Report

Current Biology

The Taurine Transporter *Eaat2* Functions in Ensheathing Glia to Modulate Sleep and Metabolic Rate

Highlights

- *Eaat2* promotes wakefulness in *Drosophila* by limiting the length of sleep bouts
- *Eaat2* functions acutely in the ensheathing glia of adult flies to regulate sleep
- Increased sleep in *Eaat2*-deficient flies is accompanied by reduced metabolic rate
- Eaat2-mediated transport of taurine may regulate sleep and metabolism

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In Brief

Stahl, Peco, et al. show that *Excitatory amino acid transporter 2* (*Eaat2*) promotes wakefulness and metabolic rate in fruit flies. *Eaat2* operates in ensheathing glia and can transport taurine across cell membranes, revealing a new mechanism for how glial transporters may influence neural circuits in the brain controlling sleep-wake regulation.

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The Taurine Transporter *Eaat2* Functions in Ensheathing Glia to Modulate Sleep and Metabolic Rate

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SUMMARY

Sleep is critical for many aspects of brain function and is accompanied by brain-wide changes in the physiology of neurons and synapses [1, 2]. Growing evidence suggests that glial cells contribute to diverse aspects of sleep regulation, including neuronal and metabolic homeostasis [3–5], although the molecular basis for this remains poorly understood. The fruit fly, *Drosophila melanogaster*, displays all the behavioral and physiological characteristics of sleep [1, 2], and genetic screening in flies has identified both conserved and novel regulators of sleep and wakefulness [2, 6, 7]. With this approach, we identified *Excitatory amino acid transporter 2* (*Eaat2*) and found that its loss from glia, but not neurons, increases sleep. We show that *Eaat2* is expressed in ensheathing glia, where *Eaat2* functions during adulthood to regulate sleep. Increased sleep in *Eaat2*-deficient flies is accompanied by reduction of metabolic rate during sleep bouts, an indicator of deeper sleep intensity. Eaat2 is a member of the conserved EAAT family of membrane transport proteins [8], raising the possibility that it affects sleep by controlling the movement of ions and neuroactive chemical messengers to and from ensheathing glia. *In vitro*, Eaat2 is a transporter of taurine [9], which promotes sleep when fed to flies [10]. We find that the acute effect of taurine on sleep is abolished in *Eaat2* mutant flies. Together, these findings reveal a wake-promoting role for *Eaat2* in ensheathing glia through a taurine-dependent mechanism.

RESULTS AND DISCUSSION

To identify novel regulators of sleep, we used *Actin5c*-GAL4 (*Act5c*) to screen 918 Transgenic RNAi Project (TRiP) lines, which allow for knockdown of mRNA expression of individual genes via upstream activating sequence (UAS)-controlled RNAi [11]. Upon knockdown of *Excitatory amino acid transporter 2* (*Eaat2*) (*Act5c>Eaat2*-RNAi), flies slept more during the daytime compared to control flies targeting a *Luciferase* sequence (*Act5c>Luc*-RNAi; Figures 1A and 1B). To determine whether *Eaat2* functions in neurons or glia, we first measured sleep upon pan-neuronal knockdown of *Eaat2* (*Elav>Eaat2*-RNAi), but we found that this did not alter sleep (Figures 1C and 1D). In contrast, knockdown in glia (*Repo>Eaat2*-RNAi) increased daytime sleep (Figures 1E and 1F), which phenocopied knockdown in all cells with *Act5c*-GAL4. Taken together, these findings reveal a wake-promoting role for *Eaat2* and localize this function to glial cells.

The architecture of sleep comprises bouts that vary in number and duration [12, 13]. Daytime sleep is more fragmented than nighttime sleep, with fewer individual sleep bouts that have a shorter length on average [14, 15]. Knockdown of *Eaat2* in all cells (*Act5c>Eaat2*-RNAi), or only glia (*Repo>Eaat2*-RNAi), increased the average length of daytime sleep bouts (Figures S1A and S1C) without affecting their number (Figures S1E and S1G). In addition, nighttime bout length was increased in *Act5c>Eaat2*-RNAi and *Repo>Eaat2*-RNAi flies, raising the possibility that sleep consolidation is increased, with little or no effect on total nighttime sleep (Figures S1A, S1C, S1E, and S1G). These findings suggest that *Eaat2* promotes sleep maintenance and is dispensable for sleep initiation. Awake *Eaat2*-deficient flies were as active as controls during the daytime and nighttime, indicating that they were not simply lethargic (Figures S1I and S1K). As expected, sleep architecture and waking activity was unaffected by knockdown of *Eaat2* in neurons (Figure S1B, S1F, and S1J). These results indicate that *Eaat2* increases wakefulness by shortening individual sleep bouts.

To distinguish whether *Eaat2* regulates sleep acutely or whether this effect is secondary to a role in nervous system development, we selectively knocked down *Eaat2* in glia of adult flies (*Repo>Eaat2*-RNAi) using the TARGET (temporal and regional gene expression targeting) system [16]. TARGET uses GAL80^{TS}, which binds and represses GAL4 activity at 18°C but is non-functional at 29°C. Baseline testing of flies reared and

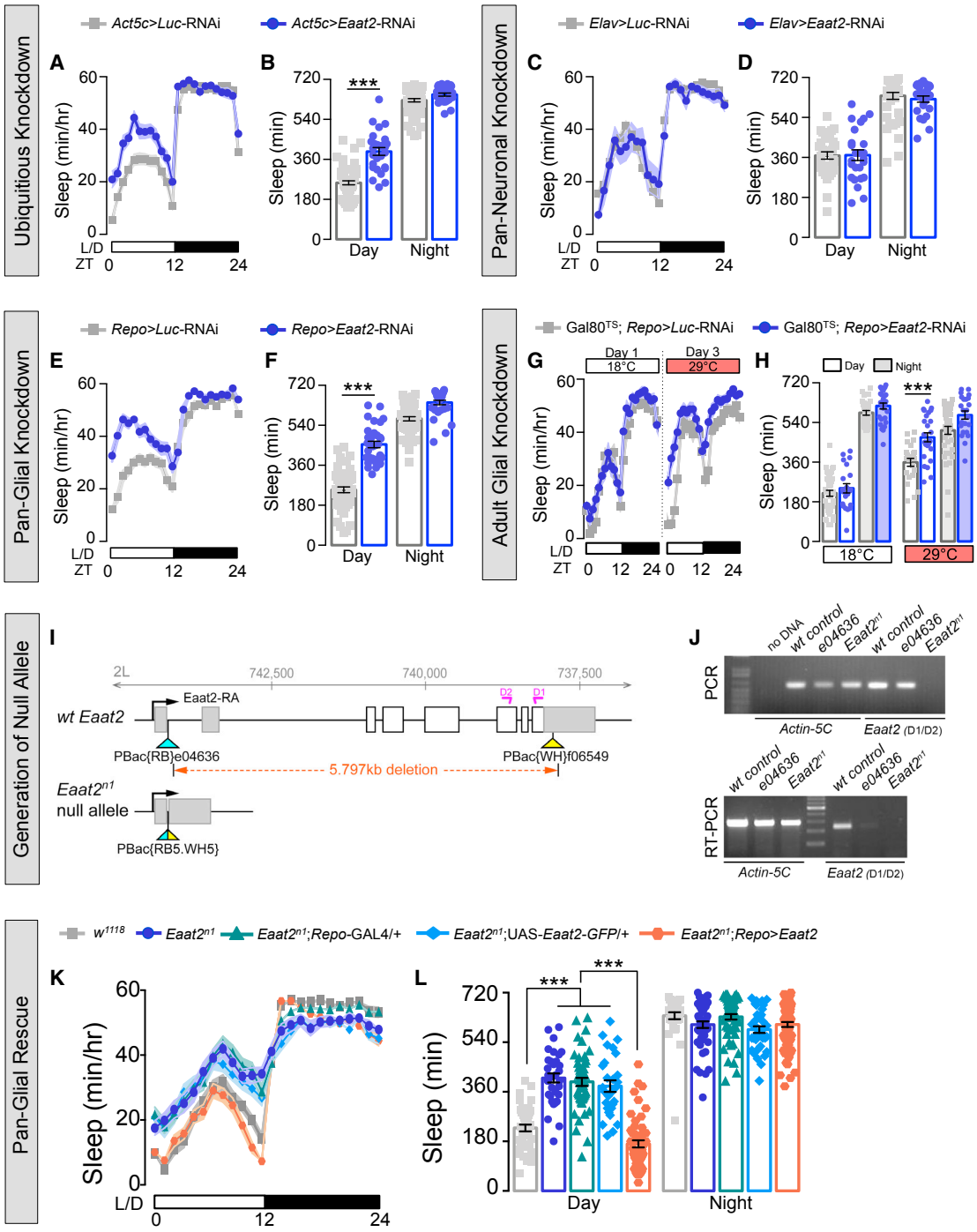

Figure 1. *Eaat2* Functions in Glia to Modulate Sleep

(A and B) 24-hr sleep profile (A) showing light/dark (L/D) conditions and quantification of sleep duration (B) for *Act5c>Eaat2*-RNAi flies (blue) and *Act5c>Luc*-RNAi controls (gray) ($t = 7.955$, $df = 83$, ***$p < 0.0001$, $n > 22$).

(C and D) 24-hr sleep profile (C) and quantification (D) of *Elav>Eaat2*-RNAi flies and *Elav>Luc*-RNAi controls ($t = 0.6551$, $df = 54$, $p > 0.9$, $n > 25$).

(E and F) Sleep profile (E) and cumulative sleep (F) in flies with pan-glial knockdown of *Eaat2* (*Repo>Eaat2*-RNAi) and controls ($t = 10.21$, $df = 78$, ***$p < 0.0001$, $n > 32$).

