Investigating the biderectional relationship between traumatic brain injury and sleep homeostatsis in *Drosophila melanogaster*

Rebecca Nicole Ray
Lake Forest College
Lake Forest, Illinois 60045

ABSTRACT

Traumatic brain injury (TBI) occurs when a sudden, severe impact to the head causes brain damage. TBI can impact health and homeostatic behaviors, like sleep. Studies have established *D. melanogaster* as a model to study TBI and sleep, so I decided to investigate the relationship between TBI and sleep using *D. melanogaster*. I found evidence of a time-dependent, disruptive effect of TBI on sleep and a protective effect of sleep against TBI outcomes, suggesting the relationship between TBI and sleep is bidirectional. I then examined what mechanism might underly this bidirectional relationship. Prior research posits that sleep helps clear reactive oxygen species (ROS) to protect against oxidative stress. To investigate whether ROS mediates the TBI-sleep relationship, I manipulated neuronal expression of the antioxidant *SOD2* and found it affected TBI outcomes and sleep. Future research is needed to clarify the role of ROS in the relationship between TBI and sleep.

DEDICATION

To my brother, Kellan, for reminding me to laugh and take each day as its own.

To my dad, Tim, for being my rock and encouraging me to chase even my craziest dreams.

To my mom, Leslie, for showing me how to love, and inspiring me to go out into the world and make it my own.

And, to my two grandparents, Sue Roby and John Opie, both of whom lived with diseases mentioned in this paper. You are the reason I fell I love with asking questions.

This is for you all, I love you.

ACKNOWLEDGEMENTS

I would like to thank Lake Forest College, the Neuroscience Program, and the Biology and Psychology Departments for supporting me in this research and for providing me the opportunity, resources, and knowledge to pursue my passion of science. I also thank my committee members, Paul Henne and Vivian Ta-Johnson, for their guidance and support throughout this journey. I would like to give special thanks to my committee chair and research mentor, Rebecca Delventhal, for her endless guidance and dedication. The experience and knowledge I have gained during my two years as her student have been invaluable, and it has been an honor to serve as a member of her lab. I would also like to thank my fellow lab members, Jorge A. Garcia, Daniel Tulchinskiy, Inés Riojas, and Salma Abdelkhalek for their support and companionship. Special thank you to Madeline Morland and Shannon Gibbons for their contributions to data collection and time committed to the success of this project. I would also like to give special thanks to Kamden T. Kuklinski for his wisdom and guidance throughout my time in the lab and to Maria Jose Orozco Fuentes and Otoha Tatami for the friendship, laughter, and love they bring to my life. You all have made this lab my home.

I would also like to thank Patrick Austin and Jill Wilkerson, my two coaches, for supporting me outside of my academic life and helping me achieve my goals of pursuing research while being a year-round collegiate athlete. You have both taught me the difficult life lessons of patience, hard work, and resilience. Finally, thank you to my parents, Leslie and Tim Ray. You have been there and supported me every step of the way, and you have never let me doubt myself. My love for you both goes beyond words, but know that I am proud to be your daughter.

Thank you to all the people, and flies, who helped make this possible.

LIST OF FIGURES

LIST OF SUPPLEMENTARY FIGURES

1. INTRODUCTION

1.1 Traumatic brain injury in humans

Traumatic brain injury (TBI) occurs when a mechanical force inflicts a sudden impact to the head which directly causes neuronal damage to brain tissue, resulting in neuropathology and dysfunction. Different classifications of TBI include closed head injury from acceleration and deceleration forces, penetration injury from a projectile, and blast injury from a blast wave. Closed head injury is the most prevalent type of TBI, and the

*This author wrote this paper as a senior thesis under the direction of Dr. Rebecca Delventhal.

force of impact can cause the brain to ricochet within the skull, which can lead to severe damage to neurons, glia, and blood vessels. The structural damage as a direct result of the impact is considered the primary injury. After the initial impact, damage to neuronal tissue can still occur hours or even days later through secondary injuries, which are caused by the cellular and molecular response to the damage brought about by the primary injury. Complications such as hemorrhage, inflammation, ischemia, hypoxia, cerebral edema, heightened intracranial pressure, and infection can occur from the primary injury and cause secondary injuries, such as oxidative damage and inadequate oxygenation (Mckee & Daneshvar, 2015).

1.1.1 Classification and severity of traumatic brain injury

Approximately 2.5 million people in the United States experience a TBI every year, and many injuries result in hospitalization (Friedan et al., 2015). While most people survive the direct consequences of the injury, between 3 and 5 million people in the U.S. are currently living with a disability that is a direct result of a TBI (Friedan et al., 2015; Zaloshnja et al., 2008), and within 5 years of rehabilitation, 1 in 5 patients will die (Corrigan et al., 2014).

Receiving a TBI often coincides with loss of consciousness, loss of memory before, after, or of the event itself, sensorimotor deficits, and states of confusion or trouble concentrating (Friedan et al., 2015). In humans, TBI is commonly quantified using the Glasgow Coma Scale (GCS), a 15-point scale measuring motor, verbal, and eye opening responses in patients potentially suffering from TBI (Sternbach, 2000). Some examples of responses we might expect a healthy individual to exhibit that may be affected after TBI include withdrawal from a painful stimulus, eye opening to a sound cue, and oriented, comprehensive speech (Sternbach, 2000). The GCS examines such responses and provides a score that can be used to diagnose injury severity, plan treatment, and signify a prognosis (Sternbach, 2000).

In general, there are considered to be three levels of TBI severity, each diagnosed using a GCS score range: mild (13-15), moderate (9-12), and severe (8 or less) (Sternbach, 2000). Importantly, all severities of TBI have been associated with long-term physical, cognitive, and behavioral complications (Mckee & Daneshvar, 2015). Mild TBI, including concussions and some blast injuries, can be caused by activities in sports, military duty, and other conditions, including epilepsy. Complete neurological recovery is often expected after mild TBI. With moderate TBI, lethargy is initially present and after a severe TBI, such as from a car crash, individuals are often in a coma, unable to respond to stimuli. The risk of secondary injury, damage brought about by the cellular response to the primary injury, is high for patients with severe TBI (Mckee & Daneshvar, 2015). In a study of over 500 TBI patients, 89% of patients with severe TBI suffered from hypotension (a drop in blood pressure) as opposed to 11% of patients with moderate TBI (Andriessen et al., 2011)approach, and treatment of traumatic brain injury (TBI. Eighty-five percent of patients with severe TBI also suffered from hypoxia (low blood oxygen levels) and had lower hemoglobin levels compared to moderate TBI patients, suggesting they were unable to transport oxygen through their blood as efficiently (Andriessen et al., 2011)approach, and treatment of traumatic brain injury (TBI. Patients with severe TBI also had higher blood glucose levels, which have been associated with worse neurological outcomes after injury (Andriessen et al., 2011; Zhao et al., 2011)approach, and treatment of traumatic brain injury (TBI. Such complications put patients with severe TBI at a much higher mortality risk. One study examining quality of life after TBI reported the mortality rate of severe TBI patients to be 35.7% compared to 17.2% and 4.1% of moderate and mild TBI patients, respectively. However, those who survive are almost always faced with long-term consequences as a result of such severe neurological damage.

1.1.2 Long-term consequences of traumatic brain injury

Apart from the damage brought about by secondary injuries, TBI can also lead to negative long-term health outcomes. Severe TBI has been linked with increased risk of dementia later in life, including increased risk of developing Parkinson's Disease and Alzheimer's Disease (Bower et al., 2003; Fleminger, 2003)MN, from 1976 through 1995. Each incident case was matched by age (+/-1 year. Individuals diagnosed with Parkinson's Disease were four times more likely to have a history of head trauma, and males who experienced a head injury were 50% more likely to develop Alzheimer's Disease (Bower et al., 2003; Fleminger, 2003)MN, from 1976 through 1995. Each incident case was matched by age (1 year. An earlier age of onset for Alzheimer's Disease has also been associated with TBI (Nemetz et al., 1999). Additionally, TBI has been linked with memory and cognitive deficits and an increase in depression diagnosis, with the risk of clinical depression being up to three times greater (Guskiewicz et al., 2005, 2007).

TBI can also lead to changes in homeostatic mechanisms and behaviors. Homeostasis encompasses the processes designed to optimize function by maintaining stability and adapting to internal and external stimuli. An example of one such mechanism is metabolism, the breakdown of glucose into expendable energy. When blood glucose levels are high, a signal is released to bring glucose into cells to be metabolized. Once blood glucose levels become low again, another signal is sent to release stored glucose back into the blood. TBI can disrupt this homeostatic balance by causing hypermetabolism and catabolism, leading to increased energy expenditure and breakdown of important molecules such as fats and proteins (Lee & Oh, 2022). Such changes in TBI patients' metabolic homeostasis can lead to malnourishment, and difficulties obtaining adequate nourishment can have a negative impact on recovery and mortality (Lee & Oh, 2022). In addition to metabolism, another homeostatic behavior that is disrupted by TBI is sleep.

1.1.3 Traumatic brain injury causes long-term sleep disruption

TBI is known to cause sleep disruption, which can further prevent proper function and rate of recovery after injury (Kalmbach et al., 2018). TBI can cause a range of sleep disruptions, including insomnia, excessive daytime sleepiness, and hypersomnia (Sandsmark et al., 2017). A meta-analysis of 21 studies reporting on sleep disturbances after TBI found that 50% of TBI patients experience some form of sleep disruption after injury, regardless of injury severity or type (Mathias & Alvaro, 2012)which may compromise recovery and quality of life. Prevalence rates vary widely, reflecting differences in the criteria and measures that are used to assess sleep, as well as sample differences. This meta-analysis examined the prevalence of general and specific, and formally and informally diagnosed, sleep disturbances following TBI in order to establish the nature and extent of these sequelae and their potential impact on recovery.\nMETHODS: Data from 21 studies, which assessed (1. In TBI patients that were 3 to 24 months post injury, excessive daytime sleepiness due to nightly sleep disruptions was reported as the most common sleep-related symptom (Verma et al., 2007)who presented with sleep-related complaints 3 months to 2 years following TBI, were studied. None had sleep complaints prior to the TBI. Orophrayngeal, chin, and TMJ examinations were considered benign. The severity of injury was assessed by the Global Assessment of Functioning (GAF. Insomnia was the second most common symptom, where half of patients with insomnia suffered from sleep onset insomnia (problems falling asleep) and half from sleep maintenance insomnia (having difficulty staying asleep) (Verma et al., 2007)who presented with sleep-related complaints 3 months to 2 years following TBI, were studied. None had sleep complaints prior to the TBI. Orophrayngeal, chin, and TMJ examinations were considered benign. The severity of injury was assessed by the Global Assessment of Functioning (GAF. A separate study also found that within 6 months after TBI, rates of sleep onset insomnia were higher in patients with worse motor, vocal, and ocular function (Kalmbach et al., 2018). Similarly, rates of short sleep duration were higher in patients with impairment of such functions at 1 and 3 months after injury (Kalmbach et al., 2018). Other studies have shown that TBI can cause an increased need for sleep. Meta-analysis data reports that 28% of TBI patients are diagnosed with hypersomnia after injury (Mathias & Alvaro, 2012)which may compromise recovery and quality of life. Prevalence rates vary widely, reflecting differences in the criteria and measures that are used to assess sleep, as well as sample differences. This meta-analysis examined the prevalence of general and specific, and formally and informally diagnosed, sleep disturbances following TBI in order to establish the nature and extent of these sequelae and their potential impact on recovery.\nMETHODS: Data from 21 studies, which assessed (1. Another study found that within six months after TBI, patients slept significantly more within 24 hours and had shorter sleep onset latency (Imbach et al., 2015). Over half the

patients also experienced excessive daytime sleepiness, reported by a sleep latency less than 8 minutes (Imbach et al., 2015). Considering the prevalence of sleep disruptions after TBI, it is clear that homeostatic behaviors such as sleep are highly affected after injury. Studying how sleep may play a role in TBI recovery is important for improving injury outcomes.

1.2 Negative impacts of sleep disruption

Sleep is a natural, reversible state of unconsciousness that is characterized by immobilization and reduced responsiveness to stimuli (Rasch & Born, 2013)(Rasch & Born, 2013). Sleep is regulated by our circadian rhythm as well as homeostatic pressures. While the circadian clock is understood to control the timing of sleep, sleep homeostasis is considered to control the duration and consolidation of sleep (Dijk et al., 1999). Sleep homeostasis also regulates the response to sleep need or sleep pressure, which is built up during long periods of wakefulness and is relieved during sleep (Dijk et al., 1999).

Sleep is a universal behavior found in nearly all organisms ranging from vertebrates like mammals, birds, and reptiles, to invertebrates like flies and worms (Cirelli & Tononi, 2008). Sleeping behavior leaves organisms vulnerable to predatory attacks and is not part of reproductive behavior necessary for species survival. Yet it continues to be conserved in nearly all organisms, so there must be an important evolutionary benefit to sleep.

1.2.1 Sleep is required for proper neurological function and health maintenance

Sleep in humans is frequently associated with learning and memory benefits, as sleep immediately after learning has been shown to improve memory while sleep deprivation impairs the ability to remember information (Rasch & Born, 2013). Slow-wave sleep in particular, characterized as stages 3 and 4 of non-REM sleep, has shown protective qualities towards memory (Alger et al., 2012)questions still remain as to the type and length of sleep necessary to best benefit declarative memories. A better understanding could lend support in one direction or another as to the much-debated role of sleep, be it passive, permissive, or active, in memory processing. The present study employed a napping paradigm and compared performance on a bimodal paired-associates task of those who obtained a 10-min nap, containing only Stages 1 and 2 sleep, to those whose nap contained slow-wave sleep (SWS. Participants allowed to enter slow-wave sleep when napping correctly recalled more words during a short-term test compared to those kept awake, and in the long-term they correctly recalled twice as many words than even participants who napped but were prevented from entering slow-wave sleep (Alger et al., 2012)questions still remain as to the type and length of sleep necessary to best benefit declarative memories. A better understanding could lend support in one direction or another as to the much-debated role of sleep, be it passive, permissive, or active, in memory processing. The present study employed a napping paradigm and compared performance on a bimodal paired-associates task of those who obtained a 10-min nap, containing only Stages 1 and 2 sleep, to those whose nap contained slow-wave sleep (SWS. In another study, when asked to learn and later recall a list of words, sleep-deprived participants forgot over 15% more words than those allowed to sleep normally (Gais et al., 2006).

We need sleep for more than just learning and memory, however. Severe sleep deprivation in any organism for extended periods of time almost certainly leads to death (Cirelli & Tononi, 2008). Rats prevented from sleeping due to persistent physical stimulation died after 2-4 weeks of no sleep (Rechtschaffen & Bergmann, 1995)but it usually requires strong stimulation which can obscure the interpretation of effects. The disk-over-water method permits chronic sleep deprivation of rats with gentle physical stimulation that can be equally applied to yoked control rats. A series of studies with this method has revealed little or no pathology in the control rats. The deprived rats show a reliable syndrome that includes temperature changes (which vary with the sleep stages that are lost. Hypothermia due to declining body temperature, catabolic tissue breakdown, and organ failure as a result of infection are all possible contributors to the certain death such severe sleep deprivation brings about (Rechtschaffen & Bergmann, 1995)but it usually requires strong stimulation which can obscure the interpretation of effects. The disk-over-water method permits chronic sleep deprivation of rats with gentle physical stimulation that can be equally applied to yoked control rats. A series of studies with this method has revealed little or no pathology in the control rats. The deprived rats show a reliable syndrome that includes temperature changes (which vary with the sleep stages that are lost. Such fatal consequences are also seen in humans, particularly those with fatal familial insomnia (FFI). FFI is a rare genetic neurodegenerative disease where individuals eventually die after sleep disruptions worsen such that no sleep is acquired at all (Montagna, 2002). Cardiac complications, hypometabolism, severe loss of slow-wave sleep, and death of up to 90% of the neurons in certain thalamic regions of the brain are all characteristics of FFI (Montagna, 2002). Similar to severe sleep deprivation in rats, FFI is fatal and individuals with FFI typically die within 72 months of disease onset (Montagna, 2002).

Outside of the rare instances where sleep loss is fatal, chronic lack of sleep can still cause numerous adverse health outcomes, including dysregulation of body temperature and body weight and increased risk of infections (Garbarino et al., 2021). A meta-analysis of over 600,000 adults from across the world reported a significant association between sleep deprivation and obesity, with a similar association found even amongst children (Cappuccio et al., 2008)and to obtain an estimate of the risk.\n\nMethods:\nWe performed a systematic search of publications using MEDLINE (1996-2007 wk 40. Another study investigating sleep-related infection risk showed that when exposed to a common cold virus, over twice as many participants who slept less than 5 hours per night became sick compared to participants who slept more than 7 hours (Prather et al., 2015). Interestingly, some evidence also suggests that sleep deprivation can even hinder our ability to develop immunity against certain pathogens (Fernandes et al., 2020).

Sleep loss is also linked with the development of chronic disease. For example, individuals with sleep apnea are at a higher risk of developing cardiovascular disease (Kasasbeh et al., 2006). Repetitive surges in heart rate and blood pressure from the cycles of hyper- and hypoventilation characteristic of the disorder can often lead to hypertension (Kasasbeh et al., 2006). One study examining sleep and risk of Type-2 diabetes reported that sleeping less than 6 hours per night predicted worse glycemic control, an indicator of heightened diabetes risk (Knutson et al., 2006)Chicago, Ill. The final analysis included 161 participants. Glycemic control was assessed by hemoglobin A1c (HbA1c. Furthermore, poor sleep is even associated with higher depression risk, and over 80% of individuals with major depressive disorder reportedly experience some form of sleep disturbance (Zimmerman et al., 2006).

Perhaps the most chronic disease of all is aging. Sleep consolidation, characterized by uninterrupted sleep without prolonged nightly awakenings, is generally high in adolescents and young adults, but sleep becomes much more fragmented in older adults (Dijk et al., 1999). For example, one study examining age-related reductions in homeostatic sleep drive found young adults, aged 20-30, slept about 7.5 hours every night on average with an average of 20 awakenings (Dijk et al., 2010)n = 44. Older adults, aged 66 and above, only slept an average of about 6.5 hours with about 28 awakenings, indicating significantly more fragmented sleep in older adults (Dijk et al., 2010)n = 44. The changes in sleep consolidation we see in older adults could be the result of a reduced homeostatic sleep drive as older adults have a reduced propensity, or sense of readiness, to fall asleep at night and spend less time in slow-wave sleep (Dijk et al., 2010)n = 44.

The age-induced gradual shift away from consolidated sleep and towards more fragmented sleep could be a contributing factor to the metabolic dysregulation, high infection risk, and development of neurodegenerative diseases that so frequently come with aging (Dijk et al., 1999). In addition to causing a higher risk for infection and obesity, prolonged sleep loss can reduce an individual's resting metabolic rate (Buxton et al., 2012)and to obtain an estimate of the risk.\n\nMethods:\nWe performed a systematic search of publications using MEDLINE (1996-2007 wk 40. Individuals with sleep complications are also at a higher risk for developing Parkinson's Disease, and increases in amyloid-beta plaques, a defining characteristic of Alzheimer's Disease, were found after only a single night of sleep deprivation (Hsiao et al., 2017; Shokri-Kojori et al., 2018).

Clearly, sleep loss has many negative consequences not only on cognitive elements like learning and memory but also on our own health and survival. The natural decline in sleep duration and consolidation we see with age is just one of the many ways sleep problems can come about. Sleep problems are a common occurrence today, and sleep disorders put millions of people at high risk of suffering from the negative health outcomes seen as a result of chronic sleep loss (CDC, 2020; Stranges et al., 2012).

Considering the vast array of negative consequences associated with acute and chronic sleep loss, it is vital that we receive an adequate amount of sleep each night. Human adults are recommended to get 7 to 9 hours of sleep every night (Watson et al., 2015). However, many adults do not routinely obtain enough sleep, and the prevalence of sleep disorders and other sleep disruptions today has brought about an epidemic of sleep loss (Chattu et al., 2018).

