Does Sleep Protect Against Oxidative Stress?

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If sleep does not serve an absolutely vital function, then it is the biggest mistake the evolutionary process has ever made.

Allan Rechtschaffen

Abstract

Sleep is a vital behaviour, and despite spending one third of our lives asleep, we are yet to define its function. A proposed function connects sleep and the build-up of reactive oxygen species (ROS) in the brain, suggesting that sleep is needed as antioxidant defence mechanism. Indeed, ROS accumulation is implicated with prolonged waking, and artificial increase of ROS in the brain can induce sleep in animals. Using the fruit fly *Drosophila melanogaster*, I aim to investigate sleep's possible role as an antioxidant defence. Acting as a ROS sensor in sleep-promoting dorsal fan-shaped (dFB) neurons is the potassium channel Shaker's β subunit Hyperkinetic (Hk). Oxidization of its cofactor NADPH causes conformational changes of the channel and thereby modulates dFB neuron activity. dFB neurons could thus act as an early-alert system for rising ROS levels in the brain.

Here, I analysed the sleep pattern, learning and lifespan of short-sleeping *Hk* mutants, and found severe deficits in learning as well as a drastically shortened lifespan. Learning, additional to sleep duration, was rescued by reinstating Hk into dFB neurons. Lifespan was affected independently of sleep duration, as pan-neuronal *Hk* expression was required to rescue lifespan.

Introduction of the ROS-limiting alternative oxidase (AOX) into mitochondria of flies affected sleep duration and architecture similarly to *Hk* mutants. However, learning and lifespan remained unaffected, showing that flies were healthy despite severely shortened sleep duration. Furthermore, *AOX* expression in *Hk* mutants rescued learning, suggesting that learning deficits after chronic sleep deprivation are caused by an accumulation of ROS.

Lifespan on the other hand seemed to be affected by loss of Hk regardless of sleep state or *AOX* expression, showing its dependence on functional Hk itself.

Summarizing, ROS plays an important role in the induction and regulation of sleep, and symptoms of chronic sleep deprivation can be counteracted via regulation of ROS production.

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12LD . . . . . 12-hour light:12-hour dark

AD . . . . . Alzheimer's disease

ADHD . . . . . attention deficit hyperactivity disorder

ADP . . . . . adenosine diphosphate

AID . . . . . auxin-inducible degron

AKR . . . . . aldo-keto reductase

AL . . . . . antennal lobe

AMP . . . . . adenosine monophosphate

AOX . . . . . alternative oxidase

APS . . . . . aversive phototaxis suppression

ASD . . . . . autism spectrum disorder

ATP . . . . . adenosine triphosphate

BDSC . . . . . Bloomington *Drosophila* Stock Center

cAMP . . . . . cyclicAMP

CNCB . . . . . Centre for Neural Circuits and Behaviour

COPD . . . . . chronic obstructive pulmonary disease

CREB . . . . . cAMP response element-binding protein

CS . . . . . Canton-S

CS+ . . . . . conditioned stimulus

CS- . . . . . unconditioned stimulus

DA . . . . . dopamine

DAM . . . . . *Drosophila* Activity Monitors

DAT . . . . . dopamine transporter

dFB . . . . . dorsal fan-shaped body

DP . . . . . dark period

EEG . . . . . electroencephalography

EGF . . . . . epidermal growth factor

EMG . . . . . electromyography

EOG . . . . . electrooculography

ETC . . . . . electron transport chain

GABA . . . . . γ-aminobutyric acid

H_2O_2 . . . . . hydrogen peroxide

HEK . . . . . human embryonic kidney

Hk . . . . . Hyperkinetic

HMT . . . . . horizontal mitochondrial transfer

I_A . . . . . A-type potassium current

$I_{non\text{-}A}$ . . . . . non-A-type potassium current

IMM . . . . . inner mitochondrial membrane

K^+ . . . . . potassium

KC . . . . . Kenyon cell

K-NAA . . . . . 1-naphthaleneacetic acid potassium salt

LP . . . . . light period

LPP . . . . . lipid peroxidation product

MB . . . . . mushroom body

MB-IR . . . . . multi-beam infrared recording

MCH 4-methylcyclohexanol

Na^+ ..... sodium

NADPH/$NADP^+$ ..... nicotinamide adenine dinucleotide phosphate

NREM ..... non-rapid eye movement

O_2^- ..... superoxide

OCT 3-octanol

OMM ..... outer mitochondrial membrane

PD ..... Parkinson's disease

PDoze ..... probability to fall asleep

PN ..... projection neuron

PWake ..... probability to wake up

REM ..... rapid eye movement

ROS ..... reactive oxygen species

SB-IR ..... single-beam infrared recording

SCAMP ..... Sleep and Circadian Analysis MATLAB Program

Sh ..... Shaker

SOD ..... superoxide dismutase

SWS ..... slow wave sleep

VDRC ..... Vienna *Drosophila* Resource Center

VLPO ..... ventrolateral preoptic area

WT ..... wild type

Chapter 1

Introduction

The enigma that is sleep has been fascinating researchers for centuries. The first known theories on the function and origin of sleep were formulated in Ancient Greece about 2600 years ago by Alcmaeon of Croton (Beare, 1906), followed by other great known philosophers and scientists such as Hippocrates, Aristotle, Galen, and René Descartes (Schulz, 2022; Whitehead, 2002). Even though it is a common and daily behaviour, as we spend one third of our lifetime asleep, the regulation of sleep and its function have remained a mystery to this day.

1.1 Defining sleep

Sleep is a complex behaviour not easily defined by one specific feature. Instead, it has to be described and measured by more than one parameter to distinguish it from other states. On a behavioural level, sleep is defined by four major observations: 1) a stereotypic or species-specific posture is assumed, 2) behavioural quiescence is maintained, 3) the arousal threshold is increased, meaning that the individual does not react to weak stimuli, and 4) the state of quiescence is reversible, either because the individual wakes up naturally through internal

changes or after a strong enough external stimulation (Campbell and Tobler, 1984). Additional to a behavioural definition of sleep, researchers developed electrophysiological criteria to quantitatively measure sleep in mammals and birds. Electroencephalography (EEG), electromyography (EMG), and (in humans) electrooculography (EOG) measurements together are used to determine sleep and different sleep states, especially in mammalian sleep research, including humans (Campbell and Tobler, 1984; Hirshkowitz, 2004). Sleep in mammals, based on EEG and EOG data, is divided into rapid eye movement (REM) sleep and non-REM (NREM) sleep. In humans, NREM sleep is typically further divided into sleep stages 1–4, depending on the frequency of EEG activity, with stage 4 being the deepest sleep stage called slow wave sleep (SWS), and stages 1–3 presenting lighter sleep stages in descending order. All sleep stages, including REM sleep, are accompanied by muscle atonia, seen in EMG measurements (reviewed in Hirshkowitz, 2004).

Despite sleep prohibiting the simultaneous execution of any other vital behaviour, such as searching for food and water, or finding a mate, and sleep leaving an individual vulnerable, unable to perceive and react to any immediate danger such as predators or other environmental threats, it seems to be evolutionarily conserved as all animals (including humans) display sleep or sleep-like behaviours (Campbell and Tobler, 1984). Sleep-like behaviour has even been found in early branches of animal phyla, in animals with only few neurons such as the nematode *Caenorhabditis elegans* (Raizen et al., 2008) and the cnidarian *Hydra vulgaris* (Kanaya et al., 2020). Sleep thus seems to be a vital behaviour as well, for the two reasons mentioned already (evolutionarily conserved, paradoxical to other vital behaviours). Chronic sleep loss causes severe health issues, from decreased vigilance (Dinges et al., 1997; Belenky et al., 2003; Huber et al., 2004; Van Dongen et al., 2003), to impaired learning and memory (Chandra et al., 2023; Cirelli et al., 2004; Li et al., 2009; Saygin et al., 2017; Seugnet et al.,

2008; Stickgold, 2005; Tempesta et al., 2017; Van Dongen et al., 2003; Walker and Stickgold, 2004; Yoo et al., 2007), a compromised immune system (Van Leeuwen et al., 2009; Spiegel et al., 2009), and even early death (Rechtschaffen et al., 1983; Shaw et al., 2002; Stephenson et al., 2007).

Generally, the timing of sleep is regulated by two separate yet interacting processes: the circadian system and the homeostatic system (Borbély, 1982). While the circadian rhythm and its control of sleep is reasonably well understood, the sleep homeostat has been comparatively understudied, with many different regulatory systems playing into it. Partially for this reason, sleep and its function are still hardly understood.

1.2 Theories on the function of sleep

There are many proposed and confirmed functions of sleep. However, in most cases it remains unknown whether these functions are opportunistic functions that took advantage of an already existing sleep mechanism, or whether they are the true evolutionarily conserved reason why sleep developed and is vital to a wide range of animal species today (reviewed in Krueger et al., 2016). One proposed function of sleep is an immune function, as sleep and sleep loss affect immune system parameters (reviewed in Imeri and Opp, 2009); injury (Singh and Donlea, 2020) or illness (Toth and Krueger, 1988) are often associated with a higher need for sleep or changed sleep patterns. Other proposed theories state that sleep is needed to reduce total caloric use, with energy stores being depleted during wakefulness and restored during sleep, or it could be needed to restore brain energy stores only, as brain glucose consumption is significantly higher during wakefulness than during NREM sleep (Buchsbaum et al., 1989). Furthermore, sleep has been proposed to serve a glym-

phatic function, evidence showing there is an enhanced flow from the brain to circulation during sleep, removing toxins and other brain products (Xie et al., 2013). Another proposed function of sleep is to restore waking-induced performance degradation, as sleep loss causes impaired cognitive and behavioural performance, and performance is restored by sleep in a dose-dependent manner (Belenky et al., 2003; Van Dongen et al., 2003). Lastly, there is the synaptic homeostasis theory, which states that SWS is required for synaptic downscaling after synaptic potentiation during wakefulness (Tononi and Cirelli, 2003).

Although many of these proposed functions indicate a clear evolutionary advantage of sleep, few can (yet) explain why an individual would have to be unconscious, as opposed to being in a state of quiet restfulness.

1.2.1 The free radical flux theory of sleep

In 1994, Reimund published a theory on the function of sleep called the free radical flux theory (Reimund, 1994). In his theory, he argues that neurons likely generate free radicals at a high rate, caused by both a high metabolic rate and their increased need for oxygen, as they are sensitive to hypoxia. Neurotransmitter metabolism creates an additional burden of hydrogen peroxide (H_2O_2) (Sinet et al., 1980). Oxygen-derived free radicals and H_2O_2, also called reactive oxygen species (ROS), can cause considerable damage to lipids, proteins, and DNA if accumulating uncontrollably, which accelerates aging and, in extreme cases, causes cell death (reviewed in Bertram and Hass, 2008).

Reimund theorizes that during wakefulness the high metabolic rate causes a net accumulation of free radicals, which accumulate until a certain threshold is reached, by which sleepiness sets in. During sleep, the decreased metabolic rate in combination with an increased efficiency of the antioxidant system, Reimund argues, causes a net depletion of free

radicals. Essentially, the free radical flux theory of sleep proposes a restorative function of sleep, with sleep acting as an antioxidant defence (Reimund, 1994).

Subsequent research on the relationship between ROS and sleep, especially in rats and mice, has been fairly inconclusive to this day. Many papers claim to find signs for increased oxidative stress in certain brain regions and other organs such as liver, heart, and gut after prolonged sleep deprivation (D'Almeida et al., 1998; Alzoubi et al., 2012; Kanazawa et al., 2016; Ramanathan et al., 2002; Silva et al., 2004; Singh and Kumar, 2008; Süer et al., 2011; Vaccaro et al., 2020), and even show that administered antioxidants are able to rescue behavioural deficits associated with sleep deprivation (Alzoubi et al., 2012; Kanazawa et al., 2016; Silva et al., 2004; Singh and Kumar, 2008; Vaccaro et al., 2020). Others, however, were not able to observe a clear relationship between sleep need and oxidative stress (D'Almeida et al., 1997; Cirelli et al., 1999; Gopalakrishnan et al., 2004). Thus, due to varying protocols and lengths of sleep deprivation, different ROS-associated parameters being analysed, and conflicting results, it has been difficult to establish a solid connection between ROS levels and sleep.

1.3 *Drosophila melanogaster* in sleep research

1.3.1 Why flies?

Drosophila melanogaster, commonly known as the fruit fly, is a frequently used model organism. Like any other animal ever studied, fruit flies show periodical epochs of rest that are classified as sleep according to standard criteria (as discussed above) (Hendricks et al., 2000; Shaw et al., 2000). However, in contrast to mammals, fly sleep cannot be assessed by measuring an EEG. Even though sleep is associated with electrophysiological changes in the fly

brain, electrophysiological recordings are only possible with a low throughput and in head-fixed flies (as opposed to freely running flies). To overcome this problem, a behaviourally measurable criterion for fly sleep has been formulated: periods of immobility of five or more consecutive minutes are counted as sleep. Evidence shows that after five minutes of immobility flies show a higher arousal threshold than briefly quiescent flies, meaning that they are more difficult to startle after the five minute mark (Hendricks et al., 2000; Shaw et al., 2000). Furthermore, longer resting flies assume a typical position in a preferred location that has been identified as a characteristic sleep posture (Hendricks et al., 2000), and flies show a homeostatic response to sleep deprivation (Hendricks et al., 2000; Shaw et al., 2000). Electrophysiological evidence supports this behavioural definition of sleep in flies (Nitz et al., 2002; Van Alphen et al., 2013; Van Swinderen et al., 2004).

Using the five minute criterion, sleep in flies has been extensively researched and further characterized during the past 20 years. Similar to humans, flies tend to sleep mostly during the night and are mostly awake during the day with a 'siesta' around noon (Andretic and Shaw, 2005; Hendricks et al., 2000; Shaw et al., 2000). Nighttime sleep seems more consolidated and deeper than daytime sleep, as daytime sleep is characterized by shorter sleep bouts from which flies are easier to rouse (Andretic and Shaw, 2005). Sleep in flies becomes shorter and more fragmented with age (Koh et al., 2006; Shaw et al., 2000), and sleep differences between male and female flies, which are pronounced in young flies, even out in older flies (Koh et al., 2006). If flies are subjected to sleep deprivation during nighttime, they typically show a robust rebound response, indicating homeostatic control of sleep additional to the circadian rhythm (Hendricks et al., 2000; Huber et al., 2004; Shaw et al., 2000). Recovery sleep after sleep deprivation is usually less fragmented and associated with a higher arousal threshold than baseline sleep (Huber et al., 2004).

Electrophysiological recordings and calcium imaging of *Drosophila* brain activity have revealed that flies undergo different sleep stages(Van Alphen et al., 2013; Tainton-Heap et al., 2021), similar to what is seen in mammals. Van Alphen et al. (2013) found a significant variability in lower frequencies depending on the time since sleep initiation, meaning that the flies seem to cycle through different states of arousability, starting with a deeper sleep phase and ending in lighter sleep. This sort of cycling through different sleep stages could be reproduced by Tainton-Heap et al. (2021) and mirrors typical sleep profiles in humans during the night (Hirshkowitz, 2004).

Additional to the fruit fly being an excellent model organism to study sleep, they also have a fast generation time, are cheap, easy to maintain, and have a relatively simple biology. Further, *Drosophila melanogaster* has an extensive genetic toolbox to manipulate genes and neurons, and a large repertoire of behavioural tests available to study sleep and hallmarks of acute or chronic sleep deprivation. Some behavioural hallmarks of chronic sleep deprivation studied in the fly are learning and memory, as well as longevity. The *Drosophila* Activity Monitoring (DAM) system (Pfeiffenberger et al., 2010) is widely used to study activity and sleep on a large scale, while odour-associated negative reinforcement assays are often used to study learning and short-term memory.

1.3.2 Conserved mechanisms of sleep regulation

Aside from similar behavioural outputs between flies, mammals, and other animals, there are many regulatory mechanisms of sleep conserved between species (reviewed in Allada and Siegel, 2008; Zimmerman et al., 2008a). One pathway often mentioned is the cyclic adenosine monophosphate (cAMP) signalling pathway and cAMP response element-binding protein (CREB). Manipulations causing elevated cAMP levels or increased CREB activity in

flies, *Caenorhabditis elegans*, or mice, result in increased wakefulness, while reduced cAMP levels or inhibited CREB activity usually result in more sleep (Graves et al., 2003; Hendricks et al., 2001; Joiner et al., 2006; Raizen et al., 2008). Similarly, the epidermal growth factor (EGF) receptor pathway acts sleep promoting in all three model organisms, meaning that an increase in EGF receptor signalling increases sleep or quiescence, while a decrease results in wakefulness (Foltenyi et al., 2007; Kramer et al., 2001; Kushikata et al., 1998; Van Buskirk and Sternberg, 2007).

Activation of receptors for γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the mammalian nervous system, has long been known to promote sleep, and many GABAergic compounds targeting $GABA_A$ receptors are being used pharmacologically to counteract insomnia (reviewed in Harrison, 2007). Neurons of a major sleep centre in the mammalian brain, the ventrolateral preoptic area (VLPO), are GABAergic and hypothesized to inhibit other, wake-promoting brain areas during sleep onset (reviewed in Saper et al., 2005). In *Drosophila*, a $GABA_A$ receptor has been shown to regulate sleep onset, as maintained GABA signalling caused flies to fall asleep more quickly (Agosto et al., 2008). In contrast to GABA, dopamine's (DA) conserved function is wake promoting in both mammals and flies. Increase in dopamine signalling, for example by inhibited dopamine uptake from the synaptic cleft (via inhibition of the dopamine transporter (DAT) in mice, or via the *fumin* mutation of DAT in flies), or an increased dopamine concentration in the brain decreases sleep in mice (Nishino et al., 1998; Wisor et al., 2001) and flies (Andretic et al., 2005; Kume et al., 2005). Additionally, the loss of dopaminergic neurons, as seen with Parkinson's disease (PD), causes daytime sleepiness (reviewed in Dauer and Przedborski, 2003)).

Another important shared feature between sleep in mammals and flies is the involvement of potassium (K^+) channels. In *Drosophila*, the K^+ channel Shaker (Sh) has been found to be

an important modulator of sleep, as *Sh* mutants often display a shortening of sleep duration (Cirelli et al., 2005). Likewise, Sh's intracellular β subunit Hyperkinetic (Hk) is needed to produce a regular sleep behaviour (Bushey et al., 2007; Kempf et al., 2019). In mice, knockout of the K^+ channel Kv1.2, a Sh homologue, causes a similar reduction in sleep, specifically NREM sleep (Douglas et al., 2007). K^+ channels and their involvement in sleep will be discussed in more detail in Chapter 2.

1.3.3 Dorsal fan-shaped body neurons: a sleep control center

Dorsal fan-shaped body (dFB) neurons are widely recognized as one of the flies' main sleep control centres (Donlea et al., 2011, 2014). dFB neurons consists of about a dozen neurons in each hemisphere, with axonal projections to layers 6 and 7 of the dorsal fan-shaped body (Hulse et al., 2021), giving them their name. dFB neurons induce sleep when electrically active (ON state), while wakefulness is favoured when dFB neurons are electrically silent (OFF state) (Pimentel et al., 2016). Dopamine exposure can inhibit dFB neurons in the ON state acutely, and prolonged dopamine exposure eventually switches dFB neurons to the OFF state (Pimentel et al., 2016). To do so, dopamine modulates two types of K^+ currents in dFB neurons in opposite directions: a voltage-dependent A-type (I_A) current and a voltage-independent non-A-type ($I_{non\text{-}A}$) current. In the ON state, I_A is upregulated, while being attenuated during the OFF state. Meanwhile, $I_{non\text{-}A}$ currents are increased in the OFF state (Pimentel et al., 2016).

The $I_{non\text{-}A}$ current in dFB neurons is dependent on the two-pore domain leak K^+ channel Sandman (Pimentel et al., 2016). Depleting Sandman from dFB neurons causes the dopamine-induced OFF state to fail, which means that dFB neurons are electrically active most of the time and flies sleep longer (Pimentel et al., 2016).

The I_A current in dFB neurons is dependent on the K^+ channel Sh and its β subunit Hk (Kempf et al., 2019; Pimentel et al., 2016). Depleting Sh or Hk from dFB neurons slows down the fast inactivation kinetics, which in turn prolongs interspike intervals, thereby reducing spike-activity of dFB neurons. On a behavioural level, Sh or Hk depletion from dFB neurons causes insomnia (Kempf et al., 2019; Pimentel et al., 2016).

The activity of dFB neurons is not only regulated by dopamine but also the internal ROS concentration. Kempf et al. (2019) shows that an acute accumulation of ROS in dFB neurons with the help of a genetically encoded photosensitizer increases their spike frequency, slows the I_A inactivation kinetics, and instantly induces sleep in flies. Chronically reducing ROS production in dFB neurons, via expression of an alternative oxidase (AOX) in mitochondria, has the opposite effect: reducing spike frequency, increasing I_A inactivation speed, and causing flies to be awake for longer (Kempf et al., 2019) (for more details on AOX see Chapter 3). The effects of internal ROS concentration on dFB neuron activity and sleep can be linked to Hk and its cofactor nicotinamide adenine dinucleotide phosphate (NADPH), which can be oxidized to $NADP^+$, with changes in $NADP^+$:NADPH ratio underlying changes in I_A current properties (Kempf et al., 2019). As the cofactor exchange rate is slow (Weng et al., 2006), any oxidant exposure can have a long-lasting effect, meaning that ROS production can be tracked over time and could be used to induce sleep when a certain threshold is reached, similarly to what Reimund proposed (Reimund, 1994) (see Section 1.2.1). Due to this unique function of dFB neurons to track ROS production, Kempf et al. (2019) hypothesized dFB neurons to act as an early warning system for rising ROS levels in the whole brain. However, it remains unknown if clearance of ROS is indeed a function of sleep, or whether ROS accumulation is merely a convenient way for dFB neurons to measure elapsed waking time.

1.4 Project hypothesis and aim

With this thesis, I aimed to unravel the relationship between ROS and sleep, the involvement of dFB neurons in this relationship, and to answer the question whether clearance of ROS is a vital function of sleep. To do so, I investigated the sleep behaviour of flies with a dysfunctional ROS-sensing machinery (Hk deficiency, Chapter 2) or a reduction in ROS production (AOX expression, Chapter 3), allowing for a side-by-side comparison of flies with reduced ROS production (AOX) or ROS detection (Hk). I tested for signs of chronic sleep deprivation in the form of learning and memory deficits, and a reduced lifespan. Evaluation of these behaviours helped me determine whether reduced ROS levels in the brain and subsequent sleep loss (Kempf et al., 2019) is damaging, harmless, or even beneficial for flies.

Chapter 2

Effects of Hyperkinetic on Sleep, Learning and Lifespan

2.1 Introduction

K^+ channels are one of the common features implicated in sleep regulation across animal phyla (Allada and Siegel, 2008; Zimmerman et al., 2008a). *Drosophila*'s K^+ channel family includes the voltage-gated I_A channel Shaker (Sh), which has a cytosolic β subunit called Hyperkinetic (Hk). The name *Shaker* originates from mutations in *Drosophila melanogaster*, based on a shaking phenotype during anaesthesia (Catsch, 1948). Around 20 years later, in 1969, the first *Hk* mutants were found to show a similar shaking phenotype (Kaplan and Trout, 1969).

Up until the 1980s, little was known about these mutated genes except that they are X chromosome-linked (Catsch, 1948). In 1983, by studying A-type potassium (I_A) currents via voltage clamp experiments, the first evidence for *Sh* being a potassium (K^+) channel was found (Salkoff, 1983). However, it took four more years before the *Sh* gene was fully se-

quenced by two independent groups (Kamb et al., 1987; Tempel et al., 1987). In 1989, Stern and Ganetzky found *Hk* mutations to have an effect on A-type K^+ currents and proposed, that Hk might either be a subunit of the Sh channel or a protein involved in its regulation (Stern and Ganetzky, 1989).

During the 1990s, sequence homology with the mammalian $K_v\beta$ subunit revealed Hk to be a K^+ channel β subunit (Chouinard et al., 1995) and suggested that Hk, like other $K_v\beta$ proteins, belongs to the aldo-keto reductase (AKR) superfamily, many members of which are NAD(P)H-dependent oxidoreductases (McCormack and McCormack, 1994; Chouinard et al., 1995). Electrophysiological analyses indicated that the Hk β subunit alters the activation and inactivation kinetics of I_A currents, suggesting an interaction between the Hk β subunit and the Sh α subunit (Wang and Wu, 1996). *Hk* mutants showed higher neuronal excitability and broadened action potentials (Yao and Wu, 1999). Additionally, the Hk β subunit only interacts with the α subunit of certain fast inactivating channels (Wang and Wu, 1996; Yao and Wu, 1999). These results supported the hypothesis that Hk influences K^+ channel function (Wang and Wu, 1996; Yao and Wu, 1999).

In 1999, the crystal structure of a mammalian K_v β subunit offered incisive clues to its potential function and mechanism (Gulbis et al., 1999). The structure revealed a classical charge-relay system with an $NADP^+$ cofactor, thereby confirming the protein's catalytic potential. The cofactor is tightly bound in a deep cleft, with only its nicotinamide ring exposed, while the subunit's substrate pocket is wide open. Even though there seems to be an obvious correspondence between the β subunit and AKRs, both of these features differentiate the two (Gulbis et al., 1999). This led Gulbis et al. (1999) to speculate that interactions of the α and β subunit of K^+ channels could link the redox chemistry of a cell to changes in membrane potential — a hypothesis later confirmed by Pan et al. (2008) (see Figure 2.1).

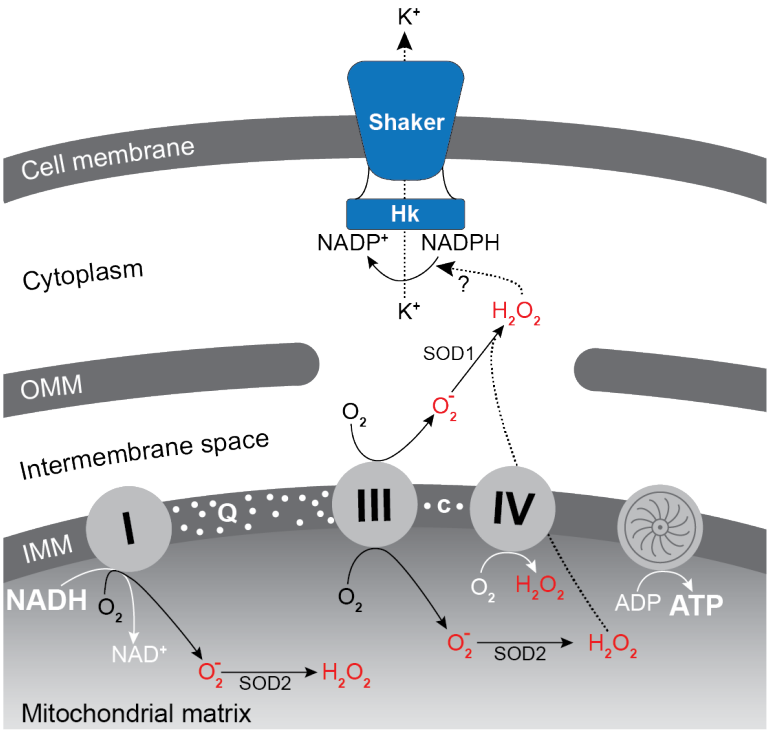

Figure 2.1: ROS production in the mitochondria changes electrophysiological activity of dFB neurons. ROS is being produced in various sites of the electron transport chain (ETC). Short-lived superoxide (O_2^-) is produced initially, being oxidized to hydrogen peroxide (H_2O_2) by mitochondrially located superoxide dismutase (SOD) 2 or cytoplasmic SOD1. If H_2O_2 is not transformed by catalase into water (not depicted), it can oxidize a whole variety of substrates, including DNA, proteins and lipids. The substrate used by Hk to oxidize its bound co-factor, NADPH, remains unknown (indicated by question mark). Oxidization of NADPH to $NADP^+$ causes conformational change to the potassium (K^+) channel Shaker, which binds Hk intracellularly. This change enables more rapid firing of the neuron, characterizing them as more active. c: cytochrome, IMM: inner mitochondrial membrane, OMM: outer mitochondrial membrane. Adapted from Kempf et al. (2019).

