GENETIC ANALYSIS OF SLEEP IN *DROSOPHILA MELANOGASTER*

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By

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Abstract

Genetic Analysis of sleep in *Drosophila melanogaster*

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Sleep is phylogenetically ubiquitous and important for survival. Although the essential role of sleep is well recognised, the molecular mechanisms that control it still remain largely unknown. In the last decade, *Drosophila* has emerged as a valid and a powerful model for sleep research, and has been extensively used to explore the underlying molecular mechanisms. The major aim of the current work was to explore natural variation in sleep and identify genetic variations that contribute to phenotypic variation and consequently important for evolution of this trait.

A biometric analysis of 16 reciprocal crosses using two inbred strains revealed an extensive phenotypic variation and substantial heritability. Interestingly, these experiments showed the significant contribution of maternal factors to variation in sleep. Subsequently, QTL mapping was carried using a set of 187 recombinant inbred lines, derived from a North-American population. Five QTL associated with different sleep parameters have been identified. Complementation tests using deficiency strains spanning the QTL intervals, and P-element insertion in candidate genes, allowed refining of the QTL interval and identifying few candidate genes, including *CG9328* and *Rab9,* which are likely to underlie these QTL.

In addition, the role of *dopamine-acetyltransferase* (*Dat*) in sleep was investigated, by generating a *dat*-GAL4 construct that allowed identifying *Dat* neurons in the brain. Genetic ablation of *Dat* neurons caused sleep fragmentation, with flies exhibiting shorter bouts of sleep. The GAL4/UAS system has also been used to explore the brain regions underlying sleep sexual dimorphism. Miss-expressing the female-specific form of *transformer* allowed feminising or masculinisation of specific regions in the brain and indicated a role of the mushroom bodies. The role of DNA methylation in sleep regulation was also investigated. Miss-expressing *Dnmt2* (DNA methyl-transferase) demonstrated that homeostasis of methylation is important for normal levels of sleep, and may explain the link between sleep and life-span.

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Chapter 1: Introduction

1.1 Introduction

Sleep is ubiquitous and is a tightly regulated phenomenon that is common to a broad range of animal species from insects to mammals (Campbell and Tobler 1984). The fact that sleep is phylogenetically widespread suggests that it must be important for survival, particularly given the high adaptive costs that are associated with it: during sleep, animals are more vulnerable to predation, they cannot forage for food nor can they take care of their young. Furthermore, sleep deprivation experiments indicate the importance of sleep for both survival and proper brain function: classic experiments in rats have shown that long-term sleep deprivation results in death (Rechtschaffen and Bergmann 2002). In humans, cognitive performance decreases with increasing amounts of sleep deprivation (Rogers, Dorrian & Dinges 2003). However, although the essential role of sleep is well recognised, the function that it serves and the mechanisms that control it still remain largely unknown.

1.1.1 Theories of sleep function

Given the nature of sleep as a form of rest, it is not surprising that some sleep theories suggest that sleep performs a restorative function (Berger and Phillips 1995), including the preservation of energy through reduced caloric expense when compared to a waking period. In endothermic animals, the decrease in body temperature during sleep contributes to energy conservation (although not as much as daily torpor or a seasonal hibernation). This theory predicts that selective pressure will be higher in small endotherms due to their higher metabolic rates (Berger and Phillips 1995). Brain glycogen stores,

which are depleted during waking periods, are replenished through sleep (Benington, Frank 2003). However, the main weakness of this theory is that the energy saved during sleep is negligible (amounting to calories equivalent to about one frankfurter bun) (Savage and West 2007). Instead, they provide strong evidence that the time spent asleep by an organism is determined by its brain's metabolic rate, and not by its body metabolic rate.

Another interesting theory suggests that sleep is related to memory and learning, since sleep is involved in synaptic plasticity and the neurophysiological processes that occur during sleep promote synaptic plasticity (Benington and Frank 2003). Processes such as the synthesis of genes and proteins that are required for synaptic plasticity actually take place during sleep (reviewed in Benington and Frank 2003). In addition, sleep deprivation has been shown to affect memory consolidation, which may lead to impaired learning abilities (Benington and Frank 2003).

Opp (2009) suggested that sleep plays an important role in the immune system because sleep deprivation has an impact on the immune response and infections that challenge the immune system change sleep patterns. Thus it has been hypothesized that sleep is a component in the response to infection and that it functions as a host defence mechanism. In addition, a correlation between total sleep time and the number of white blood cells (central to the immune response) was seen in 26 mammalian species, suggesting that sleep could increase immune competency (Preston *et al.* 2009).

Obviously, sleep may serve multiple functions. Zimmerman *et al.* 2006 analysed global gene expression during sleep and waking periods in *Drosophila* and found that a broad range of functions were associated with sleep, including lipid synthesis and nervous

system development. The suggestion that sleep might be driven by multiple mechanisms (immune response, energy conservation and neural function) may well apply to sleep for other organisms.

1.2 Characteristics of sleep

1.2.1 Behaviour

With the exception of some migratory birds that may sleep during flight and some marine animals that sleep while swimming (Rattenborg 2006), sleep is manifested in most animals as a behavioural quiescence (immobility), which is characterised by reduced motor activity (reviewed by Allada and Siegel 2008). Frequently, sleep is associated with a specific posture or a favoured resting site; gorillas, for example, prepare nests for sleep (Moorcroft 2007). Sleep is associated with a higher arousal threshold, rendering sleeping animals less responsive to external stimuli. In addition, sleep is under homeostatic regulation, which becomes more obvious when animals are sleep-deprived for extended periods. Following long waking periods, sleep-deprived animals will show a compensatory increase in sleep (even during times when the animal is usually awake). This increase in sleep time and intensity is often referred to as 'sleep rebound' and is driven by the sleep homeostasis. Another driver of sleep that underlies the regular, daily time structure is the circadian clock, which will be described below (section 1.4).

The behavioural criteria identifying sleep are somewhat more ambiguous than the neural criteria (see below), but are practically easier to implement in research and, being less species-specific, can be used to study sleep in a broader range of organisms. In addition, not all animals have the basic neuroanatomy to generate cortical waves that

signify sleep in mammals (see below). Behavioural criteria therefore provide a way of studying sleep in a broader range of organisms (Campbell and Tobler 1984).

The behavioural criteria outlined above (immobility, increased arousal threshold and homeostatic regulation) have been instrumental in identifying sleep in non-mammals such as zebrafish (Zhdanova *et al.* 2001) and in invertebrates like cockroaches (Tobler and Neuner-Jehle 1992), honeybees (Sauer *et al*. 2003, Kaiser and Steiner-Kaiser 1983) and (importantly) in *Drosophila* (which will be described in detail in section 1.6). Recently, the roundworm *C. elegans* has also been shown to have a sleep-like state called lethargus, which is present during the larval stage just before each of the four moults (Raizen *et al.* 2008). Periods of lethargus last around two hours, during which time the worms show maximal quiescence and reduced sensitivity to mechanical and olfactory stimuli.

1.2.2 Electrophysiology

Electrophysiological recording from the brains of various animals indicate that, despite its passive appearance, sleep is actually an active process, associated with characteristic neural and motor activity (reviewed in Campbell and Tobler 1984, Shaw *et al*. 2003). The recording of eye movement during sleep by an electro-oculogram (EOG) reveals two alternating states in humans (and in a number of other mammals): Rapid eye movement (REM) and Non-rapid eye movement (NREM). Intriguingly, muscle tone disappears during REM sleep (Aserinsky and Kleitman 1953), which is the reason why this stage is also called *paradoxical* sleep (PS). Because REM sleep is associated with dreaming, the reduced muscle tone may serve to block the execution of dreamed motor actions (Luppi 2005).

NREM sleep can be further divided into three stages, N1-N3, which show distinct brain wave patterns, as measured by electroencephalogram (EEG), and muscle tone, as measured by electromyography (EMG). In humans, EEG when awake consists of beta waves that have the highest frequency (> 13 Hz) and show asynchronous patterns. During relaxation periods (while awake), the brain activity changes to alpha waves, which are of a lower frequency (8-13 Hz) and display increased synchrony.

The EEG patterns change according to the behavioural state, from low amplitude, relatively fast activity of waking and rapid eye movement (REM) sleep to slow waves and spindles (oscillations of thalamic origin) of non-rapid eye movement (NREM) sleep (Figure 1.1). REM sleep EEG shows theta activity (4-7 Hz) similar to waking periods, but the muscle tone is reduced or absent. NREM sleep EEG includes slow-wave sleep, which is characterised by large slow waves (< 4.5 Hz). The most studied frequency band is the EEG delta activity (0.5-4.5 Hz) during NREM sleep, also called slow-wave activity (SWA). The greater the SWA, the deeper NREM sleep is and the more difficult it is to wake a person up when an external stimulus is applied (Luppi 2005). Delta activity is thus considered to be a marker of sleep intensity and sleep need.

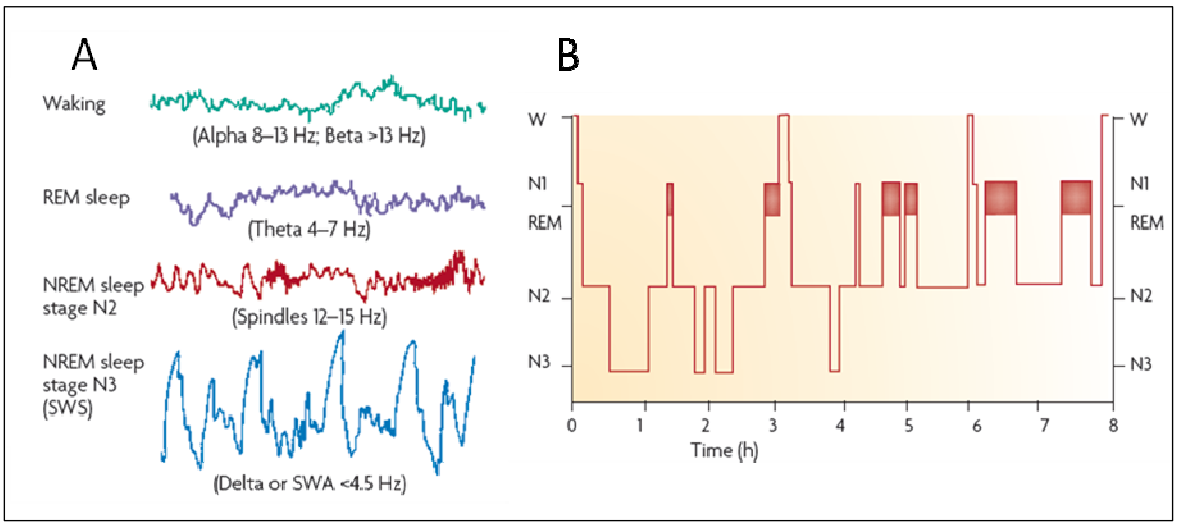


Figure 1.1: Sleep pattern in adult humans. (A) EEG traces of waking, rapid-eye movement (REM) and non-rapid eye movement (NREM) sleep. The waking EEG shows low-voltage, fast activity in the beta (>13 Hz) and alpha (8-13 Hz) ranges. NREM sleep consists of a transitional stage (not shown), followed by stage 2 (N2) where the alpha activity disappears, and followed by stage 3 (N3) or slow-wave sleep (SWS), when the EEG shows prominent slow waves. (B) The cycles of sleep stages during the night in adult humans (Figure taken from (Cirelli 2009)).

1.3 Genetics of sleep

The analysis of various sleep phenotypes, including the daily amount of REM and NREM sleep, indicates considerable variation among species, strains and individuals within a species. Although environmental factors such as light, temperature and diet may substantially contribute to the phenotypic variation, these factors do not completely account for the observed variability, suggesting that part of the variation is genetic (Franken, Malafosse & Tafti 1999).

Twin studies in the 1930s first suggested that sleep is under genetic control (reviewed in Young, Lader & Fenton 1972). The EEG patterns of monozygotic twins were shown to have a higher resemblance than those of dizygotic twins (or unrelated subjects),

confirming that this highly functional brain phenotype may be tightly regulated by genes and loosely (if at all) affected by the environment (Young, Lader & Fenton 1972). Sleep duration is another phenotype that is highly similar in twins, even if they are living apart, confirming that there is no environmental influence on this phenotype (Gedda and Brenci 1983).

1.3.1 Genetic control of sleep disorders

Many sleep disorders run in families, suggesting that there is a substantial genetic component (Kimura and Winkelmann 2007). Restless leg syndrome (RLS) causes a periodic limb movement during sleep. Most patients who suffer from this have a family history and the disorder is believed to have a strong genetic component. The affected gene(s) underlying RLS has not been identified yet, but linkage studies that attempt to find genetic markers that cosegregate with the phenotype have found three loci on chromosomes 12.q, 14.q.13-21 and 9p24-23 (p and q for short and long arms, respectively) (Kimura and Winkelmann 2007) that follow an autosomal dominant mode of inheritance. Winkelmann (2007) carried out an association study and found four loci on chromosomes 2p, 6p, 9p and 15q that were associated with RLS. One of these SNPs was located in *MEIS1*, a homeobox which is known to be involved in limb movement. Together, the four SNPs contributed more than 50% to the risk of RLS.

Narcolepsy is a disease that causes excessive daytime sleep, sudden loss of muscle tone and sleep paralysis (Taheri and Mignot 2002). In contrast to dogs and mice, where it is under genetic control, in humans this is mostly sporadic (over 95% of cases), as suggested by its low concordance in monozygotic twins (Taheri and Mignot 2002). However, a genetic factor was identified in the major histocompatibility complex (MHC) region in

narcolepsy patients, and 90-100% patients showed a strong association with human leukocyte antigen (HLA) alleles (Nishino 2007). The strong association of HLA markers, combined with a genome wide association study, suggests that narcolepsy is an autoimmune disorder (Hallmayer *et al.* 2009); however, HLA haplotypes alone cannot explain the occurrence of narcolepsy, and so other susceptibility genes may also be important (Nishino 2007).

Insomnia is one of the most common sleep disorders, with about 20% of the population suffering from chronic insomnia (Dauvilliers *et al.* 2005). Fatal familial insomnia (FFI) is a prion disease that causes sleep fragmentation, reduced sleep time, loss of circadian regulation of sleep and interference of REM sleep during wakefulness (Schenkein and Montagna 2006). FFI is an autosomal dominant inherited disease caused by a single mutation at codon 178 of the *PRNP* (PRioN Protein) gene, resulting in the substitution of asparagine for aspartic acid (Schenkein and Montagna 2006). Interestingly, a higher recurrence of a failure to initiate, maintain and terminate sleep was seen in patients homozygous (CC) for the *Clock* genotype (Hamet and Tremblay 2006).

Obstructive sleep apnea syndrome (OSAS) is a common sleep disorder which is characterised by reduced airflow during sleep, caused by a dysfunction in the thalamus region of the brain and the mechanism controlling breathing. Familial forms of OSAS have been reported suggesting a genetic basis for this disorder, and the many risk factors for OSAS, including obesity, are also under genetic control (Hamet and Tremblay 2006). A study by Kadotani (2001) has found a possible link between the *ApoE4* (*Apolipoprotein E 4*) gene and OSAS, suggesting a strong genetic component controlling the sleep apnea syndrome.

Another example of a genetic element in sleep disorders is the familial advanced sleep phase syndrome (FASPS), which runs in families and causes subjects go to sleep about 3-4 hrs earlier than unaffected people (Toh *et al.* 2001). However, sleep duration in FASP patients is normal. FASP is caused by a mutation in the *hPER2* (human *period*), a circadian clock gene (Figure 1.2). In hamsters, the short-period mutant *tau* was cloned and was found to be a mutation of the homologous casein kinase gene (Lowrey *et al.* 2000).

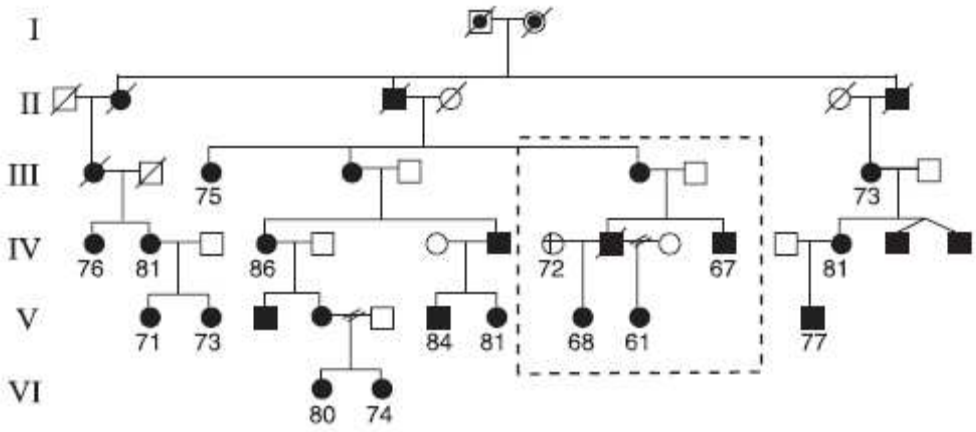


Figure 1.2: A pedigree of a family carrying Familial Advanced Sleep Phase Syndrome (FASPS). The disorder is carried as autosomal dominant trait and is caused by a mutation in hPer2 in a conserved Serine (see text). Circles, females; squares, males; filled circles and squares, affected individuals; empty circles and squares, unaffected individuals. In some individuals (enclosed by a dotted square) the ASPS phenotype does not co-segregate with the mutation. (Figure taken from (Toh *et al.* 2001)

Most mouse genes that are known to affect sleep have been identified by a reverse genetics approach, where a candidate gene is mutated and the effects of the mutation on the phenotype (e.g. sleep) are measured. Numerous mutant lines have been tested so far, confirming that these genes affect sleep (Tobler *et al.* 1996, Zhang *et al.* 1996). The study on rats used a microarray to profile the expression of ~15,000 transcripts in spontaneously

awake, sleep deprived and sleeping rats (Cirelli, Gutierrez & Tononi 2004). About 5% of the gene transcripts were differentially expressed due to their behavioural state. ~100 known genes that showed increased expression during sleep provided a molecular link of sleep to protein synthesis and neural plasticity. Quantitative Trait Loci (QTL) analyses have also successfully shown that a single mouse gene affects sleep quality and quantity (see chapter 4). Thus, genetic factors that contribute to variations in sleep allow us to investigate sleep disorders using various genetic approaches in order to localize a gene and determine its function.

1.4 The circadian clock and sleep rhythms

Sleep is among the many processes that show a regular daily rhythmicity and are driven by an endogenous pacemaker, the circadian clock. Animals that are isolated from experiencing any external cues (i.e. in laboratory conditions) show a robust rhythm of sleep, although the cycle length of this rhythm is usually slightly different from 24 hours (the extent of that difference is species-specific) and reflects the 'free-run' of the clock (hence the name 'circa-dian' = about a day). Under natural conditions, however, the clock is synchronised (entrained) to the solar cycle through various external cues and the overt periodicity is invariably 24 hours.

At the molecular level, the circadian clock is a network of clock proteins that interact with each other and auto-regulate their own expression. One of the conserved mechanisms underlying the molecular oscillations is a translation-transcription negative feedback loop in which positive elements (transcription factors), such as mouse CLOCK and BMAL1, produce heterodimers and bind to E-box elements (the canonical sequence of

CACGTG) in the promoter regions of various clock genes. Some of these genes which serve as negative elements also heterodimerise (in mice, the three PERIOD paralogues, PER1-3, and the two CRYPTOCHROMES, CRY1-CRY2) and shuttle back to the nucleus, where they repress their own transcription. The resulting reduction of the negative elements (PER, CRY) gradually leads to reduced repression and transcription of these genes is then resumed in a new circadian cycle (reviewed in Hastings, Maywood & Reddy 2008).

Studies into both mice and *Drosophila* indicate the presence of multiple, interlinked transcription loops. In mice, BMAL1 negatively autoregulates itself, while promoting transcription of PER2 and CRY (Yu, Nomura & Ikeda 2002). The orphan nuclear receptor REV-ERBα, which shows a circadian oscillation, suppresses the expression of CLOCK and BMAL1 (Preitner *et al.* 2002). Another loop is carried by the bHLH transcription factors DEC1 and DEC2, which are clock-controlled genes and are also repressors of the transcription induced by the CLOCK:BMAL1 complex (Honma *et al.* 2002). In *Drosophila*, CLOCK and CYCLE promote the transcription of two other genes, *vrille* (Blau and Young 1999) and *Pdp1ε* (Lin *et al.* 1997). In turn, Vrille inhibits and Pdp1ε promotes clock transcription.

In addition to the transcription loops, other post-transcriptional and post-translational mechanisms exist that contribute to the circadian oscillation. In fact, the circadian clock of cyanobacteria can be entirely explained by the rhythmic phosphorylation of clock proteins (Iwasaki and Kondo 2004). The phosphorylation status of the negative elements such as PER affect their stability, and consequently this introduces an important regulation of the circadian cycle. The mammalian CKIε is a kinase that phosphorylates all PER paralogues and affects their nuclear translocation (Takano *et al.* 2000). The CKIε

hamster mutant (called *Tau*) exhibits an abnormal circadian cycle (Lowrey *et al.* 2000); similarly, a mutation in the *Drosophila* orthologue (*doubletime*) disrupts the circadian behaviour (Price *et al.* 1998). Importantly, FASPS is associated with a serine to glycine mutation in human PER2, which is located in a CKIε phosphorylation site (see Figure 1.2). This mutation causes hypophosphorylation by CKIε which results in a shorter circadian cycle; in a very similar way, a single nucleotide substitution in the fifth exon of the fly *per* orthologue that changes a serine residue into an aspargine leads to a faster rhythm (18 hour) in the mutant per^{S} (Chiu *et al* 2008; Konopka and Benzer 1971).

1.5 Molecular basis of sleep

1.5.1 Hormones

The epidermal growth factor receptor (EGFR) is an important receptor for cell growth and proliferation and has been implicated in the regulation of sleep in *Drosophila*, where increased signalling of the EGFR pathway increased sleep (Foltenyi, Greenspan & Newport 2007).

In mice, studies of mutants with altered metabolic functions have shown alterations in sleep patterns. Leptin is an anorectic hormone that is produced in the adipose cells and is responsible for metabolic regulation. In order to test the link between sleep and metabolic regulation, leptin deficient mice were tested and showed the early onset of obesity and altered sleep-wake organisation (Laposky *et al.* 2006). In particular, leptin affected sleep recovery after sleep deprivation, a 10% increase in REM sleep and sleep fragmentation (Laposky *et al.* 2006) suggesting regulation of the sleep architecture by leptin. Ghrelin, the leptin counterpart hormone, induces satiation (hunger satisfaction) when present in high

quantities. Ghrelin knock-out mice showed reduced duration of NREM sleep and higher amounts of REM (Szentirmai *et al.* 2007) when compared to wild-type mice.

Growth hormones (GH) stimulate tissue growth and a deficiency of GH causes dwarfism in growing individuals. The synthesis and release of GH is controlled by two receptors, GH-releasing hormone (GHRH) and somatostatin, of which the latter inhibits the secretion of GH. GH secretion has been shown to be related to NREM sleep in humans, and NREM and GH also correlate in studies on other species. Studies conducted on rats and mice with a non-functional GHRH receptor showed a reduction in NREM sleep and a decrease in REM sleep during the light phase (Obal *et al*. 2001; Obal *et al*. 2003).

Prolactin is a peptide hormone that is primarily related to lactation. A study on cats reported that the administration of prolactin enhanced REM sleep (reviewed in Obal *et al.* 2005). In humans, high levels of prolactin in the early morning are also related to REM sleep (Obal *et al.* 2005). Prolactin knock-out mice showed a reduction in REM during the light phase (mice are nocturnal animals) (Obal *et al.* 2005), supporting the notion that prolactin is involved in REM regulation.

Melanin-concentrating hormone (MCH) is thought to be involved in feeding behaviour, energy balance and the modulation of waking (Saper, Chou & Scammell 2001). MCH producing neurons in the rat hypothalamus have been shown to be active during REM sleep rebound (reviewed in Adamantidis *et al.* 2008), while the intracerebroventricular infusion of MCH increased total sleep duration, indicating its role in sleep regulation (Verret *et al.* 2003). In order to test whether the activation of the MCH system promotes sleep, *MCH-receptor 1* gene deletion mice were tested. These animals

with genetically inactivated MCH showed a 19% increase in REM sleep during the light phase, and a greater increase in NREM sleep after total sleep deprivation (Adamantidis *et al.* 2008).

1.5.2 Neurotransmitters

Neurotransmitters such as norepinephrine, dopamine, serotonin and histamine have been shown to play a role in sleep regulation. Targeted genetic disruptions of these neurotransmitters using lesions and pharmacological approaches have validated their role in sleep/wake cycles. Targeted disruption of the gene *dopamine β-hydroxylase* (an enzyme that converts dopamine to norepinephrine) in mice showed a disruption in the sleep/wake period distribution, a decrease in REM sleep and an inability of sleep recovery after sleep deprivation. This provided genetic evidence that norepinephrine is involved in the maintenance of waking, as well as its importance for REM sleep regulation and sleep homeostasis (Ouyang *et al.* 2004).

A similar approach has been used to determine the role of histamine neurons in sleep regulation. *Histidine-decarboxylase* (a histamine synthesizing enzyme) knock-out mice displayed fragmentation of sleep and increased REM sleep during the light phase, verifying the role of histamine neurons in sleep-wake regulation (Anaclet *et al.* 2009).

Various studies have indicated that dopamine is an important sleep regulator (Monti and Monti 2007). Parkinson's disease patients show depletion in dopamine, while increasing dopamine levels intensify schizophrenic symptoms (Carlsson 1987). Disturbances in sleep occur in patients who are suffering from schizophrenia and Parkinson's disease, suggesting that dopamine plays a role in the regulation of the sleep-

wake cycle (Yamamura and Enna 1981). Dopamine transporter knock-out mice that are lacking the gene encoding dopamine transporter (DAT) show a marked increase in hyperactivity (Wisor *et al.* 2001) (for more details, see Chapter 5).

Serotonin (5-Hydroxytryptamine (HT)) is a neurotransmitter which has been implicated in the regulation of sleep (Jouvet 1969). Reducing serotonin levels pharmacologically or by ablating serotonergic cells surgically caused insomnia, suggesting that serotonin promotes sleep (Jouvet 1969). Knock-out mouse models for serotonin transporters and receptor subtypes verify this association of serotonin and sleep. Genetically engineered *Sert* (serotonin transporter) knockout mice showed a 50% increase in REM sleep over a 24 hour period (Wisor *et al.* 2003), while knock-out mouse models for the various receptor subtypes showed a difference in REM sleep when compared to their controls (Cirelli 2009). The role of serotonin in *Drosophila* sleep has also been explored, and has been demonstrated to fulfil a sleep-promoting function (Yuan, Joiner & Sehgal 2006).

1.6 *Drosophila* as a model organism for studying sleep

Despite their 250,000 brain neurons, flies are still a relatively simple model organism, compared with humans or mice. The fully sequenced genome of the fly (Adams *et al.* 2000) consists of about 14,000 genes, most of which show a high homology to their mammalian counterparts (Rubin *et al.* 2000), including genes involved in human diseases (Chien *et al.* 2002). Importantly, when expressed in flies (in their disease form), these genes lead to similar phenotypes to human diseases (Auluck and Bonini 2002). The genome of flies is less redundant when compared with that of mice or humans, and is therefore ideal for forward genetic approaches, where each gene can be mutated and

subjected to various functional analyses. *Drosophila* shares a significant number of genes that are important for neural development and function in mammals (Hewes and Taghert 2001; Yoshihara, Ensminger & Littleton 2001; Nassel 2002). These similarities suggest that vertebrates and invertebrates may share some basic genetic mechanisms for sleep, as they do for circadian rhythms, learning and memory, and aging (Finch and Ruvkun 2001; Stanewsky 2003).

Several studies have shown that sleep in fruit flies shares key features with mammalian sleep (Hendricks *et al.* 2000; Shaw *et al.* 2000). As in mammals, sleep in *Drosophila* consists of long periods of immobility (quiescence) with an increased arousal threshold at which point the fly is unresponsive to mild external stimuli. Pharmacological agents affect sleep in *Drosophila* similar to the way they do this in vertebrates, indicating a conserved underlying molecular mechanism (Hendricks *et al.* 2000; Shaw *et al.* 2000). Caffeine, for example, a popular stimulant that reduces sleep and promotes wakefulness in mammals, similarly increases wakefulness in flies (Wu *et al.* 2009). However, while the effect of caffeine in mammals is mediated by the antagonizing signalling of adenosine, in *Drosophila* caffeine promote wakefulness by blocking cAMP phosphodiesterase activity (Wu *et al.* 2009).

Along the same lines, anti-histamines induce sleep in flies, as they do in mammals (Hendricks *et al.* 2000), and modafinil, a drug that elevates histamine level in the hypothalamus, promotes wakefulness in flies (Hendricks *et al.* 2003a).

Electrophysiological studies conducted in *Drosophila* showed that, as in mammals, sleep is associated with a specific brain activity. Local-field potentials (LFPs) have been recorded in the fly brain using one electrode placed between the mushroom bodies (two

neuropils in the adult midbrain) and another electrode placed in the optic lobes (Nitz *et al.* 2002). The recorded neural activity shows a strong correlation with sleep/wake states (Figure 1.3).

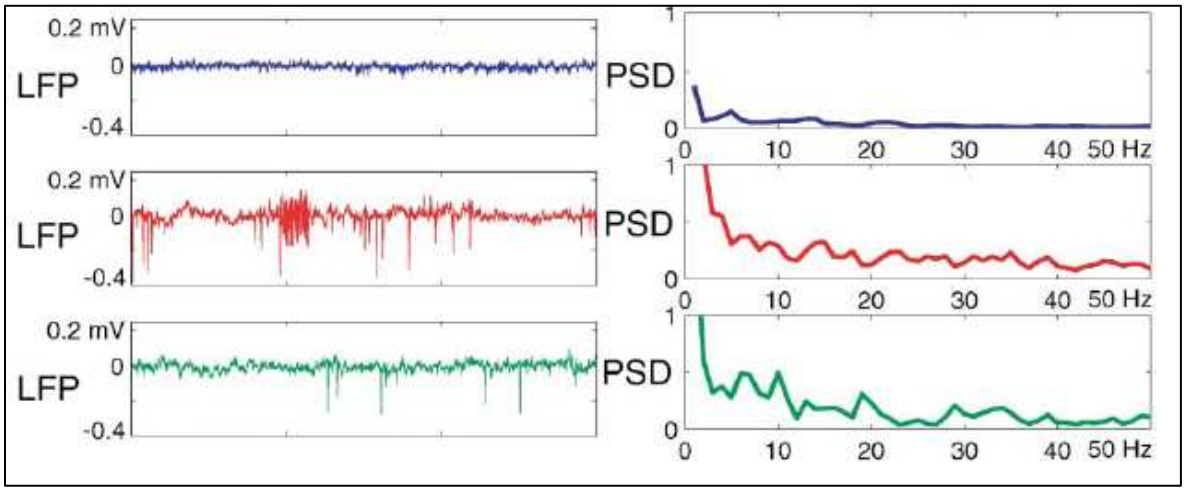


Figure 1.3: Comparisons of LFPs during active and quiescent states in *Drosophila melanogaster*. Local-field potentials (LFP) and power spectral density (PSD) associated across 5-s time bins. The period of quiescence (blue) was associated with reduced spiking activity, when compared with periods of high (red) and moderate (green) motor arousal (Figure from (Nitz *et al.* 2002)).

The *Drosophila* mushroom bodies are the principal site of learning and memory (Davis 2004) and may functionally resemble the cerebral cortex of mammals; they have also been identified as a centre for sleep regulation (Joiner *et al.* 2006; Pitman *et al.* 2006). The chemical ablation of the mushroom bodies resulted in reduced sleep (Pitman *et al.* 2006), and when cyclic-AMP dependent protein kinase (PKA) activity was enhanced in the mushroom bodies, altered sleep patterns were observed (Joiner *et al.* 2006).

Flies that are sleep deprived through the night show increased sleep during the subsequent day (Huber *et al.* 2004), indicating tight homeostatic regulation. A recent study has illustrated the usefulness of *Drosophila* for sleep research by investigating the long-

term consequences of sleep deprivation using a fly model for Parkinson's disease (Seugnet *et al.* 2009a).

As in mammals, the sleep patterns of individual flies are age dependent (Shaw *et al.* 2000). Sleep in mammals is prominent in the very young, stabilises during adolescence and adulthood, and declines in old age. The same pattern is seen in *Drosophila* (Shaw *et al.* 2000): on the first day of eclosion, the amount of sleep is high; this then declines steadily until the 3rd day when an adult pattern is reached. As the fly ages, the amount of (nocturnal) sleep declines, and by 33 days it is significantly below that found in young adults (Shaw *et al.* 2000). In addition, sleep gets more fragmented with age in flies, as it does in mammals (Koh *et al.* 2006).

What does sleep in flies look like? Early studies using videotape recording (Hendricks *et al.* 2000) or an ultrasound system (Shaw *et al.* 2000) indicated that the majority of rest bouts that last at least 5 minutes are associated with complete quiescence, while shorter intervals (less than 3% of rest bouts) are often associated with various limb movements and therefore may signify rest rather than sleep (Hendricks *et al.* 2000).

The 5-minute threshold was adopted as the benchmark for sleep studies in flies, and this provides a convenient method to measure sleep, using automated activity systems that continuously track the locomotor activity of individual flies. Each fly is housed in a glass tube, and the number of times that the fly crosses an infra-red beam is recorded (the Drosophila Activity Monitoring System by Trikinetics is a popular apparatus among fly research groups). The total amount of sleep (e.g. per day) can be easily estimated by summing the 5-minute epochs in which no activity was recorded from the fly.

The mean total accumulated sleep per day in wild-type flies (Canton-S strain) is about 720 min for females and 930 min for males (Ho and Sehgal 2005). Sleep during the dark phase is significantly longer than that recorded during the light phase and is similar for both sexes; the difference in total sleep is due to diurnal sleep, which is significantly higher in males than in females (males have a mid-day 'siesta', see Figure 1.4).

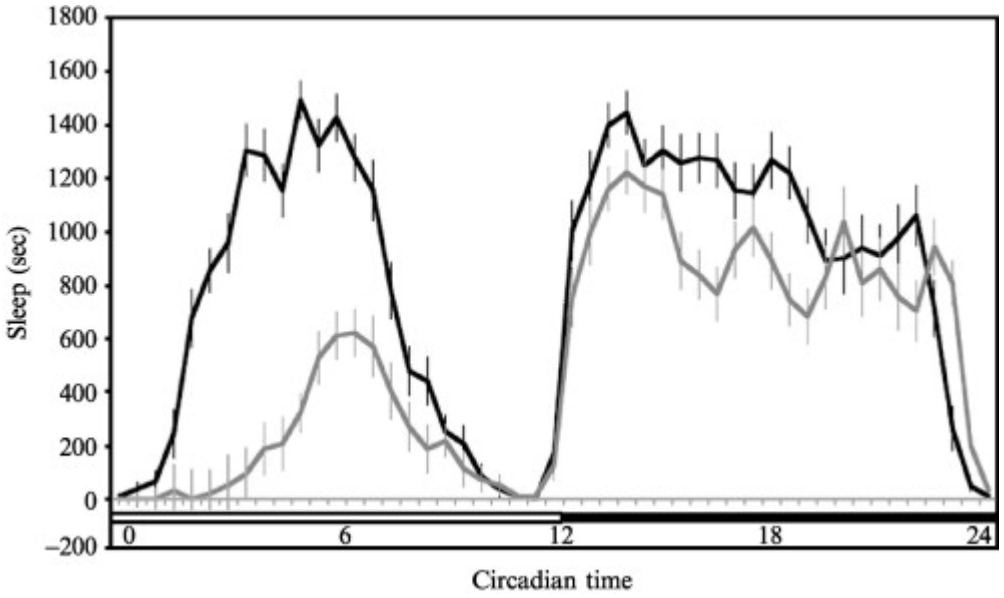


Figure 1.4: A typical sleep cycle in *Drosophila*. Average amount of sleep (in seconds) in a wild-type strain Canton-S for males (black) and females (grey) in a 24-hour day under constant conditions of 25ºC and LD: 12:12. Light (white) and dark (black) period is represented below the graph. Error bars represent standard error (Ho and Sehgal 2005).

1.7 Identifying sleep relevant genes in *Drosophila*

The relative ease of carrying a mutagenesis screen in *Drosophila* provides an efficient way of isolating sleep mutants, which would be rather expensive and time consuming using rodents. Because many of the genes show a high degree of conservation, these screens may help in identifying genes that serve similar roles in mammals; a good example is in the field of chronobiology, where *Drosophila* has played a major role in identifying many of the core genes of the circadian clock (Hendricks 2003). The human counterpart of one such gene, *period*, when it was mutated in people affected with Advance Phase Sleep Syndrome, affected the circadian rhythm but not the sleep make up (Toh *et al.* 2001) (see section 1.3). Therefore, the discovery of genes in *Drosophila* that are involved in the regulation of sleep can be applicable to studies on human sleep. The most commonly used strategies in the genome-wide search for genes that affect a phenotype of interest are quantitative trait loci (QTL) analysis, mutagenesis, molecular genetics and candidate gene studies.

1.7.1 Forward genetic-mutagenesis screen

Genetic studies of sleep focused on identifying genes that regulate sleep through mutagenesis screening in *Drosophila*. A large-scale screen of 6,000 ethylmethane sulphonate (EMS) induced mutated *Drosophila* lines (X-chromosome) and 3,000 lines carrying randomly inserted P-elements was conducted (Cirelli *et al.* 2005). This screen identified 15 lines that showed much less daily sleep than the average males and females. One of the most extreme lines, which slept for only 4-5 hours as compared to 8-10 hours sleep in wild-type flies, was named *minisleep* (*mns*). The irregular leg-shaking and wing

movement phenotype that the mutant displayed aided mapping of the mutation to the gene *Shaker*, a previously identified phenotype in the X-chromosome mutant. *Shaker* (*Sh*) encodes the α-subunit of the tetrameric potassium channel that passes voltage-dependent current.

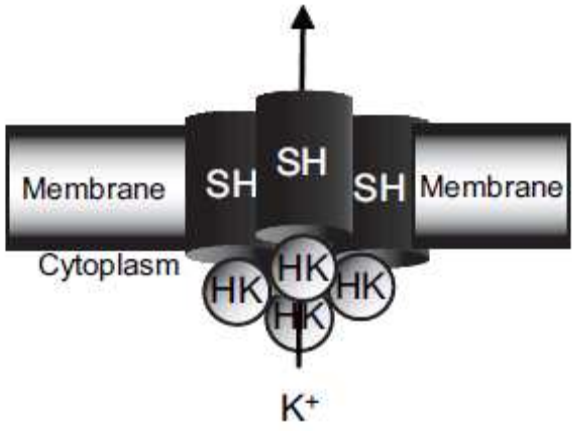


Figure 1.5: Diagram showing the *Hyperkinetic* (*Hk*) β modulatory unit attached onto the cytoplasmic side of the *Shaker* (*Sh*) pore. A fast-inactivating voltage dependent potassium (K^+) current passes through the pore. (Figure from (Bushey *et al.* 2007))

The gene *Hyperkinetic* (*Hk*) codes for a β-subunit that interacts with each of the α-subunit coded by *Shaker* (Bushey *et al.* 2007) (Figure 1.5). Loss-of-function mutations in both *Sh* and *Hk* cause a short-sleeping phenotype, impairment in learning and memory and a reduced lifespan (Cirelli *et al.* 2005; Bushey *et al.* 2007). However, sleep in *Hyperkinetic* mutants is not as reduced as it is in *Shaker* mutants, probably because the potassium current is not completely abolished, as is the case for *Shaker* flies.

Another forward genetic screen of 3,500 mutagenesis lines identified a mutation which was named *sleepless* (*sss*) (Koh *et al.* 2008). The mutants here showed a marked reduction in daily sleep (85% less in males and 80% less in females when compared to the

controls). The gene *sss* codes for a glycosyl-phosphatidylinositol anchored membrane protein. A reduction in the protein level does not affect normal sleep, but significantly reduces the sleep rebound after sleep deprivation. This study also isolated another mutant allele of *sleepless*, *quiver* that impairs the *Shaker*-dependent current. Q*uiver* flies consistently exhibit an aberrant sleep pattern (Koh *et al.* 2008). The *sss* mutants have reduced levels of the *Shaker* protein, suggesting that the short-sleeping phenotype in *sleepless* flies, at least in part, may be mediated by the *Shaker* current.

Biogenic amines like dopamine, which modulate sleep in vertebrates (see section 1.5.2), were also implicated in sleep regulation in *Drosophila* (Shaw *et al.* 2000). The mutant *fumin* (meaning sleepless in Japanese) displayed high levels of activity and reduced levels of sleep (Kume *et al.* 2005). *Fumin* flies do not show a sleep rebound in response to sleep deprivation and have a normal life span. This mutation was mapped to the *dopamine transporter* (*DAT*) gene, indicating a role for dopamine in the regulation of sleep (Kume *et al.* 2005). Soon after this, a study conducted on 5,297 EMS-induced mutant lines identified seven short-sleeping mutants, of which one was found to be another allele of *fumin* (Wu *et al.* 2008).

Harbison *et al.* (2008) screened 136 isogenic strains with P-element insertions in various genes and identified 53 insertion lines that displayed abnormal sleep patterns (either increased or reduced). This screen implicated genes involved in various biological functions, suggesting again that sleep serves multiple functions rather than a single purpose. In general, the rather low number of mutations identified in the various screens in *Drosophila* has led to the notion that the genetic architecture of sleep may be determined

by many genes each with a small effect, which seems to be the general rule for complex behaviour traits (Tafti and Franken 2002).

Another candidate gene for a potential role in sleep studies is *CREB*, which encodes the cAMP response element binding-protein (CREB). This plays an important role in learning and memory, neural plasticity and circadian rhythms in flies and mammals. In order to determine the role of CREB in *Drosophila* sleep, Hendricks *et al*. 2001 investigated mutants with CREB activity that was either higher or lower than normal. Lowering the CREB activity led to increased amounts of daily sleep, while raising it decreased the amounts of sleep, similar to the mammalian expression data for CREB (Cirelli and Tononi 2000).

1.7.2 Differential display and microarray studies

The global profiling of gene expression in fly heads, using microarrays to target differentially expressed transcripts during sleep or waking periods, provided a large number of candidate genes that may play a role in sleep (Shaw *et al.* 2000). These included genes encoding the fatty acid synthase, the metabolic protein cytochrome P450 and *dopamine N-acetlytransferase* (*Dat*).[1] Another microarray study recorded gene expression in spontaneously awake, sleep deprived and sleeping flies (Cirelli, LaVaute & Tononi 2005). Genes that were upregulated during wakefulness were particularly enriched for functions such as carbohydrate metabolism, immune response and stress response; while genes upregulated during sleep showed an over-representation of the process associated with lipid metabolism.

[1] The role of this gene in sleep will be further explored in Chapter 5.

1.7.3 Analysis of circadian clock mutants to identify candidate sleep genes

Circadian clock genes are obvious candidates for testing sleep phenotypes, since they may also be important regulators of the time structure of sleep. The response to sleep deprivation was studied in *Drosophila* core clock mutants *timeless* (tim^0) and *period* (per^0) (Hendricks *et al.* 2000). The baseline sleep pattern of the mutants was, as expected, arrhythmic in constant darkness. After six hours of sleep deprivation during the night phase, per^0 flies showed a significant increase in rest, as normal flies would.[2] In contrast, the tim^0 flies showed a significant decrease in rest. When *timeless* was rescued in tim^{07} background (a strain carrying wild-type *tim* transgene), the flies showed a significant rest rebound (Hendricks *et al.* 2000). Overall, the study showed that *timeless* has a function beyond its role in the circadian clock and may be linked to the homeostatic regulation of sleep. A mutation in another clock gene, *cycle* (cyc^{01}), also seems to have a sleep phenotype (Hendricks *et al.* 2003b). Male *cyc* mutant flies display a reduced sleep rebound after deprivation, while the mutant females exhibit a much longer duration of sleep rebound.

