Supplementary Materials and Methods

Flies. Flies were raised on a 12 h:12 h light:dark cycle on standard cornmeal-yeast agar medium at 25°C and 60% humidity. *aay*^{KG} (BDSC #14135), *stdh*^{pBac} (BDSC #11438), *aay* RNAi #3 (BDSC #38338), 23E10-Gal4 (BDSC #49032), and *ChAT*-Gal80 (BDSC #60321) were obtained from the Bloomington Drosophila Stock Center. *aay*^{KG} and *stdh*^{pBac} flies were outcrossed six times to *iso31* flies (BDSC #5905). *aay* RNAi #1 (VDRC #110661) was obtained from the Vienna Drosophila Resource Center. *aay* RNAi #2 (#3705R-3) was obtained from NIG-FLY. *elav*-Gal4, *nrv2*-Gal4, and UAS-NaChBac were used previously in the lab.

Gal4 lines used for screening with *aay* RNAi #1 are as follows: OK107 (1) (lab stock), c739 (2) (lab stock), 201y (3) (lab stock), 104y (4) (kindly provided by Dr. J Douglas Armstrong), *Dsk* (5) (lab stock), *Dh44* (6) (kindly provided by Dr. Greg Suh), *Ilp2* (7) (lab stock), *Lk* (8) (lab stock), *Pdf* (9) (lab stock), *Cry* (10) (lab stock), *tim* (11) (lab stock), *Tdc2* (7) (lab stock), *TH* (12) (lab stock), OK371 (13) (lab stock), *ChAT*-7.4 (*Cha*) (14) (lab stock), 55D01 (BDSC #39110), and *ppk* (BDSC #32079). For expression patterns of 55D01-Gal4, please refer to FlyLight expression data (http://flweb.janelia.org/cgi-bin/view_flew_imagery.cgi?line=R55D01).

Brain enrichment analysis of starvation regulated genes. Briefly, we compared averaged RNA-seq reads between control FD6 brain samples (2 replicate) vs. 9 different head samples from the Flybase database (ftp://ftp.flybase.net/releases/FB2017_06/precomputed_files/genes/gene_rpkm_report_fb_2017_06.tsv.gz). Differential gene expression was assessed by t-test, using the limma package (15).

Quantitative PCR. Individual 5–7-day-old male flies, in a 12 h:12 h light:dark cycle, were collected and sacrificed by freezing at -80°C. Heads were removed by vortexing and isolated on frozen sieves. Frozen heads were homogenized, and total RNA was extracted from 20 heads per sample with Trizol (Invitrogen). RNA (1 μg) was used for cDNA synthesis using

M-MLV Reverse Transcriptase (Promega). qPCR was performed using PrimeQ-Mastermix (Genetbio) and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The qPCR primers used in this study were as follows: 5'-AGC GAC GAT TCC CTA ATC AC-3' (forward) and 5'-ACA TTG CCA CCG AAA CCT AT-3' (reverse) for *aay*; 5'-CGT CAC ATC CGC TGC CTT TC-3' (forward) and 5'-TCA CTC CGG TTC GCT TCT GT-3' (reverse) for *stdh*; 5'-CAC CAG GAA CTT CTT GAA TCC GG-3' and 5'-AGA TCG TGA AGA AGC GCA CCA AG-3' for RpL32 as an internal control.

Generation of *aay* polyclonal antibody. A bacterial expression vector encoding HIS-AAY was transformed into BL21 competent *E. coli* cells (Enzynomics). Expression of HIS-AAY protein was induced with 0.5 mM Isopropyl-β-D-thiogalactoside (IPTG) overnight at 18°C. Cells were harvested and sonicated using six 10 second bursts. Following sonication, cells were lysed in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). Lysates were centrifuged, and the supernatant containing His-tagged AAY was purified using a Ni-NTA resin (Thermo) according to the manufacturer's instructions and dialyzed against a dialysis buffer (25 mM Tris-Cl, 300 μM NaCl, and 30% glycerol). The purified recombinant protein was injected into two rabbits.

Light-induced arousal. 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 and incubated in 12 h:12 h light:dark cycle for three days. On the fourth day, flies were transferred to 5% sucrose or 1% agar on ZT0 (lights on) and a single light pulse was given on ZT18 (3 a.m.) for 20 seconds. Flies that displayed any locomotion during 5 minutes (sleep = greater than or equal to 5 minutes of inactivity) prior to light-pulse were excluded from analysis. For each fly that was sleeping, any movement within 5 minutes of light-pulse (light-pulse bin included) was regarded as a positive response to stimulation.

Startle-induced negative geotaxis assay. 13-17 male flies (5-7 days old) raised on standard cornmeal-yeast agar medium were transferred to an empty polystyrene vial apparatus (two polystyrene vials vertically joined by tape, facing each other) without anesthesia. Flies were

allowed to acclimatize to apparatus for 10 minutes before starting assay. For each trial, flies were tapped gently, but rapidly to the bottom of the vial and the number of flies that passed the 8 cm mark (from the bottom of the vial) in 10 seconds were scored. This was repeated ten times with 1 minute rest intervals between each trial. 10 trials constituted 1 experiment and percentage of flies that passed the 8cm mark were average over 5 experiments for each genotype.

Sleep rebound. Mechanical sleep deprivation was carried out with Sleep Nullifying Apparatus (SNAP) (16). Baseline sleep was recorded on the SNAP for three days and flies were mechanically sleep deprived for 12 hours, starting from ZT12 (lights off) at day 4 until ZT0 (lights on) at day 5. Sleep rebound was measured for the initial 12 hours (ZT0 – ZT12) at day 5. Cumulative percentage sleep loss was calculated in individual flies and averaged for each genotype.

MCA treatment. For MCA treatment, five-to-seven-day-old male flies were monitored in 5% sucrose/1% agar food for 4 days. On the fifth day, PCR tubes with 1% agar supplemented with either 200 μM or 800 μM MCA (Sigma) replaced the previous food at ZT0 and flies were monitored for 24 hours. Percentage sleep change was calculated during the night-time (ZT12 - ZT24) of MCA treatment day, since the majority of the exaggerated starvation-induced sleep suppression in *stdh* mutants were observed during the night-time.

Triglyceride and protein assays. Five- to seven-day-old male flies were placed in 5% sucrose/1% agar food in groups of eight and entrained in a 12 h:12 h light:dark cycle for 3 days. On the fourth day, flies were divided into two groups: the first group was sacrificed at ZT0 (non-starved), and the second group was starved for 18 hours in 1% agar. After starvation, the second group of flies were also sacrificed. Triglyceride and protein measurements were made in whole flies according to the manufacturer's instructions using the Infinity Triglycerides kit (Thermo) and the BCA Protein Assay kit (Thermo).

Survival under starvation. To measure survival during starvation, five- to seven-day-old male flies were loaded in DAM on 5% sucrose/1% agar food and were incubated for two days for acclimatization. Starvation began on ZT0 of day 3 and locomotor activity was monitored every 60 minutes until death (locomotor count = 0) for each fly. Number of flies that died at each time point were counted manually and a Kaplan-Meier curve was drawn for each genotype.

Statistics. All statistical analyses were performed with Prism 7 (GraphPad). Pairwise comparisons were evaluated by two-tailed unpaired t-tests. One-way ANOVA followed by Tukey's post-hoc test was used when the effect of a single independent variable was assessed (e.g., genotype) among more than two genotypes. Two-way ANOVA followed by Tukey's post hoc test was used when the interactive effect of two independent variables were assessed (e.g., genotype vs. starvation and *aay* mutation vs. *stdh* mutation).