Research during the 2000s further consolidated the K_v β subunit's function as an NADPH-dependent AKR (Tipparaju et al., 2005; Long et al., 2005; Weng et al., 2006). More evidence arose that the β subunit's oxidoreductase function might correlate to its K_v current-inactivating activity (Bähring et al., 2001; Tipparaju et al., 2005, 2007). Binding of the co-factor NADPH to $K_v\beta$ was found to be critical for both $K_v\alpha$ and β interaction, as well as the inactivation of K_v currents (Tipparaju et al., 2007, 2005). Depending on whether the cofactor is in its oxidized ($NADP^+$) or reduced (NADPH) form, inactivation of the K_v current can be reduced and activation of the K_v current can be shifted – if predominantly $NADP^+$ is bound, the inactivation of K_v currents is slowed and activation is shifted to depolarized potentials

(Tipparaju et al., 2005). While much is known about the effects of cofactor exchange in the β subunit, it has been proven difficult to find the biological substrate for $K_v\beta$ that forms a redox couple with NADPH (Figure 2.1, question mark).

In *Drosophila melanogaster*, the first behavioural phenotypes described in *Hk* and *Sh* mutants — aside from the shaking phenotype — are a drastically shortened lifespan, as well as an increased metabolic rate as measured by oxygen consumption (Trout and Kaplan, 1970). About 30 years later, Wang et al. (2000) discovered hypersensitivity to oxidative stress in the form of paraquat, a ROS-inducing agent, in K^+ channel mutants, including *Sh* and *Hk* mutants. They speculated this hypersensitivity to be potentially connected to the shorter lifespan and increased metabolic rate. Furthermore, they found K^+ channel mutants to specifically affect the I_A current by greatly reducing its amplitude and lengthening the time to peak (Wang et al., 2000).

Sh was first implicated in sleep in 2005, when Cirelli et al. (2005) found that mutations in the Sh locus in *Drosophila melanogaster* reduced sleep. Additionally, they confirmed the earlier described shortened lifespan of *Sh* mutants and hypothesized that their longevity is compromised by chronic sleep loss (Cirelli et al., 2005). A learning phenotype was linked to Sh and Hk, when Bushey et al. (2007) found that all characterized *Sh* and *Hk* mutants have reduced sleep and impaired memory. They noted that carriers of weak *Hk* mutant alleles, which showed little reduction in sleep, had no memory deficits, indicating that the memory and sleep phenotypes might me linked (Bushey et al., 2007). Furthermore, they showed that *Hk* mutants reduced sleep primarily by affecting the Sh complex. On an electrophysiological level it is important to note that sleep loss and memory deficits were both associated with a complete loss of I_A current in *Sh* mutants (Bushey et al., 2007). Not long ago, Kempf and colleagues could draw a connection between *Drosophila*'s sleep-promoting dFB neu-

rons and Hk (Kempf et al., 2019). Hk was identified as the sensor of intracellular redox state changes in dFB neurons (Figure 2.1). Impaired *Hk* expression in dFB neurons caused reduced sleep in flies, but reinstatement of functional Hk into dFB neurons in an otherwise *Hk* mutant background rescued the sleep deficit (Kempf et al., 2019). Although mutations in *Hk* have been shown to cause sleep loss, memory impairment and a reduced lifespan, in this chapter I sought to investigate sleep, learning, and longevity of flies with Hk deficiency more thoroughly, in order to dissect the relationship between Hk, dFB neurons, and sleep (deprivation).

2.2 Sleep

2.2.1 The genomic Hk mutant *Hk^1*

Homozygous Hk mutant flies (*Hk^1*) as well as heterozygous controls (*Hk^1*/+), in the following referred to as WT flies, were tested for sleep behaviour using the *Trikinetics* DAM system (see Methods section 5.2). As shown before (Cirelli et al., 2005; Bushey et al., 2007, 2010), *Hk^1* flies had a shortened sleep duration, predominantly during the day (light period, LP) (Figure 2.2A–C). Loss of total sleep time added up to about 5 hours on average (Figure 2.2B), with about 4 hours lost during daytime alone (Figure 2.2C). Nighttime sleep (dark period, DP) was only mildly affected; even though nighttime sleep duration differed between *Hk^1* and WT flies, the effective nightly sleep loss averaged around 1 hour (Figure 2.2C), leaving the majority of *Hk^1* flies with 10 to 11 hours of sleep each night.

As the flies' activity during waking periods can be an indication of overall health (Andretic and Shaw, 2005), I quantified activity during waking but did not observe a decrease (Figure 2.2D). *Hk^1* flies therefore do not suffer from a walking impairment that causes exten-

sive rest periods that could be confused for sleep. On the contrary, Hk^1 flies showed a small increase in activity during LP and clear hyperactivity compared to WT flies during DP (Figure 2.2D). Both differences were statistically significant ($P<0.0001$); however results need to be interpreted carefully, as WT flies were barely awake during DP, resulting in a small sample size for waking activity during this time.

Even though nighttime sleep was not much affected, sleep architecture — defined by the number and duration of sleep bouts — changed considerably during both daytime and nighttime sleep. On average, WT flies experienced shorter but more numerous sleep episodes during LP, and longer but fewer sleep episodes during DP (Figure 2.2E–F), indicating deeper, more consolidated sleep during DP. Interestingly, during DP WT flies either exhibited very long sleep bouts lasting almost the whole of the DP ($\sim$700min), or they experienced shorter sleep bouts up to 400min long (Figure 2.2F, dark panel). For Hk^1 flies, during LP both the number of sleep bouts and their duration was decreased compared to control flies, together accounting for the loss in daytime sleep. During DP, in contrast, Hk^1 flies experienced pronounced sleep fragmentation, exhibiting more but shorter sleep bouts than WT controls (Figure 2.2F). Uninterrupted nighttime sleep was far less common than in WT controls, and most sleep bouts did not exceed 400min, significantly lowering the average sleep bout duration (Figure 2.2F). The opposite relationship held true for the number of sleep bouts (Figure 2.2E), counteracting the sleep loss that would have resulted from shorter sleep bouts by increasing the frequency of falling asleep. These results indicate that during LP, Hk^1 flies have difficulties with sleep initiation and sleep maintenance, while during DP mainly sleep maintenance is affected, causing the flies to wake up more often.

Two additional parameters were analysed to gain deeper insight into the flies' sleep profile: the probability of falling asleep (PDoze), and the probability of waking up (PWake).

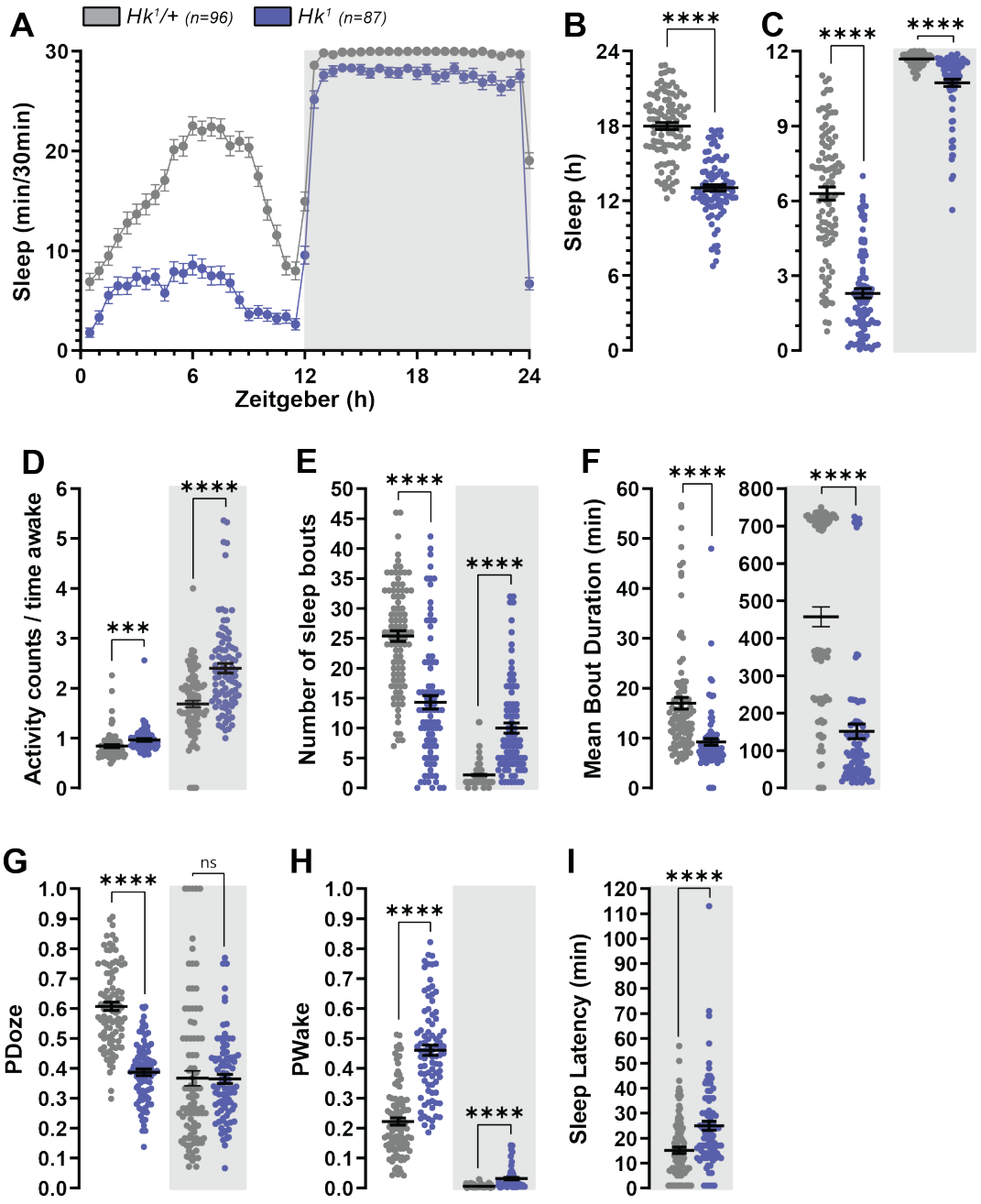

Figure 2.2: *Hk¹* flies show reduced sleep duration predominantly during LP. (A) Sleep profile (minutes sleep per 30min) of homozygous *Hk¹* flies (blue) and heterozygous controls (grey). Mean ± SEM. **(B)** *Hk* mutant flies slept significantly less than control flies during 24h ($t_{(181)}$ = 13.15, P<0.0001), **(C)** as well as during LP (white background) and DP (grey background) respectively, LP: $t_{(181)}$ = 12.13, P<0.0001, DP: $t_{(181)}$ = 6.96, P<0.0001. **(D)** Activity of flies while being awake during LP ($t_{(181)}$ = 3.47, P = 0.0006) and DP ($t_{(181)}$ = 6.15, P<0.0001). **(E)** The number of sleep bouts decreased significantly in *Hk* mutant flies during LP ($t_{(181)}$ = 7.75, P<0.0001) and increased during DP ($t_{(181)}$ = 9.43, P<0.0001). **(F)** The average duration of sleep bouts was significantly decreased during both LP ($t_{(181)}$ = 5.63, P<0.0001) and DP ($t_{(181)}$ = 9.18, P<0.0001) in *Hk* mutants. **(G)** PDoze of *Hk* mutants was significantly reduced during LP ($t_{(181)}$ = 12.38, P<0.0001) but not DP ($t_{(181)}$ = 0.08, P=0.9374). **(H)** Sleep depth of *Hk* mutants decreased during LP ($t_{(181)}$ = 11.66, P<0.0001) and DP ($t_{(181)}$ = 7.17, P<0.0001). **(I)** Latency (in min) to fall asleep during DP was increased ($t_{(181)}$ = 4.43, P<0.0001). **(B–I)** Genotypes were compared by unpaired two-sided t test. ns P>0.05, *** P<0.001, **** P<0.0001. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 87–96.

More specifically, PDoze is the probability of an active fly to become inactive in the next time bin (the next minute), while PWake is the probability of an inactive fly to become active. Wiggin et al. (2020) tried to determine a biological relevance for each parameter. They found PDoze to be a good approximate for the sleep pressure that builds up over time. If PDoze is increased, the probability of going to sleep is much higher, therefore reflecting a higher sleep pressure in the healthy fly. In mutated flies, the apparent sleep pressure in form of PDoze might be decreased. This however does not mean internal sleep pressure is not building up, but that it cannot be translated into sleep. Thus, PDoze can reflect both, sleep pressure or the ability to translate sleep pressure into sleep. PWake on the other hand seems to be a good measure of sleep depth in flies. The lower PWake, the lower the probability of flies to wake up, hence the deeper the flies are asleep. The higher PWake, the higher the probability of flies to wake up, characterizing lighter sleep phases. During LP, Hk^1 flies showed a decreased PDoze (Figure 2.2G) and a higher PWake (Figure 2.2H) compared to controls. This would suggest a lower sleep pressure reducing the probability of flies to fall asleep; and if they did, they experienced lighter sleep than controls, causing them to wake up more often. Both characteristics fit with the sleep profile seen and described before. During DP, PDoze did not differ between Hk^1 and WT flies (Figure 2.2G). This suggests that the rate by which the flies fell asleep did not differ either. PWake on the other hand was slightly but significantly increased, hinting that Hk^1 flies woke up more frequently during the night than controls (Figure 2.2). Again, both parameters fit the sleep profile described before.

Finally, the latency to fall asleep during DP (Figure 2.2I) quantifies the time between lights off and the start of the first sleep bout. As flies mainly sleep during the night, this serves as an additional measure of sleep pressure that might have built up during the day, or its translatability into sleep. Consistent with the inference from the analysis of PDoze above,

Hk^{1} flies took longer to fall asleep than WT flies (Figure 2.2I).

Taken together, all parameters point to a sleep architecture in which *Hk*^{1} flies are not able to fall asleep or maintain sleep during LP, characterized by fewer short and light sleep episodes. During DP, *Hk*^{1} flies suffer from fragmented sleep, having shorter and lighter sleep bouts than WT flies, but are able to fall asleep more frequently in order to counteract potential sleep deprivation.

2.2.2 RNAi-mediated Hk depletion from dFB neurons

Knockout of Hk in *Drosophila* is a highly disruptive manipulation. Not only is Hk abundant pan-neuronally in the brain, it is also expressed in muscle cells, including th heart (Li et al., 2022). To determine whether the absence of Hk exclusively in sleep-promoting dFB neurons only is sufficient to mimic the sleep phenotypes seen in *Hk*^{1} flies, the expression of *Hk* was knocked down in dFB neurons using the *UAS/Gal4* system (Brand and Perrimon, 1993), combining the dFB neuron-specific driver *R23E10-Gal4* with a Hk-specific RNAi line (VDRC line 47805). Sleep was measured as described before.

Knockdown of Hk in dFB neurons (*R23E10>RNAi*^{Hk}), similar to pan-neuronal Hk knockout, caused a reduction of sleep duration compared to both parental controls (*R23E10-Gal4*/+ and *UAS RNAi*^{Hk}/+) (Figure 2.3A–C), confirming previous reports (Kempf et al., 2019; Pimentel et al., 2016), while simultaneously contradicting a recent report that questions the role of dFB neurons in sleep (De et al., 2023). Similar to the sleep phenotype of *Hk*^{1} flies, sleep was most prominently lost during the day, but also significantly reduced during the night (Figure 2.3C). On average, flies with dFB-restricted Hk depletion slept about 5 hours less than their parental controls (Figure 2.3B).

Again, considering the flies' activity during wake bouts, *R23E10>RNAi*^{Hk} flies displayed

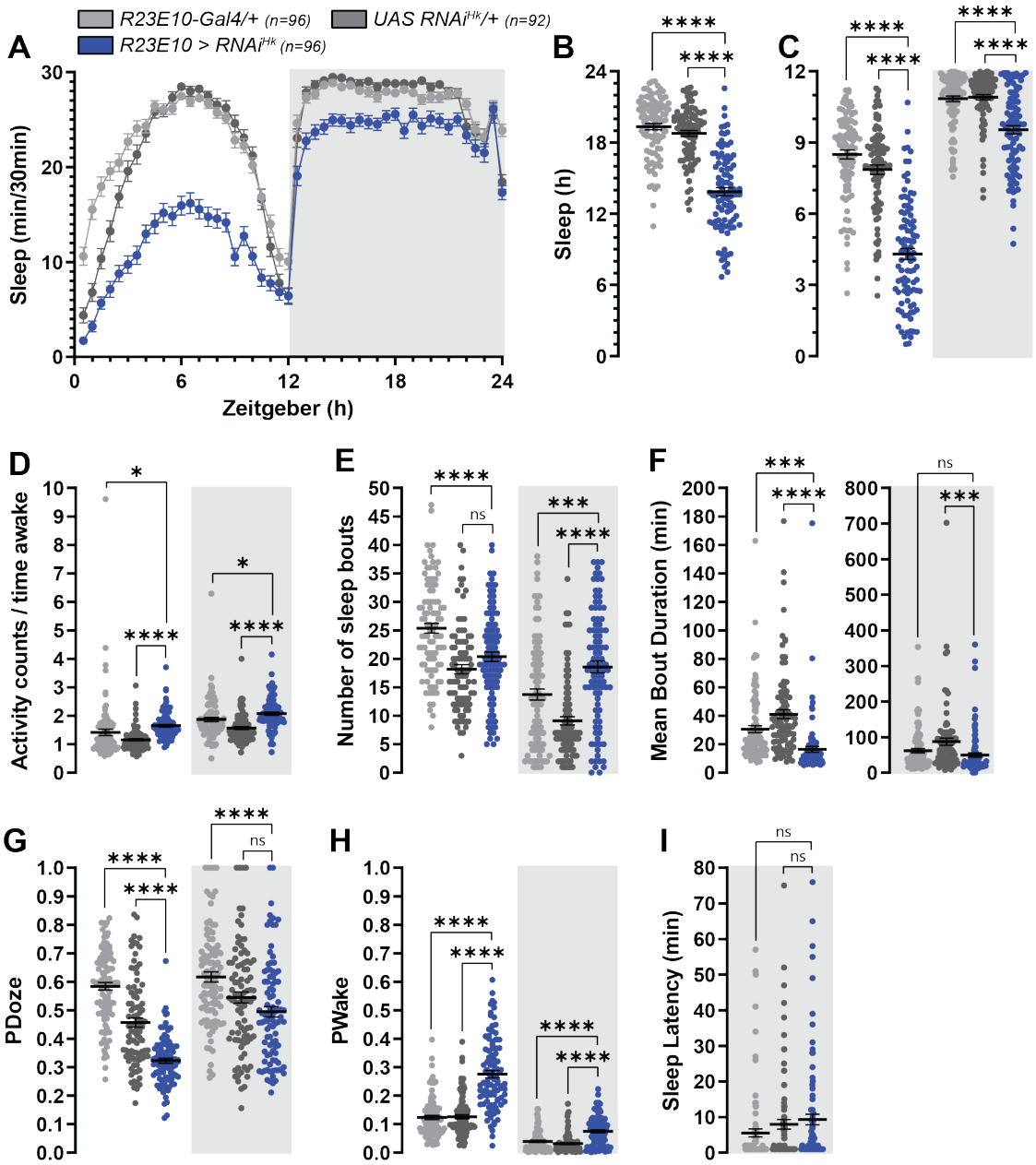

Figure 2.3: ***R23E10>RNAi^{Hk}* flies show reduced sleep duration predominantly during LP.** **(A)** Sleep profile (minutes sleep per 30min) of *R23E10>RNAi^{Hk}* flies (blue) and parental controls (grey). Mean ± SEM. **(B)** Flies expressing *R23E10>RNAi^{Hk}* in dFB neurons slept less than their parental controls during 24h ($F_{(2, 281)}$ = 111.3, P<0.0001) and **(C)** during LP (white background) and DP (grey background) respectively. LP: $F_{(2, 281)}$ = 119.9, P<0.0001, DP: $F_{(2, 281)}$ = 32.30, P<0.0001. **(D)** Activity of flies while being awake during LP ($F_{(2, 281)}$ = 11.33, P<0.0001) and DP ($F_{(2, 281)}$ = 17.28, P<0.0001). **(E)** The number of sleep bouts was significantly increased in *R23E10>RNAi^{Hk}* flies during DP ($F_{(2, 281)}$ = 25.79, P<0.0001) but not LP ($F_{(2, 281)}$ = 19.96, P<0.0001). **(F)** The average duration of sleep bouts was significantly decreased during LP ($F_{(2, 281)}$ = 22.66, P<0.0001) but not DP ($F_{(2, 281)}$ = 6.63, P = 0.0015). **(G)** PDoze of *R23E10>RNAi^{Hk}* flies was significantly reduced during LP ($F_{(2, 281)}$ = 103.0, P<0.0001) but not DP ($F_{(2, 281)}$ = 11.24, P<0.0001). **(H)** Sleep depth decreased during both LP ($F_{(2, 281)}$ = 84.60, P<0.0001) and DP ($F_{(2, 281)}$ = 29.61, P<0.0001). **(I)** Latency (in min) to fall asleep during DP was unaffected by *RNAi^{Hk}* expression ($F_{(2, 281)}$ = 2.05, P = 0.1305). **(B–I)** Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, * P<0.05, *** P<0.001, **** P<0.0001. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 92–96.

modest hyperactivity compared to their parental controls, during both LP and DP (Figure 2.3D). Though the increase in activity was statistically significant, the effect size was small.

Taking a closer look at how the sleep architecture changed upon decreased *Hk* expression, a similar picture arose as seen in *Hk* mutant flies. While the number of sleep bouts was unchanged in *R23E10>RNAi*^{Hk} flies as compared to at least one parental control during LP, the mean sleep bout duration dropped significantly (Figure 2.3E–F), accounting for the sleep loss during LP. In DP, the mean sleep bout duration of *R23E10>RNAi*^{Hk} flies was significantly shortened compared to one parental control only (Figure 2.3E), and the number of sleep bouts did significantly increase (Figure 2.3D). The combined effect was an overall modest sleep loss of about 1.5 hours during DP. The sleep phenotype of *R23E10>RNAi*^{Hk} flies thus closely resembled that of *Hk*^{1} flies.

As additional read-outs of sleep architecture, PDoze, PWake, and sleep latency were analysed. During LP, PDoze was reduced in *R23E10>RNAi*^{Hk} flies as compared to both parental controls. This reflected a lower apparent sleep pressure during the day, whereas during the night sleep pressure seemed unchanged, as PDoze did not differ from at least one parental controls (Figure 2.3F). Compared to controls, PWake on the other hand was significantly increased in *R23E10>RNAi*^{Hk} flies during both LP and DP (Figure 2.3G). This phenotype echoes the shorter sleep bout duration during both LP and DP in *R23E10>RNAi*^{Hk} flies. Sleep latency was unchanged in *R23E10>RNAi*^{Hk} flies as compared to both parental controls (Figure 2.3).

Depleting Hk from sleep-promoting dFB neurons is therefore sufficient to shorten sleep. This suggests that Hk deficiency in the rest of the brain as well as muscle cells only plays a minor role in creating the sleep phenotype seen in *Hk* (and perhaps also *Sh*) mutants.

2.2.3 Restoration of WT Hk in dFB neurons of *Hk^1* mutants

Kempf et al. (2019) reported that the rescue of functional Hk in dFB neurons in a *Hk* mutant background restores normal sleep patterns (Kempf et al., 2019). To reproduce previous findings, a fully functional copy (*Hk WT*) as well as a catalytically inactive variant of *Hk* (*Hk K289M*) were expressed in dFB neurons (using *R23E10-Gal4*) in an otherwise *Hk* mutant background (*Hk^1*), and sleep behaviour was recorded. The catalytically inactive variant was used in order to determine whether the oxidoreductase activity of the Hk subunit is essential for proper functioning of the Hyperkinetic/Shaker channel complex, and therefore sleep.

dFB neuron-specific *Hk WT* expression in a *Hk* mutant background (*Hk^1; R23E10>Hk WT*) indeed increased sleep relative to *Hk^1* flies, especially during LP (Figure 2.4A and C), but fell approximately 2h short of a full rescue to sleeping hours of WT controls (*Hk^1/+; UAS Hk WT/+* and *Hk^1/+; R23E10-Gal4/+*). dFB neuron-specific expression of the catalytically inactive *Hk K289M* variant (*Hk^1; R23E10>Hk K289M*), on the other hand, showed no such increase in sleep duration as compared to *Hk* mutants (*Hk^1; UAS Hk K289M/+* and *Hk^1; R23E10-Gal4/+*) and WT controls (*Hk^1/+; UAS Hk K289M/+* and *Hk^1/+; R23E10-Gal4/+*) (Figure 2.4B and C). Confirming previous findings (Kempf et al., 2019), I conclude that the Hk subunit needs to be catalytically active in order to regulate sleep in dFB neurons. A corollary of these findings is that *Hk^1; R23E10>Hk K289M* flies are a good control for *Hk^1; R23E10>Hk WT* flies, since their genetic components are almost identical. Additionally, the three heterozygous WT controls as well as the three *Hk* mutant controls overall showed very similar sleep behaviour. Data from the three heterozygous WT control groups and the three *Hk* mutant control groups were therefore pooled for all subsequent analyses. Behaviour for all groups individually as shown in Figure 2.4C, as well as the appropriate comparisons can be found in Supplementary Figure 6.1.