1.8 Aims of this study

The recent emergence of *Drosophila* as a model system for sleep research has already provided new insights into the underlying molecular mechanisms of this highly conserved function. Various forward genetic approaches, including microarray profiling studies, have elucidated a number of candidate genes that may be important for the

[2] The same result was reported by Shaw 2000. The fact that arrhythmic clock mutants showed a normal pattern of sleep-rebound indicated the homeostatic component of sleep that can be dissociated from circadian control.

regulation of sleep (see above). However, little is known about the genetic variations that are associated with sleep-related loci and contribute to phenotypic variation in this trait in wild populations. Identifying specific genetic variants that segregate in a natural population is essential to develop a deeper understanding of sleep mechanisms and how these have evolved.

A major aim of this project was to explore genetic variation related to sleep and to identify genes which contribute to the phenotypic variation. Rather than using mutant strains, I have focused on the sleep behaviour of normal flies derived from various wild populations. This analysis may contribute to our understanding of the evolution of this fundamental process, and will highlight the genes that are the target for natural selection.

My first step (Chapter 3) was to carry out a biometric analysis of the sleep patterns of flies derived from Italian and Dutch populations. The analysis of the phenotypic variation was performed on various reciprocal crosses (16 generations). This approach has provided a crude estimation of the contribution of various factors, both chromosomal and non-chromosomal (such as the maternal effect and cytoplasmic factors), to the inheritance of sleep. This analysis revealed a substantial phenotypic variation in sleep and indicated that the heritability of this trait is significant. In order to obtain a more detailed insight into the genetic architecture of sleep, I carried out quantitative trait loci (QTL) mapping using a set of recombinant inbred lines derived from North American populations (Chapter 4). Several significant QTL contributing to variations in sleep patterns have been identified using composite interval mapping, and these were further studied using complementation tests.

I have used various other approaches to study the unexplored aspects of sleep. Chapter 5 describes a follow up analysis of the gene *Dopamine acetyltransferase* (*Dat*), which had previously been implicated in regulating sleep in flies (Shaw *et al.* 2000). I have cloned the upstream genomic region of *Dat* (harbouring its putative promoter) and explored its role using the fly GAL4-UAS system (Brand, Perrimon 1993). This approach allowed me to ablate the *Dat* expressing neurons and to test the effects on the sleep patterns of the fly. In addition, I used the GAL4-UAS system to study the intriguing sexual-dimorphism associated with diurnal sleep in flies (Chapter 6). Mis-expression of the female-specific *transformer* gene allowed the feminising of specific regions in the male brain or masculinising specific female brain neurons. These experiments were aimed at identifying the brain neurons that mediate this sex-specific sleep phenotype. Finally, the same system (GAL4-UAS) was used to mis-express DNA methytransferase (*Dnmt2*) in the adult *Drosophila* in order to test the role of DNA methylation in sleep regulation. Overall, this study utilised a broad range of approaches, including quantitative genetics, neurogenetics and epigenetic, that take advantage of the *Drosophila* model system to study the molecular and cellular basis of sleep.

Chapter 2: General Materials and Methods

2.1 Fly stocks, media and growth conditions

2.1.1 Media and growth conditions

The flies were grown in vials or glass bottles on sugar meal (100 g agar, 462g dried yeast, 462 g sugar, 10L water, 50 mL 20% Nipagen in ethanol). The flies were kept at 18°C (with approx. life cycle of 21 days) for maintenance of stocks, and at 25°C (with approx. life cycle of 10 days) during the experiments. At both these temperatures, the light regime was 12 hours of light and 12 hours of darkness (LD 12:12). The specific fly strains are listed in each of the Results chapters.

2.2 Sleep Recording

2.2.1 Experimental Set-up

Individual male or female flies, about 3-4 days old, were placed in glass tubes which are 50 mm in diameter and about 80 mm in length. Sugar food (about 20mm tube length) was added and the tube was sealed with a small black plastic cap (TriKinetics Inc) on one end, and plugged with cotton on the other end. These tubes were then individually placed in the 32 channels of the activity monitors (TriKinetics Inc.) used to record the sleep-wake activity of single flies. An infrared beam runs through the monitor and an event is recorded each time the fly interrupts this beam.

The monitors were placed into incubators (Scientific Laboratory Supplies Ltd.) at temperature of 25°C, and light:dark (LD) cycle of 12:12 hr. Rest/activity was measured by recording individual beam crossings of individual flies, where the events were scored at regular time intervals of 5 minutes (see Introduction).

2.2.2 Data Collection and analysis

The locomotor activity monitors were connected to a PC using a Power Supply Interface Unit (TriKinetics Inc.) and the data from them was collected using the DAMSystem 302.exe software (TriKinetics Inc.).

Only the data of flies which survived till the end of the experiment were used for all further analysis. An activity plot of all individual flies was plotted using Microsoft Excel 2007. The sleep data was further processed using the R-package (2.8.0) and BeFly! a collection of macros developed by Dr. Edward Green (Dept. Genetics, Leicester). Different sleep parameters (described in the Results) were calculated using the R-package and BeFly!

2.3 The GAL4-UAS System

The GAL4/UAS system has been used in this study to test mis-expression of various genes. The principle of this technique is described below, and more details are provided in the relevant chapters (5-7).

A GAL4/UAS ectopic expression system is commonly used for targeted gene expression in *Drosophila melanogaster* (Brand, Perrimon 1993). GAL4 is an 881 amino acid transcription factor of yeast that acts as a regulator of genes (eg. GAL10 and GAL1).

GAL4 regulates its transcription by binding directly to five optimized GAL4 sites known as Upstream Activation Sequences (UAS).

Targeted gene expression in vivo has been a powerful technique to study gene function. In this system, the gene of interest, the responder, is under the control of the UAS element. Transcription of the responder gene requires GAL4, thus in its absence, the gene is transcriptionally silent. Activation of transcription was achieved when the responder lines were mated to flies expressing GAL4 (driver) in a specific pattern (Figure 2.1). Thus the progeny expressed the gene of interest that reflected the GAL4 pattern. Another way to target gene expression was done by using the GAL4 transcriptional activator and the promoter region from the gene of interest (Chapter 5). The transcription factor activated transcription of a reporter gene, RFP (Green Fluorescent Protein) which is bound to the UAS element. This resulted in expression of the RFP in tissues/cells where the gene of interest is typically expressed (Brand, Perrimon 1993).

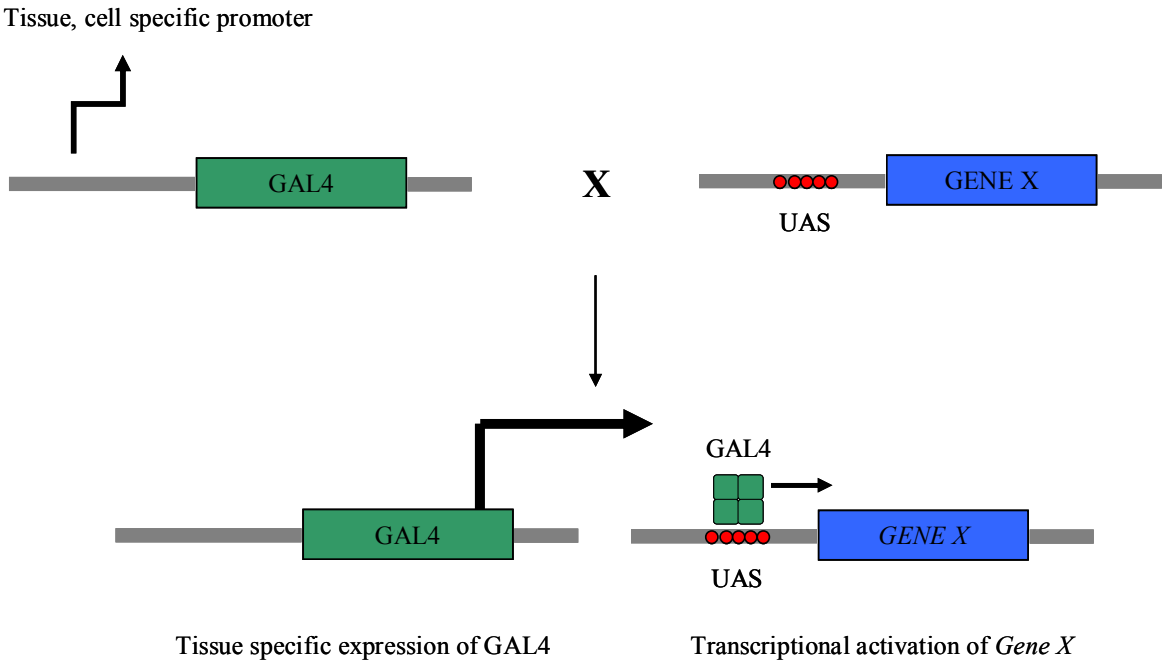


Figure 2.1: The bipartite UAS/GAL4 system in *Drosophila*. The GAL4 gene drives tissue/cell specific expression. The target gene, downstream of Upstream Activating Sequence (UAS) is silent in absence of GAL4. When these two lines were crossed, the target gene was activated where GAL4 was expressed.

The GAL4/UAS system can also be used for targeted gene knockdowns and study the effect in development and behaviour. RNA interference (RNAi) is an effective reverse-genetic approach to generate loss-of-function phenotype. The presence of double stranded RNA (dsRNA) causes posttranscriptional silencing of the corresponding gene in *Drosophila* and many other organisms. Transgene with an inverted-repeat (IR) configuration that produces hairpin loop (hpRNA) are fused to GAL4-responsive UAS. On crossing the UAS-IR and the GAL4 lines, the progeny that contain both the GAL4 and UAS elements express the IR sequence in the tissues/cells expressing GAL4 and thus silencing the gene in these tissues/cells (Figure 2.2).

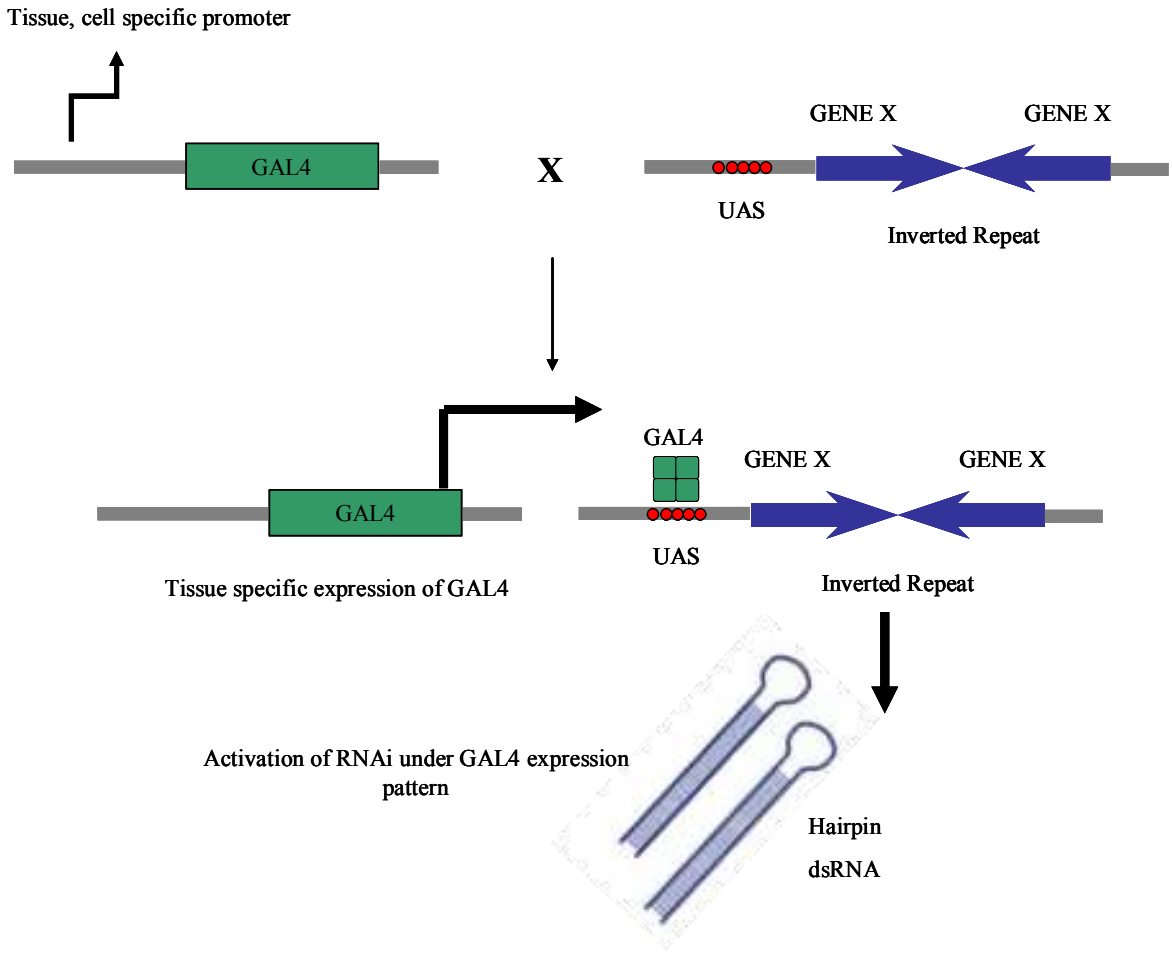


Figure 2.2: Gene knock-down using RNA interference. Transgenic lines with inverted repeat placed downstream of upstream activation sequences (UAS) promoter were crossed to GAL4 driver lines. Tissue-specific expression of inverted repeats by GAL4 protein produced hairpin loop RNA which induced RNA interference in the progeny that contained both the GAL4 and UAS elements.

2.4 DNA extraction, amplification and visualization

2.4.1 DNA extraction

A single fly was used for extraction of genomic DNA. Each fly squash was prepared using 50µl of Squishing Buffer (SB) which is composed of 10mM Tris-HCl pH 8.2, 1mM EDTA, 25mM NaCl and 200ug/ml Proteinase K (added freshly each time). The fly squashes were incubated at 37ºC for 1 hour and then at 95ºC for 2 min to inactivate Proteinase K. The DNA was then stored at -20ºC.

2.4.2 DNA amplification – Polymerase Chain Reaction (PCR)

All the PCR reactions were done using the DYAD™ DNA Engine Peltier Thermal Cycler or G-STORM GS4 thermal cycler. The reagents used for the PCR are listed in Table 2 and the standard cycling conditions are stated in Table 2.2 Primers were designed using Primer3. The list of primers used and their annealing temperature and amplicon sizes are listed in Appendix 1. The components of 11.1 X Buffer are also listed in Appendix 1.

Table 2.1: PCR components

PCR component	Concentration
DNA	Upto 500ng
11.1X	1X
Forward Primer	5pmoles
Reverse Primer	5pmoles
Taq DNA polymerase	1 unit
Water	To make final volume of 20 µl or 50 µl

Table 2.2: Standard cycling conditions

PCR Step	Temperature	Time (min:sec)
Initial Denaturation	92˚C	02:00
Denaturation	92˚C	00:30
Annealing	Specific for primer(refer Appendix 1)	0:30
Extension	72˚C	(depending on the size of the PCR fragment)
Repeat step 2, 3 and 4 for 35 cycles		
Final Extension	72˚C	10:00

2.4.3 Visualization of DNA by Standard Gel Electrophoresis

PCR fragments were separated using Agarose Gel Electrophoresis. The gels were made by melting the Seakem® LE Agarose in 1X TBE Buffer (10X TBE contains Tris 0.89, EDTA-Na^2-Salt 0.02M and Boric Acid 0.89M). Ethidium Bromide (1%) was added to the melted gel at a concentration of 5µl/100ml 1X TBE Buffer and it was allowed to set. The DNA samples were loaded onto the gel using appropriate amount of 5X Gel loading Buffer and distilled water to make a final concentration of 1X. Gel Electrophoresis was done using 1X TBE as the running buffer at a voltage of 130-140 V. The DNA fragment size was determined either by Fullranger 100bp DNA ladder (Norgen) or λ DNA (digested with *Hind*III) or ϕ DNA (digested with *Hae*III). All agarose gels were visualized under a ultra-violet Trans illuminator and the gel image was captured using a gel documentation system (Syngene).

2.4.4 PCR Purification

All PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN).

2.4.5 DNA sequencing

DNA fragments were purified and sequenced using the Protein Nucleic Acid Chemical Laboratory (PNACL) service at University of Leicester. The chromatograms were analysed using Chromas Version 1.45 (available freely http://www.technelysium.com.au/chromas.html).

2.5 Cloning Procedures

2.5.1 Bacterial Strain, growth and media conditions

The bacterial strain DH5ά (Invitrogen) of *E.coli* was used for cloning. This was grown in Luria Broth (LB) with continuous vigorous shaking at 225 rpm at a temperature of 37°C. Ampicillin was added with a working concentration of 400ug/ml to the media for appropriate selection of the bacterial strain containing the plasmid.

2.5.2 Transformation

Plasmid was transformed into chemically competent cells by heat-shock. About 5µl of plasmid DNA was added to the competent cells and incubated on ice for 30 minutes. The cells were given heat shock for 20 seconds at 37°C and then immediately placed on ice for 2 minutes. Transformed cells were allowed to grow in LB at 37°C and 225 rpm for 2 hours. The cells were then plated onto appropriate selection medium plates (for the plasmid) and incubated at 37°C. Single colony was picked from the plates and grown in LB at 37°C and 225rpm overnight.

2.5.3 Plasmid purification

Plasmid DNA was extracted from bacterial cells by performing a mini-prep using a QIAprep® Spin Miniprep kit (QIAGEN). The DNA was eluted in Elution Buffer and the concentration was measured and stored at -20°C until further use.

2.5.4 Restriction Digestion

Restriction digestion was carried out by the appropriate enzyme obtained from New England Biolabs®. 1µl (20 units) of the enzyme was used with the appropriate buffer. If necessary, 1X BSA was also added to increase the efficiency of the enzymatic reaction. Distilled water was added to make up the 20µl final volume of the reaction, which was incubated at 37°C for 30 min and heat inactivated at 65° for 15 min.

2.5.5 Ligation

The concentrations for ligation reaction were determined using the following formula/equation:

$$\text{Amount (ng) of insert} = \frac{\text{amount (ng) of vector} \times \text{size (kb) of insert}}{\text{size (kb) of vector}} \times \frac{\text{conc of insert}}{\text{conc of vector}}$$

3:1 ratio of concentration of insert to a vector was used. The ligation reaction carried Ligase buffer (New England Biolabs®) 10X, Taq DNA ligase (1µl) and water to make the final volume of 20 µl. The mixture was incubated at 65°C for 5 minutes before the Taq DNA ligase was added. Once added it was incubated at 25°C (room temperature) before leaving it at 4°C overnight.

2.6 Confocal Imaging

2.6.1 Brain dissection

Flies were collected and fixed in 4% paraformaldehyde (PFA) by incubating overnight at 4°C. The brains were washed three times for 15 min each with Phosphate Buffer Saline (PBS) on a rotating wheel. The brains were dissected under a microscope with a pair of forceps. A fly was placed in a droplet of PBS on a Petri dish with silicon layer under the microscope. Using the forceps the wings were removed. The body was separated from the head and discarded. The proboscis was removed by holding down the head with one forceps and the mouthparts with the other to pull the proboscis out. An opening inside the head was created once the proboscis was discarded. The right and left forceps in each hand were used to hold each eye through this opening and gently pulled apart with equal force to reveal the brain. The brain was cleaned by removing any connective tissue from its surface.

2.6.2 Preparation of slides

The dissected brains were carefully mounted on glass slides (VWR) with a drop of 1X PBS and covered with a glass cover slip (VWR). The cover slip was sealed with nail enamel and visualised immediately under a confocal microscope.

2.6.3 Visualisation of brains

The slides containing the brains were visualised on a laser scanning Olympus FV1000 Confocal microscope. Images of individual brains were taken under 10X or 20 X magnifications. The laser and microscopic settings were determined for each experiment.

2.7 RNA extraction

2.7.1 RNA extraction

Whole flies were homogenized in 1ml TRIzol reagent (Invitrogen) using a mortar and pestle for 60 sec. The homogenized samples were incubated at room temperature for 5 min. 200µl of chloroform was added and incubated at room temperature for 5 min. The homogenate was then centrifuged at 12,000 g for 15 min at 4ºC. The aqueous phase was transferred into a new tube and 500µl of isopropanol was added and incubated at room temperature for 10 min. The mixture was then centrifuged at maximum for 10 min at 4ºC. The pellet was washed with 500µl 70% ethanol. RNA was eluted in DEPC water. These stocks were made from fly lines by freezing and storing at -80ºC.

2.7.2 RNA quantification

RNA samples extracted from whole flies were quantified and checked for purity by using 1.5µl of the sample using spectrophotometer (Nanodrop) at 260nm. All samples had a 260/280 nm ratio of 1.8 or greater.

2.8 Real-Time Quantitative Polymerase Chain Reaction (qPCR)

2.8.1 Complementary DNA (cDNA) synthesis

RNA was treated with DNase I (2U/µl) and 5µl 10X DNase I buffer for removal of any genomic DNA. The mixture was incubated at 37ºC for 30 min. 5 µl of DNase inactivation reagent was added to the mixture and incubated at room temperature for 2 min before centrifuging the mixture at maximum speed for 1.5 min. To about 1.2 µg RNA, 250 ng random primers (Invitrogen), 2 µl of 20mM dNTP mix and 0.5µl aequorin cRNA

(exogenous control) was added. The samples were incubated at 65ºC for 5 min and then immediately chilled on ice for 1 min. cDNA mix of 5X RT buffer, 0.1 M DTT ,RNase OUT and 0.5 Superscript II RT (Invitrogen) was prepared and then was added to the RNA mix. This mixture was incubated at 25ºC for 10 min, thereafter at 42ºC for 50 min, heat activated at 70ºC for 15 min before chilling it on ice. 0.5 µl RNase H was added and incubated at 37ºC for 20 min. cDNA was eluted in elution buffer (EB) and the 1st strand was stored at -20ºC until further use.

2.8.2 cDNA quantification

cDNA samples were quantified by using 1.5 µl of the sample using spectrophotometer (Nanodrop).

2.8.3 Preparation of Standard curve samples

For the preparation of standard curve samples, PCR amplification for the gene of interest and the endogenous control gene was performed using cDNA samples (see section 2.4.2). PCR products were purified and quantified. According to the concentration of the PCR product, 300,000, 30,000, 3000, 300 and 30 copy dilutions were determined.

2.8.4 Real-Time Quantitative PCR

All the quantitative PCRs were performed using DNA Engine Opticon System (MJ Research PTC-200 DNA Engine Cycler and CFD-3200 Fluorescence Detector). The reagents used for the qPCR are listed below in Table 2.3 and the cycling conditions are listed in Table 2.4. Primers were designed using the Primer3 tool and are listed in Appendix 1.

Table 2.3: qPCR components

Component	Concentration
cDNA	Upto 500ng
Forward Primer	500nM
Reverse Primer	500nM
2X SYBR mix	10µl
Water	To make the final volume of 20µl or 50µl

Table 2.4: qPCR cycling conditions

PCR Step	Temperature	Time (min:sec)
Initial Enzyme Activation	94°C	15:00
Denaturation	94°C	00:15
Annealing	64.8°C	00:30
Extension	72°C	00:30
Repeat steps 2, 3 and 4 for 42 cycles		
Melting Curve	50 to 95°C reading every 0.2°C	

2.8.5 Data collection and Analysis

The data was analysed using the Opticon Monitor software (MJ Research) version 1.08. More details about the threshold settings will be discussed in Chapter 7.

2.9 Statistical Analysis

All graphs were made using Microsoft® Excel 2007. All statistical analyses in this report were done using R statistical software (R Development Core Team 2010). Specific statistical procedures are described in the various Result chapters.

Chapter 3: Hereditary components of natural phenotypic variation in sleep in *Drosophila melanogaster*

3.1 Introduction

While data on the genetics of sleep in *Drosophila* is accumulating, the extent of genetic variation associated with this trait in natural populations remains unknown. Some genes that are vital for sleep are likely to be under intensive directional selection and will therefore show no genetic variation; these invariant loci cannot contribute to adaptations in this trait that natural population may undergo (Harbison, Mackay & Anholt 2009). In contrast, some sleep loci may be involved in local adaptations of populations to different environments and will retain extensive genetic variation. Indeed, a recent study demonstrated that 60 generations of artificial selection for short sleepers have generated insomniac-like flies (Seugnet *et al.* 2009b). Although, selection intensity, heritability, and number of loci were not estimated in that study, the robust response to selection indicated significant genetic variation for sleep is present in these flies.

In this chapter, I have investigated the inheritance pattern of sleep between two natural strains from different geographical locations. The biometric analysis of the phenotypic variation exhibited by various reciprocal crosses provided a simple, yet effective way to estimate the contribution of two types of factors, chromosomal and non-chromosomal by contrast analysis of variance (see below). While other more advanced methods have been developed to discover the specific genetic variations underlying sleep, such as quantitative trait loci mapping and whole-genome association studies (reviewed by Mackay 2010), the biometric approach I have taken here still remains valuable in

elucidating the role of non-chromosomal transmission, such as maternal effect in the hereditary pattern of the trait.

The additive-dominance model is often the standard starting point for analysing the genetic architecture of a behavioural trait (Kearsey and Pooni 1996), even though the behavioural characters may have a very complex genetic basis. The phenotypic variation exhibited by the reciprocal crosses of the basic generations (P1,P2, the reciprocals F1 and BC) allows identifying contribution of additional factors (Figure 3.1); the relative contribution of autosomal and sex chromosome can be estimated, and non-chromosomal components like cytoplasmic and maternal factors which may be transmitted from maternal parent to progeny could be uncovered (De Belle and Sokolowski 1987). Permanent cytoplasmic factors include non-chromosomal components of the egg that persist throughout the life of the progeny and are transmitted over generations (e.g. mitochondrial DNA), while transient maternal factors include non-chromosomal components of the egg or developing embryo which dissipate during the lifetime of the progeny and are not transmitted to subsequent generations.

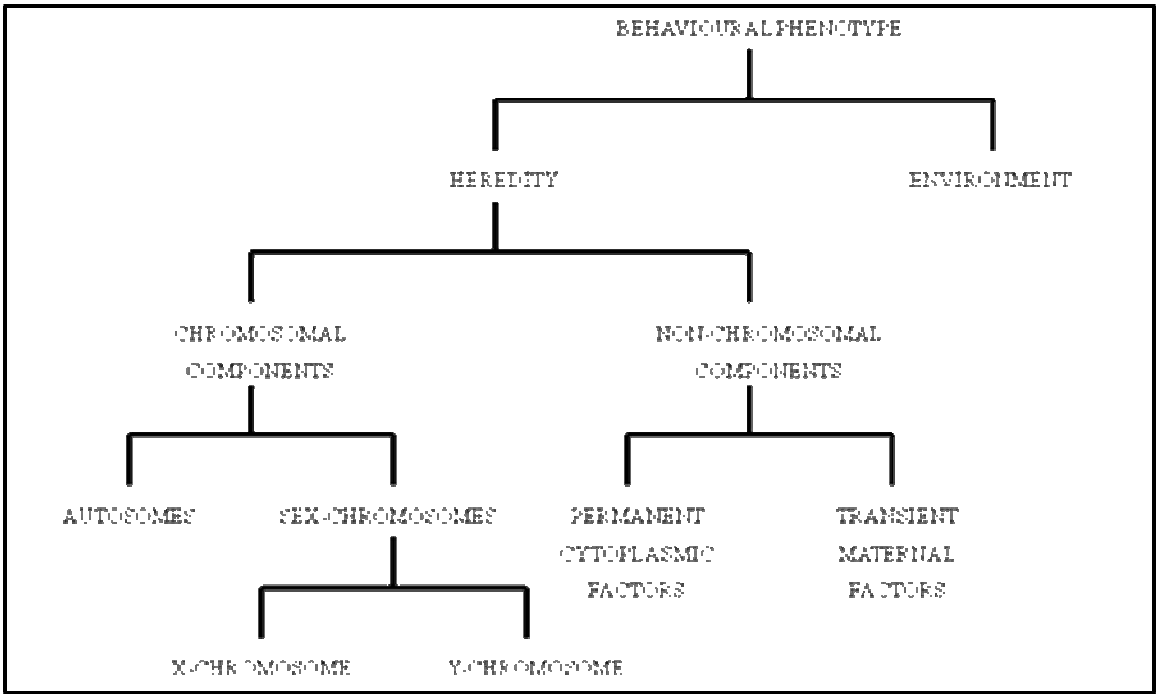


Figure 3.1: Components contributing to phenotypic variation. From (De Belle, Sokolowski 1987).

3.2 Materials and Methods

3.2.1 Fly stocks and crosses

Two inbred strains Sal and Hu, were used to investigate the inheritance pattern of sleep. These strains originated from Salice Salento (Sal) in Italy, and Houten (Hu) in the Netherlands (Tauber *et al.* 2007). One isofemale line from each population was selected and inbred by full-sib mating for 20 generations. Flies were raised and maintained on sugar media in LD 12:12 at 25°C, which was also the temperature used for the experiments. For crosses, approximately 100 flies were used (50 of each sex) in food bottles.

3.2.2 Sleep measurement

Males and virgin female age 3-4 days were placed individually in infra-red driven activity monitors (TriKinetics Inc.). Experiments were carried at 25°C with a light:dark LD 12:12 cycle. Sleep was recorded for over a period of four days. Two different parameters of sleep were measured: (i) the average daily accumulated sleep and (ii) the ratio of diurnal (12hr light) to nocturnal (12hr dark) sleep. A custom written program in R (R Development Core Team 2010) was used for calculating these parameters (Appendix 1).

3.2.3 Cross analysis

16-reciprocal cross analysis was carried out to analyse hereditary components contributing to sleep in *Drosophila*. The crossing scheme and the heritable components related to each cross are shown in Table 3.1. Contrast analysis of variance was used to test the role of various factors in sleep inheritance. In general hereditary factors are broadly divided into chromosomal and non-chromosomal components (Figure 3.1). The contrast analysis of variance allows planned comparisons between crosses differing in only one factor in consideration. The 16 reciprocal cross analysis allows to assess the contribution of the autosomes and each of the sex-chromosome, and the role of maternal contributions (paternal factors are usually considered negligible); these can be further divided to permanent cytoplasmic factors (for eg mitochondrial DNA), and transient maternal effect, which include cytoplasmic factor in the eggs such as proteins or mRNA that affect the developing embryo.

The means of different generations were analysed using contrast analysis of variance to test the importance of a particular factor. Comparing the same component (for example,

the X chromosome) on different backgrounds also allows testing the interaction among various components.

Together with the parental contrasts, dominance contrasts and comparing the F1 strains to their parents, nine contrasts were performed for male, and seven contrasts were performed for females (De Belle, Sokolowski 1987). The possible contrasts are listed below (cross nos. as in Table 3.1):

1. S vs. H (1 vs. 2, Table 3.1), to investigate the difference between parental strains;
2. Parental strains vs. F1 crosses (1 + 2 vs. 3 + 4), to test dominance;
3. F1 crosses (3 vs. 4) to test deviation from autosomal mode of inheritance;
4. Backcross to females (5 + 8 vs. 6 + 7), to explore interactions between sex chromosome and all other factors;
5. Backcross to males (9 + 12 vs. 10 + 11), to explore interaction between permanent cytoplasmic and all other factors;
6. Backcrosses (6 + 7 vs. 9 + 12) to explore interactions between transient maternal to all other factors (males only);
7. F2 crosses (13 + 15 vs. 14 + 16) for significance of Y chromosomal effects (males only);
8. Backcross to females (5 + 7 vs. 6 + 8) for significance of X-chromosomal effects (females only);
9. F2 crosses (13 + 14 vs. 15 + 16) for significance of permanent cytoplasmic factors;
10. Backcrosses (6 + 12 vs. 7 + 9) for significance of transient maternal factors (males only).

Table 3.1: 16 crosses between Houten (H) and Sal (S) *D. melanogaster* strains used to separate their hereditary components. Modified from Wahlsten (1979) and Sokolowski (1992).

Cross No.	Mother	Father		Autosomes	Female XX	Male X	Y	Permanent cytoplasmic factors	Transient cytoplasmic factors
Parental strains									
1	S	x	S	S	S	S	S	S	S
2	H	x	H	H	H	H	H	H	H
Reciprocal F1 hybrids									
3	S	x	H	F_1	F_1	S	H	S	S
4	H	x	S	F_1	F_1	H	S	H	H
Reciprocal backcrosses									
5	S	x	(SxH)	Bs	S	S	H	S	S
6	S	x	(HxS)	Bs	F_1	S	S	S	S
7	H	x	(SxH)	B_H	F_1	H	H	H	H
8	H	x	(HxS)	B_H	H	H	S	H	H
9	(SxH)	x	S	Bs	Bs	S/H	S	S	F_1
10	(HxS)	x	S	Bs	Bs	S/H	S	H	F_1
11	(SxH)	x	H	B_H	B_H	S/H	H	S	F_1
12	(HxS)	x	H	B_H	B_H	S/H	H	H	F_1
Reciprocal F2 hybrids									
13	(SxH)	x	(SxH)	F_2	F_2	S/H	H	S	F_1
14	(SxH)	x	(HxS)	F_2	F_2	S/H	S	S	F_1
15	(HxS)	x	(SxH)	F_2	F_2	S/H	H	H	F_1
16	(HxS)	x	(HxS)	F_2	F_2	S/H	S	H	F_1

3.2.4 Calculation of broad sense heritability

For an F_2 generation, the broad sense heritability is

$$h^2 = (\tfrac{1}{2}D + \tfrac{1}{4}H) / (\tfrac{1}{2}D + \tfrac{1}{4}H + E)$$

where, D is the *additive genetic variance;* H is the *dominance genetic variance*; E is the *environmental variance* V_E. E was estimated by taking the mean of the variances of the two parental generations.

The phenotypic variance of the F2 is

$$V(F_2) = \tfrac{1}{2}D + \tfrac{1}{4}H + E$$

Thus, by calculating E and $V(F_2)$, one can estimate $\tfrac{1}{2}D + \tfrac{1}{4}H$ ($=V(F_2) - E$) and calculate h^2. For more details see (Kearsey, Pooni 1996 p. 49).

3.3 Results

Sal male flies showed a significantly higher amount of daily sleep (25%, ~180 min) than Hu flies, while no significant difference between the females was detected (Table 3.2-3.3). The significantly higher diurnal/nocturnal ratio in Sal males ($p < 0.001$, Table 3.2-3.3) indicates that difference in total sleep between the strains was largely due to higher diurnal (mid-day) sleep in Sal males (whose mean daytime sleep was 486 min compared 379 min in Hu males). Broad-sense heritabilities were calculated for daily sleep (males;h^2=0.27, females; h^2=0.59) and diurnal/nocturnal ratio (males; h^2=0.65, females; h^2=-0.43[3]). These broad-sense heritability estimates for total sleep have standard error of 0.05 (for both male

[3] Negative heritability may indicate an experimental artefact that introduced environmental noise.

and female) (Dickerson 1969) indicating it is significantly different from "zero", while broad-sense heritability for D/N ratio for male has a standard error of 0.16.

Table 3.2: Daily accumulated sleep [min], Diurnal/Nocturnal ratio, standard deviation (SD) and number of males and females from crosses between Sal and Hu strains

	Males					Females				
	Daily Sleep		Diurnal/Nocturnal Ratio			Daily Sleep		Diurnal/Nocturnal Ratio		
Cross	Mean	SD	Mean	SD	n	Mean	SD	Mean	SD	n
SxS	901.56	107.31	0.908	0.184	54	764.40	105.12	0.577	0.236	47
HxH	720.15	154.15	0.719	0.284	33	716.27	116.19	0.482	0.393	23
SxH	727.92	155.98	0.702	0.133	39	627.17	121.30	0.436	0.181	24
HxS	795.39	132.47	0.894	0.321	59	683.78	109.63	0.634	0.512	47
S x (SxH)	741.67	132.50	0.668	0.203	36	629.79	133.94	0.414	0.245	34
S x (HxS)	623.88	185.23	0.707	0.223	24	650.21	129.65	0.271	0.169	19
H x (SxH)	666.46	146.20	0.638	0.223	24	666.07	96.04	0.396	0.159	15
H x (HxS)	771.48	135.85	0.675	0.172	27	710.40	100.02	0.368	0.158	20
(SxH) x S	902.52	157.94	0.865	0.188	66	699.82	122.93	0.521	0.385	39
(HxS) x S	825.76	95.13	0.769	0.179	42	683.68	109.53	0.460	0.173	40
(SxH) x H	763.02	126.61	0.688	0.241	43	729.29	92.41	0.439	0.188	33
(HxS) x H	783.41	133.52	0.792	0.238	32	764.19	130.67	0.466	0.218	33
(SxH) (SxH)	723.38	170.98	0.627	0.222	24	625.75	134.59	0.360	0.161	21
(SxH) (HxS)	716.24	158.02	0.901	0.362	67	690.16	202.85	0.606	0.239	51
(HxS) (SxH)	770.73	137.03	1.102	0.406	22	602.25	127.02	0.682	0.316	24
(HxS) (HxS)	766.05	147.47	0.712	0.234	19	749.32	123.87	0.409	0.235	23

The amount of sleep for each cross is shown in Table 3.2, and the results of the genetic analysis are shown in Table 3.3. The comparison of the reciprocal F1 hybrid males indicated a significant difference (Table 3.3, contrast 3). As males of these crosses share the same set of autosomes, some other non-autosomal components of heredity must contribute to this phenotypic difference. In the female data, this difference also existed, but was only marginally significant. The deviation from autosomal model is unlikely to be due

to the sex-chromosomes; since the contrasts testing the role of the Y (male data) and the X (female data) did not show any significant difference. In addition, there was no significant interaction between the sex chromosome and the other components, both in male and female data.

There was however, a significant contribution of the maternal factors. The male F2 data reveal a significant contribution (contrast 9), as well as a significant interaction with their genetic background (contrast 5), suggesting a role for permanent cytoplasmic factors. The male backcross generation means also indicated a significant contribution of the transient maternal factors (contrast 10), as well as a significant interaction with the genetic background (contrast 6), indicating a role for transient maternal factors.

In *Drosophila*, males usually exhibit a longer mid-day sleep ('siesta') than females (see Introduction), which may suggest in this case that the total sleep difference observed in males but not in females of the two strains, is due to increased siesta sleep in Sal males. This was consistent with the diurnal /nocturnal ratio data (Table 3.2) that show that diurnal sleep is significantly higher in Sal males, while in females the ratio is rather similar.

The contrast analysis of the diurnal /nocturnal ratio revealed a similar architecture to that seen for the daily accumulated sleep (Table 3.4) Here too, the reciprocal F1 hybrids (both males and females) exhibited significant different sleep ratios (Table 3.4, contrast 3). However, this deviation from an autosomal model is unlikely, due to the sex chromosomes, since the contrasts that tested the role of the Y (contrast 7, male data) and the X (contrast 8, female data) did not show any significant difference. There was also no evidence for significant interaction between the sex chromosome and the other components, both in

male and female data. In males, there was a significant evidence for interaction between transient maternal factors to all other factors (contrast 6).

Table 3.3: Contrast ANOVA of daily accumulated sleep of males and females between Hu (H) and Sal (S) *D. melanogaster* strains. Asterisks; *≤ 0.05, **<0.01, ***<0.000, NS; not significant.

	#	Males		Females	
Source		t	P	t	P
Model (between crosses)		125.782	< 0.000***	110.351	<0.000***
Contrasts					
S vs. H parental strains	1	6.149	<0.000***	1.572	NS
Dominance	2	2.147	0.032*	3.477	<0.000***
F_1: Non-autosomal model	3	-2.573	0.010**	-1.842	0.066
Interactions with background					
Sex-chromosome[4]	4	-0.360	NS	-0.090	NS
Permanent cytoplasmic factors	5	-2.339	0.019**	1.126	NS
Transient maternal factors[5]	6	-6.901	<0.000***		
Non-autosomal components					
Y-chromosome[2]	7	0.546	NS		
X-chromosome	8			-1.593	NS
Permanent cytoplasmic factors	9	-2.312	0.021*	-0.335	NS
Transient maternal factors[2]	10	-3.660	<0.001***		

[4] Testing Y chromosome in male data only, and X chromosome using the female data.

[5] Males only

Table 3.4: Contrast ANOVA of diurnal/nocturnal ratio of males and females between Hu(H) and Sal (S) *D. melanogater* strains. Asterisks; *≤ 0.05, **<0.01, ***<0.000, NS; not significant.

	#	Males		Females	
Source		t	P	t	P
Model (between crosses)		69.03	< 0.000***	35.57	<0.000***
Contrasts					
S vs. H parental strains	1	3.493	<0.000***	1.74	NS
Dominance	2	0.296	NS	-0.188	NS
F_1: Non-autosomal model	3	-3.7	<0.001**	-3.188	0.001**
Interactions with background					
Sex-chromosome[6]	4	-1.84	NS	0.036	NS
Permanent cytoplasmic factors	5	0.394	NS	0.927	NS
Transient maternal factors[7]	6	-2.695	<0.01*		
Non-autosomal components					
Y-chromosome[2]	7	-1.319	NS		
X-chromosome	8			0.935	NS
Permanent cytoplasmic factors	9	-0.903	NS	0.167	NS
Transient maternal factors[2]	10	-0.089	NS		

3.4 Discussion

This study was aimed at analysing the basic hereditary pattern of sleep in two strains derived from wild populations. The major difference between the strains was the amount of diurnal sleep in males, which was elevated in the Italian flies (Sal) as compared with Dutch flies. This difference may reflect thermal adaptations of the populations from

[6] Testing Y chromosome in male data only and X chromosome using the female data.

[7] Males only

which these two strains were derived, as the extent of midday sleep has been shown to be temperature dependent (increases at higher temperature), possibly to prevent desiccation (Collins, Rosato & Kyriacou 2004)). Thus, these results may suggest that the flies do not merely respond to temperature, but are genetically programmed to sleep at specific times.

F1 hybrids slept considerably less than their two parental strains (Table 3.2). This kind of underdominance may reflect a classic heterosis effect, and was observed in other behavioural studies such as *Drosophila* larval foraging (Bauer and Sokolowski 1985). The increased activity (less sleep) in the F1 progeny is not surprising as these flies are heterozygous for some deleterious alleles which may have accumulated in their highly inbred parental lines; conversely, this may also imply that the higher sleep in the parental lines may reflect ill-health of these highly inbred strains which is manifested in their lethargic behaviour. Interestingly, the observed increased activity in F1 flies is not accompanied (or not constrained) by sleep, as homeostasis theories of sleep would predict.

In this study, the inheritance pattern of sleep between two strains has been analysed. The relative contributions of additive, dominance and epistatic effects contributing to the differences in the sleep patterns was initially analysed using joint scaling tests (data not shown) (Kearsey and Pooni 1996). In addition, other factors such as cytoplasmic factors and Y-chromosome contributions were analysed using contrasts analysis of variance. The amounts and significance of factors such as additive, dominance, epistatic and maternal were estimated simultaneously using maximum likelihood method. Y-chromosome and cytoplasmic effects, and their interactions could also be included in the joint scaling models, but as more parameters are added, the analysis becomes more difficult and prone to systematic errors (Huttunen and Aspi 2003). However, the results were rather

ambiguous and inconclusive (data not shown), which led to testing the effects of these factors using a more sensitive approach of contrast analysis of variance (Huttunen and Aspi 2003).

A major finding of this study using contrast analysis of variance method is the significant contribution of the maternal factors to the phenotypic variation, a factor which is often neglected in behavioural studies. Maternal effect has been identified in behavioural traits such as, larval foraging in *D. melanogaster* (Bauer and Sokolowski 1988) and courtship song in *D. virilis* (Huttunen and Aspi 2003). Maternal effects are broadly defined as all sources of offspring phenotypic variation arising from the female parent (apart from the genes that she may be contributing and environment). In many species, maternal diet has shown to influence number, size and/or quality of her offspring. For example, in the seed bettle, the female parent can change the size of the egg according to the host (reviewed in Mousseau and Fox 1998). Female parents also control the deposition of other cytoplasmic developmental cues in the egg (eg. mRNAs, hormones). These cytoplasmic factors may be influenced by the environment experienced by the parent (eg. Photoperiod, temperature) and can lead to significant effects in the offspring (eg. Growth, behaviour) (reviewed in Mousseau and Fox 1998).

