Drosophila Hyperkinetic mutants have reduced sleep and impaired memory

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Running title: Sleep and memory effects of *Hk* mutations

Sleep is thought to be important for health, cognition, and memory[1 2 3]. Recent work has shown that fruit flies share many features of mammalian sleep[4 5], including homeostatic regulation and performance deficits after prolonged wakefulness[6]. Recently, through a systematic mutagenesis screen, we identified short-sleeping *Drosophila melanogaster* lines that carry loss-of-function mutations in *Shaker (Sh* [7]. *Sh* codes for the alpha pore-forming subunit of a fast-inactivating voltage-dependent potassium channel[8]. Since the Shaker current is potentiated by a beta modulatory subunit coded by *Hyperkinetic (Hk)* [9], we asked whether *Hk* mutations also produce a sleep phenotype. Here we demonstrate that severe loss-of-function Hk^1 and Hk^Y mutants are short sleepers, and that they are restored to normal sleep by a functional Hk^+ transgene. Moreover, by using a heat-box spatial learning paradigm, we found that Hk^1 and Hk^Y lines also have a memory deficit. To clarify whether the short sleep phenotype and the memory impairment are associated, we then tested a weaker hypomorphic allele, Hk^2. Unlike, Hk^1 and Hk^Y flies, Hk^2 flies are normal sleepers and perform normally in the heat-box. We also compared short-sleeping *Sh* lines (Sh^{mns}, Sh^{102}, Sh^M, *Df(Sh)*) with normal sleeping ones (Sh^{120}, Sh^X) and found that only the former had impaired memory. Thus, both pore-forming and modulatory subunits of the Shaker complex strongly affect sleep duration. Moreover, short sleep is associated with poor memory.

Many features of sleep are shared between mammals and fruit flies. As in mammals, sleep in *Drosophila* consists of long periods of behavioral immobility with increased arousal threshold[4, 5], is associated with changes in brain electrical activity[10] and gene expression[5, 11], is reduced by caffeine and psicostimulants[5, 12, 13], and becomes fragmented with aging[14]. In both mammals and flies sleep is also homeostatically regulated, i.e. its duration and intensity increase with the duration of prior waking[6], and sleep deprivation results in deficit in performance[6].

In a recent study we found that Sh^{mns} flies, which carry a point mutation in a conserved *Sh* domain, sleep only 3-4 hours/day, while their wild-type controls sleep 8-14 hours/day[7]. After crossing out genetic modifiers accumulated over many generations, we found that other hypomorph or amorph *Sh* alleles become short sleepers, and fail to complement the short sleeping Sh^{mns} phenotype. The *Sh* locus encodes the alpha subunit of a tetrameric potassium channel that passes a voltage-activated fast-inactivating (I_A) current[8]. Homologous channels in vertebrates have similar properties[15] and, in both mammals and flies, play a major role in the control of membrane repolarization and transmitter release[8].

The beta subunit encoded by *Hk* binds to each alpha subunit in the tetramer (Fig.1a) and its presence increases both the kinetic speed and amplitude of the potassium current through the pore[9 16], whereas *Hk* loss-of-function mutations enhance neuronal excitability[17]. To test whether flies carrying severe *Hk* loss-of-function mutations would be short sleeping we backcrossed the Hk^1 and Hk^Y mutations into different genetic backgrounds (*wCS10, CS, w1118*), identified the mutant male or female progeny (Suppl. Fig.1a-c), and then compared their sleep to that of their wild-type siblings. Hk^- male flies of all strains spent 30 to 54% less time asleep than their wild-type siblings (Fig. 1b, Suppl. Fig.1d). Homozygous mutant females also slept less than their heterozygous siblings, while daily sleep amount did not differ between heterozygous and wild type

females (Fig. 1c; Suppl. Fig.1e), indicating that mutations in *Hk* act recessively to reduce sleep. As expected, the severity of the short sleeping phenotype depended on both gender and genetic background, being greater in the *w1118* strain for males and in the *CS* strain for females (Fig. 1).

The decrease in daily sleep amount was mainly due to a decrease in the duration of sleep episodes (Suppl. Fig. 2), and was positively correlated with the decrease in daily sleep amount in both males and females (Suppl. Fig. 3). During waking, both male and female mutant flies were more active than their wild-type siblings (Suppl. Fig.1f-g). However, the decrease in daily sleep amount was negatively correlated with the increase in waking locomotor activity in females, but not in males (Suppl. Fig. 3), suggesting that the effects of *Hk* mutations on sleep and locomotor activity can be dissociated.

To determine whether mutations in *Hk* are responsible for the short sleeping phenotype 3 sets of experiments were performed. Genetic mapping indicated that both the shaking phenotype and the short sleeping phenotype mapped to a locus between *cv* and *v*, which includes the *Hk* locus (Suppl. Fig. 4a-b). Moreover, complementation analysis showed that, like homozygous Hk^{1}/Hk^{1} and Hk^{Y}/Hk^{Y} flies, Hk^{1}/Hk^{Y} flies also slept significantly less than their heterozygous siblings, i.e. the 2 mutant alleles failed to complement each other (Fig. 1c). Finally, since *Hk* acts recessively to affect sleep, it was possible to cytologically map the short sleeping phenotype in females using 3 deficiencies that include the *Hk* locus. These deficiencies failed to complement Hk^{1} and Hk^{Y} for the short sleeping phenotype (Suppl. Fig. 5). Hk^{1} and Hk^{Y} are two independently isolated mutations, and it is unlikely that both of them carry a second mutation in the same gene, different from *Hk*, but close to the *Hk* locus. Thus, these data strongly suggest that mutations in *Hk* produce a short sleeping phenotype.

The wild-type $Hk^{17K\text{-}X94}$ transgene can rescue the shaking phenotype produced by either the Hk^{1} or the Hk^{Y} mutation[9]. We compared sleep in flies carrying a copy of $Hk^{17K\text{-}X94}$ in the presence of either a mutant or a wild-type copy of *Hk* (Fig. 2a). Male Hk^{-} mutants that inherited the transgene slept significantly more than their Hk^{-} siblings (Figure 2b-c), and their daily sleep amount did not consistently differ from that of wild-type siblings that inherited the balancer chromosome *FM7a* (Fig. 2c) or the chromosome with a wild-type Hk^{+} allele (compare Fig. 1b with Fig. 2c). Importantly, the transgene did not consistently increase daily sleep amount in flies that inherited a wild-type Hk^{+} allele (Fig. 2c). Thus, a wild-type *Hk* transgene specifically rescues the short sleeping phenotype caused by a mutation in *Hk*. Male Hk^{-} mutants that inherited $Hk^{17K\text{-}X94}$ may, or may not, show a decrease in locomotor activity (data not shown), suggesting that the effects of the transgene on sleep and motor activity can be dissociated.

When subjected to 24 hours of sleep deprivation all Hk^{-} and Hk^{+} flies lost > 90% of their baseline sleep, and showed an increase in sleep duration during the 24 hours following sleep deprivation (Suppl. Fig. 6). The amount of sleep recovered varied depending on genetic background, ranging from 10 to 80% of the amount of sleep lost during sleep deprivation, but in all cases the increase in sleep duration did not differ between each Hk^{-} mutant line and its wild-type siblings (Suppl. Fig. 6a). After sleep deprivation sleep in Hk^{-} flies became also more intense, as indicated by an increase in their arousal threshold, but again Hk^{-} flies and their wild-type siblings showed similar changes (Suppl. Fig. 6b). Finally, sleep deprived Hk^{-} flies were impaired in their ability to move away from a complex stimulus, and a similar deficit in the escape response was

also observed in their wild-type siblings (Suppl. Fig. 6c). Thus, the homeostatic regulation of sleep is preserved in *Hk*$^{-}$ flies. The short sleeper phenotype persisted under constant darkness, when sleep amounts were even lower than under light-dark conditions (Suppl. Fig. 7). Moreover, under constant darkness *Hk* flies maintained a rhythmic modulation of locomotor activity with a period of ~24 hours (Suppl. Fig. 7). Thus, *Hk* mutations affect the daily sleep amount but not the homeostatic or the circadian regulation of sleep.

