) and found a role for histone methylases in regulating sleep-specific changes in gene expression. Prolonged SD leads to an overall decrease in gene expression driven by inhibitory histone methylation activity (H3K9) in the brain. The specific removal of H3K9 methylases, such as Su(var)3-9 or G9a, in neurons, interferes with this process, blocking sleep-specific changes in gene expression and canceling the fly's internal homeostatic control of sleep. In the second part of this thesis, I dissected the recently hypothesized function of sleep, in facilitating DNA repair mechanisms. DNA damages, in particular double-stranded breaks (DSBs), are known to accumulate in the brain during wakefulness and decrease during sleep. Pharmacologically induction of DSBs leads to an increased baseline sleep in flies. Moreover, the pan-neuronal reduction of DNA repair-related genes, particularly p53, promotes increased sleep: the combination of chronic SD with the knockdown of p53 leads to heightened levels of neuronal DSBs and a reduction in lifespan. Unravelling the cellular basis for sleep drive may facilitate the improvement in restorative sleep quality for individuals dealing with sleep disorders and provide valuable insights into the relationship between sleep, aging, and neurodegenerative diseases.

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List of abbreviations

ACh…..……...Acetylcholine

AD…….…….....Alzheimer disease

ADO ……..…… Adenosine

AstA…….…. Allatostatin-A

ATM…….…….…. Ataxia telangiectasia mutated protein

ATR ….……...Ataxia telangiectasia and Rad3-related protein

BER …….……...Base excision repair

BDSC ….……Bloomington Drosophila Stock Center

BRP…….……Brucopilot

CaMKII …….…….$Ca^{2+/}$calmodulin-dependent protein kinase II

circRNAs …….……...Circular RNAs

CHK1 …….……Checkpoint kinase 1

CHK2 …….……Checkpoint kinase 2

CI ……..…...Courtship index

CLK …….……...Clock protein

CO2 ….……...Carbon dioxide

CRY …….……...Cryptochrome protein

CpG …….……Cytosine-guanine dinucleotide

cVA …….……Cis-vaccenyl acetate

cv-c …….……Crossveinless-c

Ct …….……....Cycle threshold

DA …….……..Dopaminergic

DAM …….……...Drosophila Activity Monitor

DAT …….……Dopamine transporter protein

DBD …….……DNA-binding domain

DD …….……...Constant dark

DDR …….……DNA damage response

DEGs …….……...Differentially expressed genes

DG …….……...Dentate gyrus

DNMT …….…….DNA methyl transferases

DSBs …….……...Double strand breaks

dFB ……...…...Dorsal fan-shaped body neurons

EB …….……Ellipsoid body

ELAV ……..……..Embryonic Lethal, Abnormal Vision

eIF2 …….……..Gamma subunit of translation initiation factor 2

EEG …….……..Electroencephalogram

ETO …….……..Etoposide

FasII …….……..Fasciclin II

GLP …….…...…...G9a-like protein

GO …….……Gene Ontology

GRs …….……...Glucocorticoid receptors

HAT …….……..Acetyltransferase

HDAC …….……...De-acetyltransferase

HMTases …….…...Histone methyltransferases

HPA …….……...Hypothalamic-pituitary-adrenal

HR ……..Homologous recombination

ILPs ……Insulin producing cells

Jmjc ……Jumonji

KCs ……Kenyon cells

KD ……..Knocked-down

KDSC …….Kyoto Drosophila Stock Center

LD ……...Light-dark cycle

LI …….Learning index

lncRNA ……...Long non-coding RNA

LTM ……Long term memory

MB ……..Mushroom body

MeCP ……..Methyl-CpG binding proteins

miRNA/miR ……MicroRNA

MM ……. Male-male interaction

MNR ……Mre11-Rad50-NBS1

NER …….Nucleotide excision repair

NGS …….Normal Goat Serum

NHEJ ……...Non-homologous end joining

NO ……...Noduli

NREM……..Non-REM

Oat ……...Ornithine aminotransferase

OD ……...Optical density

OR ……...Odorant receptor

PAM ……Protocerebral anterior medial

Parp1……....Poly (ADP-ribose) polymerase 1 protein

PB ……...Protocerebrum bridge

PBS …….Phosphate buffered saline

PER …….Period protein

PEV …….Position-effect variegation

PFA …….Paraformaldehyde

PIKK ……...Phosphatidylinositol 3-kinase (PI3K)-related protein kinase

piRNA …….Piwi-interacting RNA

PPL1 ……....Posterior protocerebrum lateral-1

PPL2 ……Posterior protocerebrum lateral-2

Put ……Putrescine

REM ……Rapid eye movement

RNAi ……...RNA interference

ROI ……..Region of interest

RPA …….Replicative protein A

RT ……Room temperature

SCN …….Suprachiasmatic nucleus

SD ……....Sleep deprivation

SEZ ……..Subesophageal ganglion zone

Shi ……$Shibire^{ts}$

shRNA …….Short-hairpin RNA

SHY …….Synaptic homeostasis hypothesis

STM …… Short term memory

sLN_vs ……...Small ventral lateral neurons

sNPFR …….Short neuropeptide F receptor

Spe ……...Spermine

Spd ……...Spermidine

Spds ……..Spermidine synthase

SSBs …….....Single strand breaks

sss …….....Sleepless

Syn …….…..Synapsin

SWA ……....Slow wave activity

SWS ……...….Slow-wave sleep

TAD ……Transcription activation domain

TIM……..Timeless protein

TIR1…….....Transport inhibitor response 1

TOP2……Topoisomerase II

TRPA1 ……Transient receptor potential ankyrin 1

UAS ……Upstream-activating sequence

VDRC …….Vienna Drosophila Resource Center

ZT ……Zeitgeber Time

5mC …….5-methylcytosine

Chapter 1

Introduction

1.1 General overview of sleep, from mammals to flies

1.1.1 Historical definition of sleep

Sleep is a fascinating and mysterious behavior that has stimulated great interest for the past 2500 years. Approximately in the year 540 BC Alcmaeon of Croton, a Greek physician, theorized that sleep resulted from a state of unconsciousness induced by reduced blood circulation to the brain, which occurred due to blood draining from the body surface to more internal blood vessels (Huffman 2008). Half a century later, Aristotle suggested that sleep represented a cessation of consciousness within the heart, believed to be the center of sensation. Around 350 B.C., Aristotle published a volume entitled "On Sleep and Sleeplessness", starting with this opening text:

> *"With regard to sleep and waking, we must consider what they are: whether they are peculiar to soul or to body, or common to both; and if common, to what part of soul or body they appertain: further, from what cause it arises that they are attributes of animals, and whether all animals share in them both, or some partake of the one only, others of the other only, or some partake of neither and some of both."*

This text already frames fundamental questions about sleep that will be deeply debated over the next 2000 years. For a period of approximately 1600 years, limited progress was achieved in understanding the nature of sleep: only between the 18th and 19th centuries, several theories about the origin of sleep will be proposed, such as the existence of a sleep "substance" or toxin that is produced during the day and dissipated during the night, or that blood flow can influence the sleepiness. At the beginning of the 19th century, several experts started believing that the brain was the center of sleep. Constantin von Economo, among the others, was a Romanian psychiatrist who noticed that patients with viral encephalitis exhibited insomnia or excessive sleepiness (encephalitic lethargy). Upon examining the brains of dead patients, he found that each

specific region of the brain is attributed to regulating sleep or wakefulness. For instance, he observed a correlation between damage in the anterior hypothalamus and prolonged insomnia, suggesting that this specific brain region is involved in enhancing sleep. On the other hand, damages to the lateral/posterior hypothalamus were the main cause of sleepiness, suggesting that this specific region was involved in the regulation of wakefulness (Economo 1930). These findings would be confirmed by subsequent studies in mammals, such as research in rats (Nauta 1946).

One of the biggest inventions in the history of sleep science was the electroencephalogram (EEG) performed by Hans Berger for the first time in 1928, where he placed electrodes on human scalps and recorded the electrical activity of the brain. This technique greatly accelerated the expansion and progress in the field of sleep during the 20th century. In 1937, Frederic Bremer discovered that by looking at the EEG frequency, it was possible to identify if a person was awake or asleep (Bremer 1937). Furthermore, in 1953, the Russian sleep researcher Nathaniel Kleitman, together with his student Eugene Aserinsky, were the first contributors to the formal description of rapid eye movement (REM) sleep. They noticed that over the whole sleep period, there are specific stages characterized by “rapid, jerky, and binocularly” eye movement and low muscle tone. Recording of these eye movements by using the EEG, was also correlated to dreaming stages (Aserinsky and Kleitman 1953). This study was further expanded by other researchers, such as Dement (Dement and Kleitman 1957). Contemporaneously, progress in the circadian rhythm was also achieved: Erwin Bunning, approximately around 1930, proved the existence of an internal biological clock across species (including plants). This was the first study proving that sleep is a rhythmic process, governed not only by environmental cues, but also by an internal circadian clock. In addition, he demonstrated the hereditary aspect of the circadian rhythms by conducting experiments where he bred bean plants with varying periods of leaf-movement rhythms. His findings revealed the subsequent generation exhibited period lengths that were intermediate (R.-C. Huang 2018).

Collectively, all these early pioneers have contributed to establishing sleep as a new interesting subject of study and scientific field. The main subsequent progresses are described in the following section.

1.1.2 Understanding the structure of sleep

As previously mentioned, Berger was able to recognize when individuals are awake or asleep: during wakefulness, EEG records show a high-frequency amplitude pattern of activity, while this becomes slower (with larger amplitude waves) during sleep. Based on Berger's observations, researchers started describing distinct EEG sleep stages. In 1968 Allan Rechtschaffen and Antony Kales published the first EEG manual where they formalized the characterization of different stages of sleep (Kales and Rechtschaffen 1968). Based on the EEG frequency, they found two main types of sleep: REM and non-REM (NREM) sleep, which alternate forming a sleep cycle of approximately 90 minutes. On average, four to five sleep cycles occur every night, depending on the time spent in sleep and each sleep cycle is divided into one REM and four NREM stages, although nowadays NREM stages 3 and 4 are often combined in humans. NREM stages 3 and 4 are when the deepest sleep occurs and are characterized by the presence of slow-wave sleep (SWS): sleep here shows moderate muscle tone and precludes any eye movement. By contrast, the REM stage is marked by higher frequency breathing, heart rate, and blood pressure, along with an EEG pattern similar to that of a fully awake individual. This is why REM sleep is also known as "paradoxical sleep".

REM and NREM sleep stages have been identified in many mammals and also other species, such as reptiles and birds (Scott S. Campbell and Tobler 1984). However, together with its stages, there are also other markers defining sleep behavior across species. In 1913, Henri Pieron described 4 fundamental features, that will be largely mentioned and exploited in the following years by other researchers. 1) Sleep-specific posture: he observed that individuals adopt specific postures during sleep, often associated with muscle relaxation, that are different from waking postures. 2) Behavioral quiescence: during sleep, there is a reduction in involuntary motor activity and responsiveness to external stimuli. 3) Elevated arousal threshold: it takes a stronger stimulus to awaken an individual from sleep, compared to what would be needed to rouse them from a waking state. This aspect is essential to protect the quality and continuity of sleep. 4) Reversibility of sleep: individuals can transition in and out of sleep during the night (Piéron 1913). Piéron was also one of the early pioneers to propose the concept of "homeostatic sleep drive", suggesting that the need for sleep accumulates over time during wakefulness and dissipates during sleep. The four sleep features described by Piéron were found by Campbell and Tobler to be shared across

the animal kingdom, even by insects, such as the *Drosophila melanogaster*, commonly known as “fruit fly”. This discovery resulted in the use of invertebrate species in sleep studies, given their multiple advantages, such as shorter lifespan or simpler genetics. Flies, in particular, are very easy to manipulate and can be subjected to high throughput experiments (this will be largely discussed in section 1.2). In the following decades, they will be largely utilized in order to identify the main components of the fly clock drive (starting with the work of Ronald Konopka and Seymour Benzer who, in 1971, discovered *period,* the first circadian gene). Additionally, studies in *Drosophila melanogaster* have contributed essential insights of the influence of the homeostatic drive, upon sleep pressure (J. C. Hendricks et al. 2000; Shaw et al. 2000a).

1.1.3 Sleep-wake cycle: how does it work?

Sleep has been described as a behavior consisting of a periodic altered state of consciousness, characterized by physical quiescence and decreased ability to react to external stimuli (elevated arousal threshold). For more than 30 years, sleep has been thought to be regulated by the generally accepted "two process-model", first proposed by Alexander Borbely (Borbély 1982), where *Process S* and *C* work together to drive the sleep/wake cycle throughout the day. Process S represents the homeostatic driver, a predictor of *sleep pressure*, by which the need to sleep changes based on how long an individual has been asleep or awake. According to Process S, the build-up in sleep pressure will be greater after staying awake longer than usual; however, when sleep occurs, the sleep pressure reduces exponentially to a low level, allowing the individual to wake up. On the other hand, the second process, Process C, is independent of the occurrence of sleep: it refers to a circadian pacemaker regulated by specific cells in the brain, which are involved in organizing the sleep-wake cycle in a 24 hour-rhythm: this happens in sync with the 24-hour light-dark cycle of the day (Fig.1.1).

Despite being described as two distinct mechanisms, Process S and C combine and cooperate to regulate the sleep-wake cycles, depending on the characteristics of each species. For example, in diurnal mammals, wakefulness is prominent during the day and sleep occurs during the night. However, the mechanisms by which the two processes interact, are still not well understood.

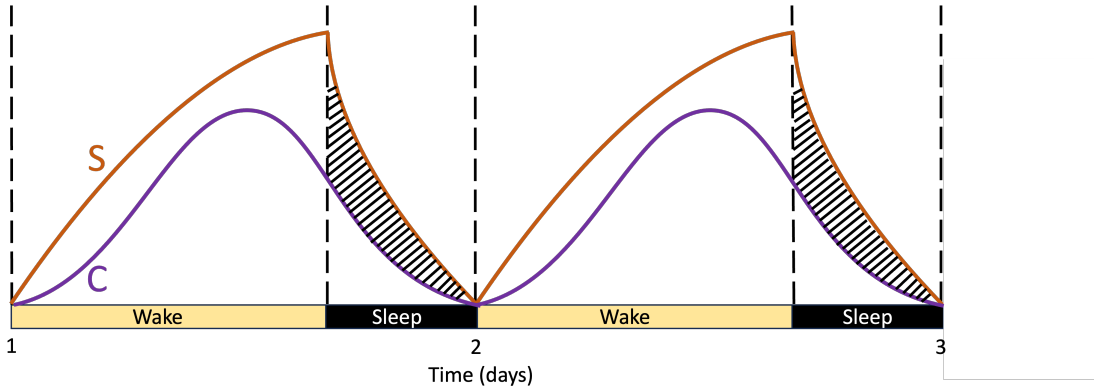


Figure 1.1 Two-processes model of sleep regulation.
Illustration of the model explaining the drive to sleep given by the combination of two processes: a circadian drive (Process C) and a homeostatic drive (Process S). Process S determines the need for sleep, depending on how long individuals have been awake: indeed, it is a predictor of sleep pressure, which increases during wake time and decreases during sleep. Process C, instead, establishes the timing for sleep, according to the light-dark phase.

1.1.4 The circadian driver of sleep regulation

Research on sleep has been mostly focused on the investigation of the molecular mechanisms related to the circadian control: in mammals for instance, the suprachiasmatic nuclei (SCN) of the hypothalamus in the brain are responsible for circadian rhythms (Mistlberger 2005). In flies, a set of symmetric small ventral lateral neurons (sLN_vs) was found to play a similar role to the mammalian SCN (Helfrich-Förster 1996). Genetic research in both groups of cells has identified a 'master circadian clock' system, made by a core of critical "clock genes", which includes positive and negative regulators of sleep. In mammals, *Bmal1* and *Clock* are members of this core: they act as positive regulators of gene transcription, controlling for instance the production of period (PER) and cryptochrome (CRY). These two interact to form a protein complex that enhances a negative feedback loop by inhibiting CLOCK-BMAL1-mediated transcription, consequently suppressing the expression of *Per* and *Cry*. Once the PER/CRY complex is degraded, the inhibition of CLOCK-BMAL1-mediated transcription is temporarily abolished: this allows the complex CLOCK-BMAL1 to activate transcription again, restarting the molecular loop every 24 hours (Fig. 1.2). In addition to this, research has found that molecular clocks are not only confined to SCN cells, but they are also expressed in several peripheral tissues, all synchronized by the "master" SCN through both chemical and neuronal signals (Fogg et al. 2014).

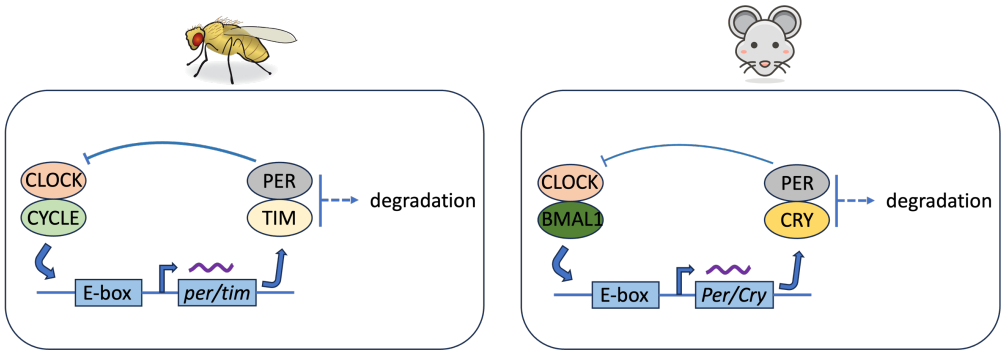


Figure 1.2. The circadian clock loop in Drosophila and mammals.
In mammals, CLOCK and BMAL1 bind together making heterodimers, which positively regulate circadian genes, such as period *(Per)* and cryptochrome *(Cry)*. PER and CRY make complexes too, regulating CLOCK-BMAL heterodimers in a negative manner. However, progressively during the day, PER and CRY are degraded and consequently, BMAL and CLOCK are activated again. This molecular loop takes 24 hours to complete. Similarities in the system are shown in flies as well, where the main core is made by CLOCK and CYCLE, homologous of mammalian CLOCK and BMAL1. Illustrations of the fly and the mouse are taken from https://commons.wikimedia.org/wiki, created under the Creative Commons license.

Furthermore, all the molecular fluctuations within the SCN are extremely synchronized to the 24-hour dark-light cycle detected by the eye. Indeed, when the retina detects light, an intracellular cascade intermediates the connection between a group of photosensitive ganglion cells and the SCN, leading to the suppression of CLOCK-BMAL1 activity. Since light detected by the eye is crucial to synchronize the timing of sleep, it has been considered as the primary 'Zeitgeber' (time-giver) (Do and Yau 2010).

Overall, the just described circadian rhythm is comparable in flies (Fig. 1.2). The activator dimer CLOCK-BMAL1 is made by the homologous CLOCK and CYCLE, which positively regulates the complex PER-TIMELESS (TIM, homologous for the mammalian CRY). There are, however, some functional divergences, such as in CRY which plays a central role in the mammalian circadian clock, with a light-independent action. In *Drosophila*, CRY is a photoreceptor that binds and blocks the negative feedback action of PER-TIM dimer, in a light-dependent manner (Griffin, Staknis, and Weitz 1999). Another fundamental factor involved in regulating the circadian process in flies is the Pigment Dispersing Factor (PDF), a neuropeptide expressed in the main circadian neurons in the *Drosophila* brain. Loss of function of *pdf* or its receptor *pdfr* has been linked to disruption in several rhythmic behaviors (Renn et al. 1999; Park et al. 2000; Lear, Zhang, and Allada 2009). It has been found to synchronize molecular oscillations among different circadian pacemaker neurons: for instance, in pdf^{01}

mutants, oscillations of *tim* and *cry* mRNAs in pacemaker neurons are diminished (Peng et al. 2003).

1.1.5 The homeostatic mechanisms regulating sleep

The homeostatic driver is what allows individuals (mammals, but some insects as well) to recover after a period of sleep deprivation. This recovery consists of an increment of sleep, also called "sleep rebound" (Cirelli and Tononi 2008), correlated to an increase in sleep intensity and/or sleep duration. Due to the restrictions enforced by the circadian clock, changes in sleep intensity usually occur more frequently than changes in sleep duration, but they are also more difficult to detect, especially in the fly. In mammals, sleep intensity modulations are associated with changes in neuronal activity, as found in the late 1930s by Frederic Bremer. This can be accurately detected using the EEG, a quantification of sleep based on brain waves, which allows a high-throughput behavioral analysis in a non-invasive way. Each sleep stage is characterized by specific EEG waves. As previously mentioned, SWS are highly relevant, as they correspond to deep sleep, during NREM stages 3 and 4. Variations in the frequency and amplitude of slow oscillations are measured and quantified as slow-wave activity (SWA) (Dijk 2009). As a reflection of sleep pressure, SWA decreases during sleep time, while it progressively rises with wake duration, reaching high peaks when entering early sleep (Allada, Cirelli, and Sehgal 2017). Many theories have been proposed to underly the mechanisms upstream of this SWA fluctuation during the wake-sleep cycle, such as the influence of specific metabolic processes, for example, the regulation of glycogen levels. Changes in glycogen levels are essential to regulate the build-up of adenosine (Ado) in the cerebral cortex, by which the brain is capable of sensing wake status (Feinberg et al. 1978; Benington and Heller 1995; Scharf et al. 2008). Research on mice has shown, in particular, that Ado levels in the basal forebrain tend to rise upon sleep deprivation and to reduce during sleep recovery (Kacsoh, Greene, and Bosco 2017).

Another mechanism possibly implicated in SWA modulation is the synaptic homeostasis hypothesis (SHY) proposed by Cirelli and Tononi (2014), which postulates that during wake, the overall synaptic strength of multiple neuronal populations increases, due to learning and memory needs. During wakefulness, SWA increases, due to the stronger connection between neurons. Instead, during sleep, where synaptic re-normalization weakens neuronal connections, SWA decreases. We should not consider

the mechanisms described above as mutually exclusive, as energy metabolism and neuronal plasticity are extremely linked to each other.

In *Drosophila*, electrophysiological measurements in the brain are technically possible, but also very challenging, due to the small-size brain. Technical progress in electrophysiology and imaging, combined with genetic and behavioral tools, has provided high temporal resolution of synaptic activity in flies. Two-photon imaging, together with genetically triggered calcium indicators, has, in particular, revolutionized the field, by allowing neuronal activity recording of specific sets of neurons (Denk, Strickler, and Webb 1990; J. W. Wang et al. 2003; Jayaraman and Laurent 2007; Ng et al. 2002). Despite its high resolution, this approach remains elaborated and very challenging. Therefore, other parameters have been used in order to define sleep homeostasis in flies, which have still contributed to progress in this field. For instance, upon sleep deprivation, flies show rebound sleep response: sleep becomes longer and more consolidated in its structure, and arousal thresholds increases (J. C. Hendricks et al. 2000; Shaw et al. 2000a; Huber, Ghilardi, et al. 2004; Alphen et al. 2013; Seidner et al. 2015). As in mammals, disruption of sleep homeostasis causes cognitive impairments in learning and memory formation (Seugnet et al. 2009; Donlea et al. 2011), and reduces mating success and aggression (Kayser et al. 2015). Thanks to a mutagenesis screen, several chemical messengers, such as neurotransmitters, have been found to be essential regulators of sleep homeostasis. Acetylcholine (ACh), for instance, is one of the most important excitatory neurotransmitters in flies: Seidner et al. (Seidner et al. 2015) found that the activation of cholinergic neurons induces wakefulness and, in specific cases, sleep rebound. *sleepless* (sss), *shaker* and *hyperkinetic* are all short-sleeping mutants, with single-gene mutations affecting cholinergic neurotransmission (Koh et al. 2008; Cirelli, LaVaute, and Tononi 2005; Bushey et al. 2007). Another fundamental neurotransmitter is dopamine: changes in the dopaminergic (DA) neurotransmission can lead to a strong reduction of sleep and arousal thresholds, and a dramatic increase of activity (Hendricks et al. 2003; Wu et al. 2008). "*fumin*" mutant flies, with a genetic mutation in the *dopamine transporter* gene (*dat*), show hyperactive behavior (Kume et al. 2005). Some studies have also linked specific brain regions to sleep homeostasis regulation: an essential function is played by the dorsal fan-shaped body (dFB) neurons: when activated, these neurons enhance sleep, often implicated in memory consolidation. On the other hand, inhibition of dFB neurons leads to decreased sleep time and diminished sleep rebound (Donlea, Pimentel, and Miesenböck 2014a; Huber, Hill, et al.

2004). Another region implicated in homeostatic sleep is the mushroom body neurons (MBs), comparable to the mammalian cerebral cortex and involved in associative learning. Neurogenetic screens revealed that MBs are able to promote both sleep and wake by activating specific subsets of these neurons (Joiner et al. 2006; Pitman et al. 2006). A deeper dissection of the main brain regions involved in sleep homeostasis is reported in section 1.2.6.

1.1.6 Consequences of sleep loss

Although the role of sleep remains unclear, there is no doubt that widespread physiological deficits occur in some organisms upon lack of sleep. Sleep-deprived rats, for instance, have shown pronounced adverse effects on overall health: indeed, they fail to keep regular temperature rhythms and they easily develop skin injuries. Also, they experience defects in their metabolism, such as weight loss (Rechtschaffen et al. 1983), and acute sleep loss causes death (Rechtschaffen et al. 1983). Furthermore, as in rats, recent data have shown deleterious deficits upon sleep loss on human metabolism, such as weight gain, obesity, diabetes (Knutson et al. 2007) and on the immune system, as well (Moldofsky et al. 1989). Also, a higher risk of cardiovascular diseases and cancer has been found in shift-workers (Haus and Smolensky 2013). In *Drosophila*, sleep-deprived wild-type flies (Huber, Hill, et al. 2004; Seugnet et al. 2008), as well as short-sleeping mutants (Smith and Harbison 2018), have shown impairments in learning and memory-processing. However, memory abilities can be restored to normal levels, by increasing sleep artificially. Similarly to flies, sleep loss in humans leads to significant neurological disturbances, mostly associated with significant cognitive impairments (Durmer and Dinges 2005; Van Dongen et al. 2003) and decision-making difficulties (Pilcher and Huffcutt 1996). Indeed, research found that the probability of learning is three times higher if the individual is allowed to sleep (Walker et al. 2002), and in addition, some tasks are not fully learned if sleep is disrupted the night after learning (Walker et al. 2003), suggesting that sleep is necessary for restructuring new memories. The mechanisms by which it influences memory, however, are still unknown and currently debated by contradictory theoretical models (Diekelmann and Born 2010; Tononi and Cirelli 2014).

1.2 Drosophila melanogaster in sleep research

1.2.1 Why are fruit flies valuable in biological research?

Drosophila melanogaster is a species of fly belonging to the Diptera order. With a body size of 2-3mm, it is usually findable in kitchens, by fermented food, mainly rotten fruit, thus explaining its most common names, as "fruit fly" or "vinegar fly". *Drosophila* is one of the most powerful model systems and has been used in biological research since the beginning of the 20th century. Compared to other animal systems, such as mice, flies are very easy and cheap to keep in laboratory conditions. Also, their life cycle takes approximately 10 days at room temperature (25°C) from fertilization to adult eclosion and they produce a large number of offspring per generation. The short generation time and high fecundity facilitate high-throughput investigations, making robust genetic tests and statistical analyses. The development period includes four main metamorphosis stages: egg, larva, pupa, and adult fly.

After the sequencing of the whole fly genome in 2000 (Adams et al. 2000), scientists concluded that approximately 60% of genes has been shared by mammals and flies: this encouraged even further use of *Drosophila* to investigate human genetic diseases, especially neurodegenerative types, like Alzheimer's and Parkinson's (Yoshihara, Ensminger, and Littleton 2001).

1.2.2 Drosophila in behavioral studies

Progresses in neurogenetic research allowed fruit flies to become one of the most exploited model systems in the study of complex behaviors. It is truly astonishing how the tiny brain of a fly can generate such a wide range of behaviors, including courtship, aggression, learning, memory, sleep, and social interactions. Already in the 1970s, Seymour Benzer started considering the fly as a fundamental unit of behaviors, finding corresponding features in human behavior. This suggested the opportunity to identify homologous genes and conserved molecular mechanisms involved in behaviors that are shared between flies and mammals. Interestingly, Benzer introduced the “forward genetics” method, one of the most commonly used genetic approaches even nowadays, where mutant individuals with peculiar behaviors are isolated, and specific genetic tools

and techniques are then applied for precise control over the genes and the neural circuits involved in that specific phenotype (Benzer 1967; 1973). Over the years, many more genetic tools were identified and published to improve the manipulation of genes and neurons in flies. Furthermore, researchers have mapped many of the fly neural circuits, making it easier to study the relationship between genes, neurons, and behavior. Finally, *Drosophila* research offers a more ethical alternative to using vertebrate animals for behavioral studies. Given their lesser size and overall reduced complexity, researchers can explore various aspects of behavior without causing harm to more sentient creatures.

1.2.3 Sleep in *Drosophila*: sleep architecture and measuring methods

Following Campbell and Tobler's observations, sleep in insects is largely defined using immobility as a proxy (S. S. Campbell and Tobler 1984). Only in the year 2000, two studies scrupulously analyzed *Drosophila* rest (Joan C Hendricks et al. 2000; Shaw et al. 2000b), concluding that flies share most of the mammalian criteria for sleep. More

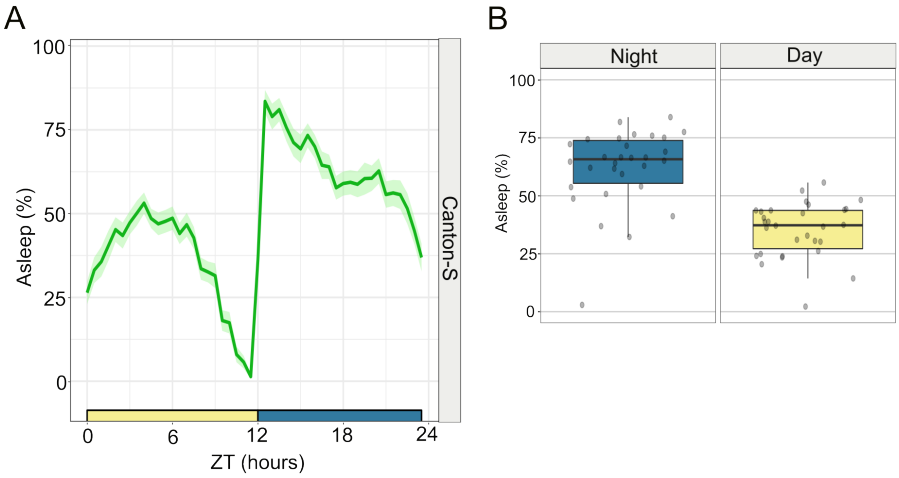


Figure 1.3. A typical sleep-time visualization in Drosophila.
A. Illustration of the usual sleep profile of a male population from Canton-S flies. The yellow portion corresponds to the day, which starts at ZT0 (Zeitgeber Time). The dark-blue portion, instead, represents the night and begins at ZT12. Indeed, in this case, the time regime is made by 12h of light and 12 of darkness (12:12 LD). Males tend to show a consistent portion of sleep during the night; they also experience a day-specific peak of sleep approximately around ZT6, commonly known as "siesta" time. **B**. Quantification of sleep amount shown in the previous graph (A), where daytime and nighttime sleep are differentiated.

specifically, these criteria are: quiescence, increased arousal threshold, specific posture and sleeping-location, reversibility, sleep rebound after sleep deprivation, and

modulation by using sleep-wake-altering substances such as caffeine. Given these behavioral homologies, the basic function of sleep could be evolutionarily preserved: indeed, today *Drosophila* represents a key model in sleep research.

Looking at the typical sleep profile in *Drosophila* (Figure 1.3), the activity is characterized by two main peaks, an evening and a morning peak, both preceding the light transition time. By contrast, sleep is mostly distributed in the middle of the day (this time is also known as fly 'siesta', (Andretic and Shaw 2005) and in the night, more uniformly spread in the latter. The sleep structure usually differentiates between day and night periods. Indeed, during the night flies tend to sleep with a lower number of bouts, which are, however, much longer than the bouts during the day (Andretic and Shaw 2005). In line with this, the activity bouts at night are significantly shorter (Andretic and Shaw 2005) and the arousal thresholds are usually higher. Furthermore, flies exhibit a significant sexual dimorphism in their sleep: male flies usually show longer periods of sleep, during both light and dark phases, while females experience higher activity during the day (Huber, Hill, et al. 2004).

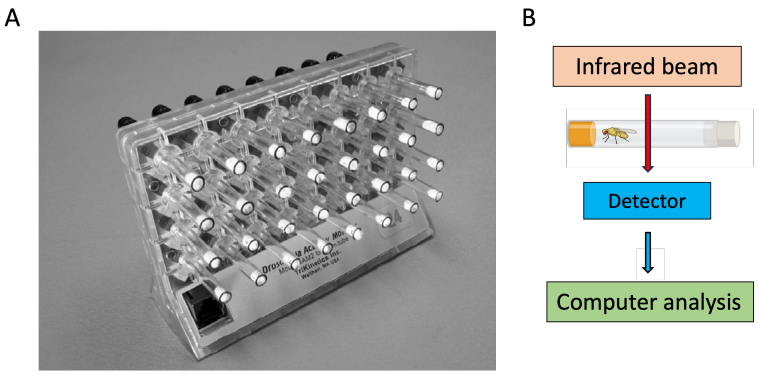


Figure 1.4. Drosophila Activity Monitor system (DAMs).
A. Photo of a DAM system (image taken from Trikinetics Users Guide 8, http://trikinetics.com/). The monitor can hold 32 glass tubes, housing individual flies. Flies move constantly along the tube, between the two ends, where respectively food medium and cotton wool are placed. **B.** Schematic illustration of the infrared beam mechanism. A beam of light sent by an infrared LED crosses the middle of the tubes and reaches an infrared detector on the opposite side. Whenever a fly walks in the middle, the beam is broken, and it does not reach the infrared detector. The activity of flies is measured based on the number of beams crossed during the whole day.

It is very important to clarify the definition of sleep in *Drosophila*: if a fly is not moving, it does not necessarily mean it is sleeping. Most research today agrees to identify sleep behavior as a period of inactivity lasting 5 min or more, without any locomotor activity (Cirelli and Bushey 2008). That is because 5 min of immobility is usually characterized

by increase of arousal threshold during both light and dark phases (Shaw et al. 2000a; Huber, Hill, et al. 2004). A decreased responsiveness to external stimuli, together with all the sleep-defining features previously mentioned, are also fundamental to distinguishing sleep from sickness behaviors, usually characterized by increased agitation, lack of coordination, or, on the other hand, lethargy, and irregular sleep patterns with fragmented sleep, which may not resemble typical sleep cycles.

To measure sleep in flies, most of the studies over the past 20 years, have taken advantage of a tool called Drosophila Activity Monitor (DAM, Fig. 1.4). This device contains several glass tubes, crossed in the middle by an infrared beam: a single fly is contained in each tube and its activity is tracked as beam-crosses over time (Pfeiffenberger et al. 2010). Although DAM remains the most widely used tool to score sleep in flies, it has a few disadvantages. In fact, the DAM paradigm is not able to exclude the possibility that a fly can still be active without crossing the midline of the tube, and because of that, sleep score can be overestimated. Currently, more advanced machines based on video tracking (Gilestro 2012) allow to have marked improvement in spatiotemporal resolution, addressing the issue of movement false negatives. An example of these tracking devices is the ethoscope, developed by the Gilestro laboratory at Imperial College London, which allows to collect in real-time high-throughput behavioral data of small animals (Gilestro 2012; Geissmann et al. 2017, see following section 1.2.4).

1.2.4 The ethoscope, an advanced tool for Drosophila sleep recording

The ethoscope is an automated tracking device of approximately 10 x 12 x 16 cm, which allows to gain in real-time high-throughput behavioral data of small animals (Geissmann et al. 2017). This tool has more advantages than the infrared-beam crossing systems, including its ability to distinguish accurately different types of behaviors, such as walking, sleep, or micromovements (Gilestro 2012). Also, sleep-related data are undoubtedly more precise, and unlike the DAM, sleep score is more accurate.

In terms of building ethoscopes, the whole process is very inexpensive, and it can be achieved simply using 3D-printing technology. The hardwares and softwares are readily available online. Two main parts make an ethoscope: an upper case with a small-independent computer (Rasberry Pi) and a camera; a lower case with an infrared-LED

strip, to keep tracking in darkness, and a behavioral arena, printed with different shapes, where flies are loaded. For simple sleep analysis, as in DAM, flies are secured in glass tubes containing food and cotton at each end.

The ethescopes are usually located in climate-controlled incubators, where they are powered by a universal serial bus (USB) cable. Multiple ethoscopes can be activated at the same time: they are connected via Wi-Fi to a local network, thanks to which they transfer data to a local storage server, the node: each machine works independently. Interestingly, the ethoscope offers the opportunity to interact with flies in real-time. The goal can be to trigger specific stimuli in flies to investigate *how* the behavior changes in response to those inputs: this approach represents a strategic tool to give important insights into neuroscience. Several modules have been designed for this purpose: the *optomotor*, for instance, is a device for mechanical stimuli (shown in Figure 1.5C) and it is the only module exploited in this study. After plugging it to the bottom of the ethoscope, the optomotor is able to trigger sleep deprivation, by rotating the tubes when flies are immobile for a certain amount of time. As a consequence of sleep deprivation, flies need normally to recover, increasing the amount of sleep (sleep rebound) the day after the sleep loss: this treatment is, therefore, a good tool to better elucidate the mechanisms of sleep homeostasis. The most important part of the optomotor is the fact that it receives input from the ethoscope depending on the active real-time state of a single fly: indeed, when a fly is detected to be asleep, the optomotor, coordinated by the ethoscope, only enhances the rotation of the motor located under the glass tube of the sleeping fly. This method avoids a few problems that are associated with previous mechanical SD methods: before the invention of the ethoscope, all those studies using the Drosophila activity monitor, DAM, had to combine the use of specific devices, such as shakers, vortexes, or vibrating surfaces, in order to sleep-deprive flies. These methods are totally autonomous and not connected to the DAMs, which are located on top of them and constantly shaken. The stimulus is continuously triggered, meaning that all the flies, no matter if asleep or awake, are disturbed. Furthermore, another phenomenon that can also occur is habituation. Flies can, in fact, become habituated to the stimulus, altering their behavior and consequently the data collection (Öztürk-Çolak et al. 2020). On the other hand, the optomotor allows to solve all these technical issues. Another example of a module is the Air-Gas-Odor (AGO), used to investigate arousal thresholds in sleeping flies, by stimulating them with air-puff of any type of odors. This is well described in French et al. 2021.

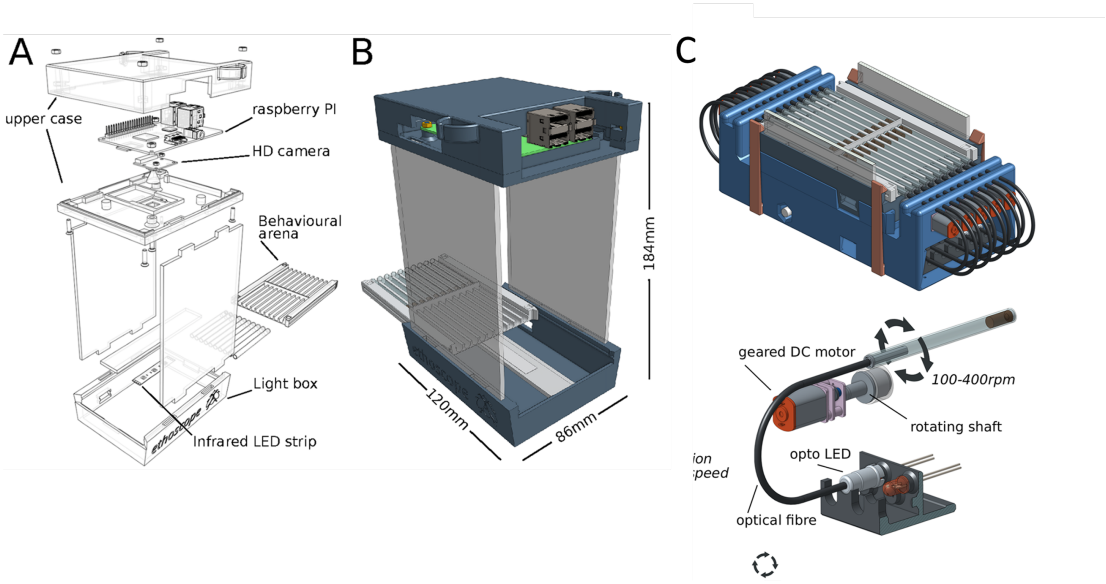


Figure 1.5. The ethoscope and optomotor module.
A-B. Schematic visualizations of the ethoscope composition. It consists of two cases, separated by plastic walls. The lower case has an infrared LED strip (essential for night-sleep tracking) and holds a behavioral arena, too. The upper case, instead, contains the rPi and HD camera. **C.** Illustration of an optomotor. This module can deliver light or physical rotation stimuli to specific flies. All the illustrations are taken from Geissmann et al. 2017.

1.2.5 Sleep rebound response in flies

As mentioned in section 1.1.5, flies normally exhibit all the behavioral hallmarks of sleep, including the ability to show sleep recovery, in response to a certain period of sleep deprivation: this increase in sleep, commonly known as rebound sleep, is marked by an increase of intensity and duration of sleep (Huber, Hill, et al. 2004). Rebound sleep normally occurs after sleep deprivation and it is more significant following extended sleep loss, such as 12 or 24 hours, compared to shortest periods, such as 6 hours. Very interestingly, this increase in sleep only makes up a small fraction of the total loss (Shaw et al. 2000a): short and long-sleeping flies can, in fact, show a similar rebound sleep. As shown by Geissman et al, flies that were chronically sleep-deprived (SD over 228 hours) exhibited a recovery sleep similar to flies sleep-deprived for only one night (Geissmann, Beckwith, and Gilestro 2019). Furthermore, rebound sleep is also characterized by an increase in arousal threshold: flies deprived of sleep for 24 hours, display impaired responsiveness to unpleasant stimuli, although they maintained a sustained locomotor activity: this excludes any unspecific effects of fatigue (Huber, Hill, et al. 2004).

It is fundamental to highlight that rebound sleep is not a categorical phenomenon. Recent studies have demonstrated that, in specific scenarios or upon precise environmental factors, this recovery sleep can be also reduced or abolished. For instance, Beckwith et al. found that a male wild-type fly normally exhibits a strong rebound sleep, after being sleep-deprived in the presence of another male fly. When paired with a female fly, males are even more deprived of sleep, due to the preference for courtship: however, in this specific scenario, they fail to exhibit any subsequent rebound, suggesting that the sexual arousal is able to modulate the sleep pressure (Beckwith et al. 2017). Another study showed that mated female flies experience a very robust rebound upon sleep deprivation by mechanical method or caffeine supply, but not by starvation (a natural method for sleep deprivation with clear consequences in virgin females and occasionally in males). This effect can be also replicated when feeding flies with a diet missing of amino acids, meaning that proteins are essential in upholding standard sleep quality. Furthermore, the same study found that the *Drosophila insulin-like peptide* (*dilp*-2) is required for the loss of sleep rebound upon starvation-induced SD (E. B. Brown et al. 2020). Although mated female flies are known to exhibit rebound upon mechanical SD, their response was shown to be less pronounced in aged flies (35 days old), compared to young flies, as reported by another paper (Vienne et al. 2016).

Overall, flies, as similar as mammals, are able to modulate and balance sleep, based on other biological needs. Therefore, understanding the regulating mechanisms of rebound sleep may be fundamental to understanding the real function of sleep. Over the years, few genes were identified to be involved in rebound sleep regulation. As already mentioned in section 1.1.5, an interesting example is *sleepless*, activator of the *Shaker* potassium channels, essential to enhance sleep by suppressing neuronal excitability (Cirelli, LaVaute, and Tononi 2005). Mutations in *sleepless* were found to affect baseline sleep and also to abolish rebound sleep after SD (Koh et al. 2008). A similar effect was shown when mutating the dopamine transporter gene (DAT) (Kume et al. 2005). Another example of a gene implicated in homeostasis regulation, is *pumilio*. *Pumilio* (*pum*) is a post-transcriptional repressor of mRNAs and is mainly involved during neuronal development and neuronal excitability. The knockdown of *pum* affects rebound sleep upon chronic, but not acute SD (De Jesús-Olmo et al. 2020). Evidence of genes triggering a larger rebound following SD, upon mutation, was also found. For instance, the enzyme arylalkylamine N-acetyltransferase 1 (AANAT1) is mainly

expressed by astrocyte cells and was found to modulate the accumulation in the brain of neurotransmitters, such as serotonin and dopamine, after SD. The loss of AANAT1 is associated with much higher recovery sleep after a night of SD, compared to normal conditions (Davla et al. 2020). Together with specific target genes, in the past decades, precise regions of the fly brain were also identified to play a key role in regulating homeostatic sleep and, consequently, the rebound response: this will be deeply dissected in the following section.

1.2.6 Neuroanatomy of sleep in flies

Recently, several regions in the fly brain have been proposed and shown to be implicated in sleep regulation: for a better understanding of this work, the most important populations of neurons involved in the sleep-related central complex will be dissected in more detail, over this section.

The central complex is made of different classes of neurons, that can be made by various subtypes of cells, expressing different neurotransmitters. Dorsal fan-shaped body neurons are one of the main components of the central complex: they are known to promote sleep, playing a fundamental role in the homeostatic sleep drive regulation. Donlea et al. found that sleep deprivation increases the sensitivity and activity of dFB neurons. Mutations in the gene *crossveinless-c* (cv-c)**,** a Rho-GTPase-activating-protein were found to reduce the excitability of the dFB, decreasing sleep and general rebound response (Donlea, Pimentel, and Miesenböck 2014b). The basic mechanism of switch ON-OFF of dFB neurons was better elucidated by the research from Pimentel et al.: they found that dopamine plays a neuromodulatory mediator of the switch, by influencing specific voltage-gated potassium channels. The genetic silencing of the dFB triggers the downregulation of the current of Shaker and Shab channels and the upregulation of Sandman channel (Pimentel et al. 2016).

Another central complex region implicated in sleep homeostasis is the ellipsoid body (EB). Indeed, rebound sleep following sleep deprivation is impaired after genetic silencing of this region (Liu et al. 2016). In more detail, the neuronal activity of a specific class of neurons named R5 (previously known as R2) is modulated in response to sleep debt and also to circadian input: slow wave oscillations are not detectable in the morning, but during the day, they increase in parallel to sleep need and are also subjected

to daily variations (Raccuglia et al. 2019). Donlea et al. found that the synaptic activity of R5 cells is influenced by the dFB neurons, through a third population of neurons, the helicon cells. These cells usually respond to visual inputs and are inhibited by the sleep-promoting neuropeptide allatostatin-A (AstA), which consequently allows sleep to occur. dFB neurons are the cells responsible for the release of inhibitory transmitters, including AstA (Donlea et al. 2018).

The mushroom bodies (MB) represent another sleep-related brain region: they consist of two paired structures with an L-like shape, located centrally in the brain at the side of the central complex. They contain approximately 2200 neurons, including the Kenyon cells (KCs), which compose the three main lobes of each symmetric structure: α/β lobe, α '/β′ lobe and γ lobe (Crittenden et al. 1998; Aso et al. 2014). Similarly to the dFB and EB, MBs play a fundamental role in homeostatic sleep. Initial studies believed that a general activation of the MBs led to sleep promotion and the ablation of these neurons was responsible for sleep reduction (Pitman et al. 2006; Joiner et al. 2006). However, the introduction of genetic tools, such as the split-GAL4, allowed to functionally manipulate more precise sets of cells, creating a highly detailed picture of the connectivity inside the MB region. This allowed, thereafter, the identification of subregions implicated in both sleep and wake-promoting activity: for instance, the genetic activation of the KCs in the α'/β' lobe, leads to a decreased sleep, while activation of the γ lobe, leads to increased sleep (Sitaraman et al. 2015). Several populations of neurons implicated in sleep regulation are known to transmit inputs into MBs: among these, one of the main classes that have received remarkable attention is the dopaminergic neuron population. Dopamine is one of the most important neurotransmitters required for the regulation of sleep and arousal. In particular, it is known to promote wakefulness. 20 types of dopaminergic neurons project into the MBs, including regions of the protocerebral anterior medial (PAM) and the posterior protocerebrum lateral-1 and 2 (PPL1 and PPL2) (Mao and Davis 2009; F. Li et al. 2020; Takemura et al. 2017). Mushroom body neurons, to conclude, are also shown to be essential in regulating learning and memory formation (Pascual and Préat 2001; Turrel, Goguel, and Preat 2018).

Another component of the central complex is the protocerebrum bridge (PB). Initial studies demonstrated that PB is implicated in locomotor-control behaviors, such as leg coordination, walking speed or locomotor handedness (Strauss et al. 1992; Strauss and

Heisenberg 1993; Triphan et al. 2010). Subsequent works highlighted another role of the PB in sleep regulation. Tomita et al., for instance, found that mutant flies with defects in the middle of the PB structure, decrease their total daily sleep compared to control flies, suggesting this specific part to be a sleep-promoting region. However, they also identified another population of neurons within the PB which, on the other hand, promotes wakefulness. In this work, they propose an elegant neuronal circuit between these 2 groups of neurons, regulated by dopamine signaling, in order to mediate sleep and wakefulness (Tomita et al. 2021).

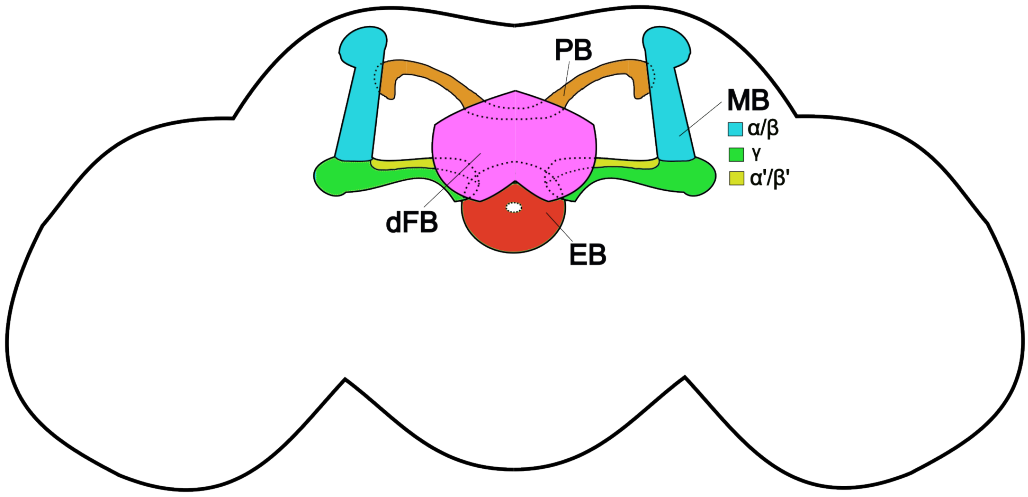


Figure 1.6. Schematic illustration of the anatomy of the main fly brain regions involved in sleep regulation. The central complex, located in the center of the adult fly brain, includes different components involved in sleep regulation: fan-shaped body (FB), ellipsoid body (EB) and protocerebrum bridge (PB). The mushroom bodies (MB) take place at the side of the central complex: the three types of lobes are also shown in different colors (α/β lobe in light blue, α '/β' in yellow, and γ lobe in green).

All the neuronal regions described above are schematically illustrated in Figure 1.6.

1.3 The emerging role of epigenetics in sleep

1.3.1 What is epigenetics?

Every single cell within an organism is nearly characterized by the same identical genetic sequence, which remains consistent throughout the lifetime. However, depending on the role they play in the organism, differentiated cells exhibit significant variations in their observable characteristics (phenotypes). All the information required to generate a fully differentiated organism, starting from a single fertilized egg, is transmitted not solely by genetics, but also by epigenetics. The first person to introduce the term "epigenetics" was the embryologist Conrad Waddington in 1942, who defined it as the "*whole complex of developmental processes*" that exists between "*genotype and phenotypes*" (Waddington, C.H 1942). In particular, in his work "The epigenotype", he referred to biological systems as "*concatenations of processes linked together in a network, so that a disturbance at an early stage may gradually cause more and more far-reaching abnormalities in many different organs and tissues*". To better elucidate this concept, he described the "epigenetic landscape" as a metaphor of a ball rolling down on an inclined surface, made of valleys and peaks, representing the pathway of different steps/decisions fundamental to make a cell develop. During its cell-fate, naïve cells gradually lose their totipotency, reaching their final differentiation stage at the bottom of the hill (Figure 1.7A). Furthermore, below the landscape, an intricate network of specific genes takes part, to give guidelines and determine the path to embark: here, genes act as pegs, essential to pull the slopes of the epigenetic landscape and designing all the different pathways occurring during differentiation (Figure 1.7B).

The second important contribution to building the current definition of epigenetics was made by the microbiologist David L. Nanney, who claimed the existence of two distinct cell regulatory systems: the "genetic system", made of a "library of specificities" which refers to the mechanisms of the replicating template DNA, and the complementary system, made of "auxiliatory mechanisms" which determines what "specificities" need to be expressed to determine a specific cell type. He defined these auxiliatory mechanisms as *epigenetic*, which can persist over many generations (Nanney 1958): "*the epigenetic systems regulate the expression of the genetically determined potentialities".*

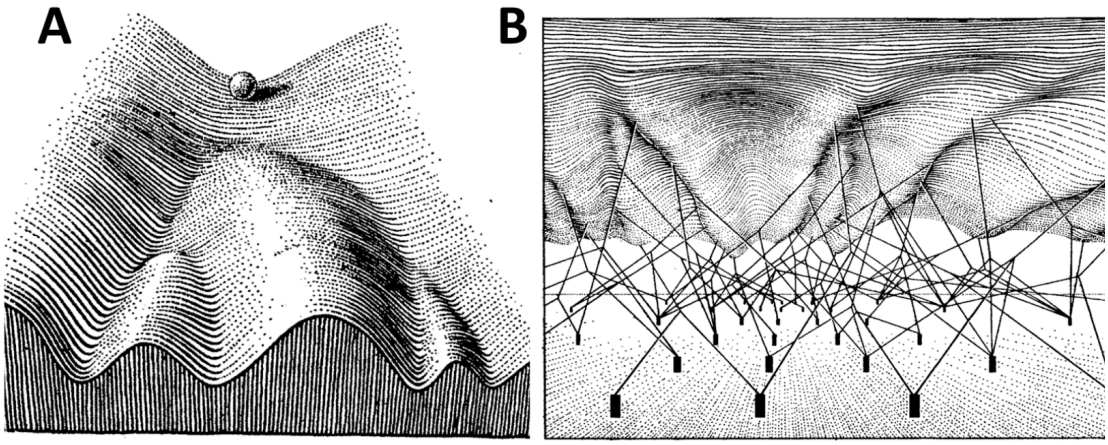


Figure 1.7. Waddington's epigenetic landscape.
A. Cell differentiation is illustrated as a metaphor of a marble falling on a surface of valleys and peaks, symbolizing distinct levels of regulation, which are essential to determine the irreversible cell-fate. **B.** Genes are shown as pegs to model the epigenetic landscape and consequently, to shape all the potential differentiation pathways. Figures adapted from Waddington, 1957, pp 19 and 26.

Waddington and Nanney published their hypothesis at the time when the role of DNA as a carrier of genetic information was just being discovered (Avery, MacLeod, and McCarty 1944; Hershey and Chase 1952) and when its structure was just published (Watson and Crick 1953). Several essential notions, that contributed to the current understanding of epigenetics, were achieved in the following years: for instance, the discovery of the X-chromosome inactivation (Lyon 1961) or the identification of the nucleosome structure (Kornberg 1974; Kornberg and Thomas 1974). However, subsequent studies performed in eucaryotes led to a new stage in the study of epigenetics: the description of histone acetylation, histone methylation, and DNA methylation (Allfrey, Faulkner, and Mirsky 1964; Riggs 1975; Holliday and Pugh 1975).

The current definition of epigenetics was officially coined during a Cold Spring Harbor meeting, which claimed that "*epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alteration in the DNA sequence"* (Berger et al. 2009). Here, it is fundamental to highlight that "heritable" refers mainly to an inherited phenotype from a dividing cell to daughter cells, and less to intergenerational inheritance. Indeed, the epigenetic marks are usually erased in gametes and restarted after fertilization, likely to prevent these signs from being transferred from parents to the offspring. Although still debated, some evidence of epigenetic inheritance across

generations has been also found over time: for instance, Gapp et al. found that injection of RNAs from traumatized male sperm into fertilized wild-type egg cells in mice leads to the maintenance of the alteration in the subsequent offspring (Gapp et al. 2014). Overall, studies on transgenerational epigenetic inheritance have been mainly performed in animal models, thus demanding whether this phenomenon is also conserved in humans. Furthermore, it is not clear if it occurs as a stochastic error or evolutionary advantage.

As proposed by Berger in 2009, epigenetic marks do not cause changes in the DNA sequence: the word *epigenetics* derives from the Greek term "*epi*", which means "above", in this context, "above the genome". Epigenetic marks are plastic and reversible and can be easily influenced by environmental factors, such as nutrition, temperature, or even air pollution. Aberrations in the epigenome can lead to alteration in fundamental processes, such as cell differentiation or generally, gene expression, by affecting the normal function of specific gene targets. Alteration in the epigenome can be associated with several disorders, including metabolic disorders, autoimmune diseases, or even cancer (Raghuraman et al. 2016; Lund and van Lohuizen 2004; Hewagama and Richardson 2009).

1.3.2 Overview of the main epigenetic signatures

As mentioned in the previous section, epigenetics is a relatively new study in biology that has revolutionized our understanding of genetics: it investigates those modifications happening "on top of " DNA, which are able to control and regulate gene expression, without causing any alteration in the sequence of the nucleotides (S. J. Brown, Stoilov, and Xing 2012). The mechanisms that produce such changes occur in many biological processes such as cell differentiation, imprinting, or X-chromosome inactivation. They have been classified into three major categories (Fig. 1.8): histone modification, DNA methylation, and noncoding RNAs regulation (Espada and Esteller 2013).

Histone modifications represent posttranslational changes that modulate the availability of transcriptional machinery to the DNA chain. It is known that histone proteins associate with each other in small units making the chromatin, called nucleosomes, which include, in more detail, a core of 8 histones surrounded by approximately 147bp of wrapped DNA. The histone octamer core includes two copies of 4 different histones:

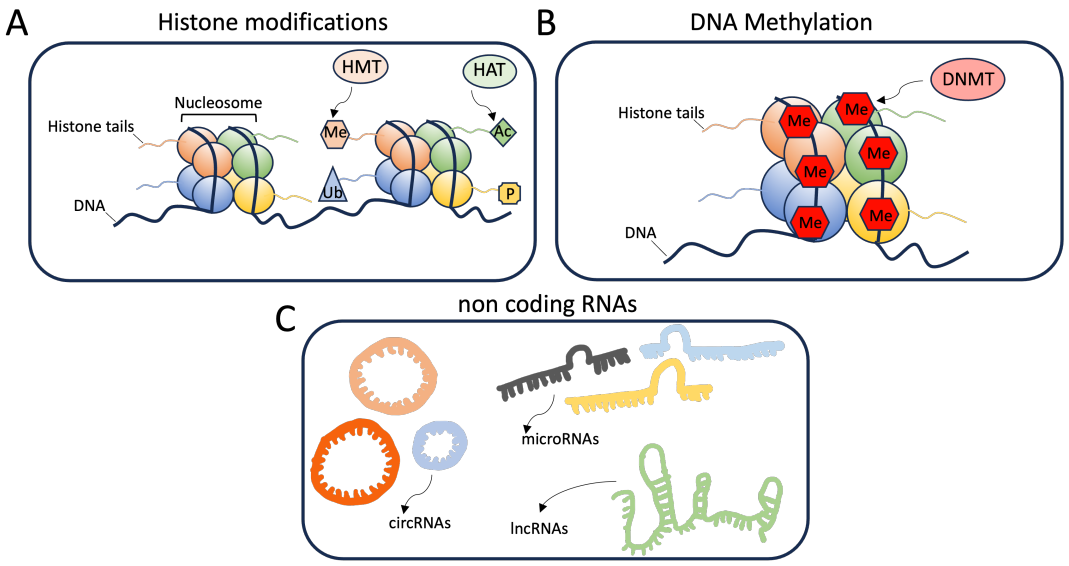


Figure 1.8. The three main groups of epigenetic modifications.
A. An example of histone modifications. Histones are proteins making complexes that allow DNA packaging. Histone tails undergo post-translational modifications, able to influence the gene expression positively or negatively. Here, a few examples are shown, such as histone methylation and acetylation, placed by histone methyltransferase and histone acetyltransferase (HMTs and HATs) enzymes, respectively. **B.** DNA methylation schematization. This modification requires the addition by DNMT enzymes of a methyl group to the carbon-5-position of a cytosine, usually followed by a guanine. This prevents the binding of transcriptional factors to promotor sequences, repressing consequently gene activation. **C.** Examples of non-coding RNAs implicated in gene regulation: long non-coding RNAs (lncRNA), microRNAs (miRNAs), and circular RNAs (circRNAs, emerged very recently thanks to Salzman et al. 2012)**.**

H3, H4, H2A, and H2B (Spencer and Davie 1999). Specific enzymes catalyze modifications on these proteins, acting at the histone N-terminal tails, on amino acids such as arginine, lysine, or tyrosine (Andrew J. Bannister and Kouzarides 2011). Depending on the residue involved, there are several types of histone modifications, which have a specific impact on gene expression. Histone acetylation for instance, positively activates gene expression, with a few exceptions for H4 histone (Kurdistani, Tavazoie, and Grunstein 2004). On the other hand, histone methylation can cause both activation and repression: this depends on the specific histone tail, the amino acid residue involved and the number of methyl groups added (Andrew J. Bannister and Kouzarides 2011; Swygert and Peterson 2014). The main histone modifications are listed and described in Table 1.1.

Table 1.1. Types of histone modification.

Histone modification	Target	Effect on the gene expression
Acetylation	Lysine amino groups of histones	Transcription activation: chromatin becomes structurally loose and less compact
Methylation	Arginine or lysine in histones	Arginine: transcription activation
		Lysine: transcription activation or inhibition, depending on the lysine target and the number of methyl groups added
Ubiquitination	Lysine of H2A and H2B histones	Transcription initiation and elongation
Phosphorylation	Serine, threonine, or tyrosine of histones	Transcription activation, chromatin condensation and DNA damage (depending on the amino residue and histone)
SUMOylation	Lysine of histones	Repression of transcription

Over the past 20 years, advances in technology, such as ChiP-sequencing or mass spectrometry, have allowed identifying the distribution of the histone modifications across the genome, highlighting the existence of many combinations of these marks, which in certain circumstances, are more likely to occur together. This suggests the occurrence of a crosstalk which could subsist between modifications on the same histone tail (*cis*), or on consecutive histones or even nucleosomes within a chromatin domain (*trans*) (T. Zhang, Cooper, and Brockdorff 2015; Strahl and Briggs 2021). The key players of the histone modifications can be separated into three distinct categories: *writers*, a group of enzymes capable of depositing the mark, *erasers*, qualified in removing the marks, and *readers*, a range of proteins containing a specific domain adept in recognizing and binding the epigenetic marks, to further mediate the outcome in chromatin remodeling or gene expression modulation. Altered regulation in any of these protein categories has been associated with disorders, such as tumorigenesis (Biswas and Rao 2017; Zhao and Shilatifard 2019): indeed, in the field of cancer drug discovery, the latest therapeutic approaches aim to target these specific alterations.

Along with histone modifications, DNA methylation is another common epigenetic marker. It involves the formation of 5-methylcytosine (5mC), by the addition of a methyl group to a cytosine-guanine dinucleotide, or to "CpG islands", containing those

dinucleotides in high concentration. Overall, the enzymes responsible for the deposition of this mark are called DNA methyltransferases (DNMT) and are divided into two classes: DNMT1 is responsible for the *maintenance* of the DNA methylation, especially after DNA replication, where newly synthesized filaments are lacking all the modifications (E. Li, Bestor, and Jaenisch 1992; Iida et al. 2002; Takeshita et al. 2011). DNMT3a and DNMT3b, instead, in cooperation with DNMT3L (Hata et al. 2002), are responsible for the *de novo* DNA methylation at previously unmethylated sites (Okano et al. 1999), such as transposons and repeat regions in the genome (Jurkowska, Jurkowski, and Jeltsch 2011). DNA methylation is commonly associated with decreased gene expression, by disrupting transcription factors binding (Brenet et al. 2011a; Watt and Molloy 1988). Indeed, the methylated DNA is recognized and bound by methyl-CpG binding proteins (MeCP), readers of this epigenetic mark: they recruit several transcription repressors which alter the chromatin structure, avoiding transcription initiation (Nan et al. 1998; Martinowich et al. 2003). Genes expression at those loci is, therefore, turned off and no transcript will be produced. Since the event of its discovery in bacteria, DNA methylation has been widely studied in different model systems, revealing all the biological processes involving this specific epigenetic mark: chromatin organization, genomic imprinting, gene expression regulation, silencing of retroviral transposable elements, development and aging (Moore, Le, and Fan 2013; P. A. Jones 2012; Ginno et al. 2020; Richardson and Yung 1999). It represents a very dynamic mark, defined by developmental and tissue-specific patterns (Horvath 2013; Davies et al. 2012).

Finally, the last class of epigenetic modifications involves non-coding RNAs, which have changed our previous overview of the central dogma of biology (DNA-RNA-protein), where RNA transcripts do not necessarily code for proteins. In humans, almost 98% of the genome does not code for proteins and initially, this proportion of DNA was labelled as “junk DNA”. However, of the 98%, approximately 90% was still transcribed into non-coding RNAs, found, over the years, to play significant functions (Ohno 1972; ENCODE Project Consortium et al. 2007; Wilusz, Sunwoo, and Spector 2009). Several studies have suggested a developmentally complex role for these RNAs during the evolution (Mattick 2004; Pang, Frith, and Mattick 2006; Taft, Pheasant, and Mattick 2007): indeed, while the number of protein-coding genes has remained relatively the same, the proportion of noncoding RNAs has remarkably expanded. These ncRNAs can act as both positive and negative regulators of gene activity. They are usually classified

by several properties, such as their structural features, the length of the transcript, or their functional activities. In addition, their expression is regulated in a tissue or developmental stage-specific manner (Srijyothi et al. 2018). Among the main classes of noncoding RNA, lncRNAs represent the most abundant class: they are able to influence gene transcription via chromatin remodeling or antisense transcription (Mercer, Dinger, and Mattick 2009; Ponting, Oliver, and Reik 2009).

1.3.2.1 A deep focus on a specific epigenetic mark: the H3K9 histone methylation

Early discoveries found that the genomic DNA, in eukaryotic nuclei, is organized in a complex structure called "chromatin", which is divided into different stained regions, heterochromatin, and euchromatin. Heterochromatin includes regions that remain condensed during the whole cell cycle, while euchromatin regions can decondense during interphase (Heitz 1928). This explains why the former is usually associated with gene silencing and the latter with active transcription.

Distinct histone modifications modulate euchromatin and heterochromatin, to make the DNA more or less accessible to the transcriptional machinery. The H3K9me is one epigenetic marker associated with the formation of heterochromatin, known to be divided into constitutive and facultative. The constitutive heterochromatin is made of repetitive and non-coding sequences located around telomeres and centromeres in all cell types. On the other hand, facultative heterochromatin involves the repression of certain genes in a cell type-specific manner (Elgin and Reuter 2013; Saksouk, Simboeck, and Déjardin 2015; Trojer and Reinberg 2007). The H3K9me is mostly known for its role in building the constitutive heterochromatin, essential for maintaining genome stability during events of chromosome segregation or recombination (Janssen, Colmenares, and Karpen 2018; Nicetto and Zaret 2019). However, more recent works based on genome-wide analysis, established a crucial role for H3K9me in regulating gene expression, especially during development, but also for the control of adult behaviors.