During LP, sleep in *Hk¹; R23E10>Hk WT* flies almost reached WT levels, while only a partial rescue was achieved during DP (Figure 2.4D). All *Hk* mutant flies showed hyperactivity during LP and DP (Figure 2.4E), resembling the phenotype seen in *Hk¹* flies (compare

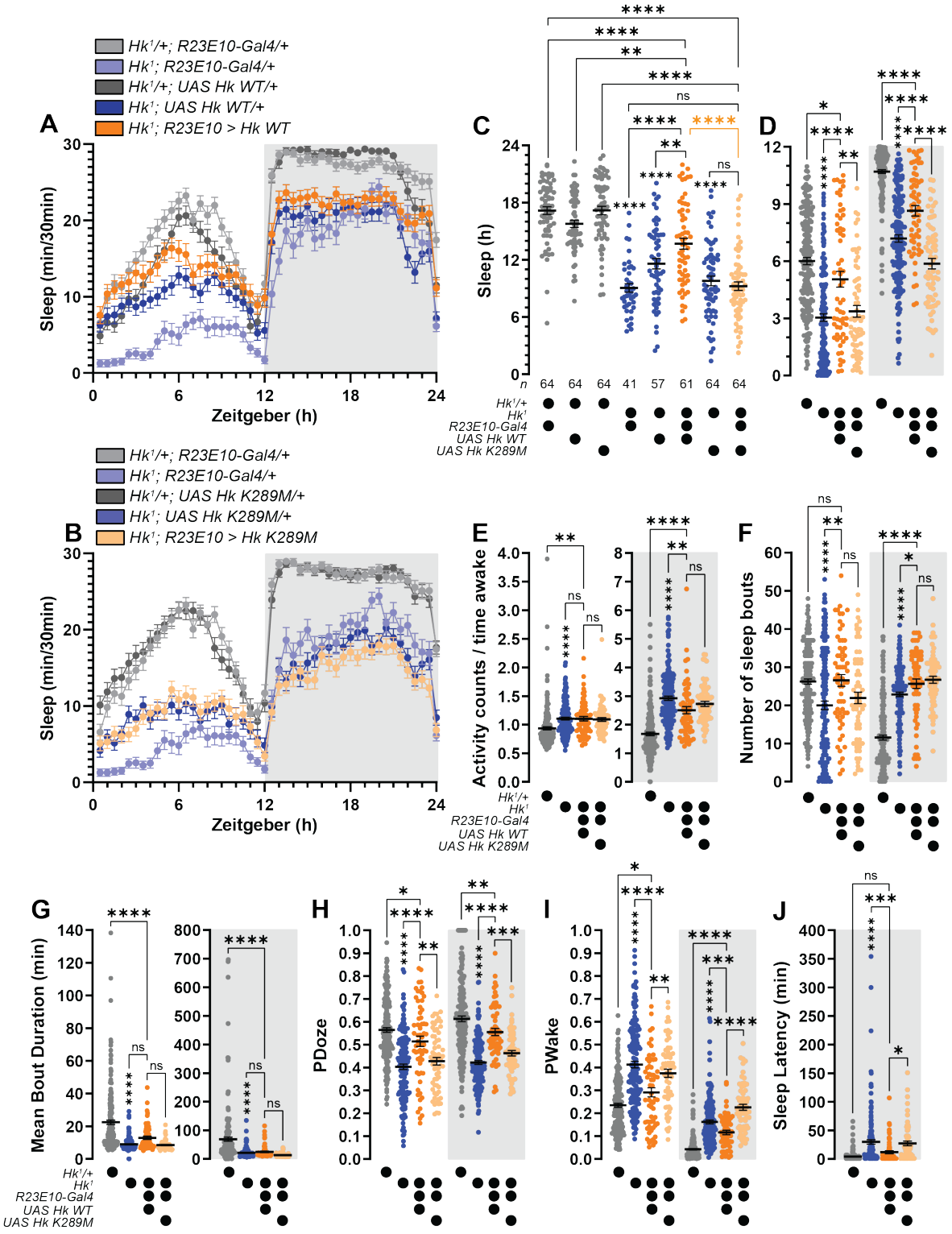

Figure 2.4: WT *Hk* expression in dFB neurons partially rescues sleep in *Hk^1* flies. **(A)** Sleep profile (minutes sleep per 30min) of *Hk^1; R23E10>Hk WT* flies (dark orange) and appropriate *Hk* mutant (blue) and heterozygous (grey) controls. Mean ± SEM. **(B)** Sleep profile (minutes sleep per 30min) of *Hk^1; R23E10>Hk K289M* flies (light orange) and appropriate *Hk* mutant (blue) and heterozygous (grey) controls. Mean ± SEM. **(C)** Total amount of sleep per day of all genotypes tested. *Hk^1; R23E10>Hk WT* flies showed a partial rescue of sleep duration as compared to *Hk* mutant flies and heterozygous controls ($F_{(7, 471)}$ = 53.45, P<0.0001). Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, ** P<0.01, **** P<0.0001. Comparisons between genotypes are indicated by connecting lines, if no line is indicated, comparison was made with the direct WT control. Two independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 41–64. **(D)** Total amount of sleep during LP (white background) or DP (grey background) of *Hk^1; R23E10>Hk WT* flies, *Hk^1; R23E10>Hk K289M* flies, and pooled *Hk* mutant and heterozygous WT controls. *Hk^1; R23E10>Hk WT* flies showed a partial rescue in sleep time during both LP ($F_{(3, 475)}$ = 42.57, P<0.0001) and DP ($F_{(3, 475)}$ = 135.4, P<0.0001). **(E)** Activity of flies while being awake during LP ($F_{(3, 475)}$ = 10.41, P<0.0001) and DP ($F_{(3, 475)}$ = 63.73, P<0.0001). **(F)** The number of sleep bouts was normalized in *Hk WT*-expressing flies during LP ($F_{(3, 475)}$ = 9.74, P<0.0001) but not during DP ($F_{(3, 475)}$ = 95.14, P<0.0001). **(G)** The average duration of sleep bouts was significantly decreased in all *Hk* mutant flies during LP ($F_{(3, 475)}$ = 40.13, P<0.0001) and DP ($F_{(3, 475)}$ = 22.15, P<0.0001). **(H)** PDoze of *Hk^1; R23E10 > Hk WT* flies was partially rescued to WT levels during both LP ($F_{(3, 475)}$ = 39.66, P<0.0001) and DP ($F_{(3, 475)}$ = 62.56, P<0.0001). **(I)** Sleep depth was partially rescued in *Hk^1; R23E10 > Hk WT* flies during LP ($F_{(3, 475)}$ = 43.75, P<0.0001) and during DP ($F_{(3, 475)}$ = 108.3, P<0.0001). **(J)** Latency (in min) to fall asleep during DP was returned to WT levels in *Hk^1; R23E10 > Hk WT* flies ($F_{(3, 475)}$ = 21.52, P<0.0001). **(D–J)** Heterozygous WT controls and *Hk* mutant controls were pooled respectively. Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Comparisons between genotypes are indicated by connecting lines, if no line is indicated, comparison was made with the direct WT control. Two independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 41–192.

with Figure 2.2D). Analysing the sleep architecture, the number of sleep bouts was rescued in *Hk^1; R23E10>Hk WT* flies during LP compared to WT controls, while during DP the number of sleep bouts was increased as in other *Hk* mutants (Figure 2.4F). The average sleep bout duration in *Hk^1; R23E10>Hk WT* flies was statistically indistinguishable from other *Hk* mutants, despite a tendency toward longer sleep bouts during both LP and DP (Figure 2.4G). Incomplete rescue in both these parameters thus explains the incomplete rescue in total sleep duration seen in *Hk^1; R23E10>Hk WT* flies.

PDoze was significantly increased during both LP and DP in *Hk^1; R23E10>Hk WT* flies as compared to *Hk* mutant controls, but did not reach WT levels (Figure 2.4H), meaning that translated sleep pressure was higher in *Hk^1; R23E10>Hk WT* flies than in *Hk* mutants. In *Hk^1; R23E10>Hk WT* flies, PWake was decreased during both LP and DP as compared to *Hk* mutant controls, but again, did not fully reach WT levels (Figure 2.4I). This shows, that

Hk^1; R23E10>Hk WT flies had close to normal sleep depth, but still suffered from slightly lighter sleep phases than WT flies.

Lastly, the sleep latency during DP was not changed in *Hk^1; R23E10>Hk WT* flies as compared to WT controls, while *Hk* mutant flies had a much longer sleep latency (Figure 2.4J). Again, this showed that sleep pressure was higher in *Hk^1; R23E10>Hk WT* flies than in *Hk* mutant controls.

These findings are in line with the previous conclusion that the sleep behaviour of *Hk^1; R23E10>Hk WT* flies resembles that of WT controls, whereas the sleep behaviour of *Hk^1; R23E10>Hk K289M* flies resembles that of *Hk^1* mutants (Kempf et al., 2019).

2.3 Learning and memory

A vast body of literature shows that sleep is important for learning and memory consolidation, and that sleep deprivation can impair either (Chandra et al., 2023; Cirelli et al., 2004; Van Dongen et al., 2003; Stickgold, 2005; Walker and Stickgold, 2004; Wiggin et al., 2021; Yoo et al., 2007). Additionally, many studies have found mutations in the Sh channel complex (or A-type K^+ channels in mammals) to similarly cause learning and memory impairments (Bushey et al., 2007; Cowan and Siegel, 1984, 1986; Giese et al., 1998). This raises the question of whether the chronic sleep deficit in K^+ channel mutants could be the reason for their apparent learning and memory impairments. In the following set of experiments I will try to determine whether rescuing the short-sleeping phenotype of *Hk* mutant flies is sufficient to restore normal learning behaviour.

Learning of flies was assessed using a negative reinforcement paradigm with two different odours (see Methods, section 5.3). For each fly, the post-training preference for the

unpunished odour was subtracted from baseline preference for said odour, resulting in a parameter reflecting the learning ability of flies, in the following called 'learning index'.

As reported before (Bushey et al., 2007), Hk^1 flies tested in the learning paradigm on average showed a deficit in their learning behaviour, as compared to WT flies (Figure 2.5A). Interestingly, the same phenotype could not be reproduced upon Hk knockdown in dFB neurons (Figure 2.5B), even though sleep loss was similar between Hk^1 flies and *R23E10>RNAi*^{Hk} flies. The learning index was reduced in *R23E10>RNAi*^{Hk} flies compared to one parental control (*R23E10-Gal4/+*, P<0.0001) but not the other (*UAS RNAi*^{Hk}*/+*, ns). This could either indicate that Hk deficiency in dFB neurons and the resulting sleep loss are not sufficient

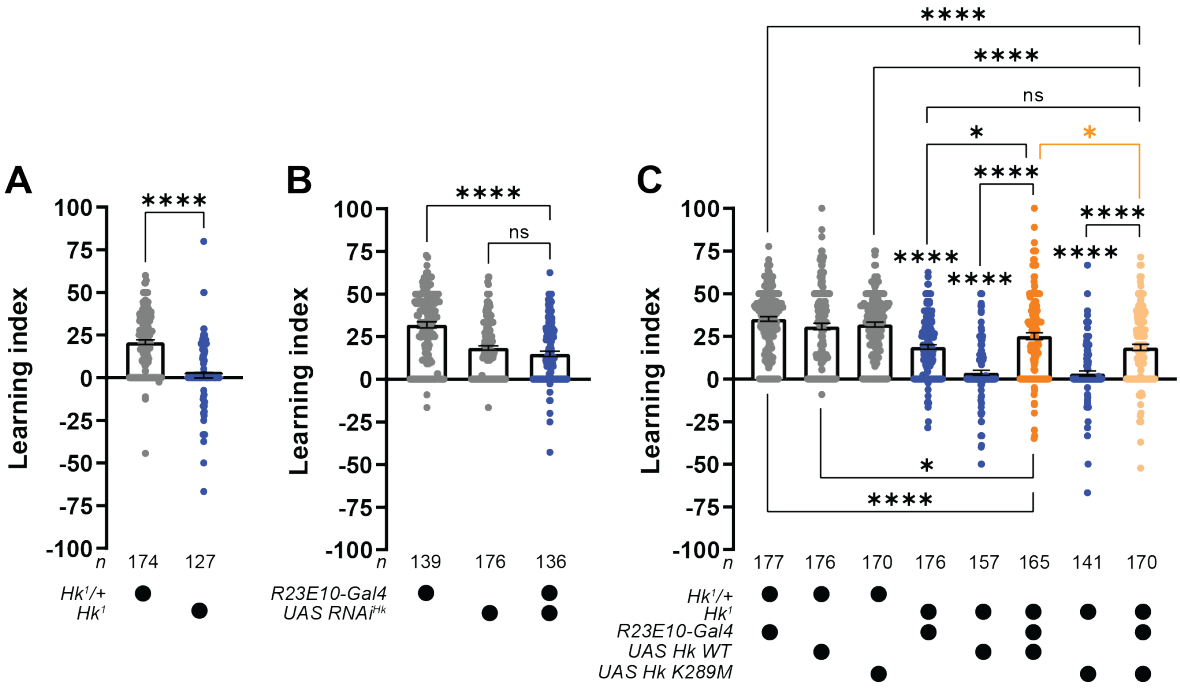

Figure 2.5: *Hk*^{1} flies show learning deficits that can be partially rescued by rescuing *Hk* expression in dFB neurons. **(A)** *Hk* mutant flies (blue) showed a significantly decreased learning index compared to heterozygous control (grey) flies ($t_{(299)}$ = 9.06, P<0.0001). **(B)** Learning index of *R23E10>RNAi*^{Hk} flies (blue) and parental controls (grey). *R23E10>RNAi*^{Hk} flies showed no learning deficit as compared to both parental controls ($F_{(2, 448)}$ = 34.06, P<0.0001). **(C)** Learning index of *Hk*^{1}*; R23E10 > Hk WT* flies (dark orange), *Hk*^{1}*; R23E10>Hk K289M* flies (light orange) and *Hk* mutant (blue) and heterozygous (grey) controls. *Hk*^{1}*; R23E10>Hk WT* flies showed partially rescued learning ($F_{(7, 1324)}$ = 58.40, P<0.0001). Genotypes were compared either by unpaired two-sided t test **(A)** or by one-way ANOVA with Holm-Šídák's multiple comparisons test **(B–C)**. ns P>0.05, * P<0.05, **** P<0.0001. Comparisons between genotypes are indicated by connecting lines, if no line is indicated, comparison was made with the direct WT control. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 127–177.

to cause severe learning deficits, which in turn would mean that the learning deficit seen in *Hk*^{1} flies is not caused by sleep loss, but rather due to a secondary function of Hk in the brain. Another explanation could be that the efficacy of *RNAi* expression might have been poor.

To determine how important sleep and functional Hk in dFB neurons are for learning without using *RNAi* expression, *Hk* expression was rescued in dFB neurons of *Hk*^{1} flies. Learning abilities of either the close-to-normal sleeping *Hk*^{1}*; R23E10>Hk WT* flies or the short sleeping *Hk*^{1}*; R23E10>Hk K289M* flies were tested and compared to heterozygous *Hk*^{1}/+ WT controls or homozygous *Hk*^{1} mutants (Figure 2.5C). *Hk*^{1}*; R23E10>Hk K289M* flies showed no improved learning index compared to at least one *Hk* mutant control (*Hk*^{1}*; R23E10-Gal4/+*), and showed clear learning deficits when compared to either WT control (*Hk*^{1}*/+; R23E10-Gal4/+* and *Hk*^{1}*/+; UAS Hk K289M/+*). *Hk*^{1}*; R23E10>Hk WT* flies as well showed a learning deficit when compared to their WT controls (*Hk*^{1}*/+; R23E10-Gal4/+* and *Hk*^{1}*/+; UAS Hk WT/+*). However, other than *Hk*^{1}*; R23E10>Hk K289M* flies, *Hk*^{1}*; R23E10>Hk WT* flies showed an improved learning index as compared to the *Hk*^{1} mutants (*Hk*^{1}*; R23E10-Gal4/+* and *Hk*^{1}*; UAS Hk WT/+*). Additionally, *Hk*^{1}*; R23E10>Hk WT* flies showed an improved learning index compared to *Hk*^{1}*; R23E10>Hk K289M* flies. The expression of catalytically competent Hk thus partially improved the learning index, similar to partially rescuing sleep duration.

2.4 Lifespan

An additional factor often associated with sleep loss and chronic sleep deprivation is a reduced lifespan (Rechtschaffen et al., 1983; Shaw et al., 2002). Again, mutations in the Sh channel complex are known to cause a shortening of lifespan in *Drosophila melanogaster*

(Bushey et al., 2010; Trout and Kaplan, 1970) as well as mammals (McCormack et al., 2002; Connor et al., 2005). For this reason, lifespan was another parameter analysed with different states of *Hk* expression in flies. Lifespan was assessed and analysed as stated in Methods section 5.4. As has been described before (Bushey et al., 2010; Trout and Kaplan, 1970), Hk^1 flies showed a drastically shorter lifespan than WT controls (Figure 2.6A-B). WT flies had a median lifespan of 62 days, whereas Hk^1 flies had a median lifespan of only 29 days (Figure 2.6A), and lived therefore less than half a long as WT flies.

To determine how important *Hk* expression in dFB neurons is for survival, Hk was rescued in dFB neurons only. Either Hk construct used before, *Hk WT* and *Hk K289M*, was expressed in dFB neurons and lifespan of flies was assessed (Figure 2.6C–E). It is important to note that all *Hk* mutant flies (Hk^1*; UAS R23E10-Gal4/+*, Hk^1*; UAS Hk WT/+*, and Hk^1*; UAS Hk K289M/+*) died significantly earlier than their WT controls (Hk^1*/+; UAS R23E10-Gal4/+*, Hk^1*/+; UAS Hk WT/+*, and Hk^1*/+; UAS Hk K289M/+*, respectively). However, neither Hk^1*; R23E10>Hk K289M* flies (Hk^1*; R23E10>Hk K289M*) nor Hk^1*; R23E10>Hk WT* flies (Hk^1*; R23E10>Hk WT*) showed an improved life expectancy compared to *Hk* mutant flies (Figure 2.6E): Hk^1*; R23E10>Hk WT* flies and Hk^1*; R23E10>Hk K289M* flies both had a median lifespan of 54 days, and their respective probability of survival closely followed that of Hk^1*; R23E10-Gal4/+* flies (median lifespan: 53 days) (Figure 2.6C-E). Overall, the decrease in life expectancy in *Hk* mutant flies as compared to WT controls was not as severe as in the absence of additional transgenes (Figure 2.6A–B), but still significant.

These results indicate that lifespan in case of *Hk* mutations might not primarily be caused by the loss of sleep but other impairments caused by the depletion of functional Hk. This means that a brain-wide rescue of Hk in otherwise *Hk* mutant flies should fully rescue lifespan. To test this, *Hk WT* or *Hk K289M* were expressed pan-neuronally in *Hk* mutant flies

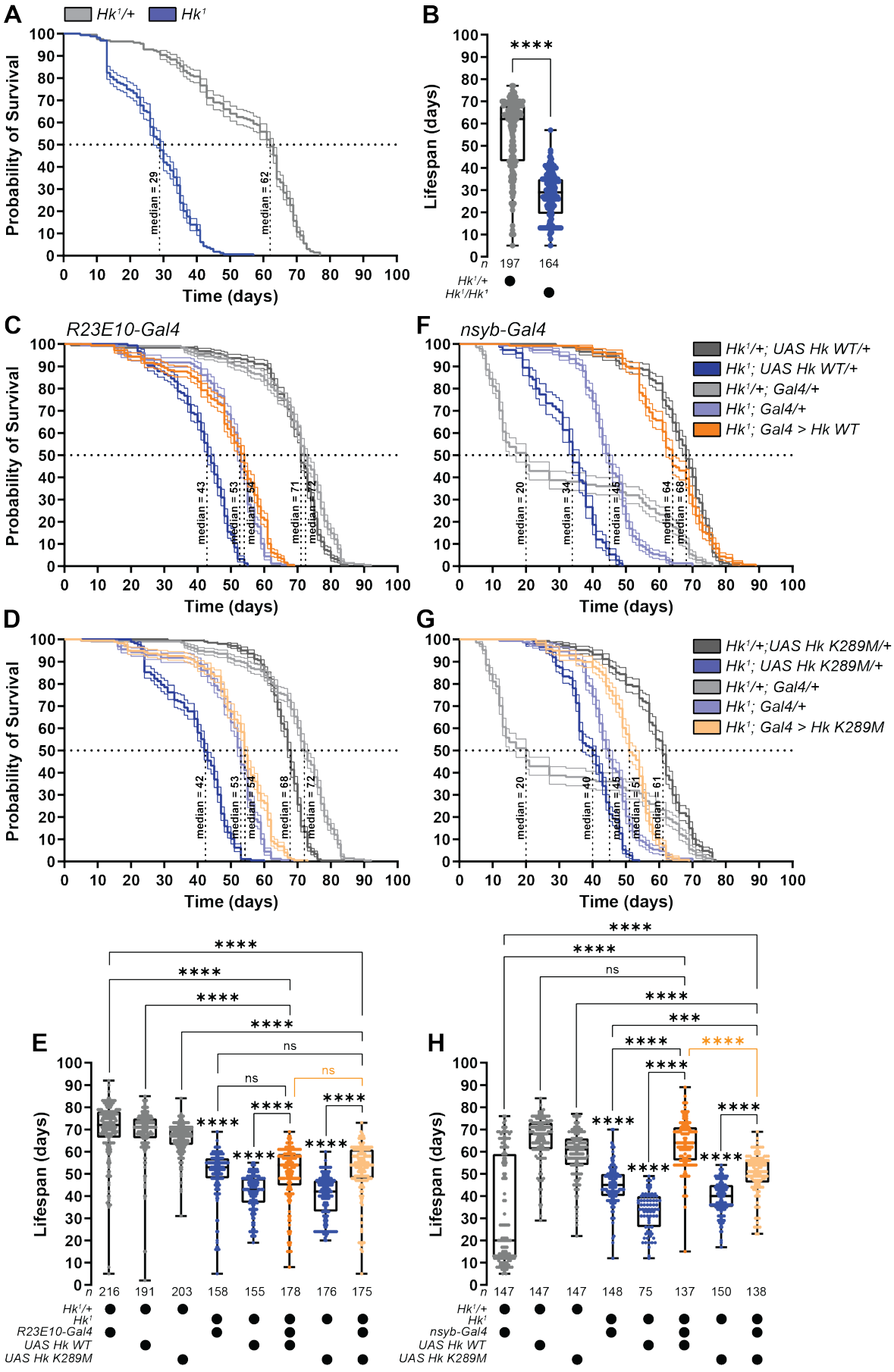
A
Hk¹/+
Hk¹
Probability of Survival
Time (days)
median = 29
median = 62
B
Lifespan (days)
****
n 197 164
Hk¹/+
Hk¹/Hk¹
C
R23E10-Gal4
median = 43
median = 53
median = 54
median = 71
median = 72
D
median = 42
median = 53
median = 54
median = 68
median = 72
F
nsyb-Gal4
Hk¹/+; UAS Hk WT/+
Hk¹; UAS Hk WT/+
Hk¹/+; Gal4/+
Hk¹; Gal4/+
Hk¹; Gal4 > Hk WT
median = 20
median = 34
median = 45
median = 64
median = 68
G
Hk¹/+;UAS Hk K289M/+
Hk¹; UAS Hk K289M/+
Hk¹/+; Gal4/+
Hk¹; Gal4/+
Hk¹; Gal4 > Hk K289M
median = 20
median = 40
median = 45
median = 51
median = 61
E
ns
n 216 191 203 158 155 178 176 175
Hk¹/+
Hk¹
R23E10-Gal4
UAS Hk WT
UAS Hk K289M
H
***
n 147 147 147 148 75 137 150 138
nsyb-Gal4

Figure 2.6: Brain-wide knockout of Hk causes a shortened lifespan that can be rescued by pan-neuronal *Hk* expression. **(A)** Survival curve of *Hk* mutant flies (blue) and heterozygous control flies (grey). **(B)** Lifespan of Hk^1 flies and heterozygous controls. *Hk* mutants lived a significantly shorter time than controls ($t_{(359)}$ = 18.09, P<0.0001). **(C)** Survival curve of *Hk^1; R23E10>Hk WT* flies (dark orange), *Hk* mutant (blue) and heterozygous (grey) controls. **(D)** Survival curve of *Hk^1; R23E10>Hk K289M* flies (light orange), *Hk* mutant (blue) and heterozygous (grey) controls. **(E)** Lifespan of Hk^1 flies with dFB-specific Hk expression and respective *Hk* mutant and heterozygous controls. Lifespan could not be rescued by dFB-specific expression of *Hk WT* in a Hk^1 background ($F_{(7, 1444)}$ = 231.7, P<0.0001). **(F)** Survival curve of *Hk^1; nsyb>Hk WT* flies (dark orange), *Hk* mutant (blue) and heterozygous (grey) controls. **(G)** Survival curve of *Hk^1; nsyb>Hk K289M* flies (light orange), *Hk* mutant (blue) and heterozygous (grey) controls. **(H)** Lifespan of Hk^1 flies with brain-wide Hk expression and respective *Hk* mutant and heterozygous controls. Lifespan was rescued by pan-neuronal expression of *Hk WT* in a Hk^1 background ($F_{(7, 1081)}$ = 148.5, P<0.0001). Genotypes were compared either by unpaired two-sided t test **(B)** or by one-way ANOVA with Holm-Šídák's multiple comparisons test **(E)** and **(H)**. ns P>0.05, **** P<0.0001. Comparisons between genotypes are indicated by connecting lines, if no line is indicated, comparison was made with the direct WT control. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, n = 155–216.

using the *nsyb-Gal4* driver and lifespan was assessed.

As seen in Figure 2.6F–H, *Hk^1/+; nsyb-Gal4/+* flies had a very low life expectancy, and overall showed other signs of poor health such as sluggish and unsteady walking, and lethargy. It remains unclear what caused this decrease in health in (genetically) WT flies, but it seemed to be a combination of the *nsyb-Gal4* driver heterozygously expressed in a Canton-S (wild type strain, CS) background (for comparison, see also Chapter 3.5). Therefore, *Hk^1/+; nsyb-Gal4/+* flies were not considered a proper WT control in the further analysis of lifespan (but will be shown in figures for the sake of completeness).

Figures 2.6F and H show that brain-wide *Hk WT* expression (*Hk^1; nsyb>Hk WT*) was able to fully rescue the lifespan of flies. *Hk^1; nsyb>Hk WT* flies with a median lifespan of 64 days did not significantly differ from WT control flies (*Hk^1/+; UAS Hk WT/+*) with a median lifespan of 68 days. Expression of the catalytically dead *Hk K289M* (*Hk^1; nsyb>Hk K289M*), however, was not able to fully rescue lifespan, even though it did increase median life expectancy by a few days (Figure 2.6G and H). *Hk^1; nsyb>Hk K289M* flies lived for a median lifespan of 51 days, which was significantly lower than the median lifespan of 61 days of their WT control (*Hk^1/+; UAS Hk K289M/+*). *Hk* mutant flies showed a median

lifespan of 34 (*Hk^1; UAS Hk WT/+*), 40 (*Hk^1; UAS Hk K289M*), and 45 days (*Hk^1; nsyb-Gal4/+*), respectively.

2.5 Discussion

The above experiments show that Hk deficiency in sleep-promoting dFB neurons is sufficient to disrupt sleep, and that restoring *Hk* expression in dFB neurons of an otherwise *Hk* mutant fly at least partially rescues the sleep phenotype. Similarly, the learning and memory deficits of Hk-deficient flies could be partially rescued upon reintroduction of Hk into dFB neurons, while lifespan stayed drastically shortened. However, a full rescue of *Hk* expression in the whole brain could reinstate normal lifespan, suggesting that the effect of the *Hk* mutation on lifespan is tied to the brain, and not to other organs (e.g. the heart). Interestingly, any rescue in sleep, learning, and lifespan required that Hk's catalytically active site remains intact.

Given a vast body of literature documenting learning and memory deficits upon sleep deprivation not only in *Drosophila melanogaster* (Li et al., 2009; Seugnet et al., 2008), but also in mammals (Saygin et al., 2017) including humans (Killgore et al., 2006; Van Dongen et al., 2003; Yoo et al., 2007), and recently even in *Caenorhabditis elegans* (Chandra et al., 2023), the results reported here were expected. However, since chronic sleep deprivation in this study is caused by a genetic mutation, it is difficult to dissect whether sleep loss is causing the significant learning deficit in Hk-deficient flies, or if Hk itself is needed in order to produce a robust learning response. K^+ channels are prominently expressed in the MB (Rogero et al., 1997; Schwarz et al., 1990), a structure important for the formation, storage and retrieval of olfactory memories (Heisenberg et al., 1985; McBride et al., 1999). Sh channels conduct A-type current in about 25% of the MB neurons (Gasque et al., 2005), and

the absence of Sh channels modifies the K^+ current profile in these neurons (Gasque et al., 2005), which suggests that the absence of Hk would cause a change as well. It has not been directly shown whether Hk deficiency in the MB leads to dysfunctionality or causes changes in K^+ currents, but *Sh* and *Hk* mutants have both been shown to cause learning impairments in both the fruit fly (Cowan and Siegel, 1984, 1986) and mice (Giese et al., 1998). Whether these impairments are caused directly by dysregulations of K^+ currents or are a secondary phenotype caused by chronic sleep loss has not been evaluated to date. However, rescuing *Hk* expression in dFB neurons can give us a clue whether Hk itself is needed for learning. dFB neurons are not known to regulate learning uncoupled from sleep duration. If learning deficits were indeed a direct consequence of Hk deficiency in the brain, *Hk* expression in dFB neurons is expected to be insufficient to rescue them. Being able to partially rescue sleep and learning simultaneously indicates learning deficits following a loss in sleep, rather than being a direct consequence of Hk deficiency. This is in line with earlier findings (Bushey et al., 2007), but additional experiments are needed to validate this hypothesis.

An intriguing possibility is that Hk in the MB may be required not only for memory but also for the regulation of sleep, in which the structure is demonstrably involved (Joiner et al., 2006; Pitman et al., 2006). Several studies report waking experience, especially including learning and memory tasks, to influence the length and quality of sleep the following night (Bushey et al., 2011; Ganguly-Fitzgerald et al., 2006; Huber et al., 2006, 2007). If flies lacking Hk in the MB are learning-impaired, this might also affect their sleep duration and pattern.

Lifespan was not extended after reintroduction of *Hk* into dFB neurons. Only pan-neuronal expression of *Hk* could rescue lifespan, indicating that the reduced life expectancy of *Hk* mutants is likely not a mere secondary symptom caused by chronically shortened

sleep, but rather caused by direct effects of the loss of Hk in other brain regions (or the whole brain). Indeed, Brew et al. (2007) and Douglas et al. (2007) showed that knockout of Kv1.2, a mammalian Shaker homologue, caused early death in mice, while McCormack et al. (2002) showed that Kvβ2 knockout causes severe seizures in mice that result in a shortened lifespan. However, Douglas et al. (2007) simultaneously showed reduced NREM sleep and increased wakefulness in KO mice.