(G and H) TARGET system multi-day sleep profile (G) and quantification (H). No difference was observed at the non-permissive 18°C (day 1, two-way ANOVA, $F_{(1, 115)} = 3.220$; $p > 0.1$, $n > 23$), but when measured again 24 hr after transfer to 29°C (day 3), daytime sleep was increased in GAL80^{TS};*Repo>Eaat2*-RNAi flies (two-way ANOVA, $F_{(1, 108)} = 21.69$; ***$p < 0.001$, $n > 23$).

(I) *Eaat2* gene structure (RNA transcript A [RA] mRNA isoform), with coding exons (white) and non-coding exons (gray). Two *piggyBac* (PBac) insertions flanking the Eaat2 locus were excised (top) to generate a null mutant (bottom).

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tested at 18°C showed no difference in sleep between *Eaat2* knockdown flies and controls (Repo>*Luc*-RNAi) (Figures 1G and 1H). However, daytime sleep was increased when flies were shifted to 29°C for 24 hr to inactivate $GAL80^{TS}$ and achieve glial-specific knockdown of *Eaat2* (Figures 1G and 1H). This indicates that *Eaat2* regulates sleep acutely during adulthood. The effect appears more moderate than constitutive knockdown, which might reflect reduced efficacy of knockdown with TARGET, the influence of testing at a higher temperature, or added requirement for *Eaat2* during development.

To verify that the *Eaat2*-RNAi sleep phenotype was not due to off-target effects, we tested flies homozygous for the piggyBac alleles $Eaat2^{e04636}$ and $Eaat2^{e00569}$ [17]. Daytime sleep was increased in both alleles compared to controls (Figures S1M and S1N), and the quantity of nighttime sleep was unaffected (Figures S1M and S1N). We generated a null allele ($Eaat2^{n1}$) by recombination-mediated excision of genomic DNA between $Eaat2^{e04636}$ and $Eaat2^{f06549}$ (Figure 1I) [18], removing the entire *Eaat2* coding region. We confirmed recombination by PCR (Figure 1J) and used RT-PCR to demonstrate loss of *Eaat2* mRNA (Figure 1J). Fecundity, viability, longevity, and time spent feeding were normal in $Eaat2^{n1}$ flies (Figures S1O–S1R). Recovery sleep after mechanical sleep deprivation was similar in $Eaat2^{n1}$ flies and w^{1118} controls, indicating that *Eaat2* is not required for homeostatic sleep rebound (Figures S1S–S1U). However, $Eaat2^{n1}$ flies displayed an increased quantity of daytime sleep (Figures 1K and 1L) and lengthened sleep bouts (Figure S1D). No differences in bout number or waking activity were detected, phenocopying RNAi knockdown flies (Figures S1H and S1L). Daytime wakefulness could be rescued to control levels by selective reintroduction of *Eaat2* to glia (*Repo>Eaat2-GFP*), but not by the *Repo*-GAL4 or UAS-*Eaat2-GFP* transgenes alone (Figures 1K and 1L).

It has been suggested that *Eaat2* is expressed in glia and neurons [19], but with immunohistochemistry (IHC) for Eaat2 in larvae, we have found it to be restricted to ensheathing glia [20]. Ensheathing glia enwrap neuropil regions in the CNS of both larvae and adults [20–23]. In larvae, their expression of mitochondrial sulfite oxidase affects glutamate homeostasis and neural circuits controlling motor behavior [24]. In adults, they eliminate axon debris in a *Drosophila* model of Wallerian neurodegeneration [23]. With anti-Eaat2 IHC in adult brains, we found Eaat2 expressed selectively in ensheathing glia. The specificity of this pattern for Eaat2 was confirmed because it was lost from $Eaat2^{n1}$-null animals compared to controls (Figures 2A–2F), though some non-specific labeling remained in the brain periphery. Eaat2-positive glia enwrapped neuropil regions throughout the brain, including those of known sleep centers such as the ellipsoid body, mushroom body, and fan-shaped body (Figures 2A–2F′; Video S1) [25–28], consistent with a role for *Eaat2* and ensheathing glia in sleep regulation. The identity of Eaat2-positive cells as ensheathing glia was confirmed by co-labeling with *R56C01-GAL4*, which selectively labels ensheathing glia in larvae [20] and in adults (Figures 2G–2H‴). We further confirmed this pattern of Eaat2 expression with $Eaat2^{MI10251}$, a GFP-expressing gene-trap in the Eaat2 locus (Figure S2A; Video S2), and via anti-Eaat2 co-labeling with the ensheathing glia marker *R56F03*-GAL4 (Figure S2B) [29].

We observed increased daytime sleep upon selective *Eaat2* knockdown in ensheathing glia using either *R56C01>Eaat2*-RNAi (Figures 2I and 2J) or *Mz0709>Eaat2*-RNAi (Figures S2C and S2D). However, *Eaat2* knockdown in astrocytes (*Alrm*-GAL4) or perineurial glia (*NP6293*-GAL4) did not affect sleep duration (Figures S2E and S2F). Re-introduction of *Eaat2-GFP* to ensheathing glia of $Eaat2^{n1}$ animals rescued daytime wakefulness to control levels (Figures 2K and 2L). While *Eaat2* is expressed in ensheathing glia distributed throughout the brain, *R56C01*-GAL4 is more restricted. With whole-brain reconstruction of ensheathing glia labeled with *R56C01*>mCD8-GFP (Figures S2G–S2J), we found some contacting the mushroom bodies and fewer in the ellipsoid body and fan-shaped body. Though *R56C01*-GAL4 is not exclusive to these areas, its ability to affect sleep by driving *Eaat2*-RNAi knockdown raises the possibility *Eaat2* functions in these regions to regulate sleep [25–27].

In contrast with *Eaat2* loss of function, overexpression of *Eaat2* in the ensheathing glia of otherwise normal flies (*R56C01>Eaat2-GFP*) reduced sleep during the daytime and nighttime (Figures 2M and 2N). This suggests that *Eaat2* levels are an important determinant of total sleep and may play a role in day and night sleep regulation. Since *Eaat2* overexpression inhibits both daytime and nighttime sleep (Figure 2N), and since *Eaat2* loss of function affects nighttime sleep architecture (Figure S1A, S1C, and S1D), we favor the idea that *Eaat2* may generally promote wakefulness and reduce sleep intensity, rather than modulate daytime sleep specifically. High levels of nighttime sleep could override any detectable role for *Eaat2* in restricting night sleep quantity.

To further test how ensheathing glia influence sleep, we disrupted their function acutely by expressing temperature-sensitive dynamin GTPase $Shibire^{TS1}$ (Shi^{TS1}) [23, 30]. In neurons, Shi^{TS1} blocks neurotransmitter release within minutes of a temperature shift [30], and it has also been shown to disrupt the function of multiple glial cell types [23, 31]. The mechanism of Shi^{TS1} action in glia is not currently understood, though it is presumed to inhibit vesicular trafficking to and from the cell surface, which has the potential to interfere with membrane transporters. At the permissive temperature (22°C), there was no effect of Shi^{TS1} with either *R56C01*-GAL4 (Figures 2O and 2P) or *Mz0709*-GAL4 (Figures S2K and S2L). However, at 31°C, daytime sleep increased compared to control flies harboring the GAL4 or Shi^{TS1} transgene alone (Figures 2O and 2P). No significant effect of Shi^{TS1} expression on nighttime sleep was detected (Figure 2O). These findings indicate that ensheathing glia function acutely to promote wakefulness.

Both sleep and glial cells have been linked to metabolic regulation, but little is known about the molecular basis for sleep-dependent changes in metabolic rate [32]. To determine the role of *Eaat2* in metabolic regulation, we used the SAMM (sleep

(J) PCR (top) and RT-PCR (bottom) to validate the $Eaat2^{n1}$ allele.
(K and L) Sleep profile (K) and cumulative analysis (L) reveals that daytime wakefulness is rescued in $Eaat2^{n1}$; *Repo>Eaat2-GFP* flies (orange) to levels of w^{1118} controls (gray) (one-way ANOVA, $F_{(4,\ 199)} = 57.36$; ***$p < 0.0001$, $n > 28$).
Error bars represent ±SEM. See also Figure S1.