The main histone-methyltransferases were identified initially from studies in *Drosophila*. Here, three different key players were described: Eggless, Su(var)3-9, and G9a, all expressed in different tissues and implicated in critical developmental stages (Schotta et al. 2002; Stabell et al. 2006; Clough, Tedeschi, and Hazelrigg 2014). Interestingly, the distribution of these proteins differs across specific areas of

chromosomes. Eggless is required during multiple stages of oogenesis, for the differentiation of germline stem cells and for piRNA cluster transcription (Clough 2014, Rangan 2011). It also plays a role in building heterochromatin at chromosome 4 (Seum et al. 2007; Tzeng et al. 2007). Su(var)3-9 was originally discovered by its first function as a suppressor of the position-effect variegation (*PEV*), a phenomenon of cis inactivation of euchromatic genes after being relocated very close or within a sequence of heterochromatin (Mis, Ner, and Grigliatti 2006; Elgin and Reuter 2013; Grigliatti 1991; E. B. Lewis 1950). It is responsible for H3K9me2-3 at centromeric and telomeric regions and more recently, has been found to bind also euchromatic genes at the exon and CDSs levels, across different tissues. Loss of Su(var)3-9 leads to H3K9 abrogation on these targets, which is not compensated by the other HMTases (Maksimov et al. 2018; Schotta et al. 2002). G9a is also a suppressor of *PEV* and is responsible for the H3K9me2 in euchromatin regions, therefore, playing a role in negatively regulating gene expression, and telomeric regions (Kato et al. 2008). The H3K9me2-3 marks are finally bound by "reader" proteins, such as heterochromatin protein 1 (HP1), also known as Su(var)2-5 (A. J. Bannister et al. 2001; Jacobs and Khorasanizadeh 2002). HP1 is able to oligomerize, forming a dimer and creating a link between two adjacent nucleosomes. More recent works found that Su(var)3-9 regulates the H3K9-binding of HP1 in the pericentromeric regions, whereas Eggless regulates its recruitment in chromosome 4 and the cytological region 2L:31 (Figueiredo et al. 2012).

In mammals, there are three main families of well-characterized H3K9 methyltransferases, with two distinct variants each: SUV39H1, SUV39H2, SETDB1, SETDB2, G9a, and G9a-like protein (GLP). While G9a and GLP are attributed to mono- and di-methylation, SUV39H1, SUV39H2, SETDB1 and SETDB2 are fundamental for di- and tri-methylation (H. Wu et al. 2010; Montavon et al. 2021). A total loss of the activity of all these enzymes in mouse embryonic fibroblasts, led to decondensation of the heterochromatin, deregulation of repeat elements, such as transposons, and, consequently, high genomic instability (Montavon et al. 2021). Other studies found the existence of a partial overlapping function between these proteins (Fritsch et al. 2010; García-Cao et al. 2004; Gauchier et al. 2019; Becker et al. 2017). Furthermore, these methylases have been found to methylate not only H3K9, but also other histones and non-histone targets. For instance, SUV39H1 and SUV39H2 have been found to methylate other chromatin-modifying proteins (Piao et al. 2015; Kudithipudi et al. 2017), SETDB1 targets cell-cycle related enzymes, while G9a/GLP methylates also

transcription factors (Feldman et al. 2006; Pless et al. 2008; Lee et al. 2010; 2011; Ling et al. 2012)

1.3.3 Histone modifications in sleep regulation

Several studies have clearly confirmed that histone modifications are able to modulate sleep. In this context, one of the main contributions is made by histone acetylation, normally involved in various neurobiological mechanisms (Schaefer and Lyko 2010; Zemach et al. 2010). Indeed, most research has found a correlation between this type of epigenetic modification and circadian rhythms: in mammals, for example, the CLOCK protein is an acetyltransferase, which targets both histones H3 and H4 (Doi, Hirayama, and Sassone-Corsi 2006; Hirayama, Cho, and Sassone-Corsi 2007) of circadian factors, such as BMAL1. Also the histone deacetylase (HDAC) SIRT1 regulates circadian histone acetylation, consequently modifying the expression of proteins like BMAL1 or PER2. Here, the deacetylate reaction is made by the consumption of NAD^+, a substrate linked to the metabolic state and transduced to the circadian clock (Nakahata et al. 2008; Asher et al. 2008). Research in *Drosophila* has found that loss of the histone acetyltransferase (HAT) Elp3 causes hyperactivity and sleep loss in adult flies (Singh et al. 2010). Similarly, the depletion of HAT Tip60, leads to sleep-wake cycle disruption, compromising the small ventro-lateral circadian pacemaker neurons (Pirooznia et al. 2012).

Despite limited knowledge, compared to acetylation, histone methylation also has an impact on sleep modulation. For instance, in mammals, the histone methyltransferase MLL1 forms a complex with CLOCK-BMAL1(Katada and Sassone-Corsi 2010), which provides a permissive chromatin state for circadian transcription. In addition to this, JARID1A, a lysine-specific demethylase, triggers the transcription of master circadian genes, such as *clock* and *bmal1*, via a demethylase activity and is able to enhance the histone acetylation on the *Per* promotor, by inhibiting HDAC1 (DiTacchio et al. 2011). In flies, a crucial role is played by Jumonji (Jmjc) factors, a protein family including several histone demethylases, which correlate to specific sleep disturbances upon depletion (Shalaby et al. 2018).

The impact of other histone modifications on sleep is currently unexplored with a few exceptions: in humans for instance, a genome-wide study identified an interesting

correlation between sleepiness and a single nucleotide polymorphism (rs2218488) in the gene *eya1*, which codes for a histone phosphatase (Gottlieb, O'Connor, and Wilk 2007). A deeper molecular analysis, however, is necessary to better elucidate the link between this enzyme and sleep regulation.

1.3.4 DNA methylation in sleep regulation

In mammals, DNA methylation is known to be involved in sleep regulation. Sleep-deprived mice, for instance, show increased expression of the two DNA methyltransferase genes *DNMT3A1* and *DNMT3A2*: this result suggests a consequentially higher level of DNA methylation upon sleep loss (Massart et al. 2014a). In addition, short-sleeping mice (< 6.8 h) have differential DNA methylation of approximately 50 genes, compared with long sleepers (>7.8; H. Huang et al. 2017). Other murine models also change the expression of genes related to DNA methylation upon sleep deprivation (Narwade, Mallick, and Deobagkar 2017). For instance, circadian genes, such as *Cry1* and *Per1,* also are subject to hypermethylation (Qureshi and Mehler 2014b). Similarly to that, in humans, *CRY2* and *CLOCK* are hypermethylated in long-term night shift workers, further supporting the notion that sleep loss has a strong impact on the methylome.

Despite the substantial evidence in mammals, little is known about the potential correlation between sleep and methylome in *Drosophila.* The main reason for this is that flies have been shown to lack any functional DNA cytosine methylation (Goll et al. 2006; Schaefer and Lyko 2010; Zemach et al. 2010; Raddatz et al. 2013). Nevertheless, the DNA hydroxy-methylase TET has recently been found to play an interesting role in flies. Although TET is implicated in DNA de-methylation in mammals (Tahiliani et al. 2009), surprisingly it seems to take part also in RNA de-methylation in flies. Wang et al. found out that in adult flies this enzyme is required for normal larval locomotion, and circadian rhythms (F. Wang et al. 2018). Also, pupal lethality follows its complete loss of function (Goll et al. 2006) (Qureshi and Mehler 2014b). This suggests that epigenetic modifiers can have alternative targets.

1.3.5 noncoding RNAs in sleep regulation

Over the last 20 years, several classes of ncRNAs have been described, but only two of them seem to impact sleep regulation: lncRNAs and microRNAs. The lncRNAs class includes long transcripts, of 200 or more nucleotides, that are required in many processes, such as gene expression regulation (Shi, Zhang, and Qin 2017). They are expressed ubiquitously, but a large portion has been found in the brain (Derrien, Guigó, and Johnson 2012). Recent studies have implicated some lncRNAs in neurological processes and others in circadian mechanisms. Research in mice has found that lncRNA116HG is mostly active during sleep and upon its loss of expression, circadian genes, such as *Clock*, *Cry1*, or *Per2,* were down-regulated (Powell et al. 2013). lncRNA116HG is also associated with Prader-Willi syndrome, characterized by sleep and circadian disturbances. In flies, Soshnev et al. have identified for the first time a lncRNA involved in the regulation of sleep behavior. They discovered a cytoplasmic lncRNA called *Yar,* which leads to defects in sleep, when disrupted: the main consequences are shorter sleep bouts during baseline sleep and reduced rebound upon sleep deprivation (Soshnev et al. 2011).

The second class of ncRNAs associated with sleep regulation are microRNAs. Compared to lncRNAs, they include very small transcripts of 20-23 nucleotides in length, able to bind to complementary regions of mRNA transcripts. This fusion is necessary to trigger a process called *RNA interference* (*RNAi*), implicated in the inhibition of mRNA translation. Similar to lncRNAs, a consistent amount of microRNAs have been found in the brain (Davis et al. 2007), where it is implicated in several processes, including circadian mechanisms (Lugli et al. 2008). Research in rats has shown that, upon sleep deprivation, specific miRNAs in the brain, such as miR-138, let-7b, or miR-125a-5p, are differentially expressed between dark and light (Davis et al. 2007; Davis, Clinton, and Krueger 2012). In addition, miR-192/ 194 and mir-494 are implicated in *Per* and *Bmal1* regulation, respectively (Soshnev et al. 2011; Davis et al. 2007). As well as in mammals, research in *Drosophila* has described several sleep-related miRNAs. For instance, miRs bantam and let-7 regulate separately, the expression level of *clock* and *clockwork orange* (a circadian transcriptional repressor) (Kadener et al. 2009; Chen et al. 2014). Furthermore, miR-263b and miR-274, specific to glial cells, tend to regulate some circadian behaviors, although target genes remain undefined (You et al. 2018). Finally, a recent work based on a library of miRNA sponges

(miRNA inhibitors), described a genetic screen where 25 miRNA were found to be related to sleep-baseline: 8 are wake-promoting and 17 sleep-promoting (Goodwin et al. 2018).

1.3.6 Why study the interplay between sleep and epigenetics?

Recent studies have highlighted the interconnection that exists between sleep and epigenetics. In the previous sections, we have dissected for instance, how sleep deprivation impairs DNA methylation in mammals, which consequently deregulates target genes, often involved in circadian circuits in both neural and non-neuronal sites (Qureshi and Mehler 2014a). Also in humans, a night of sleep deprivation has been associated with DNA methylation and tissue-specific transcriptional changes in clock genes (Cedernaes et al. 2015). Interestingly, sleep-loss consequences have been also found in other circumstances, external to sleep. A pilot work found that young women with shorter and irregular sleep show acceleration in epigenetic aging processes (Carskadon et al. 2019).

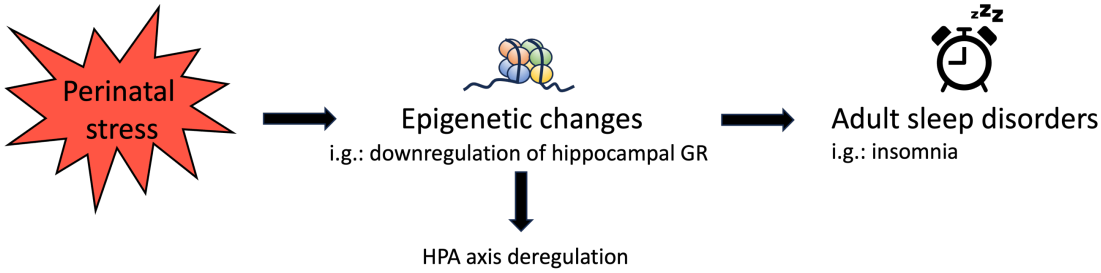


Figure 1.9. Adult sleep disorders, as a consequence of early-life stress exposure.
Schematic illustration of the correlation between sleep disorders during adulthood and stress exposure during early life (utero life and lactation period). The epigenetic downregulation of glucocorticoid receptors (GR) in the hippocampus, upon perinatal stress, causes deregulation of the hypothalamic-pituitary-adrenal (HPA) axis, which seems to persist during adulthood, correlating to sleep disturbances, like insomnia.

On the other hand, there is evidence of how external lifestyle factors, such as diet or stress, may modulate sleep through changes in the epigenetic landscape. For instance, stress factors acting during pregnancy seem to play a role in the development of sleep disorders in adult life. As shown in Figure 1.9, early stress exposure causes epigenetic downregulation of glucocorticoid receptors (GRs) in the newborn hippocampus, followed by alteration of the hypothalamic-pituitary-adrenal (HPA) axis. These changes

are proposed to be persistent also during adulthood, leading to sleep disturbances, such as insomnia (Lo Martire et al. 2020).

Another supporting piece of evidence of the lifestyle-epigenetics-sleep axis, is the consumption of coffee and its correlation to several health outcomes. Heavy consumption of coffee can lead, for instance, to insomnia (Butt and Sultan 2011). It is well known that caffeine is what improves cognitive function and increases alertness, by blocking adenosine from binding to its receptor (McLellan, Caldwell, and Lieberman 2016). In this context, epigenetic studies have found that DNA methylation plays a role as a mediator in the influence of caffeine on health. For instance, together with works on mammals, a study on humans has been recently published (Chuang et al. 2017), showing how caffeine changes DNA methylation in blood immune cells. Surprisingly, most of the methylated variations were located inside or near genes already known to associate with chronic diseases, such as Parkinson's or Alzheimer's diseases. This study provides new insights about the correlation between coffee consumption, epigenetics, and health.

Remaining in the context of cognitive diseases, interestingly, people affected by Alzheimer's disease (AD) have shown impaired levels of epigenetic marks in the hippocampus (Sanchez-Mut and Gräff 2015), alongside impairments in neurogenesis in the dentate gyrus (DG), a region of the hippocampus normally involved in learning and cognitive abilities formation (Kent and Mistlberger 2017). Among the symptoms, patients affected by AD are known to experience various sleep disturbances, such as fragmented nighttime sleep, a higher number of daytime naps, and reduced time spent in REM stages. This suggested that the cognitive deficits observed in AD patients may be related to disruption of the neurogenesis in the DG, more likely due to both epigenetic alterations and poor sleep quality (Mander et al. 2016). Despite the existence of a link between sleep and epigenetic disruptions in AD, a mutual combination of epigenetic medications and behavioral therapies (targeting the circadian clock) are usually applied to allow a delay in the progress of cognitive deficits.

All the evidence described above highlights the crosstalk between sleep and epigenetics, a newly emerging field which in the coming years, will be making significant advances in understanding the main epigenetic changes implicated in sleep disturbances. These studies will be beneficial to understand the importance, but also the risk, of the

environmental factors in the context of sleep disturbances, with the goal of developing future and novel therapies, aiming to treat sleep-related disorders and the associated cognitive deficit.

1.4 The emerging role of DNA repair in sleep

1.4.1 DNA damage and sleep in flies and mice

Although it is established that sleep occurs in every species with neural networks, its functional role remains a mystery. Recently, a new theory has emerged, believing that sleep is crucial to correct and fix imbalances and damages accumulated during the waking state (Mourrain and Wang 2019). Indeed, the neuronal activity during wake time is very costly: as we explore and learn new information, synaptic connections are intensified, leading to an increase of synaptic strength in many regions of the brain, where higher demand for energy and cellular supplies is required. In this regard, the synaptic homeostasis hypothesis (SHY), one of the first hypothesized functions for sleep, claims that sleep is needed to re-establish synaptic homeostasis (Tononi and Cirelli 2003; 2006). In association with this increased synaptic activity, there is also an accumulation of waste and toxic metabolites that need to be cleared from the brain. Therefore, an additional function for sleep in neuronal genome maintenance has been proposed, where damage in neuronal DNA can potentially act as a homeostatic driver of sleep. Bellesi et al. have shown that situations such as exploration of enriched environments, when there is heightened demand for learning and memory, lead to the accumulation of DNA double-strand breaks (DSBs) in mice brains (Bellesi et al. 2016). This conclusion was based on previous observations which found that increased neuronal activity by optogenetic stimulation was sufficient to raise the level of DSBs, in particular in the hippocampus, where most learning and memory circuits are built up (Suberbielle et al. 2013). Since DNA breaks are essential for the transcription and expression of neuronal genes, some of which are involved in synaptic plasticity, one hypothesis is that DSBs may happen during the transcriptional activation of genes related to learning and memory formation. Surprisingly, Suberbielle et al. found that these DNA breaks were repaired within 24h: in this regard, Bellesi et al. suggested that sleep helps DNA repair, by upregulating DNA repair genes.

1.4.2 DNA damage and sleep in zebrafish

The correlation between sleep and DNA damage has also been confirmed by Zada et al. in zebrafish, who found, indeed, an increase in DSBs during sleep deprivation. They found that fish normally experience a cyclic fluctuation of breaks over 24h, with higher levels during the wake phase and lower levels during the night. Interestingly, imaging of larval brains showed that sleep enhances neuronal chromosome dynamics in a homeostatic (and not circadian) dependent manner. During the day, chromosome dynamics remained low (D. Zada et al. 2019). In addition, they inhibited these chromosome dynamics by increasing the expression of the lamina-associated polypeptide 2 (Lap2β), a protein that directly interacts with lamins, further anchoring the chromatin to the nuclear lamina. As a consequence of this inhibition, they found a pronounced increased number of DSBs specifically during the night, further suggesting that chromosome dynamics may correlate with the reduction of DSBs during the night. Moreover, to validate the specific function for sleep in neuronal nuclei, they observed that both chromosome dynamics and DSBs did not differ between day and night in other tissues, such as the peripheral endothelial or the Schwann cells.

To investigate the reciprocal correlation between sleep and DNA damage, Zada et al. also questioned whether DSBs may cause changes in sleep. In order to achieve this, they exposed zebrafish larvae to etoposide (Eto), a drug that induces DSBs, and found that sleep time was increased in Eto-treated larvae, compared to controls. Two hours after the treatment, when sleep was still detected to be elevated, chromosome dynamics increased and DSBs decreased in number. These data suggest again that the accumulation of damages (described previously to be specific for wakefulness) may enhance sleep, which leads to a higher level of chromosome dynamics and subsequently reduces the number of DSBs (D. Zada et al. 2019). Finally, they confirmed that during sleep, the DNA damage response (DDR) activity increases, following the rising level of chromosome dynamics. In particular, the DDR initiator poly (ADP-ribose) polymerase 1 (Parp1), normally involved in the recruitment of downstream DDR proteins, becomes more active upon sleep deprivation. According to Zada et al., Parp1 promotes sleep: in fact, the inhibition of Parp1 leads to a reduction of the sleep-dependent chromosome dynamics and the activity of the DNA repair (David Zada et al. 2021). These findings provide further evidence of DNA damage as a homeostatic driver for sleep.

1.4.3 The molecular mechanisms in DNA repair

In the previous section, we introduced insights into the correlation between sleep and the DNA damage response (DDR). Together with Parp1, found by Zada et al. to enhance sleep, DDR involves a set of responses mediated by different groups of other enzymes. Each type of damage triggers a specific pathway of cascades. For instance, double-strand breaks, marked by phosphorylation of H2Av, are sensed by the Mre11-Rad50-NBS1 (MNR) complex, which rapidly promotes the activation of the kinases ataxia telangiectasia mutated protein (ATM) (Lavin et al. 2005; Shiloh 2003). ATM recruits

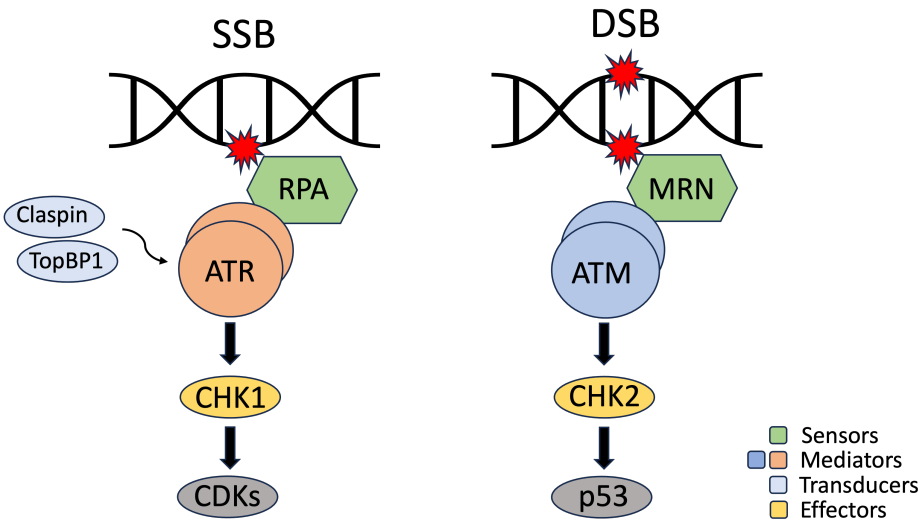


Figure 1.10. Schematic illustration of the DNA damage response pathways.
ATM and ATR are key players for the DSBs and SSBs repair activation, respectively. Both DNA damage responses are regulated by several proteins which can be differentiated in sensors, mediators, transducers, and effectors, depending on their function.

targets such as Checkpoint kinase 2 (CHK2), together with the transcriptional factor p53, which orchestrates different cellular stress responses, such as DNA repair, senescence, and apoptosis (Bruno et al. 2006; Meulmeester et al. 2005; Lakin and Jackson 1999). Single strand breaks (SSBs) are recognized by the sensor replicative proteins A (RPA), which allow the activation of the ataxia telangiectasia and Rad3-related protein (ATR) (Zou and Elledge 2003; Choi et al. 2010). Thanks to the cooperation of TopBP and Claspin, other partner proteins, ATR allows the phosphorylation of CHK1. Downstream of CHK1, there are several CDK proteins, essential to regulate the cell cycle progression (Fig. 1.10, X. Li et al. 2013).

1.4.4 ATM, ATR, and circadian influence

ATM, ubiquitously expressed, takes part to the phosphatidylinositol 3-kinase (PI3K)-related protein kinase family (PIKK), which is normally involved in cell-homeostasis processes, such as DNA damages response and cell growth (C. A. Lovejoy and Cortez 2009). In baseline conditions, ATM is not crucial for cell functioning, and it is stored under an inactive homodimer shape. However, it becomes essential under stressful conditions, which facilitate the phosphorylation and the split of the homodimer into separate active monomers (X. Wang et al. 2016). ATM is implicated in the activation of several DSB repair pathways, such as homologous recombination (HR), non-homologous end joining (NHEJ), and base excision repair (BER) (Chang et al. 2017; Ciccia and Elledge 2010; Chou et al. 2008). On the other hand, ATR also belongs to the PIKK family, but differently to ATM, represents a fundamental protein for cell survival. Indeed, the depletion of ATR leads to embryonic lethality in mice and general cell death in human cells (E. J. Brown and Baltimore 2000; Eric J. Brown and Baltimore 2003; Cortez et al. 2001), probably due to its essential role in regulating cell cycle progression. ATR recruits members to allow the nucleotide excision repair (NER) (Kang and Leem 2014).

In the last decade, several studies conducted in mice have highlighted a circadian component influence in the regulation of the DDS. For instance, the ATM-CHK2 kinase function in promoting the activation of p53 is also mediated by PER proteins, which aids the phosphorylation of CHK2 (Gery et al. 2006). Oklejewicz et al., instead, found that in the presence of DSBs, the circadian light-dark phase is shifted, due to ATM-mediated damage signaling (Oklejewicz et al. 2008). Another study conducted in mouse fibroblasts and liver cells found that upon UV-induced damages, the ATR-CHK1 activation is triggered by the interaction between CHK1 and CRY1/TIM, which builds up a unique molecular complex (Kang and Leem 2014). In general, the influence of the circadian clock system in the rhythmicity of ATM-CHK2 and AT-CHK1 is fundamental to allow maintaining genome stability. Indeed, alterations in the circadian clock can trigger replication stress and reduce the DNA repair capacity by causing genome instability and in certain circumstances, cancer. For instance, both up and down-regulation of *bmal1* is associated with alteration in the expression of p21, a downstream target of p53, correlated with aberrant DNA synthesis and cancer initiation (Yeh et al. 2014; Gréchez-Cassiau et al. 2008; Angelousi et al. 2019).

1.5 Aim of this work

Based on the notions described in this introduction, in the next chapters, I will be dissecting the connection between sleep and genome dynamics, by focusing on two different DNA levels, and using *Drosophila melanogaster* as model system. First, I will describe the implication of a specific type of histone modification, the H3K9 methylation, in the context of sleep regulation (Chapter 3). Second, I will be further proving the emerging correlation between sleep and DNA repair, by giving evidence of DNA damages, in particular DSBs, as homeostatic drive for sleep (Chapter 4). Combining the study of behavior with strategical genetic manipulations, I will assess the potential role of specific changes in DNA, at different levels, in sleep regulation.

Chapter 2
Material and Methods

2.1 Fly stock and handling

The *RNAi* lines used in this project were obtained from the Vienna Drosophila Research Center (VDRC) (see Table 1), while GAL4 drivers and control strains were obtained from the Bloomington Drosophila Stock Center (BDSC) and the Kyoto Drosophila Stock Center (KDSC). Before any experimental use, all the stocks were backcrossed using the strain w1118. Flies were raised at room temperature on vials with standard corn and yeast media, under 12 h light:12 h dark (LD) conditions. Behavioral experiments were performed using 2-3 days old male flies: one day in advance of the actual experiment, they were moderately anesthetized using CO_2, placed in glass tubes (70mm length x 5mm external diameter x3mm internal diameter), and moved into the ethoscopes, which were stored in incubators set at 60% humidity and 25°C. 10 days old flies were used, instead, for immunostaining experiments.

2.2 GAL4-UAS system-mediated *RNAi*

The *GAL4-UAS* system, introduced for the first time by Brand and Perrimon in 1993 (Brand and Perrimon 1993), represents a powerful genetic tool, with the aim to enhance the expression of target genes in a specific spatiotemporal manner. It is based on the action of the GAL4 protein, a transcription factor originally discovered in yeast and normally not expressed in flies: GAL4 is able to activate specific target genes, by recognizing sequences on their promotor regions, called UAS (Upstream Activation Sequences) (Duffy 2002).

To replicate this simple mechanism also in *Drosophila*, two different types of lines were made: the GAL4 line, expressing the GAL4 protein under the control of a tissue-specific promotor; the UAS line, with the UAS sequence upstream of a gene of interest. These

lines, taken individually, cannot enhance the transcriptional activation. Only after crossing them, it allows the offspring to express GAL4, which will bind the UAS and consequently activate the transcription of the linked gene (Duffy 2002). GAL4-UAS system can be exploited to manipulate genes in different ways, by over-expression or knock-down. One of the main advantages is the possibility to induce *RNAi* to knock-down specific genes (mRNAs).

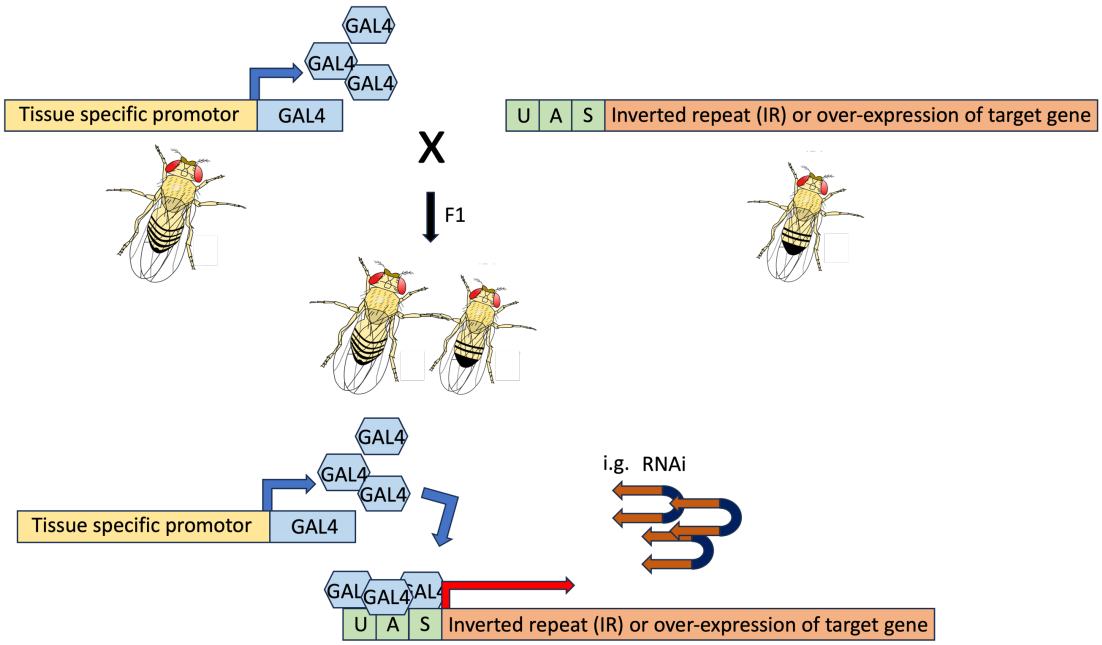


Figure 2.1. The *RNAi* through the GAL4-UAS system.
Schematic illustration of the genetic fly lines normally crossed to achieve the *RNAi* of a gene target: a GAL4 driver, expressing the yeast transcriptional factor GAL4, under the regulation of a tissue-specific promotor; a *UAS* line, containing an insertion with an inverted repeat (RI) of the gene target, downstream from UAS sequences that are recognized and activated by GAL4. Only the offspring generated by crossing these two lines, will enhance the formation of RNA harpins triggering the gene silencing, specifically in the tissue where GAL4 is expressed. Fly illustrations are taken from https://commons.wikimedia.org/wiki, created under the Creative Commons license.

As shown in Figure 2.1, the UAS line will generate a transcript made of inverted repeats with a sequence complementary to a gene of interest. The nature of that sequence will lead to the formation of double-stranded *RNAi* hairpins, which results in silencing of complementary transcripts. In this project, the GAL4-UAS system was mainly exploited to trigger the knockdown of specific target genes, but also to enhance the blockage or activation of specific sets of neurons (this approach will be better described in paragraph 2.4). As illustrated in Figure 2.2, the resulting offspring was subjected to baseline sleep and to rebound sleep analysis, triggered by different SD methods, which are described in the next paragraphs: dynamic SD, starvation and male-male interaction.

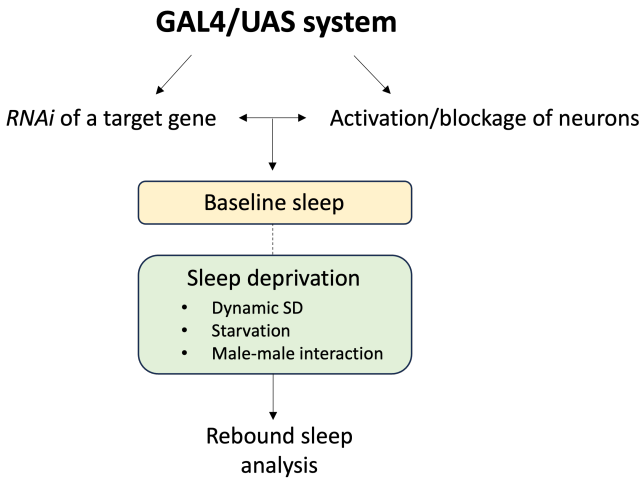


Figure 2.2. Schematic illustration of the main purposes of using the GAL4/UAS system in this study.

Finally, a list of the fly stocks used in this project is given in the following Table:

Table 2.1. List of fly lines used in this study.

GAL4	**Source**	**Expression pattern**
nSyb-GAL4	Dionne Lab (Imperial College London, UK)	pan-neurons
Elav-GAL4	Bloomington Stock Center	pan-neurons
R72G06-GAL4	Bloomington Stock Center (#39792)	dFB
AstA-GAL4	Bloomington Stock Center (#51978)	Allatostatin-A neuropeptide expressing neurons
ple-GAL4	Bloomington Stock Center (#8848)	dopaminergic neurons
repo-GAL4	Bloomington Stock Center	glial cells
GH298-GAL4	Bloomington Stock Center (#37294)	olfactory interneurons
PDF-GAL4	Bloomington Stock Center	PDF expressing neurons
sNPFR-GAL4	Bloomington Stock Center (#46547)	sNPFR expressing neurons
R52B10-GAL4	Bloomington Stock Center (#69657)	protocerebral bridge
R30G03-GAL4	Bloomington Stock Center (#49646)	EB
R93F07-GAL4	Bloomington Stock Center (#40658)	EB, dFB
R69F08-GAL4	Bloomington Stock Center (#39499)	EB
R58H05-GAL4	Bloomington Stock Center (#39198)	EB, bulb
R24C12-GAL4	Bloomington Stock Center (#49076)	adult antenna lobe
Su(var)3-9-GAL4	Kyoto Stock Center (#104-393)	Su(var)3-9 expressing neurons
TDC2-GAL4	Bloomington Stock Center (#9313)	octopaminergic neurons
nSyb-GeneSwitch	Bloomington Stock Center (#80699)	pan-neurons
MB010B-GAL4	Bloomington Stock Center (#68293)	MB
UAS/ RNAi	**Source**	

Su(var)3-9-RNAi	Vienna Drosophila Resource Center (#39378)
Eggless-RNAi	Vienna Drosophila Resource Center (#101677)
G9A-RNAi	Vienna Drosophila Resource Center (#25473)
TRPA1^{ts}	Southall Lab (Imperial College London, UK)
Shi ^{ts}	Bloomington Stock Center
eIF2γ-RNAi	Bloomington Stock Center (#31619)
Syn-RNAi	Vienna Drosophila Resource Center (#1095871)
BRP-RNAi	Huang Lab (Freie Universität Berlin, Germany)
ATM-RNAi	Vienna Drosophila Resource Center (#22502)
p53-RNAi	Vienna Drosophila Resource Center (#10692)
Mutant/ wt strains	**Source**
Canton Special (CS)	Stanewsky Lab (UCL, UK)
fumin mutant	Jepson Lab (UCL, UK)
Su(var)3-9- GFP	Brennecke Lab (IMBA, Vienna BioCenter)

2.3 Sleep deprivation methods

2.3.1 Mechanical sleep deprivation

In order to trigger sleep deprivation mechanically, ethoscopes were combined to a module, called *optomotor*. This module employs servo motors, able to rotate tubes housing individual flies. The rotation will occur only when flies are sleeping and the duration, speed, and direction of the rotation can be easily modulated, in order to adjust the quality of the stimulus. In all the behavioral experiments performed, sleep deprivation was scheduled with a 120 seconds immobility trigger, for a period of 24 hours. The motors were also programmed to rotate for 1 second. Rebound sleep between ZT0 and ZT3 of day 3 was measured and comparisons between genotypes were performed using statistical tests outlined in section 2.5.

2.3.2 Sleep suppression by starvation

With the aim of sleep-depriving flies by starvation, after one day of baseline sleep, flies were transferred to tubes containing only 2% agarose (Sigma #A6236), at ZT0 of day

2, by flipping and tapping the two tubes together. After 24 hours, flies were moved back to the tubes with normal food, before the onset of ZT0 of day 3: this last manipulation was done in darkness with red lights, a color that flies cannot detect, and very moderately, to avoid arousal as much as possible. In order to exclude any artifact due to the manipulation, a secondary group of flies per genotype was made as a control. In this condition, flies were transferred to new tubes with normal food on the second day and moved back to previous tubes on the third day. Rebound sleep between ZT0 and ZT3 of day 3 was measured in control and starved groups and comparisons between genotypes were performed using statistical tests outlined in section 2.5.

2.3.3 Sleep suppression by Male-Male interaction

Single male flies were placed in glass tubes and loaded into the ethoscope. Baseline sleep was recorded for 1-2 days. On the day of the interaction, white-eyed male flies (w^{1118}) were added as intruders, at ZT0, by one side of the tubes already containing single male flies and without CO_2 anesthesia. After approximately 23h, at ZT23, intruders were removed in darkness with red lights, and rebound sleep was measured later, between ZT0 and ZT3. In order to exclude any artifact due to the manipulation, a secondary group of flies per genotype was made as a control and housed in the same ethoscopes. Tubes containing these control flies underwent the same manipulation, however, no intruder was added. Comparisons between genotypes were performed using statistical tests outlined in section 2.5.

2.4 Temperature-sensitive experiments

In this project, two different temperature-sensitive fly lines have been used: TRPA1 and Shibire. TRPA1 (Transient receptor potential ankyrin 1) is a voltage-gated cation channel that is only activated at the specific temperature of 29°C. Below this threshold, it is kept inactive (Laursen et al. 2015). This channel, normally expressed in a small set of warmth-activated neurons (AC neurons), (Hamada et al. 2008), has been largely used to depolarize and activate specific neurons in a temporally restricted manner (Pulver et al. 2009; Roessingh and Stanewsky 2017; Berni, Muldal, and Pulver 2010). On the other hand, *Shibire^{ts}* (*Shi*) is a temperature-sensitive allele of the protein GTPase dynamin that

is fundamental in endocytosis events for synaptic vesicle recycling, essential to allow the fission of the vesicle from the membrane (van der Bliek and Meyerowitz 1991). This specific allele allows normal endocytosis at temperature <22°C, but at higher temperature prevents synaptic vesicle recycling, which causes inhibition of synaptic transmission. Therefore, the overexpression of *Shi^{ts}* has been used to trigger synaptic blockade of specific sets of neurons (Kosaka and Ikeda 1983; Kitamoto 2001).

In this project, crosses involving uas-TRPA1^{ts} and uas-Shi^{ts} lines, have been raised at 18°C until hatching. During experimental recordings, the temperature inside incubators was set and maintained at 22°C for a baseline day to avoid premature activation and then manually increased to 29°C, for the duration of the whole sleep-deprivation experiment.

2.5 Statistical analyses

All the data obtained in this project were analyzed using the bioinformatics software R. For behavioral analysis, the main R package used was *Rethomics* (Geissmann et al. 2019). However, plots were also generated using *ggplot2* package. For comparisons, the statistical analysis was performed by using the pairwise Wilcoxon rank sum test for multiple variables, where the p-values were adjusted using the False Discovery Rate (FDR) method. Sleep and activity plots display the proportion of time spent in sleeping or moving within 30-minute intervals, averaging each animal. Individuals who died over experiments were opportunely removed from the analysis. For the ethograms, the intermediate lines represent the mean, while the shades areas indicate the bootstrapped 95% confidence interval (Benjamini and Hochberg 1995). For the boxplots, the intermediate lines represent the median, the top and bottom edges of the box (hinges) indicate the first and third quartiles and the whiskers reach the maximum and minimum values that fall within 1.5 times the interquartile range from the quartiles. The number of animals used in each experiment is reported in all the figures. Log-Rank tests were applied between conditions for lifespan assays. The following convention was used to mark statistical significances: $p>0.05$: n.s.; $p<0.05$: *; $p<0.01$: **; $p<0.001$: ***. All

the experiments were performed not less than two times and data analysis integrated all the replicates.

2.6 Immunohistochemistry

2.6.1 Sample preparation and immunostaining

Before dissecting, flies were anesthetized on ice. Brains were dissected and stored in 0.1M phosphate buffered saline (PBS) (Sigma #P4417) and then incubated in 4 % Paraformaldehyde (PFA, Sigma #158127) solution for 20 minutes at room temperature to fix the tissue. Brain tissues were washed three times for 10 minutes in 500 ul 0.1M PBS with 0.03% Triton X (PBST) (Sigma # 9002-93-1): for a better rinse, tubes

Table 2.2. List of antibodies used for experiments.

Primary antibodies				
Antigene	Raised in	Conc.	Source	Cat#
nc82/BRP	mouse	1:10	DSHB	nc82
GFP	rabbit	1:200	Abcam	ab290
PDF	mouse	1:200	DSHB	PDF C7
DCP1	rabbit	1.100	Cell Signaling	9578S
H3K9 trime	rabbit	1:300	Abcam	ab8898
H2aV	mouse	1:10	DSHB	UNC-93
FasII	mouse	1:10	DSHB	1D4
Syn	mouse	1:10	DSHB	3C11

Secondary antibodies				
Antigene	Fluorophore	Conc.	Source	Cat#
rabbit	Alexa 568	1:250	Abcam	ab175471
mouse	Alexa 647	1:250	Abcam	Ab150115
chicken	Alexa 488	1:250	Abcam	Ab150169

containing brains were placed in the wheel (25 rpm). Brain tissues were then blocked for 1 hour at room temperature in 500 ul blocking solution, made by diluting pure Normal Goat Serum (NGS) (Life technologies #16210-064) in PBST. To get stained, the tissues were incubated in primary antibody solution (blocking buffer with diluted primary antibodies) at 4 degrees for two nights: as previously, tubes containing brains

were placed in the wheel for a better antibody penetration. After 3 times of PBST wash, the tissues were incubated in secondary antibody solution at 4 degrees for two nights and covered with foil to keep away from light. After 3 times of PBST wash, the brain tissues were mounted on slides with drops of vectashield (Vector Laboratories #WZ-93952-27) for confocal imaging. All the antibodies used in this study with corresponded concentrations are listed in Table 2.2.

2.6.2 Confocal imaging and quantification

LeicaTM SP8 Inverted confocal microscope was used to acquire images of whole-mount-stained fly brains. Alex Fluor 568, 488, and 647 were used as fluorescent dyes. Images were collected with Leica lasX software, using a resolution of 1024×1024. Confocal stacks of brains were taken at 40× magnification and Z stack stepwise: 4 μm. ImageJ software (FiJi distribution, Schindelin et al. 2012) was used to process and analyze the images.

For images where the fluorescence intensity had to be quantified, 10 z-stacks focused on the front brain were selected, starting from the top of the antennal lobes, where most of the cell nuclei are localized: the maximum intensity projection of selected stacks was generated and the average pixel intensity was measured in the respective region of interest (ROI), which was considered as the whole brain. An opportune normalization was made by drawing a region in the background, in order to measure and subtract the intensity outside the brain. At least two biological repeats were performed per experiment: each experimental replicate was, first, normalized separately, by calculating an average intensity value for each condition and dividing all individual intensity values by its respective mean value, to ensure that each replicate is internally comparable. Once replicates were normalized, they were combined, by taking the average of their normalized values, and finally plotted using R. Images shown in figures were converted to the color scale “fire”, which ranges from low intensity (blue) to high intensity (red).

2.7 Pharmacological treatment and fitness assays

2.7.1 Etoposide exposure

In this study, we triggered the accumulation of DNA double-strand breaks by exposing flies to Etoposide. Etoposide is an anti-tumor drug which acts as inhibitor of the DNA topoisomerase II (Cayman Chemical: CAY12092). DNA topoisomerase II are enzymes involved in numerous DNA processes, able to remove superhelical twists from DNA and resolve knots or tangles in the genetic material, by cleavage and re-ligation of the double strand. Etoposide binds and stabilizes the topoisomerase-DNA complex, a transient intermediate in the catalytic cycle of topoisomerase II, thus preventing re-ligation. The accumulation of cleavage complexes induces the generation of permanent DNA strand breaks (Meresse et al. 2004; Montecucco, Zanetta, and Biamonti 2015).
In this study, Etoposide was dissolved in DMSO (VWR #83673.230) to generate 50mM concentrated stocks and subsequently mixed with flyfood to reach a 2.5 mM concentration. Before exposure to the drug, male flies were housed for 2 days in normal food, then starved for 1 day in agar tubes to allow a suitable consumption of the drug in the next step. On day 4th they were swapped in drug food and after 27h placed back into normal food tubes. Behavior was tracked for the following 5 days. Appropriate controls were also made exposing flies to food with only DMSO, the solvent used to dissolve Etoposide.

2.7.2 Courtship assay

Courtship assay was implemented by using special 3D printed arenas, containing 6 chambers sealed by glass cover slides. Naive males were collected and aged for 1-2 days, and virgin or pre-mated females were also aged for 2-3 days and placed in groups of 10 animals until the onset of the experiment. For training session, females were placed in separate chambers, sealed with glass slides, and left for at least 1 hour to recover from anesthesia. Using an aspirator, males were then transferred into the same chambers. Male-female pairs were left undisturbed during the training period (1h for learning) and recorded using ethoscopes: over this time, males will court and be rejected by the pre-mated females. At the end of the training, males were gently separated from pre-mated

females without anesthesia and then housed in new chambers containing new pre-mated females. For learning, trained naive males were immediately tested and recorded for 15 min.

Videos were manually scored by observing stereotypical courtship behaviors, characterized by orienting towards and following the female, tapping, extending, vibrating the wing, licking, and attempting copulation. The courtship index (CI) was calculated as the percentage of time that the male tries to court during the first 10 min of the testing period.

2.8 GeneSwitch system

The GeneSwitch system is a modified version of the GAL4/UAS system, which allows spatial and temporal control of the transgene expression. The UAS sequences are here regulated by a chimeric GAL4 protein (GAL4-progesterone-receptor fusion protein), which becomes active only in the presence of the steroid RU486 (mifepristone, Abcam # ab120356), usually added to food (Fig. 2.2). In this study, the GeneSwitch system has

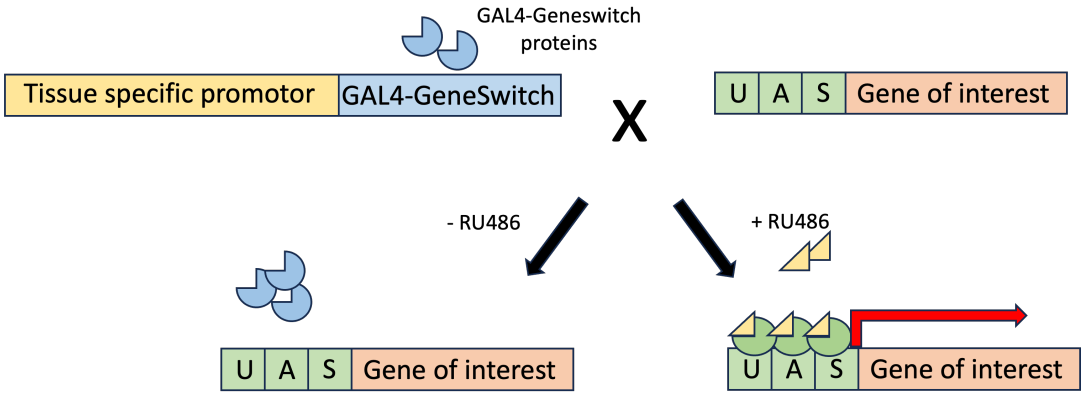


Figure 2.3. Schematic illustration of the GeneSwitch mechanism of action.
The GAL4-GeneSwitch protein, without the presence of the substrate RU486, is kept in inactive status and is not able to bind the UAS sequences. In the other hand, the addition of RU486, activates the protein, which now can bind the UAS sequence and transcribe the downstream gene.

been exploited in order to induce the activation of Su(var)3-9 knockdown, specifically in the adult stage. Flies were therefore raised in normal food until emerging and transferred then into food containing RU486, previously dissolved into dimethyl sulfoxide (DMSO), to reach a final concentration of 500uM. This specific concentration was chosen based on previous works (Scialo et al. 2016; Roman et al. 2001). Flies were

kept in tubes containing food + RU486 for the whole experiment recording. Furthermore, appropriate controls were also made by exposing flies, with the same genotypes, to food with only DMSO, the solvent used to dissolve RU486. This condition was expected to not enhance the *RNAi*, as the GAL4-Geneswitch protein is not able to bind the UAS sequences, without the presentence of its substrate.

2.9 RNA-related methods

2.9.1 RNA extraction and DNAase treatment

Heads, brains, or bodies from samples were dissected in PBS and transferred to 200ul of Trizol (Life Technologies #15596026), immediately. Tissue homogenization was performed by using the TissueLyser II system, for rapid and efficient disruption through high speed-shaking. After the addition of 200ul 100% chloroform (VWR #85853.320), samples were centrifuged for 15 mins at 12000g at 4°C and upper phases were transferred to new tubes, where 200ul of isopropanol (Sigma #l9516) was added. Following 30 mins on ice, samples were then centrifuged at 21000g for 30 mins. The supernatant was then discarded, and RNA pellet was washed and centrifuged with 80% Ethanol (VWR #20821.330) for 5 mins. RNA was re-suspended in 15ul of RNAase free H_2O and measured with a calibrated spectrophotometer (NanoDrop). Only samples with optical density (OD) ratios (A260/A280) of 1.9-2.2 were considered for DNAase treatment.

DNAase treatment was performed by adding 1ul of DNase I and 1ul of DNase I reaction buffer (Invitrogen #AM1906) to 8ul of each sample, 1ug concentrated. After incubating at RT for 15 mins, 1ul 25mM EDTA was added to all samples, which were then incubated at 65°C for another 15 mins. DNA-free RNA samples were immediately stored at -80°C.

2.9.2 cDNA synthesis

For reverse transcription, the Superscript III kit (Termofisher #18080093) was used. Briefly, a first mix with 1ul of oligo(dt) (50uM, Invitrogen #100023441) and 1ul of

dNTPs (10 mM, Thermo Scientific#R0192), was added to 1ug of DNA-free RNA, diluted in 10ul (see previous section). Denaturation was enhanced for 3 min at 65°C, followed by 2-5min on ice. A second mix was then prepared with the following ingredients: 4ul of 5X buffer, 1ul of DTT, and 1ul of Superscript III Reverse Transcriptase. 6ul of the above-described mix was added to each RNA sample and the reaction was performed for 50min at 50°C, followed by 15min at 70°C. cDNA was diluted to a final volume of 50ul and 2ul of diluted cDNA was then used per each qPCR reaction.

2.9.3 qPCR and data collection

Primers of specific target sequences were designed using the Primer3 algorithm and then reviewed for sequence complementarity on the *Drosophila* genome, using the BLAST tool of UCSC Genome Browser (https://genome.ucsc.edu/cgi-bin/hgBlat?command=start). All primers used in this project are listed in Table 2.3.

Table 2.3. Primers used in this work.

Target	5'-3' Sequence
elF2g Fw	GCCATATCCGGCGTGCAGACTG
elF2g Rv	CATCCGATACGAAGCTGGCCGG
Su(var)3-9 Fw	CACGGCAAATCCACGGTGGTCA
Su(var)3-9 Rv	CTGTCGCTGCTGCTTGGAGGTC
BRP Fw	CTACCGATGCCGATACCAATAC
BRP Rv	GTGTGTGTCCCTGTGTTTATCT
Actin5C	CCGACCGTATGCAGAAGGAG
Actin5C	TGGAAGGTGGACAGCGAAG

The SYBR Green dye (Applied Biosystems #4385612) was used in order to detect the amplification of cDNA in real-time. SYBR Green can, in fact, bind double-strand DNA, emitting a fluorescent signal. Each qPCR reaction was made with the following mix: 10ul of SYBR Green mix buffer, 0.4ul of 5uM primer Fw, 0.4ul of 5uM primer Rv, 2ul of cDNA, and 7.2 ul of H20. All reactions were performed in three technical replicates, including one reaction with no cDNA, as a negative control to evaluate contamination. At least two biological replicates were performed per experiment. Thermal cycling conditions are shown in Table 2.4.

Cycle threshold (Ct) values were generated for each reaction. These values reflect the number of cycles that are necessary for the fluorescent signal to exceed the baseline threshold, usually set to zero, and they are inversely proportional to the original

Table 2.4. Thermal cycling conditions used in this work.

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

expression level of the target gene: greater Ct level (above 36 cycles) corresponds to lower amount of target DNA in the sample. Data from each qPCR reaction were then collected and analyzed in RStudio. The "Delta-Delta Ct" method was used as a normalization method, where Ct values of target genes were compared to those of reference/housekeeping genes (actin, in this specific study) whose expression levels are not supposed to change between different conditions. Table 2.5 shows the main steps of the calculation applied in this study, for comparisons between experimental and control conditions. Pairwise t-tests were applied to evaluate statistical differences.

Table 2.5. ΔΔCT calculation.

Step N.	Formula
1	ΔCT = CT(target gene)−CT(reference gene)
2	ΔΔCT = ΔCT(experimental sample)−ΔCT(control sample)
3	2^- (ΔΔCT)

2.9.4 RNA seq analysis

RNA samples were sent to GENEWIZ for library preparation and Illumina sequencing, where the following services were applied: 2x 150bp sequencing and 10M read pairs, per sample. Raw data in FASTQ format were collected and processed. After removing the adapters using TrimGalore!, reads were counted and mapped to the *Drosophila* genome, using Kallisto. Kallisto is a fast program for quantifying transcript abundances (Bray et al. 2016). It is based on a novel algorithm, that avoids the alignment step and allows the identification of transcripts which a read is compatible with. This process, called *pseudoalignment*, can be more accurate and quicker than other existing

quantification tools, which instead, are based on alignment counting. Differential expression of genes between experimental and control conditions was then analyzed in R, using the DEseq2 package. After adjusting for multiple comparisons, only genes with p-value < 0.05 and log2foldchange – or + 1.5, were considered downregulated or upregulated, respectively, in the experimental condition. Gene ontology (GO) was generated using the online tool WebGestalt.

Chapter 3

Results: the role of H3K9me in sleep

3.1 Sleep and histone modifications: a screening of potential new sleep- related genes

As already mentioned in section 1.3.3, several studies have already outlined the emerging correlation between histone modifications and sleep regulation. Besides the fundamental impact of some specific epigenetic marks, such as the acetylation, very little is known about the contribution of other types of histone modifications. In order to address this gap, I started this project by performing a screen of several epigenetic modifiers, with the goal to identify potential new candidates involved in sleep. The first aim was to analyze the baseline sleep upon knockdown of enzymes implicated in different types of histone modifications, to evaluate any differences with the appropriate parental controls (data not shown). The screen led me to finally focus the attention on a class of modifiers, implicated in the H3K9methylation. In *Drosophila* there are three main H3K9methyltranferases: Su(var)3-9, G9a, and Eggless, all expressed in different tissues and implicated mainly in the heterochromatin formation during critical developmental stages (see section 1.3.7 for more detail). Besides the crucial work of these enzymes during embryogenesis, recent studies highlighted the implication of a few of them during the adult stage, too. For example, G9a was shown to affect learning formation, foraging, and courtship behavior, upon depletion (Anreiter, Kramer, and Sokolowski 2017; Kramer et al. 2011). However, the over-expression of G9a in G9a-mutant flies was sufficient to rescue any cognitive defect. This is one piece of evidence that encouraged me to hypothesize that H3K9me could still modulate the chromatin state in the adult brain, influencing adult behaviors, possibly including sleep.

I took advantage of the *GAL4-UAS* system to trigger the knockdown of all three H3K9 methylases specifically in the nervous system, to avoid unspecific effects given their ubiquitous expression. Therefore, I proceeded to generate genetic crosses, including parental controls, made by crossing *Canton-S* flies, the wild-type strain mainly used in

my lab, with the reciprocal *UAS* or *GAL4* lines. More specifically, three sets of crosses were generated per each candidate: 1) UAS-*RNAi x GAL4*; 2) *CS x GAL4*; 3) *CS x* UAS-*RNAi*. When possible, the behavior of both female and male offspring was recorded by the ethoscopes. Among the three enzymes, Su(var)3-9 appeared to be the main key player in the context of sleep regulation.

3.2 Su(var)3-9 and sleep investigation

3.2.1 Su(var)3-9 and baseline sleep

Su(var)3-9 is one of the most important H3K9methyltranferases in *Drosophila*. It acts as a suppressor of the *PEV* (a phenomenon of inactivation of euchromatic genes after being relocated very close or within a sequence of heterochromatin) and it is responsible for the deposition of the H3K9me marker on heterochromatin regions and also specific euchromatin genes (for more info see section 1.3.7). After knocking down Su(var)3-9 pan-neuronally, using nSyn-GAL4 as a driver, I observed an increase of the baseline sleep in the *RNAi* flies, when compared to the parental controls, showing, interestingly, an evident difference among the two sexes. Indeed, as shown in Figure 3.1, knocked down (KD) female flies experienced a raising of sleep amount during both dark and light phases. Male KD flies, instead, slept more specifically during the day upon the transition from light to dark. Statistical comparisons can be found in more detail, in Table 3.1.

I then looked at the sleep architecture, to evaluate any changes in the quality of the sleep. I found that, independently from sex, the increase in baseline sleep was given only by longer sleep episodes. Sleep bout numbers in the *RNAi* were similar to the control, suggesting that the increased sleep in the KD was not fragmented, but in contrast, very consolidated, perhaps reflecting a deep sleep state (Figure 3.2 and Table 3.2 for statistical comparisons). These results encouraged me to proceed on a deeper investigation of Su(var)3-9, to identify any potential role in the context of the circadian and homeostatic sleep control.

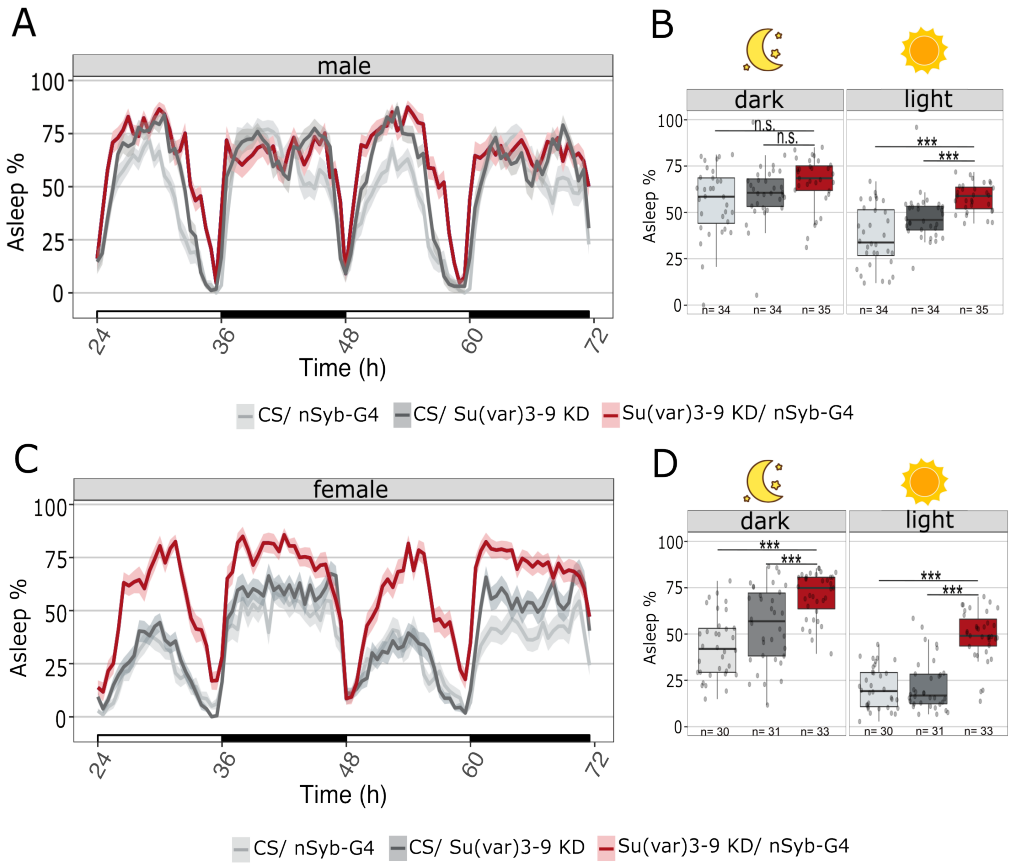


Figure 3.1. Su(var)3-9 *RNAi* flies sleep more than controls.
A,C. Baseline sleep profiles of *RNAi* (red line) and parental control flies (grey lines), between day 1 and day 3 (24h-72h), in males and females, respectively. Su(var)3-9 RNAi/ nSyb GAL4 flies have shown an increase in sleep, compared to nSyb GAL4/CS (+) and Su(var)3-9 RNAi/CS (+) flies. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **B,D.** Quantification of sleep amounts, where light and dark phases sleep are measured separately. Statistically significant changes between *RNAi* and controls are shown during daytime in males and during both light and dark phases in females. In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s. $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from three replicates and the *N* of the animals per each group is reported within the figure.

Table 3.1. Statistical differences in sleep mean between Su(var)3-9 *RNAi* flies and controls.

female, light phase			**male, light phase**		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/nSyb-G4	Su(var)3-9 KD/nSyb-G4	6.4e-12	CS/nSyb-G4	Su(var)3-9 KD/nSyb-G4	7.6e-08
CS/Su(var)3-9 KD	Su(var)3-9 KD/nSyb-G4	9.8e-10	CS/Su(var)3-9 KD	Su(var)3-9 KD/nSyb-G4	5.0e-05
female, dark phase			**male, dark phase**		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/nSyb-G4	Su(var)3-9 KD/nSyb-G4	1.3e-09	CS/nSyb-G4	Su(var)3-9 KD/nSyb-G4	0.14393
CS/Su(var)3-9 KD	Su(var)3-9 KD/nSyb-G4	0.00105	CS/Su(var)3-9 KD	Su(var)3-9 KD/nSyb-G4	0.06349

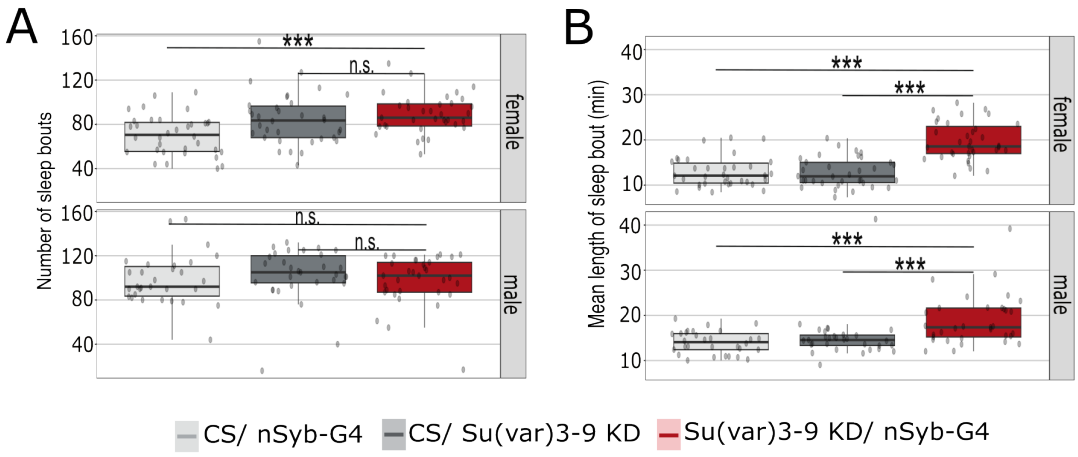


Figure 3.2. Su(Var)3-9 *RNAi* flies show longer sleep episodes.
Quantification of the number **(A)** and the length **(B)** of sleep bouts in Su(var)3-9 *RNAi* (red boxplot) and control flies (grey boxplots), where females and males are measured separately. Statistically significant differences are shown only in B, where *RNAi* flies exhibit longer sleep bouts than controls. No significative differences were detected in the number of sleep episodes. In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s. $p>0.05$, from pairwise Wilcoxon rank sum test.

Table 3.2. Statistical differences in sleep parameters between Su(var)3-9 *RNAi* flies and controls.

female, number of sleep bouts			**male, number of sleep bouts**		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/nSyb-G4	Su(var)3-9 KD/nSyb-G4	0.00038	CS/nSyb-G4	Su(var)3-9 KD/nSyb-G4	0.45301
CS/Su(var)3-9 KD	Su(var)3-9 KD/nSyb-G4	0.23647	CS/Su(var)3-9 KD	Su(var)3-9 KD/nSyb-G4	0.22997
female, mean length			**male, mean length**		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/nSyb-G4	Su(var)3-9 KD/nSyb-G4	2.4e-09	CS/nSyb-G4	Su(var)3-9 KD/nSyb-G4	5.9e-05
CS/Su(var)3-9 KD	Su(var)3-9 KD/nSyb-G4	2.4e-09	CS/Su(var)3-9 KD	Su(var)3-9 KD/nSyb-G4	0.00027

3.2.2 Su(var)3-9 and the circadian sleep

In *Drosophila*, the circadian neurons play an essential role in determining the locomotor activity program (Shang, Griffith, and Rosbash 2008; Cavanaugh et al. 2014), characterized by peaks of activity during dawn and dusk, almost before the light transitions. As shown in the activity profile of Figure 3.3A, Su(var)3-9 KD flies preserved normal morning and evening peaks, suggesting that the clock drive might be not impaired from the knockdown. To further investigate this aspect, I changed the light conditions and placed the *RNAi* flies, with the parental controls, in constant darkness (DD). In these conditions, wild-type flies are known to show weaker morning peaks but persist evening peaks, reoccurring approximately every 23.8h. Clock mutants such as *period*, on the other

hand, are normally shown to be completely arrhythmic in DD (Grima et al. 2004; Stoleru et al. 2004). In the actogram from Figure 3.3B, flies were left for 2 days in LD (12 h light:12 h dark) and the following 15 days in DD cycle (24 h dark). For the whole length of the experiment, the knock-down of Su(var)3-9 did not cause any change in the rhythm of the sleep behavior, besides the lower power shown in Figure 3.3C, given by the general higher amount of sleep in the *RNAi* flies. Finally, to exclude the lack of role of

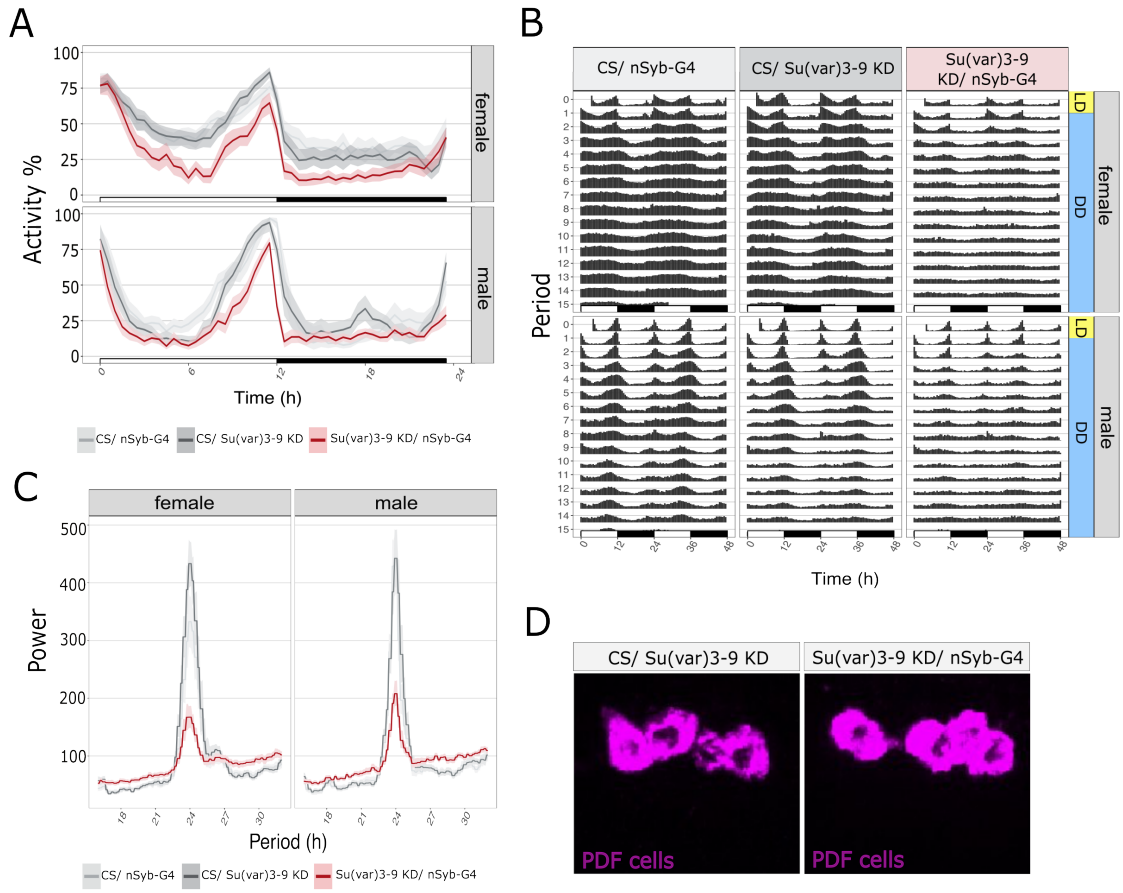


Figure 3.3. Su(var)3-9 is not involved in circadian period.
A. Su(var)3-9 *RNAi* flies show normal activity peaks during light transitions (lights-on morning and lights-off transition). Female and male activities are shown separately. **B.** Representative actograms in Su(var)3-9 *RNAi* flies and parental controls, in DD condition: any evidence of arrhythmicity was detected, for both female and male flies. **C.** Power analysis of the circadian period of activity in DD in all the genotypes. **D.** Staining of the PDF-expressing neurons, showing no morphological abnormalities in the *RNAi* flies, compared to controls.