1.2.2 Common sleep disorders and prevalence of sleep disruption

There are various types of sleep disorders that occur at different frequencies and present different patterns of sleep disruption. Insomnia is the most common, affecting about 30% of adults worldwide, and is subdivided into two types: sleep onset (the inability to initiate sleep) and sleep maintenance (the inability maintain sleep) (Bhaskar et al., 2016; Stranges et al., 2012; Thorpy, 2012). Sleep apnea affects around 15% of individuals and occurs when breathing passages become constricted or closed during sleep, causing excessive snoring or gasping for air, disrupted sleep, and sleepiness during the day (Thorpy, 2012; Young et al., 2009). Hypersomnia is characterized by excessive daytime sleepiness and longer periods of nocturnal sleep (Dauvilliers & Buguet, 2005; Thorpy, 2012). Narcolepsy also includes excessive sleepiness during the day and often coincides with bouts of sudden loss of muscle tension (Thorpy, 2012). Restless legs syndrome causes sensations of "creeping" in the legs that causes an unpleasant feeling, along with aches and pains that can make falling asleep difficult. Prevalence of hypersomnia, narcolepsy, and restless legs syndrome are estimated to be below 10% of the general population (Dauvilliers & Buguet, 2005; Ohayon et al., 2012; Partinen et al., 2012).

Perhaps not diagnosable, but another common, more recent form of sleep disruption occurs as a result of excessive use of electronic screens during the day and before sleeping, particularly in adolescents. A 2011 poll by the National Sleep Foundation found that 97% of young adults had one or more electronic device available for use in their bedroom (Gradisar et al., 2013). One study investigating how electronic use before bedtime affected sleep reported nearly all participants used some type of electronic device (or more than one) within 1 hour of their bedtime (Hysing et al., 2015)and sleep deficiency rising in adolescents constitutes a major public health concern. The aim of the present study was to investigate daytime screen use and use of electronic devices before bedtime in relation to sleep. Design: A large cross-sectional population-based survey study from 2012, the youth@hordaland study, in Hordaland County in Norway. Setting: Cross-sectional general community-based study. Participants: 9846 adolescents from three age cohorts aged 16–19. The main independent variables were type and frequency of electronic devices at bedtime and hours of screen-time during leisure time. Outcomes: Sleep variables calculated based on selfreport including bedtime, rise time, time in bed, sleep duration, sleep onset latency and wake after sleep onset.\nResults: Adolescents spent a large amount of time during the day and at bedtime using electronic devices. Daytime and bedtime use of electronic devices were both related to sleep measures, with an increased risk of short sleep duration, long sleep onset latency and increased sleep deficiency. A dose–response relationship emerged between sleep duration and use of electronic devices, exemplified by the association between PC use and risk of less than 5 h of sleep (OR=2.70, 95% CI 2.14 to 3.39. Excessive electronic use, characterized as over the recommended 2 hours per day, both during the day and in the hour before bedtime was positively correlated with higher sleep deficiency and longer sleep onset latency (American Academy of Pediatrics, 2001; Hysing et al., 2015)such as violent or aggressive behavior, substance use, sexual activity, obesity, poor body image, and decreased school performance. In addition to the television ratings system and the v-chip (electronic device to block programming. While an increase in sleep deficiency and sleep onset latency could likely be a result of sleep simply being replaced with time spent looking at a screen, there are other mechanisms that could explain why excessive electronic use can cause sleep disruptions. Exposure to bright light at night can cause the circadian rhythm to reset and delay the onset of sleep (Khalsa et al., 2003). Even more interesting is the possibility of persistent psychophysiologic (learned) insomnia, which can cause individuals to associate their bed or bedroom with electronic use, resulting in heightened arousal instead of relaxation once lying in bed (Hauri & Fisher, 1986; Hysing et al., 2015)somatized tension and negative conditioning. Twenty-two patients diagnosed as PPI were compared on sleep and psychological questionnaires to 22 normal subjects (Ns. Despite the differing mechanistic possibilities, it's clear that excessive electronic use heightens the risk of sleep disruption. Due to the vast prevalence of electronics today, such disruptive effects pose a greater risk of spreading to far more people worldwide than diagnosable sleep disorders are capable of (Gradisar et al., 2013).

With the prevalence of chronic sleep disorders and sleep problems such as those brought about by bedtime electronic use, sleep loss has become a frequent and widespread occurrence. An assessment by the CDC's Behavioral Risk Factor Surveillance System found that 35-37% of adults in the United States have short sleep duration, defined by whether the recommended number of sleep hours is met, which for adults is a minimum of 7 (CDC, 2020). A study by the World Health Organization also found sleep problems in adults aged 50 and over from 7 countries across Africa and Asia (Stranges et al., 2012). It's estimated that by 2030, over 260 million people will suffer from sleep difficulties (Stranges et al., 2012).

It is clear that sleep disorders and general sleep disruptions are prevalent today. Considering the connection of sleep to TBI and injury-related impairment, understanding how sleep problems may affect recovery after TBI is important for developing preventative strategies and treatments to improve rehabilitation for TBI patients.

1.1.3 Sleep disruptions affect injury recovery and outcomes

TBI increases the risk of sleep problems after injury (see section 1.1). Given that sleep disruption is known to cause an array of negative health consequences, however, it is possible for sleep problems to have an adverse effect on TBI outcomes. One study examined the prevalence of impaired function in TBI patients with prior sleep problems (Kalmbach et al., 2018). Patients with TBI-induced disabilities ranging from severe, characterized by being conscious but unable to function independently, to upper-moderate, those struggling with work, leisure, and social activities but capable of being independent, were considered functionally impaired (Kalmbach et al., 2018; Wilson et al., 2021). Difficulty with both falling asleep and staying asleep prior to injury was associated with an increased risk of functional impairment (Kalmbach et al., 2018). Specifically, patients who had insomnia prior to injury were eight times more likely to have worse functional impairment after TBI than those without sleep problems before injury (Kalmbach et al., 2018). Another study looking at sleep disruptions as a predictor of TBI outcomes also found poor sleep to be associated with worse outcomes and longer rehabilitation (Sandsmark et al., 2017). TBI patients who did not suffer from sleep difficulties in the first 2 weeks post-injury were nearly 6 times more likely to have better functional outcomes after injury (Sandsmark et al., 2017).

Altogether, there is significant evidence to suggest sleep disruption and sleep loss in humans can be detrimental to TBI recovery. TBI is also capable of causing more sleep difficulties, further perpetuating the negative effects of sleep problems on TBI outcomes. Considering this bidirectional relationship, understanding the physiological purpose of sleep may offer insight into why sleep loss can affect TBI outcomes and how TBI may interfere with sleep regulation.

1.2.4 A physiological need for sleep

Considering the universality of sleep behavior across the animal kingdom and the severe, sometimes fatal, consequences of sleep loss, there must be some physiological need for sleep beyond higher cognition. There have been many theories as to why such a need exists. One theory proposes sleep is required for energy conservation where, sim-

ilar to hibernation, sleep offers a period of time where activity and energy expenditure are suppressed (Siegel, 2005). Another theory suggests sleep is needed for synaptic homeostasis, specifically the pruning of synapses formed during wakefulness to maximize the brain's function and performance (Brown & Naidoo, 2010). Sleep has also been thought to regulate protein synthesis in the brain, as inhibition of neuronal protein synthesis has been found to increase sleep (Methippara et al., 2009).

Another theory of a physiological need for sleep is the Free Radical Flux Theory, which suggests sleep is required for alleviating oxidative stress that builds up during wakefulness and causes cell damage (Reimund, 1994). The theory proposes that sleep is needed to maintain neural tissue stability, which is threatened by oxidative stress and the energy-demanding nature of the brain (Reimund, 1994). The brain is a very metabolically active organ, so it produces large amounts of reactive oxygen species (ROS), which are small molecular byproducts of metabolism that can cause cellular damage by reacting with DNA, proteins, and lipids (Phaniendra et al., 2015). The Free Radical Flux Theory proposes that sleep duration is directly correlated with the burden of ROS accumulation based on the metabolic rate of a certain species, where a higher metabolic rate increases ROS accumulation (Reimund, 1994). The theory suggests that endogenous antioxidant mechanisms in the brain that are meant to clear ROS cannot keep up with the amount of ROS generated throughout the day alone, so sleep is needed to reduce ROS levels before oxidative stress and cellular damage can occur (Reimund, 1994). Without sleep, it's more likely that ROS, given its reactive nature, will begin to accumulate and cause cellular damage.

1.3 Reactive oxygen species

Reactive oxygen species (ROS) are free radicals that can occur naturally within an organism or be the result of environmental exposure. A free radical is an atom or molecule whose outermost valence shell has one or more unpaired electrons and is, therefore, very unstable and reactive

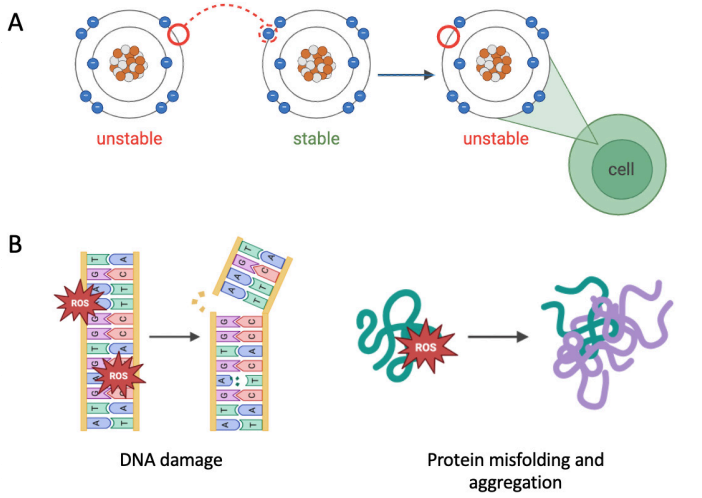

Figure 1. Molecular structure of ROS and cellular damage. **A.** The chemical process behind ROS within cells. An unstable free radical with an unpaired electron steals an electron from a stable molecule. The stable molecule then loses that electron and becomes a radical itself. **B.** ROS-induced DNA damage and protein misfolding/aggregation. ROS can cause single- and double-stranded breaks in the phospholipid backbone of DNA and oxidize nucleotides. ROS can also cause proteins to misfold, resulting in protein aggregation and loss of proper protein function (Juan et al., 2021).

The superoxide radical, one of the most common types of ROS, is formed in mitochondria when an extra electron is added to a dioxygen molecule (O_2) (Figure 2) (Andrés et al., 2023; Phaniendra et al., 2015)both the reactive oxygen species (ROS. Antioxidants like superoxide dismutase (SOD) 2 help neutralize and transform superoxide into hydrogen peroxide through a dismutation reaction. In this reaction, SOD catalyzes the reaction between two superoxide radicals and hydrogen ions to produce hydrogen peroxide and water, effectively neutralizing the superoxide radical. (Pha-

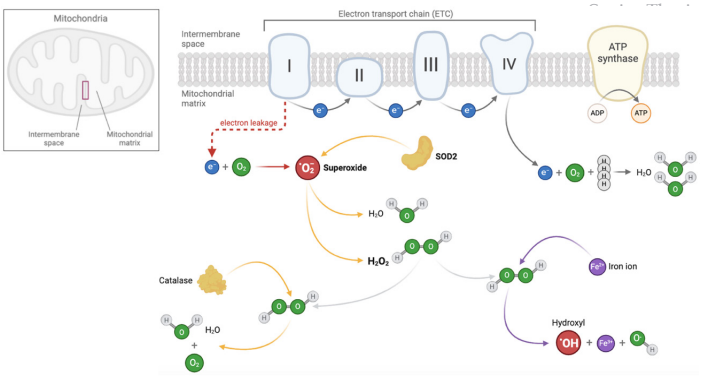

Figure 2. The production and neutralization of superoxide in mitochondria. Normal function of the electron transport chain with in the mitochondrial matrix and intermembrane space transfers electrons to help generate adenosine triphosphate (ATP), which the cell uses for energy. Most electrons react with oxygen and hydrogen molecules to produce water (H_2O) at the end of the ETC. However, an estimated 0.2-2% of electrons leak from the ETC, most prominently from complexes I and III, and react with oxygen molecules to form superoxide radicals (Zhao et al., 2019). The antioxidant superoxide dismutase 2 (SOD2) catalyzes the reaction to neutralize superoxide radicals and produce hydrogen peroxide (H_2O_2) and water (Wang et al., 2018). The hydrogen peroxide nonradical can react with iron ions (Fe^{2+}) to produce hydroxyl radicals ($OH^{\cdot}$) (Phaniendra et al., 2015). To prevent hydrogen peroxide from forming hydroxyl radicals, the antioxidant catalase helps degrade hydrogen peroxide into water and oxygen (Phaniendra et al., 2015).

1.3.1 ROS-induced cellular damage

ROS are capable of inflicting damage on many vital cellular components. Radicals can alter the molecular structure of DNA by causing single and double strand breaks in the phosphate backbone and oxidizing nucleotides (Juan et al., 2021; Sharma et al., 2016)the generation of DSBs at low levels of ROS is still controversial. In the present study, we show that H2O2 at biologically-relevant levels causes a marked increase in oxidative clustered DNA lesions (OCDLs. They can also lead to lipid peroxidation, which inhibits normal membrane function and permeability (Wong-ekkabut et al., 2007). Additionally, proteins can be denatured by radicals, altering their structure and function (Butterfield et al., 1998). Exposure of rodents to acute normobaric hyperoxia for up to 24 h results in oxidative modifications in cytosolic proteins and loss of activity for the oxidation sensitve enzymes glutamine synthetase and creatine kinase. Cytoskeletal protein spin labeling also reveals synaptosomal membrane protein oxidation following hyperoxia. These changes are similar to the changes seen in senescent brains, compared to young adult controls. The antioxidant spin trapping compound N-tertbutyl-α-phenylnitrone (PBN.

Most endogenous ROS are formed in the mitochondria since that is where the electron transport chain (ETC) takes place (Figure 2) (Phaniendra et al., 2015). The ETC is vital for creating the electrochemical gradient necessary to produce energy in the form of adenosine triphosphate (ATP) for the cell to use. Electron transfer is an important part of this process. However, electrons can leak from the complexes within the chain and react with other molecules, most notably oxygen, to produce ROS. Because of its proximity to the ETC and ROS production, mitochondrial DNA is much more vulnerable to oxidative damage than nuclear DNA (Phaniendra et al., 2015). Damage to mitochondrial DNA holds severe consequences for the survival of a cell as the genes encoded by mitochondrial DNA are vital for proper organelle function and metabolic processes (Habbane et al., 2021).

In addition to the mitochondria, ROS can be found in the Endoplasmic Reticulum (ER). A main function of the ER is to fold proteins synthesized in the cell and secrete them in order for proteins to carry out their specific functions. ROS can be generated in the ER during the process of folding proteins using a mechanism called oxidative protein folding (Malhotra et al., 2008; Tu & Weissman, 2004). However, like with many locations within the cell where ROS is generated, the ER is susceptible to oxidative stress if too much ROS is accumulated. ROS and oxidative stress have been associat-

ed in the misfolding and inefficient secretion of proteins from the ER (Malhotra et al., 2008). When proteins processed by the ER are misfolded and are unable to be secreted, they aggregate within the ER and cause ER stress (Brown & Naidoo, 2010). Cells under ER stress have shown higher levels of oxidative stress markers than cells with normal ER function (Malhotra et al., 2008). Additionally, reducing ROS levels using antioxidant treatment lessens ER stress by increasing protein secretion (Malhotra et al., 2008). Such evidence suggests that ER stress and oxidative stress go hand in hand.

1.3.2 Nonpathological roles of ROS

While there is overwhelming evidence that accumulation of ROS is detrimental, ROS at low levels does have a nonpathological purpose. Cells require a certain concentration of electrons, or redox state, in order to properly function (Valko et al., 2007)e.g. nitric oxide, NO(*. Similar to how pH is regulated in a cellular environment, cells tightly regulate their redox state, which is determined by the rate at which ROS are produced versus neutralized by antioxidants (Valko et al., 2007)e.g. nitric oxide, NO(*. When kept at low levels and within the range of the required redox state, ROS function as important signaling molecules (Valko et al., 2007)e.g. nitric oxide, NO(*. Regulated increases in ROS disrupt the balance of the redox state, and this imbalance in redox state acts as a signal to manage various important cellular functions (Valko et al., 2007) e.g. nitric oxide, NO(*. These include gene expression, pathogenic defense, cardiovascular growth and development, cell adhesion, and even sensation of blood-oxygen concentration (Galter et al., 1994; Griendling et al., 2000; Keisari et al., 1983; Valko et al., 2007)but inhibit their DNA binding activity in vitro. We now show that both the activation of NF kappa B and the inhibition of its DNA binding activity is modulated in intact cells by the physiological oxidant glutathione disulphide (GSSG.

The innate immune response is also closely tied with redox signaling. Important components of the immune system, such as macrophages, are activated by ROS, and low ROS production during phagocytosis has been shown to cause hypersensitivity to infections (Morris et al., 2022; Pollock et al., 1995). ROS-induced damage also provides signals required for apoptosis (Valko et al., 2007)e.g. nitric oxide, NO(*. Apoptosis, or programmed cell death, is important for elimination of damaged cells. However, internally regulated apoptosis is also necessary for proper development and survival of cells, and changes in ROS levels help regulate this programmed death (Hengartner, 2000). Despite its nonpathological roles, ROS is mostly viewed as a pathological molecule due to its induction of oxidative stress at high levels. To avoid oxidative stress, cellular systems designed to neutralize ROS and prevent ROS accumulation are vital to cell health and survival.

1.3.3 Antioxidants

Antioxidants are molecules that help neutralize free radicals before cellular damage can be inflicted. Antioxidants give radicals a source of electrons to prevent them from taking electrons from more important molecules in the cell, such as the DNA phosphate backbone. We can acquire antioxidants exogenously through our diet and endogenously through sources within our bodies. Vitamin E, vitamin C, and beta-carotene are all examples of exogenous antioxidants that we obtain by consuming fresh fruits and vegetables, oils, and certain fatty foods (Pham-Huy et al., 2008). After we consume exogenous antioxidants, we absorb them through our gastrointestinal tract where they are transported to our cells (Halliwell, Zhao, et al., 2000; Okagu & Udenigwe, 2022)and contain a complex mixture of antioxidants (including ascorbate, carotenoids, vitamin E and other phenolics such as the flavonoids.

However, we get most of the antioxidants our bodies require endogenously (Pham-Huy et al., 2008). Endogenous antioxidants can come in the form of non-enzymes produced by our endocrine glands, like melatonin and uric acid, or as enzymes that are encoded in our DNA, like superoxide dismutase and catalase (Pham-Huy et al., 2008). There are three different types of superoxide dismutase (SOD) in particular, each encoded by a different gene (Flynn & Melov, 2013). Each type can be found in different parts of the cell: SOD1 is found mostly in the cytoplasm, SOD2 in the mitochondria, and SOD3 in the extra-cellular matrix (Flynn & Melov, 2013). The presence of SOD2 within the mitochondria puts it in close proximity to where the mitochondrial ETC produces free radicals (Flynn & Melov, 2013; Phaniendra et al., 2015). Because of this, SOD2 plays a very important role in neutralizing superoxide anions into their non-radical hydrogen peroxide counterparts before any damage can be done to important mitochondrial components and the surrounding the cell.