Behavioral studies in humans have shown a link between sleep and memory[18]. We used the heat-box spatial learning paradigm[19 20 21] to assess learning and memory in *Hk*$^{-}$ mutants. In this paradigm, single flies are housed inside a dark chamber that, during several training sessions, can be quickly heated to an elevated temperature every time the fly runs to one-half of the chamber, and cools down as soon as the fly runs back. Training sessions alternate with testing periods when the whole box remains cool. During testing normal flies show a progressively greater preference for the previously non-heated side of the box, an indication of learning and, for some time afterwards, continue to avoid the previously punished side even after training stops, an indication of memory. During training Hk^{1} and Hk^{Y} mutants and their wild-type siblings were equally sensitive to the high temperature (39°C) and, as expected, spent most of the time in the non-heated side of the box (Fig. 3a-b, while bars). All flies also learned to prefer the non-heated side of the chamber (Fig. 3a-b, grey bars), with no differences between mutant flies and siblings (stat). After training, however, Hk^{1} and Hk^{Y} mutants lost their preference for the unpunished side more quickly than controls (Fig. 3a-b, black bars). We then tested flies carrying Hk^{2}, a weaker hypomorphic *Hk* allele[22], and found that they were normal sleepers relative to their wild-type siblings (values here; Suppl. Fig. 8). Hk^{2} flies showed both normal learning and memory in the heat-box (Fig. 3c). Since these results suggested a link between short sleeping phenotype and memory decay, we took advantage of the existence of both short-sleeping (Sh^{mus}, Sh^{102}, Sh^{M}, *Df(Sh)*) and normal sleeping mutant *Sh* lines (Sh^{120}, Sh^{X}), and tested them in the heat-box. We found that, consistent with the results for *Hk* lines, all short sleeping *Sh* lines showed a quicker memory decay than their wild-type siblings, while all normal sleeping *Sh* lines also performed normally in the heat-box (Fig. 4a-b). Spontaneous locomotor activity in the heat-box, instead, was similar in all lines (Fig. 4c)

These and previous data[7] indicate that voltage-dependent potassium channels play a key role in regulating sleep duration in flies. In mammals, there are at least 16 genes coding for Shaker or Shaker-like channels, and 4 genes coding for Kv beta regulatory subunits[23 24], and their functions differ depending on subunit composition, anatomical distribution, and electrophysiological properties[24]. Sleep duration is normal in *Kv3.2* knock-out mice[25], but reduced in *Kv.3.1/Kv3.3* knockout mice, whose inability to maintain consolidated sleep is most likely due to motor dysfunctions[26]. A promising candidate that needs to be tested is *Kv1.2*, which shows the strongest sequence homology with *Sh*, is highly expressed in the mammalian thalamocortical system[27], and has been implicated in the severe insomnia of a patient affected by Morvan's syndrome[28].

Of all *Hk*$^{-}$ and *Sh*$^{-}$ flies that we tested, only the short sleeping lines could not perform well in the heat-box, suggesting that their daily sleep amount is not enough to maintain a normal waking performance in this operant task. Ion channels mutations, however, are pleiotropic, and *Hk* mutations are no exception. Hk^{1} and Hk^{Y} flies, for

example, are not only short sleeping, but shake under ether anesthesia[22], and show an abnormal visually-induced jumping response[29]. It is likely that in addition to shaking and jumping, also the short sleeping phenotype is a consequence of an increase in neuronal excitability, because differences in neuronal excitability are at the core of the distinction between the sleeping and the awake brain. It is also likely that the hyperexcitability mediating each of these phenotype occurs in different neuronal circuits. Indeed, the shaking phenotype is known to depend on the motoneurons of the ventral thoracic ganglion[30], while the locus of the jumping response is in the central nervous system (CNS [31]). The *Drosophila* brain areas mediating the short sleeping phenotype remain unknown, but recent evidence suggests that the mushroom bodies could play an important role. The pleiotropy of *Hk* mutations raises the issue of whether the impaired memory retention of Hk^1 and Hk^Y flies could be due, rather than to insufficient sleep, to a generalized change in neuronal excitability in the CNS, or to a change specific to those CNS neuronal circuits involved in spatial learning. While it may be impossible to completely rule out this possibility (all 3 phenotypes are recessive), the fact that normal sleeping Hk^2 flies performed well but still showed the shaking and jumping phenotypes suggest that the impaired memory in the heat-box paradigm is more related to the reduced sleep amount than to other consequences of *Hk* mutations.

In conclusion, these molecular and genetic data add to the behavioral evidence in humans that sleep is important for performance, learning, and memory, and that the latter suffer when total acute sleep deprivation or chronic sleep restriction occur[32].

Acknowledgments
Supported by a grant from the United States Defense Advanced Research Projects Agency.

Figure legends

Figure 1. ***Mutations in Hk reduce sleep in male and female flies***. **a.** Schematic showing the Hyperkinetic (HK) beta modulatory unit attached on the cytoplasmic side to the Shaker pore (SH). A fast-inactivating voltage dependent potassium (K^+) current passes through the pore. **b-c.** Minutes of sleep over a 24-h period (mean ± SEM) for male (b) and female (c) flies. The number of tested flies is indicated below each column. *, $p<0.001$; **, $p<0.0001$ (unpaired Student's T-test).

Figure 2: ***A wild-type Hk transgene rescues the short sleeping phenotype.*** **a**. Crossing scheme. The *Hk17K-X94* transgenic construct includes the *w+* transgene, and therefore flies that inherited the transgene were identified based on eye color in a *w-* (*w1118* or *wCS10*) background. **b**. Daily time course (in 30-min intervals) of the amount of sleep in Hk^- males (black line), and in Hk^- males carrying the transgene (grey line). The color code is according to the scheme in panel a. **c**. Daily sleep amounts (mean ± SEM, min of sleep/24 hours) and activity index (number of beam crossings/min, during waking). The numbers of tested flies is indicated below each column. The color code is according to the scheme in panel a. *, $p<0.05$; **, $p<0.001$ (unpaired Student's T-test). The analysis included a FM7a balancer chromosome dominantly marked with *Bar1* (*B1*) in addition to recessive markers (*y31d sc8 wa vOf*). This FM7a balancer has a Hk^+ allele, since in female flies it does not produce a shaking phenotype in combination with either Hk^1 or Hk^Y. FM7a was selected among all the X-chromosome balancers tested because it does not significantly affect daily sleep amount in a *w1118* background.

Figure 3. ***Hk Mutations that reduce sleep also impairs performance in the heat-box learning paradigm.*** **a.** Performance in the heat-box for Hk^1, Hk^Y and Hk^2 as compared to their wild-type siblings. Performance index (PI) is the amount of time flies spend on one side divided by the total time. PI can range from -1 (all time is spent on the heated, punished side), to +1 (all time is spent on the non-heated side), while 0 indicates no preference for either side. Before the experiments temperature (T) sensitivity was tested in all lines and did not differ between mutant and wild-type siblings (see Methods). From left to right: the white bar represents the 10-min pre-training period when both sides of the chamber are kept at 22°C; the thick grey bars represent five 1-min periods of training (punished side is heated at 39°C, the other side remains at 22°C); the thin black bars represent 1-min periods of testing (both sides at 22°C). During training PI is a measure of T sensitivity, and did not differ between mutant and wild-type siblings (T sensitivity was also tested between 22 and 40°C before the experiments, and did not differ between lines). During testing PI measures learning (when testing periods alternate with training periods), or memory (after the training periods have ended). All flies learned to prefer the non-heated side of the chamber (* $p<0.05$, signrank test), with no differences between mutant flies and siblings. After training, however, short sleeping (Hk^1, Hk^Y), but not normal sleeping (Hk^2) flies lost their preference for the unpunished side more quickly. **b.** The extinction coefficient measures the speed at which flies lose their preference for the unpunished side, and was calculated by dividing PI for each fly by the total time the flies have a significant preference for that side: the bigger the extinction, the sooner the

preference is lost. The N of crossings is a measure of spontaneous locomotor activity in the 10-min pre-training period.

