

Calcineurin and Its Regulator Sra/DSCR1 Are Essential for Sleep in *Drosophila*

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Sleep is a fundamental biological process for all animals. However, the molecular mechanisms that regulate sleep are still poorly understood. Here we report that sleep-like behavior in *Drosophila* is severely impaired by mutations in *sarah* (*sra*), a member of the Regulator of Calcineurin (RCAN) family of genes. Sleep reduction in *sra* mutants is highly correlated with decreases in Sra protein levels. Pan-neuronal expression of *sra* rescues this behavioral phenotype, indicating that neuronal *sra* function is required for normal sleep. Since Sra regulates calcineurin (CN), we generated and examined the behavior of knock-out mutants for all *Drosophila* CN genes: *CanA-14F*, *Pp2B-14D*, and *CanA1* (catalytic subunits), and *CanB* and *CanB2* (regulatory subunits). While all mutants show at least minor changes in sleep, *CanA-14F^{KO}* and *CanB^{KO}* have striking reductions, suggesting that these are the major CN subunits regulating sleep. In addition, neuronal expression of constitutively active forms of CN catalytic subunits also significantly reduces sleep, demonstrating that both increases and decreases in CN activity inhibit sleep. *sra* sleep defects are suppressed by CN mutations, indicating that *sra* and CN affect sleep through a common mechanism. Our results demonstrate that CN and its regulation by Sra are required for normal sleep in *Drosophila* and identify a critical role of Ca²⁺/calmodulin-dependent signaling in sleep regulation.

Introduction

Although sleep or sleep-like states have been observed in most animal species studied to date, the basic role of sleep is still not well understood. Previous studies identifying sleep mutants have shown that changes in sleep are associated with changes in neuronal activity. In *Drosophila*, mutations that decrease activity of the *shaker* voltage-gated K⁺ channel increase neuronal activity and decrease sleep (Cirelli et al., 2005). In addition, activity of various neurotransmitters and neuropeptides, including octopamine (Crocker and Sehgal, 2008), dopamine (Kume et al., 2005), and pigment-dispersing factor (pdf) (Parisky et al., 2008; Chung et al., 2009), are associated with decreased sleep in flies, while activity of others, including GABA (Agosto et al., 2008; Parisky et al., 2008) and serotonin (Yuan et al., 2006), are associated with increased sleep. However, intracellular molecular components mediating the effects of these molecules on sleep have not readily been

identified. Ca²⁺/calmodulin (CaM) signaling is known to be critical for synaptic activity since it regulates activity of Ca²⁺/CaM-dependent protein kinases and phosphatases essential for plasticity, but a role of Ca²⁺/CaM signaling in sleep has not previously been described.

Calcineurin (CN) is a highly conserved Ca²⁺/CaM-dependent protein phosphatase, implicated in synaptic plasticity in mammals (Rusnak and Mertz, 2000). CN is composed of a heterodimer of an ~60 kDa catalytic subunit (CnA) and an ~19 kDa EF-hand Ca²⁺-binding regulatory subunit (CnB). Both subunits are essential for phosphatase activity. Besides Ca²⁺ and CaM, CN activity also depends on the regulator of CN (RCAN) family of proteins (Hilioti and Cunningham, 2003; Davies et al., 2007). RCAN proteins were initially identified as CN inhibitors that bind directly to CnA subunits to inhibit activity. However, RCAN knock-outs in fungi, *Drosophila*, and mice exhibit phenotypes similar to CN mutants, indicating that these proteins function both as CN activators and inhibitors (Görlach et al., 2000; Vega et al., 2003; Mehta et al., 2009). In *Drosophila*, the *sra* gene has been shown to encode the only known RCAN protein as assayed by homology searches, biochemical interactions, and functional genetic interactions (Ejima et al., 2004; Horner et al., 2006; Takeo et al., 2006).

Here we show that both *sra* mutants and specific CN subunit gene mutants have severe sleep defects, indicating that Ca²⁺/CaM signaling plays a critical role in sleep regulation. These results suggest a mechanistic connection linking synaptic plasticity and sleep.