A recent study in *Drosophila* has identified a link between sleep and metabolism (Harbison and Sehgal 2009). Following sleep deprivation, a significant change in energy stores (level of glycogen and triglycerides) was observed which was strain and sex-dependent. One may then speculate, that maternal factors (transient) transferred in the egg cytoplasm may have a major impact on progeny metabolism. This in turn, may influence the foraging and sleep pattern of the progeny.

The information provided by the biometric analysis I used here is rather limited. More advanced methods (albeit more demanding) were developed in recent years that potentially can identify the specific genetic polymorphisms underlying the phenotypic variation, including QTL analysis and association studies (see Chapter 1 and 4). A QTL mapping of sleep using RI lines from North-American population is presented in Chapter 4.

Chapter 4: Mapping sleep quantitative trait loci (QTL) in *Drosophila*

4.1 Introduction

Sleep is a complex phenotype which is manifested at various levels (e.g. physiological, molecular), and at least in vertebrates, consists of various states (i.e. REM and NREM sleep; see Introduction). The various measures of sleep are *quantitative* in nature and are likely to be controlled by large number of genes (albeit with small effect), and affected by environmental factors and gene-environment interactions. This is consistent with the fact that only relatively small number of candidate genes have been identified in mutagenesis screens in *Drosophila* (see Introduction), suggesting that the genetic architecture may not be explained by a few major genes.

Quantitative trait loci (QTL) mapping is an approach which is well suited for studying complex phenotypes (Box 4.1). QTL mapping is aimed at identifying the loci (e.g. genes) that underlies the phenotypic variation in the trait, and therefore may indicate those loci that are the target of selection and important for evolution of the trait. This is in contrast to the conventional mutagenesis screens, which may yield important genes underlying the trait, but not necessarily important for its evolution; mutations isolated by mutagenesis do not necessarily constitute the loci underlying phenotypic variation and important for evolution of the trait. QTL mapping and mutagenesis are therefore complementary approaches.

QTL mapping has been widely used in agriculture studies where genes affecting traits such as yield, resistance, fruit size, have been targeted (Mauricio 2001). It has also

been useful in identifying QTLs important for health, such as loci involved in asthma, obesity, hypertension, diabetes and other complex phenotypes in mammals (Korstanje and Paigen 2002). QTL studies in humans have identified genes affecting Alzheimer's disease (*APP*), ovarian and breast cancer, (*BRCA1* and *BRCA2*) cholesterol levels, and blood pressure (reviewed in Korstanje and Paigen 2002). Recently, a QTL study in inbred mice identified eight QTLs in addition to some previously reported for mouse life span (Lang *et al.* 2010).

Sleep has been explored by QTL studies in rodents. Many aspects of sleep and sleep EEG (electroencephalographic) activity differ dramatically among inbred strains of mice (reviewed in Shaw and Franken 2003). QTL analysis in 25 recombinant inbred mice strains identified a significant QTL that accounted for 49% variation in the increase of delta power slow-wave sleep, following sleep deprivation (Franken, Chollet & Tafti 2001). The QTL was named *Dps1*. Later, Mackiewicz *et al.* 2008 analysed the haplotype structure and managed to further reduce the QTL from ~34 Mb (236 genes) to 13 Mb (44 genes). They have also measured expression level of genes within the QTL, and sequenced the promoter region of various genes. The analysis indicated that a SNP in the promoter region of *Homer1* is likely to be the causative variation underlying this QTL. Interestingly, the *Drosophila Homer1* ortholog has been proposed to play important role in synaptogenesis, signal transduction, and is expressed in a broad range of tissues but is highly enriched in the CNS. *Homer* null mutants exhibit increased spontaneous activity (Diagana *et al.* 2002) indicating its role in locomotor control and behaviour plasticity.

Drosophila with its compact genome and well characterized genetics has also been used in QTL mapping, in various areas, including locomotor behaviour (Jordan, Morgan &

Mackay 2006). QTL have been identified underlying complex traits such olfaction (Fanara *et al.* 2002), courtship (Gleason, Nuzhdin & Ritchie 2002), longevity (Mackay 2002), and male mating behaviour (Moehring and Mackay 2004). A recent study of aggression in *Drosophila* has identified QTL on chromosome 2 and 3 and on further fine-mapping of these loci, indicated four candidate genes (Edwards and Mackay 2009).

My analysis of sleep in flies from natural populations (Chapter 3) revealed an extensive phenotypic variation and indicated that the heritability of this trait is substantial. Here, I used QTL mapping to identify specific loci underlying the variation in sleep, in a population of RI lines derived from wild population in California. Several QTL have been identified and were subsequently narrowed down by deficiency and mutation complementation tests.

Box 4.1: QTL mapping

The experimental design

Here I briefly outline the principles of QTL mapping (For the mathematical framework see (Lynch and Walsh 1998). Most experimental crosses are based on two highly inbred (isogenic) strains, that ideally, but not necessarily differ substantially in a quantitative trait of interest. These lines are then crossed and their F1 offspring are either intercrossed to generate F2 offspring population, or back crossed to one of their parents to generate back-cross population. The trait is measured in individuals of these populations, aiming at identifying genome regions where the genotype is associated with the phenotype. The complete genotype of the parental genomes is usually unknown; instead, a large number of molecular markers spanning the genome are established (microsatellites and

SNP are popular). These markers are used to genotype the individuals whose phenotype was measured (note that because two isogenic parental lines have been used, maximum two alleles for each marker are segregating). Consequently, data for QTL mapping include: (i) the phenotype measurements, (ii) the genotypes of the individuals and (iii) a genetic map of the markers that specify position and genetic distance between the markers.

A third QTL mapping population is based on recombinant inbred lines (RIL). Here, many brother-sister pairs of F2 offsprings are mated (in plants, F2 are simply selfed) and their progeny is used to establish a new line. These lines are made isogenic by full-sib mating for 10-20 generations resulting in RIL which are unique combinations of the parental genomes (see figure 4.1). There are two major advantages for using RIL over the F2 or back-cross populations: First, the same set of RIL can be used for mapping different phenotypes while genotyping is carried only once. Secondly, trait is measured by number of individuals from each RIL and this additional information on variation within RIL increases enormously the power of the analysis.

Statistical analysis

Conceptually, single-marker analysis is the simplest. For each marker Mi (i=1:n), the individual phenotype measures are grouped based on their genotype at that marker, and the mean values are compared. The difference between the means will depend on how close the QTL is to the marker, and the QTL effect *a.* In case of a complete linkage of the marker and the QTL (unfortunately, a rare event), the difference between the genotypes will be 2**a*. The main problem with single marker analysis is that the size of the QTL and its distance from the marker are confounded (Falconer, Mackay 1996 Introduction to

Quantitative Traits, Book). The 'interval mapping' approach has circumvented this problem (Lander, Botstein 1989). Here, the likelihood of a QTL between each two adjacent markers is calculated. The likelihood is calculated based on conditional probabilities of the genotype, the QTL effect and at different recombination frequencies. The likelihood includes a link function that allows estimating the phenotypes based on the other estimated. The ratio between the maximal likelihood estimator (MLE), and the likelihood of a null model ('no QTL present in the interval') is the likelihood ratio (or the log Odds, LOD) which is calculated along fixed intervals between the markers (e.g. 10 cM) and is graphically plotted (see figure 4.1). The significance threshold of the LOD scores can be determined by permutations (shuffling) of the data, where phenotypes are randomly associated with the genotypes in the dataset. The process is repeated (e.g. n=1000) and a genome-wide LOD distribution is obtained. The 5% percentile of the distribution may serve as a significance threshold.

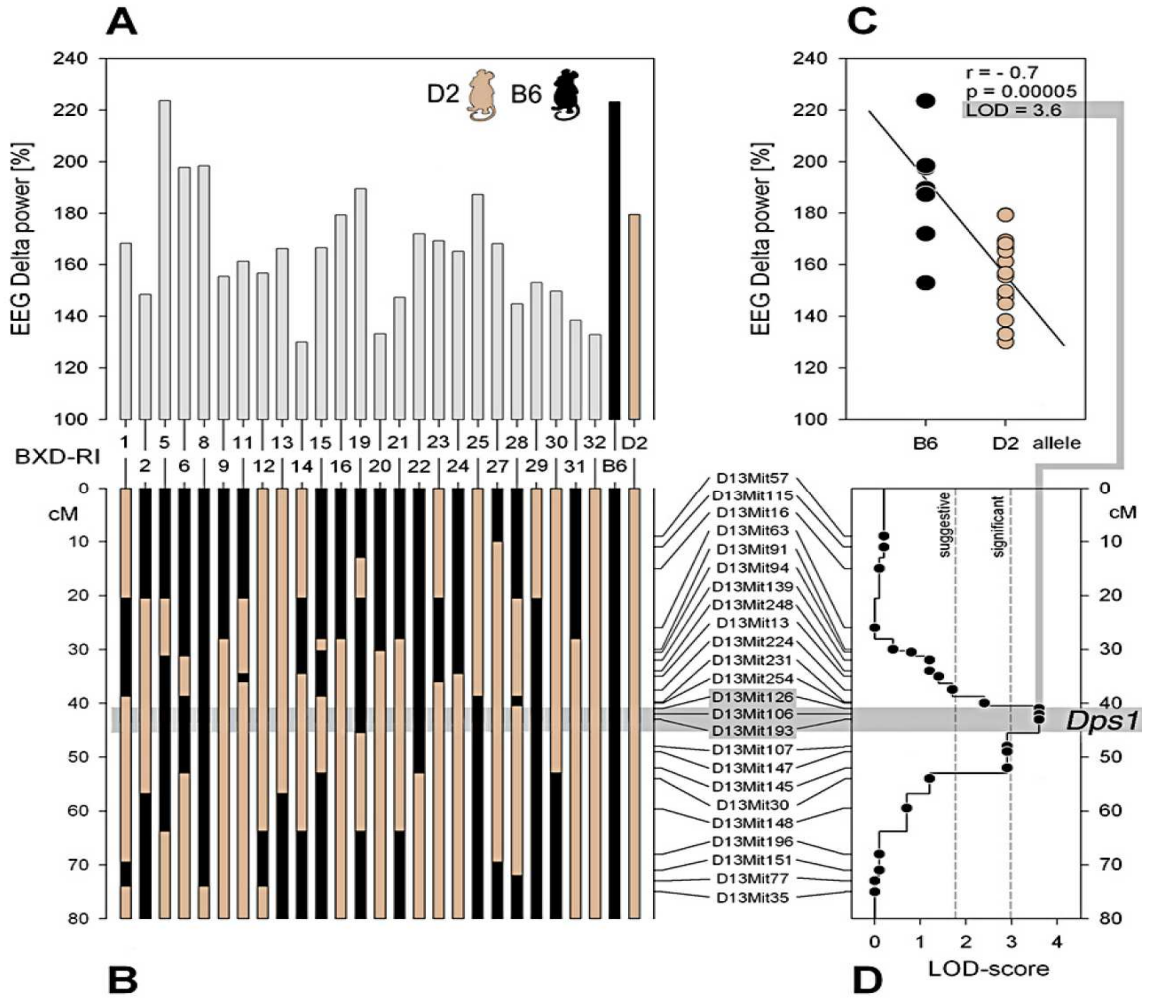


Figure 4.1: Quantitative Trait Loci (QTL) analysis illustrated here for sleep in two inbred strains of mice. **A**. Two parental strains D2 (brown) and B2 (black) were used for generating 25 RI lines (BxD-RI, gray) and the EEG delta power was measured after 6-h sleep deprivation. **B**. The RI strains were generated by sib- mating a pair of F2 animals and inbreeding for many generations (e.g. 20). Each of the RI lines is a 'mosaic' of the parental genomes, and the genotype is determined using a set of markers spanning the genome (n=25 in this example). **C.** Grouping the phenotype values of the RI lines based on the genotype of three successive markers (marked in gray bar in panel B) yields the maximal difference between the alleles. **D**. A LOD score plot indicating a significant QTL spanning these three markers (named *Delta power in sleep-1*; Dps1) (Franken and Tafti 2003).

Refining the QTL by complementation tests

Once QTL regions have been identified, fine-scale mapping using complementation tests may be carried out to narrow down the QTL interval and identify candidate genes. In *Drosophila*, one can take advantage of the availability of deficiency strains that carry small deletions at defined cytological regions. These Df lines are usually homozygous lethal, and are thus maintained against a balancer (Bal) chromosome which suppresses recombination and contains a dominant visible marker, allowing easy detection of whether F1 progeny received a wild-type or deficiency (Df) chromosome. A set of deficiency lines with overlapping segments within the candidate region can help to determine which part of the interval underlies the QTL. Each of the two parental lines (P1 and P2) is crossed to deficiency lines to create a class of offsprings: *Df/P1, Df/P2, Bal/P1 and Bal/P2*. Using a two-way ANOVA, quantitative complementation can be detected when (*Df/ P1 - Df/ P2*) = (*Bal/ P1 - Bal/ P2*) indicating no QTL present in that interval, and when the QTL does reside within the interval, quantitative failure to complement will be observed as (*Df/ P1 - Df/ P2*) $\neq$ (*Bal/ P1 - Bal/ P2*) (Figure 4.2). The same approach can be used with null mutants to test complementation to specific candidate genes in the QTL (Fanara *et al.* 2002).

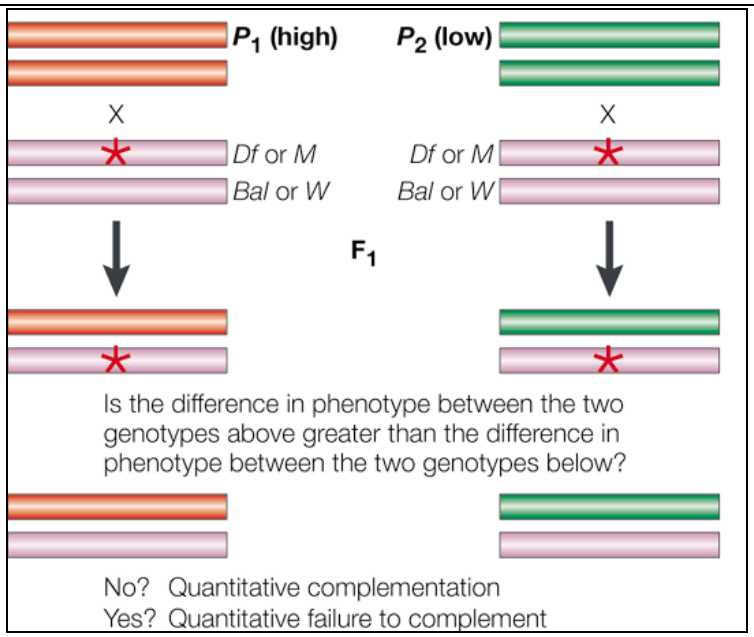


Figure 4.2: Quantitative complementation tests. These tests require Df (deficiency) or M (mutant) and Bal (balancer) or W (wild-type) allele at the candidate locus, and two QTL alleles. The strains that contain different QTL alleles are crossed to a strain that contains either Df or M and a strain that contains Bal or W allele, and the trait phenotype is measured in the resulting progeny (figure from (Mackay 2001)).

One drawback of deficiency mapping methods is that failure to complement may be observed due to epistatic interactions rather than allelism (Service 2004). There could be interaction between loci and the deficiency and the wild-type chromosomes (outside the QTL), or there maybe interaction between loci on the balancer and the wild-type chromosomes.

4.2 Materials and Methods

4.2.1 Genome Scan for QTL affecting sleep

4.2.1.1 Fly stocks

The set of RI strains was generated by Sergey Nuzhdin's lab at the University of Southern California. Briefly, the parental lines were derived from two isofemale strains collected from an orchard in Winters, California (38°N, 121°W) during 2001. These two lines were isogenized by 40 generations of inbreeding. These parental lines were expanded to a set of 500 isogenic lines which were then randomly mated for 15 generations. Each inter-mated line was sib-crossed for 15 generations to make the final set of RILs, 187 of which were used here. These lines were SNP genotyped at 31, 34, and 37 intronic and intergenic markers, respectively, along the X, second, and third chromosomes, using a multiplex oligoligation assay (A. Genissel and S. V. Nuzhdin, personal communication). These RIL have been previously used for QTL mapping (Bergland *et al.* 2008).

For the complementation tests, deficiency (Df) stocks were obtained from Bloomington *Drosophila* Stock Centre.

Table 4.1 provides the list of all deficiencies tested with their cytological locations. Virgin female flies (High or Low lines) were crossed to male flies containing deficiencies (Df/Bal). F1 Males of each of the four genotypes (Df/H, Bal/H, Df/L, and Bal/L) were collected and tested.

Table 4.1: Stocks used for deficiency complementation mapping of QTL affecting sleep.

Chromosome 2			Chromosome 3		
QTL	**Genotype**	**Cytological location**	**QTL**	**Genotype**	**Cytological location**
37F-38A	Df(2L)r10	35D1;36A6-7	87A-89B	Df(3R)T-32	86D9;87C3-4
	Df(2L)cact-255rv64	35F-36A;36D		Df(3R)ea	88E7-13;89A1
	Df(2L)TW137	36C2-4;37B9-C1		Tp(3;Y)ry506-85C	87D1-2;88E5-6
	Df(2L)TW130	37B9-C1;37D1-D2		Df(3R)sbd105	88F9-89A1;89B9-10
	Df(2L)VA12	37C2-5;38B2-C1		Df(3R)P115	89B7-8;89E7
				Df(3R)DG2	89E1-F4;91B1-B2
37C-38B	Df(2L)ED1226	37B9;37E3			
	Df(2L)Exel8041	37D7;37F2			
	Df(2L)BSC301	37D1;37E5			
	Df(2L)Exel6044	37F2;38A3			
	Df(2L)Exel6045	38A3;38A7			
	Df(2L)Exel7077	38A7;38B2			

4.2.1.2 Sleep measurements

All experiments were performed at 25°C, under 12:12 LD cycles for four days (see section 2.2). From each RI line, 25 males were tested. Three sleep variables were measured: daily accumulated sleep (average of four days), bout duration (median of all bouts recorded over 4 days, typically 200-240 bouts), and the diurnal/nocturnal (DN) sleep ratio (averaged over 4 days).

4.2.1.3 Quantitative genetic analysis

Analysis of Variance (ANOVA) of sleep phenotypes was carried using the R statistical software (R Development Core Team 2010) . Note that variation between the RILs is entirely genetic, while variation within RILs entirely reflects the environmental variation (V_E). Broad sense (h^2) heritabilities were calculated as $\sigma^2_B /(\sigma^2_B + \sigma^2_W)$, σ^2_B and σ^2_E are the between and within variance components ($\sigma^2_W=MS_W$, $\sigma^2_B=(MS_B - MS_W)/k$). The pairwise correlations between sleep variables were calculated using Pearson's method as $r_G= cov(x,y)/ (\sigma_X\sigma_Y)$, where cov(x,y) is the product of covariance between line means for traits x and y and σ_X and σ_Y are the square roots of the among line variance components for each trait separately.

4.2.1.4 QTL analysis

Genome scans for QTL was performed for accumulated sleep, diurnal/nocturnal ratio (LD) ratio, and sleep bout duration using composite interval mapping as implemented in R/qtl software (Broman *et al.* 2003). The mean phenotype values of each RIL have been used. The linkage map was constructed using Kosambi function. QTL analysis was performed using Haley-Knott regression, which is a fast approximation of interval mapping (Haley and Knott 1992), as recommended by Broman and Sen 2009. Genotype probabilities were estimated at 1 cM intervals, and 2.5 cM for a two QTL scan (testing for epistasis). Genotyping error of 0.001 was assumed following the manual recommendations. Genome wide significance threshold was calculated by permutations tests (n=800), using p=0.05 for detecting significant QTL.

4.2.2 Quantitative complementation tests to deficiencies and P-element mutations

4.2.2.1 Statistical analysis

The parental strains that were used for generating the RIL have been lost. Instead, I have used two RILs (308, 325) that showed a significant phenotypic difference and resembled the genotypes of the parental lines. These lines (H and L), were crossed to each of the deficiency lines and sleep of the four progeny genotypes (Df/H, Df/L, Bal/H, and Bal/L) was tested (n=32 flies of each genotype). Quantitative failure to complement the QTL occurs when the difference between the H/L lines is greater in the deficiency background than in the balancer chromosome background. This was analysed by two-way factorial analysis of variance (ANOVA):

$$Y = L + G + L \times G + E$$

where L is effect of line (H or L), G is effect of genotype (Df or Bal), L x G is the interaction term, and E is error variance. A significant L x G (ANOVA, $P \leq 0.05$) interaction term, where the difference between (H/Df and L/Df) is greater than the difference between (H/Bal and L/Bal) is interpreted as quantitative failure to complement (Pasyukova, Vieira & Mackay 2000). Once a deficiency was identified spanning the QTL, the process has been repeated with smaller deficiencies within that region.

4.2.2.2 P-element insertion complementation tests

In addition, quantitative complementation tests was also carried using p-element insertion mutants to test candidate genes in the QTL region, which were indicated to be involved in sleep, in microarray studies (Zimmerman *et al.* 2006; Cirelli, LaVaute &

Tononi 2005). Table 4.2 provides the list of mutants that were tested (all stocks were obtained from Bloomington stock centre).

Table 4.2: The list of genes that were tested for failure to complement and their cytological locations.

Significant QTL	**Gene tested**	**Genotype**	**Cytological location**
37C-38B	*Side*	y[1]w[67c23];P{y[+t7.7]w[+mC]=wHy}Side[DG30408]	37B9-37B10
	Diaphanous	P{ry[+t7.2]=PZ}dia[1]CG9323[04138]cn[1]/CyO;ry[506]	38E7-38E8
	CG9328	y[1];P{y[+mDint2]w[BR.E.BR]=SUPorP}CG9328[KG09432]; ry[506]	38E10-38F1
	CG9338	y[1]w[67c23];P{y[+mDint2]w[BR.E.BR]=SUPorP}CG9338[KG00683]	38F3-38F3

4.3 Results

4.3.1 Sleep phenotypes and genetic variation in RI lines

There was a significant variation among the 187 RI lines for each of the three sleep phenotypes ($p \leq 0.0001$) (Table 4.3, Figure 4.3). Broad-sense heritabilities of total sleep and D/N (but not bout duration) were substantial (Table 4.3). There was a positive correlation between total sleep and bout duration ($r_G = 0.45$, p-value ≤ 0.0001) (Figure 4.4A), and a negative correlation between total sleep and D/N ratio ($r_G = -0.32$, p-value ≤ 0.0001) (Figure 4.4C). However, the relatively low correlations suggested that these traits are mediated by non-overlapping genetic factors.

Table 4.3: ANOVA for each sleep trait. Mean values (± standard error) for each trait are given.; MS, mean square; H^2, broad sense heritability

Trait	Mean		MS	$F_{(186,4443)}$	*P-value*	H^2
Total sleep	839.81± 9.6 min	line	378788	16.35	< 0.000	0.38
		residuals	23168			
D/N ratio	0.88± 0.015	line	1.08	10.425	< 0.000	0.28
		residuals	0.10			
Bout duration	15.70± 0.40 min	line	600	2.4812	< 0.000	0.05
		residuals	242			

4.3.2 Genome scan for QTL affecting sleep

Composite interval mapping was used to localise QTL affecting sleep (Zeng 1994). Five significant QTL affecting total sleep, bout duration and D/N ratio were identified based on the permutation-derived genome-wide significance threshold. The QTL regions ranged from 800kb to 5 Mb encompassing 70- 437 candidate genes. Two QTL regions contribute for accumulated sleep and bout duration, while one to Diurnal/Nocturnal ratio (Table 4.4, Figure 4.5).

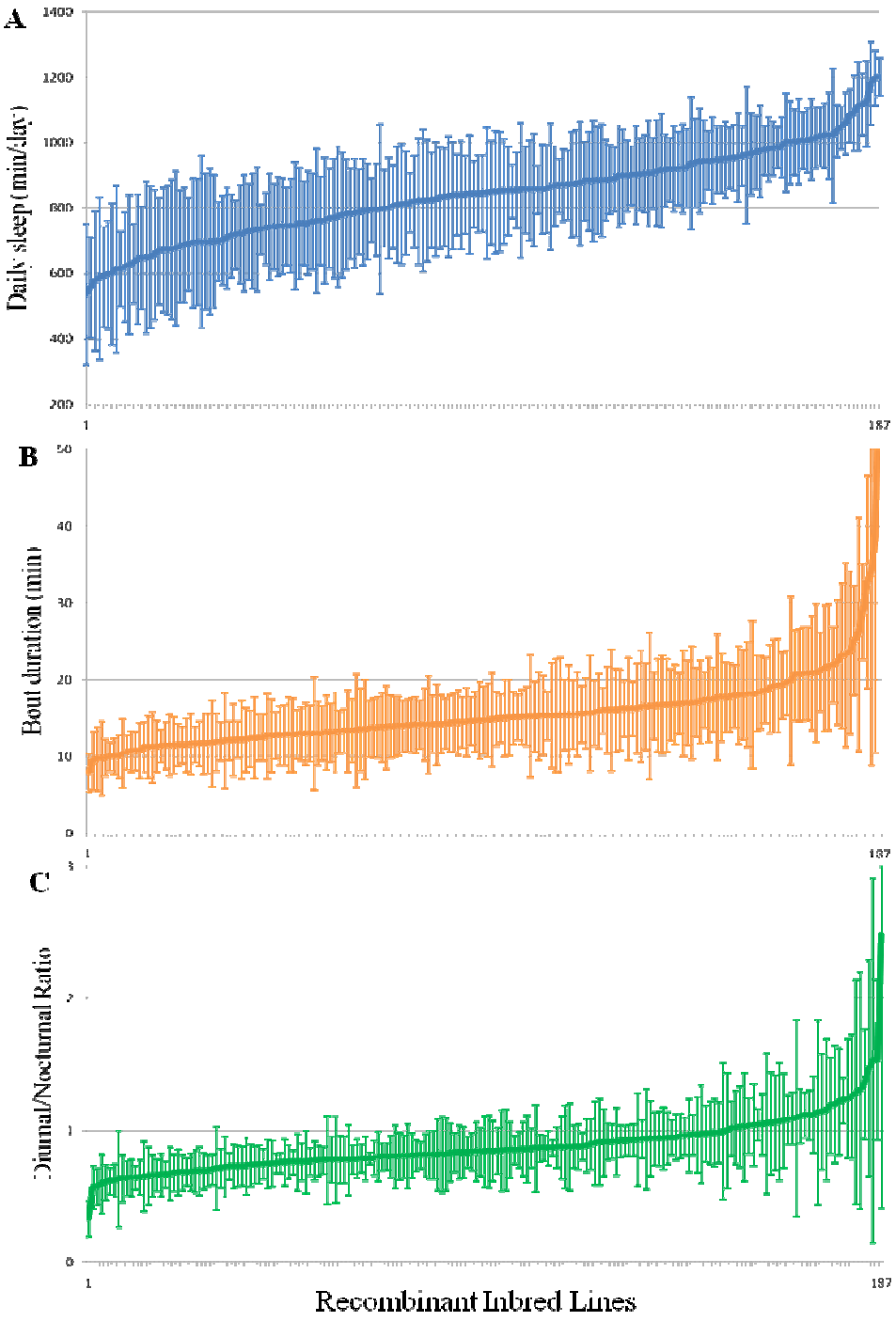


Figure 4.3: Range of variation of sleep variables in 187 RI strains. (A) Daily accumulated sleep, (B) bout duration and (C) D/N ratio. The means are sorted from lowest to highest. Error bars represent SD.

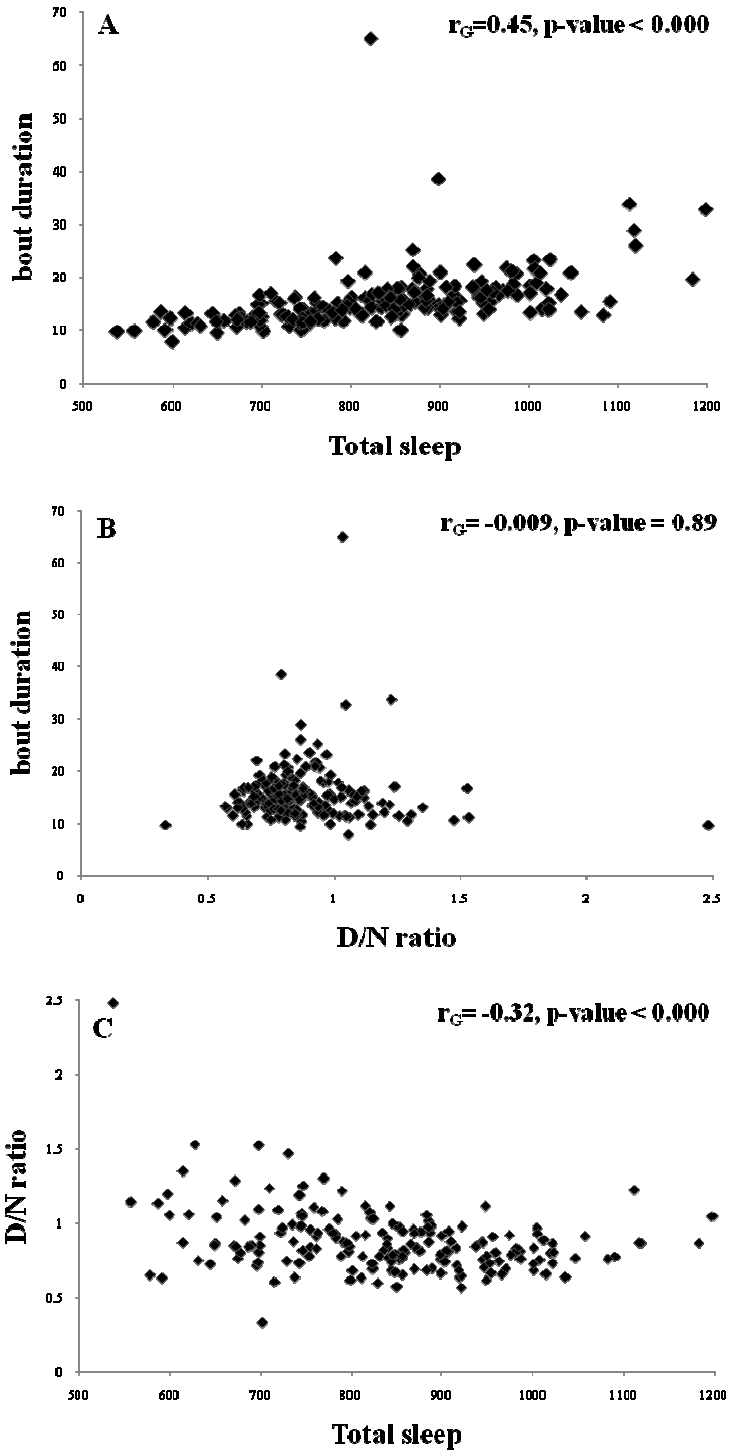


Figure 4.4: Correlations among lines for total sleep and bout duration (A), D/N ratio and bout duration (B) and total sleep and D/N ratio(C).

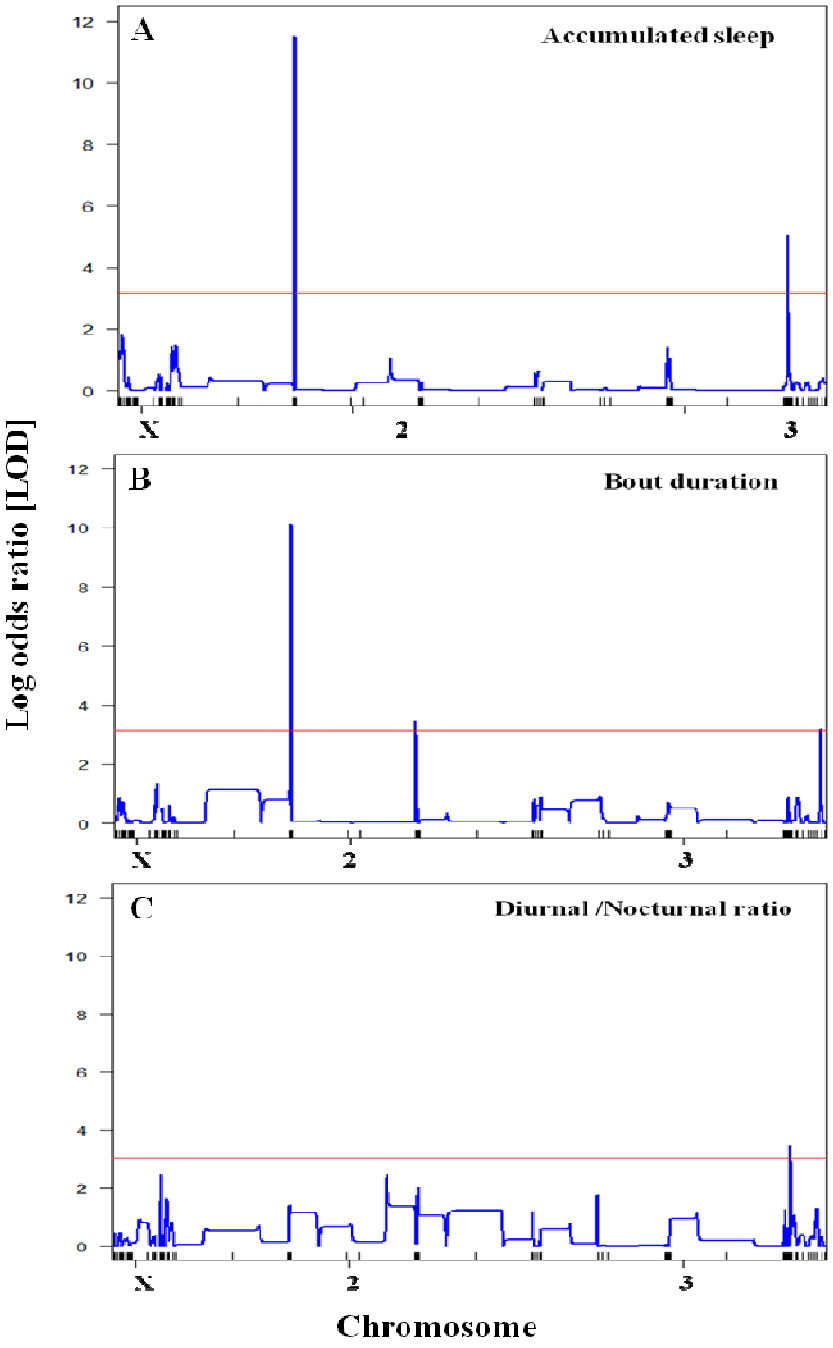


Figure 4.5: Composite interval mapping of sleep QTL. Log of odds (LOD) scores and significant thresholds plotted against chromosome location for (A) accumulated sleep, (B) bout duration and (C) D/N ratio. Horizontal lines (red) represent significance thresholds for each trait and the bars on the X-axis represent the location of the markers on the chromosomes.

Table 4.4: Summary of QTL mapping results. Log of odds score (LOD). Size of the QTL interval given by 95% confidence limits CL(=1.5 LOD support interval) and the QTL effect.

Trait	QTL	Marker	QTL interval	Ch	LOD	% variation	Effect
Daily sleep	1	38A	37C-38C	2L	10.81	22.49	-50 min
	2	88D	86C-89B	3R	4.44	8.48	34 min
Bout duration	3	38A	35E-38C	2L	10.25	26.65	-1.8 min
	4	50A	47D-50A	2R	3.51	8.88	0.9 min
D/N ratio	5	88D	87A-90E	3R	3.46	5.87	-0.05

4.3.3 Deficiency complementation mapping

I used deficiency complementation mapping to refine the size of each QTL interval (Table 4.5, Figure 4.7). Five overlapping deficiencies spanning QTL1 (37C;38C) and six deficiencies spanning QTL2 (region 86C;89B) were used for complementation tests (Figure 4.6). All three sleep variables were analysed in all tests. Within QTL1, one of the deficiencies (region 37C;38A, ~ 650kb) showed a failure to complement for total sleep (p-value = 0.05) (Table 4.5, Figure 4.7) and was treated as a new QTL interval that was further tested by smaller deficiencies (Figure 4.8).

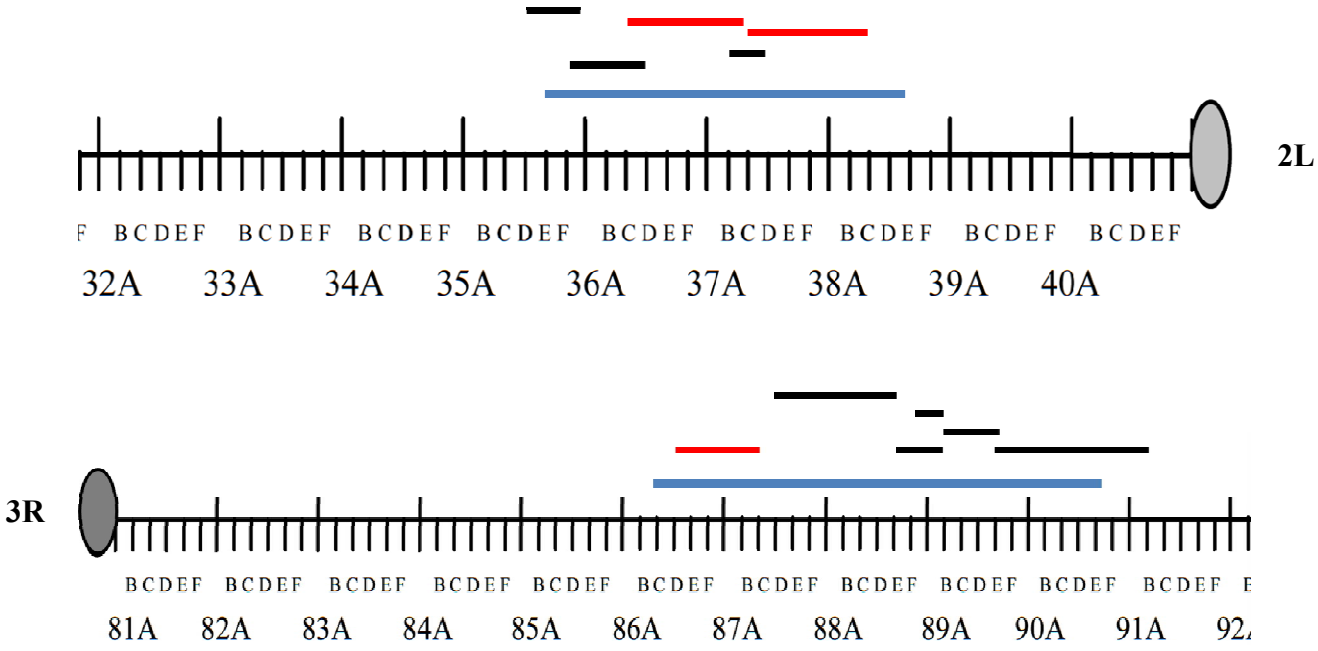


Figure 4.6: The significant regions from the QTL mapping (blue) that were used for deficiency complementation tests for chromosome 2L and 3R. Deficiencies that were tested are denoted in black, while those that showed failure to complement are in red. The grey circles represent centromere of the chromosome.

Table 4.5: P-values from ANOVA for deficiencies that showed failure to complement. L (line effect); G, genotype effect (*Df* or *Bal*); LxG, interaction. Significance values are indicated by asterisks. *=0.05, **=0.01, ***=0.0001. Grey box indicates significance for *Bal* which does not indicate failure to complement. NS, not significant

Genotype	Cytological Position	Total sleep			D/N ratio			Bout duration		
		L	G	LxG	L	G	LxG	L	G	LxG
37C-38C										
Df(2L)r10	35D1;36A6-7	***	NS	NS	NS	NS	NS	***	NS	NS
Df(2L)cact-255rv64	35F-36A;36D	***	NS	NS	***	***	NS	***	NS	NS
Df(2L)TW137	36C2-4;37B9-C1	**	**	NS	**	**	NS	*	***	0.0002***
Df(2L)TW130	37B9-C1;37D1-D2	*	*	NS	*	***	NS	**	NS	NS
Df(2L)VA12	37C2-5;38B2-C1	***	***	0.05*	**	***	NS	***	*	NS
87A-89B										
Df(3R)T-32	86D9;87C3-4	*	***	<0.000***	***	***	***	**	***	<0.000***
Df(3R)ea	88E7-13;89A1	***	***	*	**	***	*	***	***	NS
Tp(3;Y)ry506-85C	87D1-2;88E5-6	*	NS	NS	NS	***	NS	NS	NS	NS
Df(3R)sbd105	88F9-89A1;89B9-10	***	**	NS	NS	NS	NS	*	***	NS
Df(3R)P115	89B7-8;89E7	NS	NS	NS	**	NS	NS	*	NS	NS
Df(3R)DG2	89E1-F4;91B1-B2	***	*	NS	NS	NS	NS	**	*	NS

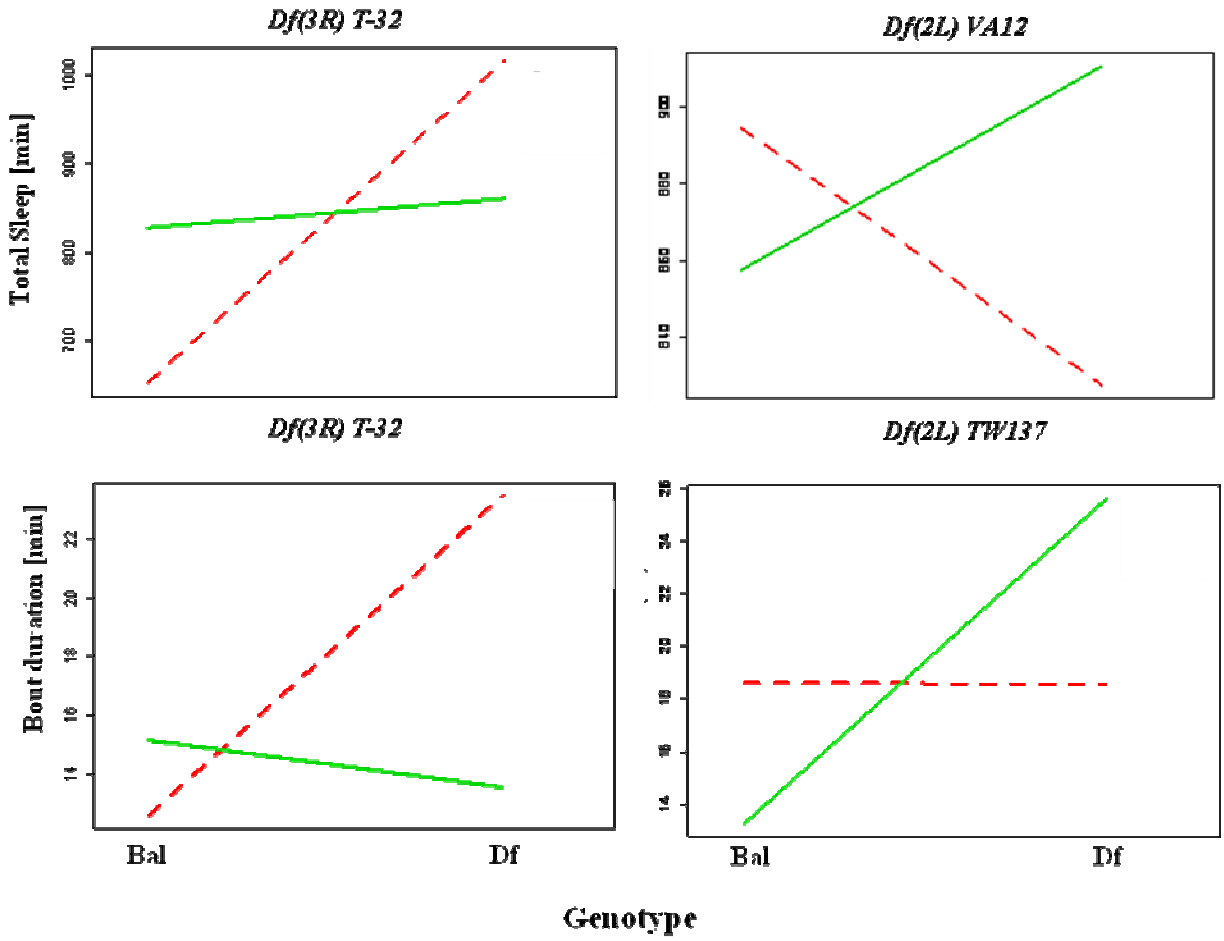


Figure 4.7: Deficiency complementation tests for the deficiencies that showed failure to complement for total sleep (Top) and bout duration (bottom). Green and red lines represent high and low lines respectively.