Su(var)3-9 in the clock neurons, I decided to trigger the knock-down specifically in the PDF neurons, known to regulate the circadian rhythm (see section 1.1.4). The knockdown of Su(var)3-9 in this class of cells did not impair the behavior of the flies (this will be better discussed in section 3.3.1). Furthermore, I dissected adult brains from *RNAi* and control flies and stained brain tissues with PDF-antibody. As shown in Figure 3.3D, the knockdown did not lead to changes in the anatomical morphology of the PDF cells. All

these results taken together, suggested that Su(var)3-9 is not implicated in the circadian drive for sleep regulation.

While performing DD experiments, I noticed, however, that several Su(var)3-9 KD flies died by the end of the recordings. Therefore, I also decided to monitor their lifespan over a longer period, such as 30 days. In order to do so, I placed groups of 10 flies in distinct vials and created three different conditions: females, males, and mixed-sex groups. I generated the same starting number of vials for both Su(var)3-9 KD and the parental control CS/Su(var)3-9 and manually counted the survivors every week over a month. As

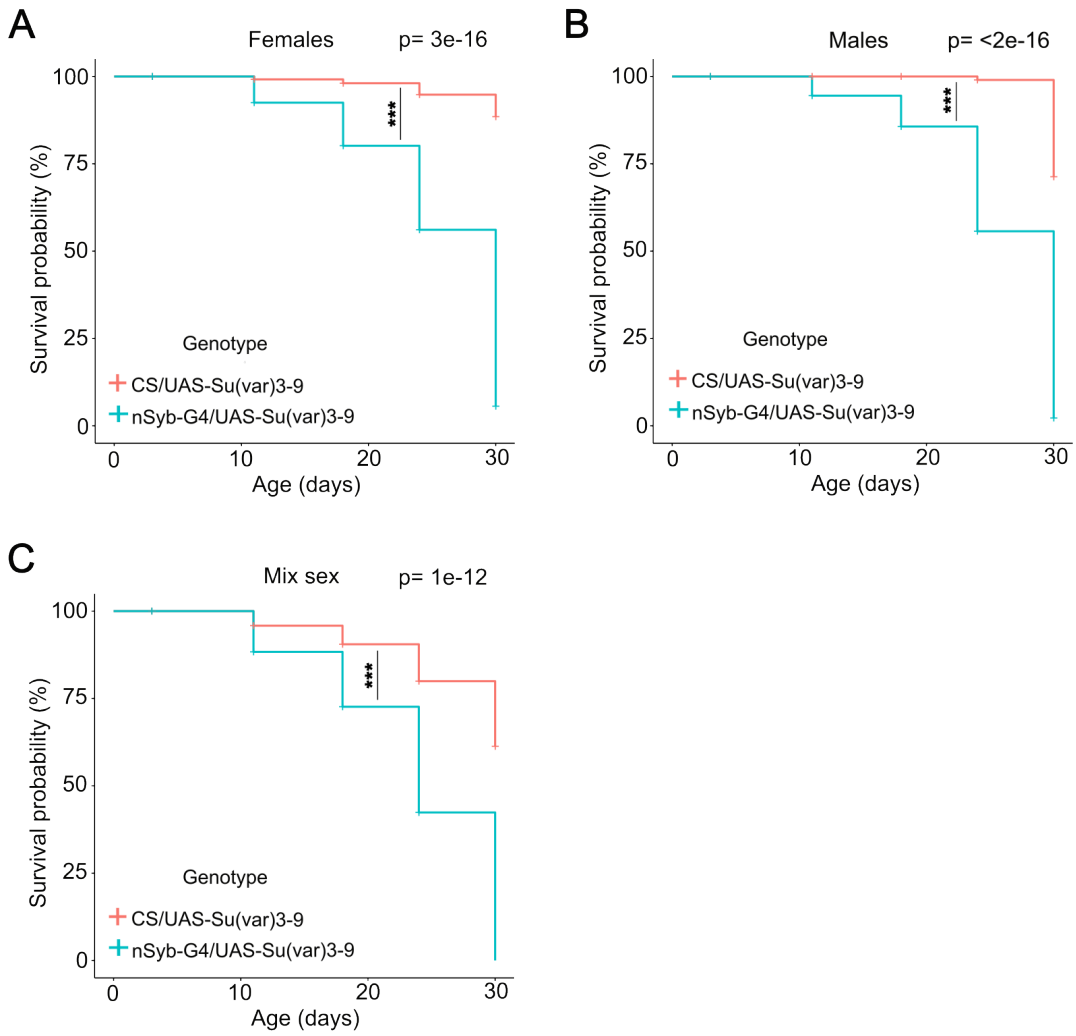


Figure 3.4. The pan-neuronal knockdown of Su(var)3-9 reduced lifespan in aged flies.
Lifespan of Su(var)3-9 knocked-down flies (light-blue line) and parental controls (red line), over three conditions: female **(A)**, male **(B)**, and mixed-sex groups **(C).** Y-axis represents the survival probability, X-axis is the age of the flies, over a month. Long-Rank tests were performed for statistical comparisons between conditions, where * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$.

shown in Figure 3.4, in all three conditions, *RNAi* flies kept a high survival probability until age 10 days: however, after that, they exhibited a very drastic reduction of survivors, more concentrated between age 25 and 30, at the end of which only a tiny number of individuals survived. On the other hand, control flies showed, overall, a high survival trend in all the conditions. These data suggest that pan-neuronal knockdown of Su(var)3-9 has a significant impact on the longevity of the flies. As already mentioned, Su(var)3-9 is known to be involved in heterochromatin formation and transcriptional silencing, and its knockdown may lead to dysregulation of gene expression patterns critical for maintaining cellular homeostasis and mitigating the effects of aging. The drastic reduction in survival probability observed in Su(var)3-9 KD flies indicates a significant physiological challenge that the insects face, likely due to increased genomic instability, altered epigenetic regulation, or impaired stress response pathways. This finding may have important implications for the interpretation of results obtained in studies involving Su(var)3-9 knockdown, discussed in the next paragraphs. In particular, it highlights the importance of employing appropriate controls and experimental designs to accurately discern the specific effects of Su(var)3-9 manipulation from nonspecific stress-related effects on experimental outcomes. The content of paragraph 3.5 will be fundamental to further give potential insights into the correlation between sleep and the H3K9methylation, without passing through the knock-down of Su(var)3-9.

3.2.3 Su(var)3-9 and the homeostatic sleep

From the previous section, I concluded that Su(var)3-9 does not play any role in the regulation of the circadian clock. On the other hand, another fundamental mechanism implicated in sleep is the homeostatic drive, which determines “how long” and “how deep” individuals need to sleep (sleep pressure). In order to assess whether Su(var)3-9 *RNAi* flies are differently sensitive to sleep pressure, I sleep-deprived flies for 1 day and measured their ability to rebound on the day following the manipulation. To achieve that, I used different approaches, already known from the literature to disturb sleep in different ways: mechanical sleep deprivation, starvation, and male-male interaction.

Starting from the sleep deprivation (SD) by dynamic, I combined the ethoscope with a further device called optomotor: thanks to this module, a glass tube was rotated every time the fly housed inside, was detected from the camera to be immobile for at least 2

min. After 24h of SD, I allowed flies to recover and sleep, undisturbed from any manipulation. During this day, I measured the rebound sleep in the first 3 hours of the early morning (ZT0-ZT3). As shown in Figure 3.5B, this SD regime successfully disrupted sleep across all the genotypes. However, only parental controls were able to compensate for the loss of sleep by rebounding on the day of recovery: Su(var)3-9 *RNAi* flies, instead, failed to show any increase in sleep. This was evident in both female and male flies, as shown in Figures 3.5A and C. Statistical comparisons can be found in more detail, in Table 3.3.

I proceeded then with the other SD methods. In order to enhance starvation, on the day of the actual manipulation, I transferred flies into tubes containing only agar and water and waited for 24 hours. Over this time, wild-type flies are normally known to increase their locomotor activity, becoming hyperactive and suppressing sleep (Yang et al. 2015; Yu et al., 2016.). Furthermore, they show rebound sleep, after being reintroduced into normal food: for instance, this is very consistent in female flies, particularly virgins but not mated, as extensively shown by a previous PhD student in the Gilestro Lab, Hannah Jones (H. Jones 2022). Therefore, for this experiment, I decided to subject to starvation only virgin females. As predicted, all the genotypes exhibited a lower amount of sleep during starvation, although not very pronounced in the parental controls. However, on the recovery day, when flies were transferred back into normal food before the light transition, all the genotypes, including Su(var)3-9 *RNAi* flies, showed an increase in rebound sleep during the first three hours of the morning, as shown in Figure 3.6A-C and Table 3.4. A similar result was achieved with the third sleep-suppression method: the male-male interaction, in socially naive animals. *Drosophila* males are known to reveal agonistic and aggressive behavior, when encountering other males, with the consequent result of reducing the amount of sleep (Beckwith and French 2019). On the day following the sleep deprivation, all the genotypes again displayed a clear increase in sleep (Figure 3.6B-D and Table 3.4).

Putting these results together, the knockdown of Su(var)3-9 seems to affect specifically only the rebound related to dynamic SD and not the ones related to starvation or MM interaction. Based on recent data collected in the Gilestro laboratory, we have hypothesized a theory where the rebound in relation to starvation and MM may be driven by other forms of stress and not just the actual lack of sleep. Given the assumption that Su(var)3-9 plays a role specifically in sleep, this may explain the increase of sleep upon

starvation or MM in the *RNAi* flies, where other mechanisms may play a key role, in order to enhance the rebound.

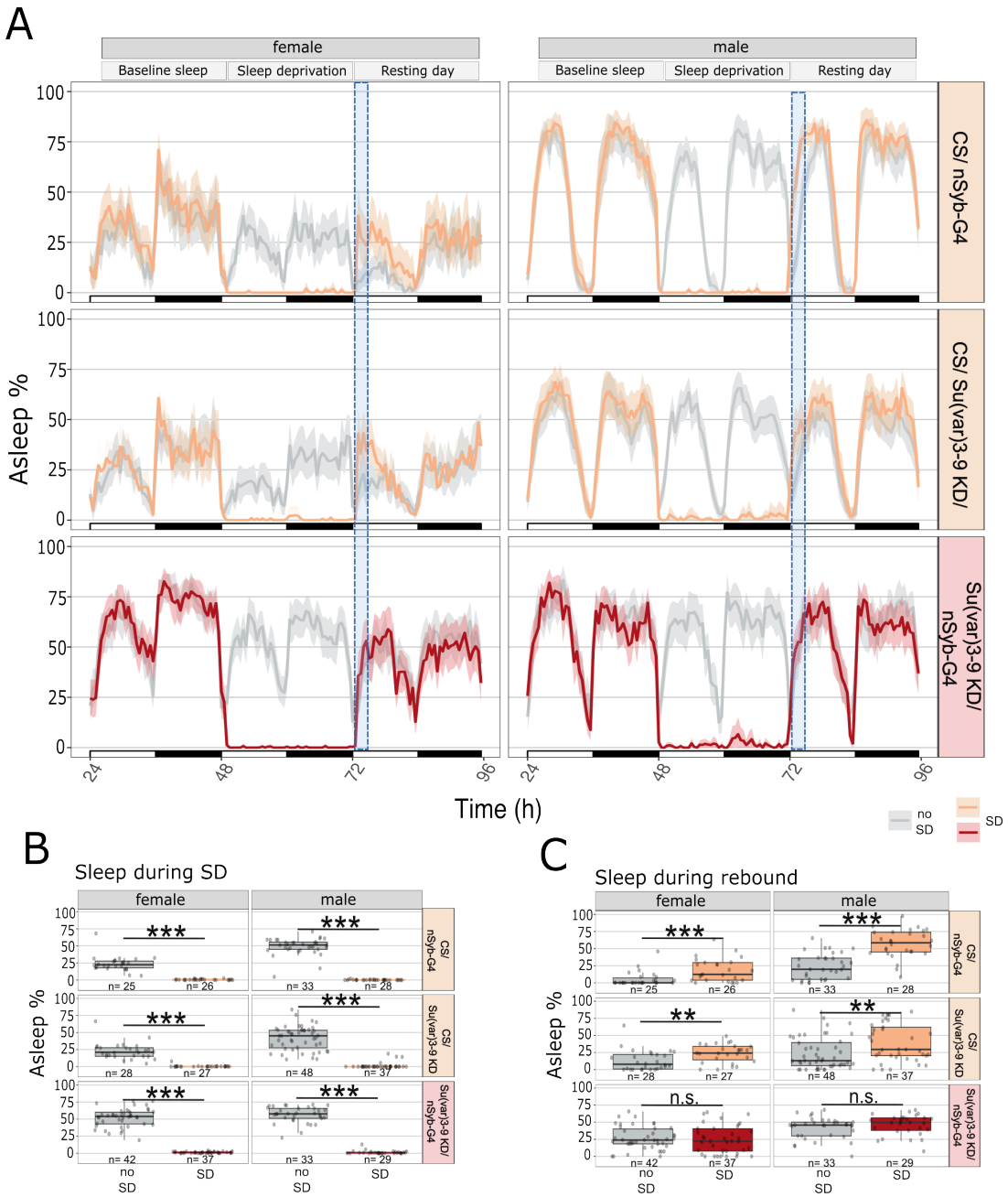


Figure 3.5. Su(var)3-9 *RNAi* flies do not show rebound sleep upon dynamic SD.
A. Ethogram over three days in a row, where mechanical sleep deprivation occurs on the second day. Per each genotype, two groups of flies are shown: undisturbed control group (grey line) and sleep-deprived group (orange lines for parental controls and red lines for *RNAi* flies). As expected, SD groups from both genetic controls exhibit higher sleep amounts on the early morning of recovery day (third day, highlighted in blue). Su(var)3-9 KD flies, instead, do not show rebound sleep. Females and males from each genotype are measured separately. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **B**. Quantification of sleep during mechanical sleep deprivation, which reduced sleep efficiently across all the genotypes. **C**. Quantification of rebound sleep during the first 3 hours of the early morning, following SD manipulation. Statistically significant changes in sleep rebound have been found in the two genetic controls, after comparing SD to no SD groups. In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from three replicates and the *N* of the animals per each group is reported within the figure.

Table 3.3. Statistical differences between SD and no SD flies in sleep during SD and rebound, from Su(var)3-9 *RNAi* flies and controls.

female, sleep during SD			**male, sleep during SD**		
no SD	SD	p-value	no SD	SD	p-value
CS/nSyb-G4	CS/nSyb-G4	1.6e-09	CS/nSyb-G4	CS/nSyb-G4	2.1e-11
CS/Su(var)3-9 KD	CS/Su(var)3-9 KD	3.3e-10	CS/Su(var)3-9 KD	CS/Su(var)3-9 KD	8.9e-14
Su(var)3-9 KD/nSyb-G4	Su(var)3-9 KD/nSyb-G4	3.9e-13	Su(var)3-9 KD/nSyb-G4	Su(var)3-9 KD/nSyb-G4	2.7e-11
female, sleep during rebound			**male, sleep during rebound**		
no SD	SD	p-value	no SD	SD	p-value
CS/nSyb-G4	CS/nSyb-G4	0.00106	CS/nSyb-G4	CS/nSyb-G4	2.1e-06
CS/Su(var)3-9 KD	CS/Su(var)3-9 KD	0.00380	CS/Su(var)3-9 KD	CS/Su(var)3-9 KD	0.00925
Su(var)3-9 KD/nSyb-G4	Su(var)3-9 KD/nSyb-G4	0.60478	Su(var)3-9 KD/nSyb-G4	Su(var)3-9 KD/nSyb-G4	0.18666

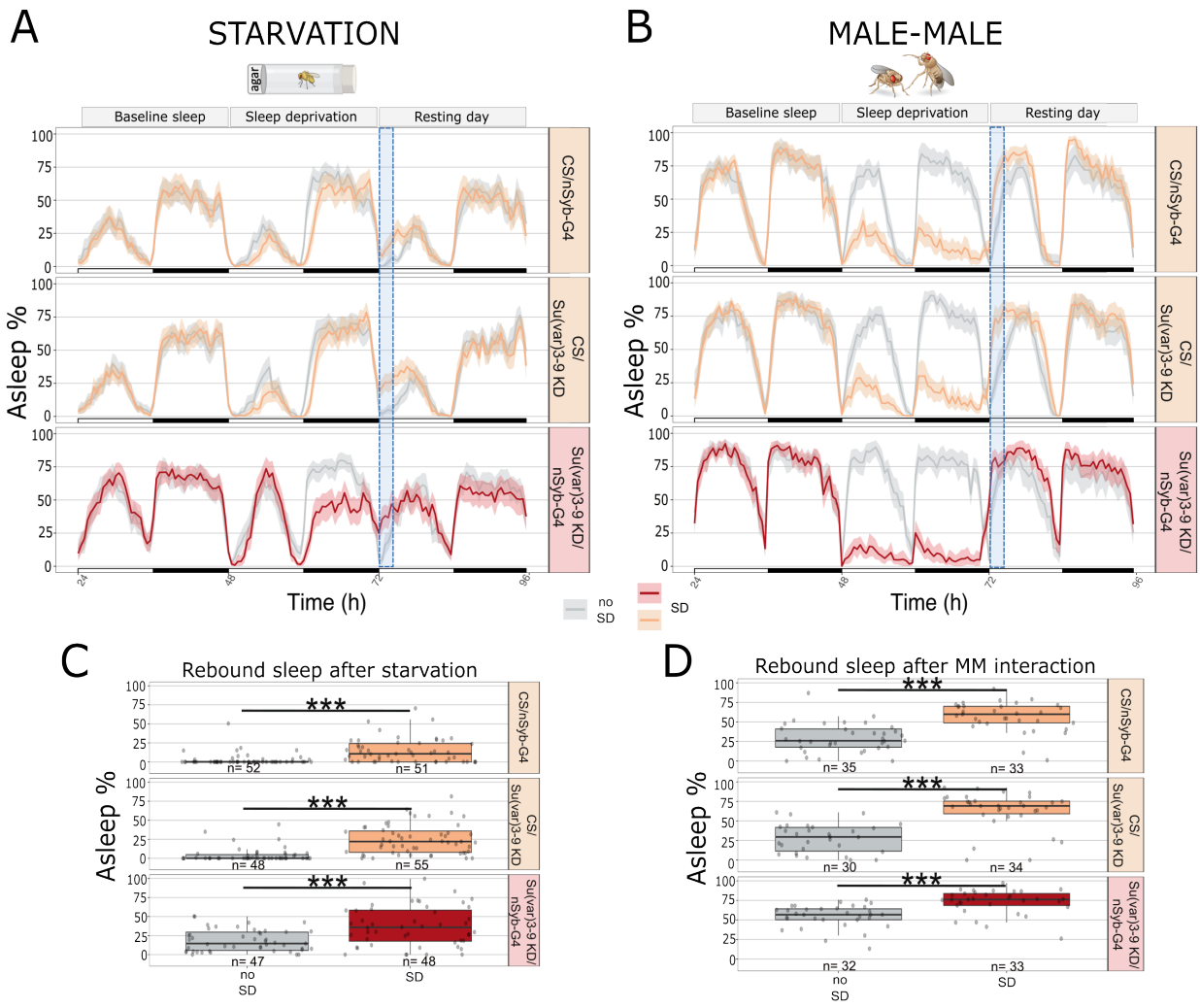


Figure 3.6. Su(var)3-9 *RNAi* flies do show rebound sleep upon starvation and male-male interaction. **A, B.** Ethograms over three days in a row, where sleep deprivation due to, respectively, starvation (A) or MM interaction (B), occurs on the second day. Per each genotype, two groups of flies are shown: undisturbed control group (grey line) and sleep-deprived group (orange lines for parental controls and red lines for *RNAi* flies). SD groups from all the genotypes, including Su(var)3-9 KD, exhibit higher sleep amounts on the early morning of resting day (third day, highlighted in blue). Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **C, D.** Quantification of rebound sleep during the first 3 hours of the early morning, following starvation (C) and MM interaction (D), respectively. Statistically significant changes in sleep rebound have been found in all the genotypes, after comparing SD to no SD groups. In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from three replicates and the *N* of the animals per each group is reported within the figure.

Table 3.4. Statistical differences between SD and no SD flies in rebound sleep upon starvation and MM, from Su(var)3-9 RNAi flies and controls.

female, sleep during rebound (STARVATION)			**male, sleep during rebound (MALE-MALE)**		
no SD	SD	p-value	no SD	SD	p-value
CS/nSyb-G4	CS/nSyb-G4	5.3e-07	CS/nSyb-G4	CS/nSyb-G4	7.0e-07
CS/Su(var)3-9 KD	CS/Su(var)3-9 KD	3.5e-10	CS/Su(var)3-9 KD	CS/Su(var)3-9 KD	7.0e-07
Su(var)3-9 KD/nSyb-G4	Su(var)3-9 KD/nSyb-G4	0.00045	Su(var)3-9 KD/nSyb-G4	Su(var)3-9 KD/nSyb-G4	5.3e-06

3.2.4 Verifying the Su(var)3-9 *RNAi* line #39378 BL

The knockdown of Su(var)3-9 affects the baseline and the homeostatic sleep in flies, suggesting a promising new epigenetic regulator of sleep. However, before proceeding with more analysis and experiments, I found it fundamental to test the fly line used so far, to exclude any unspecific effects, such as off-targets from the *RNAi*. After deep research from the literature, I found that Su(var)3-9 gene is normally fused with the locus of another gene, the *gamma subunit of translation initiation factor eIF2*. Indeed, the two genes share the first two exons and their protein synthesis is produced by alternative splicing. This is a unique gene structure, only found in *Drosophila* and other insects (Krauss and Reuter 2000). In humans, eIF-2 γ and SUV29H1 genes, are located in distant regions of the X chromosome (Ehrmann et al. 1998).

The *RNAi* line for Su(var)3-9 used in this study (#39378 BL) was produced by insertion of the transgenic transposable element 15258. As shown in Figure 3.7, this P element drives the expression of an inverted repeat, which targets the second exon of Su(var)3-9, also in common with all the eIF2γ isoforms. This may suggest that any effects given by using the line could be a combination of the knockdown of both Su(var)3-9 and eIF2γ.

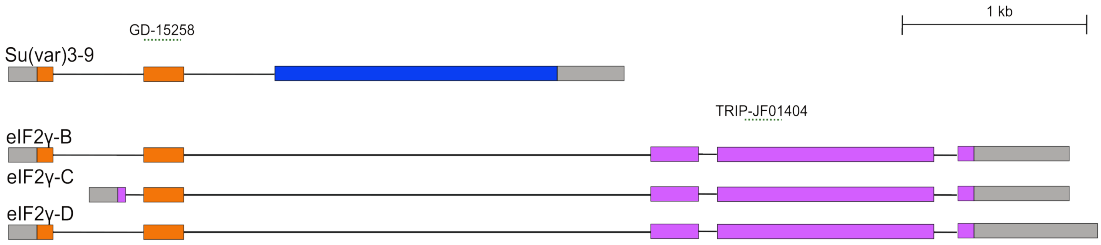


Figure 3.7. *Su(var)3-9* and *eIF2γ* locus genes share two exons.
Schematic illustration of the gene locus for *Su(var)3-9*, sharing the first two exons (in orange) with the *eIF2γ* gene: respective proteins are produced by alternative splicing. Two annotations are also reported: GD-15258 refers to the insertion of the P element (responsible for line #39378 BL, used in this project) on the second exon of Su(var)3-9 and eIF2γ isoforms. TRIP-JF01404 refers to the insertion of the P element (line #31619 BL) on exon 4 of eIF2γ.

Unfortunately, no *RNAi* lines specific for Su(var)3-9 only, are available: none of the inverted repeats already designed target the third exon, the only one specific for Su(var)3-9 and not included in eIF2γ. However, I decided to validate the accuracy of the collected data, by different approaches. First, I found available a TRIP *RNAi* line specific for eIF2γ

(#31619 BL, TRIP-JF01404): as reported in Figure 3.7, here the inverted repeat targets the exon 4, one of the exons this time specific for eIF2γ and not shared with Su(var)3-9. Therefore, the next step was to test this line, by setting up the crosses with nSyb-GAL4 and by subjecting the offspring to SD stimuli. As shown in Figure 3.8A-B, both parental controls and KD flies experienced rebound responses upon sleep deprivation: this was true for both female and male flies. Furthermore, the baseline sleep was also not affected by the *RNAi*, as reported in Figure 3.8C-D (see Table 3.5 for statistical comparisons). These data do not recapitulate what achieved so far with line #39378, suggesting that the specific knockdown of Su(var)3-9 is responsible for the increased baseline sleep and the depletion of the rebound.

After testing the *RNAi* line specific for eIF2γ, I next decided to validate the efficiency of the knockdown from the original line #39378, by monitoring the mRNA expression level in the *RNAi* flies and parental controls. I performed real-time qPCR in brains and bodies (no heads), to validate the specificity of the knockdown in the neuronal tissue. As shown in Figure 3.9B, no reduction of Su(var)3-9 or eIF2γ was detected in the body of any genotypes, as expected. On the other hand, in the brains of the KD flies, a reduced mRNA level of Su(var)3-9, but not eIF2γ, was finally measured, confirming again, that the specific knockdown of Su(var)3-9 and not eIF2γ, is responsible for the sleep phenotypes observed in the *RNAi* flies (Figure 3.9A).

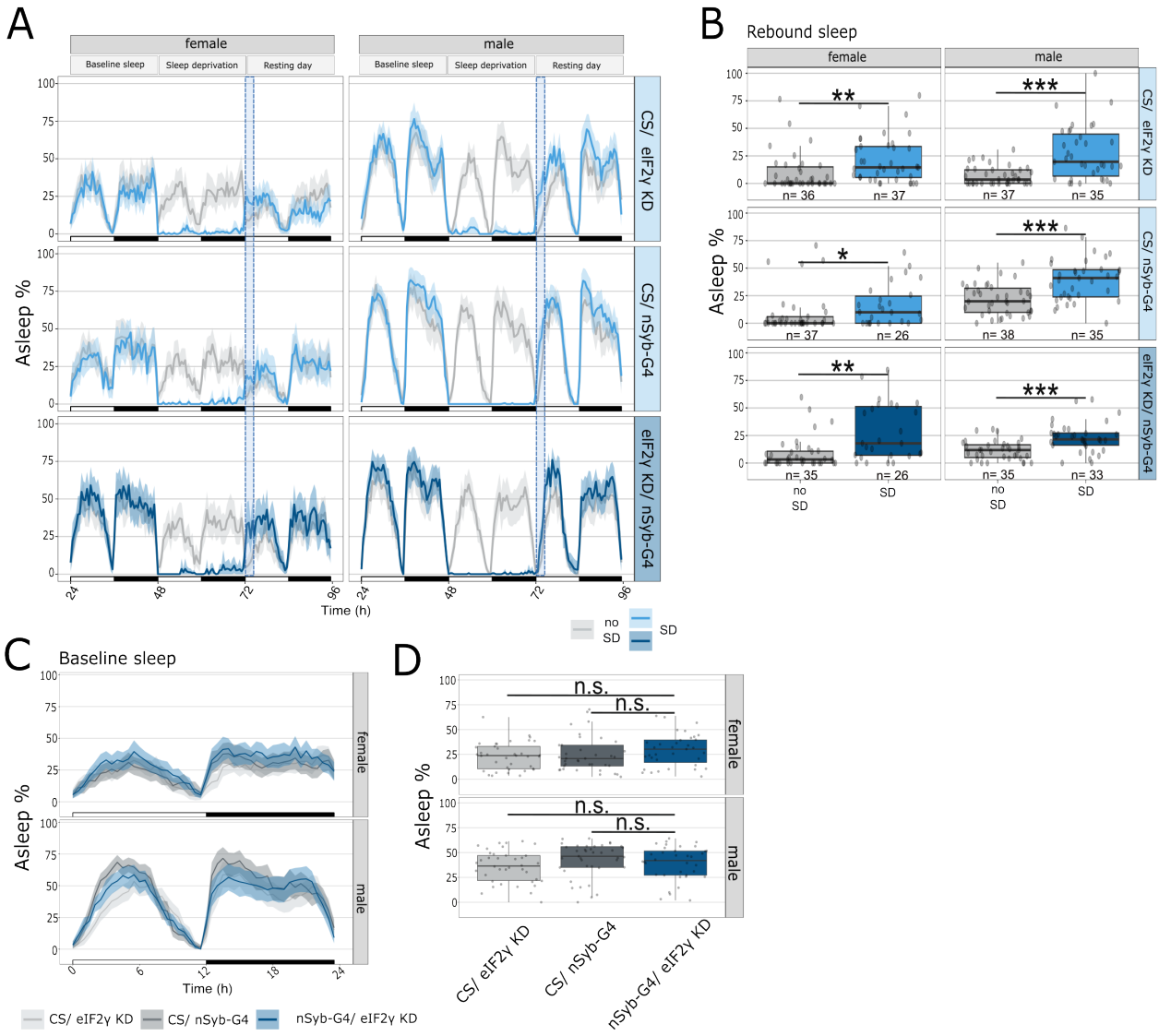


Figure 3.8. eIF2γ knockdown does not affect baseline and homeostatic sleep.
A. Ethogram over three days in a row, where sleep deprivation occurs on the second day. Per each genotype, two groups of flies are shown: undisturbed control group (grey line) and sleep-deprived group (light blue lines for parental controls and dark blue lines for *RNAi* flies). SD groups from all the genotypes, including eIF2γ KD, exhibit higher sleep amounts in the early morning of the resting day (third day, highlighted in blue). Female and male flies are analyzed separately. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **B.** Quantification of rebound sleep during the first 3 hours of the early morning, following SD. Statistically significant changes in sleep rebound have been found in all the genotypes, after comparing SD to no SD groups. **C.** Ethogram wrap (average) of three days of baseline sleep in no SD flies. Parental controls are shown in grey lines, while KD is in blue. **D.** Quantification of the total baseline sleep, showing no differences between genotypes. In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from two replicates and the *N* of the animals per each group is reported within the figure.

Table 3.5. Statistical differences between SD and no SD flies in rebound sleep and between genotypes in baseline sleep, from eIF2γ RNAi flies and controls.

female, sleep during rebound			male, sleep during rebound		
no SD	SD	p-value	no SD	SD	p-value
CS/nSyb-G4	CS/nSyb-G4	0.04621	CS/nSyb-G4	CS/nSyb-G4	0.00039
CS/ eIF2γ KD	CS/ eIF2γ KD	0.00391	CS/ eIF2γ KD	CS/ eIF2γ KD	0.00012
eIF2γ KD/nSyb-G4	eIF2γ KD/nSyb-G4	0.00207	eIF2γ KD/nSyb-G4	eIF2γ KD/nSyb-G4	0.00024
female, baseline sleep over three days			**male, baseline sleep over three days**		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/nSyb-G4	eIF2γ KD/nSyb-G4	0.17582	CS/nSyb-G4	eIF2γ KD/nSyb-G4	0.34758
CS/ eIF2γ KD	eIF2γ KD/nSyb-G4	0.17180	CS/ eIF2γ KD	eIF2γ KD/nSyb-G4	0.34858

After confirming that eIF2γ is not correlated to sleep regulation, I finally decided to further validate the knockdown of Su(var)3-9, by monitoring the level of the H3K9methylation. Indeed, as a regulator of this mark, I expected a reduction of the

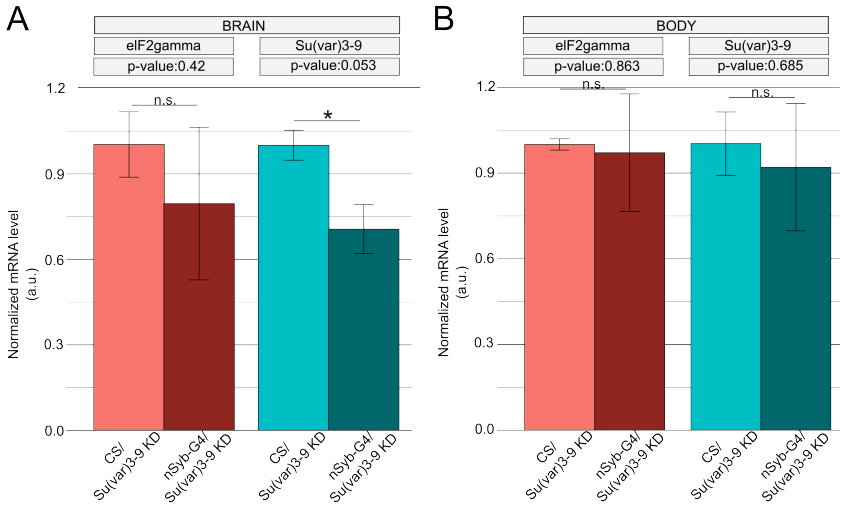


Figure 3.9. qPCR in adult brains and bodies upon knockdown of Su(var)3-9.
A. Reduced mRNA levels of Su(var)3-9, but not of eIF2γ, were measured in adult brains of Su(var)3-9 KD/nSyb-G4 flies (dark red and blue bars), compared to the parental control CS/Su(var)3-9 KD (light red and blue bars). Any change was, instead, detected in the body of the same flies (**B**). Data shown is from two biological replicates.

H3K9me signal, upon *RNAi*. With the aim to investigate so, I dissected adult brains from the following genotypes: 1) nSyb-G4/Su(var)3-9 *RNAi*, 2) CS/Su(var)3-9 *RNAi*. I then proceeded by performing immunostaining, using an antibody anti-H3K9me. As visible in Figure 3.10, focusing on the front part of the brain where most of the cell bodies are localized, the knockdown of Su(var)3-9 drops the deposition of the H3K9me in the neuronal cell body nuclei, confirming that the *RNAi* line is well-working.

All these data, taken together, suggested a potential specificity of line #39378, thus promoting Su(var)3-9 as a new epigenetic regulator of sleep.

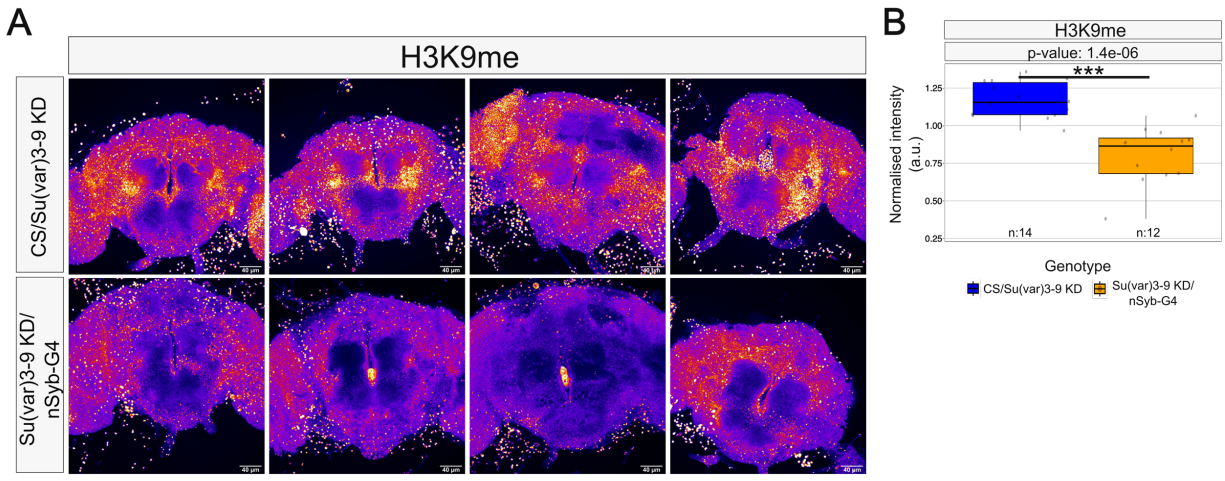


Figure 3.10. The knockdown of Su(var)3-9 decreases the H3K9me level in adult brains.
A. Confocal projection of adult front brains stained with ab anti-H3K9me, in Su(var)3-9 KD/nSyb-G4 and CS/Su(var)3-9 KD flies. A reduced signal was detected in the cell nuclei, upon knockdown. **B.** Quantification of the normalized intensity, where *** indicates $p<0.0001$, from pairwise Wilcoxon rank sum test.

3.2.5 Is Su(var)3-9 required for sleep specifically in the adult stage?

Su(var)3-9 is known to play a critical role during developmental stages. Therefore, I asked whether the effects in sleep due to the knockdown of Su(var)3-9, described in sections 3.2.1 and 3.2.3, are related to impairments during the development or adult phase. In order to investigate so, I took advantage of the GeneSwitch system, a slightly different version of the UAS-GAL4 system, where the UAS sequences are recognized and regulated by a chimeric GAL4 protein (GAL4-progesterone-receptor fusion protein), which becomes active only in the presence of the steroid RU486 (mifepristone), usually added to food. With the aim to enhance the activation of Su(var)3-9 knockdown in all neurons specifically in the adult stage, I exploited the nSyb-GeneSwitch as a GAL4 line: all the crosses were placed and raised in normal food. Only after hatching, adult flies were then transferred into food containing RU486 at 500 uM, for the experiment recording: this will promote the knockdown specifically during adulthood. Furthermore, appropriate controls were also made by exposing flies, with the same genotypes, to food with only DMSO, the solvent used to dissolve RU486. This condition was expected to not enhance the *RNAi*,

as the GAL4-Geneswitch protein is not able to bind the UAS sequences, without the presentence of its substrate.

In order to highlight any effect on sleep homeostasis, I subjected flies to dynamic sleep deprivation, combining again the ethoscopes with the optomotors. As shown in Figure

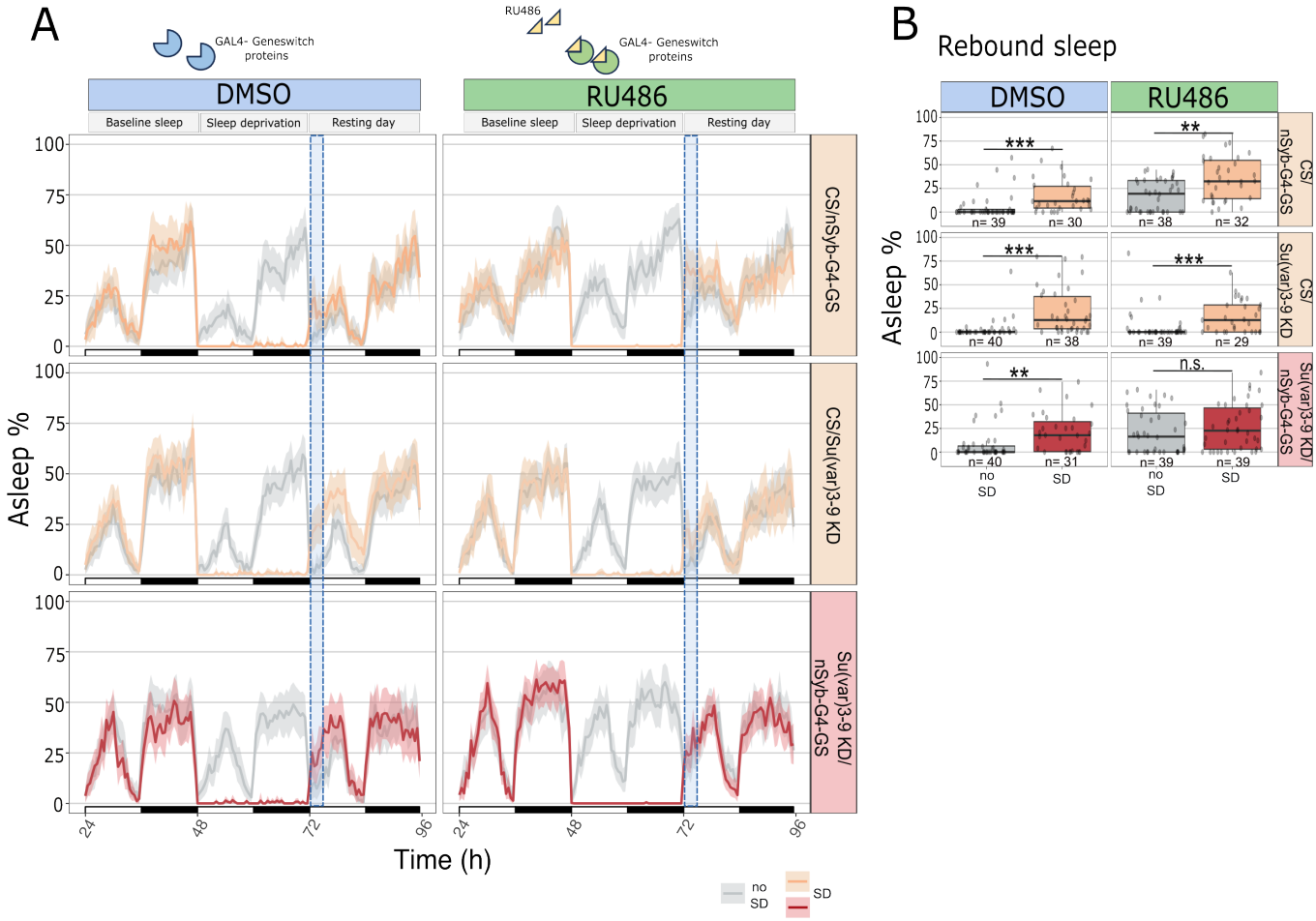


Figure 3.11. Su(var)3-9 plays a role in homeostatic sleep regulation during the adult stage.
A. Ethogram over three days in a row, where sleep deprivation occurs on the second day. The same experiment was performed in two different food conditions: DMSO only and RU486. Per each genotype, two groups of flies are shown: undisturbed control group (grey line) and sleep-deprived group (orange lines for parental controls and red lines for *RNAi* flies). On DMSO condition, SD groups from all the genotypes, including Su(var)3-9 KD, exhibit higher sleep amounts in the early morning of resting day (third day, highlighted in blue). On RU486 condition, instead, Su(var)3-9 KD flies do not show any rebound. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **B.** Quantification of rebound sleep during the first 3 hours of the early morning, following SD. In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from two replicates and the *N* of the animals per each group is reported within the figure.

Table 3.6. Statistical differences between SD and no SD flies in rebound sleep, upon knockdown of Su(var)3-9 in adult stage.

DMSO, sleep during rebound			RU486, sleep during rebound		
no SD	SD	p-value	no SD	SD	p-value
CS/nSyb-G4-GS	CS/nSyb-G4-GS	0.00010	CS/nSyb-G4-GS	CS/nSyb-G4-GS	0.00199
CS/ Su(var)3-9 KD	CS/ Su(var)3-9 KD	1.9e-06	CS/ Su(var)3-9 KD	CS/ Su(var)3-9 KD	0.00064
Su(var)3-9 KD /nSyb-G4-GS	Su(var)3-9 KD /nSyb-G4-GS	0.00392	Su(var)3-9 KD/nSyb-G4-GS	Su(var)3-9 KD/nSyb-G4-GS	0.39778

3.11, in the condition of food containing DMSO only, all the genotypes experienced rebound sleep on the day following the SD. This was expected, even for the Su(var)3-9 RNAi/nSyb-G4-GS flies: indeed, with no presence of RU486, GAL4 was not activated, and the knockdown of Su(var)3-9 was not actually enhanced in this specific condition. However, very interestingly, when adding RU486 to the food, only the parental controls still showed a rebound after SD. Su(var)3-9 KD flies, instead, did not compensate for any loss of sleep during the resting day (see Table 3.6 for statistical comparisons). These results recapitulate the same data collected with the normal pan-neuronal driver nSyb-GAL4, suggesting that Su(var)3-9 is implicated in sleep homeostasis regulation, specifically during the adult stage.

3.2.6 Summary

- The pan-neuronal knockdown of Su(var)3-9 leads to increased baseline sleep in both male and female flies (more pronounced in the latter), compared to parental controls: this increase was characterized by longer sleep episodes, while sleep bout numbers remained similar to controls.
- Su(var)3-9 is not implicated in circadian regulation: KD flies exhibited normal morning and evening peaks and their rhythm was preserved in DD conditions. Staining in the PDF-expressing neuron did not show abnormalities in the morphology when comparing KD to control flies.
- Su(var)3-9 regulates the homeostatic sleep: upon one day of dynamic SD, both female and male KD flies failed to show the usual rebound response, observed instead in the parental controls. Normal sleep rebounds were detected when sleep-depriving flies by starvation or male-male interaction.

- The *RNAi* line used in this project to enhance the knockdown of Su(var)3-9, specifically reduces the mRNA level of Su(var)3-9 and the H3K9me level in the neuronal tissue, upon comparisons to controls.
- Su(var)3-9 is implicated in sleep homeostasis regulation, specifically during adult stage.

3.3 What cells in the adult brain require Su(var)3-9 for sleep regulation?

3.3.1 The effect of Su(var)3-9 knockdown on specific sets of neurons

In the previous sections, I showed that the pan-neuronal knockdown of Su(var)3-9 causes a general increase of the baseline sleep and abolishes the rebound response upon dynamic SD: the latter seems to depend on a specific role of Su(var)3-9 during the adult stage. The next step was then to investigate these behaviors, by performing a GAL4 screen, in order to identify more precise sets of neurons which could recapitulate what was achieved so far by knocking down Su(var)3-9 in the whole neuronal brain tissue. From the list of

Table 3.7. List of drivers with lethal consequences.

Driver	Notes
R93F07-G4	Pupal lethality. Few flies emerged and died immediately
GH298-G4	Larval lethality
REPO-G4	Larval lethality
PLE-G4	Larval lethality
R69F08-G4	Larval lethality
R58H05-G4	Adult-semi lethality
R24C12-G4	Adult-semi lethality

GAL4 dissected over the screen, I necessarily had to exclude the following drivers, due to the absence of viable adult flies upon crossing with the Su(var)3-9 *RNAi* line: R93F07-GAL4 (expressed in the ellipsoid and fan-shaped body), GH298-GAL4 (antennal lobe, olfactory neurons, GABAergic neurons), REPO-GAL4 (glial cells), Ple-GAL4 (dopaminergic neurons), R69F08-GAL4 (ellipsoid body), R58H05-GAL4 (ellipsoid body and bulb) and R24C12-GAL4 (antennal lobe). More precise details about their lethal phenotypes are shown in Table 3.7.

Other GAL4 lines were also tested: these lines were viable upon crossing with Su(var)3-9 and, therefore, their behavior was recorded and analyzed. The lines in question are: Asta-GAL4 (AstA peptide -expressing neurons), R52B10-GAL4 (protocerebral bridge), PDF-GAL4 (PDF peptide -expressing neurons), sNPFR-GAL4 (short neuropeptide F receptor -expressing neurons) and TDC2-GAL4 (octopaminergic neurons). Appropriate crosses were generated, and the offspring were subjected to dynamic SD.

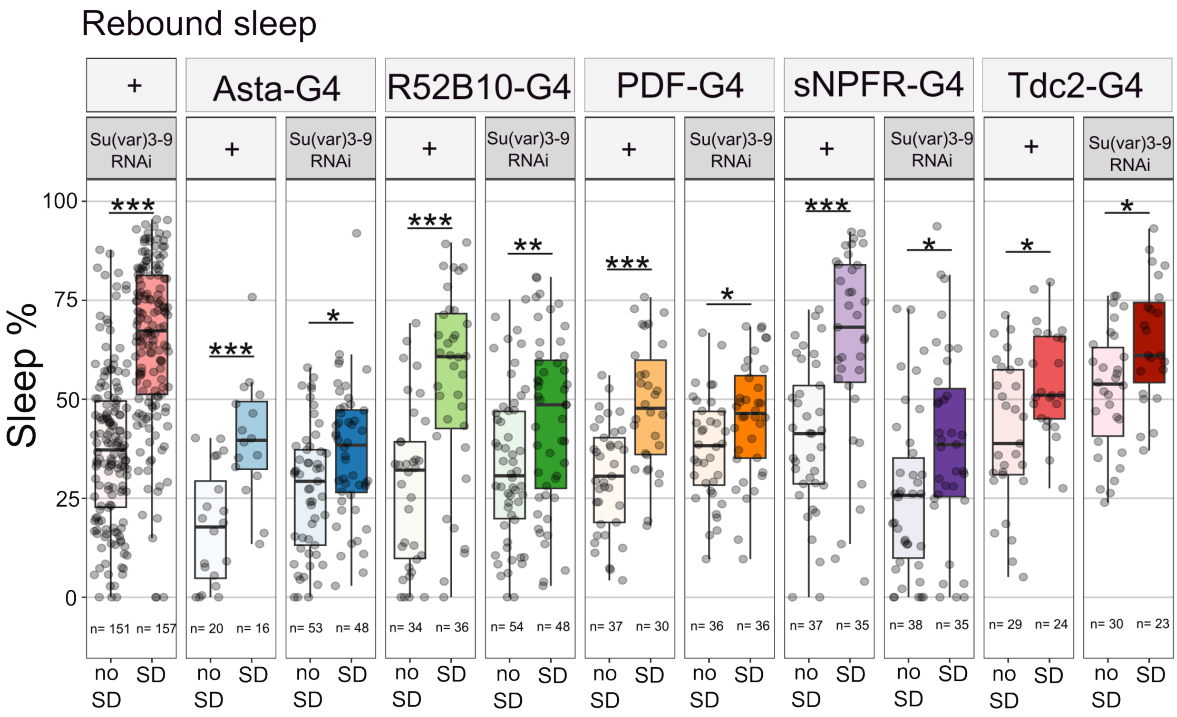


Figure 3.12. UAS-Su(var)3-9 RNAi does not impair rebound response when crossed to Asta, R52B10, PDF, sNPFR, and TDC2-GAL4 lines.
Quantification of rebound sleep during the first 3 hours of the early morning, following SD. Each GAL4 section includes the parental control and the actual KD. The parental control for the *RNAi* line is reported at the very left of the graph. Per each genotype, two groups of flies are shown: undisturbed control group (no SD) and sleep-deprived group (SD). Parental controls generated by crossing respective GAL4 or *RNAi* lines with Canton-S flies, are denoted with "+". * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from two replicates and the *N* of the animals per each group is reported within the figure.

In Figure 3.12, the rebound response of these flies is shown: together with the parental controls, the knockdown of Su(var)3-9 in these 5 classes of neurons did not impair the rebound response, as all the flies exhibited an increase of sleep upon SD (see Table 3.8 for statistical comparisons). Furthermore, the baseline sleep was also not affected (data not shown). These results suggest that the expression of Su(var)3-9 in any of these neurons is not required for sleep regulation.

Table 3.8. Statistical differences between SD and no SD flies in rebound sleep upon knockdown of Su(var)3-9 in different neuronal populations.

sleep during rebound		
no SD	SD	p-value
CS/ Su(var)3-9 KD	CS/ Su(var)3-9 KD	< 2e-16
CS/ Asta-G4	CS/ Asta-G4	0.00115
Su(var)3-9 KD / Asta-G4	Su(var)3-9 KD / Asta-G4	0.01573
CS/R52B10-G4	CS/R52B10-G4	7.7e-05
Su(var)3-9 KD /R52B10-G4	Su(var)3-9 KD /R52B10-G4	0.01204
CS/PDF-G4	CS/ PDF-G4	0.00015
Su(var)3-9 KD / PDF-G4	Su(var)3-9 KD / PDF-G4	0.03065
CS/ sNPFR-G4	CS/ sNPFR-G4	0.00013
Su(var)3-9 KD / sNPFR-G4	Su(var)3-9 KD / sNPFR-G4	0.02207
CS/ Tdc2-G4	CS/ Tdc2-G4	0.02476
Su(var)3-9 KD / Tdc2-G4	Su(var)3-9 KD / Tdc2-G4	0.01888

Despite these negative results, I finally found three more lines, which have shown an interesting behavior. The drivers in question are R72G06-GAL4 (fan-shaped body and superior intermediate protocerebrum), R30G03-GAL4 (ellipsoid body, prow, and bulb) and MB010B-GAL4 (mushroom body). They will be discussed separately in the next three sections.

3.3.2 Su(var)3-9 and R72G06-GAL4 driver, marker for ExlF2 cells

R72G06-GAL4 labels a specific class of neurons called “ExFl2”, known to mainly project to the dorsal Fan-Shaped body, in layer 6. These neurons have already been shown to promote sleep (Liu et al. 2016), by acting as sleep “effectors”: their specific activation directly increases sleep pressure, suggesting a crucial key role in sleep homeostasis regulation (Donlea et al. 2011; Donlea, Pimentel, and Miesenböck 2014b). Going into detail about the molecular mechanism of action, neurotransmitters such as serotonin and dopamine, regulate the activation of the ExFl2 neurons: after being active, they inhibit a

class of helicon cells, which also project their dendrites into the dorsal FB, layer 6. These cells respond to visual inputs and are usually implicated in promoting locomotion. Their axons, instead, project to the R5 neurons of the Ellipsoid Body, previously called R2. The R5-dFB-helicon circuit plays a sort of loop, where helicon cells favorite locomotion by transferring visual inputs to R5. R5 neurons, after being active, lead to build-up sleep pressure and activate ExFl2, probably via neurotransmitters. This inhibits the helicon

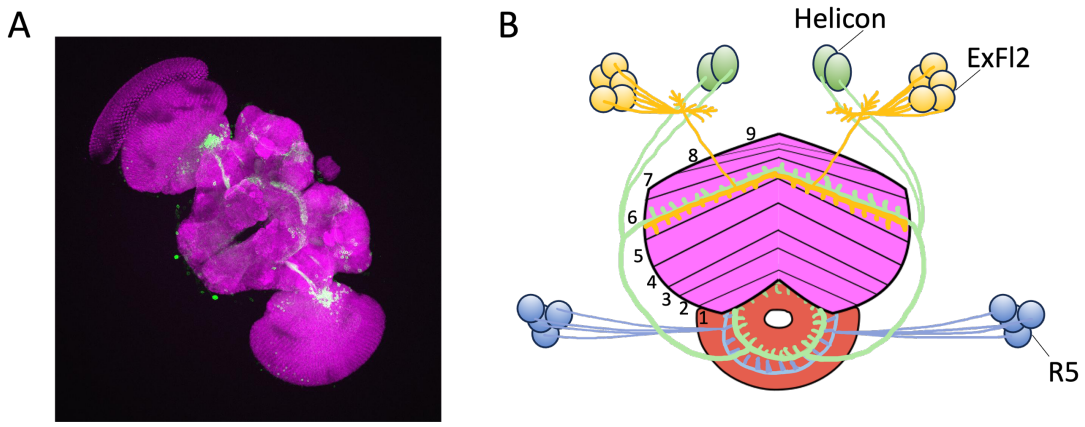


Figure 3.13. The ExFl2 neurons.
A. Expression pattern of the R72G06-GAL4, marker for the ExFl2 cells, mainly included in the fan-shaped body neurons and in the superior intermediate and ventral lateral protocerebrum (Jenett et al. 2012). **B.** Schematic illustration of the R5-dFB-helicon circuit.

cells, blocking the cascade of visual signals to R5, which stops the locomotion. Consequently, it follows a reduction of the excitation of the R5, driven partially by the helicon cells (Figure 3.13, Donlea et al. 2018).

ExFl2 neurons clearly play a fundamental role in homeostatic sleep. While performing the GAL4-screen, I crossed the Su(var)3-9 *RNAi* line to R72G06-GAL4, previously described to label ExFl2 cells. As shown in Figure 3.14, the KD flies failed to show a rebound response, after a day of sleep deprivation, differentially to both parental controls, which exhibited an increase of sleep in both female and male flies. These data remind, very interestingly, of the homeostatic phenotype observed upon enhancing the *RNAi* with nSyb-GAL4, suggesting that Su(var)3-9 may play a homeostatic role for sleep regulation, specifically in the ExFl2 neurons. However, no changes in the baseline sleep were detected after comparisons, meaning that other populations of cells are probably responsible for this effect. Statistical comparisons of the rebound response are shown in Table 3.9.

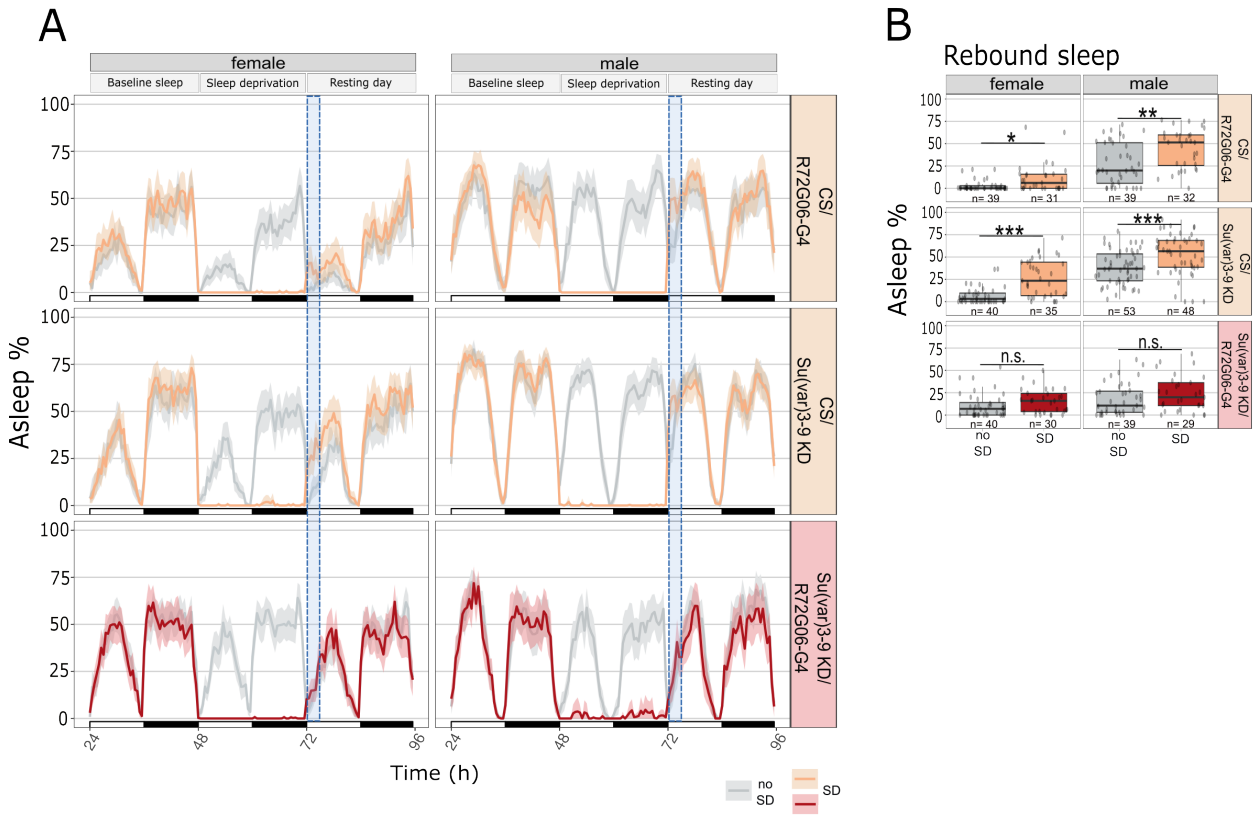


Figure 3.14. Su(var)3-9 plays a role in homeostatic sleep regulation in the ExFl2 neurons.
A. Ethogram over three days in a row, where mechanical sleep deprivation occurs on the second day. Per each genotype, two groups of flies are shown: undisturbed control group (grey line) and sleep-deprived group (orange lines for parental controls and red lines for *RNAi* flies). As expected, SD groups from both genetic controls exhibit higher sleep amounts in the early morning of recovery day (third day, highlighted in blue). Su(var)3-9 KD flies, instead, do not show rebound sleep. Females and males from each genotype are measured separately. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **B.** Quantification of rebound sleep during the first 3 hours of the early morning, following SD. In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from three replicates and the *N* of the animals per each group is reported within the figure.

Table 3.9. Statistical differences between SD and no SD flies in rebound sleep upon knockdown of Su(var)3-9 in ExFl2 neurons.

female, sleep during rebound			**male, sleep during rebound**		
no SD	SD	p-value	no SD	SD	p-value
CS/R72G06-G4	CS/ R72G06-G4	0.01986	CS/ R72G06-G4	CS/ R72G06-G4	0.00434
CS/Su(var)3-9 KD	CS/Su(var)3-9 KD	1.8e-05	CS/Su(var)3-9 KD	CS/Su(var)3-9 KD	0.00126
Su(var)3-9 KD/ R72G06-G4	Su(var)3-9 KD/ R72G06-G4	0.09772	Su(var)3-9 KD/ R72G06-G4	Su(var)3-9 KD/ R72G06-G4	0.18024

In order to better elucidate the correlation between Su(var)3-9 and the ExFl2 neurons, I decided to conditionally activate for 12h during the night, the ExFl2 cells, by exploiting the heat-activated cation channel TrpA1 (Hamada et al. 2008). Thus, I recorded the

behavior over 2.5 days and measured sleep, particularly during the night of the thermoshock. As previously published by Liu et al 2016, the thermosensitive activation of the ExFl2 cells, given by placing flies uas-TrpA1^{ts}/ R72G06-G4 at 29°C, leads to an increase of the baseline sleep, compared to the parental controls. This increase ceases

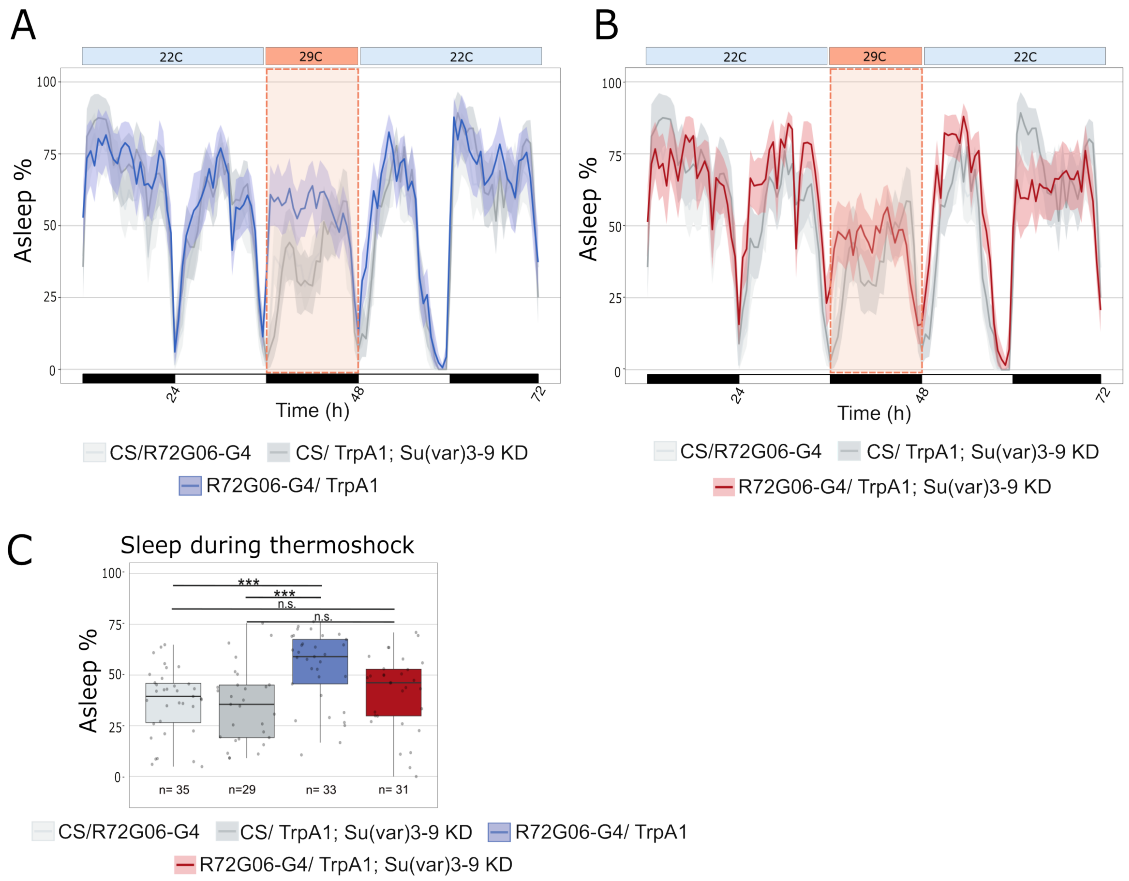


Figure 3.15. Su(var)3-9 knockdown inhibits the increase of sleep upon activation of the ExFl2 neurons.
A, B. Ethograms over 2.5 days in a row, where a thermoshock is given on the second night, by increasing the temperature from 22C to 29C. Parental controls are shown in grey lines, while UAS-TrpA1/R72G06-G4 and UAS-TrpA1; UAS-Su(var)3-9 KD/R72G06-G4 are respectively in blue and red lines. Upon Su(var)3-9 KD, the usual increase in sleep related to the activation of the ExFl2 neurons, is suppressed. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **C.** Quantification of sleep during the night of TrpA1 activation. In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from two replicates and the *N* of the animals per each group is reported within the figure.

as soon as the temperature was brought back to 22C, as shown in Figure 3.15A-B. The following step was then to reproduce the same experiment, combining, however, the knockdown of Su(var)3-9. When simultaneously activating the ExFl2 neurons and triggering the *RNAi* of Su(var)3-9 in the same cells, flies failed to show the usual increase in sleep (Figure 3.15C-D, see Table 3.10 for statistical comparisons). These data suggest that the knockdown of Su(var)3-9 is probably responsible for suppressing the ExFl2

neuronal activity in the context of sleep regulation, although the molecular mechanisms causing this effect remain unclear.

Table 3.10. Statistical differences between genotypes in sleep during thermoshock, ± knockdown of Su(var)3-9 in ExFl2 neurons.

sleep during thermoshock, -Su(var)3-9 KD			sleep during thermoshock, +Su(var)3-9 KD		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/R72G06-G4	TrpA1/ R72G06-G4	0.00041	CS/ R72G06-G4	Su(var)3-9 KD-TrpA1/ R72G06-G4	0.19830
CS/Su(var)3-9 KD	TrpA1/ R72G06-G4	0.00065	CS/Su(var)3-9 KD-TrpA1	Su(var)3-9 KD-TrpA1/ R72G06-G4	0.14218

3.3.3 Su(var)3-9 and R30G03-GAL4 driver, marker for R2 neurons

R30G03-GAL4 is a driver which labels the ellipsoid body, specifically the R2 (or R5) neurons (Figure 3.16). As already described in the previous section, together with the ExFl2, R2 cells are also fundamental to generating a homeostatic sleep drive. Indeed, other works previously showed that the inhibition of the R2, drastically reduces the rebound response upon sleep deprivation (Liu et al. 2016): R2 neurons act upstream of ExFl2, in order to regulate sleep homeostasis.

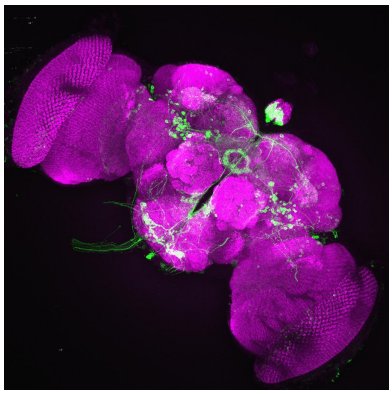


Figure 3.16. Expression pattern of R30G03-GAL4. Figure adapted from Jenett et al. 2012.

