Serine metabolism in the brain regulates starvation-induced sleep suppression in *Drosophila melanogaster*

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Sleep and metabolism are physiologically and behaviorally intertwined; however, the molecular basis for their interaction remains poorly understood. Here, we identified a serine metabolic pathway as a key mediator for starvation-induced sleep suppression. Transcriptome analyses revealed that enzymes involved in serine biosynthesis were induced upon starvation in *Drosophila melanogaster* brains. Genetic mutants of *astray* (*aay*), a fly homolog of the rate-limiting phosphoserine phosphatase in serine biosynthesis, displayed reduced starvation-induced sleep suppression. In contrast, a hypomorphic mutation in a serine/threonine-metabolizing enzyme, *serine/threonine dehydratase* (*stdh*), exaggerated starvation-induced sleep suppression. Analyses of double mutants indicated that *aay* and *stdh* act on the same genetic pathway to titrate serine levels in the head as well as to adjust starvation-induced sleep behaviors. RNA interference-mediated depletion of *aay* expression in neurons, using cholinergic Gal4 drivers, phenocopied *aay* mutants, while a nicotinic acetylcholine receptor antagonist selectively rescued the exaggerated starvation-induced sleep suppression in *stdh* mutants. Taken together, these data demonstrate that neural serine metabolism controls sleep during starvation, possibly via cholinergic signaling. We propose that animals have evolved a sleep-regulatory mechanism that reprograms amino acid metabolism for adaptive sleep behaviors in response to metabolic needs.

starvation | sleep regulation | serine

Sleep and metabolism are interconnected processes that modulate each other, which is best exemplified during sleep deprivation conditions. During acute sleep deprivation, large amounts of lysolipids are found in the mouse cortex, indicating that membrane phospholipids are degraded during sleep loss (1). Metabolites such as tryptophan, serotonin, and taurine are increased in human plasma samples during acute sleep deprivation, and these increases may be associated with its antidepressive effects (2). While information from such metabolic studies of acute sleep deprivation is important, prolonged partial sleep loss is more prevalent in modern society. In rats and humans, prolonged sleep deprivation decreases two specific metabolites, diacylglycerol 36:3 and oxalic acid, which are restored to baseline levels after recovery sleep (3). Other studies further show that chronic sleep deprivation reduces blood leptin levels, induces hunger, and alters food choice in humans with a preference for sweet, salty, and high-carbohydrate foods (4, 5). These observations suggest that quantity of sleep affects metabolic processes and behaviors for reasons that are as yet unclear.

Conversely, changes in dietary composition influence the quantity and quality of sleep. Relative amounts of dietary sugar modulate arousal threshold and control sleep architecture in *Drosophila* (6–8). Gustatory perception of lower sugar concentration increases the number of sleep episodes whereas metabolic sensing of higher nutritional values in dietary sugar suppresses sleep partitioning (6). In addition, meal size and nutritional content contribute to postprandial sleep. Flies that eat more generally sleep more, and protein-rich foods promote sleep after meal consumption via leucokinin receptor neurons (9). The aforementioned studies highlight the important notion that various aspects of sleep can be regulated by diverse metabolic pathways.

The starvation state is an excellent model that illustrates the interaction between metabolism and sleep. As a behavioral response to starvation, animals suppress sleep and enhance locomotion to forage for new food sources (10, 11). Since the discovery that fruit flies suppress sleep during starvation (12), new genes and neural circuits are being identified that can modulate sleep during starvation. For example, circadian clock genes, *Clock* and *cycle*, limit starvation-induced sleep suppression in *cryptochrome*-expressing cells (12). Also, translin, a highly conserved RNA/DNA-binding protein, promotes wakefulness in leucokinin neurons during starvation (13). On the other hand, transgenic activation of gustatory neurons that sense sweetness is sufficient to restore sleep in starved flies (7, 8). However, despite recent advances, the molecular cues linking starvation and sleep remain largely unknown.

To identify genes that regulate starvation-induced sleep suppression, we profiled differentially expressed genes (DEG) in starved fly brains. DEG analysis identified genes involved in serine biosynthesis to be up-regulated in the brain poststarvation. Functional studies using genetic manipulation of key enzymes in the serine biosynthesis pathway revealed an essential role for serine in modulating sleep during starvation.

Significance

Foraging and sleep are two conflicting behaviors in starved animals; however, it remains elusive how metabolic status governs sleep drive. In this study, we show that a biosynthetic pathway for the amino acid serine is transcriptionally up-regulated by starvation in adult fly brains. The behavioral response to genetic manipulation of key enzymes involved in serine metabolism supports the sleep-suppressing effect of serine in response to starvation. In a society where daily diet is becoming increasingly important to the sleep quality of individuals, our study defines an amino acid metabolic pathway that underlies adaptive sleep behaviors upon dietary stress.