Supplementary References

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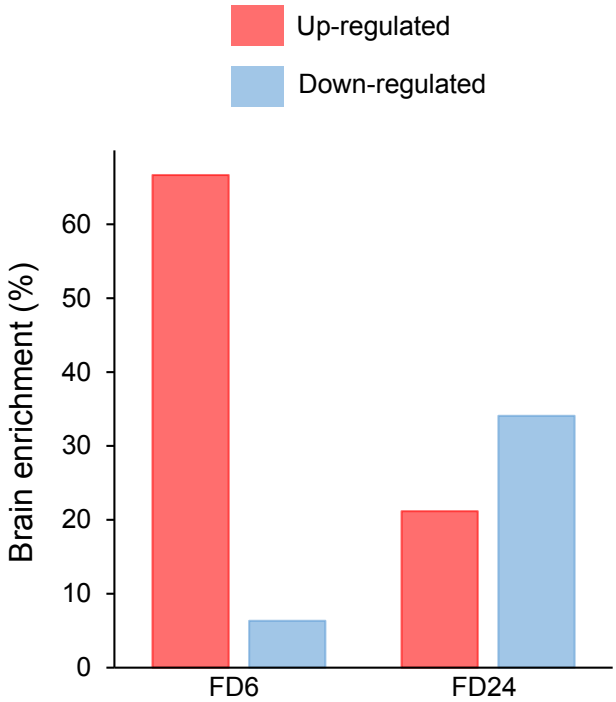
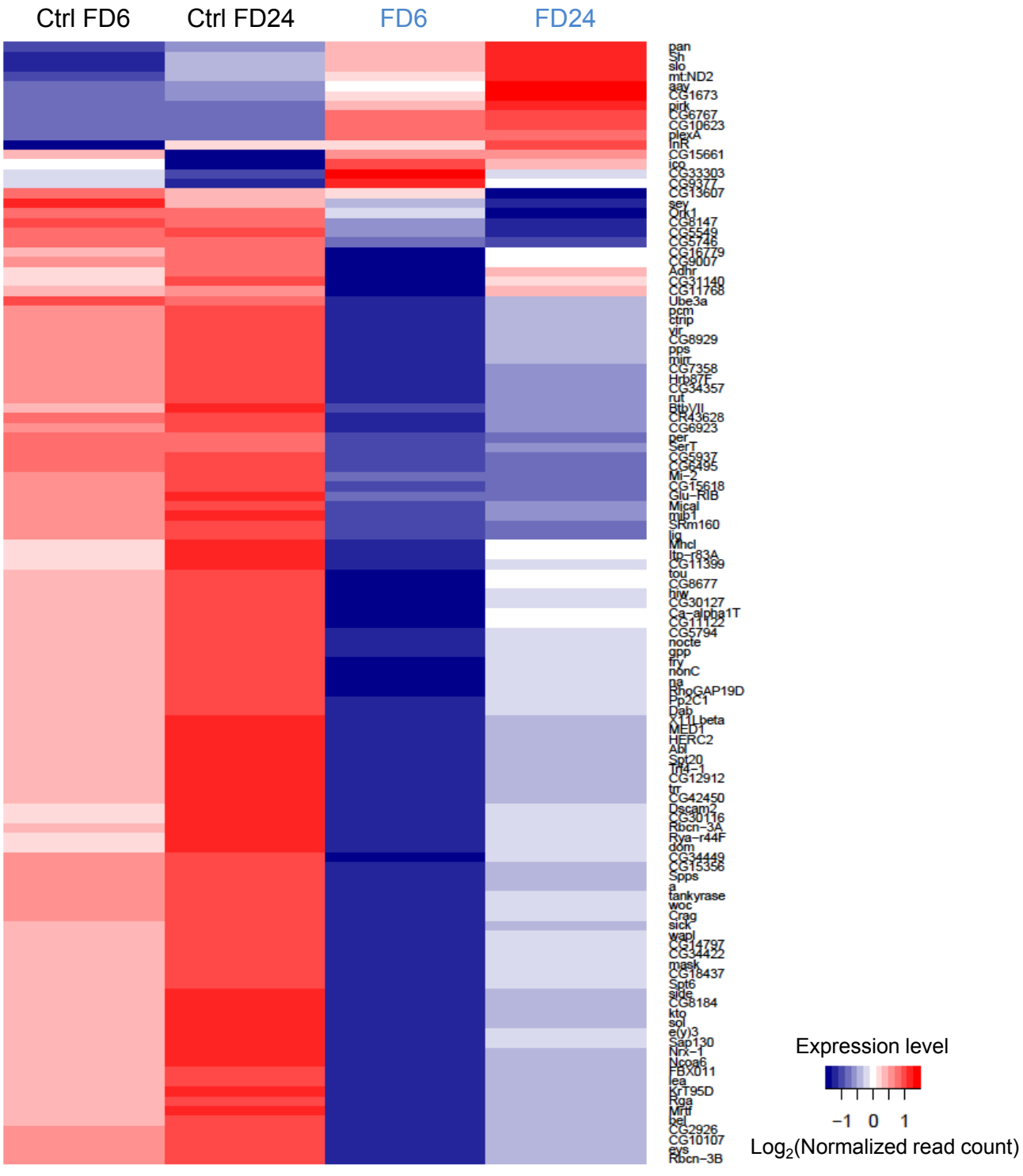
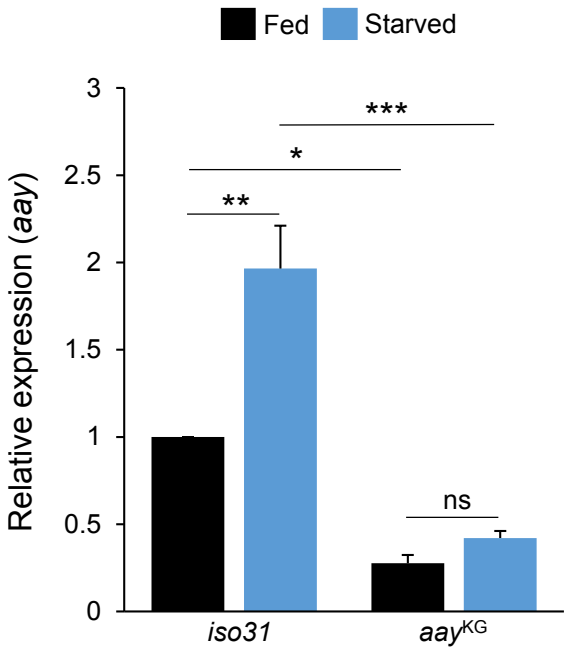


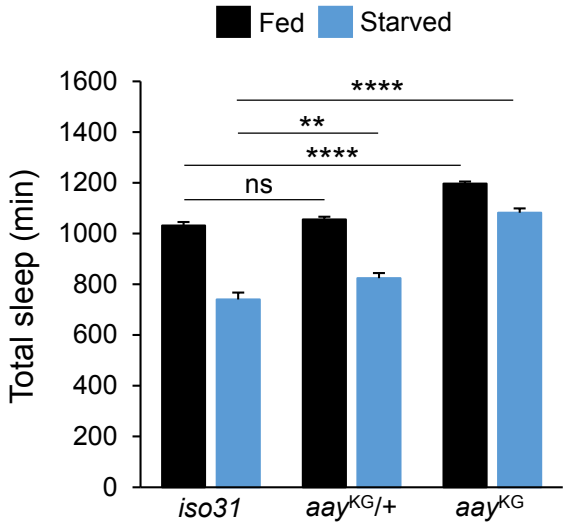
Supplementary Figure 1. Brain enrichment of genes regulated by short-term and long-term starvation. Genes up-regulated by short-term starvation (FD6) tended to be enriched more in the brain than genes up-regulated by long-term starvation (FD24), whereas the opposite trend was evident in down-regulated genes.