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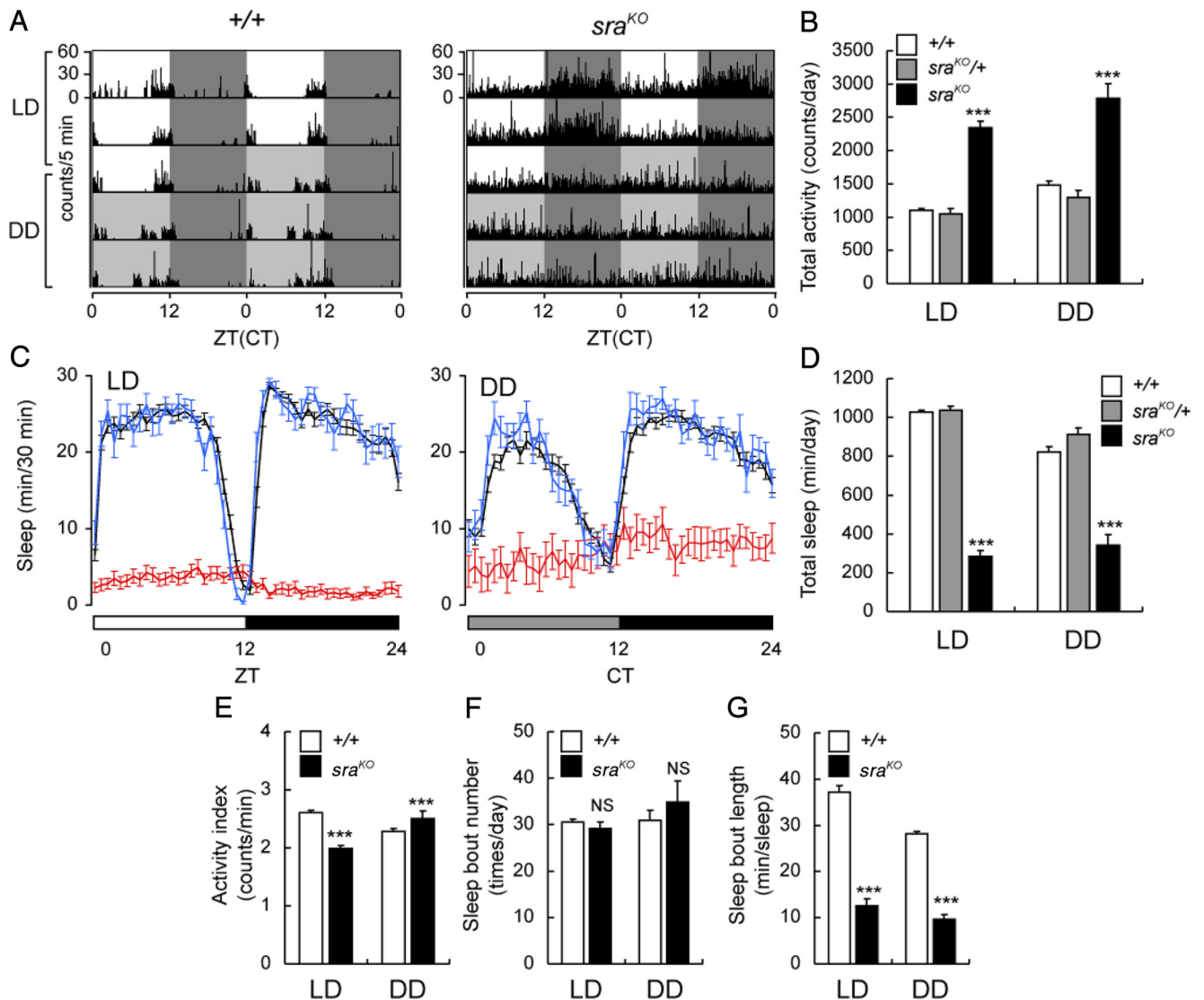


Figure 1. *sra*^{KO} mutants exhibit increased locomotor activity and decreased sleep. **A**, Locomotor activity in representative wild-type (+/+) and *sra*^{KO} flies in LD and DD conditions (3 d each in LD and DD conditions) are double plotted, such that each horizontal line corresponds to 2 d. Data are plotted as number of activity counts (beam crossings) per 5 min. **B**, Total daily activity of +/+, *sra*^{KO/+}, and *sra*^{KO} flies plotted in LD and DD conditions. **C**, Daily sleep profiles of +/+ (black), *sra*^{KO/+} (blue), and *sra*^{KO} (red) flies in LD and DD conditions. The amount of sleep per 30 min bin is plotted versus a daily time course. Light and dark phases and subjective day and night are indicated by white, black, and gray bars, respectively. **D**, Total daily sleep of +/+, *sra*^{KO/+}, and *sra*^{KO} flies in LD and DD conditions. **E–G**, Waking activity index (**E**), sleep bout number (**F**), and sleep bout length (**G**) in +/+ and *sra*^{KO} mutants. Data in **B** and **D** were analyzed by one-way ANOVA followed by Tukey's *post hoc* analyses. Data in **E–G** were analyzed by *t* test. In all cases, NS indicates no significant difference and *** indicates *p* < 0.001. Error bars indicate SEMs.

Materials and Methods

Fly stocks and maintenance. Flies were reared on conventional corn meal, yeast, glucose, agar medium at 25°C. All stocks were outcrossed to our wild-type, *w*(CS) line at least six times to normalize genetic backgrounds (Dura et al., 1993). *elav-GAL4* (BL-8765; *PjGAL4-elav.Lj2*) was obtained from the Bloomington Stock Center. *sra*^{KO} (Takeo et al., 2006), *sra*^{GS3080} (Ejima et al., 2004), and *UAS-sra* (Ejima et al., 2004) lines have been described previously.

Measurement of locomotor activity and sleep. Two- to four-day-old male flies were placed individually in glass tubes (length 6.5 cm; inner diameter, 3 mm) containing ~1 cm of food at one end and a cotton stopper on the other end. Locomotor activity was measured using a *Drosophila* activity monitor (DAM) (Trikinetics), which records how often flies cross an infrared beam located near the center of each tube. Crossings per minute were recorded over 3 d in LD conditions followed by 3 d in DD conditions. Data were processed using a Microsoft Excel-based program described previously (Hendricks et al., 2003). Sleep was defined as 5 or more minutes of continual inactivity and was plotted in 30 min time bins. A waking activity index was calculated by

dividing total daily activity by the length of the active period, which was defined as the total number of minutes where a fly exhibited at least one activity count. Analyses were performed using at least 19 flies per genotype.

