mRNA expression in mouse hypothalamus and basal forebrain during influenza infection: a novel model for sleep regulation

Ming Ding and Linda A. Toth
Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, Illinois

Submitted 10 January 2005; accepted in final form 12 December 2005

Ding, Ming, and Linda A. Toth. mRNA expression in mouse hypothalamus and basal forebrain during influenza infection: a novel model for sleep regulation. Physiol Genomics 24: 225–234, 2006. First published January 10, 2006; doi:10.1152/physiolgenomics.00005.2005.—After influenza infection, C57BL/6J mice develop increased slow-wave sleep (SWS) during the dark phase of the day-night cycle, whereas BALB/cByJ mice develop decreased SWS during the light phase. A previous analysis of CXB recombinant inbred mice revealed a quantitative trait locus (QTL) designated Srilp (sleep response to influenza, light phase) that was related to expression of the BALB/cByJ sleep phenotype. Srilp was localized to the 10- to 12-cM region of mouse Chr 6 between D6Mit74 and D6Mit188. Temt (theioe S-methyltransferase), which is located at region B3 of Chr 6, is a potential candidate gene for Srilp. We evaluated the expression of Temt and other Srilp candidate genes in hypothalamus and basal forebrain of uninfected and influenza-infected C57BL/6J and BALB/cByJ mice. We report here that Temt expression varies significantly with respect to mouse strain, health status, brain region, and day-night phase. C57BL/6J mice show day-night variation in Temt expression in hypothalamus, but BALB/cByJ mice do not. Temt expression in basal forebrain is much higher in C57BL/6J mice than in BALB/cByJ mice. During influenza infection, both C57BL/6J and BALB/cByJ mice show reduced Temt mRNA in basal forebrain at 30 h postinoculation, but expression remains much lower in the BALB/cByJ strain. In contrast, prostaglandin-D-synthase (Ptgds) and lipocalin 2 (Lcn2) mRNA increase in basal forebrain of both strains after influenza infection. Administration of the TEMT inhibitor sinefungin reduces sleep in uninfected BALB/cByJ mice and attenuates influenza-induced sleep enhancement in C57BL/6J mice. These data suggest that strain- and infection-related alterations in sleep may be influenced by Temt expression and perhaps by subsequent effects on prostaglandin metabolism.

**Experimental Procedures**

**Mice.** All animal procedures performed in this study were approved by the Laboratory Animal Care and Use Committee of the Southern Illinois University School of Medicine. Adult male C57BL/6J and BALB/cByJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were maintained at 22°C on a 12:12-h light-dark (LD) cycle. Mice were inoculated intranasally with either uninfected BALB/cByJ mice and attenuates influenza-induced sleep enhancement in C57BL/6J mice. These candidate genes in hypothalamus and basal forebrain of uninfected and influenza-infected C57BL/6J and BALB/cByJ mice.

**EXPERIMENTAL PROCEDURES**

**Mice.** All animal procedures performed in this study were approved by the Laboratory Animal Care and Use Committee of the Southern Illinois University School of Medicine. Adult male C57BL/6J and BALB/cByJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were maintained at 22 ± 1°C on a 12:12-h light-dark (LD) cycle. Mice were inoculated intranasally with either uninfected BALB/cByJ mice and slows sleep in uninfected BALB/cByJ mice and attenuates influenza-induced sleep enhancement in C57BL/6J mice. These data suggest that strain- and infection-related alterations in sleep may be influenced by Temt expression and perhaps by subsequent effects on prostaglandin metabolism.

**Temt; Ptgds; Lcn2; sinefungin; selenium; prostaglandin**
During surgery, mice were maintained under a heat lamp, and the eyes were protected with a bland ophthalmic ointment. Four insulated stainless steel wires (Plastics One, Roanoke, VA) barren at the tips were positioned parallel to and under the skull in bilateral frontal and parietotemporal positions. EEG electrodes (Plastics One) were placed subcutaneously overlying the nuchal muscles. All electrodes were inserted into a headstage pedestal (Plastics One) that was secured to the skull with dental acrylic. During the same surgery, a transmitter (Data Sciences, St. Paul, MN) for telemetric monitoring of temperature and locomotor activity was implanted intraperitoneally. At this time, mice received 1 ml of saline intraperitoneally to maintain hydration and to lubricate the transmitter and the abdominal cavity. Another milliliter of saline was administered intraperitoneally the next morning. The analgesic ibuprofen was administered via the drinking water (1 mg/ml), beginning 1 day before surgery and continuing for 7 days after surgery. After surgery, mice were housed in individual cages in temperature-controlled, sound-attenuated chambers maintained at 22 ± 1°C on a 12:12-h LD cycle. Softened food was provided for 5 days after surgery.

For the anticipated experimental use, mice were connected via a flexible, light-weight tether to a six-channel commutator that permits monitoring of EEG and EMG signals while allowing unrestricted movement by the animal. This acclimation period has proven sufficient for the development of stable patterns of sleep and activity (38). One of the EEG electrodes was made continuous with cable shielding and served as a ground; two of the remaining three electrodes were referenced in the combination that provided optimal differentiation of the three vigilance states (wakefulness, SWS, and REMS).

