
Faculty of Biology
ATUT PhD Programme in Biology

# The Role of Adenosine Receptor in the Living Processes of *Drosophila melanogaster*

Debarati Bhattacharya

Doctoral dissertation
Conducted under the supervision of
Prof. dr hab. Elżbieta Pyza
Department of Cell Biology and Imaging
Institute of Zoology and Biomedical Research,
Faculty of Biology

Krakow, Poland
2023

**Contents**

**List of Abbreviation**

| | |
|---|---|
| ***AMP*** | ***Adenosine monophosphate*** |
| ***APNEA*** | *Aminophenylethyladenosine* |
| ***AR*** | *Adenosine receptor* |
| ***ATL313*** | *4-{3-[6-amino-9-(5-cyclopropylcarbamoyl-3,4-dihyroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl}-piperidine-1-carboxylic acid methyl ester.* |
| ***AZ*** | *Active Zone* |
| ***BRP*** | *Bruchpilot protein* |
| ***cAMP*** | *Cyclic adenosine monophosphate* |
| ***CGRP*** | *Calcitonin gene-related peptide* |
| ***CGS21680*** | *5´-N´-ethylcarboxyaminoadenosine* |
| ***CLK*** | *CYC-CLOCK-CYCLE heterodimer* |
| ***CPA*** | *Cyclopentyl-adenosine* |
| ***CRY*** | *Cryptochrome protein* |
| ***dAdoR*** | *Drosophila Adenosine receptor gene* |
| ***DAMS*** | *Drosophila Activity Monitoring System* |
| ***DANs*** | *dopaminergic neurons* |
| ***DD*** | *constant darkness* |
| ***dFB*** | *dorsal fan-shaped body* |
| ***DM*** | *Diabetes Mellitus* |
| ***DN*** | *dorsal neurons* |
| ***DS*** | *daytime sleep* |
| ***GABA*** | *gamma-aminobutyric acid* |
| ***GPCRs*** | *G protein-coupled receptors* |
| ***HD*** | *Huntington's Disease* |
| ***HTT*** | *Huntington protein* |
| ***IgE*** | *Immunoglobulin E* |
| ***IHC*** | *Immunohistochemistry* |
| ***IOP*** | *Intra Ocular Pressure* |
| ***IP3*** | *inositol triphosphate* |
| ***LD*** | *Light- Dark* |

| | |
|---|---|
| ***LL*** | *constant Light* |
| ***LMC*** | *Large Monopolar Cells* |
| ***LNVs*** | *ventral lateral neurons* |
| ***MAPK*** | *Mitogen-activated protein kinases* |
| ***MB*** | *mushroom bodies* |
| ***NECA*** | *5'-N-ethylcarboxamidoadenosine* |
| ***NS*** | *Nighttime sleep* |
| ***OSNs*** | *Olfactory Sensory Neurons* |
| ***PDE*** | *phosphodiesterase* |
| ***PER*** | *PERIOD protein* |
| ***PKA*** | *cAMP-dependent protein kinase A* |
| ***REM*** | *rapid eye movement* |
| ***R-PIA*** | *-[R (-) N6-(2-phenyl-isopropyl) adenosine]* |
| ***s-LNvs*** | *small ventral lateral neurons* |
| ***SWS*** | *Slow Wave Sleep* |
| ***TIM*** | *TIMELESS protein* |
| ***TS*** | *Total sleep* |
| ***VLPA*** | *Ventro-Lateral Preoptic Area* |
| ***ZT*** | *ZeitgeberTime* |

## Figure Legends

**Table Legends**

**Abstract**

*Drosophila melanogaster* possesses a single adenosine receptor called dAdoR, whose functions in living processes are still unknown. By overexpressing or silencing *dAdoR* in photoreceptors, neurons, or glial cells, I examined fruit flies' survival, fitness, sleep, and locomotor activity.

In the survival assay, I observed that *dAdoR* overexpression causes early deaths in younger flies (1-10 days old), while silencing prevents early deaths and stabilizes median survival. However, I observed that experimental flies with silencing of this gene show a reduced overall lifespan. I found that overexpression of adenosine receptors in neurons and glial cells improves the fitness of older flies (60 days old), while silencing deteriorates their fitness.

In sleep experiments, I observed that *dAdoR* overexpression increases day sleep (*siesta*) in photoreceptors and night sleep in glial cells, while in all neurons, it increases total sleep. However, silencing of *dAdoR* did not show significant changes in sleep.

To study synaptic mechanism of behavioural changes, I examined the level of presynaptic protein Bruchpilot (BRP), and its daily pattern in the fly's first optic neuropil (lamina), after *dAdoR* silencing in photoreceptors or glial cells. I confirmed that the BRP protein oscillates in the tetrad synapses and shows significant changes at the beginning (ZT1) of the day and in the middle of the night (ZT16). During the evening peak of locomotor activity (ZT13), the protein level was highest in both experimental and control flies. However, after silencing of *dAdoR* in glial cells, I observed that BRP level changes only at the beginning and the middle of the day (ZT1 and ZT4).

In the final part of my thesis, I studied the possible effects of caffeine on functioning of adenosine receptors in sleep regulation, ageing, and behaviour. In wild-type flies I found that caffeine affects more strongly female flies, and it influences *siesta*. Also, caffeine is unable to disrupt the circadian clock when *dAdoR* is overexpressed or silenced (in all neurons, *pdf*-expressing clock neurons, *tim*-expressing neurons or *th*-expressing dopaminergic neurons). I observed that caffeine treatment decreases *siesta* when *dAdoR* is overexpressed in all neurons, *tim*-expressing neurons or in *th*-expressing dopaminergic neurons. In turn, *dAdoR* silencing increases *siesta.* This shows that adenosine receptors are involved in the regulation of *siesta*.

**Keywords:** adenosine, locomotor activity, sleep, synaptic plasticity, circadian rhythms, caffeine.

**Streszczenie**

*Drosophila melanogaster* posiada pojedynczy receptor adenozynowy, zwany dAdoR, którego funkcje w procesach życiowych są wciąż nieznane. Poprzez nadekspresję lub wyciszenie dAdoR w fotoreceptorach, neuronach i komórkach glejowych zbadaliśmy przeżywalność, kondycję, sen i aktywność lokomotoryczną muszek owocowych.

W teście przeżywalności zaobserwowaliśmy, że nadekspresja *dAdoR* powoduje wyższą śmiertelność u młodych osobników (w wieku 1-10 dni), podczas gdy wyciszenie zapobiega przedwczesnej śmierci.. Zaobserwowaliśmy jednak, że eksperymentalne szczepy z wyciszeniem tego genu wykazują zmniejszoną ogólną długość życia. Odkryliśmy, że nadekspresja receptorów adenozynowych w neuronach i komórkach glejowych poprawia sprawność starszych osobników (60 dniowych), podczas gdy wyciszenie *dAdoR* obniża ich sprawność.

W badaniach snu zaobserwowaliśmy, że nadekspresja *dAdoR* w fotoreceptorach i komórkach glejowych zwiększa sen odpowiednio w dzień (sjesta) i w nocy, podczas gdy nadekspresja we wszystkich neuronach zwiększa długość całkowitego snu (w dzień i w nocy). Natomiast wyciszenie *dAdoR* nie wykazało znaczących zmian we śnie.

Aby zbadać wpływ ekspresji *dAdoR* na poziomie komórkowym, na plastyczność synaptyczną, zbadaliśmy poziomi dobowe zmiany presynaptycznego białka Bruchpilot (BRP) w pierwszym neuropilu (lamina) płata wzrokowego mózgu *D. melanogaster*, poprzez wyciszenie *dAdoR* w fotoreceptorach lub komórkach glejowych. Potwierdziliśmy, że białko BRP oscyluje w synapsach tetradycznych i wykazuje istotne zmiany poziomu na początku dnia (ZT1) oraz w środku nocy (ZT16). Podczas wieczornego szczytu aktywności lokomotorycznej (ZT13) poziom białka był najwyższy zarówno u owadów doświadczalnych, jak i kontrolnych. Natomiast po wyciszeniu *dAdoR* w komórkach glejowych zaobserwowaliśmy, że poziom BRP zmienia się tylko na początku i w połowie dnia (ZT1 i ZT4).

W końcowej części mojej pracy magisterskiej zbadaliśmy wpływ kofeiny na receptory adenozynowe w regulacji snu, starzeniu się i zachowaniu. Wykorzystując dziki szczep Canton-S *D. melanogaster* stwierdziliśmy, że kofeina działa silniej u samic niż u samców i wpływa na czas trwania sjesty. Jednakże kofeina nie zaburza molekularnego mechanizmu zegara okołodobowego

gdy poziom dAdoR jest podwyższony lub obniżony we wszystkich neuronach lub tylko w wybranych grupach neuronów: neuronach zegarowych z ekspresją *pdf*, neuronach z ekspresją *tim,* lub w neuronach dopaminergicznych). Zaobserwowaliśmy, że suplementacja diety kofeiną skraca sjestę, gdy *dAdoR* ulega nadekspresji (we wszystkich neuronach, neuronach *tim*-pozytywnych i neuronach dopaminergicznych). Z kolei wyciszenie *dAdoR* wydłuża sjestę. To wskazuje, że w regulacji czasu sjesty zaangażowane są receptory adenozyny.

**Słowa kluczowe:** adenozyna, aktywność lokomotoryczna, sen, plastyczność synaptyczna, rytmy okołodobowe, kofeina

# 1. Introduction

## 1.1. Origin of Adenosine

The first evidence of adenosine as a **life-preserving** molecule was found in 1981. Adenosine was recognized as a cell density signal that can induce the formation of fruiting bodies, after starvation, in the bacterium *Myxococcus xanthus* (yellow slime coccus), a rod-shaped Gram-negative bacterium [1].

## 1.2. What is Adenosine?

Adenosine is an endogenous agonist of adenosine receptors. It is a ribonucleoside composed of adenine bound to ribose [**Fig. 1.1**]. Adenosine is produced by the metabolism of adenosine triphosphate (ATP). Throughout the body, adenosine performs multiple functions, including vasoconstriction [2–4] or vasodilation [5] of veins and arteries, T cell proliferation and cytokine production [6], neuroprotection in ischemic [7], hypoxic and oxidative stress events [8], in synaptic plasticity [9], inhibition of lipolysis [10], and it stimulates bronchoconstriction[11]. In addition to this, it acts as a neuromodulator in the nervous system and helps in neurotransmitter release [12].

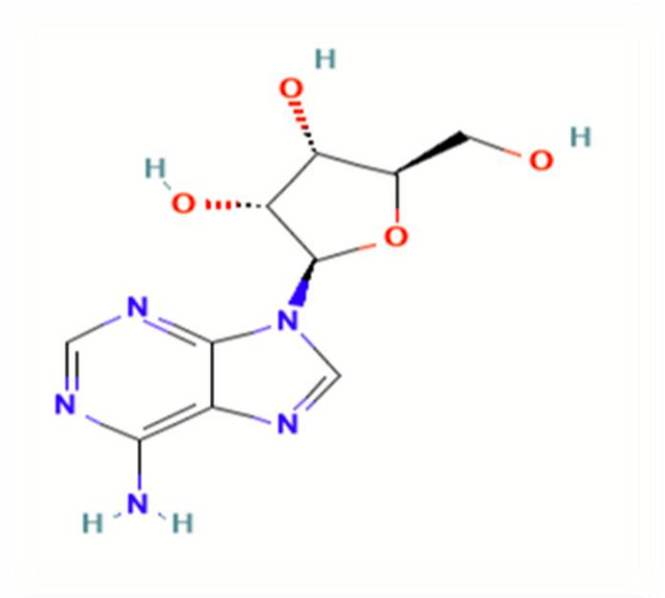


**Fig. 1.1.** 2D structure of adenosine [13].

Adenosine has several agonists and antagonists that have been proven useful in the development of new drugs and clinical applications. For example, adenosine agonists are useful in the prevention of reperfusion injury after cardiac ischemia or stroke, as well as in the treatment of

hypertension and epilepsy, whereas adenosine antagonists are effective in the treatment of renal failure and are used as cognition enhancers [14].

### 1.3. Adenosine Metabolism

Adenosine is produced both intracellularly and extracellularly. Intracellularly, it is produced from 5 ′-adenosine monophosphate (5′-AMP) by the action of the enzyme 5′-nucleotidase. Adenosine then follows several metabolic/synthetizing pathways [**Fig. 1.2**]. It is metabolized to inosine and hypoxanthine by adenosine deaminase and to uric acid by xanthine oxidase. Adenosine is transported out of the cell to the extracellular space by specific bidirectional nucleoside transporters [15]. These transporters are classified into equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs) [16].

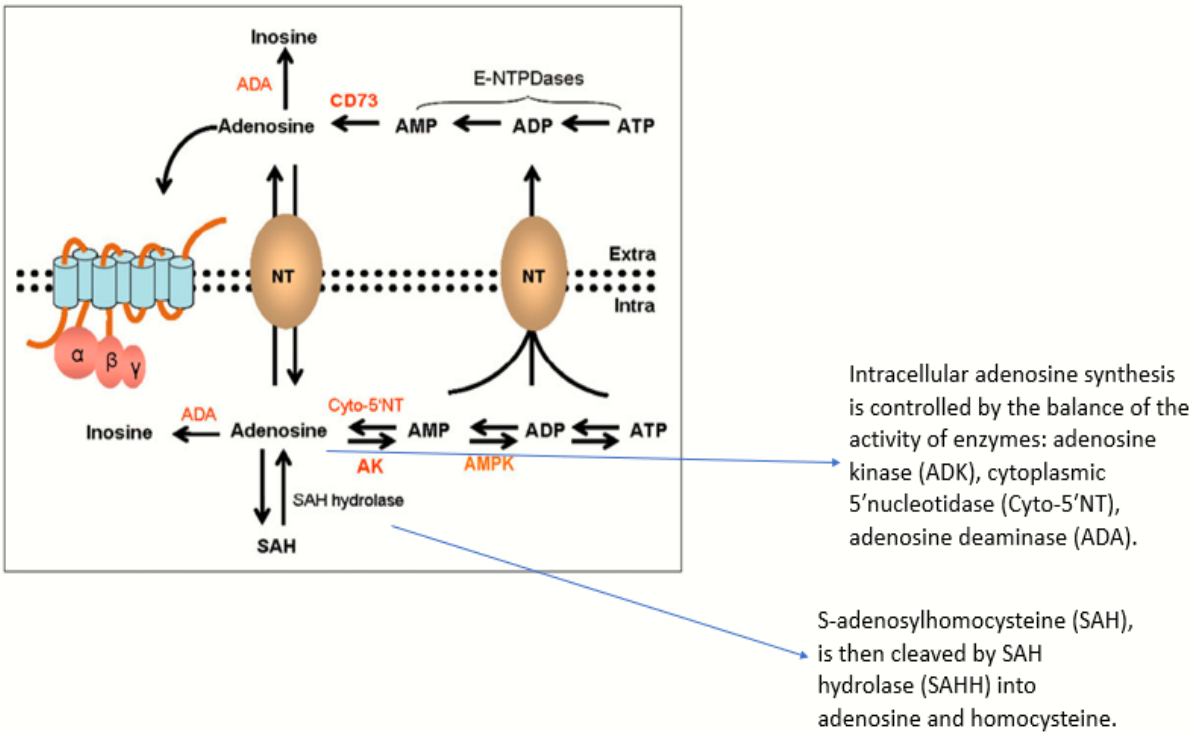


**Fig. 1.2.** Adenosine synthesis and metabolic pathways inside and outside the cell [17].

ENTs are passive bidirectional transporters that transport adenosine across the plasma membrane on its concentration gradient. CNTs are active $Na^+$ dependent transporters that transport adenosine against its concentration gradient [18]. Adenosine can be converted back to 5′-AMP by the enzyme

adenosine kinase and then to ADP and ATP. Extracellular 5′-AMP is produced by degradation of ATP (by ectonucleotidase) and cyclic AMP (by ecto-cyclic AMP phosphodiesterase) [15,19]. During resting periods, extracellular and intracellular levels of adenosine are very similar. In pathophysiological conditions (inflammation, ischemia, and hypoxia), the level of this nucleoside increases. This extra adenosine is removed and transported through the ENTs. Inside the cell, adenosine is deaminated to inosine through adenosine deaminase (ADA) or phosphorylated to AMP by adenosine kinase (AK). During physiological conditions, adenosine is preferentially transformed to AMP, while in pathological states it is converted to inosine [20,21].

### 1.4. Adenosine Receptors and Their Molecular Structure

The action of adenosine is mediated through specific cell surface receptors. These receptors are known as adenosine receptors (ARs) and belong to the family of G-protein coupled receptors. All of them have a very similar molecular architecture. Each receptor consists of a core domain of seven alpha helixes, each helix is 20 to 27 amino acids long. The core domain is linked by three intracellular and three extracellular loops [22] with an extracellular amino-terminus and an intracellular carboxy-terminus. The *N*-terminal domain has *N*-glycosylation sites that influence the trafficking of the receptor to the plasma membrane, while the carboxy-terminus contains serine and threonine residues that serve as phosphorylation sites for protein kinases and enable receptor desensitization. In addition, the carboxy-terminus and the third intracellular loop enable the coupling of ARs to G-proteins [23,24].

Adenosine receptors are divided into four types, named A1, A2A, A2B, and A3 receptors [**Fig. 1.3**], which are widely distributed in all tissues and organs. These receptors have distinct localization, signal transduction pathways, and different means of regulation upon exposure to agonists. All four adenosine receptors have been cloned from many mammalians and some from non-mammalian species. A2AR has a longer C-terminus tail composed of 122 amino acids, while the C-terminal domains of the A1, A2B, and A3 receptors are made up of 30 – 40 amino acids [25]. The A1 and A2A receptors possess a high affinity for adenosine, while A2B and A3A show a lower affinity [26]. The classification of these receptors is based on their differential coupling with adenylyl cyclase to regulate cyclic AMP levels. A1 and A3 are coupled to $G_{i/o}$ proteins, while A2A and A2B are coupled to $G_{s/olf}$ proteins [27]. Activation of the A2A and A2B receptors increases

cyclic AMP production, resulting in activation of protein kinase A (PKA) and phosphorylation of the cyclic AMP response element binding protein (CREB). In turn, activation of the A1 and A3A receptors inhibits cyclic AMP production and decreases PKA activity and CREB phosphorylation [7,16,27]. The A1 and A3 share 49 % sequence similarity, whereas the A2A and A2B receptors are 59% identical. The A3 receptor was the first to be isolated and pharmacologically characterized [24]. Recent discovery is the A2C receptor found in fish and amphibians [25].

### 1.5.Adenosine Receptor Distribution and Functions

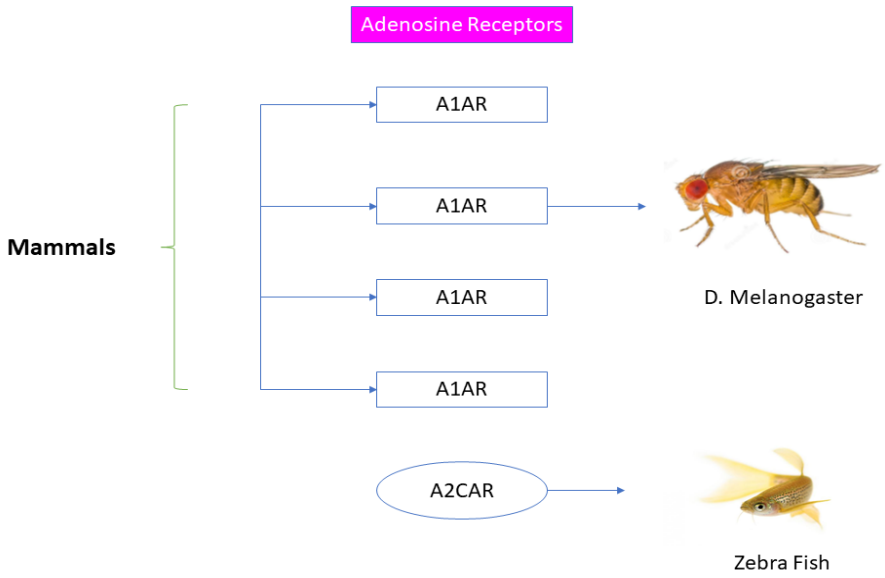


**Fig. 1.3.** A schematic chart showcasing the different types of ARs.

#### (a) A1AR.

The adenosine A1 receptor (A1AR) is present throughout the body. In the central nervous system (CNS), A1ARs are widely distributed in neurons, in the brain cortex, the hippocampus, and the cerebellum [8]. These receptors are also present in astrocytes [26], oligodendrocytes [27], and microglia [28]. In neurons, A1ARs are localized in high number in synaptic regions, where they modulate the release of neurotransmitters, such as glutamate, acetylcholine, serotonin, and GABA[7]. This receptor has an inhibitory function in most tissues in which it is expressed. The availability of the AlAR canine cDNA sequence resulted in the subsequent cloning of AlAR from rats [29,30], bovine

[31,32], human [33–35] , and rabbit [36] cDNA libraries. The various clones encode a protein of 326 amino acids (except for rabbit A1AR, which has 328 amino acids). The amino acid identity of the species homologues of the AlAR is approximately 87 and 92%. AlAR is known to inhibit adenylyl cyclase [37,38]. In the brain, it slows metabolic activity through a combination of actions. Presynaptically, it reduces synaptic vesicle release, while postsynaptically, it stabilizes magnesium on the NMDA (N-methyl-D-aspartate) receptor.

**(b) A2AR.**

The A2AR has a wide-spread distribution and occurs both centrally and peripherally. Its highest expression is in the striatum, the olfactory tubercle, and in the immune system, while lower expression has been reported in the cerebral cortex, hippocampus, heart, lung, and blood vessels. This type of receptor is expressed in pre- and postsynaptic neurons, astrocytes, microglia, and oligodendrocytes, where it orchestrates several functions related to excitotoxicity, neuronal glutamate release, glial activity, blood-brain barrier (BBB) permeability, and peripheral immune cell migration. A2AR has been cloned from canine [39], rat [40], and human [41] cDNA libraries. The total amino acid identity of the rat and human to the canine is 82 and 93%, respectively. A2AR has been associated with activation of adenylyl cyclase through the Gs protein [37,38]. A2ARs can increase activity of NMDA receptors leading to excitotoxicity [42]. In the case of synucleinopathy, signalling of the A2A receptor causes the reduction in cognition and neurodegeneration [43]. An increase in the amount of A2AR in microglia results in neurodegeneration. A2AR is involved in the modulation of MAPK signalling [44,45]. A2AR also interacts with different accessory proteins, such as D2-dopamine receptors, actinin, nucleotide site opener (ARNO), ubiquitin-specific protease (USP4), and translin-associated protein X (TRAX) through its long COOH terminus.

**(c) A2BAR**

A2BAR was first identified and cloned in the rat hypothalamus [46] and the human hippocampus [47]. The proposed structure of A2BAR is the typical structure of the G protein-coupled receptor (GPCR) [48]. A2ARs are divided into two subtypes based on differences observed for agonist binding (high affinity, A2AR; low affinity, A2BAR) and differences in anatomical distribution (striatum, A2AR; other regions of the brain, A2BAR) [49,50]. A2BAR has been cloned from brain cDNA libraries in rats [45,46] and humans [47]. In the transmembrane domain regions, both rat and

human A2BARs show 73% amino acid identity with the A2AR of the respective species. The A2BAR consists of approximately 80 amino acid residues fewer than the A2AR and is similar in size to the A1AR and A3AR. This receptor subtype mediates the secretory action of adenosine in mast cells [51]. A2BAR binds to adenosine with low affinity [8,52,53]. A2BAR can be coupled to several intracellular signalling pathways and plays physiological functions that differ from those of A2AR [53,54,63,55–62]. It plays a role in the modulation of inflammation and immune responses in selected pathologies such as cancer and diabetes, as well as renal, lung, and vascular diseases. The expression of A2BAR increases under different harmful conditions such as hypoxia, inflammation, and cell stress. It is highly expressed in the periphery, where it has been found in the bowel, bladder, lung, vas deferens, and different cell types, including fibroblasts, smooth muscle, endothelial, immune, alveolar epithelial, chromaffin, taste cells, and platelets. In the brain they are found in astrocytes, neurons, and microglia [56,64].

The main signalling pathway of A2BAR involves adenylyl cyclase (AC) that leads to an increase in intracellular cAMP levels and causes activation of PKA and other cAMP effectors, such as Epac [65–71]. However, the A2B AR-Gq-PLC pathway also mediates several crucial functions of A2BAR [72–74]. A2BAR also couples with the MAPK (Mitogen-activated protein kinase) and arachidonic acid signalling pathways and regulates membrane ion channels through βγ subunits of G-protein [48,75–77].

**(d) A3AR**

Researchers published the sequence of a clone isolated from the rat testis cDNA library that encoded a protein of 320 amino acids that has a total amino acid identity of 47 and 42% for the canine AlAR and A2AR, respectively [24]. The same clone was independently isolated from the rat brain cDNA library and analyzed due to its sequence similarity to existing cloned ARs [78]. The clone stably expressed in CHO cells (Chinese hamster ovary) showed a pharmacological profile not typical of any characterized A1AR or A2AR.

The affinity for the agonist ligands was much lower for this cloned receptor. Even xanthine derivatives bound very poorly to the cloned receptor. Stimulation of the receptor with NECA or R-PIA resulted in a pertussis toxin-sensitive inhibition of forskolin-stimulated adenylyl cyclase

activity. Based on these properties and the sequence similarity, the clone was identified as a unique AR type and was termed A3AR.

The sheep [79] and human [80] A3ARs were subsequently cloned, and both revealed a 72% overall amino acid identity with the rat A3AR; the sheep and human subtypes are more like each other than the rat homologue. The A3AR is like the AlAR in size; it is composed of 317 – 320 amino acids, depending on the species. Unlike other cloned ARs, A3AR possesses a consensus site for N-linked glycosylation at both the amino terminus and the second extracellular loop.

The species homologues of the A3AR also differ substantially in the binding of various agonist ligands, although this binding has typically lower affinity than in case of AlAR. There are also significant differences between species regarding the tissue distribution of mRNA for A3AR. The most abundant amount of the A3AR is found in the rat testes [24,78]. Rat tissues containing moderate amounts of A3AR are found in the lung, kidney and heart, while lower levels are detected in brain regions including the cortex, striatum and olfactory bulb. For many tissues, the expression level of A3AR, assessed by radioligand binding, is unknown and its physiological role has not been determined. However, A3AR is present at higher levels in the lungs in all species, hinting at its role in mediating allergic responses in the pulmonary system. Administration of the A3AR agonist Ni-(3-iodobenzyl)-5'-N-methy1carbixamidoadenosine (IB-MECA) in mice, can induce decrease in locomotor activity [80]. **Table 1.1** shows the summarized functions of these four types of receptors.

**Table 1.1. Types of adenosine receptors and their functions**

| Type | Functions |
|---|---|
| **A1AR** | • It helps in vasoconstriction in mammals.<br>• It inhibits chloride transport in the rectal glands of shark.<br>• The number of these receptors increases in the case of sleep deprivation.<br>• The high A1AR in the KO mouse model helps to improve cognitive performance, sleep, and it helps to overcome depressive behaviour.<br>• An increase in A1AR in the neocortex results in epilepsy in humans.<br>• Activation of A1AR in cats and rabbits reduces IOP (Intra Ocular Pressure)<br>• A1AR agonists inhibit the trigeminal nerve, suppressing the release of CGRP and protecting against cluster migraines and headaches in rats, cats, and humans. |

| | |
|---|---|
| | • Activation of A1AR causes the disappearance of t-LTD in the mouse hippocampus and plays a role in synaptic plasticity.<br>• It helps in gills vasoconstriction in cold-adapted Antarctic teleost fish.<br>• The A1AR agonist CPA induces bronchoconstriction in allergic rabbits by increasing airway resistance.<br>• It plays a role in fertilization. The A1AR agonist can mimic the IP3 receptors (present in sperm) which get activated upon depolarization of sperm membrane caused by an influx of calcium into the sperm head. This extracellular calcium influx is necessary for the acrosomal reaction.<br>• It plays an important role in thermoregulation. The use of an A1AR agonist results in dose-related hypothermia, while an A1AR antagonist such as theophylline can decrease the hypothermic effect.<br>• A1AR is involved in breathing movements in sheep fetus. During anemia, the level of A1AR increases in the rostral midbrain causing breathing problems. Recovery of breathing is attributed to downregulation of A1AR in the rostral midbrain.<br>• It can inhibit cAMP production in the rabbit kidney. |
| **A2AR** | • It is present in the VLPA region and is known to promote SWS (slow wave sleep) in pigeons.<br>• Administration of the CGS21680 A2AR agonist to the rat forebrain promotes SWS and paradoxical sleep.<br>• Helps in vasodilation in mammals.<br>• It facilitates chloride transport in shark rectal glands.<br>• It helps in fast melanin dispersion and skin darkening in teleost fish (guppy and catfish).<br>• It helps in the relaxation of cerebral arteries in cats by stimulating the accumulation of cAMP.<br>• The application of an A2AR agonist increases IOP resulting in ocular hypertension in the retina of cats and rabbits.<br>• A2AR helps in pulmonary vasodilation in lambs. These receptors become functional by the $128^{th}$ day of gestation in the pulmonary circulation of lamb fetus.<br>• A2AR helps in GABA neuron migration in hippocampal development of rodents.<br>• It plays a neuroprotective role in HD (Huntington's Disease) mouse models. Stimulation of A2AR triggers an anti-apoptotic effect in a rat neuron-like cell line (PC12). The CGS21680 A2AR agonist increases proteasome activity and prevents the accumulation of mutant HTT (huntingtin) aggregates. |

| | |
|---|---|
| | • Application of the A2AR agonist ATL313 in A2AR deficient mice can protect the liver from ischemia-reperfusion injury (IRI) |
| **A2BAR** | • Mice lacking A2BAR demonstrate heightened susceptibility to IgE antigen-induced anaphylaxis due to enhanced mast cell activation. The A2B adenosine receptor functions as a critical regulator of signalling pathways within the mast cell, which act together to limit the magnitude of mast cell response when antigen is encountered.<br>• A2BAR is highly expressed in various types of tumor cells or tissues and promotes tumor cell proliferation. A2BAR was found to be overexpressed in colorectal carcinoma cells, and inhibition of A2BAR blocked proliferation of colon cancer cells.<br>• It plays a role in mediating the progression of diabetic nephropathy.<br>• It can protect against renal fibrosis. In mice, genetic deletion of A2BAR protects against renal fibrosis.<br>• It provides renal protection. A2BAR reduces neutrophil-dependent TNF-α production and suppresses inflammation [54]; A2BAR has been suggested to function as a critical regulator in DM (Diabetes mellitus). A2BAR activation increases insulin resistance by increasing the production of proinflammatory mediators such as IL-6 and C-reactive protein [81].<br>• Blockade of A2BAR in mice reduced glucose production in liver and enhanced glucose disposal into skeletal muscle and brown adipose tissue [81]. |
| **A2CAR** | • Olfaction and chemo sensation in zebrafish.<br>• It can locate adenosine and adenine nucleotides in olfactory sensory neurons (OSNs).<br>• It has been found in fish (both freshwater and saltwater) and amphibians.<br>• It is absent in reptiles, birds and mammals. |
| **A3AR** | • Activation of A3AR before ischemia helps in lung protection.<br>• A3AR expressed on eosinophils in the human lungs inhibits eosinophil chemotaxis and protects against diseases such as asthma and rhinitis.<br>• In the feline model, it decreases the severity of lung reperfusion injury.<br>• In allergic rabbits use of the A3AR agonist, APNEA fails to cure bronchoconstriction.<br>• It plays a role in cerebral protection and is quite protective against heart infarctions.<br>• In the kidney, the A3AR agonist IB-MECA can worsen renal injury, but the use of the A3AR antagonist MRS-1191 confers renal protection after ischemia and reperfusion. |

### 1.6.Adenosine receptor of *drosophila melanogaster*

In *Drosophila melanogaster,* a single *dAdoR* gene (CG9753) [82] was described and dAdoR is a GPCR that activates adenylate cyclase [83] leading to cAMP production and calcium signalling just like the A2B receptor in mammals. The *D. melanogaster* receptor (dAdoR) has a sequence similarity of 38% in the N-terminal region comprising 350 amino acids as A2AR in humans and 70 % sequence similarity with *Anopheles gambiae* (392aa) and *Apis mellifera* (462aa) [82]. The expression of *dAdoR* in different cell lines and tissues shows a notable variation. The highest expression has been reported in the nervous system. In larvae, *dAdoR* is expressed in the optic lobes, ring glands, imaginal discs, and salivary glands [82,83]. Insect *AdoR* activation is essential for energy metabolism during development. It has also been found that overexpression can be lethal for larvae and pupae if *dAdoR* is overexpressed in all tissues, while tissue-specific expression results in decreased mortality [82]. However, changes in a diet can prevent this lethality. For example, a 10% addition of sucrose to the diet can protect flies from lethality [84].

In *D. melanogaster*, *dAdoR* signalling plays a role in synaptic plasticity, stress response, immune protection, and hematopoiesis [85–87]. Adenosine signalling can modify the pathogenic effects of polyglutamine in a *Drosophila* model of Huntington's disease [88].

The fruit fly is a perfect model species for studying the molecular mechanisms involved in sleep. Sleep is easily measured in *D. melanogaster*, which decreases sensory responsiveness during sleep, and it tends to show a rebound after sleep deprivation [89–92]. After the discovery of *dAdoR* in *Drosophila*, adenosine could be further studied for its role in rest/activity regulation and other processes.

### 1.7.*Drosophila* as a Model Species in Neuroscience

For the last 100 years, the fruit fly *D. melanogaster* has been used as a model to answer many questions. The advantages it offers for experimental studies are impossible in humans and other vertebrates. The genetic tools for neuronal circuit analysis, cost-effectiveness of culturing, short development, ease of cell biological manipulations, and relevance to human physiology have made it as an important model organism. One aspect of biology in which *D. melanogaster* has contributed extensively is neurobiology. The adult *Drosophila* nervous system has about 150 000 neurons and a remarkable complex behavioural repertoire. This nervous system continually allows

for new surprises about its capabilities. Headless flies that retain only their nerve cord are capable of complex reflexive behaviour, including grooming and righting of the body if it is inverted [93].

The *D. melanogaster* genome has been completely sequenced. Starting with the discovery of the *period* gene in 1971 [94], *Drosophila* has been a model organism in the study of complex behaviours, such as courtship [95], aggression [96], feeding [97], drug addiction [98], learning and memory [99], circadian rhythms [100], and sleep [101].

*Drosophila* has a bilaterally symmetric brain that is joined to the ventral nerve cord innervating the thorax and abdomen. The homologies between the fly's and mammalian brain regions are less obvious, because of the evolution of brain organization that is adapted to their respective lifestyles. However, ontogeny and localized gene expression show a division of the developing brain region into a protocerebrum, deutocerebrum, and tritocerebrum, which appear evolutionarily homologous to the forebrain, midbrain, and hindbrain regions of vertebrates [102]. Recent evidence also identifies homology between the main neurosecretory regions of the brains and the hypothalamus-pituitary system of vertebrates [103].

### 1.8.Neurotransmitter Systems

The similarities between the fly and human nervous systems extend also to the main neurotransmitter systems and channels [104], which are the target of many pharmacological interventions relevant to neuropsychiatric conditions.

Acetylcholine is the main excitatory neurotransmitter in the central nervous system (CNS) of flies, in contrast to its more limited role in the mammalian CNS. Glutamate is the excitatory neurotransmitter at the *Drosophila* neuromuscular junction (NMJ), but it has a more limited role in the *Drosophila* brain than in the mammalian CNS. *Drosophila* has a glutamate uptake transporter in glial cells [105] and a variety of ionotropic glutamate receptors [104] that respond to some of the same ligands as mammalian receptors, including N-methyl D-aspartate (NMDA) [106] receptors. Flies also have metabotropic (G-protein-coupled) glutamate receptors that respond to mammalian receptor ligands [107,108].