Elimination or suppression of SOD2 is known to cause damage to many cellular components and tissues (Flynn & Melov, 2013). With the loss of SOD2, ROS production can go unchecked, leading to oxidative stress and further health problems. In humans, decreased *SOD2* expression has been found to be correlated with cardiovascular complications in patients with sickle cell disease (Dosunmu-Ogunbi et al., 2022). Also, mutant mice that had a 48-55% decrease in SOD2 activity developed dilated cardiomyopathy, a condition that is characterized by enlarged heart cavities and thinner wall thickness and can be fatal (Li et al., 1995). Loss of SOD2 can also lead to metabolic dysfunction. Mice with deficient SOD2 activity showed an increase in susceptibility to neurotoxins, which can elicit mitochondrial dysfunction, leading to the generation of more ROS (Andreassen et al., 2001). With more ROS and less SOD2 readily available to neutralize radicals, chances of oxidative stress increase (Andreassen et al., 2001).

Antioxidants and their role in preventing oxidative stress may also have an effect on neurodegenerative disease. For example, the progression of Alzheimer's Disease can be worsened by oxidative stress (Flynn & Melov, 2013; Leuner et al., 2012). Parts of the ETC, specifically complex I, functionally deteriorate with age, and complex I-derived ROS can trigger formation of amyloid-beta plaques, key pathological features in Alzheimer's Disease. One study found an increase in amyloid-beta plaques *in vitro* within just 2 hours of complex I dysfunction and ROS release (Leuner et al., 2012). Additionally, another study found that post-mortem samples of Alzheimer's Disease patients had increased oxidative stress markers (Sultana et al., 2006). This evidence could imply that oxidative stress is a factor in inducing Alzheimer's Disease, but it could also suggest that Alzheimer's pathology itself leads to an increase in oxidative stress. Similarly, SOD2 is upregulated early on in the progression of Alzheimer's Disease, but it is unknown if SOD2 is directly related to Alzheimer's Disease progression or if it is upregulated in response to an increase in oxidative stress (Flynn & Melov, 2013). Still, antioxidants may play a role in preventing Alzheimer's Disease progression. One study found amyloid-beta plaque formation was significantly suppressed after treating cells with deficient complex I using the antioxidant vitamin C (Leuner et al., 2012).

While antioxidants are found throughout our bodies, the Free Radical Flux Theory posits that there may not be sufficient amounts to clear all the ROS that are produced during periods of high activity, like wakefulness, that then accumulate (Reimund, 1994). Depleted levels of antioxidants have been reported during periods of extended wakefulness, along with increases in oxidative stress (Trivedi et al., 2017). Considering this evidence, an additional process to clear ROS, characterized by periods of little activity, may be necessary to prevent oxidative stress. That process could be sleep (Reimund, 1994).

1.4 Reactive oxygen species and sleep

If sleep is important for clearing ROS, then it could be that with sleep loss comes the inability to protect against oxidative stress. A study examining sleep and oxidative stress found that women under mild sleep restriction for 6 weeks had a 78% increase in oxidative stress when measuring oxidative stress markers in endothelial cells (Shah et al., 2023)which is more pronounced in female than male persons. We reported recently first causal evidence that mild, prolonged sleep restriction mimicking \"real-life\" conditions impairs endothelial function, a key step in the development and progression of cardiovascular disease, in healthy female persons. However, the underlying mechanisms are unclear. In model organisms, sleep restriction increases oxidative stress and upregulates antioxidant response via induction of the antioxidant regulator nuclear factor (erythroid-derived 2. This protective effect of sleep against oxidative stress was also found in flies. Short-sleeping fruit flies exhibited an increase in sensitivity to oxidative stress such that when exposed to H_2O_2 and paraquat, a chemical known to cause oxidative stress, the flies had significantly shorter survival (Hill et al., 2018). Additionally, increasing sleep

in flies through genetic and pharmacological methods caused a greater resistance to oxidative stress, resulting in higher survival rates when exposed to H_2O_2 and paraquat (Hill et al., 2018). Also, ROS accumulated only in the gut of the fly after sleep deprivation, suggesting the oxidative stress response may be tissue specific (Vaccaro et al., 2020)2020. Increases in oxidative stress and lipid peroxidation, a consequence of oxidative stress, were also found in rats after sleep deprivation (Silva et al., 2004). Interestingly, sleep deprived rats who had increased oxidative stress also displayed deficits in memory, as shown by reduced avoidance of an environment with a learned association to an aversive shock stimulus (Silva et al., 2004). Such evidence suggests oxidative stress as a possible mechanism for how sleep deprivation impairs cognitive processes like memory.

With the connection between sleep and oxidative stress, it is no surprise that sleep is also related to the expression of oxidative stress response genes. Short-sleeping flies showed an increase in expression of antioxidants and mitochondrial stress genes (Hill et al., 2018). The same findings were also seen in mice under sleep deprivation for 72 hours (Lungato et al., 2013). However, another study found antioxidant levels were decreased in rats after 5 days of sleep deprivation (Everson et al., 2005). This inconsistency illustrates the fact that expression of oxidative stress response genes may be time dependent, as opposing effects on expression can be seen after differing lengths of sleep deprivation.

In addition to oxidative stress genes, sleep can also affect expression of ER stress genes, which respond to accumulation of misfolded proteins within the ER. During periods of sleep loss, ER stress genes, such as binding immunoglobulin protein (BiP), are upregulated (Naidoo et al., 2007). Additionally, transgenic flies that overexpressed BiP had increased rates of recovery sleep following sleep deprivation, suggesting expression of ER stress genes may play a role in regulating sleep (Naidoo et al., 2007). However, considering the role of ROS in generating ER stress, ROS may be the underlying mechanism behind this association between sleep and expression of ER stress genes (Brown & Naidoo, 2010; Malhotra et al., 2008)how protein misfolding and oxidative stress impact each other has not been explored. We have analyzed expression of coagulation factor VIII (FVIII. It is possible that during sleep deprivation, ER stress is activated by ROS that has not been cleared due to sleep loss, and the same ROS accumulation that activates ER stress and causes ER stress gene overexpression could increase recovery sleep.

1.4.1 Reactive oxygen species as a regulator of sleep homeostasis

Considering sleep is protective against oxidative stress, ROS can act as a regulator of sleep such that as ROS levels fluctuate, so does the need for sleep. In flies with neuronal overexpression of antioxidant genes SOD1 and SOD2, sleep duration was significantly shorter, suggesting a lesser need to clear ROS (Hill et al., 2018). Another study found rats injected with low levels of *tert*-butyl hydroperoxide (TBHP), a reagent known to promote ROS production, had significantly increased sleep (Ikeda et al., 2005). Interestingly, the levels of TBHP used were enough to induce sleep without causing oxidative damage (Ikeda et al., 2005). This finding supports the concept that ROS, as a molecule found naturally within organisms, can be nonpathological at low levels and may play an important role in signaling when and how much sleep is needed. Considering ROS as a molecule to regulate sleep could offer a mechanistic explanation for how TBI and sleep impact each other. If ROS acts as a regulator of sleep and a consequence of sleep loss, and sleep can impact TBI recovery, it is possible that ROS is directly correlated with TBI outcomes.

1.5 Reactive oxygen species and traumatic brain injury

TBI has been found to induce oxidative stress. In one study examining humans with TBI, markers for oxidative stress found in cerebral spinal fluid were 9 times higher than that of uninjured patients (Bayir et al., 2002). Rats with TBI also exhibited increased oxidative stress markers at 6, 24, and 72 hours after TBI delivery (Tyurin et al., 2000), indicating the effect of TBI on ROS is conserved. Interestingly, another study in humans found the concentration of serum free thiols, an antioxidant and known biomarker of oxidative stress, to be reduced in TBI patients within 24 hours of receiving the injury (Visser et al., 2022). However, this reduction could be the result of depleted antioxidant stores due to more ROS needing to be cleared. Interestingly, serum free thiol concentration was also associated with worse recovery at 6 months post-injury, with higher oxidative stress levels in patients with incomplete recovery than those who recovered completely (Visser et al., 2022). This finding suggests higher ROS levels may be detrimental to TBI recovery.

There are numerous ways that TBI can impact ROS production. TBI can disrupt metabolic homeostasis, increasing the metabolic rate and in turn producing more ROS (Fesharaki-Zadeh, 2022; Lee & Oh, 2022). With ischemia, a possible secondary injury of TBI, an inadequate blood supply prevents proper amounts of oxygen from reaching damaged parts of the brain. Lack of proper oxygen supply disrupts the ability for mitochondria to produce adenosine triphosphate (ATP), the primary energy source for cells, resulting in mitochondrial dysfunction and the excessive production of ROS (Andrabi et al., 2020). Widespread release of the excitatory neurotransmitter glutamate as a result of the physical impact of a TBI can also cause ROS production (Eastman et al., 2020)including posttraumatic epilepsy (PTE. Excessive glutamate can induce neurotoxicity, causing a major influx of calcium ions into neurons and overloading neuronal mitochondria with calcium (Peng et al., 2019)how ROS burdens can influence neural circuit integrity remains unclear. Here, we investigate the impact of excitotoxicity induced by depletion of Drosophila Eaat1, an astrocytic glutamate transporter, on locomotor central pattern generator (CPG. This mitochondrial overload leads to the generation of excess amounts of ROS in an injured brain (Peng et al., 2019)how ROS burdens can influence neural circuit integrity remains unclear. Here, we investigate the impact of excitotoxicity induced by depletion of Drosophila Eaat1, an astrocytic glutamate transporter, on locomotor central pattern generator (CPG.

TBI also has the potential of dysregulating endogenous antioxidant mechanisms as a way of inducing ROS increase. In fruit flies given TBIs, a known oxidative stress response gene, GstD2, was found to be upregulated (Katzenberger et al., 2016). However, antioxidant levels in human TBI patients were found to be depleted in the days after injuries occurred (Bayir et al., 2002). This finding was supported by another study done in rats, which found depleted antioxidant levels immediately after TBI, however normal antioxidant levels returned 72 hours after injury (Tyurin et al., 2000). It is possible that endogenous levels of antioxidants are decreased for a period of time as a result of neutralizing higher levels of ROS than normal. The excessive production of ROS after TBI along with possible temporary depletion of the antioxidant systems necessary to remove ROS can lead to further problems, such as the breakdown of the blood-brain barrier, sensorimotor deficits, and chronic inflammation as a result of cellular and tissue damage induced by oxidative stress (Fesharaki-Zadeh, 2022).

Considering the cellular havoc that can be caused by ROS and oxidative stress – and the risks associated with both TBI and sleep disruptions – it is important to consider how ROS may play a mediating role in the relationship between TBI and sleep. Investigating this relationship could lead to a better understanding of how sleep impacts TBI outcomes and the importance of sleep in TBI prevention and recovery. However, studying these questions in humans poses a challenge as human TBIs can only be ethically studied after they occur naturally.

Instead, we can use *Drosophila melanogaster*, also known as the common fruit fly, to experimentally address these questions in a controlled manner. Fruit flies have been well established as models of human diseases. About 65% of all known genes related to diseases in humans exist in flies as functional homologs (similar versions of a gene) (Ugur et al., 2016). Because of this, we can use flies to 2study human diseases more precisely than is possible in humans, by examining the effect of gene manipulation on survival and behavior. Decades of using flies as model organisms provides us with a large number of assays and tools to study correlates of human diseases and behaviors, including TBI and sleep (Hendricks et al., 2000; Katzenberger et al., 2013; Ugur et al., 2016).

1.6 Modeling traumatic brain injury in *D. melanogaster*

There are many different models that have been developed to deliver TBIs to fruit flies (Barekat et al., 2016; Behnke et al., 2021; Kat-

zenberger et al., 2013)there has been a growing appreciation that even repetitive, milder forms of TBI (mTBI. Some models deliver a head-specific injury, which can be useful for ensuring that any injury related phenomenon is a result of injury to the brain specifically (Behnke et al., 2021). Other models deliver full body injuries, which may offer a more realistic injury paradigm similar to those experienced by humans, since forces that cause human TBI are rarely delivered to the head only (Barekat et al., 2016; Katzenberger et al., 2013)there has been a growing appreciation that even repetitive, milder forms of TBI (mTBI.

In this study, I use a modified model of the high-impact trauma (HIT) device developed by Katzenberger et al. (2013)resulting in immediate and long-term consequences including physical, behavioral, and cognitive problems. Despite the importance of TBI as a major health issue, our understanding of the underlying cellular and molecular mechanisms is limited. To unravel these mechanisms, we have developed a model of TBI in the fruit fly,\n Drosophila melanogaster\n , where we can apply many powerful experimental tools. The main features of human TBI also occur in flies, suggesting that the underlying mechanisms are conserved. Our studies demonstrate the value of a fly model for understanding the consequences of TBI and may ultimately enable development of therapies for their prevention and treatment. Traumatic brain injury (TBI as a way of delivering full body TBIs to many flies simultaneously (Figure 3). In this model, flies are subjected to a repeated number of strikes to achieve injury through rapid acceleration and deceleration within a vial.

We can use fruit flies to model TBI in mammals because many injury outcomes seen in mammalian TBI models are reproducible in flies. Some of these characteristics include acute death, shortened lifespan, and decline in locomotor activity. In flies that received a TBI with the HIT device, acute mortality was increased and flies had a significantly shorter lifespan and worse locomotor impairment (Delventhal et al., 2022; Katzenberger et al., 2013)resulting in immediate and long-term consequences including physical, behavioral, and cognitive problems. Despite the importance of TBI as a major health issue, our understanding of the underlying cellular and molecular mechanisms is limited. To unravel these mechanisms, we have developed a model of TBI in the fruit fly,Drosophila melanogaster, where we can apply many powerful experimental tools. The main features of human TBI also occur in flies, suggesting that the underlying mechanisms are conserved. Our studies demonstrate the value of a fly model for understanding the consequences of TBI and may ultimately enable development of therapies for their prevention and treatment. Traumatic brain injury (TBI. In other studies that looked at head-specific delivery of repeated mild TBI, injured flies showed a smaller increase in acute mortality, indicating TBI severity was indeed mild (Behnke et al., 2021). However, they still found significant deficits in lifespan and long-term locomotor ability in injured flies (Behnke et al., 2021). Additionally, other TBI-induced pathologies found in humans that are reproducible in flies include increased sensitivity to TBI in older flies, activation of the innate immune pathway, increased phosphorylation of Tau protein, and immediate ataxia (Barekat et al., 2016; Corrigan et al., 2014; Friedan et al., 2015; Kalmbach et al., 2018; Katzenberger et al., 2013)2018; Katzenberger et al., 2013. Injured flies also show increases in vacuolization, which is used as a measure of whole-brain neurodegeneration (Behnke et al., 2021; Bower et al., 2003; Fleminger, 2003).

Like in humans, TBI has also been found to cause sleep disruption in flies. Mild TBI given to flies caused sleep-maintenance insomnia, or more fragmented sleep, one week after injury (Barekat et al., 2016)there has been a growing appreciation that even repetitive, milder forms of TBI (mTBI. Also after injury, flies showed alterations in their circadian locomotor activity, where injured flies showed no rhythmic activity pattern (Barekat et al., 2016)there has been a growing appreciation that even repetitive, milder forms of TBI (mTBI.

1.7 Modeling sleep in *D. melanogaster*

Like TBI, we can also use flies as a model to study sleep. Although sleep is found in nearly all organisms, the behavior can look different across the animal kingdom (Cirelli & Tononi, 2008). A study by Hendricks et al. (2000) proposed a list of criteria for an inactive state in flies to be considered sleep-like or synonymous to sleep in humans. The criteria included rhythmically controlled periods of uninterrupted immobility, a posture or resting place specific to a species, a higher difficulty becoming aroused or alert, and a homeostatic regulated period of rebound sleep in response to longer periods of wakefulness (Hendricks et al., 2000).

These criteria can be seen fulfilled when considering the sleeping behavior of other animals, including humans. A circadian pacemaker rhythmically controls the timing and consolidation of our sleeping periods, sending signals to initiate sleep at night and to promote wakefulness during the day (Dijk et al., 1999). Humans take up a typical horizontal position when sleeping, with the most common position being sleeping on our sides (Skarpsno et al., 2017). While we sleep, we become less responsive to stimuli unless they reach above our arousal threshold (Berry & Gleeson, 1997). Arousal stimuli can range from external, like an alarm clock, to internal, such as lack of oxygen in individuals with sleep apnea (Berry & Gleeson, 1997). Finally, during long periods of wakefulness, our homeostatic regulator builds up a higher sleep pressure and we subsequently exhibit longer sleep duration in the form of rebound sleep to make up for the sleep loss (Dijk et al., 1999).

Detailed observations of resting behavior in flies indicated that flies met these same criteria for a sleep-like state. The number of resting flies at a time followed a rhythmic pattern, with a peak during the subjective night and an increase in activity at the start of the subjective day. Resting flies took up a specific posture, usually turning away from their food supply in a prone or supported position, and had small, sporadic movements of their proboscis, abdomen, or extremities that were not recognized as a patterned motor behavior. Resting flies were not disturbed by awake flies that ran into them and were not awoken by light mechanical stimulus created by tapping on the vial, indicating a higher arousal threshold. Finally, after a period of sleep deprivation, the number of sleeping flies increased. Evidence of rebound sleep in flies after periods of sustained wakefulness suggest the presence of a homeostatic regulatory mechanism in flies (Hendricks et al., 2000).

Such observation of flies' resting behavior showed all criteria were fulfilled to consider rest in flies a sleep-like state. With a circadian sleep and activity pattern, characteristic resting posture, decreased responsiveness to stimuli, and rebound sleep after deprivation, sleep in flies can be considered analogous to that of mammals (Hendricks et al., 2000). In addition to meeting the criteria for a sleep-like state, flies also showed similar responses to stimulants as seen in humans (Hendricks et al., 2000). Sleep decreased after flies consumed caffeine, and feeding flies an adenosine agonist, cyclohexyladenosine (CHA), increased their sleep (Hendricks et al., 2000). Numerous studies have used flies to examine sleep behavior and sleep related phenomenon, solidifying flies as a model organism to study sleep (Allada et al., 2017; Koh et al., 2006; Robertson & Keene, 2013).

1.7.1 A genetic basis for sleep in *D. melanogaster*

Certain genes in the fly genome have been identified as sleep regulating genes. Forward genetic screens have identified a series of fly mutants that display shortened sleep duration while maintaining their circadian rhythm. For example, a loss-of-function mutation of the gene *sleepless* has been shown to decrease sleep in flies by dysregulating nicotinic acetylcholine receptors (nAChRs) and the potassium ion channel Shaker (Wu et al., 2014)the molecular basis of its control is still largely a mystery. We previously showed that the quiver/sleepless (qvr/sss. Similarly, mutations of the genes *fumin*, an allele for the *Drosophila dopamine transporter*, and *redeye,* which encodes for a nAChR subunit, also result in shortened sleep in flies (Kume et al., 2005; Shi et al., 2014)but the underlying molecular mechanisms are not well understood. It has been reported that the Drosophila rest/activity cycle has features in common with the mammalian sleep/wake cycle, and it is expected that use of the fly genetic model will facilitate a molecular understanding of sleep and arousal. Here, we report the phenotypic characterization of a Drosophila rest/activity mutant known as fumin (fmn.

Flies with a mutation of the gene *insomniac* (*inc*) also display a shorter sleep duration (Stavropoulos & Young, 2011). *Inc* encodes for an adaptor of the Cullin-3 ubiquitin ligase complex, which is an important part of the protein degradation process (Stavropoulos & Young, 2011).

When using a knockout mutation, where the gene is eliminated from the genome, one study found *inc* mutant flies had a reduction of sleep to less than 350 minutes per day compared to more than 900 minutes per day in control flies (Stavropoulos & Young, 2011). The study also found that when using an RNAi knockdown of *inc*, where expression of the gene is just reduced, sleep was shortened to the same degree as knockout mutants only when *inc* was knocked down in the neurons of flies (Stavropoulos & Young, 2011). Additionally, restoring *inc* function in knockout mutants subsequently rescued normal sleep-wake behavior, suggesting proper *inc* function is necessary for normal sleep duration. Importantly, no other morphological or behavioral changes in *inc* knockdown mutants were found, indicating the *inc* gene is only involved in sleep regulation (Stavropoulos & Young, 2011). Manipulation of the expression of sleep regulating genes like *inc* has been used in previous literature to manipulate sleep and study the effect of sleep loss in flies (Hill et al., 2018).