One way to dissect this relationship is to introduce a dFB-specific knockdown of Hk. With that, a learning deficit as well as a decrease in lifespan could be linked to dFB neurons specifically, and be associated with loss of sleep in general. Even though, as reported previously (Kempf et al., 2019; Pimentel et al., 2016), a robust sleep loss was seen in *R23E10>RNAi*^{Hk} flies, no learning deficit could be reported. At first glance, this result seems contradictory to what has been reported before, here (see Figure 2.5A) and in other literature (Bushey et al., 2007; Cowan and Siegel, 1984, 1986). However, we need to keep in mind that RNAi expression and efficiency is not as strong and long-lived as a null mutation. Sleep recordings of all flies were conducted with 4–5 day old flies, while for learning experiments flies were aged up to 10 days after eclosion. In some cases, RNAi expression in older flies can be less efficient. It is possible, that at age 7–10 days flies already recovered enough sleep to show no learning deficit when tested. For future experiments, it is crucial to measure expression efficacy, and to test sleep and learning of age-matched RNAi-expressing flies, to avoid further confusion and make the interpretation of results more straightforward.

Lastly, it is important to note that the (partial) rescue in sleep and learning only occurred with the catalytically functional version of Hk. This adds to an increasing list of evidence proving the importance and significance of Hk's AKR activity (Bähring et al., 2001; Fogle et al., 2015; Kempf et al., 2019; Pan et al., 2008; Weng et al., 2006). The results presented

here show that behavioural output is coupled to functional redox-sensing activity of Hk. A mutation that disrupts its proper functioning cannot rescue shortened sleep, learning deficits, and reduced survival rates of flies.

Chapter 3

Effects of the Alternative Oxidase on Sleep, Learning, and Lifespan

3.1 Introduction

Impairment of Hk-dependent ROS-sensing, as seen in the previous chapter, is one way of reducing ROS-induced dFB neuron activity (Kempf et al., 2019), leading to sleep loss; another way is to cap ROS production. Conveniently, introducing the alternative oxidase (AOX) into the *Drosophila melanogaster* genome reduces ROS production in the mitochondria.

AOX was discovered in connection with thermogenic cyanide-resistant respiration, which was first described in the protist *Paramecium caudatum* (Lund, 1918). Since the prediction of a second oxidase, in addition to complex IV (cytochrome c oxidase), in 1950 (James and Beevers, 1950) in *Arum spadix*, the existence of AOX has been confirmed in different species of plants (Bendall and Bonner, 1971; Elthon and McIntosh, 1987; Storey and Bahr, 1969), eukaryotic protists such as algae (Dinant et al., 2001) and jacobids (McDonald and Vanlerberghe, 2006), fungi (Henry et al., 1974; Lambowitz and Slayman, 1971), proteobac-

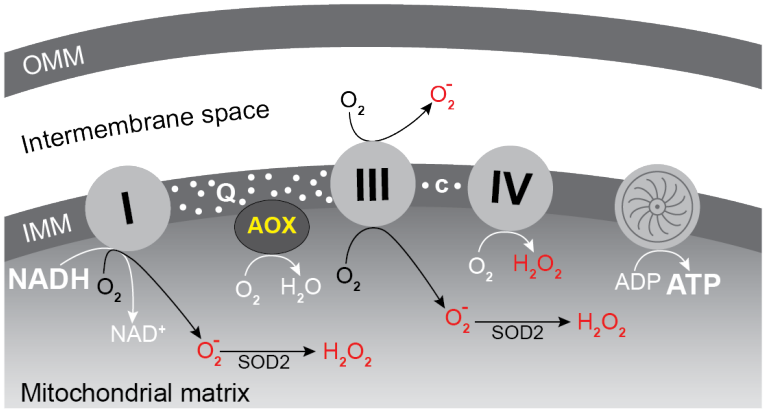

Figure 3.1: The alternative oxidase in the mitochondrial respiratory chain. The alternative oxidase (AOX, here depicted in yellow) is anchored in the inner mitochondrial membrane (IMM), where it uses electrons (white dots) from the ubiquinone pool (Q) to reduce molecular oxygen to water. c: cytochrome, H_2O_2: hydrogen peroxide, O_2^-: superoxide, OMM: outer mitochondrial membrane, SOD2: superoxide dismutase 2. Adapted from Kempf et al. (2019).

teria (McDonald and Vanlerberghe, 2005; Venter et al., 2004), and in various phyla of metazoa (McDonald and Vanlerberghe, 2004; McDonald et al., 2009). Vertebrate and arthropod species, however, seem to have lost this gene during evolution (McDonald et al., 2009).

AOX is located in the mitochondrial inner membrane (IMM). There, it taps into the ubiquinone pool and uses free electrons to reduce molecular oxygen to water, bypassing complexes III and IV of the electron transport chain (ETC) (Figure 3.1). AOX therefore acts as a sort of safety valve, preventing electron spillover that would produce ROS in case of a saturated electron transport chain and/or low adenosine diphosphate (ADP) levels. Thus, mitochondrially-produced ROS levels are significantly decreased in cells which express AOX. ROS production outside of the mitochondria however cannot be prevented by AOX (Maxwell et al., 1999).

With the discovery of metazoan AOX, first experiments on *AOX* expression in model systems were conducted. AOX was first successfully expressed in human embryonic kidney (HEK) cells (Hakkaart et al., 2006), and not long after in whole organisms such as fruit flies (Fernandez-Ayala et al., 2009) and mice (El-Khoury et al., 2013). In both organisms, AOX

was successfully targeted to the IMM, improved cyanide resistance, limited mitochondrial ROS production, and seemed to have no obvious deleterious effects (El-Khoury et al., 2013; Fernandez-Ayala et al., 2009). Additionally, in various disease models, AOX expression could improve or counteract effects of the respective disease, such as Alzheimer's disease (AD) (El-Khoury et al., 2016) and PD (Fernandez-Ayala et al., 2009), smoke-induced lung dysfunction (Giordano et al., 2019), and sepsis (Mills et al., 2016), to name a few.

Using one of the developed AOX fly models, Kempf et al. (2019) established a link between ROS-sensing in dFB neurons and sleep. Their work suggests that Hk in dFB neurons acts as a ROS sensor, causing flies to fall asleep when ROS levels reach a critical threshold. On the other hand, flies expressing *AOX* lost several hours of daily sleep, indicating that a lower ROS production and therefore a slower build-up of critical ROS levels caused the flies to stay awake. As extensive ROS accumulation is known to be harmful and cause cell death (reviewed in Ghosh et al., 2018), the question arises whether sleep might be needed to counteract and reduce ROS levels that build up over the course of waking hours (Reimund, 1994). In other words, is ROS accumulation simply a convenient measure of elapsed waking time, or do flies (and possibly other animals) need sleep in order to relieve the ROS burden that has built up during waking?

In the following set of experiments, I will make use of an established *Drosophila* model of *AOX* expression (Andjelković et al., 2015) and measure two behavioural signs of chronic sleep deprivation, learning ability and lifespan. This will help me assess whether reducing mitochondrial ROS production and consequentially overall sleep time, is harmful to the flies. Moreover, this will enable me to directly compare the results to the effects of Hk deficiency on sleep, learning, and lifespan.

3.2 Sleep

3.2.1 *AOX* expression in dFB neurons

To dissect the relationship between sleep and ROS, the mitochondria-targeted, ROS-reducing *AOX* (*UAS AOX 7.1;* Andjelković et al., 2015) was expressed in dFB neurons of flies, using the dFB-targeting driver *R23E10-Gal4*. Sleep was measured and quantified as described before.

Expression of *AOX* in dFB neurons (*R23E10>AOX 7.1*) reduced overall sleep duration, as observed previously (Kempf et al., 2019). The sleep loss was most pronounced during LP, but also present during DP (Figure 3.2A–C). On average, *R23E10>AOX 7.1* flies lost 5–6 hours of sleep per day, as compared to either parental control (*R23E10-Gal4/+* and *UAS AOX 7.1/+*) (Figure 3.2B). 3–4 hours of sleep were lost during LP, the other 2 hours were lost during DP (Figure 3.2C). This pattern of reduced sleep duration resembled sleep loss caused by loss of functional Hk (see Figure 2.2 for reference).

Similar to *Hk* mutant flies, *R23E10>AOX 7.1* flies showed an increased activity during both LP and DP, indicating hyperactivity when the flies were awake (Figure 3.2D).

When further analysing parameters of sleep architecture, like the number of sleep bouts, average bout duration, PDoze, and PWake, again a similar pattern to what was observed in *Hk* mutant flies could be detected. While during LP both the number and the duration of sleep bouts were reduced in *R23E10>AOX 7.1* flies compared to parental controls, during DP, the shortening of sleep bouts was counterbalanced by an increase in the number of sleep bouts (Figure 3.2E–F). Even though the parental controls were not always identical in terms of displayed behaviour, *R23E10>AOX 7.1* flies showed significant and unidirectional changes in sleep parameters compared to both controls. The same was seen analysing PDoze and

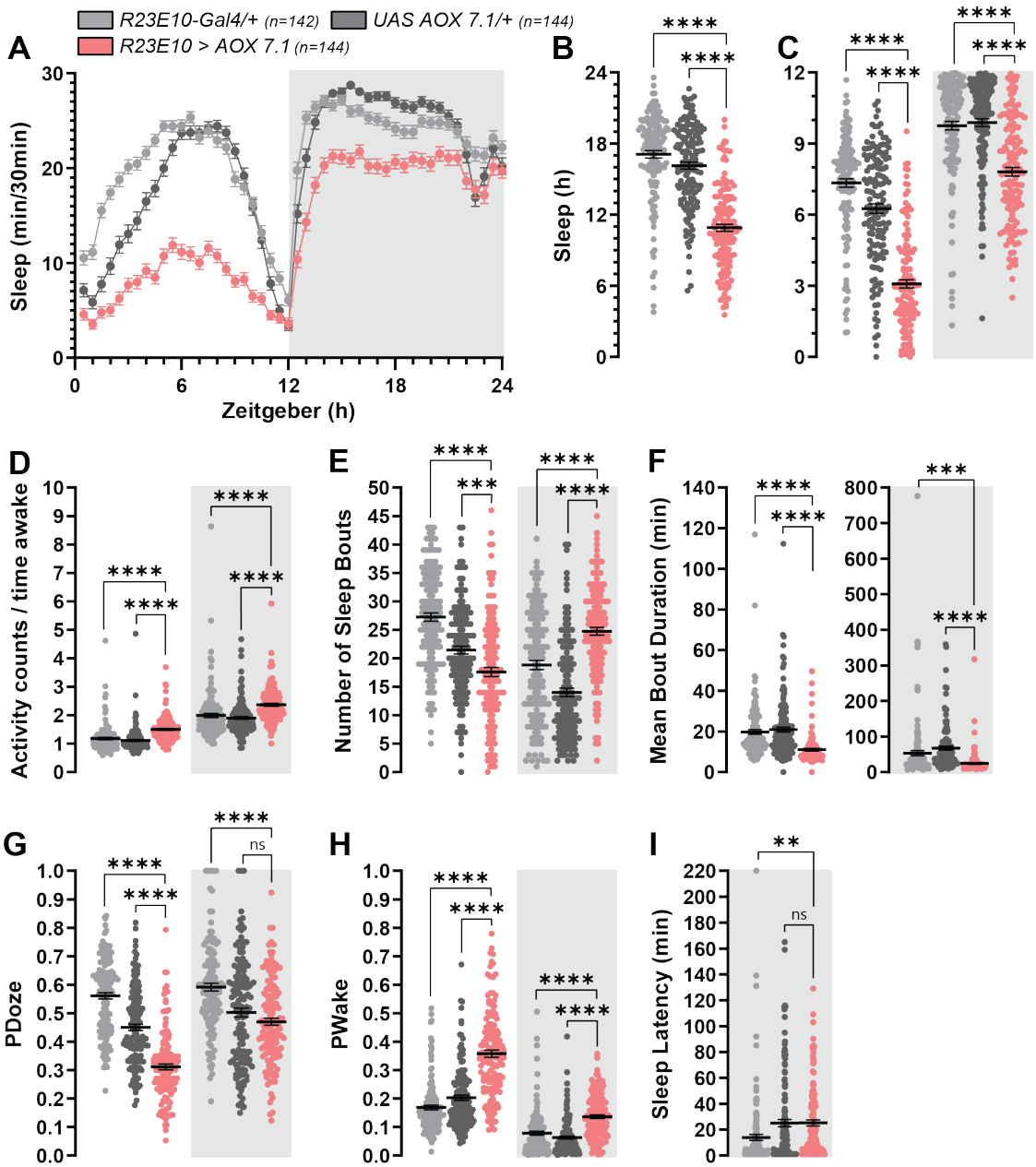

Figure 3.2: ***AOX* expression in dFB neurons reduces sleep duration similar to Hk deficiency.** **(A)** Sleep profile (minutes sleep per 30min) of *R23E10>AOX 7.1* flies (red) and parental controls (grey). Mean ± SEM. **(B)** Flies expressing *AOX* in dFB neurons slept less than their parental controls during 24h ($F_{(2, 427)}$ = 124.5, P<0.0001) and **(C)** during LP (white background) and DP (grey background) respectively. LP: $F_{(2, 427)}$ = 145.2, P<0.0001, DP: $F_{(2, 427)}$ = 43.80, P<0.0001. **(D)** Activity of flies while being awake during LP ($F_{(2, 427)}$ = 30.27, P<0.0001) and DP ($F_{(2, 427)}$ = 18.14, P<0.0001). **(E)** The number of sleep bouts was significantly decreased in *AOX*-expressing flies during LP ($F_{(2, 427)}$ = 43.27, P<0.0001) but increased during DP ($F_{(2, 427)}$ = 52.99, P<0.0001). **(F)** The average duration of sleep bouts was significantly decreased during LP ($F_{(2, 427)}$ = 28.23, P<0.0001) and DP ($F_{(2, 427)}$ = 16.98, P<0.0001) upon *AOX* expression. **(G)** Sleep pressure of *AOX*-expressing flies was significantly reduced during LP ($F_{(2, 427)}$ = 132.1, P<0.0001) but not DP ($F_{(2, 427)}$ = 20.27, P<0.0001). **(H)** Sleep depth decreased during both LP ($F_{(2, 427)}$ = 100.5, P<0.0001) and DP ($F_{(2, 427)}$ = 39.24, P<0.0001) upon *AOX* expression. **(I)** Latency (in min) to fall asleep during DP was unaffected by *AOX* expression ($F_{(2, 427)}$ = 6.81, P = 0.0012). **(B–I)** Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 142–144.

PWake. During LP, PDoze was severely reduced in *R23E10>AOX 7.1* flies compared to parental controls, while remaining unchanged during DP compared to at least one parental control (Figure 3.2G). This reflected a lowered sleep drive during LP, which could explain the reduced number of sleep bouts. PWake on the other hand was significantly increased during both LP and DP (Figure 3.2H) in *R23E10>AOX 7.1* flies. This indicates lighter sleep, causing the flies to wake up more often than their parental controls, and was in agreement with the shortened length of sleep bouts seen during both LP and DP in *R23E10>AOX 7.1* flies. One difference between the sleep phenotypes seen in *R23E10>AOX 7.1* flies and *Hk* mutant flies was the sleep latency. Contrary to *Hk* mutants, *R23E10>AOX 7.1* flies showed no increase in sleep latency during DP as compared to at least one parental control (Figure 3.2I). This was in line with PDoze being unchanged during DP, meaning that flies had little trouble falling asleep quickly when lights turned off during DP. It also further indicates that a reduction in ROS production via *AOX* expression mainly disrupts daytime sleep, while nighttime sleep might be regulated via different or additional mechanism(s).

The results shown here demonstrate that reduction of ROS production via overexpression of *AOX* causes a loss in total sleep time, accompanied by changes in other sleep parameters highly similar to sleep phenotypes observed in *Hk* mutant flies (for comparison, see chapter 2.2).

3.2.2 Pan-neuronal *AOX* expression

Since expression of *AOX* in dFB neurons alone caused a severe reduction in sleep time, I asked whether brain-wide expression might further reduce sleep. Therefore, I used the pan-neuronal driver *nsyb-Gal4* to drive *AOX* expression. Sleep behaviour was measured and assessed as described before.

Pan-neuronal expression of *AOX* (*nsyb>AOX 7.1*) caused a similarly strong sleep loss as seen in flies with dFB-driven *AOX* expression (Figure 3.3A–C). Compared to both parental controls (*nsyb-Gal4/+* and *UAS AOX 7.1/+*), pan-neuronal *AOX* expression caused a sleep loss of about 5 hours on average. Most of the sleep was lost during LP (around 3–4 hours), and only little sleep was lost during DP (1–2 hours) (Figure 3.3C). Contrary to other phenotypes seen before, no hyperactivity was detected in *nsyb>AOX 7.1* flies compared to parental controls, during both LP and DP (Figure 3.3D).

Further analysing parameters of sleep architecture, a similar pattern as seen in *R23E10> AOX 7.1* flies was observed. *nsyb>AOX 7.1* flies showed fewer and shorter sleep bouts during LP compared to both parental controls. During DP, sleep bouts in *nsyb>AOX 7.1* flies were also shorter, but the number of sleep bouts increased slightly (Figure 3.3E–F). Analysing PDoze and PWake, it became clear that the sleep depth changed gravely in *nsyb>AOX 7.1* flies, but the apparent sleep pressure did not (Figure 3.3G–H). PDoze, as a measure of sleep pressure was not significantly different in *nsyb>AOX 7.1* flies as compared to at least one parental control, during DP, and during LP, although significant, the difference was marginally small (Figure 3.3G). This was the first incident in which no change in PDoze could be seen during LP. However, it seemed that one of the parental controls, *UAS AOX 7.1/+*, showed a highly decreased PDoze compared to the other parental control *nsyb/+*. This went alongside a reduced number of sleep bouts in the *UAS* control when compared to the *Gal4* control. This already reduced baseline in PDoze and the number of sleep bouts might be the cause of not seeing a further drop in PDoze in *nsyb>AOX 7.1* flies. PWake, as a measure of sleep depth, was increased in *nsyb>AOX 7.1* flies during both LP and DP, indicating a generally lighter sleep (Figure 3.3H). This goes hand in hand with the shorter length of sleep bouts seen in *nsyb>AOX 7.1* flies during both LP and DP. Lastly, sleep latency during

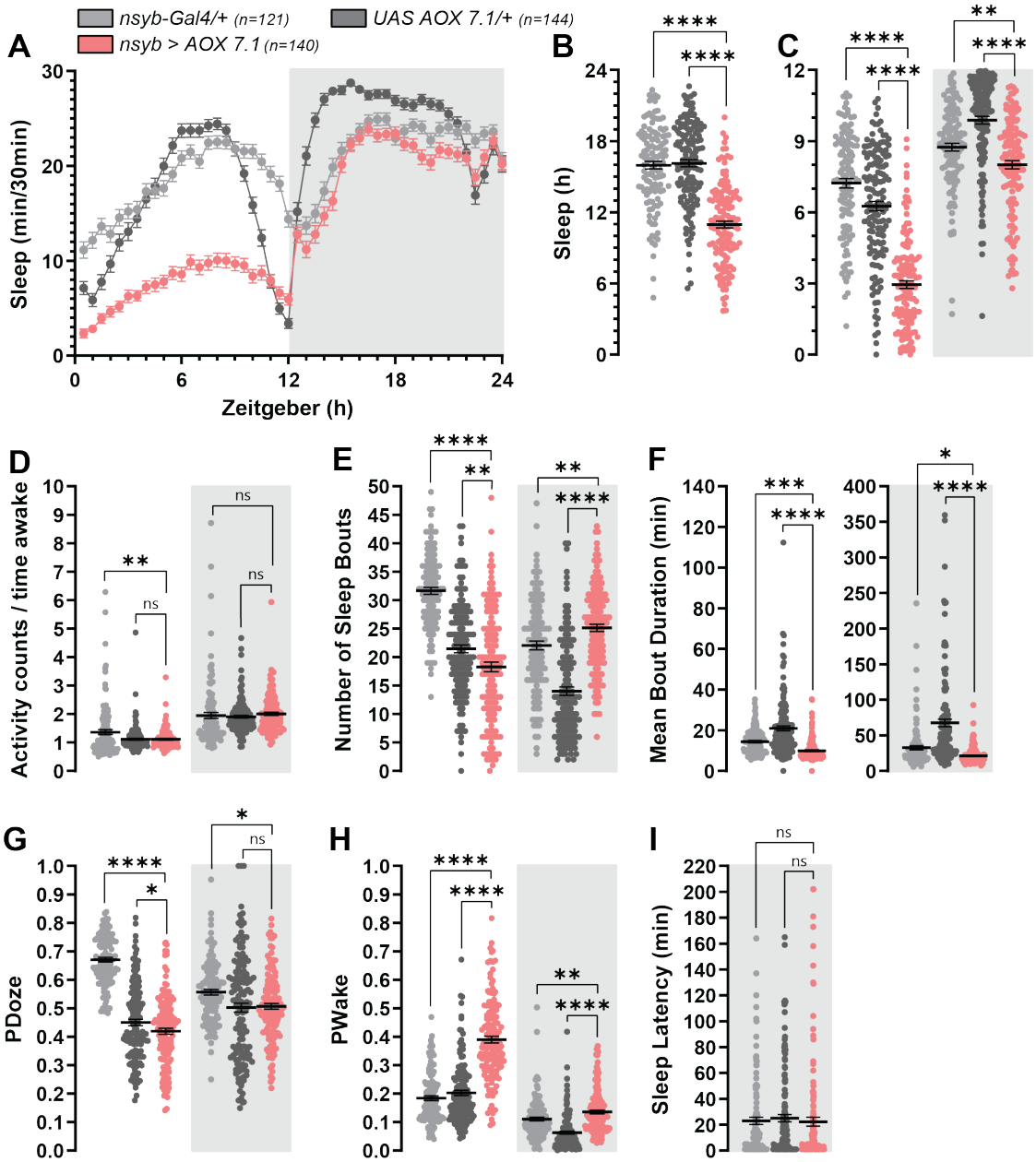

Figure 3.3: Brain-wide *AOX* expression primarily shortens daytime sleep. **(A)** Sleep profile (minutes sleep per 30min) of *nsyb>AOX 7.1* flies (red) and parental controls (grey). Mean ± SEM. **(B)** Flies expressing *AOX* pan-neuronally slept less than their parental controls during 24h ($F_{(2, 402)}$ = 99.21, P<0.0001) and **(C)** during LP (white background) and DP (grey background) respectively. LP: $F_{(2, 402)}$ = 141.5, P<0.0001, DP: $F_{(2, 402)}$ = 34.22, P<0.0001. **(D)** Activity of flies while being awake during LP ($F_{(2, 402)}$ = 7.01, P = 0.0010) and DP ($F_{(2, 402)}$ = 0.54, P = 0.5860). **(E)** The number of sleep bouts was significantly decreased in *AOX*-expressing flies during LP ($F_{(2, 402)}$ = 85.61, P<0.0001) but increased during DP ($F_{(2, 402)}$ = 68.79, P<0.0001). **(F)** The average duration of sleep bouts of *nsyb>AOX 7.1* flies was significantly decreased during LP ($F_{(2, 402)}$ = 44.92, P<0.0001) and DP ($F_{(2, 402)}$ = 46.60, P<0.0001). **(G)** Sleep pressure of *AOX*-expressing flies was significantly reduced during LP ($F_{(2, 402)}$ = 166.8, P<0.0001) but not DP ($F_{(2, 402)}$ = 5.41, P = 0.0048). **(H)** Sleep depth of *nsyb>AOX 7.1* flies decreased during both LP ($F_{(2, 402)}$ = 126.8, P<0.0001) and DP ($F_{(2, 402)}$ = 39.77, P<0.0001). **(I)** Latency (in min) to fall asleep during DP was unaffected by *AOX* expression ($F_{(2, 402)}$ = 0.23, P = 0.7930). **(B–I)** Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 121–144.

DP was analysed, but no changes could be observed upon *AOX* expression (Figure 3.3I).

Taken together it seemed that pan-neuronal expression of *AOX* was not able to reduce sleep even further than with dFB-specific expression. This indicates that targeting dFB neurons had the maximal effect on sleep that could not be pushed further by including more neurons. This, however, does not exclude other neurons to be involved in the ROS-sensing sleep circuit, only that targeting the dFB neurons is likely to have the strongest impact.

What is striking and important to notice is that parental controls showed very different phenotypes for certain parameters, which complicates the interpretation of these results. However, whenever a significant difference between *AOX*-expressing flies and both of their parental controls was found, the phenotype-shift was unidirectional as compared to both parental controls.

3.2.3 Mushroom body-specific *AOX* expression

Since targeting a small subset of neurons was enough to drastically shorten sleep in *AOX*-expressing flies, I asked whether targeting a different subset of neurons implicated in sleep regulation (Joiner et al., 2006; Pitman et al., 2006), would have a similar effect. To test this, *AOX* was expressed in Kenyon cells (KCs) of the MB, using the MB-specific driver *MB247-Gal4*. Sleep duration and other associated parameters were recorded and analysed as previously described.

Expression of *AOX* in the MB (*MB247>AOX 7.1*) overall caused a sleep loss of 3–4 hours compared to parental controls (*MB247-Gal4/+* and *UAS AOX 7.1/+*), as seen before predominantly during LP (Figure 3.4A–C). *MB247>AOX 7.1* flies therefore show a milder sleep phenotype than *R23E10>AOX 7.1* or *nsyb>AOX 7.1* flies.

Activity of *MB247>AOX 7.1* flies was not significantly increased during both LP and DP,

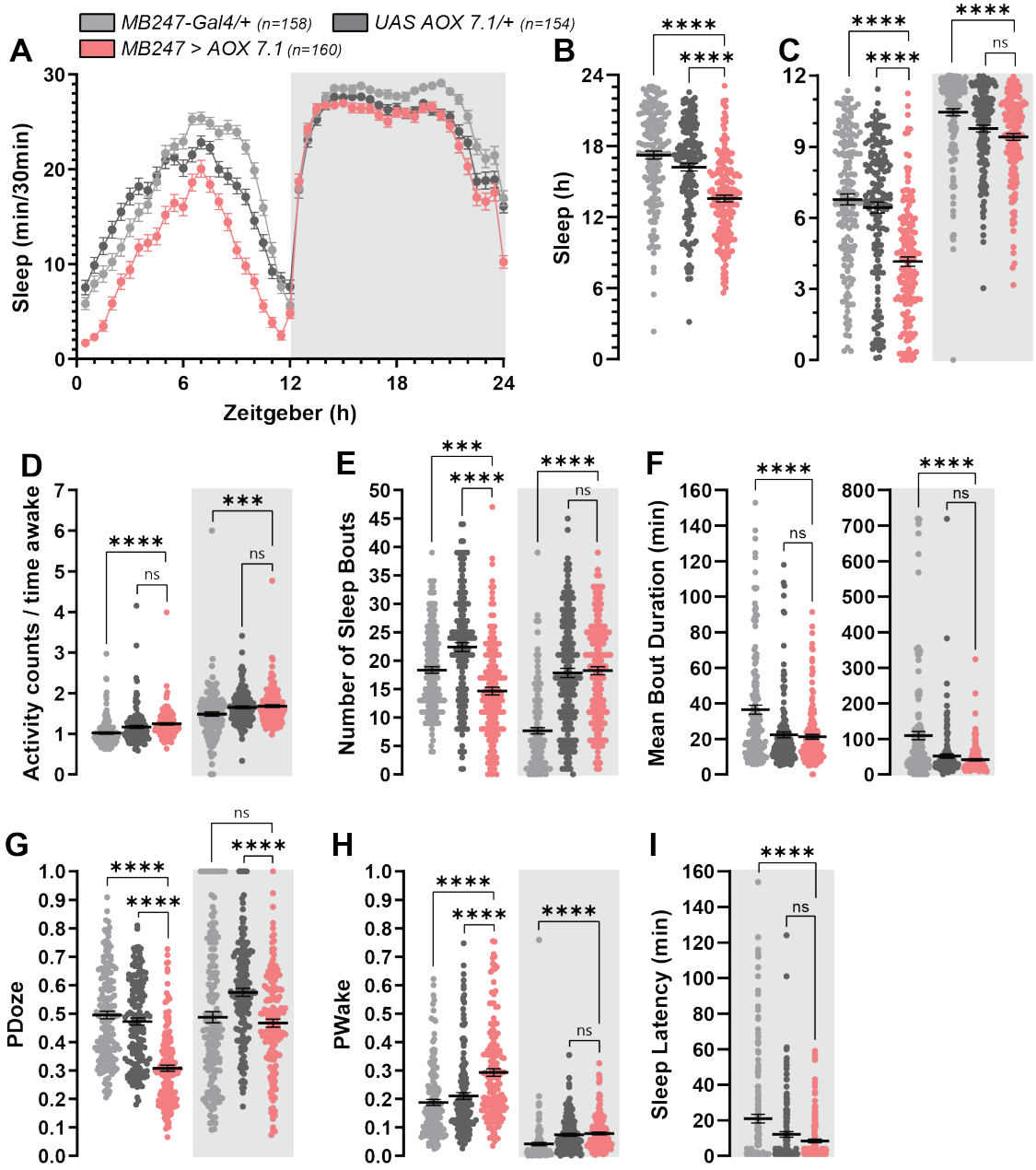

Figure 3.4: *AOX* expression in MB neurons exclusively shortens daytime sleep. **(A)** Sleep profile (minutes sleep per 30min) of *MB247>AOX 7.1* flies (red) and parental controls (grey). Mean ± SEM. **(B)** Flies expressing *AOX* in MB neurons slept less than their parental controls during 24h ($F_{(2, 469)}$ = 36.98, P<0.0001) and **(C)** during LP (white background), but not during DP (grey background). LP: $F_{(2, 469)}$ = 42.92, P<0.0001, DP: $F_{(2, 469)}$ = 13.98, P<0.0001. **(D)** Activity of flies while being awake during LP ($F_{(2, 469)}$ = 14.73, P<0.0001) and DP ($F_{(2, 469)}$ = 7.18, P = 0.0009). **(E)** The number of sleep bouts was significantly decreased in *AOX*-expressing flies during LP ($F_{(2, 469)}$ = 32.47, P<0.0001) but unchanged during DP ($F_{(2, 469)}$ = 73.98, P<0.0001). **(F)** The average duration of sleep bouts of *MB247>AOX 7.1* flies was unchanged during both LP ($F_{(2, 469)}$ = 20.37, P<0.0001) and DP ($F_{(2, 469)}$ = 21.67, P<0.0001). **(G)** Sleep pressure of *AOX*-expressing flies was significantly reduced during LP ($F_{(2, 469)}$ = 74.14, P<0.0001) but not DP ($F_{(2, 469)}$ = 12.69, P<0.0001). **(H)** Sleep depth of *MB247>AOX 7.1* flies decreased during LP ($F_{(2, 469)}$ = 21.82, P<0.0001) but not during DP ($F_{(2, 469)}$ = 15.24, P<0.0001). **(I)** Latency (in min) to fall asleep during DP was unchanged upon *AOX* expression ($F_{(2, 469)}$ = 13.63, P<0.0001). **(B–I)** Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, *** P<0.001, **** P<0.0001. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 154–160.

compared to at least one parental control (Figure 3.4D). Analysing the sleep architecture, I found different phenotypes than observed previously. During LP, the number of sleep bouts was decreased in *MB247>AOX 7.1* flies when compared to controls, while it was unchanged during DP compared to at least one parental control (Figure 3.4E). However, the average sleep bout duration did not change during either LP or DP (Figure 3.4F). During LP, PDoze and PWake were significantly decreased and increased, respectively, while neither parameter was changed during DP in *MB247>AOX 7.1* flies (Figure 3.4G–H). Lastly, sleep latency during DP was unchanged in *MB247>AOX 7.1* flies as compared to parental controls (Figure 3.4I).