Western blot analysis. Two- to four-day-old flies were frozen in liquid N₂ and heads were isolated on dry ice. Protein extracts were made by crushing heads in sample loading buffer (50 mM Tris Cl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). Two and a half head equivalents per lane were subjected to 10% PAGE and transferred to nylon membranes. Blots were probed with rabbit anti-Sra antibody (1:1000) (Takeo et al., 2006) or mouse anti- α -tubulin antibody (1:1000) (Sigma-Aldrich) and appropriate HRP-conjugated secondary antibodies (Promega). Chemiluminescent signals were obtained using ECL Western blotting reagents (GE Healthcare) and detected using x-ray film (Bio-rad Laboratories). Densitometric analyses were performed using ImageJ software (<http://rsb.info.nih.gov/ij/>).

Generation of knock-out lines. CN gene knock-out lines were generated using the ends-out gene targeting technique (Gong and Golic, 2003). Briefly, a knock-out cassette (containing *FRTs* and I-SceI recognition

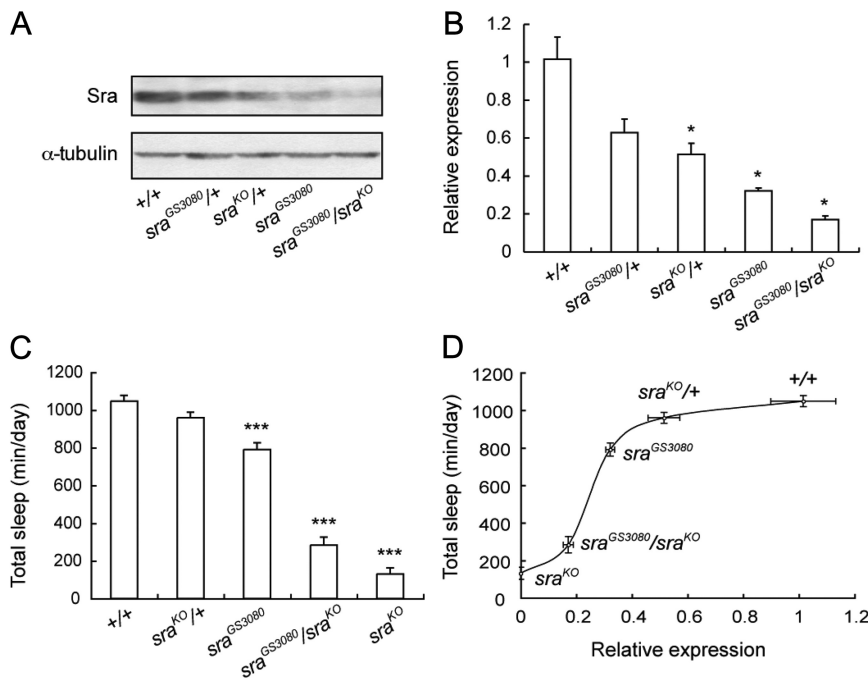


Figure 2. Sleep in *sra* mutants is correlated with amounts of Sra protein. **A**, A representative Western blot measuring Sra protein amounts in indicated *sra* mutants. The same blot probed with anti- α -tubulin is shown for comparison. **B**, Relative amounts of Sra normalized to α -tubulin in *sra* mutants. One-way ANOVA indicates significant difference in Sra levels due to genotype. * indicates a significant difference ($p < 0.05$) compared to +/+, as assayed by Tukey's *post hoc* analysis. **C**, Total daily sleep plotted for various *sra* mutants. One-way ANOVA indicates significant differences in sleep due to genotype. *** indicates significant difference ($p < 0.001$) compared to +/+ as assayed by Tukey's *post hoc* analysis. **D**, Total daily sleep plotted as a function of Sra protein amounts.

sequences surrounding a 5' homologous region, a w^+ marker, and a 3' homologous region) was integrated non-homologously into the genome to generate *P{donor}* lines. The knock-out cassette was excised and linearized by crossing the *P{donor}* lines to a [70FLP][70I-SceI] line, which contains heat-inducible FLP recombinase and I-SceI endonuclease transgenes, and heat shocking the progeny 3–5 d after egg laying. Candidate homologous knock-out lines were identified as described previously (Takeo et al., 2006) and confirmed by PCR. *P{donor}* plasmids containing the knock-out cassettes were generated by cloning ~2 kb of sequence both upstream and downstream of the knock-out locus into the polylinker of *p{EndsOut2}* (Takeo et al., 2006). Primer sequences used for amplifying all upstream and downstream sequences are available upon request. The *mini-white* gene was inserted in between upstream and downstream sequences in all knock-out constructs, except for the *CanA1^{KO}* construct, which used the *hsp::white* gene with the *GMR* enhancer from *pGX-attP* (Huang et al., 2009). Two *loxP* sites were inserted around the *mini-white* or *white* genes to facilitate removal of the marker after identification of knock-out lines. In addition, an *attP* site was inserted 3' to the downstream *loxP* site to allow future integration of constructs into the knock-out site.

Generation of UAS-*CanA^{act}* lines. Constitutively active forms of CnA genes were generated as previously described for *Pp2B-14D* (Sullivan and Rubin, 2002). Briefly, *CanA1^{act}* was made by inserting a stop codon at amino acid residue 456 (out of 622), *Pp2B-14D^{act}* was made by inserting a stop at residue 456 (out of 570) and *CanA-14F^{act}* was made by inserting a stop at residue 450 (out of 584). PCR was performed using 3' primers incorporating a stop codon at the appropriate location and flanking restriction sites. Amplified genes were cloned into a *pUASTattB* vector (Bischof et al., 2007), which contains an *attB* site that was used for ϕ C31-dependent integration at the *attP* site (2L-22A) of *ZH-attP-22A* flies (Bischof et al., 2007). Transformants were outcrossed to *w*(CS) flies for six generations to normalize genetic background.

