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# A neuron-glia interaction involving GABA Transaminase contributes to sleep loss in *sleepless* mutants

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## Abstract

Sleep is an essential process and yet mechanisms underlying it are not well understood. Loss of the *Drosophila quiver/sleepless* (*qvr*/*sss*) gene increases neuronal excitability and diminishes daily sleep, providing an excellent model for exploring the underpinnings of sleep regulation. Here, we used a proteomic approach to identify proteins altered in *sss* brains. We report that loss of *sleepless* post-transcriptionally elevates the CG7433 protein, a mitochondrial γ-aminobutyric acid transaminase (GABAT), and reduces GABA in fly brains. Loss of *GABAT* increases daily sleep and improves sleep consolidation, indicating that *GABAT* promotes wakefulness. Importantly, disruption of the *GABAT* gene completely suppresses the sleep phenotype of *sss* mutants, demonstrating that GABAT is required for loss of sleep in *sss* mutants. While SSS acts in distinct populations of neurons, GABAT acts in glia to reduce sleep in *sss* flies. Our results identify a novel mechanism of interaction between neurons and glia that is important for the regulation of sleep.

### Keywords

sleep; GABA transaminase; glia; mitochondria; quiver/sleepless; *Drosophila*

## Introduction

Sleep remains among the most poorly understood of all biological phenomena, and disruptions of sleep are associated with many neuropsychiatric and neurological disorders (1). However, little is known about the molecular mechanisms that underlie sleep. Although wake-promoting and sleep-promoting networks in the brain appear to be involved in



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**Conflict of Interest**
The authors declare no conflict of interest.

switching behavioral states (2), the mechanisms by which different inter-cellular signals interact with each other and/or respond to physiological states of the brain to drive wake or sleep is largely unknown.

With any such process about which little is known, the best approach is a random, unbiased one that makes no assumptions about the molecules that may be involved. To this end, researchers have undertaken forward genetic screens in model organisms to identify sleep-regulating genes. One such screen in *Drosophila* led to the identification of *sleepless* (*sss*), a small GPI-anchored protein whose loss results in dramatic reductions in daily sleep, frequently >80% (3). The *sleepless* mutation is an allele of the *quiver*[1] locus identified previously by Wu and colleagues (4) in screening for mutants hypersensitive to reactive oxygen species. SSS protein was found to be a positive regulator of the voltage-gated potassium channel, Shaker, so in the absence of SSS, neural excitability is increased (5, 6). Loss of Shaker also increases excitability and reduces daily sleep (7). However, the *Shaker* mutants exhibit a much smaller decrease in sleep than *sleepless* mutants, suggesting that loss of Shaker function does not account for all the mechanisms underlying the sleep phenotype of *sleepless*.

We took an unbiased approach towards identifying the mechanisms underlying the sleep phenotype of *sss* mutants by conducting proteomic analysis of brain protein abundance. Our data reveal a novel connection between *sss* and the mitochondrial metabolism of γ-aminobutyric acid (GABA) by GABA transaminase (GABAT) in glial cells. *sss* fly brains display Increased GABAT and reduced GABA levels. We show that GABAT is a wake-promoting factor in *Drosophila*, and loss of GABAT completely suppresses the phenotype of the *sss* mutant and restores sleep. Tissue specific rescue experiments reveal that GABAT is required in glia to promote wakefulness in response to neuronal loss of *sleepless*. Interestingly, changes in GABAT are implicated in epilepsy and in neuropsychiatric disorders, which may account for some of the sleep abnormalities reported in these disorders (8).

## Materials and Methods

### Fly strains and DNA constructs

All fly stocks were maintained on standard molasses-cornmeal-yeast food. An isogenic $w^{1118}$ (iso31) strain was used as wild type in this study(3). $sss^{P1}$, Δ*sss* (Δ40), and UAS-*sss* were described by Koh et al(3, 5). The Gad1-GAL4, VGAT-GAL4, and Repo-GAL4 were gifts from Gero Miesenböck, Julie Simpson, and Vanessa Auld. The F01602 insertion was ordered from the Exelixis Collection at Harvard Medical School and the PL00338 insertion from the Bloomington Stock Center. Both F01602 and PL00338 stocks were outcrossed into the iso31 background for 7 generations. Wild type control strains (Con PL00338 and Con F01602) were established from siblings of PL00338 and F01602 heterozygotes prior to the cross for homozygosity.

To generate the gGABATtvh genomic transgene, a 4.9 kb HpaI/HindIII DNA fragment from the Bac clone BACR13N10 (BACPAC Resources Center) containing CG7433 was inserted into a pattB vector (9) with flanking FRT sites (Fig. 1B). To disable the *Tom20* gene in the

construct, the Lys6 residue of *Tom20* was mutated to an amber stop codon (AAA->TAA) by two primers, TomutF, 5' GTA ATA TGA TTG AAA TGA ACT AAA CTG CAA TCG GCA TTG 3', and tomutR, 5' CAA TGC CGA TTG CAG TTT AGT TCA TTT CAA TCA TAT TAC 3'. A 29 amino acid thrombin-V5-His6 tandem tag (LVPRGSGKPI PNPLLGLDST RTGHHHHHH) was then added to the C-terminus of CG7433. The final pattB-FRT-GABATtvh construct was used to generate transgenic flies with the phiC31 technique (9), which targets the transgene to the zh-attP-96E locus on the third chromosome (Rainbow Transgenic Flies, Inc). Transformants were confirmed with respect to insertion sites at the zh-attP-96E locus by genomic DNA PCR (with two primers: attBlanding5', 5' GAT CCA CTA GTG TCG ACG ATG 3' and ZH96E, 5' CGA AAT GTC GGC ATA TTG TG 3') and outcrossed to iso31 background for 7 generations.

To generate *GABAT* DNA constructs for GABAT constitutive overexpression in S2 cells, we slightly modified the pIZ-V5/His A vector (Life Technologies, Grand Island, NY) to generate a second NotI site after the V5-His6 tag to generate the DNA vector pIZ-VHn. The primers XbaI-BglII_CG7433f (5' aaa tct aga tct GAA ATT GAT AAA ATC CGA AC 3') and XbaI-CG7433r (5' aaa TCT AGA ATA CCT TGA AGA ACC TTG 3') were used to amplify the *Drosophila CG7433* cDNA clone RH42429 (*Drosophila* Genomics Resource Center) and the XbaI fragment of the PCR product was inserted into pIZ-VHn to generate pIZ-GABATvh where the *Drosophila* GABAT is tagged with a V5-His6 tag at the C-terminus. To generate the UAS-GABATvh transgene, the 1.8 kb NotI DNA fragment from pIZ-GABATvh containing the entire V5-His6 tagged CG7433 cDNA was subcloned into the NotI site of pUAST-attB (9) to generate a UAS-GABATvh construct, which was used to generate UAS-GABATvh transgenic flies with the transgene targeted to the 3$^{rd}$ chromosome at the zh-attP-96E site.

## Circadian behavior and sleep assays

To evaluate the locomotor activity rhythm, circadian period and sleep, flies were loaded in glass tubes containing standard molasses-cornmeal-yeast food, except for the experiments to rescue *sss* with GAL4 driven UAS-*sss* expression, where 5% sucrose with 2% agar was the food. For the gene-switch (GS) experiments or for the experiments where effects of drugs were tested, RU486 or drugs were added to the food as indicated. Flies were first entrained at 25°C in 12:12LD for two days and locomotor activity was recorded using the *Drosophila* Activity Monitoring System (Trikinetics, Waltham, MA) for 4 days in 12:12LD or > 7 days in constant darkness (DD) at 25°C. Activity counts were collected in 1 min bins and analyzed using ClockLab (Actimetrics, Wilmette, IL). Rhythmicity was determined initially by $\chi^2$ periodogram analysis for flies that had more than 5 days of activity data; flies were deemed arrhythmic if the activity data revealed no significant period and FFT (fast Fourier transform) values were lower than 0.01. For sleep analysis, only flies that survived the entire experiment were included in data analysis. pySolo (10) or a Matlab based custom software (3) was used for sleep analysis with the sleep definition being continued immobility of at least 5 minutes. Two methods were used to test for statistical difference in total (daily) sleep, daytime sleep, nighttime sleep, sleep bout number, sleep latency, and activity index in among genotypes. We used Student's *t*-test for pair-wise comparison between two genotypes and One-way ANOVA with Tukey's test for comparison among three or more

genotypes. These tests were done using JMP 9 or Graphpad Prism 5 software. As sleep bout length is not normally distributed, it cannot be depicted as an average. Thus, we determine how sleep is distributed among bouts of different length. We use the Mann-Whitney U (MWU) test in Microsoft Excel to compare two genotypes in terms of how their sleep bout numbers are distributed among four different duration categories (5-15, 15-50, 50-150, and >150 minutes) and then use the Bonferroni method to adjust for multiple genotype comparison. Fraction of sleep time in each of the four categories of sleep bout duration is then plotted for qualitative comparison.

