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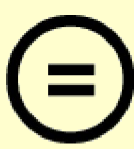

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Master's Thesis

Threonine Enhances Sleep Drive via a GABAergic Pathway in *Drosophila*

Yoonhee Ki

Department of Biological Sciences

Graduate School of UNIST

2018

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Yoonhee Ki

Department of Biological Sciences

Graduate School of UNIST

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A thesis/dissertation
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Master of Science

Yoonhee Ki

January 12, 2018

Approved by

Advisor
Chunghun Lim

Threonine Enhances Sleep Drive via a GABAergic Pathway in *Drosophila*

Yoonhee Ki

This certifies that the thesis/dissertation of Yoonhee Ki is approved.

January 12, 2018

signature

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Chunghun Lim

signature

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Chan Young Park

signature

____

Jae-Ick Kim

ABSTRACT

Amino acids are often used as sleep-inducing supplements, yet the neural basis underlying sleep regulation remains unclear. Here we employed *Drosophila* as a genetic model to demonstrate that threonine facilitates sleep onset via a specific GABAergic pathway. Feeding wild-type flies with sucrose supplemented with individual amino acids differentially affected their sleep behaviors. Glycine, a co-agonist for the N-methyl-D-aspartate receptor, lengthened the average duration of sleep bouts and thus improved the sleep quality, consistent with its effects on human sleep. On the other hand, threonine markedly increased the daily amount of sleep and shortened latency to sleep onset in a dose-dependent manner. Threonine-fed flies also fell asleep faster than control-fed flies when their sleep was disturbed in midnight which implicates the SPET is regulated in time of day independent manner. Circadian clock components are reported to have intimate relationship with sleep behavior. However, our genetic ablation of clock component revealed that the sleep-promoting effects of a threonine (SPET) is independent of clock components. GABA-transaminase (GABAT) is a mitochondrial enzyme that metabolizes GABA in glial cells so that it results in increased GABA in brain in the absence of this enzyme. Genetic ablation or pharmacological inhibition of GABA-transaminase masked SPET. Pharmacological inhibition of GABA reuptake by feeding nipecotic acid (NipA) also abolished SPET. A transcriptional reporter for intracellular Ca2+ levels revealed that a threonine diet led to excitation of a specific subset of GABAergic neurons, whereas a conditional blockade of the synaptic transmission in GABAergic neurons suppressed SPET. Transgenic RNA interference of $GABA_B$ receptor in neurons suppressed SPET whereas RNA interference of $GABA_B$ in glia fully sustains it. It further implicated metabotropic GABA receptors in the neural output pathway of SPET. Finally, we have elevated the endogenous threonine levels by genetic down-regulation of threonine metabolizing enzyme. Hypomorphic mutants of *threonine 3-dehydrogenase* (*CG5955*) had elevated threonine levels and showed shorter time for latency to sleep in both natural and sleep-disturbed condition. Pan-neuronal knock down of CG5955 by RNA interference was sufficient for enhancing sleep drive. Taken together, these findings reveal a neural mechanism underlying how animals adaptively adjust their sleep behaviors based on a specific diet and define a novel sleep-regulatory pathway that intimately links essential threonine metabolism to the control of sleep drive. Given genetic elevation of endogenous threonine levels facilitates sleep onset, threonine can be considered as an endogenous sleep enhancer.

Keywords: sleep regulation | threonine | GABA

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ABBREVIATIONS

ABL : Average Sleep Bout Length

AKB : Alpha-Ketobutyric Acid, 2-Keto Butyrate

AL : Antennal Lobe

BBB : Blood Brain Barrier

BCA : Bicinchoninic Acid Assay

BHB : Beta-Hydroxybutyric Acid

CalexA : Calcium-dependent nuclear import of the transcriptional activator LexA

DAM : Drosophila Activity Monitor

EOS : Ethanolamine O-Sulfate

GABA : Gamma-Aminobutyric Acid

GABAT : GABA-transaminase

Gad : Glutamic Acid Decarboxylase

GFP : Green Fluorescent Protein

GHB : Gamma-Hydroxybutyrate

LH : Lateral Horn

LK : Leucokinin

LKr : Leucokinin Receptor

LN : Lateral Neuron

LTA : L-Threonine Aldolase

mACT : middle Antenno-cerebral Tract

NAD : Nicotinamide Adenine Dinucleotide

NADH : Nicotinamide Adenine Dinucleotide Hydrate

NipA : Nipecotic Acid

NMDAR : N-methyl-D-aspartate Receptor

REMs : Rapid Eye Movements

NPF : Neuropeptide Y

PBS : Phosphate-Buffered Saline

PCR : Polymerase Chain Reaction

PDF : Pigment Dispersing Factor

Rdl : Resistance to Dieldrin

RNA : Ribonucleic Acid

SPET : Sleep-Promoting Effects of Threonine diet

THIP : 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol

VN : Ventral Neuron

ZT : zeitgeber time

I. INTRODUCTION

1.1 Sleep

Sleep is somewhat life-threatening physiology of animals since during sleep stage they dramatically lose consciousness and sensibility which then become helpless about the environmental threats. But evolutionary pressure has allowed sleep to be here. There must be an exceedingly important role of sleep beyond its huge opportunity costs. Obviously deprivation of sleep causes several physiological defects. It has been known that poor sleep causes mood and emotion changes as well as impaired motor function (*1*). Also deprivation of sleep affects every aspects of cognition problems including diminished ability for alertness (*2*), decision making (*3*), long term memory consolidation (*4*), learning (*5*) and executive function (*6*). The alterations of immunity (*7*) and metabolism (*8*) are also parts of symptoms. Chronic total sleep deprivation, ultimately, brings a death (*9*). Despite its wide range of impacts on life, not much is known about its architecture.

The circadian clock and sleep homeostasis are two key regulators that shape daily sleep behaviors in animals (*10*). In stark contrast to the homeostatic nature of sleep, the internal machinery of sleep is vulnerable to external (e.g., environmental change) and internal (e.g., genetic mutation) conditions that lead to adaptive changes in sleep behaviors. The sleep behavior is conserved among mammals, insects, and even lower eukaryotes (*11, 12*).

1.2 Role of inhibitory neurotransmitter GABA in sleep

To date, a number of sleep-regulatory genes and neurotransmitters have been identified in animal models as well as in human (*13-15*). For instance, gamma-aminobutyric acid (GABA) is a non-proteinogenic amino acid which acts as a prominent inhibitory neurotransmitter in brain. Early study found that the global central increase of GABA by either infusion of GABA or inhibition of GABA transaminase (GABAT) increased slow-wave sleep in cats (*16, 17*). In humans it has been shown that increase of GABA decreased latency to sleep as well as the amount of waking (*18*) while it has no or little effects on the duration of sleep stages (*19, 20*). The sleep regulatory role of GABA has been well established via the action of GABA(A) receptor. Muscimol, the GABA(A) agonist, significantly enhanced low frequencies of electroencephalogram (EEG) during slow-wave sleep (*21, 22*). Similarly, another agonist of GABA(A) receptor gaboxadol (THIP) could increase non-rapid eye movement sleep (non-REMS) and the enhancement of slow-wave activity in both rats and humans (*23, 24*). In the structural view point, gallopin et al., found that anterior hypothalamic neurons which were known to regulate sleep are GABAergic (*25*).

The sleep regulatory role of GABA is now well documented and is also conserved through

invertebrate to vertebrate (*26*). Recent study showed that in the absence of GABAT internal GABA level was increased which results in sleep promotion in *drosophila* (*27*) which is comparable to humans. The long-sleep phenotype in GABA-T mutants accompanies higher sleep consolidation and shorter latency to sleep onset, consistent with the observations that pharmacological enhancement of GABAergic transmission facilitates sleep in flies and mammals, including humans (*17, 18, 20*). In addition, *resistance to dieldrin* (*Rdl*), a *Drosophila* homolog of the ionotropic GABA receptor, suppresses wake-promoting circadian pacemaker neurons in adult flies to exert sleep-promoting effects (*28-31*) . Similarly, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP), an agonist of the ionotropic GABA receptor, promotes sleep in insects and mammals (*23, 24, 32*) while downregulation of GABAB-R2 in the large PDF neurons (l-LNv) could reduce sleep maintenance during the second half of the night (*33*).

1.3 Possibilities of amino acids as sleep medications

Many sleep medications modulate GABAergic transmission. A prominent side effect of anti-epileptic drugs relevant to GABA is also drowsiness (*34*). Conversely, glycine supplements improve sleep quality in a way distinct from traditional hypnotic drugs, minimizing deleterious cognitive problems or addiction (*35, 36*). N-methyl-D-aspartate receptor (NMDAR), one of the ionotropic glutamate receptors, has been identified as a neural substrate for glycine-dependent sleep promotion (*37, 38*). Because glycine and D-serine act as co-agonists of the glutamate receptor, we hypothesized that other amino acids might display neuromodulatory effects, particularly on sleep behaviors.

1.4 Drosophila as a model animal for sleep study

To dissect out molecular and neural components important for sleep regulation of amino acids, the employment of a proper sleep model and its quantitative analyses in genetically traceable organisms are essential. Since the first identification of the voltage-gated potassium channel *Shaker* as a sleep-regulatory gene in *Drosophila* (*39*), fruit flies have been one of the most advantageous genetic models to dissect molecular and neural components important for sleep homeostasis and sleep plasticity. In flies, any period of immobility lasting more than 5 minutes is considered as sleep episode (*40, 41*). This operational definition of sleep is based on differential arousal threshold after a given period of immobility, and is widely accepted for measuring fly sleep using the standard Drosophila Activity Monitor (DAM) System. Amount of sleep minutes, then, is the most straightforward sleep parameter which can be calculated by summing up each length of sleep episodes. This system further provides more sophisticated sleep parameters such as average sleep bout length (ABL) and latency to sleep onset. Not only the parameters interact intimately, but also they are regulated independently in coincidence with the complexity of sleep behavior.

1.5 Threonine as a novel sleep regulating molecules

Threonine is a ketogenic amino acid where catabolism of the amino acid consists of three independent pathways result in two ketones (L-2-amino-acetoacetate, 2-ketobutyrate) and glycine. In this study we found a novel sleep-regulating molecule by screening 20 amino acids on sleep behavior in a quantitative manner in *drosophila*. Threonine supplements during behavior assay gradually increases total sleep amount and decreases latency to sleep both in a day by day and a dose-dependent manner. Several lines of our genetic and pharmacological evidence suggest that GABAergic transmission mediates the SPET. While circadian clocks have been thought to control the latency to sleep, we demonstrated that the SPET neither requires circadian pacemaker neurons nor the functionality of circadian clocks. Importantly, genetic engineering that elevates endogenous levels of threonine was sufficient to enhance sleep drive. Taken together, we propose threonine as a novel sleep-regulatory molecule that promotes sleep primarily by facilitating wake to sleep transition

1.6 Summary

In this study, we employed a *Drosophila* genetic model of sleep behaviors to discover sleep-promoting effects of a threonine diet (SPET). Although circadian clocks may govern the timing of sleep onset, we demonstrated that SPET relies on a clock-independent GABAergic pathway to adaptively affect sleep onset latency. Moreover, genetic elevation of endogenous threonine levels was sufficient to enhance the sleep drive. We, thus, propose threonine as a novel sleep-regulatory molecule that facilitates the transition from arousal to sleep.

II. MATERIALS AND METHODS

2.1 Fly Stocks.

Flies were raised on standard cornmeal-yeast-agar medium at 25˚C. The strains, including w1118 (BL5905), GABA-T[PL](BL19461), Df(3L)BSC731 (BL26829; GABA-T deficiency), Df(3L)BSC839 (BL27917; *CG5955* deficiency), Pdfr[BG00979] (BL12523), ELAV-Gal4 (BL458), GAD1-Gal4 (BL51630), REPO-Gal4 (BL7415), UAS-GABA-B-R1 RNAi (BL51817), and UAS-*CG5955* RNAi (BL64566), were obtained from the Bloomington *Drosophila* Stock Center. *CG5955*[GS20382] (201409), Rdl[1] (106453), and Rdl[MD-RR] (106444) were obtained from the Kyoto Stock center. Per[01], Clk[Jrk], Pdf-Gal4, Cry-Gal4, to-Gal4, UAS-TNT, UAS-shibirets, and UAS-mLexA-VP16-NFAT have been described previously (25, 26, 47-51).

