miR-190 acts during pupation to influence adult sleep in Glu^{ACh} cells

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Dedicatoria

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Abstract

miR-190 Acts During Pupation to Influence Adult Sleep in Glu^{ACh} Cells

A Dissertation Presented to the Faculty

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By Emmanuel José Rivera-Rodríguez

Sleep is a widely conserved behavior, known to influence many cognitive and physiological functions. Studies have implicated microRNAs in the regulation of gene expression changes that control sleep behavior. The Griffith lab screened a library of 143 microRNA sponges using the fruit fly *Drosophila melanogaster*, in order to find microRNAs involved in sleep regulation (Goodwin et al., 2018). This screen showed miR-190 to be involved in the regulation of sleep. Flies expressing the miR-190-sponge (miR-190-SP) in a pan-neuronal manner showed decreased and fragmented sleep, as well as changes in other sleep parameters. Spatial and temporal mapping of its locus of action demonstrates that *miR-190* is required during the pupal stage, in a population of neurons that transcribe both glutamatergic and cholinergic genes, to which we refer to as Glu^{ACh} cells. Thermogenetic activation of this population of cells by means of dTRPA1 results in a decrease and fragmentation of sleep, phenocopying miR-190 knockdown. At the molecular level, RNA sequencing of both pupal brains and adult heads showed that pan-neuronal expression of miR-190-SP induces an upregulation of multiple genes, among those several cholinergic genes, as well as *midline*, a T-box

transcription factor previously shown to operate as a suppressor of glutamatergic fate. *Glu^{ACh} > mid* overexpression phenocopied *miR-190* knockdown with animals showing a decrease in total sleep and sleep fragmentation. Additionally, our RNAseq results show downregulation expression of dopaminergic genes upon sleep deprivation of miR-190-SP flies and altered neuronal activity in specific dopaminergic cells. Taken together, our results suggest that *miR-190* acts during development in Glu^{ACh} cells to allow their glutamatergic differentiation, as well as regulating neuronal activity of the sleep/arousal neuronal circuit, ultimately establishing the *Drosophila* adult sleep behavior.

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Chapter 1

Introduction

Sleep is a Conserved Behavior with Essential Functions

Humans have been curious about sleep throughout history. Descriptions of sleep and concepts such as insomnia, snoring and narcolepsy can be found in human records dating to the time of ancient Egypt, in the sixteenth century (Reviewed in Asaad, 2015). Sleep is a highly evolutionarily conserved behavior, known to be present in most animal species studied (Reviewed in Campbell and Tobler, 1984). Episodes of sleep are defined by a series of criteria including: behavioral quiescence and characteristic posture, increased arousal threshold, rapid reversibility, circadian distribution, homeostatic regulation, changes in genetic expression, and changes in brain electrical activity (Campbell and Tobler, 1984; Flanigan, 1973; Gilestro et al., 2009; Joiner, 2016; Kaiser, 1988; Shaw et al., 2000; Tobler, 1983).

The fact that sleep must take place even when it puts the organism at risk in its environment, with possible dangers such as predators, suggests it plays a critical role. Even though an ultimate function for sleep remains to be elucidated, its relevance can be observed by looking at disruptions arising from sleep loss. For example, evidence from both mammals and invertebrates demonstrates negative effects of sleep deprivation on learning and memory (Melnattur et al., 2021; Seugnet et al., 2008; Walker and Stickgold, 2004). In humans, sleep deprivation has been linked to increased anxiety and depression, according to self-reported symptom studies (Babson et al., 2010). In the most severe scenario, chronic sleep deprivation can lead to death, as has

been shown in several organisms, including rats (Rechtschaffen et al., 2002), flies (Shaw et al., 2002), cockroaches (Stephenson et al., 2007), and humans (Montagna, 2002).

Sleep and Gene Expression

Various approaches including gene knockouts, point-mutations, and genome-wide association studies have identified a plethora of genes involved in the regulation of sleep. Genes with sleep regulatory function can be classified into many different categories such as transcription factors, neuropeptides, kinases, among others. For example, in mice, deletion of the transcription factor *Bmal1* disrupts several aspects of sleep, with changes including increased total sleep, decreased sleep homeostatic response, and abnormal circadian distribution of sleep and wake episodes (Laposky et al., 2005). In fruit flies, the neuropeptide F (NPF) has been shown to be linked to sleep. Males flies over-expressing NPF show increased nighttime sleep and altered sleep homeostatic response upon sleep deprivation (He et al., 2013). Lastly, in mice, the Ca^{2+}/calmodulin-dependent protein kinase II has been shown to be involved in regulating sleep, as knockout of the subunits *Camk2a* and *Camk2b*, results in a significant decrease in total sleep (Tatsuki et al., 2016).

Drosophila as a Model for Sleep

The study of sleep has benefited from the use of the fruit fly *Drosophila melanogaster* as a model. Findings show that *Drosophila* have a sleep-like state (Hendricks et al., 2000; Shaw et al., 2000). This sleep-like state has been found to

share similarities with mammalian sleep including: a specific posture, extended periods of quiescence distributed in a circadian manner, increased arousal threshold upon stimulus, homeostatic regulation, and changes in brain electrophysiological activity (Hendricks et al., 2000; Shaw et al., 2000; van Alphen et al., 2013). *Drosophila* sleep can be assessed by means of locomotor activity tracking assays and tools such as the *Drosophila Activity Monitor System* (DAM). In this assay, individual flies are placed in a clear tube bisected by an infrared beam that is interrupted when the fly moves, which is counted as a bout of locomotor activity (Pfeiffenberger et al., 2010). A fly is considered to be asleep when showing 5 minutes of quiescence or more. This definition of sleep was determined by the observation that after five minutes of rest, animals present increased arousal threshold upon mechanical stimulation (Shaw et al., 2000). *Drosophila* sleep is characterized by a consolidated period of sleep during the night, and high levels of wake during the beginning and end of the daytime, with a “siesta” during the middle of the day (Hendricks et al., 2000).

Neurotransmitters and Sleep in *Drosophila*

Evidence demonstrates different effects of neurotransmitters on the regulation of sleep in *Drosophila*. According to current data, neurotransmitters can be generally classified in several different categories: sleep-promoting (serotonin and GABA), wake-promoting (dopamine, octopamine, histamine), and neurotransmitters involved in both (acetylcholine and glutamate) (Reviewed in Ly et al., 2018), although there are clearly exceptions (Liu et al., 2019). This literature review will focus specifically on the sleep

regulatory function of acetylcholine and glutamate, and the wake-promoting neurotransmitter dopamine.

In *Drosophila*, the neurotransmitter acetylcholine is known to be primarily excitatory, with expression in the protocerebrum, mushroom bodies, and central complex (Buchner et al., 1986; Salvaterra and Kitamoto, 2001; Yasuyama and Salvaterra, 1999). It has been shown that acetylcholine is responsible for providing excitatory inputs onto the wake-promoting large ventrolateral neurons (l-LNvs), demonstrated by whole-cell recordings (McCarthy et al., 2011). In these experiments it was observed that treatment with nicotinic acetylcholine receptor (nAChR) agonists (acetylcholine and nicotine), induced depolarization and action potential firing of l-LNvs, which was reversed by application of nAChR antagonists (curare and α-BuTX) (McCarthy et al., 2011). Additionally, acetylcholine induces wakefulness by acting on a cluster of wake promoting mushroom body neurons by means of G-protein coupled signaling. VAChT-RNAi expression in MB surface/posterior and γ neurons leads to an increase in sleep, suggesting a wake-promoting effect of acetylcholine in those neurons (Yi et al., 2013). On the other hand VAChT-RNAi expression in MB α/β leads to a decrease in sleep, suggesting a sleep-promoting role of acetylcholine in those neurons (Yi et al., 2013).

Evidence has also demonstrated a role of the neurotransmitter glutamate in the regulation of sleep in *Drosophila*, having been shown to induce both wake and sleep in a loci-specific manner (Reviewed in Ly et al., 2018). Adult-specific activation of *Drosophila* CNS glutamatergic cells by means of the voltage-gated sodium channel NaChBac, leads to an increase in wake during both daytime and nighttime, with longer wake episodes (Zimmerman et al., 2017). Additionally, inhibition of CNS glutamatergic

cells by means of the potassium channel shaker (EKO), leads to an increase in nighttime sleep (Zimmerman et al., 2017). On the other hand, several studies have investigated the sleep-promoting effects of glutamatergic signaling. Evidence demonstrates that pan-neuronal knockdown of the NMDA type glutamate receptor gene *Nmdar1*, leads to a significant decrease in total sleep (Tomita et al., 2015). In support of these results, treatment with the NMDAR antagonist MK-801, also leads to a decrease in sleep (Tomita et al., 2015). Other results show that increased expression the vesicular glutamate transporter *vglut*, leads to an increase in both daytime and nighttime sleep (Robinson et al., 2016). Additionally, these effects were found to be dependent on both NMDAR and AMPAR signaling, as expression of RNAi against the NMDAR subunits NR1 and NR2, as well as the AMPA receptor GluRI, lead to a rescue of the observed sleep behavior upon increased *vglut* expression (Robinson et al., 2016). Glutamatergic signaling has also been shown to be involved in the regulation of homeostatic compensatory sleep upon sleep deprivation (Liu et al., 2016). In these experiments, ellipsoid body R2 cell-specific mRNA isolation followed by qPCR, demonstrated an increase of the NMDAR subunit *dNR1*. Additionally, immunostaining of ellipsoid body R2 cells after sleep deprivation showed an increase in *dNR2* NMDAR subunit. Lastly, knockdown of either *dNR1* and *dNR2* by mean of RNAi, led to a suppression of sleep rebound after sleep deprivation (Liu et al., 2012). Taken together these results demonstrate loci-specific role of glutamatergic signaling, with effects on both wake and sleep.

Studies demonstrate the presence of neurotransmitters shown to be important for wake promotion in *Drosophila*, among those Dopamine (Reviewed in Ly et al., 2018). In

fruit flies, projections from clusters of dopaminergic neurons have been shown to innervate the central complex and mushroom bodies, important regions for sleep regulation (Donlea et al., 2011; Friggi-Grelin et al., 2003; Joiner et al., 2006; Liu et al., 2016; Mao and Davis, 2009; Pitman et al., 2006). Manipulation of dopaminergic levels by genetic mutations or pharmacology supports the role of dopaminergic signaling on sleep regulation. Flies expressing the mutant allele *fumin*, which affects the dopamine transporter *dDAT* gene and disrupts dopamine clearance (Makos et al., 2009), show deficits in sleep with increased arousal (Kume, 2005).

The role of dopamine in *Drosophila* sleep has also been supported by pharmacological studies. Treatment with the dopamine synthesis inhibitor 3IY leads to an increase in sleep during daytime (Andretic et al., 2005). On the other hand, increasing dopaminergic signaling by means of methamphetamine treatment, leads to an increase of arousal (*Andretic et al., 2005*). Lastly, earlier evidence demonstrates an arousal effect of caffeine in *Drosophila* (Hendricks *et al.*, 2000; Shaw *et al.*, 2000) to be mediated by the dopamine receptor dDA1 in mushroom bodies (Andretic et al., 2008). In this study, the resistance to caffeine, induced by the mutant *dumb1* (dDA1 deficient), is rescued by transgenic expression of WT dDA1 (Andretic et al., 2008).

Several cell clusters have been identified to be important in the observed dopamine-dependent regulation of sleep in *Drosophila*. For example, PPL1 and PPM3 are clusters of dopaminergic neurons that innervate dFSB, and their activation by means of dTRPA1 leads to a significant decrease in nighttime sleep (Liu et al., 2012b). Later evidence confirmed the signaling between dopaminergic cells and the dFSB, by means of optogenetic activation of TH-cells combined with electrophysiological

recording from dFSB. In these experiments, optogenetic activation of TH-GAL4^{+} cells by means of *csChrimson*, lead to a decrease in dFSB firing, concomitant with an increase wake-behavior (Pimentel et al., 2016).

micro-RNAs: Biogenesis, Mechanism of Action and Functions

microRNAs are endogenous short transcripts (˜23 nucleotides) of non-coding RNA, with post-transcriptional regulatory functions on gene expression of their targets (Ambros, 2004; Bartel, 2009; Huntzinger and Izaurralde, 2011). Pri-microRNAs are hairpin-like microRNA precursors, transcribed by the RNA-Polymerase II. A pri-microRNA is cleaved by the nuclear RNase drosha, producing a ˜70 nucleotide pre-microRNA, which is then exported into the cytoplasm (Lee et al., 2003). In the cytoplasm the pre-microRNA's stem loop is cleaved by the enzyme Dicer, leaving a short double-stranded microRNA precursor (Hutvágner et al., 2001). Then either a 5' or 3' microRNA "guide" strand is loaded into the Ago protein of the RNA induced silencing complex (RISC), while the remaining "passenger" strand is usually degraded (Bhayani et al., 2012; Hutvágner *et al.*, 2001; Lee *et al.*, 2003; Meijer et al., 2014). Once loaded into the RISC complex a microRNA is ready to effect its regulatory function on its target mRNA products.