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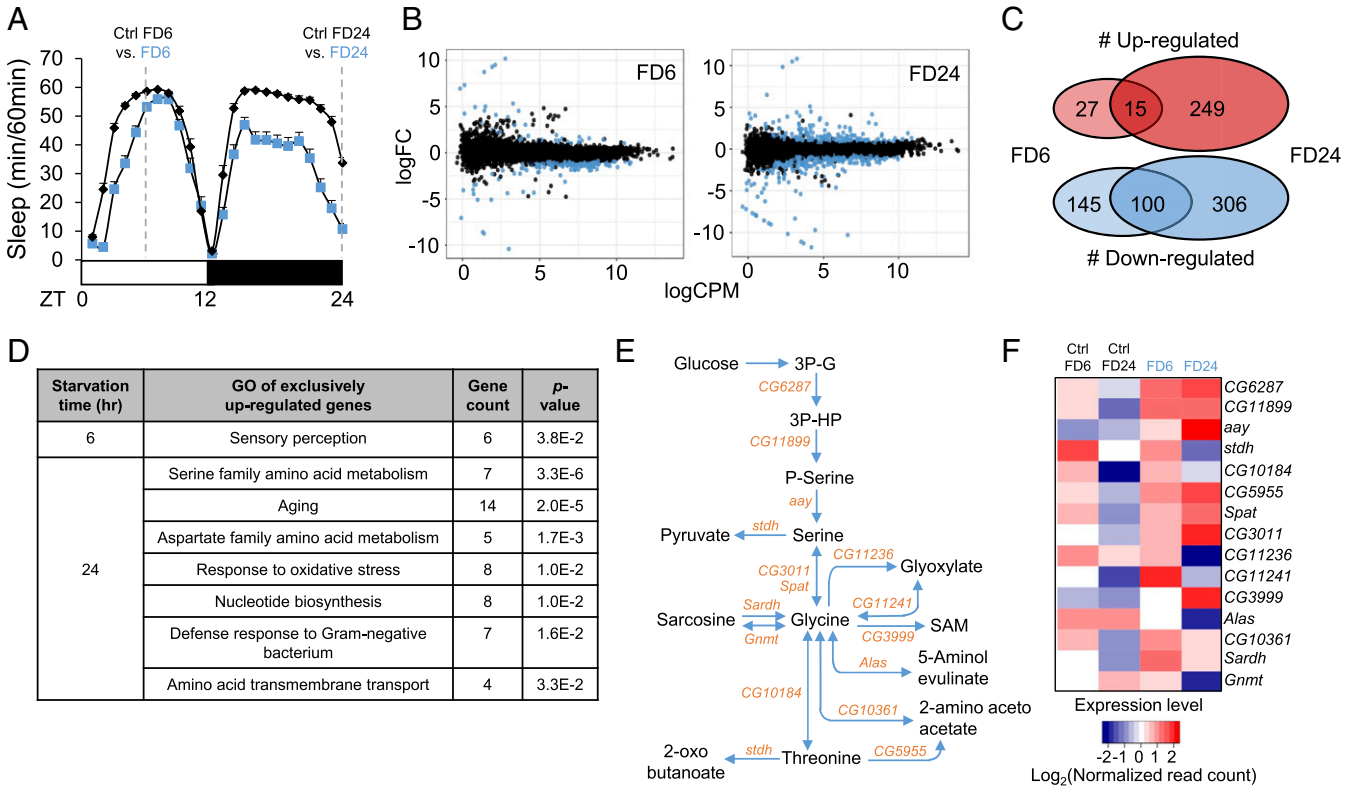
Results

Transcriptome Analyses of Starved *Drosophila* Brains. Physiological responses to starvation include differential gene expression (14, 15). We reasoned that some of those starvation-sensitive genes may play a specific role in sleep regulation under starved conditions. To identify sleep-regulatory genes that respond to starvation in the central nervous system but not in peripheral tissues, we decided to perform RNA sequencing (RNA-seq) (16) on the brains of *Drosophila melanogaster*. Accordingly, we searched for DEGs in fly brains starved for 6 [food deprivation 6 h (FD6)] and 24 h [FD 24 h (FD24)] (Fig. 1*A* and Dataset S1).

Comparison of RNA expression profiles between starved and nonstarved brains revealed 287 and 670 DEGs [false discovery rate (FDR) < 0.05 threshold] after 6 and 24 h of starvation, respectively (Fig. 1*B*). Among the DEGs, 42 genes were up-regulated and 245 genes were down-regulated in FD6, while 264 genes were up-regulated and 406 genes were down-regulated in FD24 (Fig. 1*C*). Gene ontology (GO) analysis revealed that genes up-regulated exclusively during short-term starvation (FD6) were mainly relevant to sensory perception, while genes up-regulated exclusively during long-term starvation (FD24) were involved in biological processes such as amino acid metabolism, nucleotide metabolism, and amino acid transmembrane transport (Fig. 1*D*). In contrast, the GO classifications of down-regulated genes in either FD6 or FD24 samples were relatively diverse (Dataset S2). Consistent with the GO analysis, the starvation-induced genes in FD6 samples showed higher enrichment in brains (~65%) than those in FD24 samples (~20%) when their relative expression was compared between our control brain samples and previously published head samples (*SI Appendix, Supplementary Materials and Methods*) that contain peripheral tissues including fat body, sensory organs, and the compound eye, besides the brain (*SI Appendix*, Fig. S1 and Dataset S3). Finally, the regulation of 115 genes overlapped between FD6 and FD24 (*SI Appendix*, Fig. S2), and GO analysis of these genes revealed various biological processes to be enriched including mating behavior, axon development/neuron projection morphogenesis, and regulation of cell communication.