1.8 The current study

The currently study aims to use *D. melanogaster* as a model to investigate the role of oxidative stress in the potential bidirectional relationship between TBI and sleep. This study also aims to examine how antioxidants could be protective or detrimental to TBI recovery and sleep following injury.

To do this, I first looked at how TBI affected sleep homeostasis over time by measuring changes in sleep phenotypes across different time points after TBI delivery. I measured sleep duration (total time spent asleep) and sleep fragmentation (frequency and length of sleep bouts) in flies with and without TBI. Based on previous data in both humans and flies (Barekat et al., 2016; Imbach et al., 2015; Kalmbach et al., 2018; Verma et al., 2007)2015; Kalmbach et al., 2018; Verma et al., 2007, I expected to see significant changes in sleep homeostasis in injured flies, including sleep fragmentation and sleep duration. Changes in sleep after TBI would indicate that TBI-induced damage disrupts important homeostatic regulators of sleep. Indeed, I found that in the short-term, at 48 hours and 1 week post-TBI, flies' sleep was more fragmented. However, in the long-term, 2 and 4 weeks post-TBI, flies progressed to seeping more, suggesting that sleep disruption after TBI changes over time.

To further examine the potential bidirectional relationship between TBI and sleep, I looked at whether sleep has a protective or detrimental effect on TBI outcomes. To accomplish this, I gave a TBI to short-sleeping flies with a genetic neuronal knockdown of the sleep-regulator gene *inc* (Figure 6A) (Stavropoulos & Young, 2011). After TBI delivery, I measured acute mortality as a readout of TBI recovery. To our knowledge, no previous research has investigated how manipulating sleep prior to TBI affects recovery in flies. However, studies on humans show that sleep problems prior to TBI resulted in worse recovery after injury (Kalmbach et al., 2018). Therefore, I expected to see worse TBI recovery in short-sleeping flies, represented by higher acute mortality. Indeed, I found acute mortality after TBI to be significantly higher in short-sleeping flies. Higher acute mortality in short-sleeping flies suggests sleep has a protective effect against TBI consequences while sleep loss is detrimental to TBI recovery.

Finally, to investigate how oxidative stress and ROS accumulation may play a role in TBI recovery and if TBI-related oxidative stress contributes to the changes in sleep homeostasis we see after injury, I manipulated the expression of the antioxidant gene *SOD2* in the neurons of flies. Genetic knockdown and overexpression of *SOD2* were used as a method of increasing and decreasing ROS levels, respectively. In using this method, we assume that a decrease in neuronal *SOD2* expression would increase ROS levels in the brain and overexpression of neuronal *SOD2* would decrease ROS (Figure 7A,8A). I hypothesized that flies with neuronal *SOD2* knockdown would have worse TBI outcomes and would sleep more as a result of a heightened need to clear accumulated ROS. I also predicted that flies with neuronal *SOD2* overexpression would have better recovery from TBI and would sleep less due to lower ROS levels. Data supporting these theories would suggest that oxidative stress is detrimental to TBI recovery and is the molecular basis for why TBI causes sleep disruption in injured flies. Such data would also show that antioxidants are protective against TBI and prevent sleep disruption from occurring after injury. However, I found that *SOD2* overexpression was detrimental against TBI outcomes while *SOD2* knockdown showed no significant effect. I also found that altering *SOD2* expression had a disruptive effect on sleep. While these results did not align with what I predicted, they do suggest that neuronal expression of *SOD2* and the levels of ROS in the brain have an influence on TBI outcomes and sleep.

2. METHODS AND MATERIALS

2.1 *Drosophila* strains and husbandry

Male flies were used in all sleep, lifespan, and locomotor assays. Wildtype w^{1118} *iso31* flies were used for sleep characterization after TBI. To obtain a short-sleeping fly using RNAi knockdown of *insomniac* (*inc*) in neurons, female virgin flies with the pan-neuronal driver *elav*-Gal4 were crossed with male flies carrying the UAS-*incRNAi* transgene. Parental controls were obtained by crossing the *elav*-Gal4 driver and UAS-*incRNAi* lines with their wildtype background line that they were outcrossed to, *wiso31*. Similarly, for RNAi knockdown of *SOD2*, the *elav*-Gal4 driver line was crossed with UAS-*SOD2RNAi* and each were crossed to *wiso31* for parental controls. For *SOD2* overexpression lines, the *elav*-Gal4 driver line was crossed with flies carrying the UAS-*SOD2* transgene and parental controls were obtained by crossing each to *wiso31*.

All flies were raised and kept on prepared glucose medium (Archon Scientific) for *Drosophila* and housed in incubators (Percival Scientific) at 25 ºC with a 12h:12h light:dark diurnal cycle. Male and female flies were permitted to mate for up to 72 hours post-eclosion before being separated, after which only male flies were kept and transferred to fresh food every 48-72 hours.

2.2 Traumatic brain injury delivery and conditions

TBI delivery was conducted using an adapted version of the high-impact trauma (HIT) device developed by Katzenberger et al. (2013) resulting in immediate and long-term consequences including physical, behavioral, and cognitive problems. Despite the importance of TBI as a major health issue, our understanding of the underlying cellular and molecular mechanisms is limited. To unravel these mechanisms, we have developed a model of TBI in the fruit fly,\n Drosophila melanogaster\n , where we can apply many powerful experimental tools. The main features of human TBI also occur in flies, suggesting that the underlying mechanisms are conserved. Our studies demonstrate the value of a fly model for understanding the consequences of TBI and may ultimately enable development of therapies for their prevention and treatment.\n \n ,\n \n Traumatic brain injury (TBI (Figure 3). The adapted device consisted of a wooden frame with a metal spring attached, to which a plastic, shatter-resistant vial was tightly fitted to one end using Velcro tape. At rest, the vial sat on a foam pad that was level with the bottom of the wooden frame. To give a TBI, or deliver strikes, each vial was loaded with 40-45 flies and attached to the end of the spring. Using a handheld quick release archery trigger, a string was used to pull the spring back to a specified deflection angle of 90°. Once the vial was resting against a metal rod positioned at 90°, the quick release trigger was opened, releasing the string and the spring, and the vial slammed against the pad, delivering one strike.

A

B

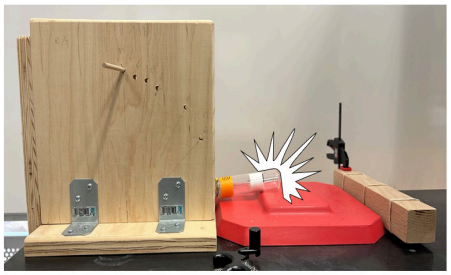

Figure 3. High-impact trauma (HIT) device. **A.** Engaged position immediately prior to strike. The loaded HIT vial (flies are not present in this example) is attached to a spring. A string is used to pull the spring back until it rests against a rod placed at a 90° deflection angle. **B.** Resting position immediately after strike. The HIT vial smacks onto a pad, causing the flies to ricochet within the vial. Adapted from Kuklinski senior thesis (2023).

2.3 Acute mortality index

The acute mortality index (MI) was calculated by dividing the number of flies dead in each vial within 24-48 hours after HIT by the total number of flies in the vial and converting to a percentage (see equation below). Dead flies were excluded from subsequent experiments, and the remaining flies were maintained by transferring to fresh

$$Acute\ mortality = \frac{Number\ of\ dead\ flies}{Total\ number\ of\ flies}\ x\ 100$$

2.4 Sleep assay

Sleep analysis was obtained by measuring flies' activity using Drosophila Activity Monitors (DAMs) (TriKinetics, Inc). The DAMs allowed for the activity of individual flies to be monitored continually for a desired period of time. Each DAM contained 32 channels, into each of which a small 5mm tube containing a single fly was inserted (Figure 4). One end of each vial contained glucose growth medium sealed from the outside using wax and the opposing end was closed off using a small segment of yarn. DAM vials were typically prepared the same day flies were loaded, but in some instances, vials were prepared 1-2 days before and kept refrigerated with a plastic wrap covering to prevent medium from drying out. Refrigerated vials were left at room temperature for at least 10 minutes before flies were loaded to prevent sudden exposure to a chilled environment. Upon loading, the vials were secured with a rubber band to prevent the vials from shifting or falling out of the DAM. Then DAMs were placed in a 25˚ C incubator and connected to a computer running DAMSystem3 software (TriKinetics, Inc) to record activity data. Activity data was summed into 1 minute bins for each channel for analysis. Channel files were analyzed using PHASE (Phase, Activity, and Sleep under Entrainment), a MATLAB-based program developed by Persons et al. (2022). In *Drosophila*, one bout of sleep is characterized as at least 5 minutes of inactivity (Shaw et al., 2000). Based on this, PHASE analysis produced the total sleep duration, number of sleep bouts, and the average bout duration for each day as well as during day and night.

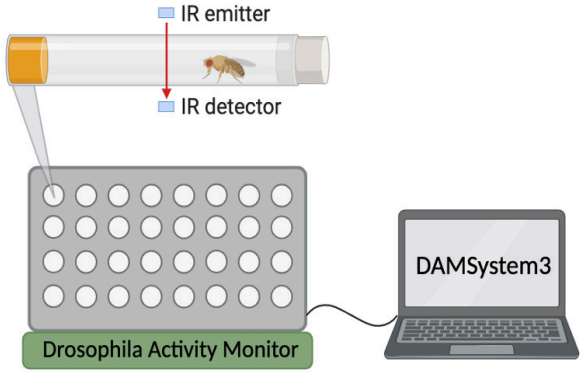

Figure 4. Drosophila Activity Monitor (DAM) (TriKinetics, Inc). A single monitor with 32 ports is loaded with 5mm tubes each containing a single fly. The monitor sends an infrared (IR) beam through the center of each tube. Each time a fly crosses the beam it is counted as a beam break by the DAMSystem 3 software (TriKinetics, Inc). Activity is measured based on the number of beam breaks within 1 minute for a single fly.

All sleep assays were conducted for a total of 8 days, with the first and last partial days excluded from analysis because they did not represent a full 24-hour period. Therefore, each dataset included sleep data from 6 full days (144 hours), beginning at the start of "lights-on" in the 12h:12h L:D cycle. Flies that were found dead after the 8 days were excluded from analysis. For sleep after TBI in *wiso31* flies, sleep was measured 48 hours, 1 week, 2 weeks, and 4 weeks after TBI delivery (Figure 5A). Sleep analysis was performed on uninjured *elav>incRNAi* flies 4 days post-eclosion to confirm the short-sleeping phenotype prior to TBI delivery (Figure 6B). All other sleep experiments were conducted at 1 week after TBI (Figures 7,8).

2.5 Lifespan assay

Starting 48 hours after TBI delivery, vials containing 15-40 flies were transferred to new vials containing fresh food medium every 2-3 days. During each transfer, the number of dead flies was recorded.

2.6 Locomotor assay

Vials containing 10-30 flies were transferred to empty vials without the use of CO_2 exposure. Another empty vial was secured atop using transparent tape to create a 20 mm high climbing vial. Flies were allowed to acclimate within the climbing vials for 10 minutes prior to the locomotor task while the vials were positioned on their sides. For the assay, 5-7 climbing vials at a time were secured vertically in a clear plastic apparatus within which humidity and temperature were maintained between 20-23˚C and 40-60% relative humidity. If the initial relative humidity was below 45%, a damp paper towel was placed inside the apparatus to raise the humidity within.

In the beginning of the assay, the apparatus was tapped down firmly against a padded surface at least 5 times to gather the flies at the bottom of the vial. Flies were allowed to climb up the vials for 75 seconds before being tapped down again. The apparatus was tapped down a total of 5 times over the course of 6 minutes. Video recordings of each experiment were made for later analysis. After the assay, climbing vials were removed and flies were briefly anesthetized with CO_2 to allow for the total number of flies in each climbing vial to be recorded. Flies were then immediately transferred to new vials containing fresh food medium. Locomotor activity of the same cohort of flies was measured at 48 hours, 2 weeks, and 4 weeks after injury. Video recordings of each assay were manually analyzed to count the number of flies that climbed past the halfway point on the vial, about 10 mm, in 20 seconds. Locomotor ability was quantified by calculating a percentage of flies that were at the halfway point or above (pass rate), and then averaged across the five trials for each vial.

2.7 Statistical analyses

Statistical analyses were performed by GraphPad Prism (version 10.2.1). Acute mortality index in *wiso31* flies was analyzed using an unpaired *t*-test. All other acute mortality index analyses used a Two-Way ANOVA with a Tukey's multiple comparisons test for post-hoc analysis in *elav>incRNAi* flies and Šídák's multiple comparisons test for post-hoc analysis in *elav>SOD2 and elav>SOD2RNAi* flies. The type of post-hoc analysis used for acute mortality index was based on the number of genotype conditions included in the analysis (3 conditions for *elav>incRNAi* flies and 2 for *elav>SOD2 and elav>SOD2RNAi*). Sleep duration, bout number, and aver

age bout duration after TBI were analyzed using a Two-Way ANOVA, and post-hoc analysis was obtained through a Šídák's multiple comparisons test for *wiso31* flies and an Uncorrected Fisher's LSD test for *elav>SOD2* and *elav>SOD2RNAi* flies. Here, the type of post-hoc analysis used was based on the type of comparisons made across conditions (only between injury conditions for *wiso 31* flies, across injury and genotype conditions for *elav>SOD2* and *elav>SOD2RNAi* flies). Addition-

ally, sleep duration in uninjured *elav>incRNAi* flies was analyzed using an unpaired *t*-test. Lifespan data were analyzed with a Mantel-Cox long-rank test. Climbing data were analyzed using a Two-Way ANOVA with a Tukey's multiple comparisons test for post-hoc analysis.

3. RESULTS

3.1 TBI affects short- and long-term sleep homeostasis

Previous studies show that TBI significantly alters sleep behavior in flies and humans (Barekat et al., 2016; Imbach et al., 2015; Kalmbach et al., 2018; Verma et al., 2007)2015; Kalmbach et al., 2018; Verma et al., 2007. I wanted to see how sleep homeostasis changed over time following a TBI. In order to test this, I induced TBI in wildtype flies and measured sleep duration, bout number, and average bout duration at 48 hours, 1 week, 2 weeks, and 4 weeks post-TBI (Figure 5A).

Injured flies had significantly increased acute mortality, which is expected (Figure 5B). At 48 hours and 1 week post-TBI, injured (TBI) flies slept for the same amount of time on average as uninjured control flies, but injured flies had significantly more bouts and shorter bout duration, indicating higher sleep fragmentation (Figure 5C,D,E). However, by 2 and 4 weeks post-injury, TBI flies display significantly longer sleep duration. No significant difference in number of bouts or bout duration was found between TBI and NH flies at 2 weeks post-TBI. However, at 4 week post-TBI, TBI flies had significantly fewer bouts and longer bout duration, indicating higher sleep consolidation. These findings show that TBI alters sleep homeostasis, but that the effect changes over time. Injured flies have more fragmented sleep in the short-term and progress to having longer, more consolidated sleep in the long-term. Given the evidence that TBI causes changes in sleep homeostasis, I wanted to know if altered sleep prior to TBI had an effect on TBI outcomes.

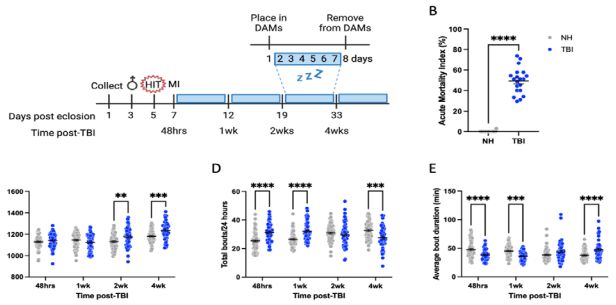

Figure 5: TBI disrupts sleep behavior over time. **A.** Experimental timeline depicting flies collected within 0-2 days of age, males separated 24 hours later, injured at 5 days of age, and sleep assays conducted at 48 hours, 1, 2, and 4 weeks later. **B.** Acute mortality of flies used in the following sleep analysis; uninjured controls (NH) are marked in gray, injured flies (TBI) are marked in blue. TBI flies had significantly higher acute mortality than uninjured control flies, $p < 0.0001$, as analyzed by a Welch's corrected unpaired *t*-test to determine the effect of injury on acute mortality. Sample size was 19-20 vials of 30-45 flies per condition. **C.** Average total sleep duration per 24 hours at 48 hours, 1 week, 2 weeks, and 4 weeks post-TBI. Sleep duration was not significantly different between TBI and uninjured flies at 48 hours and 1 week post-TBI, but was significantly increased at 2 weeks ($p < 0.01$) and 4 weeks ($p < 0.001$) post-TBI. Data for all sleep measurements (C,D,E) were analyzed using a two-way ANOVA to determine the main effect and Šídák's multiple comparisons test for post-hoc analysis. Each data point represents an individual fly. Sample size was 64 flies per condition **D.** Average total number of sleep bouts per 24 hours at 48 hours, 1 week, 2 weeks, and 4 weeks post-TBI. Number of sleep bouts was significantly increased in TBI flies at 48 hours ($p < 0.0001$) and 1 week ($p < 0.0001$) post-TBI compared to uninjured controls. No significant difference in bout number was found at 2 weeks, but a significant decrease in bout dumber was found in TBI flies at 4 weeks post-TBI, $p < 0 .0005$. Sleep bouts were quantified as the number of inactive periods lasting at least 5 minutes. **E.** Average duration of each sleep bout per 24 hours at 48 hours, 1 week, 2 weeks, and 4 weeks post-TBI. Average bout duration was significantly shorter in TBI flies at 48 hours ($p < 0.0001$) and 1 week ($p < 0.0005$) post-TBI when compared to uninjured controls. No significant difference was found at 2 weeks post-TBI, but at 4 weeks average bout duration was significantly longer in TBI flies, $p < 0.0001$. Average bout duration was measured in average minutes per bout.

3.2 Short-sleeping flies have worse acute survival following TBI

To further understand the relationship between TBI and sleep, I examined whether sleep has a protective or detrimental effect on TBI outcomes. Previous research indicates that prior sleep problems in patients with TBI predict worse recovery (Kalmbach et al., 2018). To investigate this relationship in flies, I used the Gal4-UAS system to knockdown the sleep-regulating gene *insomniac* (*inc*) in the neurons of flies, which previous research has established as a way to obtain short-sleeping flies (*elav>incRNAi)* (Figure 6A) (Stavropoulos & Young, 2011). The Gal4-UAS system allows us to control the expression of certain genes in specific tissues. The pan-neuronal driver *elav* drives expression of *Gal4* which binds to the upstream activating sequence (*UAS*), producing an RNAi transgene downstream that binds to endogenous *inc* mRNA, preventing translation of *inc* in neurons only.

Analysis of sleep duration confirmed short-sleeping phenotype in uninjured *elav>incRNAi* flies (Figure 6B). I then gave *elav>incRNAi* flies a TBI and quantified injury outcomes by measuring acute mortality 48 hours post-TBI. Short-sleeping flies had a significantly higher acute mortality than the *UAS* parental control, but not the *Gal4* control, post-TBI. However, the sample size of *Gal4* control flies was much smaller than *UAS* controls and there is no significant difference in acute mortality between *UAS* and *Gal4* parental controls (Figure 6C). These results indicate shortened sleep worsens TBI outcomes, suggesting sleep has a protective effect against TBI.

In addition to acute mortality, I examined locomotor ability as a measure of TBI outcomes in short-sleeping flies. *Elav>incRNAi* flies were also tested in a climbing assay, and I measured the percentage of flies that climbed halfway up the apparatus (data not shown). However, the sample size I used to measure locomotor ability was incomplete (1-6 vials of 11-29 flies for each condition). Prior research done in the lab established a sample size of 10-15 vials as the minimum needed for statistical significance. Subsequent repeats of this assay, with a sample size of 10-15 vials, are required for adequate analysis of how shortened sleep affects locomotor ability after TBI.