Figure 4. ***Only Sh mutations that reduce sleep also increase extinction in the heat-box learning paradigm.*** Extinction coefficient and N of crossings in short sleeping and normal sleeping *Sh* lines.

Supplementary Figure 1. a. ***Crossing scheme used to generate Hk^+ and Hk^- male flies***, which were separated based on the non-shaking and shaking phenotypes, respectively. **b.** Crossing scheme used to generate females in the *CS* background. Heterozygous females were crossed to Hk^- males to generate heterozygous or homozygous mutant female progeny, which were separated based on the non-shaking and shaking phenotypes, respectively. **c.** Crossing scheme used to generate the females in the w^{1118} background. The segregation away from 2 piggyBac insertions located in the intron downstream of the first *Hk* promoter, *PBac{XP}d01140* (this figure) and *PBac{RB} e00640* (not shown) was used to determine the genotype. Flies that inherit these insertions (Hk^+/*PBac*) have colored eyes (due to the presence of a w^+ transgene), do not shake, and show daily sleep amount and activity levels similar to those of siblings that inherit a wild-type chromosome (data not shown). Heterozygous females were crossed to Hk^- mutant males, and the white eyed homozygous mutant female progeny was compared to colored eyed heterozygous female siblings. Also, to determine whether Hk^- acts dominantly to reduce sleep, heterozygous females were crossed to Hk^+ males to produce heterozygous (*w1118* Hk^-/*w1118* Hk^+) female (grey bar), which were compared to homozygous Hk^+ (*w1118 PBac{XP}d01140/ w1118* Hk^+) females (grey slashed bar) siblings. **d-e.** Daily time course (in 30-min intervals) of the amount of sleep in Hk^+ and Hk^- male (d) and female (e) flies in different genetic backgrounds. Strains *wCS10, CS*, and *w1118* were chosen because they differ significantly in daily sleep amount, but none of them carries a single mutation that can account for this difference (presence or absence of a functional *w+* transgene does not affect daily sleep amount, data not shown). White and black bars under the x axis indicate the light and dark period, respectively. The number of tested flies is the same as in Fig. 1. **f-g** The activity index for males (f) and female (g) flies over a 24 h period (number of beam crossings/min, during waking). The numbers of tested flies is indicated below each column. *, $p<0.05$; **, $p<0.001$ (unpaired Student's T-test).

Supplementary Figure 2. ***The effects of Hk mutations on sleep and waking parameters depend on strain and gender.*** Duration (in min) and number of sleep and waking episodes over a 24 h period (mean ± SEM), in male (**a**) and female (**b**) flies (same flies shown in Fig. 1). *, $p<0.05$; **, $p< 0.001$; (Student's T-test).

Supplementary Figure 3. ***The decrease in sleep amount is positively correlated with the decrease in the duration of sleep episodes.*** On the Y axis Δ indicates the difference, in the indicated variable, between wild-type and mutant sibling. The X axis shows the difference in sleep (min/24 h) between wild-type and mutant sibling.

Supplementary Figure 4a. ***Genetically mapping of the short sleeping phenotype associated with Hk1 and HkY to the Hk locus on the X-chromosome.*** **a.** Crossing

scheme: heterozygous females (y cho cv v f/*y+ cho+ cv+ Hk1 v+ f+*) were crossed to *w1118* males to generate recombinant male flies. *cv* and *v* that are the flanking markers most proximal to *Hk* on the X-chromosome **b.** Normal distribution plot for the recombinant classes shown in a. Consistent with the fact that the short sleeping phenotype maps to the *Hk* locus, the recombinants that inherited the Hk^1 mutation on average slept less compared to sibling populations that inherited a wild-type allele (Hk^+). **c.** Comparison between the recombination frequency found in this study (Rf) and published data (http://flybase.bio.indiana.edu).

Supplementary Figure 4b. Same as Suppl. Figure 4a, except that the short sleeping phenotype was mapped to the Hk^Y allele.

Supplementary Figure 5. ***Deficiencies including the Hk locus fail to complement the short sleeping phenotype.*** **a.** Schematic representation of the region of the X chromosome containing the *Hk* locus (9B5). As expected, deficiency lines *Df(1)W5, Df(1)ras59,* and *Df(1)ED7005,* which include the *Hk* locus, produced a shaking phenotype when crossed to either Hk^1 or Hk^Y, while deficiencies that did not include 9B5 (*Df(1)ED699, Df(1)HC13*) did not. **b.** Crossing scheme used to perform complementation tests with Hk^1 and Hk^Y. **c.** Daily sleep amounts (mean ± SEM, min of sleep/24 hours) and activity index (number of beam crossings/min, during waking). As in b, black bars represent the $Df(Hk)/Hk^-$ combination while grey bars represent the Hk^-/Hk^+ combination. The deficiency and Hk^- allele tested is indicated below the siblings pairs. The numbers of tested flies is indicated below each column. *, $p<0.05$; **, $p<0.001$ (unpaired Student's T-test).

Supplementary Figure 6. ***The response to sleep deprivation is similar in Hk⁻ mutants and their wild-type siblings.*** **a.** Hk^1 and Hk^Y males and their Hk^+ siblings were tested in the *w1118* and *CS* background. The 2 left bars in each group represent the percentage of sleep lost during 24 hours of sleep deprivation (compared to baseline sleep = 100%), while the 2 right bars indicate the percentage of sleep recovered during the 24 hours after sleep deprivation (recovered = number of minutes flies overslept relative to baseline during the first 24 h after sleep deprivation, expressed as % of sleep lost during sleep deprivation). Only sleep deprived flies that lost ≥90% of baseline sleep were included in this analysis. The number of tested flies is indicated below each column (same number for lost and gained). With the exception of Hk^Y flies in the *w1118* background, all flies slept longer the day after sleep deprivation than during baseline ($p<0.05$; paired Student's T-test). **b.** Arousal threshold was measured as the percentage of sleeping flies (immobile for at least 5 min) that did not show an escape response after the delivery of a complex stimulus of low intensity[6]. A similar percentage (60-70%) of male Hk^1 flies and wild-type siblings are non-responsive during baseline, and their number increases significantly and to a similar extent after sleep deprivation. Values for baseline (bl.) and recovery after sleep deprivation (recov.) refer to the first 6 hours of the dark period. **c.** To assess performance after sleep deprivation awake flies were tested for their ability to respond to a complex stimulus. As before[6], performance was measured as the percentage increase in the number of beam crossings during the minute after the delivery of the stimulus relative to the minute prior to the stimulation. All flies were active (awake) the minute prior to the

delivery of the stimulus. Values (mean±SEM) refer to the first 6 hours of the light period before (bl.) and after (recov.) sleep deprivation. *, $p<0.05$; **, $p<0.001$ (unpaired Student's T-test).

Supplementary Figure 7. ***The circadian regulation of sleep is similar in Hk⁻ mutants and their wild-type siblings.*** Males were raised and kept in 12:12 light-dark (LD) cycle for one week, and then moved to constant darkness (DD) for two weeks. Hk1 and HkY mutants were tested in the CS background and compared to Hk+ siblings. Crossing scheme used to generate flies is the same as in figure 1a. Panels a through d depict flies in LD conditions while panels e through h depict flies that have been in DD for over 5 days. **a.** Left, daily time course (in 30-min intervals) of the amount of sleep taken as the average of the last two days in LD. Right, daily sleep amounts (mean ± SEM, min of sleep/24 hours) and activity index (number of beam crossings/min, during waking) taken from the last two days in LD. The number of tested flies is indicated below each column. **b.** Autocorrelation analysis of locomotor behavior as calculated in wild-type and Hk^- flies taken over the last 4 days in LD cycle. The order of flies tested is the same as in c. **c.** Actograms depicting activity over days 6-10 in DD. The grey bar under the plots represents the subjective day period, the black bar represents subjective night. The estimated period is 24.0 hours in wild-type flies and 24.1 hours in Hk^- flies. Autocorrelation analysis for individual Hk^- flies indicates that >90% are rhythmic, similar to wild-type flies (data not shown).