2.2 Sleep Analyses.

All behavioral tests were performed using individual male flies, unless otherwise indicated. Each fly was housed in a 65 × 5 mm glass tube containing 5% agarose with 2% sucrose (behavior food). Locomotor activity was recorded using the *Drosophila* Activity Monitor system (Trikinetics) under 12-h LD cycles at 25°C and quantified by the number of infrared beam crosses per minute. Sleep bouts were defined as no activity for ≥5 min. Sleep parameters were analyzed using an Excel macro (52). For amino acid supplements, each amino acid was dissolved at the indicated concentrations in the behavior food. For oral administration of GABA-T or GABA transporter inhibitors, EOS (Tokyo Chemical Industry) or NipA (Sigma) was directly dissolved to 10 mM in behavior food containing the indicated concentrations of threonine. Flies were pre-fed on amino acid- and/or inhibitor-containing behavior food for 3.5 days and their sleep behaviors were monitored for 24 h.

2.3 Measurement of Sleep Latency after Arousal.

Arousal threshold after mechanical stimulus was measured as described previously (53) with minor modifications. Briefly, locomotor activities were recorded in standard LD cycles, while behavioral test tubes containing individual male flies were scraped with a thin wood stick at zeitgeber (ZT) 16 (lights-on at ZT0; lights-off at ZT12). Mechanical stimuli used in our tests include: 1) scraping sound and vibration without direct scraping (weak stimulus), 2) gentle scraping (medium stimulus), and 3) hard scraping repeated 3–4 times (strong stimulus). Flies were defined as aroused if they displayed inactivity for > 5 min prior to the stimuli but showed any locomotor response within 10 min. The percentage of aroused flies were calculated for each experiment and averaged for each

group from three independent experiments. Latency to sleep onset after arousal was individually calculated and averaged for each group. To calculate the percentage of light-aroused flies and sleep latency after arousal, LD-entrained flies were exposed to an 1-min light pulse at ZT16 instead of the mechanical stimuli.

2.4 Video Analyses of Locomotor Activity.

Male flies were pre-fed on control or amino acid-containing behavior food for four LD cycles at 25°C. After brief anesthetization, flies were individually placed into 6-well plates with an approximate height of 2 mm. After 25 min of habituation, time-lapse images were obtained at 10 Hz using HandyAVI software (AZcendant). Approximately 3000 frames (corresponding to a 5-min video recording) were analyzed using ImageJ software to quantify the locomotor activity of each fly. Positional changes in X- and Y-axes were measured from two consecutive frames, and a difference larger than three pixels was considered as movement.

The parameters used in the assay were calculated as follows:

Total distance = sum(sqrt(x_change(:)^2+y_change(:)^2)); (in cm)

MovingTime = (number of moved-frames)*(sec/frame); (in sec)

MovingSpeed = Total distance*1000/MovingTime; (in mm/sec)

MovingBouts = number of transition from non-moved to moved

Averaged moving bout length = MovingTime/MovingBouts

2.5 Whole-Brain Imaging.

Transgenic flies were pre-fed on control or amino acid-containing behavior food for four LD cycles at 25°C prior to imaging experiments. Whole brains were dissected in phosphate-buffered saline (PBS) and fixed in PBS containing 3.7% formaldehyde. Fixed brains were washed three times in PBS containing 0.3% Triton X-100 (PBS-T), blocked in PBS-T containing 0.5% normal goat serum, and then incubated with mouse anti-GFP (NeuroMab) and rabbit anti-GABA (Sigma) antibodies for 2 days at 4°C. After washing three times in PBS-T, brains were further incubated with anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 antibodies (Jackson ImmunoResearch) for 1 day at 4°C, washed three times with PBS-T, and then mounted in VECTASHIELD mounting medium (Vector Laboratories). Confocal images were acquired using a Multi-Photon Confocal Microscope (LSM780NLO, Carl Zeiss) with Plan-Apochromat 40x/1.3 Oil lens and analyzed using ImageJ software.

2.6 Quantitative PCR.

Total RNA was purified from 10 flies per each genotype (five males and five females) using Trizol Reagent, according to the manufacturer's instructions (Thermo Fisher Scientific). cDNA was prepared from DNase I-treated RNA samples using the M-MLV Reverse Transcriptase reagent (Promega) and random hexamers. Diluted cDNA samples were quantitatively analyzed by SYBR Green-based Prime Q-Mastermix (GeNet Bio) and gene-specific primers using the LightCycler 480 real-time PCR system (Roche). To validate the efficiency of transgenic RNA interference, total RNAs from head or body extracts were analyzed similarly.

Primer sets were used as following sequences:
qPabp5b: 5'-ATCTCCCACAGGACGTCAAC-3';
qPabp3b: 5'-GCGACGAAGAGAAGGATCAC-3';
q*CG5955*_F: 5'-TTCTGATCACAGGTGGCTTG-3';
q*CG5955*_R: 5'-CGATCTTCTGGAGACCCTTG-3';

2.7 Threonine Measurement.

Quantitative measurement of threonine was performed as described previously (54) with minor modifications. Briefly, 30 female flies were homogenized in 200 μL of PBS containing 0.05% Triton X-100. Whole-body extracts were clarified twice by centrifugation, and total proteins in the extracts were quantified using the Pierce BCA Protein Assay Kit according to manufacturer's instructions (Thermo Fisher Scientific). After boiling, soluble extracts were further clarified by centrifugation and subjected to an enzymatic reaction. Each reaction mixture included 40 μL of 5× HEPES reaction buffer (500 mM HEPES pH 8.0, 1 mM NADH, 0.25 mM pyridoxal 5-phosphate, and 5 mM dithiothreitol), 160 μL of soluble body extracts, and 1 U of alcohol dehydrogenase (Sigma). In parallel, control reactions with a serial dilution of threonine stock solution (16 mM) were used to generate a standard curve for quantification. The enzymatic reactions were set up in a 96-well microplate (Corning) and incubated for 30 min at 4°C followed by 10-min incubation at 25°C. Absorbance at 340 nm was measured for each reaction mixture using an Infinite M200 microplate reader (Tecan) before 1 μL of bacterially purified L-threonine aldolase (LTA) was added to each reaction mixture. The reaction mixture was further incubated at 37°C for 5 min and post-LTA absorbance was measured to calculate decreases in NADH levels.

2.8 Protein Purification of L-threonine Aldolase.

The coding sequence of LTA was PCR-amplified from genomic DNA of Pseudomonas aeruginosa (a gift from R.J. Mitchell) and cloned into a modified pDuet vector (a gift from C. Lee). Bacterial

purification of His-tagged LTA proteins using Ni-NTA Agarose (Qiagen) was performed as described previously (55). Purified proteins were dialyzed using a dialysis buffer (50 mM NaH2PO4, pH 8.0, 10 μM pyridoxal 5-phosphate, and 1 mM dithiothreitol), diluted in 50% glycerol, quantified using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), and stored at −80°C prior to use.

Genomic sequence of LTA is amplified with the following primer set:

pLTA_F 5'-GATC GGATCCATGACCGATCACACCCAACAG-3'

pLTA_R 5'-GATC AAGCTTTCAGGCGCCCATCACCAG-3'

III. RESULTS

3.1 Threonine Diet Increases Sleep Bout Numbers and Shortens Sleep Latency to Promote Sleep.

3.1.1 Threonine enhances sleep drive by shortening latency to sleep.

To determine if amino acid supplements modulate sleep in *Drosophila*, we quantitatively assessed sleep behaviors in wild-type flies fed 5% sucrose containing 17.5 mM of each amino acid in 12-h light:12-h dark (LD) cycles at 25°C. As expected, glycine supplementation enhanced the sleep quality by significantly lengthening the average duration of sleep episodes (Fig. 1). The strongest impact on sleep quantity and quality was observed with cysteine supplementation. However, the cysteine-fed flies showed significant defects in moving speed which could be a representation of sickness. Therefore, compromised locomotion and lethality due to a cysteine diet led to its exclusion from further analyses (Fig. 3). Intriguingly, threonine supplementation potently elevated total sleep amount by increasing the number of sleep bouts. Unlike glycine, it does not accompany with the increase in average duration of sleep episodes. In addition, sleep latency after lights-off was specifically shortened by threonine compared with that by other amino acids. SPET is dose-dependent and observed in both male and female flies (Fig. 2). This implicates the ubiquitous roll of threonine on sleep as well as the threonine as a direct sleep regulating factor.

3.1.2 Sleep-promoting effects of threonine is not result from locomotion defects.

Among various sleep-relevant parameters we measured, waking activity indicates an activity/min. This parameter is partially indicative for locomotive defects. To address the locomotive defects effects on SPET, we used 2-dimentional locomotive tracking method. Although a threonine diet led to lower waking activity, a video recording of fly locomotor activities with time frames of higher resolution confirmed that the threonine supplement did not impair general locomotion since it does not affects moving speed of flies (Fig. 3). Moreover, tryptophan or histidine supplements similarly lowered waking activity but did not affect sleep behaviors comparable to threonine. Thus, we conclude that sleep-promoting phenotype of threonine is genuine while phenotype from cysteine is resulting from locomotion defects.

essential
non-essential

Sleep Amount (min)
Sleep Latency (min)
Waking Activity (beam crosses/min)
Sleep Bout Number
ABL (min/sleep bout)

control Thr Phe Trp Met Val Leu Ile His Lys Arg Tyr Gly Ala Glu Asp Ser Pro Gln Asn Cys

102.8

Fig. 1. Threonine diet facilitates sleep onset to promote sleep. Wild-type male flies were loaded on to 5% sucrose food containing 17.5 mM of each amino acid (day 0) and entrained in LD cycles at 25ºC. Total sleep amount, latency to sleep onset after lights-off, activity counts while awake, total sleep bouts number and average sleep bout length were calculated from individual flies on day 4 and averaged per each amino acid. Essential amino acids were indicated by a grey background. Error bars indicate SEM (n=29–213). *P < 0.05, **P < 0.01, ***P < 0.001 to control (black bars) as determined by Student’s t-test.

Fig. 2. Sleep-promoting effects of a threonine diet are dose-dependent and observed in both male and female flies. (A-B) Wild-type male (A) or female (B) flies were loaded on to 5% sucrose food containing the increasing amount of threonine (day 0) and entrained in LD cycles at 25ºC. Sleep behaviors in individual flies were analyzed similarly to the data presented in Fig. 1. Data represent average +/- SEM (n=11–213). *P < 0.05, **P < 0.01, ***P < 0.001 to control (black bars) as determined by Student's t-test.

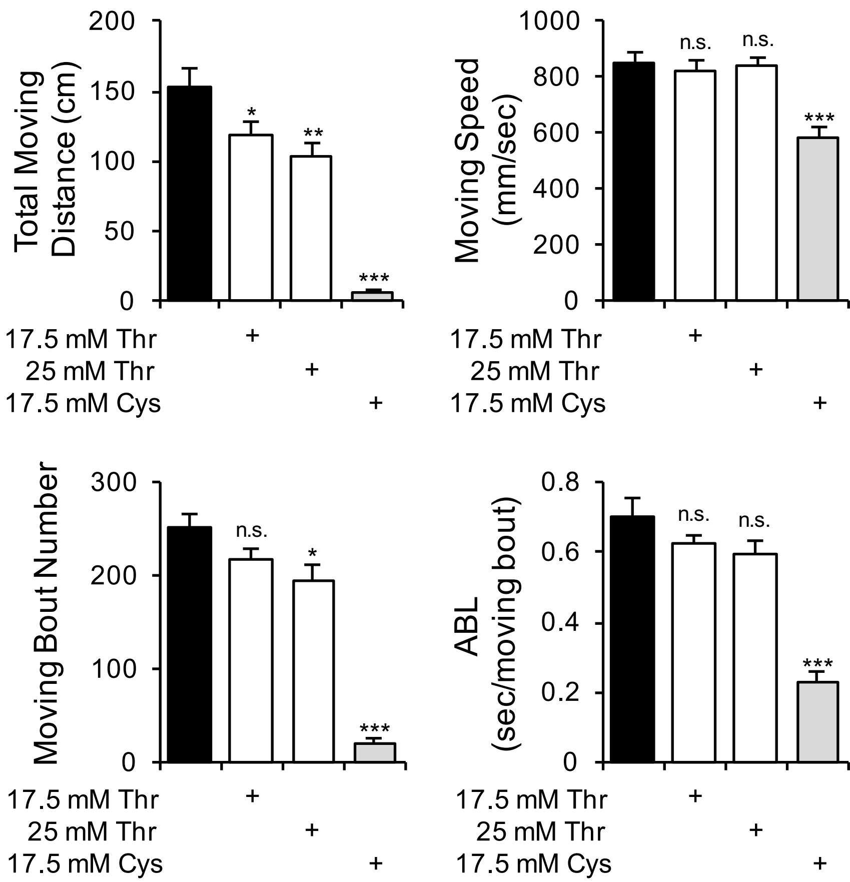


Fig. 3. A threonine diet does not impair general locomotion. Wild-type male flies were loaded on to 5% sucrose food containing 17.5 mM of each amino acid (day 0) and entrained in LD cycles at 25ºC. Locomotor activities in individual flies were video-recorded for 5 min on day 4. Total moving distance, moving speed, the number of moving bouts, and averaged moving bout length (ABL) were calculated from individual flies and averaged per each condition. Error bars indicate SEM (n=34–76). n.s., not significant; *P < 0.05, **P < 0.01, ***P < 0.001 to control (black bars) as determined by Student's t-test.