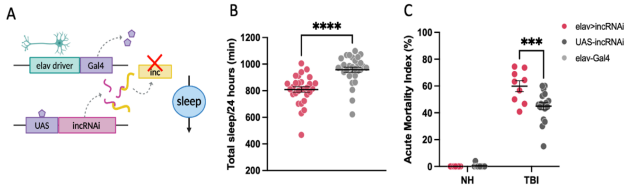

Figure 6: Short-sleeping flies exhibit worse TBI outcomes. **A.** Neuronal knockdown of the *inc* gene using *incRNAi* and the *Gal4-UAS* system. **B.** Average total sleep duration per 24 hours at 4 days post-eclosion prior to injury. Short-sleeping flies with neuronal knockdown of *inc* are marked in pink, *UAS* parental controls are in dark gray, and *Gal4* parental controls are in light gray. *elav>incRNAi* flies had significantly shorter sleep duration ($p < 0.0001$), confirming the short-sleeping phenotype. Data was analyzed using an unpaired *t*-test to determine baseline sleep duration in flies with neuronal *inc* knockdown. Each point on the graph represents an individual fly. The sample size was 32 flies per condition **C.** Acute mortality index of *elav>incRNAi* flies post-TBI. Acute mortality was significantly higher in *elav>incRNAi* TBI flies than in *UAS* TBI controls, $p < 0.0005$. No significant difference in acute mortality was found between *elav>incRNAi* TBI flies and *Gal4* TBI controls or across uninjured conditions. A two-way ANOVA was used to determine the main effect and a Tukey's multiple comparisons test was run for post-hoc analysis. Sample size was 4-18 vials of 30-45 flies per condition.

3.3 Neuronal *SOD2* knockdown leads to sleep fragmentation, but has no impact on TBI outcomes

My results show evidence of a bidirectional relationship be-

tween TBI and sleep. Considering prior research connecting ROS to TBI outcomes and sleep homeostasis, I sought to examine whether ROS mediates the bidirectional relationship between TBI and sleep. To investigate how ROS may play such a role, I first looked at how increasing ROS levels affected TBI outcomes. To raise ROS levels, I knocked down the antioxidant gene *SOD2* using the same Gal4-UAS system and *elav* driver as previously described (*elav>SOD2RNAi*) and measured acute mortality and lifespan post-TBI (Figure 7A).

I found no significant difference in acute mortality following TBI between *elav>SOD2RNAi* flies and *UAS* controls (Figure 7B). Similarly, *elav>SOD2RNAi* had no significant effect on TBI-induced reduction in lifespan when compared to *UAS* controls, though the effect neared significance ($p = .089$) (Figure 7C). However, uninjured *elav>SOD2RNAi* flies did have a significantly shorter lifespan than *UAS* control flies. While these results don't indicate neuronal knockdown of *SOD2* has any major effect on TBI outcomes, it does suggest that increasing ROS levels in neurons by decreasing expression of SOD2 is detrimental to the long-term survival of flies.

I then looked at how neuronal *SOD2* knockdown affected sleep one week after injury. I first found that sleep duration did not significantly differ across injury or *SOD2* knockdown conditions (Figure 7D). However, did I find that *elav>SOD2RNAi* flies had a significantly higher bout number and average bout duration post-TBI when compared to uninjured flies of the same genotype (Figure 7E,F). This result is consistent with the phenotype observed earlier in *wiso31* wild-type flies where TBI resulted in sleep fragmentation (Figure 5D, E). However, no significant differences in bout number or average bout duration were found between injured and uninjured *UAS* control flies. This is in contrast to the sleep fragmentation seen in injured *elav>SOD2RNAi* flies. This result may suggest that ROS levels have an effect on sleep fragmentation, but only in the context of TBI. Considering the effect of neuronal *SOD2* knockdown on sleep fragmentation post-TBI was also found in *wiso31* wildtype flies (Figure 5D,E), an increase in ROS could be an underlying mechanism behind how TBI leads to more fragmented sleep.

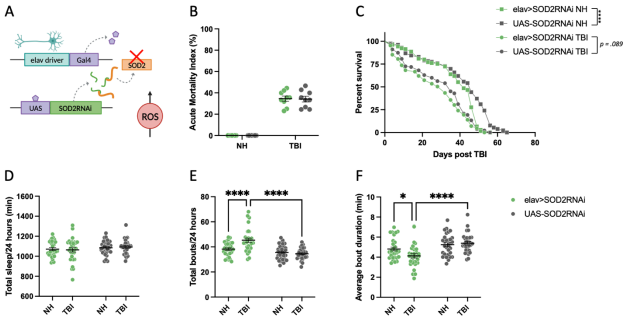

Figure 7: Neuronal *SOD2* knockdown has no significant effect on TBI outcomes but leads to sleep fragmentation after TBI. **A.** Neuronal knockdown of the *SOD2* gene using *SOD2RNAi* and the *Gal4-UAS* system. **B.** Acute mortality index of *elav>SOD2RNAi* flies used in the following lifespan and sleep assays. Flies with neuronal knockdown of *SOD2* are marked in green and *UAS* parental controls are in dark gray. No significant difference in acute mortality was found across injury and *SOD2* knockdown conditions. Data was analyzed using a two-way ANOVA to determine the main effect and Šídák's multiple comparisons test for post-hoc analysis. Sample size was 7-9 vials containing 30-45 flies. **C.** Lifespan of *elav>SOD2RNAi* flies starting at 48 hours post-TBI. *elav>SOD2RNAi* NH flies had a significantly shorter lifespan compared to *UAS* NH control flies, $p < 0.0001$. No significant difference in lifespan between *elav>SOD2RNAi* TBI flies and *UAS* TBI controls was found. Lifespan was analyzed using a Mantel-Cox long-rank test to determine the effect of *SOD2* knockdown between TBI and NH conditions. Each condition is represented by a sample size of 125-135 flies. **D.** Average sleep duration per 24 hours at 1 week post-TBI. No significant difference in sleep duration was found across injury and *SOD2* knockdown conditions. Data for all sleep measurements (D,E,F) was analyzed using a two-way ANOVA to determine the main effect and an Uncorrected Fisher's LSD test for post-hoc analysis. Each data point represents an individual fly. Sample size was 32 flies per condition. **E.** Average total number of sleep bouts per 24 hours at 1 week post-TBI. *elav>SOD2RNAi* TBI flies had a significant increase in bout number compared to *elav>SOD2RNAi* NH flies ($p < 0.0001$) and *UAS* TBI control flies ($p < 0.0001$). No significant difference in bout number was found between *UAS* TBI and NH flies. Sleep bouts were quantified as the number of inactive periods lasting at least 5 minutes. **F.** Average duration of each sleep bout per 24 hours at 1 week post-TBI. Average bout duration was significantly longer in *elav>SOD2RNAi* TBI flies than in *elav>SOD2RNAi* NH flies ($p < .05$) and *UAS* TBI controls ($p < 0.0001$). No significant difference in average bout duration between *UAS* TBI and NH flies was found. Average bout duration was measured in average minutes per bout.

3.4 Neuronal *SOD2* overexpression shortens sleep and worsens TBI outcomes

To get a more complete understanding of how levels of ROS might impact the bidirectional relationship between sleep and TBI, I also looked at the effect of decreasing ROS on TBI outcomes and sleep. To reduce ROS levels, I used a neuronal overexpression of *SOD2* (*elav>SOD2*) (Figure 8A). Using the Gal4-UAS system again, I used *elav* to further drive the expression of endogenous *SOD2* in neurons. Similar to previous experiments, I used acute mortality and lifespan as a measure of TBI outcomes.

Surprisingly, I found significantly higher acute morality following TBI in *elav>SOD2* flies than in *UAS* control flies (Figure 8B). I also found that lifespan was significantly decreased in both injured and uninjured *elav>SOD2* flies when compared to injured and uninjured *UAS* controls, respectively (Figure 8C). These results suggest that neuronal *SOD2* overexpression is detrimental for TBI outcomes, as seen by higher acute mortality and shorter lifespan.

Given the known protective effect of antioxidants against cellular and tissue damage (Andreassen et al., 2001; Li et al., 1995), this detrimental effect of antioxidant overexpression on TBI outcomes is somewhat surprising. However, the impact of antioxidant expression on sleep is also important to consider. Results from this study as well as others indicate sleep as a protective factor against TBI. Overexpression of antioxidants, including *SOD2*, have also been found to decrease sleep duration (Hill et al., 2018). Antioxidant-induced changes in sleep homeostasis could disrupt the protective effect of sleep on TBI, leading to worse TBI outcomes.

Considering this, I examined whether neuronal *SOD2* overexpression had an effect on sleep post-TBI. I measured sleep duration, number of bouts, and average bout duration just at 1 week post-TBI. I found that sleep was significantly decreased in uninjured *elav>SOD2* flies compared to uninjured *UAS* control flies (Figure 8D), confirming prior findings from Hill et al., 2018 that antioxidant overexpression decreases sleep. Additionally, only *elav>SOD2* TBI flies showed a significant increase in sleep duration post-TBI, when compared to uninjured elav>SOD2 flies. *Elav>SOD2* TBI flies also showed a significant increase in bout number compared to both uninjured *elav>SOD2* NH flies and injured *UAS* TBI controls (Figure 8E). Unlike what we see at one week post-TBI in wild-type flies, there was no significant difference in sleep bout number or duration between *UAS* TBI and NH controls (Figure 8F). A decrease in baseline sleep prior to TBI due to neuronal *SOD2* overexpression could explain why TBI outcomes were worse in flies with neuronal *SOD2* overexpression.

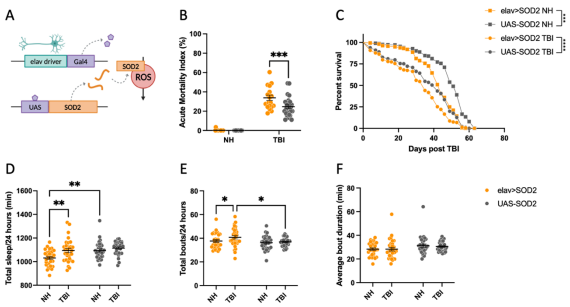

Figure 8: Neuronal *SOD2* overexpression worsens TBI outcomes

and shortens sleep. **A.** Neuronal overexpression of the *SOD2* gene using the *Gal4-UAS* system. **B.** Acute mortality index of *elav>SOD2* flies used in the following lifespan and sleep assays. Flies with neuronal overexpression of *SOD2* are marked in orange and *UAS* parental controls are in dark gray. *elav>SOD2* TBI flies had significantly higher acute mortality than *UAS* TBI controls, $p < 0.0005$. No significant difference in acute mortality was found between *elav>SOD2* uninjured flies and *UAS* uninjured controls. Analysis was done using a two-way ANOVA to determine the main effect and Šídák's multiple comparisons test for post-hoc analysis. Sample size was 18-25 vials containing 30-45 flies. **C.** Lifespan of *elav>SOD2* flies starting at 48 hours post-TBI. *elav>SOD2* TBI flies had a significantly shorter lifespan than *UAS* TBI flies, $p < .0005$. Lifespan of *elav>SOD2* uninjured flies was also significantly shorter compared to *UAS* uninjured controls, $p < 0.0001$. Lifespan was analyzed using a Mantel-Cox long-rank test to determine the effect of *SOD2* overexpression between TBI and NH conditions. Each condition is represented by a sample size of 160-360 flies. **D.** Average sleep duration per 24 hours at 1 week post-TBI. Sleep duration in *elav>SOD2* uninjured flies was significantly shorter compared to *elav>SOD2* TBI flies ($p < 0.005$) and *UAS* uninjured controls ($p < 0.005$). No significant difference in sleep duration was found between *UAS* TBI and uninjured flies. Data for all sleep measurements (D,E,F) was analyzed using a two-way ANOVA to determine the main effect and an Uncorrected Fisher's LSD test for post-hoc analysis. Each data point represents an individual fly. The sample size was 32 flies for each condition. **E.** Average total number of sleep bouts per 24 hours at 1 week post-TBI. *elav>SOD2* TBI flies had significantly more bouts when compared to *elav>SOD2* NH flies ($p < 0.05$) and *UAS* TBI controls ($p < 0.05$). No significant difference in bout number was found between *UAS* TBI and NH flies. Sleep bouts were quantified as the number of inactive periods lasting at least 5 minutes. **F.** Average duration of each sleep bout per 24 hours at 1 week post-TBI. No significant difference in average bout duration was found across injury and *SOD2* overexpression conditions. Average bout duration was measured in average minutes per bout.

4. DISCUSSION

In the current study, I used *D. melanogaster* to examine the role of oxidative stress in the bidirectional relationship between TBI and sleep homeostasis. I also asked whether ROS has a protective or detrimental effect on TBI outcomes by altering neuronal expression of a key antioxidant gene, *SOD2*. My results show that TBI causes short- and long-term changes in sleep homeostasis. Injured flies exhibited more fragmented sleep in the short-term but transitioned to sleeping more in the long-term. I also found evidence that sleep has a protective effect against TBI-induced acute mortality. Furthermore, manipulating neuronal antioxidant expression showed differing effects on TBI outcomes. *SOD2* overexpression had detrimental effects on acute mortality and lifespan while *SOD2* knockdown showed no effect on TBI outcomes. Similarly, alterations in neuronal antioxidant expression had distinct effects on sleep homeostasis. *SOD2* overexpression shortened the sleep duration of uninjured flies while *SOD2* knockdown increased sleep fragmentation in injured flies. Overall, my results show evidence for a bidirectional effect between TBI and sleep homeostasis. My manipulations of neuronal antioxidant expression also suggest that levels of oxidative stress can affect TBI outcomes and sleep post-injury. However, further research is required to elucidate the extent to which oxidative stress plays a mediating role in the relationship between TBI and sleep.

4.1 The relationship between TBI and sleep homeostasis is bidirectional

To begin to understand the relationship between TBI and sleep homeostasis in *D. melanogaster*, I measured changes in sleep homeostasis at different timepoints post-TBI (Figure 5A). I found a significant increase in sleep fragmentation among injured wildtype flies at 48 hours and one week post-TBI (Figure 5C-E). At two and four weeks post-TBI, however, injured flies no longer had fragmented sleep and instead showed a significant increase in sleep duration. My results clearly indicate a time-dependent, disruptive effect of TBI on sleep homeostasis after injury, where flies have more fragmented sleep in the short-term but progress to sleeping more in the long-term. These results are consistent with prior studies done in humans that show TBI can disrupt sleep duration and cause sleep fragmentation after injury (Kalmbach et al., 2018; Mathias & Alvaro, 2012; Sandsmark et al., 2017; Verma et al., 2007)2018; Mathias & Alvaro, 2012; Sandsmark et al., 2017; Verma et al., 2007. However, to my knowledge, no evidence of a time-dependent effect that produces distinct short-term and long-term changes in TBI-induced sleep disruption has been identified in humans. The results of my study highlight the question of what molecular changes may occur over time to cause such progressive changes in sleep homeostasis after TBI.

Such molecular changes could be the result of ROS. As previously mentioned (see section 1.3.2), ROS is implicated in the immune system as a nonpathological molecule (Morris et al., 2022). It is possible that ROS is initially required for the immune response in the short-term after TBI. This necessary dysregulation of the normal ROS balance in the brain could dysregulate homeostatic sleep mechanisms, leading to more fragmented sleep in the short-term. In the long-term, however, continuously high levels of ROS can lead to oxidative stress and cellular damage (Phaniendra et al., 2015). If sleep is needed to clear ROS, then perhaps flies with a TBI progress to sleeping more in the long-term in order to clear the accumulating ROS that's no longer needed for the immune response. These fluctuations in ROS levels could be what is driving the changes in sleep disruption we see over time after TBI.

Based on my findings indicating a detrimental effect of TBI on sleep, I then examined whether sleep had an effect on TBI outcomes. I used a genetic neuronal knockdown of the *insomniac* (*inc*) gene to obtain a short-sleeping fly. After receiving a TBI, injured short-sleeping flies had a significantly higher acute mortality than injured normal-sleeping flies (Figure 6C). My result suggests sleep has a protective effect against TBI outcomes because sleep loss enhanced the detrimental effect of TBI on acute mortality. This is also consistent with previous studies reporting worse function and recovery in TBI patients with prior sleep problems (Kalmbach et al., 2018). Furthermore, when considering what is going on molecularly, it is important to recognize ROS as a double-edged sword with its nonpathological roles and pathological potential. It is possible that in this case, we decrease sleep to the extent that ROS levels go unchecked, allowing ROS levels to supersede those required for immune activation and instead begin to cause cellular damage. This accumulation of ROS as a result of sleep loss could be what leads to an increase in acute mortality after TBI. Altogether, my results indicate that the relationship between TBI and sleep homeostasis in *D. melanogaster* is bidirectional, such that TBI disrupts sleep and sleep loss is detrimental to TBI recovery.

4.2 Altering neuronal *SOD2* expression affects TBI outcomes

4.2.1 Neuronal *SOD2* knockdown does not affect TBI-induced mortality

To examine whether oxidative stress plays a mediating role in the relationship between TBI and sleep, I first used a genetic neuronal knockdown of the antioxidant *SOD2* to increase the levels of neuronal ROS. I found that knockdown of *SOD2* had no significant effect on acute mortality after TBI but did significantly shorten the lifespan on uninjured flies (Figure 7B,C). This is consistent with prior evidence that shows neuronal knockdown of *SOD2* in flies shortens lifespan (Martin et al., 2009) RNAi-mediated silencing of the mitochondrial antioxidant manganese superoxide dismutase (SOD2. I also observed a trend towards a decreased lifespan in injured flies when *SOD2* was knocked down that approached statistical significance ($p = 0.089$). Reduced antioxidant levels and oxidative stress have been associated with a vast array of cellular damage and health consequences (Andreassen et al., 2001; Flynn & Melov, 2013; Leuner et al., 2012; Li et al., 1995). My results showing a lifespan decrease in uninjured and trending decrease in injured flies with *SOD2* knockdown support a negative effect of ROS on overall health and TBI outcomes. However, considering this, the lack of effect of neuronal *SOD2* knockdown on acute mortality is surprising. It's possible that in the short-term, an increase in ROS is not detrimental to TBI recovery. Again, as mentioned previously (see section 1.3.2), ROS is necessary for important immune-related functions, like macrophage activation (Morris et al., 2022). Perhaps decreasing antioxidant expression has beneficial effects in the hours and days immediately after receiving a TBI by increasing ROS signaling and ROS-activated components of the immune system (Morris et al., 2022).

In addition to effects on TBI outcomes, I also found that SOD2 knockdown increases sleep fragmentation in injured flies at one week post-injury. This phenotype reflects the fragmentation of sleep I found in wildtype flies after TBI (Figure 5D,E), although I do not observe this TBI-induced phenotype in the control flies with normal SOD2 expression, likely due to differences in genetic background between strains. It's possible that the sleep fragmentation I see 1-2 weeks after TBI is related to a short-term decrease in antioxidant expression or increase in ROS production. Unlike previous studies, however, I found no effect of increased ROS levels on sleep duration (Ikeda et al., 2005). This may be due to the fact that I only knocked down expression of *SOD2* in neurons, and some literature suggests other tissues may have more of an impact on ROS-regulated sleep than the brain (Vaccaro et al., 2020)2020.

4.2.2 Possible tissue dependent effects of oxidative stress

In this study, I manipulated the expression of *SOD2* specifically in neurons. However, some prior research suggests the damaging effects of ROS production and oxidative stress are tissue specific. A study by Vaccaro et al., 20202020 investigating the effect of sleep loss on oxidative stress found increases in oxidative stress markers only within in the gut of sleep deprived flies. No ROS accumulation or markers of oxidative stress were found in the brain after sleep deprivation. Additionally, overexpression of antioxidants, including *SOD2*, in the gut extended the lifespan of sleep deprived flies by almost 30 days. When the same antioxidants were overexpressed in neurons, sleep deprived flies retained their shortened lifespan (Vaccaro et al., 2020)2020.

