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Use-Dependent Plasticity Regulates Sleep Need in Drosophila Melanogaster

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USE-DEPENDENT PLASTICITY REGULATES SLEEP NEED IN *DROSOPHILA MELANOGASTER*

by

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Sleeping Together: Using social interactions to understand the role of sleep in plasticity

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I. Introduction

Although research animals in laboratory environments are often housed individually in relatively simple enclosures, their wild counterparts must interact in more complex environments outside of a controlled setting. In nature, they must reliably find food, avoid enemies and predators, interact socially with conspecifics and compete for potential mates. While standardized lab environments allow researchers to easily control environmental and social influences, manipulating the social environment of research animals can be a powerful experimental tool. Social experience alters the expression of genes related to synaptic function and plasticity, induces elaborations in the morphology of neural structures throughout the brain (Volkmar and Greenough, 1972; Greenough et al., 1978; Technau, 2007), improves cognitive and behavioral performance (Pham et al., 1999a;

Toscano et al., 2006) and alters subsequent sleep (Ganguly-Fitzgerald et al., 2006). In this review, we discuss the use of social enrichment/isolation as an experimental paradigm to study plastic mechanisms in the brain and to investigate the relationship between sleep and synaptic plasticity.

Although specific details regarding the conditions of "enriched" environments may differ between studies (e.g. – some enriched environments included novel objects that were periodically changed, some consisted of bare enclosures), all include enhanced social exposure. Thus, while non-social factors may contribute to some experimental results, it is likely that many of the experimental outcomes can be attributed to the enhanced social exposure. Greenough et al (1978) compared the effect of two different social enrichment conditions on synaptic ultrastructure. In this study, the first enrichment condition consisted of a large cage in which 12 rats were housed and presented with toys that were changed daily as well as a 30 minute opportunity to explore a different "toy-filled field". In the second enrichment condition, two rats were housed together in a standard laboratory cage. After 30 days in these conditions, rats were sacrificed and tissue from the occipital cortex was inspected for post-synaptic densities containing subsynaptic plate perforations, a marker of increased synaptic strength. While tissue from both enriched groups exhibited approximately 25% more subsynaptic plate perforations than tissue from isolated siblings, there was no difference detected between enriched conditions indicating that social interaction alone, not exposure to novel objects or a spatially larger enclosure,

was likely sufficient to induce the changes in synaptic structure. Similarly, studies of environmental enrichment using the fruit fly *Drosophila melanogaster* have found significant changes in neural structure and behavior that are associated with social interactions and cannot be attributed to differences in enclosure volume or other physical differences in the housing conditions (Heisenberg et al., 1995; Ganguly-Fitzgerald et al., 2006).

II. Social Enrichment induces plastic mechanisms

Following a period of enriched social experience, elaborations in neural circuitry have been characterized in a number of vertebrate and invertebrate species. Neurons in the visual cortex of socially-enriched rats have an increased number of dendritic branches (Volkmar and Greenough, 1972) and show ultrastructural evidence of strengthened synaptic connections (Greenough et al., 1978). Significant increases in the number of hippocampal synapses in socially enriched rats have also been reported (Briones et al., 2006). Structural elaboration of individual cerebellar Purkinje cells in monkeys housed in a social environment suggests that the plastic effects of social enrichment on neural structures are evolutionarily conserved from rodents to primates (Floeter and Greenough, 1979). Elevated levels of neuronal growth factors may promote the increased dendritic elaboration and synaptogenesis that have been observed in response to social enrichment; exposure to an enriched social environment has been associated with increased expression of a number of neurotrophic factors

including *nerve growth factor* (*NGF*) (Torasdotter et al., 1998; Pham et al., 1999a; Ickes et al., 2000), *brain-derived growth factor* (*BDNF*) (Young et al., 1999; Ickes et al., 2000), and *glial-cell-derived neurotrophic factor* (*GDNF*) (Young et al., 1999; Faherty et al., 2005). Neurotrophic factors, particularly *NGF* and *BDNF*, play an important role in synaptic plasticity (Gómez-Palacio-Schjetnan and Escobar, 2008; Hennigan et al., 2009) and impaired neurotrophin signaling results in memory impairments (De Rosa et al., 2005; Walz et al., 2005; Bekinschtein et al., 2007; Heldt et al., 2007).

The observations of structural plasticity in response to social enrichment seem to be widely conserved across species. Structural plasticity in response to enriched environments has been observed not only in mammals, but also in several invertebrate species. Honeybees progress through a series of social roles within the hive structure that are associated with experience-dependent changes in the structure of brain regions including the Mushroom Bodies (MBs) (Withers et al., 1993) and Antennal Lobes (Winnington et al., 1996). Although the mechanisms that control the transition between social roles are not well characterized, these transitions are associated with extensive changes in gene expression profiles, indicating a role for genetic regulation (Whitfield et al., 2003). While genetic studies are difficult to conduct using honeybees, the fruit fly *Drosophila melanogaster* is a widely used genetic model for the study of behavioral genetics. Indeed, social experience does induce significant changes in neuropil structure throughout the *Drosophila* brain (Heisenberg et al., 1995; Technau, 2007). In

these studies, the number of Mushroom Body fibers was significantly elevated in socially enriched animals compared to wild-type siblings housed in isolation or deprived of visual or olfactory stimuli during social interactions. It should be noted that while it is not yet clear whether the increased number of fibers results from increased branching of projections in existing neurons or the birth of new neurons. Interestingly, recent studies showed newly born Kenyon Cells in the Mushroom Bodies of socially-enriched wild-type flies (Technau, 2007). While little other evidence has been found for neurogenesis in the Mushroom Bodies of adult *Drosophila*, observations of newly born neurons have been made in the Mushroom Bodies of other insect species following social enrichment. BrdU labeling was used to reveal a significant elevation in the rate of neurogenesis in the Mushroom Bodies of adult crickets that were housed in a socially enriched environment for several days (Scotto Lomassese et al., 2000; Scotto-Lomassese et al., 2002) and subsequent investigations found this elevation to be mediated via a nitric oxide-dependent signaling mechanism (Cayre et al., 2005). Similar observations have been made in mice. Social enrichment resulted in an overall increase in the number of cells in the hippocampus including a significantly higher number of newly generated neurons and astroytes compared to socially isolated controls (Kempermann et al., 1997). Based on their measurements, Kempermann *et al.* (1997) estimated that, on average, socially enriched mice possessed approximately 40,000 more dentate gyrus granule cells per hemisphere than their isolated siblings. These data suggest not only that the morphology of existing neurons can be altered by social experience, but that the

generation of new neurons can be enhanced in response to the social enrichment.

As mentioned above, an increase in the number of Mushroom Body fibers were found in socially enriched flies of a number of different wild-type strains. Interestingly, no change in fiber number was found in flies with mutant alleles of the classical memory genes *rutabaga* or *dunce* (Balling et al., 2007). Together, these important studies suggest that complex sensory cues that accompany social interactions in the fly induce plastic mechanisms that alter the structure of the Mushroom Bodies, an important structure for associative memory in the fly. Further investigations have found that deprivation of visual stimuli by housing flies in complete darkness also alters the structure of several other neuropils involved in visual processing including the lamina, medulla, and lobula plate (Barth et al., 1997) as well as the volume of the central complex and Mushroom Body calyces (Barth and Heisenberg, 1997). Interestingly, Barth *et al* (1997) describe a critical window for the effects of visual stimuli on the volume of the early visual system that is restricted to the first 4-5 days after eclosion; housing flies in complete darkness starting 5 days after eclosion had no effect on the volume of the lamina. Conversely, another study showed that social experience can alter the volume of the MB calyces and medulla for at least 16 days after eclosion (Heisenberg et al., 1995). Together, these findings suggest that experience-dependent plasticity may be controlled by two separate mechanisms. First, the early visual system (e.g. – photoreceptor projections into the lamina)

may be shaped by visual experience in the first few days after eclosion to optimize the ability of the brain to receive visual stimuli. Second, another type of plasticity that can encode memories throughout adult life can exhibit experience-dependent morphological changes for a much longer period of time and may allow downstream associative centers (including the MB) which are influenced by more complex, multisensory stimuli (including social interactions). This kind of temporal division can also be observed in mammalian models. While ocular dominance plasticity in the visual cortex can be robustly observed during a critical period early in life (reviewed in (Berardi et al., 2000), structural changes in other brain regions including the hippocampus can be observed much later in life (reviewed in (Lee and Son, 2009).

III. Functional Effects of Social Enrichment

Exposure to complex social environments not only alters neural circuitry at the structural level, but also alters the physiological functioning of synapses in circuits throughout the brain. Hippocampal slices from rats housed in an enriched environment, for example, exhibit robust long-term potentiation (LTP) and long-term depression (LTD) in the Schaffer Collateral Pathway, while rats housed in social isolation demonstrate relative impairments for both LTP and LTD at this synapse (Duffy et al., 2001; Artola et al., 2006). Other studies, however, have found that hippocampal synapses in the medial perforant path can become significantly potentiated during social enrichment to the extent that

LTP can be occluded (Green and Greenough, 1986; Foster et al., 1996). Similar studies have found that mice housed in social isolation in lab conditions exhibit impaired LTP in the cingulate cortex when compared to tissue from wild mice that had developed in a natural, socially complex environment (Zhao et al., 2009). Along with the physiological impairments observed in socially isolated animals, exposure to a socially enriched environment increases the expression of glutamatergic AMPA receptors throughout the hippocampus (Foster et al., 1996), providing molecular evidence that glutamatergic synapses in the hippocampus can become highly potentiated during exposure to complex social environments. Because technical limitations have prevented the widespread use of electrophysiology to characterize the activity patterns of *Drosophila* central neurons until recently, very little is known about whether social enrichment induces similar physiological changes in the adult fly. Recent studies have, however, found that social enrichment significantly alters the excitability of motorneurons in larvae (Ueda and Wu, 2009).

Given the structural elaborations and physiological enhancements induced by socially enriched environments, it might be expected that these conditions improve the behavioral and cognitive performance of animals exposed to complex social interactions. Indeed, wild-type animals housed in an enriched environment demonstrate significant improvements in hippocampus-dependent memory in both the Morris Water Maze (Pham et al., 1999b; Briones et al., 2006; Toscano et al., 2006) and Contextual Fear Conditioning (Duffy et al., 2001).

Social enrichment seems to not only improve spatial memory in healthy animals, but may also aid recovery from brain damage following traumatic brain injury, neonatal hypoxia-ischemia, excitotoxic injury and early exposure to lead poisoning; in all of these conditions, enriched animals exhibited significant improvements in behavioral assays and normalized physiological functioning compared to socially isolated controls (Young et al., 1999; Koh et al., 2005; Kline et al., 2007; Cao et al., 2008; Hoffman et al., 2008; Pereira et al., 2008; Pereira et al., 2009). Additionally, many of the cognitive benefits of social enrichment have also been observed in rodent models of neurodegenerative disease. In a rodent model of Parkinsonism, social enrichment during adulthood prevents the death of dopaminergic neurons in the substantia nigra (Faherty et al., 2005). Similar studies using a transgenic mouse model for Alzheimer's Disease found that socially isolated mice expressing a mutant form of the human *Amyloid Precursor Protein* (*APP*) exhibited more rapid decline in memory in the fear conditioning paradigm as well as a dramatic acceleration in amyloid-beta plaque deposition relative to socially housed animals that expressed the same mutant transgene (Dong et al., 2004). Importantly, recent studies of human populations have indicated that increased cognitive activity, including social interaction, throughout life may reduce the risk of developing Alzheimer's disease later in life (Carlson et al., 2008). Together, these data suggest that exposure to social enrichment may induce neuroprotective mechanisms to maintain normal functioning in the face of genetic defects that would otherwise induce impairment. While the mechanisms that mediate these effects are not well

characterized, the same neurotrophic signals that are elevated in response to social enrichment have been found to have neuroprotective effects in disease models for neurodegeneration; infusion of *BDNF* into the entorhinal cortex of aged mice expressing transgenic *APP* restores performance in the Morris Water Maze assay and restored expression of synaptic markers in the hippocampus (Nagahara et al., 2009). Similar effects have been observed by increasing *NGF* signaling (De Rosa et al., 2005) and there is preliminary evidence that these pathways may be effective therapeutic targets for humans afflicted with Alzheimer's disease (Tuszynski et al., 2005). Although the consequences of social interactions on aging have not been well studied in *Drosophila*, recent experiments have suggested that social enrichment can delay premature death in flies mutant for *Cu/Zn superoxide dismutase* (*CuZnSOD*) (Ruan and Wu, 2008) suggesting a phylogenetically conserved role for the functional benefits of social interactions. It is important to note that lifespan extension was not observed in socially enriched wild-type flies indicating that flies lacking *CuZnSOD*, which has been implicated in mechanisms associated with a number of aging-related neurodegenerative disorders including Parkinson's, Huntington's and Alzheimer's diseases, may be especially sensitive to beneficial effects of socially complex environments and further implicates social enrichment as a potential intervention for the alleviation of neurodegenerative disorders (Ruan and Wu, 2008).

IV. Using social enrichment to investigate functions of sleep

A. Sleep & Plasticity

Although sleep is a biological process that is necessary for survival in vertebrates and invertebrates, the underlying biological functions of sleep are currently unknown. A growing body of literature, however, suggests an important and evolutionarily conserved role for sleep in the processing and consolidation of new memories. For example, hippocampal ensembles that were activated together when rats navigated a novel maze were re-activated in an identical manner when the animals slept later (Wilson and McNaughton, 1994). Further studies have found that similar re-activation can be observed in the visual cortex and that replay in these two regions is coordinated to replay the same experience (Euston et al., 2007; Ji and Wilson, 2007). There is also evidence for replay of waking experience during sleep in humans. Subjects were recruited to play the video game Tetris for several hours over the course of three days; over the course of the study, a majority of the subjects reported hypnogogic imagery associated with playing the game (Stickgold et al., 2000). This type of imagery seemed to be associated with the process of learning how to play the game because subjects who had less previous Tetris experience were the most likely to report Tetris-related imagery during dreams at night. This replay of newly formed associations during sleep seems to facilitate the processing and consolidation of those memories. In one recent study, subjects were taught to play a card game while being exposed to a specific odor cue. When subjects were re-exposed to

the same odor cue during slow-wave sleep that night, hippocampal activation was significantly elevated along with significantly improved hippocampus-dependent declarative memory of the card game the next day (Rasch et al., 2007). Additionally, human performance in motor learning tasks stabilized with repeated trials over the course of the day, then dramatically improved following a night of sleep (Walker et al., 2005; Stickgold and Walker, 2007) and sleep deprivation impaired consolidation of long-term memory in rodents and flies (Graves et al., 2003; Ganguly-Fitzgerald et al., 2006). Indeed, sleep is significantly altered by previous experience and is important for the consolidation of recently acquired memories.

In the decade since the establishment of *Drosophila* as a model system for the study of sleep (Hendricks et al., 2000; Shaw et al., 2000), the fly has been successfully used to identify a number of candidate genes and pathways that are involved in sleep regulation. Among these pathways is the cAMP/PKA signaling cascade, which has classically been associated with learning and memory in the fly (Dudaí et al., 1983). Several genetic mutants that induce a downregulation in cAMP signaling result in decreased sleep time and, conversely, manipulations that increase cAMP signaling yield elevated sleep compared to genetic controls (Hendricks et al., 2001). Although the circuitry that controls sleep in the fly is largely unknown, additional investigations have found that disruption of the cAMP/PKA signaling cascade within the Mushroom Bodies, a structure that is important for associative processing, can strongly modulate sleep time (Joiner et

al., 2006).

Using a forward genetic screen to identify genes that alter sleep time, Cirelli et al found that flies mutant for the voltage-dependent potassium channel *Shaker (Sh)* exhibit a robust decrease in sleep when compared to wild-type controls (Cirelli et al., 2005). Similarly, several mutants for the beta modulatory subunit *Hyperkinetic (Hk)*, which influences *Sh* conductance, also sleep less than background controls (Bushey et al., 2007). When these mutant flies were tested for memory using the heat-box assay (Putz and Heisenberg, 2002), flies with mutant alleles for *Sh* or *Hk* that resulted in decreased sleep time also exhibited memory impairments while alleles that did not change sleep also had no significant effect on memory performance (Bushey et al., 2007). Although these results are purely correlational, they provide evidence that genetic mechanisms that influence sleep in the fly may be tightly intertwined with pathways that are important for learning and memory.

B. Social Enrichment Increases Sleep

Despite the evidence for a relationship between plasticity and sleep, little is known about the mechanisms by which sleep and plasticity interact. As described above, social enrichment is a simple experimental manipulation that induces robust plasticity in circuits throughout the brain. Given that the social environment can induce structural changes in the brain in both mammals and

invertebrates, it may be possible to begin to elucidate the underlying molecular mechanisms linking sleep and plasticity using the power of *Drosophila* genetics.

With that in mind, we evaluated flies that were housed in a socially enriched environment since they are likely to experience increased visual, olfactory pheromonal and auditory signals compared to siblings that are housed individually in social isolation. Moreover interactions between individuals in a social environment are likely to increase the incidence of other behaviors including flying, jumping, geotaxis and grooming, to name a few (Ganguly-Fitzgerald et al., 2006). We hypothesized that increased exposure to these events would likely produce changes in structural plasticity within the brain which would increase sleep need. To test this hypothesis wild-type flies were housed in a socially enriched environment with ~35-40 siblings for five days. As seen in Figure 1 socially enriched flies sleep approximately 2 hours/day more than siblings that have been socially isolated for five days (Ganguly-Fitzgerald et al., 2006; Donlea et al., 2009) (Figure 1). The increase is observed in both single-sex vials (male-male and female-female) and in mixed vials (male-female). Interestingly, social enrichment not only increases sleep time but, importantly, increases sleep consolidation. That is, isolated flies exhibit short-sleep bouts during the day which do not appear to be restorative (Seugnet et al., 2008). In contrast socially enriched flies maintain sleep bout durations more typical of that seen during night-time sleep (Ganguly-Fitzgerald et al., 2006). Thus, social enrichment increases sleep consolidation sufficiently to permit the restorative

properties of sleep.

Although the number of interactions that can potentially occur in the socially enriched environment is difficult to quantify precisely, it is clear that compared to their isolated siblings the brains of socially enriched flies must adapt and respond to a larger variety of stimuli. In that regard, it is worth noting that the change in sleep does not appear to be induced by other factors such as the volume of the enclosure or abiotic factors indirectly caused by enrichment. For example, blind and olfactory defective flies do not respond to social enrichment with an increase in sleep indicating that being in close proximity with 35-40 siblings for 5 days is not sufficient to induce a sufficient change in neuronal plasticity as to induce an increase in sleep (Ganguly-Fitzgerald et al., 2006). Similarly, changes in sleep are not observed in enriched animals that are mutant for classical memory genes that also influence structural plasticity such as the adenylyl cyclase *rutabaga* or the cAMP phosphodiesterase *dunce*. Finally, the increase in sleep following social enrichment is directly proportional to the number of flies housed in the enriched environment (Ganguly-Fitzgerald et al., 2006). Together these observations indicate that the increase in sleep is likely due to social interactions inducing changes in neuronal plasticity.

An alternative hypothesis is that sleep is disrupted during social enrichment such that the subsequent increase in sleep simply reflects enhanced homeostatic drive not changes in plasticity. However, as mentioned above flies mutant for memory

genes do not exhibit an increase in sleep even though one would expect that their sleep would be as disrupted during social enrichment as that hypothesized to occur in wild-type flies. Interestingly, flies mutant for the circadian clock gene *period* (*per*^{01}) exhibit a sleep rebound that is ~3 times larger than wild-type flies indicating that they are very responsive to even small changes in sleep (Figure 2). Despite their sensitivity to sleep loss, however, *per*^{01} flies do not increase their sleep following social enrichment (Figure 2). Thus, if social enrichment resulted in disrupted sleep as the alternative hypothesis suggests, *per*^{01} flies would show a larger increase in sleep than that seen in wild-type flies. Since *per*^{01} flies do not show an increase in sleep following social enrichment, we believe that the genetic mechanisms that control sleep homeostasis following sleep deprivation are dissociable from the mechanisms underlying increased sleep following social enrichment.

C. Circuits and genes that influence the response to social enrichment

To determine whether social enrichment may be dependent upon plastic mechanisms that are invoked following social interactions, we conducted a mini-brain screen to restore *rutabaga* functioning in specific circuits in an otherwise *rutabaga* mutant background. We used the bipartite yeast-GAL4 system to express *rutabaga* in ~35 circuits (Brand and Perrimon, 1993). Surprisingly, when *rutabaga* was rescued in ventral lateral neurons (LN_Vs), 16 cells that comprise the circadian clock, the increase in sleep following social enrichment was

restored (Donlea et al., 2009). Recent studies from several independent groups have reported that the LN_Vs play an important role in sleep-wake regulation in the fly (Agosto et al., 2008; Parisky et al., 2008; Sheeba et al., 2008). That is, manipulations that increase the excitability of the LN_Vs result in large increases in waking behavior. A subset of the LN_Vs innervates the medulla where they are uniquely situated to modulate the processing of simple and complex visual information. Together these data suggest that environmentally induced plasticity in the LN_Vs may be important for changes in sleep following social enrichment.

Having identified a circuit, the LN_Vs, that is required for the social environment to induce an increase in sleep, we began to evaluate candidate genes that coordinate the changes in neuronal plasticity that are induced by social experience within this circuit. Although the clock gene *period* is widely expressed throughout the fly brain, specific rescue of *period* within clock cells was able to restore plasticity induced sleep in an otherwise per^{01} mutant background (Donlea et al., 2009). This observation is particularly interesting given that the *period* gene has been shown to play a non-circadian role in memory consolidation (Sakai et al., 2004). Similarly, the transcription factor *blistered* (*bs*) is both transcriptionally elevated in the brain following social enrichment and is required in the LN_Vs for the social environment to modify sleep time. It is worth noting that the mammalian homolog of *blistered*, *Serum Response Factor*, induces transcription of a genetic program that mediates synaptic potentiation and is necessary both for *in vitro* assays of plasticity such as LTP and LTD as well as *in*

vivo assays of behavioral plasticity (Ramanan et al., 2005; Etkin et al., 2006). To further explore the role of *bs* in coordinating social enrichment and sleep, we evaluated genes that are known to be regulated by *bs*. One such gene, *Epidermal growth factor receptor* (*Egfr*), is transcriptionally activated by social enrichment in wild-type flies. When *Egfr* was expressed in the LN_Vs of a *bs* mutant, the increase in sleep following social enrichment was rescued. Thus, we have identified 4 genes that operate within a specific circuit that is known to both influence sleep-wake regulation and that is able to coordinate the interaction between social environment and sleep.

D. Social environment alters synaptic terminals

As mentioned above, Heisenberg and colleagues conducted a series of elegant studies demonstrating that it is possible to quantify environmentally induced structural changes in the brains of adult flies. To determine whether the social enrichment paradigm was able to induce structural changes in the brain, we expressed a GFP-tagged construct of the post-synaptic protein *discs-large* (UAS-*dlg*WT-gfp) in the LN_Vs. As seen in Figure 3, the number of synaptic terminals was significantly increased after 5 days of social enrichment. Similar results were obtained using a GFP tagged pre-synaptic marker. These results compliment those reported by Heisenberg and colleagues and demonstrate that the social environment is able to induce quantifiable changes in brain structures within a circuit that is known to play a role in sleep regulation. Since we have not

demonstrated that the labeled synaptic terminals are functional, it is possible that the increased number of GFP-tagged terminals is an artifact of the environmental manipulation and does not reflect functional changes that influence sleep time. To test this possibility, UAS-*dlg*WT-gfp was expressed in a *bs* mutant background and flies were exposed to social enrichment. As seen in Figure 3, the number of synaptic terminals was not altered in socially enriched *bs* mutants. That is, flies that are not capable of responding to the social environment with an increase in sleep do not display structural changes in the number of synaptic terminals. These data suggest that in the absence of functional plasticity, the environment is not associated with changes in structural plasticity.

E. Social enrichment vs. Long-term memory

Although structural plasticity and increased sleep following social enrichment require expression of several genes that are necessary for the formation and consolidation of associative memories, it is difficult to determine whether the increase in sleep is truly dependent upon plastic mechanisms. We hypothesized that if the increase in sleep following social enrichment is dependent upon plasticity related processes then the circuits and genes that have been identified for social enrichment should also play a role in the consolidation of long-term memory. To test this hypothesis we utilized Courtship Conditioning, an associative assay in which male flies learn to alter their courtship behavior based

on previous exposure to unreceptive courtship targets. In courtship conditioning training, male flies are paired with mated female flies that are unreceptive to further copulation attempts or with male flies that have been genetically altered to express aphrodisiac pheromonal cues. During this training period the subject male will proceed through stereotypical courtship behaviors in an attempt to woo the unreceptive courtship trainer, but is ultimately unable to copulate and forms an operant association between courtship rejection and normally aphrodisiac pheromonal cues (Gailey et al., 1984). Following training, male subject flies are returned to individual tubes. Subsequent memory is probed by exposing trained males to a normally attractive courtship target; if a trained male retains memory of the training experience, he will spend less time courting during the test period than his naïve brothers. When wild-type male flies are subjected to a spaced training protocol consisting of three 1-hour training periods with a pheromonally-feminized *Tai2* male fly, they exhibit robust reductions in courtship for at least 48 hours (Ganguly-Fitzgerald et al., 2006). Although acquisition and consolidation of memory following Courtship Conditioning requires similar genetic and neural mechanisms as memories formed in other associative assays (Siwicki and Ladewski, 2003), Courtship Conditioning requires male flies to process complex, naturalistic visual and pheromonal cues and to interpret social behaviors and postures and, thus, seems more directly comparable to the types of neural processing that might occur during social enrichment than occurs in many other assays.

Following a spaced training protocol that induces long-term memories, male flies exhibit a significant increase in sleep. The increase in sleep appears necessary for memory consolidation since four-hours of sleep deprivation eliminates subsequent LTM. Interestingly, sleep deprivation immediately following training not only eliminates LTM, but it also blocks the increase in sleep typically observed following the spaced-training protocol. This observation suggests that the increase in sleep following training is likely due to molecular processes associated with memory consolidation; once the memory has been disrupted there is no need for more sleep. Consistent with their role in experience-dependent changes in sleep, flies mutant for *period* and *bs* do not show increases in sleep following training and show no evidence for LTM after 48 h. Importantly, expression of either wild-type *per* or wild-type *bs* within the subset of clock neurons that are required for social-enrichment, restores both the increase in sleep and LTM consolidation following Courtship Conditioning in *per* and *bs* mutant backgrounds respectively. Thus, the genes and circuits that have been identified as playing a role in mediating the effects of social enrichment on sleep are also required for the increase in sleep following the formation of LTM. Together with the data demonstrating that social enrichment results in an increase in the number of synaptic terminals, it appears as if the social environment alters sleep time by modifying molecular processes associated with synaptic plasticity.

F. Synaptic Homeostasis & Sleep

Although the direct effects of sleep at the synapse are largely unknown, a recent hypothesis has suggested that a function of sleep may be to downscale synaptic connections throughout the brain (Tononi, 2003; Tononi and Cirelli, 2005). According to this hypothesis, experiences during waking drive patterns of activity in neural circuits that potentiate synapses and increase the strength and number of neural connections. If this kind of potentiation were allowed to continue unchecked, we could ultimately run into some severe consequences; energy requirements for the brain would grow, space available for new synaptic connections would disappear, and the signal strength between synaptic connections would reach a saturation point. To prevent these problems, Tononi and Cirelli have proposed that synchronized patterns of activity during sleep facilitate global synaptic downscaling to allow for normal functioning each morning (Tononi, 2003; Tononi and Cirelli, 2005). In support of this hypothesis they have found that the levels of proteins that are associated with synaptic potentiation are increased during waking and decreased during sleep in both flies and rats (Vyazovskiy et al., 2008; Gilestro et al., 2009). Although these studies provide molecular data that are consistent with the down-scaling hypothesis, they do not address whether sleep acts to reduce synaptic connections at the level of individual terminals.

Given the ability of the environment to increase synaptic terminals in the LN_Vs, social enrichment is uniquely suited to test the hypothesis that synaptic

homeostasis is major role of sleep. If the synaptic homeostasis model is correct, then flies that were exposed to social enrichment for 5 days but are prevented from sleeping should show a persistence in the number of synaptic terminals. However, if consolidation is accomplished through synaptic potentiation then flies that are sleep deprived following social enrichment should display a decrease in the number terminals. Following social enrichment flies were allowed to either sleep *ad libitum* for 48 hours or were sleep deprived for 48 hours. The number of synaptic terminals in projections from the large LN_Vs (l-LN_Vs) was then quantified. We found that the sleep-deprived flies retained an elevated number of l-LN_V terminals following social enrichment, but that flies who could sleep *ad libidum* no longer had any significant change in terminal number when compared to their socially isolated siblings (Donlea et al., 2009). These data are consistent with the downscaling hypothesis and indicate that flies sleep more when LN_V terminal number has been elevated by plastic mechanisms and that this increased sleep acts to reduce the number of terminals back to a baseline level.

At first glance, the increase in sleep following social enrichment appears somewhat paradoxical. That is, social enrichment increases the number of synaptic terminals in the LN_Vs while the activation of the LN_Vs strongly promote waking (Sheeba et al., 2008). How can these two observations be reconciled? During social enrichment, we hypothesize that complex sensory signals induce a prolonged elevation of LN_V activity (Figure 4). The strength of these sensory signals results in an increase in the strength of synaptic connections between

visual input circuits and the LN_Vs that is mediated by Hebbian mechanisms (Figure 4B). The experience-dependent increase in synaptic strength seems to be implemented as an increase the number of synaptic terminals in the LN_Vs over the course of social enrichment. After LN_V activity is elevated by several days of enriched social experience, it is possible that homeostatic mechanisms decrease the overall excitability of the LN_Vs to prevent chronic hyper-excitation and hold firing rate around a baseline set-point; similar homeostatic functioning has been previously described in *Drosophila* central synapses (Kazama and Wilson, 2008). Once enriched flies are moved into sleep monitors following social enrichment, complex sensory stimuli are removed and the lowered excitability of the LN_Vs reduces the firing rate below that of previously socially isolated controls (Figure 4C). This lowered firing rate reduces the wake-promoting signal from these neurons and results in increased sleep for several days until LN_V excitability can be homeostatically elevated to restore a normalized firing rate. Coincidentally, this model could also account for the downscaling of synaptic terminal number following social enrichment; if overall excitability of the LN_Vs is reduced when flies are removed from social enrichment and complex sensory stimuli are removed, input to each individual terminal could become less likely to drive action potentials in the LN_Vs and, as a result, Hebbian mechanisms might weaken the synaptic connections by decreasing terminal numbers. Although many of the specific mechanisms of this model have not been thoroughly tested, this model provides a framework by which our structural evidence for plasticity in the LN_Vs can be reconciled with previous studies

indicating that neural activity in the LN_Vs reduces sleep time.

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Figures

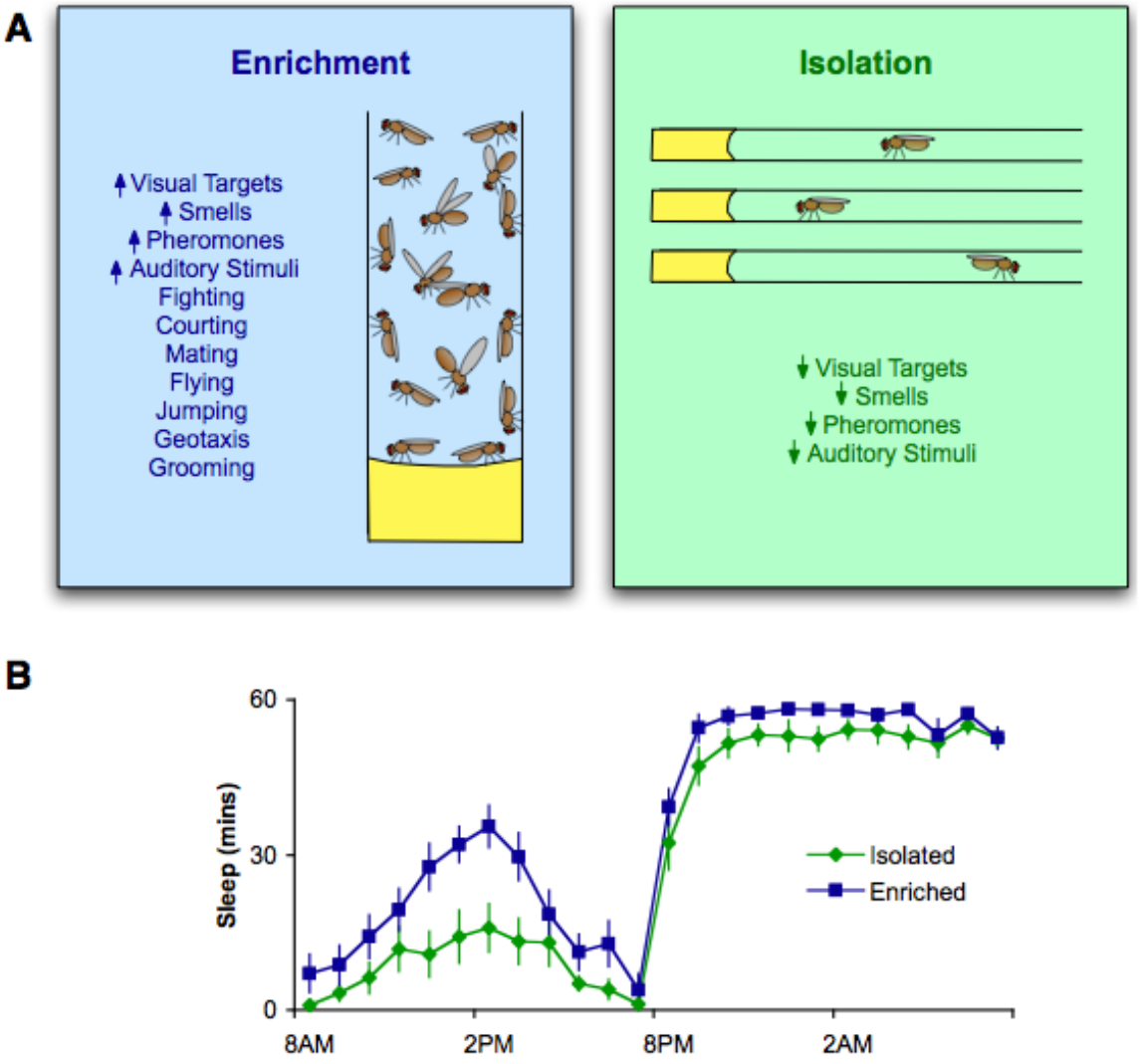

Figure 1. Exposure to an enriched social environment increases sleep in *Drosophila*.

A) Flies are housed for five days either in a socially enriched vial with ~40 other siblings or in small vials in social isolation. During this time, flies in the socially enriched environment are exposed to complex visual, olfactory, pheromonal and auditory stimuli that are generated by other flies and fight, court, and mate with

conspecific flies. Socially isolated animals, however, are not exposed to these sensory cues and have no social interactions with other flies. B) When transferred to sleep monitors after 5 days of enrichment/isolation, socially enriched females sleep ~120 minutes/day more than their socially isolated sisters.

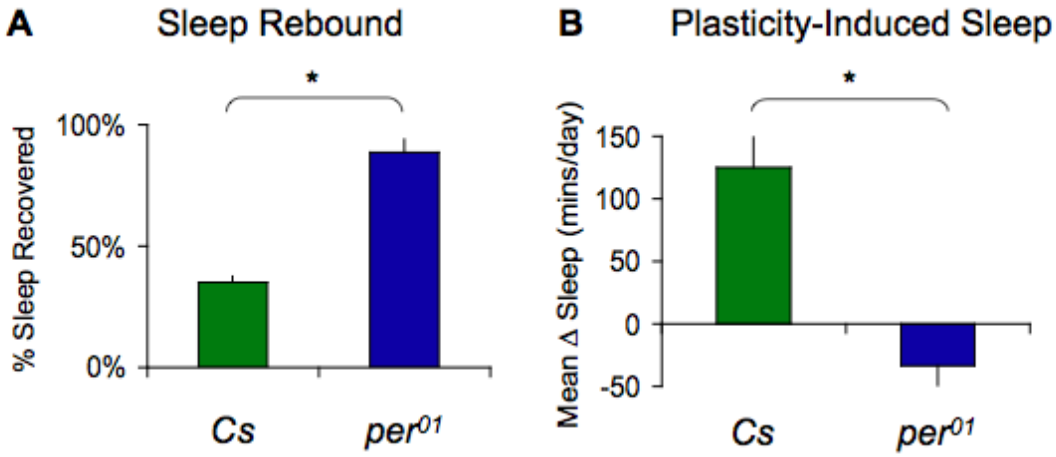

Figure 2. Flies mutant for *per*^{01} are extremely sensitive to sleep loss but do not respond to social enrichment with an increase in sleep.

A) Following 12 h of sleep deprivation Cs flies recover ~30% of their lost sleep while *per*^{01} mutants recover >90%. % sleep recovered is calculated for each individual as a ratio of the minutes of sleep gained above baseline during the 24 h of recovery divided by the total min of sleep lost during 12 h of sleep deprivation. B) Following five days of social enrichment, *Cs* flies sleep significantly more than their siblings that were housed in social isolation while sleep is unaffected by social enrichment in *per*^{01} mutants. Increased sleep after social enrichment is shown as a difference in daytime sleep amount between socially enriched and socially isolated siblings.

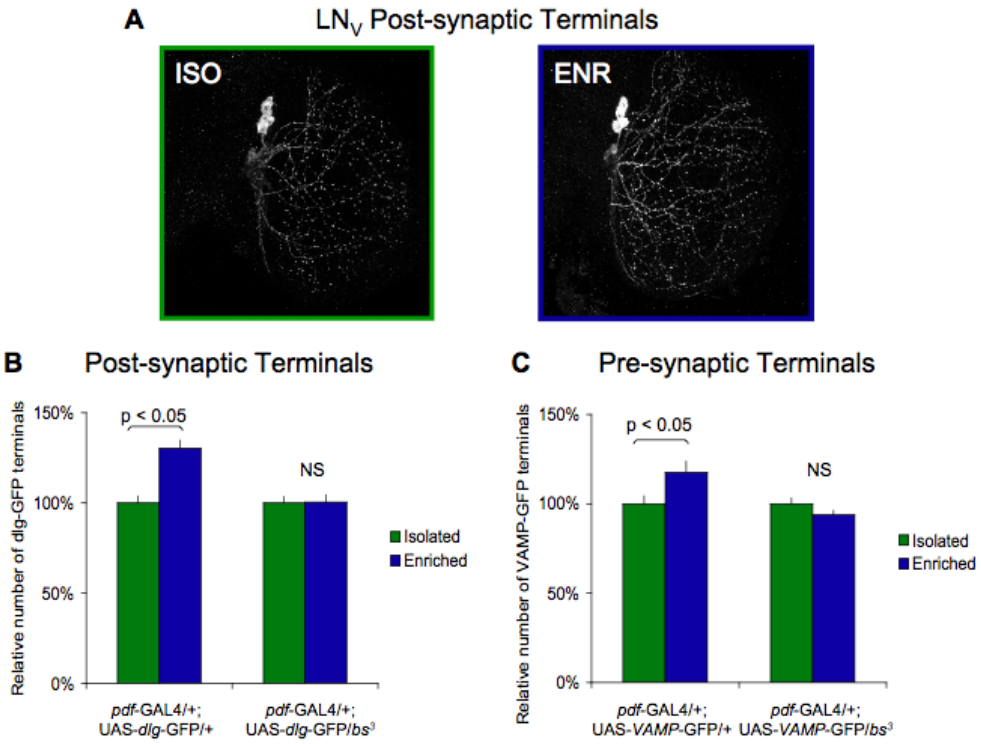

Figure 3. Flies mutant for *bs* show no increase in LN_V terminals following exposure to social enrichment.

