The Granuphilin homolog *bitesize* regulates sleep and longevity in *Drosophila melanogaster*

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"Above all, don't fear difficult moments. The best comes from them."

Rita Levi-Montalcini

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Abbreviations

APL	anterior paired lateral neurons
Btsz	Bitesize
Drosophila	*Drosophila melanogaster*
DAM	*Drosophila* activity monitoring system
DAN	dopaminergic neuron
DAT	Dopamine Transporter
DILP	*Drosophila* Insulin-like peptide
DopR	Dopamine Transporter
DPM	dorsal paired medial
EtOH	Ethanol
FOXO	Forkhead box-containing protein, subfamily O transcription factor
GABA	gamma-aminobutyric acid
gDNA	genomic DNA
GFP	green-fluorescent-protein
IGF	Insulin-like Growth Factor
IIS	Insulin/Insulin-like Growth Factor signalling pathway
KC	Kenyon cells
Meth	methamphetamine hydrochloride
mg	milligram
ml	milliliter
MNCs	Median neurosecretory cells
NREM	non-rapid eye movement
PAM	protocerebral anterior medial
PBS	phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDK1	Phosphoinositide-dependent kinase 1
PI3K	Phosphoinositide-3-kinase
PIP2	Phosphatidylinosiol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
q-RT-PCR	Quantitative Real-Time PCR
REM	rapid eye movement
RNAseq	RNA sequencing
SHD	Synaptotagmin-like protein homology domain
SWS	slow-wave-sleep
SYA	Sugar-yeast-agar
T2D	type 2 diabetes
µl	microliter
wDah	$white^{Dahomey}$
3IY	3-Iodo L-tyrosine

Summary

In a RNAseq based screen of long-lived *dilp 2-3,5* mutants, the *btsz* locus was identified as a potential downstream effector. The function of Granuphilin, the mammalian homolog of *btsz,* has been well described to mediate the exocytosis of insulin-containing granules in pancreatic beta cells via interaction with Rab27a (Yi *et al.* 2002, Torii *et al.* 2004, Gomi *et al.* 2005). In contrast, the function of Btsz, specifically the P1 isoform containing the highly conserved SHD domain, is barely described in *Drosophila.* A previous study conducted in our lab revealed, that Btsz P1 is exclusively expressed in the mushroom body (Weigelt 2018), a brain region that has been implicated in the regulation of sleep and olfactory learning (Joiner *et al.* 2006). Strikingly, deletion of *btsz P1* resulted in a significant lifespan extension in male and female flies and increased sleep length in young male flies (Weigelt 2018). In this study we showed that deletion of *btsz P1* significantly influenced sleep patterns in both, male and female flies, in line with the observed lifespan extension in both sexes. Notably, *btsz P1Δ* mutants also exhibited an improved sleep quality during ageing, which was accompanied by ameliorated deterioration in locomotion during ageing, indicating that *btsz P1Δ* mutants do not only live longer, but also show a delayed onset of a characteristic physiological decline. Since *btsz P1Δ* mutants phenocopy *dilp 2-3,5* mutants in extended lifespan, reduced fecundity and weight, and prolonged nocturnal sleep, we wondered if Btsz P1 acts on the insulin-signalling pathway. Therefore, we performed genetic epistasis experiments and combined both mutations. The combination of both mutations, however, did not result in additive effects regarding lifespan and sleep, but rather resulted in detrimental effects. In addition, Btsz P1 did not localize in insulin-secreting neurons, suggesting that Btsz P1 influences insulin-signalling probably indirectly. To further investigate whether Btsz P1 regulates other sleep-regulating pathways, we modulated several sleep-regulating signalling-pathways pharmacologically and genetically. Sleep analysis revealed that *btsz P1Δ* mutants are resistant to interventions on the dopamine-signalling pathway, implying that Btsz P1 potentially acts in dopaminergic neurotransmission. Brain immunostainings revealed that Btsz P1 is not expressed in dopaminergic neurons, but in neurons expressing DopR, further supporting our sleep data. Also, analysis of the single cell expression atlas of the ageing *Drosophila* brain strengthened our hypothesis that Btsz P1 probably executes its function in neurons expressing DopR, since expression of *btsz P1* was detected in the same neuronal clusters as DopR. Interestingly, Ca-alpha 1T, a channel that has been implicated in the regulation of sleep, colocalised with Btsz P1 in neurons expressing DopR. In addition, *Ca-alpha 1T null* mutants were resistant to methamphetamine treatment, reminiscent to *btsz P1Δ* mutants, rising the hypothesis that Btsz P1 might influence the exocytosis of Ca-alpha 1T to the transmembrane to restore-ion currencies in order to regulate the neuronal excitability.

Zusammenfassung

In einem RNAseq-basierten Screening von langlebigen *dilp 2-3,5*-Mutanten wurde der *btsz*-Locus als potenzieller nachgeschalteter Effektor identifiziert. Die Funktion von Granuphilin, dem Säugetier-Homolog von Btsz, in der Regulation der Exozytose von insulinhaltigen Granula in pankreatischen Betazellen über die Interaktion mit Rab27a ist bereits in der Literatur beschrieben (Yi *et al.* 2002, Torii *et al.* 2004, Gomi *et al.* 2005). Im Gegensatz dazu ist die Funktion von Btsz, insbesondere der P1-Isoform, die für die hoch konservierte SHD-Domäne kodiert, in *Drosophila* weitestgehend unbekannt. Eine frühere Studie, die in unserem Labor durchgeführt wurde, zeigte, dass Btsz P1 ausschließlich im Pilzkörper exprimiert wird (Weigelt 2018), einer Hirnregion, die verantwortlich für Regulierung von Schlaf und olfaktorischem Lernen ist (Joiner *et al.* 2006). Auffallend ist, dass die Deletion von *btsz P1* zu einer signifikanten Verlängerung der Lebensspanne bei männlichen und weiblichen Fliegen und zu einer erhöhten Schlafdauer bei jungen männlichen Fliegen führt (Weigelt 2018). In dieser Studie konnten wir zeigen, dass die Deletion von *btsz P1* nicht nur das Schlafverhalten bei männlichen, sondern auch bei weiblichen Fliegen signifikant beeinflusst, was im Einklang mit der beobachteten Verlängerung der Lebensspanne bei beiden Geschlechtern steht. Bemerkenswert ist, dass *btsz P1Δ*-Mutanten auch eine verbesserte Schlafqualität während des Alterns aufwiesen, die mit einer abgemilderten Verschlechterung der Motorfunktion während des Alterns einherging, was darauf hindeutet, dass *btsz P1Δ*-Mutanten nicht nur länger leben, sondern auch einen retardierten Beginn der altersassoziierten physiologischen Beeinträchtigungen zeigen. Da *btsz P1Δ*-Mutanten phänotypisch ähnlich zu *dilp 2-3,5*-Mutanten in Bezug auf eine verlängerte Lebensspanne, eine geringere Fruchtbarkeit und ein geringeres Gewicht sowie einen verlängerten Nachtschlaf sind, fragten wir uns, ob Btsz P1 auf den Insulin-Signalweg einwirkt. Daher führten wir genetische Epistase-Experimente durch und kombinierten beide Mutationen. Die Kombination beider Mutationen führte jedoch nicht zu additiven Effekten in Bezug auf Lebensspanne und Schlaf, sondern eher zu gegensätzlichen Phänotypen. Darüber hinaus war Btsz P1 nicht in Insulin-sezernierenden Neuronen exprimiert, was darauf hindeutet, dass Btsz P1 den Insulin-Signalweg wahrscheinlich eher indirekt beeinflusst. Um weiter zu untersuchen, ob Btsz P1 in andere schlafregulierende Signalwege involviert ist, haben wir mehrere schlafregulierende Signalwege pharmakologisch und genetisch moduliert. Die Schlafanalyse ergab, dass *btsz P1Δ*-Mutanten gegen Eingriffe in den Dopamin-Signalweg resistent sind, was darauf hindeutet, dass Btsz P1 möglicherweise in die dopaminerge Neurotransmission involviert ist. Immunfärbungen des Gehirns zeigten, dass Btsz P1 nicht in dopaminergen Neuronen, sondern in Neuronen, die DopR exprimieren, lokalisiert ist, was unsere Schlafdaten weiter unterstützt. Auch die Analyse des Einzelzellexpressionsatlas des alternden *Drosophila*-Gehirns bestätigte unsere Hypothese, dass Btsz P1 seine Funktion wahrscheinlich in Neuronen ausübt, die DopR exprimieren, da die Expression von Btsz P1 in denselben neuronalen Clustern wie DopR nachgewiesen wurde.

Interessanterweise co-lokalisierte Ca-alpha 1T, ein Kanal, der mit der Regulierung des Schlafs in Verbindung gebracht wird, mit Btsz P1 in Neuronen, die DopR exprimieren. Darüber hinaus waren Ca-alpha 1T-Null-Mutanten resistent gegen Methamphetamin-Behandlung, was phänotypisch ähnlich zu *btsz P1Δ*-Mutanten ist und die Hypothese aufkommen lässt, dass Btsz P1 die Exozytose von Ca-alpha 1T an die Transmembran beeinflussen könnte, um die Ionenverteilung in zuvor aktivierten Neuronen wiederherzustellen und so die neuronale Erregbarkeit zu regulieren.

INTRODUCTION

1. Introduction

1.1 Ageing – an intricate mystery to solve

Ageing can be defined as a progressive decline in physiological integrity and function during adulthood, which correlates with an increased risk of death (Harman 1991). Ageing is a multidimensional process, which involves an increasingly well specified set of mechanisms. Advancing age is a major risk factor for numerous diseases, such as cancer, diabetes, neurodegenerative and cardiovascular diseases (Niccoli and Partridge 2012). Hence, the ageing process and the underlying mechanisms are a promising target to promote health during late life (Kennedy *et al.* 2014, Hodgson *et al.* 2020, Li *et al.* 2021, Cox 2022, Guo *et al.* 2022).

1.1.1 Extending lifespan – not only a blessing but also a challenge?

Human lifespan has greatly increased, mainly as a result of improvements in medicine, hygiene and food supply. Human lifespan has doubled since 1800 (Finch 2010), resulting in a global population with an age over 60 years that was in 2017 twice as large as in 1980 and which is predicted to double again by 2050 (United-Nations 2018). Concomitantly, in 2020 the number of people with an age of 60 years and older was greater than that of children with an age of five years or younger. According to predictions of the World Health Organization, the number of people aged 80 years and older will reach 426 million by 2050, due to an expected threefold increase from 2020 to 2050 (WHO 2022). However, healthspan has not increased proportionally to lifespan, causing more years of life that are accompanied and dominated by morbidities (Crimmins 2015). The gap between healthspan and lifespan not only affects people on an individual level, but also poses enormous economic and social burdens, underlining the profound need for a deeper understanding of ageing in order to delay its onset, compress morbidity and extend healthspan (Crimmins 2015, Garmany *et al.* 2021) .

A new era of ageing research has elucidated nine mechanisms of ageing, which have been classified as the nine hallmarks of ageing in 2013 (López-Otín *et al.* 2013). These hallmarks, namely genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered cellular communication, are not only present during normal ageing, but can also be manipulated to accelerate or delay the ageing process (López-Otín *et al.* 2013). As a result of a growing body of ageing research during the past decade, disabled macro autophagy, chronic inflammation and dysbiosis were recently added to the hallmarks of ageing (López-Otín *et al.* 2023). The importance of ageing hallmarks varies between different kinds of organisms and between different tissues in the same organism, and they

are not independent of, for instance, DNA damage leading to cellular senescence, which can cause inflammation.

1.1.2 The Insulin/ IGF pathway and its influence on ageing

The hallmark of dysregulated nutrient sensing has garnered significant scientific attention, especially in relation to modifications within the evolutionarily conserved Insulin/Insulin-like Growth Factor (IGF) signalling network (IIS). The IIS network not only regulates nutrient sensing and growth but also exerts control over metabolism, stress-resistance, and reproduction (Fontana *et al.* 2010, Partridge *et al.* 2011). Strikingly, experimental reductions in insulin-signalling have consistently induced remarkable extensions of life- and healthspan across diverse species, spanning from yeast to humans (Vanfleteren and De Vreese 1995, Tissenbaum and Ruvkun 1998, Clancy *et al.* 2001, Holzenberger *et al.* 2003, van Heemst *et al.* 2005). Noteworthy, the increase in lifespan is accompanied by a delayed onset of age-related pathologies, highlighting the pivotal role of the IIS network in promoting overall healthspan (Bonkowski *et al.* 2006, Cohen *et al.* 2006, Selman *et al.* 2008, Wessells *et al.* 2009). The profound significance of the IIS-network was initially unravelled through the introduction of mutations in the nematode worm *Caenorhabditis elegans* in the *age-1* and *daf-2* genes, which encode homologs of phosphoinositide-3-kinase (PI3K) and the Insulin receptor, respectively. These genetic alterations resulted in an unprecedented two-fold increase in lifespan (Kenyon *et al.* 1993, Kimura *et al.* 1997). Subsequent investigations have further elucidated that the observed extension of lifespan, achieved by downregulating the activity of the insulin/IGF-1-like receptor in *Caenorhabditis elegans*, is entirely reliant on daf-16, the sole worm ortholog of the highly conserved Forkhead box-containing protein, subfamily O transcription factor (FOXO) (Kenyon *et al.* 1993).

In order to delineate further implications of the IIS network, *Drosophila melanogaster* (*Drosophila*) has proved a suitable model organism for understanding the molecular mechanisms of ageing, as a result of its relatively short lifespan and the variety of available techniques for genetic modifications (Piper and Partridge 2018). In *Drosophila*, seven insulin-like peptides (DILPs) have been identified (Grönke *et al.* 2010), which act as Insulin receptor agonists and undergo transcriptional (Casas-Tinto *et al.* 2007) and post-transcriptional regulation through proteolytic processing (Fernandez *et al.* 1995). The expression of DILPs is controlled in a spatio-temporal manner during development and adulthood, implying evolutionary diversification of function (Grönke *et al.* 2010) and altered inducibility upon environmental stimuli (Toivonen and Partridge 2009). In adult flies DILP2, DILP3 and DILP5, which have functional relevance in carbohydrate homeostasis, stress response, fecundity and ageing (Rulifson *et al.* 2002, Broughton *et al.* 2005, Grönke *et al.* 2010), are synthesised by a subset of median neurosecretory cells (MNCs) in the fly brain (Brogiolo *et al.* 2001, Ikeya *et al.* 2002). However, only the

expression of DILP2 and DILP3 is restricted to the MNCs, and ovarian follicle cells and Malpighian tubules exhibit abundant expression of DILP5 (Ikeya *et al.* 2002) (Figure 1A). The fat body in the head and abdomen is the main organ producing DILP6, which has been implicated in both the regulation of carbohydrate and lipid storage and resistance to oxidative stress. Interestingly, fat-body-specific overexpression of DILP6 was reported to reduce *dilp2* and *dilp5* mRNA levels and DILP2 secretion into the haemolymph (Bai *et al.* 2012). Expression of DILP7 can be observed in a subset of postmitotic neurons of the abdominal ganglia in the ventral nerve cord innervating the hindgut (Miguel-Aliaga *et al.* 2008, Nässel and Broeck 2016) and has been proposed to influence the decision-making process in egg-laying, based on the nutritional state (Yang *et al.* 2008). DILP1 expression in the MNCs is predominantly restricted to the pupal stage during development where it promotes an increase in metabolic rate. In the adult stage DILP1 has also been suggested to affect metabolism and stress resistance (Liao *et al.* 2020).

The role of DILPs in longevity has been intensively studied. Several studies have elucidated that genetic manipulation of the expression levels of DILP2,3,5, and 6 positively affects lifespan through various mechanisms (Broughton *et al.* 2005, Grönke *et al.* 2010, Bai *et al.* 2012, Post *et al.* 2018).

Upon binding of DILPs autophosphorylation of the Insulin receptor is induced, which subsequently leads to the phosphorylation of the Insulin receptor substrate Chico (Figure 1B). Phosphorylation of Chico results in the activation of PI3K, which converts phosphatidylinosiol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3), which functions as a second messenger. This step is negatively regulated by the lipid phosphatase and tensin homologue PTEN, which catalyses the dephosphorylation of PIP3 to PIP2. The accumulation of PIP3 at the plasma membrane leads to the activation of several kinases, including the phosphoinositide-dependent kinase 1 (PDK1). Activated PDK1 in turn recruits, phosphorylates, and activates the protein kinase B (AKT), resulting in the inhibition of FOXO's translocation to the nucleus and its transcriptional activity through its phosphorylation. Blocking transcriptional activity of FOXO leads to changes in target gene expression that are involved in growth, proliferation, stress resistance and metabolism (Toivonen and Partridge 2009, Semaniuk *et al.* 2021). In line with previous findings, modulation of the IIS pathway via heteroallelic, hypomorphic form of the Insulin receptor (Tatar *et al.* 2001), loss of Chico (Clancy *et al.* 2001), increased expression of PTEN and overexpression of FOXO (Hwangbo *et al.* 2004) all result in a lifespan extension.

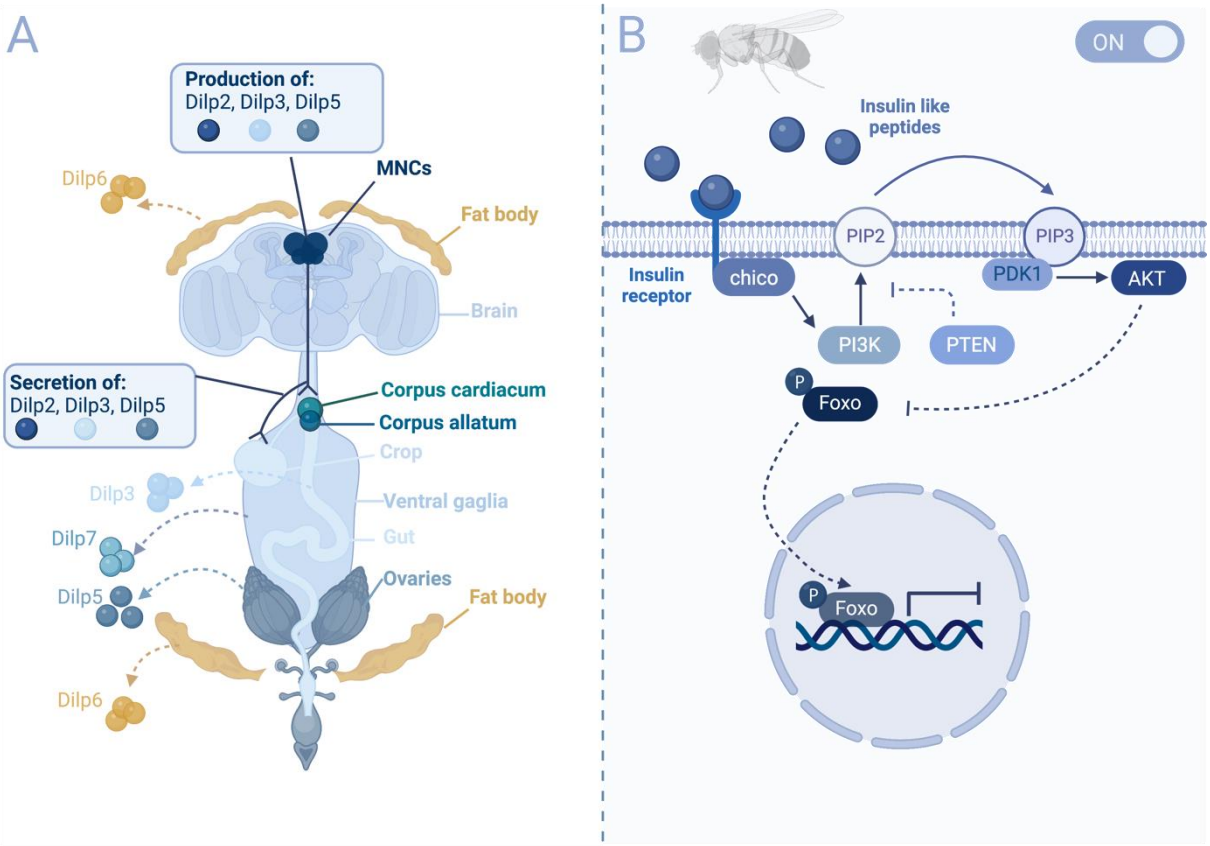


Figure 1: Schematic representation of Insulin/IGF signalling in *Drosophila*.
A) Adapted from (Nässel and Broeck 2016) and (Toivonen and Partridge 2009) depicts tissues and cell clusters that are involved in the synthesis and secretion of DILPs in adult female flies. (B) Simplified illustration of the insulin/IGF signalling network in *Drosophila* in an activated state. Created with Biorender.

1.1.3 The Insulin/ IGF pathway and its influence on sleep

Insulin/IGF signalling influences a variety of physiological functions, inter alia: sleep. A growing body of research has highlighted the connection between insulin-signalling and regulation of sleep. For instance, a recent study reported that sleep in *Drosophila* is regulated by insulin-signalling in clock-neurons (Yamaguchi *et al.* 2022). Interestingly, *Dilp 2-3,5* mutants also exhibited improved sleep quality during ageing (Metaxakis *et al.* 2014). In addition, sleep duration is influenced by insulin-like peptides and the corresponding receptor (Cong *et al.* 2015), supported by a study revealing that the expression of a constitutive Insulin receptor regulates sleep (Palermo *et al.* 2022). In mammals, sleep is also regulated by insulin/IGF signalling. Insulin-like growth factor I modulates sleep through hypothalamic orexin neurons in mice (Zegarra-Valdivia *et al.* 2020). However, more research is needed to better understand the connection between insulin-signalling and sleep and the potential implications.

1.2 Sleep and Ageing

1.2.1 An introduction to sleep

Sleep is a reversible, recurring state of altered consciousness characterised by reduced activity, relaxed postural muscles, and decreased responsiveness to external stimuli (Rasch and Born 2013). It can be classified into two main types: rapid eye movement (REM) sleep and non-rapid eye movement (NREM) sleep. NREM sleep further consists of three or four distinct stages, depending on the classification (Moser *et al.* 2009) (Figure 2). During the night, sleep cycles between the different sleep stages. In the first sleep cycles deep sleep is more prominent and decreases during the night. In contrast, periods of REM sleep extend during the night and dominate the last sleep cycles (Colten 2006).

REM sleep is characterised by increased activity in motor and sensory areas of the brain, as well as enhanced blood flow in specific brain regions. During REM sleep, heart rate, blood pressure, and respiration exhibit greater variability compared to NREM sleep. Muscular tone is absent in REM sleep, and it is a common occurrence for individuals to experience dreams during this stage (Colten 2006). REM occupies approximately 10 minutes within the first sleep cycle, but the duration increases up to 50 minutes in the final cycle. Electroencephalogram (EEG) activity during REM sleep is similar to the EEG activity during the wake state and is defined by low-voltage and high frequency activity. In contrast, NREM sleep is associated with reduced brain activity, heart rate, blood pressure, sympathetic nerve activity, respiration, and blood flow to the brain (Purves and Williams 2001, Colten 2006, Chokroverty 2009, Moser *et al.* 2009, Medic *et al.* 2017).

NREM sleep is divided into four stages (N1-N4). N1, which typically lasts 1-7 minutes during the initial sleep cycle and accounts for 3-8 % of total sleep time, represents the transition from wakefulness to a light sleep stage. This stage is characterised by slow eye movements, hypnic jerks and decreased EEG activity with (4–8 Hz) and increased amplitude (50–100 µV), defined as theta waves. In N2, muscles are more relaxed, pulse and respiration become more regular, and eye movements are scarce. N2 occupies approximately 45-55 % of total sleep time and lasts for 10-25 minutes per cycle. Sleep spindles, with oscillations of 10-15 Hz (50-150 V) that occur repeatedly and last for a few seconds, are characteristic for N2. N3/4, which is (depending on the literature) mostly subdivided from N3 into N3 and N4, is also known as deep sleep and has the highest threshold for arousal and contributes to 20-25 % of total sleep time. N3 is defined by slower waves at 2–4 Hz (100–150 µV), with further decreasing oscillations at 0.5–2 Hz (100–200 µV), which are described as slow waves or delta waves. (Purves and Williams 2001, Colten 2006, Chokroverty 2009, Moser *et al.* 2009).

Throughout the night, transitions between different sleep stages occur in a cyclic manner. Each cycle begins with N1, progresses through N2 and N3/4, and culminates in REM sleep. However, the composition of these cycles can vary within a single night, indicating dynamic changes in the sleep

architecture (Purves and Williams 2001, Colten 2006, Chokroverty 2009, Moser *et al.* 2009).

1.2.2 How does sleep change during ageing?

The architecture of sleep, both at the macro level (involving sleep duration and sleep stages) and the micro level (encompassing the quantity and quality of brainwave oscillations, or brainwaves characterised by rhythmic neural activity), undergoes significant transformations throughout the ageing process. These changes are particularly evident from adulthood to around 60 years of age in healthy individuals. Macro changes observed during ageing include shorter periods of REM sleep, deeper non-REM stages, decreased sleep efficiency, and prolonged periods of lighter non-REM stages (Kales *et al.* 1967, Zepelin *et al.* 1984, Ohayon *et al.* 2004, Conte *et al.* 2014, Vienne *et al.* 2016) (Figure 2).

Stage	Brain Waves*	Characteristics	Proportion**	Changes during Ageing
N1	**Theta waves** Frequency (4-8 Hz) Amplitude (50-100 µV)	Stage between waking and sleep, slow eye movement, hypnic jerks, can be easily disturbed by noises	59.4 ± 29.2 min per night 15.3 ± 7.7 % per night	• Less time spent asleep during the night • Earlier bedtimes • Sleep latency • Sleep fragmentation • Early awakenings • Reduction of deep sleep stages • More time spent inlight sleep stages • Reduced sleep efficiency
N2	**Spindles** Frequency (10-15 Hz) Amplitude (50-150 µV)	Light sleep, muscles relax, limbs feel heavy, pulse and breathing become regular, no eye movement	195.4 ± 48.4 min per night 49.2 ± 9.3 % per night	
N3	**Slower Waves** Frequency (2-4 Hz) Amplitude (100-150 µV)	Deep sleep, low blood pressure, restorative stage, awakenings and arousals are rare	62.7 ± 40.4 min per night 16.1 ± 10.8 % per night	
N4	**Delta Waves** Frequency (0.5-2 Hz) Amplitude (100-200 µV)			
R	**Similar to awake (beta waves)** Frequency (15-60 Hz) Amplitude (~30 µV)	Dreaming stage, rapid eye movements, low muscle tone	77.1 ± 23.4 min per night 19.4 ± 5.1 % per night	

Figure 2: Sleep stages and their characteristics in humans in the context of ageing.
Sleep constitution and its changes during ageing. Sleep can be divided in REM (R) and NREM sleep, the latter is further subdivided in N1-4. During the night, sleep cycles between the different sleep stages. *The different sleep stages are characterised by distinct patterns of brain waves, which differ in frequency and amplitude (Purves and Williams 2001). The composition of a sleep cycle changes within one night and REM sleep proportion extends during the night. During aging sleep architecture changes and aged people spent more time awake, in lighter NREM stages and less time in REM sleep. **Proportions and lengths of the presented sleep stages were assessed by using the guidelines of the American Academy of Sleep Medicine (Moser *et al.* 2009). Created with Biorender.

The micro architecture of sleep also displays alterations during the ageing process. A decline in the density and amplitude of slow wave activity (N3/4), already prominent in middle-aged adults, becomes more pronounced in older adults. These changes are primarily observed in the prefrontal cortex and during the initial non-REM cycles (Dijk *et al.* 1989, Landolt *et al.* 1996, Carrier *et al.* 2011, Mander *et al.* 2013). Additionally, sleep spindles, generated through interactions between corticothalamic networks and the reticular nucleus of the thalamus, exhibit a reduction in spectral power with age (De Gennaro and Ferrara 2003). Consequently, sleep spindles, in terms of their number and structure, serve as early indicators of ageing, detectable even in middle-aged individuals (Martin *et al.* 2013).

Sleep-related complaints in older adults are not solely attributable to insomnia, which affects approximately 25 to 50 % of individuals aged 65 to 79 years (Mellinger *et al.* 1985, Foley *et al.* 1995), but also to diminished sleep quality. Reduced sleep quality encompasses lighter and more fragmented sleep, as well as delayed sleep onset (Buysse 2014, Gulia and Kumar 2018). In addition to the natural ageing process, various factors contribute to lower sleep quality in older individuals, including depression, anxiety, and neurodegenerative disorders (Yu 2010, Palagini *et al.* 2013, Maglione *et al.* 2014, Smagula *et al.* 2015, Zhai *et al.* 2015).

Multimorbidity, defined as the coexistence of at least two significant chronic diseases, affects 50 % of individuals aged 65 and older and can lead to polypharmacy, which is associated with sleep-related complaints (Kaufman *et al.* 2002). Polypharmacy, which is in epidemiologic studies often outlined as taking five or more medications, often results in daytime sleepiness and other sleep disorders (Miner and Kryger 2017). Specific medications can exacerbate primary sleep disorders, adversely impacting sleep architecture. For instance, certain antidepressants can worsen restless leg syndrome and periodic limb movements during sleep, while opiates and benzodiazepines have negative effects on sleep-disordered breathing. Beta blockers can increase sleep fragmentation by suppressing melatonin secretion. Furthermore, certain drugs can modulate the duration of REM sleep and even induce REM sleep behaviour disorder. Additionally, some medications may disrupt sleep due to their diuretic properties or side effects such as coughing. Altogether, aged individuals suffering from multimorbidity are especially susceptible for drug-drug or drug-disease interactions, which can lead to a cascade effect to treat side effects that are caused by other medications (Miner and Kryger 2017). However, sleep disruptions are not only triggered by certain medications, but frequently caused by waking to void (nocturia), resulting in awakenings and shortened sleep periods (Duffy *et al.* 2016). Consequently, a substantial proportion of the elderly population experiences sleep disorders and insomnia complaints, which are closely associated with declining health, reduced quality of life and elevated mortality risk (Zielinski *et al.* 2016).

1.2.3 How can sleep affect health?

1.2.3.1 The influence of sleep on health parameters

The importance of sleep is evident not least from the fact that it is considered the third pillar of health alongside exercise and nutrition. Sleep has multifaceted influence on human health and a variety of vital functions, including restoration, memory consolidation, cognitive performance, energy conservation, brain waste clearance, modulation of immune response and many others, thus promoting overall health (Zielinski *et al.* 2016).

Sleep is crucial for proper cognitive and neurological function and has lead researchers to the conclusion that "sleep is of the brain, by the brain and for the brain" (Hobson 2005). Sleep significantly impacts memory consolidation and storage. As an example, during slow-wave-sleep (SWS), neuronal representations in the hippocampus that are encoded during the wake state get reactivated and thus the redistribution of those memories to the neocortical sites for long term storage are facilitated. In addition, these events are also suggested to induce permanent synaptic changes for memory stabilization (Diekelmann and Born 2010). Furthermore, sleep promotes up- and downscaling of synapses in a use-dependent manner to enable adaptation to change in environment (Tononi and Cirelli 2014, Palagini *et al.* 2022). Notably, sleep and mental health have a strong bidirectional connection (Palagini *et al.* 2022). A meta-analysis of randomised controlled trials has demonstrated that improving sleep has significant beneficial effects on mental health, depression, and anxiety, underlining the importance of sleep not only on a physiological, but also on an emotional level (Scott *et al.* 2021).

To date many studies have outlined a bidirectional connection between immune function and sleep as well. Nocturnal sleep affects adaptive immunity through the regulation of cytokine production, thereby mediating inflammatory homeostasis. Also, innate immunity is affected by an increase in natural killer cell activity that is dependent on nocturnal sleep (Irwin 2015). In the event of infection, sleep enhances host defences and can therefore reduce the risk of infection, improve infection outcomes and vaccine response (Besedovsky *et al.* 2019) .

Sleep also affects metabolic regulation and endocrine release. The control of glucose and nocturnal release of hormones is dependent on different sleep stages (Gronfier and Brandenberger 1998, Steiger 2003, Morselli *et al.* 2010). A recent study suggests that a substantial part of human metabolome is regulated in a sleep stage-specific way, with unique metabolic responses observed during REM sleep, N3 sleep and wake, concluding that sleep stages play a crucial role in optimizing metabolic circuits for human health (Nowak *et al.* 2021).

1.2.3.2 The connection between sleep and diseases

Sleep, an indispensable pillar of health, intensely influences various physiological systems and overall health. Sleep deprivation and various sleep disorders have been convincingly demonstrated to have a substantial influence on the formation and progression of diseases, as well as overall health. These interactions are frequently bidirectional and can intensify each other, resulting in cascade of health burdens.

During ageing, people often experience insufficient quality and quantity of sleep, which is often reflected by difficulties with initiating sleep onset, maintaining sleep, prolonged awakenings during the night and early-morning awakenings without being able to initiate sleep again. In sum, these symptoms also lead to reduced total sleep time and sleep efficiency. Insomnia might be diagnosed, if the symptoms occur over a period of time and are in coexistence with complaints during daytime, such as fatigue, reduced energy, daytime sleepiness, impairments in attention, concentration, memory and behavioural problems or mood disturbances (Brewster *et al.* 2018). Symptoms of insomnia affect more than 50 % of older adults and are often accentuated by psychological or physiological comorbidities and medication. It is suggested that 50 % of chronic sleep problems in the ageing population are undiagnosed. However, it is important to note that insomnia and other chronic sleep complaints are not a natural result of the ageing process, even though there are typical, non-disease-related alterations in sleep patterns during ageing (Miner and Kryger 2017). Also, daytime drowsiness and primary sleep disorders, such as sleep-disordered breathing, restless leg syndrome and REM sleep behaviour disorder, are more prominent among older adults (Crowley 2011, Suzuki *et al.* 2017). The high prevalence of sleep complaints and sleep disorders explains the growing body of research that tries to unravel the relationships between insufficient sleep and the development and progression of diseases that pose enormous health burden to the ageing society.

Sleep disturbances in the elderly, such as short and long sleep duration or abnormal sleep duration, are often associated with impaired cognitive performance and several neurobiological disorders. Interestingly, sleep disturbances increase the risk of dementia and cortical thinning (Wennberg *et al.* 2017). Sleep disturbances can serve as an evaluation tool to predict worse outcomes in patients with dementia, including more severe cognitive and neuropsychiatric symptoms and lower quality of life. Furthermore, the kind of sleep disruption can be utilized as an early indication and prognostic tool for various types of dementia (Wennberg *et al.* 2017). For example, insomnia has been associated to Alzheimer dementia. The aggregation of beta amyloid often occurs already two decades before the disease diagnosis. Short sleep duration can exacerbate the accumulation of beta amyloid by increasing the production and disturbing the elimination by the glymphatic system (Mayer *et al.* 2021).

