Neuropeptides in Neural Circuits and Behavior

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Neuropeptides in Neural Circuits and Behavior

A dissertation presented

by

Hannah Elizabeth Somhegyi

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

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in the subject of

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Neurons comprise major elements of the nervous system, but do not function in isolation. All nervous system function requires neurons to communicate with each other to integrate, relay, and output information. Neuropeptides are a class of signaling molecules commonly used throughout the nervous system to transfer information between neurons; however, their actions in neuronal circuits and roles in behavior are less understood compared to those of small molecule neurotransmitters. To examine the role of neuropeptides in circuits and behavior we performed two complementary studies.

To investigate the role of neuropeptides in neural circuits, we studied how neuropeptides might influence primary olfactory circuit function. We find that all olfactory neurons investigated within a single Drosophila olfactory microcircuit contain dense-core vesicles (DCVs), suggesting that most olfactory neurons express neuropeptides in addition to neurotransmitters. We show that many DCVs localize to presynaptic sites, suggesting that cotransmission of neurotransmitters and neuropeptides plays a prevalent role in this circuit. We find that projection neuron (PN) presynaptic sites contain more DCVs than expected by chance when olfactory receptor neurons (ORNs) are postsynaptic. These data suggest that PNs may use neuropeptides to regulate signaling within a single olfactory channel. This provides an additional modulatory mechanism that can increase the flexibility of olfactory circuit function.

We also explored how neuropeptides influence behavior by identifying a novel role for a neuropeptide in sleep behavior. We find that the neuropeptide Corazonin (Crz) is released from
~14 neurons in the pars lateralis to promote wake behavior in Drosophila. We show that the cell cycle protein, Cyclin A, localizes to presynaptic sites within these cells and promotes wake likely via Crz release. In addition, we provide evidence that Cyclin A functions with other repurposed cell cycle machinery to promote wake. Finally, we find that these cells integrate signals from several wake-promoting neuropeptides. This work identifies a novel network of sleep molecules, including the neuropeptide Corazonin, and an integrating center of wake-promoting signals. Together, these studies provide valuable insight into the role of neuropeptides in circuit function and behavior.
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LIST OF ABBREVIATIONS

Ach acetylcholine
AL antennal lobe
APC/C anaphase-promoting complex/cyclosome
AST-A allastatin A-type peptide
ATP adenosine triphosphate
ATR allatotropin
cAMP cyclic adenosine monophosphate
Cdc20 Fizzy
Cdh1 Fizzy-related
Cdk1 Cyclin-dependent kinase 1
Cdk2 Cyclin-dependent kinase 2
Crz Corazonin
CrzR Corazonin receptor
CycA Cyclin A
CycB Cyclin B
DAR2 Drosophila Allostatin A receptor 2
DCV dense-core vesicle
DH31 diuretic hormone 31
DH31R diuretic hormone 31 receptor
DH44 diuretic hormone 44
DH44R1 diuretic hormone 44 receptor 1
DH44R2 diuretic hormone 44 receptor 2
DN1 dorsal neuron 1
DN2 dorsal neuron 2
DN3 dorsal neuron 3
DTK *Drosophila* tachykinin-related peptide
DTKR *Drosophila* tachykinin-related peptide receptor
GABA gamma-Aminobutyric acid
GFP green fluorescent protein
GRASP Reconstitution Across Synaptic Partners
LD 12-hour light/12-hour dark
LN local interneuron
LNd dorsal lateral neuron
MG multi-glomerular
MIP myoinhibitory peptide
MPN modulatory proctolin neuron
Myr-GFP myristoylated green fluorescent protein
NPF neuropeptide F
ORN olfactory receptor neuron
PDF pigment-dispersing factor
PDFR pigment-dispersing factor receptor
PER Proboscis Extension Response
PL *pars lateralis*
PN projection neuron
Rca1 Regulator of Cyclin A
RNAi RNA interference
s-LNv small lateral ventral neuron
l-LNv large lateral ventral neuron
sNPF short neuropeptide F
sNPFR1 short neuropeptides F receptor
STAT3 Signal transducer and activator of transcription 3
STG stomatogastric ganglion
TARA TARANIS
UAS-Dcr2 UAS-Dicer2
VMAT2 vesicular monoamine transporter 2
ZT Zeitgeber time
Chapter 1

INTRODUCTION
Underlying every behavior, thought, and perception is a neuronal circuit. Circuits are composed of elements, *i.e.* neurons, and their sites of connections, *e.g.* synapses. The function of a circuit is influenced at many levels. For example, intracellular proteins regulate the activities of specific neurons to direct sensitivity and reliability of individual components. Furthermore, the molecules used to mediate communication between neurons can influence the speed as well as area of interaction. Finally, the wiring diagram, *i.e.* how neurons are connected, specifies and organizes the computations performed by the network. Thus, to understand how complex behaviors are performed it is useful to investigate the various levels of neuronal circuit function.

To gain insight into these different aspects of neuronal circuit function, we focus our work on two levels of regulation. In Chapter 2, we investigate the role of co-transmission in *Drosophila* olfactory circuit function. We find that all olfactory neurons studied within a glomerulus contain DCVs in addition to synaptic vesicles, suggesting that these neurons may express both neuropeptides and neurotransmitters and may use a combination of these signals to communicate with other olfactory neurons. We find that certain synapse types are enriched with DCVs, suggesting that olfactory neurons may direct neuropeptide signaling to specific postsynaptic partners. Finally, we explore ways in which this directed signaling may influence olfactory circuit function.

In Chapter 3, we investigate proteins important for regulating neuronal function as well as the neuropeptides involved in *Drosophila* sleep behavior. We find that the cell cycle protein Cyclin A (CycA), together with other cell cycle proteins, function in ~14 neurons in the *pars lateralis* (PL) to promote cellular activity and wake behavior. We identify a novel function of the neuropeptide Crz in sleep behavior by showing that it relays wake information from the PL. Finally, we find that these cells integrate signals from several wake-promoting neuropeptides.
Together, this work highlights two levels of influence on circuit function: the molecules (e.g. neuropeptides) used by neurons to communicate and the proteins involved in regulating individual neuronal activity.

1.1 Neuropeptides in co-transmission

Neurons comprise major elements of the nervous system, but do not function in isolation. All nervous system function (e.g. smell, sleep, memory, etc.) requires neurons to communicate with each other to integrate, relay, and output information. There are many ways in which neurons communicate, and each method affects the quality of the message being sent. For example, neurons can talk using electrical synapses (i.e. gap junctions) or chemical synapses. Electrical synapses are mostly bidirectional and are typically faster than chemical synapses. Chemical synapses utilize a variety of substances (e.g. neurotransmitters, biogenic amines, neuropeptides, and gases), and each of these can have distinct effects (e.g. excitatory vs. inhibitory) on postsynaptic partners. In addition, neurons can release more than one type of transmitter (i.e. co-transmission), which adds further circuit flexibility. This section focuses on the role of signaling molecules, particularly in the case of co-transmission, on neuronal circuit function. We highlight examples of co-transmission, with emphasis placed on those from primary olfactory circuits, and explore how co-transmission supports circuit flexibility.

1.1.1 Rethinking Dale’s Principle: evidence for co-transmission

When neurotransmitters were first being discovered, the prevailing idea was that neurons use only one transmitter. Eccles (1957) established the idea of “one neuron-one transmitter”, widely referred to as Dale’s Principle, by interpreting a statement made by Dale that “a neuron is a
metabolic unit and operates at all its synapses by the same chemical transmission mechanisms” (Dale, 1935; Eccles, 1957; Sámano et al., 2012). However, over the late sixties and seventies, scientists discovered the first pieces of evidence suggesting that neurons can express more than one transmitter (Furshpan et al., 1976; Jaim-Etcheverry and Zieher, 1969; Lands, 1976; Owman, 1964; Patterson and Chun, 1974). In 1976, Burnstock confronted the concept of “one neuron-one neurotransmitter” and introduced the co-transmission hypothesis, stating that neurons can contain two or more transmitters in the same presynaptic nerve ending (Burnstock, 1976). It is now generally accepted that many neurons express multiple types of transmitters (Burnstock, 2004).

There are three general classes of co-transmitter neurons: neurons that release fast-acting, small-molecule neurotransmitters (e.g. glutamate, GABA); neurons that release a neurotransmitter and a slow-acting monoamine (e.g. dopamine); and neurons that release a neurotransmitter and a neuromodulator (e.g. neuropeptides, neurotrophins, ATP) (Vaaga et al., 2014). To further complicate the picture, neurons can also release more than two types of transmitters (Vaaga et al., 2014). Here, we will focus on the functional implications of neurotransmitter and neuropeptide co-transmission.

Neuropeptides, such as pigment-dispersing factor (PDF) and neuropeptide Y, are short peptides generally ranging from 3-100 amino acids in length (Salio et al., 2006). Unlike neurotransmitters, which are stored in synaptic vesicles, neuropeptides are housed in compartments called DCVs. Neuropeptides are synthesized and packaged into DCVs in the soma, and are then trafficked to their sites of release. Conversely, neurotransmitters are made and packaged into synaptic vesicles at presynaptic sites. Both neurotransmitters and neuropeptides can be released from presynaptic sites; however, neuropeptides can also be
released from non-synaptic sites, such as from the soma or dendrites. Finally, while neuropeptides can function broadly as circulating hormones (i.e. volume transmission), they can also act locally like neurotransmitters. In support of local function, there is considerable overlap between expression patterns of peptide-containing processes and their respective receptors in several regions of the brain (van den Pol, 2012). Furthermore, peptidases can actively break down peptides extracellularly to restrict neuropeptide action to local targets. These and other data led van den Pol to formulate the *local diffusion* hypothesis, suggesting that neuropeptides released by most neurons act locally on synaptic partners and on immediately adjacent neurons (van den Pol, 2012). In further support of this hypothesis, small lateral ventral neurons (s-LNvs), which release the major circadian pacemaker PDF, undergo time-dependent remodeling of their axonal projections. The axonal rearrangement of s-LNvs suggests that their connectivity to postsynaptic partners is dynamic and necessary for function (Gorostiza et al., 2014). These data are consistent with PDF acting locally, because rearrangement would be unnecessary in the case of volume transmission. In conclusion, while neurotransmitters and neuropeptides are both signaling molecules, there are a number of important differences between them that can lead to distinct physiological effects.

There are many functional implications of co-transmission. Co-transmitters can be released from the same or distinct presynaptic sites to allow a single neuron to release different combinations of transmitters onto postsynaptic partners (Figure 1.1, A) (Blitz and Nusbaum, 1999; Hattori et al., 1991; Kueh and Jellies, 2012; Landry et al., 2003; Sámano et al., 2006; 2012; Sossin et al., 1990; Sulzer and Rayport, 2000). For example, motor pattern selection in the stomatogastric ganglion (STG) of the crab *Cancer borealis* occurs during activation of modulatory proctolin neuron (MPN), which co-transmits proctolin and GABA. This selection
Figure 1.1: Co-transmission of neurotransmitters and neuropeptides increases circuit flexibility.

(A) Neurons may have neurotransmitter-containing, synaptic vesicles (red circles) present at all presynaptic sites, but preferentially direct neuropeptide-containing, DCVs (teal circles) to only a subset of sites. This would restrict co-transmission to only the teal colored postsynaptic partner. Conversely, the other postsynaptic partners (gray) would receive only neurotransmitter-mediated input. (B) Neuropeptide release tends to require a higher firing rate than that required for the release of small-molecule transmitters, so neuronal activity levels can direct which transmitters are released. During low presynaptic activity (left) typically only neurotransmitters are released, whereas higher presynaptic activity (right) generally allows the release of both neurotransmitters and neuropeptides. (C) Co-transmission can also be state dependent. For example, presynaptic state changes can trigger the action of an axo-axonic synapse. When the axo-axonic synapse is not active (left) state 1; e.g. starved), both transmitters are released. Conversely, when the axo-axonic synapse is active (right) green molecules released) state 2; e.g. fed) neuropeptide release is inhibited.
Figure 1.1: (Continued) Co-transmission of neurotransmitters and neuropeptides increases circuit flexibility.
requires the release of proctolin from MPN onto the STG network and GABA from MPN onto projection neurons in the commissural ganglia, which demonstrates a spatial and functional segregation of co-transmitter actions within MPN (Blitz and Nusbaum, 1999). In addition, neuropeptide release tends to require higher firing rates than those needed for the release of small-molecule transmitters, so neuronal activity levels can direct which transmitters are released (Figure 1.1, B) (Adams and O'Shea, 1983; Bishop et al., 1987; Cropper et al., 1990; Whim and Lloyd, 1989; 1990; Wilim et al., 1996). For example, weak activation of a cell may trigger the release of neurotransmitters, but only during periods of prolonged stimulation may neuropeptides be able to be released as well (Nusbaum et al., 2017). Co-transmission can also be state dependent. For example, presynaptic activity can be influenced by internal state changes, resulting from an axo-axonic synapse. When the axo-axonic synapse is not active (state 1; e.g. starved), both transmitters are released. Conversely, when the axo-axonic synapse is active (state 2; e.g. fed) peptide release is inhibited (Figure 1.1, C) (DeLong et al., 2009; Ko et al., 2015; Root et al., 2011). Here, the presence of two co-transmitters allows the cell to integrate and relay state-dependent information. Finally, neuropeptides usually act on GPCRs, whereas neurotransmitters typically, although not always, activate ligand-gated ion channels. Thus, the neuropeptides and neurotransmitters generally act on varying timescales, relatively long and short, respectively (Nusbaum and Blitz, 2012). Together, co-transmission alters the computational capabilities of neuronal circuits by enhancing coding space through spatial and temporal mechanisms.
Figure 1.2: Anatomy of Drosophila olfactory processing.

(A) Each hemisphere of the brain contains an antennal lobe (solid black circle), which is composed of sub-compartments called glomeruli (dashed black circles). Olfactory receptor neurons expressing the same odorant receptor (yellow) detect odorants and send axons to the same glomerulus. They form cholinergic synapses onto projection neurons (teal), which relay this information to higher-order brain regions. Local interneurons interconnect glomeruli, and synapse onto olfactory receptor neurons and projection neurons. They play an important role in lateral processing. (B) The same olfactory circuit is depicted but in a simplified model.
1.1.2 The *Drosophila* antennal lobe as a model for studying circuit implications of co-transmission

The fly senses olfactory cues in its antenna, and sends this information to the antennal lobes (ALs) (Figure 1.2). In the adult fly, there are about 50 glomeruli, the basic unit of organization in the olfactory system, which each receive feed-forward sensory input from olfactory receptor neurons. All ORNs projecting to the same glomerulus express the same odorant receptor, and therefore have similar odor responses (Hallem and Carlson, 2006; Vosshall et al., 2000). ORNs form cholinergic synapses onto projection neurons, which relay AL signals to higher-order brain regions (Stocker et al., 1990). Several “sister” PNs receive input from ORNs within each glomerulus and have highly correlated patterns of activity (Kazama and Wilson, 2009).

The fly AL is far from being a simple relay station for sensory input. Rather, early signal modulation takes place by means of lateral processing from a heterogeneous population of mostly GABAergic, local interneurons (LNs) that innervate multiple glomeruli (Chou et al., 2010). In addition, olfactory neurons are innervated by several extrinsic neurons. It is well established that subsets of olfactory neurons express the receptors for and receive input from extrinsic inputs (*e.g.* serotonin, octopamine, CCHamide, SIFamide, and NPF-expressing neurons) (Busch et al., 2009; Carlsson et al., 2010; Farhan et al., 2013; Lee et al., 2017; Martelli et al., 2017; Sizemore and Dacks, 2016). These inputs have been shown to relay internal state information (*e.g.* hunger) by altering neuronal excitability and network properties of olfactory neurons.

In further support of early signal processing, several studies have shown that neuropeptides in addition to their well-known neurotransmitters, GABA or acetylcholine (Ach), are expressed in some primary olfactory neurons and local interneurons in insect antennal lobes.
and the mammalian olfactory bulb (Carlsson et al., 2010; Fusca et al., 2015; Gutiérrez-Mecinas et al., 2005; Lizbinski and Dacks, 2018; Lizbinski et al., 2017; Nässel and Homberg, 2006), indicating that there are intrinsic sources of neuromodulation in these circuits (Lizbinski and Dacks, 2018). For example, in different moth species, allatostatin A-type peptide (AST-A), allatotropin (ATR), myoinhibitory peptide (MIP), FMRFamide-related peptides, and tachykinin-related peptides (DTKs) are expressed in olfactory neurons (Berg et al., 2006; Utz et al., 2007). DTKs, especially, seem to be ubiquitous in the insect AL, as their expression has been observed in cockroach, honeybee, moth, and fly ALs (Schachtner et al., 2005; Winther et al., 2006; 2003).

