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Activation of Egf-r/ERK by Rhomboid signaling regulates the consolidation and maintenance of sleep in *Drosophila*

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The dissertation of Krisztina Foltenyi is approved,

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ABSTRACT OF THE DISSERTATION

Activation of Egf-r/ERK by Rhomboid signaling regulates the consolidation and maintenance of sleep in *Drosophila*

by

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The function of sleep remains a major mystery despite 200 years of research on the subject. Recently, the fruit fly *Drosophila* has been shown to display a behavior that has the essential characteristics of sleep and has thus become a model for the study of sleep's function. The EGFR/ErbB signaling pathway is well conserved in the central

roles it plays in many signaling events during development in worms, flies, and mice. In mammals EGFR/ErbB signaling has also been shown to play a role in circadian regulation of activity. In the current study, its role in the regulation of sleep in *Drosophila* is examined.

The findings reported here show a novel role for Egf-r/ERK signaling in sleep consolidation and maintenance in *Drosophila melanogaster*. In the adult fruit fly, Egf-r is expressed ubiquitously throughout the nervous system and the current analysis revealed that overexpression of Egf-r pathway signaling components Rho and Star causes an acute, reversible and dose-dependent increase in sleep that tightly parallels an increase in phosphorylated ERK in the head and brain. Inhibition of Egf-r with a dominant-negative form of the receptor was able to suppress the increase in sleep levels produced by Rho and Star, demonstrating that the signal is mediated by the Egf-r pathway.

In contrast to the increase in sleep amount after Rho overexpression, inhibiting it lead to a significant decrease in sleep. Importantly, this decrease in sleep was due to a dramatic shortening of the duration of sleep episodes accompanied by an elevation of sleep bout number. This observation suggests a state of increased sleep need, but an inability to maintain the sleep state. Therefore, the function of Rho may be to keep a fly asleep once the state has been initiated.

The inhibition of sleep depends on interfering with *rhomboid* expression in the pars intercerebralis, a part of the fly brain that is functionally analogous to the hypothalamus in vertebrates, which is a region of the mammalian brain well established to be a regulator of arousal. These studies suggest that sleep and its regulation by Egf-r signaling may be ancestral to insects and mammals.

Chapter 1

Introduction to Sleep and the Model Organism *Drosophila melanogaster*

1.1 Definitions and theories of sleep

The modern view of sleep was defined by Henri Pieron in 1913 as having three main characteristics. First, it is a biological necessity, since an animal deprived of sleep will eventually die. Second, it has its own intrinsic homeostatic rhythm, independent of other circadian ques. Third, it is characterized by an absence of motor functioning, and a decrease in responsiveness to environmental stimuli (Pieron, 1913). Other characteristics of sleep include a species-specific posture combined in many instances with a favored resting place such as a nest or burrow, and finally, the sleep state should be distinguishable from coma or injury by being rapidly reversible with proper stimuli (Campbell and Tobler, 1984).

Many theories exist on why we sleep. The simplest of these is a basic survival argument that proposes that sleep evolved as a mechanism of protection from predation or other dangers during diurnal lighting or temperature conditions that an organism is poorly adapted for (Allison and Cicchetti, 1976). Although there is no denying that sleep can prevent an animal from being active at inappropriate times, it would nonetheless seem that being unaware of the environment is at least as risky as running around in suboptimal conditions, so this theory is at best paradoxical. Actually, the evolution of the circadian rhythm would be sufficient to explain this view of sleep, since then an animal could solve the problem by staying put, but still aware of its surroundings. Better yet, one of the most convincing observations that this basic theory of sleep is insufficient to

explain the phenomenon is something researchers could have simply observed on themselves: that a missed night's sleep produces a sleep debt that needs to be made up even when external conditions are such that would normally favor activities that, in the present state of our understanding, have a much clearer survival value than sleep (Siegel, 2005).

Another hypothesis is that sleep is a form of restoration or replenishment. Although this seems to be intuitively true, nobody has found exactly what is being restored or replenished. For example, there is more oxygen consumed during REM sleep than while awake. There is also not as much energy conservation taking place during sleep as once thought, in fact it is only a 15% savings, something that could be easily made up with just a little bit of food. Besides, an animal that is awake can also conserve energy. There is, however, evidence that sleep deprivation in the rat correlates with increased oxidative stress and membrane disruption in some parts of the brain, but mostly in the hypothalamus, a part of the brain that has the highest rate of protein synthesis and presumably the highest generation rate of reactive oxygen species (Siegel, 2005). So the restorative effect of sleep is definitely needed, although it might not have been the primal cause for the evolution of the behavior.

Another famous but equally controversial theory is that sleep is necessary for memory consolidation, and thus learning. But although sleep deprivation has obvious effects on lucidity and task performance, an essential role for sleep in memory consolidation remains unproven. For example, humans on antidepressants or with brainstem lesion-induced suppression of REM sleep do not show any memory deficits, and further studies also failed to demonstrate any correlation between REM sleep time

and learning ability in humans or other species (Siegel, 2001; Vertes, 2004). In many of these studies, when non-REM sleep deprivation was used as control, there was a similar failure to produce deficits in cognition. Therefore, it is equally unlikely that this form of sleep has a role in memory consolidation (Siegel, 2001).

In sum, there really is no satisfactory theory yet on why we sleep. The observations that come the closest are that overall, sleep does save energy and reverses changes in brain function induced by waking activities (Siegel, 2005).

1.2 Misconceptions about sleep

The most common misconception about sleep is that it is a passive state, or that it is synonymous with being unconscious. Neuroscience began with the premise that sleep is like switching off a machine, as a way to spend time in between necessary waking activities. The first person to propose that sleep was an active state with its own set of laws is the neuroscientist and psychologist Sigmund Freud. Since then it has been clearly demonstrated that sleep is a highly organized, deliberate process during which the brain remains active, and body functions such as anabolism, growth and immune processes are at higher levels than in waking. In mammals, sleep is marked by brainwave patterns unlike any seen during the waking state, distinguished by their low frequencies and high synchronicity (in fact, brainwaves produced during sleep are the longest electromagnetic waves known in nature, with one wavelength having the distance from the Earth to the Moon, or even further). But the brain state that most clearly demonstrates that sleep is an active process is during the rapid eye movement (REM) phase, a time when the conjuring

of dreams makes for a brain that actually exhibits a higher amount of activity then when the dreamer is awake.

Thus, even though there is an external appearance of sleep being a restful state, and it probably is for some organs and muscles, on a cognitive level it is a highly active process that is most certainly the key to the true need for sleep. All other functions that have also been implicated as reasons for sleep such as immunity, temperature regulation and dietary metabolism co-evolved to take advantage of the sleep state, but probably could have functioned just as well without it. Even such proposals as sleep being needed for the cells of the brain to recover from oxidative stress, to repair and replace essential cellular components and to deal with the bi-products of waking metabolic activity (Siegel, 2005) seem overly simplistic and limited by our own conscious abilities, because there is no reason why a cell would not be powerful enough to do those things simultaneously, just like digestion of the previous meal doesn't stop while eating another one.

The above mentioned functions probably go awry from sleep deprivation simply because they are so interrelated with sleep after millions of years of co-evolution, that they can give the illusion of being the very reason for sleep. In other words, just because sleep deprivation affects a certain function, it doesn't mean that the function explains sleep. The bottom line is that after a century of research, sleep is still a mystery, and for this reason we turned to the humble, but genetically powerful *Drosophila* to help decipher how sleep is regulated, and hopefully in the future help researchers shed light on why is it that we sleep.

1.3 Sleep in *Drosophila*

Sleep is universal to all mammals, although the amount and position of sleep vary greatly between different species. For example, humans sleep lying down, elephants stand up, and the hippopotamus sleeps under water. Cows stand up even during REM sleep, and can keep their eyes open while sleeping. Dolphins continue swimming since only half of their brain sleeps at a time. Elephants sleep only 3.3 hours, humans 7 hours, rats 13.2 hours (Campbell and Tobler, 1984). This demonstrates that even though nature has gone to great lengths to restructure the expression of sleep to fit the particular life of an animal, losing it altogether is not an option.

How far back in evolution can we go and still find sleep? REM sleep is only known to exist in birds and mammals, but it is difficult to determine if their common ancestors such as reptiles or amphibians also have it due to the anatomy of their brain. Nonetheless, these animals do sleep, as do fish and even the cockroach (Campbell and Tobler, 1984). In fact, for the first time slow waves in the brains of sleeping crayfish have been recorded, demonstrating that the superficial appearance of sleep behavior in an invertebrate actually correlates with similar changes in brain activity observable in mammals (Ramon et al., 2004). Recently, driven mostly by a practical curiosity, researchers that included John Newport took a closer look at whether other members of the *Arthropoda* phylum also sleep by analyzing the behavior of the fruit fly *Drosophila melanogaster*. They found that these creatures exhibited the same canonical behavioral hallmarks of sleep as previously defined in mammals.

Whether under light/dark conditions or constant darkness, *Drosophila* on a daily basis showed extended durations of immobility lasting up to 10 hours, during which time they had a preferred resting location close to their source of food, with a relaxed posture conveyed by lowering their abdomens to the floor (Figure 1.1) (Hendricks et al., 2000; Shaw et al., 2000).

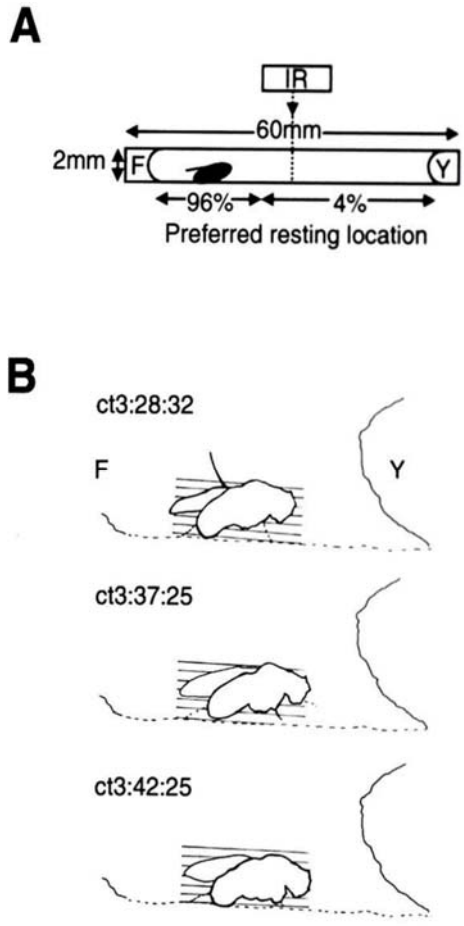

Figure 1.1 *Drosophila* prefers to sleep near food, and has a preferred sleep posture (A)Depicts the dimensions of the tubes used for activity assays, and the location *Drosophila* prefers for sleeping near the food. (B) Time lapse video showing a fly lowering itself to the floor for sleeping. This figure was adapted from (Hendricks et al., 2000)

Furthermore, we observed that the circadian mutant, *timeless*, also continued to rest in consolidated segments in constant darkness, but under a rhythm that might be revealing the sleep homeostat independent from the consolidating effects of the circadian clock (compare Figure1.2A with 1.2B). This observation further supported that *Drosophila* has a deeper drive towards periods of inactivity other than a simple circadian command to restrict movement during hours of darkness.

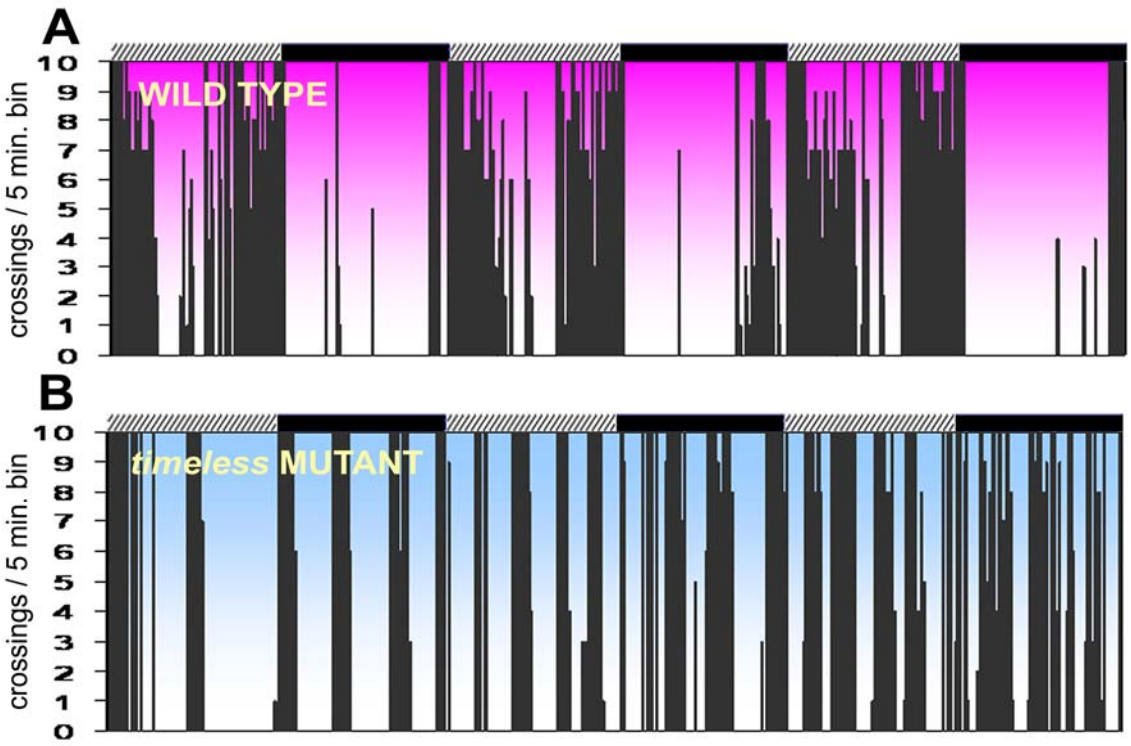

Figure 1.2 Wild-type and circadian mutant flies both sleep in constant darkness
Representative actograms for three days of a single fly from labeled genotype. Flies were entrained to a 12 hour light:dark cycle, and then placed into constant darkness for monitoring. Hatched bars above actograms represent subjective light, black bars subjective dark conditions

This state of rest started looking more and more like sleep with the discovery that these periods of immobility were accompanied by an increased arousal threshold, demonstrating a disconnection from the environment characteristic of mammalian sleep. Furthermore, when the flies were deprived of sleep there was a homeostatic sleep pressure created to make up the lost sleep, and this was independent of the possible stress created from the deprivation technique itself (Hendricks et al., 2000; Shaw et al., 2000). When flies were continuously deprived of sleep without a chance to make up for it, they eventually died. Another interesting discovery was that young flies that are just a few days old sleep a lot more than older flies, and older flies have more fragmented sleep. This age-dependent decline in sleep levels is also the ontological touchstone of mammalian sleep (Koh et al., 2006).

To determine whether neuronal or neurotransmitter mechanisms are also conserved between flies and mammal, researchers tested whether *Drosophila* responds to the same pharmacological agents known to modulate arousal in mammals. Sleep levels decreased with agents such as caffeine, and metamphetamine, and increased with anti-histamines (Andretic et al., 2005; Hendricks et al., 2000; Huber et al., 2004; Shaw et al., 2000). The study of specific neuromodulatory neurotransmitter systems such as dopamine (Andretic et al., 2005; Kume et al., 2005) and serotonin (Joiner et al., 2006; Yuan et al., 2006; Pitman et al., 2006) also showed strong parallels with what is known about these systems in mammals (Aszalos, 2006; Boutrel and Koob, 2004; Dzirasa et al., 2006; Espana and Scammell, 2004), with the two systems seemingly playing a balancing act between the states of arousal versus quiescence.

More recently, it was shown that gene transcription in *Drosophila* is modified by behavioral states, with genes related to different functional categories expressed during waking and sleeping (Cirelli et al., 2005; Zimmerman et al., 2006), as had previously been shown in rats (Cirelli and Tononi, 2000). The only signaling pathway studied thus far in Drosophila sleep is the 3',5'-monophosphate (cAMP)/CREB, and it was found to play a possible role in the restorative function of sleep, since the flies needed more sleep if the pathway's activity was below normal (Ganguly-Fitzgerald et al., 2006; Hendricks et al., 2001).

As a final correlation between mammalian and Drosophila sleep, researchers discovered that if you insert electrodes into the middle of the fly brain, the electrical activity in the brain changes when flies sleep (Nitz et al., 2002). In sum, sleep seems to be an ancestral state conserved between Arthropods and mammals, and with its relative simplicity *Drosophila* seems promising to help unlock the mystery of why sleep evolved.

1.4 The role of Egf-r and ERK in sleep in mammals

It has been shown that the artificial stimulation of RTKs by the application of ligands to the brain has the capacity to increase sleep levels in mammals. These include the insulin receptor (Obal et al., 1998), tropomyosin-related kinase (trkA and trkC) receptors (Yamuy et al., 2005), and the epidermal growth factor receptor (Egf-r), a member of the ErbB family of RTKs (Kushikata et al., 1998). In mammals, EGF has been isolated from the brain (Schaudies et al., 1989), and its receptors are widely distributed throughout the central nervous system (Wiedermann et al., 1988). The brain-

wide activation of EGFR through the administration of EGF to the brain of rabbits was shown to first cause an increase in non-rapid eye movement (non-REM) sleep, followed by an increase in REM sleep as well at higher doses of EGF (Kushikata et al., 1998). Nevertheless, whether EGF normally plays a role in sleep regulation was not addressed by this study.

A later study further demonstrated that the Egf-r ligand, TGF-α, is a rhythmically transcribed and secreted neuropeptide by the suprachiasmic nucleus (SCN), the circadian control center of the mammalian brain. The researchers showed that active behaviors were altered and sleep timing became irregular by aberrant Egf-r signaling. This was achieved by eliminating the rhytmicity of SCN TGF-α secretion, or with the use of a hypomorphic *Egf-r* allele (Kramer et al., 2001). These studies demonstrated that the ectopic activation of the ErbB family of RTKs can affect the overall levels and timing of when an animal sleeps, but did not demonstrate that the ErbB family of RTKs plays a direct role in endogenous sleep.

The downstream effector or Egf-r signaling is ERK, although at least 20 other neuromodulatory transmitters and peptides besides Egf-r ligands are also known to regulate ERK activation. Nonetheless, the role of ERK in sleep has never been investigated, although there is one study that showed that levels of phosphorylated ERK decrease in the rat hippocampus after sleep deprivation (Guan et al., 2004). The hippocampus is a region of the brain required for the processing of memories (Born et al., 2006), therefore this result could possibly connect ERK to a role in sleep through ERK's functions in synaptic changes associated with memory consolidation, and the state of sleep could make these processes more efficient. This possible effect of sleep on ERK

activation is not far-fetched, since outside the context of sleep, ERK has been demonstrated to play crucial roles in synaptic plasticity involved in development, learning and memory within many model systems, including *Drosophila* (Hoeffer et al., 2003; Sanyal et al., 2002; Sweatt, 2004; Thomas and Huganir, 2004).

1.5 The Egf-r pathway and how we used it to study sleep in the fruit fly

The ErbB family of RTKs in mammals consists of four members that can form either homodimers or heterodimers. In vertebrates, these ligands bind to specific family members, with ErbB-1 (EGFR) binding EGF and TGF-α, while ERbB-(3) and (4) bind the neuregulins. In contrast, the *Drosophila* ErbB family has only one member, Egf-r (EGFR or ErbB-1 in mammals). The *Drosophila* Egf-r pathway has been extensively characterized in developmental contexts, with four known activating ligands. One of them, Vein, is considered to be a neuregulin homologue, and is produced in a soluble form that does not need any further processing. However, the other three (Spitz, Gurken and Keren), all TGF-α homologues, are produced as membrane-bound precursors that need further processing by the proteins Star and one member of the Rhomboid family (Urban et al., 2002). Star is a trans-membrane cargo receptor believed to translocate uncleaved ligand from the endoplasmic reticulum (ER) to the Golgi. In the Golgi, a member of the serine protease family of Rhomboids (Rho) cleaves the membrane-bound ligand into its soluble form, reviewed in (Shilo, 2003; Shilo, 2005), and summarized in Table 1.1.

Table 1.1 Core elements in Egf-r activation. Adopted from (Shilo, 2005)

	Species and function	
Element	***Drosophila***	**Human/Mouse**
EGF receptor	**EGFR**	**EGFR**
		ERBB2 (does not bind ligand)
		ERBB3 (has an inactive kinase domain)
		ERBB4
EGF ligands	**Spitz (acts in most EGF signaling situations)**	**EGF (activates EGFR)**
	Keren (function unknown)	**TGFα (activates EGFR)**
	Gurken (functions in oogenesis)	**Heparin-binding EGF (activates EGFR)**
	Vein (functions in a restricted manner)	**Amphiregulin (activates EGFR)**
		Betacellulin (activates EGFR)
		Epiregulin (activates EGFR)
		Epigen (activates EGFR)
		Neuregulin 1 (activates ERBB3 and ERBB4)
		Neuregulin 2 (activates ERBB3 and ERBB4)
		Neuregulin 3 (activates ERBB3 and ERBB4)
		Neuregulin 4 (activates ERBB3 and ERBB4)
Ligand processors	**Rhomboid 1 (commonly cleaves Spitz)**	**ADAM17/TACE (cleaves TGFα)**
	Rhomboid 2/BRHO/STET (cleaves Spitz in male and female germline)	
	Rhomboid 3/Roughoid (functions predominantly in eye development)	
	Star (traffics ligand precursors from the ER)	

Overproduction of Rho has been demonstrated to result in ectopic secretion of activated ligand, leading to a potent stimulation of Egf-r signaling. We used Rhomboid-1 alone and in combination with the upstream carrier Star to address the question of whether Egf-r signaling is directly involved in sleep in the fruit fly.

We found that triggering the Egf-r pathway induces excessive sleep in *Drosophila*, and that this behavioral change correlates with the activation of extracellular signal regulated kinase (ERK), a well-established downstream effector of Egf-r signaling.

Inhibiting the pathway by cell-specific inhibition of Rho expression had the opposite effect of decreasing sleep levels and disrupting sleep consolidation. Finally, we demonstrate that this decrease in sleep is dependent on the inhibition of Rho in the pars intercerebralis, a part of the fly brain believed to be functionally analogous to the hypothalamus and pituitary gland in mammals.