Moreover, sleep fragmentation has been linked to decreased cortical thickness in the lateral orbitofrontal cortex and inferior frontal gyrus (Ancoli-Israel *et al.* 2003, Lim *et al.* 2016, Wennberg *et al.* 2017). Similarly, frontotemporal gray matter atrophy has been suggested to be accelerated by either self-reported short or long sleep duration (≤ 7 hours or ≥ 7 hours) (Spira *et al.* 2016). Comparable to dementia, also Parkinson's disease is frequently associated to sleep disorders and disturbances. In general, 90 % of patients suffering from neurodegenerative disease and more specifically 60 to 95 % of patients suffering from Parkinson's disease, experience sleep disorders, which deteriorate motor symptoms and cognition (Stavitsky *et al.* 2012, Mayer *et al.* 2021).

Individuals experiencing sleep problems are also at an elevated risk of developing depression or anxiety disorders. It is estimated that 90 % of individuals with depression also experience sleep issues (Tsuno *et al.* 2005). While it was previously believed that sleep problems were merely symptoms of depression, recent evidence suggests a bidirectional relationship. Sleep problems, including insomnia and excessive daytime sleepiness, are risk factors for depression in older individuals (Jaussent *et al.* 2011).

Many studies in the past years also revealed a negative effect of sleep disturbances on the immune function and the impact of sleep deprivation on the progression of autoimmune diseases. Sleep deprivation can potentially lead to a breakdown of immunologic self-tolerance. Notably, various non-apnea sleep disorders, including insomnia, have been associated with a higher risk of developing autoimmune conditions (Hsiao *et al.* 2015, Garbarino *et al.* 2021). Sleep deprivation also compromises the ability of the host defence to protect against infections with bacteria and viruses. Accordingly, participants afflicted with sleep disorders had a 1.23 times higher risk of developing herpes zoster compared to the control group (Chung *et al.* 2016). Moreover, sleep-deprived individuals, particularly those with habitual short sleep (less than or equal to 5 hours) as opposed to 7-8 hours of sleep, are more susceptible to respiratory infections, as observed in both cross-sectional and prospective studies (Patel *et al.* 2012, Prather and Leung 2016, Garbarino *et al.* 2021). Additionally, experimental viral exposures have demonstrated increased vulnerability to infections in sleep-deprived individuals (Cohen *et al.* 2009, Prather *et al.* 2015). Likewise, adolescents with shorter sleep duration (around 6 hours) in comparison to longer sleep duration (around 7 hours) face an elevated risk of common acute illnesses such as cold, flu-like and gastroenteritis (Orzech *et al.* 2014, Garbarino *et al.* 2021). Strikingly, also the importance of adequate sleep in maintaining an effective anti-tumour response has gained recognition. Various epidemiological studies have indicated a potential link between short sleep duration and an increased risk of certain cancers, such as breast, colorectal, and prostate cancer,

although the results were occasionally conflicting (Kakizaki *et al.* 2008, Kakizaki *et al.* 2008, Jiao *et al.* 2013, Cao *et al.* 2019, Garbarino *et al.* 2021).

There is a significant link between poor sleep quality and the risk of developing type 2 diabetes (T2D). Notably, sleep disturbances have far-reaching consequences that extend beyond mere quality of life, exerting discernible effects on metabolic processes. Chronic, partial sleep deprivation confers an elevated susceptibility to T2D. For instance, men reporting short sleep duration (≤ 5 hours per night) exhibited a twofold higher risk of developing T2D, whereas those with prolonged sleep duration (> 8 hours per night) faced a more than threefold increased risk (Mallon *et al.* 2005, Yaggi *et al.* 2006). In addition to associations, investigations into the mechanistic underpinnings have highlighted intriguing aspects. A study exploring the impact of a single night of sleep deprivation in healthy individuals demonstrated a remarkable induction of insulin resistance and reduced peripheral insulin sensitivity when sleep was restricted to a mere 4 hours per night (Donga *et al.* 2010). Similarly, a correlation between short sleep duration and increased fasting glucose was identified. The study reported that patients sleeping less than 6 hours per night had a threefold higher likelihood of experiencing impaired fasting glucose, which was probably mediated by increased insulin resistance, in comparison to study participants sleeping 6 to 8 hours per night (Rafalson *et al.* 2010).
Furthermore, in the context of calorie-restricted obese adults, a controlled sleep deprivation protocol of one hour per night over five consecutive nights resulted in an attenuated proportion of fat mass loss, despite comparable overall weight reduction achieved by calorie-restricted individuals with adequate sleep (Wang *et al.* 2018). These findings suggest that sleep deprivation might play a crucial role in modulating body composition during weight loss interventions.

In conclusion, sleep-related issues, such as insufficient or excessive sleep duration, daytime drowsiness, sleep-disordered breathing, prolonged sleep latency, and daytime dysfunction, significantly increase the risk of various physiological conditions. The adverse impact on overall health is strikingly evident in a study revealing that patients diagnosed with sleep disorders have approximately 16 doctor visits and receive 40 medication prescriptions annually, in contrast to 12 visits and 22 prescriptions for those without diagnosed sleep disorders (Huyett and Bhattacharyya 2021).
Moreover, the prevalence of sleep disorders is a matter of concern, with approximately 7.1 % or roughly 14 million Americans suffering from at least one sleep disorder. However, this number may be an underestimation as insufficient sleep syndrome, obstructive sleep apnea, and insomnia report prevalences of 11 %, 3-17 %, and 10 % respectively, underscoring the seriousness of sleep-related issues (Huyett *et al.* 2021).

The financial implications of sleep disorders are also substantial, as evidenced by the estimated costs reaching approximately $94.9 billion for adults in the United States diagnosed with sleep disorders alone (Huyett and Bhattacharyya 2021). Additionally, research on community-dwelling women in their 90s indicated higher healthcare costs among those with self-reported sleep problems, The mean health-care costs increased gradually in participants with no impairment from $10,745 to $15,332 in participants with impairments in three to five evaluated sleep health dimensions (satisfaction, daytime sleepiness, timing, latency and duration) demonstrating a clear association between poor sleep quality and overall health, with significant economic implications (Ensrud *et al.* 2020). Therefore, it is evident that sleep disorders not only negatively impact quality of life but also contribute significantly to compromised physical and mental health, imposing considerable economic challenges.

Considering the importance of sufficient sleep and adequate sleep hygiene for general health, disease prevention and reduced disease progression, it is crucial to recognize and manage sleep disruptions as modifiable risk factors. Implementing targeted strategies to promote improved sleep and addressing underlying mechanisms could lead to advanced disease management. Further research is indispensable for unravelling these mechanisms and developing tailored strategies to improve sleep quality and consequently, health and disease outcomes.

1.2.4 The influence of sleep on lifespan

Studies have already revealed that impaired sleep quality has significant adverse effects on overall health. Moreover, sleep disorders are associated with higher rates of morbidity and mortality. However, the potential impact of sleep on lifespan has not been extensively investigated. It is hypothesized that older individuals may exhibit specific adaptations to age-related changes in sleep patterns (Mazzotti *et al.* 2014). So far, sleep in long-lived individuals has been mostly assessed using self-report questionnaires in epidemiological studies (Gu *et al.* 2010, Chang-Quan *et al.* 2012). Examination of sleep quality in centenarians could already confirm a positive correlation between sleep quality, survival and successful ageing (Tafaro *et al.* 2007). Another study examined sleep patterns and biochemical profiles in oldest old (85-105 years), older old (60-70 years) and young (20-30 years) adults by using full-night polysomnography, sleep electroencephalogram spectral analysis, one week of actigraphy (evaluation of rest and activity by measuring gross motor activity) and peripheral blood collection. The oldest individuals in this study displayed reduced sleep efficiency and REM sleep compared to the older adults. However, the percentage of stage N3 sleep, and delta power remained similar across both groups. Interestingly, oldest old participants also demonstrated a consistent and regular sleep-wake schedule and had a more favourable lipid profile with higher levels of HDL-cholesterol and lower levels of triglycerides compared to the older adults (Mazzotti *et al.* 2014).

A recent study suggests that individuals aged 30 years, having all five low-risk sleep factors (sleep duration of 7-8 h/day, difficulty falling asleep ≤ 2 times/week, trouble staying asleep ≤ 2 times/week, no sleep medication use, and waking feeling rested ≥ 5 days/week) was associated with a 4.7-year increase in life expectancy for men (52.2 years vs. 47.5 years) and a 2.4-year increase for women (56.7 years vs. 54.3 years) when compared to those with none or only one low-risk sleep factors (Li and Qian 2023). However, comprehensive studies to identify potential causal mechanisms that might link improved sleep quality and positive lifespan effects are still missing. Due to the inherent challenges of longitudinally measuring sleep, especially with polysomnography, over extended periods and linking it to potentially underlying mechanisms, suitable animal models are required to gain valuable insights about the connection of sleep and lifespan.

1.2.5 Studying sleep in model organisms in the context of ageing

Mice have emerged as a valuable model organism for investigating sleep. Utilizing electroencephalogram measurements in mice offers detailed insights not only into sleep duration but also into different sleep stages. Interestingly, ageing mice exhibit sleep patterns that share similarities with human ageing, such as pronounced sleep fragmentation and increased wakefulness during their rest phase (Welsh *et al.* 1986). Similarly, during their active phase, 12-month-old mice experience enhanced sleep fragmentation and overall sleep time compared to their younger 3-month-old counterparts, mirroring human naps (Soltani *et al.* 2019). Furthermore, older mice (22-24 months) struggle to sustain long periods of wakefulness or non-rapid eye movement (NREM) sleep. However, intriguing differences also emerge when comparing the sleep architecture of mice in the context of ageing to humans. Even though older mice tend to sleep more particularly during the dark period, which is comparable to the increased daytime sleep in humans, also total sleep duration is increased in old mice in contrast to declining total sleep time in humans (Mander *et al.* 2017, Soltani *et al.* 2019). Additionally, mice display increased delta power and slow-wave density in the prefrontal cortex (Soltani *et al.* 2019). Moreover, characteristics of slow waves in older mice, such as increased amplitude, steeper slopes, and fewer multipeak waves, suggest higher sleep pressure, in contrast to elderly humans. This distinction complicates the direct comparison of sleep changes during aging between mice and humans (Panagiotou *et al.* 2017).

Drosophila is also a useful model organism for sleep studies and to investigate sleep disorders due to its reflection of fundamental features defining sleep. Flies exhibit changes in synapse plasticity, expression of genes involved in energy metabolism and responses to stress dependent on sleep or wakefulness, while the arousal threshold increases during night (Ho and Sehgal 2005). Furthermore, sleep in flies is under homeostatic and circadian control and can be modulated by pharmacological

agents (Borbely 1982). Similar to humans, flies show increased activity and wakefulness during the day under light conditions. In contrast, dark conditions induce a state of less activity and sustained periods of quiescence (Peschel and Helfrich-Förster 2011). Like humans, flies display an age-dependent decrease in sleep quality characterised by increased sleep fragmentation, shorter sleep periods and an increase in daytime sleep, making *Drosophila* a suitable model to study sleep also in the context of ageing (Vienne *et al.* 2016). Interestingly, a deep sleep like sleep state has been recently identified in *Drosophila*, which is characterised by repetitive stereotypically extensions and retractions of the proboscis. The deep sleep stage was accompanied by an increased waste clearance and reduced injury-induced mortality (van Alphen *et al.* 2021).

Several methods have been established to measure sleep in *Drosophila*. Initially, sleep was defined as a period of inactivity enduring at least five minutes. Based on that definition, actigraphy was used to assess sleep by monitoring the activity of captured flies through interruptions of an infrared beam. This technique, which is also known as *Drosophila* activity monitoring system (DAM), enables the assessment of activity, sleep-like states and in addition circadian rhythm in a great scale and cost-efficient manner (Figure 3A). A drawback of this approach is the inability to distinguish between sleep and quiescence and therefore total sleep time might be overestimated. A more precise but also more elaborate method is to measure sleep by video tracking. Since a deep sleep stage in *Drosophila* was recently identified and linked to repetitive extensions and retractions of proboscis, this system allows to obtain more detailed information also about the sleep depth in tethered flies (Shafer and Keene 2021, van Alphen *et al.* 2021). Besides, more elegant, and complex methods have evolved to measure sleep and sleep intensity more precisely by assessing local field potentials across broad regions of the fly brain (Figure 3B). Sleep was linked to decreased overall brain activity and a reduced frequency of oscillations (7-10 Hz), which was connected to transitional sleep. Furthermore, deeper sleep intensities were observed during sleep bouts lasting longer than 15 and 30 minutes (Alphen *et al.* 2013, Shafer and Keene 2021). Comparable to mammals, flies also exhibit reduced whole-body metabolic rates, especially after longer periods of sleep, and therefore measurement of metabolic rate is another suitable method to study sleep (Figure 3C). Additionally, the quantification of arousal threshold has gained importance, since it is a relatively easy way to obtain information about sleep depth in flies individually (Figure 3D). Different stimuli (e.g., olfactory, or mechanical) have been established that can be used in different intensities to evaluate sleep depth. Experiments have revealed that the arousal threshold increases after 5 minutes of sleep onset to ~ 15 minutes of sleep, suggesting the existence of different sleep stages that are linked to different arousal thresholds (Alphen *et al.* 2013, Faville *et al.* 2015, Shafer and Keene 2021).

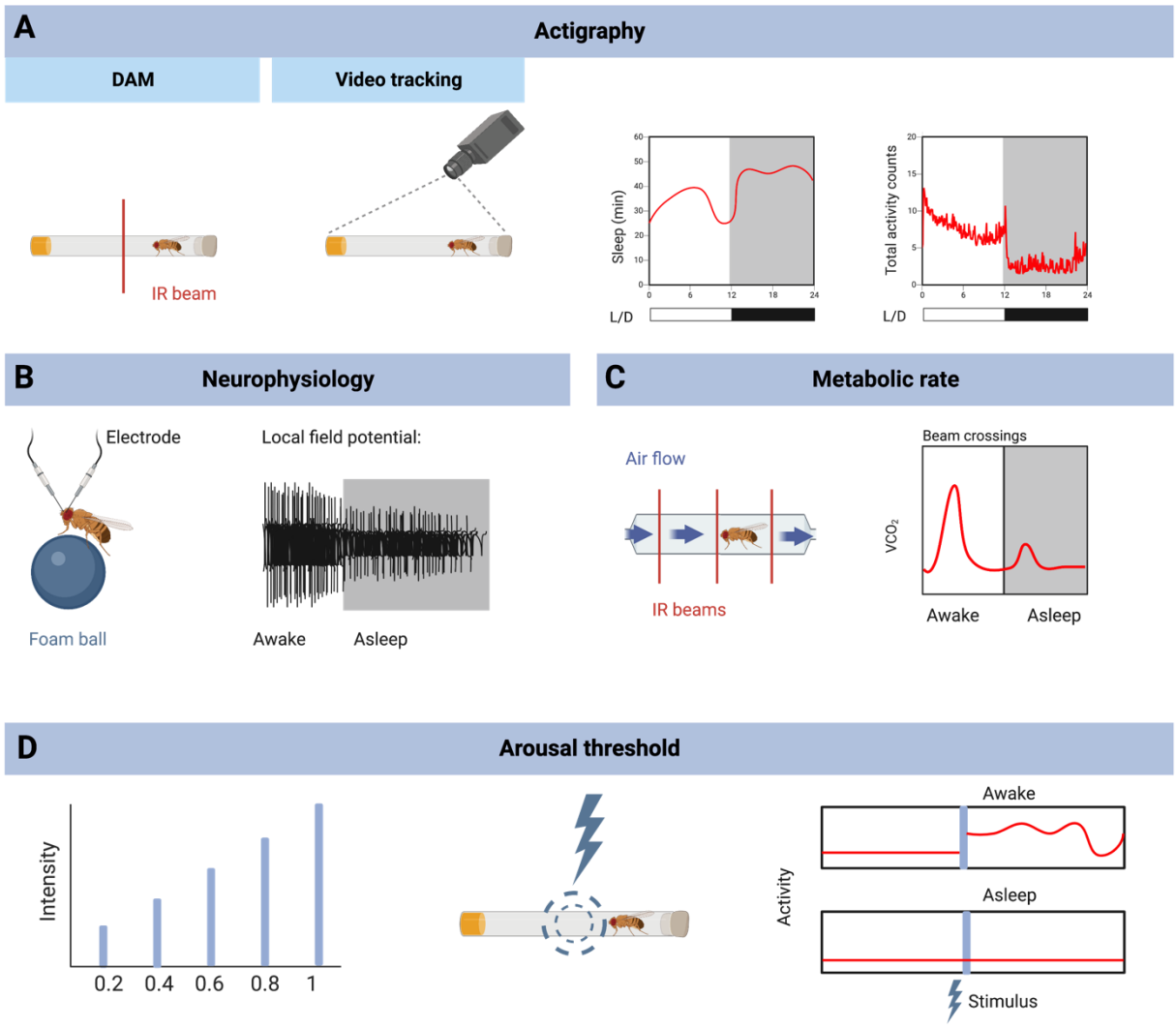


Figure 3: Techniques to assess sleep in *Drosophila*.
Adapted from (Shafer and Keene 2021). (A) Activity monitoring (DAM) and video tracking are established actigraphy methods. Sleep is defined as period of quiescence for at least 5 minutes. DAM assesses activity by measuring disruptions of an infrared (IR) beam. Video tracking can be analysed manually or with software. Actigraphy assesses sleep and activity and the respective length of sleep and activity bouts. (B) Neurophysiological techniques are used to record local field potential in individual flies. Locomotor activity can be assessed simultaneously by measuring the tethered fly's movement on the ball. (C) Simultaneous measurement of locomotor activity with IR beams and CO_2 output gives information about metabolic rate during wake and sleep states. (D) Arousal threshold is measured by using different intensities of stimuli and simultaneous tracking of locomotor activity. Altered activity upon varying stimuli intensities indicates depth of sleep. Created with Biorender.

1.2.5.1 The regulation of sleep in *Drosophila*

In-depth investigations of sleep regulation in *Drosophila* have discovered seven neurotransmitters involved in the regulation of sleep-wake states. Dopamine, octopamine, and histamine are key players in promoting wakefulness, while serotonin and gamma-aminobutyric acid (GABA) primarily promote sleep. On the other hand, acetylcholine and glutamate have dual functions, with distinct effects on sleep or wake-states dependent on specific brain circuits (Ly et al. 2018). These neurotransmitters also play crucial roles in signalling-pathways within neurons that innervate the mushroom body, a region involved in olfactory learning and the regulation of sleep. For instance, dorsal medial paired neurons release GABA onto wake-promoting α'/β' mushroom body neurons, promoting sleep through the action of Serotonin and GABA (Haynes et al. 2015). Conversely, protocerebral anterior medial (PAM) and PPL1 clusters of dopaminergic neurons innervate the mushroom body and contribute to wakefulness (Aso et al. 2014, Sitaraman et al. 2015). To better understand the mechanisms controlling sleep, various nutritional, pharmacological, and genetic interventions have been employed in *Drosophila* studies (Yang et al. 2015). Thus, starvation-induced increased activity serves as a proxy for octopamine-signalling in flies. Notably, dopamine-signalling emerges as a major regulator of sleep and activity in flies (Figure 4). Upon activation, dopaminergic neurons undergo membrane polarization, leading to the opening of voltage-dependent calcium channels and subsequent calcium influx into the presynaptic cell. This process triggers the release of dopamine-containing vesicles into the synaptic cleft via exocytosis (Greer et al. 2005, Yamamoto and Seto 2014). Dopamine then binds to one of the four G-Protein coupled dopamine receptors (Dop1R1, Dop1R2, Dop2R, and DopEcR) in *Drosophila*, initiating a signalling cascade that mediates its effects (Gotzes et al. 1994, Sugamori et al. 1995, Feng et al. 1996, Han et al. 1996, Hearn et al. 2002). The activation of Dop1 receptors by dopamine results in cAMP activation, while the binding of dopamine to Dop2 receptors leads to its inactivation (Li et al. 2000, Yamamoto and Seto 2014). Dopamine is transported back into the presynaptic cells by the dopamine transporter (DAT). DAT is inhibited by methamphetamine hydrochloride (Meth) (Kume et al. 2005, Ueno and Kume 2014). The dopamine transporter (DAT) is responsible for re-uptake of dopamine into presynaptic cells. When inhibited by Meth DAT prevents dopamine re-uptake, leading to increased dopamine levels in the synaptic cleft. This results in constitutive activation of dopamine receptors and subsequently increased activity in both humans and flies (Kume et al. 2005, Hart et al. 2007). Consistent with the pharmacological effect of Meth, *fumin* (Japanese word for sleepless) mutant flies that carry a point mutation in the dopamine transporter exhibit hyperactivity and reduced sleep (Kume et al. 2005). The synthesis of dopamine also plays a crucial role in dopamine-signalling activity. The pharmacological inhibitor 3-Iodo L-tyrosine (3IY) inhibits tyrosine hydroxylase, the enzyme catalysing the rate-limiting reaction from L-Tyrosine to L-Dopa, a precursor of dopamine. Flies treated

with 3IY exhibit increased sleep due to lower dopamine secretion (Bainton et al. 2000, Yamamoto and Seto 2014).

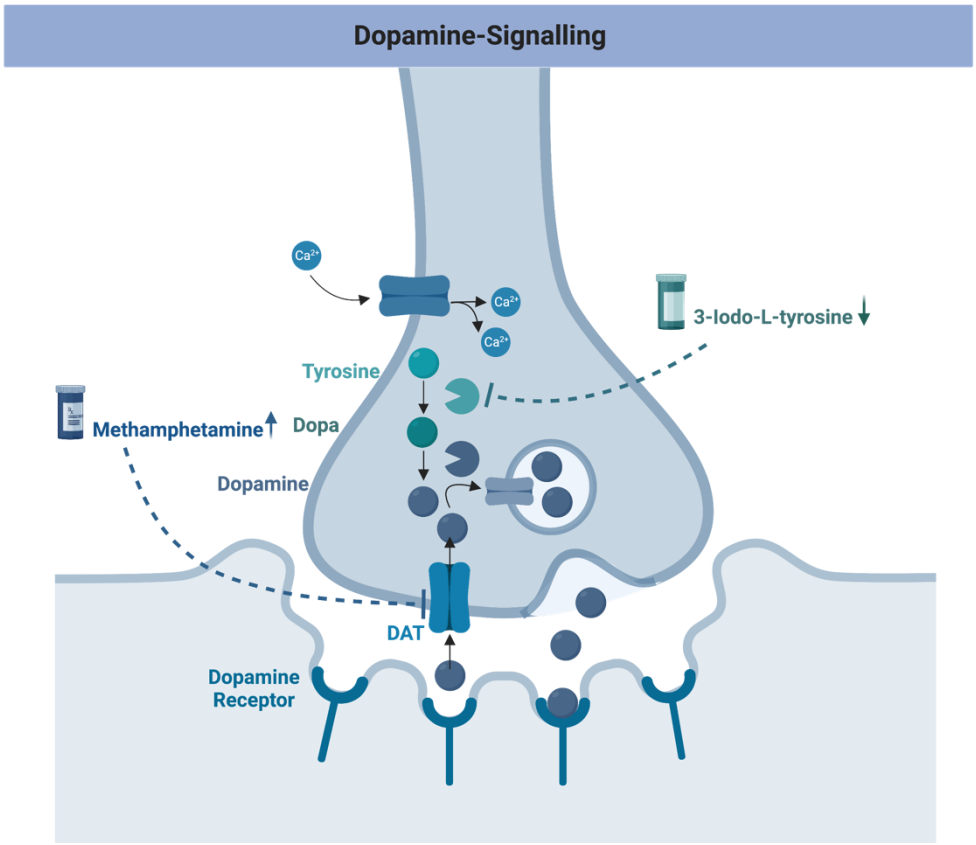


Figure 4: Pharmacological interventions on dopamine-signalling.
Tyrosine undergoes catalysis to be converted into Dopa, a precursor of Dopamine, which is then further catalysed to form Dopamine. Dopamine is stored in presynaptic vesicles. Upon presynaptic cell depolarization, the dopamine-containing vesicles fuse with the membrane and release Dopamine through exocytosis. Dopamine subsequently binds to the dopamine receptor on the postsynaptic cell, initiating a signalling cascade. The dopamine transporter (DAT) then transports Dopamine back into the presynaptic cell, facilitating its recycling. Pharmacological interventions such as methamphetamine can be used to activate dopamine-signalling, whereas treatment with the tyrosine hydroxylase inhibitor 3IY results in reduced dopamine-signalling activity. Created with Biorender.

1.2.5.2 The influence of sleep on lifespan in *Drosophila*

Despite significant progress in understanding the regulation of sleep in *Drosophila*, extensive research on the underlying mechanisms, and the establishment of various pharmacological and genetic tools to facilitate in-depth sleep research, the impact of sleep on lifespan remains unknown. It is still debated if sleep quality and length might correlate to lifespan. Several mutants (*insomnia-like, Hk^{1}, Hk^{Y}* and *Hk^{2}*) exhibiting a decreased sleep duration also have a shortened lifespan (Seugnet *et al.* 2009, Bushey *et al.* 2010). In line with these findings, the previously described long-lived *dilp 2-3,5* exhibited improved sleep quality during ageing with increased nocturnal sleep length and daytime activity and reduced sleep fragmentation (Metaxakis *et al.* 2014). In contrast, a study that investigated lifespan of eight representative fruit fly lines from the Sleep Inbred Panel, showed that flies with short-sleep periods

had an increased lifespan, accompanied by a reduced ageing rate compared to flies with long-sleep (Thompson *et al.* 2020). However, the tested flies displayed relatively short lifespans and were only compared to one another and not to a control group with an intermediate sleep length. Another publication claims that sleep deprivation is not necessarily detrimental for lifespan (Geissmann *et al.* 2019). Nevertheless, sleep deprivation was previously reported to be associated with a severe physiological and cognitive decline (McDermott *et al.* 2003, Seugnet *et al.* 2009, Holth *et al.* 2019). Therefore, the observed findings might be explained by the underlying laboratory conditions that are less challenging compared to a natural environment. In addition, it is plausible that the tested flies were not completely sleep deprived and still had micro-periods of sleep (Geissmann *et al.* 2019). The contradictory findings underline that to date it remains elusive whether improved sleep quality might contribute to longevity.

1.2.5.3 A Potential role of Bitesize in insulin-signalling?

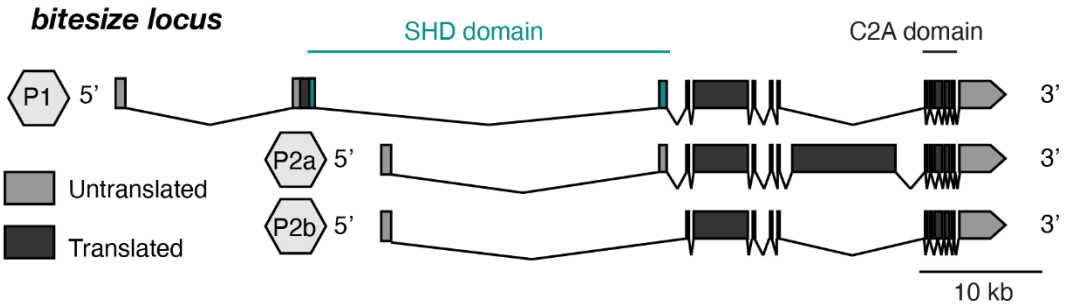


Figure 5: The *bitesize* locus in *Drosophila*.
Adapted from (Weigelt 2018). The *bitesize* locus encodes nine different transcripts that originate from two different promoters, termed P1 and P2. Only P1 isoforms encode for the highly conserved SHD domain (turquoise boxes).

In an RNA-sequencing based screen in long lived *dilp2,3-5* mutants (Weigelt *et al.* 2020), the *bitesize* gene locus was identified as a regulated gene and potential downstream effector of IIS. Apart from Synaptotagmin itself, the *bitesize* (*btsz*) gene encodes for the only Synaptotagmin-like protein in *Drosophila*. Two promoters, termed P1 and P2 control the expression of several isoforms, whereas only the transcript originating from the P1 promoter carries the highly conserved Synaptotagmin-like protein homology domain (SHD) (Figure 5). In mammals, the SHD domain is crucial for the interaction of Granuphilin, the homologue of Bitesize, with the GTP-bound form of Rab27a. The Rab27a/Granuphilin complex regulates the exocytosis of insulin-containing granules via interaction with Syntaxin 1a in pancreatic beta cells (Torii *et al.* 2004) (Figure 6). Interestingly, Bitesize P1 was also downregulated on a protein level in *dilp2,3-5* mutants (Weigelt 2018). Besides the SHD domain, the fly orthologue *btsz* also codes for two C2 domains that are located at the carboxyl terminal. C2 domains

often function as calcium sensors by targeting proteins to the membrane in a calcium-dependent manner, but C2 domains also have calcium-independent phospholipid binding properties. To date, only a few studies have investigated the *bitesize* gene and mostly focused on the P2 isoforms. These isoforms have been shown to interact with Moesin and thereby organizing actin in a local domain, ultimately contributing to the stabilization of E-cadherin in primary embryonic epithelia (Pilot *et al.* 2006). In addition, another study demonstrated that several *bitesize* transcripts originating from the P2 promotors are localized to the apical membrane, especially in follicle cells and eye imaginal discs. Furthermore, mutations of *bitesize* resulted in a smaller cell size and number, thus leading to prolonged development and smaller flies (Serano and Rubin 2003). The most recent study in 2023 suggested that *bitesize* isoforms lacking the Moesin Binding Domain also influence actin remodelling by direct interaction of the C-terminal end of Bitesize with F-actin bundles and thereby ensuring proper embryo development during syncytial stages (Yeh *et al.* 2023).

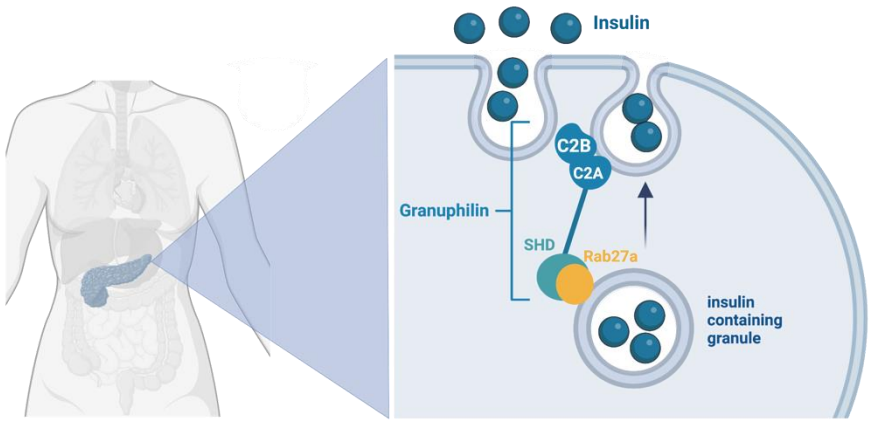


Figure 6: The function of Granuphilin in insulin secretion.
In mammals Granuphilin is expressed in pancreatic beta cells, where it mediates the exocytosis of insulin. Granuphilin interacts via its SHD domain with the vesicle trafficking protein Rab27a. Additionally, Granuphilin contains two C2 domains that ensure targeting Granuphilin to the membrane. Created with Biorender.

To address the function of the Btsz P1 protein in the context of sleep and longevity, our lab previously generated a mutant that carries a 160 bp deletion around the ATG start codon of the P1 isoform. Interestingly, *btsz P1Δ* mutants weighed less, laid fewer eggs and were longer lived than wild type control flies (Weigelt 2018), reminiscent of IIS mutant flies. Bitesize P1 is exclusively expressed in the brain. By using a green-fluorescent-protein (GFP)-reporter line, we could show that the protein is specifically expressed in the mushroom body and antennal lobes (Weigelt 2018). Interestingly, expression of Btsz P1 colocalized with Rab27, which is described as an interaction partner of the mammalian orthologue. Since the mushroom body is crucial for the regulation of sleep, activity and sleep was measured in *btsz P1Δ* mutants. Notably, male *btsz P1Δ* mutants were significantly less active and slept significantly more than wild type controls, indicating a potential role of Btsz P1 in the regulation of sleep. However, the underlying mechanism of a Btsz P1 dependent regulation of sleep and how improved sleep might contribute to an extension in lifespan remains to be elucidated.

1.3 Aims of this Study

Deciphering the role of the Bitesize protein in the regulation of sleep and longevity

- Analysis of the Bitesize P1-dependent regulation of sleep in male and female flies
- Characterisation of the function of Bitesize P1 in the regulation of sleep during ageing
- Examination on the influence of Bitesize P1 on healthspan
- Investigation of the underlying signalling pathway by which Bitesize P1 regulates sleep
- Identification of potential interaction partners of Bitesize P1

MATERIAL AND METHODS

2. Material and Methods

2.1 Fly work

2.1.1. Fly maintenance

Flies were kept in glass bottles or plastic vials on food containing sugar-yeast-agar (SYA) (Table 1) at 25 °C, 65 % humidity and a 12-hour light-dark cycle. Food was prepared by boiling sugar, yeast, and agar, followed by cooling to 60 °C. Nipagin (Methyl 4-hydroxybenzoate) and propionic acid were added subsequently to prevent bacterial and fungal infections.

Table 1: Ingredients 1x SYA

Ingredients	Quantity
Sugar	5 %
Yeast	10 %
Agar	1.5 %
Nipagen (10 % in EtOH)	0.3 %
Propionic Acid	0.03 %

Experimental flies were maintained in populations of 25 male or female flies per vial. Flies were transferred to new vials with fresh food three times a week. As control, *white*^{Dahomey} (*wDah*) flies were used, which were generated in our laboratory by backcrossing the *w*^{1118} allele into the outbred wild type *Dahomey* strain (Grönke *et al.* 2010). All flies in the experiment carried the endosymbiotic bacterium *Wolbachia pipientis*.

To generate experimental flies, males and females of the desired genotypes were crossed in cages containing grape-juice plates. A small amount of fresh yeast paste was added to the grape juice-agar plate to stimulate egg laying. Eggs were collected over a 16–20-hour period into 15 ml falcon tubes containing phosphate-buffered saline (PBS). Eggs were allowed to sediment by gravity, and the supernatant was removed. 20 µL of the eggs were then dispensed to fresh bottles using a cut pipette tip. 24 hours after eclosion, adult flies were transferred to fresh bottles and allowed to mate for 2 days unless specified otherwise. Mated male and female flies were sorted under light anaesthesia using CO_2. Virgins were sorted on glass petri dishes containing ice.