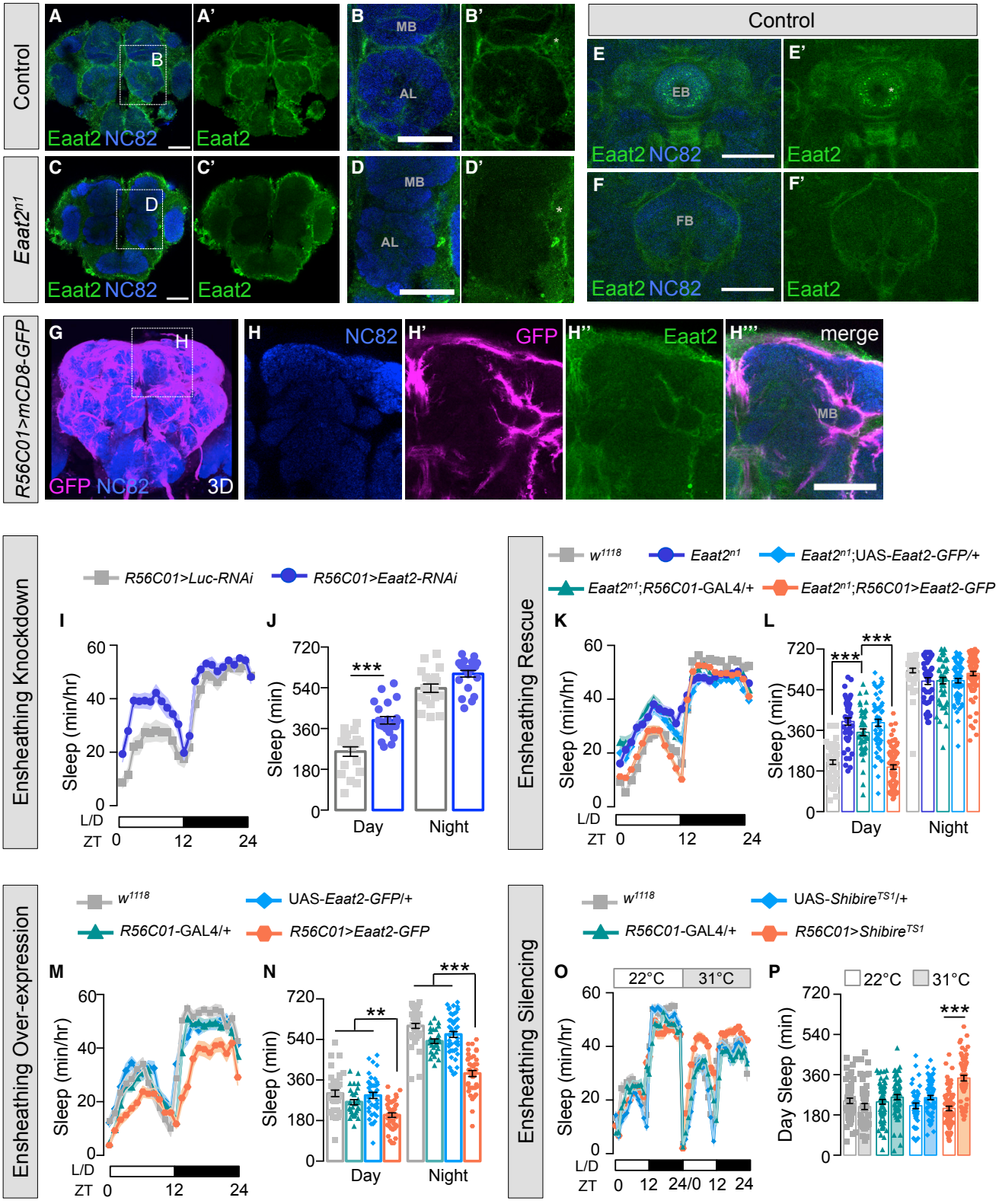

Figure 2. The Sleep-Suppressing Function for *Eaat2* Localizes to Ensheathing Glia

(A–F) Localization of Eaat2 (green) and neuropil (NC82, blue) in adult brains. Single confocal z slices are shown; scale bar, 50 μm. Asterisks (*) denote background immunoreactivity.

(A and B) Eaat2 expression in control flies in whole brain (A) and in a closer view (B) that highlights the antennal lobe (AL) and mushroom body (MB).

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and activity metabolic monitor) system, which allows for simultaneous measurement of sleep and CO_2 output from single flies [33] (Figure 3A). Consistent with standard *Drosophila* activity monitors, daytime sleep of *Eaat2*^{n1} flies was increased in the SAMM system (Figures S3A and S3B). This was accompanied by lower metabolic rate (CO_2 output) during daytime, but not nighttime (Figures 3B–3D). The daytime sleep and metabolic phenotypes were both rescued by restoring *Eaat2* to ensheathing glia of *Eaat2*^{n1} flies (*R56C01>Eaat2-GFP*) (Figures S3A, S3B, and 3B–3D), revealing that the reduced metabolic rate of *Eaat2*^{n1} flies can be localized to loss of *Eaat2* from ensheathing glia.

To examine the relationship between *Eaat2*, sleep and metabolic rate, we measured CO_2 output during individual daytime and nighttime sleep bouts. Whole-body metabolic rates drop during prolonged nighttime sleep bouts, providing a physiological readout of sleep that may be a proxy for sleep intensity [33]. Control flies did not reduce their CO_2 output over the first 20 min of daytime sleep bouts, suggesting that daytime sleep is physiologically different from nighttime sleep (Figures 3E and 3F). However, *Eaat2*^{n1} flies did reduce their CO_2 output over prolonged (20 min) daytime sleep bouts (Figures 3E and 3F), which was rescued by restoring *Eaat2* to ensheathing glia (Figure 3E). This raises the possibility that loss of *Eaat2* increases sleep intensity during the day and suggests that the lower daytime metabolic rate of *Eaat2*^{n1} mutant flies is not exclusively due to increased sleep duration. During nighttime, both control and *Eaat2*^{n1} flies reduced CO_2 output during individual sleep bouts (Figure 3F). Sleep intensity in flies also correlates with elevated arousal threshold and a reduction in 11–40 Hz activity oscillations within the brain [34], which could be used to further define behavioral and physiological changes that accompany the sleep phenotype of *Eaat2*^{n1} flies.

When *Eaat2* was overexpressed in ensheathing glia (*R56C01>Eaat2-GFP*), reduced sleep during both day and night (Figures S3C and S3D) was accompanied by elevated whole-body metabolic rate (Figures 3G–3I). Therefore, *Eaat2* levels alone appear sufficient to modulate both sleep and metabolic rate. However, sleep-dependent effects on metabolic rate were unaltered in flies overexpressing *Eaat2* (Figures 3J and 3K), suggesting that the increased metabolic rate observed in *Eaat2*-overexpressing flies is primarily due to changes in basal metabolic rate or to sleep loss.

Eaat2 has high affinity for taurine *in vitro*, and taurine levels are altered in whole-brain lysates of *Eaat2* mutant flies, suggesting that Eaat2 influences taurine availability *in vivo* [9, 19]. In humans, taurine is one of few serum and urinary metabolites consistently upregulated upon sleep deprivation [35, 36], but it remains to be determined whether taurine levels change in the brain under these conditions. Interestingly, flies fed high doses of taurine increase their total sleep by up to 50% [10], although the neurobiological basis for this effect is unknown. To explore whether the ability of Eaat2 to promote daytime wakefulness is related to taurine transport, we tested control flies and *Eaat2*^{n1} mutants for the effects of taurine feeding on sleep. Taurine robustly promoted daytime sleep in control flies, without affecting sleep in *Eaat2*^{n1} flies (Figures 4A–4C). Further, selectively restoring *Eaat2* in ensheathing glia (*Eaat2*^{n1}; *R56C01>Eaat2-GFP*) rescued the effect of taurine feeding on sleep regulation (Figures 4D–4F). There was no effect of taurine feeding on nighttime sleep, though this could be due to a ceiling effect (Figures 4A and 4D). These findings suggest *Eaat2* mediates these effects directly, since only in the presence of *Eaat2* does taurine promote sleep. In the SAMM system, taurine feeding also increased daytime sleep of *w*^{1118} controls, but not of *Eaat2*^{n1} flies (Figures S4A and S4B). Accordingly, metabolic rate was reduced in taurine-fed *w*^{1118} controls, but not in taurine-fed *Eaat2*^{n1} flies (Figures 4G and 4H). Strikingly, taurine-fed controls suppressed metabolic rate over the first 20 min of daytime sleep bouts, phenocopying *Eaat2*^{n1} mutants, and suggesting that taurine increases sleep depth (Figure 4I). Together, these findings reveal that the effects of taurine feeding on sleep are accompanied by changes in metabolic rate.

These results add wakefulness to the growing list of *Drosophila* behaviors in which glial cells play an important regulatory role, including circadian rhythms and locomotor activity [37], courtship [38], and memory [32–34]. Our findings show *Eaat2* potently impacts daytime sleep and influences sleep architecture both day and night. A previous study found that *Eaat2* transcript levels may fluctuate in fly heads according to time of day [39], raising the possibility that changes in *Eaat2* levels might influence circadian regulation of sleep.

We identify a role for *Eaat2* in the modulation of sleep and sleep-dependent changes in metabolic rate, suggesting that ensheathing glia influence neural circuits regulating these processes. Tools to monitor neural activity in these circuits could be used to understand whether and how Eaat2 and ensheathing glia influence the physiological state of sleep-associated neurons during sleep and wakefulness. GABAergic neurons in *Drosophila* are sleep promoting, as they are in mammals [40],

(C and D) Loss of Eaat2 in *Eaat2*^{n1} mutants imaged in whole brain (C) and high magnification (D).
(E and F) Eaat2 in ensheathing glia ellipsoid body (EB; E and E′) and fan-shaped body (FB; F and F′).
(G and H) 3D reconstitution of a confocal z stack (ImageJ/Fiji) (G) and single confocal slices (H) showing co-labeling of Eaat2 (green) and *R56C01>mCD8-GFP* (magenta) surrounding neuropil (NC82, blue). Scale bar, 50 μm.
(I and J) Sleep profile (I) and cumulative analysis (J) revealed that daytime sleep was increased in *R56C01>Eaat2*-RNAi (blue) compared to controls (gray) ($t = 5.587$, $df = 39$, ***$p < 0.0001$, $n > 19$).
(K and L) Sleep profile (K) and cumulative analysis (L) revealed rescue of *Eaat*^{n1} mutants with Eaat2 in ensheathing glia (*Eaat2*^{n1};*R56C01>Eaat2-GFP*, orange) (one-way ANOVA, $F_{(4, 280)} = 59.34$; ***$p < 0.0001$, $n > 45$).
(M and N) Sleep profile (M) and cumulative analysis (N) revealed that flies overexpressing *Eaat2* in ensheathing glia (*R56C01>Eaat2-GFP*, orange) sleep less during the daytime and nighttime than *w*^{1118} (gray) flies and controls (green, light blue) (one-way ANOVA, $F_{(3, 147)} = 13.21$; **$p < 0.001$, ***$p < 0.0001$, $n > 30$).
(O and P) Sleep profile (O) and cumulative analysis (P) revealed that daytime sleep was increased in flies expressing *Shi*^{TS1} in ensheathing glia (R56C01>*Shi*^{TS1}, orange) at 31°C and was unchanged in *w*^{1118} (gray) and controls (green, blue) (two-way ANOVA, $F_{(3, 461)} = 6.374$; ***$p < 0.001$, $n > 43$). No differences were observed when tested at 22°C (daytime) or at nighttime when tested at either 22°C or at 31°C ($p > 0.1$, $n > 43$).
Error bars represent ±SEM. See also Figure S2 and Videos S1 and S2.