Results

sra^{KO} mutants exhibit increased locomotor activity and loss of sleep

While studying Ca^{2+} signaling in egg activation in *Drosophila*, we noticed that *sra*^{KO} mutants move continuously and do not become quiescent. To characterize this behavioral phenotype more precisely, we measured the activity of these mutants using a *Drosophila* activity monitor. When wild-type flies are raised in 12 h light/dark (LD) conditions, they are preferentially active during the times around lights on and lights off (Fig. 1A) (Hendricks et al., 2000; Shaw et al., 2000). After light entrainment, this locomotor behavior continues in continual dark conditions (DD) in a circadian manner (Konopka and Benzer, 1971). In contrast, *sra*^{KO} flies move throughout the 24 h day (Fig. 1A) and are approximately twofold more active than wild-type flies in both LD and DD conditions (Fig. 1B). Heterozygous *sra*^{KO}/+ mutants have identical activity to wild-type flies, indicating that this increased activity is a recessive phenotype.

The continual movement of *sra*^{KO} flies suggested that they may have decreased sleep. From previous work, *Drosophila* sleep has been defined as 5 or more minutes of continuous inactivity, since these periods are associated with increased arousal threshold, a characteristic of sleep (Hendricks et al., 2000; Shaw et al., 2000). Compared to wild-type flies, *sra*^{KO} flies showed a dramatic loss of sleep, to ~30% of wild-type amounts (Figs. 1C,D, 2C, 3B). This result was also observed in DD conditions.

To determine whether general hyperactivity could explain the increased daily activity and decreased sleep in *sra*^{KO} mutants, we calculated locomotor activity, specifically during waking in wild-type and *sra*^{KO} mutants. Waking activity was slightly decreased in LD conditions in *sra*^{KO} mutants, while it was only slightly increased in DD conditions (Fig. 1E). Thus the increase in daily activity of *sra*^{KO} mutants is due to increased time spent in a waking state, rather than to increased activity during waking.

Total sleep amounts depend upon both the frequency of sleep bouts and the length of time of each sleep bout. The number of daily sleep bouts was the same in *sra*^{KO} mutants and wild-type flies, in both LD and DD conditions (Fig. 1F). In contrast, the average sleep bout length in *sra*^{KO} mutants was decreased to ~30% that of wild type, in LD and DD conditions (Fig. 1G), indicating that decreased sleep in *sra*^{KO} was due to a decrease in sleep maintenance, rather than to a decrease in sleep onset.

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Sleep is correlated with levels of Sra protein

To verify that the *sra*^{KO} sleep phenotype is due to mutation of the *sra* locus, we measured sleep in a second mutant, *sra*^{GS3080}, which has a *P*-element transposon insertion in the intron of the *sra* gene (Ejima et al., 2004). This insertion reduces Sra protein levels to 30% of wild-type levels (Fig. 2A,B). Surprisingly, sleep was only mildly reduced in *sra*^{GS3080} mutants (Fig. 2C), suggesting that relatively low amounts of Sra are sufficient for normal sleep. We tested this idea by measuring Sra protein levels (Fig. 2B) and daily amounts of sleep (Fig. 2C) in several *sra* homozygous, heterozy-

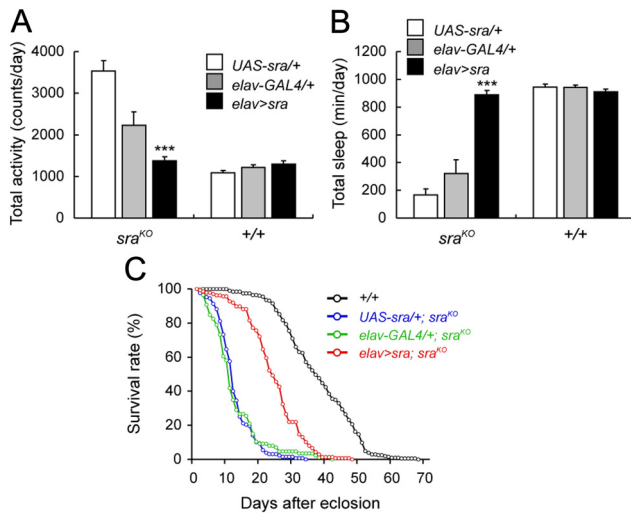


Figure 3. Expression of *sra* in neurons rescues activity, sleep and lifespan phenotypes of *sra*^{KO} mutants. **A**, Daily activity of UAS-*sra*^{+/+}; *sra*^{KO} and *elav-GAL4*^{+/+}; *sra*^{KO} flies is significantly higher than wild type, while activity in *elav>sra*; *sra*^{KO} flies is reduced back to wild-type levels. Neuronal expression of *sra* in a wild-type background has no effect on activity. **B**, Neuronal expression of *sra* rescues *sra*^{KO} sleep defects (*elav>sra*; *sra*^{KO}), while it has no effects in a wild-type background. Driver alone and UAS-*sra* alone controls do not rescue *sra*^{KO} sleep defects. In **A** and **B**, *** indicates significant differences between *elav>sra* and both UAS-*sra* alone and *elav-GAL4* alone controls in the *sra*^{KO} background ($p < 0.001$). No significant differences were detected in the +/+ background. **C**, Survival curves for male wild-type (+/+), *elav>sra*; *sra*^{KO}, *elav-GAL4*^{+/+}; *sra*^{KO}, and UAS-*sra*^{+/+}; *sra*^{KO} flies. Neuronal expression of *sra* partially rescues the reduced lifespan of *sra*^{KO} mutants. Wild-type males have an average lifespan of 37.82 ± 0.76 d, while *elav>sra*; *sra*^{KO}, *elav-GAL4*^{+/+}; *sra*^{KO}, and UAS-*sra*^{+/+}; *sra*^{KO} flies have average lifespans of 24.30 ± 0.70 , 12.60 ± 0.83 , and 12.65 ± 0.48 d, respectively.