2.1.2 Transgenic fly lines

In this study, we used the following transgenic fly lines (Table 2). All constructs or mutations were backcrossed into the *wDah* wild-type strain for a minimum of six generations.

Table 2: Transgenic fly lines used in this study

Fly line	Source
btsz P1Δ	Carina Weigelt (Weigelt 2018)
Ca-alpha1T del	Bloomington #68200
Ca-alpha1T-Gal4	Bloomington #68201
GFP::Btsz P1	Carina Weigelt (Weigelt 2018)
Dilp 2-3,5	(Grönke *et al.* 2010)
Dilp2-Gal4	Bloomington #37516
fumin	Kazuhiko Kume (Kume *et al.* 2005)
TH-Gal4	Bloomington #51982
UAS-mCherry	Partridge Lab (Javier Moron)
wDah	(Grönke *et al.* 2010)

2.1.3 Food for sleep experiments

Sleep experiments were conducted using standard food (1x SYA) or drug-containing food. The following drug concentrations were used, if not indicated otherwise in the figure legends (Table 3).

Table 3: Drug concentrations used for sleep experiments

Drug	Dilution	Final Concentration	Food	Control
3IY	500 mg in 100 ml SYA	5 mg/ml	1x SYA	1x SYA
Meth	100 mg in 1000 µl EtOH	1 mg/ml	1x SYA	1x SYA + EtOH
Gaboxadol	Stock solution: 100 mg Gaboxadol in 4.5 ml ddH_2O Experiment: 10 mg in 450 µl ddH_2O in 50 µl 1x SYA	0.2 mg/ml	1x SYA	1x SYA + ddH_2O

Starvation assays were performed using food containing 1 % agarose in ddH2O, with 1x SYA serving as the control food.

2.1.4 Lifespan analysis

To assess the lifespan of flies, mating was conducted for two days prior to the experiment, unless otherwise specified. Flies were sorted into either glass vials (10 flies per vial) or plastic vials (20 flies per vial). 15 replicates per genotype were used for glass vials and 8-10 replicates for plastic vials. Flies were transferred to vials containing fresh sugar-yeast-agar food three times a week, and deaths were scored.

2.1.5 Development assay and body weight

For the development assay, 25 eggs laid within a 6-hour time window at 25 °C were collected and transferred to a plastic vial per condition. After eclosion, male and female flies were counted twice a day, and their weight was measured. 10 biological replicates per genotype were used, totalling 250 eggs.

To assess body weight, groups of two flies were temporarily anesthetized using CO_2 and weighed using an ME235S analysis balance (Sartorius Mechatronics). A total of 20 flies for each sex per genotype was measured.

2.1.6 Fecundity

To determine fecundity, 20 female flies were transferred to fresh glass or plastic vials, where they were allowed to lay eggs overnight (approximately 16 hours). The number of eggs laid was manually scored for each replicate (n=10).

2.1.7 Climbing Assay

For the climbing assay, flies were acclimated in climbing plastic tubes for one hour prior to the experiment. 20 flies were used per genotype. The flies were then transferred to the first tube of the climbing apparatus (Benzer 1967), which consisted of six compartments in total. The flies were tapped to the bottom of the vial and allowed to climb a distance of 15 cm for 20 seconds. After the climbing period, flies that reached the upper vial were transferred to the second compartment. This process was repeated five times. The climbing index (CI) (Greene *et al.* 2003) was calculated based on the number of flies in each compartment, resulting in a score (lowest = 0 = all flies in vial 1, highest = 1 = all flies in vial 5).

2.1.8 Activity Measurement

In order to analyse the sleep and activity patterns of adult flies, the DAM2 activity monitor system was employed (Trikinetics). Individual seven-day-old male or virgin female flies were single-housed in plastic or glass tubes containing fresh SYA with or without drugs. To prevent food from drying out, the tubes were sealed with paraffin. To mitigate potential batch effects based on the flies' positions in the chamber, their placement was randomized. The flies' activity was monitored continuously for at least four days, with 32 flies per treatment group used in each experiment, if not indicated otherwise. After one day and one night of habituation in a 12-hour light and 12-hour dark cycle (LD), activity was measured for at least three consecutive days. In the figures, the second day and night of the experiment are representatively depicted.

The DAM system measures the activity of each fly using an infrared beam that passes through each tube. Every 5 minutes, the Trikinetics Software tracks how often the beam is interrupted by the flies' movements. To analyse the various sleep parameters, an R script (R.1.7) developed previously in the lab by Jonathan Paulitz was utilized (unpublished). In flies, sleep is defined as a period without movement lasting at least five minutes (Shaw *et al.* 2000, Huber *et al.* 2004). All 5-minute periods without movement per day or night were summed up to calculate the total sleep duration. Sleep periods without interruptions are termed "sleep bouts”. The R script thus counted the number of sleep bouts per day or night without disruptions. To determine the uninterrupted sleep duration, the total sleep duration was divided by the number of sleep bouts, resulting in the sleep bout length, which serve as an indicator for sleep quality. Conversely, the number of beam interruptions indicates activity. Total activity was analysed by summing up all activity counts during the day or night. To calculate the activity duration, all active 5-minute intervals were summed up. Activity bouts were analysed by calculating all active periods without sleep disruptions. The activity bout length was then calculated by dividing the activity duration by the number of activity bouts (Huber *et al.* 2004).

2.2 Molecular biology methods

2.2.1 Extraction of genomic DNA

2.2.1.1 Extraction of genomic DNA of single legs

In order to genotype flies through PCR, genomic DNA (gDNA) of single legs was isolated. To collect legs, flies were anaesthetised on a CO_2 and one leg per fly was collected using forceps. Then legs were transferred to a 96-well plate, containing 50 µl of Squishing buffer (Table 4) with freshly added Proteinase K at a final concentration of 200 µg/ml. The 96-well plate was then incubated Veriti 96-well thermal cycler (Applied Biosystems) at 37 °C for 30 minutes, following a step at 95 °C for 5 min to inactivate Proteinase K. When not used immediately for PCR, the 96-well plate containing the gDNA was sealed and stored at 4°C.

2.2.1.2 Extraction of genomic DNA of whole flies

To genotype flies through PCR, genomic DNA was isolated from individual whole flies. First, single flies were homogenized with a pipette tip in a tube containing 50 µl of Squishing buffer (Table 4) with freshly added Proteinase K at a final concentration of 200 µg/ml. Subsequently, the homogenate was incubated at 37 °C for 30 minutes. After inactivating the Proteinase K at 95 °C for 5 minutes, the fly homogenate was centrifuged for 15 minutes at 16,000 x g, and the resulting supernatant containing gDNA was either used immediately for PCR or stored in a new tube at 4°C until further use.

Table 4: Ingredients for 500 ml Squishing Buffer

Ingredients	Quantity	Concentration
Tris Base (pH 8.2)	0.61 g	20 mM
EDTA	0.15 g	1 mM
NaCl	0.73 g	25mM

2.2.2 Polymerase Chain Reaction (PCR)

Standard genotyping PCRs were conducted using HotStar Taq Plus MasterMix (Qiagen) as per the manufacturer's instructions for the amplification of a gene of interest. Therefore, 1 µl (gDNA extracts from whole flies) or 7 µl (gDNA extracts from fly legs) of the DNA template without addition of water was used for each PCR reaction.

Table 5: Ingredients for PCR per sample

Ingredients	Quantity
Mastermix (2x)	10 µl
Forward Primer (10 µM)	0.5 µl
Reverse Primer (10 µM)	0.5 µl
DNA	1 µl
Loading Dye	2 µl
Water	6 µl
Total Volume (per sample)	**20 µl**

The following primers were used for genotyping:

Table 6: Primers used for genotyping.

Name	Target	Fwd/Rev	Sequence
CW109	bitesize (first coding exon)	fwd	TGGAATTTGCAGAATATTCACGG
CW119	bitesize (first coding exon)	rev	CGTCTCTGTTGAGCACCTCG
CW440	fumin wild type allele	fwd	GCCCTCTTTCCCTATGCAGT
CW441	fumin wild type allele	rev	CAGCGTAACGATCCAGCAGA
CW442	fumin mutated allele	fwd	ATGCGATTCTCATGTCACCT
CW443	fumin mutated allele	rev	AGGGAAAACAGTCCAGCTAC
AJ019	Gal4 construct	fwd	AACAACTGGGAGTGTCGCTA
AJ020	Gal4 construct	rev	TGGAACCTGACTCGAAGACC
AJ021	Ca-alpha1TΔ	fwd	CCCTTTGTCCCCTGATCAGT
AJ022	Ca-alpha1TΔ	rev	CTGTGCGTGTGTGGTCATAG
AJ029	Ca-alpha1T Gal4	fwd	CAAACACAGACACAGACGCA
AJ030	Ca-alpha1T Gal4	rev	GTGGTGTTGGTTGTGGTGTT
AJ033	pValium10 in Rab27 RNAi	fwd	CGCAGCTGAACAAGCTAAAC
AJ034	pValium10 in Rab27 RNAi	rev	GACCTTCAACCACCTTGCAG

gDNA was transferred into a 96-well plate and the PCR was run in a Veriti 96-well thermal cycler (Applied Biosystems) according to the following protocol with 35 cycles of step 2-4.

Table 7: PCR cycles

Step	Temperature	Duration
1) Initial Denaturation	95 °C	15 min
2) Denaturation	94 °C	15 sec
3) Annealing	55 °C	30 sec
4) Extension	72 °C	2 min
5) Final Extension	72 °C	2 min

2.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse the PCR fragments. The gel consisted of 1.5 % agarose, 1x TAE buffer, and 10 µl of SYBR SAFE DNA Stain (Invitrogen) per 100 ml to visualize the DNA. The DNA fragments were separated using Sub Cell GT horizontal electrophoresis cells (BioRad) at 120-130 mV for 40-60 minutes. As a reference for DNA fragment sizes, we used the 50 bp Hyperladder (Bioline).

Table 8: Ingredients TAE Buffer (50x)

Ingredients	Quantity
Tris Base	242 g
Acetic Acid (1 M)	57.1 ml
EDTA (0.5 M)	100 ml
ddH_2O	Ad 1000 ml

2.2.4 RNA extraction

To extract total RNA, Trizol (Invitrogen) was used. During the whole protocol samples were kept on ice. First, 100 µl Trizol were added, and samples were homogenized using a handgun and RNase-free pestles. Next, 650 µl Trizol were added, and samples were mixed. After adding 200 µl Chloroform, samples were vortexed for 15 sec each and incubated on ice for 3 min. A centrifugation step at maximum speed at 4 °C for 15 min followed. Next, the upper aqueous layer containing RNA was transferred to a new RNase free tube. One volume Isopropanol was added, then samples were mixed, and 1/10 volume of Sodium Acetate were added. Subsequently, RNA was precipitated at -80 °C for at least 45 min. After precipitation, frozen samples were centrifuged at maximum speed at 4 °C for 30 min. Isopropanol was removed and the pellet was washed two times with 700 µl cold 70 % RNase free EtOH and centrifuged at maximum speed at 4 °C for 15 min each. Subsequently, most EtOH was removed, and samples were centrifuged briefly again. Residual EtOH was removed with a 10 µl pipette and the pellet was briefly airdried. Finally, RNA was resuspended in 20 µl RNAse free H_2O, if DNase

treatment (ThermoFisher) was not required. RNA concentration was determined using the Qubit BR RNA assay (ThermoFisher).

2.2.5 cDNA Synthesis

To generate cDNA of mRNA the SuperScript III first-strand synthesis kit (Invitrogen) with oligo(Dt)$_{20}$ primers was employed. 600 ng of total RNA was utilized for cDNA synthesis.

2.2.6 Quantitative Real-Time PCR (q-RT-PCR)

For q-RT-PCR of mRNA, PowerUp SYBR Green Master Mix (ThermoFisher) was used following the manufacturer's instructions. q-RT-PCR was performed using QuantStudio7 (ThermoFisher). Relative expression (fold induction) was calculated using the ΔΔCT method with Rpl32 as the normalization control.

Table 9: Primer used for q-RT-PCR

Name	Target	Fwd/Rev	Sequence
SOL268	Rpl32	fwd	ATATGCTAAGCTGTCGCACAAATGG
SOL269	Rpl32	rev	GATCCGTAACCGATGTTGGGCA
CW049	Btsz P1	fwd	AGTCGGTGAGGCAGAGAGAT
CW071	Btsz P1	rev	GGTTGTTGTGTGGCATCGAG
AJ031	Rab27	fwd	AGGAGCGCTTCCGTTCACTA
AJ032	Rab27	rev	GCCGTCTCCAGGAAGCTCTT

2.2.7 RNA sequencing

RNA was extracted using Trizol (Invitrogen) followed by a DNase (Qiagen) treatment. Quality control was performed by using RNA ScreenTapes in the Agilent 4200 TapeStation System (Agilent). Three biological replicates were used per genotype and PolyA-enriched libraries were generated at the Max-Planck-Genome-Centre Cologne (Germany). RNA sequencing was performed with a Hiseq 3000, with 16 million single-end reads per sample, and 150 bp length. The raw sequence reads underwent alignment using HISAT2 (Kim *et al.* 2015) version 2.1.0 against the BDGP6 (release 90) reference genome. To filter multi-mapped reads, SAMtools (Li *et al.* 2009) was used. Visualization and analysis of data were conducted using SeqMonk, Genome Workbench, and R, leveraging specific Bioconductor packages including EdgeR (Robinson *et al.* 2010), topGO, and org.Dm.eg.db. Differentially expressed genes were considered significant with an adjusted p-value of less than 0.05, and no specific fold change cut-off was applied. In cases where not otherwise specified, the pool of expressed genes served

as the background for all functional enrichment analyses including expression data. Analysis was performed by Nathalie Jauré.

2.3 Confocal imaging

2.3.1 Immunostaining of dissected fly tissue

Flies were anesthetized using ice and then transferred to silicon plates. Tissues were dissected with fine tweezers (No. 5 Dumont) in PBS and collected in 1.5 ml Eppendorf tubes. The tissues were fixed in 4 % paraformaldehyde in PBS at 4 °C on a nutating mixer for 2 hours. The subsequent steps were also carried out on a nutating mixer. Tissues were washed in 1 ml of PBT (PBS + 0.5 % Triton-X) six times, each time for 30 minutes. After that, the tissues were blocked in 1 ml of blocking buffer (PBT + 5 % fetal bovine serum + 0.01 % sodium azide) at room temperature for 1 hour to prevent unspecific binding. Primary antibodies were used and diluted in the blocking buffer, as indicated in the following table.

Table 10: Dilution of primary antibodies

Primary Antibody	Species	Dilution	Company	RRID
GFP	Chicken	1:250	Life Technologies	AB_2534023
TH	Mouse	1:100	Immunostar	AB_572268
mCherry	Rabbit	1:200	Abcam	AB_2571870

Following an overnight incubation at 4 °C, tissues underwent six washing steps with PBT, each lasting 30 minutes. Subsequently, the tissues were incubated with suitable Alexa Fluor secondary antibodies (diluted 1:1000 in blocking buffer) overnight at 4 °C. Afterwards, the tissues were washed six times with PBT for 30 minutes each, following an incubation in 50 % glycerol in PBS for 30 minutes. For mounting on a microscope slide, Vecta Shield Antifade Mounting Medium with DAPI (VectorLabs) was used. The imaging was performed using a Leica SP8-DSL confocal microscope.

Table 11: Secondary antibodies

Secondary Antibody	Species	Against	Company	RRID
Alexa 488	Goat	Chicken	Molecular Probes	AB_142924
Alexa 568	Goat	Mouse	Molecular Probes	AB_2534072
Alexa 633	Goat	Rabbit	Molecular Probes	AB_141419

2.3.2 Confocal imaging of endogenous expression

Brains were manually dissected in PBS and fixed with 4 % paraformaldehyde for 15 min at room temperature in the dark. Subsequently, brains were washed two times with PBT (PBS + 0,5 % Triton-X) for 15 min each. Brains were then incubated in 50 % glycerol in PBS for 30 min in the dark. Finally, brains were mounted on slides with a spacer to avoid tissue damage in VectaShield Antifade Mounting Medium with DAPI (Vectorlabs). Imaging of endogenous GFP or mCherry was done using a Leica SP8-X or Leica SP8-DLS confocal microscope.

2.4 Statistical analysis

GraphPad Prism was employed for statistical analysis, and specific statistical tests are indicated in the figure legends. Two-way ANOVA was consistently followed by a multiple comparison post-hoc test. Lifespan experiments were recorded in Excel (Microsoft), and the log-rank test was utilized for statistical analysis. Significance levels were determined as follows: * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The Granuphilin homolog *bitesize* regulates sleep and longevity in *Drosophila melanogaster*

3. The Granuphilin homolog *bitesize* regulates sleep and longevity

3.1 Mutation of *bitesize* increases sleep in male and female flies

Bitesize P1 is expressed in the mushroom body of adult flies, a brain region that is involved in the regulation of sleep. In line with this finding, mutation of *btsz P1* resulted in increased sleep length and reduced activity in male flies. Interestingly, male and female *btsz P1Δ* mutants were also long lived compared to wild type flies (Weigelt 2018). However, it remains unknown whether the sleep phenotype of *btsz P1Δ* mutants is conserved in both sexes and whether it is associated with the observed lifespan extension. Studies have indicated that activity patterns are sexually dimorphic, with female flies exhibiting primarily nocturnal sleep compared to male flies which sleep during day and night, suggesting a sex-specific difference in the regulation of sleep (Koh *et al.* 2006). Therefore, we measured activity and sleep of 7-day old female virgin and male flies (Figure 7). Daytime activity was significantly reduced in male but not female *btsz P1Δ* mutants compared to control flies (Figure 7A). During the night, female and male *btsz P1Δ* flies were significantly less active than wild type flies (Figure 7B). Consistent with the findings of Koh *et al*., female flies slept in general less during the day compared to male flies, regardless of the genotype. However, significant differences in total sleep were observed in both sexes between *btsz P1Δ* mutants and wild type flies, with *btsz P1Δ* flies sleeping significantly more than *wDah* flies (Figure 7C). During the night, female and male flies slept comparably long in both genotypes, however, female, and male *btsz P1Δ* mutants slept longer than wild type flies (Figure 7D). There was no significant interaction between genotype and sex using 2-Way ANOVA analysis, suggesting that the *btsz P1* mutation affects sleep in a similar manner in both female and male flies.

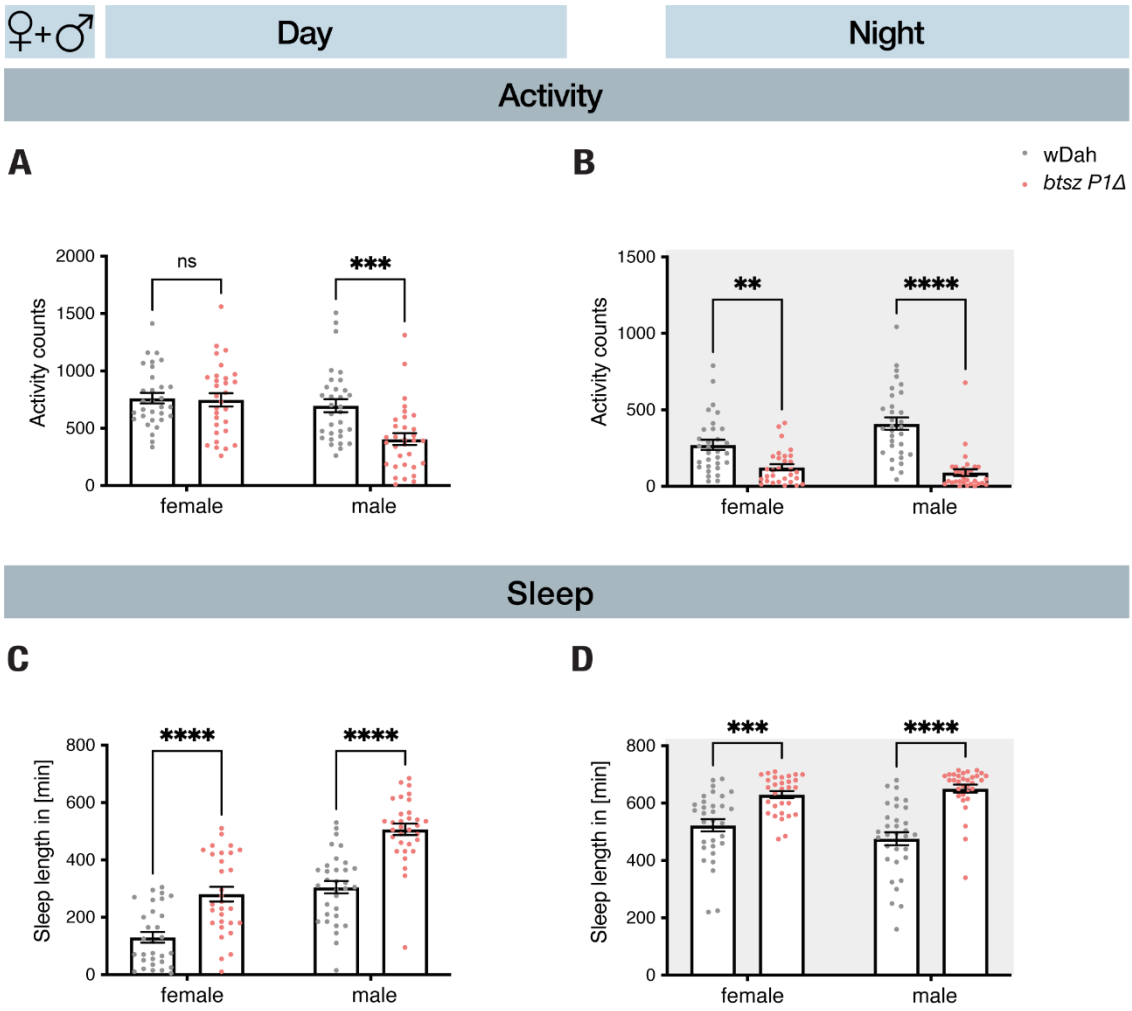


Figure 7: Female and male *btsz P1Δ* mutants exhibit reduced activity and increased sleep.
(A) During the night, 7-day old male *btsz P1Δ* mutants were less active than the respective wild type control (Interaction: *, gender: **** < 0.0001, genotype: ** < 0.01). (B) During the night, 7-day old female virgin and male *btsz P1Δ* flies were significantly less active than *wDah* control flies (Interaction: ** < 0.01, gender: ns., genotype: **** < 0.0001). (C) During the day, female flies of both genotypes slept less compared to their male counterpart. But female and male *btsz P1Δ* mutants slept significantly longer than their respective wild type control (Interaction: ns., gender: **** < 0.0001, genotype: **** < 0.0001). (D) During the night, female flies exhibited similar total sleep length compared to their male counterpart. However, significant differences with an increased total time spent asleep were observed in both, female and male *btsz P1Δ* mutants compared to wild type flies (Interaction: ns., gender: ns., genotype: **** < 0.0001). 2-way ANOVA with multiple comparison Tukey's post hoc test was performed for statistical analysis. Columns and errors bars represent mean and SEM (Mean + SEM). n=32 male or female flies per condition.

3.2 The role of Btsz P1 in the regulation of sleep during ageing

Mutation of Btsz P1 strongly impacted the macro architecture of sleep in young flies. However, whether this effect persists in older flies remains unknown. In both *Drosophila* and humans, sleep quality typically declines with age, characterised by increased sleep fragmentation in old flies and aged individuals (Koh *et al.* 2006, Metaxakis *et al.* 2014, Mander *et al.* 2017). Moreover, the clear separation between daytime activity and nocturnal sleep diminishes with age, as daytime sleep becomes more prominent (Mander *et al.* 2017). Given that sleep fragmentation and age-related sleep disorders significantly affect quality of life and overall health in humans, we asked whether the extended longevity observed in *btsz P1Δ* mutants is associated with improved sleep quality during ageing. To explore this, we conducted sleep experiments including young (day 2 and 5), middle-aged (day 25), and old (day 45) male flies (Jahn, 2019).

We measured total activity during day (Figure 8A) and night (Figure 8B). Total activity during the day was relatively stable with age, with no significant differences between the different age groups, both in *btsz P1Δ* mutants and wild type flies (Figure 8A). Nighttime activity did not significantly change with age in wild type males (Figure 8B). In contrast, nighttime activity constantly increased with advancing age in *btsz P1Δ* mutants, resulting in a significant difference between day 2 and 45 ($p^* < 0.05$). While young and middle-aged *btsz P1Δ* exhibited lower activity levels than wild type flies, at old age they showed comparable activity levels. A decrease in total activity in old age might indicate reduced mobility and should be considered when analysing total sleep.

Next, we assessed total sleep duration during day (Figure 8C) and night (Figure 8D). Notably, *btsz P1Δ* flies exhibited extended daytime sleep compared to wild type flies, and this prolonged sleep pattern persisted as the flies aged (Figure 8C). During nighttime, wild type flies experienced a decrease in sleep duration between days 2 and 25, with no significant further decline in older flies. Remarkably, young and middle-aged *btsz P1Δ* mutants maintained longer sleep durations, with middle-aged mutant flies showing total sleep levels similar to young wild type flies. However, sleep duration in *btsz P1Δ* mutants also declined during ageing, aligning their sleep duration with that of old wild type flies. In summary, *btsz P1Δ* flies exhibited increased daytime sleep duration, and increased nocturnal sleep persisted in young and middle-aged mutants.

An additional measure of sleep quality is sleep bout length, which reflects the duration of uninterrupted sleep periods. Sleep fragmentation is a common age-related symptom. Sleep bout length is calculated by dividing total sleep duration by the number of sleep bouts. During the day (Figure 8E), young *btsz P1Δ* mutants (2 and 5 days old) displayed longer sleep bout lengths, with 100.5

and 90.0 minutes respectively, compared to wild type flies with 42.1 and 42.3 minutes per bout. In middle-aged wild type flies, sleep bout lengths increased similarly to overall sleep duration, which indicates more daytime sleep in advancing age similar to humans. However, in old wild type flies, sleep bout length slightly decreased compared to middle-aged wild type flies, despite of increased sleep length, indicating more disrupted day time sleep. During the night (Figure 7F), young and middle-aged *btsz P1Δ* mutants exhibited increased sleep bout length compared to wild type flies. Notably, the constant decline in nighttime sleep bout length in wild type flies already occurred between days 2 and 25, with minimal further change by day 45. In contrast, sleep bout length in *btsz P1Δ* mutants remained relatively stable up to day 25 and only declined later in life. At 45 days, no significant difference in nocturnal sleep bout length was observed between *btsz P1Δ* mutants and wild type flies. In summary, wild type flies experienced a decline in sleep bout length early on, while *btsz P1Δ* mutants maintained longer uninterrupted sleep periods.

In summary, both wild type flies and *btsz P1Δ* mutants displayed relatively stable activity patterns during day and night. Similar to humans, total sleep duration increased during the day and decreased during the night already in middle-old wild type flies, whereas the decline in nocturnal sleep duration was delayed in *btsz P1Δ* mutants. Similarly, sleep fragmentation in *btsz P1Δ* mutants started to become prominent later on in life compared to wild type flies. Thus, *btsz P1Δ* mutants display a delayed on-set of age-associated sleep disturbances.

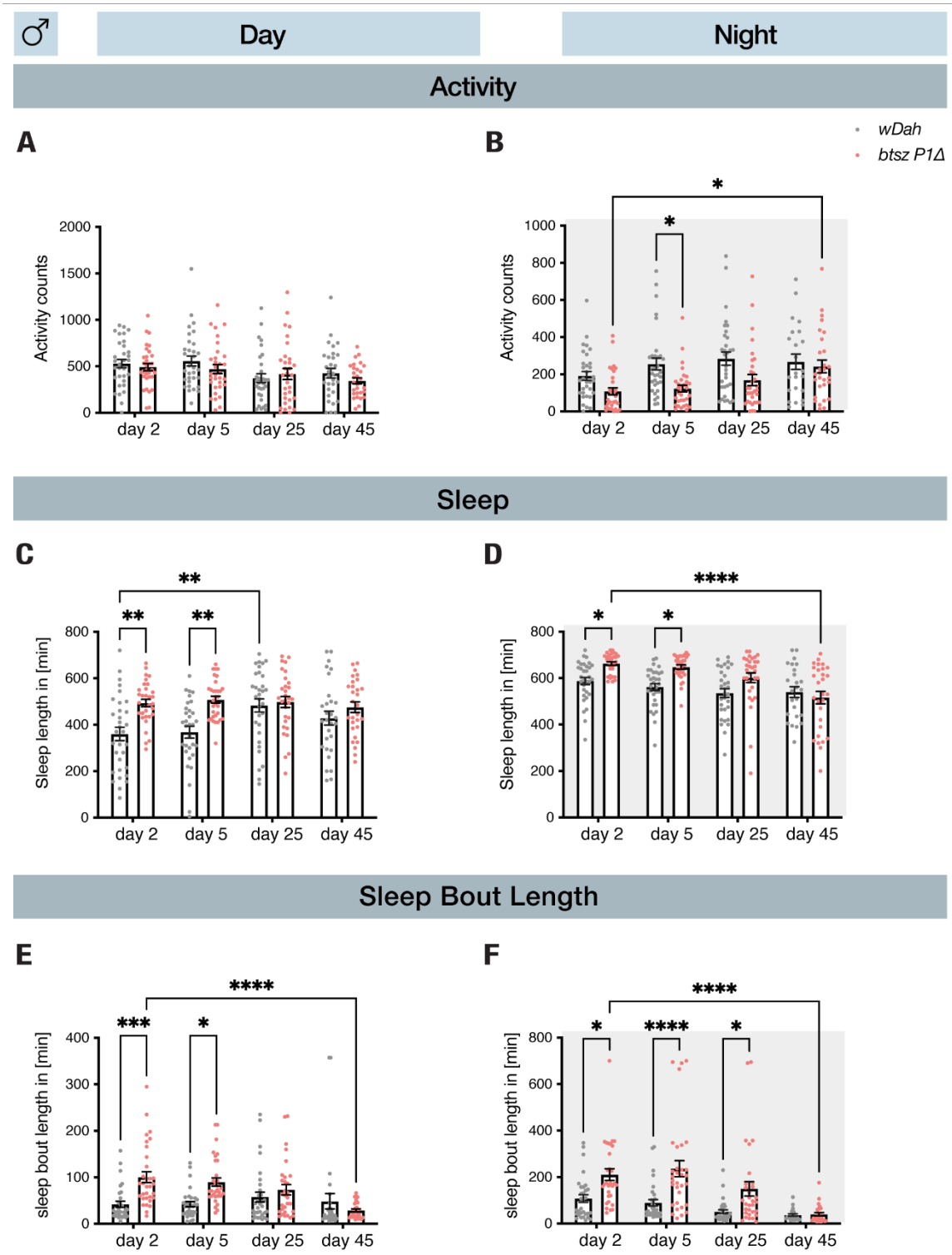


Figure 8: *btsz P1Δ* flies sleep more and have a delayed on-set of sleep disturbances during ageing.
Total activity, total sleep length and sleep bout length of 2-, 5-, 25- and 45-day old male flies was analysed. (A) Daytime activity slightly decreased in both genotypes during ageing (Interaction: ns., age: ** < 0.01, genotype: ns.). (B) Nocturnal activity constantly increased in advancing age in *btsz P1Δ* mutants, but the activity levels of young and middle-aged flies were lower than in wild type flies. In wild type flies total activity also increased in young and middle-aged flies, but decreased again in old flies, resulting in similar activity levels in wild type and *btsz P1Δ* flies (Interaction: ns., age: ** < 0.01, genotype: **** < 0.0001). (C) Total sleep length of *btsz P1Δ* mutants increased during the day compared to wild type flies during all ages (Interaction: * < 0.05, age: * < 0.05, genotype: **** < 0.0001). (D) Wild type flies showed a decrease in sleep length from day 2 to 25, which did not decline further. *btsz P1Δ* flies had an increased sleep length in young and middle old flies, but sleep length decreased in old age (Interaction: * < 0.05, age: **** < 0.0001, genotype: **** < 0.0001). (E) Analysis of sleep bout length during the day revealed that young *btsz P1Δ* flies had elevated sleep bout length during the day, but

Sleep bout length declined during ageing in old *btsz P1Δ* mutants, whereas sleep bout length in wild type flies was relatively stable (Interaction: *** < 0.001, age: ** < 0.01, genotype: *** < 0.001). (F) Nocturnal sleep bout length started at a higher level in *btsz P1Δ* mutants and declined more slowly during ageing in comparison to wild type flies. Sleep bout length of wild type and *btsz P1Δ* flies was similar in old flies (Interaction: * < 0.5, age: **** < 0.0001, genotype: **** < 0.0001). 2-way ANOVA with multiple comparison Tukey's post hoc test was performed. Columns and errors bars represent mean and SEM. (Mean + SEM). n=32 male flies per condition, however some of the flies had to be censored due to inactivity on multiple days, resulting in n=31 for 25-day old *btsz P1Δ* flies, n=25 for 45-day old wild type flies, n=29 for 45-day old *btsz P1Δ* flies. The results have also been described in a previous study (Jahn, 2019).