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Chapter 2

Overproduction of Egf-r ligand increases sleep levels and phosphorylated ERK in fly heads

2.1 Overproduction of secreted Egf-r ligand increases sleep levels

To test the role of neural Egf-r as one of the pathways regulating aspects of sleep and arousal, we asked whether the activation of the Egf-r pathway could modulate sleep levels in *Drosophila*. For this purpose, I made use of the binary UAS/GAL4 expression system (Brand and Perrimon, 1993) to express upstream Egf-r pathway components known to activate the receptor. I found that heat shock (*hs*-Gal4) induction of Rhomboid-1 (Rho) and Star resulted in an increase in sleep levels throughout the circadian cycle compared to baseline (Figure 2.1A,C,D). A typical experimental course for *hs*-Gal4>*rho,Star* flies is shown in Figure 1A. Exposure of 4-5 day old female flies to 60' heat shock at 37ºC lead to significantly higher sleep levels for the following two days. The effect on sleep was most pronounced during the light period immediately following Rho and Star expression, a time when flies are normally highly active (compare before and after heat shock in Figures 2.1C and 2.1D). By the third night after heat shock, sleep levels had dropped to below normal and recovery from this compensatory decrease in sleep required another 3-4 days. Thus, ectopic activation of Egf-r by elevated ligand production increases periods of inactivity, demonstrating that the receptor can affect behavior in adult *Drosophila*.

I found that the increase in sleep was dose-dependent, since expressing Rho and Star at higher levels with two copies of the heat shock driver further suppressed locomotor activity, resulting in flies that on average spent more than 50 min of each hour asleep (Figure 2.1D). In fact, with this genotype there was enough uninduced leakage from the heat shock promoter to cause a gradual increase in sleep during the course of

several days even at ambient temperatures (Figure 2.2). Rho alone was sufficient to cause an increase in sleep (Figure 2.1B), but to a lesser extent than when co-expressed with Star. Although Star is an indispensable component of Rho mediated Egf-r signaling, it has been shown by others that Rho levels are normally limiting for this pathway (Guichard et al., 1999; Sturtevant et al., 1993). Ectopic misexpression of Star has been demonstrated to have at most a marginal effect on Egf-r activation, and therefore cannot activate Egf-r signaling on its own (Urban et al., 2002). In sum, activation of Egf-r signaling increases sleep in a dose-dependent manner ranging from the smallest effect by Rho alone, to the strongest with Rho and Star driven by two copies of *hs*-Gal4.

As a final test within this group of experiments, I also tested the effect of overexpressing a soluble form of the Egf-r ligand Spitz (s-Spitz) that does not require processing by Rho. Driving this highly potent ligand also caused an increase in sleep levels (Figure 2.1E), although most of these flies did not survive for longer than 3-4 days after heat shock. Upon death they had grossly inflated abdomens, indicating that the systemic activation of Egf-r signaling in a Rho and Star independent fashion is too severe for the animals. In fact, this cross was difficult to begin with, having to be done at 18ºC, and even so only few survivors hatched out of each vial. The overexpression of s-Spitz in throughout the *Drosophila* body with the heat shock method can potentially turn normally non Egf-r signaling cells into activators of the receptor. This is not the case when Rho and Star are overexpressed, since then Egf-r signaling can only be enhanced by cells in which unprocessed ligand is normally present. But the data is included to demonstrate that s-Spitz does have the same effect on sleep as the much gentler way of activating Egf-r through Rho and Star.

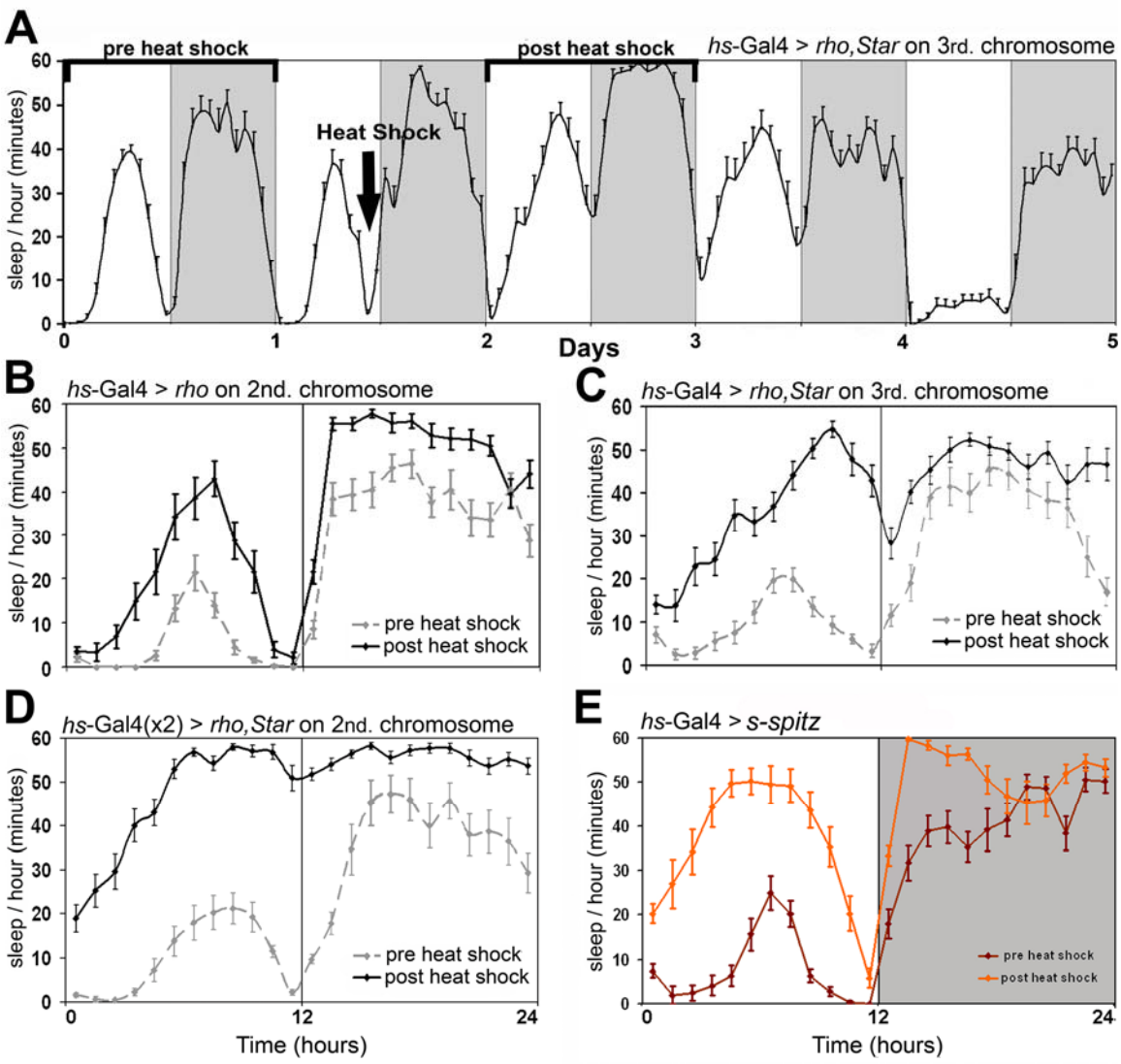

Figure 2.1 Overexpression of Egf-r ligand increases sleep in a dose-dependent manner. (A) Five consecutive days showing sleep levels for *hs*-Gal4>*rho,Star* flies beginning from the second full day after loading 2 day old flies on monitors. Shading represents lights off. First bracket above day 1 depicts baseline sleep, the second bracket above day 3 post heat shock sleep. The second day was considered a time of manipulation and recovery, and was not included in any of the further analyses in these experiments. Arrow denotes a 60 min, 37ºC heat shock. Behavior was assayed at 23ºC. Each data point is the group average for that hour, bars represent ±SEM. (B-E) Average activity traces of the effects of Rho with Star on sleep levels (B-D), and a secreted Egf-r ligand, s-Spitz (E). (B) hs-Gal4>*rho* (n=20) (C) A different *hs*-Gal4>*rho,Star* experiment as that in (A) (*n=20*). (D) *hs*-Gal4(×2)>*rho,Star* (*n=22*). (E) *hs*-Gal4>*s-spitz* (n=21)

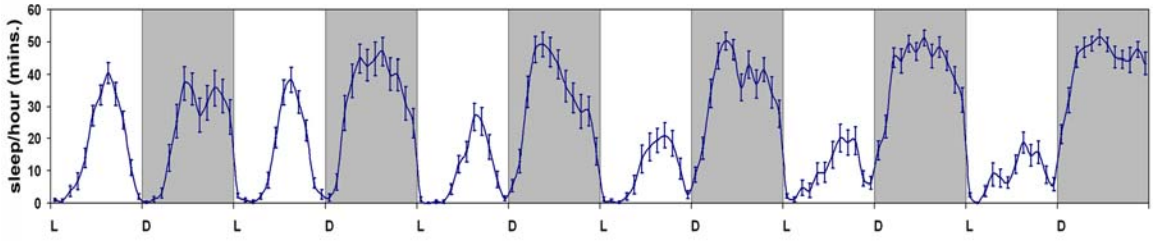

Figure 2.2 *hs*-Gal4(x2)>*rho,Star* flies gradually increase their sleep levels without heat shock.
Group average ±SEM. L=Lights on; D,shading=lights off

The most dramatic difference among the groups assayed in the Rho and Star overexpression experiments is in their increase in daytime sleep, since an increase in nighttime sleep is harder to detect due to its intrinsically higher levels. The high daytime sleep levels were generally due to the combined effect of increased sleep bout number (Figure 2.3C) and bout duration, especially in the maximum length of sleep bouts during the day (Table 2.1). This observation suggests that Egf-r signaling has a somnolent effect on the animals, manifested in increased attempts to sleep rather than being active, and maintaining the sleep state for much longer times without interruption. As expected from the large increase in total sleep levels, the group where Rho and Star were overexpressed by two heat shock drivers (*hs*-Gal4(×2)>*rho,Star*) exhibited the strongest overall alteration in sleep, with nearly a 4-fold rise in sleep bout duration and an 8-fold increase in maximum sleep bout length relative to controls ($p \leq 0.0001$ Wilcoxon/Kruskal-Wallis tests, Table 2.1). In fact, sleep bout lengths became so long for this group, that their sleep bout number actually declined compared to the experimental groups. The change in sleep patterns for this group was most poignantly demonstrated by flies that slept through 8

Table 2.1 Change in sleep parameters and counts per minute due to heat shock expression of Egf-r pathway signaling components

HS-Gal4 expression of Egf-r activators and controls [a] (minutes)																																
sleep parameter		total day sleep			total night sleep			bout number day			bout number night			bout duration day				bout duration night				max bout dur. day				max bout dur. night				counts per minute		
genotype	N	ave	±SEM	[b]	ave	±SEM	[b]	ave	±SEM	[b]	ave	±SEM	[b]	med	10%	90%	[c]	med	10%	90%	[c]	med	10%	90%	[c]	med	10%	90%	[c]	ave	±SEM	[b]
hs-Gal4 x w^{1118}	80	59	8.2	B	29	12.6	A	-1.2	0.54	A	1.6	0.8	AB	9.8	-1.6	44.1	A	-4.6	-100	108	A	30	-5	155	A	-23	-224	329	A	-0.23	0.06	AB
hs-Gal4>*rho II*	46	173	18.2	C	142	14	B	2.4	0.69	BC	-3.9	0.6	C	18	-1	99.2	A	71.4	-11	531	B	70	-3	229	B	238	5	482	B	-0.11	0.06	A
hs-Gal4>*rho, Star* on III	62	254	14.3	D	189	16	B	4.8	0.88	C	3.4	0.9	AB	14	-2	36.2	A	1.5	-61	98.7	A	88	11.5	188	B	37.5	-187	344	A	-0.54	0.04	D
hs-Gal4 (x2)>*rho, Star on II*	47	394	17.6	E	193	24	B	2.8	0.81	BC	4.8	0.9	A	36	-19	84.2	C	-13.9	-141	147	A	240	75	433	C	-15	-212	315	A	-0.47	0.1	CD
hs-Gal4>rho^{H281Y}	55	62	14.8	B	15	16	A	0.65	0.54	AB	2.1	1	AB	8.5	-4.3	56	B	-8.9	-161	131	A	25	-12	177	A	-50	-399	278	A	-0.37	0.05	BCD
w^{1118} x *rho,Star on III*	48	-0.1	9.3	A	5	16	A	0.71	0.67	AB	-0.33	0.8	BC	-0.1	-9.8	9.5	D	-0.76	-23	50	A	0	-106	86	D	-10	-71	174	A	-0.26	0.04	ABC
w^{1118} x rho^{H281Y}	60	36	6.3	AB	-5	12	A	1.4	0.39	B	0.38	0.8	B	3.8	-8.8	15.7	B	-6.1	-150	109	A	5	-15	40	A	0	-188	174	A	-0.29	0.04	ABC

Hs-Gal4 expression of Egf-r inhibibitors and their controls [a] (minutes)																																
sleep parameter		total day sleep			total night sleep			bout number day			bout number night			bout duration day				bout duration night				max bout dur. day				max bout dur. night				counts per minute		
genotype	N	ave	±SEM	[b]	ave	±SEM	[b]	ave	±SEM	[b]	ave	±SEM	[b]	med	10%	90%	[c]	med	10%	90%	[c]	med	10%	90%	[c]	med	10%	90%	[c]	ave	±SEM	[b]
hs-Gal4 x w^{1118}	80	59	8.2	A	29	12.6	A	-1.2	0.54	A	1.6	0.8	ABC	9.8	-1.6	44.1	A	-4.6	-100	108	A	30	-5	155	A	-23	-224	329	A	-0.23	0.06	BCD
hs-Gal4>*rho,S,Egf-rDN(x2)*	43	55	22	A	-91.6	24	B	0.42	1	AB	5.2	1.6	A	4	-7.3	31.9	B	-26	-133	18.8	A	15	-60	122	A	-80	-488	152	A	-0.18	0.05	CD
hs-Gal4>*Egf-rDN(x2)*	26	87	19.2	A	8	12	A	1.2	0.91	AB	1.15	1	ABC	6.2	-27	56.5	B	-31.2	-526	327	A	40	-145	173	A	-95	-418	316	A	-0.27	0.07	ABCD
w^{1118} x *Egf-rDN(x2)*	46	68	12.1	A	-11	15	A	0.09	0.59	AB	-0.87	0.9	BC	5.1	-8.6	34.1	B	0.1	-85	154	A	15	-38	185	A	-23	-312	312	A	-0.06	0.05	D

[a] Each data point is the difference in sleep between heat shocked flies compared to their pre-heat shock levels, so as to remove the contribution of the differences in pre heat-shock baseline sleep between the genotypes

[b] Letters next to each genotype represent statistical category compared with the other genotypes in that category.

All parametric (normally distributed) data represented as average ±SEM, statistical significance was determined using Tukey-Kramer HSD test $p \leq 0.05$.

Non-parametric data represented as median with the 10% and 90 % quantiles. Statistical significance was determined using Wilcoxon/Kruskal-Wallis test for independent groups/pairs. For the first set, α' was set to 0.0024 with the Bonferroni adjustment for 21 comparisons ($p \leq 0.05$)
For the second set, the Bonferroni adjustment was set at 0.0083 for 6 comparisons.

uninterrupted hours during the light cycle from late morning through the active period just before lights off.

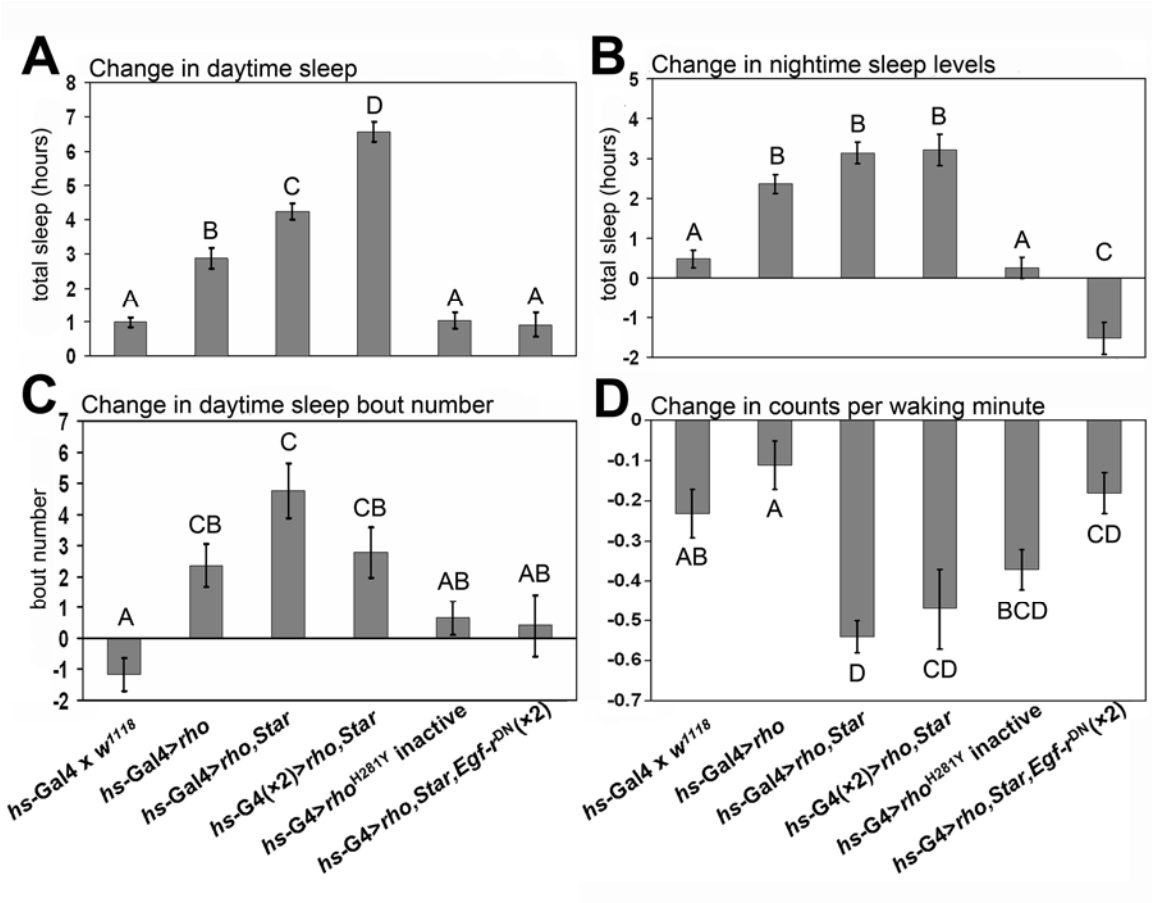

Figure 2.3 Rho and Star overexpression increases sleep without decreasing waking activity (A,B) Histograms showing the change in total average daytime (A) and nighttime (B) sleep for each genotype (labeled below C and D). To remove the differences of the different genetic background in baseline sleep, the pre heat shock sleep level of each genotype was subtracted from its sleep level after heat shock so that only the differences due to the heat shock remained. Letters above bars represent statistically significant groups as determined by Tukey-Kramer HSD test for normally distributed data ($p<0.05$), groups not sharing any letters are statistically distinguishable. (C) Change in daytime sleep bout number. (D) Change in counts per waking minute after heat shock. Note that statistical groups A,B,C and D are all represented by at lest one of the conrols (*hs*-Gal4 × w^{1118} and *hs*-Gal4 > rho^{H281Y} in the panel, others are in Table 2.1)

2.2 Rho and Star overexpressing flies are normal in their response to the environment and locomotor activity.

To evaluate that the Rho and Star overexpressing flies (*hs*-Gal4(×2)>*rho,Star*) were not just sick or paralyzed, I measured the percentage of flies that responded to the change in lighting conditions following the first day after heat shock. I found that 91% of the flies that had spent most of that afternoon asleep were startled into activity by crossing the infrared monitor beam at least once when the lights were shut off (Figure 2.4). The same reaction was observed at the end of the night, when lights went from off to on. Normal flies also show a consistent, immediate response to lights-off or lights-on with a burst of locomotor activity, even if the lights go on in the middle of the night when they are sleeping. This result demonstrates the rapid reversibility of this state, one of the identifying characteristics of sleep discussed in the introduction (section 1.1). If these flies had been comatose or otherwise debilitated, they would not have responded to the stimulus. It is also interesting to note in Figure 2.4 the lack of any anticipation of either lights off or on during the first 24 hours of the actogram, demonstrating that Egf-r signaling is powerful enough to suppress any input from the circadian for heightened levels of activity at dusk or dawn (compare to the control in Figure 2.4), although the circadian phase itself is not shifter *per se* (discussed later).

To further verify that all experimental groups of flies were also normal during periods of activity, I calculated the change in counts per waking minute and determined that even though all genotypes had a transient decrease in their counts after heat shock, no experimental group differed statistically from all of the controls ($p>0.05$, Tukey-

Kramer HSD Test, Figure 2.3, Table 2.1). These latter controls affirm that the activation of Rho/Egf-r signaling in the adult fly causes a dose-dependent increase in sleep levels and sleep consolidation without any detectable adverse effects on locomotion or responsiveness to environmental changes.

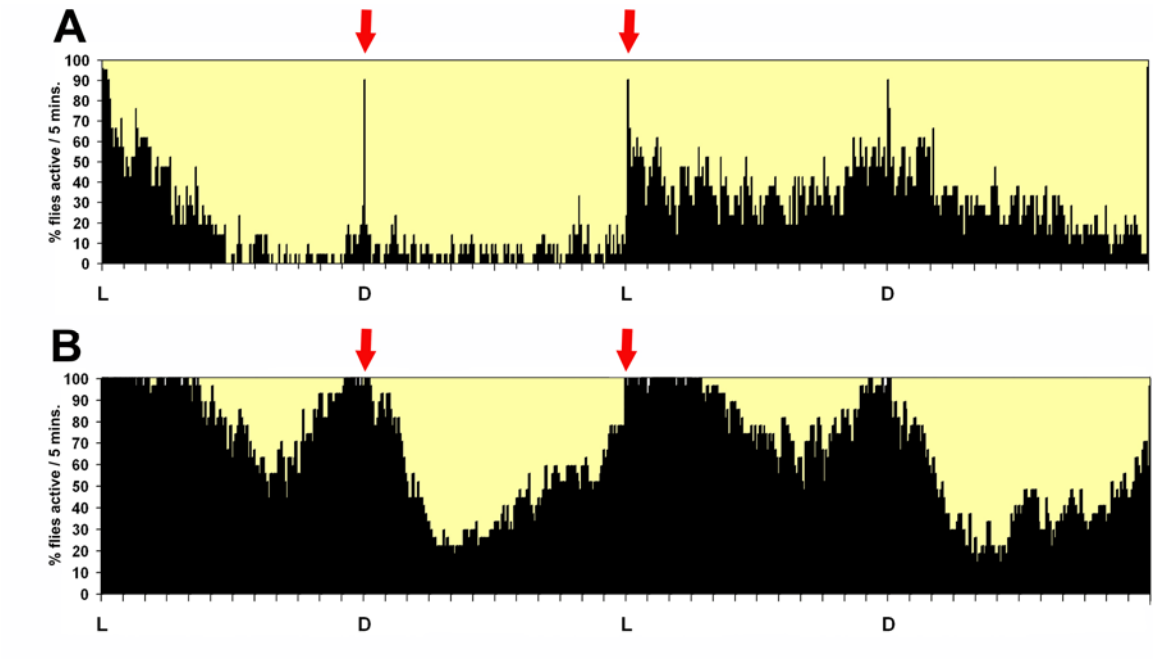

Figure 2.4. Flies overexpressing Rho and Star react to change in lighting conditions
(A) Same *hs*-Gal4(×2)>*rho,Star* flies as in Figure 1C are shown for the first two whole days after heat shock. Data is showing the % of flies active within a single 5 min. collection bin. Red arrow marks the point of lights off and on after a day and night most of the flies spent in inactivity, demonstrating that they did react to this environmental change. (B) Their untreated control is presented for comparing activity levels. The immediate effect of lights-off is obscured for this group due to high activity before and after such transitions.