A microRNA will target and bind specific mRNAs by means of Watson-Crick base complementarity (Reviewed in Huntzinger and Izaurralde, 2011). The microRNA contains a "seed" sequence within its 5'end, containing 2-7 nucleotides. The microRNA seed sequence recognizes a partially complementary binding on the target mRNA, usually located in the 3'-UTR region of the mRNA (Reviewed in Bartel, 2009; Huntzinger

and Izaurralde, 2011). Once bound to its target mRNA, a microRNA will inhibit protein expression by either translational inhibition or mRNA destabilization and degradation (Lee et al., 1993; Lim et al., 2005; Wightman et al., 1993). Evidence has shown that a microRNA can affect mRNA translation either during initiation (Humphreys et al., 2005; Pillai et al., 2005), or during post-initiation stages of translation (Olsen and Ambros, 1999; Seggerson et al., 2002). Alternatively, microRNAs have also been shown to inhibit protein expression through mRNA degradation either by endonucleolytic cleavage of the mRNA target (Yekta et al., 2004) or by degrading their targets by means of the 5'-to-3' mRNA decay pathway (Behm-Ansmant, 2006; Giraldez et al., 2006; Rehwinkel, 2005). Evidence demonstrates that mRNA target degradation plays a major role in the translational repression in animals, accounting for 75-84% of the microRNA-dependent reduction of protein production (Guo et al., 2010; Hendrickson et al., 2009).

Previous evidence shows microRNAs to be involved in a plethora of biological processes including development, cancer, and sleep, among others (Reviewed in Carthew et al., 2017; Macfarlane and R. Murphy, 2010; O'Connell et al., 2010; Wienholds and Plasterk, 2005). For example, in *C. elegans*, the gene *lin-14* is involved in the developmental cell fate determination and loss-of-function mutations in *lin-14* leads to abnormal early expression of cell fates, that should occur later in development (Olsen and Ambros, 1999). Studies show that the levels of LIN-14 protein are high during early L1 developmental stage and then decrease between later L1 and L2 stages, allowing for the proper cell fate determination (Ambros and Horvitz, 1987). Later evidence demonstrated that the downregulation of LIN-14 is controlled by the microRNA *lin-4*, which is expressed at the end of the L1 developmental stage, giving rise to the

proper cell fate determination (Feinbaum and Ambros, 1999). microRNAs have also been shown to be involved in the regulation of development in other organisms, such as in the case of the first microRNA identified in fruit flies, *bantam*. In flies, *bantam* is expressed in imaginal disks, a larval tissue that gives rise to adult body parts such as antennas, wings, legs, and eyes. *Bantam*'s expression in imaginal disks allows for proper body part development by promoting cell-proliferation and cell apoptosis inhibition by downregulation of the proapoptotic gene *hid* (Brennecke et al., 2003; Hipfner et al., 2002). In the context of the nervous system, microRNAs have been shown to be linked to processes for neuronal development, with specific functions such as neuronal maturation, connectivity and plasticity (Reviewed in McNeill and David, 2012). For example, the murine brain-enriched microRNA *miR-137* has been shown to play a role in early neuronal differentiation through the regulation of the gene *cdk6* and the neuronal marker *tuj1* (Silber et al., 2008). Other functions of *miR-137* include the regulation of neuronal maturation, through the regulation the gene *mib1* (Smrt et al., 2010). Additionally, in *Drosophila melanogaster*, loss of the microRNAs *let-7, miR-100, and miR-125*, have been shown to prevent the development and maturation of neuromuscular junctions (NMJs), via regulation of the transcription factor *abrupt* (Caygill and Johnston, 2008; Sokol et al., 2008).

Studies have shown microRNAs to be involved in the progression of diseases, such as cancer, in humans (Calin et al., 2002; Calin et al., 2004; Iorio and Croce, 2012). Early evidence linking microRNAs with cancer development comes from studies aiming to identify tumor suppressor genes at chromosome 13q14, which is often found deleted in chronic lymphocytic leukemia (Calin et al., 2002). It was found in this study that

instead of containing a protein-coding tumor suppressor gene, the 13q14 chromosomal region contains the two microRNA genes *miR-15a* and *miR-16-1*. The involvement of these 2 microRNAs in chronic lymphocytic leukemia was also confirmed by the fact that in approximately 69% of cases studied, *miR-15a* and *miR-16-1* appeared to be knocked out or knocked down (Calin et al., 2002). Further studies of different human cancer cases demonstrated increased expression of microRNAs linked to the negative regulation of tumor suppressor genes (Calin et al., 2004). In such a scenario, increased expression of the aforementioned microRNAs leads to a downregulation of tumor suppressor genes and hence progression of tumor development. Additionally, microRNAs important for oncogene suppression are often found in chromosomal loci prone to deletion or mutations (Reviewed in Iorio and Croce, 2012). The involvement of microRNAs in the progression of cancer has made these post-transcriptional regulators key biomarkers in the classification, diagnosis, prognosis and treatment of the condition in humans (Reviewed in Iorio and Croce, 2012; Jansson and Lund, 2012).

MicroRNAs function has also been linked to the regulation of sleep in different organisms. For example, early evidence from microarray experiments demonstrated that sleep deprivation induces locus-specific changes in microRNA expression in the rat brain (Davis et al., 2007). Later studies also demonstrated that the activity of microRNAs can alter sleep, measured as EEG delta-wave activity in rats (Davis et al., 2012). In *Drosophila*, microRNAs have been shown to be involved in the regulation of sleep behaviors. For example, the microRNA *miR-276a* has been shown to regulate sleep and circadian rhythmicity by targeting the *npfr, tim,* and *DopR1* genes (Chen and Rosbash, 2016; Zhang et al., 2021a; Zhang et al., 2021b). Results show that *miR-276a*

knockout and overexpression leads to either increased or decreased sleep, respectively (Zhang et al., 2021a; Zhang et al., 2021b). Additionally, it has been shown that misregulation of *miR-276a* by means of genetic knockout or overexpression leads to disruptions in molecular and behavioral circadian rhythmicity (Chen and Rosbash, 2016). Also, evidence demonstrates that in *Drosophila*, the microRNA *miR-92a* regulates sleep behavior by means of suppression of neuronal excitability and targeting of *sirt2* expression, with locus-specific effects of *miR92a* misregulation on total sleep (Chen and Rosbash, 2017).

The study of microRNA function has benefited from the use the microRNA inhibitors, microRNA-sponges (Loya et al., 2009). microRNA-sponges are transgenic RNA sequences containing repetitive sequences complementary to specific microRNAs (Loya et al., 2009). When expressed, a microRNA-sponge binds to its target microRNA, hence inhibiting the microRNA from interacting with its target mRNA, and ultimately leading to a misregulation of the targets (Loya et al., 2009). The Van Vactor laboratory engineered a library of 143 microRNA-sponges targeting highly expressed and evolutionarily conserved microRNAs (Fulga et al., 2015). This library has been further utilized for addressing the regulatory function of microRNAs in several biological processes and functions such as learning and memory (Busto et al., 2015), wing development and morphology (Fulga et al., 2015), glia-dependent circadian regulation (You et al., 2018), and fly locomotor behaviors (Donelson et al., 2020). Utilizing this microRNA-sponge library, the Griffith lab has worked to better understand the regulatory functions of microRNAs on sleep. With this purpose Goodwin et al. (2018) screened the library of 143 microRNA sponges, aiming to find microRNAs involved in sleep. Several

microRNAs were found to be linked to *Drosophila* sleep, with 8 microRNAs identified as wake-promoting, and 17 others identified as sleep-promoting (Goodwin et al., 2018). Results from this study demonstrated a role of the microRNA *let-7* on sleep promotion, with knockdown by means of let-7-sponge or CRISPR knockout, leading to developmental and adult specific effects on sleep. Additionally *let-7* sleep-promoting effects were mapped to mushroom body neurons by means of GAL4/UAS locus-specific let-7-sponge expression (Goodwin et al., 2018). This screen also suggested a sleep-promoting role of the microRNA *bantam* (Goodwin et al., 2018). Our recent work further characterized *bantam* sleep regulatory function, with results showing that *bantam* acts to promote early nighttime sleep in a cluster of bilateral Mushroom Body Output Neurons (MBONs) (Hobin et al., 2021). Additionally, our results suggested a role of *kelch* and *CCHamide-2 receptor* as mediators of *bantam* sleep regulatory function (Hobin et al., 2021). Our screen also showed a sleep-promoting function of the microRNA *miR-190* (Goodwin et al., 2018). Either global or pan-neuronal knockdown of *miR-190* by means of a miR-190-sponge, leads to a decrease in total sleep (Goodwin et al., 2018).

The microRNA *miR-190* has been studied in the context of different types of human diseases, and particularly cancer (Reviewed in Yu and Cao, 2019). *MiR-190* has been shown to be involved in different stages of cancer development, from proliferation to metastasis and drug resistance, throughout the regulation of a myriad of mRNA targets (Reviewed in Yu and Cao, 2019). In *Drosophila*, *miR-190* has been shown be important for Hypoxia Inducible Factors (HIF)-dependent responses to hypoxia, through the regulation of the gene *fatiga* (De Lella Ezcurra et al., 2016). Aside from our results

demonstrating a sleep-promoting role of the microRNA *miR-190* in *Drosophila*, hitherto no other research has further studied the role of this microRNA on the regulation of sleep behaviors.

Thesis Briefing

The aim of the project described in this thesis is to better understand the sleep-promoting role of the microRNA *miR-190* in *Drosophila melanogaster*. This project specifically **(1)** maps its locus of action in the fly brain **(2)** identifies *miR-190's* window of time for sleep regulation, and **(3)** seeks to understand *miR-190's* mechanism of action for sleep regulation through mRNA target identification and assessment of its effect on loci-specific neuronal activity.

The role of microRNAs is known to be evolutionarily conserved, and microRNAs have been linked to the regulation of sleep in humans, with studies showing altered microRNA expression profiles concomitant with sleep pathologies (Holm et al., 2014). The current work shines light on the genetic regulation of sleep and may help elucidate potential therapeutic targets for the understanding and treatment of sleep disorders and its comorbidities in humans.

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Chapter 2

miR-190 Acts in the Pupal Nervous System to Regulate Neurotransmitter Identity in Glu^{ACh} Cells and Sleep Behavior

microRNAs are endogenous, short transcripts of non-coding RNA, with post-transcriptional regulatory functions (Huntzinger and Izaurralde, 2011). microRNAs have been shown to regulate sleep behaviors in an evolutionarily conserved manner (Chen and Rosbash, 2016; 2017; Davis et al., 2007; Davis et al., 2012; Zhang et al., 2021a; Zhang et al., 2021b). The study of microRNAs function has benefitted from the use of microRNA-sponges (Donelson et al., 2020; Fulga et al., 2015; Loya et al., 2009; You et al., 2018). The Griffith lab screened a library of 143 micro-RNA-sponges (Fulga et al., 2015; Goodwin et al., 2018), aiming to find microRNAs involved in the regulation of sleep behaviors in *Drosophila melanogaster*. Various microRNAs were identified to be linked to sleep behavior, with 8 microRNAs found to be wake-promoting and 17 others identified as sleep promoting (Goodwin et al., 2018). Among the sleep-promoting microRNAs identified by Goodwin et al. (2018), we find the microRNA *miR-190*. The current project seeks to further unravel *miR-190* regulatory function on sleep.

miR-190 in Neurons Regulates Multiple Sleep Parameters

To knockdown the function of *miR-190* we used a transgenic miRNA sponge as previously described (Fulga et al., 2015; Goodwin et al., 2018). To determine whether the role of *miR-190* on sleep was neuronal, miR-190-SP was expressed under the pan-neuronal driver *nsyb-GAL4*. Flies pan-neuronally expressing the miR-190-SP showed a