3.3 Btsz P1 mutants show improved climbing ability at old age

During ageing, physiological functions such as locomotion decline in humans, often caused by functional defects in the nervous system and musculature. Interestingly, *Drosophila* also exhibits age-related functional deficits, such as deteriorated locomotor ability. Locomotor ability in *Drosophila* can be assessed by evaluating negative geotaxis, flying and spontaneous walking. Startle-induced climbing (in the following referred to as climbing assay) is a well-established assay to measure negative geotaxis in *Drosophila* (Jones and Grotewiel 2011). To assess locomotor ability during ageing, we tested female and male flies on a weekly basis over a time course of six weeks. The climbing assay revealed that the locomotor ability of female and male wild type flies already started to decline significantly on day 7 of the experiment, which was not the case for female *btsz P1Δ* flies (Figure 9). Interestingly, climbing ability seems to deteriorate more strongly in female than in male wild type flies after day 21. During ageing, female and male *btsz P1Δ* flies had a significantly improved climbing ability, which was remarkably prominent from day 28 onwards, indicating that *btsz P1Δ* flies are less susceptible to the

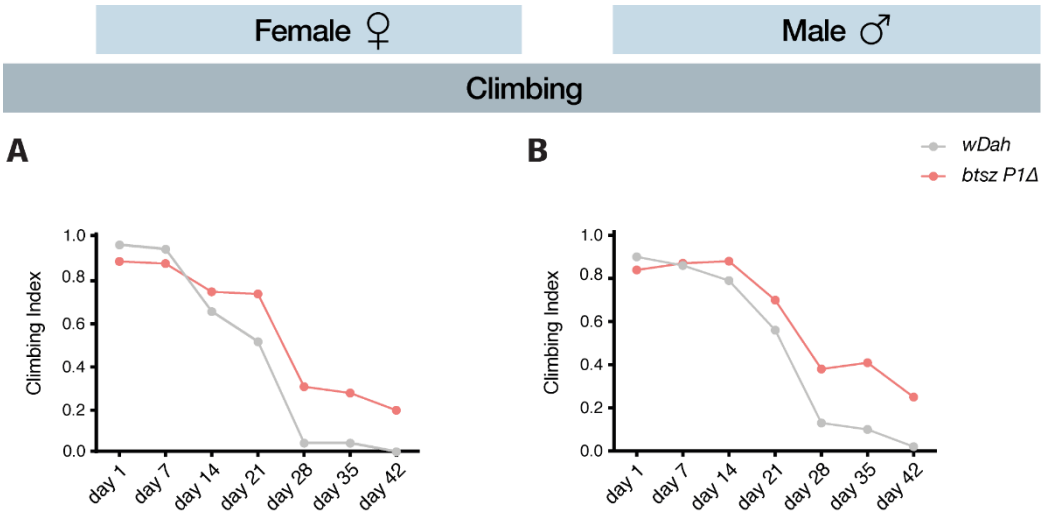


Figure 9: *btsz P1Δ* mutants exhibit an improved climbing ability during ageing.
Climbing assays of female (A) and male (B) flies during ageing revealed that *btsz P1Δ* mutants exhibit improved locomotion abilities compared to wild type controls (Interaction: **** < 0.0001., age: **** < 0.0001, genotype: **** < 0.0001) n=20. 2-way ANOVA with multiple comparison Tukey's post hoc test was performed for statistical analysis. The results were previously described in (Jahn 2019).

age-related decline in locomotor ability. The results also suggest that the observed sleep phenotypes of *btsz P1Δ* mutants are not caused by deficits in locomotor ability.

3.4 What is the underlying signalling-pathway by which Btsz P1 regulates sleep?

3.4.1 Mutation of Btsz P1 does not result in additive phenotypic effects in *dilp 2-3,5* mutants

Long-lived *dilp 2-3,5* mutant flies carry a deletion affecting insulin-like peptides 2, 3, and 5, leading to diminished insulin-signalling. Given the role of the mammalian homolog of Btsz P1 in insulin exocytosis and the downregulation of Btsz P1 protein levels in *dilp 2-3,5* mutants, we aimed to investigate the potential involvement of Btsz P1 in the insulin-signalling pathway. To this end, we used *btsz P1Δ, dilp 2-3,5* double mutant flies, aiming to ascertain whether the combined impact of both mutations contributes additively to the observed phenotypic outcomes.

Fecundity and lifespan experiments were conducted for control, *btsz P1Δ, dilp 2-3,5* and *btsz P1Δ, dilp 2-3,5* double mutants female flies. The fecundity assay confirmed that both *btsz P1Δ* and *dilp 2-3,5* 5 single mutants displayed a significant reduction in egg laying, consistent with previous findings (Figure 10A) (Weigelt 2018). Female wild type flies demonstrated an average egg laying of 13 eggs within 24 hours, whereas *btsz P1Δ* females laid approximate 8 eggs during the same timeframe. The most significant decline in fecundity was observed in *dilp 2-3,5* females, which only laid 1 egg within 24 hours. Fecundity was also reduced in *btsz P1Δ, dilp 2-3,5* when compared to *wDah* controls; however, this reduction was not significantly altered in comparison to *dilp 2-3,5* single mutants, since the fecundity was already low with less than 1 egg/ 24 hours. Therefore, a comparison between *dilp 2-3,5* single mutants and *btsz P1Δ, dilp 2-3,5* is not conclusive. Next, we conducted a lifespan experiment and showed that *btsz P1Δ* and *dilp 2-3,5* single mutants had an extended lifespan with median lifespans of 66.5 and 73.5 days, respectively, in comparison to wild type flies, which had a median lifespan of 59.5 days (Figure 10B)**.** In contrast, the combination of both mutations led to a reduction in median lifespan to 54.5 days. Thus, double mutants did not show additive effects on lifespan but were short lived.

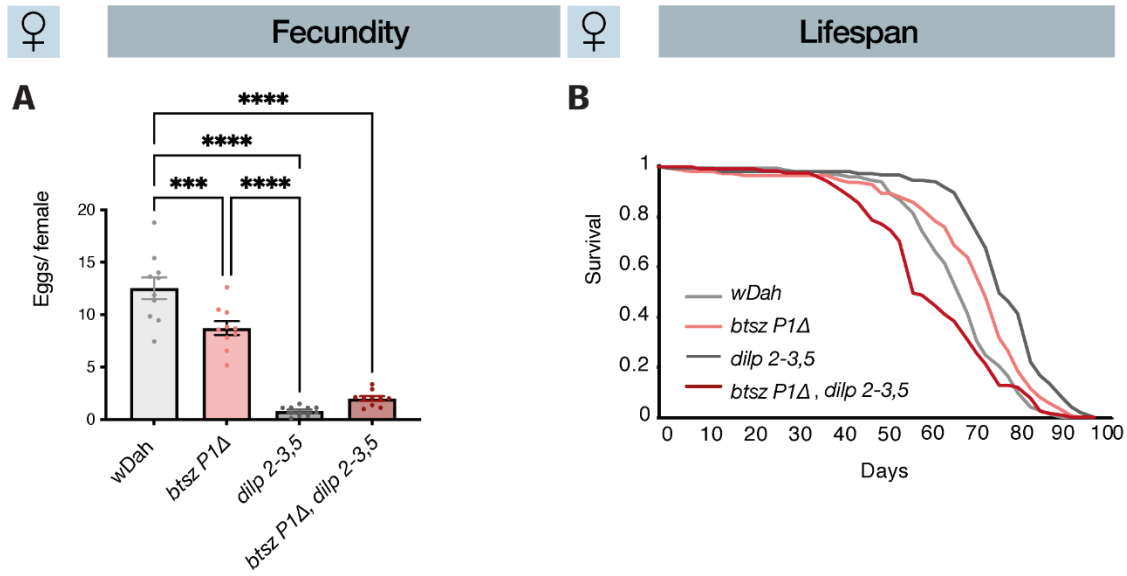


Figure 10: No additive effects on fecundity and lifespan are found in *btsz P1Δ*, *dilp 2-3,5* double mutants.
(A) Wild type flies laid in average 12.54 eggs per female in 24 hours, whereas the *btsz P1Δ* mutant flies laid 8.73 eggs. *dilp 2-3,5* mutants exhibited the strongest reduction in fecundity in comparison to wild type flies with 0.83 eggs, followed by *btsz P1Δ*, *dilp 2-3,5* double mutants with 1.01 eggs. Interaction (****p < 0.0001). For statistical analysis Two-Way-ANOVA was used, n=10. (B) *wDah* females had a median lifespan of 59,5 days, while the lifespan of *btsz P1Δ* and *dilp 2-3,5* mutants was extended (66.5 and 73.5 days, respectively). In contrast, double mutants are short lived (median lifespan of 54.5 days), n=150. For statistical analysis log-rank test was performed

Dilp 2-3,5 female virgin flies exhibit increased night sleep length (Metaxakis *et al.* 2014), reminiscent of *btsz P1Δ* mutants. To address whether the combination of both mutations result in even stronger reduced activity and a further increased sleep duration, we performed sleep experiments with 7-day old male *wDah*, *btsz P1Δ, dilp 2-3,5* and *btsz P1Δ, dilp 2-3,5* double mutants.

During the day, *btsz P1Δ* flies displayed significantly diminished activity levels, consistent with previous findings (Figure 11A). Intriguingly, *dilp 2-3,5* mutants exhibited comparable activity levels to *btsz P1Δ* flies. In contrast, it has been reported that 10-day old female virgin flies exhibit hyperactivity during the day, which is postulated to be mediated by FOXO and increased octopamine levels (Metaxakis *et al.* 2014), indicating a sexual dimorphism in the regulation of activity in *dilp 2-3,5* mutants. Surprisingly, the combination of both mutations resulted in activity levels that did not differ significantly from wild type control flies. During the night, both *btsz P1Δ* and *dilp 2-3,5* mutants showed a trend towards reduced activity (Figure 11B). Conversely, *btsz P1Δ, dilp 2-3,5* mutants were hyperactive during the night and had significantly increased activity levels exceeding the other tested genotypes.

In line with previous experiments, *btsz P1Δ* flies slept significantly longer than *wDah* flies during the day (Figure 11C) and night (Figure 11D). A comparable sleep pattern was also observed in *dilp 2-3,5* mutants. However, the combination of both mutations abolished this effect, resulting in sleep levels

similar to wild type flies. In summary, *btsz P1Δ, dilp 2-3,5* double mutants did not recapitulate the reduced sleep phenotypes of *btsz P1Δ* and *dilp 2-3,5* mutants, but instead neutralized each other.

In order to draw conclusions about the quality of sleep, the length of the individual sleep bouts, meaning the length of each sleep period without disruption, was also evaluated. As expected, *btsz P1Δ,* showed a significantly longer sleep bout length in comparison to all other tested genotypes during day (Fig. 11E). During the night, *btsz P1Δ,* flies exhibited improved sleep quality with an average sleep bout length of 300 min per sleep bout. *dilp 2-3,5* also demonstrated significantly increased sleep bout length of around 200 min, as shown previously (Metaxakis *et al.* 2014, Weigelt 2018), whereas both, wild type flies and *btsz P1Δ, dilp 2-3,5* mutants slept less than 100 min without disruption (Fig. 11F). Since sleep bout length of double mutants, did not differ significantly from wild type flies, the hyperactivity of *btsz P1Δ, dilp 2-3,5* did not negatively affect sleep periods, indicating that of *btsz P1Δ, dilp 2-3,5* mutants were not active over longer periods, but were more active within the respective wake periods.

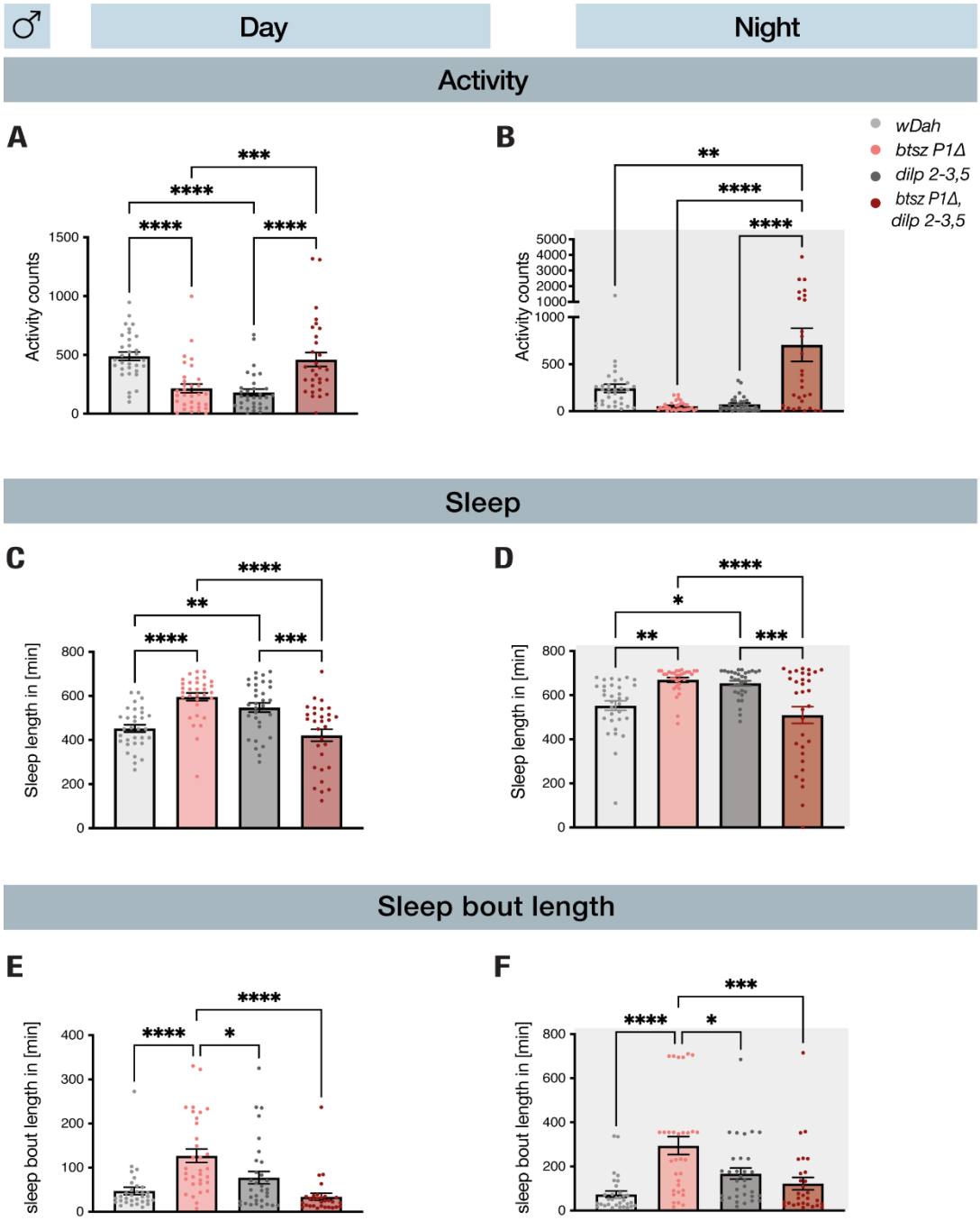


Figure 11: *btsz P1Δ, dilp 2-3,5* double mutants do not exhibit additive effects on activity and sleep parameters. We measured activity and sleep of 7-day old male wild type, *btsz P1Δ*, *dilp 2-3,5* and *btsz P1Δ*, *dilp 2-3,5* mutants. (A) During the day, total activity was significantly reduced in *btsz P1Δ* and *dilp 2-3,5* mutants, compared to wild type flies. The activity levels of *btsz P1Δ*, *dilp 2-3,5* mutants were comparable to wild type flies. (B) During the night, a trend towards reduced activity was observed in *btsz P1Δ* and *dilp 2-3,5* single mutants. In contrast, activity levels were significantly elevated in double mutants compared to the other tested genotypes. (C) During the day (D) and during the night, *btsz P1Δ* and *dilp 2-3,5* mutants slept significantly longer than wild type flies and double mutants. *btsz P1Δ*, *dilp 2-3,5* mutants had a comparable sleep length compared to wild type flies. (E) During the day *btsz P1Δ* mutants showed a prolonged sleep bout length compared to *wDah, dilp 2-3,5* and *btsz P1Δ, dilp 2-3,5* flies. (F) At night, *btsz P1Δ* mutants demonstrated extended sleep bouts in comparison to wild type flies, *dilp 2-3,5,* and *btsz P1Δ*, *dilp 2-3,5* mutants. Ordinary one-way ANOVA with multiple comparison Tukey's post hoc test, with single pooled variance, was performed. Columns and errors bars represent mean and SEM, (Mean + SEM). Significance is indicated as followed. p* < 0.05, p** < 0.01, p*** < 0.001, p**** < 0.0001) Results of the sleep experiment are based on n=32 male flies per condition, three of *btsz P1Δ*, *dilp 2-3,5* mutants had to be censored due to inactivity on multiple days, resulting in n=29.

Strikingly, *btsz P1Δ* flies phenocopy several characteristics reminiscent of *dilp 2-3,5* mutans: *btsz P1Δ* flies show a reduced body weight, developmental delay, and an extended lifespan (Grönke *et al.* 2010, Weigelt 2018). In the adult fly, Btsz P1 is specifically expressed in the mushroom body, but its expression during development remains unexplored. To investigate whether Btsz P1 might be expressed in insulin-producing cells during development, potentially explaining the observed developmental effects, we analysed localization of Btsz P1 using an endogenously GFP-tagged Btsz P1 (GFP::Btsz P1) in L3 larvae (Weigelt 2018). Insulin producing cells were visualized by using a dilp2-Gal4 driver (Rulifson *et al.* 2002) in combination with UAS-mCherry. GFP::Btsz P1 was expressed in the mushroom body during development, consistent with its expression in adult flies. Expression was not solely restricted to the mushroom body but was also detected in the central nerve cord (Figure 12). Dilp 2 expression in insulin-producing cells (Ikeya *et al.* 2002), did not co-localize with Btsz P1 expression, suggesting that Btsz P1 is not expressed within insulin-producing neurosecretory cells. Thus, unlike granuphilin in mammals, *Drosophila bitesize* is unlikely to modify insulin-signalling by directly regulating the secretion of insulin like peptides.

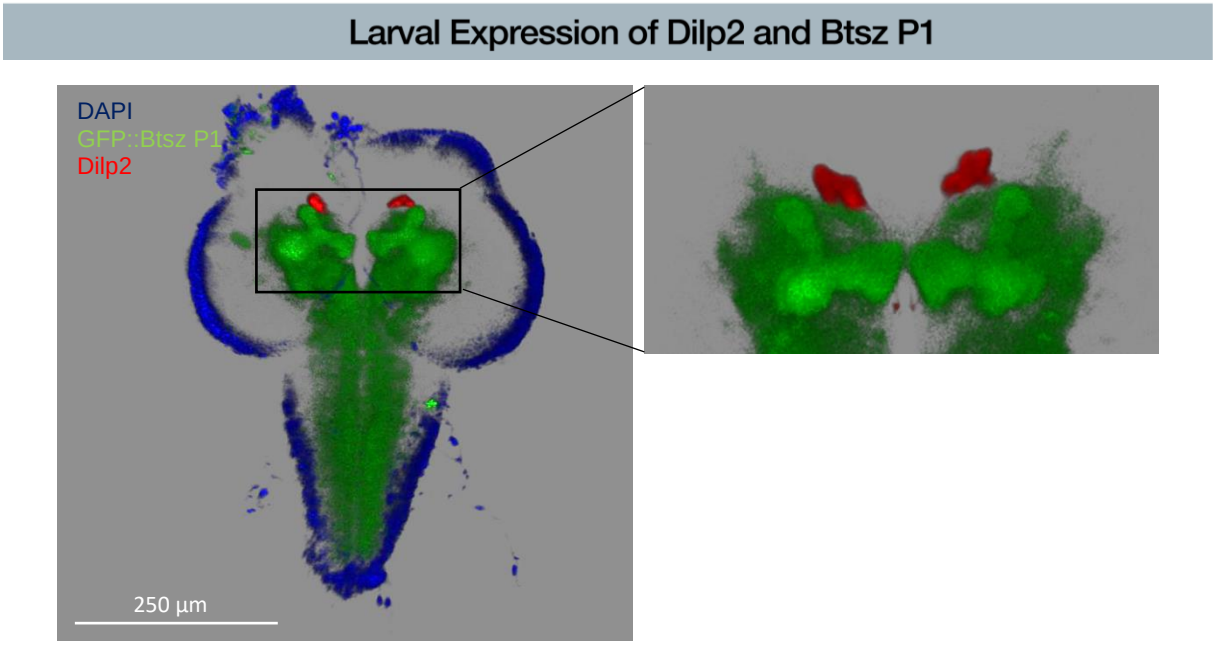


Figure 12: Btsz P1 is not expressed in insulin-producing cells during development.
3D projection of antibody-stainings of Dilp2-Gal4>UAS-mCherry, GFP:Btsz P1 L3 larval brains revealed that Btsz P1 is expressed in the mushroom body and in the central nerve cord. Insulin producing cells cluster symmetrically in two groups but did not colocalize with Btsz P1 positive cells. (blue: DAPI, green: GFP::Btsz P1, red: insulin-like peptide 2). Results were previously shown in (Jahn 2019).

3.4.2 Investigations of sleep-regulating pathways in *btsz P1Δ* mutants

3.4.2.1 No changes in octopamine-singalling are observed in *btsz P1Δ* mutants

Since Btsz P1 does not directly regulate the secretion of insulin-like peptides, we next asked whether Btsz P1 might be involved in the exocytosis of sleep regulating neurotransmitters. Seven neurotransmitters have been described to regulate sleep in *Drosophila*, including octopamine (Crocker and Sehgal, 2008), the biological equivalent to norepinephrine in mammals (Li *et al.* 2016). Octopamine-signalling is enhanced during periods of starvation, leading to elevated activity levels (Yang *et al.* 2015). Flies with reduced octopamine-signalling do not increase their activity in response to starvation, which can be used as a proxy to measure octopamine-signalling. In order to address whether octopamine-signalling is altered in *btsz P1* mutants, we examined activity and sleep patterns of fully fed and starved *wDah* and *btsz P1Δ* males. In line with prior experiments, activity levels of *btsz P1Δ* mutants were decreased during both day and night under normal feeding conditions (Figure 13A + B). When exposed to starvation, the activity of wild type flies increased significantly during the night but not during the day (Figure 13B). The activity of *btsz P1Δ* mutants also increased significantly upon starvation, during both day and night, suggesting that octopamine-signalling is not impaired in these mutants. Next, we analysed sleep in both *wDah* controls and *btsz P1Δ* mutants. Corresponding with activity measurements, starvation induced a comparable reduction in sleep for both *wDah* and *btsz P1Δ* mutants during the day and night (Figure 13C + D). Statistical analysis using a 2-way ANOVA unveiled a significant interaction between genotype and starvation treatment for activity levels and daytime sleep, implying that *btsz P1Δ* mutants exhibit an intensified starvation response. In summary, *btsz P1Δ* mutant males were able to increase their activity in response to starvation, indicating that octopamine-signalling was not impaired in *btsz P1Δ* mutants.

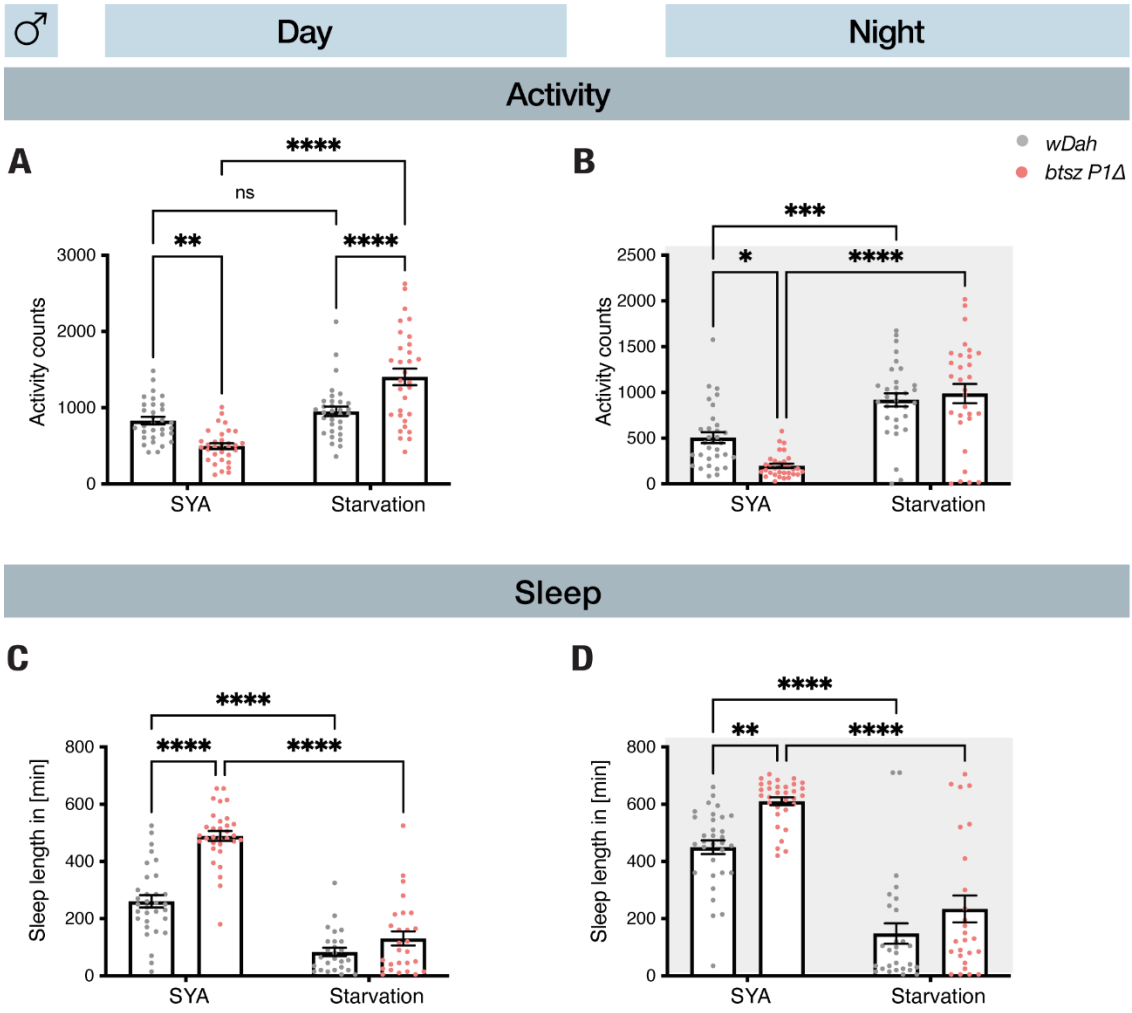


Figure 13: male *btsz P1Δ* mutants exhibit increased activity levels upon starvation.
We measured sleep and activity in 7-day old male wild type flies and *btsz P1Δ* mutants under fully fed conditions and starvation. (A) During the day, only *btsz P1Δ* mutants reacted with elevated activity upon starvation (Interaction: **** < 0.0001, diet: **** < 0.0001, genotype: ns.). (B) During the night, both wild type and *btsz P1Δ* flies reacted with significantly increased activity levels upon starvation (Interaction: *** < 0.001., diet: **** < 0.0001, genotype: ns.). (C) During the day, sleep length was significantly reduced in both tested genotypes upon starvation (Interaction: **** < 0.0001, diet: **** < 0.0001, genotype: **** < 0.0001). (D) During the night, starvation significantly reduced sleep length in wild type flies and *btsz P1Δ* mutants (Interaction: ns., diet: **** < 0.0001, genotype: *** < 0.001.). 2-way ANOVA with multiple comparison Tukey's post hoc test was performed. Columns and errors bars represent mean and SEM. (Mean + SEM). Results of the sleep experiment are based on n=32 male flies per condition. Flies that were inactive for multiple days were censored.

Since activity levels can differ between male and female flies, we also measured activity and sleep patterns in female virgin wild type and *btsz P1Δ* flies. In line with the male data, activity was significantly elevated in *btsz P1Δ* mutants during the day upon starvation, whereas no significant changes were observed in wild type control flies (Figure 14A). During the night, starvation resulted in increased activity levels in both genotypes (Figure 14B). Day sleep length was increased in *btsz P1Δ* mutant females compared to wild type flies and decreased significantly upon starvation (Figure 15C).

In contrast to males, where both wild type and *btsz P1Δ* flies exhibited reduced sleep levels, day sleep was not reduced by starvation in female flies. Since male flies sleep longer during the day than female flies, starvation might fail to further reduce the short sleep duration in wild type females. During the night, similar effects to males were observed, with reduced sleep length in both wild type flies and *btsz P1Δ* mutants, indicating that octopamine-signalling is not disturbed by the *btsz P1Δ* mutation, either in male or in female flies.

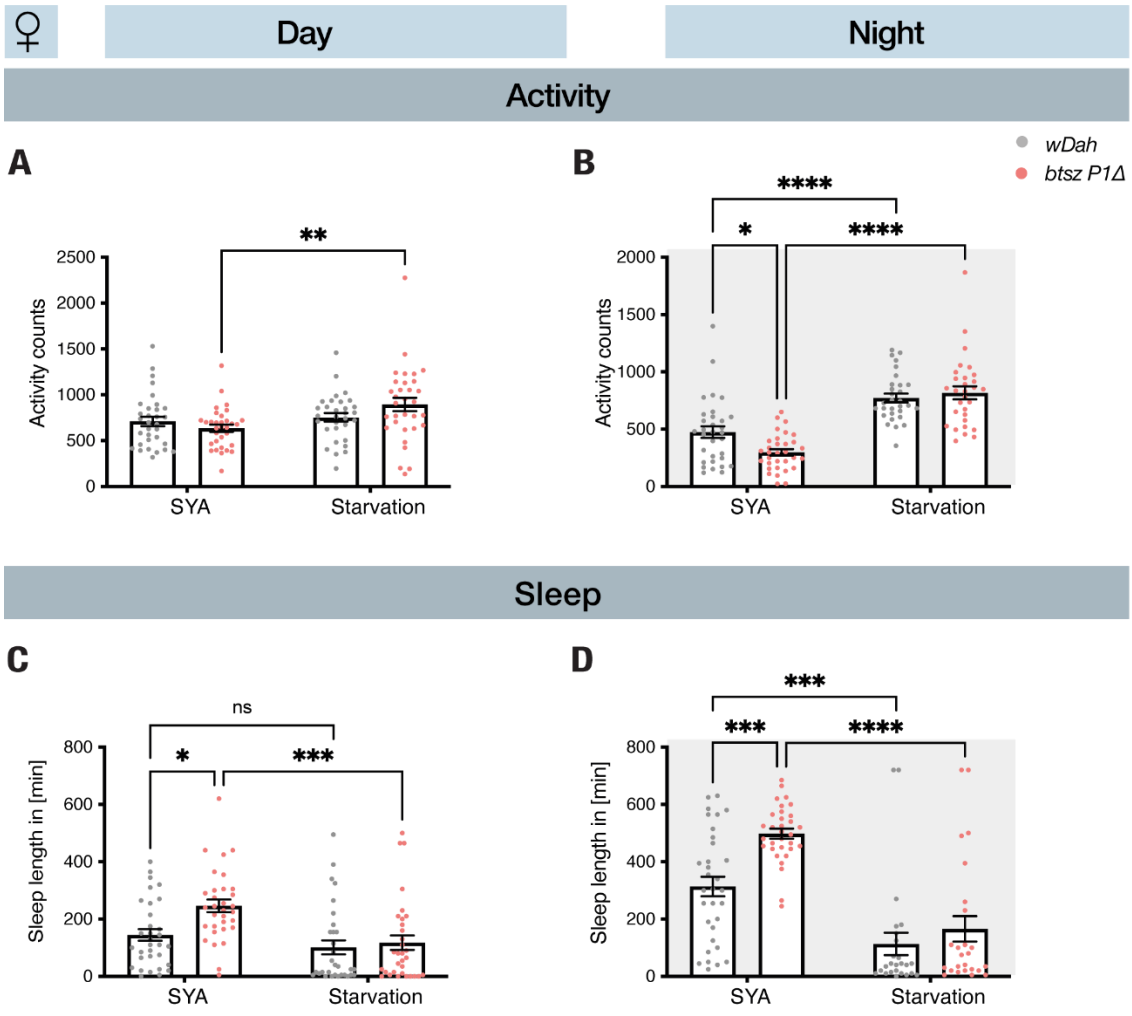


Figure 14: female *btsz P1Δ* mutants react with increased activity levels to starvation.
We measured sleep and activity in 7-day old female wild type flies and *btsz P1Δ* mutants under fully fed conditions and starvation. (A) During the day, *btsz P1Δ* mutants reacted with increased activity to starvation (Interaction: ns., diet: ** < 0.01, genotype: ns.). (B) During the night, starvation raised activity levels in both wild type and *btsz P1Δ* flies (Interaction: ns., diet: *** < 0.001, genotype: * < 0.05). (C) During the day, solely *btsz P1Δ* mutants reacted with decreased sleep length upon starvation (Interaction: * < 0.05, diet: **** < 0.0001, genotype: ns.). (D) During the night, starvation resulted in significantly shortened time spent asleep in both tested genotypes (Interaction: ns., diet: **** < 0.0001, genotype: *** < 0.001). 2-way ANOVA with multiple comparison Tukey's post hoc test was performed. Columns and errors bars represent mean and SEM. (Mean + SEM). Results of the sleep experiments are based on n=32 female virgin flies per condition. Some of the flies had to be censored due to inactivity on multiple days.

3.4.2.2 No impairments in GABA-signalling are found in *btsz P1Δ* mutants

Several microcircuits innervating the *Drosophila* mushroom body have been identified that are crucial to balance and tightly regulate sleep and wake. GABA-signalling is one of the neurotransmitters regulating sleep and wake microcircuits located in the mushroom body. Dorsal paired medial neurons (DPM) are sleep-promoting inhibitory neurons, which stimulate sleep by secretion of GABA onto wake-promoting mushroom body α'/β' neurons. Activation of DPM was linked to elevated levels of chloride in mushroom body neurons (Haynes *et al.* 2015). Besides DPM neurons, anterior paired lateral neurons (APL) have been linked to promote sleep by inhibiting wake-promoting Kenyon cells (KC) through GABA (Haynes *et al.* 2015, Driscoll *et al.* 2021). Several pharmacological agents are available to manipulate GABA-signalling in *Drosophila.* Gaboxadol, a selective extrasynaptic GABA receptor agonist (Wafford and Ebert 2006), which has clinical implications in treating insomnia in humans, has been reported to induce sleep and promote deep sleep, accompanied by elevated arousal threshold in flies (Berry *et al.* 2015, van Alphen *et al.* 2021).

In order to test whether the *btsz P1* mutation can influence GABAergic neurotransmission, we treated 7-day old female virgin wild type flies and *btsz P1Δ* mutants with 0.2 mg/ml Gaboxadol (van Alphen *et al.* 2021) and measured activity and sleep. During the day, both wild type and *btsz P1Δ* flies showed decreased activity levels upon Gaboxadol treatment (Figure 15A), while during the night only wild type flies showed a significant decrease in activity levels (Figure 15B). This might be due to the already very low activity levels of *btsz P1Δ* mutants that cannot further be reduced by Gaboxadol administration. 2-way ANOVA analysis indicated no significant interaction between genotype and Gabodaxol treatment, suggesting that mutants show a similar response to Gabodaxol as wild type flies. Sleep length was significantly increased in both tested genotypes upon Gaboxadol treatment, with even greater effects in *btsz P1Δ* mutants (Figure 15C). During the night, no significant differences in sleep length were observed in wild type and *btsz P1Δ* flies upon Gaboxadol administration (Figure 15D). Since Gaboxadol primarily promotes deep sleep and induces a rapid sleep onset, it cannot be ruled out that potential changes in sleep depth or arousal threshold were induced by drug administration, which are not detectable by the DAM system. In summary, Gaboxadol treatment particularly influenced activity and sleep length, both in wild type and *btsz P1Δ* flies, suggesting that GABAergic neurotransmission is not impaired in *btsz P1Δ* mutant flies.