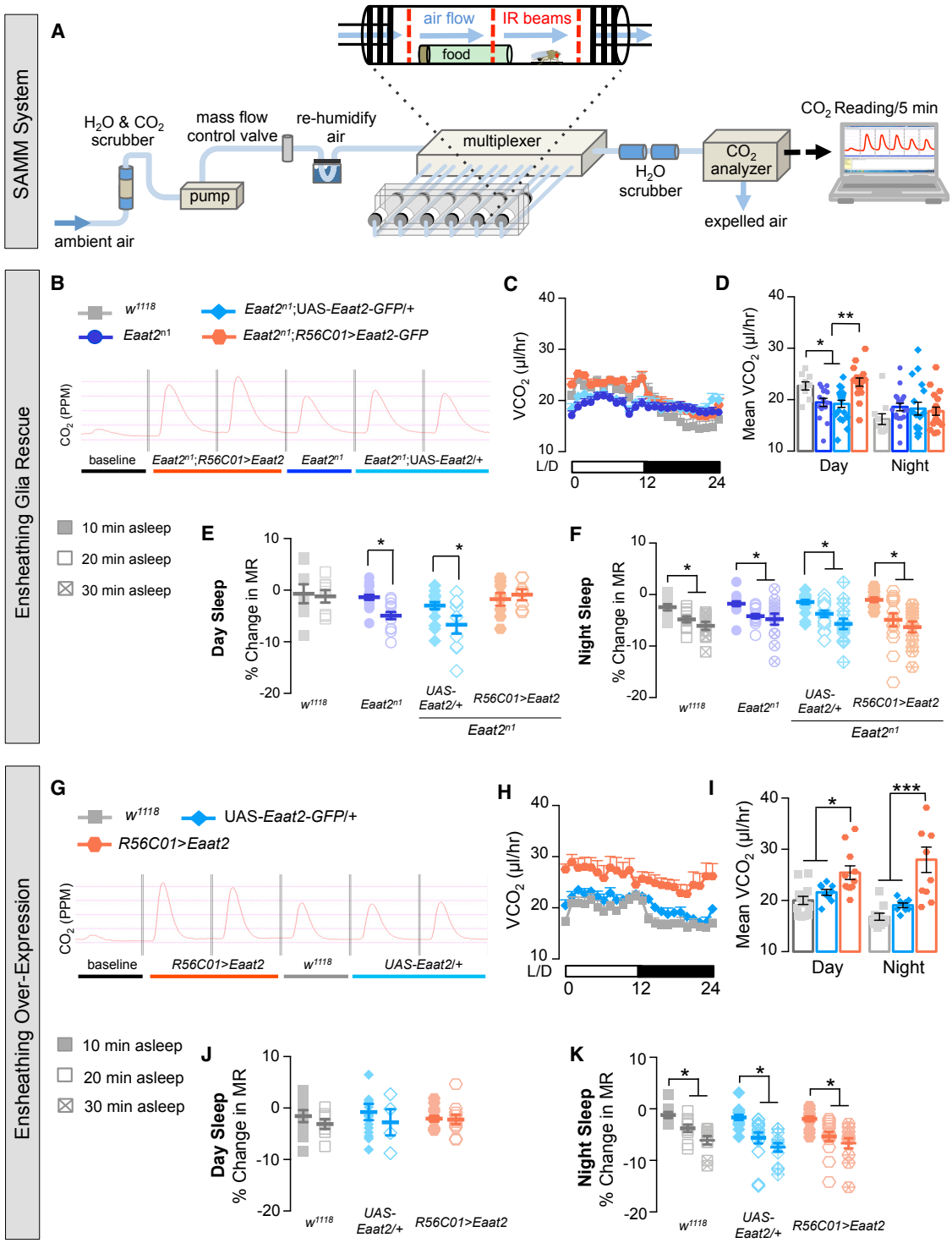

Figure 3. *Eaat2* Functions in Ensheathing Glia to Regulate Sleep-Dependent Changes in Metabolic Rate

(A) Diagram of SAMM system.

(B) Representative 5-min CO_2 measurements from rescue flies (orange) and controls (dark blue and light blue).

(C and D) Metabolic profile (C) and cumulative quantification (D) revealed that metabolic rate was reduced during daytime in flies homozygous for $Eaat2^{n1}$ (dark blue and light blue) compared to w^{1118} flies (gray) and *Eaat2*-rescued flies (orange) (one-way ANOVA, $F_{(3, 56)} = 7.967$; *$p < 0.05$, **$p < 0.01$, $n > 10$). Nighttime metabolic rate of $Eaat2^{n1}$ flies did not differ from that of controls (one-way ANOVA, $F_{(3, 56)} = 0.8807$; $p > 0.4$, $n > 10$).

(E) A drop in metabolic rate during daytime sleep bouts occurred in $Eaat2^{n1}$ flies (dark blue and light blue) (two-way ANOVA, $F_{(1, 77)} = 7.732$; **$p < 0.01$, $n > 10$), but not in w^{1118} or *Eaat2*-rescued flies (orange) ($p > 0.97$, $n > 10$).

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and glial metabolism of GABA regulates *Drosophila* sleep [3]. In mammals, taurine is a GABA receptor agonist [41], although to our knowledge this has not yet been determined for GABA receptors in flies. We speculate that taurine-feeding could promote sleep in *Drosophila* by increasing GABA-receptor-mediated transmission or inhibitory tone and that *Eaat2*-deficient animals may be refractory to further activation of GABA receptors due to impaired transport of endogenous taurine into ensheathing glia. Whether glial-dependent taurine transport is a critical and conserved regulator of sleep awaits further study, but it is interesting to note that, in mice, taurine may underlie tonic inhibition of neurons in the ventrobasal thalamus of mice [42], a brain region that may be important in regulating sleep-wake transitions in mammals [43]. The identification of *Eaat2* as a modulator of sleep provides an avenue for investigating how neuron-glia interactions contribute to sleep regulation and sleep-dependent changes in metabolism.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Measuring metabolic rate with the SAMM system
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Analysis of fly sleep behavior
  - Analysis of fly metabolic rate

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and two videos and can be found with this article online at https://doi.org/10.1016/j.cub.2018.10.039.

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AUTHOR CONTRIBUTIONS

All authors contributed to the experimental design, data analysis, and writing of the manuscript. Experiments were performed by B.A.S., E.P., S.D., N.A.C.M., and K.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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(F) Metabolic rate reduced in all genotypes during nighttime sleep bouts (two-way ANOVA, $F_{(2, 147)} = 36.09$; *$p < 0.05$, $n > 10$).
(G) Sample 5-min CO_2 measurements for flies overexpressing Eaat2 in ensheathing glia and controls.
(H and I) Metabolic profile (H) and cumulative quantification (I) revealed that metabolic rate was elevated in flies overexpressing *Eaat2* in ensheathing glia (orange) relative to *w*^{1118} (gray) and controls (light blue) during the daytime (one-way ANOVA, $F_{(2, 30)} = 8.544$; *$p < 0.05$, ***$p < 0.001$, $n > 9$) and nighttime (one-way ANOVA, $F_{(2, 30)} = 14.13$; **$p < 0.01$, ***$p < 0.00)1$, $n > 9$).
(J) Metabolic rate during daytime sleep bouts in flies overexpressing *Eaat2* (orange) and controls (gray, blue) (two-way ANOVA, $F_{(2, 55)} = 25.01$; $p > 0.7$, $n > 11$).
(K) Metabolic rate was reduced during nighttime sleep bouts (two-way ANOVA, $F_{(2, 104)} = 30.24$; *$p < 0.05$, $n > 11$), but the effect was similar across all genotypes ($p > 0.3$, $n > 11$).
Error bars represent ±SEM. See also Figure S3.