### 2D-DIGE proteomic analysis

4-6 days old wild type and *sss*Δ flies were entrained to 12:12LD at 25°C and collected in dark immediately before ZT0 (Zeitgeber Time 0, when lights are turned on). About 700 brains (˜350 each from males and females) from each genotype were dissected in PBS buffer (pH7.4) on ice and stored at −80°C before protein extraction. Protein extraction, CyDye Fluor labeling, 2D-DIGE, data imaging, and statistical analysis of protein levels with DeCyder were performed at the Proteomic Core Facility of the University of Pennsylvania, as described by Marouga *et al* (11). Briefly, after protein extraction, a portion of protein was partially labeled (1-3% of total protein being labeled) with three CyDyes for each 2D-gel for imaging and statistical analysis: 50 μg wild type protein was labeled with Cy3, 50 μg *sss*Δ protein was labeled with Cy5, and 25 μg wild type plus 25 μg *sss*Δ protein was labeled with Cy2. To increase the protein amount for protein spot identification, 300 μg wild type plus 300 μg of *sss*Δ protein was loaded onto the third 2D-gel in addition to the CyDye labeled samples. The first dimension was run on pH3-10NL isoelectric focusing stripes (Amersham) and the second dimension on 30 cm x 30 cm SDS-PAGE gels (Jule Inc). After electrophoresis, Cy3, Cy5 and Cy2 images were scanned and the volumes of protein spots were analyzed using DeCyder software. The Cy2 signal was used as internal control across all three independent gels. The *sss*Δ:wild type volume ratios for each protein spot were determined relative to the Cy2 signal as internal standard and were used to calculate changes in average abundance and statistical probability by Student's *t*-test across all three gels. Protein spots with an average of more than 1.5 fold difference and statistical significance ($P<0.01$) were marked and some of them were selected to be picked up by robot, trypsin-digested, and subjected to LC tandem mass-spectrometry for protein identification. The tandem mass spectrometry data were then analyzed by the Sequest software against the *Drosophila* proteome database to identify protein candidates corresponding to the spots. CG7433 was identified as a likely candidate gene from five spots.

### Amino acid analysis

Flies were entrained at 12:12LD, 25°C and collected for brain dissection before the lights-on transition (ZT0). Twenty brains from each genotype were homogenized in 100 μl water on ice and subsequently deproteinized by passing through a filter with a 10,000-Dalton molecular weight cut-off (Millipore, Bedford, MA) via centrifugation. Flow-through for each genotype (40 μl) was then subjected to phenylisothiocyanate (PITC) derivatization. After the samples were cleaned up and dried, they were dissolved in 100 μl Pico-Tag diluent according to the Pico-Tag manual from the manufacturer (Waters, Milford, MA). GABA (10 nmole) and a few other L-amino acid standards were also treated in parallel with the brain

samples to identify elution time of these amino acids. A Pico-Tag free amino acid analysis column (3.9 x 300 mm) was attached to a Waters HPLC system and run at 1 ml/min at ambient temperature. After equilibrating with Buffer A [70 mM sodium acetate (pH 6.45 with 10% acetic acid)–acetonitrile (975: 25)] (Waters), for each run, 50 μl of brain samples or standards were injected onto the column and eluted with standard gradients consisting of combinations of Buffer A and Buffer B [water–acetonitrile–methanol (40: 45: 15)], as recommended for physiologic amino acids by the manufacturer. At room temperature, GABA standards were eluted by 100% buffer A at 7.31 ± 0.06 minutes (mean ± stdev).

## Immunohistochemistry

To staining for GABAT expression with MitoSOX in Schneider 2 cells, *Drosophila* Schneider 2 (S2) cells were cultured in Schneider's *Drosophila* Medium (Invitrogen) at 25°C with standard techniques. Before transfection, cells were dislodged into fresh medium and diluted to $10^6$ cells/ml. pIZ-GABATvh (50 ng/$10^6$ cells) were prepared to transfect the S2 cells with Effectene Transfection Reagent (Qiagen) according to the manufacturer's protocol. After transfection, the cells were dispended $0.5\times10^6$ cells/well onto a Lab-Tek chambered coverglass (Fisher Scientific, Pittsburgh, PA). Forty eight hours later, the cells were then incubated with 1 μM MitoSOX in PBS buffer (pH 7.4) for 5 minutes to mark for mitochondria. After washing with PBS buffers, the cells were fixed with 4% para-formaldehyde (PFA) in PBS for 30 min at room temperature. After three 5 min washes with 0.3% Triton X-100 in PBS, samples were incubated with 5% normal donkey serum (NDS) in PBS-Triton X-100 and with primary antibodies rabbit anti-V5 (Bethyl Laboratories) in 5% NDS (1:1000 dilution) for 1 hour each at room temperature. After three 20 min washes, cells were incubated with donkey FITC anti-rabbit secondary antibody (1:300 dilution) (Jackson ImmunoResearch Laboratories) in 5% NDS for 2h at room temperature, followed by extensive washes. Cells were mounted with Vectashield mounting medium (Vector) and protein staining (FITC) was compared with MitoSOX staining using a Leica TCS SP5 confocal microscope. Untransfected cells without a FITC signal served as negative controls that only stain with MitoSOX.

To co-stain GABATvh and GAD1-YFP in the brains, $GAD1^{CPTI000977}$/+;gGABATtvh/+ transheterozygous flies were dissected in PBS buffers. After fixing with 4% para-formaldehyde (PFA) in PBS for 30 min at room temperature, the brains were washed with PBS extensively and sequentially incubated with 5% NDS in PBS-Triton X-100 and with Alexa Fluor 647 conjugated V5 mouse monoclonal antibody (Invitrogen) (1:1000 dilution) in 5% NDS followed by extensive washes. The brains were mounted with Vectashield mounting medium (Vector) and V5 staining for GABATtvh (Alexa Fluor 647) was compared with the YFP signal from GAD1-YFP using a Leica TCS SP5 confocal microscope.

## Quantitative real-time PCR and Western blot analysis

Flies were collected for brain dissection in 12:12LD, 25°C less than 30 minutes before the lights-on transition (ZT0). Brains were dissected on ice and total RNA was isolated using Trizol isolation system (Invitrogen) from the brain, and cDNAs were synthesized by using a high-capacity cDNA Archive kit (Life Technologies, Grand Island, NY). Quantitative real-

time PCR (qPCR) was performed using an ABI prism 7100 with a SYBR Green kit (Life Technologies).

For Western blot analysis with fly heads, flies were collected on dry ice before lights-on (ZT0) in 12:12LD, 25°C and fly heads were separated for protein extraction using standard cell lysis protocol (12). After SDS-PAGE, proteins were transferred onto nitrocellulose membrane and processed for antibody incubation. Primary antibodies mouse anti-V5, and mouse anti-β-Actin were used at 1:3000 dilution. Following enhanced chemiluminescence, images were taken (12) via a Kodak image station.

## Results

### GABA transaminase is increased in *sleepless* mutants

To identify mechanisms underlying the sleep phenotype of *sleepless* (*sss*) flies, we screened for proteins mis-regulated in *sss* brains. We collected wild type and *sss* flies at the end of the night, following entrainment to 3 days of 12hr-light:12hr-dark (12:12LD) cycles, dissected ~700 brains for each genotype and subjected the extracts to two dimensional Differential In Gel Electrophoresis (2D-DIGE) (13). A >1.5 fold change and statistically significant ($P<0.01$) difference in abundance between the two genotypes was used as a cut-off to select 20 protein spots for identification by LC tandem mass spectrometry analysis (LC-MS/MS). A gene annotated CG7433 was identified as a leading candidate in five of these 20 spots, showing a 2-3 fold increase in the *sss* mutant (Fig. 1A). To confirm that the *sss* mutation leads to an increase in CG7433, we generated a transgene of the CG7433 genomic locus with a Thrombin-V5-His6 tandem tag (TVH) attached to the C-terminus of the coding sequence (Fig. 1B). This genomic transgene, termed gCG7433tvh, was introduced into wild type and *sss* mutant flies and levels of the epitope tagged transgenic CG7433 protein (CG7433tvh) were assayed by western blot analysis using an anti-V5 antibody. Levels of the CG7433tvh protein were increased in the brains of both male and female *sss* flies, based upon western blots (Fig. 1C, average of 54% increase, $P<0.05$) and protein purification from fly heads (data not shown), confirming the proteomic results that CG7433 is increased in *sss* mutants. However, CG7433tvh levels showed no detectable increase in flies deficient for *Shaker* (data not shown), which could be due to the weaker sleep phenotype of *Shaker* or a Shaker-independent effect of *sleepless* on CG7433.