A) LN_V projections in socially isolated pdf-GAL4/+; UAS-dlg-GFP/+ flies (left, representative image) contain fewer GFP-positive terminals than those of socially enriched siblings (right, representative image). B) Following 5 days of social enrichment, wild-type controls exhibit a significant increase in the number of LN_V post-synaptic terminals labeled with *dlg*-GFP (left), but no change in the number of post-synaptic terminals can be detected in flies carrying a mutant allele for *bs* (right). C) Similarly, an increased number of LN_V pre-synaptic terminals labeled with *VAMP*-GFP can be measured in wild-type control flies (left), but no change in pre-synaptic terminal number was observed in flies with the bs^3 mutation (right).

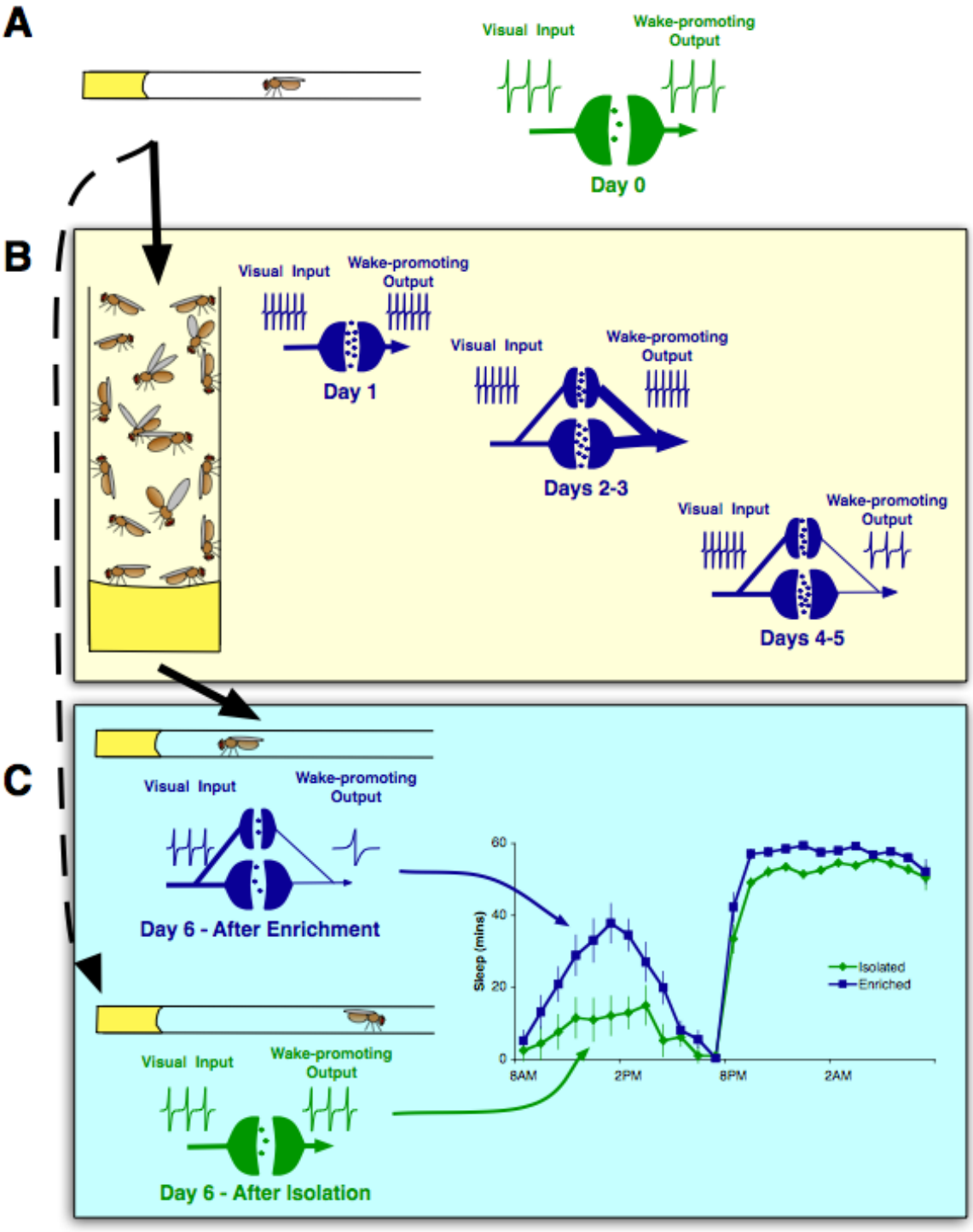

Figure 4. Proposed model for homeostatic regulation of synaptic terminals during social enrichment.

A) Under baseline conditions, wake-promoting output from the LN_Vs is modulated by signals originating from visual circuits in the medulla. B) When exposed to a socially enriched environment, complex sensory stimuli likely drives increased activity in primary visual circuits that provide input to the LN_Vs. As a result, activity in the LN_Vs would become more strongly correlated with input signals from sensory circuits and, as a result, synaptic connections to the LN_Vs become potentiated as new terminals are constructed. Following several days of hyperactivity and increased potentiation, homeostatic mechanisms may be induced in the LN_Vs to reduce overall firing rate and prevent chronic hyperexcitation. C) Upon transfer to sleep monitors, flies are withdrawn from the complex visual stimuli that are associated with a socially enriched environment. As a result, wake-promoting output from the LN_Vs is decreased and socially enriched flies sleep more than their siblings that had been housed in social isolation.

References

Agosto J, Choi JC, Parisky KM, Stilwell G, Rosbash M, Griffith LC (2008) Modulation of GABA(A) receptor desensitization uncouples sleep onset and maintenance in Drosophila. Nat Neurosci.

Artola A, von Frijtag JC, Fermont PC, Gispen WH, Schrama LH, Kamal A, Spruijt BM (2006) Long-lasting modulation of the induction of LTD and LTP in rat hippocampal CA1 by behavioural stress and environmental enrichment. Eur J Neurosci 23:261-272.

Balling A, Technau GM, Heisenberg M (2007) Are the structural changes in adult Drosophila mushroom bodies memory traces? Studies on biochemical learning mutants. J Neurogenet 21:209-217.

Barth M, Heisenberg M (1997) Vision affects mushroom bodies and central complex in Drosophila melanogaster. Learn Mem 4:219-229.

Barth M, Hirsch HV, Meinertzhagen IA, Heisenberg M (1997) Experience-dependent developmental plasticity in the optic lobe of Drosophila melanogaster. J Neurosci 17:1493-1504.

Bekinschtein P, Cammarota M, Igaz LM, Bevilaqua LR, Izquierdo I, Medina JH (2007) Persistence of long-term memory storage requires a late protein synthesis- and BDNF- dependent phase in the hippocampus. Neuron 53:261-277.

Berardi N, Pizzorusso T, Maffei L (2000) Critical periods during sensory development. Curr Opin Neurobiol 10:138-145.

Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401-415.

Branson K, Robie AA, Bender J, Perona P, Dickinson MH (2009) High-throughput ethomics in large groups of Drosophila. Nat Meth.

Briones TL, Suh E, Jozsa L, Woods J (2006) Behaviorally induced synaptogenesis and dendritic growth in the hippocampal region following transient global cerebral ischemia are accompanied by improvement in spatial learning. Exp Neurol 198:530-538.

Bushey D, Huber R, Tononi G, Cirelli C (2007) Drosophila Hyperkinetic mutants

have reduced sleep and impaired memory. J Neurosci 27:5384-5393.

Cao X, Huang S, Ruan D (2008) Enriched environment restores impaired hippocampal long-term potentiation and water maze performance induced by developmental lead exposure in rats. Developmental psychobiology 50:307-313.

Carlson MC, Helms MJ, Steffens DC, Burke JR, Potter GG, Plassman BL (2008) Midlife activity predicts risk of dementia in older male twin pairs. Alzheimer's & dementia : the journal of the Alzheimer's Association 4:324-331.

Cayre M, Malaterre J, Scotto Lomassese S, Holstein GR, Martinelli GP, Forni C, Nicolas S, Aouane A, Strambi C, Strambi A (2005) A role for nitric oxide in sensory-induced neurogenesis in an adult insect brain. Eur J Neurosci 21:2893-2902.

Cirelli C, Bushey D, Hill SL, Huber R, Kreber R, Ganetzky B, Tononi G (2005) Reduced sleep in Drosophila Shaker mutants. Nature 434:1087-1092.

Cottone LA, Adamo D, Squires NK (2004) The effect of unilateral somatosensory stimulation on hemispheric asymmetries during slow wave sleep. Sleep 27:63-68.

Dankert H, Wang L, Hoopfer E, Anderson DJ, Perona P (2009) Automated monitoring and analysis of social behavior in Drosophila. Nat Meth:1-7.

De Rosa R, Garcia AA, Braschi C, Capsoni S, Maffei L, Berardi N, Cattaneo A (2005) Intranasal administration of nerve growth factor (NGF) rescues recognition memory deficits in AD11 anti-NGF transgenic mice. Proc Natl Acad Sci USA 102:3811-3816.

Dong H, Goico B, Martin M, Csernansky CA, Bertchume A, Csernansky JG

(2004) Modulation of hippocampal cell proliferation, memory, and amyloid plaque deposition in APPsw (Tg2576) mutant mice by isolation stress. Neuroscience 127:601-609.

Donlea JM, Ramanan N, Shaw PJ (2009) Use-dependent plasticity in clock neurons regulates sleep need in Drosophila. Science 324:105-108.

Dudaí Y, Uzzan A, Zvi S (1983) Abnormal activity of adenylate cyclase in the Drosophila memory mutant rutabaga. Neurosci Lett 42:207-212.

Duffy SN, Craddock KJ, Abel T, Nguyen PV (2001) Environmental enrichment modifies the PKA-dependence of hippocampal LTP and improves hippocampus-dependent memory. Learn Mem 8:26-34.

Etkin A, Alarcón JM, Weisberg SP, Touzani K, Huang YY, Nordheim A, Kandel ER (2006) A role in learning for SRF: deletion in the adult forebrain disrupts LTD and the formation of an immediate memory of a novel context. Neuron 50:127-143.

Euston DR, Tatsuno M, McNaughton BL (2007) Fast-forward playback of recent memory sequences in prefrontal cortex during sleep. Science 318:1147-1150.

Faherty CJ, Raviie Shepherd K, Herasimtschuk A, Smeyne RJ (2005) Environmental enrichment in adulthood eliminates neuronal death in experimental Parkinsonism. Brain Res Mol Brain Res 134:170-179.

Ferveur JF (2005) Cuticular hydrocarbons: their evolution and roles in Drosophila pheromonal communication. Behav Genet 35:279-295.

Floeter MK, Greenough WT (1979) Cerebellar plasticity: modification of Purkinje cell structure by differential rearing in monkeys. Science 206:227-229.

Foster TC, Gagne J, Massicotte G (1996) Mechanism of altered synaptic strength due to experience: relation to long-term potentiation. Brain Res 736:243-250.

Gailey DA, Jackson FR, Siegel RW (1984) Conditioning Mutations in DROSOPHILA MELANOGASTER Affect an Experience-Dependent Behavioral Modification in Courting Males. Genetics 106:613-623.

Ganguly-Fitzgerald I, Donlea JM, Shaw PJ (2006) Waking experience affects sleep need in Drosophila. Science 313:1775-1781.

Gilestro GF, Tononi G, Cirelli C (2009) Widespread changes in synaptic markers as a function of sleep and wakefulness in Drosophila. Science 324:109-112.

Gómez-Palacio-Schjetnan A, Escobar ML (2008) In vivo BDNF modulation of adult functional and morphological synaptic plasticity at hippocampal mossy fibers. Neurosci Lett 445:62-67.

Graves LA, Heller EA, Pack AI, Abel T (2003) Sleep deprivation selectively impairs memory consolidation for contextual fear conditioning. Learn Mem 10:168-176.

Green EJ, Greenough WT (1986) Altered synaptic transmission in dentate gyrus of rats reared in complex environments: evidence from hippocampal slices maintained in vitro. J Neurophysiol 55:739-750.

Greenough WT, West RW, DeVoogd TJ (1978) Subsynaptic plate perforations: changes with age and experience in the rat. Science 202:1096-1098.

Heisenberg M, Heusipp M, Wanke C (1995) Structural plasticity in the Drosophila brain. J Neurosci 15:1951-1960.

Heldt SA, Stanek L, Chhatwal JP, Ressler KJ (2007) Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. Mol Psychiatry 12:656-670.

Hendricks JC, Finn SM, Panckeri KA, Chavkin J, al. e (2000) Rest in Drosophila Is a Sleep-like State. Neuron.

Hendricks JC, Williams JA, Panckeri KA, Kirk D, Tello MK, Yin JC, Sehgal A (2001) A non-circadian role for cAMP signaling and CREB activity in Drosophila rest homeostasis. Nat Neurosci 4:1108-1115.

Hennigan A, Callaghan CK, Kealy J, Rouine J, Kelly AM (2009) Deficits in LTP and recognition memory in the genetically hypertensive rat are associated with decreased expression of neurotrophic factors and their receptors in the dentate gyrus. Behav Brain Res 197:371-377.

Hoffman AN, Malena RR, Westergom BP, Luthra P, Cheng JP, Aslam HA, Zafonte RD, Kline AE (2008) Environmental enrichment-mediated functional improvement after experimental traumatic brain injury is contingent on task-specific neurobehavioral experience. Neurosci Lett 431:226-230.

Huber R, Ghilardi MF, Massimini M, Tononi G (2004) Local sleep and learning. Nature 430:78-81.

Huber R, Esser SK, Ferrarelli F, Massimini M, Peterson MJ, Tononi G (2007) TMS-Induced Cortical Potentiation during Wakefulness Locally Increases Slow Wave Activity during Sleep. PLoS ONE 2:e276.

Ickes BR, Pham TM, Sanders LA, Albeck DS, Mohammed AH, Granholm AC (2000) Long-term environmental enrichment leads to regional increases in

neurotrophin levels in rat brain. Exp Neurol 164:45-52.

Iwasaki N, Karashima A, Tamakawa Y, Katayama N, Nakao M (2004) Sleep EEG dynamics in rat barrel cortex associated with sensory deprivation. Neuroreport 15:2681-2684.

Ji D, Wilson MA (2007) Coordinated memory replay in the visual cortex and hippocampus during sleep. Nat Neurosci 10:100-107.

Joiner WJ, Crocker A, White BH, Sehgal A (2006) Sleep in Drosophila is regulated by adult mushroom bodies. Nature 441:757-760.

Kattler H, Dijk DJ, Borbély AA (1994) Effect of unilateral somatosensory stimulation prior to sleep on the sleep EEG in humans. Journal of sleep research 3:159-164.

Kazama H, Wilson RI (2008) Homeostatic matching and nonlinear amplification at identified central synapses. Neuron 58:401-413.

Kempermann G, Kuhn HG, Gage FH (1997) More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493-495.

Kent C, Azanchi R, Smith B, Formosa A, Levine JD (2008) Social Context Influences Chemical Communication in D. melanogaster Males. Curr Biol.

Kline AE, Wagner AK, Westergom BP, Malena RR, Zafonte RD, Olsen AS, Sozda CN, Luthra P, Panda M, Cheng JP, Aslam HA (2007) Acute treatment with the 5-HT(1A) receptor agonist 8-OH-DPAT and chronic environmental enrichment confer neurobehavioral benefit after experimental brain trauma. Behav Brain Res 177:186-194.

Koh S, Chung H, Xia H, Mahadevia A, Song Y (2005) Environmental enrichment

reverses the impaired exploratory behavior and altered gene expression induced by early-life seizures. J Child Neurol 20:796-802.

Krupp J, Kent C, Billeter J, Azanchi R, So A, Schonfeld J, Smith B, Lucas C, Levine JD (2008) Social Experience Modifies Pheromone Expression and Mating Behavior in Male Drosophila melanogaster. Curr Biol.

Lee E, Son H (2009) Adult hippocampal neurogenesis and related neurotrophic factors. BMB Rep 42:239-244.

Lyamin OI, Manger PR, Ridgway SH, Mukhametov LM, Siegel JM (2008) Cetacean sleep: an unusual form of mammalian sleep. Neuroscience and biobehavioral reviews 32:1451-1484.

Miyamoto H, Katagiri H, Hensch T (2003) Experience-dependent slow-wave sleep development. Nat Neurosci 6:553-554.

Nagahara AH, Merrill DA, Coppola G, Tsukada S, Schroeder BE, Shaked GM, Wang L, Blesch A, Kim A, Conner JM, Rockenstein E, Chao MV, Koo EH, Geschwind D, Masliah E, Chiba AA, Tuszynski MH (2009) Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. Nat Med 15:331-337.

Parisky KM, Agosto J, Pulver SR, Shang Y, Kuklin E, Hodge JJ, Kang K, Liu X, Garrity PA, Rosbash M, Griffith LC (2008) PDF cells are a GABA-responsive wake-promoting component of the Drosophila sleep circuit. Neuron 60:672-682.

Pereira LO, Strapasson AC, Nabinger PM, Achaval M, Netto CA (2008) Early enriched housing results in partial recovery of memory deficits in female, but not in male, rats after neonatal hypoxia-ischemia. Brain Res 1218:257-266.

Pereira LO, Nabinger PM, Strapasson AC, Nardin P, Gonçalves CA, Siqueira IR, Netto CA (2009) Long-term effects of environmental stimulation following hypoxia-ischemia on the oxidative state and BDNF levels in rat hippocampus and frontal cortex. Brain Res 1247:188-195.

Pham TM, Söderström S, Winblad B, Mohammed AH (1999a) Effects of environmental enrichment on cognitive function and hippocampal NGF in the non-handled rats. Behav Brain Res 103:63-70.

Pham TM, Ickes B, Albeck D, Söderström S, Granholm AC, Mohammed AH (1999b) Changes in brain nerve growth factor levels and nerve growth factor receptors in rats exposed to environmental enrichment for one year. Neuroscience 94:279-286.

Putz G, Heisenberg M (2002) Memories in drosophila heat-box learning. Learn Mem 9:349-359.

Ramanan N, Shen Y, Sarsfield S, Lemberger T, Schütz G, Linden DJ, Ginty DD (2005) SRF mediates activity-induced gene expression and synaptic plasticity but not neuronal viability. Nat Neurosci 8:759-767.

Rasch B, Büchel C, Gais S, Born J (2007) Odor cues during slow-wave sleep prompt declarative memory consolidation. Science 315:1426-1429.

Robin C, Daborn PJ, Hoffmann AA (2007) Fighting fly genes. Trends Genet 23:51-54.

Ruan H, Wu CF (2008) Social interaction-mediated lifespan extension of Drosophila Cu/Zn superoxide dismutase mutants. Proc Natl Acad Sci USA 105:7506-7510.

Sakai T, Tamura T, Kitamoto T, Kidokoro Y (2004) A clock gene, period, plays a key role in long-term memory formation in Drosophila. Proc Natl Acad Sci USA 101:16058-16063.

Scotto Lomassese S, Strambi C, Strambi A, Charpin P, Augier R, Aouane A, Cayre M (2000) Influence of environmental stimulation on neurogenesis in the adult insect brain. J Neurobiol 45:162-171.

Scotto-Lomassese S, Strambi C, Aouane A, Strambi A, Cayre M (2002) Sensory inputs stimulate progenitor cell proliferation in an adult insect brain. Curr Biol 12:1001-1005.

Seugnet L, Suzuki Y, Vine L, Gottschalk L, Shaw PJ (2008) D1 Receptor Activation in the Mushroom Bodies Rescues Sleep-Loss-Induced Learning Impairments in Drosophila. Curr Biol.

Shaw PJ, Cirelli C, Greenspan RJ, Tononi G (2000) Correlates of sleep and waking in Drosophila melanogaster. Science 287:1834-1837.

Sheeba V, Fogle KJ, Kaneko M, Rashid S, Chou YT, Sharma VK, Holmes TC (2008) Large Ventral Lateral Neurons Modulate Arousal and Sleep in Drosophila. Curr Biol.

Siwicki KK, Ladewski L (2003) Associative learning and memory in Drosophila: beyond olfactory conditioning. Behav Processes 64:225-238.

Stickgold RJ, Walker MP (2007) Sleep-dependent memory consolidation and reconsolidation. Sleep Med 8:331-343.

Stickgold RJ, Malia A, Maguire D, Roddenberry D, O'Connor M (2000) Replaying the game: hypnagogic images in normals and amnesics. Science 290:350-353.

Technau GM (2007) Fiber number in the mushroom bodies of adult Drosophila melanogaster depends on age, sex and experience. J Neurogenet 21:183-196.

Tononi G (2003) Sleep and synaptic homeostasis: a hypothesis. Brain Res Bull 62:143-150.

Tononi G, Cirelli C (2005) Sleep function and synaptic homeostasis. Sleep medicine reviews 10:49-62.

Torasdotter M, Metsis M, Henriksson BG, Winblad B, Mohammed AH (1998) Environmental enrichment results in higher levels of nerve growth factor mRNA in the rat visual cortex and hippocampus. Behav Brain Res 93:83-90.

Toscano CD, McGlothan JL, Guilarte TR (2006) Experience-dependent regulation of zif268 gene expression and spatial learning. Exp Neurol 200:209-215.

Tuszynski MH, Thal L, Pay M, Salmon DP, U HS, Bakay R, Patel P, Blesch A, Vahlsing HL, Ho G, Tong G, Potkin SG, Fallon J, Hansen L, Mufson EJ, Kordower JH, Gall C, Conner J (2005) A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. Nat Med 11:551-555.

Ueda A, Wu CF (2009) Effects of Social Isolation on Neuromuscular Excitability and Aggressive Behaviors in Drosophila: Altered Responses by Hk and gsts1, Two Mutations Implicated in Redox Regulation. J Neurogenet:1-17.

Villella A, Hall JC (2008) Neurogenetics of courtship and mating in Drosophila. Adv Genet 62:67-184.

Volkmar FR, Greenough WT (1972) Rearing complexity affects branching of dendrites in the visual cortex of the rat. Science 176:1445-1447.

Vyazovskiy V, Borbély AA, Tobler I (2000) Unilateral vibrissae stimulation during waking induces interhemispheric EEG asymmetry during subsequent sleep in the rat. Journal of sleep research 9:367-371.

Vyazovskiy VV, Cirelli C, Pfister-Genskow M, Faraguna U, Tononi G (2008) Molecular and electrophysiological evidence for net synaptic potentiation in wake and depression in sleep. Nat Neurosci.

Walker MP, Stickgold R, Alsop D, Gaab N, Schlaug G (2005) Sleep-dependent motor memory plasticity in the human brain. Neuroscience 133:911-917.

Walz R, Roesler R, Reinke A, Martins MR, Quevedo J, Izquierdo I (2005) Short- and long-term memory are differentialy modulated by hippocampal nerve growth factor and fibroblast growth factor. Neurochem Res 30:185-190.

Whitfield CW, Cziko AM, Robinson GE (2003) Gene expression profiles in the brain predict behavior in individual honey bees. Science 302:296-299.

Wilson MA, McNaughton BL (1994) Reactivation of hippocampal ensemble memories during sleep. Science 265:676-679.

Winnington AP, Napper RM, Mercer AR (1996) Structural plasticity of identified glomeruli in the antennal lobes of the adult worker honey bee. J Comp Neurol 365:479-490.

Withers GS, Fahrbach SE, Robinson GE (1993) Selective neuroanatomical plasticity and division of labour in the honeybee. Nature 364:238-240.

Yasuda T, Yasuda K, Brown RA, Krueger JM (2005) State-dependent effects of light-dark cycle on somatosensory and visual cortex EEG in rats. Am J Physiol Regul Integr Comp Physiol 289:R1083-1089.

Young D, Lawlor PA, Leone P, Dragunow M, During MJ (1999) Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. Nat Med 5:448-453.

Zhao MG, Toyoda H, Wang YK, Zhuo M (2009) Enhanced synaptic long-term potentiation in the anterior cingulate cortex of adult wild mice as compared with that in laboratory mice. Mol Brain 2:11.

Chapter 2:

Waking Experience Affects Sleep-need in *Drosophila*

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Summary: The complexity and intensity of social interaction and/or information gleaned during prior waking stably changes subsequent sleep in *Drosophila*.

Adapted from:

Ganguly-Fitzgerald I, Donlea J, Shaw PJ. Waking experience affects sleep need in Drosophila. Science. 2006 Sep 22;313(5794):1775-81.

Abstract

Sleep is a vital, evolutionarily-conserved phenomenon, whose function is unclear. Although mounting evidence supports a role for sleep in the consolidation of memories, until present, a molecular connection between sleep, plasticity and memory formation has been difficult to demonstrate. We establish *Drosophila* as a model to investigate this relationship and demonstrate that the intensity and/or complexity of prior social experience stably modifies sleep-need and architecture. Furthermore, this experience-dependent plasticity in sleep-need is subserved by the dopaminergic and cAMP signaling pathways and a unique subset of 17 long-term memory genes.

Manuscript

Sleep is critical for survival, observed in man, mouse and fly (*1-3*), and yet, its function remains unclear. While studies suggest that sleep may play a role in the processing of waking information (*4, 5*), a direct molecular link between waking experience, plasticity and sleep remains undemonstrated. We have taken advantage of *Drosophila* genetics and the behavioral and physiological similarities between fruit-fly and mammalian sleep (*2, 3*) to investigate the molecular connection between experience, sleep, and memory.

Drosophila is uniquely suited to explore the relationship between sleep and plasticity. First, flies sleep. This is evidenced by consolidated periods of quiescence associated with reduced responsiveness to external stimuli and homeostatic regulation – the increased need for sleep that follows sleep deprivation (*6*). Second, *Drosophila* has been successfully used to elucidate conserved mechanisms of plasticity. For example, exposure to enriched environments, including social environment, impacts the number of synapses and the size of regions involved in information processing in vertebrates and *Drosophila* (*7, 8*). In the fly, these structural changes occur in response to experiential information received within a week of emergence from pupal cases (*9*). While brain plasticity is not limited to this period, the first week of emergence does coincide with the development of complex behaviors in *Drosophila*, including sleep. Hence, day-time sleep, which accounts for about 40% of total sleep in adults is highest immediately after eclosion and stabilizes to adult levels 4 days post-emergence (*3*).

To assess the impact of waking experience during this period of brain and behavioral development, individuals from the wild-type *C-S* strain were exposed to either social-enrichment or impoverishment immediately at eclosion and tested individually for sleep, five days later (Fig 1A). Socially-enriched individuals (E), exposed to a group of 30 or more males and females (1:1 sex ratio) prior to being tested, slept significantly more than their socially-impoverished (I) siblings, who

were housed individually (Fig 1B, C; $p < 0.005$). This difference in sleep (ΔSleep(E)) was restricted to day-time sleep. Socially-enriched individuals consolidated their day-time sleep into longer bouts of ~60 min compared to their isolated siblings, who slept in 15 min bouts (Fig 1D, $p < 0.0001$). In contrast, night-time sleep was unaffected by prior social experience (Fig 1B, C; $p = 0.4328$), corresponding with observations that day-time sleep is more sensitive to sex, age, genotype and environment, compared to night-time sleep (*10*). This effect of social experience on sleep persisted over a period of days (Fig 1E). Moreover, it was a stable phenotype: when socially-enriched, longer-sleeping individuals and socially-impoverished, shorter-sleeping siblings were sleep-deprived for 24 hours, they defended their respective pre-deprivation baseline sleep quotas by returning to these levels following a normal homeostatic response (Fig 1F; S1).

Experience-dependent modifications in sleep have long been observed in humans, rats, mice and cats (*11-13*). But what is the nature of the experiential information that modifies sleep-need in genetically-identical *Drosophila*? Differences in sleep-need in socially-enriched and socially-impoverished individuals were not a function of the space to which they were exposed: flies reared in 2cc tubes slept the same as those reared in 40cc vials (Fig 1H; $p = 0.5407$). Neither did it arise out of differences in reproductive state or sexual activity between the 2 groups: socially-impoverished mated and virgin individuals slept the same (Fig 1*I*; $p = 0.9450$), as did socially-enriched individuals from

mixed-sex or single-sex groups (Fig S2). Further, differences in sleep were not a reflection of differences in overall activity (infra-red beam breaks) between the two groups (Fig 1J; p = 0.9369). While social context can reset biological rhythms (*14*), mutations in *clock* (*Clk*^{jerk}) , *timeless* (*tim*^{01}) and *cycle* (*cyc*^{01}) disrupt circadian rhythms but had no effect on experience-dependent responses in sleep-need (Fig 1F).

Since social interaction requires sensory input, we next evaluated fly strains that were selectively impaired in vision, olfaction and hearing. Blind *norpA* homozygotes, failed to display a response in sleep to waking experience: sleep-need in socially-impoverished and enriched *norpA* mutants was unaltered (Fig 1G; p= 0.8385). In contrast, *norpA/+* heterozygotes with restored visual acuity slept more when previously socially-enriched (Fig 1F; p < 0.0001). Attenuating visual signals by rearing wild-type (*C-S*) flies in darkness also abolished the effect of waking experience on sleep (Fig 1G; p = 0.7198). Compromising the sense of smell, while retaining visual acuity, also blocked experience-dependent changes in sleep-need: socially-enriched *smellblind*^{1} mutants slept the same as their impoverished siblings (Fig 1G; p = 0.8478). As confirmation, we specifically silenced neurons carrying olfactory input to the brain (*Or83b-Gal4*/*UAS-TNT*;(*15*)) and observed that sleep in these flies was not affected by prior waking experience (Fig 1F; p = 0.7569). Auditory cues, however, did not affect the relationship between experience and sleep (Fig 1F; p < 0.0001). Finally, sleep-need in individual *Drosophila* increased with the size of the social group to which

they were previously exposed (Fig 1K). Socially-isolated flies slept the least, while those exposed to social groups of 4, 10, 20, 60 and 100 (1:1 sex ratio) showed proportionately increased day-time sleep-need (Fig 1K). When rendered blind, however, flies did not display this relationship between sleep-need and the intensity of prior social interactions (See *norpA* mutants in Fig 1K).

If sensory information received during a critical period of juvenile development directs the maturation of the sleep-need, then sleep-time and consolidation should be unresponsive to environmental changes in the adult. Alternatively, if experience-dependent modifications in sleep reflect ongoing plastic processes, this phenomenon would persist in the adult. We observed that sleep in flies was modified by their most recent social experience regardless of early exposure. Socially-impoverished adults demonstrated an increase in sleep-need when exposed to social-enrichment (I⇒E) prior to being assayed (Fig 2A-C). Conversely, socially-enriched flies became shorter-sleepers following exposure to social-isolation (E⇒I; Fig 2D, E). Moreover, repeated switching of exposure between the 2 social environments consistently modified sleep, reflecting an individual's most recent experience (Fig S3).

An estimation of neurotransmitter levels in whole brains revealed that short-sleeping, socially-impoverished individuals contained 3 times less dopamine compared to their longer-sleeping, socially-stimulated isogenic siblings (Fig 2F). Silencing or ablating the dopaminergic circuit in the brain (*TH-Gal4/UAS-TNT*

and *TH-Gal4/UAS-Rpr*;(*16*)) specifically abolished response to social-impoverishment in individuals that were reared in social-enrichment (Fig 2H). We obtained similar results when endogenous dopamine levels were aberrantly increased, by disrupting the monoamine catabolic enzyme, arylalkylamine N-acetyltransferase, (in Dat^{lo} mutants) (*17*) (Fig 2H). Hence, abnormal up or down-regulation of the dopaminergic system prevented behavioral plasticity in longer-sleeping socially-enriched individuals when switched to social-impoverishment .

Our observation that dopaminergic transmission affects experience-dependent plasticity in sleep-need is particularly compelling, given its role as a modulator of memory (*18*). We thus screened mutations in 49 genes implicated in various stages of learning and memory (*19-21*) to assess their impact on experience-dependent changes in sleep-need. Of these, only mutations in short- and long-term memory genes affected experience-dependent plasticity in sleep-need (Fig 3). Mutations in *dunce* (dnc^1) and *rutabaga* (rut^{2080}) have opposite effects on intracellular levels of cAMP, but are both correlated with short-term memory loss. In dnc^1 mutants, waking experience had no impact on subsequent sleep-need (Fig 3A). This effect was partially rescued in dnc^1/+ heterozygotes, but complete rescue was only achieved when a fully-functional *dunce* transgene (*22*) was introduced into the null mutant background (Fig 3A). rut^{2080} on the other hand, reminiscent of aberrant dopaminergic modulation, selectively abolished the ability of socially-enriched adults to demonstrate decreases in sleep following exposure to social-impoverishment (Fig 3A). Similarly, of the 43 long-term memory genes

screened, 17 (~40%) specifically disrupted the change in sleep-need in socially-enriched adults following exposure to social-impoverishment (Fig 3B). For example, over-expression of the *Drosophila CREB* gene repressor, *dCREB-b*, resulted in socially-enriched flies that continued to be long-sleepers even following exposure to social-impoverishment at maturity (Fig 3B). As a control, over-expression of the *dCREB-a* activator yielded wild-type phenotypic read-out (Fig 3C). It is noteworthy that not all long-term memory mutants disrupted the relationship between experience and sleep. Instead, the particular subset of genes identified, only half of which are expressed in the mushroom bodies (*21*), may specifically contribute to pathways that underlie sleep-dependent consolidation of memories.

Finally, to assess the correlation between sleep and memory, males flies trained for a courtship conditioning task that generated long-term memories were measured for sleep following training. Males whose courtship attempts are thwarted by non-receptive, recently-mated females or by males expressing aphrodisiac pheromones, form long-term associative memories as evidenced by subsequently reduced courtship of a receptive virgin female (K. Siwicki; (*23*)). Trained males that formed long-term memories slept significantly more than their untrained siblings and wake-controls (that were sleep-deprived while the experimental flies were being trained; Fig 4A-D). Exposure to a virgin female did not alter sleep-need. As before, this increase in sleep was associated with longer day-time sleep bouts in trained individuals compared to controls (Fig 4C).

Further, sleep-deprivation for 4 h immediately following training abolished training-induced changes in sleep-bout duration (24± 4 in trained vs. 18 ± 3 in naïve controls, p = 0.3617), as well as, courtship memory (Fig 4E). While these results are intriguing, invertebrate memory is particularly sensitive to extinction by mechanical perturbations. However, gentle handling that ensured wakefulness, but not mechanical stimulation, immediately following training also abolished subsequent courtship memory (Fig S4). Furthermore, sleep-deprivation *per se* did not affect the formation of long-term memory: trained flies that were allowed to sleep unperturbed for 24 h and then subjected to 4h of sleep-deprivation retained courtship memory (Fig 4E).

In summary, we demonstrate a rapid and dynamic relationship between prior social experience, memory consolidation and sleep in a genetically tractable model organism, *Drosophila melanogaster*. In particular, we report that experienced-dependent changes in sleep-need require dopaminergic modulation, cAMP signaling and a particular subset of long-term memory genes - supporting the hypothesis that sleep and neuronal activity may be inexorably intertwined. These observations are particularly compelling given two recent studies (*24, 25*)demonstrating a central role of the mushroom bodies in sleep regulation and emphasize the importance for establishing Drosophila as a model system to investigate the molecular pathways underlying sleep and plasticity.

SUPPORTING ONLINE MATERIAL:

Culture methods: All fly stocks were reared and tested on dark karo-syrup-yeast-agar medium, at 25° C, 65% humidity and a 12 h light:dark schedule.

Fly strains: Canton-Special (*C-S*), as wild-type ; *Clk*^{jrk} circadian rhythm mutant; *norpA*^{P41}/*norpA*^{P41} visually-impaired EMS mutant (*1*); olfactory EMS mutant, *smellblind*^{1} (*sbl*^{1}) generously donated by Joel Levine (*2*); homozygous *Dat*^{lo} / *Dat*^{lo} strain (*3*) ; Deficiency stock, *Df*(2R)Px2/SM5, which uncovers the *Dat*^{lo} locus; *5G23*/*CyO* an EMS mutant with auditory-specific deficits and it's background strain, *40A-G13*. Strains generously donated by Daniel Eberl (*4*); *rutabaga*^{2080} single *P*-element insertional mutant; *dunce*^{1} single *P*-element insertional mutant; heat-shock dunce strain containing the transposon, *hspdnc54* kindly donated by Ron Davis; *dCREBa* (activator) and *dCREBb* (repressor) strains kindly donated by Jerry Yin; 41 single *P*-element insertional mutants (Hartford mutants) generated in the *w*^{118}*/CJ-1* background strain that specifically disrupt long-term memory in the fly, along with the background strain generously donated by Tim Tully (*5*); Tai2 males naturally express elevated levels of pheromones that elicit robust courtship in wild-type C-S males (*6*).

Gal4-UAS strains: The Gal4 strains contained transposons bearing the promoter region of a gene of interest directing the expression of the yeast transcription factor gene, *Gal4*. Hence, the *Pdf-Gal4* strain bore the promoter of the *Pdf* (*Pigment dispersing factor*) gene, thus expressing the Gal4 protein in all cells that

would naturally express *Pdf*; the *Or83b-Gal4* strain contained the promoter of a ubiquitously expressed odourant receptor gene, *Or83b* (generously donated by Leslie Vosshall); and the *TH-Gal4* transgenic strain, contained a transposon had the Gal4 gene spliced to an upstream sequence of the dopamine biosynthetic enzyme, *tyrosine hydroxylase* (*TH*) promoter. The UAS strains contained transposons that contained the Gal4-DNA binding sequence, *UAS*, spliced to the *tetanus toxin light chain* (*TeTxLC*) gene, or the apoptotic gene, *rpr* (*reaper*). Binding of Gal4 to the *UAS* sequence occurs when *Gal4* strains are crossed to *UAS* strain, thus directing the expression of *TeTxLC* or *rpr* in specified neurons, resulting in the blocking of evoked neurotransmitter release or elimination of cells, respectively.

Experimental paradigm for juvenile social exposure: Late stage pupae were gently dislodged from the sides of vials in which they were developing using a moistened paint brush. They were divided into 2 groups: those, that were placed individually in small plastic tubes with fly food (isolated group); and those, that were sexed and placed in plastic fly vials with food in groups of 30-60, in a 1:1 sex ratio (socialised group, see Fig 1A). Emerging flies from both treatments were collected upon the completion of day 4 post-emergence and measured individually, for sleep parameters.