Such evidence introduces an important concept in the context of this study. I expected to see increased ROS production in the brain, which is considered one of the most metabolically active organs, after TBI and for neuronal ROS accumulation to have an effect on survival. However, it's possible that an organ regularly exposed to high levels of ROS due to its high metabolic activity may have a more efficient activation of its antioxidant mechanisms and stricter regulation over the balance between ROS and antioxidant levels. Perhaps manipulating antioxidant expression in the gut would result in more potent effects on not only sleep but also TBI outcomes. Knocking down *SOD2* in the gut of flies may elicit a more pronounced detrimental effect on TBI outcomes which we expect to see considering the damaging effect of ROS (Flynn & Melov, 2013).

4.2.3 Neuronal *SOD2* overexpression worsens TBI outcomes

In addition to knocking down *SOD2*, I used a different genetic manipulation to increase *SOD2* expression in neurons and reduce neuronal ROS levels. I found that *SOD2* overexpression led to higher acute mortality after injury and shortened lifespan, suggesting a detrimental effect of *SOD2* overexpression on TBI outcomes (Figure 8B,C). Considering the protective effect of antioxidants like SOD2 against ROS accumulation and oxidative stress, this detrimental effect on TBI was surprising. Antioxidants have been shown to be important for preventing cellular and tissue damage, and SOD2 deficiency has been linked with neurotoxicity and cardiac complications (Andreassen et al., 2001; Li et al., 1995). Antioxidants also possibly play a role in ameliorating the damaging effect of oxidative stress on neurodegeneration (Leuner et al., 2012).

There are various explanations for why *SOD2* overexpression has such a detrimental effect on TBI outcomes. An important consideration is how *SOD2* overexpression affects sleep after injury. When I measured sleep in flies with *SOD2* overexpression one week after they received a TBI, I found *SOD2* overexpression had a significant effect on sleep duration (Figure 8D). Specifically, in uninjured flies with *SOD2* overexpression, sleep duration was significantly decreased compared to injured flies with *SOD2* overexpression. Uninjured flies with *SOD2* overexpression also had significantly shorter sleep duration uninjured flies with normal *SOD2* expression. Such decrease in sleep in the presence of increased *SOD2* levels supports the Free Radical Flux Theory, which suggests a decrease in ROS would subsequently lessen the need for sleep (Reimund, 1994). Importantly, as I and other studies have previously shown, sleep has a protective effect against negative TBI outcomes (Kalmbach et al., 2018; Sandsmark et al., 2017). Therefore, a decrease in sleep as a result of antioxidant overexpression could prevent sleep from enacting its protective qualities and consequently lead to worse TBI outcomes.

Higher antioxidant levels are also not always beneficial. Some studies report that antioxidant supplementation has no effect on health outcomes or can even worsen health (Poljsak et al., 2013). A meta-analysis of 19 clinical trials found higher all-cause mortality rates in humans who consumed high doses of vitamin E supplements, specifically at doses higher than 150 International Units (IU) per day (Miller et al., 2005)several trials of high-dosage vitamin E supplementation showed non-statistically significant increases in total mortality.\nPURPOSE: To perform a meta-analysis of the dose-response relationship between vitamin E supplementation and total mortality by using data from randomized, controlled trials.\nPATIENTS: 135,967 participants in 19 clinical trials. Of these trials, 9 tested vitamin E alone and 10 tested vitamin E combined with other vitamins or minerals. The dosages of vitamin E ranged from 16.5 to 2000 IU/d (median, 400 IU/d. This dosage is far below the current recommended upper limit for vitamin E intake per day: 1,100-1,500 IU. Another study found an association between high mortality risk and common antioxidative supplements, including vitamin B, iron, copper, and magnesium (Mursu et al., 2011)long-term health consequences of many compounds are unknown.\n\nMethods\nWe assessed the use of vitamin and mineral supplements in relation to total mortality in 38 772 older women in the Iowa Women's Health Study, mean age 61.6 years at baseline in 1986. Supplement use was self-reported in 1986, 1997 and 2004. Through December 31, 2008, 15 594 deaths (40.2%. While exogenous antioxidants may act through different mechanistic pathways than endogenous antioxidants like SOD2, these findings raise questions surrounding the possible adverse effects of antioxidant gene overexpression. Again, as mentioned previously, ROS at low levels has an important nonpathological role in vital cell functions, such as cell signaling and immune response regulation (Valko et al., 2007)e.g. nitric oxide, NO(*. ROS are required for the activation of immune cells like macrophages and help regulate functions such as apoptosis, which is necessary for cellular health and development (Hengartner, 2000; Morris et al., 2022)ROS/RNS production, and the activity of cellular antioxidants in the activation and performance of macrophages, dendritic cells, neutrophils, T-cells, B-cells, and natural killer cells; (b. It's possible that high levels of antioxidants disrupt the ROS-antioxidant balance that is vital for proper cell functioning. In fact, one study found transgenic mice that overexpressed intra- and extracellular *GSH peroxidase* antioxidants had disrupted thermoregulation and died faster when exposed to higher temperatures (Mirochnitchenko et al., 1995). Interestingly however, transgenic mice that overexpressed *SOD1*, another superoxide dismutase like *SOD2*, showed no change in thermoregulation (Mirochnitchenko et al., 1995). It's clear that the balance between ROS and antioxidant levels is delicate. Based on the nonpathological roles of ROS, especially in immune functions that help respond to TBI-induced damage, it's possible that a minimum level of ROS is required for proper recovery from TBI, especially in the short-term. By overexpressing *SOD2* in neurons, such protective functions of ROS could be inhibited. Further research into how ROS might be necessary for TBI recovery is needed.

4.3 Effects of TBI mimic those of aging

Consequences of TBI often appear to mimic health problems that occur as a natural consequence of aging. It is possible that TBI causes long-term health complications by speeding up the aging process. Similar cognitive deficits, neurodegeneration, cardiovascular complications, metabolic problems, and of course, changes in sleep homeostasis are seen in both TBI patients and older adults (Dijk et al., 1999; Friedan et al., 2015). Interestingly, this congruent effect on sleep homeostasis was replicated in my study. I found sleep fragmentation in injured flies in the short-term post-TBI, similar to the increase in fragmented sleep experienced by humans as they age.

To further investigate this phenomenon, I compared sleep homeostatic phenotypes between injured flies at 48 hours post-TBI (seven days old) and uninjured flies at four weeks post-TBI (33 days old). To my surprise, I found no significant difference in sleep bout duration or bout number (sleep fragmentation) between injured, younger flies and uninjured, older flies (Supplementary Figure 1). This same effect was

also seen when I compared injured, younger flies to uninjured, "middle-aged" flies at two weeks post-TBI (19 days old). These results support the notion that sleep disruption after TBI is similar to that seen during aging. This suggests a possible similarity in the mechanisms that lead to sleep disruption after TBI and that occur during aging.

One such mechanism could be ROS accumulation and oxidative stress. Studies have shown that expression of oxidative stress response genes become diminished with age. One study found that adults over 90 had a 17% decrease in SOD1 levels compared to adults aged 55-59 (Kozakiewicz et al., 2019). Levels of catalase and glutathione peroxidase, two other types of antioxidants, also showed a 20% and 27% decrease between the two age groups, respectively (Kozakiewicz et al., 2019). Based on the Free Radical Flux Theory, if a function of sleep is to prevent oxidative stress by clearing accumulating ROS, aging individuals or people with a TBI may be more susceptible to oxidative stress if they experience a loss of sleep. The inability to obtain enough sleep to prevent oxidative stress could lead to further negative health consequences.

4.4 Future studies

In light of the results of this study, future studies are needed to clarify some aspects of the mechanistic relationship between TBI, sleep, and oxidative stress. First, I used only one type of mechanism to manipulate sleep in flies which leaves open the possibility that the effects I observed are specific to the underlying mechanism I used to shorten sleep, rather than broadly reflective of the relationship between sleep and TBI. In this study I obtained short-sleeping flies through a neuronal knockdown of *insomniac* (*inc*) using the Gal4-UAS system (*elav>incRNAi*). I would like to use other short-sleeping mutants, including *sleepless*, *fumin*, and *redeye*, to ensure the protective effect of sleep against TBI is not dependent on the RNAi knockdown mechanism and is not related to only *inc* expression. The *inc* gene encodes for an adaptor of the Cullin-3 ubiquitin ligase complex, an important part of the protein degradation process (Hendricks et al., 2000). It is possible that the effect of *inc* knockdown on TBI is tied to the dysfunction of the Cullin-3 adaptor protein and not the dysregulation of sleep. If the TBI-induced acute mortality of these alternate short-sleeping mutants is not increased relative to their controls, then the increased acute mortality I saw with neuronal knockdown of *inc* may be the direct result of *inc* gene dysfunction, rather than reduced sleep. However, if all short-sleeping mutants with different molecular mechanisms also show an increase in acute mortality after TBI, then I could more strongly conclude that the loss of sleep itself is detrimental to TBI outcomes, not the mechanisms by which sleep loss occurs.

Second, I used only acute mortality as a measure of TBI outcome severity in short-sleeping flies. Since TBI affects not only acute survival but long-term survival as well (Katzenberger et al., 2013), I would like to examine the lifespan of short-sleeping flies after TBI. Examining how sleep loss also affects lifespan after TBI will provide another measure of TBI recovery and give more insight into the extent of the protective effect of sleep against TBI outcomes. If the effect of TBI on lifespan in injured short-sleeping flies is similar to effect seen in flies with knocked down or overexpression *SOD2*, more conclusions could be drawn about how oxidative stress mediates the effect of sleep on TBI. Additionally, repeated climbing analysis of short-sleeping flies after TBI would also demonstrate the effect of sleep loss on TBI-induced locomotor decline. Considering short-sleeping flies have a higher acute mortality after TBI, I would expect them to also exhibit a worse deficit in TBI-induced locomotor decline. This would strengthen the results of my study by suggesting sleep not only has a protective effect against TBI in the short-term, but also required for proper TBI recovery long-term.

Third, I aim to investigate how increasing sleep in flies affects TBI outcomes. Considering sleep loss is detrimental to TBI recovery, perhaps sleeping more could increase resilience against TBI. I attempted to address this question by using the dorsal Fan-Shaped body (dFB) Gal4 to drive expression of the neuron-activating bacterial channel (NaChBac) UAS transgene. This is a known mechanism used to increase sleep in *Drosophila* by activating sleep-inducing neurons in the dorsal Fan-Shaped body region of the brain (Hill et al., 2018) (Supplementary Figure 8A,B). However, *dFB>NaChBac* flies exhibited extreme abdominal bloating after eclosion, which had been anecdotally observed in prior research and is presumably due to unrelated disruption in fluid homeostasis. When I attempted to give the flies a TBI using the HIT device, the majority of the flies had ruptured abdomens due to the bloating and died almost immediately. Too few flies remained alive to continue with the experiment (Supplementary Figure 8C). Obtaining a healthy, long-sleeping fly through a different mechanism would allow me to investigate how increasing sleep affects TBI outcomes. One mechanism to increase sleep that was mentioned previously is the use of the adenosine agonist, cyclohexyladenosine (CHA) (Hendricks et al., 2000). Flies that were fed CHA had a significant increase in sleep while maintaining their normal circadian pattern (Hendricks et al., 2000). Another alternative mechanism for increasing sleep in flies includes knocking down *Excitatory amino acid transporter 2* only in glial cells (Stahl et al., 2018). This method only increases daytime sleep, however, so it would be important to consider how changes in daytime sleep versus nighttime sleep affect TBI. Another study showed mutant flies with desensitized $GABA_A$ receptors had an increase in total sleep duration (Agosto et al., 2008). However, this increase was small and may not be substantial enough to have significant effects on TBI outcomes (Agosto et al., 2008). Regardless, considering sleep's protective effect against TBI, we would expect to see less severe TBI outcomes and better recovery in flies that slept more.

Next, I will examine sleep phenotypes of flies with *SOD2* overexpression and knockdown at two and four weeks post-TBI to further clarify whether the effect of *SOD2* expression on sleep is similar to that seen after TBI. I chose to measure sleep at one week post-TBI in flies with *SOD2* overexpression and knockdown because my work characterizing sleep after injury showed consistent results at one week post-TBI. However, my results also showed that sleep disruption after TBI changes over time. In order to continue investigating oxidative stress in relation to sleep and TBI and see how ROS might affect sleep over time, I would like to see how sleep changes at two and four weeks post-TBI in flies with *SOD2* overexpression and knockdown. Specifically, I would like to see whether the transition from fragmented sleep to sleeping more in injured wildtype flies is also seen in flies where *SOD2* is knocked down or overexpressed. Similar changes in TBI-induced sleep disruption over time between wildtype flies and flies with manipulated *SOD2* expression could indicate that ROS is involved in the relationship between sleep and TBI.

Finally, I will measure the expression of antioxidants after TBI. This will allow me to determine how TBI affects antioxidant gene expression and from that infer how TBI affects ROS production. As stated previously, TBI can disrupt antioxidant expression (Bayir et al., 2002; Katzenberger et al., 2016; Visser et al., 2022). Additionally, sleep loss has been linked with increases in antioxidant expression (Hill et al., 2018). Using qRT-PCR, I will measure neuronal expression of various antioxidant and oxidative stress genes 48 hours after injury. An increase in antioxidant expression after TBI would indicate that ROS production is elevated after injury. In addition to measure antioxidant expression, directly examining the levels of ROS in the fly brain after TBI is important to see how TBI directly leads to ROS production and accumulation. ROS molecules are inherently difficult to detect due to their unstable nature. However, previous studies have used dihydroethidium (DHE), which normally fluoresces blue but emits red fluorescence when oxidized by ROS, to directly measure ROS levels (Vaccaro et al., 2020)2020. Directly quantifying ROS levels in the brain would allow me to more accurately measure how TBI affects ROS accumulation. This investigation is vital for a full understanding of how ROS and oxidative stress may play a role in the relationship between sleep and TBI.

4.5 Alternative explanations

While previous literature provides solid evidence linking oxidative stress to both sleep homeostasis and TBI, it is possible other mechanisms play an influential role in the relationship between TBI and sleep. As mentioned previously, sleep is governed by both homeostatic regulation and the circadian rhythm (Dijk et al., 1999). It is possible that, in addition to homeostasis, TBI causes changes to circadian regulation which subsequently disrupts sleep. In fact, one study found that the normal, diurnal oscillation in expression of the circadian clock genes *Clock* and *Per2* was absent in human TBI patients (Zhanfeng et al., 2019). Overall expression of *Clock* and *Per2* was also decreased after TBI (Zhanfeng et al., 2019). Another study

also found alterations in the timing and decreased expression of circadian clock genes *Clock, Per1, and Per2,* in mice given a TBI (Korthas et al., 2022). These findings suggest TBI has a disruptive impact on the diurnal pattern of circadian clock gene expression which is vital for proper circadian regulation. Circadian dysfunction can lead to problems initiating and maintaining sleep, which can lead to further TBI complications (Korthas et al., 2022). Importantly, disrupted circadian gene expression has been shown to affect the innate immune system (Jerigova et al., 2022). TBI also activates the innate immune system, which is an important part of the body's response to the damage brought about by TBI (Katzenberger et al., 2013). Depletion of circadian genes has been associated with increased white-blood cell recruitment, and mice with depletion of the circadian gene *Bmal1* had a higher risk of developing inflammatory-related diseases (Huo et al., 2017; Nguyen et al., 2013). Disruption of the innate immune system as a result of circadian dysfunction could prevent the immune system from properly responding to TBI-induced damage, leading to further health complications.

However, some studies suggest that TBI is not strongly associated with circadian rhythm. In mice with a TBI, no change in circadian behavior (wheel-running) was found despite having a decrease in circadian clock gene expression (Korthas et al., 2022)severity and pathology have different sleep and circadian rhythm disruptions, we performed a detailed sleep and circadian analysis of the high-frequency head impact TBI model (a mouse model that mimics sports-related head impacts. Additionally, in human TBI patients, no disruption of rhythmic melatonin production was observed, despite TBI patients having more decreased, fragmented sleep (Duclos et al., 2019).

Regardless, it's possible that TBI induces disruption of circadian clock gene expression, which can contribute to sleep problems and further TBI-related health consequences. However, circadian dysregulation is likely not the only factor that causes the changes in sleeping behavior we see after TBI.

Additionally, when considering sleep problems in humans with TBI, it's important to consider how sleep problems in TBI patients could be caused by other injury-related consequences that are not a direct result of brain damage or gene expression alterations. Collateral damage to the neck, back, or other parts of the body that occurred during the TBI-inducing event can often result in pain which can interfere with sleep (Paolicchi et al., 2000). Pain medications such as opioids given after injury can also cause sleep disruptions (Robertson et al., 2016). While the effect of pain and medication use on sleep in TBI patients is important to consider, these factors highlight aspects of the relationship between sleep and TBI that are established in model organisms, such as *D. melanogaster*, but are not generalizable to a human population. Fruit flies have nociceptors, receptors required for encoding stimuli related to pain or tissue damage (Boivin et al., 2023). However, whether the subjective experience of pain in invertebrates like flies is analogous to pain experienced by humans and other vertebrates remains unclear. Additionally, fruit flies are not typically exposed to drugs like opioids after a TBI and therefore are not susceptible to the sleep-disrupting effects of such pharmaceuticals. Considering my evidence that a bidirectional relationship between sleep and TBI can be replicated in flies, it is not likely that pain or medication use is the sole factor mediating the relationship between sleep and TBI.

Overall, the generalizability of the bidirectional relationship between sleep and TBI found in flies to humans should be carefully considered. TBI induced and controlled in a laboratory setting does not directly replicate the life events experienced by humans that result in TBI. Additionally, while sleeping behavior in flies has been shown to be analogous to sleep in humans, the higher cognitive demands of the human brain may make humans more sensitive to alterations in sleeping behavior and TBI-induced neuronal damage (Hendricks et al., 2000).

4.6 Conclusions

My study shows evidence for a bidirectional relationship between TBI and sleep homeostasis in *D. melanogaster*. I also found evidence to suggest an effect of neuronal antioxidant expression on TBI outcomes and sleep post-injury. Further research needs to be done to clarify how oxidative stress may play a mediating role in the relationship between TBI and sleep.

Note: Eukaryon is published by students at Lake Forest College, who are solely responsible for its content. The views expressed in Eukaryon do not necessarily reflect those of the College.