Tissue specific mRNA profiling indicated that CG7433 expression is highly enriched in the adult brain and thoracic-abdominal ganglia (14). It is also broadly expressed in neuropil-associated glia and subperineurial glia during late embryonic stages (15). Analysis of the coding sequence revealed that CG7433 encodes a protein that has a 23-amino-acid N-terminal mitochondrial signal peptide (16) and its primary amino acid sequence is highly similar to both human and pig γ-aminobutyric acid transaminases (GABAT) (56% identity) (Fig. S1). Pig GABAT requires pyridoxal 5'-phosphate (PLP) as a co-factor for its enzymatic activity and it homodimerizes via two cysteines to form a [Fe2-S2] cluster (17). BLAST analysis indicates that all 8 amino acids required for PLP binding and the two cysteine residues required for homodimerization are conserved between CG7433, pig, and human GABATs (Fig. S1).

We next expressed CG7433 with a C-terminal V5-His6 tag in *Drosophila* S2 cells and confirmed that it colocalizes with a mitochondrial dye, MitoSox (Fig. 1D). GABATs typically break down GABA in the mitochondria by removing an amino group to form succinic semialdehyde (SSA). To further confirm the predicted function of CG7433 as a GABA transaminase, we performed amino acid analysis to evaluate GABA levels in brain extracts of wild type flies, $sss^{P1}$ mutants and two putative mutant alleles of CG7433 (PL00338 and F01602). The PL00338 allele consists of a *piggybac* GAL4 enhancer trap insertion in the 5' untranslated region of CG7433 and F01602 contains an Exelixis *piggybac* WH insertion in the last exon of CG7433, disrupting 20 amino acids prior to the C-terminus (Fig. 1B) (18, 19). The $sss^{P1}$ mutation dramatically reduces brain GABA levels to about ~30% of those in wild type flies, while PL00338 and F01602 boost GABA levels ~2 and 3 fold respectively (Fig. 1E). These data support the identification of CG7433 as a GABA transaminase (GABAT) and so we refer to it as such hereafter, and refer to the genomic transgene gCG7433tvh and the two alleles, PL00338 and F01602, as gGABATtvh, $gabat^{PL}$ and $gabat^{F}$, respectively.

Notably, GABAT is detected as multiple spots horizontally on 2D gels (Fig. 1A), likely due to post-translational modifications that lead to significant changes in isoelectric point but little change in molecular weight. Analysis of *GABAT* mRNA levels from brains by reverse transcription quantitative PCR revealed no significant difference between wild type and *sss* flies (Fig. 1F). This supports the idea that the altered GABAT levels in *sss* mutants also occur at a translational or post-translational level. On the other hand, *GABAT* mRNA levels are negligible in the $gabat^{PL}$ mutant (1% of wild type level) and reduced to 35% in $gabat^{F}$. Thus, $gabat^{PL}$ is overall a more severe allele than $gabat^{F}$. However, because the transposon in the $gabat^{F}$ allele is located in the last exon of the protein coding region, the reduced GABAT mRNA is predicted to produce a truncated protein. Through these experiments we established that GABAT levels are increased post-transcriptionally and GABA levels are decreased in the brains of *sss* flies. Conversely, disruption of the *GABAT* gene increases GABA in fly brains.

## GABA transaminase is a negative regulator of sleep

GABA is a major inhibitory neurotransmitter that promotes sleep in both mammals and *Drosophila* (2, 20-22). However, it is not known how a mitochondrial enzyme such as GABAT transaminase might affect sleep. Since $gabat^{PL}$ and $gabat^{F}$ mutants have increased GABA in the brain, we asked if they promote sleep. Sleep was assayed in the *gabat* mutant flies and their respective genetic background controls (con PL00338 and con F01602), derived from outcrossing at 25°C, in the presence of standard 12:12LD cycles (3). *GABAT* mutant flies showed significant increases in both daytime and nighttime sleep, amounting to an increase in total daily sleep time of >3 hrs for each of the two mutants, $gabat^{F}$ and $gabat^{PL}$ (Fig. 2A and 2B). Strikingly, mutant flies also showed a dramatic increase in the fraction of sleep spent in long (>150min/sleep episode) sleep episodes, with such episodes accounting for 50-60% of total daily sleep for the mutants compared with approximately 20% for wild type controls (Fig. 2C). In addition, the mutants exhibited a marked decrease in sleep episode number at night (Fig. 2D). Longer episode length and reduced episode number together indicate better consolidation of sleep at night in the *gabat* mutants. During the

daytime, an increase in episode number also contributes to the increased sleep of *gabat*$^{F}$ flies. Lastly, we also measured sleep latency, or the time the flies take to fall asleep after lights-off at ZT12, an indicator of mechanism underlying sleep initiation (23). The *GABAT* mutant flies showed significantly reduced sleep latency compared with their respective wild type controls (Fig. 2E). However, when awake, these mutants showed at least as much locomotor activity as controls (Fig. 2F), suggesting that they are not hypoactive. To summarize, loss of GABA transaminase promotes daily sleep amount and sleep consolidation in *Drosophila*.

Next we examined if the gGABATtvh genomic transgene could rescue the increased sleep in the *gabat*$^{PL}$ mutant. Indeed, introduction of the genomic GABAT transgene reduced sleep of *gabat*$^{PL}$ to levels similar to or lower than those of wild type controls (Fig. 2G). In addition, the mutants carrying the genomic transgene showed increased sleep bout number and reduced sleep bout duration, demonstrating a reduction in sleep consolidation (Fig. 2H and data not shown). Finally, sleep latency of the *gabat*$^{PL}$ mutant was also restored to wild type levels (Fig. 2I). These data demonstrate that loss of the GABAT protein accounts for the altered sleep time and sleep architecture in *gabat*$^{PL}$ flies. Thus, GABAT transaminase is a wake-promoting molecule in *Drosophila melanogaster*.

To exclude the possibility that a dysregulated circadian clock causes the sleep phenotypes in *gabat* mutants (24), we tested the locomotor behavior of *gabat* mutants in constant darkness. Although *gabat*$^{F}$ flies showed some arrhythmicity, most *gabat*$^{PL}$ flies were rhythmic and neither genotype showed statistical differences in circadian period from wild type controls (Fig. 3H and Table S1). The lack of a circadian phenotype in *gabat*$^{PL}$ , which is a very severe allele of *gabat* and has a strong sleep phenotype, indicates that clock disruption does not cause the sleep phenotype. More likely, the excessive sleep in some *gabat*$^{F}$ flies masks an underlying circadian rhythm. Double mutant analysis (see below) supported the idea that circadian rhythms are largely unimpaired in *gabat* flies. These findings also indicate that the GABAergic inputs required for maintenance of circadian rhythms in *Drosophila* (21, 25) are not directly impacted by loss of GABAT.

### Disruption of *GABAT* restores sleep specifically in *sss* mutants

To test if the severely reduced sleep in *sss* is due to elevated GABAT levels, we crossed *gabat* mutations into a *sss*$^{P1}$ background to reduce GABAT. Indeed, the *gabat*$^{F}$ mutation suppressed the sleep phenotype of *sss*$^{P1}$ mutants, completely restoring nighttime sleep to levels observed in the *gabat*$^{F}$ mutants alone (Fig. 3A and 3B). In addition, the *gabat*$^{F}$;*sss*$^{P1}$ double mutants showed an increase in the time spent in long sleep episodes (>150 min/sleep episode) and reduced nighttime sleep episode number, equivalent to that in *gabat*$^{F}$ flies (Fig. 3C and 3D, respectively). The increased sleep latency in *sss* flies was also completely suppressed in the *sss*$^{P1}$;*gabat*$^{F}$ double mutant to the level of *gabat*$^{F}$ mutants (Fig. 3E). Even though stronger suppression of the short sleep phenotype was observed when the stronger *gabat*$^{PL}$ allele was coupled with *sss*$^{P1}$ (Fig. S2), only the data for *sss*$^{P1}$;*gabat*$^{F}$ double mutants are shown here for the sake of easier comparison with data from *dat*$^{fmn}$;*gabat*$^{F}$ double mutants (see details below, Fig. 4).