Experimental paradigm for adult social exposure: 4 day old flies reared in socially-deprived conditions, were divided into a control group that remained

isolated for another 4 days adult life, and the test group that was switched to enriched conditions on day 5, by pooling 30-60 individuals (1:1 male: female ratio) into a single vial for the same period (Fig 2). Similarly, group-reared, socially-enriched 4 day old adults were half maintained in enriched conditions (controls), and half isolated for a period of 4 days. At the end of adult exposure, flies were tested for sleep parameters. This procedure attempts to distinguish between the effects of early rearing from adult exposure, on subsequent sleep.

Behavioural measurements: Activity in the fly was assessed using the Drosophila Activity Monitoring System as previously described (*7*). Sleep was defined as periods of quiescence (no beam breaks) lasting 5 minutes or long. Activity data for each genotype was collected for a total of 3 undisturbed full days (72 h) and averaged.

Statistics and comparisons: In every case, comparisons were made between age-matched individuals exposed to the 2 treatment conditions within each particular genotype. Student's t test or an ANOVA with a *post hoc* Tukey test was applied when appropriate, for all comparisons. Bonferroni's correction was considered for multiple comparisons.

Induction of transgenes using heat: The *hsp-dnc54* strain was reared at 37°C and tested for sleep at 25°C. *dCREB-a* and *d-CREB-b* transgenic strains were placed 37°C for the period of adult exposure (Days 5-8) to social-enrichment or

impoverishment and subsequently tested at 25°C.

High Performance Liquid Chromatography Analysis: Age-matched *C-S* flies were exposed to 4 days of social-enrichment or impoverishment and subsequently divided into 2 groups. One group was measured for sleep parameters while the other group was dissected to collect whole brains. 20 whole brains were quickly dissected on ice and collected in 100 μl HPLC buffer. Samples were sent to Dr. Raymond F Johnson, Neurochemistry Core Lab, Nashville, for HPLC analyses.

Male courtship conditioning assay: This assay was developed in Kathy Siwicki's Lab. Wild-type *C-S* males were collected at eclosion and housed individually until they were between 4-6 days old. They were then individually paired with a single Tai2 trainer male (Tai2 males naturally express elevated levels of pheromones that elicit robust courtship behavior in wild-type *C-S* males) for a 1h training session. Predictably, during this time, naïve *C-S* males attempt to court the unreceptive Tai2 males. Three 1h training sessions were interspersed with 1h rest periods in order to facilitate the formation of long-term associative memories. At the end of training, *C-S* male subjects were placed in fresh tubes of food and monitored for sleep parameters.

Memory consolidation was measured by testing 'trained' *C-S* males with sexually-receptive virgin *C-S* females 48 hours after the end of the last training session. Males that have successfully formed long-term associative memories court virgin receptive females significantly less than naive controls. Courtship

Index (CI) for each male was calculated by dividing the time spent courting (the sum of the lengths of all of the courtship bouts) by the total length of the test.

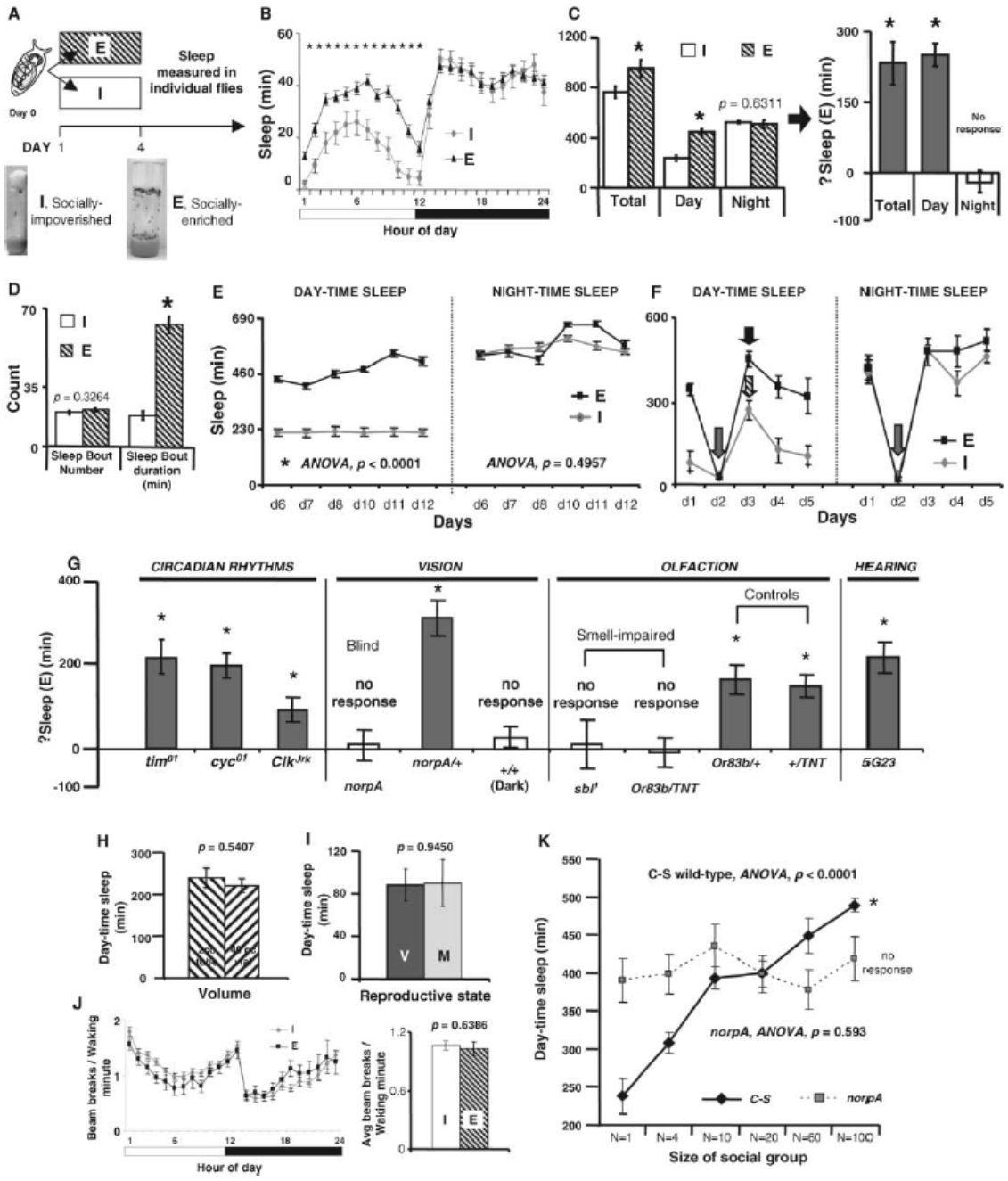

Fig. 1. Social experience changes *Drosophila* sleep patterns **A** Experimental paradigm for Juvenile Exposure. **B** Sleep per hour, over a 24h period. **C** Total sleep, Day-time sleep, Night-time sleep and ΔSleep(E), the response in sleep to social-enrichment, calculated as a difference in sleep between E individuals and their I siblings. **D** Day-time sleep bout number and duration. E, *n*=51; I, *n*=24. **E**

Sleep during 12 days post-social exposure. I, *n*=39; E, *n*=48. **F** Sleep following 24 h of SD. I, *n*=16; E, *n*=16. **G** ∆Sleep(E) in circadian, olfactory, visual and auditory mutants. **H** Sleep in flies reared in a 2cc tube and a 40cc fly vial. **I** Sleep in socially-impoverished virgins (V) and mated (M) flies. **J** Activity per waking minute each hour over 24 h. **K** Day-time sleep in *C-S* and blind mutants, exposed to increasingly larger social groups. N denotes total group size. * $p < 0.001$.

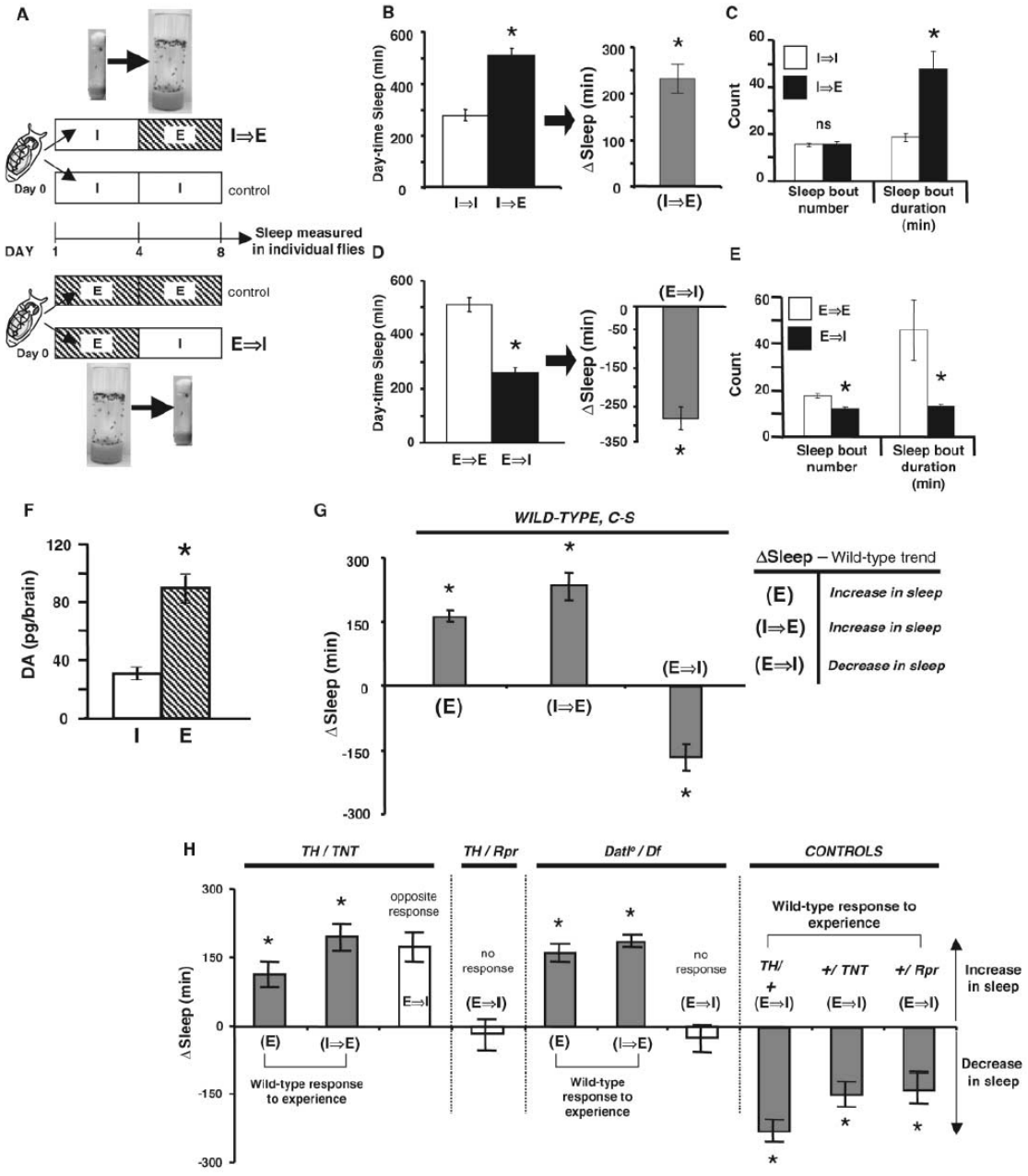

Fig. 2. Sleep-need and Dopamine Levels. **A** Experimental paradigm for Adult Plasticity. **B – E** Day-time sleep, sleep response (ΔSleep) and bout number and duration in I⇒E (*n*=20) and E⇒I (*n*=55) flies compared to their respective age-matched controls (I⇒I, *n*=25; E⇒E, *n*=23). **F** Dopamine content in whole brains. **G** ΔSleep in *C-S* flies. **H** ΔSleep in strains with aberrant dopaminergic

transmission. In the case of E⇒I *TH/TeTxLC*, flies show an aberrant increase in sleep. *$p < 0.005$.

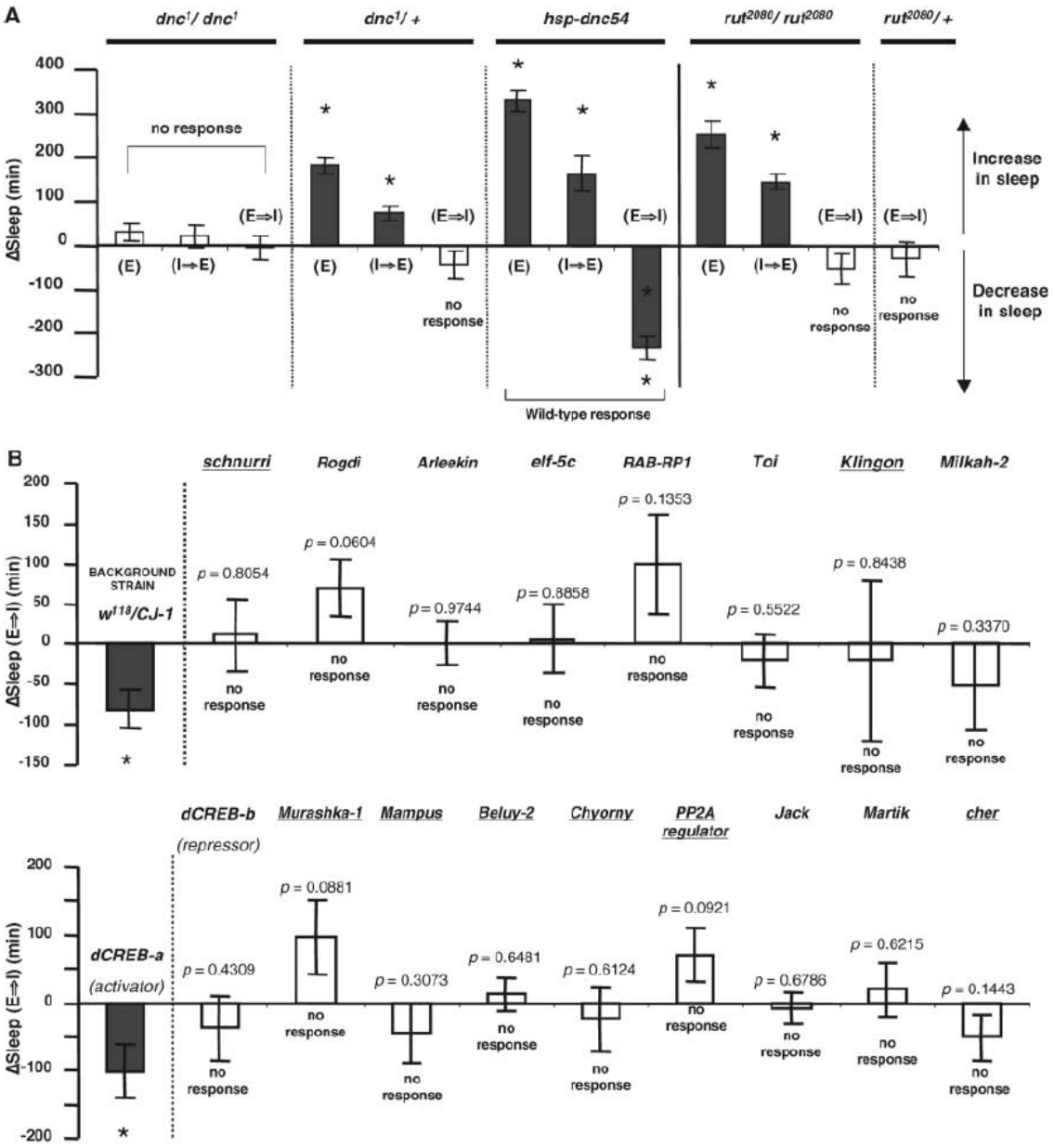

Fig. 3. Long and short-term memory mutants are resistant to experience-dependent changes in sleep. **A** ΔSleep in short-term memory mutants, *dnc*^{1} *dnc*^{1}*/+, hsp-dnc54* and *rut*^{2080}. **B** ΔSleep(E⇒I) in *w*^{118}*/CJ-1* wild-type background strain, *dCREB-a* (memory activator)and *dCREB-b* (memory repressor) heat-inducible strains and 17 long-term memory mutants. Underlined genes are not expressed in the mushroom bodies. *$p < 0.001$.

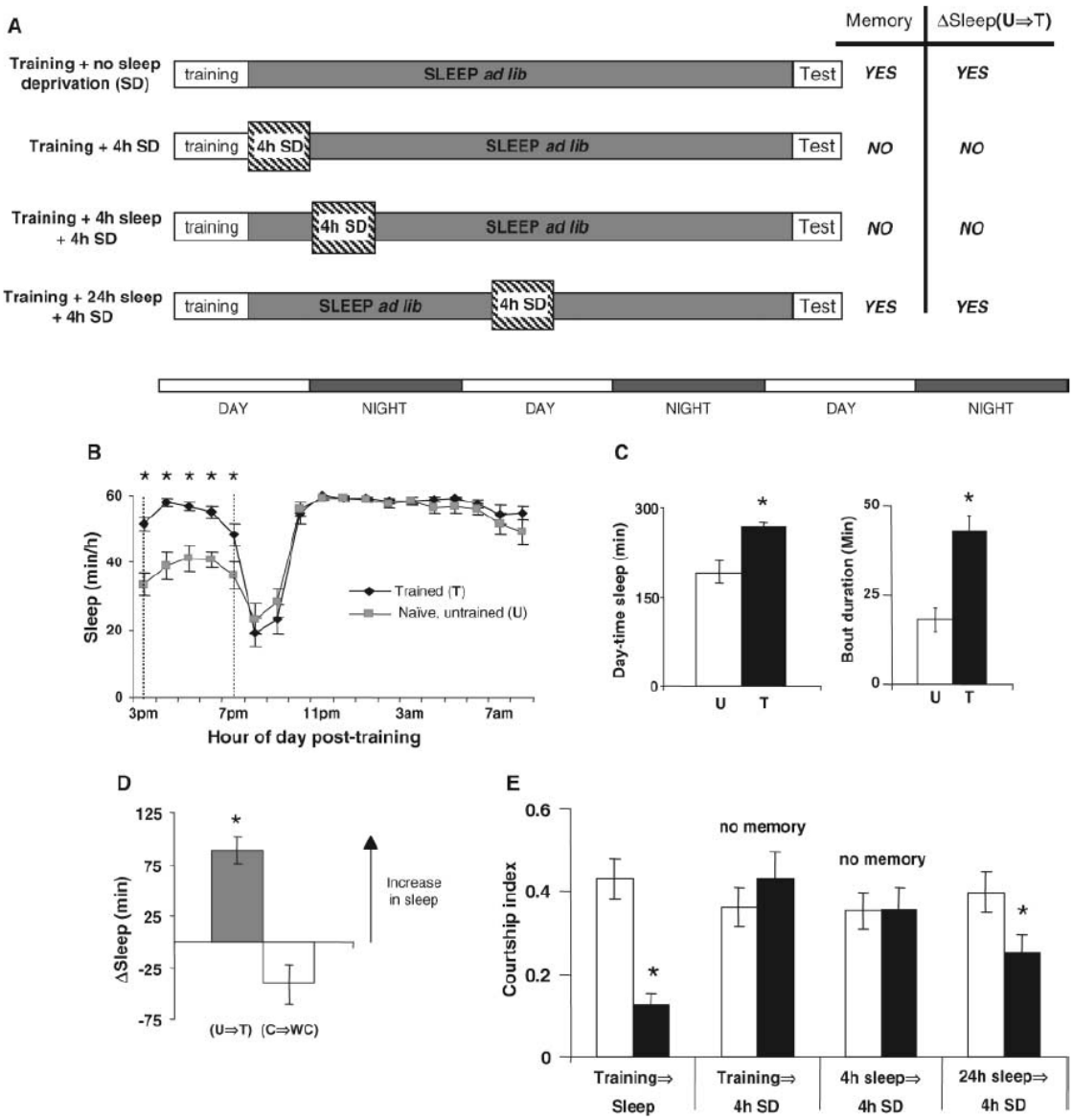

Fig. 4. Formation of associative memories is correlated with post-training increases in sleep. **A** Schematic of experimental design. **B** Sleep following training for courtship conditioning in trained (T) and untrained (U) individuals. **C** Day-time sleep and bout duration. **D** ΔSleep in trained and untrained flies (ΔSleep(U⇒T)) compared to ΔSleep in untrained wake and unperturbed controls (ΔSleep(WC⇒C)). **E** Courtship index (ratio of the percentage of time spent courting to total time of exposure) in T and U flies, after training; and following

SD.

References and Notes

1. S. S. Campbell, I. Tobler, *Neurosci Biobehav Rev* 8, 269 (1984).

2. J. C. Hendricks *et al.*, *Neuron* 25, 129 (2000).

3. P. J. Shaw, C. Cirelli, R. J. Greenspan, G. Tononi, *Science* 287, 1834 (2000).

4. C. Smith, *Behav Brain Res* 69, 137 (1995).

5. R. Stickgold, *Nature* 437, 1272 (2005).

6. R. Huber *et al.*, *Sleep* 27, 628 (2004).

7. H. van Praag, G. Kempermann, F. H. Gage, *Nat Rev Neurosci* 1, 191 (2000).

8. M. Heisenberg, M. Heusipp, C. Wanke, *J Neurosci* 15, 1951 (1995).

9. A. Balling, G. M. Technau, M. Heisenberg, *J Neurogenet* 4, 65 (1987).

10. K. S. Ho, A. Sehgal, *Methods Enzymol* 393, 772 (2005).

11. S. Ribeiro, V. Goyal, C. V. Mello, C. Pavlides, *Learn Mem* 6, 500 (1999).

12. P. Maquet *et al.*, *Nat Neurosci* 3, 831 (2000).

13. H. Miyamoto, H. Katagiri, T. Hensch, *Nat Neurosci* 6, 553 (2003).

14. J. D. Levine, P. Funes, H. B. Dowse, J. C. Hall, *Science* 298, 2010 (2002).

15. M. C. Larsson *et al.*, *Neuron* 43, 703 (2004).

16. F. Friggi-Grelin *et al.*, *J Neurobiol* 54, 618 (2003).

17. D. Brodbeck *et al.*, *DNA Cell Biol* 17, 621 (1998).

18. P. S. Goldman-Rakic, *Adv Pharmacol* 42, 707 (1998).

19. J. Dubnau, T. Tully, *Annu Rev Neurosci* 21, 407 (1998).

20. J. C. Yin, T. Tully, *Curr Opin Neurobiol* 6, 264 (1996).

21. J. Dubnau *et al.*, *Curr Biol* 13, 286 (2003).

22. B. Dauwalder, R. L. Davis, *J Neurosci* 15, 3490 (1995).

23. S. M. McBride *et al.*, *Neuron* 24, 967 (1999).

24. W. J. Joiner, A. Crocker, B. H. White, A. Sehgal, *Nature* 441, 757 (2006).

25. J. L. Pitman, J. J. McGill, K. P. Keegan, R. Allada, Nature 441, 753 (2006).

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Chapter 3:

Use-dependent plasticity in clock neurons regulates sleep need

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Abstract

Sleep is important for memory consolidation and is responsive to waking experience. Unfortunately the underlying mechanisms are unknown. The circadian clock regulates sleep and influences memory. Thus, clock circuitry is uniquely positioned to coordinate interactions between processes underlying memory and sleep-need. We have previously shown that flies increase sleep both following exposure to an enriched social environment and after protocols that induce long-term memory. Here we show that flies mutant for the adenylyl cyclase *rutabaga* (*rut*), the clock gene *period* (*per*), and the *Drosophila* homologue of *Serum Response Factor*, *blistered* (*bs*), are deficient for experience-dependent increases in sleep. Rescue of each of these genes within the *Pigment Dispersing Factor* (*pdf*)-expressing ventral lateral neurons (LN_Vs) restores increased sleep following social enrichment. Rescue of wild-type *bs* or *per* within the LN_Vs restores long-term memory after Courtship Conditioning. Social experiences that induce increased sleep are associated with an increase in the number of synaptic terminals in the LN_V projections into the medulla. Moreover, the number of synaptic terminals is reduced during sleep and this decline is prevented by sleep deprivation. These results are consistent with the hypothesis that the function of sleep is for synaptic down-scaling and demonstrates that the clock plays a fundamental role in plasticity and sleep.

Although sleep is a process that is necessary for survival the function(s) of sleep are unknown (*1,2*). Sleep is regulated by circadian influences and is important for consolidation of Long-Term Memories (LTM) (*3-5*). Additionally, LTM is modulated by circadian mechanisms (*6,7*). Because the relationship between sleep, memory, and circadian rhythms seem to be phylogenetically conserved, *Drosophila* can be used to elucidate mechanisms that coordinate these processes. *Drosophila* show an increase in daytime sleep following exposure to socially enriched environments (*5*). Similarly, an increase in sleep following courtship conditioning is necessary for LTM (*5*).

Increased sleep after social enrichment is dependent upon genes that are required for learning and memory, including genes that alter cAMP signaling (*5*). Although newly eclosed flies that are mutant for the adenylyl cyclase *rutabaga* (rut^{2080}) show increased sleep after social enrichment, 3-4 day old adult *rut* mutants do not respond to changes in the social environment (Fig. S1). Elevating wild-type *rut* in adult flies using a RU486 inducible driver rescued experience-dependent increases in sleep in adult *rut* mutants (Fig. S1); vehicle treated siblings showed no increase in sleep (Fig. S1). To identify circuits that mediate experience-dependent increases in sleep, we used a series of GAL4 lines to drive wild-type *rut* expression in brain circuits (Fig. 1A). Figure S2 illustrates the data analysis used to quantify each GAL4 rescue. Expression of UAS-*rut* using *pdf*-GAL4 restored the increase in daytime sleep and daytime sleep bout duration, although to a lesser extent than *GSelav* (Fig. 1B-E). The

expression pattern of *pdf*-GAL4 is limited to the ventral lateral neurons (LN_Vs), a group of clock neurons that express *Pigment-Dispersing Factor* (*pdf*) (*8, 9*). Although *pdf* is the only known output from the LN_Vs, flies mutant for *pdf* show a wild-type increase in sleep (Fig. S3).

Given this role of clock cells, we examined the clock gene *period* (*per*) which is expressed in the LN_Vs and is required for LTM (*6*). Rescue of wild-type *per* using a 7.2 Kb fragment of the *per* genomic sequence (per^{01}; *per*+7.2-2) restored expression of PER at CT0 within the LN_Vs as well as the dorsal lateral neurons, LN_Ds, (Fig. 1F); mutant flies carrying a null mutation, per^{01}, expressed no PER (Fig. 1G). While per^{01} mutants showed no increase in sleep following social enrichment, per^{01};*per*+7.2-2 flies displayed normal experience-dependent increases in sleep (Fig. 1H). per^{01} mutants have no LTM when tested 48-hours after training and only show a transient increase in sleep (Fig. 1I, J). per^{01};*per*+7.2-2 flies displayed LTM (Fig. 1K) and increases in sleep (Fig. 1L,M). Although *per* levels are low in mutants for *Clock* and *cycle*, both acquire LTM (*6*) and increase sleep following social enrichment (*5*). Thus, only a very small amount or *per* may be required to support increased sleep and LTM.

To further investigate the role of synaptic plasticity in clock cells, we used the *Drosophila* homolog for *Serum Response Factor* (*SRF*), *blistered* (*bs*). In mice, *SRF* is essential for activity-induced gene expression and plays an important role in synaptic long-term potentiation (*10*) and in contextual habituation (*11*). *bs* retains a 93% identity with *SRF* within the DNA-binding MCM1-ARG80-

Agamous-Deficiens-SRF (MADS) domain (*12*). Social enrichment elevated the transcription of *bs* in wild-type *Canton-S* (*Cs*) flies (Fig. 2A). Mutants carrying a p-element inserted into the *bs* gene (P{GAL4}bs^{1348}) do not increase sleep following social enrichment (Fig. 2B). This deficit was also found in flies carrying either of two other mutant alleles for *bs* (bs^{2} and bs^{3}), was present in flies that are homozygous for mutant *bs* alleles, and flies that have been outcrossed to either *Cs* or to flies carrying the In(2LR)Px^{4} deficiency (Fig. 2C). The P-element insertion in bs^{1348} preserves the MADS domain; similar N-terminal truncated mutant SRF acts as dominant negative (*13*). BS is expressed throughout the brain, including *pdf*-expressing LN_Vs (Fig. 2D-F). When UAS-*egfp* was driven by P{GAL4}bs^{1348}, expression was restricted to a small number of neurons including the LN_Vs (Fig. 2G-I). Expression of *bs* using P{GAL4}bs^{1348} to drive either of two wild-type *bs* (UAS-*bs*) constructs rescued experience-dependent increases in sleep (Fig. 2J). Moreover, inducing *bs* expression within the LN_Vs using *pdf*-GAL4 increased sleep following social enrichment (Fig. 2K).

To establish whether expression of *bs* is required for LTM, we tested flies carrying the P{GAL4}bs^{1348} mutant allele using courtship conditioning. While *P{GAL4}*bs^{1348}*/+* flies acquire short-term memory (Fig. 2L), LTM was impaired (Fig. 2M, left). Rescue of wild-type *bs* using P{GAL4}bs^{1348} restored LTM (Fig. 2M, right). Next, we used the GAL4 repressor *cry*-GAL80 to block UAS-*bs* expression within the LNs. While UAS-*bs*/+; *cry*-gal80/+ control flies showed significant courtship suppression (Fig. 2N, left), P{GAL4}bs^{1348}/UAS-*bs*; *cry*-

GAL80/+ flies had no LTM (Fig. 2N, right) suggesting a role for the LNs although we cannot exclude a role of the DNs. Although *SRF* deletion in mouse forebrain results in neurons with abnormal morphology (*14*), the morphology of LN_Vs in mutant P{GAL4}*bs*^{1348}/+ flies (Fig. S4A) did not differ from that of LN_Vs in P{GAL4}*bs*^{1348}/UAS-*bs* rescue flies (Fig. S4B). All three mutants for *bs* had intact circadian rhythms and showed anticipatory activity prior to light-dark transitions; only *bs*^{3} flies show an altered period under constant darkness (Fig. S5A-F). These findings suggest that there are no developmental abnormalities in the LNvs in *bs* mutants.

Hypomorphic alleles for *bs* prevent proper wing development through interactions with *Epidermal growth factor receptor* (*Egfr*) signaling (*15-17*). Because *Egfr* alters sleep in *Drosophila* (*18*), interactions between *bs* and *Egfr* may regulate responses to social experience. Following social enrichment, transcription of *Egfr* was significantly elevated in *Cs* flies (Fig. 3A). The *Egfr* genomic sequence contains several CArG elements that can be bound by *bs* to promote transcription (Fig. 3B) and transcription of *Egfr* was significantly reduced in *bs* mutants (Data not shown). Thus, we used P{GAL4}*bs*^{1348} to drive expression of a constitutively active *Egfr* construct (UAS-*Egfr**). Although P{GAL4}*bs*^{1348}/+ mutants showed no change in sleep after social enrichment (Fig. 3C), activation of *Egfr* in P{GAL4}*bs*^{1348}/+;UAS-*Egfr**/+ flies increased sleep (Fig. 3D-E). Conversely, the expression of a dominant-negative construct for *Egfr* (UAS-*Egfr*^{DN}) using *pdf*-GAL4 prevented increases in sleep following social enrichment

while parental controls (*pdf*-GAL4/+ and UAS-*Egfr*^{DN}/+) were wild-type (Fig. 3F). A recent theory proposes that a function of sleep is to downscale synaptic connections (*19*). Moreover, structural plasticity can be induced by environmental manipulation in *Drosophila* (*20*). To quantify the effect of social enrichment on the number of post-synaptic terminals in LN_V projections, we used *pdf*-GAL4 to drive expression of a GFP-tagged construct of the post-synaptic protein *discs-large* (UAS-*dlg*WT-gfp). After five days of social enrichment, LN_V projections into the medulla of *pdf*-GAL4/+;;UAS-*dlg*WT-gfp/+ flies contained significantly more GFP-positive terminals (Fig. 4A-C). Although we have not demonstrated that the labeled synaptic terminals are functional, these tools have been used to quantify synapses (*21*). The expression of the UAS-*dlg*WT-GFP marker did not alter synaptic function in a wild-type background (*20*) and did not prevent the increase in sleep when expressed using pdf-GAL4 following social enrichment (Fig. S6). To test the effect of waking on synapse number, socially isolated *pdf*-GAL4/+;;UAS-*dlg*WT-gfp/+ flies and their enriched siblings were either allowed to sleep *ad libitum* or were sleep deprived for 48 hours after social enrichment. While the number of *dlg*-GFP positive terminals remained elevated in sleep deprived socially enriched flies, terminal number was significantly reduced in siblings that were allowed to sleep (Fig. 4D). Similarly, the number of pre-synaptic terminals in LN_V projections into the medulla using a GFP-tagged construct of the pre-synaptic protein *synaptobrevin* (UAS-*VAMP*-GFP) in *pdf*-GAL4/+;UAS-*VAMP*-GFP/+ flies was increased (Fig. 4E-G). Following 48 hours of recovery, socially enriched *pdf*-GAL4/+;UAS-*VAMP*-GFP/+ flies had a reduced

number of *VAMP*-GFP positive pre-synaptic terminals relative to their sleep deprived siblings (Fig. 4H). A recent study has reported a clock-dependent remodeling in the axonal terminals of the PDF circuit that is highest during the day (*22*). Recent data indicates that hyper-excitation of a subset of the LN_Vs suppresses sleep in *Drosophila (23-25).* Together with our results these data suggest that the PDF circuit is well suited to test the hypothesis that sleep acts to down-scale synaptic connections that are potentiated during waking experience. Given these findings, we hypothesize that complex sensory signals induce a prolonged elevation of LN_V activity during social enrichment. The strength of these signals might then result in an increase in synaptic strength by Hebbian mechanisms. We presume that several days of enriched social experience elevates LN_V activity. Thus, we hypothesize that synaptic-homeostatic mechanisms decrease the excitability of the LN_Vs to prevent chronic hyper-excitation and hold firing rate around a baseline set-point; similar homeostatic functioning has been previously described in *Drosophila* central synapses (*29*). Once socially enriched flies are moved into sleep monitors, complex sensory stimuli would be removed and the lowered excitability of the LN_Vs would reduce the baseline firing rate below that of socially isolated controls. This lowered firing rate might then permit an increase in sleep. Simultaneously, reducing the excitability of the LN_Vs might also allow for the down-scaling of LN_V synaptic connections after social enrichment. Together with the data presented by Sheeba *et al* (*28*), our data begin to describe a neuronal circuit for experience dependent changes in sleep.

In summary, we demonstrate that the LN_Vs, a subset of the *Drosophila* circadian circuitry, are required for increased sleep following social experience and for consolidation of long-term memories. These behavioral responses are regulated by *rut*, *per*, and *bs* expression within the LN_Vs. Importantly, the role of the LN_Vs in behavioral plasticity is independent of circadian mechanisms. Finally, we show that social experience increases synaptic terminal number in LN_V projections into the medulla and that subsequent sleep downscales the number of synaptic terminals. Together, these data further support a fundamental function for sleep in synaptic plasticity and memory consolidation.