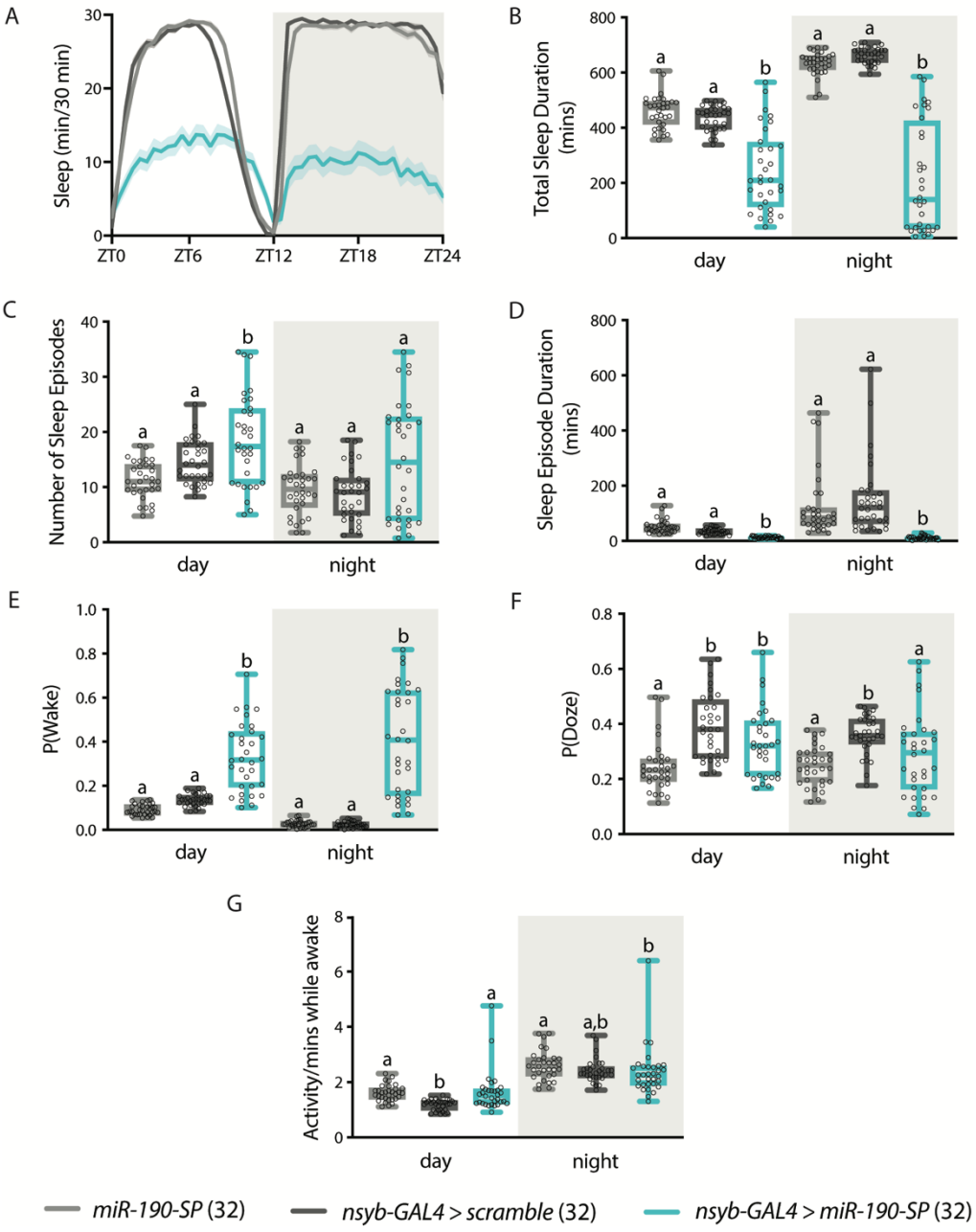

Figure 2.1. miR-190-SP in neurons disrupt normal sleep behavior. LD sleep data for *miR-190-SP* (UAS control) (light gray), *nsyb-GAL4 > scramble* (scramble control) (dark gray) and *nsyb-GAL4 > miR-190-SP* (aqua). (A) Sleep per 30 mins across 24 hours of LD. (B) Mean total sleep during both day and night relative to UAS control and scramble control. (C-D) Number of sleep episodes and sleep episode duration during daytime and nighttime. (E-F) Sleep pressure shown as P(Wake) and P(Doze). (G) Daytime and nighttime activity while awake. Whiskers represent maxima and minima. Circle data points representing individuals overlaid box-and-whiskers. Letters indicate groups of statistical similarity ($p<0.05$) for one-way ANOVA with Tukey's multiple comparisons test or Kruskal-Wallis with Dunn's multiple comparisons test. Numbers of individuals per group are reported next to the genotype.

significant decrease in total sleep, both during the day and night (Figure 2.1A and 2.1B). sleep is determined by structural parameters including number of sleep episodes and sleep episode duration. We observed changes in these structural parameters, upon pan-neuronal expression of miR-190-SP. *nsyb-Gal4 > miR-190-SP* flies showed a fragmented sleep behavior, with a significant increase in number of sleep episodes during the day (Figure 2.1C) and a significant decrease in duration during both day and night (Figure 2.1D), compared to controls. To determine an effect of *miR-190* knockdown on sleep pressure, P(Wake) and P(Doze) were calculated (Wiggin et al., 2020). Interestingly, these animals show an increase P(Wake), a parameter associated with high dopaminergic tone, but not P(Doze) (Figure 2.1E-F). The observed changes in sleep behavior upon *miR-190* pan-neuronal knockdown were not due to an increase in locomotor activity (Figure 2.1G). Taken together, these results demonstrate a neuronal role of *miR-190* in the regulation of sleep. These effects were found to be neuronal-specific, as expressing miR-190-SP in glial cells by means of a *repo-GAL4* had no effects on sleep (data not shown).

miR-190 acts in Glu^{ACh} cells to Regulate Sleep

To map the effect of *miR-190* to a specific population of cells, we inhibited the microRNA's function in a neuron type-specific manner using GAL4 drivers. Cholinergic cells are known to have generally wake-promoting effects (Seidner et al., 2015; Wu et al., 2014). To address whether *miR-190* acts to regulate sleep in cholinergic cells we expressed miR-190-SP under the cholinergic driver *chat-GAL4*.

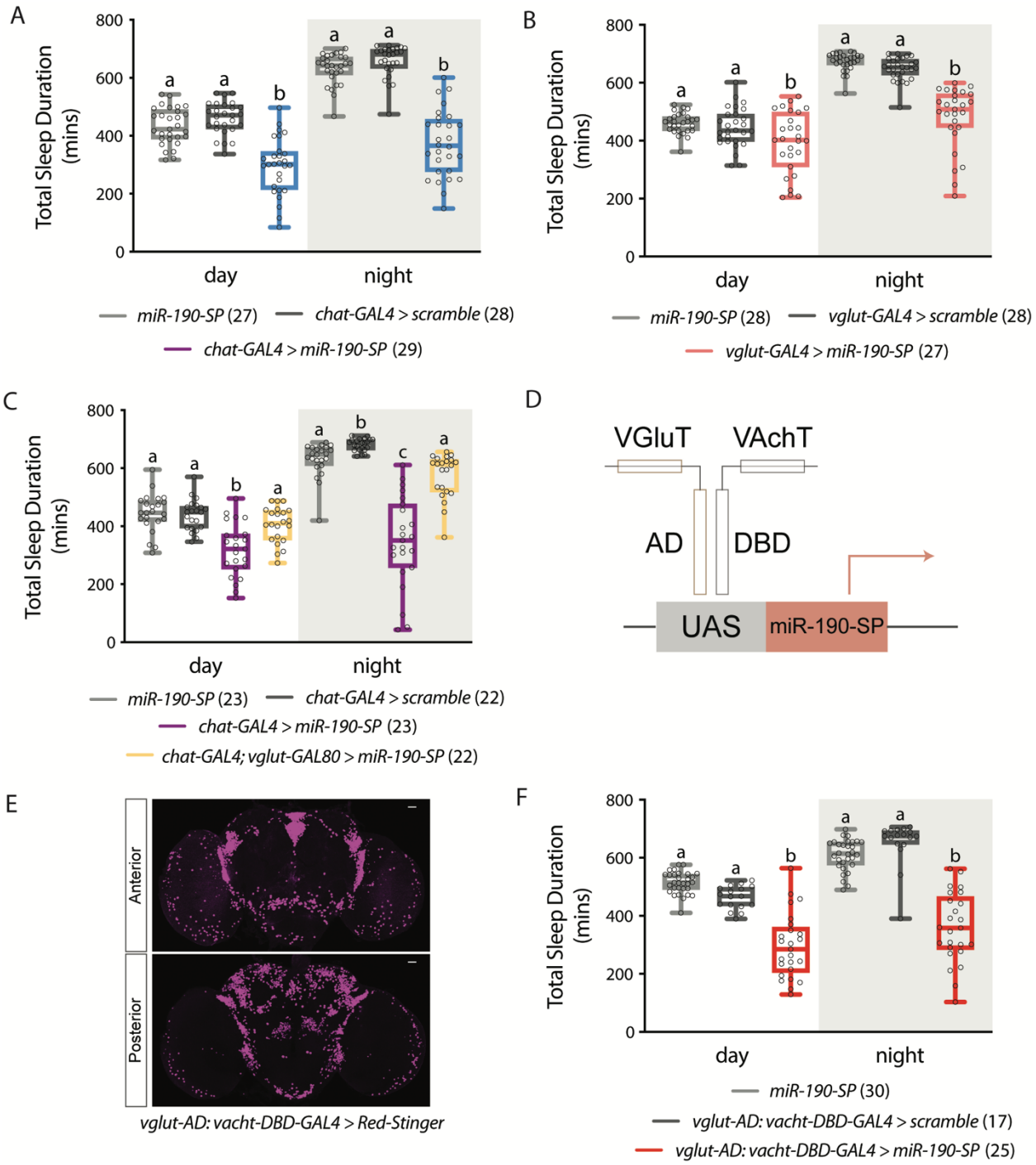

Figure 2.2. *miR-190* acts in Glu^{ACh} cells to regulate sleep. Mean total sleep during both day and night upon *miR-190-SP* expression under the cholinergic chat-GAL4 (A) or the glutamatergic driver vglut-GAL4 (B) relative to UAS control and scramble control. (C) Mean daytime and nighttime total sleep for animals simultaneously expressing vglut-GAL80 and miR-190-SP under chat-GAL4, relative to *chat-GAL4 > miR-190-SP,* UAS and scramble controls. (D) vglutAD:vachtDBD-GAL4 design. Split-GAL4 activation and DNA-binding domains were inserted into *VAChT* and *VGluT* genes by means of CRISPR. (E) Expression of Red-Stinger under vglutAD:vachtDBD-GAL4 for visualization of Glu^{ACh} cells. (F) Mean daytime and nighttime total sleep for animals expressing miR-190-SP under vglutAD:vachtDBD-GAL4. Whiskers represent maxima and minima. Circle data points representing individuals overlaid box-and-whiskers. Letters indicate groups of statistical similarity ($p<0.05$) for one-way ANOVA with Tukey's multiple comparisons test or Kruskal-Wallis with Dunn's multiple comparisons test. Numbers of individuals per group are reported next to the genotype.

When expressing the miR-190-SP in cholinergic cells we observed results that very closely phenocopied *miR-190* pan-neuronal knockdown. *chat-GAL4 > miR-190-SP* flies showed a significant decrease in total sleep during both daytime and nighttime (Fig. 2.2A, Supplementary Figure 1.A). Similar to cholinergic cells, glutamatergic cells have been shown to induce wake-promoting effects in the *Drosophila* brain (Zimmerman et al., 2017). Interestingly, expression of miR-190-SP in glutamatergic cells by means of the *vglut-GAL4* driver also replicated pan-neuronal and cholinergic inhibition of *miR-190* function. *vglut-GAL4 > miR-190-SP* flies showed decreased total sleep during both day and night (Fig. 2.2B, Supplementary Figure 1.B). Previous evidence had demonstrated that some subsets glutamatergic cells in the ventral ganglion express genes of the cholinergic transmitter system (Lacin et al., 2019). To address whether *miR-190* acts in such population of cells, flies were designed to express miR-190-SP under the *chat-GAL4* driver, while simultaneously expressing *vglut-GAL80*, to inhibit GAL4 in $VGluT^{+}$ cells. *chat-GAL4; vglut-GAL80 > miR-190-SP* animals showed a rescue in their sleep behavior, with no changes in total sleep relative to control groups (Figure 2.2C, Supplementary Figure 1.C), suggesting that there may be a population of cells in the adult brain that express both cholinergic and glutamatergic genes.

To more precisely capture the *miR-190* locus of action, split-GAL4 domains (AD and DBD) were inserted into the *VGluT* and *VAChT* genes by means of CRISPR

(Figure 2.2D). Neurons targeted by this split-GAL4 combination will be referred to as Glu^{ACh} cells (Figure 2E). Expression of miR-190-SP in Glu^{ACh} cells results in a strong

sleep phenotype, with a decrease in total sleep during both day and night (Figure 2.2F, Supplementary Figure 1.D).