Intriguingly, *astray* (*aay*), a fly homolog of the rate-limiting phosphoserine phosphatase involved in serine biosynthesis (17, 18), was in the top 10 genes induced by long-term starvation. Therefore, we further examined the expression of genes involved in the serine metabolic pathway (Fig. 1*E*). Heat-map analysis revealed that genes involved in serine synthesis (*CG6287*, *CG11899*, and *aay*) were all up-regulated after 6 and 24 h of starvation (Fig. 1*F*).

***aay*-Dependent Serine Synthesis Is Required for Starvation-Induced Sleep Suppression.** To determine genetically whether serine biosynthesis is important for starvation-relevant sleep regulation, we examined sleep behaviors in flies harboring a transgenic P-element insertion (KG05974, referred to as aay^{KG} from hereafter) upstream of the 5′UTR of *aay* that reduced *aay* mRNA levels to ~30% of *iso31* control flies (Fig. 2*A* and *SI Appendix*, Fig. S3). Western blot analysis confirmed that AAY protein levels were significantly elevated in *iso31* wild types after 24 h of starvation, while aay^{KG} suppressed AAY expression (Fig. 2 *B* and *C*), thereby validating aay^{KG} as a hypomorphic allele of *aay*. Consistent with AAY induction, starvation significantly elevated



D

Starvation time (hr)	GO of exclusively up-regulated genes	Gene count	*p*-value
6	Sensory perception	6	3.8E-2
24	Serine family amino acid metabolism	7	3.3E-6
	Aging	14	2.0E-5
	Aspartate family amino acid metabolism	5	1.7E-3
	Response to oxidative stress	8	1.0E-2
	Nucleotide biosynthesis	8	1.0E-2
	Defense response to Gram-negative bacterium	7	1.6E-2
	Amino acid transmembrane transport	4	3.3E-2

Fig. 1. Transcriptome analyses of starved *Drosophila* brains identify up-regulation of serine biosynthesis pathway. (*A*) Schematic diagram depicting RNA-seq experimental design. Wild-type flies were fed 5% sucrose/1% agar (Ctrl) or deprived of sucrose for 6 and 24 h (FD6 and FD24). (*B*) Scatter plots demonstrate log counts per million (cpm) vs. log fold-change (FC) in expression of brains starved for 6 and 24 h. Blue dots represent DEGs (FDR < 0.05). (*C*) Venn diagram showing the number of genes that are regulated during short-term (FD6) and long-term (FD24) starvation. (*D*) Gene ontology analysis of genes that are up-regulated exclusively in FD6 and FD24. (*E*) Schematic diagram of the serine metabolic pathway. (*F*) Heat-map of expression level of genes involved in the serine metabolic pathway in fed (Ctrl FD6 and Ctrl FD24) and starved conditions (FD6 and FD24). Colors indicate the log2 values of normalized read counts.

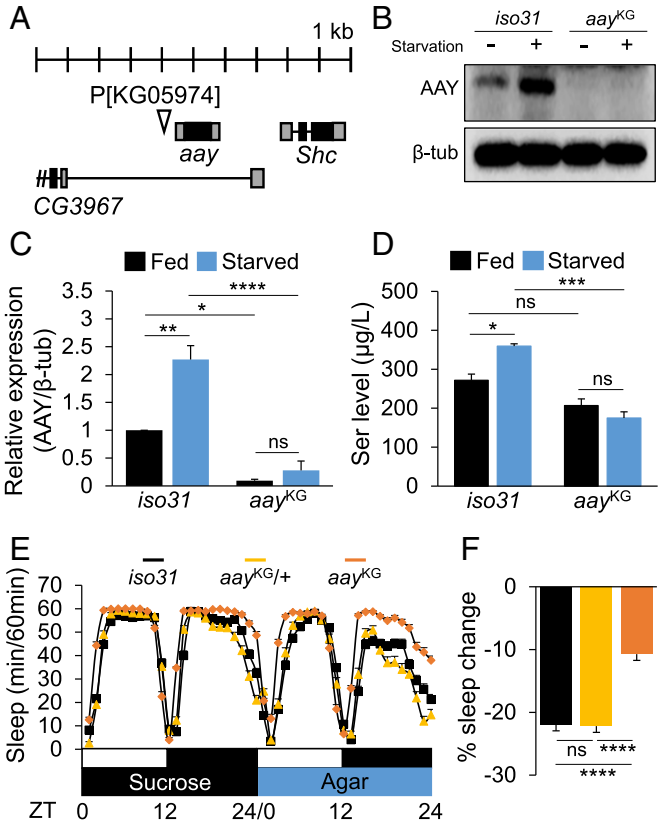


Fig. 2. Starvation-induced *aay* expression elevates free serine levels in heads and supports sleep suppression during starvation. (*A*) Schematic diagram illustrating KG05974 insertion near the 5′UTR of *aay* locus. (*B*) Western blot of head extracts in *iso31* and aay^{KG} flies during fed and starved conditions. (*C*) Quantification of Western blots ($n = 3$) [$F_{(1,8)} = 12.92$; $P = 0.0070$]. (*D*) Serine concentrations in head extracts of *iso31* control and aay^{KG} flies during fed and starved conditions ($n = 3$) [$F_{(1,8)} = 14.53$; $P = 0.0052$]. (*E*) Average sleep traces of *iso31* control (black; $n = 88$), aay^{KG}/+ (light orange; $n = 80$), and aay^{KG} (orange; $n = 78$) flies. (*F*) Percentage sleep change during starvation in *iso31* and aay^{KG}/+ controls vs. aay^{KG} flies. ns, $P > 0.05$; *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$; ****$P < 0.0001$. All error bars represent SEM.

