Alterations in EEG activity and sleep after influenza viral infection in GHRH receptor-deficient mice

Jeremy A. Alt,1 Ferenc Obal, Jr.,2 T. R. Traynor,1 Janos Gardi,3 Jeannine A. Majde,1 and James M. Krueger1
1Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, Washington State University, Pullman, Washington 99164-6520; and 2Department of Physiology and 3Endocrine Unit, University of Szeged, A. Szent-Györgyi Medical Center, 6720 Szeged, Hungary
Submitted 23 December 2002; accepted in final form 17 February 2003

Alterations in EEG activity and sleep after influenza viral infection in GHRH receptor-deficient mice. J Appl Physiol 95: 460–468, 2003. First published February 21, 2003; 10.1152/japplphysiol.01190.2002.—Viral infections induce excess non-rapid eye movement sleep (NREMS) in mice. Growth hormone-releasing hormone receptor (GHRH receptor) was previously identified as a candidate gene responsible for NREMS responses to influenza challenge in mice. The dwarf lit/lit mouse with a nonfunctional GHRH receptor was used to assess the role of the GHRH receptor in viral-induced NREMS. After influenza A virus infection the duration and intensity (electroencephalogram (EEG) delta power) of NREMS increased in heterozygous mice with the normal phenotype, whereas NREMS and EEG delta power decreased in homozygous lit/lit mice. Lit/lit mice developed a pathological state with EEG slow waves and enhanced muscle tone. Other influenza-induced responses (decreases in rapid eye movement sleep, changes in the EEG high-frequency bands during the various stages of vigilance, hypothermia, and decreased motor activity) did not differ between the heterozygous and lit/lit mice. GH replacement failed to normalize the NREMS responses in the lit/lit mice after influenza inoculation. Decreases in NREMS paralleled hypothermia in the lit/lit mice. Lung virus levels were similar in the two mouse strains. Lit/lit mice had a higher death rate after influenza challenge than the heterozygotes. In conclusion, GHRH signaling is involved in the NREMS response to influenza infection.

growth hormone-releasing hormone receptor; growth hormone; fever; non-rapid eye movement sleep; rapid eye movement sleep; lit/lit mice; electroencephalogram

EXCESS SLEEPINESS, FATIGUE, and fever are common symptoms of many infectious diseases. Intranasal inoculation of influenza virus in mice results in lethal pneumonitis and profound increases in non-rapid eye movement sleep (NREMS) lasting 3 days or more, whereas rapid eye movement sleep (REMS), body temperature (Tb), and motor activity are decreased (16, 17, 44, 46, 48). Influenza-induced enhancements in NREMS are strain dependent because C57BL/6 mice display these responses, whereas infected BALB/c mice do not (46). Promotion of NREMS is due, in part, to the release of somnogenic proinflammatory cytokines in response to influenza infection (25). A quantitative trait loci analysis indicated that the growth hormone-releasing hormone receptor (GHRH receptor) gene is a candidate gene for the NREMS increase induced by influenza challenge in mice (48).

GHRH is a sleep-regulatory substance. Intracerebroventricular, intraperiocular, or systemic administration of GHRH increases NREMS and enhances electroencephalogram (EEG) slow-wave activity during NREMS in rats, mice, and rabbits. GHRH also increases the time spent in deep NREMS characterized by intense EEG slow-wave activity in humans (34, 41, 49). Conversely, NREMS decreases when GHRH is inhibited with a competitive antagonist, by means of immunoneutralization, or via the negative feedback loops in the somatotropic axis (34). NREMS is also reduced in transgenic or mutant mice and rats with decreased GHRH production or a defect in GHRH receptor signaling (34). It seems that GHRH promotes sleep via the preoptic region of the anterior hypothalamus (53). The GHRHergic mechanism is involved in the enhancement of NREMS elicited by at least one somnogenic proinflammatory cytokine, interleukin-1β (IL-1), because immunoneutralization of GHRH greatly attenuates the NREMS response to IL-1 (33).

Lit/little (lit/lit) mice have a point mutation in the GHRH receptor gene resulting in the replacement of a single amino acid in the receptor protein (20, 27). This receptor is not capable of binding GHRH (19). Consequently, secretion of both growth hormone (GH) and insulin-like growth factor 1 (IGF-1) (a GH-dependent hormone and paracrine) is low, and the mice are dwarfs. Previously, we showed that spontaneous NREMS and REMS are lower in the lit/lit mice compared with heterozygous controls (32). Infusion of GH into lit/lit mice normalizes REMS but does not alter NREMS. This finding suggests that the REMS deficit is due to the lack of GH and/or IGF-1, whereas the reduction of NREMS results from the malfunction of

Address for reprint requests and other correspondence: J. M. Krueger, Washington State Univ., College of Veterinary Medicine, Dept. of VCAPP, PO Box 646520, Pullman, WA 99164-6520 (E-mail: krueger@vetmed.wsu.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
hypothalamic GHRH receptors. Therefore, lit/lit mice were used in this study to determine whether the deficiency in GHRH signaling alters the sleep response to influenza infection. We report that lit/lit mice fail to produce normal NREMS responses to virus challenge and this alteration is not corrected by GH administration.