To further understand whether the activity of Glu^{ACh} cells had an effect on sleep, we activated them by means of the heat-activated transient receptor potential ion channel dTRPA1 (Hamada et al., 2008), either during pupation or adulthood to determine if their activity was important for setting sleep levels. To activate Glu^{ACh} cells during pupation, animals were raised at 18°C until the onset of pupation when they were exposed to 29°C in intervals of 1 hour for a total of 4 times a day, until they reached

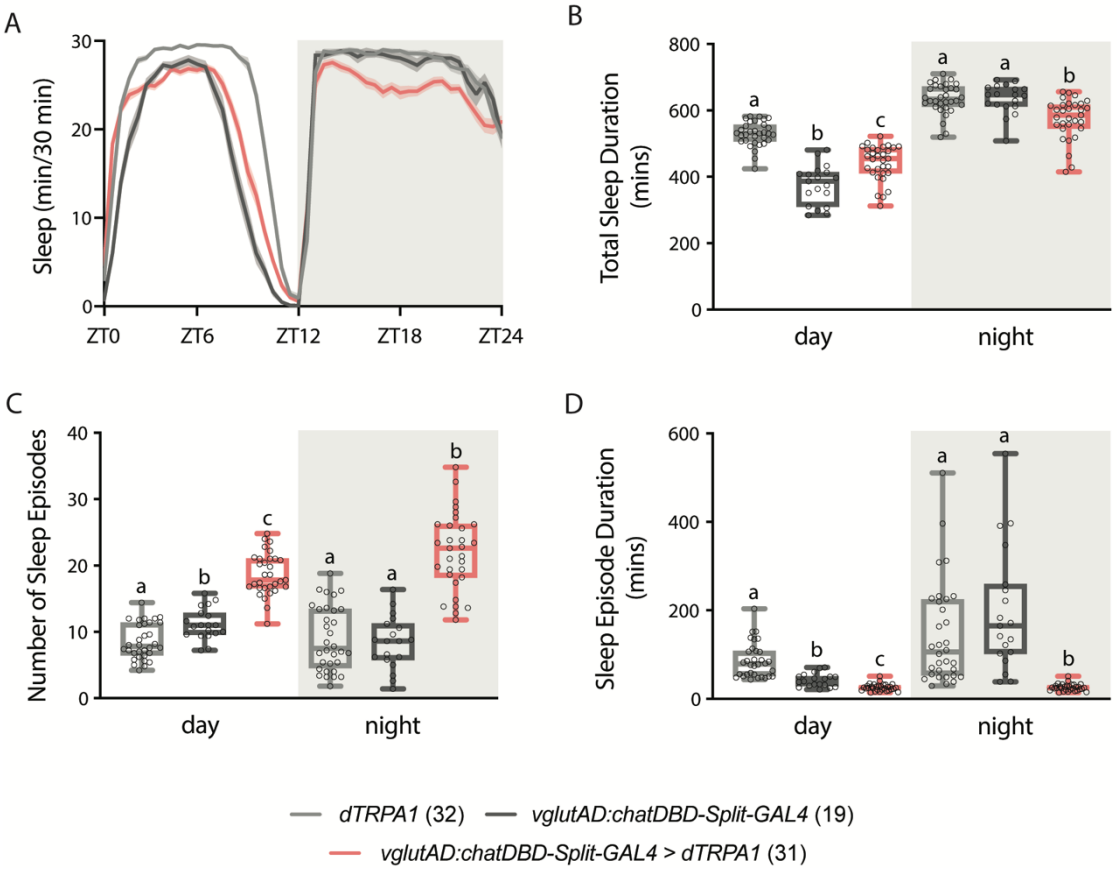

Figure 2.3. Pupal activation of Glu^{ACh} cells decreases nighttime sleep and induces sleep fragmentation. (A) Sleep per 30 mins across 24 hours of LD. (B) Mean total sleep during both day and night relative to UAS control and scramble control. (C-D) Number of sleep episodes and sleep episode duration during daytime and nighttime. Whiskers represent maxima and minima. Circle data points representing individuals overlaid box-and-whiskers. Letters indicate groups of statistical similarity ($p<0.05$) for one-way ANOVA with Tukey's multiple comparisons test or Kruskal-Wallis with Dunn's multiple comparisons test. Numbers of individuals per group are reported next to the genotype.

adulthood. Pupal activation of Glu^{ACh} cells led to a decrease in total sleep during the night (Figure 2.3A-B) and an increase in sleep fragmentation, with more sleep episodes of shorter duration during the day and night (Figure 2.3C-D). To induce activation of Glu^{ACh} cells during adulthood, animals were raised at 18°C until reaching adulthood when they were exposed to 29°C in intervals of 1 hour for a total of 4 times a day, during the first 4 days of adulthood. Adult activation of Glu^{ACh} cells lead to an increase in sleep fragmentation with more sleep episodes of shorter duration during both daytime

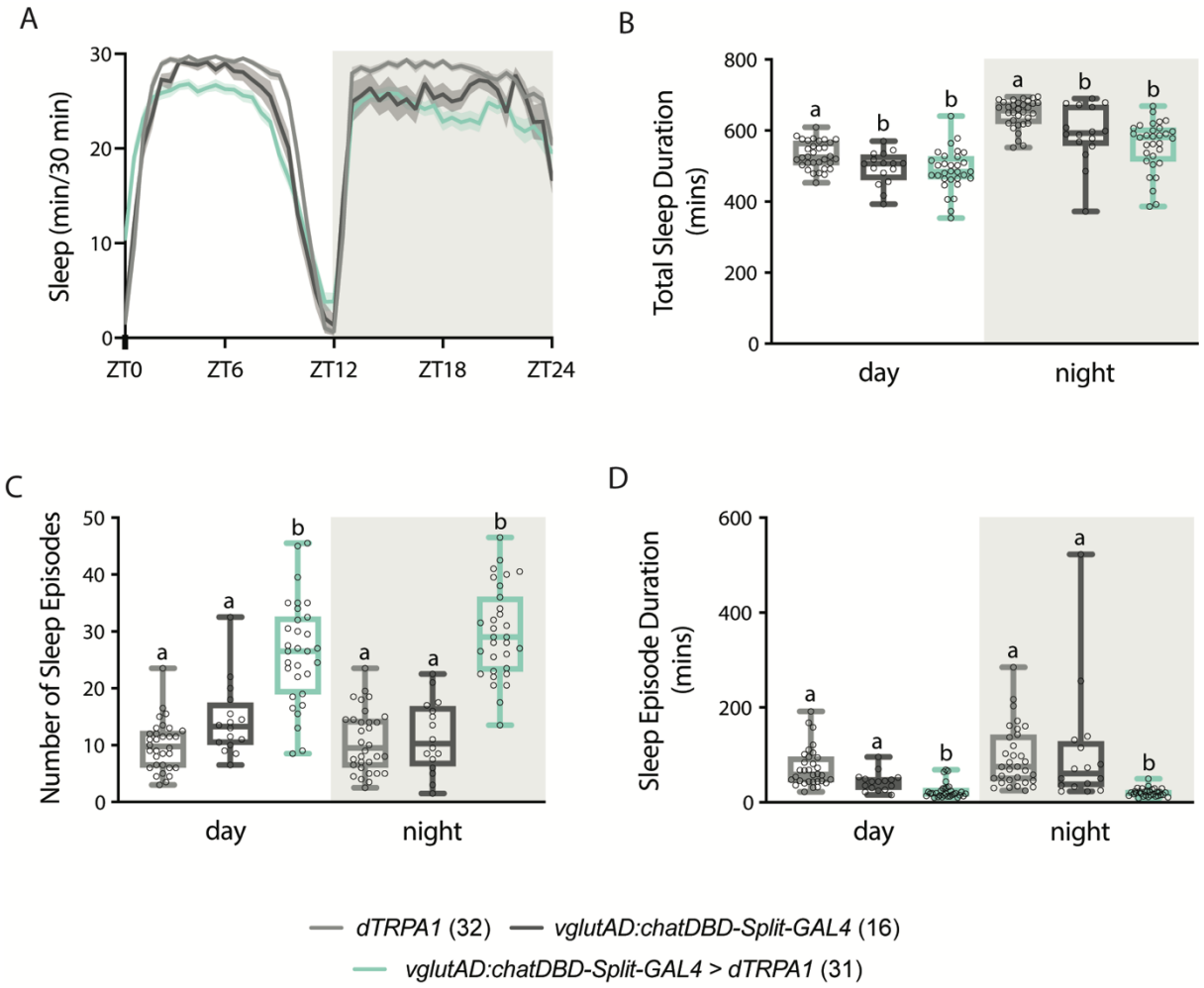

Figure 2.4. Adult activation of Glu^{ACh} cells induces sleep fragmentation. (A) Sleep per 30 mins across 24 hours of LD. (B) Mean total sleep during both day and night relative to UAS control and scramble control. (C-D) Number of sleep episodes and sleep episode duration during daytime and nighttime. Whiskers represent maxima and minima. Circle data points representing individuals overlaid box-and-whiskers. Letters indicate groups of statistical similarity ($p<0.05$) for one-way ANOVA with Tukey's multiple comparisons test or Kruskal-Wallis with Dunn's multiple comparisons test. Numbers of individuals per group are reported next to the genotype.

and nighttime (Figure 2.4C-D). Taken together, these results suggest that *miR-190* acts in a cluster of cells with transcriptionally active *ChaT* and *VGluT* genes which exhibit sleep-regulating activity and that its role may be regulated by neuronal firing.

miR-190 acts during pupation to affect sleep

Sleep regulators may act either during development, on the formation/maturation of circuits important for sleep (Chakravarti Dilley et al., 2020; Xie et al., 2019), or acutely during adulthood (reviewed in Crocker and Sehgal, 2010). To determine the window in which *miR-190* acts we expressed miR-190-SP pan-neuronally using the *nsyb-GAL4* driver, while simultaneously expressing the ubiquitous temperature-sensitive GAL4 inhibitor *tubulin-GAL80^{ts}* (McGuire et al., 2003). *nsyb-Gal4; tub-Gal80^{ts} > miR-190-SP* flies express miR-190-SP at 29°C but not at 18°C. To limit the expression of miR-190-

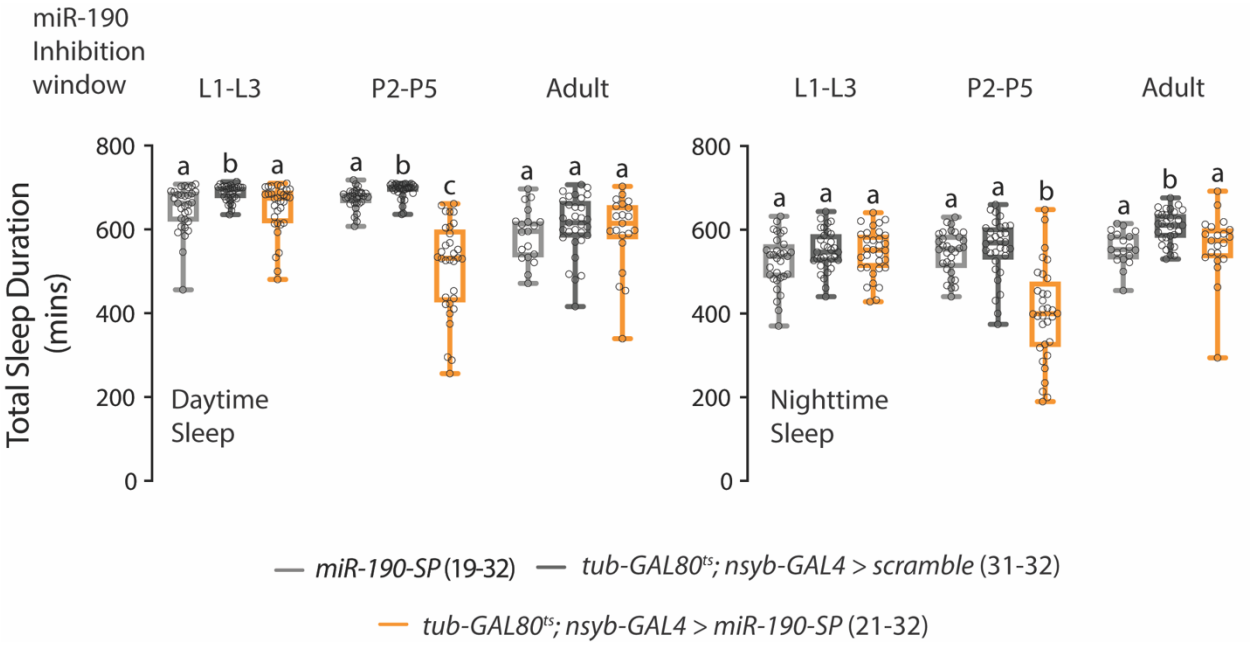

Figure 2.5. *miR-190* acts during pupation to affect sleep. Mean daytime and nighttime total sleep for flies pan-neuronally expressing miR-190-SP during early development (L1-L3), pupal stage (P2-P5) or adulthood; by means of tub-GAL80^{ts}. Whiskers represent maxima and minima. Circle data points representing individuals overlaid box-and-whiskers. Letters indicate groups of statistical similarity ($p<0.05$) for one-way ANOVA with Tukey's multiple comparisons test or Kruskal-Wallis with Dunn's multiple comparisons test. Numbers of individuals per group are reported next to the genotype.