While performing the GAL4 screen, I also crossed UAS-Su(var)3-9-*RNAi* to R30G03-GAL4. Similar to the knockdown of Su(var)3-9 in the ExFl2 neurons, also in this case, KD flies did not show any rebound response upon SD, in contrast to both parental controls

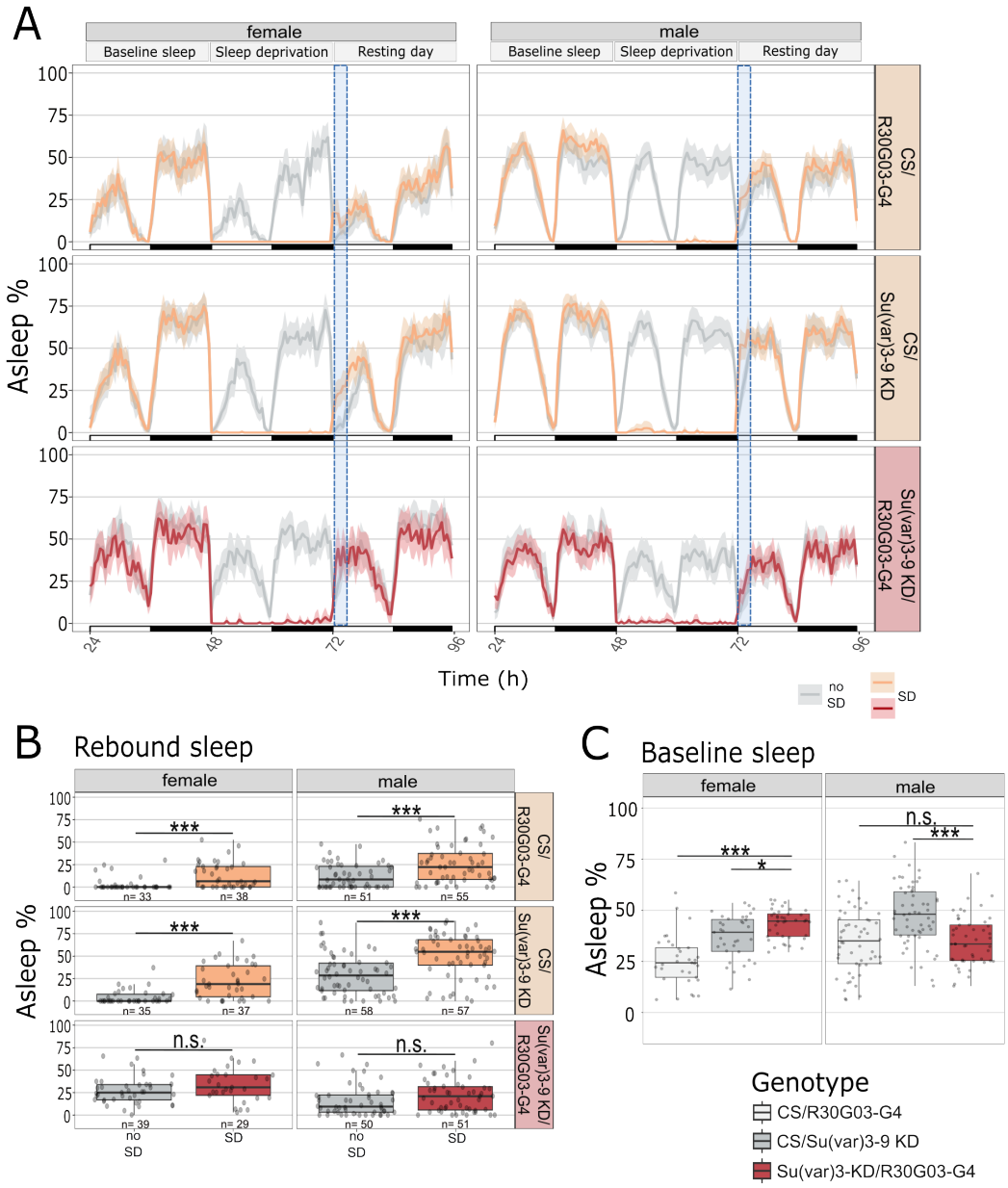


Figure 3.17. Su(var)3-9 plays a role in homeostatic sleep regulation in the R2 neurons.
A. Ethogram over three days in a row, where mechanical sleep deprivation occurs on the second day. Per each genotype, two groups of flies are shown: undisturbed control group (grey line) and sleep-deprived group (orange lines for parental controls and red lines for *RNAi* flies). As expected, SD groups from both genetic controls exhibit higher sleep amounts in the early morning of recovery day (third day, highlighted in blue). Su(var)3-9 KD flies, instead, do not show rebound sleep. Females and males from each genotype are analyzed separately. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **B.** Quantification of rebound sleep during the first 3 hours of the early morning, following SD. **C.** Quantification of the baseline sleep over three days in no SD flies, where female KD flies experience higher sleep, in comparison to parental controls. In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from three replicates and the *N* of the animals per each group is reported within the figure.

(Figure 3.17 A-B). Furthermore, female, but not male, *RNAi* flies, exhibited higher baseline sleep, as reported in Figure 3.17C and Table 3.11. This effect seems to partially recapitulate the increase in baseline sleep observed after knocking down Su(var)3-9 pan-

neuronally. However, it is also not in line with the findings from Liu et al 2016, where upon inactivation of the R2, they did not observe any significant impairment of the baseline sleep.

Table 3.11. Statistical differences between SD and no SD flies in rebound sleep, upon knockdown of Su(var)3-9 in R2 neurons.

female, sleep during rebound			**male, sleep during rebound**		
no SD	SD	p-value	no SD	SD	p-value
CS/R30G03-G4	CS/ R30G03-G4	0.00041	CS/ R30G03-G4	CS/ R30G03-G4	0.00097
CS/ Su(var)3-9 KD	CS/ Su(var)3-9 KD	1.4e-05	CS/ Su(var)3-9 KD	CS/ Su(var)3-9 KD	3.0e-05
Su(var)3-9 KD /R30G03-G4	Su(var)3-9 KD / R30G03-G4	0.15140	Su(var)3-9 KD/ R30G03-G4	Su(var)3-9 KD / R30G03-G4	0.09332
female, baseline sleep over three days			**male, baseline sleep over three days**		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/ R30G03-G4	Su(var)3-9 KD / R30G03-G4	8.7e-09	CS/ R30G03-G4	Su(var)3-9 KD / R30G03-G4	0.94036
CS/ Su(var)3-9 KD	Su(var)3-9 KD / R30G03-G4	0.01282	CS/ Su(var)3-9 KD	Su(var)3-9 KD / R30G03-G4	7.5e-05

3.3.4 Su(var)3-9 and MB010B-GAL4

MB010B-GAL4, unlike the other drivers used in this project, is a split-GAL4. A split-GAL4 operates akin to the UAS-GAL4 system, where the DNA-binding domain (DBD) and the transcription activation domain (TAD) of GAL4, combine and attach to heterodimerizing leucine zippers, as demonstrated by Luan et al., (Luan et al. 2006). Only when TAD and DBD coexist within a cell, they synergize to restore the GAL4 transcriptional activity (Dionne et al. 2018). This is a very powerful technique, exploited to target specific single cells or specific cell types.

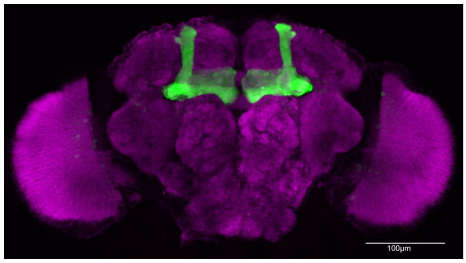


Figure 3.18. Expression pattern of MB010B-GAL4. Figure adapted from Vogt et al. 2014.

The MB010B-GAL4 labels the subunits α/β, α'/β' and γKenyon Cells of the mushroom body neurons, known to control several aspects of behaviors, such as sleep, memory, learning, and sensory processing (Figure 3.18, Heisenberg et al. 1985; Pitman et al. 2006;

Aso et al. 2014; L. P. C. Lewis et al. 2015; Kirkhart and Scott 2015). As applied for the other drivers, I crossed the usual UAS-Su(var)3-9 *RNAi* line to MB010B-GAL4. The knockdown in this class of neurons prevented the increase of sleep upon SD, only in male and not female flies, as shown in Figure 3.19A-B, probably suggesting some sexual differences in the regulation of sleep, for this specific brain region. Furthermore, given the essential role played by the mushroom body neurons in learning and memory formation, I decided to validate the potential presence of cognitive defects in the *RNAi* flies, by performing Courtship assays. Courtship conditioning is one of the most widely used paradigms to elucidate mechanisms underlying different forms of learning and memory. It relies on the presence of a specific pheromone called cis-

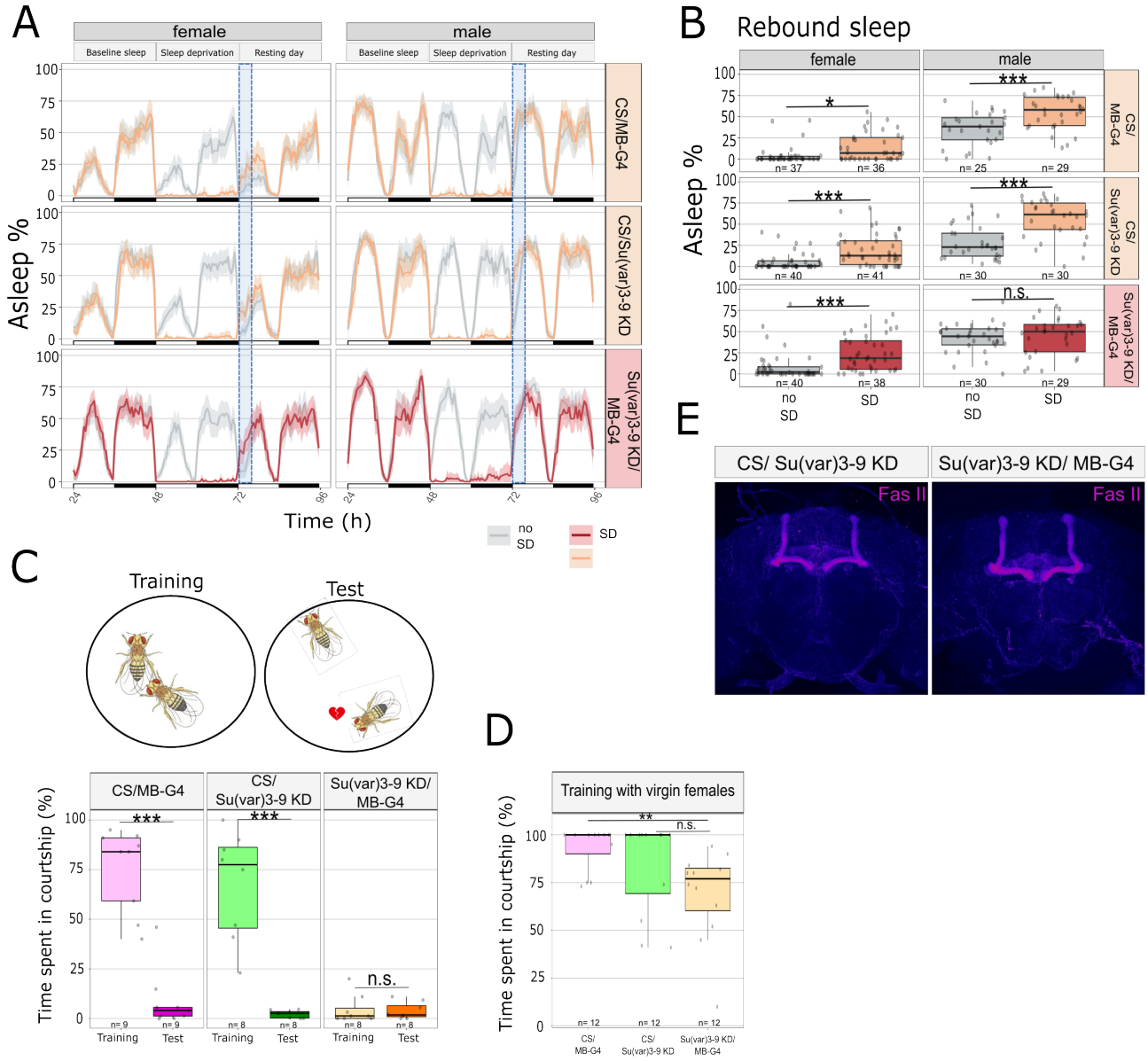


Figure 3.19. Su(var)3-9 KD in the MB partially affects homeostatic rebound sleep and courtship behavior.
A. Ethogram over three days in a row, where mechanical sleep deprivation occurs on the second day. Per each genotype, two groups of flies are shown: undisturbed control group (grey line) and sleep-deprived group (orange lines for parental controls and red lines for *RNAi* flies). As expected, SD groups from both genetic controls exhibit higher sleep amounts in the early morning of recovery day (third day, highlighted in blue). Male Su(var)3-9 KD flies, instead, do not show rebound sleep, while females behave normally as controls. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **B**. Quantification of rebound sleep during the first 3 hours of the early morning, following SD. **C**. Percentage of time spent on courtship behavior. No significant differences were detected in the KD flies, when comparing Training with Test sessions. **D**. Courtship during training sessions with virgin females. No differences were detected across the genotypes. **E**. Staining of the FasII-expressing neurons, showing no morphological abnormalities in the *RNAi* flies, compared to controls. In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test and the *N* of the animals per each group is reported within the figure. Fly illustrations are taken from https://commons.wikimedia.org/wiki, created under the Creative Commons license.

Table 3.12. Statistical differences in rebound sleep and courtship parameters upon knockdown of Su(var)3-9 in MB neurons.

female, sleep during rebound			**male, sleep during rebound**		
no SD	SD	p-value	no SD	SD	p-value
CS/MB-G4	CS/ MB-G4	0.01506	CS/ MB-G4	CS/ MB-G4	0.00138
CS/ Su(var)3-9 KD	CS/ Su(var)3-9 KD	0.00044	CS/ Su(var)3-9 KD	CS/ Su(var)3-9 KD	3.3e-05
Su(var)3-9 KD / MB-G4	Su(var)3-9 KD / MB-G4	0.00103	Su(var)3-9 KD/ MB-G4	Su(var)3-9 KD / MB-G4	0.36612

Courtship Index			**Courtship during training with virgin female**		
Trianing	Test	p-value	Genotype 1	Genotype2	p-value
CS/ Su(var)3-9 KD	CS/ Su(var)3-9 KD	0.0002	CS/ MB-G4	Su(var)3-9 KD / MB-G4	0.655
Su(var)3-9 KD / MB-G4	Su(var)3-9 KD / MB-G4	0.8903	CS/ Su(var)3-9 KD	Su(var)3-9 KD / MB-G4	0.068
CS/MB-G4	CS/ MB-G4	0.0001			

vaccenyl acetate (cVA) on the female abdomen. This pheromone, which is deposited by the male during copulation, naturally reduces courtship behavior, especially when combined with rejection by the female. The courtship behavior of male flies can be easily observed, by quantifying stereotypical movements, such as orienting towards and following the female, tapping, extending, vibrating the wing, licking, and attempting copulation. Very interestingly, male flies learn to differentiate between receptive virgin and non-receptive mated females. Indeed, after experiencing sexual rejection, they exhibit reduced courtship behavior towards non-receptive females (Koemans et al. 2017; Hall 1994; McBride et al. 1999). In order to exploit this behavior to provide insights into learning, naive male flies were trained with mated females for 1 hour and then,

immediately tested and recorded for 15 mins. As shown in Figure 3.19C, both parental controls spent much less time in courtship during tests, in comparison to training sessions, suggesting a high learning index. However, the *RNAi* flies exhibited a very short time spent in courtship, during both training and test sessions, making not possible the calculation of the learning index. In order to better elucidate this result, I decided to perform a control training, by pairing males with virgin flies. As reported in Figure 3.19D, this time the *RNAi* flies tried to court as similar to control flies, meaning a high courtship index. Statistical comparisons are shown in Table 3.12.

Recent studies have shown that the suppression of courtship during training with mated females can be associated with the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a fundamental player in several aspects of neuronal functions (Griffith 2004). Suppression during training seems to be triggered by the expression of CaMKII in the cholinergic antennal lobe neurons, cells located downstream of the olfactory receptor neurons (Mehren and Griffith 2004). The olfactory receptor neurons are connected to the courtship circuitry by the integration of olfactory cues, such as pheromones or endogenous fly odors. Given all these assumptions, it may be possible that the knockdown of Su(var)3-9 leads to a different perception of the fly odors. In this context, I need to mention that Shase Jafari et al (2021) demonstrated that Su(var)3-9, together with dLsd1, regulates the heterochromatin formation in order to direct the odorant receptor (OR) expression (Jafari et al. 2021). Therefore, the knockdown of Su(var)3-9 may cause an upregulation of OR, which consequently, allows flies to differently integrate olfactory cues, such as the pheromone cVa, and, perhaps, to better distinguish mated from virgin flies.

Finally, I dissected adult brains from *RNAi* and control flies and stained tissues with an antibody anti-Fasciclin II (FasII), a protein preferentially expressed in the MB, very strongly in the α, β lobes and weakly in the γ lobes. As shown in Figure 3.19E, the knockdown did not lead to changes in the anatomical morphology of the mushroom body cells, excluding any developmental impairment.

3.3.5 Summary

Su(var)3-9 is a key player for sleep regulation in specific sets of neurons:

- ExFl2 neurons. The knockdown of Su(var)3-9 in these cells, abolishes the rebound response upon 1 day of dynamic SD. In addition, *RNAi* flies do not experience the increase of baseline sleep, usually observed upon activation of the ExFl2, promoting Su(var)3-9 as a regulator of the sleep-related function of these neurons.
- R2 neurons, known to act upstream of ExFl2 cells. The knockdown of Su(var)3-9 in these cells abolishes the rebound response upon SD. Furthermore, the baseline sleep in female *RNAi* flies appeared also increased, compared to parental controls', resembling the effects observed upon pan-neuronal knockdown.
- MB neurons. The knockdown of Su(var)3-9 in these cells causes impairments in the rebound response upon SD, only in male flies. During training of courtship assays, male *RNAi* flies exhibited a high courtship index when paired with virgin females, but not with mated females, suggesting in these flies, perhaps, a more sophisticated mechanism to sense the mating status of females.

3.4 More insights about the knockdown of Su(var)3-9 and the other H3K9 methylases: Eggless and G9a

3.4.1 Su(var)3-9 and the synaptic plasticity

According to the synaptic homeostasis hypothesis (SHY), proposed by G.Tononi and C.Cirelli (Tononi and Cirelli 2003; 2006), sleep is a fundamental need for the brain, required to regulate the total amount of synaptic strengths, which usually accumulate during wakefulness, driven by ongoing learning, in order to allow connections throughout the brain. However, this condition consequently requires an increased demand of cellular needs for supplies and energy, which can lead to stressful conditions. Furthermore, at the end of the day, the increased synaptic strengths may saturate the opportunity to learn, driving the need for synaptic renormalization. This is supposed to occur during sleep, where the brain goes into "offline" mode, disconnecting from the external environment, restoring synaptic plasticity and, therefore, the ability to learn (Figure 3.20).

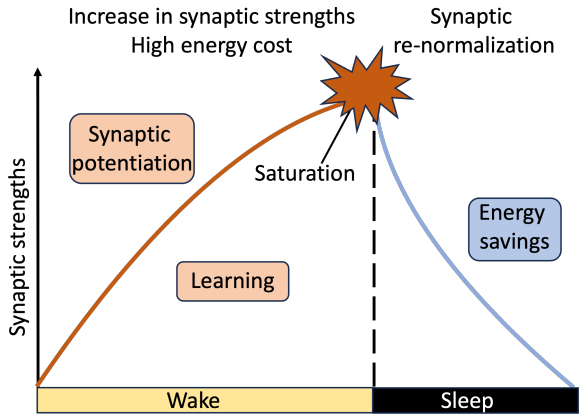


Figure 3.20. Illustration of the synaptic homeostasis hypothesis (SHY).

According to SHY, in both mammals and flies, periods of wakefulness cause an increase in synaptic markers, such as Bruchpilot (BRP) and Synapsin (Syn), two of the main pre-synaptic components (Vyazovskiy et al. 2008; Gilestro, Tononi, and Cirelli 2009), while sleep decreases their level. This oscillation seems to be completely independent of the time of the day.

In order to evaluate a potential correlation between Su(var)3-9 and the level of the above-mentioned synaptic proteins, I decided to enhance the pan-neuronal knockdown of Su(var)3-9, to subject the flies to 1 day of sleep deprivation and quantify the protein level by immune-staining. As shown in Figure 3.21, both genotypes CS/Su(var)3-9 KD and nSyb-G4/Su(var)3-9 KD, exhibited higher levels of BRP and Syn upon SD, following the findings of Gilestro 2009. However, very interestingly, the *RNAi* flies showed an increase in the baseline level of both synaptic scaffolding markers, when comparing the no SD condition to the equivalent parental control (Table 3.13).

At this stage, I believe it important to mention the study of S.Huang et al, in 2020, who found that sleep in flies can be related to the status of the presynaptic active zone (S. Huang et al. 2020). In their work, they show that a brain-wide upregulation of BRP can drive the upregulation of other scaffolding proteins, which has a crucial impact on sleep regulation. Indeed, the increase of *brp* copies leads to an increase in baseline sleep in a dosage-dependent manner: the more copies they were triggering, the higher was the baseline-sleep increase. Given all these assumptions, I started hypothesizing that the raised level of the synaptic proteins BRP and Syn in the Su(var)3-9 *RNAi* flies, could be

then responsible for the increased baseline sleep observed and discussed in section 3.2.1, as one of the main sleep perturbations driven by the knockdown. In order to validate this

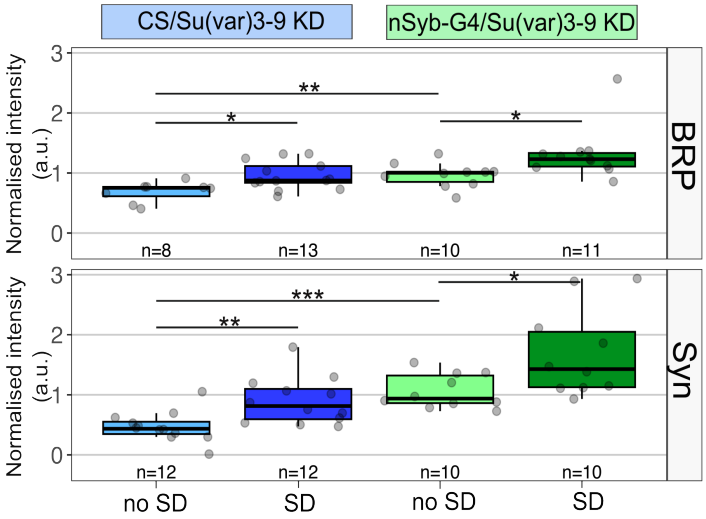


Figure 3.21. The Su(var)3-9 knockdown increases BRP and Syn baseline level.
Quantification of normalized intensity of BRP and Syn, from confocal projection of adult front brains. Per each genotype, intensities from SD and no SD flies are shown. * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$, from pairwise Wilcoxon rank sum test.

Table 3.13. Statistical differences of BRP and Syn intensity between SD and no SD flies and between Su(var)3-9 RNAi and controls.

BRP intensity			Syn intensity		
Genotype1	Genotype2	p-value	Genotype1	Genotype2	p-value
CS/ Su(var)3-9 KD no SD	CS/ Su(var)3-9 KD SD	0.03828	CS/ Su(var)3-9 KD no SD	CS/ Su(var)3-9 KD SD	0.00446
CS/ Su(var)3-9 KD no SD	nSyb-G4/Su(var)3-9 KD no SD	0.00779	CS/ Su(var)3-9 KD no SD	nSyb-G4/ Su(var)3-9 KD no SD	0.00043
nSyb-G4/ Su(var)3-9 KD no SD	nSyb-G4/ Su(var)3-9 KD SD	0.01238	nSyb-G4/ Su(var)3-9 KD no SD	nSyb-G4/ Su(var)3-9 KD SD	0.03054

hypothesis, I decided to trigger the pan-neuronal knockdown of BRP, simultaneously with the knockdown of Su(var)3-9 and record for sleep. Flies were also subjected to SD and homeostatic sleep was analyzed. Figure 3.22A shows the sleep pattern of 5 different genotypes: CS/nSyb-G4 and CS/UAS-BRP KD_UAS-Su(var)3-9 KD, the two parental controls; BRP KD-nSyb-G4 (which triggers the *RNAi* of BRP only); Su(var)3-9 KD-nSyb-G4 (which triggers the *RNAi* of Su(var)3-9 only); BRP-Su(var)3-9 KD (triggering the *RNAi* of both Su(var)3-9 and BRP). Regarding the homeostatic sleep, the addition of

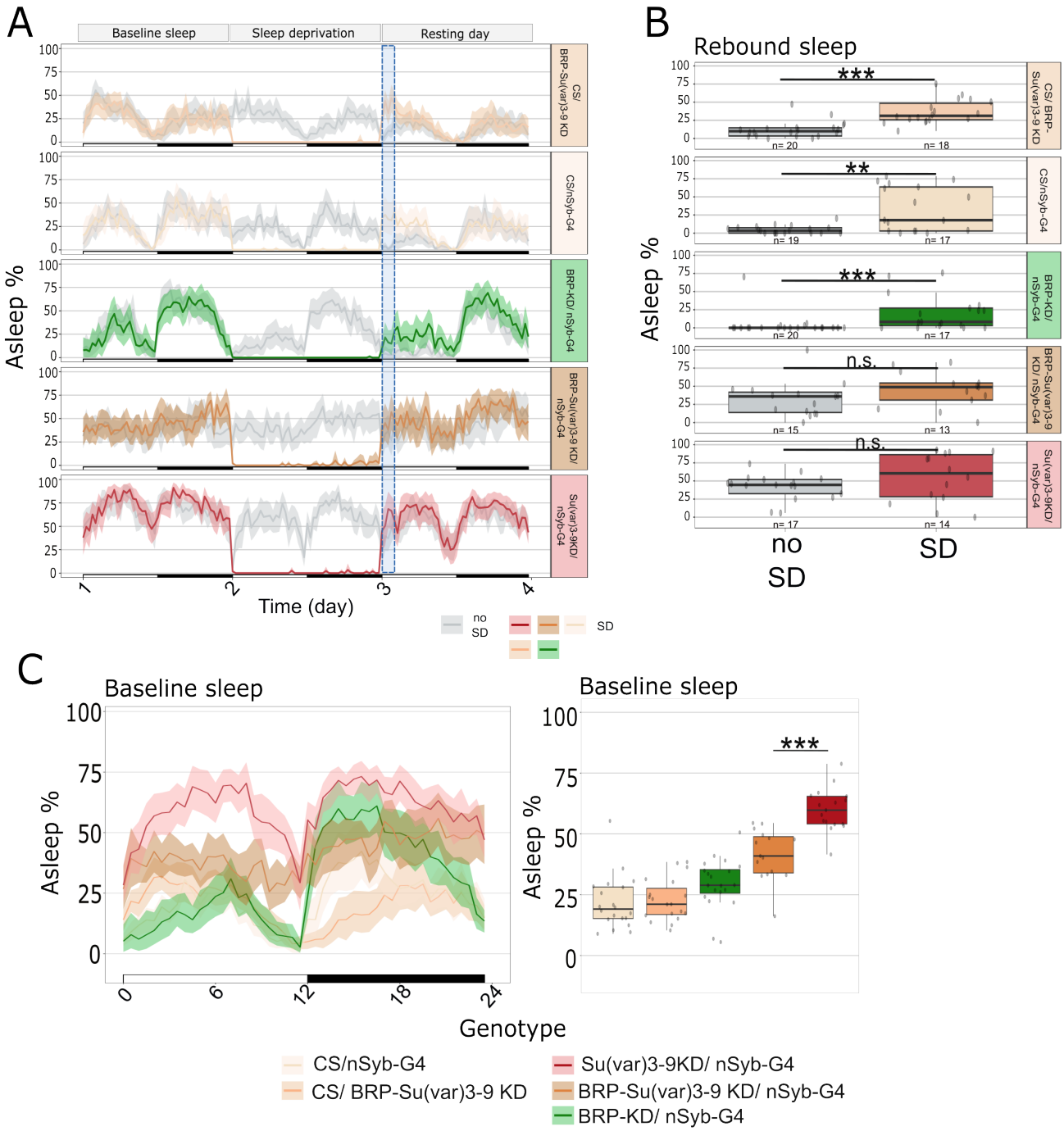


Figure 3.22. BRP knockdown reduces the baseline sleep in Su(var)3-9 KD flies.
A. Ethogram over three days in a row, where sleep deprivation occurs on the second day. Per each genotype, two groups of flies are shown: undisturbed control group (grey line) and sleep-deprived group (colored line). Rebound sleep is highlighted in blue. Only female flies were analyzed. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **B.** Quantification of rebound sleep during the first 3 hours of the early morning, following SD. Statistically significant changes in sleep rebound have been found in parental controls and BRP (only) KD flies, after comparing SD to no SD groups. **C.** Ethogram wrap (average) of three days of baseline sleep in no SD flies and quantification of the total baseline sleep, showing significant differences between BRP-Su(var)3-9 KD and Su(var)3-9 KD flies (p-value: 6.0e-06). In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from two replicates and the *N* of the animals per each group is reported within the figure.

Table 3.14. Statistical differences between SD and no SD flies in rebound sleep, from Su(var)3-9 - BRP RNAi and reciprocal controls.

Sleep during rebound		
no SD	SD	p-value
CS/ BRP-Su(var)3-9 KD	CS/ BRP-Su(var)3-9 KD	9.1e-05
CS/ nSyb-G4	CS/ nSyb-G4	0.01259
BRP KD/ nSyb-G4	BRP KD/ nSyb-G4	0.00100
Su(var)3-9 KD/ nSyb-G4	Su(var)3-9 KD/ nSyb-G4	0.28394
BRP-Su(var)3-9 KD/ nSyb-G4	BRP-Su(var)3-9 KD/ nSyb-G4	0.15019

the BRP knockdown in Su(var)3-9 KD flies, was not sufficient to mitigate the effect of the rebound response, as these flies, similarly to Su(var)3-9 KD flies, did not increase their sleep at the end of the sleep deprivation. BRP KD flies only, instead, exhibited normal rebound, as the parental controls (Figure 3.22B, Table 3.14). Despite the absence of changes in homeostatic sleep, different was the situation in terms of baseline sleep. The knockdown of both BRP and Su(var)3-9 led to a lower increase in baseline sleep, compared to what was previously observed in the Su(var)3-9 KD flies, suggesting a partial rescue of the phenotype (Figure 3.22C-D). Besides the lower amount of sleep, however, it was also easy to notice that the double knockdown flattened the activity peaks, which normal flies clearly show around the light transitions (morning and evening peaks). In order to evaluate any arrhythmicity, I decided to monitor all genotypes in constant darkness (DD). Over 14 days in DD, both parental controls and the single knockdowns of BRP and Su(var)3-9, have shown normal rhythmicity, displaying the usual activity peaks approximately at the same time of the day. However, BRP-Su(var)3-9 *RNAi* flies seem to slightly shift the activity bouts over the days, suggesting a circadian clock rhythm with a 27h-period (Figure 3.23B). Furthermore, on day 11, the activity pattern becomes completely flat, with no evidence of peaks. This may suggest that the addition of BRP knockdown in Su(var)3-9 *RNAi* flies may aggravate the phenotype, causing impairments in the circadian system, which was not affected when triggering the knockdown of Su(var)3-9 only.

Finally, I decided to re-apply the same approach described above, by combining the knockdown of Synapsin, instead of BRP, to Su(var)3-9 *RNAi* flies. As similar as BRP, the addition of Syn knockdown did not improve the homeostatic response after 1 day of SD (Figure 3.24A-B). Apart from the two parental controls, all the *RNAi* flies, including

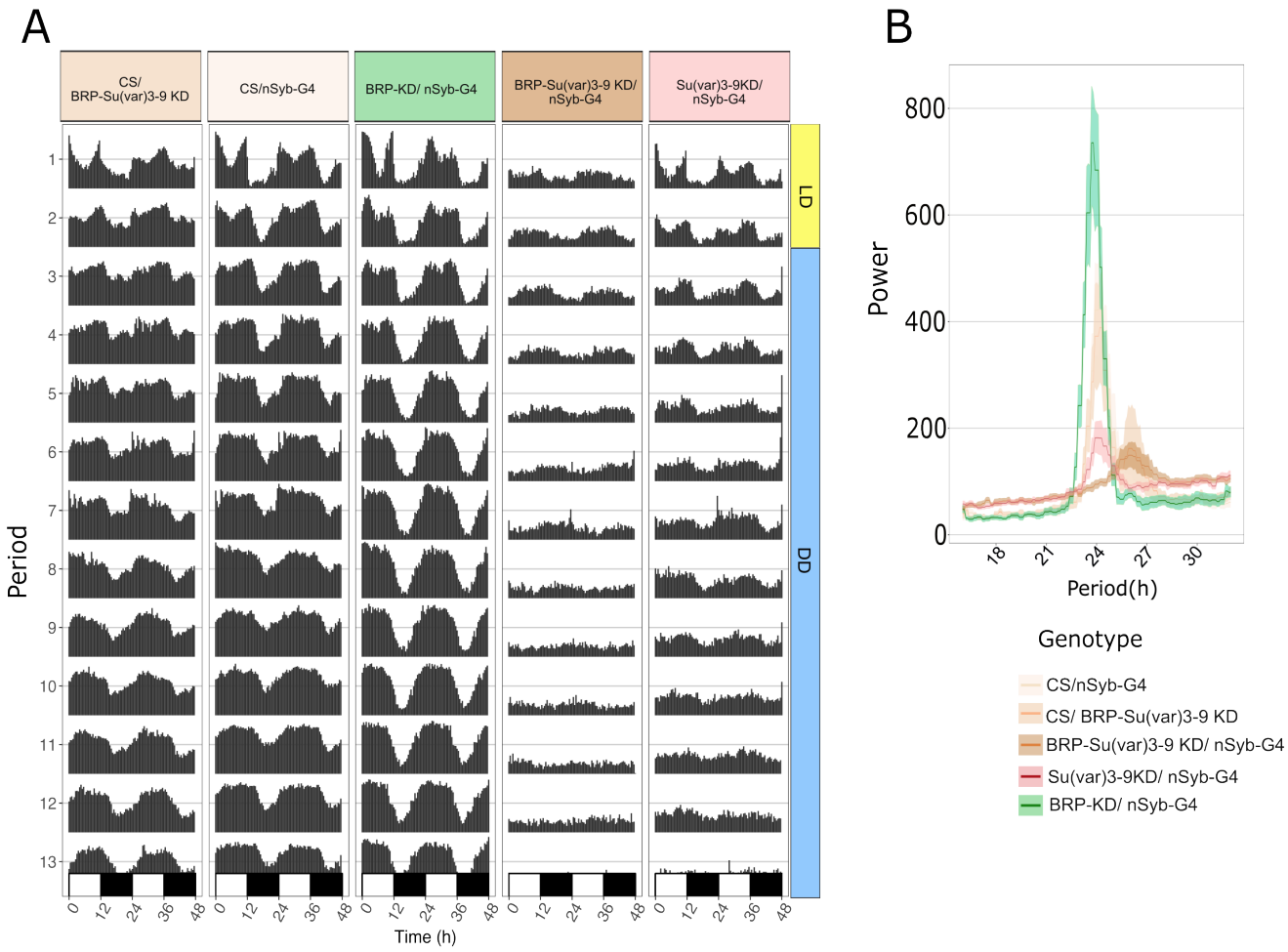


Figure 3.23. The knockdown of BRP in Su(var)3-9 RNAi flies flattens the activity peaks in DD. **A.** Representative actograms in Su(var)3-9 *RNAi* flies, BRP-Su(var)3-9 *RNAi*, BRP *RNAi,* and parental controls, in DD condition: BRP-Su(var)3-9 *RNAi* flies progressively show flattened activity peaks over the days. **B.** Power analysis of the circadian period of activity in DD in all the genotypes, showing a period of 27h in BRP-Su(var)3-9 *RNAi* flies.

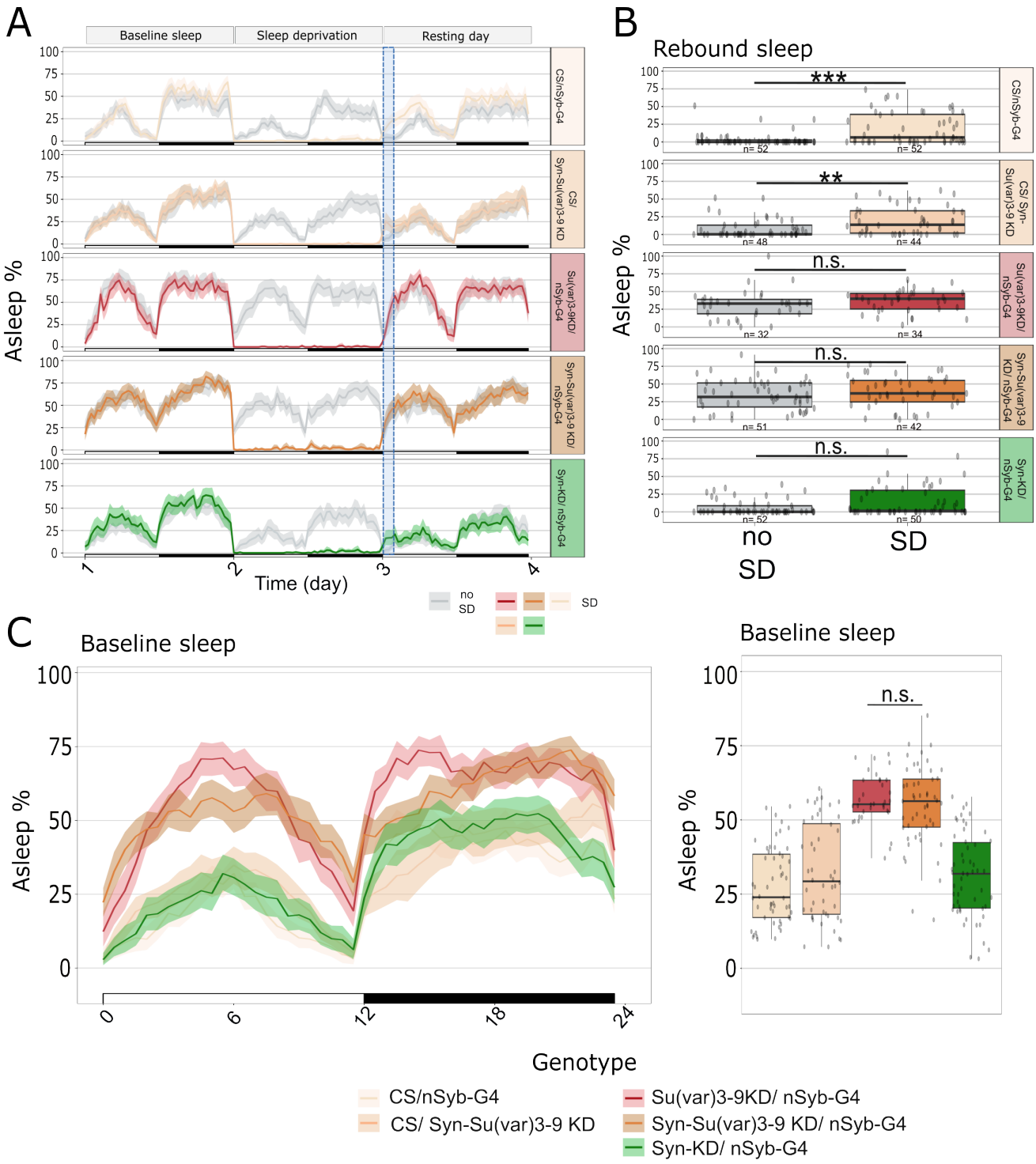


Figure 3.24. The knockdown of Syn does not rescue the sleep impairments in Su(var)3-9 *RNAi* flies. **A.** Ethogram over three days in a row, where sleep deprivation occurs on the second day. Per each genotype, two groups of flies are shown: undisturbed control group (grey line) and sleep-deprived group (colored line). Rebound sleep is highlighted in blue. Only female flies were analyzed. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **B.** Quantification of rebound sleep during the first 3 hours of the early morning, following SD. Statistically significant changes in sleep rebound have been found in parental controls only, after comparing SD to no SD groups. **C.** Ethogram wrap (average) of three days of baseline sleep in no SD flies and quantification of the total baseline sleep, where no differences were detected when comparing Su(var)3-9 KD/nSyb-G4 to Syn-Su(var)3-9 KD/nSyb-G4 flies (p-value: 0.72) In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from three replicates and the *N* of the animals per each group is reported within the figure.

Table 3.15. Statistical differences between SD and no SD flies in rebound sleep, from Su(var)3-9 -Syn RNAi and reciprocal controls.

Sleep during rebound		
no SD	SD	p-value
CS/ Syn-Su(var)3-9 KD	CS/ Syn-Su(var)3-9 KD	0.00365
CS/ nSyb-G4	CS/ nSyb-G4	3.6e-05
Syn KD/ nSyb-G4	Syn KD/ nSyb-G4	0.06820
Su(var)3-9 KD/ nSyb-G4	Su(var)3-9 KD/ nSyb-G4	0.09184
Syn-Su(var)3-9 KD/ nSyb-G4	Syn-Su(var)3-9 KD/ nSyb-G4	0.27594

Syn KD/nSyb-G4, did not rebound (Table 3.15). Furthermore, there were no changes in terms of baseline sleep, when comparing Su(var)3-9 KD to Syn-Su(var)3-9 KD, suggesting that the knockdown of Syn was not enough to rescue the usual sleep impairments observed in Su(var)3-9 *RNAi* flies (Figure 3.24 C-D). All these results may suggest that the higher level of BRP and Syn in Su(var)3-9 *RNAi* flies, does not represent the only factor driving the increase of the baseline sleep. The knockdown of Su(var)3-9 probably causes the alteration of multiple mechanisms, that playing together, lead to severe consequences.

3.4.2 Sleep and other H3K9 methyltransferases: Eggless and G9a

Until now, I proceeded by perturbating the H3K9me, throughout the knockdown of Su(var)3-9, and subsequentially performing sleep analysis. However, as largely discussed in section 1.3.7, we know that in *Drosophila* there are also two more H3K9 histone methyltransferases, Eggless and G9a. In order to build a full picture of the contribution of the H3K9me in sleep regulation, I decided to analyze the sleep pattern, after triggering the pan-neuronal knockdown of Egg and G9a, distinctively. G9a KD/nSyb-G4 flies were fully viable, while the knockdown of Egg caused an adult semi-lethality: only half of the flies hatched and immediately died, preventing any possible sleep recording. Therefore, for Eggless only, I necessarily had to make the crosses using Elav-GAL4, another neuronal GAL4, which drives the expression of GAL4 during an earlier developmental stage, compared to nSyb-G4. As previously performed, flies were subjected to sleep deprivation, during sleep recording.

As reported in Figure 3.25, the knockdown of both Eggless and G9a did not lead to any impairment in baseline sleep: indeed, over three days, *RNAi* flies slept as similar as the parental controls. Very interesting is, however, the homeostatic sleep situation. While Egg *RNAi* flies exhibited an increase in sleep on the day following sleep deprivation, suggesting that this methyltransferase enzyme is probably not involved in sleep regulation, the knockdown of G9a showed no evidence of rebound response upon SD, in male flies. Female KD flies behaved as normal as parental controls, suggesting again, some kind of sexual difference (Figure 3.25C-D and Table 3.16). These results, taken

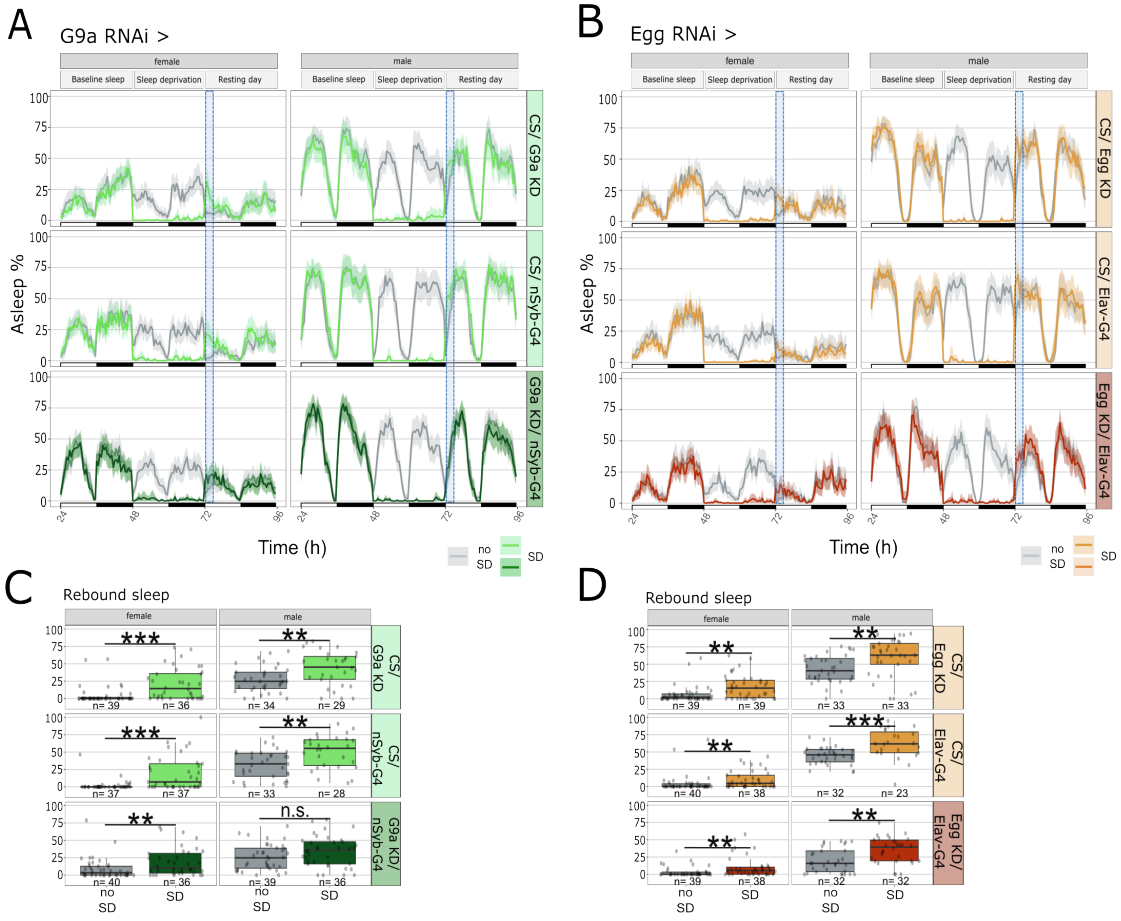


Figure 3.25. The knockdown of G9a, but not Egg, impairs the rebound response in male flies.
A, B. Ethograms over three days in a row, show the sleep pattern in G9a (A) and Egg (B), respectively. Sleep deprivation occurs on the second day: for each genotype, two groups of flies are shown: undisturbed control group (grey line) and sleep-deprived group (colored line). Rebound sleep is highlighted in blue. Females and males are analyzed separately. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **C, D.** Quantifications of rebound sleep during the first 3 hours of the early morning, following SD in G9a (C) and Egg (D) flies, respectively. In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from three replicates and the *N* of the animals per each group is reported within the figure.

Table 3.16. Statistical differences between SD and no SD flies in rebound sleep, from Egg or G9a RNAi and reciprocal controls.

G9a, female, sleep during rebound			G9a, male, sleep during rebound		
no SD	SD	p-value	no SD	SD	p-value
CS/nSyb-G4	CS/nSyb-G4	0.00013	CS/nSyb-G4	CS/nSyb-G4	0.00319
CS/ G9a KD	CS/ G9a KD	0.00012	CS/ G9a KD	CS/ G9a KD	0.01274
G9a KD / nSyb-G4	G9a KD / nSyb-G4	0.01579	G9a KD/ nSyb-G4	G9a KD / nSyb-G4	0.10115
Egg, female, sleep during rebound			**Egg, male, sleep during rebound**		
no SD	SD	p-value	no SD	SD	p-value
CS/Elav-G4	CS/ Elav -G4	0.00628	CS/ Elav -G4	CS/ Elav -G4	0.00086
CS/ Egg KD	CS/ Egg KD	0.00140	CS/ Egg KD	CS/ Egg KD	0.00505
Egg KD / Elav -G4	Egg KD / Elav -G4	0.00181	Egg KD/ Elav -G4	Egg KD / Elav -G4	0.00323

together, may suggest that G9a, and not Egg, plays a role in the homeostatic sleep regulation, possibly cooperating with Su(var)3-9, in this function. In order to validate this hypothesis, I decided to trigger the knockdown of the same targets in the ExFl2 neurons, cells where Su(var)3-9 has already been shown to operate, to regulate the homeostatic response. The aim here is, therefore, to see if G9a is also required for sleep in this specific population of neurons. Together with G9a, I also induce the knockdown of Egg, as a negative control: here I was not expecting any perturbation, following the results with Elav-G4. Only male flies were recorded this time.

As shown in Figure 3.26, the knockdown of G9a specifically in the ExFl2 cells, triggered by exploiting the R72G06-G4 driver, phenocopied the results previously collected with nSyb-G4: the *RNAi* flies, in fact, were not rebounding upon SD, suggesting again impairments in the homeostatic response. On the other hand, Egg KD flies were able to normally recover on the day following SD, confirming again that Egg probably does not play any role in sleep regulation (Figure 3.26B and Table 3.17). G9a, instead, seems to be another good candidate for regulating homeostatic sleep. The fact that both G9a and Su(var)3-9 were shown to play the same role in the same class of neurons, leads to hypothesize that the two enzymes may collaborate for the completion of this function. Although there is no evidence of these methyltransferases in cooperating during the adult stage, there are certainly studies confirming their overlapping roles in the deposition of the H3K9me during development. Mis et al, for instance (2006), demonstrated the existence of a

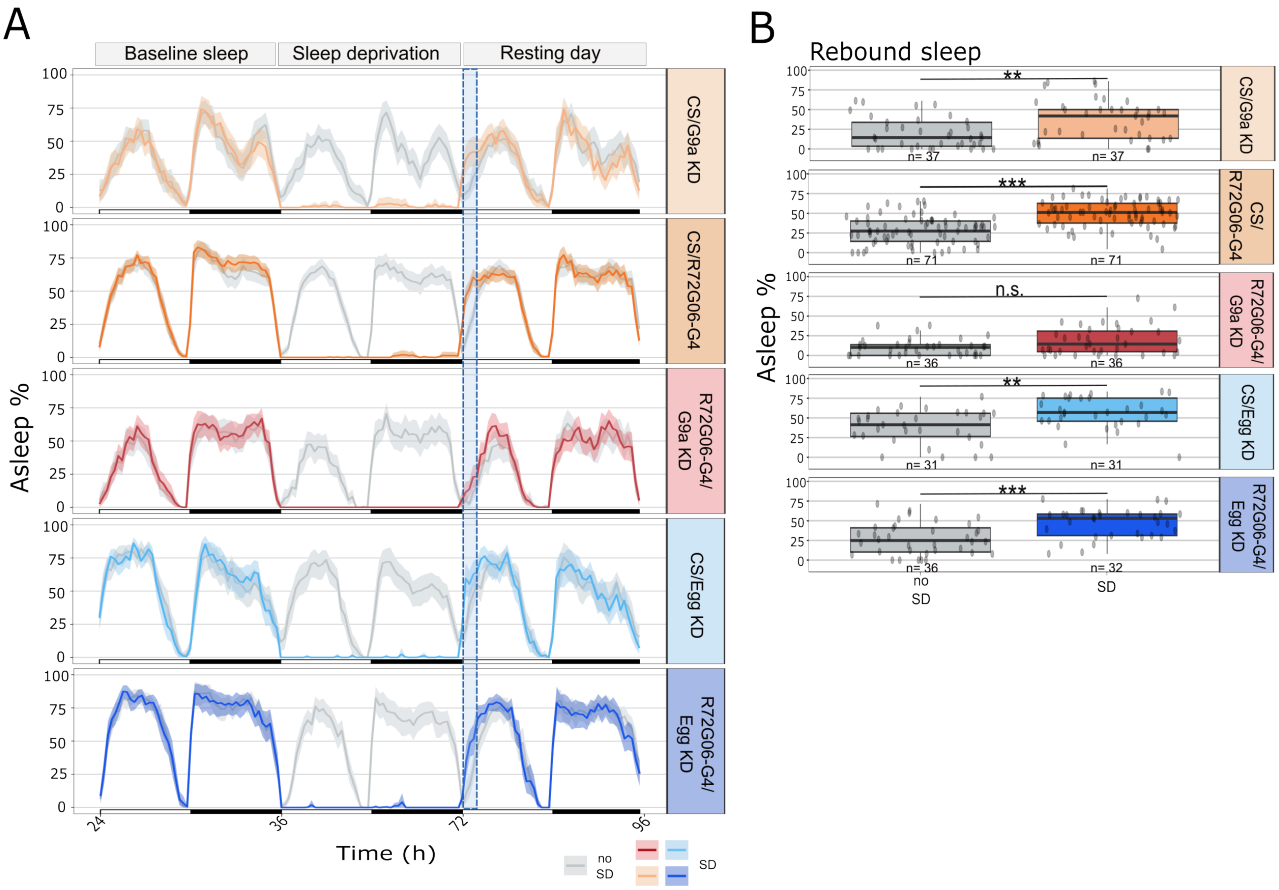


Figure 3.26. G9a, but not Egg, controls the rebound response in the ExFL2 neurons.
A. Ethogram over three days in a row, where sleep deprivation occurs on second day: per each genotype, two groups of flies are shown: undisturbed control group (grey line) and sleep-deprived group (colored line). Rebound sleep is highlighted in blue. Only males are analysed. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **B.** Quantification of rebound sleep during the first 3 hours of the early morning, following SD. In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from two replicates and the *N* of the animals per each group is reported within the figure.

Table 3.17. Statistical differences between SD and no SD flies in rebound sleep, upon knockdown of G9a in the ExFL2 neurons.

Sleep during rebound		
no SD	SD	p-value
CS/ Egg KD	CS/ Egg KD	0.00294
CS/ G9a KD	CS/ G9a KD	0.00264
CS/ R72G06-G4	CS/ R72G06-G4	2.8e-09
R72G06-G4/ Egg KD	R72G06-G4/ Egg KD	0.00039
R72G06-G4/ G9a KD	R72G06-G4/ G9a KD	0.05770

genetic interaction between G9a and Su(var)3-9, across specific developmental stages. They found that the double mutation for both proteins led to very low viability: among 706 embryos collected, only 8% managed to survive, reaching the adult stage. The single

mutants for G9a and Su(var)3-9 had, instead, 38% of viability. They also found that there appears to be a synergistic interaction between the two proteins, more pronounced during larval stages, since only 15.8% of the double mutants managed to move from larval to pupal stadium, against the 60.8% and 71.3% of the single mutants for G9a and Su(var)3-9, respectively (Mis, Ner, and Grigliatti 2006).

It is still unclear if G9a and Su(var)3-9 play a synergistic role during adulthood, too. However, my data suggest that this could be potentially possible. More future works are required, to better elucidate this hypothesis.

3.4.3 Summary

- The pan-neuronal knockdown of Su(var)3-9 leads to higher baseline levels of synaptic proteins, such as BRP and Syn, in *RNAi* flies compared to parental controls.
- The simultaneous knockdown of Su(var)3-9 and BRP does not recover the rebound response upon SD, but partially rescues the baseline sleep level. However, *RNAi* flies appeared arrhythmic.
- The simultaneous knockdown of Su(var)3-9 and Syn does not rescue any effects previously observed in Su(var)3-9 *RNAi* flies.
- The knockdown of G9a in pan-neurons and more specifically in ExFl2 cells, also abolishes rebound sleep upon SD, suggesting a potential collaboration between G9a and Su(var)3-9 in the role of homeostatic sleep regulation. On the other hand, the knockdown of Eggless does not disturb sleep.

3.5 Perturbations in sleep and consequential effects in H3K9me

3.5.1 Sleep deprivation and H3K9me

Thus far we proceeded by introducing perturbations at the H3K9me level, knocking down the three main histone modifiers involved in the deposition of this marker, and then

looking at the sleep behavior upon KD. This strategy allowed me to identify in Su(var)3-9 mainly, but also in G9a, two more players involved in sleep control. In section 1.3.6, I described how histone modifications can be easily influenced by external factors in the environment. Therefore, as opposed to what was performed so far, I also asked whether perturbations in sleep may modulate and change the level of the H3K9me, in the brain of adult flies. Although it is already clear how sleep disturbances, such as sleep deprivation, can change epigenetic markers, like histone acetylation, the impact on H3K9 histone methylation has not been explored, yet.

In order to better elucidate this area, I subjected wild-type flies (Canton-S) to different lengths of sleep deprivation: 1,3,5 and 7 days of SD. On the day when the SD was meant to end, I immediately dissected the adult fly brains, avoiding flies to recover from the loss of sleep by rebound response, and avoiding any restoration of accumulated synaptic strengths. Appropriate controls of flies not sleep-deprived were also dissected in order to make opportune comparisons. After dissection, adult brains were immuno-stained with two antibodies: anti-BRP and anti-H3K9me. Confocal projections of frontal brains are shown in Figure 3.27: as expected positive control for the sleep deprivation, the level of BRP increased, almost in parallel to the length of the SD. This is pretty much in line with previous findings from Gilestro et al 2009, where they found an accumulation of synaptic markers, such as BRP, upon SD, because of the net strengthening of synapses (Gilestro, Tononi, and Cirelli 2009). As mentioned above, I also stained the same brains with ab anti-H3K9me: as expected and reported in Figure 3.27B, this shows colocalization with DAPI, in the nuclei of the cell bodies. Here, the circumstances were very interesting: acute SD, given by 1 or 3 days of mechanical stimuli, did not impair or change the level of this epigenetic marker. However, a prolonged SD, such as 5 or 7 days, drastically increased the level of H3K9me, upon comparison to control individuals (Figure 3.27C and Table 3.18). These results suggest that only a more chronic perturbation of sleep may modulate the regulation of the gene expression, by increasing the H3K9me and, consequently, increasing the number of down-regulated genes. This hypothesis will be better elucidated and discussed in section 3.5.4, which describes the output from an RNA-seq analysis.

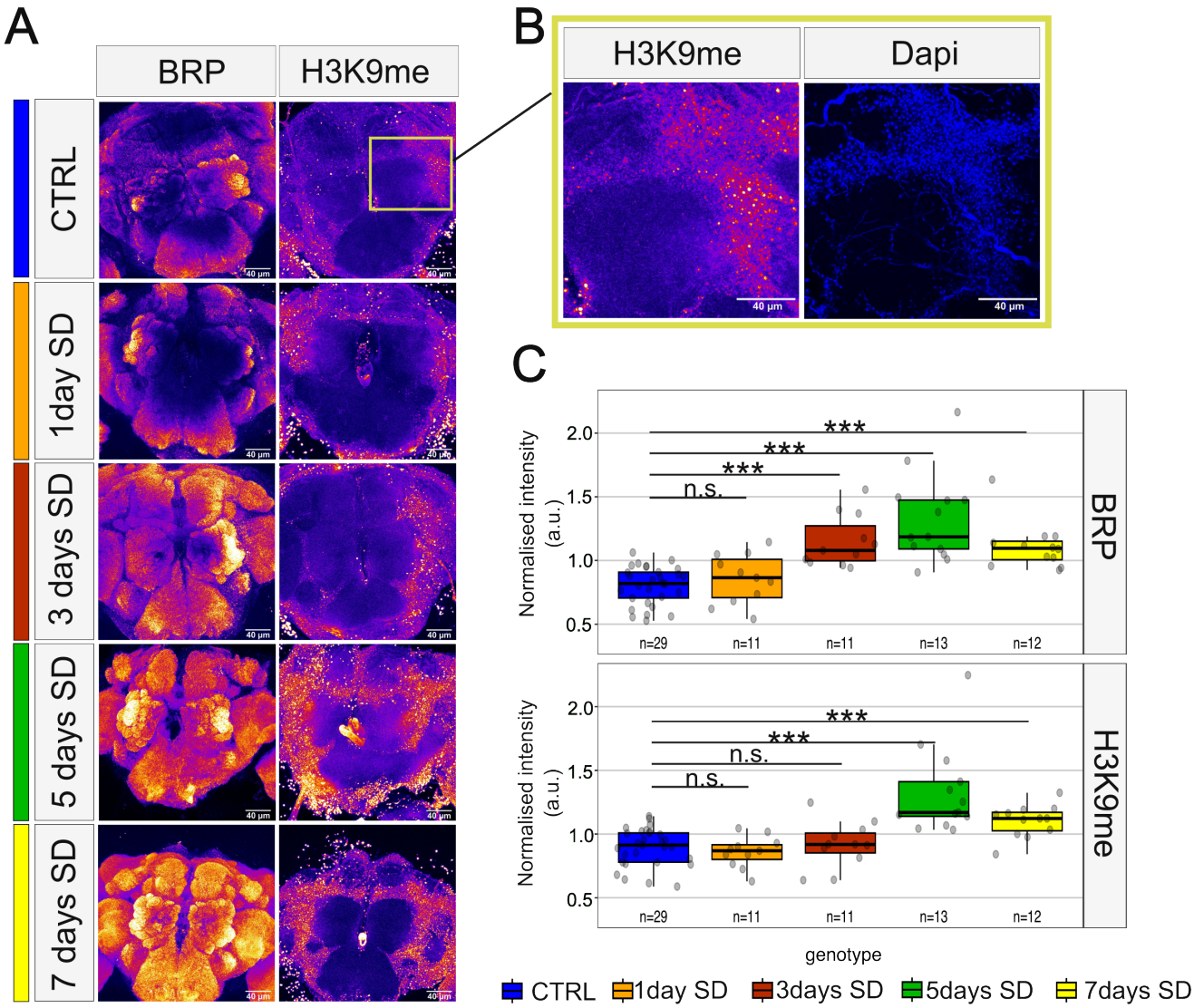


Figure 3.27. Chronic dynamic SD causes an increase in the H3K9me level.
A. Confocal projection of adult front brains stained with ab anti-H3K9me and anti-BRP, in sleep-deprived and control flies. An increased level of both BRP and H3K9me was detected in the brains, upon SD (only chronic SD for H3K9me). **B.** Magnified section of A, showing the colocalization of the signals between H3K9me and DAPI, in the nuclei of the cell bodies. **C.** Quantification of the normalized intensity, where *** indicates p<0.001; n.s p>0.05, from pairwise Wilcoxon rank sum test.

Table 3.18. Statistical differences in BRP and H3K9me intensity, between CTRL and SD groups.

BRP intensity			H3K9me intensity		
Genotype1	Genotype2	p-value	Genotype1	Genotype2	p-value
CTRL	1 day SD	0.53102	CTRL	1 day SD	0.49527
CTRL	3 days SD	3.6e-06	CTRL	3 days SD	0.84457
CTRL	5 days SD	2.5e-07	CTRL	5 days SD	3.2e-07
CTRL	7 days SD	0.00072	CTRL	7 days SD	0.00040

3.5.2 H3K9me in *fumin* mutants

From the previous section, I found that only a prolonged dynamic SD leads to a higher level of H3K9me. This exciting finding was never discovered or remotely explored before. Therefore, further experiments were needed to give more confirmation. The first step was to dissect and analyze the adult brains of a short-sleeping mutant, called *fumin*. *fumin*, meaning "sleepless' in Japanese, is characterized by a constant hyperactive behavior, due to a mutation in the *dopamine transporter* gene (*dat*). The loss of *dat* and, consequently, the persistent elevated level of dopamine, is the main responsible of this constant wakefulness. Furthermore, these flies have shown a decreased arousal threshold and no rebound response upon sleep deprivation. Their circadian rhythm, however, was not impaired (Kume et al. 2005; Kume 2006).

The reason why I decided to focus on this specific fly strain is because it represents a genetically driven way of chronic sleep deprivation. Without even stimulating flies by dynamic SD, I dissected adult brains from both *fumin* and Canton-S flies, used as control, and stained again the brains with anti-BRP and anti-H3K9me. As shown in Figure 3.28A-B, *fumin* flies exhibited higher levels of BRP, compared to CS flies. This was already discovered by S.Huang et al. (2020), who were analyzing a few short-sleeping mutants, including *fumin* (S. Huang et al. 2020). Their study found that sleep deficits can cause widespread changes in the organization of synapse molecules, such as BRP, mainly in the presynaptic active zone.

Together with BRP, also the staining for H3K9me has shown increased levels of this epigenetic marker in *fumin* mutants, differently to CS, as shown in Figure 3.28C-D. This result further confirms the idea that chronic sleep deprivation, genetically or dynamically driven, causes higher deposition of this specific histone methylation in the nuclei of the neuronal tissue.

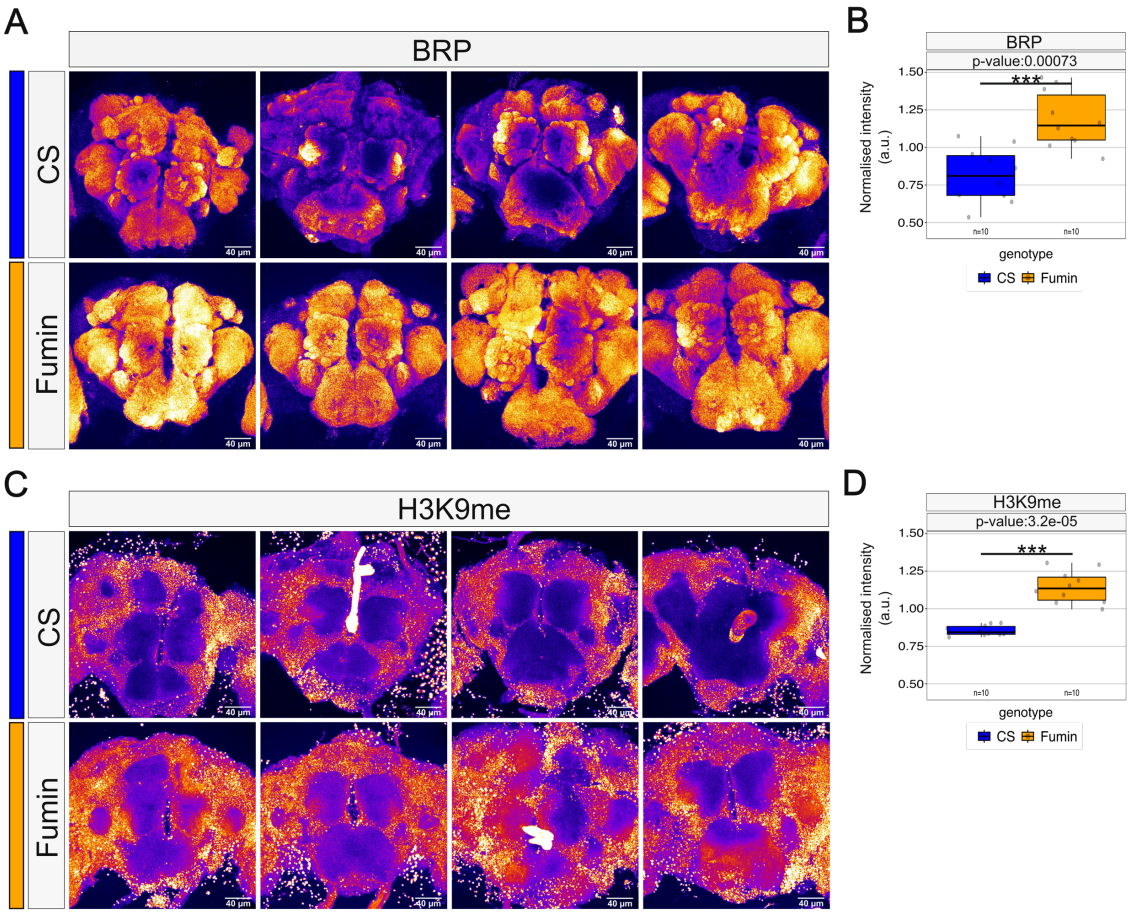


Figure 3.28. *fumin* flies show higher BRP and H3K9me levels.
A-C. Confocal projections of adult front brains from CS and *fumin*, stained with ab anti-BRP (A) and anti-H3K9me (C). An increased level of both BRP and H3K9me was detected in the brains of *fumin* flies. **B-D.** Quantifications of the normalized intensity, where *** indicates $p<0.001$, from pairwise Wilcoxon rank sum test.