3.1.3 Orally administrated threonine helps sleep drive in a time of day independent manner.

The increase of sleep often considered as more deep sleep than usual. To determine if SPET involves an increase in arousal threshold (i.e., sleep depth), we quantified arousal responses to sensory stimuli. To test the arousal threshold, a range of mechanical stimuli is introduced in the middle of night and the percentage of aroused flies was calculated for each strength of stimuli. Control- and threonine-fed flies displayed no significant differences in the percentage of aroused flies when awakened by a range of mechanical stimuli in the middle of night (Fig. 4). However, latency to the first sleep episode after mechanical awakening was substantially shortened in threonine-fed flies. Similar results were obtained when nighttime sleep was interrupted by a pulse of light (Fig. 4). So we can conclude that threonine could promote latency to sleep onset in a various condition including light-awakened, mechanically-awakened as well as clock dependent sleep onset. Taken together, these data suggest that a higher sleep drive but not a change in sleep depth contributes to SPET.

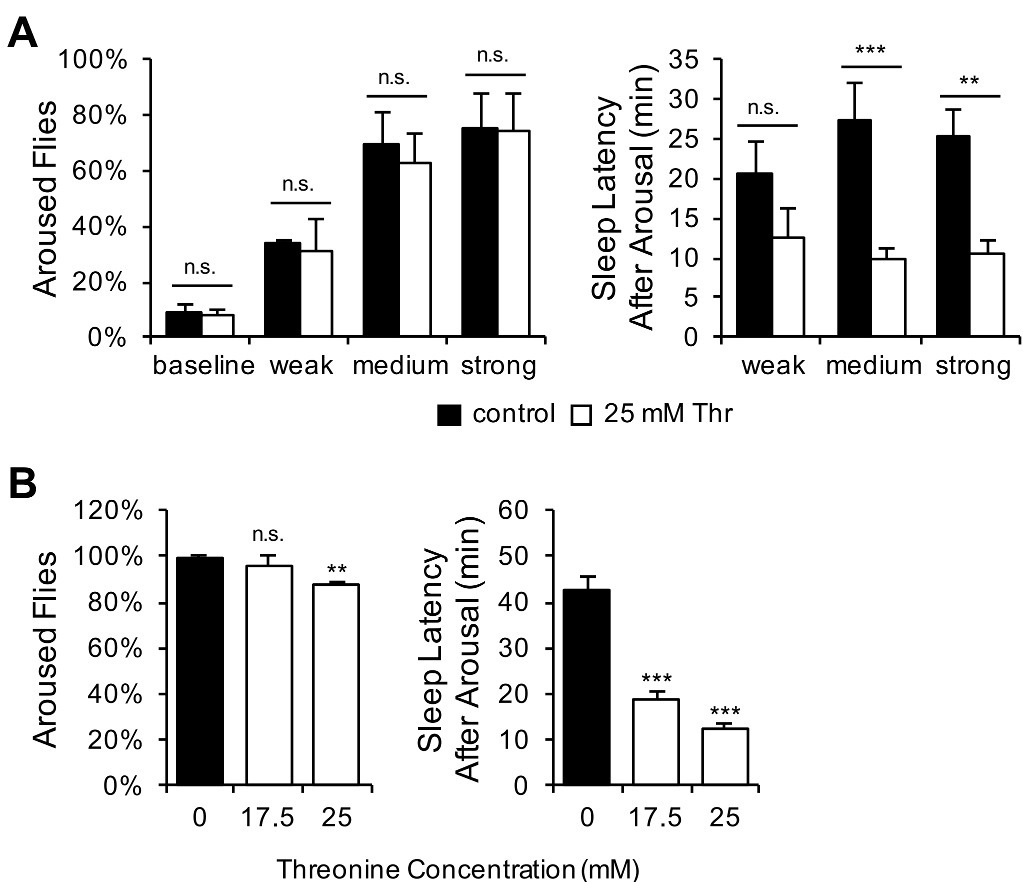
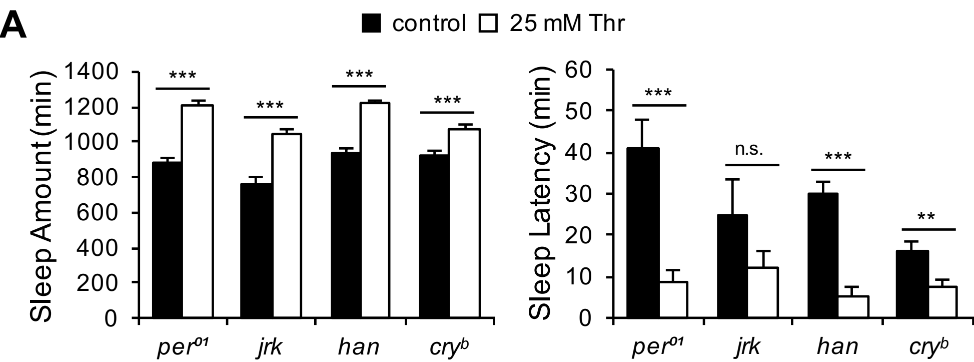


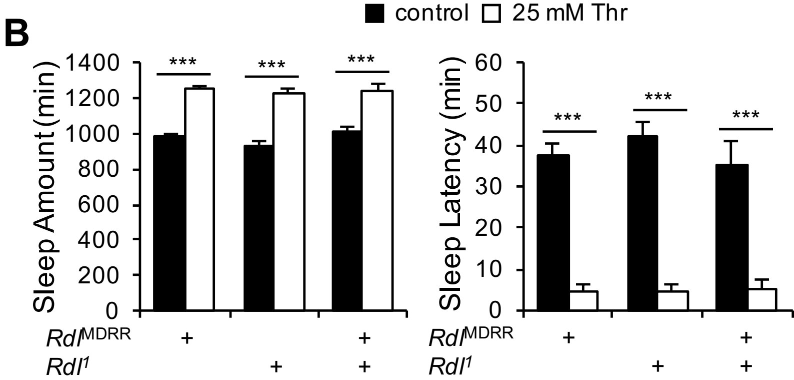
Fig. 4. A threonine diet causes a higher sleep drive. (A) Control- and threonine-fed flies were awakened by a range of mechanical stimuli 4 hours after lights-off on day 4. Aroused flies were defined as described in Methods. The percentage of aroused flies per each condition was averaged from three independent experiments. Sleep latency after arousal was calculated from individual flies and averaged per each condition (n=12–27). Error bars indicate SEM. Two-way ANOVA detected significant effects of threonine supplementation on sleep latency after arousal ($F[1,119] = 20.43$, $P < 0.0001$) but not on % aroused flies ($F[1,16] = 0.227$, $P = 0.6402$). n.s., not significant; **$P < 0.01$, ***$P < 0.001$ as determined by Bonferroni's multiple comparisons. (B) Wild-type male flies were loaded on to 5% sucrose food containing the indicated amount of threonine (day 0) and entrained in LD cycles at 25ºC. Control- and threonine-fed flies were exposed to an 1-min light pulse at ZT16 (i.e., 4 hours after lights-off) on day 4. The percentage of light-aroused flies per each condition was averaged from three independent experiments. Sleep latency after light-arousal was calculated from individual flies and averaged per each condition (n=61–74). Error bars indicate SEM. n.s., not significant; **$P < 0.01$, ***$P < 0.001$ to control (black bars) as determined by Student's t-test.

3.2 Genetic or Pharmacological Elevation of Synaptic GABA Masks SPET.

3.2.1 Sleep-promoting effects of threonine is a clock-independent feature.

Rdl and *wide awake* (*wake*) are two evolutionarily conserved genes that have been implicated in the regulation of sleep latency in *Drosophila* (12, 15). *WAKE* silences wake-promoting circadian clock neurons to promote sleep onset in a manner that is dependent on the circadian neuropeptide Pigment-dispersing factor (Pdf) and its receptor, *Pdfr*. In addition, *WAKE* genetically and biochemically interacts with *RDL* to control sleep latency. Therefore, we questioned whether the effects on sleep latency observed with SPET involved this clock-dependent pathway for facilitating sleep onset. *Han* is a pdf receptor mutant while *Per01*, *jrk* and *cryb* are hypomorphic mutants of core clock components. However, neither genetic mutations in clock-relevant genes nor transgenic manipulation of circadian pacemaker neurons by blocking synaptic transmission impacted sleep latency in SPET (Fig. 5).





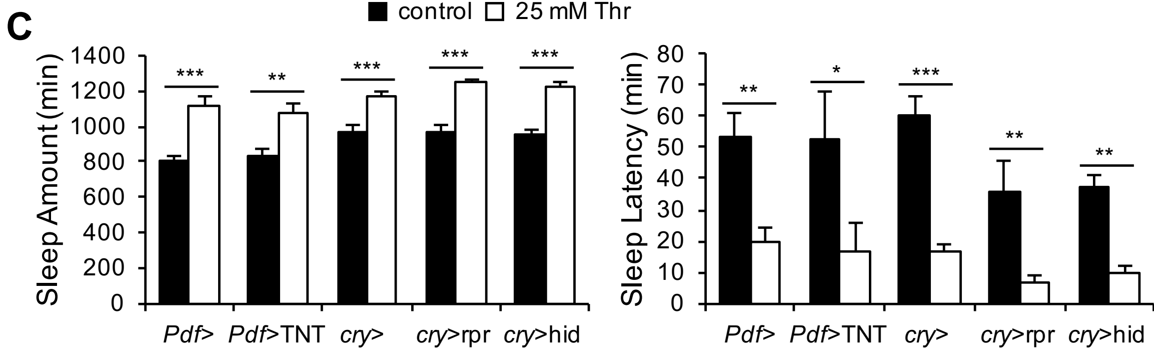


Fig. 5. Neither mutations in clock-relevant genes nor transgenic manipulation of circadian pacemaker neurons impact on SPET. (A) Circadian clock mutants (per01, jrk, han, and cryb) were loaded on to 5% sucrose food containing 25 mM of threonine (day 0) and entrained in LD cycles at 25ºC. Sleep behaviors in individual flies were analyzed similarly to the data presented in Fig. 1. Data represent average +/- SEM (n=19–46). n.s., not significant; **P < 0.01, ***P < 0.001 as determined by Student's t-test. (B) SPET was comparable between trans-heterozygous Rdl mutants and their heterozygous controls. Two-way ANOVA detected no significant interaction between genotype and threonine supplementation (sleep amount, F[2,130] = 0.6833, P = 0.5067; sleep latency, F[2,130] = 0.4653, P = 0.6290). Error bars indicate SEM (n=12–30). ***P < 0.001 as determined by Sidak's multiple comparisons test. (C) Neither blocking of synaptic transmission in Pdf-expressing clock

neurons (Pdf>TNT) nor genetic ablation of cry-expressing clock neurons (cry>rpr or cry>hid) affected SPET. Two-way ANOVA detected no significant interaction of threonine supplementation with Pdf>TNT (sleep amount, F[1,49] = 0.4167, P = 0.5216; sleep latency, F[1,49] = 0.01406, P = 0.9061) or with cry ablation (sleep amount, F[2,101] = 1.094, P = 0.3387; sleep latency, F[2,101] = 1.153, P = 0.3197). Error bars indicate SEM (n=8–20). *P < 0.05, **P < 0.01, ***P < 0.001 as determined by Sidak's multiple comparisons test.