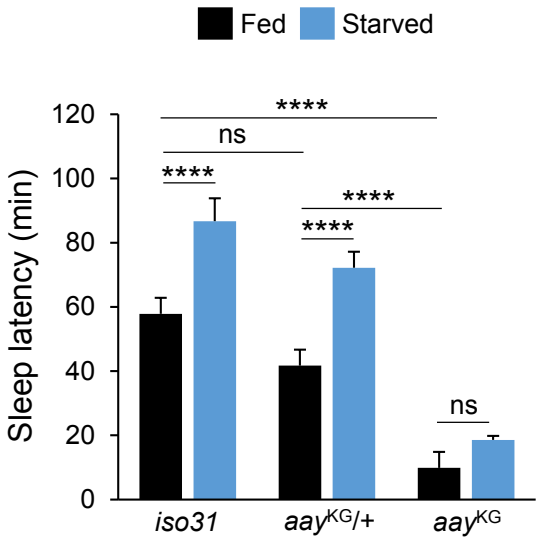
Supplementary Figure 2. Heat-map of overlapping genes between FD6 and FD24. Heat-map shows the expression level of the overlapping 115 genes in fed (Ctrl FD6 and Ctrl FD24) and starved conditions (FD6 and FD24). Colors indicate the log2 values of normalized read counts for gene expression.



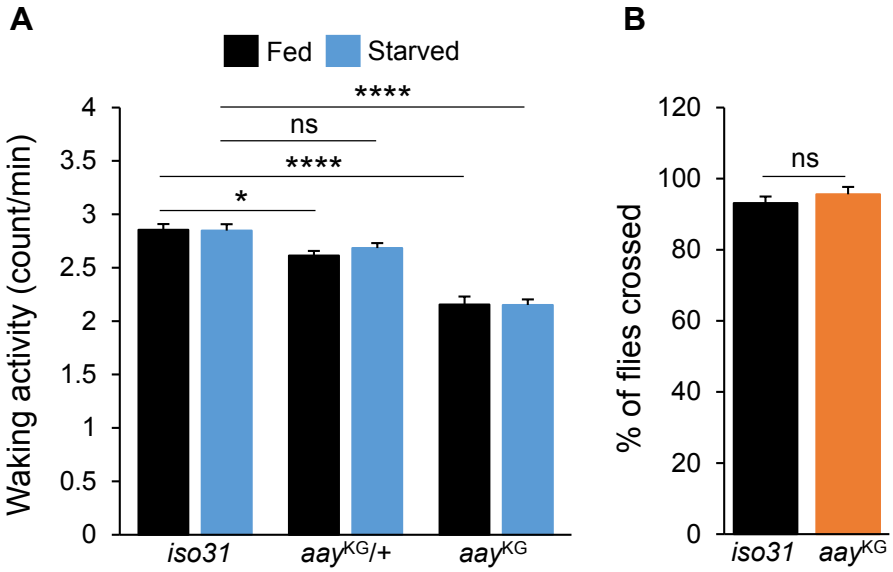
Supplementary Figure 3. *aay* mRNA quantification in head extracts of *iso31* control and *aay* mutants. *aay* mRNA expression was induced upon starvation in *iso31* control flies, while the KG05974 insertion reduced *aay* expression (n=3). Starvation*genotype [$F_{(1,8)}$=10.55; P=0.0117]. Two-way ANOVA followed by Tukey post-hoc analysis was used for comparison. ns, P>0.05; *P<0.05; **P<0.005; ***P<0.0005. All error bars represent SEM.



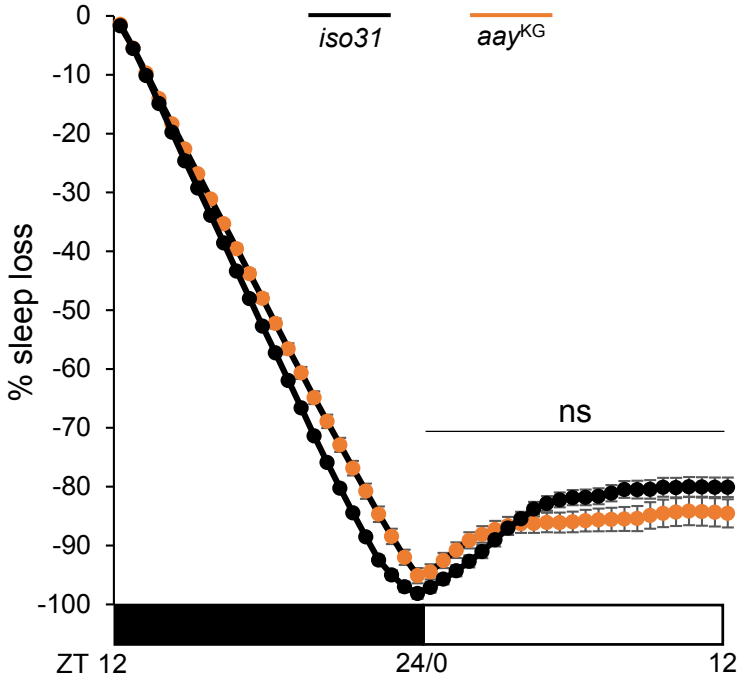
Supplementary Figure 4. Total sleep duration of *aay* mutants in fed and starved conditions. Baseline sleep is increased in *aay* homozygous mutants during fed condition (black) compared to *iso31* and *aay* heterozygous controls. Two-way ANOVA followed by Tukey post-hoc test was used for multiple comparison. ns, P>0.05; **P<0.005; ****P<0.0001. All error bars represent SEM.



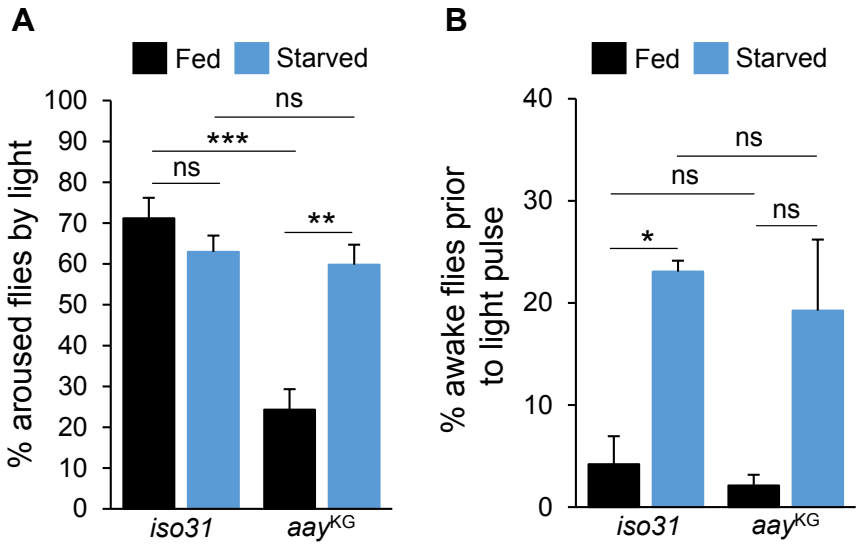
Supplementary Figure 5. Sleep onset during fed and starved conditions in *aay* mutants. *aay* mutants exhibit decreased sleep latency at night-time (ZT12) during fed condition compared to *iso31* and *aay* heterozygous controls. Furthermore, *aay* mutants do not show signs of delayed sleep onset during starvation. Starvation*genotype [$F_{(2,486)}$=4.248; P=0.0148]. Two-way ANOVA followed by Tukey post-hoc test was used for multiple comparison. ns, P>0.05; ****P<0.0001. All error bars represent SEM.