Two studies assessed the impact of the TEMT inhibitor sinfungin (Sigma Chemical, St. Louis, MO) (3) on sleep. In the first study, the sleep, activity, and temperature patterns of C57BL/6J mice were monitored for 24 h without treatment (day 1). The next morning, mice were lightly anesthetized with methoxyfluorane immediately after light onset and were then inoculated intranasally with 25 PFU of the influenza strain A/HKX31. Sleep, temperature, and locomotor activity were monitored for the next 3 days. At the time of inoculation (day 2) on the next 2 days (days 3 and 4), mice were injected intraperitoneally with sinfungin (4 or 8 mg/kg) or with an equivalent volume of vehicle (pyrogen-free saline). Mice were euthanized, and tissues were collected on day 5. In the second study, sleep patterns of BALB/cByJ mice were monitored for 24 h without treatment (day 1). Immediately after light onset on day 2, mice were injected intraperitoneally with sinfungin (4 or 8 mg/kg) or saline and monitoring continued. Mice received additional injections of sinfungin or saline on days 3 and 4. Mice were euthanized for tissue collection on day 5.

Sleep data acquisition and determination of vigilance states. The EEG signals were passed through delta (1–4 Hz) and theta (4–8 Hz) filters (Coulbourn Instruments, LeHigh Valley, PA) and into a data acquisition system (Quality Software, Springfield, IL) that samples, digitizes, and stores signals at 16 Hz. EMG signals were similarly processed without filtering. Temperature and locomotor activity were telemetrically recorded via transmission from the intraperitoneal transmitter to a receiver positioned under the animal’s cage (Data Sciences). All data were continuously sampled and stored on a computer.

Computer-assisted methodology employing custom software was used to assign vigilance states to each 10-s epoch of the recording period. First, EEG tracings were visually examined to determine a threshold delta-wave amplitude (DWA) associated with SWS for each animal. Thresholds for EMG associated with periods of movement and for ratios of theta-delta band amplitudes associated with REMS were also determined. The data for each mouse were then scored in 10-s intervals for the entire experiment. A mouse was considered to be in a state of SWS whenever the average DWA for any two consecutive intervals exceeded the SWS threshold in association with a low-amplitude EMG signal. REMS was identified by low-amplitude EEG and EMG signals that occur in association with a high ratio of theta-delta amplitudes. At all other times, mice were considered to be awake. All computer-scored data were visually reviewed to verify the accuracy of the computerized scoring. Sleep data were summarized in 2-h intervals.

Tissue collection. mRNA for the genes Temt, Ghrhr, Crhr2, Nyp, Adcyap1r1, Lcn2 (lipocalin 2), and Ptgds (prostaglandin D synthase) was assessed in basal forebrain and, in some cases, hypothalamus. These brain regions were selected because of their well-documented association with the regulation of sleep (33). Hypothalamus was removed from the base of the brain based on visual landmarks. Basal forebrain was removed from the slice delineated by coronal cuts made approximately at the level of and 1-mm rostral to the optic chiasm (~0.014 mm anterior to and 0.34 mm posterior to bregma) (28). Basal forebrain was removed from the slice based on the visual landmarks of the anterior commissure, third ventricle, striatum, and olfactory tubercle. For PCR experiments, tissues from two to five mice were pooled for analysis, and three to seven independent replications were performed.

Virus titers. Pulmonary virus titers were measured using a plaque formation assay. Monolayers of Madin-Darby canine kidney fibroblasts were incubated with serial 10-fold dilutions of lung homogenate. An agar/media suspension was poured over infected cells, which were then incubated at 37°C for 6–7 days. The numbers of plaques present in the monolayers were then counted. Data were expressed in terms of PFU per lung. Significant strain variation in titers was not observed at either time point (titer ×10−3 at 2.5 h postinoculation: BALB/cByJ = 29 ± 5 PFU, n = 44; C57BL/6J = 29 ± 7 PFU, n = 36) (titer ×10−3 at 42 h postinoculation: BALB/cByJ = 9 ± 2 PFU, n = 15; C57BL/6J = 20 ± 7 PFU, n = 15).

RT-PCR analysis. Hypothalamus and basal forebrain were placed in RNALater (Ambion, Austin, TX) immediately after dissection. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and was digested with DNase I (Invitrogen). cDNA was synthesized from total RNA using Superscript (Invitrogen). Expression of Temt was estimated by RT-PCR using a primer pair designed according to the cDNA sequence of murine Temt (43). The sense primer was 5′-GCTAGAATGGAGGGCAAGGT-3′ (nucleotides 30–135) and the antisense primer was 5′-AGCTTGGAGGCAAAGCTTIA-3′ (nucleotides 854–873). Gapdh (glyceraldehyde-3-phosphate dehydrogenase) was used as reference or “housekeeping” gene; the sense primer was 5′-ACACGCCGCTCTCATTTGAC-3′ (nucleotides 130–147), and the antisense primer was 5′-GAAGACACCAGTGAATCCTCC-3′ (nucleotides 316–336) (BC083149). The PCR mix (10 μl) consisted of 1 μl of cDNA, 1 μl of each 10-pmol primer, reaction buffer, 200 μM each dNTPs, 2.5 mM MgCl2, and 0.5 U Taq polymerase (Invitrogen). Reactions were carried out in a thermocycler for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C, with the exception of Ptgds, for which only 20 cycles were used. PCR products were electrophoretically separated on agarose gel and visualized with ethidium bromide under UV illumination.