gous, and transheterozygous mutants. As seen in Figure 2D, reducing Sra to 50% of wild-type levels does not significantly affect sleep. Decreasing Sra further, to 30% of wild-type levels, has a mild effect, while a further reduction to 20% results in a precipitous decrease in time spent asleep. Thus, relatively large reductions in Sra function are necessary to affect sleep, and decreased sleep is highly correlated with reduced levels of Sra protein.

Neuronal expression of *sra* rescues *sra*^{KO} phenotypes

If increased locomotor activity and decreased sleep in mutants are due to reduced amounts of Sra, these phenotypes should be rescued by expression of wild-type *sra*. To test this, we expressed a UAS-*sra*⁺ transgene pan-neuronally using an *elav-GAL4* driver in a *sra*^{KO} background. While control *sra*^{KO} lines containing UAS-*sra*⁺ alone or *elav-GAL4* alone had over twice the locomotor activity of wild-type flies (compare Fig. 3A with Fig. 1B), activity was reduced back to the wild-type levels in *elav-GAL4*/*UAS-sra*⁺; *sra*^{KO} flies (*elav>sra*; *sra*^{KO}). Similarly, sleep was completely restored to wild-type amounts in this genotype (Fig. 3B). Overexpression of *sra*⁺ in a wild-type background did not affect activity or sleep, demonstrating that reduced activity and increased sleep is due to rescue, rather than to a nonspecific effect of *sra*⁺ overexpression.

sra^{KO} mutants have a significantly reduced lifespan

We observed that *sra*^{KO} flies tend to die much earlier than wild-type flies. Sleep is thought to be an essential biological function, since sleep deprivation can lead to death (Rechtschaffen et al., 1983; Shaw et al., 2002). In addition, several previously identified sleep mutants have decreased lifespans, suggesting that sleep and organismal lifespan may be linked (Shaw et al., 2002; Cirelli et al.,

2005; Seugnet et al., 2009) (but also see Kume et al., 2005). Thus we measured the lifespan of *sra*^{KO} mutants and determined that it was significantly reduced compared to wild type. While wild-type flies have mean (\pm SEM) lifespans of 37.82 ± 0.76 d for males and 37.36 ± 0.76 d for females, *sra*^{KO} mutants (*elav-GAL4*^{+/+}; *sra*^{KO}) live an average of only 12.60 ± 0.83 d for males (Fig. 3C), and 12.81 ± 0.48 d for females (data not shown). This shortened lifespan can be partially rescued by expressing *sra* in neurons (*elav>sra*; *sra*^{KO}), indicating that, similar to sleep, lifespan is dependent on neuronal Sra function.

Calcineurin regulates sleep and locomotor activity

Why do *sra* mutants sleep less than wild-type flies? Sra's role as a CN regulator (Horner et al., 2006; Takeo et al., 2006) suggested that CN may play a role in regulating sleep. To test this possibility, we generated individual knock-outs of all *Drosophila* CN genes and measured sleep and locomotion. In *Drosophila*, three genes, *CanA-14F*, *Pp2B-14D*, and *CanA1*, encode catalytic subunits, and two genes, *CanB* and *CanB2*, encode regulatory subunits. We generated knock-outs of all of these genes by homologous recombination (Fig. 4). As seen in Figure 5A, all knock-out lines affected total daily locomotor activity except for *CanB2*^{KO}. In addition, all lines significantly affected daily amounts of sleep (Fig. 5D). However, the extent to which individual CN gene knock-outs affected locomotion and sleep varied widely. Among the regulatory subunits, *CanB2*^{KO} had the smallest effect on these behaviors (a 10% decrease in the amount of sleep). In contrast, knock-out of the other regulatory subunit, *CanB*, dramatically increased locomotion and decreased the amount of sleep (Fig. 5A, C, D, F) (an ~50% decrease in the amount of sleep). These phenotypes were similar to those of *sra*^{KO} flies. Interestingly, while *CanB*^{KO} mutants had decreased sleep amounts to ~50% of wild-type in both daytime and nighttime in LD conditions, in DD conditions, these mutants showed a further decrease in sleep during the subjective day to 20% of wild type. While we are not certain why this occurs, this result suggests that decreasing CN activity enhances sleep in the presence of light (Fig. 5F).

Among the catalytic subunit mutants, *Pp2B-14D*^{KO} flies had a small but significant 25% increase in locomotion and a 10% decrease in the amount of sleep. *CanA1*^{KO} flies had an opposite effect of slightly decreasing total activity and slightly increasing the amount of sleep. However, we believe this apparent sleep effect is artifactual, due to reduced mobility of *CanA1*^{KO} flies. *CanA-14F*^{KO} flies showed the largest effects on activity and sleep, increasing locomotor activity approximately threefold and decreasing the amount of sleep over twofold (Fig. 5A, B, D, E). Similar to *CanB*^{KO} mutants, *CanA-14F*^{KO} flies also sleep less during the subjective day in DD conditions than they do in LD conditions (Fig. 5E), again suggesting that while CN plays an important role in sleep in general, it may also have a second, more specific role in suppressing sleep in the presence of light.