SP to the pupal stage, animals were raised at 18°C until pupation, then were shifted to 29°C until day 2 post eclosion when they were shifted to 25°C and tested for sleep behavior. Pan-neuronal-pupal expression of miR-190-SP phenocopied the constitutive pan-neuronal knockdown of *miR-190*, with flies showing a significant decrease in total sleep during both day and night compared to controls (Figure 2.5, Supplementary Figure 2). miR-190-SP expression prior to pupation or during adulthood showed no statistically significant effects on the amount of sleep. These results suggest a role of *miR-190* on the establishment of adult sleep levels during pupation.

miR-190 is required for downregulation of cholinergic genes

miRNAs carry out their role by operating as post-transcriptional regulators of specific target mRNAs, either inhibiting their translation or promoting their degradation (Jonas and Izaurralde, 2015). To identify *miR-190* mRNA targets for sleep regulation we performed paired-end RNA sequencing. *nsyb-GAL4 > miR-190-SP; GAL80^{ts}* animals were raised at 18ºC to suppress miR-190-SP expression, and temperature was raised to 29ºC after pupal day 2 to induce miR-190-SP expression, knocking down miR-190 in a pupal-specific manner. On the last day of pupation (pharate stage) brains

Gene	Developmental Stage	Fold-Change	P-Value
chat	Pupae	3.08	0.0005
vacht	Pupae	1.71	0.14
midline	Pupae	300	0.0022
spab	Adult	1.95	0.0143

Table 2.1. Upregulated cholinergic related genes upon miR-190-SP pan-neuronal expression in pupal or adult brains. Genes upregulated upon miR-190-SP pan-neuronal expression during pupation or adulthood. Fold-change and P-value significance for edgeR differential expression analysis relative to scramble controls is shown.

were dissected and RNA was extracted for RNAseq (Kim et al., 2017; Ma et al., 2021). RNAseq was also performed using RNA extracted from *nsyb-GAL4 > miR-190-SP* adult heads. EdgeR was used to analyze differential gene expression and RNAseq data was sorted using the following criteria: (1) predicted *miR-190* targets; (2) transmitter-specific markers (2) and (3) transmitter-enriched transcription factors. Among the *miR-190* direct targets differentially expressed we found *Chat*, the synthetic enzyme for ACh (3.08-fold, P=0.00051 in pupae) and *spab*, which operates in complex with the cholinergically-enriched transcription factors *CG10656* and *knot* (Shokri et al., 2019) (1.95-fold,

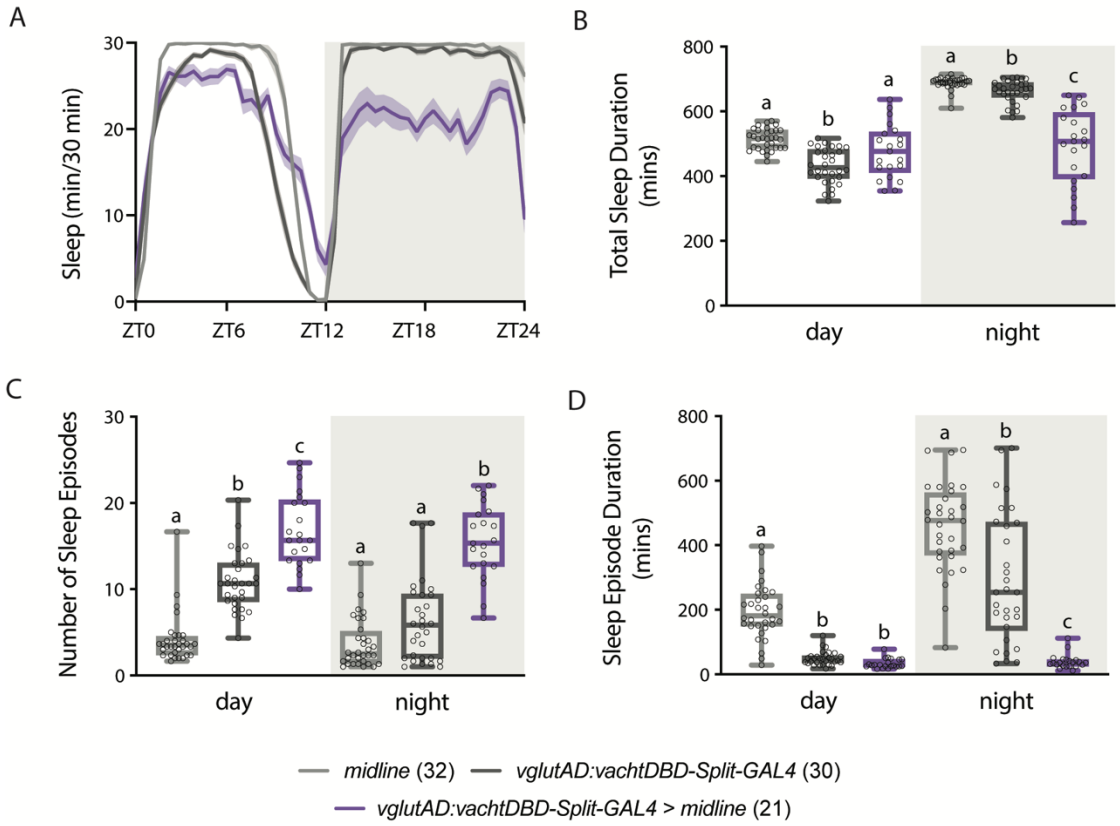

Figure 2.6. *midline* over-expression in Glu^{ACh} cells partially phenocopies *miR-190* knockdown. (A) Sleep per 30 mins across 24 hours of LD. (B) Mean total sleep during both day and night relative to UAS control and scramble control. (C-D) Number of sleep episodes and sleep episode duration during daytime and nighttime. Whiskers represent maxima and minima. Circle data points representing individuals overlaid box-and-whiskers. Letters indicate groups of statistical similarity ($p<0.05$) for one-way ANOVA with Tukey's multiple comparisons test or Kruskal-Wallis with Dunn's multiple comparisons test. Numbers of individuals per group are reported next to the genotype.

P=0.01439 in adults) (Table 2.1). Additionally, we observed an upregulation of *midline*, a transcription factor predicted to be a suppressor of glutamatergic fate (Estacio-Gomez et al., 2020; Leal et al., 2009) (300-fold, P=0.022 in pupae) (Table 2.1). These were considered of interest, given our sleep behavior results, which demonstrated that knockdown of miR-190 in cholinergic, glutamatergic, and Glu^{ACh} cells lead to a disrupted sleep behavior (Figure 2.2). Interestingly,

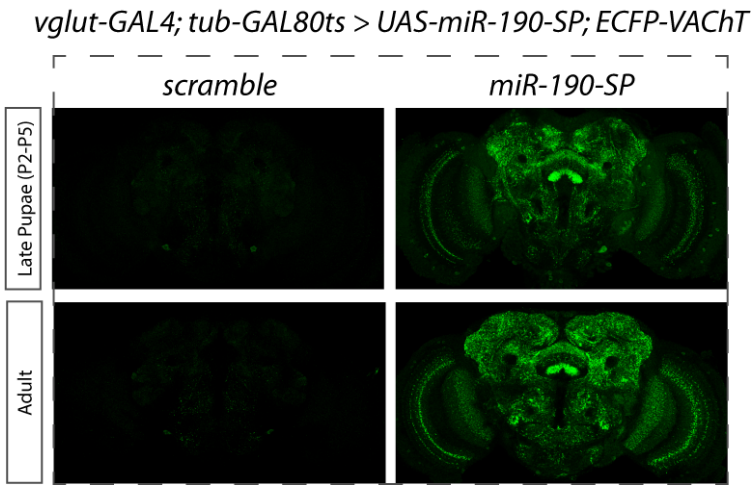

Figure 2.7. *miR-190* acts in Glu^{ACh} neurons to suppress VAChT translation. ECFP signal is increased upon both pupal and adult expression of miR-190-SP under the glutamatergic driver vglut-GAL4.

mid over expression in Glu^{ACh} cells partially phenocopies *miR-190* KD, having a decrease in total sleep during the nighttime (Figure 2.6. A-B) and an increase in sleep fragmentation, with animals showing significantly more sleep episodes (Figure 2.6C) of shorter duration (Figure 2.6D) during both day and night.

Additionally, we observe that expression of miR-190-SP in glutamatergic cells induced an increase in ECFP-VAChT signal in FSB neurons, relative to scramble control (Figure 2.7). These results demonstrate that under normal conditions, *miR-190* acts to suppress translation of VAChT in Glu^{ACh} neurons.

Taken together these data suggests a role of *miR-190* for the suppression of cholinergic identity in Glu^{ACh} cells. Our results suggest that under normal conditions

miR-190 acts to suppress cholinergic identity through the downregulation of cholinergic targets and the suppressor of neuronal glutamatergic fate determination *midline*.

miR-190 inhibition alters dopaminergic tone

Our previous results indicated that *miR-190* pan-neuronal knockdown leads to deficits in sleep rebound (Goodwin *et al.*, 2018). It has also been shown that there are changes in gene expression after sleep deprivation (Gilestro *et al.*, 2009). To determine if the response to SD is disrupted by *miR-190* inhibition we performed RNAseq heads from *nsyb-GAL4 > miR-190-SP* (and scramble controls) at ZT0 after 12 hours of sleep deprivation (non-deprived flies were also used as controls). Our results showed a decrease in expression of dopaminergic genes after SD in animals pan-neuronally expressing miR-190-SP,

Gene	Developmental Stage	Fold-Decrease	P-Value
vmat	Adult	2	0.0297
dop1r1	Adult	1.8	0.0023
dat	Adult	1.9	0.0447
dop2r	Adult	1.6	0.0904

Table 2.2. Downregulated dopaminergic genes upon miR-190-SP pan-neuronal expression and Sleep Deprivation in adult brains. Dopaminergic genes downregulated upon sleep deprivation and constitutive pan-neuronal expression of miR-190-SP. Fold-change and P-value significance for edgeR differential expression analysis relative to scramble controls is shown.

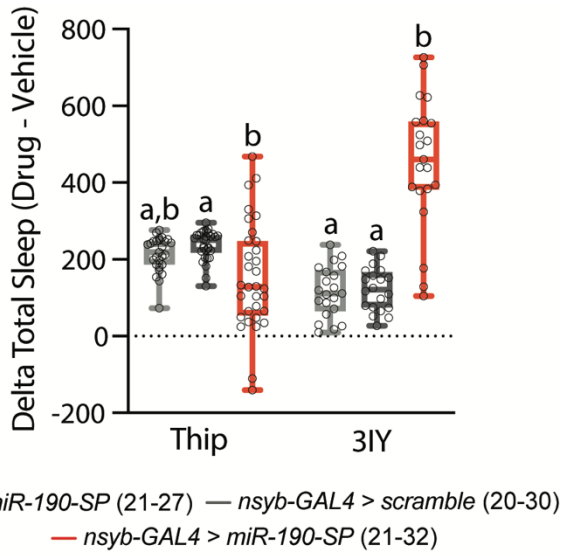

Figure 2.8. miR-190-SP flies are specifically sensitive to dopamine signaling. Flies were fed with either THIP (0.1 mg/mL) or 3IY (0.3125 mg/mL). Whiskers represent maxima and minima. Circle data points representing individuals overlaid box-and-whiskers. Letters indicate groups of statistical similarity ($p<0.05$) for one-way ANOVA with Tukey's multiple comparisons test. Numbers of individuals per group are reported next to the genotype.

but not in controls. Among these genes were the vesicular monoamine transporter VMAT (2.0-fold, P=0.02971), the dopamine transporter DAT (1.9-fold, P=0.04471), and a dopamine receptor, Dop1R1 (1.8-fold, P=0.00238) (Table 2.2). These results suggests that the low levels of total sleep in miR-190-SP animals may be due to increase baseline levels of dopaminergic signaling (Supplementary Table 1). Our data suggests that upon sleep deprivation, elevated levels of dopaminergic signaling would have to be decreased to allow animals to sleep rebound, and this compensation could be achieved by decreasing expression of dopaminergic genes.

To assess whether the low levels of baseline sleep in miR-190-SP expressing animals were due to aberrant high levels of DA signaling, *nsyb-GAL4 > miR-190-SP* (and their respective scramble controls) were fed the dopaminergic synthesis inhibitor 3-Iodo-Tyrosine (3IY), previously shown to be a strong somnogen (Andretic et al., 2005). Feeding 3IY lead to an increase in total sleep for *nsyb-GAL4 > miR-190-SP* but not for their respective controls (Figure 2.8). To discard the possibility of the sleep rescue coming from a parallel pathway independent of *miR-190*'s effect and not DA specific, we fed flies with the $GABA_A$ agonist Gaboxadol (THIP). Feeding of THIP did not have significantly different effects on miR-190-SP and scramble groups (Figure 2.8). Additionally, our HPLC analysis of bioamine levels in brains showed no difference in steady-state levels (Figure 2.9), suggesting that the proposed increase in DA signaling is limited specifically to the sleep circuit rather than globally. miR-190-SP animals show no hyperactive locomotor activity (Figure 2.1G), which would be expected to see with global increases of DA signaling such as that observed upon amphetamine treatment (Andretic *et al.*, 2005).