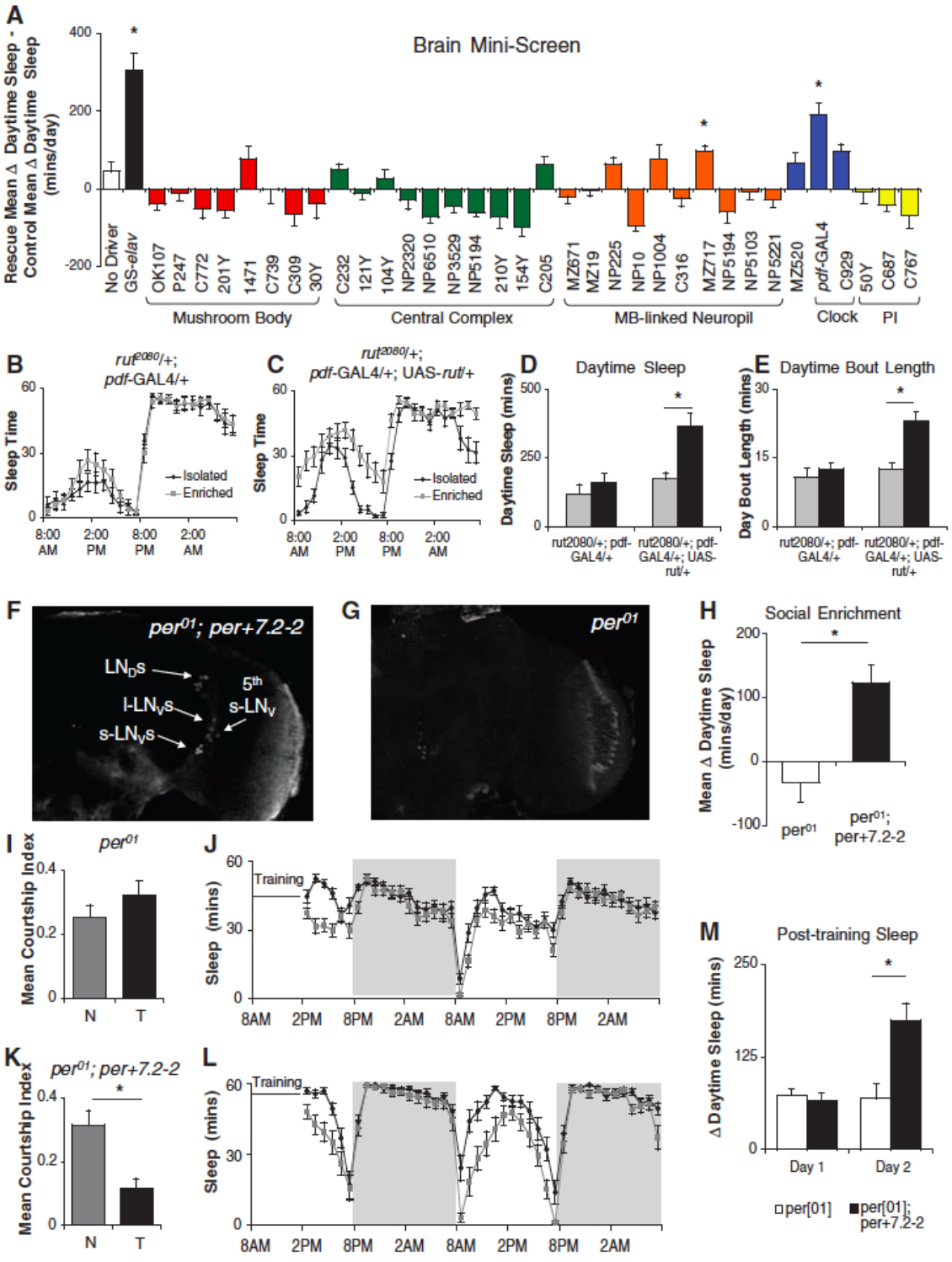
A
Brain Mini-Screen
Rescue Mean Δ Daytime Sleep - Control Mean Δ Daytime Sleep (mins/day)
400
200
0
-200
No Driver
GS-elav
OK107
P247
C772
201Y
1471
C739
C309
30Y
C232
121Y
104Y
NP2320
NP6510
NP3529
NP5194
210Y
154Y
C205
MZ671
MZ19
NP225
NP10
NP1004
C316
MZ717
NP5194
NP5103
NP5221
MZ520
pdf-GAL4
C929
50Y
C687
C767
Mushroom Body
Central Complex
MB-linked Neuropil
Clock
PI
B
rut2080/+; pdf-GAL4/+
Sleep Time
Isolated
Enriched
8:00 AM
2:00 PM
8:00 PM
2:00 AM
C
rut2080/+; pdf-GAL4/+; UAS-rut/+
D
Daytime Sleep
Daytime Sleep (mins)
rut2080/+; pdf-GAL4/+
rut2080/+; pdf-GAL4/+; UAS-rut/+
E
Daytime Bout Length
Day Bout Length (mins)
F
per01; per+7.2-2
LNDs
5th s-LNv
l-LNvs
s-LNvs
G
per01
H
Social Enrichment
Mean Δ Daytime Sleep (mins/day)
per01
per01; per+7.2-2
I
per01
Mean Courtship Index
N
T
J
Training
Sleep (mins)
8AM
2PM
8PM
2AM
K
per01; per+7.2-2
L
M
Post-training Sleep
Δ Daytime Sleep (mins)
Day 1
Day 2
per[01]
per[01]; per+7.2-2

Fig. 1 – Clock cells regulate experience-dependent increases in sleep

(A) Data for each GAL4 line (GAL4> *rut*^{2080}/+;;UAS-*rut*/+) is compared to its parental line (GAL4:*rut*^{2080}). No increase in ΔDaytime Sleep is observed in the absence of GAL4 [(*rut*^{2080}/+;;UAS-*rut*/+)-(*rut*^{2080}/+); white bar]. The mean ΔDaytime Sleep for *Gs-elav*-GAL4 (RU+ vs. RU-) is shown to facilitate comparisons (Black). One-way ANOVA for genotype, $F_{(33,903)}$=9.09. (*p<.05 with correction for 34 comparisons; n ≥ 16 for all groups). (B-C) Sleep following social enrichment in mutant *rut*^{2080}/+; *pdf*-GAL4/+ flies and *rut*^{2080}/+; *pdf*-GAL4/+; UAS-*rut*/+ (n = 16 in each group). (D-E) Average daytime-sleep and sleep bout-duration in *rut*^{2080}/+; *pdf*-GAL4/+ mutant and *rut*^{2080}/+; *pdf*-GAL4/+; UAS-*rut*/+ rescue flies. Two-way ANOVA reveals a genotype by condition interaction for sleep ($F_{(1,57)}$ = 4.44, p = 0.03) and bouts ($F_{(1,57)}$ = 6.59, p = 0.013) (*p<.05, planned pair-wise comparisons with a Tukey correction; n=14-16 for all groups). (F-G) PER immunohistochemistry of *per*^{01};*per*+7.2-2 and *per*^{01} mutants. (H) ΔDaytime Sleep in *per*^{01} mutants and *per*^{01};*per*+7.2-2 flies (p = 0.0002, n = 30-32 each group). (I) Male *per*^{01} mutants do not exhibit a reduction in courtship 48 hours after a spaced Courtship Conditioning (N = Naïve, T = Trained, n = 13-14). (J) *per*^{01} flies only exhibit a transient increase in sleep immediately following training. (K) *per*^{01};*per*+7.2-2 flies exhibit suppression of courtship 48 hours after training (p = 0.001, n = 13 each group). (L) *per*^{01};*per*+7.2-2 males, also show sustained increases in sleep for two days. (M) ΔDaytime Sleep is quantified for *per*^{01} and *per*^{01};*per*+7.2-2 males. *per*^{01};*per*+7.2-2 males. Two-way ANOVA reveals a genotype by day interaction $F_{(1,39)}$ = 7.48, p = 0.009. (*p<.05 planned

pair-wise comparisons with a Tukey correction; n = 13-14 each group)

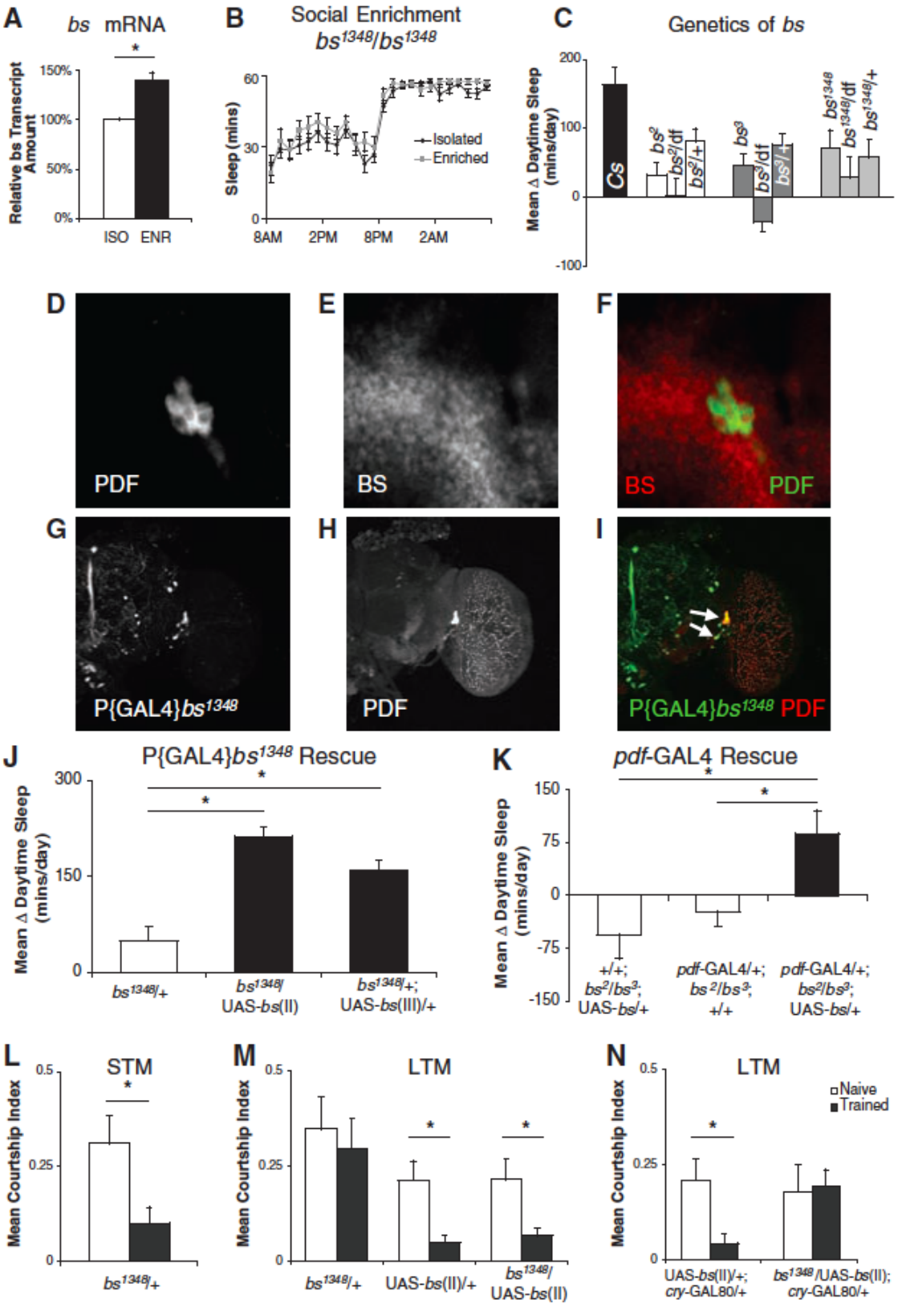
A
bs mRNA
Relative bs Transcript Amount
150%
100%
50%
0%
ISO
ENR
B
Social Enrichment
bs1348/bs1348
Sleep (mins)
60
30
0
8AM
2PM
8PM
2AM
Isolated
Enriched
C
Genetics of bs
Mean Δ Daytime Sleep (mins/day)
200
100
0
-100
Cs
bs2
bs2/df
bs2/+
bs3
bs3/df
bs3/+
bs1348
bs1348/df
bs1348/+
D
PDF
E
BS
F
BS
PDF
G
P{GAL4}bs1348
H
PDF
I
P{GAL4}bs1348 PDF
J
P{GAL4}bs1348 Rescue
Mean Δ Daytime Sleep (mins/day)
300
150
0
bs1348/+
bs1348/ UAS-bs(II)
bs1348/+; UAS-bs(III)/+
K
pdf-GAL4 Rescue
Mean Δ Daytime Sleep (mins/day)
150
75
0
-75
-150
+/+; bs2/bs3; UAS-bs/+
pdf-GAL4/+; bs2/bs3; +/+
pdf-GAL4/+; bs2/bs3; UAS-bs/+
L
STM
Mean Courtship Index
0.5
0.25
0
bs1348/+
M
LTM
Mean Courtship Index
0.5
0.25
0
bs1348/+
UAS-bs(II)/+
bs1348/ UAS-bs(II)
N
LTM
Naive
Trained
Mean Courtship Index
0.5
0.25
0
UAS-bs(II)/+; cry-GAL80/+
bs1348/UAS-bs(II); cry-GAL80/+

Fig. 2 –*blistered* regulates experience-dependent increases in sleep.

(A) *bs* transcripts are significantly elevated in socially enriched *Cs* flies compared to their isolated siblings ($p = 0.03$). (B) Flies homozygous for the P{GAL4}bs^{1348} insertion do not respond to social enrichment with an increase in sleep ($n = 16$ each group). (C) Mean ΔDaytime Sleep is absent in bs^2 and bs^3 mutants and persists when each allele (bs^2, bs^3, or P{GAL4}bs^{1348}) is outcrossed to Cs or with the Deficiency In(2LR)Px^4. One-way Anova for genotype ($F_{(9,124)} = 2.73$, $p = 0.04$, $n = 32$ each group). (D) Expression of UAS-egfp using *pdf*-GAL4 labels the cell bodies of LN_Vs. (E) Immunohistochemistry using anti-BS (1:1000). (F) Co-localization of BS and *pdf*-GAL4 indicates that BS is expressed in *pdf*-expressing LN_Vs. (G) P{GAL4}bs^{1348} was used to drive UAS-egfp and the expression pattern was evaluated using confocal microscopy. (H) Brains of P{GAL4}bs^{1348}*/+;UAS-egfp* flies were co-labeled with anti-PDF antibody (1:10000) (I) Co-localization of GFP with PDF indicates that P{GAL4}bs^{1348} drives GAL4 expression in *PDF*-expressing LN_Vs (arrows). (J) The failure to respond to social enrichment with an increase in sleep can be rescued by combining P{GAL4}bs^{1348} with either of two separate UAS-*bs* alleles. One-way ANOVA for genotype ($F_{(2,45)} = 22.86$, $p = 1.4 \times 10^{-7}$, *p<.05 Bonferroni post-hoc test, $n = 16$ each group). (K) Expressing wild-type *bs* using *pdf*-GAL4 in an otherwise *bs* mutant background (*pdf*-GAL4/+; bs^2/bs^3;UAS-*bs*) rescues increases in ΔDaytime Sleep); parental lines are shown in white. The lower Δsleep vs. 2J may indicate a partial rescue. One-way ANOVA for genotype ($F_{(2,45)} = 6.30$, $p = 0.003$, *p<.05 Bonferroni post-hoc test, $n = 16$ each group). (L) Courtship suppression in P{GAL4}bs^{1348} mutants tested five

minutes after Courtship Conditioning (White bars represent naïve males, Dark bars represent trained males) ($p = 0.02$, $n = 13$ each group). (M) LTM is disrupted in P{GAL4}bs^{1348}/+ mutants tested 48 hours after training and rescued by expression of UAS-*bs.* Two-way ANOVA reveals a genotype by condition interaction ($F_{(2,72)} = 16.73$, $p = 0.000$) (*$p<.05$, planned pair-wise comparisons with a Tukey correction; n=13 for all groups). (N) Blocking GAL4 expression in clock cells using *cry*-GAL80 prevents rescue of LTM. Genotype by Condition interaction ($F_{(1,48)} = 2.32$, $p = 0.14$) (*$p<.05$, planned pair-wise comparisons with a Tukey correction n=13 for all groups).

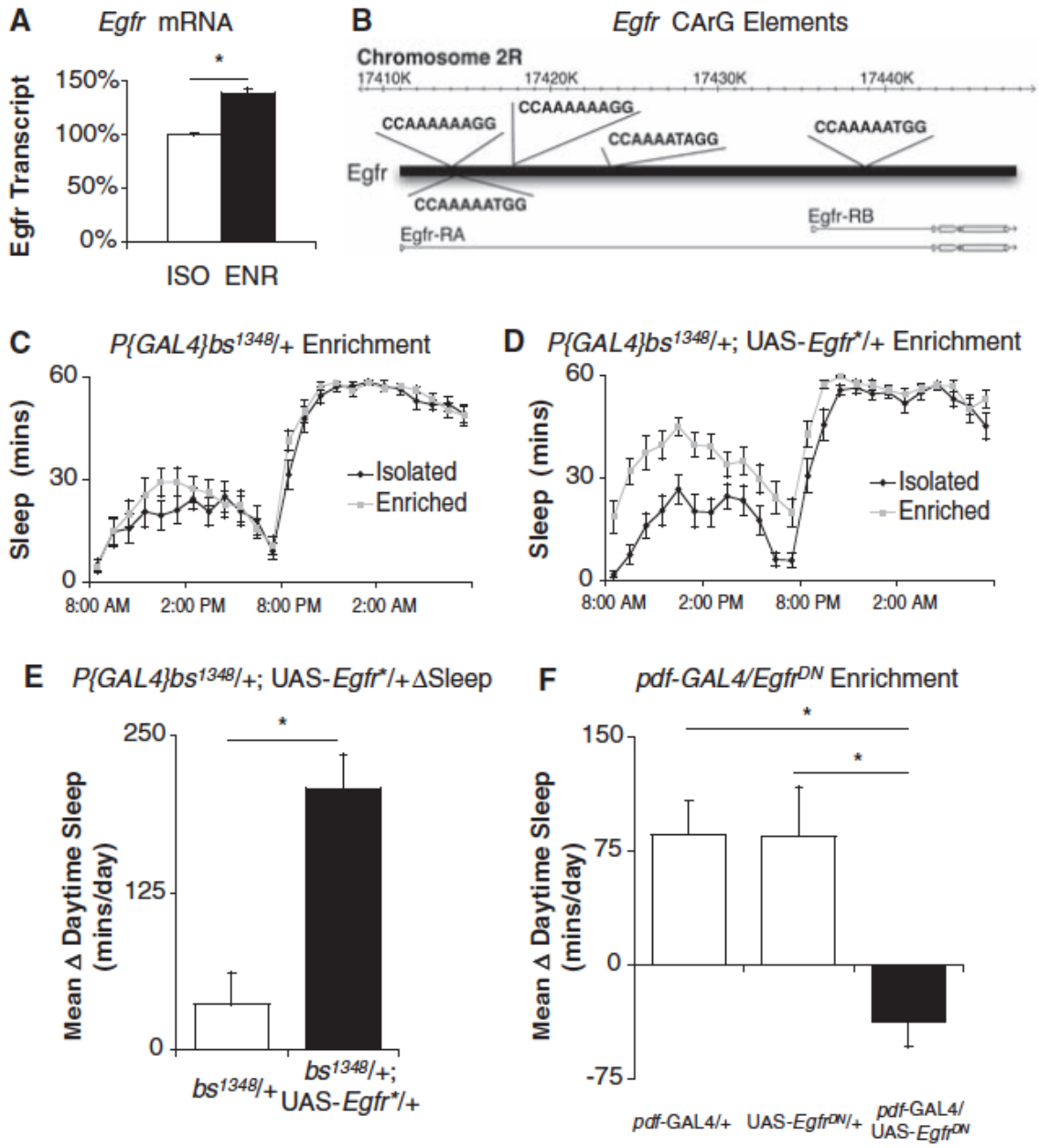

Fig. 3 – *Egfr* mediates experience-dependent sleep.

(A) Transcription of *Egfr* is significantly elevated in socially enriched *Cs* flies compared to their isolated siblings as measured by qPCR. (B) Genomic *Egfr* sequence contains several SRF-binding CArG elements. (C-D) Driving the expression of UAS-*Egfr** with P{GAL4}*bs*^{1348} restores ΔSleep after social enrichment. (E) Summary of the response for data shown in C-D (*p = 7x10^{-5}, n

= 16 each group). (F) No change in ΔSleep is observed in *pdf*-GAL4/UAS-*Egfr*^{DN}. One-way ANOVA for genotype $F_{(2,44)} = 7.75$, p = 0.001, (*p<.05, Bonferroni correction, n = 15-16 each group).

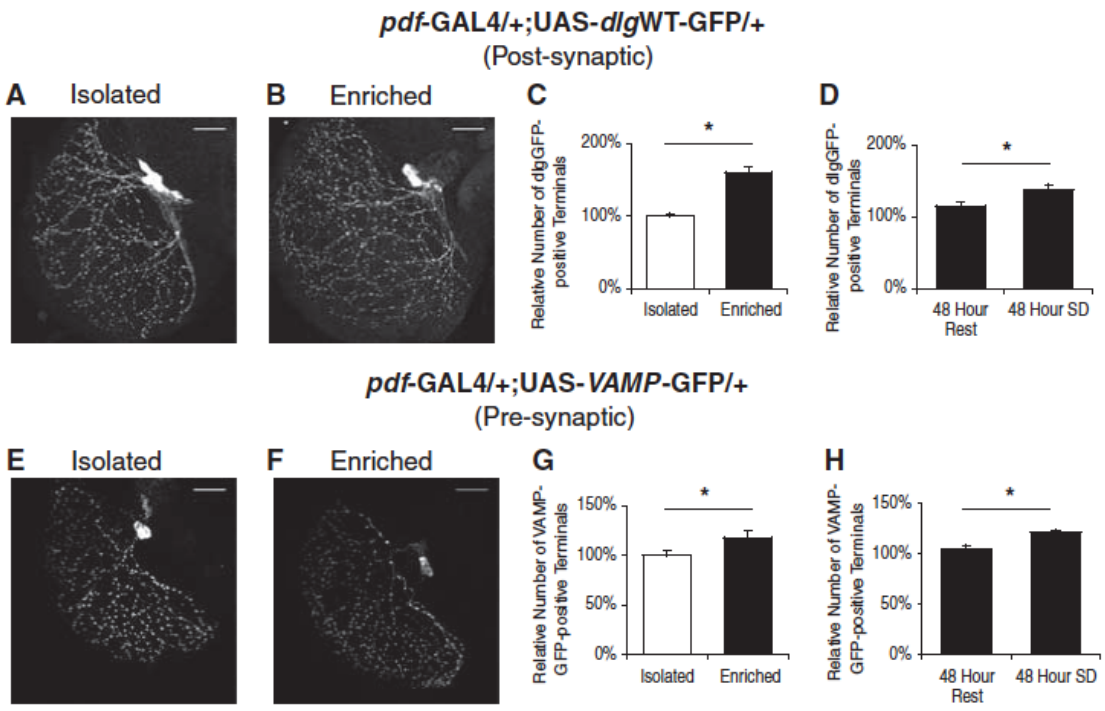

Fig. 4 – LN_V synapse number in medulla

(A-B) LN_V projections in socially enriched *pdf*-GAL4/+;; UAS-*dlg*WT-gfp/+ flies contain more GFP-positive terminals than their socially isolated siblings when evaluated using confocal microscopy. (C) Relative quantification of *dlg*-GFP-immunopositive terminals in socially isolated *pdf*-GAL4/+;; UAS-*dlg*WT-gfp/+ flies v.s. socially enriched siblings ($p = 1.5x10^{-9}$, n = 33-34 each group). (D) *dlg*-GFP positive terminals 48 hours following social enrichment in sleep deprived flies and their normally sleeping siblings. (p = 0.003, n = 15-18 each group). (E-F) Social enrichment of *pdf*-GAL4/+; UAS-*VAMP*-GFP/+ flies induces a modest increase of LN_V terminals relative to isolated siblings. (G) Relative quantification of *VAMP*-GFP-positive terminals in socially isolated *pdf*-GAL4/+;UAS-*VAMP*-gfp/+ flies and their socially enriched siblings (p = 0.03, n = 16-18 each group). (H) UAS-*VAMP*-gfp positive terminals 48 hours following social enrichment in sleep deprived flies

and their normally sleeping siblings ($p = 0.01$, $n = 18$ each group).

Figure S1

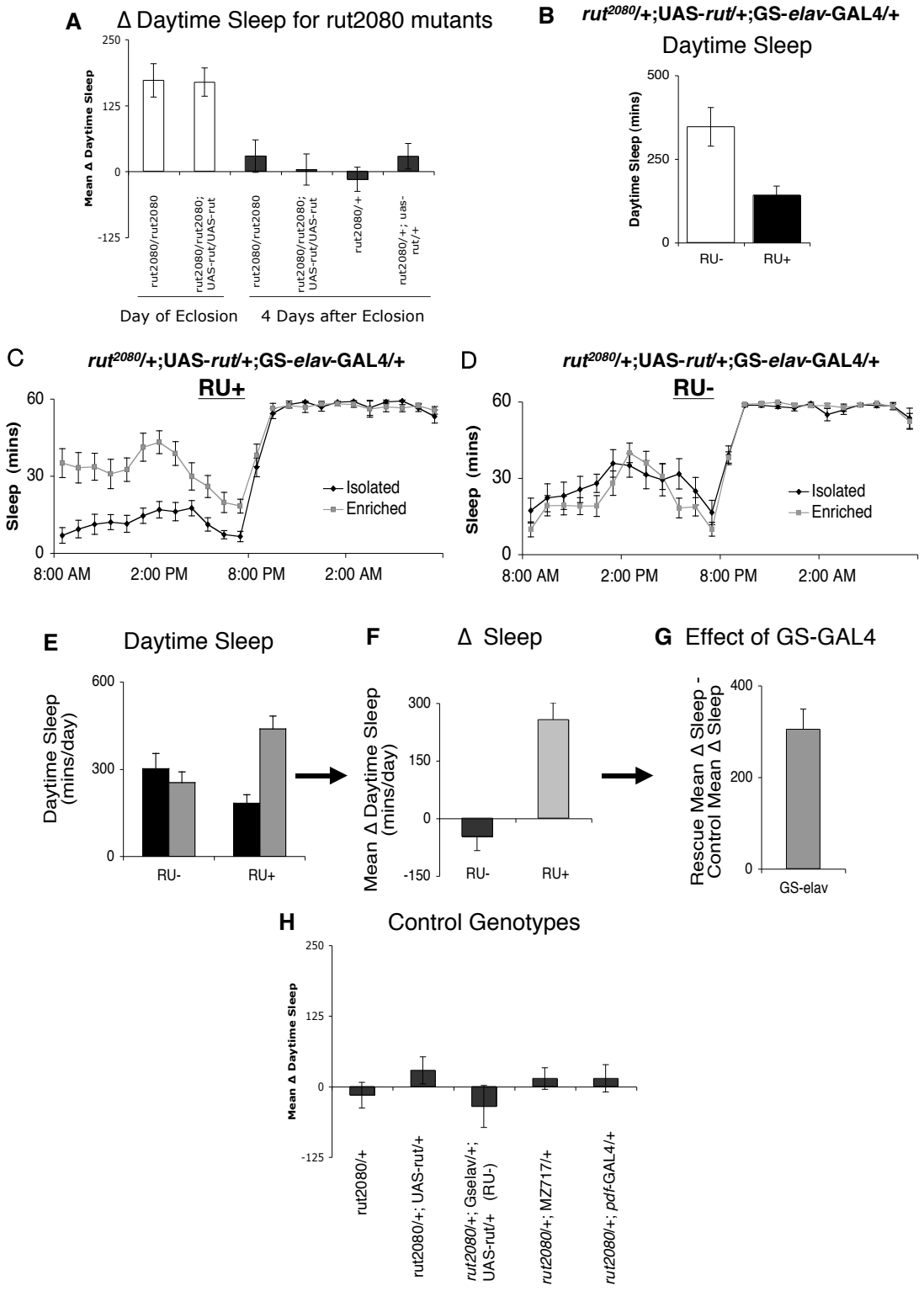

Figure S1 – Pan-neuronal expression of *rut* rescues experience-dependent increases in sleep. (A) Although *rut*$_{2080}$ mutants show increased sleep when enrichment starts within a day after eclosion (white), there is no change in sleep

when 3-4 day old flies that are homozygous or heterozygous for *rut*$_{2080}$ are socially enriched (grey). (B) The GeneSwitch system was used to express wild-type *rut* throughout the brains of flies otherwise mutant for *rut* using *GS-elav*-GAL4. Vehicle control (RU-) fed *rut*$_{2080}$/+;*UAS-rut*/+;*GS-elav*-GAL4/+ flies display a significant increase in daytime sleep compared to siblings fed RU486 (RU+; 100_g/ml) (*p,<.05; n=16 each group). A previous report has shown that rutabaga mutants sleep more than controls (*6*). (C-D) *GS-elav*-GAL4 was also effective in rescuing experience dependent increases in sleep in 3-4 day old flies. No change in sleep was observed in siblings fed vehicle. (E) To quantify the change in sleep induced by expressing UAS-*rut*$_{wt}$ with a specific GAL4 driver, we first measure the amount of daytime sleep over each of 3 days for socially isolated (Black) and socially enriched groups (grey). In this example, data are shown for induced (RU+) and vehicle control (RU-) for *rut*$_{2080}$/+; GS-*elav*-GAL4/+; UAS-*rut*/+. 2(RU+, RU-) by 2(Isolated, Enriched) ANOVA reveals a significant main effect for condition ($F_{(1,60)} = 6.474$, $p = 0.014$), a significant RU by condition interaction ($F_{(1,60)} = 13.511$, $p = 0.001$), but no significant main effect for RU ($F_{(1,60)} = 0.693$, $p = 0.408$). Thus, in the isolated condition, RU+ rescues wild-type baseline sleep whereas in the enriched condition RU+ rescues the wild-type response to changes in the social environment. (F) The average daytime sleep for the isolated group is subtracted from the daytime sleep for each individual socially-enriched sibling. The difference is referred to as ΔSleep. (G) Finally, the Mean ΔSleep score for the negative control group in 1F (RU-, dark grey) is subtracted from the ΔSleep value for each individual enriched fly in the

induced rescue group 1F (RU+, light grey) to calculate a mean and standard error for the wild-type *rut* expression with that specific GAL4 driver. (H) None of the heterozygous parental lines respond to social enrichment. The three *rut*$_{2080}$*/+GAL4/+* lines that were able to rescue experience dependent changes in sleep (Figure 1A) are shown.

Figure S2

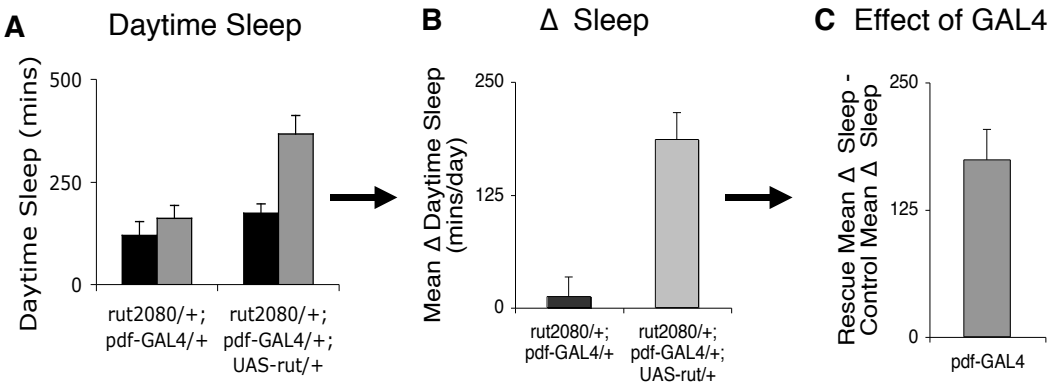

Figure S2 – Effect of wild-type *rut* rescue on experience-dependent increases in sleep. (A) To quantify the change in sleep induced by expressing UAS-*rut*^{wt} with a specific GAL4 driver, we first measure the amount of daytime sleep over each of 3 days for socially isolated (Black) and socially enriched groups (grey). In this example, data are shown for rescue (right) and negative control (left) for *pdf*-GAL4 rescue. Two-way ANOVA reveals a genotype by condition interaction ($F_{(1,57)}$ = 4.44, p = 0.03). (B) The average daytime sleep for the isolated group is subtracted from the daytime sleep for each individual socially-enriched sibling. The difference is referred to as Δ Sleep. (C) Finally, the Mean ΔSleep score for the negative control (*rut*^{2080}/+; *pdf*-GAL4/+) is subtracted from the Mean ΔSleep value for the experimental rescue (*rut*^{2080}/+; *pdf*-GAL4/+; UAS-*rut*/+) to quantify the effect of wild-type *rut* expression with that specific GAL4 driver.

Figure S3

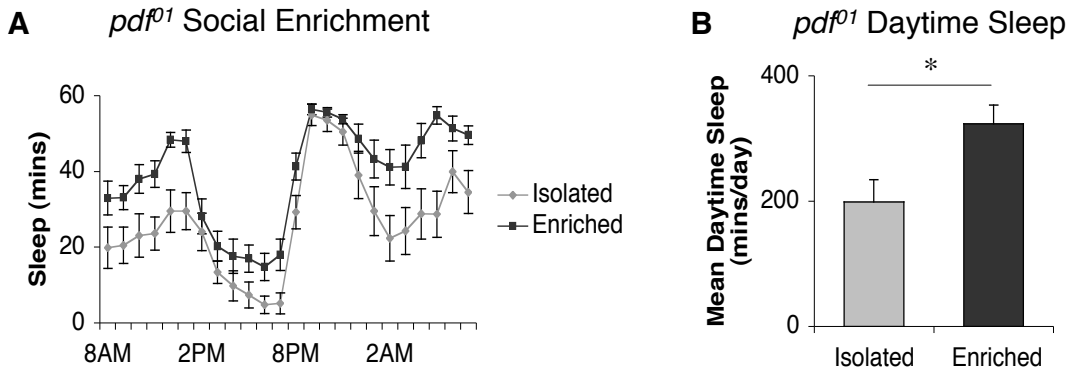

Figure S3 – *Pigment Dispersing Factor* is not required for experience-dependent increase in sleep. (A) Socially enriched flies carrying a null mutation for *pdf* (*pdf*^{01}) show a significant increase in sleep compared to their socially isolated siblings. Data is shown for a representative day (n=15-16). **(B)** Average daytime sleep is increased in socially enriched *pdf*^{01} flies relative to their socially isolated siblings (p=0.01, n=15-16). * signifies p<0.05. P-value was generated using a Student's T-test.

Figure S4

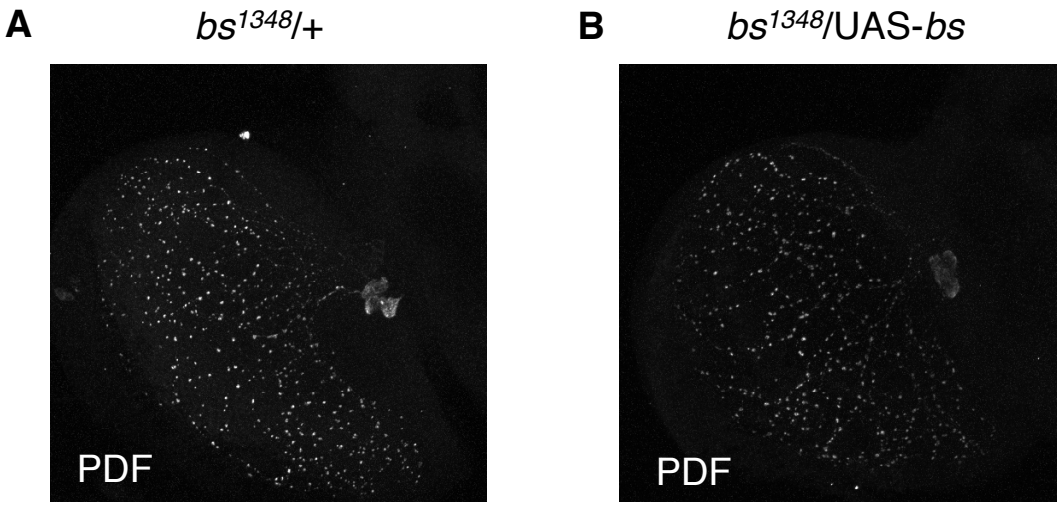

Figure S4 – LN_V morphology is normal in flies mutant for *blistered*. **(A)** Immunohistochemistry with anti-PDF (1:10000) in P{GAL4}*bs*^{1348}/+ mutant flies. **(B)** Staining of P{GAL4}*bs*^{1348}/UAS-*bs* flies with anti-PDF (1:10000). No difference in PDF distribution is noticeable between P{GAL4}*bs*^{1348}/+ mutants and P{GAL4}*bs*^{1348}/UAS-*bs* flies.

Figure S5

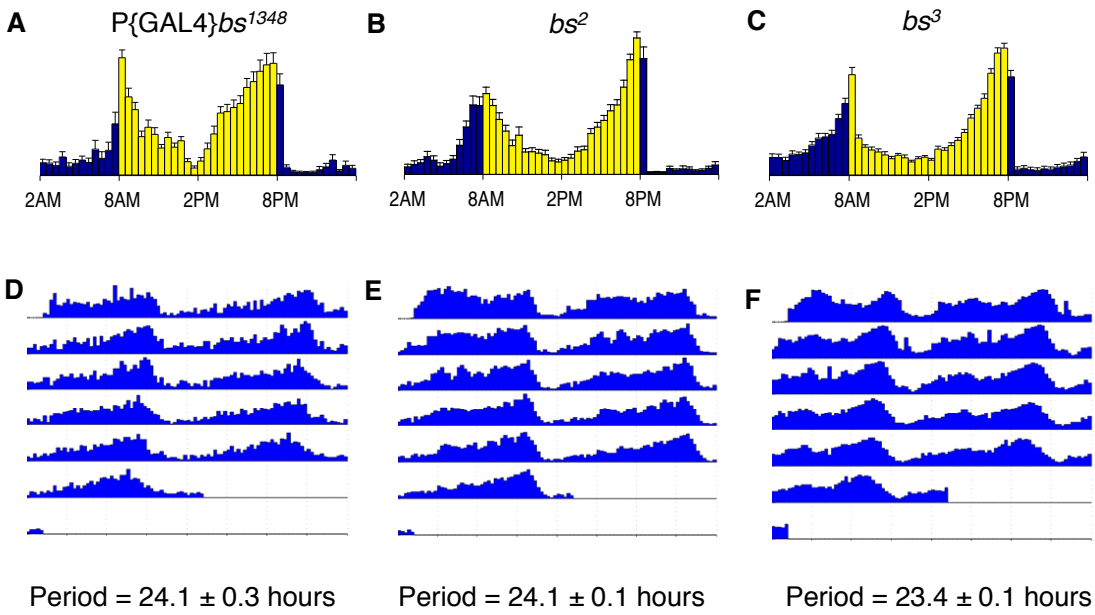

Figure S5 - *blistered* is not required for circadian behavioral rhythms
(A) Flies in a P{GAL4}*bs*^{1348} mutant background show intact anticipatory locomotor activity peaks prior to lights-on and lights-off on a 12hr:12hr light:dark schedule (LD) (n=24). **(B)** Under LD conditions, flies in a *bs*^{2} mutant background demonstrate anticipatory activity prior to light transitions (n=32). **(C)** *bs*^{3} mutant flies transiently increase locomotor activity before light transitions under LD (n=32). **(D)** Flies in a P{GAL4}*bs*^{1348} mutant background free-run on a 24.1±0.11 hour locomotor activity period under constant darkness (DD) (n=24). **(E)** Flies carrying the *bs*^{2} mutant allele also free-run on a 24.1±0.1 hour period in DD (n=26). **(F)** Under constant darkness, flies in a *bs*^{3} mutant background free-run on a 23.4±0.1 hour locomotor period (n=32).

Acknowledgements

We thank Matthew Thimgan, Giulio Tononi and Paul Taghert for helpful comments on this manuscript. This study was funded in part by NIH Grants R01-NS051305-01A1 and T32 GM008151. J.D. designed and performed the experiments, analyzed data and wrote the paper. P.S. designed the experiments, analyzed data and wrote the paper. N.R. designed the experiments and wrote the paper.

References

A. Rechtschaffen, *et al*, *Science* 221, 182 (1983).

P. J. Shaw, *et al*, *Nature* 417, 287 (2002).

M. P. Walker, *et al*, *Neuron* 35, 205 (2002).

L. Graves, *et al*, *Learn. Mem.* 10, 168 (2003).

I. Ganguly-Fitzgerald, *et al, Science* 313, 1775 (2006).

T. Sakai, *et al, Proc. Natl. Acad. Sci. USA.* 101, 16058 (2004).

S. Decker, *et al, Proc. Natl. Acad. Sci. USA.* 104, 15905 (2007).

M. Heisenberg, *et al, J. Neurogenet.* 2, 1 (1985).

S. E. McGuire, *et al, Science* 293, 1330 (2001).

W. J. Joiner, *et al, Nature* 441, 757 (2006).

J. L. Pitman, *et al, Nature* 441, 753 (2006).

K. Foltenyi, *et al, Nat. Neurosci.* 10, 1160 (2007).

C. Helfrich-Forster, U. Homberg, *J. Comp. Neurol.* 337, 177 (1993).

S. C. Renn, *et al*, *Cell* 99, 791 (1999).

C. Helfrich-Forster, *J. Comp. Physiol [A]*. 182, 435 (1998).

N. Ramanan, *et al, Nat. Neurosci.* 8, 759 (2005).

A. Etkin, *et al, Neuron* 50, 127 (2006).

M. Affolter, *et al, Development* 120, 743 (1994).

B. Knoll, *et al, Nat. Neurosci.* 9, 195 (2006).

D. Fristrom, *et al, Development* 120, 2661 (1994).

J. Montagne, *et al, Development* 122, 2589 (1996).

F. Roch, *et al, Development* 125, 1823 (1998).

M. A. Wilson, B. L. McNaughton, *Science* 265, 676 (1994).

D. Ji, M. A. Wilson, *Nat. Neurosci.* 10, 100 (2007).

G. Tononi, C. Cirelli, *Brain Res. Bull.* 62, 143 (2003).

G. Tononi, C. Cirelli, *Sleep Med. Rev.* 10, 49 (2006).

V. Sheeba *et al, Curr. Biol.* 18, 1 (2008).

Y. Zhang *et al*, *Neuron* 53, 201 (2006).

H. Kazama, R. I. Wilson, *Neuron* 58, 401 (2008).

M. Hamblen *et al, J. Neurogenet.* 3, 249 (1986).

Materials & Methods

Flies

Flies were cultured at 25˚C in 50-60% relative humidity for a 12h:12h light:dark cycle on yeast, dark corn syrup, and agar food as described previously (Shaw *et*

al, 2002). Lights came on at 8:00 AM. Canton-S, *rut*^{2080} and *rut*^{2080};UAS-*rut* flies were obtained from T. Zars (University of Missouri-Columbia), 104Y-GAL4, 121Y-GAL4, NP2320-GAL4, NP6510-GAL4, 210Y-GAL4, 154Y-GAL4, and C205-GAL4 flies were obtained from M. Heisenberg (University of Wurzburg), NP3529-GAL4, NP5194-GAL4, MZ671-GAL4, MZ19-GAL4, NP225-GAL4, NP10-GAL4, NP1004-GAL4, NP5103-GAL4, and NP5221-GAL4 flies were obtained from the Drosophila Genetic Resource Center (Kyoto Institute of Technology), C316-GAL4 and MZ717-GAL4 flies were obtained from S. Waddell (University of Massachusetts), MZ520-GAL4 and C929-GAL4 flies were obtained from F. Rouyer (CNRS), *per*^{01} and *per*^{01};*per*$^{+7.2-2}$ flies were obtained from J. C. Hall (Brandeis University), Tai2 flies were obtained from K. Siwicki (Swarthmore College), OK107-GAL4, P247-GAL4, 201Y-GAL4, 1471-GAL4, C739-GAL4, C309-GAL4, C232-GAL4, *bs*^{2}, *bs*^{3}, *P{GAL4}bs*^{1348}, In(2LR)*Px*^{4} flies were obtained from the Bloomington Stock Center (Bloomington, IN), UAS-*bs*(II) and UAS-*bs*(III) flies were obtained from Z. Han (University of Michigan), 50Y-GAL4, C767-GAL4, C687-GAL4, *pdf*-GAL4 and *cry*-GAL80 flies were obtained from P. Taghert (Washington University in St. Louis), UAS-*Egfr** and UAS-*Egfr*^{DN} flies were obtained from J. Skeath (Washington University in St. Louis), UAS-*dlgWT*-GFP flies were obtained from B. Lu (Stanford University) and UAS-*VAMP*-GFP flies were obtained from A. DiAntonio (Washington University in St. Louis).