2.3 Increased sleep requires functional Rho and Egf-r signaling

To further support that activation of Egf-r is responsible for the sleep increase, I co-expressed Rho and Star along with a dominant negative form of Egf-r (Egf-r^{DN}). This form of the receptor is still capable of binding ligand and dimerizing with the wild-type receptor, but since it is missing the catalytic cytoplasmic domain, blocks the Egf-r signal from propagating into the interior of the cell (Freeman, 1996). The increase in sleep due to Rho and Star could be completely suppressed by Egf-r^{DN} (Figure 2.3A), indicating that the effect is mediated by Egf-r. In fact, the total nighttime sleep actually shows a significant decrease in this group (Figure 2.3B, $p<0.05$, Tukey-Kramer HSD test), an effect not achievable with the overexpression of Egf-r^{DN} on it own (Table 2.1, discussed later). Since Egf-r^{DN} acts downstream of Rho and Star, it does not influence the total level of initial overexpression of these two processing proteins. Thus, the successful suppression of Rho and Star induced sleep with Egf-r^{DN} also signifies that this must be due to the functional activities or Rho and Star, and not simply a by-product of their high levels.

To further ensure that the increase in sleep is due to the catalytic activity of Rho and not to a toxic side-effect such as induced Golgi fragmentation (Lee et al., 2001), I tested a point mutant of the protease in which a histidine residue is exchanged for tyrosine in the catalytic domain of the protein, rendering it unable to cleave ligand (Urban et al., 2001). Overexpression of Rho^{H281Y} using heat shock failed to increase sleep levels (Figures 2.3A,B and Figure 2.5), most clearly demonstrated by the lack of activity suppression during evening hours (with a slight increase in daytime sleep likely to be a

residual effect of the heat shock itself, as also seen in the *hs*-Gal4×w^{1118} control, Figure 2.3A). I confirmed that the UAS-rho^{H281Y} construct was expressed by analyzing extracts from *hs*-Gal4>rho^{H281Y} flies on Western Blots (Figure 2.5 inset) with a Rho antibody. Since studies in development show that misexpression of wild-type Rho in wings by driver MS1096 causes an almost 100% conversion of the wing membrane to vein tissue due to ectopic release of secreted Egf-r ligand (Figure 3.1), I also tested the mutant Rho construct in the same setting. I found that the wings of MS1096-Gal4>rho^{H281Y} flies were indistinguishable from MS1096-Gal4 alone, thus confirming that this mutant form of Rho is inactive in vivo, but does not interfere with normal Rho functioning. These last set of results demonstrate that both Rho and Egf-r have to be functional to achieve the increased sleep levels observed with the ectopic activation of Egf-r signaling.

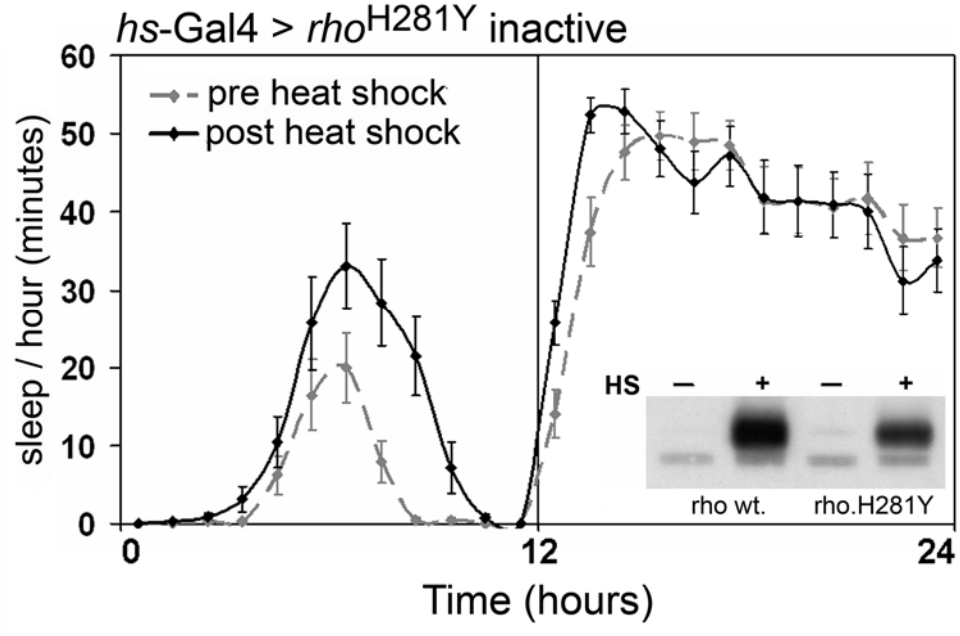

Figure 2.5 A catalytically inactive point mutant of Rho has no effect on sleep levels *hs*-Gal4>rho^{H281Y} (n=28). (inset) control Western Blot with anti-Rho with *hs*-Gal4>*rho* and *hs*-Gal4>rho^{H281Y} before and after heat shock.

2.4 Increase in sleep is not due to an alteration of the circadian phase

There are two possible ways in which the increase in sleep due to Rho and Star could be generated. First, this increase in sleep could be due to a direct effect on the sleep mechanism or homeostat. Second, it could be an effect of the circadian clock, i.e., the clock has been shifted to a phase when flies are normally asleep, similar to the resetting of the circadian clock because of a light flash at night, for example (Myers et al., 1996). I reasoned that if the latter were the case, entrained flies assayed in constant dark conditions after the induction of Rho and Star would have a visible shift in the phase of their circadian rhythm once their activity levels returned to normal. I found that this was not the case, since even though heat shocked flies overexpressing Rho and Star had a transient decrease in the amplitude of their rhythm due to excessive sleep, once their activity levels returned to normal, the phase of their rhythm was in exactly the same position as in their non-heat shocked controls after five days in constant darkness (Figure 2.6). This result demonstrates that the effect of Rho and Star on sleep levels is through the direct modulation of a sleep-related function, and not the result of a circadian phase shift.

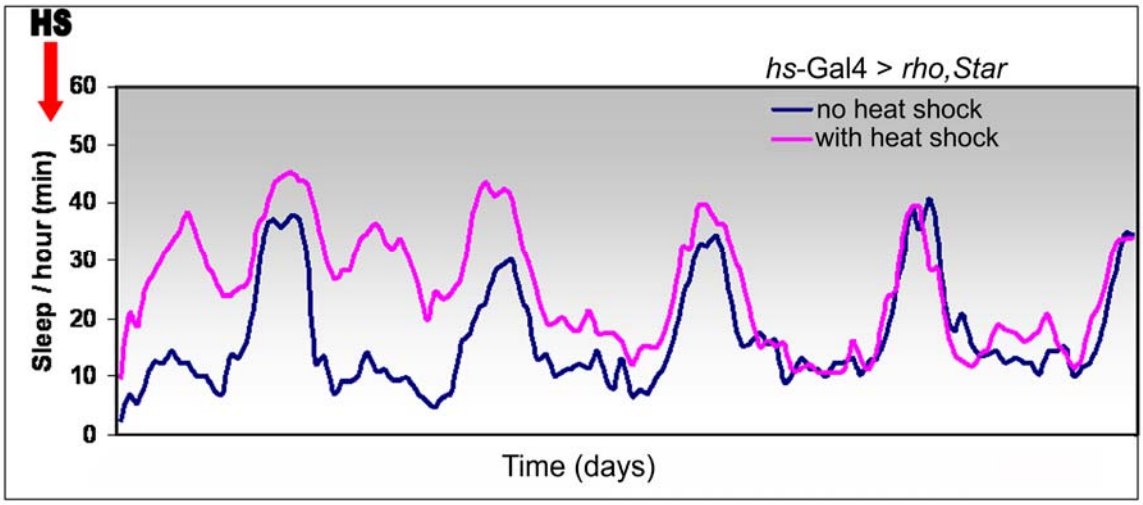

Figure 2.6 Circadian phase is not shifted in Rho and Star overexpressing flies
Hs-Gal4>*rho,Star* flies were reared, loaded onto monitors, and allowed to adjust to monitors for three days all under 12 hour L:D cycle so as to be fully entrained. They were heat shocked at the time point depicted by the red arrow, and at the following normal lights off the lights were switched off permanently so as to monitor them under a free running circadian. The heat shocked vs. not treated group had the same circadian phase after 5 days in complete darkness.

2.5 Increased sleep correlates with an increase in phosphorylated ERK in fly heads

Since it is well documented that one of the downstream signaling effects of Egf-r is the phosphorylation and activation of ERK (ppERK), I wondered if the duration of elevated ppERK has a similar time course as the increased sleep in flies overexpressing Rho and Star. To test this question, I monitored the sleep levels of *hs*-Gal4>*rho,Star* flies, and, in parallel, collected samples for the analysis of ERK activation in whole fly heads. The result of this experiment was that the increase in sleep in the Rho and Star overexpressing flies follows the same time course as ERK activation. Furthermore, the time course of ppERK does not correlate well with the longer persistence of elevated Rho

protein levels (Figures 2.7). The faster kinetics of the attenuation of ppERK levels compared to Rho might be due to a molecular or behavioral feed-back mechanism that negatively regulates Egf-r and ERK signaling. Such feedback could be a possible explanation for the compensatory decrease in sleep observed two days after Rho and Star induction (Figures 1.1A and 2.8A,B).

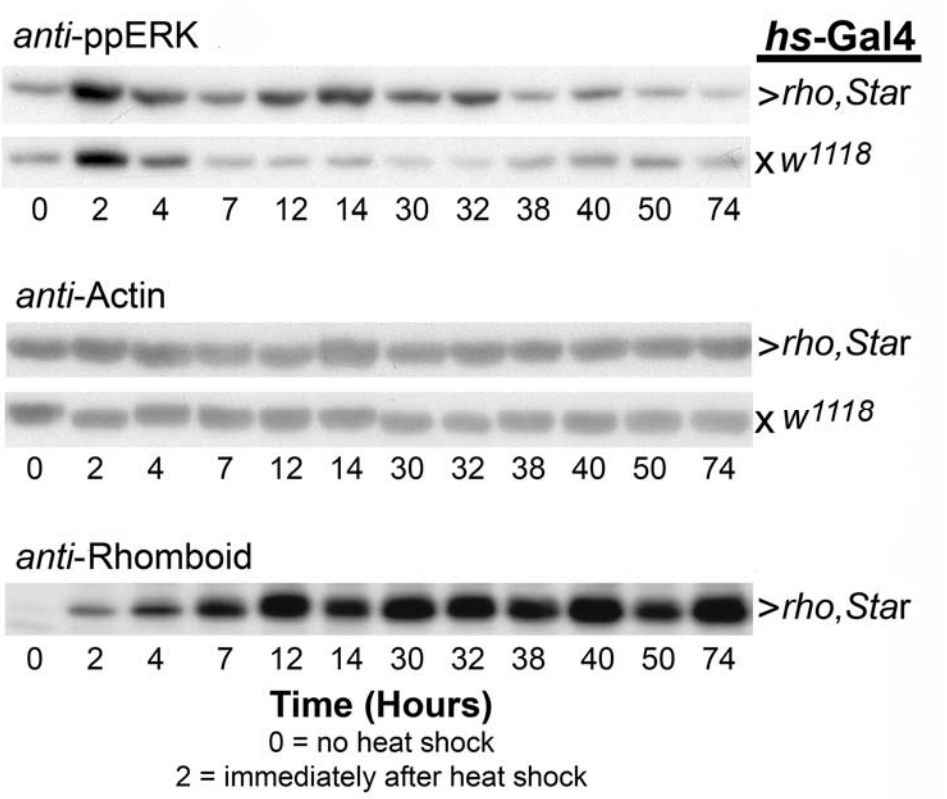

Figure 2.7 Phosphorylated ERK levels increase in fly heads after Rho and Star induction Immunoblots for the data quantified in Figure 2.8, using antibodies against ppERK, Actin and Rho. For each data point 15 heads were pooled and 5 ug (~ ½ female fly head) loaded onto the gel. Flies in monitors for Figure 2.8, and flies in vials for the data shown above were heat shocked together and then separated for further monitoring and collection. The experiment was repeated twice with similar results.

Sleep levels for flies overexpressing Rho and Star are shown for three consecutive days in Figure 2.8A, starting from one hour before the administration of heat shock. Subtracting out the untreated baseline sleep levels for this genotype reveals that the flies are clearly sleeping more than normal by the 8th hour after heat shock, and the effect continues for up to another 34 hrs (Figure 2.8B). Immediately following heat shock treatment there was a spike in ppERK in both the experimental and control groups due to the stress of the heat shock itself (Ng and Bogoyevitch, 2000), but only the flies expressing Rho and Star continue to show high levels of ppERK after the flies have recovered from the heat stress (Figures 2.7 and 2.8C). In contrast, the *hs*-Gal4×w^{1118} control group only displayed the initial ppERK spike, all other fluctuations exhibited by ppERK levels in this group are mild, and correspond to general diurnal effects of the circadian rhythm on ERK activation as reported by others (Williams et al., 2001).

To determine whether the activation of ERK is tightly correlated with the induction of sleep through the EGF receptor, I further analyzed ERK activation in some of the other genotypes I had tested for sleep behavior (Figures 2.1B and 2.3).

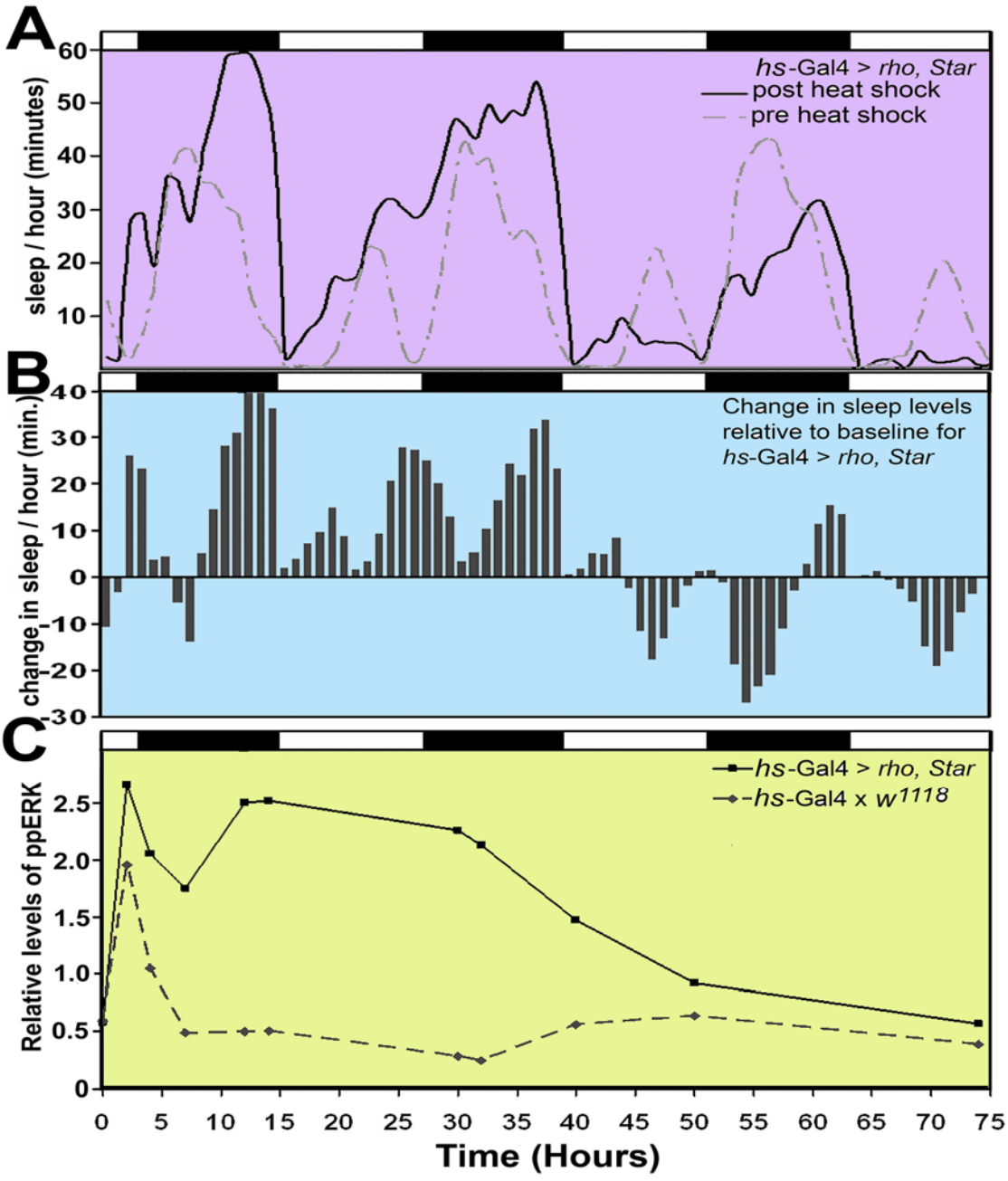

Figure 2.8 Increased sleep correlates with increased levels of activated ERK (ppERK)
(A) A trace showing mean population (*n=19*) sleep levels starting from 1 hr before heat shock administration, and the following three days for *hs*-Gal4>*rho,Star* flies (black line). The grey line is the non heat shocked baseline for the same genotype. Time is labeled below panel C, bars above panel A show light (white) or dark (black) conditions. (B) Same data as in A, with no heat shock baseline subtracted from the experimental to show the change in sleep amount. (C) Levels of ppERK normalized to the actin loading control from immunoblots prepared from fly heads collected at marked times during the course of the experiment (Figure 2.7).

I found that Rho alone, in addition to elevating sleep levels (Figures 2.1B and 2.3), is also sufficient to increase ppERK levels (Figure 2.9, line 2). However, it does take longer for Rho alone to cause the increase in ppERK as compared to when it is co-expressed with Star (compare Figure 2.9, lines 1 and 2), reflecting the known synergism between Rho and Star in activating their ligand substrate. Additionally, I found that overexpression of the mutant Rho^{H281Y}, or when $Egf\text{-}r^{DN}$ was co-expressed with Rho and Star, ppERK levels did not change (Figure 2.9, lines 3 and 4). Thus, these experiments demonstrate that active Egf-r signaling is required for the hyper phosphorylation of ERK kinase, and that there is a marked parallel between the time course of activated ERK and increased sleep levels.

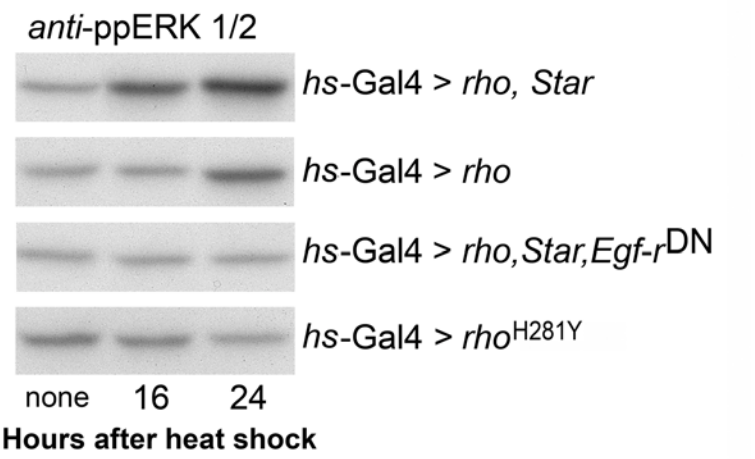

Figure 2.9 Active Egf-r signaling is required for the increase in ERK phosphorylation. Immunoblots stained against ppERK at 0,16 and 24 hrs after heat shock. *Hs*-Gal4>*rho, Star* (line 1) was repeated, and was considered a positive control for the others. *Hs*-Gal4>*rho* (line 2). *Hs*-Gal4>*rho,Star,Egf-r*^{DN} (line 3), *Hs*-Gal4>*rho*^{H281Y} (line 4).

2.6 Rho and Star induce phosphorylated ERK in the fly brain

In the Western Blot experiments described above there was a substantial increase in ppERK levels in fly head homogenates after Rho and Star induction. I was therefore curious whether it could be possible to visualize the anatomical location of the observed increase in ppERK in the fly brain. Immunohistochemsitry was performed at the time point showing the greatest difference in behavior and ppERK levels between experimental and control flies, i.e., the normal evening activity period at ~23 hrs after heat shock (Figure 2.1A, 2.1C and 2.8C), with heat treated *hs*-Gal4×w^{1118} flies serving as the control. The most striking regional difference between the brains of flies overexpressing Rho and Star and control brains (compare Figure 2.10A with 2.10B) was a highly elevated level of ppERK in an axonal tract that projects through the dorsal protocerebrum, median bundle and the tritocerebrum. I further confirmed that this was indeed a single, continuous track by 3-D imaging using the Volocity program. These results demonstrate that the increase in ppERK levels due to Rho and Star seen on Western Blots can be detected *in situ*, and implicate these regions of the brain as playing a possible role in sleep in the fly.