In 2010, Carlsson et al. performed a screen for neuropeptides in the *Drosophila* AL and mapped the identity of the cells to which some of the identified neuropeptides belong. Consistent with previous reports (Nässel et al., 2008), short neuropeptide F (sNPF) was found to be expressed in axon terminals of ORNs belonging to 13 glomeruli. They also observed expression of DTK, MIP, and AST-A in non-overlapping LN clusters, suggesting that these are three discrete subpopulations of LNs. Although this work identified a number of neuropeptides previously unknown to be expressed by olfactory neurons in *Drosophila*, it is likely not conclusive. Together, these data indicate that some olfactory neurons express neuropeptides in addition to neurotransmitters, which supports circuit flexibility.

While the role of neurotransmitters in information transfer and processing across olfactory neurons is well established (Wilson, 2013), only a few studies have carefully investigated the functional role of neuropeptide release from ORNs and LNs (Ignell et al., 2009; Ko et al., 2015; Root et al., 2011; Winther et al., 2006). In 2009, Ignell et al. found that DTKs released from LNs signal onto ORN DTK receptors (DTKRs), which are expressed in all glomeruli (Birse et al., 2005), to cause presynaptic inhibition by reducing calcium influx and
neurotransmitter release in ORNs. This peptidergic presynaptic inhibition of ORNs is detected behaviorally only at high odorant concentrations, suggesting that this feedback mechanism may serve as a way to modulate dynamic range in sensitivity to relevant odors (Ignell et al., 2009). As mentioned before, a subset of ORNs release sNPF. sNPF plays a role in starvation-dependent presynaptic facilitation of DM1 ORNs. ORNs express insulin receptors, and, when insulin levels are low during starvation periods, sNPF receptor (sNPFR1) transcription in ORNs is increased. This increase in ORN sNPFR1 enhances autocrine signaling in these cells, which in turn facilitates ORN neurotransmitter release. Thus, DM1 ORNs in hungry flies show a higher sensitivity to odorants than in fed flies. DM1 is hardwired for innate odor attraction (Semmelhack and Wang, 2009). This mechanism optimizes olfactory representation to enhance food finding (Root et al., 2011). In 2015, Ko et al. extended findings from these studies by demonstrating that the same insulin signal, encoding hunger state, affects ORNs from distinct glomeruli differently. While DM1 is hardwired for odor attraction, DM5 is hardwired for odor aversion (Semmelhack and Wang, 2009). Starved flies exhibit increased sensitivity to attractive odors and reduced sensitivity to aversive odors (Inagaki et al., 2014; Root et al., 2011; Wu et al., 2005). Given that insulin often encodes hunger state information, it would follow that DM1 and DM5 ORNs would respond differently to insulin. As mentioned above, in starved flies where insulin levels are low, DM1 ORNs show increased sNPFR levels which leads to increased ORN Ach release (presynaptic facilitation) (Root et al., 2011). Conversely, reduced insulin signaling causes increased expression of DTKRs in DM5 ORNs, which receive input from DTKs released from LNs, to cause a decrease in ORN Ach release (presynaptic inhibition) (Ko et al., 2015). Together, these peptidergic modulations resulting from a starved state cause the fly to be more attracted and less aversive to odors, which is hypothesized to encourage the fly to seek out even
potentially noxious food. In the antennal lobe, neuropeptides released from ORNs and LNs shape local neural circuit activity to incorporate information about the animal’s internal state.

1.1.3 Using electron microscopy to investigate neuropeptide expression and directed targeting

Work in the past 10 years has revealed that some ORNs and LNs express neuropeptides in addition to neurotransmitters (Carlsson et al., 2010; Fusca et al., 2015; Gutièrrez-Mecinas et al., 2005; Lizbinski et al., 2017; Nässel and Homberg, 2006; Ni et al., 2008). However, it is unclear whether all or just a subset of ORNs and LNs release both small-molecule and peptide transmitters. Similarly, do any PNs express neuropeptides? To investigate the prevalence of co-transmission in olfactory neurons, we leveraged a recently reconstructed local olfactory circuit (Tobin et al., 2017) (work continued by Asa Barth-Maron, unpublished). Tobin et al. used large-scale serial section electron microscopy to elucidate the synaptic connections between olfactory neurons in the *Drosophila* DM6 glomerulus. They collected ~1900 thin sections from a single female fly brain, and imaged them using electron microscopy (~4 x 40 nm/voxel). The images were aligned to create a volumetric dataset comprising the anterior portion of the brain, including the antennal lobes. ORNs, PNs and LNs were manually reconstructed in the DM6 glomerulus, which was independently identified by three experts. Each segment and synapse of every reconstructed neuron was identified by at least two independent annotators. Using this approach, the authors reconstructed the local olfactory circuit in the DM6 glomerulus. This dataset provides a unique opportunity to investigate the presence of DCVs in a complete local circuit at a resolution that allows us to assay the position of individual DCVs, which may contain neuropeptides (Nusbaum and Blitz, 2012; Nusbaum et al., 2017), in reconstructed DM6 ORNs, PNs, and LNs.
In this work, we hypothesize that DCVs contain neuropeptides, and use the location of DCVs as a proxy for the putative position of neuropeptides. Although there is ample support for this hypothesis (Nusbaum and Blitz, 2012; Nusbaum et al., 2017), it is important to understand the limitations of this approach. While we hypothesize that DCVs within the olfactory neurons studied contain neuropeptides, these compartments may contain other kinds of signaling molecules. For example, vesicular monoamine transporter 2 (VMAT2) has been shown to localize to some large DCVs (Li et al., 2005; Liu et al, 1994; Waites et al., 2001; Krantz et al., 2000; Nirenberg et al., Grygoruk et al., 2014). VMAT2 is an integral membrane protein that packages monoamines such as dopamine, norepinephrine, serotonin, and histamine into organelles. Thus, it is possible that the DCVs identified in this study, which we hypothesize to contain neuropeptides, may also contain monoamines. Future work is needed to validate the hypotheses generated in this project.

Few studies have carefully investigated the anatomical location of DCVs in reconstructed neuronal circuits (Eichler et al., 2017; Schlegel et al., 2016). In addition, while there are a number of studies that have provided evidence for segregation of transmitters to specific presynaptic sites, few have shown that this segregation results in directed targeting of DCVs to certain postsynaptic partners. Thus, our work aims to leverage the reconstructed, local olfactory circuit to investigate the presence of DCVs as well as whether they are preferentially targeted to sites with specific postsynaptic partners.
1.2 Neuropeptides in sleep behavior

Neuropeptides are a large and diverse group of signaling molecules that play important roles in regulating a multitude of processes. Acting as neurotransmitters, neuromodulators, hormones, and/or growth factors, neuropeptides have been shown to be involved in many behavioral actions such as those associated with social interactions, feeding, courtship, and sleep (Schoofs et al., 2017). Often multiple neuropeptides work in concert to regulate specific behaviors. In addition, many neuropeptides appear to have more than a single function. To better understand how neuropeptides influence behavior, we focused on Drosophila sleep behavior as a model system.

Sleep is an essential behavior that is conserved widely across species. While it is clear that sleep is genetically controlled (Sehgal and Mignot, 2011), the exact mechanisms underlying this behavior remain elusive. We are only beginning to uncover the intracellular and signaling molecules involved in sleep behavior.

1.2.1 Sleep across species

Until recently, sleep studies have been primarily restricted to humans and other mammalian species. This is largely because the definition of sleep states based on altered brain electrical activity was not easily applied to animals lacking well-defined cortices. In 1984, Campbell and Tobler proposed specific behavioral criteria to identify sleep-like states in bees, cockroaches, and several species of fish and amphibians (Campbell and Tobler, 1984). Their behavioral definition states that a sleep-like state is (1) a period of quiescence associated with a species-specific posture, (2) accompanied by an increase in arousal threshold, (3) reversible, (4) homeostatically regulated to ensure proper levels of sleep occur, and (5) controlled by the circadian clock. However, Campbell and Tobler’s behavioral definition of sleep was controversial, and it wasn’t
until the past decade that scientists revisited this definition to show that non-mammalian species including zebrafish (*Danio rerio*) (Yokogawa et al., 2007; Zhdanova et al., 2001), worms (*Caenorhabditis elegans*) (Raizen et al., 2008), and fruit flies (*Drosophila melanogaster*) (Hendricks et al., 2000; Shaw, 2000) exhibit sleep-like states.

It is now well-established that behavioral criteria alone are sufficient to define a sleep-like state (Sehgal and Mignot, 2011). Zebrafish show rest-activity rhythms that are synchronous with the day-night cycle, being quiescent during the night (Yokogawa et al., 2007). In *C. elegans*, quiescence is restricted to lethargus periods, which occur during development after each of the four larval stages, and in the adult stage during satiated periods (Cho and Sternberg, 2014; Raizen et al., 2008). In 2000, two groups (Hendricks et al., 2000; Shaw, 2000) suggested that the sustained periods of rest fruit flies exhibit are sleep-like states. During these reversible, quiescent bouts, flies exhibit a stereotyped sleeping posture (behavioral criteria 3 & 1). Shaw et al. demonstrated that flies show decreased responsiveness to visual, tactile, and olfactory stimuli if they had been quiescent for 5 minutes or more, which defines a sleep bout, indicating that these periods of quiescence are associated with increased arousal thresholds (behavioral criteria 2). In addition, when flies were kept awake using various deprivation methods, they exhibit compensatory increases in rest the next day. This observation suggests that quiescence in flies is homeostatically regulated (behavioral criteria 4). Finally, it is clear from studies of clock gene mutants that fly sleep is regulated by a circadian mechanism (behavioral criteria 5). Together, these findings indicate that rest in flies meets the five behavioral criteria of a sleep-like state. Additional evidence is provided by recent observations that flies exhibit electrophysiologically distinct stages of sleep intensity (van Alphen et al., 2013), fly sleep can be pharmacologically manipulated by the same neurochemicals that affect mammalian sleep (Hendricks et al., 2000;
Shaw, 2000), and fly sleep quality decreases with age similarly to what is observed for human sleep (Sehgal and Mignot, 2011). These data strongly suggest that study of sleep in flies has implications for our understanding of human sleep.

1.2.2 *Drosophila* is a powerful system for elucidating the underpinnings of sleep

The powerful genetic toolkit of *Drosophila* makes this organism ideally suited for probing the genetic basis of sleep. The fly nervous system can be easily visualized, and the molecular genetic tools available allow for unparalleled opportunities to dissect gene function and manipulate neuronal circuitry (Venken et al., 2011). In addition, sleep can be reliably assayed in an unbiased and automated manner by monitoring fly locomotor activity using the *Drosophila* Activity Monitoring System (Trikinetics) (Shaw, 2000). Fruit flies have already proven to be a successful system in which to answer complex biological questions such as sleep (e.g. the molecular basis of the circadian clock) (Hardin, 2011). In support of this, several genetic screens have identified many sleep genes with conserved roles across species since the discovery of fly sleep in 2000 (Hendricks et al., 2000; Sehgal and Mignot, 2011; Shaw, 2000).

1.2.3 Neuropeptides involved in sleep behavior

More than 25 neuropeptides have been implicated in sleep regulation (Dubowy and Sehgal, 2017; Richter et al., 2014). There does not appear to be one master regulatory neuropeptide devoted solely to regulating sleep and wakefulness. Rather, many neuropeptides are important for sleep and also appear to also have additional roles in processes such as feeding and reward. As the lists of genes and neuropeptides involved in sleep behavior continue to grow, it is becoming clearer that sleep regulation is orchestrated by a complex set of genes and neurons.
More work is needed to uncover additional neuropeptides involved in sleep as well as how their effects on sleep are regulated. The study of sleep in *Drosophila* has allowed us to leverage the power of forward genetics to identify many novel sleep factors. Furthermore, studying the mechanisms by which these factors regulate sleep provides insight into the molecular network as well as neuronal circuits involved in sleep regulation. We took this approach by investigating the mechanism by which the sleep factor, CycA (Rogulja and Young, 2012), regulates sleep, and from this work identified a novel sleep neuropeptide called Crz.

### 1.2.4 Rca1 and Cyclin A promote sleep in *Drosophila*

Through a neuronal-specific RNAi interference (RNAi) screen, Rogulja and Young identified two cell cycle proteins, regulator of Cyclin A1 (Rca1) and its main target CycA (Dong et al., 1997), that function in post-mitotic neurons to promote sleep in *Drosophila* (Rogulja and Young, 2012). UAS-RNA interference (*UAS-RNAi*)-mediated knockdown of *Rca1* using the pan-neuronal elavGal4 driver results in reduced total amount of daily sleep, and more frequent, shortened sleep episodes than those seen in wild type flies. Similar phenotypes were observed when CycA was depleted (*elav>CycA-RNAi*), indicating that both Rca1 and CycA promote sleep in *Drosophila*. It took significantly longer for *elav>CycA-RNAi* flies to fall asleep after the lights went off than controls, suggesting that the wake-sleep transition is disrupted in these flies. To test whether the homeostatic mechanism is intact in *elav>CycA-RNAi* flies, Rogulja and Young examined the flies’ responses to sleep deprivation. The observed reduced sleep rebound suggests that CycA is necessary for proper function of the sleep homeostat. Conversely, no circadian clock defects were observed, as evidenced by normal circadian rhythmicity, as well as normal molecular oscillations of the circadian clock in constant darkness. Together, these data suggest
that CycA coordinates sleep behavior in part by regulating homeostatic and circadian inputs to the sleep system.

To investigate where CycA functions to promote sleep, antibodies were used to determine where CycA is expressed in the brain. The CycA protein was detected in only ~40-50 cells in the adult fly brain (Figure 1.3). In contrast to many other known sleep factors showing broad localization (Cirelli et al., 2005; Stavropoulos and Young, 2011), this limited expression suggests that CycA may act as a critical switch in the regulation of sleep. There are ~150 clock neurons organized into six major clusters: three dorsal clusters (DN1, DN2, and DN3), a dorsal-lateral cluster (LNd), and two groups of ventral-lateral neurons (small, s-LNv and large, l-LNv). Many of the CycA cells are intermingled with circadian clock neurons, but only some are clock cells themselves (2 LNds) (Figure 1.3) (Rogulja and Young, 2012). s-LNvs are major circadian pacemaker neurons (Grima et al., 2004; Stoleru et al., 2004), known to regulate sleep (Parisky et al., 2008). They send projections near the dorsal cluster of CycA cells, and, along with l-LNvs and other cells, produce the neuropeptide PDF which is a major regulator of the clock output (Helfrich-Forster, 2004). Interestingly, all CycA cells express the PDF receptor (Rogulja and Young, 2012). This arrangement suggests an intriguing circuit mechanism through which dorsal neurons expressing CycA might receive information from circadian clock cells.

1.2.5 Rca1 and Cyclin A are cell cycle proteins

Cell division is a highly complex process. Cells must accurately replicate and segregate DNA and divide. Failure to reliably complete this process can result in serious consequences (e.g. cancer). Therefore, cells have evolved a complex, interconnected network of biochemical events to maintain faithful spatial and temporal control of cell division. The cell cycle has four major
Figure 1.3: Cyclin A is expressed in neurons adjacent to clock cells.

A schematic representation of an adult brain with the location of CycA (pink), clock (green), and clock cells expressing CycA (olive) is illustrated. Two LNd clock neurons express CycA (olive) (Rogulja and Young, 2012). Dashed boxes enclose CycA cells that may receive direct input from clock cells called s-LNvs.
phases: G1, a growth phase where cells become “committed” to divide; S, a DNA and centrosome duplication phase; G2, a phase where DNA replication fidelity is confirmed; and M, a phase during which cell division (mitosis) occurs (Sherr, 2004). A family of proteins, known as cyclins, directs the progression through the cell cycle. Distinct cyclins regulate each stage of the cycle by activating specific cyclin dependent kinases (Cdks) to control the biological events associated with each phase (Bloom and Cross, 2007). Several proteins, such as Cdk inhibitors and ubiquitin ligases, regulate Cyclin/kinase complexes, which ensure that the cycle progresses in an ordered fashion.