As in vertebrates, GABA (gamma-aminobutyric acid) is the principal inhibitory neurotransmitter in flies, found throughout the brain. Flies have ionotropic GABA-A receptors, G-

protein-coupled GABA-B receptors, and a vesicular GABA transporter [109]. The pharmacology of these is similar, but not identical, to that of vertebrate receptors. In humans, the dopaminergic system is the target of many addictive substances. *Drosophila* also has a dopaminergic system, comprising over a hundred neurons organized in 15 clusters per adult brain hemisphere [110]. The pharmacology of the *Drosophila* neurotransmitter system is sufficiently conserved to see the effects of substances, including cocaine. *Drosophila* also has a serotoninergic system that shows quite similar, although not identical pharmacological properties to humans [111,112]. With around 40 serotoninergic neurons per brain hemisphere [113], it plays a roles in several behaviours including feeding [114], sleep, and aggression.

*Drosophila* has at least 15 classes of vertebrate-like neuropeptide receptors. Those with potential relevance to neuropsychiatric disorders include galanin, oxytocin/vasopressin, tachykinins, neuropeptide Y (NPY), thyrotropin-releasing hormone (TRH), bombesin/GRP, nociceptin, gastrin/cholecystokinin [115–117]. Many of them found in *Drosophila* may directly influence behaviour. Known examples include *amnesiac* gene coding AMN, a neuropeptide-like mammalian pituitary adenylate cyclase-activating protein (PACAP), involved in both olfactory memory and sleep [118,119]; pigment dispersing factor (PDF) expressed in lateral neurons (LNs) that regulates sleep and circadian rhythms, and neuropeptide Y (NPY), involved in behaviours including tolerance to alcohol and aggression.

*Drosophila* is also an instrumental animal model for the study of neuropsychiatric disorders [120]. The fruit fly shows a variety of sophisticated behaviours, which can be compared to 'simple' human behaviours (that can be altered in neuropsychiatric disorders), including behaviours as fundamental as Pavlovian learning and sleep. The powerful genetic and circuit analysis tools of *Drosophila* allow investigation of the mechanisms of these behaviours and how they can be altered in *Drosophila*. Higher-order human behaviours, which are impaired in some of the most devastating neuropsychiatric disorders, can be also simplistically modelled in flies due to similarity of the neurobiological mechanism between flies and humans [120].

### 1.9.Circadian Rhythms in *Drosophila melanogaster*

Circadian rhythms were first described in *Drosophila* in 1935 by German zoologists, Hans Kalmus and Erwin Bünning. They have been detected in physiological processes, behaviour and in sleep-wake cycle in all animal species, as well as in humans.

*Drosophila* shows many different and easily measurable circadian patterns of behaviour. This clock-controlled behaviour includes eclosion [94], olfactory sensitivity [121], egg laying [122], courtship [123,124], gustatory sensitivity [125], and learning and memory [126]. *D. melanogaster* shows two peaks of activity: in the morning and in the evening. This rhythmic activity persists under constant conditions, however, only with one peak in the subjective evening. Factors such as light and temperature are well known to entrain the *Drosophila* clock [127,128]. Another important reason to study circadian rhythms in *Drosophila* is the simplicity of the organization of neuronal networks that allows easy detection of the function of single cells or clusters in the brain. These advantages have led scientists to study the circadian clock of *Drosophila.*

At the beginning of the 1970s, Seymore Benzer and his student Ron Konopka were screening flies with abnormal circadian behaviour [94]. They isolated strains that showed long (29 h) or short (19 h) period (29 h) of eclosion or were arrhythmic. They found that all three fly strains had a mutation in the same gene locus, located on the X chromosome. This led to the discovery of the first 'clock gene' [94]. It was called *period*, and the mutants were designated *period* Long, *period* Short, or *period* 01. The *period* gene is also well conserved in mice, zebrafish and humans [129–132]. After a decade, researchers discovered novel clock genes, including *timeless* (*tim*), *Clock* (*Clk*), *cycle* (*cyc*) and *cryptochrome* (*cry)*. These genes and their product proteins play a key role in the molecular mechanism of the circadian clock [133]. It has been reported that the clock genes *per* and *tim* are important in governing the circadian rhythms of female mating activity in *Drosophila* [134]. Recent studies on clockwork shows that the $per^s$ domain is important in temperature compensation of the *Drosophila* circadian clock [135].

### 1.10. Circadian Clock of *Drosophila melanogaster*

The *D. melanogaster* circadian clock consists of 150 neurons. These clock neurons are divided into seven main groups, named after their anatomical position [**Fig. 1.4**]. Three neuronal groups located dorsally are known as dorsal neurons 1–3 ($DN_{1–3}$), the other four groups that are located laterally are known as lateral neurons dorsal (LNd), large ventral (l-LNv), posterior (LPN), and small ventral (s-LNv). Furthermore, there are a few hundred glial cells expressing clock proteins such as PER and TIM in the fly's brain [136–138]. The clock neurons are further subdivided into different subgroups according to their protein content, size, and/or function. DN1 cells consist of about 16 cells. Two of those cells, DN1a, do not express the transcription factor TIM, but they

express the neuropeptide IPN-amide and the blue light photoreceptor cryptochrome [139]. Cryptochrome is expressed in two to six other DN1 cells that are located more posteriorly, namely the DN1p [138]. With only two cells, the $DN_2$ cluster is the smallest among clock neurons, while the 40 $DN_3$ neurons form the largest group. Again, $DN_3$ neurons show a variety of cell body sizes and can also be subgrouped [127].

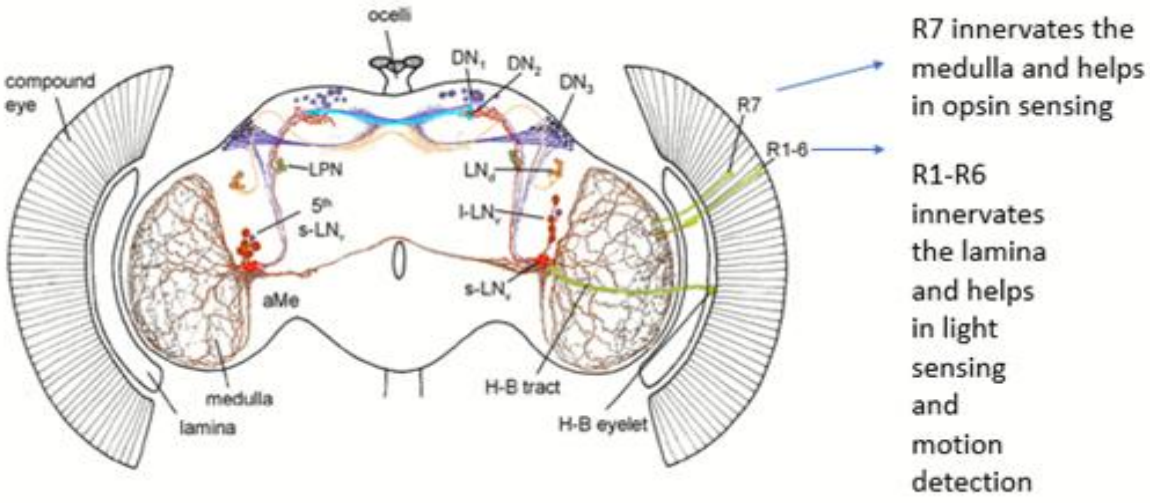


**Fig. 1.4.** Diagram of the *Drosophila* brain displaying the major clock cells and their arborizations. The major classes of photoreceptors, the R1-6, R7/8, and Hoffbauer-Buchner (H-B) cells of the compound eye and the eyelet respectively, act as light inputs [136].

LNvs function as M (morning) oscillators, while the LNds and DNs function as E (evening) oscillators [140,141]. Further studies restricted the location of the M oscillator to the s-LNvs and revealed that the fifth, PDF negative, 5th s-LNv is a part of the E oscillator [141,142]. s-LNvs are the main circadian pacemaker cells because they are mandatory to maintain rhythmic locomotor behaviour in constant darkness (DD).

About four l-LNvs and four of the five s-LNvs express the neuropeptide pigment-dispersing factor (PDF). The fifth s-LNv is close to the l-LNvs and lacks PDF [142,143]. The LNds are located more dorsally. This heterogeneous cell group comprises six cells, all expressing different neurotransmitters, such as acetylcholine and ion transport peptide, the long or short form of neuropeptide F [144]. The last lateral neuronal group is the LPN (lateral posterior neuron) group.

These neurons seem to be closely connected to the temperature entrainment of the circadian clock [138,139,145].

## 1.11. Molecular Mechanism of the Circadian Clock

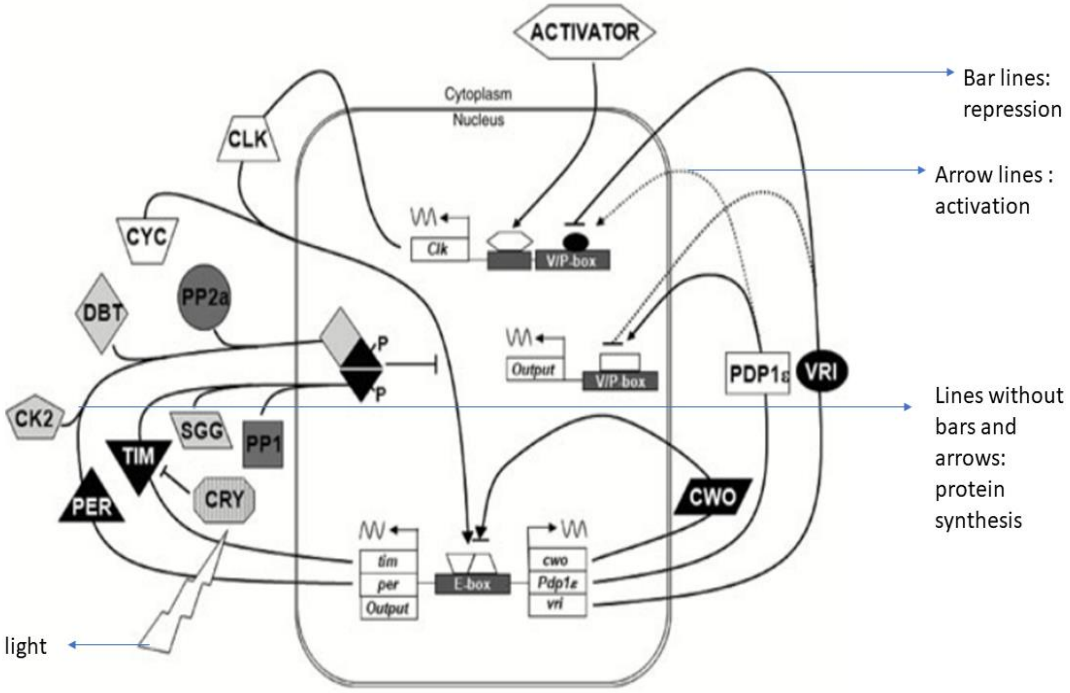


**Fig. 1.5.** Transcriptional feedback loops of the circadian clock in *Drosophila* [146]. Genes are depicted in black. V/P- boxes and E boxes are depicted in grey. The lightning bolt shows blue light activation of CRY.

In *D. melanogaster,* circadian genes and proteins produce the 24-hour cycle of rest and activity. The synchronization of the clock is caused by light perceived by the compound eyes, ocelli, Hofbauer-Buchner eyelets (HB eyelets) and cryptochrome (CRY), the circadian photoreceptor of the fruit fly. The core clock mechanism comprises two interdependent feedback loops [**Fig. 1.5**], namely the PER/TIM loop and the CLK/CYC loop [127].

## 1.12. PER-TIM Loop

In the *per/tim* loop, the *per*, *tim* and *clockwork orange* (*cwo*) transcription is activated from ~ZT4(middle of the day) to ~ZT16(later part of the night) by CLK-CYC, a heterodimeric basic

helix loop helix (bHLH) / PAS (PER-ARNT-SIM) protein complex that binds E-boxes in the *per*, *tim*, and *cwo* promoters [147–152]. PER and TIM proteins begin to accumulate at ~ ZT12, approximately 6 – 8 h after their respective mRNAs. This delay is due to phosphorylation-induced destabilization of PER by DBT kinase (double-time; a homolog of mammalian casein kinase 1e) that remains bound to PER [153–155]. PER-DBT then binds TIM to form DBT-PER-TIM complexes that accumulate in the cytoplasm [156–158]. Phosphorylation of PER by casein kinase 2 (CK2) and TIM by SHAGGY (SGG), a homolog of mammalian glycogen synthase kinase 3, promotes nuclear localization of PER-DBT and TIM. PER and TIM phosphorylation are counterbalanced by PP2a- and PP1-mediated dephosphorylation, respectively, which stabilize PER and TIM and alter their nuclear localization [69,159].

### 1.13. CLK-CYC Loop

#### (a) Repression of CLK-CYC- mediated transcription

Once in the nucleus, the PER-TIM-DBT complexes (or the PER-DBT complexes) then bind to CLK, promote DBT-dependent CLK phosphorylation, and release of CLK-CYC from E box to inhibit transcription of *per* and *tim* from ~ZT16 to ~ZT4 [160–163]. This PER-mediated repression is reinforced by the binding of CWO (clockwork orange) to E1, to displace CLK-CYC [164–167]. Once CLK-CYC is released from E1, H3K9 acetylation and H3K4 trimethylation of *per* and *tim* promoter sequences decrease in concert with reduced transcriptional activity [168]. The binding of CLK-CYC to E1 E-boxes initiates activation of target gene transcription [150,163]. CLK-CYC binding promotes chromatin modifications, including the acetylation of histone H3 lysine 9 (H3K9) and the trimethylation of histone H3 lysine 4 (H3K4), which in turn enhance the binding of transcription machinery and activate transcription [168].

#### (b) Reactivation of CLK-CYC-dependent transcription

As light is on at ZT0 /the beginning of the day, the blue-light photoreceptor CRY binds to TIM and promotes its proteasome-dependent degradation [169–173]. This light-dependent degradation of TIM accounts for phase advances and delays that reset the oscillator to local time [158,174–176]. Progressive phosphorylation of PER by DBT in the nucleus ultimately triggers its degradation in the proteasome by ~ZT4 [155,177,178]. PER degradation is triggered by DBT-dependent

phosphorylation of PER S47, which is bound by the SLIMB F box protein and targeted to the ubiquitin-proteasome pathway [177,178]. Once PER is degraded, hypophosphorylated CLK accumulates and displaces CWO from E box to initiate another cycle of *per*, *tim* and *cwo* transcription. The CLK/CYC also controls rhythmic transcription of *vrille* (*vri*) and *PAR domain protein1ε* (*Pdp1ε*), which control cyclical expression of *Clk* [149,179,180], and rhythmically expressed output genes, the so-called *clock-controlled genes* (*ccg*) [164,166,181]. In the *Clk* feedback loop, CLK-CYC activates *vri* transcription between ~ZT4 and ZT16 by binding to E box of the *vri* promoter [148,182]. The VRI protein accumulates in phase with the *vri* mRNA, finally reaching a peak of abundance at ~ZT14. As VRI level rises, VRI binds to VRI/PDP1*ε* box (V/P box) of the *Clk* promoter, thereby repressing *Clk* transcription [182,183]. As PER-TIM-DBT complexes feedback to inhibit CLK-CYC-dependent transcription from ~ ZT16 to ZT4, *vri* mRNA and protein decline to low levels. The loss of VRI-dependent repression allows activation of *Clk* transcription by PDP1*ε*, and *Clk* mRNA rises to high levels during the late evening and early morning. Once PER/TIM complexes are degraded during midday, the next cycle of *vri* transcription and VRI-dependent repression is initiated.

### 1.14. Entrainment of the Circadian Clock

The circadian clock is entrained by several input factors, known as the '*Zeitgebers*', that synchronize the clock to daily changes in the environment [184]. These factors involve light [127,185,186], temperature [187], social cues [188], or even magnetism [128].

The clock responds to light in two ways, the first through the visual pathway mediated by the light-sensitive proteins in the eye called rhodopsins (mainly rhodopsin Rh1 in R1-R6 photoreceptors is involved in the entrainment of the clock), which are crucial in activating the M (morning) and E (evening) oscillators [189]. The morning oscillator (M) is responsible for the morning peak of activity and is accelerated by light. The other oscillator – the evening oscillator (E) – is inducing the evening peak of activity and is slowed by light. The M oscillator cells maintain circadian rhythms under DD conditions and during short winter days, while the E oscillator cells maintain circadian rhythms in LL and under long summer days.

The second way of entrainment is through the blue-light sensitive cryptochrome (CRY). The CRY protein is a circadian photoreceptor that sets the circadian clock phase in response to light.

It is present in the compound eyes of flies and in the clock neurons of the brain [139,190]. Once activated by light, CRY causes degradation of TIM. At the beginning of the night, a light pulse reduces the TIM level and takes the clock backwards (phase delay), while at the end of the night, a light pulse reduces the high TIM level in the nucleus and takes the clock forwards (phase advance) [158,174,176].

In the fruit fly, temperature entrains the clock under constant darkness or constant light conditions. Until now, two genes have been known to influence circadian temperature reception. These are the *norpA* (*no receptor potential*) gene [191] and *nocte* [192]. It has also been found that sensory organs called the chordotonal organs are involved in temperature entrainment [193]. These organs send information directly to peripheral clock neurons or clock neurons in the brain.

Social cues can reset the circadian rhythms in *D. melanogaster*. Wild-type fruit flies transmits and receives cues (olfactory signals) which influences the circadian time [188]. The endogenous circadian clock is highly influenced by magnetic fields. Recent work shows that circadian periods get shortened in *Drosophila* when exposed to a magnetic field [194]. This sensitivity to magnetic field is due to the light activation of the CRY [129]. CRY is considered as an important circadian photopigment in both mammals [195] and flies.

## 1.15. The Role of Circadian Clock in Regulating the Presynaptic Active Zone Protein Bruchpilot in the *Drosophila* First Optic Neuropil

The visual system both in the larval and adult stages is known to exhibit plasticity. Synaptic plasticity is brought about by external stimuli from the environment and by internal stimuli [196,197]. The visual system of *Drosophila* consists of the retina and optic lobes. The optic lobe is composed of the lamina, medulla, lobula, and lobula plate. The first neuropil (lamina) of the fly's optic lobe provides a good system for studying various processes in the nervous system, including synaptic plasticity. The lamina is built of synaptic units called cartridges, comprising photoreceptor terminals and other type of neurons. The cartridges are ensheathed by epithelial glial cells, which like all glial cell subtypes in the visual system, are characterized by the expression of the *reversed polarity* gene (*repo*) encoding a paired-like homeodomain protein [198–200].

The retina consists of 750 ommatidia. Each ommatidium is composed of eight photoreceptors (R1 to R8). R1-R6 innervate the lamina layer, while R7 and R8 innervate the medulla layer. R1 to

R6 are responsible for light and motion detection, R7 expresses UV-sensitive opsins, and R8 expresses blue and green-sensitive opsins [201]. R8 is also believed to be involved in the colour detection function.

The lamina, or the first optic neuropil receives light signals from the retina and processes them before sending them to the medulla. In the lamina, most of the numerous synapses are tetrad synapses formed between the photoreceptor terminals and four lamina post-synaptic cells. Two of these, L1 and L2 monopolar cells, are known to exhibit circadian morphological plasticity. They change their diameter twice during the day in *Drosophila*, i.e., at the beginning of the day and at the beginning of the night [202,203]. They also change their shape from conical to cylindrical. These changes are visible in both LD (light dark) 12:12 and DD (constant darkness). The morphological changes seen in L1 and L2 during the day/night cycle seem to mirror the daily pattern of locomotor activity of *Drosophila* [204].

These changes also suggest that this type of morphological plasticity is generated by the circadian clock. In constant light (LL) the axon size of both monopolar cells (L1 and L2) is larger, while in DD (constant darkness) the axon size is smaller than in LD12:12. The changes in the size of neurons are offset by three epithelial glial cells that surround the cartridge [204]. In *Drosophila*, L1 and L2 are responsible for the differences in diameter of the distal and proximal parts of lamina cartridges. The diameter of the proximal cartridges has been observed to be smaller than distal ones [205]. Not only the axons, but also the dendrites and nuclei of L2 are known to exhibit circadian plasticity [206,207]. At the beginning of the day, when flies are highly active, the L2 dendrites increase in size, while during the night they shrink [207]. Apart from tetrad synapses, the feedback synapses formed between L2 and the photoreceptor terminals [208], are also plastic, changing in number in LD12:12 and DD [209]. During periods of rest, they modulate the photoreceptor activity and increase their sensitivity under low light conditions [210,211].

The *Drosophila* synapses consist of a pre-synaptic element called T-bar which is made up of proteins and one of them is the scaffolding protein Bruchpilot (BRP). BRP is homologous to the mammalian ELKS/CAST family of synaptic proteins [212] that facilitate efficient vesicle release [213]. The T bar is composed of two BRP isoforms, 190 kD (BRP-190) and 170 kD (BRP-170) [212]. Both of them are recognized by the anti-BRP monoclonal antibody NC82 [214]. This protein is known to exhibit circadian plasticity. In WT flies the level of BRP oscillates during the day with two peaks,

at the beginning of the day and at the beginning of the night [215,216]. This oscillation resembles the bimodal locomotor activity pattern seen in *Drosophila,* with a low morning and a high evening peak of activity [187]. The morning peak of BRP is under the control of light, whereas the evening peak is under the control of the circadian clock. During the night, BRP expression depends on PER and TIM proteins [216], while during the day on CRY protein. CRY regulates synaptic plasticity in the visual system. It is light sensitive and during the early morning it binds to TIM and BRP resulting in ubiquitination and subsequent degradation [217].

### 1.16. Sleep in *Drosophila*

*Drosophila* has previously contributed to understanding the conserved mechanism of sleep regulation in mammals and flies [89,91,218,219]. In *Drosophila,* sleep is regulated by two mechanisms: circadian and homeostatic [220]. Sleep timing is regulated by the circadian clock, while homeostatic regulation helps sleep-deprived flies for sleep rebound which is crucial for restorative functions.

Sleep is sexually dimorphic; males sleep more than females, especially during the day [221–223]]. Interestingly, sleep is also present in larvae and is important for neurogenesis [224]. Being a diurnal species, *Drosophila* sleeps primarily at night [225]. Total sleep in flies refers to sleep that occurs during the light and dark periods [226].

Nighttime sleep is defined as a state of 5 minutes of inactivity [89,91] characterized by an increased arousal threshold. Nighttime sleep is governed by the circadian clock [227,228]. During warm summer temperatures, *Drosophila* also takes mid-day naps during the day called *siesta*. It is an adaptive response to minimize exposure to heat. This behavioural plasticity is not governed by the circadian clock [229]. However, it is partly governed by the thermal sensitive splicing of the 3'-terminal introns found in the key circadian clock gene [230–232]. The splicing of this intron (called dmpi8; *D. melanogaster per* intron 8) is progressively more efficient as daily temperatures decrease, leading to an increase in *per* mRNA levels [230].

The fly's brain contains many sleep-regulating centers [**Fig. 1.6**]. The important sleep centers are confined to the dorsal and central regions of the brain and involve mushroom bodies (MB) and the central complex (CC). The CC is composed of four interconnected substructures, the protocerebral bridge (PB), the fan-shaped body (FB), the ellipsoid body (EB), and the noduli (NO), and is a higher center that controls locomotor behaviour [226,233–236].

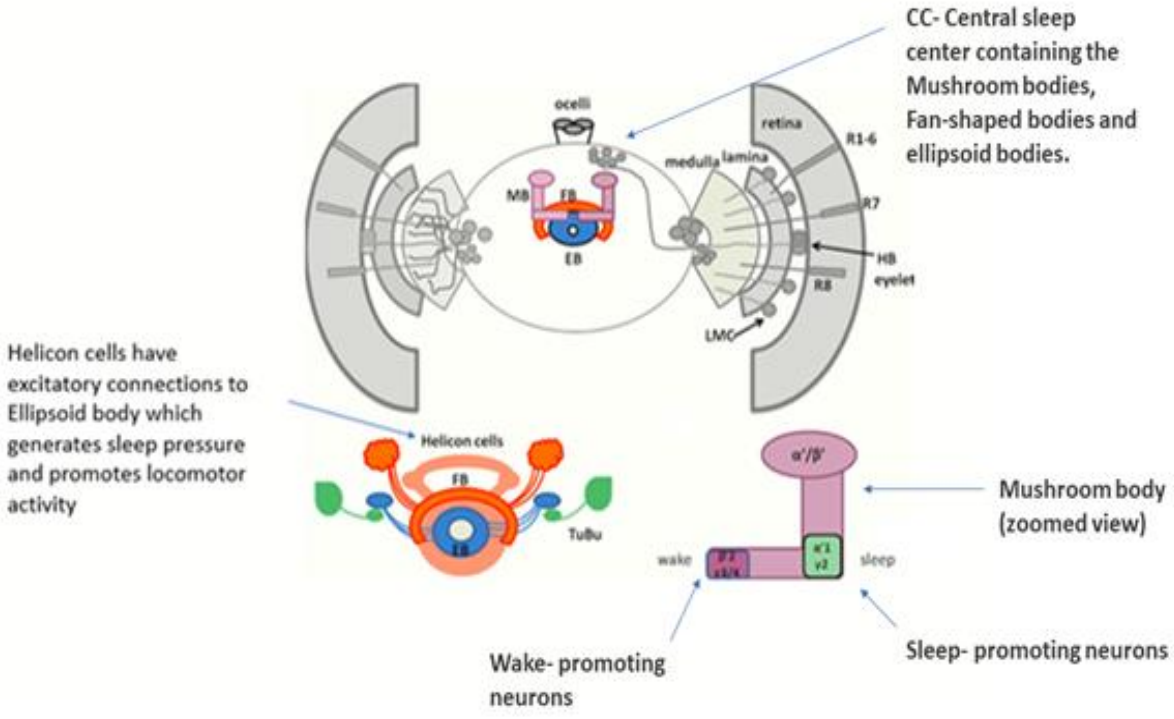


**Fig. 1.6.** A schematic representation of *Drosophila* sleep centers and structures mediating light signals in *Drosophila* brain [237]. Purple colour shows the mushroom body (MB), green colour shows the Tubercular bulbar neurons (TuBu).

The mushroom bodies were first identified in 2006 [233,234]. They are essential bilateral structures in the fly brain involved in learning and memory [238]. In mushroom bodies, Kenyon cells (KCs) and mushroom body output neurons (MBONs) promote sleep, while dopaminergic neurons (DAs) participate in wake promotion [239,240].

In *Drosophila*, clock neurons expressing the Pigment-Dispersing Factor (PDF) peptide (lateral neurons) [241] play an essential role in controlling circadian rhythms in behavior [140,141] and in promoting wake [242–244]. Another group of clock neuron involved in regulating sleep are dorsal neurons. They form synapses with tubercular-bulbar neurons [245,246].

In the brain of *Drosophila*, the fan-shaped body (FB) participates in the homeostatic regulation of sleep [247]. The dorsal FB (dFB) neurons form inhibitory synapses with helicon cells [248] that control the homeostatic sleep drive [226].

The activation of these neurons helps to convert short-term memory (STM) to long-term memory (LTM) [249]. dFB neurons are modulated by dopaminergic inputs [250–253]. Dopamine causes these neurons to switch ON and OFF [252]. This switching mechanism is quite similar to that observed in humans which resembles sleep and wakefulness [254]. Sleep deprivation increases the excitability of dFB neurons [249].

Other regions that regulate sleep in *Drosophila* involve the pars intercerebralis (PI), a neuroendocrine center in the fly's brain [255,256]. The PI is connected to the clock network and is an important component of the circadian output pathway for the rest/activity rhythm [257]. In addition, dorsal-paired medial (DPM) neurons, which innervate the MB, are involved in sleep promotion [258]. Even the glia is involved in sleep regulation in *Drosophila* [259–264].

Sleep is crucial in improving fitness [265] and immune functions [266], but at the same time, it can be a costly behavioural state since resting flies engage less in other activities such as foraging and mating.

Sleep is also affected by several factors like temperature, food, age, mating, social experience, and light. High temperature (29°C) can reduce day sleep, a phenomenon termed prolonged morning wakefulness (PMW) [246], while low temperature results in increased sleep during the day and reduced sleep during the night [231,267,268].

Feeding is correlated with sleep since food deprivation or hunger can cause sleep deprivation. In starvation, flies show reduced sleep [269]. DAs are regulated in response to both starvation and ingestion of high concentrations of sucrose (1 M), which can promote arousal and suppress sleep [270,271]. Feeding is completely under the control of the circadian clock in both flies [271] and mammals [272].

Sleep is also related to dietary composition. Proteins, salt, and sucrose can all promote post-prandial sleep [271]. Postprandial sleep is regulated by the leucokinin receptor (Lkr) neurons, which arborise in the suboesophageal ganglion (SOG), in the lateral horn (LH) and the fan-shaped body (FB), areas of the brain known to regulate feeding, processing of olfactory information, and control of sleep, respectively.

Another group of neurons called the allatostatin A-positive (AstA) neurons located in the postero-lateral protocerebrum (PLP) are known to promote sleep [273]. Gustatory information is also important in sleep/wake behaviour. The gustatory receptor neurons (GRNs) can help flies to detect food substances like sugar, amino acids, bitter-tasting substances or dangerous plant metabolites. These neurons project to the superior medial protocerebrum (SMP) [274], an area where many sleep-related neurons arborise [239,275]. Lack of GRN stimulation can suppress sleep during starvation. For example, flies with impaired sugar sensing show increased locomotion [276] and sleep suppression [277] when fed arabinose (a non-nutritive sugar). While activation of sweet GRNs by TRPA1 was sufficient to induce sleep in starved flies [277,278].

### 1.17. Neurotransmitters in Sleep

In *D. melanogaster* seven neurotransmitters are involved in the sleep/wake regulation. The neurotransmitters that promote sleep are serotonin and gamma-aminobutyric acid (GABA) and the wake promoting neurotransmitters are dopamine, octopamine, and histamine. While some neurotransmitters like acetylcholine and glutamate have dual roles as a sleep promoter and inhibitor.

### 1.18. Caffeine and Sleep

The structure of caffeine was described near the end of the 19th century by Hermann Fischer. It is similar to that of adenosine. Caffeine is metabolized in the liver by the cytochrome P450 oxidase enzyme system into three dimethylxanthines: **paraxanthine**, which increases lipolysis, leading to elevated levels of glycerol and free fatty acids in blood plasma; **theobromine**, which dilates blood vessels and increases urine volume; and **theophylline**, which relaxes smooth muscles of the bronchi, and is used to treat asthma. Caffeine is a toxic plant alkaloid. It has a bitter taste and is considered toxic to insects. For example, it has been shown to cause the mortality of *Drosophila* larvae [279]. Despite this, caffeine is one of the most commonly used psychoactive stimulants in the world. It has both positive and negative effects. Caffeine not only promotes arousal, it also promotes alertness after sleep loss [280] and helps improve mood in humans [281]. Apart from this, caffeine has some known negative effects on humans ranging from insomnia, nausea, and heart palpitations. It is also known to influence metabolic rate [282,283], locomotor activity [284–286] and

learning abilities [287,288]. Caffeine also influences the circadian clock and is known to cause lengthening of the circadian period in humans [289], rodents [290,291], and *Drosophila* [292].

Since caffeine is popular for causing sleep loss, its effect on sleep has been widely studied in humans, rodents, and *Drosophila.* Caffeine consumption during the day causes a reduction in 6-sulfatoxymelatonin (the main metabolite of melatonin) at night, which is one of the mechanisms by which sleep is interrupted [293]. Caffeine has several cellular targets: ryanodine receptors, GABA receptors, glycine receptors and phosphodiesterase, which could be related to its effect on sleep [294]. However, its most important biological targets are adenosine receptors. This is because caffeine attaches to the same receptors to which adenosine would normally attach, and it causes removal of the adenosinergic tonus, thereby becoming an adenosine blocker.

Caffeine acts on both A1AR and A2AR and has different affinities for different adenosine receptors. Therefore, caffeine produces distinct impacts on tissues depending on the level of expression and the type of adenosine receptor. Despite the highest affinity of caffeine for the adenosine A2A receptor, the most prominent acute effects of caffeine are attributed to the antagonism of the adenosine A1 receptor. However, chronic caffeine consumption results in tolerance of adenosine A1 receptors to caffeine (in this condition the effects of caffeine on adenosine A1 receptors are negligible) and its action on adenosine A2A receptors becomes predominant [295,296].

Several studies on vertebrates have attempted to encode the role of caffeine in sleep. Studies on caffeine ingestion and nighttime sleep loss in *Drosophila* have also been studied in detail. The use of *Drosophila* in caffeine studies has been possible mainly due to a single adenosine receptor that is 30% similar to the mammalian A2AR. Due to a single receptor, chances of observing abnormalities of sleep upon caffeine treatment become clear and high. The bitter taste of caffeine [297], which is recognized by gustatory receptors (Gr33a, Gr66a, and Gr93a), can suppress appetite [298] in flies, leading to sleep loss [299,300].

Sleep loss at night can be independent of adenosine receptors [292]. *Null* mutants of *dAdoR* have been found to show less nighttime sleep (similar to controls), confirming that the adenosine receptor in the fruit fly is not involved in the wake-promoting effect of caffeine [292]. This wake-up promotion is facilitated by dopamine neurons.

In mice lacking the A2A receptor, caffeine does not increase wakefulness [301]. However, to date, no study has tried to decode this mechanism by which caffeine exerts its action. These differences in the effect of caffeine on vertebrates and invertebrates point to the possibility of an underlying mechanism being highly different between mammals [301,302] or other vertebrates [303] and fruit flies. The A1AR plays a role in sleep deprivation while A2AR in sleep promotion. For example, in rat's injection of the A2AR agonist in ventral striatum promotes slow-wave sleep (SWS) [304]. A2AR inhibits histaminergic neurons [305], causes excitation of sleep-active neurons in the ventrolateral preoptic nucleus [306], and modulates the release of acetylcholine in the pontine reticular formation [307], causing increased SWS and REM sleep.

Fruit flies subjected to different doses of caffeine show changes in locomotor activity and nighttime sleep. The effects are more pronounced in female flies. In wild-type and transgenic flies lacking functional dAdoR, caffeine is known to inhibit night sleep. It has been shown that wake promotion is independent of a functional AdoR and caffeine acts through the cAMP/PKA pathways antagonizing PDE (phosphodiesterase) [292].

Adenosine, in contrast, is a well-known sleep promoter. Increased levels of adenosine can increase sleep. Ado (adenosine) kinase, deaminase, and transport inhibitors decrease wakefulness and increase sleep [308–310]. Studies have shown that the use of an adenosine agonist can increase SWS [311] while the use of an adenosine antagonist decreases sleep and promotes wakefulness [304,311].

Although sleep is quite conserved between fruit flies and mammals [91,218,219,312], low conservation of neuropeptides and molecular pathways responsive to caffeine are not conserved in flies [292,313–315]. Unfortunately, we are still behind in fully understanding the mechanism of *AdoR* signalling in flies treated with caffeine.

Hence, the use of high-throughput genetic tools and screening techniques, as well as the development of a more selective antagonist that can cause a complete blockade of adenosine, will help us to understand the molecular pathways responsive to caffeine and to understand its role in the daytime and nighttime sleep.

## 1.19. The Aim of the Thesis

After the discovery of a single adenosine receptor in *Drosophila melanogaster* (dAdoR), the opportunity appeared to further study the function of adenosine by using this powerful model. The functions of AdoR in *Drosophila* are not yet known; therefore, my study aimed to examine the influence of overexpression or silencing of *dAdoR* in photoreceptors, neurons, and glial cells, on flies' survival, fitness, daytime (*siesta*) and nighttime sleep, and locomotor activity.

As functioning of synapses is crucial to maintain fitness, longevity and behaviour in *Drosophila melanogaster*, the second objective of the thesis was to check the daily pattern of expression (the daily level) of the presynaptic active zone protein Bruchpilot (BRP) in flies with silenced expression of *dAdoR*. The abundance of BRP in the photoreceptor terminals of *Drosophila* first visual neuropil or lamina is well known to change in a circadian manner. I wanted to know whether lower expression of *dAdoR* can alter the BRP level and circadian pattern of changes in BRP abundance.

Adenosine is an important sleep-promoting agent in mammals. Hence, I wanted to check whether it plays a similar role in *Drosophila,* which in such a case could be a very convenient model for studying this phenomenon. As sleep in both mammals and *Drosophila* is influenced by the same stimulants and hypnotics, I used caffeine, a major biological target of the adenosine receptor in the study. The objective of this part of the study was to check age-dependent changes in sleep of wild-type (WT) flies after feeding them with caffeine, and its (caffeine) influence on daytime (*siesta*) and nighttime sleep, as well as the circadian clock, after overexpression or silencing of *dAdoR* in transgenic flies.