Expression of other genes was assessed using identical methods with the following primer pairs: for Ptgds, the sense primer was 5′-TGGCTGTTTGTTCACATTGG-3′ (nucleotides 2–21); and the antisense primer was 5′-ACAGAACAGGGCTCTGCAAGTGA-3′ (nucleotides 681–700) (NM_008963); for Ghrhr, the sense primer was 5′-TCCGTGTGACAGCTCTGTTG-3′ (nucleotides 689–708) and the antisense primer was 5′-CCACGACTCATGGTCCTCCA-3′ (nucleotides 890–909) (XM_132546); for Crhr2, the sense primer was 5′-CAGGGTTTCTTGTGTCGTT-3′ (nucleotides 1239–1258), and the antisense primer was 5′-GTGGTGTCGTAGTGTGTTGAA-3′ (nucleotides 1392–1411) (NM_009953); for Nyp, the sense primer was 5′-CAGGTTCTTCTCAGGGCCACC-3′ (nucleotides 9–28), and the antisense primer was 5′-ACACAAACAGGGAAAAGGC3′ (nucleotides 484–503) (NM_023346); for Adcyap1r1, the sense primer was 5′-TGGAGGTTGTTGACTGACT-3′ (nucleotides 397–416), and the antisense primer was 5′-TAGCGAGCTTGTAGAGGGC3′.
(nucleotides 925–944) (NM_007407); for Lcn2, the sense primer was 5‘-ATGTCACTCTCTCCATCTGCC-3‘ (nucleotides 276–295), and the antisense primer was 5‘-ACACGCTCTTTGTTCTTCA-3‘ (nucleotides 521–540) (NM_008491).

Quantitative real-time PCR analysis. mRNA for Temt, Ptgsds, Lcn2, Crrh2, and Ghrhr was quantified using quantitative real-time PCR (qPCR) with fluorescence detection. For Temt, the sense primer was 5’-GCGCTGCAATCCTCTACAGG-3’ (nucleotides 133–152), and the antisense primer was 5’-GACACGCTTTGTTCTTCA-3’ (nucleotides 361–382) (45). For Ptgsds, the sense primer was 5’-CAGTGGTAGGACAATTCTACG-3’ (nucleotides 416–435), and the antisense primer was 5’-CGACGCTCTTGTTCTTCA-3’ (nucleotides 643–662) (NM_008493). For Lcn2, Ghrhr, and Crrh2, the primer pairs described above were used for qPCR.

qPCR was performed using a Smart Cycler real-time PCR instrument (Cepheid, Sunnyvale, CA) and the iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA). The PCR mix (26.5 μl) consisted of 1 μl of cDNA, 1 μl with 10 pmol of each primer, and 12.5 μl of SYBR Green mix. qPCR was performed with 45 cycles of 30 sec at 62°C and 1 min at 72°C. The expression of genes was analyzed using Smart Cycler Software version 1.2f (Cepheid Sunnyvale, CA).

Detected target transcripts were normalized to the endogenous housekeeping gene Gapdh. Gapdh mRNA varied significantly between hypothalamus and basal forebrain (ANOVA: mean effect of brain region, $F = 10.117, p = 0.004$), but this variation was not robust in magnitude. Cycles required for threshold detection of Gapdh mRNA were 14.25 ± 0.09 in hypothalamus ($n = 4–5$) and 14.44 ± 0.19 in basal forebrain ($n = 4–5$). Gapdh mRNA did not vary significantly as a function of mouse strain, day-night phase, or health status.

Polymorphism analysis for Temt and Crrh2. DNA was extracted from the livers of C57BL/6J and BALB/cByJ mice. Six primer pairs were designed according to the genome sequence of murine Temt published at the National Center for Biotechnology Information (National Institutes of Health) homepage (NT_039343), and seven primer pairs were designed for Crrh2 genomic sequence from intron 3 to intron 9 (NW-000250). The PCR mix (10 μl) consisted of 1 μl of DNA, 1 μl of each 10-pmol primer, reaction buffer, 200 μM each of the dNTPs, 2.5 mM MgCl2, and 0.5 U Taq polymerase (Invitrogen). Reactions were carried out in a thermocycler for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C. PCR products were purified from the agarose gel using a GeneCLean II kit (Q.BIO gene, Carlsbad, CA). The University of Iowa DNA Core Facility performed sequence analysis using the BigDye Terminator v3.1 Cycle Sequencing kit (ABI Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 Genetic Analyzer.

Polymorphisms were identified by comparing sequences from C57BL/6J and BALB/cByJ mice using GeneRunner software.

For genotyping, genomic DNA from each of the 13 strains of CXB RI mice was purchased from the Jackson Laboratory. Temt and Crrh2 primer pairs were designed according to the genome sequence of murine Temt and Crrh2. For Temt, the sense primer was 5’-ACCTCAGCCACTTGGCTCAG-3’ (nucleotides 3274–3293), and the anti-sense primer was 5’-TGGCTTTTGTGGTTTTT-3’ (nucleotides 3465–3484) (NT_039343). For Crrh2, the sense primer was 5’-ACTCTGCTGGCTTCTTCT-3’ (nucleotides 31361–31380), and the antisense primer was 5’-TTTCTAAGGACAAAGGGCCTA-3’ (nucleotides 31478–31497) (NW-000250). PCR was performed as described above. PCR products were separated by electrophoresis on 12% polyacrylamide gel and visualized with ethidium bromide under UV illumination.