Taken together, these results showed that *AOX* expression in the MB mostly affected daytime sleep but not nighttime sleep. It therefore seemed to depict an overall milder phenotype of what was seen in *R23E10>AOX 7.1* and *nsyb>AOX 7.1* flies. Furthermore, it seemed that mainly sleep initiation was affected rather than sleep maintenance, causing the milder sleep phenotype as compared to dFB-specific or brain-wide *AOX* expression.

Again, parental controls showed different behaviours for certain parameters. But as described before, all significant differences seen between the experimental group and the parental controls were unidirectional and therefore unlikely just an effect of genetic background.

3.2.4 *AOX* expression in projection neurons of the antennal lobe

Next, I asked whether *AOX* expression in other brain regions that are not associated with initiation or regulation of sleep, would cause the same effect in sleep reduction as seen before. Therefore, *AOX* was expressed in projection neurons (PNs) of the antennal lobe (AL), known for its importance in olfaction, using the AL-specific driver *GH146-Gal4*. Sleep was

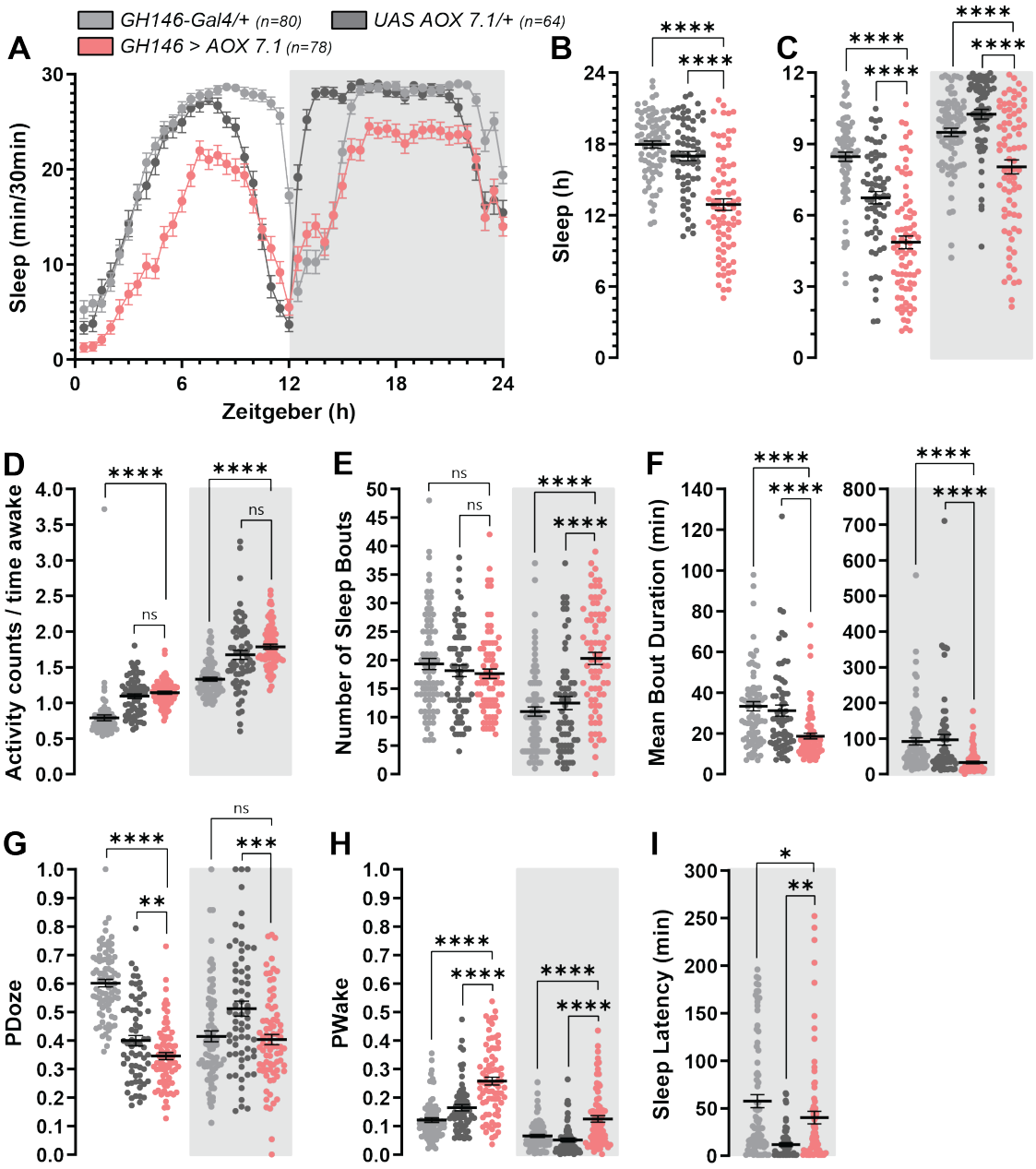

Figure 3.5: ***AOX* expression in PNs of the antennal lobe shortens sleep.** **(A)** Sleep profile (minutes sleep per 30min) of *GH146>AOX 7.1* flies (red) and parental controls (grey). Mean ± SEM. **(B)** Flies expressing *AOX* in PNs slept less than their parental controls during 24h ($F_{(2, 219)}$ = 47.28, P<0.0001) and **(C)** during LP (white background) and DP (grey background), respectively. LP: $F_{(2, 219)}$ = 59.19, P<0.0001, DP: $F_{(2, 219)}$ = 23.22, P<0.0001. **(D)** Activity of flies while being awake during LP ($F_{(2, 219)}$ = 36.32, P<0.0001) and DP ($F_{(2, 219)}$ = 34.81, P<0.0001). **(E)** The number of sleep bouts was unchanged in *AOX*-expressing flies during LP ($F_{(2, 219)}$ = 0.93, P = 0.3973) but increased during DP ($F_{(2, 219)}$ = 26.19, P<0.0001). **(F)** The average duration of sleep bouts of *GH146>AOX 7.1* flies was decreased during both LP ($F_{(2, 219)}$ = 14.33, P<0.0001) and DP ($F_{(2, 219)}$ = 12.86, P<0.0001). **(G)** Apparent sleep pressure of *AOX*-expressing flies was significantly reduced during LP ($F_{(2, 219)}$ = 93.65, P<0.0001) but not during DP ($F_{(2, 219)}$ = 7.60, P = 0.0006). **(H)** Sleep depth of *GH146>AOX 7.1* flies decreased during both LP ($F_{(2, 219)}$ = 41.58, P<0.0001) and DP ($F_{(2, 219)}$ = 22.30, P<0.0001). **(I)** Latency (in min) to fall asleep during DP was not affected by *AOX* expression ($F_{(2, 219)}$ = 14.31, P<0.0001). **(B–I)** Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 64–80.

measured and analysed as previously described.

Surprisingly, *AOX*-expressing flies (*GH146>AOX 7.1*) showed a strong shortening of sleep time when compared to either parental control (*GH146-Gal4/+* and *UAS AOX 7.1/+*), during both LP and DP (Figure 3.5A–C). The total sleep loss of *GH146>AOX 7.1* flies during a whole day averaged around 4–5 hours, with about 2–3.5 hours lost during LP, and around 1.5–2 hours lost during DP. *GH146>AOX 7.1* flies showed normal activity during LP, and a tendency for hyperactivity during DP (Figure 3.5D). However, the increase in activity was not significant compared to at least one parental control.

In contrast to flies with MB-specific *AOX* expression, shortening of sleep in *GH146>AOX 7.1* flies mainly resulted from a shortening of sleep bouts, while the number of sleep bouts either stayed the same (during LP) or even increased (during DP) (Figure 3.5E–F). These phenotypes resembled what was observed for flies with dFB-specific or pan-neuronal *AOX* expression. Also phenotypes for PDoze and PWake followed this pattern. *GH146>AOX 7.1* flies showed a slight decrease in PDoze during LP, but no change during DP as compared to one parental control (Figure 3.5G). PWake on the other hand was increased during both LP and DP (Figure 3.5H), explaining the occurrence of shorter sleep bouts as flies are more likely to wake up from lighter sleep. The sleep latency during DP was not affected in *GH146>AOX 7.1* flies (Figure 3.5I).

These results pose the question whether *AOX* expression, no matter where in the brain, might be able to reduce sleep in a significant manner. As sleep patterns between *AOX*-expressing flies were not always the same depending on where *AOX* was expressed, different subsets of neurons might have distinct and differently strong effects on sleep. Further experiments are needed to carefully dissect the network of sleep-inducing neurons and the site of ROS-sensing.

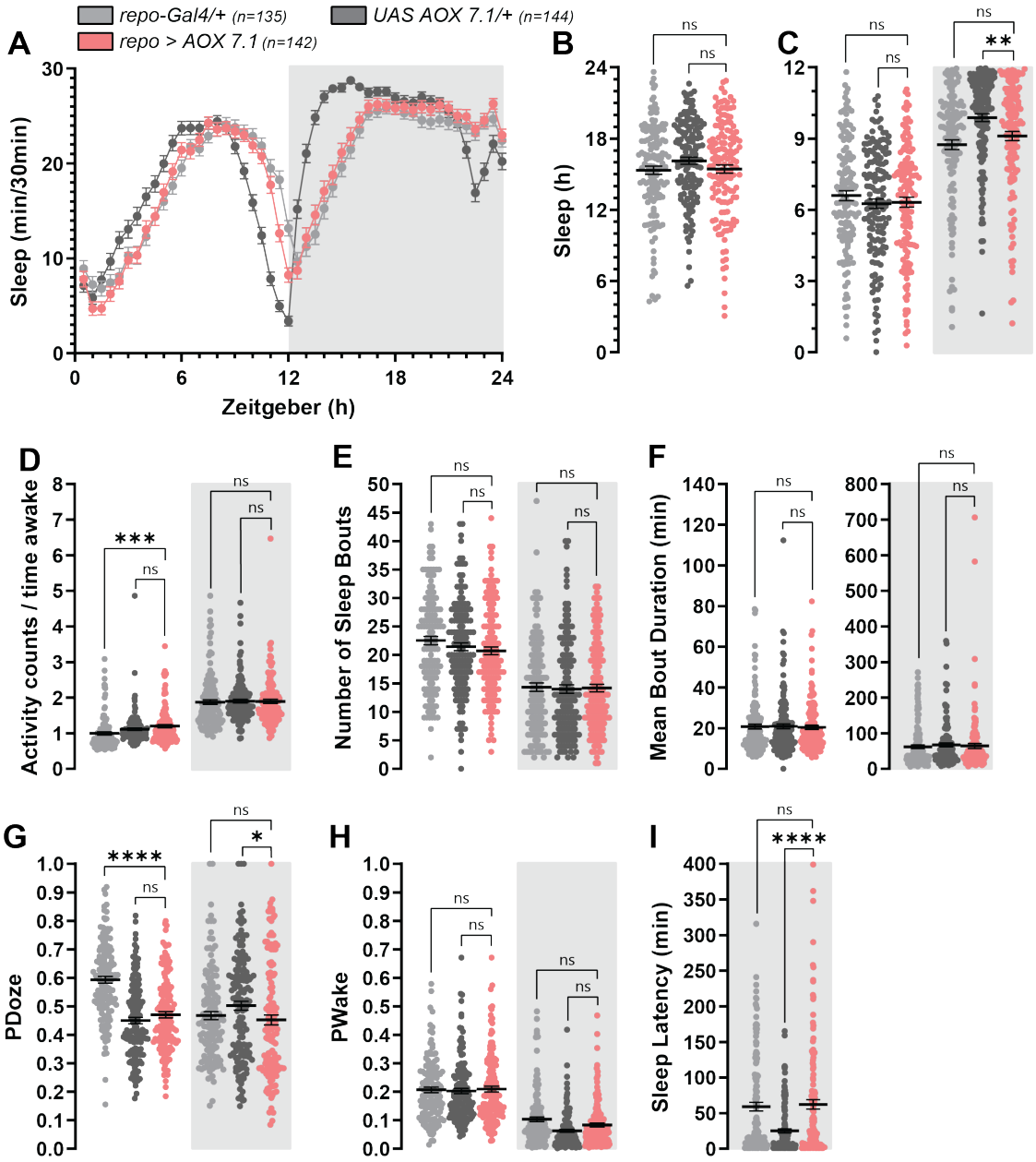

Figure 3.6: ***AOX* expression in glial cells does not affect sleep.** **(A)** Sleep profile (minutes sleep per 30min) of *repo>AOX 7.1* flies (red) and parental controls (grey). Mean ± SEM. **(B)** *AOX* expression in glial cells had no effect on sleep in 24h ($F_{(2, 418)}$ = 1.77, P = 0.1713) and **(C)** during LP (white background) and DP (grey background) respectively. LP: $F_{(2, 418)}$ = 0.77, P = 0.4657, DP: $F_{(2, 418)}$ = 9.55, P<0.0001. **(D)** Activity of flies while being awake during LP ($F_{(2, 418)}$ = 6.41, P = 0.0018) and DP ($F_{(2, 418)}$ = 0.06, P = 0.9388). **(E)** The number of sleep bouts was unchanged in *AOX*-expressing flies during LP ($F_{(2, 418)}$ = 1.61, P = 0.2003) and DP ($F_{(2, 418)}$ = 0.06, P = 0.9431), **(F)** similar to the average duration of sleep bouts. LP: $F_{(2, 418)}$ = 0.04, P = 0.9575, DP: $F_{(2, 418)}$ = 0.19, P = 0.8273. **(G)** Sleep pressure of *AOX*-expressing flies was unaffected during LP ($F_{(2, 418)}$ = 45.57, P<0.0001) and DP ($F_{(2, 418)}$ = 2.76, P = 0.0644). **(H)** Sleep depth was unchanged upon *AOX* expression during both LP ($F_{(2, 418)}$ = 0.14, P = 0.8715) and DP ($F_{(2, 418)}$ = 8.847, P = 0.0002). **(I)** Latency (in min) to fall asleep during DP was unaffected by *AOX* expression ($F_{(2, 418)}$ = 14.85, P<0.0001). **(B–I)** Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 135–144.

3.2.5 *AOX* expression in glial cells

Recent evidence implicates glial cells in sleep regulation (Blum et al., 2021; Gerstner et al., 2017; Goodwin et al., 2018; Haydon, 2017). To test if glial cells play a role in the ROS-dependent aspect of sleep control, *AOX* was expressed in glial cells using the glia-specific driver *repo-Gal4*, and sleep was recorded.

Interestingly, no difference in sleep between *AOX*-expressing flies (*repo>AOX 7.1*) and their parental controls (*repo-Gal4/+* and *UAS AOX 7.1/+*) could be detected, neither during LP nor during DP (Figure 3.6A–C). Consequentially, no differences in other sleep parameters, such as the number of sleep bouts, their average length, PDoze, PWake, and sleep latency were observed (Figure 3.6E–I). *repo>AOX 7.1* flies also did not show any activity impairments, neither hyper- nor hypoactivity (Figure 3.6D).

Taking these results together, *AOX* expression in proximity to neurons was not enough to change sleep regulation and sleep patterns in flies. *AOX* had to be expressed in neurons directly, thereby lowering ROS production in the targeted neurons, in order to cause a reduction in sleep.

3.2.6 *AOX* expression in the gut

Another organ that has been implicated in sleep in connection with ROS is the gut. It has been reported that chronic sleep deprivation causes ROS to accumulate in the gut of flies and mice, and that animals are more vulnerable to additional ROS exposure after sleep deprivation (Vaccaro et al., 2020). However, it remained unknown whether this relationship is bidirectional. In other words, can a reduced ROS accumulation in the gut influence sleep behaviour? To test this, *AOX* was expressed in enterocytes using the gut-specific driver

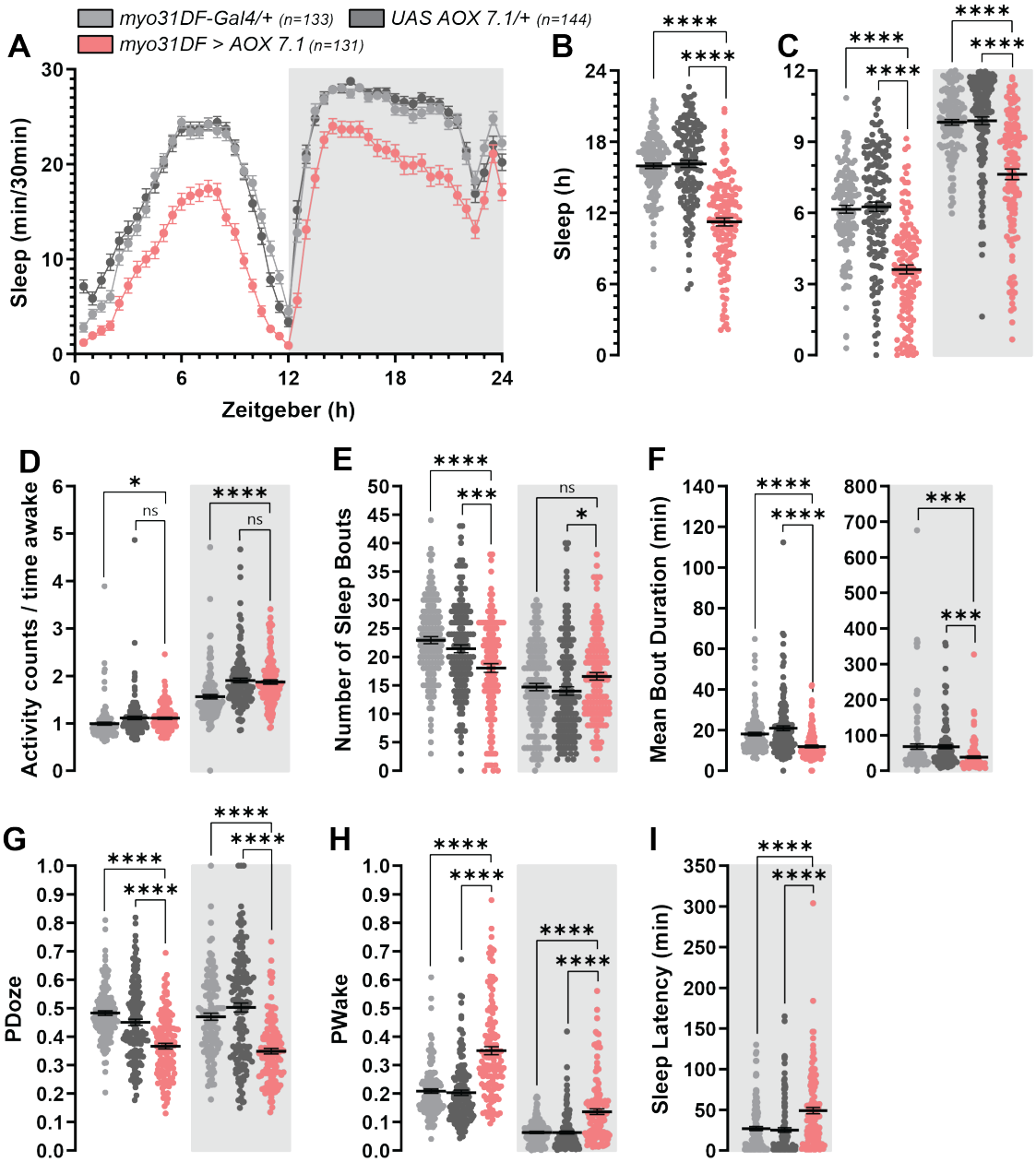

Figure 3.7: *AOX* expression in enterocytes shortens day- and nighttime sleep. **(A)** Sleep profile (minutes sleep per 30min) of *myo31DF>AOX 7.1* flies (red) and parental controls (grey). Mean ± SEM. **(B)** Flies expressing *AOX* in the gut slept less than their parental controls during 24h ($F_{(2, 405)}$ = 90.83, P<0.0001) and **(C)** during LP (white background) and DP (grey background) respectively. LP: $F_{(2, 405)}$ = 65.53, P<0.0001, DP: $F_{(2, 405)}$ = 55.15, P<0.0001. **(D)** Activity of flies while being awake during LP ($F_{(2, 405)}$ = 4.948, P = 0.0075) and DP ($F_{(2, 405)}$ = 16.51, P<0.0001). **(E)** The number of sleep bouts was significantly decreased in *AOX*-expressing flies during LP ($F_{(2, 405)}$ = 12.71, P<0.0001) but unchanged during DP ($F_{(2, 405)}$ = 3.79, P = 0.0233). **(F)** The average duration of sleep bouts in *myo31DF>AOX 7.1* flies was significantly decreased during LP ($F_{(2, 405)}$ = 23.49, P<0.0001) and DP ($F_{(2, 405)}$ = 8.92, P = 0.0002). **(G)** Sleep pressure of *AOX*-expressing flies was significantly reduced during LP ($F_{(2, 405)}$ = 35.99, P<0.0001) and DP ($F_{(2, 405)}$ = 38.63, P = 0.0048). **(H)** Sleep depth of *myo31DF>AOX 7.1* flies decreased during both LP ($F_{(2, 405)}$ = 60.43, P<0.0001) and DP ($F_{(2, 405)}$ = 39.76, P<0.0001). **(I)** Latency (in min) to fall asleep during DP was increased upon *AOX* expression ($F_{(2, 405)}$ = 18.19, P<0.0001). **(B–I)** Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, * P<0.05, *** P<0.001, **** P<0.0001. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 131–144.

myo31DF-Gal4 and sleep was recorded.

AOX expression in the gut (*myo31DF>AOX 7.1*) caused an average reduction of sleep duration by about 4 hours as compared to both parental controls (*myo31DF-Gal4/+* and *UAS AOX 7.1/+*) (Figure 3.7A–B). Interestingly, other than what has been observed before, the sleep loss was evenly distributed between LP and DP (Figure 3.7C). No significant changes in activity could be observed in *myo31DF>AOX 7.1* flies as compared to at least one parental control (Figure 3.7D).

Further investigating sleep parameters, it was found that *myo31DF>AOX 7.1* flies mostly showed similar tendencies than flies with dFB-specific *AOX* expression regarding number and length of sleep bouts, and PDoze and PWake (Figure 3.7E–H). However, one difference was the decrease of PDoze during DP of *myo31DF>AOX 7.1* flies compared to controls (Figure 3.7G), which was not seen in *R23E10>AOX 7.1* or *nsyb>AOX 7.1* flies. Additionally, the number of sleep bouts did not increase in *myo31DF>AOX 7.1* flies compared to controls (Figure 3.7E), as was seen in dFB-specific or pan-neuronal *AOX* expression. Another difference seen was the increased sleep latency during DP in *myo31DF>AOX 7.1* flies as compared to their parental controls (Figure 3.7I).

These results suggest that changed ROS levels in the gut can indeed influence sleep behaviour of flies. A first step has been made in understanding how a change in ROS production in the gut is communicated to the brain and eventually translated into sleep or wakefulness (Titos et al., 2023), yet much remains to be uncovered.

3.3 Learning and memory

As described in an earlier chapter (see Chapter 2.3), *Hk* mutant flies displayed severe learning deficits that could be partially corrected by rescuing *Hk* expression in dFB neurons. Since *AOX* expression, especially in dFB neurons, caused sleep loss resembling sleep loss in Hk-deficient flies, I asked whether *AOX*-expressing flies would show learning deficits as well. To test this, *AOX* was expressed in dFB neurons (*R23E10-Gal4*), MB neurons (*MB247-Gal4*), the PNs (*GH146-Gal4*), pan-neuronally (*nsyb-Gal4*), in glial cells (*repo-Gal4*), and in the gut (*myo31DF-Gal4*), and learning ability was tested in a negative reinforcement paradigm. The learning index, as described before, was used as output to approximate learning ability.

As seen in Figure 3.8, learning was not impaired, irrespective of where *AOX* was ex-

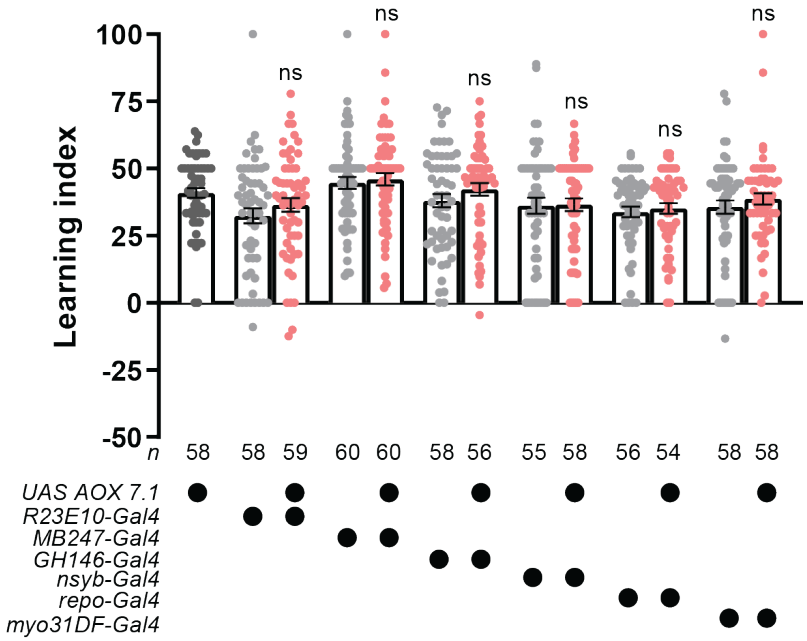

Figure 3.8: *AOX* expression in various subsets of neurons, in glial cells, and in the gut does not affect learning ability. Learning index of flies expressing *AOX* in different subsets of neurons (*R23E10-Gal4, MB247-Gal4, GH146-Gal4*), the whole brain (*nsyb-Gal4*), glial cells (*repo-Gal4*), or the gut (*myo31DF-Gal4*) (pink), and parental controls (grey). *AOX* expression showed no effect on learning ability of flies, R23E10: $F_{(2, 172)} = 3.14$, P = 0.0458, MB247: $F_{(2, 175)} = 1.50$, P = 0.2255, GH146: $F_{(2, 169)} = 0.96$, P = 0.3833, nsyb: $F_{(2, 168)} = 1.26$, P = 0.2871, repo: $F_{(2, 165)} = 4.11$, P = 0.0182, myo31DF: $F_{(2, 171)} = 1.55$, P = 0.2156. Three independent experiments were conducted, one representative experiment is shown here. Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05. Individual dots represent individual flies, bars represent mean ± SEM, n = 54–60.

pressed; no differences in learning index could be detected compared to parental controls (*UAS AOX 7.1/+* and respective *Gal4* control). *AOX* expression therefore dissociates any sleep loss from memory deficits.