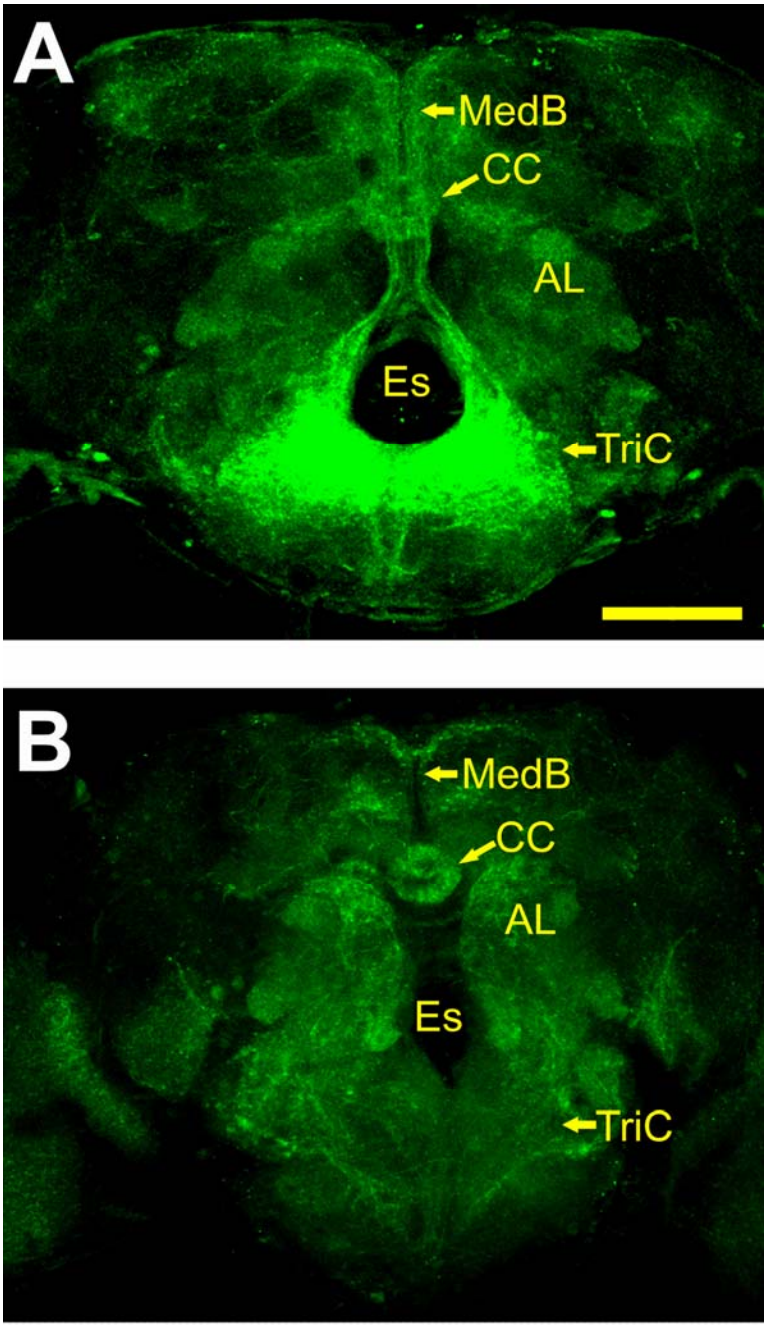

Figure 2.10 Heat shock expression of Rho and Star alters the distribution and amount of activated ERK in the fly brain.
(A) Cumulative Z-series stack of a whole-mount *hs*-Gal4>*rho,Star* brain 23 hrs after heat shock, stained against ppERK (green) (B) *Hs*-Gal4×w^{1118} control, also heat shocked and collected at the same time. A confocal slice through the level of the central complex showing equal staining in both conditions served as the staining control (shown embedded in the projections). Secondary antibody did not stain anything in the brain (data not shown) AL = Antennal Lobe, CC = Central Complex, Es = Eosophagus, MedB = Median Bundle, OL = Optic Lobe, TriC = Tritocerebrum. Scale bar equals 100 μm.

2.7 Materials & Methods

Drosophila stocks and conditions

Flies were raised on yeast/molasses/agar food, and assayed at 23ºC under 12 hr light: 12 hr dark conditions. Stocks *w**;;Hs-Gal4, *w**;UAS-*rho*;, *w**;;UAS-*rho*,UAS-*Star* were obtained from A. Guichard and E. Bier (UCSD, La Jolla, CA). w^{1118} (#5905), *w**;UAS-*Egf-r*^{DN};UAS-*Egf-r*^{DN} were obtained from the Bloomington Stock Center (Bloomington, IN). Rho^{H281Y} was identified with in an *in-vivo* EMS screen with the use of a leaky HS-*rho* construct (A. Guichard, personal communication), we cloned this mutant Rho into pUAST and made *w**;UAS-*rho*^{H281Y} stock (Rainbow Transgenic Fly Service, Newbury Park, CA). Since studies in development show that ubiquitous misexpression of wild-type Rho in wings causes a wide-spread conversion of the wing membrane to vein tissue (Sturtevant et al., 1993), we also tested our construct in the same setting. We found that misexpression of Rho^{H281Y} in wings (MS1096>*rho*^{H281Y}) did not produce a phenotype distinguishable from the MS1096×w^{1118} control, therefore confirming that Rho^{H281Y} is an inactive form of Rho.

Activity data collection and analysis

Female flies were assayed with the TriKinetics *Drosophila* activity-monitoring (Waltham, MA) system on 5% sucrose/1%a gar. Sleep was measured as previously described in 5-min bins (Andretic et al., 2005; Shaw et al., 2000). Statistical analysis was performed with JMP software. Normality was determined with the Wilks-Shapiro test

(Shapiro and Wilk, 1965), parametrically distributed data was analyzed with one-way ANOVA with the Tukey-Kramer Honestly Significant Test as the post-hoc analysis, and non-parametric data was analyzed with Wilcoxon/Kruskal-Wallis ANOVA tests with the appropriate α level determined according to the Bonferroni correction for multiple independent comparisons ($p<0.05$)(Sokal and Rohlf, 1995).

Heat shock conditions

For heat shock, incubators already containing the flies were turned up to 37ºC, and T was monitored with an internal T probe to ensure it rose to 37ºC (~20 mins.), and then timed for 1 hr, after which T was lowered back to 23ºC. In total, flies spent 60 min at 37ºC. After heat shock we made certain that flies were not stuck to the tubes, and if they were and could not be shaken off, the fly was removed from the experiment. For heat shock experiments, prior to loading with flies, the tubes with sucrose/agar food were dried in a container with desiccant overnight at 37ºC to reduce moisture condensation during heat shock.

Western Blot

The following lysis buffer will extract endogenous Rho from fly heads, and was used for all experiments: 2.5% CHAPS, 50 mM KCl, 120 mM NaCl, 2 mM DTT, 20 mM Tris-HCl pH 7.5, protease inhibitor cocktail with EDTA(Roche), phosphatase inhibitor cocktails I and II when needed (Sigma). Flies were frozen in liquid nitrogen and 15 heads/sample were collected on ice. They were lysed in 15 µl/head using a motorized pestle, then kept on ice for 45 mins with intermittent vortexing. Total protein

concentration was measured using the Bradford method (CHAPS does not interfere). Rho is highly hydrophobic, so any samples in which Rho was examined were not heated. Transfers were performed without SDS. Rho antibody was used at 1:3000 (from E. Bier, UCSD, La Jolla, CA), anti pp-ERK (Sigma) 1:5000, and anti-actin (JLA20, Developmental Studies Hybridoma Bank) 1:10,000 and were probed overnight at 4ºC in 5% milk/TBST. Bands intensities were quantified with the NIH software program, ImageJ.

Immunohistochemistry

ppERK: Hs-Gal4>*rho,Star* and Hs-Gal4×w^{1118} flies were heat shocked while on activity monitors, and collected 23 hours later, at Zeitgeber Time 10. Flies were collected in batches of 7 by removing activity tubes from monitors and directly plunging them cap-down into ice. They were then individually shaken out onto ice, heads and probosci removed, and fixed in glass vials with 8% paraformaldehyde(PF)/PBS (no detergent) for 40 minutes on a rotating shaker at 50 rpm. Brains were dissected and stained with anti-ppERK (Sigma) at 1:200 in 0.2% BSA, 0.3% TritonX-100, 0.3% deoxycholate in PBS pH 7.4. Brains were mounted in FluoroGuard™ Antifade Reagent (Biorad) with #1 cover slips as spacers. Images of fluorescently labeled brains were acquired on a Leica SP2-AOBS (Leica Microsystems, Wetzlar, Germany) scanning confocal microscope with 20× and/or 40× objective lenses. The tritocerebral-median bundle-protocerebral ppERK signal was confirmed to be a single, continuous unit by 3-D imaging using the Improvision® program Volocity® (Coventry, England, data not shown).

Parts of this chapter were adapted from segments of the following submitted paper, of which I was the primary researcher and author:

Foltenyi, K., Greenspan, R.J., and Newport, J.W. (submitted Nov., 2006) Activation of Egf-r/ERK by Rhomboid signaling regulates the consolidation and maintenance of sleep in *Drosophila*. *Cell* (submitted).

2.8 References

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Chapter 3

Inhibition of Rho in the pars intercerebralis decreases sleep

3.1 Introduction to *rho*^{DN} construct used to inhibit Rho function

To interfere with Rho functioning, I made use of a *rho* RNAi construct identified in an in-vivo mutagenesis screen by Annabel Guichard (Guichard et al., 2002). This construct consists of a full-length *rho* palindrome with UAS sequences on both ends, separated by another *rho* fragment which includes the presence of natural introns to facilitate RNAi folding (Figure 3.1E). Egf-r activation is required for the formation of wing veins during development, and this activation is Rho dependent (compare Figures 3.1A and 3.1B). When the *rho* RNAi construct (*rho*^{DN}) was expressed in wings, it inhibited *rho* expression, and thus erased wing veins due to the consequent lack of Egf-r activation (compare Figure 3.1A and 3.1C).

I also confirmed that this RNAi construct is functional in neurons and targets Rho by showing it could reduce endogenous Rho levels by driving *rho*^{DN} with the pan-neural driver *elav*-Gal4, and assaying for Rho protein in heads by Western Blot (Figure 3.2). To determine if endogenous Rho is required for normal sleep, I made use of UAS-*rho*^{DN} to reduce or block Rho activity in the nervous system of the fly.

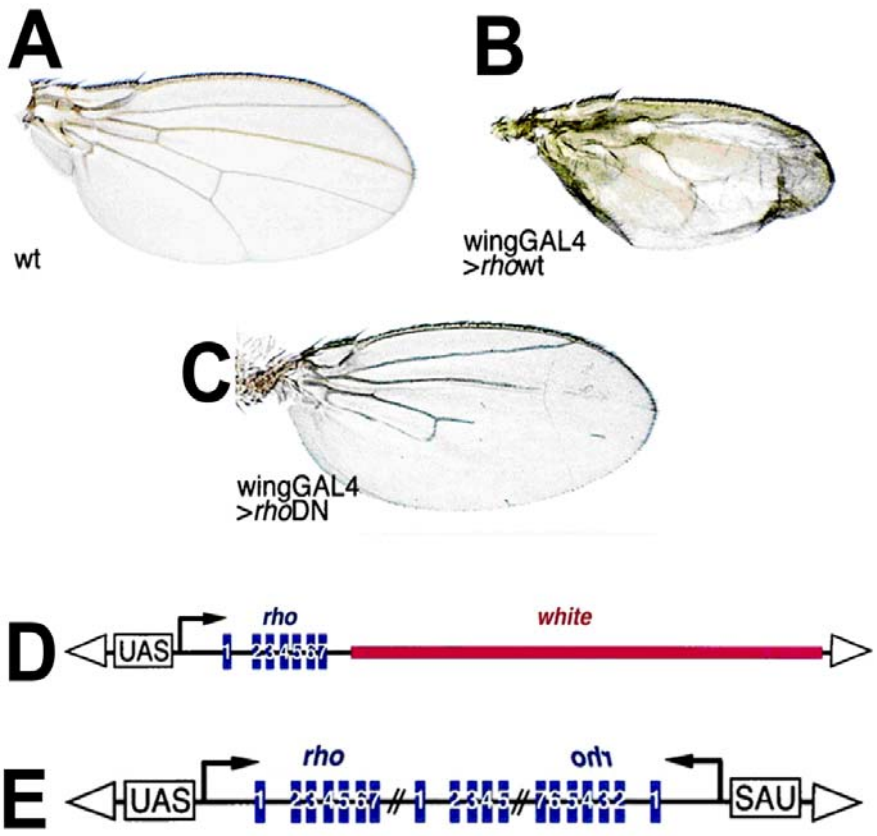

Figure 3.1 Structure and function of the *Rhomboid* RNAi construct.

(A-C) Wings of the following genotypes: wing-Gal4 used was MS1096. (*A*) wild type; (*B*) wing-GAL4>UAS-*rho*^{wt}; (*C*) wing-GAL4>UAS-*rho*^{DN}; (*D,E*) Structures of wild-type and mutant UAS-*rho* construct. Blue boxes indicate the transmembrane domains of the Rho protein. Triangles indicate the inverted terminal repeats of the P element. (*D*) Wild-type pUAS-*rho*^{wt}; (*E*) pUAS-*rho*^{DN}. This figure is adapted from (Guichard et al., 2002)

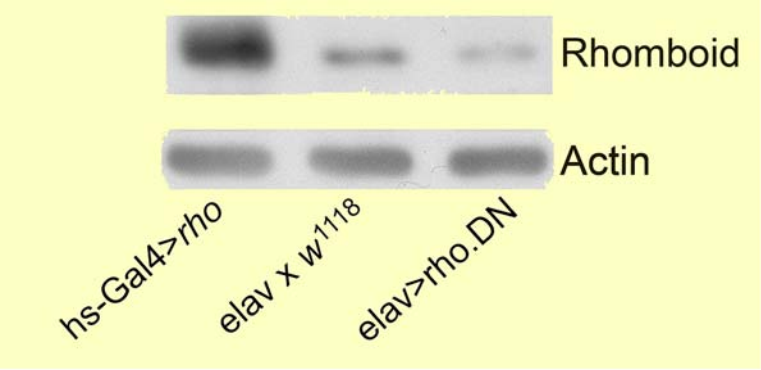

Figure 3.2 *Rhomboid* RNAi reduces Rhomboid protein levels in neurons
Immunoblot against Rho in *elav*>*rho*^{DN} and controls. *Hs*-Gal4>*rho* is included as a positive control to show the location of the Rho band. Actin loading control is included.

3.2 Inhibition of Rho decreases sleep

Although ectopic activation of Egf-r by Rho induces sleep in the fly, these experiments did not address whether the pathway is necessary for normal sleep patterns. I drove expression of *rho*^{DN} in all neurons using *elav*-Gal4, and found a dramatic decrease in sleep levels (Figure 3.3D). But *elav*-Gal4>*rho*^{DN} exhibited developmental defects that included lethality when flies are reared above 25ºC, and at lower temperatures induced missing facets in the anterior portion of the eye, as well as lowering the counts per waking minute in the adults (Table 3.2). Nonetheless, this result demonstrated a possible requirement of Rho in sleep, therefore we screened for more restricted neural drivers to identify a specific brain region where Rho might mediate its effect on sleep without the negative developmental effects seen with *elav*-Gal4>*rho*^{DN}.

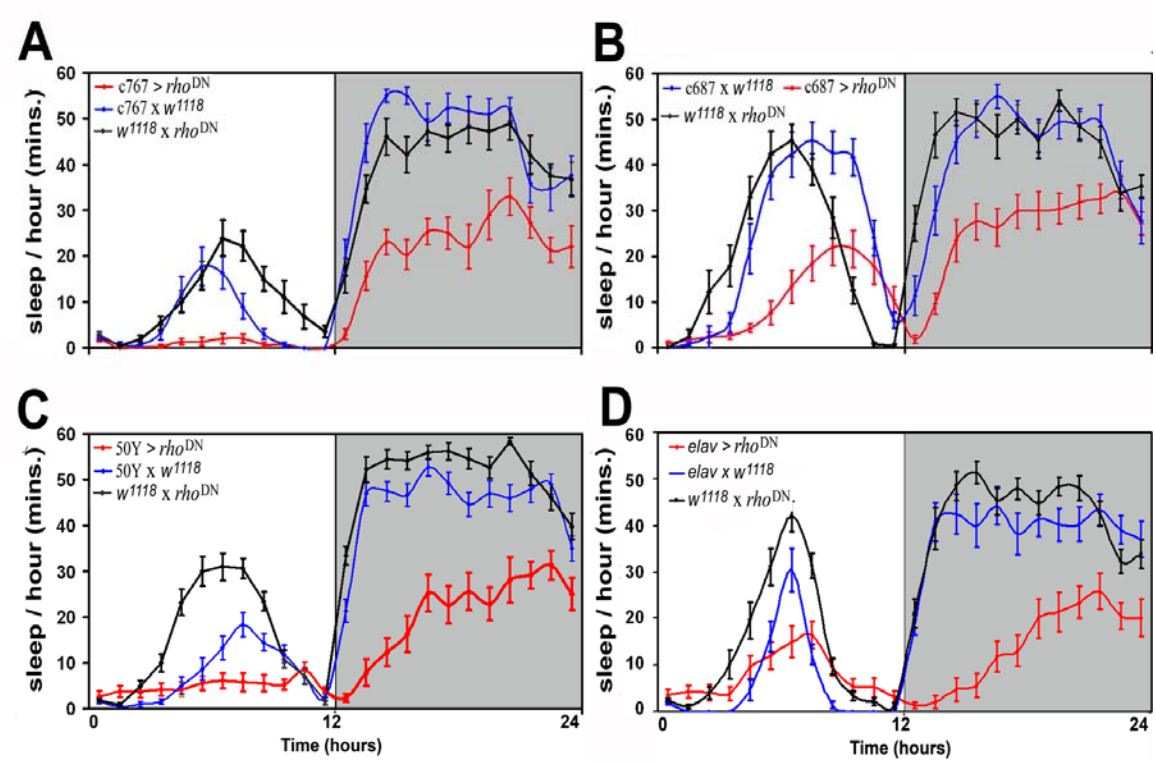

Figure 3.3 Directed *rho* RNAi expression reduces sleep.
Representative traces of sleep levels for one 24 hr LD cycle for flies with UAS-*rho*^{DN} (*rho* RNAi) driven by specified Gal4 drivers (red), and their controls. Blue lines represent the driver crossed to *w*^{1118} controls, and black lines UAS-*rho*^{DN}×*w*^{1118} controls. All time intervals shown are the fourth or fifth day after the start of monitoring of 1-2 day old female flies. Points represent group average ±SEM. Shading represents lights off. (A) c767>*rho*^{DN} (*n=15*). (B) 50Y>*rho*^{DN} (*n=19*) (C) c687>*rho*^{DN} (*n=25*). (D) *elav*>*rho*^{DN}

Table 3.1 List of 49 Gal4 drivers tested with *rho*^{DN} for an effect on sleep levels

Gal4 line	Effect on sleep*	Gal4 line	Effect on sleep*
30Y	no	c507	no
36Y	no	c588	no
50Y	**yes**	c601	no
52Y	no	c687	**yes**
59Y	no	c767	**yes**
64Y	no	c774	no
91Y	no	c814	no
95Y	no	c929	XXX**
106Y	no	*appl*-Gal4	no
116Y	no	*cry*-Gal4	no
129Y	no	*Elav*-Gal4	**yes**
146Y	no	Feb194	XXX**
201Y	no	*GMR*-Gal4	no
210Y	no	Jan191	no
287a	no	Jan229	no
c005	no	Kurs45	no
c041	no	Kurs58	XXX**
c062	no	Mai289	no
c119	no	Mai301	no
c279	no	*Nervana2*-Gal4	no
c309	no	*per*-Gal4	no
c319	no	*repo*-Gal4	no
386Y	**yes**	Sep54	no
c420	no	*tim*-Gal4	no
c448	no		

*All effects on sleep were a decrease in sleep levels compared to both Gal4×w^{1118} and w^{1118}×*rho*^{DN} controls.

**Gal4×w^{1118} control slept less than 30 mins. per hr. at night, results were considered inconclusive.

I screened 48 other driver lines corresponding to a variety of brain regions with *rho*^{DN} (Table 3.2), and found four lines with a significant effect on sleep: c767, 50Y, c687, and 386Y (Figure 3.3A,B,C Tables 3.1 and 3.2). None of the other tested drivers produced clear sleep defects, including drivers with expression patterns in the eye, optic lobes, mushroom bodies or glial cells. Unlike in the experiment where Egf-r signaling was enhanced, flies where Egf-r signaling is compromised showed the greatest effect on sleep levels during the night. Further analysis of the *rho*^{DN} induced change in nighttime sleep levels in *elav*-Gal4 and three of the most restricted drivers, namely c767, 50Y and c687 (386Y is broadly expressed in most peptidergic cells (Taghert et al., 2001)), revealed that the duration of sleep bouts was dramatically shortened, accompanied by an increase in the number of times flies attempted sleep as compared to their controls (Figure 3.4B,C and Table 3.2). These changes indicate that these flies did have a sleep need, but were unable to maintain the sleep state. For all three of the restricted drivers, the plots for total nighttime sleep, bout number and bout duration (Figure 3.4A-C) are statistically indistinguishable ($p>0.01$, Kruskal-Wallis Test, $\alpha'=0.0085$), suggesting that the same mechanism is being impaired by *rho*^{DN} in all three cases. Moreover, drivers c767, 50Y, c687 and 386Y expressing *rho*^{DN} did not demonstrate any lethality or observable developmental abnormalities, and locomotor activity (counts per waking minute) was normal relative to one or both of their respective controls (Table 3.2).