Statistical analysis. Values are presented as means ± SE. A mixed-model ANOVA was used to assess the overall level of significance across experimental groups. The within-subject variable was brain region. Between-subject variables were mouse strain, LD phase, and health status. Unpaired t-tests were performed to compare mean differences between two different groups. $P < 0.05$ was considered to be statistically significant. Bonferroni corrections for multiple comparisons were used as appropriate. SPSS software was used for statistical analysis.

RESULTS

Assessment of mRNA expression of Temt and other genes using RT-PCR. RT-PCR was performed on hypothalamus and basal forebrain of uninfected and influenza-infected C57BL/6J and BALB/cByJ mice euthanized at 30 h postinoculation (i.e., during the light phase at a time that corresponds to the expression of the Srilp phenotype). The PCR band for Temt cDNA was clearly visible in hypothalamus and basal forebrain of uninfected mice. Band intensity from basal forebrain of BALB/cByJ mice was weaker than that of C57BL/6J mice (Fig. 1). Compared with uninfected mice, Temt band intensities in the basal forebrain were lower in both strains of infected mice (Fig. 1). Band intensity in the hypothalamus did not vary markedly as a function of health status, but expression was lower in C57BL/6J mice compared with BALB/cByJ mice.

Band intensities of Ptgsds in hypothalamus were similar in uninfected C57BL/6J and BALB/cByJ mice and increased in both strains after infection (Fig. 1). In basal forebrain, band intensity was greater in uninfected C57BL/6J mice compared with uninfected BALB/cByJ mice, but intensities were similar in both strains after influenza infection (Fig. 1).

In uninfected mice, the Lcn2 band intensity was weaker in basal forebrain of BALB/cByJ mice compared with C57BL/6J mice (Fig. 1). After influenza infection, band intensities of Lcn2 were increased in both hypothalamus and basal forebrain of both strains.

mRNA levels were also assessed for the genes Ghrhr, Crrh2, Npy, and Adcyap1r1. However, these genes did not display significant differences in mRNA levels as a function of mouse strain, brain region, or health status (data not shown).

Validation and quantification of Temt expression using qPCR. qPCR was used to confirm and quantify differences in Temt mRNA that were revealed by RT-PCR. When normalized for Gapdh, Temt mRNA varied significantly as a function of mouse strain, brain region, LD phase, and health status (Fig. 2, Table 1) (ANOVA for within-subject variables: mean effect of brain region, $F = 179.644, P < 0.001$; region/strain/LD phase/health interaction, $F = 14.423, P = 0.001$) (ANOVA for between-subject variables: mean effect of strain, $F = 17.288, P < 0.001$; mean effect of LD phase, $F = 69.071, P < 0.001$; phase/health interaction, $F = 6.771, P = 0.015$; strain/phase/health interaction, $F = 11.358, P = 0.002$).

In uninfected mice, Temt expression in basal forebrain was markedly lower in BALB/cByJ vs. C57BL/6J mice during both light (0.09 ± 0.02 vs. 1.01 ± 0.14, $n = 7, P = 0.00002$) and dark (0.11 ± 0.04 vs. 1.23 ± 0.19, $n = 6, P = 0.0002$) phases (Fig. 2B). In hypothalamus of uninfected mice, Temt expression was equivalent in both strains during the dark phase, but was sixfold higher in BALB/cByJ vs. C57BL/6J mice during the light phase (5.96 ± 0.83 vs. 1.00 ± 0.18, $n = 5, P = 0.0004$) (Fig. 2A). Thus Temt expression showed significant variation as a function of LD phase in hypothalamus of C57BL/6J mice (dark vs. light: 6.79 ± 0.39 vs. 1.00 ± 0.18, $n = 4–5, P < 0.001$) but not in BALB/cByJ mice (Fig. 2A).

Temt expression in hypothalamus of C57BL/6J mice was not significantly altered by influenza infection at either 30 or 42 h after inoculation compared with uninfected mice at the same
Fig. 1. RT-PCR of Temt, Ptgds, and Lcn2 mRNA in hypothalamus and basal forebrain of uninfected and influenza-infected C57BL/6J and BALB/cByJ mice at 30 h after inoculation. The 871-, 210-, and 246-bp bands represent RT-PCR products for Temt, Gapdh, Ptgds, and Lcn2, respectively. M represents molecular markers of a 100-bp ladder. Lanes 1, 2, 3, and 4: hypothalamus. Lanes 5, 6, 7, and 8: basal forebrain. Lanes 1, 3, 5, and 7: tissues from uninfected C57BL/6J and BALB/cByJ mice. Lanes 2, 4, 6, and 8: tissues from influenza-infected C57BL/6J and BALB/cByJ mice. This study was repeated 5 times with similar results. RT-PCR was run for 35 cycles for Temt, Lcn2, and Gapdh genes and for 20 cycles for Ptgds gene.

Time point (Fig. 2A). However, expression in basal forebrain of C57BL/6J mice was significantly lower at 30 h after inoculation (0.41 ± 0.05 vs. 1.01 ± 0.14, n = 7, P = 0.001) (Fig. 2B).