3.4 Lifespan

Hk-deficient flies, along with other short-sleeping mutants, displayed severely reduced lifespans (see chapter 2.4; Bushey et al., 2010). In the following, I analysed whether short-sleeping, *AOX*-expressing flies show the same shortening of lifespan.

R23E10>AOX 7.1 flies, despite having shown severe sleep loss, not only did not have a reduced lifespan, they even showed a slight but significant increase in longevity (Figure 3.9A). Mainly due to the prevention of early mortality, the median survival time of flies with *AOX* expression (*R23E10>AOX 7.1*) was prolonged by three days (median: 79 days) as compared to both parental controls (*R23E10-Gal4/+* and *UAS AOX 7.1/+*; median: 76 days). The population's maximum lifespan was not increased however.

Flies with MB-specific (*MB247>AOX 7.1*), pan-neuronal (*nsyb>AOX 7.1*), glia-specific (*repo>AOX 7.1*), or gut-specific (*myo31DF>AOX 7.1*) *AOX* expression likewise showed no decrease in lifespan as compared to either parental control (*Gal4 driver/+* and *UAS AOX 7.1/+*) (Figure 3.9B–E). *nsyb>AOX 7.1* partially overcame the distinctive early mortality associated with the *nsyb-Gal4* driver; however, their overall lifespan was not significantly different from at least one parental control.

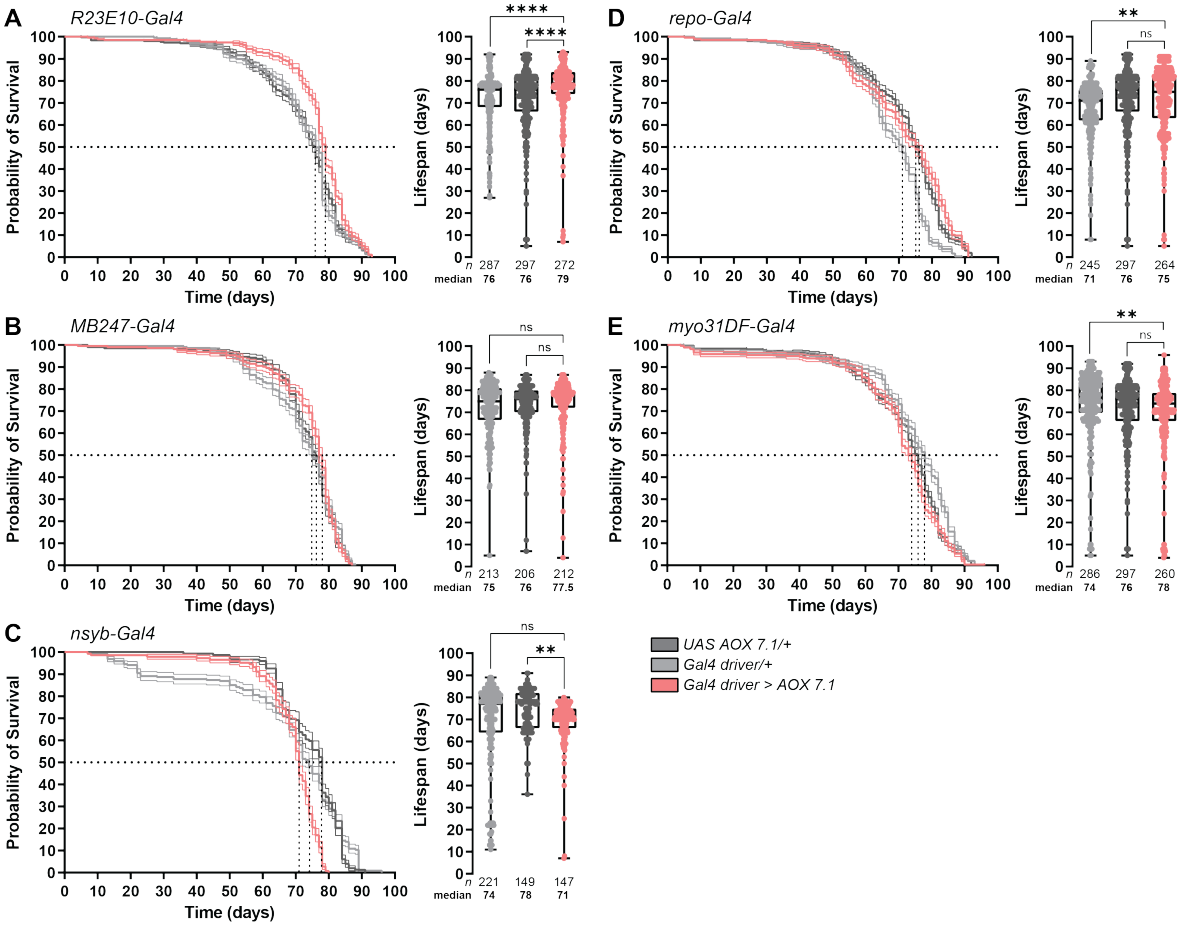

Figure 3.9: ***AOX* expression in sleep-promoting dFB neurons prolongs lifespan.** **(A)** Survival curve and lifespan of flies expressing *AOX* in dFB neurons. *AOX* expression prolonged lifespan of flies significantly ($F_{(2, 853)}$ = 12.15, P<0.0001). **(B)** Survival curve and lifespan of flies expressing *AOX* in MB neurons. *AOX* expression neither prolonged nor shortened lifespan of flies ($F_{(2, 628)}$ = 1.00, P = 0.3674). **(C)** Survival curve and lifespan of flies expressing *AOX* pan-neuronally. *AOX* expression neither prolonged nor shortened lifespan of flies ($F_{(2, 515)}$ = 7.54, P = 0.0006). **(D)** Survival curve and lifespan of flies expressing *AOX* in glial cells. *AOX* expression neither prolonged nor shortened lifespan of flies ($F_{(2, 803)}$ = 7.13, P = 0.0009). **(E)** Survival curve and lifespan of flies expressing *AOX* in the gut. *AOX* expression neither prolonged nor shortened lifespan of flies ($F_{(2, 840)}$ = 4.00, P = 0.0186). **(A–E)** Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, ** P<0.01, **** P<0.0001. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, n = 206–297.

3.5 *AOX* expression in *Hk*^{1} mutants

As Hk's role in dFB neurons has been proposed to involve sensing of ROS generated during waking (Kempf et al., 2019), I asked whether the detrimental effects of Hk deficiency-induced sleep deprivation, such as learning deficits and reduced longevity, could be counteracted by reducing ROS levels in either dFB neurons or the whole brain. To test this, I expressed *AOX* in a *Hk* mutant background. Since *AOX* expression produced sleep loss

without affecting learning or lifespan, it was conceivable that reducing the ROS burden with the help of AOX might alleviate the learning deficits and shortened lifespan of flies lacking Hk, thus proving that both symptoms are primarily caused by the flies' inability to sense increased ROS levels and counteract them by sleeping.

3.5.1 Sleep of *Hk^1* mutants with dFB-restricted *AOX* expression

AOX was expressed in *Hk* mutant flies using the dFB-specific *Gal4* driver *R23E10-Gal4* and sleep behaviour was assessed. *Hk* mutant flies with dFB-specific *AOX* expression (*Hk^1; R23E10 >AOX 7.1*) showed shortened sleep compared to WT controls (*Hk^1/+, UAS AOX 7.1/+* and *Hk^1/+; R23E10-Gal4/+*), but overall slept for a comparable time as *Hk* mutant controls (*Hk^1; UAS AOX 7.1/+* and *Hk^1; R23E10-Gal4/+*) (Figure 3.10A–B). As both WT parental controls and both *Hk* mutant parental controls showed very similar sleep architecture, these controls were pooled for the analysis of additional sleep parameters. Behaviour for all groups individually as shown in Figure 3.10B, as well as the appropriate comparisons can be found in Supplementary Figure 6.2.

Interestingly, the distribution of sleep in LP and DP differed between *Hk* mutant flies with and without *AOX* expression (Figure 3.10A and C). Expression of *AOX* slightly increased sleep during LP but further decreased sleep during DP. Waking activity showed no differences between the three groups during LP, but was increased in *Hk* mutant flies during DP, reflecting phenotypes seen before in *Hk* mutants as well as *R23E10>AOX 7.1* flies.

During LP, *AOX* expression did not change the mean bout duration of *Hk* mutants (Figure 3.10F), while the number of sleep bouts slightly increased, but was still significantly reduced compared to WT control flies (Figure 3.10E). During DP, *AOX* expression further increased the already higher number of sleep bouts in *Hk* mutants, while the average duration of sleep

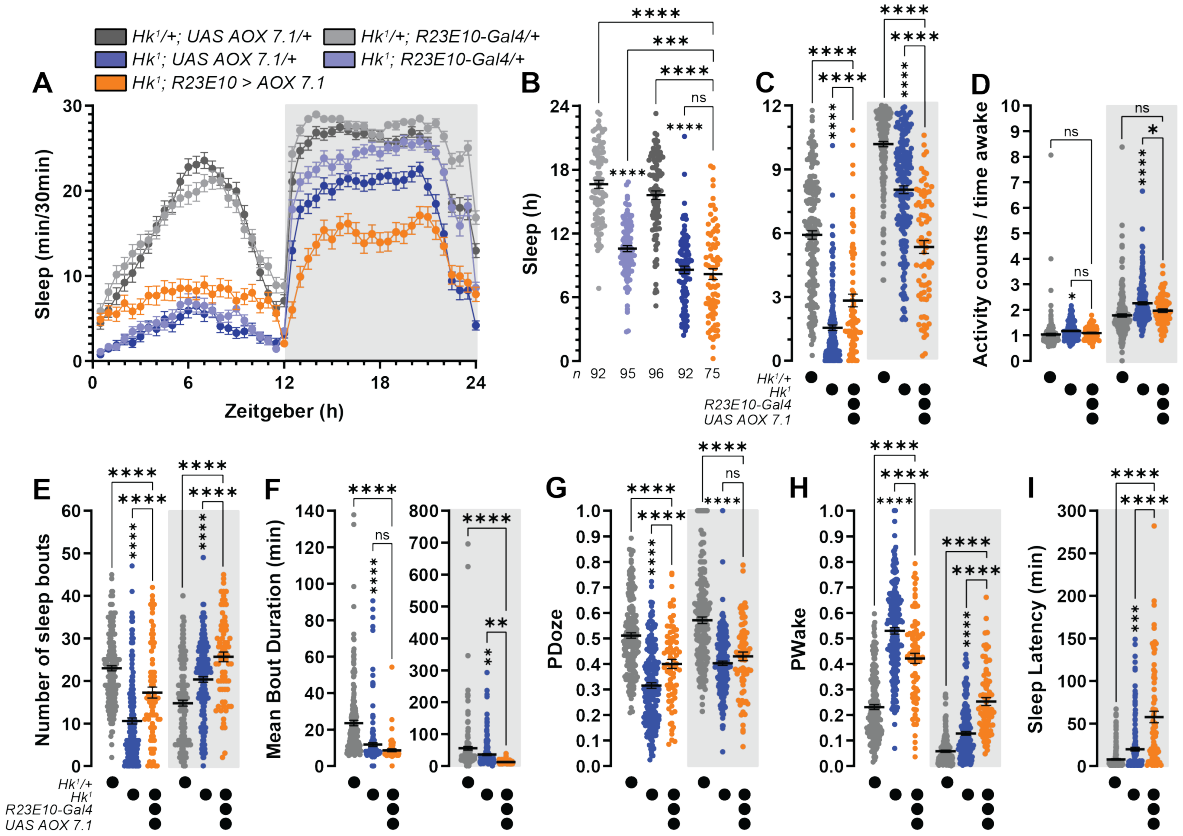

Figure 3.10: dFB-specific *AOX* expression in a *Hk* mutant background affects daytime sleep differently than nighttime sleep. **(A)** Sleep profile (minutes sleep per 30min) of *Hk^1; R23E10>AOX 7.1* flies (orange), *Hk* mutant controls (blue) and heterozygous WT controls (grey). Mean ± SEM. **(B)** *Hk* mutant flies with *AOX* expression in dFB neurons overall slept less than WT controls, but lost no additional sleep compared to *Hk* mutant flies ($F_{(4, 445)}$ = 106.9, P<0.0001). Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, **** P<0.0001. Comparisons between genotypes are indicated by connecting lines, if no line is indicated, comparison was made with the direct WT control. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 75–96. **(C)** *AOX* expression slightly increased sleep during LP (white background) and decreased sleep during DP (grey background) compared to *Hk* mutant controls. LP: $F_{(2, 447)}$ = 169.3, P<0.0001, DP: $F_{(2, 447)}$ = 132.9, P<0.0001. **(D)** Activity of flies while awake during LP ($F_{(2, 447)}$ = 3.95, P = 0.0199) and DP ($F_{(2, 447)}$ = 16.24, P<0.0001). **(E)** The number of sleep bouts was significantly decreased in *Hk* mutant flies during LP ($F_{(2, 447)}$ = 78.22, P<0.0001) but increased during DP ($F_{(2, 447)}$ = 39.15, P<0.0001). **(F)** The average duration of sleep bouts was significantly decreased in *Hk* mutants during LP ($F_{(2, 447)}$ = 34.25, P<0.0001) and DP ($F_{(2, 447)}$ = 14.86, P<0.0001). **(G)** PDoze of *Hk* mutant flies was significantly reduced during LP ($F_{(2, 447)}$ = 79.48, P<0.0001) and DP ($F_{(2, 446)}$ = 64.04, P<0.0001). **(H)** Sleep depth decreased during both LP ($F_{(2, 447)}$ = 169.8, P<0.0001) and DP ($F_{(2, 447)}$ = 127.0, P<0.0001) in *Hk* mutant flies. **(I)** Latency (in min) to fall asleep during DP was increased upon *AOX* expression in a *Hk* mutant background ($F_{(2, 447)}$ = 66.05, P<0.0001). **(C–I)** Both WT controls and both *Hk* mutant controls were pooled respectively. Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, * P<0.05, ** P<0.01, **** P<0.0001. Comparisons between genotypes are indicated by connecting lines, if no line is indicated, comparison was made with the direct WT control. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 75–188.

bouts was further shortened (Figure 3.10F). Following the trend seen in the number of sleep bouts, during LP, PDoze was increased upon *AOX* expression in *Hk* mutants, while PWake was reduced, but both parameters did not reach WT levels (Figure 3.10G–H). Similarly following the trend seen in the average sleep bout duration, during DP, PWake was significantly increased upon *AOX* expression in *Hk* mutants (Figure 3.10). Additionally, sleep latency was lengthened in *AOX*-expressing *Hk* mutants (Figure 3.10I).

Taking all these parameters together, expressing *AOX* in a *Hk* mutant background seemed to slightly increase the flies' ability to fall asleep during LP. During DP, *Hk* mutant flies' ability to fall asleep similarly increased upon *AOX* expression, while the sleep depth remained impaired, causing flies to wake up frequently. Sleep, especially nighttime sleep, was thus further fragmented in *Hk^1; R23E10>AOX 7.1* flies as compared to *Hk* mutant controls.

3.5.2 Sleep of *Hk^1* mutants with pan-neuronal *AOX*

AOX was expressed in *Hk* mutant flies using the pan-neuronal *Gal4* driver *nsyb-Gal4* and sleep behaviour was assessed. Similar to what was observed with dFB-specific *AOX* expression in a *Hk* mutant background, expression of *AOX* in the whole brain (*Hk^1; nsyb>AOX 7.1*) did not change overall sleep amount as compared to *Hk* mutant controls (*Hk^1; nsyb-Gal4/+* and *Hk^1; UAS AOX 7.1/*) (Figure 3.11A–B). Again, for further analysis of sleep parameters, both heterozygous WT controls (*Hk^1/+; nsyb-Gal4/+* and *Hk^1/+; UAS AOX 7.1/+*) and both *Hk* mutant controls were pooled. Behaviour for all groups individually as shown in Figure 3.11B, as well as the appropriate comparisons can be found in Supplementary Figure 6.3.

Analysing LP and DP individually, *AOX* expression did change the amount of time spent asleep in *Hk* mutants. During LP, *AOX* expression caused a very slight but significant increase in sleep, while during DP sleep duration was even further reduced (Figure 3.11A

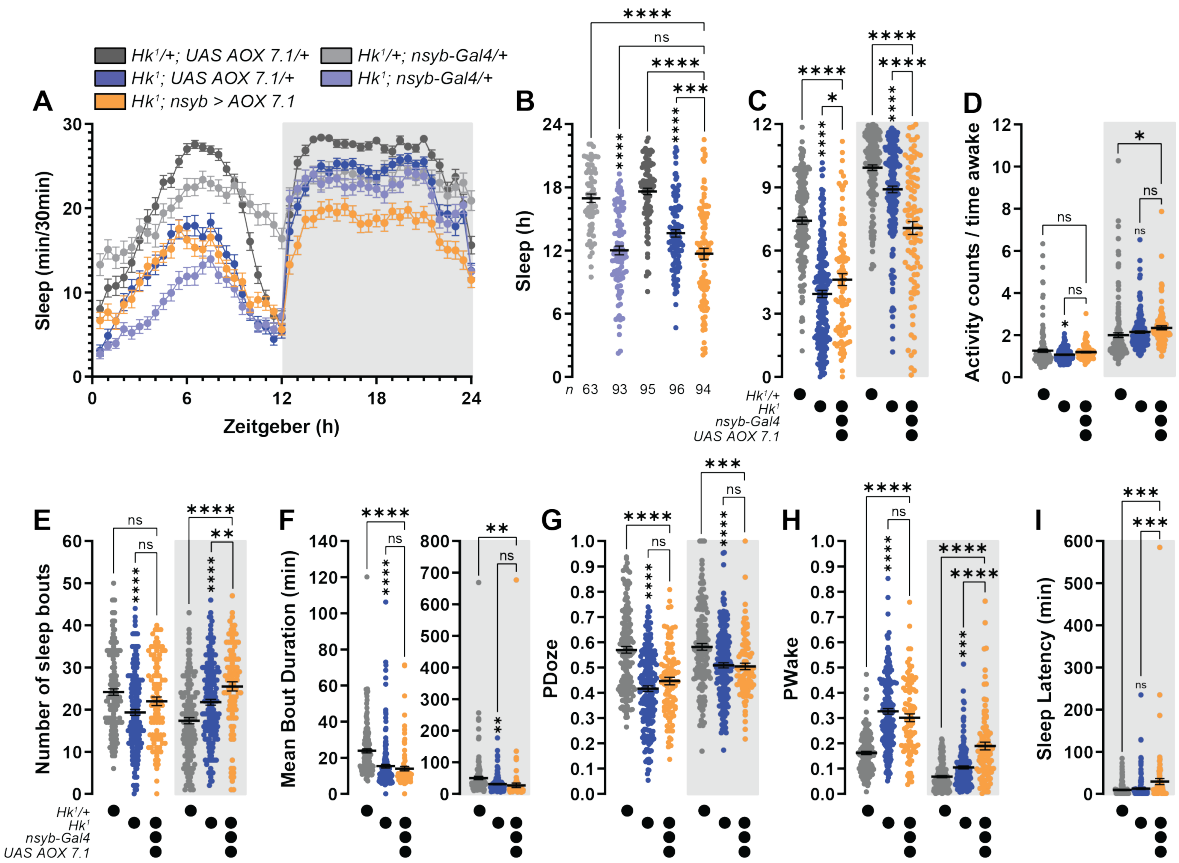

Figure 3.11: Brain-wide *AOX* expression in a *Hk* mutant background does neither rescue nor worsen short sleep phenotype. **(A)** Sleep profile (minutes sleep per 30min) of *Hk[1]; nsyb>AOX 7.1* flies (orange), *Hk* mutant controls (blue) and heterozygous WT controls (grey). Mean ± SEM. **(B)** *Hk* mutant flies with neuronal *AOX* expression overall slept less than WT controls, but lost no additional sleep compared to *Hk* mutant flies ($F_{(4, 436)}$ = 45.09, P<0.0001). Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, *** P<0.001, **** P<0.0001. Comparisons between genotypes are indicated by connecting lines, if no line is indicated, comparison was made with the direct WT control. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 63–96. **(C)** *AOX* expression slightly increased sleep during LP (white background) and decreased sleep during DP (grey background) compared to *Hk* mutant controls. LP: $F_{(2, 438)}$ = 99.78, P<0.0001, DP: $F_{(2, 438)}$ = 49.45, P<0.0001. **(D)** Activity of flies while awake during LP ($F_{(2, 438)}$ = 4.27, P = 0.0146) and DP ($F_{(2, 438)}$ = 3.12, P = 0.0453). **(E)** The number of sleep bouts was unchanged in *AOX*-expressing *Hk* mutant flies compared to both controls during LP ($F_{(2, 438)}$ = 10.81, P<0.0001) but increased during DP ($F_{(2, 438)}$ = 22.51, P<0.0001). **(F)** The average duration of sleep bouts was significantly decreased in *Hk* mutants during LP ($F_{(2, 438)}$ = 22.95, P<0.0001) and DP ($F_{(2, 438)}$ = 7.63, P = 0.0006). **(G)** PDoze of *Hk* mutant flies was significantly reduced during LP ($F_{(2, 438)}$ = 44.84, P<0.0001) and DP ($F_{(2, 438)}$ = 12.97, P<0.0001). **(H)** Sleep depth decreased during both LP ($F_{(2, 438)}$ = 76.48, P<0.0001) and DP ($F_{(2, 438)}$ = 51.56, P<0.0001) in *Hk* mutant flies. **(I)** Latency (in min) to fall asleep during DP was increased upon *AOX* expression in a *Hk* mutant background ($F_{(2, 438)}$ = 9.24, P = 0.0001). **(C–I)** Both WT controls and both *Hk* mutant controls were pooled respectively. Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Comparisons between genotypes are indicated by connecting lines, if no line is indicated, comparison was made with the direct WT control. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 94–189.

and C), similar to sleep phenotypes observed with dFB-specific *AOX* expression. The flies' waking activity was unchanged in all groups (Figure 3.11D).

Sleep duration was affected most gravely by changes in the number of sleep bouts. During DP, *AOX* expression caused a higher number of sleep bouts as compared to *Hk* mutant and WT controls, while bout duration stayed the same compared to *Hk* mutants (Figure 3.11E and F). However, it has to be noted that a significant reduction in bout duration during DP is observed upon removal of the single outlier in *AOX*-expressing flies. During LP, both the number and duration of sleep bouts remained unchanged (Figure 3.11E and F). PDoze showed no difference upon *AOX* expression, while PWake increased during DP only (Figure 3.11G and H). Lastly, nighttime sleep latency was increased in *AOX*-expressing flies as compared to both *Hk* mutant and WT controls (Figure 3.11I), indicating the flies' inability to fall asleep.

Taking these results together, the slight increase in sleep seen in *AOX*-expressing flies during LP can be explained by an insignificantly increased number of sleep bouts, while the decrease of sleep during DP is explained by a shortening of bout duration. This is also in line with the increased PWake, which hints towards lighter sleep from which flies can be roused more easily. The sleep phenotypes seen upon brain-wide *AOX* expression in a *Hk* mutant background follow sleep patterns seen upon dFB-specifc *AOX* expression in a *Hk* mutant background.

3.5.3 Learning and memory

AOX was expressed in a *Hk* mutant background either in dFB neurons only (using *R23E10-Gal4*) or pan-neuronally (using *nsyb-Gal4*), and the learning index of flies was assessed using odour-based negative reinforcement.

AOX expression in dFB neurons (*Hk*^{1}*; R23E10>AOX 7.1*) could not fully rescue learning deficits seen in *Hk* mutant flies (*Hk*^{1}*; R23E10-Gal4/+* and *Hk*^{1}*; UAS AOX 7.1/+*) when compared to WT controls (*Hk*^{1}*/+; R23E10-Gal4/+* and *Hk*^{1}*/+; UAS AOX 7.1/+*) (Figure 3.12A). However, learning behaviour slightly but significantly improved with *AOX* expression compared to *Hk* mutants without *AOX* expression.

Next, to test whether increasing the range of *AOX* expression further improves learning abilities, *AOX* was expressed pan-neuronally. First, it has to be noted that one of the WT control groups, *Hk*^{1}*/+; nsyb-Gal4/+*, showed a very low learning index. Overall, these flies seemed to have a poor health, locomotor impairments, and reduced life expectancy (see Figure 3.13C and D). Therefore, in the following analysis of learning behaviour (and later lifespan) these flies will be mostly excluded.

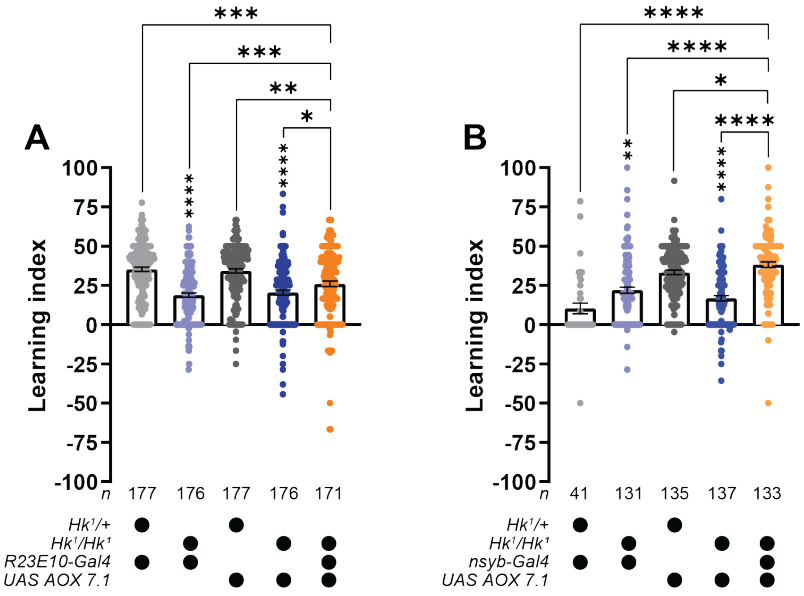

Figure 3.12: *Hk*^{1} flies show learning deficits that can be rescued by brain-wide *AOX* expression. **(A)** Learning index of *Hk*^{1}*; R23E10>AOX 7.1* flies (orange), *Hk* mutant controls (blue), and heterozygous WT controls (grey). *AOX* expression in dFB neurons of *Hk* mutant flies caused a partial rescue of learning index ($F_{(4, 872)}$ = 26.90, P<0.0001). **(B)** Learning index of *Hk*^{1}*; nsyb>AOX 7.1* flies (orange), *Hk* mutant controls (blue), and heterozygous WT controls (grey). *AOX*-expressing flies showed a full rescue of learning deficit ($F_{(4, 572)}$ = 31.85, P<0.0001). **(A** and **B)** Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Comparisons between genotypes are indicated by connecting lines, if no line is indicated, comparison was made with the direct WT control. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 41–177.

As seen in Figure 3.12B, pan-neuronal expression of *AOX* (*Hk^1; nsyb>AOX 7.1*) can rescue the learning deficit seen in *Hk* mutant flies (*Hk^1; nsyb-Gal4/+* and *Hk^1; UAS AOX 7.1/+*) and even slightly improve learning behaviour compared to the WT control (*Hk^1/+; UAS AOX 7.1/+*). This suggests, that *Hk* itself is not required for learning, but that ROS status and the correct sensing of and correction for ROS levels in neurons might be essential for learning.