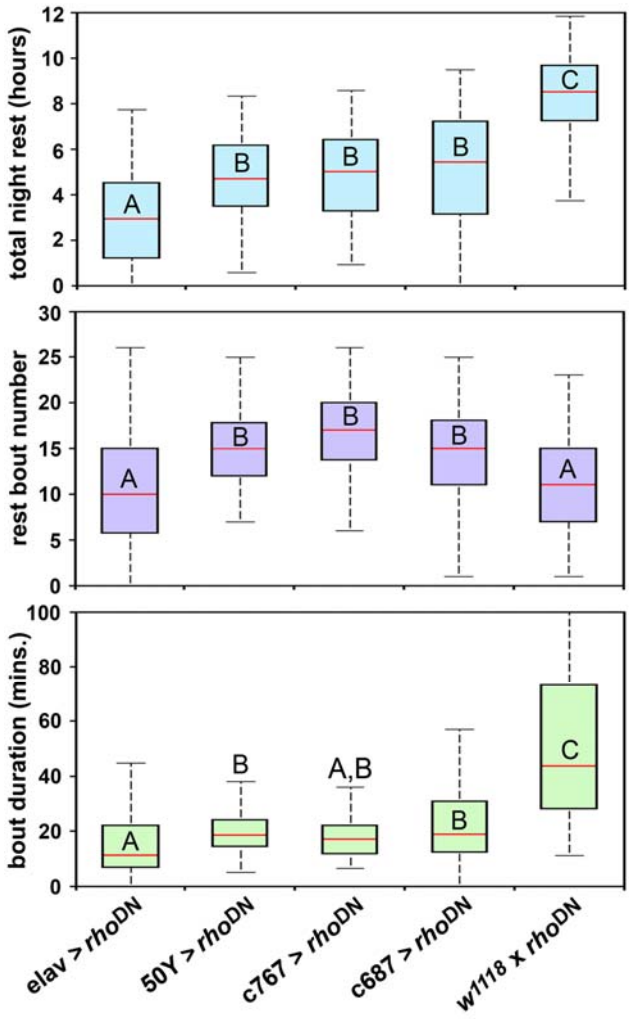

Figure 3.4 Boxplots for three nighttime sleep parameters shown for *elav*>, 50Y>, C767>,c687>*rho*^{DN} and their common control

The data are represented this way because of the non-parametric distribution of bout duration. Lines within boxes represent the median, upper and lower box limits the 75% and 25% quantiles, and whiskers the 95% and 5% quantiles. Letters represent statistically significant groups as determined by 10 independent group-wise or pair-wise comparisons using Wilcoxon/Kruskal-Wallis Test, (α'=0.005). (A) Total nighttime sleep levels. (B) Number of nighttime sleep bouts. (C) Sleep bout duration (see also Table 3.2).

Table 3.2 Change in sleep parameters and counts per minute due to *rhomboid* RNAi expression in the nervous system

Gal4 drivers expressing *rhomboid* RNA interference compared individualy to their own set of controls assayed simultaneously

sleep parameter		total day rest				total night rest				bout number day				bout number night				bout duration day				bout duration night				bout max day				bout max night				counts per minute			
genotype	N	med	10%	90%	a	med	10%	90%	a	med	10%	90%	a	med	10%	90%	a	med	10%	90%	a	med	10%	90%	a	med	10%	90%	a	med	10%	90%	a	med	10%	90%	a
c687>*rho*^{DN}	97	**65**	5	249	A	**325**	118	490	A	**9**	1	16.2	A	**15**	6	22	A	**6.4**	2.5	19	A	**19**	10	44.1	A	**15**	5	66	A	**65**	20	220	A	**1.54**	1.1	2.4	A
c687x*w*^{1118}	70	**223**	76	395	B	**508**	347	650	B	**9**	6	15.9	A	**10**	2.1	18	B	**20**	8.3	54	B	**48**	20	228	B	**80**	15	283	B	**198**	65	610	B	**1.98**	1.3	2.9	B
w^{1118}x*rho*^{DN}	79	**210**	100	325	B	**575**	385	665	B	**13**	7	19	B	**9**	2	17	B	**15**	8.8	36	C	**64**	24	315	B	**45**	20	150	B	**285**	85	635	B	**1.58**	1.1	2	A
50Y>*rho*^{DN}	71	**40**	0	164	A	**280**	106	410	A	**7**	1	20	A	**15**	9.1	20	A	**5**	0	9.7	A	**18.5**	9.7	29.2	A	**10**	0	25	A	**55**	20	125	A	**1.54**	0.9	2.4	A
50Yx*w*^{1118}	78	**67.5**	20	170	A	**520**	414	605	B	**8.5**	3	18.1	A	**11**	6.9	19	B	**7.1**	4.2	11	B	**47.3**	23	87.2	B	**15**	5	35	B	**163**	65	340	B	**1.67**	1.2	2.3	A
w^{1118}x*rho*^{DN}	65	**135**	38	264	B	**520**	355	669	B	**13**	6	18.4	B	**11**	3	19	B	**9.3**	5.7	23	C	**49.2**	20	221	B	**25**	10	92	C	**200**	55	592	B	**1.48**	1	2.1	A
c767>*rho*^{DN}	73	**5**	0	83	A	**300**	122	470	A	**2**	0	13	A	**17**	12	23	A	**5**	0	7.5	A	**17.2**	8.3	31.6	A	**5**	0	15	A	**50**	20	135	A	**1.63**	1.1	2.4	A
c767x*w*^{1118}	79	**55**	5	155	B	**550**	390	650	B	**8**	1	15	B	**13**	5	19	B	**6.7**	3.3	13	B	**42.1**	20	132	B	**15**	5	40	B	**180**	70	465	B	**1.6**	1.1	2.4	A
w^{1118}x*rho*^{DN}	54	**150**	28	315	C	**495**	390	568	C	**13**	5	18	C	**13**	8	20	B	**11**	5.3	23	C	**36.5**	23	67	B	**28**	8	68	C	**128**	55	285	C	**1.48**	1	2.2	A
elav>*rho*^{DN}	81	**105**	15	285	A	**175**	10	355	A	**12**	3	24	A	**10**	2	19	A	**7.9**	5	16	A	**11.3**	5	38.3	A	**20**	5	60	A	**45**	5	155	A	**1.04**	0.6	1.7	A
*elav*x*w*^{1118}	69	**55**	0	135	B	**445**	245	630	B	**6**	1	11	B	**11**	4	18	A	**6.8**	0	27	A	**36.7**	14	144	B	**15**	0	75	A	**155**	45	485	B	**2.08**	1.6	2.7	B
w^{1118}x*rho*^{DN}	89	**205**	85	305	C	**500**	310	620	B	**10**	6	16	A	**11**	5	17	A	**18**	7	51	B	**43.2**	19	103	B	**70**	15	235	B	**170**	50	450	B	**1.65**	1.2	2.3	C

Gal4 drivers expressing *rhomboid* RNA interference compared to each other and their grouped common control

sleep parameter		total day rest				total night rest				bout number day				bout number night				bout duration day				bout duration night				bout max day				bout max night				counts per minute			
genotype	N	med	10%	90%	a	med	10%	90%	a	med	10%	90%	a	med	10%	90%	a	med	10%	90%	a	med	10%	90%	a	med	10%	90%	a	med	10%	90%	a	med	10%	90%	a
50Y>*rho*^{DN}	71	**40**	0	163	B	**280**	106	409	B	**7**	1	20	B	**15**	9.2	20	B	**5**	0	9.7	B	**18.5**	9.6	29.2	B	**10**	0	25	B	**55**	20	125	AB	**1.54**	0.9	2.4	A
c767>*rho*^{DN}	73	**5**	0	83	A	**300**	122	470	B	**2**	0	13	C	**17**	12	23	B	**5**	0	7.5	A	**17.2**	8.3	31.6	AB	**5**	0	15	A	**50**	20	135	AB	**1.63**	1.1	2.5	A
c687>*rho*^{DN}	97	**65**	5	249	BC	**325**	118	490	B	**9**	1	16	B	**15**	6	22	B	**6.4**	2.5	19	BC	**19**	10	44	B	**15**	5	66	C	**65**	20	220	B	**1.54**	1.1	2.4	A
elav>*rho*^{DN}	81	**105**	15	285	C	**175**	10	355	A	**12**	3	24	A	**10**	2	19	A	**7.9**	5	16	C	**11.3**	5	38	A	**20**	5	60	C	**45**	5	155	A	**1.04**	0.6	1.7	B
w^{1118}x*rho*^{DN}	256	**175**	59	307	D	**510**	340	630	C	**12**	6	17	A	**11**	5	18	A	**13**	6.3	36	D	**43.6**	21	129	C	**40**	10	172	D	**170**	55	502	C	**1.61**	1.1	2.2	A

All data represented as median and 10% and 90% quantiles, units are in minutes, except for last column

Letters after each group represent the statistical category each member of the group belongs to, groups not sharing any common letters are statistically significant from each other.

In the first set, groups were compared using Wilcoxon/Kruskal-Wallis test, with an α'=0.017 cut off for 3 independent comparisons

Groups were compared with the same test in the second set, with an α'=0.005 cut off for 10 group or pair-wise comparisons

3.3 Flies expressing *rho*^{DN} are unable to regain lost sleep after sleep deprivation

I tested the effect of sleep deprivation on flies expressing *rho*^{DN}, to see whether they are able to mount a sleep rebound in a state of increased sleep need (Figure 3.5). Deprivation was performed by vibrating the monitors at 3-4 minute intervals during the course of the 12 hour night, a technique that is capable of fully depriving the flies of their sleep. 50Y-,c767-,c687>*rho*^{DN} flies lost less sleep than their controls because they slept less to begin with, not because they adapted to the stimulus. Nonetheless, flies expressing *rho*^{DN} recovered only ~16% of their lost sleep, while the control groups recovered ~ 40%, indicating that *rho*^{DN} flies do have some ability to sleep more in response to deprivation, although not to the same extent as the controls. Another observed difference between 50Y-,c767-,c687>*rho*^{DN} and the controls was in the time course of the recovery, with most of it occurring during the second half of the day for the flies expressing *rho*^{DN}, unlike the immediate initiation of recovery observed in the controls (compare slopes of the recovery curves between hours 12 and 18). This delay to rebound indicates that these flies were in a heightened state of arousal after the deprivation ended, and could not settle down. These sleep deprivation experiments are consistent with the former findings that impairing Rho function in flies makes them short sleepers that cannot maintain a prolonged sleep state and have difficulty settling down after being stimulated.

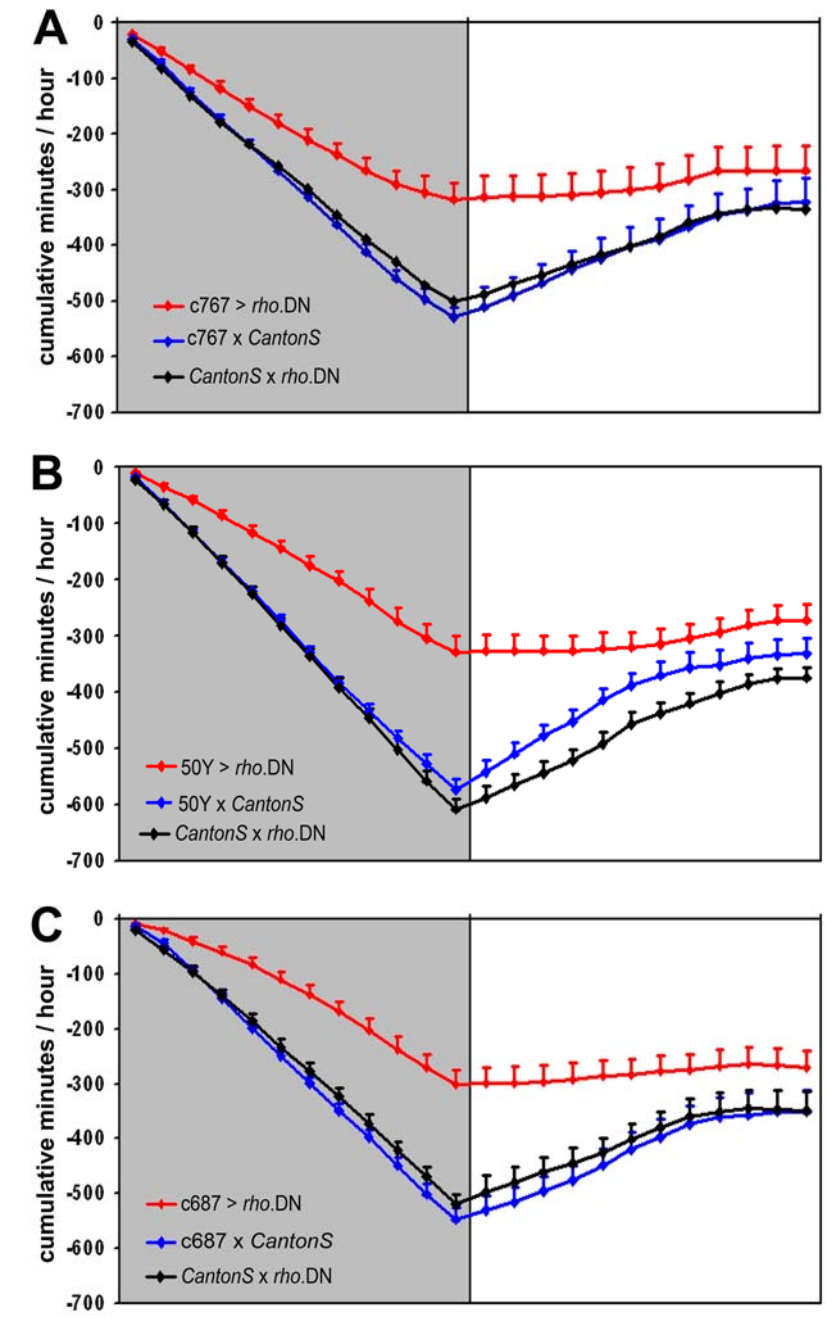

Figure 3.5 c767, 50Y, c687 flies expressing *rho*^{DN} are unable to recover from sleep deprivation

An example of the effect of sleep deprivation on c767-,50Y-, and c687>*rho*^{DN} flies. The first 12 hrs (0-11) represent cumulative sleep lost, the second 12 hrs. (12-24) are cumulative sleep recovered.

3.4 Inhibition of sleep with *rho*^{DN} does not result in a circadian phase shift

To balance the circadian rhythm experiment done with flies overexpressing Rho and Star, I wanted to determine if inhibiting Rho also had the same result of not altering the circadian phase. In order to do this experiment, c767>*rho*^{DN} flies were raised under normal light:dark conditions, so that they will be entrained to this cycle before loading them unto monitors. After monitor loading, they were allowed to stay in the same lighting conditions for one day, and on the following night after normal lights off, the lights were unplugged so that the flies were now in constant darkness. Although there is still a clear effect of *rho*^{DN} on sleep levels under constant dark conditions, the phase of the circadian remained aligned with the controls (Figure 3.5). This result demonstrates that whether Egf-r signaling is hyperactivated (Figure 2.6) or hypo activated, there is no effect on the intrinsic circadian clock itself, therefore the effects on sleep levels are not due to a circadian phase shift.

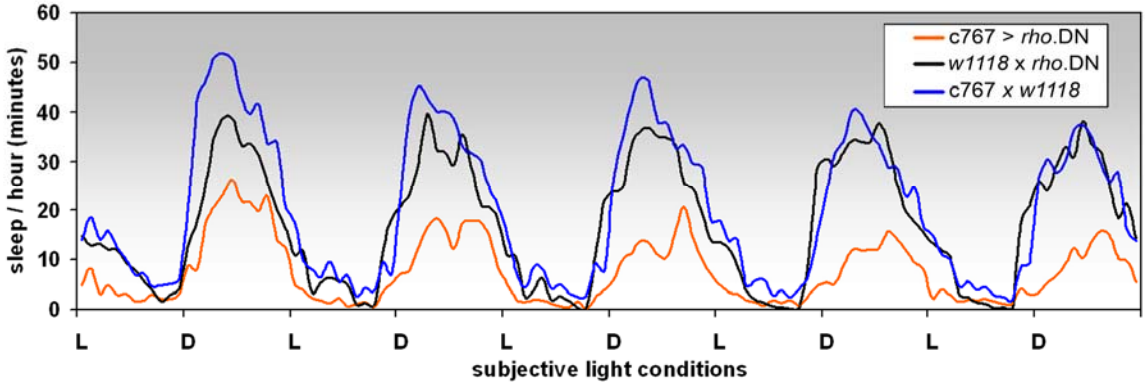

Figure 3.6 Inhibiting Rho in c767 cells does not shift the circadian phase Flies were monitored in constant darkness. Subjective lighting conditions are depicted on the x axis (n=32 for all groups).

3.5 Ablation of c767 cells results in a transient decrease in sleep

The concern regarding expressing *rho*^{DN} with drivers that are active during development, is that the effect seen on sleep levels are a consequence of developmental abnormalities. Genomic lesions in *rho* are known to be lethal, and specifically, its expression is necessary for some aspects of neural development. Therefore, my concern was not unwarranted, although the expression of *rho*^{DN} in only a few restricted cells is in of itself much gentler than the genomic mutants researchers routinely publish behavioral studies on without addressing the possibility that the problem might be developmental.

Unfortunately, when I attempted to use a temperature sensitive version of the Gal4 inhibitor *tub*-Gal80ts, the Gal80 had an unexpected interaction with the *rho* RNAi construct that blocked the folding of the double-stranded transcript, and instead, allowed the transcript to be translated into active Rho protein. I visualized this effect by driving *rho*^{DN} combined with *tub*-Gal80ts with the wing driver MS1096, where instead of seeing an unaffected vein structure due to the presumed blockage of *rho*^{DN} by Gal80ts, I actually observed the appearance of many extra and thickened veins, indicating the activation of Egf-r signaling rather than the inhibition normally seen with *rho*^{DN} (see Appendix A.1 for further details). So not only was *tub*-Gal80ts unable to fully suppress Gal4, but what leaked through was somehow translated into active Rho protein from the full-length double-stranded *rho* construct. I determined that this was not due to driver strength, i.e. *rho*^{DN} levels, but to the presence of the *tub*-Gal80ts construct itself (see Appendix A.1). The mechanism of the interaction between the *rho*^{DN} transcript and Gal80 is unknown,

but the bottom line is that this approach was unusable for a developmental study of the effects of Rho inhibition. But is was comforting to know that at least with the studies where we activated Egf-r signaling, I could temporally regulate the overexpression of Rho after development was finished.

Another approach I could take to the question of developmental defects was to ablate the cells of the drivers by expressing the caspases Head Involution Defective (hid), and Reaper (rpr), also only after development was complete. In order to do this, I combined the Gal4 drivers with *tub*-Gal80ts, and also UAS-*hid*,UAS-*rpr* and *tub*-Gal80ts, so as to have two copies of *tub*-Gal80ts in each cross to ensure suppression of Gal4. But even with two copies of *tub*-Gal80ts, the only viable cross at 18ºC were ones involving the driver c767. c767>*hid,rpr* was to a low level viable without *tub*-Gal80ts as well, so their behavior was also observed on activity monitors.

Driving HID, Rpr with c767 without *tub*-Gal80ts resulted in flies that in the beginning of their adult lives slept far below the level of the controls, but slowly increased sleep during the course of five-six days to ultimately match control levels (Figure 3.6A). Inhibiting c767 Gal4 activity with *tub*-Gal80ts during development by rearing flies at 18ºC, loading them into monitors and then keeping them at 29ºC for three days to denature Gal80ts and allowing for the expression of HID and Rpr, also ultimately led to flies that slept transiently below control levels (Figure 3.6B). This decrease in sleep was not due to the flies spending three day at the stressful temperature of 29ºC, since the controls were treated in exactly the same manner, and they slept at normal levels. Moreover, when the same genotype was monitored at a constant 23ºC without

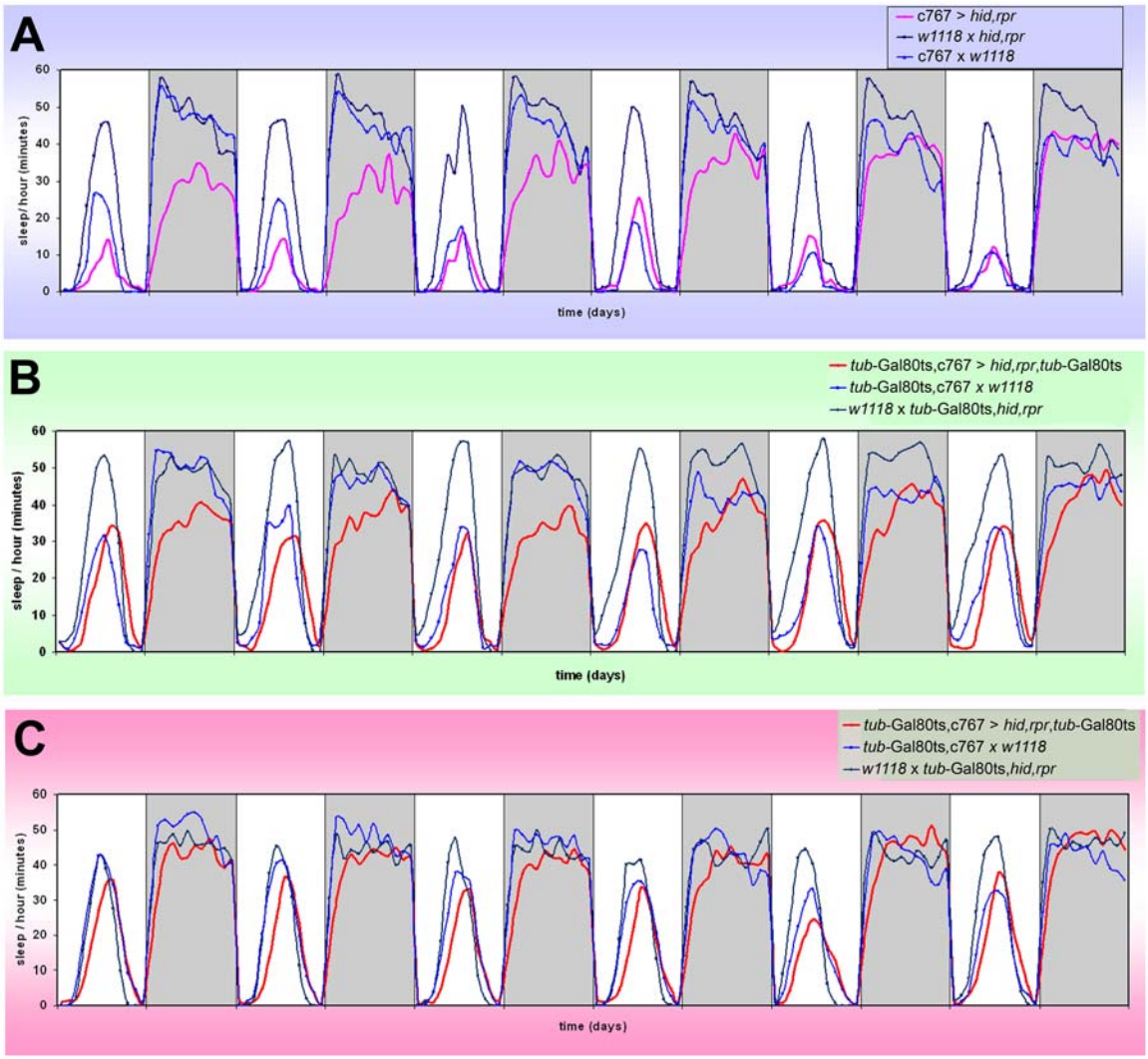

Figure 3.7 Ablating c767 cells with hid and rpr transiently decreases sleep
(A) Sleep levels of c767>hid,rpr flies show through the age of 3-8 days. (B)*tub*-Gal80ts,c767>*hid,rpr,tub*-Gal80ts flies shown after three days at 29ºC to allow for the ablation of c767 cells. The trace starts from the third day after recovery from the high temperature exposure.(C) Same genotype and same age flies as the ones in panel B, but without exposure to 29ºC, with c767 cells presumably still intact.

ever de-regulating Gal80, the sleep level of the flies never dropped to below the level of the controls, indicating that the drop in sleep was due to the ablation of the c767 driver cells (compare Figures 3.6B and 3.6C).