## 2. MATERIAL AND METHODS

**Table 2.1** shows the equipment used in the research.

**Table 2.1. Technical Equipment**

| Instrument | Manufacturer |
|---|---|
| Cryostat LEICA CM1850 UV | Leica Biosystems, USA |
| Eppendorf Centrifuge 5424 R | Eppendorf AG, Germany |
| Digital Dry Bath Incubator MD-01N | Major Science, USA |
| Invitrogen Sample mixer (Hula Mixer) | Thermo Fisher Scientific, USA |
| The LSM 780 Laser Scanning Confocal Microscopy | Carl Zeiss, Germany |
| GmCLab Fixed speed Mini Centrifuge | Gilson, UK |
| Classic Advanced Vortex Mixer | VELP Scientifica, Italy |
| LED 60-TB Compound Microscope | Motic, Europe |
| MOV-112S Sterilizer | Sanyo, Japan |
| Versatile Environmental Test Chamber (MLR 351) | Sanyo, Japan |
| RADWAG Weighing Balances | USA |
| Consort High tech pH meter C830 series | Daihan Scientific, Korea |
| IKA RH Basic 2 Magnetic Hot Plate Stirrer | Akribis Scientific Limited, UK |
| Thermolyne Roto-Mix 50800 orbital shaker | Thermolyne, Canada |

**Table 2.2** shows the chemicals and reagents used in the research.

**Table 2.2. Chemicals and Reagents**

| **NAME** | **MANUFACTURER** |
|---|---|
| CRYOMATRIX | Thermo scientific, USA |
| CAFFEINE | Sigma-Aldrich, China |
| VECTASHIELD | Vector Laboratories, USA |
| BSA (BOVINE SERUM ALBUMIN) | Sigma-Aldrich, USA |
| $Na_2HPO_4$ | POCH, Poland |
| $NaH_2PO_4$ | POCH, Poland |
| SUCROSE | Sigma-Aldrich, USA |
| NaCl | POCH, Poland |
| GELATIN FROM PORCINE SKIN | Sigma-Aldrich, USA |

## 2.1. *Drosophila* Maintenance

**Table 2.3** shows the ingredients used to prepare the standard cornmeal medium. All fly stocks were raised in this standard cornmeal medium. For all the experiments the flies were kept at 25°C and 60% RH (relative humidity) in LD 12:12 light-dark cycle.

**Table 2.3. Medium for *Drosophila* culture**

| Ingredients | Proportion |
|---|---|
| Water | 2000 ml |
| Honey | 100 ml |
| Agar | 10 mg |
| Molasses | 50 ml |
| Baker Yeast | 14 mg |
| Ethanol | 30 ml |
| 10% p-Hydroxy-benzoic acid methyl ester | 3 mg |
| Cornmeal | 120 mg |

## 2.2.Fly Strains and Crosses for Behavioural Experiments

For experiments, we used Canton-S wild-type flies and the following transgenic strains: *GMR-Gal4w*$^{1118}$; *P{GMR-GAL4.w-}2/CyO*, kindly received from the laboratory of Prof. Ralf Stanewsky, *elav-Gal4, P{GAL4-elav.L}2/CyO*, from Bloomington Drosophila Stock Center, No 8765 [316], *repo-Gal4* (*w*$^{1118}$*; P{Gal4}) repo-/TM3,Sb*$^{1}$, from Bloomington Drosophila Stock Center, No 7415 [317], *UAS-AdoR* (kindly received from the laboratory of Dr. Eva Dolezelova [82]), *UAS-VALIUM10*, *P {UAS-GFP.VALIUM10} att P2;* from Bloomington Drosophila Stock Center (35786) [318], and two strains of *UAS-AdoR*$^{RNAi}$ (1386 and 1385 from Vienna Drosophila Resource Centre; VDRC).

I targeted the retina photoreceptors, all neurons and glial cells using *GMR-Gal4*, the pan-neuronal *elav-Gal4* and the pan-glial *repo-Gal4* drivers, respectively. For overexpression experiments, these driver lines were crossed with the *UAS-AdoR* strain or with Canton-S to obtain a control group of individuals. For silencing experiments, the driver strains were crossed with the *UAS-AdoR*$^{RNAi}$ strains or with the *UAS-VALIUM* to obtain a control group of individuals. Experiments were carried out on males and females of the first generation (F1) of these crosses.

### 2.3. Fly Strains and Crosses for BRP Measurements

I used genetic crosses between *GMR-Gal4* and *UAS-AdoR*$^{RNAi}$, as well as *repo-Gal4* and *UAS-AdoR*$^{RNAi}$ to study the effect of silencing of *dAdoR* in the eye photoreceptors and lamina glia. To generate genetic controls, I crossed *GMR-Gal4* with *UAS-VALIUM* and *repo-Gal4* with *UAS-VALIUM*. F1 male flies (approximately 7-10 days old) from the crosses were collected and kept under LD 12:12 and DL 12:12 conditions for 1 week and decapitated at ZT1, ZT4, ZT13 and ZT16, where ZT0 means the beginning of the day in LD 12:12 and ZT12 means the beginning of the night. The flies used for the DL experiments were decapitated in dim red light, using a dissecting microscope equipped with red exit filters on fibre optic light guides [319].

### 2.4. Fly Strains for Experiments with Caffeine

For experiments, I used Canton-S wild-type flies and the following transgenic strains: *elav-Gal4, P{GAL4-elav.L}2/CyO*, from Bloomington Drosophila Stock Center, No 8765 [316], *pdf-GAL4, P{Pdf-GAL4.U}*[241], *tim-Gal4*, *P{Gal4-tim.E}* [320], *th-Gal4, P{ple-GAL4.F}* from Bloomington Drosophila Stock Center [321], *UAS-AdoR* (received from the laboratory of Dr. Eva Dolezelova [82]), *UAS-VALIUM10*, *P {UAS-GFP.VALIUM10} att P2*; from Bloomington Drosophila Stock Center, No 35786 [318]. I targeted all neurons, *pdf*-expressing clock neurons, *tim*-expressing neurons, and *th*-expressing dopaminergic neurons, respectively. These driver strains were crossed with the *UAS-AdoR* strain to overexpress *AdoR* (*elav*/ *pdf*/*tim*/ *th -Gal4>UAS-AdoR*) and with Canton-S flies (*elav*/ *pdf*/*tim*/ *th -Gal4>CS*) to obtain sibling controls verifying genetic background. For silencing studies, the driver strains were crossed with the *UAS-AdoR*$^{RNAi}$ strain (*elav*/ *pdf*/*tim*/ *th -Gal4>UAS-AdoR*$^{RNAi}$) and with the strain *UAS-VALIUM* (*elav*/ *pdf*/*tim*/ *th -Gal4>UAS-VALIUM*) to obtain individuals with silenced expression of *AdoR* and the control, respectively.

### 2.5. Survival Assay

In the survival assay, 1-day-old males and females (n = 30 for each) were placed in vials [**Fig. 2.1**] with cornmeal medium. Dead individuals were counted every day until the end of the experiment when all flies died. To maintain optimal rearing conditions, the flies were transferred to fresh food every two days. It was done without using $CO_2$, which is often used to anesthetize flies but can also affect their activity.

Survival is presented as a Kaplan-Meier curve in which the percentage of survived flies is plotted against their age. The statistically significant differences between the experimental and control groups were analyzed using the log-rank test (Mantle-Cox). The median survival (MS) of each group designates the day that 50% of the flies were dead.

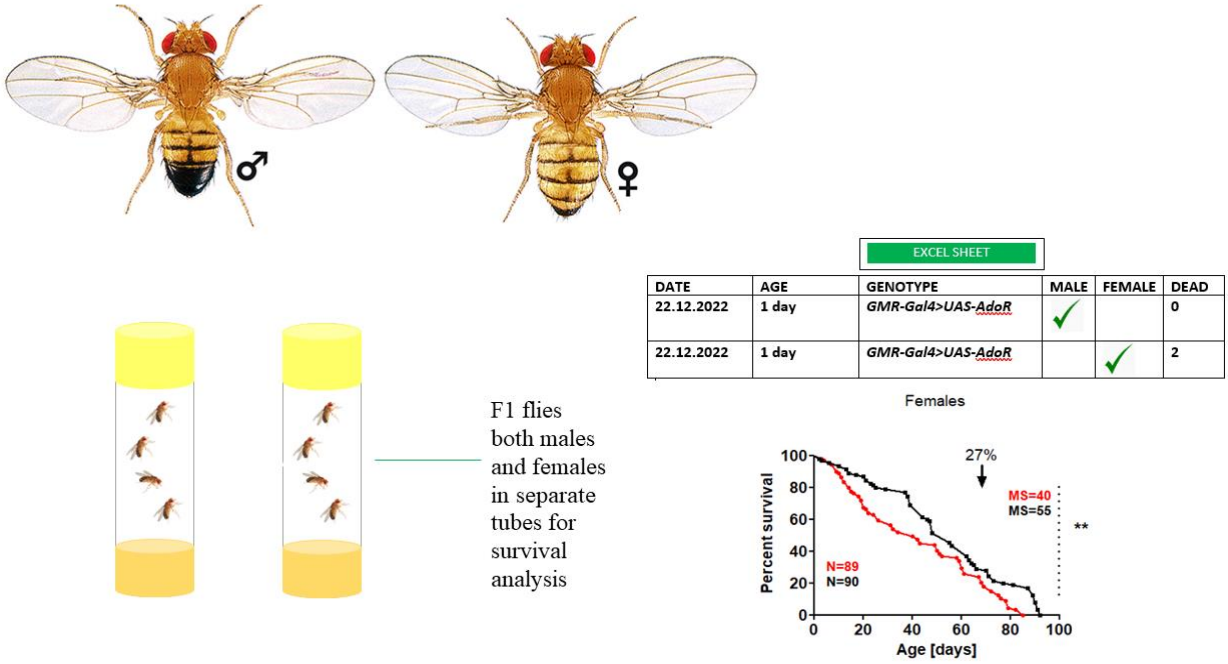


**Fig. 2.1.** Illustration of survival assay for the experimental and control groups.

### 2.6.Climbing Assay

*Drosophila* is naturally inclined to climb against gravity (negative geotaxis). For the geotactic climbing assay [318], which tests the locomotion and fitness of flies, 30 males or females were loaded into 140 ml, empty vials (φ=3.5 cm) with a line marked 5 cm above the bottom of the vial [**Fig. 2.2**]. During the test, the vial was vigorously tapped to force flies to descend to the bottom to subsequently take advantage of their natural tendency to climb upward against gravity. The test was always carried out at the beginning of the day (one hour after the beginning of the light phase in LD12:12, at ZT1) when flies are typically very active. Experiments were carried out in a dark room to avoid increased movement in response to light stimuli or other distractions. The number of flies that climbed above the line in 15 seconds was recorded. The climbing abilities of 7, 14, 30, and 60-day-old flies were evaluated in three trials.

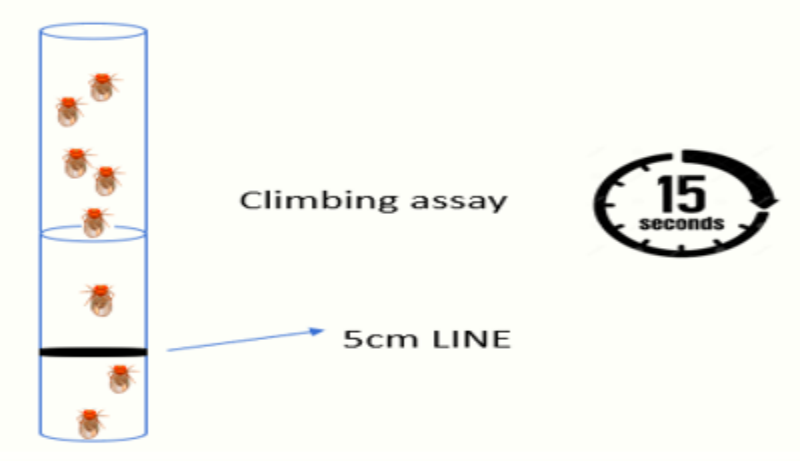


**Fig. 2.2.** Schematic representation of the climbing assay.

### 2.7.Locomotor Activity and Sleep

To measure locomotor activity and sleep, I used the *Drosophila* Activity Monitoring System (DAMS) (TriKinetics, Waltham, MA, USA) [**Fig. 2.3**]. Males 1-2 day old were used for the experiment. Locomotor activity and sleep of flies were recorded for 2 weeks [7 days in LD12:12 and 6 days in constant darkness (DD)] using the *Drosophila* Activity Monitoring System (DAMS) (TriKinetics, Waltham, MA, USA).

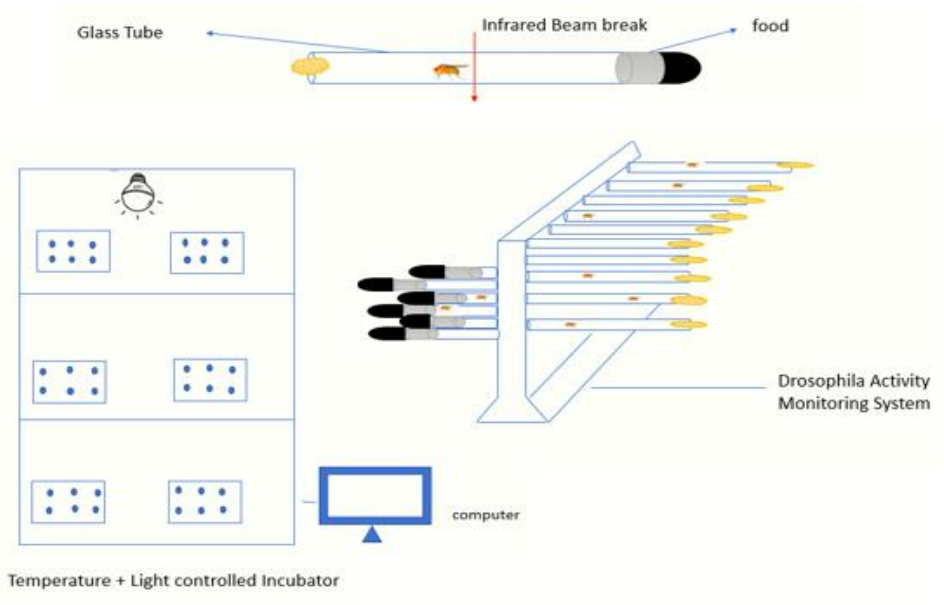


**Fig. 2.3.** Drosophila activity monitoring system.

Each DAM monitor has 32 channels for 32 tubes that hold one fly each. The tubes with fly food at one end was plugged with a plastic plug while the other end was closed using a sponge plug to prevent flies from escaping. The monitors were then housed in temperature, humidity, and light-sensitive incubators for continuous recording of their activity and sleep [323]. The DAM system records infrared beam breaks by flies that walk inside glass tubes. The data from DAMS monitors were periodically uploaded to a computer.

Locomotor activity was continuously recorded, and sleep was checked on the second day of the recording. Since sleep in *Drosophila* is defined as a 5-min period of inactivity [324], I counted the number of 5-min bins per hour of fly immobility for sleep analysis. Graphs for sleep profile show ZT (*Zeitgeber* Time) where ZT0 refers to lights on and ZT12 refers to lights off. Sleep profile shows the amount of sleep averaged for the second day of recording, in 60-min bins. **Table 2.4** shows the agar-sucrose medium in which flies were kept during the experiment.

**Table 2.4. Medium during recording locomotor activity of flies**

| Ingredients | Proportion |
|---|---|
| Water | 100 ml |
| Sugar | 5 mg |
| Agar | 2 mg |

### 2.8.Caffeine Exposure

The desired concentrations i.e., 0.1 mg/ml, 0.5 mg/ml and 1 mg/ml, of caffeine were dissolved in distilled water to make stock solutions and then mixed into the melted agar-sugar food (5% sucrose and 1% agar) used to feed flies during the recording of locomotor activity [**Fig. 2.3**].

### 2.9.Immunohistochemistry

7 to 10-days old males were immobilized with $CO_2$ and decapitated in a drop of fixative; 4% formaldehyde (PFA) in 0.1M phosphate buffer (PB). Flies were decapitated 4 times during the day: at the beginning of the day (ZT1) and the night (ZT13) and in the middle of the day (ZT4) and the night (ZT16) (in LD 12:12, ZT0 denotes the end of the night/the beginning of the day and ZT12 denotes the end of the day/the beginning of the night). For each time point (ZT), 30 flies were sacrificed. Decapitation during the dark part of the cycle, at ZT13 and ZT16, was conducted

in dim red light, using a dissecting microscope equipped with red exit filters on fibre optic light guides [319].

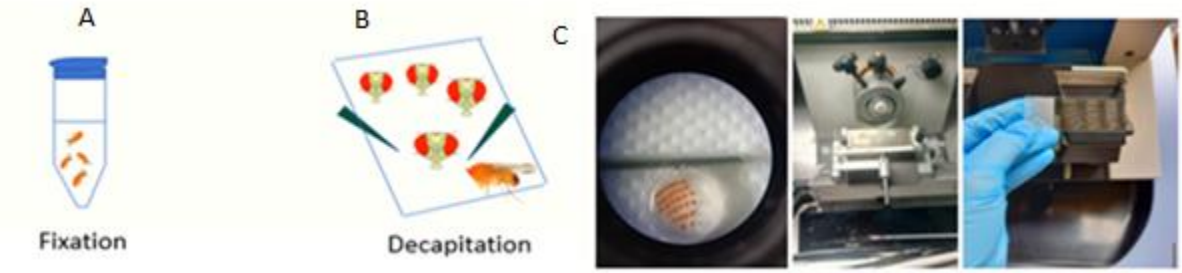


**Fig. 2.4. (A)** Fixation, **(B)** decapitation procedures, and **(C)** Photographs show (from left to right): decapitated and fixed heads arranged in cryomatrix, holder with block of embedded heads inside the cryostat chamber, sectioning of frozen Drosophila heads and arranging sections on slides.

After tissue fixation and cryoprotecting infiltration in a 25% sucrose solution, head cryosections [**Fig. 2.4 (A – B)**] were cut [**Fig. 2.4 C**], washed [**Fig. 2.5**] and incubated with mouse Mab NC82 (Developmental Studies Hybridoma Bank [DSHB], IA) [**Table 2.5**], which recognizes the C-terminus of BRP protein [212,213].

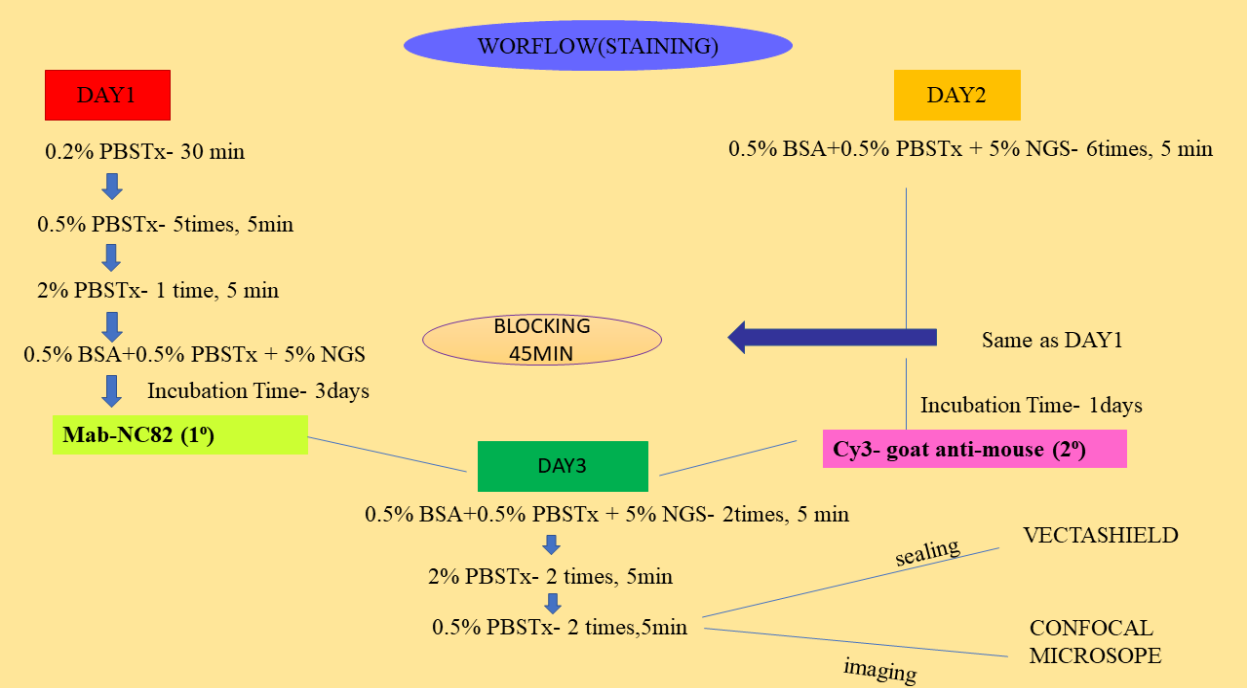


**Fig. 2.5**. Workflow staining chart showing the steps of washing for three different days along with antibody incubation.

After several washes in 0.1M sodium phosphate buffer saline (PBS) [**Fig. 2.4 (C)**] containing 0.02% Triton-X (Sigma), sections were incubated with goat anti-mouse Cy3 [**Table 2.5**] conjugated secondary antibodies (Jackson ImmunoResearch Laboratories).

**Table 2.5. Antibodies used for immunohistochemistry**

| Antibody Type | Dilution | Host Species | Manufacturer |
|---|---|---|---|
| Primary antibody Mab NC82 (anti-Bruchpilot) | 1:10 | Mouse | DSHB Hybridoma USA |
| Secondary antibody | 1:500 | Goat | Jackson Immuno Research Laboratories USA |

The preparations were examined using a Zeiss LSM 780 Meta confocal microscope after final washing and mounting in Vectashield medium (Vector).

## 2.10. Immunoquantification

Images of the first optic neuropil (lamina) from different ZTs, showing cartridges in the longitudinal sections, (7 – 10 images/individual per ZT) were collected using identical image acquisition parameters.

The fluorescence intensity of the BRP-specific immunolabeling (brightness) in the distal and proximal parts of 7 – 10 lamina cartridges per individual [**Table 2.6**] was evaluated using Image J software (NIH, Bethesda) as the Mean Grey Value that corresponds to the sum of the grey values of all pixels in a selected area divided by the number of pixels within the selection (Image J divides the range of grey values between Min and Max in 16-bit images into 256 bins). The average Mean Grey Value for each image/individual was used for statistical analysis of differences occurring between $AdoR^{RNAi}$ flies and their controls at different time points.

**Table 2.6. Software used for the experiments**

| Name of Software | Use |
|---|---|
| SHINY R-DAMS | Analysis of Sleep, Locomotor activity and Circadian Rhythm |
| GraphPad Prism Version 5 | Statistical Analysis of data |
| Image J | Immunoquantification |

## 2.11. Statistical Analysis

**(a) Mann-Whitney *U* (Student t-test) -** a non-parametric test was used to check statistically significant differences between two groups, for example - experimental vs. control (caffeine experiment for locomotor and sleep analysis).

**(b) Kruskal – Wallis (K-W)** – a non-parametric test after one-way ANOVA was used for evaluation of immunohistochemistry labelling (for specific time points to check statistically significant differences between experimental and control groups). It was also used for analysis of climbing assay, locomotor activity and sleep data after overexpressing or silencing of *dAdoR*.

**(c) Dunn's Multiple Comparison Test** - a *post hoc* test was used for determining statistically significant differences between groups.

**(d) Mantle-Cox (Log-rank) Test** - used to check survival of experimental and control groups at each observed time event.

The significance of the differences between the groups in each test was set at $p < 0.05$. The error bars represent SDs. Each experiment was repeated three times. Stars on graphs: * $p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$.

# 3. Results

## 3.1. The Increased dAdoR in Photoreceptors, Neurons, and Glial Cells Affects the Survival of Flies

The Kaplan-Meier survival curves of males and females with *dAdoR* overexpressed in photoreceptors, all neurons, and glial cells were significantly different from the curves of control flies [**Fig. 3.1 (A)**]. The differences in survival were analyzed using the log-rank test (**Table 3.1**).

In the F**ig. 3.1 (A – F)**] arrows accompanied by percentages indicate the median survival (MS) decrease (down) or increase (up). Overproduction of *dAdoR* mRNA in photoreceptors decreased the median survival (MS) and lifespan of both males and females [**Fig. 3.1 (A – B)**]. The 50% of males and females of *GMR-Gal4 >UAS-AdoR* lived 42 and 40 days, respectively, while males and females of the control groups lived 55 days.

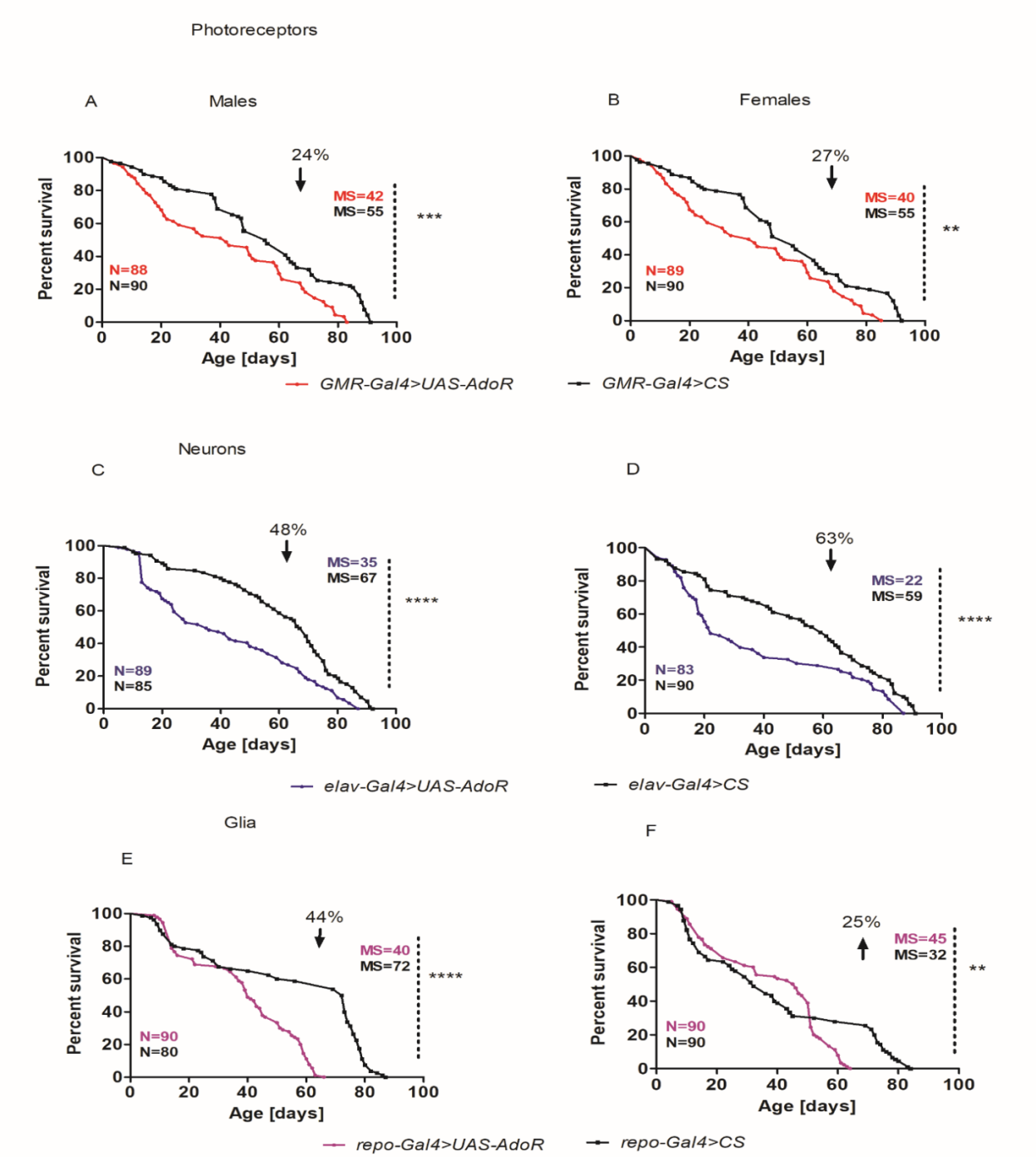


**Fig. 3.1.** Kaplan-Meier survival curves for males and females with overexpressed *dAdoR* in photoreceptors (red), neurons (blue) and glia (magenta). N values depicted for three independent repetitions. For males experimental and controls, (**A**) photoreceptors (n = 88; n = 90), (**B**) neurons (n = 89; n = 85), (**C**) glia (n = 90; n = 80). For females, (**D**) photoreceptors (n= 89; n = 90), (**E**) neurons (n = 83; n = 90), (**F**) glia (n = 90; n = 90).

As the Kaplan-Meier survival curves show, *dAdoR* overexpression decreased the survival of 1 – 50 days old flies rather than the older flies. Besides, the strongest effect was observed when *dAdoR* overexpression was induced in all neurons and not just in photoreceptor cells [**Fig. 3.1 (C – D)**]. Therefore, the MS for *elav-Gal4 >UAS-AdoR* flies was even more reduced.

It was 35 and 22 days for males and females, respectively. The maximum of lifespan of *GMR-Gal4 >UAS-AdoR* and *elav-Gal4> UAS-AdoR* flies was 80 days, which was 10 days shorter than the control flies.

**Table 3.1. Statistics for the survival results.**

| **Groups Compared** | **Overexpression Log-rank test statistics** | **Overexpression Log-rank test *p-value*** | **Silencing Log-rank test statistics** | **Silencing Log-rank test *p-value*** |
|---|---|---|---|---|
| *GMR-Gal4>UAS-AdoR vs GMR-Gal4>CS* | 16.4 | <0.0001 | | |
| *GMR-Gal4 >UAS-AdoR $^{RNAi}$ vs GMR-Gal4>UAS-VALIUM* | | | 5.0 | <0.05 |
| *GMR-Gal4>UAS-AdoR vs GMR-Gal4>CS* | 11.4 | <0.001 | | |
| *GMR-Gal4>UAS-AdoR$^{RNAi}$ vs GMR-Gal4>UAS-VALIUM* | | | 10.1 | <0.01 |
| *elav-Gal4>UAS-AdoR vs elav-Gal4>CS* | 18.5 | <0.0001 | | |
| *elav-Gal4>UAS-AdoR$^{RNAi}$ vs elav-Gal4>UAS-VALIUM* | | | 7.7 | <0.01 |
| *elav-Gal4>UAS-AdoR vs elav-Gal4>CS* | 9.6 | <0.01 | | |
| *elav-Gal4>UAS-AdoR$^{RNAi}$ vs elav-Gal4>UAS-VALIUM* | | | 6.7 | <0.01 |
| *repo-Gal4>UAS-AdoR vs repo-Gal4>CS* | 47,6 | <0.0001 | | |
| *repo-Gal4>UAS-AdoR$^{RNAi}$ vs repo-Gal4>UAS-VALIUM* | | | 10.7 | <0.01 |
| *repo-Gal4>UAS-AdoR vs repo-Gal4>CS* | 7.1 | <0.01 | | |
| *repo-Gal4>UAS-AdoR$^{RNAi}$ vs repo-Gal4>UAS-VALIUM* | | | 5.2 | <0.05 |
| **MALES** | | | | |
| **FEMALES** | | | | |

Interestingly, the influence of *dAdoR* overexpression on survival of flies was quite different when overexpression was induced in glial cells. Here, the main effect of rapid decrease of survival in comparison with control flies was observed in older flies that lived more than 40 days [**Fig. 3.1 (E)**]. Consequently, the maximum of individual lifespan was reduced to 66 and 64 days for males and females [**Fig. 3.1 (E – F)**], respectively. In control flies, the maximum of individual lifespan was 84 days for males and 89 days for females.

## 3.2. The Decreased dAdoR in Photoreceptors, Neurons, and Glial Cells Mildly Affects the Survival of Flies

The Kaplan-Meier survival curves of males and females with the silenced expression of *dAdoR* in photoreceptors, all neurons or glial cells were also different from the curves of control flies [**Fig. 3.2 (B)**]. Although I saw significant differences caused by silencing of *dAdoR,* however the overall survival was shorter than what I observed with overexpression of *dAdoR* [**Fig. 3.2 (A)**]. The median survival for males of *GMR-Gal4 >UAS-AdoR*$^{RNAi}$ (males with silenced *dAdoR* in photoreceptors) and males in the control group was almost the same: 40 and 41 days, respectively. However, the survival of males with silenced *dAdoR* in photoreceptors that were not yet 40 days old was better than that in males of the control group of the same age. In turn, the survival of older males with silenced *dAdoR* in photoreceptors was worse than that of control males of the same age [**Fig. 3.2 (A)**]. The differences in MS were analyzed using the log-rank test (**Table 3.1**).

In the case of females with silenced *dAdoR* in photoreceptors [**Fig. 3.2 (B)**], the initial positive effect of *dAdoR* silencing on survival was also observed, but for a shorter time, it lasted only until the flies were 20 days old. The MS of experimental and control flies was 28 and 42 days, respectively. The survival of older females with silenced *dAdoR* was worse than that of females from the control group. Therefore, it appears that flies with silenced *dAdoR* were protected from early death for some time. Later, however, their mortality was higher than control individuals and quite rapid. Flies of the *GMR-Gal4 >UAS-AdoR*$^{RNAi}$ strain showed a shorter lifespan. In summary, males survived up to 62 days compared to 78 days in the control and females lived 67 days compared to 78 days in the control [**Fig. 3.2 (A – B)**].

MS of flies with silenced *dAdoR* in neurons was 30 days for males (control 48 days) and 39 days for females (control 45 days) [**Fig. 3.2 (C – D)**].

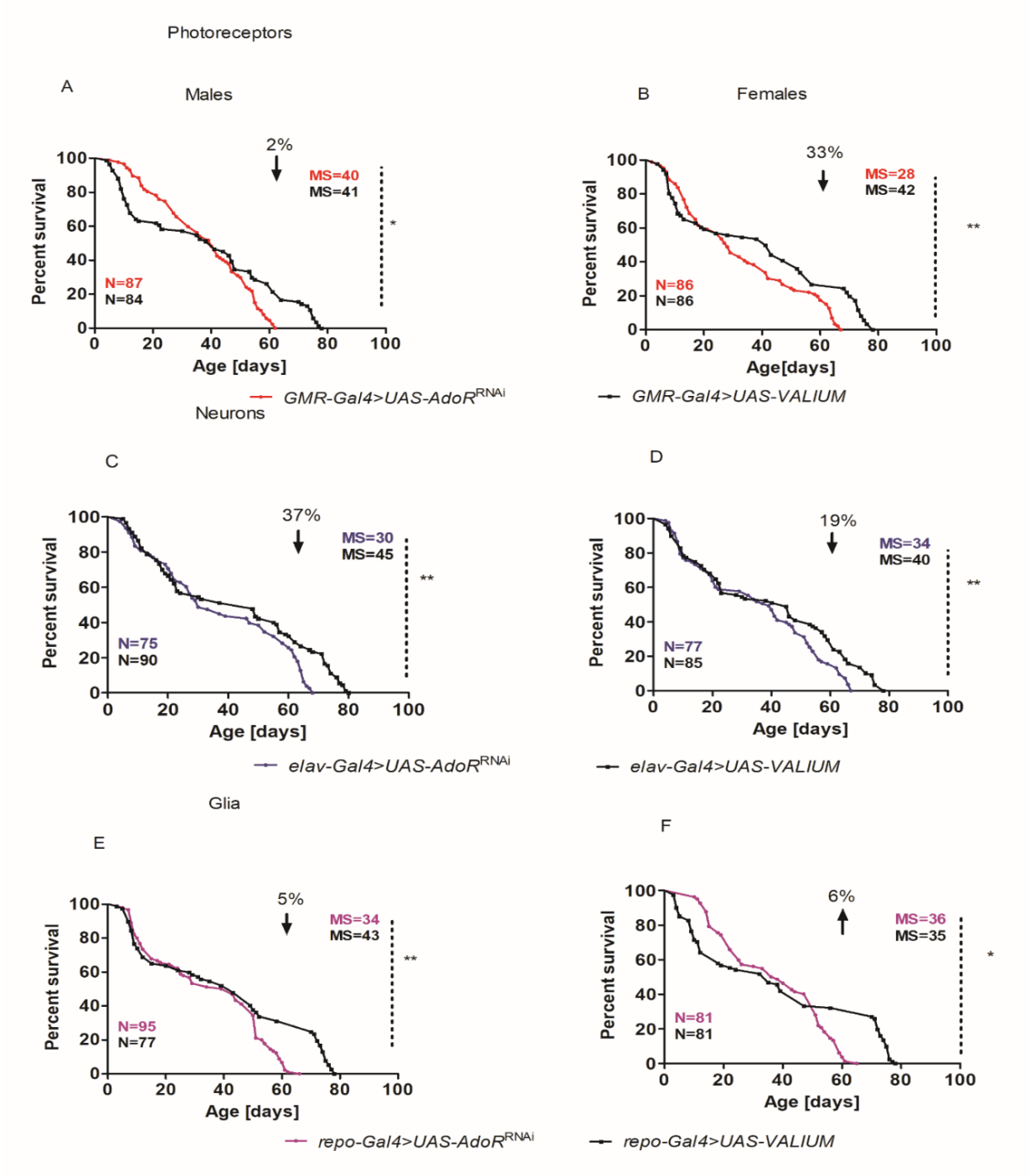


**Fig. 3.2.** Kaplan-Meier survival curves for males and females with the silenced expression of *dAdoR* in photoreceptors (red), neurons (blue) and glia (magenta) N values depicted for three independent repetitions. For males experimental and controls, (**A**) photoreceptors (n = 87; n = 84), (**B**) neurons (n = 75; n = 90), (**C**) glia (n = 95; n = 77). For females, (**D**) photoreceptors (n = 86; n = 86), (**E**) neurons (n = 77; n = 85), (**F**) glia (n = 81; n = 81).