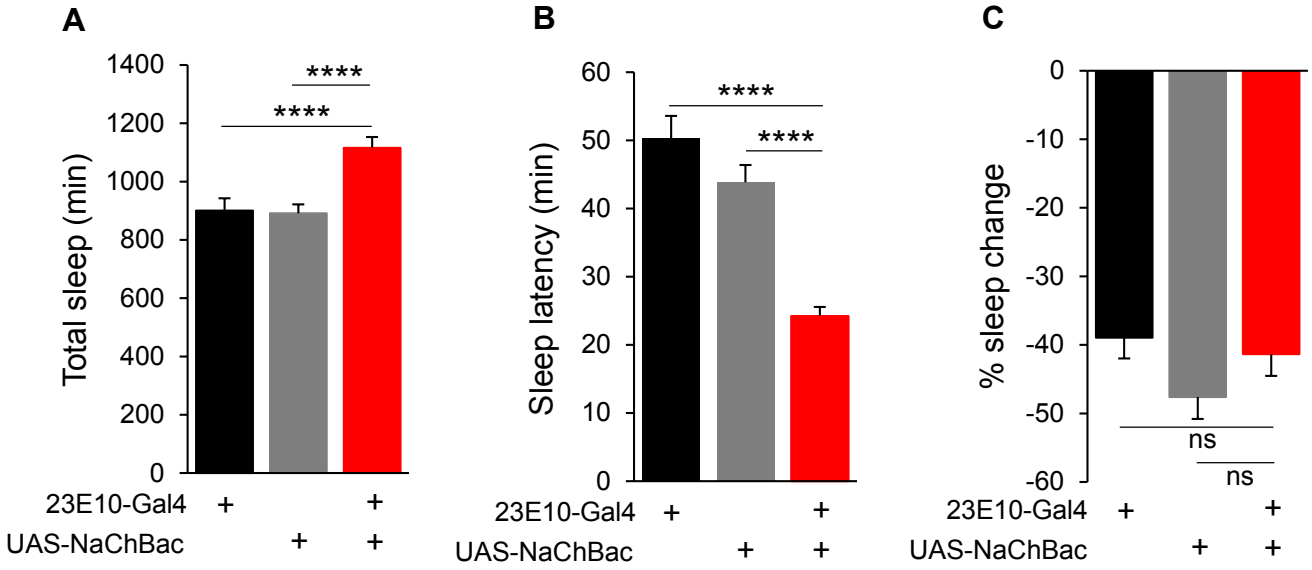
Supplementary Figure 6. Waking activity and locomotor performance in *aay* mutants. (A) Waking activity in *iso31* (n=88), aay mutant heterozygotes (n=80), and aay mutants (n=78) in fed and starved conditions. Two-way ANOVA followed by Tukey post-hoc analysis was used for comparison. (B) *aay* mutants show comparable climbing performance to *iso31* control flies during fed condition (n=5). [t=0.9021; P=0.3934]. Unpaired t-test was used for comparison. ns, P>0.05; *P<0.05; ****P<0.0001. All error bars represent SEM.



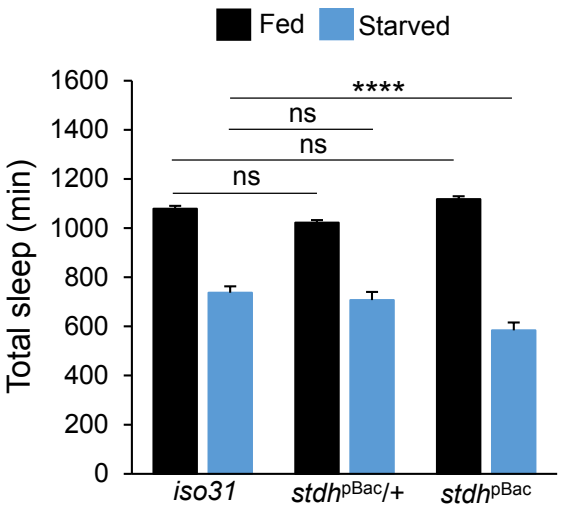
Supplementary Figure 7. Sleep rebound in *aay* mutants after mechanically-induced sleep deprivation. *aay* mutant female flies (orange, n=62) exhibit similar sleep rebound (ZT24/0 - ZT12) to *iso31* control flies (black, n=67) after 12 hours of mechanically induced sleep-deprivation during the previous night (ZT12 - ZT24/0). Cumulative sleep loss was calculated in individual flies and averaged for each genotype. Unpaired t-test was used for comparison in each time point during the sleep rebound period. ns, P>0.05.



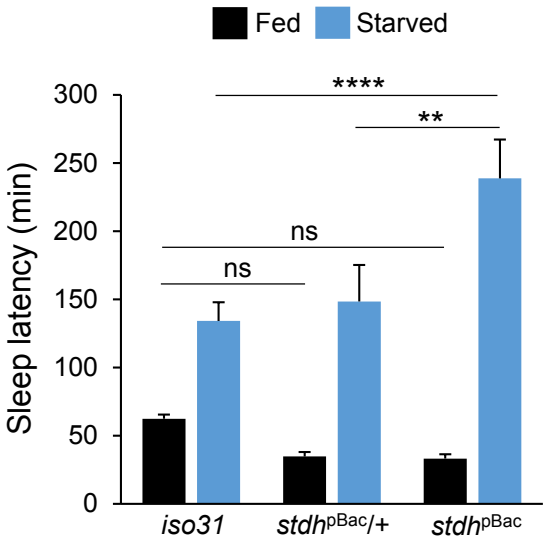
Supplementary Figure 8. *aay* mutation affects arousability differentially in fed and starved conditions. (A) Percentage of flies awakened by brief (20 seconds) light stimulus was lower in *aay* mutants during fed condition. However, *aay* mutants showed comparable arousability to *iso31* flies during starvation (n=3). Across the 3 trials, 91 and 73 *iso31* flies were sleeping before light pulse during fed and starved conditions, respectively. In addition, 80 and 73 *aay* mutants were sleeping before light pulse during fed and starved conditions, respectively. (B) Percentage of flies that were awake before light pulse during fed and starved conditions. Two-way ANOVA followed by Tukey post-hoc analysis was used for comparison. ns, $P>0.05$; *$P<0.05$; **$P<0.005$; ***$P<0.0005$. All error bars represent SEM.



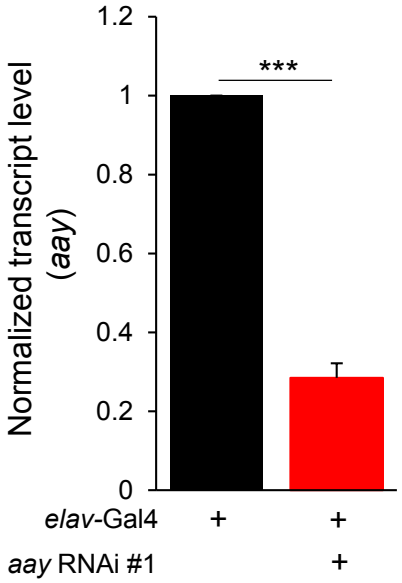
Supplementary Figure 9. High sleep drive does not correlate to starvation-induced sleep suppression. (A) Activation of 23E10 neurons (23E10-Gal4 > UAS-NaChBac; n=62) increases daily sleep amount in flies compared to controls (23E10-Gal4/+; n=42 and UAS-NaChBac/+; n=41) in fed condition. (B) Flies with activation of 23E10 neurons exhibited decreased sleep latency at night-time (ZT12) compared to controls in fed condition. (C) 23E10 neuron-activated flies exhibit similar starvation-induced sleep suppression to controls. One-way ANOVA followed by Tukey post-hoc test was used for multiple comparison. ns, P>0.05; ****P<0.0001. All error bars represent SEM.