As the cell transitions from G1 to S phase, CycA associates with and activates cyclin dependent kinase 2 (Cdk2), via its conserved C-terminal cyclin box domain, to initiate DNA replication (Coverley et al., 2002). Late in S phase, CycA binds to a different Cdk, Cdk1, and remains associated through the early stages of M phase to facilitate entry into mitosis (Hochegger et al., 2008). CycA is later degraded during mitosis and G1 through ubiquitin-dependent pathways. Fizzy (Cdc20) and Fizzy-related (Cdh1) target the N-terminal destruction box motif of CycA to promote degradation via a multisubunit ubiquitin ligase called the anaphase-promoting complex/cyclosome (APC/C) during early mitosis and late mitosis to G1, respectively (Sigrist and Lehner, 1997; Sigrist et al., 1995; Zachariae and Nasmyth, 1999). Furthermore, Rca1, which is homologous to the mammalian early mitotic inhibitor (Reimann et al., 2001; Zielke et al., 2006), is a negative regulator of Fizzy-related-dependent APC/C activity (Grosskortenhaus and Sprenger, 2002). Thus, Rca1 prevents premature degradation of CycA and allows for normal entry into mitosis.
1.2.6 Repurposing cell cycle proteins

To carry out the wide array of diverse biological processes, eukaryotic cells have evolved functionally distinct roles for many proteins. The term ‘moonlighting protein’ was coined by Constance Jeffery to describe proteins that play multiple cellular roles (Jeffery, 1999). This concept of reusing proteins to function in independent molecular pathways is not uncommon. Examples include: phosphoglucose isomerase, a cytosolic glycolytic enzyme that can act as an extracellular cytokine when secreted; STAT3, which functions as a transcription factor and electron transport chain enzyme; and presenilin, a catalytic component of the multiprotein γ-secretase enzyme complex and cytoskeletal protein (Huberts and van der Klei, 2010). Similarly, many cell cycle proteins not only regulate fundamental cell cycle processes but also exert additional cell cycle independent functions in neurons (Herrup, 2013). In mice, Cyclin E functions in post-mitotic neurons to control synapse formation and function in a cell cycle-independent manner (Odajima et al., 2011). In addition, Ced20-APC/C regulates dendritic growth and branching, whereas Cdh1-APC/C controls axon growth and patterning in mammals (Kim et al., 2009; Puram et al., 2011; Yang et al., 2009). Furthermore, Cdh1-APC/C also regulates glutamate receptor trafficking to modulate synaptic strength in post-mitotic neurons in flies and worms (Juo and Kaplan, 2004). These and other data indicate that the functions of cell cycle proteins are not always restricted to the process of cell division. Rather, many cell cycle have post-mitotic functions.

1.2.7 Investigating the mechanism by which Cyclin A regulates sleep

Despite decades of effort, our understanding of how we sleep remains incomplete. Further elucidation of the molecular pathways and neural circuits underlying sleep behavior will shed
light on this phenomenon. To understand how CycA influences sleep, we investigate the molecular and circuit mechanisms by which CycA regulates sleep. To probe the molecular mechanisms, we identify proteins functioning in the CycA sleep pathway as well as a novel sleep neuropeptide called Crz. To address how CycA functions at the circuit level, we profile the neuronal networks in which CycA functions. Together, this work provides exciting advances to our understanding of how we sleep.
1.3 References


Chapter 2

Neurotransmitter and Neuropeptide Co-transmission in Olfactory Neurons May Support Circuit Flexibility

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Author contributions:

H.E.S. and W.C.A.L conceived the ideas and designed the experiments. H.E.S performed the experiments, analyzed the data, wrote the chapter, and made the figures.
2.1 Introduction

To understand how complex behaviors are performed, it is useful to uncover their underlying circuit mechanisms. This involves identifying the neurons and their sites of connection to create a ‘connectome’ of a neuronal circuit. Synapses are typically defined as sites of neurotransmitter release in serial-section electron microscopy datasets. For example, presynaptic sites are identified by the presence of neurotransmitter-containing synaptic vesicles, and postsynaptic partners are defined as the cells adjacent to release sites. This approach allows one to identify all of the input and output sites in a given circuit. However, these criteria make the assumption that neurons communicate through neurotransmitters alone and that the details of individual synaptic events are relatively insignificant.

Although connectomes have become invaluable tools in neuroscience, they do not provide a complete picture of the connections between neurons. This is in part because many neurons release neuropeptides in addition to neurotransmitters (Nusbaum et al., 2017). Thus, focusing only on neurotransmitter-mediated connections overlooks the flexibility neuropeptide signaling adds to circuit function. There are many possible mechanisms for which cotransmission of neurotransmitters and neuropeptides provides opportunities for circuit flexibility. However, some of the proposed mechanisms have more support than others (Nusbaum et al., 2017).

For example, there is a lot of evidence suggesting that neuropeptide release tends to require a higher firing rate than that needed for the release of neurotransmitters (Adams and O'Shea, 1983; Bishop et al., 1987; Cropper et al., 1990; Whim and Lloyd, 1989; 1990; Wilim et al., 1996) (Figure 1.1, B). Following low levels of presynaptic activity, only neurotransmitters are typically released. Conversely, during periods of increased presynaptic activity, both
neurotransmitters and neuropeptides are generally released. The different release properties of neurotransmitters and neuropeptides supports a neuron’s ability to encode differing levels of presynaptic activity.

It has also been postulated that co-transmitters can be released from the same or distinct presynaptic sites to target different postsynaptic partners (Figure 1.1, A). For example, a neuron may have synaptic vesicles at all presynaptic sites, but only target DCVs to a subset of sites. By directing DCVs to a subset of presynaptic sites, the neuron would be able to target neuropeptides to specific postsynaptic partners. Although there is a lot of support for neurons targeting different transmitters (mostly different neurotransmitters) to particular presynaptic sites (Blitz and Nusbaum, 1999; Hattori et al., 1991; Kueh and Jellies, 2012; Landry et al., 2003; Sámano et al., 2006; 2012; Sossin et al., 1990; Sulzer and Rayport, 2000), fewer studies have looked at the segregation of neurotransmitters and neuropeptides to our knowledge (Sámano et al., 2006; Vega et al., 2010). Furthermore, only a limited number of studies provide anatomical support for transmitter segregation at the electron microscopic level (Zhang et al., 2015). In addition, how a neuron may be able to preferentially target DCVs to particular presynaptic sites remains largely unknown (Sámano et al., 2012).

To further address whether neurons direct DCVs to particular presynaptic sites, we turned to the *Drosophila* olfactory circuit (Figure 1.2). The fly senses olfactory cues in its antenna, and sends this information to the antennal lobes. Each antennal lobe is composed of sub-compartments called glomeruli. Glomeruli receive feed-forward sensory input from primary sensory neurons, called ORNs. All ORNs projecting to the same glomerulus express the same odorant receptor. This allows each glomerulus to function as an individual odor response channel. ORNs form cholinergic synapses onto secondary neurons, called PNs, which relay
signals to higher-order brain regions. The fly antennal lobe is far from being a simple relay station for sensory input. Rather, LNs interconnect glomeruli and form synapses onto ORNs and PNs to partake in lateral processing.

To investigate the details of this circuit, Tobin et al., 2017 (efforts continued by Asa Barth-Maron, unpublished) used serial section electron microscopy to reconstruct neurons in the DM6 glomerulus of the fly. They traced olfactory neurons in this dataset to unveil the connectomic details of this circuit. The feed-forward olfactory circuit, whereby ORNs detect olfactory cues and transfer this information to PNs, which relay these signals to higher order brain regions is well established (Figure 1.2, B). Interestingly, in further support of early signal processing, this connectomic work directly showed that each glomerulus is not just a simple feed-forward circuit, but also rather a rich hub of recurrent and reciprocal connections (Figure 2.1). Their findings indicate that PN dendrites don’t just receive input, but also signal back onto LNs, ORNs, and other PNs. There is currently little known about the functional implications of PN signaling onto ORNs.

Work in the past 10 years has revealed that some ORNs and LNs express neuropeptides in addition to neurotransmitters (Carlsson et al., 2010; Fusca et al., 2015; Gutiérrez-Mecinas et al., 2005; Lizbinski et al., 2017; Nässel and Homberg, 2006; Ni et al., 2008). Co-transmission could provide an additional way in which early signal processing takes place in this circuit.

Although many olfactory neurons appear to release neuropeptides, it is unclear whether all or just a subset of ORNs and LNs release both small-molecule and peptide transmitters. Similarly, do any PNs express neuropeptides? In addition, are neuropeptides present at presynaptic sites? Localization of neuropeptides and neurotransmitters at the same presynaptic sites suggests neurons may use co-transmission to signal to other cells. To address these
Figure 2.1: Connections in the *Drosophila* olfactory circuit are bidirectional.

The *Drosophila* olfactory circuit is not a simple feed-forward circuit, but instead a rich center of bidirectional and recurrent (synapses with other cells of the same type, *e.g.* projection neurons synapsing onto other projection neurons) connections. The thickness of the arrows corresponds to the proportion of the total outputs from each cell type.
questions, we assayed the presence of DCVs in the previously completed DM6 microcircuit (Tobin et al., 2017). We identified DCVs, which may contain neuropeptides (Nusbaum and Blitz, 2012; Nusbaum et al., 2017), in reconstructed DM6 ORNs, PNs, and LNs.

We find that all neurons investigated contain DCVs, suggesting that they all may express both neurotransmitters and neuropeptides. We also show that many DCVs localize to presynaptic sites, suggesting that co-transmission of neurotransmitters and neuropeptides may play a prevalent role in this circuitry. We find that DCVs are enriched at specific PN presynaptic sites (e.g. PN → ORN), which could preferentially direct putative PN neuropeptide signaling to ORN postsynaptic partners. These data suggest that PNs may use neuropeptides to modulate signaling within a single glomerular channel. Finally, we find that presynaptic sites with ORN partners are clustered on PN branches. We hypothesize that this clustering may facilitate preferential DCV targeting to certain presynaptic sites in PNs. Together our data suggest that peptidergic synaptic actions may provide additional modulatory mechanisms to increase the flexibility of olfactory circuit function.
2.2 Results

2.2.1 All ORNs, PNs, and LNs screened contain dense-core vesicles

To investigate DCV distributions within and across olfactory neurons we leveraged a previously collected connectomic dataset, whereby one glomerulus on both sides of an adult female fly brain was comprehensively reconstructed. Tobin et al., reconstructed all 53 DM6 ORNs axons, as well as all 5 DM6 PN dendrites they target (Tobin et al., 2017). In addition, the processes of 37 LNs extending into DM6 glomeruli have also been traced (Asa Barth-Maron, unpublished). This dataset allowed us to test whether these ORNs, PNs and LNs express neuropeptides in addition to neurotransmitters (Figure 2.2, A & B). We screened 20 randomly selected ORNs (11 right and 9 left), all 5 PNs, and 37 LN fragments for DCVs (Figure 2.2, B & C). We looked for DCVs in ORN and PN processes reconstructed both in and beyond DM6 (entire neurons were not reconstructed), whereas we screened for DCVs only in LN processes located within DM6 (Figure 2.2, B). We measured a number of DCV features (e.g. fill type, docked/not-docked to plasma membrane, diameter, localization) and analyzed features across cells.

Across these cells, we identified 13,371 DCVs with a median diameter of 74 nm (Figure 2.2, D), which is consistent with our criteria and supports that the identified compartments are DCVs (van de Bospoort et al., 2012). The identified DCVs came in many flavors: dense-core extending to the edges of the compartment (filled) with dark or medium electron density, or space surrounding the dense-core and the vesicular membrane (halo), which can be medium or light in density (Figure 2.2, E). The majority of DCVs across cell types had a light halo appearance (Figure 2.2, F). Differences in DCV fill have been observed in other studies (Eichler et al., 2017; Schlegel et al., 2016), and could reflect different types of neuropeptides contained or partial release of signaling molecules (e.g. Kiss-and-run release) (Tsuboi et al., 2004).
Figure 2.2: All ORNs, PNs, and LNs screened contain dense-core vesicles.

(A) DCVs were screened in 11 right ORNs (dashed yellow neuron), 9 left ORNs (solid yellow neuron), 2 right PNs (green neuron, right), 3 left PNs (green neuron, left), and 37 left, DM6 LN neurons (pink neurons). Approximate DM6 glomeruli boundaries are outlined using dashed red lines. (B) Electron microscopy section of anterior fly brain. DM6 glomeruli are outlined in red. Adapted from Tobin et al., 2017. (C) Example DCV (solid red arrow) localizing to a presynaptic site, which is characterized by the presence of synaptic vesicles and a t-bar (open red arrow). (D) Histogram of all of the identified DCVs’ diameters in nanometers (nm). The median DCV diameter was 74 nm. (E) Representative DCVs showing the diversity of appearances. DCVs had either electron density extending to the edge of the compartment (filled) or not (halo). DCVs of each also appeared to have different overall densities (light, medium, or dark). (F) Percent of DCVs displaying these four appearances across cell types are shown. (G) The number of DCVs within DM6 normalized by each neuron’s path length within DM6 is displayed across cell types. Each dot represents data from one cell type. ORNs L and ORNs R reflect data from each of the glomeruli, left and right DM6, respectively. There are two LNs that do not have DCVs in processes within DM6, but do in processes extending into other glomeruli.
Figure 2.2: (Continued) All ORNs, PNs, and LNs screened contain dense-core vesicles.
Across the 20 ORNs screened, we observed little variability in DCV features (data not shown), suggesting that DM6 ORNs partake in similar signaling via these compartments. Thus, we felt that labeling DCVs in only 20 ORNs was sufficient for our analyses. The limited variability in DCV features across ORNs is consistent with literature suggesting that ORNs projecting the same glomerulus are of the same “type” and have similar response properties (Wilson, 2013). Thus, it is likely that ORNs projecting to the DM6 glomerulus may participate in similar neuropeptide signaling.

All neurons except for two LNs contained DCVs in DM6 processes (Figure 2.2, G). Although these two neurons did not contain DCVs within DM6 processes, they did contain DCVs in processes extending into other glomeruli (data not shown). These data suggest that all screened neurons may express neuropeptides in addition to neurotransmitters. Within DM6, LNs contained more DCVs and showed greater variability in DCV number across LNs (mean: 1.60 DCVs/µm, SD: +/- 0.83 DCVs/µm) than that for ORNs (L mean: 0.32 DCVs/µm, L SD: +/- 0.14 DCVs/µm; R mean: 0.38 DCVs/µm, R SD: +/- 0.16 DCVs/µm) and PNs (mean: 0.21 DCVs/µm, SD: +/- 0.14 DCVs/µm) (Figure 2.2, G). These data suggest that LNs are the dominant contributor of neuropeptide signaling in this circuit, and that some LNs may play a greater role in this than others. Furthermore, they also suggest that DM6 PNs and ORNs may express neuropeptides, which had previously never been reported.

2.2.2 Many dense-core vesicles localize to presynaptic sites

DCVs are transported from the soma to their release sites. To investigate where their contents may be exocytosed, we sought to identify where DCVs are located within the olfactory neurons. We find that DCVs are not randomly distributed, but are primarily located within the DM6
glomeruli in ORNs and PNs (no processes outside of DM6 were screened for LNs) (Figure 2.3, A & B; Figure A.1; Figure A.2). 95.2% and 97.2% of DCVs were located within DM6 on average in ORNs and PNs, respectively (Figure 2.3, B), despite only an average of 63.6% of total reconstructed ORN path lengths (average total path length: 455.9 µm) and 91.8% of those for PNs (average total path length: 2,511.5 µm) fell within DM6 (Figure 2.3, C). These data suggest that DCV position is not randomly distributed and DCV localization may reflect sites of function and release.

DCVs can be released from both presynaptic and non-synaptic sites. Like DCVs, most presynaptic sites of ORNs and PNs are primarily restricted to DM6 glomeruli (Figure 2.3, A; Figure A.1; Figure A.2). These observations led us to test whether some DCVs localize to neurotransmitter-releasing, presynaptic sites. If so, this would suggest these neurons may release both neurotransmitters and neuropeptides from the same presynaptic sites (Nusbaum et al., 2017; Schlegel et al., 2016). We found that ~60% of DCVs localized to presynaptic sites across the cell types (Figure 2.3, D), suggesting that these neurons may engage in co-transmission of neurotransmitters and neuropeptides from some presynaptic sites.

To assay whether DCVs are present at all presynaptic sites or just a subset, we measured the percent of presynaptic sites that contain at least one DCV across neurons. We found that nearly all presynaptic sites (93% on average) across LNs contained at least one DCV (Figure 2.3, E; Figure A.3), suggesting LNs may co-transmit neurotransmitters and neuropeptides from most of their presynaptic sites. Conversely, only a subset of ORN and PN presynaptic sites (39% and 27% on average, respectively) contained at least one DCV (Figure 2.3, E; Figure A.1, Figure A.2).
**Figure 2.3:** Many dense-core vesicles localize to presynaptic sites.