Supplementary Figure 8. ***The Hk2 mutation is associated with normal daily sleep amount.*** **a**. Daily time course (in 30-min intervals) of the amount of sleep in Hk^2 and Hk^+ males in two different genetic backgrounds. The number of tested flies is the same as in b. **b.** Daily sleep amounts (mean ± SEM, min of sleep/24 hours) and activity index (number of beam crossings/min, during waking). The number of tested flies is indicated below each column. *, $p<0.001$; **, $p<0.0001$ (unpaired Student's T-test).

Methods

Animals

Flies (1-2 week old) were cultured and tested at 21°C, 68% humidity, on yeast, dark corn syrup and agar food. Hk^1 is a point mutation induced by ethyl methanesulfonate[33]. Flies carrying Hk mutant alleles, Hk+ transgene and balancers had been crossed at least four generations into the respective background. Heterozygous Hk- mutant females (FM7/Hk) were crossed to males (w; Hk+/+) with the transgene . Male progeny that inherited the wild-type $Hk^{17K\text{-}X94}$ transgene could be descriminated by the w+ marker gene within this P element construct.

Locomotor activity, sleep, and measures of sleep intensity

Experiments included 1 day of adaptation, 2 baseline days, 1 sleep deprivation (SD) day and 2 recovery days after SD. At the beginning of the experiment, individual flies were placed in the Drosophila Activity Monitor System (DAMS, Trikinetics) inside glass tubes with enough food for 1 week of recording. Monitors were housed inside environmental chambers (ThermoForma) where temperature and humidity were kept constant. Data

analysis was performed by a custom-designed software developed in our laboratory[6] and based on Statistica (StatSoft). The data were further analyzed using Matlab (Mathworks). Sleep and wakefulness were determined for consecutive 1-min epochs. Wakefulness was defined as any period of at least 1 min characterized by activity (≥ 1 count/min). Based on previous work[6], sleep was defined as any period of uninterrupted behavioral immobility (0 counts/min) lasting > 5 min. The duration of sleep episodes was calculated by counting the number of consecutive 1-min epochs of sleep. Brief awakenings were defined as 1-min epochs with at least one count preceded and followed by 1-min epochs with no counts.

Escape response to a complex stimulus

Flies were exposed to a complex stimulus consisting of a combination of noise and vibration. Flies remained inside a DAMS monitor, which was inserted into a custom-made frame specifically designed for the test[6]. The stimulus was produced by a flap vigorously pushed for a few seconds against the glass tubes housing the flies. Such stimulus was delivered once every hour at either side of the tubes via a computer-controlled motor, and flies were tested for a total of 48 hours, including one baseline day and the first recovery day after SD. Previous studies have shown that most flies move away from the stimulus (and by doing so cross the infrared beam) if prior to its delivery they had been actively moving around. By contrast, most flies do not show an escape response if they had been immobile for 5 minutes before the stimulus was delivered[6]. Thus, the percentage of non-responsive flies is used as a measure of the arousal threshold to distinguish awake flies from sleeping flies.

Escape response to heat

Single flies were placed inside a heat box where position and movements of the fly were recorded and displayed on line[6]. Flies were first adapted to the chamber for 30 min. Temperature on either side of the chamber was then alternately increased by 4°C every min from baseline value to 44°C (1min each at 24, 28, 32, 36, 40 and 44°C). The latency to crossing the infrared beam, i.e. the time a fly needed to move to the cooler side of the chamber, was measured for each temperature step. Latencies for all temperature steps were averaged for each fly. Most flies took < 8 sec to move to the cold side of the chamber. Thus, on average, the total heat exposure for each fly lasted for 30-40 sec. Flies were tested during the first 2 hours after the end of SD and at the same time of day during baseline. Pilot studies showed that the response to heat does not habituate in flies tested during 2 consecutive baseline days.

Sleep deprivation

During SD, flies remained in the DAMS monitor, which was placed vertically inside a framed box able to rotate along its major axis under the control of a motor[6]. The box could rotate 180°C clock-wise or counter-clock-wise (2-3 revolutions/min). At the nadir of each rotation, the monitor was dropped 1 cm. This caused the flies to fall from their current position to the bottom of the tube. Previous studies[6] had shown that this method is effective in reducing total sleep time by > 90%. Since locomotor activity during SD was continuously recorded, the extent of sleep loss could be calculated for each individual fly.

Heat box Experimental Setup

The conditioning apparatus was built in the workshops of the Biocenter and is a modified version of the one used in[19]. It consists of an array of 15 chambers (26 × 4 × 2 mm) operated in parallel, each with Peltier elements on top and bottom allowing for fast heating and cooling. The Peltier elements cover the whole length of the chamber. A control circuit and a thermo sensor keep the chamber at a defined temperature. Glass side walls enable transmission and detection of infrared light from a LED source (invisible to the flies). The fly casts a shadow on a bar code reader on the opposite side of the chamber. The position signal of the bar code reader is sent to the computer with a frequency of 10 Hz. Experiments are performed in complete darkness. Chambers are cleaned with a pipe cleaner every day before experiments. Measurements are performed on at least three days to avoid effects of daily variability. The different groups in one graph are measured strictly in parallel.

Statistical analysis

Two-way ANOVAs with factors "day" (e.g. baseline vs recovery) and "line" were used to analyze the data. Contrasts were tested by post-hoc t-test if the main factor or interaction reached significance.