MATERIALS AND METHODS

Virus

Allantoic fluid from specific pathogen-free chicken embryos infected with influenza A/Puerto Rico/8/34 (H1N1) (PR8) was prepared under nonpyrogenic conditions by Specific Pathogen-Free Avian Supply. The virus was washed four times and purified (7), suspended in Dulbecco’s phosphate-buffered saline with Ca\(^{2+}\) and Mg\(^{2+}\) plus 0.2% bovine albumin (DPBS-A) (Sigma Chemical) at 200 µg/ml of viral protein (Bio-Rad protein assay), and stored at −80°C. Viral samples were diluted 1:10 in DPBS-A, heat inactivated by boiling for 10 min, and tested for endotoxin contamination by the Limulus lysate gel-clot assay (BioWhittaker); no endotoxin was detected (detection limit = 0.125 endotoxin units/ml). The stock virus was also tested for mycoplasma and acholeplasma species by nested PCR by use of a kit (American Type Culture Collection); the stock was negative. The starting titer of the purified virus was 1 × 10\(^{6}\) median tissue culture infectious doses (TCID\(_{50}\)) as determined by 50% end-point dilution in triplicate in Madin-Darby canine kidney (MDCK) cells cultured with trypsin at 72 h. The virus was diluted 1:2 in Hank’s balanced salt solution containing Ca\(^{2+}\) and Mg\(^{2+}\) (GIBCO BRL), and mice were inoculated intranasally within 10 min of light onset at 0800 with 5 µl/mouse after light methoxyflurane (Metofane, Schering-Plough Animal Health) inhalation anesthesia.

Animals

Heterozygous male C57BL/6 (C57BL/6J-Ghrhr-lit–/+) (n = 44), and homozygous male lit/lit mice (n = 46) 2–4 mo old were purchased from Jackson Laboratories. The difference in body weight between the two groups (heterozygous mice 22.7 ± 0.66 g, lit/lit mice 11.4 ± 0.30 g; P < 0.001) was significant. The mice were housed individually in environmental chambers. The chambers were on a 12:12-h light-dark cycle (lights on at 0800) with an ambient temperature of 29 ± 1°C. Food and water were available ad libitum. All animal procedures conformed with the principles set forth in our animal use protocol, which was approved by our IACUC.

Surgery and Recordings

Mice in groups 1 and 2 (see below) were provided with EEG and electromyogram (EMG) electrodes as previously described (32). After surgery, mice were given 7 days to recover. During this period, mice were connected to lightweight recording cables for habituation to the experimental conditions. Signals from the amplifiers were digitized at 128 Hz and collected on a computer. NREMS, REMS, and wakefulness were visually scored off-line in 10-s epochs by using criteria previously reported (32). The time spent in each vigilant state was calculated in 1-h intervals and for the 12-h light and dark cycles. On-line fast Fourier transformation was performed on EEG data in 2-s epochs. Fast Fourier transformation spectral analysis generated power values from 0.25 to 63.5 Hz in 0.5-Hz bins that were integrated for 1-Hz bands. The power values were averaged for every 10 s. Mean power spectra between 1 and 20 Hz were obtained from 1.0 Hz power values in artifact-free uninterrupted 10-s NREMS, REMS, or wake epochs (and state U; see below) during each day of recording and were averaged for each mouse. The integrated power was also determined for each state of vigilance in the delta band (0.25–4 Hz), theta band (4.25–8 Hz), alpha band (8.25–12 Hz), and the sigma/beta band (12.5–20) for statistical comparisons among the recording days.

Experimental Protocols

Group 1, sleep responses to virus. Starting at light onset, EEG and EMG were recorded for 2 consecutive days to obtain baseline values of spontaneous sleep-wake activity in heterozygous (n = 14) and lit/lit mice (n = 13). On the third experimental day mice were inoculated. EEG and EMG recordings continued for the next 4 days.