(A) An ORN skeleton is shown in gray. Positions of DCVs (pink), presynaptic sites (green), and postsynaptic sites (blue) are shown. Approximate outlines of DM6 glomeruli are highlighted in dashed red lines. (B) The percent of DCVs within the DM6 glomeruli across neurons are shown. (C) The percent of total neuron path length located within DM6 across cells is depicted. (D) The relative locations of DCVs across cell types are shown. (E) The percent of presynaptic or (F) postsynaptic sites containing at least one DCV per neuron across cell types are displayed. (Gi-Giii) Histograms of the number of DCVs at each presynaptic site are shown for (Gi) ORNS, (Gii) PNs, and (Giii) LNs. (Hi-Hiii) Histograms of the number of DCVs at each postsynaptic site are displayed for (Hi) ORNS, (Hii) PNs, and (Hiii) LNs. There are no PN postsynaptic sites that contain DCVs.
Figure 2.3: (Continued) Many dense-core vesicles localize to presynaptic sites.
In contrast, few DCVs were located within postsynaptic sites, suggesting that there may be a comparatively limited amount of DCV content release from these sites (Figure 2.3, D). Consistent with this, only a small fraction of postsynaptic sites contain at least one DCV in ORNs, LNs, and PNs (Figure 2.3, F). Instead, the remaining DCVs tended to localize to nonsynaptic sites (Figure 2.3, D). This is not surprising, because DCVs can be released from nonsynaptic sites in addition to classical presynaptic sites (Nässel, 2009). Of presynaptic sites that contain at least one DCV, ORN presynaptic sites had a median of one DCV/site, PNs had 2 DCVs/site, and LNs had 6 DCVs/site (Figure 2.3, Gi–Giii), suggesting that LNs may release more neuropeptides from presynaptic sites than ORNs and PNs. Conversely, of the postsynaptic sites that contain at least one DCV, ORNs contained a median of one DCV/site, PNs had 0 DCVs/site and LNs had one DCV/site (Figure 2.3, Hi–Hiii), which is consistent with previous findings that only a small fraction of DCVs localize to postsynaptic sites (Figure 2.3, D). Together, these data suggest that many neuropeptides may be released from presynaptic sites of ORNs, PNs, and LNs, and are consistent with olfactory neurons using co-transmission of neurotransmitters and neuropeptides to signal to other cells from some presynaptic sites.

2.2.3 PN → ORN presynaptic sites are enriched with dense-core vesicles

Given that only a subset of ORN and PN presynaptic sites contained at least one DCV, we wondered whether these neurons preferentially direct DCVs to particular synapse types (Figure 1.1, A). For example, PNs might want to differentially signal onto ORNs within their glomerular channel versus LNs broadcasting across glomerular channels. A potential mechanism for this would be to release neuropeptides from PN presynaptic sites preferentially when there are ORN postsynaptic partners. To test whether DCVs are enriched at presynaptic sites with particular
Figure 2.4: PN → ORN presynaptic sites are enriched with DCVs.

(A) The percent of all ORN presynaptic sites containing at least one DCV is shown in gray. Each dot corresponds to one neuron. The percent of presynaptic sites with at least one DCV and at least one ORN, PN, or multi-gglomerular (MG) postsynaptic partner are shown in yellow, teal, and pink, respectively. Multi-gglomerular neurons include LNs. The same data is shown for (B) PN and (C) LN presynaptic sites.
postsynaptic partners, we probed the percent of presynaptic sites with at least one DCV for particular presynaptic and postsynaptic pairs. We found that PN presynaptic sites with at least one ORN postsynaptic partner were more likely to contain DCVs than expected by chance (Figure 2.4, B), suggesting that DCVs are enriched at PN → ORN presynaptic sites. Conversely, we did not observe any differences in the percent of ORN or LN presynaptic sites containing DCVs across different postsynaptic partner types (Figure 2.4, A & C). The enrichment of DCVs at PN presynaptic sites with at least one ORN partner suggests that PNs may be signaling back onto ORNs with both neurotransmitters and neuropeptides. This provides a mechanism whereby the circuit may be able to modulate activity in single glomerular channels.

2.2.4 PN presynaptic sites with ORN partners are clustered

These data provide direct evidence that PNs preferentially target DCVs to particular presynaptic sites, which supports the model previously mentioned (Figure 1.1, A). How is preferential DCV targeting to specific presynaptic sites achieved? DCVs are trafficked from the soma to their release sites, and how they know where to go remains an open question. We wondered whether neurons might able to facilitate DCV delivery by clustering presynaptic targets. For example, a neuron could restrict all sites with ORN partners to a particular branch. Conversely, neurons could instead randomly distribute these sites throughout the branches, which would require DCVs to be trafficked across several branches using a less efficient delivery system.

To test whether PNs cluster presynaptic sites with ORN partners, we visualized PN DM6 branches containing presynaptic sites in a way that allowed us to resolve individual presynaptic sites as well as the branches they belong to (Figure 2.5). We found clustering among many PN presynaptic sites with ORN postsynaptic partners. To assay the significance of the observed
**Figure 2.5:** PN presynaptic sites with ORN partners are clustered.

For each PN (name labeled above), branches extending into DM6 from the point of entry (gray circle) are depicted using a hierarchical layout. Only branches containing presynaptic sites are shown, and the path length of the neuron corresponds to its length in Y. Using this visualization approach we can see each presynaptic site as well as the branch it belongs to. Presynaptic sites with at least one ORN postsynaptic partner are red, whereas presynaptic sites without ORN postsynaptic partners are outlined in black. *p* values from clustering metric are shown for each neuron.
Figure 2.5: (Continued) PN presynaptic sites with ORN partners are clustered.
clustering we developed a scoring metric (see Experimental Procedures). 4 out of the 5 PNs showed significantly increased clustering than expected by chance (Figure 2.5). Only PN3 LS did not show significant clustering (Figure 2.5), but this is likely because there are not many presynaptic sites with ORN postsynaptic partners in this neuron. Consistent with previous data, we see a similar clustering pattern of presynaptic sites that contain at least one DCV (Figure 2.6). Together, these data strongly suggest that PNs cluster sites with at least one ORN postsynaptic partner. We hypothesize that this may be a way to facilitate DCV trafficking to its target presynaptic sites.
**Figure 2.6**: PN presynaptic sites with dense-core vesicles are clustered.

For each PN (name labeled above), branches extending into DM6 from the point of entry (gray circle) are depicted using a hierarchical layout. Only branches containing presynaptic sites are shown, and the path length of the neuron corresponds to its length in Y. Using this visualization approach we can see each presynaptic site as well as the branch it belongs to. Presynaptic sites with at least 1 DCV are orange, whereas presynaptic sites without DCVs are green. *p* values from clustering metric are shown for each neuron.
Figure 2.6: (Continued) PN presynaptic sites with dense-core vesicles are clustered.
2.3 Discussion

2.3.1 Many olfactory neurons may express both neurotransmitters and neuropeptides

We took advantage of a previously reconstructed local olfactory circuit (Tobin et al., 2017) to investigate the abundance and distribution of DCVs within neurons. We found that all screened DM6 ORNs, PNs, and LNs contain both synaptic vesicles and DCVs, suggesting that these neurons may express both neurotransmitters and neuropeptides (van den Pol, 2012). This is the first evidence that DM6 ORNs may express neuropeptides. Previously, only a subset of ORNs, not including DM6 ORNs, had been found to release sNPF (Carlsson et al., 2010; Nässel et al., 2008). It is possible that DM6 ORNs express sNPF at low levels undetectable using immunofluorescence (Carlsson et al., 2010), and/or other neuropeptides. Future work is needed to identify which (if any) neuropeptides are expressed in DM6 ORNs. Our data also provide the first evidence that any PNs may express neuropeptides. Identification of the neuropeptides expressed by DM6 PNs will provide insight into the functional implications of putative PN neuropeptide signaling. Finally, we found that all LNs extending processes in DM6 contain DCVs. LNs contain more DCVs on average than ORNs or PNs, suggesting that LNs may be the major contributors of neuropeptide release in this circuit. In addition, LNs display the greatest variability in DCV number across cells as compared to that for ORNs and PNs, suggesting that LNs may engage in varying degrees of neuropeptide signaling. LNs are a heterogeneous cell population (Wilson, 2013); thus, this variability in DCV abundance across LNs may reflect different LN subtypes. Previous work has shown that LNs can express DTK, MIP, and/or AST-A in Drosophila (Carlsson et al., 2010). However, more work is needed to determine if the LNs in this study express these and/or other neuropeptides. Together, our data suggest that all DM6
olfactory neurons may partake in co-transmission of neurotransmitters and neuropeptides, which enhances microcircuit flexibility and coding capacity.

2.3.2 Anatomical evidence for directed dense-core vesicle targeting

Unlike neurotransmitters, neuropeptides are produced and packaged in the soma and transported to sites of release, which can be both synaptic and non-synaptic regions (van den Pol, 2012). Consistent with other reports in *Drosophila*, we found that DCVs are not randomly distributed and can localize to presynaptic sites (Eichler et al., 2017; Schlegel et al., 2016). While our data are consistent with some DCV contents being released from non-synaptic sites, they suggest that most DCV contents may be released from conventional presynaptic sites in DM6 olfactory neurons. Our findings are in contrast to some mammalian studies showing that DCVs are preferentially associated with non-synaptic regions (Zhu et al., 1986). However, these investigations did not sample all DCVs within a given neuron, and therefore may not be representative of the overall spatial distribution of DCVs within cells.

We found that 93% of LN presynaptic sites on average contain DCVs, suggesting that LNs may co-transmit neuropeptides and neurotransmitters from most of their presynaptic sites. Conversely, only 39% and 27% of presynaptic sites contained at least one DCV on average in ORNs and PNs, respectively. Given that not all ORN and PN presynaptic sites contain DCVs, we hypothesized that these neurons may preferentially direct DCVs to presynaptic sites with postsynaptic partners of a particular type. We found that PN presynaptic sites were more likely to contain DCVs than expected by chance when at least one ORN was postsynaptic, suggesting that neuropeptides released from PNs may preferentially target ORNs. Although we recognize that neuropeptides may act on distant in addition to local targets (Nässel, 2009), we hypothesize that
the putative neuropeptides released from presynaptic sites preferentially target postsynaptic partners in this context. Consistent with this, the observation that not all presynaptic sites contain DCVs, suggests that where these putative neuropeptides are localized is important for their function. Thus, we hypothesize that PNs may preferentially release neuropeptides onto ORN postsynaptic partners.

This is the first evidence to our knowledge of preferential DCV targeting to presynaptic sites with specific postsynaptic partners. While several studies have suggested that this may occur (Blitz and Nusbaum, 1999; Hattori et al., 1991; Kueh and Jellies, 2012; Landry et al., 2003; Sámano et al., 2006; 2012; Sossin et al., 1990; Sulzer and Rayport, 2000), most of this work lacks the resolution and circuit details required to directly test this model. Only a few papers have investigated DCV position within a reconstructed neuronal circuit (Eichler et al., 2017; Schlegel et al., 2016); however, these groups did not test whether presynaptic sites with particular postsynaptic partners are enriched with DCVs. Thus, this work provides an exciting advancement in our understanding of putative neuropeptide signaling and illustrates the richness of electron microscopy datasets.

2.3.3 PNs may use neuropeptides to modulate signaling within a single glomerular channel

While the role of small-molecule neurotransmitters in information transfer and processing within olfactory neurons is well established (Wilson, 2013), only a few studies have carefully investigated the functional role of neuropeptide release from ORNs and LNs (Ignell et al., 2009; Ko et al., 2015; Root et al., 2011; Winther et al., 2006). LNs play important roles in lateral processing (Wilson, 2013). However, LNs innervate many glomeruli, so it is likely that their activity affects multiple glomerular channels. Conversely, ORNs and PNs of a given type project
only to one glomerulus (Figure 1.2, A). Thus, in PNs preferentially targeting DCVs to sites with at least one ORN partner, they may be able to direct the release of putative neuropeptides onto ORNs. This provides a mechanism whereby the circuit may able to modulate activity within single glomerular channels. DM6 ORNs respond to pentanoic acid and are involved in odor lateralization (Gaudry et al., 2013). There is currently little known about the function of PN → ORN signaling; however, our data suggest that PNs may use co-transmission of neurotransmitters and neuropeptides to signal onto ORNs. Future work is needed to investigate the effects of this signaling on neural circuit function and behavior.

2.3.4 Clustering of specific presynaptic site types may facilitate dense-core vesicle delivery

How differential targeting of DCVs to presynaptic sites is achieved remains to be answered. To address this question, we tested whether certain presynaptic sites are clustered. We observed a consistent clustering of PN presynaptic sites with ORN postsynaptic partners, which are the sites we showed to be enriched with DCVs. We hypothesize that this clustering may facilitate preferential DCV delivery to specific presynaptic sites. Whether postsynaptic partner type specifies DCV clustering, or vise versa remains an open question.
2.4 Experimental procedures

Identifying dense-core vesicles

DCVs were identified in DM6 olfactory neurons previously reconstructed in a serial section transmission electron microscopy dataset comprising the anterior half of a female *Drosophila* brain (Tobin et al., 2017) (Asa Barth-Maron, unpublished). Using criteria based on the literature, we defined DCVs as compartments that are 60 – 200 nm in diameter and contain an electron-dense core (van de Bospoort et al., 2012; Wang et al., 2016). We screened through traced neurons twice each using CATMAID (http://www.catmaid.org) (Saalfeld et al., 2009), and manually identified DCVs. DCV appearance was measured qualitatively as being filled with dark or medium electron density or halo with medium or light electron density (Figure 2.2, E). Presynaptic and postsynaptic sites refer to synaptic connections representing fast, chemical synapses previously identified (Tobin et al., 2017) (Asa Barth-Maron, unpublished). Data were extracted with custom and previously written (https://github.com/schlegelp/PyMaid) Python scripts using the CATMAID API (http://catmaid.readthedocs.io/en/stable/api.html).

Quantifying the normalized number of DCVs within each neuron

For each neuron, we divided the total number of DCVs within DM6 by its DM6 path length in microns.

Identifying DCVs within presynaptic and postsynaptic sites

To probe DCV position, we first needed to define the size of presynaptic and postsynaptic sites. We randomly sampled 30 presynaptic and 10 postsynaptic sites for each neuron and measured the approximate diameter of the synaptic vesicle pool (distance from t-bar to furthest synaptic
vesicle in the plane where the t-bar is most obvious) and terminal (distance from each side of the membrane at the postsynaptic ending) for each site type, respectively (Figure 2.7, A–D). The mean presynaptic site sizes were 616 nm, 881 nm, and 720 nm, for ORNs, PNs, and LNs, respectively. The mean postsynaptic site sizes were 404 nm, 337 nm, and 444 nm, for ORNs, PNs, and LNs, respectively.

We determined the closest synaptic site for each DCV by identifying the site with the closest path length distance. We then measured the Euclidian distance between the DCV to that synaptic site. If this distance was less than the average presynaptic or postsynaptic size for the given cell type (e.g. ORN, PN, or LN), then the DCV was assigned to that site. If not, the DCV was assigned to a non-synaptic site.

Given the variability of presynaptic and postsynaptic sizes, we wanted to be sure that using average site sizes for a given cell type was appropriate. To test this, we computed the number of DCVs/presynaptic site for all presynaptic sites we had measurements for using individual presynaptic size criteria and mean presynaptic site size criteria. We found a strong correlation in the number of DCVs/presynaptic site obtained using the two measurement methods (Figure 2.7, E & F). These data suggest that there is little difference in measurements when using either presynaptic size method, and that using average presynaptic or postsynaptic size criteria is a valid approach.

Quantifying the number of DCVs within presynaptic and postsynaptic sites

For each presynaptic and postsynaptic site, we counted the number of DCVs with distances to it that were shorter than the mean presynaptic or postsynaptic site size for the given cell type. In
FIGURE 2.7: Defining presynaptic and postsynaptic site sizes.

(A & B) A representative presynaptic site with its two postsynaptic partners is shown. (A) Presynaptic site size was defined as the distance (yellow) from the t-bar (red arrow) to the synaptic vesicle furthest away in the plane where the t-bar was most obvious. (B) Postsynaptic site size was defined as the diameter (yellow) of the postsynaptic terminal. (C) Presynaptic site sizes of 30 randomly sampled presynaptic sites across all 62 neurons screened for DCVs. Data are shown for neurons from each cell type. (D) Postsynaptic site sizes of 10 randomly sampled postsynaptic sites across all 62 neurons screened for DCVs. Data are shown for neurons from each cell type. (E) The number of DCVs per presynaptic site for each site that we have presynaptic site measurements for is shown using individual site size criteria (y-axis) and mean site size criteria (x-axis). There is a strong positive correlation, suggesting that there is little difference in the data obtained using the two approaches. (F) The same data are shown as in (E) with the number of DCVs per presynaptic site obtained using individual site size criteria minus that obtained using mean site size criteria. 73% of the presynaptic sites had the same result with the two different criteria.
FIGURE 2.7: (Continued) Defining presynaptic and postsynaptic site sizes.
the rare cases that a DCV fell within two synaptic sites, it was assigned to the closest synaptic site. Each DCV was assigned to only one synaptic site.