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Figure 1

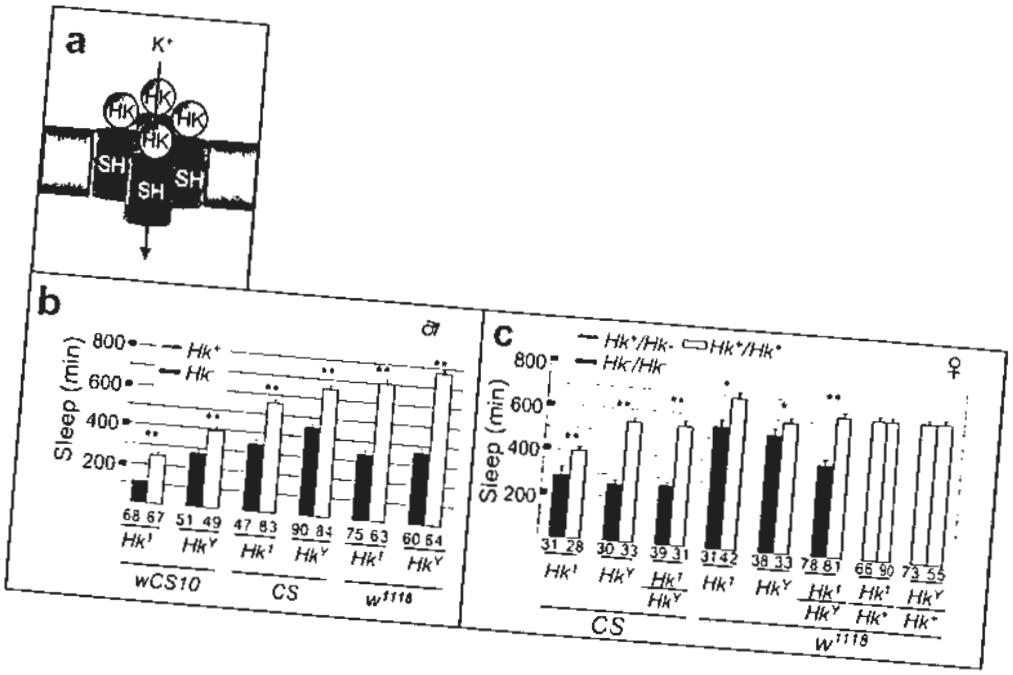


Figure 2

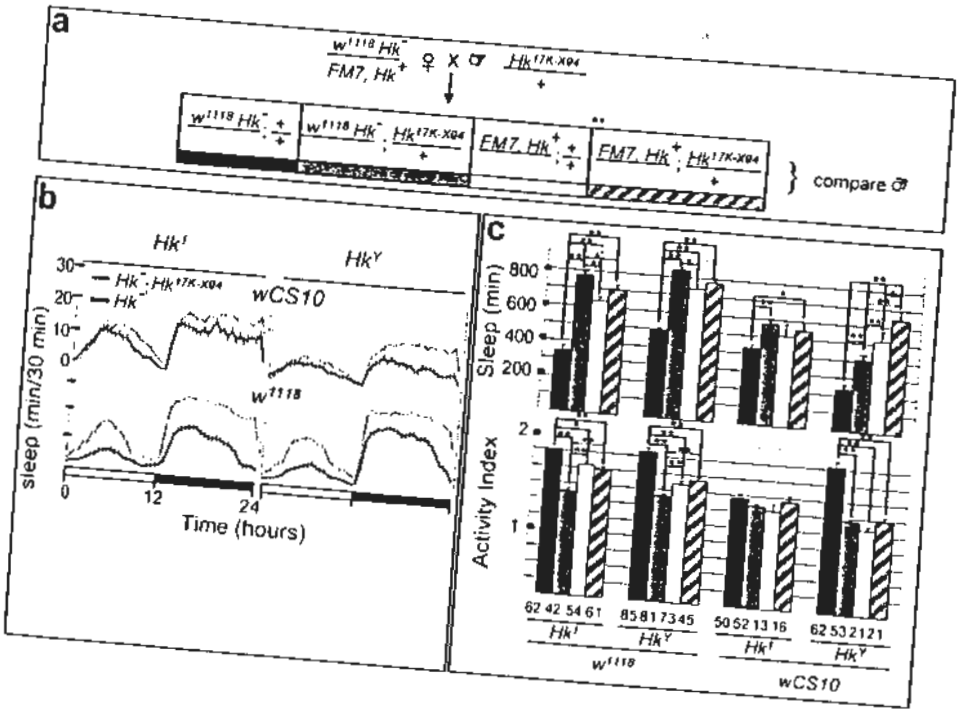


Figure 3

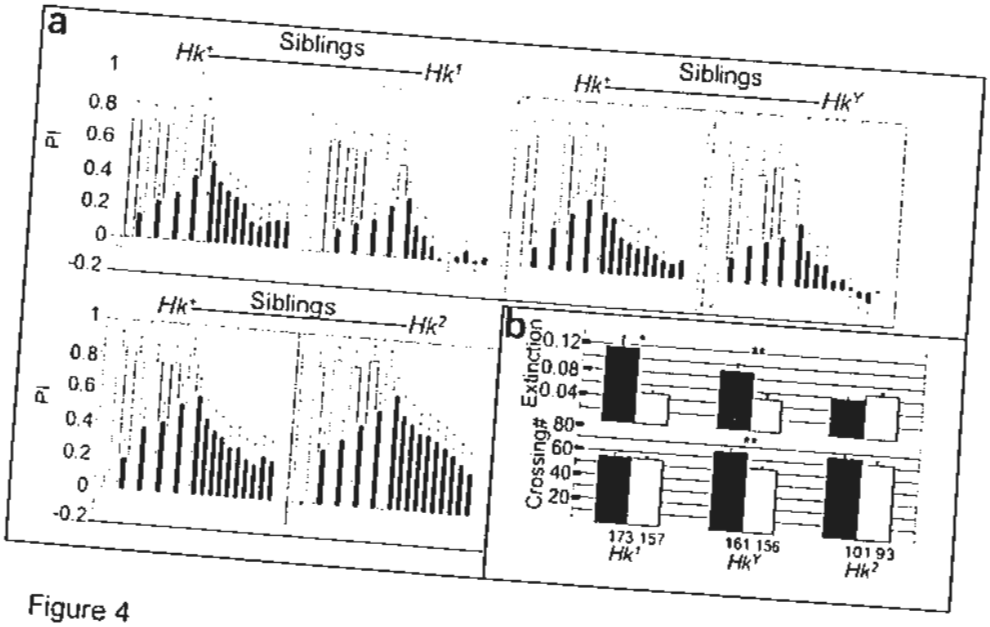
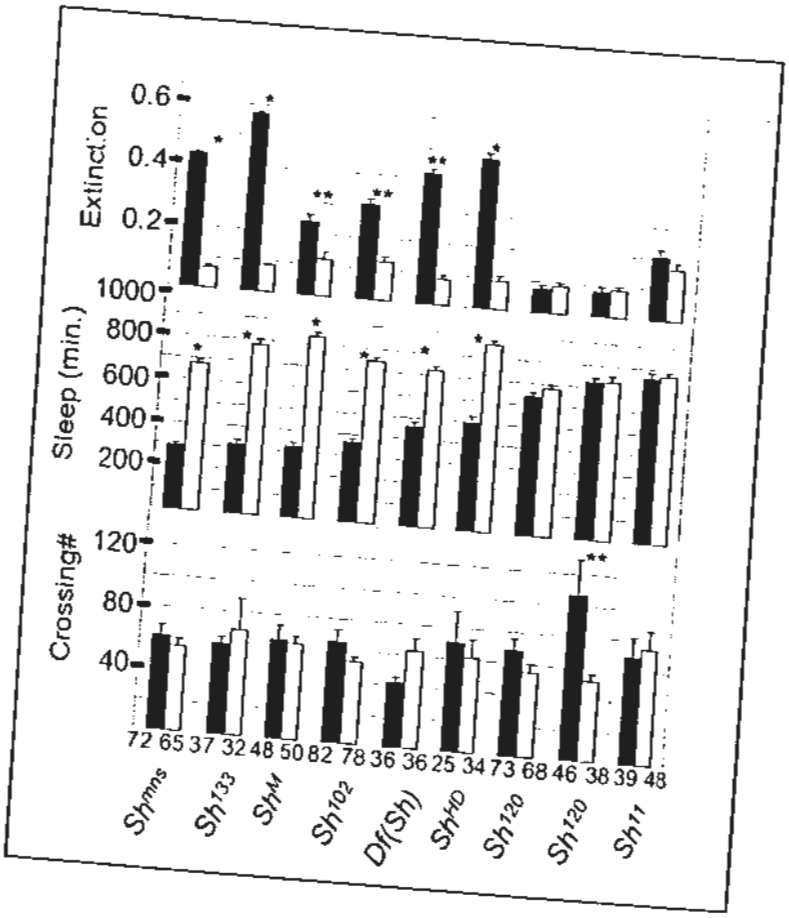
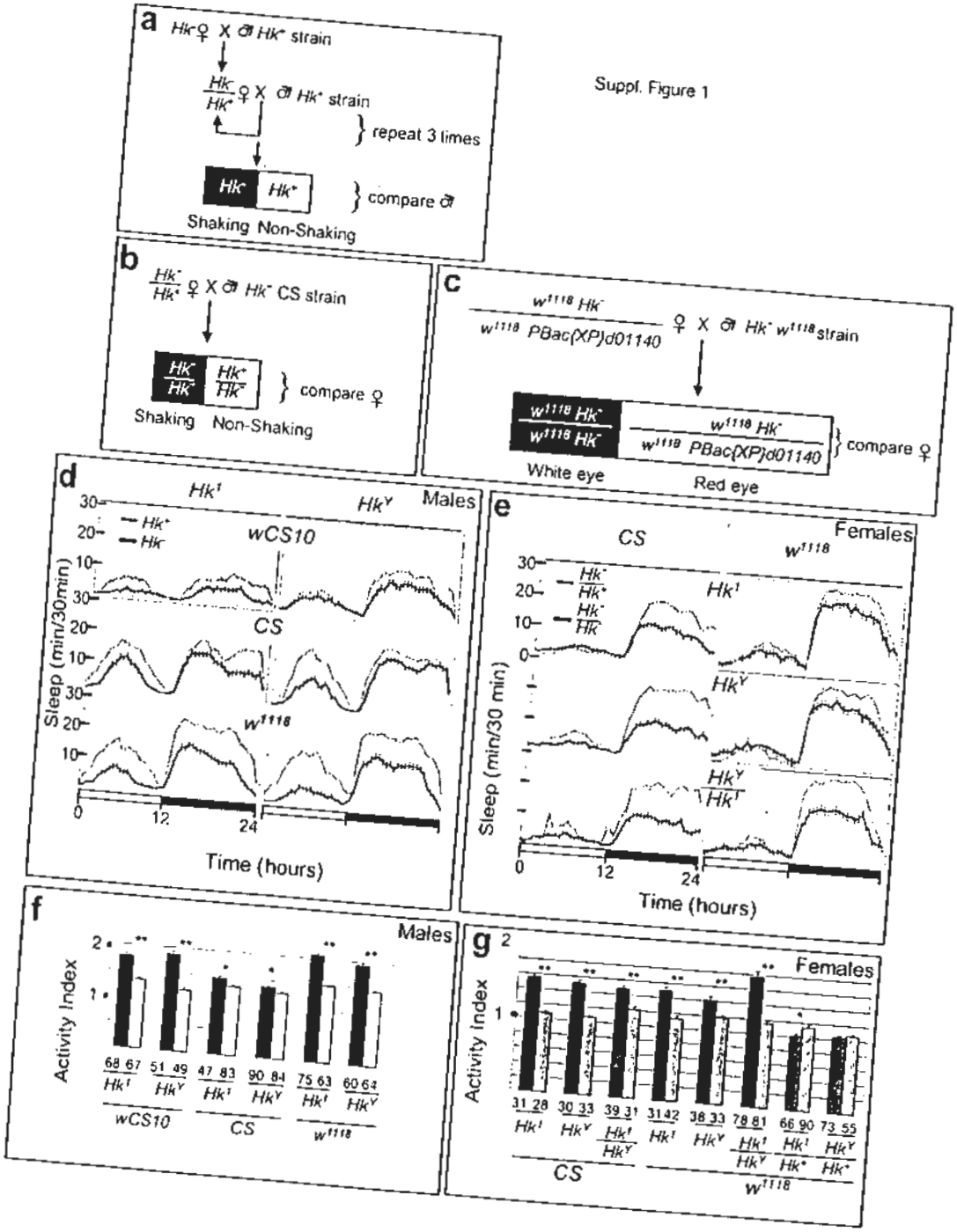