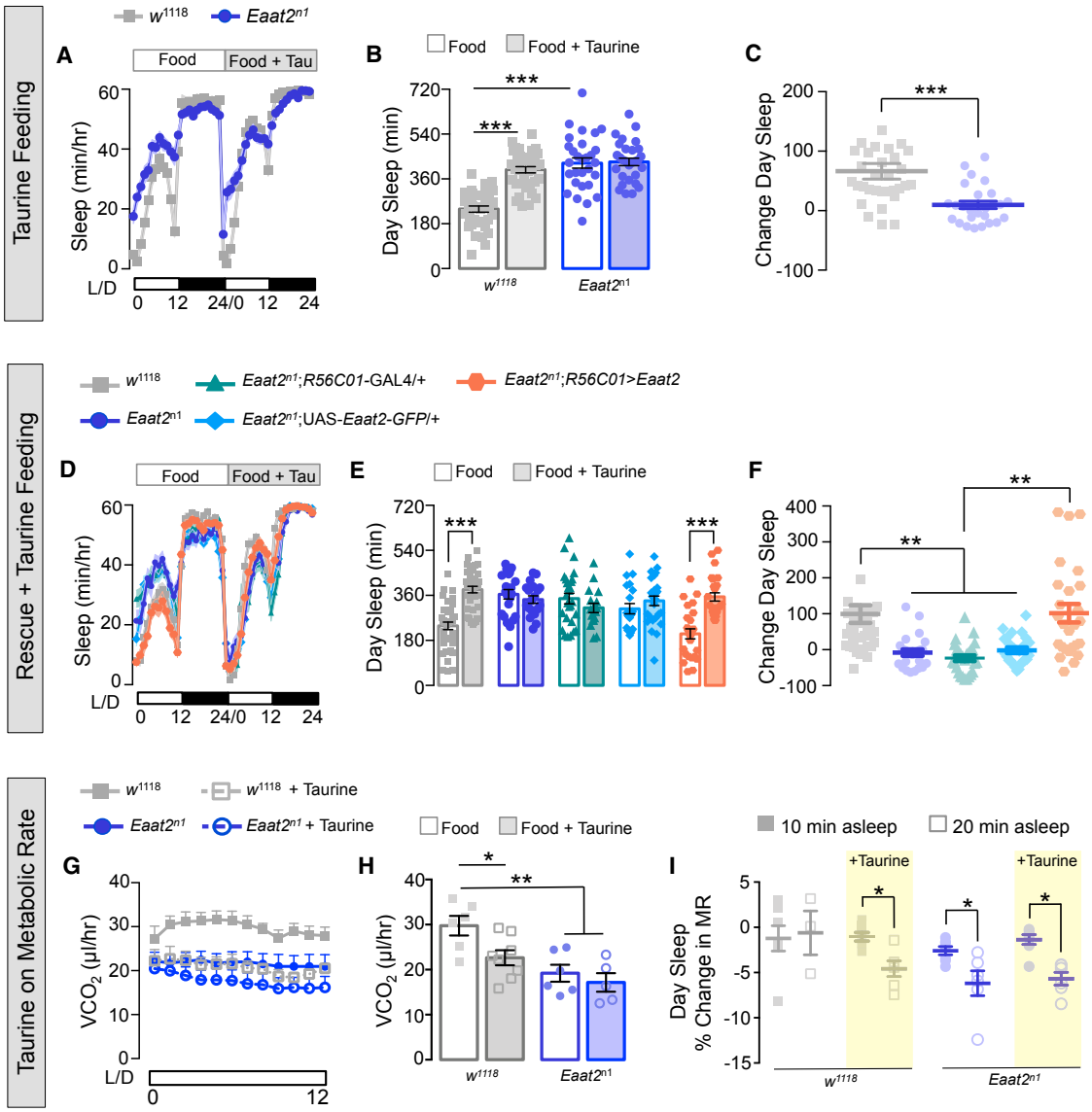

Figure 4. *Eaat2* Modulates Sleep by Regulating Taurine Transport

(A and B) Sleep profile (A) and cumulative quantification (B) reveal that taurine (100 mM) feeding increased sleep in w^{1118} controls (gray) but had no effect on sleep in $Eaat2^{n1}$ flies (blue; two-way ANOVA, $F_{(3, 128)} = 38.41$; ***$p < 0.0001$, $n > 29$).

(C) Percentage change in sleep (day 2 over day 1) upon taurine feeding ($t = 3.611$, $df = 56$, ***$p < 0.001$, $n > 29$).

(D and E) Sleep profile (D) and cumulative analysis (E) of daytime sleep revealed that restoration of *Eaat2* to ensheathing glia rescued the sleep-inducing effect of taurine. Taurine feeding increased sleep in rescued flies (orange) and w^{1118} controls (gray) (two-way ANOVA, genotype factor: $F_{(4\ 251)} = 4.050$; treatment factor, $F_{(1, 251)} = 21.03$; *$p < 0.0001$, $n > 17$).

(F) Percentage change in sleep (day 2 over day 1) upon taurine feeding (one-way ANOVA, $F_{(4, 138)} = 10.39$; **$p < 0.01$, ***$p < 0.001$, $n > 25$). There was no difference between rescued flies (orange) and controls ($p > 0.9$, $n > 25$).

(G and H) Metabolic profile (G) and quantification (H) revealed that metabolic rate was lower in taurine-fed w^{1118} flies (dashed gray line) compared to untreated w^{1118} flies (solid gray line) but was unchanged upon taurine feeding of $Eaat2^{n1}$ flies (blue; two-way ANOVA, genotype factor: $F_{(1, 20)} = 15.30$; **$p < 0.01$, $n > 5$; treatment factor: $F_{(1, 20)} = 6.887$; *$p < 0.05$, $n > 5$).

(I) Percentage change in metabolic rate during daytime sleep bouts lasting 10 min (solid squares) or 20 min (open squares). Taurine feeding altered metabolic rate in w^{1118} flies, but not in $Eaat2^{n1}$ flies (two-way ANOVA, time asleep factor: $F_{(1, 41)} = 14.11$; *$p < 0.05$, $n > 4$).

Error bars represent ±SEM. See also Figure S4.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Guinea pig anti-Eaat2	[20]	N/A
Rabbit polyclonal anti-GFP	Torrey Pines Biolabs	TP401
Mouse monoclonal anti-bruchpilot (nc82)	Developmental Studies Hybridoma Bank	nc82; RRID: AB_2314866
Goat anti-guinea pig Alexa Fluor 647	Jackson ImmunoResearch	106-605-003
Goat anti-mouse Rhodamine Red-X	Jackson ImmunoResearch	115-295-146
Goat anti-rabbit Alexa Fluor 488	Thermo Fisher Scientific	A-11008
Chemicals, Peptides, and Recombinant Proteins		
Paraformaldehyde 16% solution EM Grade	EMS	15710
SlowFade Diamond Antifade Mountant with DAPI	Thermo Fisher Scientific	S36964
Bloomington Formulation Nutri-fly food	Genesee Scientific	66-113
Sucrose	Fisher Scientific	S3-500
Agar	Fisher Scientific	BP1423-500
Taurine	Sigma Aldrich	T0625
Indicating Drierite, 8 mesh	Hammond Drierite	23025
Ascarite II	Acros Organics	81133-20-2
Critical Commercial Assays		
RNEasy Plus Mini Kit	QIAGEN	74134
One-Step RT-PCR Kit	QIAGEN	210210
Drosophila Activity Monitors (DAMs)	TriKinetics	DAM2
Experimental Models: Organisms/Strains		
w^{1118} fly strain	Bloomington Drosophila Stock Center (BDSC)	6326
P{Act5C-GAL4-w}E1	BDSC	25374
P{TRiP.JF01355}	BDSC	31603
P{TRiP.HMS01998}	BDSC	40832
P{GMR56C01-Gal4}attP2	BDSC	Former 54513
P{GMR56F03-GAL4}attP2	BDSC	39157
UAS-mCD8::GFP	BDSC	32186
Mi{MIC}Eaat2^{MI10251}	BDSC	54513
PBac{RB}Eaat2^{e00569}	Harvard Exelixis collection	e00569
PBac{WH}Eaat2^{f06549}	Harvard Exelixis collection	f06549
PBac{RB}Eaat2^{e04636}	Harvard Exelixis collection	e04636
UAS-*Eaat2-GFP*	[21]	N/A
Alrm-GAL4	[25]	N/A
NP6293-GAL4	[23]	N/A
Mz0709-GAL4	[44]	N/A
Eaat2^{n1}	This paper	N/A
Software and Algorithms		
ImageJ/FijI	NIH	v1.52 e
Microsoft Office Powerpoint	Microsoft	v2007
Microsoft Office Excel	Microsoft	v2007
DAMs Activity Software	TriKinetics	v3.08
Drosophila Counting Macro	[12]	v5.19.9 2010
Prism	Graphpad software	v6

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
DART Tracking Software	BFK Labs	v2018
ExpeData	Sable Systems International	ExpeData-P
Other		
Olympus confocal laser-scanning microscope	Olympus	BX-63 Fluoview FV1000
Fly Tracking Box	BFK Labs	Full-enclosure
Locomotor Activity Monitor	TriKinetics	Custom
Subsampler/Pump	Sable Systems International	SS4
Mass Control Valve	Sierra Instruments	840L-2-OV1-SV1-E-V1-S1
Flow Multiplexer	Sable Systems International	MUX
CO2/H2O Analyzer	Li-Cor, Sable Systems International	LI-7000
MultiTube Vortexer	VWR	VX2500
Repeat-cycle relay switch	Macromatic	TR631122

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Alex C. Keene (keenea@fau.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila stocks

The biological model used throughout this study is the fruit fly, *Drosophila melanogaster*. Fly strains were obtained from Bloomington (BL) Stock Center: *Act5c*-GAL4 (P{Act5C-GAL4-w}E1; BL#25374), UAS-*Luc*-RNAi (P{TRiP.JF01355}; BL#31603), UAS-*Eaat2*-RNAi (P{TRiP.HMS01998}; BL#40832), *R56C01*-GAL4 (P{GMR56C01-Gal4}; former BL#54513), R56F03-GAL4 (P{GMR56F03-Gal4}; BL#39157), UAS-mCD8::GFP (BL#32186) and Mi{MIC}*Eaat2*^{MI10251}(BL#54513); Harvard Exelixis collection: *Eaat2*^{e00569} (PBac{RB}*Eaat2*^{e00569}), *Eaat2*^{f06549} (PBac{WH}*Eaat2*^{f06549}), *Eaat2*^{e04636} (PBac{RB}*Eaat2*^{e04636}); and published sources (UAS-*Eaat2-GFP* [19]; *Alrm*-GAL4 [23]; *NP6293*-GAL4 [21]; *Mz0709*-GAL4) [45]. The background control line used in this study is *w*^{1118} (BL#6326), and all experimental strains were either generated in a *w*^{1118} background strain, or outcrossed to *w*^{1118} for 6 generations prior to analysis.

METHOD DETAILS

Generation of the *Eaat2*^{n1} flies

The *Eaat2*^{n1} null allele was generated by Flippase (FLP)-mediated recombination between two *piggyBac* (PBac) transposon insertions in the *Eaat2* locus carrying FLP Recognition Target (FRT) sites: PBac{RB}^{e04636} and PBac{WH}^{f06549} [17, 18]. Recombination of these transposons removed the entire coding region of *Eaat2* and left a residual hybrid element of ~6.1 kilobases that was ineligible for removal by further transposition.

Single fly DNA preparation and PCR validation

For genomic DNA templates for PCR, single flies were placed in a 0.5 mL microcentrifuge tube with 50 μL of freshly-prepared squishing buffer (25mM NaCl, 1mM EDTA pH8.0, 10mM Tris pH8.2, 0.2mg/mL Proteinase K) and mashed for a few seconds with a pipette tip. Incubation at 30 min at 37°C allowed digestion and 2 min at 95°C for inactivation of the enzyme. 2 μL of DNA template were used for PCR in a 25 μL reaction volume with Taq polymerase (BioBasic), along with specific oligonucleotide primers. To screen for successful *Eaat2* deletion events, element-specific (i.e., piggyBac) and genome-specific primers were used and revealed i) absence of PBac element ends, ii) presence of residual element ends, and finally iii) deletion of *Eaat2* coding sequence (primers D1/D2). *Actin5C* specific primers were used to confirm presence of template DNA in all samples. Eaat2 primers D1: 5′-AAGTCCTC CATCGTGGTGTC-3′; D2: 5′-GCATGAACGAGAAACTCAAGG-3′; *Actin5C* primers: 5′-GAGCGCGGTTACTCTTTCAC-3′; 5′-ATCCC GATCCTGATCCTCTT-3′.