Behavioral Analysis

Sleep in *Drosophila* was measured as previously described (*2*). Briefly, flies

were placed into 65-mm long glass tubes and sleep parameters were continuously evaluated throughout all experiments by using the Trikinetics *Drosophila* activity monitoring system (www.Trikinetics.com). Locomotor activity was recorded in 1-minute bins and periods of quiescence that lasted for 5-minutes or longer were categorized as sleep.

1-4 day old flies were divided into a socially isolated group, which were individually housed in 65-mm glass tubes, and a socially enriched group, consisting of 35-40 female flies housed in a single vial as previously described (*5*). After five days of social enrichment/isolation, flies were placed into clean 65-mm glass tubes and sleep was recorded for three days using the Trikinetics activity monitoring system. Mean Δ Sleep was generated by quantifying sleep time for socially isolated and socially enriched flies for three full days. Average daytime sleep for socially isolated siblings was subtracted from the amount of daytime sleep for each individual socially enriched fly for each of three days. Daily Δ Sleep values were averaged for each socially enriched fly. Finally, the mean of the individual Δ Sleep values was used to quantify the increase in sleep for each group.

Courtship conditioning memory was measured in 4-6 day old males as previously described (*5*). Short-term memory was assayed in a 10-minute test starting 5 minutes after a 1-hour training period with a pheromonally-feminized Tai2 male. Long-term memory was observed in a 10-minute test starting 48 hours after a

spaced training period (3 x 1-hour training periods each separated by 1-hour) with a Tai2 male. For long-term memory experiments, flies were placed into clean 65-mm glass tubes after training and sleep was recorded using the Trikinetics activity monitoring system. Courtship Index is scored as the percentage of a 10 minute test exposure to a Tai2 male that the subject male spends engaged in courtship behavior.

Circadian rhythms were observed in male flies housed in 65mm glass tubes on 4% sucrose, 2% agar medium. Locomotor activity was measured using Trikinetics activity monitors. Free-running periods were determined by chi-squared periodogram analysis using MATLAB as previously described (*30*).

Quantitative PCR

Total RNA was isolated from fly heads by using TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Reverse-transcription (RT) reactions were carried out in parallel on Dnase I-digested total RNA as described (*2*). RT products were stored at –80˚C until use. PCRs to measure levels of artificial transcript were performed to confirm uniformity of RT within sample groups and between samples. All reverses were performed in triplicate. At least two quantitative PCR replications were performed for each condition. Values were expressed as a percentage of socially isolated animals and were evaluated by using a Student's T-Test.

Immunohistochemistry

Brains were removed from the head casing and fixed in 4% paraformaldehyde in phosphate buffer solution (PBS) (1.86 mM NaHPO, 8.41 mM NaHPO, and 175 mM NaCl) for 1 hour and washed in PBS. Following a 2-hour pre-incubation in 3% normal goat serum in PBS-TX (PBS containing 0.3% Triton X-100), brains were washed in PBS-TX. Brains were incubated in the following primary antibody concentrations in PBS-TX: 1:1000 Rat anti-PER (gift from P. Taghert, Washington University), 1:1000 Rabbit anti-GFP (Invitrogen), 1:10,000 Guinea Pig anti-PDF (gift from P. Taghert, Washington University), washed in PBS-TX and incubated in the appropriate fluorescent secondary antibodies (Invitrogen). Confocal stacks were acquired with a 1µm slice thickness using an Olympus FV500 laser scanning confocal microscope and processed using ImageJ. Immunopositive terminals were counted using the ImageJ binary thresholding algorithm. All samples that were directly compared were processed in parallel and imaged using identical microscope settings.

Supplemental References

1. P. J. Shaw, *et al*, *Nature* 417, 287 (2002).
2. P. J. Shaw, *et al, Science* 287, 1834 (2000).
3. M. Barth, M. Heisenberg, *Learning & Memory* 4, 219 (1997).
4. I. Ganguly-Fitzgerald, et al, Science 313, 1775 (2006).
5. M. Hamblen et al, J. Neurogenet. 3, 249 (1986).
6. J.C. Hendricks et al, Nat Neurosci. 4 1108 (2001).

Chapter 4:

Increased dopamine signaling delays functional senescence in behavioral and structural plasticity.

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Introduction

Healthy aging is associated with deficits in cognitive processes, including memory formation, in humans (DASELAAR *et al.* 2003), monkeys (DUMITRIU *et al.* 2010), dogs (LANDSBERG 2005), mice (PELEG *et al.* 2010), rats (WINOCUR 1998), worms(MURAKAMI *et al.* 2005) and flies(TAMURA *et al.* 2003; YAMAZAKI *et al.* 2007). Although sleep facilitates memory formation in young people(WALKER *et al.* 2005), sleep-dependent memory-consolidation is impaired with aging (SPENCER *et al.* 2007). Thus, age related deficits in sleep-dependent plasticity provide an example of functional senescence; that is, the age-related deterioration in physiological status that interferes with the ability to maintain youthful-functioning during aging (GROTEWIEL *et al.* 2005).

In recent years, it has become increasingly clear that manipulations that lead to an extended lifespan may not improve functional senescence (BHANDARI *et al.* 2007; GROTEWIEL *et al.* 2005). As a consequence, more effort has been focused on elucidating the underlying molecular mechanisms that can lengthen the healthspan of the organism even if these mechanisms do not extend lifespan (BHANDARI *et al.* 2007; IWASA *et al.* 2010). For example, while caloric restriction and mutant alleles for the G-protein coupled receptor *methuselah* significantly lengthen lifespan in *Drosophila*, they do not delay the onset of behavioral senescence as measured by geotactic and olfactory avoidance assays (BHANDARI *et al.* 2007; COOK-WIENS and GROTEWIEL 2002). Conversely, mutant alleles for *DC0*, the catalytic subunit of PKA, delay senescence for a form of

consolidated memory, but do not alter lifespan in *Drosophila* (YAMAZAKI *et al.* 2007). While lengthening lifespan is an important goal of aging research, there is evidence that elderly individuals are less interested in living longer than in maintaining their cognitive abilities until late in life (GROTEWIEL *et al.* 2005; PHELAN *et al.* 2004). Given these data, pursuing interventions that can delay aspects of behavioral senescence may not only provide additional insight into the regulation of aging, but also identify manipulations that can maximize quality of life during aging.

To evaluate functional senescence in sleep-related plasticity in *Drosophila*, we have developed a high throughput assay to evaluate sleep following social enrichment (DONLEA *et al.* 2009; GANGULY-FITZGERALD *et al.* 2006). This protocol is based upon the observation that exposure to enriched environments, including social environment, impact the number of synapses and the size of regions involved in information processing in mammals and flies (GREENOUGH *et al.* 1978; PHAM *et al.* 1999; TECHNAU 2007; TOSCANO *et al.* 2006; VOLKMAR and GREENOUGH 1972). Indeed, following five days of exposure to a socially enriched environment, young flies exhibit structural plasticity in projections from the *Pigment dispersing factor* (*PDF*)-expressing ventral lateral neurons (LN_Vs)(DONLEA *et al.* 2009), a cluster of wake-promoting cells (PARISKY *et al.* 2008; SHANG *et al.* 2008; SHEEBA *et al.* 2008), and behavioral plasticity as measured by a significant increase in sleep time (DONLEA *et al.* 2009; GANGULY-FITZGERALD *et al.* 2006). Furthermore, recovery sleep following social enrichment

is required to down-scale the number of LN_V terminals back to baseline levels (DONLEA *et al.* 2009), indicating that increased sleep following social enrichment is directly related to the structural plasticity induced by social experience.

In the current study, we find that both behavioral and structural plasticity following social enrichment decline with age in *Drosophila*. We identify dopaminergic signaling as a target for altering plasticity with age; young flies with impaired dopaminergic signaling exhibit deficits in both structural and behavioral plasticity and, conversely, senescence is delayed in aged flies with elevated dopamine levels. Our results also indicate that elevated expression of the transcription factor *blistered* (*bs*) in the LN_Vs delays senescence of plasticity following social enrichment. Together, these data suggest that observing sleep following social enrichment can provide a productive model for identifying mechanisms of plasticity that degrade with age.

Results

Plasticity-Induced Sleep declines with age

Previous studies have indicated that although *Drosophila* can survive more than 2 months in a laboratory environment, the ability of flies to properly form memories degrades within 20 days of eclosion (NECKAMEYER *et al.* 2000; TAMURA *et al.* 2003; YAMAZAKI *et al.* 2007). Thus, the fly provides a model for studying the effects of physiological aging on brain plasticity. Some of the mechanisms associated with the loss of plasticity with age have been identified by directly

measuring the effect of age on associative memory formation (NECKAMEYER *et al.* 2000; TAMURA *et al.* 2003; YAMAZAKI *et al.* 2007). However, these experiments are not trivial and thus, the field could benefit from the development of additional high throughput behavioral assays for evaluating the effects of aging on plasticity. We have recently developed a high throughput assay to evaluate sleep following social enrichment. In this procedure, flies are exposed to either social enrichment, consisting of 35-40 flies maintained in a 50mL vial, or social isolation, consisting of flies being housed individually in Trikinetics tubes, for 5 days (DONLEA *et al.* 2009; GANGULY-FITZGERALD *et al.* 2006). Flies are placed into their respective conditions beginning on day 5 after eclosion through the beginning of day 10. Sleep is not evaluated on day 10 to allow the socially enriched animals to adapt to the Trikinetics tubes and to minimize the influence of handling on sleep (socially isolated flies are also placed into fresh tubes). Sleep is quantified for 3 days between day 11 and day 14. Using this protocol we have shown that socially enriched flies exhibit a significant increase in daytime sleep compared to their socially impoverished siblings (ΔDaytime Sleep) and that this increase in sleep is associated with structural plasticity in the LN_Vs, a cluster of wake-promoting neurons in the fly brain (DONLEA *et al.* 2009). Moreover, we have shown that the response to social enrichment depends upon the expression of genes that are necessary for memory formation and synaptic plasticity (DONLEA *et al.* 2009). In the current study, we examine whether responses to a socially enriched environment may provide a new paradigm for investigating the effects of aging on plasticity.

To begin, we examined sleep in flies of various ages following five days of social isolation or social enrichment. As previously reported (GANGULY-FITZGERALD *et al.* 2006), when 5 day old female flies are exposed to social enrichment for 5 days, they exhibit an increase in sleep compared to siblings that were socially isolated (Fig. 1A). If, however, we expose female flies to social enrichment for 5 days beginning on day 20 after eclosion, no change in sleep can be detected compared to siblings that were socially isolated (Fig. 1B). To more precisely analyze the effects of aging on plasticity-induced changes in sleep, we measured sleep after 5 days of social enrichment in male and female flies at ages between 5 days and 45 days after eclosion. As shown in Figure 1C, male and female flies both exhibit robust increases in sleep following social enrichment at 5-10 days after eclosion compared to age-matched isolated siblings. However, female flies show no change in sleep when exposed to social enrichment beginning on 15 and 20 days after eclosion. Interestingly, male flies subsequently exhibit a gradual loss of plasticity-induced sleep until 45 days of age.

In young flies, social enrichment not only increases sleep time, it also increases sleep consolidation. Thus, we compared the lengths of sleep bouts in isolated young (11 day old) and aged (26 day old) *Cs* females after 5 days of social isolation or enrichment. As seen in Fig. 1D, while daytime sleep bout length is increased in young *Cs* females following social enrichment, no change in daytime bout length can be observed in aged females. A similar trend emerges when we

examine the effect of social enrichment on sleep bout length during the night (Fig. 1E). Indeed, night-time sleep bouts are lengthened after social enrichment in young females, but not in aged females and night-time sleep bouts are not changed between young and aged flies after isolation. Thus, older female flies do not increase total sleep or sleep consolidation following exposure to an enriched social environment. Note that the plasticity-induced sleep degrades in female flies in our study at approximately the same age that previous studies have reported age-related changes in *amnesiac*-dependent memory (TAMURA *et al.* 2003; YAMAZAKI *et al.* 2007). Thus, the loss of plasticity-induced changes in sleep might be connected to age-related loss of memory formation.

While degradation of plasticity-induced sleep is observed at a similar age as age-related memory loss, it is possible that this deficit could be caused by changes in extraneous factors such as an inability to sleep or to impaired social interactions, rather than the loss of plasticity *per se*. As seen in Figure 1 D, the length of sleep bouts during the day and night are not statistically different between isolated young females (11 day old) and isolated aged females (26 day old) (Light Bars), indicating that aging does not directly alter sleep consolidation between 11 and 26 days after eclosion. Although aged flies retain the ability to sleep in a similar manner as younger flies, it is also possible that the loss of plasticity-induced sleep could be caused by a loss in social interactions with age or to age-related deficits in reproductive status. To test the latter hypothesis, we examined the ability of young and aged females to produce offspring and found

that flies of both ages reproduce (Fig. 1F). Thus 25 day old flies retain the ability to successfully mate and lay viable eggs. To examine whether 25 day old females exhibit any age-related locomotor impairments that might alter social interactions during enrichment, we also examined group locomotor activity of young and aged females during social enrichment and found that aged flies retain intact locomotor activity (Fig. 1G). Together, these data indicate that aged females retain intact locomotor activity and are able to interact socially with other flies.

In humans, social lifestyles are believed to influence cognitive declines during aging (FABRIGOULE *et al.* 1995; WANG *et al.* 2002). Thus we asked whether rearing flies in a socially enriched environment throughout their lives would alter their response to social enrichment 20 days after eclosion compared to socially isolated siblings. Flies were reared in either social isolation or social enrichment for their first 20 days. When sleep is measured after 5 days of social enrichment or isolation, no plasticity-induced sleep is observed in either group (Fig. 1H). Thus, the loss of plasticity-induced sleep with age seems to be dissociable from the previous social history of the fly.

Recently, studies have begun to explore the relationship between lifespan and functional senescence defined as age-related declines in functional status (GROTEWIEL *et al.* 2005). Several studies have found that manipulations that increase lifespan may not alter functional senescence in behavior (BHANDARI *et*

al. 2007; IWASA *et al.* 2010). Thus, we asked whether a manipulation that can extend lifespan would also be able to extend functional senescence as measured by maintaining youthful response to social enrichment during aging. Flies were housed at 18°C, a temperature known to prolong lifespan, or maintained at 25°C throughout their lives. At 20 days of age flies were placed into either social isolation or enrichment for 5 days. Sleep was quantified in socially isolated and socially enriched flies at 25°C to avoid confounding effects of temperature on sleep. As seen in Figure 1I, flies reared at 18˚C for days 1-20 behave similarly to their siblings reared at 25˚C from eclosion and do not response to social enrichment with an increase in sleep. Thus, functional senescence in plasticity-induced sleep is separable from extension of lifespan.

Loss of *dDA1* signaling in the LN_vs degrades plasticity-induced sleep in young flies

Dopaminergic signaling has been found to be necessary for memory formation (SCHWAERZEL *et al.* 2003) and has also been shown to decline with age in *Drosophila* (IMAI et al. 2008; NECKAMEYER et al. 2000), indicating that altered dopaminergic signaling might underlie age-related impairments in plasticity. Although previous data from our lab found that genetically disrupting dopaminergic signaling alters plasticity-induced sleep in young flies, it is unclear whether these effects are a result of eliminating dopaminergic signaling chronically throughout development. To determine whether the acute depletion of dopamine can prevent plasticity-induced sleep in the adult fly, we exposed

flies to the *Tyrosine Hydroxylase* inhibitor 3-iodo L-tyrosine (3IY) between day 5-10 after eclosion. Flies were fed 3IY while they were housed in social isolation or enrichment and then returned to standard food in the morning of day 10, allowed to recovery for ~18 h before sleep was evaluated for 3 days. As seen in Figure 2A, acutely disrupting DA for 5 days prevented the increase in sleep typically seen following social enrichment. Examination of behavior during social enrichment using group activity monitors revealed that locomotor activity did not differ between 3IY-fed and vehicle-fed controls (Supplemental Figure S1A,B). Although, 3IY treatment modestly increases sleep during the 5 day exposure, sleep parameters were not statistically different from vehicle-fed controls at the beginning of testing and after ~18 on standard food (Supplemental Figure S1). Thus, it is unlikely that the impaired response to social enrichment can be attributed to drug induced changes in sleep or locomotion.

To more specifically examine the role dopamine in mediating behavioral plasticity in response to social enrichment, we evaluated transcript levels for the *Drosophila D1-type dopamine receptor* (*dDA1*) using qPCR. As seen in Figure 2B. *dDA1* transcripts are upregulated by ~50% in mRNA extracted heads of 10 socially enriched females compared to their isolated controls (Figure 2C). To determine the extent to which the dDA1 receptor might be involved in mediating the response to social enrichment we evaluated behavioral plasticity in flies fed the D1-antagonist SCH23390. As seen in Figure 2B, no increase in sleep was observed in socially enriched flies that had been maintained on SCH23390 for 5

days and allowed to to recover for ~18 h before sleep was evaluated. As above, no changes in locomotor activity during enrichment were observed SCH23390 fed flies compared to controls (Supplemental Figure 2A,B). Importantly, SCH23390 did not alter sleep parameters either during the 5 day exposure or at the beginning of the 3 day recording period (Supplemental Figure 2). These data reinforce the interpretation above that the effects of DA signaling on behavioral plasticity cannot be explained by non-specific effects of drugs on sleep or locomotion. Thus, dopaminergic signaling plays an important role in behavioral plasticity following exposure to an enriched social environment in young *Drosophila*.

Since SCH23390 has been shown to activate both the *dDA1* and the *dopamine receptor enhanced in Mushroom Bodies* (*DAMB*), we tested whether *dDA1* signaling is required for plasticity-induced sleep in young flies by analyzing sleep after social enrichment in flies carrying two independent mutant alleles for *dDA1*. Flies that are homozygous either for the $dumb^2$ allele, a hypomorphic allele formed by a piggyBac insertion (KIM *et al.* 2007b), or for the $dumb^3$ allele, a p-element insertion that drives the expression of GAL4 (SEUGNET *et al.* 2008), show no change in sleep after social enrichment (Figure 2D, left, center). Importantly, rescue of *dDA1* expression by using the GAL4 driver inserted into the first intron of *dDA1* in the $dumb^3$ allele to drive expression of the UAS-element inserted into the first intron of *dDA1* in the $dumb^2$ allele of $dumb^2/dumb^3$ flies restores increased sleep following social enrichment during days 5-9 after eclosion

(Figure 2D, right). Since previous reports indicate that *dumb*^{3}-GAL4 expresses ectopically, we used the *dumb*^{3}-GAL4 driver to express a membrane-bound GFP reporter and found wide expression throughout the brain, including in PDF-immunopositive LN_Vs (Figure 2E). Given the involvement of the LN_Vs in the regulation of plasticity-induced changes in sleep, we specifically tested the role of *dDA1* expression in the LN_Vs by using *pdf*-GAL4 to rescue *dDA1* expression in a *dumb*^{2} mutant background (*pdf*-GAL4;;*dumb*^{2}). As seen in Figure 2F, rescue of *dDA1* in the LN_Vs of *pdf*-GAL4;;*dumb*^{2} flies partially restores plasticity-induced sleep while *dumb*^{2} flies exhibit a mutant phenotype (Figure 2F). Given that, *dumb*^{3}-GAL4 expresses in many areas outside the LNvs, we obtained an RNAi construct targeted for *dDA1 (*DIETZL *et al. 2007)* and expressed it in the LN_Vs using pdf-GAL4 (*pdf*-GAL4/+; UAS-*dDA1*^{RNAi}/+). We found that socially enriched parental controls (*pdf*-GAL4/+ and UAS-*dDA1*^{RNAi}/+) exhibited an increase in sleep compared to isolated siblings while *pdf*-GAL4/+; UAS-*dDA1*^{RNAi}/+ flies did not exhibit behavioral plasticity in response to social enrichment. (Figure 2G). Together, with the data presented above our results indicate that disrupting *dDA1* signaling pharmacologically, with classic mutants and with RNAi blocks the response to social enrichment and that these effects are mediated, in part, through the LN_Vs.

Depleting dopamine prevents structural plasticity during social enrichment

Previously, we have found that exposure to a socially enriched environment not only increases sleep, but also induces structural plasticity as measured by an

increase in the number of synaptic terminals in LN_V projections into the medulla (DONLEA *et al.* 2009). Thus, we asked whether disrupting behavioral plasticity using 3IY would also disrupt structural plasticity. Structural plasticity was evaluated using both a synaptic terminal marker *dlgGFP* and immunoreactivity using PDF immunohistochemistry to specifically label PDF terminals. As shown in Figure 3A-C, terminals labeled with *dlgGFP* closely overlap with PDF-immunopositive varicosities in *pdf*-GAL4/+; UAS-*dlgGFP*/+, indicating that *dlgGFP* and PDF-immunohistochemistry seem to label overlapping synaptic terminals in the LN_Vs. To determine whether these markers would reveal similar changes in structural plasticity following social enrichment both with and without 3IY, we quantified both dlgGFP-positive and PDF-immunopositive terminals in 10 day old *pdf*-GAL4/+; UAS-*dlgGFP*/+ flies after social enrichment. Interestingly, 10 day old *pdf*-GAL4/+; UAS-*dlgGFP*/+ flies exhibit both a significant increase in punctae labeled by *dlgGFP* (Figure 3D, left) and an increase in punctae quantified using PDF-immunohistochemistry (Figure 3E, left). Importantly, 3IY, which blocks behavioral plasticity is also associated with a lack of structural plasticity as measured by both *dlgGFP*-labeling and PDF-immunohistochemistry. Thus, behavioral plasticity is highly correlated with structural plasticity using two independent markers of terminals. Importantly, these data indicate that PDF immunohistochemistry can be used to evaluate structural plasticity following social enrichment in a variety of transgenic lines without having to co-express UAS-*dlgGFP*.

Elevation of *dDA1* signaling in aged flies restores plasticity-induced sleep

The molecular mechanisms underlying the loss of plasticity-induced sleep with age have not been identified. Since dopaminergic signaling regulates behavioral plasticity in young flies and dopamine levels decline with age, we asked whether we could restore youthful responses to social enrichment by restoring dopamine levels. 20-day old female Cs flies were fed 2mg/mL of L-3,4-dihydroxyphenylalanine (L-DOPA) for 5 days during social isolation or enrichment. As seen in Supplemental Figure 3, chronic L-DOPA administration did not substantially alter either sleep parameters or group activity. Interestingly, L-DOPA succeeded in restoring the youthful response of 26 day old animals to the socially enriched environment as measured by a significant increase in ΔDaytime sleep and these effects were also seen for measurements of sleep consolidation (Figure 4A,B). Thus, acute L-DOPA administration in older flies restores behavioral plasticity.

To determine whether L-DOPA would be able to restore structural plasticity in addition to behavioral plasticity following social enrichment, we quantified *dlgGFP*-positive punctae. First, we asked whether aging was associated with an increase in LN_V terminals independent of social enrichment. As seen in Supplemental Figure 4, age did not alter *dlgGFP* puctae in the LN_Vs. Next we fed aged *pdf*-GAL4/+; UAS-*dlgGFP*/+ flies vehicle or L-DOPA while being enriched beginning on day 20. As seen in Figure 4C,D aged vehicle-fed controls did not show an increase in LN_V terminals. However, *pdf* -GAL4/+; UAS-*dlgGFP*/+ flies

that were fed L-DOPA displayed a significant increase in *dlgGFP* positive terminals following social enrichment (Figure 4 C,D). In all, these data suggest that it is possible to restore youthful responses in both behavioral and structural plasticity pharmacologically by acutely elevating dopamine levels for 5 days in aged females.

As described above, dopamine seems to act primarily through *dDA1* receptors to modulate plasticity-induced sleep. To explore whether specifically increasing levels of *dDA1* signaling in the LN_Vs restores plasticity to the same degree as elevating dopamine levels, we over-expressed *dDA1* receptors in the LN_Vs (*pdf*-GAL4/+;; *dumb*^{2}/+) and measured sleep in 26 day old females after social enrichment. While aged *pdf*-GAL4/+ and *dumb*^{2}/+ parental controls showed no increase in sleep following social enrichment, aged *pdf*-GAL4/+;; *dumb*^{2}/+ flies demonstrate a significant increase in daytime sleep and sleep consolidation in response to social enrichment at the same age (Figure 4E,F). Thus, enhanced *dDA1* signaling in the LN_Vs delays the age-dependent loss of behavioral plasticity.

Increased expression of *blistered* delays loss of plasticity in aging flies

The response to social enrichment depends upon the expression of a variety of genes involved in synaptic plasticity, including the transcription factor *blistered* (*bs*) (DONLEA *et al.* 2009). Indeed, transcript levels of *bs* are increased following social experience in young flies that exhibit both LN_V structural plasticity and a

subsequent increase in sleep. To examine whether altered *bs* transcription is correlated with loss of plasticity during aging, we used qPCR to examine the effect of social enrichment on *bs* transcript levels. We evaluated *bs* transcript levels in young females, aged females, and, since L-DOPA can restore behavioral and structural plasticity during aging, aged females fed L-DOPA during social enrichment. As seen in Figure 5A, young females display a significant increase in *bs* transcripts compared to their age-matched isolated siblings. However, *bs* transcripts were not increased in aged females following social enrichment (Figure 5A, center). Importantly, 5 days of L-DOPA administration during social enrichment partially restored the youthful response of *bs* transcript levels compared to isolated controls (Figure 5A, right). These data suggest that the induction of *bs* transcription in response to social enrichment may mediate the plastic response to experience. Thus, we examined whether genetic over-expression of *bs* in the LN_Vs could delay the loss of plasticity-induced sleep in aged flies. As seen in Figure 5B,C flies over-expressing *bs* in the LN_Vs (*pdf*-GAL4/+; UAS-*bs*/+) retain the ability to increase sleep time and sleep consolidation following social enrichment at 26 days old, while age-matched parental controls (*pdf*-GAL4/+ and UAS-*bs*/+) are not able to generate a plastic response. To determine whether the expression of *bs* in LNvs could also restore youthful changes in structural plasticity, we examined *dlgGFP* labeled punctae in *pdf*-GAL4/+; UAS-*bs*/UAS-*dlgGFP* flies following social enrichement. As seen in Figure 5 D,E the age-matched parental control (*pdf*-GAL4/+; UAS-*dlgGFP*/+) did not respond to social enrichment with an increase in *dlgGFP*

positive terminals in the LNvs. However, 25 day *pdf*-GAL4/+; UAS-*bs*/UAS-*dlgGFP* flies showed a significant increase in terminals following social enrichment. Thus, elevated expression of *bs* specifically in the LN_Vs can delay age-dependent declines in behavioral and structural plasticity following social experience.

Discussion

Loss of neural plasticity is a consequence of aging that is widely conserved across a variety species (BURKE and BARNES 2005). We demonstrate that measuring the responses of *Drosophila* following exposure to a socially enriched environment provides a high-throughput assay for measuring deficits in plasticity that occur during aging. Importantly, young flies (10 day old) exhibit structural plasticity in the LN_Vs and an increase in sleep time and consolidation following social enrichment, but older flies (20-25 day old) no longer exhibit either of these plastic responses. Our previous studies indicate that the mechanisms regulating LN_V structural plasticity and subsequent increases in sleep may overlap (DONLEA and SHAW 2009; DONLEA *et al.* 2009). Indeed, aging seems to reduce both structural and behavioral plasticity to similar extents following social enrichment. Furthermore, administration of L-DOPA and over-expression of *bs* in aged females can restore youthful levels of both structural and behavioral plasticity. These data support the hypothesis that the increases in sleep time and consolidation that we observe in young flies following social enrichment are a consequence of the structural plasticity induced by social experience.

Because dopaminergic signaling degrades with age in flies (IMAI *et al.* 2008; NECKAMEYER *et al.* 2000), we tested whether loss of DA impairs plasticity. Indeed, pharmacologic and genetic disruption of dopaminergic signaling through the *dDA1* receptor in a cluster of wake-promoting neurons, the LN_Vs, eliminates plasticity in young flies. Together with a recent study indicating that expression of *dDA1* in the LN_Vs can modulate locomotor activity (LEBESTKY *et al.* 2009), our data indicates that dopaminergic signaling into the LN_Vs alters sleep regulation in young flies, particularly after social experience.

Conversely, elevating dopaminergic signaling either by administration of L-DOPA or by over-expression of *dDA1* in the LN_Vs restores plasticity following social enrichment to aged flies, indicating that interventions targeting dopaminergic signaling may provide a strategy for delaying the onset of functional senescence. We also demonstrate that using these manipulations may provide insight into other genetic mechanisms that can alter plasticity with age. In particular, we found that aging results in altered transcriptional regulation of *bs* in response to social enrichment. This dysregulation is partially restored in aged animals by administration of L-DOPA, indicating that the altered transcriptional response of *bs* to social experience may be related to the ability to induce a plastic response, not to aging alone. Indeed, over-expression of *bs* permits aged flies to respond to social enrichment with plastic changes in LNv structure and sleep.

Despite previous studies in humans that have identified a significant effect of age on the relationship between sleep and plasticity (DASELAAR *et al.* 2003), the lack of a genetic animal model for these effects has complicated the identification of underlying mechanisms. Our data provide novel evidence that the effects of age on plasticity-induced changes in sleep are conserved from humans to the fruit fly and establish the fly as a model to study the mechanisms that contribute to deficits in plasticity-induced sleep that accumulate with age. Using this model, we demonstrate that heightened dopaminergic signal or elevated expression of *bs* can delay plasticity-related senescence. In combination with studies using associative conditioning assays, examining the responses to social enrichment may contribute to future investigation of the effects of aging on sleep regulation and on plasticity.

Methods

Flies

The flies were cultured at 25˚C with 50-60% relative humidity and kept on a diet of yeast, dark corn syrup, molasses, sucrose and agar under a 12-hour light:12-hour dark cycle. *Canton-S* flies were obtained from T. Zars (University of Missouri, Columbia), *dumb*^{2} flies were obtained from K. Han (Pennsylvania State University), *dumb*^{3} flies were obtained from Bloomington Stock Center, *pdf*-GAL4 flies were obtained from P. Taghert (Washington University, St. Louis MO), UAS-*dlgWT*-GFP flies were obtained from B. Lu (Stanford University), and UAS-*bs*(II) flies were obtained from Z. Han (University of Michigan).

Behavioral Analysis

Drosophila sleep and activity patterns were assessed as described previously (SHAW *et al.* 2000). In summary, flies were placed into individual 65 mm tubes and all activity was continuously measured through the Trikinetics Drosophila Activity Monitoring System (www.Trikinetics.com). Locomotor activity was measured in 1-minute bins and sleep was defined as periods of quiescence lasting at least 5 minutes.

Social Enrichment

To standardize the environmental conditions during critical periods of brain development, all flies were collected upon eclosion and maintained in same-sex vials containing 30 flies. Flies were divided into a socially isolated group, which were individually housed in 65-mm glass tubes, and a socially enriched group, consisting of 40-45 female flies housed in a single vial as previously described(GANGULY-FITZGERALD *et al.* 2006). Locomotor activity during social enrichment was measured using a *Drosophila* Population Monitor (Trikinetics, Waltham MA). After five days of social enrichment/isolation, flies were placed into clean 65-mm glass tubes and sleep was recorded for three days as described above. To calculate the mean and standard error for ΔSleep in the experimental group we first calculate the grand mean of the daytime sleep for the isolated group, averaged over three days, and then subtracted it from the average daytime sleep observed for each individual socially-enriched sibling. The

difference is referred to as ΔSleep.

Fecundity

Female flies were individually housed in 65-mm glass tubes with single 4 day old *Cs* males for 2 days, then transferred to clean tubes. 4 days later, each tube was observed for the presence of newly hatched larvae.

Pharmacology

3IY (10 mg/mL), SCH23390 (1 mg/mL), and L-DOPA (2 mg/mL) were dissolved in standard lab media and fed to flies for 5 days while they were housed in social isolation or enrichment. Following social enrichment or isolation, flies were returned to standard media with no drug while ΔSleep following social enrichment was measured.

Quantitative PCR

Total RNA was isolated from fly heads by using TRIzol following the manufacturer's protocol. Reverse-transcription (RT) reactions were carried out in parallel on *Dnase* I-digested total RNA as described(SEUGNET *et al.* 2006). RT products were stored at –80˚C until use. PCRs to measure levels of artificial transcript were performed to confirm uniformity of RT within sample groups and between samples. All reverses were performed in triplicate. At least two quantitative PCR replications were performed for each condition. Values were expressed as a percentage of socially isolated animals and were evaluated by

using a Student's T-Test. Sequences for each of the primers used were as follows: *rp49* F - aagaagcgcaccaagcacttcatc, *rp49* R - tctgttgtcgataccctttgggctt, *bs* F – gacggagctcagctacaaca, *bs* R – gaggtaggcgatcggtcat, *dDA1* F - agcgattgcggatctctt, *dDA1* R - caaaattgcgctccaaag. Data are presented as mean ± SEM.

Immunohistochemistry

Brains were removed from the head casing and fixed in 4% paraformaldehyde in phosphate buffer solution (PBS) (1.86 mM NaHPO, 8.41 mM NaHPO, and 175 mM NaCl) for 1 hour and washed in PBS. Following a 2-hour pre-incubation in 3% normal goat serum in PBS-TX (PBS containing 0.3% Triton X-100), brains were washed in PBS-TX. Brains were incubated in the following primary antibody concentrations in PBS-TX: 1:1000 Rat anti-PER (gift from P. Taghert, Washington University), 1:1000 Rabbit anti-GFP (Sigma), 1:10,000 Guinea Pig anti-PDF (gift from P. Taghert, Washington University), washed in PBS-TX and incubated in the appropriate fluorescent secondary antibodies.

Confocal microscopy

Confocal stacks were acquired with a 1µm slice thickness using a laser scanning confocal microscope and processed using ImageJ. All samples that were directly compared were processed in parallel and imaged using identical microscope settings. Quantitative analysis of pre- and post-synaptic terminals was conducted on socially isolated and socially enriched flies expressing UAS-*dlgGFP* under

control of the *pdf*-GAL4 driver. Immunopositive terminals were counted using the ImageJ binary thresholding algorithm. The number of synaptic terminals for all of the socially isolated flies was used to generate a grand mean. The grand mean of the isolated flies was used to normalize each individual enriched brain. The individual normalized values were then used to calculate the mean and standard error for the group. The mean and standard error for socially isolated flies were calculated by normalizing to their own group mean. The normalized values for each group were then evaluated using a independent sample t-test.

Statistics

All comparison were done using a Student's T-test or, if appropriate, ANOVA and subsequent modified Bonferroni comparisons unless otherwise stated. All statistically different groups are defined as $p < 0.05$.