To test whether the *sss* $^{P1}$ phenotype is caused by high GABAT expression in adults, and not only during development, we conditionally inhibited the enzyme in adult male flies using the pharmacological agent ethanolamine O-sulfate (EOS), which is a specific, competitive, and catalytic inhibitor of GABAT (26). 10mM EOS treatment partially rescued nighttime sleep in sss$^{P1}$ (Fig. 3F), suggesting that the *sss* $^{P1}$ phenotype is at least in part due to increased GABAT activity in adults. Importantly, these data indicate that the relevant GABA/GABAT circuits are in place in *sss* adults for the drug to have an effect. Results from a genetic rescue experiment of sss$^{P1}$, using an inducible panneuronal driver (*elav*-GS) to express *sss* specifically in adults, corroborated these findings. Partial rescue of the *sss* phenotype was obtained, supporting an adult function and perhaps also an effect during development (Fig. 3G).

Although *sss* flies have a functional clock (Fig. 3H), they display weak activity rhythms, most likely because of the reduced sleep (3). We found that *gabat*$^{F}$ also strengthens circadian activity rhythms of *sss*$^{P1}$ and improves rhythmic activity in constant darkness with no apparent change in circadian period (Fig. 3H and Table S1). However, neither *gabat* mutant rescued the ether-induced leg shaking phenotype of *sss*$^{P1}$ (data not shown), clearly indicating that different mechanisms underlie the leg shaking and loss of sleep phenotypes of *sss*.

Since *gabat* increases sleep and *sss* decreases sleep, independent and additive effects of the two mutants should produce only a moderate restoration of sleep in the *gabat*$^{F}$;*sss*$^{P1}$ double mutant compared with *sss*. The complete and epistatic suppression of night-time sleep and overall sleep quality of *sss* by mutations in *gabat* indicates that an additive effect is unlikely. In other words, the two genes appear to be in the same sleep-regulating pathway with the effect of the *gabat* mutant being epistatic to that of *sss*. Nevertheless, we investigated the possibility of additive effects by testing if *gabat* mutants restore sleep in a different severe short-sleeping mutant *dat*$^{fmn}$, which has a single nucleotide change that disrupts the dopamine transporter gene (27).

Since *dat*$^{fmn}$;*gabat*$^{PL}$ flies are homozygous-lethal, we compared the behavior of *dat*$^{fmn}$;*gabat*$^{F}$ flies with that of each of the two single mutants. Male *dat*$^{fmn}$;*gabat*$^{F}$ flies exhibited ~6 hrs of daily sleep, which represents a significant increase over ~1.5 hrs in *dat*$^{fmn}$ flies, but only accounting for ~30-40% of the daily sleep seen in wild type control flies (Fig. 4A and 4B). Meanwhile, the increase in sleep in the *dat*$^{fmn}$;*gabat*$^{F}$ flies was largely during the daytime, with the increase in nighttime restricted mostly to the very early night; over the course of the night, sleep in the double mutant decreased to levels seen in *dat*$^{fmn}$ single mutants (Fig. 4A). Furthermore, sleep in *dat*$^{fmn}$;*gabat*$^{F}$ flies was as or even more fragmented than in *dat*$^{fmn}$ flies, in contrast to *gabat* mutants and *sss*$^{P1}$;*gabat*$^{F}$ double mutants where long sleep bout durations are indicative of well-consolidated sleep (compare Fig. 3C with Fig. 4C). In fact, the increased sleep in *dat*$^{fmn}$;*gabat*$^{F}$ over *dat*$^{fmn}$ was largely due to an increase in sleep bout number (Fig. 4D). Interestingly, although it was largely ineffective in rescuing other aspects of sleep architecture, *gabat*$^{F}$ rescued sleep latency in the *dat*$^{fmn}$ flies (Fig. 4E). As latency to sleep is measured after lights-off in the early evening, increased sleep at this time may have accounted for the decreased latency in the double mutant. These data indicated that incorporation of *gabat*$^{F}$ in the short sleeping *dat* mutant only restores sleep latency and not any other sleep parameters.

The differential rescue of the various sleep parameters of *dat*$^{fmn}$ mutants by *gabat*$^{F}$ indicates that genetic interactions between *DAT* and *GABAT* are complicated. In the double mutant *dat*$^{fmn}$;*gabat*$^{F}$ flies, the sleep promoting effect of *gabat*$^{F}$ mutation dominates to reduce sleep onset and sleep amount during the early evening (Fig. 4A). However, the wake promoting *dat*$^{fmn}$ dominates in shortening the sleep bout length throughout the night and reducing sleep amount during the late evening. On the other hand, all sleep parameters of *sss* mutants are completely suppressed by the *gabat* mutants (Fig. 3). If high levels of GABAT (and low GABA) were always a consequence of short sleep, disruption of *gabat* would be expected to suppress all short-sleeping mutants. Given that GABAT promotes wakefulness (Fig. 2) and loss of GABAT strongly suppresses the short-sleep phenotype of *sss* (Fig. 3) but not that of *dat*$^{fmn}$ (Fig. 4), we conclude that elevated GABAT is relevant for the sleep loss of *sss* mutants.

## SSS likely acts in GABAergic neurons to promote sleep

To identify the cellular link between SSS and GABAT, we next examined whether restoring SSS expression in GABA producing neurons rescues sleep in *sss* mutants. In *Drosophila*, GABA is synthesized by glutamic acid decarboxylase 1 (Gad1) and packaged presynaptically into vesicles by the vesicular GABA transporter (VGAT). It is widely accepted that once GABA is released, it can be re-taken up by pre-synaptic neurons for recycling or by apposed glial cells for degradation (28).

We showed previously that *sss* cDNA, expressed panneuronally or in cholinergic cells with the UAS-GAL4 system, rescues the sleep phenotype of *sss* mutants (5). In light of the suppression of *sss* by *gabat*$^{F}$, we drove expression of UAS-*sss* in GABAergic neurons of *sss*$^{P1}$ animals using either a Gad1-GAL4 (29) or a VGAT-GAL4 transgene (30). Both GABAergic GAL4 drivers were able to significantly restore daily sleep to 80% and 90% that of wild type, respectively (Fig. 5A and 5B), with effects on both daytime and nighttime sleep. The restored sleep was due to both lengthened sleep bout duration and increased bout number (Fig. 5C and 5D). Lastly, SSS expression in GABAergic cells completely rescued sleep latency to wild type levels (Fig. 5E). Thus, directing UAS-*sss* expression to GABAergic cells using either Gad1-GAL4 or VGAT-GAL4 can rescue the *sss* mutant. The rescue by VGAT-GAL4 is consistently better than that by Gad1-GAL4 as it restores higher levels of nighttime sleep and longer sleep bout duration. Since expression of SSS in either GABAergic or cholinergic neurons almost completely rescues the sleep phenotypes of *sss*, we suggest that at least a subset of SSS expressing neurons are both GABAergic and cholinergic.

We also tried to rescue *sss* by driving expression of SSS in GABAT cells, using the GAL4 enhancer trap allele of *GABAT* (PL00338), and in glia, using Repo-GAL4. Other than a slight (2-hour) increase in daytime sleep driven by Repo-GAL4, neither driver rescued the *sss* short sleep phenotype. Both drivers failed to rescue the following sleep parameters of the *sss* mutant—nighttime sleep, sleep bout duration, nighttime sleep bout number, and sleep latency (Fig. 5A-E). Therefore, while SSS may function in GABAergic cells, it does not act to modulate sleep in GABAT cells, nor in glia. Thus, loss of *sleepless* may act non-cell-autonomously to up-regulate GABAT levels.

### GABAT acts in glia to modulate sleep in *sss* mutants

To further confirm the non-cell-autonomous regulation of GABAT by the loss of *sleepless*, we next asked where GABAT expression is required for the short sleep phenotype of *sss* mutants. To this end, we generated a UAS transgene of V5-His6 tagged GABAT (UAS-GABATvh) and drove its expression in different cells of the $sss^{P1}$;$gabat^{F}$ double mutant using various GAL4 drivers. As expected, presence of the UAS-GABATvh transgene alone had minimal effects on sleep in $sss^{P1}$;$gabat^{F}$ flies (Fig. 6). However, restoring the expression of GABAT in GABAT cells with the GAL4 enhancer trap allele, PL00338, diminished nighttime sleep to ~35% that of *sss*;*gabat* (Fig. 6A and 6B, compare orange with blackxs), indicating partial restoration of the *sss* phenotype (Fig 6A, compare orange with red). In addition to a reduction in total sleep, the sleep was more fragmented and sleep latency increased, even though the number of sleep bouts remained unchanged (Fig. 6C to 6E). These data demonstrate that expression of GABAT in its native pattern of expression can restore a *sss*-like phenotype in *sss*;*gabat* double mutants. Strikingly, restoring the expression of GABAT in glial cells with Repo-GAL4 was equally effective in reducing sleep of the *sss*;*gabat* double mutants (Fig. 6A and 6B, compare black, orange and blue). The restoration in glia was also accompanied by fragmented sleep bouts and increased sleep latency (Fig. 6C to 6E). On the other hand, expression of GABAT by the Gad1-GAL4 driver did not restore the short-sleeping phenotype of $sss^{P1}$ (Fig. 6, grey), although Gad1-Gal4 driven expression of SSS is sufficient to rescue the $sss^{P1}$ mutant (Fig. 5). These data indicate that GABAT expression in glia is required to promote wakefulness in the *sss* mutant. Thus, SSS is likely required in GABAergic neurons for normal sleep and loss of SSS non-cell-autonomously modulates GABA transaminase in glia, indicating that a neuron-glia interaction mediates the loss of sleep in *sss*.