3.5.4 Lifespan

Finally, lifespan of *AOX*-expressing *Hk* mutants was assessed. As mentioned before, for reasons unknown *Hk^1/+; nsyb-Gal4/+* flies seemed unhealthy and showed a very high death rate during the first 20 days after eclosion. Therefore, despite being genetically WT flies, they were mostly disregarded when interpreting data (Figure 3.13C and D).

Despite the successful rescue in learning behaviour, neither dFB-specific, nor pan-neuronal expression of *AOX* in an otherwise *Hk* mutant context could rescue lifespan (Figure 3.13). Flies with *AOX* expression in dFB neurons had a median lifespan of 47 days (*Hk^1; R23E10> AOX 7.1*, while *Hk* mutant controls showed median lifespans of 52 days (*Hk^1; UAS AOX 7.1/+*) and 54 days (*Hk^1; R23E10-Gal4/+*), respectively. WT controls however lived around 20 days longer, with median lifespans of 70 (*Hk^1/+; UAS AOX 7.1/+*) and 77 days (*Hk^1/+; R23E10-Gal4/+*), respectively (Figure 3.13A and B). Flies with pan-neuronal *AOX* expression showed a similar pattern. WT controls had a median lifespan of 73 days (*Hk^1/+; UAS AOX 7.1/+*), while *Hk* mutant flies died about 20 days earlier with a median lifespan of 50 days (both *Hk^1; nsyb-Gal4/+* and *Hk^1; UAS AOX 7.1/+*). *AOX* expression in a *Hk* mutant background decreased median lifespan further to 49 days (*Hk^1; nsyb>AOX 7.1*) (Figure 3.13C and D).

It seems that functional Hk might not be needed for learning performance, but is essential for survival of the flies. *AOX* expression could not improve lifespan even partially; on the contrary it seemed to even further reduce lifespan especially in case of dFB-specific expression (Figure 3.13A and B).

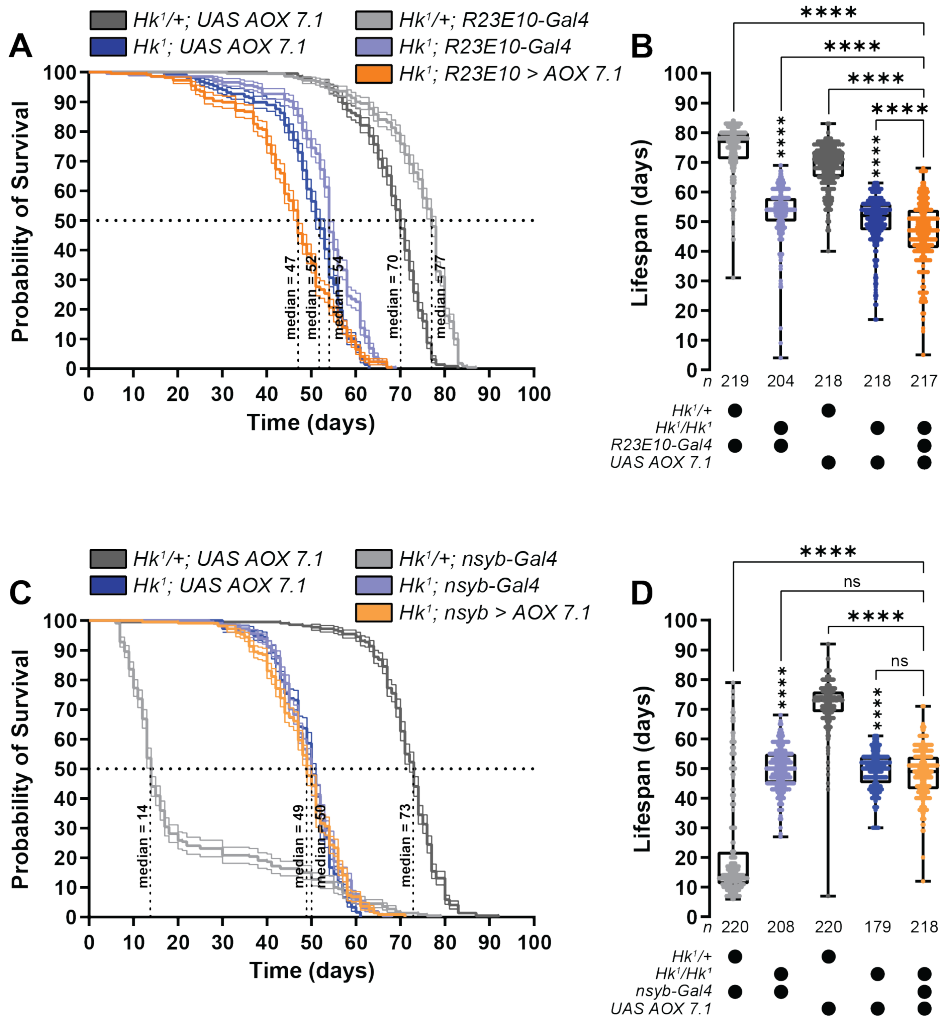

Figure 3.13: Brain-wide knockout of Hk causes a shortened lifespan in flies independent of *AOX* expression. (A) Survival curve of *Hk[1]; R23E10>AOX 7.1* flies (orange), *Hk* mutant controls (blue), and heterozygous WT controls (grey). **(B)** Lifespan of *AOX*-expressing flies and controls. *AOX* expression in dFB neurons cannot rescue early death as seen in *Hk* mutants ($F_{(4, 1071)}$ = 386.5, P<0.0001). **(C)** Survival curve of *Hk[1]; nsyb>AOX 7.1* flies (orange), *Hk* mutant controls (blue), and heterozygous WT controls (grey). **(D)** Lifespan of *AOX*-expressing flies and controls. Lifespan could not be rescued by brain-wide expression of *AOX* in a *Hk[1]* background ($F_{(4, 1040)}$ = 587.6, P<0.0001). **(B** and **D)** Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, **** P<0.0001. Comparisons between genotypes are indicated by connecting lines, if no line is indicated, comparison was made with the direct WT control. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, n = 179-220.

3.6 Discussion

In this chapter, I described my findings that expression of *AOX*, and consequential reduction of ROS production, in neurons and the gut can drastically shorten daily sleep time without affecting learning ability or lifespan, two major behaviours often reported to be severely affected by chronic sleep deprivation (Cirelli et al., 2004; Li et al., 2009; Shaw et al., 2002).

The sleep behaviour seen upon *AOX* expression, especially when targeting dFB neurons, closely resembled sleep patterns seen in *Hk* mutant flies. A possible explanation for the similar sleep phenotypes is that both manipulations act via the same pathway, as AOX is limiting ROS levels, while Hk acts as a ROS sensor (Kempf et al., 2019). In contrast to *Hk* mutant flies, though, *AOX*-expressing flies displayed no learning deficits or reduced lifespan, despite their shortened sleep, suggesting a difference in the quality of sleep reduction between both conditions. The obvious difference lies in the level of ROS production and thereby resulting total ROS concentration in the targeted cells. In *Hk* mutant flies, cells cannot sense ROS levels and therefore, even if critical ROS levels are reached that should induce sleep via the Sh channel complex, sleep cannot be initiated. In *AOX*-expressing flies, critical levels of ROS might take longer to reach, causing flies to stay awake for longer.

ROS has long been implicated with sleep and, more specifically, sleep deprivation. While Reimund theorized about the role of ROS in sleep and sleep regulation (Reimund, 1994), other groups put his theory to the test. Many studies on mice and rats found increased ROS products, especially lipid peroxidation products (LPPs), or reduced antioxidant defences after short-term or long-term sleep deprivation (summarized in Villafuerte et al., 2015). Reduced levels or activity of glutathione and sodium dismutase (SOD), two antioxidants, were reported after prolonged sleep deprivation (D'Almeida et al., 1998; Alzoubi et al., 2012;

Kanazawa et al., 2016; Ramanathan et al., 2002; Süer et al., 2011), often restricted to certain brain areas such as the hippocampus (Alzoubi et al., 2012), prefrontal cortex (Ramanathan et al., 2002), striatum (Kanazawa et al., 2016), or hypothalamus (D'Almeida et al., 1998). On the other hand, treating sleep-deprived animals with antioxidants can reduce ROS levels, and drastically improves other behaviours associated with sleep deprivation, such as learning abilities (Alzoubi et al., 2012; Silva et al., 2004). Other groups, focusing on flies, found a positive correlation between expression of the antioxidant SOD and lifespan (Orr and Sohal, 1994; Parkes et al., 1998; Sun and Tower, 1999). Orr and Sohal (1994) found that overexpression of SOD extends lifespan of flies by a third, prolongs the mortality rate doubling time, and delays the loss in physical performance. Parkes et al. (1998) showed a lifespan extension of 40% by overexpressing the human SOD1, while Sun and Tower (1999) were able to extend the flies' lifespan by 50%. More recent reports using fly models showed both an increased sensitivity to oxidative stress (Hill et al., 2018) in short sleeping mutant flies, and an increase of ROS in the gut, with consequential early death after prolonged sleep deprivation (Vaccaro et al., 2020). Additionally, overexpression of antioxidants could reduce sleep, reduce ROS levels, and prevent early death (Hill et al., 2018; Vaccaro et al., 2020). All these findings suggest that sleep might have antioxidant properties and increases an organisms resistance to oxidative stress, which builds up during prolonged waking. This is supported also by results reported in this chapter, as expression of *AOX*, effectively reducing ROS production (Fernandez-Ayala et al., 2009), produces normal learning and lifespan despite a short-sleeping phenotype. It is important to note that *AOX* expression in itself does not extend lifespan (Sanz et al., 2010). The lifespan extension as compared to other short-sleeping flies thus seems to be based on the reduced need to sleep.

In line with these results is also the theory of waste clearance as a function of sleep. It

is know that waste and metabolite clearance from the brain of mice (Xie et al., 2013) and flies (Van Alphen et al., 2021) is increased during sleep. Van Alphen et al. (2021) found proboscis movements in flies and underlying waste clearance from the brain to be highly associated with a deep sleep stage, characterized by a higher arousal threshold. The number of proboscis movements could be increased during longer sleep bouts, which indicates that sleep is highly advantageous to clear the brain from metabolites and toxins (Van Alphen et al., 2021). One of these waste products could be ROS, accumulating in the brain during wakefulness.

In an extension of findings (Kempf et al., 2019), ROS sensing does not seem to be confined to dFB neurons. As seen here, *AOX* expression not only in dFB neurons, but also the MB, the AL, and even the gut caused shortened sleep in flies. One could suspect a leaky expression of the *UAS AOX 7.1* transgene, causing AOX to always be present in dFB neurons and therefore causing the sleep phenotype. However, expression of *AOX* in glial cells caused no sleep phenotype, rendering a leaky expression of *AOX* an unlikely cause of the reduced sleep duration. It rather seems that no matter where in the brain *AOX* is expressed, flies will consequentially sleep chronically less with little consequences to their learning ability or lifespan. Additionally, the same can be achieved when targeting *AOX* expression to enterocytes in the fly gut. This result takes findings by Vaccaro et al. (2020) one step further: they found a gradual accumulation of ROS in the gut of flies and mice after prolonged sleep deprivation, and could show that gut-targeted antioxidant expression prevents ROS accumulation and rescues lifespan during prolonged sleep deprivation. My results show that this relationship also holds the other way around: limiting ROS production in the gut can shorten sleep and reduce sleep need without consequences for learning or lifespan.

Even though *AOX* expression elsewhere than dFB neurons caused a shortened sleep phe-

notype, expression of *AOX* in the whole brain did not cause additional loss in sleep compared to dFB-specific expression. Although it is difficult to directly compare the two conditions due to different genetic backgrounds that might affect behaviour, the overall similarity of the sleep pattern as well as the length of total sleep indicate that sleep cannot be further reduced by *AOX* expression than what is seen in dFB-specific expression. In comparison, sleep reductions with expression in other types of neurons were much milder. The lack of an additive effect on sleep could be a sign that sleep phenotypes seen upon targeting MB neurons and AL PNs are caused by mechanisms working in the same pathway as dFB neurons.

As both *Hk* mutants as well as *AOX*-expressing flies experienced a shortened sleep time, and both manipulations presumably act in the same signalling pathway of sleep induction, I was interested to see which influence *AOX* expression had on sleep, learning and lifespan of Hk^{1} flies. Both with dFB-restricted and brain-wide expression, the presence of AOX subtly changed the sleep pattern of Hk^{1} flies, while maintaining a similar reduction in total sleep time. When directly comparing values of sleep parameters, sleep patterns of *AOX*-expressing Hk^{1} flies resembled sleep of *AOX*-expressing flies more than sleep of Hk^{1} flies, consistent with AOX acting upstream of Hk.

In addition to changing the sleep pattern, dFB neuron-restricted *AOX* expression in a *Hk* mutant background was sufficient to rescue learning deficits. This suggests that learning deficits seen in *Hk* mutants are at least in part secondary to the chronic sleep deprivation of these flies rather than caused by the deficiency of Hk in dFB neurons or other brain structures implicated in learning (like the MB). This finding strengthens earlier results of increased sleep duration and restored learning upon reinstatement of Hk in dFB neurons (see chapters 2.2 and 2.3).

Despite a successful rescue of learning, lifespan was not restored in *Hk* mutants upon

AOX expression. Since even brain-wide *AOX* expression could not rescue lifespan, it is highly probable that at least parts of the shortened lifespan seen in *Hk* mutant flies are caused either directly by the deficiency of Hk or the shortened sleep phenotype, independent of ROS levels, or by effects outside the brain. Combining these results with earlier findings, where reinstatement of Hk in dFB neurons was insufficient to rescue lifespan despite increased sleep time, while pan-neuronal expression of WT *Hk* could rescue lifespan (see chapter 2.4), it becomes clear that functional Hk is likely necessary to sustain a WT lifespan.

Chapter 4

Conclusions

4.1 Main Findings

With the experiments described in this thesis, I cannot yet fully answer whether Reimund was right about the role of sleep as an antioxidant defence (Reimund, 1994). However, we are inching closer to the answer.

In chapter 2, I showed that Hk deficiency, even if targeted to only dFB neurons, causes shortened sleep, learning deficits, and a shortened lifespan. Sleep and learning could be rescued by reinstating Hk in dFB neurons, suggesting that the learning deficit seen in *Hk* mutants is a consequence of chronic sleep loss. Lifespan on the other hand was impacted by Hk deficiency outside of the context of chronic sleep deprivation, as only a reinstatement of Hk into the whole brain rescued lifespan. Furthermore, I found that the catalytically active site of Hk is essential for both normal sleep time (and consequently learning), and a healthy lifespan.

In chapter 3, I showed that expression of *AOX*, and the consequential reduction in mitochrondrial ROS production, effectively reduce sleep time to a similar degree as a strong

mutation in *Hk*. This effect is not limited to, but most effective when targeting dFB neurons. Strikingly, chronic sleep loss generated in this manner affected neither learning nor lifespan, implying that flies can exist and perform healthily with lower than WT levels of sleep if neuronal ROS production is also capped. Additionally, I showed that learning deficits seen after chronic sleep loss are linked to ROS accumulation, as expression of *AOX* prevented learning deficits in *Hk* mutant flies.

4.1.1 Does sleep protect against oxidative stress?

Results presented in this thesis make a strong case for ROS playing an important role in the induction of sleep and regulation of time spent asleep, and show that symptoms of chronic sleep deprivation can be counteracted via regulation of ROS production.

Yet, several aspects of sleep regulation, ROS, and further consequences of chronic sleep deprivation will need to be investigated in future work. Therefore, I will discuss some limitations of this study in more detail and describe future research proposals in the following section.

4.2 Limitations and Future Research

While some questions about the nature of the relationship between ROS and sleep could be answered in this thesis, new questions arose during the process and others have remained unaddressed.

Hk acts as a ROS sensor by virtue of its cofactor NADPH being oxidized to $NADP^+$, but the nature of the other half reaction — the coupled reduction of an endogenous Hk substrate — remains unknown. The substrate is unlikely a short-lived ROS such as superoxide (O_2^-)

or H_2O_2, as both are highly reactive and are not expected to leave the mitochondria and reach the target site at the cell membrane without reacting with other components within the cell. A more likely candidate substrate for Hk are the aforementioned LPPs. Contrary to O_2^-, H_2O_2, and other primary produced ROS, LPPs are relatively long-lived and involved in a wide range of signalling cascades (Ayala et al., 2014; Esterbauer et al., 1991). AKRs other than Hk are known to catalyse the reduction of lipid peroxidation-derived phospholipid aldehydes (Spite et al., 2007), and Hk itself seems to prefer reducing hydrophobic aldehydes over ketones if presented with both (Tipparaju et al., 2008). Further research might reveal potential biological substrates for Hk.

A somewhat puzzling discovery are flies with dFB-restricted *AOX* expression that show no deficits in learning and no shortened lifespan. This is surprising, as ROS levels are expected to build up in the whole brain, but in case of dFB-restricted expression, ROS would still be able to accumulate normally everywhere except dFB neurons. The accumulation of ROS is predicted to cause learning deficits and a shortened lifespan, as seen with *Hk* mutants and other, chronically sleep-deprived flies (Bushey et al., 2007, 2010; Cirelli et al., 2005). Finding the reason for this unexpected result would go beyond the scope of this thesis, but future experiments might help solving the matter. One potential explanation is leakiness of the *UAS AOX 7.1* transgene. Very low background expression of *AOX* has been detected in *UAS AOX 7.1*-carrying flies, despite the lack of a *Gal4*-driver (see Supplementary Figure 6.4). This background expression does not seem strong enough to induce any changes in sleep behaviour, as glia-targeted *AOX* expression causes no changes in sleep duration or architecture. However, this background expression might be strong enough to cause a relevant decrease in ROS production in the brain, sufficient to prevent learning deficits and early death of chronically short-sleeping flies. This hypothesis could be tested by suppres-

sion of *AOX* expression everywhere except dFB neurons, using the *Gal4*-repressor Gal80 (Suster et al., 2004). Another explanation might be that AOX, present in dFB neurons, is getting transported to other regions in the brain. As AOX is sitting in the IMM, a possible mechanism facilitating AOX-trafficking could be horizontal mitochondrial transfer (HMT) (for an overview, see Dong et al., 2023). Although HMT has not yet been demonstrated in *Drosophila melanogaster*, it is a proven concept in mammalian research, including during the development of and in the fully-formed mammalian nervous system (Dong et al., 2023; Marti Gutierrez et al., 2022). If HMT were to occur in *Drosophila*, AOX could be trafficked and distributed throughout the brain, even if expression is restricted to dFB neurons. However, whether HTM exists in the fruit fly is highly speculative at this point. Whether AOX is being trafficked throughout the brain, due to HTM or other processes unknown, could possibly be investigated by visualizing AOX distribution in the brain of ageing flies.

Expression of *AOX* seems to only bring advantages for fruit flies: they can sleep for a shorter amount of time in a lighter sleep stage, therefore have more time to find food and reproduce, and are more easily aroused when asleep, making them more vigilant against any sort of external danger. Additionally, AOX was found to counteract the effects of many detrimental diseases (El-Khoury et al., 2016; Fernandez-Ayala et al., 2009; Giordano et al., 2019; Mills et al., 2016). Still, the *AOX* gene was lost during evolution and is missing in flies (and mammals, including humans) (McDonald et al., 2009). If AOX was safe and presented such a great advantage for survival, why would the early ancestors of arthropods and vertebrates have lost the gene? Here, the only behaviours evaluated were sleep, odour-associated learning, and lifespan. Even though learning and lifespan did not seem affected by the chronic loss in sleep, other behaviours or less obvious cognitive functions might be affected in *AOX*-expressing flies. Other groups have reported *AOX*-expressing flies to develop

normally and be able to reproduce, though they hypothesized a slight drop in the efficiency of catabolism to cause the evolutionary loss of AOX (Fernandez-Ayala et al., 2009). Several years later, as a result of *AOX* expression, the same group did report a defect in male reproductive function (Saari et al., 2017), and developmental failure under certain nutritional conditions (Saari et al., 2019). In mammals, AOX has been reported to interfere with oxygen sensing, which can have life-threatening consequences (Dogan et al., 2018; Sommer et al., 2020). Thus, although *AOX*-expressing flies observed in this study did not show any obvious negative side-effects, AOX, especially if present in all mitochondria, might not be as harmless as it seems. Further research, especially by analysing additional markers of chronic sleep deprivation, is needed in order to fully establish a strictly positive impact of AOX and reduced ROS production on the flies' performance, and therefore cement the role of sleep as antioxidant. Some of these markers could be additional learning and memory assays, testing short-term and long-term memory, together with memory consolidation of flies. It would be important to test learning and memory with different tasks, to determine whether phenotypes are specific to the task or stimulus used for the training, or a general phenomenon. Next to olfactory conditioning (such as used in this thesis), visual conditioning (e.g. aversive phototaxis suppression (APS) assay) (van Swinderen, 2011) and courtship conditioning (Koemans et al., 2017) are only some of the possible assays to test *Drosphila* learning and memory more thoroughly. Importantly, the fan-shaped body of flies is required for context-dependent navigation (Hulse et al., 2021); it would be interesting to investigate if *AOX* expression in dFB neurons has an effect on navigational strategies of flies. Information from these different behavioural assays could paint a more complete picture of the flies' cognitive abilities in conditions of chronic sleep loss due to *AOX* expression.

To ensure AOX does not cause any developmental defects, one could use an inducible

expression system for future experiments. One system that seems appropriate, as it is compatible with the Gal4 system and does neither affect sleep nor lifespan by itself, is the auxin-inducible expression system (McClure et al., 2022). It depends on Gal80, which represses expression of *Gal4* (Suster et al., 2004), being bound to two auxin-inducible degron (AID) sequences. If flies are being fed the 1-naphthaleneacetic acid potassium salt (K-NAA) auxin, Gal80 will be degraded, allowing Gal4-dependent expression of transgenes (McClure et al., 2022). Additional to ruling out developmental effects of AOX, especially lifespan experiments could benefit from an inducible system. When expressing a transgene, experimental flies often show a boost in lifespan compared to their parental controls, which makes for an inappropriate comparison. Here, the inducible system could help, by simply comparing the induced with the uninduced flies, both groups sharing the same genotype.

Although lifespan was one of the major readouts in this study, sleep was only assessed in younger flies aged five to seven days. In *AOX*-expressing flies, a major loss in sleep was observed during that age, which seemingly did not affect lifespan. However, whether the loss in sleep in aged flies was as severe as seen in young flies remains unknown, yet easily testable in additional experiments, possibly with the auxin-inducible expression system.

Another interesting result is that mainly daytime sleep was affected from sleep loss caused by either Hk deficiency or *AOX* overexpression, while nighttime sleep was only mildly affected, primarily in the form of sleep fragmentation. This suggests that ROS signalling via dFB neurons is predominantly mediating daytime sleep, while a different mechanism might be responsible for (or overrule ROS-dependent dFB neuron control of) nighttime sleep. This other sleep circuit seems to operate independently of ROS signalling, as even brain-wide manipulations of ROS sensing or ROS accumulation did not severely affect nighttime sleep duration. This would explain why flies frequently woke up during nighttime,

as if unable to maintain a constant sleep state, but did not lose much total sleep, as they fell asleep as frequently. It would be interesting to investigate this ROS-independent sleep induction and determine, how the two circuits connect and influence each other.

In addition to these specific open questions, there are some general technical limitations to sleep research in flies. All sleep behaviour presented in this thesis was recorded using single-beam infrared recording (SB-IR) of fly movement. As movement is only recorded at the half point of a 60mm long tube, flies are able to walk and move around a significant distance without ever crossing the infrared beam, which can cause a higher estimate of sleep in the final analysis (Garbe et al., 2015; Zimmerman et al., 2008b). A solution to this could be multi-beam infrared recording (MB-IR), or video-tracking of flies (Garbe et al., 2015; Zimmerman et al., 2008b). While MB-IR does not seem to pick up any differences in sleep duration in regards to SB-IR, it is more sensitive to changes in sleep pattern and architecture, making it more sensitive to qualitative changes in sleep (Garbe et al., 2015). Video-tracking, additionally to recording all locomotor movements, can pick up on small movements like flicking of wings, proboscis movements, and other subtle adjustments to the sleep posture of flies (Zimmerman et al., 2008b). It could also be useful to track sleep of flies in differently shaped environments, as recording chambers do not have to be restricted to a tube-shape (Garbe et al., 2015). Although both MB-IR and video-tracking seem more sensitive to detect changes in sleep pattern, sleep phenotypes presented in this thesis were strong enough to be picked up by SB-IR. It would be interesting to further analyse differences in sleep behaviour using video-tracking, but for the purpose and aim of this thesis SB-IR was sufficiently sensitive.

Most systems used to record sleep in flies, be they SB-IR, MB-IR, or video-tracking, are based on single fly recordings, with flies housed individually in tubes or wells. Tracking

of fly behaviour is easier that way; however, flies are social animals and their sleep pattern changes according to their social experience (Chi et al., 2014; Donlea et al., 2009; Ganguly-Fitzgerald et al., 2006; Lone et al., 2016). An enriched waking experience, whether due to learning (Donlea et al., 2009) or interaction with other flies (Donlea et al., 2009; Ganguly-Fitzgerald et al., 2006; Lone et al., 2016), increases and consolidates sleep mainly in male flies (Lone et al., 2016). Even social experience as larvae can influence sleep in the adult flies; sleep of females is more consolidated when they were crowded at the larval stage (Chi et al., 2014). Furthermore, population sleep is slightly distinct from individual sleep, and depends on population size as well as the ratio of female:male flies (Liu et al., 2015). For experiments described in this study, flies were housed in same-sex groups until 48 hours before sleep was recorded. This might not mimic 'natural' sleep conditions, but facilitates comparison of sleep between groups and eliminates possible confounding factors while still giving flies the socially enriched environment during their early days after eclosion.

Another factor influencing sleep duration and architecture is the flies' sex and mating status (for female flies) (Chi et al., 2014; Garbe et al., 2016; Isaac et al., 2010; Lamaze et al., 2017; Lone et al., 2016). Generally, males sleep longer than females (Chi et al., 2014), and female virgins sleep longer than mated females, while sleep returns to baseline around 10 days post-mating (Garbe et al., 2016; Isaac et al., 2010). In both cases, males vs females and virgin vs mated, differences in sleep are mainly observed during daytime (Chi et al., 2014; Garbe et al., 2016; Isaac et al., 2010; Lamaze et al., 2017). Additionally, certain environmental conditions seem to affect only one or the other sex, such as temperature and socializing as adults mainly affecting male flies (Lamaze et al., 2017; Lone et al., 2016), and crowding as larvae and mating status mainly affecting females (Chi et al., 2014; Garbe et al., 2016; Isaac et al., 2010). To exclude possible confounding factors, in this study only

mated female flies were used for all behaviours tested. However, it could be interesting to see whether changes in sleep described in this thesis can also be observed in male and virgin female flies.

4.3 Impact and Applicability of this Research

Research presented in this thesis is fairly fundamental and hardly immediately applicable for human benefit. Yet it presents important insights into the working mechanisms of sleep and brings us a step closer to solving the mystery of the function of sleep. Shedding light on the molecular and cellular regulatory processes of sleep, especially if universal to all animals including humans, will have great benefits for future research on health conditions associated with or based on sleep disruptions. In particular neurodevelopmental disorders, such as attention deficit hyperactivity disorder (ADHD) (Lee et al., 2014), autism spectrum disorder (ASD) (Baker and Richdale, 2015), Down syndrome (Bassell et al., 2015), and many others are known to cause sleep disorders. Interestingly, treating sleep problems and improving sleep quality can improve other symptoms of the respective disorder and improve overall quality of life (Brand et al., 2015; Hiscock et al., 2015). Additionally, sleep has been implicated in a wide range of other health issues, from AD (Peter-Derex et al., 2015) and epilepsy (Roliz and Kothare, 2022), to chronic obstructive pulmonary disease (COPD) (Tsai, 2017) and cardiovascular disease (Khan and Aouad, 2022). Further, immune responses are highly related to quality of sleep, while sleep can vice versa be modified by the immune system (e.g. increased sleep need when feeling ill) (Besedovsky et al., 2019). Thus, any gain in knowledge on sleep, how to regulate it, and how to improve sleep quality, has the potential to help countless patients suffering from a broad spectrum of ailments in the future.