**Quantifying presynaptic site clustering**

We developed a metric to quantify clustering of presynaptic sites with at least one ORN postsynaptic partner. To compute a clustering value for each neuron, we computed a score for each presynaptic site with at least one ORN postsynaptic partner. We obtained each score by measuring the path length distance from the given presynaptic site to every other presynaptic site in the cell. If the site to which we measured the distance to had at least one ORN postsynaptic partner then this distance value was made positive, otherwise it was made negative. We added the reciprocal of all these distances, to more heavily weight the presynaptic sites that are closer to the site of interest than those that are further away, to produce a score for the given presynaptic site. Finally, to obtain the final clustering value for each neuron, we computed the median of the all the presynaptic sites with ORN partners’ scores. Using this approach, the more positive the clustering value is the more clustering of presynaptic sites with ORN postsynaptic partners exists. A similar approach was taken for sites with at least one DCV.

To determine whether the measured score is significantly higher than chance, we shuffled the presynaptic sites’ partner identities and computed clustering values 10,000 times for each neuron. From these data, we determined 99% and 95% confidence intervals, and if the observed clustering value fell above either interval then support was given for the sites being significantly clustered. Using these distributions, we computed a \( p \) value for each neuron.
Statistics

Statistical analysis was performed using custom Python scripts and GraphPad Prism. Graphs were generated using custom and modified, previously written (https://github.com/schlegelp/PyMaid), Python scripts and GraphPad Prism, and edited using Adobe Illustrator. Friedman tests with Dunn’s multiple comparisons post-tests were used to test DCV targeting to presynaptic sites with specific postsynaptic partners. For all other comparisons between three groups, One-way ANOVAs with Tukey’s post-tests were used. A Pearson correlation coefficient was computed for number of DCVs/presynaptic site data obtained using individual and mean presynaptic site size criteria. All data are shown as mean ± SEM. Results are as displayed as: ns = not significant, * = \( p \leq 0.05 \), ** = \( p \leq 0.01 \), and *** = \( p \leq 0.001 \).

Code

All code is available at www.github.com/somhegyi/LeeLabScripts.
2.5 Acknowledgements

Tobin et al. and Asa Barth-Maron reconstructed the DM6 neurons screened for DCVs. Logan Thomas developed the clustering metric. Many thanks to Philipp Schlegel for help with code and answering questions about identifying DCVs in electron microscopy datasets. Thanks to Michael Nusbaum for providing relevant references and engaging with conversation about co-transmission. We would also like to thank Wilson lab members for helpful discussions about this project.
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Chapter 3

CYCLIN A FUNCTIONS IN THE PARS LATERALIS TO PROMOTE WAKE

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Author contributions:

H.E.S. and D.R. conceived the ideas and designed the experiments. H.E.S performed the experiments, analyzed the data, wrote the chapter, and made the figures.
3.1 Introduction

Sleep is one of the most enigmatic phenomena in biology. This behavior may seem maladaptive, but the conservation of sleep across nearly all animal species suggests that sleep serves a vital purpose (Sehgal and Mignot, 2011). Sleep is known to play a role in several important processes, such as memory consolidation (Stickgold and Walker, 2005) and metabolic homeostasis (Porkka-Heiskanen, 2013) and is essential for normal lifespan (Hendricks et al., 2000; Rechtschaffen et al., 1983). In addition, sleep disorders, such as insomnia and sleep apnea, are extremely common, and can have profound effects on human health (Sehgal and Mignot, 2011). How and why do animals sleep?

While it is clear that sleep is genetically controlled (Sehgal and Mignot, 2011), the exact mechanisms underlying this behavior remain elusive. The powerful genetic toolkit of Drosophila makes this organism ideally suited for probing the genetic basis of sleep. Rest in flies exhibits many of the hallmarks of mammalian sleep: consolidated periods of immobility, an increased arousal threshold, and different stages of sleep intensity (Hendricks et al., 2000). Over 15 years of studies using this model have greatly enhanced our understanding of the molecules and neuronal circuits involved in sleep behavior, yet our understanding of how sleep is controlled is still incomplete.

Recently, Rogulja and Young discovered that two cell cycle proteins, Rca1 and its main target CycA, function in post-mitotic neurons to promote sleep in Drosophila (Rogulja and Young, 2012). In contrast to known sleep factors, CycA is expressed in only ~40–50 cells in the adult fly brain. This limited expression suggests that CycA might act as a critical switch in the regulation of sleep. The goal of this study was to investigate the molecular and circuit mechanisms by which CycA regulates sleep behavior.
Although knockdown of CycA in all neurons causes decreased sleep, we found that the effect of CycA on sleep depends on the cellular context. Knockdown of CycA in ~14 wake-promoting neurons in the PL, a neurosecretory center analogous to the mammalian hypothalamus (de Velasco, 2007; Saper et al., 2005), resulted in increased sleep. We found that PL CycA likely drives wake by promoting the release of a novel sleep neuropeptide called Crz. CycA localizes to presynaptic sites and appears to promote PL activity through interactions with other cell cycle machinery, Cyclin B (CycB), Cdk1, and Cdk2. Finally, we found that RNAi against receptors of wake-promoting peptides diuretic hormone 31 (DH31), diuretic hormone 44 (DH44), or PDF in PL cells caused increased sleep, suggesting that CycA-expressing PL cells integrate multiple wake signals. Together, this work identifies a new network of sleep molecules and an integrating center of wake-promoting signals.
3.2 Results

3.2.1 PL Cyclin A promotes wake

To investigate in which cells CycA functions to regulate sleep, we identified Gal4 lines that label subpopulations of CycA-expressing cells. Knockdown of CycA in ~14 neurons in the PL using PL-Gal4 (PL-Gal4 + UAS-CycA-RNAi, denoted “PL>CycA-RNAi”) increased sleep in males (Choi et al., 2008) (Figure 3.1, A & B). This effect was strongest on day sleep, although a small increase in night sleep was also observed (Figure 3.1, C & D). Sleep bout length was increased in PL>CycA-RNAi flies compared to those of controls (Figure 3.1, E), suggesting that PL CycA is important for sleep maintenance. Sleep bout number and sleep delay (time of the first sleep bout after the lights are turned off) were unaffected in PL>CycA-RNAi flies (Figure 3.1, F & G), suggesting that CycA in PL cells does not affect sleep initiation in males. Similar results were observed in females except that sleep delay was significantly reduced in PL>CycA-RNAi females when compared to controls (Figure B.1, A–F). Finally, to confirm that PL CycA promotes wake, we knocked down Rca1, which is known to stabilize CycA levels (Dong et al., 1997), in PL neurons. Consistent with this, we observed increased sleep when Rca1 was knocked down compared to that of control flies (Figure 3.1, H). Together, these results indicate that CycA expressed in PL cells promotes wake and regulates sleep maintenance in both males and females. In addition, CycA may also regulate sleep initiation in females.

The sleep phenotype of PL>CycA-RNAi is opposite of that observed when CycA was knocked down using the pan-neuronal elav-Gal4 (Rogulja and Young, 2012) (Figure 3.2, A, B, & F). In addition, knockdown of CycA in a subset of circadian cells caused decreased sleep, while CycA-RNAi in the PI or in a pair of dorsal anterior lateral cells (Chen et al., 2012) caused
**Figure 3.1:** PL Cyclin A promotes wake.

**A** CycA (pink) is expressed in PL cells, labeled by Myr-GFP. Detailed image of the region surrounded by the white dashed line is shown on the bottom right. Sites of colocalization are marked with red arrows. **B** Knockdown of CycA using RNAi in PL cells \((UAS-Dcr2/UAS-Dcr2;\ PL-Gal4/UAS-CycA-RNAi, n = 229)\) increased the duration of total sleep compared to that of the parental control animals tested as hemizygotes \((UAS-Dcr2;\ PL-Gal4 (n = 83), \ UAS-Dcr2; \ UAS-CycA-RNAi (n = 103))\). **C** Day sleep and **D** night sleep were increased in \(UAS-Dcr2/UAS-Dcr2;\ PL-Gal4/UAS-CycA-RNAi\) animals compared to controls. **E** Sleep bout length was increased in \(UAS-Dcr2/UAS-Dcr2;\ PL-Gal4/UAS-CycA-RNAi\) flies, but **F** number of daily sleep bouts and **G** sleep delay were unaffected. **H** Knockdown of Rca1 using RNAi in PL cells \((UAS-Dcr2/UAS-Rca1-RNAi;\ PL-Gal4 (n = 90))\) caused increased sleep when compared to controls \((UAS-Dcr2;\ PL-Gal4 (n = 53), \ UAS-Rca1-RNAi (n = 96))\). All data are from male flies.
FIGURE 3.1: (Continued) PL Cyclin A promotes wake.
**Figure 3.2**: The effect of Cyclin A on sleep depends on the cellular context.

(A) Schematic of CycA-expressing cells adapted from (Rogulja and Young, 2012). Various Gal4s labeling subsets of CycA cells are highlighted (R22D11-Gal4 (purple), G0431-Gal4 (green), *Tim(UAS)-*Gal4 (orange), PL-Gal4 (pink), and *elav*-Gal4 is a pan-neuronal driver). 

(B) Knockdown of *CycA* using *elav*-Gal4 caused reduced sleep [*elav*-Gal4; *UAS-Dcr2/UAS-Dcr2; *UAS-CycA-RNAi* (*n* = 36)] compared to controls [*elav*-Gal4; *UAS-Dcr2* (*n* = 30), *UAS-Dcr2; UAS-CycA-RNAi* (*n* = 138)].

(C) Knockdown of *CycA* using R22D11-Gal4 caused increased sleep [R22D11-Gal4/*UAS-Dcr2; UAS-CycA-RNAi* (*n* = 36)] compared to controls [R22D11-Gal4 (*n* = 70), *UAS-Dcr2; UAS-CycA-RNAi* (*n* = 75)].

(D) Knockdown of *CycA* using G0431-Gal4 caused increased sleep [*UAS-Dcr2/UAS-Dcr2; G0431-Gal4/UAS-CycA-RNAi* (*n* = 115)] compared to controls [*UAS-Dcr2; G0431-Gal4* (*n* = 117), *UAS-Dcr2; UAS-CycA-RNAi* (*n* = 99)].

(E) Knockdown of *CycA* using *tim(UAS)-*Gal4 caused reduced sleep [*tim(UAS)-*Gal4/*UAS-Dcr2; *UAS-CycA-RNAi* (*n* = 117)] compared to controls [*tim(UAS)-*Gal4/*UAS-Dcr2* (*n* = 116), *UAS-CycA-RNAi* (*n* = 109)].

(F) Knockdown of *CycA* using PL-Gal4 caused increased sleep [*UAS-Dcr2/UAS-Dcr2; PL-Gal4/UAS-CycA-RNAi* (*n* = 229)] compared to controls [*UAS-Dcr2; PL-Gal4* (*n* = 83), *UAS-Dcr2; UAS-CycA-RNAi* (*n* = 103)] (same data as in Figure 2.1, B). All data are from male flies.
FIGURE 3.2: (Continued) The effect of Cyclin A on sleep depends on the cellular context.
increased sleep (Figure 3.2, A, C, D & E). These data indicate that CycA can regulate sleep in different ways depending on the cellular context.

To test whether the effect of PL>\textit{CycA-RNAi} on sleep is due to altered sleep behavior and not general mobility defects we measured waking activity (average activity per minute awake). We observed a significant decrease in waking activity in PL>\textit{CycA-RNAi} flies compared to that of controls (Figure 3.3, A), suggesting that PL>\textit{CycA-RNAi} animals are less active than control flies when awake. This decrease could be due to general movement defects or increased sleep pressure even when awake. For example, as narcoleptics tend to have trouble staying awake (Peever and Fuller, 2017), PL>\textit{CycA-RNAi} flies may have difficulty maintaining their wake state. First, to determine whether PL>\textit{CycA-RNAi} animals have general movement defects, we assayed climbing, courtship, and startle response behaviors and found no effect when \textit{CycA} was knocked down in PL cells (Figure 3.3, B–D). These results suggest that PL>\textit{CycA-RNAi} does not affect general mobility, but does alter sleep behavior. Second, to address whether \textit{CycA} knockdown affects the development of PL cells, we examined the cell morphology of PL cells in PL>\textit{CycA-RNAi} flies using \textit{UAS-CD8-GFP}. We observed no difference in PL cell morphology in PL>\textit{CycA-RNAi} flies when compared to that of the control (Figure 3.3, E & F). Together, these data suggest that PL>\textit{CycA-RNAi} disrupts sleep behavior and does not cause general mobility or developmental defects.

### 3.2.2 PL neuronal activity promotes wake

To investigate how the activity of PL cells affects sleep, we used PL-Gal4, which almost exclusively labels CycA-expressing PL neurons (Figure 3.1, A), to drive the expression of the non-selective cation channel, \textit{NaChBac} (Nitabach et al., 2006). Activation of PL cells using
**Figure 3.3**: PL>\textit{CycA-RNAi} flies do not exhibit general mobility or developmental defects.

(A) Knockdown of \textit{CycA} in using RNAi in PL neurons \([UAS-Dcr2/UAS-Dcr2; PL-Gal4/UAS-CycA-RNAi (n = 229)]\) decreased activity per waking minute compared to those of the parental control animals \([UAS-Dcr2; PL-Gal4 (n = 83), UAS-Dcr2; UAS-CycA-RNAi (n = 103)]\). (B) There was no difference in climbing ability for \(UAS-Dcr2/UAS-Dcr2; PL-Gal4/UAS-CycA-RNAi\) flies \((n = 11)\) compared to control animals \([UAS-Dcr2; PL-Gal4 (n = 11), UAS-Dcr2; UAS-CycA-RNAi (n = 10)]\). (C) No difference was observed in courtship index (proportion of time spent courting or mating) for \(UAS-Dcr2/UAS-Dcr2; PL-Gal4/UAS-CycA-RNAi (n = 18)\) and \(UAS-Dcr2; PL-Gal4 (n = 18)\). \(UAS-Dcr2; UAS-CycA-RNAi\) flies have light eyes and do not court. (D) Knockdown of \textit{CycA} using RNAi in PL cells \([UAS-Dcr2/UAS-Dcr2; PL-Gal4/UAS-CycA-RNAi (n = 161)]\) had no difference in startle response (activity / minute for 3 minutes following 2V startle) compared to those of parental control animals \([UAS-Dcr2; PL-Gal4 (n = 224), UAS-Dcr2; UAS-CycA-RNAi (n = 147)]\). (E) Representative images of PL cell morphology visualized using CD8-GFP are shown. There was no obvious difference in neuronal morphology between control [left, \textit{UAS-CD8-GFP}; PL-Gal4]) and PL>\textit{CycA-RNAi} flies [right, \textit{UAS-CD8-GFP/UAS-Dcr2}; PL-Gal4/\textit{UAS-CycA-RNAi}]. (F) Quantification of PL morphology are shown \([UAS-CD8-GFP; PL-Gal4 (n = 25), UAS-CD8-GFP/UAS-Dcr2; PL-Gal4/UAS-CycA-RNAi (n = 22)]\). All data are from male flies.
FIGURE 3.3: (Continued) PL>\textit{CycA-RNAi} flies do not exhibit general mobility or developmental defects.
Figure 3.4: Cyclin A is necessary for the wake promoting effect of PL neuronal activation.

(A) Activation of PL cells [UAS-NaChBac; PL-Gal4 (n = 72)] caused decreased sleep compared to parental controls [PL-Gal4 (n = 39), UAS-NacChBac (n = 40)]. (B) Silencing of PL cells [UAS-TNT-G, UAS-Kir2.1; PL-Gal4 (n = 44)] caused increased sleep compared to parental controls [PL-Gal4 (n = 79), UAS-TNT-G, UAS-Kir2.1 (n = 73). (C) CycA-RNAi suppresses the wake-promoting effect of PL>NaChBac [UAS-NaChBac/UAS-Dcr2; UAS-CycA-RNAi/PL-Gal4 (n = 100), UAS-NaChBac/UAS-CD8-GFP; UAS-Dcr2/PL-Gal4 (n = 108)] compared to controls [PL-Gal4 (n = 150), UAS-NaChBac/UAS-CD8-GFP; UAS-Dcr2 (n = 64), UAS-CD8-GFP; UAS-Dcr2/UAS-CycA-RNAi (n = 96), UAS-Dcr2/UAS-CD8-GFP; UAS-CycA-RNAi/PL-Gal4 (n = 100)]. UAS-CD8-GFP is used to control for the number of UAS constructs present across conditions. All data are from male flies.
FIGURE 3.4: (Continued) Cyclin A is necessary for the wake promoting effect of PL neuronal activation.
UAS-NaChBac caused decreased sleep (Figure 3.4, A). Consistent with this, silencing of PL neurons using tetanus toxin (UAS-TNT-G) (Sweeney et al., 1995) and an inward rectifying potassium channel (UAS-Kir2.1) (Baines et al., 2001) resulted in increased sleep (Figure 3.4, B). These data indicate that PL neuronal activity promotes wake, and were confirmed by an independent group (Afonso et al., 2016). Because PL>CycA-RNAi caused increased sleep, we hypothesized that CycA activity is required for the wake-promoting effect of PL activation. To test this hypothesis, we knocked down CycA while activating PL cells. Consistent with our hypothesis, we observed a partial suppression of the wake-promoting effect of PL cell activation when CycA was knocked down (Figure 3.4, C). These data indicate that CycA functions downstream of PL activation and is required for the wake-promoting effect of PL activity.