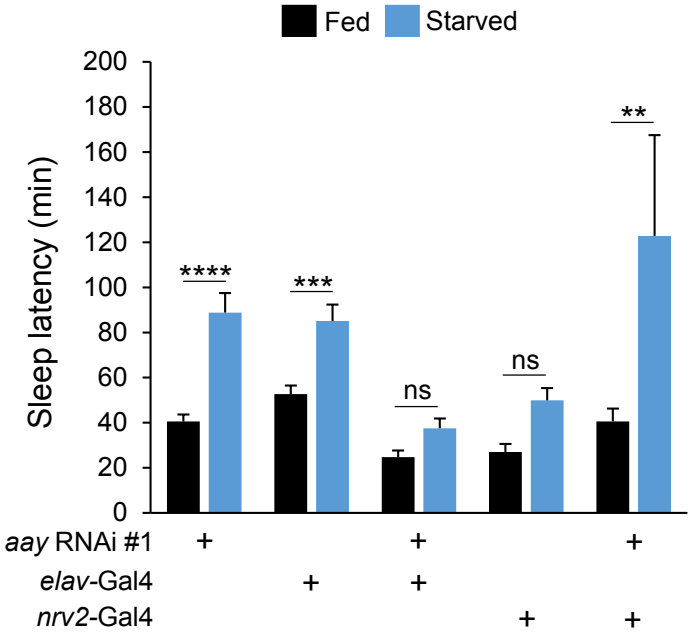
Supplementary Figure 10. Total sleep duration of *stdh* mutants in fed and starved conditions. Baseline sleep was not affected in *stdh* homozygous mutants in fed condition (black) compared to *iso31* and *stdh* heterozygous controls. Two-way ANOVA followed by Tukey post-hoc test was used for multiple comparison. ns, P>0.05; ****P<0.0001. All error bars represent SEM.



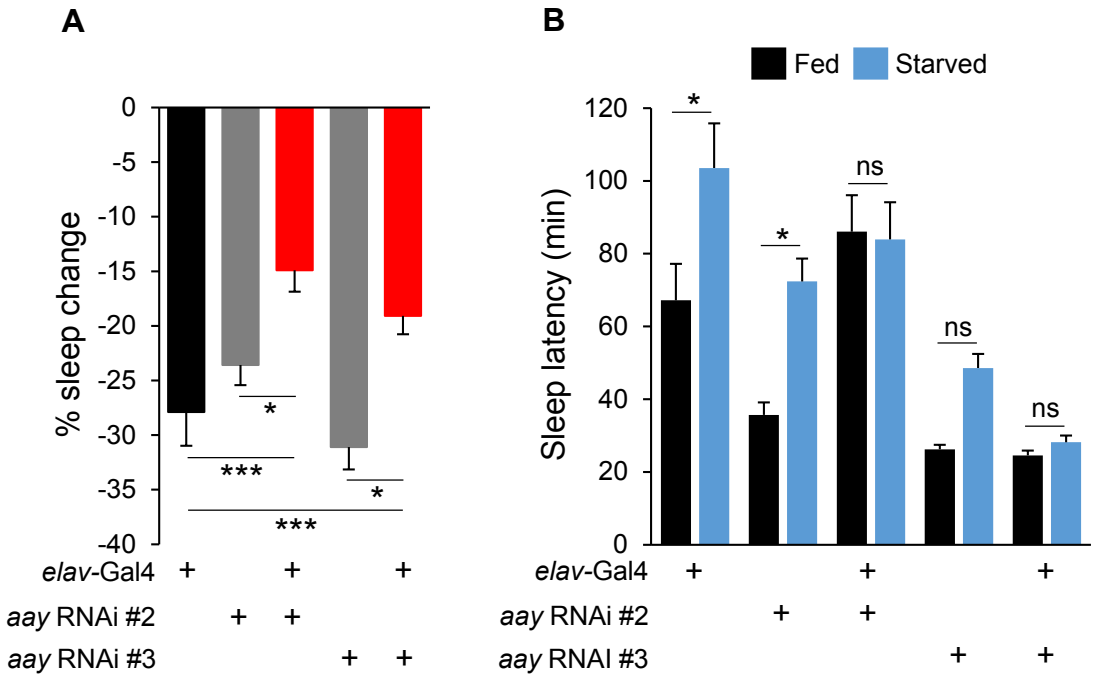
Supplementary Figure 11. Sleep onset during fed and starved conditions in *stdh* mutants. *stdh* mutants show increased sleep latency at night-time (ZT12) compared to *iso31* and *stdh* heterozygote controls during starvation. Starvation*genotype [$F_{(2,504)}$=8.377; P=0.0003]. Two-way ANOVA followed by Tukey post-hoc test was used for multiple comparison. ns, P>0.05; **P<0.005; ****P<0.0001. All error bars represent SEM.



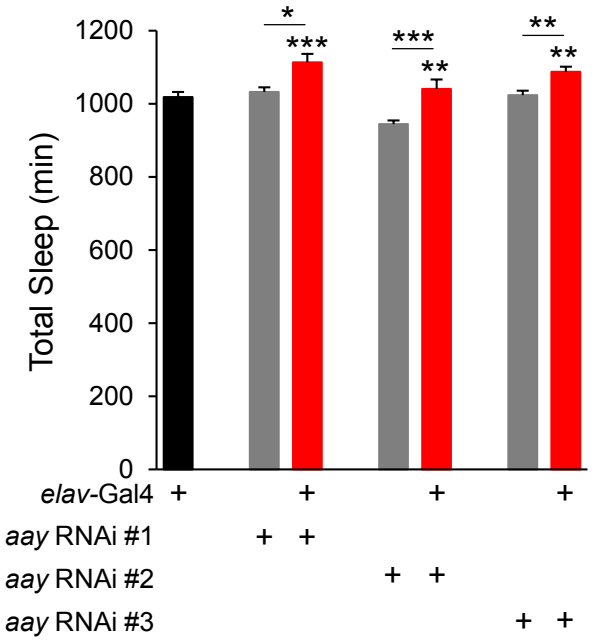
Supplementary Figure 12. *aay* is expressed in neurons. Quantification via qPCR revealed that *aay* transcript levels are significantly reduced when RNAi transgene #1 was expressed pan-neuronally (n=3). [t=13; P=0.0002]. Unpaired t-test was used for comparison. ***P<0.0005. Error bar represents SEM.