3.5.3 The modulation of H3K9me after rebound sleep

Previously, I revealed how the H3K9me level can be modulated by sleep deprivation, in the condition of prolonged stimuli. Indeed, acute SD was not found to affect this specific epigenetic marker. Given these assumptions, the next step was to understand if the increased H3K9me level, triggered by chronic SD, was a temporary or a more permanent change in the chromatin of the neuronal nuclei. In order to investigate so, I subjected CS flies to, at least, 5 days of SD, the shortest period of sleep perturbation essential to induce the change. At the end of the dynamic stimulus, I then allowed flies to fully recover, by giving 1 or 3 days of resting period. Over this time, they could behave normally, probably recovering from sleep loss. Fly adult brains were finally dissected

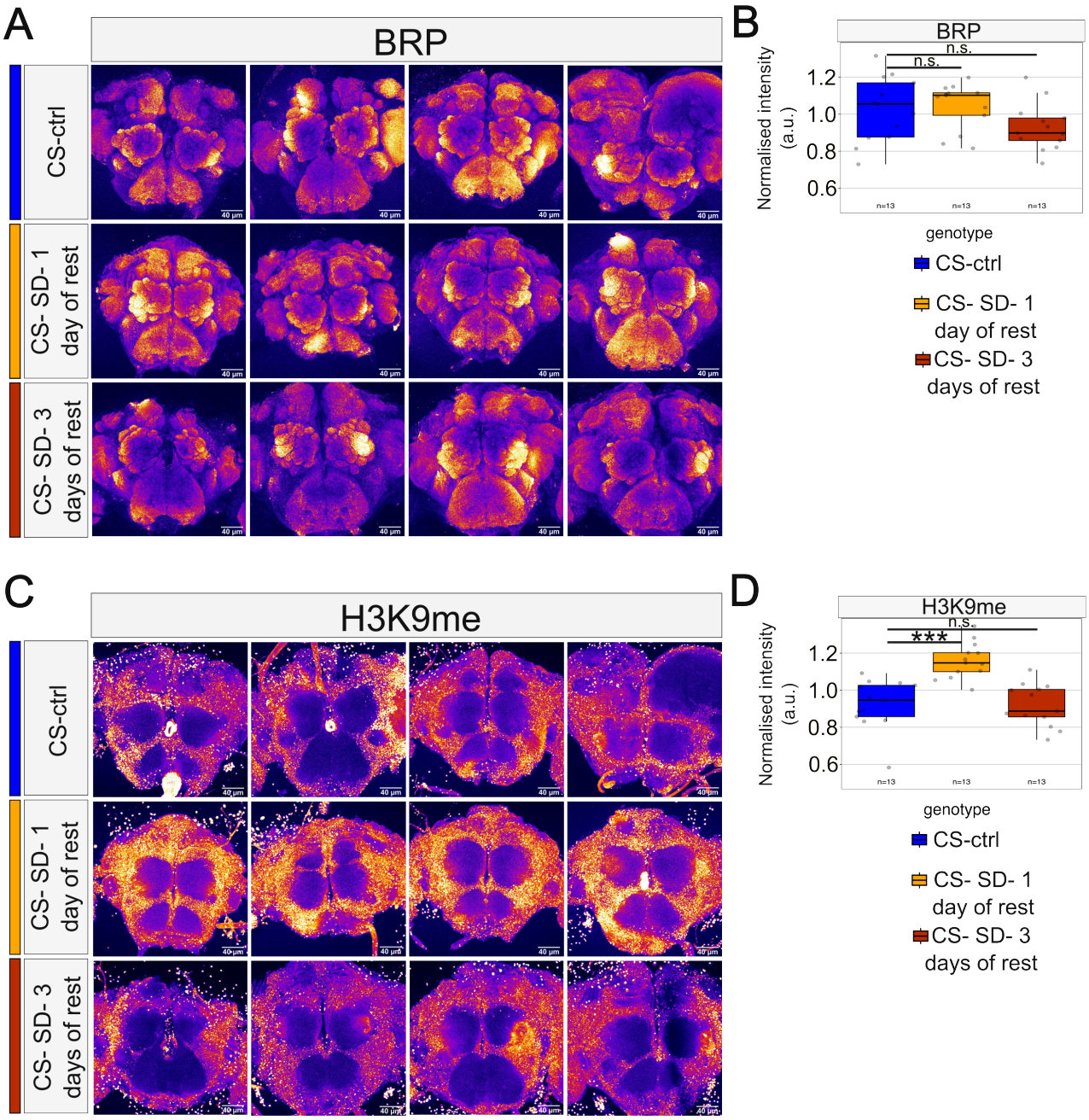


Figure 3.29. CS flies exhibited higher H3K9me levels also upon 1 day of rest, after chronic SD. **A-C.** Confocal projections of adult front brains from CS, stained with ab anti-BRP (A) and anti-H3K9me (C). An increased level of H3K9me was still detected in the neuronal nuclei of flies subjected to SD, followed by 1 day of rest only. **B-D.** Quantifications of the normalized intensity, where *** indicates $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test.

Table 3.19. Statistical differences in BRP and H3K9me intensity, between CTRL and rested groups.

BRP intensity			H3K9me intensity		
Genotype1	Genotype2	p-value	Genotype1	Genotype2	p-value
CTRL	SD-1 day of rest	0.85826	CTRL	SD-1 day of rest	9.7e-05
CTRL	SD-5 days of rest	0.20830	CTRL	SD-5 days of rest	0.81231

at the end of the resting period (1 or 3 days after SD) and immunostaining was performed using ab anti-BRP and anti-H3K9me. Appropriate controls of flies no sleep-deprived were also included, in order to allow suitable comparisons.

One day of rest, following prolonged sleep deprivation, was already enough to restore the usual level of BRP. Indeed, as shown in Figure 3.29A-B, no difference was detected when comparing no SD flies to those who have undergone the actual manipulation. These results were expected, as Gilestro et al. showed similar data: a few hours of recovery upon SD were already sufficient to decline BRP level, suggesting that sleep is fundamental to downregulate the expression of synaptic proteins (Gilestro, Tononi, and Cirelli 2009). Aside from BRP, different was the outcome for H3K9me. Upon chronic SD, one day only of recovery was not enough to re-establish and decline the H3K9me level: these flies still experienced an extremely pronounced increment of this epigenetic marker, as shown in Figure 3.29C-D. However, prolonged SD followed by 3 days of resting period, allowed for full restoration of the usual level of H3K9me: flies subjected to this specific condition did not exhibit differences when compared to controls (see Table 3.19 for statistical comparisons). These data suggest that changes in the H3K9me, due to sleep disturbances, are only temporary and can be easily modulated by allowing individuals to rest and sleep for a longer period than 1 day.

3.5.4 Differential expressed genes (DEGs) upon SD and knockdown of Su(var)3-9

3.5.4.1 Acute vs chronic SD

Thus far I have given new insights about a potential interconnection between H3K9methylation and sleep: perturbations in the main H3K9methyltranferas enzymes, in particularly Su(var)3-9 and G9a, lead to impairments in baseline and homeostatic sleep. On the other hand, sleep deficits due, for instance to chronic sleep deprivation, alter the level of the H3K9me deposition in the brains of SD flies, although only in a temporary way, when long recovery is allowed. In order to further analyze the linkage between sleep and this epigenetic marker, I decided to perform an RNA seq analysis, to look more deeply into the changes in the gene expression. In Figure 3.30, an illustrated scheme of

the strategy applied for this purpose, is shown. 10 days old flies were collected from the following genotypes: CS/ Su(var)3-9 *RNAi* and nSyb-G4/ Su(var)3-9 *RNAi* flies. A sleep

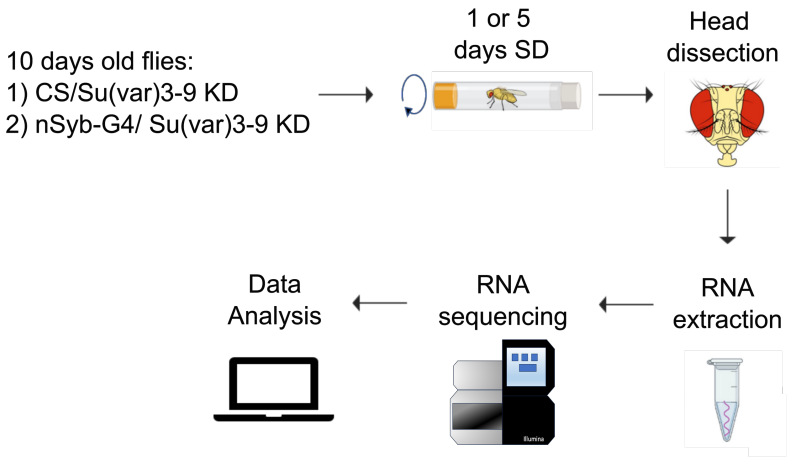


Figure 3.30. Illustration of the RNA seq experiment.
Illustrations are taken from https://commons.wikimedia.org/wiki, created under the Creative Commons license.

deprivation of 1 or 5 days was triggered, in order to mimic both acute and chronic SD. Heads from all conditions, including appropriate controls of flies no sleep-deprived, were collected and RNA was opportunely extracted. Finally, samples were sent for sequencing and data were analyzed, in order to make comparisons and identify potential differentially expressed genes (DEGs). Starting from acute SD, 1 day of sleep deprivation did not seem to affect too much the gene expression in CS/Su(var)3-9 KD flies: only a few genes were de-regulated when comparing SD to no SD individuals (one downregulated and 4 up-regulated genes, Figure 3.31). This outcome is very different from what was previously observed in other studies (Cirelli, LaVaute, and Tononi 2005; Zimmerman et al. 2006), where higher numbers of targets were claimed to be deregulated. However, it is necessary to highlight that many differences can be spotted in the experimental and analysis methods applied in these works, compared to those that have been used in this project. For instance, the sleep deprivation method exploited in Cirelli's study involves the use of a shaker, where flies are constantly subjected to dynamic stimuli. In my study, instead, the SD is given by a more specific stimulus that is only triggered when an individual is detected to be asleep. This avoids any unspecific effect due to habituation or a more stressful environment. In Zimmermann, different was the tissue source, since they extrapolated RNA from brains, while whole heads have been used here, and different was the age of the flies and the time when individuals were sacrificed. All these data, taken together, may explain the disparate results between this and other studies.

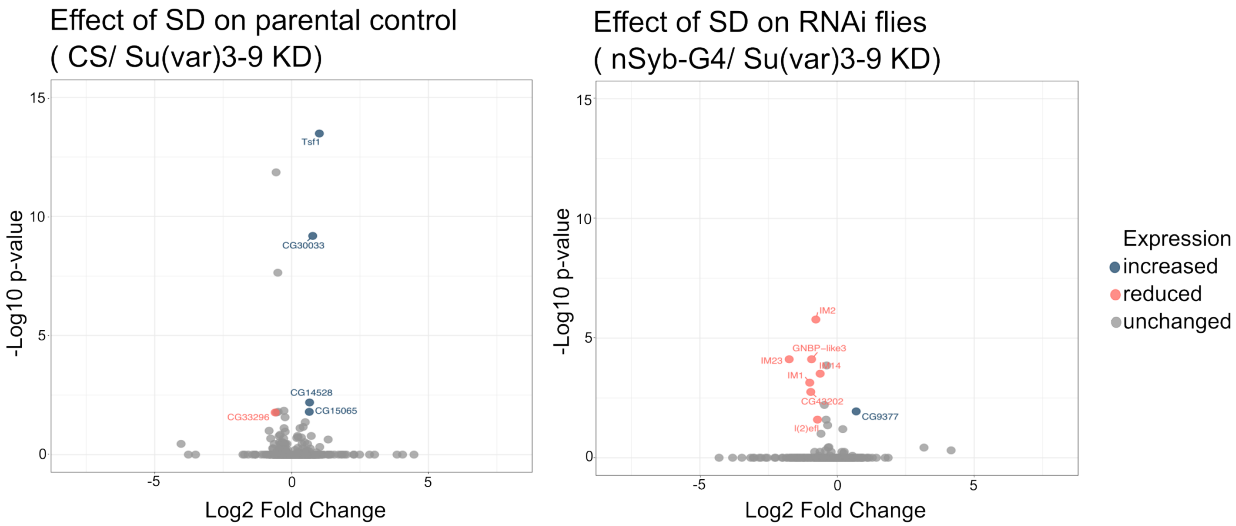


Figure 3.31. RNA-seq in Su(var)3-9 and parental control flies, upon acute SD.
Volcano plots of differentially expressed genes in Su(var)3-9 *RNAi* (right side graph) and control flies (left side graph), after 1 day of sleep deprivation. Red dots are the down-regulated genes, while blue dots are the up-regulated genes. Unchanged genes are shown in grey. The x-axis represents the log2 (fold-change), while the y-axis is the -log10 (p-value) values of the DEGs. Only candidates with p-value < 0.05 and log2 Fold change < -1.5 or >1.5, were considered statistically significant different.

Going back to the results from the RNA seq upon acute SD, not many changes were also detected in the *RNAi* flies: 7 genes were found to be downregulated, while only 1 gene was up-regulated in the SD flies, compared to no SD. Despite the low number of de-regulated genes in both parental control and KD flies, no matches were identified when comparing the targets found to be mis-regulated in the two genotypes. However, in both conditions, most of those targets were mainly involved in defense and immune responses. All the above-mentioned DEGs are listed in Table 3.20. We can conclude that the acute SD impacts the gene expression only minimally: this was true for both *RNAi* and parental control flies.

More interesting was, instead, the result from the RNA seq upon chronic SD. As shown in Figure 3.32, 5 days of sleep deprivation causes the deregulation of many more target genes, compared to the outcome from the acute SD: approximately 85 genes were found to be down-regulated in the parental control CS/ Su(var)3-9 KD, when comparing SD to no SD flies. No up-regulated genes were identified. These exciting results could be possibly connected with my previous findings about the H3K9methylation level. Indeed, previously, I have shown that chronic sleep deprivation can lead to an increased level of

this specific epigenetic sign. Higher H3K9me level could also mean, consequently, higher number of down-regulated genes, which is what is visible from the RNA-seq outcome upon chronic SD. Further analysis, however, is definitely required in the future to validate the interconnection between those results. For instance, a CHIP-seq specific for the H3K9me will be beneficial in order to show that the loci match the lower expressed genes.

Table 3.20. List of DEGs upon acute SD in Su(var)3-9 RNAi and parental controls.

CS/ Su(var)3-9 upon SD	
Up-regulated	Notes
CG14528	Predicted to be involved in protein processing.
CG15065	Predicted to be involved in defense response.
CG30033	Expressed in adult fat body
Tsf1	Involved during the immune response.
Down-regulated	Notes
CG33296	Predicted to enable neurotransmitter:sodium symporter activity.

nSyb-G4/ Su(var)3-9 upon SD	
Up-regulated	Notes
CG9377	Predicted to be involved in proteolysis
Down-regulated	Notes
IM2	Involved in defense response.
GNBP-like3	Involved in defense response to other organism.
IM23	Involved in antibacterial humoral response.
IM14	Involved in antifungal humoral response.
IM1	Involved in defense response.
CG43202	resistance to bacterial infection
l(2)efl	Predicted to enable unfolded protein binding activity.

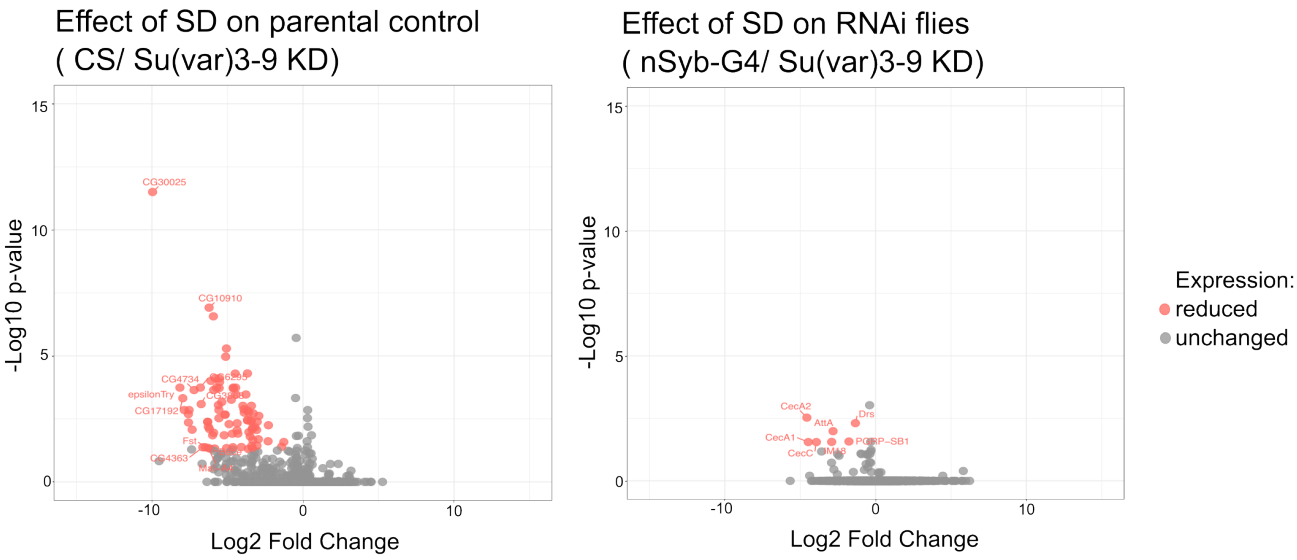


Figure 3.32. RNA-seq in Su(var)3-9 RNAi and parental control flies, upon chronic SD.
Volcano plots of differentially expressed genes in Su(var)3-9 *RNAi* (right side graph) and control flies (left side graph), after 5 days of sleep deprivation. Red dots are the down-regulated genes, while unchanged genes are shown in grey. No up-regulated genes were detected. The x-axis represents the log2 (fold-change), while the y-axis is the -log10 (p-value) values of the DEGs. Only candidates with p-value < 0.05 and log2 Fold change < -1.5 or >1.5, were considered statistically significant different.

In relation to these discoveries, interesting were also the data in the Su(var)3-9 *RNAi* flies. As shown in the right side of Figure 3.32, there are evidently, fewer down-regulated genes (only 7), upon chronic SD, in the KD flies. This could be potentially in line with previous results: given the lower expression of Su(var)3-9 enzyme, *RNAi* flies have exhibited lower levels of H3K9me, even in baseline conditions. This could mean that, upon chronic SD, these flies cannot respond by elevating the H3K9me level, which as a consequence, cannot lead to an increase in the number of downregulated target genes. However, as already mentioned, this remains a supposition and further experiments are required for a better validation.

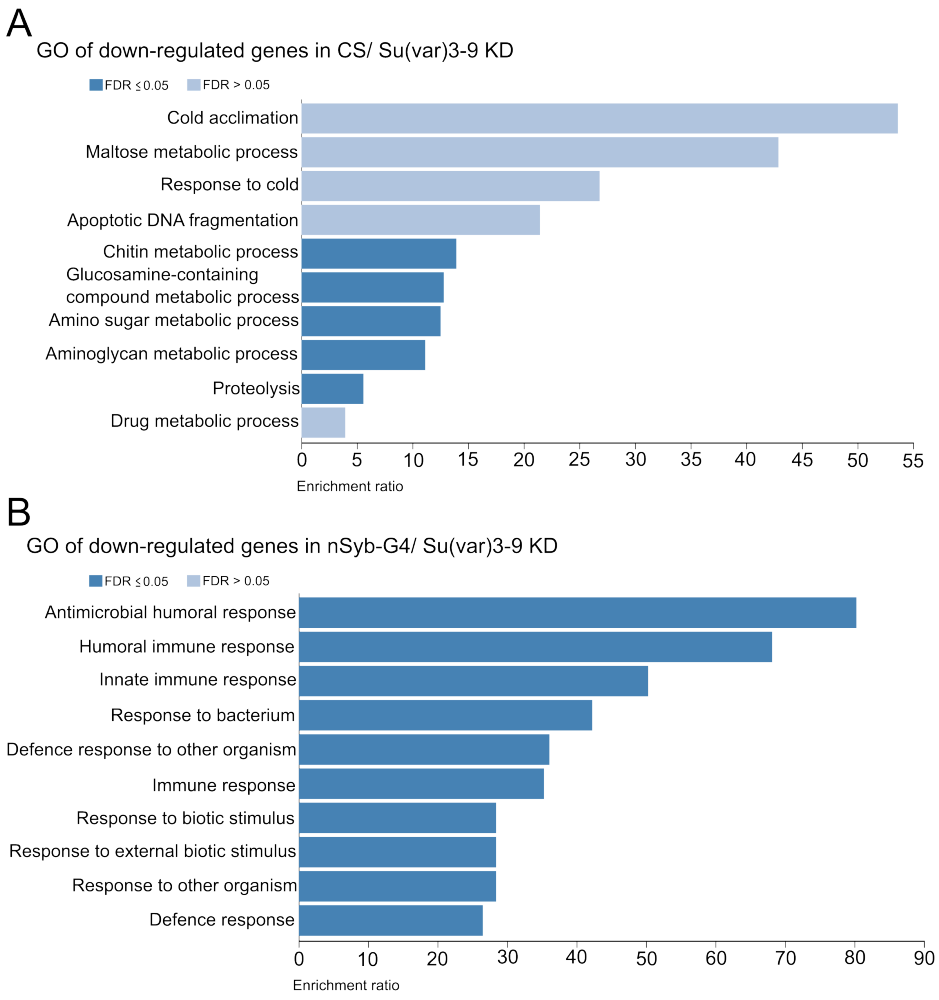


Figure 3.33. Gene Ontology enrichment analysis of down-regulated differentially expressed genes, upon chronic SD.
GO for BP (biological processes) in CS/Su(var)3-9 KD (A) and nSyb-G4/Su(var)3-9 KD (B). The bars represent distinct ontology categories, and the length relies on the number of DEGs included per category. The two graphs were generated using the WebGestalt tool.

In order to explore a bit more in detail the biological processes in which these genes are involved, I also performed some Gene Ontology (GO) analysis: Figure 3.33 shows that in CS/Su(var)3-9 flies, the de-regulated genes are mainly involved in metabolic processes. In the *RNAi* flies (Figure 3.33B), instead, all the targets entirely relate to immune or general defense response. These last data remind the outcome from the RNA seq in the KD flies, upon acute SD, suggesting that immune response is probably not influenced by the H3K9methylation.

Taken together these results support the idea that only a more prolonged SD can bring severe effects on the regulation of gene expression in control flies, while acute SD does not have a dramatic impact. This may support a study from 2019 (Noya et al. 2019), according to which the mRNA level, mainly of synaptic transcripts, is controlled by the circadian rhythm, while their proteins are regulated in a post-transcriptional manner, by the sleep-wake cycles, depending therefore, on the activity-rest period of the individual. Collectively, this may suggest that the acute SD does not modulate the transcript level, but it probably influences the translation of the synaptic proteome, in relation to the vigilance state. However, when prolonging the period of sleep deprivation, it is plausible that circadian and homeostatic drives interact, communicating with each other and influencing the mRNA level, under higher sleep pressure.

Overall, my study provides new insights into the dynamic interplay between H3K9 methylation and sleep, where I show that only chronic SD significantly impacts H3K9me, leading to alterations in gene expressions. However, we cannot exclude all the pleiotropic effects of histone methylation extending beyond sleep, potentially influencing cognitive function, metabolic processes, mood and psychiatric disorders, and developmental and aging processes. Understanding these pleiotropic phenotypes and their interactions can provide a comprehensive insight into the multifaceted role of histone methylation in health and disease, highlighting the importance of considering these factors in sleep research and therapeutic interventions.

3.5.4.2 The effects of Su(var)3-9 knockdown in DEGs

Finally, I also analyzed the RNA seq data to monitor the effect of the knockdown of Su(var)3-9, by comparing the *RNAi* with the parental control, upon baseline condition (no

SD). The pan-neuronal *RNAi* of Su(var)3-9 leads to a down-regulation of 522 genes and an up-regulation of 265 genes. After performing GO analysis, shown in Figure 3.34, it was clear that the main affected biological processes were involved in metabolism regulation. Among the down-regulated GO categories, I found of particular interest, the polyamine metabolic process category (Figure 3.34C).

Polyamines are organic compounds, specifically, polycationic alkylamines, usually produced from L-ornithine or from decarboxylation of amino acids. They are involved in several biological processes, such as the synthesis and stabilization of both nucleic acids and proteins, cell signaling, cell growth, development, etc. (Hesterberg, Cleveland, and Epling-Burnette 2018; Pegg 2016). The most important polyamines are putrescine (put), spermidine (spd) and spermine (spe). Very recently, it has been found that polyamine can influence behaviors, such as sleep. For instance, Bedont et al (2023) show that both

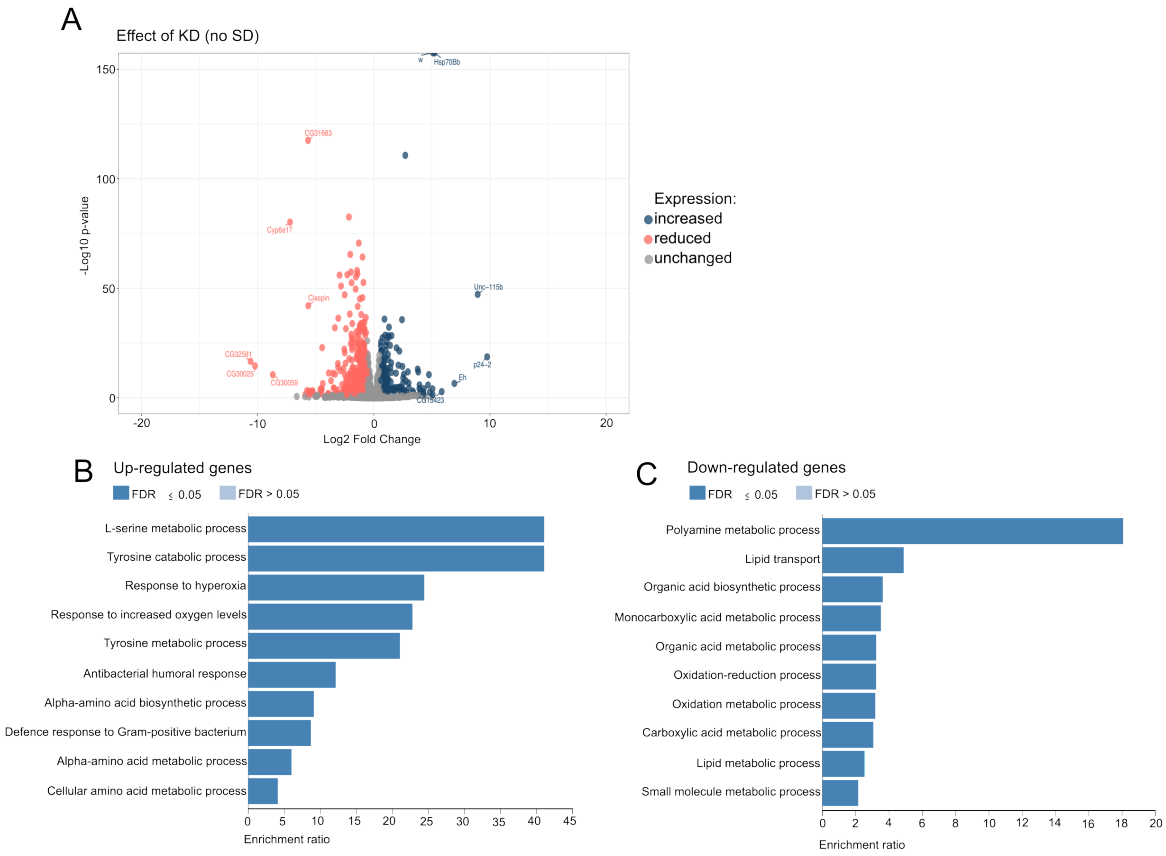


Figure 3.34. The Su(var)3-9 knockdown in pan-neurons causes the deregulation of many genes.
A. Volcano plot of differentially expressed genes, upon KD of Su(var)3-9. Red dots are the down-regulated genes, while blue dots are the up-regulated genes. Unchanged genes are shown in grey. The x-axis represents the log2 (fold-change), while the y-axis is the -log10 (p-value) values of the DEGs. Only candidates with p-value < 0.05 and log2 Fold change < -1.5 or >1.5, were considered statistically significant different. **B-C.** GO in BP (biological processes) for up-regulated genes(A) and down-regulated genes (B).

The bars represent distinct ontology categories, and the length relies on the number of DEGs included per each category. The two graphs were generated using the WebGestalt tool.

knockdown and mutation of the spermidine synthase (spds), the enzyme converting putrescine to spermidine, increases total sleep (Bedont et al. 2023). Furthermore, flies fed with putrescine exhibit a similar increase in sleep, suggesting that this specific polyamine is probably one of the main players in sleep regulation. This study was also in line with other previous results, such as Kanaya et al. 2020, showing sleep gain upon knockdown of the ornithine aminotransferase (oat) (Kanaya et al. 2020).

Given all these assumptions, very interestingly, Su(var)3-9 *RNAi* flies exhibited down-regulation of several components of the polyamine pathway, including *spds* or *oat*. As suggested by Bedont, it is plausible that the deregulation of polyamines may have contributed to change the baseline sleep level in the *RNAi* flies. It is good to remind at this stage, that the knockdown of Su(var)3-9 causes an increase of baseline sleep, following the results from the knockdown of *spds* or *oat*, above mentioned to lead to higher baseline sleep, too. More investigations are required in the future, in order to test if polyamines and Su(var)3-9 are truly connected and responsible for sleep regulation.

3.5.5 The influence of chronic SD in Su(var)3-9 level

Thus far I have given new insights about a potential interconnection between H3K9methylation and sleep: in particular, I have shown how a more prolonged sleep deprivation leads to an increased level of this specific epigenetic marker. The next step was then, to understand if the methyltransferase enzymes, specifically Su(var)3-9, could mirror the level of H3K9me. Given the increase of H3K9me, I hypothesized that the activity of Su(var)3-9 needed to be even more intense, over chronic SD. In order to validate this hypothesis, I decided to monitor and quantify by immune-staining, Su(var)3-9 protein, in the brains of chronically sleep-deprived flies. Unfortunately, it was not possible to find any available antibody specific to Su(var)3-9. Therefore, I decided to resort to a different strategy. Instead of subjecting sleep deprivation to Canton-S flies, I performed the experiment using a transgenic fly stock, kindly donated by Brenneke J. This fly has a GFP peptide, fused to the C-terminal of Su(var)3-9. This allows the generation of a Su(var)3-9-GFP fused protein, where Su(var)3-9 can be easily detected

by GFP-staining. A detailed scheme of the transgenic construct used to express the above-mentioned protein is reported in Figure 3.35.

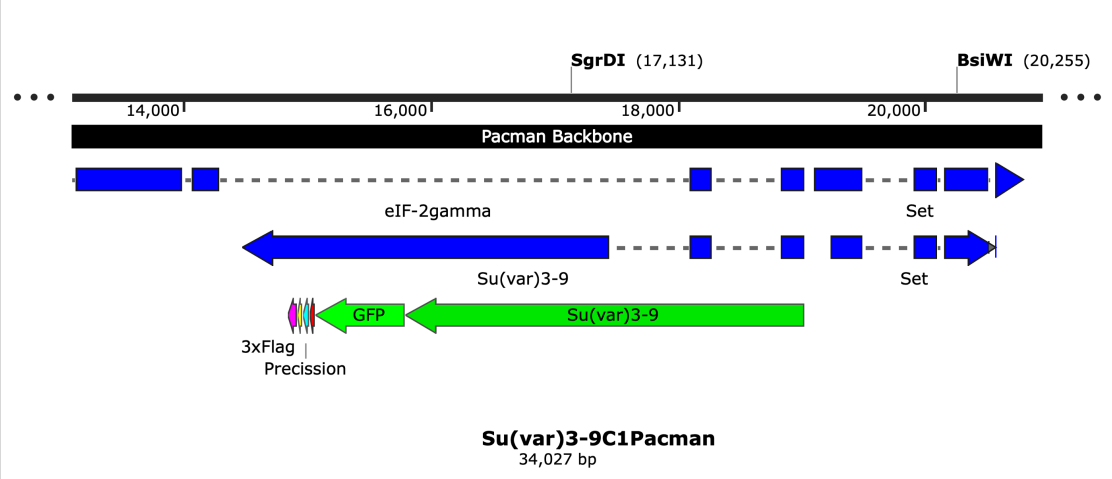


Figure 3.35. Transgenic construct to generate Su(var)3-9-GFP fused protein.
Partial map of the Pacman clone CH322-164L18, containing the Su(var)3-9 locus with C-terminal GFP_Precission_V5_3xFLAG. Reproduced from SnapGene software (www.snapgene.com).

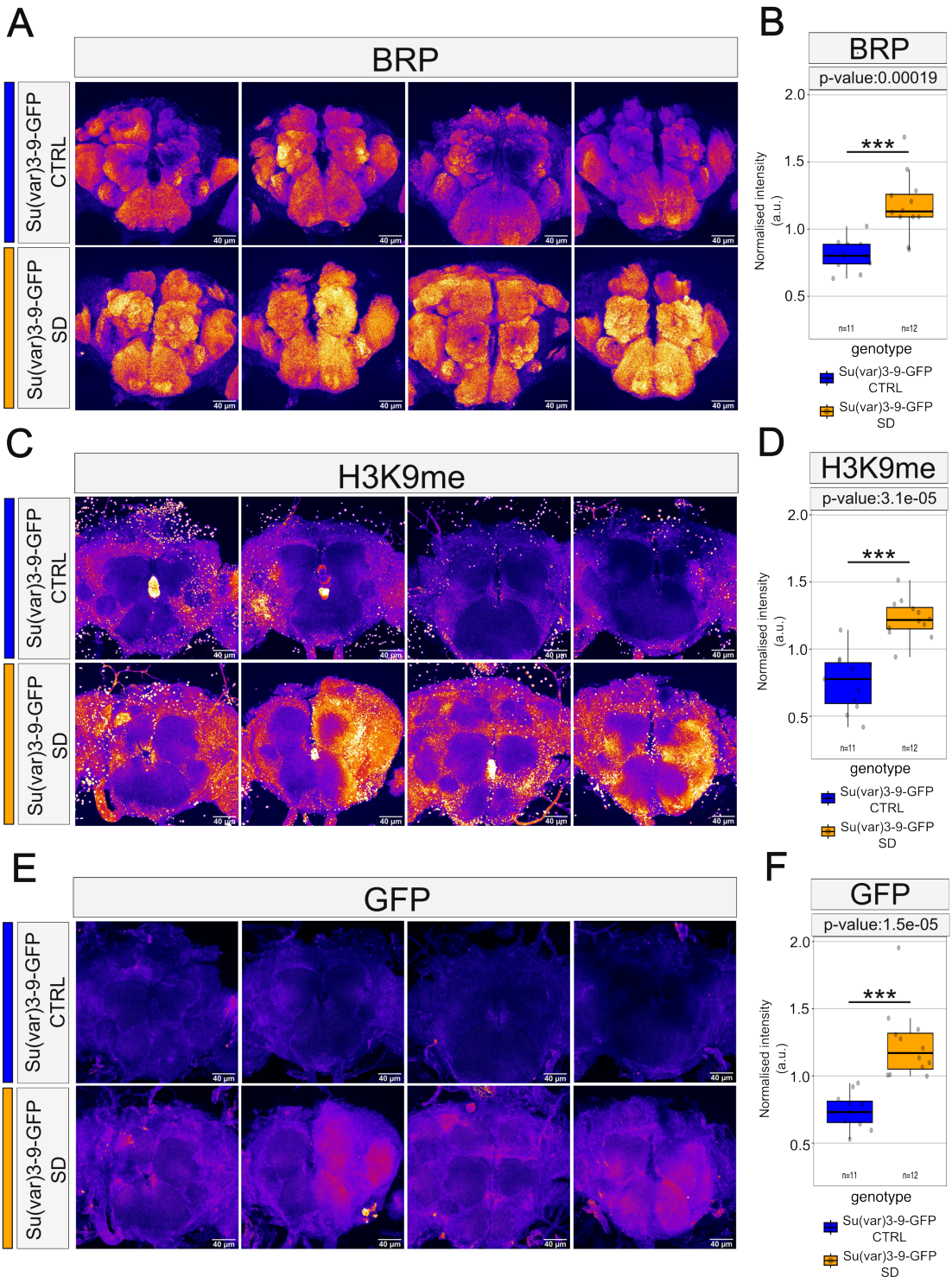


Figure 3.36. Su(var)3-9-GFP tagged flies exhibited higher BRP, H3K9me, and Su(var)3-9 protein levels upon chronic SD.
A,C,E. Confocal projections of adult front brains from Su(var)3-9-GFP tagged flies, stained with ab anti-BRP (A), anti-H3K9me (C) and anti-GFP (E). An increased level of all three targets was detected in the brains of flies subjected to SD. **B-D-F.** Quantifications of the normalized intensity, where *** indicates $p<0.001$, from pairwise Wilcoxon rank sum test.

Su(var)3-9-GFP flies were, therefore, sleep deprived for 5 days, and adult brains were dissected and immune-stained using ab anti-GFP, together with anti-H3K9me and BRP,

as positive controls for the SD. As shown in Figure 3.35 (A-B-C-D), SD flies exhibited higher BRP and H3K9me levels, as expected. In addition, the level of Su(var)3-9 was detected to be increased: this result suggests that, over prolonged SD, there might be a higher activity of Su(var)3-9, generated by an increase of its protein level (Figure 3.36 E-F).

Besides the increased protein level, I wanted to further dissect the impact that chronic SD has on Su(var)3-9 transcripts regulation. Data from RNA seq showed that the mRNA level of Su(var)3-9 was not impaired upon chronic SD, in the parental control CS/ Su(var)3-9 KD. It is fundamental to remember that the RNA seq was performed in whole heads: in order to further validate this result, I decided to quantify by qPCR, the transcript level in brain tissues. As shown in Figure 3.37, together with Su(var)3-9, I decided also to monitor BRP mRNA level: in both cases, no change was detected when comparing

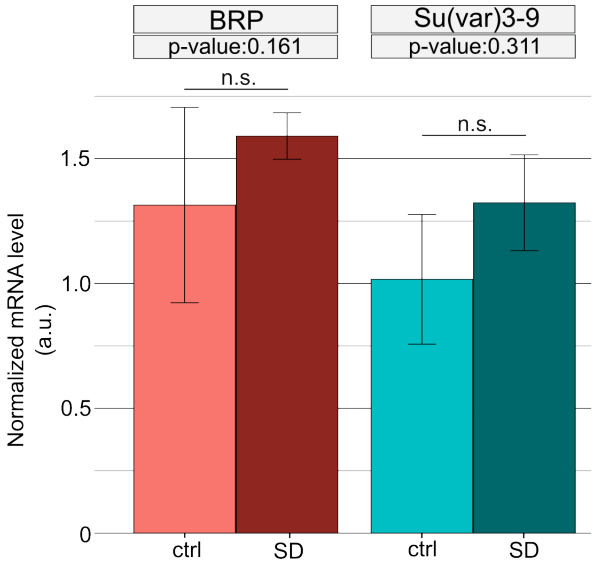


Figure 3.37. qPCR in adult brains of CS flies, upon chronic SD.
Transcript levels of BRP and Su(var)3-9 are shown, respectively in red and blue bars (no SD in light color, SD in dark color). No changes were detected when comparing SD to no SD flies. Data shown is from two biological replicates: per each replicate, 50 brains were dissected in each condition.

SD to no SD condition. This outcome is in line with the work from Nova et al. 2019, already mentioned previously, where they found that, while transcript expression is modulated by the circadian clock, sleep/wake cycles influence the protein expression via a post-transcriptional regulation (Noya et al. 2019). The strong sleep pressure generated

by the chronic SD may be, therefore, the main player responsible for the increase of BRP and Su(var)3-9 proteins, leaving their transcript levels in a baseline condition.

3.5.6 Behavioral effects upon inhibition of Su(var)3-9-expressing cells

Given the evident correlation between Su(var)3-9 and sleep, I wanted to focus more on detail in the identification of the brain areas where Su(var)3-9 is mostly expressed. In this regard, I was able to find a GAL4 line, which is driving the expression of GAL4 specifically in the cells expressing Su(var)3-9. The first step was to cross this line to an UAS-mcd8-GFP line (membrane-bound GFP reporter gene), to allow the Su(var)3-9 visualization by immunostaining, using ab anti-GFP. Figure 3.38 shows the BRP and GFP colocalization. Su(var)3-9 seems to be widely expressed, showing neuronal projections into specific recognizable regions. For instance, it is clear the expression in some lobes of the MB (seen in section 3.3.4 to have a partial sleep phenotype upon Su(var)3-9 KD in this region) and in the insulin-producing cells (ILPs), implicated in metabolic homeostasis and life span regulation, and in response to different types of stress (Broughton et al. 2005; Clancy et al. 2001). In particular, the expression pattern of 3 to 7 DILPs (2-3-5) resembles the expression pattern of Su(var)3-9 shown in Figure 3.38: mainly in the dorsal protocerebrum and the dorsal part of the median bundle (Giannakou and Partridge 2007). Interestingly, from the RNA seq analysis, most of the ILPs were detected to be de-regulated upon knockdown of Su(var)3-9: in particular, ILP-2,3 and 5 were found to be downregulated, while ILP-6 was up-regulated. Ablation of ILPs was found in another work (Cong et al. 2015), to decrease total sleep in mutant flies: unfortunately, this result seems not to be in line with my results (probably given the different circumstances), suggesting that the sleep phenotypes observed in Su(var)3-9 KD, are not depending on the insulin-pathway. Indeed, from section 3.3.1, I have shown that the specific KD of Su(var)3-9 in the sNPFs neurons, known to be implicated in insulin signaling, did not impair sleep in *RNAi* flies, further excluding this pathway from my investigation.

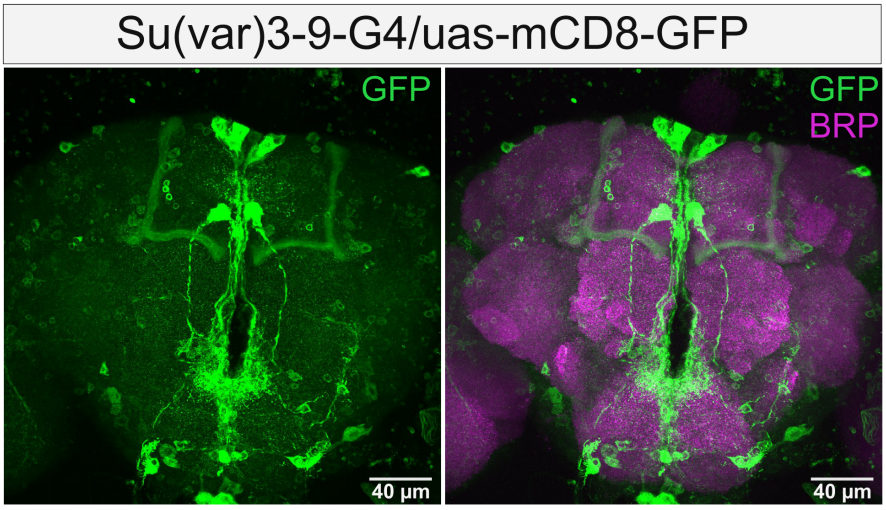


Figure 3.38. Expression pattern of Su(var)3-9-GAL4.
Confocal projections of adult front brains in Su(var)3-9-GAL4/ uas-mCD8-GFP, stained with ab anti-GFP (green) and anti-BRP (magenta).

Finally, from Figure 3.38, other cells around the subesophageal ganglion zone (SEZ) were also visualized, although it was not possible to precisely define the identity of these set of cells.

The next step was then, to test the Su(var)3-9-GAL4 line: the goal was to inhibit the neurons covered by this driver and record the behavior of the flies, to see if there was any recapitulation of the sleep-phenotypes observed upon knockdown of Su(var)3-9. The strategy exploited was to over-express the thermosensitive allele *shibire*^{ts} (Shi^{ts}), a GTPase required for endocytosis, which allows synaptic transmission at <22° C, but reduces neurotransmitter release at 29°C, causing synapse inhibition (Damke et al. 1994; Kosaka and Ikeda 1983; Duffy 2002b). The idea was to trigger the inhibition of synapse release in the adult phase: flies were, therefore, raised at 18°C and only for experimental recording, they were given 1 day of baseline sleep at 22°C and three days at 29°C, during which sleep deprivation of 1 day was also subjected, in order to validate any homeostatic sleep effect. As shown in Figure 3.9 C-D, at 22°C, when synapse blockage was technically not enhanced yet, Su(var)3-9-GAL4/uas-Shi^{ts} flies already experienced higher baseline sleep, compared to the parental controls: indeed, both male and female showed higher sleep, specifically during the light phase. After increasing the temperature to 29°C, the rise in total sleep was more pronounced in female flies: they slept more than controls over the whole day, during the day and night time (Figure 3.39E-F). This result recapitulates

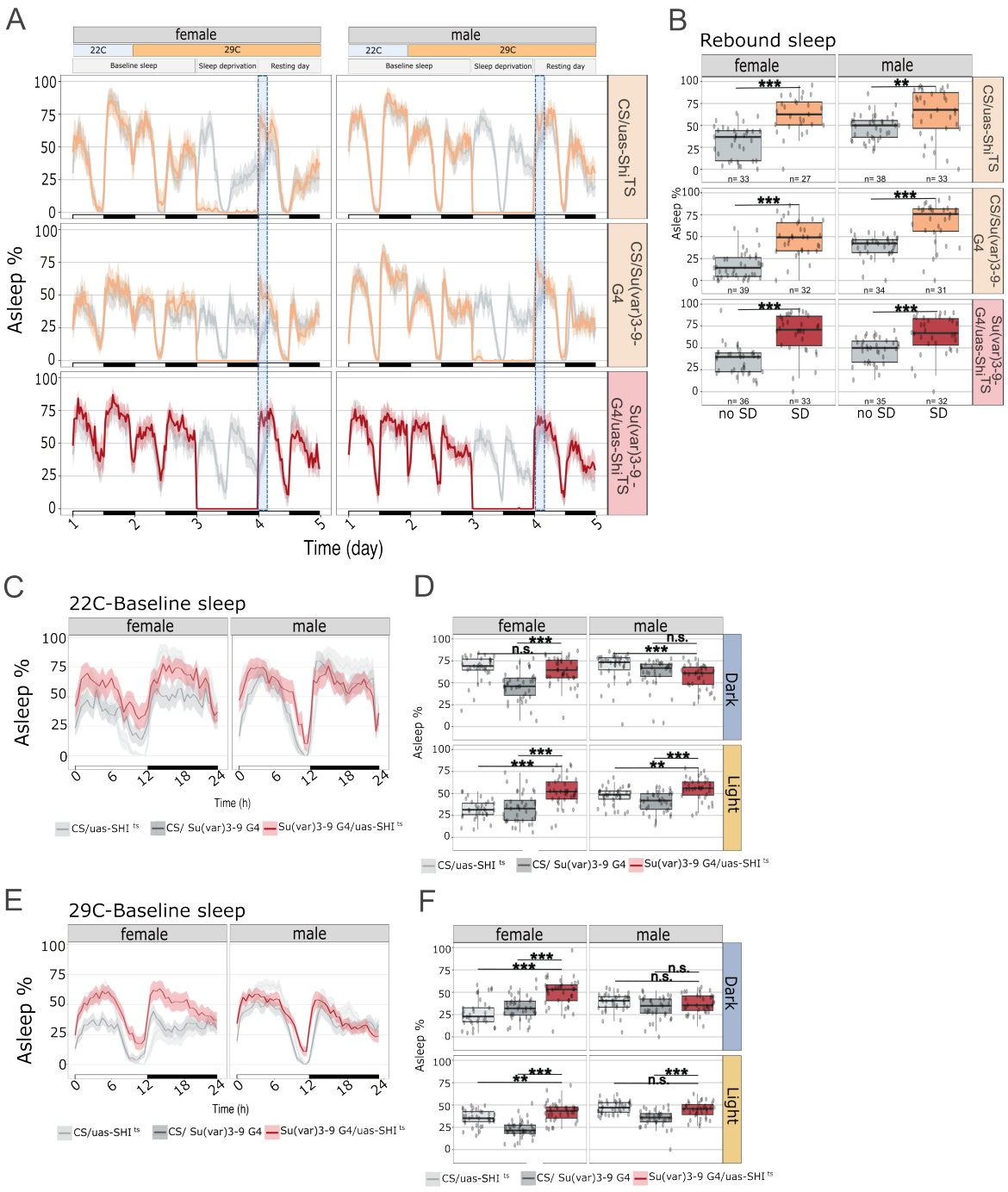


Figure 3.39. The inhibition of Su(var)3-9-expressing cells only partially resembles Su(var)3-9 *RNAi* effects.

A. Ethogram over three days in a row, where sleep deprivation occurs on the second day. Per each genotype, two groups of flies are shown: undisturbed control group (grey line) and sleep-deprived group (colored line). Rebound sleep is highlighted in blue. Female and male flies were analyzed separately. Intermediate lines indicate the mean while shaded areas represent the bootstrapped 95% confidence interval. **B.** Quantification of rebound sleep during the first 3 hours of the early morning, following SD. Statistically significant changes in sleep rebound have been found in all the genotypes, after comparing SD to no SD groups. **C-E.** Ethogram wraps (average) of baseline sleep in no SD flies, before (C) and after (E) the increase of temperature**. D-F.** Quantification of the total baseline sleep, in relation to Figures C and E, respectively. Light and dark phases are analyzed separately. In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from two replicates and the *N* of the animals per each group is reported within the figure.

Table 3.21. Statistical differences between SD and no SD flies in rebound sleep, and between genotypes in baseline sleep, ± inhibition of Su(var)3-9-expressing cells.

female, sleep during rebound			**male, sleep during rebound**		
no SD	SD	p-value	no SD	SD	p-value
CS/UAS-shi^{ts}	CS/UAS-shi^{ts}	4.1e-06	CS/UAS-shi^{ts}	CS/UAS-shi^{ts}	0.00314
CS/ Su(var)3-9-G4	CS/ Su(var)3-9-G4	1.8e-07	CS/ Su(var)3-9-G4	CS/ Su(var)3-9-G4	1.6e-05
UAS-shi^{ts} / Su(var)3-9-G4	UAS-shi^{ts} / Su(var)3-9-G4	1.7e-06	UAS-shi^{ts} / Su(var)3-9-G4	UAS-shi^{ts} / Su(var)3-9-G4	0.00042
female, baseline sleep 22°C, light phase			**male, baseline sleep 22°C light phase**		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/UAS-shi^{ts}	UAS-shi^{ts} / Su(var)3-9-G4	8.2e-07	CS/UAS-shi^{ts}	UAS-shi^{ts} / Su(var)3-9-G4	0.00248
CS/ Su(var)3-9-G4	UAS-shi^{ts} / Su(var)3-9-G4	4.9e-05	CS/ Su(var)3-9-G4	UAS-shi^{ts} / Su(var)3-9-G4	6.0e-06
female, baseline sleep 22°C, dark phase			**male, baseline sleep 22°C, dark phase**		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/UAS-shi^{ts}	UAS-shi^{ts} / Su(var)3-9-G4	0.26596	CS/UAS-shi^{ts}	UAS-shi^{ts} / Su(var)3-9-G4	0.00013
CS/ Su(var)3-9-G4	UAS-shi^{ts} / Su(var)3-9-G4	4.6e-05	CS/ Su(var)3-9-G4	UAS-shi^{ts} / Su(var)3-9-G4	0.12677
female, baseline sleep 29°C, light phase			**male, baseline sleep 29°C, light phase**		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/UAS-shi^{ts}	UAS-shi^{ts} / Su(var)3-9-G4	0.00869	CS/UAS-shi^{ts}	UAS-shi^{ts} / Su(var)3-9-G4	0.26386
CS/ Su(var)3-9-G4	UAS-shi^{ts} / Su(var)3-9-G4	1.3e-10	CS/ Su(var)3-9-G4	UAS-shi^{ts} / Su(var)3-9-G4	0.00016
female, baseline sleep 29°C, dark phase			**male, baseline sleep 29°C, dark phase**		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/UAS-shi^{ts}	UAS-shi^{ts} / Su(var)3-9-G4	4.9e-07	CS/UAS-shi^{ts}	UAS-shi^{ts} / Su(var)3-9-G4	0.42091
CS/ Su(var)3-9-G4	UAS-shi^{ts} / Su(var)3-9-G4	1.0e-05	CS/ Su(var)3-9-G4	UAS-shi^{ts} / Su(var)3-9-G4	0.19311

the same effect observed upon Su(var)3-9 *RNAi* in female baseline sleep. However, strange was the situation for males: in fact, after switching the temperature from 22 to 29°C, no increase in sleep was detected when comparing Su(var)3-9-GAL4/uas-Shi^{ts} flies to controls. In addition, upon 1 day of sleep deprivation, flies from all conditions were able to respond to the dynamic stimulus, by increasing rebound sleep during the following early morning (Figure 3.39A-B). Statistical comparisons are reported in Table 3.21.

These data taken together suggest that the synapse blockage of Su(var)3-9-expressing neurons, only partially recapitulate the sleep impairments observed mainly in the baseline sleep of Su(var)3-9 KD flies. Furthermore, no similarities were found in terms of homeostatic sleep deficits. One explanation could be that, besides the reduced neurotransmitters released in Su(var)3-9-GAL4/uas-Shi^{ts} flies, Su(var)3-9 protein is still

fully functional in this condition, meaning that no differentially expressed genes should be detected if comparing to parental controls. Therefore, DEGs in Su(var)3-9 *RNAi* flies may explain the deregulation of molecular pathways involved for instance in sleep homeostasis regulation. Further investigations are required in the future to better elucidate this missing piece.

3.5.7 Summary

- Chronic, but not acute, sleep deprivation, increases the level of H3K9methylation in the nuclei of neuronal cell bodies, compared to not-sleep-deprived fly brains.
- *fumin* mutant flies, known to exhibit a constant hyperactive behavior, also showed higher levels of H3K9methylation in the neuronal tissue, compared to Canton-S flies.
- The increase of H3K9methylation upon chronic SD is a reversible phenomenon: 3 days of rest, following 5 days of SD, are sufficient to restore the normal level of this epigenetic mark.
- The pan-neuronal *RNAi* of Su(var)3-9, interferes with the process of decrease in gene expression, normally observed upon chronic (but not acute) sleep deprivation in control flies.
- The pan-neuronal *RNAi* of Su(var)3-9 leads to a down-regulation of 522 genes and up-regulation of 265 genes, targets mainly involved in the metabolic processes, such as polyamine regulation processes.
- Chronic SD enhances the protein (and not mRNA) level of Su(var)3-9.
- The synapse blockage of Su(var)3-9-expressing neurons, only partially recapitulates the sleep impairments observed in the baseline sleep of Su(var)3-9 KD flies. Furthermore, no similarities were found in terms of homeostatic sleep deficits.

Chapter 4
Results: the role of DNA damage/repair in sleep

4.1 Introduction

Sleep is evolutionarily conserved across the animal kingdom. Besides this, the precise function of sleep is still not well understood, remaining an uncovered question in the field of neuroscience. Revealing the aim of sleep will be fundamental, not only to further elucidate the molecular pathways driving this phenomenon, but also to elucidate the complex correlations between sleep and aging process, and neurodegenerative diseases.

Among the hypothesized theories in the field, there is one which hypothesizes that sleep is required to re-establish homeostasis in the brain, by repairing all the unbalances occurring over wakefulness. Indeed, as already described in the introduction, the neuronal activity allowing learning and memory formation during the wake phase comes to a cost: the progressive accumulation of synaptic strengths requires more consumption of energy and cellular supplies, which, at the of the day, causes synaptic saturated levels, unfunctional for memory formation. This recalls the need to return to a sustainable level: sleep time is when synaptic strengths are reset (Tononi and Cirelli 2003). In addition, sleep is also thought to clear, the brain from toxins and metabolic waste, accumulated during awake (Xie et al. 2013).

Together with these theories, there is another hypothesis emerged very recently, which hypothesizes that DNA damage may contribute to driving sleep. Indeed, neuronal activity during enriched wakefulness can cause DNA double-stranded breaks (DSBs). In both mice and flies, these breaks are believed to alleviate the structural constraints which limit the transcription of genes associated with immediate neural plasticity (Bellesi et al. 2016; Madabhushi et al. 2015). In this context, it is essential to highlight that repairing DNA damages in neurons is much slower, compared to dividing cells, suggesting that these

damages may easily accumulate in the brain (Nouspikel and Hanawalt 2000). Several studies in various animals, such as zebrafish, mice, and partially in flies as well, have shown that normally during wakefulness there is an accumulation of DNA damages, which drastically decreases over night-time, during sleep (Bellesi et al. 2016; D. Zada et al. 2019; Cheung et al. 2019). A challenging aspect of understanding the purpose of sleep lies in discerning whether it serves as a fundamental function in its own right or if it capitalizes on extended periods of rest for other beneficial purposes. Nevertheless, there is substantial evidence pointing towards a reciprocal relationship between DNA damages and sleep. Zada et al. found that the deliberate induction of DNA double-stranded breaks through pharmacological means causes an increase in sleep in zebrafish. Interestingly, they found that specifically during sleep there is increase of chromosome dynamics which results in facilitating the repair of DSBs. On the other hand, sleep deprivation has been shown to reduce the expression of DNA repair genes and increase the occurrence of DBSs in human blood cells (Cheung et al. 2019; D. Zada et al. 2019). All these findings, taken together, suggest a complex interplay between sleep and the repair of DNA damages: this was mainly explored in mice and zebrafish, while less described in *Drosophila.* With the aim to further investigate the mechanisms underlying the axis sleep-DNA damage, I decided to investigate in more detail this field in flies too, aiming to give more proof of evidence.

4.2 The reciprocal correlation between sleep and DNA damage

4.2.1 Sleep deprivation and the effects in DNA damage

Bellesi et al. already found that an enriched wake environment, full of climbing, social interactions, and flights, can increase the DSBs in fly brains. This method does not preclude that the DNA damage may be the result of other forms of stress and not just the lack of sleep itself. For this reason, the first step was to subject flies to a proper and more specific form of sleep deprivation. As previously described, I combined the ethoscopes with optomotors, devices able to induce the rotation of tubes containing single flies, only when these are detected to be asleep by the computer. The idea was to enhance SD for 1

or 5 days in Canton-S flies, dissecting adult brains immediately at the end of dynamic stimulus, to avoid flies rebounding and resting. Brains tissues were then, immune-stained using ab anti-H2Av and anti-DCP1. H2Av, the only H2A variant in *Drosophila*, represents a chimeric version of H2AX and H2AZ variant families, typical of mammals (Baldi and Becker 2013). As similar as H2AX function, also H2Av is mostly implicated and required in the repair of DNA damages: indeed, in response to DSBs, the C-terminal tail of H2Av is usually phosphorylated at Ser137 (Madigan, Chotkowski, and Glaser 2002). H2Av can be spread in the chromatin, but only the H2Av located close to the damage will be phosphorylated. The lack of H2Av phosphorylation can cause the accumulation of apoptotic marks, as shown by Madigan 2002. Furthermore, as similar as H2AZ, H2Av is also implicated in chromatin-mediated regulation of the transcription (van Daal and Elgin 1992; Santisteban, Kalashnikova, and Smith 2000; Adam et al. 2001). In conclusion, H2Av staining can be used to detect signs of DSBs.

On the other hand, DCP-1 is an effector caspase, one of the first discovered members of the family of ICE/CED-3 proteases, involved in apoptosis and general cell death programming. Very recently was also found to be involved in the autophagy regulation (DeVorkin et al. 2014). Staining for DCP-1 can be exploited to mark any sign of apoptosis.

As shown in Figure 4.1A-B, flies subjected to sleep deprivation, no matter the length of the stimulus, experienced a higher number of DSBs and consequently a high level of H2Av marker: 5 days of SD leads to an even more drastic increase of H2Av, found in the nuclei from brain tissues. A zoomed-in section is shown in Figure 4.1E. Regarding the DCP-1 staining, very interestingly, 1 day of SD did not bring any change, compared to no sleep-deprived individuals. However, a chronic SD, finally causes a higher DCP-1 signal, meaning higher apoptotic marks (Figure 4.1B-C and Table 4.1). These results, taken together, suggest that even a shorter period of SD, such as 1 day, can lead to an increased number of DSBs. However, only the accumulation of many more damages, due to a chronic lack of sleep, may trigger to a caspase cascade, as initiation of apoptosis.

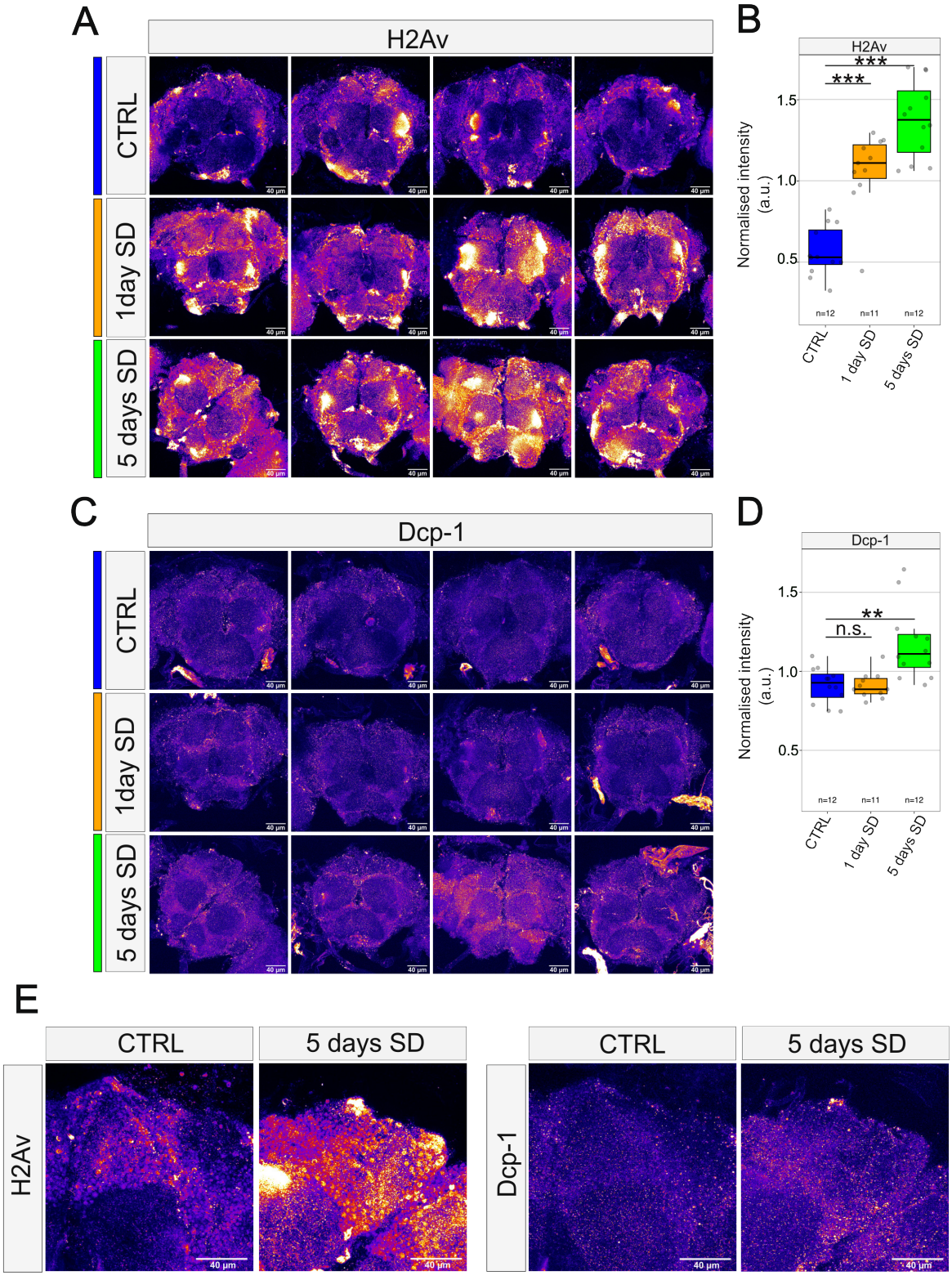


Figure 4.1. General sleep deprivation causes increased number of DSBs, while only chronic SD leads to initiation of apoptosis.
A-C. Confocal projections of adult front brains from CS flies stained with ab anti-H2Av (A), anti-DCP-1 (C). An increased level of both targets was detected in the brains of flies subjected to chronic SD. Acute SD only raises the H2Av level. **B-D.** Quantifications of the normalized intensity, where *** indicates $p<0.001$ and n.s $p>0.05$, from pairwise Wilcoxon rank sum test. **E.** Magnified section from Figure A, showing in more detail DSBs and apoptotic marks in control and 5-days sleep-deprived flies.

Table 4.1. Statistical differences in H2Av and DCP-1 intensity, between CTRL and SD groups.

H2Av intensity			DCP-1 intensity		
Genotype1	Genotype2	p-value	Genotype1	Genotype2	p-value
CTRL	1 day SD	0.00031	CTRL	1 day SD	0.83275
CTRL	5 days SD	5.5e-06	CTRL	5 days SD	0.00143

4.2.2 H2Av signal, a marker for DNA damages, in dFB neurons

From the previous section, I found that sleep deprivation promotes the accumulation of DSBs and in the case of a more prolonged dynamic stimulus, even the accumulation of apoptotic markers. While analyzing the immunostaining described in Figure 4.1, I noticed that the H2Av signal was not only restricted in the neuronal cell bodies, mostly located in the front part of the brain, but it was also detected in the central complex, specifically in the dorsal fan-shaped body. As shown in Figure 4.2, it is quite clear that the staining of H2Av is approximately localized around layers 6 and 7 of dFB. Furthermore, in the SD conditions, both 1 and 5 days in length, there are clearly more H2Av markers, compared to the control. Although the increase of H2Av in dFB of SD flies confirms what was already observed in the previous section, I found odd this particularly data, given the

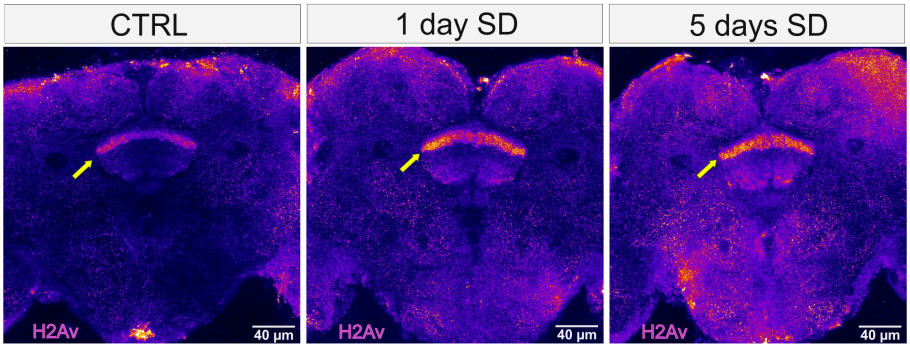


Figure 4.2. H2Av signals in the dFB neurons.
Confocal projections of adult brains from CS flies, focused on the central complex region. Brain tissues were stained with ab anti-H2Av. As pointed out in yellow arrows, H2Av markers are shown approximately around layers 6 and 7 of dFB, with higher intensity in the sleep-deprived conditions, compared to controls.

peculiar localization of H2Av. Indeed, as a histone protein, I was expecting to find this variant of H2A only in the nuclei of cell bodies. Although it is not clear yet from the literature what is the exact localization of H2Av in the adult brain, it is a certain fact that during embryogenesis, the balance in the nuclei of histone proteins, including the variant H2Av, is regulated by the lipid droplets. Lipid droplets represent cytoplasmic organelles, which mainly work as storage of fat and proteins from various cellular compartments,

including histones (Z. Li et al. 2014). Balancing the histone levels is crucial for several processes, such as genome stability and transcription regulation. In the specific case of H2Av, regulating the right ratio of this histone is fundamental to avoid hypersensitivity to H2Av overexpression, which can lead to an increase in DNA damages and eventually to embryonic death. Zhihuan li et al. proposed the hypothesis where this buffering system may be not just typical of the embryonic stage. Only more recently, lipid droplets were, indeed, also found in the adult brains (Ralhan et al. 2021). Here, these storages have been found to control the response to cellular stress, like oxidative stress or starvation, in order to maintain the health of the brain. Lipid droplets were found in various cells, such as neurons, microglia, and astrocytes. As similar as the embryonic stage, in the adult brain, these droplets were found to store different types of proteins, including potentially H2Av, although there are no current studies confirming this hypothesis. As a plausible explanation of my results, it is possible that H2Av is normally stored in the lipid droplets across the central complex, which act as a homeostatic driver upon sleep pressure, to regulate the distribution of this histone variant when required. This explanation remains only a hypothesis and further validations would be required in the future.

4.2.3 The pharmacological induction of DSBs and the effects in sleep

From previous sections, it was obvious that dynamic sleep deprivation can lead to the accumulation of DSBs and, eventually, initiation of apoptosis, in the cell bodies of adult brain tissues. This was probably happening because, assuming sleep functioning as a DNA repair driver, there were no right requirements and conditions to allow the complete repair of damages, progressively accumulating due to the lack of sleep.