Compared with uninfected mice, influenza-infected BALB/cByJ mice showed significantly less Temt mRNA in both hypothalamus (3.32 ± 0.24 vs. 5.96 ± 0.83, n = 5, P = 0.02) and basal forebrain (0.03 ± 0.003 vs. 0.09 ± 0.02, n = 7, P = 0.01) at 30 h after infection but not at 42 h (Fig. 2, A and B). Furthermore, at the 30-h postinoculation time point, Temt mRNA in basal forebrain was significantly lower in infected BALB/cByJ mice compared with infected C57BL/6J mice (0.03 ± 0.003 vs. 0.41 ± 0.05, n = 7, P < 0.001) (Fig. 2B).

Ptgds mRNA in basal forebrain and hypothalamus. qPCR was used to analyze basal forebrain and hypothalamus for Ptgds mRNA. This analysis was performed on five or six independent samples per experimental group, with each sample consisting of pooled basal forebrain from two mice. When normalized for Gapdh, Ptgds mRNA varied significantly as a function of brain region and health status (Fig. 3) [ANOVA for region (within-subjects variable): region/infection interaction, F = 7.10, P = 0.012] (ANOVA for between-subject variables in basal forebrain: mean effect of infection status, F = 26.302, P < 0.001; mean effect of LD phase, F = 11.224, P = 0.002; strain/infection interaction, F = 8.152, P = 0.007; strain/phase/infection interaction, F = 6.072, P = 0.018) (ANOVA for between-subject variables in hypothalamus: mean effect of LD phase, F = 13.713, P = 0.001; strain/infection interaction, F = 5.226, P = 0.028).

Uninfected BALB/cByJ and C57BL/6J mice had equivalent levels of Ptgds mRNA in basal forebrain and hypothalamus during the light phase (basal forebrain: 0.83 ± 0.07 vs. 1.00 ± 0.09, n = 6; hypothalamus: 0.88 ± 0.28 vs. 1.00 ± 0.30, n = 5). However, as with Temt, Ptgds mRNA levels showed significant variation as a function of LD phase in basal forebrain of C57BL/6J mice (dark vs. light: 2.03 ± 0.39 vs. 1.00 ± 0.09, n = 6, P = 0.03) but not BALB/cByJ mice (Fig. 3B). Ptgds mRNA levels were significantly higher during the dark phase in hypothalamus of both strains (C57BL/6J: 3.44 ± 0.66 vs. 1.00 ± 0.30, n = 5–6, P = 0.01; BALB/cByJ: 2.57 ± 0.52 vs. 0.88 ± 0.28, n = 5–6, P = 0.03) (Fig. 3A).

Inoculation with influenza virus significantly increased Ptgds mRNA in basal forebrain of both strains of mice at 30 h after inoculation (C57BL/6J: 1.95 ± 0.18 vs. 1.00 ± 0.09, n = 6, P = 0.0009; BALB/cByJ: 1.99 ± 0.28 vs. 0.83 ± 0.07, n = 6, P = 0.0026). Only BALB/cByJ mice showed increased expression at the 42-h time point (4.03 ± 1.02 vs. 0.97 ± 0.07, n = 5 or 6, P = 0.009). The magnitude of the effect varied as a function of time in infected BALB/cByJ mice (30 vs. 42 h: 1.99 ± 0.28 vs. 4.03 ± 1.02, n = 5 or 6) but not in infected C57BL/6J mice. Significant differences were not detected in hypothalamus of either strain at 30 or 42 h after inoculation (Fig. 3A).

Lcn2 mRNA in basal forebrain and hypothalamus. Infection with influenza virus induced a robust increase in Lcn2 mRNA in basal forebrain and hypothalamus that varied in magnitude as a function of mouse strain and health status but not brain region (ANOVA for between-subject variables in hypothalamus: mean effect of strain, F = 5.863, P = 0.028; mean effect of infection status, F = 8.12, P = 0.012; strain/infection interaction, F = 5.845, P = 0.028) (ANOVA for between-subject variables in basal forebrain: mean effect of strain, F = 10.70, P = 0.003; mean effect of infection status, F = 31.72, P < 0.001; strain/infection interaction, F = 10.73, P = 0.003) (Fig. 4). In uninfected mice, Lcn2 expression in basal forebrain was lower in BALB/cByJ mice than in C57BL/6J mice (0.43 ± 0.15 vs. 1.00 ± 0.18, n = 7, P = 0.032) (Fig. 4B) but in hypothalamus was higher in BALB/cByJ mice (2.94 ± 0.58 vs. 1.00 ± 0.14, n = 5, P = 0.01) (Fig. 4A). At 30 h after influenza infection, expression of Lcn2 was several hundred-fold higher in basal forebrain and hypothalamus of both strains of mice.
During infection, Lcn2 expression in basal forebrain: C57BL/6J: 311 ± 86 vs. 1.00 ± 18, n = 7, P = 0.0044; BALB/cByJ: 1.138 ± 240 vs. 0.43 ± 0.15, n = 7, P = 0.0005; hypothalamus: C57BL/6J: 230 ± 80 vs. 1.00 ± 0.14, n = 5, P = 0.02; BALB/cByJ: 2.791 ± 1.056 vs. 2.94 ± 0.58, n = 5, P = 0.03). During infection, Lcn2 expression in