A similar trend was observed in the maximum survival of flies with the silenced *dAdoR* gene in neurons. The maximum of lifespan was 68 days for males compared to 80 days for the control, and for females, it was 67 days compared to 78 days for the control. In the case of glial cells, the scenario was reflecting the same trend observed after silencing of *dAdoR* in the retina photoreceptors and neurons. The silenced *dAdoR* in glia decreased the lifespan to 68 days in males compared to 78 days in the controls and 65 days in females compared to 78 days in the control. After silencing of *dAdoR* in glial cells, MS was 34 days for males (control 39 days) and 35 days for females (control 36 days) [**Fig. 3.2 (E – F)**].

### 3.3.dAdoR Affect the Fitness of Flies when Overexpressed in Neurons and Glia

To detect any changes in locomotion and fitness of flies with overexpressed or silenced *dAdoR*, the climbing behaviour of flies was assessed in the commonly used behavioural assay, the negative geotaxis test. Typically, climbing abilities of flies decline with age, and this was also observed in my experiments [**Fig. 3.3 (A – D)**, **Fig. 3.4 (A – D)** Mann-Whitney t-test].

However, overexpression of *dAdoR* in the *Drosophila* neurons **[Fig. 3.3 (C – D)]** significantly improved the climbing ability of 60-day old males, as well as 30 and 60-day old females. In turn, silencing of *dAdoR* in neurons **[Fig. 3.4 (C – D)]** had the opposite effect. It decreased the climbing ability of 60 days old males as well as 30- and 60-days old females.

Overexpression of *dAdoR* in glia showed that middle-aged male flies **[Fig. 3.3 (E)]** have better climbing ability than the control. However, this could not be further visible in 60 days old males and it was not seen in 30- or 60-days old females **[Fig. 3.3 (F)]**. The climbing ability of 60 days - old females significantly declined.

Silencing of *dAdoR* in glia caused a further decline in the climbing of 60 days old males and females **[Fig. 3.4 (E – F)].** On the contrary, the higher and lower level of adenosine receptors only in photoreceptors did not show statistically significant differences compared to the control **[Fig. 3.3 (A – B), Fig. 3.4 (A – B)].**

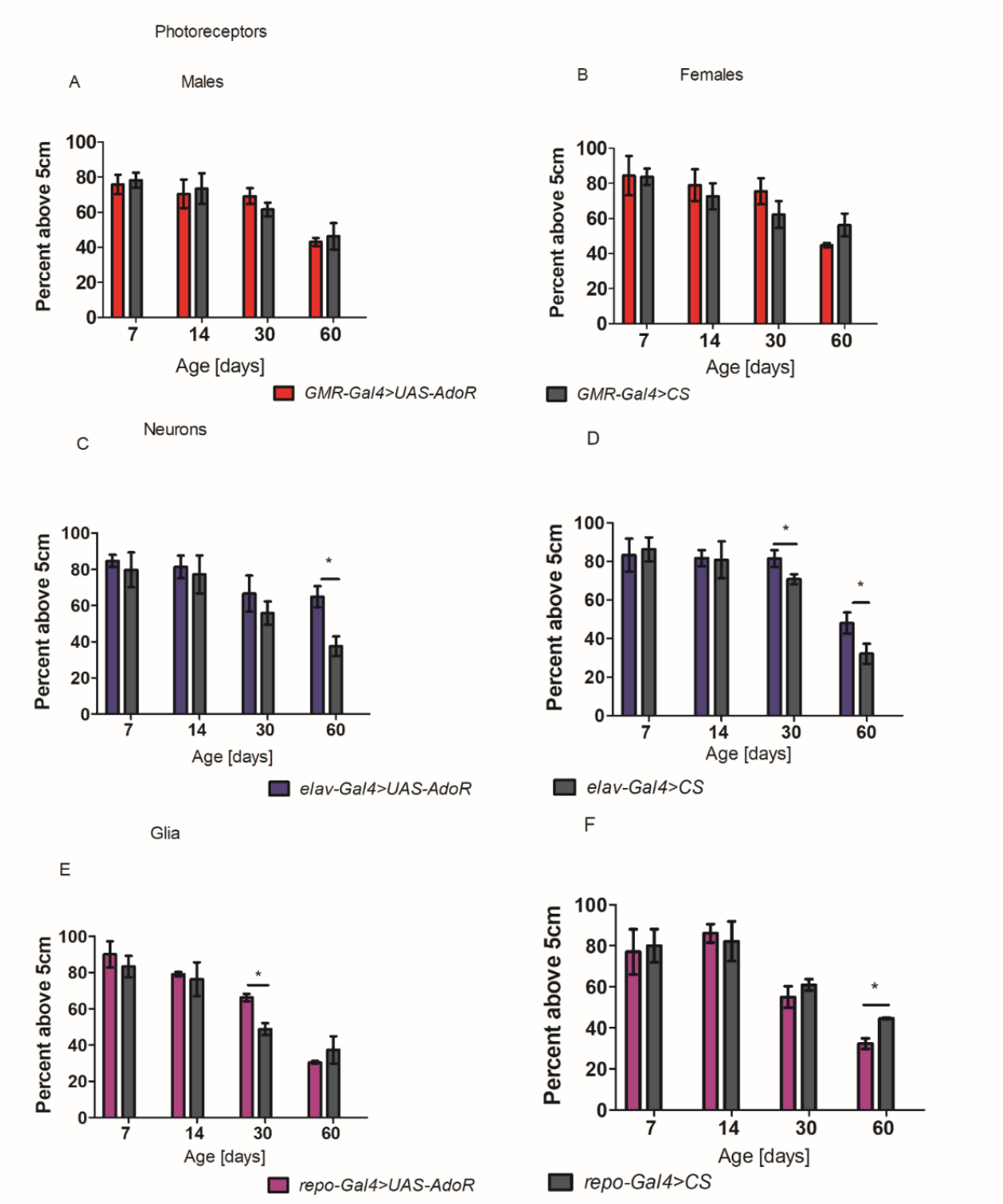


**Fig. 3.3.** Climbing ability of males and females with *dAdoR* overexpression in photoreceptors (**A-B**) neurons (**C-D**) and glial cells (**E-F**), data represents means ± SD, for three independent repetitions. Here, n = 3 indicates the means for each genotype for different ages.

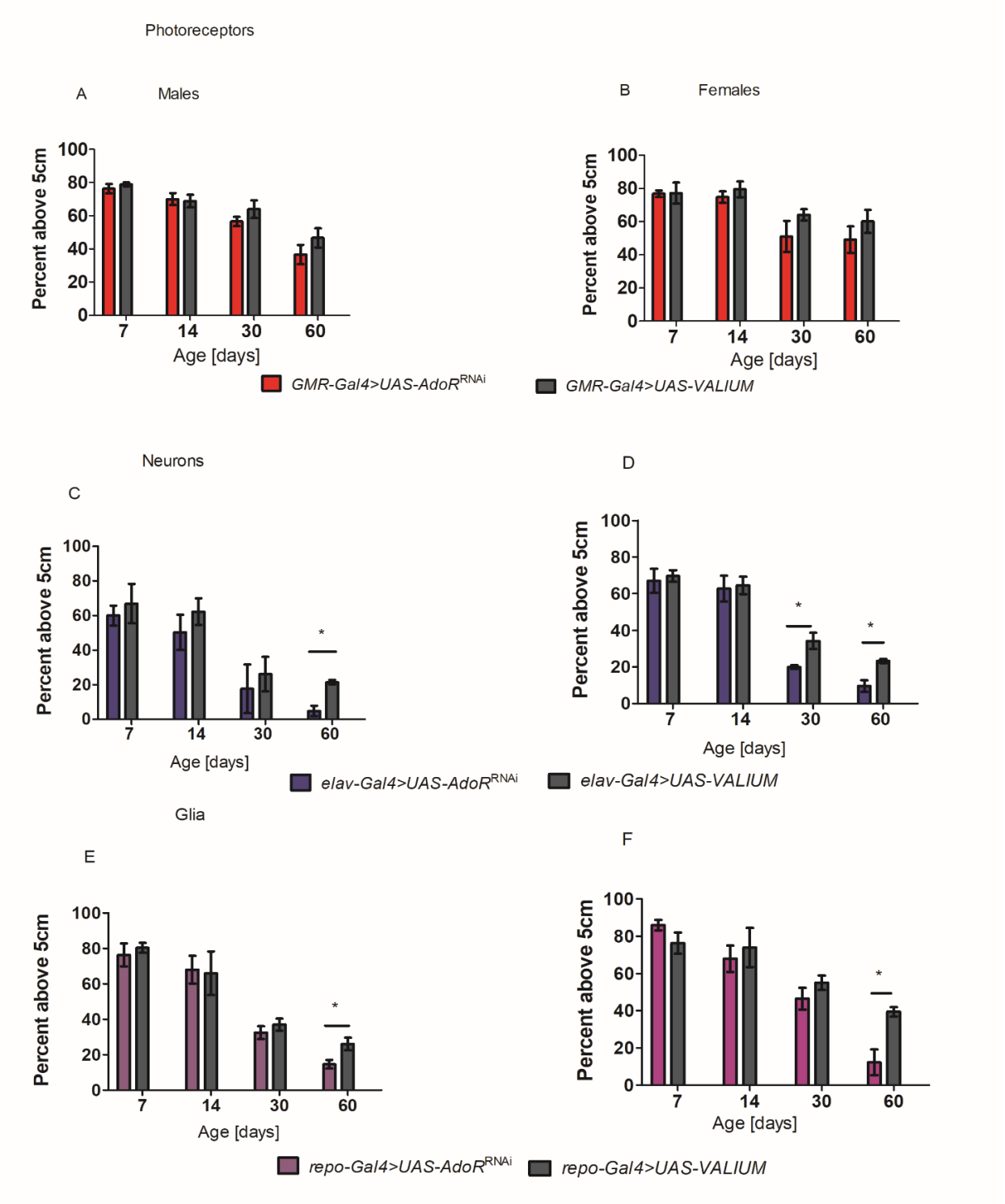


**Fig. 3.4.** Climbing ability of males and females with *dAdoR* silencing in photoreceptors (**A**-**B**), neurons (**C**-**D**) and glial cells (**E**-**F**), data show means ± SD, for three independent repetitions. Here, n=3 indicates the means for each genotype for different ages.

### 3.4.Adenosine Receptor Signalling in Photoreceptors, Neurons, and Glial Cells Affects *Drosophila* Sleep

Changes in sleep of males with overexpression of *dAdoR* in photoreceptors, neurons and glial cells were analyzed on the second day of activity recording in LD12:12. The results obtained indicate that adenosine receptor signalling in photoreceptors, neurons, and glial cells influences day (*siesta*) and night sleep in *Drosophila* [**Fig. 3.5**] when intensified due to the receptor overexpression.

When *dAdoR* was overexpressed in the retina photoreceptors, the total sleep of flies (*GMR-Gal4>UAS-AdoR*) did not change significantly, however, their daytime sleep was significantly longer (Mann-Whitney *U*-test, $p< 0.0001$), while nighttime sleep was shorter (Mann-Whitney *U*-test, $p < 0.05$) than the daytime and nighttime sleep of *GMR-Gal4>CS* control flies [**Fig. 3.5 (A)**]. There were increasingly more sleep counts in the average sleep profile of the experimental flies from ZT1 to ZT5, at ZT6, and ZT7.

Later the number of sleep counts was decreased, but until ZT9 it was still higher than in control flies [**Fig. 3.5 (A)**]. Therefore, the significant differences between the experimental flies and their sibling controls occurred during the light part of the cycle, when the photoreceptors are active [**Fig. 3.5 (B)**].

When *dAdoR* was overexpressed in all neurons, the flies (*elav-Gal4>UAS-AdoR*) slept significantly more than control flies during the light and the dark part of the 24 h cycle [**Fig. 3.5 (C)**]. Their total sleep was significantly longer (Mann-Whitney *U*-test, $p < 0.0001$) due to a 35% increase in daytime sleepiness and a 36% increase in nighttime sleep (Mann-Whitney *U*-test, $p < 0.0001$). There were also more sleep counts in the average sleep profile of the experimental flies than in the profile of their sibling controls during both the day (ZT0-ZT12) and the night (ZT12-ZT0) [**Fig. 3.5 (D)**].

Overexpression of *dAdoR* in glial cells [**Fig. 3.5 (E)**], like in neurons, induced a significant increase in both daytime (Mann-Whitney *U*-test, $p < 0.05$) and nighttime sleep (48% increase: Mann-Whitney *U*-test, $p < 0.05$) of *repo-Gal4>UAS-AdoR* flies. In consequence, the total sleep of flies increased by 35%; Mann-Whitney *U*-test, $p < 0.05$). However, both daytime and nighttime sleep increased only by 21 and 48%, respectively.

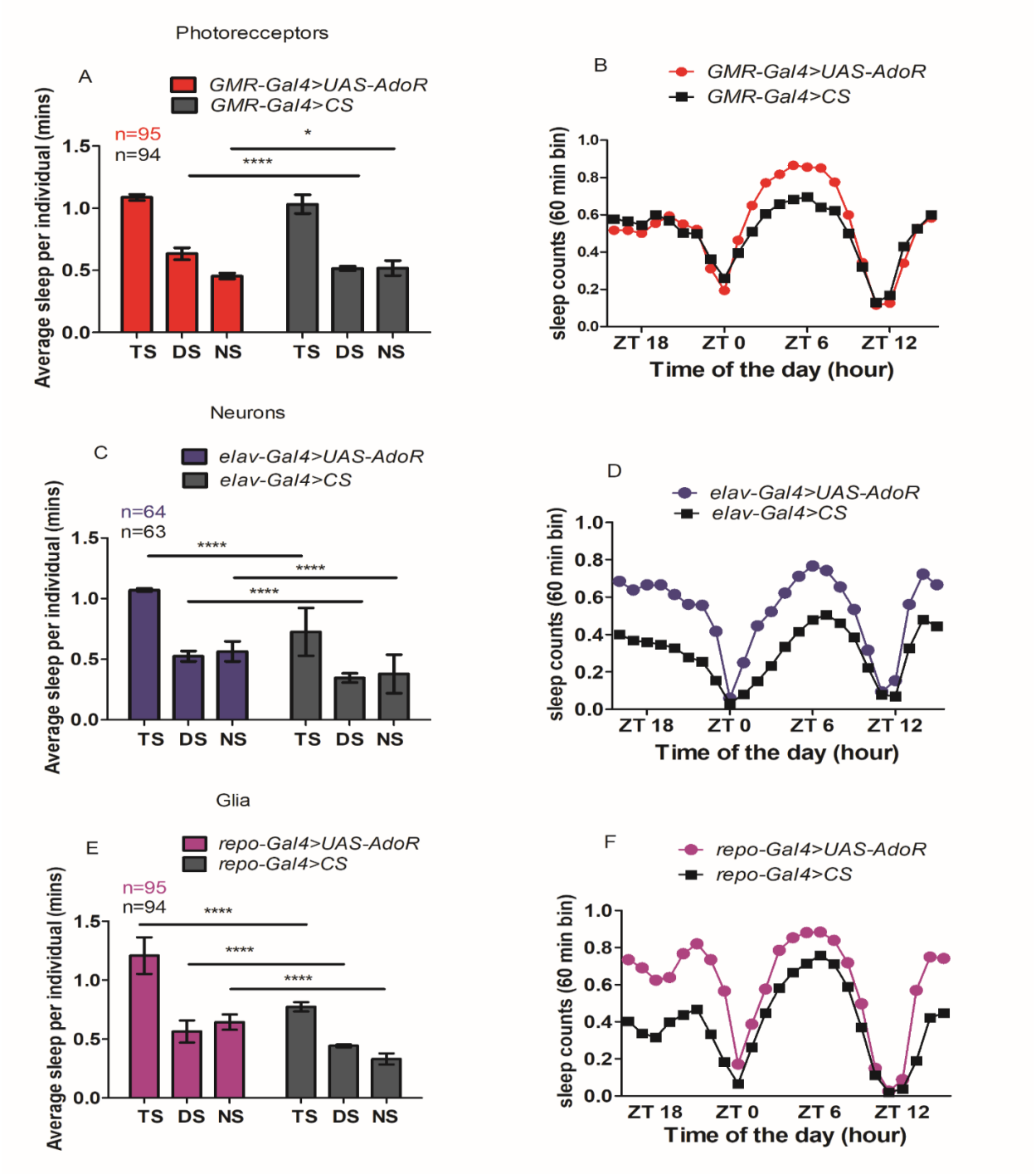


**Fig. 3.5.** The means ± SD of total (TS), daytime (DS) and nighttime (NS) sleep (**A**, **C**, **E**) as well as the daily sleep profiles (**B**, **D**, **F**) of males with *dAdoR* overexpression in photoreceptors (red), neurons (blue) and glia (magenta), N values depicted for three independent repetitions for experimental and controls in (**A**) photoreceptors (n = 95; n = 94), (**B**) neurons (n = 64; n = 63) and (**C**) glia (n = 95; n = 94).

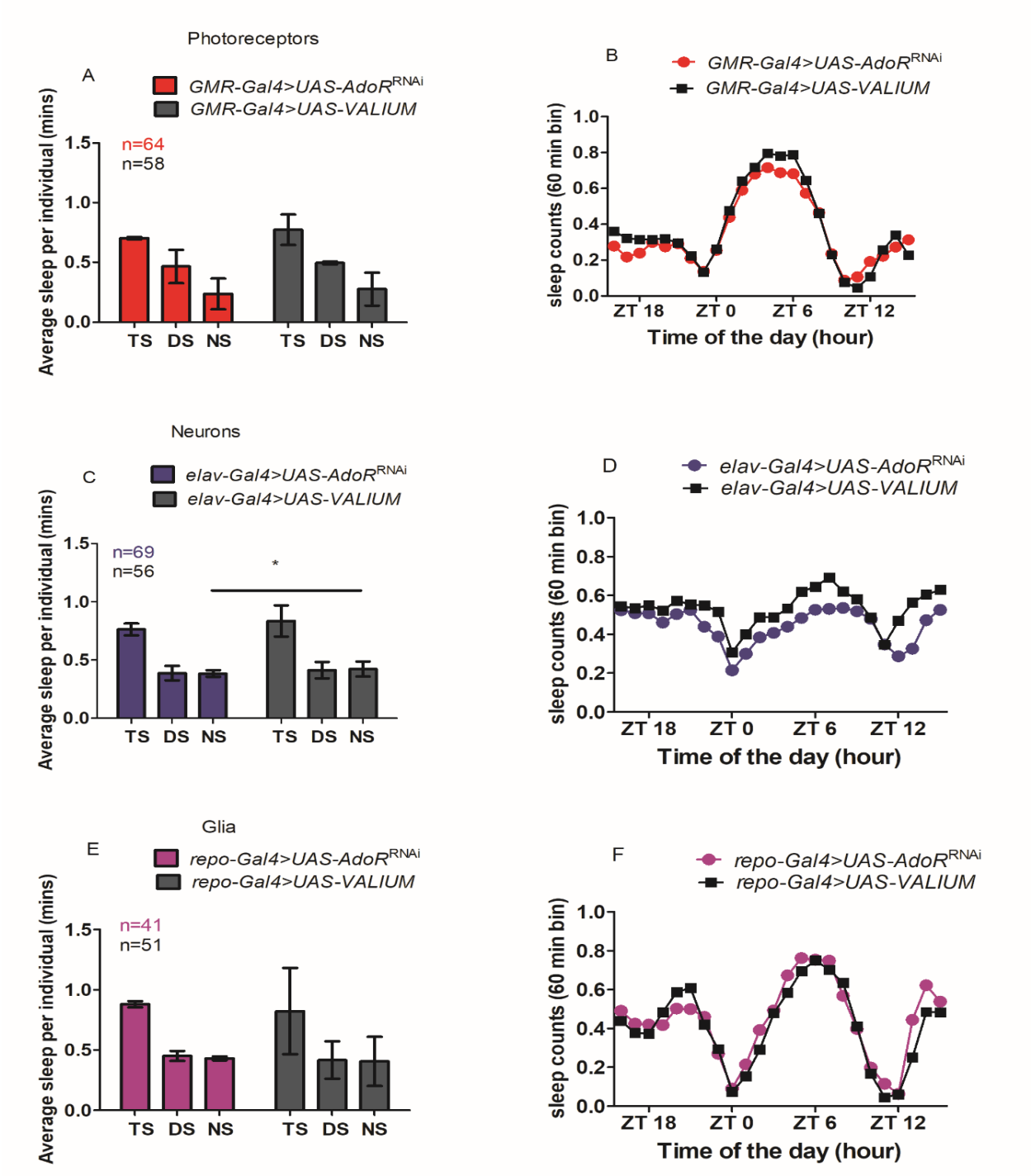


**Fig. 3.6.** The means ± SD of total sleep (TS), daytime sleep (DS) and nighttime sleep (NS) (**A**, **C**, **E**), as well as the daily sleep profiles (**B**, **D**, **F**) of males with *dAdoR* silencing. N values depicted for three independent repetitions for experimental and controls in(**A**) photoreceptors (n=64; n=58), (**B**) neurons (n=69; n=56), and (**C**) glia (n=41; n=51).

There were also more sleep counts at all time points of the night (ZT13-ZT23) in the average sleep profile of the experimental flies than in their sibling controls [**Fig. 3.5 (F)**]. The circadian clock was not distorted by the overexpression of *dAdoR* in any of the cell types, as the period of the free-running rhythm of locomotor activity of flies in constant darkness (DD) was similar to that of control individuals.

**Table 3.2** shows the summarized results for *dAdoR* overexpression and silencing in photoreceptors, neurons, and glia.

**Table. 3.2. Summarized results for *dAdoR* overexpression and silencing.**

| | **Strains** | ***GMR-GAL4*** | | | | | | ***elav-GAL4*** | | | | | | ***repo-GAL4*** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Gender | Male | | | Female | | | Male | | | Male | | | Male | | | Female | | |
| | Age groups | Y | M | O | Y | M | O | Y | M | O | Y | M | O | Y | M | O | Y | M | O |
| **OE** | Survival | | + | + | + | + | + | - | + | + | - | + | + | + | + | - | + | + | - |
| | Climbing | * | * | * | * | * | * | - | - | + | - | + | + | - | + | - | * | - | - |
| | Sleep | + | | | | | | + | | | | | | + | | | | | |
| **SL** | Survival | + | + | - | + | + | - | + | + | - | + | + | - | + | + | - | + | + | - |
| | Climbing | * | * | - | * | * | * | * | * | - | | * | - | * | * | * | * | * | - |
| | Sleep | * | | | | | | - | | | | | | * | | | | | |
| | Immuno-histochemistry | - | | | | | | | | | | | | - | | | | | |

**OE**-overexpression, **SL**- silencing, **Y**- young flies, **M**- middle-aged flies, **O**- old flies, "+"-positive effect, "**-**" negative effect, "*" – no effect.

The genetic crosses of the driver (GAL4) lines with *UAS- AdoR* construct produced F1 that revealed a clear increase in the amount of sleep in comparison with control siblings in a cell-specific fashion **[Fig. 3.5 (A – F)]**. However, when the same driver lines were crossed with *UAS-AdoR*$^{RNAi}$ silencing construct, the F1 showed almost no sleep differences from the control flies.

The silencing of *dAdoR* in photoreceptors, neurons or glial cells did not cause significant changes in the amount of sleep (total, day and night sleep) **[Fig. 3.6 (A** and **E)]**, except for a small decrease of the nighttime sleep that was observed in *elav-Gal4>UAS- AdoR*$^{RNAi}$ flies [**Fig. 3.6 (C)**]. There was no change in the length of the circadian period of the locomotor activity rhythm.

### 3.5. The Effect of *dAdoR* Silencing in Photoreceptors

Since my studies revealed the decrease in *Drosophila* fitness after silencing of *dAdoR* in neurons or glial cells, I decided to check the influence of silencing on the functioning of *Drosophila* synapses by examining the abundance of the presynaptic scaffolding protein Bruchpilot (BRP).

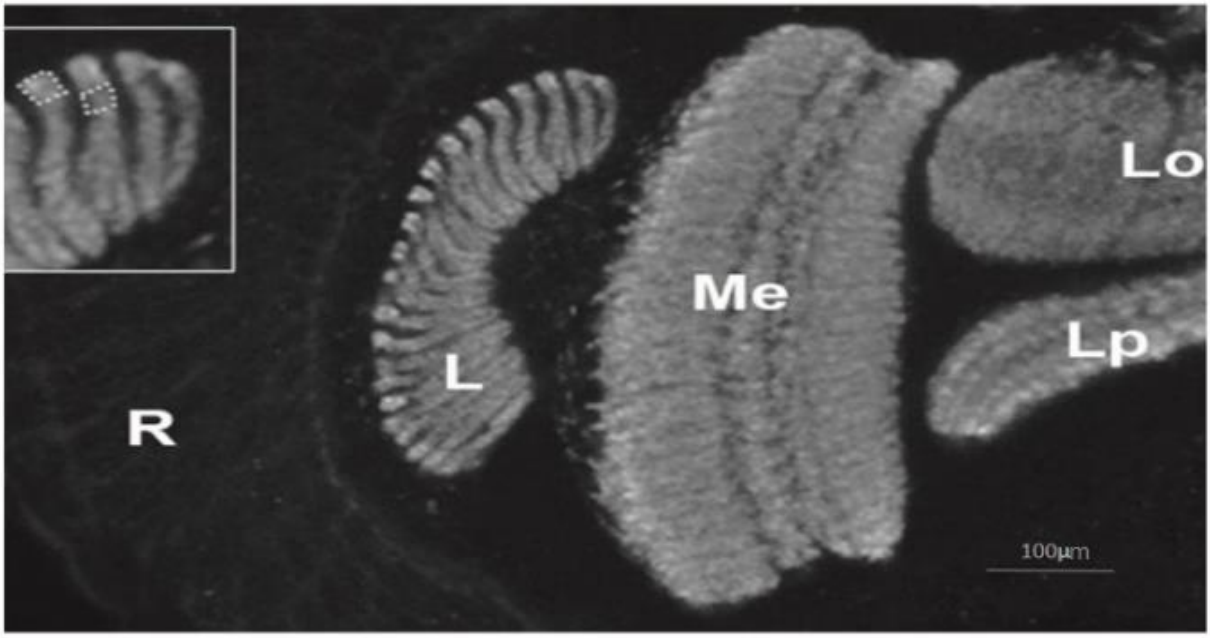


**Fig. 3.7.** Confocal image showing the BRP abundance in the visual system of *Drosophila* as revealed by immunostaining with Mab NC82. R - retina, L- the first optic neuropil (lamina), Me - the second neuropil (medulla), the third neuropils: Lo – lobula, Lp – lobula plate. Scale bar: 100 µm. The intensity of BRP-related fluorescence (corresponds to the BRP abundance) in cartridges is represented as the Mean Grey Value. The left upper corner shows a zoomed view of a lamina cartridge in which the distal and proximal part was highlighted using the Image J software used for measuring the BRP protein fluorescence.

I chose to monitor the level of BRP in the synaptic units (cartridges) of the first optic neuropil or lamina **[Fig. 3.7]**, where BRP level shows daily and circadian fluctuations [216]. I examined BRP level at four-time points (ZT1, ZT4, ZT13, and ZT16) and in flies with *dAdoR* silencing in the lamina photoreceptor terminals, which are the main neuronal components of the lamina cartridges, as well as in glial cells, which enwrap each cartridge.

The results showed that the level of BRP-related fluorescence in the distal **[Fig. 3.8. (A)]** and proximal **[Fig. 3.8 (B)]** parts of the lamina cartridges changes significantly during the day in

control (distal part: K-W test, H=13.76, p<0.001; proximal part: K-W test, H=13.88, p<0.001) and in experimental flies with the silenced expression of *AdoR* in photoreceptors (distal part: K-W test, H=27.34, p<0.0001; proximal part: K-W test, H=28.01, p<0.0001).

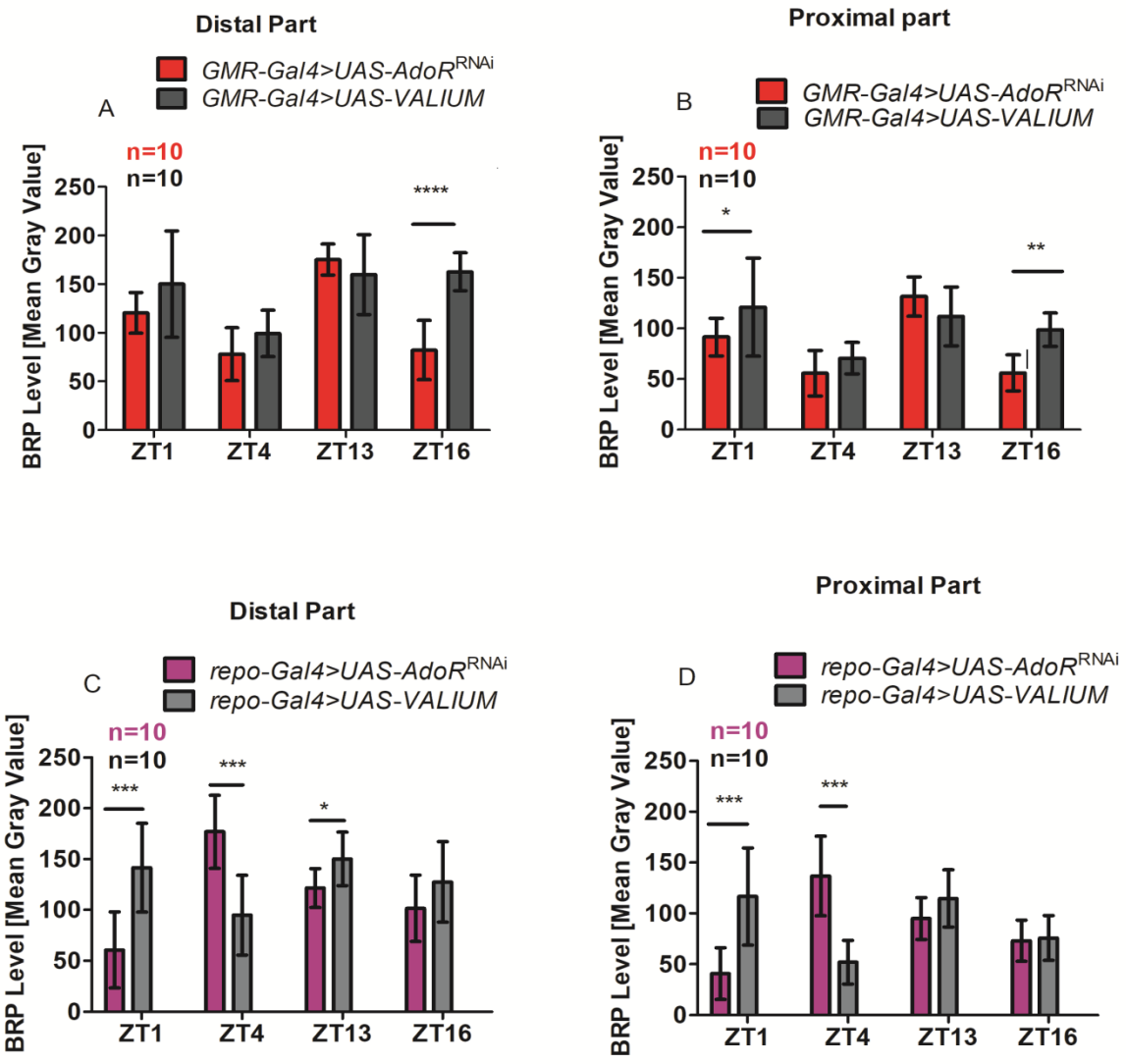


**Fig. 3.8.** BRP abundance in the distal and proximal lamina of *Drosophila* strains with *dAdoR* silencing in photoreceptors (**A-B**) and glial cells (**C-D**). The data shows mean ± SD values for three independent repetitions. Here, n =10 represents lamina cartridges of each individual repetition per time point.

In the distal lamina of control flies **[Fig. 3.8 (A)]**, the lowest level of fluorescence was observed in the middle of the day, at ZT4. There were statistically significant differences between

ZT4 and ZT1 ($p<0.05$), ZT13 ($p<0.01$) and ZT16 ($p<0.05$). The experimental group with silenced *dAdoR* in the photoreceptors revealed low fluorescence not only at ZT4 but also at ZT16.