free serine levels in an *aay*-dependent manner ($P = 0.0052$, by two-way ANOVA) (Fig. 2*D*).

Importantly, aay^{KG} flies showed a modest increase in baseline sleep during fed condition (*SI Appendix*, Fig. S4), while starvation-induced sleep suppression was substantially compromised compared with isogenic *iso31* wild-type or aay^{KG}/+ controls (Fig. 2 *E* and *F*). Furthermore, aay^{KG} flies exhibited shorter latency to night-time sleep onset in fed condition, as well as an inability to increase the sleep latency during starvation (*SI Appendix*, Fig. S5). Waking activity was modestly reduced in aay^{KG} flies, compared with either *iso31* or aay^{KG}/+ controls (*SI Appendix*, Fig. S6*A*). However, negative geotaxis assay (*SI Appendix*, *Supplementary Materials and Methods*) revealed that aay^{KG} flies showed climbing activity comparable to *iso31* control flies (*SI Appendix*, Fig. S6*B*), arguing against the idea that sleep phenotypes observed in aay^{KG} flies are due to their sickness or defects in general locomotion. In addition, we mechanically sleep-deprived wild-type and aay^{KG} flies during the night-time (ZT12–ZT24) and analyzed their sleep rebound on the following day (ZT0–ZT12) (*SI Appendix*, *Supplementary Materials and Methods*). Under these conditions, *iso31* control and aay^{KG} flies exhibited similar sleep rebound (*SI Appendix*, Fig. S7), indicating that *aay* mutants have intact sleep homeostasis to modulate sleep behaviors upon sleep deprivation, but their sleep response to starvation was specifically disrupted.

We next contemplated the possibility that deeper sleep depth in aay^{KG} flies might be responsible for their sleep phenotypes in either fed or starved conditions. When we assessed arousal threshold by a light pulse at night (ZT18) (*SI Appendix*, *Supplementary Materials and Methods*), aay^{KG} flies displayed decreased arousability compared with *iso31* control flies during fed condition (*SI Appendix*, Fig. S8*A*). Accordingly, the higher arousal threshold in aay^{KG} flies is consistent with their longer baseline sleep and possibly higher sleep drive in fed condition. By contrast, *aay* mutants exhibited similar arousability to *iso31* control flies during starvation (*SI Appendix*, Fig. S8*A*). It is thus unlikely that *aay* mutants exhibit reduced starvation-induced sleep suppression due to their deeper sleep. These results also indicate *aay*-dependent and *aay*-independent regulation of arousal threshold in fed and starved conditions, respectively. Nonetheless, we do not exclude the possibility that a smaller pool of sleeping flies during starvation influences the behavioral assessment of arousability in different genetic backgrounds (*SI Appendix*, Fig. S8*B*). Using an independent long-sleeping transgenic line, we further validated that higher baseline sleep in fed condition does not necessarily cause resistance to starvation-induced sleep suppression (*SI Appendix*, Fig. S9).

***aay* and *stdh* Function Along a Common Genetic and Metabolic Axis for Sleep Regulation.** *CG8129* likely encodes a fly homolog of *serine/threonine dehydratase* (referred to as *stdh* hereafter) that breaks down serine and threonine to pyruvate and 2-oxobutanoic acid, respectively (19–21). We isolated a transgenic fly with a piggyBac insertion in the intron of *stdh* (C04459, referred to as $stdh^{pBac}$ from hereafter), which strongly reduced *stdh* mRNA levels in homozygous mutants (Fig. 3 *A* and *B*). Hypomorphic mutant phenotypes were biochemically validated by higher serine levels in $stdh^{pBac}$ flies compared with *iso31* control in either fed or starved conditions (Fig. 3*C*). We detected no significant increase in threonine levels in $stdh^{pBac}$ flies (Fig. 3*D*), although much lower concentrations of free threonine in fly heads might have been limiting for its detectability in our biochemical analyses. We found that $stdh^{pBac}$ flies showed baseline sleep comparable to *iso31* wild-type or $stdh^{pBac}$/+ flies in fed condition (*SI Appendix*, Fig. S10), while sleep suppression by starvation was exacerbated (Fig. 3 *E* and *F*). In addition, latency to sleep onset was increased in $stdh^{pBac}$ flies compared with controls during starvation, but not in fed condition (*SI Appendix*, Fig. S11). Collectively, these data thus suggest that *stdh* specifically limits sleep suppression during starvation, without affecting baseline sleep.