Given the well-established role of dopamine as the main neurochemical effector of arousal (Andretic *et al.*, 2005), at least partially via inhibition of the dFSB circuit (Liu *et al.*, 2012; Ueno et al., 2012), our data posits that in the miR-190SP flies there is an elevated baseline dopaminergic activity, which posits a need to downregulate dopaminergic signaling in the dFSB upon SD. To investigate the effect of *miR-190* on the neuronal activity of the dFSB we utilized the genetically encoded calcium indicator GCaMP. GCaMP was expressed in in dopaminergic projections onto the dFSB be means of the *TH-LexA* driver, while pan-neuronally expressing miR-190-SP under the elav-GAL4 driver. *elav-GAL4; TH-LexA > miR-190-SP; GCaMP* flies showed a significantly higher ΔF/F0 than their respective scramble control, upon stimulation with the cholinergic agonist carbachol (Figure 2.10A-B). Taken together our data suggests that *miR-190* knock-down effects on sleep may be due to an increase in dopaminergic signaling locally in the dFSB. Summarizing, our results demonstrate that *miR-190* is required in neurons for its sleep-regulatory functions. We specifically demonstrate that *miR-190* acts during pupation to establish the adult sleep behavior. Our results map *miR-190* sleep regulatory function to a peculiar and previously undescribed population of neurons with co-transcription of both glutamatergic and cholinergic markers. The current work shows that in these cells, *miR-190* acts to suppress cholinergic markers and hence promote glutamatergic differentiation. Additionally, our

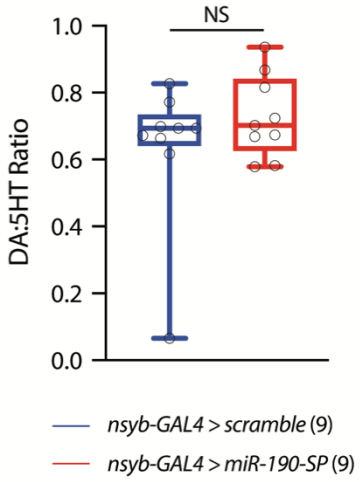

Figure 2.9. miR-190-SP expression has no effects on global levels of DA. *nsyb-GAL4 > miR-190-SP* (and *scramble*) male fly brains were dissected and used for HPLC against DA. A ratio between DA and 5HT was calculated. Whiskers represent maxima and minima. Circle data points representing individuals overlaid box-and-whiskers. NS represents p>0.05 for Mann-Whitney test.

results show that *miR-190* acts to suppress neuronal activity in dopaminergic projections upon dFSB cells.

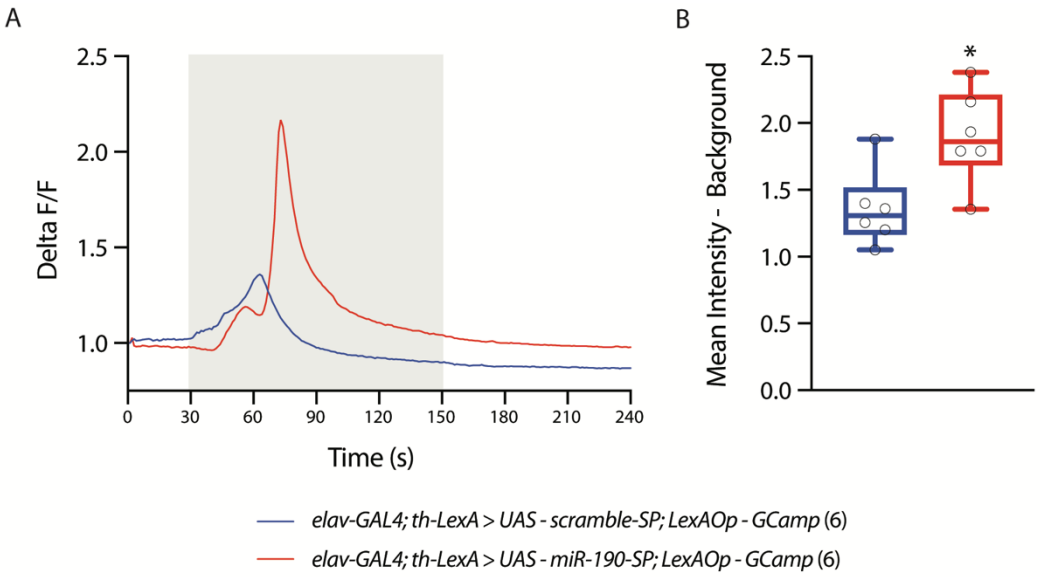

Figure 2.10. Pan-neuronal miR-190-SP expression leads to increase neuronal activity in TH+ projections onto the dFSB. (A) GCaMP signals were recorded from TH+ projections on the dFSB, while simultaneously perfusing AHL for 30 seconds (baseline), followed by Carbachol [100 μM] perfusion for 120 seconds, followed by a AHL wash for 90 seconds. (B) Delta F/F was calculated from baseline signal and signal during Carbachol perfusion. Whiskers represent maxima and minima. Circle data points representing individuals overlaid box-and-whiskers. Asterisk represents statistical significance ($p<0.05$) for Mann-Whitney test.

Experimental Procedures

Drosophila Lines

UAS lines include *UAS-miR-190-SP* and *UAS-scramble-SP* (Fulga et al., 2015), *20XUAS-IVS-dTRPA1* (Hamada et al., 2008), *UAS-mid2.12* (Miskolczi-McCallum et al., 2005), and *UAS-Red-Stinger*. LexAop strains include *LexAop-GCamp6f* (Bloomington #44277). GAL4 strains include *nsyb-GAL4, elav-GAL4* (Bloomington #458), *chat-GAL4* (Bloomington #60317), *vglut-GAL4* (Bloomington #38078). A *th-LexA* line was used (Dana et al., 2014). GAL80 lines include *tubulin-GAL80^{ts}* (McGuire et al., 2003), and *vglut-GAL80* (Bloomington #58448). Split-GAL4 lines include *vglut-AD* (Bloomington #82986) and *vacht-DBD* which was generated by cloning its DNA sequence into the *Drosophila* expression vector pBS-KS-attB2-SA(1)-T2A-Gal4DBD-Hsp70 which was then integrated into Mi{MIC}ChAT[MI08244] on chromosome 3.

Drosophila Husbandry

Flies were grown on standard cornmeal-dextrose-yeast agar medium at 25°C in a 12-hr LD cycle. To express miR-190-SP during development or adulthood with *tubulin-GAL80^{ts}*, flies were raised at either 18°C or 29°C. To avoid lethality due to constitutive activation of Glu^{ACh} cells with dTRPA1 during either development or adulthood, flies were exposed to 4x 1h periods of 29°C, each 29°C period was followed by 2h periods of 18°C.

For pharmacological experiments animals were grown on standard cornmeal-dextrose-yeast agar medium until adulthood. 3-5 days old animals were loaded in DAM tubes

containing either Thip (Gaboxadol) or 3-Iodo-Tyrosine, diluted in a food mixture of 2% agar and 5% sucrose, at a concentration of 0.1 mg/mL and 0.3125 mg/mL, respectively. Drug concentrations were chosen in such a way that sleep behavior of control animals was not disrupted.

Sleep Assays

Sleep was measured using the Drosophila Activity Monitoring (DAM) system (Trikinetics, Waltham, MA), as previously described (Donelson et al., 2012). 3-5 days post-eclosion flies were loaded into glass tubes with a food mixture of 2% agar and 5% sucrose. Temperature was kept at 25°C for the duration of the experiment unless differently specified. Flies were raised and tested under a 12:12 Light:Dark cycle. Flies showing periods of more than 5 minutes of immobility were classified as sleeping (Hendricks et al., 2000). Flies were entrained for 2 days, and sleep was averaged across 3-5 days.

Analysis Software and Statistical Testing

Sleep data was analyzed using the Sleep and Circadian Analysis MATLAB Program (SCAMP) (Donelson et al., 2012). Statistical analysis was performed with GraphPad Prism. For all sleep parameters a D'Agostino & Pearson test was used to determine normality of data. If data was normally distributed it was analyzed using a T-test or ANOVA (depending on the number of groups) with Tukey test for multiple comparisons. If data was not normally distributed it was analyzed using a Mann-Whitney or Kruskal-Wallis test with a Dunn's test for multiple comparisons.

Immunohistochemistry

Fly brains were dissected in cold Schneider Insect Medium (S2). Brains were fixed with 2% paraformaldehyde (PFA) in S2 at room temperature for 55 minutes. After fixation brains were washed 4x with phosphate buffered saline with 0.5% Triton X-100 (PBST) while nutating. Brains were then placed in blocking solution (PBST with 5% normal goat serum (NGS; Invitrogen) for 1.5 hours at room temperature while nutating. Blocking solution was removed and brains were incubated in primary antibodies diluted in 5% NGS for 4 hours at room temperature followed by 2 overnights of incubation at 4°C while nutating. Brains were then washed 4x in 0.5% PBST and incubated with secondary antibodies diluted in 5% NGS for 4 hours at room temperature followed by 3 overnights of incubation at 4°C while nutating. Brains were washed in 4x in 0.5% PBST as previously described and then fixed in 4% PFA for 4 hours while nutating. Brains were washed in 4x in 0.5% PBST and mounted on slides using Vectashield Mounting Medium (Vector Laboratories). Slides were imaged on a Leica SP5 confocal microscope under 20x or 60x objective lens. Sum intensity projections were generated using FIJI software.

The primary antibodies used were: rabbit anti-GFP (1:1000, Thermo Fisher), mouse anti-GFP (1:200, Sigma) and mouse anti-Brp (1:100, DSHB). The secondary antibodies used were Alexa Fluor 488 anti-mouse/rabbit antibody (Invitrogen), and Alexa Fluor 635 anti-mouse/rabbit antibody (Invitrogen), both at 1:200 dilutions.

Paired-end RNA Sequencing

tub-GAL80^{ts}; nsyb-GAL4 > miR-190-SP and *tub-GAL80^{ts}; nsyb-GAL4 > scramble-SP* flies were raised at 18C and shifted to 29C 2 days after pupation onset, prior to dissection at pharate stage. Pharate brains were dissected in cold AHL with TTX. Total RNA was extracted using Trizol. mRNA was selected using Dynabeads mRNA DIRECT Purification Kit (Thermofisher). Paired-end sequence libraries were generated from the purified mRNA using NEXTflex Rapid Directional qRNA-Seq kit (Illumina) in accordance with the manufacturer's instructions. These libraries were quantified using an Agilent TapeStation and sequenced using a NextSeq 500 system (Illumina) with paired-end reads of 75bp. Three replicates were collected for each genotype. STAR was used to map the sequenced reads to the dm6 genome. The edgeR software package was used to perform Differential Gene Expression analysis on the mapped reads (Robinson et al., 2010).

High-Performance Liquid Chromatography (HPLC)

HPLC was performed with the collaboration with Jackeline Whitney Parker, from the Hirsh lab at the University of Virginia. Brains from 3-5 days old male flies were dissected and used for HPLC. Brains were transferred to a 1.5mL tube, containing 50uL of 50mM citric acid. Brains were quick-spined and homogenized. Homogenized supernatant was transferred to a spin filter and centrifuged at 11K rpm for 4 minutes. After samples were prepared, those were placed in dry ice and sent to the Hirsh lab for analysis. 40uL of sample were loaded and measures were taken using ChromeNav. DA peak values were normalized to 5HT.

GCaMP6f Imaging

Imaged flies were males, 3-5 days post-eclosion. *elav-GAL4; TH-LexA > miR-190-SP; GCamp6f* brains were dissected in cold AHL and loaded into a recording chamber submerged in AHL. GCaMP signals were recorded while simultaneously perfusing AHL for 30 seconds (baseline), followed by Carbachol [100 μM] perfusion for 120 seconds, followed by a AHL wash for 90 seconds. Imaging data was acquired using a Zeiss 880 LSM confocal microscope with a 40x objective, at an acquisition rate of 1Hz (1 second exposure), a 1824x1424 resolution, and 4x4 binning. GCaMP signals were recorded from TH^+ projections onto dFSB. ΔF/F was calculated from baseline signal and signal during Carbachol perfusion. Data was analyzed with a Mann-Whitney test using Prism GraphPad.

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Chapter 3

Discussion

Previous work has linked microRNAs to the regulation of several biological processes and behaviors, including sleep (Chen and Rosbash, 2016; 2017; Davis et al., 2007; Davis et al., 2012; Zhang et al., 2021a; Zhang et al., 2021b). To find specific microRNAs involved in the regulation of sleep our lab performed a screen using a library of microRNA sponges targeting specific microRNAs (Fulga et al., 2015; Goodwin et al., 2018). The screening performed by Goodwin et al. (2018) identified 33 microRNAs involved in the regulation of sleep, with 8 microRNAs identified as wake-promoting and 17 others identified as sleep promoting. Among the sleep promoting microRNAs identified in by Goodwin et al. (2018), we find the microRNA *miR-190*. Our early findings showed that either global or pan-neuronal knockdown of *miR-190* leads to disruptions in sleep behavior, with flies showing decreased total sleep and increased sleep fragmentation relative to scramble controls (Goodwin et al., 2018). The current work aimed to further understand miR-190's role on the regulation of sleep and arousal. My work specifically looked into determining: **(1)** *miR-190's* locus of action for sleep regulation, **(2)** *miR-190's* requirement for establishing sleep levels and architecture during development, adulthood or both and **(3)** *miR-190's* mRNA target identification and mechanism of action.