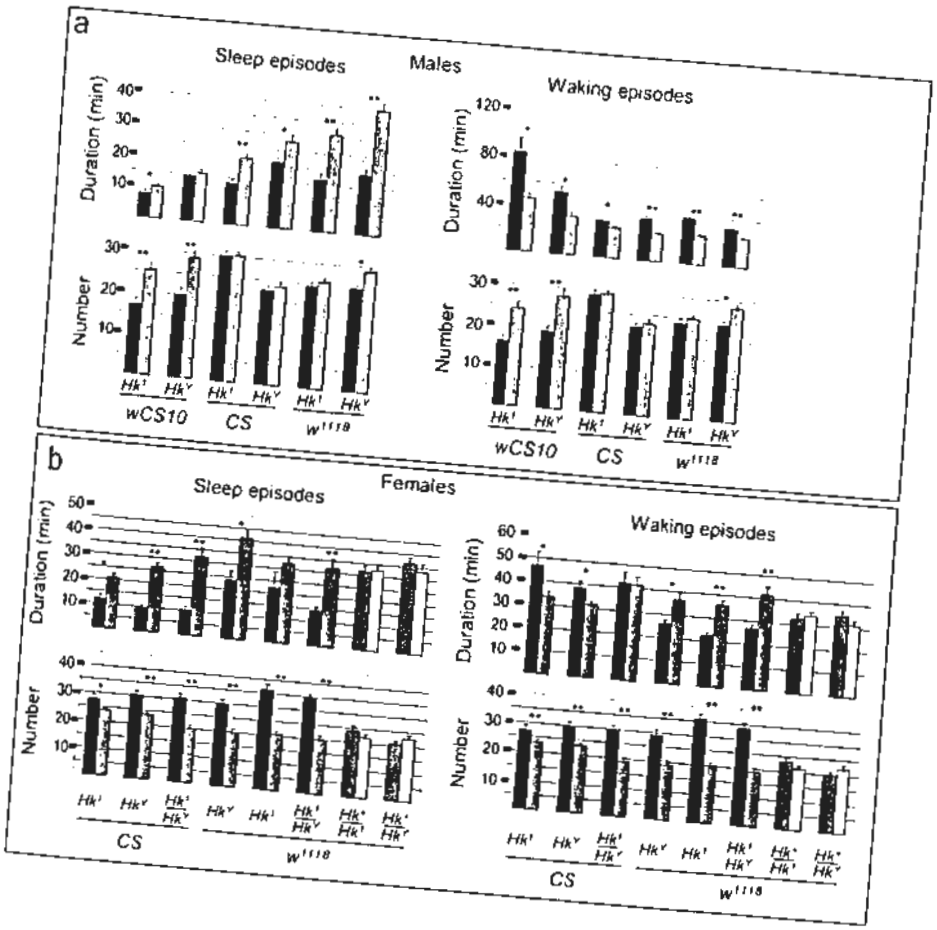
Figure 4



Suppl. Figure 1



Suppl Figure 2



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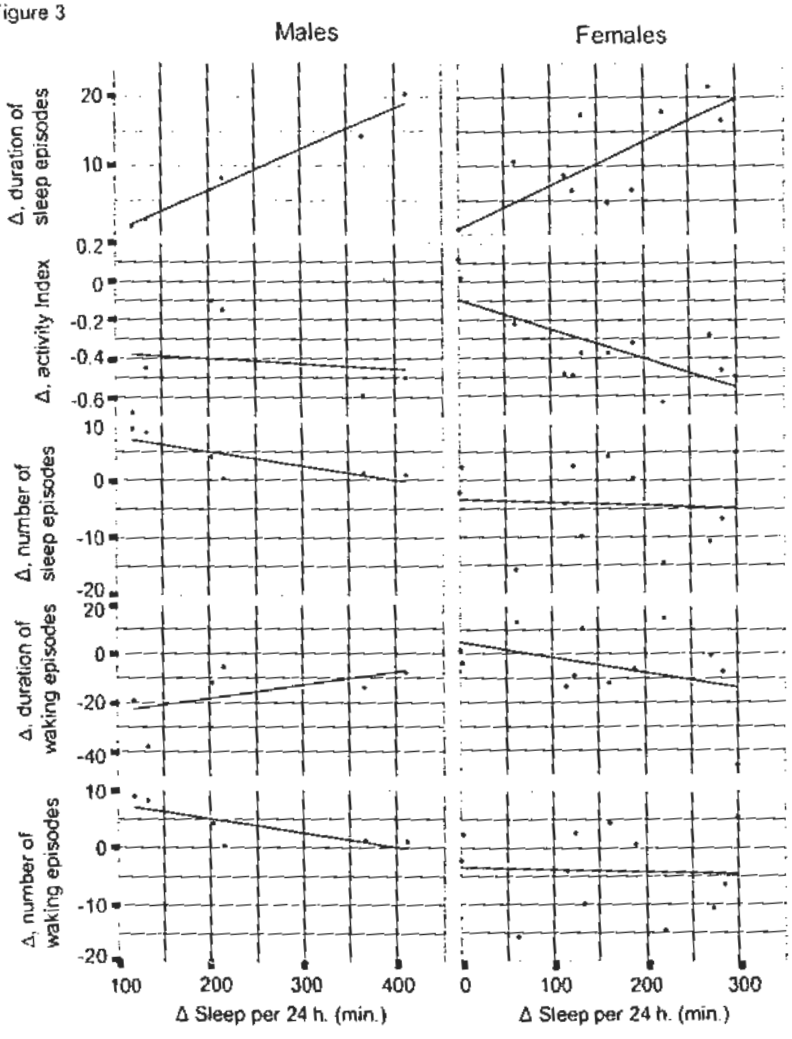
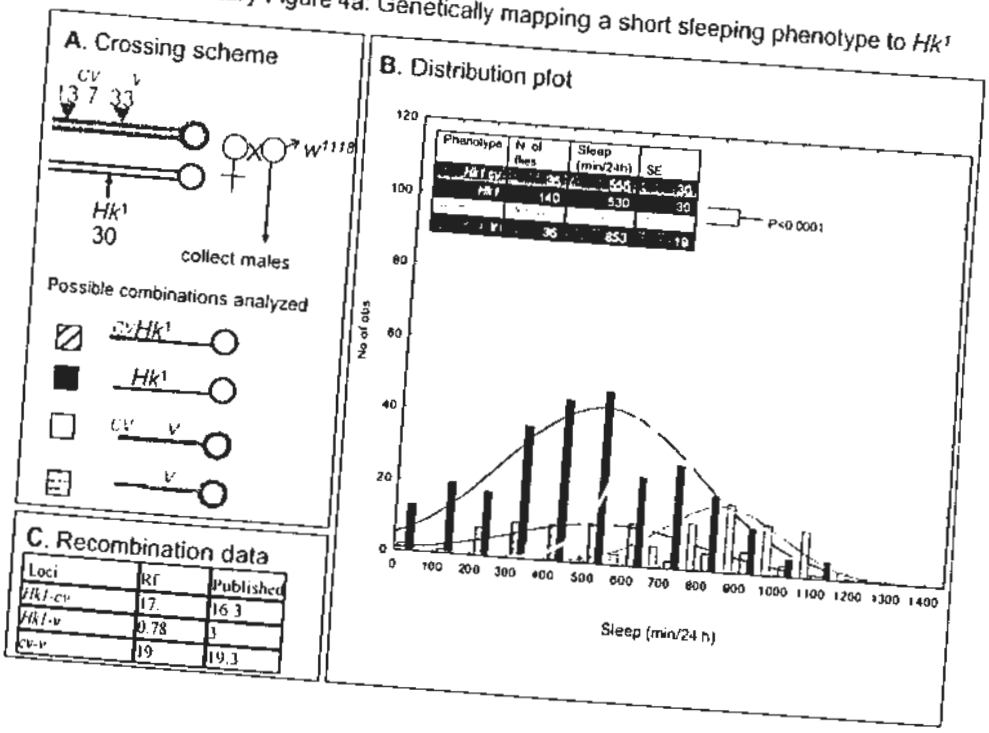
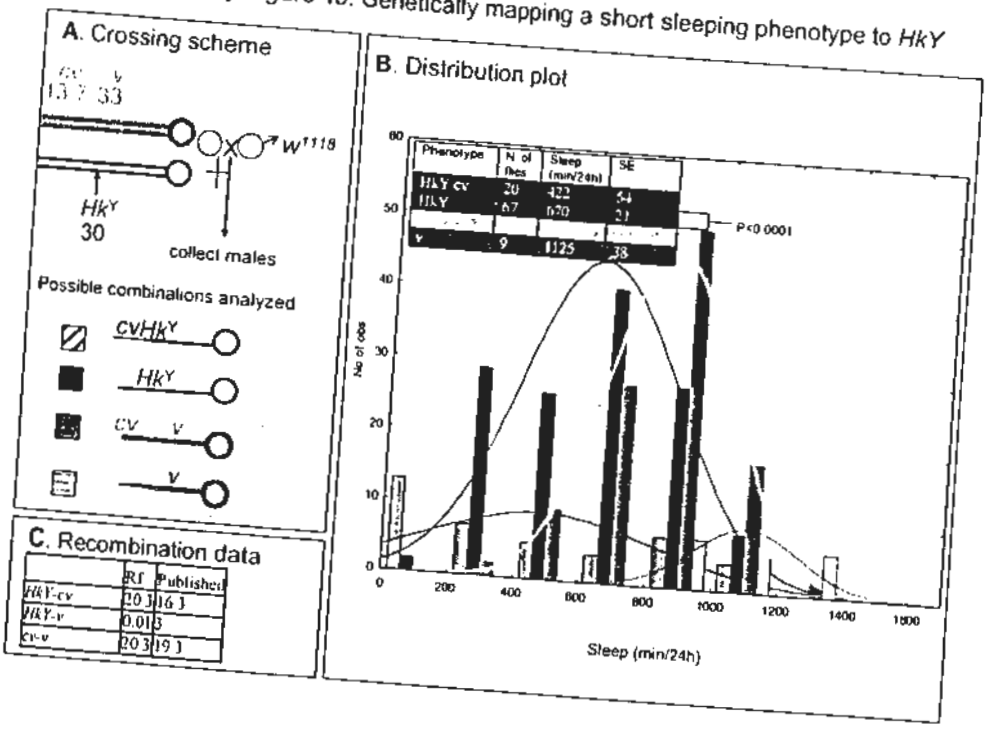


		R^2 value	Significance F
Duration of sleep episodes	Male	0.974	0.000255
	Female	0.786	0.00335