Acknowledgements

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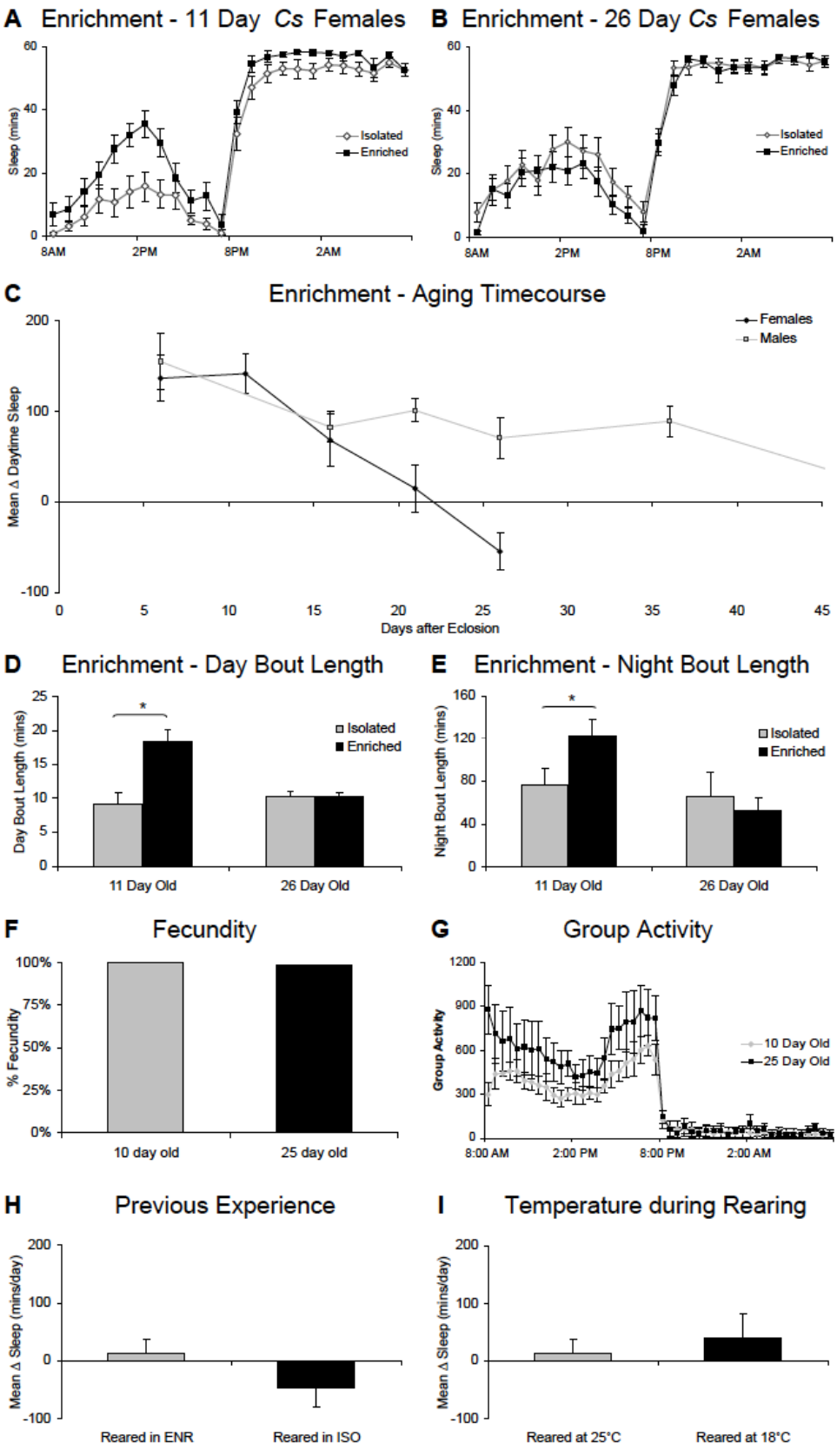

A Enrichment - 11 Day Cs Females
Sleep (mins)
Isolated
Enriched
8AM
2PM
8PM
2AM
B Enrichment - 26 Day Cs Females
C Enrichment - Aging Timecourse
Females
Males
Mean Δ Daytime Sleep
Days after Eclosion
D Enrichment - Day Bout Length
Day Bout Length (mins)
11 Day Old
26 Day Old
E Enrichment - Night Bout Length
Night Bout Length (mins)
F Fecundity
% Fecundity
10 day old
25 day old
G Group Activity
Group Activity
10 Day Old
25 Day Old
8:00 AM
2:00 PM
8:00 PM
2:00 AM
H Previous Experience
Mean Δ Sleep (mins/day)
Reared in ENR
Reared in ISO
I Temperature during Rearing
Reared at 25°C
Reared at 18°C

Figure 1 – Aging prevents increased sleep following social enrichment

A-B) *Cs* females exhibit increases in sleep after being exposed to social enrichment at 11 days after eclosion (A, p=0.001, Student's T-test, n=16 each group), but not at 26 days after eclosion (B, p=0.41, Student's T-test, n=16 each group). **C)** Although *Cs* males show a gradual decrease in Δ Sleep between 5 and 45 days after eclosion (One-way ANOVA, $F_{(5,169)}$=2.54, p=0.025, n=16-48 each group), *Cs* females show no change in sleep after social enrichment by 20 - 25 days of age (One-way ANOVA, $F_{(4,154)}$=11.69, p=2.57x10^{-8}, n=32 each group). **D)** 11 day old *Cs* females exhibit longer daytime sleep bouts after social enrichment (left), while social enrichment has no effect on daytime bout length in 26 day old *Cs* females (right). Two-way ANOVA reveals a significant age x condition interaction ($F_{(1,1,1,60)}$=9.10, p=0.004, *p<0.05 modified Bonferroni Post-hoc test, n=16 each group). **E)** 11 day old *Cs* females exhibit longer nighttime sleep bouts after social enrichment (left), while social enrichment has no effect on nighttime bout length in 26 day old *Cs* females (right). Two-way ANOVA reveals a significant main effect for age ($F_{(1,1,1,60)}$=5.87, p=0.018, *p<0.05 modified Bonferroni Post-hoc test, n=16 each group). **F)** 26 day old *Cs* females retain intact locomotor activity while housed in social enrichment compared to 11 day old *Cs* females. Two-way ANOVA reveals a significant main effect for age ($F_{(1,46,46)}$=32.93, p=3.76x10^{-8}). **G)** *Cs* females show no difference in the ability to produce viable offspring at 25 days compared to 10 days after eclosion (Student's T-test, p=0.31). **H)** Continuous housing in social isolation or social enrichment from eclosion to 20 days after eclosion has no effect on mean Δ

Sleep in 26 day old *Cs* females (Student's T-test, p=0.54, n=16 each group). **I)** 26 day old *Cs* females show no increase in sleep after social enrichment when reared at 18˚C or at 25˚C (Student's T-test, p=0.17, n=16 each group).

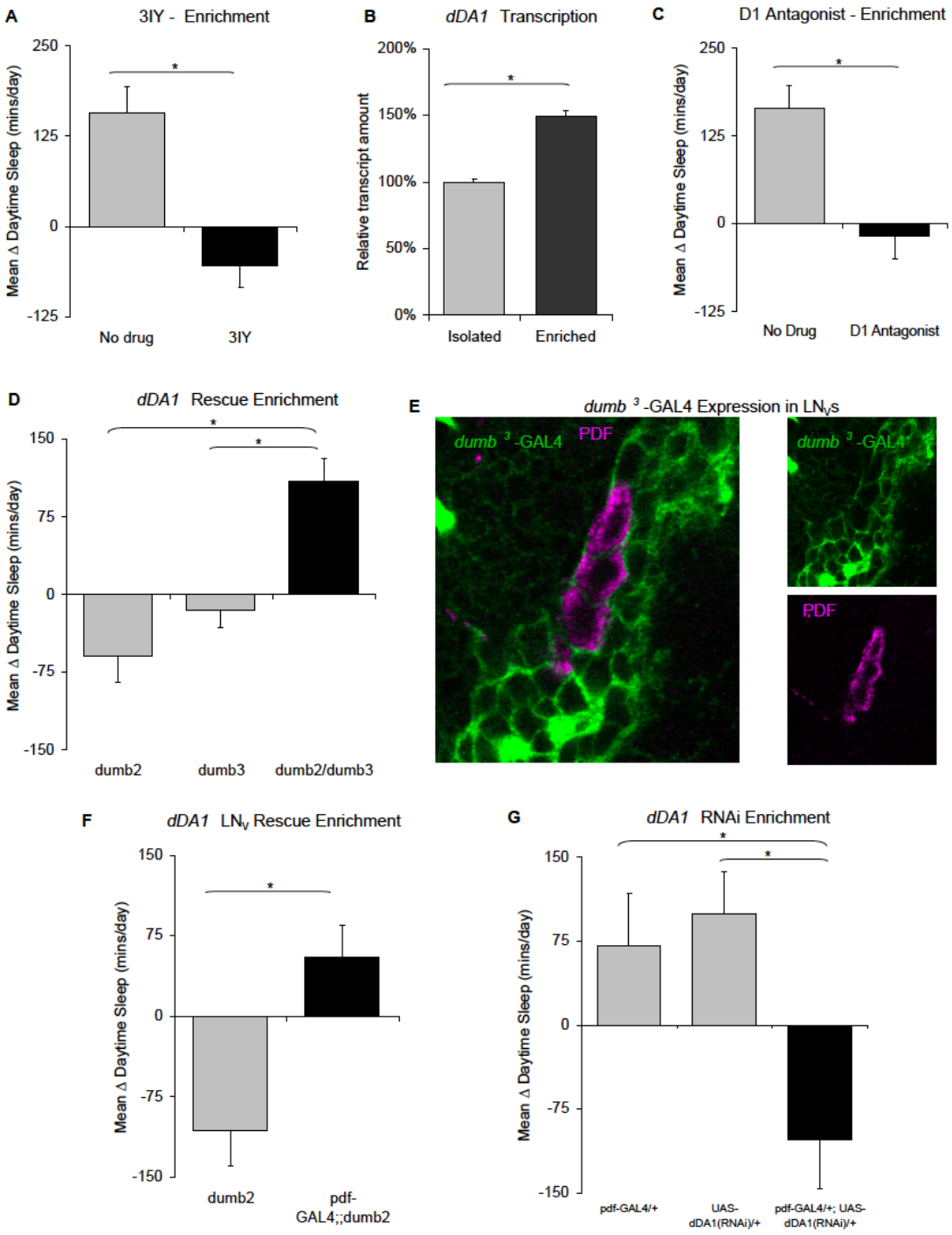


Figure 2 – Dopaminergic signaling is required for increased sleep following social enrichment

A) While vehicle-fed 11 day old *Cs* females exhibit increased daytime sleep

following social enrichment (left), 11 day old *Cs* females fed 10 mg/mL 3IY during enrichment/isolation show no subsequent change in daytime sleep (right) ($p=8.135x10^{-5}$, Student's T-Test, n=16 each group). **B)** Abundance of *dDA1* mRNA is significantly elevated in the heads of 10 day old *Cs* females following social enrichment (p=0.003, Two-tailed Student's T-Test, n=2 each group). **C)** Vehicle-fed 11 day old *Cs* females exhibit increased daytime sleep following social enrichment (left), but 11 day old *Cs* females fed a D1-antagonist (1 mg/mL SCH23390) during enrichment/isolation show no subsequent change in daytime sleep (right) (p=0.0004, Two-tailed Student's T-Test, n=16 each group). **D)** Flies that are homozygous for either of two independent *dDA1* mutant alleles show no increase in sleep after social enrichment (*dumb*^{2}, left, or *dumb*^{3}, center), while *dumb*^{2}*/dumb*^{3} rescue flies exhibit a wild-type increase in sleep after exposure to a socially enriched environment. One-way ANOVA for genotype ($F_{(2,89)}$=16.81, $p=6.38x10^{-7}$, *p<0.05 modified Bonferroni post-hoc test, n=29-32 each group). **E)** A membrane-bound GFP reporter indicates that *dumb*^{3}-GAL4 is expressed widely in the brain, including in *pdf*-expressing LN_Vs (UAS-CD8::GFP/+;*dumb*^{3}/+). **F)** Although *dumb*^{2} mutants do not increase their sleep after social enrichment (left), rescue of *dDA1* expression exclusively in the LN_Vs (*pdf*-GAL4;;*dumb*^{2}, right) restores increased sleep after enriched social experience (p=0.0011, Two-tailed Student's T-Test, n=16 each group). **G)** Flies expressing an RNAi construct for *dDA1* in the LN_Vs (*pdf*-GAL4/+; UAS-*dDA1*^{RNAi}/+) show no increase in sleep following social enrichment (right). Parental controls (*pdf*-GAL4/+, left; UAS-*dDA1*^{RNAi}/+, center) exhibit increased daytime sleep following social

enrichment. One-way ANOVA for genotype ($F_{(2,45)}$=6.60, p=0.003, *p<0.05 modified Bonferroni post-hoc test, n=16 each group).

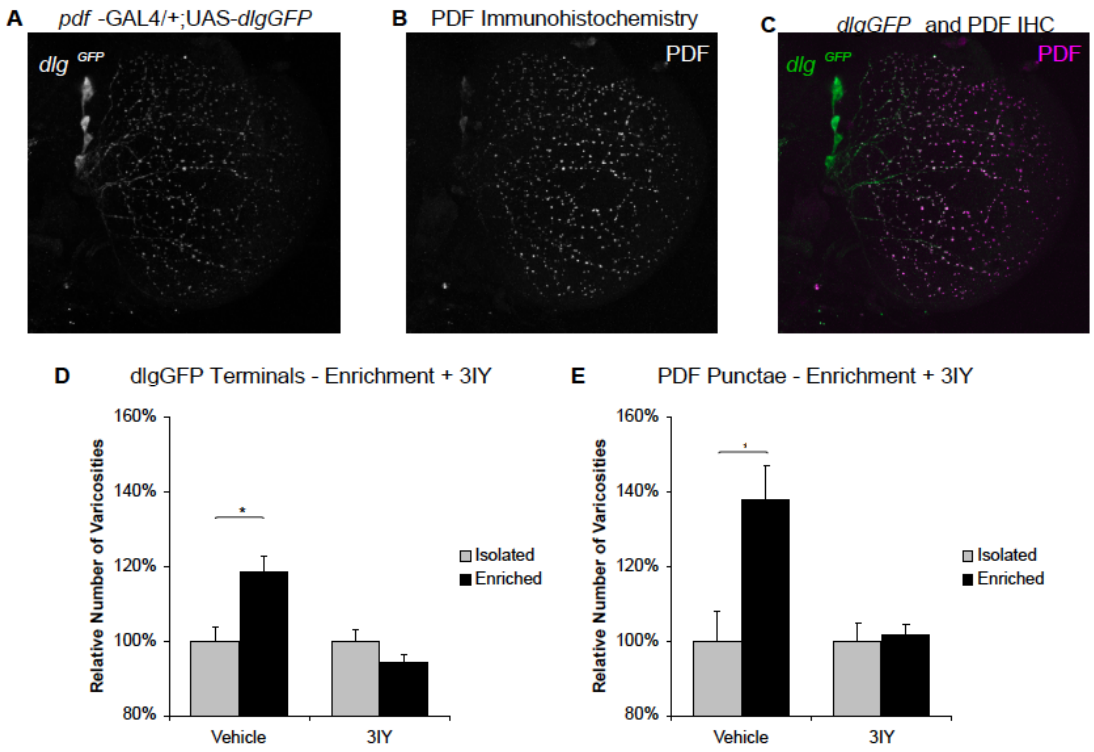


Figure 3 – Depletion of dopamine prevents structural plasticity following social enrichment

A-C) Colocalization of PDF and *dlg*^{GFP} in LN_V projections of *pdf*-GAL4/+; UAS-*dlg*^{GFP}/+ flies. **D)** Vehicle-treated 10 day old *pdf*-GAL4/+; UAS-*dlg*^{GFP}/+ flies showed an increase in the number of *dlg*^{GFP}-positive varicosities (left), but siblings fed 10 mg/mL 3IY show no change in *dlg*^{GFP}-positive varicosities (right) following social enrichment. Two-way ANOVA reveals a significant treatment x condition interaction ($F_{(1,1,1,40)}$=17.644, p=0.0001, *p<0.05 modified Bonferroni post-hoc test, n=11 each group). **E)** Vehicle-fed flies exhibit a significant increase in PDF-immunopositive punctae following social enrichment (left), while enrichment has no effect on the number of PDF-positive punctae in siblings fed 10 mg/mL 3IY (right). Two-way ANOVA reveals a significant treatment x

condition interaction ($F_{(1,1,1,40)}$=7.535, p=0.009, *p<0.05 modified Bonferroni post-hoc test, n=11 each group).

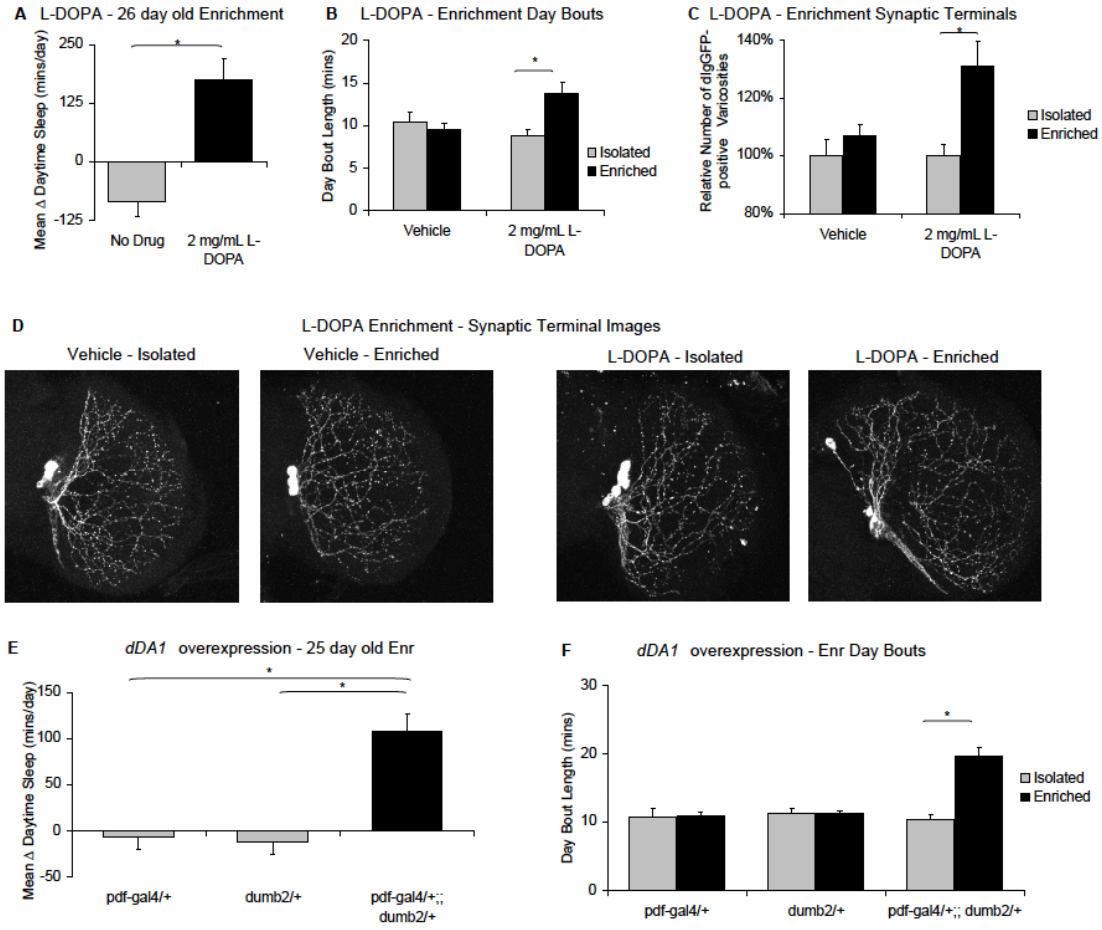


Figure 4 – Elevated dopaminergic signaling restores increased sleep after social enrichment in aged flies

A) 26 day old *Cs* females show a significant increase in sleep following social enrichment when fed 2 mg/mL L-DOPA during isolation/enrichment (right) while vehicle-fed controls show no change in sleep. ($p=4.06x10^{-5}$, Two-tailed Student's T-test, n=16 each group) **B)** 26 day old *Cs* females exhibit a significant increase in daytime sleep bout length following social enrichment when fed L-DOPA (right), while vehicle controls show no change in bout length (left). Two-way ANOVA reveals a significant condition x treatment interaction ($F_{(1,1,1,60)}=8.84$, $p=0.004$, *$p<0.05$ modified Bonferroni Post-hoc test, n=16 each group). **C-D)** L-

DOPA administration to 20 day old *pdf*-GAL4/+; UAS-*dlg*^{GFP}/+ flies induces an increase in LN_V terminal number following social enrichment while vehicle-treated controls show no change in LN_V terminals after social enrichment (C). Two-way ANOVA reveals significant main effect for condition (Isolated vs. Enriched) ($F_{(25,1)}$=11.55, p=0.0023, *signifies p<0.05 modified Bonferroni Post-hoc test, n=13 each group). Representative images from vehicle-fed and L-DOPA treated brains are shown in (D). **E)** 26 day old flies over-expressing *dDA1* in the LN_Vs (*pdf*-GAL4/+;; *dumb*^{2}/+, right) demonstrate a robust increase in daytime sleep after social enrichment while 26 day old genetic control flies show no change in sleep after social enrichment (*pdf*-GAL4/+, left; *dumb*^{2}/+, center). One-way ANOVA ($F_{(2,189)}$=18.74, p=3.75x10^{-8}, *p<0.05 modified Bonferroni Post-hoc test, n=64 each group) **F)** Over-expression of *dDA1* in the LN_Vs induces increased daytime bout length after social enrichment in 26 day old flies (*pdf*-GAL4/+;; *dumb*^{2}/+, right). 26 day old parental controls show no change in day bout length after social enrichment (*pdf*-GAL4/+, left; *dumb*^{2}/+, center). Two-way ANOVA reveals significant genotype x condition interaction ($F_{(1,2,2,336)}$=10.81, p=2.95x10^{-5}, *p<0.05 modified Bonferroni Post-hoc test, n = 50-64 each group).

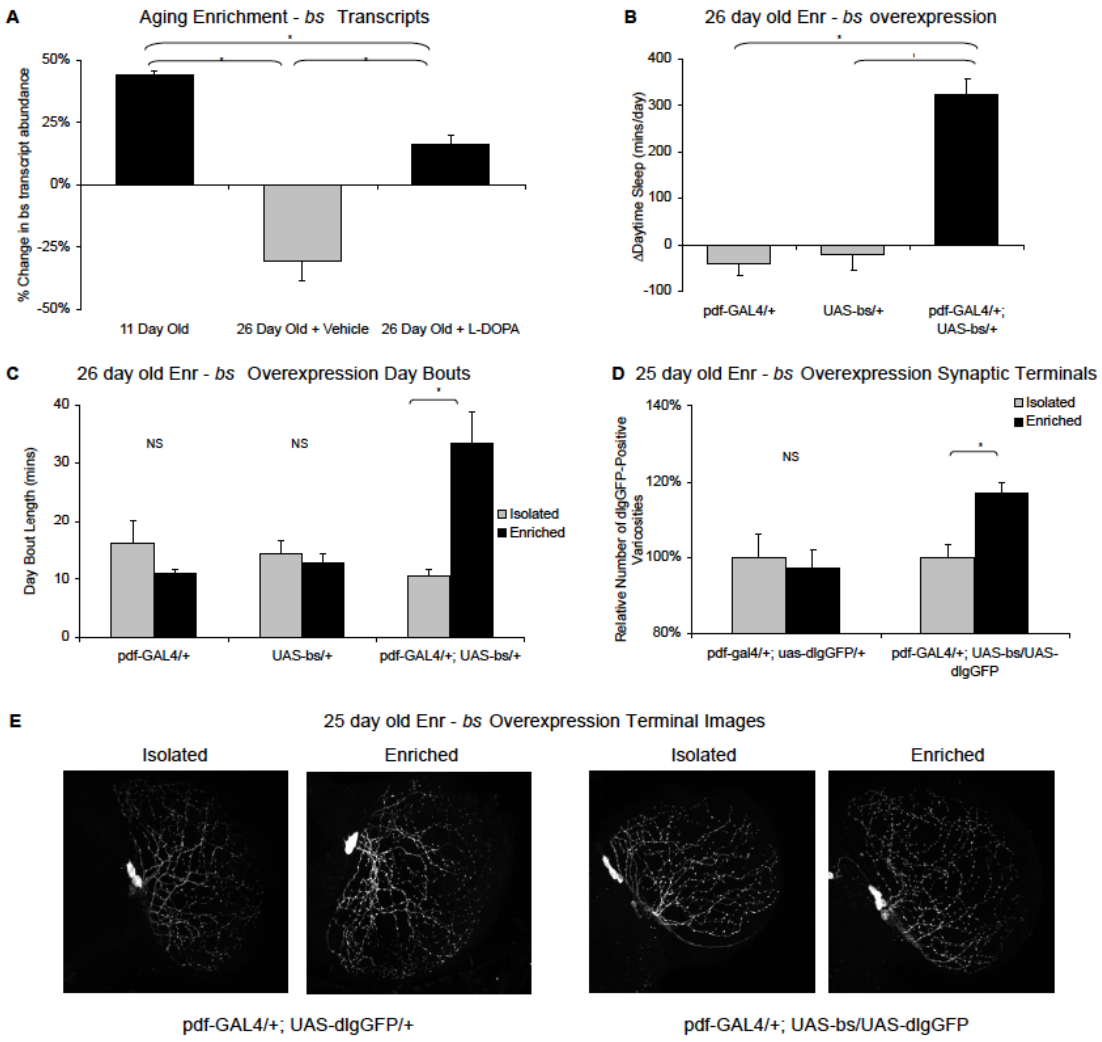
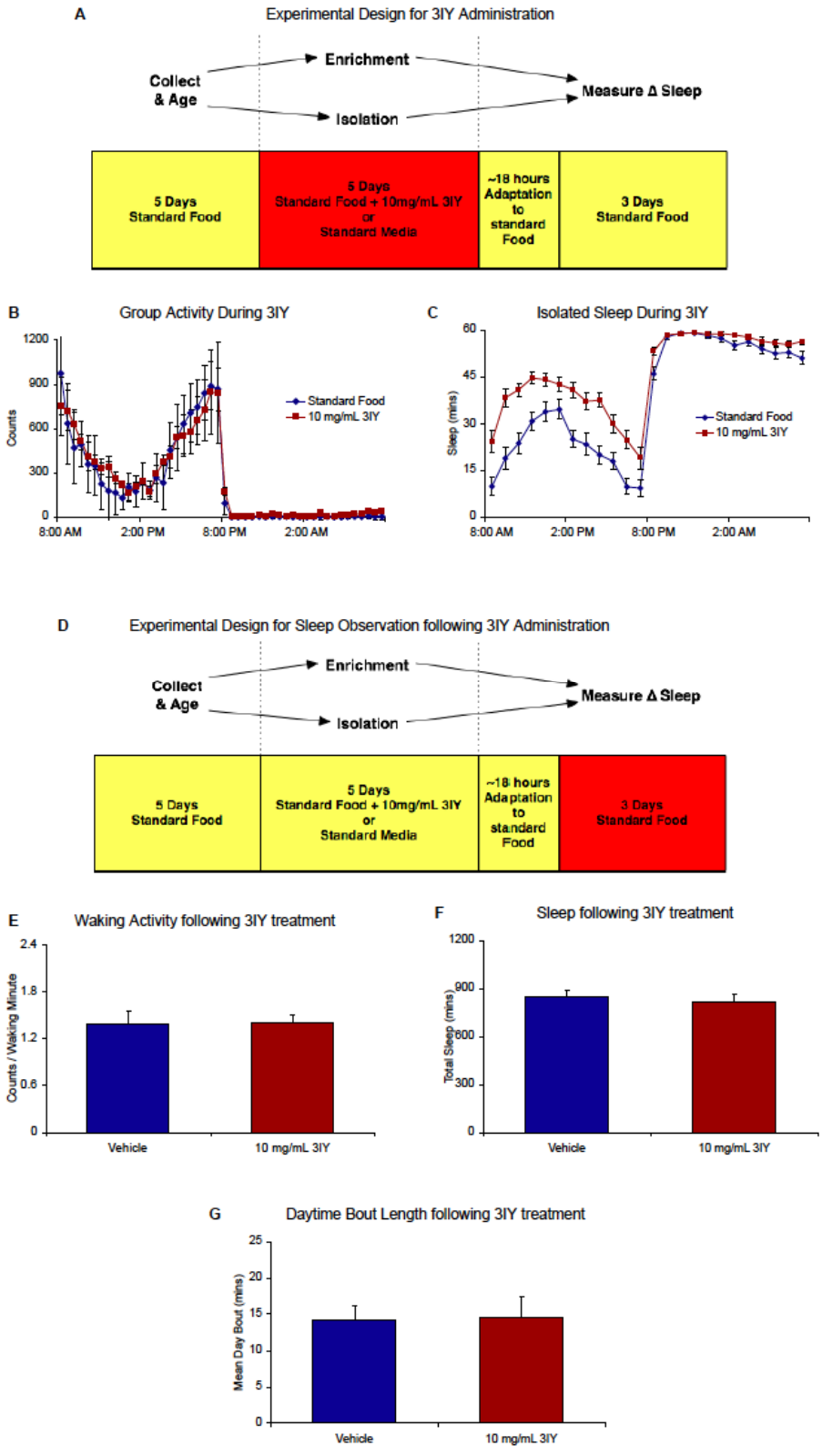


Figure 5 – Overexpression of *blistered* in the LN_Vs restores plasticity in aged flies.

A) Abundance of *bs* transcripts is significantly elevated when *Cs* females are housed in socially enriched conditions 11 days after eclosion (left), but not when social enrichment begins at 26 days of age (center). Administration of 2 mg/mL L-DOPA during social enrichment restores elevated *bs* transcript abundance in 26 day old *Cs* females (right). One-way ANOVA ($F_{(2,3)}$= 131.76, p = 0.0012, *p<0.05 modified Bonferroni post-hoc test, n=2 each group) **B)** Over-expression of *bs* in the LN_Vs restores increased daytime sleep after social enrichment in 26

day old females (*pdf*-GAL4/+; UAS-*bs*/+, right), while 26 day old control flies show no change after social enrichment (*pdf*-GAL4/+, left; UAS-*bs*/+, center). One-way ANOVA ($F_{(2,43)} = 43.15$, $p = 5.26x10^{-11}$, *p<0.05 modified Bonferroni post-hoc test, n=14-16 each group). **C)** *bs* over-expression in the LN_Vs results in increased daytime bout length after social enrichment of 26 day old females (*pdf*-GAL4/+; UAS-*bs*/+, right) while social enrichment has no effect on bout length in 25 day old controls (*pdf*-GAL4/+, left; UAS-*bs*/+, center). 2-Way ANOVA reveals significant genotype x condition interaction ($F_{(1,2,2,83)}$=13.99, p=5.1x10-6, *p<0.05 modified Bonferroni Post-hoc test, n=14-16 each group). **D-E)** While 26 day old control flies (*pdf*-GAL4/+; UAS-dlg^{GFP}/+) show no change in the number of LN_V terminals following social enrichment (D, left), 26 day old flies over-expressing *bs* in the LN_Vs (*pdf*-GAL4/+; UAS-*bs*/UAS-dlg^{GFP}) exhibit a significant increase in the number of LN_V terminals (D, right). 2-Way ANOVA reveals significant genotype x condition (Isolated vs Enriched) interaction ($F_{(1,1,1,40)} = 7.73$, $p = 0.008$, *p<0.05 modified Bonferroni post-hoc test, n = 11 each group). Representative images from control (*pdf*-GAL4/+; UAS-dlg^{GFP}/+) and *bs*-overexpressing (*pdf*-GAL4/+; UAS-*bs*/UAS-dlg^{GFP}) flies are shown in (E).

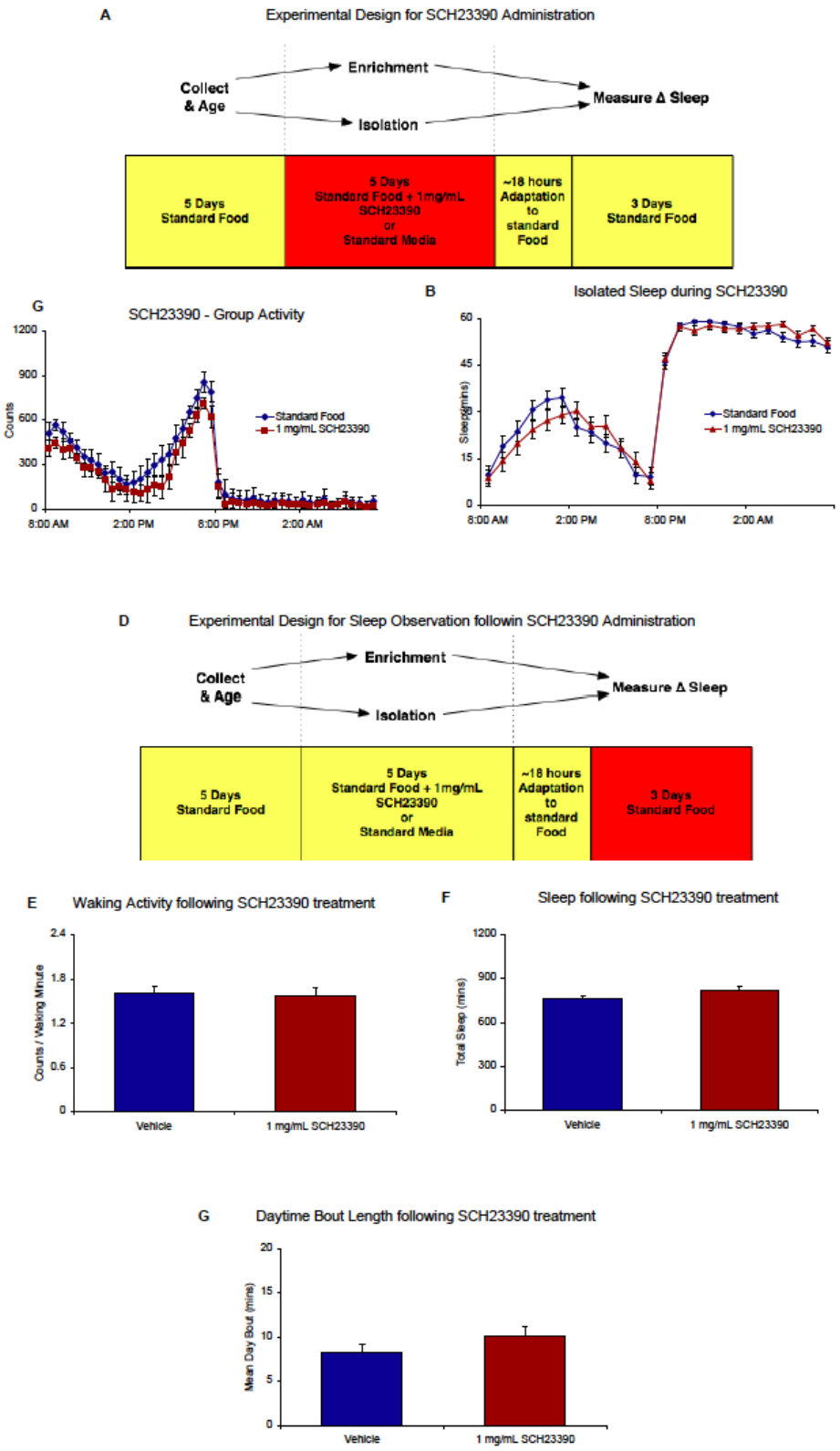
Supplemental Figure 1



Supplemental Figure 1 – Sleep during and after 3IY administration

A) Experimental design for dopamine pharmacology during social enrichment. Data presented in panels B-C represent sleep and activity data collected from isolated or enriched flies during pharmacological treatments. **B)** Group activity during social enrichment is not significantly altered in flies fed 10 mg/mL 3IY. T-test for total locomotor activity p=0.92, n=3 each group. **C)** Sleep is increased during social isolation in flies that are administered 10 mg/mL 3IY. Student's T-test for total sleep time $p=4.5x10^{-6}$, n=30-32 each group. **D)** Experimental design for the measurement of ΔSleep following administration of 3IY to socially isolated or enriched flies. Data presented in panels E-G was collected after all flies were allowed to adapt to fresh Trikinetics tubes containing standard food for ~18 hours. **E)** The intensity of waking activity as measured by counts/waking minute is not altered in isolated flies previously fed 3IY compared to vehicle-treated controls. Student's T-Test p=0.44, n=13-14. **F)** Following ~18 hours on standard food, no difference in sleep time is detected between socially isolated flies previously fed 3IY and their vehicle-treated siblings. Student's T-test for total sleep p=0.53, n=13-14. **G)** After ~18 hours of adaptation to standard food, no change in daytime sleep bout length is observed in isolated flies previously administered 3IY relative to their vehicle-fed controls. Student's T-test n=0.94, n=13-14.

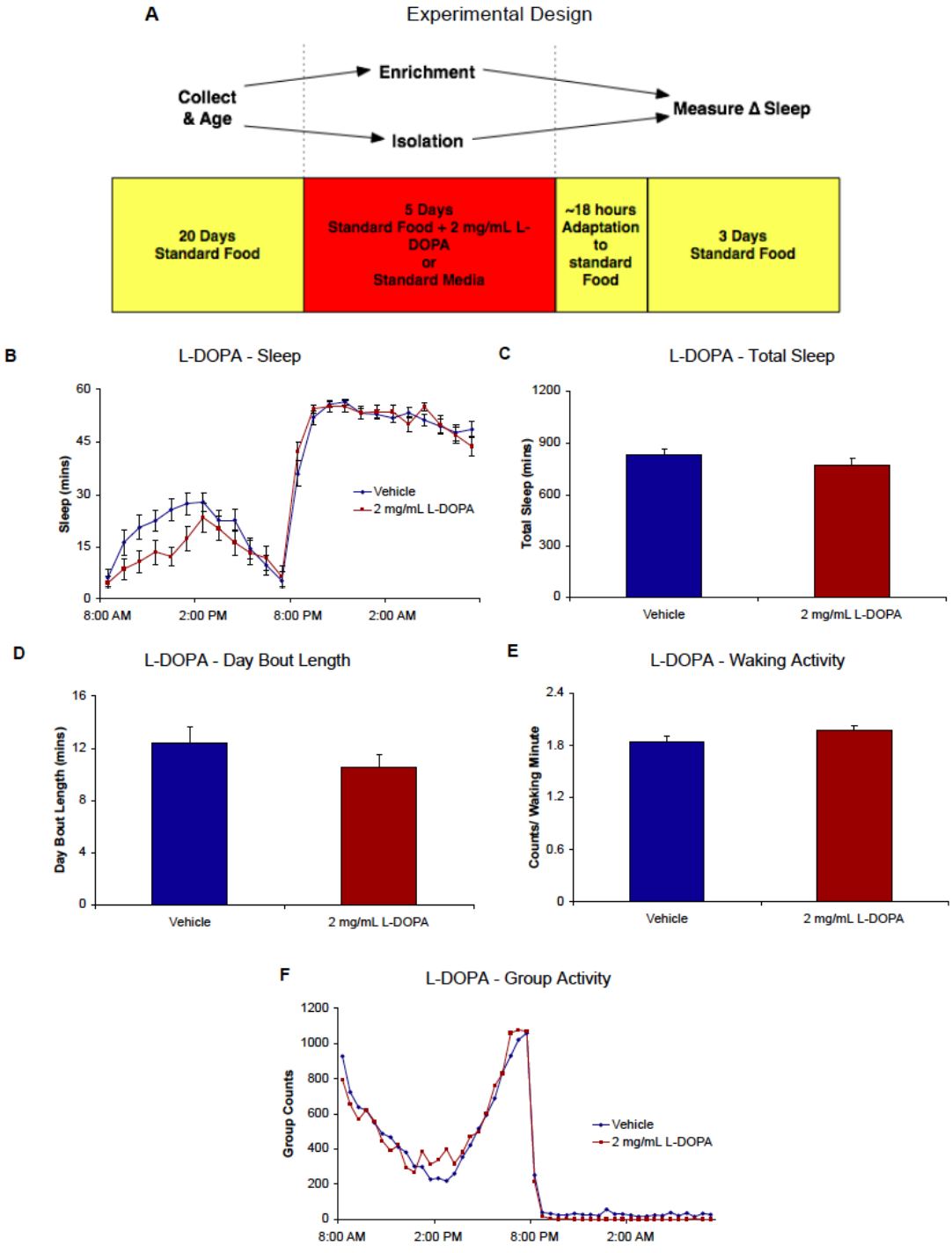
Supplemental Figure 2



Supplemental Figure 2 – Sleep during and after administration of SCH23390

A) Experimental design for dopamine pharmacology during social enrichment. Data presented in panels B-C were collected while flies were administered 1 mg/mL SCH23390 during social isolation or enrichment. **B)** Administration of 1 mg/mL SCH23390 to 5 day old *Cs* females during social enrichment does not alter locomotor activity. T-test for total locomotor activity p=0.46, n=3 each group. **C)** Sleep time is not altered in flies during administration of SCH23390. Student's T-test for total sleep time p=0.80, n=30-32 each group. **D)** Experimental design for measuring sleep following administration of SCH23390 during social isolation or enrichment. Panels E-G represent data collected after flies adapted to fresh Trikinetics tubes containing standard food for ~18 hours. **E)** Intensity of waking activity is not altered in socially isolated flies allowed to adapt to standard food for 18 hours after administration of SCH23390 compared to vehicle-treated controls. Student's T-test p=0.86, n=16 each group. **F)** No change in total sleep time can be detected in socially isolated flies fed standard food for ~18 hours after administration of SCH23390 compared to vehicle-treated controls. Student's T-test p=0.18, n=16 each group. **G)** Daytime sleep bout length is not altered in socially isolated flies that are fed standard food for ~18 hours after treatment with SCH23390 relative to vehicle-fed controls. Student's T-test p=0.25, n=16 each group.