Specifically knocking down miR-190's function in glutamatergic cells, by means of expression of miR-190-SP under a *vglut-GAL4* driver, phenocopied *miR-190* pan-neuronal knockdown, with animals showing a dramatic decrease in total sleep and

increased sleep fragmentation. Interestingly the same effect was observed when expressing miR-190-SP under the cholinergic driver *chat-GAL4*. These results prompted us to ponder the possibility of *miR-190* acting in a population of cell with both glutamatergic and cholinergic neurotransmitter identity. To address the possibility of *miR-190* acting in neurons with dual neurotransmitter machinery, we designed split-GAL4 drivers. Using CRISPR we engineered split-GAL4 domains (AD and DBD) into the *VAChT* and *VGluT* genes. Interestingly, expression of miR-190-SP under this *vglut: vacht-split-GAL4* combination, phenocopied the sleep behavior observed when expressing the sponge under the aforementioned pan-neuronal, glutamatergic, and cholinergic drivers. We refer to the cells targeted by this split-GAL4 combination as Glu^{ACh} neurons. Given that no overlap between the cholinergic and glutamatergic populations had been reported in the adult brain, we assessed whether the effect of the sponge was due to expression at an earlier time in development, by limiting *nsyb > miR-190-SP* expression with tub-$GAL80^{ts}$. Our results showed that miR-190-SP expression specifically during pupation, but not during earlier or later stages, elicited statistically significant changes in sleep that phenocopied the constitutive knockdown of *miR-190* in all neurons, or specifically in the neurons targeted by the *vglut-GAL4* and *chat-GAL4* drivers. These results coincided with other findings from our lab which demonstrated that Glu^{ACh} cells co-transcribe glutamatergic and cholinergic genes from pupation to adulthood (Y. Zhang, unpublished). Additionally, our results demonstrate that expression miR-190-SP in Glu^{ACh} neurons leads to an increase in *VAChT::ECFP* signal, relative to scramble control. These findings are in agreement with our results from RNA sequencing that demonstrate an upregulation of the cholinergic genes *chat, vacht,* and

spab, and the suppressor of glutamatergic differentiation *mid* (Estacio-Gomez et al., 2020; Leal et al., 2009). Taken together these results strongly suggest that miR-190 acts in Glu^{ACh} cells to inhibit their cholinergic differentiation and promote glutamatergic cell fate.

The drastic reduction of sleep seen with knockdown of *miR-190* implies an aberrant configuration of the sleep circuits involved in the determination of daily sleep levels and arousal. Additionally, our results show that *nsyb-GAL > miR-190-SP* animals can only generate very low levels of compensatory sleep in response to sleep deprivation (Goodwin et al., 2018). This diminished response to sleep deprivation could be explained by the fact these animals have a low baseline sleep, hence the minutes they can be deprived are lower as well, leading to a lower sleep compensatory rebound. However, it was not clear whether this diminished compensatory sleep response could be completely explained by a floor effect. To determine whether the response to sleep deprivation is affected by inhibition of *miR-190* we performed RNA sequencing from heads of sleep deprived animals pan-neuronally expressing miR-190-SP. We observed that sleep deprived animals pan-neuronally expressing miR-190-SP showed a significant decrease in several dopaminergic related genes such as *vmat*, *dop1r1* and *dat*, relative to scramble controls. Given that dopamine is the principal neurochemical mediator of arousal (Andretic et al., 2005), and its known to act through the dFSB circuit (Liu et al., 2012; Ueno et al., 2012), our data suggested that knockdown of *miR-190* leads to an increase in baseline dopaminergic signaling, which posits a need to suppress dopaminergic signaling upon sleep deprivation, for the animal to compensate for sleep loss. Our results demonstrated as well that the observed low levels of baseline

sleep observed upon *miR-190* knockdown could be explained by an increase in baseline dopaminergic signaling. This idea was supported by the fact that treatment with the dopamine synthesis inhibitor 3IY resulted in a rescue of the sleep behavior. Additionally, our assessment of neuronal activity of TH+ projections on the dFSB, by means of the calcium indicator GCaMP, demonstrated higher responses to carbachol stimulation in miR-190-SP expressing animals, relative to controls. HPLC analysis of bioamine levels from whole brains did not detect any global changes in DA levels, suggesting a locus-specific effect of *miR-190* on dopaminergic signaling. This is also supported by the fact that miR-190-SP expressing animals don't show a hyperlocomotor behavior, as has been observed with global increases in dopamine levels, for example, by means of amphetamine treatment (Andretic et al., 2005).

The Model:

This work suggests a mechanism for the establishment of adult sleep and arousal baseline. In this model, the microRNA *miR-190* acts in a specific group of pupal neurons to inhibit their expression of cholinergic proteins, hence determining their glutamatergic fate. Our experiments demonstrate that inhibition of *miR-190* result in the upregulation of several cholinergic target genes during pupation, concomitant with a disrupted adult sleep behavior, with decreased total sleep and increased sleep fragmentation. In this section I will postulate a model for the role of *miR-190* on the regulation of sleep by acting on the development of the neuronal sleep circuitry.

Studies demonstrate that the core sleep homeostasis circuit also serves as the most potent sleep-generating circuit in *Drosophila* (Donlea et al., 2011). In this sleep-

generating circuit the intrinsic neurons of the dFSB (exF12) operate as the node for integration between sleep promoting signals and the arousal system (Figure 3.1). Inputs from the arousal system into the dFSB are carried by a small number PPL1 dopaminergic neurons, which act on exF12 cells to inhibit their neurotransmitter release (Liu et al., 2012; Ueno et al., 2012). On the other hand, exF12 also receive sleep promoting inputs such as excitatory glutamatergic inputs from the clock and superior medial protocerebrum (Ni et al., 2019). Additional pro-sleep inputs onto exF12 cells also includes feedback from the ellipsoid body which, interestingly, receives input from exF12 and operates as a sensory integrator (Donlea et al., 2018). The nature of pro-sleep inputs from the ellipsoid body onto exF12 remains to be determined. Evidence has shown exF12 cells release both GABA and peptides (Donlea et al., 2018; Ni et al.,

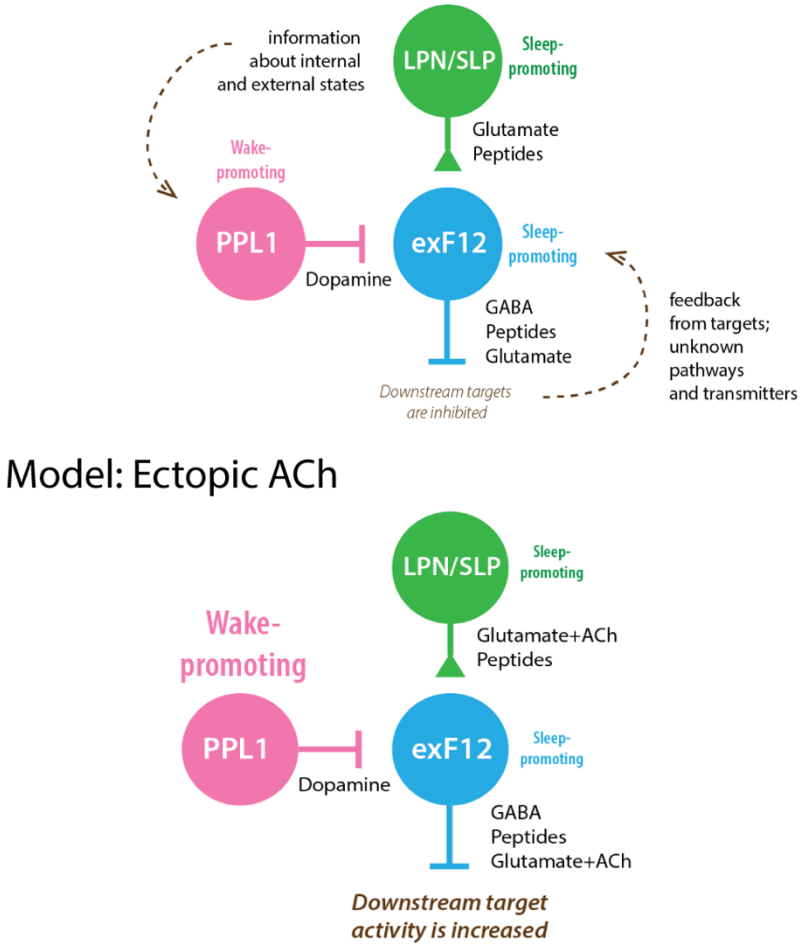

Figure 3.1. Core sleep homeostasis circuit Wildtype is shown at top. Hypothesized changes after pupal inhibition of *miR-190* are shown at bottom.

2019). Additionally, results from our FP marking system have demonstrated these cells to be glutamatergic (Figure 3.2), suggesting they could act to inhibit downstream targets via GluCl or mGluR. Also, exF12 cells are included in the Glu^{ACh} cells (Figure 3.3), making them candidates for being part of the circuit involved in establishing adult sleep levels. The second candidate group in the proposed circuit are the excitatory inputs from the clock and superior protocerebrum, which release both glutamate and peptides. Whether these cells are part of the Glu^{ACh} cluster remains to be elucidated.

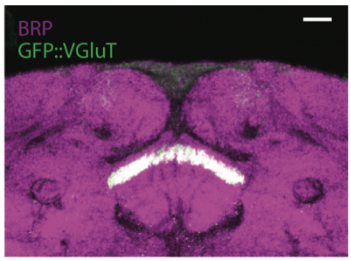

Figure 3.2. exF12 cells are glutamatergic. IHC from *84C10-GAL4 > FLP; FRT-stop-FRT-GFP-VGluT* adult brains. FLP recombination in dFSB cells removes a stop cassette from the VGluT::GFP allele and marks them as $VGluT^{+}$.

A model is proposed to explain how disruptions in *miR-190* result in a lower adult sleep baseline (Figure 3.1). This model postulates that Glu^{ACh} cells become abnormally cholinergic upon pupal knockdown of *miR-190*. Lack of suppression of the cholinergic release machinery in exF12 cells may result in inappropriate ACh release (usually excitatory in insects), which together with glutamate and GABA would diminish the ability of exF12 cells to inhibit downstream targets. In such conditions, dopaminergic inputs would have a much higher effect on sleep given the lack of opposing sleep-promoting drive.

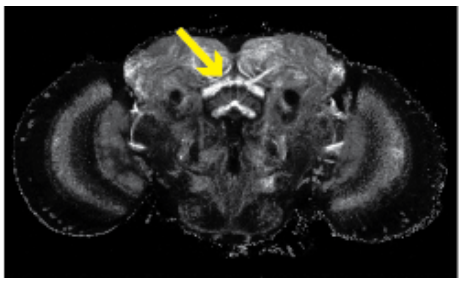

Figure 3.3. Glu^{ACh} neurons are a sleep controlling subset of adult glutamatergic cells. IHC from *vglutAD: vachtDBD-Split-GAL4 > myr-GFP*. Cell membrane labeling shows neutropils innervated by Glu^{ACh} cells. Yellow arrow marks dFSB.

Several approaches could be used to further test models for *miR-190* regulation of sleep in *Drosophila*. One would be to identify direct and indirect targets of *miR-190* with altered expression upon *miR-190* downregulation, specifically in Glu^{ACh}

cells. To understand the effects of *miR-190* inhibition on the expression of target genes, we could perform FACS sorting to isolate Glu^{ACh} cells from both pupal and adult brains, upon expression of miR-190-SP, followed by RNAseq. As performed with our bulk RNAseq experiments, putative targets of *miR-190* for the establishment of sleep/arousal baseline, would be confirmed by means of overexpression and knockdown of those within Glu^{ACh} cells. Changes in sleep behavior after manipulation of putative targets would be addressed by means of measurements of locomotor activity, using the DAM system.

It would also be important to investigate the normal role of Glu^{ACh} cells in adult sleep behavior. This could be achieved by making use of neuronal activators (dTRPA1, CsChrimson) and inactivators (shi^{ts}, GtACR1), expressed within Glu^{ACh} cells, by means of our split-GAL4 drivers. Such experiments have been attempted as part of our recent work. However, our data shows that our Glu^{ACh} split-GAL4 drivers also target cells within the VNC, potentially involved in locomotion. When we activated Glu^{ACh} cells by means of dTRPA1 we observed increased sleep fragmentation (data not shown). However, we have also observed that strong activation of Glu^{ACh} cells by means of dTRPA1, also leads to animals having seizures and ultimately dying with constant activation. Hence, when expressing neuronal activators or silencers within Glu^{ACh} cells, our sleep behavior results may be contaminated by mere changes on locomotion (data not shown). To avoid this, we are currently building lines that have both miR-190-SP and *tsh-GAL80* a transgene that suppresses GAL4 action within the ventral nerve chord (VNC), aiming to suppress potential activation of motor neurons contained within the VNC (Chen et al., 2018). Additionally, the specific role of exF12 cells within the Glu^{ACh} cluster might be

addressed by utilizing exF12 to target the expression of the aforementioned neuronal activators and inhibitors. These experiments will inform whether cells within the Glu^{ACh} cluster are involved in the regulation of sleep and arousal, and if they effect a specific sleep or wake-promoting function.