3.2.2 Flies having higher GABA levels does not show additive sleep-increase by threonine.

Intriguingly, we found that mutants trans-heterozygous for a hypomorphic allele over chromosomal deficiency in the genetic locus of GABA-T did not exhibit SPET compared with heterozygous controls (Fig. 6). However, it is possible that short sleep latency in GABA-T mutants masks SPET because of a floor effect. Therefore, we tested if oral administration of the GABA-T inhibitor ethanolamine O-sulfate (EOS) could decrease SPET. EOS treatment increased the amount of daily sleep and shortened sleep latency modestly in control flies but substantially suppressed SPET (Fig. 6). GABA-T is a mitochondrial enzyme that metabolizes GABA into succinic semialdehyde (8), thereby suppressing GABAergic transmission. Accordingly, we reasoned that SPET might involve a GABA-dependent sleep drive, and therefore, increases in synaptic GABA levels caused by GABA-T mutation probably masks SPET. This idea was further supported by our observation that nipecotic acid (NipA), which blocks GABA reuptake from synaptic clefts (24), comparably suppressed SPET (Fig. 6). These genetic and pharmacological data together suggest that threonine supplementation enhances GABAergic transmission to mediate SPET.

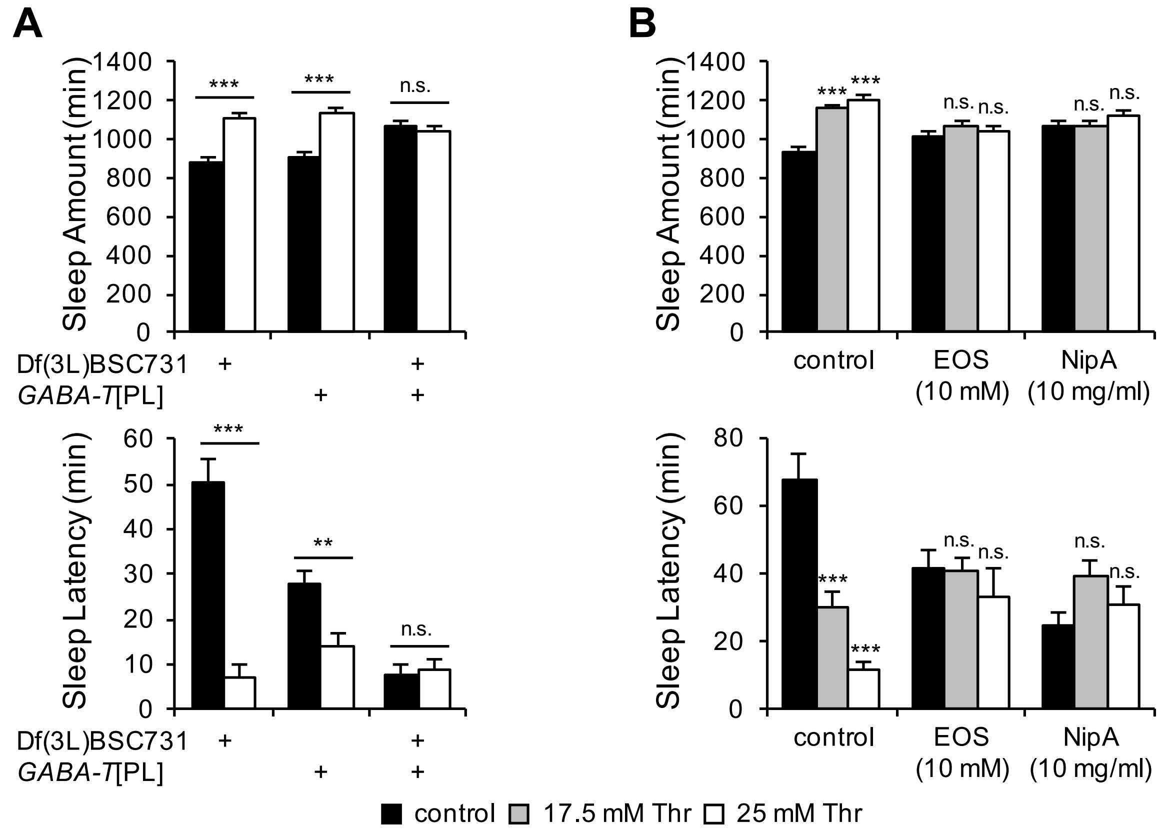


Fig. 6. Genetic or pharmacological elevation of GABA masks SPET. (A) SPET was desensitized in GABA-T trans-heterozygous mutants compared with their heterozygous controls. Sleep behaviors in individual flies were analyzed similarly to the data presented in Fig. 1. Data represent average +/- SEM (n=15–36). Two-way ANOVA detected significant interactions between GABA-T mutation and threonine supplementation on both sleep amount ($F[2,147] = 12.21$, $P < 0.0001$) and sleep latency ($F[2,147] = 26.53$, $P < 0.0001$). (B) Co-administration of GABA-T inhibitor (EOS) or GABA transporter inhibitor (NipA) with threonine blocked SPET. Where indicated, 10 mM of EOS or NipA was added to behavior food to pharmacologically increase GABA levels. Data represent average +/- SEM (n=22–37). Two-way ANOVA detected significant interactions of threonine supplementation with EOS (sleep amount, $F[2,155] = 14.07$, $P < 0.0001$; sleep latency, $F[2.155] = 11.2$, $P < 0.0001$) or with NipA (sleep amount, $F[2,162] = 13.09$, $P < 0.0001$; sleep latency, $F[2.162] = 26.58$, $P < 0.0001$)). n.s., not significant; **$P < 0.01$, ***$P < 0.001$ to controls (black bars) as determined by Tukey's multiple comparisons test.

3.3 SPET Implicates GABAergic Excitation and Transmission.

3.3.1 Threonine diet specifically excites a subset of GABAergic neurons.

To determine if the threonine diet activates GABAergic neurons, we utilized a transcriptional reporter of intracellular Ca2+ levels as a quantitative proxy for neural activity. The transgenic Ca2+ indicator employs calcium-dependent nuclear import of the transcriptional activator LexA (CaLexA) to drive the expression of green fluorescent protein (GFP) (25). Accordingly, we reasoned that this strategy is ideal for monitoring long-term changes in neural activity on a threonine diet because threonine supplementation exhibited cumulative effects on baseline sleep in LD cycles (data not shown). Confocal microscopy of adult fly brains revealed the strongest GFP expression by CaLexA in neurons projecting into antennal lobes (AL), medial antenno-cerebral tract (mACT), and lateral horn (LH) (Fig. 7), probably reflecting differences in baseline Ca2+ levels among glutamate decarboxylase 1 (GAD1)-expressing GABAergic neurons. More importantly, threonine, but not arginine, supplementation induced CaLexA signals in a subgroup of GABAergic neurons lateral to antennal lobes (LN) (Fig. 8), indicating their specific excitation by the threonine diet.

Fig. 7. Transcriptional Ca^{2+} reporter showed baseline Ca^{2+} level dependent expression pattern which is distinct from nuclear GFP expression. Confocal imaging of nuclear GFP (top, green) or a transcriptional reporter for intracellular Ca2+ (CaLexA-induced GFP) (bottom, green) expressed in GABAergic neurons by a transgenic GAD1-Gal4 driver. Adult fly brains were immuno-stained with mouse anti-GFP and rabbit anti-GABA antibodies (magenta). AL, antennal lobe; D, dorsal; LH, lateral horn; LN, lateral interneurons; mACT, medial antenno-cerebral tract; V, ventral; VN, ventral interneurons.

A GAD1>CaLexA GABA

laternal neurons (LN) ventral neurons (VN)

control

25 mM Thr

D V

V D

B

GFP-positive LN

GFP-positive VN

***

n.s.

25 mM Thr +

25 mM Arg +

Fig. 8. Threonine diet excites a specific subset of GABAergic neurons. (A) Representative images of LN and VN in control- or threonine-fed flies. Arrow heads indicate threonine-induced expression of GFP by CaLexA in LN. (B) The number of LN or VN expressing CaLexA-induced GFP was quantified in each hemisphere from control-, threonine-, or arginine-fed flies. Data represent average +/- SEM (n=14–32). n.s., not significant; **P < 0.01 as determined by Student's t-test.

3.3.2 GABAergic transmission via a metabotropic GABA receptor contributes to SPET.

To further validate the involvement of GABAergic transmission in SPET, we expressed a shibire^{ts1} transgene (26) in GAD1-expressing GABAergic neurons. The shibire^{ts1} is a temperature-sensitive mutant allele in a *Drosophila* homolog of dynamin that interferes with synaptic vesicle recycling and thus, blocks synaptic transmission at restrictive (29°C) but not permissive (21°C) temperatures. We indeed observed that the synaptic blockade of GABAergic neurons at 29˚C significantly suppressed SPET (Fig. 9), indicating that SPET requires pre-synaptic output from GABAergic transmission. Moreover, RNA interference (RNAi)-mediated depletion of a metabotropic GABA receptor R1 in neurons, but not in glial cells, desensitized SPET (Fig. 10). Taken together, these data suggest that SPET implicates the excitation of GABAergic neurons and their neural transmission via a specific subtype of GABA receptors.

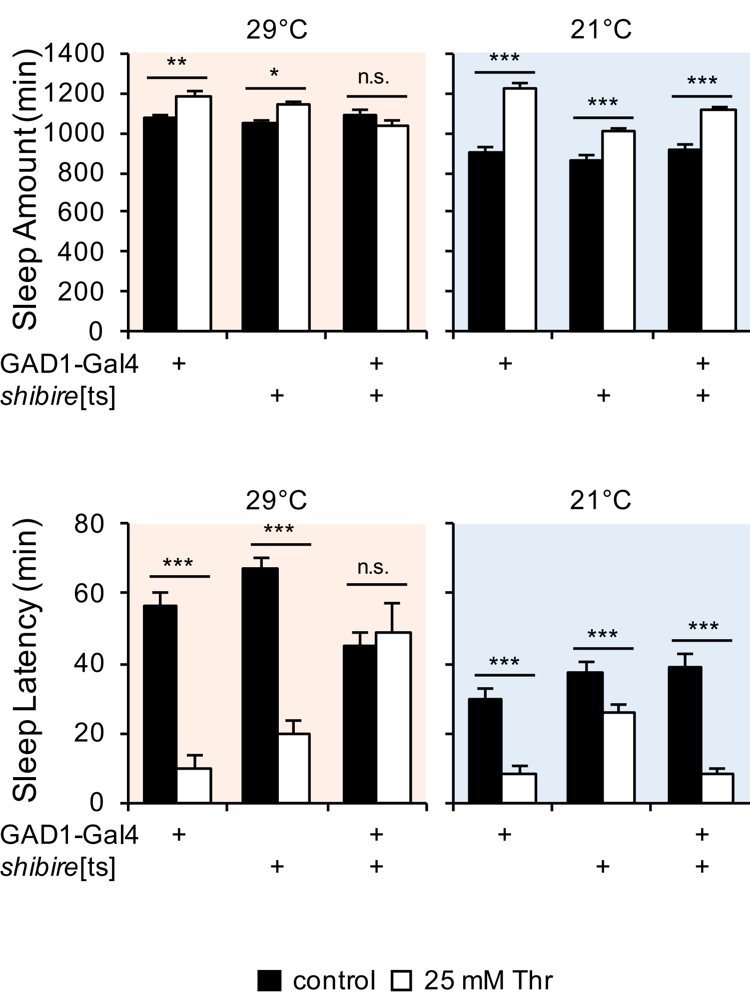


Fig. 9. A thermogenetic blockade of GABAergic transmission masked SPET. Transgenic flies were entrained in LD cycles at restrictive (29ºC) or permissive (21ºC) temperature. Sleep behaviors in individual flies were analyzed similarly to the data presented in Fig. 1. Data represent average +/- SEM (n=20–32 for 29ºC; n=29–41 for 21ºC). Two-way ANOVA detected significant interactions between genotypes and threonine supplementation on sleep amount ($F[2,163] = 9.811$ $P < 0.0001$) and sleep latency ($F[2,163] = 18.08$, $P < 0.0001$) at 29ºC. n.s., not significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as determined by Sidak's multiple comparisons test.