Reverse transcriptase-PCR (RT-PCR)

RT-PCR was performed on total RNA isolated from homozygous adult flies of the following genotypes: control *w*^{1118}, *Eaat2*^{e04636}, and *Eaat2*^{n1} (RNEasy Plus Mini Kit and One-Step RT-PCR Kit, QIAGEN). The *Eaat2* primer set (D1/D2) amplified a band of 479 base pairs (bp) in controls, but not in *Eaat2*^{n1} mutants. The amplicon bridges an intron–exon junction to distinguish cDNA from genomic DNA templates. An *Actin5C* fragment of 586 bp was amplified to ensure cDNA template integrity.

Immunostaining of adult CNS and microscopy

Brains were dissected in cold sodium phosphate buffer (PB; pH 7.2) and transferred into PLP (2% Paraformaldehyde in PB with Lysine) for 1 hr at room temperature [46]. All subsequent steps were performed on a Nutator at room temperature in Eppendorf LoBind microcentrifuge tubes (Sigma-Aldrich, #Z666548). After three washes (15 min each) with PB 0.3% Triton X-100 (PBT), the tissues were blocked in 5% normal goat serum (Jackson Laboratories) in PB 0.5% Triton X-100 for 1 hr. Tissues were incubated with anti-Eaat2 antiserum (1:3000 [20]), anti-BRP (1/5; #nc82 Developmental Studies Hybridoma Bank), anti-GFP (1/1000; Torrey Pines Biolabs, #TP401) for 4 hr at room temperature followed by 2 × overnight at 4°C; washed 4 × 30 min in PBT, then incubated with secondary antibodies 2 × overnight at 4°C: goat anti-guinea pig (Alexa Fluor 647, Jackson ImmunoResearch, #106-605-003), goat anti-mouse (Rhodamine Red-X, Jackson ImmunoResearch #115-295-146), and goat anti-rabbit (Alexa Fluor 488, Thermo Fisher Scientific, #A11008), washed 4 × 30min in PBT, followed by a final wash in PB. Tissues were mounted in SlowFade Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific, #S36964). Fluorescence images were acquired with an Olympus BX-63 Fluoview FV1000 confocal laser-scanning microscope, and processed using Fiji.

Fly sleep behavior

The *Drosophila* Activity Monitoring system (DAMs; Trikinetics) detects activity by monitoring infrared beam crossings of single flies. Beam-break counts are used to calculate amount of sleep and associated sleep metrics, by identifying 5-min bouts of quiescence using the *Drosophila* Sleep Counting Macro [13]. Flies were briefly anesthetized with CO_2, loaded individually in DAMs tubes with standard fly food (Bloomington Formulation, Nutri-fly, #66-113, Genesee Scientific), and allowed to acclimate for 24-hr. Baseline sleep was then measured for 24-hr (12-hr light (L)/12-hr dark (D)). Flies were maintained in incubators (Model #DR-41NL, Percival) at 25°C with ~50% relative humidity for the entire experiment duration in accordance with previously published protocols [44, 47]. All behavioral experiments utilized 5-8 day old, mated female flies. Taurine (Sigma Aldrich, # T0625) was dissolved in dH_2O at 50 mg/mL, then mixed to 100 mM (final) in cooled, standard fly food (Bloomington Formulation, Nutri-fly, #66-113, Genesee Scientific), similar to a prior report [10]. To determine the acute effects of taurine on sleep duration, baseline sleep was measured for 24-hr on standard fly food in DAMs, then flies were transferred to fly food plus taurine for an additional 24-hr.

Fecundity and longevity

For fecundity experiments, groups of 100 flies (50 males and 50 females) of each genotype were housed in breeding chambers and allowed to acclimate and mate for 24-hr. Flies were transferred to grape food plates with yeast paste and allowed to lays eggs for 3-hr. Eggs were counted immediately, and the resulting pupae were counted days later. Longevity was measured by loading individual flies in DAMs with food of 5% sucrose in 1% agar, transferring flies to fresh food each week. Activity was monitored continuously, and death recorded when activity ceased.

Sleep deprivation and rebound

For sleep deprivation experiments, flies were shaken while housed in DAMs monitors. Monitors are attached to a custom-milled base that is mounted on a vortexer (Fisher Scientific, MultiTube Vortexer). Mechanical shaking stimulus was applied to the vortexer with a repeat-cycle relay switch (Macromatic, #TR631122), wherein flies were shaken for ~3-4 s every min for 12-hr from ZT12 (darkness onset) through ZT0 (light onset), as previously described [47, 48]. Sleep rebound was measured the following day from ZT0-ZT12.

Acute versus developmental effects with TARGET

Acute versus developmental effects were determined using the previously published Temporal And Regional Gene Targeting (TARGET) system using $GAL80^{TS}$ [16]. $GAL80^{TS}$, a temperature-sensitive inhibitor of GAL4, was combined with glial-specific *Repo*-GAL4 to selectively inhibit *Eaat2* (UAS-*Eaat2*-RNAi) during either development or adulthood. At the permissive temperature (18°C) *Eaat2*-RNAi is not expressed to achieve knockdown. When shifted to 29°C, $GAL80^{TS}$ cannot repress GAL4, *Eaat2*-RNAi is expressed, and *Eaat2* is selectively knocked down in glia of adult flies. Conversely, by rearing flies at 29°C throughout development, then maintaining flies at 18°C following eclosion, *Eaat2*-RNAi is expressed in glia during development, but not adulthood.

Fly tracking and feeding behavior

Individual flies were loaded into 65mm plastic DAMs tubes mounted on white polyamide platforms housed in light-controlled DART system tracking boxes equipped with infrared cameras for tracking fly position ([49]; BFK Labs). Flies were acclimated for 24-hr, then recorded for 24-hr at 15 frames per second. Video recordings, fly position tracking, arena/specific regions (e.g., near food) are controlled/analyzed using DART software (BFK labs). For feeding analyses comparing w^{1118} and $Eaat2^{n1}$ flies, the DAM tubes were divided into 3 equal regions (Figure S1R) and the fraction of time individual flies spent in the region housing the food was sampled once every 60 s and averaged the mean position of individuals every hr. This yielded 24 hr readings for each genotype representing the fraction of time each genotype (n = 20 individuals) spent feeding. Since the measure relies on the frequency of individuals of a given genotype feeding in the region, and this same population of individuals with the same genotype is sampled repeatedly over 24-hr, it is relevant to note that the "n" for this analysis represents a repeated sampling of fraction time each hr for each genotype.

Measuring metabolic rate with the SAMM system

Measures of metabolic rate were determined using the Sleep and Activity Metabolic Monitor (SAMM) system, using the methodology as described previously [34]. In brief, CO_2 output was measured using ExpeData Pro (Sable Systems International v.1.8.4) from chambers carrying individual flies. Simultaneously, sleep/activity was measured with a custom-designed activity monitor (Trikinetics). CO_2 was sampled once every 5 min from each chamber, which corresponds to the convention that a bout of sleep in *Drosophila* corresponds to 5 min of monitored inactivity. Since only five flies plus baseline can be run in the SAMM system, 1-2 flies of each genotype were assayed in each run in a randomized order.

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis of fly sleep behavior

We performed a Student's t test, one-way ANOVA or two-way ANOVA with Sidak's correction for multiple comparisons, when appropriate, using InStat software (GraphPad Software v.6.0). The two-tailed p value for significance is denoted at $p < 0.05$. The "n" refers to the number of biological samples tested unless otherwise stated.

Analysis of fly metabolic rate

The hourly volume of VCO_2, (μL/hr) was calculated by summing the 12 readings within one hr. The mean VCO_2 is the average of the 12 daytime or 12 nighttime hourly readings for a single fly. The "n" reported refers to the number of individual flies assayed. Statistical analyses using InStat software (GraphPad v.6.0) employed either a Student's t test or one-way ANOVA with Sidak's multiple comparison correction.

Sleep-dependent changes in metabolic rate were determined as previously described [33]. Data was analyzed using two-way ANOVA with Sidak's correction for multiple comparisons, comparing the initial percent change in metabolic rate during prolonged sleep bouts (10 min sleep bin, normalized to first 5 min bin) to each subsequent sleep bin (daytime: 20 min bin, nighttime: 20 and 30 min bins). For daytime sleep bouts, this analysis was limited to 20 min asleep, because very few control flies ($n \leq 2$ of measured individuals) had sleep bouts longer than 20 min during daytime.