Activity Index	Male	0.0176	0.802
	Female	0.511	0.00896
Number of sleep episodes	Male	0.633	0.0583
	Female	0.00501	0.827
Duration of waking episodes	Male	0.309	0.252
	Female	0.156	0.203
Number of waking episodes	Male	0.631	0.0591
	Female	0.00319	0.862

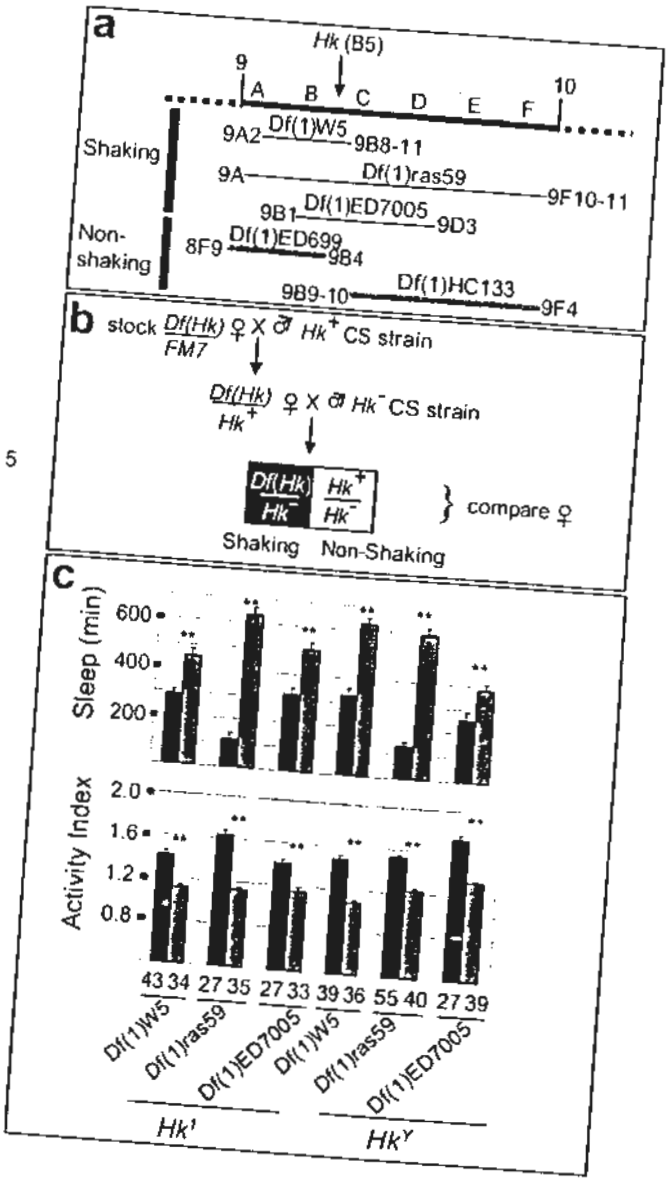
Supplementary Figure 4a: Genetically mapping a short sleeping phenotype to *Hk*¹