The gradual increase in sleep levels was not observed when only Rho was inhibited in these cells instead of their complete ablation, but the two perturbations probably have a very different effect on the biology of the fly. Nonetheless, these results demonstrate that c767 cells do play a role in sleep levels, even if the fly brain eventually finds a way to compensate for this defect

3.6 50Y, c767 and c687 are active in the Rho-expressing pars intercerebralis

Since 50Y, c767 and c687 driving *rho* RNAi had similar effects on nighttime sleep patterns, I examined whether their expression patterns also included a common group of cells. A comparison of the three expression patterns revealed that all three inserts drive expression prominently in a set of neurons in the pars intercerebralis (PI), which project into the tritocerebrum (Figure 3.8). The tritocerebrum is the same region seen heavily stained for ppERK after heat shock induction of Rho and Star (Figure 2.10). Driver 386Y has been previously published, and includes a much larger set of neurosecretory cells than 50Y, c767 and c687 (Taghert et al., 2001).

To further confirm that there is overlap between the drivers in the PI region, those with the narrowest expression patterns, 50Y and c767, were used simultaneously to express GFP, and cell bodies in the PI were counted. 50Y has 14-15 Gal4 expressing cells in the PI, c767 has 11-12, and in brains expressing both drivers there are 18-21 cells labeled with GFP, therefore there is a likely overlap in 6-7 cells. There is also a much brighter GFP signal in 6 cells when using both drivers, further confirming the possibility that the two drivers overlap in those cells (Figure 3.8D). Although these 6-7 cells are not

necessarily the only ones mediating the abnormal sleep effect of UAS- *rho*^{DN}, this analysis confirms that there is nonetheless a direct overlap between 50Y and c767.

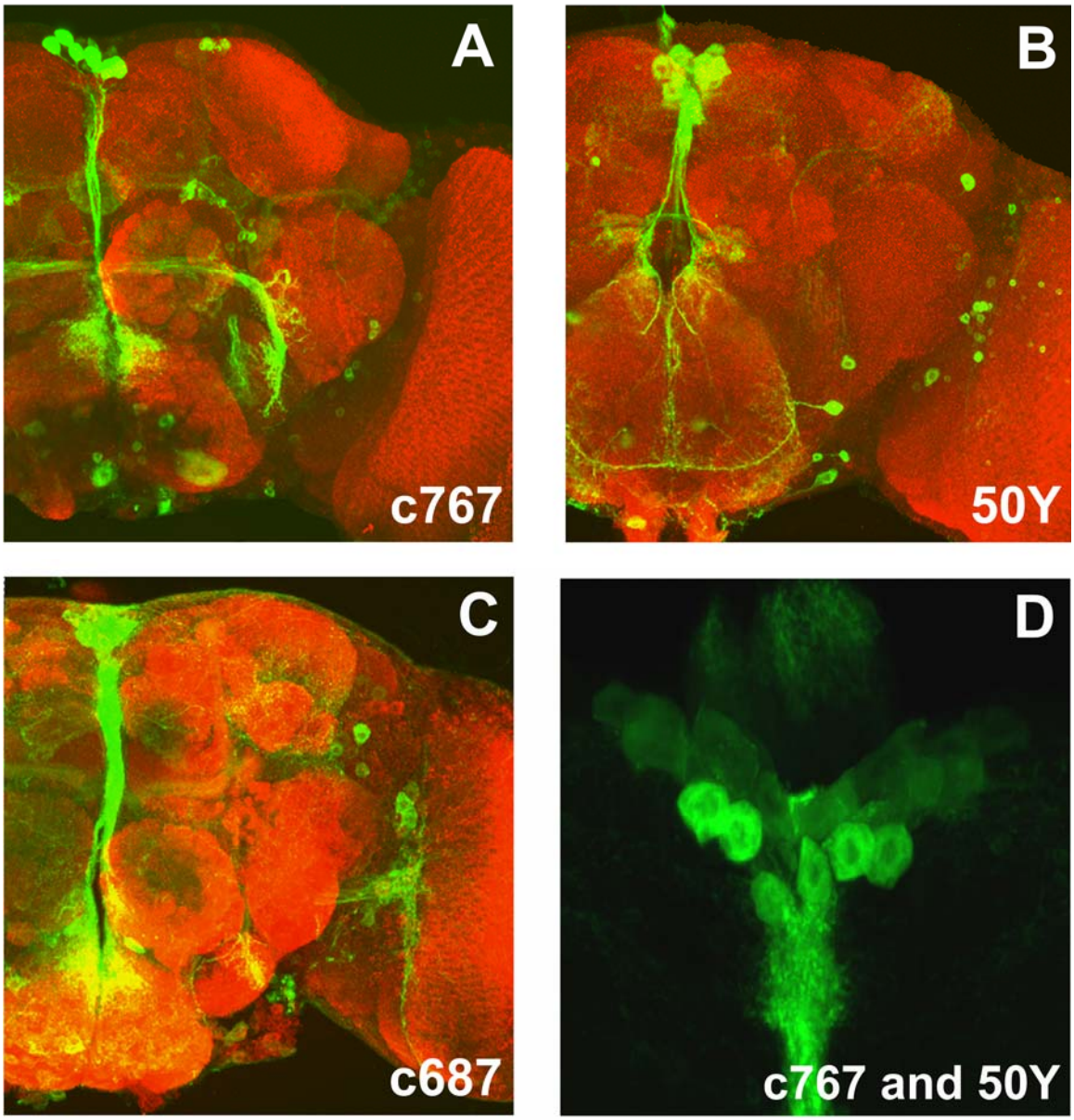

Figure 3.8 Whole-mount brains showing the expression pattern of drivers c767, 50Y and c687

(A-C) Neurons were imaged with a membrane-bound form of GFP expressed under the control of a UAS promoter (UAS-mCD8::GFP.L) (green). The cells at the top are a part of the pars intercerebralis (PI), whose axons innervate the tritocerebrum. The neuropil staining antibody nc82 was used to visualize overall brain structure (red). (D) A close up of the PI region of a c767,50Y>*CD8::GFP* brain. Note 6 bright cells, a possible region of c767 and 50Y overlap.

Next, I asked whether endogenous *rho* is expressed in these PI cells. Using fly brains of genotype 50Y>*LacZ*, I found that *rho* is present in many of the large PI cells (Figure 3.9, red), and some overlap with 50Y cells marked by LacZ (Figure 3.9B,C, green). DAPI DNA staining revealed that *rho* is not present in all cells, and that in those cells that do express *rho*, the RNA transcript is restricted to the cytoplasm, as expected (Figure 3.9C). To strengthen confidence in the specificity of the *rho* RNA expression pattern, I stained w^{1118} brains for Rho protein with anti-Rho antibody (Figure 3.9D), and observed similar results in the PI (note that Rho is also expressed in other parts of the brain).

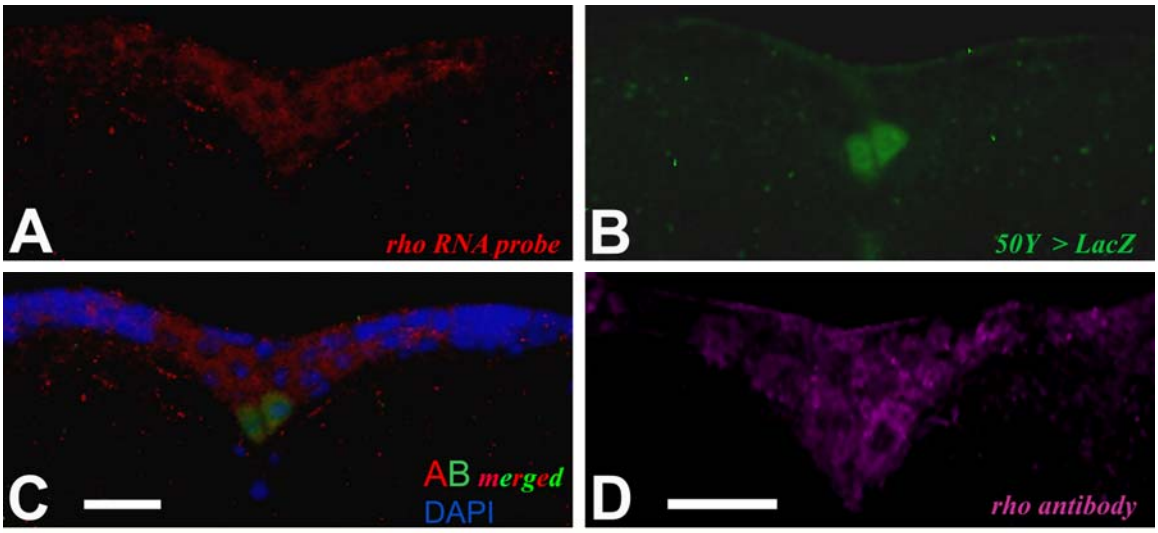

Figure 3.9 Rho is expressed in pars intercerebralis (PI) cells.
(A-C) A 2 µm frontal section from the PI region of a 50Y>*LacZ* brain co-stained for *rho* with antisense RNA (A, red), and anti-β-Gal (B, green), and were merged in (C) along with DAPI staining (blue), to show the overlap between *rho*, 50Y, and cell nuclei. (D) The PI region in an independently stained w^{1118} brain with anti-Rho showing the same pattern as the *rho in situ* hybridization (A). Scale bars represent 20 µm.

3.7 Cell of the pars intercerebralis terminate on areas containing unphosphorylated ERK

Given the overlap of the Gal4 drivers in the PI, I asked whether these cells projected into areas containing inactive ERK, which could potentially be activated with appropriate cellular signals.

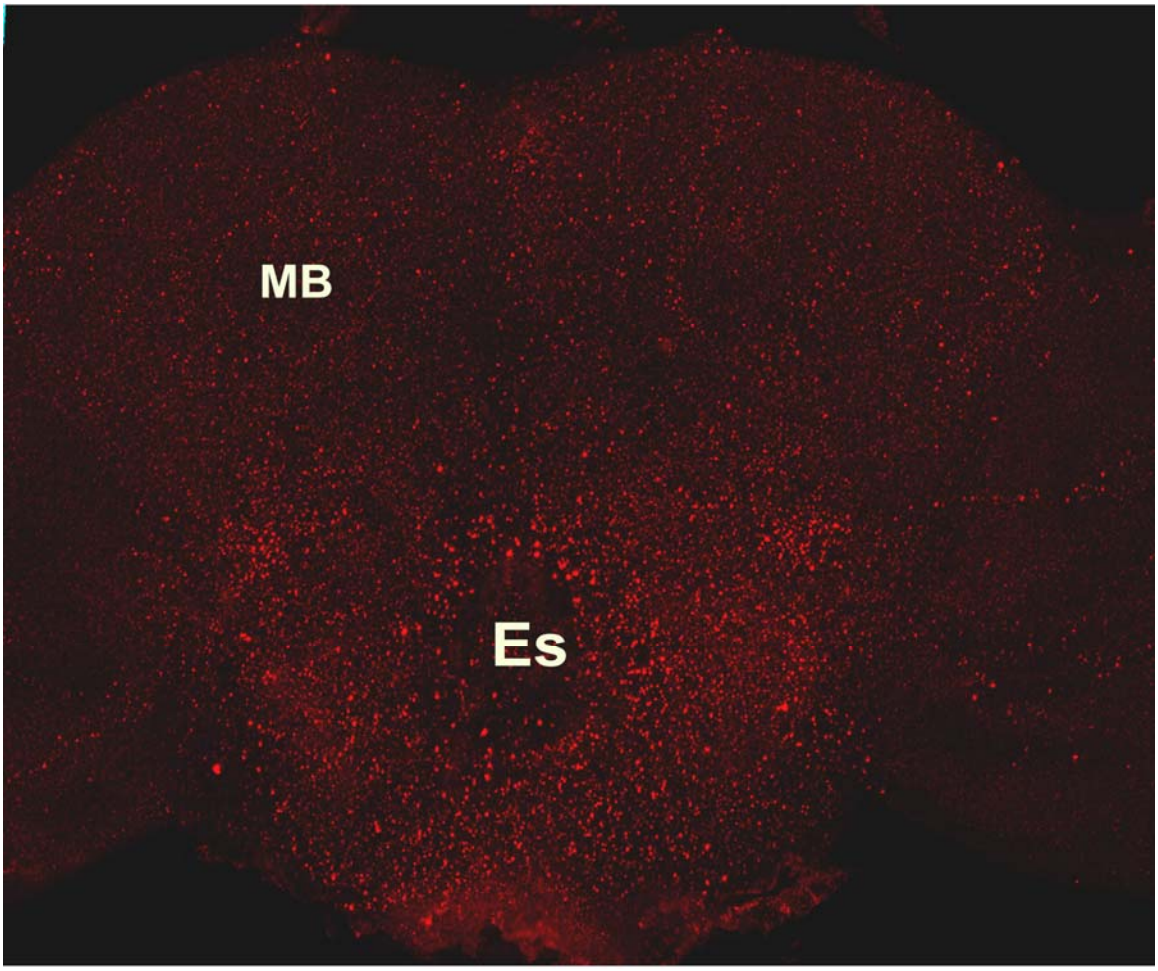

Figure 3.10 Unphosphorylated ERK in the brain
A whole-mount dorsal view of a brain labeled with an antibody specific for the unphosphorylated form of the ERK activation loop. Es=eosophagus, MB=mushroom body

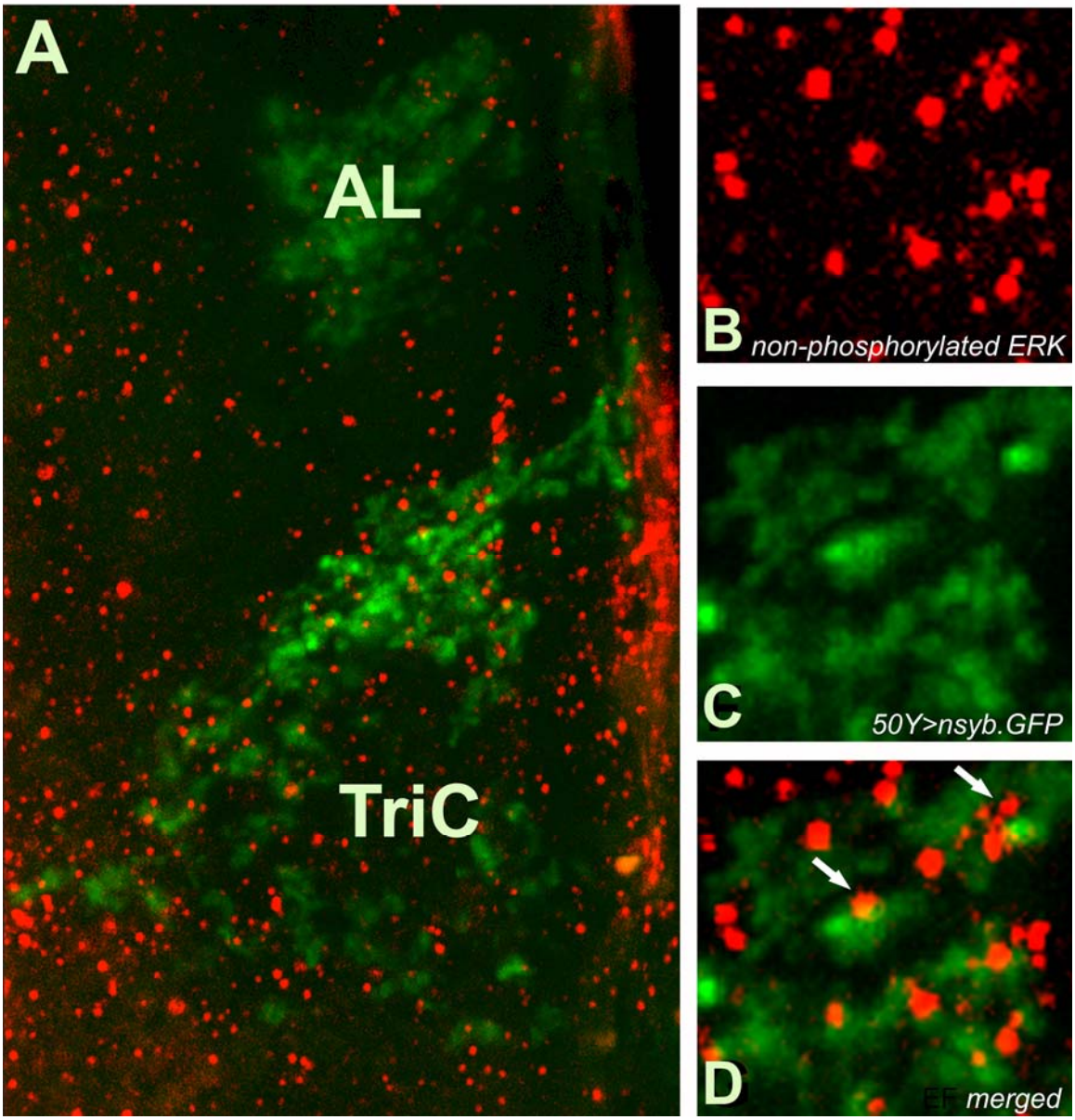

Figure 3.11 Cell of the PI terminate on areas containing unphophorylated ERK
GFP tagged neural Synaptobrevin was used to visualize individual synaptic terminals of 50Y PI cells (50Y>*nsyb.GFP*). (A) A cumulative Z-series of the tritocerebrum showing penetration by PI cells from driver 50Y (green), and staining with the non-phosphorylated ERK antibody (red). AL=antennal lobes, Tric=tritocerebrum. (B-D) A close-up of the dorsal tritocerebrum at the highest density of PI cell innervation showing individual synapses of the 50Y PI cellls. (D) B and C merged. The two structures are adjacent, but do not overlap within the same plane (arrows). The images in A-D are a projection of a 9 μm thick tissue, collected at 1 μm intervals.

To reveal the global localization of ERK in the fly brain, I labeled whole-mount brains with an antibody specific for the non-phosphorylated form of the ERK activation loop. This is the exact same sequence used for making the antibody against the phosphorylated form of ERK used in Chapter 2, except that ERK does not have to be activated in order for the antibody to detect it. This staining pattern revealed that ERK is expressed heavily throughout the central portion of the fly brain, localized to areas that appear to be synapses (Figure 3.10).

When brains were doubly labeled with the non-phosphorylated ERK antibody as well as with a GFP tagged form of neural Synaptobrevin driven by 50Y, the terminals of the PI cells innervating the tritocerebrum were seen to terminate juxtaposed to, but not superimposed on structures carrying ERK (Figure 3.11), suggesting a pre- and post-synaptic relationship between the two areas.

3.8 Expressing dominant-negative Egf-r with drivers 50Y, c767 and c687 does not affect sleep

To further investigate the relationship between the PI cells and Egf-r signaling, I over expressed Egf-r^{DN} with 50Y, c767 and c687, and found that there was no detectable effect on sleep levels (Figure 3.12). This result demonstrates that Rho signaling is most likely not an autocrine mechanism in these cells. If it were, inhibiting the Egf-r receptor should have the same effect as inhibiting Rho expression within the same cells.

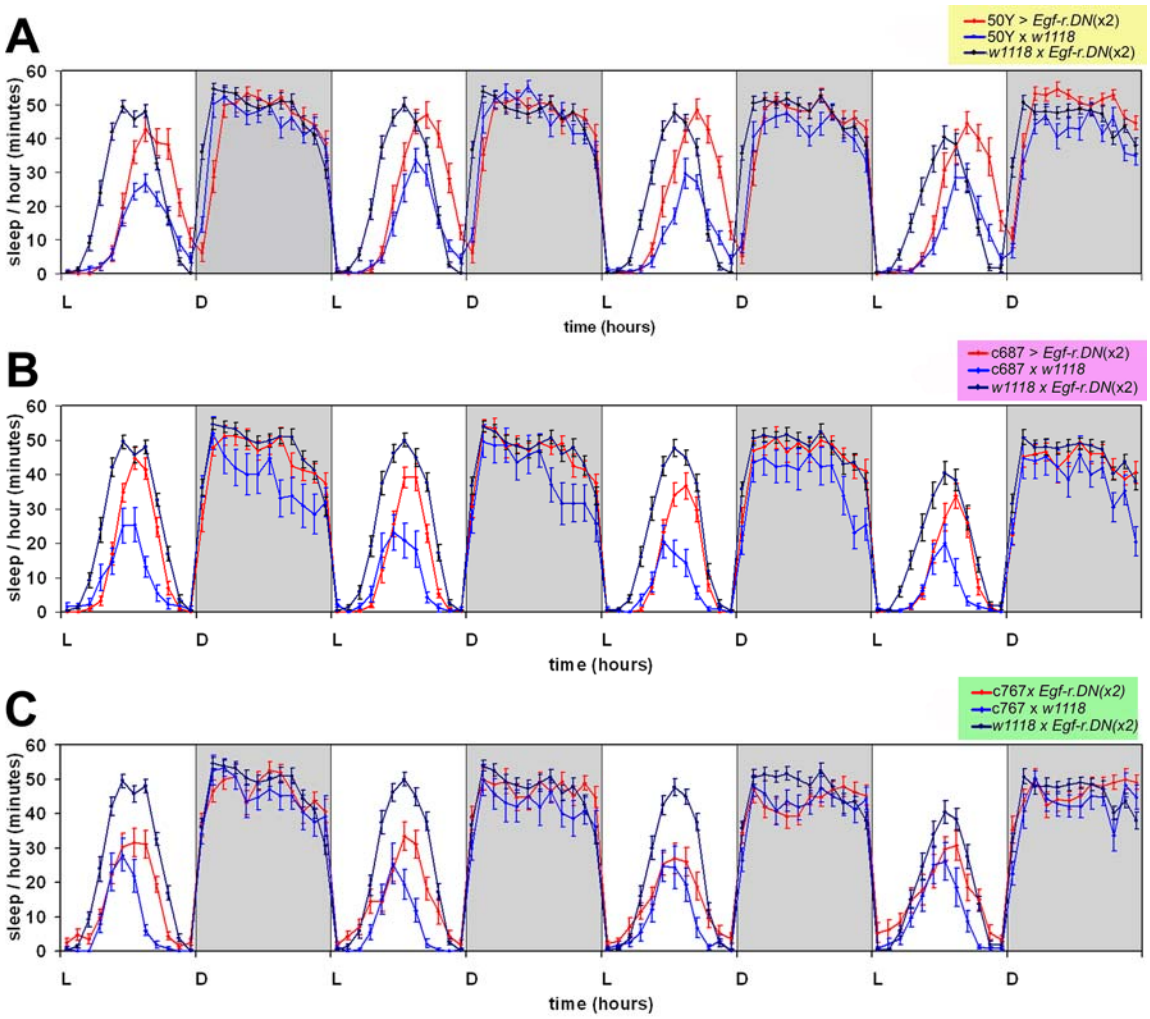

Figure 3.12 Expressing Egf-r^{DN} with drivers 50Y, c767 and c687 does not affect sleep
Gal4 drivers were crossed to a stock carrying two copies of UAS-Egf-rDN. Graphs show four days of activity monitoring. L=lights on, D=lights off.