There were statistically significant differences between ZT13 when the fluorescence intensity was highest, ZT4 ($p<0.001$), and ZT16 ($p<0.01$). Therefore, the pattern of daily changes in the BRP abundance varied in the distal lamina of control and experimental flies. The highest (49%) and statistically significant difference between the experimental and control group was in the middle of the night, at ZT16 (49%; Mann-Whitney *U*-test, $U=5$, $p < 0.001$) **[Fig. 3.8. (A)].**

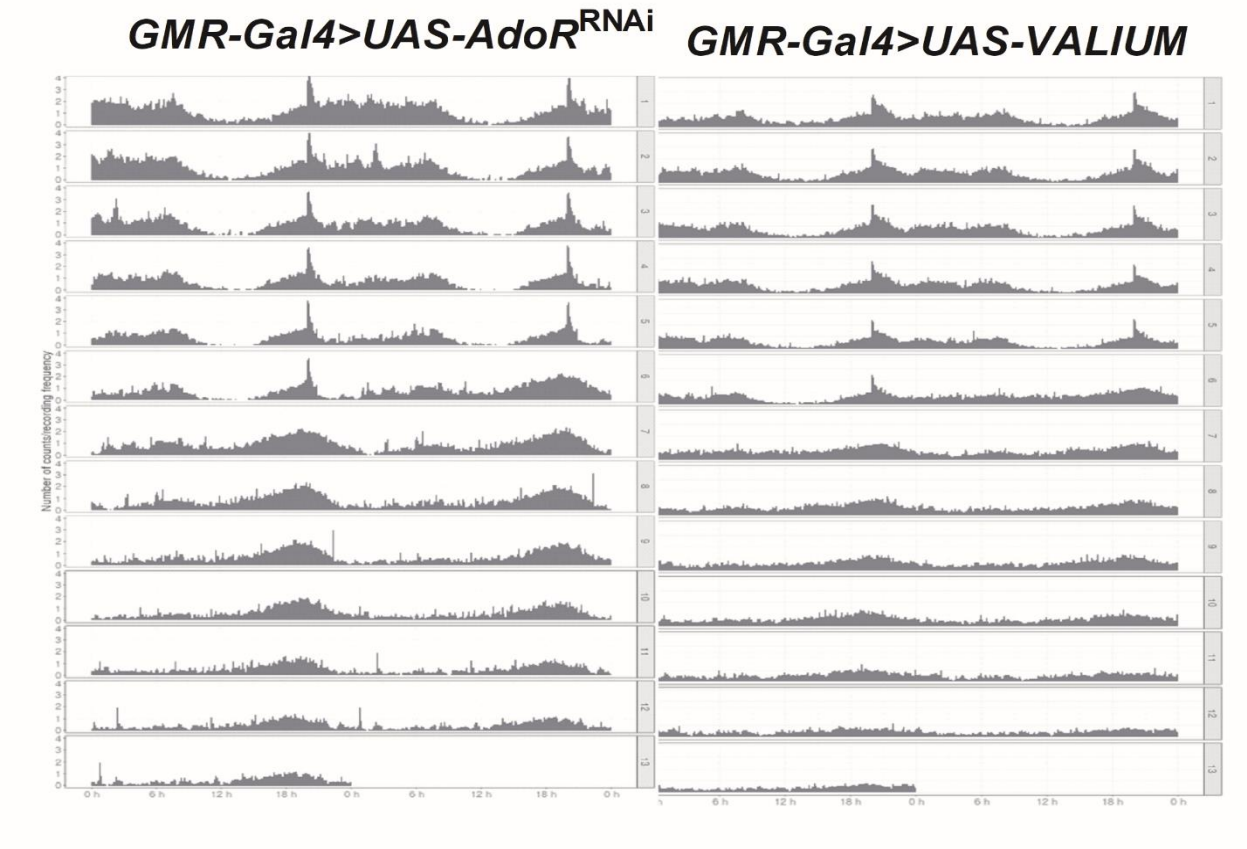


**Fig. 3.9.** Double-plotted actograms of a fly with silencing of *dAdoR* in photoreceptors (left actogram) and a control fly (right actogram).

At the proximal depth of the lamina [**Fig. 3.8 (B)**], ZT-dependent changes in the level of BRP-related fluorescence between the control and experimental flies were very similar to those in the distal lamina. In control flies, the lowest level of fluorescence intensity was at ZT4 (it was significantly lower than at ZT1 and ZT13, for both $p<0.01$), whereas in the experimental flies it was low at ZT4 and at ZT16 (in both cases it was significantly lower than at ZT13, $p< 0.001$).

However, significant differences between control and experimental flies occurred not only in ZT16 (44%; Mann-Whitney U-test, U=5, $p < 0.001$) but also in ZT1 (24.5%; Mann-Whitney U-test, U=26, $p < 0.05$).

The obtained results showed that silencing of *dAdoR* does impact the level of the synaptic active zone protein BRP, which is crucial to maintain synaptic transmission in the nervous system and in turn affects behaviour and other processes. The higher expression of BRP at the beginning of the day and night is correlated with two peaks in locomotor activity of *Drosophila* **(Fig. 3.9).**

### 3.6.The Effect of *dAdoR* Silencing in Glial Cells

The level of BRP-related fluorescence in the distal **[Fig. 3.8 (C)]** and proximal parts **[Fig. 3.8 (D)]** of the lamina cartridges changed significantly between time points during the day in experimental flies with silenced expression of *dAdoR* in glial cells (distal part: K-W test, H=24.04, $p<0.0001$; proximal part: K-W test, H=23.88, $p<0.0001$). The control flies also revealed significant changes in the fluorescence intensity at different time points during the day (distal part: K-W test, H=65, $p<0.05$; proximal part: K-W test, H=17.51, $p<0.001$).

In the distal lamina of control flies **[Fig. 3.8 (C)]**, for *dAdoR* silencing in photoreceptors, the lowest fluorescence intensity was observed at ZT4, and the statistically significant difference occurred only between ZT4 and ZT13; $p <0.05$). In turn, the experimental group with silenced *dAdoR* in glial cells showed the lowest fluorescence intensity at ZT1 and there was the statistically significant difference between ZT1 and ZT4 ($p< 0.001$) when the fluorescence intensity was the highest. In the experimental group, the fluorescence intensity was also significantly higher at ZT4 than at ZT16 ($p< 0.01$). Consequently, the pattern of daily changes of the BRP level in the distal lamina in control and experimental flies with silenced *dAdoR* in glial cells varied greatly. The statistically significant differences between experimental and control groups occurred during the day at ZT1 (57%; Mann-Whitney *U*-test, $U=7$, $p < 0.001$) and ZT4 (46%; Mann-Whitney *U*-test, $U=7$, $p < 0.001$), and also at the beginning of the night at ZT13 (19%; Mann-Whitney *U*-test, $U=20$, $p < 0.05$).

In the proximal part of the lamina **[Fig. 3.8 (D)]**, ZT-dependent changes in the level of BRP-related fluorescence intensity in control and experimental flies were very similar to those in the

distal lamina. The lowest fluorescence was observed at ZT4. It was significantly lower than at ZT1 ($p<0.01$) and at ZT13 ($p<0.01$). The experimental group with silenced *dAdoR* in glial cells again showed the lowest fluorescence intensity at ZT1 and the highest at ZT4. There were statistically significant differences between ZT1 and ZT4 ($p< 0.001$), as well as between ZT1 and ZT13 ($p<0.05$). The fluorescence intensity at ZT4 was also significantly higher than at ZT16 ($p< 0.05$). In the proximal part of the lamina, significant changes between control and experimental flies with silenced *dAdoR* in glia occurred only during the day at ZT1 (65%; Mann-Whitney *U*-test, $U=6$, $p < 0.001$) and ZT4 (62%; Mann-Whitney *U*-test, $U=6$, $p < 0.001$) **[Fig. 3.8(D)]**.

### 3.7. *Siesta* and Nighttime Sleep After Feeding with Caffeine

I compared daytime sleep and nighttime sleep in control flies. The result showed **[Fig. 3.10]** that in control flies there is a statistically significant difference between daytime and nighttime sleep (18%; Mann-Whitney *U*-test, $U=1940$, $p > 0.0001$). I then checked the effect of different concentrations of caffeine and compared daytime and nighttime sleep in treated flies. I did not observe significant differences between daytime and nighttime sleep in males treated with 0.1 **[Fig. 3.10 (A)]** and 0.5 mg/ml of caffeine **[Fig. 3.10 (C)]**. Interestingly, I found that increasing the concentration to 1 mg/ml **[Fig. 3.10 (E)]** in the feeding medium caused significant differences between daytime and nighttime sleep by approximately 24% (Mann-Whitney *U*-test, $U=1678$, $p < 0.0001$) in treated flies. This difference was more robust for the treated flies compared to 16% in controls (Mann-Whitney *U*-test, $U=2061$, $p < 0.0001$).

In the given **Table 3.3** the changes observed in TS, DS and NS in flies fed with different concentrations of caffeine along with their respective controls are indicated using downward arrow (blue) for decrease and upward arrow (red) for increase.

**Table 3.3. Sleep after feeding males with different concentrations of caffeine.**

| | **Caffeine** | | |
|---|---|---|---|
| Males | 0.1mg/ml | 0.5mg/ml | 1mg/ml |
| **Total sleep** | ↓ | ↑ | ↑ |
| **Daytime sleep** | ↓ | ↑ | ↑ |
| **Nighttime sleep** | ↓ | ↓ | ↓ |

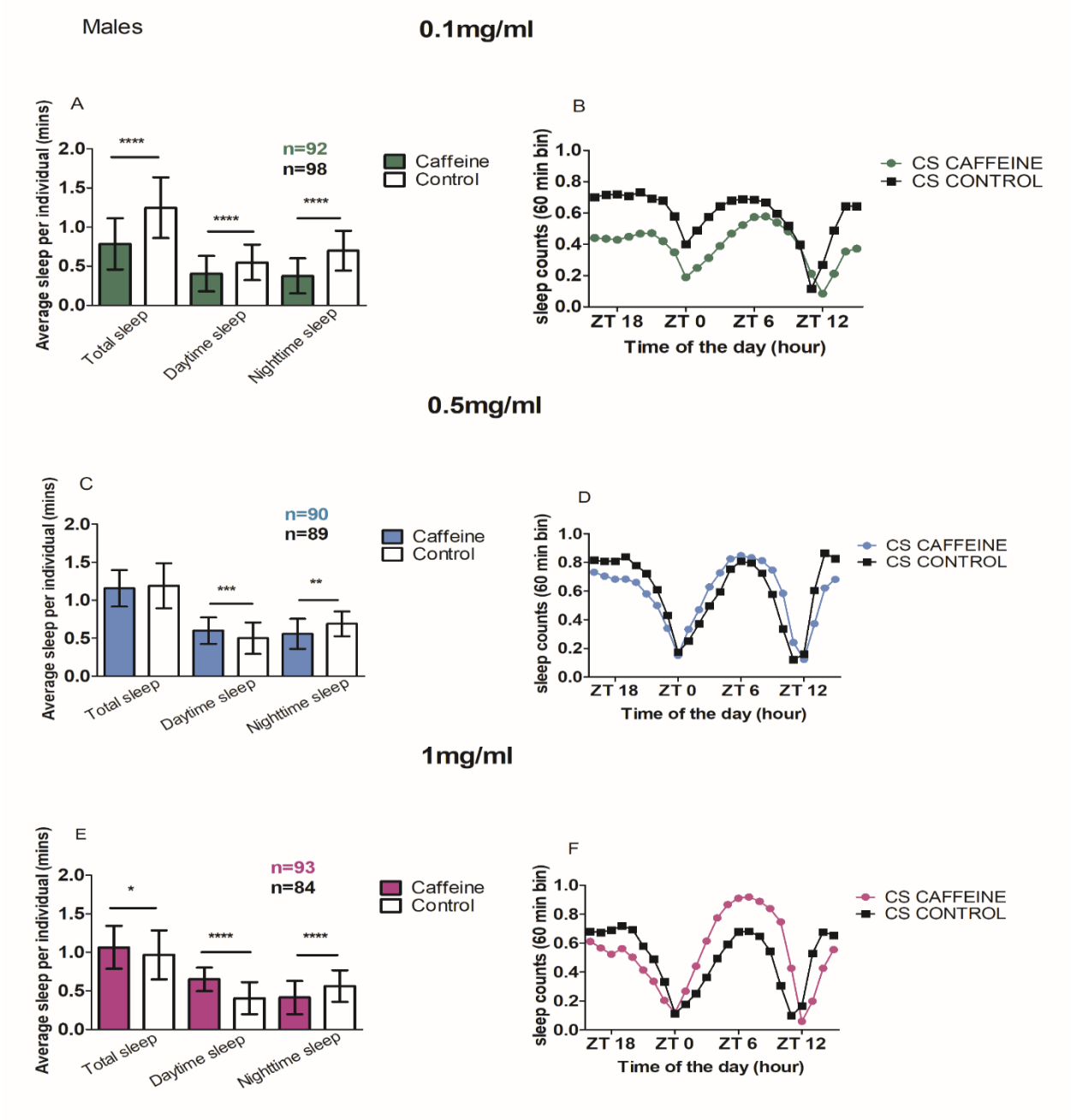


**Fig. 3.10.** The effect of different concentrations of caffeine on the total (TS), daytime (DS), and nighttime sleep (NS) (**A**, **C**, **E**) and sleep structure (**B**, **D**, **F**) of young (3 days old) male flies. N values depicted for three independent repetitions for caffeine treated flies and their respective controls for **(A)** 0.1 mg/ml (n = 92; n = 98), for **(B)** 0.5 mg/ml (n = 90; n = 89), and for (C) 1 mg/ml (n = 93; n = 84).

Next, I compared the effect of each concentration on total sleep, daytime sleep, and nighttime sleep. I observed major differences in total sleep which were concentration dependent. The low

concentration of 0.1 mg/ml caused a significant reduction of total sleep by 74% in the treated flies (Mann-Whitney *U*-test, $U$=1722, $p < 0.0001$) compared to controls, while higher concentrations of caffeine caused a significant increase of total sleep by 11% for 0.5 mg/ml (Mann-Whitney *U*-test, $U$=1199, $p < 0.001$) and by 10% for 1 mg/ml (Mann-Whitney *U*-test, $U$=3226, $p < 0.05$) in the treated flies when compared to controls.

I observed that the concentration was important in producing changes in daytime sleep or *siesta* in flies. The low concentration (0.1 mg/ml) caused the statistically significant decrease in daytime sleep by 14% (Mann-Whitney *U*-test, $U$=2999, $p < 0.0001$). Examination of the sleep profile **[Fig. 3.10 (B)]** showed that sleep was considerably reduced during the day (ZT0-ZT6). Similarly, increasing the concentration led to a statistically significant increase in daytime sleep by 20% (Mann-Whitney *U*-test, $U$=786, $p < 0.0001$) for 0.5 mg/ml and 39% (Mann-Whitney *U*-test, $U$=1369, $p < 0.0001$) for 1 mg/ml. Examination of the sleep profile showed higher sleep from ZT0-12 for both concentrations of caffeine, 0.5 **[Fig. 3.10 (D)]** and 1 mg/ml **[Fig. 3.10 (F)].**

Although I observed that the concentration of caffeine is important in producing various changes in total and daytime sleep of treated flies, I also found that caffeine caused a significant decrease in nighttime sleep in the case of all concentration used. Sleep decreased by 32% (Mann-Whitney *U*-test, $U$=1583, $p < 0.0001$) after exposure to 0.1 mg/ml. The sleep profile **[Fig. 3.10 (B)]** showed sleep reduction between ZT12-ZT23. For 0.5 mg/ml, sleep decreased by 8% (Mann-Whitney *U*-test, $U$=1360, $p < 0.05$) when compared to controls.

The sleep profile **[Fig. 3.10 (D)]** showed a reduction in sleep only at ZT18. Furthermore, for 1 mg/ml sleep was reduced by 24% (Mann-Whitney *U*-test, $U$=1678, $p < 0.0001$). The sleep profile **[Fig. 3.10 (F)]** showed sleep reduction between ZT13-19, at ZT22 and ZT23, while sleep was similar to the controls at ZT20.

### 3.8.Effects of Caffeine on Sleep in Females

The difference between daytime and nighttime sleep (**Table 3.4**) in female controls was statistically significant (21%) (Mann-Whitney *U*-test, $U$=2261, $p < 0.0001$) (**Fig. 3.11**). Similarly, I observed small but statistically significant differences in daytime and nighttime sleep in the treated flies with 0.1 mg/ml (6%) (Mann-Whitney *U*-test, $U$=2833, $p < 0.05$) and 1 mg/ml (8%) of

caffeine (Mann-Whitney *U*-test, *U*=2444, $p < 0.001$). However, I found no significant differences between daytime and nighttime sleep in flies treated with 0.5 mg/ml of caffeine.

The changes observed in TS, DS and NS (**Table 3.4**) in flies fed with different concentrations of caffeine are indicated using arrow (blue) for decrease, downward arrow (red) for increase while no changes are abbreviated as "nc".

**Table 3.4. Sleep after feeding females with different concentrations of caffeine.**

| | **Caffeine** | | |
|---|---|---|---|
| Females | 0.1mg/ml | 0.5mg/ml | 1mg/ml |
| **Total sleep** | ↓ | ↑ | nc |
| **Daytime sleep** | nc | ↑ | ↑ |
| **Nighttime sleep** | ↓ | ↓ | ↓ |

Next, I measured the total sleep and noticed interesting changes in the treated flies. Flies treated with 0.1 mg/ml of caffeine showed a decrease in total sleep by 25% (Mann-Whitney *U*-test, *U*=2325, $p < 0.0001$) compared to controls [**Fig. 3.11(A)**].

Total sleep increased, however, in flies treated with 0.5 mg/ml of caffeine by 23% (Mann-Whitney *U*-test, *U*=432, $p < 0.0001$) compared to controls but I did not find significant changes in total sleep when females were treated with 1 mg/ml of caffeine. Total sleep was almost similar to the control (Mann-Whitney *U*-test, *U*=2820, $p > 0.05$).

I observed that the low concentration of 0.1 mg/ml caffeine did not cause significant changes in daytime sleep (5%) (Mann-Whitney *U*-test, *U*=3545, $p > 0.05$). Examination of the sleep profile **[Fig. 3.11 (B)]** showed that sleep in the experimental flies was similar to controls during the day between ZT0-ZT12.

In addition, I detected that daytime sleep increased in flies by 29% (Mann-Whitney *U*-test, *U*=252, $p < 0.0001$) after 0.5 mg/ml [**Fig. 3.11(C)]** and by 20% after 1 mg/ml [**Fig. 3.11(E)]** concentrations of caffeine (Mann-Whitney *U*-test, *U*=1399, $p < 0.0001$). The sleep profile **[Fig. 3.11 (D, F)]** of flies exposed to both concentrations of caffeine showed longer sleep during the day between ZT0-ZT12.

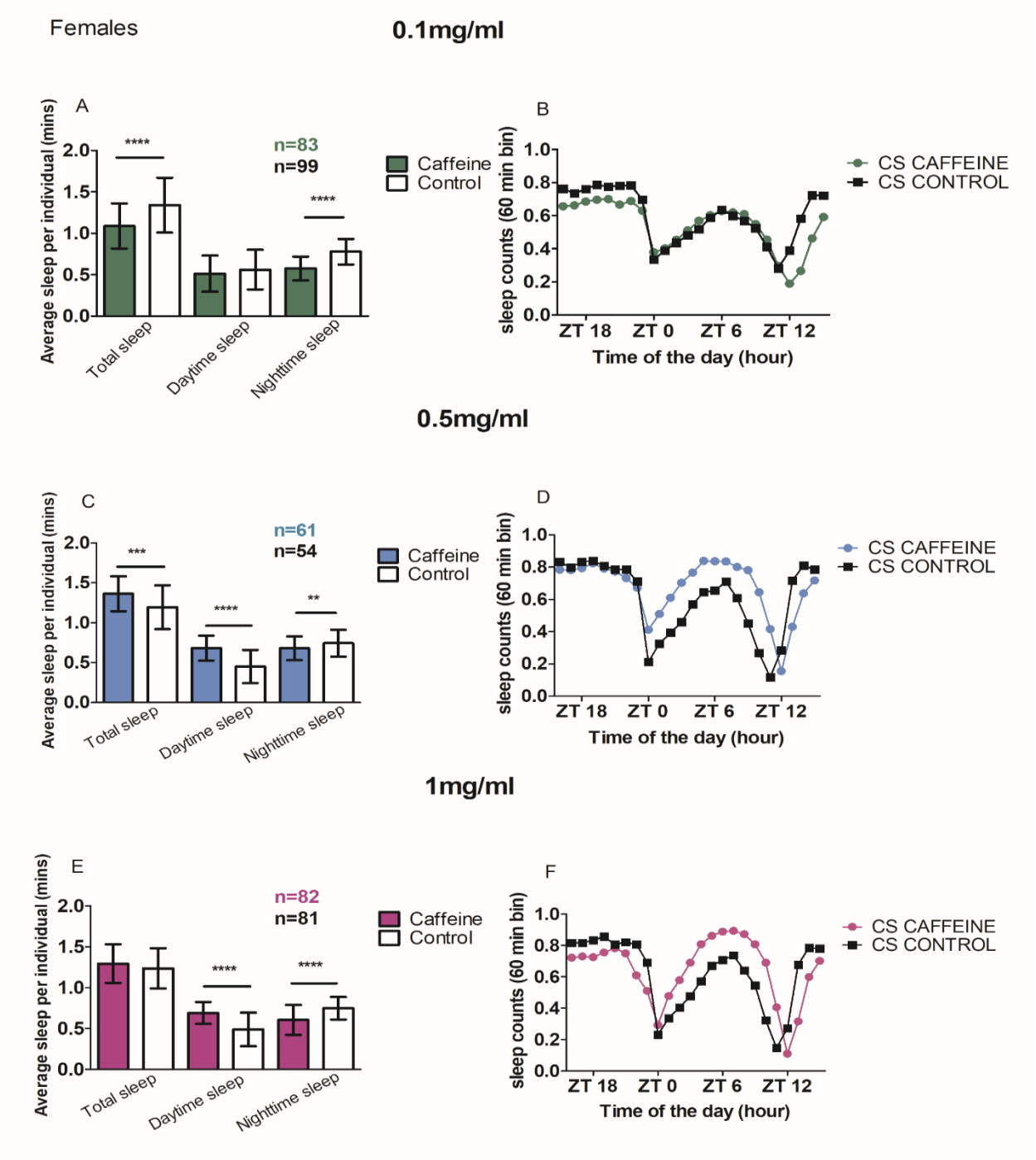


**Fig. 3.11.** The effect of different concentrations of caffeine on the total (TS), daytime (DS), and nighttime sleep (NS) (**A**, **C**, **E**) and sleep structure (**B**, **D**, **F**) of young (3 days old) female flies. N values depicted for three independent repetitions for caffeine treated flies and their respective controls for **(A)** 0.1 mg/ml (n = 83; n = 99), for **(B)** 0.5 mg/ml (n = 61; n = 54), and for **(C)** 1 mg/ml (n = 82; n = 81).

Next, I observed that nighttime sleep decreased after exposure to all concentration used. However, the examination of the sleep profile **[Fig. 3.11 (B)]** showed that sleep during ZT13-ZT23 was reduced after applying the concentration of 0.1 mg/ml caffeine, while sleep after the exposure to 0.5 mg/ml **[Fig. 3.11 (D)]** decreased only at the beginning of the night, at ZT13. For 1 mg/ml of caffeine exposure, the nighttime sleep was reduced from the beginning of night (ZT13) to the later part of the night (ZT23) **[Fig. 3.11 (F)]**.

### 3.9.Effects of Caffeine on Nighttime Sleep in Males

While considering the male controls of 3 days, 30 days, 50 days old, significant differences in total sleep (K-W test, H=12. 61, $p<0.001$) were only observed between 30 days and 50 days old males **[Fig. 3.12 (A)]**. Total sleep between 30 days and 50 days old flies varied by 29% (Dunn's multiple comparison test, $p<0.001$). The flies treated with 1 mg/ml of caffeine, had significant differences in total sleep in all age group studied (K-W test, H=7. 551, $p<0.05$). Total sleep varied between 3 days and 50 days old males by 29% and between 3 days and 30 days old by 31% (Dunn's multiple comparison test, $p<0.05$).

Although I detected these age-dependent changes in controls and caffeine-treated flies, the total sleep of 3 days old caffeine-treated flies was significantly higher by 10% compared to 3 days old controls (Mann-Whitney *U*-test, $U=3226$, $p<0.05$). Unlike these changes, a comparison of total sleep in 30 days and 50 days old caffeine-treated flies with their controls showed no differences. **Table 3.5** shows the summarized results. (+) = caffeine treated group; (-) = without caffeine.

**Table 3.5. Sleep in males after 1 mg/ml of caffeine exposure.**

| Males | **Caffeine** | | | **Control for flies of different age** | | |
|---|---|---|---|---|---|---|
| Age (days) | 3 | 30 | 50 | 3 | 30 | 50 |
| **Total sleep** | * | * | * | nc | * | * |
| **Daytime sleep** | nc | nc | * | * | * | * |
| **Nighttime sleep** | nc | nc | nc | * | * | * |

**Table 3.5** shows effect on TS, DS, and NS in experimental and control male flies for different ages (3, 30, and 50 days old) after 1 mg/ml of caffeine exposure. Here, * refers to the changes observed, whereas no changes are abbreviated as "nc".

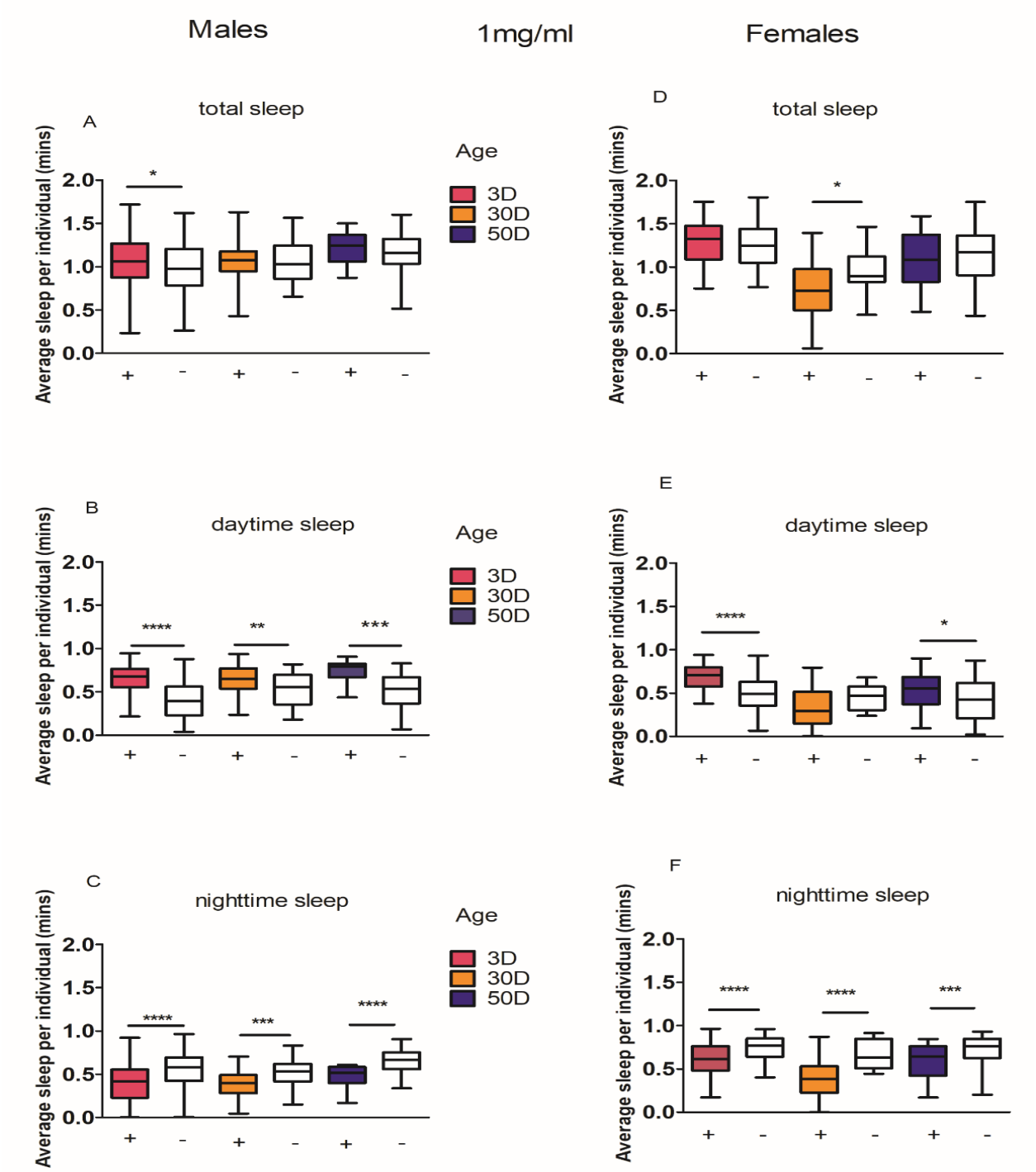


**Fig. 3.12.** The effect of 1mg/ml of caffeine on total, daytime, and nighttime sleep of male (**A**, **B**, **C** - left) and female flies (**D**, **E**, **F** - right). N values depicted for three independent repetitions for caffeine and controls in 3 days old males (n = 93; n = 84), 30 days old males (n = 46; n = 28), 50 days old (n = 20; n = 50). For females 3 days old (n = 81; n = 81), 30 days old (n = 48; n = 28), 50 days old (n = 39; n = 49).

I found significant differences in daytime sleep or *siesta* in different age groups of controls (K-W test, H=12. 28, p<0.001). Daytime sleep [**Fig. 3.12 (B)**] showed statistically significant differences between 3 days and 30 days old males by 28% (p<0.001) and between 3 days and 50 days old by 24% (p<0.001). However, the flies treated with caffeine revealed a significant difference in *siesta* (K-W test, H=7. 083, p<0.05) only between the age group 3-days and 50 days old (by 29%, p<0.05).

Comparisons between caffeine treated group and controls showed higher *siesta* by 39% in 3 days (Mann-Whitney *U*-test, *U*=1369, p<0.0001), 11% in 30 days (Mann-Whitney *U*-test, *U*=423, p<0.007) and by 23% in 50 days (Mann-Whitney *U*-test, *U*=166, p < 0.0001) old flies.

Similarly, I found age-dependent significant differences in nighttime sleep in control flies (K-W test, H=13. 19, p<0.001). Differences were observed between 3 days and 50 days old flies by 24% and between 30 days and 50 days old by 36% (Dunn's multiple comparison test, p<0.001). Although I detected differences in nighttime sleep **[Fig. 3.12 (C)]** in controls, I did not observe age-dependent changes in nighttime sleep of caffeine-treated flies (K-W test, H=3. 7525, p>0.05). Nighttime sleep in caffeine treated group comparing with controls was 15% shorter the in 3 days (Mann-Whitney *U*-test, *U*=2414, p<0.0001) 12% in 30 days old males (Mann-Whitney *U*-test, *U*=350, p<0.0005) and 18% in 50 days old (Mann-Whitney *U*-test, *U*=173, p<0.0001).

### 3.10. Females are More Sensitive to Caffeine-Induced Age-Dependent Changes in Sleep

In female controls, total sleep was **[Fig. 3.12 (D)]** significantly different (K-W test, H=12. 27, p<0.0001) only between 3 days and 30 days old individuals (Dunn's multiple comparison test, p<0.001). While in caffeine-treated flies, significant differences occurred in all age group females (3, 30, 50 days old) (K-W test, H=66. 37, p<0.0001). Total sleep varied between 3 days and 30 days old flies by 72%, between 3 days and 50 days old by 27%, and between 30 and 50 days old by 44% (Dunn's multiple comparison test, p<0.0001). In females, total sleep was significantly decreased by 21% only between the 30 days caffeine-treated group and the control (Mann-Whitney *U*-test, *U* =205, p<0.014). Then I measured daytime sleep (*siesta*) in controls **[Fig. 3.12 (E)]** and observed no significant differences in all age group studied (K-W test, H=2. 867, p>0.05). However, in the caffeine-treated group, I detected robust changes in *siesta* (K-W test, H=63. 79, p<0.0001). Daytime sleep increased by 70% between females 3 days and 30 days old while it

increased to 35% between females in age of 3 days, 30 days and 50 days (Dunn's multiple comparison test, p<0.0001). In addition to these changes, a comparison of *siesta* in flies treated with caffeine with controls showed a statistically significant increase only for 3 days old flies by 20% (Mann-Whitney *U*-test, *U*=1399, p<0.0001), and for 50 days old by 11% (Mann-Whitney *U*-test, *U*=704.5, p<0.017). No changes in *siesta* were observed for 30 days old females. **Table 3.6** shows effects of 1 mg/ml of caffeine exposure on TS, DS, and NS in experimental female flies comparing with the control in different age groups (3, 30, and 50 days old). Here, * refers to the changes observed whereas no changes are abbreviated as "nc".

**Table 3.6. Sleep in females after 1mg/ml of caffeine exposure.**

| Females | **Caffeine** | | | **Control** | | |
|---|---|---|---|---|---|---|
| Age (days) | 3 | 30 | 50 | 3 | 30 | 50 |
| **Total sleep** | * | * | * | **nc** | * | **nc** |
| **Daytime sleep** | * | * | * | **nc** | **nc** | **nc** |
| **Nighttime sleep** | * | * | * | **nc** | **nc** | **nc** |

The differences in nighttime sleep **[Fig. 3.12 (F)]** were not significant in the control group in flies of different age (K-W test, H=2. 617, p>0.05). However, after caffeine treatment, nighttime sleep was significantly reduced by 43% between flies in age 3 days, 30 days and 50 days old (Dunn's multiple comparison test, p<0.0001). Comparisons between caffeine treated flies and controls showed a significant reduction of nighttime sleep in 3 days old flies by 14% (Mann-Whitney *U*-test, *U*=1787, p<0.0001), 30 days old by 27% (Mann-Whitney *U*-test, *U*=111, p<0.0001) and 50 days old by 14% (Mann-Whitney *U*-test, *U*=543.5, p<0.0003).

### 3.11. Caffeine is Unable to Influence *Siesta* when *dAdoR* is Overexpressed

After caffeine treatment (0.5 mg/ml), [**Fig. 3.13 (A)**] in flies with *dAdoR* overexpression in neurons (**Table 3.7**), the total sleep was decreased by 57% comparing with the control (Mann-Whitney *U*-test, *U*=0.0, p < 0.0001). Moreover, caffeine treatment decreased the daytime sleep by 42% (Mann-Whitney *U*-test, *U*=0.0, p < 0.0001) and nighttime sleep by 15% (Mann-Whitney *U*-test, *U*=81.0, p < 0.03) in *elav-Gal4>UAS-AdoR*. Furthermore, caffeine did not influence total sleep in *elav-Gal4>CS* (+) and *elav-Gal4>CS* (-) (Mann-Whitney *U*-test, *U*=246.0, p > 0.05). The statistically significant increase (Mann-Whitney *U*-test, *U*=203.5, p< 0.02) of daytime sleep by 7% and (Mann-

Whitney *U*-test, $U$=172.5, $p < 0.005$) nighttime sleep by 13% was observed in comparing with controls [**Fig. 3.13 (A)]**.

Next, I checked sleep after overexpression of *dAdoR* in *pdf*-expressing neurons which are clock ventral lateral neurons (LNv). I observed no changes in total sleep, daytime sleep, and nighttime sleep **[Fig. 3.13 (B)]**. Similarly, in *tim*-expressing neurons, including clock and non-clock neurons, I observed that caffeine treatment significantly decreased total sleep and nighttime sleep after overexpressing *dAdoR*. Total sleep between *tim-Gal4>UAS-AdoR* (+) and *tim-Gal4>UAS-AdoR* (-) was significantly reduced by 14% (Mann-Whitney *U*-test, $U = 149.5$, $p < 0.005$), while nighttime sleep was reduced by 16% (Mann-Whitney *U*-test, $U$=112.0, $p < 0.0004$). However, total sleep and nighttime sleep after caffeine treatment in the group *tim-Gal4>CS* did not show significant changes comparing with the control *tim-Gal4>CS*. Unlike these changes, caffeine did not influence *siesta* in *tim*-expressing neurons after overexpression of *dAdoR* **[Fig. 3.13 (C)]**.

**Table 3.7. Summary of *dAdoR* overexpression in different groups of neurons after treatment with 0.5 mg/ml of caffeine.**

| N | Genotype | Total sleep | Daytime sleep | Nighttime sleep |
|---|---|---|---|---|
| 16<br>39 | *elav-Gal4>UAS-AdoR (+) vs.*<br>*elav-Gal4>UAS-AdoR (-)* | ↓ | ↓ | ↓ |
| 16<br>16 | *elav-Gal4>CS (+) vs.*<br>*elav-Gal4>CS (-)* | nc | ↑ | ↓ |
| 67<br>70 | *th-Gal4 >UAS-AdoR (+) vs.*<br>*th-Gal4 >UAS-AdoR (-)* | ↓ | nc | ↓ |
| 31<br>23 | *th-Gal4 >CS* (+) vs.<br>*th-Gal4 >CS* (-) | nc | nc | nc |
| 22<br>24 | *tim-Gal4 >UAS-AdoR* (+) vs.<br>*tim-Gal4 >UAS-AdoR* (-) | ↓ | nc | ↓ |
| 15<br>23 | *tim-Gal4 >CS* (+) vs.<br>*tim-Gal4 >CS* (-) | nc | nc | nc |
| 24<br>32 | *pdf-Gal4 >UAS-AdoR* (+) vs.<br>*pdf-Gal4 >UAS-AdoR* (-) | nc | nc | nc |
| 23 | *pdf-Gal4 >CS* (+)<br>*pdf-Gal4 >CS* (-) | nc | nc | ↓ |

N shows the sample size for each genotype for three independent repetitions. The genotype column shows the comparison between caffeine treated (+) and control (-) group. Changes observed in TS, DS and NS are indicated using downward arrows (blue) for decrease and upward arrows (red) for increase while no changes are abbreviated as “nc”.