Table 1. Ratios of Temt mRNA in hypothalamus and basal forebrain of uninfected and influenza-infected mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hypothalamus Infected/uninfected (30-h PI)</th>
<th>P value</th>
<th>Light/dark uninfected (30-h PI)</th>
<th>P value</th>
<th>Basal Forebrain Infected/uninfected (30-h PI)</th>
<th>P value</th>
<th>Light/dark uninfected (30-h PI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>0.62 (n=5, 5)</td>
<td>0.09</td>
<td>0.15 (n=5, 4)</td>
<td>&lt;0.0001</td>
<td>0.41 (n=7, 7)</td>
<td>0.001</td>
<td>0.82 (n=7, 6)</td>
<td>0.35</td>
</tr>
<tr>
<td>BALB/cByJ</td>
<td>0.56 (n=5, 5)</td>
<td>0.02</td>
<td>0.88 (n=5, 4)</td>
<td>0.64</td>
<td>0.29 (n=7, 7)</td>
<td>0.01</td>
<td>0.82 (n=7, 6)</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Mice were inoculated immediately after light onset with either allantoic fluid or influenza virus and were euthanized at 30 or 42 h after inoculation (i.e., at the midpoint of the light phase or dark phase for uninfected mice). Ratios are based on the mean values of Temt expression for each experimental group. ANOVA were conducted based on the actual expression data for each sample, as illustrated in Fig. 2. PI, postinoculation.
substitutions were detected in introns 1 and 2, and 26- and 11-bp deletions were detected in intron 2 of BALB/cByJ mice. These intron deletions and substitutions were located within the COOH-terminal domains of the Temt DNA molecule, which are responsible for specific recognition and binding of the target DNA sequences (32). In contrast, the NH2-terminal domains carry the catalytic sites. With regard to the Crhr2 molecule, a 5-bp repeat deletion (CAAAA) was detected in intron 6 of BALB/cByJ mice.

The 26-bp deletion in Temt intron 2 and the 5-bp deletion in Crhr2 intron 6 of BALB/cByJ mice were used as novel markers for genotyping the CXB set of RI mice (Fig. 5, Table 2). The resultant strain distribution patterns (SDPs) revealed that the Crhr2 and Temt markers share the genotype SDP of D6Mit384 (27.5 cM) but differ from the phenotype SDP of Srilp, which corresponds most strongly with D6Mit316 (28.0 cM) (41).

**TEMT inhibition and sleep.** Sleep was monitored in C57BL/6J mice that were infected with influenza virus and also treated with either pyrogen-free saline (vehicle) or with the TEMT inhibitor sinefungin (4 mg·kg⁻¹·day⁻¹). During the 72-h postinfection period, infected mice that were treated with vehicle (n = 6) developed enhanced sleep, as reported previously (38) (paired t-test, P = 0.03). However, the responses of mice treated with sinefungin (n = 9) were significantly attenuated compared with mice treated with saline (P = 0.038) (Fig. 6). Sinefungin treatment did not significantly alter the hypothermia that accompanies influenza infection in mice (data not shown). Lung viral titers were not significantly altered by sinefungin treatment.

Administration of sinefungin to uninfected C57BL/6J mice (4 mg·kg⁻¹·day⁻¹; n = 3), which have relatively high amounts of Temt mRNA, did not influence time spent in SWS (data not shown). In contrast, administration of sinefungin (4 or 8 mg·kg⁻¹·day⁻¹; n = 6 and 9, respectively) to uninfected BALB/cByJ mice, which have relatively low Temt mRNA, induced a modest and marginally significant reduction in time spent in SWS compared with saline-treated mice (n = 5) (1-way ANOVA: F = 3.521, P = 0.053) (Fig. 7). Sinefungin treatment did not significantly alter core temperature or locomotor activity (data not shown). Analysis of basal forebrain collected from these mice at 72 h after infection revealed that sinefungin administration significantly reduced levels of Temt mRNA in basal forebrain (relative levels in mice that received saline, 4 mg·kg⁻¹·day⁻¹, or 8 mg·kg⁻¹·day⁻¹ were, respectively, 1.00 ± 0.16, 0.73 ± 0.11, and 0.40 ± 0.08; 1-way ANOVA: F = 6.812, P = 0.012) (Fig. 8). These findings were validated using RT-PCR (Fig. 8).

**DISCUSSION**

Our previous analysis of sleep during influenza infection in CXB RI mice revealed a QTL, Srilp, associated with the

---

**Fig. 5.** Temt genotypes in CXB recombinant inbred (RI) mice. Genomic DNAs were amplified by PCR with intron 2-specific primers. Products were subjected to 12% polyacrylamide gel electrophoresis. Arrows indicate C57BL/6J (212 bp) and BALB/cByJ (186 bp) alleles. Temt genotypes of CXB RI mice were determined by comparing their PCR products to those of C57BL/6J and BALB/cByJ mice.
BALB/cByJ phenotype of reduced sleep during the light phase (40). The 95% confidence interval that defines Srilp contains the gene Temt. A comparison of C57BL/6J and BALB/cByJ mice revealed significantly less Temt mRNA in basal forebrain of the BALB/cByJ strain. At 30 h after inoculation with influenza virus (i.e., during the light phase), Temt mRNA was significantly lower in basal forebrain of both strains. This infection-related reduction, coupled with low basal levels, resulted in an extremely low relative level of Temt mRNA in infected BALB/cByJ mice (Fig. 2B).