3.2.3 Corazonin mediates the wake-promoting effect of PL neuronal activity

To better understand how CycA in PL cells promotes wake, we investigated which molecule(s) are released from these cells to regulate sleep. PL neurons are known to express the neuropeptide Crz (Lee et al., 2007), and we confirmed that all CycA-expressing PL cells are stained by Crz antibodies (Figure 3.5, A). To test whether Crz regulates sleep, we knocked down Crz using two independent RNAi lines in PL cells. PL>Crz-RNAi resulted in increased sleep as compared to controls, suggesting that Crz promotes wake in males (Figures 3.5, B–D). Similar results were observed when Crz was knocked down in females (Figure B2, A–C). Reduced Crz protein levels resulting from RNAi-mediated knockdown were confirmed by immunohistochemistry. We observed a significant reduction in Crz antibody staining in PL>Crz-RNAi brains when compared to control brains (Figures 3.5, E & F), suggesting that the sleep increase observed in PL>Crz-RNAi flies is due to the reduction of Crz. Together, these data indicate that Crz promotes wake.
Figure 3.5: Corazonin mediates the wake-promoting effect of PL neuronal activity.

(A) Antibodies targeting CycA (pink) and Crz (green) show colocalization in PL cell bodies (solid red arrows) and in puncta (open red arrows) along processes. (B) Knockdown of Crz using two distinct RNAi lines caused increased sleep \([\text{UAS-Dcr2/} U\text{A}S-\text{Crz-RNAi A; UAS-Dcr2/PL-Gal4 (}n = 119), \text{UAS-Dcr2; UAS-Crz-RNAi B/PL-Gal4 (}n = 71)\)] compared to parental controls \([\text{UAS-Dcr2; PL-Gal4 (}n = 207), \text{UAS-Crz-RNAi A; UAS-Dcr2 (}n = 134), \text{UAS-Crz-RNAi B (}n = 61)\)]]. We also observed increased (C) day and (D) night sleep in \(\text{Crz-RNAi}\) animals. (E) To test the specificity of the RNAi constructs, we stained control [left, \(\text{UAS-Dcr2; PL-Gal4}\)] and knockdown [right, \(\text{UAS-Dcr2/UAS-Crz-RNAi A; UAS-Dcr2; PL-Gal4}\)] brains for antibodies targeting Crz. We observed a reduction in antibody staining in \(\text{PL>Crz-RNAi}\) brains. (F) Quantification of Crz antibody fluorescence intensity for control [\(\text{UAS-Dcr2; PL-Gal4 (}n = 6)\)] and knockdown [\(\text{UAS-Dcr2/UAS-Crz-RNAi A; UAS-Dcr2/PL-Gal4 (}n = 10)\)] brains is shown. (G) \(\text{Crz-RNAi}\) suppresses the wake-promoting effect of \(\text{PL>NaChBac}\) \([\text{UAS-NaChBac/UAS-Dcr2; UAS-Crz-RNAi A/PL-Gal4 (}n = 93), \text{UAS-NaChBac/UAS-CD8-GFP; UAS-Dcr2/PL-Gal4 (}n = 154)\)] compared to controls [\(\text{PL-Gal4 (}n = 184), \text{UAS-NaChBac/UAS-CD8-GFP; UAS-Dcr2 (}n = 66), \text{UAS-CD8-GFP; UAS-Dcr2/UAS-Crz-RNAi A (}n = 138), \text{UAS-Dcr2/UAS-CD8-GFP; UAS-Crz-RNAi A/PL-Gal4 (}n = 75)\)]]. \(\text{UAS-CD8-GFP}\) is used to control for the number of \(\text{UAS}\) constructs present across conditions. All data are from male flies.
Figure 3.5: (Continued) Corazonin mediates the wake-promoting effect of PL neuronal activity.
We observed a significant decrease in waking activity in PL>Crz-RNAi flies (Figure 3.6, A), suggesting that PL>Crz-RNAi flies, like PL>CycA-RNAi animals, are less active than control flies when awake. We confirmed that PL>Crz-RNAi animals do not exhibit general movement defects, by finding that PL>Crz-RNAi does not affect climbing, courtship, and startle response behaviors (Figure 3.6, B–D). These results suggest that the sleep increase observed when Crz is knocked down is due to altered sleep behavior and not defects in general mobility. We also examined the cell morphology of PL cells in PL>Crz-RNAi flies using UAS-CD8-GFP to determine whether Crz knockdown affects cellular development. We observed no difference in PL cell morphology in PL>Crz-RNAi flies when compared to the control (Figure 3.6, E & F). Together, these data suggest that the effect of PL>Crz-RNAi on sleep behavior is due to altered sleep and not mobility or developmental defects.

Finally, to determine whether Crz mediates the wake-promoting effect of PL neuronal activation, we knocked down Crz while activating PL cells using UAS-NaChBac. PL>Crz-RNAi partially suppressed the decreased sleep phenotype of PL activation (Figure 3.5, G). These data suggest that PL cells promote wake at least in part via Crz release. The partial suppression may be due to incomplete knockdown of Crz (Figures 3.5, E & F), or PL cells releasing other molecules in addition to Crz to regulate sleep. PL cells are also known to release sNPF, a neuropeptide important for sleep regulation (Chen et al., 2013; Kapan et al., 2012). However, we did not observe any effect on sleep behavior when sNPF was knocked down using RNAi (data not shown). Together, these data demonstrate that Crz is a novel sleep-related neuropeptide that mediates much of the wake-promoting effect of PL neuronal activation.

We noticed that CycA puncta in PL cells colocalize with Crz, Brp-short, and Syt-GFP puncta (Figure 3.5, A; data not shown), indicating that CycA localizes to presynaptic sites.
FIGURE 3.6: PL>Crz-RNAi flies do not exhibit general mobility or developmental defects.

(A) Knockdown of Crz using RNAi in PL cells [UAS-Dcr2/UAS-Dcr2; UAS-Crz-RNAi A/PL-Gal4 (n = 119)] decreased activity per waking minute compared to that of the parental control animals [UAS-Dcr2; PL-Gal4 (n = 145), UAS-Dcr2; UAS-Crz-RNAi A (n = 134)]. (B) There was no difference in climbing ability for UAS-Dcr2/UAS-Dcr2; UAS-Crz-RNAi A/PL-Gal4 flies (n = 9) compared to control animals [UAS-Dcr2; PL-Gal4 (n = 10), UAS-Dcr2; UAS-Crz-RNAi A (n = 10)]. (C) No difference was observed in courtship index (proportion of time spent courting or mating) for UAS-Dcr2/UAS-Dcr2; UAS-Crz-RNAi A/PL-Gal4 (n = 18), UAS-Dcr2; PL-Gal4 (n = 18), and UAS-Dcr2; UAS-Crz-RNAi A (n = 18). (D) Knockdown of Crz using RNAi in PL cells [UAS-Dcr2/UAS-Dcr2; UAS-Crz-RNAi A; PL-Gal4 (n = 109)] caused no difference in startle response (activity / minute for 3 minutes following 2V startle) compared to that of the parental control animals [UAS-Dcr2; PL-Gal4 (n = 225), UAS-Dcr2; UAS-Crz-RNAi A (n = 90)]. (E) Representative images of PL cell morphology visualized using CD8-GFP are shown. There was no obvious difference in neuronal morphology in control flies [left, UAS-CD8-GFP; PL-Gal4] compared to PL>Crz-RNAi flies [right, UAS-CD8-GFP/UAS-Dcr2; UAS-Crz-RNAi A/PL-Gal4]. (F) Quantification of PL morphology is shown [UAS-CD8-GFP; PL-Gal4 (n = 23), UAS-CD8-GFP/UAS-Dcr2; UAS-Crz-RNAi A/PL-Gal4 (n = 20)]. All data are from male flies.
Figure 3.6: (Continued) PL>Crz-RNAi flies do not exhibit general mobility or developmental defects.
Similar results were also obtained from an independent group (Afonso et al., 2015). Given this localization pattern and the observation that knockdown of either \textit{CycA} or \textit{Crz} in PL cells cause increased sleep, we hypothesize that CycA facilitates the release of Crz to promote wake.

3.2.4 Cyclin A may work with other cell cycle proteins in PL neurons to promote wake

To address how PL CycA promotes wake we considered whether other cell cycle proteins are also repurposed to regulate sleep. Does CycA function with other cell cycle machinery or does it work with an independent molecular network in PL cells? To investigate whether other cell cycle proteins play a role in sleep behavior, we knocked down various cell cycle proteins using RNAi. PL knockdown of another cell cycle cyclin, \textit{CycB} resulted in increased sleep (Figure 3.7, A), suggesting that CycB, like CycA, promotes wake in PL cells. Cdk1 interacts with and mediates the role of both CycA and CycB during mitosis (Follette and OFarrell, 1997). PL knockdown of \textit{Cdk1} using RNAi caused increased sleep (Figure 3.7, B), suggesting that CycA and CycB interact with Cdk1, as during mitosis, to promote wake. These data are supported by another group’s finding that overexpression of Cdk1 in PL neurons causes decreased sleep (Afonso et al., 2015). CycA has been shown to also interact with Cdk2 and is necessary for DNA replication during S phase in mammals (Girard et al., 1991; Pagano et al., 1992). Although the role of CycA in S phase is less clear in \textit{Drosophila}, ectopic CycA expression can induce S phase, suggesting that CycA may be capable of interacting with Cdk2 in \textit{Drosophila} (Lehner et al., 1991; Thomas et al., 1994). Consistent with this, knockdown of \textit{Cdk2} in PL cells resulted in increased sleep (Figure 3.7, C). These data suggest that Cdk2 may interact with CycA to promote wake in PL cells. To further test whether the Cdks mediate the role of CycA in sleep regulation, we ectopically expressed tagged Cdk1 and Cdk2 in PL cells. CycA has previously been shown to
FIGURE 3.7: PL Cyclin A may work with other cell cycle proteins to promote wake.

(A) Knockdown of CycB using three distinct RNAi lines caused increased sleep [UAS-Dcr2; UAS-CycB-RNAi A/PL-Gal4 (n = 91), UAS-Dcr2; UAS-CycB-RNAi B/PL-Gal4 (n = 78), UAS-Dcr2; UAS-CycB-RNAi C/PL-Gal4 (n = 67)] compared to parental controls [UAS-Dcr2; PL-Gal4 (n = 144), UAS-CycB-RNAi A (n = 87), UAS-CycB-RNAi B (n = 76), UAS-CycB-RNAi C (n = 66)]. (B) Cdk1 knockdown using two distinct RNAi lines caused increased sleep [UAS-Dcr2; UAS-Cdk1-RNAi A/PL-Gal4 (n = 38), UAS-Dcr2; UAS-Cdk1-RNAi B/PL-Gal4 (n = 73)] compared to parental controls [UAS-Dcr2; PL-Gal4 (n = 152), UAS-Cdk1-RNAi A (n = 43), UAS-Cdk1-RNAi B (n = 73)]. (C) Knockdown of Cdk2 caused increased sleep [UAS-Dcr2; UAS-Cdk2-RNAi/PL-Gal4 (n = 84)] compared to parental controls [UAS-Dcr2; PL-Gal4 (n = 103), UAS-Cdk2-RNAi (n = 77)]. (D) Colocalization of CycA (pink) and Cdk1-myc (green) puncta (red arrows) in PL neurons. PL-Gal4 is driving the expression of Cdk1-myc. (E) Colocalization of CycA (pink) and Cdk2-myc (green) puncta (red arrows) in PL neurons. PL-Gal4 is driving the expression of Cdk2-myc. All data are from male flies.
FIGURE 3.7: (Continued) PL Cyclin A may work with other cell cycle proteins to promote wake.
colocalize with ectopically expressed Cdk1 puncta (Afonso et al., 2015). We confirmed this finding and also found that CycA puncta colocalize with Cdk2 puncta (Figure 3.7, D & E), suggesting that CycA may bind to and interact with Cdk1 and/or Cdk2 at PL presynaptic sites to promote wake. Together, these data suggest that CycA acts with other repurposed cell cycle proteins to regulate sleep in PL neurons.

3.2.5 PL neurons receive input from several wake-promoting peptides

To understand the role of PL neurons in the known sleep circuitry of Drosophila, we probed potential inputs to PL cells. PL neurons are located in close proximity to master clock cells called s-LNvs, which release a wake-promoting neuropeptide called PDF (Choi et al., 2005; Parisky et al., 2008). We hypothesized that PL cells may lie downstream of s-LNvs. Using pdf-LexA and PL-Gal4, we detected a GFP Reconstitution Across Synaptic Partners (GRASP) (Feinberg et al., 2008) signal in the region of possible contact (Figure 3.8, A), suggesting that these cells are synaptically connected. To elucidate the direction of this interaction, we knocked down PDFR in PL neurons and observed an increased sleep phenotype (Figure 3.8, B). These data suggest that s-LNvs signal directly to PL cells via PDF.

PL cells are known to express receptors for two other neuropeptides implicated in sleep, circadian rhythms, and metabolism called DH31 and DH44 (Cavanaugh et al., 2014; Johnson et al., 2005; Kunst et al., 2014). In addition, PL processes are in close proximity to those of DH31 and DH44 neurons (Cavanaugh et al., 2014; Kunst et al., 2014). There are two known DH44 receptors, DH44R1 and DH44R2, and one DH31 receptor, DH31R1 (Dus et al., 2015; Johnson et al., 2005). PL knockdown of any of these receptors resulted in increased sleep (Figure 3.8, C–E), suggesting that PL cells receive input from DH31 and DH44 wake-promoting neuropeptides.
FIGURE 3.8: PL neurons receive input from multiple wake-promoting neuropeptides.

(A) Schematic of CycA-expressing PL neurons and the projection patterns of sLNVs (labeled by pdf-LexA). Reconstituted GRASP signal (green) observed using pdf-LexA and PL-Gal4 adjacent to CycA (red). (B) Knockdown of PDFR [UAS-Dcr2; UAS-PDFR-RNAi/PL-Gal4 (n = 115)] caused increased sleep compared to controls [UAS-Dcr2; PL-Gal4 (n = 76), UAS-PDFR-RNAi (n = 103)]. (C) Knockdown of DH31R [UAS-Dcr2; UAS-DH31R-RNAi/PL-Gal4 (n = 60)] caused increased sleep compared to controls [UAS-Dcr2; PL-Gal4 (n = 101), UAS-DH31R-RNAi (n = 59)]. (D) Knockdown of DH44R1 [UAS-Dcr2; UAS-DH44R1-RNAi/PL-Gal4 (n = 91)] caused increased sleep compared to controls [PL>Dcr2 (n = 95), UAS-DH44R1-RNAi (n = 82)]. (E) DH44R2 knockdown using two distinct RNAi lines caused increased sleep [UAS-Dcr2; UAS-DH44R2-RNAi A/PL-Gal4 (n = 80), UAS-Dcr2; UAS-DH44R2-RNAi B/PL-Gal4 (n = 81), UAS-Dcr2; UAS-DH44R2-RNAi C/PL-Gal4 (n = 125)] compared to parental controls [UAS-Dcr2; PL-Gal4 (n = 170), UAS-DH44R2-RNAi A (n = 40), UAS-DH44R2-RNAi B (n = 61), UAS-DH44R2-RNAi C (n = 106)]. All data are from male flies.
Figure 3.8: (Continued) PL neurons receive input from multiple wake-promoting neuropeptides.
Together, these results suggest that PL neurons integrate several wake-promoting signals. Furthermore, Crz has been implicated in metabolism (Kubrak et al., 2016), so it’s possible that these cells may play a role in the intersection between sleep and metabolic pathways (Kapan et al., 2012).
3.3 Discussion

3.3.1 PL Cyclin A promotes wake

Although knockdown of CycA in all neurons causes decreased sleep (Rogulja and Young, 2012), we found that the effect of CycA on sleep depends on the cellular context. Knockdown of CycA in ~14 wake-promoting neurons in the PL resulted in increased sleep. These data suggest that PL CycA promotes wake. We also showed that PL knockdown of Rca1, which stabilizes CycA levels (Dong et al., 1997), causes increased sleep. These data further support the role of PL CycA in promoting wake and also strongly suggest that Rca1 is expressed and promotes wake in PL neurons. We and others have shown that activation of PL neurons decreases sleep (Afonso et al., 2015), suggesting that PL neurons promote wake. Furthermore, we provided evidence that the wake-promoting effect of PL activation requires CycA activity.