We next generated a double-mutant line harboring both aay^{KG} and $stdh^{pBac}$ alleles to determine if *aay* and *stdh* control sleep during starvation via a common sleep-regulatory pathway. We found that the double mutants exhibited reduced starvation-induced sleep suppression, similar to *aay* single mutants (Fig. 4 *A* and *B*), indicating that *aay* mutation masks the starvation-dependent exaggeration of sleep suppression in $stdh^{pBac}$ flies ($P = 0.0144$, by two-way ANOVA). To further examine if starvation-induced sleep suppression was relevant to serine levels, we assessed the individual levels of all detectable amino acids in the heads of *iso31* control and each mutant during fed and starved conditions (*SI Appendix*, Table S1). While significant changes in the amount of several amino acids were detectable in different genetic or nutrient conditions, serine levels were most consistent with the starvation-induced sleep suppression phenotypes of each single or double mutant. In fact, *aay* mutation masked the elevated serine levels in $stdh^{pBac}$ flies ($P = 0.0009$, by two-way ANOVA) (Fig. 4*C*). Possible explanations for other amino acids dysregulated in *aay* or *stdh* mutants (e.g., glutamate, glycine, and lysine) could include the partial overlap of their metabolic pathways with *aay*- or *stdh*-mediated biochemical reactions or physiological compensation for

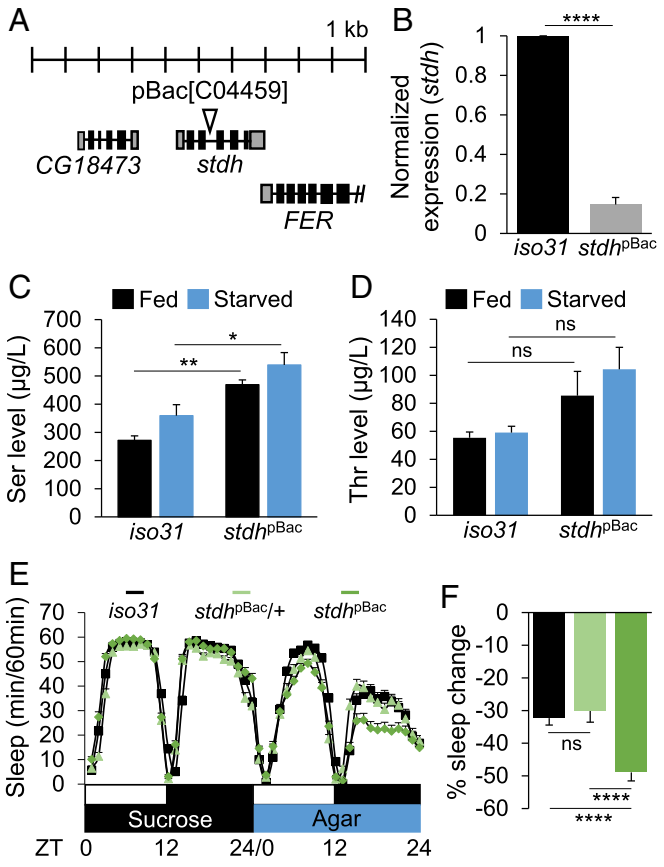


Fig. 3. *stdh* metabolizes free serine and limits starvation-induced sleep suppression. (*A*) Schematic diagram illustrating C04459 insertion in the intron of the *stdh* locus. (*B*) *stdh* transcript levels are strongly repressed in *stdh*^{pBac} flies ($n = 3$). (*C*) Serine and (*D*) threonine concentrations in head extracts of *iso31* control and *stdh*^{pBac} flies during fed and starved conditions ($n = 3$). (*E*) Average sleep traces of *iso31* (black; $n = 91$), *stdh*^{pBac}/+ (light green; $n = 81$), and *stdh*^{pBac} flies (green; $n = 83$). (*F*) Percentage sleep change during starvation in *iso31* and *stdh*^{pBac}/+ controls vs. *stdh*^{pBac} flies. ns, $P > 0.05$; *$P < 0.05$; **$P < 0.005$; ****$P < 0.0001$. All error bars represent SEM.

the genetic loss of *aay*- or *stdh*-dependent amino acid metabolism. Also, a relatively small number of biological replications ($n = 3$) do not exclude the possibility of experimental variations. Regardless, we clearly observed that the starvation-induced sleep suppression phenotypes in each mutant strongly correlated to serine levels. Collectively, these data demonstrate that *aay* and *stdh* function along a common genetic and metabolic axis to modulate sleep during starvation.

Serine Metabolic Pathway Implicates Cholinergic Signaling via Nicotinic Acetylcholine Receptors in Sleep Suppression During Starvation. To understand the neural basis of how the serine metabolic pathway contributes to starvation-relevant sleep behaviors, we determined which *aay*-expressing cells are important for starvation-induced sleep suppression. We first confirmed that pan-neuronal expression of an RNA interference (RNAi) transgene against *aay* significantly decreased *aay* mRNA levels in fly head extracts (*SI Appendix*, Fig. S12). Consistent with a role of neuronal *aay* in sleep regulation, pan-neuronal, but not pan-glial depletion of *aay*, phenocopied *aay*^{KG} flies during starvation (Fig. 5*A* and *SI Appendix*, Fig. S13). Similar results were obtained using two additional RNAi transgenes that target different regions of the *aay* transcript (*SI Appendix*, Fig. S14), strongly suggesting that the RNAi phenotypes are not due to off-target effects. In addition, pan-neuronal depletion of *aay* transcripts by all three RNAi transgenes caused longer sleep duration in fed condition (*SI Appendix*, Fig. S15), similar to *aay*^{KG} flies.