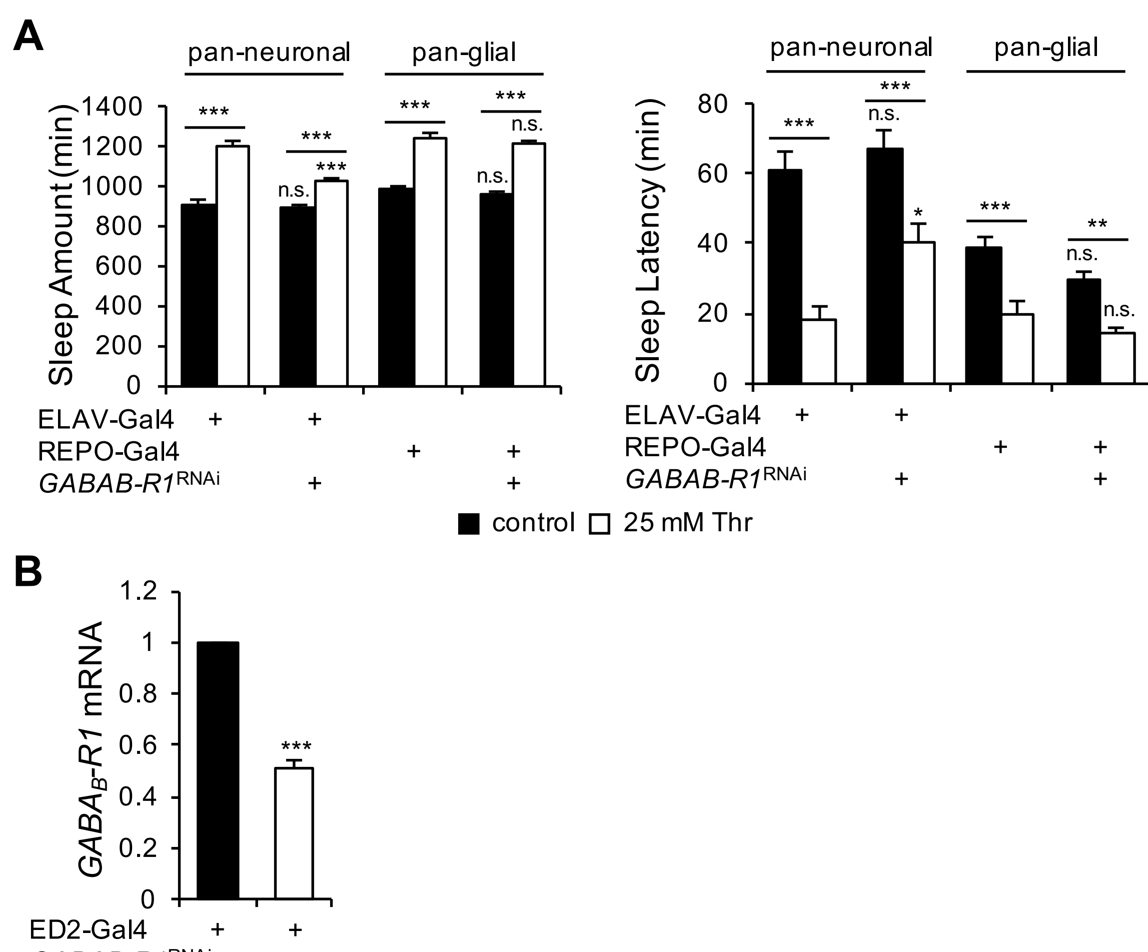
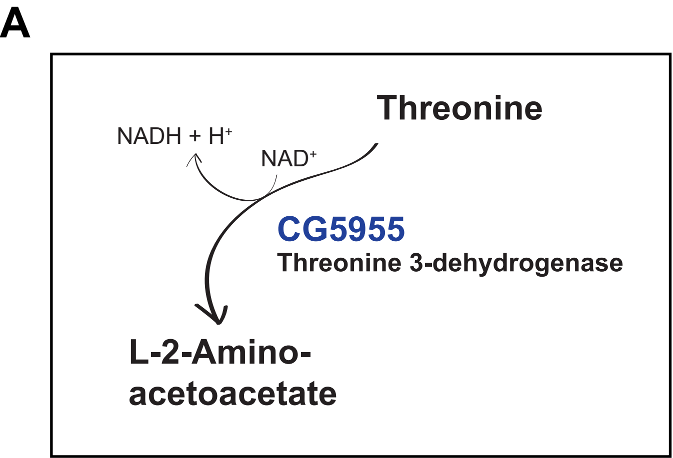


Fig. 10. Pan-neuronal knock down of metabotropic receptor partially blocks SPET. (A) Pan-neuronal (ELAV-Gal4) but not pan-glial (REPO-Gal4) deletion of a metabotropic GABA receptor (GABAB-R1) by transgenic RNA interference (RNAi) significantly suppressed SPET. Sleep behaviors in individual flies were analyzed similarly to the data presented in Fig. 1 except that sleep analyses on day 3 were shown. Data represent average +/- SEM (n=26–41). Two-way ANOVA detected significant interactions between genotype (pan-neuronal RNAi) and threonine supplementation (sleep amount, $F[1,131] = 13.37$, $P = 0.0004$; sleep latency, $F[1,131] = 2.381$, $P = 0.1252$). No significant interaction was observed between pan-glial RNAi and threonine supplementation. n.s., not significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ to Gal4 heterozygous controls as determined by Tukey's multiple comparisons test. (B) Validation of transgenic RNAi for GABAB-R1. The RNAi transgene for GABAB-R1 was overexpressed by pan-neuronal (ED2) Gal4 driver. Total RNA was extracted from 50 fly heads, treated with DNase I, and reverse-transcribed to synthesize cDNA samples. Relative mRNA levels of GABAB-R1 or polyA-binding protein (normalizing control) were measured by quantitative realtime PCR using gene-specific primers. Relative expression was then calculated by normalizing to the relative mRNA level in Gal4 heterozygous controls (black bars, set as 1). Data represent average +/- SEM (n=3). ***$P < 0.001$ as determined by Student's t-test.

3.4 Genetic Increase in Threonine Levels Facilitates Sleep Onset.

Because SPET was evident with dietary threonine at relatively high concentrations, we next determined if elevation in physiological threonine levels could act as an endogenous promoter of sleep. We reasoned that genetic mutations in threonine-metabolizing enzymes might cause an increase in steady-state levels of endogenous threonine. *CG5955* is a fly homolog of *threonine 3-dehydrogenase* that converts threonine and NAD+ into L-2-amino-acetoacetate, NADH, and H+ (Fig. 11). We found a transposable P-element insertion in the proximal promoter region of the *CG5955* locus that decreased relative levels of *CG5955* mRNA (Fig. 11). *CG5955* mutants trans-heterozygous for the hypomorphic allele over the chromosomal deficiency indeed displayed a higher ratio of threonine to protein levels than heterozygous controls (Fig. 12). Although mutations in *CG5955* did not potently affect daily sleep amounts, sleep latency was significantly shortened (Fig. 12). Pan-neuronal depletion of *CG5955* expression was sufficient for shortening sleep latency (Fig. 13), mimicking the sleep phenotype in *CG5955* trans-heterozygous mutants. Taken together, these data demonstrate that genetic elevation in endogenous levels of threonine, particularly in the neurons, is sufficient to drive sleep, further supporting threonine as a sleep enhancer.



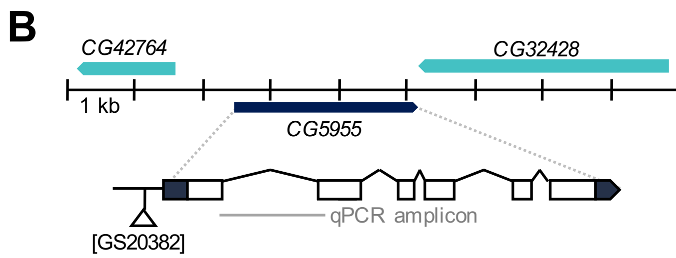


Fig. 11. Schematics of threonine catabolizing enzyme pathway and its genetic locus. (A) A threonine metabolic pathway catalyzed by *threonine 3-dehydrogenase* (*CG5955*). (B) An insertional mutant allele of P element ([GS20382]) in the *CG5955* locus. An amplicon used in quantitative PCR is depicted by a grey line.

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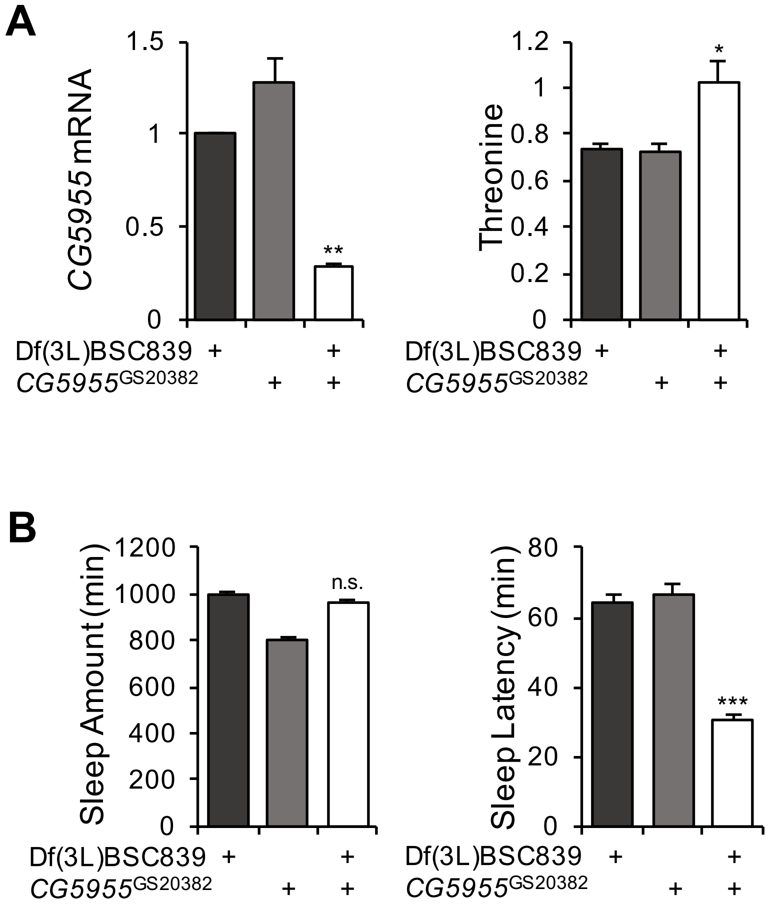
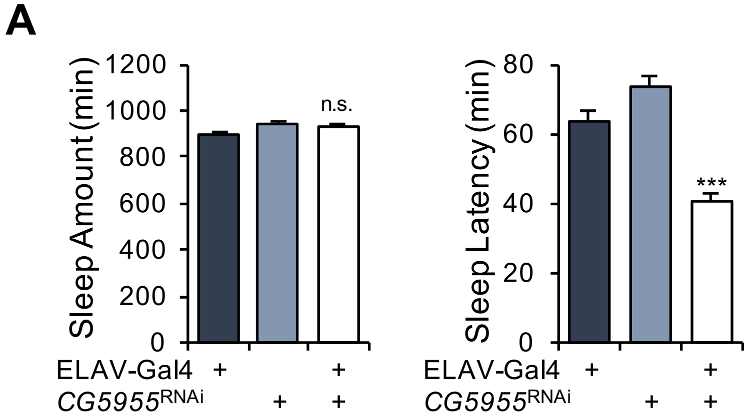


Fig. 12. Mutations in *threonine 3-dehydrogenase* elevate endogenous threonine levels and shorten sleep latency. (A) Trans-heterozygous mutations in *CG5955* decreased relative levels of *CG5955* mRNA (left, normalized to polyA-binding protein mRNA levels) but elevated those of endogenous threonine (right, normalized to protein levels). Data represent average +/- SEM (n=3). (B) Trans-heterozygous mutations in *CG5955* shortened sleep latency. Total sleep amount and latency to sleep onset after lights-off were calculated from individual flies in LD cycles at 25ºC and averaged per each genotype. Error bars indicate SEM (n=122–151). n.s., not significant; *P < 0.05, **P < 0.01, ***P < 0.001 to heterozygous controls as determined by one-way ANOVA, Tukey's multiple comparisons test.