Our results indicate that all CN subunits, with the possible exception of *CanA1*, contribute to daily locomotion and sleep. However, they also indicate that the functions of CN subunits are not identical, and suggest that the *CanA-14F* catalytic and the *CanB* regulatory subunits are the most important in controlling sleep amounts.

Constitutively active calcineurin inhibits sleep

If decreasing CN reduces sleep, we reasoned that increasing CN activity might increase sleep. Ca^{2+} /CaM-independent, constitutively active CN can be made by truncating the C-terminal Ca^{2+} /CaM binding regions and autoinhibitory domains of CnA

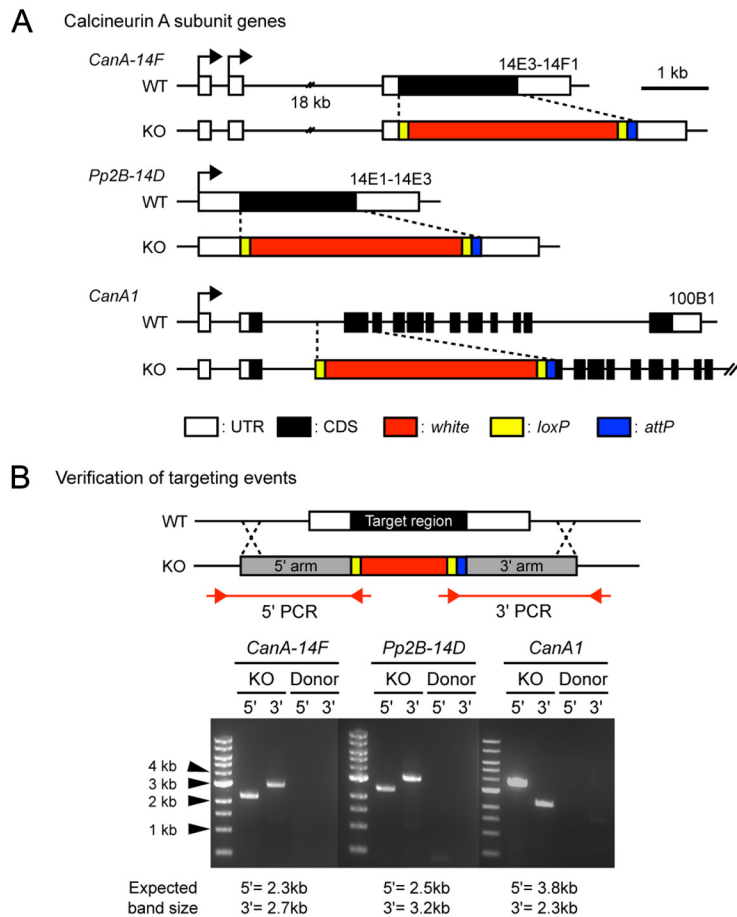


Figure 4. Generation of CN gene knock-out mutants. **A**, Schematic diagram of genomic loci for wild-type and knock-out alleles of *CanA-14F*, *Pp2B-14D*, and *CanA1*. *CanA-14F^{KO}* and *Pp2B-14D^{KO}* constructs precisely remove all of the protein coding regions of the corresponding genes, while the *CanA1^{KO}* construct removes the second and third exons. **B**, Verification of knock-outs. Knock-out lines were confirmed by PCR, shown schematically in the upper panel. Two separate PCRs were performed per knock-out candidate, one corresponding to the 5' end of the knock-out locus and the second corresponding to the 3' end. Internal primers were made bridging the *loxP* and *white* regions, while external primers were made to areas outside the homologous region. Knock-out lines show bands of the expected size (KO), while *P{donor}* lines, where the knock-out cassette is integrated at a non-homologous site, show no bands (Donor). *CanB^{KO}* and *CanB2^{KO}* were made using the same strategy, and will be described elsewhere.

catalytic subunits (O'Keefe et al., 1992). Thus, we constructed *UAS-CanA^{act}* transgenic lines, which express constitutively active versions of each of the *CanA* catalytic subunit genes under *GAL4* control. We crossed our lines to an *elav-GAL4* line and measured sleep in the progeny. Neuronal expression of all three *CanA^{act}* transgenes caused significant increases in activity and decreases in sleep, indicating that both increases and decreases in CN activity increase locomotor activity and decrease sleep (Fig. 5G).

Genetic interaction between *sra* and calcineurin

Mutations in both *sra* and CN genes increase locomotion and decrease amounts of sleep. Are these two phenotypes linked or independent? To answer this question, we made double mutants containing *CanA-14F^{KO}* or *Pp2B-14D^{KO}* and *sra* hypomorphic mutations, to see whether effects on sleep were purely additive or whether we could see genetic interactions between these mutations. In this experiment, we used *sra^{GS3080}/sra^{KO}* transheterozygotes, which sleep approximately one-third of the amount that wild-type flies sleep (Fig. 2C), allowing for easy identification of both increases and decreases in sleep. While *sra^{GS3080}/sra^{KO}* flies have reduced sleep, both *CanA-14F^{KO}* (Fig. 5G) and *Pp2B-14D^{KO}* (Fig. 5H)

mutations partially suppressed this phenotype. In contrast, single *CanA-14F^{KO}* or *Pp2B-14D^{KO}* mutations decreased amounts of sleep. These results indicate that sleep phenotypes in *sra* and CN gene mutants occur through a common genetic pathway, and that *Sra* antagonizes CN under our experimental conditions. Increased CN activity in *sra* hypomorphic mutants leads to a decrease in the amount of sleep, which can be suppressed by deleting one of the CN catalytic subunits.