We next tested a number of Gal4 transgenes to deplete *aay* expression in specific subsets of neurons and map a neural locus important for *aay*-dependent sleep regulation. While most sleep-relevant Gal4 drivers did not show any significant sleep phenotypes during starvation, *aay* depletion by either *ChAT* 7.4-Gal4 (*Cha*) (22) or 55D01-Gal4, an enhancer transgene from a genetic locus of *vesicular acetylcholine transporter*, significantly reduced starvation-induced sleep suppression (Fig. 5*B*), without affecting baseline sleep in fed condition (*SI Appendix*, Fig. S16). We further employed a *ChAT*-Gal80 transgene (23) that blocks the expression of the *aay* RNAi transgene possibly in cholinergic neurons. Nonetheless, starvation-induced sleep suppression was significantly reduced by the pan-neuronal depletion of *aay*, even in the presence of the *ChAT*-Gal80 transgene (*SI Appendix*, Fig. S17). Although these Gal4 and Gal80 transgenes might not be expressed exclusively in cholinergic neurons, our observations suggest the hypothesis that *aay*-dependent serine biosynthesis in cholinergic neurons might be necessary, but not sufficient, for regulating sleep during starvation.

To examine the possible implication of cholinergic signaling in sleep regulation by the serine metabolic pathway, we tested if oral administration of mecamylamine (MCA) (*SI Appendix, Supplementary Materials and Methods*), a nicotinic acetylcholine receptor antagonist (24), could affect starvation-induced sleep suppression. Interestingly, MCA rescued the exaggerated sleep suppression in starved *stdh*^{pBac} flies during night-time in a dose-dependent manner, while it had no significant effects on *iso31*

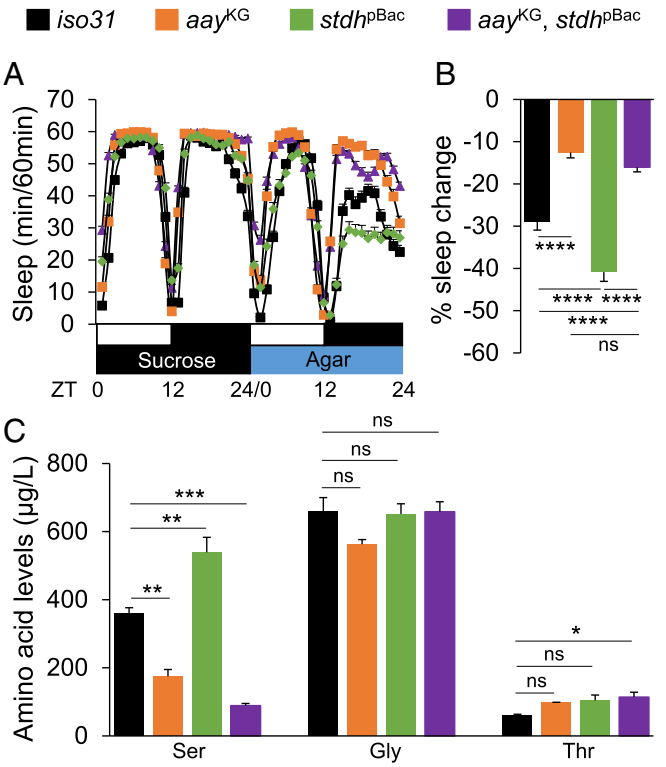


Fig. 4. *aay* and *stdh* act in the same genetic pathway to titrate free serine levels and control sleep during starvation. (*A*) Average sleep traces of *iso31* (black; $n = 116$), *aay*^{KG} (orange; $n = 81$), *stdh*^{pBac} (green; $n = 104$), and double mutants (purple; $n = 160$). (*B*) *aay*^{KG} masks the exaggerated starvation-induced sleep suppression of *stdh*^{pBac} flies [$F_{(1,457)} = 6.035$; $P = 0.0144$]. (*C*) Comparison of serine, glycine, and threonine levels in head extracts of *iso31* and mutants in starved condition ($n = 3$). ns, $P > 0.05$; *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$; ****$P < 0.0001$. All error bars represent SEM.