Together, these activity results along with the non-phosphorylated ERK staining pattern, suggest that the Rho positive cells of the PI serve as pre-synaptic elements which activate Egf-r and ERK in postsynaptic neurons within the tritocerebrum. This observation is consistent with mammalian systems in which members of the ErbB family and ERK have been found to directly interact with a post-synaptic density protein (PSD-

95) (Sabio et al., 2004), and *Drosophila* may have a similar arrangement (see Discussion).

3.9 Materials & Methods

Drosophila stocks and conditions

Flies were raised on yeast/molasses/agar food, and assayed at 23ºC under 12 hr light: 12 hr dark conditions. *W**;;UAS-*rho*^{DN} was obtained from A. Guichard and E. Bier (UCSD, La Jolla, CA). 50Y, c687 and c767 were obtained from D. Armstrong. *Elav*-Gal4, w^{1118} (#5905), *w**;UAS-*Egf-r*^{DN};UAS-*Egf-r*^{DN}, *w**;UAS-*mCD8::GFP.L*, *w**;;UAS-*n-syb.eGFP* were obtained from the Bloomington Stock Center (Bloomington, IN).

Activity data collection and analysis

Sleep deprivation experiments were performed on a vibrating platform with 10 second vibrations at 3-4 min intervals, and processed as previously described (Shaw et al., 2002). For further information refer to section 2.7

Immunohistochemistry

Rho and inactive ERK: Heads were fixed in 6 % Paraformaldehyde/PBS for 1 hour. Anti-Rho was used at 1:500. The exclusively non-phosphorylated ERK antibody was used at 1:500 (Sigma). For all antibodies except ppERK, the following blocking buffer was used: 0.3% Tx-100, 0.3% deoxycholate, 5% normal goat serum, 0.2% BSA in PBS. For microscopy refer to section 2.7.

***Rhomboid in situ* hybridization with β-Galactosidase immunohistochemistry**

Heads and probosci of 50Y>*LacZ* flies were removed on ice, and fixed for 1 hr on a shaker at 50 rpm in 6% Paraformaldehyde/PBS pH 9.5 to enhance signal (Basyuk et al., 2000). Heads were washed for 3x10 mins. in PBS pH 7.4. Brains were dissected in PBS, pH 7.4. Brains were dehydrated in ethanol series: 5 mins. each 30%, 50%, 70%, 2x 100%, no detergent. They were then rehydrated 5 mins. each in 70%, 50%, 30%, then 3x 4 mins. in PBS 0.1% Tween-20 (PBT). Brains were permeabilized with 10 μg/ml proteinase K in PBT for 1 minute. Proteinase K reaction was stopped with 3x3 min. rinses in PBT + 10 mg/ml glycine, then rinsed 5x5 mins. in PBT. They were then re-fixed in 4% paraformaldehyde for 20 mins. The rest of the procedure followed standard *in situ* hybridization methods. DNP labeled *rho* anti-sense RNA was a gift from David Kosman and William McGinnis (UCSD, La Jolla, CA). Anti-β-Galactosidase was used at 1:1000 (Promega).

Parts of this chapter were adapted from segments of the following submitted paper, of which I was the primary researcher and author:

Foltenyi, K., Greenspan, R.J., and Newport, J.W. (submitted Nov., 2006) Activation of Egf-r/ERK by Rhomboid signaling regulates the consolidation and maintenance of sleep in *Drosophila*. *Cell* (submitted).

3.10 References

Guichard, A., Srinivasan, S., Zimm, G., and Bier, E. (2002). A screen for dominant mutations applied to components in the Drosophila EGF-R pathway. Proc Natl Acad Sci U S A *99*, 3752-3757.

Sabio, G., Reuver, S., Feijoo, C., Hasegawa, M., Thomas, G. M., Centeno, F., Kuhlendahl, S., Leal-Ortiz, S., Goedert, M., Garner, C., and Cuenda, A. (2004). Stress- and mitogen-induced phosphorylation of the synapse-associated protein SAP90/PSD-95 by activation of SAPK3/p38gamma and ERK1/ERK2. Biochem J *380*, 19-30.

Taghert, P. H., Hewes, R. S., Park, J. H., O'Brien, M. A., Han, M., and Peck, M. E. (2001). Multiple amidated neuropeptides are required for normal circadian locomotor rhythms in Drosophila. J Neurosci *21*, 6673-6686.

Chapter 4

Discussion, Conclusions and Implications

4.1 Summary of findings

The findings reported in this thesis show a novel role for Egf-r/ERK signaling in sleep consolidation and maintenance in *Drosophila melanogaster*. In the adult fruit fly, Egf-r is expressed ubiquitously throughout the nervous system (Schejter et al., 1986), where its only known function is a role in the maintenance and survival of neurons (Botella et al., 2003). My analysis revealed that overexpression of Egf-r pathway signaling components Rho and Star causes an acute, reversible and dose-dependent increase in sleep that tightly parallels an increase in phosphorylated ERK in the head.

In contrast to the increase in sleep amount after Rho overexpression, inhibiting it lead to a significant decrease in sleep. Importantly, this decrease in sleep was due to a dramatic shortening of the duration of sleep episodes accompanied by an elevation of sleep bout number. This observation suggests that flies have an increased need for sleep, but are unable to stay asleep, perhaps analogous to insomnia in humans. Therefore, I propose that the Egf-r pathway might function in sleep maintenance.

Previous experiments on Egf-r signaling in mammalian systems have demonstrated that continuous ectopic activation of the ErbB family of receptor tyrosine kinases in the central nervous system suppresses waking activities such as wheel-running, grooming and feeding in the hamster, and can enhance spontaneous sleep in rabbits (Kramer et al., 2001; Kushikata et al., 1998; Snodgrass-Belt et al., 2005). The study performed on hamsters found that constant release of an ErbB ligand into the tissues surrounding the mammalian circadian center (subparaventricular zone), the suprachiasmic nucleus (SCN), resulted in the disruption of the rhytmicity of such

behaviors as sleep, body temperature and general movement. However, the amount of time the animals spent engaging in these activities was still approximately normal. Together, the rabbit and hamster studies suggest that ErbB signaling could be involved in sleep. My results demonstrate that *Drosophila* also experiences increased sleep with over stimulation of Egf-r signaling as in rabbits, but in addition I find that Egf-r signaling is necessary for maintenance of the sleep state.

4.2 Overexpression of Rho, Star and Egf-r receptor mutants

Overexpression of the Egf-r ligand processors Rho and Star resulted in a dose-dependent increase in sleep levels. Based on behavioral and biochemical data, the time course of the excessive sleep spans approximately 40 hours following the 8th hour after heat induction of Rho and Star. In many instances, I observed the increase in sleep to be followed by a subsequent compensatory decrease in sleep levels, from which flies take several days to return to baseline sleep levels. There could be a biochemical or a behavioral explanation for this sleep decrease. Biochemically, it is clear from the results of the Western Blot on activated ERK vs. Rhomboid protein levels (Figure 2.7), that Egf-r signaling is down-regulated in response to over activation, and it might take the fly several days to recalibrate its Egf-r levels (see Appendix A.3), and thus normal signaling from this pathway. Behaviorally, the compensatory decrease in sleep could be due to a feed-back of the excessive sleep on the sleep homeostat itself, just as sleep deprivation causes a sleep debt and subsequent rebound by the same mechanism.

Inhibition of Egf-r with a dominant-negative form of the receptor (Egf-r^{DN}) was able to suppress the increase in sleep levels produced by Rho and Star, along with phosphorylation of ERK in fly heads. This reversal of Rho and Star mediated changes in sleep when they are co-expressed with Egf-r^{DN} demonstrates that the signal is mediated by the Egf-r pathway. In fact, the co-expression of Rho, Star and two copies of Egf-r^{DN} resulted in a statistically significant drop in sleep levels compared to the controls. This decrease was not observed when Egf-r^{DN} was over expressed on its own. One explanation for this effect could be that since Egf-r is expressed ubiquitously throughout the nervous system, it might have roles in other behaviors that may be independent from Rho activation (such as Vein, discussed later). Thus, inhibiting Egf-r everywhere could result in a behavioral output that has an undefined interference with sleep behavior. On the other hand, co-expressing Egf-r^{DN} with Rho and Star could have created a sensitized background for the action of Egf-r^{DN} by activating a feed-back loop that achieves a down regulation of wild-type Egf-r by increasing its ubiquitination and degradation (Meisner et al., 1997; Sturtevant et al., 1994; Waterman et al., 1999) only in specific neurons that are in a position to be modulated by Rho.

A result not mentioned in any of the preceding chapters is that when a constitutively active form of Egf-r was expressed (Egf-r.lambdatop), there was a decrease in sleep levels compared to baseline (Appendix A.4). This result demonstrates most clearly that in studies of behavior one has to be very careful about where a process is being manipulated, and not take it for granted that something applies on a global level. For example, Egf-r and ERK (Figure 3.10) are close to being ubiquitous throughout the nervous system. In that case, many neurons responsible for other behaviors than sleep

would give a completely different behavioral output if, for example, ERK signaling was in some form modulated within other cells than the ones responsive to Rho and Star. In this light, studying a mutant of ERK (*rolled in Drosophila*) would be completely futile, since who could predict what the behavioral consequence of a mutant ERK expressed in all of the locations seen on Figure 3.10 would be? I have learned that when it comes to behavior, the location of the switch is as important as what the switch controls, and results that at first might seem contradictory are not necessarily so.

4.3 Egf-r regulation of sleep bypasses the circadian

According to Borbély's two-process model, sleep onset is regulated by two main inputs: the circadian rhythm which regulates the timing of sleep independent of prior sleep and waking and determines the alternation of periods with high and low sleep propensity. The other component is the sleep homeostat that determines sleep need, and is regulated by actual time spent awake or asleep (Borbely and Achermann, 1999). In both *Drosophila* and mammals, the circadian component has been identified as residing in specific areas of the brain, which are located in clusters of neurons dispersed in a non-centralized fashion within the *Drosophila* brain (Kaneko and Hall, 2000), but have been centralized to the SCN locus within the hypothalamus in mammals (Mignot et al., 2002). The sleep homeostat is not understood in either organism, researchers have yet to identify what factor is being measured, and where (Mignot et al., 2002).

My results demonstrate that the regulation of sleep by Rho does not influence the phase of the circadian rhythm, whether Rho is over- of under expressed. That is, neither

does the alteration in Rho protein change the period of the circadian rhythm, but the consequent change in sleep behavior also does not feed back onto circadian timekeeping processes. Only the level and consolidation of sleep is being influenced, not its circadian timing. In the mammalian studies, the Egf-r signals were originating from the SCN, but that is not the case in this study since inhibiting Rho expression with *rho*^{DN} in the circadian regulatory cells with *period*- or *timeless*-Gal4 did not change sleep patterns (Table 3.1). This does not mean that Egf-r signaling is not regulated by inputs from the circadian rhythm, but it is not a part of the circadian machinery itself. Therefore, the effects observed on sleep regulation by aberrant Egf-r signaling were most likely a signal coming from a region of the brain that lies downstream of circadian control and is involved in the sleep process itself.

4.4 Brain regions involved in Egf-r mediated sleep

The brain regions that appear to be involved in the influence of Rho/Egf-r/ERK signaling on sleep are the pars intercerebralis (PI), median bundle, and tritocerebrum, illustrated in Figure 4.1. Although the mushroom body is the only region of the Drosophila brain that has been previously reported as having an effect on sleep (Joiner et al., 2006; Pitman et al., 2006; Yuan et al., 2006), I did not observe Rho expression in the mushroom body, nor did inhibiting Rho with UAS-*rho*^{DN} in this structure have any effect on sleep levels (Table 3.1).

Cells of the PI send out axonal projections through the median bundle and then bifurcate, innervating the tritocerebrum or running alongside the oesophageal canal to

innervate the endocrine gland called the corpora cardiaca (Rajashekhar and Singh, 1994). My results indicate that the PI cells innervating the CC are not the ones responsible for the observed decrease in sleep, since Gal-4 drivers active in these PI cells (Siegmund and Korge, 2001), did not produce a significant drop in sleep levels when expressing *rho* RNAi (Table 3.1).

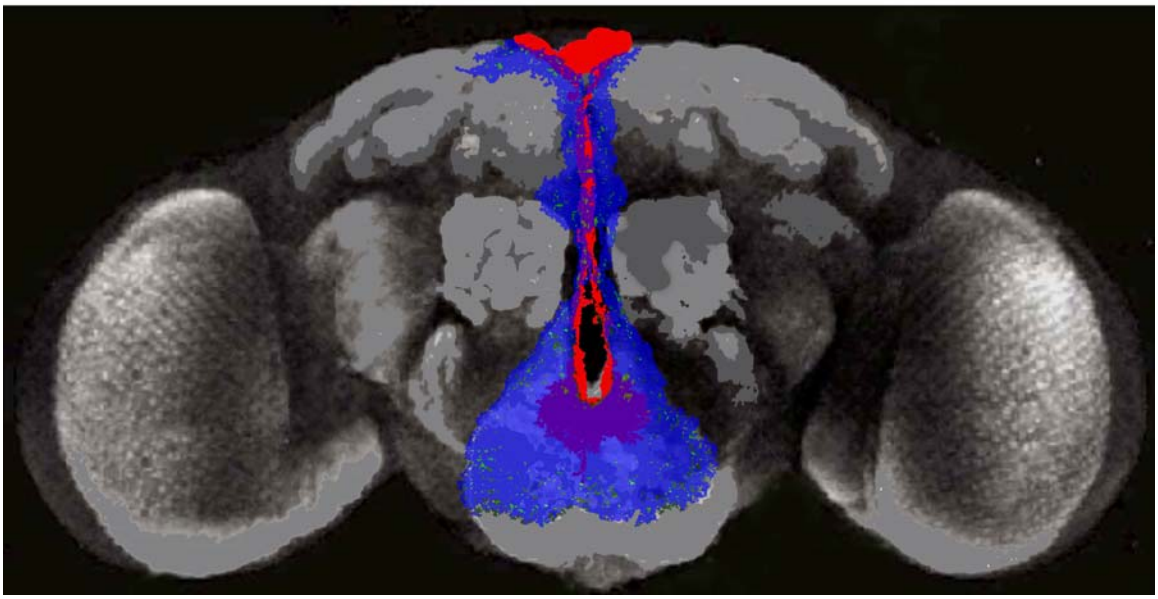

Figure 4.1 Brain regions involved in Rhomboid/Egf-r/ERK sleep
Composite false colored assembled image of c767>*mCD8::GFP.L* driver pattern in the PI, median bundle and tritocerebrum (bright red), activated ERK (ppERK) in response to Rho and Star overexpression (blue), overlaid onto an image of a *Drosophila* brain. The expression patterns were "grabbed" from the appropriate stained brains by the "magic wand" tool in Photoshop, and adjusted to fit onto the scale of the portrayed brain.

Since the PI anatomically receives projections from cells expressing the circadian proteins Period and Timeless, it has been previously proposed that cells of the PI might receive circadian inputs that, in turn, result in the rhythmic release of neuropeptides

(Kaneko and Hall, 2000), a function crucial for the circadian aspect of sleep regulation if neuroendocrine signaling is involved. My results complement this proposal in the sense that a PI role in sleep would likely respond to circadian input.

The PI together with the CC have been suggested to be the developmental equivalent of the mammalian hypothalamic-pituitary axis (Chang et al., 2001; De Velasco et al., 2004; Veelaert et al., 1998). The hypothalamus and PI are also functionally analogous, that is in both insects and vertebrates, neurosecretory neurons located in the anteromedial brain produce peptide hormones that are transported along axons to a peripheral gland. In *Drosophila*, this gland is the corpora cardiaca located in the thorax, in vertebrates it is the pituitary gland located directly beneath the base of the hypothalamus (Figure 4.2). However, the hypothalmus regulates many other functions that do not involve signaling to the pituitary, but to other parts of the brain including the brainstem and the cortex (Figure 4.2). My results also show that the regulation of sleep in *Drosophila* by the PI is most likely not mediated though signaling to the CC, but to the direct modulation of other brain centers, such as the tritocerebrum and its dorsal protocerebral projections, suggesting that the PI shares some functional homology with the hypothalamus in its involvement in regulating arousal.

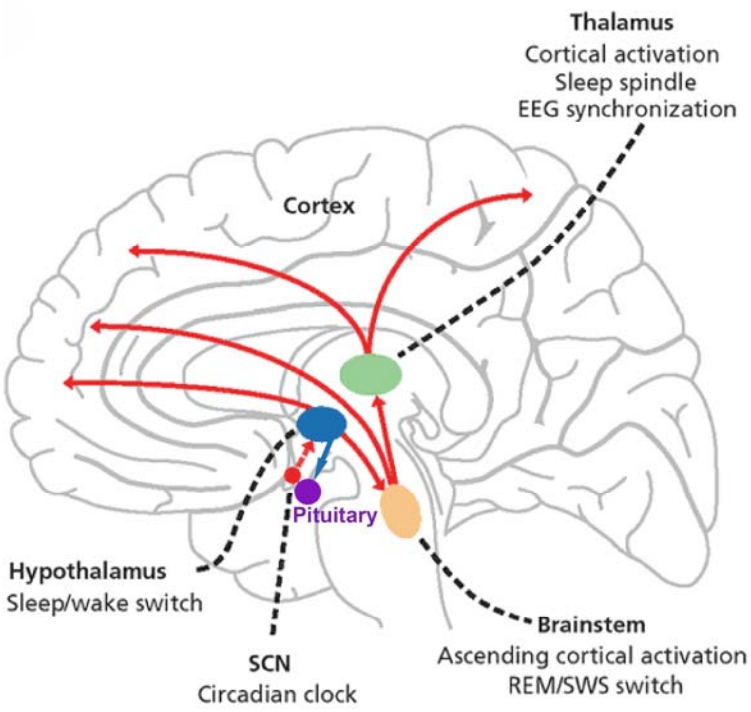

Figure 4.2 Major brain centers regulating arousal in the human brain
Distinct roles of the brainstem, thalamus, hypothalamus and cortex in vigilance control (red arrows). The anatomical relation of the pituitary gland to the hypothalamus is also shown. This figure was adapted from (Mignot et al., 2002).

The hypothalamus is a major center in the mammalian brain for the regulation of arousal (Kilduff and Peyron, 2000; Mignot et al., 2002; Saper et al., 2001; Saper et al., 2005), and the SCN, which is a part of the hypothalamus, has already been shown to regulate activity through Egf-r signaling. Our results, however demonstrate that the disruption of Egf-r ligand production affects sleep through the PI and not the circadian control center of the *Drosophila* brain. This suggests the possibility that the Egf-r or ErbB regulation of arousal in the vertebrate hypothalamus signals through a yet

undiscovered locus within the hypothalamus other than the SCN. It is also possible that through evolution the function of Egf-r signaling already present in the PI/hypothalamic precursor fused with circadian control centers as they coalesced into one locus within the vertebrate hypothalamus. This signaling would then have been further modified to regulate aspects of waking behavior rather than the direct modulation of sleep.

At this time it is not possible to say which of the above two scenarios is more likely to be true. It would be of interest to investigate further if any other loci within the hypothalamus also secrete ErbB ligands, or if other projections from the SCN are important in sleep regulation. Focused vertebrate studies done thus far have only investigated Egf-r signaling in the subparaventricular zone, a region located immediately adjacent to the SCN, and this region did not have an affect on total sleep levels. However, the study performed in rabbits where EGF was administered diffusely to the whole brain found that the animals increased the amount and intensity of both non-REM and REM sleep. These studies do hint at an involvement of ErbB signaling in the regulation of arousal other than the SCN and/or the subparaventricular zone is highly likely in vertebrates, just as we have found Egf-r signaling to function in *Drosophila*.

4.5 What could ERK be doing in cells receiving the Egf-r activating signal?

In the fly, a single member of the Egf-r family binds both the TGF-α-like family of ligands (Spitz, Gurken, Keren), and also the neuregulin-like ligand Vein (Shilo, 2005). In vertebrates, these ligands bind to specific ErbB family members, with ErbB-1 (Egf-r) binding EGF and TGF-α, while ErbB-3 and ErbB-4 bind the neuregulins (Yarden and

Sliwkowski, 2001). In mammalian systems, ErbB-2 and ErbB-4 have been shown to co-fractionate, co-immunoprecipitate and to co-localize in cultured rat hippocampal neurons with the post-synaptic density protein PSD-95 (also known as SAP90), while being excluded from pre-synaptic terminals *in vivo* (Garcia et al., 2000; Huang et al., 2000). Similarly, ERK co-localizes with, and directly phosphorylates PSD-95, as is the case with the ErbB receptor family members (Suzuki et al., 1999; Suzuki et al., 1995). In the fly, Egf-r has also been found to interact with the post-synaptic density protein Discs Large (Dlg), the *Drosophila* homologue of PSD-95 (Humbert et al., 2003), and our results also suggest that the synaptic terminals of the PI cells innervate the tritocerebrum adjacent to cells containing the non-phosphorylated, inactive form of ERK.

ERK appears to have an ever-growing array of targets in innumerable cell types. Recently, it has been shown that the ERK signaling cascade is indispensable for its role in synaptic plasticity, where it has been shown to activate transcription factors and cytoplasmic kinases for the regulation of protein synthesis, gene expression and receptor trafficking (Sweatt, 2004). Importantly, the role of ERK signaling in synaptic plasticity has been conserved among *Aplysia, Drosophila,* and mammals (Hoeffer et al., 2003). Most recently, ERK has been shown to directly phosphorylate the pore-forming α-subunit of the A-type potassium channel Kv4.2, a member of the Shal-type (Shaker-like) family (Adams et al., 2000; Schrader et al., 2006). This broadens the role of ERK beyond the realm of cell proliferation, differentiation, and even long-term memory consolidation, and suggests it may also contribute to the more immediate alterations of the electrical properties of the neuronal membrane.