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Supplemental Information

The Taurine Transporter *Eaat2* Functions in Ensheathing Glia to Modulate Sleep and Metabolic Rate

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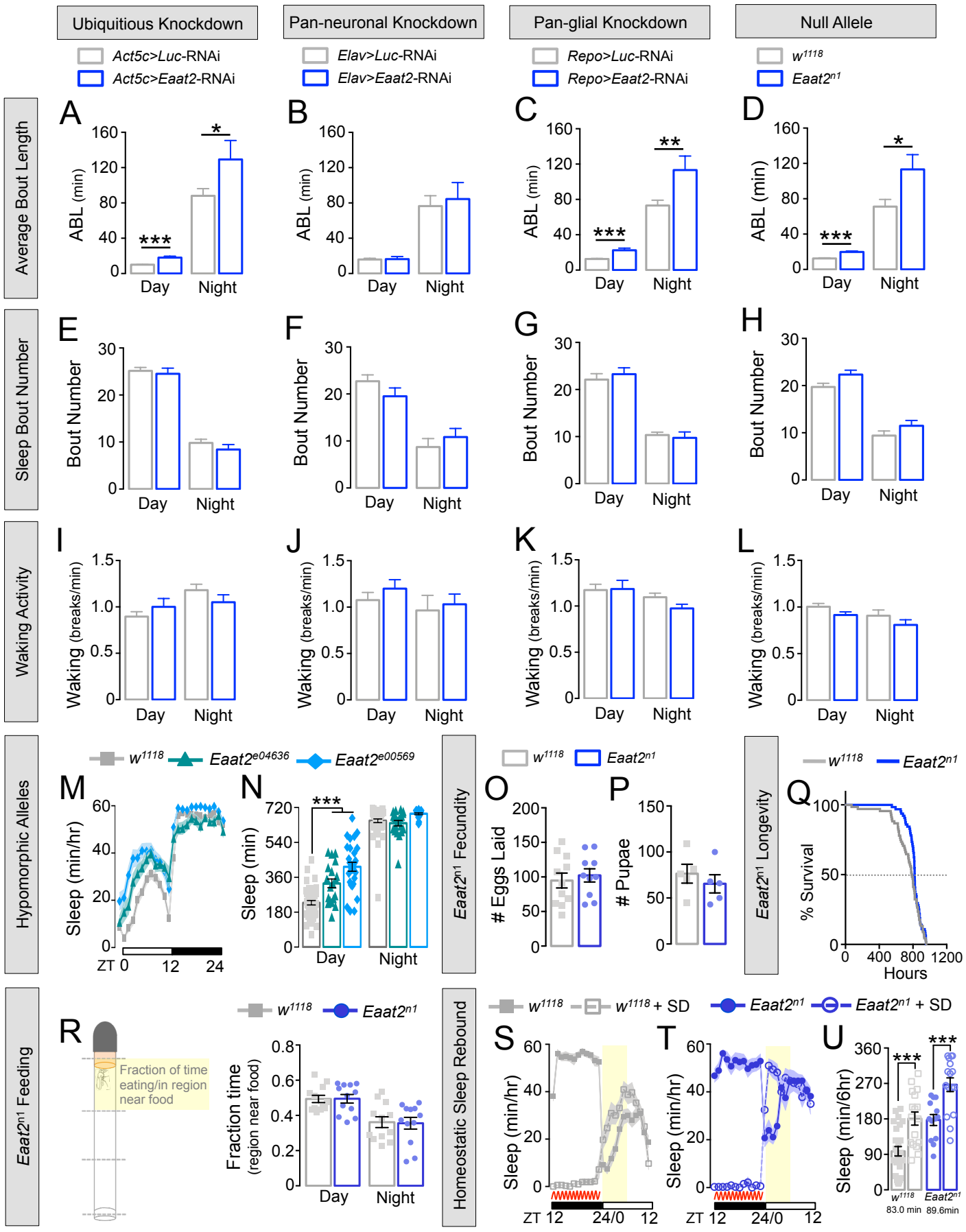
Ubiquitious Knockdown
Act5c>Luc-RNAi
Act5c>Eaat2-RNAi
Pan-neuronal Knockdown
Elav>Luc-RNAi
Elav>Eaat2-RNAi
Pan-glial Knockdown
Repo>Luc-RNAi
Repo>Eaat2-RNAi
Null Allele
w1118
Eaat2n1
Average Bout Length
Sleep Bout Number
Waking Activity
Hypomorphic Alleles
Eaat2n1 Fecundity
Eaat2n1 Longevity
Eaat2n1 Feeding
Homeostatic Sleep Rebound
ABL (min)
Bout Number
Waking (breaks/min)
Day
Night
Sleep (min/hr)
ZT 0 12 24
Sleep (min)
Eggs Laid
Pupae
% Survival
Hours
Fraction time (region near food)
Fraction of time eating/in region near food
w1118 + SD
Eaat2n1 + SD
ZT 12 24/0 12
Sleep (min/6hr)
83.0 min
89.6min

Figure S1. *Eaat2* functions in glia to modulate sleep, related to Figure 1. (**A-D**) Effect of Eaat2 loss-of-function on the average length of sleep bouts. (A) Ubiquitous knockdown with *Act5c>Eaat2*-RNAi (blue) increased bout length in both day and night relative to *Act5c>Luc*-RNAi (grey) controls (day: t= 7.065, df= 74, ***P<0.001; night: t=2.200 df=74, *P<0.05; n>22). (B) Bout length was unaffected by pan-neuronal knockdown with Elav-GAL4 (P>0.3, n>16), but (C) it was lengthened both day and night upon Eaat2 knockdown in glial cells with Repo-GAL4 (day: t= 5.164, df= 90, ***P<0.0001, night: t=2. 909 df= 90, **P<0.01; n>26), and (D) in $Eaat2^{n1}$ flies (blue) relative to w^{1118} controls (grey; day: t= 6.362 df=104, ***P<0.0001; night: t=2.073 df=104, *P<0.05; n>51). (**E-H**) Bout number was unaffected across all genotypes during both day and night sleep (P>0.1). (**I-L**) Waking activity indicated normal movement and did not differ between experimental and control flies across genotypes (P>0.1). **(M**, **N**) Daytime sleep was elevated in $Eaat2^{e04636}$ (green) and $Eaat2^{e00569}$ (light blue) flies, compared to w^{1118} controls (one-way ANOVA, $F_{(2, 91)}$= 32.02; ***P<0.001, n>20), and no differences were detected during the night (P>0.06). (**O, P**) Fecundity is unaffected in $Eaat2^{n1}$ flies (blue) relative to w^{1118} controls (grey), with no significant differences detected in egg laying (O) (P>0.6, n>10) or pupae number (P) (P>0.4, n>5). (**Q**) Analysis of longevity reveals a modest increase in lifespan for $Eaat2^{n1}$ flies (820.8 hrs, blue) compared to w^{1118} flies (785.7 hrs, grey; Chi Square= 6.343, df= 1, *P<0.01). (**R**) Video tracking revealed no differences in feeding between $Eaat2^{n1}$ (blue) and w^{1118} (grey) controls (P>0.9, n>12). (**S-U**) Flies were sleep-deprived from ZT12-ZT24 (red line) and recovery sleep measured the following day (ZT0-ZT12). During the recovery day, sleep rebound is apparent in both $Eaat2^{n1}$ (blue; t=3.897 df=27, ***P<0.001, n>13) and w^{1118} individuals (grey; t=4.151 df=41, ***P<0.001, n>17). Although differences in baseline sleep are apparent among genotypes, the amount of recovery sleep in sleep-deprived $Eaat2^{n1}$ and w^{1118} flies (open circles), each compared to their respective undisturbed control, is comparable (closed circles; 89.6 min and 83.0 min, respectively).

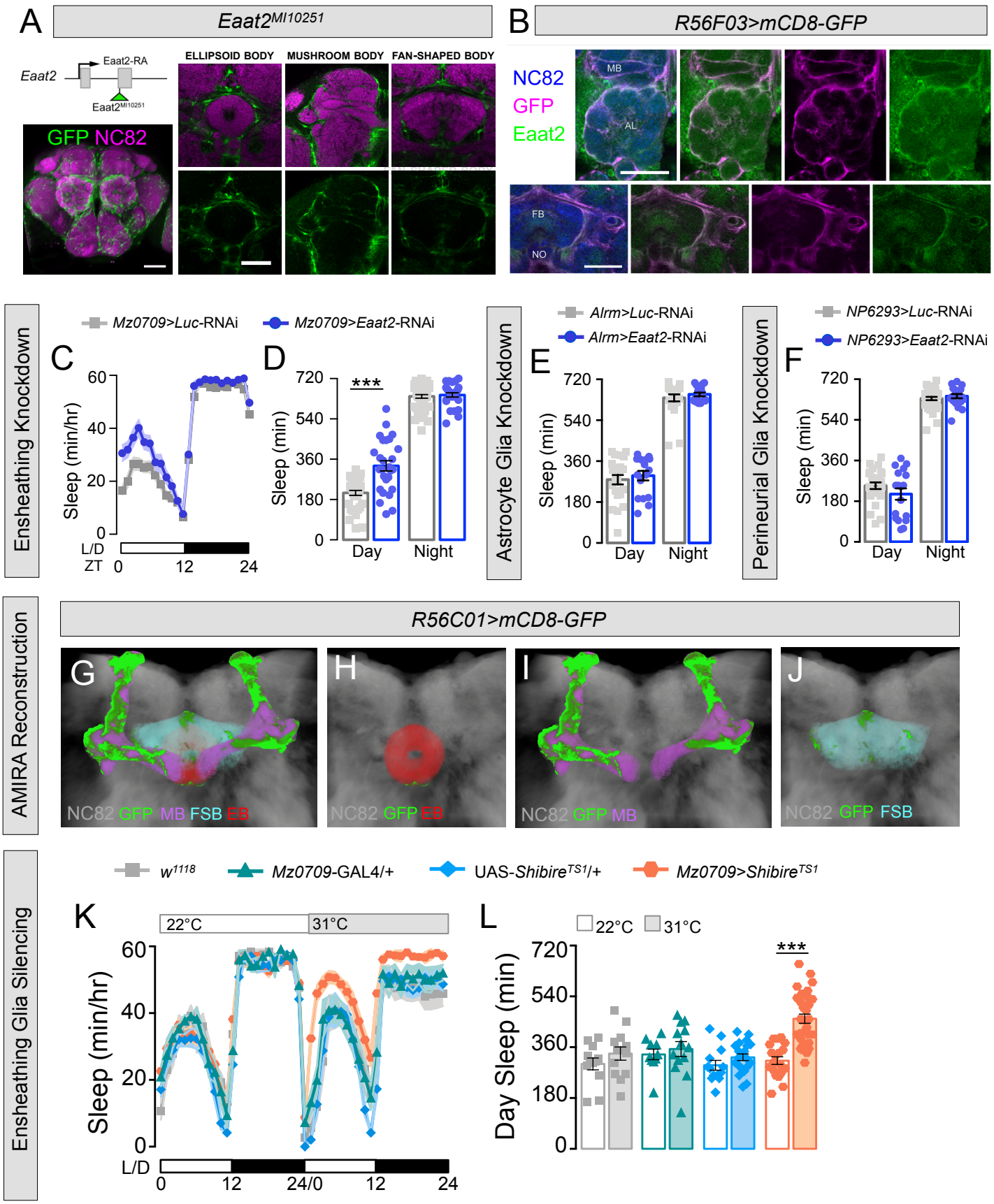
A
Eaat2MI10251
Eaat2
Eaat2-RA
Eaat2MI10251
GFP NC82
ELLIPSOID BODY
MUSHROOM BODY
FAN-SHAPED BODY
B
R56F03>mCD8-GFP
NC82
GFP
Eaat2
MB
AL
FB
NO
Ensheathing Knockdown
Mz0709>Luc-RNAi
Mz0709>Eaat2-RNAi
C
Sleep (min/hr)
L/D
ZT 0 12 24
D
Sleep (min)
***
Day
Night
Astrocyte Glia Knockdown
Alrm>Luc-RNAi
Alrm>Eaat2-RNAi
E
Sleep (min)
Day
Night
Perineurial Glia Knockdown
NP6293>Luc-RNAi
NP6293>Eaat2-RNAi
F
Sleep (min)
Day
Night
AMIRA Reconstruction
R56C01>mCD8-GFP
G
NC82 GFP MB FSB EB
H
NC82 GFP EB
I
NC82 GFP MB
J
NC82 GFP FSB
Ensheathing Glia Silencing
w1118
Mz0709-GAL4/+
UAS-ShibireTS1/+
Mz0709>ShibireTS1
K
22°C
31°C
Sleep (min/hr)
L/D
0 12 24/0 12 24
L
22°C
31°C
Day Sleep (min)
***