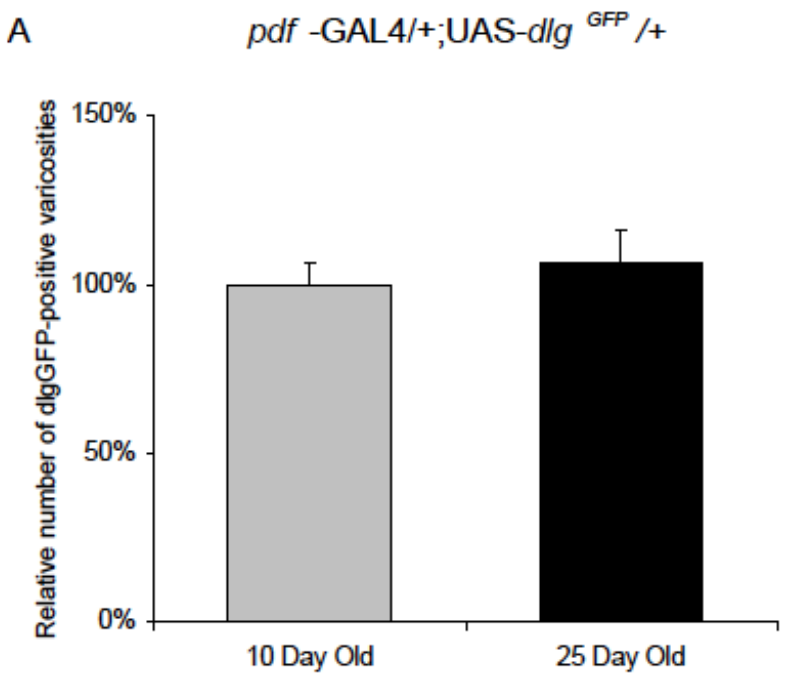
Supplemental Figure 3



Supplemental Figure 3 – No effect of L-DOPA administration on sleep in aged *Cs* females

A) Experimental design for L-DOPA administration during social isolation or enrichment in aged *Cs* females. Data presented in panels B-F were collected from socially isolated or enriched flies during administration of 2 mg/mL L-DOPA. **B-C)** No change in total sleep time of 20 day old, socially isolated *Cs* females during administration of 2 mg/mL L-DOPA compared to vehicle-fed siblings. (p=0.29, Two-tailed Student's T-test, n=23-25). **D)** Administration of 2 mg/mL L-DOPA to 20 day old *Cs* females during isolation has no significant effect on daytime bout length. (p=0.27, Two-tailed Student's T-test, n=23-35). **E)** Intensity of waking activity is not altered by administration of 2 mg/mL L-DOPA to 20 day old *Cs* females during social isolation (p=0.15, Two-tailed Student's T-test, n=23-35). **F)** Amount of locomotor activity is not altered in socially-enriched groups of 20 day old *Cs* females during administration of 2 mg/mL L-DOPA. Two-way ANOVA reveals no significant main effect for treatment ($F_{(46,1)}$=0.46, p=0.50).

Supplemental Figure 4



Supplemental Figure 4 – No effect of age on number of LN_V synaptic terminals

A) The number of *dlg*^{GFP}-positive varicosities in LN_Vs (*pdf*-GAL4/+;UAS-*dlg*^{GFP}/+) does not differ between 10 day old and 25 day old flies. (p=0.67, Two-tailed Student's T-test, n=9-10 each group).

References

BHANDARI, P., M. A. JONES, I. MARTIN and M. S. GROTEWIEL, 2007 Dietary restriction alters demographic but not behavioral aging in Drosophila. Aging Cell **6:** 631-637.

BURKE, S. N., and C. A. BARNES, 2005 Neural plasticity in the ageing brain. Nat Rev Neurosci **7:** 30-40.

COOK-WIENS, E., and M. S. GROTEWIEL, 2002 Dissociation between functional senescence and oxidative stress resistance in Drosophila. Exp. Gerontol. **37:** 1347-1357.

DASELAAR, S. M., D. J. VELTMAN, S. A. R. B. ROMBOUTS, J. G. W. RAAIJMAKERS and C. JONKER, 2003 Neuroanatomical correlates of episodic encoding and retrieval in young and elderly subjects. Brain **126:** 43-56.

DIETZL, G., D. CHEN, F. SCHNORRER, K.-C. SU, Y. BARINOVA *et al.*, 2007 A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature **448:** 151-156.

DONLEA, J., and P. J. SHAW, 2009 Sleeping Together: Using Social Interactions to Understand the Role of Sleep in Plasticity. Adv Genet **68:** 57-81.

DONLEA, J. M., N. RAMANAN and P. J. SHAW, 2009 Use-dependent plasticity in clock neurons regulates sleep need in Drosophila. Science **324:** 105-108.

DUMITRIU, D., J. HAO, Y. HARA, J. KAUFMANN, W. G. M. JANSSEN *et al.*, 2010 Selective changes in thin spine density and morphology in monkey prefrontal cortex correlate with aging-related cognitive impairment. J

Neurosci **30:** 7507-7515.

GANGULY-FITZGERALD, I., J. M. DONLEA and P. J. SHAW, 2006 Waking experience affects sleep need in Drosophila. Science **313:** 1775-1781.

GREENOUGH, W. T., R. W. WEST and T. J. DEVOOGD, 1978 Subsynaptic plate perforations: changes with age and experience in the rat. Science **202:** 1096-1098.

GROTEWIEL, M. S., I. MARTIN, P. BHANDARI and E. COOK-WIENS, 2005 Functional senescence in Drosophila melanogaster. Ageing Res Rev **4:** 372-397.

IMAI, Y., S. GEHRKE, H. Q. WANG, R. TAKAHASHI, K. HASEGAWA *et al.*, 2008 Phosphorylation of 4E-BP by LRRK2 affects the maintenance of dopaminergic neurons in Drosophila. EMBO J **27:** 2432-2443.

KIM, Y. C., H. G. LEE and K. A. HAN, 2007 D1 Dopamine Receptor dDA1 Is Required in the Mushroom Body Neurons for Aversive and Appetitive Learning in Drosophila. J Neurosci **27:** 7640.

LANDSBERG, G., 2005 Therapeutic agents for the treatment of cognitive dysfunction syndrome in senior dogs. Prog Neuropsychopharmacol Biol Psychiatry **29:** 471-479.

LEBESTKY, T., J.-S. C. CHANG, H. DANKERT, L. ZELNIK, Y.-C. KIM *et al.*, 2009 Two different forms of arousal in drosophila are oppositely regulated by the dopamine D1 receptor ortholog DopR via distinct neural circuits. Neuron **64:** 522-536.

MURAKAMI, H., K. BESSINGER, J. HELLMANN and S. MURAKAMI, 2005 Aging-dependent and -independent modulation of associative learning behavior

by insulin/insulin-like growth factor-1 signal in Caenorhabditis elegans. J Neurosci **25:** 10894-10904.

NECKAMEYER, W. S., S. WOODROME, B. HOLT and A. MAYER, 2000 Dopamine and senescence in Drosophila melanogaster. Neurobiol. Aging **21:** 145-152.

PARISKY, K. M., J. AGOSTO, S. R. PULVER, Y. SHANG, E. KUKLIN *et al.*, 2008 PDF cells are a GABA-responsive wake-promoting component of the Drosophila sleep circuit. Neuron **60:** 672-682.

PELEG, S., F. SANANBENESI, A. ZOVOILIS, S. BURKHARDT, S. BAHARI-JAVAN *et al.*, 2010 Altered histone acetylation is associated with age-dependent memory impairment in mice. Science **328:** 753-756.

PHAM, T. M., B. ICKES, D. ALBECK, S. SÖDERSTRÖM, A. C. GRANHOLM *et al.*, 1999 Changes in brain nerve growth factor levels and nerve growth factor receptors in rats exposed to environmental enrichment for one year. Neuroscience **94:** 279-286.

SCHWAERZEL, M., M. MONASTIRIOTI, H. SCHOLZ, F. FRIGGI-GRELIN, S. BIRMAN *et al.*, 2003 Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. J Neurosci **23:** 10495-10502.

SEUGNET, L., J. BOERO, L. GOTTSCHALK, S. P. DUNTLEY and P. J. SHAW, 2006 Identification of a biomarker for sleep drive in flies and humans. Proc Natl Acad Sci USA **103:** 19913-19918.

SEUGNET, L., Y. SUZUKI, L. VINE, L. GOTTSCHALK and P. J. SHAW, 2008 D1 Receptor Activation in the Mushroom Bodies Rescues Sleep-Loss-Induced Learning Impairments in Drosophila. Curr. Biol.

SHANG, Y., L. C. GRIFFITH and M. ROSBASH, 2008 Feature Article: Light-arousal and circadian photoreception circuits intersect at the large PDF cells of the Drosophila brain. Proc Natl Acad Sci USA.

SHAW, P. J., C. CIRELLI, R. J. GREENSPAN and G. TONONI, 2000 Correlates of sleep and waking in Drosophila melanogaster. Science **287:** 1834-1837.

SHEEBA, V., K. J. FOGLE, M. KANEKO, S. RASHID, Y. T. CHOU *et al.*, 2008 Large Ventral Lateral Neurons Modulate Arousal and Sleep in Drosophila. Curr. Biol.

SPENCER, R. M. C., A. M. GOUW and R. B. IVRY, 2007 Age-related decline of sleep-dependent consolidation. Learn Mem **14:** 480-484.

TAMURA, T., A. S. CHIANG, N. ITO, H. P. LIU, J. HORIUCHI *et al.*, 2003 Aging specifically impairs amnesiac-dependent memory in Drosophila. Neuron **40:** 1003-1011.

TECHNAU, G. M., 2007 Fiber number in the mushroom bodies of adult Drosophila melanogaster depends on age, sex and experience. J. Neurogenet. **21:** 183-196.

TOSCANO, C. D., J. L. MCGLOTHAN and T. R. GUILARTE, 2006 Experience-dependent regulation of zif268 gene expression and spatial learning. Exp Neurol **200:** 209-215.

VOLKMAR, F. R., and W. T. GREENOUGH, 1972 Rearing complexity affects branching of dendrites in the visual cortex of the rat. Science **176:** 1445-1447.

WALKER, M. P., R. J. STICKGOLD, D. ALSOP, N. GAAB and G. SCHLAUG, 2005

Sleep-dependent motor memory plasticity in the human brain. Neuroscience **133:** 911-917.

WINOCUR, G., 1998 Environmental influences on cognitive decline in aged rats. Neurobiol. Aging **19:** 589-597.

YAMAZAKI, D., J. HORIUCHI, Y. NAKAGAMI, S. NAGANO, T. TAMURA *et al.*, 2007 The Drosophila DC0 mutation suppresses age-related memory impairment without affecting lifespan. Nat Neurosci.

Chapter 5:

Conclusions and Future Issues

Although the function of sleep is unknown, the most compelling of the current theories regarding the function of sleep pertain to the role of sleep in plasticity or memory consolidation (Tononi and Cirelli, 2003; Stickgold and Walker, 2005). Unfortunately, many of these hypotheses are based upon assumptions that are difficult to evaluate using more traditional approaches (see below). Our data demonstrate that *Drosophila* can be used as a genetic model for investigating the relationship between sleep and plasticity. More importantly, we have developed an efficient, high throughput assay, exposure to enriched social environments, which has been validated using an associative memory assay, courtship conditioning, and the quantification of synaptic markers. We have identified a specific cluster of sleep regulatory neurons (LN_Vs) and 6 genes that are required within these cells for that are necessary for increases in sleep following social enrichment. Since aging is, perhaps, the single most important physiological variable that can modify both sleep and plasticity, we have also investigated the role of aging in responses to a socially enriched environment. Indeed, physiological aging degrades both structural and behavioral plasticity following social enrichment in wild-type flies. Furthermore, we have found that plasticity can be restored to aged flies by elevating the expression of two genes, *dDA1* and

blistered, that are also necessary for increased sleep after social enrichment in young flies. Together, these data suggest that the close relationship between sleep and plasticity that has previously been identified in humans (Walker et al., 2005; Huber et al., 2007), cats (Frank et al., 2001; Aton et al., 2009), and mice (Graves et al., 2003; Vecsey et al., 2009), is also conserved in the genetic model organism *Drosophila*.

Despite the contributions of these studies, several issues regarding the relationship between sleep and plasticity remain to be addressed. First, it remains unclear whether sleep affects the whole brain equally or whether sleep can work locally in the brain to alter specific circuits. Although sleep has been classically characterized as a unitary behavioral state that alters global physiology, processes that are associated with sleep are regulated on a local, circuit-dependent basis in the brain. Several species of marine mammals, for instance, can exhibit electrophysiological patterns of sleep in one brain hemisphere while the other hemisphere appears to be awake (reviewed in (Lyamin et al., 2008). Recent studies have also found that sleep-associated patterns of activity can be significantly altered in local circuits by previous activity. That is, if a circuit is stimulated locally during waking then the intensity of Slow Wave Activity (SWA) during subsequent sleep is elevated in that circuit (Kattler et al., 1994; Vyazovskiy et al., 2000; Miyamoto et al., 2003; Cottone et al., 2004; Huber et al., 2004; Iwasaki et al., 2004; Yasuda et al., 2005). Conversely, if afferent activity to a cortical region is suppressed during the day, then SWA is

locally reduced during sleep (Huber et al., 2007). In all, these studies indicate that although sleep is a global behavioral state, specific circuits in the brain may "sleep" differently depending on their specific need and previous activity.

Our data suggest that plasticity in the LN_Vs can induce a robust increased sleep in response to social enrichment while other circuits that are known to play a role in learning and memory are much less effective (Donlea et al., 2009). Interestingly, Gilestro *et al* have reported that levels of the synaptic active zone protein *Bruchpilot* are dramatically elevated throughout much of the brain following 16 h of waking (Gilestro et al., 2009). These data suggest that synapses in a wide variety of circuits in the fly brain may become potentiated during waking. Indeed, rescue of *rutabaga* in all neurons results in the strongest increase in sleep following social enrichment consistent with the observation that waking influences many circuits besides just the LN_Vs. Thus, while the morphology of the LN_Vs and their projections make them well suited for quantifying synaptic terminals, the relationship between synaptic homeostasis and sleep will be enhanced through the identification of additional circuits with discrete expression patterns that can be modulated by experimental interventions. That is, while we favor the use of social environment to alter neuronal plasticity, other interventions may be useful for identifying additional circuits that can then be used to determine whether they are downscaled during sleep to the same degree as reported with this paradigm. Based on the vertebrate literature described above, it is likely that the degree of downscaling

that is observed in each region will be proportional to the amount of local stimulation during waking. Further examination of synaptic homeostasis will be necessary to identify the mechanisms that mediate synaptic down-scaling during sleep and to determine whether homeostatic down-scaling might occur on a circuit-dependent basis during sleep.

Importantly, although we have found evidence of synaptic-downscaling during sleep, the functional role of this downscaling remains entirely unknown. Presumably, neural circuits can only support a limited amount of potentiation before the metabolic demands of the enhanced connections preclude further elaboration. The conditions at which social enrichment potentiates synaptic connections to this saturation point are not well characterized, and, as a result, future studies will be required to examine behavioral and metabolic consequences of saturated synaptic connections. Our studies indicate that exposure to social enrichment may provide a useful paradigm for the study of these consequences. Presumably, if synaptic-downscaling is required after social enrichment to restore proper neurological functioning, then behavioral examination of flies that have been enriched in a very large social group may be unable to properly form new memories or may exhibit metabolic abnormalities. Because we have not yet thoroughly tested these hypothesis, future studies will be required to examine the consequences of synaptic potentiation that during waking experience.

A second question is raised by the plasticity induced by social enrichment; specifically, the types of social interactions that might occur during social enrichment are not well established. Although *Drosophila* has been used as a model system for behavioral genetics over the past several decades, relatively little is known about the naturalistic social behavior of the fly. Courtship and aggression behaviors that are used by male flies have been studied in detail and thoroughly reviewed elsewhere (Robin et al., 2007; Villella and Hall, 2008), but social enrichment using only females induces a robust plastic response. The types of social interactions that occur in a female-only environment are not well characterized and the behavioral consequences of these plastic responses are entirely unknown. In order to better understand which neural circuits are likely to be altered by social enrichment as well as their functional roles, it would be helpful to better describe the types of social interactions that might occur during social enrichment. Two recent studies (Branson et al., 2009; Dankert et al., 2009) have utilized automated tracking algorithms to identify and score the social behavior of flies either in pairs or in a large group. The “ethomics” approach that these groups have begun to utilize might allow for rapid large-scale quantification of social behavior and to identify possible genetic and neural mechanisms that underlie these behaviors. As currently designed, the algorithms used in these two studies complement each other fairly well; the Ctrax software designed by Branson and colleagues is capable of tracking the movements of individual flies within larger groups over long periods of time while the CADABRA software suite utilized by Dankert and colleagues provides more detailed analysis of individual

actions related to known courtship or aggression behaviors. These algorithms, however, are not optimally designed for the identification of novel types of interactions between individual animals; Ctrax does not provide detailed analysis of behavior and CADABRA is only capable of identifying pre-defined behavioral characteristics. Ultimately, these software tools may provide efficient tools for dissecting the mechanisms controlling known types of behavior, but only careful observation by human investigators is likely to allow for the identification and characterization of currently undescribed social interactions within groups of flies.

Although these interactions have not yet been characterized, it is likely that they are mediated, at least in part, through pheromonal communication. These chemical cues consist of a number of hydrocarbon compounds that are embedded in the waxy surface of the abdominal cuticle and are known to be crucial for the initiation and regulation of courtship behavior (Ferveur, 2005). Recent studies indicate that social context can significantly alter the composition of these hydrocarbon cues in male flies (Kent et al., 2008; Krupp et al., 2008). Interestingly, these studies found that altered pheromone production in response to social experience may have important consequences for future behavior; exposure to a genetically heterogeneous group induces wild-type males to alter the expression of courtship-related pheromonal cues and also results in increased mating frequency (Krupp et al., 2008). Given the important role of chemosensation during enrichment in the induction of subsequent plasticity, it is possible that interactions via chemical cues comprise an important component of

the social interactions that induce plastic changes in the brain. Further studies are needed to better characterize the neural circuits that are affected by pheromonal communication and the behavioral consequences of these signals on sleep.

In conclusion, these studies provide a basis for further examining the role of synaptic plasticity in sleep using *Drosophila*. Modulation of synaptic connections during sleep is necessary for the consolidation of long-term memories in the fly and seems to downscale synaptic connections that are potentiated by waking experience. Currently, however, it remains unclear what the functional benefits of sleep-related plasticity are. A recent hypothesis has suggested that synaptic downscaling during sleep may act to prevent the saturation of synaptic connections and to reduce the metabolic requirements of neurological functioning (Tononi and Cirelli, 2003), but these predictions have not yet been directly tested. Although our studies have not yet fully addressed these issues, they provide an experimental framework upon which hypothesis relating to the role of sleep in synaptic plasticity may be more directly examined.

References

Aton S, Seibt J, Dumoulin M, Coleman T, Shiraishi M, Frank M, Manzoni O (2009) The Sedating Antidepressant Trazodone Impairs Sleep-Dependent Cortical Plasticity. PLoS ONE 4:e6078.

Branson K, Robie AA, Bender J, Perona P, Dickinson MH (2009) High-throughput ethomics in large groups of Drosophila. Nat Meth.

Cottone LA, Adamo D, Squires NK (2004) The effect of unilateral somatosensory stimulation on hemispheric asymmetries during slow wave sleep. Sleep 27:63-68.

Dankert H, Wang L, Hoopfer E, Anderson DJ, Perona P (2009) Automated monitoring and analysis of social behavior in Drosophila. Nat Meth:1-7.

Donlea JM, Ramanan N, Shaw PJ (2009) Use-dependent plasticity in clock neurons regulates sleep need in Drosophila. Science 324:105-108.

Ferveur JF (2005) Cuticular hydrocarbons: their evolution and roles in Drosophila pheromonal communication. Behav Genet 35:279-295.

Frank MG, Issa NP, Stryker MP (2001) Sleep enhances plasticity in the developing visual cortex. Neuron 30:275-287.

Gilestro GF, Tononi G, Cirelli C (2009) Widespread changes in synaptic markers as a function of sleep and wakefulness in Drosophila. Science 324:109-112.

Graves LA, Heller EA, Pack AI, Abel T (2003) Sleep deprivation selectively impairs memory consolidation for contextual fear conditioning. Learn Mem 10:168-176.

Huber R, Ghilardi MF, Massimini M, Tononi G (2004) Local sleep and learning. Nature 430:78-81.

Huber R, Esser SK, Ferrarelli F, Massimini M, Peterson MJ, Tononi G (2007) TMS-Induced Cortical Potentiation during Wakefulness Locally Increases Slow Wave Activity during Sleep. PLoS ONE 2:e276.

Iwasaki N, Karashima A, Tamakawa Y, Katayama N, Nakao M (2004) Sleep EEG dynamics in rat barrel cortex associated with sensory deprivation. Neuroreport 15:2681-2684.

Kattler H, Dijk DJ, Borbély AA (1994) Effect of unilateral somatosensory stimulation prior to sleep on the sleep EEG in humans. Journal of sleep research 3:159-164.

Kent C, Azanchi R, Smith B, Formosa A, Levine JD (2008) Social Context Influences Chemical Communication in D. melanogaster Males. Curr Biol.

Krupp J, Kent C, Billeter J, Azanchi R, So A, Schonfeld J, Smith B, Lucas C, Levine JD (2008) Social Experience Modifies Pheromone Expression and Mating Behavior in Male Drosophila melanogaster. Curr Biol.

Lyamin OI, Manger PR, Ridgway SH, Mukhametov LM, Siegel JM (2008) Cetacean sleep: an unusual form of mammalian sleep. Neuroscience and biobehavioral reviews 32:1451-1484.

Miyamoto H, Katagiri H, Hensch T (2003) Experience-dependent slow-wave sleep development. Nat Neurosci 6:553-554.

Robin C, Daborn PJ, Hoffmann AA (2007) Fighting fly genes. Trends Genet 23:51-54.

Stickgold R, Walker MP (2005) Memory consolidation and reconsolidation: what is the role of sleep? Trends Neurosci 28:408-415.

Tononi G, Cirelli C (2003) Sleep and synaptic homeostasis: a hypothesis. Brain Res Bull 62:143-150.

Vecsey CG, Baillie GS, Jaganath D, Havekes R, Daniels A, Wimmer M, Huang T, Brown KM, Li X-Y, Descalzi G, Kim SS, Chen T, Shang Y-Z, Zhuo M, Houslay MD, Abel T (2009) Sleep deprivation impairs cAMP signalling in the hippocampus. Nature 461:1122-1125.

Villella A, Hall JC (2008) Neurogenetics of courtship and mating in Drosophila. Adv Genet 62:67-184.

Vyazovskiy V, Borbély AA, Tobler I (2000) Unilateral vibrissae stimulation during waking induces interhemispheric EEG asymmetry during subsequent sleep in the rat. Journal of sleep research 9:367-371.

Walker MP, Stickgold RJ, Alsop D, Gaab N, Schlaug G (2005) Sleep-dependent motor memory plasticity in the human brain. Neuroscience 133:911-917.

Yasuda T, Yasuda K, Brown RA, Krueger JM (2005) State-dependent effects of light-dark cycle on somatosensory and visual cortex EEG in rats. Am J Physiol Regul Integr Comp Physiol 289:R1083-1089.

Appendix 1:

foraging alters resilience/vulnerability to sleep disruption and starvation in *Drosophila*

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Abstract

Recent human studies suggest that genetic polymorphisms may allow an individual to maintain optimal cognitive functioning during sleep deprivation. If such polymorphisms were not associated with additional costs, it is likely that selective pressures would allow these alleles to spread through the population such that an evolutionary alternative to sleep would emerge. To determine whether there are indeed costs associated with resiliency to sleep loss, we challenged natural allelic variants of the *foraging* gene (*for*), which results in altered levels of *Protein kinase G* (*Pkg*), with either sleep deprivation or starvation. Flies with high levels of *PKG* (for^R) do not display deficits in short-term memory following 12 h of sleep deprivation. However, short-term memory is significantly disrupted when for^R flies are starved overnight. In contrast, flies with low levels of PKG (for^S, for^{S2}) show substantial deficits in short-term memory following sleep deprivation but retain their ability to learn after 12 h of starvation. Importantly, for^R flies which initially exhibit enhanced waking in response to starvation die more rapidly when starved than do for^{s2} flies that initially respond to starvation with an increase in sleep. Finally, we found that for^R phenotypes cold be largely recapitulated in for^S flies by selectively increasing the level of PKG in the alpha/beta lobes of the mushroom bodies, a structure known to regulate both sleep and memory. Together these data indicate that while the expression of *for* may appear to provide resilience in one environmental context; it may confer an unexpected vulnerability in other situations. Understanding how these trade-offs confer resilience or vulnerability to specific environmental

challenges may provide additional clues as to why no evolutionary alternative to sleep has emerged.

Introduction

Although sleep is a behavioral state that is conserved across a diverse range of species, the biological functions of sleep remain unknown. Sleep deprivation has been shown to negatively impact cognition, but individual responses to sleep loss can vary significantly within a population (VAN DONGEN *et al.* 2004; VAN DONGEN *et al.* 2005). Recent studies suggest that a portion of this variability may be influenced by genetic factors. For example, polymorphisms for PERIOD 3 (*PER3*), a circadian clock gene, can predict the magnitude of cognitive impairment and sleep homeostasis in response to a night of sleep deprivation in humans (VIOLA *et al.* 2007). While these genetic contributions may attenuate impairments following sleep deprivation, the tradeoffs that may be associated with resistance to sleep loss remain unknown. Presumably, the potential costs must be substantial. That is, if a natural occurring polymorphism could protect an individual from sleep loss with no adverse consequences, it is likely that selective pressures would allow this allele to spread through the population and an evolutionary alternative to sleep would emerge. Sleep, however, remains widespread throughout the animal kingdom (SIEGEL 2005). As a result, it is likely that the price of protection from sleep loss that can be conferred by a specific polymorphism may also induce a cost when manifest in a different environmental context. To date, putative costs of resiliency to sleep loss have not been identified in humans or any model organism.

foraging (*for*), which codes for *Protein Kinase G* (*PKG*), is maintained in wild-type populations as a genetic polymorphism that results in either high or low levels of *PKG* activity(OSBORNE *et al.* 1997). The allele associated with higher levels of PKG ("rover", for^R) results in flies with longer foraging trails between food patches, while the allele associated with lower levels of PKG ("sitter", for^S) results in flies with shorter foraging trails. Different foraging patterns are beneficial in discrete situations so neither allele has achieved a consistent advantage and consequently both have persisted over time (FITZPATRICK *et al.* 2007). Interestingly, *for* is highly pleiotropic and is known to influence many behaviors in multiple species (SOKOLOWSKI 2010) including sleep (LANGMESSER *et al.* 2009; RAIZEN *et al.* 2008a) and learning and memory (HENDEL *et al.* 2005; MERY *et al.* 2007; WANG *et al.* 2008) to name only a few. With respect to learning and memory, recent studies have shown that for^R flies perform better on short-term memory tasks than for^S flies, while for^S flies have better long-term memory acquisition (6). These differences suggests that the *for* alleles may confer strikingly different strategies for survival, with clear advantages and disadvantages in distinct environments (MERY *et al.* 2007; PAPAJ and SNELL-ROOD 2007).

Sleep deprivation is known to result in robust cognitive impairments in humans(CHUAH *et al.* 2006; FREY *et al.* 2004), rodents (GRAVES *et al.* 2003; PALCHYKOVA *et al.* 2006), bees (HUSSAINI *et al.* 2009) and flies (BUSHEY *et al.* 2007; GANGULY-FITZGERALD *et al.* 2006; LI *et al.* 2009; SEUGNET *et al.* 2009c;

SEUGNET *et al.* 2008b). However, the extent to which prolonged waking will result in cognitive impairments is strongly influenced by the environmental context. For example, while starvation is known to induce wakefulness in many animals (BORBELY 1977; DANGUIR and NICOLAIDIS 1979; FARADJI *et al.* 1979; RASHOTTE *et al.* 1998; WILLIE *et al.* 2001), including flies (KEENE *et al.* 2010; THIMGAN *et al.* In Press), recent studies from our lab indicate that wakefulness induced by starvation is not accompanied by cognitive impairments (THIMGAN *et al.* In Press). Given that *foraging* has been implicated in memory and sleep as well as energy storage and responses to food deprivation it is likely that the naturally existing *foraging* polymorphisms will differ in their ability to maintain cognitive functioning during sleep loss. Indeed, a recent study has reported that *foraging* alters the amount of waking observed during starvation (KEENE *et al.* 2010). However, neither sleep homeostasis, survival, nor cognitive behavior were evaluated in for^R and for^S flies following starvation. As a consequence, it remains unclear whether the alternate waking-strategies exhibited by for^R and for^S flies result in functional outcomes that may provide a selective advantage or disadvantage during food loss. Since the physiological demands of sleep deprivation are likely to differ from those observed during starvation, it is unlikely that the adaptations that are beneficial in one environment will be effective in the other. Thus, we hypothesized that behavioral responses of *foraging* alleles that may confer an advantage to sleep deprivation would be deleterious during starvation.

Results

We hypothesized that polymorphisms in *foraging* would influence the response to sleep deprivation as measured by both sleep homeostasis, the increase in sleep seen following sleep loss, and short-term memory. Since diet strongly modulates the behavior of the *foraging* alleles, we first asked whether for^R flies would sleep significantly longer than for^{S2} mutants when tested under our laboratory conditions as described previously (RAIZEN *et al.* 2008). As seen in supplementary Figure S1, under our dietary conditions for^R flies sleep significantly longer than for^{S2} mutants. Surprisingly, total sleep time in for^S flies, which have intermediate levels of PKG, is statistically identical to that seen in for^R flies (Fig. S1). Thus, it appears that the levels of *foraging* that are required to increase total sleep time is lower than that observed in for^R flies.

Next, we exposed for^R, for^S and for^{S2} flies to 12 hours of sleep deprivation during their primary sleep period using the Sleep Nullifying Apparatus (SNAP). As seen in Figure 1A, for^R flies did not compensate for lost sleep during 48 h recovery while both for^S flies and for^{S2} mutants displayed a wild-type sleep rebound. The lack of a homeostatic response seen in for^R flies may represent either an adaptation that allows animals to better withstand the negative effects of waking, or it may indicate that *foraging* disrupts regulatory processes thereby preventing flies from obtaining needed sleep. Since deficits in short-term memory are a robust consequence of sleep loss (LI *et al.* 2009; SEUGNET *et al.* 2009c; SEUGNET *et al.* 2008b), we evaluated performance in the APS in for^R, for^S and for^{S2} flies

following 12 hours of sleep deprivation. In the APS, flies are individually placed in a T-maze and allowed to choose between a lighted and darkened chamber (LE BOURG and BUECHER 2002; SEUGNET *et al.* 2009a). During 16 trials, flies learn to avoid the lighted chamber that is paired with an aversive stimulus (quinine/humidity). The performance index is calculated as the percentage of times the fly chooses the dark vial during the last 4 trials of the 16 trial test (SEUGNET *et al.* 2009b; SEUGNET *et al.* 2008b). As seen in Figure 1B, for^{R} flies maintain their ability to learn following sleep deprivation while for^{S} flies are significantly impaired; for^{S2} mutants showed impaired performance in the APS both under baseline conditions and following sleep deprivation. for^{R}, for^{S} and for^{S2} flies did not differ in sensory thresholds as measured by either the Photosensitivity Index (PI-percentage of photopositive choices in 10 trials in the absence of quinine) or the Quinine Sensitivity Index (QSI-time in seconds flies reside on the non-quinine side of a chamber). Together, these data indicate that for^{R} flies are resistant to sleep deprivation, while for^{S} and for^{S2} flies remain vulnerable to the negative effects of extended waking.

The deleterious effects of waking are absent when waking is induced by starvation (THIMGAN *et al.* In Press). Given that *foraging* alters the response to food deprivation, we hypothesized that for^{R}, for^{S} and for^{S2} mutants would show different vulnerabilities to starvation. As seen in Figure 1C, when for^{R} flies are placed into recording tubes with agar and water (starvation) they exhibit an immediate and sustained increase in waking behavior and show no evidence of a

sleep rebound when placed back on to their standard diet 12 h later. Interestingly, while the wake-promoting effects of starvation are absent in *for*^{S} flies as previously described (KEENE *et al.* 2010), *for*^{S2} mutants respond to starvation with a significant increase in sleep (Figure 2C). Thus, *for*^{R} flies exhibit a seemingly wild-type response to starvation (THIMGAN *et al.* In Press). If *for*^{R} flies are resistant to the negative effects of waking, they should maintain their ability to learn in the APS as they did following sleep deprivation. However, *for*^{R} flies display impaired short-term memory following waking induced by starvation (Figure 1D). Surprisingly, *for*^{S2} mutants, which exhibit impaired short-term memory both under baseline conditions and after sleep deprivation, recover their ability to learn when starved. Thus, *for*^{S2} mutants sleep more and display normal cognitive behavior following starvation while *for*^{R} flies display an unexpected vulnerability in short-term memory when waking is induced by the absence of food.

Although waking up to forage during starvation would enhance the opportunity to find food it requires additional energy expenditure. In contrast, sleeping would minimize the ability to find food but would likely conserve energy. Thus, we asked whether the alternate behavioral strategies exhibited by *for*^{R} and *for*^{S2} mutants would be associated with changes in survival during starvation. The average difference in the LD50 in hours to starvation between *for*^{R} and *for*^{S2} mutants was 10.25±3.19 (p=.01, one-sample t-test, n=4 replicates); a representative example of survival during starvation is shown in Fig. 1E. Interestingly, *for*^{S2} mutants live

longer during starvation than *for*^{R} flies despite having significantly lower triglyceride stores (Fig. 1F). Thus, while *for*^{R} flies appear resistant to the behavioral consequences of sleep deprivation, *for*^{S} and *for*^{S2} flies appear more suited to withstand the challenge of overnight starvation.

Given that sleep plays a role in memory consolidation (WALKER and STICKGOLD 2006) and that *foraging* polymorphisms have been shown to independently alter both sleep and memory (MERY *et al.* 2007; RAIZEN *et al.* 2008a) we examined the relationship between sleep and plasticity in *for*^{R}, *for*^{S} and *for*^{S2} flies. Previous studies have shown that enriched social environments impact the number synapses in mammals and flies and that these changes are associated with alterations in sleep (BALLING *et al.* 1987; DONLEA and SHAW 2009; VAN PRAAG *et al.* 2000). Thus, we evaluated sleep in *for*^{R}, *for*^{S} and *for*^{S2} flies after they had been exposed to either social enrichment, which consists of ~60 flies maintained in a 50mL vial, or social isolation, which consists of flies being housed individually in Trikinetics tubes, for 5 days (DONLEA *et al.* 2009; GANGULY-FITZGERALD *et al.* 2006). Surprisingly, neither *for*^{S} nor *for*^{S2}, which have normal LTM using olfactory conditioning (MERY *et al.* 2007), display an increase in sleep following social enrichment (Fig 2A). In contrast, *for*^{R} flies, which have impaired LTM using olfactory conditioning, maintain behavioral plasticity following social enrichment (Fig. 2A). Together, these data suggest that *foraging* may play a unique role when plasticity is induced in a social context.

To test this hypothesis, we evaluated LTM in male flies using a spaced training protocol in a courtship conditioning assay that results in decreased courtship behavior for at least 48 hours after training (DONLEA *et al.* 2009; GANGULY-FITZGERALD *et al.* 2006). As seen in Fig. 2B, for^R and for^S flies display a significant reduction in courtship 48 h following spaced training (T) compared to their naïve siblings (N) indicating that they developed LTM. In contrast, for^{S2} males show no reduction in courtship indicating that they have impaired memory consolidation (Fig. 2C, right). Note that while naïve courtship was low in for^S males, it was not so low as to preclude the development of LTM. Moreover, naïve courtship was also low in for^{S2} mutants that are in the same genetic background as for^R. Thus, it is likely that the reduced level of naïve courtship is due to the *foraging* polymorphism and not due to genetic background. The observation that for^R flies show both long-term behavioral plasticity in response to social enrichment and LTM following courtship conditioning suggests that *foraging* may be particularly relevant for plasticity induced in a social context.

We have previously shown that sleep is increased following courtship conditioning in wild-type flies and that LTM is disrupted if flies are sleep deprived immediately following training (GANGULY-FITZGERALD *et al.* 2006). As seen in Fig 2C, male for^R flies sleep significantly more following spaced training than their naïve siblings. Similarly, for^{S2} mutants, which did not develop LTM, did not increase their sleep following training consistent with previous reports that courtship behavior in the absence of LTM formation does not alter sleep

(GANGULY-FITZGERALD *et al.* 2006). Given that *for*^{S} flies developed an LTM, it is unclear why they did not show an increase in post-training sleep (Fig. 2B). However, one explanation may be that the changes in behavior were too small to effectively induce changes in sleep. We next asked whether post-training sleep deprivation would disrupt memory consolidation in *for*^{R} and *for*^{S} flies. Interestingly, 4 h of sleep deprivation immediately following spaced training did not disrupt LTM in *for*^{R} flies while LTM was disrupted in *for*^{S} flies. Thus, *foraging* appears to allow memory consolidation to proceed in the absence of sleep.