5. REFERENCES

Agosto, J., Choi, J. C., Parisky, K. M., Stilwell, G., Rosbash, M., & Griffith, L. C. (2008). Modulation of GABAA receptor desensitization uncouples sleep onset and maintenance in Drosophila. *Nature Neuroscience*, *11*(3), 354–359. https://doi.org/10.1038/nn2046

Alger, S. E., Lau, H., & Fishbein, W. (2012). Slow wave sleep during a daytime nap is necessary for protection from subsequent interference and long-term retention. *Neurobiology of Learning and Memory*, *98*(2), 188–196. https://doi.org/10.1016/j.nlm.2012.06.003

Allada, R., Cirelli, C., & Sehgal, A. (2017). Molecular mechanisms of sleep homeostasis in flies and mammals. *Cold Spring Harbor Perspectives in Biology*, *9*(8), 1–20. https://doi.org/10.1101/cshperspect.a027730

American Academy of Pediatrics. (2001). Children, adolescents, and television. *Pediatrics*, *107*(2), 423–426. https://doi.org/10.1542/peds.107.2.423

Andrabi, S. S., Yang, J., Gao, Y., Kuang, Y., & Labhasetwar, V. (2020). Nanoparticles with antioxidant enzymes protect injured spinal cord from neuronal cell apoptosis by attenuating mitochondrial dysfunction. *Journal of Controlled Release*, *317*, 300–311. https://doi.org/10.1016/j.jconrel.2019.12.001

Andreassen, O. A., Ferrante, R. J., Dedeoglu, A., Albers, D. W., Klivenyi, P., Carlson, E. J., Epstein, C. J., & Beal, M. F. (2001). Mice with a partial deficiency of manganese superoxide dismutase show increased vulnerability to the mitochondrial toxins malonate, 3-nitropropionic acid, and MPTP. *Experimental Neurology*, *167*(1), 189–195. https://doi.org/10.1006/exnr.2000.7525

Andrés, C. M. C., Pérez de la Lastra, J. M., Andrés Juan, C., Plou, F. J., & Pérez-Lebeña, E. (2023). Superoxide anion chemistry—Its role at the core of the innate immunity. *International Journal of Molecular Sciences*, *24*(3), 1–44. https://doi.org/10.3390/ijms24031841

Andriessen, T. M. J. C., Horn, J., Franschman, G., Van Der Naalt, J., Haitsma, I., Jacobs, B., Steyerberg, E. W., & Vos, P. E. (2011). Epidemiology, severity classification, and outcome of moderate and severe traumatic brain injury: A prospective multicenter study. *Journal of Neurotrauma*, *28*(10), 2019–2031. https://doi.org/10.1089/neu.2011.2034

Barekat, A., Gonzalez, A., Mauntz, R. E., Kotzebue, R. W., Molina, B., El-Mecharrafie, N., Conner, C. J., Garza, S., Melkani, G. C., Joiner, W. J., Lipinski, M. M., Finley, K. D., & Ratliff, E. P. (2016). Using Drosophila as an integrated model to study mild repetitive traumatic brain injury. *Scientific Reports*, *6*(1), 1–14. https://doi.org/10.1038/srep25252

Bayir, H., Kagan, V. E., Tyurina, Y. Y., Tyurin, V., Ruppel, R. A., Adelson, P. D., Graham, S. H., Janesko, K., Clark, R. S. B., & Kochanek, P. M. (2002). Assessment of antioxidant reserves and oxidative stress in cerebrospinal fluid after severe traumatic brain injury in infants and children. *Pediatric Research*, *51*(5), 571–578. https://doi.org/10.1203/00006450-200205000-00005

Behnke, J. A., Ye, C., Setty, A., Moberg, K. H., & Zheng, J. Q. (2021). Repetitive mild head trauma induces activity mediated lifelong brain deficits in a novel Drosophila model. *Scientific Reports*, *11*(1), 1–15. https://doi.org/10.1038/s41598-021-89121-7

Berry, R. B., & Gleeson, K. (1997). Respiratory arousal from sleep: Mechanisms and significance. *Sleep*, *20*(8), 654–675. https://doi.org/10.1093/sleep/20.8.654

Bhaskar, S., Hemavathy, D., & Prasad, S. (2016). Prevalence of chronic insomnia in adult patients and its correlation with medical comorbidities. *Journal of Family Medicine and Primary Care*, *5*(4), 780–784. https://doi.org/10.4103/2249-4863.201153

Boivin, J.-C., Zhu, J., & Ohyama, T. (2023). Nociception in fruit fly larvae. *Frontiers in Pain Research*, *4*, 1–11. https://doi.org/10.3389/fpain.2023.1076017

Bower, J. H., Maraganore, D. M., Peterson, B. J., McDonnell, S. K., Ahlskog, J. E., & Rocca, W. A. (2003a). Head trauma preceding PD: A case-control study. *Neurology*, *60*(10), 1610–1615. https://doi.org/10.1212/01.wnl.0000068008.78394.2c

Bower, J. H., Maraganore, D. M., Peterson, B. J., McDonnell, S. K., Ahlskog, J. E., & Rocca, W. A. (2003b). Head trauma preceding PD: A case-control study. *Neurology*, *60*(10), 1610–1615. https://doi.org/10.1212/01.WNL.0000068008.78394.2C

Brown, M. K., & Naidoo, N. (2010). The UPR and the anti-oxidant response: Relevance to sleep and sleep loss. *Molecular Neurobiology*, *42*(2), 103–113. https://doi.org/10.1007/s12035-010-8114-8

Butterfield, D. A., Koppal, T., Howard, B., Subramaniam, R., Hall, N., Hensley, K., Yatin, S., Allen, K., Aksenov, M., Aksenova, M., & Carney, J. (1998). Structural and functional changes in proteins induced by free radical-mediated oxidative stress and protective action of the antioxidants *N-tert* -Butyl-α-phenylnitrone and vitamin E [a]. *Annals of the New York Academy of Sciences*, *854*(1), 448–462. https://doi.org/10.1111/j.1749-6632.1998.tb09924.x

Buxton, O. M., Cain, S. W., O'Connor, S. P., Porter, J. H., Duffy, J. F., Wang, W., Czeisler, C. A., & Shea, S. A. (2012). Adverse metabolic consequences in humans of prolonged sleep restriction combined with circadian disruption. *Science Translational Medicine*, *4*(129), 1–19. https://doi.org/10.1126/scitranslmed.3003200

Cappuccio, F. P., Taggart, F. M., Kandala, N.-B., Currie, A., Peile, E., Stranges, S., & Miller, M. A. (2008). Meta-analysis of short sleep duration and obesity in children and adults. *Sleep*, *31*(5), 619–626.

CDC. (2020). *Adults – Sleep and sleep disorders*. CDC. https://www.cdc.gov/sleep/data-and-statistics/Adults.html

Chattu, V., Manzar, Md., Kumary, S., Burman, D., Spence, D., & Pandi-Perumal, S. (2018). The global problem of insufficient sleep and its serious public health implications. *Healthcare*, *7*(1), 1–16. https://doi.org/10.3390/healthcare7010001

Cirelli, C., & Tononi, G. (2008). Is sleep essential? *PLoS Biology*, *6*(8), 1605–1611. https://doi.org/10.1371/journal.pbio.0060216

Corrigan, J. D., Cuthbert, J. P., Harrison-Felix, C., Whiteneck, G. G., Bell, J. M., Miller, A. C., Coronado, V. G., & Pretz, C. R. (2014). US population estimates of health and social outcomes 5 years after rehabilitation for traumatic brain injury. *The Journal of Head Trauma Rehabilitation*, *29*(6), 1–9. https://doi.org/10.1097/

HTR.0000000000000020

Dauvilliers, Y., & Buguet, A. (2005). Hypersomnia. *Dialogues in Clinical Neuroscience*, *7*(4), 347–356.

Delventhal, R., Wooder, E. R., Basturk, M., Sattar, M., Lai, J., Bolton, D., Muthukumar, G., Ulgherait, M., & Shirasu-Hiza, M. M. (2022). Dietary restriction ameliorates TBI-induced phenotypes in Drosophila melanogaster. *Scientific Reports*, *12*(1), 1–16. https://doi.org/10.1038/s41598-022-13128-x

Dijk, D.-J., Duffy, J. F., Riel, E., Shanahan, T. L., & Czeisler, C. A. (1999). Ageing and the circadian and homeostatic regulation of human sleep during forced desynchrony of rest, melatonin and temperature rhythms. *Journal of Physiology*, *516*(2), 611–627.

Dijk, D.-J., Groeger, J. A., Stanley, N., & Deacon, S. (2010). Age-related reduction in daytime sleep propensity and nocturnal slow wave sleep. *Sleep*, *33*(2), 211–223. https://doi.org/10.1093/sleep/33.2.211

Dosunmu-Ogunbi, A., Yuan, S., Shiwarski, D. J., Tashman, J. W., Reynolds, M., Feinberg, A., Novelli, E. M., Shiva, S., & Straub, A. C. (2022). Endothelial superoxide dismutase 2 is decreased in sickle cell disease and regulates fibronectin processing. *Function*, *3*(2), 1–12. https://doi.org/10.1093/function/zqac005

Duclos, C., Dumont, M., Paquet, J., Blais, H., Van der Maren, S., Menon, D. K., Bernard, F., & Gosselin, N. (2019). Sleep-wake disturbances in hospitalized patients with traumatic brain injury: Association with brain trauma but not with an abnormal melatonin circadian rhythm. *Sleep*, *43*(1), 1–8. https://doi.org/10.1093/sleep/zsz191

Eastman, C., D'Ambrosio, R., & Ganesh, T. (2020). Modulating neuroinflammation and oxidative stress to prevent epilepsy and improve outcomes after traumatic brain injury. *Neuropharmacology*, *172*, 1–30. https://doi.org/10.1016/j.neuropharm.2019.107907

Everson, C. A., Laatsch, C. D., & Hogg, N. (2005). Antioxidant defense responses to sleep loss and sleep recovery. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *288*(2), 374–383. https://doi.org/10.1152/ajpregu.00565.2004

Fernandes, E. R., Barbosa, M. L., Amaral, M. P., Apostolico, J. de S., Sulczewski, F. B., Tufik, S., Andersen, M. L., Boscardin, S. B., Keller, A. C., & Rosa, D. S. (2020). Sleep disturbance during infection compromises Tfh differentiation and impacts host immunity. *iScience*, *23*(10), 1–27. https://doi.org/10.1016/j.isci.2020.101599

Fesharaki-Zadeh, A. (2022). Oxidative stress in traumatic brain injury. *International Journal of Molecular Sciences*, *23*(21), 1–19. https://doi.org/10.3390/ijms232113000

Fleminger, S. (2003). Head injury as a risk factor for Alzheimer's disease:

The evidence 10 years on; a partial replication. *Journal of Neurology, Neurosurgery & Psychiatry*, *74*(7), 857–862. https://doi.org/10.1136/jnnp.74.7.857

Flynn, J. M., & Melov, S. (2013). SOD2 in mitochondrial dysfunction and neurodegeneration. *Free Radical Biology and Medicine*, *62*, 4–12. https://doi.org/10.1016/j.freeradbiomed.2013.05.027

Friedan, T. R., Houry, D., & Baldwin, G. (2015). *Report to Congress on traumatic brain injury in the United States: Epidemiology and rehabilitation*. Centers for Disease Control and Prevention.

Gais, S., Lucas, B., & Born, J. (2006). Sleep after learning aids memory recall. *Learning & Memory*, *13*(3), 259–262. https://doi.org/10.1101/lm.132106

Galter, D., Mihm, S., & Dröge, W. (1994). Distinct effects of glutathione disulphide on the nuclear transcription factor kappa B and the activator protein-1. *European Journal of Biochemistry*, *221*(2), 639–648. https://doi.org/10.1111/j.1432-1033.1994.tb18776.x

Garbarino, S., Lanteri, P., Bragazzi, N. L., Magnavita, N., & Scoditti, E. (2021). Role of sleep deprivation in immune-related disease risk and

outcomes. *Communications Biology*, *4*(1), 1–17. https://doi.org/10.1038/s42003-021-02825-4

Gradisar, M., Wolfson, A. R., Harvey, A. G., Hale, L., Rosenberg, R., & Czeisler, C. A. (2013). The sleep and technology use of Americans: Findings from the National Sleep Foundation's 2011 Sleep in America Poll. *Journal of Clinical Sleep Medicine : JCSM : Official Publication of the American Academy of Sleep Medicine*, *9*(12), 1291–1299. https://doi.org/10.5664/jcsm.3272

Griendling, K. K., Sorescu, D., Lassègue, B., & Ushio-Fukai, M. (2000). Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *20*(10), 2175–2183. https://doi.org/10.1161/01.ATV.20.10.2175

Guskiewicz, K. M., Marshall, S. W., Bailes, J., McCrea, M., Cantu, R. C., Randolph, C., & Jordan, B. D. (2005). Association between recurrent concussion and late-life cognitive impairment in retired professional football players. *Neurosurgery*, *57*(4), 719–726. https://doi.org/10.1227/01.NEU.0000175725.75780.DD

Guskiewicz, K. M., Marshall, S. W., Bailes, J., Mccrea, M., Harding, H. P., Matthews, A., Mihalik, J. R., & Cantu, R. C. (2007). Recurrent concussion and risk of depression in retired professional football players. *Medicine & Science in Sports & Exercise*, *39*(6), 903–909. https://doi.org/10.1249/mss.0b013e3180383da5

Habbane, M., Montoya, J., Rhouda, T., Sbaoui, Y., Radallah, D., & Emperador, S. (2021). Human mitochondrial DNA: Particularities and diseases. *Biomedicines*, *9*(10), 1–11. https://doi.org/10.3390/biomedicines9101364

Halliwell, B., Clement, M. V., & Long, L. H. (2000). Hydrogen peroxide

in the human body. *FEBS Letters*, *486*(1), 10–13. https://doi.org/10.1016/s0014-5793(00)02197-9

Halliwell, B., Zhao, K., & Whiteman, M. (2000). The gastrointestinal tract: A major site of antioxidant action? *Free Radical Research*, *33*(6), 819–830. https://doi.org/10.1080/10715760000301341

Hauri, P., & Fisher, J. (1986). Persistent psychophysiologic (learned) insomnia. *Sleep*, *9*(1), 38–53. https://doi.org/10.1093/sleep/9.1.38

Hendricks, J. C., Finn, S. M., Panckeri, K. A., Chavkin, J., Williams, J. A., Sehgal, A., & Pack, A. I. (2000). Rest in drosophila Is a sleep-like state. *Neuron*, *25*(1), 129–138. https://doi.org/10.1016/S0896-6273(00)80877-6

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature*, *407*(6805), 770–776. https://doi.org/10.1038/35037710

Hill, V. M., O'Connor, R. M., Sissoko, G. B., Irobunda, I. S., Leong, S., Canman, J. C., Stavropoulos, N., & Shirasu-Hiza, M. (2018). A bidirectional relationship between sleep and oxidative stress in Drosophila. *PLOS Biology*, *16*(7), 1–22. https://doi.org/10.1371/journal.pbio.2005206

Hsiao, Y.-H., Chen, Y.-T., Tseng, C.-M., Wu, L.-A., Perng, D.-W., Chen, Y.-M., Chen, T.-J., Chang, S.-C., & Chou, K.-T. (2017). Sleep disorders and an increased risk of Parkinson's disease in individuals with non-apnea sleep disorders: A population-based cohort study. *Journal of Sleep Research*, *26*(5), 623–628. https://doi.org/10.1111/jsr.12545

Huo, M., Huang, Y., Qu, D., Zhang, H., Wong, W. T., Chawla, A., Huang, Y., & Tian, X. Y. (2017). Myeloid Bmal1 deletion increases monocyte recruitment and worsens atherosclerosis. *The FASEB Journal*, *31*(3), 1097–1106. https://doi.org/10.1096/fj.201601030R

Hysing, M., Pallesen, S., Stormark, K. M., Jakobsen, R., Lundervold, A. J., & Sivertsen, B. (2015). Sleep and use of electronic devices in adolescence: Results from a large population-based study. *BMJ Open*, *5*(1), 1–7. https://doi.org/10.1136/bmjopen-2014-006748

Ikeda, M., Ikeda-Sagara, M., Okada, T., Clement, P., Urade, Y., Nagai, T., Sugiyama, T., Yoshioka, T., Honda, K., & Inoué, S. (2005). Brain oxidation is an initial process in sleep induction. *Neuroscience*, *130*(4), 1029–1040. https://doi.org/10.1016/j.neuroscience.2004.09.057

Imbach, L. L., Valko, P. O., Li, T., Maric, A., Symeonidou, E.-R., Stover, J. F., Bassetti, C. L., Mica, L., Werth, E., & Baumann, C. R. (2015). Increased sleep need and daytime sleepiness 6 months after traumatic brain injury: A prospective controlled clinical trial. *Brain*, *138*(3), 726–735. https://doi.org/10.1093/brain/awu391

Jerigova, V., Zeman, M., & Okuliarova, M. (2022). Circadian disruption and consequences on innate immunity and inflammatory response. *International Journal of Molecular Sciences*, *23*(22), 1–23. https://doi.org/10.3390/ijms232213722

Juan, C. A., Pérez de la Lastra, J. M., Plou, F. J., & Pérez-Lebeña, E. (2021).

The chemistry of reactive oxygen species (ROS) revisited: Outlining their

role in biological macromolecules (DNA, lipids and proteins) and induced pathologies. *International Journal of Molecular Sciences*, *22*(9), 1–31. https://doi.org/10.3390/ijms22094642

Kalmbach, D. A., Conroy, D. A., Falk, H., Rao, V., Roy, D., Peters, M. E., Van Meter, T. E., & Korley, F. K. (2018). Poor sleep is linked to impeded recovery from traumatic brain injury. *Sleep*, *41*(10), 1–9. https://doi.org/10.1093/sleep/zsy147

Kasasbeh, E., Chi, D. S., & Krishnaswamy, G. (2006). Inflammatory aspects of sleep apnea and their cardiovascular consequences. *Southern Medical Journal*, *99*(1), 58–67, 68–69, 81. https://doi.org/10.1097/01.smj.0000197705.99639.50

Katzenberger, R. J., Ganetzky, B., & Wassarman, D. A. (2016). Age and diet affect genetically separable secondary injuries that cause acute mortality following traumatic brain injury in *Drosophila*. *G3 Genes|Genomes|Genetics*, *6*(12), 4151–4166. https://doi.org/10.1534/g3.116.036194

Katzenberger, R. J., Loewen, C. A., Wassarman, D. R., Petersen, A. J., Ganetzky, B., & Wassarman, D. A. (2013). A *Drosophila* model of closed head traumatic brain injury. *Proceedings of the National Academy of Sciences*, *110*(44), 4152–5159. https://doi.org/10.1073/pnas.1316895110

Keisari, Y., Braun, L., & Flescher, E. (1983). The oxidative burst and related phenomena in mouse macrophages elicited by different sterile inflammatory stimuli. *Immunobiology*, *165*(1), 78–89. https://doi.org/10.1016/S0171-2985(83)80048-5

Khalsa, S. B. S., Jewett, M. E., Cajochen, C., & Czeisler, C. A. (2003). *A phase response curve to single bright light pulses in human subjects*. 945–952. https://doi.org/10.1113/jphysiol.2003.040477

Knutson, K. L., Ryden, A. M., Mander, B. A., & Van Cauter, E. (2006). Role of sleep duration and quality in the risk and severity of Type 2 diabetes mellitus. *Archives of Internal Medicine*, *166*(16), 1768–1774. https://doi.org/10.1001/archinte.166.16.1768

Koh, K., Evans, J. M., Hendricks, J. C., & Sehgal, A. (2006). A *Drosophila* model for age-associated changes in sleep:wake cycles. *Proceedings of the National Academy of Sciences*, *103*(37), 13843–13847. https://doi.org/10.1073/pnas.0605903103

Korthas, H. T., Main, B. S., Harvey, A. C., Buenaventura, R. G., Wicker, E., Forcelli, P. A., & Burns, M. P. (2022). The effect of traumatic brain injury on sleep architecture and circadian rhythms in mice—A comparison of high-frequency head impact and controlled cortical injury. *Biology*, *11*(7), 1–18. https://doi.org/10.3390/biology11071031

Kozakiewicz, M., Kornatowski, M., Krzywińska, O., & Kędziora-Kornatowska, K. (2019). Changes in the blood antioxidant defense of advanced age people. *Clinical Interventions in Aging*, *14*, 763–771. https://doi.org/10.2147/CIA.S201250

Kume, K., Kume, S., Park, S. K., Hirsh, J., & Jackson, F. R. (2005). Dopamine is a regulator of arousal in the fruit fly. *The Journal of Neuroscience*, *25*(32), 7377–7384. https://doi.org/10.1523/JNEUROSCI.2048-05.2005

Lee, H. Y., & Oh, B.-M. (2022). Nutrition management in patients with traumatic brain injury: A narrative review. *Brain & Neurorehabilitation*, *15*(1), 1–13. https://doi.org/10.12786/bn.2022.15.e4

Leuner, K., Schütt, T., Kurz, C., Eckert, S. H., Schiller, C., Occhipinti, A., Mai, S., Jendrach, M., Eckert, G. P., Kruse, S. E., Palmiter, R. D., Brandt, U., Dröse, S., Wittig, I., Willem, M., Haass, C., Reichert, A. S., & Müller, W. E. (2012). Mitochondrion-derived reactive oxygen species lead to enhanced amyloid beta formation. *Antioxidants & Redox Signaling*, *16*(12), 1421–1433. https://doi.org/10.1089/ars.2011.4173

Li, Y., Huang, T.-T., Carlson, E. J., Melov, S., Ursell, P. C., Olson, J. L., Noble, L. J., Yoshimura, M. P., Berger, C., Chan, P. H., Wallace, D. C., & Epstein, C. J. (1995). Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nature Genetics*, *11*(4), 376–381. https://doi.org/10.1038/ng1295-376

Lungato, L., Marques, M. S., Pereira, V. G., Hix, S., Gazarini, M. L., Tufik, S., & D'Almeida, V. (2013). Sleep deprivation alters gene expression and antioxidant enzyme activity in mice splenocytes. *Scandinavian Journal of Immunology*, *77*(3), 195–199. https://doi.org/10.1111/sji.12029

Malhotra, J. D., Miao, H., Zhang, K., Wolfson, A., Pennathur, S., Pipe, S. W., & Kaufman, R. J. (2008). Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. *Proceedings of the National Academy of Sciences*, *105*(47), 18525–18530. https://doi.org/10.1073/pnas.0809677105

Martin, I., Jones, M. A., Rhodenizer, D., Zheng, J., Warrick, J. M., Seroude, L., & Grotewiel, M. (2009). *Sod2* knockdown in the musculature has whole-organism consequences in *Drosophila*. *Free Radical Biology and Medicine*, *47*(6), 803–813. https://doi.org/10.1016/j.freeradbiomed.2009.06.021

Mathias, J. L., & Alvaro, P. K. (2012). Prevalence of sleep disturbances, disorders, and problems following traumatic brain injury: A meta-analysis. *Sleep Medicine*, *13*(7), 898–905. https://doi.org/10.1016/j.sleep.2012.04.006

Mckee, A. C., & Daneshvar, D. H. (2015). The neuropathology of traumatic brain injury. In *Handbook of Clinical Neurology* (Vol. 127, pp. 45–66). Elsevier. https://doi.org/10.1016/B978-0-444-52892-6.00004-0

Methippara, M. M., Bashir, T., Kumar, S., Alam, N., Szymusiak, R., & McGinty, D. (2009). Salubrinal, an inhibitor of protein synthesis, promotes deep slow wave sleep. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, *296*(1), 178–184. https://doi.org/10.1152/ajpregu.90765.2008

Miller, E. R., Pastor-Barriuso, R., Dalal, D., Riemersma, R. A., Appel, L. J., & Guallar, E. (2005). Meta-analysis: High-dosage vitamin E supplementation may increase all-cause mortality. *Annals of Internal Medicine*, *142*(1), 37–46. https://doi.org/10.7326/0003-4819-142-1-200501040-00110

Mirochnitchenko, O., Palnitkar, U., Philbert, M., & Inouye, M. (1995). Thermosensitive phenotype of transgenic mice overproducing human glutathione peroxidases. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(18), 8120–8124.