Another important aim would be to understand whether the observed changes in sleep behavior upon miR-190-SP expression are caused by changes in levels of cholinergic transmission. Our results demonstrate an increase in VAChT::ECFP levels increase upon miR-190-SP expression in Glu^{ACh} cells (Figure 2.7). However, we found that overexpression of VAChT alone is not sufficient to recapitulate miR-190-SP sleep phenotype (data not shown). Future work could address whether knockdown of *miR-190* also elicits changes in the levels of other cholinergic proteins such as ChAT. This could be achieved by designing a ChaT::ECFP reporter to be expressed together with miR-190-SP, followed by IHC. Additionally, future work could address whether overexpression of the vesicular acetylcholine transporter (VAChT), together with the choline acetyltransferase (ChAT), within the Glu^{ACh} cluster, is sufficient to phenocopy miR-190-SP phenotype.

To further unravel our model, it would also be important to identify abnormal cholinergic outputs from exF12 cells. To do this, we could express the ATP-gated cation channel P2X2 in Glu^{ACh} cells (together with miR-190-SP or scramble-SP), while GCaMP is expressed in Helicon cells, target of exF12 cells, by means of the *R24B11-LexA* driver. In *Glu^{ACh} > scramble* animals application of ATP should evoke release from Glu^{ACh} neurons onto Helicon cells, causing inhibition in the latter (Donlea et al., 2018). On the other hand, if expression of miR-190-SP in Glu^{ACh} cells alters the balance and

identity of their signal, making them cholinergic, the activity of Helicon cells may increase. The neurotransmitter identity of the altered signals could be addressed by means of pharmacology, utilizing ACh, GABA and glutamate blockers. The same principle could be applied to determine abnormal neuronal signaling between LPN/SLP and exF12 cells (Ni et al., 2019).

Another aspect to be further studied is the involvement of the transcription factor *midline* in the sleep regulatory effects of *miR-190*. Our RNAseq results show that pupal knockdown of *miR-190* results in an upregulation of *midline*. Additionally, upregulation of *midline* in Glu^{ACh} cells results in a partial phenocopy of the miR-190-SP sleep phenotype, causing a decrease in nighttime sleep. To further understand *midline* involvement, we could express an RNAi against midline while simultaneously expressing the miR-190-SP in Glu^{ACh} cells. If the effects of *miR-190* knockdown on sleep are due to an abnormal effect of higher levels of midline, when expressing miR-190-SP concomitant with a *midline* RNAi, we should see a rescue in the sleep behavior. Our results from VAChT::ECFP with miR-190-SP expression demonstrate an increase in VAChT levels in Glu^{ACh} cells. To demonstrate the involvement of *midline* in the VAChT increase observed upon *miR-190* knockdown, we could express miR-190-SP together with *midline* RNAi and perform IHC to determine whether a VAChT increase is observed, relative to controls. A previous study demonstrates that *midline* acts to determine neuronal fate in the CNS through the regulation of the regulation of the *Drosophila* segmentation gene *even-skipped* (Doe et al., 1988; Leal et al., 2009). However, our results from whole-brain RNAseq doesn't show changes in *even-skipped*. FACS sorting of Glu^{ACh} cells from miR-190-SP expressing animals, followed by RNAseq

could highlight changes of downstream targets of *midline* potentially involved in proper neuronal fate determination of Glu^{ACh} cells, and hence *Drosophila* adult sleep behavior.

Taken together, our work demonstrates the involvement of the microRNA miR-190 in the establishment of sleep and arousal behaviors. Future work remains to be done to further unravel the specific neuronal circuit where the microRNA operates to effect its role on sleep and identify the molecular targets of miR-190 within this specific locus. Our work shines light both on the effects of microRNA on both the regulation of sleep and development. This work may help better understand mechanisms of regulation of sleep in humans, allowing us to identify potential therapeutic targets, as well as better understanding sleep disorders and comorbidities.

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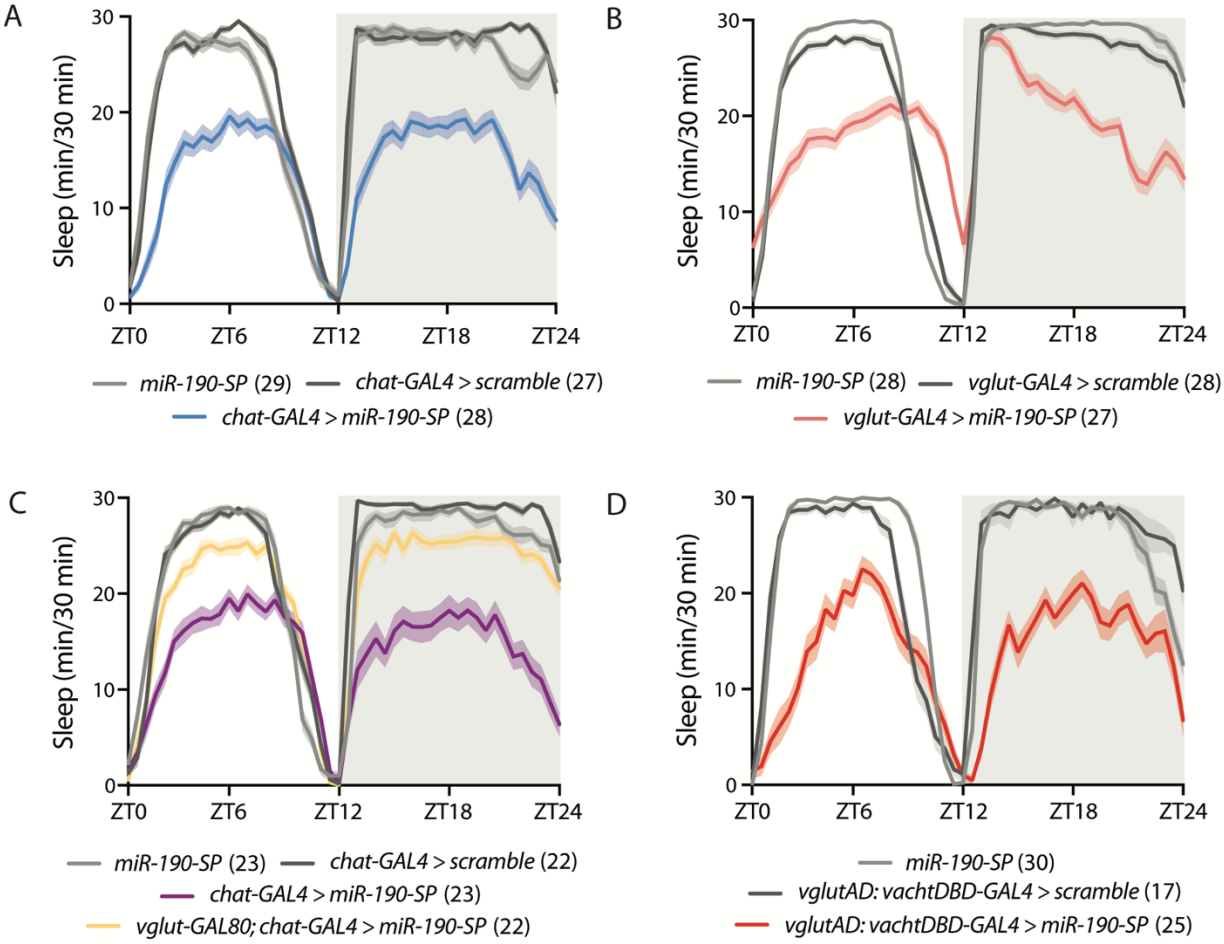
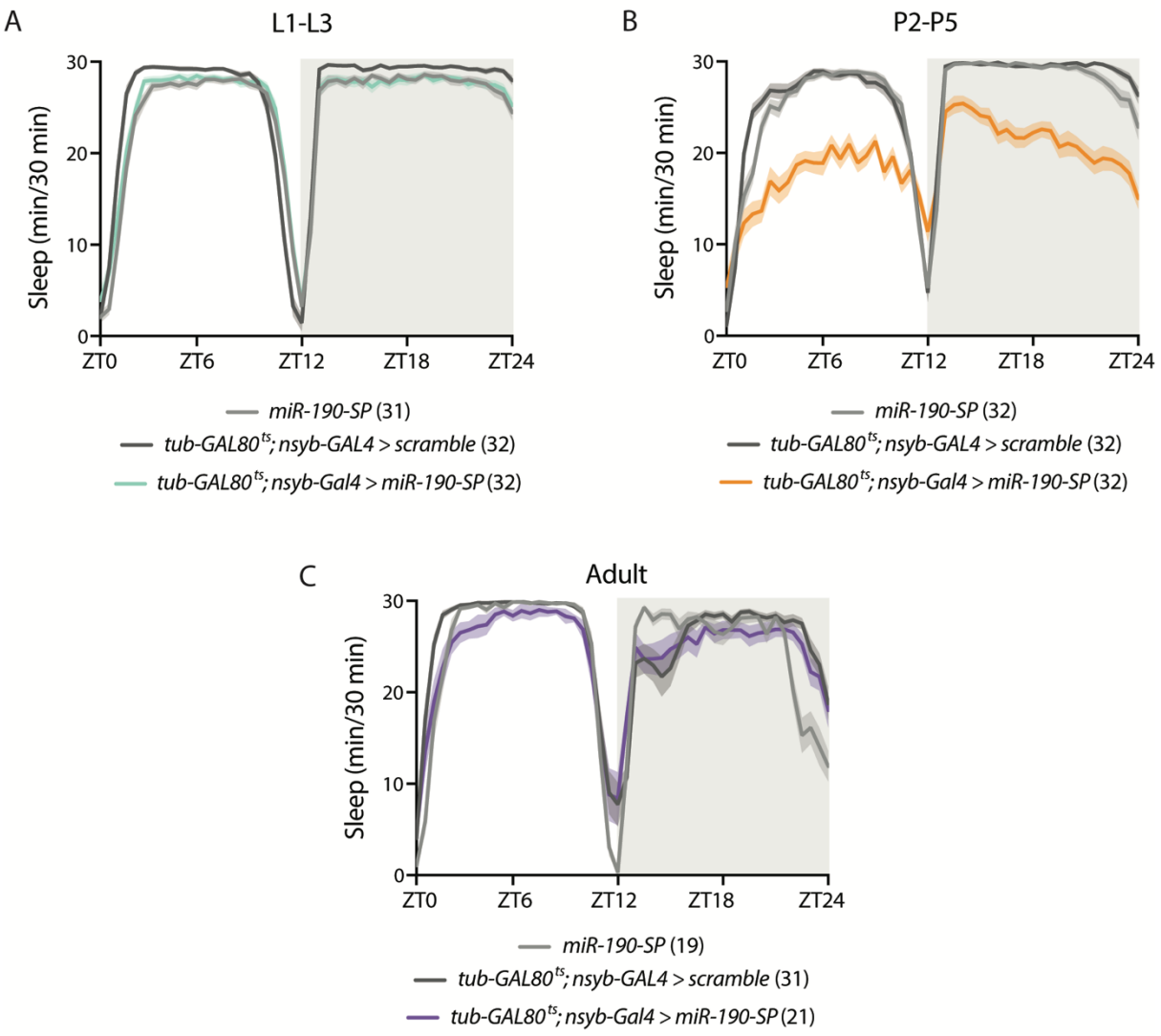
Supplementary Figures



Supplementary Figure 1. S30 Plots showing that *miR-190* acts in Glu^{ACh} cells to regulate sleep. In (A-D), LD sleep data for UAS control and scramble control is shown in light gray and dark gray, respectively. (A) data for chat-*GAL4 > miR-190-SP* is shown in blue. (B) Data for vglut-*GAL4 > miR-190-SP* is shown in pink. (C) Data for vglut-*GAL4 > miR-190-SP* is shown in pink. (D) Data for chat-*GAL4 > miR-190-SP* is shown in purple, data for *vglut-GAL80; chat-GAL4 > miR-190-SP* is shown in yellow. (D) Data for *vglutAD: vachtDBD-GAL4 > miR-190-SP* is shown in orange.



Supplementary Figure 2. S30 Plots showing that *miR-190* acts in the pupal brain to regulate sleep. In (A-C), LD sleep data for UAS control and scramble control is shown in light gray and dark gray, respectively. *tub-GAL80^{ts}; nsyb-GAL4 > miR-190-SP* flies pan-neuronally expressed miR-190-SP during either L1-L3, P2-P5, or adulthood, upon exposure to 29°C. (A) Data for miR-190-SP expressing animals during L1-L3 stages is shown in aqua. (B) Data for miR-190-SP expressing animals during P2-P5 stages is shown in orange. (C) Data for miR-190-SP expressing animals during adulthood is shown in purple.

Gene	*nsyb-GAL4 > scramble* non-sleep deprived	*nsyb-GAL4 > miR-190-SP* non-sleep deprived	*nsyb-GAL4 > scramble* sleep deprived	*nsyb-GAL4 > miR-190-SP* sleep deprived
vmat	3801.33	6136	5312.33	2621.66
dop1r1	670	1541.66	1000	558.66
dat	198.33	371.66	250.33	151.66
dop2r	336.33	831.33	580.66	377

Supplementary Table 1. Pan-neuronal expression of miR-190-SP leads to an upregulation in dopaminergic genes upon baseline sleep, and downregulation upon sleep deprivation. Raw reads from RNA sequencing performed from whole brains from non-sleep deprived and sleep deprived *nsyb-GAL4 > scramble* and *nsyb-GAL4 > miR-190-SP* flies.