Group 2, effects of GH replacement on the responses to influenza virus. Osmotic minipumps (ALZET 1002, 0.25 µl/h for 14 days, weight after loading: 0.5 g; Durect) were implanted subcutaneously in the dorsum of heterozygous (n = 8) and lit/lit mice (n = 9). Minipumps were filled with pyrogen-free saline (PFS) for the heterozygous mice, mouse GH (mGH) in PFS delivering 11 µg mGH per day in 4 lit/lit mice, and rat GH (rGH) delivering 24 µg rGH per day in 8 lit/lit mice. (mGH and rGH were gifts from Dr. A. F. Parlow of The National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases). These GH infusions are biologically active in lit/lit mice (31, 32). The minipumps did not interfere with the activity of the mice. One week after implantation, the lit/lit mice had gained 1.9 ± 0.22 g weight, which was significantly higher than the weight gain of the heterozygous mice implanted with minipumps delivering physiological saline (0.8 ± 0.23) (32). Spontaneous sleep-wake activity was recorded for 1 or 2 days on day(s) 8 and/or 9 of GH infusion. Then mice were inoculated with virus, and EEG and EMG recordings continued for 4 more days. In the morning on day 5 after infection, blood samples were collected from three lit/lit mice infused with 11 µg mGH/day, four lit/lit mice infused with 24 µg rGH, and nine heterozygous mice infused with PFS. An additional eight heterozygous mice and two lit/lit mice, which were not infected, were killed as controls. Blood was centrifuged, and plasma was removed and stored at −20°C. IGF-1 was measured in duplicate by means of a rat IGF-1 enzyme immunoassay kit (Diagnostic Systems Laboratories).

Group 3, Tb determination. A separate group of heterozygous (n = 8) and lit/lit mice (n = 9) were implanted with intraperitoneal Mini-Mitter transmitters (catalog no. 100-0002; weight: 1.6 g) for recording motor activity and Tb, by using DataQuest III software (Mini-Mitter). After 5 days of habituation, spontaneous activity and Tb were recorded for 48 h. Activity counts and the distribution of activity did not differ between the two groups of mice. Data from these 2 days were averaged and used as baseline values. Mice were then given virus at light onset, and motor activity and Tb were recorded over the next 4 days.

Group 4, viral titers in lung and brain. Lung and brain viral titers were determined in a separate group of heterozygous (n = 7) and lit/lit mice (n = 8). Mice were inoculated at light onset with virus. Twenty-four hours later lung and brain tissues were quickly dissected and immediately placed on dry ice. Samples were stored at −80°C. Viral titers were expressed as TCID\(_{50}\) in MDCK cells at 72 h as described by Monterio et al. (30).

Group V, histology. In a separate group of heterozygous mice (n = 3) and lit/lit mice (n = 4), histopathological
changes in the lungs were evaluated 24 h after viral challenge. Lungs were fixed in 10% formalin and embedded in paraffin, and sections were stained with hematoxylin and eosin. Histopathological changes in the lungs were quantified as the proportion of the lungs containing edema and leukocyte infiltration. Control mice received heat-inactivated virus.

Statistics

Two-way analyses of variance (ANOVA) for repeated measures and for independent measures were used to compare 1-Hz power spectra among days and between groups, respectively, where the day and the frequency were the two factors. The two-way repeated-measures ANOVA program discarded animals with missing data, and therefore the sample sizes decreased by the animals that died in the course of the experiment and the animals that did not produce at least 40 artifact-free 10-s epochs of a particular state of vigilance on a day. Changes in the power in the various frequency bands and variations in sleep states and Tb were compared by means of one-way ANOVA for repeated measures within a group of animals. Post hoc pairwise comparisons were performed by means of the Student-Newman-Keuls test. Pearson product-moment correlation was calculated between postinfection days and Tb or duration of NREMS. Student’s t-test was used for intergroup comparisons of body weight and duration of sleep states on the baseline day. The Cox proportional hazards test was used to analyze differences in death rates between strains of mice. An α level of $P < 0.05$ was considered significant in all tests performed.

RESULTS

EEG Responses, Groups 1 and 2

During spontaneous sleep, NREMS, and REMS were characterized by intense delta and highly regular theta activity, respectively, in both heterozygous and lit/lit mice (Fig. 1). The relative theta activity was high during waking but not as regular as during REMS. The waking power spectra also contained significant power in the low-frequency range. Although epochs with obvious artifacts were discarded, it is possible that movement artifacts of low frequency contributed to delta power during wakefulness. Total power varied greatly among the individual mice, and significant differences could not be detected between the groups. Although the delta power during NREMS at night tended to be smaller in the lit/lit mice than in heterozygous mice, previous experiments showed that EEG power did not differ among heterozygotic and lit/lit mice with or without GH replacement (32). Therefore, power values from lit/lit mice with (group 2) and without GH (group 1) were pooled for statistical purposes and for Fig. 1.