I observed that caffeine significantly decreases total sleep in the group *th-Gal4> UAS-AdoR* (+) comparing with the control *th-Gal4> UAS-AdoR* (-) by 15% (Mann-Whitney *U*-test, $U$=1632, $p < 0.001$), while total sleep did not show significant differences between the group *th-Gal4> CS* (+) and *th-Gal4> CS* (-). Nighttime sleep was significantly decreased by 17% in *th-Gal4> UAS-AdoR* (+) comparing with *th-Gal4> UAS-AdoR* (-) (Mann-Whitney *U*-test, $U$=1255, $p < 0.0001$), whereas no significant changes were observed between the groups *th-Gal4> CS* (+) and *th-Gal4> CS* (-) **[Fig. 3.13 (D)]**. Caffeine did not have any effect on *siesta* in flies after overexpressing *dAdoR* in dopaminergic neurons.

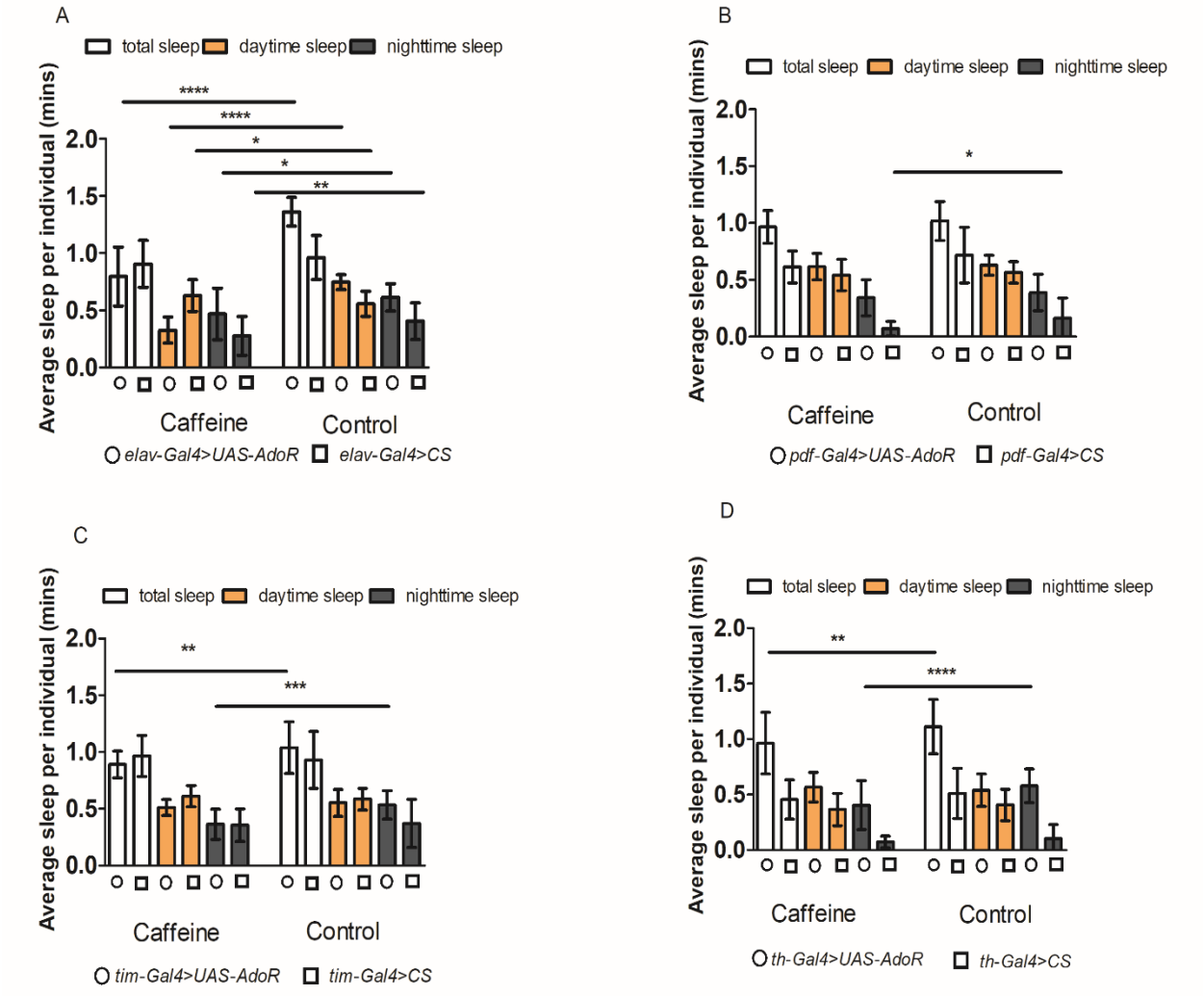


**Fig. 3.13.** The effect of 0.5 mg/ml of caffeine on total, daytime, and nighttime sleep in transgenic flies with *dAdoR* overexpression in (A) all neurons, (B) *pdf*-expressing (clock) neurons, (C) *tim*-expressing neurons, and (D) *th*-expressing dopaminergic neurons.

### 3.12. Caffeine Influences Night Sleep and *Siesta* when *dAdoR* is Silenced

I measured sleep after exposure to caffeine flies with silencing *dAdoR* in all neurons and found no significant changes in total and nighttime sleep. I observed, however, that *siesta* or daytime sleep was significantly increased by 13% in *elav-Gal4>UAS-AdoR*$^{RNAi}$ (+) treated with caffeine comparing with *elav-Gal4>UAS-AdoR*$^{RNAi}$ (-) (Mann-Whitney *U*-test, $U = 87.00$, $p < 0.022$).

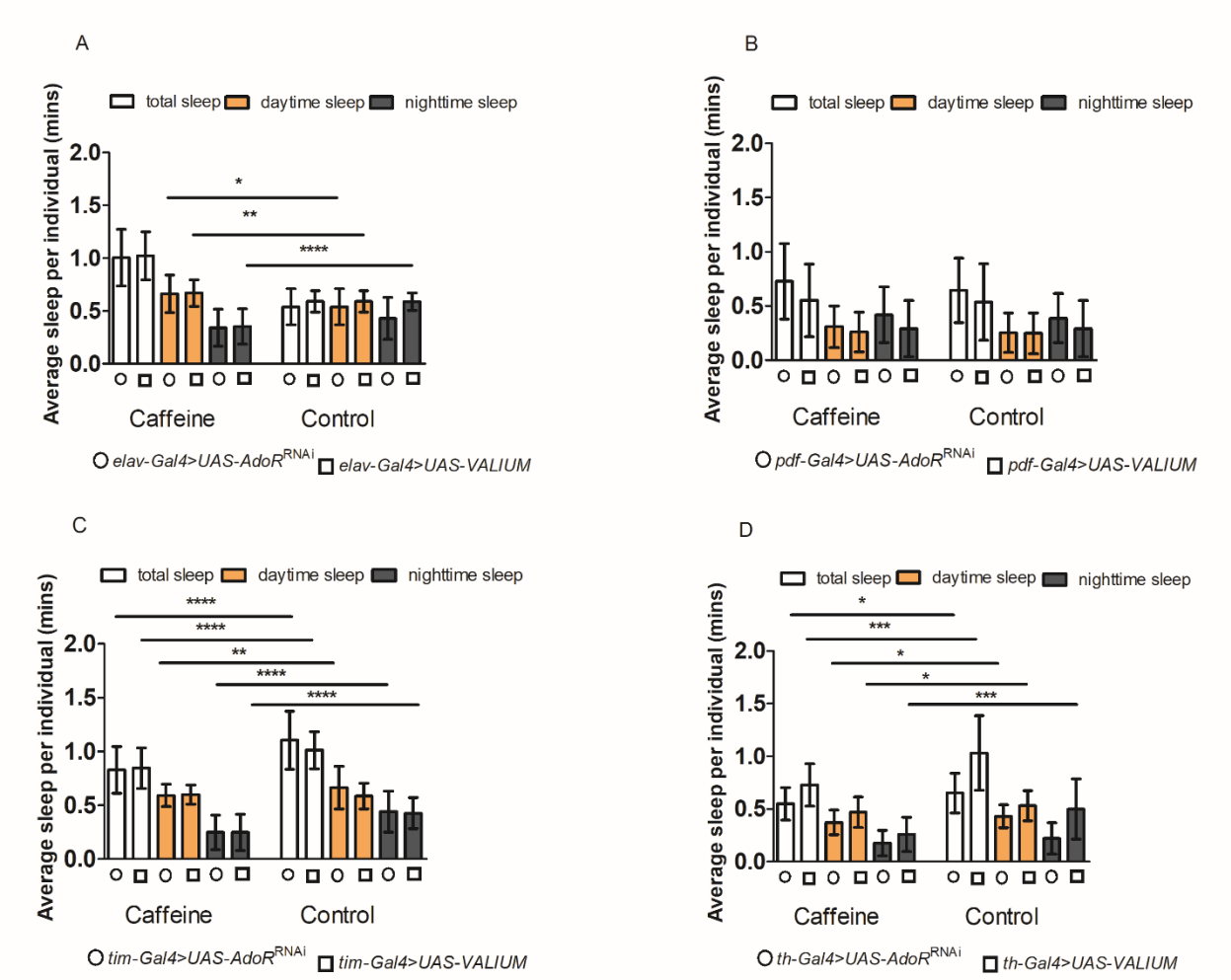


**Fig. 3.14.** The effect of 0.5 mg/ml of caffeine on total, daytime, and nighttime sleep in transgenic flies with *dAdoR* silencing in (A) all neurons (B) *pdf*-expressing (clock) neurons (C) *tim*-expressing neurons (D) *th*-expressing dopaminergic neurons.

The daytime sleep in *elav-Gal4>UAS-VALIUM* (+) comparing with the control *elav-Gal4>UAS-VALIUM* (-) increased by 7% (Mann-Whitney *U*-test, $U = 257.5$, $p < 0.005$) but nighttime sleep was significantly reduced by 23% (Mann-Whitney *U*-test, $U = 257.5$, $p < 0.005$)

[**Fig. 3.14 (A)**]. In *pdf*-expressing clock neurons, silencing of *dAdoR* did not cause changes in total, daytime, and nighttime sleep after caffeine treatment [**Fig. 3.14 (B)**].

The silencing of *dAdoR* in *tim*-expressing neurons caused a decrease in total, daytime, and nighttime sleep. Total sleep was reduced by 28% in the group *tim-Gal4> UAS-AdoR*$^{RNAi}$ (+) comparing with *tim-Gal4> UAS-AdoR*$^{RNAi}$ (-) (Mann-Whitney *U*-test, $U = 383.\ 0$, $p < 0.0001$), while in the control group *tim-Gal4>UAS-VALIUM* (+) the total sleep was reduced by 26% when compared with *tim-Gal4>UAS-VALIUM* (-) (Mann-Whitney *U*-test, $U = 484.0$, $p < 0.0001$).

The daytime sleep in the group *tim-Gal4> UAS-AdoR*$^{RNAi}$ (+) was reduced by 7% comparing with the control *tim-Gal4> UAS-AdoR*$^{RNAi}$ (-) (Mann-Whitney *U*-test, $U = 555.\ 5$, $p < 0.0065$). No changes in the daytime sleep were observed in the groups *tim-Gal4>UAS-VALIUM* (+) and *tim-Gal4>UAS-VALIUM* (-). The nighttime sleep was also reduced in the group *tim-Gal4> UAS-AdoR*$^{RNAi}$ (+) treated with caffeine comparing with the untreated group *tim-Gal4> UAS-AdoR*$^{RNAi}$ (-) by 53% (Mann-Whitney *U*-test, $U = 367.0$ $p < 0.0001$).

**Table 3.8. Summary of *dAdoR* silencing in different groups of neurons after treatment with 0.5 mg/ml of caffeine exposure.**

| N | Genotype | Total sleep | Daytime sleep | Nighttime sleep |
|---|---|---|---|---|
| 21 | *elav-Gal4>UAS-AdoR*$^{RNAi}$ *(+) vs.* | nc | ↑ | nc |
| 14 | *elav-Gal4>UAS- AdoR*$^{RNAi}$ *(-)* | | | |
| 56 | *elav-Gal4>UAS-VALIUM (+) vs.* | nc | ↑ | ↓ |
| 16 | *elav-Gal4> UAS-VALIUM (-)* | | | |
| 38 | *th-Gal4 >UAS- AdoR*$^{RNAi}$ *(+) vs.* | ↓ | ↓ | nc |
| 46 | *th-Gal4 >UAS- AdoR*$^{RNAi}$ *(-)* | | | |
| 32 | *th-Gal4 > UAS-VALIUM (+) vs.* | ↓ | ↓ | ↓ |
| 42 | *th-Gal4 > UAS-VALIUM (-)* | | | |
| 40 | *tim-Gal4 >UAS- AdoR*$^{RNAi}$ *(+) vs.* | ↓ | nc | ↓ |
| 42 | *tim-Gal4 >UAS- AdoR*$^{RNAi}$ *(-)* | | | |
| 39 | *tim-Gal4 > UAS-VALIUM (+) vs.* | nc | nc | nc |
| 47 | *tim-Gal4 > UAS-VALIUM (-)* | | | |
| 57 | *pdf-Gal4 >UAS- AdoR*$^{RNAi}$ *(+) vs.* | nc | nc | nc |
| 63 | *pdf-Gal4 >UAS- AdoR*$^{RNAi}$ *(-)* | | | |
| 63 | *pdf-Gal4 > UAS-VALIUM (+)* | nc | nc | nc |
| | *pdf-Gal4 > UAS-VALIUM (-)* | | | |

N shows the sample size for each genotype for three independent repetitions. The genotype column shows the comparison between caffeine treated (+) and control (-) group. Changes observed in TS,

DS and NS are indicated using downward arrows (blue) for decrease and upward arrows (red) for increase while no changes are abbreviated as “nc”.

There was 20% reduction in nighttime sleep in the group of *tim-Gal4>UAS-VALIUM* (+) comparing with *tim-Gal4>UAS-VALIUM* (-) (Mann-Whitney *U*-test, $U = 351.0$, $p < 0.0001$) [**Fig. 3.14 (C)]**. In dopaminergic neurons silencing of *dAdoR* (**Table 3.8**) caused a decrease in total sleep by 11% (Mann-Whitney *U*-test, $U = 605.0$, $p < 0.0113$) and in *siesta* by 30% (Mann-Whitney *U*-test, $U = 618.0$, $p < 0.0108$) in the group *th-Gal4> UAS-AdoR*$^{RNAi}$ (+) when compared to *th-Gal4> UAS-AdoR*$^{RNAi}$ (-).

Interestingly, caffeine treatment did not decrease nighttime sleep in the same groups. In addition to these changes, I found that total sleep, *siesta*, and nighttime sleep in *th-Gal4>UAS-VALIUM* (+) comparing with *th-Gal4>UAS-VALIUM* (-) were significantly decreased. Total sleep was reduced by 31%; Mann-Whitney *U*-test, $U = 338.0$, $p < 0.0001$), *siesta* by 7% (Mann-Whitney *U*-test, $U = 503.5$, $p < 0.0334$) and nighttime sleep by 23% (Mann-Whitney *U*-test, $U = 347.5$, $p < 0.0002$) [**Fig.3.14 (D)]**.

## 4. Discussion

The survival rate, revealing the general strengths and vigor of flies, showed that enhanced adenosine signalling is harmful to young flies, as it leads to increased mortality at the initial stage of life of adult flies. This finding is consistent with results of other authors showing that an excess of extracellular adenosine causes the death of *Drosophila* larvae and pupae [325]. However, I also observed an increased survival of middle-aged individuals with overexpressed *dAdoR*. Therefore, it appears that flies that survive through this initial stage can ultimately live longer than control flies (more than 80 days).

In contrast, flies with silenced *dAdoR*, although protected against early death, later showed higher mortality than control flies. Their survival decreased quite abruptly **(Fig. 3.2).** These results imply that there is a certain physiological level of adenosine and/or its receptors, possibly different at different stages of life (low in young flies and high in old flies), which once exceeded or not adequate, influences many biological processes and ultimately survival. Therefore, it is not surprising that chronic overproduction of adenosine has been found to occur in several pathological

conditions [326]. The climbing assay is a simple assay that enables quick and easy screening for climbing deficits of flies, thus providing information on their physiological ageing [327]. However, it cannot distinguish between neuronal and motor deficits. It is commonly known that the fitness of organisms declines with age [328]. To understand what cell types are affected and by what mechanism, I examined behavioural changes related to the up-regulation and downregulation of *AdoR*.

In this study, I found that adenosine signaling is a detrimental factor in maintaining fitness. In humans, the role of adenosine in maintaining fitness has also been pointed out. Athletes involved in various sports require interval training to achieve peak performance, and this training induces higher levels of adenosine production due to the increased rate of utilization of ATP. In my studies on fitness in the experimental group of 60-day-old flies, I found a drastic decline of fitness with the silenced *dAdoR* gene. In rodents, the cardiovascular protection provided by A2AR reduces with age [329]. In flies, the decrease of fitness was reversed in older and middle-aged females and older males after increasing dAdoR levels in all neurons. The higher level of *dAdoR* mRNA in glial cells, after overexpression of *dAdoR* in all glia, was also beneficial for the fitness of flies. These results suggest that adenosine influences the adaptive responses necessary for improved performance [330]. Oral administration of adenosine to athletes improved their strength, lean body mass, blood flow, and increased power and performance [331]. I observed a similar effect after enhancing adenosine signalling in older flies, but more studies are needed to explain the mechanisms of the observed effect. These changes occurred when enhance adenosine signalling was in neurons and glial cells.

However, in the case of photoreceptors, I observed no changes in fitness when *dAdoR* was overexpressed or silenced. But in survival and sleep as well as in cellular studies I found significant differences. It can be attributed to the fact that *GMR-Gal4* (a driver for the eye development) shows a broad expression profile. It has also been reported that *GMR* is expressed in the wing and leg imaginal discs, trachea, etc., in addition to the eye discs [316]. It is also present in neurons of the ventral ganglia in the second and third instar larvae [332,333].

The fruit fly is a diurnal species and shows a five-minute state of immobility or inactivity called sleep, which mostly occurs at night [91,312]. Flies also decrease their mobility during the day, and this daytime sleep is called *siesta*. Adenosine is a popular and well-known somnogenic agent that

increases sleep in mammals [334]. A2AR receptors are excitatory and are known to promote sleep. Adenosine A2A receptor agonists, administered to the subarachnoid space adjacent to the basal forebrain area, help to induce sleep [304].

A well-known fact is that sleep in the fruit fly and mammals has similarities that appear to be evolutionarily conserved. One of the hypotheses about sleep function is that it is a time for the process of synaptic plasticity [335]. Like the application of adenosine, the A1 receptor agonist cyclohexyl adenosine was found to increase sleep in both flies and mammals[89]. My results showed that an excess *dAdoR* mRNA induces more sleep at night and increases total sleep in flies, just as in rodents while suppressing the *dAdoR* gene expression does not affect sleep. *dAdoR* overexpression also affects *siesta*, which was shorter than in the control. In parallel, my results showed a decrease in night locomotor activity in flies with overexpression of *dAdoR*. I also observed that most flies with overexpression or silencing of *dAdoR* were rhythmic in locomotor activity and period of the rhythm was similar in experimental and control flies.

Adenosine has many physiological functions throughout the nervous system [336]. As a neuromodulator, it plays a role in the fine-tuning of synaptic transmission [337]. Although both A1 and A2 receptors are involved in such actions, it seems more concrete that A2AR is a key player in the regulation of neuromuscular transmission [338]. In flies, overexpression of *dAdoR* in glial cells also affected sleep, however, only total sleep and nighttime sleep were longer, but the *siesta* did not change. Upon silencing the *AdoR* gene in clock neurons, there was no effect on the sleep of fruit flies. This lack of effect may be due to weak signalling that occurred due to the down-regulation of the *dAdoR* gene.

The obtained results suggest a greater role for adenosine and its receptors in neuron-glial crosstalk in *Drosophila.* Adenosine is responsible for the interaction between glial cells and neurons. An increase in the *dAdoR* mRNA in glia and neurons promotes sleep, but the *siesta* decreases only after a higher level of *dAdoR* mRNA in neurons but not in the glia. It means that night sleep is regulated by adenosine and its receptors, but shorter *siesta* could be an effect of longer sleep during the night regulated by neurons, or the regulation of *siesta* is by a mechanism independent of dAdoR.

Based on the changes observed in the fly survival and fitness test, I was curious to know about the role of the presynaptic protein BRP after *dAdoR* silencing in the fruit fly's retina photoreceptors or in glial cells of the fruit fly and whether it is related to changes of fitness in flies with silenced *dAdoR*.

My results showed that BRP abundance still maintains its oscillation in tetrad synapses of the lamina and shows the daily rhythm even after *dAdoR* silencing. However, the level of this protein mostly decreases after silencing of *dAdoR* in both photoreceptors and glial cells. It indicates that lower expression of BRP in experimental flies after silencing of *dAdoR* may have an impact on fitness. Earlier studies have shown that lower expression of BRP affects the ultrastructure of synaptic active zones (AZ) in *Drosophila* [212]. Another study reported that flies carrying pan-neuronal *brp-RNAi* showed locomotor defects during the climbing assay [212]. Flies with lower expression of BRP could not sustain stable flight and crashed to the ground (hence, Bruch pilot)[212].

A similar effect was observed in the climbing test in the present study. BRP is important to maintain the structure of AZ and proper neurotransmission at the *Drosophila* neuromuscular junction (NMJ) [212]. Therefore, it is obvious that flying with lower BRP results in an unstable flight. In control flies, the BRP level increases during the first part of the day. This increase is attributed to the blue-light-sensitive protein CRY (cryptochrome) that is responsible for the degradation of BRP, resulting in its higher synthesis [339,340]. I observed two peaks, first during the beginning of the day (ZT1) and second during the beginning of the night (ZT13). This daily rhythm exhibits a bimodal pattern of two peaks called the morning peak and the evening peak observed previously in WT flies in BRP abundance in the lamina and in locomotor activity of the fruit fly [216].

These changes correspond to the rhythmic changes previously reported in the lamina photoreceptor terminals (R1 – R6), as well as their postsynaptic partners L1 and L2 large monopolar cells (LMC). These LMCs swell during the day and shrink at night in *Musca domestica* [202], while in *Drosophila* they swell twice during the day, at the beginning of the day and the beginning of the night [203].

In my study, I focused on the lamina because it provides a convenient part of the brain for studying various processes in the central nervous system, such as synaptic plasticity. The observations from the longitudinal sections of the lamina cartridges showed that the level of BRP

protein oscillates in the distal and proximal depth of the lamina. The level of this protein in the distal lamina was comparatively higher than in the proximal lamina in both the experimental and control groups. Although I observed cyclical changes in the level of the BRP protein at other time points, at ZT13 I found that the level of this protein was high in both experimental and control groups, when flies exhibit high locomotor activity.

Furthermore, I observed the lowest expression of BRP in the lamina of the experimental group at the beginning of the day (ZT1) when *dAdoR* was silenced in glia cells. This resulted in the lack of the morning peak of BRP. The absence of the morning peak indicates an involvement of glial cells via dAdoR on synaptic transmission in tetrad synapses during the day. While at the beginning of the night, BRP level was lower than in the controls, but it is still higher than that observed at the beginning of the day. In the later part of the night, the level of BRP in both the distal and proximal lamina did not show any significant changes between the experimental and control groups. This means that BRP level can still oscillate despite the silencing of *dAdoR*. However, it does not resemble the bimodal activity pattern, which I observed in the case of photoreceptors for the experimental group but in the controls, the BRP level showed a bimodal pattern. This also means that the working mechanism of the adenosine receptor is different in the photoreceptors and glia cells present in the visual system of *Drosophila*. Furthermore, it is seen that BRP is crucial for maintaining fitness and locomotion in *Drosophila.*

*Drosophila* is a good model organism because of the ease of genetic manipulations that give rise to extreme phenotypes that help us to understand several mechanisms, especially signalling. Secondly, I can study age-dependent changes in *Drosophila* which can be useful for studies in humans. *Drosophila* has a single AR which is 30% similar to the mammalian A2AR. In *Drosophila*, the adenosine signalling mechanism has been studied in the case of synaptic plasticity [341], hyperplastic epithelial tumor growth [342] and immune responses [343].

Sleep is conserved in flies and mammals. Sleep in both is even influenced by the same stimulants and hypnotics [91,312]. However, the signalling mechanism of caffeine on sleep in flies has not been studied in details. Compared to vertebrates, the adenosine signalling system is quite simple in *Drosophila*. Due to a single type of adenosine receptor, the chance of observing abnormalities of sleep becomes clear and high. I treated flies of different age (3 days, 30 days, and 50 days old) with various concentration of caffeine (0.1, 0.5, and 1 mg/ml).

In my studies with WT (wild type) flies we found that caffeine affects total sleep differently in young males and females (3 days old). Total sleep in flies exposed to the low concentration of 0.1mg/ml showed a decrease in both males and females. In turn increasing the concentration to 0.5 and 1 mg/ml caused a significant increase in total sleep in males. Whereas, in young females, the higher dose of 1 mg/ml caused no changes in total sleep. In addition to these changes observed in total sleep, we found interesting changes in *siesta*.

Male *siesta* is more sensitive to the concentration of caffeine in food. In males, *siesta* was reduced after the low concentration of 0.1 mg/ml of caffeine while increase in *siesta* occurred after the higher concentrations 0.5 and 1 mg/ml. In females, the concentration of 0.1 mg/ml was unable to influence *siesta*. My results indicate that caffeine affects sleep, especially total and daytime sleep (*siesta*) depending on the concentration and gender.

Unlike total sleep and daytime sleep, caffeine's role in significantly decreasing nighttime sleep is more robust and reproducible at all concentrations. Next, I compared the age-dependent changes for controls and caffeine-treated groups in WT flies.

Total sleep in male controls showed age-dependent differences only between 30 days and 50 days old. Whereas, in the caffeine group I observed changes in total sleep for all age flies (3 days, 30 days, 50 days). Unlike these changes in total sleep, the controls showed an increase in *siesta* and nighttime sleep with age. While in caffeine treated group, age-dependent changes in *siesta* were observed only between 3 days and 50 days of flies whereas, no changes in nighttime sleep were observed with age.

The comparison between groups (caffeine vs. control) showed higher total sleep only in 3 days old caffeine-treated flies. *Siesta* was increased and nighttime sleep was decreased for 3 days, 30 days, and 50 days caffeine-treated flies in comparing with controls.

Unlike the observed changes in males, age-dependent differences in female controls were detected only in total sleep while daytime and nighttime sleep showed no changes. In caffeine-treated females total sleep was increased in 3 days and 50 days old while in middle-age flies (30 days old) it was decreased. There was an increase of *siesta* in flies in all age studied. Apart from this, nighttime sleep was significantly reduced with age. These changes suggest that caffeine influence age-dependent changes which are more robust in females than in males.

Comparisons between-groups of caffeine treated and control showed a decrease of total sleep only in middle-age females (30 days), while daytime sleep was significantly higher in 3 days and 50 days old caffeine-treated flies. In addition to these changes, nighttime sleep between all age caffeine-treated flies and controls showed a significant decrease.

Based on the changes in WT flies, I examined sleep in transgenic flies with overexpression and silencing of *dAdoR* in all neurons, *pdf*-expressing neurons, *tim*-expressing neurons, and dopaminergic neurons. The main reason of these experiments was to focus on the clock neurons because they are involved in sleep timing and duration in *Drosophila melanogaster*. The previous studies of other authors on caffeine showed its role in clock distortion and circadian period lengthening in both flies [292] and mammals [289–291]. My results suggest no abnormalities or circadian period lengthening in all groups of transgenic flies. Circadian period lengthening was observed only in WT flies after caffeine treatment. This could be attributed to the fact that caffeine causes a partial blockade of adenosine receptors [344].

When *dAdoR* was overexpressed in all neurons, caffeine caused a significant decrease in total sleep, *siesta*, and nighttime sleep. Whereas, in *pdf*-expressing neurons, I observed no changes in sleep, in *tim*-expressing neurons and dopaminergic neurons, I found a decrease in total and nighttime sleep with no changes in *siesta*.

Interestingly, after silencing *dAdoR* in all neurons, I saw that caffeine caused an increase in *siesta* but did not influence total sleep and nighttime sleep. Again, in *pdf*-expressing neurons, no changes occured. In *tim*-expressing neurons, there was an overall decrease in sleep while in dopaminergic neurons there was a decrease in total sleep and *siesta*, while nighttime sleep remained unchanged.

Caffeine is known to interact with the dopaminergic system to exert some behavioural effects [345,346]. This action is likely mediated by the inhibition of A2A adenosine receptor, which is primarily localized in dopamine-rich areas of the brain [296]. The changes in sleep after overexpression and silencing of *dAdoR* in dopaminergic neurons can be explained through the mechanism of formation of the A2AR- D2R heterodimer [347–350]. Shorter total sleep and *siesta* after *dAdoR* silencing can occur when caffeine interferes with A2AR and increases D2R, leading to wake promotion [351,352]. Similarly, increased expression of adenosine receptor may interfere with

the expression of D2R (dopaminergic receptor D2) resulting in no changes in *siesta* when *dAdoR* is overexpressed.

My results suggest that adenosine receptors are involved in regulating *siesta*. Furthermore, the changes produced by caffeine were not robust and did not cause any distortion of the circadian clock. This could be due to the involvement of adenosine receptors. The study on A2AR knockout mice has shown that caffeine causes only 50% blockage of adenosine receptors [296]. Overexpression and silencing of *dAdoR* in *pdf*-expressing neurons had no effect on sleep or the clock although PDF is an important neurotransmitter of the circadian clock that provides a signal to synchronize the clock [353].

The results with WT and transgenic *Drosophila* strains showed that caffeine affects nighttime sleep, regardless of its concentration in food or age of flies. Furthermore, the influence on nighttime sleep is independent of adenosine receptors. In humans, caffeine consumption causes a reduction of 6-sulfatoxymelatonin (the main metabolite of melatonin) in the following night [293]. Therefore, caffeine consumption and its effect on melatonin secretion are also known to be one of the causes of night sleep disruption. Whereas *Drosophila* studies have reported that caffeine-induced arousal at night is facilitated by the dopamine receptor DA1 [354,355], which is expressed in mushroom bodies (MB). Arousal increases because caffeine is known to activate the cAMP-PKA (Protein Kinase A) pathway, leading to increase cAMP level and sleep fragmentation in WT flies and flies lacking a functional adenosine receptor [292]. However, other studies on *Drosophila* have claimed that changes in feeding behaviour are responsible for caffeine-mediated sleep loss [298].

In my studies on sleep after *dAdoR* overexpression in all neurons, I saw an increase in nighttime sleep and shorter *siesta*. Whereas my results on caffeine treatment of transgenic flies showed that after *dAdoR* overexpression in all neurons *siesta* gets shorter, while in *tim*-expressing neurons and dopaminergic neurons it does not change. It means that caffeine is unable to influence *siesta* when *dAdoR* is overexpressed.

In addition, silencing of *dAdoR* resulted in changes of *siesta*. To conclude I found that in *Drosophila* caffeine exerts its effect by antagonizing adenosine receptors. These changes in the length of *siesta* involve adenosine receptors.

## 5. Conclusions

In the past, it was suggested that if there is a discovery of adenosine receptors in the fruit fly, then studying its role in sleep would be a golden opportunity. The adenosine receptor in *Drosophila* has been discovered, but its role in sleep and the regulation of other processes is still unknown.

In my study, I found that the overexpression of *dAdoR* promotes sleep during the night and decreases activity, just as in vertebrates. The study provided a new and different perspective on the role of *dAdoR* in the promotion of fitness and survival based on tissue specificity and showcased the role of *dAdoR* in the middle-aged survival of fruit flies. I conclude that ageing influences the expression of *dAdoR* in neurons that delays the senescence of negative geotaxis in middle-aged flies with progression in old age. In addition to this, the invertebrate *AdoR* is a key player in fine-tuning communication between neurons and glia. What happens to adenosine signalling when flies are old is unknown. This is probably an effect of the decrease in ATP and adenosine production with ageing. The effect of *dAdoR* could be strain-specific and tissue-specific, but my study showed important functions of adenosine receptors in the regulation of sleep, longevity, fitness, locomotor activity and synaptic plasticity.

In turn my experiments on caffeine, showed that the mode of action of caffeine on sleep physiology and behaviour is highly dependent on its mode of administration. I found that the caffeine concentration, sex and age of flies, their genetic background, and A2AR-D2R antagonism are some of the factors that can modulate the effect of caffeine on daytime and nighttime sleep. All these helped to examine more deeply the caffeine signalling mechanism in *Drosophila*, which may be important for clinical trials of adenosine receptor-based therapy.

## 6. Summary

The present work summarizes the functions of the *Drosophila* adenosine receptor (dAdoR) in the living processes of this species such as lifespan, fitness, sleep, and locomotor activity. My results indicate that *dAdoR* overexpression is highly beneficial not only in increasing sleep but also, in improving fitness in older flies. However, its effect on the lifespan has a moderate effect on older flies and is negative for younger flies. On the contrary, silencing of *dAdoR* decreases the fitness and sleep of flies but has a positive impact on the lifespan of younger flies. This decrease in fitness can be attributed to the level of the BRP protein levels, which is influenced after *dAdoR* silencing in photoreceptors and glial cells. I found that lower level of BRP is co-related with decrease of fitness, reduced locomotor activity, and unstable flight in the transgenic lines. This shows that adenosine is important in maintaining the fitness. Since fly's sleep and mammalian sleep share several similarities and are influenced by the same stimulants and hypnotics, the molecular pathway by which caffeine affects sleep in the presence of adenosine receptors is still unknown. Since sleep timing in flies is influenced by the circadian clock, I observed that the action of caffeine on clock neurons of transgenic flies with overexpression or silencing was less robust. Since caffeine is known to cause clock distortion, I believe that the presence of adenosine receptors prevented this action. Although the role of caffeine to decrease nighttime sleep is universally known, my studies showed that adenosine receptors are involved in regulating *siesta* in *Drosophila.*

## 7. References


1. Shimkets, L. J. & Dworkin, M. Excreted adenosine is a cell density signal for the initiation of fruiting body formation in Myxococcus xanthus. *Dev. Biol.* **84**, 51–60 (1981).
2. Osswald, H., Spielman, W. S. & Knox, F. G. Mechanism of adenosine-mediated decreases in glomerular filtration rate in dogs. *Circ. Res.* **43**, 465–469 (1978).
3. Pawlowska, D., Granger, J. P. & Knox, F. G. Effects of adenosine infusion into renal interstitium on renal hemodynamics. *Am. J. Physiol.* **252**, F678–F682 (1987).
4. Berthold, H., Just, A., Kirchheim, H. R., Osswald, H. & Ehmke, H. Renal haemodynamic responses to exogenous and endogenous adenosine in conscious dogs. *J. Physiol.* **510**, (1998).
5. Fredholm, B. B., Irenius, E., Kull, B. & Schulte, G. Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. *Biochem. Pharmacol.* **61**, (2001).
6. Haskó, G., Linden, J., Cronstein, B. & Pacher, P. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat. Rev. Drug Discov.* **7**, 759–770 (2008).
7. Cunha, R. A. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochem. Int.* **38**, 107–125 (2001).
8. Fredholm, B. B., IJzerman, A. P., Jacobson, K. A., Linden, J. & Müller, C. E. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and Classification of Adenosine ReceptorsAn Update. *Pharmacol. Rev.* **63**, 1–34 (2011).
9. de Mendonça, A. & Ribeiro, J. A. Adenosine and synaptic plasticity. *Drug Dev. Res.* **52**, 283–290 (2001).
10. Van der Graaf, P. H. *et al.* Mechanism-based pharmacokinetic-pharmacodynamic modeling of antilipolytic effects of adenosine A(1) receptor agonists in rats: prediction of tissue-dependent efficacy in vivo. *J. Pharmacol. Exp. Ther.* **290**, 702–709 (1999).
11. Bouma, M. G., Stad, R. K., van den Wildenberg, F. A. & Buurman, W. A. Differential regulatory effects of adenosine on cytokine release by activated human monocytes. *J. Immunol.* **153**, 4159–4168 (1994).
12. Sebastião, A. M. & Ribeiro, J. A. Fine-tuning neuromodulation by adenosine. *Trends Pharmacol. Sci.* **21**, 341–346 (2000).