We next compared the protein expression patterns of GABAT and GAD1, using the tagged GABATtvh transgene and a transposon insertion, carrying a yellow fluorescent protein (YFP) fluorescent marker, in the Gad1 gene, CPTI000977 (31). The CPTI000977 insertion truncates the GAD1 protein and fuses a YFP reporter to its C-terminus (GAD1-YFP). Fluorescent images of brains stained for GABATtvh and GAD1-YFP are largely different from each other (Fig. 6F and 6G). GABATvh is often found neighboring, but not overlapping with the GAD1-YFP signal (Fig. 6G), suggesting that in *Drosophila* GABAT is largely expressed in non-GABAergic cells. Interestingly, CG1732, the GABA:sodium symporter, required for GABA re-uptake from extra-cellular space in *Drosophila*, is expressed in a subset of lateral glial cells in the ventral nerve cord at embryonic stage 15-16 and in glia-like cells in adult brains (32, 33). Expression of CG1732 has thus far only been reported in glia, which is consistent with the idea that GABAT acts in glia to break down GABA following re-uptake. Together the expression data support our behavioral analysis that expression of GABAT in non-GABAergic cells, specifically glia, is required for the short sleep phenotype in *sleepless* mutants.

## Discussion

GABA is an inhibitory neurotransmitter that promotes sleep in both mammals and *Drosophila*. In fact, benzodiazepines, a class of $GABA_A$ receptor blockers, are widely

prescribed for insomnia (34). However, little is known about the regulation of GABA signaling in the context of sleep:wake cycles. Our data demonstrate that a mitochondrial enzyme, GABA transaminase, expressed in glia regulates GABA levels to affect sleep. GABA transaminase catalyzes GABA turnover to succinic semialdehyde, which in turn is turned over into succinic acid by succinic semialdehyde dehydrogenase, recycling the carbon backbone to the Krebs cycle (35). We note that the role of mitochondria in the control of GABA levels is generally under-appreciated, but our work here indicates that it provides an important link between cellular metabolism and GABA-regulated processes such as sleep.

Notably, it was an unbiased approach that led us to the discovery of the *Drosophila* CG7433 gene, which turns out to encode a GABA transaminase (GABAT) upregulated in *sss* brains. We propose that increased GABAT is a mechanism underlying the loss of sleep in *sss*, rather than a mere consequence of sleep loss, for the following reasons. Firstly, disruption of the *GABAT* gene results in increased GABA levels, total sleep, and sleep consolidation, indicating that GABAT itself is a *bona fide* negative regulator of sleep. Secondly, *sss* mutant brains show increased GABAT and decreased GABA levels, which are predicted to decrease sleep and/or sleep consolidation. Furthermore, increased GABAT as a homeostatic response to the loss of sleep in *sss* is unlikely as it would only exacerbate the sleep loss. Lastly, disruption of *GABAT* completely and specifically represses the sleep phenotype of $sss^{P1}$, confirming both that GABAT has a role in sleep regulation and that GABAT acts downstream of SSS to modulate sleep. Another sleep promoting molecule downstream of GABAT activity is γ-hydroxybutyric acid (GHB) (36). Due to low similarity of the primary sequence of this enzyme among different species, we are uncertain if *Drosophila* contains succinic semialdehyde reductase, which catalyzes a product of GABAT, SSA, into GHB. However, increased GABAT activity in *sss* and loss of GABAT in *gabat* would be predicted to increase and reduce downstream GHB levels, and hence sleep, respectively, contrary to the observed sleep phenotypes of *sss* and *gabat*. We therefore exclude GHB as relevant in the mechanism underlying the sleep phenotypes of *sss* and *gabat*.

Latency to sleep is used as a clinical measurement of sleepiness for human patients, and was shown by Griffith and colleges to be regulated by the kinetics of $GABA_A$ receptor signaling in *Drosophila* (21). Patients with sleep onset insomnia have prolonged latency to sleep. We show that latency to sleep onset is altered in both *sss* and *gabat* mutants, severely lengthened in the *sss* mutants (Fig. 3E) and reduced in *gabat* mutants (Fig. 2E), supporting the idea that GABAT affects $GABA_A$ signaling to regulate sleep. In addition, although the *gabat* mutants, like other sleep mutants, accumulate modifiers of the baseline sleep phenotype over time, their reduced latency to sleep remains intact (data not shown). Interestingly, the *gabat* sleep latency phenotype even dominates in both double mutant backgrounds – $sss^{P1}$; $gabat^{F}$ as well as $dat^{fmn}$;$gabat^{F}$, in which loss of *gabat* is coupled with potentiated dopamine signaling (Fig. 4E). The latter suggests that the sleep promoting effect of *gabat* is dominant to the wake promoting effect of dopamine at lights-off (ZT12, when sleep latency was measured). The reduced sleep during the later night and the short duration of sleep bouts in $dat^{fmn}$;$gabat^{F}$ animals (Fig. 4A and 4C) indicate that the potentiated dopamine signaling

dominates over *gabat* as the night proceeds. This is vastly different to the increased sleep throughout the night in *gabat* or *sss*;*gabat* mutant flies (Fig. 2A and Fig. 3A).

We showed previously that *sss* mutants can be rescued by expression of SSS in cholinergic neurons. The present study, indicating a role for GABAergic neurons is not inconsistent with the previous result, as co-expression of GABA and acetylcholine has been reported previously (37). We suggest that loss of *sss* in cholinergic cells increases cholingeric signaling, which may generally reduce sleep. However, the effect on GABAT reported here likely leads to a lasting and profound sleep loss.

Our data indicate that loss of SSS non-cell-autonomously affects glial GABAT. It is not clear how this cell non-autonomy is achieved, especially given that GABAT is a mitochondrial protein. As loss of SSS causes hyperactivity of neurons that normally express it, this may lead to higher energy demands on surrounding glial cells via a glia-neuron metabolic shuttle, such as the lactate-pyruvate shuttle (38), and affect mitochondrial metabolism in glia. A computational model simulating an astrocyte–GABAergic neuron cellular complex predicts that substantially increasing GABAergic neuron activity would lead to both increased lactate release from glia to neurons and increased GABA turnover to SSA in glia (i.e. increased GABAT activity) (32). We note that increased excitability of GABAergic neurons in *sss* mutants may not only increase GABAT in glia, but could also ultimately cause depletion of GABA from the GABAergic neurons. Indeed, repetitive stimulation of GABAergic neurons is reported to deplete GABA and reduce the number of recycling vesicles (39). Mammalian data suggest that GABA can also be released from glia in a tonic fashion through an anion channel (40). Should this be the case, modulating GABAT activity would regulate the glial GABA level and therefore its tonic release to the extra-neuronal space, providing a means to regulate sleep through tonic GABA inhibition (41).

An alternative explanation for the increased GABAT is that increased activity of GABAergic neurons triggers a homeostatic response in neighboring glia. As noted earlier, we did not observe increased GABAT in *Shaker* mutants although these also have high neural activity and display decreased sleep. It is important to note though that the sleep phenotype of *Shaker* mutants is much weaker than that of *sss* flies(3, 7). It is not even known if Shaker and SSS act in the same cells to regulate sleep, although SSS affects Shaker activity at the larval neuromuscular junction in a cell-autonomous manner (5). Even if they do act in the same cells, effects of SSS may be greater as it appears to affect channels other than just Shaker (unpublished observations). Regardless of the differences between SSS and Shaker, an effect of neural activity on GABAT in glia is probably not restricted to *sss* mutants. High neural activity in epilepsy is associated with sleep disruption (42) and, interestingly, it is commonly treated by an inhibitor of GABAT, vigabatrin(43). To date there are only 3 reported cases of patients with GABAT deficiency, compared with hundreds of cases for deficiencies of other enzymes of the GABA shunt such as glutamic acid decarboxylase and succinic semialdehyde dehydrogenase (44, 45), hindering genetic and clinical studies on the human enzyme. These patients exhibited elevated GABA concentrations in the plasma, the cerebrospinal fluid (44) or the basal ganglia (45), and displayed symptoms that include hypotonia and lethargy. Vigabatrin, an irreversible