The EEG during NREMS changed significantly after infection in heterozygous and lit/lit mice. The changes in EEG power among the 5 recording days [heterozygous: $F(4,88) = 2.512, P < 0.05$; lit/lit mice: $F(4,88) = 45.533, P < 0.0001$] varied with the frequency (day × frequency interaction, heterozygous: $F(76,16729) = 6.888, P < 0.0001$; lit/lit mice $F(76,1672) = 13.643, P < 0.0001$). By day 3, heterozygous mice exhibited more EEG delta power during NREMS after infection than on the baseline day [$F(4,97) = 8.07, P < 0.0001$] (Fig. 1). Analysis of 12-h time blocks showed that delta power increased significantly during both the light and the dark periods on day 3 and also during the light period on day 4. Power in the higher frequency bands decreased (theta: $F(4,97) = 3.23, P < 0.02$; alpha: $F(4,97) = 7.62, P < 0.0001$; and sigma/beta: $F(4,97) = 14.2, P < 0.0001$). Reductions in the sigma/beta power were significant on days 2–4, the power in the alpha band decreased on days 3–4, and theta power decreased on day 4.

A major difference was observed in the infection-induced variations in delta power during NREMS between the heterozygous and lit/lit mice (Fig. 1). In contrast to the heterozygous mice, EEG delta power decreased in the lit/lit mice [$F(4,94) = 12.7, P < 0.0001$]. The reductions in delta power were significant on each postinfection day although the power of the
EEG waves with the lowest frequencies (1–2 Hz) tended to return to normal on days 3 and 4. The direction of the alterations in EEG power in the other frequency bands was similar to, although larger in magnitude, than the changes in the heterozygous mice. Thus EEG power decreased in the theta band \( F(4,94) = 46.1, P < 0.0001 \), significant on each day], the alpha band \( F(4,94) = 60.6, P < 0.0001 \), significant on days 2–4], and the sigma/beta band \( F(4,94) = 86.3, P < 0.0001 \), significant on days 2–4).

During scoring of the sleep records of the infected mice (particularly in \( \text{lit/lit} \) mice; see below), periods were noted in which the EEG and EMG differed from signals characteristic of normal states of vigilance. This state was defined as pathological and denoted as \( U \). Isolated spikes or trains of slow waves with modest amplitudes appeared on a relatively flat EEG background. The power spectrum in \( \text{state U} \) (Fig. 1A in the \( \text{lit/lit} \) mice) and NREMS differed \( F(1,24) = 55.5, P < 0.001 \), NREMS on day 3 was used for comparison]. The power peaked between 3 and 4 Hz, and it was significantly smaller than the power during NREMS in each frequency band. The EMG in \( \text{state U} \) differed fundamentally from the muscle activity during NREMS because the muscle tone increased in \( \text{state U} \), often exceeding the EMG activity in quiet wakefulness.

The EEG during REMS remained highly regular during the experiment. The power spectra among the 5 days indicated significant variations in the heterozygous \( F(4,84) = 2.469, P < 0.05 \) and in the \( \text{lit/lit} \) mice \( F(4,28) = 3.28, P < 0.025 \). The interactions between the day and frequency factors were highly significant \( \text{heterozygous: } F(76,1596) = 8.079, P < 0.001; \text{lit/lit} \) mice: \( F(76,532) = 10.164, P < 0.0001 \), suggesting differential day-dependent alterations in power in the individual frequencies. Delta power during REMS did not change in the control mice. Slight increments in delta power occurred in the \( \text{lit/lit} \) mice on days 3 and 4 \( F(4,78) = 13.3, P < 0.001 \). However, the EEG during REMS remained largely free from pathological slow waves and spikes. Although the 1-Hz resolution did not allow accurate determination of the frequency of the theta peak, it was obvious that the peak power inside of the theta range shifted to lower frequencies starting on \( \text{day 2} \) in both \( \text{lit/lit} \) and heterozygous mice. However, the theta power did not decrease. In fact, owing to a widening of the theta peak in the spectra, significant increments were observed in the power in the theta range on days 2–4 in the controls \( F(4,96) = 10.5, P < 0.0001 \) and the \( \text{lit/lit} \) mice \( F(4,78) = 18.3, P < 0.0001 \). Power decreased slightly in the alpha \( \text{controls: } F(4,96) = 8.68, P < 0.0001, \text{lit/lit} \) mice: \( F(4,78) = 18.3, P < 0.0001 \) and sigma/beta bands \( \text{heterozygous: } F(4,96) = 4.17, P < 0.005, \text{lit/lit} \) mice: \( F(4,78) = 19.7, P < 0.0001 \) on days 2–4.