Figure S2. The sleep-suppressing function for *Eaat2* localizes to ensheathing glia, related to Figure 2. (A) *Eaat2*^{MI10251} is a GFP-expressing gene-trap in the *Eaat2* locus. GFP expression (green) in the adult brain is reminiscent of ensheathing glia surrounding regions of neuropil (NC82, magenta). Whole brain panel (left) is an average projection of a confocal Z-stack (Fiji). Magnified panels are single confocal Z-slices showing ensheathing glia around known sleep-wake centers. Scale bars, 50μm. See Video S2. (**B**) anti-Eaat2 (green) co-labels ensheathing glia (R56F03>mCD8-GFP, magenta). Top row are magnified views at the level of the mushroom body (MB) and antennal lobe (AL); bottom row is at the level of the fan-shaped body (FB). Scale bars = 50μm. (**C, D**) 24-hr sleep profile (C) and quantification (D) showing that knockdown of *Eaat2* in ensheathing glia (*Mz0709>Eaat2*-RNAi, blue) significantly increases daytime sleep, compared to (*Mz0709>Luc*-RNAi, grey) controls (t=5.178 df=62, P< 0.0001, n>27)). (**E, F**) Sleep is unaffected when *Eaat2* is knocked down in astrocytes (E) (P>0.4, n>17) or perineurial glia (F) (P>0.1, n>18). **(G-J)** Whole-brain reconstruction (AMIRA) with genetically-labeled ensheathing glia (*R56C01>mCD8-GFP*) counterstained with anti-BRP (NC82; grey). *R56C01*-GAL4 labeled glia (green) are broadly distributed throughout the brain, and reconstruction revealed them at sleep centers, in low numbers near the ellipsoid body (H, red), and in higher numbers near the mushroom bodies (I, purple), and the fan-shaped body (J, cyan). (**K**, **L**) Multi-day sleep profile (K) and quantification (L) shower daytime sleep was greater in flies expressing *Shi*^{TS1} in ensheathing glia (*Mz0709>Shi*^{TS1}, orange) at 31°C than controls that were either *w*^{1118} (grey), *Mz709*-GAL4 (green), or UAS-*Shi*^{TS1} (green) flies (two-way ANOVA, $F_{(3, 125)}$= 19.11; **P<0.01, ***P<0.001, n>12). No differences in daytime sleep were detected between any groups when tested at 22°C (P>0.6, n>12).

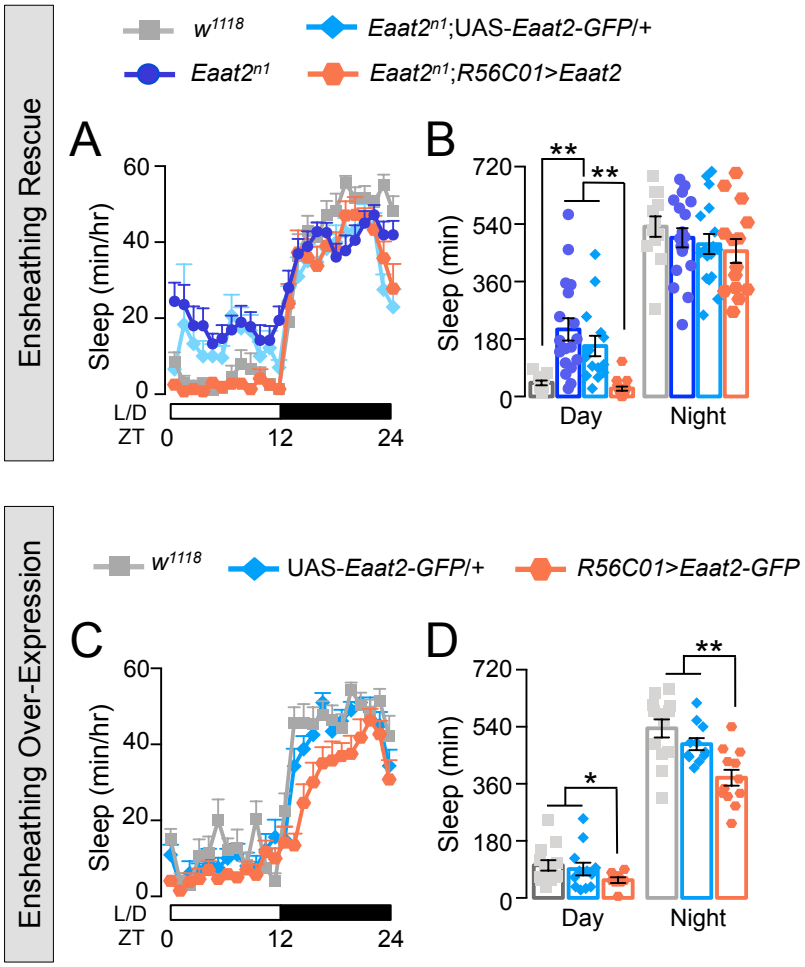
Ensheathing Rescue
w1118
Eaat2n1
Eaat2n1;UAS-Eaat2-GFP/+
Eaat2n1;R56C01>Eaat2
A
Sleep (min/hr)
60
40
20
0
L/D
ZT 0
12
24
B
Sleep (min)
720
540
360
180
0
**
**
Day
Night
Ensheathing Over-Expression
w1118
UAS-Eaat2-GFP/+
R56C01>Eaat2-GFP
C
Sleep (min/hr)
60
40
20
0
L/D
ZT 0
12
24
D
Sleep (min)
720
540
360
180
0
*
**
Day
Night

Figure S3. *Eaat2* functions in ensheathing glia to regulate sleep-dependent changes in metabolic rate, related to Figure 3. (**A, B**) 24-hr sleep profile (A) and quantification (B) of flies in the SAMM system, confirming increased sleep of *Eaat2*^{n1} flies (dark blue) and their rescue with Eaat2 expression in ensheathing glia (orange) (day: one-way ANOVA: $F_{(3, 56)} = 7.967$; **P<0.01, n>9; night: P>0.4, n>9). (**C, D**) 24-hr sleep profile (C) and quantification (D) of flies in the SAMM system, showing that flies over-expressing *Eaat2* in ensheathing glia sleep less than controls (day: one-way ANOVA: F (2, 30) = 8.544; P<0.05, n>9; night: one-way ANOVA: $F_{(2, 25)} = 14.13$; P<0.01, n>9).

Taurine on Metabolic Rate

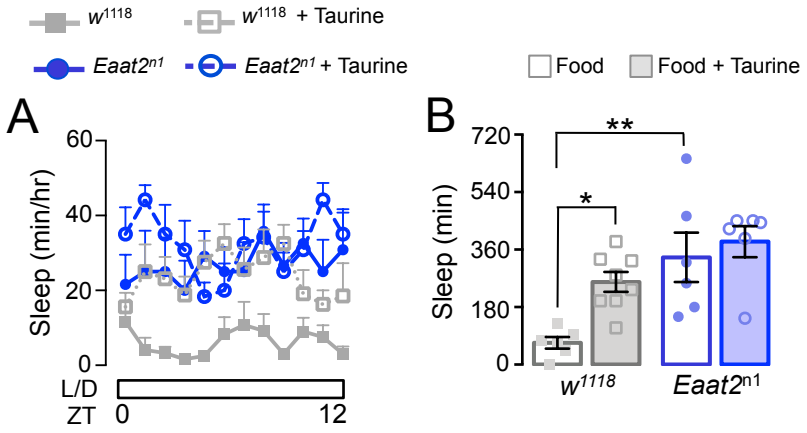

Figure S4. *Eaat2* modulates sleep by regulating taurine transport, related to Figure 4. (**A**, **B**) 12-hr sleep profile (A) and quantification (B) of $Eaat2^{n1}$ (blue) and w^{1118} (grey) flies that were fed taurine (open) or were not (solid). (**B**) Quantification of daytime and nighttime sleep in taurine fed (solid bars) compared to standard food (open bars; two-way ANOVA: Genotype factor, $F_{(1, 22)} = 17.44$; $^{**}P<0.01$, n>6; Treatment factor, $F_{(1, 22)} = 6.455$; $^{*}P<0.05$, n>6).