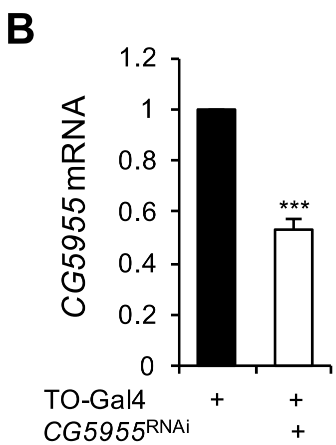


Fig. 13. Pan-neuronal depletion of *CG5955* by RNAi shortened sleep latency. (A) Total sleep amount and latency to sleep onset after lights-off were calculated from individual flies in LD cycles at 25ºC and averaged per each genotype. Error bars indicate SEM (n=122–151). n.s., not significant; *P < 0.05, **P < 0.01, ***P < 0.001 to heterozygous controls as determined by one-way ANOVA, Tukey's multiple comparisons test. (B) Validation of transgenic RNAi for *CG5955*. The RNAi transgene for *CG5955* was overexpressed by fat body-specific (TO) Gal4 driver. Total RNA was extracted from 10 whole flies, treated with DNase I, and reverse-transcribed to synthesize cDNA samples. Relative mRNA levels of *CG5955* or polyA-binding protein (normalizing control) were measured by quantitative realtime PCR using gene-specific primers. Relative expression was then calculated by normalizing to the relative mRNA level in Gal4 heterozygous controls (black bars, set as 1). Data represent average +/- SEM (n=3). ***P < 0.001 as determined by Student's t-test.

IV. DISCUSSION

4.1 Sleep plasticity by threonine diet does not share previously reported sleep latency regulating pathway.

Studies on sleep homeostasis have been establishing molecular and cellular machinery that sustains baseline sleep as well as compensates for sleep deficits. On the other hand, sleep plasticity could be considered as a process of how the sleep machinery interacts intimately with external sleep-regulatory factors (e.g., light, temperature, or starvation) to re-shape the architecture of animal sleep accordingly. Using a *Drosophila* genetic model, we investigated plasticity in sleep behaviors induced by a threonine diet and elucidated an underlying neural mechanism of SPET. Wake-promoting circadian pacemaker neurons are important for sleep latency at the transition from light to dark (15). In addition, expression of WAKE in clock neurons and its association with RDL is a key mechanism in the circadian control of sleep onset (12-15). However, our evidence indicates that dietary threonine facilitates sleep onset in a light- or circadian-clock-independent manner. Moreover, we found that SPET operates via a specific GABAergic pathway implicating a metabotropic GABA receptor, thereby defining a novel pathway for control of sleep latency.

4.2 Threonine-induced sleep regulation is distinct from previously reported sleep plasticity relevant to food intake.

Sleep is a complex yet sensitive physiology which is highly vulnerable to both environmental and genetic conditions. The sleepiness after a meal (postprandial sleep) gates a relationship between food intake represented by metabolism and sleep. Previous studies demonstrated that the availability and quality of nutrients affects sleep behaviors in *Drosophila*. Sucrose contents in food and their gustatory perception dominate over dietary protein to affect daily sleep (27-29). Starvation promotes arousal in a manner dependent on the circadian clock genes Clock and cycle (30) as well as neuropeptide F (NPF), a fly ortholog of mammalian neuropeptide Y(31). In addition, starvation-induced suppression of daily sleep requires a subset of circadian pacemaker neurons that expresses NPF. We observed, however, that NPF-expressing neurons are dispensable for SPET (Fig. 14). Other studies indicate that protein may be one of the nutrients contributing to postprandial sleep drive in *Drosophila*, suggesting its relevance to SPET (32, 33). Although leucokinin (Lk) and Lk receptor (Lkr) play an important role in postprandial sleep and starvation-induced arousal (34), we found that SPET was comparable between control and their hypomorphic mutant flies (Fig. 15). Therefore, SPET and its neural basis reveal a sleep-regulatory mechanism distinct from those involved in sleep plasticity relevant to food intake.

4.3 Structural similarities of threonine derivative AKB and GABAergic derivatives.

What is the molecular basis of SPET? One hypothesis is that a molecular sensor specifically expressed in a subset of GABAergic neurons responds to an increase in threonine levels, activates GABAergic transmission, and shortens latency to sleep onset. Alternatively, but not exclusively, structural similarities among threonine, GABA, and their metabolic derivatives (e.g., alpha-ketobutyrate, a derivative of threonine metabolism; gamma-hydroxybutyrate, a direct derivative of GABA) may explain the implication of GABAergic transmission in SPET (Fig.16).

4.3.1 Beta-hydroxybutyric acid upregulates GABAergic signals.

GABAergic signaling has been implemented in treating various neuronal diseases such as sleep, mood change and epilepsy by targeting GABA receptors. The efficacy of ketogenic diet on medically refractory epilepsy has also been discussed through GABAergic aspect. Though the involvement of GABAergic is not clear, ketogenic diet could succeed to improve sleep quality as well (*42*). Notably, beta-hydroxybutyrate (BHB), one of the ketone bodies having anti-convulsive effects, has been reported to be extensively related with GABAergic signaling. Early studies have shown that the GABA was increased upon BHB application possibly via facilitating transamination of aspartate to yield glutamate which then metabolized into GABA by the action of glutamate decarboxylase (Gad) (*43, 44*). After a decade from then two other possibilities for GABAergic regulation of BHB has been arose. In 2009, Yuka and his colleagues showed BHB-dependent inhibition of GABAT activity in cultured astrocytes (*45*). Following research demonstrated rather direct involvement of BHB in GABA synthesis where BHB became a substrate for Gad1 and turns into GABA (*46*).

4.3.2 Binding affinities of gamma-hydroxybutyrate and beta-hydroxybutyrate on GABA receptors.

Gamma-hydroxybutyrate(GHB) is a substance occurred from deamination of GABA. In 1964, Laborit, in his review paper, classified the effects of GHB into hypnotic, hypothermic, anticonvulsant and anesthetic (*47*). The mechanism of GHB effects is not clear but it can bind to GABAB receptor and seemed to require GABAB receptor to exert its effects (*48, 49*). Only a single study provided by Nathan et al., in 2012, showed high affinity of GHB about α4βδ GABAA receptor subunit so far (*50*), nevertheless, the relationship between GHB and GABA receptors is quite evident. Thus, there is no such an evidence of GABA receptor binding affinity for BHB yet, it nevertheless could be an another possible scenario for GABAergic regulation of BHB. In fact, early electrophyolosical study, which have based on the structural similarities between BHB and GABA, showed BHB could induce stimulus-evoked IPSPs on hippocampal tissue slice (*51*), though it is somewhat controversial for now.

4.3.3 Alpha-ketobutyric acid is a threonine derivative which has high structural similarity with beta-hydroxybutyrate

Threonine is a ketogenic amino acid where catabolism of the amino acid consists of three independent pathways result in two ketone bodies (L-2-amino-acetoacetate, 2-ketobutyrate) and

glycine. Intriguingly, in 1987, Ronald and Eric evidently showed that, in the NADH-linked enzymatic assay, high concentration of 2-ketobutyrate(AKB) interferes the enzymatic reaction between BHB and BHB dehydrogenase probably due to the structural similarities between AKB and BHB (*52*). To our surprise, as like other molecules (GABA, BHB, GHB) having anti-convulsiveness several studies have reported the anti-convulsive effects of L-threonine also (*53-55*) which thereby, suggests the relationship between threonine and GABA possibly mediated via AKB.

Given that dietary GABA could results in increase of threonine (*56*), the GABAergic link shown here could rather be cyclic effects among molecules, nonetheless the intimate relationship between four butyric acid-derivatives is discernable.

4.4 Threonine could systemically change redox status which alters properties of GABA receptors

Just like ketogenic diet alters the redox status which is represented by [NAD^+]/[NADH] ratio, a ketogenic amino acid, threonine, could alter the redox status. Both type of GABA receptors(ionotropic/metabotropic) are structurally diverse since they are constituted from various receptor subunits. The structural diversity might result in functional plasticity. In other word, the context dependent regulation could be the key to GABAergic physiology. Among others, there is an evidence that the $GABA_A$ receptor is modulated by redox reagent (*57, 58*). During the wakefulness, brain consumes energy in every single second for every single activity which is followed by gradual dropping in NAD/NADH ratio (*59*). It's instinctive to think that animals have evolved to have sleep arranging mechanism corresponding to the ratio to buffer NADH attenuation due to awake status. There is not much known about NAD/NADH in sleep regulation. Nonetheless, a research of NAD/NADH dependent regulation of core clock component, CLOCK/BMAL1, supports the idea (*60*). In this sense, the redox state change could help animals to become asleep state possibly by embracing the GABAergic signaling. Hence, even if 2-ketobutyrate can binds to GABA receptors, it might require the redox power supplied via metabolism of threonine to be physiologically functional.

4.5 High permeability of threonine via blood brain barrier among amino acids.

It's noteworthy that the brain proportion of threonine is only required for the SPET [Fig.10 and Fig.13]. Previous study discussed about the poor permeability of glycine and serine crossing blood brain barrier (BBB) but threonine seemed doing much better than them. BBB is a highly selective membrane constituted of epithelial cells to prevent any unpleasant visitors from blood stream and is also conserved in insects(*61*). Water, gas and small lipid-soluble molecule could enter the brain by passive diffusion otherwise active transport is needed. It seems like glycine and serine are too much soluble to cross the BBB while water solubility of glutamate is too low to travel through blood reaching brain [Fig. 19]. The solubility and the size of threonine together could make threonine to

cross the BBB well. Alternatively, peripherally metabolized threonine signal results in the BBB to be more permeable (i.e. inflammatory signaling) so that entrance of hydrophilic molecule being generous. Or, it would be about the transporters residing BBB which requires further investigation.

4.6 Existence of threonine deaminase remarks importance of threonine metabolism

Amino acid metabolism involves the transfer of the amino group between amino acids and alpha-keto acids by various transaminases and thus, relies on the availability of the amino acid pool. On the other hand, a group of amino acids, including glutamate, glycine, serine, and threonine, have specific deaminases that selectively remove their amino group (*62*). The presence of specific deaminases is indicative of active mechanisms for fine control in metabolism and possibly in other physiological processes. This idea is further supported by the fact that glutamate, glycine, and serine are neuromodulators important for brain function, including sleep regulation. Because serine, glycine, and threonine together constitute a metabolic pathway (Fig.17), SPET may be indirectly due to glycine- or serine-dependent activation of sleep-promoting NMDAR (*37, 38*). However, sleep-modulatory effects of dietary glycine and threonine were distinct from each other. In addition, neuronal depletion of NMDAR did not substantially compromise SPET (Fig. 18). Accordingly, we speculate that threonine may act as a neuromodulator, similar to other amino acids with specific deaminases.

4.7 Ending remarks

Given our genetic evidence that threonine probably acts as an endogenous sleep enhancer and that serine, in contrast, has wake-promoting functions, particularly in starvation conditions (Sonn et al., manuscript submitted), we define the essential metabolic pathway of serine-glycine-threonine as a key module for sleep regulation involving metabolic sleep cues. Future studies should address if specific sleep needs affect the threonine metabolic pathway as a homeostatic mechanism of sleep drive. In addition, it will be interesting to determine if SPET is conserved among other animals, including humans.

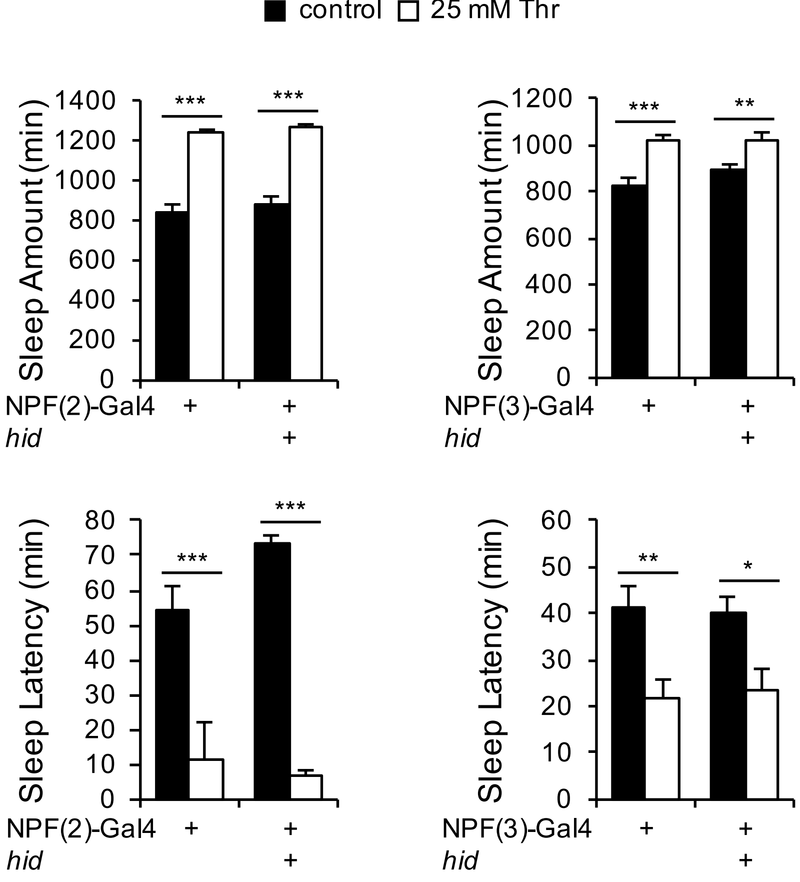


Fig. 14. Genetic ablation of NPF-expressing neurons does not compromise SPET. Head involution defective (hid), a pro-apoptotic gene, was overexpressed in NPF-expressing neurons by two independent Gal4 drivers. Sleep behaviors in individual transgenic flies were analyzed similarly to the data presented in Fig. 1. Data represent average +/- SEM (n=19–24). Two-way ANOVA detected no significant interaction of threonine supplementation with NPF(2) ablation (sleep amount, $F[1,83] = 0.09440$, $P = 0.7594$; sleep latency, $F[1,83] = 3.045$, $P = 0.0847$) or with NPF(3) ablation (sleep amount, $F[1,86] = 1.482$, $P = 0.2268$; sleep latency, $F[1,86] = 0.06404$, $P = 0.8008$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as determined by Sidak's multiple comparisons test.