Chapter 5

Methodology

5.1 Fly strains and husbandry

All flies were kept on standard agar food (yeast, cornmeal, molasses; see Supplementary Methods and Supplementary Table 6.1) and housed at 25°C under 12-hour light:12-hour dark (12LD) cycles. Fly strains used and mentioned in this thesis were acquired or created by combining stocks from Bloomington *Drosophila* Stock Center (BDSC), Vienna *Drosophila* Resource Center (VDRC), or the Centre of Neural Circuits and Behaviour (CNCB) stock collection. The *UAS AOX 7.1* stock was a kind gift from Howard Jacobs' lab (Andjelković et al., 2015).

For a better overview, fly strains used and created for this study are listed in Table 5.1.

5.2 Sleep measurement

Mated female flies were collected upon eclosion and aged in vials of 20 flies. 4–5 day old flies were inserted individually into 65mm long tubes with food on one end (sealed with

Table 5.1

Strains of *Drosophila melanogaster* and their sources			
***Drosophila* strain**	**Source**		Chapter
R23E10-Gal4	BDSC # 49032		3.2–3.4
nsyb-Gal4	CNCB stock collection		3.2–3.4
MB247-Gal4	CNCB stock collection		3.2–3.4
GH146-Gal4	CNCB stock collection		3.2–3.4
myo31DF-Gal4	CNCB stock collection		3.2–3.4
repo-Gal4	BDSC # 7415		3.2–3.4
Hk^1	BDSC # 3562		2.2–2.4
UAS AOX 7.1	Gift from Jacobs lab (Andjelković et al., 2015)		3.2–3.4
UAS Hk WT	BDSC # 8627		
UAS Hk K289M	BDSC # 86269		
UAS $RNAi^{Hk}$	VDRC # 47805 GD		2.2–2.3
Canton-S (CS)	CNCB stock collection		
Genotype created	**Source strains**		Chapter
Hk^1;; R23E10-Gal4	Hk^1	R23E10-Gal4	2.2–2.4, 3.5
Hk^1;; nsyb-Gal4	Hk^1	nsyb-Gal4	2.2–2.4, 3.5
Hk^1; UAS Hk WT	Hk^1	UAS Hk WT	2.2–2.4
Hk^1; UAS Hk K289M	Hk^1	UAS Hk K289M	2.2–2.4
Hk^1;; UAS AOX 7.1	Hk^1	UAS AOX 7.1	3.5

BDSC: Bloomington *Drosophila* Stock Center
CNCB: Centre for Neural Circuits and Behaviour
VDRC: Vienna *Drosophila* Resource Center

wax to prevent from drying out) and a cotton plug on the other. Tubes with flies were then loaded into TriKinetics *Drosophila* Activity Monitors (DAM) (TriKinetics Inc., Waltham MA, USA) and housed at 25°C under 12LD cycles. The DAM system uses infrared beams to record activity of the flies whenever they move through the midpoint of the tube, thereby causing a beam break. Bouts of inactivity lasting for five minutes and longer were classified as sleep, according to common definitions (Hendricks et al., 2000; Shaw et al., 2000). Activity of flies was recorded for up to three days (three 12LD cycles), data presented throughout the thesis are measurements taken on day 2. Immobile flies or flies that had died during the three days of monitoring were excluded from further analysis. Activity data was binned into 1min and 30min bins, and using the Sleep and Circadian Analysis MATLAB Program

(SCAMP) (version: 2020_v3), activity data was processed and total sleep duration, waking activity, number of sleep episodes, average sleep bout duration, probabilities to fall asleep or awake, respectively, and sleep latency were extracted for further analysis.

5.3 Learning experiments

Learning experiments were conducted as described before (Claridge-Chang et al., 2009). Mated female flies were collected upon eclosion and aged in vials of 20 flies. 7–10 day old flies were inserted individually into recording chambers wide enough for the flies to walk bidirectionally. The chambers allowed flow-through of air and odour from the two opposing sides (left and right), with the floor and ceiling of the chamber being coated in an electrically conductive material to allow shock punishment of flies. The experimental protocol lasted for a total of 15 minutes, including 2 minutes of pre-training test, 5 minutes of training, and 2 minutes of post-training test. The two tests consisted of 4-methylcyclohexanol (MCH; 1ppm) and 3-octanol (OCT; 20 ppm) flowing into the chamber from opposite sides. The total flow speed used was 5 l_n/min, distributed over 20 chambers. During training, first only MCH was given three times for 20s, followed by an electric shock of 50V at the end of each odour period, lasting for 2s (conditioned stimulus, CS+). In between these shock periods, the flies were exposed to clean air for 20s. After MCH exposure, flies were given a short resting period until the same procedure was repeated with OCT exposure, however, no shock was applied (unconditioned stimulus, CS-). In between tests and training, the flies were given short resting periods with clean air. After the training protocol, the flies were given a resting period of at least 5 minutes before the post-training test started.

During the whole protocol, fly movements and positions along the length of the chamber

were being recorded using video-tracking. Analysis of the flies' movements and decision making was performed in MATLAB (The MathWorks), using a customized script. For the learning index, the difference in percentage decisions for CS- during the pre-training test and the post-training test were calculated per fly. Flies that made fewer than two decisions during either test phase were excluded from the analysis.

5.4 Lifespan assay

Upon eclosion, mated female flies were collected and kept in vials containing standard food. Lifespan assays were usually started with 15 flies per vial and 5 vials per genotype (75 flies per genotype). Three times a week (every two to three days), flies were flipped into new vials containing fresh food, simultaneously counting dead flies. Flies that escaped during this process, were stuck in food, or got accidentally harmed by the experimenter were excluded from the analysis. Lifespan was determined beginning from the day of eclosion (day 0). All lifespan experiments were repeated at least three times.

5.5 Statistical analysis

All data were processed and analysed using GraphPad Prism 10. For comparison between two groups, two-sided t tests were used to compare group means. For comparison between three or more groups, one-way ANOVA followed by Holm-Šídák's multiple comparisons test was used.

Chapter 6

Supplementary Material

6.1 Supplementary Methods

6.1.1 Fly food preparation

Ingredients and amounts for the preparation of fly food can be found in Supplementary Table 6.1. Tegosept and ethanol were combined and mixed until tegosept had dissolved. To make the paste, about 5L water was mixed with cornmeal and yeast until the mixture was thin and smooth with no or few lumps. To dissolve the agar, the rest of the water was mixed with the agar in a large boiler, while increasing the temperature to 90°C. Then the paste and molasses was added, mixed in, and the mixture was sterilized by gradually bringing it to a boil (up to 94°C) and maintaining it for at least 5 minutes. After mixture was cooled to 72°C the tegosept dissolved in ethanol, and the acid were added. The final product was distributed into vials and cooled to solidify.

6.1.2 RNA extraction

For RNA extractions, 4 bodies and heads of flies were used per genetic condition. To separate limbs from bodies, tubes with the flies were placed in liquid nitrogen for 10-20s and vortexed for 10s repeatedly. Bodies of flies were collected, 200µL of TRIzol was added to each sample, homogenized thoroughly, and incubated at room temperature for 5 minutes. Samples then were centrifuged at 4ºC for 5–10 minutes at 12,000g. Supernatant was collected, 40µL of chloroform was added to each sample, mixed, and placed at room temperature for 5 minutes. The TRIzol/chloroform tubes were then centrifuged at 4ºC for 15 minutes at 12,000g. After the centrifuge run, the colourless layer was collected and 100µL of isopropanol was added to each sample, mixed, and placed at room temperature for 10 minutes. The samples were centrifuged again at 4ºC for 10 minutes at 12,000g. The supernatant was discarded and 200µL of 75% ethanol was added to the pellet. Samples were centrifuged for 5 minutes at 7,500g at 4ºC, the ethanol was discarded, and the pellet was let dry until transparent. Depending on the size of the pellet, a certain volume of nuclease free water was added, and samples were placed at 55ºC for 10 minutes.

RNA concentrations, 260/280 ratio, and 260/230 ratio were measured before proceeding with generating cDNA.

6.1.3 cDNA generation

gDNA digestion was performed on all samples using ezDNase enzyme (Invitrogen, Thermo Fisher Scientific), before cDNA was generated using the SuperScript™ IV VILO™ Master Mix (Invitrogen, Thermo Fisher Scientific). Per genetic condition, 500ng of RNA was used.

6.1.4 qPCR

qPCR was performed using AOX-targeting and, for reference, GAPDH-targeting primers (see Table 6.2). For the reactions, 20µL of a mixture of 1x SYBR Green, 500nM primer, and cDNA diluted 1:400 were used per well, each reaction was made in triplet. LightCycler 480 (Roche) was used to measure PCR reactions.

6.2 Supplementary Tables

Table 6.1

Fly food ingredients	
Reagent	**Amount in 32L water**
Agar	216g
Yeast	800g
Cornmeal	2000g
Molasses	1.2L
Acid Mix	134.4mL
Tegosept	44.8g
Ethanol	224mL

Table 6.2

List of primers	
Name	**Primer Sequence**
AOX forward	GGAAGAGCGATGCAAGATGG
AOX reverse	CCGTCCAGTGTATGTACCGA
GAPDH forward	AAAAAGCTCCGGGAAAAGG
GAPDH reverse	AATTCCGATCTTCGACATGG

Table 6.3

List of reagents		
Reagent	**Supplier**	**Product ID**
10x ezDNase Buffer	Thermo Fisher Scientific (Invitrogen)	11766051
2-propanol	Merck (Sigma-Aldrich)	67-63-0
3-Octanol (OCT)	Merck (Sigma-Aldrich)	589-98-0
4-Methylcyclohexanol	Merck (Sigma-Aldrich)	589-91-3
Ambion™ Nuclease-Free Water	Thermo Fisher Scientific (Ambion)	AM9937
Chloroform	Merck (Sigma-Aldrich)	67-66-3
Ethanol absolute	Merck (Sigma-Aldrich)	64-17-5
ezDNase enzyme	Thermo Fisher Scientific (Invitrogen)	11766051
LightCycler 480 SYBR Green I Master	Roche	04707516001
SuperScript™ IV VILO™ Master Mix	Thermo Fisher Scientific (Invitrogen)	11756050
SuperScript™ IV VILO™ No RT Control	Thermo Fisher Scientific (Invitrogen)	11756050
TRIzol Reagent	Thermo Fisher Scientific (Ambion)	15596026

6.3 Supplementary Figures

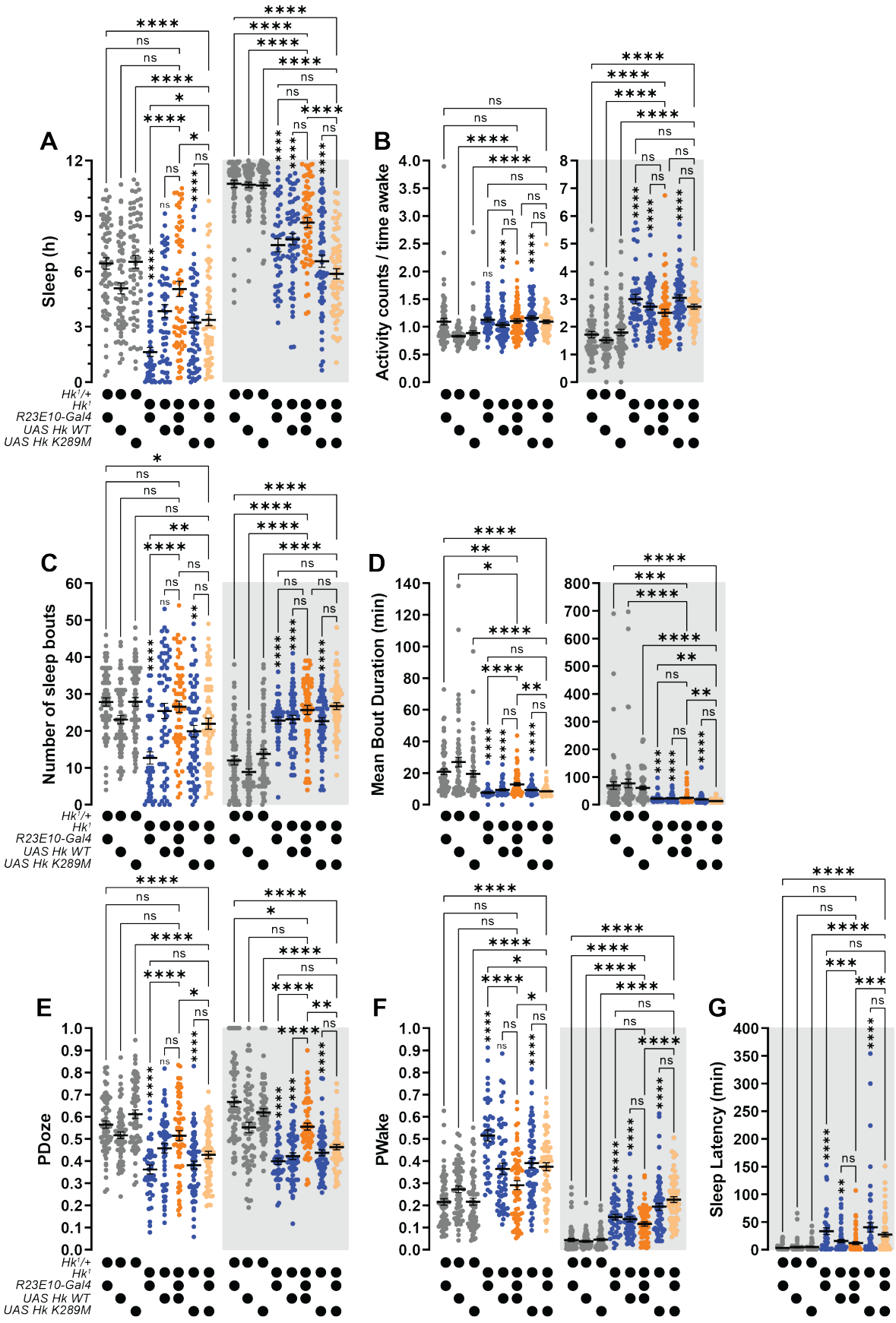
A
Sleep (h)
B
Activity counts / time awake
C
Number of sleep bouts
D
Mean Bout Duration (min)
E
PDoze
F
PWake
G
Sleep Latency (min)
Hk1/+
Hk1
R23E10-Gal4
UAS Hk WT
UAS Hk K289M

Figure 6.1: Sleep of *Hk^{1}* flies with dFB-specific *Hk* expression. **(A)** Total duration of sleep during LP (white background) or DP (grey background) of *Hk^{1}; R23E10 > Hk WT* flies, *Hk^{1}; R23E10 > Hk K289M* flies, and *Hk* mutant and heterozygous WT controls. *Hk^{1}; R23E10 > Hk WT* flies showed a partial rescue in sleep duration during both LP ($F_{(7, 471)}$ = 23.74, P<0.0001) and DP ($F_{(7, 471)}$ = 60.61, P<0.0001). **(B)** Activity of flies while being awake during LP ($F_{(7, 471)}$ = 9.15, P<0.0001) and DP ($F_{(7, 471)}$ = 28.51, P<0.0001). **(C)** The number of sleep bouts was normalized in *Hk WT*-expressing flies during LP ($F_{(7, 471)}$ = 9.74, P<0.0001) but not during DP ($F_{(7, 471)}$ = 43.10, P<0.0001). **(D)** The average duration of sleep bouts was significantly decreased in all *Hk* mutant flies during LP ($F_{(7, 471)}$ = 19.44, P<0.0001) and DP ($F_{(7, 471)}$ = 9.75, P<0.0001). **(E)** PDoze of *Hk^{1}; R23E10 > Hk WT* flies was partially rescued to WT levels during both LP ($F_{(7, 471)}$ = 21.73, P<0.0001) and DP ($F_{(7, 471)}$ = 31.80, P<0.0001). **(F)** Sleep depth was partially rescued in *Hk^{1}; R23E10 > Hk WT* flies during LP ($F_{(7, 471)}$ = 24.67, P<0.0001) and during DP ($F_{(7, 471)}$ = 50.20, P<0.0001). **(G)** Latency (in min) to fall asleep during DP was returned to WT levels in *Hk^{1}; R23E10 > Hk WT* flies ($F_{(7, 471)}$ = 12.28, P<0.0001). **(A–G)** Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Comparisons between genotypes are indicated by connecting lines, if no line is indicated, comparison was made with the direct WT control. Two independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 41–64.

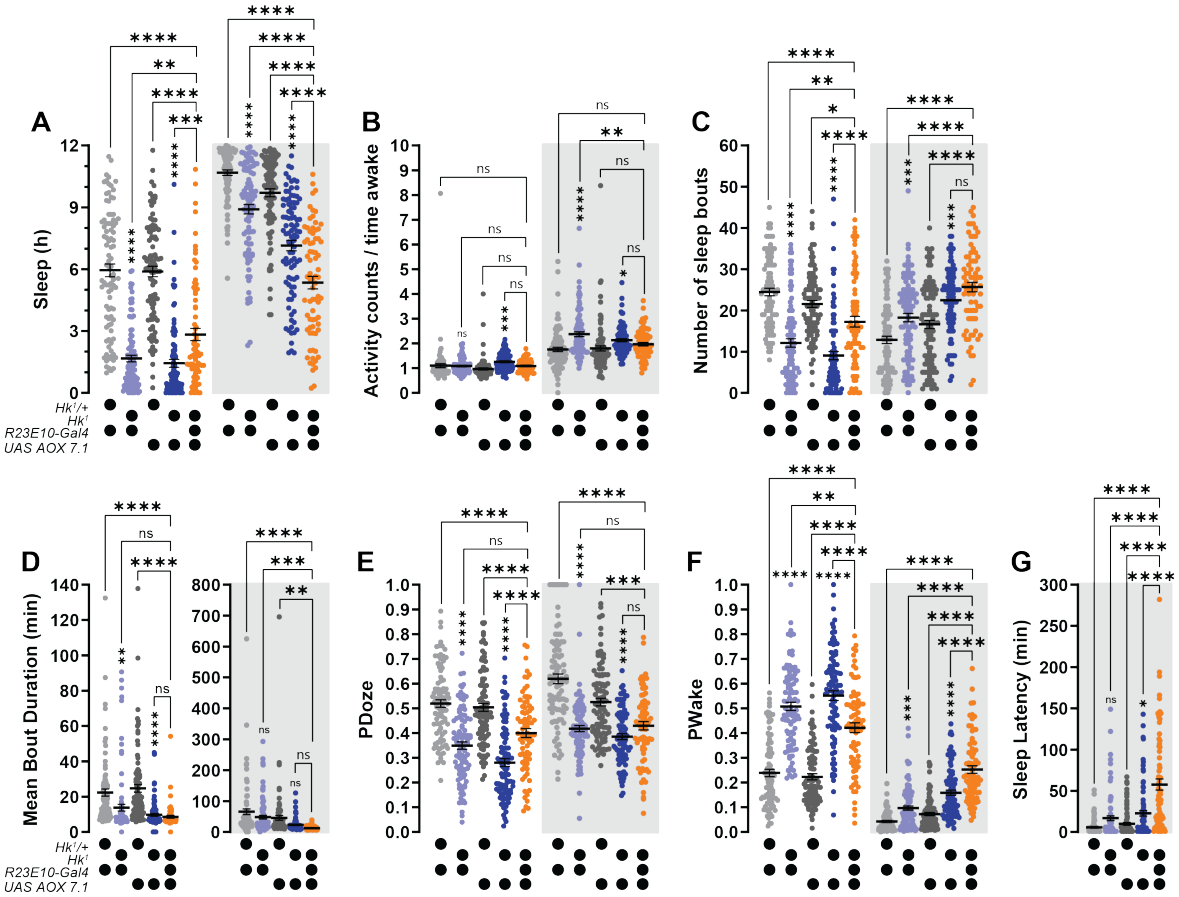

Figure 6.2: Sleep of *Hk*^{1} flies with dFB-specific *AOX* expression. **(A)** *AOX* expression slightly increased sleep during LP (white background) and decreased sleep during DP (grey background). LP: $F_{(4, 445)} = 84.50$, $P<0.0001$, DP: $F_{(4, 445)} = 82.75$, $P<0.0001$. **(B)** Activity of flies while awake during LP ($F_{(4, 445)} = 4.63$, $P = 0.0011$) and DP ($F_{(4, 445)} = 9.20$, $P<0.0001$). **(C)** The number of sleep bouts was significantly decreased in *Hk* mutant flies during LP ($F_{(4, 445)} = 42.07$, $P<0.0001$) but increased during DP ($F_{(4, 445)} = 24.58$, $P<0.0001$). **(D)** The average duration of sleep bouts was significantly decreased in *Hk* mutants during LP ($F_{(4, 445)} = 18.20$, $P<0.0001$) and DP ($F_{(4, 445)} = 11.06$, $P<0.0001$). **(E)** PDoze of *Hk* mutant flies was significantly reduced during LP ($F_{(4, 445)} = 43.07$, $P<0.0001$) and DP ($F_{(4, 445)} = 39.40$, $P<0.0001$). **(F)** Sleep depth was decreased during both LP ($F_{(4, 445)} = 86.32$, $P<0.0001$) and DP ($F_{(4, 445)} = 74.69$, $P<0.0001$) in *Hk* mutant flies. **(G)** Latency (in min) to fall asleep during DP was increased upon *AOX* expression in a *Hk* mutant background ($F_{(4, 445)} = 33.69$, $P<0.0001$). **(A–G)** Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns $P>0.05$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. Comparisons between genotypes are indicated by connecting lines, if no line is indicated, comparison was made with the direct WT control. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 75–96.

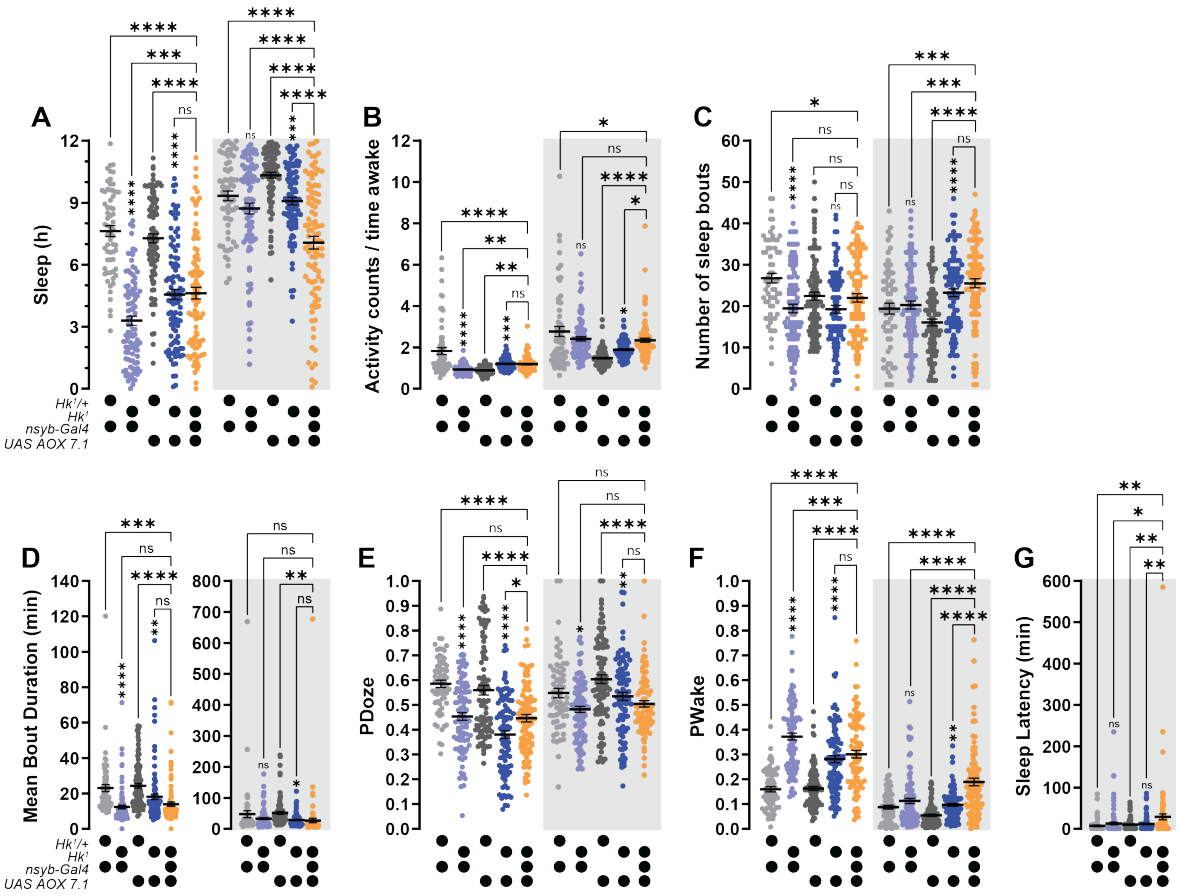

Figure 6.3: Sleep of *Hk*^{1} flies with brain-wide *AOX* expression. **(A)** *AOX* expression did not affect sleep during LP (white background) but decreased sleep during DP (grey background) compared to *Hk* mutant flies. LP: $F_{(4, 436)}$ = 55.13, P<0.0001, DP: $F_{(4, 436)}$ = 27.38, P<0.0001. **(B)** Activity of flies while awake during LP ($F_{(4, 436)}$ = 32.87, P<0.0001) and DP ($F_{(4, 436)}$ = 21.22, P<0.0001). **(C)** The number of sleep bouts was unchanged in *AOX*-expressing *Hk* mutant flies compared to controls during LP ($F_{(4, 436)}$ = 7.36, P<0.0001) but increased during DP ($F_{(4, 436)}$ = 13.81, P<0.0001). **(D)** Compared to WT controls, the average duration of sleep bouts was significantly decreased in *Hk* mutants during LP ($F_{(4, 436)}$ = 13.97, P<0.0001) but not DP ($F_{(4, 436)}$ = 3.95, P = 0.0037). **(E)** PDoze of *Hk* mutant flies was significantly reduced during LP ($F_{(4, 436)}$ = 25.90, P<0.0001) and DP ($F_{(4, 436)}$ = 9.58, P<0.0001). **(F)** Sleep depth decreased during both LP ($F_{(4, 436)}$ = 46.52, P<0.0001) and DP ($F_{(4, 436)}$ = 27.62, P<0.0001) in Hk mutant flies. **(G)** Latency (in min) to fall asleep during DP was increased upon *AOX* expression in a *Hk* mutant background ($F_{(4, 436)}$ = 4.69, P = 0.0010). **(A–G)** Genotypes were compared by one-way ANOVA with Holm-Šídák's multiple comparisons test. ns P>0.05, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Comparisons between genotypes are indicated by connecting lines, if no line is indicated, comparison was made with the direct WT control. Three independent experiments were conducted before data was pooled. Individual dots represent individual flies, bars represent mean ± SEM, n = 63–96.

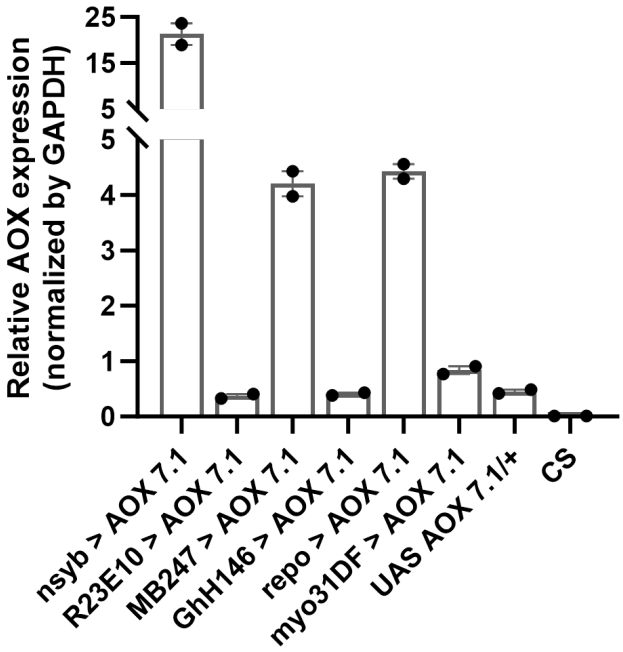

Figure 6.4: Relative *AOX* expression using different drivers. Relative *AOX* expression, normalized to housekeeper gene *GAPDH* expression, using the drivers *nsyb-Gal4*, *R23E10-Gal4*, *MB247-Gal4*, *GH146-Gal4*, and *myo31DF-Gal4*. For reference, flies with the undriven *UAS AOX 7.1* construct, and Canton-S (CS) WT flies were used. Data of two replicates were pooled, with n=4 flies per replicate.

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