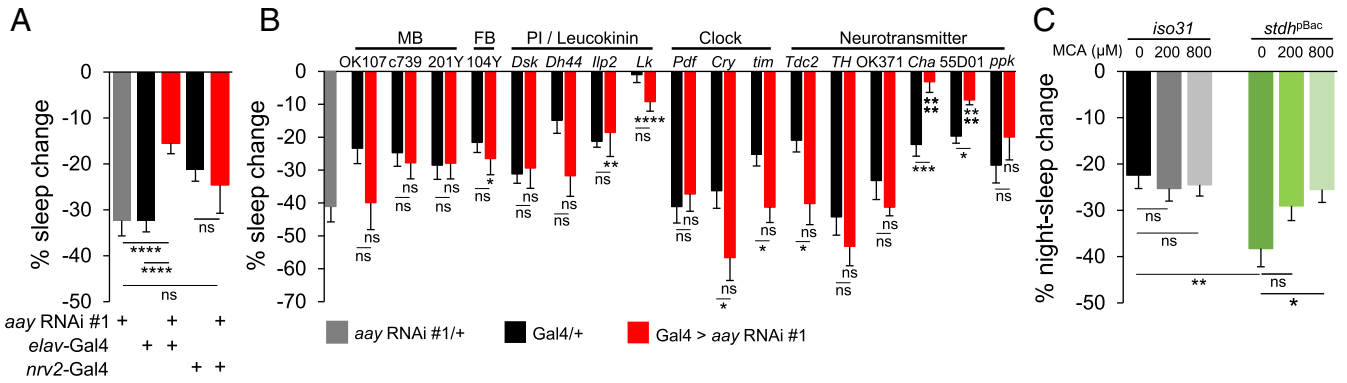


Fig. 5. Sleep regulation by the serine metabolic pathway implicates cholinergic signaling. (*A*) Percentage sleep change during starvation in flies with pan-neuronal and glial knockdown of *aay* transcripts (*aay* RNAi #1/+, $n = 37$; *elav*-Gal4/+, $n = 46$; *elav*-Gal4 > *aay* RNAi #1, $n = 42$; *nrv2*-Gal4/+, $n = 29$; *nrv2*-Gal4 > *aay* RNAi #1, $n = 21$). (*B*) Percentage sleep change during starvation when *aay* transcripts are depleted in mushroom body (MB), fan-shaped body (FB), pars intercerebralis (PI), *leucokinin*, clock, and various neurotransmitter-related regions in the brain ($n = 7$–33). (*C*) Dose-dependent effect of MCA administration on *stdh*^{pBac} flies during starvation. The 200 μM MCA effect: [$F_{(1,281)} = 3.637$; $P = 0.0575$] and 800 μM MCA effect: [$F_{(1,284)} = 6.071$; $P = 0.0143$]. *iso31* 0 μM, $n = 71$; *iso31* 200 μM, $n = 79$; *iso31* 800 μM, $n = 81$; *stdh*^{pBac} 0 μM, $n = 66$; *stdh*^{pBac} 200 μM, $n = 69$; and *stdh*^{pBac} 800 μM, $n = 70$. ns, $P > 0.05$; *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$; ****$P < 0.0001$. All error bars represent SEM.

control flies (Fig. 5*C* and *SI Appendix*, Fig. S18). These data indicate that the exaggerated sleep-suppressing effect of *stdh* mutation during starvation requires nicotinic acetylcholine receptor function, thereby supporting a possible role for cholinergic signaling in serine metabolism-dependent sleep regulation during starvation.

Discussion

The genetic and cellular components that link sleep and metabolism have been unveiled by traditional genetic approaches in animal models including *Drosophila* (8, 25, 26); yet there are likely more uncharacterized genes and pathways that regulate this important process. We have demonstrated that starvation induces serine biosynthesis in the transcript, enzymes, and free amino acid levels. Genetic mutants of *aay* and *stdh* exhibited decreased and increased serine levels, respectively, and a positive correlation between serine levels and starvation-induced sleep suppression was evident. Collectively, our molecular and genetic evidences implicate endogenous serine in the brain as a wake-promoting signal during starvation.

It has been documented that the lack of food availability immediately suppresses *Drosophila* sleep (13), suggesting that sensory processes might be involved in sleep suppression at the initial stages of starvation. In fact, our GO enrichment analyses revealed that short-term starvation selectively induces the transcripts of several genes implicated in sensory perception in the brain. On the other hand, prolonged starvation elevates the brain expression of genes involved in various cellular processes such as amino acid metabolism and nucleotide biosynthesis, indicating that long-term starvation might not be simply a severer version of short-term starvation. We thus speculate that starvation-induced sleep suppression might be regulated by two distinctive processes: a fast-acting mechanism perceives the lack of food via a sensory input and temporally suppresses sleep, while slow metabolic processes sustain suppressed sleep states to ensure that food sources with nutritional value are obtained.

What will be the mechanistic basis by which endogenous serine suppresses sleep during starvation? Fat storage and expenditure have been associated with starvation resistance and locomotor activities during starvation (27), yet our biochemical analyses indicate that the sleep-modulatory effects of serine upon starvation are unlikely to involve alterations in lipid metabolism (*SI Appendix*, Figs. S19 and S20). Intriguingly, amino acid transporters in the brain are emerging as important players in the direct regulation of various neural activities and relevant physiological functions (28–30). Therefore, we reason that a dedicated transporter might facilitate the mobilization of serine to its site of action to suppress sleep during starvation. It is noteworthy that long-term starvation selectively induces the brain expression of genes implicated in amino acid transmembrane transport, which possibly sensitizes *Drosophila* sleep to the wake-promoting effects of serine. This hypothesis partially explains why *stdh* mutants, which have high basal levels of serine in heads, could exhibit their sleep suppression phenotypes specifically in starved condition.