inhibitor of mammalian GABAT used clinically, is known to induce sleepiness in humans (46), and promote non-rapid-eye-movement (NREM) sleep in amygdala-kindled rats (47). Thus, GABAT may have a conserved role in the regulation of sleep. Our data, generated as a result of an unbiased proteomic analysis, provide a mechanistic basis and an animal model to understand the role of GABAT in sleep regulation. Importantly, our studies show that GABAT can be regulated through a cell-non-autonomous and post-transcriptional mechanism.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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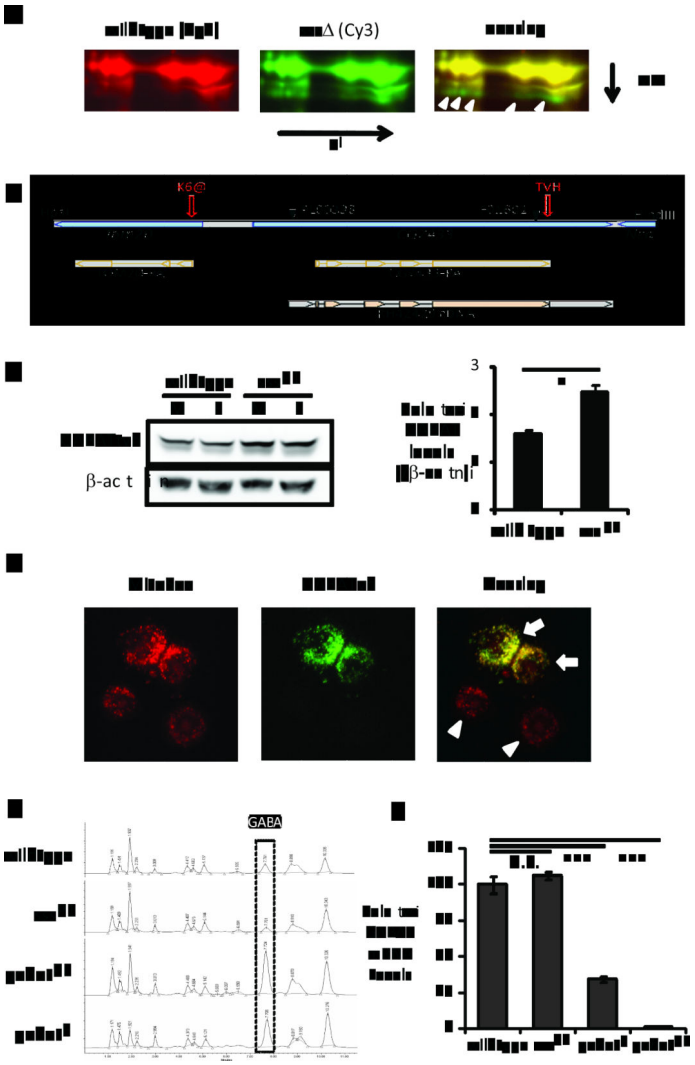


**Figure 1.**

GABAT is increased and GABA is decreased in *sss* flies. (**A**) 2D-DIGE of *sss* and wild type brain proteins. Left, proteins in wild type brains stained with Cy3. Middle, proteins in *sss* mutants stained with Cy5. Right, overlay image of Cy5 and Cy3 staining. Shown in the image is only a zoom-in crop of the 2D-DIGE images. The yellow color denotes similar protein levels and the green color indicates proteins increased in *sss* mutants. White open arrows point to the protein spots that are increased in *sss* and identified by mass spectrometry as CG7433 positive. (**B**) Schematic of the genomic DNA structure around gene CG7433 (GABAT). Shown as a bar with a black border is the 4.9 kb genomic DNA from the Bac clone BACR13N10, flanked by HpaI and HindIII sites. The shaded diagram at the bottom depicts the proteins of the *CG7433* and the neighboring *Tom20* gene, as well as the *CG7433* cDNA clone RH42429. Closed arrowheads show the positions of the two transposon insertions, PL00338 and F01602. Indicated by the red open arrows are two modifications to the gCG7433tvh genomic construct: a thrombin-V5-His6 tandem tag (TVH) added to the C-terminus of the CG7433 open reading frame (ORF) and the mutation of the Lys6 residue of TOM20 to an amber stop codon (K6@) to disable the *Tom20* gene.

(**C**) Western blot analysis (Left) and quantitation (Right) of GABATtvh protein derived from the gGABATtvh genomic transgene. V5 staining detected higher GABATtvh protein levels in the *sss*$^{P1}$ mutants than in wild type for both male (M) and female (F) flies. Actin staining was used as a loading control. Shown in the right chart is the quantification of the left panel, the averages of GABAT/beta-actin ratios from both male and female samples in either a wild type or *sss* genetic background. *: $P<0.05$ (Student's t-test). (**D**) Localization of epitope tagged *Drosophila* GABAT to mitochondria in S2 cells. MitoSOX marks mitochondria (left panel, red) and V5 staining (middle panel, green) denotes the expression of GABATvh. Note that the lower two cells (white arrow heads) are negative control cells exhibiting only MitoSOX staining but not V5, indicating the cells were not transfected with pIZ-GABATvh. (**E**) HPLC amino acid analysis to compare GABA levels from fly brains. X-axis is elution time. Y-axis is absorbance at 254 nm, indicating the level of PITC-conjugated amino acids. The boxed peaks are GABA levels from the different genotypes shown on the left. (**F)** Reverse transcription quantitative PCR analyzing brain *GABAT* mRNA levels. *GABAT* mRNAs are first normalized to β-actin mRNA levels then to those in wild type flies which were set as 100%. *GABAT* mRNA levels from *sss*$^{P1}$ were not different from those in wild type ($P=0.385$), while the *GABAT* mRNAs from *gabat*$^{F}$ and *gabat*$^{PL}$ were significantly reduced to 34% and 1%, respectively ($P<0.001$, One-way ANOVA with Tukey's test).