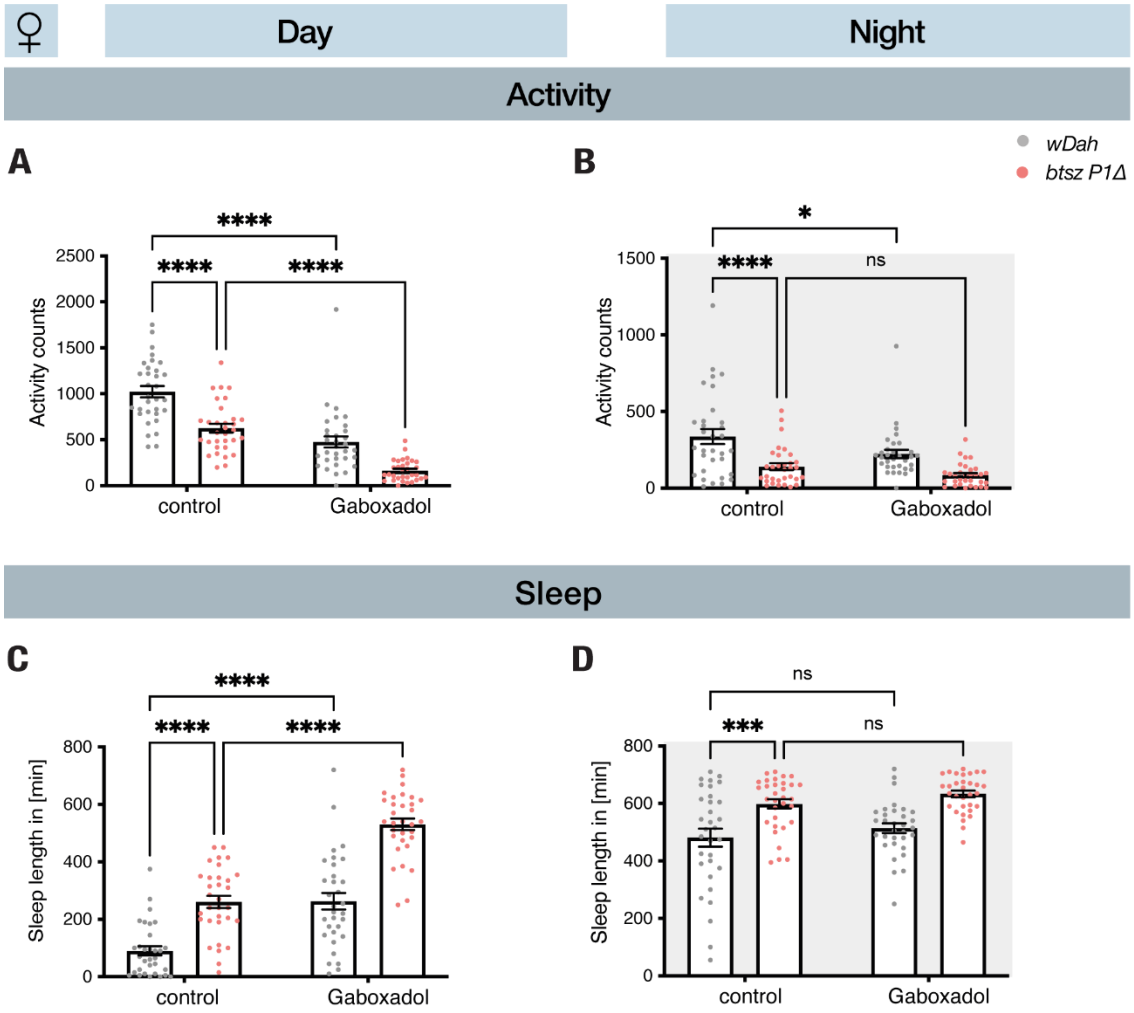


Figure 15: Gaboxadol increases activity and sleep in wild type and *btsz P1Δ* flies during the day.
We assessed activity and sleep levels of 7-day old female virgin flies upon 0.2 mg/ml Gaboxadol administration and control food. (A) During the day, Gaboxadol treatment induced a significant decrease in activity in both wild type flies and *btsz P1Δ* mutants (Interaction: ns., treatment: **** < 0.0001, genotype: **** < 0.001). (B) During the night, activity levels were significantly reduced in *wDah* flies upon Gaboxadol treatment, whereas activity levels of *btsz P1Δ* mutants remained stable (Interaction: ns., treatment: ** < 0.01, genotype: **** < 0.001). (C) During the day, Gaboxadol administration significantly increased sleep length in both tested genotypes (Interaction: * < 0.05, treatment: **** < 0.0001, genotype: **** < 0.001). (D) During the night, sleep levels remained unaltered in both genotypes upon Gaboxadol treatment (Interaction: ns., treatment: ns., genotype: **** < 0.001). 2-way ANOVA with multiple comparison Tukey's post hoc test was performed. Columns and errors bars represent mean and SEM. (Mean + SEM). Results of the sleep experiment are based on n=32 female virgin flies per condition.

3.4.2.3 Mutation of *btsz P1* blocks dopamine-signalling

Dopaminergic neurotransmission is crucial for the interaction and regulation of sleep- and wake-promoting neurons in the mushroom body. Dopamine has wake promoting properties in *Drosophila,* similar to humans. Two well described clusters of dopaminergic neurons, PPL1 and PPM3, project to the central complex, which innervates the dorsal fan-shaped body, a brain region that is crucial for sleep homeostasis (Sitaraman *et al.* 2015, Sitaraman *et al.* 2015, Driscoll *et al.* 2021). The majority of dopamine-signalling in the fly brain is attributed to two distinct groups of neurons known as PAM and PPL, comprising around 130 to 150 neurons in total. These neurons constitute the major neuromodulatory input to the mushroom body (Driscoll *et al.* 2021). The mushroom body contains 2000 KC that project parallel axonal fibers to create two vertical and three horizontal lobes, with dendrites organised within the calyx. The lobes are innervated by 22 mushroom body output neurons, which receive input from the KC and form discrete sections. Each of these sections is influenced by one or more of the 20 subgroups of dopaminergic neurons (DANs), establishing modulatory connections (Aso *et al.* 2014, Cohn *et al.* 2015, Aso and Rubin 2016, Hattori *et al.* 2017, Handler *et al.* 2019, Driscoll *et al.* 2021). A recent study revealed that the pathways downstream of PAM neurons that innervate the γ5 and β′2 compartments of the mushroom body regulate wakefulness through DopR1 and DopR2 receptors expressed in downstream KC and the mushroom body output neurons (Driscoll *et al.* 2021). Several pharmacological and genetic interventions have been implemented that manipulate dopaminergic signalling in order to elucidate dopaminergic neurotransmission in *Drosophila.* For instance, 3IY is used, since it inhibits dopamine synthesis and consequently reduces dopamine-dependent wake-promoting signals (Andretic *et al.* 2005).

To investigate whether the *btsz P1Δ* mutation affects dopamine-signalling, we conducted sleep experiments with young (7-day old) wild type and *btsz P1Δ* mutant males fed with 5 mg/ml 3IY. During the day, 3IY treatment did not affect activity levels in wild type or *btsz P1Δ* mutants (Figure 16A). In contrast, during the night, 3IY treatment induced a significant reduction in activity levels in wild type flies, whereas the activity levels of *btsz P1Δ* mutants remained unaltered (Figure 16B). As anticipated, wild type flies responded to the 3IY treatment by displaying a notable increase in sleep duration during the day on the first day of the experiment (data not shown). On the second day, the same trend was observed, but it was not significant (Figure 16C). During the night, the impact on wild type flies was more pronounced, leading to a significant increase in total sleep duration with 3IY treatment up to the sleep length of *btsz P1Δ* mutants. In contrast, sleep length of *btsz P1Δ* mutants was not further increased upon 3IY treatment (Figure 16D), indicating that dopamine-signalling might be altered in *btsz P1Δ* mutants. Consistent with this hypothesis, the 2-way ANOVA analysis of night sleep showed a significant interaction between genotype and 3IY treatment.

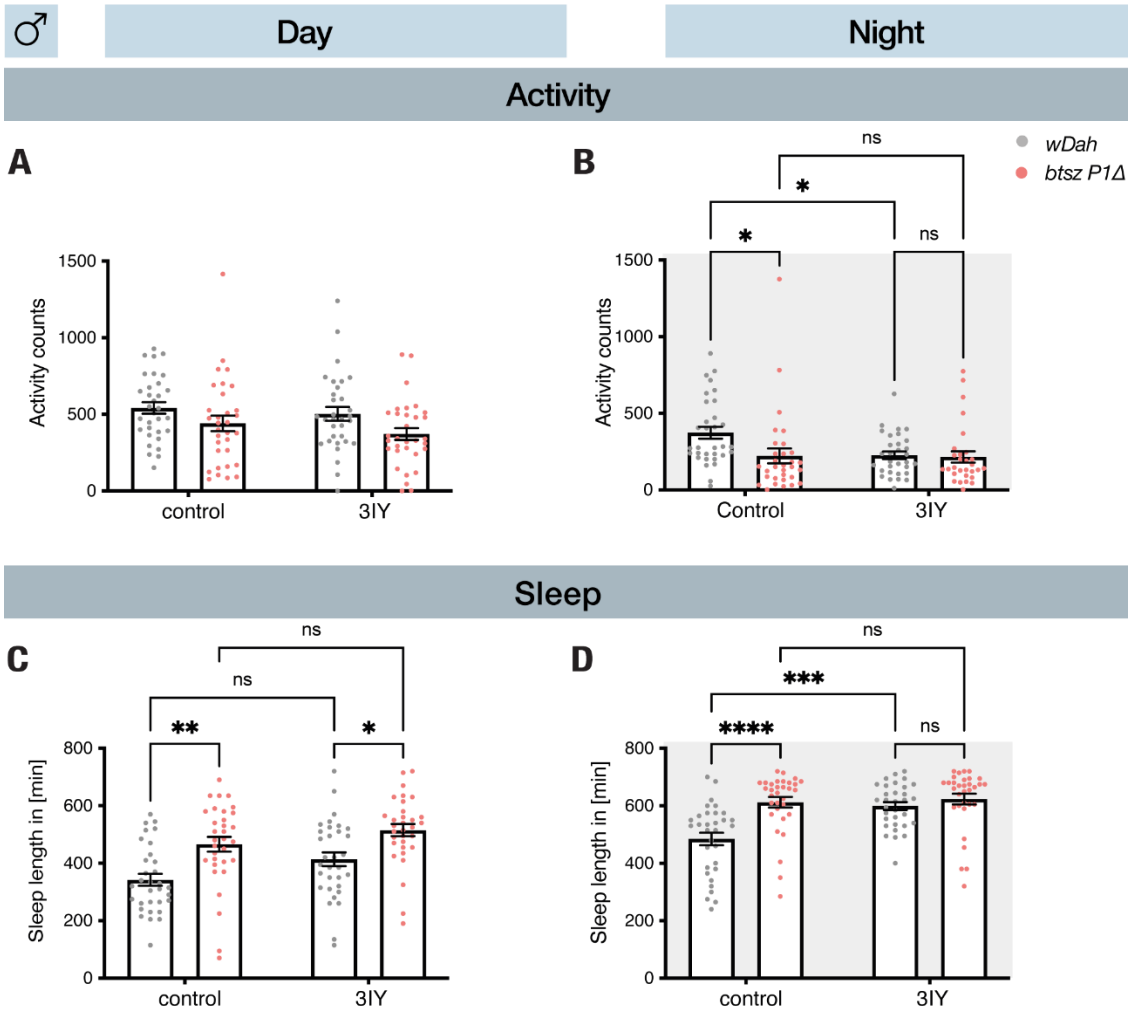


Figure 16: male *btsz P1Δ* mutants are resistant to 3IY treatment.
We assessed activity and sleep in 7-day old male wild type and *btsz P1Δ* flies treated with 5 mg/ml 3IY or control food. (A) During the day, 3IY treatment did not affect activity levels in both tested genotypes (Interaction: ns., treatment: ns., genotype: ** < 0.01). (B) During the night, 3IY treatment resulted in a significant reduction of activity levels in wild type flies, whereas activity remained unchanged in *btsz P1Δ* mutants (Interaction: ns., treatment: ** < 0.01, genotype: **** < 0.0001). (C) During the day, total sleep duration was not influenced by 3IY treatment in none of the tested genotypes (Interaction: ns., treatment: * < 0.05, genotype: * < 0.05). (D) During the night, 3IY treatment significantly increased total sleep duration in control flies. In contrast, *btsz P1Δ* mutants exhibited no changes in total sleep length (Interaction: ** < 0.01, treatment: *** < 0.001, genotype: **** < 0.0001.). 2-way ANOVA with multiple comparison Tukey's post hoc test was performed. Columns and errors bars represent mean and SEM. (Mean + SEM). Results of the sleep experiment are based on n=32 male flies per condition. Flies that were inactive for multiple days were excluded from the analysis.

We also tested the effects of 3IY treatment on female virgin flies. However, as we did not see the reported effects of 3IY treatment on activity or sleep patterns in control flies, the results were not conclusive (Supplementary Figure 1).

Next, we addressed whether mutation of *btsz P1* blocks dopamine-signalling in presynaptic dopaminergic neurons or in downstream neurons. Therefore, we treated flies with Methamphetamine hydrochloride, an inhibitor of the dopamine transporter that prevents dopamine re-uptake into the presynaptic cell (Kume *et al.* 2005, Ueno and Kume 2014). This, in turn, leads to an elevation in dopamine-signalling and increased activity levels (Andretic *et al.* 2005). We treated 7-day old wild type and *btsz P1Δ* males with 1 mg/ml and, in a separate experiment, to 2 mg/ml Meth for the entirety of the sleep experiment. Surprisingly, both our male control flies and *btsz P1Δ* mutant flies demonstrated no significant increase or decrease in activity or sleep duration upon Meth treatment (Supplementary Figure 2). This finding diverged from the outcomes of previous sleep experiments conducted with female flies (Andretic *et al.* 2005, Metaxakis *et al.* 2014). As there is a lack of literature concerning the effects of Meth on sleep in male flies, we subsequently used virgin female flies for all experiments involving the effects of Meth on sleep and activity.

In females, Meth administration resulted in a significant increase in daytime activity in wild type flies (Figure 187A). In contrast, Meth treatment did not significantly increase day activity in *btsz P1Δ* mutant females (Figure 17A). During the night, the effect of Meth treatment was more pronounced in wild type flies with strongly increased activity (Figure 17B). In contrast, nighttime activity was not increased by Meth treatment in *btsz P1Δ* mutant females (Figure 17A+B). Consistent with prior studies, female wild type flies exhibited a pronounced reduction in day and night sleep in response to Meth treatment, whereas *btsz P1Δ* remained unaffected by Meth administration (Figure 17C+D). 2-way ANOVA analysis showed a significant interaction between Meth and genotype ($p < 0.01$), indicating that *btsz P1Δ* mutants reacted differently to Meth treatment compared to wild type flies.

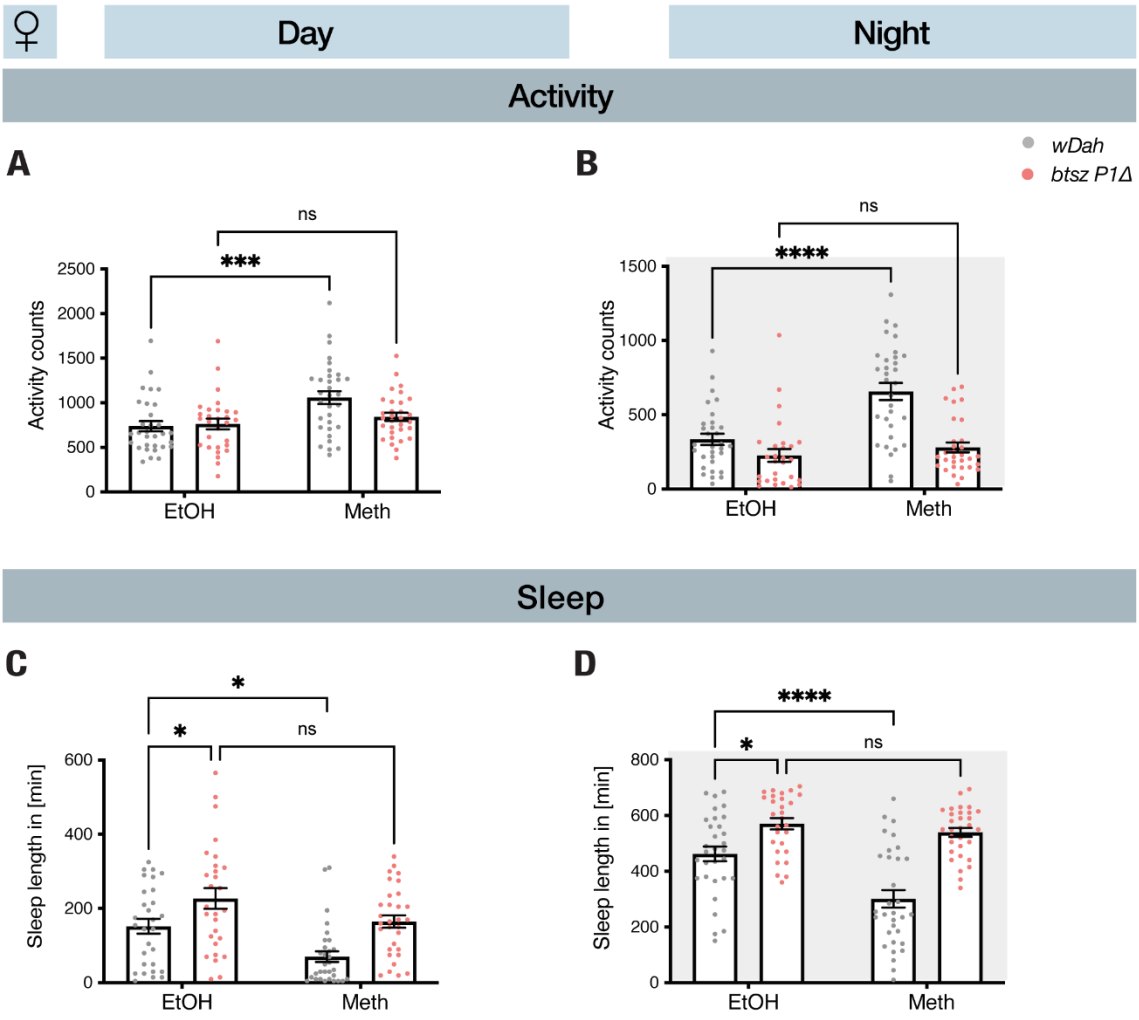


Figure 17: female *btsz P1Δ* mutants are resistant to Meth treatment.
We assessed activity and sleep in 7-day old female virgin wild type and *btsz P1Δ* flies treated with 1 mg/ml Meth or Ethanol. (A) During day and (B) during night, wild type flies reacted with a significant activity increase upon Meth treatment, whereas treated *btsz P1Δ* did not show activity changes. (A) (Interaction: ** < 0.01, treatment: *** < 0.001, genotype: ns.), (B) (Interaction: ** < 0.01, treatment: **** < 0.0001, genotype: **** < 0.0001). (C) *btsz P1Δ*, flies slept significantly longer than wild type flies. Meth treatment did not affect mutant flies, but significantly reduced daytime sleep length of female wild type flies (Interaction: ns., treatment: *** < 0.001, genotype: **** < 0.0001). (D) During the night, female *wDah* slept significantly less after Meth exposure. In contrast, administration of Meth had no effects on *btsz P1Δ* mutants, which slept significantly longer than *wDah*. (Interaction: ** < 0.01, treatment: *** < 0.001, genotype: **** < 0.0001). 2-way ANOVA with Tukey's multiple comparison post hoc test was per formed Columns and errors bars represent mean and SEM. (Mean + SEM). These results are based on n=32 female virgin flies per treatment. Flies that were inactive for multiple days were excluded from the analysis.

To further verify our pharmacological data, we used a genetic approach to study the interaction of Btsz P1 and dopamine-signalling. Consistent with the pharmacological effect of Meth, *fumin* (Japanese for sleepless) mutant flies that carry a mutation in the dopamine transporter are hyperactive and show a decrease in sleep (Kume *et al.* 2005). To investigate if *btsz P1Δ* can block *fumin* induced hyperactivity, we generated *btsz P1Δ, fumin* double mutants and performed sleep experiments with 7-day old male flies. In accordance with the Meth experiments and published data (Kume *et al.* 2005), *fumin* mutants were significantly more active than wild type controls during day and night (Figure 18A+B). As expected, *btsz P1Δ* mutants showed significantly decreased activity during day and night (Figure 18A+B). Interestingly, mutation of *btsz P1* was able to rescue the daytime and nocturnal hyperactive states of *fumin* mutants and even further reduced them (Figure 18A+B). Hence, the activity of *btsz P1Δ*, *fumin* double mutants did not differ significantly from *btsz P1Δ* mutants. Similar effects were also observed for total sleep duration. Consistent with previous findings, *btsz P1Δ* mutants slept significantly longer during day and night than the respective wild type controls, whereas *fumin* mutants displayed the opposite effect and slept significantly less than wild type controls during day and night (Figure 18C+D). During the day, *btsz P1Δ*, *fumin* double mutants showed increased sleep length comparable to wild type flies. Strikingly, during the night, total sleep duration of *btsz P1Δ*, *fumin* double mutants was similar to the sleep duration of *btsz P1Δ* mutants, indicating that *btsz P1Δ* can block the hyperactivity phenotype of *fumin* mutants. Altogether, the results confirm the finding that *btsz P1Δ* acts downstream of dopaminergic signalling.

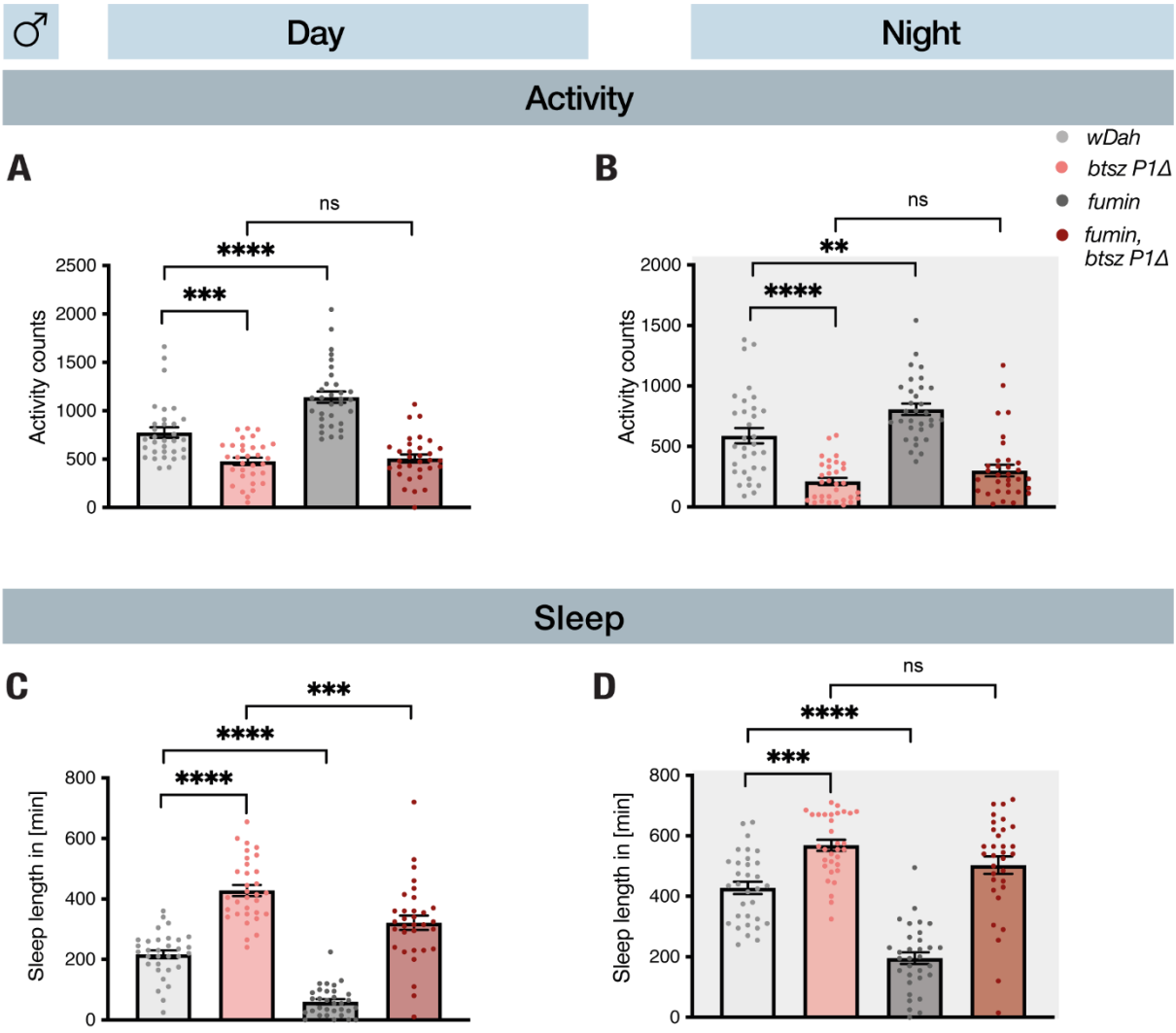


Figure 18: Mutation of *btsz P1* rescues the hyperactivity phenotype of *fumin* mutants.
(A) During the day, *btsz P1Δ* mutant males were significantly less active compared to wild type males. Opposite effects were observed in *fumin* mutants, which were hyperactive. Combination of both mutations resulted in activity levels which did not differ significantly from *btsz P1Δ* mutants (Interaction: *** < 0.001). (B) During the night, activity levels were significantly reduced in *btsz P1Δ* mutants und elevated in *fumin* mutants compared to wild type flies. *btsz P1Δ, fumin* double mutants exhibited comparable activity levels to *btsz P1Δ* mutants (Interaction: *** < 0.001). (C) During the night, total sleep duration was significantly increased in *btsz P1Δ* mutants and decreased in *fumin* mutants. Double mutants displayed a comparable sleep duration to wild type flies (Interaction: ns.). (D) During the night, total sleep duration was elevated in *btsz P1Δ* mutants and decreased in *fumin* mutants compared to wild type flies. *btsz P1Δ, fumin* double mutants showed a similar sleep length to *btsz P1Δ* mutants (Interaction: *** < 0.001). 2-way ANOVA with Tukey's multiple comparison post hoc test was per formed Columns and errors bars represent mean and SEM. (Mean + SEM). These results are based on n=32 male flies per condition. Flies that were inactive for multiple days were excluded from the analysis.

Since pharmacological interventions only induced the expected effect in one of the sexes in the previously described experiments, we wondered if the genetic interaction of *btsz P1Δ* and *fumin* can also be observed in female flies. Therefore, we also assessed sleep and activity in 7-day old female virgin flies. In short: the female data is consistent with the results from the male flies. The combination of both *btsz P1Δ* and *fumin* mutations, resulted in a rescue of *fumin* mutants' hyperactivity during day and night and even further decreased activity levels to *btsz P1Δ* mutants' level (Figure 19A+B). During the day, double mutants exhibited an intermediate phenotype with no significant difference in total sleep duration compared to wild type control flies (Figure 19C). During the night, *btsz P1Δ, fumin* double mutants displayed a total sleep duration comparable to *btsz P1Δ* mutants. The results show that mutation of *btsz P1* causes similar effects in both male and female flies. In addition, the genetic interaction indicates that Btsz P1 probably executes its function downstream of the dopamine transporter.

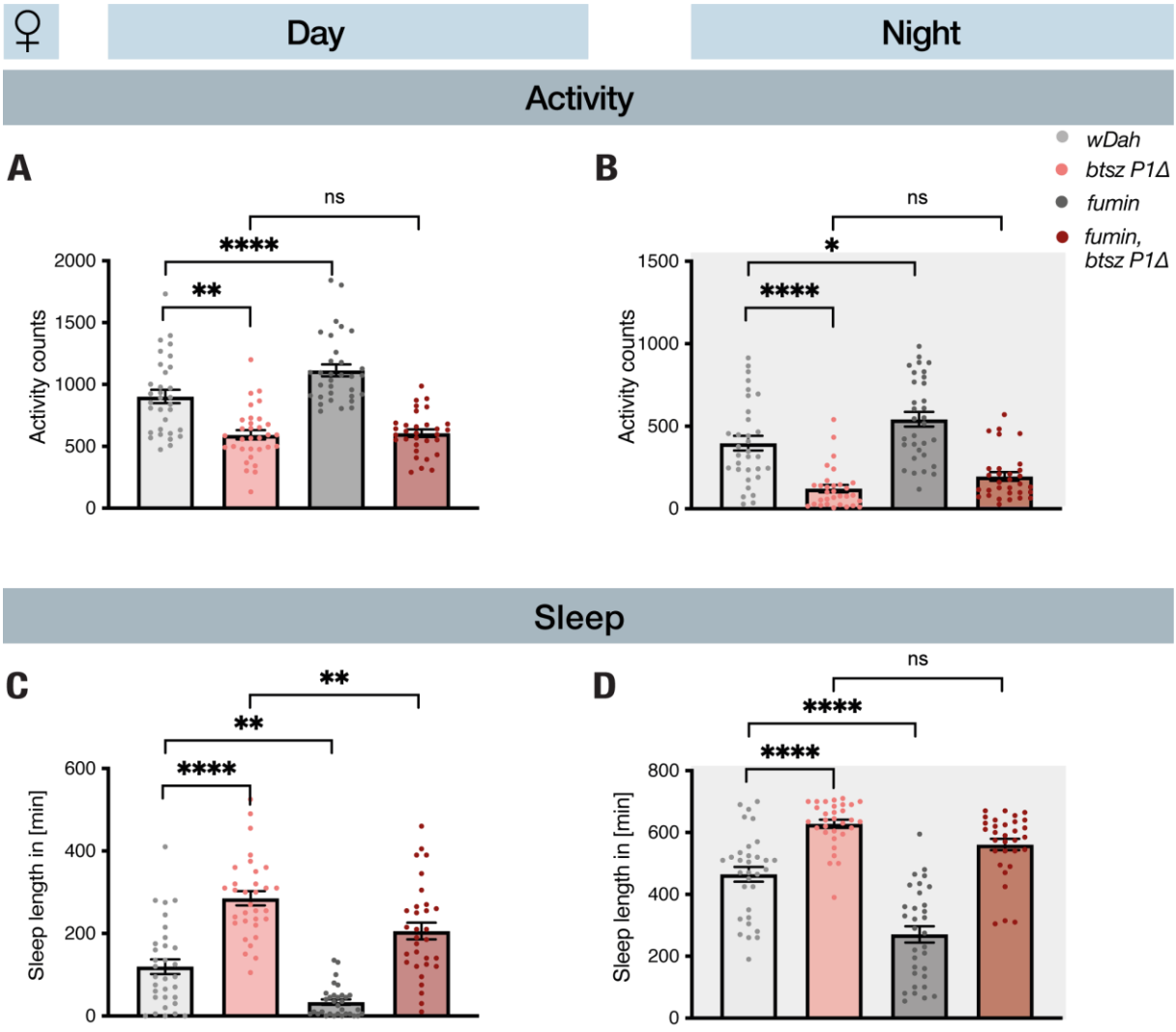


Figure 19: mutation of *btsz P1* rescues the hyperactivity phenotype of female *fumin* mutants.
(A) During the day, *btsz P1Δ* mutants were significantly less active compared to wild type flies. Opposite effects were observed in *fumin* mutants, which were hyperactive. Combination of both mutations resulted in activity levels which did not differ significantly from *btsz P1Δ* mutants (Interaction: * < 0.05). (B) During the night, activity levels were significantly reduced in *btsz P1Δ* mutants und elevated in *fumin* mutants compared to wild type flies. *btsz P1Δ, fumin* double mutants exhibited comparable activity levels to *btsz P1Δ* mutants (Interaction: ns.). (C) During the night, total sleep duration was significantly increased in *btsz P1Δ* mutants and decreased in *fumin* mutants. Double mutants displayed a comparable sleep duration to wild type flies (Interaction: ns.). (D) During the night, total sleep duration was elevated in *btsz P1Δ* mutants and decreased in *fumin* mutants compared to wild type flies. *btsz P1Δ, fumin* double mutants showed a similar sleep length to *btsz P1Δ* mutants (Interaction: ** < 0.01). 2-way ANOVA with Tukey's multiple comparison post hoc test was per formed Columns and errors bars represent mean and SEM. (Mean + SEM). These results are based on n=32 female virgin flies per condition. Flies that were inactive for multiple days were excluded from the analysis.

The finding that *btsz P1Δ* mutants are resistant to both 3IY and Meth treatment and that *btsz P1Δ* is able to rescue the hyperactivity phenotype of *fumin* mutants suggest that dopamine-signalling is disrupted after the release of dopamine into the synaptic cleft. Thus, we next tested whether Btsz P1 is expressed in dopamine-producing or -receiving neurons. Dopaminergic neurons are characterised by the expression of Tyrosine Hydroxylase (TH). To investigate a potential co-localization, we crossed flies carrying the dopaminergic neuron-specific TH-Gal4 driver line, with UAS-mCherry, GFP::Btsz flies. Brains of adult flies were dissected and whole mount antibody stainings using α-GFP and α-mCherry specific antibodies were performed. Btsz P1 signal (green) was detected as previously described in the mushroom body and antennal lobes. Clusters of dopaminergic neurons (red) were found in proximity to the mushroom body, which are described in the literature as dorsolateral and dorsomedial neurons (Friggi-Grelin *et al.* 2003) (Figure 20A). Dopaminergic neurons clustered also in KC, but no colocalization was found (Figure 20B), suggesting that Btsz P1 does not act in dopaminergic neurons. We next tested whether Btsz P1 is expressed in cells receiving dopaminergic input. Like Btsz P1, the Dop1R is expressed in the mushroom body (Landayan *et al.* 2018), which is particularly important for the wake-promoting effects of dopamine (Li *et al.* 2000), indicating that Btsz P1 might interact with Dop1R or influence signal transduction in Dop1R expressing cells. To test this hypothesis, whole mount immunostainings of adult flies, carrying a Dop1R1 driver and a UAS-mCherry construct in a GFP::Btsz P1 background, were performed. Dop1R1 mediates its function in the MB γ-lobe, in which we also detected mCherry signal (Silva *et al.* 2020). In this part of the MB the Btsz P1 signal (green) and Dop1R1 signal (red) overlay, consistent with the hypothesis that Btsz P1 might mediate its function in cells receiving dopaminergic inputs (Figure 20C). We also performed RNA sequencing of 7-day old female wild type flies and *btsz P1Δ* mutants, which will be explained in more depth in the following chapter. RNA sequencing revealed that Dop1R1 expression is significantly increased in *btsz P1Δ* mutants compared to wild type flies (Figure 20D). This might indicate a potential compensatory effect to impaired dopamine-signalling in neurons receiving dopaminergic inputs.