3.3.2 A network of cell cycle proteins function in PL neurons to promote wake

CycA localizes to presynaptic sites and appears to promote PL activity through possible interactions with other cell cycle machinery, CycB, Cdk1, and Cdk2. Knockdown of CycB, Cdk1, or Cdk2 cause increased sleep, suggesting that these cell cycle proteins are present in PL neurons and promote wake. Cyclins regulate the cell cycle by working through cyclin dependent kinases. CycA interacts with Cdk1 during mitosis and has been shown to function in S-phase with Cdk2 in mammals (Follette and OFarrell, 1997; Pagano et al., 1992). We confirmed that ectopic expression Cdk1 colocalizes with CycA puncta (Afonso et al., 2015), suggesting that CycA may directly interact with Cdk1 in PL neurons. In addition, we also showed that ectopically expressed Cdk2 colocalizes with CycA puncta as well, suggesting that CycA may also directly interact with Cdk2 to promote wake. Together, our results suggest that CycA
functions with Cdk1 and/or Cdk2 to promote sleep. Future work is needed to identify the targets of Cdk1 and/or Cdk2 kinase activity, which will provide insight into how the PL Cdks promote wake. In addition, we showed that CycB, which is known to interact with Cdk1 during the cell cycle (Follette and OFarrell, 1997), promotes wake, but more work is needed to understand if and how CycA and CycB interact with each other to regulate sleep (Figure 3.9).

Consistent with our findings, Afonso et al. showed that overexpression of a hyperactive version of Cdk1 (Cdk1-AF) causes decreased sleep. Our data showing that PL>\textit{Cdk1-RNAi} results in increased sleep supports this finding, and, together, strongly suggest that Cdk1 promotes wake in PL cells. Interestingly, only overexpression of Cdk1-AF and not wild type Cdk1 resulted in decreased sleep (Afonso et al., 2015). Cdk1-AF is a mutant Cdk1 with mutations in inhibitory phosphorylation sites (Ayeni et al., 2014). This result suggests that Cdk-inhibitory kinases, Wee1 and Myt1, and activating phosphatases, String and Twine (Lee and Orr-Weaver, 2003), may be expressed in PL cells. Consistent with this, we saw staining in PL neurons with antibodies targeting Wee1, String, and Twine in PL cells (we do not have Myt1 antibodies; data not shown). These data suggest that several other cell cycle proteins may also be expressed in PL neurons and have post-mitotic roles in sleep regulation.

Afonso et al. identified a sleep-promoting role for an additional cell cycle protein, TARANIS (TARA), which is a \textit{Drosophila} homolog of a family of mammalian transcriptional coregulators that play a role in cell cycle progression. Knockdown of TARA activity using mutants or pan-neuronal RNAi caused decreased sleep, suggesting that TARA promotes sleep. Interestingly, they showed that TARA physically interacts with CycA in \textit{Drosophila} S2 cells, and that CycA levels are decreased in \textit{tara} mutants. These and other data suggest that TARA regulates CycA levels post-transcriptionally. Consistent with their pan-neuronal knockdown,
FIGURE 3.9: PL neurons integrate multiple wake-promoting signals and drive wake.

We have shown that CycA functions in PL neurons to promote wake via the release of Crz. CycA likely acts with other cell cycle proteins, CycB, Cdk1, and Cdk2 to regulate wake. Finally, we showed that PL cells integrate wake-promoting signals via the receptors PDFR, DH31R, DH44R1, and DH44R2.
they found that knockdown of \textit{tara} using PL-Gal4 results in decreased sleep. This finding is at odds with our work, because we showed that \textit{CycA} knockdown in PL cells causes increased sleep. If TARA stabilizes \textit{CycA} levels in PL cells, we would expect PL>\textit{CycA-RNAi} to cause decreased sleep. It is possible that TARA affects other proteins in addition to \textit{CycA} thereby masking the sleep phenotype of \textit{CycA} reduction. Conversely, the observation that pan-neuronal \textit{tara} knockdown results in a stronger decreased sleep phenotype than specific knockdown in PL cells suggests that TARA acts in non-PL neurons as well. It is possible that the reduction of \textit{CycA} protein levels occurs through a non-autonomous mechanism. The authors showed that \textit{CycA} levels are reduced in \textit{tara} mutants, but not in PL>\textit{tara-RNAi} animals. Thus, it is unclear how TARA and \textit{CycA} interact in PL neurons.

3.3.3 A novel role for the neuropeptide Corazonin in sleep regulation

We identified a novel role for the neuropeptide Crz in sleep regulation by showing that Crz functions in PL neurons to promote wake. Interestingly, we observed a large reduction in Crz antibody staining in PL>\textit{CycA-RNAi} animals compared to controls (data not shown). This suggests that Crz protein levels are reduced when \textit{CycA} is knocked down in PL neurons. Previously work has shown that activation of PL neurons causes reduced Crz antibody staining (Zhao et al., 2010), suggesting that PL activation causes the majority of Crz to be released making it less detectable using antibodies. However, we were unable to reproduce these results (data not shown), so it is difficult to interpret these findings. Our behavior data are consistent with \textit{CycA} promoting the release of Crz. It is possible that when \textit{CycA} is reduced, Crz is unable to be released and is then targeted for degradation, which would cause a reduction in antibody staining. More work is needed to understand how reduced \textit{CycA} levels cause lowered Crz
protein levels. Regardless, these data provide further support for a molecular interaction between CycA and Crz.

3.3.4 The role of PL neurons in sleep and metabolism

Crz has previously been shown to play a role in stress response and metabolism. Crz signaling during metabolic stress coordinates increased food intake and diminishes energy stores to regain metabolic homeostasis (Kapan et al., 2012; Kubrak et al., 2016; Zhao et al., 2010). These and other data are consistent with the hypothesis that Crz is released during states of stress and metabolic deficits, and may serve as a signal for physiological stress (Boerjan et al., 2010; Veenstra, 2009). Several studies have revealed that sleep and metabolism are physiologically connected (Yurgel et al., 2014). For example, flies and mammals are known to suppress sleep when starved to presumably forage for food (Danguir and Nicolaidis, 1979; Keene et al., 2010). Our observations suggesting that Crz promotes wake are consistent with this general observation and Crz’s known role in metabolism. Furthermore, these data suggest that PL neurons may be an important intersection between sleep and metabolism. To better understand how PL neuronal activity is regulated, we investigated putative sources of input.

Sleep is rhythmically regulated by the circadian clock, but it is not entirely clear how the clock and sleep systems interact. In CycA neuronal knockdown animals, the clock is fully functional (Rogulja and Young, 2012), suggesting that the observed defects in sleep result from a flaw in the clock output. Interestingly, master clock cells called s-LNvs produce PDF, a major regulator of the clock output, and send axonal projections near PL neurons (Choi et al., 2005; Griffith, 2013). This observation suggests an intriguing circuit mechanism through which PL neurons receive information from circadian clock cells. In support of this hypothesis, we
observed reconstituted GRASP signals between PL and PDF neurons. Furthermore, PL>PDF-RNAi caused increased sleep, suggesting that PDF cells signal directly to PL neurons. Together, these data suggest that PL neurons receive wake-promoting PDF signals from s-LNvs.

PL neurons are known to express receptors for two diuretic hormones, DH31 and DH44 (Johnson et al., 2005). Both DH31 and DH44 have been shown to mediate nutritive stress responses and promote wake (Cavanaugh et al., 2014; Kunst et al., 2014; Vanderveken and O'Donnell, 2014; Veenstra, 2009). For example, DH44 neurons are activated by nutritive sugars found in the hemolymph, suggesting that DH44 acts as a post-ingestive nutrient sensor. DH44 signaling stimulates rapid proboscis extension reflex, which reinforces the ingestion of nutritive foods, and enhances gut motility, which would facilitate digestion of nutritive foods (Dus et al., 2015). In addition, DH44 is a circadian output molecule and has been shown to promote wake (Cavanaugh et al., 2014; Cavey et al., 2016). DH31 and DH44 stimulate the production of cAMP (Cabero et al., 2002; Veenstra, 2009), suggesting that these molecules would promote PL neuronal activity. Consistent with this, we found that knockdown of DH31R, DH44R1, or DH44R2 in PL neurons caused increased sleep, which is similar to the increased sleep phenotype observed in silenced PL neurons. These data suggest that PL neurons detect signals from DH31 and DH44 neuropeptides to promote wake. Furthermore, these data provide the first evidence that PL neurons express DH44R2.

This work supports a mechanism whereby PL neurons integrate wake-promoting and metabolic distress information, and promote the release of Crz, which triggers wake. We also show that several cell cycle proteins are expressed and function in PL neurons to promote wake presumably by facilitating the release of Crz. This study generates a number of exciting implications for how sleep and metabolic systems may interact.
3.4 Experimental procedures

Fly genetics

Flies were raised on standard cornmeal/molasses food at 25°C in a 12-hour light/12-hour dark (LD) cycle. All transgenes were introgressed into the wild type iso31 background by backcrossing for at least 5 generations. In all experiments, Gal4 and UAS parental controls were tested as hemizygotes.

Fly stocks

UAS-RNAi lines (CycA (5940R-1); Rca1 (10800R-1); CycB (3510R-3 [A]); DH31R (17043R-4), and DH44R2 (12370R-2)) were obtained from NIG-Fly Stock center. elav-Gal4, UAS-NaChBac, UAS-TNT-G, UAS-Myr-GFP, UAS-Kir2.1, UAS-CD8-GFP, UAS-Dicer2 (UAS-Dcr2), UAS-Cdk1-myc, UAS-Cdk2-myc, and CyO/Sp;LexAop-spGFP11, UAS-spGFP was obtained from the Bloomington Stock Center. UAS-RNAi lines (Crz (106876 [A], 30670 [B]); CycB (109611 [B], 43772 [C]); Cdk1 (41839 [A], 106130 [B]); Cdk1 (104959); DH44R1 (110708); PDFR (106381); DH44R2 (43313 [B], 102292 [c]); and R22D11-Gal4 is from the Vienna Stock Center. PL-Gal4 contains a Crz promoter fragment (504A311-249) and was a gift from J. Park. Tim(UAS)-Gal4 is a gift from Amita Sehgal. G0431-Gal4 is a gift from Ann-Shyn Chiang.

Sleep assay

1-3 days old flies were tested for sleep at 25°C. Locomotor activity levels were monitored using the Drosophila Activity Monitoring System (Trikinetics). For sleep measurements, activity counts were collected in one minute bins for 7 days in LD (data from the first day was not scored). Sleep was identified as at least 5 minutes of inactivity using a sliding window. All the
sleep parameters were determined using custom MATLAB software. ~30 flies/genotype were
run in each experiment, and data from 2-3 experiments are shown. Day sleep is scored as sleep
during Zeitgeber time (ZT) 0–12, while night sleep is scored as ZT 12–24. Total sleep is scored
as sleep during ZT 0-24. Sleep delay is the time until the first sleep bout after the lights go off at
ZT 12.

Immunohistochemistry and microscopy
Fly heads were fixed in PBST and 4% paraformaldehyde at room temperature for 20 minutes,
rinsed in PBST, and then dissected. After washing with PBST three times (15 minutes each), the
brains were incubated with primary antibodies (diluted in PBST) at 4˚C for 48 hours. After three
PBST washes (15 minutes each), the brains were incubated with secondary antibodies (diluted in
PBST) at 4˚C for 24 hours. Following three washes with PBST (15 minutes each), the brains
were mounted with VectaShield (Vector Labs) on glass slides using standard procedures.
Confocal sections were acquired using an Olympus Fluoview 1000 microscope or an Olympus
Fluoview 12000 microscope at 0.5-3 µm intervals. Maximum intensity projections of image
stacks were obtained in FIJI (Schindelin et al., 2012).

Antibodies
*Primary:* Goat anti-CycA NT antibody was obtained from Santa Cruz (sc-15869, 1:100 dilution).
The Crz (rabbit) antibody was a gift from Jan Veenstra (1:100 dilution). C-Myc (mouse) was
obtained from Developmental Studies Hybridoma Bank (9E10, 1:100 dilution). For GRASP
experiments, mouse anti-GFP clone 3E6 (CAT# A11120, Invitrogen, 1:2,000 dilution) primary
antibody was used. Otherwise we used rabbit anti-GFP (CAT# A-11122, Invitrogen, 1:1,000
String (rabbit) antibodies (1:100 dilution) were a gift from Eric Wieschaus (Di Talia et al., 2013). String (guinea pig) antibodies (1:100 dilution) were a gift from Yukiko Yamashita (Inaba et al., 2011). Twine (rat) antibodies (1:100 dilution) were a gift from Eric Wieschaus (Di Talia et al., 2013). Wee1 (rat) antibodies (1:100 dilution) were a gift from Eric Wieschaus (Di Talia and Wieschaus, 2012).

Secondary: Alexa Fluor 488 donkey anti-rabbit is from Invitrogen (CAT# A21206, 1:400 dilution). Alexa Fluor 488 donkey anti-mouse is from Invitrogen (CAT# A21202, 1:400 dilution). Alexa Fluor 555 donkey anti-goat was from Invitrogen (CAT# A21432, 1:400 dilution). Alexa Flour 488 donkey anti-guinea pig is from Jackson Immuno Research (CAT# 706-545-148, 1:400 dilution). Alexa Fluor 488 donkey anti-rat is from Invitrogen (CAT# A21208, 1:400 dilution).

Courtship assay

Courtship assays were performed and videotaped in cylindrical courtship chambers (10 mm diameter and 3 mm height) at 23°C and ambient humidity. Each male fly (3–6 days old) was paired with a w^{1118} virgin female fly in each chamber. We manually scored the courtship index as the fraction of time during which the male fly was engaged in mating behaviors (courtship and copulation) within 5 min once the male fly started courting. We scored a bout of courtship as initiated when the male oriented toward the female, began tracking her, and unilaterally extended his wing to sing to her. We scored a bout as terminated if the male did not sing in the subsequent 30 seconds or turned away from the female. If the male fly did not court within the first 15 minutes of each assay, the courtship index was scored as 0. This description was adapted from (Zhang et al., 2016).
Startle response assay

We measured average activity (number of beam crossings) per minute for 3 minutes following 2V vibration stimulation. Stimulation was performed during a 10-hour period beginning 1.5 hours after lights went off at ZT 12. Stimulation occurred repeatedly every 40-50 minutes during this period. 10 data points were obtained from each fly.

Climbing assay

About 5 sets of 10 flies were collected for each genotype and put into two empty food vials connected at their open sides (vials were sealed with tape). We conducted the experiment one hour after collection to allow flies to acclimate and recover from anesthesia. Vials were manually tapped to make the flies drop to the bottom. The percent of flies to climb ≥ 8 cm in 10 seconds following the tap were measured for each trial. Trials were repeated 10 times with at least one minute of rest in between trials. Percentages across all 10 trials were averaged to give one value for each set of flies per experiment. Experiments were conducted on two different occasions to provide data from a total of ~10 sets of flies per genotype.

Quantification of PL neuronal morphology

Neuronal morphology was scored blind using maximum intensity projections of confocal stacks taken 1-3 μm apart of PL neurons visualized with PL>CD8-GFP. Morphology was scored as “normal”, no processes extending down the midline, or extra branching.
Statistics

Statistical analysis was performed using GraphPad Prism. Graphs were generated using GraphPad Prism and edited using Adobe Illustrator. For comparisons between three or more groups, One-way ANOVAs with Tukey’s post-tests were used. For comparisons between two groups, Mann-Whitney tests (Figure 3.3, C) or unpaired t tests with Welch’s corrections (Figure 3.5, F) were used. All data are shown as mean ± SEM. Results are as displayed as: ns = not significant, * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, and **** = p ≤ 0.0001.
3.5 Acknowledgements

Stephen Zhang and Michelle Frank wrote the custom MATLAB software used to extract sleep behavior data. Stephen Zhang helped perform the courtship assays. Iris Titos Vivancos helped conduct the startle response experiments. We would like to thank Jan Veenstra, Eric Wieschaus, Amita Sehgal, and Yukiko Yamashita for kindly providing reagents.
3.6 References


Chapter 4

CONCLUSIONS AND FUTURE DIRECTIONS
Nervous system function requires neurons to communicate with each other to integrate, relay, and output information. Neuropeptides are a class of signaling molecules commonly used throughout the nervous system to transfer information between neurons; however, their actions in neuronal circuits and roles in behavior are less understood compared to those of small molecule neurotransmitters. We examined the role of neuropeptides in neural circuits and behavior by performing two complementary studies. First, we investigated the role of putative co-transmission of neurotransmitters and neuropeptides in *Drosophila* olfactory circuit function. Second, we investigated the proteins important for regulating neuronal function as well as the neuropeptides involved in *Drosophila* sleep behavior. Our findings provide insight into how neuropeptides may influence neural circuit function and behavior.