Given that the MBs modulate both sleep and memory (JOINER *et al.* 2006; MCBRIDE *et al.* 2000; PITMAN *et al.* 2006; SEUGNET *et al.* 2008b), we hypothesized that *for* signaling in the MBs would phenocopy *for*^{R} and confer resistance to sleep deprivation. Sleep homeostasis and performance in the APS were evaluated following 12 h of sleep deprivation in flies over-expressing *for* in the MBs of otherwise *for*^{S} homozygous background. As seen in figure 3A,B when *for* is overexpressed primarily in the alpha/beta lobes of the MB using the C739 or 30Y GAL4 drivers, sleep rebound is significantly attenuated. Thus, expressing *for* in the MBs recapitulates the sleep rebound phenotype observed in *for*^{R} flies. Surprisingly, overexpression of *for* using the 201Y GAL4 driver, which expresses predominantly in the gamma lobes and only weakly in the alpha/beta lobes, does not significantly alter sleep rebound (Fig. 3C). As mentioned above, a low sleep rebound could represent either an adaptation that allows animals to better withstand the negative effects of waking, or a disruption in regulatory processes

that prevent flies from obtaining needed sleep. Consistent with the *for*^{R} phenotype described above, overexpressing *for* using c739 or 30Y in an otherwise *for*^{S} background also prevented deficits in short-term memory following sleep deprivation (Fig. 3 D, E). No differences in sensory thresholds were observed between genotypes (Supplemental Table 1). Unfortunately, 201y*/+;for*^{S} control flies were altered in the APS under baseline conditions, such the effect of MB gamma lobe overexpression using 201y on short-term memory after sleep deprivation could not be assessed (data not shown). These data indicate that *for* activity in the Mushroom Bodies, particularly the MB alpha/beta lobes, recapitulates the sleep resistance phenotype observed in *for*^{R} flies.

A previous report indicates that *for*^{R} flies and flies overexpressing *for* within the MB using c739, 30y and 201y GAL4 drivers have impaired LTM following olfactory conditioning (MERY 2007). However, the data presented above indicate that *for*^{R} flies can generate normal LTM following courtship conditioning. To further define the role of for in LTM induced by courtship conditioning, we expressed *for* in the MBs of an otherwise *for*^{S} background. Consistent with the results reported for olfactory conditioning, expressing *for* in the MB alpha/beta lobes using C739 or 30y significantly disrupted LTM (Fig. 3F,G). However, in contrast to its effect on olfactory conditioning, expressing *for* using 201y did not disrupt LTM (Fig. 3 H). Once again, these data indicate that while the expression of *for* may appear to provide resilience in one environmental context (sleep deprivation); it may confer an unexpected vulnerability in other situations (LTM).

Finally, we asked whether *for* overexpression in the MBs would phenocopy the for^R response to starvation. Indeed, expressing *for* using either C739 or 30Y in an otherwise for^S background results in a for^R response to starvation (Fig. 4A,B). However, when *for* is expressed primarily in the gamma lobes using the 201y GAL4 driver the change in sleep during starvation does not differ from parental controls (Fig. 4C). Thus, expressing *for,* primarily in the alpha/beta lobes but not gamma lobes, recapitulates many of the for^R phenotypes. In for^R flies, the increased waking observed during starvation is associated with reduced survival. Thus, we asked whether the increased waking observed in starved c739 or 30Y flies overexpressing *for* would alter survival. As seen in figure 4D,E, survival was not altered during starvation when *for* was expressed using C739 or 30Y. These latter results may not be surprising when considering the observation that for^S flies are in a different genetic background and have substantially larger triglyceride stores than that seen in for^R flies (Fig. 1F). However, survival during starvation was increased when using 201y to express *for* in the gamma lobes (Fig. 4F). Although determining precisely how driving expression of for within the gamma lobes extends survival is beyond the scope of the current investigation, these data suggest that the MBs gamma lobes may play a role in controlling and/or responding to metabolic signals. In any event, these data show that the localized expression of *for* within the MBs can alter both short-term and long-term susceptibility to starvation.

Discussion

Our results show not only that the naturally-occurring *foraging* polymorphism modulates sleep homeostasis, but also demonstrates that resistance to sleep loss has an opposing effect on resistance to starvation. Importantly, these data indicate that for^R flies are resistant to the negative effects of waking as measured by the absence of a sleep rebound, and the ability both to retain intact STM and to consolidate LTM during sleep loss. Furthermore, the role of *foraging* can be largely recapitulated in a sitter background by expressing wild-type *foraging* in the alpha/beta lobes of the MBs. These results provide the first evidence that sensitivity to starvation may be an indirect cost of resiliency to sleep loss and that a natural polymorphism can alter sleep homeostasis in *Drosophila.*

While roles for the MBs in memory formation (HAN *et al.* 1992) and sleep (JOINER *et al.* 2006; PITMAN *et al.* 2006) have been well characterized, our data demonstrate that the MBs can also modulate behavioral responses to starvation in *Drosophila*. Specifically, the over-expression of *for* in the MB alpha-beta lobes of otherwise for^S flies causes flies to lose sleep during overnight starvation. Interestingly, over-expression of *for* primarily in the MB gamma lobes has no effect on the behavior of flies during overnight starvation, but prolongs the survival of chronically starved flies indicating a branch-specific role for the effects of *for* on responses to starvation. The neural mechanisms mediating these responses are not known, but the nutritional state of the animal may be relayed to the mushroom bodies through a cluster of dopaminergic neurons that receive

input from neurons expressing *Drosophila* neuropeptide F (NPF) (KRASHES *et al.* 2009). NPF is an ortholog to the mammalian neuropeptide Y, a regulator of feeding behavior (TATEMOTO *et al.* 1982). In the fly, elevated expression of NPF increases foraging behaviors in larvae (WU *et al.* 2003) and over-expression of an NPF receptor (*npfr1*) causes well-fed larvae to eat bitter food that they typically avoid unless they are food deprived (WU *et al.* 2005). Furthermore, Krashes et al., (2009) elegantly demonstrated that signaling from NPF-expressing neurons to *npfr1* in a single cluster of dopaminergic neurons, the MB-MP neurons, that project into the MBs can modulate the retrieval of appetitive associations, potentially by modulating the output of the MB alpha/beta and gamma lobes (KRASHES *et al.* 2009). Neuroanatomical analysis of these dopaminergic MB-MP cells indicates that they may send separate sets of projections into the heel of the MB and onto the MB alpha/beta lobe neurons (KRASHES *et al.* 2009), potentially providing branch-specific signals of nutrient availability into the MB alpha/beta and gamma lobes. Although components of the circadian clock have also been implicated in the regulation of sleep during starvation (KEENE *et al.* 2010), our findings suggest that nutrient sensing pathways signal may through the MBs to modulate survival strategies during food deprivation.

While the biological functions of sleep remain unknown, it has been proposed that sleep plays an important role in energy conservation. Although the amount of energy that is directly saved by sleeping remains controversial, it is possible

that patterns of neural activity during sleep allow for greater efficiency and reduced energy usage by neural circuits during subsequent waking. Indeed, recent data indicates that waking experience can result in an increase in the strength of synaptic connections in the brain during waking, and that subsequent sleep allows the brain to downscale synaptic strength (DONLEA *et al.* 2009; GILESTRO *et al.* 2009). Although the metabolic consequences of this type of potentiation are not known, our data indicate that for^R flies, which absorb nutrients more quickly and utilize both carbohydrates and lipids from the hemolymph more rapidly than for^S flies (KENT *et al*. 2009), are better equipped to maintain cognitive functioning following sleep deprivation. Importantly, previous data that *for* activity is significantly higher in the head than the rest of the body (BELAY *et al.* 2007) suggest that *for* signaling may be specifically suited to utilize energy stores for the metabolic needs of the brain. These results indicate that the elevated usage of energy stores that has been observed in for^R flies might have two effects; first, to provide sleep-deprived brains with an increased energy supply that can temporarily maintain proper neurobiological functioning despite elevated energy demands and, second, to more rapidly draw down energy stores during starvation. Indeed, studies from our lab indicate modulating lipid metabolism can alter the homeostatic response to sleep deprivation (THIMGAN *et al*, In Press). While the current understanding of the mechanisms that might connect localized *for* signaling in neural circuitry, including the MBs, to the utilization of energy stores elsewhere in the fly are largely unknown, future

investigations of these mechanisms may provide insight into the processes underlying the tradeoff between resistance to sleep loss and starvation.

Materials and Methods

Flies

The flies were cultured at 25C with 50-60% relative humidity and kept on a diet of yeast, dark corn syrup and agar under a 12-hour light:12-hour dark cycle.

Behavioral Analysis

Drosophila sleep and activity patterns were assessed as described previously(SHAW *et al.* 2000). In summary, flies were placed into individual 65 mm tubes and all activity was continuously measured through the Trikinetics *Drosophila* Activity Monitoring System (www.Trikinetics.com). Locomotor activity was measured in 1-minute bins and sleep was defined as periods of quiescence lasting at least 5 minutes.

Sleep Deprivation – 4-7 day old females were placed into individual 65 mm tubes and the sleep-nullifying apparatus (SNAP) was used to sleep deprive these flies for 12 hours during the dark phase (lights out to lights on) as previously described(SHAW *et al.* 2002). All sleep and activity was monitored using the Trikinetics *Drosophila* Activity Monitoring System. Sleep rebound was calculated by comparing sleep immediately post-deprivation to baseline sleep values collected immediately prior to sleep deprivation for each individual fly.

Overnight Starvation – 4 – 7 day old females were placed into individual 65 mm tubes and fed normal fly media for two days to obtain baseline. During overnight starvation, flies were transferred into individual tubes containing a 1% agar gel for 12 hours during the dark phase and returned to normal fly media the next morning at lights-on as previously described (THIMGAN *et al.* In Press). Locomotor activity was monitored throughout the experiment as described above. Cumulative sleep lost and then gained was calculated by comparing sleep during baseline to the starvation day and two subsequent recovery days.

Long-term Starvation – 4-7 day old females were placed into individual 65 mm tubes containing a 1% agar media. Locomotor activity was monitored as described above and the time of death was defined as the hour following the last recorded locomotor activity. The percent of flies alive was calculated by dividing the flies alive at that time-point by the total number of flies in the group.

APS Short-term memory – One-week-old female flies were placed into a T-maze paradigm as previously described(SEUGNET *et al.* 2008). In summary, the flies were forced to choose between a light and a dark tube. Flies are instinctively drawn to light; however, the lighted tube also contains a filter paper wetted with 10^{-1}M quinine hydrochloride solution, a highly aversive stimulus. Over 4 blocks of 4 trials, the number of times the fly chooses the dark tube is recorded. The performance index is defined as the number of times the fly chooses the dark

chamber in the last block of 4 trials. For learning experiments following a 12-hour sleep deprivation, the deprivation continued until the learning experiment was performed.

Courtship Conditioning – 4–6 day old males were trained as previously described(GANGULY-FITZGERALD *et al.* 2006). The males were exposed to pheremonally-feminized *Tai2* males in a spaced training protocol consisting of three one-hour training sessions, each separated by one hour. Forty-eight hours later, trained and naive males were exposed to *Tai2* males for a 10-minute testing period. The Courtship Index is defined as the percent of time that each subject fly spends in courtship behavior during the 10-minute testing period.

Social Enrichment - To standardize the environmental conditions during critical periods of brain development, all flies were collected upon eclosion and maintained in same-sex vials containing 30 flies for 3 days. 3-4 day old flies were divided into a socially isolated group, which were individually housed in 65-mm glass tubes, and a socially enriched group, consisting of 40-45 female flies housed in a single vial as previously described(GANGULY-FITZGERALD *et al.* 2006). After five days of social enrichment/isolation, flies were placed into clean 65-mm glass tubes and sleep was recorded for three days as described above. To calculate the mean and standard error for ΔSleep in the experimental group we first calculate the grand mean of the daytime sleep for the isolated group, averaged over three days, and then subtracted it from the average daytime sleep

observed for each individual socially-enriched sibling. The difference is referred to as ΔSleep.

Triglyceride Measurements – Ten females flies were collected 10 days post-eclosion, frozen and stored at -80^{o}C. Frozen samples were weighed and homogenized in a 2:1 solution of methanol:chloroform, then suspended in starting reagent for Infinity (ThermoElectron) triglyceride reagent. Triglyceride levels were detected using colorometric detection according to the manufacturer's specifications. Standard curves for known triglycerides were run in parallel and used to quantify the experimental lipid levels. Results are the average of three to six separated trials for each genotype.

Statistics - All comparison were done using a Student's T-test or, if appropriate, ANOVA and subsequent modified Bonferroni tests unless otherwise stated. All statistically different groups are defined as $p < 0.05$.

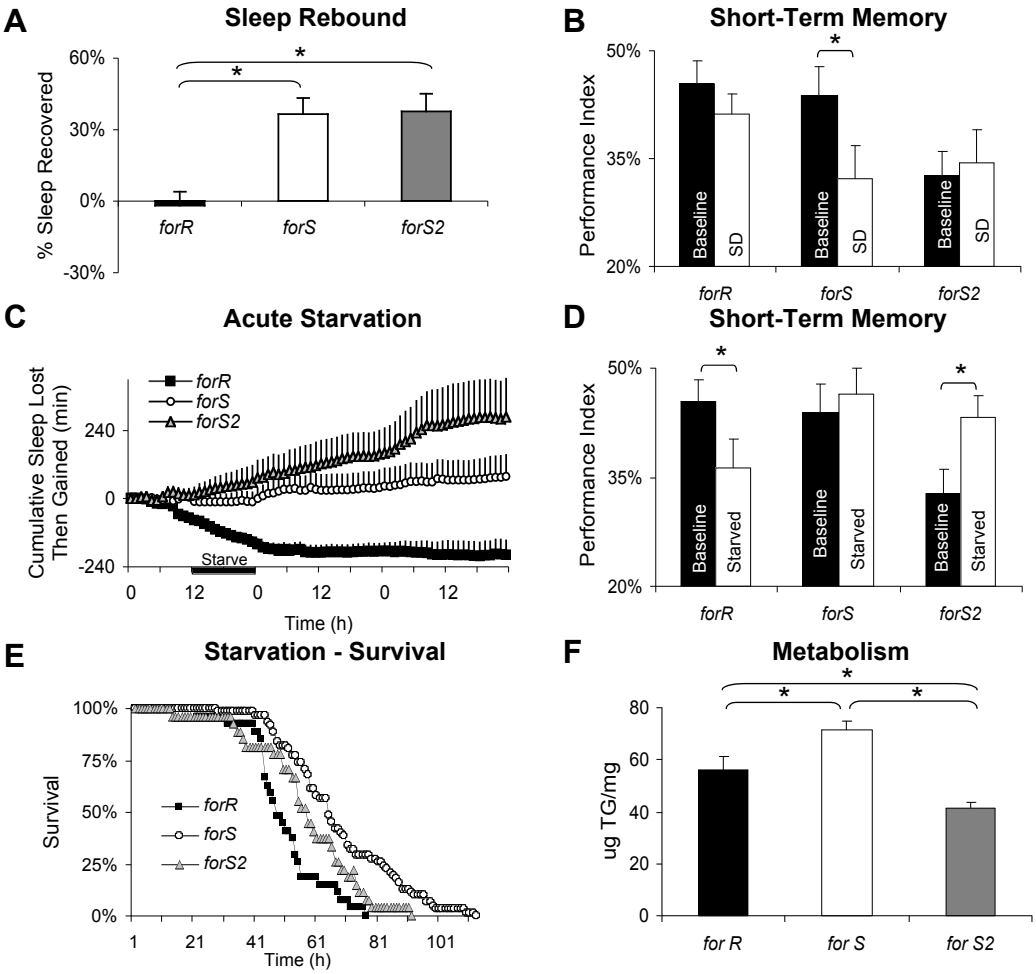


Figure 1 – *foraging* locus differentially confers resilience/vulnerability to sleep deprivation and starvation.

(A) *for*^{R} flies do not compensate for 12 hours of sleep deprivation with a subsequent increase in sleep (black bar) while both *sitters* (*for*^{S}, white) and *for*^{S2} mutants (gray) exhibit a wild-type sleep rebound. % sleep recovered is calculated for each individual as a ratio of the minutes of sleep gained above baseline during the 48 h of recovery divided by the total min of sleep lost during 12 h of sleep deprivation. One way ANOVA $F_{[2,139]}$=10.702, p=7.6x10^{-5}; n=45-52 each group; * p<0.05 modified Bonferroni Test. **(B)** Learning is impaired in *for*^{S} flies following 12 h of sleep deprivation while *for*^{S2} mutants display learning

impairments both during baseline and following sleep deprivation. In contrast, *for*^{R} flies maintain their ability to learn following sleep deprivation. The performance index for APS is calculated as the number of photonegative choices during the last 4 trials of a 16 trial test, a higher score indicates learning. Two way ANOVA reveals significant main effect for Genotype ($F_{[2,61]}$=4.065, p=0.022); n=10 each group; *p < 0.05 modified Bonferroni Test. **(C)** When placed into starvation prior to lights-out, *for*^{R} flies display significantly less sleep than during the previous baseline night and do not exhibit a sleep rebound when placed back onto food the following morning. Neither *for*^{S} flies nor *for*^{S2} mutants respond to starvation with an increase in waking. Cumulative sleep lost or gained during starvation; a negative slope indicates sleep lost, a positive slope indicates sleep gained; when the slope is zero recovery is complete. One way ANOVA $F_{[2,42]}$=12.253, p=1x10^{-18}, n=14-16 each group. **(D)** Short-term memory is impaired in *for*^{R} flies when waking is induced by starvation. *for*^{S} flies, which do not lose sleep, maintain baseline learning following a night of starvation. Surprisingly, *for*^{S2} mutants learn following a night of starvation. Two way ANOVA reveals significant Genotype X Condition interaction ($F_{[2,59]}$ = 4.552, p = 0.015, n = 10 in each group). * p < 0.05 modified Bonferroni Test. **(E)** *for*^{S} mutants survive longer than *for*^{R} and *for*^{S2} flies during chronic starvation (n>27/group). **(F)** *for*^{R} flies have lower organismal triglyceride levels than sitter *for*^{S} flies, but higher than *for*^{S2} mutants. One way ANOVA $F_{[2,9]}$ = 17.036, p = 0.001 *p < 0.05 modified Bonferroni test. Data are presented as mean ± SEM.

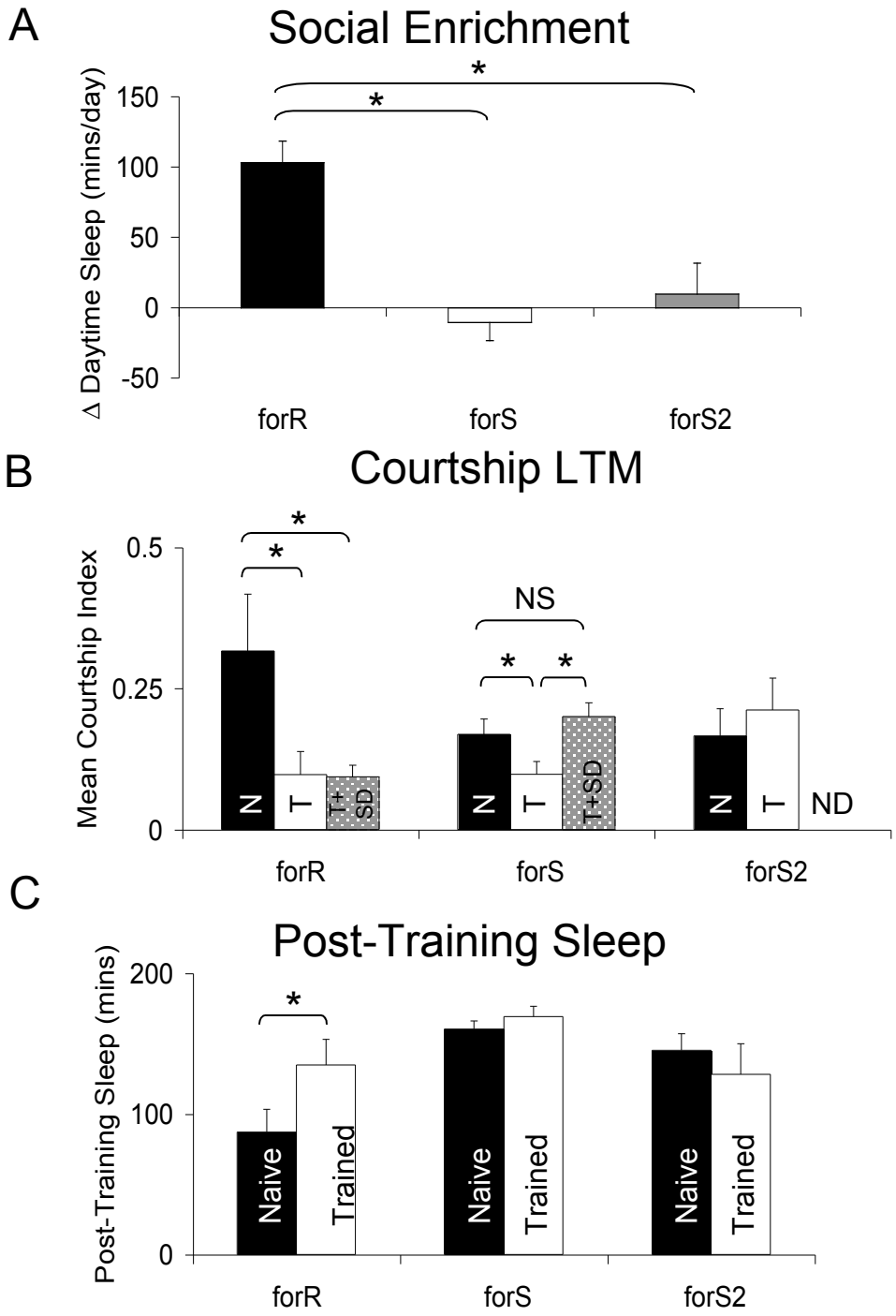


Figure 2 – Sleep deprivation does not prevent consolidation of long-term memory in *for*^{R} flies.

(A) *for*^{R} females exhibit a significant increase in sleep following social enrichment relative to isolated siblings while *for*^{S} and *for*^{S2} females show no change in sleep

following social enrichment. One-way ANOVA ($F_{[2,44]}$ = 12.180, p = $6.18x10^{-5}$, n=15-16/group). * p<0.05 modified Bonferroni test. **(B)** Following spaced training for courtship conditioning, for^{R} males sleep significantly more than their naïve siblings; *p=0.03. In contrast, neither for^{S} nor for^{S2} males display an increase in sleep following training. **(C)** for^{R} flies show reduced courtship 48 hours after a spaced training protocol (Trained, T) compared to Naïve (N) siblings indicating intact long-term memory (LTM). Sleep deprivation (T+SD) for 4 h immediately following training does not alter LTM; One-way ANOVA $F_{[2,48]}$=6.927, p=0.002, *p<.01 modified Bonferroni Test. Trained for^{S} flies also exhibit reduced courtship 48 hours after training however no reduction in courtship is seen when for^{S} flies are exposed to 4 h of sleep deprivation immediately following training; One-way ANOVA $F_{[2,77]}$=4.551, p=0.014, *p<.05 modified Bonferroni Test. for^{S2} mutants show no change in courtship 48 hours after training indicating a failure to develop LTM; courtship was not evaluated in for^{S2} flies following SD (ND) (independent t-test, p=.55) . Data are presented as mean ± SEM

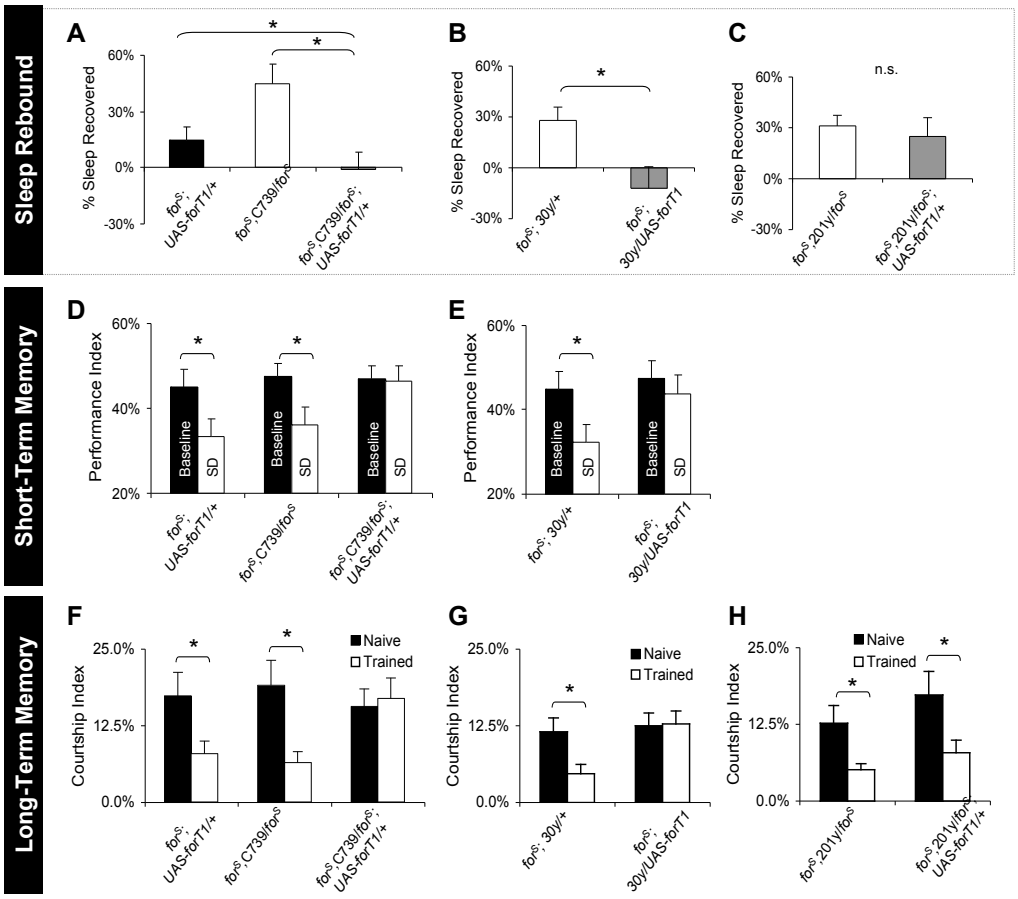


Figure 3 – Over expression of *foraging* in the MB alpha and beta lobes confers resilience to sleep deprivation but disrupts LTM formation.

(A-C) When *UAS-for* is expressed in the MB alpha and beta lobes using c739-GAL4/*forS* and 30y-GAL4/*forS* drivers (*w; for^S,c739/for^S;UAS-for/+* (A) and *w;for^S;30y/UAS-for* (B)) no sleep rebound is observed following 12 h of sleep deprivation while the parental lines (*w;for^S,c739/for^S*, *w;for^S;UAS-for/+* and *w;for^S;30y/+*) display a normal sleep rebound. In contrast, flies expressing *UAS-for* in the MB gamma lobes (*w;for^S,201y/for^S;UAS-for* (C)) and their parental controls (*w;for^S,201y/for^S*) exhibit a wild-type response to sleep deprivation; One way ANOVA $F_{[6,90]}$=5.841, p=3.5 x10^{-5}, n=11-16/group, *p<0.05 modified Bonferroni test. **(D-E)** As expected, *w;for^S,c739/for^S*, *w;for^S;UAS-for/+* and *w;*

for^{S}; *30y* parental lines display significant reductions in performance in the APS following 12 h of sleep deprivation while both *w; for*^{S}*,c739/for*^{S}*; UAS-for/+* (D) and *w; for*^{S}*; 30y/UAS-for* (E) flies retain short-term memory following sleep deprivation; One way ANOVA, $F_{[1,66]}$=8.866, p=0.004, n=6-9/group, * p < 0.05 modified Bonferroni test. **(F-H)** Courtship conditioning fails to induce LTM in flies expressing UAS-*for* in the MB alpha and beta lobes (*w; for*^{S}*,c739/for*^{S}*; UAS-for/+* (F) and *w; for*^{S}*; 30y/UAS-for* (G)) but is evident in the parental lines as indicated by reduced courtship 48 h post-training. Expressing *UAS-for* in MB gamma lobes does not alter LTM (*w*;*for*^{S},*201y*/*for*^{S};*UAS-for*/+, (H)); Two way ANOVA reveals significant Genotype X Condition interaction, $F_{[6,194]}$ = 16.638, p=1.4 x10^{-11}, * p < 0.05 modified Bonferroni Test. Data are presented as mean ± SEM.

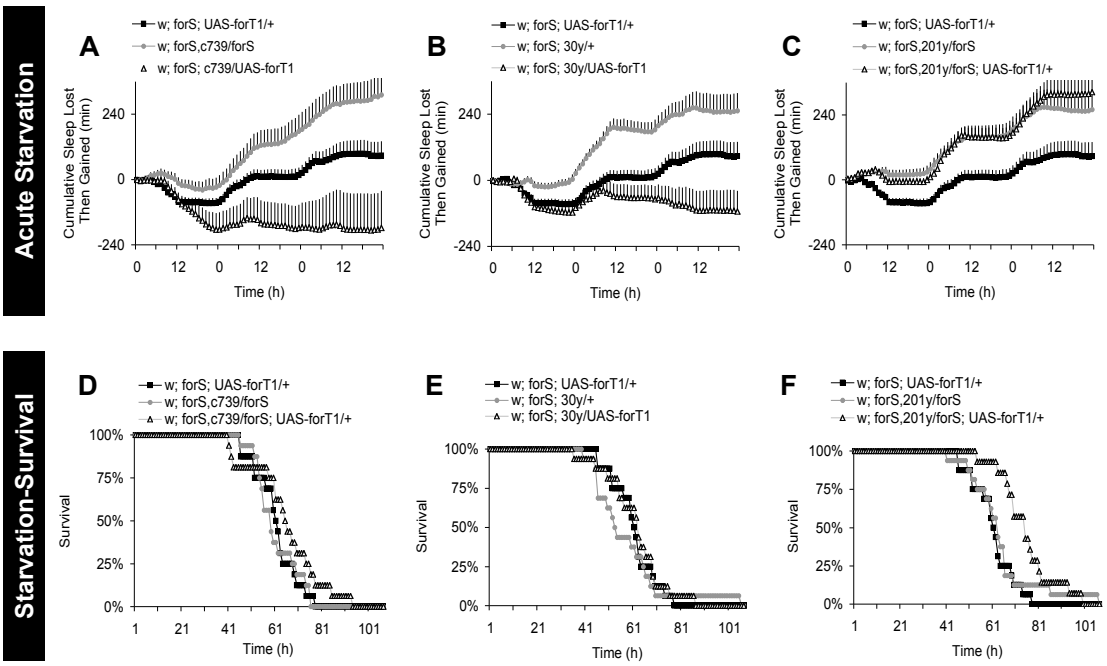
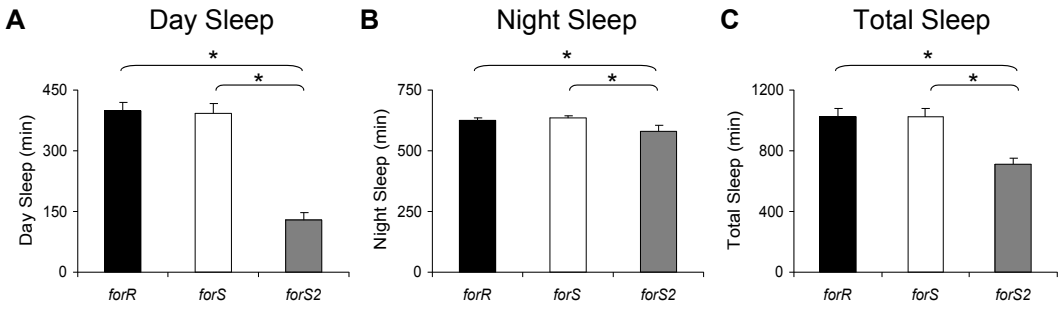


Figure 4 – *for* overexpression in the MBs alters response to starvation.

(A-C) When *UAS-for* is expressed in the MB alpha and beta lobes using c739-GAL4/*for*^{S} (A) and 30y-GAL4/*for*^{S} (B) (*w; for*^{S}*,c739/for*^{S}*;UAS-for/+* and *w; for*^{S}*; 30y/UAS-for*) flies exhibit a *for*^{R} like response to starvation while the parental lines (*w; for*^{S}*,c739/for*^{S}, *w; for*^{S}*; UAS-for/+* and *w; for*^{S}*; 30y/+*) retain the *for*^{S} phenotype. In contrast, flies expressing *UAS-for* in the MB gamma lobes (*w; for*^{S}*,201y/for*^{S}*;UAS-for/+*) and their parental controls (*w; for*^{S}*,201y/for*^{S}) exhibit a *for*^{S} response to starvation (C); repeated measures ANOVA reveals a significant Genotype X hour interaction for c739 ($F_{[142,3440]}$=12.523, p=1.0 x10^{-15}, n=14-24/group), 30y ($F_{[142,2982]}$=7.87, p=1.0 x10^{-15}, n=14-16/group) and 201y, respectively. Interaction contrasts failed to reveal a significant Genotype by hour interaction between *w; for*^{S}*,201y/for*^{S}*; UAS-for/+* and *w; for*^{S}*,201y/for*^{S} ($F_{[71,1775]}$=1.197, p=0.135, n=14-15/group). Data are presented as mean ± SEM.

(D-F) Survival during chronic starvation is not altered when *UAS-for* is expressed in the MB alpha/beta lobes (D-E) but is increased when *UAS-for* is expressed in the MB gamma lobes (F).



Supplemental Figure 1 – Polymorphisms at the *foraging* locus alter sleep in *Drosophila*.

(A) for^{S2} mutant show significantly less overall sleep than *rover* (for^{R}) or *sitter* (for^{S}) flies (One-way ANOVA $F_{[2,87]}=54.44$, $p=4.65x10^{-18}$, n=30/group, *p<.05 modified Bonferroni Test). **(B)** for^{S2} mutants show significantly less daytime sleep than *rover* or *sitter* flies (One-way ANOVA $F_{[2,87]}=3.07$, p=0.05, n=30/group, *p<.05 modified Bonferroni Test). **(C)** for^{S2} mutants show significantly less nighttime sleep than *rover* or *sitter* flies (One-way ANOVA $F_{[2,87]}=35.69$, $p=4.81x10^{-12}$, n=30/group, *p<.05 modified Bonferroni Test).

References

BELAY, A. T., R. SCHEINER, A. K. SO, S. J. DOUGLAS, M. CHAKABORTY-CHATTERJEE *et al.*, 2007 The foraging gene of Drosophila melanogaster: Spatial-expression analysis and sucrose responsiveness. J Comp Neurol **504:** 570-582.

DONLEA, J. M., N. RAMANAN and P. J. SHAW, 2009 Use-dependent plasticity in clock neurons regulates sleep need in Drosophila. Science **324:** 105-108.

FITZPATRICK, M. J., E. FEDER, L. ROWE and M. B. SOKOLOWSKI, 2007 Maintaining a behaviour polymorphism by frequency-dependent selection on a single gene. Nature **447:** 210-212.

GANGULY-FITZGERALD, I., J. M. DONLEA and P. J. SHAW, 2006 Waking experience affects sleep need in Drosophila. Science **313:** 1775-1781.

GILESTRO, G. F., G. TONONI and C. CIRELLI, 2009 Widespread changes in synaptic markers as a function of sleep and wakefulness in Drosophila. Science **324:** 109-112.

HAN, P. L., L. R. LEVIN, R. R. REED and R. L. DAVIS, 1992 Preferential expression of the Drosophila rutabaga gene in mushroom bodies, neural centers for learning in insects. Neuron **9:** 619-627.

HUSSAINI, S. A., L. BOGUSCH, T. LANDGRAF and R. MENZEL, 2009 Sleep deprivation affects extinction but not acquisition memory in honeybees. Learn Mem **16:** 698-705.

JOINER, W. J., A. CROCKER, B. H. WHITE and A. SEHGAL, 2006 Sleep in Drosophila is regulated by adult mushroom bodies. Nature **441:** 757-760.

KEENE, A. C., E. R. DUBOUÉ, D. M. MCDONALD, M. DUS, G. S. B. SUH *et al.*, 2010 Clock and cycle Limit Starvation-Induced Sleep Loss in Drosophila. Current Biology**:** 1-7.

KENT, C. F., T. DASKALCHUK, L. COOK, M. B. SOKOLOWSKI, and R. J. GREENSPAN, 2009 The Drosophila foraging gene mediates adult plasticity and gene-environment interactions in behaviour, metabolits, and gene expression in response to food deprivation. PLoS Genet **5:** e1000609.

KRASHES, M. J., S. DASGUPTA, A. VREEDE, B. WHITE, J. D. ARMSTRONG *et al.*, 2009 A neural circuit mechanism integrating motivational state with memory expression in Drosophila. Cell **139:** 416-427.

MERY, F., 2007 Aging and its differential effects on consolidated memory forms in Drosophila. Exp Gerontol **42:** 99-101.

MERY, F., A. T. BELAY, A. K.-C. SO, M. B. SOKOLOWSKI and T. J. KAWECKI, 2007 Natural polymorphism affecting learning and memory in Drosophila. Proc Natl Acad Sci USA **104:** 13051-13055.

OSBORNE, K. A., A. ROBICHON, E. BURGESS, S. BUTLAND, R. A. SHAW *et al.*, 1997 Natural behavior polymorphism due to a cGMP-dependent protein kinase of Drosophila. Science **277:** 834-836.

PITMAN, J. L., J. J. MCGILL, K. P. KEEGAN and R. ALLADA, 2006 A dynamic role for the mushroom bodies in promoting sleep in Drosophila. Nature **441:** 753-756.

RAIZEN, D. M., J. E. ZIMMERMAN, M. H. MAYCOCK, U. D. TA, Y. J. YOU *et al.*, 2008 Lethargus is a Caenorhabditis elegans sleep-like state. Nature.

SEUGNET, L., Y. SUZUKI, L. VINE, L. GOTTSCHALK and P. J. SHAW, 2008 D1 Receptor Activation in the Mushroom Bodies Rescues Sleep-Loss-Induced Learning Impairments in Drosophila. Curr. Biol.

SHAW, P. J., C. CIRELLI, R. J. GREENSPAN and G. TONONI, 2000 Correlates of sleep and waking in Drosophila melanogaster. Science **287:** 1834-1837.

SHAW, P. J., G. TONONI, R. J. GREENSPAN and D. F. ROBINSON, 2002 Stress response genes protect against lethal effects of sleep deprivation in Drosophila. Nature **417:** 287-291.

SIEGEL, J. M., 2005 Clues to the functions of mammalian sleep. Nature **437:** 1264-1271.

SOKOLOWSKI, M. B., 2010 Social interactions in "simple" model systems. Neuron **65:** 780-794.

TATEMOTO, K., M. CARLQUIST and V. MUTT, 1982 Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature **296:** 659-660.

THIMGAN, M., Y. SUZUKI, L. SEUGNET, L. GOTTSCHALK and P. J. SHAW, In Press Lipid Metabolism Genes Prevent Impairment Following Sleep Loss. PLoS Biology.

VIOLA, A. U., S. N. ARCHER, L. M. JAMES, J. A. GROEGER, J. C. LO *et al.*, 2007 PER3 Polymorphism Predicts Sleep Structure and Waking Performance. Curr. Biol.

WALKER, M. P., and R. STICKGOLD, 2006 Sleep, memory, and plasticity. Annu Rev Psychol **57:** 139-166.

WU, Q., T. WEN, G. LEE, J. H. PARK, H. N. CAI *et al.*, 2003 Developmental control of foraging and social behavior by the Drosophila neuropeptide Y-like system. Neuron **39:** 147-161.

WU, Q., Z. ZHAO and P. SHEN, 2005 Regulation of aversion to noxious food by Drosophila neuropeptide Y- and insulin-like systems. Nat Neurosci **8:** 1350-1355.