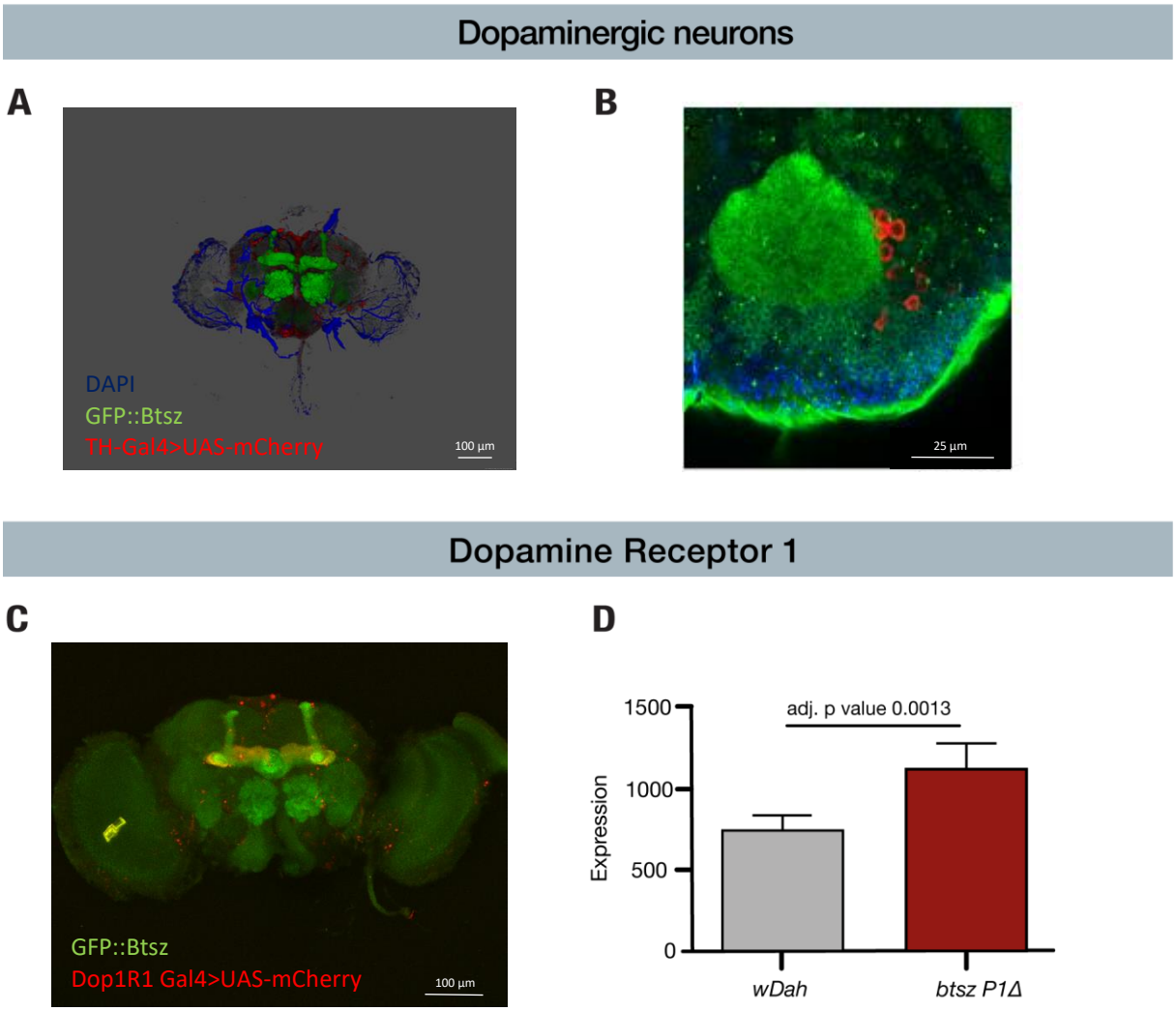


Figure 20: Btsz P1 does not co-localize with dopaminergic neurons, but rather seems to act in dopamine-receiving neurons.
(A) Stainings of TH-Gal4> UAS-mCherry, GFP:: Btsz P1 brains were performed. GFP:: Btsz P1 (green) did not colocalize with dopaminergic neurons (red). (B) Dopaminergic cells clustered as described and were found close to Btsz P1 positive cells. (C) An overlay of cells expressing Dop1R1 (red) and Btsz P1 positive cells (green) was found in dissected brains of Dop1R1 Gal4> UAS-mCherry, GFP:: Btsz P1 flies. (E) RNA sequencing of 7-day old female flies revealed that Dop1R1 expression is significantly increased in *btsz P1Δ* mutants compared to wild type flies.

Consistent with the phenotype of *btsz P1Δ* mutant flies, dopamine-deficient flies, which are not able to release dopamine, also show extended sleep length (Riemensperger *et al.* 2011). To exclude that changes in total dopamine levels or other neurotransmitters are causal for the observed sleep phenotype of *btsz P1Δ* mutants, we measured dopamine, octopamine and serotonin levels using mass spectrometry-based metabolomics in heads of 7-day old wild type and *btsz P1Δ* mutant females. The analysis revealed no significant differences in dopamine levels in *btsz P1Δ* mutants (Figure 21A), consistent with the hypothesis that Btsz P1 potentially mediates its function in dopamine-receiving cells. We observed a slight increase in octopamine levels in *btsz P1Δ* mutants (Figure 21B), which has wake-promoting properties. However, the elevated octopamine levels do not seem to have functional consequences on activity levels, since activity is reduced in *btsz P1Δ* mutants. Levels of serotonin also did not differ significantly in *btsz P1Δ* mutants compared to wild type flies (Figure 21C). In conclusion, the observed phenotypes were not caused by changes in levels of the tested neurotransmitters.

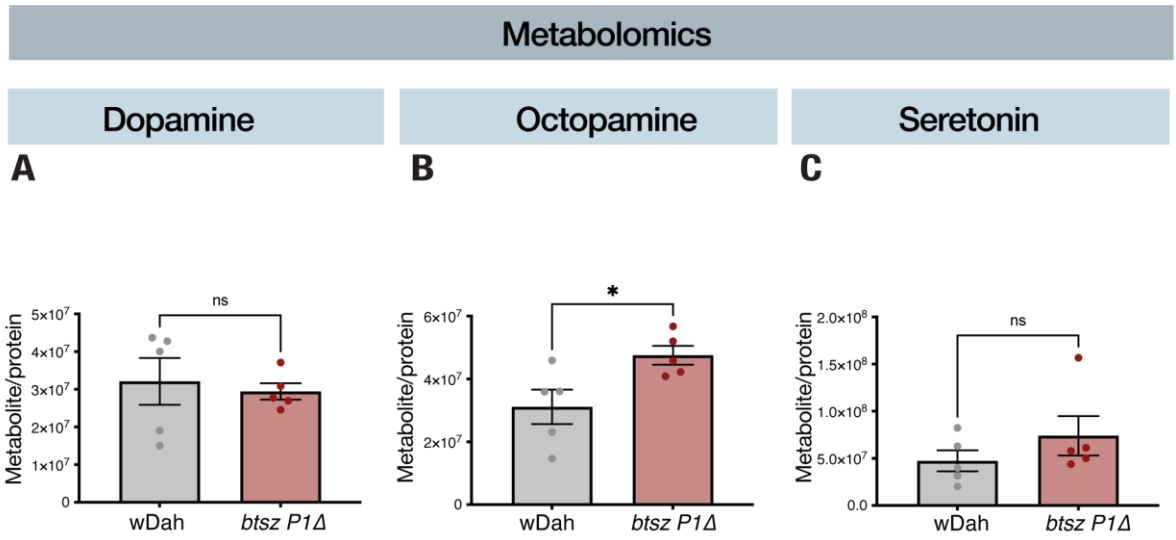


Figure 21: Dopamine levels are not increased in *btsz P1Δ* mutants.
Determination of (A) dopamine, (B) octopamine and (C) serotonin levels in heads of 7-day old female flies by mass-spectrometry. No significant changes of total dopamine and serotonin level were observed in *btsz P1Δ* mutants compared to wild type flies (A+C). (B) Octopamine levels were significantly increased in *btsz P1Δ* mutants ($p^* < 0.05$). Unpaired and two-tailed t-test was performed for statistical analysis. n= 5 replicates with 25 fly heads each. These results were previously described in (Jahn 2019).

3.4.3 RNA sequencing of *btsz P1Δ* mutants

To gain insights into the molecular changes associated with the mutation of *btsz P1*, we performed RNA sequencing (RNAseq) on heads of 7-day old wild type and *btsz P1Δ* females. Multidimensional scaling of the tested samples revealed that replicates of each genotype group clustered together, indicating similarity within the biological replicates and dissimilarity between the genotypes (Figure 22A). In total, the expression of 9399 genes was detected, of which 497 were significantly down-regulated (depicted in blue) and 491 significantly up-regulated (depicted in red) in *btsz P1* mutants compared to control flies (Figure 22B).

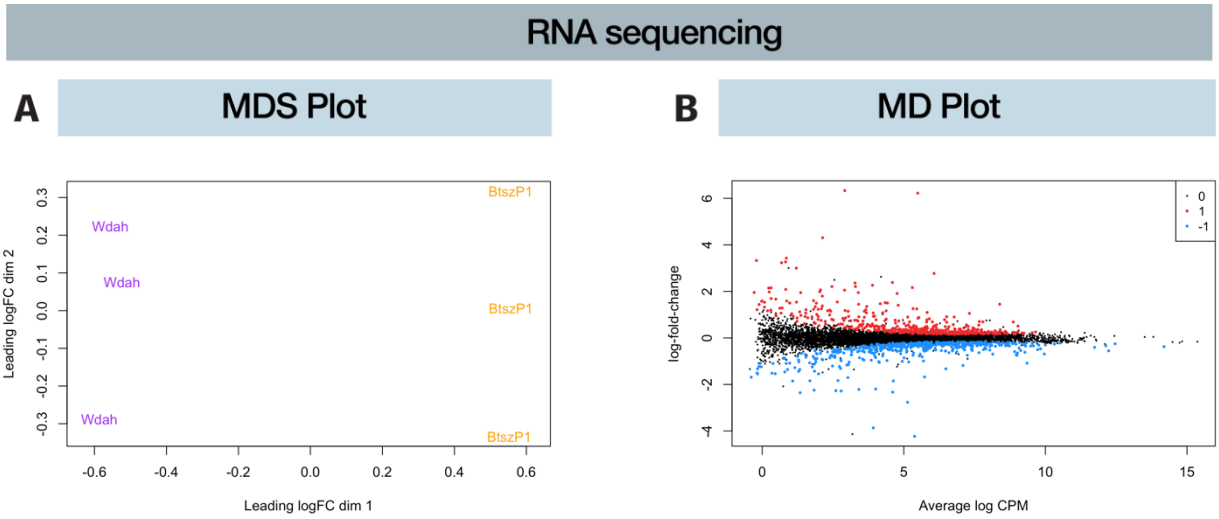


Figure 22: RNAseq analysis of *btsz P1Δ* mutants.
(A) Multidimensional scaling of the tested samples depicts distinct clusters for wild type flies and *btsz P1Δ* mutants. (B) Mean difference plot visualizes all 491 significantly up (red) and 497 downregulated (blue) differentially expressed genes. RNAseq was based on three biological replicates per sample, containing each 25 heads of the respective genotype. Bioinformatic analysis was conducted by Nathalie Jauré.

We next performed GO term enrichment for differentially expressed genes between *btsz P1Δ* mutants and wild type flies and filtered for the 20 first most significant terms (Figure 23A). Since sleep influences a variety of physiological functions, it was not surprising that terms that are not directly related to sleep were identified. The most significant term was oxidation-reduction process with 81 differentially expressed genes. Interestingly, also G protein coupled receptor (GPCR) signalling and drug metabolic processes were listed, with 37 and 44 differentially expressed genes, respectively. However, the GO term sleep (GO:0030431) with 69 annotated genes, was not significantly enriched. Only 7 genes related to this GO term were found to be regulated (Supplementary Table 1), explaining why the term was not listed. Though, the GO term “circadian rhythm” was found to be significant (Supplementary Table 2). We already tested in previous experiments whether the circadian rhythm of *btsz P1Δ* mutants is

disturbed and assessed therefore activity patterns in a 12-hour light and 12-hour dark cycle, which was switched after 3 days to constant darkness (Supplementary Figure 3). Despite of the loss of the characteristic peak in activity upon light switches in both genotypes, *btsz P1Δ* mutants were able to sustain a rhythm with increased activity during the day and decreased activity during the night without light cues, comparable to wild type flies. The results indicate that circadian rhythm is not affected in *btsz P1Δ* mutants. When we checked for other significant GO terms, the term “circadian rhythm” was listed for upregulated genes (Supplementary Table 2). Within that term, we found several genes that have also been reported to be associated with the regulation of sleep. As an example, Shab, which encodes for the structural alpha subunit of a delayed rectifier K^+ channel (Flybase) and is expressed in the dorsal fan-shaped body, has been reported to be down-regulated upon Dop1R2 dependent dopaminergic transmission. Interestingly, downregulation of Shab was also linked to the upregulation of the two-pore-domain potassium channel, Sandman, which hyperpolarizes neurons and has been implicated in the regulation of sleep (Pimentel *et al.* 2016, Holland 2018). Also, TfAp-2, a transcription factor, which was upregulated, is involved in the regulation of sleep. Neuronal knock-down of TfAP-2 abolishes night-time sleep almost completely in *Drosophila* (Kucherenko *et al.* 2016). Moreover, upregulated genes such as *pdf* and channels such as unc 79, which is expressed in the mushroom body, also have been reported to regulate sleep and sleep duration (Parisky *et al.* 2008, Murakami *et al.* 2021). The enzyme arylalkylamine N-acetyltransferase 1 (AANAT1), which also occurred in the term circadian rhythm, has been reported to limit the accumulation of dopamine and serotonin upon sleep deprivation (Davla *et al.* 2020). Zhang *et al.* reported that over-expression of the potassium channel Ork1, an upregulated gene of the term circadian rhythm, increases sleep duration (Zhang *et al.* 2017). Hence, several of the listed genes in the term “circadian rhythm” have been described in the regulation of sleep und serve as interesting candidates to further study the mechanism of Btsz P1- dependent regulation of sleep.

Since we were interested in in potential implications that are caused by differentially regulated GPCR-signalling and potential alterations in dopamine-signalling, we visualized with Cnet plot linkages of enriched genes of GPCR signalling (Figure 23B). Remarkably, Dop1R1 occurred as an enriched gene, providing further evidence for our hypothesis that alterations in sleep in *btsz P1Δ* mutants are caused by changes in dopaminergic signalling.

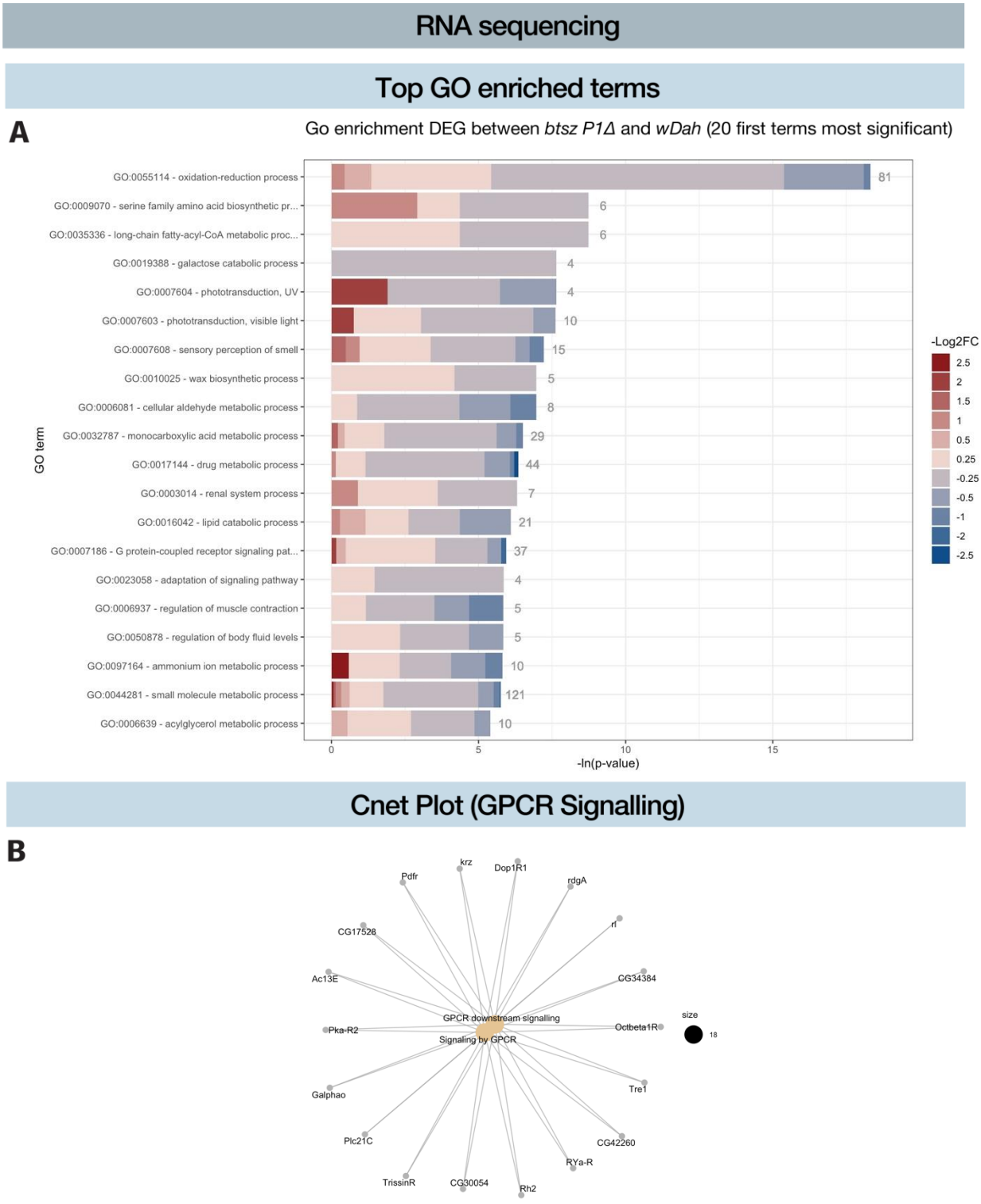


Figure 23: Top GO enriched terms of *btsz P1Δ* mutants compared to *wDah* and GPCR Signalling related Cnet plot. (A) Top Go enriched terms between *btsz P1Δ* mutants and *wDah* flies. The 20 first most significant terms are listed. (B) GPCR signalling was differentially regulated in *btsz P1Δ* mutants compared to wild type flies with Dop1R1 as differentially regulated gene. RNAseq is based on three biological replicates per sample, containing each 25 heads of the respective genotype. Bioinformatic analysis was conducted by Nathalie Jauré.

3.4.4 In which cell clusters does Btsz P1 mediate its function?

Previous experiments suggested that Btsz P1 might act in cells that receive dopaminergic inputs. Thus, we hypothesized that Btsz P1 might mediate its function in the downstream-signalling cascade of the dopamine receptor, but in which specific cell cluster Btsz P1 acts, and which steps of the signalling-cascade might be regulated, and which other molecules might be involved in this process is currently unknown. The two pore-domain potassium channel Sandman is also involved in the regulation of sleep in dopamine-receiving cells (Pimentel *et al.* 2016). Interestingly, this channel is internalized after signal transduction, recycled, and transported back to the post-synaptic membrane via vesicle exocytosis (Pimentel *et al.* 2016). As Btsz P1 contains a highly conserved SHD domain, which is required for the regulation of exocytosis of vesicles in mammals (Gomi *et al.* 2005), we speculated that Btsz P1 might be involved in the recycling process of a channel that is involved in the regulation of sleep. In order to identify potential candidate channels, we used the single cell transcriptome atlas of the ageing *Drosophila* brain (Davie *et al.* 2018). First, we identified cell clusters in which Btsz P1 is annotated as gene marker (Table 12). The analysis, which was conducted by Nathalie Jauré, confirmed the expression pattern in the KCs, α/β and α'/β' lobes of the mushroom body. In addition, expression was detected in the dorsal fan-shaped body which has been implicated in dopamine-dependent regulation of sleep (Liu *et al.* 2012). Moreover, *btsz P1* was abundant in subperineurial glia, which have been suggested to regulate sleep and activity patterns through oscillators (Damulewicz *et al.* 2022). Also, protocerebral clusters in which *btsz* was identified as a gene marker, have been recently linked to the regulation of sleep. Interestingly, this neuronal circuit has also been linked to dopaminergic neurotransmission (Tomita *et al.* 2021).

Table 12: Cell clusters in the *Drosophila* brain that express *btsz P1* annotated as gene marker.
To understand the cell specific expression of the *btsz* gene, the dataset from the publication 'A Single-Cell Transcriptome Atlas of the Aging Drosophila Brain' (Davie *et al.* 2018) was used. The table summarises gene markers for each clusters identified using Seurat, an R package specialised in single-cell data analysis. From this table all clusters having *btsz* annotated as a gene marker were filtered

Cluster number	Cell type	Note
8	G-KC	Gamma kenyon lobe
22	α/β lobe	
28	α'/β' lobe	
61	dfb	Dorsal fan-shaped body neurons
66	OCTY	Subperineurial glia
74	NA	
80	Poxn	Cells of the protocerebral cluster

Since we postulated that Btsz P1 might executive its function in cells receiving dopaminergic input, we next identified cell clusters that co-express *btsz* and dopamine receptors (Table 13). Interestingly, we observed expression of Dop1R1 and Dop1R2 in KCs, and α/β and α'/β' lobes of the mushroom body, consistent with the results from the whole-brain immunostainings. In addition, expression of Dop1R1 was detected in dorsal fan-shaped body neurons. Remarkably, when checking for other gene markers of those cluster, Rab27 the interaction partner of the mammalian homolog, was also identified.

Table 13: Cell clusters in the *Drosophila* brain that co-express *btsz P1* and DopRs.

Cluster number	Cell type	Note	Additional markers
8	G-KC	Gamma kenyon lobe	Dop1R1, Dop1R2, Rab27, Ca-α 1T
22	α/β lobe		Dop1R1, Dop1R2, Rab27
28	α'/β' lobe		Dop1R1, Dop1R2
61	dfb	Dorsal fan-shaped body neurons	Dop1R1
66	OCTY	Subperineurial glia	
74	NA		
80	Poxn	Cells of the protocerebral cluster	

3.4.4.1 Does Btsz P1 regulate sleep by interaction with Ca-alpha1T?

We then identified channels and other relevant gene markers, which clustered in the same cells (Supplementary Table 1). One of those channels, which caught our interest, is calcium – alpha 1T (Ca-α 1T), which has been already described in the context of regulation of sleep. In mammals, which express several T-type Ca^{2+} channels, this group of channels are described as sleep stabilizers involved in the generation of the delta rhythms of deep sleep (Jeong *et al.* 2015). Noteworthy, Ca-α 1T mutants demonstrate an increased sleep duration, reminiscent of the *btsz P1Δ* mutants' sleep phenotype (Jeong *et al.* 2015). Interestingly, RNAseq analysis revealed that Ca-α 1T was up-regulated in *btsz P1Δ* mutant brains (data not shown). To validate our findings and provide further evidence that Ca-α 1T

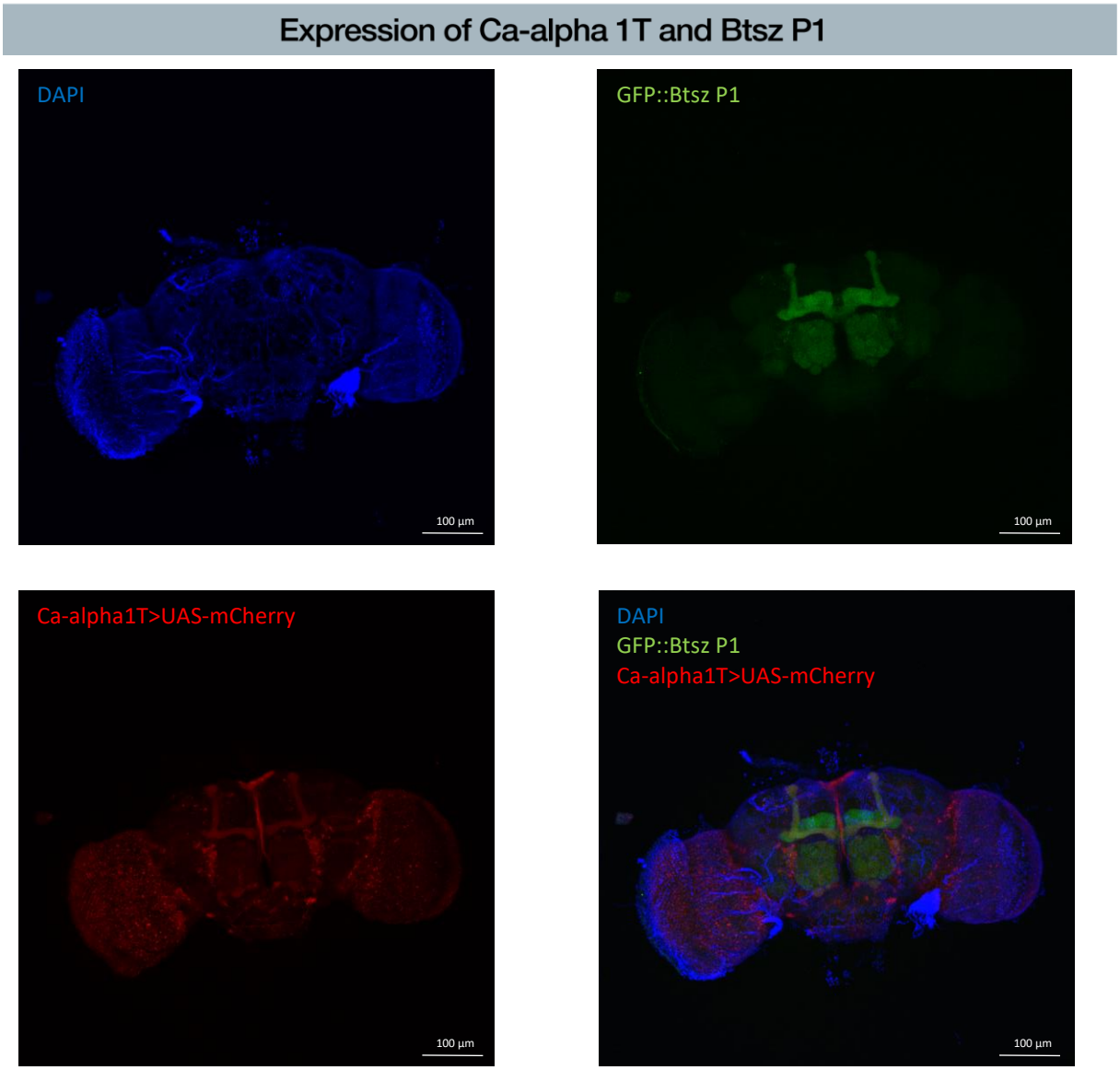


Figure 24: Ca-α 1T colocalizes with Btsz P1 in the α/β of the mushroom body.
Confocal imaging of endogenous expression in female Ca-α 1T > UAS-mCherry, GFP:: Btsz P1 brains was performed. Maximum projection revealed that GFP:: Btsz P1 (green) colocalized with cells expressing Ca-α 1T in the the α and β lobe of the mushroom body .

and Btsz P1 might act in the same pathway, we dissected brains of flies expressing a Ca-α 1T> UAS-mCherry construct in a GFP::Btsz P1 background and performed confocal imaging of endogenous expression. Remarkably, we detected Ca-α 1T signal (red) in the α and β lobe of the mushroom body, where Btsz P1 (green) is expressed as well (Figure 24). Thus, Ca-α 1T is a candidate channel that might be involved in *btsz* mediated regulation of sleep.

We then conducted sleep experiments with female virgin flies that carried a Gal4 knock-in allele, including a termination sequence, which probably results in a null-allele (*Ca-alpha1T null*) and flies carrying a deletion (*Ca-alpha1TΔ)*, which was postulated to result in a loss of function and exposed the flies to Meth treatment. Meth administration resulted in elevated activity levels in *Ca-alpha1TΔ* mutants during the day and during the night (Figure 25A+B).

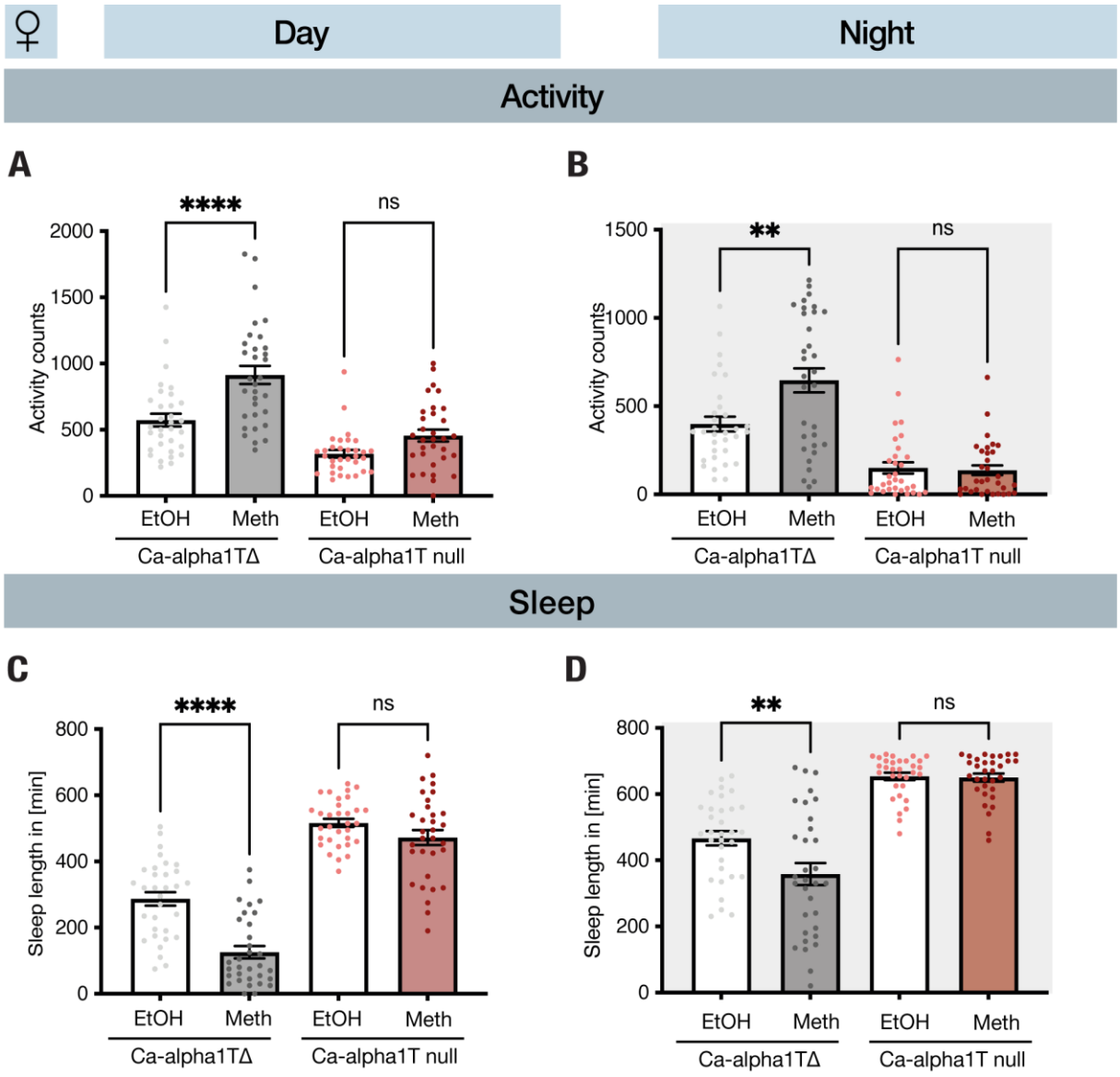


Figure 25: Knock-out of Ca-alpha 1T leads to resistance to Meth treatment.
We assessed sleep and activity of 7-day old female virgin flies and exposed *Ca-alpha1T null* and *Ca-alpha1TΔ* mutants to 1 mg/ml Meth or Ethanol, respectively. During day (A) and during night (B), activity levels were significantly increased in *Ca-alpha1TΔ* mutants upon Meth treatment compared to the EtOH control, whereas activity levels remained unchanged in *Ca-alpha1T null* flies. Sleep duration during the day (C) and during the night (D) was significantly reduced in *Ca-alpha1TΔ* mutants upon Meth exposure. In contrast, total sleep duration did not differ in *Ca-alpha1T null* flies upon Meth treatment compared to the EtOH control. Ordinary one-way ANOVA with multiple comparison Tukey's post hoc test was performed. Columns and errors bars represent mean and SEM. (Mean + SEM). Results of the sleep experiment are based on n=32 female virgin flies per condition. Flies that were inactive for multiple days were excluded from the analysis.

In contrast, activity of *Ca-alpha1T null* flies was not increased upon Meth treatment, reminiscent of *btsz P1Δ* mutants. In general, the activity levels in *Ca-alpha1T null* mutants were lower, however these results are preliminary since the flies were not backcrossed. In line with the activity levels, the sleep duration of *Ca-alpha1TΔ* mutants significantly declined upon Meth treatment during day and night, whereas total sleep duration of *Ca-alpha1T null* was not affected by the Meth treatment (Figure 25C+D). Thus, these results are consistent with the hypothesis that Ca-alpha 1T could be involved in Btsz P1-dependent regulation of sleep. In the future, experiments should be conducted to determine if the localisation of Ca-alpha 1T within the neuron is regulated by Btsz P1 and whether Ca-alpha 1T influences the activity state of dopaminergic neurons in a Btsz-P1 dependent manner.