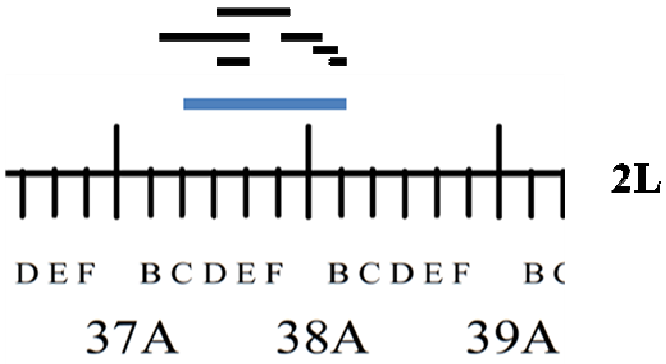


Figure 4.8: Complementation deficiency mapping for the region (37C-38B) that showed failure to complement on chromosome 2L. The region is denoted in blue, while the deficiencies covering the region are in black.

Table 4.6: P-values from ANOVA for deficiencies that showed failure to complement. Significance is indicated by asterisks, *≤0.05, **≤0.01, ***≤0.0001. NS, not significant

Genotype	Cytological Position	Total sleep			D/N ratio			Bout duration		
		L	G	LxG	L	G	LxG	L	G	LxG
37C-38B										
Df(2L)ED1226	37B9;37E3	*	***	NS	*	NS	NS	*	***	NS
Df(2L)Exel8041	37D7;37F2	*	***	NS	NS	***	NS	NS	*	NS
Df(2L)BSC301	37D1;37E5	*	*	NS	***	***	NS	*	NS	NS
Df(2L)Exel6044	37F2;38A3	*	NS	NS	NS	***	NS	*	*	NS
Df(2L)Exel6045	38A3;38A7	*	**	NS	*	***	NS	NS	NS	NS
Df(2L)Exel7077	38A7;38B2	***	**	NS	***	**	NS	***	NS	NS

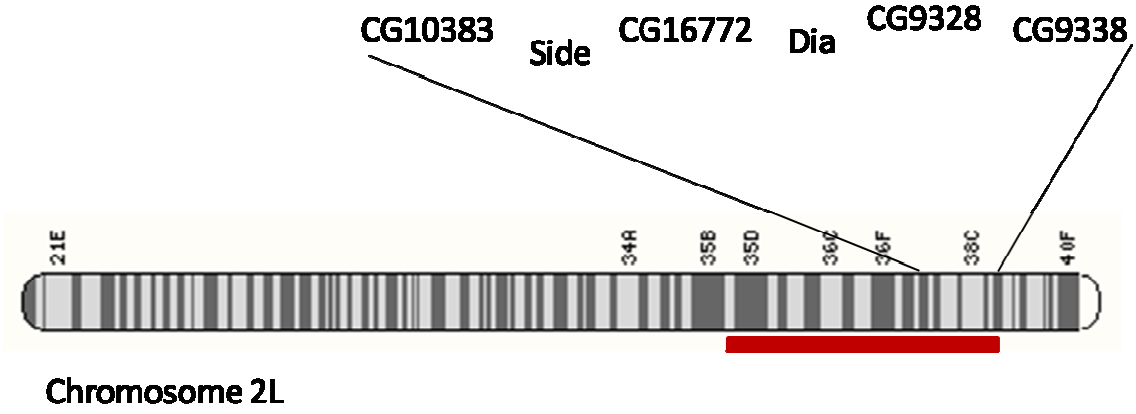


Figure 4.9: Position of candidate genes along the QTL region (35D-38C). The red shaded area denotes the QTL region along Chromosome 2L. Four genes were tested in complementation tests.

Table 4.7: P-values from ANOVA for candidate genes that showed failure to complement. Significance is indicated below by asterisks, *≤ 0.05, **≤0.01, ***≤0.0001. NS, not significant

Genotype	**Cytological Position**	**Total sleep**			**D/N ratio**			**Bout duration**		
		L	**G**	**LxG**	**L**	**G**	**LxG**	**L**	**G**	**LxG**
37C-38B										
Side	37B9-37B10	***	***	NS	***	***	NS	NS	***	NS
Diaphanous	38E7-38E8	***	NS	NS	***	***	NS	NS	NS	NS
CG9338	38F3-38F3	***	**	NS	NS	NS	NS	NS	NS	NS
CG9328	38E10-38F1	**	*	NS	***	***	0.02*	NS	NS	NS

4.3.4 P-element insertion complementation tests

Analysis of data from previous sleep microarray studies (Zimmerman *et al.* 2006; Cirelli, LaVaute & Tononi 2005) revealed 6 genes differentially expressed in fly heads during sleep, which were located in the QTL interval 35D;38C (Figure 4.9). These genes served as candidates and were tested by complementation tests using P-element insertion mutants. Four genes, to which insertion mutants were available (*dia*, *CG9328*, *Side* and *CG9338*) were tested (Table 4.2). The *CG9328* mutant failed to complement the natural alleles for sleep behaviour (Table 4.7, Figure 4.10). The gene product of *CG9328*, located at 38E10-38F1 has not been characterised yet, but in one of the sleep microarray studies (Cirelli, LaVaute & Tononi 2005), *CG9328* transcript levels in fly heads showed a daily cycle, with expression peaking during the night. It is important to note that *CG9328* failed to complement the allelic variation for D/N ratio, although this QTL was originally associated with total sleep and bout duration only (Table 4.4).

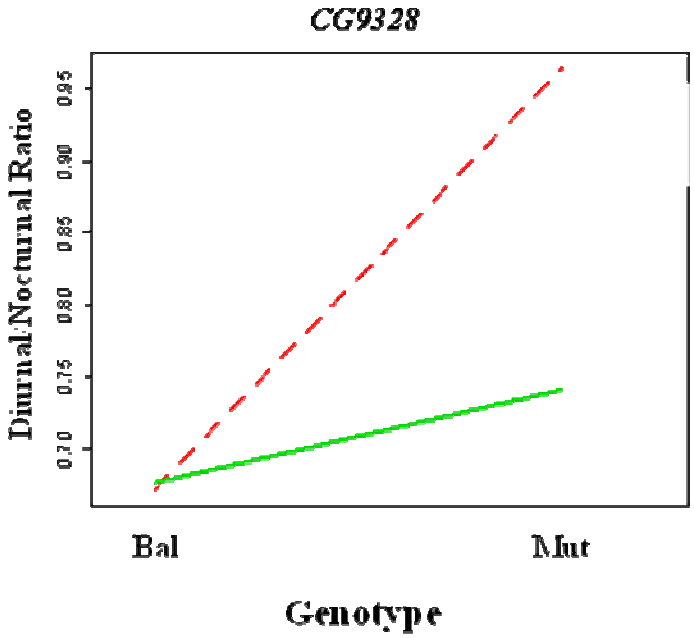


Figure 4.10: Interaction plot of *CG9328* for D/N ratio in a complementation test. Green and red lines represent high and low lines respectively.

4.4 Discussion

Here I have used QTL mapping and complementation tests to identify putative region and candidate genes affecting variation in sleep using a set of recombinant inbred lines derived from a wild-population. The analysis indicated two QTL for total sleep, two QTL for bout duration, and a single QTL for D/N ratio (Table 4.4, Figure 4.5). There is an extensive overlap between the genomic intervals of QTL1 and QTL3 for total sleep and for bout duration, suggesting that the same loci contribute to variation in both traits, which is consistent with the positive genetic correlation between these two traits (Figure 4.4).

Complementation tests revealed four deficiencies that failed to complement the natural alleles (Table 4.6). The fact that two of these deficiencies were in QTL1 suggest that this interval can be further fragmented to multiple QTL, a common observation in QTL mapping (e.g. Moehring and Mackay 2004), hinting that peaks in the LOD curve (Figure 4.5) underestimate the real number of sleep QTL.

Indeed, a recent association study in *Drosophila* using 40 RI strains (Harbison *et al.* 2009) identified large number of genes associated with sleep. The study was based on a large set of markers (SFP, single feature polymorphism, referring to probes of Affymetrix arrays that showed variable signal when hybridised with DNA), and variation in transcript levels (QTT, quantitative trait transcript). SFP in 134 genes were significantly associated with various sleep traits, as well as 1659 transcripts (QTT), although the latter may be indirectly driven by the sleep QTL. This suggested that sleep is driven by a large number of loci, possibly with small effect. The discrepancy between the large number of loci identified by Harbison *et al.* and the few QTL identified in the current study is probably due to the lower resolution of the markers used here. Interestingly, analysis of the genes located within the interval that the deficiency failed to complement (2L:37C-38B, Figure 4.7) revealed a single gene, *CG17549,* that was included in Harbison's SFP list of 157 genes associated with *night bout number*. Analysis of Harbison's larger list of QTT (1659 transcripts) revealed the gene *Akt1* located in QTL5 (3R: 87A-89B), and additional 7 genes (*CG10447*, *CG9987*, *CG10165*, *Rab9*, *CG10262*, *CG31797* and *CG16772*) that reside within the interval uncovered by the deficiency (2L:37C-38B).

Out of these genes, *Rab9* is particularly interesting (*Rab1* was also included in Harbison's 157 gene list). Rab proteins are part of the Ras superfamily of small GTP-binding proteins and are involved in various aspects of intracellular vesicular transport, including vesicle formation and fusion, and release of the vesicle content. Rab1 mediates transport between the endoplasmic reticulum and Golgi (reviewed by Sudhof 2004). Mackiewicz *et al*. 2007 studied changes in gene expression during spontaneous sleep and sleep deprivation in the mouse using microarray, and found sleep-specific up-regulation of 8 Rab genes, including *Rab1b.* Thus, *Rab9* is a strong candidate for QTL1, and since it shows sleep-associated variation in transcript level, it is likely that the causative sequence variation is in the upstream regulatory region of the gene.

My attempt to further narrow down the interval (37C-38B) was unsuccessful. None of the smaller deficiencies spanning this QTL region showed a failure to complement. It is possible, that this QTL consists of multiple small-effect loci (e.g. Cirelli and Bushey 2008), and when smaller regions within the QTL are tested in complementation test, their individual effect is too small to be detected. The interaction terms tested multiple times in quantitative complementation tests can also result in false positives. Multiple comparisons can be controlled by Bonferroni correction method to reduce the error rates. In addition, epistasis with loci on the deficiency and balancer chromosome cannot only produce spurious significant complementation tests (see Box 4.1 Service 2004), but can also cause false negative tests if the epistasis effect on the trait oppose the QTL effect, reducing the statistical power to detect it.

Also, variation in marker density is observed throughout the linkage map which could result in 'ghost' QTLs. In particular, QTL on chromosome 2 for daily sleep and bout duration have a significant high LOD score (Table 4.4, Figure 4.5), which could be an over-representation owing to the poor marker density on chromosome 2. Deficiency complementation tests performed on this region (37C-38B) also did not reveal any significant regions. Thus a better marker resolution on chromosome 2 is required, which would increase the QTL detection power.

The region 37C-38B was also interrogated by mutation complementation tests, targeting specific candidate genes. Two microarray studies (Zimmerman *et al.* 2006, Cirelli, LaVaute & Tononi 2005) identified genes showing differential expression during sleep and wakefulness. Mutants of four of these genes that mapped to this region have been tested by complementation tests (Table 4.7), and insertion in *CG9328* has failed to complement the natural alleles (Figure 4.10). Other 8 genes that showed differential expression during sleep (Zimmerman *et al.* 2006; Cirelli, LaVaute & Tononi 2005) were mapped to the QTL on chromosome 2L and 3R (including *DopR*) are yet to be tested.

Further studies on *CG9328*, would be an ideal way forward to confirm its role in sleep. Sequence analyses in the two parental lines would provide evidence that variation, if any in *CG9328*, contributes to the variation in sleep behaviour between these strains. The region 86D-87C on chromosome 3 also showed failure to complement the natural alleles and needs to be further tested with deficiency complementation tests.

Apart from complementation tests, another classical method to confirm the existence of QTL is to introgress the putative QTL into a homozygous background by backcrossing for multiple generations. This method has been applied to *Drosophila* bristle number studies (Lyman and Mackay 1998; Long *et al.* 2000) where existence of all QTLs affecting the trait were confirmed. However, the introgressed region has about 20cM linked genome to the naturally derived chromosome, therefore only confirming the existence of QTL but do not refine their map positions (Mackay 2001).

In most organisms, high-resolution recombination mapping is necessary in order to further reduce the size of a candidate region, which has been done to successfully identify a gene (*fw2*.2) involved in tomato fruit size (Frary *et al.* 2000). This technique can be time consuming and difficult since a large number of recombinants and a large number of molecular markers are needed in order to narrow down candidate regions to a manageable size. Markers are also needed for each interval tested, the smaller the refinement, the greater the number of markers. Thus, the use of recombinant isogenic lines makes the task of mapping more manageable since behavioural assays are only performed for informative recombinants and all flies of a single line are of the same genotype and therefore a particular genotype can be tested multiple times. However, in *Drosophila* the use of deficiency lines to narrow down the region and reduce the potential number of candidate genes is highly beneficial.

QTL mapping is a powerful tool for detecting regions/genes affecting variation in a trait, but is limited by the sample size used. Multiple QTL for sleep behaviour have been

identified but it could be possible that additional genes with a smaller effect may be present and by increasing the number of individuals tested per line, this could be identified. Also, only the genes within the mapped region with a mutant stock available can be tested, and thus it is possible that the additional genes in this region that may be contributing to the variation in sleep behaviour may go undetected.

Chapter 5: Dopaminergic control of sleep in *Drosophila*

5.1 Introduction

Neuromodulators such as dopamine (DA), serotonin, histamine, noradrenaline and orexin have shown to be important in a broad range of processes including the regulation of mammalian sleep/wake cycles (Boutrel and Koob 2004; Siegel 2004) (see Chapter 1). The effect of dopamine (and other wakefulness-promoting substances) is mediated by specific DA receptors (D1 and D2) (reviewed in Qu *et al.* 2010). However, evidence for the role of DA in sleep and wakefulness has only recently started to emerge (see below), suggesting in general that DA is a wake-promoting agent.

For example, in rats, the levels of extracellular DA are lower in the light phase (when typically asleep) than during the dark phase (Isaac and Berridge 2003; Feenstra, Botterblom & Mastenbroek 2000; Lena *et al.* 2005). Consistent with this observation, administering of D1 receptor agonists promotes wakefulness and reduces slow-wave and REM sleep (Isaac and Berridge 2003; Rye and Freeman 2008), while D1 antagonists promote sleep (Ongini *et al*. 1993; Rye and Freeman 2008). D2 receptors agonists exert more complex effects. A low dose of the D2 agonists enhances sleep, while higher doses enhance wakefulness (Python, de Saint Hilaire & Gaillard 1996; Lagos *et al.* 1998; Olive, Seidel & Edgar 1998). Another study in rats (Lu, Jhou & Saper 2006) showed that approximately half of the DA neurons in the ventral periaqueductal gray matter (vPAG)

expressed Fos protein during wakefulness or wakefulness induced by environmental stimulation, but none expressed Fos during sleep.

The primary mode of removal of DA is by re-uptake of DA into the presynaptic terminal via DA transporter (DAT). In mice, a DA transporter (DAT) knock-out (KO) strain exhibits reduced NREM sleep and increased consolidation of activity bouts during wakefulness (Wisor *et al.* 2001). Another study tested the role of D2 receptor, in D2(R) knock-out mice (Qu *et al.* 2010). The KO mice showed a significant decrease in wakefulness, with an increase in both NREM and REM sleep. These effects were also observed in wild-type animals in which the D2R antagonist raclopride was administered, demonstrating the specific role of D2R in maintenance of wakefulness (Qu *et al.* 2010).

Research in *Drosophila*, indicates that DA may play a similar important role in neurotransmission and behaviour in flies, as it does in vertebrates. For example, wakefulness promoting drugs such as cocaine and amphetamines that inhibit the re-uptake of dopamine, and increasing the concentration of dopamine in the brain, were tested in flies (McClung and Hirsh 1998; Andretic, van Swinderen & Greenspan 2005). Flies administered with cocaine exhibited hyperactive behaviour and reduced average resting time (McClung and Hirsh 1998). Substances such as methamphetamine (METH) have shown similar results of increased wakefulness in *Drosophila* (Andretic, van Swinderen & Greenspan 2005). Flies that were exposed to the inhibitory drug 3-iodo-tyrosine that inhibits the activity of the enzyme TH in dopamine synthesis (see Box 5.1, Figure 5.1) showed a significant increase in the amount of sleep (Andretic, van Swinderen & Greenspan 2005), consistent with the notion that wakefulness is positively correlated with

dopamine levels in the brain. This further corroborated that DA levels are correlated with wakefulness. Also, caffeine induced wakefulness involves both adenosinergic and dopaminergic systems (Andretic *et al.* 2008). Flies that were fed with caffeine showed that the wake promoting effect, required functional dopamine transporter as well as *Drosophila* D1 dopamine receptor (*dDA1*) (Andretic *et al.* 2008).

The recently isolated sleep mutant *fumin* (*fmn*) further indicated the role of the dopaminergic system (Kume *et al.* 2005). Flies with this mutation, which mapped to the DA transporter gene (DAT), exhibited high level of activity and a sleepless phenotype (Kume *et al.* 2005). Another study which tested 2977 mutant lines showed a similar short-sleeping phenotype in a few lines, and failure to complement *fumin* identified a novel allele of DAT (Wu *et al.* 2008). Normally, presynaptic DAT mediate the removal of DA from the synaptic cleft, suggesting that the mutant phenotypes are caused by the elevated levels of dopamine. This mirrors the situation in $DAT^{-/-}$ mice in which hyperactivity and shortening of the rest phase was observed (Jones *et al.* 1999). These observations reinforce the importance of monoaminergic, and specifically of dopaminergic, signalling in regulation of sleep.

5.1.1 Role of Dopamine N-acetylatransferase (Dat) *in sleep*

Interestingly, the gene *Dopamine acetyltransferase* (*Dat*) has been identified in a microarray study of genes differentially expressed in sleep and waking (Shaw *et al.* 2000). Transcript levels of *Dat* (whose product is involved in catabolism of DA) were up-regulated during waking as compared to sleep. This may parallel the finding in rats where

arylsulfotransferase, another enzyme implicated in catabolism of DA, shows a similar increase during waking (Cirelli and Tononi 1998). Further experiments were conducted on Dat^{lo} mutants where activity of the enzyme was deficient (Shaw *et al.* 2000). Flies homozygous for the mutation Dat^{lo} exhibited normal sleep, but a significantly magnified rebound following a 12-hr sleep deprivation. Shaw *et al.* 2000 speculated that catabolism of monoamines might be one of the functions of sleep, counteracting the effects of high DA (and other monoamines) released during waking.

Little is known about the expression of *Dat* in the fly brain (Brodbeck *et al.* 1998). Using the UAS-GAL4 system (see section 2.3), *Dat* expressing neurons, which might be involved in sleep regulation have been identified. Briefly, the upstream sequence of the gene, presumably harbouring the *Dat* promoter, was fused to a GAL4 sequence (a yeast protein). The *Dat*-GAL4 transgene would drive the expression of GAL4 in a transcriptional pattern that mirrors the expression of *Dat* (or the specific splice-form controlled by that promoter-see below). I used the *Dat*-GAL4 transgene to drive the expression of UAS-RFP to identify DA neurons, and (UAS-*hid*,UAS-*rpr*) to ablate these neurons and test their role in sleep.

Box 5.1

Biogenic amines

Biogenic amines are metabolic products of amino acids, and are found in numerous tissues of vertebrates and invertebrates species. In particular, they are present in the central nervous system (CNS) in distinct populations of neurons where they control neural activity. Dopamine (DA) is one of the major amines present in the CNS along with adrenaline and noradrenaline. It modulates various behaviours including movement, cognition, motivation, and sleep-wake behaviour (Durstewitz, Kroner & Gunturkun 1999). DA belongs to the family of catecholamine's that acts primarily as a neurotransmitter, and is synthesized in a common pathway along with other neurotransmitters like norepinephrine and epinephrine (Figure 5.1).

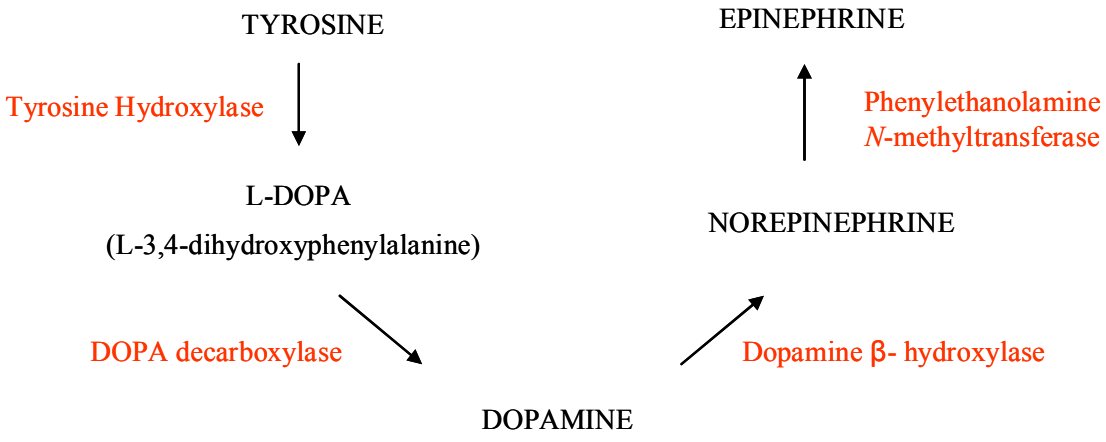


Figure 5.1: Biogenic amine synthesis pathway. Dopamine is synthesized from the amino acid tyrosine. Tyrosine is then modified by tyrosine hydroxylase (TH) to form DOPA. DOPA decarboxylase then removes a carbon dioxide to from dopamine, which in turn serves as a precursor to form norepinephrine and epinephrine.

Box 5.1 continued

Dopamine N-acetyltransferase (*Dat*)

Dopamine N-acetyltransferase (*Dat*) gene encodes for an enzyme from a family of enzymes, the arylalkylamine N-acetyl transferase (aaNAT) which performs diverse physiological functions, including breakdown of monoamines, sclerotization and production of melatonin (Brodbeck *et al.* 1998; Hintermann, Jeno & Meyer 1995). The *Drosophila* orthologue, although showing weak homology to vertebrates' aaNAT, acetylates tryptamine, serotonin, and dopamine with the same affinity, and is probably involved in catabolism of all these neurotransmitters (Hintermann, Jeno & Meyer 1995).

The cloning of *Dat* has revealed two isoforms that are the product of alternative splicing (Brodbeck *et al.* 1998). The two isoforms differ only in their 5' end (Figure 5.2). The two protein isoforms are differentially expressed during development. DAT B protein (240 aa) is detected from 8 h up to the adult stages at relatively constant levels, while DAT A (275 aa) protein is expressed during the late pupal stages and in adults (Brodbeck *et al.* 1998) In the Dat^{lo} mutant, which has been isolated by Maranda & Hodgetts (1977), the expression of *Dat* B (but not the other isoform) is abolished. The mutation arises due to an insertion of mobile genetic elements downstream of exon 1, leading to a premature termination of transcription within the mobile element.

Box 5.1 continued

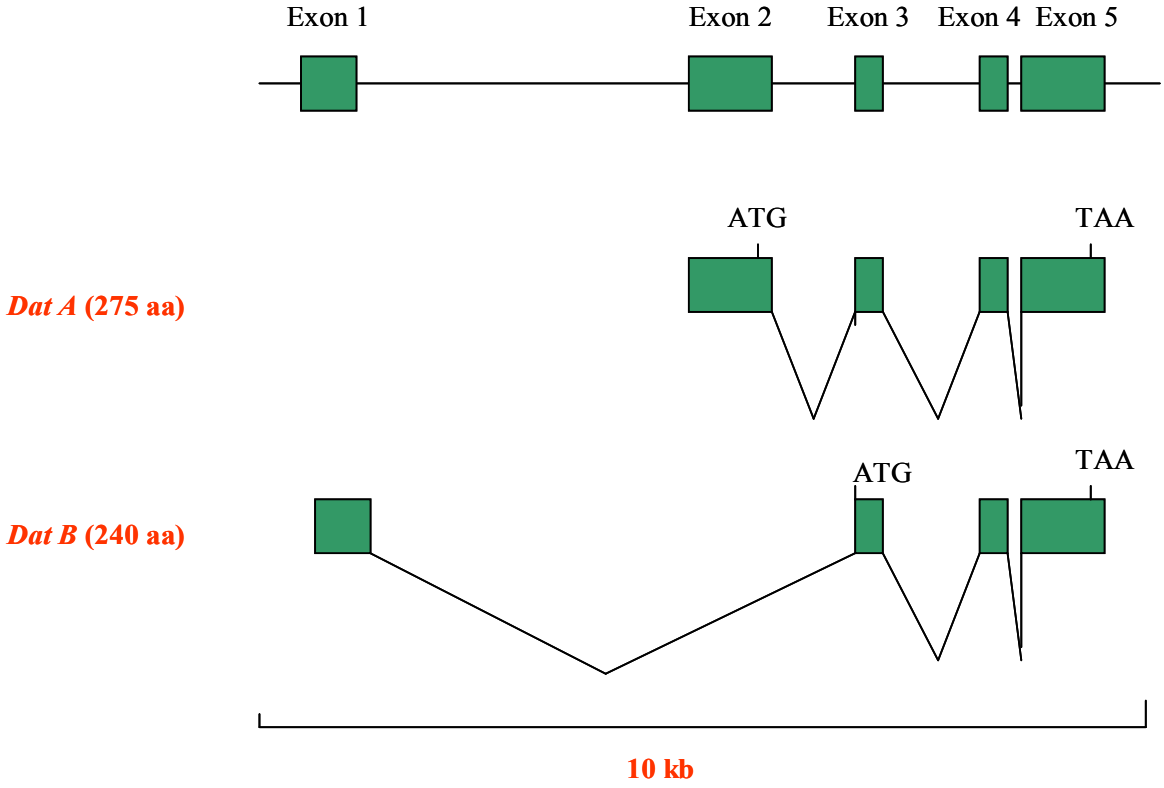


Figure 5.2: Genomic organization of *Dat* . The gene consists of five exons that span 10kb giving rise to two gene products, *Dat A* (isoform A) and *Dat B* (isoform B). The two isoforms vary in their respective first exons. Exon 1 contains no ATG followed by an open reading frame (ORF), so it was suggested that the translation of *Dat B* occurs utilizing an inframe methionine codon 105bp downstream of the ATG of *Dat A* (Brodbeck *et al.* 1998).

5.2 Materials and Methods

5.2.1 Fly stocks and maintenance

The strain w^{1118} was used for generating the transgenic lines. UAS-RFP (Bloomington stock no. 8547) was used for visualization of neurons, and UAS-*head-involution defective* (UAS-*hid*) and UAS-*reaper* (UAS-*rpr*) (Zhou *et al.* 1997) responder line was used for ablation of *Dat* neurons. Flies were maintained in vials on standard food (see section 2.2) under constant conditions of 25°C and Light:Dark 12:12 cycle during the experiments.

5.2.2 Cloning strategy

The complete intergenic region (1.9kb) between *Dat* and *CG4065* (co-ordinates: 2R: 20025108, 20027327), encompassing the putative *Dat* promoter was amplified by PCR (Roche long template PCR system with thermostable Taq polymerase with proofreading activity) using genomic DNA (from Canton-S wild-type strain) as a template. The primers *Dat*GAL4 5' (forward) and *Dat*GAL4 3' (reverse) (Table 5.1) were used for PCR. The amplification was verified by agarose gel electrophoresis (Figure 5.4). The integrity of the insert was also verified by sequencing, and the alignment to the database sequence can be found in appendix 3.

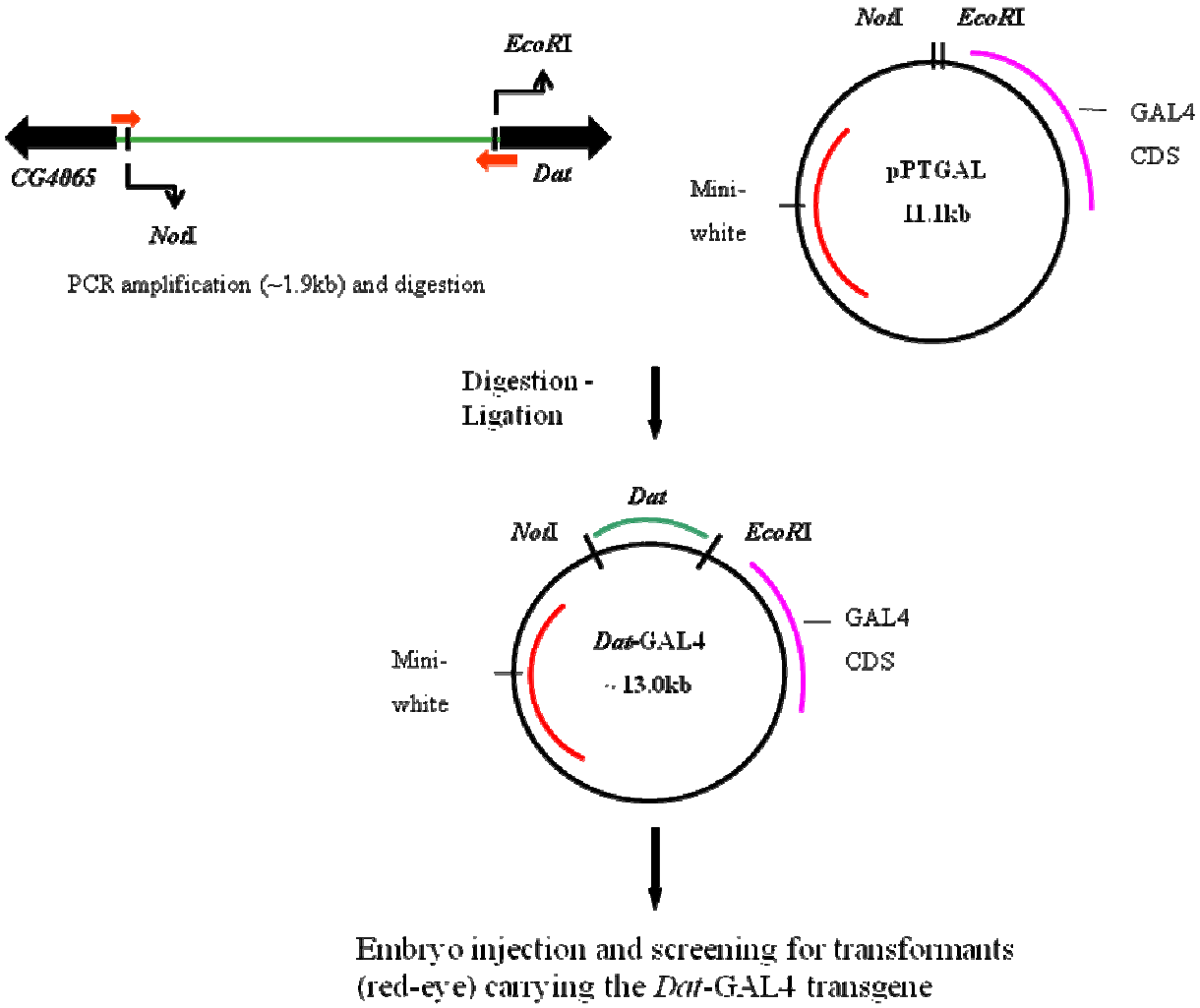


Figure 5.3: Cloning strategy to produce the *Dat*-GAL4 construct. The 1.92 kb fragment was amplified (primers are shown in red), digested by *Eco*RI and *Not*I, and cloned into the pPTGAL vector, which was digested with the same enzymes. The re-constructed plasmid (~13kb) was injected into w^{1118} embryos, which were subsequently screened for transformants (red eyes).

Table 5.1: List of primers.

Primer Name	Primer Sequence
*Dat*GAL4 5’	CACTCGTCCTGTTCCAGCT
*Dat*GAL4 3’	AGAATCTTATATGTACTCGAATGCTA
*Dat*GAL4_NotI 5’	GTTGCTCACCTTCCGTTCAT
*Dat*GAL4_NotI 3’	TCGTCACTTGGTCACACTGG
*Dat*GAL4_EcoRI 5’	GCAGAGAGATTGGGATTGGA
*Dat*GAL4_EcoRI 3’	GAATTCTTCTCGCGATTTGG

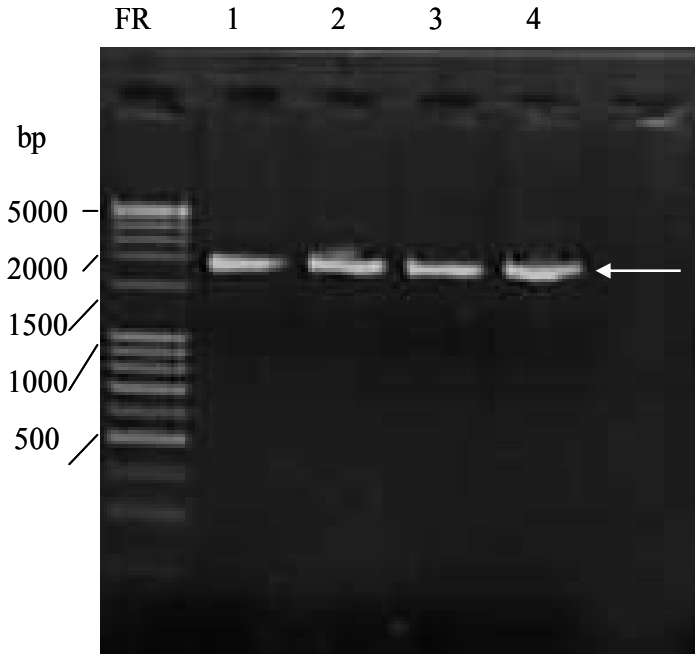


Figure 5.4: PCR amplification of the *Dat* upstream fragment. Gel electrophoresis of the PCR products (4 replicates, lanes 1-4) reveals a single band of 1.9 kb (see arrow), indicating a successful amplification. Full ranger (FR) marker containing DNA fragments of known sizes (base pairs) is seen on the left.

The amplified region was digested with *Not*I and *EcoR*I (20 units of each) in a 20 µl reaction (see section 2.5) targeting two natural restriction sites at 2R:20025250 and 20027147 respectively. The fragment was purified and cloned into the vector plasmid pPTGAL (Sharma *et al.* 2002) using the same restriction sites. This vector allows us to test and to visualize the promoter activity by using the binary reporter system GAL4/UAS (Brand and Perrimon 1993) (Figure 5.3). The plasmid contains the gene encoding the GAL4 transcription factor and a reporter gene, in this case, the gene *white* that encodes red eyes in *Drosophila melanogaster*. The sequences for pPTGAL and the *Dat* region can be found in appendix 3.

The integrity of the construct was confirmed by both restriction digestion and by PCR. Figure 5.5 shows the agarose gel image of the restriction digestion of the *Dat*-GAL4 construct with *EcoR*I and *Not*I. Two bands of DNA fragments were obtained, one corresponding to the vector (11 kb) and the other to the insert (~ 2kb). Figure 5.6 shows the agarose gel image of the two PCRs that were set up spanning the *EcoR*I and the *Not*I sites. DNA fragments of appropriate size were obtained confirming the integrity of *Dat*-GAL4 construct.

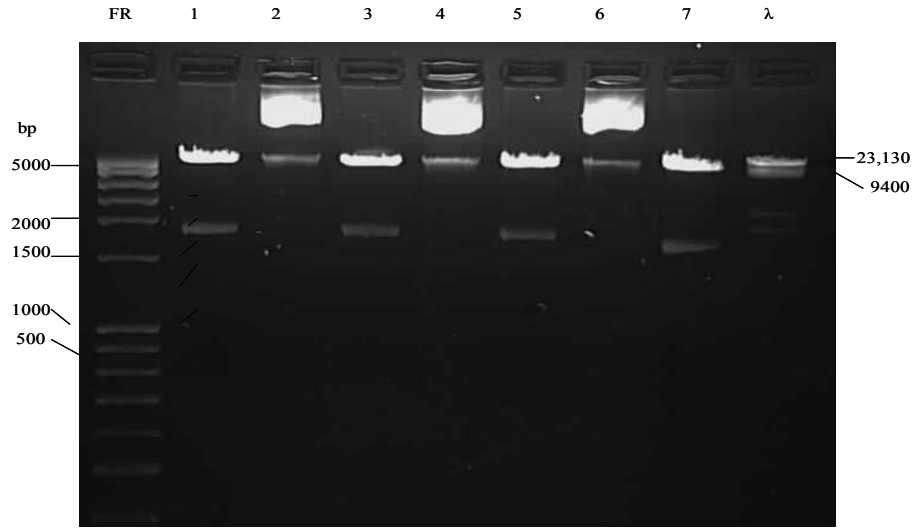


Figure 5.5: Gel image of the restriction digestion of *Dat*-GAL4 construct. Full ranger and λ markers containing DNA fragments of known sizes (bp) are seen on extreme left and right respectively. Lanes 2, 4 and 6 contains the uncut control plasmid while lanes 1, 3, 5, and 7 contain the restriction digestion products. The vector is seen as a band 11.1 kb and the insert as ~2 kb.

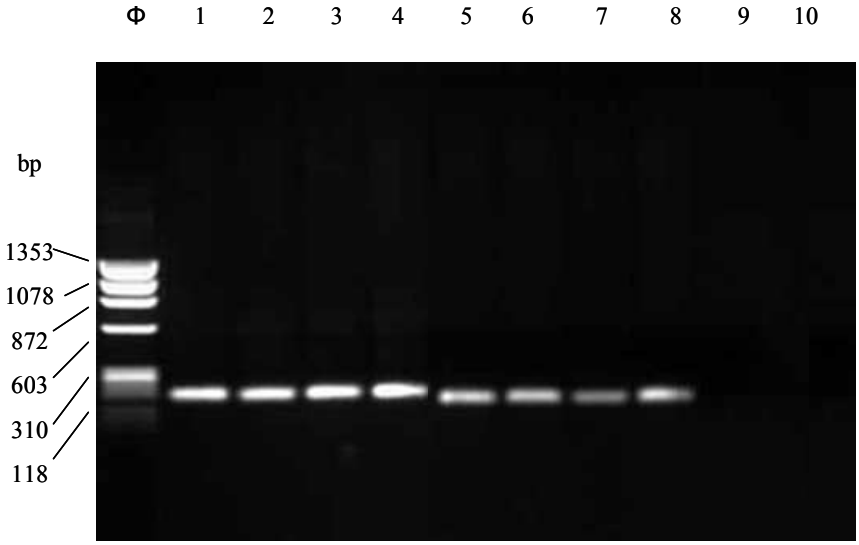


Figure 5.6: PCR amplification of the *Dat*-GAL4 construct. Φ marker containing DNA fragments of known sizes (bp) is seen on extreme left. Lanes 1- 4 contain DNA fragments (185bp) showing successful amplification spanning *Not*I restriction site and lanes 5- 8 contain DNA fragments (153bp) showing successful amplification spanning the *EcoR*I site. Lanes 9 and 10 show negative controls for both PCRs respectively.

Germ-line transformation of w^{1118} was carried using standard techniques (BestGene Inc, Chino Hills, CA). Five independent transgenic lines were recovered and the insertions were mapped by crossing to a double balancer stock (*w;CyO/Sco;MKRS/TM6B)*

5.2.3 Confirmation of Dat-GAL4 construct and transgenic lines

A PCR amplification strategy was designed to verify the *Dat*-GAL4 transgene in the transformant lines. DNA was extracted (see section 2.4.1) using a single fly from each transgenic line. Two PCR were performed using primers spanning the *Not*I and *Eco*RI sites of the construct: *Dat*GAL4_NotI 5' (forward) and *Dat*GAL4_NotI 3' (reverse), *Dat*GAL4_EcoRI 5' (forward) and *Dat*GAL4_EcoRI 3' (reverse) respectively (Table 5.1).

5.2.4 Dat-GAL4 expression in brains

To determine the neural expression, *DatGAL4* lines were crossed with UAS-*RFP*. Four-day old flies from the resulting progeny were fixed overnight at 4°C in 4% paraformaldehyde. Flies were washed in 1XPBS. Brains were dissected in PBS and mounted in 1XPBS and imaged by confocal microscopy. Fluorophore (RFP) was excited at 488-nm argon laser and emissions were collected at 583 nm (see section 2.6 in Chapter 2).

5.2.5 Sleep analysis

Each of the transgenic lines was crossed with UAS-*hid,*UAS-*rpr*;+;+ to measure effect of ablation of *Dat* expressing neurons on sleep patterns. UAS-*hid,*UAS-*rpr* virgin females were crossed with *Dat*-GAL4 or w^{1118} males in vials, and the sleep patterns of the progeny was assessed. Three-four day old flies were used to monitor their sleep/wake cycle using Drosophila Activity Monitoring System (Trikinetics) at 25°C in 12:12 L:D

conditions for a total of four days. Data was collected in 5-min bins and analysed using R (http://www.R-project.org) and BeFly!. For statistical tests R statistical software tool was used. Different parameters of sleep were analysed: (i) average daily sleep (ii) average day time sleep (iii) average night time sleep (iii) sleep bout duration (iv) number of sleep bouts. Males and females from each transgenic line were analysed separately since sleep is sexually dimorphic (see section 1.4.2). A comparison between the *Dat*-GAL4>UAS-*hid,*UAS-*rpr* and the background controls (flies carrying a single transgene) was carried using Analysis of Variance (ANOVA). Tests in which the null hypothesis was rejected were followed by Tukey post-hoc test. The effect of the ablation on sleep was declared real, when the difference from each of the controls was significant.

5.3 Results

5.3.1 Confirmation of DatGAL4 construct in transgenic lines

Five transformant lines carrying independent insertion of *Dat*-GAL4 were isolated, and the presence of the transgene was verified by PCR (Figure 5.7). Four insertions (1M-3M, 5M) were mapped to the third chromosome and one (4M) to the X chromosome. In order to confirm the insertion of *Dat*-GAL4 construct in the transgenic lines, the same PCR strategy was performed across the *Not*I and *EcoR*I sites of the construct. Figure 5.7 shows the gel image of the PCR amplification of the five transgenic lines. DNA fragments of the appropriate size were obtained, confirming the presence of the *Dat*-GAL4 construct in the transgenic lines obtained.