Montagna, P. (2002). Agrypnia Excitata: A generalized overactivity

syndrome and a useful concept in the neurophysiopathology of sleep. *Clinical Neurophysiology*, *113*(4), 552–560. https://doi.org/10.1016/S1388-2457(02)00022-6

Morris, G., Gevezova, M., Sarafian, V., & Maes, M. (2022). Redox regulation of the immune response. *Cellular & Molecular Immunology*, *19*(10), 1079–1101. https://doi.org/10.1038/s41423-022-00902-0

Mursu, J., Robien, K., Harnack, L. J., Park, K., & Jacobs, D. R. (2011). Dietary supplements and mortality in older women: The Iowa Women's Health Study. *Archives of Internal Medicine*, *171*(18), 1625–1633. https://doi.org/10.1001/archinternmed.2011.445

Naidoo, N., Casiano, V., Cater, J., Zimmerman, J., & Pack, A. I. (2007). A role for the molecular chaperone protein BiP/GRP78 in Drosophila sleep homeostasis. *Sleep*, *30*(5), 557–565. https://doi.org/10.1093/sleep/30.5.557

Nemetz, P. N., Leibson, C., Naessens, J. M., Beard, M., Kokmen, E., Annegers, J. F., & Kurland, L. T. (1999). Traumatic brain injury and time to onset of Alzheimer's Disease: A population-based study. *American Journal of Epidemiology*, *149*(1), 32–40. https://doi.org/10.1093/oxfordjournals.aje.a009724

Nguyen, K. D., Fentress, S. J., Qiu, Y., Yun, K., Cox, J. S., & Chawla, A. (2013). Circadian gene Bmal1 regulates diurnal oscillations of Ly6Chi inflammatory monocytes. *Science (New York, N.Y.)*, *341*(6153), 1–15. https://doi.org/10.1126/science.1240636

Ohayon, M. M., O'Hara, R., & Vitiello, M. V. (2012). Epidemiology of restless legs syndrome: A synthesis of the literature. *Sleep Medicine Reviews*, *16*(4), 283–295. https://doi.org/10.1016/j.smrv.2011.05.002

Okagu, I. U., & Udenigwe, C. C. (2022). Transepithelial transport and cellular mechanisms of food-derived antioxidant peptides. *Heliyon*, *8*(10), 1–12. https://doi.org/10.1016/j.heliyon.2022.e10861

Paolicchi, J. M., Jayakar, P., Dean, P., Yaylali, I., Morrison, G., Prats, A., Resnik, T., Alvarez, L., & Duchowny, M. (2000). Predictors of outcome in pediatric epilepsy surgery. *Neurology*, *54*(3), 642–642. https://doi.org/10.1212/WNL.54.3.642

Partinen, M., Saarenpää-Heikkilä, O., Ilveskoski, I., Hublin, C., Linna, M., Olsén, P., Nokelainen, P., Alén, R., Wallden, T., Espo, M., Rusanen, H., Olme, J., Sätilä, H., Arikka, H., Kaipainen, P., Julkunen, I., & Kirjavainen, T. (2012). Increased incidence and clinical picture of childhood narcolepsy following the 2009 H1N1 pandemic vaccination campaign in Finland. *PloS One*, *7*(3), 1–9. https://doi.org/10.1371/journal.pone.0033723

Peng, J.-J., Lin, S.-H., Liu, Y.-T., Lin, H.-C., Li, T.-N., & Yao, C.-K. (2019). A circuit-dependent ROS feedback loop mediates glutamate excitotoxicity to sculpt the Drosophila motor system. *eLife*, *8*, 1–31. https://doi.org/10.7554/eLife.47372

Persons, J. L., Abhilash, L., Lopatkin, A. J., Roelofs, A., Bell, E. V.,

Fernandez, M. P., & Shafer, O. T. (2022). PHASE: An open-source program for the analysis of ***Drosophila*** Phase, Activity, and Sleep Under Entrainment. *Journal of Biological Rhythms*, *37*(4), 455–467. https://doi.org/10.1177/07487304221093114

Pham-Huy, L. A., He, H., & Pham-Huyc, C. (2008). Free radicals, antioxidants in disease and health. *International Journal of Biomedical Science*, *4*(2),

89–96. https://doi.org/10.59566/IJBS.2008.4089

Phaniendra, A., Jestadi, D. B., & Periyasamy, L. (2015). Free radicals: Properties, sources, targets, and their implication in various diseases. *Indian Journal of Clinical Biochemistry*, *30*(1), 11–26. https://doi.org/10.1007/s12291-014-0446-0

Poljsak, B., Šuput, D., & Milisav, I. (2013). Achieving the balance between ROS and antioxidants: When to use the synthetic antioxidants. *Oxidative Medicine and Cellular Longevity*, *2013*, 1–12. https://doi.org/10.1155/2013/956792

Pollock, J. D., Williams, D. A., Gifford, M. A., Li, L. L., Du, X., Fisherman, J., Orkin, S. H., Doerschuk, C. M., & Dinauer, M. C. (1995). Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nature Genetics*, *9*(2), 202–209. https://doi.org/10.1038/ng0295-202

Prather, A. A., Janicki-Deverts, D., Hall, M. H., & Cohen, S. (2015). Behaviorally assessed sleep and susceptibility to the common cold. *Sleep*, *38*(9), 1353–1359. https://doi.org/10.5665/sleep.4968

Rasch, B., & Born, J. (2013). About sleep's role in memory. *Physiological Reviews*, *93*(2), 681–766. https://doi.org/10.1152/physrev.00032.2012

Rechtschaffen, A., & Bergmann, B. M. (1995). Sleep deprivation in the rat by the disk-over-water method. *Behavioural Brain Research*, *69*(1–2), 55–63. https://doi.org/10.1016/0166-4328(95)00020-T

Reimund, E. (1994). The free radical flux theory of sleep. *Medical Hypotheses*, *43*(4), 231–233. https://doi.org/10.1016/0306-9877(94)90071-X

Robertson, J. A., Purple, R. J., Cole, P., Zaiwalla, Z., Wulff, K., & Pattinson, K. T. S. (2016). Sleep disturbance in patients taking opioid medication for chronic back pain. *Anaesthesia*, *71*(11), 1296–1307. https://doi.org/10.1111/anae.13601

Robertson, M., & Keene, A. C. (2013). Molecular mechanisms of age-related sleep loss in the fruit fly—A mini-review. *Gerontology*, *59*(4), 334–339. https://doi.org/10.1159/000348576

Sandsmark, D. K., Elliott, J. E., & Lim, M. M. (2017). Sleep-wake disturbances after traumatic brain injury: Synthesis of human and animal studies. *Sleep*, 1–18. https://doi.org/10.1093/sleep/zsx044

Shah, R., Shah, V. K., Emin, M., Gao, S., Sampogna, R. V., Aggarwal, B., Chang, A., St-Onge, M.-P., Malik, V., Wang, J., Wei, Y., & Jelic, S. (2023). Mild sleep restriction increases endothelial oxidative stress in female

persons. *Scientific Reports*, *13*(1), 15360. https://doi.org/10.1038/s41598-023-42758-y

Sharma, V., Collins, L. B., Chen, T., Herr, N., Takeda, S., Sun, W., Swenberg, J. A., & Nakamura, J. (2016). Oxidative stress at low levels can induce clustered DNA lesions leading to NHEJ mediated mutations. *Oncotarget*, *7*(18), 25377–25390. https://doi.org/10.18632/oncotarget.8298

Shaw, P. J., Cirelli, C., Greenspan, R. J., & Tononi, G. (2000). Correlates of sleep and waking in *Drosophila melanogaster*. *Science*, *287*(5459), 1834–1837. https://doi.org/10.1126/science.287.5459.1834

Shi, M., Yue, Z., Kuryatov, A., Lindstrom, J. M., & Sehgal, A. (2014). Identification of Redeye, a new sleep-regulating protein whose expression is modulated by sleep amount. *eLife*, *3*, e01473. https://doi.org/10.7554/eLife.01473

Shokri-Kojori, E., Wang, G.-J., Wiers, C. E., Demiral, S. B., Guo, M., Kim, S. W., Lindgren, E., Ramirez, V., Zehra, A., Freeman, C., Miller, G., Manza, P., Srivastava, T., De Santi, S., Tomasi, D., Benveniste, H., & Volkow, N. D. (2018). β-Amyloid accumulation in the human brain after one night of sleep deprivation. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(17), 4483–4488. https://doi.org/10.1073/pnas.1721694115

Siegel, J. M. (2005). Clues to the functions of mammalian sleep. *Nature*, *437*(7063), 1264–1271. https://doi.org/10.1038/nature04285

Silva, R. H., Abílio, V. C., Takatsu, A. L., Kameda, S. R., Grassl, C., Chehin, A. B., Medrano, W. A., Calzavara, M. B., Registro, S., Andersen, M. L., Machado, R. B., Carvalho, R. C., Ribeiro, R. de A., Tufik, S., & Frussa-Filho, R. (2004). Role of hippocampal oxidative stress in memory deficits induced by sleep deprivation in mice. *Neuropharmacology*, *46*(6), 895–903. https://doi.org/10.1016/j.neuropharm.2003.11.032

Skarpsno, E. S., Mork, P. J., Nilsen, T. I. L., & Holtermann, A. (2017). Sleep positions and nocturnal body movements based on free-living accelerometer recordings: Association with demographics, lifestyle, and insomnia symptoms. *Nature and Science of Sleep*, *9*, 267–275. https://doi.org/10.2147/NSS.S145777

Stahl, B. A., Peco, E., Davla, S., Murakami, K., Caicedo Moreno, N. A., Van Meyel, D. J., & Keene, A. C. (2018). The taurine transporter Eaat2 functions in ensheathing glia to modulate sleep and metabolic rate. *Current Biology*, *28*(22), 3700–3708. https://doi.org/10.1016/j.cub.2018.10.039

Stavropoulos, N., & Young, M. W. (2011). Insomniac and Cullin-3 regulate sleep and wakefulness in drosophila. *Neuron*, *72*(6), 964–976. https://doi.org/10.1016/j.neuron.2011.12.003

Sternbach, G. L. (2000). The Glasgow Coma Scale. *The Journal of Emergency Medicine, 9*(1), 67–71. https://doi.org/10.1016/s0736-4679(00)00182-7

Stranges, S., Tigbe, W., Gómez-Olivé, F. X., Thorogood, M., & Kandala, N.-B. (2012). Sleep problems: An emerging global epidemic? Findings from the INDEPTH WHO-SAGE Study among more than 40,000 older adults from 8 countries across Africa and Asia. *Sleep*, *35*(8), 1173–1181. https://doi.org/10.5665/sleep.2012

Sultana, R., Perluigi, M., & Butterfield, D. A. (2006). Protein oxidation and lipid peroxidation in brain of subjects with Alzheimer's Disease: Insights into mechanism of neurodegeneration from redox proteomics. *Antioxidants & Redox Signaling*, *8*(11–12), 2021–2037. https://doi.org/10.1089/ars.2006.8.2021

Thorpy, M. J. (2012). Classification of sleep disorders. *Neurotherapeutics*, *9*(4), 687–701. https://doi.org/10.1007/s13311-012-0145-6

Trivedi, M. S., Holger, D., Bui, A. T., Craddock, T. J. A., & Tartar, J. L. (2017). Short-term sleep deprivation leads to decreased systemic redox metabolites and altered epigenetic status. *PLoS ONE*, *12*(7), 1–13. https://doi.org/10.1371/journal.pone.0181978

Tu, B. P., & Weissman, J. S. (2004). Oxidative protein folding in eukaryotes. *The Journal of Cell Biology*, *164*(3), 341–346. https://doi.org/10.1083/jcb.200311055

Tyurin, V. A., Tyurina, Y. Y., Borisenko, G. G., Sokolova, T. V., Ritov, V. B., Quinn, P. J., Rose, M., Kochanek, P., Graham, S. H., & Kagan, V. E. (2000). Oxidative stress following traumatic brain injury in rats: Quantitation of biomarkers and detection of free radical intermediates. *Journal of Neurochemistry*, *75*(5), 2178–2189. https://doi.org/10.1046/j.1471-4159.2000.0752178.x

Ugur, B., Chen, K., & Bellen, H. J. (2016). *Drosophila* tools and assays for the study of human diseases. *Disease Models & Mechanisms*, *9*(3), 235–244. https://doi.org/10.1242/dmm.023762

Vaccaro, A., Kaplan Dor, Y., Nambara, K., Pollina, E. A., Lin, C., Greenberg, M. E., & Rogulja, D. (2020). Sleep loss can cause death through accumulation of reactive oxygen species in the gut. *Cell*, *181*(6), 1307–1328. https://doi.org/10.1016/j.cell.2020.04.049

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology*, *39*(1), 44–84. https://doi.org/10.1016/j.biocel.2006.07.001

Verma, A., Anand, V., & Verma, N. P. (2007). Sleep disorders in chronic traumatic brain injury. *Journal of Clinical Sleep Medicine*, *03*(04), 357–362. https://doi.org/10.5664/jcsm.26856

Visser, K., van der Horn, H. J., Bourgonje, A. R., Jacobs, B., de Borst, M. H., Vos, P. E., Bulthuis, M. L. C., van Goor, H., & van der Naalt, J. (2022). Acute serum free thiols: A potentially modifiable biomarker of oxidative stress following traumatic brain injury. *Journal of Neurology*, *269*(11), 5883–5892. https://doi.org/10.1007/s00415-022-11240-6

Wang, Y., Branicky, R., Noë, A., & Hekimi, S. (2018). Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. *The Journal of Cell Biology*, *217*(6), 1915–1928. https://doi.org/10.1083/jcb.201708007

Watson, N. F., Badr, M. S., Belenky, G., Bliwise, D. L., Buxton, O. M., Buysse, D., Dinges, D. F., Gangwisch, J., Grandner, M. A., Kushida, C.,

Malhotra, R. K., Martin, J. L., Patel, S. R., Quan, S., & Tasali, E. (2015). Recommended amount of sleep for a healthy adult: A joint consensus statement of the American Academy of Sleep Medicine and Sleep Research Society. *SLEEP*, *38*(6), 843–844. https://doi.org/10.5665/sleep.4716

Wilson, L., Boase, K., Nelson, L. D., Temkin, N. R., Giacino, J. T., Markowitz, A. J., Maas, A., Menon, D. K., Teasdale, G., & Manley, G. T. (2021). A manual for the Glasgow Outcome Scale-Extended Interview. *Journal of Neurotrauma*, *38*(17), 2435–2446. https://doi.org/10.1089/neu.2020.7527

Wong-ekkabut, J., Xu, Z., Triampo, W., Tang, I.-M., Peter Tieleman, D., & Monticelli, L. (2007). Effect of lipid peroxidation on the properties of lipid bilayers: A molecular dynamics study. *Biophysical Journal*, *93*(12), 4225–4236. https://doi.org/10.1529/biophysj.107.112565

Wu, M., Robinson, J. E., & Joiner, W. J. (2014). SLEEPLESS is a bifunctional regulator of excitability and cholinergic synaptic transmission. *Current Biology*, *24*(6), 621–629. https://doi.org/10.1016/j.cub.2014.02.026

Young, T., Palta, M., Dempsey, J., Peppard, P. E., Nieto, F. J., & Hla, K. M. (2009). Burden of sleep apnea: Rationale, design, and major findings of the Wisconsin Sleep Cohort study. *WMJ: Official Publication of the State Medical Society of Wisconsin*, *108*(5), 246–249.

Zaloshnja, E., Miller, T., Langlois, J. A., & Selassie, A. W. (2008). Prevalence of long-term disability from traumatic brain injury in the civilian population of the United States, 2005. *The Journal of Head Trauma Rehabilitation*, *23*(6), 394–400. https://doi.org/10.1097/01.HTR.0000341435.52004.ac

Zhanfeng, N., Hechun, X., Zhijun, Z., Hongyu, X., & Zhou, F. (2019). Regulation of circadian clock genes on sleep disorders in traumatic brain injury patients. *World Neurosurgery*, *130*, 475–486. https://doi.org/10.1016/j.wneu.2019.06.122

Zhao, Q.-J., Zhang, X.-G., & Wang, L.-X. (2011). Mild hypothermia therapy reduces blood glucose and lactate and improves neurologic outcomes in patients with severe traumatic brain injury. *Journal of Critical Care*, *26*(3), 311–315. https://doi.org/10.1016/j.jcrc.2010.08.014

Zhao, R.-Z., Jiang, S., Zhang, L., & Yu, Z.-B. (2019). Mitochondrial electron transport chain, ROS generation and uncoupling (Review). *International Journal of Molecular Medicine*, *44*(1), 3–15. https://doi.org/10.3892/ijmm.2019.4188

Zimmerman, M., McGlinchey, J. B., Young, D., & Chelminski, I. (2006). Diagnosing major depressive disorder I: A psychometric evaluation of the DSM-IV symptom criteria. *The Journal of Nervous and Mental Disease*, *194*(3), 158–163. https://doi.org/10.1097/01.nmd.0000202239.20315.16