3.5 Does granuphilin regulate sleep in mammals?

We wondered whether Btsz P1-dependent regulation of sleep might be conserved in mammals. To date, the mammalian homolog of Btsz P1, Granuphilin, has not been associated with the regulation of sleep. Literature exclusively focusses on the function of Granuphilin in the docking of granules and exocytosis of insulin in pancreatic beta-cells. To address this question we first checked if expression of Granuphilin (Gene Symbol Sytl4) or other Synaptotagmin-like genes, can be detected in the mouse hypothalamus, a key region for the regulation of sleep in mammals (Mignot *et al.* 2002). Synaptotagmin-like protein 2 (Esyt2), Rabphilin (Rph), Synaptotagmin 1 (Syt1), Synaptotagmin 4 (Syt4), Synaptotagmin 7 (Syt12), Synaptotagmin 14 (Syt14), Synaptotagmin α (Sytα) and Synaptotagmin β (Sytβ) belong to the group of Synaptotagmins and Synaptotagmin-like proteins, similar to Bitesize (Flybase). Next, we conducted bioinformatical analysis of a single cell RNAseq data set of mouse hypothalamus (Chen *et al.* 2017) and filtered for the expression of the previously listed genes of the Synaptotagmins and Synaptotagmin-like family. Interestingly, expression of Synaptotagmin genes was observed in distinct cell clusters of the hypothalamus, with differences in expression of the listed genes and cell clusters (Supplementary Figure 4+5). Remarkably, also expression of Granuphilin was detected in the hypothalamus, primarily in myelinating oligodendrocyte. Since our finding indicate that Btsz P1 acts in cells expressing DopR in flies, we also checked for the expression of genes that were listed as homologs of the *Drosophila* DopR. Hexokinase-1 (Hk1), Hexokinase-2 (Hk2), Dopamine Receptor D1a (Drda1), Potassium Two Pore Domain Channel Subfamily K Member 1 (Kcnk1) and Klf transcription factor (Klf16) were annotated as homologs. In addition, we also checked for the expression of Rab27a and Rab27b. Notably, expression of all of the listed genes was found in the hypothalamus (Supplementary Figure 6+7). However, the abundance of expression alone is not sufficient to provide evidence that the Btsz P1-dependent regulation of sleep might be conserved in mammals. Therefore, we checked for co-expression of the Btsz homologs with DopR homologs, and Btsz homologs with Rab27a or Rab27b (Supplementary Table 4). Strikingly, the

analysis revealed that co-expression was mainly observed in the cell clusters: GABA13 and GABA8, followed by GABA15 and Glu7. These cell clusters should be investigated in more depths in future studies, to further examine a potential role of Granuphilin and other Btsz homologs in mammals.

In summary, we identified a role of the Synaptotagmin-like protein Btsz P1 in the regulation of sleep in male and female flies. Mutation of *btsz P1* positively modulates sleep and locomotion during ageing. *Btsz P1Δ* mutants were blocked in pharmacological and genetic interventions that activate dopamine-signalling, suggesting that Btsz P1 is likely to mediate its function in neurons receiving dopaminergic inputs. Since the mammalian homolog of Btsz P1 regulates exocytotic processes, we postulate that Btsz P1 might regulate the exocytosis of channels regulating ion currencies in neurons expressing DopR. However, the exact mechanism remains elusive and future studies are required to unravel the molecular function of Btsz P1 and potential implications in mammals.

DISCUSSION

4. Discussion

The *btsz* gene was originally identified as a potential downstream effector of IIS signalling in an RNAseq-based screen of long-lived *dilp 2-3,5* mutant flies (Weigelt 2018). In mice, Granuphilin, the mammalian homolog of *btsz*, mediates the exocytosis of insulin granules via interaction with Rab27a (Yi *et al.* 2002, Torii *et al.* 2004, Gomi *et al.* 2005). However, the role of the *Drosophila* Btsz protein, particularly the P1 isoform containing the conserved SHD domain, is largely unexplored. A prior study from our lab showed that the Btsz P1 isoform has exclusive expression in the mushroom body, a brain region linked to sleep and olfactory learning (Joiner *et al.* 2006). Remarkably, mutation of *btsz P1* extends lifespan in male and female flies and increases sleep duration in young male flies (Weigelt 2018).

I have now shown that mutation of *btsz P1* increases sleep length in both male and female flies, consistent with the extended lifespan in both sexes. Intriguingly, the combination of *dilp 2-3,5* and *btsz P1Δ* mutations did not result in additive effects, but rather detrimental outcomes for lifespan and sleep. Pharmacological and genetic experiments showed that *btsz P1* modulates sleep via the dopamine-signalling pathway. Brain immunostaining and analysis of single cell data sets indicate that Btsz P1 is not expressed in dopaminergic neurons, but in DopR-expressing neurons. In DopR expressing neurons Btsz P1 colocalised with Ca-alpha 1T, a channel linked to the regulation of sleep. Notably, *Ca-alpha 1T null* mutants exhibited resistance to Meth treatment, similar to *btsz P1Δ* mutants. Thus, Btsz P1 may regulate neuronal excitability of dopamine-receiving cells by influencing Ca-alpha 1T exocytosis for ion currency restoration at the transmembrane level mutants. Consequently, Btsz P1 may regulate neuronal excitability of dopamine-receiving cells by influencing Ca-alpha 1T exocytosis for ion currency restoration at the transmembrane level.

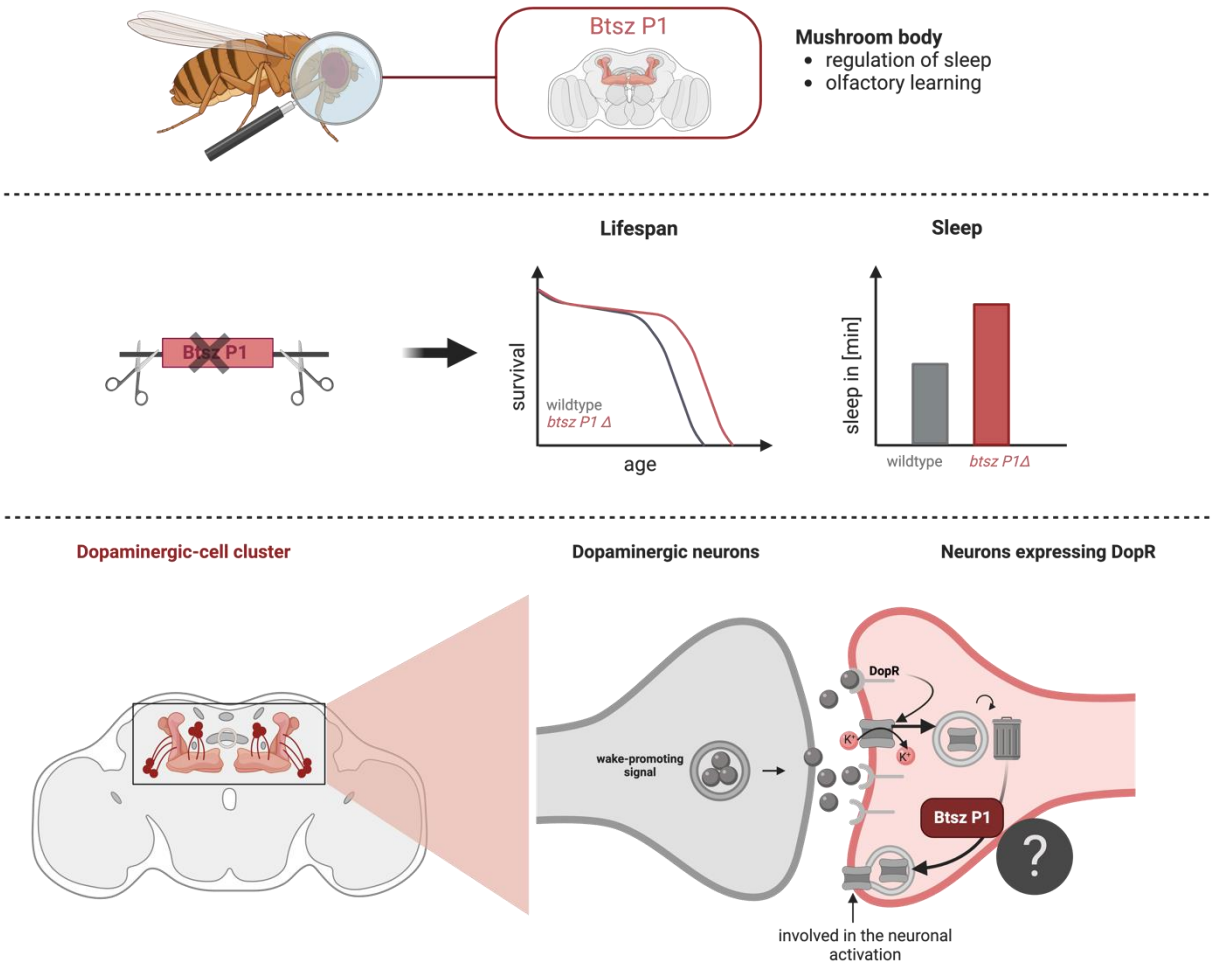


Figure 26: Schematic of the role of *bitesize* in *Drosophila*

4.1 Sex-specific responses to drug treatments

Although *btsz P1* mutants showed similar sleep phenotypes in male and female flies, we observed sex-specific differences in response to the drug treatments. Male wild type flies did not exhibit hyperactivity upon meth treatment, even when treated with a dose of 2 mg/ml, which is eight-times higher than the dose of 0.25 mg/ml, which was sufficient to increase activity and shorten sleep in wild type females. This might be due to the reduced food uptake of male flies compared to female flies and accordingly lower drug uptake. Drug uptake could be directly measured by mass spectrometry on fly brains, to exclude this possibility. Notably, studies investigating other dopamine-dependent behaviours in flies reported that meth affects male courtship behaviour already at a dosage of 0.85 mg/ml (Andretic *et al.* 2005). Thus, it is also possible that Meth does not affect sleep in male flies due to reasons other than reduced drug uptake. Consistently, most studies that measured sleep in Meth treated flies used females (Andretic *et al.* 2005, Andretic *et al.* 2008, Metaxakis *et al.* 2014). Sex specific responses to drug treatments have previously been described (Devineni and Heberlein 2012, Highfill *et al.* 2019, Baker *et al.* 2021). For example, *Drosophila* males display increased hyperactivity post ethanol consumption and greater resistance to ethanol-induced sedation compared to females, underlining sex-specific differences upon ethanol administration (Devineni and Heberlein 2012). Another possible explanation for the ineffectiveness of meth in males is the finding that young female flies exhibited lower dopamine levels than males (Bednářová *et al.* 2017). Thus, females might be more sensitive to Meth treatment, because of their reduced baseline dopamine-levels. Notably, sex-specific effects upon Meth administration are not restricted to *Drosophila* but have also been observed in Sprague-Dawley rats. Female rats show a more pronounced and sustained locomotor activity in response to Meth treatment than males (Milesi-Hallé *et al.* 2007). In line with these findings, recent research involving healthy human study participants indicated that Meth consumption led to increased reaction time solely in women and greater subjective ratings of vigor and reduced sedation, suggesting sexually dimorphic effects of Meth treatment also in humans (Mayo *et al.* 2019). Importantly, mutation of the dopamine transporter, a genetic manipulation acting similarly to Meth treatment, did not cause sex-specific effects on activity and sleep, indicating that the observed differences are likely caused by sex-specific alterations in pharmacokinetics and not by functional differences between male and female flies in the dopamine-signalling. In summary, while the underlying mechanisms that cause the sex specific response upon Meth treatment are currently elusive, the stronger response of females to Meth treatment seems to be evolutionarily conserved and in future studies it would be interesting to use the fly as model system to dissect the associated mechanisms.

A sex-specific response was also observed for 3IY, which inhibits dopamine production via inhibition of tyrosine hydroxylase (Goldstein and Weiss 1965). However, in contrast to meth, 3IY treatment induced increased sleep duration in male but not female flies. This finding is in contrast to a study that reported increased sleep upon 3IY treatment during the day in female flies (Andretic *et al.* 2005). Drug concentration (5mg/ml) was the same between the studies, but differences in genetic background (*Canton-S* vs *wDah*) might explain the discrepancy. To gain a comprehensive understanding, future investigations should determine whether differences between males and females arise from inadequate 3IY uptake or whether the sleep phenotype resulting from 3IY treatment is not shared by female flies. A metabolomic analysis of fly heads should be conducted to ascertain whether the same quantity of 3IY is present in males and females, excluding differences in drug consumption as the potential cause of these observations.

In conclusion, the sleep phenotype of *btsz P1Δ* mutants was consistent between sexes. However, sex-specific distinctions emerged in response to Meth and 3IY treatment, warranting attention in future research. Hence, sleep studies involving drug interventions should encompass both sexes, involve various dosages, and use genetic manipulations for a comprehensive understanding.

4.2 The influence of sleep on ageing and healthspan

Presently, the precise role of the *bitesize* gene in *Drosophila* remains poorly comprehended, in stark contrast to its mammalian homolog Granuphilin, which has a well-described role in insulin-signalling (Gomi *et al.* 2005). Consequently, it remains unclear whether the prolonged sleep patterns observed in *dilp 2-3,5* and *btsz P1Δ* flies contribute to reported extended lifespan and improved healthspan (Metaxakis *et al.* 2014, Weigelt 2018). Previous research has indicated that long-lived *dilp 2-3,5* mutants exhibit an improved sleep quality, not only at a young age, but also during ageing (Metaxakis *et al.* 2014), consistent with the hypothesis that improved sleep quality might contribute to enhanced healthspan and, potentially, lifespan. Our sleep experiments, with young, middle-aged and old-aged male flies, emphasise that the progression of age-related sleep deterioration is delayed in *btsz P1Δ* mutants. A prolonged sleep bout duration was observed in young and middle-aged *btsz P1Δ* male flies, although intriguingly, it did not persist in older flies. To gain a more comprehensive understanding, further exploration is needed within the 25- to 45-day age range in males. This investigation could shed light on the timing of age-related sleep disturbances and unravel the underlying factors contributing to the reduction in both total sleep length and sleep bout length in ageing *btsz P1Δ* flies. In line with the findings of the sleep experiments, male and female *btsz P1Δ* mutants exhibited improved locomotor function during ageing, indicating ameliorated physiological decline. The reduced locomotor ability of wild type flies during ageing might influence the sleep analysis, which is based on

activity movements. Consequently, more sophisticated techniques are required in the future to gain more comprehensive understanding about the sleep quality during ageing and to ensure a better distinction between sleep and inactive states. Parameters such as sleep rebound following sleep deprivation could be assessed in future studies, since reports suggest that mutants with diminished sleep length also exhibit decreased sleep rebound (Donlea *et al.* 2014). Moreover, it would be valuable to explore alterations in arousal threshold (Faville *et al.* 2015), as sleep quality encompasses not only sleep duration and bout length but also sleep depth. To further evaluate potential alterations of deep sleep during ageing in *btsz P1Δ*, proboscis extension assays are a suitable method to distinguish not only inactive states from sleep, but to additionally provide information about deep sleep stages and the respective duration and latency (van Alphen *et al.* 2021). We hypothesise that alterations in sleep depth could diverge during ageing between wild type flies and *btsz P1Δ* mutants, influencing healthspan and potentially lifespan.

4.2.1 The potential molecular consequences of high and poor sleep quality

To date, the relationship between sleep quality and lifespan remains a topic of debate (Kume *et al.* 2005, Geissmann *et al.* 2019). In *Drosophila*, mutations in genes associated with sleep disruption such as *insomnia-like, Hk^1, Hk^Y*, and *Hk^2* are associated with a decreased lifespan, indicating that poor sleep quality can reduced survival in flies (Seugnet *et al.* 2009, Bushey *et al.* 2010). In contrast, another fly study did not find a clear association between sleep length and survival (Geissmann *et al.* 2019). However, this finding requires caution as the studied flies were in controlled lab conditions, and it was not possible to exclude micro-periods of sleep. Additionally, sleep deprivation has led to considerable physiological and cognitive decline and even to toxic protein aggregation in laboratory animals (McDermott *et al.* 2003, Seugnet *et al.* 2009, Holth *et al.* 2019), which will be disadvantageous in a natural environment. The discovery of a deep sleep stage in *Drosophila*, which has been linked to waste clearance, accentuates the physiological importance of sleep. Blocking the characteristic deep-sleep induced proboscis extension increases injury-related mortality and decreases waste clearance (van Alphen *et al.* 2021). Moreover, sleep has been suggested to have a reciprocal relationship with reactive oxygen species in neurons. Mutants with short sleep duration have been shown to display an elevated vulnerability to acute oxidative stress. Strikingly, prolonged sleep duration induced by pharmacological or genetic methods has been linked to increased survival rates after oxidative challenges (Hill *et al.* 2018). Another study further supported these findings by exposing flies and mice to sleep deprivation. Sleep deprivation was not only associated with accumulation of reactive oxygen species, specifically in the gut, but also increased mortality (Vaccaro *et al.* 2020).

Further research is needed to elucidate the influence of sleep on ageing and its impact on health and lifespan. To further investigate the role of sleep during ageing and its importance regarding healthspan, additional experiments should be performed. Potentially, the impaired sleep quality during ageing could be improved by treatment with Gaboxadol. To assess the effects of improved sleep quality on the ageing process, lifespan, climbing ability and waste clearance should be investigated in wild type flies that are exposed to Gaboxadol throughout the ageing process. Additionally, the ability to induce deep sleep stages and waste clearance potential during the ageing process should be examined in *btsz P1Δ* mutant flies, to test whether the increased sleep length of *btsz P1Δ* mutants also correlates with an improved sleep quality and potentially increased waste clearance. In addition, it should be tested whether the prolonged sleep duration in *btsz P1Δ* mutants has protective effects against the accumulation of reactive oxygen species.

4.3 Does Btsz P1 influence insulin-signalling?

Reduction of insulin-signalling has been shown to extend lifespan in diverse species including *Drosophila*, e.g. *dilp 2-3,5* mutants that lack *Drosophila* insulin-like peptides 2, 3 and 5 and are 30 % longer lived (Grönke *et al.* 2010). Similarly, flies with a mutation in Btsz P1, phenocopy insulin mutant phenotypes including an extended lifespan, reduced fecundity and delayed development (Weigelt 2018). These results suggested that Btsz P1 might be involved in insulin-signalling similar to its mammalian homologue Granuphilin, that mediates the exocytosis of insulin (Yi *et al.* 2002). However, immunostainings in larval and adult brains demonstrated that Btsz P1 is not expressed in insulin-secreting neurons, suggesting that Btsz P1 does not influence insulin-signalling in *Drosophila* by regulating the exocytosis of insulin-like peptides. Surprisingly, *btsz P1Δ, dilp 2-3,5* double mutants were short lived compared to the corresponding single mutants and hyperactive at night. It might be that the combination of the *btsz P1Δ* and *dilp 2-3,5* mutations results in a further reduction in insulin-signalling, which could be detrimental and cause the observed phenotypes. Consistent with this hypothesis, a too severe reduction in insulin-signalling can shorten lifespan and beneficial effects on lifespan are usually observed at intermediate levels (Cohen and Dillin 2008). How *btsz* might affect insulin-signalling is currently unclear. Btsz P1 is expressed in the mushroom body, which is a target for insulin-like peptides and expresses the Insulin receptor (Nässel *et al.* 2013). There is currently no evidence that Btsz P1/Granuphilin is involved in the recycling of the Insulin receptor or in the regulation of signalling-pathways that are targeted by insulin-like peptides. In future it needs to be tested if insulin-like peptides are localized in the mushroom body and might be affected by the mutation of *btsz P1*

4.4 A potential role of Btsz P1 in sleep-regulating pathways?

4.4.1 Btsz P1 does not seem to influence octopamine-signalling

Octopamine, dopamine, and histamine are well-known neurotransmitters that promote wakefulness in flies (Crocker and Sehgal 2008, Liu *et al.* 2012, Ueno *et al.* 2012, Ueno and Kume 2014, Kasture *et al.* 2018). In the central nervous system of *Drosophila*, octopaminergic cells are abundant, projecting into various regions, including the calyx of the mushroom body (Sinakevitch and Strausfeld 2006). Mutants with disrupted octopamine synthesis exhibit increased sleep (Crocker and Sehgal 2008), prompting us to investigate whether octopamine-signalling is affected in *btsz P1Δ* mutants.

To explore potential alterations in octopamine-signalling, we subjected both wild type and *btsz P1Δ* flies to starvation, a condition known to induce hyperactivity through increased octopamine-signalling (Yang *et al.* 2015, Li *et al.* 2016). Remarkably, the response of *btsz P1Δ* mutants to starvation closely

resembled that of wild type flies, with both genotypes exhibiting increased activity, suggesting that the deletion of Btsz P1 does not disrupt octopamine release. However, the response of *btsz P1Δ* mutants towards starvation was even stronger. Interestingly, mass spectrometry measurement revealed increased octopamine levels in *btsz P1Δ* mutants, which potentially could explain the intensified starvation response in *btsz P1Δ* mutants.

Remarkably, flies carrying a mutation in the octopamine receptor display increased sleep compared to controls. Though, starvation induced increased activity in these mutants (Erion *et al.* 2012), resembles the extended sleep and increased activity levels observed in *btsz P1Δ* mutants upon starvation. In contrast to *btsz P1Δ* mutations, octopamine receptor mutants did not supress sleep upon starvation, despite increased activity levels. In addition, expression of the octopamine receptor is not restricted to the mushroom body but has also been reported throughout the adult brain and ventral nerve cord (McKinney *et al.* 2020), further suggesting that Btsz P1 does not directly influence octopamine-signalling.

4.4.2 GABA-signalling is not impaired in *btsz P1Δ* mutants

Numerous microcircuits within the *Drosophila* mushroom body have been identified as essential for maintaining a balanced and finely controlled sleep-wake cycle. Among these, neurotransmitter systems, GABA signalling plays a crucial role in regulating sleep and wake microcircuits located within the mushroom body. Dorsal paired medial neurons promote sleep by releasing GABA onto the α'/β' wake-promoting neurons, which results in the neurons' inhibition. Additionally, anterior paired lateral neurons have been associated with sleep promotion by inhibiting wake-promoting KC through GABA-signalling (Haynes *et al.* 2015, Driscoll *et al.* 2021). Therefore, we treated wild type flies and *btsz P1Δ* mutants with the GABA-agonist Gaboxadol and analysed sleep, to test whether alterations in GABA-signalling were responsible for the observed sleep phenotype of *btsz P1Δ* mutants. Sleep analysis revealed that *btsz P1Δ* mutants reacted with an expected prolonged sleep duration to Gaboxadol treatment, similar to wild type flies, indicating that GABA-signalling alone is not impaired in *btsz P1Δ* mutants.

However, research has elucidated an intricate interplay between GABAergic neurons and dopamine receptors in the context of aversive and appetitive memory (Karam *et al.* 2020). Presynaptic dopaminergic neuronal activity is tightly regulated by a timed inhibitory feedback loop. For the consolidation of appetitive memory, a feedback loop involving dopamine neurons expressing a GABA receptor and GABAergic mushroom body output neurons expressing both DopR2 and Dop1R2 is indispensable (Pavlowsky *et al.* 2018, Karam *et al.* 2020). This feedback loop regulates presynaptic

dopamine neuron activity, preventing over-activation of dopamine-signalling (Perisse *et al.* 2016, Pavlowsky *et al.* 2018, Karam *et al.* 2020).
For aversive memory, DopR2 signalling in GABAergic neurons, particularly in the anterior paired lateral neurons that connect to the mushroom body, is crucial for restraining GABAergic inhibition (Zhou *et al.* 2019, Karam *et al.* 2020). Therefore, examination of memory formation in *btsz P1Δ* mutants could give more detailed information about neuronal circuits in which *btsz P1* might mediate its function.

4.4.3 Altered dopamine-signalling explains the sleep phenotype of *btsz P1Δ* mutants

Dopaminergic cells form clusters, including the PAM and PPL1 clusters, project to the mushroom body and thereby regulate wake and sleep states (Aso *et al.* 2014, Sitaraman *et al.* 2015). The wake-promoting impact of dopamine has been extensively explored via pharmacological and genetic approaches. For instance, the tyrosine hydroxylase inhibitor 3IY results in reduced dopamine synthesis and in consequence increased daytime sleep (Andretic *et al.* 2005). Correspondingly, tyrosine hydroxylase deletion caused by frameshift mutations resulted in both increased daytime and nighttime sleep while raising arousal threshold (Riemensperger *et al.* 2011).

Interestingly, male *btsz P1Δ* mutants did not show differences in total sleep length and activity during day or night upon 3IY treatment in contrast to wild type flies, indicating that dopamine-signalling might be impaired in *btsz P1Δ* mutant flies. Mass spectrometry analysis revealed that total dopamine levels were not changed in heads of *btsz P1Δ* mutants, suggesting that Btsz P1 does not affect dopamine synthesis, but probably acts downstream. To further support the generated pharmacological evidence in male flies, a genetic approach with female flies carrying mutation in the tyrosine hydroxylase and the *btsz P1Δ* mutation should be conducted, to overcome sex-dependent pharmacokinetic differences and prove that the observations are conserved in both sexes.

To further investigate dopamine-signalling in *btsz P1Δ* mutants we performed sleep experiments under the administration of Meth. Meth inhibits DAT in *Drosophila* (Freyberg *et al.* 2016) and mammals (Xie and Miller 2009), resulting in intensified dopamine-signalling. Meth treatment reduced sleep in female wild type flies, in line with prior findings (Andretic *et al.* 2005). Strikingly, *btsz P1Δ* mutants did not display meth-induced hyperactivity. This finding was verified by an independent genetic approach in male and female flies, in which the hyperactivity phenotype of *fumin* mutants (the genetic equivalent to meth treatment) was blocked by the mutation of *btsz P1*. Meth and the mutation of DAT (*fumin*) act downstream of dopamine synthesis in the synaptic cleft, where the re-uptake of dopamine to the presynaptic cell is blocked. Resistance of *btsz P1Δ* mutants to both 3IY and meth treatment implies that dopamine-signalling is disrupted in *btsz P1Δ* mutants after the release of dopamine into the

synaptic cleft, e.g., in dopamine-receiving cells. Consistent with this hypothesis, Btzs P1 was only detected in dopamine receiving neurons and not in dopaminergic cells itself, further suggesting that Btsz P1 affects signal transduction after binding of dopamine to the dopamine receptor. Similar to the expression pattern of Btsz P1, Dop1R is expressed in the mushroom body (Landayan *et al.* 2018), which is particularly important for the wake-promoting effects of dopamine (Li *et al.* 2000), suggesting that Btsz P1 might interact with Dop1R or influences signal transduction in Dop1R expressing cells. This hypothesis is further supported by the sleep phenotype of DopR mutants. DopR mutants (*dumb*), which carry a hypomorphic allele for DopR, exhibit impaired arousal and increased sleep, similar to *btsz P1Δ* mutants (Faville *et al.* 2015). Interestingly, RNAseq revealed that DopR is up-regulated in *btsz P1Δ* mutants, probably as a compensatory effect to reduced dopaminergic-signalling. In line with our hypothesis, *btsz P1* expression was detected in cell clusters with DopR as gene marker.

Strikingly, dopaminergic signalling in the mushroom body is not only implicated in the regulation of arousal and sleep, but also in learning, memory formation, temperature preference and courtship behaviour (Neckameyer 1998, Bang *et al.* 2011, Karam *et al.* 2020). Considering the potential function of Btsz P1 in cells expressing DopR the investigation of other dopamine-regulated behaviours could be interesting in the future. It has been reported that Dop1R1-signalling in the mushroom body is responsible for encoding long-term memory and in acquisition of memory by integrating high levels of dopaminergic input with odour-evoked calcium signals, thus ultimately resulting in increased activity of mushroom body output neurons (Tomchik and Davis 2009, Berry *et al.* 2012, Karam *et al.* 2020). Subsequently, as released dopamine levels decrease, Dop1R2, acting in the same neurons as Dop1R1, induces the process of forgetting (Berry *et al.* 2012, Karam *et al.* 2020). This paradoxical phenomenon is believed to occur due to the distinct activation profiles of these two receptors (Berry *et al.* 2012, Karam *et al.* 2020). Interestingly, increased sleep-drive after learning has been reported to result in decreased dopaminergic activity, thus enhancing memory retention (Berry *et al.* 2015). Hence, potential changes in olfactory learning in *btsz P1Δ* mutants should be investigated in the future.

In addition, it has been shown that dopaminergic signalling regulates male courtship behaviour. For instance, it has been reported that Dop1R1 mutants display male-male courtship behaviour (Chen *et al.* 2012). In line with these findings, mutation of Dop1R1, which is expressed in the mushroom body, resulted in reduced courtship towards less appealing females, which was a result of delayed courtship initiation and extended periods between courtship bouts (Lim *et al.* 2018). Consequently, in future studies courtship behaviour of *btsz P1Δ* mutants should be examined as well.

4.5 In which neuronal subset does Btsz P1 mediates its function?

Numerous neuronal classes have been identified to influence sleep (Liu *et al.* 2012, Sitaraman *et al.* 2015, Ly *et al.* 2018), which project to or are part of the mushroom body. Notably, the wake-promoting effects observed in flies upon activation were attributed to dopaminergic-neuron populations within the PAM and PPL1 clusters that target the mushroom body (Sitaraman *et al.* 2015). However, Btsz P1 expression was not detected in the PAM or PPL1 clusters, indicating that other cells might contribute to the sleep phenotype of *btsz P1Δ* mutants, which potentially receive dopaminergic inputs of those clusters. Recently, a study mapped the pathways from PAM neurons that connect to the γ5 and β'2 MB compartments. The study revealed that the state of wakefulness is controlled by both DopR1 and DopR2 receptors in the subsequent KCs and mushroom body output neurons (Driscoll *et al.* 2021). In addition, both wake-promoting and sleep-promoting microcircuits have been identified (Sitaraman *et al.* 2015), which could also potentially be impacted in *btsz P1Δ* mutants. By utilizing the data of the single cell expression atlas regarding the respective cell clusters in which *btsz P1* expression is annotated, together with relevant publications, the relevant cell cluster in the Btsz P1-dependent regulation of sleep should be further investigated and identified. Since no RNAi lines specifically targeting the *btsz P1* isoform are available and previously generated RNAi lines were not efficient, Dop1R1 RNAi lines could be used alternatively. Therefore, the recently generated Btsz P1^{null} Gal4 line could be used to specifically drive Dop1R1 RNAi expression in neurons expressing *btsz P1.* Additionally, upon potential identification of other proteins that are potentially involved in the Btsz P1- dependent regulation of sleep, potential interaction partners could be down-regulated using RNAi under expressional control of *btsz P1.*

4.6 The search for Btsz P1' partners in crime

A study has shown that the two pore-domain potassium channel Sandman is also involved in the regulation of sleep in dopamine-receiving cells. Interestingly, this channel is internalized after signal transduction, recycled, and transported back to the post-synaptic membrane via vesicle exocytosis (Pimentel *et al.* 2016). As Btsz P1 contains a highly conserved SHD domain, which is required for the regulation of exocytosis of vesicles in mammals (Gomi *et al.* 2005), we postulated that Btsz P1 might regulate the exocytosis of sleep-regulating channels in order to control neuronal excitability.

To further elucidate the exact mechanisms, we filtered the single cell expression atlas for different channels that have been implicated in the regulation of sleep or that are co-expressed with Btsz P1 and DopR and identified Ca-alpha 1T, as a potential candidate that might be involved in Btsz P1-dependent regulation of sleep. Ca-alpha 1T has previously been associated with the regulation of sleep. In mammals, where various T-type Ca^{2+} channels exist, this group of channels is recognized as sleep stabilizers involved in generating the delta rhythms of deep sleep (Jeong *et al.* 2015). Interestingly, Ca-α 1T mutants display prolonged sleep duration, reminiscent of the sleep pattern observed in *btsz P1Δ* mutants (Jeong *et al.* 2015). Furthermore, our RNAseq analysis revealed an up-regulation of Ca-α 1T in *btsz P1Δ* mutant heads. We validated the expression of Ca-α 1T with whole mount immunostainings and performed sleep experiments under meth exposure. Interestingly, *Ca-α 1T null* mutants were resistant to meth treatment, similar to *btsz P1Δ* mutants, indicating that both proteins might act on the same pathway. In future studies, the localisation of Ca-α 1T should be investigated in wild type conditions compared to *btsz P1Δ* background, in order to investigate if Btsz P1 potentially influences the transmembrane localisation of Ca-α 1T. Remarkably, Ca-α 1T was annotated in a cell cluster with Rab27 expression, a postulated interaction partner of Btsz P1 in mediating exocytosis, based on the mammalian data. A recent study has reported that lifespan is modulated by Rab27 in α/β posterior neurons of the fly mushroom body (Lien *et al.* 2020), resembling the lifespan phenotype of *btsz P1Δ* mutants. However, sleep patterns were not investigated in this study. In future experiments, sleep patterns of Rab27 mutants should be further investigated and whether Rab27 and Btsz P1 mediate via interaction the exocytosis of Ca-α 1T in order to regulate sleep.