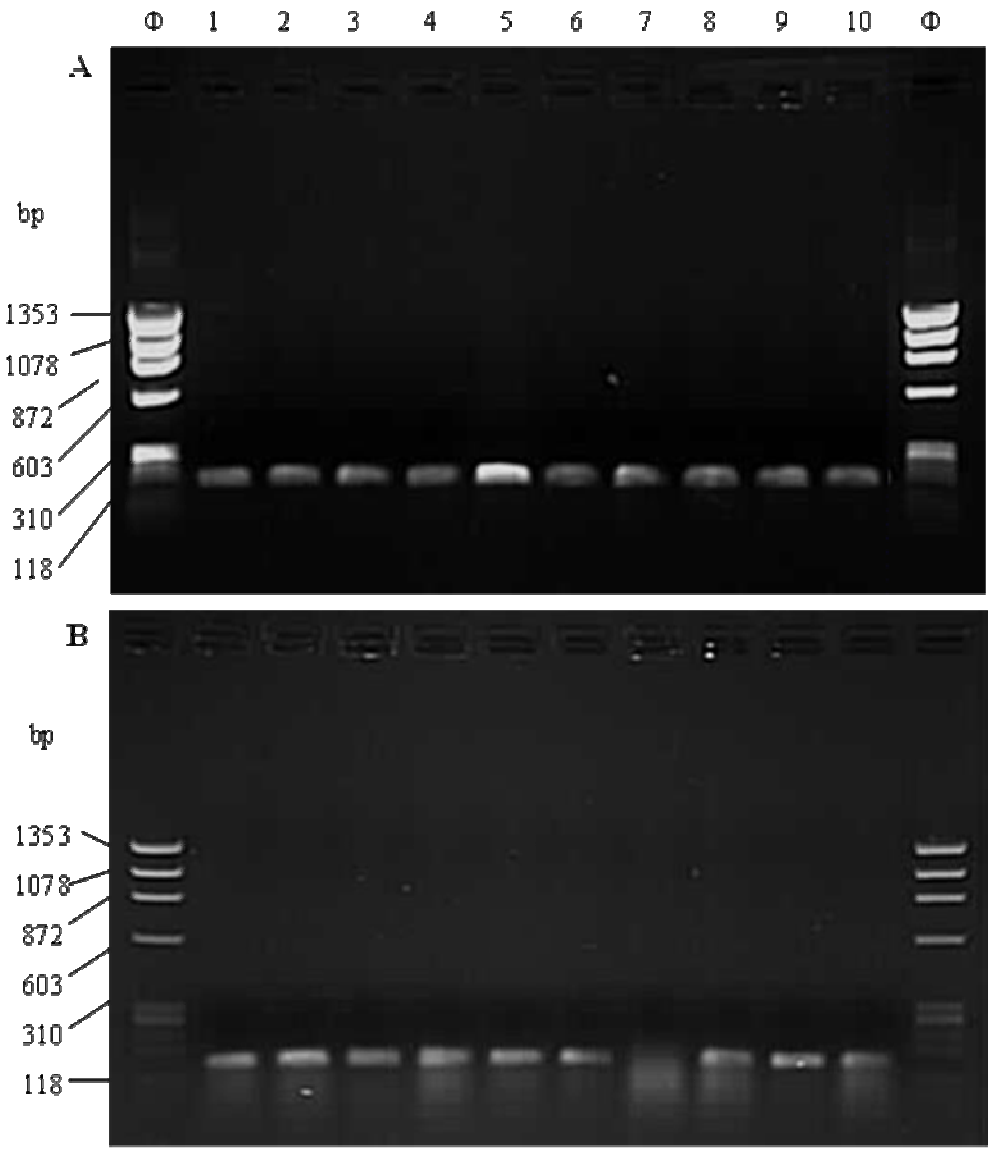


Figure 5.7: *Dat*-GAL4 is present in all transgenic flies. **A.** PCR amplification of DNA extracted from single transgenic flies using the primers *Dat*GAL4_NotI 5'and DatGAL4_NotI 3' (see Methods), flanking the *Not*I sites. The DNA ladder (Φx) is shown on both sides. Lanes 1-2: 1M, 3-4:2M, 5-6:3M, 7-8:4M, 9-10:5M. The expected fragments size (153bp) is present in all lanes. **B**. Same as the upper panel, showing the PCR using the *Dat*GAL4_EcoRI 5' and *Dat*GAL4_EcoRI 3' primers, spanning the *Eco*RI site.

5.3.2 Analysis of Dat expressing neurons

5.3.2.1 *Where are dopaminergic neurons located?*

To identify *Dat* expressing neurons, the reporter construct UAS-RFP was driven with each of the *Dat*-GAL4 drivers, which resulted in a robust fluorescent signal in the brain (Figure 5.8), although there is substantial variation between the insertion, and even between samples from the same insertion. One of the insertions (2M) showed a strong non-specific signal from the mushroom bodies and was therefore removed from further analysis. Dopaminergic neurons in the brain have been previously identified in *Drosophila* and clusters of these neurons are listed in Table 5.2 (Nassel and Elekes 1992). I have adopted this nomenclature, which has been used in other studies as well (e.g. (Drobysheva *et al.* 2008)).

Table 5.2: Nomenclature of dopaminergic neurons (Nassel and Elekes 1992)

Neuron clusters	Nomenclature (Nassel and Elekes 1992)
PAL	Dorsolateral anterior protocerebral
PPL1	Dorsolateral posterior protocerebral
PPL2	Lateral posterior protocerebral
PPM1	Dorsomedial posterior protocerebral
PPM2, PPM3	Dorsomedial posterior protocerebral
SE	Subesophageal ganglion
VUM	Ventral unpaired medial neurons

Figure 5.8 shows the expression of RFP in the fly brains driven by different GAL4 driver lines. In the transformant 1M, (Figure 5.8A), three clusters of neurons can be seen that may be associated with PPM1, PPL1, and PPM2/3 clusters (Figure 5.8F). The cluster corresponding to PPM1 is also present in another brain from the same transgenic line (see Figure 5.8B), with an additional cluster in the subesophageal ganglion region. In the brain sample driven by 3M, possible *Dat* neurons might be of the PPL2 and SE clusters (see arrow Figure 5.8C), while in the other sample from the same line, clusters of PPM1, PPL2 and SE/VUM may also be seen (Figure 5.8D). In the transgenic line 4M, expression of RFP is seen in the neurons that may be part of the PPM2 cluster (Figure 5.8E), while in the other brain from the same cross (Figure 5.8G) a small number of neurons (see arrow) above the gut and in the subesophageal region are labelled. In the transgenic line 5M, fluorescence is seen from neurons that may be a part of the PPL2 and PPM2 clusters (Figure 5.8 H-I).

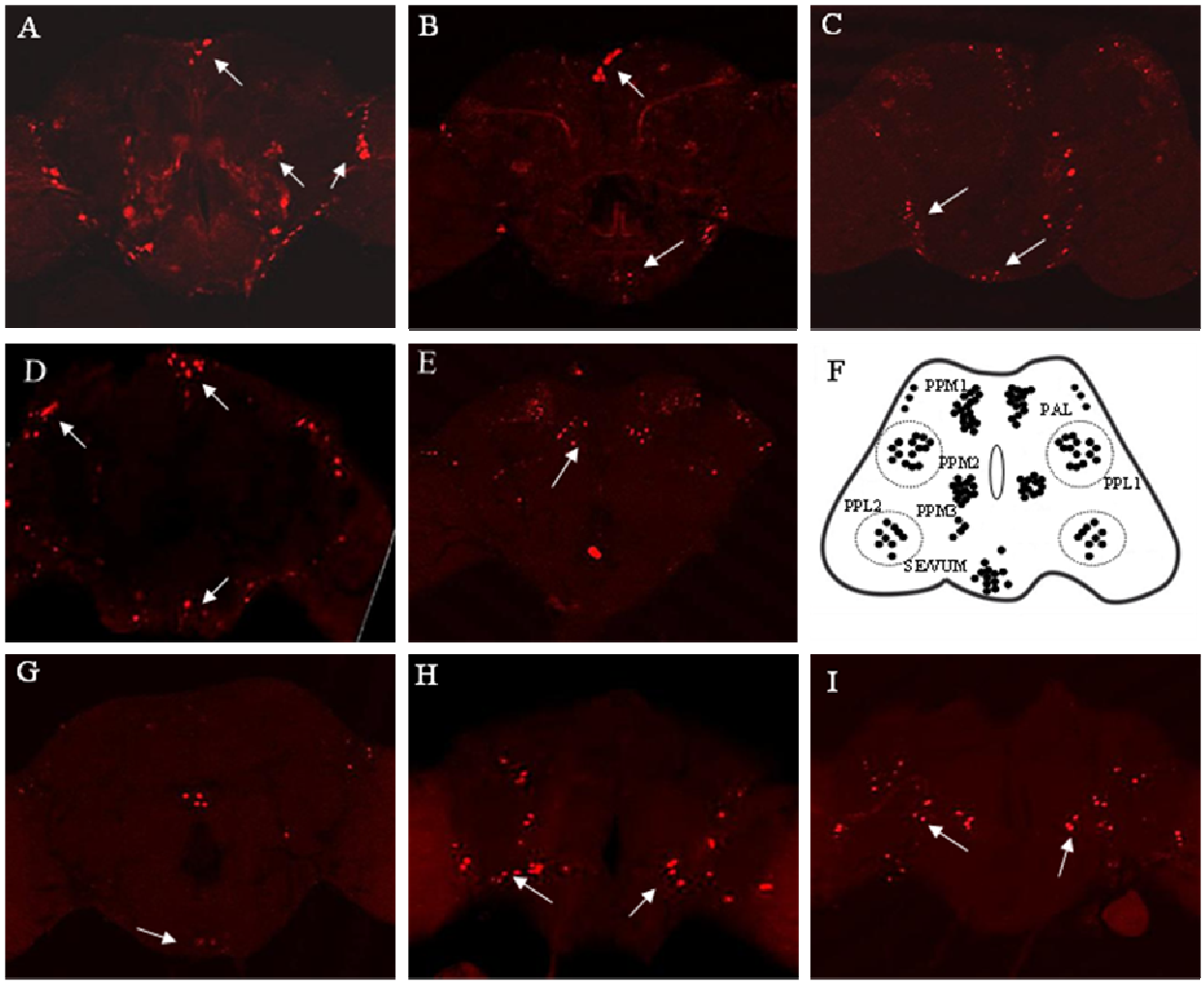


Figure 5.8: Confocal images showing RFP fluorescence in adult brain. Two independent examples are shown for each insertion: 1M (A-B), 3M (C-D), 4M (E-G) and 5M (H-I). The localization of previously identified clusters of DA neurons (identified using tyrosine hydroxylase (TH)[8] antibodies) is illustrated in (F) (Drobysheva *et al.* 2008). See Table (Table 5.2) for cluster names.

[8] TH catalyses the synthesis of DOPA, the precursor of DA.

5.4.2.2 Ablation of Dat neurons

The ablation of *Dat* neurons in *Dat*-GAL4>UAS-*hid,*UAS-*rpr* was verified by crossing these lines with the UAS-RFP strain, and analysing their brains by confocal imaging (transformant 4M was excluded from subsequent analysis because the insertion was inconveniently inserted in the X Chr.). No fluorescence was present in the brains that were analysed from the transformant lines (1M, 3M and 5M) (Figure 5.9), suggesting a complete elimination of *Dat* expressing neurons. Expression was examined using the same confocal settings in about 10 brains per genotype, in two independent experiments.

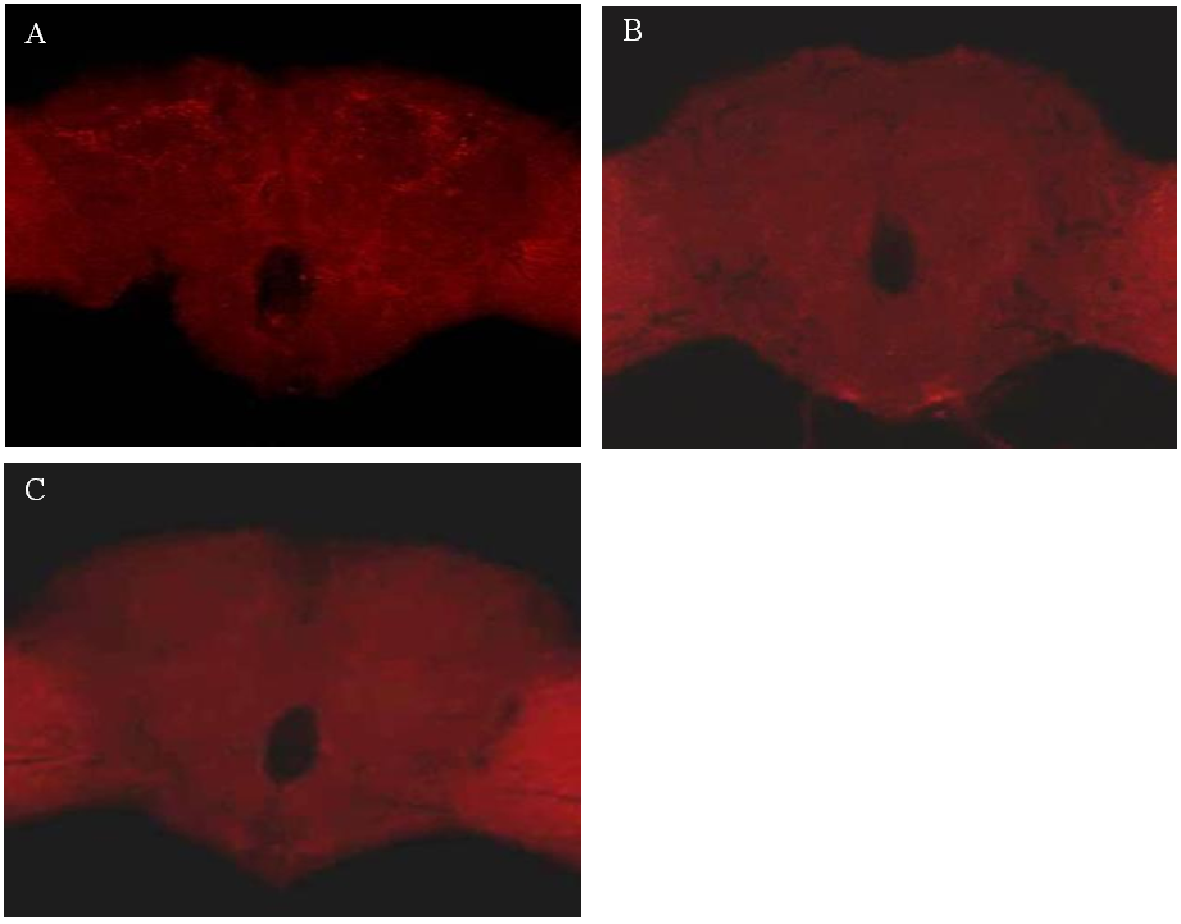


Figure 5.9: Genetic ablation of *Dat* neurons. (A-C)Three representative examples using the transformant lines 1M, 3M and 5M respectively are shown.

5.3.2.2 *Does ablating Dat expressing neurons have any effect on sleep?*

To determine the effect of ablating *Dat* neurons on sleep, the transgenic lines were crossed to UAS-*hid,*UAS-*rpr*;+;+, and the F1 progeny were assessed for their sleep patterns. Of all the transgenic lines tested (1M, 3M and 5M) , sleep was slightly, but consistently reduced, particularly during midday in male flies (Figure 5.10 B,D,F) as compared to the background controls. Statistical analysis (ANOVA) (Table 5.3) showed a significant difference between *Dat*-GAL4>UAS-*hid*,UAS-*rpr* and the controls. In contrast, the female data did not show the same trend (Figure 5.10 A,C,E).

Table 5.3: Statistical overview (ANOVA) of transformant lines with their background controls for midday siesta sleep (males only). P-value *≤0.05, **<0.01, ***<0.001

Males	Midday siesta sleep
1M	$F_{(2,63)} = 13.01^{***}$
3M	$F_{(2,56)} = 7.25^{**}$
5M	$F_{(2,62)} = 18.96^{***}$

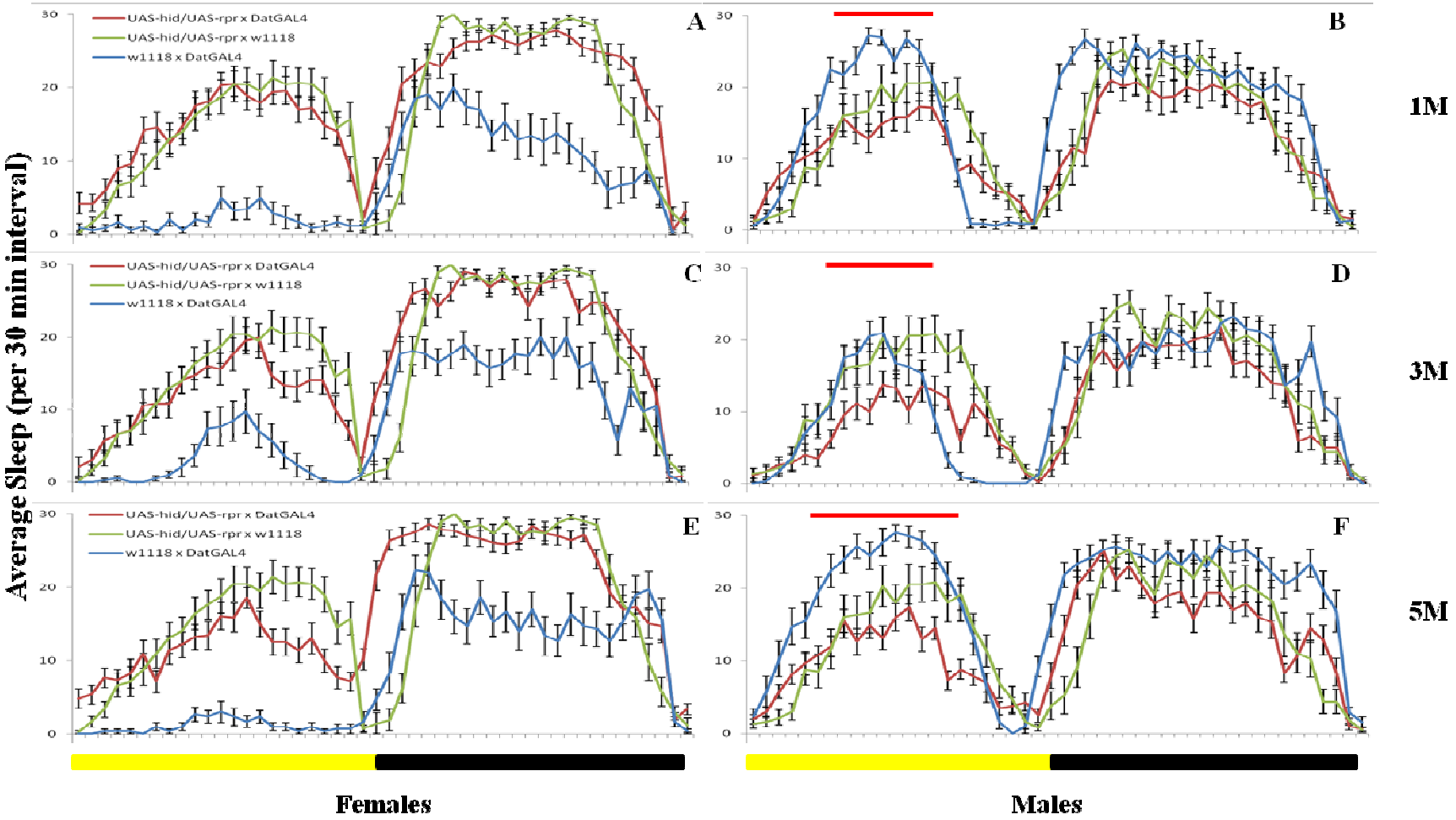


Figure 5.10: Sleep profile in 30 min intervals (mean ± S.E.M) over a day. The left panel shows data for female, while the right panel shows data for male flies. The bar below the X-axis indicates 12-hour light (yellow) and 12-hour dark (black) periods. The sleep profile of UAS-*hid*, UAS-*rpr*/*Dat*-GAL4 (red) versus background controls (green and blue) is shown (n= 56 – 63). (A-B) shows the profile of 1M, (C-D) and (E-F) shows profiles of 3M and 5M lines respectively. The red bar denotes the midday siesta in males. Error bars representS.E.M.

Further analysis of other sleep parameters was performed (see section 5.2.5). A comparison between the background controls and the *Dat*-GAL4>UAS-*hid*,UAS-*rpr* genotype using Analysis of Variance (ANOVA), revealed a significant variation in all the lines tested (see Table 5.4). Tukey's *post-hoc* tests were followed comparing the treatment to control genotypes, and no significant difference in average daily sleep between the genotypes (Figure 5.11-13A) was observed. However, there was a significant increase in average bout number in the GAL4/UAS flies (both males and females) in all three GAL4 drivers (Figure 5.11-13B). Intriguingly, this was also accompanied by a significant decrease in bout duration in males (but not in females), in all three GAL4 drivers (Figure 5.11-13C). Overall, these results suggest that ablating *Dat* expressing neurons leads to a fragmented sleep pattern which may be manifested as hyperactivity.

Table 5.4: Statistical overview (ANOVA) of transformant lines with their background controls for different parameters of sleep. P-value *$\leq$0.05, **<0.01, ***<0.001

Males	Daily sleep	No. of sleep bouts	Sleep bout duration
1M	$F_{(2,63)}= 3.9^{*}$	$F_{(2,63)} = 8.3^{***}$	$F_{(2,63)} = 6.33^{**}$
3M	$F_{(2,56)} = 6.11^{**}$	$F_{(2,56)} =16.69^{***}$	$F_{(2,56)} =15.19^{***}$
5M	$F_{(2,62)} = 12.95^{***}$	$F_{(2,62)} = 20.59^{***}$	$F_{(2,62)} = 12.01^{***}$
Females			
1M	$F_{(2,62)} = 40.4^{***}$	$F_{(2,62)} = 10.3^{***}$	$F_{(2,62)} = 12.0^{***}$
3M	$F_{(2,60)} = 39.8^{***}$	$F_{(2,60)} = 14.36^{***}$	$F_{(2,60)} = 4.4^{**}$
5M	$F_{(2,61)} = 38.3^{***}$	$F_{(2,61)} = 5.22^{**}$	$F_{(2,61)} = 9.2^{***}$

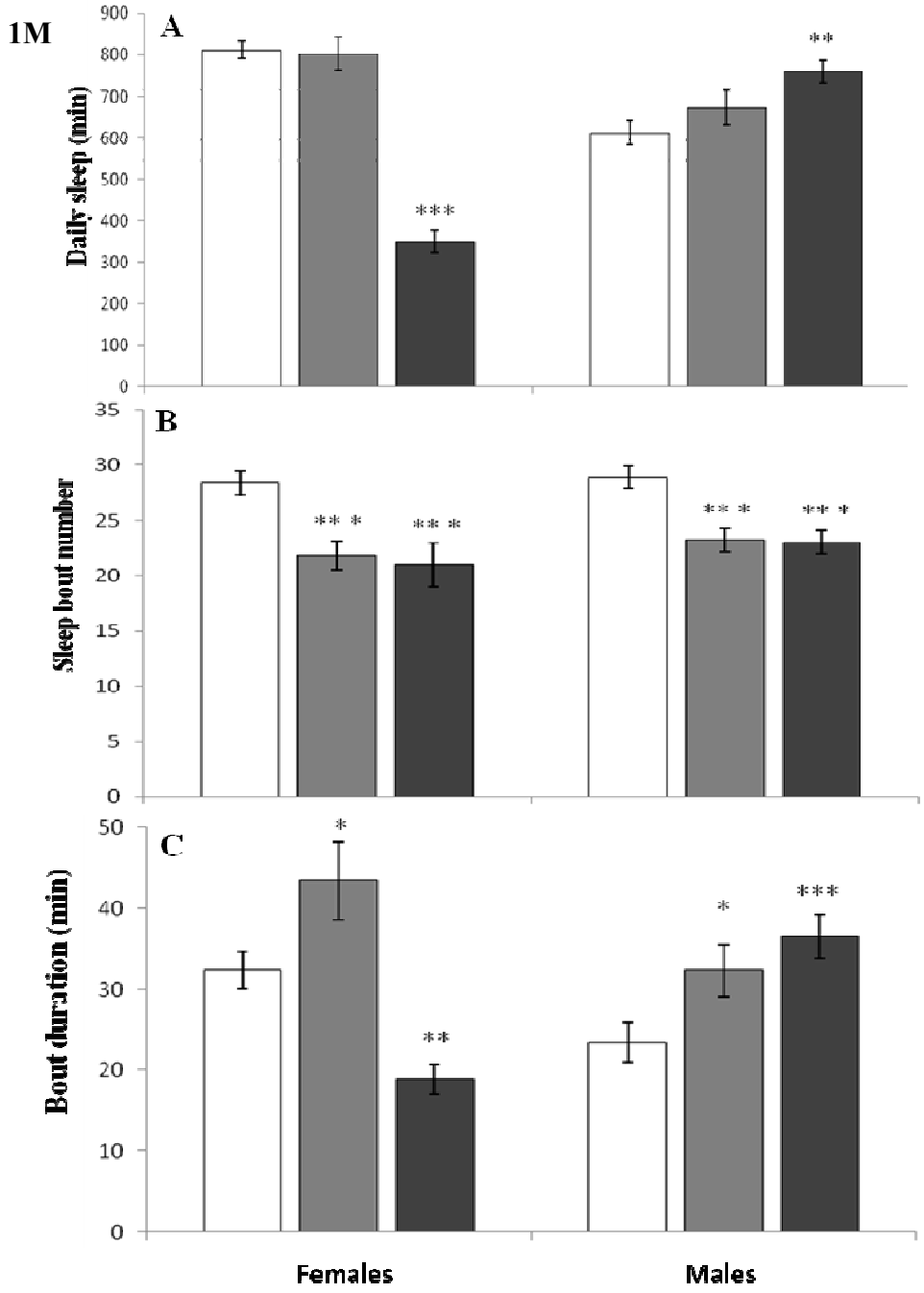


Figure 5.11: Sleep phenotype of transformant flies (1M) with ablated *Dat* neurons. Average daily sleep amount (A) with 12:12 LD conditions. Sleep intensity (B-C): average sleep bout length (B) and average number of sleep bouts (C). White bars, GAL4/UAS-*hid*,UAS-*rpr*; grey bars, UAS/+; black bars, GAL4/+. Males and females as indicated at the bottom. Asterisks, GAL4/UAS combination significantly different from GAL4/+ and UAS/+ controls (Tukey post hoc, *P < 0.05, **P < 0.01, ***P< 0.001). Error bars indicate SEM.

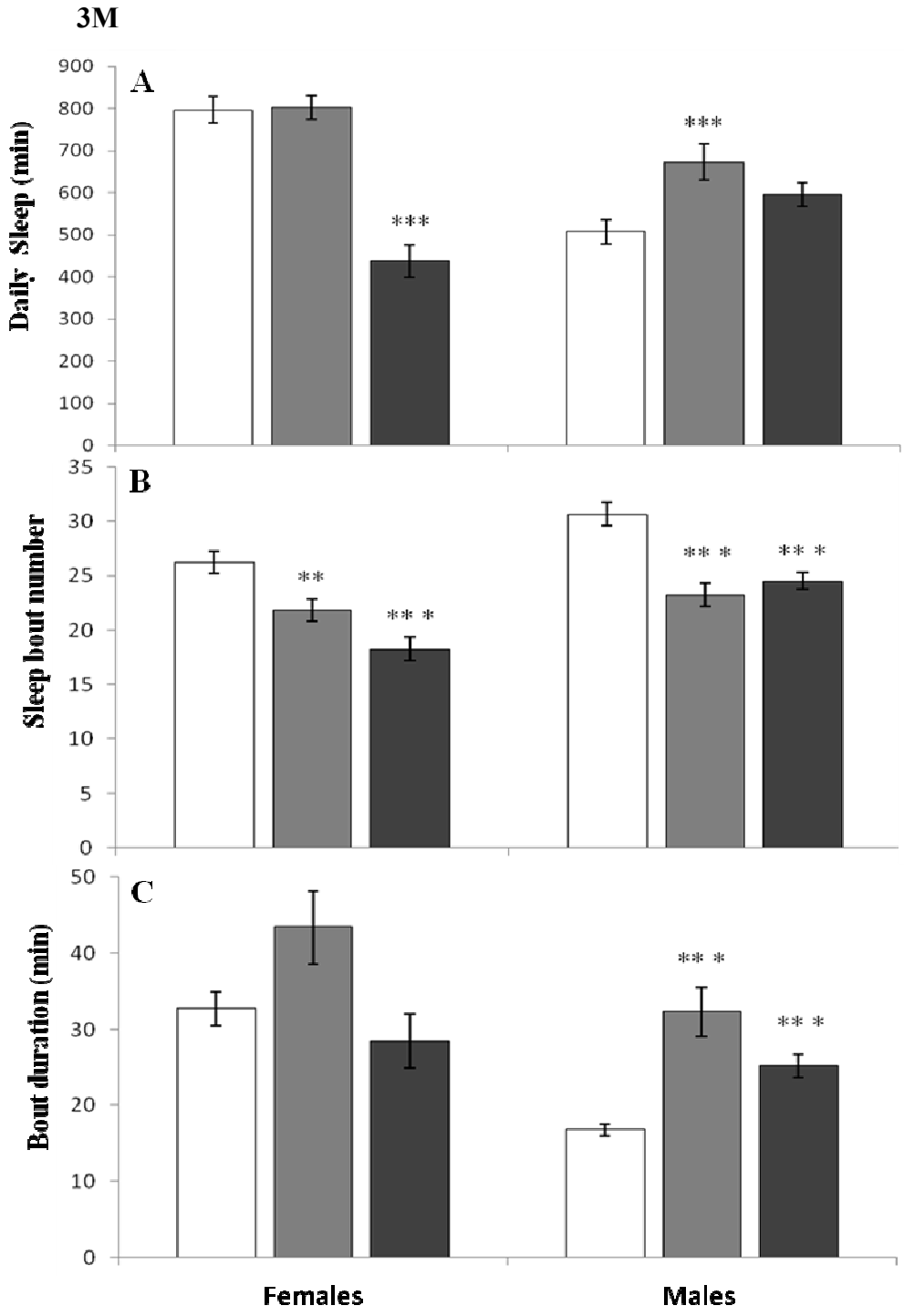


Figure 5.12: Sleep phenotype of transformant flies (3M) with ablated *Dat* neurons. (Caption same as Figure 5.11, Tukey post hoc, *P < 0.05, **P < 0.01, ***P< 0.001).

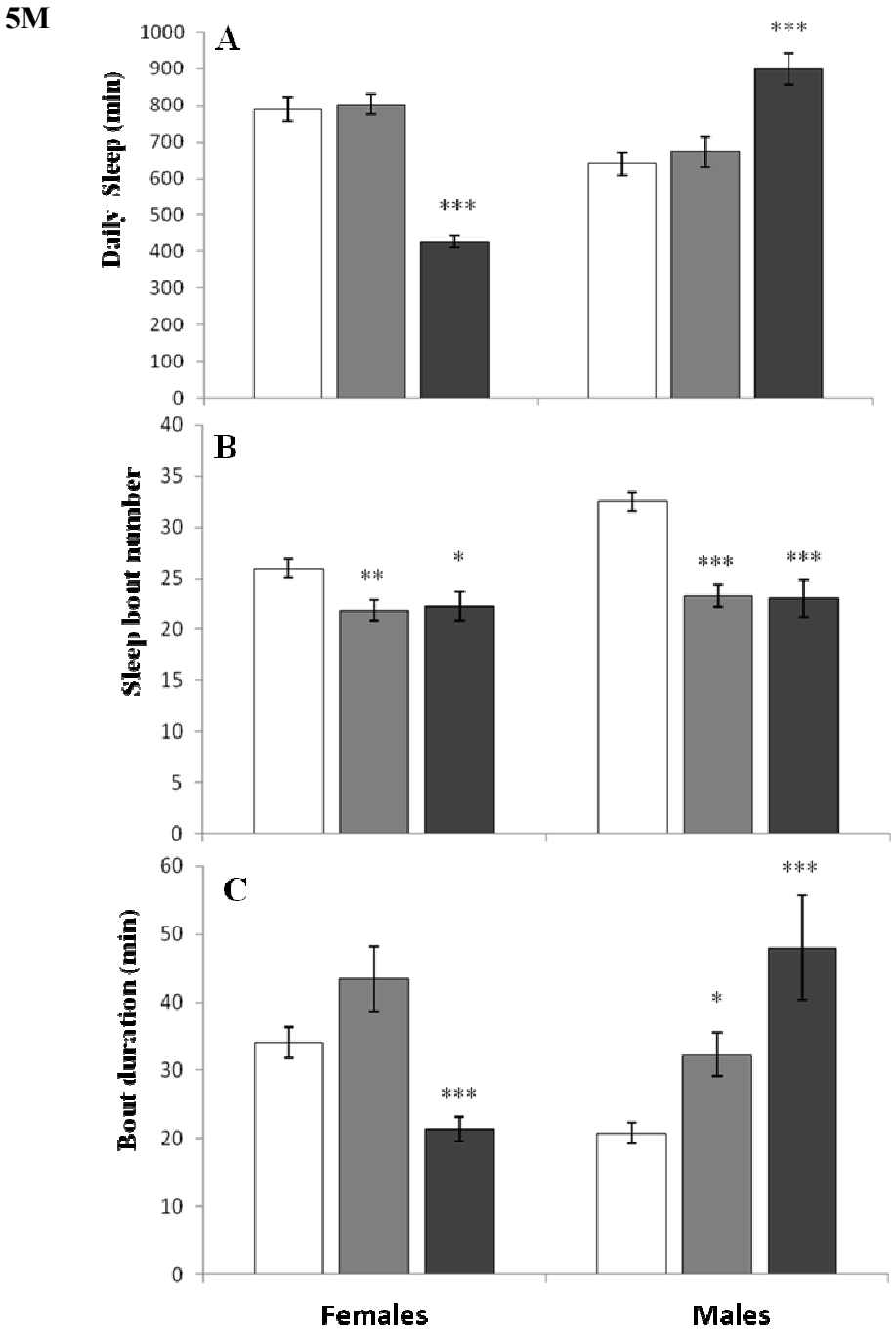


Figure 5.13: Sleep phenotype of transformant flies (5M) with ablated *Dat* neurons. (Caption same as Figure 5.11, Tukey post hoc, *P < 0.05, **P < 0.01, ***P< 0.001)

5.4 Discussion

Experiments described in this chapter were aimed at generating a GAL4 transgene that would aid identifying the *Dat* expressing neurons in the brain and testing their role in sleep. A 1.9 kb fragment upstream of *Dat* was cloned and *Dat*-GAL4 transgenic strains were generated. The alternative splicing that occurs at this locus (Figure 5.2) probably involves two promoters associated with each of the splicing products, since the expression of only DatB is abolished in Dat^{lo} mutant, while expression of DatA remains intact (Brodbeck *et al.* 1998). Because Dat^{lo} is caused by a transposon insertion in the first intron, it is also likely that the promoter of *DatB* is located further downstream within this intron. Since only the upstream sequence has been used for constructing the *Dat*-GAL4, it is likely that this driver includes the promoter of *DatA*, and the expression pattern that this transgene driver reflects only that splice variant (e.g. *Dat**A***-GAL4).

The expression pattern revealed by this driver is rather complicated and inconclusive (Figure 5.8). While fluorescence seems to be specific, and several clusters of cells are clearly visible (e.g. Figure 5.8 A,B), reproducibility between the different transgenic lines (and even within lines) is poor. The difference between transformant strains may reflect the different genomic regions where the transgene has been inserted (i.e. position effect). This was clearly the case with the transformant 2M in which a strong and non-specific signal from the mushroom body was observed (see appendix 3). It was also difficult to associate the labelled neurons with the previously characterized DA neurons which are traditionally labelled by Tyrosine Hydroxylase (TH) antibodies. It is however possible that *Dat*A and TH are not co-expressed in the same neurons (see below). Nevertheless, the *Dat*A-GAL4 driver is an

important tool in understanding the DA circuitry, and together with a future GAL4 driver that carries the DatB promoter, will allow studying the expression of both splice forms, which apparently does not overlap (Brodbeck *et al.* 1998).

Ablation of the *Dat*A neurons disrupted sleep with flies exhibiting shorter bouts of sleep (Figure 5.11-13B); although daily amounts of sleep did not change. In particular, female flies showed significant differences in their background controls for daily amounts of sleep. This could be attributed to the different w^{1118} strains used for the transformant lines (Rosato and Kyriacou 2006). In comparison, daily amounts of sleep in Dat^{lo} mutants also remains normal, but sleep rebound is significantly elevated (Shaw *et al.* 2000). The sleep structure of these mutants (bout number, duration) has not been reported. Nevertheless, the results here suggest that accumulating DA leads to hyperactivity and lack of sleep maintenance which fits well with the general dogma about the role of DA in sleep and waking (see section 5.1)[9]. But this interpretation is only valid if synthesis of DA (in which TH is important) and catabolism (mediated by DAT protein) is carried out in different cells; otherwise, ablation of *Dat* neurons will eliminate all dopaminergic pathways (including synthesis), and DA levels will decrease (in which case increase in sleep is expected). Immunocytochemistry experiments using TH antiserum in *Dat*A-GAL4>RFP flies will allow testing whether the two proteins are co-expressed or if the processes are taking place in different sets of neurons (*Dat* antibodies are currently not available). It will also allow better classification of the putative *Dat* neurons and confirm the presence of *Dat* specific neurons.

[9] Yet another explanation could be that apart from *Dat* neurons other non-DA related factors in these cells may be important for sleep. In addition, *Dat* and the upstream gene *CG4065* point in opposite directions, so potentially, the intergenic region may harbour a bidirectional promoter, and the expression of both genes will overlap. Thus, the observed sleep phenotype may be caused by *CG4065* rather than *Dat*.

Interestingly, the effect of ablation of *Dat*A neurons is sexually dimorphic, with males, but not females, showing lower midday sleep levels (Figure 5.10), and shorter bout durations. This may be related to the fact that *Dat* enzyme activity is higher per unit of body weight in males than in females (Maranda, Hodgetts 1977). This may suggest that DA is important in the general sexual dimorphism associated with sleep in flies (see chapter 1), a process which will be further explored in Chapter 6.

Finally, the generation of a mushroom body (MB) GAL4 driver, although coincidental might be useful for future studies. Interestingly, flies with ablated MB exhibited rather normal sleep, with only night time sleep being longer in females, compared to the control lines (see appendix 3). These results fail to support the recent study of (Pitman *et al.* 2006) where they used three MB GAL4 drivers to silence synaptic transmission (using *shibire*) in the MBs, which caused *reduced* sleep (although only males were analysed in that study). The discrepancy between the two studies might be due to non-overlapping expression pattern, or the difference in way the cells were silenced.

Chapter 6: Analysis of sleep sexual dimorphism in *Drosophila*

6.1 Introduction

Studies in various organisms indicate consistent sleep differences between males and females. For example, (i) frequency of sleep spindles is elevated in women compared with men (Gaillard and Blois 1981), (ii) women sleep longer, when deprived from external cues, in lab conditions (Wever 1984) and (iii) *slow wave sleep* (SWS) is more frequent in women than in men (middle-age subjects) (Reynolds *et al.* 1990). Sleep patterns are also sexually dimorphic in mice (Sinton, Valatx & Jouvet 1981) and in rats (reviewed in Fang and Fishbein 1996).

In *Drosophila*, the pattern of sleep is also sexually dimorphic, with pronounced mid-day sleep ('siesta') in males, but not in female (see Introduction). A recent study by Catterson *et al*. (2010) has shown that diet has a major impact on sleep patterns, in a way which was also sex-dependant. Feeding males with dietary yeast extracts increased their locomotor activity and shortened both diurnal and nocturnal sleep, while females responded to this diet with reduced daytime locomotor activity and a more fragmented nocturnal sleep. The female-specific sleep pattern seems to be present only in inseminated females (Isaac *et al.* 2010). This led to the discovery that the sex-peptide, which males transfer to the female during copulation, modulates the female behaviour and promotes their mid-day waking.

Sex determination in *Drosophila* has been extensively studied (see below) and genetic tools are available allowing manipulation of specific target tissues. Here, I have used the UAS-

GAL4 system to feminize male specific regions of the brain and masculinise female specific neurons, trying to identify the sleep circuits that may be controlling this sexually dimorphic behaviour in flies.

The main determinant of sex in *Drosophila* is the ratio of X chromosome to the number of autosomes (X:A). The information about sex ratio is facilitated by maternal and genetic components, and then passed through a cascade of genes namely, *Sex-lethal* (*Sxl*), *transformer* (*tra*), *transformer-2* (*tra2*), *doublesex* (*dsx*) and *fruitless* (*fru*) (Figure 6.1) (reviewed by Schutt and Nothiger 2000).

The ratio of X:A determines the on/off mode of *Sxl* gene. In females, where the X:A ratio is 1, Sxl is active and produces SXL protein, and causes splicing of the *tra* pre mRNA such that TRA protein is produced. Along with TRA, when the protein product of *tra-2* gene is also present, pre-mRNA of *dsx* gene is spliced into its female-specific form encoding the DSX^F protein and activates the female sexual differentiation and represses the male sexual differentiation. While in males, where the sex ratio is 0.5, no SXL is made, so the *tra* pre-mRNA is spliced into its male-specific form, which does not produce TRA protein. Although TRA2 is present in males, it cannot act without active TRA, so the *dsx* pre m-RNA is spliced into male-specific form, producing DSX^M form and activates male sexual differentiation (reviewed by Schutt and Nothiger 2000).

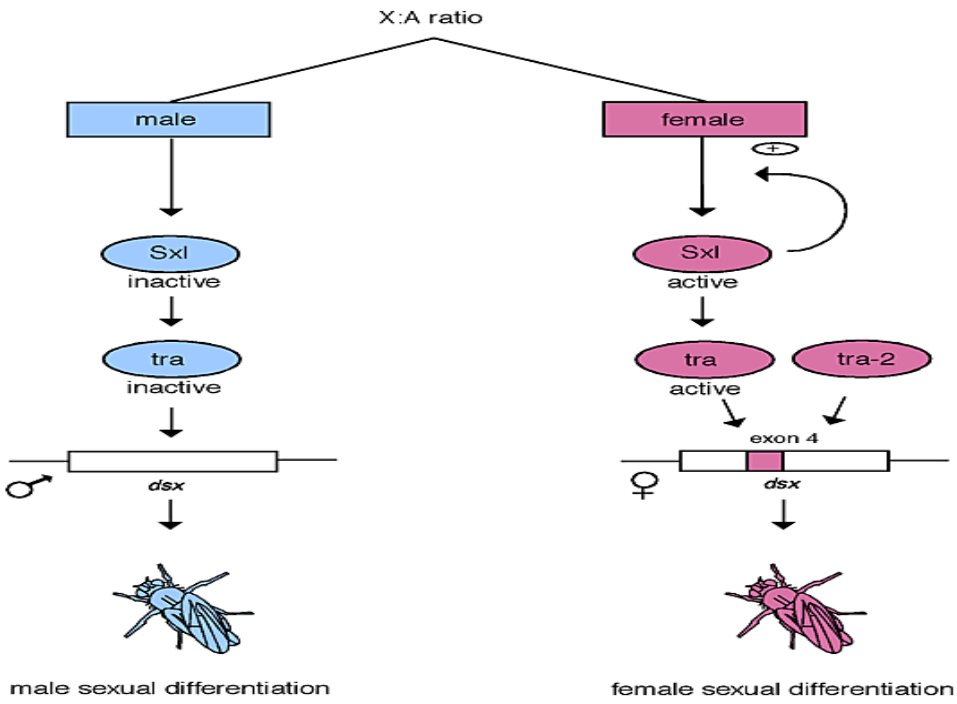


Figure 6.1: Sex determination in *Drosophila*. In somatic cells, the ratio of X chromosome to the autosomes (X:A) regulates the activity of *Sex-lethal* (*Sxl*) which in turn activates *transformer* (*tra*). *tra* along with its splice-form *transformer-2* (*tra 2*) controls alternative RNA splicing of *doublesex* (*dsx*) which determines whether the gonad develops into male or a female.

The *transformer* gene is one of a set of regulatory genes responsible for somatic sexual differentiation in females and has no function in males (McKeown, Belote & Baker 1987). Ectopic expression of the female form of *tra* RNA causes chromosomal males to develop as females (McKeown, Belote & Boggs 1988). The female spliced form of *tra* switches cells in an otherwise male, to female pattern of development, and this has been achieved using the UAS-GAL4 binary system in *Drosophila*. Lines with GAL4 constructs mobilized to different genomic locations are crossed to those carrying reporter feminizing gene *upstream activating sequence-transformer* (UAS-*tra*). The *tra* is then activated in all the tissues expressing GAL4,

creating tissue-specific feminization (Ferveur *et al.* 1995). This system has been used to identify the regions of the brain responsible for male courtship behaviour (Ferveur *et al.* 1995, Ferveur *et al.* 1997). A similar approach has also been used to masculinise female specific tissues, using *tra-2* RNA interfering construct (UAS-*tra2*-IR) (Lazareva *et al.* 2007).

6.2 Materials and Methods

6.2.1 Fly stocks, crosses and maintenance

The genotypes and stock IDs are listed in Table 6.1. A UAS-*tra* transgenic line was used to over-express *tra* under GAL4 control to switch cells in an otherwise male fly to a female pattern of development, creating tissue-specific feminization. Three transgenic strains carrying UAS-*tra*-RNAi or UAS-*tra2*-RNAi were used for masculinisation of female tissues: UAS-*tra*-IR and UAS-*tra2*-IR were obtained from Vienna *Drosophila* RNAi Centre, and another UAS-*tra2*-IR[2] strain was a gift from Prof. John Belote at Syracuse University. UAS-*dicer2* transgenic flies were used to enhance the efficiency of RNAi in some lines (specified when used).