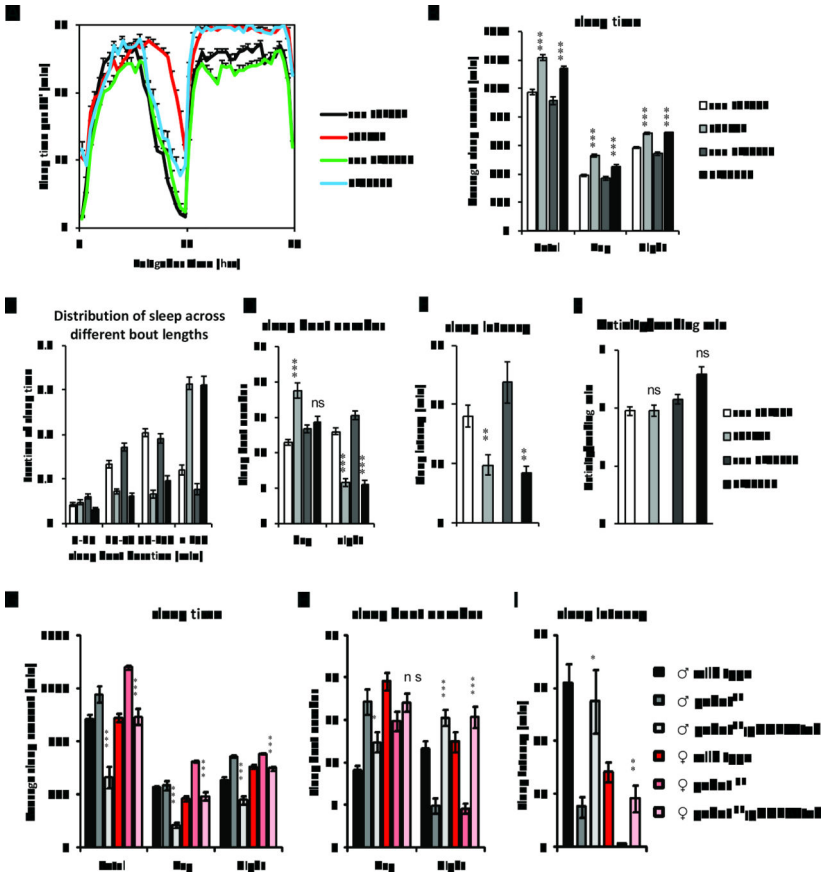


**Figure 2.**

Loss of *GABAT* promotes sleep. (**A**-**F**) Male *GABAT* mutants and control flies from outcrossing were monitored for sleep behavior in 12hr light:12hr dark cycles (12:12LD) at 25°C. Daily sleep profiles (**A**) and parameters of sleep behavior (**B**-**F**) were compared between each of the two *GABAT* mutants ($gabat^{F}$ and $gaba^{PL}$) and their respective wild type genetic background controls (Con F01602 and Con PL00338). (A) X-axis denotes the time of day, with Zeitgeber Time (ZT) 0-12 denoting lights-on and ZT12-24 denoting lights-off. The Y-axis shows the amount of sleep per 30 minute bins. (**G**-**I**) A gGABATtvh transgene was used to rescue the sleep phenotype of PL00338 under 12:12LD at 25°C. Sleep time (**G**), sleep bout number (**H**), and sleep latency (**I**) were compared among male (♂) and female (♀) flies from wild type control (iso31), *gabat* mutant ($gabat^{PL}$), and genomic rescue ($gabat^{PL}$;gGABATtvh) flies. For (**B**-**F**), Student's *t*-test was performed between each *GABAT* mutant and its respective control for all sleep parameters, except for the distribution of sleep bouts based on their duration, where the Mann-Whitney U (MWU) test was used. The mutants exhibited increased average total, daytime, and night time sleep (all $P<0.001$) (**B**), increased proportion of sleep in longer sleep episodes (Mann-Whitney U test, both $P<0.0001$) (**C**), increased number of sleep bouts (daytime, F01602 vs Con F01602 $P<0.001$, PL00338 vs Con PL00337, not significant; nighttime, both $P<0.001$) (**D**) and decreased sleep latency (both $P<0.01$) (**E**). However, the waking activity (**F**) was not significantly different between mutants and controls. For (**G**-**I**),One-way ANOVA with Tukey's test was used to examine the differences in sleep time, bout number, and latency for male and female

flies respectively. Asterisks show statistical significance between *gabat*$^{PL}$ and *gabat*$^{PL}$,gGABATtvh (***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; ns, not significant).

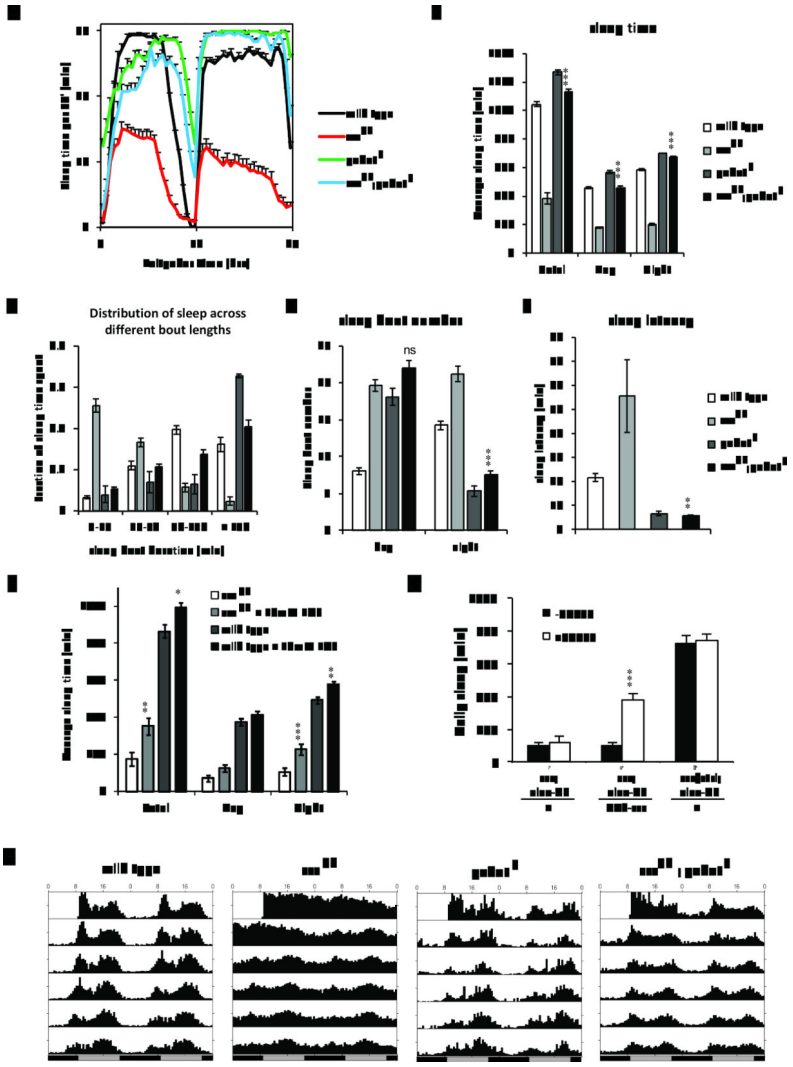


**Figure 3.**

Disruption of *gabat* completely suppresses the short sleep phenotype of *sss*$^{P1}$ flies. To determine the effect of the *gabat* mutation on the *sss* phenotype, *sss*$^{P1}$;*gabat*$^{F}$ male flies were compared with *sss*$^{P1}$, *gabat*$^{F}$ and wild type control flies for daily sleep profiles (**A**), sleep time (**B**), distribution of sleep with respect to the length of sleep episodes (**C**), number of sleep bouts (**D**), and sleep latency (**E**). (**F**) Inhibition of GABAT in adults partially rescues the *sss* mutant. We treated wild type and *sss* flies with an inhibitor specific for GABAT, EOS, and compared day, night, and total sleeptime. *sss*$^{P1}$ and wild-type males (between 1-5 days of age) were fed either 10mM EOS or control food for 5 days. We averaged sleeptime of flies from days 3-5 to ensure that the drug was ingested. (G) The *sss*$^{P1}$ phenotype results from adult and developmental effects. To restrict *sss* expression to adults, we used an *elav*-GS driver to conditionally rescue *sss*$^{P1}$ adults. Daily sleep was monitored in females in the presence (white bar) or absence (black bar) of 500μM RU486. Heterozygous (*sss*/ctrl) flies were included as positive controls. (**H**) *gabat*$^{F}$ improves circadian rest:activity rhythms of *sss*$^{P1}$. Male flies were first entrained to 12:12 Light:Dark cycles at 25°C for 4 days before the flies were transferred into constant darkness for assay of free-running rhythms. The figure

shows average 30 minute activity bins from flies of the respective groups recorded for 7 days in continuous darkness (DD), starting from day 1 (DD1). One-way ANOVA with Tukey's test was used to examine the differences in sleep time, bout number, and latency. Asterisks depict the results of statistical tests between $sss^{P1}$ and $sss^{P1}$;$gabat^{F}$ (**B**-**E**) or control and EOS-treated $sss^{P1}$ and wild-type flies (**F**) ***, $P<0.001$; **, $P<0.01$; ns, not significant. MWU test was used to test for differences in sleep bout distribution. $gabat^{F}$ rescued the short sleep bout duration phenotype of sss (MWU test with Bonferroni adjustment, $sss^{P1}$ vs $sss^{P1}$;$gabat^{F}$ $P<0.0001$; $gabat^{F}$ vs $sss^{P1}$;$gabat^{F}$, $P=0.92$). Unpaired t-tests with Bonferroni correction were used to test for differences in daily sleep in (**G**). *** $P<0.001$.

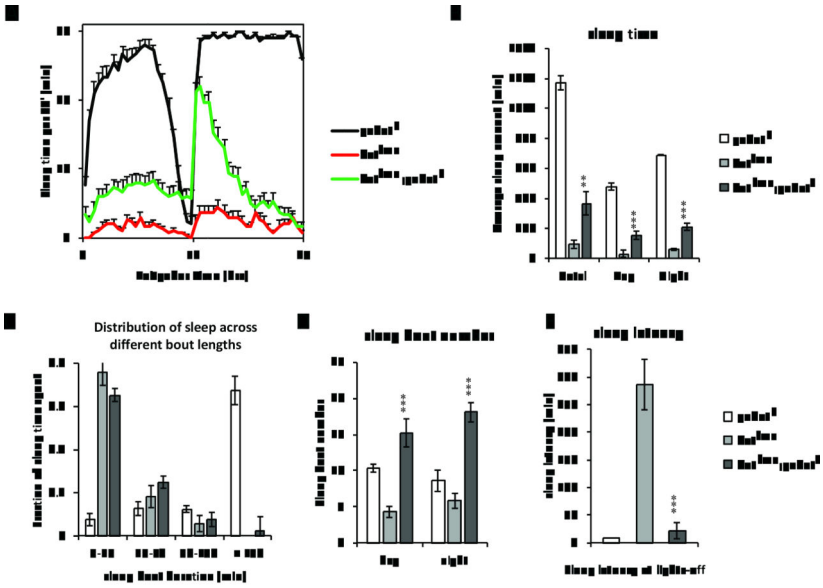


**Figure 4.**

Disruption of *gabat* does not restore wild type sleep in *dat*$^{fmn}$ flies. The double mutant *dat*$^{fmn}$;*gabat*$^{F}$ male flies were compared with *dat*$^{fmn}$ and *gabat*$^{F}$ flies for daily sleep profiles (**A**), sleep time (**B**), distribution of sleep with respect to the length of sleep episodes (**C**), number of sleep bouts (**D**), and sleep latency (**E**). Statistics was applied as in Figure 3. Asterisks indicate results of one-way ANOVA followed by a posthoc Tukey's test. ***, $P<0.001$; **, $P<0.01$. While *gabat*$^{F}$ showed longer sleep bout duration than both *dat*$^{fmn}$ and *dat*$^{fmn}$;*gabat*$^{F}$ flies (MWU test with Bonferroni adjustment, $P<0.0001$ for both), *dat*$^{fmn}$;*gabat*$^{F}$ did not show differences from *dat*$^{fmn}$ in sleep bout duration (MWU test with Bonferroni adjustment, $P=0.273$).