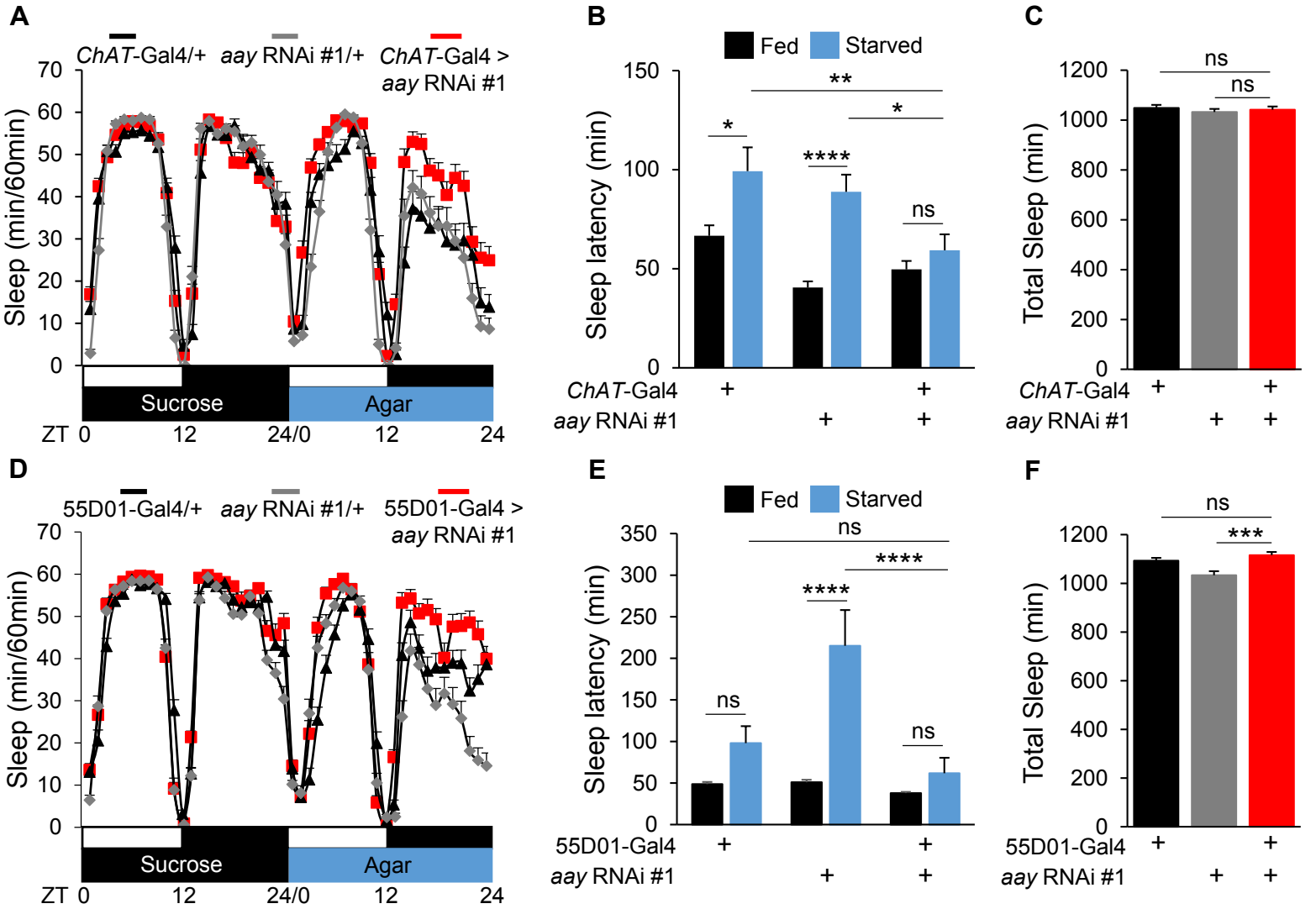
Supplementary Figure 13. Sleep onset during fed and starved conditions in flies with pan-neuronal depletion of *aay*. Targeted knockdown of *aay* in neurons confers resistance to increase of sleep latency at night-time (ZT12) during starvation. Starvation*genotype [$F_{(2,244)}$=5.31; P=0.0055]. In contrast, flies with knockdown of *aay* in glia exhibited a delay in sleep onset during starvation. Starvation*genotype [$F_{(2,168)}$=2.05; P=0.1319] Two-way ANOVA followed by Tukey post-hoc test was used for multiple comparison. ns, P>0.05; **P<0.005; ***P<0.0005; ****P<0.0001. All error bars represent SEM.



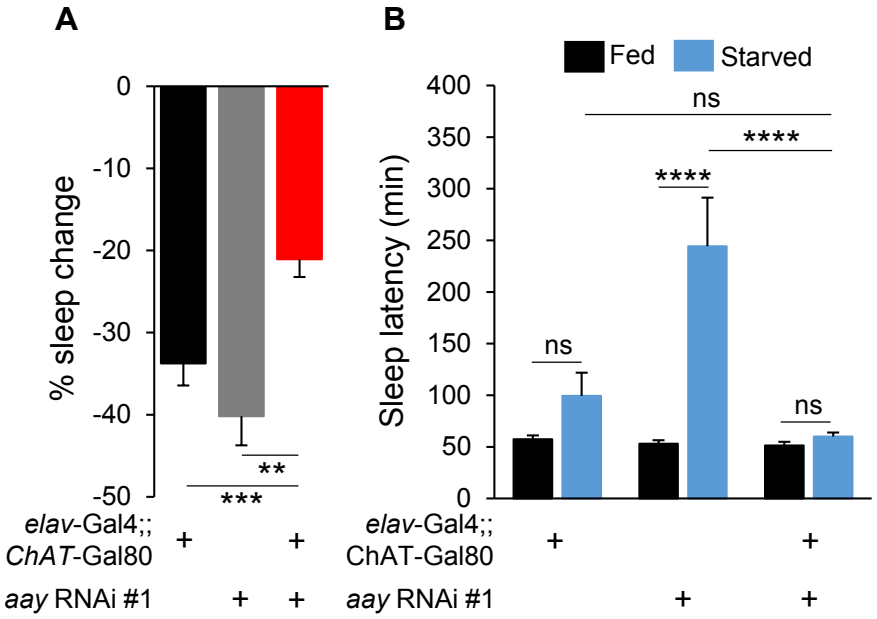
Supplementary Figure 14. Pan-neuronal knockdown of *aay* with two additional RNAi transgenes. (A) Pan-neuronal knockdown of *aay* with RNAi #2 (*elav*-Gal4 > *aay* RNAi #2; n=41) and RNAi #3 (*elav*-Gal4 > *aay* RNAi #3; n=59) reduced starvation-induced sleep suppression in flies compared to its controls (*elav*-Gal4/+; n=48, *aay* RNAi #2/+; n=61, and *aay* RNAi #3/+; n=61). One-way ANOVA followed by Tukey post-hoc test was used for multiple comparison. (B) Knockdown of *aay* in neurons confer resistance to increase of sleep latency at night-time (ZT12) during starvation. Starvation*RNAi #2 [$F_{(2,294)}$=3.271; P=0.0394]. Starvation*RNAi #3 [$F_{(2,330)}$=3.819; P=0.0229]. Two-way ANOVA followed by Tukey post-hoc test was used for multiple comparison. ns, P>0.05; *P<0.05; ***P<0.0005. All error bars represent SEM.