Four enhancer-trap strains, 103Y, 30Y, 121Y (Gatti Sylvain, Jean-François Ferveur & Jean-René Martin 2000) and *Voila*-GAL4 (Balakireva *et al.* 1998) with expression patterns in the mushroom bodies, central complex and a small cluster in pars intercerebralis (PI) were obtained from Jean-François Ferveur at the University of Dijon . Two *elav*-GAL4 strains were obtained from Bloomington Stock centre driving expression of the gene of interest in the nervous system and Kenyon cells. *repo*-GAL4 and 1471-GAL4 strains with expression patterns in glial cells and in the gamma lobes of MBs respectively were obtained from

Bloomington Stock centre. *takeout*-GAL4 driving expression in the fat body as well as in a subset of cells within the maxillary palps and antennae was used (Dauwalder *et al.* 2002). Ubiquitous expression of all UAS constructs was driven by *actin*-GAL4. The genotypes, stock IDs and expression patterns are listed in Table 6.2.

In addition, flies carrying a single transgene for both GAL4 and UAS constructs were crossed to w^{1118} and their F1 progeny were tested as controls. All stocks and experimental crosses were maintained at 25°C with a Light:Dark 12:12 cycle.

Table 6.1: List of UAS constructs

Stock ID	Strain
4590	w[1118]; P{w[+mC]=UAS-tra.F}20J7
v2560	w[1118]; P{GD764}v2560 (UAS-traIR)
v8868	w[1118]; P{GD768}v8868 (UAS-tra2IR)
	w P{UAS-tra2-IR} [61A]; +; P{UAS-tra2-IR} [82A]
v60008	w^{1118};P{UAS-dicer2,w^{+}};+

Table 6.2: List of GAL4 strains

Stock ID	Strain	GAL4 Expression
	actin-GAL4	Ubiquitous
8760	w[*]; P{w[+mC]=GAL4-elav.L}	Nervous system and Kenyon cells
8765	P{w[+mC]=GAL4-elav.L}2/CyO	Nervous system and Kenyon cells
9465	w[1118]; P{w[+mW.hs]=GawB}1471	Γ lobes of MBs
7415	w[1118]; P{w[+m*]=GAL4}repo/TM3,Sb[1]	Glia cells
	Takeout-GAL4	Fat bodies in adult heads
Jean-François Ferveur	121Y	MB, PI, CC
	30Y	MB, PI, CC
	103Y	MB, PI, CC
	Voila-GAL4	MBs, PI

MB: Mushroom Body, CC: Central complex, PI: Pars intercerebralis

6.2.2 Sleep assay

Three-four day old flies were used to monitor their sleep/wake cycle using the DAMS (TriKinetics) at 25°C in 12:12 LD conditions for a total of four days. Only virgin females were used for all experiments. Data was collected in five- min bins and then pooled into 30-min bins for further analysis (see Chapter 2). 'Siesta' sleep time interval was defined for each GAL4 line carrying a single transgene. This time interval (typically, between 5-8hr after light on, for specific times of each strain see appendix 4) was used to calculate average sleep for the lines carrying the GAL4/UAS transgenes and the control genotype carrying a single transgene (UAS or GAL4). The sexually dimorphic behaviour was verified in all control lines. In the feminizing experiments, where the female-spliced form of *tra* was expressed in males, siesta sleep was calculated in the feminized males and in females, and compared to their background

controls. For masculinisation of the females, RNAi constructs of *tra* and *tra-2* were expressed in females, and siesta sleep was assessed in males and masculinised females, and compared to their background controls. In each experiment, the sleep scores of the three genotypes was compared by one-way ANOVA, and tests indicating significant difference were followed by Tukey *post-hoc* tests, comparing each of the control to the GAL4/UAS genotype. Statistical tests were carried with the statistical software R (R Development Core Team 2010).

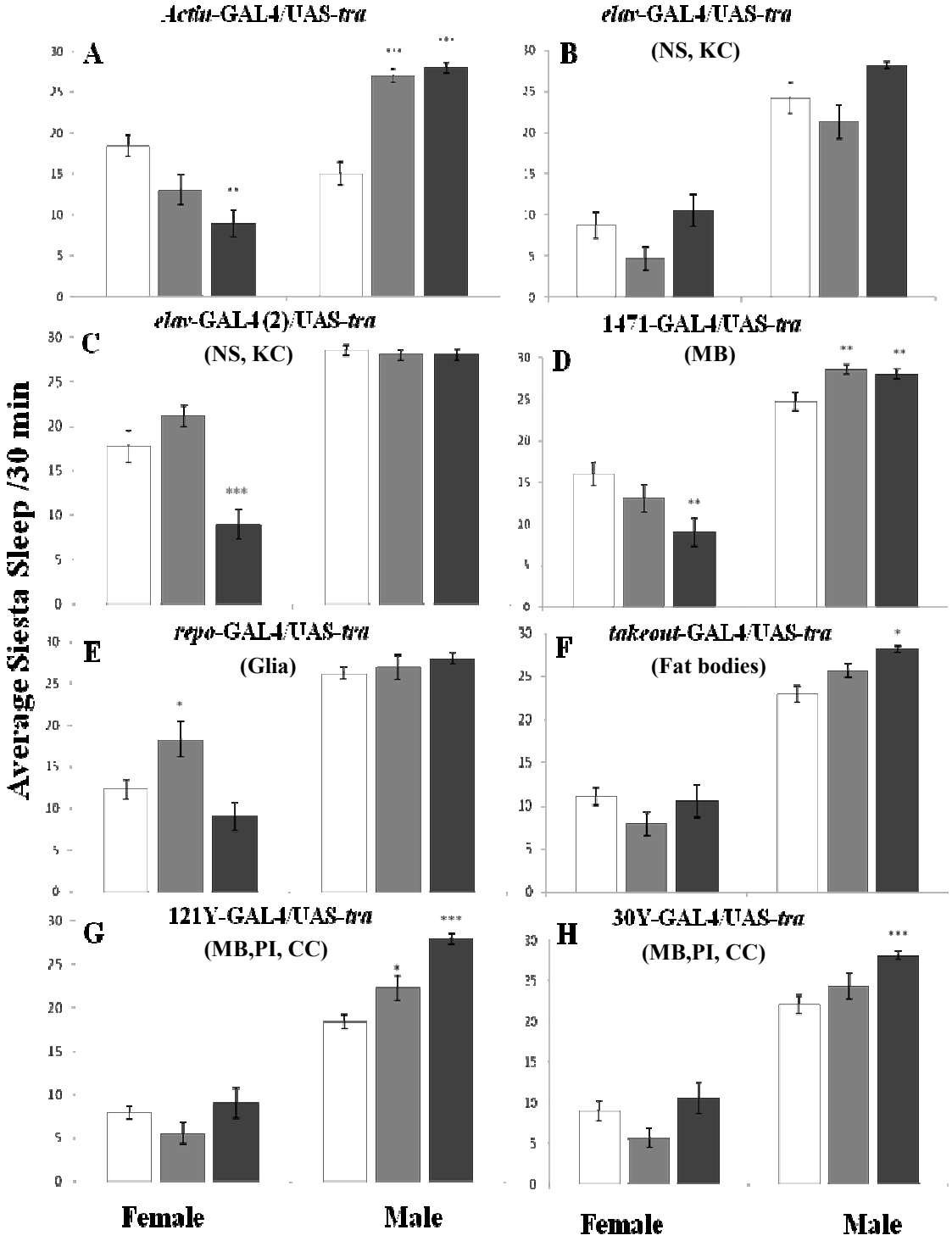
6.3 Results

6.3.1 Genetic feminization of male brain and its consequences on sleep behaviour

Average siesta sleep/ 30 min was calculated for male and female flies as a way to measure their sex specific sleep pattern.

To test if expressing the female splice-form of *tra* in males led to a female specific sleep pattern, a UAS-*tra* construct was expressed ubiquitously in all cells of a male fly using the *actin*-GAL4 driver. Feminized males showed a significant reduction (~ 50%) of sleep, compared with males carrying a single transgene (either UAS or GAL4) (Figure 6.2A). Intriguingly, over-expressing *tra* in females induced an increase in siesta sleep. Overall, the use of the ubiquitous *actin* driver demonstrated the utility of this approach.

Subsequently, nine different GAL4 drivers were used to express *tra* in various areas of the fly brain. Expression of UAS-*tra* in mushroom bodies by the GAL4 drivers 1471 and 121Y led to feminized male sleep behaviour (reduction of mid-day sleep) (Figure 6.2D,G) when compared to their controls (although not as low as in normal females, or *actin*-feminised males).


Actin-GAL4/UAS-tra
A
elav-GAL4/UAS-tra
(NS, KC)
B
elav-GAL4 (2)/UAS-tra
(NS, KC)
C
1471-GAL4/UAS-tra
(MB)
D
repo-GAL4/UAS-tra
(Glia)
E
takeout-GAL4/UAS-tra
(Fat bodies)
F
121Y-GAL4/UAS-tra
(MB,PI, CC)
G
30Y-GAL4/UAS-tra
(MB,PI, CC)
H
Average Siesta Sleep /30 min
Female
Male
Female
Male

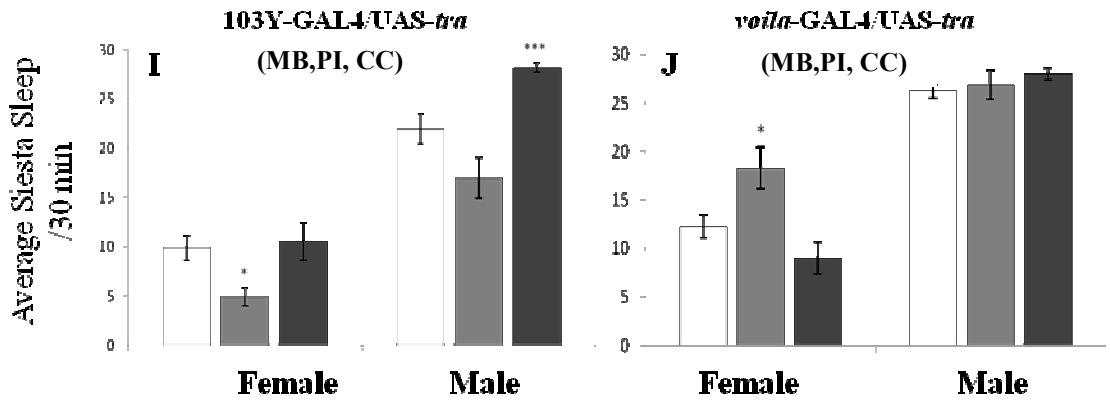


Figure 6.2: Siesta sleep in feminized males. The histograms represent the average siesta sleep/30 min during a specific siesta period. The data shown in each box represents siesta sleep for the GAL4/UAS genotypes (white, n= ≥ 20 for all GAL4 lines; males and females) and the single transgene control genotypes (GAL4/+; grey, UAS/+; black) for both sexes. Asterisks represent experimental genotype (GAL4/UAS) significance levels compared to control genotypes (GAL4/+ and UAS/+). TukeyHSD *post hoc* tests were performed (*P < 0.05, **P < 0.01, ***P< 0.001). Error bars represent S.E.M. The genotype in each experiment is shown above each panel. Expression patterns as indicated, NS: nervous system; KC: Kenyon cells; MB: Mushroom body; CC: Central complex; PI: pars intercerebralis

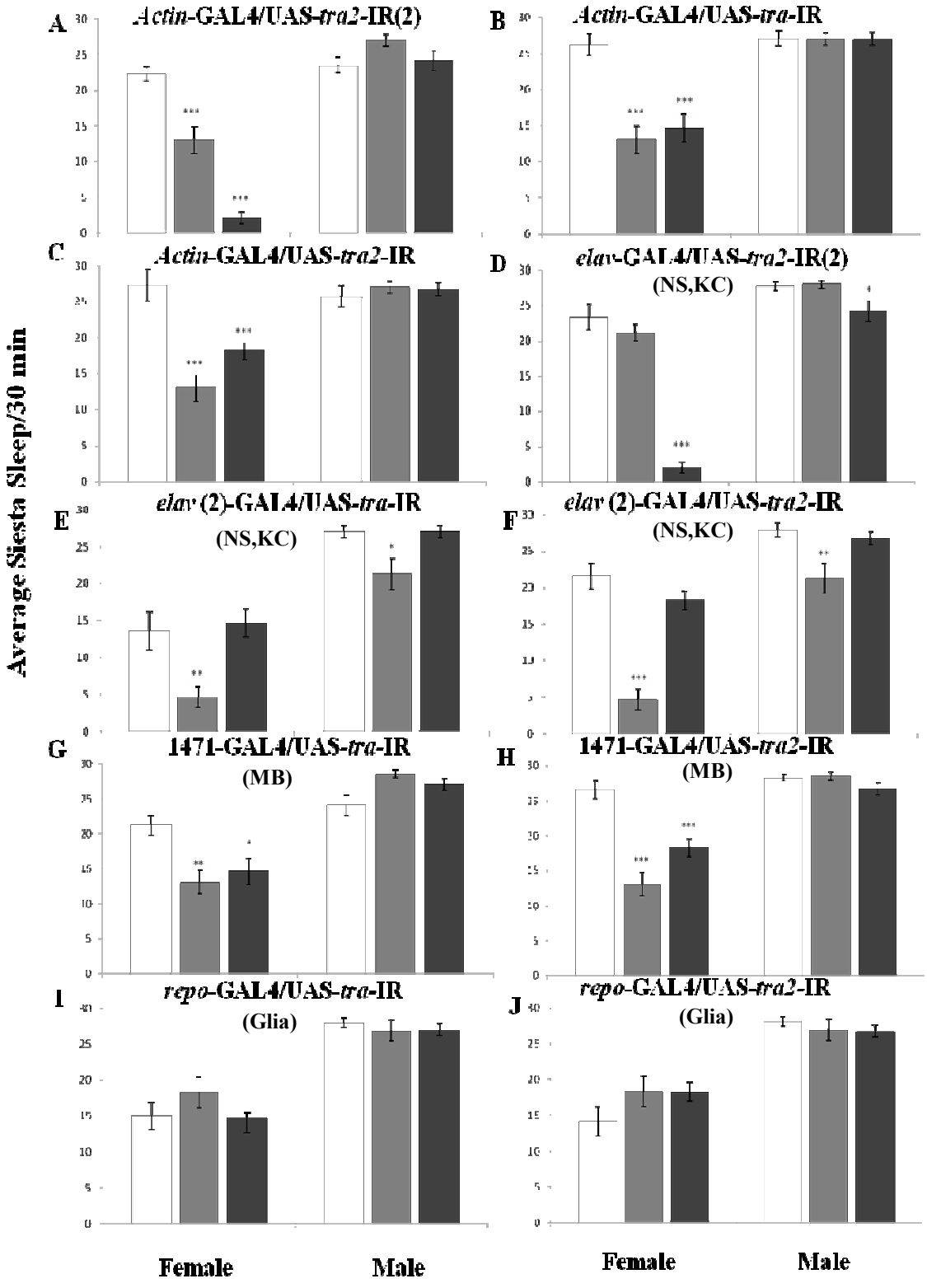
6.3.2 Masculinisation of female

To test whether masculinisation of these brain circuits would lead to a male specific sleep pattern in females (mid-day sleep), the same GAL4 drivers were used to express a *tra* and *tra2* interfering RNAi construct (UAS-*tra*-IR and UAS-*tra2*-IR) and their average siesta sleep was measured (Figure 6.3).

Using the *actin*-GAL4 driver to ubiquitously express the UAS controlled RNAi constructs of *tra* and *tra2* caused a significant increase of about 50% siesta sleep compared to their single transgene control females (Figure 6.3A,B,C), indicating that male specific behaviour can be induced in an otherwise female fly. Masculinising females using the 1471 GAL4 driver to knockdown *tra* (Figure 6.3G) or *tra2* (Figure 6.3H) significantly increased

male like siesta sleep. Knockdown of *tra2* by *takeout* driver induced increased siesta sleep in females (Figure 6.3K) and a similar increase was observed when using the 121Y GAL4 driver to knockdown *tra* (Figure 6.3L) or *tra2* (Figure 6.3M).

Using the 30Y driver to knockdown either *tra* (Figure 6.3N) or *tra2* (Figure 6.3O) also induced a male-like siesta sleep. Driving the knockdown of *tra* with the 103Y driver also caused a significant increase in siesta sleep in females (Figure 6.3P), but not with the UAS-*tra2* transgene (Figure 6.3Q); note however, that the level of siesta sleep is already high in the UAS/+ control females.


A
Actin-GAL4/UAS-tra2-IR(2)
B
Actin-GAL4/UAS-tra-IR
C
Actin-GAL4/UAS-tra2-IR
D
elav-GAL4/UAS-tra2-IR(2)
(NS,KC)
E
elav (2)-GAL4/UAS-tra-IR
(NS,KC)
F
elav (2)-GAL4/UAS-tra2-IR
(NS,KC)
G
1471-GAL4/UAS-tra-IR
(MB)
H
1471-GAL4/UAS-tra2-IR
(MB)
I
repo-GAL4/UAS-tra-IR
(Glia)
J
repo-GAL4/UAS-tra2-IR
(Glia)
Average Siesta Sleep/30 min
Female
Male
Female
Male

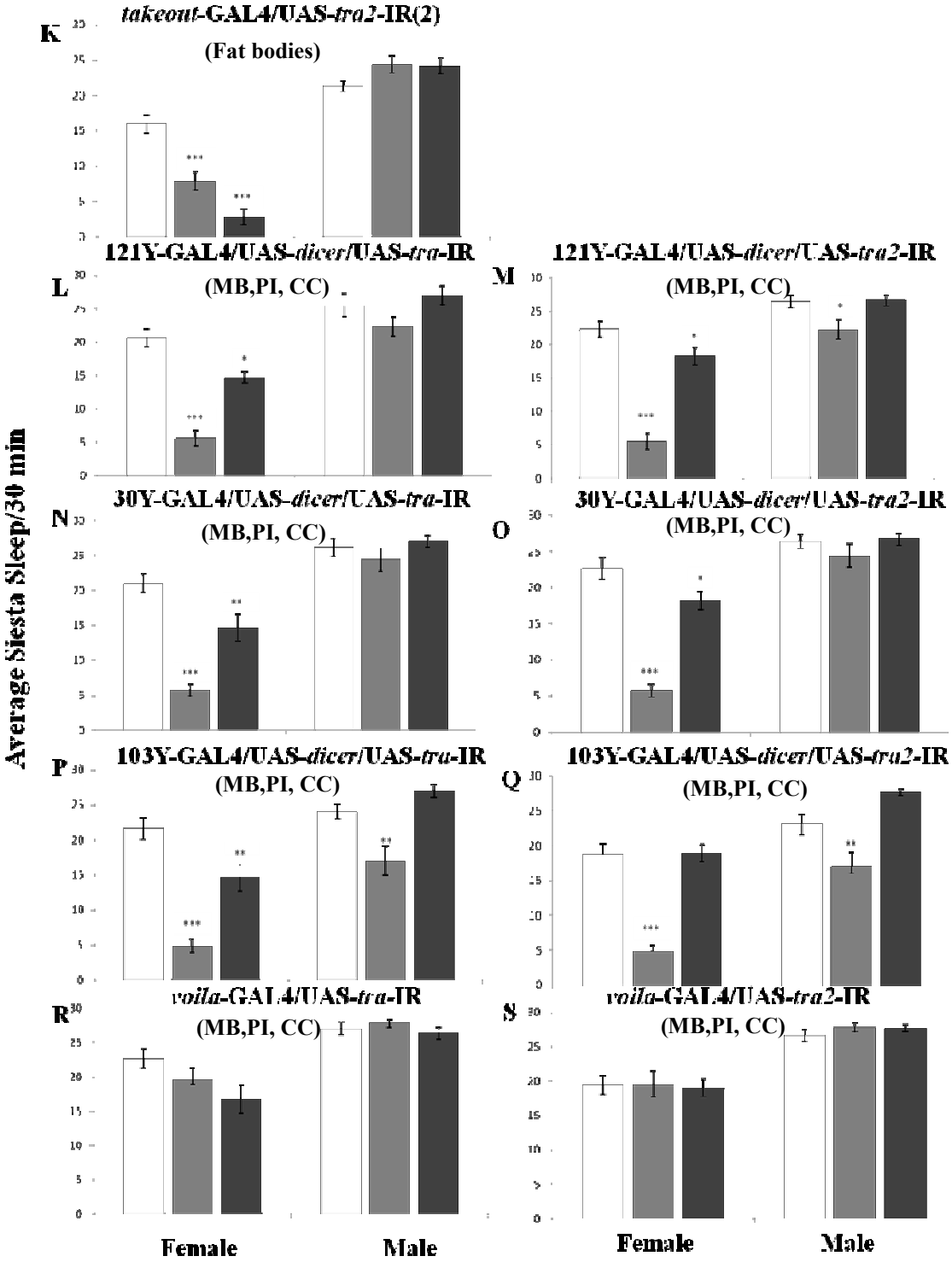

K
takeout-GAL4/UAS-tra2-IR(2)
(Fat bodies)
L
121Y-GAL4/UAS-dicer/UAS-tra-IR
(MB,PI, CC)
M
121Y-GAL4/UAS-dicer/UAS-tra2-IR
(MB,PI, CC)
N
30Y-GAL4/UAS-dicer/UAS-tra-IR
(MB,PI, CC)
O
30Y-GAL4/UAS-dicer/UAS-tra2-IR
(MB,PI, CC)
P
103Y-GAL4/UAS-dicer/UAS-tra-IR
(MB,PI, CC)
Q
103Y-GAL4/UAS-dicer/UAS-tra2-IR
(MB,PI, CC)
R
voila-GAL4/UAS-tra-IR
(MB,PI, CC)
S
voila-GAL4/UAS-tra2-IR
(MB,PI, CC)
Average Siesta Sleep/30 min
Female
Male
Female
Male

Figure 6.3: Siesta sleep in masculinised females. The histograms represent the average sleep/30 min during specific siesta period. The data shown in each box represents siesta sleep for the GAL4/UAS genotypes (white, n= $\geq$ 20 for all GAL4 lines; males and females) and the single transgene control genotypes (GAL4/+; grey, UAS/+; black) for both sexes. Asterisks represent experimental genotype (GAL4/UAS) significance levels compared to control genotypes (GAL4/+ and UAS/+). TukeyHSD *post hoc* tests were performed (*P < 0.05, **P < 0.01, ***P< 0.001). Error bars represent S.E.M. The genotype in each experiment is shown above each panel. Expression patterns as indicated, NS: nervous system; KC: Kenyon cells; MB: Mushroom body; CC: Central complex; PI: pars intercerebralis.

6.4 Discussion

The genetic feminization or masculinisation of specific tissues or cells using the GAL4-UAS system has been a powerful approach to identify cellular basis of sexual dimorphic traits in *Drosophila*. Here I have used a set of GAL4 drivers with a defined expression pattern to identify cells that may be underlying the difference in male-female mid-day sleep.

Feminization of males using the 1471-GAL4 and 121-GAL4 drivers induced a female-like reduced siesta (Figure 6.2D,G), while the masculinisation of females using these drivers and also 30Y-GAL (and possibly 103Y), triggered the male-like increased siesta (Figure 6.3).

Three of these lines 121Y, 30Y and 103Y (and Voila) have been previously implicated in controlling a sexually dimorphic behaviour (locomotion) (Gatti Sylvain, Jean-François Ferveur & Jean-René Martin 2000); females show significantly shorter inter-bout intervals than males. The overlap of the expression patterns of these lines was restricted to a small cluster in the pars-intercerbralis, which therefore was suggested as a candidate for the location of that circuit. Here, however, the Voila driver (and possibly 103Y) did not have any effect on reversing sleep, while another mushroom body driver 1471-GAL4 did. The overlap between

these driver lines is wider and mainly consists of mushroom bodies (MB), which have recently been implicated in the regulation of sleep (Joiner *et al.* 2006; Pitman *et al.* 2006). Testing additional GAL4 drivers with expression in the MB, will aid indentifying the specific neurons underlying sexual dimorphism. This can be combined with the GAL80 enhancer trap, to repress the GAL4 expression, to drive feminization or masculinisation in a subset of cells of the drivers described here, refining the candidate regions (Suster *et al.* 2004). This approach has been very successful in refining the brain neurons that constitute the circadian clock in *Drosophila* (e.g. (Stoleru *et al.* 2004)).

Interestingly, knockdown of *tra2* by the *takeout*-GAL4 driver also induced siesta in females, indicating a role for the fat body. Previous studies showed that *to* is under circadian control and is involved in the regulation of feeding as well as adaptation to starvation (Sarov-Blat *et al.* 2000; Meunier, Belgacem & Martin 2007). Thus, it is possible that the sleep sexual dimorphism is mediated by *to* (and the fat body) indirectly, so feminizing or masculinising the fat body change the feeding status of the animal, and consequently its foraging behaviour. This idea fits well the recent studies that show a direct link between sleep pattern and feeding (Catterson *et al.* 2010).

Finally, it is interesting to note that the work presented here was based on sleep differences between males and *virgin* females. The clear difference between the sexes is in apparent contradiction to Isaac *et al.* (2010) who reported that virgin females show male-like siesta and switch to mid-day activity following mating because of the effect of the sex-peptides (SP) transferred by the males. The discrepancy between the studies may be related to the strain used, but it seems that other factors in addition to the SP contribute to the decreased

mid-day sleep of females. It is also possible, that using mated females here would have increased the observed gender differences, and consequently the power to detect smaller effects of the feminization or masculinisation.

Chapter 7: Role of DNA methylation in sleep regulation

7.1 Introduction

There is a growing recognition that epigenetic modifications, including DNA methylation, play an important regulatory role in a broad range of processes (reviewed in Richardson 2003). Various studies have demonstrated the critical function DNA methylation serves. For example, DNA methylation is essential for embryonic development in mice (Li, Bestor & Jaenisch 1992), as well as in *Xenopus* (Stancheva, Hensey & Meehan 2001). Developmental abnormalities are also observed in plants with reduced levels of DNA methylation (Finnegan, Peacock & Dennis 1996). In humans, changes in DNA methylation have also been linked to human diseases such as cancer (Jones and Baylin 2002). In general, DNA methylation at the promoter region of a gene may reduce the level of transcription leading to gene silencing (reviewed in Richardson 2003). It has been suggested that environment stimuli can also change the level of DNA methylation, and consequently the level of gene transcription (Jaenisch and Bird 2003).

Accumulating evidence suggests that epigenetic modifications are also important in neural function and behaviour (Levenson and Sweatt 2005). Importantly, several studies have shown that light entrainment of the mammalian circadian clock induces histone acetylation at the promoter region of *per1* and *per2*, attenuating their transcription (Naruse *et al.* 2004). Also, CLOCK, a major protein of the circadian pacemaker has histone acetyltransferase (HAT) activity (Doi, Hirayama & Sassone-Corsi 2006). Recently, the role of ELP3 (a member of the protein complex which has HAT activity) in neurodevelopment and behaviour in

Drosophila was studied (Singh *et al.* 2010). Knockdown of Elp3 by dsRNAi resulted in various morphological synaptic changes which were associated with hyperactivity and loss of sleep in the adult flies.

Could DNA methylation also play a role in *Drosophila* sleep? Until recently, DNA methylation was not considered significant in *Drosophila* (Lyko 2001). The overall level of DNA methylation is rather low and only 1% of cytosine residues are methylated. The highest level of methylation is measured during the early embryo stage. Also, DNA methylation in *Drosophila* occurs at CpT or CpA dinucleotides, rather than CpG which is methylated in other organisms, making it difficult to detect with conventional CpG specific assays.

In vertebrates (as well as in some insect groups) DNA methylation is catalysed by three DNA methyltransferases, Dnmt1-3 (Goll and Bestor 2005). Dnmt1 and Dnmt3 differ in their N-terminal domains that mediate protein-protein interactions, while Dnmt2 lacks this domain and retains only the methyltransferase domain (Figure 7.1). Dnmt1 mediates copying the methylation pattern of the parental strand to the newly synthesized daughter strand (maintenance methyltransferase). Dnmt3 prefers unmethylated DNA as its substrate and establishes methylation patterns during embryogenesis (Schaefer and Lyko 2007). Analysis of the *Drosophila* genome revealed only a single DNA methyltransferase orthologue, *dDnmt2* (Lyko *et al.* 2000). Dnmt2, whose function is largely unknown, is the most conserved DNA methyltransferase, and the only one present in the *Drosophila* genome (Kunert *et al.* 2003; Narsa Reddy *et al.* 2003). Overexpression of *Dnmt2* gene caused hypermethylation at CpT/A dinucleotides, while depletion of the gene had no effect on the viability of the embryos or any apparent phenotype in adult flies (Kunert *et al.* 2003).

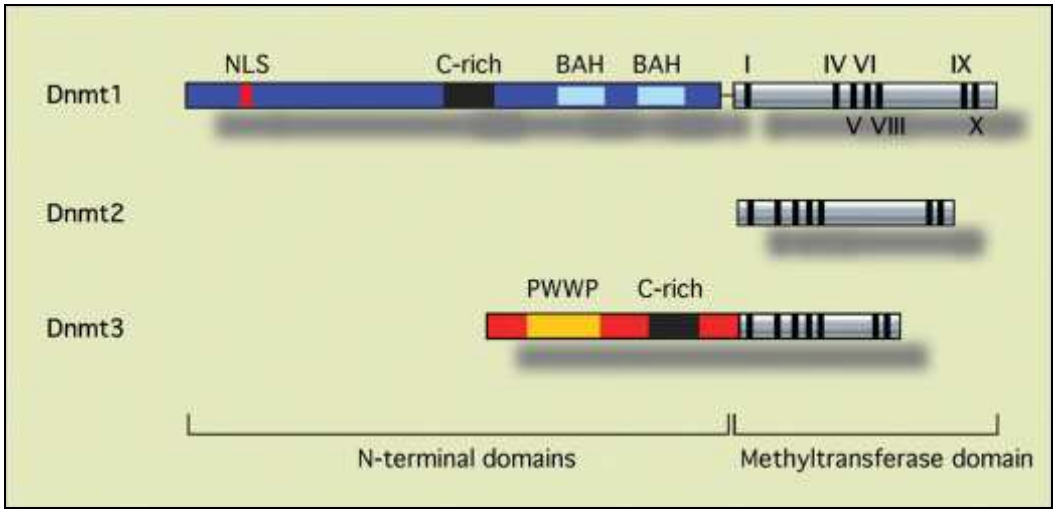


Figure 7.1: Structure of DNA methyltransferases. The 3 groups of methytransferases share the C-terminal, also known as the methyltransferase domain. Dnmt1 and Dnmt3 proteins also have an N-terminal, while Dnmt2 protein lacks this terminal and consists only of the methytransferase domain. NLS: nuclear localization signal, C-rich: cysteine rich, BAH: bromo adjacent homology domain, PWWP: Pro-Trp-Trp-Pro domain (Schaefer and Lyko 2007).

Recently, Lin *et al.* (2005) have over-expressed *dDnmt2* using the GAL4-UAS binary system and found a significant increase in life span of the flies (Lin *et al.* 2005). Interestingly, study by Koh *et al.* (2006) demonstrated a link between sleep and aging in *Drosophila* with sleep becoming more fragmented with age (Koh *et al.* 2006). Bushey *et al*. (2010) found that life span in short-sleep *Hyperkinetic* mutants was significantly reduced compared with wild-type flies. Here, I tested whether DNA methylation plays a role in sleep by over-expressing and knocking-down *dDnmt2* using GAL4-UAS system. The results suggest that homeostasis of methylation is important for normal levels of sleep.

7.2 Materials and Methods

7.2.1 Fly stocks and maintenance

Three UAS-*dDnmt2* transgenic strains were used for over-expression of *dDnmt2* (gift from Dr. Che-Kun James Shen at Institute of Molecular Biology, Taiwan ROC). Transgenic strains carrying UAS-*dDnmt2*-RNAi were used to silence the gene. These included two from the Vienna *Drosophila* RNAi Centre (Stock no. 37815 and 37816), and two strains from the National Institute of Genetic Fly Stocks (NIG-FLY) in Japan (Stock no. 10692R-2 and 10692R-3). Ubiquitous expression of all UAS constructs was driven by *actin*-GAL4. The genotype of the flies is listed in Table 7.1.

GAL4 directed expression was tested in flies hemizygous for the UAS and GAL4 constructs. In addition, the GAL4 and UAS strains were crossed to w^{1118} and their F1 progeny (carrying the transgene, but not the balancer chromosome) was tested as controls. Flies were reared under constant conditions at 25°C and light:dark cycle of 12:12 hr, and were maintained in vials containing standard food (section 2.1).

Table 7.1: List of UAS-*dDnmt2* lines

Stock no.	Genotype
v37815	w^{1118};;UAS-*dDnmt2*-RNAi/TM3
v37816	w^{1118};;UAS-*dDnmt2*-RNAi
NIG-Fly 10692R-2	w^{1118};UAS-*dDnmt2*-RNAi;
NIG-Fly 10693R-3	w^{1118};;UAS-*dDnmt2*-RNAi
Dr.Che-Kun	w^{1118},UAS-*dDnmt2* (2-3);;
James Shen Laboratory	w^{1118};UAS-*dDnmt2* (4-5)/CyO;
	w^{1118};;UAS-*dDnmt2* (6-4)/TM6B

7.2.2 Quantitative Polymerase Chain Reaction (qPCR)

To verify the miss-expression of *dDnmt2*, quantitative real-time PCR was carried out by using a standard protocol (see section 2.8). Total RNA was isolated from adult flies from three different genotypes UAS-*dDnmt2*/+, UAS-*dDnmt2*/*actin*-GAL4 and *actin*-GAL4/+ using Trizol (Invitrogen) followed by treatment with DNaseI (Ambion). RNA was quantified and cDNA was synthesized by reverse transcription (see section 2.8). RNA samples were spiked with 82 pg of *aequorin* cRNA that served as exogenous reference gene (Gilsbach *et al.* 2006). qPCR was performed by using Brilliant® II SYBR® Green QPCR Master Mix (Stratagene). Primers for qPCR were: *dDnmt2* 5'CAAAAGAGTGCTGGTCATGG 3' (forward) and 5' AAAGCGGTGTGTATGCAGAG 3' (reverse) and for *aequorin*: 5' TTGACGAGATGGTCTACAAGGCATC 3' (forward) and 5' GAAGGCTTCTACAGCATCTTTGTGTCGT 3' (reverse). Three replicates were performed with each sample. PCR was run for 42 cycles [94°C (15 min), 94°C (15 sec), 64.8°C (30 sec) 72°C(30 sec)], followed by a melting curve program (50 to 95°C) to confirm the absence of

primer-dimers. A five-fold log-scale dilution standard curve was generated for *dDnmt2* using known amounts of cDNA for absolute quantification for each sample. qPCR reaction for the exogenous *aequorin* control gene was also performed to allow standardization of expression of *dDnmt2* gene. A five-fold dilution log-scale dilution standard curve was also generated for *aequorin* by using its cDNA.

7.3 Results

7.3.1 Over-expression of dDnmt2

Ubiquitous over-expression of UAS-*dDnmt2* transgene was driven by *actin*-GAL4 using three independent UAS strains (Figure 7.2). Elevated levels of sleep are observed during the early hours of the day in all lines tested, in both males and females, except for the males of line 6-4 (Figure 7.2F). This is particularly clear in females, showing a marked increase in mid-day sleep compared to both controls. Various sleep parameters were further tested by ANOVA (Table 7.2). The means and the *post-hoc* analysis (TukeyHSD) are shown in Figure 7.3-7.5. Daily sleep was significantly increased in both males and females, relative to their background controls (Figure 7.3-7.5A); most likely due to increase in daytime sleep (Figure 7.3-7.5B). There is also a moderate increase in nocturnal sleep (significant in lines 2-3 and 4-5). There is also some evidence for increase in bout number (line 4-5 females, Figure 7.4 C), and an increase in bout duration (line 2-3 both males and females, Figure 7.3F). Line 2-3 also showed significant consolidated nighttime sleep, where their sleep bouts were decreased along with an increase in bout duration (not shown).

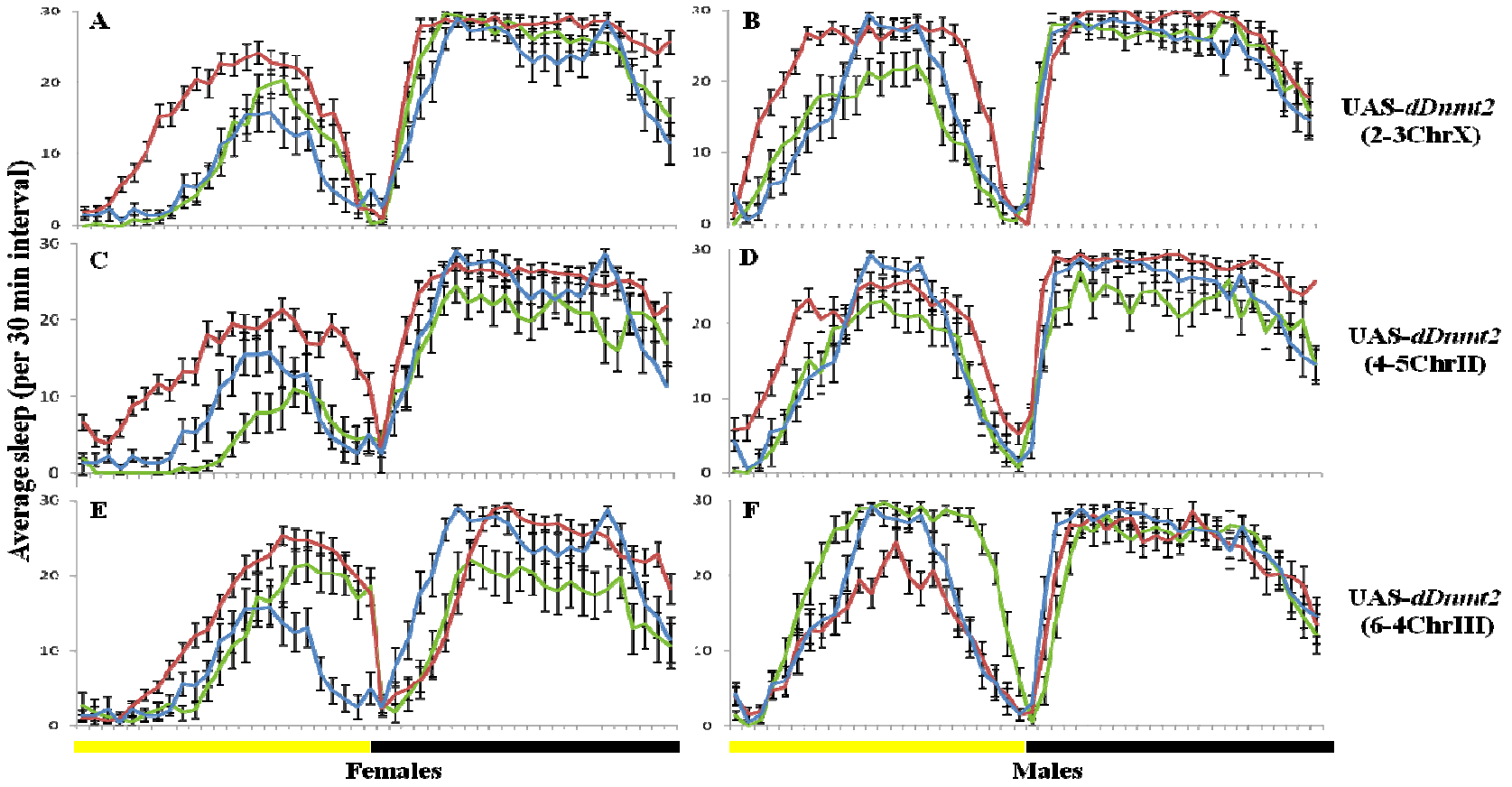


Figure 7.2: Over-expressing *dDnmt2* increases sleep. Sleep profiles are shown in 30 min intervals (mean ± S.E.M) over a period of 24 hr. The left and right panels show the data for female and male flies respectively. The bar below the X-axis indicates 12-hour light (yellow) and 12-hour dark (black) periods. The sleep profile of *actin*-GAL4/UAS-*dDnmt2* (red) versus background controls (GAL/+; blue and UAS/+; green) (n= 52-68) is shown. Female flies show increased amount of day-time sleep compared to their controls in all three UAS lines tested.

Table 7.2: ANOVA of sleep phenotypes comparing *actin>dDnmt2* with their two background controls (flies carrying either the UAS or the GAL4 transgene only) P-value *≤0.05, **<0.01, ***<0.001

	P-value				
Males	Daily sleep	Day time Sleep	Night time Sleep	Number of bouts	Bout duration
UAS-*dDnmt2* (2-3)	$F_{(2,52)}=13.4^{***}$	$F_{(2,52)}=26.51^{***}$	$F_{(2,52)}= 0.78$	$F_{(2,52)}= 2.67$	$F_{(2,52)}= 4.65^{*}$
UAS-*dDnmt2* (4-5)	$F_{(2,60)}=9.63^{***}$	$F_{(2,60)}=10.8^{***}$	$F_{(2,60)}= 4.89^{**}$	$F_{(2,60)}= 7.9^{***}$	$F_{(2,60)}= 4.35^{*}$
UAS-*dDnmt2*(6-4)	$F_{(2,58)}=3.57^{*}$	$F_{(2,58)}=13.97^{***}$	$F_{(2,58)}= 1.18$	$F_{(2,58)}= 6.95^{**}$	$F_{(2,58)}= 6.42^{**}$
Females					
UAS-*dDnmt2* (2-3)	$F_{(2,66)}=49.75^{***}$	$F_{(2,66)}=53.01^{***}$	$F_{(2,66)}=11.9^{***}$	$F_{(2,66)}= 1.24$	$F_{(2,66)}= 4.96^{**}$
UAS-*dDnmt2* (4-5)	$F_{(2,68)}= 34.79^{***}$	$F_{(2,68)}=62.82^{***}$	$F_{(2,68)}= 5.08^{**}$	$F_{(2,68)}= 18.12^{***}$	$F_{(2,68)}= 0.76$
UAS-*dDnmt2*(6-4)	$F_{(2,66)}= 9.62^{***}$	$F_{(2,66)}=20.89^{***}$	$F_{(2,66)}=8.77^{***}$	$F_{(2,66)}= 4.07^{*}$	$F_{(2,66)}= 0.6$

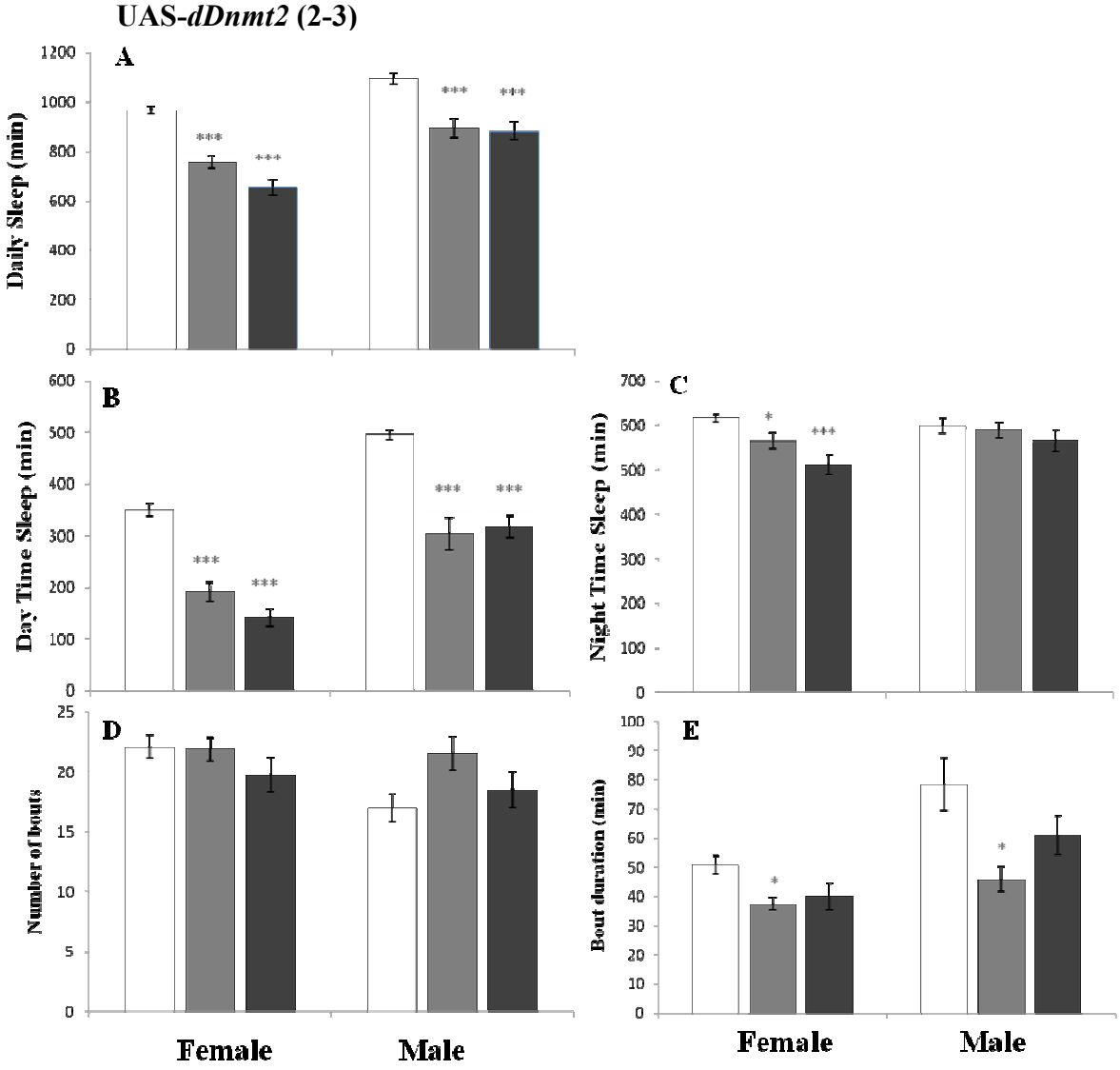


Figure 7.3: Sleep phenotypes following *dDnmt2* over-expression (2-3). Averages of daily sleep (A), daytime sleep (B) and nighttime sleep (C). Sleep intensity was measured as average sleep bouts (D) and average length of sleep bout (E). White bars indicate *actin*-GAL4/UAS-*dDnmt2*; grey bars indicate UAS/+; black bars indicate GAL4/+. Male and female flies as indicated at the bottom. Asterisk, GAL4/UAS combination significantly different from GAL4/+ and UAS/+ controls (TukeyHSD, *P < 0.05, **P < 0.01, ***P< 0.001). Error bars indicate SEM.