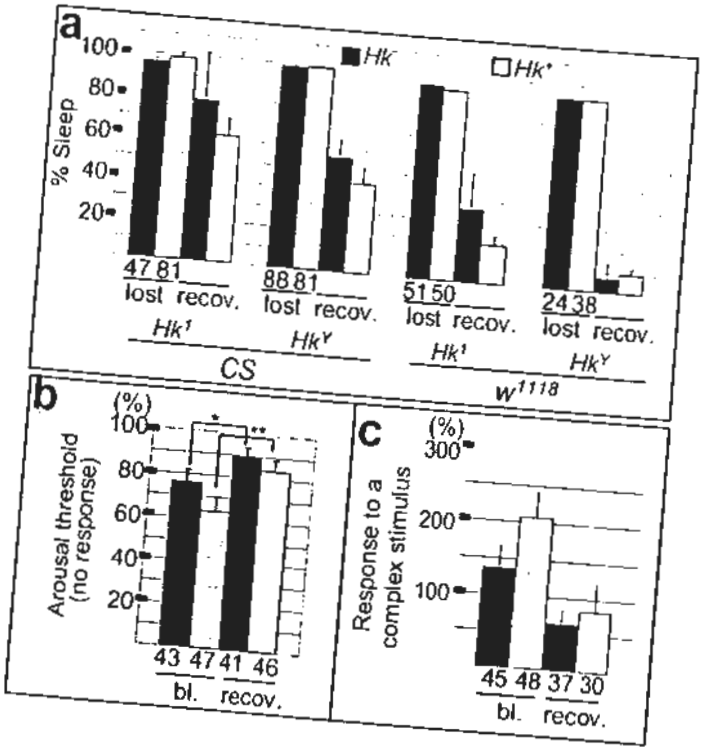
Supplementary Figure 4b: Genetically mapping a short sleeping phenotype to *HkY*

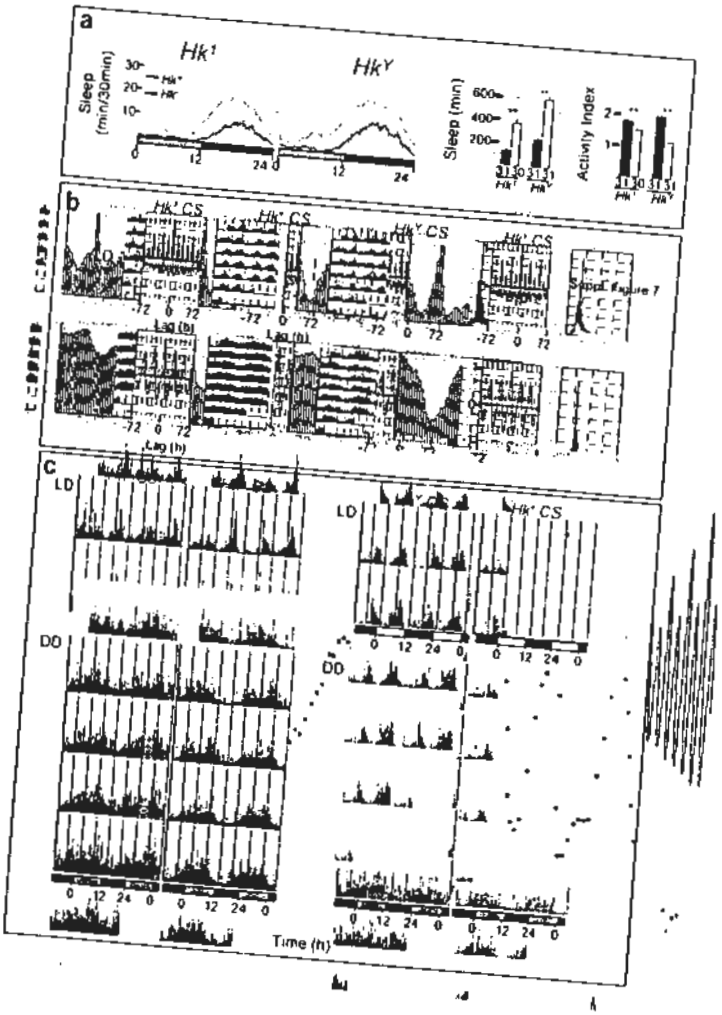


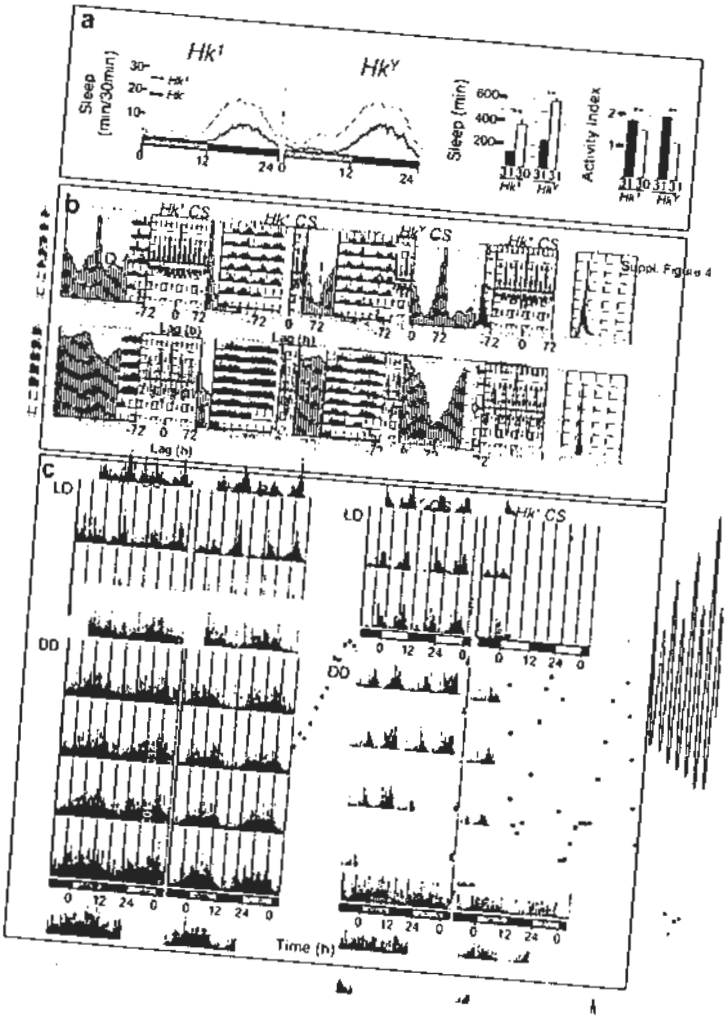
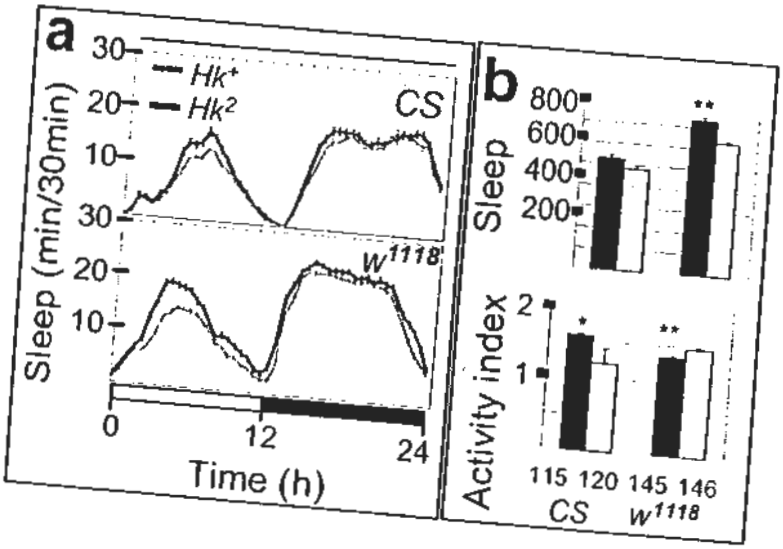
Suppl. Figure 5



Suppl. Figure 6







Suppl. Figure 8