Our data, taken together with the known biological function of TEMT, suggest that Temt could contribute to regulation of sleep. TEMT is one of three mammalian \textit{S}-adenosylmethionine-dependent methyltransferases. The enzymatic action of TEMT is to catalyze the transfer of a methyl group from \textit{S}-adenosylmethionine to selenium, sulfur, or tellurium, yielding the corresponding methylmonium compound (43). Selenium is an antioxidant trace element that is widely distributed throughout the body but is found in particularly high concentrations in the brain (7). TEMT appears to be the sole enzyme responsible for synthesis of the final methylation product of selenium, the trimethylselenium ion, in mice (26). Trimethylselenium ion is excreted in the urine by a variety of animals, including humans, implying widespread occurrence of the enzyme. TEMT is abundant in liver and lung (26). Our data reveal that Temt gene expression also occurs in mouse hypothalamus and basal forebrain. Thus TEMT may be important for catalyzing the final metabolic step for disposal of excess selenium in brain as well as in the periphery (26). Furthermore, Temt mRNA levels are not uniform across different brain structures and are influenced by both light-dark phase and health status.

Inorganic tetravalent selenium compounds are potent, specific, noncompetitive, and reversible inhibitors of brain prostaglandin D synthase (PGDS) (18). PGDS is an important sleep-modulatory enzyme under physiological conditions; has been purified from the brains of rats, frogs, and humans; and is the enzyme responsible for the synthesis of prostaglandin (PG) D2 in brain (15, 16). PGD2 is a major prostanooid in the mammalian brain as well as a major endogenous sleep-promoting substance in mice, rats, and monkeys and probably in humans (15). PGD2 promotes sleep via both the ventral rostral basal forebrain (24) and the anterior hypothalamus (22). Administration of inorganic selenium compounds modifies sleep, anesthesia, and hypothalamic phospholipid regulation in rats and mice (9, 19, 36).

We speculate that the low Temt mRNA we observed in basal forebrain of influenza-infected BALB/cByJ mice could sequentially produce accumulation of inorganic selenium, inhibition of PGDS, reduced PGD2, and reduced sleep (Fig. 9). Consistent with this idea, several studies have shown that, under normal conditions, C57BL/6 mice demonstrate modestly greater amounts of both SWS and REMS than do BALB/c mice, particularly during the light (resting) phase of the 24-h cycle (8, 11, 31, 35, 38, 42). To test the relationship between TEMT and sleep, we examined the impact of the nucleoside competitive inhibitor of TEMT (32). Consistent with our model, treatment of mice with sinefungin attenuates the characteristic sleep enhancement of influenza-infected C57BL/6J mice and reduces sleep in uninfected BALB/cByJ mice. In contrast, administration of sinefungin does not change the temperature or activity responses of uninfected BALB/cByJ mice.

**Table 2. Strain distribution patterns of genotypes and Srilp phenotypes of CXB RI mice**

<table>
<thead>
<tr>
<th>Strain Distribution Patterns</th>
<th>CXB Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Srilp phenotype</td>
<td></td>
</tr>
<tr>
<td>Market/gene genotypes</td>
<td>cM (Chr 6)</td>
</tr>
<tr>
<td>D6Mit71</td>
<td>31.0</td>
</tr>
<tr>
<td>D6Mit316</td>
<td>28.0</td>
</tr>
<tr>
<td>Srilp</td>
<td>C</td>
</tr>
<tr>
<td>Crhr2</td>
<td>27.5</td>
</tr>
<tr>
<td>Temt</td>
<td>27.5</td>
</tr>
<tr>
<td>Temt</td>
<td>27.5</td>
</tr>
<tr>
<td>Crhr2</td>
<td>27.5</td>
</tr>
<tr>
<td>Phenotype/genotype designations: B = C57BL/6J; C = BALB/cByJ</td>
<td>B = C57BL/6J; C = BALB/cByJ</td>
</tr>
</tbody>
</table>

Genotypes of Temt and Crhr2 in CXB RI mice were determined by PCR using polymorphisms that differentiate C57BL/6J and BALB/cByJ mice. These genotypes narrowed the QTL region of Srilp to a location between D6Mit316 and D6Mit384. Chr, chromosome; RI, recombinant inbred; QTL, quantitative trait locus.

Fig. 6. Slow-wave sleep (SWS) in influenza-infected C57BL/6J mice with and without sinefungin administration. Data are expressed as mean differences from baseline values for each mouse. Bars denote means ± SE for percentage of time spent in SWS during sequential light and dark phases after influenza inoculation at time 0. Injections were administered at 0, 24, and 48 h. Shaded bars, saline administration (n = 6); open bars, sinefungin administration (4 mg/kg) (n = 9).
mice or influenza-infected C5BL/6J mice. Thus the impact of sinefungin appears to be specific to modulation of sleep.