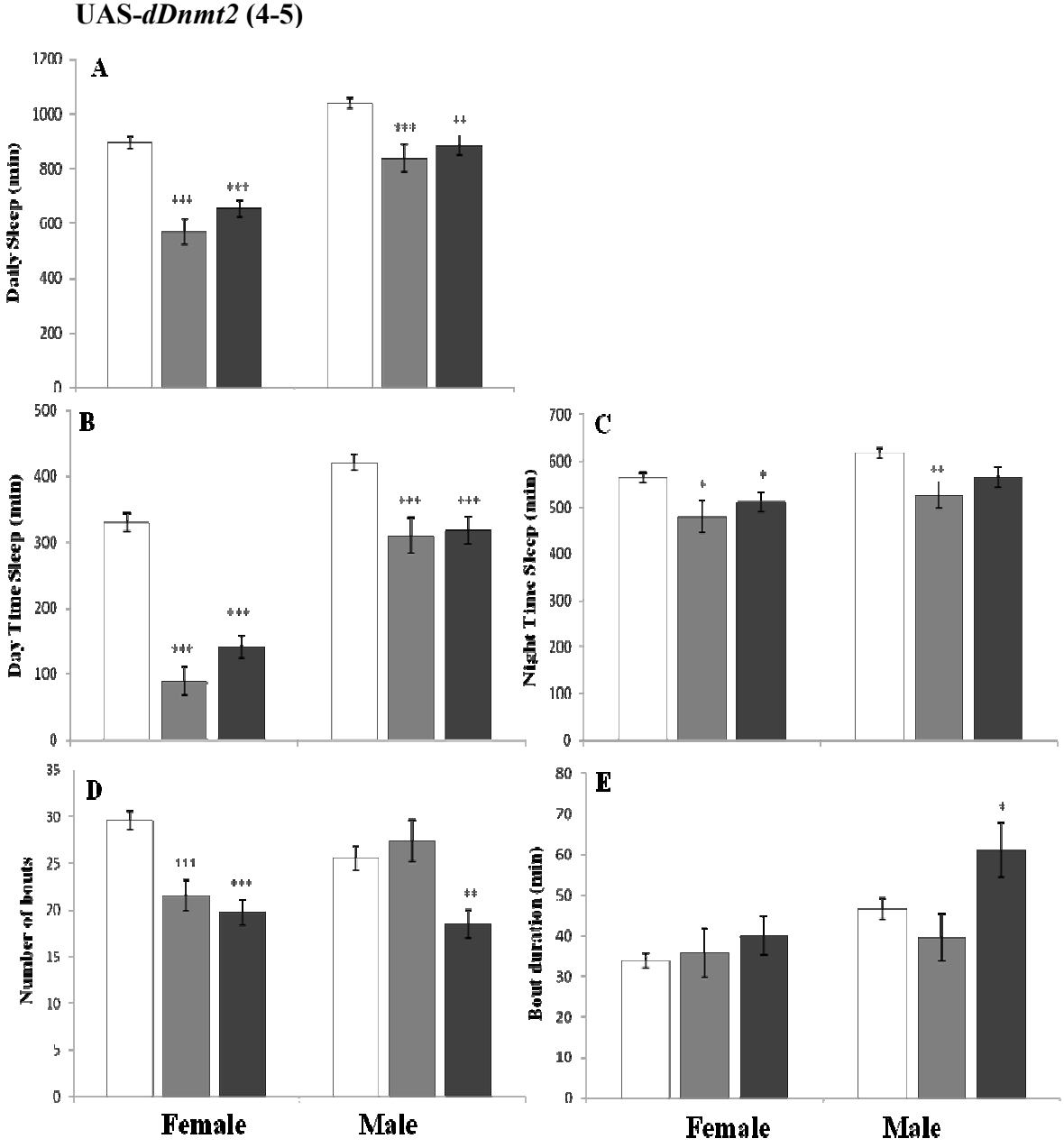


Figure 7.4: Sleep phenotypes following *dDnmt2* over-expression (4-5). Figure legend same as 7.3. (TukeyHSD, *P < 0.05, **P < 0.01, ***P< 0.001). Error bars indicate SEM.

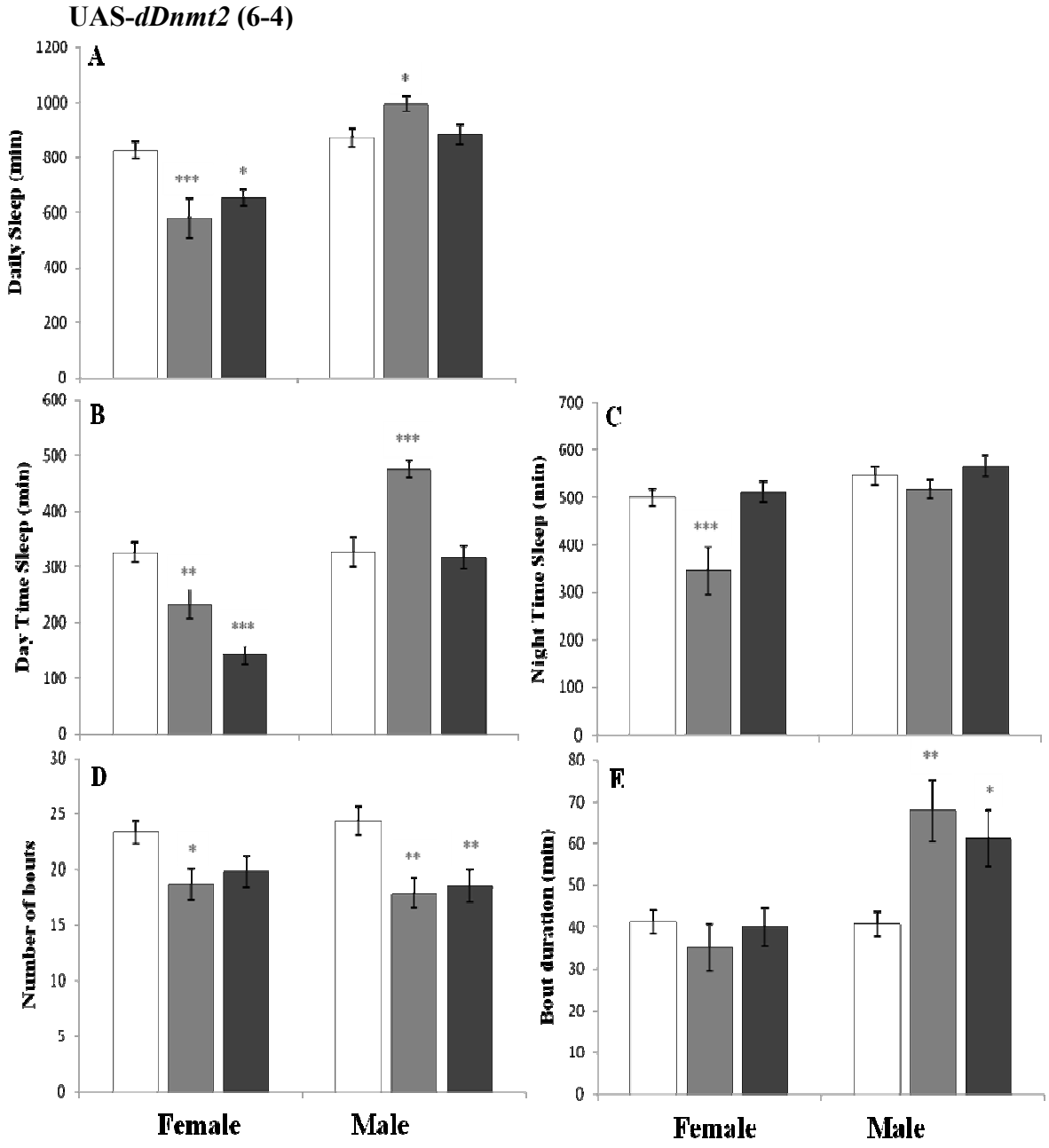


Figure 7.5: Sleep phenotypes following *dDnmt2* over-expression (6-4). Figure legend same as 7.3. (TukeyHSD, *P < 0.05, **P < 0.01, ***P< 0.001). Error bars indicate SEM.

7.3.2 Knockdown of dDnmt2

To investigate the effects of DNA methylation on sleep, the function of *dDnmt2* was reduced with *dDnmt2*-RNAi by using the GAL4/UAS system. Figure 7.6 shows the sleep profiles of flies carrying the various *dDnmt2*-RNAi transgenes. Average sleep is higher during the first hours of the day in all lines tested, and this was particularly clear in females. Also, increase in mid-day sleep was seen compared to both controls.

ANOVA of various sleep parameters, comparing the *dDnmt2* knockdown lines with its background controls, indicated a significant variation between the genotypes (Table 7.3). This was followed by Tukey *post-hoc* test, allowing the comparison of UAS/GAL4 progeny to the UAS/+ and GAL4/+ for each sleep variable in males and females. Females with the *dDnmt2* knockdown showed increase in daily sleep, which was characterized by an increase in diurnal sleep (Figure 7.7-7.10 A, B). The significant increase in sleep was accompanied by an increase in number of sleep bouts, although this was statistically significant in line 2R-3. (Figure 7.7-7.10D). Intriguingly, there is a suggestive evidence for a increase in the average sleep bout duration in females, which may indicate increase in sleep consolidation (lines 37816, 2R-2 Figure 7.7, 7.10E).

Table 7.3: ANOVA of sleep phenotypes comparing *actin>dDnmt2*-RNAi with their two background controls (flies carrying either the UAS or the GAL4 transgene only) P-value *≤0.05, **<0.01, ***<0.001

				P-value	
Males	Daily sleep	Day time sleep	Night time sleep	Number of bouts	Bout Duration
1069-2R-3	$F_{(2,60)}= 1.5$	$F_{(2,60)}= 6.97^{**}$	$F_{(2,60)}= 0.07$	$F_{(2,60)}= 6.61^{**}$	$F_{(2,60)}= 1.79$
1069-2R-2	$F_{(2,63)}= 7.6^{***}$	$F_{(2,63)}= 10.33^{***}$	$F_{(2,63)}= 2.93$	$F_{(2,63)}= 5.6^{**}$	$F_{(2,63)}= 6.45^{**}$
dDnmt2-RNAi (37815)	$F_{(2,89)}= 10.91^{***}$	$F_{(2,89)}= 27.74^{***}$	$F_{(2,89)}= 0.06$	$F_{(2,89)}= 3.54^{*}$	$F_{(2,89)}= 0.5$
dDnmt2-RNAi (37816)	$F_{(2,93)}= 6.7^{**}$	$F_{(2,93)}= 16.67^{***}$	$F_{(2,93)}= 1.2$	$F_{(2,93)}= 0.03$	$F_{(2,93)}= 0.3$
Females					
1069-2R-3	$F_{(2,52)}= 28.79^{***}$	$F_{(2,52)}= 52.91^{***}$	$F_{(2,52)}= 6.97^{**}$	$F_{(2,52)}= 6.61^{**}$	$F_{(2,52)}= 0.18$
1069-2R-2	$F_{(2,61)}= 55.75^{***}$	$F_{(2,61)}= 68.21^{***}$	$F_{(2,61)}= 13.85^{***}$	$F_{(2,61)}= 2.5$	$F_{(2,61)}= 6.16^{**}$
dDnmt2-RNAi (37815)	$F_{(2,61)}= 39.43^{***}$	$F_{(2,61)}= 126.71^{***}$	$F_{(2,61)}= 0.5$	$F_{(2,61)}= 1.01$	$F_{(2,61)}= 6.38^{**}$
dDnmt2-RNAi (37816)	$F_{(2,69)}= 55.20^{***}$	$F_{(2,69)}= 101.1^{***}$	$F_{(2,69)}= 5.75^{**}$	$F_{(2,69)}= 2.79$	$F_{(2,69)}= 9.51^{***}$

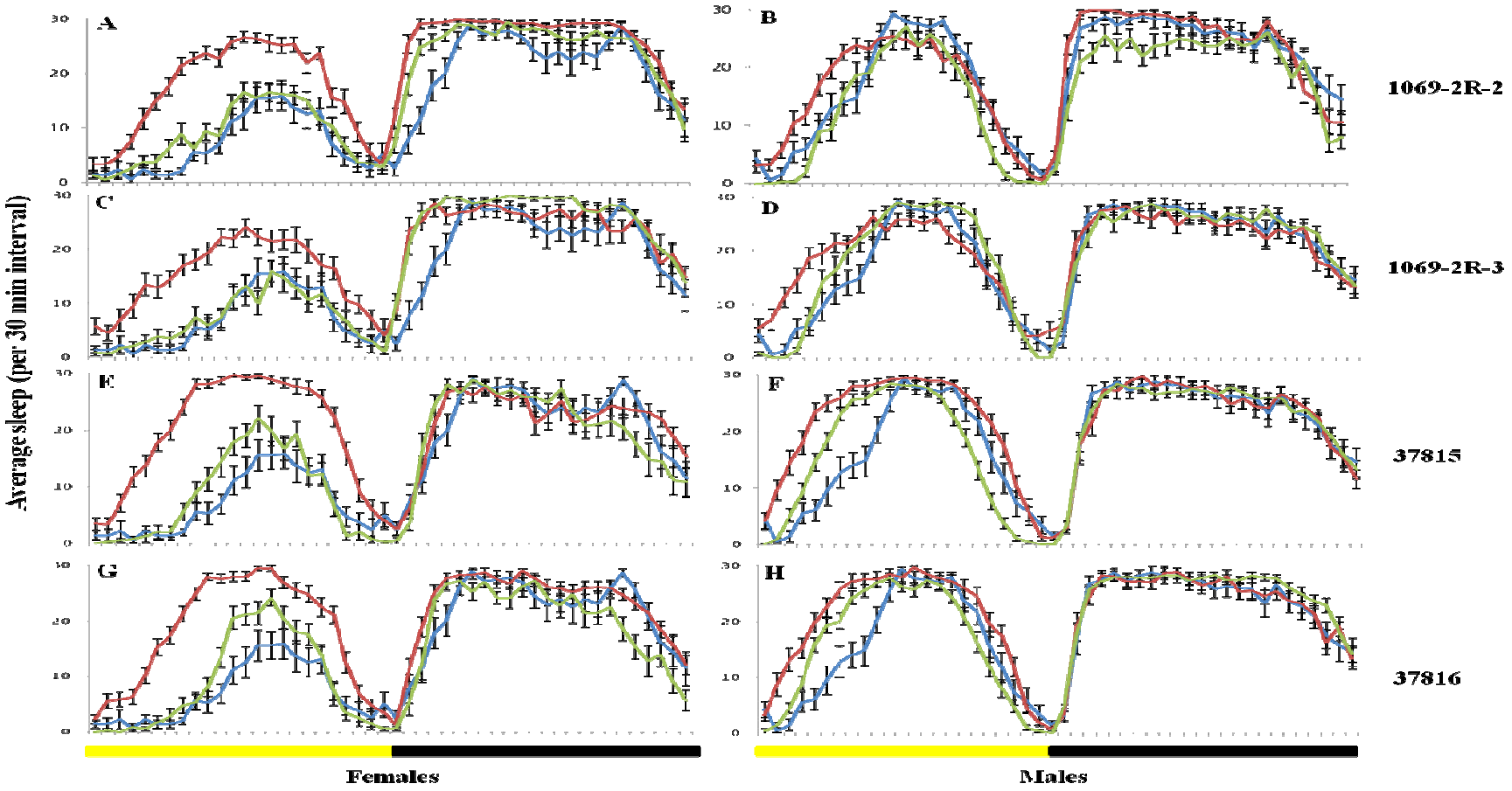


Figure 7.6: Sleep profile in 30 min intervals (mean ± S.E.M) over a period of 24 hr in *dDnmt2* knockdown flies. The left and right panels show the data for female and male flies respectively. The bar below the X-axis indicates 12-hour light (yellow) and 12-hour dark (black) periods. The sleep profile of *actin*-GAL4/UAS-*dDnmt2*-RNAi (red) versus background controls (UAS/+; green and GAL4/+; blue) (n= 52-80) is shown.

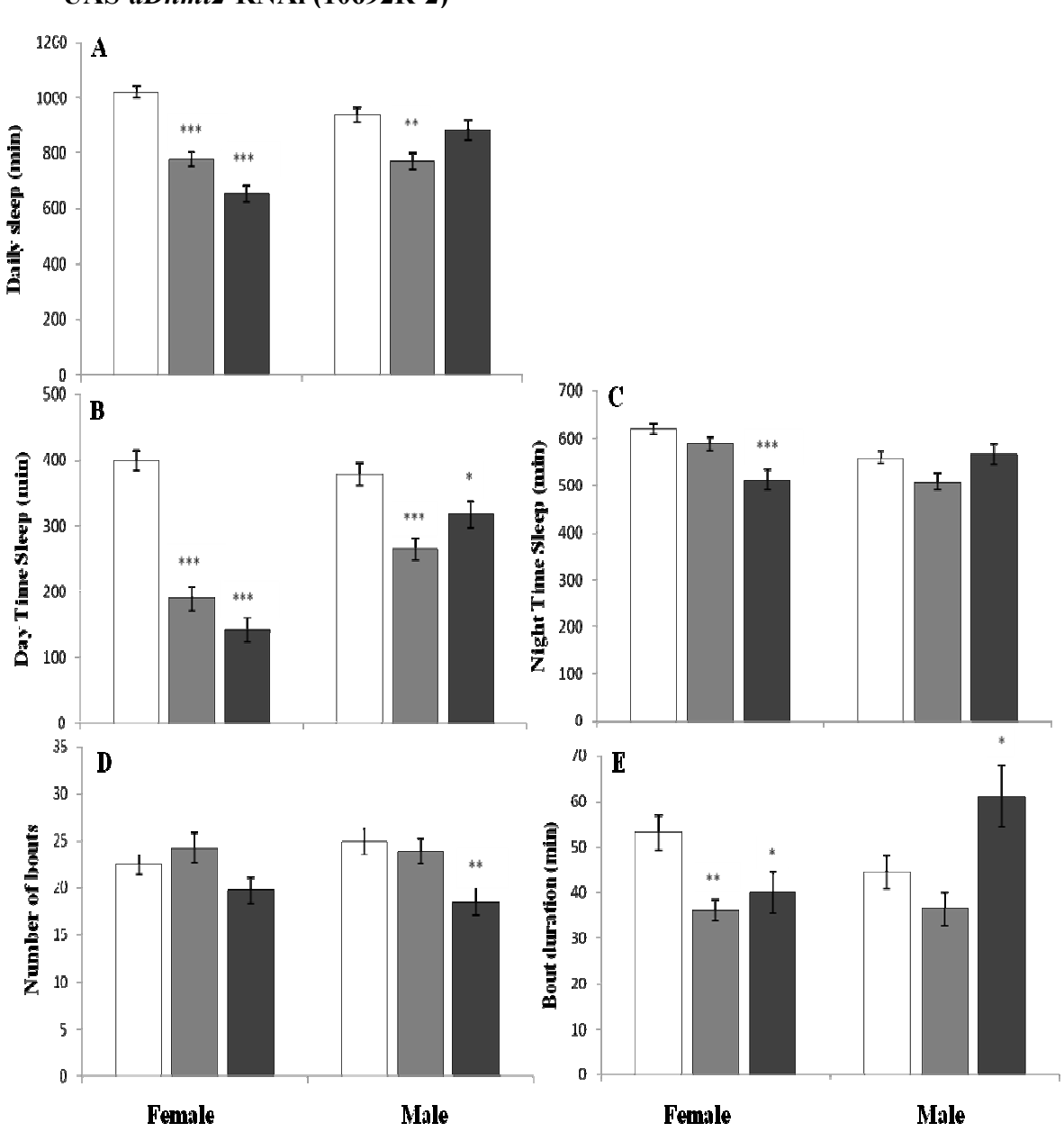


Figure 7.7: Sleep phenotype of *dDnmt2* knockdown flies (2R-2). Averages of daily sleep (A), daytime sleep (B) and nighttime sleep (C). Sleep intensity was measured as average sleep bouts (D) and average bout length (E). White bars indicate *actin*-GAL4/UAS-*dDnmt2*-RNAi; grey bars indicate UAS/+; black bars indicate GAL4/+. Male and female flies as indicated at the bottom. Asterisks, GAL4/UAS combination significantly different from GAL4/+ and UAS/+ controls (Tukey, *P < 0.05, **P < 0.01, ***P< 0.001). Error bars indicate SEM.

UAS-*dDnmt2*-RNAi (10692R-3)

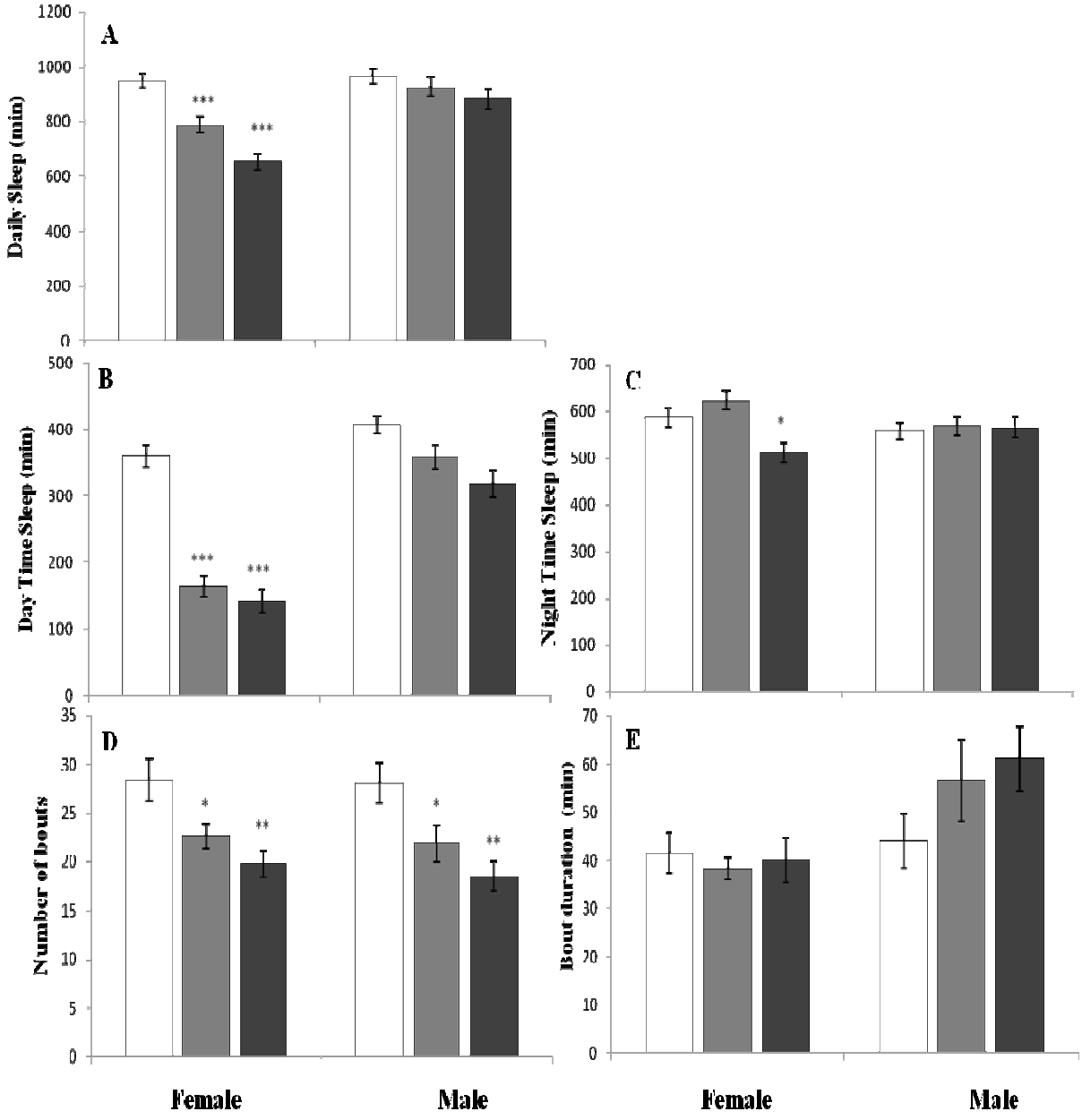


Figure 7.8: Sleep phenotype of *dDnmt2* knockdown flies (2R-3). Figure legend same as 7.7. (TukeyHSD, *P < 0.05, **P < 0.01, ***P< 0.001). Error bars indicate SEM.

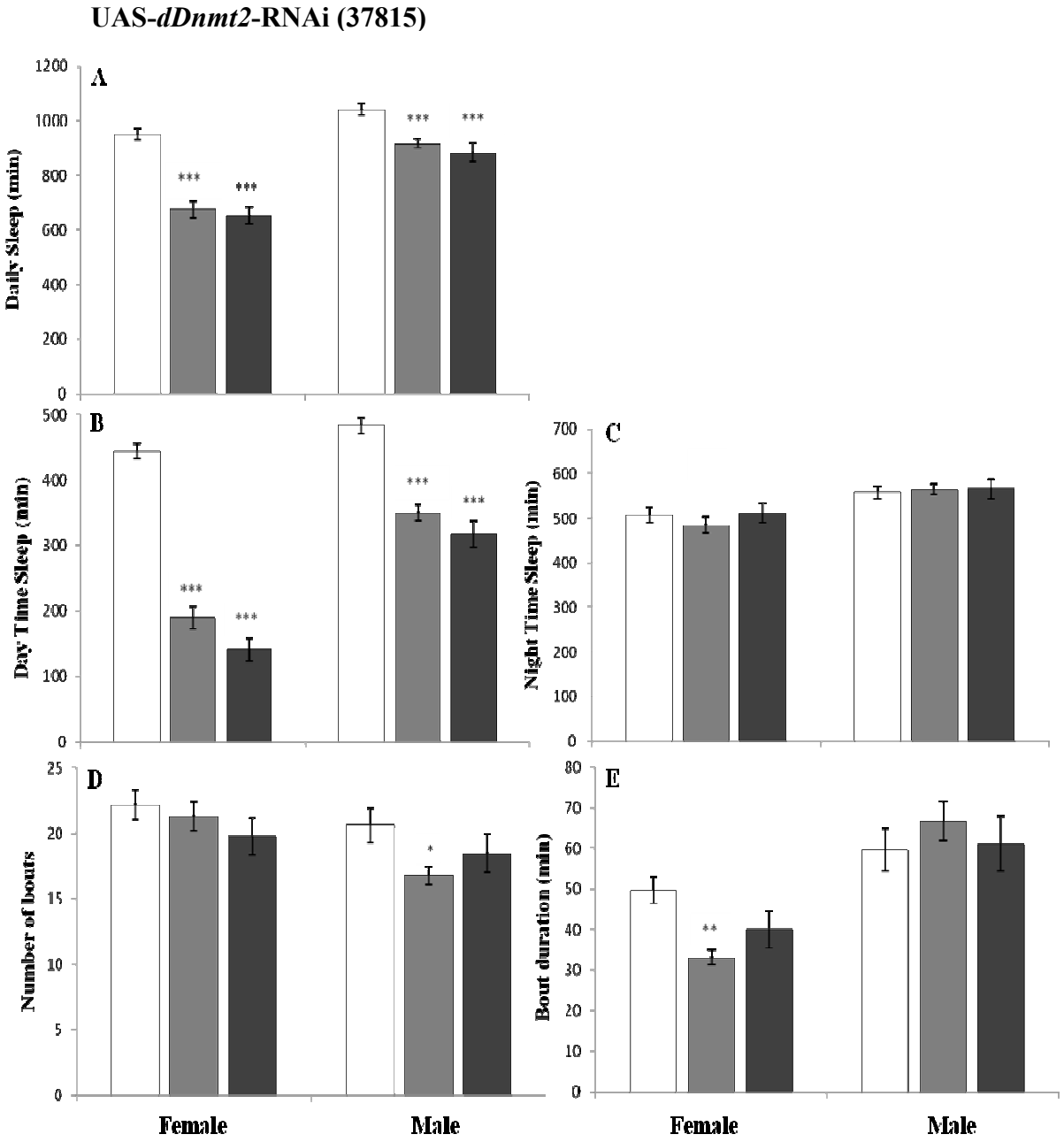


Figure 7.9: Sleep phenotype of *dDnmt2* knockdown flies (37815). Figure legend same as 7.7. (TukeyHSD, *P < 0.05, **P < 0.01, ***P< 0.001). Error bars indicate SEM.

UAS-*dDnmt2*-RNAi (37816)

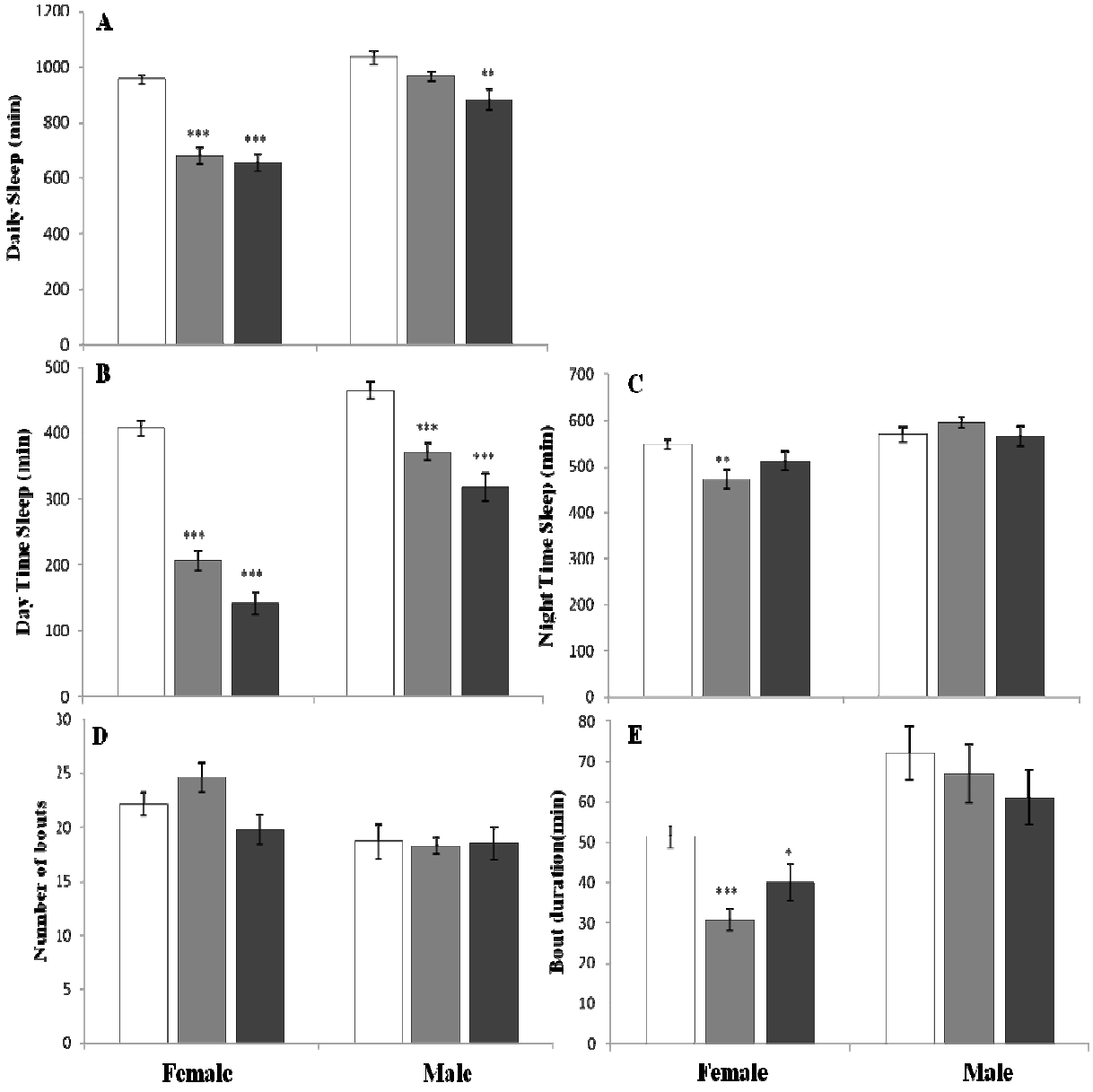


Figure 7.10: Sleep phenotype of *dDnmt2* knockdown flies (37816). Figure legend same as 7.7. (TukeyHSD, *P < 0.05, **P < 0.01, ***P< 0.001). Error bars indicate SEM.

7.3.3 Verifying dDnmt2 misexpression by qPCR

Relative expression of *dDnmt2* to the exogenous control gene *aequorin* was quantified to verify its knockdown and over-expression. PCR efficiency was calculated by plotting the threshold cycle value C(t) vs log10 concentration of the template used. Standard curves for both primer sets suggested efficient amplification, as indicated by correlation coefficients and linear regression slopes. Primers for *dDnmt2* and *aequorin* gave correlation coefficients of 1.00 and 0.99 respectively, with slopes of 0.30 for *dDnmt2* and 0.32 for *aequorin* (see appendix 5).

The standard curve method, using *aequorin* as an exogenous control allowed determining relative expression levels of *dDnmt2*. Figure 7.11 shows the relative expression of *dDnmt2* in the over-expressing lines in comparison to their background controls. RT-PCR data for three UAS-*dDnmt2* over-expressing lines driven by *actin*-GAL4 resulted in higher amounts of *dDnmt2*. Knock down of *dDnmt2* in multiple RNA interference lines was also verified by RT-PCR (Figure 7.12)

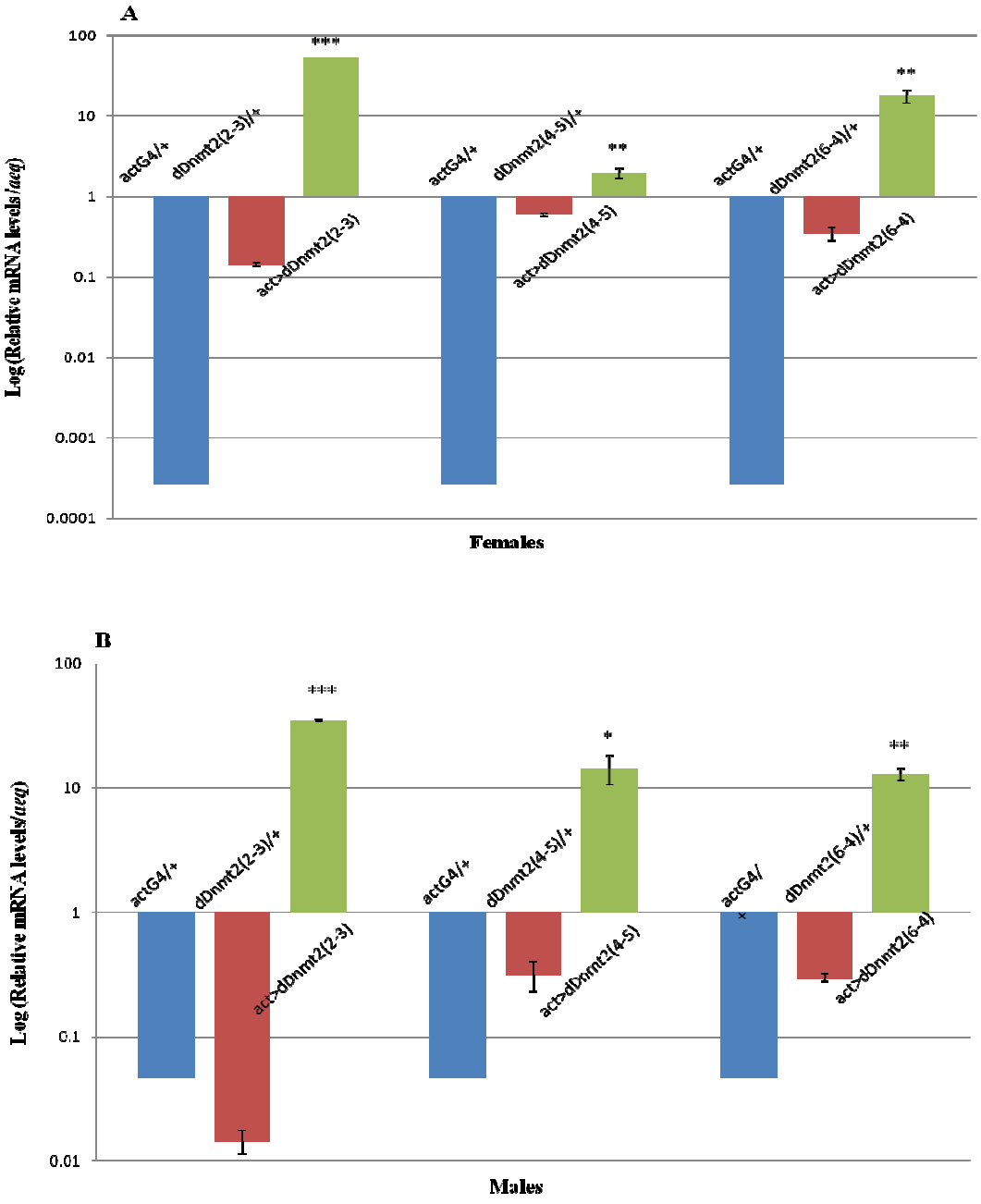


Figure 7.11: Quantitative RT-PCR analysis to verify expression levels of *dDnmt2*. **(A)** mRNA levels of *dDnmt2* relative to the endogenous control aequorin resulted in higher amounts of *dDnmt2* in the three independent *actin>dDnmt2* (green) lines compared to the UAS (red)and GAL4 (blue) controls for females. TukeyHSD *post hoc* test was performed comparing the UAS/GAL4 combination to their controls. Asterisks denote *P < 0.05, **P < 0.01, ***P< 0.001. Error bars indicate S.E.M. **(B)** Males, same as above.

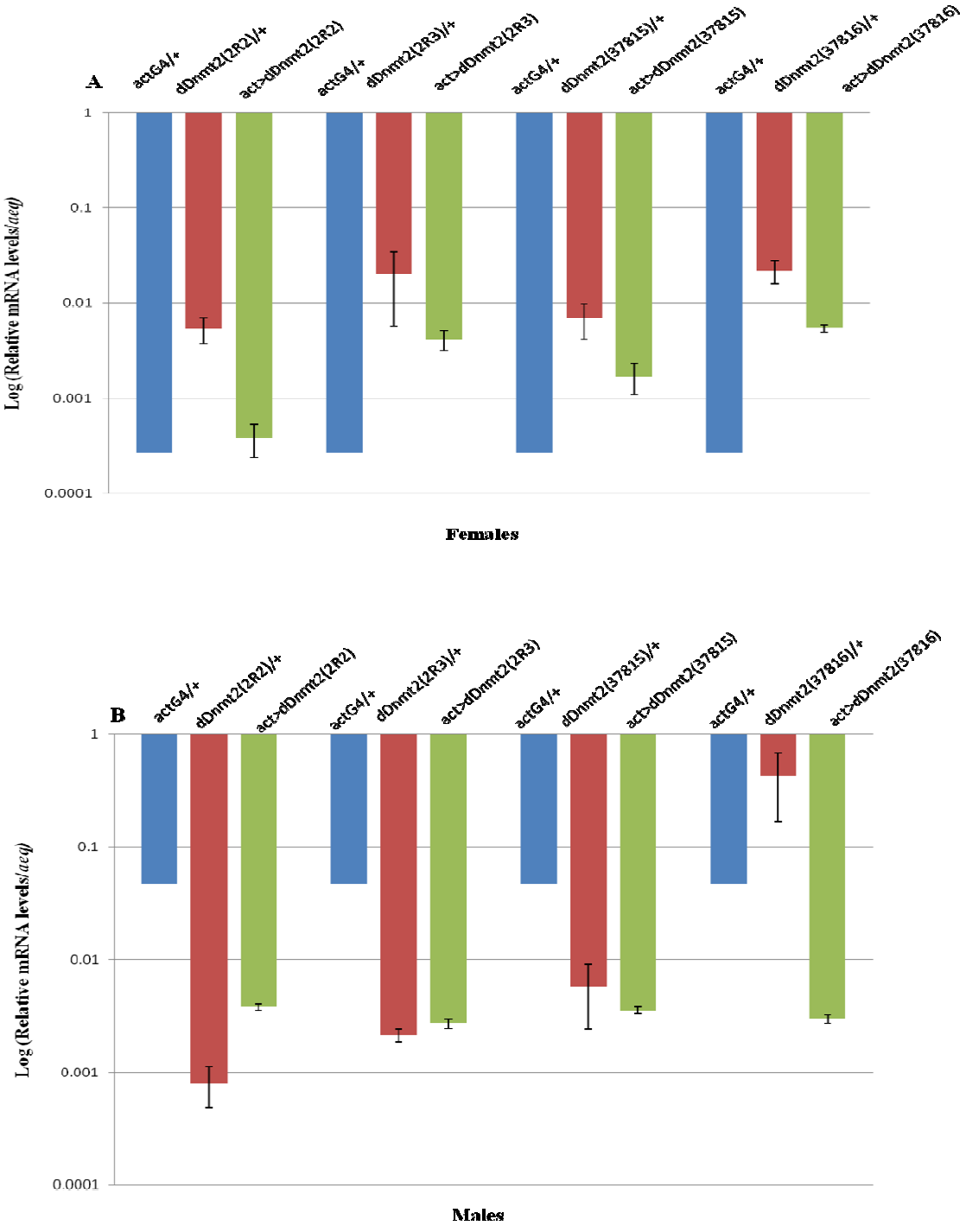


Figure 7.12: Characterization of *Drosophila* lines with *dDnmt2* knockdown using quantitative RT-PCR. **(A)** mRNA levels from flies expressing *actin>dDnmt2*-RNAi (green) and *actin*-GAL4/+ (blue); UAS-*dDnmt2*-RNAi/+ (red) are shown with marked knockdown of *dDnmt2* in females from all four lines. No significant differences were detected among the experimental and control lines. Error bars indicate S.E.M. **(B)** Males, same as above.