4.7 What is the molecular consequence of deletion of *btsz P1*?

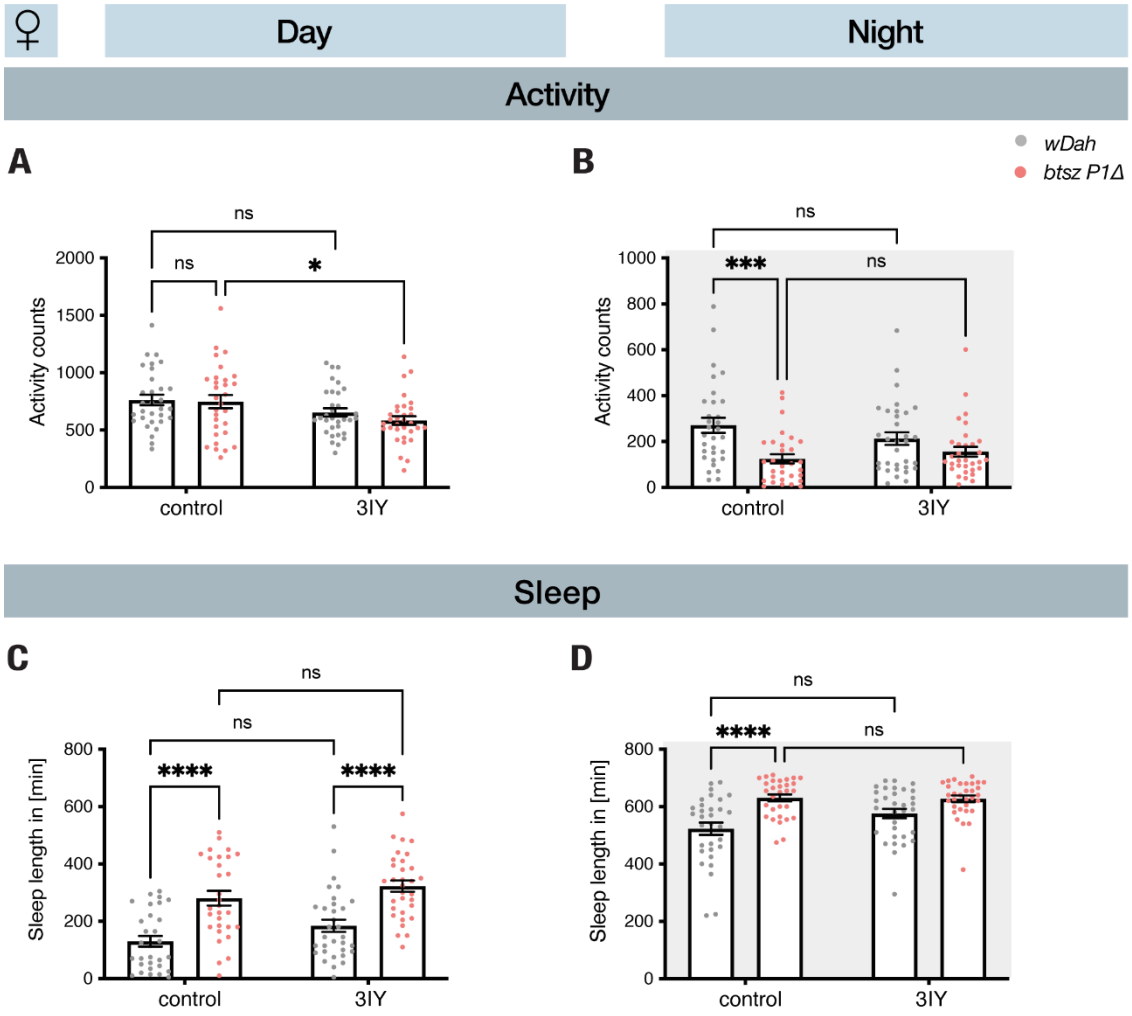
To date, the molecular consequences of deletion of *btsz P1* remain elusive since no suitable antibody is available. Since the deletion of *btsz P1* only affects 160 bp around the first coding exon and subsequent additional start codons in the sequence can be used, it is unlikely that the mutation causes a complete loss-of function of the *btsz P1* gene. Transcripts containing parts of the first coding exon and the second coding exon, which are specific to the P1 isoform have been detected in the RNAseq of *btsz P1Δ* mutants. Therefore, diminished expression of the highly conserved SHD domain, mediating the exocytosis in mammals, can be ruled out as a possible explanation for the observed phenotypes. Consequently, deletion of *btsz P1* probably causes either a hypomorphic allele or gain of function mutation due to a truncated protein. *btsz P1* and the isoforms originating from the P2 promoter, share the C2A domain and C-terminal end. Only the first coding exon and the SHD domain are specific for *btsz P1.* In addition, *btsz P2* expression is also abundant in the brain, resulting in no suitable antibodies for the specific detection of Btsz P1. Since Btsz P1 specifically cannot be detected on a protein level without a suitable antibody, we are currently not able to ultimately state if deletion of *btsz P1* might result in a truncated protein with alternating functions. Genetic complementation assays, with *btsz P1 null* alleles will be required to gain more information about the genetic characteristics of the mutation.

4.8 Is the function of Btsz P1 potentially conserved in mammals?

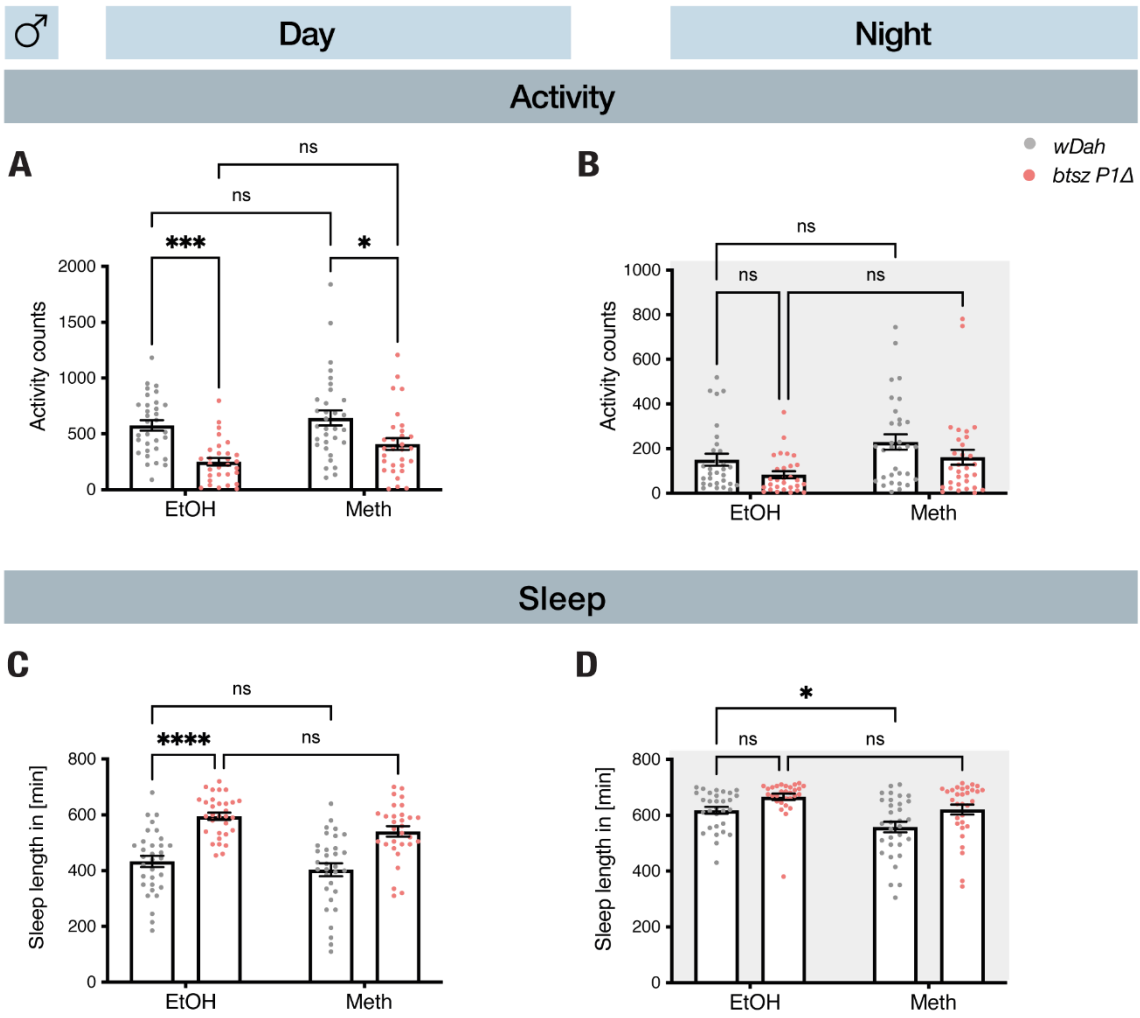
Granuphilin, the mammalian homolog of Btsz P1, has been intensively studied in the context of the regulation of insulin containing granules via Rab27a in pancreatic beta cells (Yi *et al.* 2002, Torii *et al.* 2004, Gomi *et al.* 2005). It is currently unknown, whether Granuphilin plays a role in the regulation of sleep or the insulin-signalling pathway in the brain. Initially, Granuphilin expression was not detected in the brain probably due to technical limitations and detection issues of immunoblotting and Northern blotting (Wang *et al.* 1999, Yi *et al.* 2002). However, Granuphilin expression, as well as expression of other Btsz P1 homologs, was detected by single-cell RNAseq of the mouse hypothalamus (Chen *et al.* 2017). In addition, Granuphilin expression in the brain pons was detected using in situ hybridisation (Allen Brain Atlas). Despite its expression in specific brain areas, the function of Granuphilin in the brain has not been analysed so far. The role of Btsz P1 in the regulation of sleep might generate evidence that Granuphilin potentially has functional implications in the brain. Further studies are needed to investigate whether the Btsz P1-dependent regulation of sleep is potentially conserved in mammals.

SUPPLEMENT

5. Supplement



Supplementary Figure 1: female flies did not react upon 3IY treatment.
(A) During the day, 3IY treatment did not result in a reduction in activity in wild type flies (Interaction: ns., treatment: ** < 0.01, genotype: ns.). (B) During the night, no significant differences upon 3IY treatment were observed in both genotypes (Interaction: ns., treatment: ** < 0.01, genotype: ns.). (C) During the day, sleep duration was unaltered upon 3IY treatment in both genotypes (Interaction: ns., treatment: * < 0.05, **** < 0.0001). (D) During the night, 3IY treatment did not result in changes of total sleep duration (Interaction: ns., treatment: ns., genotype: **** < 0.0001.). 2-way ANOVA with multiple comparison Tukey's post hoc test was performed. Columns and errors bars represent mean and SEM. (Mean + SEM). Results of the sleep experiment are based on n=32 male flies per condition, however some of the flies had to be censored due to inactivity on multiple days.



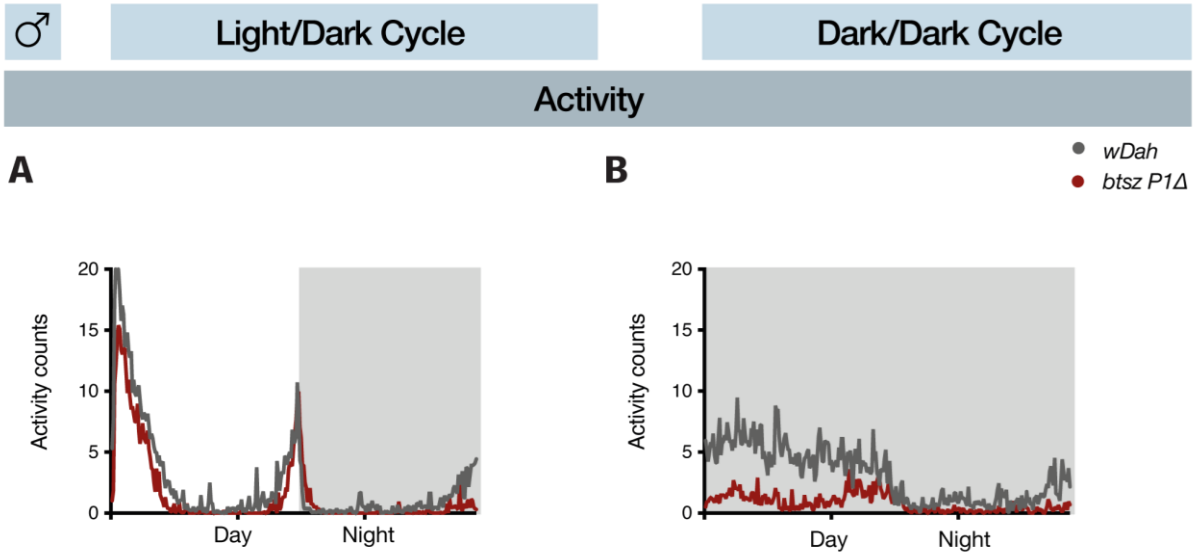
Supplementary Figure 2: Neither wild type nor *btsz P1Δ* males reacted to meth treatment.
(A) During day and night (B) both tested genotypes did not show activity chances upon Meth treatment (A) (Interaction: ns., treatment: * < 0.05, genotype: **** < 0.0001), (B) (Interaction: ns., treatment: ** < 0.01, genotype: * < 0.05). (C) During the day, untreated *btsz P1Δ* mutants slept longer than wild type flies. No decrease of sleep length upon Meth treatment was observed in wild type and *btsz P1Δ* males (Interaction: ns., treatment: ** < 0.01, genotype: *** < 0.001). (D) During the night, Meth treatment did not affect both tested genotypes (Interaction: ns., treatment: * < 0.05, genotype: **** < 0.0001). 2-way ANOVA with multiple comparison Tukey's post hoc test was performed. Columns and errors bars represent mean and SEM. (Mean + SEM). Results of the sleep experiment are based on n=32 male flies per condition, however some of the flies had to be censored due to inactivity on multiple days. The results have previously been reported in (Jahn 2019).

Supplementary Table 1: Differentially expressed genes of the GO term "sleep".
(up-regulated indicated in red, down-regulated indicated in blue)

Gene	Gene product	GO class	Annotation extension
FBgn0051676		GO:0030431	InterPro
FBgn0002719	Malic enzyme	GO:0030431	FlyBase
FBgn0032022		GO:0030431	InterPro
FBgn0032899		GO:0030431	InterPro
FBgn0036697	rogdi	GO:0030431	UniProt
FBgn0032725	Nedd8 ubiquitin like	GO:0030431	Flybase
FBgn0033872		GO:0030431	InterPro

Supplementary Table 2: Genes annotated to the GO term circadian rhythm

GO_id	GO_term	GO_pval	GO_gene	GO_logfc	GO.Gene_symbol
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0262593	2.08407283655335	Shab
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0261953	0.925311535780704	TfAP-2
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0260753	0.894851160826418	Pdfr
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0259938	0.263782823722551	cwo
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0039536	0.25186500404042	unc80
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0038870	0.250906322682879	Oga
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0038693	0.247575534522497	unc79
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0038118	0.242152231323907	timeout
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0023407	0.234107888319863	B4
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0023178	0.23103087881996	Pdf
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0022382	0.208880899487738	Pka-R2
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0019643	0.189547319190911	AANAT1
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0017561	0.189517432822695	Ork1
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0014019	0.187446002556908	Rh5
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0004611	0.181320577452606	Plc21C
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0003997	-0.232943953816358	hid
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0003429	-0.312348920903107	slo
GO:0007623	GO:0007623 - circadian rhythm	5.80914299031403	FBgn0000459	-0.541344777687688	disco



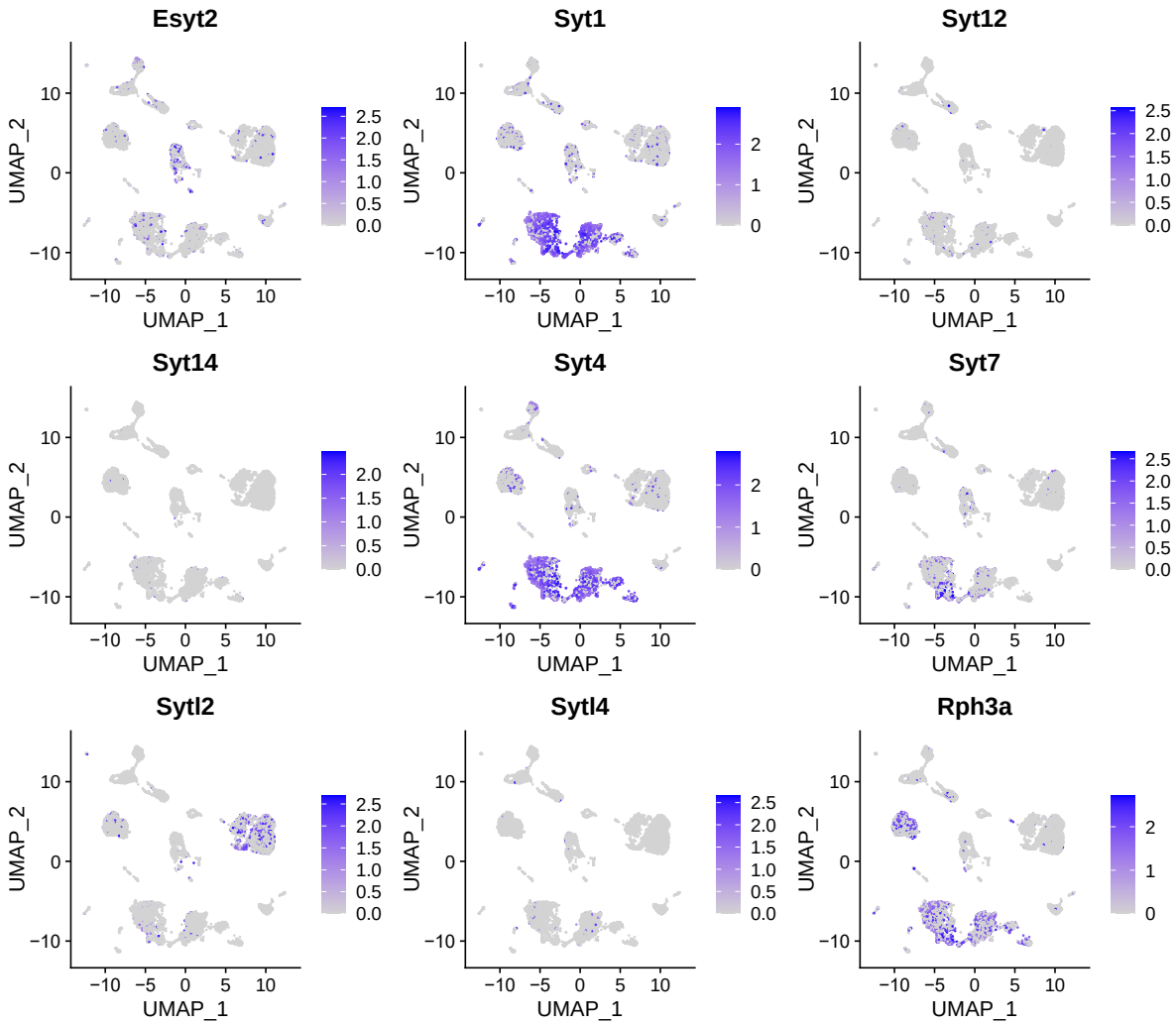
Supplementary Figure 3: Circadian rhythm was not disturbed in *btsz P1Δ* mutants.
We assessed activity of 7-day old male wild type flies and *btsz P1Δ* mutants for three days in a 12-hour light and 12-hour dark cycle. After three days we switched the sleep chamber to constant darkness. (A) In a 12-hour light and 12-hour dark cycle, characteristic activity peaks can be observed in both genotypes which align with light-switches. (B) In constant darkness activity increases during the day and reduces during the night in both genotypes.

Supplementary Table 3: Gene markers identified in cell clusters expressing *btsz*

Gene Symbol	Cluster ID	Annotation
Dop1R2	8	G-KC
Dop1R1	8	G-KC
dlg1	8	G-KC
nAChRalpha6	8	G-KC
sdt	8	G-KC
slo	8	G-KC
Shawl	8	G-KC
metro	8	G-KC
Rab27	8	G-KC
Rdl	8	G-KC
fwe	8	G-KC
Ca-beta	8	G-KC
nAChRalpha5	8	G-KC
pyd	8	G-KC
Elk	8	G-KC
nAChRalpha1	8	G-KC
cac	8	G-KC
Ca-alpha1D	8	G-KC
Ktl	8	G-KC
Rab27	22	a/b-KC
Dop1R2	22	a/b-KC
Rdl	22	a/b-KC
slo	22	a/b-KC
dlg1	22	a/b-KC
Dop1R1	22	a/b-KC
pHCl-1	22	a/b-KC
metro	22	a/b-KC
sdt	22	a/b-KC
Elk	22	a/b-KC
Shawl	22	a/b-KC
pyd	22	a/b-KC
Task6	22	a/b-KC
KCNQ	22	a/b-KC
Ca-beta	22	a/b-KC
nAChRalpha5	22	a/b-KC

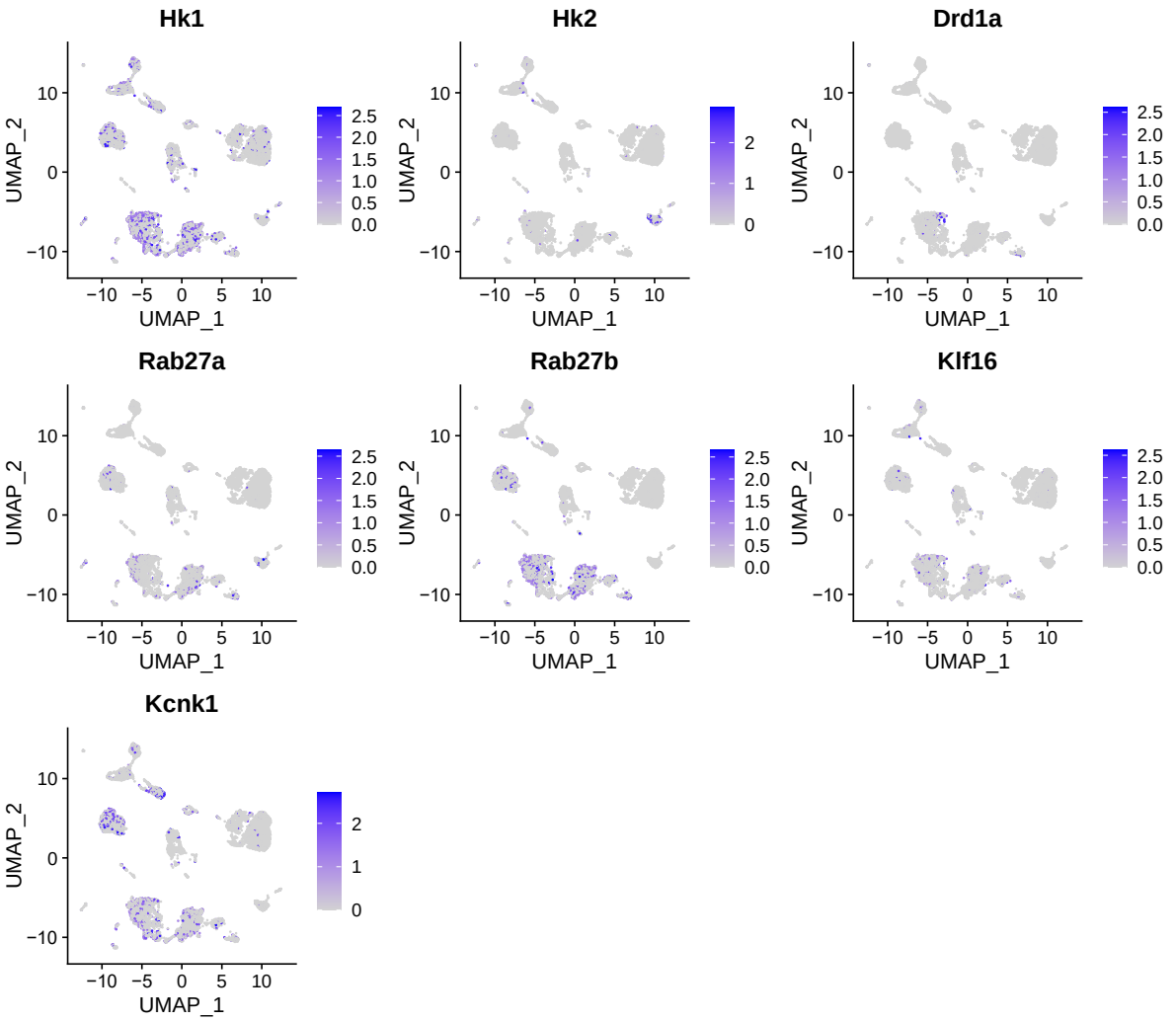
fwe	22	a/b-KC
GluClalpha	22	a/b-KC
Dop1R2	28	a'/b'-KC
dlg1	28	a'/b'-KC
nAChRalpha6	28	a'/b'-KC
metro	28	a'/b'-KC
Dop1R1	28	a'/b'-KC
porin	28	a'/b'-KC
nAChRbeta1	28	a'/b'-KC
Rdl	28	a'/b'-KC
nAChRalpha5	28	a'/b'-KC
cac	28	a'/b'-KC
Elk	28	a'/b'-KC
Task7	28	a'/b'-KC
unc80	28	a'/b'-KC
fwe	61	dFB
CG4587	61	dFB
CASK	61	dFB
stj	61	dFB
porin	61	dFB
Shal	61	dFB
nAChRalpha7	61	dFB
Dop1R1	61	dFB
Ktl	61	dFB
twz	61	dFB
Irk2	66	Subperineurial Glia
ort	74	Unannotated
Ca-alpha1T	74	Unannotated
para	74	Unannotated
porin	74	Unannotated
Teh2	74	Unannotated
Ih	74	Unannotated
CG4587	74	Unannotated
Teh1	74	Unannotated
Lcch3	74	Unannotated
Ktl	74	Unannotated
stj	74	Unannotated
CASK	74	Unannotated

nAChRalpha4	74	Unannotated
unc80	74	Unannotated
fwe	74	Unannotated
Ca-beta	74	Unannotated
Rdl	80	Poxn
5-HT7	80	Poxn
nAChRalpha7	80	Poxn
sdt	80	Poxn
slo	80	Poxn
porin	80	Poxn
Ca-beta	80	Poxn
cac	80	Poxn
Elk	80	Poxn

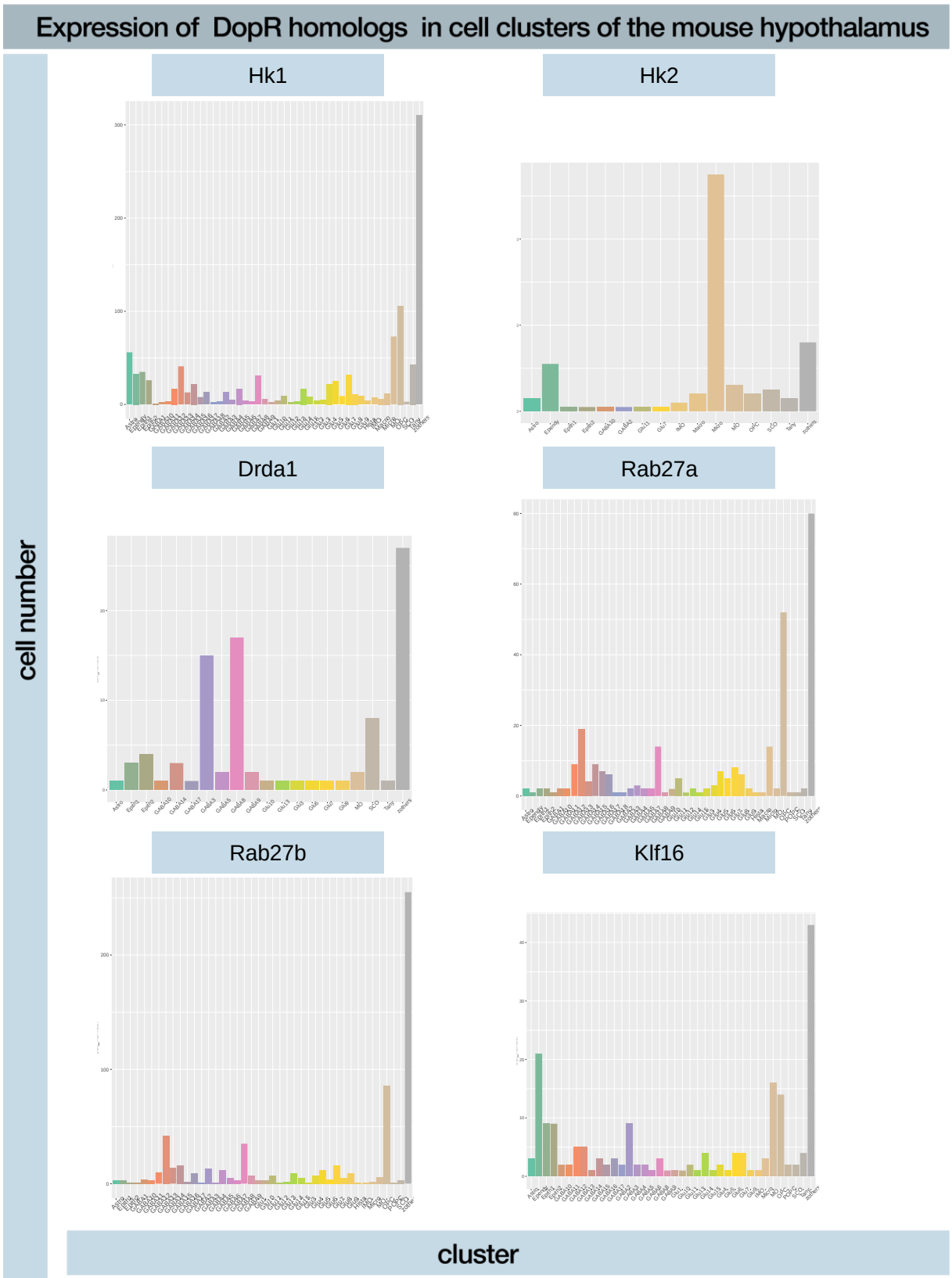


Supplementary Figure 4: Expression of *btsz* homologs in the mouse hypothalamus.
Expression of *btsz* homologs (based on Flybase), extended Synaptotagmin-like protein 2 (Esyt2), Synaptotagmin 1 (Syt1), Synaptotagmin 12 (Syt12), Synaptotagmin 14 (Syt14), Synaptotagmin 4 (Syt4), Synaptotagmin 7 (Syt7), Synaptotagmin-like protein 2 (Sytl2), Synaptotagmin-like protein 4 (Sytl4) and Rabphilin 3a (Rph3a) in the mouse hypothalamus. Bioinformatic analysis of the single cell RNAseq data set (Chen *et al.* 2017) was conducted by Nathalie Jauré.

Supplementary Figure 5: Expression of *btsz* homologs in different cell types of the hypothalamus.
Expression of *btsz* homologs (based on Flybase), extended Synaptotagmin-like protein 2 (Esyt2), Synaptotagmin 1 (Syt1), Synaptotagmin 12 (Syt12), Synaptotagmin 14 (Syt14), Synaptotagmin 4 (Syt4), Synaptotagmin 7 (Syt7), Synaptotagmin-like protein 2 (Sytl2), Synaptotagmin-like protein 4 (Sytl4) and Rabphilin 3a (Rph3a) in different cell types of the mouse hypothalamus. Bioinformatic analysis of the single cell RNAseq data set (Chen *et al.* 2017) was conducted by Nathalie Jauré.



Supplementary Figure 6*:* Expression of *Drosophila* DopR homologs and Rab27/b in the mouse hypothalamus. Expression of homologs of the Drosophila DopR. Hexokinase-1 (Hk1), Hexokinase-2 (Hk2), Dopamine Receptor D1a (Drda1), Rab27a/b, Klf transcription factor (Klf16) and Potassium Two Pore Domain Channel Subfamily K Member 1 (Kcnk1) in cell clusters of the mouse hypothalamus. Bioinformatic analysis of the single cell RNAseq data set (Chen et al. 2017) was conducted by Nathalie Jauré.



Supplementary Figure 7: Expression of *Drosophila* DopR homologs and Rab27/b in cell clusters of the mouse hypothalamus.
Expression of homologs of the *Drosophila* DopR. Hexokinase-1 (Hk1), Hexokinase-2 (Hk2), Dopamine Receptor D1a (Drda1), Rab27a/b, Klf transcription factor (Klf16) and Potassium Two Pore Domain Channel Subfamily K Member 1 (Kcnk1) in different cell types of the mouse hypothalamus. Bioinformatic analysis of the single cell RNAseq data set (Chen *et al.* 2017) was conducted by Nathalie Jauré

Supplementary Table 4: Co-expression of Btsz homologs, DopR homologs and Rab27a/b.
Bioinformatic analysis was conducted by Nathalie Jauré.

Genes	Cell Cluster	Cell Number Frequency
Hk1 and Esyt2	others	33
Hk1 and Syt1	GABA13	29
	Glu5	21
	Glu7	27
	others	250
Hk1 and Syt12	others	23
Hk1 and Syt4	GABA13	32
	GABA15	21
	GABA8	26
	Glu7	25
	others	241
Hk1 and Syt7	others	70
Hk1 and Rph3a	oligodendrocyte precursor cells	37
	others	133
Hk1 and Esyt2	others	33
Hk1 and Syt1	GABA13	29
	Glu5	21
	Glu7	27
	others	250
Hk1 and Syt12	others	23
Hk1 and Syt4	GABA13	32
	GABA15	21
	GABA8	26
	Glu7	25
	others	241
Hk1 and Syt7	others	70
Hk1 and Rph3a	oligodendrocyte precursor cells	37
	others	133
Hk1 and Esyt2	others	23
Drd1a and Syt4	others	21
Hk1 and Syt7	others	70
Rab27a and Syt1	others	65
Rab27a and Syt4	others	64
Rab27a and Syt7	others	7
Rab27a and Rph3a	others	35
Rab27b and Esyt2	others	27
Rab27b and Syt1	GABA13	35
	GABA8	31
	others	212
Rab27b and Syt12	others	22
Rab27b and Syt4	GABA13	35
	GABA8	31
	others	212
Rab27b and Syt7	others	54
Rab27b and Rph3a	oligodendrocyte precursor cells	35
	others	130
Rab27b and Esyt2	others	27
Klf16 and Syt4	others	31
Rab27b and Syt7	others	54
Klf16 and Rph3a	others	21

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Contributions

RNAseq library preparation and sequencing were performed at the Max Planck Genome Center Cologne. Dr. Carina Weigelt prepared samples for RNA sequencing. Bioinformatic analysis of the RNAseq data set, as well as analysis of the stated single-cell data sets were conducted by Dr. Nathalie Jauré. Mass spectrometry was performed in the Proteomics Facility of the Max Planck Institute for Biology of Ageing and the analysis was carried out by Dr. Patrick Giavalisco. Microscopy imaging was conducted in the FACS and Imaging Core Facility of the Max Planck Insitute for Biology of Ageing. Transgenic flies were generated by Dr. Carina Weigelt and microinjection was performed by Jacqueline Eßer. Prof. Linda Partridge and Dr. Sebastian Grönke supervised my research.

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Declarations

Declaration for the doctoral thesis (dissertation)
according to the doctoral regulations published 12th March 2020

Non-official English translation of the "Erklärung zur Dissertation"
(*The German version must be included in the doctoral thesis*)

"I hereby declare that I have completed the present dissertation independently and without the use of any aids or literature other than those referred to. All passages that have been taken, either literally or in sense, from published and unpublished works, are marked as such. I declare that this dissertation has not been submitted to any other faculty or university; that - apart from the partial publications and included articles and manuscripts listed below - it has not yet been published, and that I will not publish the dissertation before completing my doctorate without the permission of the PhD Committee. I am aware of the terms of the doctoral regulations. In addition, I hereby declare that I am aware of the "Regulations for Safeguarding Good Scientific Practice and Dealing with Scientific Misconduct" of the University of Cologne, and that I have observed them during the work on the thesis project and the written doctoral thesis. I hereby commit myself to observe and implement the guidelines mentioned there in all scientific activities. I assure that the submitted electronic version is identical to the submitted printed version".

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1. Accessibility of data and materials

The dissertation involves the acquisition of primary data or the analysis of such data or the reproducibility of the results presented in the dissertation requires the availability of data analyses, experimental protocols or sample material.

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2. Previous doctoral examinations

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I have not been convicted of a deliberate criminal offence in the preparation or commission of which the status as a doctoral candidate was abused.

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