13. Pubchem. National Center for Biotechnology Information. *PubChem Compound Summary for CID 60961, Adenosine" PubChem* 1 https://pubchem.ncbi.nlm.nih.gov/compound/Adenosine. (2022).
14. Jacobson, K. A., Van Galen, P. J. M. & Williams, M. Adenosine receptors: pharmacology, structure-activity relationships, and therapeutic potential. *J. Med. Chem.* **35**, 407–422 (1992).
15. Latini, S. & Pedata, F. Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J. Neurochem.* **79**, 463–484 (2001).
16. Paes-De-Carvalho, R. Adenosine as a signaling molecule in the retina: biochemical and developmental aspects*. **74**, 437–451 (2002).
17. Ham, J. & Evans, B. A. J. An emerging role for adenosine and its receptors in bone homeostasis. *Frontiers in Endocrinology* vol. 3 at https://doi.org/10.3389/fendo.2012.00113 (2012).
18. Ritzel, M. W. *et al.* Molecular cloning and functional expression of cDNAs encoding a human Na+-nucleoside cotransporter (hCNT1). *Am. J. Physiol. Physiol.* **272**, C707–C714 (1997).
19. Zimmermann, H. Extracellular metabolism of ATP and other nucleotides. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **362**, 299–309 (2000).
20. Godinho, R. O., Duarte, T. & Pacini, E. S. A. New perspectives in signaling mediated by receptors coupled to stimulatory G protein: The emerging significance of cAMP effux and extracellular cAMP-adenosine pathway. *Frontiers in Pharmacology* vol. 6 at https://doi.org/10.3389/fphar.2015.00058 (2015).
21. Borea, P. A., Gessi, S., Merighi, S. & Varani, K. Adenosine as a Multi-Signalling Guardian Angel in Human Diseases: When, Where and How Does it Exert its Protective Effects? *Trends Pharmacol. Sci.* **37**, 419–434 (2016).
22. Fredholm, B. B. *et al.* Structure and function of adenosine receptors and their genes. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **362**, 364–374 (2000).
23. Baldwin, J. M. Structure and function of receptors coupled to G proteins. *Curr. Opin. Cell Biol.* **6**, (1994).
24. Meyerhof, W., Müller-Brechlin, R. & Richter, D. Molecular cloning of a novel putative G-protein coupled receptor expressed during rat spermiogenesis. *FEBS Lett.* **284**, 155–160

(1991).

25. Wakisaka, N. *et al.* An Adenosine Receptor for Olfaction in Fish. *Curr. Biol.* **27**, 1437-1447.e4 (2017).
26. Biber, K., Klotz, K. N., Berger, M., Gebicke-Härter, P. J. & Van Calker, D. Adenosine A1 receptor-mediated activation of phospholipase C in cultured astrocytes depends on the level of receptor expression. *J. Neurosci.* **17**, 4956–4964 (1997).
27. Othman, T., Yan, H. & Rivkees, S. A. Oligodendrocytes Express Functional A1 Adenosine Receptors That Stimulate Cellular Migration. *Glia* **44**, 166–172 (2003).
28. PJ Gebicke-Haerter, F. C. J. T. H. N. M. B. D. V. C. Both adenosine A1- and A2- receptors are required to simulate microglial proliferation. *Neurochem* **29**, 37–42 (1996).
29. Mahan, L. C. *et al.* Cloning and expression of an A1 adenosine receptor from rat brain. *Mol. Pharmacol.* **40**, 1–7 (1991).
30. Reppert, S. M., Weaver, D. R., Stehle, J. H. & Rivkees, S. A. Molecular Cloning and Characterization of a Rat A1-Adenosine Receptor that is Widely Expressed in Brain and Spinal Cord. *Mol. Endocrinol.* **5**, 1037–1048 (1991).
31. Tucker, A. L., Linden, J., Robeva, A. S., D'Angelo, D. D. & Lynch, K. R. Cloning and expression of a bovine adenosine A1 receptor cDNA. *FEBS Lett.* **297**, 107–111 (1992).
32. Otah, M. E. & Stiles, G. L. Adenosine Receptors. *Annu. Rev. Physiol.* **54**, 211–225 (1992).
33. Libert, F. *et al.* Cloning and functional characterization of a human Al adenosine receptor. *Biochem. Biophys. Res. Commun.* **187**, 919–926 (1992).
34. Townsend-Nicholson, A. & Shine, J. Molecular cloning and characterisation of a human brain A1 adenosine receptor cDNA. *Mol. Brain Res.* **16**, 365–370 (1992).
35. Ren, H. & Stiles, G. L. Characterization of the human A1 adenosine receptor gene. Evidence for alternative splicing. *J. Biol. Chem.* **269**, 3104–3110 (1994).
36. Bhattacharya, S., Dewitt, D. L., Burnatowska-Hledin, M., Smith, W. L. & Spielman, W. S. Cloning of an adenosine Al receptor-encoding gene from rabbit. *Gene* **128**, 285–288 (1993).
37. Londos, C., Cooper, D. M. & Wolff, J. Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci.* **77**, 2551–2554 (1980).
38. Calker, D. van, Müller, M. & Hamprecht, B. Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.* **33**, 999–1005 (1979).

39. Maenhaut, C. *et al.* RDC8 codes for an adenosine A2 receptor with physiological constitutive activity. *Biochem. Biophys. Res. Commun.* **173**, 1169–1178 (1990).
40. Fink, J. S. *et al.* Molecular cloning of the rat A2 adenosine receptor: selective co-expression with D2 dopamine receptors in rat striatum. *Mol. Brain Res.* **14**, 186–195 (1992).
41. Furlong, T. J., Pierce, K. D., Selbie, L. A. & Shine, J. Molecular characterization of a human brain adenosine A2 receptor. *Mol. Brain Res.* **15**, (1992).
42. Besancon, E., Guo, S., Lok, J., Tymianski, M. & Lo, E. H. Beyond NMDA and AMPA glutamate receptors: emerging mechanisms for ionic imbalance and cell death in stroke. *Trends Pharmacol. Sci.* **29**, 268–275 (2008).
43. Hu, Q. *et al.* Aberrant adenosine A2A receptor signaling contributes to neurodegeneration and cognitive impairments in a mouse model of synucleinopathy. *Exp. Neurol.* **283**, 213–223 (2016).
44. Nanoff, C. & Stiles, G. L. Solubilization and Characterization of the A2-Adenosine Receptor. *J. Recept. Res.* **13**, 961–973 (1993).
45. Stehle, J. H. *et al.* Molecular cloning and expression of the cDNA for a novel A2-adenosine receptor subtype. *Mol. Endocrinol.* **6**, 384–393 (1992).
46. Rivkees, S. A. & Reppert, S. M. RFL9 encodes an A2b-adenosine receptor. *Mol. Endocrinol.* **6**, 1598–1604 (1992).
47. Pierce, K. D., Furlong, T. J., Selbie, L. A. & Shine, J. Molecular cloning and expression of an adenosine A2b receptor from human brain. *Biochem. Biophys. Res. Commun.* **187**, 86–93 (1992).
48. Feoktistov, I. & Biaggioni, I. Adenosine A2B Receptors. *Pharmacol. Rev.* **49**, 381–402 (1997).
49. Daly, J. W., Butts-Lamb, P. & Padgett, W. Subclasses of adenosine receptors in the central nervous system: Interaction with caffeine and related methylxanthines. *Cell. Mol. Neurobiol.* **3**, 69–80 (1983).
50. Bruns, R. F., Lu, G. H. & Pugsley, T. A. Characterization of the A2 adenosine receptor labeled by [3H]NECA in rat striatal membranes. *Mol. Pharmacol.* **29**, (1986).
51. Marquardt, D. L., Walker, L. L. & Heinemann, S. Cloning of two adenosine receptor subtypes from mouse bone marrow-derived mast cells. *J. Immunol.* **152**, 4508–4515 (1994).
52. Beukers, M. W., den Dulk, H., van Tilburg, E. W., Brouwer, J. & Ijzerman, A. P. Why Are

A2B Receptors Low-Affinity Adenosine Receptors? Mutation of Asn273 to Tyr Increases Affinity of Human A2B Receptor for 2-(1-Hexynyl)adenosine. *Mol. Pharmacol.* **58**, 1349–1356 (2000).

53. Yang, D. *et al.* The A2B adenosine receptor protects against inflammation and excessive vascular adhesion. *J. Clin. Invest.* **116**, 1913–1923 (2006).
54. Grenz, A., Homann, D. & Eltzschig, H. K. Extracellular adenosine: A safety signal that dampens hypoxia-induced inflammation during ischemia. *Antioxidants and Redox Signaling* vol. 15 at https://doi.org/10.1089/ars.2010.3665 (2011).
55. Johnston-Cox, H. A., Koupenova, M. & Ravid, K. A2 Adenosine Receptors and Vascular Pathologies. *Arterioscler. Thromb. Vasc. Biol.* **32**, 870–878 (2012).
56. Koupenova, M., Johnston-Cox, H. & Ravid, K. Regulation of Atherosclerosis and Associated Risk Factors by Adenosine and Adenosine Receptors. *Curr. Atheroscler. Rep.* **14**, 460–468 (2012).
57. Eckle, T. *et al.* Cardioprotection by ecto-5′-nucleotidase (CD73) and A2B adenosine receptors. *Circulation* **115**, 1581–1590 (2007).
58. Morello, S. & Miele, L. Targeting the adenosine A2b receptor in the tumor microenvironment overcomes local immunosuppression by myeloid-derived suppressor cells. *Oncoimmunology* **3**, (2014).
59. Patel, L. & Thaker, A. The effects of adenosine A2B receptor inhibition on VEGF and nitric oxide axis-mediated renal function in diabetic nephropathy. *Ren. Fail.* **36**, 916–924 (2014).
60. Tak, E. *et al.* CD73-dependent generation of adenosine and endothelial Adora2b signaling attenuate diabetic nephropathy. *J. Am. Soc. Nephrol.* **25**, 547–563 (2014).
61. Eisenstein, A., Patterson, S. & Ravid, K. The Many Faces of the A2b Adenosine Receptor in Cardiovascular and Metabolic Diseases. *J. Cell. Physiol.* **230**, 2891–2897 (2015).
62. Tang, J., Jiang, X., Zhou, Y. & Dai, Y. Effects of A2BR on the biological behavior of mouse renal fibroblasts during hypoxia. *Mol. Med. Rep.* **11**, 4397–4402 (2015).
63. Vecchio, E. A. *et al.* Ligand-Independent Adenosine A2B Receptor Constitutive Activity as a Promoter of Prostate Cancer Cell Proliferation. *J. Pharmacol. Exp. Ther.* **357**, 36–44 (2016).
64. Otsuki, T. *et al.* A3 adenosine receptor-mediated p53-dependent apoptosis in Lu-65 human lung cancer cells. *Cell. Physiol. Biochem. Int. J. Exp. Cell. Physiol. Biochem. Pharmacol.*

**30**, 210–220 (2012).

65. Peakman, M. -C & Hill, S. J. Adenosine A2B-receptor-mediated cyclic AMP accumulation in primary rat astrocytes. *Br. J. Pharmacol.* **111**, 191–198 (1994).
66. Murakami, S. *et al.* Adenosine regulates the production of interleukin-6 by human gingival fibroblasts via cyclic AMP/protein kinase A pathway. *J. Periodontal Res.* **35**, 93–101 (2000).
67. Sitaraman, S. V *et al.* Neutrophil-epithelial crosstalk at the intestinal lumenal surface mediated by reciprocal secretion of adenosine and IL-6. *J. Clin. Invest.* **107**, 861–869 (2001).
68. Lynge, J., Schulte, G., Nordsborg, N., Fredholm, B. B. & Hellsten, Y. Adenosine A2B receptors modulate cAMP levels and induce CREB but not ERK1/2 and p38 phosphorylation in rat skeletal muscle cells. *Biochem. Biophys. Res. Commun.* **307**, 180–187 (2003).
69. Fang, Y. & Olah, M. E. Cyclic AMP-dependent, protein kinase A-independent activation of extracellular signal-regulated kinase 1/2 following adenosine receptor stimulation in human umbilical vein endothelial cells: Role of exchange protein activated by cAMP 1 (Epac1). *J. Pharmacol. Exp. Ther.* **322**, (2007).
70. Darashchonak, N., Koepsell, B., Bogdanova, N. & Von Versen-Höynck, F. Adenosine A2B receptors induce proliferation, invasion and activation of cAMP response element binding protein (CREB) in trophoblast cells. *BMC Pregnancy Childbirth* **14**, (2014).
71. He, J., Zhao, Y., Deng, W. & Wang, D. Netrin-1 Promotes Epithelial Sodium Channel-Mediated Alveolar Fluid Clearance via Activation of the Adenosine 2B Receptor in Lipopolysaccharide-Induced Acute Lung Injury. *Respiration* **87**, 394–407 (2014).
72. Gao, Z., Chen, T., Weber, M. J. & Linden, J. A2B Adenosine and P2Y2 Receptors Stimulate Mitogen-activated Protein Kinase in Human Embryonic Kidney-293 Cells: CROSS-TALK BETWEEN CYCLIC AMP AND PROTEIN KINASE C PATHWAYS*. *J. Biol. Chem.* **274**, 5972–5980 (1999).
73. Linden, J., Auchampach, J. A., Jin, X. & Figler, R. A. The structure and function of A1 and A2B adenosine receptors. *Life Sci.* **62**, 1519–1524 (1998).
74. Panjehpour, M., Castro, M. & Klotz, K. N. Human breast cancer cell line MDA-MB-231 expresses endogenous A 2B adenosine receptors mediating a Ca 2+ signal. *Br. J.*

*Pharmacol.* **145**, 211–218 (2005).

75. Jim﷒nez, A. I. *et al.* Potentiation of ATP calcium responses by A2B receptor stimulation and other signals coupled to Gs proteins in type-1 cerebellar astrocytes. *Glia* **26**, 119–128 (1999).
76. Schulte, G. & Fredholm, B. B. The Gs-coupled adenosine A2b receptor recruits divergent pathways to regulate ERK1/2 and p38☆. *Exp. Cell Res.* **290**, 168–176 (2003).
77. Donoso, M. V., López, R., Miranda, R., Briones, R. & Huidobro-Toro, J. P. A2B adenosine receptor mediates human chorionic vasoconstriction and signals through arachidonic acid cascade. *Am. J. Physiol. - Hear. Circ. Physiol.* **288**, (2005).
78. Zhou, Q. Y. *et al.* Molecular cloning and characterization of an adenosine receptor: The A3 adenosine receptor. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 7432–7436 (1992).
79. Linden, J. Cloned adenosine A3 receptors: Pharmacological properties, species differences and receptor functions. *Trends Pharmacol. Sci.* **15**, 298–306 (1994).
80. Salvatore, C. A., Jacobson, M. A., Taylor, H. E., Linden, J. & Johnson, R. G. Molecular cloning and characterization of the human A3 adenosine receptor. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 10365–10369 (1993).
81. Figler, R. A. *et al.* Links between Insulin resistance, adenosine A2B receptors, and inflammatory markers in mice and humans. *Diabetes* **60**, 669–679 (2011).
82. Dolezelova, E., Nothacker, H. P., Civelli, O., Bryant, P. J. & Zurovec, M. A Drosophila adenosine receptor activates cAMP and calcium signaling. *Insect Biochem. Mol. Biol.* **37**, 318–329 (2007).
83. Kucerova, L. *et al.* Characterization of the Drosophila adenosine receptor: The effect of adenosine analogs on cAMP signaling in Drosophila cells and their utility for in vivo experiments. *J. Neurochem.* **121**, 383–395 (2012).
84. Buch, S., Melcher, C., Bauer, M., Katzenberger, J. & Pankratz, M. J. Opposing Effects of Dietary Protein and Sugar Regulate a Transcriptional Target of Drosophila Insulin-like Peptide Signaling. *Cell Metab.* **7**, 321–332 (2008).
85. Bajgar, A. *et al.* Extracellular Adenosine Mediates a Systemic Metabolic Switch during Immune Response. *PLoS Biol.* **13**, 1–23 (2015).
86. Knight, D. *et al.* Equilibrative nucleoside transporter 2 regulates associative learning and synaptic function in Drosophila. *J. Neurosci.* **30**, 5047–5057 (2010).

87. Poernbacher, I. & Vincent, J. P. Epithelial cells release adenosine to promote local TNF production in response to polarity disruption. *Nat. Commun.* **9**, 1–10 (2018).
88. Lin, Y. H. *et al.* Adenosine Receptor and Its Downstream Targets, Mod(mdg4) and Hsp70, Work as a Signaling Pathway Modulating Cytotoxic Damage in Drosophila. *Front. Cell Dev. Biol.* **9**, (2021).
89. Hendricks, J. C. *et al.* Rest in Drosophila Is a Sleep-like State. *Neuron* **25**, 129–138 (2000).
90. Nitz, D. A., van Swinderen, B., Tononi, G. & Greenspan, R. J. Electrophysiological Correlates of Rest and Activity in Drosophila melanogaster. *Curr. Biol.* **12**, 1934–1940 (2002).
91. Shaw, P. J., Cirelli, C., Greenspan, R. J. & Tononi, G. Correlates of sleep and waking in Drosophila melanogaster. *Science* **287**, 1834–1837 (2000).
92. Van Swinderen, B., Nitz, D. A. & Greenspan, R. J. Uncoupling of Brain Activity from Movement Defines Arousal States in Drosophila. *Curr. Biol.* **14**, 81–87 (2004).
93. Ashton, K., Wagoner, A. P., Carrillo, R. & Gibson, G. *Quantitative Trait Loci for the Monoamine-Related Traits Heart Rate and Headless Behavior in Drosophila melanogaster*. https://academic.oup.com/genetics/article/157/1/283/6048367 (2001).
94. Konopka, R. J. & Benzer, S. Clock Mutants of Drosophila melanogaster. *Proc. Natl. Acad. Sci.* **68**, 2112–2116 (1971).
95. Yamamoto, D. & Koganezawa, M. Genes and circuits of courtship behaviour in Drosophila males. *Nat. Rev. Neurosci.* **14**, 681–692 (2013).
96. Kavitz Edwad A, and F. M. de la P. Aggression in Drosophila. *Behav. Neurosci.* **129**, 549–563 (2015).
97. Bhumika & Singh, A. K. Regulation of feeding behavior in Drosophila through the interplay of gustation, physiology and neuromodulation. *Frontiers in Bioscience - Landmark* vol. 23 at https://doi.org/10.2741/4686 (2018).
98. Kaun, K. R., Devineni, A. V & Heberlein, U. Drosophila melanogaster as a model to study drug addiction. *Hum. Genet.* **131**, 959–975 (2012).
99. Kahsai, L. & Zars, T. Learning and Memory in Drosophila: Behavior, Genetics, and Neural Systems. in *Recent advances in the use of Drosophila in neurobiology and neurodegeneration* (ed. Atkinson, N.) vol. 99 139–167 (Academic Press, 2011).
100. Franco, D. L., Frenkel, L. & Ceriani, M. F. The underlying genetics of Drosophila circadian

behaviors. *Physiology* vol. 33 at https://doi.org/10.1152/physiol.00020.2017 (2018).

101. Ly, S., Pack, A. I. & Naidoo, N. The neurobiological basis of sleep: Insights from Drosophila. *Neurosci. Biobehav. Rev.* **87**, 67–86 (2018).
102. Reichert, H. A tripartite organization of the urbilaterian brain: Developmental genetic evidence from Drosophila. *Brain Res. Bull.* **66**, 491–494 (2005).
103. Tessmar-Raible, K. The evolution of neurosecretory centers in bilaterian forebrains: insights from protostomes. *Semin. Cell Dev. Biol.* **18**, 492–501 (2007).
104. Littleton, J. T. & Ganetzky, B. Ion Channels and Synaptic Organization: Analysis of the Drosophila Genome. *Neuron* **26**, 35–43 (2000).
105. Rival, T. *et al.* Decreasing glutamate buffering capacity triggers oxidative stress and neuropil degeneration in the Drosophila brain. *Curr. Biol.* **14**, 599–605 (2004).
106. Cattaert, D. & Birman, S. Blockade of the central generator of locomotor rhythm by noncompetitive NMDA receptor antagonists in Drosophila larvae. *J. Neurobiol.* **48**, (2001).
107. Sinakevitch, I., Grau, Y., Strausfeld, N. J. & Birman, S. Dynamics of glutamatergic signaling in the mushroom body of young adult Drosophila. *Neural Dev.* **5**, 10 (2010).
108. Zhang, H. T., Crissman, A. M., Dorairaj, N. R., Chandler, L. J. & O'Donnell, J. M. Inhibition of cyclic AMP phosphodiesterase (PDE4) reverses memory deficits associated with NMDA receptor antagonism. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* **23**, 198–204 (2000).
109. Enell, L., Hamasaka, Y., Kolodziejczyk, A. & Nässel, D. R. γ-Aminobutyric acid (GABA) signaling components in Drosophila: Immunocytochemical localization of GABAB receptors in relation to the GABAA receptor subunit RDL and a vesicular GABA transporter. *J. Comp. Neurol.* **505**, 18–31 (2007).
110. Mao, Z. & Davis, R. L. Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: Anatomical and physiological heterogeneity. *Front. Neural Circuits* **3**, (2009).
111. Demchyshyn, L. L. *et al.* Cloning, expression, and localization of a chloride-facilitated, cocaine-sensitive serotonin transporter from Drosophila melanogaster. *Proc. Natl. Acad. Sci.* **91**, 5158–5162 (1994).
112. Petersen, C. I. & DeFelice, L. J. Ionic interactions in the Drosophila serotonin transporter identify it as a serotonin channel. *Nat. Neurosci.* **2**, 605–610 (1999).

113. Sitaraman, D. *et al.* Serotonin is necessary for place memory in *Drosophila*. *Proc. Natl. Acad. Sci.* **105**, 5579–5584 (2008).
114. Vargas, M. A., Luo, N., Yamaguchi, A. & Kapahi, P. A role for S6 kinase and serotonin in postmating dietary switch and balance of nutrients in D. melanogaster. *Curr. Biol.* **20**, 1006–1011 (2010).
115. Hewes, R. S. & Taghert, P. H. Neuropeptides and neuropeptide receptors in the Drosophila melanogaster genome. *Genome Res.* **11**, 1126–1142 (2001).
116. Nässel, D. R. & Homberg, U. Neuropeptides in interneurons of the insect brain. *Cell Tissue Res.* **326**, 1–24 (2006).
117. Hauser, S. L. & Oksenberg, J. R. The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration. *Neuron* **52**, 61–76 (2006).
118. DeZazzo, J., Xia, S., Christensen, J., Velinzon, K. & Tully, T. Developmental expression of an amn+ transgene rescues the mutant memory defect of amnesiac adults. *J. Neurosci.* **19**, (1999).
119. Liu, W., Guo, F., Lu, B. & Guo, A. amnesiac regulates sleep onset and maintenance in Drosophila melanogaster. *Biochem. Biophys. Res. Commun.* **372**, 798–803 (2008).
120. O'Kane, C. J. Drosophila as a Model Organism for the Study of Neuropsychiatric Disorders. in *Molecular and Functional Models in Neuropsychiatry* (ed. Hagan, J. J.) 37–60 (Springer Berlin Heidelberg, 2011). doi:10.1007/7854_2010_110.
121. Krishnan, B., Dryer, S. E. & Hardin, P. E. Circadian rhythms in olfactory responses of Drosophila melanogaster. *Nature* **400**, 375–378 (1999).
122. Manjunatha, T., Dass, S. H. & Sharma, V. K. Egg-laying rhythm in Drosophila melanogaster. *J. Genet.* **87**, 495–504 (2008).
123. Fujii, S., Krishnan, P., Hardin, P. & Amrein, H. Nocturnal Male Sex Drive in Drosophila. *Curr. Biol.* **17**, 244–251 (2007).
124. Fujii, S. & Amrein, H. Ventral lateral and DN1 clock neurons mediate distinct properties of male sex drive rhythm in *Drosophila*. *Proc. Natl. Acad. Sci.* **107**, 10590–10595 (2010).
125. Chatterjee, A., Tanoue, S., Houl, J. H. & Hardin, P. E. Regulation of Gustatory Physiology and Appetitive Behavior by the Drosophila Circadian Clock. *Curr. Biol.* **20**, 300–309 (2010).
126. Lyons, L. C. & Roman, G. Circadian modulation of short-term memory in drosophiia.

*Learn. Mem.* **16**, 19–27 (2009).

127. Boothroyd, C. E. & Young, M. W. The In(put)s and Out(put)s of the Drosophila Circadian Clock. *Ann. N. Y. Acad. Sci.* **1129**, 350–357 (2008).
128. Yoshii, T., Ahmad, M. & Helfrich-Förster, C. Cryptochrome mediates light-dependent magnetosensitivity of Drosophila's circadian clock. *PLoS Biol.* **7**, e1000086 (2009).
129. Toh, K. L. *et al.* An h*Per2* Phosphorylation Site Mutation in Familial Advanced Sleep Phase Syndrome. *Science (80-. ).* **291**, 1040–1043 (2001).
130. Xu, P., Atkinson, R., Jones, D. N. M. & Smith, D. P. Drosophila OBP LUSH Is Required for Activity of Pheromone-Sensitive Neurons. *Neuron* **45**, 193–200 (2005).
131. Delaunay, F., Thisse, C., Thisse, B. & Laudet, V. Differential regulation of Period 2 and Period 3 expression during development of the zebrafish circadian clock. *Gene Expr. Patterns* **3**, 319–324 (2003).
132. Pando, M. P., Pinchak, A. B., Cermakian, N. & Sassone-Corsi, P. A cell-based system that recapitulates the dynamic light-dependent regulation of the vertebrate clock. *Proc. Natl. Acad. Sci.* **98**, 10178–10183 (2001).
133. Rosbash, M. & Hall, J. C. The molecular biology of circadian rhythms. *Neuron* **3**, 387–398 (1989).
134. Sakai, T. & Ishida, N. Circadian rhythms of female mating activity governed by clock genes in *Drosophila*. *Proc. Natl. Acad. Sci.* **98**, 9221–9225 (2001).
135. Joshi, R., Cai, Y. D., Xia, Y., Chiu, J. C. & Emery, P. PERIOD Phosphoclusters Control Temperature Compensation of the Drosophila Circadian Clock. *Front. Physiol.* **13**, (2022).
136. Helfrich-Förster, C. *et al.* Development and morphology of the clock-gene-expreasing lateral neurons of Drosophila melanogaster. *J. Comp. Neurol.* **500**, 47–70 (2007).
137. Taghert, P. H. & Shafer, O. T. Mechanisms of clock output in the Drosophila circadian pacemaker system. *J. Biol. Rhythms* **21**, 445–457 (2006).
138. Shafer, O. T., Helfrich-Förster, C., Renn, S. C. P. & Taghert, P. H. Reevaluation of Drosophila melanogaster's neuronal circadian pacemakers reveals new neuronal classes. *J. Comp. Neurol.* **498**, 180–193 (2006).
139. Yoshii, T., Todo, T., Wülbeck, C., Stanewsky, R. & Helfrich-Förster, C. Cryptochrome is present in the compound eyes and a subset of Drosophila's clock neurons. *J. Comp. Neurol.* **508**, 952–966 (2008).

140. Stoleru, D., Peng, Y., Agosto, J. & Rosbash, M. Coupled oscillators control morning and evening locomotor behaviour of Drosophila. *Nature* **431**, 862–868 (2004).
141. Grima, B., Chélot, E., Xia, R. & Rouyer, F. Morning and evening peaks of activity rely on different clock neurons of the Drosophila brain. *Nature* **431**, (2004).
142. Rieger, D., Shafer, O. T., Tomioka, K. & Helfrich-Förster, C. Functional Analysis of Circadian Pacemaker Neurons in Drosophila melanogaster. *J. Neurosci.* **26**, 2531–2543 (2006).
143. Kaneko, M., Helfrich-Fö, C. & Hall, J. C. *Spatial and Temporal Expression of the period and timeless Genes in the Developing Nervous System of Drosophila: Newly Identified Pacemaker Candidates and Novel Features of Clock Gene Product Cycling*. (1997).
144. Johard, H. A. D. *et al.* Peptidergic clock neurons in Drosophila: Ion transport peptide and short neuropeptide F in subsets of dorsal and ventral lateral neurons. *J. Comp. Neurol.* **516**, 59–73 (2009).
145. Miyasako, Y., Umezaki, Y. & Tomioka, K. Separate Sets of Cerebral Clock Neurons Are Responsible for Light and Temperature Entrainment of Drosophila Circadian Locomotor Rhythms. *J. Biol. Rhythms* **22**, 115–126 (2007).
146. Hardin, P. E. Chapter 5 - Molecular Genetic Analysis of Circadian Timekeeping in Drosophila. in *The Genetics of Circadian Rhythms* (ed. Brody, S.) vol. 74 141–173 (Academic Press, 2011).
147. Allada, R., White, N. E., So, W. V., Hall, J. C. & Rosbash, M. A mutant Drosophila homolog of mammalian clock disrupts circadian rhythms and transcription of period and timeless. *Cell* **93**, (1998).
148. Blau, J. & Young, M. W. Cycling vrille Expression Is Required for a Functional Drosophila Clock. *Cell* **99**, 661–671 (1999).
149. Cyran, S. A. *et al.* vrille, Pdp1, and dClock form a second feedback loop in the Drosophila circadian clock. *Cell* **112**, (2003).
150. Darlington, T. K. *et al.* Closing the Circadian Loop: CLOCK-Induced Transcription of Its Own Inhibitors *per* and *tim*. *Science (80-. ).* **280**, 1599–1603 (1998).
151. Hao, H., Allen, D. L. & Hardin, P. E. *A Circadian Enhancer Mediates PER-Dependent mRNA Cycling in Drosophila melanogaster*. *MOLECULAR AND CELLULAR BIOLOGY* vol. 17 https://journals.asm.org/journal/mcb (1997).

152. Rutila, J. E. *et al.* CYCLE Is a Second bHLH-PAS Clock Protein Essential for Circadian Rhythmicity and Transcription of Drosophila period and timeless. *Cell* **93**, 805–814 (1998).
153. Price, J. L. *et al.* double-time Is a Novel Drosophila Clock Gene that Regulates PERIOD Protein Accumulation. **94**, 83–95 (1998).
154. Kloss, B. *et al.* The Drosophila Clock Gene double-time Encodes a Protein Closely Related to Human Casein Kinase Iε. *Cell* **94**, 97–107 (1998).
155. Kloss, B., Rothenfluh, A., Young, M. W. & Saez, L. Phosphorylation of PERIOD Is Influenced by Cycling Physical Associations of DOUBLE-TIME, PERIOD, and TIMELESS in the Drosophila Clock. *Neuron* **30**, 699–706 (2001).
156. Curtin, K. D., Huang, Z. J. & Rosbash, M. Temporally regulated nuclear entry of the Drosophila period protein contributes to the circadian clock. *Neuron* **14**, 365–372 (1995).
157. Gekakis, N. *et al.* Isolation of timeless by PER protein interaction: Defective interaction between timeless protein and long-period mutant PERL. *Science (80-. ).* **270**, (1995).
158. Zeng, H., Qian, Z., Myers, M. P. & Rosbash, M. A light-entrainment mechanism for the Drosophila circadian clock. *Nature* **380**, 129–135 (1996).
159. Sathyanarayanan, S., Zheng, X., Xiao, R. & Sehgal, A. Posttranslational Regulation of Drosophila PERIOD Protein by Protein Phosphatase 2A. *Cell* **116**, 603–615 (2004).
160. Bae, K., Lee, C., Sidote, D., Chuang, K. & Edery, I. Circadian Regulation of a *Drosophila* Homolog of the Mammalian *Clock* Gene: PER and TIM Function as Positive Regulators. *Mol. Cell. Biol.* **18**, 6142–6151 (1998).
161. Lee, C., Bae, K. & Edery, I. The Drosophila CLOCK Protein Undergoes Daily Rhythms in Abundance, Phosphorylation, and Interactions with the PER–TIM Complex. *Neuron* **21**, 857–867 (1998).
162. Lee, C., Bae, K. & Edery, I. PER and TIM Inhibit the DNA Binding Activity of a*Drosophila* CLOCK-CYC/dBMAL1 Heterodimer without Disrupting Formation of the Heterodimer: a Basis for Circadian Transcription. *Mol. Cell. Biol.* **19**, 5316–5325 (1999).
163. Yu, W., Zheng, H., Houl, J. H., Dauwalder, B. & Hardin, P. E. PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. *Genes Dev.* **20**, 723–733 (2006).
164. Kadener, S., Stoleru, D., McDonald, M., Nawathean, P. & Rosbash, M. Clockwork Orange is a transcriptional repressor and a new Drosophila circadian pacemaker component. *Genes*

*Dev.* **21**, 1675–1686 (2007).

165. Lim, C. *et al.* clockwork orange Encodes a Transcriptional Repressor Important for Circadian-Clock Amplitude in Drosophila. *Curr. Biol.* **17**, 1082–1089 (2007).
166. Matsumoto, A. *et al.* A functional genomics strategy reveals clockwork orange as a transcriptional regulator in the Drosophila circadian clock. *Genes Dev.* **21**, 1687–1700 (2007).
167. Richier, B., Michard-Vanhée, C., Lamouroux, A., Papin, C. & Rouyer, F. The Clockwork Orange Drosophila Protein Functions as Both an Activator and a Repressor of Clock Gene Expression. *J. Biol. Rhythms* **23**, 103–116 (2008).
168. Taylor, P. & Hardin, P. E. Rhythmic E-box binding by CLK-CYC controls daily cycles in per and tim transcription and chromatin modifications. *Mol. Cell. Biol.* **28**, 4642–4652 (2008).
169. Ceriani, M. F. *et al.* Light-Dependent Sequestration of TIMELESS by CRYPTOCHROME. *Science (80-. ).* **285**, 553–556 (1999).
170. Koh, K., Zheng, X. & Sehgal, A. JETLAG Resets the Drosophila Circadian Clock by Promoting Light-Induced Degradation of TIMELESS. *Science (80-. ).* **312**, 1809–1812 (2006).
171. Naidoo, N., Song, W., Hunter-Ensor, M. & Sehgal, A. A Role for the Proteasome in the Light Response of the Timeless Clock Protein. *Science (80-. ).* **285**, 1737–1741 (1999).
172. Peschel, N., Veleri, S. & Stanewsky, R. Veela defines a molecular link between Cryptochrome and Timeless in the light-input pathway to Drosophila's circadian clock. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 17313–17318 (2006).
173. Peschel, N., Chen, K. F., Szabo, G. & Stanewsky, R. Light-Dependent Interactions between the Drosophila Circadian Clock Factors Cryptochrome, Jetlag, and Timeless. *Curr. Biol.* **19**, 241–247 (2009).
174. Hunter-Ensor, M. & Ousley, A. *Regulation of the Drosophila Protein Timeless Suggests a Mechanism for Resetting the Circadian Clock by Light*. *Cell* vol. 84 (1996).
175. Lee, C., Parikh, V., Itsukaichi, T., Bae, K. & Edery, I. Resetting the *Drosophila* Clock by Photic Regulation of PER and a PER-TIM Complex. *Science (80-. ).* **271**, 1740–1744 (1996).
176. Myers, M. P., Wager-Smith, K., Rothenfluh-Hilfiker, A. & Young, M. W. Light-Induced

Degradation of TIMELESS and Entrainment of the *Drosophila* Circadian Clock. *Science (80-. ).* **271**, 1736–1740 (1996).