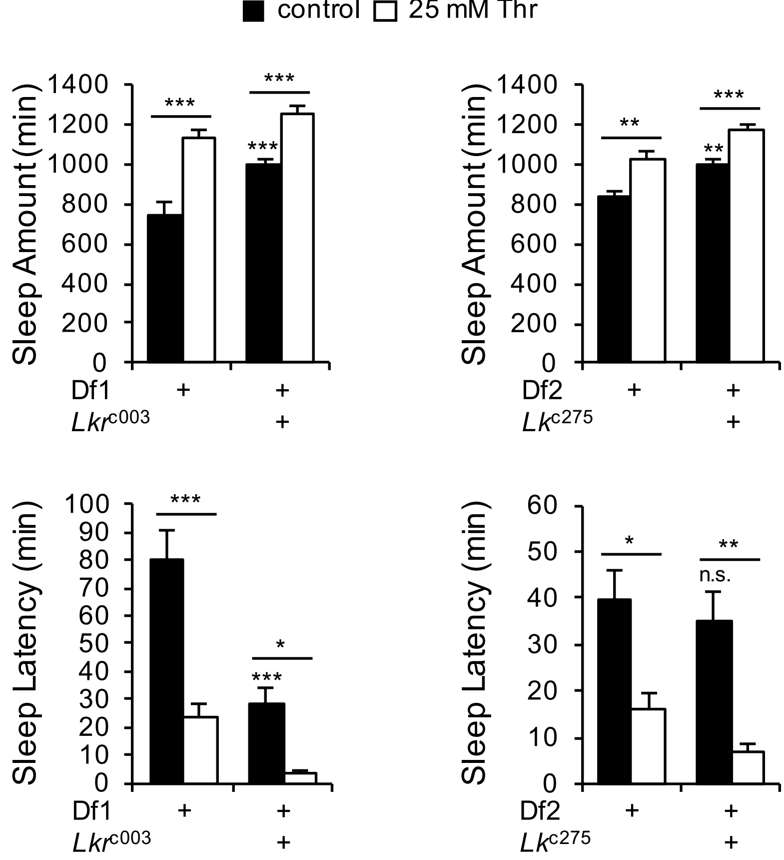


Fig. 15. Trans-heterozygous mutations in Lk or Lkr gene do not abolish SPET. Sleep behaviors in individual flies were analyzed similarly to the data presented in Fig. 1. Data represent average +/- SEM (n=14–31). Two-way ANOVA detected no significant interaction between Lk mutation and threonine supplementation (sleep amount, $F[1,82] = 0.03376$, $P = 0.8547$; sleep latency, $F[1,82] = 0.1483$, $P = 0.7012$). Significant interaction of Lkr mutation with threonine supplementation on sleep latency ($F[1,63] = 6.821$, $P = 0.0112$) but not on sleep amount ($F[1,63] = 2.043$, $P = 0.1578$) is probably due to a floor effect. n.s., not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ to heterozygous controls fed on the same food as determined by Sidak's multiple comparisons test.

3D model (JSmol)	Name / Formula	Description
	Butyric acid C_3H_7COOH	• Backbone
	α-ketobutyric acid $C_4H_6O_3$	• A threonine derivative • Interferes with β-hydroxybutyrate dehydrogenase
	β-hydroxybutyric acid $C_4H_8O_3$	• Ketone body • A preferred substrate for GABA synthesis • Inhibits GABA-T activity • Increases GABAergic signaling
	γ-hydroxybutyric acid $C_4H_8O_3$	• A precursor of GABA, glutamate, and glycine • Acts on γ-hydroxybutyric acid receptor • A weak agonist of GABAB receptor • Targets α4βδ GABAA receptor at high affinity
	γ-aminobutyric acid $C_4H_9NO_2$	• A major inhibitory neurotransmitter (GABA) • Sleep-promoting effects • Anti-convulsive effects

Fig. 16. Structural and functional relevance of a-ketobutyric acid, a threonine derivative, to GABA and GABA derivatives. Space filling model of each chemical was adopted from Wikipedia (https://en.wikipedia.org/)

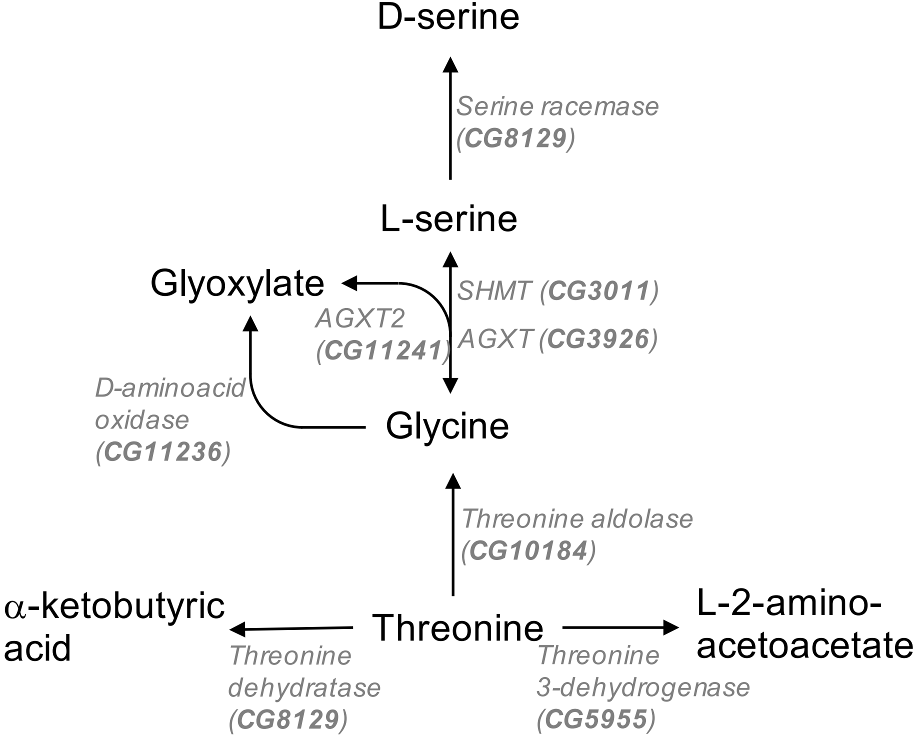


Fig. 17. A metabolic pathway of serine, glycine, and threonine. A schematic diagram of enzymes (*Drosophila* homologs) and biochemical reactions in serine, glycine, and threonine metabolism was modified from KEGG pathway database (http://www.genome.jp/kegg/pathway.html). SHMT, serine hydroxymethyltransferase; AXGT, alanine:glyoxylate aminotransferase.

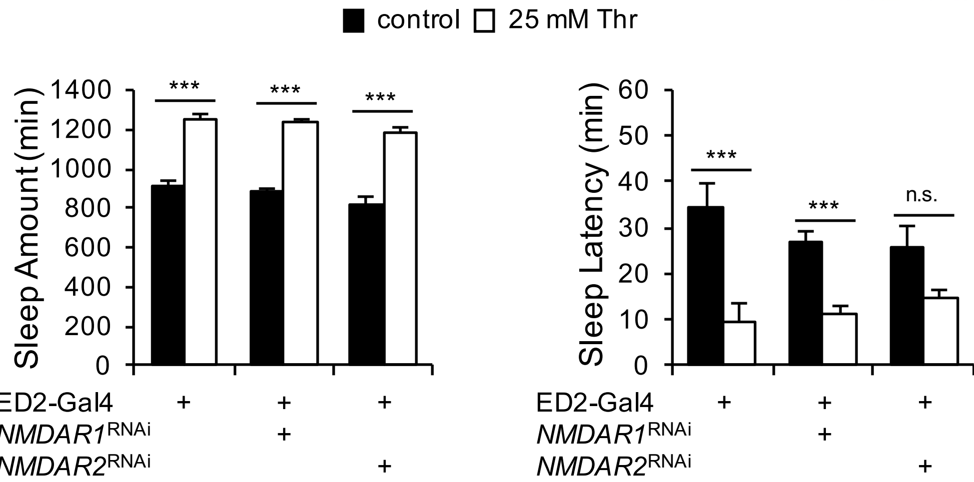


Fig. 18. Pan-neuronal depletion of Nmdar1 or Nmdar2 does not mask SPET. Sleep behaviors in individual flies were analyzed similarly to the data presented in Fig. 1. Data represent average +/- SEM (n=7–26). Two-way ANOVA detected no significant interaction of threonine supplementation with Nmdar1 depletion (sleep amount, $F[1,77] = 0.007919$, $P = 0.9293$; sleep latency, $F[1,77] = 2.369$, $P = 0.1279$) or with Nmdar2 depletion (sleep amount, $F[1,44] = 0.1550$, $P = 0.6957$; sleep latency, $F[1,44] = 1.966$, $P = 0.1679$). n.s., not significant; ***$P < 0.001$ as determined by Sidak's multiple comparisons test.

Name	MW (g/mol)	Solubility in water (mg/L) at 25℃	Nature	Reference
Alanine	89.094	164000	aliphathic	YALKOWSKY,SH & DANNENFELSER,RM (1992)
Arginine	174.204	182000	basic	YALKOWSKY,SH & DANNENFELSER,RM (1992)
Asparagine	132.119	29400	polar, neutral	YALKOWSKY,SH & DANNENFELSER,RM (1992)
Aspartate	133.103	5390	acidic	YALKOWSKY,SH & DANNENFELSER,RM (1992)
Cysteine	121.154	277000	polar, neutral	BEILSTEIN
Glutamate	146.122	**8570**	acidic	Yalkowsky, S.H., He, Yan, Jain, P. Handbook of Aqueous Solubility Data Second Edition. CRC Press, Boca Raton, FL 2010, p. 157
Glutamine	146.146	41300	polar, neutral	YALKOWSKY,SH & DANNENFELSER,RM (1992)
Glycine	75.067	**249000**	unique	YALKOWSKY,SH & DANNENFELSER,RM (1992)
Histidine	155.157	45600	basic	YALKOWSKY,SH & DANNENFELSER,RM (1992)
isoleucine	131.175	34400	aliphathic	YALKOWSKY,SH & DANNENFELSER,RM (1992)
Leucine	131.175	21500	aliphathic	YALKOWSKY,SH & DANNENFELSER,RM (1992)
Lysine	146.19	>1000000	basic	Gerhartz, W. (exec ed.). Ullmann's Encyclopedia of Industrial Chemistry. 5th ed.Vol A1: Deerfield Beach, FL: VCH Publishers, 1985 to Present., p. VA2 63
Methionine	149.208	56600	aliphathic	YALKOWSKY,SH & DANNENFELSER,RM (1992)
Phenylalanine	165.192	26900	aromatic	YALKOWSKY,SH & DANNENFELSER,RM (1992)
Proline	115.132	162000	unique	O'Neil, M.J. (ed.). The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. Cambridge, UK: Royal Society of Chemistry, 2013., p. 1393
Serine	105.093	**425000**	polar, neutral	YALKOWSKY,SH & DANNENFELSER,RM (1992)
Threonine	119.12	**97000**	polar, neutral	YALKOWSKY,SH & DANNENFELSER,RM (1992)
Tryptophan	204.229	13400	aromatic	YALKOWSKY,SH & DANNENFELSER,RM (1992)
Tyrosine	181.191	453	aromatic	CRC HANDBOOK
Valine	117.148	58500	aliphathic	YALKOWSKY,SH & DANNENFELSER,RM (1992)
GABA	103.12	**1300000**	polar, neutral	Yalkowsky, S.H., He, Yan, Jain, P. Handbook of Aqueous Solubility Data Second Edition. CRC Press, Boca Raton, FL 2010, p. 157