7.4 Discussion

The aim of this study was to analyse the importance of DNA methylation for sleep. Over-expression of *dDnmt2* resulted in increase of daily sleep, which was mainly due to increase in day-time sleep (particularly in females), and there is some suggestion that this increase in sleep was associated with longer bout durations (Figure 7.3). It is possible that the observed increase in life span following over-expression of *dDnmt2* (Lin *et al.* 2005) was mediated through increase in sleep, as other studies have already showed a link between sleep and longevity (Bushey *et al.* 2010).

Given the positive effect of DNA methylation on life span (Lin *et al.* 2005), why this trait has not been selected through evolution, and only minor level of methylation is currently present in *Drosophila*? It is possible that increasing levels of DNA methylation may interfere with other fitness traits such as reproduction and development time that counteract the effect on longevity.

Furthermore, knockdown of *dDnmt2* (Figure 7.7-7.10) also led to an increase in sleep, which here too was associated with increase of bout duration (Figure 7.7-7.10E) Also, DNA methylation levels in *Drosophila* are highest in young embryos, while later stages reveal distinctly low levels (Lyko 2001). This profile closely mirrors the expression pattern of *dDnmt2* (Lyko 2001). Thus knockdown of already low levels of *dDnmt2*, may not affect any behavioural phenotype or show any apparent abnormality.

Thus, these results suggest that *homeostasis* of DNA methylation level is important for normal sleep performance, and any deviation from this balance may be detrimental for

the fly. Studies in mammals have shown that aging is associated with both increases and decreases in methylation, a process which is tissue and gene dependent (reviewed by Richardson 2003).

In addition, the effect of *dDnmt2* miss-expression on sleep was sex-specific, suggesting that DNA methylation may target different loci in males and females, and may contribute to the sexual dimorphism in this trait (Chapter 6).

It also worth noting that using a ubiquitous GAL4 driver such as *actin* has different implications for over-expression and dsRNAi silencing. While silencing was targeted in all tissues normally expressing *dDnmt2* (although qPCR has not verified this), over-expression of *dDnmt2* occurred in tissues that normally do not express *dDnmt2*. Thus, it is possible that over-expression and silencing occurred in non-overlapping subset of cells, and therefore may drive different processes although exhibiting a similar phenotype (more sleep).

A more direct approach might be using specific drivers that target genes or brain structures that are known to be involved in sleep. This will also help understanding whether DNA methylation has a direct impact on sleep (regulating expression level of sleep genes) or indirectly by, for example changing expression level of genes that are involved in metabolism.

Chapter 8: General Discussion

Drosophila has been a powerful model organism in many research areas, offering a relatively simple system in terms of its genome and nervous system size. The implicit rationale was that research in the fly will allow insights that can be translated into working hypotheses to be tested in more complicated systems (i.e. vertebrates). A classic example for this approach is chronobiology, where the first circadian gene (*period*) was identified in *Drosophila* (Konopka and Benzer 1971) and later inspired the identification of the mammalian orthologs. In contrast, sleep research in *Drosophila* is relatively young, compared to research in mammals. Studies on genetics of sleep in human subjects were carried out as early as in the 1930s (reviewed in Young, Lader & Fenton 1972). In fact, most of the studies during 2000-2005 in *Drosophila* were aimed at establishing the fly as a valid model for sleep research, and demonstrating that rest in flies share many similarities with mammalian sleep, including sleep-specific brain electrophysiology, similar effect of pharmacological agents and similar role of biogenic amines (see Introduction)[10]. Only in recent years, sleep research in *Drosophila* has entered into a new phase in which new avenues are being explored, taking advantage of the powerful tools available for this model system for genetic dissection. Some of the experiments described here may further open new avenues for sleep research, including the genetic variation associated with sleep in wild populations (Chapters 3-4), and the role of maternal factors (Chapter 3), epigenetics (Chapter 7) and cellular basis for sleep sexual dimorphism (Chapter 6). Results from this

[10] Suggesting that the mouse is an excellent model system to study the fly (!)

broad study confirm the complexity of behaviours such as sleep which may be manifested by multi-level processes and takes the advantage of *Drosophila* to explore various different aspects of sleep. However, conducting a large study like this one, may possibly lose its focal point to explore sleep using a particular method.

Quantitative genetics was one of the areas where *Drosophila* was intensively used, and QTL mapping has been widely employed in the analysis of genetic variation of morphological traits such as sensory bristle number and wing shape (reviewed by Mackay 2001). Only recently, the methodology has been recruited for genetic dissection of behavioural phenotypes such as aggression (Edwards and Mackay 2009), courtship (Gleason, Nuzhdin & Ritchie 2002), locomotion (Jordan, Morgan & Mackay 2006), and recently for sleep (Harbison *et al.* 2009) . Indeed, QTL mapping and genome-wide association studies are particularly suitable for analysing complex phenotypes such as behavioural traits (see Chapter 4, Introduction), which apparently are driven by large number of genes. Although genome scans may initially indicate small number of QTL, for example only 4 QTL were suggested for male mating behaviour (Moehring, Mackay 2004), and 4 QTL for locomotor behaviour (Jordan, Morgan & Mackay 2006), and 5 sleep QTL identified here (Figure 4.5), subsequent deficiency mapping in each of these studies indicated a more complicated genetic architecture with multiple loci nested in the original QTL. The "large number of loci" hypothesis was also supported by quantitative genetic analysis using p-element insertions. Harbison and Sehgal (2008) analysed sleep in a set of 136 P-element insertion lines and found 21 mutants (15%) that showed aberrant sleep patterns. Yamamoto *et al.* (2008) studied startle-induce locomotion (a phenotype which

obviously depends on the sleep/waking state of the fly) and found a staggering 267 insertion lines out of 720 (37%) that had effect on this phenotype. Global expression profiling by microarrays also suggested large number of genes associated with sleep: Cirelli *et al.* (2005) identified 263 genes that showed sleep related differential expression, and a similar number was identified by Zimmerman *et al.* (2006). Overall it is likely that sleep is driven by large number of genes, although it is important to distinguish between those loci which will be under strong purifying selection and consequently monomorphic, and those that will be polymorphic and under some sort of balancing selection. Naturally, only polymorphic loci are targeted in QTL screens, and those are the loci that will be contributing to phenotypic variation and the evolution of sleep (see Chapter 4). Complementation tests using deficiency strains and P-element insertion lines identified *Rab9* and *CG9328* genes respectively. Sequence analyses of these genes in the parental strains would reveal that variation (if any) in these genes contribute to variation between the two parental strains. In addition, targeted gene knockdown in the parental strains and analysing sleep patterns will also validate its role. Other candidate genes (Appendix 2) overlapping the QTL regions can be tested using quantitative complementation tests and validate their role (if any) in sleep.

Experiments conducted in Chapter 5 were a follow-up study describing the role of *Dopamine acetyl-transferase* in sleep and identifying dopaminergic neurons by generating *Dat*-GAL4 transgenic strains. Dopaminergic clusters were identified, but staining *Dat*-GAL4 brains with Tyrosine Hydroxylase (TH) would confirm these clusters. Also, generating a transgenic strain for the other *Dat* isoform and staining neurons of both

isoforms would be important in identifying dopaminergic circuitry and ablating these neurons would aid validating the role of *Dat* in sleep in *Drosophila melanogaster*.

Using UAS-GAL4 approach, sleep sexual dimorphism was also explored (Chapter 6). Different brain specific drivers were used to identify regions important for sexual dimorphism. In particular, mushroom bodies (MB) were found to be underlying sleep sexual dimorphism, however additional drivers expressing in MBs would narrow down specific clusters. Additionally, studying morphological traits may also aid in identifying male and female specific characteristics that may be responsible for sex specific sleep behaviour. The role of DNA methylation in sleep regulation was also investigated (Chapter 7) which has been previously reported for life-span in *Drosophila melanogaster* (Lin *et.al* 2005) Overexpression of *Dnmt2*, revealed that *homeostasis* of DNA methylation is important for normal levels of sleep, explaining link between sleep and life-span.

Studying natural populations provides an opportunity to learn the extent of genetic variation as well as the "real world" phenotypic variation, which may be different, and more informative, than the behaviour of standard lab strains. The difference in sleep between SAL and Hu strains (Chapter 3) fits well with their geographical origin, but a systematic study of large number of strains should be carried to test whether this spatial correlation exists. The same populations have been used to study natural variation in the circadian clock gene *timeless* (Tauber *et al.* 2007) , which involves two alleles segregating in European and North American populations. The frequency of the alleles follows a

latitudinal cline[11] and the two TIM isoforms mediate different sensitivity to circadian light entrainment. The study of *timeless* demonstrated how studying natural variation can provide a better understanding of the mechanism, as well as the evolution of the trait. To gain new insights on the real phenotypic variation, the next step would be to look at natural strains, under natural conditions. Indeed, experiments testing the circadian activity of flies monitored outdoors show a very different picture from the one seen in the lab (Bhutani, 2009, PhD Thesis). The natural social environment is also very different from the usual conditions in the laboratory, and has a major impact on behaviour and sleep. Flies exposed to group of flies slept more than socially deprived flies (Ganguly-Fitzgerald, Donlea & Shaw 2006a), and the sleep pattern of pairs of flies dramatically changes in heterosexual pairs compared with homosexual pairs, with males becoming nocturnal rather than diurnal (Fujii *et al.* 2007). A future challenge would be to understand the gene-environment (GxE) interactions associated with sleep, and for this purpose, monitoring flies in more natural conditions will allow the flies to exhibit a wider phenotypic range.

The effect of social experience on sleep adds another dimension for the regulation of sleep, which seems to involve multiple mechanisms, including gene transcription and translation, neural activity and possibly epigenetic changes (e.g. Chapter 7). This largely mirrors the regulation of the circadian clock, which is also mediated by multi-level mechanisms (reviewed in Cermakian, Sassone-Corsi 2000). Presumably, the presence of

[11] Although the cline itself reflects the spreading of the new allele, rather than a selection process.

multiple mechanisms contributes to the robustness of the circadian system, allowing the pacemaker to endure environmental perturbations. As for sleep, the various mechanisms may reflect the fact that sleep serves various functions (See Introduction), and/or the fact that sleep is a complex phenotype composed of many different traits. A recent QTL study in mice for example, interrogated 20 sleep-wake traits (Winrow *et al.* 2009). In this context, it may also be noted that the utility of *Drosophila* as a model for sleep research is not infinite. Some mammalian traits such as EEG or REM sleep cannot be studied in the fly. Whether flies can dream, remains an open question, although some recent studies (reviewed by Swinderen 2005) suggest that flies may be useful for understanding consciousness.

Appendix 1

The components of 11.1 X PCR buffer are listed below.

Component	Concentration	Volume (676 µl)	Final Concentration
Tris. HCl (pH 8.8)	2M	167µl	45mM
Ammonium Sulphaste	1M	83 µl	11mM
Magnesium Chloride	1M	33.5 µl	4.5mM
2-mercaptoethanol	100%	3.6 µl	6.7mM
EDTA (pH 8)	10mM	3.4 µl	4.4mM
dATP	100mM	75 µl	1mM
dGTP	100mM	75 µl	1mM
dCTP	100mM	75 µl	1mM
dTTP	100mM	75 µl	1mM
BSA	10mg/ml	85 µl	113µg/ml

Primer sequences and their annealing temperatures (AT) used in this project are listed in the table below.

Primer Name	Primer Sequence	Amplicon Size	AT
DatGAL4 5’	CACTCGTCCTGTTCCAGCT	2.1kb	65°C
DatGAL4 3’	AGAATCTTATATGTACTCGAATGCTA		65°C
DatGAL4_NotI 5’	GTTGCTCACCTTCCGTTCAT	185bp	65°C
DatGAL4_NotI 3’	TCGTCACTTGGTCACACTGG		65°C
DatGAL4_EcoRI 5’	GCAGAGAGATTGGGATTGGA	153bp	65°C
DatGAL4_EcoRI 3’	GAATTCTTCTCGCGATTTGG		65°C
dnmt2 5’	CAAAAGAGTGCTGGTCATGG	113bp	64.8°C
dnmt2 5’	AAAGCGGTGTGTATGCAGAG		64.8°C
aeq 5’	TTGACGAGATGGTCTACAAGGCATC	100bp	64.8°C
aeq 3’	GAAGGCTTCTACAGCATCTTTGTGTCGT		64.8°C

R-script to calculate sleep:

```
# Eran Tauber (c) 2007

# sleep.R is R program that takes activity data (TriKinetix) and calculate different sleep #(rest) properties. Data are from 4 days in LD at 5 min bins.

# data provided with activity in columns, with header (e.g. fly name).
# the data file should be stored as c:/localdata/sleep.txt
# the exact number of flies in the file should be provided to R when asked
# the result file will be found in the same folder as "sleep.out"
# Data will be appended to the result file every time the analysis run,
# the program can be run using the Source command from the File menu.
# the data file will be deleted at the end of the analysis
# Please acknowledge the use of the program.
cat("\n","sleep.R was written by Eran Tauber (c) 2007","\n","\n","\n","\n") # prompt
cat("\n","                Press Enter to continue...","\n") # prompt
dummy.variable <- scan(n=1)
data <- read.delim("c:/localdata/sleep.txt", header = FALSE, sep ="\t")

cat("\n","Hello! How many flies we're going to analyse today?","\n") # prompt
user.input <- scan(what = 'numeric', n=1) # get input from user


# the rle function counts the runs of 0s
# we count for each L and D separately
L1.sum <- numeric() # to initiate variable
```

```
L2.sum <- numeric() # to initiate variable
L3.sum <- numeric() # to initiate variable
L4.sum <- numeric() # to initiate variable
D1.sum <- numeric() # to initiate variable
D2.sum <- numeric() # to initiate variable
D3.sum <- numeric() # to initiate variable
D4.sum <- numeric() # to initiate variable
total.sleep <- numeric()
LDratio <- numeric() # ratio between light and dark sleep (strength of siesta)
median.interval <- numeric() # median of sleep bout

# check here how many data to analyse
for (i in 1: as.numeric(user.input)) {
L1 <- rle(data[2:145,i]==0) # we skip the first lines with the headers
L2 <- rle(data[290:433,i]==0)
L3 <- rle(data[578:721,i]==0)
L4 <- rle(data[866:1009,i]==0)
D1 <- rle(data[146:289,i]==0)
D2 <- rle(data[434:577,i]==0)
D3 <- rle(data[722:865,i]==0)
D4 <- rle(data[1010:1153,i]==0)

# the runs of zeros (value=true)
L1.sum[i] <- sum(L1$lengths[L1$values == TRUE])
L2.sum[i] <- sum(L2$lengths[L2$values == TRUE])
L3.sum[i] <- sum(L3$lengths[L3$values == TRUE])
L4.sum[i] <- sum(L4$lengths[L4$values == TRUE])
D1.sum[i] <- sum(D1$lengths[D1$values == TRUE])
D2.sum[i] <- sum(D2$lengths[D2$values == TRUE])
D3.sum[i] <- sum(D3$lengths[D3$values == TRUE])
D4.sum[i] <- sum(D4$lengths[D4$values == TRUE])

total.sleep[i] <- sum(L1.sum[i],L2.sum[i],L3.sum[i],L4.sum[i],D1.sum[i],D2.sum[i],D3.sum[i],D4.sum[i])

LDratio[i] <- mean(L1.sum[i]/D1.sum[i], L2.sum[i]/D2.sum[i], L3.sum[i]/D3.sum[i], L4.sum[i]/D4.sum[i])

median.interval[i] <- median(c(L1$lengths[L1$values == TRUE],L2$lengths[L2$values ==
TRUE],L3$lengths[L3$values == TRUE],L4$lengths[L4$values == TRUE],D1$lengths[D1$values ==
TRUE],D2$lengths[D2$values == TRUE],D3$lengths[D3$values == TRUE],D4$lengths[D4$values ==
TRUE]))

}

sleep <- data.frame(t(data[1, ]),total.sleep, LDratio, median.interval)

write.table(sleep, "c:/localdata/sleep.out", append = TRUE, sep = "\t")
file.remove("c:/localdata/sleep.txt")
q("no")
```

Appendix 2

Candidate genes from different microarray and association studies that overlap with the deficiencies that failed to complement are listed below.

	Cirelli *et al.* 2005	Zimmerman *et al.* 2006	Harbison *et al.* 2009
Df(2L)TW137 Df(2L)VA12		CG10383 CG16772 CG9338	CG17549[1] Rab9[2] CG9987[2] CG31797[2] CG10165[2] CG16772[2] CG10447[2] CG10262[2]
Df(3R)T-32	DopR CG6218 CG14852 CG31344	CG7530 CG11686	CG6672[1] Ugt35b[1] Akt1[2] CG14701[2] CG6719[2] CG10898[2] CG14711[2] CG14722[2] CG17202[2] CG18549[2] CG18616[2]

1: SFP, single feature polymorphism, referring to probes of Affymetrix arrays that showed variable signal when hybridised with DNA

2: variation in transcript levels (QTT, quantitative trait transcript)

Appendix 3

Sequence of pPTGAL

Available from:

https://dgrc.cgb.indiana.edu/files/repository/1225%20pPTGAL%20-%20sequence.doc?id=5c21a374b453bffb9a6564e8b57386a6

Sequence of *Dat* (Promoter region)

2R: 20025108, 20027327

```
   1 CCAGCCGTGCATCGGGTACTGGGTGATGTCCTCCGCAGCACTGCCGCTCCGCATGGTGCG
  61 CTGCACATCCGGATCCAGAAATCCGCACTCGCCCACGCTCCACTCGTCCTGTTCCAAGCT
 121 ACTGGCCCGGGCAGCTGCCGCCGCGGCCGCCGTGGCAGCCTGGAAGTCCGGAGGCTCTGC
 181 AACGCTGctgtggatatgcgtatgtatgtgcatttagatggttatgctctgtatgtaggc
 241 acgtctgtgggttcttgccggtggtctgttgctcacCTTCCGTTCATGGTGCTCGGATCT
 301 GCCTCCGCCGGCAAGGTCGAGGGATACATCTTTTGTACCTATATTCAACAGGCGGTCAAC
 361 CTGTAGCTCGATGTGTTACAAATTTTCTTTTATTTATAAAAGATATTTTTAATGAATTAA
 421 AATATGTAACGTCCAGTGTGACCAAGTGACGAACggtcagccatgcctctatgttgcaaa
 481 tatacctcttggtaccaaacaagtcataatttctatttctaagttcatttttgattttg
 541 cggagataatcaatggctatatgattattagaaatctatagaacagttcattaatttaaa
 601 gaagagtagttcattgatacagtcttaaataggtataagcataaatgccttcagtatttt
 661 tcagcccgccaacatccaacttggcagcactggcgaagttaacagtgtactgttttaatt
 721 tgggatgttttgaaagcatttaaaagaaagtcagcaaagatttatgtggtgcttttatgt
 781 tttcccatgatttcagcttagaatttctgtaagttacccataaaacaagtgagttagcat
 841 tcaaaatgtggaacttggagccttggtggcttttaagctgcactgttaagcttaacagcc
 901 acgaccgttacaactgttaccaagttcaaatgctgagtcaggtgaaaattgaagtaaaca
 961 agcaaagcttagttcattggactgcggctggcccattaactaaacaaaatgactgaaagt
```

1021 gcaagccagcgttatctgagacccagacgaccgcctagaccgaacagaacagttccgcgg

1081 ttcaaaaaacacggatcaaagcacgagctctctttagtctcacttaattatgtttgctcc

1141 gatttgactaaagcaaataaagtatgccttgaaccgaggcattctacaaccttatcagac

1201 gcgtttgttcccgctgttatcggaaacggaaaagctcgcttgcgctttcactccgcggtt

1261 tcactccggatttcgagcaacgcggcgcaacgcccgccccacggattttggggttgaccg

1321 ccaccccgagtgttacaagtatccaagttgcgagtgggagctgagcccgaggggttggat

1381 tggtttgtttaccacggaggccccactttcactcgctcgctctccccaaatcacaatctt

1441 tctctgcctcggctcccaaatctctcttgctagtggcgatttactcaaatcagaacgctc

1501 atttaaacggaaggttacaatgttcccataaacagtggattcttcttatcaatacattca

1561 tttatgtatttattaaaacttttagatggaatataagtttaaatattttaagacttttaa

1621 gatattttgaatatgttatttgatttctttaaaagtggtaatttcgtgatttatttaaaa

1681 ataattacattgcttgccaagaaagcgcctggagttatgcaacatatttgattgtgttaa

1741 aaatgttatccccttagtatattaatttatataacttattcgaatgcgattcccttaaat

1801 ccccacttaaatccactgattacttcctccgctgacattgagtagtctgcagagagaact

1861 tggtagcccgaggaggaaacccactcaatgaatgagcagaacgcagaacggatgggcaga

1921 gagattgggattggaagcgactgagttttgagttcgaccggcgaactatttaagcaacg

1981 ctcatttgctcattgcatttCAATGTGTTATCGTTGGCCATTAAAAAATAACACGAAAAC

2041 GTTGGTCCCAAATCGCGAGAAGAATTCCACCTCCCTAGCATTCGAGTACATATAAGATTC

2101 TCAAGCCTGCAAAAGCTGGGCATCATCATTTCAAAAACGTGCTAACGGTTCACTTGGTCG

2161 GTCGAATCGGAACGAATCGGGCGAAAGTCTCCAACACAAATTCCGAAATTTAACGCTTCG

Dat (putative promoter region) was cloned into pPTGAL vector at the *EcoR*I and *Not*I restriction sites. The plasmid map is shown in Figure 1. The insert (promoter region) was sequenced and aligned to the database sequence in order to confirm its integrity. This sequence alignment can be seen in Figure 2.

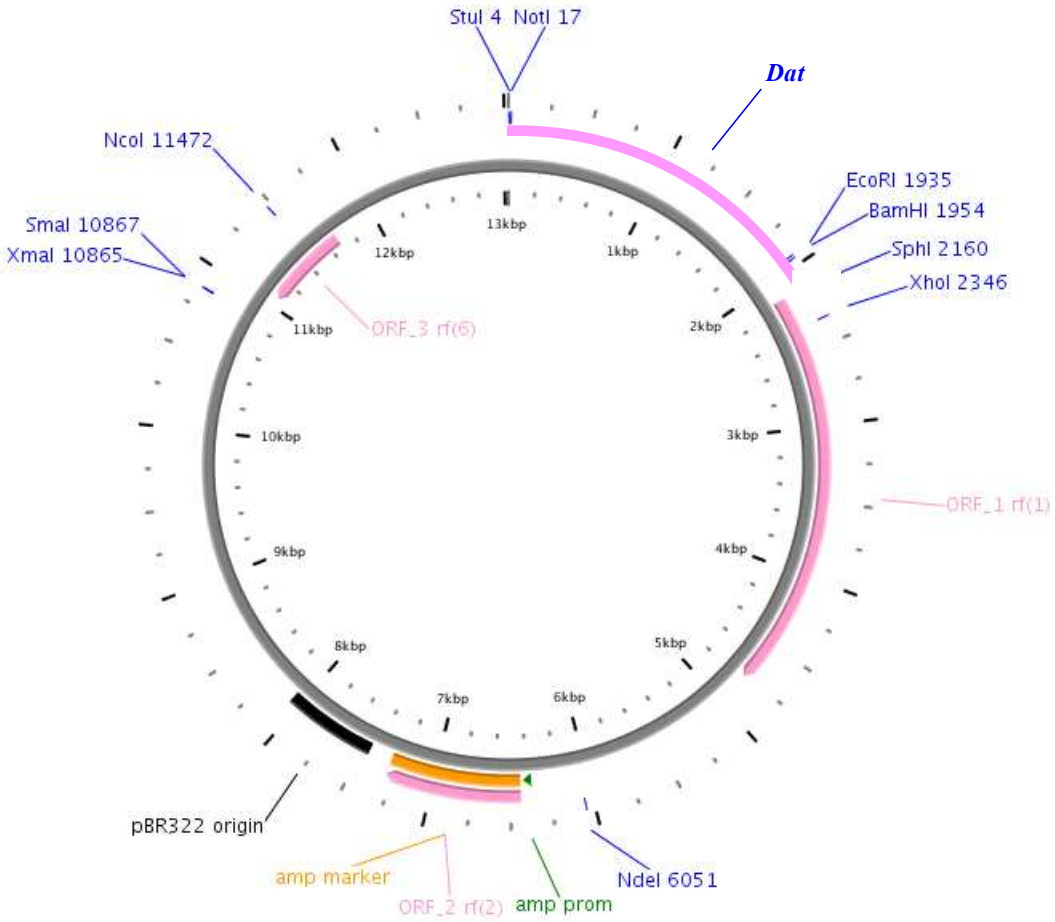


Figure 1: Plasmid map containing putative promoter of *Dat*

```
Query  1     CGGGCAGCTGCCGCCGCGGCCGCCGTGGCAGCCTGGAAGTCCGGAGGCTCTGCAACGCTG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  128   CGGGCAGCTGCCGCCGCGGCCGCCGTGGCAGCCTGGAAGTCCGGAGGCTCTGCAACGCTG  187
Query  61    CTGTGGATATGCGTATGTATGTGCATTTAGATGGTTATGCTCTGTATGTAGGCACGTCTG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  188   CTGTGGATATGCGTATGTATGTGCATTTAGATGGTTATGCTCTGTATGTAGGCACGTCTG  247
Query  121   TGGGTTCTTGCCGGTGGTCTGTTGCTCACCTTCCGTTCATGGTGCTCGGATCTGCCTCCG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  248   TGGGTTCTTGCCGGTGGTCTGTTGCTCACCTTCCGTTCATGGTGCTCGGATCTGCCTCCG  307
Query  181   CCGGCAAGGTCGAGGGATACATCTTTTGTACCTATATTCAACAGGCGGTCAACCTGTAGC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  308   CCGGCAAGGTCGAGGGATACATCTTTTGTACCTATATTCAACAGGCGGTCAACCTGTAGC  367
Query  241   TCGATGTGTTACAAATTTTCTTTTATTTATAAAAGATATTTTTAATGAATTAAAATATGT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  368   TCGATGTGTTACAAATTTTCTTTTATTTATAAAAGATATTTTTAATGAATTAAAATATGT  427
Query  301   AACGTCCAGTGTGACCAAGTGACGAACGGTCAGCCATGCCTCTATGTTGCAAATATACCT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  428   AACGTCCAGTGTGACCAAGTGACGAACGGTCAGCCATGCCTCTATGTTGCAAATATACCT  487
Query  361   CTTGGTACCAAACAAGTCATAATTTTCTATTTCTAAGTTCATTTTTGATTTTGCGGAGAT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  488   CTTGGTACCAAACAAGTCATAATTTTCTATTTCTAAGTTCATTTTTGATTTTGCGGAGAT  547
Query  421   AATCAATGGCTATATGATTATTAGAAATCTATAGAACAGTTCATTAATTTAAAGAAGAGT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  548   AATCAATGGCTATATGATTATTAGAAATCTATAGAACAGTTCATTAATTTAAAGAAGAGT  607
Query  481   AGTTCATTGATACAGTCTTAAATAGGTATAAGCATAAATGCCTTCAGTATTTTTCAGCCC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  608   AGTTCATTGATACAGTCTTAAATAGGTATAAGCATAAATGCCTTCAGTATTTTTCAGCCC  667
Query  541   GCCAACATCCAACTTGGCAGCACTGGCGAAGTTAACAGTGTACTGTTTTAATTTGGGATG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  668   GCCAACATCCAACTTGGCAGCACTGGCGAAGTTAACAGTGTACTGTTTTAATTTGGGATG  727
Query  601   TTTTGAAAGCATTTAAAAGAAAGTCAGCAAAGATTTATGTGGTGCTTTTATGTTTTCCCA  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  728   TTTTGAAAGCATTTAAAAGAAAGTCAGCAAAGATTTATGTGGTGCTTTTATGTTTTCCCA  787
Query  661   TGATTTCANNTTAGAATTTCTGGNNNTTACCCATaaannnaGTGANTTANNATTCATAAT  720
             ||||||||  ||||||||||||    ||||||||||||   |||||  |||  |||||  |||
Sbjct  788   TGATTTCAGCTTAGAATTTCTGTAAGTTACCCATAAAACAAGTGAGTTAGCATTCAAAAT  847
Query  721   GTGNRACTTGGANCCTTGGTGGCTTTTAANCTGCNCNGNTAANCTTAACAGCCACNRCCG  780
             |||  ||||||||  ||||||||||||||||||  ||||  |  |  |||  ||||||||||||  ||||
Sbjct  848   GTGGAACTTGGAGCCTTGGTGGCTTTTAAGCTGCACTGTTAAGCTTAACAGCCACGACCG  907
Query  781   TTACAACTGTTACCAAGTTCAAATGCTGAGTCAGGTGAAAATTGAAGTAAACAAGCAAAG  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  908   TTACAACTGTTACCAAGTTCAAATGCTGAGTCAGGTGAAAATTGAAGTAAACAAGCAAAG  967
Query  841   CTTAGTTCATTGGACTGCGGCTGGCCCATTAACTACAC-AAATGACTGAAAGTGCAAGCC  899
             ||||||||||||||||||||||||||||||||||  ||  ||||||||||||||||||||
Sbjct  968   CTTAGTTCATTGGACTGCGGCTGGCCCATTAACTAAACAAAATGACTGAAAGTGCAAGCC
1027
Query  900   AGCGTTATCTGAGACCCAGACGACCGCCTAGACCGAACAGAACAGTTCCGCGGTTCAAAA  959
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1028  AGCGTTATCTGAGACCCAGACGACCGCCTAGACCGAACAGAACAGTTCCGCGGTTCAAAA  1087
Query  960   AACACGGATCAAAGCACGAGCTCTCTTTAGTCTCACTTAATTATGTTTGCTCCGATTTGA  1019
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1088  AACACGGATCAAAGCACGAGCTCTCTTTAGTCTCACTTAATTATGTTTGCTCCGATTTGA  1147
```

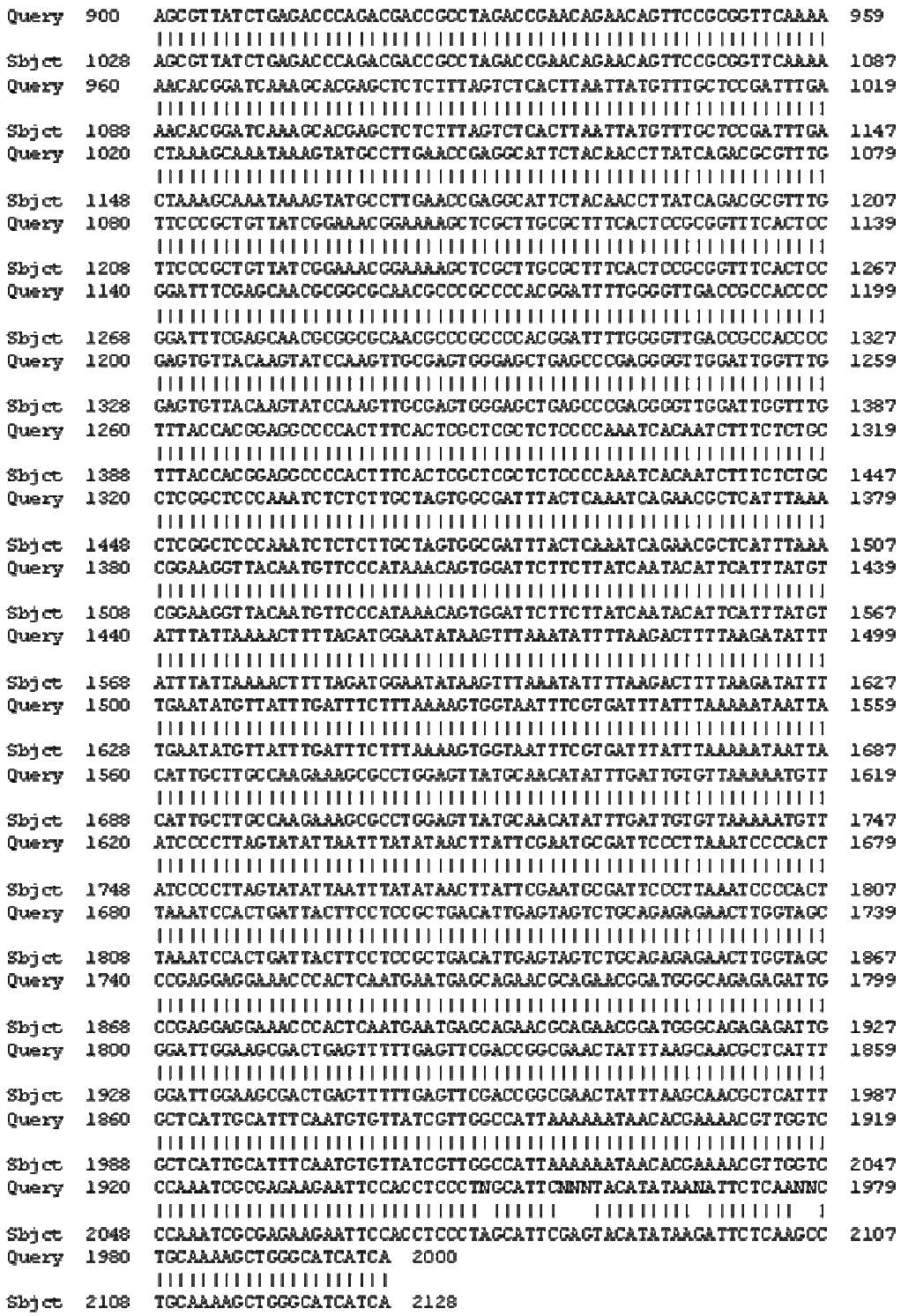


Figure 2: Sequence alignment of *Dat* (query) to database (sbjct) sequence.

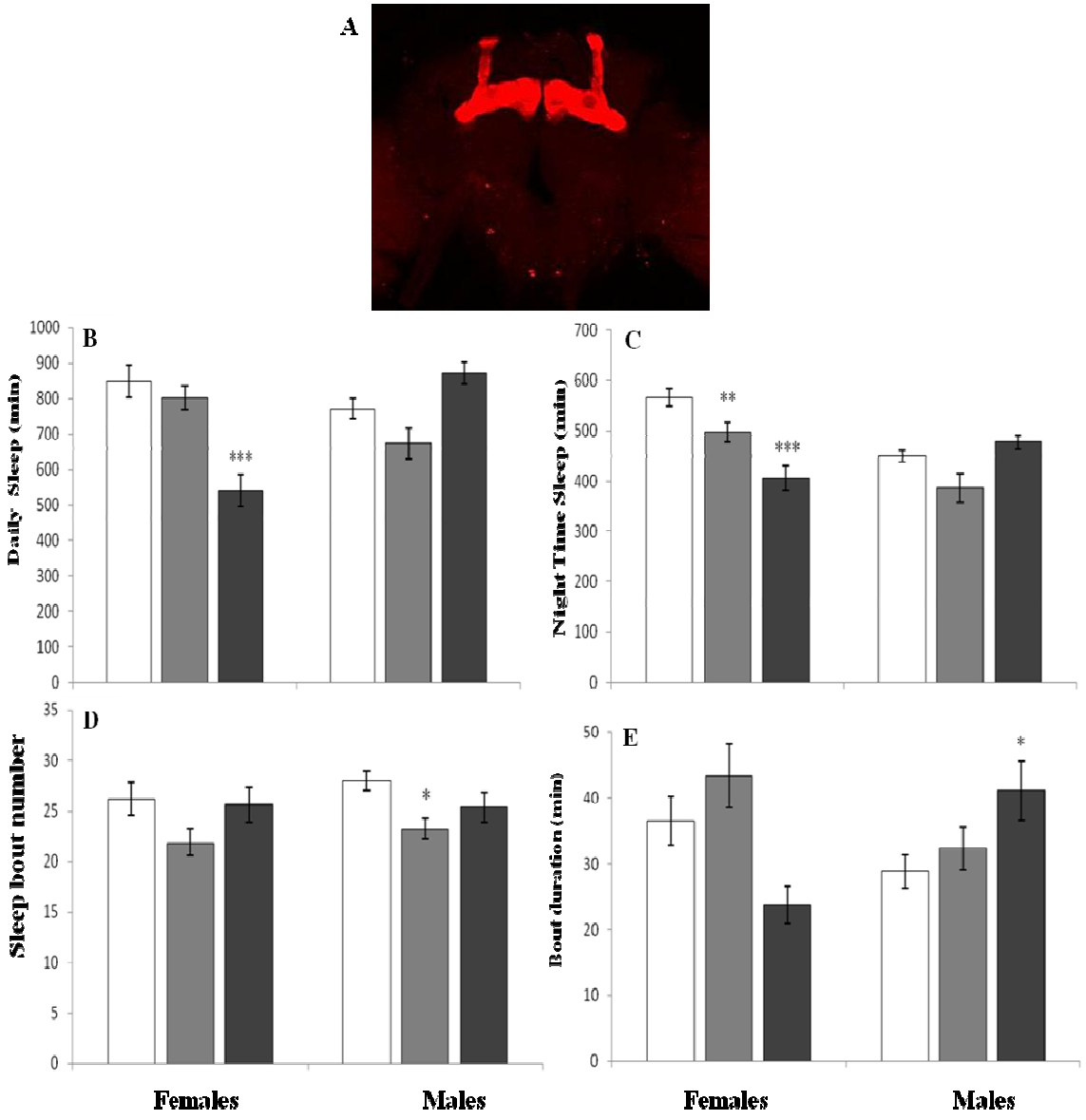


Figure 3: Confocal image showing RFP fluorescence in an adult fly brain in the transformant line 2M (A). Sleep phenotype of 2M with *Dat* ablated neurons (B-E). White bars, GAL4/UAS-hid/rpr; grey bars, UAS/+; black bars, GAL4/+. Males and females as indicated at the bottom. Asterisk, GAL4/UAS combination significantly different from GAL4/+ and UAS/+ controls (Tukey *post hoc*, *P < 0.05, **P < 0.01, ***P< 0.001). Error bars indicate SEM.

Appendix 4

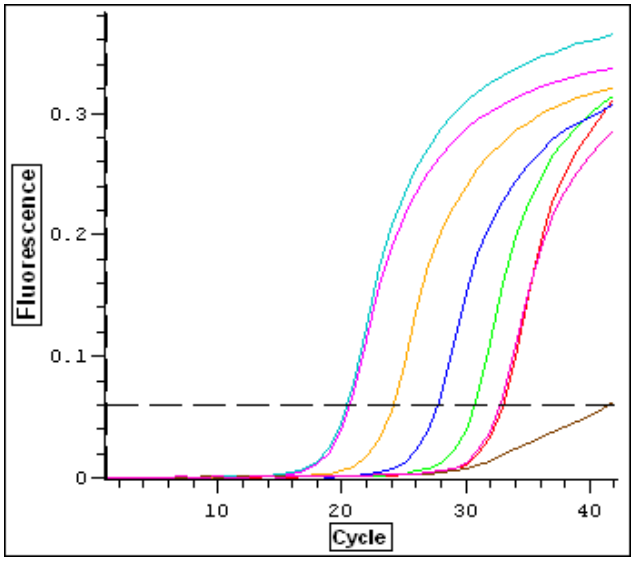
Table 1: Siesta time interval for each strain carrying a single transgene (GAL4/+) is listed below.

Strain	Siesta Interval (ZT)
actin-GAL4	5.5-9
*elav*GAL4	5-9
*elav*GAL4[2]	5-9
1471GAL4	4.5-8
repo-GAL4	5-9
takeout-GAL4	5-9
121Y	5-8
30Y	5.5-8.5
103Y	5.5-8.5
voila-GAL4	4-9

Appendix 5

Standard curves for both primer sets *aequorin* (Figure 1) and *dDnmt2* (Figure 2) suggesting efficient amplification is shown below.

A



B

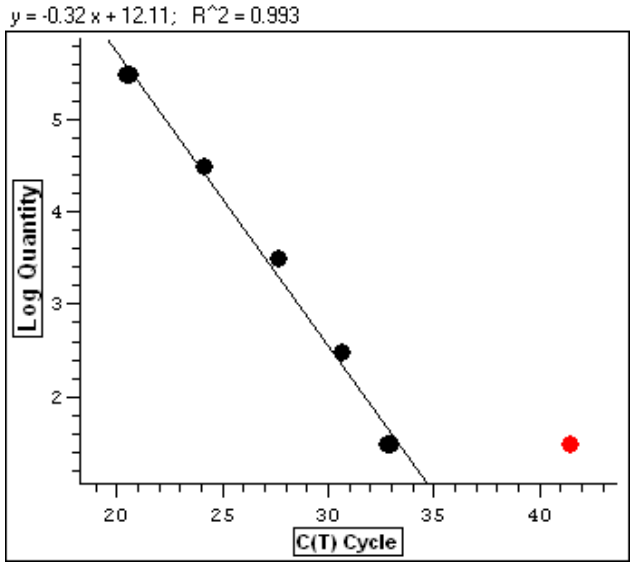


Figure 1: (A) Amplification profile of standard *aequorin* real-time PCR. Serial 5-fold dilutions per reaction were amplified for 42 cycles. (B) Standard curves for *aequorin* real

time PCR. Ct values were plotted against log10 concentration of template. The correlation coefficient was 0.993, and the slope was 0.32.

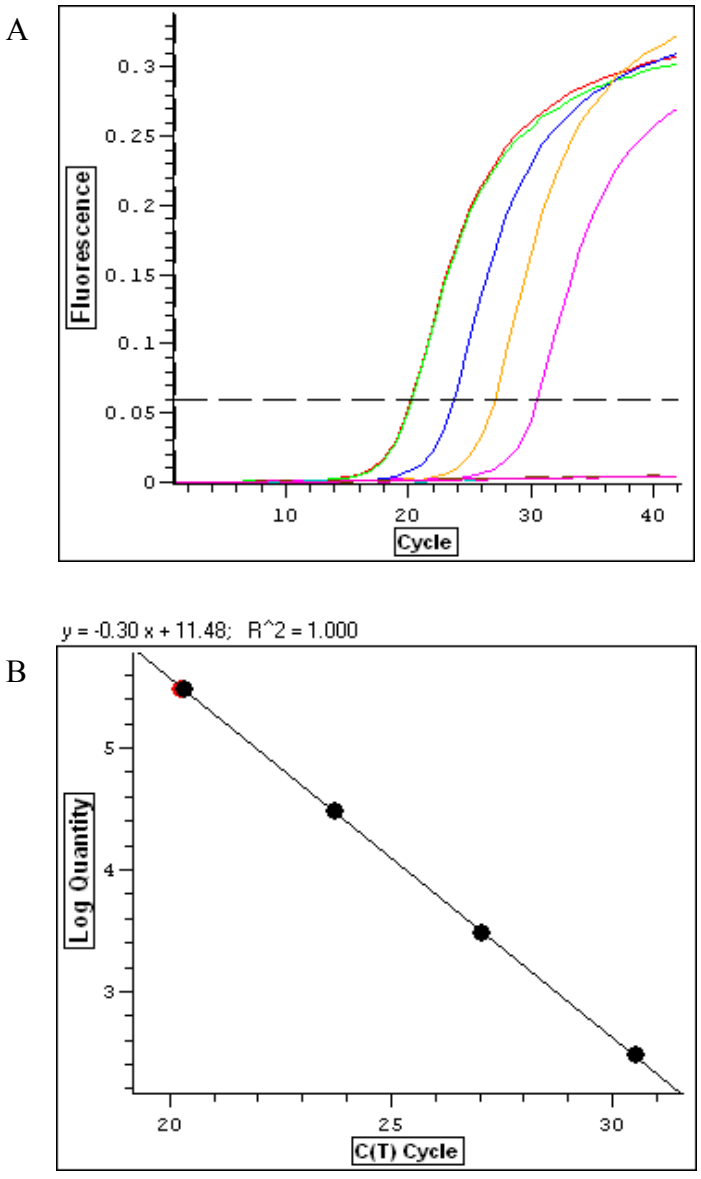


Figure 2: (A) Amplification profile of *dDnmt2* real-time PCR. Serial 5-fold dilutions per reaction were amplified for 42 cycles. (B) Standard curves for *dDnmt2* real time PCR. Ct values were plotted against log10 concentration of template. The correlation coefficient was 1, and the slope was 0.30.

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