The waking EEG was not fundamentally altered in the heterozygous mice after viral challenge. There were no significant variations among the power spectra for the 5 days of recording, although the day \( \times \) frequency interaction was statistically significant \( F(76,1672) = 2.258, P < 0.0001 \). The power in the alpha band decreased \( F(4,97) = 6.72, P < 0.0001 \) on days 2–4. Power did not change significantly in the other frequency bands. In contrast, waking power spectra varied significantly among the recording days in the \( \text{lit/lit} \) mice \( F(4,92) = 11.083, P < 0.0001; \) interaction: \( F(76,1748) = 720.121, P > 0.0001 \). Reduction in the power of the alpha band \( F(4,95) = 12.7, P < 0.0001 \), which was significant on each postinfection day, was associated with progressive increases in delta power \( F(4,95) = 16.7, P < 0.0001 \) in the \( \text{lit/lit} \) mice. This increase was significant on days 3 and 4, and it resulted from an invasion of the waking EEG by spikes and slow waves characteristic of \( \text{state U} \). In some mice, intense motor activity rather than EEG distinguished wakefulness from \( \text{state U} \) on \( \text{day 4} \).

Sleep Responses, Group 1

On the baseline day, heterozygous and the \( \text{lit/lit} \) mice exhibited a normal diurnal rhythm of sleep with more NREMS and REMS during the light period than at night (Fig. 2). As reported previously (32), the \( \text{lit/lit} \) mice spent less time in NREMS \( P < 0.0001 \) and REMS \( P < 0.0005 \) than the heterozygous mice during the light period. A decrease in NREMS was also observed during the dark period \( P < 0.05 \), whereas REMS did not differ at night between the heterozygous and \( \text{lit/lit} \) mice.

Viral challenge greatly altered NREMS in both strains of mice. Significant variations in NREMS occurred during both the light \( F(4,97) = 15.3, P < 0.0001 \) and the dark periods \( F(4,95) = 23.28, P < 0.0001 \) postinfection in heterozygous mice. NREMS was enhanced starting during the dark period on \( \text{day 1} \) postinfection. Thereafter, the time spent in NREMS increased significantly in each light or dark period except the light period on \( \text{day 3} \), which did not differ from baseline. Infection also elicited changes in NREMS during the light periods \( F(4,45) = 44.37, P < 0.0001 \) and dark periods \( F(4,42) = 20.54, P < 0.0001 \) in the \( \text{lit/lit} \) mice. However, NREMS decreased in the \( \text{lit/lit} \) mice after infectious challenge (Fig. 2). Significant decreases in NREMS started during the light period on \( \text{day 2} \) postinfection and continued until the death of the mouse or until the end of the experiment. Only very short periods (less than 1 min) of normal NREMS occurred on \( \text{day 4} \) when these NREMS epochs usually preceded REMS. Although the number of U epochs increased significantly in both the light \( F(3,32) = 9.11, P < 0.0001 \) and dark \( F(3,76) = 8.44, P < 0.0001 \) periods in the control mice, the total time spent in this state remained low. In contrast, the time occupied by \( \text{state U} \) increased progressively in the \( \text{lit/lit} \) mice [light: \( F(3,32) = 57.94, P < 0.0001 \); dark: \( F(3,29) = 14.02, P < 0.0001 \)], and the U state gradually replaced normal NREMS (Fig. 2).

REMS was suppressed in heterozygous and \( \text{lit/lit} \) mice after viral challenge (Fig. 2). This effect started on \( \text{day 2} \), and it was prominent during the light period \( \text{controls: } F(4,97) = 23.73, P < 0.0001; \text{lit/lit} \) mice: \( F(4,45) = 17.38, P < 0.0001 \). After \( \text{day 2} \), REMS

\text{J Appl Physiol} \cdot \text{VOL 95} \cdot \text{AUGUST 2003} \cdot \text{www.jap.org}
remained lower than baseline in both groups during the light period except that REMS on day 3 did not differ from baseline in the lit/lit mice. Significant variations among the recording days occurred in REMS during the dark period in the heterozygous mice \([F(4,94) = 3.962, P = 0.01]\). However, post hoc comparisons identified only a single postinfection night that differed from baseline night (day 1), and REMS was slightly increased on this night. Although significant variations were detected in REMS among the dark periods in the lit/lit mice \([F(4,42) = 2.891, P = 0.05]\), REMS time during individual dark periods did not differ statistically from the baseline night (in part because the sample size decreased owing to the death of many animals as the experiment progressed).

**Effects of GH Infusion: IGF-1, Group 2**

The mean \(\pm SE\) concentration of IGF-1 in the plasma was \(389.3 \pm 15.31\) ng/ml in the uninfected heterozygous mice. After influenza challenge, IGF-1 concentration reached \(446.8 \pm 25.9\) ng/ml, but the increase was not significant. In the two untreated lit/lit mice, the plasma IGF-1 concentrations were 24.95 and 27.80 ng/ml. At 14 days after the onset of GH infusion (day 5 postinfection) plasma IGF-1 concentrations rose to a mean of 97.9 ng/ml (minimum 56.8, maximum 161.5 ng/ml) in three lit/lit mice infused with 11 \(\mu\)g/day rGH and to a mean of 316.6 ng/ml (minimum 218.9, maximum 486.5 ng/ml) in four lit/lit mice supplemented with 24 \(\mu\)g/day rGH.