Serine is a major precursor to D-serine (31), which in turn acts as a potent coagonist of the *N*-Methyl-D-aspartic acid (NMDA) receptor (32, 33). However, it is unlikely that serine promotes starvation-induced sleep suppression via NDMA receptors because NMDA receptors are known to promote sleep in flies (34). Besides acting as a precursor to D-serine synthesis, serine is also used for synthesizing bioactive lipids such as phosphatidylserine, which are enriched in the inner leaflet of neural plasma membranes (35). Interestingly, phosphatidylserine increases acetylcholine release from cortical slices of rats (36, 37). Given the potential implication of nicotinic acetylcholine receptors downstream of *stdh*-dependent sleep effects, we reason that serine might support cholinergic signaling to wake flies up during starvation.

Cholinergic neurons comprise a large portion of the fly brain, and it has been shown that each cholinergic subpopulation mediates different aspects of sleep regulation. For example, cholinergic mushroom body α/β core neurons promote baseline sleep, whereas neighboring cholinergic α/β surface/posterior and γ neurons suppress baseline sleep (38). On the other hand, a cholinergic subpopulation that expresses the gene *pickpocket* promotes homeostatic rebound sleep (39). Interestingly, our neural mapping similarly revealed locus-specific effects of *aay* on sleep behaviors. While pan-neuronal *aay* contributes to sleep behaviors in either fed or starved conditions, *aay* depletion in a narrower population of cells using cholinergic Gal4 drivers reduced sleep suppression during starvation, but did not affect baseline sleep in fed condition. These data suggest the possibility that *aay* might differentially modulate sleep, depending on the neuronal loci and availability of food.

In summary, we have identified that starvation-induced elevation of serine biosynthesis in neurons plays a crucial role in sleep regulation during starvation, possibly via signaling through nicotinic acetylcholine receptors. Our study stands out from previous studies in that we have focused purely on metabolic pathways in neurons that can regulate sleep behaviors under metabolic stress conditions. Further investigation of serine-mediated

sleep suppression and its downstream neurological effectors will provide a platform on which molecular and neural links between sleep and metabolism can be elucidated.

Methods

Behavioral Analysis. Sleep behavior was measured using the *Drosophila* Activity Monitor (DAM) system (Trikinetics). Five- to seven-day-old male flies were individually loaded into 65 × 5 mm glass tubes plugged with 5% sucrose/1% agar in PCR tubes for 4 d. On the fifth day, PCR tubes with 1% agar replaced previous food at ZT0 (lights on), and flies were starved for 24 h within the DAM to measure sleep during starvation. Unless mentioned otherwise, percentage sleep change was calculated as the change in sleep during 24 h of starvation compared with the previous fed day. Data were analyzed using a custom written Excel macro (40).

RNA-Seq and Differential Gene Expression Analysis. Two biological replicates for each experimental condition (Ctrl FD6, Ctrl FD24, FD6, and FD24) were used to perform RNA-seq analysis. Eighty brains per experimental condition were manually dissected in PBS, and brains were kept in ice-cold RNA-Later ICE (Invitrogen) during the duration (2 h per group) of brain collection. Control and starved brain samples at each time point were collected on different days, to exclude possible circadian time-dependent effects on gene expression. Total RNA was extracted using TRIzol (Invitrogen). Library construction was carried out using a TruSeq RNA Prep kit (Illumina). RNA-seq was performed on a Hiseq2500 (Illumina) at DNA Link, resulting in 20–60 million 101-bp reads per sample. TopHat 2.0.9 (41) was used to align RNA-seq reads to the BDGP5.77 reference genome. Cufflinks 2.1.1 (42) was used to estimate read count for the BDGP5.77 transcriptome model.

Western Blot Analysis. For Western blot analysis, 12 h:12 h light:dark entrained male flies were frozen at −80 °C, and 20 heads were separated and lysed in lysis buffer (25 mM Tris-Cl pH 7.5, 300 mM NaCl, 1 mM PMSF, 1 mM DTT, 0.5% Nonidet P-40, and protease/phosphatase inhibitor mixture) and resolved by SDS/PAGE. For probing of Western blots, rabbit anti-AAY (*SI Appendix, Supplementary Materials and Methods*) and mouse anti-tubulin (Cell Signaling) were both used at 1:2,000. HRP-conjugated secondary antibodies (Cell Signaling) were used at 1:20,000 and visualized with ECL Plus (Thermo). Western blots were quantified using Image Studio Lite.

Amino Acid Analysis. Individual 5–7-d-old male flies, entrained in 12 h:12 h light:dark cycles, were collected. Twenty heads were separated and homogenized in a total of 600 μL of HPLC-grade water. Samples were centrifuged at 4 °C for 10 min at 12,000 × *g*, and 400 μL of supernatant was retrieved. Subsequently, 1:1 trichloroacetic acid was added to deproteinize samples. n-hexane was added to remove lipids and nonpolar substances, and the bottom phase was passed through a 0.2-μM syringe filter to remove trace impurities. Ion exchange resin #2622 column (Hitachi) was attached to the Hitachi L-8900 Amino Acid Analyzer (Hitachi), and ninhydrin-derivatized amino acids were detected with a UV detector.

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