On the other hand, as already observed in zebrafish, I was also supposing the existence of a reciprocal correlation between sleep and DNA damage in *Drosophila*, too. In their work, Zada et al 2019 found that the pharmacological induction of DSBs caused a following increase in sleep in fish (D. Zada et al. 2019). This result was never proved in flies. Therefore, I decided to test it first, using a similar approach. In order to induce DSBs, I fed flies with Etoposide, an anti-tumor drug acting as an inhibitor of the DNA topoisomerase II, normally implicated in the cleavage and re-ligation of DNA double-strand, during several DNA processes (Figure 4.3). The binding of the Etoposide prevents the re-ligation step, leading to the accumulation of permanent DSBs. No guidelines

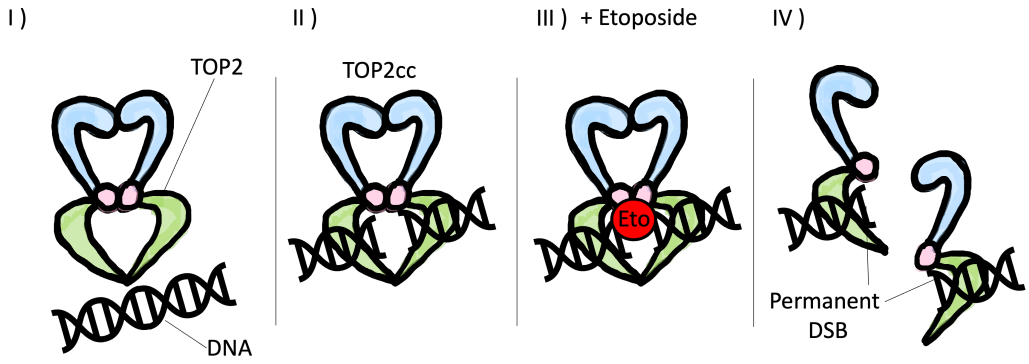


Figure 4.3. Schematic illustration of the Etoposide's mechanism of action.
Under normal circumstances, the topoisomerase 2 (TOP2) binds the DNA, to facilitate the creation and release of DNA double-strand breaks, by passing through a TOP2-DNA cleavage complex (TOP2cc). However, the addition of the Etoposide leads to the trapping of the Top2cc. In case the latter is not properly repaired, TOP2 will be removed by nucleases and the DSBs will remain permanent (W. Zhang et al. 2021).

in the literature were available on how to treat adult flies with Etoposide: therefore, I started running a few tests. The very first approach was to dissolve the drug in the usual fly food, reaching 1mM of concentration. Canton-S flies were fed with food + Etoposide for 3 hours, preceded by 15 h of starvation (they were placed into tubes containing only agar and water), in order to make them likely to eat more food the following day. At the end of the three hours of treatment with the drug, flies were moved back into normal food, and behavior was recorded for the following days. Furthermore, together with CS, I also decided to run the same test in *fumin* mutants. These flies, as mentioned in section 3.5.2, are known to be very hyperactive, showing little amount of sleep over the whole day. Given their sleep pattern, I thought that it would have been easier to see any etoposide effect on sleep, if any. As shown in Figure 4.4A-B, the treatment with Etoposide (Eto) did not lead to any change in terms of sleep amount: no increase of sleep was detected when comparing flies fed with food + Eto, to flies fed with food + DMSO (the solvent used to dissolve the drug). This result was true for both CS and *fumin* flies. Assuming that the concentration of the drug was too little, or the length of the treatment was too short, I decided to change a few experimental settings: I increased the concentration to 2.5mM, extended the treatment time to approximately 30h, and finally, also extended the starvation period to 24h (only 20h for *fumin*, given the lethality effect due to longer starvation periods). As shown in Figure 4.4C-D, this time the outcome was more intriguing: CS flies treated with Eto exhibited higher total sleep amount, compared to control flies (DMSO), over the days following the treatment. This increase

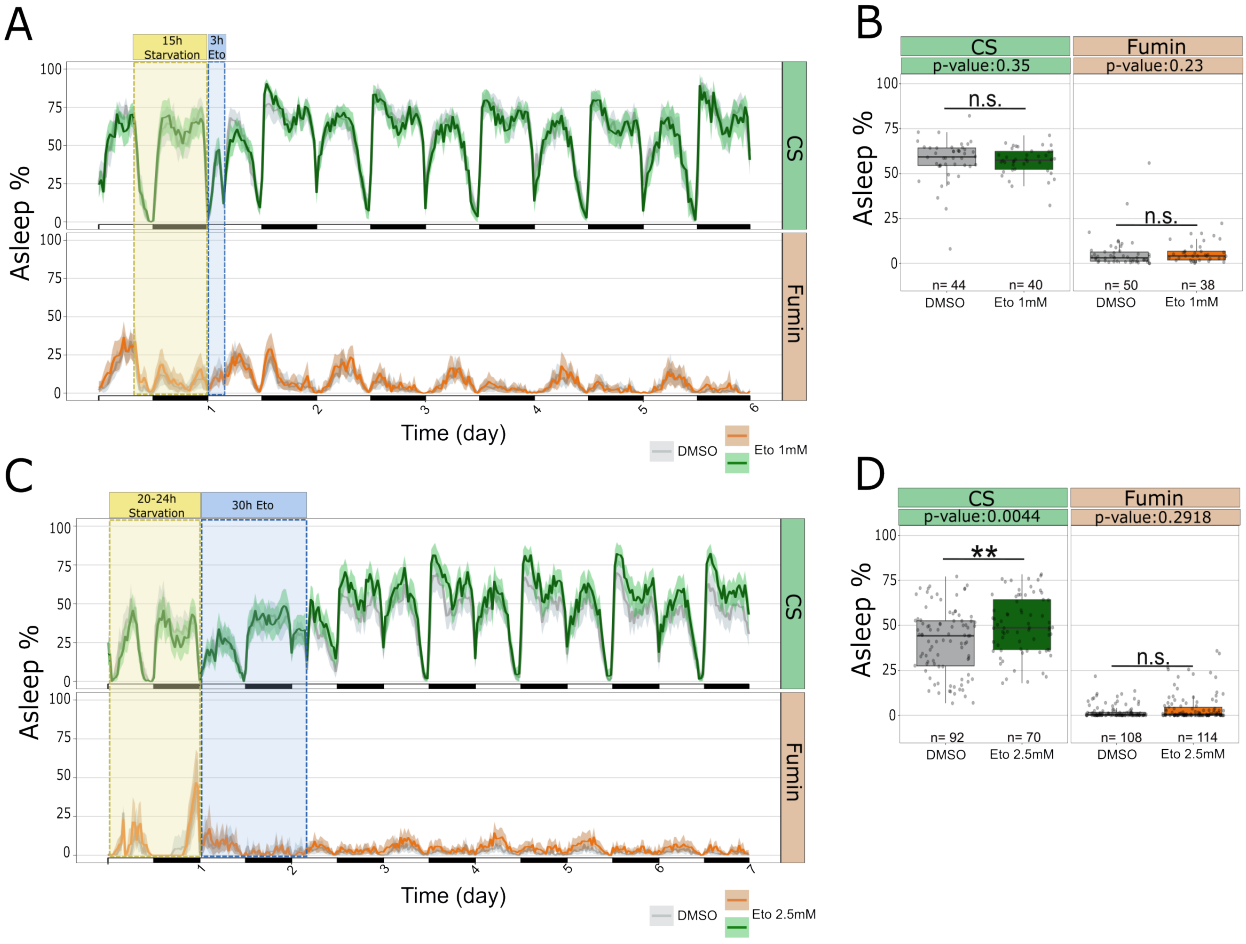


Figure 4.4. Treatment with Etoposide 2.5mM increases sleep amount in Canton-S flies.
A,C. Baseline sleep profiles of Canton-S (green lines) and *fumin* (orange lines) male flies. Appropriate controls of flies fed with food+DMSO are shown in grey lines After a starvation period, highlighted in yellow (15h in A and 20-24h in C), a treatment with the Etoposide, highlighted in blue, was following (3h in A and 30h in C). Immediately after the treatment, flies were moved back into normal food. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **B,D**. Quantifications of total sleep amounts, over the days following the Eto treatment. Significative changes between treated and control flies are shown only in CS, fed with 2.5mM Eto (D). In all figures, ** indicates a $p < 0.005$; n.s. $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from three replicates and the *N* of the animals per each group is reported within the figure.

seems to mainly occur during the night phase. These results indicate that CS flies fed with Eto may have accumulated a very high number of DSBs: as a consequence, they spent more time in sleep over the following days, probably with the aim to repair those damages and restore the health in the brain tissue. This reminds findings in fish from Zada et al 2019, further suggesting that the reciprocal correlation between sleep and DNA damages is possibly conserved across the species.

On the other hand, *fumin* flies did not experience the same behavior: in fact, individuals treated with the drug did not increase total sleep, compared to controls. This may suggest that the genetic mutation occurring in these flies, perhaps masks or obscures any effect given by the etoposide, which is, therefore, not enough to enhance sleep amount.

4.2.4 The pharmacological induction of DSBs in female flies

Similar to fish, the pharmacological induction of DSBs in flies also led to an increase in total sleep over the days following the treatment with the drug. For this experiment, I was only testing male flies. Therefore, the next step was to apply the same approach to female Canton-S flies, asking whether the response to accumulated DSBs was conserved between the sexes.

Similarly to males, female CS flies were starved for 24h and treated with Etoposide for approximately 30h. The following day, flies were transferred back to tubes containing normal food, and sleep was monitored over the following days. As shown in Figure 4.5A-B, 2.5mM concentrated Etoposide did not lead to any impairments or change in the sleep amount of treated flies, compared to controls. The next step was therefore to increase the concentration of the drug, bringing it from 2.5mM to 5mM. As reported in Figure 4.5C-D, with this different experimental condition, treated female flies finally exhibited higher total sleep amounts: the rising of sleep was spread over the whole day, during both light and dark phases. However, differently from males, the response in sleep to accumulated DSBs was only experienced over the first 3 days following the treatment with the drug. After this period, the increase of sleep ended, making the sleep pattern similar to control flies. To better elucidate these results, it is essential to highlight that female flies used in this experiment were collected after mating: as already known from the literature, mated females require higher consumption of food, to allow efficient egg production (Camus et al. 2018; Bradley and Simmons 1997). This means that the absence of perturbations upon 2.5mM Etoposide treatment is certainly not due to lower food intake. Furthermore, studies conducted in my lab (data not published), have discovered that mated females lack any rebound sleep response upon starvation or dynamic sleep deprivation, suggesting that biological drives, such as the mating status, are able to balance or modulate the sleep needs. Therefore, it is possible that DSBs accumulated in mated females generate a higher

threshold compared to male flies, that only a double Etoposide concentration is able to exceed, leading to a raised level of sleep amount.

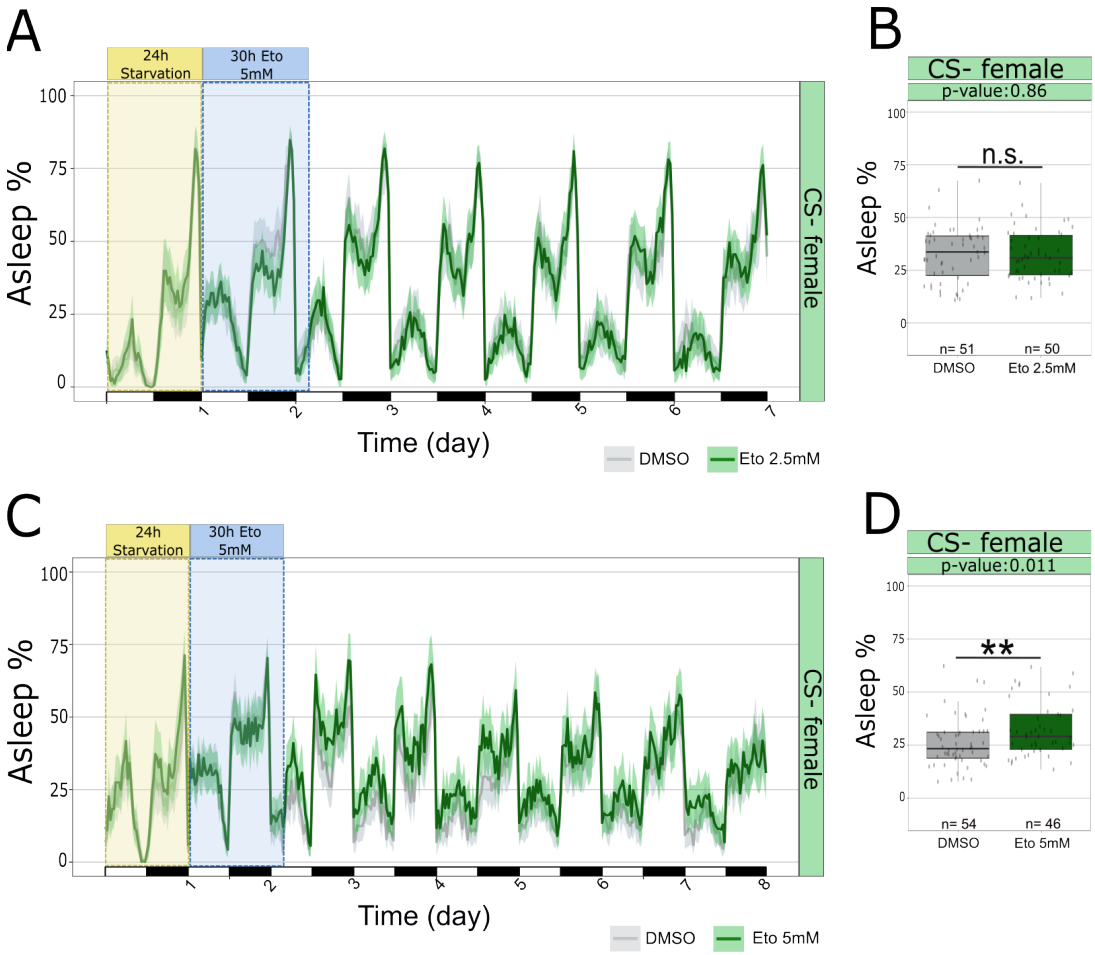


Figure 4.5. Treatment with Etoposide 5mM, but not 2.5mM, increases sleep amount in female Canton-S flies.
A, C. Baseline sleep profiles of Canton-S female flies. After a starvation period, highlighted in yellow (24h), a treatment with the Etoposide, highlighted in blue, was followed (30h). Immediately after the treatment, flies were moved back into normal food. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **B, D.** Quantifications of total sleep amounts, over the days following the Eto treatment. Significant changes between treated and control flies are shown only in flies fed with 5mM Eto (D). In all figures, ** indicates a $p < 0.005$; n.s. $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from three replicates and the *N* of the animals per each group is reported within the figure.

4.2.5 The influence of dFB neurons to regulate sleep in response to DSBs

Upon a certain threshold level of DSBs, sleep is enhanced with the aim to allow essential nuclear maintenance which allows, by prolonged off-line periods, repair of DNA damages. This suggests that DNA damages may act, in neurons, as a homeostatic driver

for sleep. The next step was, therefore, to better elucidate the mechanisms responsible for this sleep response. Several neuronal regions, mainly located in the central complex of the adult brain tissue, are already known to regulate homeostatic sleep in response to environmental stimuli. The idea is that there is a sort of communication between the accumulated DSBs and any of these neuronal regions, in order to trigger increase of sleep. Among these regions, the dFB neurons are known to represent one of the master players in homeostatic sleep drive. Therefore, I decided to inhibit this class of neurons and feed the same flies with Etoposide, in order to see their reaction in sleep, upon pharmacologically induced-DSBs.

In order to switch off the dFB, I over-expressed the thermosensitive allele *shibire^{ts},* already introduced and described in section 2.4, in the cells covered by the driver R72G06-GAL4, known to target particularly the FB. As described by Sha Liu (2016), the normal synaptic inhibition of these neurons, causes a decrease in sleep, mostly at nighttime (Liu et al. 2016). After setting up the crosses, flies were raised at 18°C and moved to 29°C upon hatching, for the whole experimental period of recording. The increase in temperature is what determines the actual activation of *Shi* and, consequently, the inhibition of the targeted cells (dFB). Together with UAS-shi^{ts}/R72G06-GAL4 flies, also the parental control CS/ UAS-shi^{ts} was tested. In order to induce DSBs, the same experimental settings described in the previous sections were applied: flies were starved for 24h and then treated with 2.5mM Etoposide for approximately 30h. Immediately after the drug feeding, flies were moved back into normal food and sleep was quantified over the following days.

As shown in Figure 4.6, CS/ UAS-shi^{ts} flies increased their total sleep amount over the days following the treatment with Etoposide. This result confirms what was previously shown, where also Canton-S flies increased their total sleep upon drug-feeding. On the other hand, UAS-shi^{ts}/R72G06-GAL4 flies exhibited a different behavior. They reduced their normal baseline sleep, confirming previous studies, but more interestingly, they did not respond to the Etoposide treatment by increasing sleep. Indeed, there were no significant changes when comparing Eto-treated with DMSO-treated flies, as shown in Figure 4.6B. These data may suggest that dFB neurons may be truly responsible for the sleep response to the accumulated DNA damages. This would be in line with another study, which claims that the FB region is also responsive to other harmful stimuli, such

as electric and heat shock (Hu et al. 2018). However, more analysis is required in order to validate this hypothesis.

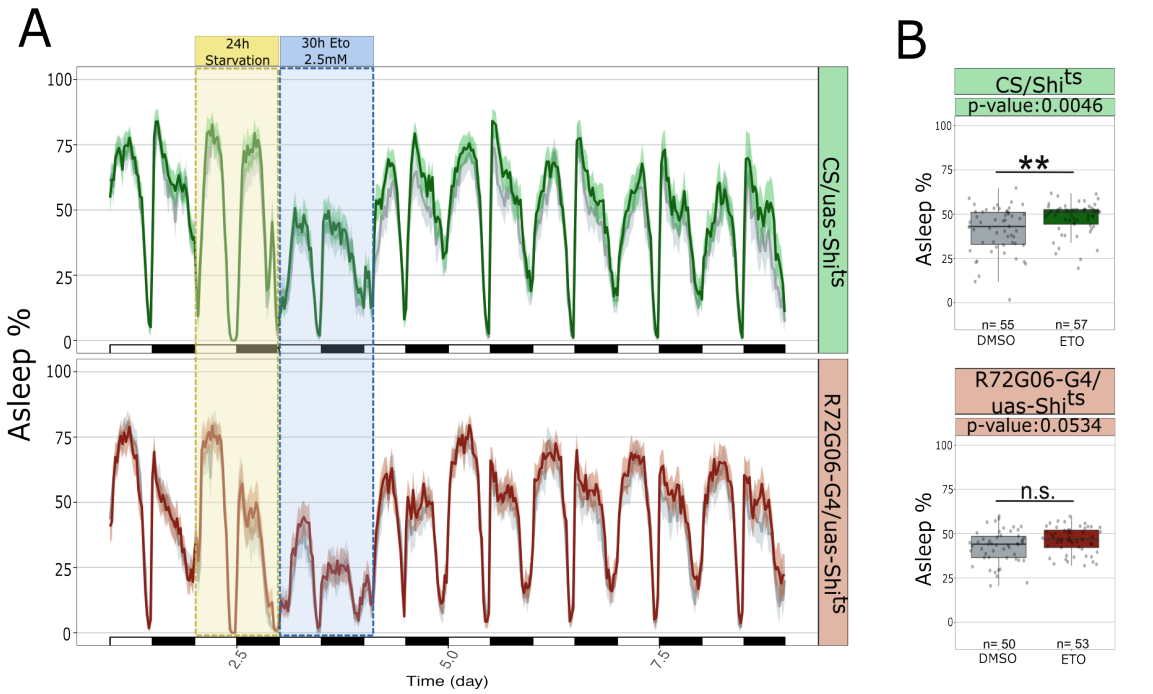


Figure 4.6. Treatment with Etoposide 2.5mM did not increase sleep amount in UAS-shits/R72G06-GAL4 flies.
A. Baseline sleep profile of CS/ UAS-shi^{ts} (green line) and UAS-shi^{ts}/R72G06-GAL4 (red line) male flies. Appropriate controls of flies fed with food+DMSO only are shown in grey lines. After a starvation period, highlighted in yellow (24h), a treatment with the Etoposide, highlighted in blue, was followed (30h). Immediately after the treatment, flies were moved back into normal food. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **B**. Quantification of total sleep amounts, over the days following the Eto treatment. Significative changes between treated and control flies are shown only in CS/ UAS-shi^{ts}, fed with 2.5mM Eto (D). In all figures, ** indicates a $p < 0.005$; n.s. $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from three replicates and the *N* of the animals per each group is reported within the figure.

Finally, some brains from all the described conditions were also immediately dissected at the end of the drug treatment and immune-staining experiments with ab anti-H2Av and anti-DCP-1 were performed. Figure 4.7 shows that CS/ UAS-shi^{ts} flies increased both levels of H2Av and DCP-1 upon treatment with Etoposide, as expected, meaning a higher level of DSBs and initiation of apoptosis. Very different was, instead, the situation for UAS-shi^{ts}/R72G06-GAL4 flies. No differences in both H2Av and DCP1 levels were detected when comparing flies treated with the drug to controls. This was peculiar and unexpected (see Table 4.2 for statistical comparisons). Furthermore, as shown in the zoomed-in section in Figure 4.7B, the H2Av baseline distribution in UAS-shi^{ts}/R72G06-

GAL4 flies (no treated condition) was dissimilar to that one in the parental control. In the latter, in fact, H2Av seems to be restricted in the nuclei of the cell bodies, while in the UAS-shi^{ts}/R72G06-GAL4 flies, the distribution appears more spread, showing some

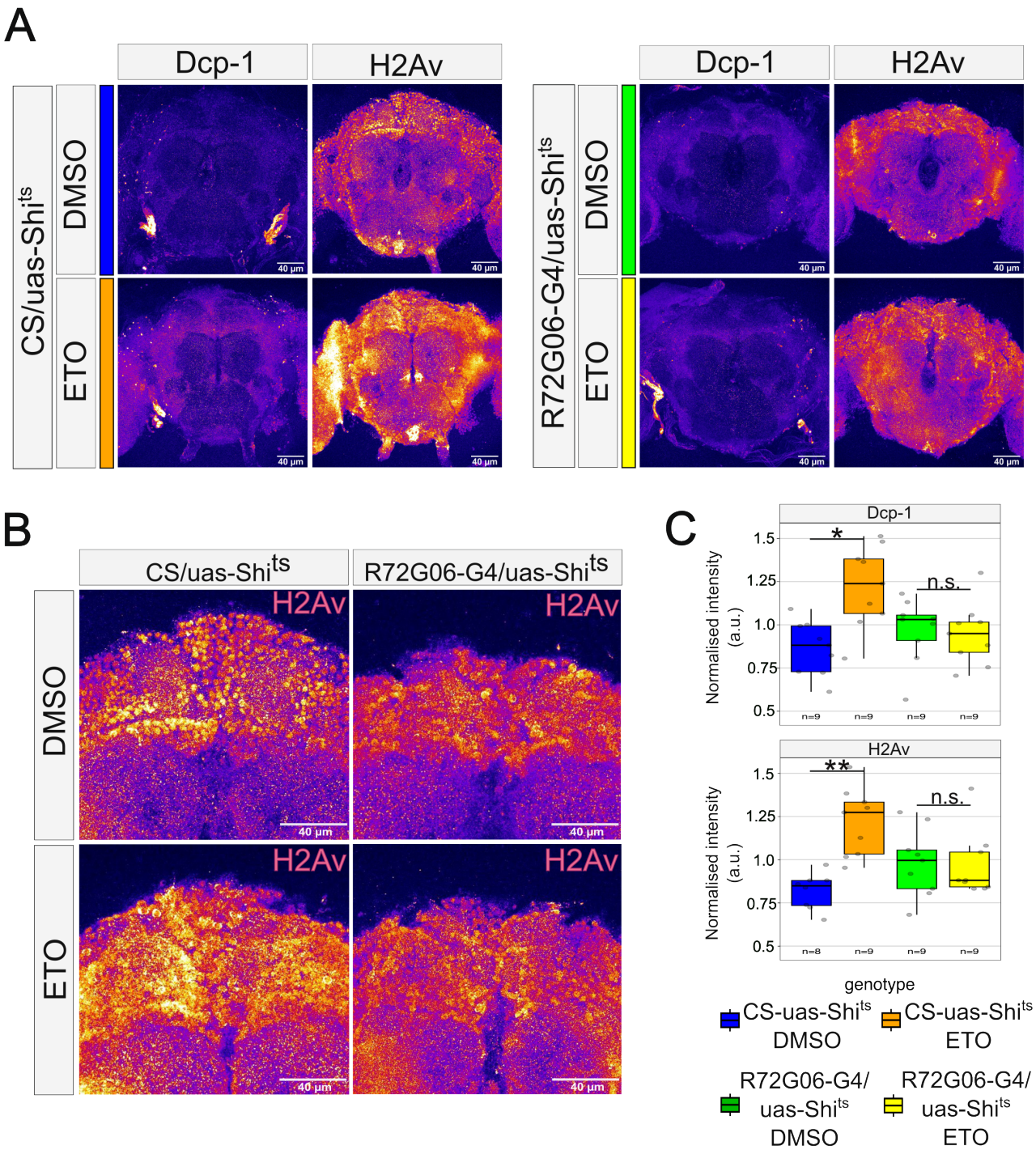


Figure 4.7. Etoposide treatment increases number of DSBs and enhances apoptosis in CS/ UAS-shits but not in UAS-shits/R72G06-GAL4 flies.
A. Confocal projections of adult front brains from CS/ UAS-shi^{ts} and UAS-shi^{ts}/R72G06-GAL4 flies, stained with ab anti-H2Av and anti-DCP-1. An increased level of both targets was detected in the brains of CS/ UAS-shi^{ts} flies, only. **B.** Magnified section from Figure A, showing in more detail H2Av signals in all

conditions. **C.** Quantifications of the normalized intensity, where * indicates a p<0.05; ** p < 0.005; *** p<0.001; n.s p>0.05, from pairwise Wilcoxon rank sum test.

Table 4.2. Statistical differences in H2Av and DCP-1intensity, upon ETO treatment, between UAS-shits/R72G06-GAL4 and parental controls.

H2Av intensity			DCP-1 intensity		
DMSO	ETO	p-value	Genotype1	Genotype2	p-value
CS/UAS-shi^{ts}	CS/UAS-shi^{ts}	0.0046	CS/UAS-shi^{ts}	CS/UAS-shi^{ts}	0.0193
R72G06-G4/UAS-shi^{ts}	R72G06-G4/UAS-shi^{ts}	0.9659	R72G06-G4/UAS-shi^{ts}	R72G06-G4/UAS-shi^{ts}	0.6945

signals localized also in the cytoplasm, reminding more likely the H2Av distribution in the parental control subjected to Etoposide. The outcome of this experiment is very complicated to interpret. First, it is unclear why the synaptic inhibition of the dFB neurons should not allow the accumulation of the DSBs upon drug treatment and why the baseline distribution of H2Av in the same flies is not just restricted to the nuclei of the cell bodies, as it is for parental control. However, the fact that UAS-shi^{ts}/R72G06-GAL4 flies do not experience increased DSBs, may explain their behavior, in particularly why they do not respond to Etoposide treatment by increasing their total sleep. Although FB neurons are known to regulate feeding behavior, here we can probably exclude the idea that the inhibition of these cells may reduce the total food intake (meaning minor effects due to Etoposide). Indeed, other studies, such as Sareen P 2021 and Musso P 2021, already found that the inhibition of a specific set of FB cells does not impair total food consumption, but only the preference of choice, if any (Sareen, McCurdy, and Nitabach 2021; Musso, Junca, and Gordon 2021). In any case, more research is required, in order to better elucidate these results and the potential role of the dFB neurons in sleep regulation upon DNA damages.

4.2.6 Summary

- General sleep deprivation causes an increased number in DSBs, while only chronic SD leads to initiation of apoptosis in the brain of adult flies. H2Av signals, markers for DNA damages, were also detected in the Fan-shaped body neurons.
- Pharmacological induced DSBs trigger increase of sleep in male Canton-S flies.

- Female Canton-S flies require a higher dosage of Etoposide to increase their baseline sleep.
- Treatment with Etoposide did not increase sleep amount upon synaptic blockage of the Fan-shaped body neurons (UAS-shi^{ts}/R72G06-GAL4 flies).

4.3 Consequences upon pan-neuronal knockdown of DNA repair-related genes

4.3.1 p53 and ATM *RNAi*: the effects in sleep

Thus far, I have demonstrated that the accumulation of DNA damages can act as a homeostatic driver of sleep: pharmacologically induced DSBs, in fact, lead to increased baseline sleep in flies treated with the drug. Although I could detect a higher level of H2Av (marker for DSBs) in the cell bodies of brain tissues from drug-treated flies, differentially to controls, from these results I cannot exclude any impairments in other tissues, too. In fact, by feeding flies with Etoposide, known to be an anti-tumor drug, it is possible that the DSBs are triggered not only in the brain, but elsewhere such as the gut tissue. For this reason, I found it fundamental to test a second strategy aiming to induce DSBs specifically in the neuronal tissue. In order to do so, I exploited the GAL4-UAS system to investigate the *RNAi* of two DNA-repair-related genes, specifically *p53* and *ATM*, in the brains of adult flies. Knocking down these targets should trigger accumulated unrepaired DNA damages in neurons. From section 1.4.4, we already described the DNA repair pathway, explaining the usual cascades triggered by different types of DNA damages. In Figure 4.8, an illustrated schematization of the DNA repair in response to double-stranded breaks (DSBs) is shown: the protein kinase ATM (ataxia telangiectasia mutated) acts upstream of a cascade where p53 plays a key role in regulating cell cycle arrest, DNA repair and apoptosis in the case of cells where accumulated damages could not be repaired. Given all this information, I decided to induce and test the knockdown of ATM or p53, using the pan-neuronal driver nSyb-GAL4. After hatching, baseline sleep of flies was recorded for 2 days in a row. Furthermore, three different ages were considered and analyzed, 3, 17, and 28 days old, in order to see if aging would aggravate

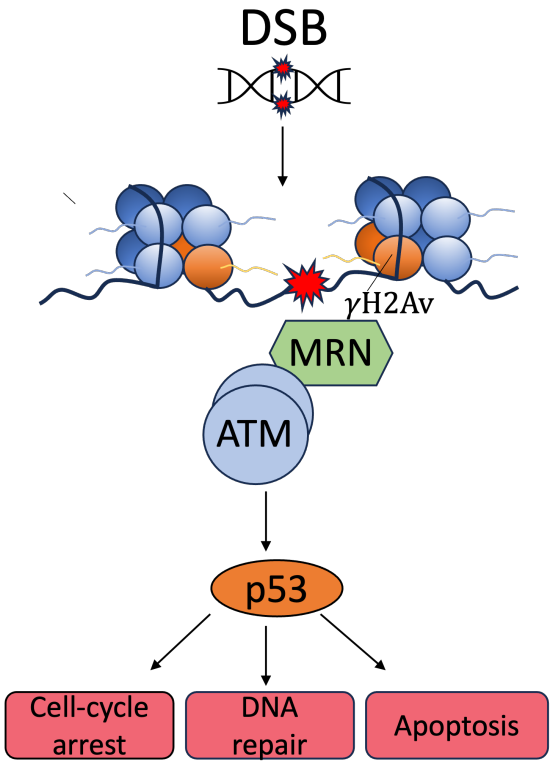


Figure 4.8. Schematic illustration of ATM and p53 activation in response to DSBs.
The histone variant H2Av is phosphorylated (γH2Av) upon DSBs, acting as a scaffold for the MRN complex (Mre11-Rad50-Nbs1), which recruits and activates the kinase ATM. ATM, consequently, phosphorylates targets, such as p53, implicated in several processes, including DANN repair.

the phenotype, in case of any, due to higher accumulation of damages. Figure 4.9 shows that the ATM knockdown did not lead to any change in the baseline sleep of young flies (3 days old), compared to their parental controls. On the other hand, p53 *RNAi* flies exhibited higher sleep over the whole day. Only older flies (28 days old) experienced longer sleep specifically during the light phase, for both ATM and p53 knockdown. Peculiar and singular was the behavior of middle-aged ATM *RNAi* flies, showing reduced sleep during the night phase. To summarize, these results suggest that p53 knocked-down flies sleep more than usual, no matter the age, while the *RNAi* of ATM leads to higher baseline sleep, only during the light phase in older flies (see Table 4.3 and Table 4.4 for statistical comparisons). I found these data very promising and in line with my previous findings. The increase of sleep seen here, upon knockdown of these two DNA repair-related genes, reminds, in fact, of the effects observed by feeding flies with Etoposide food. Before proceeding with more studies, however, I thought it essential to better validate the two knockdowns. Indeed, I assumed that p53 and ATM *RNAi*, supposedly

characterized by less efficient DNA repair, needed to increase their sleep in order to compensate for the elevated number of DNA damages.

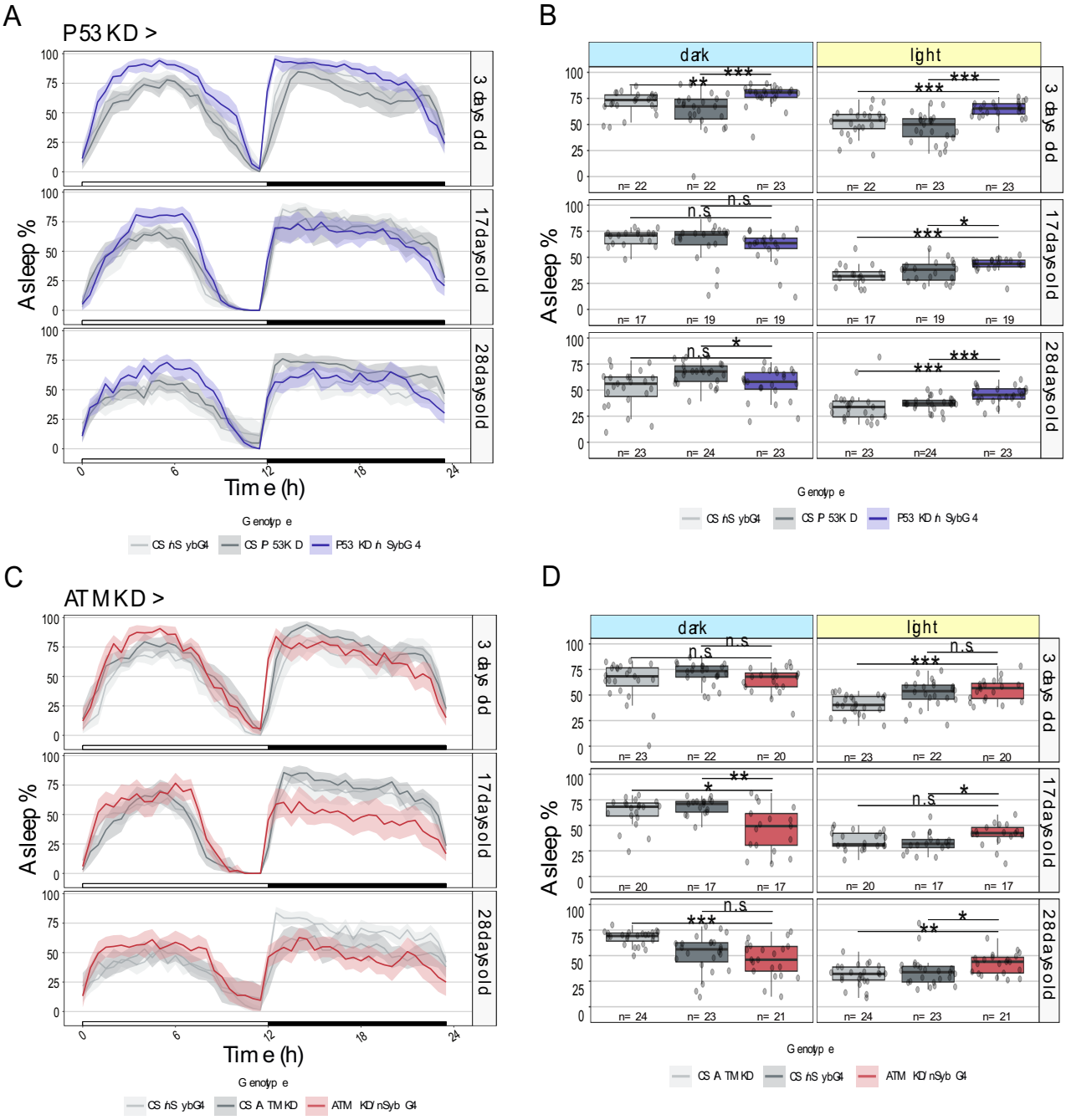


Figure 4.9. Increased baseline sleep was triggered upon p53 KD and also in old ATM KD flies during light phase.

A, C. Ethogram wraps (average) of baseline sleep upon p53 KD (A) and ATM KD (C). Parental controls are shown in grey lines, while *RNAi* flies in colored lines (blue for p53 and red for ATM). Baseline sleep of three different ages, is also analyzed and shown in separate graphs. Intermediate lines indicate the mean, while shaded areas represent the bootstrapped 95% confidence interval. **B, D.** Quantification of the total baseline sleep, extrapolated from figure A and C, respectively. Light and dark phases are analyzed separately. In all figures, * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test. Data shown is from two replicates and the *N* of the animals per each group is reported within the figure.

Table 4.3. Statistical differences in baseline sleep, between p53 RNAi and parental controls, across different ages.

p53, 3 days old, dark			p53, 3 days old, light		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/UAS-p53	UAS-p53 / nSyb-G4	0.00066	CS/UAS-p53	UAS-p53 / nSyb-G4	5.0e-06
CS/ nSyb-G4	UAS-p53 / nSyb-G4	0.00692	CS/ nSyb-G4	UAS-p53 / nSyb-G4	0.00066
p53, 17 days old, dark			**p53, 17 days old, light**		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/UAS-p53	UAS-p53 / nSyb-G4	0.24113	CS/UAS-p53	UAS-p53 / nSyb-G4	0.01591
CS/ nSyb-G4	UAS-p53 / nSyb-G4	0.08948	CS/ nSyb-G4	UAS-p53 / nSyb-G4	0.00049
p53, 28 days old, dark			**p53, 28 days old, light**		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/UAS-p53	UAS-p53 / nSyb-G4	0.02846	CS/UAS-p53	UAS-p53 / nSyb-G4	0.00049
CS/ nSyb-G4	UAS-p53 / nSyb-G4	0.54813	CS/ nSyb-G4	UAS-p53 / nSyb-G4	0.00016

Table 4.4. Statistical differences in baseline sleep, between ATM RNAi and parental controls, across different ages.

ATM, 3 days old, dark			ATM, 3 days old, light		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/UAS-ATM	UAS- ATM/ nSyb-G4	0.59764	CS/UAS-ATM	UAS- ATM/ nSyb-G4	0.00022
CS/ nSyb-G4	UAS- ATM / nSyb-G4	0.06984	CS/ nSyb-G4	UAS- ATM / nSyb-G4	0.56347
ATM, 17 days old, dark			**ATM, 17 days old, light**		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/UAS-ATM	UAS- ATM/ nSyb-G4	0.02356	CS/UAS-ATM	UAS- ATM/ nSyb-G4	0.05657
CS/ nSyb-G4	UAS- ATM / nSyb-G4	0.00523	CS/ nSyb-G4	UAS- ATM / nSyb-G4	0.01848
ATM, 28 days old, dark			**ATM, 28 days old, light**		
Genotype 1	Genotype 2	p-value	Genotype 1	Genotype 2	p-value
CS/UAS-ATM	UAS- ATM/ nSyb-G4	2.0e-05	CS/UAS-ATM	UAS- ATM/ nSyb-G4	0.00276
CS/ nSyb-G4	UAS- ATM / nSyb-G4	0.28595	CS/ nSyb-G4	UAS- ATM / nSyb-G4	0.01430

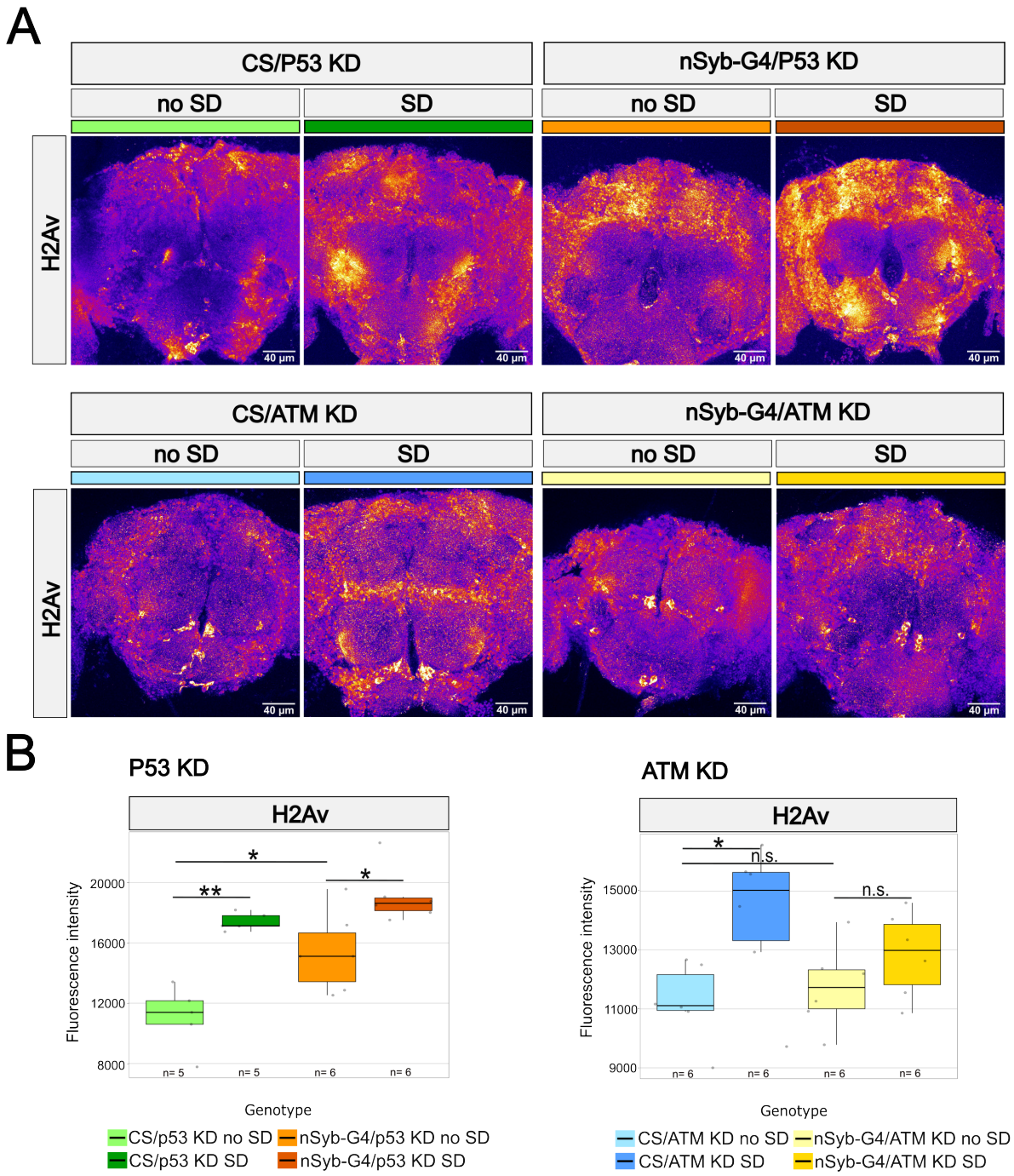


Figure 4.10. Sleep deprivation increases DNA damages in the brains of p53 *RNAi*, but no ATM *RNAi* flies.

A. Confocal projections of adult front brains from CS/ UAS-p53 KD, nSyb-G4/UAS-p53 KD, CS/ UAS-ATM KD and nSyb-G4/UAS-ATM KD flies, stained with ab anti-H2Av. An increased level of H2Av signal was detected in the neuronal nuclei of all SD conditions, excepting nSyb-G4/UAS-ATM KD. **B.** Quantifications of the normalized intensity, where * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$, from pairwise Wilcoxon rank sum test.

Table 4.5. Statistical differences in H2Av intensity, between genotypes upon knockdown of p53 and ATM.

p53, H2Av intensity			ATM, H2Av intensity		
Genotype 1	Genotype 1	p-value	Genotype1	Genotype2	p-value
CS/UAS-p53 SD	CS/UAS-p53 no SD	0.0125	CS/UAS-ATM SD	CS/UAS-ATM no SD	0.0531
nSyb-G4/UAS-p53 SD	nSyb-G4/UAS-p53 no SD	0.0461	nSyb-G4/UAS-ATM SD	nSyb-G4/UAS-ATM no SD	0.2793
CS/UAS-p53 no SD	nSyb-G4/UAS-p53 no SD	0.0253	CS/UAS-ATM no SD	nSyb-G4/UAS-ATM no SD	0.7232

Thereby, I hypothesized that prolonged sleep deprivation should probably aggravate the knockdown effect, due to an even higher accumulation of DSBs. To better investigate this hypothesis, I first subjected both knocked-down flies and their reciprocal parental controls, to 5 consecutive days of dynamic SD. At the end of the stimulus, flies were immediately collected, and adult brains were dissected, to perform immunostaining experiments. Brains were stained with anti-H2Av, in order to monitor the level of DNA damages. As shown in Figure 4.10, the parental control CS/p53 KD exhibited increased number of DSBs upon chronic SD, consistent with my previous findings. Also the knockdown of p53, upon SD, experienced increased H2Av compared to no SD condition: however, the most interesting aspect, here, is the fact that when comparing CS/p53 KD and nSyb-G4/p53 KD in each condition (no SD and SD, distinctively), it is evident the in both cases the *RNAi* of p53 drastically aggravates the phenotype, meaning that the knockdown alone already accumulates the DNA damages (Figure 4.10B and Table 4.5). This means that, for instance, in nSyb-G4/p53 SD flies, the elevated signal of H2Av is given by the sum of the actual sleep deprivation and the knockdown effects. These results in p53 can probably also explain the data from the behavioral analysis: given their higher baseline level of DNA damages, these flies need to compensate by increasing sleep, trying constantly to attempt and activate DNA repair mechanisms, which paraphs cannot be accomplished properly. At this step, more experiments are required to better elucidate the consequences of the knockdown of p53.

In the case of ATM, the circumstances were different. As normally expected, CS/ ATM KD fly brains have shown higher levels of H2Av upon chronic SD, compared to undisturbed fly brains. However, peculiar was the situation for the knockdown: ATM *RNAi* flies, in fact, did not show a higher accumulation of DNA damages upon sleep deprivation. Furthermore, when comparing nSyb-G4/ ATM KD to the parental control

CS/ ATM KD flies, in no SD condition, no changes were detected, meaning that the knockdown of ATM alone did not induce DSBs in the brain tissues of KD flies. These data result complicated to interpret and somehow resemble previously described data, where the synaptic inhibition of dFB did not enhance the accumulation of DSBs, upon treatment with Etoposide. It remains unclear exactly what can prevent the increase of DNA damages, despite the influence of a stressful condition, such as drug treatment or chronic SD.

4.3.2 p53 and ATM *RNAi*: the effects in lifespan upon chronic SD

It has been known that prolonged sleep deprivation does not have a significant impact on the lifespan of *Drosophila Melanogaster*. Geissman et al. (2019) triggered sleep deprivation in flies every day for their whole life, without observing lethality upon comparisons to undisturbed flies (Geissmann et al. 2019). This may suggest perhaps that additional stressors are required together with sleep deprivation to cause lethality. In the previous section, I observed that the knockdown of p53 aggravated and increased the accumulation of total DNA damages, usually observed upon chronic SD only. These results drove me to hypothesize that the *RNAi* of DNA repair-related genes, alongside chronic SD, would reduce the lifespan of KD individuals, due to accumulated DSBs that cannot be properly repaired, given the interfered DNA repair mechanisms usually occurring during sleep.

The knockdown of both p53 and ATM was enhanced by exploiting the GAL4/UAS system. After hatching, flies were subjected to 5 consecutively days of sleep deprivation. Appropriate controls of undisturbed flies were also included. Immediately after the SD, flies were placed into new tubes and their lifespan was manually scored over more than a month. As shown in Figure 4.11A, p53 KD flies that were not sleep-deprived, did not show any change in the lifespan, compared to both parental controls CS/p53 KD and CS/nSyb-G4. However, sleep-deprived knocked-down flies, instead, exhibited a much lower lifespan: significative changes were observed not only when compared to SD parental controls, but also when comparing knocked-down SD to knocked-down no SD flies, suggesting that the actual combination of the effects from chronic SD and the knockdown, results deleterious for the flies. These results are very promising and confirm

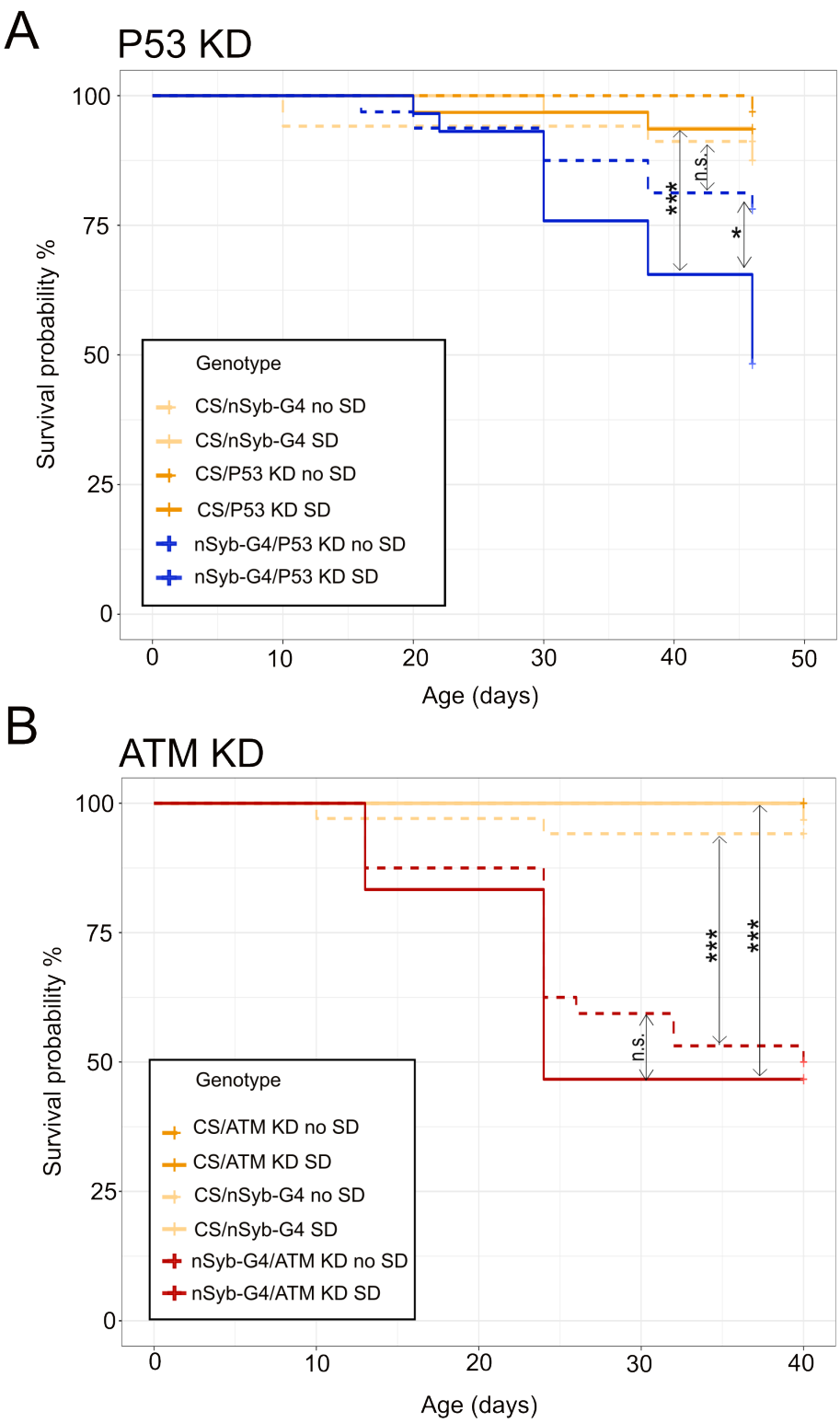


Figure 4.11. Chronic SD reduced lifespan in p53 RNAi but not in ATM RNAi flies.
The lifespan of p53 (**A**) and ATM (**B**) knocked-down flies, together with parental controls. SD conditions are shown with continuous lines, while no SD (undisturbed flies) are shown with dash lines. The Y-axis represents the survival probability, while X-axis is the age of the flies, over more than a month. Log-Rank tests were performed for statistical comparisons between conditions, where * indicates a $p<0.05$; ** $p < 0.005$; *** $p<0.001$; n.s $p>0.05$.

Table 4.6. Statistical differences in lifespan, between genotypes upon knockdown of p53 and ATM.

p53, lifespan			ATM, lifespan		
Genotype 1	Genotype 1	p-value	Genotype1	Genotype2	p-value
CS/UAS-p53 no SD	nSyb-G4/UAS-p53 no SD	0.02	CS/UAS-ATM no SD	nSyb-G4/UAS-ATM no SD	3e-06
CS/UAS-p53 SD	nSyb-G4/UAS-p53 SD	1e-04	CS/UAS- ATM SD	nSyb-G4/UAS-ATM SD	4e-06
nSyb-G4/UAS-p53 SD	nSyb-G4/UAS-p53 no SD	0.02	nSyb-G4/UAS-ATM SD	nSyb-G4/UAS-ATM no SD	0.7
CS/nSyb-G4 SD	nSyb-G4/UAS-p53 SD	7e-04	CS/nSyb-G4 SD	nSyb-G4/UAS-ATM SD	1e-05
CS/nSyb-G4 no SD	nSyb-G4/UAS-p53 no SD	0.2	CS/nSyb-G4 no SD	nSyb-G4/UAS-ATM no SD	8e-05

my hypothesis where additional stressors to chronic SD can lead to lethal consequences. On the other hand, ATM *RNAi* flies, in both SD and no SD conditions, reduced their lifespan compared to the parental controls. Furthermore, no significative changes were detected when comparing sleep-deprived *RNAi* flies to *RNAi* that slept undisturbedly (Figure 4.11B). This may mean that the drastic reduction in the lifespan of ATM KD flies is solely due to the effects of the knockdown and is not conditioned by chronic sleep deprivation, suggesting that the impairments related to the ATM *RNAi* are already deleterious. We can conclude that prolonged sleep deprivation affects lifespan in p53 *RNAi* flies, while more complicated are the circumstances for ATM *RNAi* flies.

4.3.3 Summary

- The pan-neuronal knockdown of DNA repair-related genes, in particularly p53, leads to increased baseline sleep. p53 *RNAi* flies, in fact, have shown higher sleep over their whole life, in particular during the light phase. These flies also experienced a higher accumulation of DSBs in the brains, which may act as a sleep-pressure driver. The knockdown of ATM only increased sleep in aged flies: strangely, chronic SD did not lead to the accumulation of DNA damages.
- p53 *RNAi* flies experienced reduced lifespan, upon chronic sleep deprivation, compared to parental controls, suggesting that the accumulated un-repaired damages may be the main cause of the reduced longevity. ATM *RNAi* flies experienced a drastic reduction in longevity, which was caused by the effects of the actual depletion of ATM and not the chronic SD.

Chapter 5

Discussion

5.1 H3K9methylation and sleep

5.1.1 Su(var)3-9, a key player in methylating sleep

In the past decade, the emerging role of histone modifications in sleep regulation has come to the forefront. As covered in the introduction, while significant progress has linked epigenetic markers, such as histone acetylation, to sleep, there are other modifications that remain less explored and understood in this context, and addressing this gap requires more comprehensive research. Therefore, this project is dedicated to exploring the possible involvement of specific histone modifiers in sleep, with a particular focus on key enzymes associated with H3K9 methylation in *Drosophila Melanogaster*, including G9a, Eggless, but mainly Su(var)3-9, the main subject of this exploration. This research revolved around examining the profound effects and involvement in sleep regulation of Su(var)3-9, known to be a primary agent for H3K9 methylation.

The project starts by knocking down Su(var)3-9 pan-neuronally: *RNAi* flies immediately exhibited a notably affected baseline sleep, compared to parental controls. In particular, these flies experienced a higher sleep amount on average, which was more remarkable in females than in males. Indeed, female flies showed a prominent rise in sleep during both light and dark phases, while males only during the transition from light to dark. This was the first episode of sexual differentiations I came across while pursuing my project. Interestingly, the increase in sleep was characterized by longer sleep episodes, while bout numbers remained as similar as control, suggesting therefore a more consolidated sleep in the *RNAi* flies, probably reflecting a deep sleep state. Further analysis allowed me to highlight that the higher level of synaptic marker proteins, such as BRP or Syn, detected in the *RNAi* flies, is only partially responsible for the increased baseline sleep. In this regard, I mentioned the intriguing work from Huang et al., where they found that the

increase of *brp* copies leads to an increase in baseline sleep in a dosage-dependent manner: the more copies they were triggering, the higher the baseline sleep increase. Given this observation, I induced the knockdown of Su(var)3-9, together with BRP, in order to observe a potential mitigation of the phenotype in the baseline sleep. Removing BRP and simultaneously depleting Su(var)3-9, helped in reducing the total sleep, compared to Su(var)3-9 *RNAi* only flies. However, these flies appeared also slightly arrhythmic on DD exposure, making the scenario more complicated. The depletion of Syn, instead, only further aggravated the phenotypes. These data suggested that there are probably other key players, downstream of Su(var)3-9 KD, more responsible for the increased baseline sleep.

Another layer of interest was added when exploring the contributions of Su(var)3-9 across the two main drives of sleep: the circadian and the homeostatic drive. My data suggested excluding any implication of Su(var)3-9 in the regulation of the circadian rhythm: *RNAi* flies, in fact, exhibited the usual morning and evening peaks, and no changes in rhythm were observed in DD condition, or changes in sleep by knocking-down Su(var)3-9 specifically in the circadian neurons, the PDF-expressing cells. However, different were the circumstances for the homeostatic sleep: upon sleep deprivation, wild-type flies are known to respond by showing a rebound sleep on the day following the SD, to partially recover from the sleep loss (J. C. Hendricks et al. 2000; Shaw et al. 2000a). This was always displayed by both parental controls, CS/UAS-Su(var)3-9 *RNAi* and CS/nSyb-GAL4 flies. By exploiting the main method of sleep deprivation applied in this project (dynamic SD), flies were subjected to a mechanical rotation of the glass tube, every time they were detected to be asleep. When sleep deprived by dynamic, Su(var)3-9 *RNAi* flies failed to show any rebound response, suggesting impairments in the homeostatic sleep regulation upon depletion of this specific H3K9 methylase. Interestingly, the sleep rebound upon other SD methods, such as starvation or male-male interaction, was not affected. This last result, together with previous data collected in my group, may suggest that dynamic SD is a more specific method for triggering sleep disturbance and stress due to the actual lack of sleep, while sleep responses to starvation and MM are probably driven by other mechanisms and form of stresses, perhaps more related to metabolic changes and aggression behavior, respectively (Keene et al. 2010; Hasegawa et al. 2017; Gilestro, Tononi, and Cirelli 2009; Beckwith et al. 2017; Machado et al. 2017). Although

this remains only a hypothesis, it comes in handy to highlight the role that Su(var)3-9 may play specifically in sleep.

The next step was to validate whether the knockdown of Su(var)3-9 was causing impairments in sleep from developmental or specifically from adult stages. Accumulated evidence has, in fact, highlighted the impact that epigenetic regulation has on animal adult behavior: in *Drosophila,* in particular, I already mentioned the key role played by G9a (another H3K9 methylase fundamental during development), in learning and memory formation, both short term memory (STM) and long term memory (LTM) and also foraging behavior (Kramer et al. 2011; Anreiter, Kramer, and Sokolowski 2017). Another example is the histone demethylase (HDM) KDM5, known to regulate STM and LTM (Zamurrad et al. 2018). With the aim to understand whether Su(var)3-9 is also implicated specifically in adult behavior regulation, I exploited the GeneSwitch GAL4 system, which allows to temporally-control the expression of GAL4. Knocking-down Su(var)3-9 specifically only in the adult stage, recapitulates the previous findings in the context of homeostatic sleep: *RNAi* flies, in fact, did not show any rebound sleep upon SD. However, the baseline sleep remained unchanged, suggesting that Su(var)3-9 needs to be pan-neuronally depleted from an earlier stage, to create a more catastrophic scenario, which can lead to impairments in the total amount of sleep. This is probably in line with previous studies in mammals, showing that sleep disturbances in young individuals are very often linked to perturbations of epigenetic marks occurring during development, in relation to environmental factors, such as alcohol consumption or depression during pregnancy (Pesonen et al. 2009; El-Sheikh et al. 2012; Toffol et al. 2019).

5.1.2 Su(var)3-9 in the central complex and MB

Thanks to a meticulous screen of GAL4 lines, I was able to identify three main brain regions requiring Su(var)3-9 for sleep regulation: dFB, EB, and MB, all known to be involved in homeostatic sleep. The knockdown of Su(var)3-9 in all three regions abolished the rebound response upon dynamic SD: this was observed in both female and male flies after knocking down Su(var)3-9 in dFB and EB. However, in MB, only male flies exhibited this peculiar behavior, while females showed a rebound response similar to parental controls. This was another sign of sexual differentiation: in fact, I previously mentioned differences in the baseline sleep observed between females and males upon

pan-neuronal knockdown of Su(var)3-9. It was already found that epigenetics can be responsible for a general sexual dimorphism: epigenetic modifications are fundamental for both the establishment and maintenance of sex differences in the brain and behavior (McCarthy et al. 2009; McCarthy and Nugent 2015). To give an example, Kurian et al., for instance, found that a decreased level of the methyl-CpG-binding protein 2 (MeCP2) in the amygdala of newborn rats, by small interfering RNAs, leads to differences in behaviors. Male young rats usually tend to play more than female: however, this sex difference was completely abolished upon reduction of MeCP2, as male rats reduced their social play behavior. (Kurian et al. 2008). Another study compared the distribution of H3K4me between male and female mice throughout the genome, finding approximately 248 genes with increased H3K4me in females: very interestingly, most of these target genes are associated with synaptic function and when the authors were evaluating their expression by RT-PCR, they did not detect differential expressions when comparing females to males. This very surprising result suggested that both males and females severely regulate these genes, but with different epigenetic mechanisms (Shen et al. 2015). Given all these observations, I am, therefore, not surprised to find differences in the behavior of male and female flies upon knockdown of Su(var)3-9.

Focusing on the role of Su(var)3-9 in the central complex, I was able to determine two specific sets of cells in which the H3K9 methylase is expressed: ExFl2 and R5 neurons. Here, I highlighted a redundant function in sleep homeostasis regulation: the knockdown of Su(var)3-9 in both types of neurons led to the absence of rebound sleep. Interestingly, the genetic activation of ExFl2 cells failed to induce the increase in sleep (previously observed by Liu et al. 2016) when simultaneously triggered with Su(var)3-9 KD, suggesting an essential role of the methylase in the sleep-related-functioning of ExFl2 cells. On the other hand, the knockdown in the R5 cells also affected the baseline sleep, given that female *RNAi* flies increased their total sleep, compared to parental controls (although male baseline sleep remained unchanged, suggesting another example of sexual differentiation). As reported in the introduction, it is good to remind that ExFl2 and R5, located in the dFB and EB, respectively, were shown to be interconnected: indeed, R5 cells regulate sleep passing throughout the ExFl2 neurons (Donlea et al. 2018; Liu et al. 2016). Mapping the neural circuitry and synaptic connections within these regions, possibly using optogenetics or chemogenetics, could reveal how Su(var)3-9-mediated H3K9 methylation integrates signals to regulate sleep. The differential impact on males

and females in these regions also warrants investigation into sex-specific neural network configurations.

In addition, I showed that also G9a, another fundamental H3K9 methylase in Drosophila, seems to play a role in sleep regulation specifically in the ExFl2 cells. In fact, its knockdown in these neurons abolished again the rebound response upon 1 day of dynamic SD, in male flies. The knockdown of Eggless, instead, did not affect sleep in any manner, not even upon pan-neuronal depletion. These results are pretty much in line with previous findings: while few works have published the key roles played by G9a in different behaviors, such as learning, memory, courtship, and foraging (Kramer et al. 2011; Anreiter, Kramer, and Sokolowski 2017), any evidence about Eggless in the context of behavioral regulation, has been ever published. Furthermore, despite the lack of evidence about a potential cooperation between Su(var)3-9 and G9a in the adult stage, the existence of a genetic interaction between these two enzymes during specific developmental stages was already discovered by Mis et al. (Mis, Ner, and Grigliatti 2006). Therefore, this study could be the very first evidence of Su(var)3-9 in adult behavior, played, perhaps, with the cooperation of G9a.

Finally, I tried to test the capacity of *RNAi* flies to learn, upon knockdown of Su(var)3-9 specifically in the MB region. The method applied was courtship conditioning, largely described in section 3.3.4. Unfortunately, the outcome of the experiment did not allow me to fully calculate the learning index: KD flies, in fact, resulted in completely opposite behaviors during training sessions, depending on the status of the female they were paired with. When paired with mated females, KD male flies exhibited a very low courtship index (CI), while when paired with virgin females, they were much more attracted to them, showing a higher CI. I propose that the knockdown of Su(var)3-9 could deregulate the expression of olfactory receptors, fundamental to sense pheromones such as cVa, typically released by mated females, allowing flies to differently integrate olfactory cues and, perhaps, to better distinguish mated from virgin females. In this regard, from the RNA seq analysis comparing Su(var)3-9 *RNAi* to parental controls, one olfactory receptor, the Or63a, appeared to be upregulated upon knockdown. Although Or63a is known to respond to volatile chemicals, its precise function in the context of courtship is not clear yet and more investigations are required to understand whether this candidate may be partially responsible for the Su(var)3-9 *RNAi* mating behaviors.

5.1.3 A real crosstalk between H3K9 methylation and sleep

The plot took a more interesting twist when I started hypothesizing the existence of a reciprocal crosstalk between sleep and H3K9 methylation. Thus far I described the effects in sleep regulation upon depletion of the three main H3K9 methylases in flies, G9a, Eggless, and Su(var)3-9, with deeper attention to the latter. But, on the other hand, what are the effects of H3K9 methylation upon sleep disturbances? In order to fully understand the reciprocal correlation between sleep and this specific epigenetic mark, I subjected wild-type flies to different periods of SD and monitored the level of H3K9me in the brain of SD flies, compared to no SD. The outcome was very fascinating: while acute SD (1 or 3 days of mechanical stimulation) did not seem to trigger any perturbation, a more prolonged SD, such as 5 or 7 days in a row, drastically increased the level of H3K9me in the neuronal cell bodies. To confirm the theory that chronic SD has an evident impact on H3K9me, I dissected and analyzed brains from a short-sleeping mutant, *fumin*, known to be constantly hyperactive, finding that also these flies are normally characterized by increased H3K9me compared to the baseline level in Canton-S flies. Recent studies have already highlighted evidence of perturbations in the epigenetic landscape upon sleep deprivation: research in mammals found increase of DNA methylation upon sleep loss (Massart et al. 2014; Narwade, Mallick, and Deobagkar 2017); short-sleeping individuals (<6.8h) also experienced an altered DNA methylation pattern, specifically in 52 genes, compared to long-sleeping individuals (>7.8h) (Wong et al. 2015). In terms of histone modifications, sleep deprivation reduces histone acetylation levels, known to trigger transcriptional activation: upon this condition, Duan et al., for instance, found increased expression of histone deacetylase 2 (HDAC2) and decreased level of histone acetyltransferase CBP in mice. This leads to general declined acetylation in H3 and H4, with consequential deficits in learning and cognitive impairments (Duan et al. 2016). These examples are listed here to prove the existence of a real crosstalk between sleep and epigenome. However, until now there was no evidence of the impact of sleep deprivation specifically in the H3K9 methylation. Therefore, this project led to a new discovery, which may help in generating biomarkers that can be associated with prolonged-forced wakefulness and sensitivity to sleep loss.

Another significant question was to understand whether this change in the epigenome could return to its original state after sleep recovery following the SD. Therefore, I sleep-deprived flies for at least 5 days and gave them 1 or 3 days of resting period, before dissecting their brains. Interestingly, only flies that were allowed to recover for 3 days in a row, were able to reduce their H3K9me back to baseline level, while flies that could only rest for 1 day, kept an increased H3K9me. Unfortunately, the current research on how much permanent epigenetic changes are upon SD is very poor. As suggested by Gaine et al., future studies are definitely required to explore and understand if these alterations can be reverted to baseline levels upon normal sleep patterns (Gaine, Chatterjee, and Abel 2018). Therefore, once again, this study offers a new insight into the duration of an altered histone modification, the H3K9 methylation, in relation to chronic SD. It is not clear yet if the amount of recovery sleep needed to restore the baseline level is dependent on the gravity of the sleep loss: it is possible that longer SD stimuli, such as 10 days, need more than 3 days of resting period, in order to re-establish the epigenetic balance.