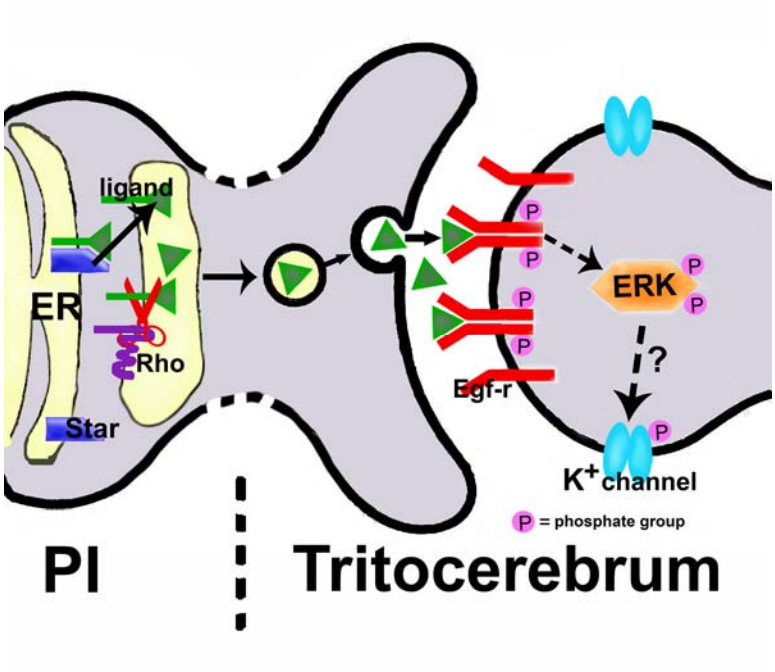

Figure 4.3 A model for the pathway leading to ERK activation in the tritocerebrum
Cartoon model of Egf-r ligand activation and secretion by Star and Rho in a PI cell, and activation of Egf-r and ERK at a synapse in the tritocerebrum.

Based on my findings and the published reports on the functions of Egf-r, I propose the following cellular mechanism for sleep regulation in *Drosophila* (Figure 7): Star and Rho in the PI produce and secrete ligand which activates Egf-r located at the post-synaptic membrane of neurons in the tritocerebrum, leading to the activation of ERK within these cells. Based on the difference in staining pattern between inactive ERK clustering near synapses, and active ERK located out in the axons, the activated ERK at least in part translocates from the post-synaptic membrane and spreads out into the axons that fill out the tritocerebrum and other locations to which these cells project. Due to a lack of ppERK in the cell bodies of these neurons and to the reversible nature of the sleep

behavior, it is unlikely that these cells are undergoing long-term synaptic structural changes associated with changes in gene expression. Instead, I propose that the action of ppERK occurs at the synapse and/or in the axon, where it is possibly altering the gating of neural receptors or channels. Thus, the membrane properties of the cells are altered in such a fashion as to modify their excitability or synaptic signaling to other neurons. This modification results in an altered brain state that ultimately manifests itself in the sleep behavior of the animal. Such a model would be consistent with a previously described mutation in the potassium channel *shaker* (Kv1.4), shown to be incapable of getting much sleep (Cirelli et al., 2005).

4.6 Final model of my work

To summarize my work in the Newport Lab, there are basically two opposing states of arousal I found within behaving *Drosophila*, demonstrating the sufficiency and necessity of Egf-r signaling in sleep: (1) Rhomboid overexpression produces a secreted ligand to Egf-r receptors in the brain. This leads to the phosphorylation and activation of Egf-r, which in turn activates ERK within receiving cells. Although at this point the final target of the Egf-r pathway within the receiving neurons is unknown, it does lead to flies that maintain sleep bouts for much longer periods that wild-type, with the overall result of a drastic increase in total sleep levels, most obvious during the daytime. (2) Inhibition of Rhomboid expression in all neurons or a sub-set of them within the pars intercerebralis leads to compromised levels of Egf-r signaling, and presumably ERK activation. This

inhibition of Egf-r signaling results in flies that have a drastic decrease in sleep levels, with highly fragmented and short sleep episodes, most evident during the night.

This study and others performed by numerous labs demonstrate that sleep is a real phenomenon in *Drosophila*. Since the behavioral manifestation of sleep in Drosophila shares many similarities with sleep in vertebrates, the function of sleep in *Drosophila* most likely also shares the same purpose as sleep in higher organisms. This is not surprising, since sleep most certainly evolved out of a fundamental mechanism of neural function common to arthropods and mammals (or as many believe even non-bilateral organisms), although this function remains to be identified. Perhaps this study will also help researchers to get a step closer to that goal, since it demonstrates that it may be possible to move sleep research out of the realm of gross anatomical or neurotransmitter system manipulations, and even to some extent the uncertainty of genetic mutants, and instead focus on identifying sleep effectors within specific target cells of the sleep regulatory loci.

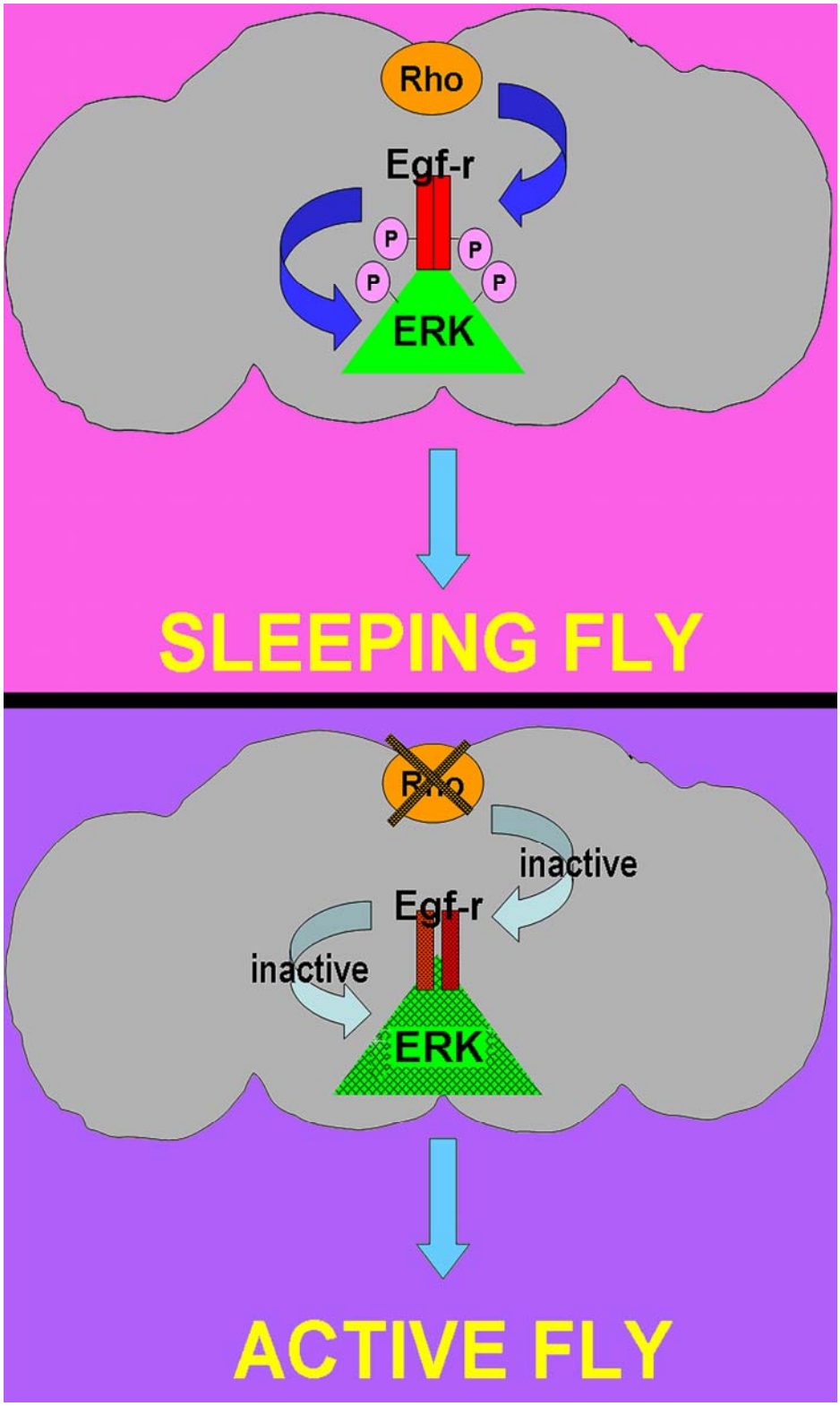

Figure 4.4 Cartoon model demonstrating sufficiency and necessity of Rho signaling as a regulator of sleep in *Drosophila.*

Parts of this chapter were adapted from segments of the following submitted paper, of which I was the primary researcher and author:

Foltenyi, K., Greenspan, R.J., and Newport, J.W. (submitted Nov., 2006) Activation of Egf-r/ERK by Rhomboid signaling regulates the consolidation and maintenance of sleep in *Drosophila*. *Cell* (submitted).

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Appendix

A.1 Expressing *rho*^{DN} along with *tub*-Gal80ts interferes with RNAi folding

As mentioned in section 3.5, performing experiments with *rho*^{DN} in a temporally regulated fashion with *tub*-Gal80ts was not possible due to an interference with the folding of the double-stranded RNAi. I combined UAS-*rho*DN with *tub*-Gal80ts and used the wing Gal4 driver MS1096 to confirm that the Gal80ts was suppressing the missing vein phenotype created when *rho*^{DN} is expressed with MS1096 alone (Figure A1B). To my surprise, the result of MS1096>r*ho*^{DN},*tub*-Gal80ts was the creation of extra veins, meaning that an ectopic expression of wild-type Rho was somehow occurring in this genotype, instead of the inhibition of endogenous Rho (Figure A.1C series). The only possible explanation for this is that since *rho*^{DN} actually consists of a palindrome of full-length *rhomboid* (Figure 3.1), the presence of Gal80ts does not only not fully inhibit Gal4, but also interferes with the folding of the transcribed construct, allowing for its translation into active Rho. Most likely this is not due simply to a low level of *rho*^{DN} present in this genotype, since driving *rho*^{DN} with other weaker wing drivers (1348, 71B and C) did not produce any extra veins (Figure A.1D,E,G). These results demonstrate that if the *rho*^{DN} construct is not expressed at a sufficiently high level, it will fail to inhibit endogenous Rho, but it will not produce extra veins without the presence of *tub*-Gal80ts.

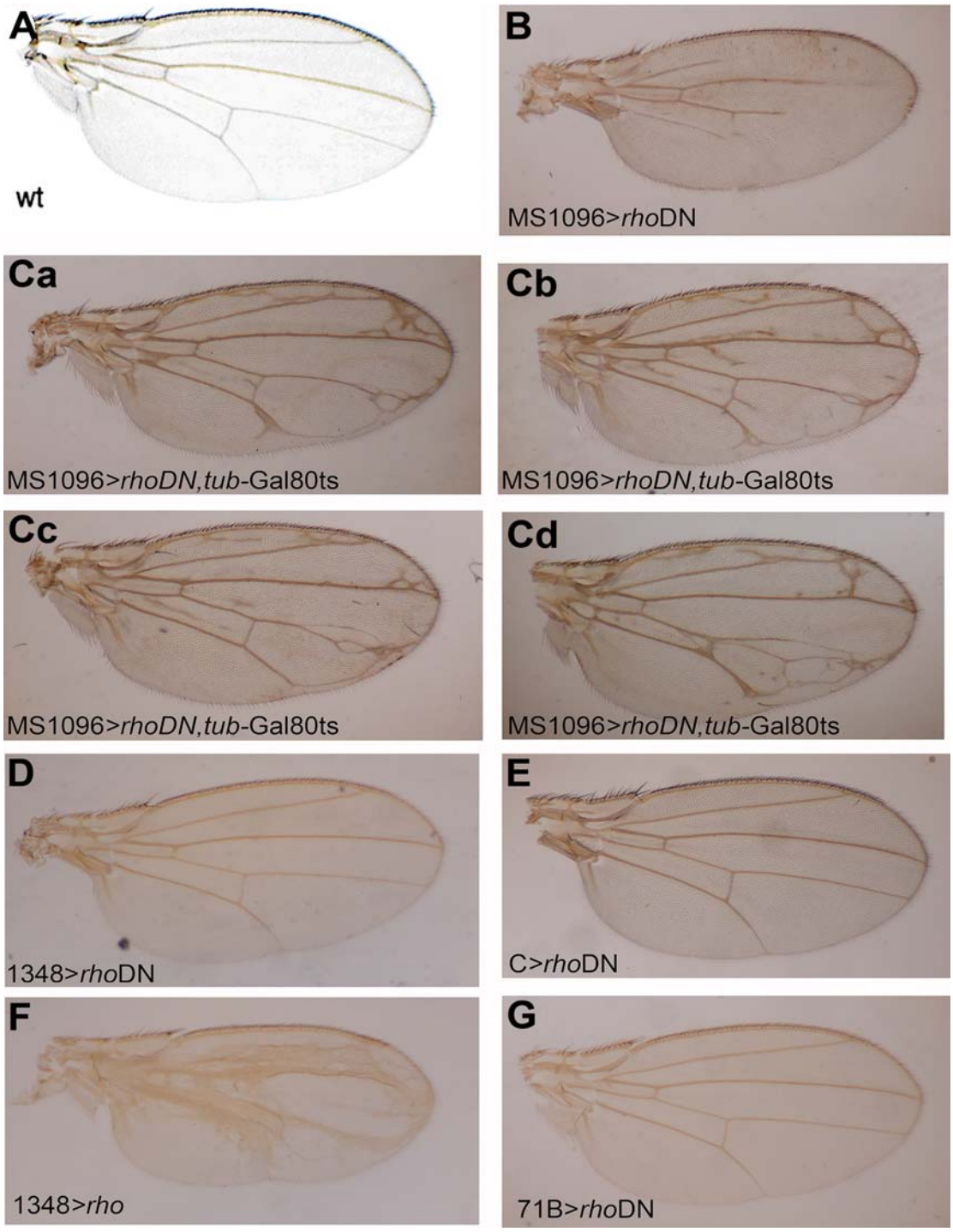

Figure A.1 Expressing *rho*^{DN} along with *tub*-Gal80ts interferes with RNAi folding (A) Wild-type wing. (B)MS1096> *rho*^{DN}. (Ca-Cd) Four different wings from MS1096>*rho*^{DN},*tub*-Gal80ts flies, to demonstrate the variety of extra-vein phenotypes. (D) 1348>*rho*^{DN}. Compare with 1348>*rho*^{wt} in F to show domain of activity in this driver. It covers an area that should produce loss of veins in D if the driver were strong enough. (E) C> *rho*^{DN} (F) 71B> *rho*^{DN}

A.2 High temperatures alter Rho and Star induced sleep behavior.

I was curious if I could verify that drivers 50Y, c767 and c687 were responsible, at least in part, to the increase in sleep seen with the heat shock overexpression of Rho and Star in Figure 2.1. But I was unable to do any experiments utilizing *tub*-Gal80ts for the temporal control of Rho and Star overexpression with drivers 50Y, c767 and c687 because having to assay flies for sleep behavior at 28ºC or 29ºC masked any possible effects on sleep. This temperature effect is demonstrated in Figure A.2 with *hs*-Gal4>*rho,Star* flies. Flies on separate monitors were heat shocked as usual, but then the monitors were split up into an incubator at 23ºC, and another one at 28ºC. During the following day and night, the flies at 23ºC exhibited the same increase in sleep behavior described in Chapter 2. On the other hand, the flies at 28ºC had an alteration in their sleep behavior due to the high temperature: During the following day, the high temperature enhanced the increase in sleep, a phenomenon seen in wild-type flies under such conditions, as if they need to have a strong siesta the same way people living in warm climates do. But during the following night (blue bracket in Figure A.2), the opposite happened: Rho and Star induced sleep was suppressed by the high temperature. This is also a phenomenon observed with wild-type flies, once again drawing a parallel between the inability of humans to sleep in hot weather at night. Overall, these results demonstrate that studying Rho induced alteration is sleep with *tub*-Gal80ts at high temperatures would produce uninterpretable and meaningless results.

To note, it was also nonsensical to try expressing Rho and Star with 50Y, c767 and c687 without *tub*-Gal80ts, since the results of the Western Blot on activated ERK vs.

Rhomboid expression clearly demonstrate that there is desensitization to the over stimulation of Egf-r signaling within two days after heat shock. Flies raised with active Rho and Star overexpression already occurring at the embryonic stage would surely lose all their sensitivity to Rho and Star by the time they are adults. I did however attempt these experiments, but saw no change in the behavior of the flies, as expected.

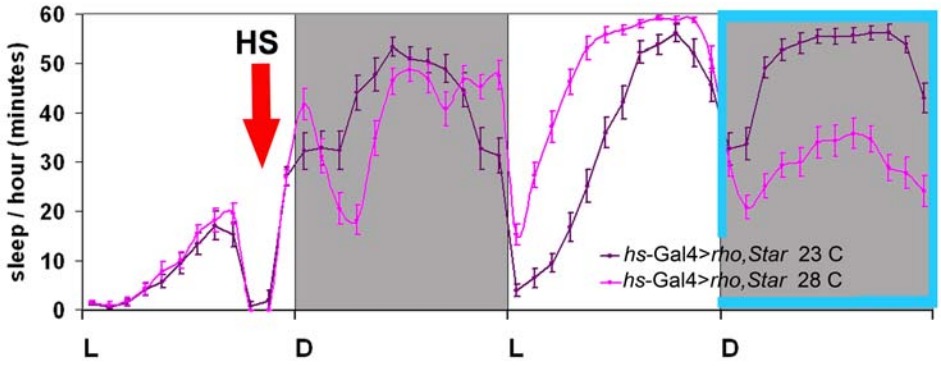

Figure A.2 High temperatures alter Rho and Star induced sleep behavior
Hs-Gal4>*rho*,*Star* flies were heat shocked at the marked time, and then split up into incubators at different temperatures. The high temperature enhances sleep levels during the day, but suppresses them at night.

A.3 Heat shock decreases Egf-r levels in the head

I was curious as to whether I could detect changes in Egf-r levels in the head of the fly in response to Rho expression. For this purpose *hs*-Gal4>*rho,Star* flies were processed for Western Blot analysis during five consecutive days following heat shock expression of Rho and Star, and I did observe a reciprocal change in protein levels with respect to Rho and Egf-r (Figure A.3). Unfortunately this drop in Egf-r protein levels occurred in later experiments with *hs*-Gal4 x w^{1118}, even though ERK is NOT hyperactivated within this control group (Figure 2.7). Therefore, the change in head Egf-r protein levels after heat shock cannot be attributed solely to Rho and Star overexpression, but has a definite component of heat stress independent of prolonged ERK activation. But nonetheless this result does demonstrate that Egf-r levels decrease after heat shock and need several days to recalibrate, although nothing more can be attributed to this effect at this resolution.

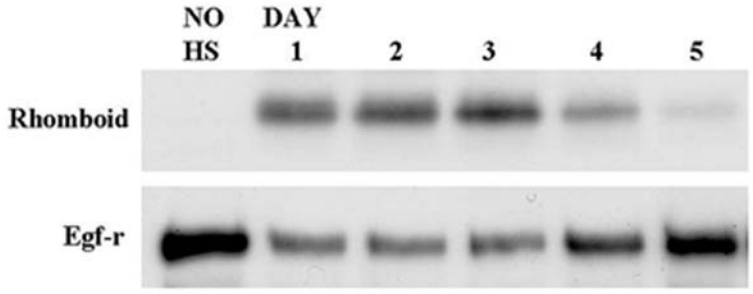

Figure A.3 Heat shock decreases Egf-r levels in the head
Hs-Gal4>*rho,Star* flies were heat shocked, and collected for five consecutive days afterwards. Heads were removed and assayed for Rhomboid and Egf-r levels by Western

Blot. Further experimentation revealed that the decrease in Egf-r levels was due, at least in part, to the heat shock itself independent of Rho overexpression.

A.4 Overexpression of a constitutively activated Egf-r decreases sleep levels

Activation of Egf-r signaling with a constitutively active Egf-r (Egf-r.lambdatop) with heat shock produced flies that had a decrease in sleep (Figure A.3), unlike when Egf-r is activated via an overproduction of ligand with Rho and Star or s-Spitz (Figure 2.1). Egf-r.lambdatop does not need activation by a ligand because its extracellular domain has been swapped by a heterologous dimerization domain from a repressor protein expressed by the Lambda bacteriophage, rendering ligand binding unnecessary for the dimerization and activation of the Egf-r receptor tyrosine kinase (Queenan et al., 1997). The effect on sleep produced by Egf-r.lambdatop is different from Rho and Star overexpression and even s-Spitz overexpression, because instead of all cells being capable of activating Egf-r in other cells as with s-Spitz, Egf-r.lambdatop potentially makes all cells capable of activating Egf-r signaling within themselves. If within these cells Egf-r.lambdatop has the adaptor proteins necessary for recruiting ERK signaling, then Egf-r.lambdatop might activate a response within cells that either don't normally use Egf-r signaling, and/or have Egf-r signaling normally not activated via Rhomboid. The combination of all of these possibilities produces flies that are unable to sleep at normal levels.

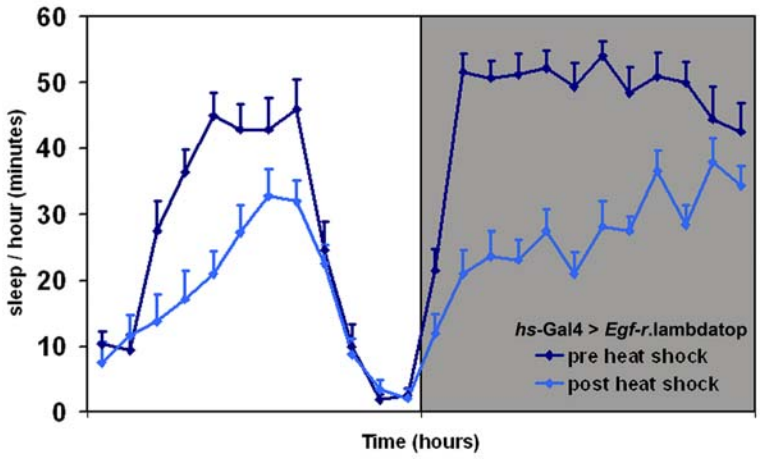

Figure A.4 Overexpression of a constitutively activated Egf-r decreases sleep levels

A.5 References

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