Discussion

Ca^{2+} /CaM signaling has been implicated in many different biological processes, but a definite role of Ca^{2+} /CaM signaling in sleep has not previously been shown. Here we demonstrate that mutations in CN genes, *CanA-14F* and *CanB*, and in the CN regulator, *sra*, strongly decrease amounts of sleep and increase total daily activity. Sleep in *sra^{KO}* mutants is reduced to almost non-existent levels.

Deletions of all individual *Drosophila* CN genes have at least minor effects on sleep. However, knock-out of one catalytic subunit gene, *CanA-14F*, and of one regulatory subunit gene, *CanB*, have particularly pronounced effects, indicating that these are the predominant subunits regulating sleep. It is possible that sleep is regulated strictly by these subunits and that the smaller effects produced by deleting the other subunits occurs indirectly, due to compensatory changes in *CanA-14F* and *CanB*. On the other hand, *sra^{KO}* mutants show more severe sleep effects than any of the single CN gene mutants, suggesting that several CN isoforms affect sleep directly to different extents, and that the *sra^{KO}* phenotypes reflect additive effects of individual CN gene knock-outs.

Although *Sra* and other RCANs are well known as CN regulators (Hilioti and Cunningham, 2003; Davies et al., 2007; Stie and Fox, 2008), it is uncertain whether RCANs function to activate or inhibit CN activity (Kingsbury and Cunningham, 2000; Vega et al., 2003; Takeo et al., 2010). In our assays, both increases and decreases in CN activity inhibit sleep, suggesting that *Sra* may affect sleep through either mechanism. However, knock-outs of *CanA-14F* or *Pp2B-14D* suppress hypomorphic *sra* sleep phenotypes, supporting a model where decreased amount of sleep in *sra* mutants is caused by increased CN activity. While this result is clear, recent work has demonstrated that *Sra* is phosphorylated at two different serine residues, and that phosphorylation at these sites determines whether *Sra* activates or represses CN activity (Vega et al., 2002; Hilioti et al., 2004; Abbasi et al., 2005; Mehta et al., 2009). Thus, although the molecular mechanisms controlling *Sra* regulation of CN need to be further clarified, our data suggest that *Sra* functions to inhibit CN activity, at least under the conditions we examined.

Increases in sleep have been associated with memory formation (Ganguly-Fitzgerald et al., 2006), while sleep deprivation has