Fig. 19. Table of general properties of amino acids

REFERENCES

1. J. J. Pilcher, A. I. Huffcutt, Effects of sleep deprivation on performance: a meta-analysis. *Sleep* **19**, 318-326 (1996).
2. H. P. Van Dongen, G. Maislin, J. M. Mullington, D. F. Dinges, The cumulative cost of additional wakefulness: dose-response effects on neurobehavioral functions and sleep physiology from chronic sleep restriction and total sleep deprivation. *Sleep* **26**, 117-126 (2003).
3. W. D. Killgore, T. J. Balkin, N. J. Wesensten, Impaired decision making following 49 h of sleep deprivation. *J Sleep Res* **15**, 7-13 (2006).
4. R. Stickgold, Sleep-dependent memory consolidation. *Nature* **437**, 1272-1278 (2005).
5. S. P. Drummond *et al.*, Altered brain response to verbal learning following sleep deprivation. *Nature* **403**, 655-657 (2000).
6. J. P. Nilsson *et al.*, Less effective executive functioning after one night's sleep deprivation. *J Sleep Res* **14**, 1-6 (2005).
7. M. Irwin *et al.*, Partial night sleep deprivation reduces natural killer and cellular immune responses in humans. *FASEB J* **10**, 643-653 (1996).
8. A. M. Weljie *et al.*, Oxalic acid and diacylglycerol 36:3 are cross-species markers of sleep debt. *Proc Natl Acad Sci U S A* **112**, 2569-2574 (2015).
9. A. Rechtschaffen, B. M. Bergmann, Sleep deprivation in the rat by the disk-over-water method. *Behav Brain Res* **69**, 55-63 (1995).
10. A. A. Borbely, A two process model of sleep regulation. *Hum Neurobiol* **1**, 195-204 (1982).
11. R. Allada, J. M. Siegel, Unearthing the phylogenetic roots of sleep. *Curr Biol* **18**, R670-R679 (2008).
12. W. J. Joiner, Unraveling the Evolutionary Determinants of Sleep. *Curr Biol* **26**, R1073-R1087 (2016).
13. R. Allada, C. Cirelli, A. Sehgal, Molecular Mechanisms of Sleep Homeostasis in Flies and Mammals. *Cold Spring Harb Perspect Biol* **9**, (2017).
14. G. Artiushin, A. Sehgal, The Drosophila circuitry of sleep-wake regulation. *Curr Opin Neurobiol* **44**, 243-250 (2017).
15. J. Tomita, G. Ban, K. Kume, Genes and neural circuits for sleep of the fruit fly. *Neurosci Res* **118**, 82-91 (2017).
16. V. T. Karadzic, [Effect of increased levels of gamma-aminobutyric acid in the central nervous system on the sleep phases in cats]. *Acta Med Iugosl* **20**, 282-290 (1966).
17. S. W. Holmes, and D. Sugden., The effects of GABAtransaminase (GABA-T) inhibition on sleep and behavior of the cat. *Sleep Res* **4**, (1975).
18. E. Schneider, B. Ziegler, H. Maxion, Gamma-aminobutyric acid (GABA) and sleep. The influence of di-n-propylacetic acid on sleep in man. *Eur Neurol* **15**, 146-152 (1977).
19. A. Fink-Jensen *et al.*, The gamma-aminobutyric acid (GABA) uptake inhibitor, tiagabine,

increases extracellular brain levels of GABA in awake rats. *Eur J Pharmacol* **220**, 197-201 (1992).

20. M. Lancel, J. Faulhaber, R. A. Deisz, Effect of the GABA uptake inhibitor tiagabine on sleep and EEG power spectra in the rat. *Br J Pharmacol* **123**, 1471-1477 (1998).
21. M. Lancel, T. A. Cronlein, J. Faulhaber, Role of GABAA receptors in sleep regulation. Differential effects of muscimol and midazolam on sleep in rats. *Neuropsychopharmacology* **15**, 63-74 (1996).
22. M. Lancel, J. Faulhaber, T. Schiffelholz, S. Mathias, R. A. Deisz, Muscimol and midazolam do not potentiate each other's effects on sleep EEG in the rat. *J Neurophysiol* **77**, 1624-1629 (1997).
23. J. Faulhaber, A. Steiger, M. Lancel, The GABAA agonist THIP produces slow wave sleep and reduces spindling activity in NREM sleep in humans. *Psychopharmacology (Berl)* **130**, 285-291 (1997).
24. M. Lancel, The GABA(A) agonist THIP increases non-REM sleep and enhances non-REM sleep-specific delta activity in the rat during the dark period. *Sleep* **20**, 1099-1104 (1997).
25. T. Gallopin *et al.*, Identification of sleep-promoting neurons in vitro. *Nature* **404**, 992-995 (2000).
26. J. E. Zimmerman, N. Naidoo, D. M. Raizen, A. I. Pack, Conservation of sleep: insights from non-mammalian model systems. *Trends Neurosci* **31**, 371-376 (2008).
27. W. F. Chen *et al.*, A neuron-glia interaction involving GABA transaminase contributes to sleep loss in sleepless mutants. *Mol Psychiatry* **20**, 240-251 (2015).
28. J. Agosto *et al.*, Modulation of GABAA receptor desensitization uncouples sleep onset and maintenance in Drosophila. *Nat Neurosci* **11**, 354-359 (2008).
29. B. Y. Chung, V. L. Kilman, J. R. Keath, J. L. Pitman, R. Allada, The GABA(A) receptor RDL acts in peptidergic PDF neurons to promote sleep in Drosophila. *Curr Biol* **19**, 386-390 (2009).
30. K. M. Parisky *et al.*, PDF cells are a GABA-responsive wake-promoting component of the Drosophila sleep circuit. *Neuron* **60**, 672-682 (2008).
31. S. Liu *et al.*, WIDE AWAKE mediates the circadian timing of sleep onset. *Neuron* **82**, 151-166 (2014).
32. S. Dissel *et al.*, Sleep restores behavioral plasticity to Drosophila mutants. *Curr Biol* **25**, 1270-1281 (2015).
33. F. Gmeiner *et al.*, GABA(B) receptors play an essential role in maintaining sleep during the second half of the night in Drosophila melanogaster. *J Exp Biol* **216**, 3837-3843 (2013).
34. S. V. Jain, T. A. Glauser, Effects of epilepsy treatments on sleep architecture and daytime sleepiness: an evidence-based review of objective sleep metrics. *Epilepsia* **55**, 26-37 (2014).
35. M. Bannai, N. Kawai, New therapeutic strategy for amino acid medicine: glycine improves the quality of sleep. *J Pharmacol Sci* **118**, 145-148 (2012).
36. K. I. Wataru YAMADERA, Shintaro CHIBA, Makoto BANNAI, Michio TAKAHASHI and Kazuhiko NAKAYAMA, Glycine ingestion improves subjective sleep quality in human

volunteers, correlating with polysomnographic changes. *Sleep and Biological Rhythms* **5**, 126-131 (2007).

37. N. Kawai *et al.*, The sleep-promoting and hypothermic effects of glycine are mediated by NMDA receptors in the suprachiasmatic nucleus. *Neuropsychopharmacology* **40**, 1405-1416 (2015).
38. J. Tomita, T. Ueno, M. Mitsuyoshi, S. Kume, K. Kume, The NMDA Receptor Promotes Sleep in the Fruit Fly, Drosophila melanogaster. *PLoS One* **10**, e0128101 (2015).
39. C. Cirelli *et al.*, Reduced sleep in Drosophila Shaker mutants. *Nature* **434**, 1087-1092 (2005).
40. J. C. Hendricks *et al.*, Rest in Drosophila is a sleep-like state. *Neuron* **25**, 129-138 (2000).
41. P. J. Shaw, C. Cirelli, R. J. Greenspan, G. Tononi, Correlates of sleep and waking in Drosophila melanogaster. *Science* **287**, 1834-1837 (2000).
42. T. Hallbook, J. Lundgren, I. Rosen, Ketogenic diet improves sleep quality in children with therapy-resistant epilepsy. *Epilepsia* **48**, 59-65 (2007).
43. Y. Daikhin, M. Yudkoff, Ketone bodies and brain glutamate and GABA metabolism. *Dev Neurosci* **20**, 358-364 (1998).
44. M. Erecinska, D. Nelson, Y. Daikhin, M. Yudkoff, Regulation of GABA level in rat brain synaptosomes: fluxes through enzymes of the GABA shunt and effects of glutamate, calcium, and ketone bodies. *J Neurochem* **67**, 2325-2334 (1996).
45. Y. Suzuki *et al.*, Beta-hydroxybutyrate alters GABA-transaminase activity in cultured astrocytes. *Brain Res* **1268**, 17-23 (2009).
46. T. M. Lund, L. F. Obel, O. Risa, U. Sonnewald, beta-Hydroxybutyrate is the preferred substrate for GABA and glutamate synthesis while glucose is indispensable during depolarization in cultured GABAergic neurons. *Neurochem Int* **59**, 309-318 (2011).
47. H. Laborit, Sodium 4-Hydroxybutyrate. *Int J Neuropharmacol* **3**, 433-451 (1964).
48. L. P. Carter, W. Koek, C. P. France, Behavioral analyses of GHB: receptor mechanisms. *Pharmacol Ther* **121**, 100-114 (2009).
49. L. P. Carter *et al.*, Novel gamma-hydroxybutyric acid (GHB) analogs share some, but not all, of the behavioral effects of GHB and GABAB receptor agonists. *J Pharmacol Exp Ther* **313**, 1314-1323 (2005).
50. N. Absalom *et al.*, alpha4betadelta GABA(A) receptors are high-affinity targets for gamma-hydroxybutyric acid (GHB). *Proc Natl Acad Sci U S A* **109**, 13404-13409 (2012).
51. P. A. Schwartzkroin, Mechanisms underlying the anti-epileptic efficacy of the ketogenic diet. *Epilepsy Res* **37**, 171-180 (1999).
52. R. A. Beyerinck, E. P. Brass, Effects of alpha-ketobutyrate and alpha-hydroxybutyrate on the enzymatic determination of acetoacetate and beta-hydroxybutyrate. *Clin Chem* **33**, 1469-1470 (1987).
53. V. P. Kui-Chung Lee, Geraldine Roberts, Elisabeth Trimble, The antispastic effect of L-threonine. *Amino Acids*, 658-663 (1990).

54. S. L. Hauser *et al.*, An antispasticity effect of threonine in multiple sclerosis. *Arch Neurol* **49**, 923-926 (1992).
55. J. H. Growdon, T. M. Nader, J. Schoenfeld, R. J. Wurtman, L-threonine in the treatment of spasticity. *Clin Neuropharmacol* **14**, 403-412 (1991).
56. Z. Li, J. Yu, Y. Peng, B. Huang, Metabolic pathways regulated by gamma-aminobutyric acid (GABA) contributing to heat tolerance in creeping bentgrass (Agrostis stolonifera). *Sci Rep* **6**, 30338 (2016).
57. A. Amato, C. N. Connolly, S. J. Moss, T. G. Smart, Modulation of neuronal and recombinant GABAA receptors by redox reagents. *J Physiol* **517 (Pt 1)**, 35-50 (1999).
58. M. E. Wilkins, T. G. Smart, Redox modulation of GABAA receptors obscured by Zn2+ complexation. *Neuropharmacology* **43**, 938-944 (2002).
59. K. A. Kasischke, H. D. Vishwasrao, P. J. Fisher, W. R. Zipfel, W. W. Webb, Neural activity triggers neuronal oxidative metabolism followed by astrocytic glycolysis. *Science* **305**, 99-103 (2004).
60. J. Rutter, M. Reick, L. C. Wu, S. L. McKnight, Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. *Science* **293**, 510-514 (2001).
61. F. Mayer *et al.*, Evolutionary conservation of vertebrate blood-brain barrier chemoprotective mechanisms in Drosophila. *J Neurosci* **29**, 3538-3550 (2009).
62. D. A. Bender, in *Introduction to nutrition and metabolism*. (CRC Press, Boca Raton, 2014), chap. 9.3.1.1, pp. 267.