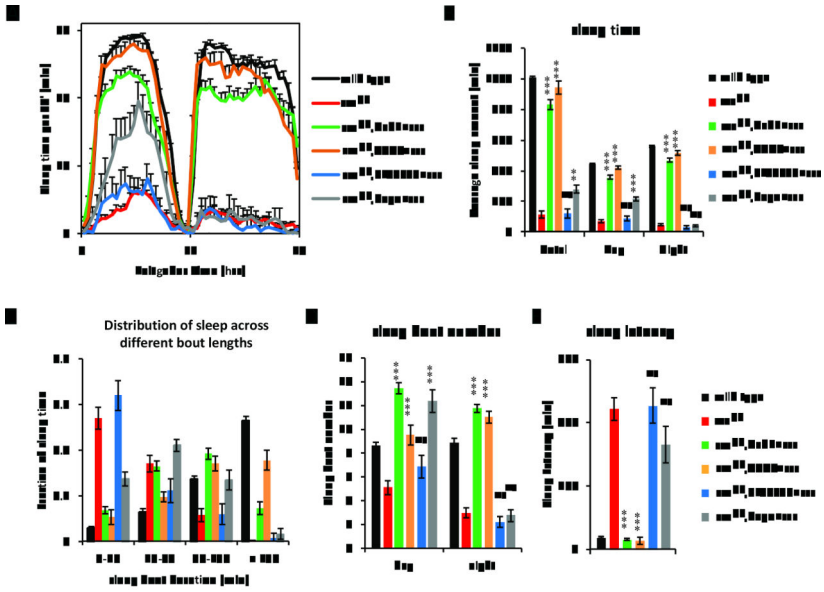


**Figure 5.**

GABAergic expression of SSS rescues *sss*$^{P1}$. To rescue sleep in *sss* flies, daily sleep behavior was tested at 12:12LD, 25°C in *sss*$^{P1}$ animals with UAS-*sss* expression driven by various GAL4 drivers in either GABAergic neurons (Gad1-GAL4, green or VGAT-GAL4, orange), GABAT expressing neurons (PL00338, blue), or glial cells (Repo-GAL4, grey). Wild type (black) and *sss*$^{P1}$ (red) animals were used as positive and negative controls, respectively. The average of 3 day behavior from three independent experiments was plotted with standard error of means. Shown in the figures are average daily sleep profiles (**A**), sleep time (**B**), distribution of sleep with respect to the length of sleep episodes (**C**), number of sleep bouts (**D**), and sleep latency (**E**). Statistics was applied as in Figure 3. Asterisks depict statistical comparisons with *sss*$^{P1}$. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; ns, not significant. Compared with *sss*$^{P1}$ controls, overexpression of SSS with either Gad1-GAL4 or VGAT-GAL4 rescues sleep bout duration (MWU test with Bonferroni adjustment, $P<0.0001$ for both drivers), while neither PL00338 nor Repo-GAL4 do (MWU test with Bonferroni adjustment, $P=0.771$ and 0.118, respectively).

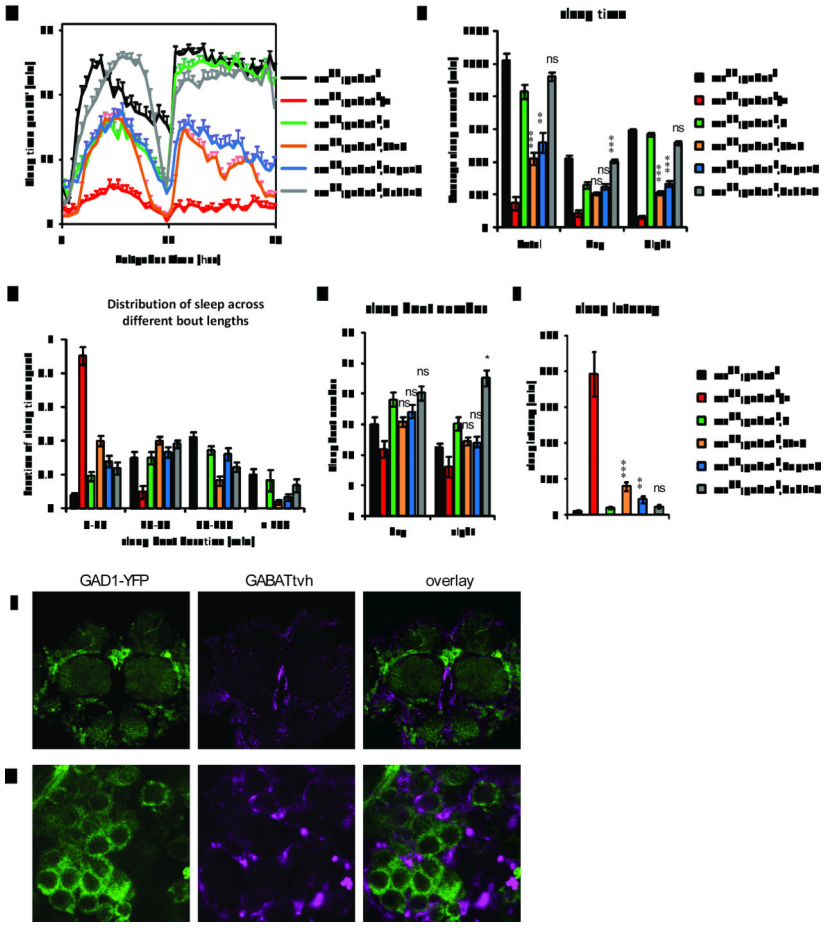


**Figure 6.**

Glial expression of GABAT partially restores the *sss*$^{P1}$ sleep phenotype in *sss*$^{P1}$;*gabat*$^{F}$ flies. Daily sleep behavior was tested at 12:12LD, 25°C in *sss*$^{P1}$;*gabat*F animals carrying UAS-GABATvh driven by GAL4 drivers in native GABAT expressing neurons (PL00338, orange), glial cells (Repo-GAL4, blue) , or GABAergic neurons (Gad1-GAL4, grey). *sss*$^{P1}$;*gabat*$^{F}$ (black) and *sss*$^{P1}$;*gabat*$^{F}$, UAS-GABATvh (green) were used as negative controls, while *sss*$^{P1}$;*gabat*$^{F}$/+ (red) animals were used as positive controls. The average of 4 day behavior was plotted with standard error of means. Shown in the figures are average daily sleep profiles (**A**), sleep time (**B**), distribution of sleep with respect to the length of sleep episodes (**C**), number of sleep bouts (**D**), and sleep latency (**E**). Statistics was applied as in Figure 3. Asterisks indicate statistical comparisons with *sss*$^{P1}$;*gabat*$^{F}$,UAS-GABATvh. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; ns, not significant. Compared with *sss*$^{P1}$;*gabat*$^{F}$,UAS-GABATvh controls, overexpression of GABAT in either PL00338 or Repo-GAL4 expressing cells fragments the sleep bout length (MWU test with Bonferroni adjustment, $P<0.0001$ for both drivers) , but not in Gad1-GAL4 expressing cells ($P=0.47$). Note, U: UAS-GABATvh; PL: PL00338; Repo: Repo-GAL4; Gad1: Gad1-GAL4. (**F-G**) Confocal imaging of GAD1-YFP and GABATtvh in *Drosophila* brains indicates distinct but often proximate staining patterns of GAD1 and GABAT expression. GABATtvh was stained by an Alexa Fluor 647 anti-V5 antibody. The YFP and Alexa Fluor 647 signals were imaged for GAD1-YFP (left panels, green) and GABATtvh (middle panels, magenta) expression

patterns with the overlay images shown in the right panels. Representative central brain (**F**) and medial lateral brain (**G**) regions are shown.