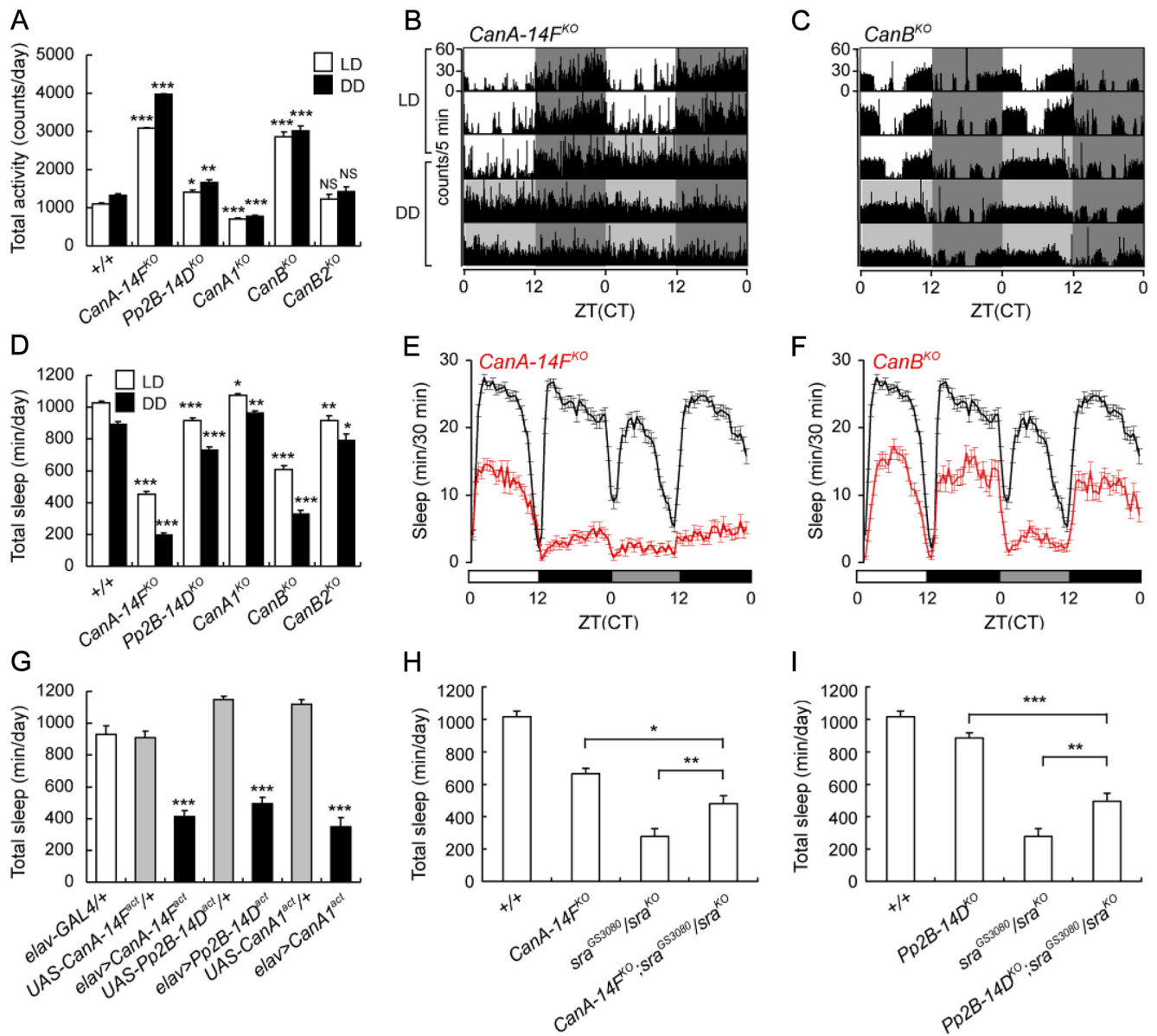


Figure 5. Both decreases and increases in CN activity inhibit sleep. **A, D**, Total daily activity (**A**) and total daily sleep (**D**) in knock-out mutants of all CN catalytic and regulatory subunit genes. Two-way ANOVA indicates significant differences in activity and sleep due to genotype, LD/DD cycle, and interaction between genotype and LD/DD cycle. All mutants, except *CanB2^{KO}*, show significant effects on activity, and all mutants show significant effects on sleep. **B, C, E, F**, Knock-outs of the *CanA-14F* catalytic subunit gene and of the *CanB* regulatory subunit gene show the most dramatic effects on activity and sleep, with approximately threefold increases in activity (**B, C**) and twofold decreases in sleep (**E, F**). **G**, Neuronal expression of all three *CanA^{act}* transgenes significantly reduces total daily sleep. One-way ANOVA indicates significant differences in sleep due to genotype. Bonferroni *post hoc* analyses indicate significant differences between wild-type and all three *elav>CanA^{act}* lines ($p < 0.001$ in all cases), but no differences between wild-type and *elav-GAL4/+* and all *CanA^{act}/+* controls. **H, I**, Genetic interaction between hypomorphic *sra* mutants (*sra^{GS3080}/sra^{KO}*) and *CanA-14F^{KO}* (**H**) or *Pp2B-14D^{KO}* (**I**). Total daily sleep amounts of wild-type, double mutants, and corresponding single mutant controls are shown. Both *CanA-14F^{KO}* and *Pp2B-14D^{KO}* suppress *sra* sleep phenotypes. In both **H** and **I**, one-way ANOVA indicates significant differences due to genotype. Bonferroni *post hoc* analyses indicate that amounts of sleep in both double mutants are significantly different from that of corresponding single mutants. Data in bar graphs and sleep profiles represent means \pm SEM; ***, **, and * indicate statistically significant differences with $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively. NS indicates that differences are not statistically significant.

been associated with inhibition of learning and memory (Seugnet et al., 2008; Li et al., 2009; Vecsey et al., 2009). Thus sleep is thought to be important for strengthening or maintaining synapses coding for memories, and decreases in sleep amounts may correlate with memory impairment. Conversely, the need to sleep may decrease when memories are not formed. Most, if not all, sleep mutants have memory defects, and CN and *sra* have also been shown to be important for memory formation and synaptic function in several species (Zeng et al., 2001; Chang et al., 2003; Lee and Ahn, 2004; Baumgärtel et al., 2008; Chang and Min, 2009; Christie-Fougere et al., 2009). Furthermore, both increases

and decreases in CN and *Sra* activity are associated with decreased memory (Mansuy et al., 1998; Foster et al., 2001; Chang et al., 2003). These results support a model where sleep and memory are linked; sleep need in CN and *sra* mutants may be decreased because memory and new memory-dependent synaptic connections are not made.

Our data are consistent with several possible roles of *Sra* and CN in sleep regulation. *Sra* and CN may be involved during development for the formation of neuronal circuits necessary for normal sleep, they may be involved acutely at the adult stage to directly regulate sleep, or both. Currently, all conditional expres-

sion systems that we have tried, including rescuing *sra* conditionally using available *sra* transgenes under heat-shock (Takeo et al., 2006) and gene switch control (Osterwalder et al., 2001) and inhibiting *sra* conditionally using RNAi transgenes, have been toxic to *sra* mutants, rescued *sra* in the absence of inducer, or failed to inhibit *sra* enough to produce sleep phenotypes. We believe that the extremely low amount of *sra* required for sleep (20–30% of wild-type amounts) is responsible for most of these problems.

Altered CN activity has been associated with several neurological disorders. For example, *Down's syndrome critical region 1* (*DSCR1*), a homolog of the *sra* gene, was identified from extensive studies of Down's syndrome (DS) patients with partial trisomy 21 (Epstein, 1995). Increased expression of *sra* is thought to alter CN activity, contributing to the mental defects observed in DS patients. In addition, a locus associated with susceptibility to schizophrenia has been mapped to *PPP3CC*, which encodes the mammalian CN gamma catalytic subunit (Gerber et al., 2003). Furthermore, mice with forebrain-specific knock-outs of CN display a spectrum of disorders strikingly similar to behaviors observed in schizophrenia patients (Miyakawa et al., 2003). Although alterations in sleep have been associated with both DS and schizophrenia, mechanistic connections between these diseases and sleep have been lacking. Our data suggest that these diseases may affect sleep through altered CN activity. It will be of great interest to determine whether both mental defects and sleep defects are caused by altered Ca^{2+} /CaM-dependent signaling in similar neuronal pathways.

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