**Effects of GH Infusion: Sleep Responses to Virus, Comparison of Group 1 and Group 2 Mice**

GH replacement did not alter baseline NREMS in the lit/lit mice, and group 2 lit/lit mice had significantly less NREMS than the heterozygous mice during both the light \([F(2,48) = 50.9, P < 0.0001]\) and the dark \([F(2,48) = 6.48, P < 0.005]\) periods. In contrast, GH
infusion enhanced and thereby normalized REMS in the lit/lit mice to a level similar to that observed in the heterozygotic mice (Fig. 2).

After viral challenge, the time spent in NREMS decreased in lit/lit mice infused with GH [light: \(F(4,43) = 32.37, P < 0.0001\); dark: \(F(4,41) = 35.7, P < 0.001\)] (Fig. 2). The time course of the virus-induced changes in NREMS after GH treatment did not differ from that observed in lit/lit mice that did not receive GH (group 1). The NREMS responses to infection were statistically identical in lit/lit mice from groups 1 and 2 and significantly different from the infection-induced enhancements in NREMS in the heterozygous mice (group 1 and 2 heterozygous mice combined) [ANOVA to compare 3 groups, light period: \(F(2,48) = 50.9, P < 0.0001\); dark period: \(F(2,48) = 6.48, P < 0.003\)].

Starting on day 2 postinfection, REMS decreased in each light period in the group 2 lit/lit mice with GH infusion \([F(4,43) = 20.9, P < 0.0001]\) (Fig. 2). REMS also varied during the dark periods among the days of recording \([F(4,41) = 11.39, P < 0.0001]\). Significant, although slight, increases in REMS occurred during the dark periods on days 1 and 2 postinfection whereas REMS decreased during the dark period on day 4. There were no significant differences in the REMS responses to infection among the heterozygous mice and among the lit/lit mice with and without GH infusion.

The pathological EEG activity with increased muscle tone also occurred in the lit/lit mice with GH infusion. The time spent in the U state increased progressively during both the light \([F(3,32) = 27, P < 0.001]\) and dark periods \([F(3,30) = 37.98, P < 0.001]\). There were no differences in the development of the U state between group 1 and group 2 lit/lit mice.

**Body Temperature: Group 3 Mice**

On the baseline day, \(T_b\) varied by 1°C between day and night in both the heterozygous and lit/lit mice (heterozygous light: 36.5 ± 0.19°C; heterozygous dark: 37.6 ± 0.23°C; lit/lit light 35.6 ± 0.12°C; lit/lit dark: 36.8 ± 0.13°C). The \(T_b\) of the lit/lit mice was lower than the \(T_b\) of the controls during both phases of the diurnal cycle \((P < 0.05)\).

Figure 3 depicts 12-h mean \(T_b\) values (group 3) and the time spent in NREMS during the 5 days of recording in heterozygous (groups 1 and 2 pooled) and lit/lit mice (group 1). After virus challenge, both strains of mice developed hypothermic responses [heterozygous light: \(F(4,28) = 16.9, P < 0.0001\); heterozygous dark: \(F(4,28) = 62.1, P < 0.0001\); lit/lit light: \(F(4,32) = 34.3, P < 0.0001\); lit/lit dark: \(F(4,32) = 99.9, P < 0.0001\)]. There was a negative correlation between the \(T_b\) and the postinfection day in both groups [heterozygous light: \(-0.63, P < 0.0001\); heterozygous dark: \(-0.76, P < 0.0001\); lit/lit light: \(-0.78, P < 0.0001\); lit/lit dark: \(-0.81, P < 0.0001\)]. In the lit/lit mice, the decline of NREMS followed the decline in \(T_b\) and was also negatively correlated with the postinfection day [light: \(-0.75, P < 0.0001\); dark: \(-0.81, P < 0.0001\)]. In contrast, a weak positive correlation was obtained between NREMS and the postinfection day in the heterozygous mice during the light period \((0.22, P < 0.05)\), whereas NREMS did not correlate with the postinfection day at night. Although the lit/lit mice had lower \(T_b\) than the heterozygotes on each postinfection day, the magnitude of the \(T_b\) drop with respect to baseline did not differ between groups.

Motor activity (group 3 mice) did not differ between the two strains of mice under basal conditions, and both strains had similar decreases after infection (data not shown).