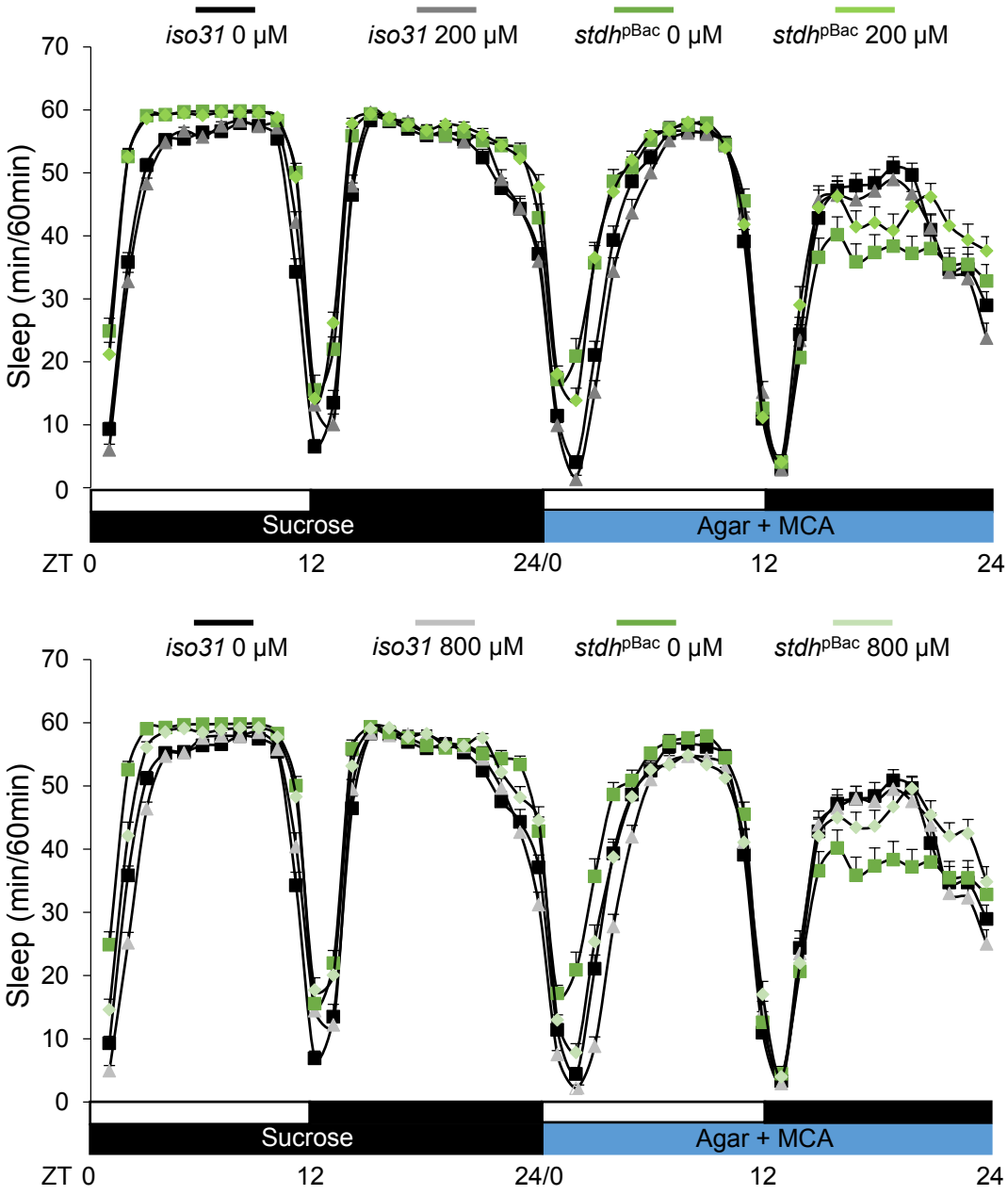
Supplementary Figure 15. Total sleep duration in flies with pan-neuronal knockdown of *aay* during fed condition. Pan-neuronal knockdown of *aay* with RNAi #1, RNAi #2, and RNAi #3 increased baseline sleep in flies during fed condition. One-way ANOVA followed by Tukey post-hoc test was used for comparison. *elav*-Gal4/+; n=94, *aay* RNAi #1/+; n=37, *elav*-Gal4 > *aay* RNAi #1; n=42, *aay* RNAi #2/+; n=61, *elav*-Gal4 > *aay* RNAi #2; n=41, *aay* RNAi #3/+; n=61, and *elav*-Gal4 > *aay* RNAi #3; n=59. ns, P>0.05; *P<0.05; **P<0.005; ***P<0.0005. All error bars represent SEM.



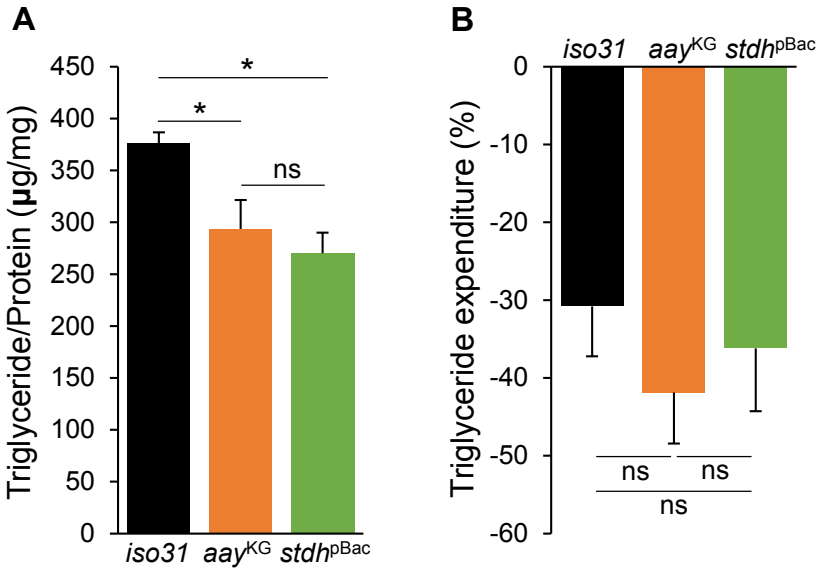
Supplementary Figure 16. *aay* knockdown with cholinergic Gal4 drivers. (A) Average sleep traces of *ChAT*-Gal4/+ (black; n=33), *aay* RNAi #1/+ (blue; n=37), and *ChAT*-Gal4 > *aay* RNAi #1 (gray; n=40) flies. (B) Flies with knockdown of *aay* with *ChAT*(*Cha*)-Gal4 do not delay sleep onset at night-time (ZT12) during starvation. Starvation*genotype [$F_{(2,214)}$=3.476; P=0.0297] (Two-way ANOVA followed by Tukey post-hoc test). (C) Knockdown of *aay* with *ChAT*-Gal4 does not increase baseline sleep during fed condition. One-way ANOVA followed by Tukey post-hoc test was used for multiple comparison. (D) Average sleep traces of 55D01-Gal4/+ (black; n=47), *aay* RNAi #1/+ (blue; n=41), and 55D01-Gal4 > *aay* RNAi #1 (gray; n=37) flies. (E) Flies with knockdown of *aay* with 55D01-Gal4 shows a tendency for resistance to sleep latency increase at night-time (ZT12) during starvation. Starvation*genotype [$F_{(2,244)}$=6.331; P=0.0021] (Two-way ANOVA followed by Tukey post-hoc test). (F) Knockdown of *aay* with 55D01-Gal4 does not increase baseline sleep in flies during fed condition. One-way ANOVA followed by Tukey post-hoc test was used for multiple comparison. ns, P>0.05; *P<0.05; **P<0.005; ***P<0.0005; ****P<0.0001. All error bars represent SEM.