Further analysis allowed me to deeply explore the origin of the increased H3K9me, finding in Su(var)3-9 a fundamental key player. By exploiting a GFP-tagged Su(var)3-9 fly line, I monitored the protein level of Su(var)3-9 upon 5 days of SD (the minimum period found to increase the H3K9me level): very interestingly, Su(var)3-9 was found to be increased in the neuronal tissue, suggesting that this enzyme is definitely responsible for the modulation of its epigenetic mark, upon chronic SD. I cannot exclude, however, also a potential contribution of the other H3K9 methylases, such as, more likely, G9a, given the promising results upon its knockdown, which suggested a presumed cooperation with Su(var)3-9. Upon chronic SD, the protein level of Su(var)3-9 is enhanced: this leads to increased H3K9me, which theoretically, should cause an increase in down-regulated genes. This was exactly the outcome of an RNAseq analysis accomplished on nSyb-G4/UAS-Su(var)3-9 *RNAi* and CS/UAS-Su(var)3-9 flies. Upon 5 days SD, CS/Su(var)3-9 exhibited an increase in downregulated target genes, compared to the same flies in no SD condition. However, the *RNAi* flies did not show such a difference, when comparing SD to no SD flies: only a tiny number of genes was downregulated upon SD. I found this result an exciting outcome, although more analyses are probably required to fully define the circumstances: despite the reduced H3K9me level in Su(var)3-9 *RNAi* fly brains, it would be interesting, for instance, to monitor the

level of the epigenetic mark in the same flies, upon chronic SD: the expectation is that, even upon the dynamic stimulus, the H3K9me level cannot be increased.
A schematic illustration of the most remarkable findings of this study is shown in Figure 5.1.

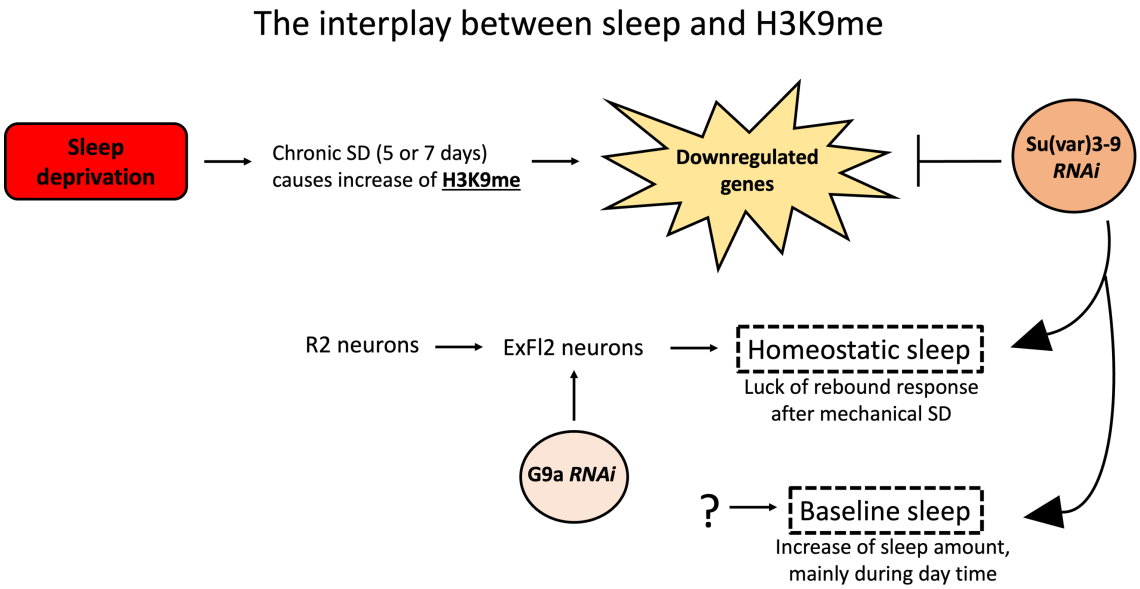


Figure 5.1. The interplay between and H3K9methylation

5.1.4 Future works and remarks

Despite these promising and impactful findings revealing H3K9me as a regulator of sleep, more extensive research and work will be required in the future to better outline and define a clearer and more complete picture. Some questions, in fact, have remained pending. The pan-neuronal knockdown of Su(var)3-9 leads to a general increase in baseline sleep: this was partially observed when depleting Su(var)3-9 specifically in the R5 neurons. However, here, the increase of baseline sleep was only detected in female and not in male flies, suggesting that other populations of neurons, together with the R5 cells, may be implicated in this function. A screening of other GAL4 lines would be beneficial to fully address this gap. Furthermore, my data showed that the simultaneous depletion of Su(var)3-9 and BRP, only partially restored the effects in baseline sleep: although these flies experienced a reduced sleep, compared to Su(var)3-9 *RNAi* only flies, their sleep was detected to be still higher than parental controls. This implies that more deregulated targets, due to the Su(var)3-9 *RNAi*, are probably responsible for the effects in baseline sleep regulation: a starting point for a deeper investigation, could be the outcome of the RNAseq, which has highlighted a detailed list of de-regulated genes in

Su(var)3-9 knocked-down flies. This will contribute to giving new insights for an in-depth molecular pathway. In addition, it would be interesting to deeply investigate the role that Su(var)3-9 was found to play specifically in dFB, EB, and MB neurons to understand the reasons for its redundant function across these regions, aiming to identify the importance of H3K9me, across the central complex, in the context of homeostatic sleep.

Another fundamental step will be to validate the hypothetical interaction between Su(var)3-9 and G9a, both found to abolish rebound sleep after 1 day of dynamic SD, upon knockdown. These two proteins were found to cooperate during development: evidence during adulthood, instead, was never published. Future experiments where Su(var)3-9 and G9a are knocked down simultaneously, may be extremely relevant to observe any aggravation of the phenotypes. Also, co-immunoprecipitations may be helpful to reveal potential interactions. Finally, a further step will be to better elucidate the effects in H3K9me upon SD. My data have exhaustively shown the crucial effects of chronic SD specifically on H3K9 tri-methylation. However, mono and di-methylation were never dissected in this project: one hypothesis that I have formulated is that acute SD (1 or 3 days) may impact H3K9 di-methylation (or mono), while chronic SD influences H3K9 tri-methylation. Immunostaining specifically for H3K9 di-me may be relevant to fully address this question. Here, it is also essential to highlight that G9a is mainly responsible for the H3K9 di-methylation: looking at the protein level of G9a over SD will be also extremely important to clarify the existence of a sort of complementation between Su(var)3-9 and G9a in sleep regulation.

5.2 DNA damage and sleep

5.2.1 Lack of sleep and accumulation of DSBs

The precise function of sleep remains an unresolved question in the field of neuroscience. In this regard, several theories have been proposed and hypothesized, and, among those, one in particular believes that sleep is fundamental to restoring homeostasis in the brain, by repairing all the unbalances occurring during wakefulness, including DNA damages. Research in various animal systems, mostly in zebrafish and mice, found that during wake time there is, in fact, an accumulation of DNA damages, which reduces in number during

sleep over night-time (Bellesi et al. 2016; D. Zada et al. 2019; Cheung et al. 2019). In addition, Zada et al. found an increase in baseline sleep in zebrafish, upon pharmacological induction of DSBs: they showed that sleep is essential to induce chromosome dynamics which allow the repair of DSBs. The transcription of DNA repair-related genes is, instead, reduced upon sleep deprivation.

This project aimed to give more insights into the reciprocal correlation between sleep and DNA damage/repair in *Drosophila Melanogaster*, where only a little progress has been achieved so far: new findings in flies may have important implications for dissecting similar processes in more complex organisms and understanding how sleep can influence DNA stability and overall health. One of the very first results was to find that flies subjected to both acute and chronic sleep deprivation experienced an increased number of DSBs. This data confirmed what was previously achieved in flies by Bellesi et al, although I believe it is fundamental to differentiate between the SD method applied in their study and the one used in this project. Indeed, in their paper, the authors placed the flies in an enriched environment, which included a very large tube, containing numerous individuals: here, flights, climbing, social interactions, etc. could occur (Bellesi et al. 2016). In my system, individual flies are sleep-deprived by using the optomotor, already discussed to be a system specifically designed to disturb only sleep. Therefore, I can state that the DSBs marked in my data could be considered as a consequence of sleep loss only and not of other conditions. On the other hand, in Bellesi's work, there are certainly more variables that may have influenced the accumulation of the damages, due to a more stressful environment. Clearly, to strengthen the findings linking sleep deprivation and DNA damage in *Drosophila melanogaster*, it would be beneficial in the future to implement more rigorous controls to exclude stress as a potential artifact. For instance, stress hormones, such as octopamine, or stress-related markers can be measured in sleep-deprived and control flies. Western blots to detect stress-related proteins (e.g., heat shock proteins) and DNA damage markers (e.g., phosphorylated H2Av or p53) should be also performed. Additionally, sleep deprived flies can be treated to mitigate stress (e.g., antioxidants or stress hormone inhibitors): if DNA damage persists despite the reduction of stress markers, it strengthens the argument that sleep deprivation is the primary cause. Finally, genetic mutants or *RNAi* lines that are deficient in stress response pathways could be also exploited. These comprehensive approaches will ensure the observed DNA damage is directly attributable to sleep deprivation.

Another new insight from my data was to find an accumulation of neuronal apoptotic marks when sleep-depriving flies, only chronically: acute SD, in fact, did not lead to this increase, after comparison to undisturbed fly brains. This interesting outcome is probably in line with previous findings: DNA repair of damages in neurons represents a very slow process, compared to more dividing cells (Barzilai, Biton, and Shiloh 2008): this means that numerous lesions can easily accumulate and if not promptly repaired, due for instance to prolonged sleep deprivation, they may lead to initiation of cell death. Acute SD, presumably, is still a stressful condition which, however, can be controlled without difficulty: the accumulated DSBs (less in number compared to those related to chronic SD) would be normally repaired, at the end of the stimuli. The accumulation of neuronal apoptotic markers in chronically sleep-deprived flies highlights the vulnerability of neurons to DNA damage and the critical role of sleep in neuronal health. This observation is consistent with the notion that neurons, being post-mitotic cells, have limited capacity for DNA repair, making them more susceptible to damage accumulation and subsequent cell death under prolonged stress conditions.

5.2.2 The reciprocal correlation between sleep and DNA damage

In the first section of this second project, I have shown the resulting effects in DSBs, upon the lack of sleep. Given the promising results, there were more questions that I started to ask: on the other way around, what are the consequences in sleep, upon induction of damages? Is there a reciprocal correlation between sleep and DNA damage? As observed in zebrafish by Zada et al., pharmacologically induced DSBs lead to an increase in baseline sleep in flies, too. Canton-S male flies fed with Etoposide, an anti-tumor drug acting as an inhibitor of the DNA topoisomerase II, were found to sleep more on the days following the treatment: this was seen when using a high dosage of Etoposide, suggesting, perhaps, that a certain amount of DSBs is required to exert sufficient homeostatic pressure for increase in sleep. In addition, this was even more pronounced in female flies, where a doubled dosage of Etoposide was necessary to enhance the change in baseline sleep, suggesting that females may be somehow more resistant than males to this specific stressful condition. In this regard, female flies were initially thought to be generally more resistant than males to various types of stresses (Pomatto, Tower, and Davies 2018): evidence confirmed, for instance, their easier resistance to starvation and desiccation

stress (Chandegra et al. 2017; Lin et al. 2023). However, studies about other forms of stress, such as oxidative stress, are much more controversial, concluding that there is no sex difference, but several factors that can influence the response to this kind of stress, like the strain or the age (Niveditha et al. 2017; Weber et al. 2012; P. C. Lovejoy et al. 2021; Belyi et al. 2020). To conclude, female and male flies experience the same sleep response, but with distinct drug concentrations, although the reasons for this difference are not clear yet.

Differently from CS, mutant *fumin* flies fed with Etoposide, did not respond to the treatment by increasing their baseline sleep: there were no differences when compared to negative controls. As previously mentioned, these flies are characterized by a mutation in the dopamine transporter gene (*dat*): the loss of *dat* causes persistently elevated levels of dopamine, responsible for their constant wakefulness. Several studies have suggested the contribution of dopamine to sleep homeostasis. Mutants for dopamine transporters in both flies and mice exhibited generally prolonged wakefulness and also reduced sleep rebound upon enriched wakefulness (Greenspan et al. 2001; Wisor et al. 2001; Kume et al. 2005; M. N. Wu et al. 2008b). The fact that *fumin* flies are not minimally influenced by Etoposide treatment, may preclude that the regulation of dopamine levels is an essential requirement to exert homeostatic pressure for an increase in sleep, upon DNA damage signalling. Interestingly, dopamine itself was linked to increased oxidative stress and DNA damages and, consequently, to the onset of neurological diseases (Fazeli et al. 2010). Therefore, it is possible that the high levels of dopamine in *fumin* mutants could interfere with the signaling pathways that trigger sleep in response to DNA damage, highlighting a potential link between neurotransmitter regulation and genomic stability. It would be very interesting in the future to monitor the lifespan of these flies upon treatment with the drug, to highlight any reduction in their longevity.

Finally, dorsal fan-shaped body neurons were found to be implicated in enhancing sleep after the accumulation of DSBs. The genetic inactivation of these cells (precisely, the ExFl2 neurons, largely discussed in the previous sections), which abolishes the sleep increase seen with DNA damage, points to a specific neural circuitry that mediates the sleep response. This finding opens avenues for exploring how different brain regions coordinate to integrate DNA damage signals and regulate sleep. Despite this very promising result, it was unclear the fact that the synaptic inhibition of the dFB neurons

did not allow the accumulation of the DSBs upon drug treatment: in addition, the baseline distribution of H2Av in the same flies was not just restricted to the nuclei of the cell bodies, as it is for the parental control. Overall, advanced techniques such as optogenetics and functional imaging could be employed to map the neural circuits involved in sleep regulation upon DNA damage. Understanding the connectivity and activity patterns of neurons in response to DNA repair signals will shed light on the neural basis of sleep homeostasis.

5.2.3 DNA repair-related genes in sleep functioning

To avoid unspecific effects of the Etoposide, I triggered the neuronal knockdown of specific DNA repair key players, p53 and ATM, to facilitate the accumulation of DSBs only in the brain tissue. p53 *RNAi* flies experienced a general increase in baseline sleep over the whole day in young flies: however, this increase was occurring predominantly during the light phase in aged flies, probably due to the general reduction of sleep that flies normally experience during aging. These results, in line with my previous findings, further suggest that the accumulation of DSBs in the brain enhances the increase of sleep, which remains consistent over the lifespan in *RNAi* flies, due to the problematic repair of the damages. This was confirmed by immunostaining experiments, which highlighted a higher baseline level of H2Av, a marker for DNA damages, in the brains of knockdown flies, compared to parental controls. Very interestingly, chronic sleep deprivation reduced the lifespan in p53 knockdown flies: this was the very first insight into lethal impacts upon SD stimuli, as Geissman et al. previously found that prolonged SD has usually minimal lethal consequences in wild-type flies (Geissmann, Beckwith, and Gilestro 2019). Therefore, according to the results of this study, it appears that flies with suppressed p53 experience an accumulation of DNA damage, which prompts an elevated requirement for sleep to support DNA repair. As a result, chronic sleep deprivation appears to elevate DNA damage levels, potentially resulting in premature mortality.

Contrary to p53 *RNAi* flies, the circumstances for ATM *RNAi* flies were more complicated to interpret. The reduction of ATM in neurons had no discernible impact on the sleep patterns of young adult flies. This outcome was surprising, given that ATM plays a pivotal role as a central regulator in the DNA damage response pathway (DDR) and triggers p53 activation: a phenotype more similar to p53-related data was, in fact,

expected. This may imply that p53 activation is more likely not orchestrated only by ATM, but, instead, occurs through alternative pathways, thereby enabling effective DNA repair and subsequently resulting in less DNA damage to prompt heightened sleep requirements. The absence of sleep response in ATM knockdown flies, was also confirmed by immunostaining, which showed that the baseline level of DNA damages remained unchanged, compared to parental controls. Unexpectedly, the same flies also did not accumulate DSBs upon prolonged SD: these results are still not fully understood. Only older flies appeared to sleep more than parental controls during the light phase, as similar as p53 *RNAi* flies: this may imply that only over aging, these flies become more sensitive to DNA damages. In addition, the lifespan of ATM *RNAi* flies was extremely affected, compared to parental controls': even flies not subjected to chronic SD experienced, in fact, a very reduced longevity, suggesting that the knockdown itself is incredibly deleterious. After all, it is already known that mutations in the ATM gene in humans lead to the development of a genetic disorder, the ataxia-telangiectasia (AT), characterized by heightened sensitivity to radiation, neural degeneration, weakened immune function, premature aging and increased susceptibility to cancer (Shiloh 2003). All available evidence strongly supports the notion that ATM plays a pivotal role in safeguarding the integrity of the genome. Considering that in this work the knockdown of ATM was only triggered in the brain (leading, therefore, to less deleterious consequences than ATM mutations), it remains still unclear why young *RNAi* flies appeared to behave normally: one explanation may lie in the partial overlapping role that ATM and ATR play in repairing DNA damages. Besides ATR and ATM have been found to be mainly required for SSBs and DSBs, respectively, there is also evidence of a more complex interplay between these two proteins (Williams and Zhang 2021; W. Li and Yanowitz 2019). It is plausible that some of the damages not promptly repaired, as a consequence of the knockdown of ATM, are still recognized and opportunely fixed by ATR: only upon aging, when the only action of the latter could be not sufficient anymore, the effects become more severe. This remains only and hypothesis and more deeper investigations are required to better elucidate the circumstances.

A schematic illustration of the most remarkable findings of this study is shown in Figure 5.2.

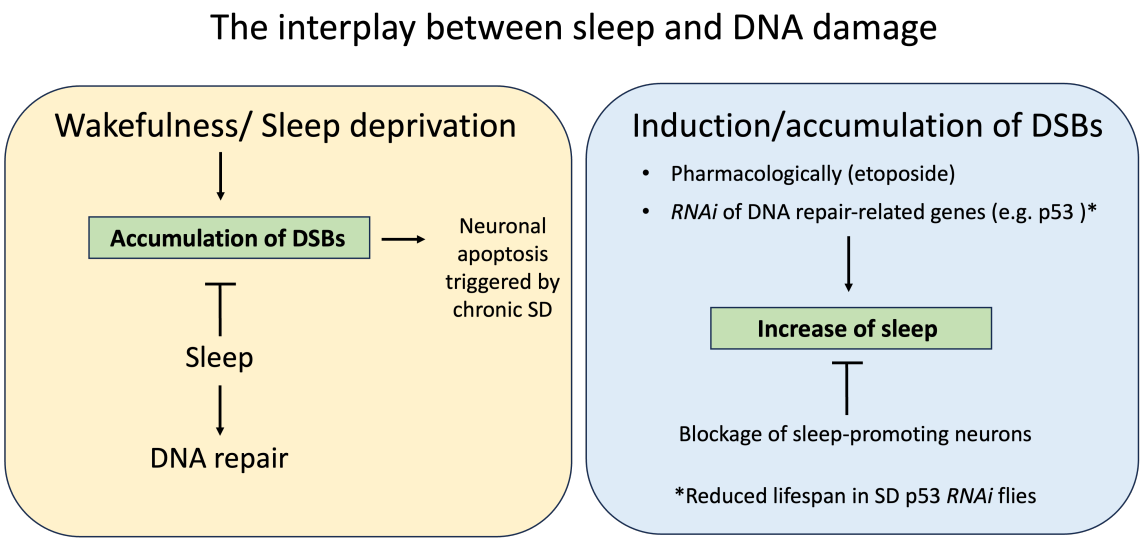


Figure 5.2. The interplay between sleep and DNA damage.

5.2.4 Future works and remarks

The knockdown of p53 and ATM occurs starting from developmental stages. To ensure that the phenotypes observed are specifically related to adult impairments, it would be beneficial to repeat the experiments by exploiting the nSyb-GAL4-GeneSwitch, already introduced in Chapter 2. Furthermore, given the fact that all the experiments for this project have been accomplished in male flies, it is fundamental to test females, too. In general, female flies tend to sleep less than males on average, showing most of the sleep episodes during the night phase. In addition, they normally exhibit greater susceptibility to SD and prolonged SD can result in some level of lethality (Geissmann, Beckwith, and Gilestro 2019). Therefore, it would be very intriguing to investigate how sleep deprivation affects the lifespan of female ATM or p53 *RNAi* flies, in comparison to their male counterparts. Initial findings from this study suggested that female flies require higher doses of Etoposide than males to impact their sleep, indicating a different resistance to the effects of DNA damage. Further exploration of this sexual dichotomy could yield valuable insights. Another future strategy would be to assess further the role in DNA repair of specific brain regions normally responsible for regulating sleep homeostasis. I already showed some preliminary results, that promote the ExFl2 neurons, located in the fan-shaped body, as key players to trigger sleep upon accumulation of DNA damages. In the future, it will be interesting to enhance the knockdown of p53 or other DNA repair-related genes, specifically in this area, and dissect the effects on sleep and lifespan of

RNAi flies, compared to controls. Finally, the ultimate objective will be to unveil a neural pathway connecting the realms of sleep and DNA repair.

5.3 Conclusions

To conclude, in this thesis, I have presented and dissected the interplay between sleep and genomic dynamics, upon changes in DNA, at two different levels. First, I have focused on sleep in the context of epigenetics, normally involving modifications localized on "top" of the DNA. At this level, I particularly investigate the correlation between sleep and H3K9 histone methylation, finding in this epigenetic mark a key role in governing sleep-related changes in gene expression. Chronic sleep deprivation, whether induced mechanically or through genetic manipulation, results in an overall reduction in gene expression, primarily driven by the activity of H3K9 methylation in the brain. In this regard, I found that the pan-neuronal knockdown of Su(var)3-9, one of the main H3K9 methylases in flies, prevents sleep-specific alterations in gene expression and disrupts the fly's internal homeostatic regulation in sleep. These results were also partially replicated upon knockdown of G9a, another fundamental methylase, suggesting, perhaps, a potential collaboration with Su(var)3-9 in this related function of sleep regulation. In the second part of this thesis, I have focused on sleep, in the context of DNA damages inside the double helix, such as double-strand breaks (DSBs). The study sheds light on the pivotal role of sleep in DNA repair, where the main progress has been achieved in other model systems, such as zebrafish. Chronic sleep deprivation leads to the accumulation of DSBs in the adult fly brain, which can enhance initiation of apoptosis. Furthermore, inducing DSBs pharmacologically in flies results in an increase in baseline sleep, further promoting DNA damage as a homeostatic drive for sleep. Very interestingly, knocking down DNA repair-related genes, particularly p53, in neurons, results in increased sleep, due to elevated levels of DNA damage in the brain. Also, chronic sleep deprivation decreases lifespan in these flies: the absence of sleep can aggravate the circumstances, due to impaired DNA repair mechanisms.

Overall, understanding the cellular mechanisms behind the regulation and function of sleep may offer opportunities to identify in the future, more specific therapies, or general biomarkers, which can enhance the quality of restorative sleep for individuals struggling

with sleep disorders and can provide key insights to better elucidate the interconnection between sleep, ageing, cognitive deficits and, eventually, neurodegenerative disorders.

Bibliography

Adam, M., F. Robert, M. Larochelle, and L. Gaudreau. 2001. 'H2A.Z Is Required for Global Chromatin Integrity and for Recruitment of RNA Polymerase II under Specific Conditions'. *Molecular and Cellular Biology* 21 (18): 6270–79. https://doi.org/10.1128/MCB.21.18.6270-6279.2001.

Adams, M. D., S. E. Celniker, R. A. Holt, C. A. Evans, J. D. Gocayne, P. G. Amanatides, S. E. Scherer, et al. 2000. 'The Genome Sequence of Drosophila Melanogaster'. *Science (New York, N.Y.)* 287 (5461): 2185–95. https://doi.org/10.1126/science.287.5461.2185.

Allada, Ravi, Chiara Cirelli, and Amita Sehgal. 2017. 'Molecular Mechanisms of Sleep Homeostasis in Flies and Mammals'. *Cold Spring Harbor Perspectives in Biology* 9 (8): a027730. https://doi.org/10.1101/cshperspect.a027730.

Allfrey, V. G., R. Faulkner, and A. E. Mirsky. 1964. 'ACETYLATION AND METHYLATION OF HISTONES AND THEIR POSSIBLE ROLE IN THE REGULATION OF RNA SYNTHESIS'. *Proceedings of the National Academy of Sciences of the United States of America* 51 (5): 786–94. https://doi.org/10.1073/pnas.51.5.786.

Alphen, Bart van, Melvyn H. W. Yap, Leonie Kirszenblat, Benjamin Kottler, and Bruno van Swinderen. 2013. 'A Dynamic Deep Sleep Stage in Drosophila'. *Journal of Neuroscience* 33 (16): 6917–27. https://doi.org/10.1523/JNEUROSCI.0061-13.2013.

Andretic, Rozi, and Paul J. Shaw. 2005. 'Essentials of Sleep Recordings in Drosophila: Moving beyond Sleep Time'. *Methods in Enzymology* 393:759–72. https://doi.org/10.1016/S0076-6879(05)93040-1.

Angelousi, Anna, Eva Kassi, Narjes Ansari-Nasiri, Harpal Randeva, Gregory Kaltsas, and George Chrousos. 2019. 'Clock Genes and Cancer Development in Particular in Endocrine Tissues'. *Endocrine-Related Cancer* 26 (6): R305–17. https://doi.org/10.1530/ERC-19-0094.

Anreiter, Ina, Jamie M. Kramer, and Marla B. Sokolowski. 2017. 'Epigenetic Mechanisms Modulate Differences in Drosophila Foraging Behavior'.

Proceedings of the National Academy of Sciences 114 (47): 12518–23. https://doi.org/10.1073/pnas.1710770114.

Aserinsky, Eugene, and Nathaniel Kleitman. 1953. 'Regularly Occurring Periods of Eye Motility, and Concomitant Phenomena, During Sleep'. *Science* 118 (3062): 273–74. https://doi.org/10.1126/science.118.3062.273.

Asher, Gad, David Gatfield, Markus Stratmann, Hans Reinke, Charna Dibner, Florian Kreppel, Raul Mostoslavsky, Frederick W. Alt, and Ueli Schibler. 2008. 'SIRT1 Regulates Circadian Clock Gene Expression through PER2 Deacetylation'. *Cell* 134 (2): 317–28. https://doi.org/10.1016/j.cell.2008.06.050.

Aso, Yoshinori, Divya Sitaraman, Toshiharu Ichinose, Karla R. Kaun, Katrin Vogt, Ghislain Belliart-Guérin, Pierre-Yves Plaçais, et al. 2014. 'Mushroom Body Output Neurons Encode Valence and Guide Memory-Based Action Selection in Drosophila'. *eLife* 3 (December):e04580. https://doi.org/10.7554/eLife.04580.

Avery, Oswald T., Colin M. MacLeod, and Maclyn McCarty. 1944. 'STUDIES ON THE CHEMICAL NATURE OF THE SUBSTANCE INDUCING TRANSFORMATION OF PNEUMOCOCCAL TYPES'. *The Journal of Experimental Medicine* 79 (2): 137–58.

Baldi, Sandro, and Peter B. Becker. 2013. 'The Variant Histone H2A.V of Drosophila--Three Roles, Two Guises'. *Chromosoma* 122 (4): 245–58. https://doi.org/10.1007/s00412-013-0409-x.

Bannister, A. J., P. Zegerman, J. F. Partridge, E. A. Miska, J. O. Thomas, R. C. Allshire, and T. Kouzarides. 2001. 'Selective Recognition of Methylated Lysine 9 on Histone H3 by the HP1 Chromo Domain'. *Nature* 410 (6824): 120–24. https://doi.org/10.1038/35065138.

Bannister, Andrew J., and Tony Kouzarides. 2011. 'Regulation of Chromatin by Histone Modifications'. *Cell Research* 21 (3): 381–95. https://doi.org/10.1038/cr.2011.22.

Barzilai, Ari, Sharon Biton, and Yosef Shiloh. 2008. 'The Role of the DNA Damage Response in Neuronal Development, Organization and Maintenance'. *DNA Repair*, DNA Damage Responses and Neurological Disease, 7 (7): 1010–27. https://doi.org/10.1016/j.dnarep.2008.03.005.

Becker, Justin S., Ryan L. McCarthy, Simone Sidoli, Greg Donahue, Kelsey E. Kaeding, Zhiying He, Shu Lin, Benjamin A. Garcia, and Kenneth S. Zaret. 2017. 'Genomic and Proteomic Resolution of Heterochromatin and Its Restriction of

Alternate Fate Genes'. *Molecular Cell* 68 (6): 1023-1037.e15. https://doi.org/10.1016/j.molcel.2017.11.030.

Beckwith, Esteban J., and Alice S. French. 2019. 'Sleep in Drosophila and Its Context'. *Frontiers in Physiology* 10:1167. https://doi.org/10.3389/fphys.2019.01167.

Beckwith, Esteban J, Quentin Geissmann, Alice S French, and Giorgio F Gilestro. 2017. 'Regulation of Sleep Homeostasis by Sexual Arousal'. Edited by Leslie C Griffith. *eLife* 6 (September):e27445. https://doi.org/10.7554/eLife.27445.

Bedont, Joseph L., Anna Kolesnik, Pavel Pivarshev, Dania Malik, Cynthia T. Hsu, Aalim Weljie, and Amita Sehgal. 2023. 'Chronic Sleep Loss Sensitizes Drosophila Melanogaster to Nitrogen Stress'. *Current Biology: CB* 33 (8): 1613-1623.e5. https://doi.org/10.1016/j.cub.2023.03.008.

Bellesi, Michele, Daniel Bushey, Mattia Chini, Giulio Tononi, and Chiara Cirelli. 2016. 'Contribution of Sleep to the Repair of Neuronal DNA Double-Strand Breaks: Evidence from Flies and Mice'. *Scientific Reports* 6 (November):36804. https://doi.org/10.1038/srep36804.

Belyi, Alexei A., Alexey A. Alekseev, Alexander Y. Fedintsev, Stepan N. Balybin, Ekaterina N. Proshkina, Mikhail V. Shaposhnikov, and Alexey A. Moskalev. 2020. 'The Resistance of Drosophila Melanogaster to Oxidative, Genotoxic, Proteotoxic, Osmotic Stress, Infection, and Starvation Depends on Age According to the Stress Factor'. *Antioxidants* 9 (12): 1239. https://doi.org/10.3390/antiox9121239.

Benington, J. H., and H. C. Heller. 1995. 'Restoration of Brain Energy Metabolism as the Function of Sleep'. *Progress in Neurobiology* 45 (4): 347–60. https://doi.org/10.1016/0301-0082(94)00057-o.

Benjamini, Yoav, and Yosef Hochberg. 1995. 'Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing'. *Journal of the Royal Statistical Society: Series B (Methodological)* 57 (1): 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.

Benzer, S. 1967. 'BEHAVIORAL MUTANTS OF Drosophila ISOLATED BY COUNTERCURRENT DISTRIBUTION'. *Proceedings of the National Academy of Sciences of the United States of America* 58 (3): 1112–19. https://doi.org/10.1073/pnas.58.3.1112.

———. 1973. 'Genetic Dissection of Behavior'. *Scientific American* 229 (6): 24–37. https://doi.org/10.1038/scientificamerican1273-24.

Berger, Shelley L., Tony Kouzarides, Ramin Shiekhattar, and Ali Shilatifard. 2009. 'An Operational Definition of Epigenetics'. *Genes & Development* 23 (7): 781–83. https://doi.org/10.1101/gad.1787609.

Berni, Jimena, Alistair M. Muldal, and Stefan R. Pulver. 2010. 'Using Neurogenetics and the Warmth-Gated Ion Channel TRPA1 to Study the Neural Basis of Behavior in Drosophila'. *Journal of Undergraduate Neuroscience Education* 9 (1): A5.

Biswas, Subhankar, and C. Mallikarjuna Rao. 2017. 'Epigenetics in Cancer: Fundamentals and Beyond'. *Pharmacology & Therapeutics* 173 (May):118–34. https://doi.org/10.1016/j.pharmthera.2017.02.011.

Bliek, A. M. van der, and E. M. Meyerowitz. 1991. 'Dynamin-like Protein Encoded by the Drosophila Shibire Gene Associated with Vesicular Traffic'. *Nature* 351 (6325): 411–14. https://doi.org/10.1038/351411a0.

Borbély, A. A. 1982. 'A Two Process Model of Sleep Regulation'. *Human Neurobiology* 1 (3): 195–204.

Bradley, T. J., and F. H. Simmons. 1997. 'An Analysis of Resource Allocation in Response to Dietary Yeast in Drosophila Melanogaster'. *Journal of Insect Physiology* 43 (8): 779–88. https://doi.org/10.1016/s0022-1910(97)00037-1.

Brand, A. H., and N. Perrimon. 1993. 'Targeted Gene Expression as a Means of Altering Cell Fates and Generating Dominant Phenotypes'. *Development (Cambridge, England)* 118 (2): 401–15.

Bray, Nicolas L., Harold Pimentel, Páll Melsted, and Lior Pachter. 2016. 'Near-Optimal Probabilistic RNA-Seq Quantification'. *Nature Biotechnology* 34 (5): 525–27. https://doi.org/10.1038/nbt.3519.

Bremer, Frédéric. 1937. *L'activité Cérébrale Au Cours Du Sommeil et de La Narcose: Contribution à l'étude Du Mécanisme Du Sommeil*. Imprimerie médicale et scientifique (Société anonyme).

Brenet, Fabienne, Michelle Moh, Patricia Funk, Erika Feierstein, Agnes J. Viale, Nicholas D. Socci, and Joseph M. Scandura. 2011a. 'DNA Methylation of the First Exon Is Tightly Linked to Transcriptional Silencing'. *PloS One* 6 (1): e14524. https://doi.org/10.1371/journal.pone.0014524.

———. 2011b. 'DNA Methylation of the First Exon Is Tightly Linked to Transcriptional Silencing'. *PloS One* 6 (1): e14524. https://doi.org/10.1371/journal.pone.0014524.

Broughton, Susan J., Matthew D. W. Piper, Tomoatsu Ikeya, Timothy M. Bass, Jake Jacobson, Yasmine Driege, Pedro Martinez, et al. 2005. 'Longer Lifespan, Altered Metabolism, and Stress Resistance in Drosophila from Ablation of Cells Making Insulin-like Ligands'. *Proceedings of the National Academy of Sciences of the United States of America* 102 (8): 3105–10. https://doi.org/10.1073/pnas.0405775102.

Brown, E. J., and D. Baltimore. 2000. 'ATR Disruption Leads to Chromosomal Fragmentation and Early Embryonic Lethality'. *Genes & Development* 14 (4): 397–402.

Brown, Elizabeth B., Kreesha D. Shah, Richard Faville, Benjamin Kottler, and Alex C. Keene. 2020. 'Drosophila Insulin-like Peptide 2 Mediates Dietary Regulation of Sleep Intensity'. *PLOS Genetics* 16 (3): e1008270. https://doi.org/10.1371/journal.pgen.1008270.

Brown, Eric J., and David Baltimore. 2003. 'Essential and Dispensable Roles of ATR in Cell Cycle Arrest and Genome Maintenance'. *Genes & Development* 17 (5): 615–28. https://doi.org/10.1101/gad.1067403.

Brown, Seth J., Peter Stoilov, and Yi Xing. 2012. 'Chromatin and Epigenetic Regulation of Pre-mRNA Processing'. *Human Molecular Genetics* 21 (R1): R90–96. https://doi.org/10.1093/hmg/dds353.

Bruno, Tiziana, Francesca De Nicola, Simona Iezzi, Daniele Lecis, Carmen D'Angelo, Monica Di Padova, Nicoletta Corbi, et al. 2006. 'Che-1 Phosphorylation by ATM/ATR and Chk2 Kinases Activates P53 Transcription and the G2/M Checkpoint'. *Cancer Cell* 10 (6): 473–86. https://doi.org/10.1016/j.ccr.2006.10.012.

Bushey, Daniel, Reto Huber, Giulio Tononi, and Chiara Cirelli. 2007. 'Drosophila Hyperkinetic Mutants Have Reduced Sleep and Impaired Memory'. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 27 (20): 5384–93. https://doi.org/10.1523/JNEUROSCI.0108-07.2007.

Butt, Masood Sadiq, and M. Tauseef Sultan. 2011. 'Coffee and Its Consumption: Benefits and Risks'. *Critical Reviews in Food Science and Nutrition* 51 (4): 363–73. https://doi.org/10.1080/10408390903586412.

Campbell, S. S., and I. Tobler. 1984. 'Animal Sleep: A Review of Sleep Duration across Phylogeny'. *Neuroscience and Biobehavioral Reviews* 8 (3): 269–300. https://doi.org/10.1016/0149-7634(84)90054-x.

Campbell, Scott S., and Irene Tobler. 1984. 'Animal Sleep: A Review of Sleep Duration across Phylogeny'. *Neuroscience & Biobehavioral Reviews* 8 (3): 269–300. https://doi.org/10.1016/0149-7634(84)90054-X.

Camus, M. Florencia, Chun-Cheng Huang, Max Reuter, and Kevin Fowler. 2018. 'Dietary Choices Are Influenced by Genotype, Mating Status, and Sex in Drosophila Melanogaster'. *Ecology and Evolution* 8 (11): 5385–93. https://doi.org/10.1002/ece3.4055.

Carskadon, Mary A., Kenneth R. Chappell, David H. Barker, Anne C. Hart, Kayla Dwyer, Caroline Gredvig-Ardito, Caitlyn Starr, and John E. McGeary. 2019. 'A Pilot Prospective Study of Sleep Patterns and DNA Methylation-Characterized Epigenetic Aging in Young Adults'. *BMC Research Notes* 12 (September):583. https://doi.org/10.1186/s13104-019-4633-1.

Cavanaugh, Daniel J., Jill D. Geratowski, Julian R. A. Wooltorton, Jennifer M. Spaethling, Clare E. Hector, Xiangzhong Zheng, Erik C. Johnson, James H. Eberwine, and Amita Sehgal. 2014. 'Identification of a Circadian Output Circuit for Rest:Activity Rhythms in Drosophila'. *Cell* 157 (3): 689–701. https://doi.org/10.1016/j.cell.2014.02.024.

Cedernaes, Jonathan, Megan E. Osler, Sarah Voisin, Jan-Erik Broman, Heike Vogel, Suzanne L. Dickson, Juleen R. Zierath, Helgi B. Schiöth, and Christian Benedict. 2015. 'Acute Sleep Loss Induces Tissue-Specific Epigenetic and Transcriptional Alterations to Circadian Clock Genes in Men'. *The Journal of Clinical Endocrinology and Metabolism* 100 (9): E1255-1261. https://doi.org/10.1210/JC.2015-2284.

Chandegra, Bhakti, Jocelyn Lok Yee Tang, Haoyu Chi, and Nazif Alic. 2017. 'Sexually Dimorphic Effects of Dietary Sugar on Lifespan, Feeding and Starvation Resistance in Drosophila'. *Aging (Albany NY)* 9 (12): 2521–28. https://doi.org/10.18632/aging.101335.

Chang, Howard H. Y., Nicholas R. Pannunzio, Noritaka Adachi, and Michael R. Lieber. 2017. 'Non-Homologous DNA End Joining and Alternative Pathways to Double-Strand Break Repair'. *Nature Reviews Molecular Cell Biology* 18 (8): 495–506. https://doi.org/10.1038/nrm.2017.48.

Chen, Wenfeng, Zhenxing Liu, Tianjiao Li, Ruifeng Zhang, Yongbo Xue, Yang Zhong, Weiwei Bai, Dasen Zhou, and Zhangwu Zhao. 2014. 'Regulation of Drosophila

Circadian Rhythms by miRNA Let-7 Is Mediated by a Regulatory Cycle'. *Nature Communications* 5 (1): 1–13. https://doi.org/10.1038/ncomms6549.

Cheung, V., V. M. Yuen, G. T. C. Wong, and S. W. Choi. 2019. 'The Effect of Sleep Deprivation and Disruption on DNA Damage and Health of Doctors'. *Anaesthesia* 74 (4): 434–40. https://doi.org/10.1111/anae.14533.

Choi, Jun-Hyuk, Laura A. Lindsey-Boltz, Michael Kemp, Aaron C. Mason, Marc S. Wold, and Aziz Sancar. 2010. 'Reconstitution of RPA-Covered Single-Stranded DNA-Activated ATR-Chk1 Signaling'. *Proceedings of the National Academy of Sciences* 107 (31): 13660–65. https://doi.org/10.1073/pnas.1007856107.

Chou, Wen-Cheng, Hui-Chun Wang, Fen-Hwa Wong, Shian-ling Ding, Pei-Ei Wu, Sheau-Yann Shieh, and Chen-Yang Shen. 2008. 'Chk2-Dependent Phosphorylation of XRCC1 in the DNA Damage Response Promotes Base Excision Repair'. *The EMBO Journal* 27 (23): 3140–50. https://doi.org/10.1038/emboj.2008.229.

Chuang, Yu-Hsuan, Austin Quach, Devin Absher, Themistocles Assimes, Steve Horvath, and Beate Ritz. 2017. 'Coffee Consumption Is Associated with DNA Methylation Levels of Human Blood'. *European Journal of Human Genetics* 25 (5): 608–16. https://doi.org/10.1038/ejhg.2016.175.

Ciccia, Alberto, and Stephen J. Elledge. 2010. 'The DNA Damage Response: Making It Safe to Play with Knives'. *Molecular Cell* 40 (2): 179–204. https://doi.org/10.1016/j.molcel.2010.09.019.

Cirelli, Chiara, and Daniel Bushey. 2008. 'Sleep and Wakefulness in Drosophila Melanogaster'. *Annals of the New York Academy of Sciences* 1129:323–29. https://doi.org/10.1196/annals.1417.017.

Cirelli, Chiara, Timothy M. LaVaute, and Giulio Tononi. 2005. 'Sleep and Wakefulness Modulate Gene Expression in Drosophila'. *Journal of Neurochemistry* 94 (5): 1411–19. https://doi.org/10.1111/j.1471-4159.2005.03291.x.

Cirelli, Chiara, and Giulio Tononi. 2008. 'Is Sleep Essential?' *PLoS Biology* 6 (8). https://doi.org/10.1371/journal.pbio.0060216.

Clancy, D. J., D. Gems, L. G. Harshman, S. Oldham, H. Stocker, E. Hafen, S. J. Leevers, and L. Partridge. 2001. 'Extension of Life-Span by Loss of CHICO, a Drosophila Insulin Receptor Substrate Protein'. *Science (New York, N.Y.)* 292 (5514): 104–6. https://doi.org/10.1126/science.1057991.

Clough, Emily, Thomas Tedeschi, and Tulle Hazelrigg. 2014. 'Epigenetic Regulation of Oogenesis and Germ Stem Cell Maintenance by the Drosophila Histone Methyltransferase Eggless/dSetDB1'. *Developmental Biology* 388 (2): 181–91. https://doi.org/10.1016/j.ydbio.2014.01.014.

Cong, Xiaona, Haili Wang, Zhenxing Liu, Chunxia He, Chunju An, and Zhangwu Zhao. 2015. 'Regulation of Sleep by Insulin-like Peptide System in Drosophila Melanogaster'. *Sleep* 38 (7): 1075–83. https://doi.org/10.5665/sleep.4816.

Cortez, D., S. Guntuku, J. Qin, and S. J. Elledge. 2001. 'ATR and ATRIP: Partners in Checkpoint Signaling'. *Science (New York, N.Y.)* 294 (5547): 1713–16. https://doi.org/10.1126/science.1065521.

Crittenden, J. R., E. M. Skoulakis, K. A. Han, D. Kalderon, and R. L. Davis. 1998. 'Tripartite Mushroom Body Architecture Revealed by Antigenic Markers'. *Learning & Memory (Cold Spring Harbor, N.Y.)* 5 (1–2): 38–51.

Daal, A. van, and S. C. Elgin. 1992. 'A Histone Variant, H2AvD, Is Essential in Drosophila Melanogaster'. *Molecular Biology of the Cell* 3 (6): 593–602. https://doi.org/10.1091/mbc.3.6.593.

Damke, H., T. Baba, D. E. Warnock, and S. L. Schmid. 1994. 'Induction of Mutant Dynamin Specifically Blocks Endocytic Coated Vesicle Formation'. *The Journal of Cell Biology* 127 (4): 915–34. https://doi.org/10.1083/jcb.127.4.915.

Davies, Matthew N., Manuela Volta, Ruth Pidsley, Katie Lunnon, Abhishek Dixit, Simon Lovestone, Cristian Coarfa, et al. 2012. 'Functional Annotation of the Human Brain Methylome Identifies Tissue-Specific Epigenetic Variation across Brain and Blood'. *Genome Biology* 13 (6): R43. https://doi.org/10.1186/gb-2012-13-6-r43.

Davis, Christopher J., Stewart G. Bohnet, Joseph M. Meyerson, and James M. Krueger. 2007. 'Sleep Loss Changes microRNA Levels in the Brain: A Possible Mechanism for State-Dependent Translational Regulation'. *Neuroscience Letters* 422 (1): 68–73. https://doi.org/10.1016/j.neulet.2007.06.005.

Davis, Christopher J., James M. Clinton, and James M. Krueger. 2012. 'MicroRNA 138, Let-7b, and 125a Inhibitors Differentially Alter Sleep and EEG Delta-Wave Activity in Rats'. *Journal of Applied Physiology* 113 (11): 1756–62. https://doi.org/10.1152/japplphysiol.00940.2012.

Davla, Sejal, Gregory Artiushin, Yongjun Li, Daryan Chitsaz, Sally Li, Amita Sehgal, and Donald J van Meyel. 2020. 'AANAT1 Functions in Astrocytes to Regulate

Sleep Homeostasis'. Edited by K VijayRaghavan and Marcos G Frank. *eLife* 9 (September):e53994. https://doi.org/10.7554/eLife.53994.

De Jesús-Olmo, Luis A., Norma Rodríguez, Marcelo Francia, Jonathan Alemán-Rios, Carlos J. Pacheco-Agosto, Joselyn Ortega-Torres, Richard Nieves, Nicolás Fuenzalida-Uribe, Alfredo Ghezzi, and José L. Agosto. 2020. 'Pumilio Regulates Sleep Homeostasis in Response to Chronic Sleep Deprivation in Drosophila Melanogaster'. *Frontiers in Neuroscience* 14 (April):319. https://doi.org/10.3389/fnins.2020.00319.

Dement, William, and Nathaniel Kleitman. 1957. 'The Relation of Eye Movements during Sleep to Dream Activity: An Objective Method for the Study of Dreaming'. *Journal of Experimental Psychology* 53 (5): 339–46. https://doi.org/10.1037/h0048189.

Denk, W., J. H. Strickler, and W. W. Webb. 1990. 'Two-Photon Laser Scanning Fluorescence Microscopy'. *Science (New York, N.Y.)* 248 (4951): 73–76. https://doi.org/10.1126/science.2321027.

Derrien, Thomas, Roderic Guigó, and Rory Johnson. 2012. 'The Long Non-Coding RNAs: A New (P)Layer in the "Dark Matter"'. *Frontiers in Genetics* 2. https://doi.org/10.3389/fgene.2011.00107.

DeVorkin, Lindsay, Nancy Erro Go, Ying-Chen Claire Hou, Annie Moradian, Gregg B. Morin, and Sharon M. Gorski. 2014. 'The Drosophila Effector Caspase Dcp-1 Regulates Mitochondrial Dynamics and Autophagic Flux via SesB'. *The Journal of Cell Biology* 205 (4): 477–92. https://doi.org/10.1083/jcb.201303144.

Diekelmann, Susanne, and Jan Born. 2010. 'The Memory Function of Sleep'. *Nature Reviews. Neuroscience* 11 (2): 114–26. https://doi.org/10.1038/nrn2762.

Dijk, Derk-Jan. 2009. 'Regulation and Functional Correlates of Slow Wave Sleep'. *Journal of Clinical Sleep Medicine* 5 (2 suppl): S6–15. https://doi.org/10.5664/jcsm.5.2S.S6.

Dionne, Heather, Karen L. Hibbard, Amanda Cavallaro, Jui-Chun Kao, and Gerald M. Rubin. 2018. 'Genetic Reagents for Making Split-GAL4 Lines in Drosophila'. *Genetics* 209 (1): 31–35. https://doi.org/10.1534/genetics.118.300682.

DiTacchio, Luciano, Hiep D. Le, Christopher Vollmers, Megumi Hatori, Michael Witcher, Julie Secombe, and Satchidananda Panda. 2011. 'Histone Lysine Demethylase JARID1a Activates CLOCK-BMAL1 and Influences the Circadian

Clock'. *Science (New York, N.Y.)* 333 (6051): 1881–85. https://doi.org/10.1126/science.1206022.

Do, Michael Tri Hoang, and King-Wai Yau. 2010. 'Intrinsically Photosensitive Retinal Ganglion Cells'. *Physiological Reviews* 90 (4): 1547–81. https://doi.org/10.1152/physrev.00013.2010.

DO, MICHAEL TRI HOANG, and KING-WAI YAU. 2010. 'Intrinsically Photosensitive Retinal Ganglion Cells'. *Physiological Reviews* 90 (4): 1547–81. https://doi.org/10.1152/physrev.00013.2010.

Doi, Masao, Jun Hirayama, and Paolo Sassone-Corsi. 2006. 'Circadian Regulator CLOCK Is a Histone Acetyltransferase'. *Cell* 125 (3): 497–508. https://doi.org/10.1016/j.cell.2006.03.033.

Donlea, Jeffrey M., Diogo Pimentel, Clifford B. Talbot, Anissa Kempf, Jaison J. Omoto, Volker Hartenstein, and Gero Miesenböck. 2018. 'Recurrent Circuitry for Balancing Sleep Need and Sleep'. *Neuron* 97 (2): 378-389.e4. https://doi.org/10.1016/j.neuron.2017.12.016.

Donlea, Jeffrey M., Matthew S. Thimgan, Yasuko Suzuki, Laura Gottschalk, and Paul J. Shaw. 2011. 'Inducing Sleep by Remote Control Facilitates Memory Consolidation in Drosophila'. *Science (New York, N.Y.)* 332 (6037): 1571–76. https://doi.org/10.1126/science.1202249.

Donlea, Jeffrey M., Diogo Pimentel, and Gero Miesenböck. 2014a. 'Neuronal Machinery of Sleep Homeostasis in Drosophila'. *Neuron* 81 (4): 860–72. https://doi.org/10.1016/j.neuron.2013.12.013.

———. 2014b. 'Neuronal Machinery of Sleep Homeostasis in Drosophila'. *Neuron* 81 (4): 860–72. https://doi.org/10.1016/j.neuron.2013.12.013.

Duan, Ruifeng, Xiaohua Liu, Tianhui Wang, Lei Wu, Xiujie Gao, and Zhiqing Zhang. 2016. 'Histone Acetylation Regulation in Sleep Deprivation-Induced Spatial Memory Impairment'. *Neurochemical Research* 41 (9): 2223–32. https://doi.org/10.1007/s11064-016-1937-6.

Duffy, Joseph B. 2002a. 'GAL4 System in Drosophila: A Fly Geneticist's Swiss Army Knife'. *Genesis* 34 (1–2): 1–15. https://doi.org/10.1002/gene.10150.

———. 2002b. 'GAL4 System in Drosophila: A Fly Geneticist's Swiss Army Knife'. *Genesis (New York, N.Y.: 2000)* 34 (1–2): 1–15. https://doi.org/10.1002/gene.10150.

Durmer, Jeffrey S., and David F. Dinges. 2005. 'Neurocognitive Consequences of Sleep Deprivation'. *Seminars in Neurology* 25 (1): 117–29. https://doi.org/10.1055/s-2005-867080.

Economo, C. V. 1930. 'SLEEP AS A PROBLEM OF LOCALIZATION'. *The Journal of Nervous and Mental Disease* 71 (3): 249.

Ehrmann, Ingrid E., Pamela S. Ellis, Sophie Mazeyrat, Sarah Duthie, Neil Brockdorff, Marie G. Mattei, Marc A. Gavin, Nabeel A. Affara, Graeme M. Brown, and Elizabeth Simpson. 1998. 'Characterization of Genes Encoding Translation Initiation Factor eIF-2γ in Mouse and Human: Sex Chromosome Localization, Escape from X-Inactivation and Evolution'. *Human Molecular Genetics* 7 (11): 1725–37.

Elgin, Sarah C. R., and Gunter Reuter. 2013. 'Position-Effect Variegation, Heterochromatin Formation, and Gene Silencing in Drosophila'. *Cold Spring Harbor Perspectives in Biology* 5 (8): a017780. https://doi.org/10.1101/cshperspect.a017780.

El-Sheikh, Mona, Ryan J. Kelly, Erika J. Bagley, and Emily K. Wetter. 2012. 'Parental Depressive Symptoms and Children's Sleep: The Role of Family Conflict'. *Journal of Child Psychology and Psychiatry* 53 (7): 806–14. https://doi.org/10.1111/j.1469-7610.2012.02530.x.

ENCODE Project Consortium, Ewan Birney, John A. Stamatoyannopoulos, Anindya Dutta, Roderic Guigó, Thomas R. Gingeras, Elliott H. Margulies, et al. 2007. 'Identification and Analysis of Functional Elements in 1% of the Human Genome by the ENCODE Pilot Project'. *Nature* 447 (7146): 799–816. https://doi.org/10.1038/nature05874.

Espada, Jesús, and Manel Esteller. 2013. 'Mouse Models in Epigenetics: Insights in Development and Disease'. *Briefings in Functional Genomics* 12 (3): 279–87. https://doi.org/10.1093/bfgp/elt005.

Fazeli, Gholamreza, Rajaraman Gnana Oli, Nicole Schupp, and Helga Stopper. 2010. 'The Role of the Dopamine Transporter in Dopamine-Induced DNA Damage'. *Brain Pathology* 21 (3): 237–48. https://doi.org/10.1111/j.1750-3639.2010.00440.x.

Feinberg, I., J. D. March, G. Fein, T. C. Floyd, J. M. Walker, and L. Price. 1978. 'Period and Amplitude Analysis of 0.5-3 c/Sec Activity in NREM Sleep of

Young Adults'. *Electroencephalography and Clinical Neurophysiology* 44 (2): 202–13. https://doi.org/10.1016/0013-4694(78)90266-3.

Feldman, Nirit, Ariela Gerson, Jia Fang, En Li, Yi Zhang, Yoichi Shinkai, Howard Cedar, and Yehudit Bergman. 2006. 'G9a-Mediated Irreversible Epigenetic Inactivation of Oct-3/4 during Early Embryogenesis'. *Nature Cell Biology* 8 (2): 188–94. https://doi.org/10.1038/ncb1353.

Figueiredo, Margarida L. A., Philge Philip, Per Stenberg, and Jan Larsson. 2012. 'HP1a Recruitment to Promoters Is Independent of H3K9 Methylation in Drosophila Melanogaster'. *PLOS Genetics* 8 (11): e1003061. https://doi.org/10.1371/journal.pgen.1003061.

Fogg, Paul C. M., John S. O'Neill, Tomasz Dobrzycki, Shaun Calvert, Emma C. Lord, Rebecca L. L. McIntosh, Christopher J. H. Elliott, Sean T. Sweeney, Michael H. Hastings, and Sangeeta Chawla. 2014. 'Class IIa Histone Deacetylases Are Conserved Regulators of Circadian Function'. *Journal of Biological Chemistry* 289 (49): 34341–48. https://doi.org/10.1074/jbc.M114.606392.

French, Alice S., Quentin Geissmann, Esteban J. Beckwith, and Giorgio F. Gilestro. 2021. 'Sensory Processing during Sleep in Drosophila Melanogaster'. *Nature* 598 (7881): 479–82. https://doi.org/10.1038/s41586-021-03954-w.

Fritsch, Lauriane, Philippe Robin, Jacques R. R. Mathieu, Mouloud Souidi, Hélène Hinaux, Claire Rougeulle, Annick Harel-Bellan, Maya Ameyar-Zazoua, and Slimane Ait-Si-Ali. 2010. 'A Subset of the Histone H3 Lysine 9 Methyltransferases Suv39h1, G9a, GLP, and SETDB1 Participate in a Multimeric Complex'. *Molecular Cell* 37 (1): 46–56. https://doi.org/10.1016/j.molcel.2009.12.017.

Gaine, Marie E., Snehajyoti Chatterjee, and Ted Abel. 2018. 'Sleep Deprivation and the Epigenome'. *Frontiers in Neural Circuits* 12 (February):14. https://doi.org/10.3389/fncir.2018.00014.

Gapp, Katharina, Ali Jawaid, Peter Sarkies, Johannes Bohacek, Pawel Pelczar, Julien Prados, Laurent Farinelli, Eric Miska, and Isabelle M. Mansuy. 2014. 'Implication of Sperm RNAs in Transgenerational Inheritance of the Effects of Early Trauma in Mice'. *Nature Neuroscience* 17 (5): 667–69. https://doi.org/10.1038/nn.3695.

García-Cao, Marta, Roderick O'Sullivan, Antoine H. F. M. Peters, Thomas Jenuwein, and María A. Blasco. 2004. 'Epigenetic Regulation of Telomere Length in

Mammalian Cells by the Suv39h1 and Suv39h2 Histone Methyltransferases'. *Nature Genetics* 36 (1): 94–99. https://doi.org/10.1038/ng1278.

Gauchier, Mathilde, Sophie Kan, Amandine Barral, Sandrine Sauzet, Eneritz Agirre, Erin Bonnell, Nehmé Saksouk, et al. 2019. 'SETDB1-Dependent Heterochromatin Stimulates Alternative Lengthening of Telomeres'. *Science Advances* 5 (5): eaav3673. https://doi.org/10.1126/sciadv.aav3673.

Geissmann, Quentin, Esteban J. Beckwith, and Giorgio F. Gilestro. 2019. 'Most Sleep Does Not Serve a Vital Function: Evidence from Drosophila Melanogaster'. *Science Advances* 5 (2): eaau9253. https://doi.org/10.1126/sciadv.aau9253.

Geissmann, Quentin, Luis Garcia Rodriguez, Esteban J. Beckwith, Alice S. French, Arian R. Jamasb, and Giorgio F. Gilestro. 2017. 'Ethoscopes: An Open Platform for High-Throughput Ethomics'. *PLoS Biology* 15 (10): e2003026. https://doi.org/10.1371/journal.pbio.2003026.

Geissmann, Quentin, Luis Garcia Rodriguez, Esteban J. Beckwith, and Giorgio F. Gilestro. 2019. 'Rethomics: An R Framework to Analyse High-Throughput Behavioural Data'. *PLOS ONE* 14 (1): e0209331. https://doi.org/10.1371/journal.pone.0209331.

Gery, Sigal, Naoki Komatsu, Lilit Baldjyan, Andrew Yu, Danielle Koo, and H. Phillip Koeffler. 2006. 'The Circadian Gene Per1 Plays an Important Role in Cell Growth and DNA Damage Control in Human Cancer Cells'. *Molecular Cell* 22 (3): 375–82. https://doi.org/10.1016/j.molcel.2006.03.038.

Giannakou, Maria E., and Linda Partridge. 2007. 'Role of Insulin-like Signalling in Drosophila Lifespan'. *Trends in Biochemical Sciences* 32 (4): 180–88. https://doi.org/10.1016/j.tibs.2007.02.007.

Gilestro, Giorgio F. 2012. 'Video Tracking and Analysis of Sleep in Drosophila Melanogaster'. *Nature Protocols* 7 (5): 995–1007. https://doi.org/10.1038/nprot.2012.041.

Gilestro, Giorgio F., Giulio Tononi, and Chiara Cirelli. 2009. 'Widespread Changes in Synaptic Markers as a Function of Sleep and Wakefulness in Drosophila'. *Science (New York, N.Y.)* 324 (5923): 109–12. https://doi.org/10.1126/science.1166673.

Ginno, Paul Adrian, Dimos Gaidatzis, Angelika Feldmann, Leslie Hoerner, Dilek Imanci, Lukas Burger, Frederic Zilbermann, et al. 2020. 'A Genome-Scale Map of DNA Methylation Turnover Identifies Site-Specific Dependencies of DNMT

and TET Activity'. *Nature Communications* 11 (1): 2680. https://doi.org/10.1038/s41467-020-16354-x.

Goll, Mary Grace, Finn Kirpekar, Keith A. Maggert, Jeffrey A. Yoder, Chih-Lin Hsieh, Xiaoyu Zhang, Kent G. Golic, Steven E. Jacobsen, and Timothy H. Bestor. 2006. 'Methylation of tRNAAsp by the DNA Methyltransferase Homolog Dnmt2'. *Science (New York, N.Y.)* 311 (5759): 395–98. https://doi.org/10.1126/science.1120976.

Goodwin, Patricia R., Alice Meng, Jessie Moore, Michael Hobin, Tudor A. Fulga, David Van Vactor, and Leslie C. Griffith. 2018. 'MicroRNAs Regulate Sleep and Sleep Homeostasis in Drosophila'. *Cell Reports* 23 (13): 3776–86. https://doi.org/10.1016/j.celrep.2018.05.078.

Gottlieb, Daniel J., George T. O'Connor, and Jemma B. Wilk. 2007. 'Genome-Wide Association of Sleep and Circadian Phenotypes'. *BMC Medical Genetics* 8 (1): S9. https://doi.org/10.1186/1471-2350-8-S1-S9.

Gréchez-Cassiau, Aline, Béatrice Rayet, Fabienne Guillaumond, Michèle Teboul, and Franck Delaunay. 2008. 'The Circadian Clock Component BMAL1 Is a Critical Regulator of p21WAF1/CIP1 Expression and Hepatocyte Proliferation'. *The Journal of Biological Chemistry* 283 (8): 4535–42. https://doi.org/10.1074/jbc.M705576200.

Greenspan, R. J., G. Tononi, C. Cirelli, and P. J. Shaw. 2001. 'Sleep and the Fruit Fly'. *Trends in Neurosciences* 24 (3): 142–45. https://doi.org/10.1016/s0166-2236(00)01719-7.

Griffin, E. A., D. Staknis, and C. J. Weitz. 1999. 'Light-Independent Role of CRY1 and CRY2 in the Mammalian Circadian Clock'. *Science (New York, N.Y.)* 286 (5440): 768–71. https://doi.org/10.1126/science.286.5440.768.

Griffith, Leslie C. 2004. 'Regulation of Calcium/Calmodulin-Dependent Protein Kinase II Activation by Intramolecular and Intermolecular Interactions'. *The Journal of Neuroscience* 24 (39): 8394–98. https://doi.org/10.1523/JNEUROSCI.3604-04.2004.

Grigliatti, T. 1991. 'Position-Effect Variegation--an Assay for Nonhistone Chromosomal Proteins and Chromatin Assembly and Modifying Factors'. *Methods in Cell Biology* 35:587–627.

Grima, Brigitte, Elisabeth Chélot, Ruohan Xia, and François Rouyer. 2004. 'Morning and Evening Peaks of Activity Rely on Different Clock Neurons of the

Drosophila Brain'. *Nature* 431 (7010): 869–73. https://doi.org/10.1038/nature02935.

H, Waddington C. 1942. 'The Epigenotype'. *Endeavour* 1:18–20.

Hall, J. C. 1994. 'The Mating of a Fly'. *Science (New York, N.Y.)* 264 (5166): 1702–14. https://doi.org/10.1126/science.8209251.

Hamada, Fumika N., Mark Rosenzweig, Kyeongjin Kang, Stefan R. Pulver, Alfredo Ghezzi, Timothy J. Jegla, and Paul A. Garrity. 2008. 'An Internal Thermal Sensor Controlling Temperature Preference in Drosophila'. *Nature* 454 (7201): 217–20. https://doi.org/10.1038/nature07001.

Hasegawa, Tatsuya, Jun Tomita, Rina Hashimoto, Taro Ueno, Shoen Kume, and Kazuhiko Kume. 2017. 'Sweetness Induces Sleep through Gustatory Signalling Independent of Nutritional Value in a Starved Fruit Fly'. *Scientific Reports* 7 (1): 14355. https://doi.org/10.1038/s41598-017-14608-1.

Hata, Kenichiro, Masaki Okano, Hong Lei, and En Li. 2002. 'Dnmt3L Cooperates with the Dnmt3 Family of de Novo DNA Methyltransferases to Establish Maternal Imprints in Mice'. *Development (Cambridge, England)* 129 (8): 1983–93. https://doi.org/10.1242/dev.129.8.1983.

Haus, Erhard L., and Michael H. Smolensky. 2013. 'Shift Work and Cancer Risk: Potential Mechanistic Roles of Circadian Disruption, Light at Night, and Sleep Deprivation'. *Sleep Medicine Reviews* 17 (4): 273–84. https://doi.org/10.1016/j.smrv.2012.08.003.

Heisenberg, M., A. Borst, S. Wagner, and D. Byers. 1985. 'Drosophila Mushroom Body Mutants Are Deficient in Olfactory Learning'. *Journal of Neurogenetics* 2 (1): 1–30. https://doi.org/10.3109/01677068509100140.

Heitz, Emil. 1928. *Das Heterochromatin Der Moose*. Bornträger.

Helfrich-Förster, Charlotte. 1996. 'Drosophilarhythms: From Brain to Behavior'. *Seminars in Cell & Developmental Biology* 7 (6): 791–802. https://doi.org/10.1006/scdb.1996.0097.

Hendricks, J. C., S. M. Finn, K. A. Panckeri, J. Chavkin, J. A. Williams, A. Sehgal, and A. I. Pack. 2000. 'Rest in Drosophila Is a Sleep-like State'. *Neuron* 25 (1): 129–38. https://doi.org/10.1016/s0896-6273(00)80877-6.

Hendricks, Joan C, Stefanie M Finn, Karen A Panckeri, Jessica Chavkin, Julie A Williams, Amita Sehgal, and Allan I Pack. 2000. 'Rest in Drosophila Is a Sleep-

like State'. *Neuron* 25 (1): 129–38. https://doi.org/10.1016/S0896-6273(00)80877-6.

Hendricks, Joan C., David Kirk, Karen Panckeri, Matthew S. Miller, and Allan I. Pack. 2003. 'Modafinil Maintains Waking in the Fruit Fly Drosophila Melanogaster'. *Sleep* 26 (2): 139–46. https://doi.org/10.1093/sleep/26.2.139.

Hershey, A. D., and M. Chase. 1952. 'Independent Functions of Viral Protein and Nucleic Acid in Growth of Bacteriophage'. *The Journal of General Physiology* 36 (1): 39–56. https://doi.org/10.1085/jgp.36.1.39.

Hesterberg, Rebecca S., John L. Cleveland, and Pearlie K. Epling-Burnette. 2018. 'Role of Polyamines in Immune Cell Functions'. *Medical Sciences (Basel, Switzerland)* 6 (1): 22. https://doi.org/10.3390/medsci6010022.

Hewagama, Anura, and Bruce Richardson. 2009. 'The Genetics and Epigenetics of Autoimmune Diseases'. *Journal of Autoimmunity* 33 (1): 3–11. https://doi.org/10.1016/j.jaut.2009.03.007.

Hirayama, Jun, Sehyung Cho, and Paolo Sassone-Corsi. 2007. 'Circadian Control by the Reduction/Oxidation Pathway: Catalase Represses Light-Dependent Clock Gene Expression in the Zebrafish'. *Proceedings of the National Academy of Sciences* 104 (40): 15747–52. https://doi.org/10.1073/pnas.0705614104.

Holliday, R., and J. E. Pugh. 1975. 'DNA Modification Mechanisms and Gene Activity during Development'. *Science (New York, N.Y.)* 187 (4173): 226–32.

Horvath, Steve. 2013. 'DNA Methylation Age of Human Tissues and Cell Types'. *Genome Biology* 14 (10): 3156. https://doi.org/10.1186/gb-2013-14-10-r115.

Hu, Wantong, Yiqing Peng, Jiameng Sun, Fang Zhang, Xuchen Zhang, Lianzhang Wang, Qian Li, and Yi Zhong. 2018. 'Fan-Shaped Body Neurons in the Drosophila Brain Regulate Both Innate and Conditioned Nociceptive Avoidance'. *Cell Reports* 24 (6): 1573–84. https://doi.org/10.1016/j.celrep.2018.07.028.

Huang, Huiyan, Yong Zhu, Melissa N. Eliot, Valerie S. Knopik, John E. McGeary, Mary A. Carskadon, and Anne C. Hart. 2017. 'Combining Human Epigenetics and Sleep Studies in Caenorhabditis Elegans: A Cross-Species Approach for Finding Conserved Genes Regulating Sleep'. *Sleep* 40 (6). https://doi.org/10.1093/sleep/zsx063.

Huang, Rong-Chi. 2018. 'The Discoveries of Molecular Mechanisms for the Circadian Rhythm: The 2017 Nobel Prize in Physiology or Medicine'. *Biomedical Journal* 41 (1): 5–8. https://doi.org/10.1016/j.bj.2018.02.003.

Huang, Sheng, Chengji Piao, Christine B. Beuschel, Torsten Götz, and Stephan J. Sigrist. 2020. 'Presynaptic Active Zone Plasticity Encodes Sleep Need in Drosophila'. *Current Biology: CB* 30 (6): 1077-1091.e5. https://doi.org/10.1016/j.cub.2020.01.019.