177. Grima, B. *et al.* The F-box protein slimb controls the levels of clock proteins period and timeless. *Nature* **420**, (2002).
178. Ko, H. W., Jiang, J. & Edery, I. Role for Slimb in the degradation of Drosophila Period protein phosphorylated by Doubletime. *Nature* **420**, 673–678 (2002).
179. Benito, J., Zheng, H. & Hardin, P. E. PDP1ε functions downstream of the circadian oscillator to mediate behavioral rhythms. *J. Neurosci.* **27**, 2539–2547 (2007).
180. Glossop, N. R. J. *et al.* VRILLE Feeds Back to Control Circadian Transcription of Clock in the Drosophila Circadian Oscillator. *Neuron* **37**, 249–261 (2003).
181. McDonald, M. J. & Rosbash, M. Microarray Analysis and Organization of Circadian Gene Expression in Drosophila. *Cell* **107**, 567–578 (2001).
182. Cyran, S. A. *et al.* vrille, Pdp1, and dClock Form a Second Feedback Loop in the Drosophila Circadian Clock. *Cell* **112**, 329–341 (2003).
183. Glossop, N. R. J. *et al.* vrille feeds back to control circadian transcription of Clock in the Drosophila circadian oscillator. *Neuron* **37**, (2003).
184. Dunlap Jay C; Loros Jennifer J; Decoursey, P. J. Molecular Biology of Circadian Pacemakers. in *Chronobiology: Biological timekeeping.* (eds. Dunlap, J. C., Loros, J. J. & DeCoursey, P. J.) 212–253 (Sinauer Associates, 2004).
185. Dubruille, R. & Emery, P. A plastic clock: How circadian rhythms respond to environmental cues in drosophila. *Molecular Neurobiology* vol. 38 at https://doi.org/10.1007/s12035-008-8035-y (2008).
186. Glaser, F. T. & Stanewsky, R. Synchronization of the Drosophila circadian clock by temperature cycles. in *Cold Spring Harbor Symposia on Quantitative Biology* vol. 72 (2007).
187. Wheeler, D. A., Hamblen-Coyle, M. J., Dushay, M. S. & Hall, J. C. Behavior in light-dark cycles of Drosophila mutants that are arrhythmic, blind, or both. *J. Biol. Rhythms* **8**, 67–94 (1993).
188. Levine, J. D., Funes, P., Dowse, H. B. & Hall, J. C. Resetting the Circadian Clock by Social Experience in *Drosophila melanogaster*. *Science (80-. ).* **298**, 2010–2012 (2002).
189. Schlichting, M., Grebler, R., Peschel, N., Yoshii, T. & Helfrich-Förster, C. Moonlight

Detection by Drosophila's Endogenous Clock Depends on Multiple Photopigments in the Compound Eyes. *J. Biol. Rhythms* **29**, 75–86 (2014).

190. Emery, P. *et al.* Drosophila CRY Is a Deep Brain Circadian Photoreceptor. *Neuron* **26**, 493–504 (2000).
191. Vaccaro, A., Birman, S. & Klarsfeld, A. Chronic jet lag impairs startle-induced locomotion in Drosophila. *Exp. Gerontol.* **85**, (2016).
192. Sehadova, H. *et al.* Temperature Entrainment of Drosophila's Circadian Clock Involves the Gene nocte and Signaling from Peripheral Sensory Tissues to the Brain. *Neuron* **64**, 251–266 (2009).
193. Peschel, N. & Helfrich-Förster, C. Setting the clock – by nature: Circadian rhythm in the fruitfly Drosophila melanogaster. *FEBS Lett.* **585**, 1435–1442 (2011).
194. Zadeh-Haghighi, H. & Simon, C. Radical pairs can explain magnetic field and lithium effects on the circadian clock. *Sci. Rep.* **12**, 269 (2022).
195. Foster, R. G., Hughes, S. & Peirson, S. N. Circadian photoentrainment in mice and humans. *Biology (Basel).* **9**, 1–45 (2020).
196. Yuan, Q. *et al.* Light-induced structural and functional plasticity in Drosophila larval visual system. *Science* **333**, 1458–1462 (2011).
197. Sugie, A. *et al.* Analyzing Synaptic Modulation of Drosophila melanogaster Photoreceptors after Exposure to Prolonged Light. *J. Vis. Exp.* (2017) doi:10.3791/55176.
198. Xiong, W. C., Okano, H., Patel, N. H., Blendy, J. A. & Montell, C. repo encodes a glial-specific homeo domain protein required in the Drosophila nervous system. *Genes Dev.* **8**, 981–994 (1994).
199. Campbell, G. *et al.* RK2, a glial-specific homeodomain protein required for embryonic nerve cord condensation and viability in Drosophila. *Development* **120**, 2957–2966 (1994).
200. Halter, D. A. *et al.* The homeobox gene repo is required for the differentiation and maintenance of glia function in the embryonic nervous system of Drosophila melanogaster. *Development* **121**, 317–332 (1995).
201. Alejevski, F. *et al.* The HisCl1 histamine receptor acts in photoreceptors to synchronize Drosophila behavioral rhythms with light-dark cycles. *Nat. Commun.* **10**, (2019).
202. Pyza, E. & Meinertzhagen, I. A. Monopolar cell axons in the first optic neuropil of the housefly, Musca domestica L., undergo daily fluctuations in diameter that have a circadian

basis. *J. Neurosci. Off. J. Soc. Neurosci.* **15**, 407–418 (1995).

203. Pyza, E. & Meinertzhagen, I. A. Daily rhythmic changes of cell size and shape in the first optic neuropil in Drosophila melanogaster. *J. Neurobiol.* **40**, 77–88 (1999).
204. Boschek, G. B. On the fine structure of the peripheral retina and lamina ganglionaris of the fly, Musca domestica. *Zeitschrift für Zellforsch. und Mikroskopische Anat.* **118**, (1971).
205. Braitenberg, V. & Hauser-Holschuh, H. Patterns of projection in the visual system of the fly II. Quantitative aspects of second order neurons in relation to models of movement perception. *Exp. Brain Res.* **16**, 184–209 (1972).
206. Pyza, E. & Górska-Andrzejak, J. External and internal inputs affecting plasticity of dendrites and axons of the fly's neurons. *Acta Neurobiol. Exp. (Wars).* **68**, 322–333 (2008).
207. Weber, P., Kula-Eversole, E. & Pyza, E. Circadian Control of Dendrite Morphology in the Visual System of Drosophila melanogaster. *PLoS One* **4**, 1–9 (2009).
208. Strausfeld, N. J. The organization of the insect visual system (Light microscopy). *Zeitschrift für Zellforsch. und Mikroskopische Anat.* **121**, 377–441 (1971).
209. Pyza, E. & Meinertzhagen, I. A. Daily and circadian rhythms of synaptic frequency in the first visual neuropile of the housefly\&\#x2019;s (*Musca domestica* L.) optic lobe. *Proc. R. Soc. London. Ser. B Biol. Sci.* **254**, 97–105 (1993).
210. Kolodziejczyk, A., Sun, X., Meinertzhagen, I. A. & Nässel, D. R. Glutamate, GABA and acetylcholine signaling components in the lamina of the Drosophila visual system. *PLoS One* **3**, 1–20 (2008).
211. Sinakevitch, I., Douglass, J. K., Scholtz, G., Loesel, R. & Strausfeld, N. J. Conserved and convergent organization in the optic lobes of insects and isopods, with reference to other crustacean taxa. *J. Comp. Neurol.* **467**, 150–172 (2003).
212. Wagh, D. A. *et al.* Bruchpilot, a Protein with Homology to ELKS/CAST, Is Required for Structural Integrity and Function of Synaptic Active Zones in Drosophila. *Neuron* **49**, 833–844 (2006).
213. Kittel, R. J. *et al.* Bruchpilot Promotes Active Zone Assembly, $Ca^{2+}$ Channel Clustering, and Vesicle Release. *Science (80-. ).* **312**, 1051–1054 (2006).
214. Matkovic, T. *et al.* The bruchpilot cytomatrix determines the size of the readily releasable pool of synaptic vesicles. *J. Cell Biol.* **202**, 667–683 (2013).
215. Woźnicka, O., Görlich, A., Sigrist, S. & Pyza, E. BRP-170 and BRP190 isoforms of

Bruchpilot protein differentially contribute to the frequency of synapses and synaptic circadian plasticity in the visual system of Drosophila. *Front. Cell. Neurosci.* **9**, 238 (2015).

216. Górska-Andrzejak, J. *et al.* Circadian expression of the presynaptic active zone protein bruchpilot in the lamina of Drosophila melanogaster. *Dev. Neurobiol.* **73**, 14–26 (2013).
217. Damulewicz, M. *et al.* Cryptochrome is a regulator of synaptic plasticity in the visual system of drosophila melanogaster. *Front. Mol. Neurosci.* **10**, (2017).
218. Ho, K. S. & Sehgal, A. Drosophila melanogaster: an insect model for fundamental studies of sleep. *Methods Enzymol.* **393**, 772–793 (2005).
219. Cirelli, C. & Tononi, G. Is Sleep Essential? *PLOS Biol.* **6**, 1–7 (2008).
220. Borbely, A. *A Two Process Model of Sleep Regulation*. (1982).
221. Elwyn Isaac, R., Li, C., Leedale, A. E. & Shirras, A. D. Drosophila male sex peptide inhibits siesta sleep and promotes locomotor activity in the post-mated female. *Proc. R. Soc. B Biol. Sci.* **277**, 65–70 (2010).
222. Khericha, M., Kolenchery, J. B. & Tauber, E. Neural and non-neural contributions to sexual dimorphism of mid-day sleep in Drosophila melanogaster: a pilot study. *Physiol. Entomol.* **41**, 327–334 (2016).
223. Wu, B. *et al.* Sexual dimorphism of sleep regulated by juvenile hormone signaling in Drosophila. *PLoS Genet.* **14**, e1007318 (2018).
224. Szuperak, M. *et al.* A sleep state in Drosophila larvae required for neural stem cell proliferation. *Elife* **7**, (2018).
225. Chiu, J. C. *et al.* Assaying Locomotor Activity to Study Circadian Rhythms and Sleep Parameters in Drosophila. *JoVE* e2157 (2010) doi:doi:10.3791/2157.
226. Liu, C., Haynes, P. R., Donelson, N. C., Aharon, S. & Griffith, L. C. Sleep in populations of Drosophila melanogaster. *eNeuro* **2**, (2015).
227. Faville, R., Kottler, B., Goodhill, G. J., Shaw, P. J. & Van Swinderen, B. How deeply does your mutant sleep? Probing arousal to better understand sleep defects in Drosophila. *Sci. Rep.* **5**, (2015).
228. van Alphen, B., Yap, M. H. W., Kirszenblat, L., Kottler, B. & van Swinderen, B. A dynamic deep sleep stage in Drosophila. *J. Neurosci. Off. J. Soc. Neurosci.* **33**, 6917–6927 (2013).
229. Yang, Y. & Edery, I. Daywake, an Anti-siesta Gene Linked to a Splicing-Based Thermostat from an Adjoining Clock Gene. *Curr. Biol.* **29**, 1728-1734.e4 (2019).

230. Majercak, J., Sidote, D., Hardin, P. E. & Edery, I. How a Circadian Clock Adapts to Seasonal Decreases in Temperature and Day Length. *Neuron* **24**, 219–230 (1999).
231. Low, K. H., Lim, C., Ko, H. W. & Edery, I. Natural Variation in the Splice Site Strength of a Clock Gene and Species-Specific Thermal Adaptation. *Neuron* **60**, 1054–1067 (2008).
232. Low Kwang Huei AND Chen, W.-F. A. N. D. Y. E. A. N. D. E. I. Natural Variation in the Drosophila melanogaster Clock Gene Period Modulates Splicing of Its 3′-Terminal Intron and Mid-Day Siesta. *PLoS One* **7**, 1–14 (2012).
233. Joiner, W. J., Crocker, A., White, B. H. & Sehgal, A. Sleep in Drosophila is regulated by adult mushroom bodies. *Nature* **441**, 757–760 (2006).
234. Pitman, J. L., McGill, J. J., Keegan, K. P. & Allada, R. A dynamic role for the mushroom bodies in promoting sleep in Drosophila. *Nature* **441**, 753–756 (2006).
235. Guo, F. *et al.* Circadian neuron feedback controls the Drosophila sleep–activity profile. *Nature* **536**, 292–297 (2016).
236. Donlea, J. M., Thimgan, M. S., Suzuki, Y., Gottschalk, L. & Shaw, P. J. Inducing sleep by remote control facilitates memory consolidation in Drosophila. *Science* **332**, 1571–1576 (2011).
237. Mazzotta, G. M., Damulewicz, M. & Cusumano, P. Better Sleep at Night: How Light Influences Sleep in Drosophila. *Frontiers in Physiology* vol. 11 at https://doi.org/10.3389/fphys.2020.00997 (2020).
238. Hige, T. What can tiny mushrooms in fruit flies tell us about learning and memory? *Neurosci. Res.* **129**, 8–16 (2018).
239. Aso, Y. *et al.* The neuronal architecture of the mushroom body provides a logic for associative learning. *Elife* **3**, e04577 (2014).
240. Sitaraman, D., Aso, Y., Rubin, G. M. & Nitabach, M. N. Control of Sleep by Dopaminergic Inputs to the Drosophila Mushroom Body. *Front. Neural Circuits* **9**, (2015).
241. Renn, S. C. P., Park, J. H., Rosbash, M., Hall, J. C. & Taghert, P. H. A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. *Cell* **99**, 791–802 (1999).
242. Parisky, K. M. *et al.* PDF Cells Are a GABA-Responsive Wake-Promoting Component of the Drosophila Sleep Circuit. *Neuron* **60**, 672–682 (2008).
243. Shang, Y., Griffith, L. C. & Rosbash, M. Light-arousal and circadian photoreception circuits

intersect at the large PDF cells of the Drosophila brain. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 19587–19594 (2008).

244. Sheeba, V. *et al.* Large ventral lateral neurons modulate arousal and sleep in Drosophila. *Curr. Biol.* **18**, 1537–1545 (2008).
245. Guo, F., Holla, M., Díaz, M. M. & Rosbash, M. A Circadian Output Circuit Controls Sleep-Wake Arousal in Drosophila. *Neuron* **100**, 624-635.e4 (2018).
246. Lamaze, A., Krätschmer, P., Chen, K.-F., Lowe, S. & Jepson, J. E. C. A Wake-Promoting Circadian Output Circuit in Drosophila. *Curr. Biol.* **28**, 3098-3105.e3 (2018).
247. Dag, U. *et al.* Neuronal reactivation during post- learning sleep consolidates long-term memory in drosophila. *Elife* **8**, (2019).
248. Donlea, J. M. *et al.* Recurrent Circuitry for Balancing Sleep Need and Sleep. *Neuron* **97**, (2018).
249. Donlea, J. M., Pimentel, D. & Miesenböck, G. Neuronal Machinery of Sleep Homeostasis in Drosophila. *Neuron* **81**, 860–872 (2014).
250. Liu, Q., Liu, S., Kodama, L., Driscoll, M. R. & Wu, M. N. Two Dopaminergic Neurons Signal to the Dorsal Fan-Shaped Body to Promote Wakefulness in Drosophila. *Curr. Biol.* **22**, 2114–2123 (2012).
251. Ueno, T. *et al.* Identification of a dopamine pathway that regulates sleep and arousal in Drosophila. *Nat. Neurosci.* **15**, 1516–1523 (2012).
252. Pimentel, D. *et al.* Operation of a homeostatic sleep switch. *Nature* **536**, 333–337 (2016).
253. Ni, J. D. *et al.* Differential regulation of the Drosophila sleep homeostat by circadian and arousal inputs. *Elife* **8**, e40487 (2019).
254. Saper, C. B., Fuller, P. M., Pedersen, N. P., Lu, J. & Scammell, T. E. Sleep state switching. *Neuron* **68**, 1023–1042 (2010).
255. Foltenyi, K., Greenspan, R. J. & Newport, J. W. Activation of EGFR and ERK by rhomboid signaling regulates the consolidation and maintenance of sleep in Drosophila. *Nat. Neurosci.* **10**, (2007).
256. Crocker, A., Shahidullah, M., Levitan, I. B. & Sehgal, A. Identification of a Neural Circuit that Underlies the Effects of Octopamine on Sleep:Wake Behavior. *Neuron* **65**, (2010).
257. Cavanaugh, D. J. *et al.* Identification of a circadian output circuit for rest: Activity rhythms in drosophila. *Cell* **157**, (2014).

258. Haynes, P. R., Christmann, B. L. & Griffith, L. C. A single pair of neurons links sleep to memory consolidation in Drosophila melanogaster. *Elife* **4**, e03868 (2015).
259. Chen, W. F. *et al.* A neuron-glia interaction involving GABA transaminase contributes to sleep loss in sleepless mutants. *Mol. Psychiatry* **20**, (2015).
260. Dissel, S. *et al.* Differential activation of immune factors in neurons and glia contribute to individual differences in resilience/vulnerability to sleep disruption. *Brain. Behav. Immun.* **47**, (2015).
261. Farca Luna, A. J., Perier, M. & Seugnet, L. Amyloid precursor protein in drosophila glia regulates sleep and genes involved in glutamate recycling. *J. Neurosci.* **37**, (2017).
262. Gerstner, J. R. *et al.* Normal sleep requires the astrocyte brain-type fatty acid binding protein FABP7. *Sci. Adv.* **3**, e1602663 (2017).
263. Vanderheyden, W. M. *et al.* Astrocyte expression of the Drosophila TNF-alpha homologue, Eiger, regulates sleep in flies. *PLoS Genet.* **14**, e1007724 (2018).
264. Seugnet, L. *et al.* Notch signaling modulates sleep homeostasis and learning after sleep deprivation in Drosophila. *Curr. Biol.* **21**, 835–840 (2011).
265. Potdar, S., Daniel, D. K., Thomas, F. A., Lall, S. & Sheeba, V. Sleep deprivation negatively impacts reproductive output in Drosophila melanogaster. *J. Exp. Biol.* **221**, (2018).
266. Besedovsky, L., Lange, T. & Haack, M. The Sleep-Immune Crosstalk in Health and Disease. *Physiol. Rev.* **99**, 1325–1380 (2019).
267. Ishimoto, H., Lark, A. & Kitamoto, T. Factors that differentially affect daytime and nighttime sleep in Drosophila melanogaster. *Front. Neurol.* **FEB**, 1–5 (2012).
268. Parisky, K. M., Agosto Rivera, J. L., Donelson, N. C., Kotecha, S. & Griffith, L. C. Reorganization of Sleep by Temperature in Drosophila Requires Light, the Homeostat, and the Circadian Clock. *Curr. Biol.* **26**, 882–892 (2016).
269. Keene, A. C. *et al.* Clock and cycle Limit Starvation-Induced Sleep Loss in Drosophila. *Curr. Biol.* **20**, 1209–1215 (2010).
270. Catterson, J. H. *et al.* Dietary modulation of Drosophila sleep-wake behaviour. *PLoS One* **5**, (2010).
271. Scott, K. *et al.* Postprandial sleep mechanics in Drosophila. (2016) doi:10.7554/eLife.19334.001.
272. Panda, S. Circadian physiology of metabolism. *Science (80-. ).* **354**, 1008–1015 (2016).

273. Chen, W.-F. *et al.* A neuron–glia interaction involving GABA transaminase contributes to sleep loss in sleepless mutants. *Mol. Psychiatry* **20**, 240–251 (2015).
274. Talay, M. *et al.* Transsynaptic Mapping of Second-Order Taste Neurons in Flies by trans-Tango. *Neuron* **96**, 783-795.e4 (2017).
275. Donlea, J. M. *et al.* Recurrent Circuitry for Balancing Sleep Need and Sleep. *Neuron* **97**, 378-389.e4 (2018).
276. Yang, Z. *et al.* Octopamine mediates starvation-induced hyperactivity in adult *Drosophila*. *Proc. Natl. Acad. Sci.* **112**, 5219–5224 (2015).
277. Hasegawa, T. *et al.* Sweetness induces sleep through gustatory signalling independent of nutritional value in a starved fruit fly. *Sci. Rep.* **7**, 14355 (2017).
278. Linford, N. J., Ro, J., Chung, B. Y. & Pletcher, S. D. Gustatory and metabolic perception of nutrient stress in *Drosophila*. *Proc. Natl. Acad. Sci.* **112**, 2587–2592 (2015).
279. Srinivasan, A. & Kesavan, P. C. Toxic effects of caffeine on growth and metamorphosis of the larvae of Musca domestica. *J. Toxicol. Environ. Health* **2**, 569–576 (1977).
280. Malinauskas, B. M., Aeby, V. G., Overton, R. F., Carpenter-Aeby, T. & Barber-Heidal, K. A survey of energy drink consumption patterns among college students. *Nutr. J.* **6**, 35 (2007).
281. Smith, A. Effects of caffeine on human behavior. *Food Chem. Toxicol. an Int. J. Publ. Br. Ind. Biol. Res. Assoc.* **40**, 1243–1255 (2002).
282. Cox, G. R. *et al.* Effect of different protocols of caffeine intake on metabolism and endurance performance. *J. Appl. Physiol.* **93**, 990–999 (2002).
283. Rumpler, W. *et al.* Oolong tea increases metabolic rate and fat oxidation in men. *J. Nutr.* **131**, 2848–2852 (2001).
284. Nikodijević, O., Jacobson, K. A. & Daly, J. W. Locomotor activity in mice during chronic treatment with caffeine and withdrawal. *Pharmacol. Biochem. Behav.* **44**, 199–216 (1993).
285. Holtzman, S. G. Complete, reversible, drug-specific tolerance to stimulation of locomotor activity by caffeine. *Life Sci.* **33**, 779–787 (1983).
286. Barraco, R. A., Coffin, V. L., Altman, H. J. & Phillis, J. W. Central effects of adenosine analogs on locomotor activity in mice and antagonism of caffeine. *Brain Res.* **272**, 392–395 (1983).
287. Angelucci, M. E. *et al.* The effect of caffeine in animal models of learning and memory.

*Eur. J. Pharmacol.* **373**, 135–140 (1999).

288. Wright, G. A. *et al.* Caffeine in floral nectar enhances a pollinator's memory of reward. *Science* **339**, 1202–1204 (2013).
289. Burke, T. M. *et al.* Effects of caffeine on the human circadian clock in vivo and in vitro. *Sci. Transl. Med.* **7**, 305ra146 (2015).
290. Oike, H., Kobori, M., Suzuki, T. & Ishida, N. Caffeine lengthens circadian rhythms in mice. *Biochem. Biophys. Res. Commun.* **410**, 654–658 (2011).
291. van Diepen, H. C. *et al.* Caffeine increases light responsiveness of the mouse circadian pacemaker. *Eur. J. Neurosci.* **40**, 3504–3511 (2014).
292. Wu, M. N. *et al.* The effects of caffeine on sleep in Drosophila require PKA activity, but not the adenosine receptor. *J. Neurosci.* **29**, 11029–11037 (2009).
293. Shilo, L. *et al.* The effects of coffee consumption on sleep and melatonin secretion. *Sleep Med.* **3**, 271–273 (2002).
294. Mustard, J. A. The buzz on caffeine in invertebrates: effects on behavior and molecular mechanisms. *Cell. Mol. Life Sci.* **71**, 1375–1382 (2014).
295. Ballarin, M., Fredholm, B. B., Ambrosio, S. & Mahy, N. Extracellular levels of adenosine and its metabolites in the striatum of awake rats: Inhibition of uptake and metabolism. *Acta Physiol. Scand.* **142**, (1991).
296. Fredholm, B. B., Bättig, K., Holmén, J., Nehlig, A. & Zvartau, E. E. Actions of Caffeine in the Brain with Special Reference to Factors That Contribute to Its Widespread Use. *Pharmacol. Rev.* **51**, 83–133 (1999).
297. Sellier, M.-J., Reeb, P. & Marion-Poll, F. Consumption of bitter alkaloids in Drosophila melanogaster in multiple-choice test conditions. *Chem. Senses* **36**, 323–334 (2011).
298. Keebaugh, E. S., Park, J. H., Su, C., Yamada, R. & Ja, W. W. Nutrition influences caffeine-mediated sleep loss in drosophila. *Sleep* **40**, (2017).
299. Lee, Y., Moon, S. J. & Montell, C. Multiple gustatory receptors required for the caffeine response in Drosophila. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 4495–4500 (2009).
300. Moon, S. J., Köttgen, M., Jiao, Y., Xu, H. & Montell, C. A taste receptor required for the caffeine response in vivo. *Curr. Biol.* **16**, 1812–1817 (2006).
301. Huang, Z.-L. *et al.* Adenosine A2A, but not A1, receptors mediate the arousal effect of caffeine. *Nat. Neurosci.* **8**, 858–859 (2005).

302. Yanik, G., Glaum, S. & Radulovacki, M. The dose-response effects of caffeine on sleep in rats. *Brain Res.* **403**, 177–180 (1987).
303. Aho, V. *et al.* Homeostatic response to sleep/rest deprivation by constant water flow in larval zebrafish in both dark and light conditions. *J. Sleep Res.* **26**, (2017).
304. Satoh, S. Region-dependent difference in the sleep-promoting potency of an adenosine a2A receptor agonist. *Eur. J. Neurosci.* **11**, 1587–1597 (1999).
305. Hong, Z.-Y. *et al.* An adenosine A2A receptor agonist induces sleep by increasing GABA release in the tuberomammillary nucleus to inhibit histaminergic systems in rats. *J. Neurochem.* **92**, 1542–1549 (2005).
306. Gallopin, T. *et al.* The endogenous somnogen adenosine excites a subset of sleep-promoting neurons via A2A receptors in the ventrolateral preoptic nucleus. *Neuroscience* **134**, 1377–1390 (2005).
307. Coleman, C. G., Baghdoyan, H. A. & Lydic, R. Dialysis delivery of an adenosine A2A agonist into the pontine reticular formation of C57BL/6J mouse increases pontine acetylcholine release and sleep. *J. Neurochem.* **96**, 1750–1759 (2006).
308. Porkka-Heiskanen, T. *et al.* Adenosine: A mediator of the sleep-inducing effects of prolonged wakefulness. *Science (80-. ).* **276**, 1265–1267 (1997).
309. Radek, R. J., Decker, M. W. & Jarvis, M. F. The adenosine kinase inhibitor ABT-702 augments EEG slow waves in rats. *Brain Res.* **1026**, 74–83 (2004).
310. Radulovacki, M., Virus, R. M., Djuricic-Nedelson, M. & Green, R. D. Adenosine analogs and sleep in rats. *J. Pharmacol. Exp. Ther.* **228**, 268–274 (1984).
311. Schwierin, B., Borbély, A. A. & Tobler, I. Effects of N6-cyclopentyladenosine and caffeine on sleep regulation in the rat. *Eur. J. Pharmacol.* **300**, 163–171 (1996).
312. Hendricks, J. C. *et al.* Rest in Drosophila is a sleep-like state. *Neuron* **25**, 129–138 (2000).
313. Lin, F. J. *et al.* Effect of taurine and caffeine on sleep-wake activity in Drosophila melanogaster. *Nat. Sci. Sleep* **2**, 221–231 (2010).
314. Andretic, R., Kim, Y.-C., Jones, F. S., Han, K.-A. & Greenspan, R. J. *Drosophila* D1 dopamine receptor mediates caffeine-induced arousal. *Proc. Natl. Acad. Sci.* **105**, 20392–20397 (2008).
315. Coelho, A. *et al.* Cytochrome P450-dependent metabolism of caffeine in Drosophila melanogaster. *PLoS One* **10**, e0117328 (2015).

316. Li, W. Z., Li, S. L., Zheng, H. Y., Zhang, S. P. & Xue, L. A broad expression profile of the GMR-GAL4 driver in Drosophila melanogaster. *Genet. Mol. Res.* **11**, 1997–2002 (2012).
317. Halter D. A; Urban J; Rickert C; Ner S.S; Ito K; Travers A. A; Technau G M. The homeobox gene repo is required for the differentiation and maintenance of glia function in the embryonic nervous system of Drosophila Melanogaster. *Development* **121**, 317–332 (1995).
318. Ni, J. Q. *et al.* A drosophila resource of transgenic RNAi lines for neurogenetics. *Genetics* **182**, 1089–1100 (2009).
319. Pyza, E. & Meinertzhagen, I. A. Neurotransmitters regulate rhythmic size changes amongst cells in the fly's optic lobe. *J. Comp. Physiol. A* **178**, 33–45 (1996).
320. Emery, P., So, W. V., Kaneko, M., Hall, J. C. & Rosbash, M. Cry, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell* **95**, (1998).
321. Friggi-Grelin, F. *et al.* Targeted gene expression in Drosophila dopaminergic cells using regulatory sequences from tyrosine hydroxylase. *J. Neurobiol.* **54**, (2003).
322. Benzer, S. Genetic dissection of behavior. *Sci. Am.* **229**, (1973).
323. Rosato, E. & Kyriacou, C. P. Analysis of locomotor activity rhythms in Drosophila. *Nat. Protoc.* **1**, 559–568 (2006).
324. Huber Reto; Hill Sean L; Holladay Carie; Biesiadecki Melissa;Tononi Giulio; Cirelli Chiara. Sleep homeostasis in Drosophila melanogaster. *Sleep* **27**, 628–639 (2004).
325. Dolezal, T., Dolezelova, E., Zurovec, M. & Bryant, P. J. A role for adenosine deaminase in Drosophila larval development. *PLoS Biol.* **3**, 1213–1224 (2005).
326. Borea, P. A., Gessi, S., Merighi, S., Vincenzi, F. & Varani, K. Pathological overproduction: the bad side of adenosine. *Br. J. Pharmacol.* **174**, 1945–1960 (2017).
327. Jones, M. A. & Grotewiel, M. Drosophila as a model for age-related impairment in locomotor and other behaviors. *Exp. Gerontol.* **46**, 320–325 (2011).
328. Rhodenizer, D., Martin, I., Bhandari, P., Pletcher, S. D. & Grotewiel, M. Genetic and environmental factors impact age-related impairment of negative geotaxis in Drosophila by altering age-dependent climbing speed. *Exp. Gerontol.* **43**, 739–748 (2008).
329. Willems, L., Ashton, K. J. & Headrick, J. P. Adenosine-mediated cardioprotection in the aging myocardium. *Cardiovascular Research* 245–255 at https://doi.org/10.1016/j.cardiores.2004.11.008 (2005).

330. Simpson, R. E. & Phillis, J. W. Adenosine in exercise adaptation. *Br. J. Sports Med.* **26**, 54–58 (1992).
331. Purpura, M. *et al.* Oral Adenosine-5′-triphosphate (ATP) Administration Increases Postexercise ATP Levels, Muscle Excitability, and Athletic Performance Following a Repeated Sprint Bout. *J. Am. Coll. Nutr.* **36**, 177–183 (2017).
332. Freeman, M. Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. *Cell* **87**, 651–660 (1996).
333. Ray, M. & Lakhotia, S. C. The commonly used eye-specific sev-GAL4 and GMR-GAL4 drivers in Drosophila melanogaster are expressed in tissues other than eyes also. *J. Genet.* **94**, 407–416 (2015).
334. Porkka-Heiskanen, T., Alanko, L., Kalinchuk, A. & Stenberg, D. Adenosine and sleep. *Sleep Med. Rev.* **6**, 321–332 (2002).
335. Tononi, G. & Cirelli, C. Sleep function and synaptic homeostasis. *Sleep Med. Rev.* **10**, 49–62 (2006).
336. Latini, S. & Pedata, F. Adenosine in the central nervous system: Release mechanisms and extracellular concentrations. *Journal of Neurochemistry* at https://doi.org/10.1046/j.1471-4159.2001.00607.x (2001).
337. Phillis, J. W. & Wu, P. H. The role of adenosine and its nucleotides in central synaptic transmission. *Prog. Neurobiol.* **16**, 187–239 (1981).
338. Nascimento, F. *et al.* Adenosine A2A receptors activation facilitates neuromuscular transmission in the pre-symptomatic phase of the SOD1(G93A) ALS mice, but Not in the symptomatic phase. *PLoS One* **9**, 1–10 (2014).
339. Damulewicz, M. & Pyza, E. The clock input to the first optic neuropil of Drosophila melanogaster expressing neuronal circadian plasticity. *PLoS One* **6**, (2011).
340. Damulewicz, M., Woźnicka, O., Jasińska, M. & Pyza, E. CRY-dependent plasticity of tetrad presynaptic sites in the visual system of Drosophila at the morning peak of activity and sleep. *Sci. Rep.* **10**, 18161 (2020).
341. Knight, D. *et al.* Equilibrative nucleoside transporter 2 regulates associative learning and synaptic function in Drosophila. *J. Neurosci.* **30**, 5047–5057 (2010).
342. Sidorov, R., Kucerova, L., Kiss, I. & Zurovec, M. Mutation in the Drosophila melanogaster adenosine receptor gene selectively decreases the mosaic hyperplastic epithelial outgrowth

rates in wts or dco heterozygous flies. *Purinergic Signal.* **11**, 95–105 (2015).

343. Zuberova, M., Fenckova, M., Simek, P., Janeckova, L. & Dolezal, T. Increased extracellular adenosine in Drosophila that are deficient in adenosine deaminase activates a release of energy stores leading to wasting and death. *DMM Dis. Model. Mech.* **3**, 773–784 (2010).
344. Costenla, A. R., Cunha, R. A. & De Mendonça, A. Caffeine, adenosine receptors, and synaptic plasticity. in *Journal of Alzheimer's Disease* vol. 20 (2010).
345. Cauli, O. & Morelli, M. Caffeine and the dopaminergic system. *Behav. Pharmacol.* **16**, (2005).
346. Svenningsson, P. & Fredholm, B. B. Exciting news about A2A receptors. *Neurology* **61**, S10-1 (2003).
347. Guitart, X. *et al.* Equilibrative nucleoside transporter ENT1 as a biomarker of Huntington disease. *Neurobiol. Dis.* **96**, 47–53 (2016).
348. Azdad, K. *et al.* Dopamine D2 and adenosine A2A receptors regulate NMDA-mediated excitation in accumbens neurons through A2A-D2 receptor heteromerization. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* **34**, 972–986 (2009).
349. Trifilieff, P. *et al.* Detection of antigen interactions ex vivo by proximity ligation assay: endogenous dopamine D2-adenosine A2A receptor complexes in the striatum. *Biotechniques* **51**, 111–118 (2011).
350. Ferré, S. & Sebastião, A. M. Dissecting striatal adenosine-cannabinoid receptor interactions. New clues from rats over-expressing adenosine A2A receptors. *Journal of neurochemistry* vol. 136 897–899 at https://doi.org/10.1111/jnc.13520 (2016).
351. Wisor, J. P. *et al.* Dopaminergic role in stimulant-induced wakefulness. *J. Neurosci. Off. J. Soc. Neurosci.* **21**, 1787–1794 (2001).
352. Kaasinen, V., Aalto, S., Någren, K. & Rinne, J. O. Expectation of caffeine induces dopaminergic responses in humans. *Eur. J. Neurosci.* **19**, 2352–2356 (2004).
353. Peng, Y., Stoleru, D., Levine, J. D., Hall, J. C. & Rosbash, M. Drosophila Free-Running Rhythms Require Intercellular Communication. *PLOS Biol.* **1**, null (2003).
354. Kim, Y. C., Lee, H. G. & Han, K. A. D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in Drosophila. *J. Neurosci.* **27**, 7640–7647 (2007).

355. Nall, A. H. *et al.* Caffeine promotes wakefulness via dopamine signaling in Drosophila. *Sci. Rep.* **6**, 20938 (2016).

## 8. Statement

I declare that I have generated this thesis without any help and without using sources other than the ones cited. I also guarantee that this thesis has not been submitted in an identical or similar form to a different examination board. Statements or figures that were added literally or modified from other sources have been marked.

## 9. Acknowledgement

I would like to express my sincere gratitude to the Jagellonian University, Krakow Poland, for accepting me as a Doctoral student under the ATUT PhD Program in Biology 2018 and for giving me this golden opportunity to complete this degree.

I thank my supervisor Prof. Dr. hab. Elzbieta Pyza for training me to conduct future research as an independent scientist. For four years I have been trained in all the new techniques and have gathered a repository of knowledge about this model organism and the application of *Drosophila* in the field of neuroscience and mankind.

I extend my warmest thanks to Dr. Jolanta Gorska- Andrzejak, my associate supervisor, who has trained me to learn the confocal microscopy.

I give a big thanks to Dr. hab. Joanna Rutkowska (Head of Power PhD) & our secretary, Ms Anna Stec for their help and cooperation with the PhD process.

I extend my warmest thanks to Dr. Mikko Jussola who has allowed me to work in his lab at Sheffield University and complete my PhD internship.

I also thank my colleagues, friends, and members of Jagellonian University for their help during my studies.

I am forever thankful to my family for their unconditional support and motivation during the PhD. And finally, I extend my gratitude to God who wrote this beautiful chapter of my destiny.