### 4.1 Investigating the functional and behavioral implications for putative PN → ORN co-transmission

We leveraged a recently reconstructed local olfactory circuit to investigate potential circuit effects of neuropeptide signaling (Tobin et al., 2017). We found that all olfactory neurons studied within the DM6 glomerulus contain DCVs in addition to synaptic vesicles. These data suggest that these neurons may express both neuropeptides and neurotransmitters and may use a combination of these signals to communicate with other olfactory neurons. We find that certain synapse types are enriched with DCVs (*e.g.* PN → ORN), suggesting that olfactory neurons may preferentially direct putative neuropeptide signaling to specific postsynaptic partners. We hypothesize that PN peptidergic signaling onto ORNs may support single glomerular channel modulation, as compared to the effects of the more broadly acting LNs. Finally, we find that PN presynaptic sites with at least one ORN postsynaptic partner are significantly clustered on
neuronal branches. We hypothesize that this may facilitate DCV delivery to specific presynaptic sites.

Neuropeptides are housed in DCVs, so we used this anatomically identifiable compartment as a proxy for neuropeptides. Although an extensive body of literature supports this assumption (Nusbaum and Blitz, 2012; Vaaga et al., 2014; van den Pol, 2012), future work is needed to confirm that these olfactory neurons express neuropeptides. In addition, it is possible that these DCVs contain signaling molecules other than neuropeptides, such as monoamines (Li et al., 2005; Liu et al., 1994; Waites et al., 2001; Krantz et al., 2000; Nirenberg et al., Grygoruk et al., 2014). Future work is needed to validate our hypothesis that the DCVs identified in this study contain neuropeptides. Furthermore, it would be informative to know which putative neuropeptides DM6 ORNs, PNs, and LNs express. We are currently using antibodies targeting various neuropeptides to stain brains with subsets of these olfactory neurons labeled using GFP. Colocalization of GFP and neuropeptide antibody staining would suggest that the given neuropeptide is expressed in that set of neurons. Recently, Liqun Luo’s group used single-cell RNA sequencing to profile the transcriptomes of *Drosophila* PNs (Li et al., 2017). Neuropeptide transcripts present in such a dataset should be used as candidates in a screen to identify the putative neuropeptides expressed in DM6 PNs.

We found that PN presynaptic sites with at least one ORN postsynaptic partner are more likely to contain DCVs than expected by chance. To confirm that ORNs receive PN peptidergic signals, it would be informative to test whether ORNs express the receptors of putative PN neuropeptide(s). This could be assayed using antibodies targeting neuropeptide receptors. Expression of these receptors in ORNs would suggest that PNs signal onto ORNs using both neurotransmitters and neuropeptides.
It is important to note that most presynaptic sites are adjacent to multiple postsynaptic profiles in this dataset. ORNs make up a higher percent of total postsynaptic partners when PN presynaptic sites contain at least one DCV than that for all PN presynaptic sites (data not shown). However, these data do not exclude the possibility that PNs may also release DCV contents onto LNs and PNs as well as ORNs. It would be informative to test whether LNs and PNs express the receptors for putative PN neuropeptides. In addition, it would be interesting to look at which LNs receive inputs from PN presynaptic sites with DCVs. LNs are a heterogeneous group of neurons; thus, it is possible that particular LN subtypes are adjacent to PN presynaptic sites with DCVs. Future work is needed to address these questions.

This work led to the hypothesis that peptidergic PN signaling onto ORNs may support single glomerular channel modulation. To address this hypothesis, we would like to test the physiological effects of this signaling. For example, it would be informative to assay ORN activity (using calcium indicators) while blocking putative PN neuropeptide signaling. Signaling can be blocked by knocking down neuropeptide expression in PNs or its receptor in ORNs. If ORN activity is affected by these manipulations it would further suggest that ORNs receive peptidergic inputs from PNs. For example, it is possible that putative PN $\rightarrow$ ORN peptidergic signaling may increase ORN activity and facilitate neurotransmitter release. Conversely, this signaling may cause ORNs to hyperpolarize, which would decrease neurotransmitter release. One can test these possibilities by assaying PN activity using calcium indicators or electrophysiological approaches following neuropeptide-signaling perturbation.

It is currently unclear when PNs would release neuropeptides; thus, we don’t know under which physiological conditions a phenotype would be observed. Peptidergic signaling typically requires higher levels of activity to be released than neurotransmitters alone (Vaaga et al., 2014).
It is possible that PNs may release neuropeptides following high levels of odor stimulation. Thus, it may be useful to perform this experiment in the presence of pentanoic acid, which is detected by DM6 ORNs (Gaudry et al., 2013). Alternatively, it is possible that PNs may release neuropeptides during changes in internal brain state. For example, PNs may release neuropeptides onto ORNs only when the animal is starved. Thus, it would be informative to perform these experiments on animals in various physiological states.

To further test the hypothesis that peptidergic PN signaling onto ORNs affects circuit function, it would be useful to assay its role in behavior. Pentanoic acid is a short-chain fatty acid that triggers a Proboscis Extension Response (PER) in flies (Tauber et al., 2017). Does knockdown of PN \( \rightarrow \) ORN peptidergic signaling affect PER behavior? It would be interesting to test this question when the animal is in different physiological states (e.g. starved vs. fed).

Our data are consistent with PN neurons expressing neuropeptides. Do PNs in other glomeruli express neuropeptides? If so, do they release the same putative neuropeptides? Does this signaling have the same physiological effect? Recently, Jing Wang’s group showed that the effect of peptidergic signaling on ORNs in response to insulin signaling was different in two distinct glomeruli (Ko et al., 2015). Activation of DM1 causes attraction, whereas that for DM5 causes aversive behavior. Interestingly, in the starved state when insulin signaling is low, DM1 ORN sNPF autocrine signaling is increased. This facilitates ORN neurotransmitter release and results in enhanced attractive behavior. Conversely, in the same state, DM5 ORNs increased the expression of the receptor of DTK (DTK is released from LNs), which causes a decrease in ORN neurotransmitter release and lowered aversive behavior. As a result, due to differing responses to peptidergic signaling in two distinct glomeruli, the animal is attracted to odors, even when potentially noxious, in a starved state to presumably facilitate feeding behavior. It will be
informative to test whether putative PN neuropeptide signaling causes distinct or comparable effects in various glomeruli, similar that seen in response to insulin signaling.

Together, this work suggests that co-transmission of neuropeptides and neurotransmitters may be a prevalent phenomenon in primary olfactory circuits. In addition, it provides some of the first concrete evidence that neurons can preferentially direct DCVs to specific presynaptic sites. How PNs are able to achieve this remains an exciting open question to be addressed. Co-transmission enhances circuit flexibility, and it will be interesting to see which additional processes and computations co-transmission supports in olfactory circuits.
4.2 Understanding how Cyclin A may facilitate Corazonin release to promote wake

Our work identified a new network of sleep molecules and an integrating center of wake-promoting signals. We found that knockdown of Cyclin A (CycA) in ~14 wake-promoting neurons in the PL resulted in increased sleep, suggesting that PL CycA promotes wake. We show that the neuropeptide Crz mediates the wake-promoting effect of PL neuronal activation, which is consistent with CycA promoting wake in PL neurons by facilitating the release of Crz. We provide evidence that CycA localizes to presynaptic sites and likely acts with other cell cycle machinery, such as CycB, Cdk1, and Cdk2, to regulate sleep. Finally, we show that PL neurons receive several wake-promoting signals. Together, this work suggests that multiple cell cycle proteins are implicated in sleep regulation and that PL neurons integrate several wake-promoting neuropeptides.

Our data are consistent with CycA promoting the release of Crz. To gain insight into how CycA functions in PL neurons, it would be informative to have a more detailed understanding of where CycA is localized. For example, does CycA localize to DCVs? Immuno-electron microscopy can be used to test this question. If CycA localizes to DCVs then this would suggest that CycA might be directly involved in neuropeptide release. It would also be useful to assay the number of fusing DCVs in wild type and PL>CycA-RNAi brains. This could be tested by activating PL neurons using Chrimson and immediately fixing them by high-pressure freezing, which would enhance the overall number of DCV fusion events. If the number of fusing DCVs is reduced in PL>CycA-RNAi brains when compared with that of wild type then this would suggest that CycA promotes DCV fusion, and therefore Crz release. Since CycA likely acts through direct interaction with Cdk1 and/or Cdk2 to regulate both the cell cycle and sleep, it would be useful to identify Cdk1 and/or Cdk2 phosphorylation targets. These substrates may modulate
synaptic ion channel modulation or DCV fusion. Interestingly, the cell cycle protein Cdk5 has been shown to facilitate synaptic vesicle fusion at mammalian synapses (Verstegen et al., 2014). Identification of Cdk1 and/or Cdk2 targets will provide mechanistic insight into how these proteins promote wake in PL neurons.

Knockdown of Crz while activating PL neurons resulted in a partial suppression of the reduced sleep phenotype observed when driving PL activity with NaChBac. Partial suppression could be due to incomplete knockdown of Crz or PL cells releasing other molecules in addition to Crz to regulate sleep. Although PL cells are also known to release sNPF, a neuropeptide important for sleep regulation (Chen et al., 2013; Kapan et al., 2012), we did not observe any effect on sleep behavior when sNPF was knocked down using RNAi (data not shown). It is possible that PL neurons co-release neuropeptides and neurotransmitters. Ach is one of the most common excitatory neurotransmitters in the fly brain. Future work is needed to test whether PL neurons use a neurotransmitter, such as Ach, in addition to Crz to relay wake signals to other brain regions.

The Crz receptor (CrzR) is expressed in the fat body and heart as well as in the central nervous system (Veenstra, 2009). Preliminary results suggest that PL neurons express CrzR. RNAi-mediated knockdown of CrzR using PL-Gal4 resulted in increased sleep (data not shown), suggesting that Crz may signal onto PL neurons in an autocrine manner. This supports a mechanism whereby Crz-neurons recruit other like cells to promote wake. We are currently working to identify the downstream targets of PL neurons. One could use CrzR-Gal4s or antibodies targeting CrzR to aid in the identification of the neurons downstream of PL cells. CrzR-Gal4 appears to be expressed in s-LNvs; however, we did not observe a defect in sleep behavior when we knocked down CrzR in PDF neurons. These data suggest that Crz does not
signal onto s-LNvS. CrzR-Gal4 also labels neurons in the subesophageal ganglion/tritocerebrum, which is located in the ventral region of the fly brain. It would be informative to identify Gal4s labeling these cells and test whether knockdown of CrzR in these neurons affects sleep behavior. Furthermore, it will be interesting to test whether PL neurons use Crz to signal to downstream neurons and/or an additional transmitter such as Ach.

Crz has been previously implicated in metabolism and stress responses (Kapan et al., 2012; Kubrak et al., 2016; Zhao et al., 2010). These and other data are consistent with the hypothesis that Crz is released during states of stress and metabolic deficits, and may serve as a signal for physiological stress (Boerjan et al., 2010; Veenstra, 2009). The different functional roles of Crz suggest that Crz-expressing PL neurons may be an important intersection between sleep and metabolic systems. We showed that RNAi against receptors of PDF, DH31, or DH44 in PL neurons caused increased sleep. These data suggest that PL neurons integrate wake-promoting signals. PL neurons also express the AST-A receptor (DAR2) (Veenstra, 2009). AST-A neuronal signaling reduces feeding and promotes sleep (Chen et al., 2016; Hergarden et al., 2012). While DH31 and DH44 stimulate the production of cAMP (Cabero et al., 2002), AST-A acts through a G; (Veenstra, 2009); thus, it is reasonable to expect that knockdown of DAR2 in PL neurons would increase their activity and cause decreased sleep. Crz has been recognized as an important molecule involved in relaying metabolic stress information (Kubrak et al., 2016; Veenstra, 2009). Future work is needed to identify all of the outputs of these neurons and how they influence sleep and metabolic pathways.

This work relied heavily on RNAi to reduce gene function. Although RNAi is a powerful approach, RNAi-mediated knockdown of gene expression is rarely complete and varies depending on the level of Gal4 expression. In addition, short hairpin RNAs can have off-target
effects (Ma et al., 2006). To confirm our findings, it would be useful to employ other methods that allow for cell type-specific gene manipulation, such as FlpStop (Fisher et al., 2017). FlpStop uses insertional mutagenesis to create conditional null alleles and reports gene manipulation by labeling targeted cells. For example, if FlpStop-mediated knockdown of CycA produces similar results as those obtained when using RNAi then this would provide further support for the role of CycA in sleep behavior.

We found that the morphology of PL neurons was not obviously disrupted in CycA-knockdown brains, suggesting that the effect of CycA on sleep is due to its post-mitotic functions and not because of developmental defects. To confirm this finding it would be useful to knockout CycA later in development (e.g. at adult stage) when the PL cells are fully developed. To temporally and spatially restrict CycA knockout, one could combine the Split Gal4 and FlpStop approaches (Fisher et al., 2017; Luan et al., 2006). For example, it is possible to create a Split Gal4 using the PL and heat shock promoters (Golic and Lindquist, 1989). Flies carrying the FlpStop cassette in a CycA intron could then undergo a short heat shock as adults, which would result in the knockout of CycA in PL neurons post-development. If these flies display a similar sleep defect as PL>CycA-RNAi flies then this would suggest that the effect of CycA on sleep is due to its post-mitotic functions.

Together, this work advances our knowledge of how neuropeptides influence neuronal circuits and behavior, and has important implications for our understanding of sensory perception and the regulation of internal brain state.
4.3 References


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Appendix A

SUPPLEMENTARY FIGURES FOR CHAPTER 2
FIGURE A.1: Distribution of dense-core vesicles in DM6 ORNs.

Skeletons of all (A) right and (B) left ORNs screened. Positions of DCVs (pink), presynaptic sites (green), and postsynaptic sites (blue) are shown. Approximate outlines of DM6 glomeruli are highlighted with dashed red lines.
Figure A.1: (Continued) Distribution of dense-core vesicles in DM6 ORNs.

B: Left ORNs

ORN 1  ORN 5  ORN 6  ORN 11
ORN 12  ORN 13  ORN 15  ORN 17
ORN 18  ORN 19  ORN 21

ORN 2  ORN 3  ORN 4  ORN 7
ORN 8  ORN 9  ORN 10  ORN 14
ORN 16

DCV  Presynaptic site  Postsynaptic site
Figure A.2: Distribution of dense-core vesicles in DM6 PNs.

Skeletons of all (A) right and (B) left PNs screened. Positions of DCVs (pink), presynaptic sites (green), and postsynaptic sites (blue) are shown. Approximate outlines of DM6 glomeruli are highlighted with dashed red lines.
Figure A.3: Distribution of dense-core vesicles in DM6 LNs.

Skeletons of all LNs screened within DM6. Positions of DCVs (pink), presynaptic sites (green), and postsynaptic sites (blue) are shown. LNs 40 and 79 contain neither DCVs nor presynaptic sites within DM6 processes, but do in processes extending outside of DM6.
FIGURE A.3: (Continued) Distribution of dense-core vesicles in DM6 LNs.
Appendix B

SUPPLEMENTARY FIGURES FOR CHAPTER 3
Figure B.1: PL Cyclin A promotes wake in females.

(A) Knockdown of CycA in using RNAi in PL cells [UAS-Dcr2/UAS-Dcr2; PL-Gal4/UAS-CycA-RNAi (n = 164)] increased the duration of total sleep compared to parental controls [UAS-Dcr2; PL-Gal4 (n = 174), UAS-Dcr2; UAS-CycA-RNAi (n = 101)]. (B) Day sleep and (C) night sleep were increased in UAS-Dcr2/UAS-Dcr2; PL-Gal4/UAS-CycA-RNAi animals compared to controls. (D) Sleep bout length was increased in UAS-Dcr2/UAS-Dcr2; PL-Gal4/UAS-CycA-RNAi flies, but (E) number of sleep bouts was not. (F) Sleep delay was decreased in UAS-Dcr2/UAS-Dcr2; PL-Gal4/UAS-CycA-RNAi animals compared to controls. All data are from female flies.
FIGURE B.1: (Continued) PL Cyclin A promotes wake in females.
FIGURE B.2: Corazonin promotes wake in females.

(A) Knockdown of Crz using RNAi lines caused increased sleep \([UAS-Dcr2/UAS-Crz-RNAi A; UAS-Dcr2/PL-Gal4 \ (n = 105)]\) compared to parental controls \([UAS-Dcr2; PL-Gal4 \ (n = 183), UAS-Crz-RNAi A; UAS-Dcr2 \ (n = 87)]\). We observed increased (C) night but not (B) day sleep in Crz-RNAi animals. All data are from female flies.