**Virus Levels, Lung Histology, and Mortality**

Edema and infiltration of leukocytes were prevalent in both the heterozygous and lit/lit lungs 24 h after challenge. Lung histopathology did not differ between the two strains of mice. Lung viral titers for lit/lit \((10^{10.91 ± 2.38} \text{TCID}_{50})\) were not significantly different from those for heterozygous mice \((10^{6.87 ± 0.20} \text{TCID}_{50})\) 24 h postinfection. Viable virus was not detected in the brain 24 h after infectious challenge in either mouse strain.
The number of deaths was lower in the heterozygous mice (groups 1 and 2; 5 of 24, 21%) than in the lit/lit mice of group 1 without GH (9 of 13, 69%) \( P < 0.05 \), Holm corrected, Cox proportional hazards analysis. In group 2 GH-treated lit/lit mice, 5 of 12 died during the 4-day postinfection period. However, the sample size was too low to evaluate whether GH had a protective effect in the lit/lit mice.

**DISCUSSION**

In response to influenza virus infection, heterozygous mice displayed severe hypothermia and alterations in sleep-wake activity and the EEG in the various states of vigilance. The \( T_b \) and sleep changes in the heterozygous mice correspond to those described previously in wild-type C57BL/6 mice and other mouse strains (8, 16, 17, 44, 46) and are consistent with the normal phenotype of the heterozygous mice. Some of the infection-induced alterations are anticipated consequences of hypothermia. These alterations include the changes in EEG power in the various frequency bands (with the exception of enhanced delta power during NREMS) and the decreases in REMS. Thus REMS decreases when the temperature of the hypothalamus is lowered (39). The intensity of EEG activity in various frequency bands decreases in response to passive cooling (1) and in hypothermic animals entering hibernation (11). The peak of theta power during REMS shifts to lower frequencies when \( T_b \) decreases in hamsters (10). These changes, which are attributed to hypothermia, were at least qualitatively similar in the lit/lit mice to those in the heterozygous mice although they tended to be more severe in the former because the \( T_b \) of the lit/lit mice was also lower. Heterozygous and lit/lit mice differed fundamentally in their NREMS responses to influenza challenge. The responses of heterozygous mice that cannot be attributed to hypothermia include increases in NREMS duration and enhancements in delta power during NREMS. EEG slow-wave activity during NREMS is a marker of sleep intensity (35). Simultaneous increases in NREMS duration and selective enhancements in EEG delta power during NREMS are signs of strong stimulation of sleep mechanisms. Stimulation of hypothalamic heat sensors is regarded as an important input for NREMS, and this mechanism is proposed to work in the range of normal \( T_b \) (29). Withdrawal of this input and stimulation of cold sensors in hypothermia are anticipated to suppress NREMS (39). In fact, normal NREMS time decreased in parallel with \( T_b \) in the lit/lit mice, and, instead, the pathological state \( U \) emerged. Heterozygous mice spent more time in normal NREMS, and their NREMS was more intense than NREMS in the lit/lit mice with identical \( T_b \). That heterozygous mice were protected against hypothermia-associated decreases in NREMS and development of state \( U \) is perhaps a more important difference between the lit/lit and intact mice than the actual rises in NREMS in the latter group.

Increased NREMS in association with infection is thought to be driven by proinflammatory cytokines, either those released from the infected target tissue or those induced in the brain (25). Release of an array of cytokines occurs in response to influenza virus in mice (21). Many proinflammatory cytokines promote NREMS after systemic or intracerebral administration (25). IL-1\( \beta \) and TNF-\( \alpha \) are the best documented somnogenic cytokines, and both are induced by influenza virus. TNF-\( \alpha \) may enhance NREMS through mechanisms such as stimulation of intracerebral PGD\(_2\) production and inhibition of locus ceruleus activity (12, 43), whereas IL-1 may reduce glutamatergic transmission via an adenosine-dependent mechanism (28), modulation of serotonergic activity (22), and direct stimulation of the sleep-active neurons in the basal forebrain by IL-1\( \beta \) (2). In addition, IL-1\( \beta \) also stimulates the sleep-promoting GHRHergic system in the hypothalamus (33). GHRH acts on GABAergic neurons in the hypothalamus, and the same GABAergic neurons are also responsive to IL-1\( \beta \) (9). Preliminary in vitro findings suggest that IL-1\( \beta \) upregulates GHRH receptors (42). It seems that normal functioning of the GHRH and GHRH receptor complex is essential for both the maintenance and the increase of NREMS in the hypothermic state induced by influenza virus. Malfunctions of the GHRH receptor results in deficiencies in the entire somatotopic axis. GH infusion, however, failed to alter NREMS in the infected lit/lit mice although the same infusion was effective in increasing IFN-\( Y \) concentrations, stimulating weight gain, and normalizing baseline REMS (32). It seems, therefore, that stimulation of the intrahypothalamic GHRHergic system is responsible for the promotion of NREMS in heterozygous mice, and, because of the lack of functioning GHRH receptors, lit/lit mice are not able to withstand hypothermia-induced suppression of normal NREMS. The pathological state \( U \) in the lit/lit mice displays some features of NREMS (delta waves in the EEG) despite the flat EEG background and the high muscle tone. This state may reflect NREMS promotion in a condition when the GHRHergic system is defective and normal NREMS mechanisms are disorganized. Cytokines and other sleep-regulatory substances that are stimulated by cytokines may act locally within cortical neuronal groups and/or thalamocortical units and enhance local sleeplike activity (26), but because coordinating mechanisms in the basal forebrain are inactivated by hypothermia and the lack of GHRH, a disorganized state may occur.