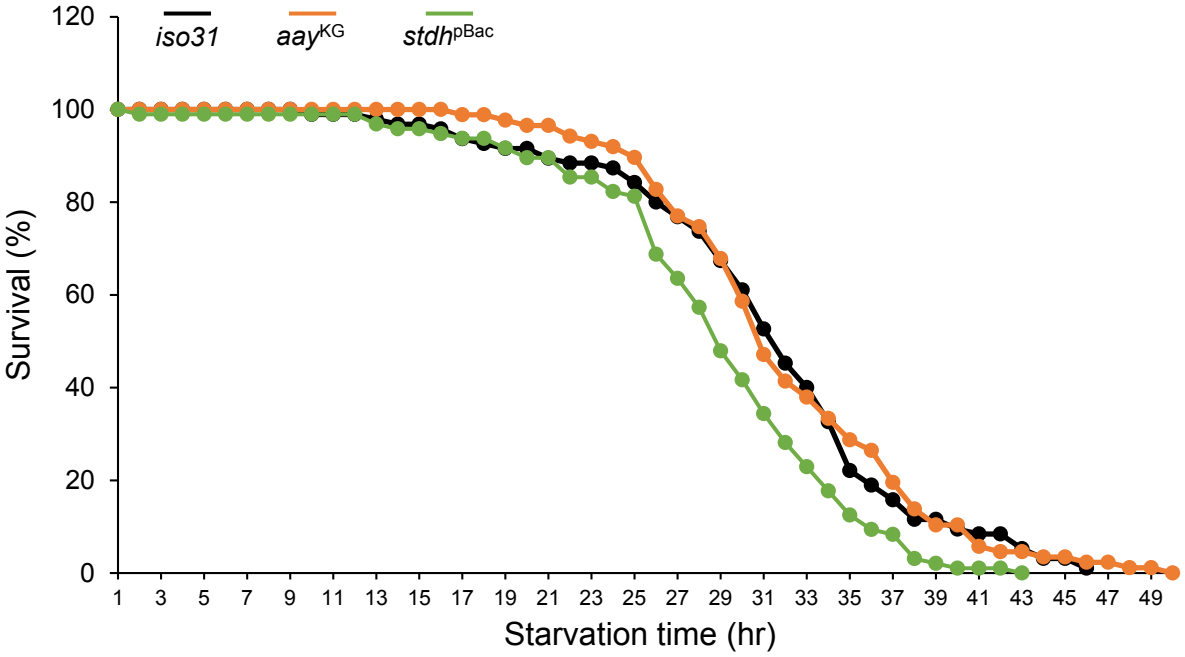
Supplementary Figure 17. *aay* function in cholinergic neurons might not be sufficient for starvation-induced sleep suppression. (A) Flies with knockdown of *aay* with the *elav*;;*ChAT*-Gal80 driver (*elav*;;*ChAT*-Gal80 > *aay* RNAi #1; n=44) exhibit reduced starvation-induced sleep suppression compared to controls (*elav*;;*ChAT*-Gal80/+; n=43 and *aay* RNAi #1/+; n=34). One-way ANOVA followed by Tukey post-hoc test was used for multiple comparison. (B) Delay in sleep onset at night-time (ZT12) was not evident in *elav*;;*ChAT*-Gal80 > *aay* RNAi #1 flies. [$F_{(2,236)}$=12.78; P<0.0001]. Two-way ANOVA followed by Tukey post-hoc test used for multiple comparison. ns, P>0.05; **P<0.005; ***P<0.0005; ****P<0.0001. All error bars represent SEM.



Supplementary Figure 18. Dose-dependent effect of MCA on exaggerated sleep suppression of *stdh* mutants during starved condition. Average sleep traces of flies in fed condition (5% sucrose/1% agar) followed by treatment of 200 and 800 μM MCA during starvation (1% agar + MCA).



Supplementary Figure 19. Fat storage and expenditure of *iso31* and serine metabolic mutants. (A) *aay* and *stdh* mutants exhibited decreased triglyceride levels compared to *iso31* control flies in fed condition (n=4). Triglyceride levels were normalized to protein levels. (B) Triglyceride expenditure was calculated as percentage change of normalized triglyceride content in fed vs. starved conditions. Triglyceride expenditure in serine metabolic mutants were comparable to *iso31* control flies after 18 hours of starvation (n=4). One-way ANOVA followed by Tukey post-hoc test was used for multiple comparison. ns, P>0.05; *P<0.05. All error bars represent SEM.



Supplementary Figure 20. Survival upon starvation of serine metabolic mutants. Kaplan-Meier starvation survival plot of *iso31* (black; n=95), *aay* mutants (orange; n=87), and *stdh* mutants (green; n=96). *aay* mutants exhibited comparable survival (P=0.7822), while *stdh* mutants exhibited slightly sensitive survival upon starvation compared to *iso31* control flies (P=0.0016). Log-rank (Mantel-Cox) test was used for statistical analyses.

	iso31		*aay*^{KG}		*stdh*^{pBac}		*aay*^{KG}, *stdh*^{pBac}	
	Fed (µg/L)	**Starved (µg/L)**	**Fed (µg/L)**	**Starved (µg/L)**	**Fed (µg/L)**	**Starved (µg/L)**	**Fed (µg/L)**	**Starved (µg/L)**
Asp	600 ± 81	718 ± 62 ns	458 ± 82 ns	521 ± 62 ns	510 ± 63 ns	530 ± 40 ns	265 ± 20 *	402 ± 67 ns
Thr	55 ± 4	59 ± 4 ns	75 ± 7 ns	98 ± 0 ns	85 ± 17 ns	104 ± 15 ns	84 ± 1 ns	114 ± 14 *
Ser	271 ± 15	359 ± 17 *	206 ± 5 ns	174 ± 20 **	469 ± 38 ***	539 ± 44 **	169 ± 10 *	89 ± 6 ***
Asn	156 ± 20	199 ± 25 ns	198 ± 17 ns	195 ± 16 ns	176 ± 32 ns	177 ± 32 ns	131 ± 10 ns	262 ± 17 ns
Glu	1186 ± 50	1464 ± 79 *	1476 ± 27 *	1560 ± 93 ns	965 ± 89 ns	903 ± 127 *	988 ± 8 ns	781 ± 53 **
Gln	566 ± 95	901 ± 110 ns	279 ± 34 ns	769 ± 132 ns	575 ± 129 ns	875 ± 107 ns	539 ± 69 ns	1426 ± 250 ns
Gly	643 ± 4	660 ± 39 ns	513 ± 23 **	563 ± 13 ns	571 ± 25 ns	651 ± 30 ns	550 ± 9 *	657 ± 29 ns
Ala	3601 ± 227	3769 ± 308 ns	3659 ± 314 ns	3600 ± 351 ns	3056 ± 210 ns	2866 ± 249 ns	2822 ± 127 ns	2325 ± 122 *
Met	51 ± 7	63 ± 10 ns	50 ± 4 ns	66 ± 3 ns	43 ± 23 ns	47 ± 8 ns	37 ± 1 ns	77 ± 14 ns
g-ABA	424 ± 28	458 ± 17 ns	388 ± 69 ns	423 ± 25 ns	399 ± 36 ns	389 ± 30 ns	378 ± 8 ns	332 ± 15 *
b-Ala	289 ± 36	443 ± 87 ns	383 ± 69 ns	474 ± 102 ns	203 ± 23 ns	203 ± 8 ns	119 ± 15 ns	294 ± 59 ns
Lys	99 ± 5	134 ± 6 *	199 ± 21 **	201 ± 4 ns	74 ± 12 ns	110 ± 11 ns	70 ± 6 ns	367 ± 47 ***
His	642 ± 17	819 ± 23 **	717 ± 26 ns	832 ± 14 ns	885 ± 60 *	1029 ± 51 ns	762 ± 63 ns	1077 ± 137 ns
Arg	1734 ± 77	2028 ± 40 *	1755 ± 110 ns	1915 ± 124 ns	1950 ± 80 ns	1911 ± 179 ns	1426 ± 33 ns	1338 ± 72 *

Supplementary Table 1. Amino acid levels in head extracts of *iso31*, *aay*, *stdh*, and double mutants during fed and starved conditions. Amino acid levels were compared between *iso31* control and metabolic mutants during fed and starved conditions (n=3). Unpaired t-test was used for comparison between fed and starved conditions in *iso31*, indicated in green. One-way ANOVA followed by Tukey's post-hoc test was used for multiple comparison between *iso31* and genetic mutants in fed (black) and starved conditions (red). ns, P>0.05; *P<0.05; **P<0.005; ***P<0.0005. All error numbers represent SEM.