Huber, Reto, M. Felice Ghilardi, Marcello Massimini, and Giulio Tononi. 2004. 'Local Sleep and Learning'. *Nature* 430 (6995): 78–81. https://doi.org/10.1038/nature02663.

Huber, Reto, Sean L. Hill, Carie Holladay, Melissa Biesiadecki, Giulio Tononi, and Chiara Cirelli. 2004. 'Sleep Homeostasis in Drosophila Melanogaster'. *Sleep* 27 (4): 628–39. https://doi.org/10.1093/sleep/27.4.628.

Huffman, Carl. 2008. 'Two Problems in Pythagoreanism'. In *The Oxford Handbook of Presocratic Philosophy*, edited by Patricia Curd and Daniel W. Graham, 0. Oxford University Press. https://doi.org/10.1093/oxfordhb/9780195146875.003.0010.

Iida, Tetsuo, Isao Suetake, Shoji Tajima, Hiroshi Morioka, Satoshi Ohta, Chikashi Obuse, and Toshiki Tsurimoto. 2002. 'PCNA Clamp Facilitates Action of DNA Cytosine Methyltransferase 1 on Hemimethylated DNA'. *Genes to Cells: Devoted to Molecular & Cellular Mechanisms* 7 (10): 997–1007. https://doi.org/10.1046/j.1365-2443.2002.00584.x.

Jacobs, Steven A., and Sepideh Khorasanizadeh. 2002. 'Structure of HP1 Chromodomain Bound to a Lysine 9-Methylated Histone H3 Tail'. *Science (New York, N.Y.)* 295 (5562): 2080–83. https://doi.org/10.1126/science.1069473.

Jafari, Shadi, Johan Henriksson, Hua Yan, and Mattias Alenius. 2021. 'Stress and Odorant Receptor Feedback during a Critical Period after Hatching Regulates Olfactory Sensory Neuron Differentiation in Drosophila'. *PLoS Biology* 19 (4): e3001101. https://doi.org/10.1371/journal.pbio.3001101.

Janssen, Aniek, Serafin U. Colmenares, and Gary H. Karpen. 2018. 'Heterochromatin: Guardian of the Genome'. *Annual Review of Cell and Developmental Biology* 34 (October):265–88. https://doi.org/10.1146/annurev-cellbio-100617-062653.

Jayaraman, Vivek, and Gilles Laurent. 2007. 'Evaluating a Genetically Encoded Optical Sensor of Neural Activity Using Electrophysiology in Intact Adult Fruit Flies'.

Frontiers in Neural Circuits 1 (November):3. https://doi.org/10.3389/neuro.04.003.2007.

Jenett, Arnim, Gerald M. Rubin, Teri-T. B. Ngo, David Shepherd, Christine Murphy, Heather Dionne, Barret D. Pfeiffer, et al. 2012. 'A GAL4-Driver Line Resource for Drosophila Neurobiology'. *Cell Reports* 2 (4): 991–1001. https://doi.org/10.1016/j.celrep.2012.09.011.

Joiner, William J., Amanda Crocker, Benjamin H. White, and Amita Sehgal. 2006. 'Sleep in Drosophila Is Regulated by Adult Mushroom Bodies'. *Nature* 441 (7094): 757–60. https://doi.org/10.1038/nature04811.

Jones, Hannah. 2022. 'Modulation of Sleep and Activity in Drosophila: A Systems Biology Approach. Genetics, Pharmacology and High-Throughput Analysis of Behaviour', March. https://doi.org/10.25560/101858.

Jones, Peter A. 2012. 'Functions of DNA Methylation: Islands, Start Sites, Gene Bodies and Beyond'. *Nature Reviews. Genetics* 13 (7): 484–92. https://doi.org/10.1038/nrg3230.

Jurkowska, Renata Zofia, Tomasz Piotr Jurkowski, and Albert Jeltsch. 2011. 'Structure and Function of Mammalian DNA Methyltransferases'. *Chembiochem: A European Journal of Chemical Biology* 12 (2): 206–22. https://doi.org/10.1002/cbic.201000195.

Kacsoh, Balint Z., Casey S. Greene, and Giovanni Bosco. 2017. 'Machine Learning Analysis Identifies Drosophila Grunge/Atrophin as an Important Learning and Memory Gene Required for Memory Retention and Social Learning'. *G3 (Bethesda, Md.)* 7 (11): 3705–18. https://doi.org/10.1534/g3.117.300172.

Kadener, Sebastian, Jerome S. Menet, Ken Sugino, Michael D. Horwich, Uri Weissbein, Pipat Nawathean, Vasia V. Vagin, Phillip D. Zamore, Sacha B. Nelson, and Michael Rosbash. 2009. 'A Role for microRNAs in the Drosophila Circadian Clock'. *Genes & Development* 23 (18): 2179–91. https://doi.org/10.1101/gad.1819509.

Kales, Anthony, and Allan Rechtschaffen. 1968. *A Manual of Standardized Terminology, Techniques and Scoring System for Sleep Stages of Human Subjects: Allan Rechtschaffen and Anthony Kales, Editors*. U. S. National Institute of Neurological Diseases and Blindness, Neurological Information Network.

Kanaya, Hiroyuki J., Sungeon Park, Ji-Hyung Kim, Junko Kusumi, Sofian Krenenou, Etsuko Sawatari, Aya Sato, et al. 2020. 'A Sleep-like State in Hydra Unravels Conserved Sleep Mechanisms during the Evolutionary Development of the Central Nervous System'. *Science Advances* 6 (41): eabb9415. https://doi.org/10.1126/sciadv.abb9415.

Kang, Tae-Hong, and Sun-Hee Leem. 2014. 'Modulation of ATR-Mediated DNA Damage Checkpoint Response by Cryptochrome 1'. *Nucleic Acids Research* 42 (7): 4427–34. https://doi.org/10.1093/nar/gku094.

Katada, Sayako, and Paolo Sassone-Corsi. 2010. 'The Histone Methyltransferase MLL1 Permits the Oscillation of Circadian Gene Expression'. *Nature Structural & Molecular Biology* 17 (12): 1414–21. https://doi.org/10.1038/nsmb.1961.

Kato, Yasuko, Masaki Kato, Makoto Tachibana, Yoichi Shinkai, and Masamitsu Yamaguchi. 2008. 'Characterization of Drosophila G9a in Vivo and Identification of Genetic Interactants'. *Genes to Cells* 13 (7): 703–22. https://doi.org/10.1111/j.1365-2443.2008.01199.x.

Kayser, Matthew S, Benjamin Mainwaring, Zhifeng Yue, and Amita Sehgal. 2015. 'Sleep Deprivation Suppresses Aggression in Drosophila'. Edited by Leslie C Griffith. *eLife* 4 (July):e07643. https://doi.org/10.7554/eLife.07643.

Keene, Alex C., Erik R. Duboué, Daniel M. McDonald, Monica Dus, Greg S. B. Suh, Scott Waddell, and Justin Blau. 2010. 'Clock and Cycle Limit Starvation-Induced Sleep Loss in Drosophila'. *Current Biology* 20 (13): 1209–15. https://doi.org/10.1016/j.cub.2010.05.029.

Kent, Brianne A., and Ralph E. Mistlberger. 2017. 'Sleep and Hippocampal Neurogenesis: Implications for Alzheimer's Disease'. *Frontiers in Neuroendocrinology* 45 (April):35–52. https://doi.org/10.1016/j.yfrne.2017.02.004.

Kirkhart, Colleen, and Kristin Scott. 2015. 'Gustatory Learning and Processing in the Drosophila Mushroom Bodies'. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 35 (15): 5950–58. https://doi.org/10.1523/JNEUROSCI.3930-14.2015.

Kitamoto, T. 2001. 'Conditional Modification of Behavior in Drosophila by Targeted Expression of a Temperature-Sensitive Shibire Allele in Defined Neurons'. *Journal of Neurobiology* 47 (2): 81–92. https://doi.org/10.1002/neu.1018.

Knutson, Kristen L., Karine Spiegel, Plamen Penev, and Eve Van Cauter. 2007. 'The Metabolic Consequences of Sleep Deprivation'. *Sleep Medicine Reviews* 11 (3): 163–78. https://doi.org/10.1016/j.smrv.2007.01.002.

Koemans, Tom S., Cornelia Oppitz, Rogier A. T. Donders, Hans van Bokhoven, Annette Schenck, Krystyna Keleman, and Jamie M. Kramer. 2017. 'Drosophila Courtship Conditioning As a Measure of Learning and Memory'. *Journal of Visualized Experiments : JoVE*, no. 124 (June), 55808. https://doi.org/10.3791/55808.

Koh, Kyunghee, William J. Joiner, Mark N. Wu, Zhifeng Yue, Corinne J. Smith, and Amita Sehgal. 2008. 'Identification of SLEEPLESS, a Sleep-Promoting Factor'. *Science (New York, N.Y.)* 321 (5887): 372–76. https://doi.org/10.1126/science.1155942.

Kornberg, R. D. 1974. 'Chromatin Structure: A Repeating Unit of Histones and DNA'. *Science (New York, N.Y.)* 184 (4139): 868–71. https://doi.org/10.1126/science.184.4139.868.

Kornberg, R. D., and J. O. Thomas. 1974. 'Chromatin Structure; Oligomers of the Histones'. *Science (New York, N.Y.)* 184 (4139): 865–68. https://doi.org/10.1126/science.184.4139.865.

Kosaka, T., and K. Ikeda. 1983. 'Reversible Blockage of Membrane Retrieval and Endocytosis in the Garland Cell of the Temperature-Sensitive Mutant of Drosophila Melanogaster, Shibirets1'. *The Journal of Cell Biology* 97 (2): 499–507. https://doi.org/10.1083/jcb.97.2.499.

Kramer, Jamie M., Korinna Kochinke, Merel A. W. Oortveld, Hendrik Marks, Daniela Kramer, Eiko K. de Jong, Zoltan Asztalos, et al. 2011. 'Epigenetic Regulation of Learning and Memory by Drosophila EHMT/G9a'. *PLOS Biology* 9 (1): e1000569. https://doi.org/10.1371/journal.pbio.1000569.

Krauss, V., and G. Reuter. 2000. 'Two Genes Become One: The Genes Encoding Heterochromatin Protein Su(Var)3-9 and Translation Initiation Factor Subunit eIF-2gamma Are Joined to a Dicistronic Unit in Holometabolic Insects'. *Genetics* 156 (3): 1157–67. https://doi.org/10.1093/genetics/156.3.1157.

Kudithipudi, Srikanth, Maren Kirstin Schuhmacher, Adam Fiseha Kebede, and Albert Jeltsch. 2017. 'The SUV39H1 Protein Lysine Methyltransferase Methylates Chromatin Proteins Involved in Heterochromatin Formation and VDJ

Recombination'. *ACS Chemical Biology* 12 (4): 958–68. https://doi.org/10.1021/acschembio.6b01076.

Kume, Kazuhiko. 2006. 'A Drosophila Dopamine Transporter Mutant, Fumin (Fmn), Is Defective in Arousal Regulation*'. *Sleep and Biological Rhythms* 4 (3): 263–73. https://doi.org/10.1111/j.1479-8425.2006.00225.x.

Kume, Kazuhiko, Shoen Kume, Sang Ki Park, Jay Hirsh, and F. Rob Jackson. 2005. 'Dopamine Is a Regulator of Arousal in the Fruit Fly'. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 25 (32): 7377–84. https://doi.org/10.1523/JNEUROSCI.2048-05.2005.

Kurdistani, Siavash K., Saeed Tavazoie, and Michael Grunstein. 2004. 'Mapping Global Histone Acetylation Patterns to Gene Expression'. *Cell* 117 (6): 721–33. https://doi.org/10.1016/j.cell.2004.05.023.

Kurian, Joseph R., Meaghan E. Bychowski, Robin M. Forbes-Lorman, Catherine J. Auger, and Anthony P. Auger. 2008. 'Mecp2 Organizes Juvenile Social Behavior in a Sex-Specific Manner'. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 28 (28): 7137–42. https://doi.org/10.1523/JNEUROSCI.1345-08.2008.

Lakin, N. D., and S. P. Jackson. 1999. 'Regulation of P53 in Response to DNA Damage'. *Oncogene* 18 (53): 7644–55. https://doi.org/10.1038/sj.onc.1203015.

Laursen, Willem J, Evan O Anderson, Lydia J Hoffstaetter, Sviatoslav N Bagriantsev, and Elena O Gracheva. 2015. 'Species-Specific Temperature Sensitivity of TRPA1'. *Temperature: Multidisciplinary Biomedical Journal* 2 (2): 214–26. https://doi.org/10.1080/23328940.2014.1000702.

Lavin, Martin F., Geoff Birrell, Philip Chen, Sergei Kozlov, Shaun Scott, and Nuri Gueven. 2005. 'ATM Signaling and Genomic Stability in Response to DNA Damage'. *Mutation Research* 569 (1–2): 123–32. https://doi.org/10.1016/j.mrfmmm.2004.04.020.

Lear, Bridget C., Luoying Zhang, and Ravi Allada. 2009. 'The Neuropeptide PDF Acts Directly on Evening Pacemaker Neurons to Regulate Multiple Features of Circadian Behavior'. *PLOS Biology* 7 (7): e1000154. https://doi.org/10.1371/journal.pbio.1000154.

Lee, Jason S., Yunho Kim, Jinhyuk Bhin, Hi-Jai R. Shin, Hye Jin Nam, Seung Hoon Lee, Jong-Bok Yoon, et al. 2011. 'Hypoxia-Induced Methylation of a Pontin

Chromatin Remodeling Factor'. *Proceedings of the National Academy of Sciences* 108 (33): 13510–15. https://doi.org/10.1073/pnas.1106106108.

Lee, Jason S., Yunho Kim, Ik Soo Kim, Bogyou Kim, Hee June Choi, Ji Min Lee, Hi-Jai R. Shin, et al. 2010. 'Negative Regulation of Hypoxic Responses via Induced Reptin Methylation'. *Molecular Cell* 39 (1): 71–85. https://doi.org/10.1016/j.molcel.2010.06.008.

Lewis, E. B. 1950. 'The Phenomenon of Position Effect'. *Advances in Genetics* 3:73–115. https://doi.org/10.1016/s0065-2660(08)60083-8.

Lewis, Laurence P. C., K. P. Siju, Yoshinori Aso, Anja B. Friedrich, Alexander J. B. Bulteel, Gerald M. Rubin, and Ilona C. Grunwald Kadow. 2015. 'A Higher Brain Circuit for Immediate Integration of Conflicting Sensory Information in Drosophila'. *Current Biology: CB* 25 (17): 2203–14. https://doi.org/10.1016/j.cub.2015.07.015.

Li, E., T. H. Bestor, and R. Jaenisch. 1992. 'Targeted Mutation of the DNA Methyltransferase Gene Results in Embryonic Lethality'. *Cell* 69 (6): 915–26. https://doi.org/10.1016/0092-8674(92)90611-f.

Li, Feng, Jack W Lindsey, Elizabeth C Marin, Nils Otto, Marisa Dreher, Georgia Dempsey, Ildiko Stark, et al. 2020. 'The Connectome of the Adult Drosophila Mushroom Body Provides Insights into Function'. Edited by Leslie C Griffith, Eve Marder, Leslie C Griffith, Jason Pipkin, and Chris Q Doe. *eLife* 9 (December):e62576. https://doi.org/10.7554/eLife.62576.

Li, Wei, and Judith L. Yanowitz. 2019. 'ATM and ATR Influence Meiotic Crossover Formation Through Antagonistic and Overlapping Functions in Caenorhabditis Elegans'. *Genetics* 212 (2): 431–43. https://doi.org/10.1534/genetics.119.302193.

Li, Xiaoman, Hongde Xu, Chongan Xu, Meina Lin, Xiaoyu Song, Fei Yi, Yanling Feng, et al. 2013. 'The Yin-Yang of DNA Damage Response: Roles in Tumorigenesis and Cellular Senescence'. *International Journal of Molecular Sciences* 14 (2): 2431–48. https://doi.org/10.3390/ijms14022431.

Li, Zhihuan, Matthew R. Johnson, Zhonghe Ke, Lili Chen, and Michael A. Welte. 2014. 'Drosophila Lipid Droplets Buffer the H2Av Supply to Protect Early Embryonic Development'. *Current Biology: CB* 24 (13): 1485–91. https://doi.org/10.1016/j.cub.2014.05.022.

Lin, Yu-Chiao, MingYang Zhang, Yu-Jen Chang, and Tsung-Han Kuo. 2023. 'Comparisons of Lifespan and Stress Resistance between Sexes in Drosophila Melanogaster'. *Heliyon* 9 (8): e18178. https://doi.org/10.1016/j.heliyon.2023.e18178.

Ling, Belinda Mei Tze, Narendra Bharathy, Teng-Kai Chung, Wai Kay Kok, SiDe Li, Yong Hua Tan, Vinay Kumar Rao, et al. 2012. 'Lysine Methyltransferase G9a Methylates the Transcription Factor MyoD and Regulates Skeletal Muscle Differentiation'. *Proceedings of the National Academy of Sciences of the United States of America* 109 (3): 841–46. https://doi.org/10.1073/pnas.1111628109.

Liu, Sha, Qili Liu, Masashi Tabuchi, and Mark N. Wu. 2016. 'Sleep Drive Is Encoded by Neural Plastic Changes in a Dedicated Circuit'. *Cell* 165 (6): 1347–60. https://doi.org/10.1016/j.cell.2016.04.013.

Lo Martire, Viviana, Danila Caruso, Laura Palagini, Giovanna Zoccoli, and Stefano Bastianini. 2020. 'Stress & Sleep: A Relationship Lasting a Lifetime'. *Neuroscience & Biobehavioral Reviews*, Prenatal Stress and Brain Disorders in Later Life, 117 (October):65–77. https://doi.org/10.1016/j.neubiorev.2019.08.024.

Lovejoy, Courtney A., and David Cortez. 2009. 'Common Mechanisms of PIKK Regulation'. *DNA Repair* 8 (9): 1004–8. https://doi.org/10.1016/j.dnarep.2009.04.006.

Lovejoy, Pamela C., Kate E. Foley, Melissa M. Conti, Samantha M. Meadows, Christopher Bishop, and Anthony C. Fiumera. 2021. 'Genetic Basis of Susceptibility to Low-Dose Paraquat and Variation between the Sexes in Drosophila Melanogaster'. *Molecular Ecology* 30 (9): 2040–53. https://doi.org/10.1111/mec.15878.

Luan, Haojiang, Nathan C. Peabody, Charles R. Vinson, and Benjamin H. White. 2006. 'Refined Spatial Manipulation of Neuronal Function by Combinatorial Restriction of Transgene Expression'. *Neuron* 52 (3): 425–36. https://doi.org/10.1016/j.neuron.2006.08.028.

Lugli, Giovanni, Vetle I. Torvik, John Larson, and Neil R. Smalheiser. 2008. 'Expression of microRNAs and Their Precursors in Synaptic Fractions of Adult Mouse Forebrain'. *Journal of Neurochemistry* 106 (2): 650–61. https://doi.org/10.1111/j.1471-4159.2008.05413.x.

Lund, Anders H., and Maarten van Lohuizen. 2004. 'Epigenetics and Cancer'. *Genes & Development* 18 (19): 2315–35. https://doi.org/10.1101/gad.1232504.

Lyon, M. F. 1961. 'Gene Action in the X-Chromosome of the Mouse (Mus Musculus L.)'. *Nature* 190 (April):372–73. https://doi.org/10.1038/190372a0.

Machado, Daniel R, Dinis JS Afonso, Alexandra R Kenny, Arzu Öztürk-Çolak, Emilia H Moscato, Benjamin Mainwaring, Matthew Kayser, and Kyunghee Koh. 2017. 'Identification of Octopaminergic Neurons That Modulate Sleep Suppression by Male Sex Drive'. Edited by Leslie C Griffith. *eLife* 6 (May):e23130. https://doi.org/10.7554/eLife.23130.

Madabhushi, Ram, Fan Gao, Andreas R. Pfenning, Ling Pan, Satoko Yamakawa, Jinsoo Seo, Richard Rueda, et al. 2015. 'Activity-Induced DNA Breaks Govern the Expression of Neuronal Early-Response Genes'. *Cell* 161 (7): 1592–1605. https://doi.org/10.1016/j.cell.2015.05.032.

Madigan, James P., Heather L. Chotkowski, and Robert L. Glaser. 2002. 'DNA Double-Strand Break-Induced Phosphorylation of Drosophila Histone Variant H2Av Helps Prevent Radiation-Induced Apoptosis'. *Nucleic Acids Research* 30 (17): 3698–3705. https://doi.org/10.1093/nar/gkf496.

Maksimov, Daniil A., Petr P. Laktionov, Olga V. Posukh, Stepan N. Belyakin, and Dmitry E. Koryakov. 2018. 'Genome-Wide Analysis of SU(VAR)3-9 Distribution in Chromosomes of Drosophila Melanogaster'. *Chromosoma* 127 (1): 85–102. https://doi.org/10.1007/s00412-017-0647-4.

Mander, Bryce A., Joseph R. Winer, William J. Jagust, and Matthew P. Walker. 2016. 'Sleep: A Novel Mechanistic Pathway, Biomarker, and Treatment Target in the Pathology of Alzheimer's Disease?' *Trends in Neurosciences* 39 (8): 552–66. https://doi.org/10.1016/j.tins.2016.05.002.

Mao, Zhengmei, and Ronald Davis. 2009. 'Eight Different Types of Dopaminergic Neurons Innervate the Drosophila Mushroom Body Neuropil: Anatomical and Physiological Heterogeneity'. *Frontiers in Neural Circuits* 3. https://www.frontiersin.org/articles/10.3389/neuro.04.005.2009.

Martinowich, Keri, Daisuke Hattori, Hao Wu, Shaun Fouse, Fei He, Yan Hu, Guoping Fan, and Yi E. Sun. 2003. 'DNA Methylation-Related Chromatin Remodeling in Activity-Dependent BDNF Gene Regulation'. *Science (New York, N.Y.)* 302 (5646): 890–93. https://doi.org/10.1126/science.1090842.

Massart, R., M. Freyburger, M. Suderman, J. Paquet, J. El Helou, E. Belanger-Nelson, A. Rachalski, et al. 2014a. 'The Genome-Wide Landscape of DNA Methylation and Hydroxymethylation in Response to Sleep Deprivation Impacts on Synaptic Plasticity Genes'. *Translational Psychiatry* 4 (January):e347. https://doi.org/10.1038/tp.2013.120.

———. 2014b. 'The Genome-Wide Landscape of DNA Methylation and Hydroxymethylation in Response to Sleep Deprivation Impacts on Synaptic Plasticity Genes'. *Translational Psychiatry* 4 (1): e347. https://doi.org/10.1038/tp.2013.120.

Mattick, John S. 2004. 'RNA Regulation: A New Genetics?' *Nature Reviews Genetics* 5 (4): 316–23. https://doi.org/10.1038/nrg1321.

McBride, S. M., G. Giuliani, C. Choi, P. Krause, D. Correale, K. Watson, G. Baker, and K. K. Siwicki. 1999. 'Mushroom Body Ablation Impairs Short-Term Memory and Long-Term Memory of Courtship Conditioning in Drosophila Melanogaster'. *Neuron* 24 (4): 967–77. https://doi.org/10.1016/s0896-6273(00)81043-0.

McCarthy, Margaret M., Anthony P. Auger, Tracy L. Bale, Geert J. De Vries, Gregory A. Dunn, Nancy G. Forger, Elaine K. Murray, Bridget M. Nugent, Jaclyn M. Schwarz, and Melinda E. Wilson. 2009. 'The Epigenetics of Sex Differences in the Brain'. *Journal of Neuroscience* 29 (41): 12815–23. https://doi.org/10.1523/JNEUROSCI.3331-09.2009.

McCarthy, Margaret M., and Bridget M. Nugent. 2015. 'At the Frontier of Epigenetics of Brain Sex Differences'. *Frontiers in Behavioral Neuroscience* 9. https://www.frontiersin.org/articles/10.3389/fnbeh.2015.00221.

McLellan, Tom M., John A. Caldwell, and Harris R. Lieberman. 2016. 'A Review of Caffeine's Effects on Cognitive, Physical and Occupational Performance'. *Neuroscience & Biobehavioral Reviews* 71 (December):294–312. https://doi.org/10.1016/j.neubiorev.2016.09.001.

Mehren, Jennifer E., and Leslie C. Griffith. 2004. 'Calcium-Independent Calcium/Calmodulin-Dependent Protein Kinase II in the Adult Drosophila CNS Enhances the Training of Pheromonal Cues'. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 24 (47): 10584–93. https://doi.org/10.1523/JNEUROSCI.3560-04.2004.

Mercer, Tim R., Marcel E. Dinger, and John S. Mattick. 2009. 'Long Non-Coding RNAs: Insights into Functions'. *Nature Reviews. Genetics* 10 (3): 155–59. https://doi.org/10.1038/nrg2521.

Meresse, Philippe, Elsa Dechaux, Claude Monneret, and Emmanuel Bertounesque. 2004. 'Etoposide: Discovery and Medicinal Chemistry'. *Current Medicinal Chemistry* 11 (18): 2443–66. https://doi.org/10.2174/0929867043364531.

Meulmeester, Erik, Yaron Pereg, Yosef Shiloh, and Aart G. Jochemsen. 2005. 'ATM-Mediated Phosphorylations Inhibit Mdmx/Mdm2 Stabilization by HAUSP in Favor of P53 Activation'. *Cell Cycle (Georgetown, Tex.)* 4 (9): 1166–70. https://doi.org/10.4161/cc.4.9.1981.

Mis, Jacek, Sarbjit S. Ner, and Thomas A. Grigliatti. 2006. 'Identification of Three Histone Methyltransferases in Drosophila: dG9a Is a Suppressor of PEV and Is Required for Gene Silencing'. *Molecular Genetics and Genomics: MGG* 275 (6): 513–26. https://doi.org/10.1007/s00438-006-0116-x.

Mistlberger, Ralph E. 2005. 'Circadian Regulation of Sleep in Mammals: Role of the Suprachiasmatic Nucleus'. *Brain Research. Brain Research Reviews* 49 (3): 429–54. https://doi.org/10.1016/j.brainresrev.2005.01.005.

Moldofsky, H., F. A. Lue, J. R. Davidson, and R. Gorczynski. 1989. 'Effects of Sleep Deprivation on Human Immune Functions'. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology* 3 (8): 1972–77. https://doi.org/10.1096/fasebj.3.8.2785942.

Montavon, Thomas, Nicholas Shukeir, Galina Erikson, Bettina Engist, Megumi Onishi-Seebacher, Devon Ryan, Yaarub Musa, et al. 2021. 'Complete Loss of H3K9 Methylation Dissolves Mouse Heterochromatin Organization'. *Nature Communications* 12 (1): 4359. https://doi.org/10.1038/s41467-021-24532-8.

Montecucco, Alessandra, Francesca Zanetta, and Giuseppe Biamonti. 2015. 'Molecular Mechanisms of Etoposide'. *EXCLI Journal* 14 (January):95–108. https://doi.org/10.17179/excli2015-561.

Moore, Lisa D., Thuc Le, and Guoping Fan. 2013. 'DNA Methylation and Its Basic Function'. *Neuropsychopharmacology* 38 (1): 23–38. https://doi.org/10.1038/npp.2012.112.

Mourrain, Philippe, and Gordon X. Wang. 2019. 'Sleep: DNA Repair Function for Better Neuronal Aging?' *Current Biology: CB* 29 (12): R585–88. https://doi.org/10.1016/j.cub.2019.05.018.

Musso, Pierre-Yves, Pierre Junca, and Michael D. Gordon. 2021. 'A Neural Circuit Linking Two Sugar Sensors Regulates Satiety-Dependent Fructose Drive in Drosophila'. *Science Advances* 7 (49): eabj0186. https://doi.org/10.1126/sciadv.abj0186.

Nakahata, Yasukazu, Milota Kaluzova, Benedetto Grimaldi, Saurabh Sahar, Jun Hirayama, Danica Chen, Leonard P. Guarente, and Paolo Sassone-Corsi. 2008. 'The NAD+-Dependent Deacetylase SIRT1 Modulates CLOCK-Mediated Chromatin Remodeling and Circadian Control'. *Cell* 134 (2): 329–40. https://doi.org/10.1016/j.cell.2008.07.002.

Nan, X., H. H. Ng, C. A. Johnson, C. D. Laherty, B. M. Turner, R. N. Eisenman, and A. Bird. 1998. 'Transcriptional Repression by the Methyl-CpG-Binding Protein MeCP2 Involves a Histone Deacetylase Complex'. *Nature* 393 (6683): 386–89. https://doi.org/10.1038/30764.

Nanney, D. L. 1958. 'EPIGENETIC CONTROL SYSTEMS*'. *Proceedings of the National Academy of Sciences of the United States of America* 44 (7): 712–17.

Narwade, Santosh C., Birendra N. Mallick, and Deepti D. Deobagkar. 2017. 'Transcriptome Analysis Reveals Altered Expression of Memory and Neurotransmission Associated Genes in the REM Sleep Deprived Rat Brain'. *Frontiers in Molecular Neuroscience* 10 (March). https://doi.org/10.3389/fnmol.2017.00067.

Nauta, W. J. H. 1946. 'Hypothalamic Regulation of Sleep in Rats; an Experimental Study'. *Journal of Neurophysiology* 9 (July):285–316. https://doi.org/10.1152/jn.1946.9.4.285.

Ng, Minna, Robert D. Roorda, Susana Q. Lima, Boris V. Zemelman, Patrick Morcillo, and Gero Miesenböck. 2002. 'Transmission of Olfactory Information between Three Populations of Neurons in the Antennal Lobe of the Fly'. *Neuron* 36 (3): 463–74. https://doi.org/10.1016/S0896-6273(02)00975-3.

Nicetto, Dario, and Kenneth Zaret. 2019. 'Role of H3K9me3 Heterochromatin in Cell Identity Establishment and Maintenance'. *Current Opinion in Genetics & Development* 55 (April):1–10. https://doi.org/10.1016/j.gde.2019.04.013.

Niveditha, S., S. Deepashree, S. R. Ramesh, and T. Shivanandappa. 2017. 'Sex Differences in Oxidative Stress Resistance in Relation to Longevity in Drosophila Melanogaster'. *Journal of Comparative Physiology B* 187 (7): 899–909. https://doi.org/10.1007/s00360-017-1061-1.

Nouspikel, T., and P. C. Hanawalt. 2000. ‘Terminally Differentiated Human Neurons Repair Transcribed Genes but Display Attenuated Global DNA Repair and Modulation of Repair Gene Expression’. *Molecular and Cellular Biology* 20 (5): 1562–70. https://doi.org/10.1128/MCB.20.5.1562-1570.2000.

Noya, Sara B., David Colameo, Franziska Brüning, Andrea Spinnler, Dennis Mircsof, Lennart Opitz, Matthias Mann, Shiva K. Tyagarajan, Maria S. Robles, and Steven A. Brown. 2019. ‘The Forebrain Synaptic Transcriptome Is Organized by Clocks but Its Proteome Is Driven by Sleep’. *Science (New York, N.Y.)* 366 (6462): eaav2642. https://doi.org/10.1126/science.aav2642.

Ohno, Susumu. 1972. ‘So Much" Junk" DNA in Our Genome. In" Evolution of Genetic Systems"’. In *Brookhaven Symposium in Biology*, 23:366–70.

Okano, M., D. W. Bell, D. A. Haber, and E. Li. 1999. ‘DNA Methyltransferases Dnmt3a and Dnmt3b Are Essential for de Novo Methylation and Mammalian Development’. *Cell* 99 (3): 247–57. https://doi.org/10.1016/s0092-8674(00)81656-6.

Oklejewicz, Małgorzata, Eugin Destici, Filippo Tamanini, Roelof A. Hut, Roel Janssens, and Gijsbertus T. J. van der Horst. 2008. ‘Phase Resetting of the Mammalian Circadian Clock by DNA Damage’. *Current Biology* 18 (4): 286–91. https://doi.org/10.1016/j.cub.2008.01.047.

Öztürk-Çolak, Arzu, Sho Inami, Joseph R. Buchler, Patrick D. McClanahan, Andri Cruz, Christopher Fang-Yen, and Kyunghee Koh. 2020. ‘Sleep Induction by Mechanosensory Stimulation in Drosophila’. *Cell Reports* 33 (9): 108462. https://doi.org/10.1016/j.celrep.2020.108462.

Pang, Ken C., Martin C. Frith, and John S. Mattick. 2006. ‘Rapid Evolution of Noncoding RNAs: Lack of Conservation Does Not Mean Lack of Function’. *Trends in Genetics* 22 (1): 1–5. https://doi.org/10.1016/j.tig.2005.10.003.

Park, J. H., C. Helfrich-Förster, G. Lee, L. Liu, M. Rosbash, and J. C. Hall. 2000. ‘Differential Regulation of Circadian Pacemaker Output by Separate Clock Genes in Drosophila’. *Proceedings of the National Academy of Sciences of the United States of America* 97 (7): 3608–13. https://doi.org/10.1073/pnas.97.7.3608.

Pascual, A., and T. Préat. 2001. ‘Localization of Long-Term Memory within the Drosophila Mushroom Body’. *Science (New York, N.Y.)* 294 (5544): 1115–17. https://doi.org/10.1126/science.1064200.

Pegg, Anthony E. 2016. ‘Functions of Polyamines in Mammals’. *The Journal of Biological Chemistry* 291 (29): 14904–12. https://doi.org/10.1074/jbc.R116.731661.

Peng, Ying, Dan Stoleru, Joel D. Levine, Jeffrey C. Hall, and Michael Rosbash. 2003. ‘Drosophila Free-Running Rhythms Require Intercellular Communication’. *PLoS Biology* 1 (1): E13. https://doi.org/10.1371/journal.pbio.0000013.

Pesonen, Anu-Katriina, Katri Räikkönen, Karen Matthews, Kati Heinonen, Juulia E. Paavonen, Jari Lahti, Niina Komsi, et al. 2009. ‘Prenatal Origins of Poor Sleep in Children’. *Sleep* 32 (8): 1086–92. https://doi.org/10.1093/sleep/32.8.1086.

Pfeiffenberger, Cory, Bridget C. Lear, Kevin P. Keegan, and Ravi Allada. 2010. ‘Locomotor Activity Level Monitoring Using the Drosophila Activity Monitoring (DAM) System’. *Cold Spring Harbor Protocols* 2010 (11): pdb.prot5518. https://doi.org/10.1101/pdb.prot5518.

Piao, Lianhua, Takehiro Suzuki, Naoshi Dohmae, Yusuke Nakamura, and Ryuji Hamamoto. 2015. ‘SUV39H2 Methylates and Stabilizes LSD1 by Inhibiting Polyubiquitination in Human Cancer Cells’. *Oncotarget* 6 (19): 16939–50. https://doi.org/10.18632/oncotarget.4760.

Piéron, Henri. 1913. *Le problème physiologique du sommeil*. Masson.

Pilcher, J. J., and A. I. Huffcutt. 1996. ‘Effects of Sleep Deprivation on Performance: A Meta-Analysis’. *Sleep* 19 (4): 318–26. https://doi.org/10.1093/sleep/19.4.318.

Pimentel, Diogo, Jeffrey M. Donlea, Clifford B. Talbot, Seoho M. Song, Alexander J. F. Thurston, and Gero Miesenböck. 2016. ‘Operation of a Homeostatic Sleep Switch’. *Nature* 536 (7616): 333–37. https://doi.org/10.1038/nature19055.

Pirooznia, Sheila K., Jessica Sarthi, Ashley A. Johnson, Meridith S. Toth, Kellie Chiu, Sravanthi Koduri, and Felice Elefant. 2012. ‘Tip60 HAT Activity Mediates APP Induced Lethality and Apoptotic Cell Death in the CNS of a Drosophila Alzheimer’s Disease Model’. *PloS One* 7 (7): e41776. https://doi.org/10.1371/journal.pone.0041776.

Pitman, Jena L., Jermaine J. McGill, Kevin P. Keegan, and Ravi Allada. 2006. ‘A Dynamic Role for the Mushroom Bodies in Promoting Sleep in Drosophila’. *Nature* 441 (7094): 753–56. https://doi.org/10.1038/nature04739.

Pless, Ole, Elisabeth Kowenz-Leutz, Maria Knoblich, Jörn Lausen, Michael Beyermann, Martin J. Walsh, and Achim Leutz. 2008. ‘G9a-Mediated Lysine Methylation Alters the Function of CCAAT/Enhancer-Binding Protein-Beta’.

The Journal of Biological Chemistry 283 (39): 26357–63. https://doi.org/10.1074/jbc.M802132200.

Pomatto, Laura C. D., John Tower, and Kelvin J. A. Davies. 2018. 'Sexual Dimorphism and Aging Differentially Regulate Adaptive Homeostasis'. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences* 73 (2): 141–49. https://doi.org/10.1093/gerona/glx083.

Ponting, Chris P., Peter L. Oliver, and Wolf Reik. 2009. 'Evolution and Functions of Long Noncoding RNAs'. *Cell* 136 (4): 629–41. https://doi.org/10.1016/j.cell.2009.02.006.

Powell, Weston T., Rochelle L. Coulson, Florence K. Crary, Spencer S. Wong, Robert A. Ach, Peter Tsang, N. Alice Yamada, Dag H. Yasui, and Janine M. LaSalle. 2013. 'A Prader–Willi Locus lncRNA Cloud Modulates Diurnal Genes and Energy Expenditure'. *Human Molecular Genetics* 22 (21): 4318–28. https://doi.org/10.1093/hmg/ddt281.

Pulver, Stefan R., Stanislav L. Pashkovski, Nicholas J. Hornstein, Paul A. Garrity, and Leslie C. Griffith. 2009. 'Temporal Dynamics of Neuronal Activation by Channelrhodopsin-2 and TRPA1 Determine Behavioral Output in *Drosophila* Larvae'. *Journal of Neurophysiology* 101 (6): 3075–88. https://doi.org/10.1152/jn.00071.2009.

Qureshi, Irfan A., and Mark F. Mehler. 2014a. 'An Evolving View of Epigenetic Complexity in the Brain'. *Philosophical Transactions of the Royal Society B: Biological Sciences* 369 (1652): 20130506. https://doi.org/10.1098/rstb.2013.0506.

———. 2014b. 'Epigenetics of Sleep and Chronobiology'. *Current Neurology and Neuroscience Reports* 14 (3): 432. https://doi.org/10.1007/s11910-013-0432-6.

Raccuglia, Davide, Sheng Huang, Anatoli Ender, M.-Marcel Heim, Desiree Laber, Raquel Suárez-Grimalt, Agustin Liotta, Stephan J. Sigrist, Jörg R. P. Geiger, and David Owald. 2019. 'Network-Specific Synchronization of Electrical Slow-Wave Oscillations Regulates Sleep Drive in Drosophila'. *Current Biology: CB* 29 (21): 3611-3621.e3. https://doi.org/10.1016/j.cub.2019.08.070.

Raddatz, Günter, Sabine Hagemann, Dvir Aran, Jörn Söhle, Pranav P. Kulkarni, Lars Kaderali, Asaf Hellman, Marc Winnefeld, and Frank Lyko. 2013. 'Aging Is Associated with Highly Defined Epigenetic Changes in the Human Epidermis'. *Epigenetics & Chromatin* 6 (1): 36. https://doi.org/10.1186/1756-8935-6-36.

Raghuraman, Sukanya, Ida Donkin, Soetkin Versteyhe, Romain Barrès, and David Simar. 2016. 'The Emerging Role of Epigenetics in Inflammation and Immunometabolism'. *Trends in Endocrinology and Metabolism: TEM* 27 (11): 782–95. https://doi.org/10.1016/j.tem.2016.06.008.

Ralhan, Isha, Chi-Lun Chang, Jennifer Lippincott-Schwartz, and Maria S. Ioannou. 2021. 'Lipid Droplets in the Nervous System'. *The Journal of Cell Biology* 220 (7): e202102136. https://doi.org/10.1083/jcb.202102136.

Rechtschaffen, A., M. A. Gilliland, B. M. Bergmann, and J. B. Winter. 1983. 'Physiological Correlates of Prolonged Sleep Deprivation in Rats'. *Science (New York, N.Y.)* 221 (4606): 182–84. https://doi.org/10.1126/science.6857280.

Renn, S. C., J. H. Park, M. Rosbash, J. C. Hall, and P. H. Taghert. 1999. 'A Pdf Neuropeptide Gene Mutation and Ablation of PDF Neurons Each Cause Severe Abnormalities of Behavioral Circadian Rhythms in Drosophila'. *Cell* 99 (7): 791–802. https://doi.org/10.1016/s0092-8674(00)81676-1.

Richardson, Bruce, and Raymond Yung. 1999. 'Role of DNA Methylation in the Regulation of Cell Function'. *Journal of Laboratory and Clinical Medicine* 134 (4): 333–40. https://doi.org/10.1016/S0022-2143(99)90147-6.

Riggs, A. D. 1975. 'X Inactivation, Differentiation, and DNA Methylation'. *Cytogenetics and Cell Genetics* 14 (1): 9–25. https://doi.org/10.1159/000130315.

Roessingh, Sanne, and Ralf Stanewsky. 2017. 'The Drosophila TRPA1 Channel and Neuronal Circuits Controlling Rhythmic Behaviours and Sleep in Response to Environmental Temperature'. *International Journal of Molecular Sciences* 18 (10): 2028. https://doi.org/10.3390/ijms18102028.

Roman, Gregg, Keita Endo, Lin Zong, and Ronald L. Davis. 2001. 'P{Switch}, a System for Spatial and Temporal Control of Gene Expression in Drosophila Melanogaster'. *Proceedings of the National Academy of Sciences* 98 (22): 12602–7. https://doi.org/10.1073/pnas.221303998.

Saksouk, Nehmé, Elisabeth Simboeck, and Jérôme Déjardin. 2015. 'Constitutive Heterochromatin Formation and Transcription in Mammals'. *Epigenetics & Chromatin* 8 (1): 3. https://doi.org/10.1186/1756-8935-8-3.

Salzman, Julia, Charles Gawad, Peter Lincoln Wang, Norman Lacayo, and Patrick O. Brown. 2012. 'Circular RNAs Are the Predominant Transcript Isoform from

Hundreds of Human Genes in Diverse Cell Types'. *PloS One* 7 (2): e30733. https://doi.org/10.1371/journal.pone.0030733.

Sanchez-Mut, Jose V., and Johannes Gräff. 2015. 'Epigenetic Alterations in Alzheimer's Disease'. *Frontiers in Behavioral Neuroscience* 9:347. https://doi.org/10.3389/fnbeh.2015.00347.

Santisteban, M. S., T. Kalashnikova, and M. M. Smith. 2000. 'Histone H2A.Z Regulats Transcription and Is Partially Redundant with Nucleosome Remodeling Complexes'. *Cell* 103 (3): 411–22. https://doi.org/10.1016/s0092-8674(00)00133-1.

Sareen, Preeti F., Li Yan McCurdy, and Michael N. Nitabach. 2021. 'A Neuronal Ensemble Encoding Adaptive Choice during Sensory Conflict in Drosophila'. *Nature Communications* 12 (1): 4131. https://doi.org/10.1038/s41467-021-24423-y.

Schaefer, Matthias, and Frank Lyko. 2010. 'Lack of Evidence for DNA Methylation of Invader4 Retroelements in Drosophila and Implications for Dnmt2-Mediated Epigenetic Regulation'. *Nature Genetics* 42 (11): 920–21; author reply 921. https://doi.org/10.1038/ng1110-920.

Scharf, Matthew T., Nirinjini Naidoo, John E. Zimmerman, and Allan I. Pack. 2008. 'The Energy Hypothesis of Sleep Revisited'. *Progress in Neurobiology* 86 (3): 264–80. https://doi.org/10.1016/j.pneurobio.2008.08.003.

Schindelin, Johannes, Ignacio Arganda-Carreras, Erwin Frise, Verena Kaynig, Mark Longair, Tobias Pietzsch, Stephan Preibisch, et al. 2012. 'Fiji: An Open-Source Platform for Biological-Image Analysis'. *Nature Methods* 9 (7): 676–82. https://doi.org/10.1038/nmeth.2019.

Schotta, Gunnar, Anja Ebert, Veiko Krauss, Andreas Fischer, Jan Hoffmann, Stephen Rea, Thomas Jenuwein, Rainer Dorn, and Gunter Reuter. 2002. 'Central Role of Drosophila SU(VAR)3-9 in Histone H3-K9 Methylation and Heterochromatic Gene Silencing'. *The EMBO Journal* 21 (5): 1121–31. https://doi.org/10.1093/emboj/21.5.1121.

Scialo, Filippo, Ashwin Sriram, Rhoda Stefanatos, and Alberto Sanz. 2016. 'Practical Recommendations for the Use of the GeneSwitch Gal4 System to Knock-Down Genes in Drosophila Melanogaster'. *PLoS ONE* 11 (8): e0161817. https://doi.org/10.1371/journal.pone.0161817.

Seidner, Glen, James E. Robinson, Meilin Wu, Kurtresha Worden, Pavel Masek, Stephen W. Roberts, Alex C. Keene, and William J. Joiner. 2015. 'Identification of Neurons with a Privileged Role in Sleep Homeostasis in Drosophila Melanogaster'. *Current Biology: CB* 25 (22): 2928–38. https://doi.org/10.1016/j.cub.2015.10.006.

Seugnet, Laurent, Yasuko Suzuki, Matthew Thimgan, Jeff Donlea, Sarah I. Gimbel, Laura Gottschalk, Steve P. Duntley, and Paul J. Shaw. 2009. 'Identifying Sleep Regulatory Genes Using a Drosophila Model of Insomnia'. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 29 (22): 7148–57. https://doi.org/10.1523/JNEUROSCI.5629-08.2009.

Seugnet, Laurent, Yasuko Suzuki, Lucy Vine, Laura Gottschalk, and Paul J. Shaw. 2008. 'D1 Receptor Activation in the Mushroom Bodies Rescues Sleep-Loss-Induced Learning Impairments in Drosophila'. *Current Biology* 18 (15): 1110–17. https://doi.org/10.1016/j.cub.2008.07.028.

Seum, Carole, Emanuela Reo, Hongzhuang Peng, Frank J. Rauscher, Pierre Spierer, and Séverine Bontron. 2007. 'Drosophila SETDB1 Is Required for Chromosome 4 Silencing'. *PLoS Genetics* 3 (5): e76. https://doi.org/10.1371/journal.pgen.0030076.

Shalaby, Nevine A., Jorge H. Pinzon, Anjana S. Narayanan, Eugene Jennifer Jin, Morgan P. Ritz, Rachel J. Dove, Heike Wolfenberg, Aylin R. Rodan, Michael Buszczak, and Adrian Rothenfluh. 2018. 'JmjC Domain Proteins Modulate Circadian Behaviors and Sleep in Drosophila'. *Scientific Reports* 8 (January). https://doi.org/10.1038/s41598-017-18989-1.

Shang, Yuhua, Leslie C. Griffith, and Michael Rosbash. 2008. 'Light-Arousal and Circadian Photoreception Circuits Intersect at the Large PDF Cells of the Drosophila Brain'. *Proceedings of the National Academy of Sciences of the United States of America* 105 (50): 19587–94. https://doi.org/10.1073/pnas.0809577105.

Shaw, Paul J., Chiara Cirelli, Ralph J. Greenspan, and Giulio Tononi. 2000a. 'Correlates of Sleep and Waking in Drosophila Melanogaster'. *Science* 287 (5459): 1834–37. https://doi.org/10.1126/science.287.5459.1834.

———. 2000b. 'Correlates of Sleep and Waking in Drosophila Melanogaster'. *Science* 287 (5459): 1834–37. https://doi.org/10.1126/science.287.5459.1834.

Shen, Erica Y., Todd H. Ahern, Iris Cheung, Juerg Straubhaar, Aslihan Dincer, Isaac Houston, Geert J. de Vries, Schahram Akbarian, and Nancy G. Forger. 2015. 'Epigenetics and Sex Differences in the Brain: A Genome-Wide Comparison of Histone-3 Lysine-4 Trimethylation (H3K4me3) in Male and Female Mice'. *Experimental Neurology* 268 (June):21–29. https://doi.org/10.1016/j.expneurol.2014.08.006.

Shi, Changhua, Ling Zhang, and Chuan Qin. 2017. 'Long Non-Coding RNAs in Brain Development, Synaptic Biology, and Alzheimer's Disease'. *Brain Research Bulletin* 132 (June):160–69. https://doi.org/10.1016/j.brainresbull.2017.03.010.

Shiloh, Yosef. 2003. 'ATM and Related Protein Kinases: Safeguarding Genome Integrity'. *Nature Reviews Cancer* 3 (3): 155–68. https://doi.org/10.1038/nrc1011.

Singh, Neetu, Meridith T. Lorbeck, Ashley Zervos, John Zimmerman, and Felice Elefant. 2010. 'The Histone Acetyltransferase Elp3 Plays in Active Role in the Control of Synaptic Bouton Expansion and Sleep in Drosophila'. *Journal of Neurochemistry* 115 (2): 493–504. https://doi.org/10.1111/j.1471-4159.2010.06892.x.

Sitaraman, Divya, Yoshinori Aso, Xin Jin, Nan Chen, Mario Felix, Gerald M. Rubin, and Michael N. Nitabach. 2015. 'Propagation of Homeostatic Sleep Signals by Segregated Synaptic Microcircuits of the Drosophila Mushroom Body'. *Current Biology: CB* 25 (22): 2915–27. https://doi.org/10.1016/j.cub.2015.09.017.

Smith, K R, and S T Harbison. 2018. '0090 Learning and Memory in Short and Long Sleeping Inbred Drosophila Melanogaster Lines'. *Sleep* 41 (suppl_1): A36–37. https://doi.org/10.1093/sleep/zsy061.089.

Soshnev, Alexey A., Hiroshi Ishimoto, Bryant F. McAllister, Xingguo Li, Misty D. Wehling, Toshihiro Kitamoto, and Pamela K. Geyer. 2011. 'A Conserved Long Noncoding RNA Affects Sleep Behavior in Drosophila'. *Genetics* 189 (2): 455–68. https://doi.org/10.1534/genetics.111.131706.

Spencer, V. A., and J. R. Davie. 1999. 'Role of Covalent Modifications of Histones in Regulating Gene Expression'. *Gene* 240 (1): 1–12. https://doi.org/10.1016/s0378-1119(99)00405-9.

Srijyothi, Loudu, Saravanaraman Ponne, Talukdar Prathama, Cheemala Ashok, and Sudhakar Baluchamy. 2018. 'Roles of Non-Coding RNAs in Transcriptional

Regulation'. *Transcriptional and Post-Transcriptional Regulation*, October. https://doi.org/10.5772/intechopen.76125.

Stabell, Marianne, Ragnhild Eskeland, Mona Bjørkmo, Jan Larsson, Reidunn B. Aalen, Axel Imhof, and Andrew Lambertsson. 2006. 'The Drosophila G9a Gene Encodes a Multi-Catalytic Histone Methyltransferase Required for Normal Development'. *Nucleic Acids Research* 34 (16): 4609–21. https://doi.org/10.1093/nar/gkl640.

Stoleru, Dan, Ying Peng, José Agosto, and Michael Rosbash. 2004. 'Coupled Oscillators Control Morning and Evening Locomotor Behaviour of Drosophila'. *Nature* 431 (7010): 862–68. https://doi.org/10.1038/nature02926.

Strahl, Brian D., and Scott D. Briggs. 2021. 'The SAGA Continues: The Rise of Cis- and Trans-Histone Crosstalk Pathways'. *Biochimica Et Biophysica Acta. Gene Regulatory Mechanisms* 1864 (2): 194600. https://doi.org/10.1016/j.bbagrm.2020.194600.

Strauss, R., U. Hanesch, M. Kinkelin, R. Wolf, and M. Heisenberg. 1992. 'No-Bridge of Drosophila Melanogaster: Portrait of a Structural Brain Mutant of the Central Complex'. *Journal of Neurogenetics* 8 (3): 125–55. https://doi.org/10.3109/01677069209083444.

Strauss, R., and M. Heisenberg. 1993. 'A Higher Control Center of Locomotor Behavior in the Drosophila Brain'. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 13 (5): 1852–61. https://doi.org/10.1523/JNEUROSCI.13-05-01852.1993.

Suberbielle, Elsa, Pascal E. Sanchez, Alexxai V. Kravitz, Xin Wang, Kaitlyn Ho, Kirsten Eilertson, Nino Devidze, Anatol C. Kreitzer, and Lennart Mucke. 2013. 'Physiologic Brain Activity Causes DNA Double-Strand Breaks in Neurons, with Exacerbation by Amyloid-β'. *Nature Neuroscience* 16 (5): 613–21. https://doi.org/10.1038/nn.3356.

Swygert, Sarah G., and Craig L. Peterson. 2014. 'Chromatin Dynamics: Interplay between Remodeling Enzymes and Histone Modifications'. *Biochimica Et Biophysica Acta* 1839 (8): 728–36. https://doi.org/10.1016/j.bbagrm.2014.02.013.

Taft, Ryan J., Michael Pheasant, and John S. Mattick. 2007. 'The Relationship between Non-Protein-Coding DNA and Eukaryotic Complexity'. *BioEssays: News and*

Reviews in Molecular, Cellular and Developmental Biology 29 (3): 288–99. https://doi.org/10.1002/bies.20544.

Tahiliani, Mamta, Kian Peng Koh, Yinghua Shen, William A. Pastor, Hozefa Bandukwala, Yevgeny Brudno, Suneet Agarwal, et al. 2009. 'Conversion of 5-Methylcytosine to 5-Hydroxymethylcytosine in Mammalian DNA by MLL Partner TET1'. *Science (New York, N.Y.)* 324 (5929): 930–35. https://doi.org/10.1126/science.1170116.

Takemura, Shin-ya, Yoshinori Aso, Toshihide Hige, Allan Wong, Zhiyuan Lu, C Shan Xu, Patricia K Rivlin, et al. 2017. 'A Connectome of a Learning and Memory Center in the Adult Drosophila Brain'. Edited by Ronald L Calabrese. *eLife* 6 (July):e26975. https://doi.org/10.7554/eLife.26975.

Takeshita, Kohei, Isao Suetake, Eiki Yamashita, Michihiro Suga, Hirotaka Narita, Atsushi Nakagawa, and Shoji Tajima. 2011. 'Structural Insight into Maintenance Methylation by Mouse DNA Methyltransferase 1 (Dnmt1)'. *Proceedings of the National Academy of Sciences* 108 (22): 9055–59. https://doi.org/10.1073/pnas.1019629108.

Toffol, Elena, Marius Lahti-Pulkkinen, Jari Lahti, Jari Lipsanen, Kati Heinonen, Anu-Katriina Pesonen, Esa Hämäläinen, et al. 2019. 'Maternal Depressive Symptoms during and after Pregnancy Are Associated with Poorer Sleep Quantity and Quality and Sleep Disorders in 3.5-Year-Old Offspring'. *Sleep Medicine* 56 (April):201–10. https://doi.org/10.1016/j.sleep.2018.10.042.

Tomita, Jun, Gosuke Ban, Yoshiaki S. Kato, and Kazuhiko Kume. 2021. 'Protocerebral Bridge Neurons That Regulate Sleep in Drosophila Melanogaster'. *Frontiers in Neuroscience* 15 (October):647117. https://doi.org/10.3389/fnins.2021.647117.

Tononi, Giulio, and Chiara Cirelli. 2003. 'Sleep and Synaptic Homeostasis: A Hypothesis'. *Brain Research Bulletin* 62 (2): 143–50. https://doi.org/10.1016/j.brainresbull.2003.09.004.

———. 2006. 'Sleep Function and Synaptic Homeostasis'. *Sleep Medicine Reviews* 10 (1): 49–62. https://doi.org/10.1016/j.smrv.2005.05.002.

———. 2014. 'Sleep and the Price of Plasticity: From Synaptic and Cellular Homeostasis to Memory Consolidation and Integration'. *Neuron* 81 (1): 12–34. https://doi.org/10.1016/j.neuron.2013.12.025.

Triphan, Tilman, Burkhard Poeck, Kirsa Neuser, and Roland Strauss. 2010. 'Visual Targeting of Motor Actions in Climbing Drosophila'. *Current Biology: CB* 20 (7): 663–68. https://doi.org/10.1016/j.cub.2010.02.055.

Trojer, Patrick, and Danny Reinberg. 2007. 'Facultative Heterochromatin: Is There a Distinctive Molecular Signature?' *Molecular Cell* 28 (1): 1–13. https://doi.org/10.1016/j.molcel.2007.09.011.

Turrel, Oriane, Valérie Goguel, and Thomas Preat. 2018. 'Amnesiac Is Required in the Adult Mushroom Body for Memory Formation'. *The Journal of Neuroscience* 38 (43): 9202–14. https://doi.org/10.1523/JNEUROSCI.0876-18.2018.

Tzeng, Tsai-Yu, Chi-Hua Lee, Li-Wei Chan, and C.-K. James Shen. 2007. 'Epigenetic Regulation of the Drosophila Chromosome 4 by the Histone H3K9 Methyltransferase dSETDB1'. *Proceedings of the National Academy of Sciences of the United States of America* 104 (31): 12691–96. https://doi.org/10.1073/pnas.0705534104.

Van Dongen, Hans P. A., Greg Maislin, Janet M. Mullington, and David F. Dinges. 2003. 'The Cumulative Cost of Additional Wakefulness: Dose-Response Effects on Neurobehavioral Functions and Sleep Physiology from Chronic Sleep Restriction and Total Sleep Deprivation'. *Sleep* 26 (2): 117–26. https://doi.org/10.1093/sleep/26.2.117.

Vienne, Julie, Ryanne Spann, Fang Guo, and Michael Rosbash. 2016. 'Age-Related Reduction of Recovery Sleep and Arousal Threshold in Drosophila'. *Sleep* 39 (8): 1613–24. https://doi.org/10.5665/sleep.6032.

Vogt, Katrin, Christopher Schnaitmann, Kristina V Dylla, Stephan Knapek, Yoshinori Aso, Gerald M Rubin, and Hiromu Tanimoto. 2014. 'Shared Mushroom Body Circuits Underlie Visual and Olfactory Memories in Drosophila'. Edited by Mani Ramaswami. *eLife* 3 (August):e02395. https://doi.org/10.7554/eLife.02395.

Vyazovskiy, Vladyslav V., Chiara Cirelli, Martha Pfister-Genskow, Ugo Faraguna, and Giulio Tononi. 2008. 'Molecular and Electrophysiological Evidence for Net Synaptic Potentiation in Wake and Depression in Sleep'. *Nature Neuroscience* 11 (2): 200–208. https://doi.org/10.1038/nn2035.

Walker, Matthew P., Tiffany Brakefield, Alexandra Morgan, J. Allan Hobson, and Robert Stickgold. 2002. 'Practice with Sleep Makes Perfect: Sleep-Dependent

Motor Skill Learning'. *Neuron* 35 (1): 205–11. https://doi.org/10.1016/s0896-6273(02)00746-8.

Walker, Matthew P., Tiffany Brakefield, Joshua Seidman, Alexandra Morgan, J. Allan Hobson, and Robert Stickgold. 2003. 'Sleep and the Time Course of Motor Skill Learning'. *Learning & Memory (Cold Spring Harbor, N.Y.)* 10 (4): 275–84. https://doi.org/10.1101/lm.58503.

Wang, Fei, Svetlana Minakhina, Hiep Tran, Neha Changela, Joseph Kramer, and Ruth Steward. 2018. 'Tet Protein Function during Drosophila Development'. *PLoS ONE* 13 (1): e0190367. https://doi.org/10.1371/journal.pone.0190367.

Wang, Jing W., Allan M. Wong, Jorge Flores, Leslie B. Vosshall, and Richard Axel. 2003. 'Two-Photon Calcium Imaging Reveals an Odor-Evoked Map of Activity in the Fly Brain'. *Cell* 112 (2): 271–82. https://doi.org/10.1016/s0092-8674(03)00004-7.

Wang, Xuejuan, Huanyu Chu, Mengjuan Lv, Zhihui Zhang, Shuwan Qiu, Haiyan Liu, Xuetong Shen, Weiwu Wang, and Gang Cai. 2016. 'Structure of the Intact ATM/Tel1 Kinase'. *Nature Communications* 7 (May):11655. https://doi.org/10.1038/ncomms11655.

Watson, J. D., and F. H. C. Crick. 1953. 'Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid'. *Nature* 171 (4356): 737–38. https://doi.org/10.1038/171737a0.

Watt, F., and P. L. Molloy. 1988. 'Cytosine Methylation Prevents Binding to DNA of a HeLa Cell Transcription Factor Required for Optimal Expression of the Adenovirus Major Late Promoter'. *Genes & Development* 2 (9): 1136–43. https://doi.org/10.1101/gad.2.9.1136.

Weber, Allison L., George F. Khan, Michael M. Magwire, Crystal L. Tabor, Trudy F. C. Mackay, and Robert R. H. Anholt. 2012. 'Genome-Wide Association Analysis of Oxidative Stress Resistance in Drosophila Melanogaster'. *PLOS ONE* 7 (4): e34745. https://doi.org/10.1371/journal.pone.0034745.

Williams, Rhys M., and Xiaodong Zhang. 2021. 'Roles of ATM and ATR in DNA Double Strand Breaks and Replication Stress'. *Progress in Biophysics and Molecular Biology* 161 (May):27–38. https://doi.org/10.1016/j.pbiomolbio.2020.11.005.

Wilusz, Jeremy E., Hongjae Sunwoo, and David L. Spector. 2009. 'Long Noncoding RNAs: Functional Surprises from the RNA World'. *Genes & Development* 23 (13): 1494–1504. https://doi.org/10.1101/gad.1800909.

Wisor, Jonathan P., Seiji Nishino, Ichiro Sora, George H. Uhl, Emmanuel Mignot, and Dale M. Edgar. 2001. 'Dopaminergic Role in Stimulant-Induced Wakefulness'. *Journal of Neuroscience* 21 (5): 1787–94. https://doi.org/10.1523/JNEUROSCI.21-05-01787.2001.

Wong, Chloe C. Y., Michael J. Parsons, Kathryn J. Lester, Joe Burrage, Thalia C. Eley, Jonathan Mill, Emma L. Dempster, and Alice M. Gregory. 2015. 'Epigenome-Wide DNA Methylation Analysis of Monozygotic Twins Discordant for Diurnal Preference'. *Twin Research and Human Genetics: The Official Journal of the International Society for Twin Studies* 18 (6): 662–69. https://doi.org/10.1017/thg.2015.78.

Wu, Hong, Jinrong Min, Vladimir V. Lunin, Tatiana Antoshenko, Ludmila Dombrovski, Hong Zeng, Abdellah Allali-Hassani, et al. 2010. 'Structural Biology of Human H3K9 Methyltransferases'. *PloS One* 5 (1): e8570. https://doi.org/10.1371/journal.pone.0008570.

Wu, Mark N., Kyunghee Koh, Zhifeng Yue, William J. Joiner, and Amita Sehgal. 2008a. 'A Genetic Screen for Sleep and Circadian Mutants Reveals Mechanisms Underlying Regulation of Sleep in Drosophila'. *Sleep* 31 (4): 465–72.

———. 2008b. 'A Genetic Screen for Sleep and Circadian Mutants Reveals Mechanisms Underlying Regulation of Sleep in Drosophila'. *Sleep* 31 (4): 465–72. https://doi.org/10.1093/sleep/31.4.465.

Xie, Lulu, Hongyi Kang, Qiwu Xu, Michael J. Chen, Yonghong Liao, Meenakshisundaram Thiyagarajan, John O'Donnell, et al. 2013. 'Sleep Drives Metabolite Clearance from the Adult Brain'. *Science (New York, N.Y.)* 342 (6156): 10.1126/science.1241224. https://doi.org/10.1126/science.1241224.

Yang, Zhe, Yue Yu, Vivian Zhang, Yinjun Tian, Wei Qi, and Liming Wang. 2015. 'Octopamine Mediates Starvation-Induced Hyperactivity in Adult Drosophila'. *Proceedings of the National Academy of Sciences* 112 (16): 5219–24. https://doi.org/10.1073/pnas.1417838112.

Yeh, Yao-Tsung, Hua Yeh, Shu-Hui Su, Jian-Sheng Lin, Kuo-Jui Lee, Huey-Wen Shyu, Zi-Feng Chen, Sheng-Yun Huang, and Shu-Jem Su. 2014. 'Phenethyl Isothiocyanate Induces DNA Damage-Associated G2/M Arrest and Subsequent

Apoptosis in Oral Cancer Cells with Varying P53 Mutations'. *Free Radical Biology & Medicine* 74 (September):1–13. https://doi.org/10.1016/j.freeradbiomed.2014.06.008.

Yoshihara, M., A. W. Ensminger, and J. T. Littleton. 2001. 'Neurobiology and the Drosophila Genome'. *Functional & Integrative Genomics* 1 (4): 235–40. https://doi.org/10.1007/s101420000029.

You, Samantha, Tudor A. Fulga, David Van Vactor, and F. Rob Jackson. 2018. 'Regulation of Circadian Behavior by Astroglial MicroRNAs in Drosophila'. *Genetics* 208 (3): 1195–1207. https://doi.org/10.1534/genetics.117.300342.

Yu, Yue, Rui Huang, Jie Ye, Vivian Zhang, Chao Wu, Guo Cheng, Junling Jia, and Liming Wang. n.d. 'Regulation of Starvation-Induced Hyperactivity by Insulin and Glucagon Signaling in Adult Drosophila'. *eLife* 5:e15693. https://doi.org/10.7554/eLife.15693.

Zada, D., I. Bronshtein, T. Lerer-Goldshtein, Y. Garini, and L. Appelbaum. 2019. 'Sleep Increases Chromosome Dynamics to Enable Reduction of Accumulating DNA Damage in Single Neurons'. *Nature Communications* 10 (1): 895. https://doi.org/10.1038/s41467-019-08806-w.

Zada, David, Yaniv Sela, Noa Matosevich, Adir Monsonego, Tali Lerer-Goldshtein, Yuval Nir, and Lior Appelbaum. 2021. 'Parp1 Promotes Sleep, Which Enhances DNA Repair in Neurons'. *Molecular Cell* 81 (24): 4979-4993.e7. https://doi.org/10.1016/j.molcel.2021.10.026.

Zamurrad, Sumaira, Hayden A. M. Hatch, Coralie Drelon, Helen M. Belalcazar, and Julie Secombe. 2018. 'A Drosophila Model of Intellectual Disability Caused by Mutations in the Histone Demethylase KDM5'. *Cell Reports* 22 (9): 2359–69. https://doi.org/10.1016/j.celrep.2018.02.018.

Zemach, Assaf, M. Yvonne Kim, Pedro Silva, Jessica A. Rodrigues, Bradley Dotson, Matthew D. Brooks, and Daniel Zilberman. 2010. 'Local DNA Hypomethylation Activates Genes in Rice Endosperm'. *Proceedings of the National Academy of Sciences of the United States of America* 107 (43): 18729–34. https://doi.org/10.1073/pnas.1009695107.

Zhang, Tianyi, Sarah Cooper, and Neil Brockdorff. 2015. 'The Interplay of Histone Modifications – Writers That Read'. *EMBO Reports* 16 (11): 1467–81. https://doi.org/10.15252/embr.201540945.

Zhang, Wenchao, Panhong Gou, Jean-Marie Dupret, Christine Chomienne, and Fernando Rodrigues-Lima. 2021. 'Etoposide, an Anticancer Drug Involved in Therapy-Related Secondary Leukemia: Enzymes at Play'. *Translational Oncology* 14 (10): 101169. https://doi.org/10.1016/j.tranon.2021.101169.

Zhao, Zibo, and Ali Shilatifard. 2019. 'Epigenetic Modifications of Histones in Cancer'. *Genome Biology* 20 (1): 245. https://doi.org/10.1186/s13059-019-1870-5.

Zimmerman, John E., Wendy Rizzo, Keith R. Shockley, David M. Raizen, Nirinjini Naidoo, Miroslaw Mackiewicz, Gary A. Churchill, and Allan I. Pack. 2006. 'Multiple Mechanisms Limit the Duration of Wakefulness in Drosophila Brain'. *Physiological Genomics* 27 (3): 337–50. https://doi.org/10.1152/physiolgenomics.00030.2006.

Zou, Lee, and Stephen J. Elledge. 2003. 'Sensing DNA Damage through ATRIP Recognition of RPA-ssDNA Complexes'. *Science (New York, N.Y.)* 300 (5625): 1542–48. https://doi.org/10.1126/science.1083430.