Hypothermia is a consistent (8, 16, 44, 46) distinguishing feature of viral influenza infection in the mouse model, whereas the infection is associated with fever in other, larger species (50). In part, cytokines might be involved in the mediation of hypothermia. In addition to TNF (24), IL-1 is also capable of eliciting long-lasting decreases in body temperature in mice (44, 51). IFN-\( \gamma \) may potentiate IL-1 and TNF production and hypothermia (5). Because of their small body size, mice tend to respond with hypothermia to stimuli that cause fever in species with larger body mass (18, 38). It
is likely that the anorexia and suppression of motor activity [characteristic responses to influenza virus, which are in part mediated by IL-1 and TNF (14)] and perhaps the pneumonia-caused hypoxia have major roles in infection-associated decreases in body temperature because they all reduce metabolic heat production. The magnitude of the $T_b$ drop did not differ between the infected heterozygous and $lit/lit$ mice. However, the $lit/lit$ mice had lower than normal baseline $T_b$ and thus the hypothermia became more severe postinfection. We assume that the low baseline $T_b$ is due to the unfavorable surface-to-body mass ratio of the dwarf mice; these animals may dissipate more heat than larger mice. In addition, the lack of GH, an anabolic hormone, may compromise the capacity of heat production. The number of deaths of the $lit/lit$ mice was significantly higher than that of the heterozygous mice. After GH treatment, the number of deaths of the $lit/lit$ mice tended to decrease. Hypothermia is assumed to be protective in mouse influenza (23), and thus hypothermia per se is unlikely to contribute to increased viral lethality in the $lit/lit$ mice. Indeed hypothermia may broadly play a protective role in animals and humans metabolically compromised by infection or other severe stresses (38). An altered immune response resulting from the GHRH deficiency might be involved in the high mortality of the $lit/lit$ mice. GH is substantially increased in the blood after acute viral infections (4). GH is permissive for proinflammatory cytokine induction by infectious stimuli (15) and thus might modulate cytokine levels in the heterozygous mice. GH and IGF-1 play a role in lymphocyte development and function (52), and cytotoxic T lymphocytes are major immune contributors to both influenza recovery and pathogenesis. Such T cells eliminate virus by killing infected cells, consequently destroying lung tissue (13). An imbalance in cytotoxic T cells could hasten the development of influenza pneumonitis in the $lit/lit$ mice. Although our histopathology studies 24-h postinfection do not suggest the presence of excessive inflammation in the $lit/lit$ animals and virus levels are not elevated, they cannot assess late events in viral clearance and elimination or the possibility that the virus disseminates outside the lung more efficiently in the $lit/lit$ mice. GH, GHRH, and IGF-1 are expressed in the normal human airway (3). If the viral infection or the virus-induced cytokines can initiate or stimulate local GHRH production, then local GHRH-GH-IGF-1 activity in the lungs may have some protective functions, and in that case the GHRH receptor defect may directly contribute to the higher mortality of the infected $lit/lit$ mice. Further experiments may determine whether GHRH has any roles in the modulation of the inflammation in the lung.

In conclusion, the results suggest that GHRH receptors have a major importance in the mediation of the sleep response to viral influenza infection. The significance of the enhancements in sleep in viral influenza is currently controversial. Correlation was found between survival and the intensity of the initial NREMS response in experimental bacterial infections (47). A previous report suggested that sleep was essential for optimal immune function in mice infected with influenza virus (6), but subsequent experiments failed to support this finding (36, 37, 45). A recent report, however, demonstrates that human subjects vaccinated in a state of sleep deprivation produced less than half the titers of anti-influenza virus IgG than subjects with normal sleep, as determined 10 days postvaccination (40). It is also unclear whether GHRH itself promotes survival of the mice. The higher mortality of the GHRH receptor-deficient mice might be related to the lack of GH and/or IGF-1 and consequent immune alterations contributing to lethal disease.

We thank K. Boda and B. Slinker for help in statistical analyses, J. Wright for advice on GH infusion, S. Bohnet for help with viral titrations, and R. Brown for excellent technical assistance.

**DISCLOSURES**

This work was supported by the National Institutes of Health (Grants NS-25378 and HD-36520) and the Hungarian National Science Foundation (OTKA 30456).

**REFERENCES**