Our data also demonstrate increased Ptgds mRNA in basal forebrain during influenza infection in both strains of mice. PGDS is a well-documented sleep-modulatory enzyme under physiological conditions (16). We speculate that this induction could reflect selenium-induced inhibition of PGDS, reduced PGD2 production, and a subsequent compensatory increase in transcription of Ptgds in response to selenium-induced PGDS inhibition and reduced PGD2 availability. On the other hand, because PGDS has a dual function (one as PGD2-producing enzyme, and another as a transporting protein; Ref. 2), increased Ptgds mRNA might also reflect an infection-related enhancement of transport of lipophilic substances. Of particular interest, PGDS binds retinoic acid (37, 43), which was recently implicated in the modulation of the EEG and the regulation of cortical synchrony during sleep (23).

Our data further reveal both the presence of Lcn2 mRNA in mouse brain and a striking increase in Lcn2 mRNA in basal forebrain and hypothalamus during influenza infection. LCN2 and PGDS are both members of the lipocalin family of proteins (5, 17, 21, 29). LCN2 protein is secreted by mouse macrophages (25) and by human neutrophils (21). Lcn2 mRNA or LCN2 protein is elevated during bacterial infections and pulmonary inflammation in mice and during severe acute respiratory syndrome (SARS) in humans (10, 30, 44). LCN2 protein may mediate processes of growth, differentiation, and inflam-

---

**Fig. 7.** SWS in uninfected BALB/cByJ mice with and without sinefungin administration. Data are expressed as mean differences from baseline values for each mouse. Bars denote means ± SE for the percentage of time spent in SWS during sequential light and dark phases after administration of sinefungin or saline. Injections were administered at 0, 24, and 48 h. Solid bars, saline, n = 5; hatched bars, 4 mg/kg sinefungin, n = 5; open bars, 8 mg/kg sinefungin, n = 9.

**Fig. 8.** Temt mRNA in basal forebrain of BALB/cByJ mice after administration of saline or sinefungin. Tissue was collected from BALB/cByJ mice euthanized 24 h after receiving the 3rd of 3 sequential daily injections of saline or sinefungin. Bars denote means ± SE for Temt mRNA as standardized to saline-treated mice (referred to as control on y-axis); n = 5–9 for each experimental group. *P < 0.03 compared with saline-treated mice. **P < 0.05 compared with mice treated with 4 mg/kg sinefungin. Bottom: RT-PCR performed on a representative set of tissues from these mice.

**Fig. 9.** Model linking Temt and sleep via regulation of selenium and prostaglandin metabolism. We hypothesize that reduced Temt expression results in decreased metabolism of inorganic selenium. Elevated levels of inorganic selenium in basal forebrain inhibit the activity of prostaglandin D synthase (PGDS), thereby decreasing the synthesis of prostaglandin (PG) D2 in basal forebrain and reducing sleep.

---

*Physiol Genomics • VOL 24 • www.physiolgenomics.org*
mation (12), and serum LCN2 has been proposed as a marker for inflammation (10, 14, 20). Thus increased Lcn2 mRNA in brain could represent a facet of the host response to peripheral inflammatory challenge, perhaps mediated via glial cells. The high homology between LCN2 and PGDS (4, 17, 34) also suggests the possibility that the two proteins may share enzymatic or transport functions.

The data presented here diminish the likelihood that Temt is the gene that underlies Srilp. Within the CXB RI panel, the SDP of a Temt polymorphism that we detected does not correspond well to the SDP of the Srilp phenotype. Also, because of their locations within introns, the functional significance of these Temt polymorphisms to the expression of the BALB/cByJ phenotype is uncertain but relatively unlikely. Nonetheless, lack of multiple functional alleles does not necessarily rule out a gene as a QTL, particularly for a complex phenotype like sleep. First, the gene of interest may be differentially regulated by upstream mechanisms. Second, common genes may be permissive but not sufficient for phenotype generation. Finally, even a major-effect gene may control a limited proportion of total phenotypic variance for a complex trait. Srilp accounts for ≈35–45% of the phenotypic variance, and several of the Srilp phenotypes assigned in Table 2 are relatively intermediate between the phenotypes of the parental strains (CXB-4, -7, -12, -13) (40). Sleep in general and the Srilp phenotype in particular are complex processes that are undoubtedly influenced by numerous genes whose alleles all exert independent or interdependent quantitative influences. Although our genotype data diminish the likelihood that Temt is the quantitative trait gene that underlies Srilp, they also narrow the confidence interval (CI) for Srilp from its original limits of 21–31 cM (95% CI) to 28–31 cM on mouse Chr 6 (Table 2). Other candidate genes located within the Srilp CI include Ghrhr, Chrh2, Npy, and Adcyap1r1. However, we did not detect significant variation in expression of these genes as a function of mouse strain, health status, or light-dark phase.

In summary, data presented here demonstrate expression of the Temt gene in mouse brain and document variation in expression as a function of mouse strain, brain region, light-dark phase, and health status. On the basis of these findings, we hypothesize that altered expression of Temt could influence patterns of sleep via effects on prostaglandin metabolism.

ACKNOWLEDGMENTS

We thank Sharon Lyons for excellent technical assistance in carrying out this work; Dr. Jiming Cheng, Division of Hematology/Oncology, Department of Internal Medicine, for helpful discussion of the data; and Drs. Stephen Markwell and Steve Verhulst, Division of Statistics and Research Consulting, and Dr. Larry Hughes, Department of Surgery, for assistance with the statistical analysis.

GRANTS

National Institutes of Health Grants NS-40220, HL-70522, and RR-17543 and the Southern Illinois University School of Medicine supported this work.

REFERENCES


